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TITLE:

Generation of Alpha-Synuclein Preformed Fibrils from Monomers and Use In vivo

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SUMMARY:

The goal of this article is to outline the steps required for the generation of fibrils from monomeric alpha-synuclein, subsequent quality control, and use of the preformed fibrils in vivo.

ABSTRACT:

Use of the in vivo alpha-synuclein preformed fibril (α-syn PFF) model of synucleinopathy is gaining popularity among researchers aiming to model Parkinson's disease synucleinopathy and nigrostriatal degeneration. The standardization of α-syn PFF generation and in vivo application is critical in order to ensure consistent, robust α-syn pathology. Here, we present a detailed protocol for the generation of fibrils from monomeric α-syn, post-fibrilization quality control

steps, and suggested parameters for successful neurosurgical injection of α-syn PFFs into rats or mice. Starting with monomeric α-syn, fibrilization occurs over a 7-day incubation period while shaking at optimal buffer conditions, concentration, and temperature. Post-fibrilization quality control is assessed by the presence of pelletable fibrils via sedimentation assay, the formation of amyloid conformation in the fibrils with a thioflavin T assay, and electron microscopic visualization of the fibrils. Whereas successful validation using these assays is necessary for success, they are not sufficient to guarantee PFFs will seed α-syn inclusions in neurons, as such aggregation activity of each PFF batch should be tested in cell culture or in pilot animal cohorts. Prior to use, PFFs must be sonicated under precisely standardized conditions, followed by examination using electron microscopy or dynamic light scattering to confirm fibril lengths are within optimal size range, with an average length of 50 nm. PFFs can then be added to cell culture media or used in animals. Pathology detectable by immunostaining for phosphorylated α-syn (psyn; serine 129) is apparent days or weeks later in cell culture and rodent models, respectively.

INTRODUCTION:

Parkinson's disease (PD) is primarily characterized postmortem by two major pathological features: widespread and progressive alpha-synuclein (α-syn) pathology, and nigrostriatal degeneration. Following injection into wildtype mice or rats, α-syn preformed fibrils (PFFs) induce progressive accumulation of pathological α-syn, which can result in protracted degeneration of substantia nigra pars compacta (SNpc) dopamine neurons over the course of many months, as well as sensorimotor deficits[1-7]. Neurons are exposed to α-syn fibrils, either via direct intracerebral injection or added to the media of cultured neurons. When the PFFs are taken into the neurons, the PFFs act to "seed" the formation of inclusions through templating, and accumulation of endogenous α-syn into phosphorylated inclusions[1,8-10]. Inclusions share similar properties to Lewy bodies: containing α-syn phosphorylated at serine 129 (pSyn), ubiquitin, and p62; possess amyloid quaternary structures as shown with positive thioflavin staining; and are resistant to proteinase K digestion[1,3,5,7,8-13]. PFF exposure leads to α-syn inclusion formation in primary and some immortalized neurons in culture, as well as mice, rats, and non-human primates in vivo[1-10,14]. It is important to note that PFFs will not lead to α-syn inclusion formation in all cell culture models and some cultured neurons will seed better than others.

Another important feature of the in vivo α-syn PFF model is the distinct sequential pathological phases that emerge over several months. In rodents, following intrastriatal injection, α-syn inclusion formation generally peaks within the SNpc and many cortical regions within 1-2 months. This aggregation peak is followed by nigrostriatal degeneration ≈2-4 months later[1,3,5,7]. These distinct pathological stages provide researchers the platform with which to study and develop strategies that 1) decrease α-syn aggregation, 2) clear already formed α-syn inclusions, and/or 3) prevent subsequent neurodegeneration. The PFF model offers distinct advantages and disadvantages as compared to neurotoxicant, transgenic, and viral vector mediated α-syn overexpression models as previously reviewed[6]. The choice of which model or approach to take should be determined by which model best suits the question the investigators are asking.

Although the PFF model has been successfully utilized by many labs, there are still groups that have experienced inconsistencies with generating fibrils and producing consistent α-syn pathology[15]. Examples of inconsistencies range from PFFs that produce little or no α-syn pathology, batch to batch seeding efficiency, and even the failure of fibrils to form. Thus, the standardization of α-syn PFF generation and in vivo application is critical in order to allow for accurate interpretations regarding the impact of novel therapeutic interventions. The following protocol outlines the steps required for the generation of PFFs from α-syn monomers, the in vitro quality control of the PFFs once formed, the sonication and measurement of PFFs prior to use, and suggestions to facilitate successful in vivo injection of PFFs into rats or mice.

PROTOCOL:

All methods involving animals have been approved by the Michigan State University Institutional Animal Care and Use Committee (IACUC).

1. Formation of α-synuclein preformed fibrils from monomers (Figure 1)

1.1 Thaw α-synuclein monomers on ice, gently resuspend by flicking the tube, and centrifuge at 15,000 x *g* for 10 min at 4 °C.

NOTE: The α-syn monomer must be specifically formulated for fibrilization. Recombinant monomers can be purchased from commercial sources or generated by protocols on site[4,10,15-16]. If purchased from commercial sources, the product must state that the α-syn monomer is specifically for the generation of fibrils. Regardless of if the monomers are purchased or generated on site, the quality control steps outlined below should be performed with each batch to ensure fibrils have formed and will seed efficiently prior to use in experiments.

1.2 Transfer supernatant to a clean 1.5 mL microcentrifuge tube and record the amount transferred.

NOTE: Be careful to avoid the pellet, which, if present, will be small.

1.3 Measure the protein concentration of the transferred supernatant by either a standard bicinchoninic acid assay (BCA), or measuring the absorbance at 280 nm with a microvolume spectrophotometer.

NOTE: The BCA assay is not as accurate for the specific measurement of α-syn and can yield results overestimating protein concentration. As a result, measuring the absorbance at 280 nm is the recommended method for determining protein concentration.

1.3.1.1 To measure with the BCA assay, follow standard BCA protocols and perform with three different dilutions of α-syn monomer. Suggested dilutions are 1:25, 1:50, and 1:100.

1.3.2.1 To measure with the A280 method, blank the reader with sterile 1x Dulbecco's phosphate buffered saline (dPBS) without calcium and magnesium, with a salt concentration of approximately 100 mM NaCl, and pH range 7.0-7.3 (**Table of Materials**).

1.3.2.2 Add 2 µL of sample to the reader and read the absorbance at 280 nm.

1.3.2.3 Use the Beer-Lambert law to determine concentration of the monomers.

$$Concentration = \left(\frac{Absorbance\ at\ 280\ nm}{(Extinction\ coefficient\ \varepsilon \times path\ length)}\right) \times Molecular\ weight$$

NOTE: Extinction coefficient ε for human α-syn is 5,960 $M^{-1}cm^{-1}$ and for mouse α-syn is 7,450 $M^{1}cm^{-1}$. Pathlength is measured in cm. Molecular weight of α-syn (14 kDa) is estimated assuming 1 Da = 1 g/mol.

1.4 Dilute the monomers with 1x dPBS to a final concentration of 5 mg/mL. Use the equation below to calculate the amount of 1x dPBS added to dilute the monomers.

$$Volume\ of\ 1x\ dPBS\ added\ to\ monomers = \left(\frac{Monomer\ concentration \times Volume\ of\ monomers}{5\ mg/ml}\right) - Volume\ of\ monomers$$

NOTE: All concentrations are in mg/mL, and volumes in µL. Monomer dilutions should be performed with 1x dPBS. Total volume used to generate fibrils should be between 100 and 500 µL to achieve reproducible fibrilization results.

1.5 Briefly vortex to mix, and centrifuge to collect all liquid at the bottom of the tube. Aliquot monomers for quality control comparison with fibrils as indicated in steps 1.8.1 – 1.8.4.

1.6 Use a tube lock or wax/plastic film (**Table of Materials**) to secure the microcentrifuge tube lid closed.

1.7 Place the tube in an orbital thermomixer with a lid for 7 days at 37 °C, shaking at 1,000 RPM (**Table of Materials**).

NOTE: At the end of the 7 days, the contents of the tube should appear turbid. The thermomixer must have a lid to prevent condensation formation on the tube lids.

1.8 Gently flick the tube to resuspend the α-syn fibrils. Aliquot fibrils for the following quality control steps as indicated in steps 1.8.1 – 1.8.4.

NOTE: Fibrils for quality control steps can be stored at RT overnight.

1.8.1 Aliquot 6 µL for the sedimentation assay.

1.8.2 Aliquot 5 µL for the thioflavin T binding assay.

1.8.3 Aliquot 2 µL for the transmission electron microscopy for fibril visualization.

1.8.4 Aliquot at least 10 µL for the endotoxin assay.

NOTE: For endotoxin assay, a commercial Limulus Amebocyte Lysate (LAL) is used, following manufacturer's instructions (**Table of Materials**). Endotoxin levels should be ≤0.5 endotoxin units/mL for the fibrils. An endotoxin removal kit can be used if this criterion is not met.

1.9 Aliquot the remaining fibrils and store. For long term storage (12-18 months), rapidly freeze on dry ice, and store at -80 °C.

NOTE: The amount to aliquot depends on the desired downstream application. In vivo use typically requires more fibrils at higher concentrations than in vitro use, and aliquot volumes should be planned accordingly. Fibrils should be stored towards the back of the freezer to prevent damage from potential freeze/thaw. The protocol can be paused here.

2. Sedimentation assay

2.1 In a clean microcentrifuge tube, add 6 µL of PFF to 54 µL of dPBS to dilute fibrils 1:10, and pipet to mix.

2.1.1 In a separate tube, add 6 µL of monomer to 54 µL of dPBS as an additional control.

2.2 Centrifuge samples at 10,000 x *g* for 30 min at RT and transfer the supernatant to a clean microcentrifuge tube.

2.3 Add 60 µL of dPBS to the remaining pellet and vortex to resuspend.

2.4 Add 30 µL of 3x sample buffer (140 µL of 50% glycerol/0.1% bromophenol blue, 40 µL of 10% SDS, and 20 µL of β-mercaptoethanol) to each tube and vortex to mix.

2.5 Incubate samples at 100 °C for 10 min and allow to cool for 5 min on ice.

2.6 Use a standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protocol to separate protein by mass. Use a protein ladder with a range that includes 14 kDa (the size band expected for monomeric α-syn).

2.7 Transfer the gel into a staining dish and cover the gel with Coomassie blue stain (0.1% Coomassie brilliant blue, 20% methanol by volume, and 10% acetic acid by volume in ddH_2O). Incubate for 3 h at RT while shaking.

2.8 Pour off the Coomassie blue stain and add enough destain (50% methanol by volume, and 10% acetic acid by volume in ddH_2O) to cover the gel. Incubate for 30 min at RT while shaking.

2.9 Pour off destain and repeat the destain step until the gel is clear and bands are visible.

2.10 Wash the gel 3 times for 5 min each by shaking in ddH_2O and image the gel.

3. Transmission electron microscopy for fibril visualization

3.1 Weigh 20 mg of uranyl acetate in a microcentrifuge tube and add 1 mL of ddH_2O to make a 2% aqueous solution. Vortex until the uranyl acetate is in solution, and allow to sit overnight.

NOTE: Uranyl acetate should be made the day before preparing samples for transmission electron microscopy. Exposure to light or agitation can cause the uranyl acetate to precipitate, as such, the tube containing the uranyl acetate solution should be covered in aluminum foil, kept in a dark place, and not shaken after the uranyl acetate is in solution.

3.2 Add 2 µL of PFF to 98 µL of dPBS to dilute fibrils 1:50, and then pipet to mix.

3.3 Prepare a clean wax/plastic film (**Table of Materials**) covered surface (approximately 50 mm x 50 mm) on the bench top. On the clean wax/plastic film (**Table of Materials**), add the following (**Figure 2**).

3.3.1 Add 4 x 10 µL drops of ddH_2O.

3.3.2 Add 1 x 10 µL drop of diluted fibrils or monomer sample.

3.3.3 Add 2 x 10 µL drops of 2% uranyl acetate.

3.4 Use fine-tipped forceps to pick up a formvar/carbon coated, mesh copper transmission electron microscopy grid (**Table of Materials**).

NOTE: Be sure to only pick up the grid by the edge, avoiding the mesh portion in the center (**Figure 2A**). If the forceps touch the mesh, the formvar/carbon coated film will be damaged, making imaging in that area of the grid impossible.

3.5 Float the grid formvar/carbon coated side (shiny side) down on the first drop of ddH_2O. Gently use the forceps to hold the grid and push down to ensure the entire coated surface is in contact with the ddH_2O. Let the grid float for 1 min.

3.6 Pick up the grid, and without touching the mesh portion of the grid, wick away the ddH_2O with filter paper.

3.7 Float the grid as above on the second drop of ddH_2O for 1 min and wick away the ddH_2O.

3.8 Float the grid on the drop of diluted fibrils for 1 min and wick away the excess as above.

3.9 Float the grid on the first drop of uranyl acetate for 1 min and wick away the excess.

3.10 Float the grid on the second drop of uranyl acetate for 1 min, wick away the excess.

3.11 Float the grid as above on the third drop of ddH_2O briefly and wick away the ddH_2O.

3.12 Float the grid as above on the fourth drop of ddH_2O briefly and wick away the ddH_2O, being sure to remove as much ddH_2O as possible.

3.13 Transfer to a grid box for storage until imaging.

NOTE: Grids should dry for at least 5 min before imaging. Grids can be imaged for at least a year after preparation and should be kept in a dry environment.

4. Thioflavin T assay

4.1 In a clean microcentrifuge tube, add 5 µL of PFF to 245 µL of dPBS to dilute fibrils 1:50, and pipet to mix.

4.2 Add 250 µL of dPBS to a separate microcentrifuge tube to serve as a negative control.

4.3 Add 5 µL of monomer to 245 µL of dPBS to serve as the monomer control.

4.4 Add 250 µL of thioflavin T in glycine buffer (25 µM thioflavin T, 100 mM glycine, 1% Triton X-100, pH 8.5) to each sample and gently mix.

4.5 Pipet 2 replicates, 200 µL each, into a black 96 well plate. Keep plate in the dark to prevent photobleaching.

4.6 Incubate for 1 h at RT and read the plate using an excitation of 450 nm and emission of 510 nm.

NOTE: Thioflavin T readings will fluctuate over time. Samples should be read at the same incubation time. If desired, multiple readings can be taken during the hour incubation and plotted over time.

5. Sonication of α-synuclein preformed fibrils

CAUTION: The sonicator and all sonication steps are performed in a culture hood to prevent exposure to fibrils that may aerosolize during sonication. The personnel performing the sonication steps should wear personal protective equipment, including gloves, clothing protection in the form of a lab coat, and a face shield while sonicating. Risk of fibril exposure can be reduced by sonicating with a cup horn sonicator, allowing the tube containing fibrils to remain closed during sonication.

NOTE: Optimal sonication parameters of fibrils are dependent on the model of sonicator used. For this reason, some optimization will need to be performed to ensure fibrils are the correct size. The sonicator used can be found in the **Table of Materials** and the parameters outlined are based on previous results with this model of sonicator. The parameters below will work for 2-4 µg/µL of PFFs in 200-400 µL of solution. Test sonication with the instrument should be performed and fibrils analyzed to ensure desired results are achieved prior to use of PFFs in experiments.

5.1 Attach a 3.2 mm diameter probe (**Table of Materials**) to the converter, and set the sonicator parameters as stated below in step 5.1.1 - 5.1.3.

5.1.1 Set amplitude to 30%.

5.1.2 Set the pulse to 01 01 (1 s on; 1 s off).

5.1.3 Set the time to 0:01:00.

NOTE: 1 min of pulsing equates to 60 pulses, and this model of sonicator (**Table of Materials**) will stop automatically. With other sonicator models, the number of pulses will need to be counted.

5.2 Thaw fibrils at RT and dilute with sterile dPBS in a culture hood.

NOTE: A clear 0.6 mL microcentrifuge tube works best for sonication and the final fibril concentration depends on intended use. Representative results shown are from a final fibril concentration of 4 µg/µL.

5.3 Wipe the probe of the sonicator with a lab tissue dampened with 70% ethanol to clean the probe. Submerge the tip of the probe in ddH_2O and pulse 10 times to further clean the probe, and then wipe dry with a lab tissue.

5.4 Place the probe tip into the tube of diluted fibrils, and position the tip at the bottom of the tube.

5.5 Sonicate for 60 pulses (1 s on; 1 s off). Move the probe up and down during each pulse to ensure all of the fibrils in the liquid are sonicated. and clean.

5.6 After 60 pulses, remove the probe from the PFFs, and add 2 µL of PFF to 98 µL of dPBS in a clean microcentrifuge tube to dilute fibrils 1:50 for sonicated fibril measurement by electron microscopy.

NOTE: Ideally, a small subset of fibrils should be measured prior to in vivo injection and a more comprehensive measurement of fibrils performed when time permits.

5.7 After sonication, briefly centrifuge PFFs for 1 s at 2,000 x *g* to collect all liquid off the sides of the tube.

CAUTION: If the tube becomes hot to the touch, stop sonicating after 30 pulses, wait 1 min, and sonicate for the final 30 pulses.

NOTE: While sonicating, keep the probe tip towards the bottom of the liquid at the start of each pulse, too close to the top of the liquid will cause sample loss.

5.8 Submerge the probe tip in 1% SDS and pulse 10 times to clean the probe. Remove the tip from the SDS, submerge in ddH_2O and pulse 10 times.

5.9 Wipe the probe with a lab tissue dampened with 70% ethanol, and then wipe the probe with a dry lab tissue. Detach the probe from the converter and store.

5.10 Wipe down all surfaces in the hood with 1% SDS, followed by 70% ethanol.

NOTE: The 1% SDS solution is used to dissociate fibrils and clean surfaces and equipment[17].

5. Transmission electron microscopy for the measurement of sonicated fibrils

NOTE: If electron microscopy is not feasible, a thioflavin T kinetics assay, and dynamic light scattering can be used as indirect measures of seeding efficiency and fibril size[4,15].

6.1 Prepare samples using the protocol from the "Electron microscopy for fibril visualization" section above.

6.2 Use a transmission electron microscope to take an image of fibrils from 6 to 10 different grid openings.

NOTE: Images should be a high enough magnification to measure fibrils, but low enough to visualize multiple fibrils simultaneously. A final magnification of approximately 75,000x should be sufficient.

6.3 Measure the length of a small subset of at least 25 fibrils prior to using fibrils in an experiment.

NOTE: These measurements can typically be performed using the imaging software associated with the microscope. For the quick validation, the person measuring should select representative fibrils and calculate the average size. Fibril length should average around 50 nm or less, with a more accurate measurement to follow.

6.4 Measure the lengths of 500+ fibrils for more comprehensive results.

NOTE: The imaging software associated with the microscope can be used for fibril measurements. Alternatively, images can be opened and fibrils measured with image processing software, using the scale bar associated with each image as a size reference with which to compare the fibril lengths.

6. Preparation of custom glass needle syringes for stereotactic injections (Figure 3)

7.1 Add approximately 10 mL of siliconizing reagent (**Table of Materials**) to a clean 50 mL beaker.

7.2 Place glass capillary tubes (length of 54 mm; outer diameter: 0.86 mm; inner diameter 0.59 mm) vertically in the beaker of siliconizing reagent and allow capillary action to draw the siliconizing reagent up the tube through the lower tube opening submerged in the siliconizing reagent.

7.3 Pipet additional siliconizing reagent into the upper tube opening that is not submerged in siliconizing reagent to completely fill the glass capillary tube.

7.4 Remove the capillary tubes from the siliconizing reagent and blot the open ends of the tubes on a paper towel to remove siliconizing reagent in the tubes. Allow capillary tubes to dry for at least 8 hours.

7.5 Place a siliconized glass capillary tube in a glass needle puller (**Table of Materials**).

7.6 Turn on the heating element and allow the attached weights to stretch the heated glass capillary tube.

7.7 Cut the pulled glass capillary tube with scissors at the thinnest point in the middle and remove the glass needle from the glass needle puller.

NOTE: The pulled needle inner and outer diameter should be approximately 80 and 100 µm, respectively. Multiple glass needles can be made at one time and stored until ready to attach to a glass syringe with attached metal needle (**Table of Materials**).

7.8 Cut a length of shrink-wrap tubing (average inner diameter 0.021” and average wall thickness 0.001”) to approximately 40 mm with scissors. Slide the shrink wrap over the metal needle of a 10 µL beveled syringe (**Table of Materials**).

7.9 Use an open flame to heat and adhere the shrink wrap to the needle while rotating the needle to apply heat evenly.

7.10 Slide the larger end of the pulled glass needle carefully over the metal needle of the syringe.

7.11 Cut a length of shrink-wrap tubing (average inner diameter 0.036” and average wall thickness 0.005”) to approximately 40 mm with scissors and carefully slide over glass needle to overlap the base of the glass needle and the metal needle of the syringe (**Table of Materials**). Use an open flame to heat the shrink-wrap to secure the glass needle to the metal needle.

7.12 Add an additional layer of shrink-wrap to secure the glass needle. Cut a length of shrink-wrap tubing (average inner diameter 0.044" and average wall thickness 0.005") to approximately 40 mm with scissors and carefully slide over glass needle to overlap the base of the glass needle and the metal needle of the syringe (**Table of Materials**). Use an open flame to heat the shrink-wrap to secure the glass needle to the metal needle.

7.13 Trim the glass needle with scissors so that the tip is approximately 8 mm long.

NOTE: The needle needs to be long enough to target the desired brain regions (required length is dependent on the dorsal/ventral coordinates).

7.14 Use steps 7.14.1 - 7.14.3 to test the needle to insure there are no leaks and there is adequate flow both in withdrawing and dispensing liquid from the glass needle.

7.14.1 Fill a 1 mL syringe with an attached 26 gauge needle with dH_2O.

7.14.2 Remove the metal plunger from the custom glass needle syringe and insert the needle of the dH_2O filled syringe into the base of the syringe. Apply pressure to dispense dH_2O from the glass needle. Inspect the interface of the glass needle and the metal needle for leaks and confirm a steady dH_2O flow.

NOTE: If needed, the glass needle can be trimmed to increase the ease of flow and additional layers of shrink-wrap added to patch any leaks from the base of the glass needle.

7.14.3 Fill a microcentrifuge tube with dH_2O. Use the custom glass needle syringe to draw in the dH_2O. Inspect the needle to confirm liquid is being taken into the syringe and that there are no bubbles.

NOTE: If there are bubbles or dH_2O is not being drawn into the needle, trimming the needle can help alleviate pressure.

7.15 Carefully store syringe with attached glass needles in the syringe boxes until needed for surgeries.

7.116 Use standard stereotaxic surgery methods for intrastriatal delivery of PFFs at optimized coordinates in mice (one site: AP +0.2 mm and ML +2.0 mm from bregma, DV -2.6 mm from dura) or in rats (two sites: AP +1.6 mm and ML +2.0 mm from bregma, DV -4.0 from dura; AP +0.1 mm and ML +4.2 mm from bregma, DV -5.0 from dura)[1,7,15,18].

NOTE: These coordinates have been used in C57BL6/C3H mice and Fischer 344 rats. When using other strains, coordinates should be optimized.

REPRESENTATIVE RESULTS:

Generation of fibrils from α-syn monomers begins with determining the concentration of the monomers. Both the BCA assay and measurement of absorbance at 280 nm (A280) can be used to measure protein content; the BCA assay results, however, suggested a higher concentration than the A280 method. PFFs derived from mouse α-syn monomer had a BCA value of 14.05 ± 0.22 and a A280 of 8.05 ± 0.03 µg/µL (**Figure 1**). Likewise, PFFs derived from human α-syn monomer also appeared to be at a higher concentration, with a BCA value of 12.95 ± 0.38 and a A280 of 7.83 ± 0.05 µg/µL (**Figure 1**). The A280 measurements are specific to α-syn based on the inclusion of the extinction coefficients and these results were used to dilute the monomers prior to 7-day incubation.

Prior to incubation, the liquid containing the α-syn monomers was clear, but should appear turbid after fibril formation. Examination with transmission electron microscopy confirmed the presence of long fibrils, measuring 10-20 nm wide (**Figure 4**). In comparison, α-syn monomers were barely visible with no discernible shape apparent (**Figure 4**). With visual confirmation of fibrillar structures, amyloid conformation of the fibrils is the next feature of PFFs that should confirmed using a thioflavin T assay. Thioflavin T exhibits enhanced fluorescence when binding to amyloid; thus, increased fluorescent signal from the samples indicates presence of amyloid. As an example, thioflavin in dPBS produced a signal of 3,287 ± 580 relative fluorescent units (RFU), mouse α-syn monomer produced a signal of 4,174 ± 158 RFU, and mouse PFFs produced a signal of 59,754 ± 6,224 RFU (**Figure 5**). In comparison, human α-syn monomer produced a similar signal of 4,158 ± 105 RFU to mouse monomer, and human PFFs produced a higher signal of 1,235,967 ± 113,747 RFU as compared to mouse PFFs (**Figure 5**). To assess the presence of pelletable fibrils, a sedimentation assay was performed. Fibrils will pellet with centrifugation. In both the mouse and human PFF samples, the supernatant fraction should have more protein in the pellet than the supernatant (**Figure 6**). In contrast, the majority of the protein from the mouse and human monomers was present in the supernatant, with little present in the pellet (**Figure 6**). With the PFFs visibly present by electron microscopy, amyloid structures present, and fibrils pelletable, the PFFs passed all in vitro quality control steps.

Both mouse and human PFFs were sonicated to produce PFFs of appropriate lengths for seeding α-syn inclusions[4,19]. PFFs were diluted to the desired concentration of 4 µg/µL and sonicated. Immediately prior to surgery, 25 representative fibrils were imaged by electron microscopy and measured to spot check the fibril size. The sonicated mouse PFFs measured 48.8 ± 3.1 nm, whereas the human PFFs measured 52.1 ± 4.4 nm in length; PFFs of both species were therefore the appropriate length (50 nm or less) to induce seeding activity. More comprehensive examination of approximately 500 fibrils revealed the average length and length distribution of the sonicated mouse fibrils. The average length was 44.4 ± 0.6 nm, with 86.6% of the PFFs measuring 60 nm or less (**Figure 4**). In comparison, human PFFs averaged 55.9 ± 1.1 nm with 69.6% of the PFFs measuring 60 nm or less (**Figure 4**).

Following intrastriatal injection of sonicated mouse PFFs into rats as previously described[3,5,7], a series of tissue sections were processed at 2 months post-surgery, when the number of inclusion containing neurons is known to peak in the SNpc, for the confirmation of phosphorylated α-syn inclusions[5]. Inclusion bearing neurons, as indicated by immunohistochemical staining for pSyn (antibody in **Table of Materials**) are present within the SNpc (**Figure 7**), as well as other regions throughout the brain which innervate the striatum (anterior olfactory nucleus, motor, cingulate, piriform, prelimbic, somatosensory, entorhinal, and insular cortices, amygdala, striatum)[1,3-5,7,20]. These inclusions share similar properties with Lewy bodies, such as binding thioflavin S, and resistance of total α-syn to proteinase K (**Figure 7**), as shown by immunohistochemical staining (antibody and proteinase K in **Table of Materials**). Confirmation of seeding within the brain indicates the in vivo quality control has been passed, and aliquots of PFFs previously frozen and saved from the same batch may be sonicated under identical parameters, with lengths validated, in larger experiments.

FIGURE AND TABLE LEGENDS:

Figure 1. Methods for the generation of α-syn fibrils. Outline of the steps required to produce fibrils from α-syn monomers. Monomers are centrifuged for 10 min (15,000 x *g*, at 4 °C). Supernatant is transferred to a clean tube and the protein concentration is determined by either the absorbance at 280 nm, or a BCA assay. Graph shows concentrations from human and mouse α-syn monomers. Columns indicate the group means, error bars represent ±1 standard error of the mean. After protein concentration is determined, α-syn monomers are diluted, briefly vortexed and incubated for 37 °C for 7 days, while shaking on an orbital mixer set at 1,000 RPM.

Figure 2. Staining methods for transmission electron microscopy. Diagram of negative staining for electron microscopy. **A)** Images depicting electron microscopy specimen grids. The grid has a dull or light side and a shiny or dark side. The shiny/dark side is coated with a formvar/carbon support film. **B)** Illustration of staining procedure. The grid is floated shiny/dark side down on the first drop of ddH_2O for 1 min and the excess is wicked away with filter paper. The process is repeated with the second drop of ddH_2O, diluted PFFs or monomers, two drops of uranyl acetate, and two additional drops of ddH_2O. Grids may be stored in a grid box until imaged. Scale bar = 3 mm.

Figure 3. Assembly of custom glass needle syringes. Diagram of the steps required to assemble glass needle attached syringes. Siliconized glass capillary tubes are pulled and cut in the middle to produce glass needles. Shrink wrap tubing is used to prepare the metal needle and form an inner seal when the glass needle is slid onto the metal needle. Two additional layers of shrink-wrap tubing that overlap the base of the glass needle and the metal needle are consecutively added and heat applied to secure the glass needle and form a water-tight seal.

Figure 4. Visualization of α-syn monomers and α-syn fibrils via transmission electron microscopy. Representative micrographs of α-syn monomers and fibrils. Top panels: Mouse and human α-syn monomer. Middle panels: Full length mouse and human α-syn PFFs. Bottom panels: Mouse and human α-syn PFFs after sonication. Bottom graph: Distribution of sonicated mouse and human α-syn PFF lengths. Scale bar = 500 nm.

Figure 5. Confirmation of amyloid structures by thioflavin T assay. Measurement of fluorescent signal from mouse and human α-syn monomer and PFF samples. Left: Results from mouse α-syn monomers and α-syn PFFs. Right: Results from human α-syn monomers and α-syn PFFs. A dPBS negative control is shown in each graph. All measurements are expressed as relative fluorescent units (RFU). Columns indicate the group means, error bars represent ±1 standard error of the mean.

Figure 6. Sedimentation assay for pelletable α-syn. Images from Coomassie stained gels. Bands shown are at approximately 14 kDa based on the protein ladder. Left: Mouse monomer and PFFs. Right: Human monomers and PFFs. For all monomer and PFF samples, the resuspended pellet (P) and supernatant (S) are shown.

Figure 7. Features of inclusions in the rat model confirming α-syn pathology. Representative micrographs from the substantia nigra pars compacta at 2 months post-injection. Left: Neurons containing pSyn and counterstained with cresyl violet. Middle: Thioflavin S positive neurons. Right: α-syn-containing inclusions resistant to proteinase K. Scale bar = 50 µm.

DISCUSSION:

Production of α-syn PFFs capable of seeding neurons and leading to Lewy body-like inclusions is dependent on multiple factors and steps. A critical factor is that the monomers used for generating fibrils need to be specifically formulated for fibrilization[4,10,15-16]. If the monomers are not formulated for fibrilization, fibrils may not form or the fibrils that do form may not produce α-syn pathology. Likewise, the buffer that the monomers are in also influence fibrilization. As such, for the best results, the salt concentration should be approximately 100 mM NaCl and pH between 7.0 and 7.3. An initial step that introduces variability is the method whereby initial protein content is determined, with measurement at A280 likely to produce more accurate results and therefore is the preferred method. The discrepancy in protein concentration can decrease the efficacy of the fibrilization process, as well as alter the assumed PFF concentration used in experiments. Both could lead to a decrease in seeding efficiency and batch variation between experiments.

Initial quality control steps will confirm critical features of the PFFs, specifically that they have a fibrillary conformation (electron microscopy), contain amyloid structures (thioflavin T assay), and are pelletable (sedimentation assay). It is important to note that the results of the thioflavin T assay will fluctuate with time and are not a direct measure of the amount of amyloid structures present, rather, the thioflavin T assay should be used only as an indicator of amyloid structures presence within the sample. Thioflavin T is typically used in in vitro assays, such as the aforementioned assay to show the fibrils contain amyloid structures. Alternatively, thioflavin S is used in tissue to detect amyloid structures, as shown in **Figure 7**. In regards to the sedimentation assay, the results show only that the PFFs are found predominantly in the pelletable fraction. As the samples are run in denaturing conditions, a single prominent band of approximately 14 kDa, the size of α-syn monomers, is present on the gels. This is unlike the multiple bands present at higher molecular weights that would be expected with PFFs if a native or non-denaturing gel was used. Lastly, successful passing of all these initial quality control steps does not guarantee α-syn inclusion seeding activity. For this reason, cell culture experiments or a small cohort of surgically-injected animals should be used to test the efficacy of the PFFs before use in larger experiments.

Sonication is a crucial step in the process and parameters will differ depending on the model of sonicator used. Sonication parameters must be applied and verified to show that short PFF fragments have been produced. Fibril size has bearing on the seeding, with shorter fibrils seeding more efficiently. Though shorter fibrils seed more efficiently, this effect plateaus and the optimal PFF length is approximately 50 nm[4,19]. It is also important not to over-sonicate the samples and expose the PFFs to excessive heat, as this may decrease seeding efficiency. These sonicated PFFs should be tested for efficacy in small cell culture or in vivo experiments prior to use in larger scale experiments. As different sonication sessions have the potential to introduce variability, experimental treatment groups should be planned accordingly.

When delivering PFFs in vivo, the localization of the injection site(s) and the total amount PFFs used can affect the number of neurons that will develop inclusions as well as the extent of neurodegeneration[7,15]. The coordinates in the protocol provide a place to start, but should be tested within the lab to ensure the desired target region develops α-syn pathology prior to use in large scale experiments. If desired, tracking dye or fluorescent beads can be used as a way to test regional targeting before using PFFs. The amount of PFFs used in vivo varies between groups, with most groups using a total between 5 to 20 µg of PFFs at one or divided between two injection sites[1-8, 15]. As the number of injection sites, location of injection sites, and amount of PFFs injected can effect results and progression of the synucleinopathy, the downstream outcomes of the parameters used should be characterized prior to using the model to test potential interventions or examining temporal features of the model.

When selecting a model to use for testing therapeutics or studying disease progression, the model used should be selected to best answer the question asked. Not all models will possess certain disease features of PD, or offer the timeframe needed to test potential interventions. The PFF model recapitulates key features of PD, such as α-syn pathology and neurodegeneration, and can lead to modest motor impairments. The model offers a predictable and protracted time-course, where inclusions form months before neurodegeneration. This allows researchers to examine and exploit the different phases throughout the protracted progression of the synucleinopathy. The current and future use of the model overall is expected to be beneficial in the study of disease progression and development of novel therapies.

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DISCLOSURES:
Joseph Patterson, Kelvin Luk, and Caryl Sortwell are currently involved in contractual arrangements with the Michael J. Fox Foundation to quality control α-syn material generated by Proteos, Inc. Coauthor Nicole Polinski, is an employee of the Michael J. Fox Foundation, which has contracted Proteos, Inc. to produce α-syn monomer. Coauthors Megan Duffy, Laura Volpicelli-Daley, and Nicholas Kanaan have nothing to disclose.

REFERENCES:

1 Luk, K. C. et al. Pathological alpha-synuclein transmission initiates Parkinson-like neurodegeneration in nontransgenic mice. *Science.* **338**, 949-953, doi:10.1126/science.1227157 (2012).

2 Luk, K. C. et al. Molecular and Biological Compatibility with Host Alpha-Synuclein Influences Fibril Pathogenicity. *Cell Reports.* **16**, 3373-3387, doi:10.1016/j.celrep.2016.08.053 (2016).

3 Paumier, K. L. et al. Intrastriatal injection of pre-formed mouse alpha-synuclein fibrils into rats triggers alpha-synuclein pathology and bilateral nigrostriatal degeneration. *Neurobiology of Disease.* **82**, 185-199, doi:10.1016/j.nbd.2015.06.003 (2015).

4 Abdelmotilib, H. et al. alpha-Synuclein fibril-induced inclusion spread in rats and mice correlates with dopaminergic Neurodegeneration. *Neurobiology of Disease.* **105**, 84-98, doi:10.1016/j.nbd.2017.05.014 (2017).

5 Duffy, M. F. et al. Lewy body-like alpha-synuclein inclusions trigger reactive microgliosis prior to nigral degeneration. *Journal of Neuroinflammation.* **15**, 129, doi:10.1186/s12974-018-1171-z (2018).

6 Duffy, M. F. et al. Quality Over Quantity: Advantages of Using Alpha-Synuclein Preformed Fibril Triggered Synucleinopathy to Model Idiopathic Parkinson's Disease. *Frontiers in Neuroscience.* **12**, 621, doi:10.3389/fnins.2018.00621 (2018).

~~7 Patterson et al. Time course and magnitude of alpha-synuclein inclusion formation and nigrostriatal degeneration in the rat model of synucleinopathy triggered by intrastriatal α-synuclein preformed fibrils. *Submitted 2019*~~

8 Luk, K. C. et al. Exogenous alpha-synuclein fibrils seed the formation of Lewy body-like intracellular inclusions in cultured cells. *Proceedings of the National Academy of Sciences of the United States of America.* **106**, 20051-20056, doi:10.1073/pnas.0908005106 (2009).

9 Volpicelli-Daley, L. A. et al. Exogenous alpha-synuclein fibrils induce Lewy body pathology leading to synaptic dysfunction and neuron death. *Neuron.* **72**, 57-71, doi:10.1016/j.neuron.2011.08.033 (2011).

10 Volpicelli-Daley, L. A., Luk, K. C., Lee, V. M. Addition of exogenous alpha-synuclein preformed fibrils to primary neuronal cultures to seed recruitment of endogenous alpha-synuclein to Lewy body and Lewy neurite-like aggregates. *Nature Protocols.* **9**, 2135-2146, doi:10.1038/nprot.2014.143 (2014).

11 Kuusisto, E., Salminen, A., Alafuzoff, I. Ubiquitin-binding protein p62 is present in

neuronal and glial inclusions in human tauopathies and synucleinopathies. *Neuroreport.* **12**, 2085-2090 (2001).

12 Neumann, M. et al. Misfolded proteinase K-resistant hyperphosphorylated alpha-synuclein in aged transgenic mice with locomotor deterioration and in human alpha-synucleinopathies. *The Journal of Clinical Investigation.* **110**, 1429-1439, doi:10.1172/JCI15777 (2002).

13 Li, J. Y. et al. Characterization of Lewy body pathology in 12- and 16-year-old intrastriatal mesencephalic grafts surviving in a patient with Parkinson's disease. *Movement disorders : official journal of the Movement Disorder Society.* **25**, 1091-1096, doi:10.1002/mds.23012 (2010).

14 Shimozawa, A. et al. Propagation of pathological alpha-synuclein in marmoset brain. *Acta Neuropathologica Communications.* **5**, 12, doi:10.1186/s40478-017-0413-0 (2017).

15 Polinski, N. K. et al. Best Practices for Generating and Using Alpha-Synuclein Pre-Formed Fibrils to Model Parkinson's Disease in Rodents. *Journal of Parkinson's Disease.* **8**, 303-322, doi:10.3233/JPD-171248 (2018).

16 Fares, M. B. et al. Induction of de novo alpha-synuclein fibrillization in a neuronal model for Parkinson's disease. *Proceedings of the National Academy of Sciences of the United States of America.* **113**, E912-921, doi:10.1073/pnas.1512876113 (2016).

17 Fenyi, A., Coens, A., Bellande, T., Melki, R., Bousset, L. Assessment of the efficacy of different procedures that remove and disassemble alpha-synuclein, tau and A-beta fibrils from laboratory material and surfaces. *Scientific Reports.* **8**, 10788, doi:10.1038/s41598-018-28856-2 (2018).

18 Geiger, B. M., Frank, L. E., Caldera-Siu, A. D., Pothos, E. N. Survivable stereotaxic surgery in rodents. *Journal of Visualized Experiments.* doi:10.3791/880 (2008).

19 Tarutani, A. et al. The Effect of Fragmented Pathogenic alpha-Synuclein Seeds on Prion-like Propagation. *Journal of Biological Chemistry.* **291**, 18675-18688, doi:10.1074/jbc.M116.734707 (2016).

20 Wall, N. R., De La Parra, M., Callaway, E. M., Kreitzer, A. C. Differential innervation of direct- and indirect-pathway striatal projection neurons. *Neuron.* **79**, 347-360, doi:10.1016/j.neuron.2013.05.014 (2013).

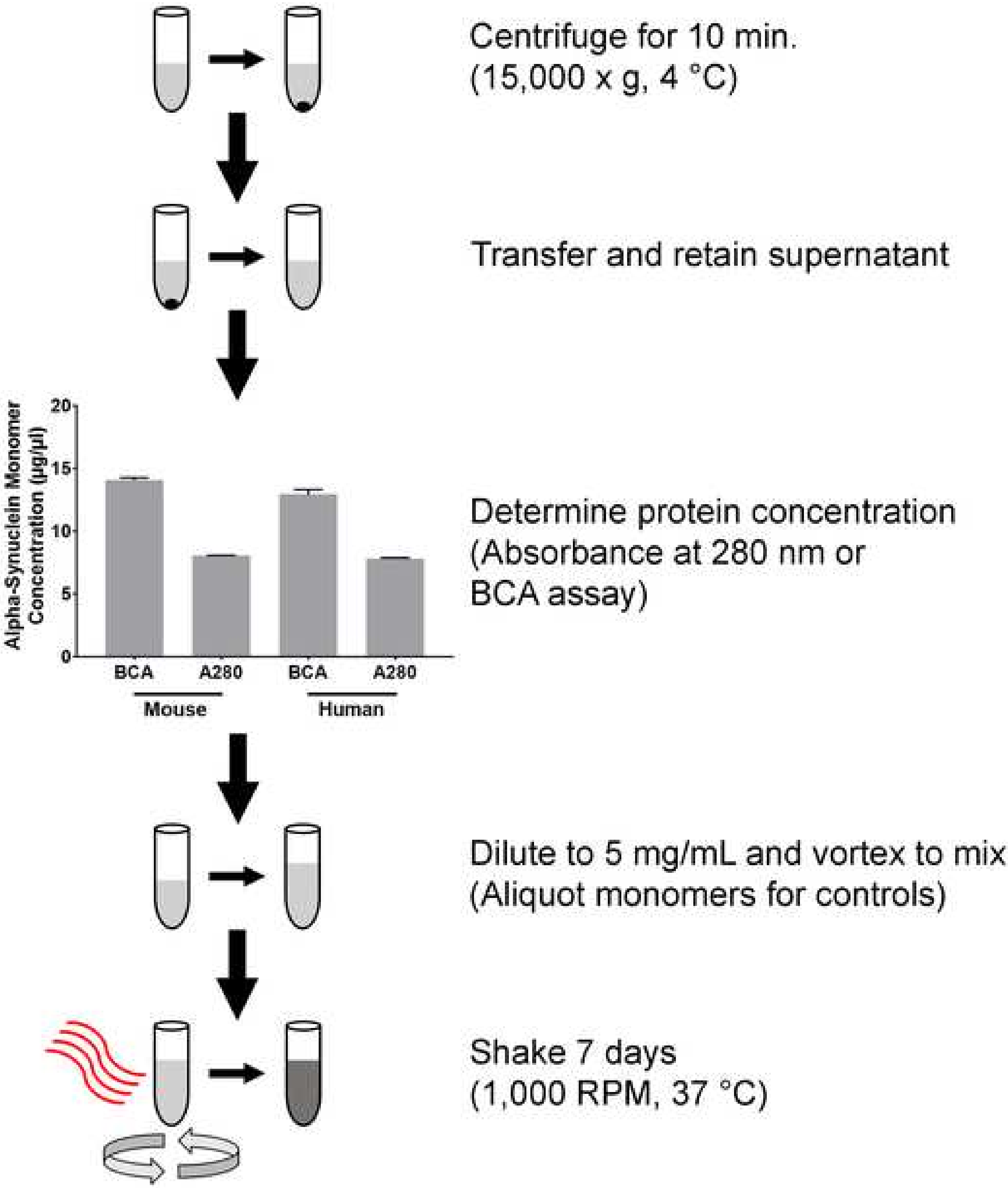

Centrifuge for 10 min.
(15,000 x g, 4 °C)
Transfer and retain supernatant
Alpha-Synuclein Monomer Concentration (µg/µl)
20
15
10
5
0
BCA
A280
BCA
A280
Mouse
Human
Determine protein concentration
(Absorbance at 280 nm or
BCA assay)
Dilute to 5 mg/mL and vortex to mix
(Aliquot monomers for controls)
Shake 7 days
(1,000 RPM, 37 °C)

Figure 2

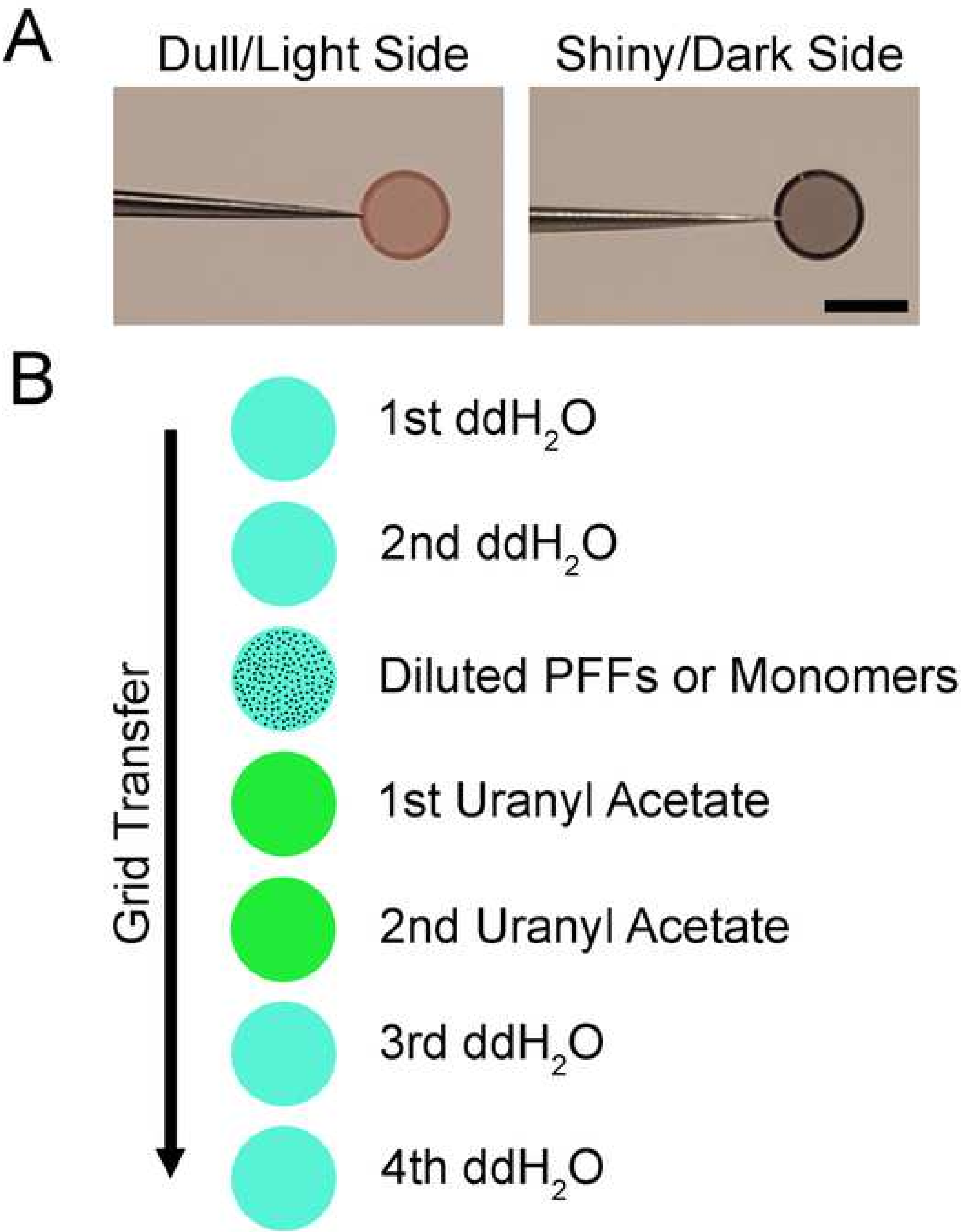
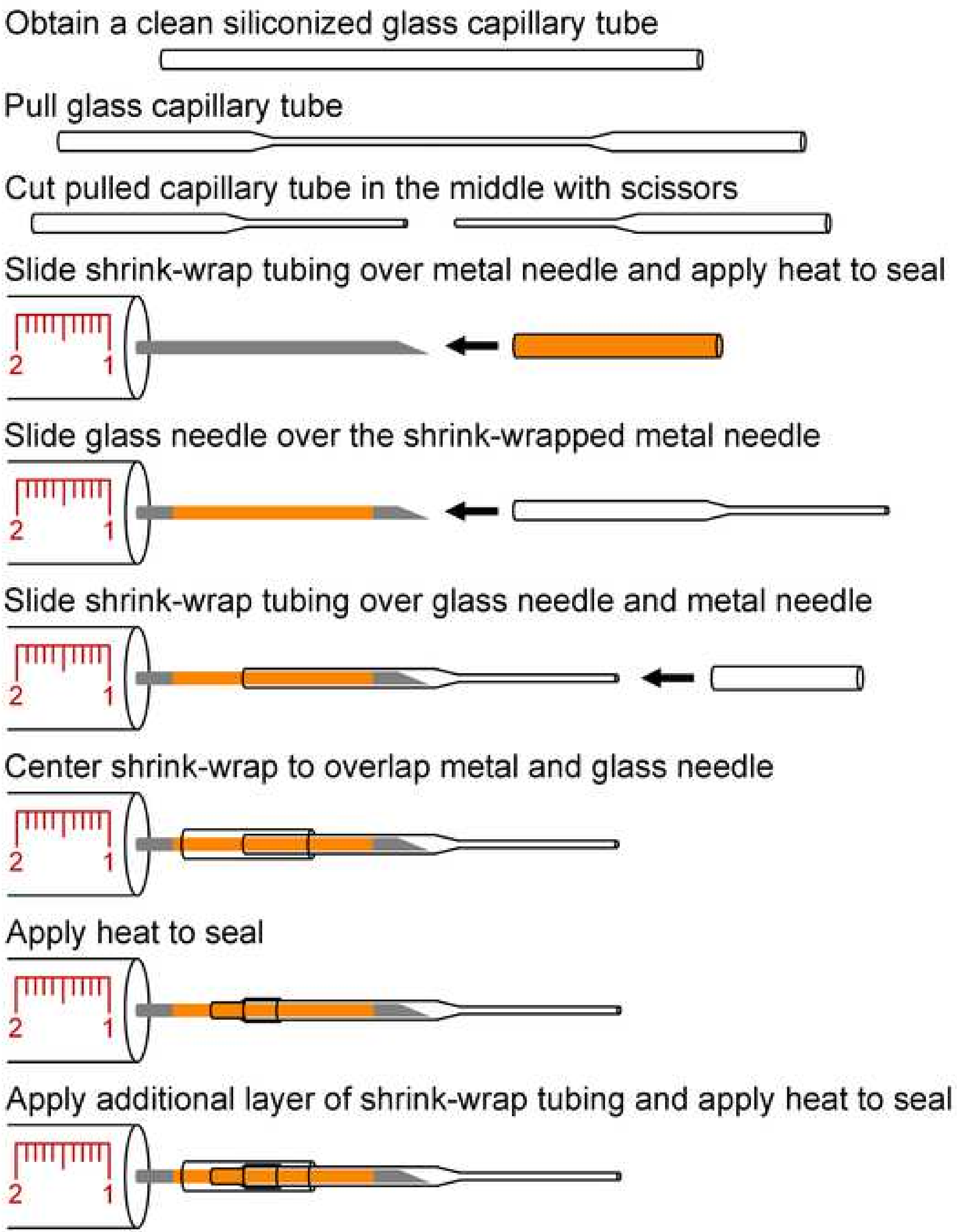

A
Dull/Light Side
Shiny/Dark Side
B
Grid Transfer
1st ddH2O
2nd ddH2O
Diluted PFFs or Monomers
1st Uranyl Acetate
2nd Uranyl Acetate
3rd ddH2O
4th ddH2O

Figure 3 Click here to access/download;Figure;JoVE figure 3.tif



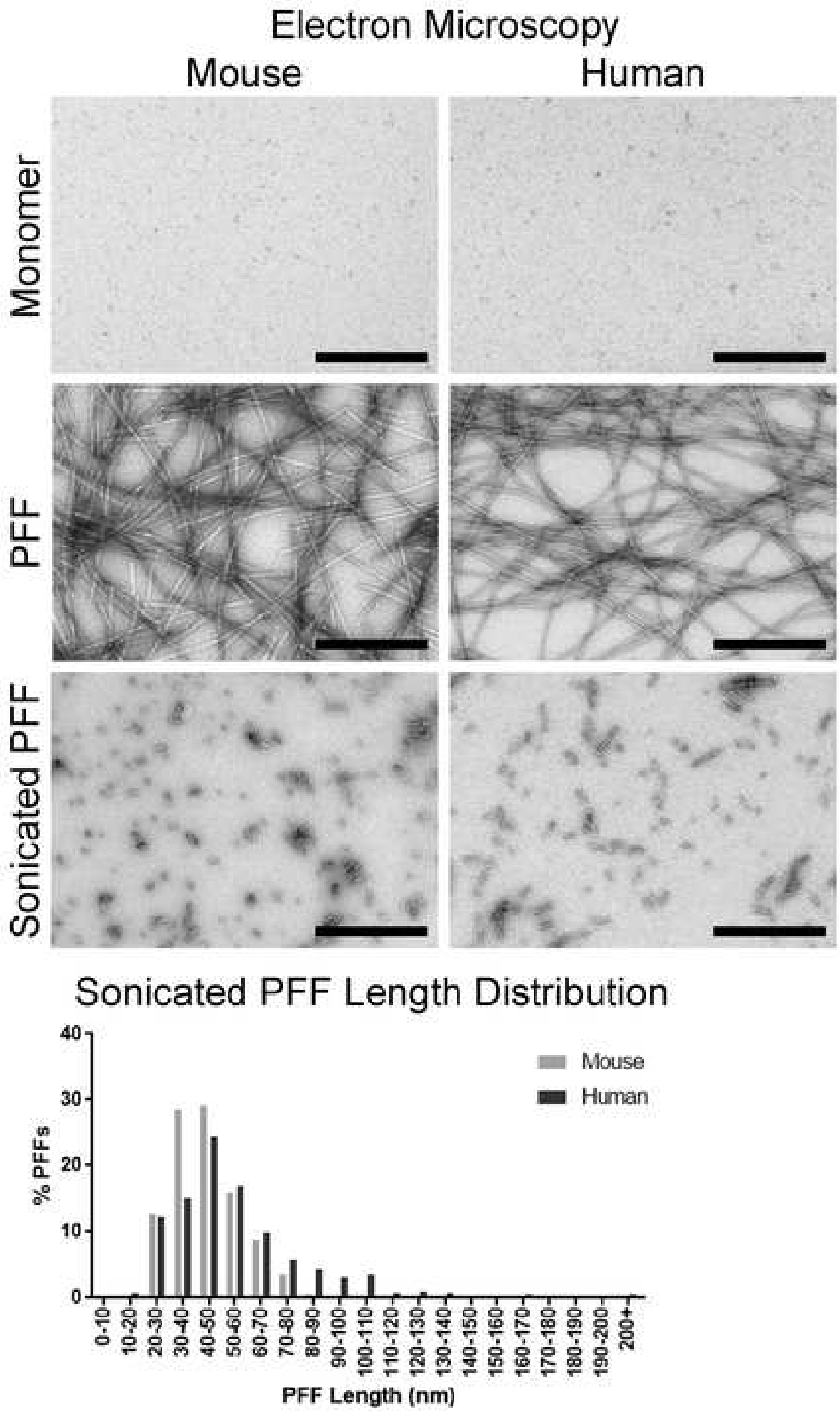

Electron Microscopy
Mouse
Human
Monomer
PFF
Sonicated PFF
Sonicated PFF Length Distribution
% PFFs
40
30
20
10
0
Mouse
Human
0-10
10-20
20-30
30-40
40-50
50-60
60-70
70-80
80-90
90-100
100-110
110-120
120-130
130-140
140-150
150-160
160-170
170-180
180-190
190-200
200+
PFF Length (nm)

Figure 5 [Click here to access/download;Figure;JoVE figure 5.tif]

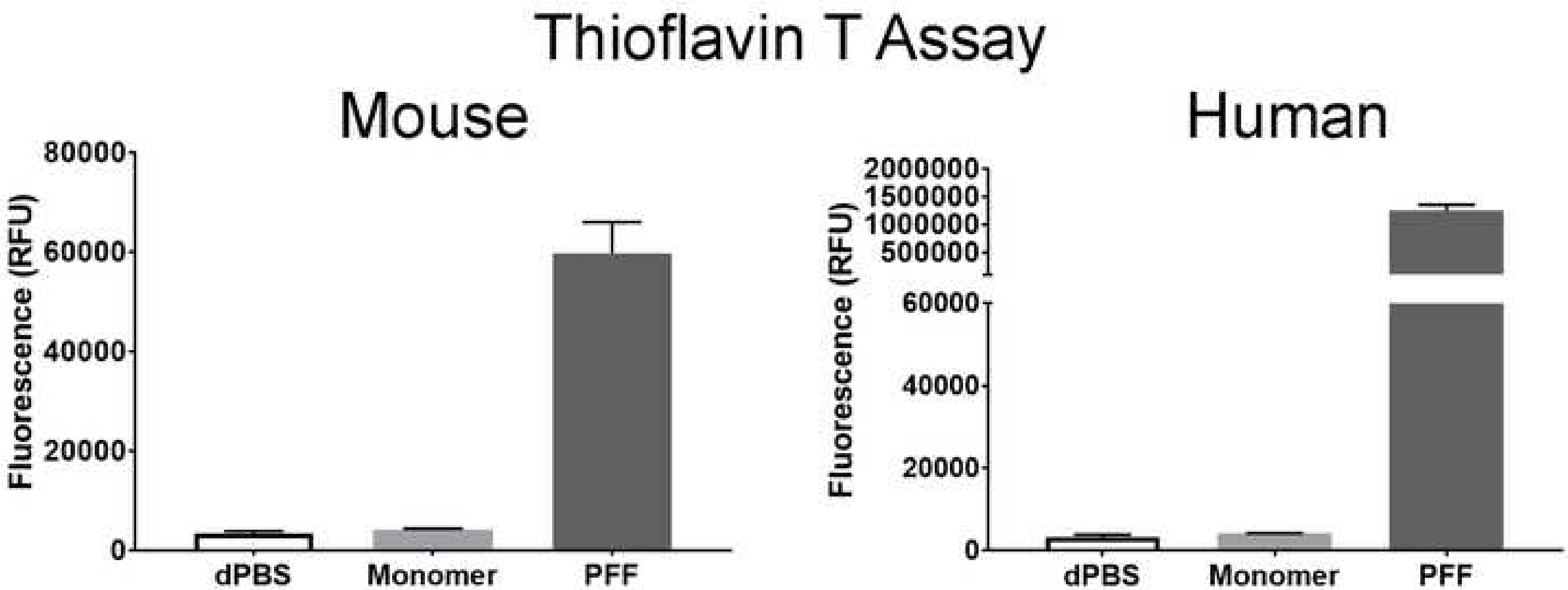
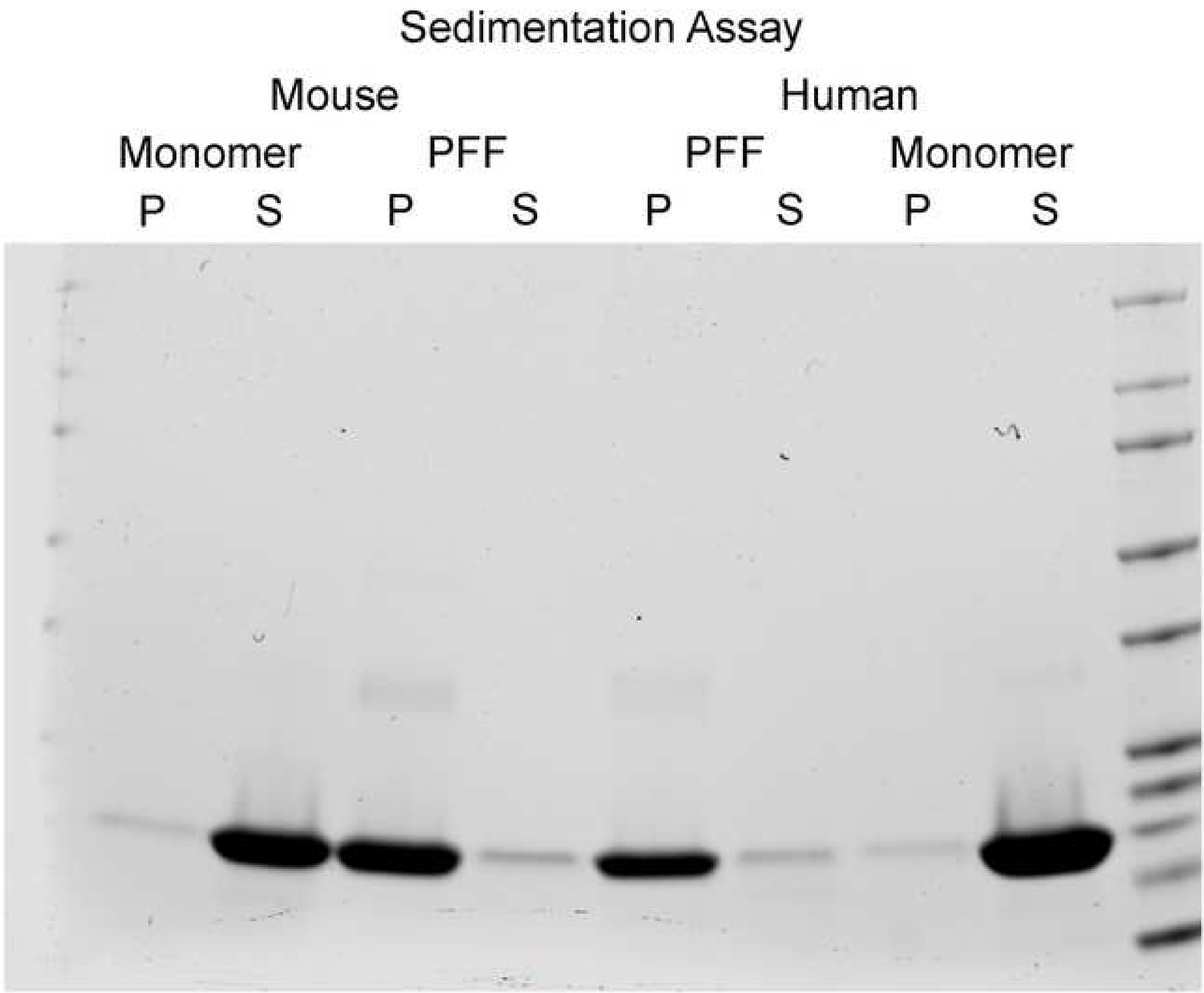
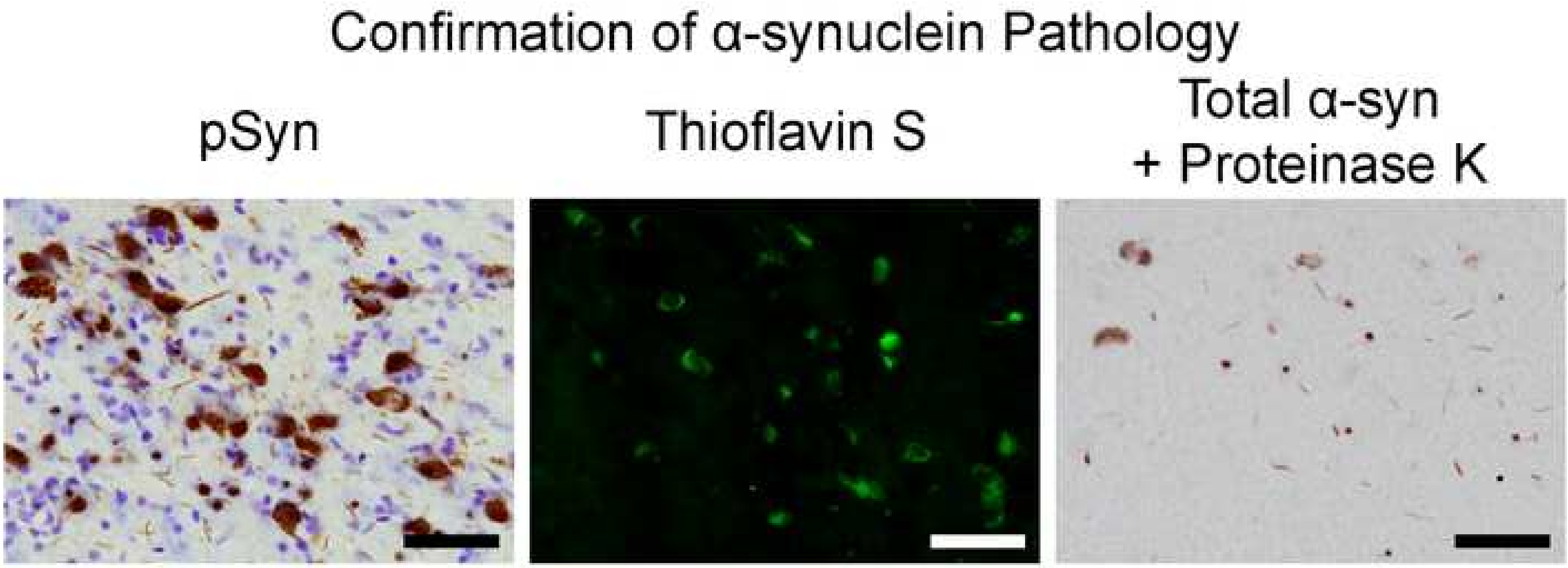


Figure 6


Sedimentation Assay
Mouse
Human
Monomer
PFF
PFF
Monomer
P
S
P
S
P
S
P
S


Confirmation of α-synuclein Pathology
pSyn
Thioflavin S
Total α-syn
+ Proteinase K

Name of Material/Equipment	Company	Catalog Number	Comments/Description
1x Dulbecco's phosphate buffered saline	Thermo Fisher (Gibco)	14190144	
Anti-alpha-synuclein (phosphorylated at serine 129) antibody	Abcam	AB184674	
Anti-alpha-synuclein antibody	Abcam	AB15530	
Bicinchonic acid	Thermo Fisher (Pierce)	PI23228	
Clear Medical Shrink Tubing (0.036" inner diameter)	Nordson Medical	103-0143	
Clear Medical Shrink Tubing (0.044" inner diameter)	Nordson Medical	103-0296	
Copper sulfate	Thermo Fisher (Pierce)	PI23224	
Eppendorf ThermoMixer C	Eppendorf	2231000574	
Eppendorf ThermoTop heated lid	Eppendorf	5308000003	
Formvar/Carbon coated electron microscopy grids	Eletron Microscopy Sciences	FCF300-Cu	
Glass capillary tube (0.53 mm outer diameter; 0.09 mm inner diameter; 54 mm length)	Drummond	22-326223	
Glass needle puller	Narishige	PC-10	
Hamilton syringe	Hamilton	80000	
Human alpha-synuclein monomer to generate preformed fibrils	Proteos	RP-003	
Mouse alpha-synuclein monomer to generate preformed fibrils	Proteos	RP-009	

Orange Medical Shrink Tubing (0.021" inner diameter)	Nordson Medical	103-0152
Parafilm M	Sigma-Aldrich	P7543
Proteinase K	Invitrogen	25530015
Qsonica 3.2 mm tip	Qsonica	4422
Qsonica Q125 sonicator	Qsonica	Q125
Thioflavin S	Sigma-Aldrich	T1892
Thioflavin T	EMD Millipore	596200
ToxinSensor Chromogenic LAL Endotoxin Assay Kit	Genscript	L00350C
Uranyl acetate	Eletron Microscopy Sciences	22400

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Dear Dr. Cao,

Thank you for your review of our manuscript entitled "Generation of alpha-synuclein preformed fibrils from monomers and use *in vivo*". Based on the editor and reviewer comments, we have added text to clarify the methods, added a figure that outlines custom glass needle assembly, changed a figure to show the gel in its entirety, and made overall changes to format the manuscript to the journal specifications. Below are our responses to the reviewers, we feel these changes strengthen the overall manuscript and are pleased to submit the edited article.

Response to Editor Comments

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.

The manuscript has been proofread again.

2) Introduction: Please expand to include the advantages of the presented method over alternative techniques with applicable references to previous studies, description of the context of the technique in the wider body of literature and information that can help readers to determine if the method is appropriate for their application.

The advantages of using the preformed fibril model as compared to other models could be, and is, it's own paper. The decision to use the model is really based on what model will be the most appropriate to answer the investigator's question(s). As such, the following statement has been added to the introduction "The PFF model offers distinct advantages and disadvantages as compared to neurotoxicant, transgenic, and viral vector mediated α-syn overexpression models as previously reviewed [6]. Though the choice of which model or approach to take should be determined by which model best suits the question the investigators are asking".

3) Please abbreviate liters to L (L, mL, µL) to avoid confusion.

The 6 cases of "ml" and 32 cases of "µl" were changed to "mL" and "µL" respectively in the text. One case of "ml" was changed to "mL" in Figure 1.

4) Please change all "RCF/rcf" units to "x g" throughout the protocol and figures.

The three cases in text and one in Figure 1 have been changed from "RCF" to "x g".

5) 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. You may use the generic term *followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Parafilm, Qsonica Q125 (https://www.sonicator.com/products/q125-sonicator), tip part #4422, Biologics Model 300 VT (http://www.biologics-inc.com/ultrasonichomogenizer-model 300vt.html), tip part # 0-120-0005, Narishige, Hamilton, Nordson Medical #103-0143, etc.*

Commercial language has been removed and replaced with "(Table of Materials)". Because of this, one of the sets of PFF sonication parameters has been omitted (sonication parameters are specific to the sonicator model used and has an effect on the final outcome).

6) Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Prior to the first protocol step, the following statement has been added "All methods involving animals have been approved by the Michigan State University Institutional Animal Care and Use Committee (IACUC)".

7) Please revise the Protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "NOTE." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

This change has been made and "note" sections added to cover the fine details associated with corresponding steps.

8) Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. 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. See examples below.

Additional details have been added throughout the manuscript, and to address the comments below.

9) 1.3.1.1: Please specify the different dilutions suggested.

A note has been added to state "*Suggested dilutions are 1:25, 1:50, and 1:100".*

10) 1.6, 3.3: Please replace commercial language (Parafilm, Parafilmed) with generic terms.

Parafilm has been removed and replaced with wax/plastic film.

11) 2.1: Does PFF here refer to the product from step 1.7?

PFF refers to the product from step 1.7 that is aliquoted for quality control steps as stated in step 1.8. This has been further clarified in step 1.8, which now states: “Gently flick the tube to resuspend the α-syn fibrils. Aliquot fibrils for the following quality control steps as indicated in steps 1.8.1 – 1.8.4.”

12) 2.1.1: Does the monomer refer to the supernatant from step 1.2? Please specify.

This is the monomer, but it is aliquoted after the concentration is determined. The readers are told to aliquot the monomers in step 1.5 (after concentration is known). To clarify this, step 1.5 now states: “Aliquot monomers for quality control comparison with fibrils as indicated in steps 1.8.1 – 1.8.4”.

13) 2.7: Please describe how to perform these steps; alternatively, provide a relevant reference.

The steps have been added starting at 2.7 and ending at 2.10.

14) 3.1: Please list an approximate volume of solution to prepare.

Exact volume given in 3.1 now. The section now states: “Weigh 20 mg uranyl acetate in a microcentrifuge tube and add 1 mL of ddH_2O for a 2% aqueous solution. Vortex until the uranyl acetate is in solution, and allow to sit overnight”.

15) 5.4: Please specify the final fibril concentration used in this protocol.

A note has been added after step 5.4 to indicate the concentration used for the representative results in the manuscript.

16) 7.1: Please describe how to coat the tube with a siliconizing reagent solution.

New steps (7.1 – 7.4) have been added to describe how to siliconize the tubes.

17) 7.3, 7.4: What is used to cut?

Scissors are used in these steps. This has been added to the protocol.

18) 7.8.1: Please describe how to test the needle.

Step 7.14 states to test the needle, and steps 7.14.1 through 7.14.3 describe how to test the needle.

19) Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

This has been done where possible.

20) After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

This has been done.

21) Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

This has been done.

22) JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

These changes have been made.

23) Figure 1: Please change "RCF" to "x g". Please use the micro symbol µ instead of u and abbreviate liters to L (L, mL, µL) to avoid confusion (i.e., µg/µL, mg/mL).

This has been changed.

24) Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please sort the items in alphabetical order according to the name of material/equipment.

This has been changed.

25) References: Please do not abbreviate journal titles.

This has been changed.

Response to Reviewer #1 Comments

1) A diagram of the assembly of the custom Hamilton-glass needle syringes for stereotactic injections would be helpful.

A diagram of the needle assembly has been added and is the new figure 3.

2) In particular, in section 7.6, how is it possible to slide the larger end of a glass tube with an ID of 0.09 mm over a metal needle that has an outer diameter of 0.47 mm (OD of a Hamilton 80000 needle)?? Wouldn't the metal needle be too thick to fit inside the glass tube? Or does the term "slide over" have a different meaning? A typical Hamilton 80000 syringe comes with a 26 gauge needle (0.47 mm OD) and even one modified with a 34 gauge needle (0.19 mm OD) still would be too large.

We agree that the dimensions of the capillary tubes in the original document are an issue. The Hamilton 80000 and product number for the Drummond glass capillary tubes listed in the table of materials was correct, the dimensions of the capillary tubes listed on the Thermo Fisher website, however, were incorrect. After contacting Drummond directly about the dimensions, we can confirm that the capillary tubes are “length of 54 mm; outer diameter: 0.86 mm; inner diameter 0.59 mm”, and the manuscript was changed accordingly to correct this error.

3) Also, the instructions for adding shrink wrap to the metal needle and glass tube was difficult to follow; for example, it is not clear what exactly is meant by the "base of a 10 ul beveled Hamilton syringe". Is the "base" where the needle goes into the barrel of the syringe?

The instructions have been changed to further clarify the steps to assemble the custom Hamilton-glass needles. A diagram has also been added as Figure 3

<u>Response to Reviewer #2 Comments</u>

1) Step 1: Please indicate total volume used for generation of fibrils, as starting volume may affect aggregation kinetics and timings. Also, indicate whether there is a minimal or maximal volume for achieving reproducible results.

A range of volumes that have been found to produce reproducible fibrilization results has been added as a note in section 1.4. The note states: “Total volume used to generate fibrils should be between 100 and 500 µL to achieve reproducible fibrilization results”.

2) Step 1.4: Please give the concentration of protein in M, as other groups may use modified versions of alpha-synuclein.

The α-syn used must be specifically formulated for fibrilization, as noted in Step 1.1. Because of this, “modified versions” of α-syn may not be able to form fibrils or may do so at a different concentration. Regardless, there is sufficient information provided in the protocol to calculate the molarity of the 5 mg/mL monomer solution if they desire.

3) Step 2.4: The actual buffer component and concentration is missing.

The buffer used is a 3X sample buffer and step 2.4 has been changed to reflect the concentration. We are confused as to the missing “buffer component” that the reviewer is asking about, as all components of the sample buffer are listed with the amounts of each component used also stated.

4) Step 2.7: Indicate if fibrillary alpha-synuclein is expected at monomer size or at higher molecular mass, or at both.

This is now clarified in the discussion. The α-syn in the sedimentation assay is run under denaturing conditions (denaturing gel, sample and running buffer contain SDS, and the samples are heated to 100 ºC and cooled prior to loading). As this is the case, we would only expect the prominent bands on the gel to be present around 14 kDa (size of the monomer).

5) Step 4.3: The volume of 25 µM thioflavin T is missing.

The volume of thioflavin T has been added to step 4.3. The directions now state “Add 250 µL thioflavin T in glycine buffer (25 µM thioflavin T, 100 mM glycine, 1% Triton X-100, pH 8.5) to each sample and gently mix”.

6) Figure 5: Please include a full gel image so it can be assessed if alpha-synuclein runs solely at monomer mass, or also at higher mass.

The sedimentation assay figure has been changed to show the entire gel and presence of higher molecular weight species addressed in the discussion.

<u>Response to Reviewer #3 Comments</u>

1) In the sedimentation assay figure 5, it would be best if the whole gel was shown. Are higher molecular weight species seen in the PFFs?

The sedimentation assay figure has been changed to show the entire gel and presence of higher molecular weight species addressed in the discussion.

2) Considering the high "stickiness" of amyloid proteins to plastic should siliconized or treated microcentrifuge tubes be used throughout the procedure?

We do not siliconize our microcentrifuge tubes or pipet tips. The microcentrifuge tubes that we used are polypropylene with a “highly polished interior for maximum sample recovery” and we have not had any noticeable issues.

3) Are less sonicated or highly sonicated PFFs equally potent in producing the expected pathology? What happens if you over-sonicate? is the material good to use?

This has been further been expended on in the discussion. Both Tarutani et al., 2016 and Abdelmotilib et al., 2017 show that fibril length can alter seeding efficiency. Shorter fibrils seed more efficiently, but this effect plateaus. As for over-sonicating, it would be expected that the heat from excessive sonication could decrease seeding efficiency and the material may not be good to use, but we have not intentionally tried to over-sonicate PFFs.

4) What concentration of PFFS and in what volume should one aim to inject in the rat or mouse brain? This is not very clear in the manuscript.

We were intentionally vague on this topic, as our lab currently has a manuscript in revision that discusses the question of concentration and volume. What has been used in the literature is now stated in the discussion.

5) Perhaps a better clarification as to the choice of Thioflavin S and Thioflavin T in 2 different assays should be included.

A better clarification in the discussion has been included as to the use of thioflavin S vs T.

Response to Reviewer #4 Comments

1) Please add a sentence to clarify in the Introduction what is meant by inconsistency with respect to other labs that have generated fibrils and produced a syn pathology. By inconsistency do you mean that some studies have been able to introduce fibrils and some not, or that there are variation in the numbers of fibrils between animals within studies?

A sentence was added to the introduction that states "Examples of inconsistencies range from PFFs that produce little or no α-syn pathology, batch to batch seeding efficiency, and even the failure of fibrils to form".

2) It is unclear from Figure 6 whether an antibody was used to visualize neurons containing pSyn, if so please list the antibody.

We have clarified in the results section that immunohistochemistry was used to visualize pSyn in the neurons in figure 7 (previously figure 6). Likewise, a pan α-syn antibody was used in the proteinase K digestion shown in figure 7. The antibodies used are now listed in the materials list.

3) The rationale for waiting two months post injection for confirmation is described in the Introduction (this is the time point when inclusion formation peaks), I would mention this briefly when describing the results as well.

The rationale for waiting two months has been added to the results, which now state: "a series of tissue sections were processed at 2 months post-surgery, when the number of inclusion containing neurons are known to peak in the SNpc, for the confirmation of phosphorylated α-syn inclusions”.

4) Also missing (or unclear) is how to decide on the concentration of fibrils to inject (or whether this is a critical point). If this is described in previous literature at least state a brief summary of how this is decided.

We were intentionally vague on this topic, as our lab currently has a manuscript in revision that discusses the question of concentration and volume. In the discussion, we now mention examples of fibril concentrations.