**Generation and sonication of α-synuclein fibrils**

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This protocol is a modification from previous published manuscripts (Patterson et al., 2019; Polinski et al., 2018; Stoyka et al., 2020; Volpicelli-Daley, Luk, & Lee, 2014).

For safe handling of fibrils please read Bousset L et al. (2016) *J Parkinsons Dis.* 6:143-51

**Reagents:**

•Monomeric α-synuclein. For in vitro primary neuron experiments, fibrils made using mouse or human recombinant α-synuclein will work. For differentiated human iPSCs, use human α-synuclein. For in vivo mouse models in which α-synuclein is endogenously expressed, use recombinant mouse α-synuclein because human α-synuclein is not as efficient in seeding α-synuclein inclusions from endogenously expressed mouse α-synuclein.

We recommend expressing and purifying α-synuclein from Escherichia coli as described (Volpicelli-Daley et al., 2014) or obtaining purified α-synuclein. For best results the α-synuclein should not have a tag (His, HA, GFP etc.). Our lab has not had success making fibrils from commercial sources.

Remove endotoxin using Pierce High Capacity Endotoxin Removal Spin columns (PI88276). Most other endotoxin removal kits have detergent which can be toxic in neuron assays.

Determine endotoxin levels using LAL endotoxin assay kit (GenScript catalog number L00350). Our values are <0.05 endotoxin units per 1 μg of protein.

Store purified α-synuclein in 10 mM Tris, pH7.5 at >10 mg/mL at -80°C

•500 mM Tris, pH7.5; sterile filtered

•500 mM KCl; sterile filtered

•8M guanidinium chloride

•1.5 mL sterile LoBind microcentrifuge tubes

**Equipment:**

Spectrophotometer

Shaker at 37°C

-80°C freezer

Benchtop centrifuge

Qsonica 700W cup horn sonicator with chiller at 15°C (other labs use Bioruptor Plus (Diagenode; Denville, NJ) with success)

Or probe tip sonicator (our lab uses Fisher FB12011). This is not recommended for *in vivo* work. If it is used for cell culture, use it in the BSL2 hood. Wear disposable lab sleeves over lab coat, Filtering Facepiece Respirator (Fisher 19-002-711), goggles. Clean hood with 1% SDS followed by water followed by 70% ethanol.

Dynamic light scattering detector such as Dynapro Nanostar (WDPN; Wyatt Technology)

OR

Access to transmission electron microscopy. Many universities such as UAB will accept EM grids and perform imaging for a fee.

**Making fibrils:**

1. When opening tubes and pipetting, perform in a BSL2 safety hood to prevent contamination
2. Thaw monomer on ice.
3. Spin monomer for 30 minutes at max speed on a benchtop centrifuge (15,000-20,000 *xg* depending on centrifuge) at 4oC. This step removes large fibrils or aggregates that may have been generated when storing and thawing high concentration of α-synuclein.
4. Keep monomer on ice at all times to prevent fibrillization.
5. We always check concentration of monomer after thawing. We have find that after a few freeze/thaws, the protein concentration becomes lower, likely because of oligomer or fibril formation (we assume path length =1). Use the spectrophotometer, A280, and Beer-Lambert law (A = ɛ \*c) to determine concentration of monomer. To measure the concentration of synuclein, ɛ for synuclein is 5960 M–1 cm–1 for human synuclein and 7450 M–1 cm–1 for mouse synuclein. Use 14.5 kDa for molecular weight.

Example from our lab:

Protein concentration written on tube says 13 mg/mL

Use 10 mM Tris, pH7.5, as blank (what the monomer is in)

A280 = 3.6

Calculate (3.6/5.96)\*14.5

= 8.7 mg/mL protein

1. Dilute monomer to 5mg/mL in 50mM Tris-HCl, 150mM KCl, pH7.5, final volume 500 μL. Shake at 37oC for 1 week; check for turbidity at end of week. If it is not turbid, it did not work.
2. Spin fibrils for 10 minutes at max speed in benchtop centrifuge at room temperature.
3. Discard supernatant and resuspend in approximately 50% of initial volume in 50mM Tris-HCl, 150mM KCl, pH7.5 (for example, if making at 500 μL, resuspend in 250 μL).
4. Measure the concentration of fibrils:
   1. Mix 5μL of fibril with 5μL of 8M guanidinium chloride (GnCl) (SAMPLE) and 5μL of 50mM Tris-HCl, 150mM KCl, pH7.5 buffer with 5μL of GnCl (BLANK).
   2. Incubate for one hour on the bench at room temperature.
   3. Use the spectrophotometer to determine concentration of fibrils using BLANK as the blank and SAMPLE to determine concentration. Note that the fibrils are diluted in GnCl, so the actual concentration will be 2X the readout.
5. Use 50mM Tris-HCl, 150mM KCl, pH7.5 to bring fibrils to 5 mg/mL.
6. Aliquot and store at -80deg C

**Sonicating Fibrils**

**Proper sonication is a key step for the fibril model to work.** For all in vivo work which involved injecting fibrils into mice or rats, we use the QSonica 700 sonicator with cup horn and tube rack for 1.5 mL polypropylene tubes with a chiller at 16°C. The cup horn sonication produces short fragments which maintain their morphology for 6-8 hours (at least) and can be stored in dry ice overnight, thawed and maintained at room temperature, and therefore remain active after overnight shipments. We found that over time, the heat generated by a probe tip sonicator causes the fibrils to form amorphous aggregates (Figure 1). This is a problem because stereotaxic surgeries can take several hours and the amorphous aggregates that form while the fibrils sit on the bench causes variability and reduces the concentration of seeding competent fragments. Another advantage of using the cup horn sonicator over probe tip is that 25 μL of fibrils can be sonicated, reducing the volume needed. This is also a closed tube system which increases safety. For neuron or cell culture work in which the fibrils are added to media and then the cells immediately after sonication, a probe tip sonicator is okay. Again, this should be performed in a BSL2 hood with all proper PPE (nanoparticle respirator, goggles, gloves etc.). The volume of fibrils to be sonicated cannot be less than 100 μL.

In all cases, we wear PPE when working with fibrils. We clean any spills with 1% SDS.



**Figure 1:** Transmission electron microscopy of α-synuclein fibrils. Immediately after probe tip or cup horn sonication, long fibrils are broken into small fragments. However, after six hours at room temperature, probe tip sonicated fibrils begin to form amorphous aggregates. With cup horn sonication performed at 16°C, the fragments after 6 hours appear similar in morphology compared to immediately after sonication. When the sonicated fibrils are placed in dry ice overnight, thawed and left at room temperature for 6 hours, the fragments appear similar to immediately after sonication, indicating that overnight shipments will maintain active fragments.

**Protocol:**

1. Pipet 25 µL fibrils (5 mg/mL) in 1.5 mL sonication tube (cat# NC0869649 Fisher Scientific).
2. Fill Qsonica water reservoir with about 900 mL water. Make sure reservoir water level height is 7 cm.
3. Attach cooling system to Qsonica and set the temperature at 15 °C.
4. Place 1.5 mL sonication tube with fibrils in Qsonica tube holder. The gap between tube bottom surface and Qsonica probe upper surface should be 1 cm. (Figure 2)



**Figure 2.** Diagram of Qsonica700 with multi-tube holder

**Sonication cycle parameters:**

We initially spent time optimizing the parameters for sonication using our Qsonica system. The following parameters may not be ideal depending on the sonicator in an individual lab, and we therefore recommend personally optimizing conditions first. The goal is to consistently obtain fragments of α-synuclein that are on average 50 nm in length (Figure 1, 3). If the fibrils are not sufficiently fragmented, the abundance of α-synuclein inclusions produced can be low and highly variable.

1. Total sonication time 15 minutes
2. Sonication pulse on for 3 seconds on and 2 seconds off
3. Amplitude 30%
4. Note: After sonication sometimes, there are few droplets inside the sample tube and sometimes not. If droplets are there take out tube and spin it at 1000 rpm for 10 seconds. Take the sample tube in safety hood and mix the PFF sample with pipette 5 times in and out (avoid introducing avoid bubbles while pipetting)
5. Sonicate sample for another 7:30 min.
6. After sonication PFF sample can be used that day or stored in -80 °C or on dry ice (for shipping) for one day but I believe sample in -80 °C should be fine for longer). After taking out from -80 °C sample is good at least for 8 hours at room temperature.

**Confirming fragmentation**

Before injecting fibrils into a large cohort of mice and waiting several months for results, researchers should ensure that their sonication protocol results in sufficient fibril fragmentation. We use a dynamic light scattering detector as a quick and reliable method to check for fragmentation. Transmission electron microscopy is another method that can be used (Figure 1).

Dynamic Light Scattering:

•Pipet 1μL sonicated PFF sample and dilute with 4 μL 1x PBS (filtered with 0.22 μm filter). Put 5 μL diluted PFF sample in disposable microcuvette (WYATT Technology) for DLS. An example of desired results is shown in Figure 3.

When done, fill cuvette with 2% SDS to decontaminate fibrils (using a squirt bottle). Let sit for at least 30 min. Use squirt bottle to rinse several times with DI water. Use filtered water for last rinse. Make sure dry before next use. The important point is to make sure the cuvette remains dust free.



**Figure 3.** Example of DLS profiles of fibrils before and after sonication. A radius of 50-70nm with minimal variability is optimal.

**Transmission electron microscopy:**

We perform our transmission electron microscopy with the help of our High Resolution Core facility. Many universities offer and EM core.

•Immediately after sonication, dilute 2 μL of sonicated fibrils with 98 μL of PBS. Spot 5 μL of diluted fibrils on glow discharged EM grid. Wait for 20 seconds and remove extra buffer by allowing it to wick onto a kimwipe. Add 8 µL uranyl acetate solution. Remove extra uranyl acetate solution after 10 seconds. Let dry and image. (Figure 1)

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