

Generation and sonication of α -synuclein fibrils

Jun 18, 2020

Generation and Sonication of α -synuclein FibrilsVijay Singh¹, Marta Castellana-Cruz², Nunilo Cremades³, Laura Volpicelli-Daley¹¹Department of Neurology, Center for Neurodegeneration and Experimental Therapeutics, University of Alabama at Birmingham, Birmingham, AL, 35294, USA.;²Centre for Misfolding Diseases, Department of Chemistry, University of Cambridge, CB2 1EW, UK.;³Institute for Biocomputation and Physics of Complex Systems (BIFI)-Joint Unit BIFI-HQFR (CSIC), University of Zaragoza, Zaragoza 50018, Spain.**1** Works for me dx.doi.org/10.17504/protocols.io.bhhrj356

Neurodegeneration Method Development Community

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ABSTRACT

Animal models that accurately recapitulate the accumulation of alpha-synuclein (α -syn) inclusions, progressive neurodegeneration of the nigrostriatal system and motor deficits can be useful tools for Parkinson's disease (PD) research. The preformed fibril (PFF) synucleinopathy model in rodents generally displays these PD-relevant features, however, the magnitude and predictability of these events is far from established. We therefore have optimized the synthesis generation of α -syn fibrils to ensure reliable, robust results. These fibrils can be added to neurons in culture, differentiated iPSCs, or injected into mice or rats. The protocol includes steps for fibril synthesis as well as sonication for fibril fragmentation which is a critical step for inducing formation of α -syn inclusions.

ATTACHMENTS

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DOI

dx.doi.org/10.17504/protocols.io.bhhrj356


PROTOCOL CITATION

Vijay Singh, Marta Castellana-Cruz, Nunilo Cremades, Laura Volpicelli-Daley 2020. Generation and Sonication of α -synuclein Fibrils. **protocols.io**
dx.doi.org/10.17504/protocols.io.bhhrj356

KEYWORDS

monomeric α -synuclein fibrils, in vitro primary neuron, human recombinant, iPSCs

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
CREATED

Jun 13, 2020

LAST MODIFIED

Jun 18, 2020

OWNERSHIP HISTORY

Jun 13, 2020  Megan FreundJun 17, 2020  Laura Volpicelli-Daley

GUIDELINES

This protocol is a modification from previously 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) An Efficient Procedure for Removal and Inactivation of alpha-Synuclein Assemblies from Laboratory Materials *J Parkinsons Dis*.6:143-51

<https://pubmed.ncbi.nlm.nih.gov/26639448/>

When opening tubes and pipetting, perform in a BSL2 safety hood to prevent contamination.

References:



Patterson, J. R., Polinski, N. K., Duffy, M. F., Kemp, C. J., Luk, K. C., Volpicelli-Daley, L. A., . . . Sortwell, C. E. (2019). Generation of Alpha-Synuclein Preformed Fibrils from Monomers and Use In Vivo. *J Vis Exp*(148).
<http://10.3791/59758>



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<http://10.1016/j.nbd.2019.104708>



Volpicelli-Daley, L. A., Luk, K. C., & Lee, V. M. (2014). 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. *Nat Protoc*, 9(9), 2135-2146.
<http://10.1038/nprot.2014.143>

Equipment:

- Spectrophotometer
- Shaker at **37 °C**
- **-80 °C** freezer
- Dynamic light scattering detector such as Dynapro Nanostar (WDPN; Wyatt Technology) or access to transmission electron microscopy
- 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.

- 1.5 mL sonication tube (cat# NC0869649 Fisher Scientific)

Materials:

- Monomeric α -synuclein



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 μ g of protein.

Store purified α -synuclein in 10 mM Tris, **pH 7.5** at >10 mg/mL at **-80 °C**.

- 8M guanidinium chloride
- 1.5 mL sterile LoBind microcentrifuge tubes
- 500 mM KCl; sterile filtered
- 500 mM Tris, **pH 7.5**; sterile filtered
- Ice
- LAL endotoxin assay kit (GenScript catalog number L00350)
- PBS
- Uranyl acetate solution
- Deionized water

- Cuvettes
- 1% SDS

SAFETY WARNINGS

Please see the Safety Data Sheet (SDS) for safety warnings and hazards before start.

When opening tubes and pipetting, perform in a BSL2 safety hood to prevent contamination.

BEFORE STARTING

Sonicating Fibril

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.

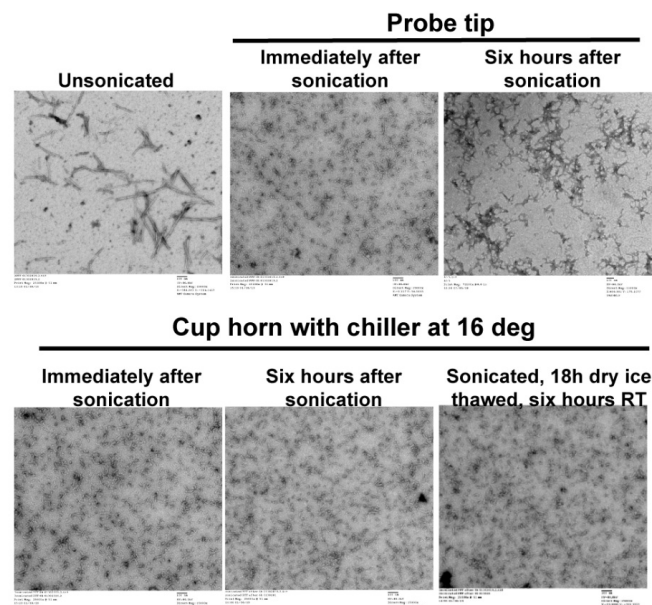





Figure 1: Transmission electron microscopy of α -synuclein fibrils. Immediately after probe tip or cup horn sonication, long fibrils are broken into small fragments. However, after six hours at room temperature, probe tip sonicated fibrils begin to form amorphous aggregates. With cup horn sonication 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.

Making fibrils

1 Thaw monomer  **On ice** .

2 

Spin monomer for  **00:30:00** at max speed on a benchtop centrifuge ( **15000 x g** -  **20000 x g** depending on centrifuge) at  **4 °C** .



This step removes large fibrils or aggregates that may have been generated when storing and thawing high concentration of α -synuclein.


3 Keep monomer  **On ice** at all times to prevent fibrillization.



We always check concentration of monomer after thawing. We have found 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 ($A = \epsilon \cdot c$) to determine concentration of monomer. To measure the concentration of synuclein, ϵ for synuclein is $5960 \text{ M}^{-1} \text{ cm}^{-1}$ for human synuclein and $7450 \text{ M}^{-1} \text{ cm}^{-1}$ 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 $(3.6/5.96) \cdot 14.5$
= 8.7 mg/mL protein

4 

Dilute monomer to  **5 mg /ml** in **[M]50 Milimolar (mM) Tris-HCL** , **[M]150 Milimolar (mM) KCL** , **pH7.5** ,
final volume  **500 μ l** .

5 Shake at  **37 °C** for  **168:00:00** (1 week); check for turbidity at end of week.



If it is not turbid, it did not work.

6 

Spin fibrils for ⌚ **00:10:00** at max speed in benchtop centrifuge at 🌡 **Room temperature** .

7 

Discard supernatant and resuspend in approximately 50% of initial volume in [M]**50 Milimolar (mM) Tris-HCL** ,
[M]**150 Milimolar (mM) KCL** , pH**7.5** (for example, if making at 🧴**500 µl** , resuspend in 🧴**250 µl**).

8 Measure the concentration of fibrils.

8.1 

Mix 🧴**5 µl** of fibril with 🧴**5 µl** of
[M]**8 Molarity (M) guanidinium chloride (GnCl) (SAMPLE)** and 🧴**5 µl** of
[M]**50 Milimolar (mM) Tris-HCL** , [M]**150 Milimolar (mM) KCL** , pH**7.5** buffer with
🧴**5 µl GnCl (BLANK)** .

8.2 

Incubate for ⌚ **01:00:00** on the bench at 🌡 **Room temperature** .

8.3 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.

9 Use [M]**50 Milimolar (mM) Tris-HCL** , [M]**150 Milimolar (mM) KCL** , pH**7.5** to bring fibrils to 🧴**5 mg /mL** .

10 Aliquot and store at 🌡 **-80 °C**

Sonicating Fibrils

11 

Pipet 🧴**25 µl** fibrils (**5 mg/mL**) in 🧴**1.5 mL** sonication tube.

12 Fill Qsonica water reservoir with about **900 mL water** .



Make sure reservoir water level height is 7 cm.

13 Attach cooling system to Qsonica and set the temperature at **15 °C** .

14 Place **1.5 mL** , therefore,sonication tube with fibrils in Qsonica tube holder.



The gap between tube bottom surface and Qsonica probe upper surface should be 1 cm.

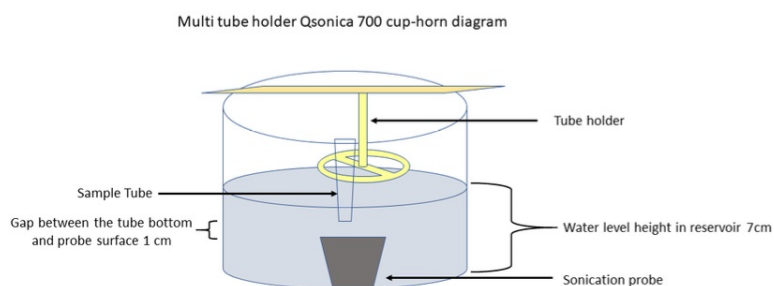


Figure 2. Diagram of Qsonica700 with multi-tube holder



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.

15 Total sonication time: **00:15:00** .

16 Sonication pulse on for **00:00:03** and off for **00:00:02** .

17 Amplitude 30%.



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 00:00:10** . Take the sample tube in safety hood and mix the PFF sample with pipette 5 times in and out (avoid introducing bubbles while pipetting).

18 Sonicate sample for another **07:30:00** .



After sonication PFF sample can be used that day or stored in **-80 °C** or on dry ice (for shipping) for one day, but a sample in -80 °C should be fine for longer. After taking out from **-80 °C** sample is good at least for **08:00:00** at **Room temperature** .

Confirming Fragmentation

19



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.

Dynamic Light Scattering:

20



Pipet **1 µl sonicated PFF sample** and dilute with **4 µl 1x PBS (filtered with 0.22 µm filter)** .

21 Put **5 µl diluted PFF sample** in disposable microcuvette (WYATT Technology) for DLS.

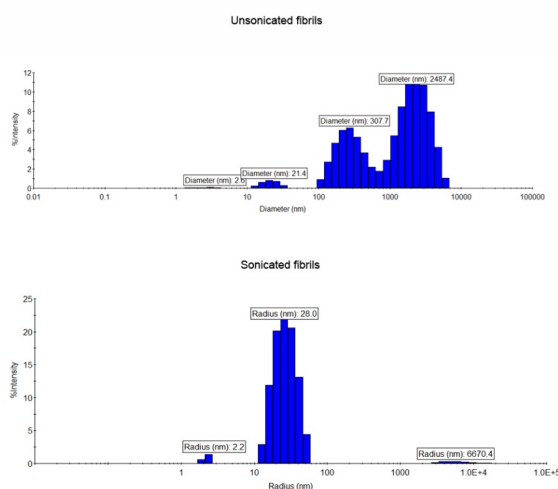



Figure 3. Example of DLS profiles of fibrils before and after sonication. A radius of 50-70nm with minimal variability is optimal.

22 When done, fill cuvette with 2% SDS to decontaminate fibrils (using a squirt bottle).

23 Let sit for at least  00:30:00 .

24 

Use squirt bottle to rinse several times with DI water.

25 

Use filtered water for last rinse.

26 Make sure dry before next use.



The important point is to make sure the cuvette remains dust free.



In the next step we perform our transmission electron microscopy with the help of our High Resolution Core facility. Many universities offer an EM core.

Transmission Electron Microscopy

27 

Immediately after sonication, dilute  2 µl of sonicated fibrils with  98 µl of PBS .

28 Spot  5 µl of diluted fibrils on glow discharged EM grid.

29 Wait for  00:00:20 and remove extra buffer by allowing it to wick onto a kimwipe.

30 

Add  8 µl uranyl acetate solution .

31 Remove extra uranyl acetate solution after  00:00:10 .



Let dry and image.