NeuroEDDU Protocols

Quality Control Characterization of α-Synuclein Preformed Fibrils (PFFs)

Authors: Esther Del Cid Pellitero, Rina Shlaifer, Wen Luo, Andrea Krahn, Emmanuelle Nguyen-Renou, Destiny-Love Manecka

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Version	Authors/Updated by	Date	Signature
V1	Destiny-Love Manecka	January 5, 2017	
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The involved functions approve the document for its intended use:

Name	Function:	Role	Date	Signature
Dr. Edward Fon	R&D	Principal Investigator		
Dr. Thomas Durcan	R&D	Group Leader, EDDU Platform		

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1 Introduction

1.1 Objectives

This protocol describes how to:

- Determine fibril length of sonicated unlabelled α-synuclein preformed fibrils (PFFs) by dynamic light scattering (DLS) and electron microscopy (EM)
- Confirm cellular uptake of fluorescently-labelled α-synuclein PFFs by immunofluorescence (IF)

1.2 Protocol Overview

The characteristics of α-synuclein PFFs must be validated prior to experimental use by performing quality control analyses of fibril length and cellular uptake (Figure 1).

Fibril length of sonicated α-synuclein PFFs is first assessed through quantification of particle size by DLS; α-synuclein monomers may be analyzed in parallel as a control. This analysis will provide the size distribution of fibrils within the sample (i.e. the proportion of fibrils in the sample of a specific length). The length of the majority of fibrils in a sample of sonicated α-synuclein PFFs should be ≤100 nm.

Then, sonicated α-synuclein PFFs are visualized by EM using negative staining to quantify fibril length and assess fibril uniformity; non-sonicated α-synuclein PFFs may be analyzed in parallel as a control. Note that α-synuclein monomers are too small to be visualized by EM (~6 nm). The average fibril length of α-synuclein PFFs should be approximately 55 nm after sonication for 30 seconds, and ≤50 nm after sonication for 60 seconds.

Cellular uptake is assessed by treating cells with fluorescently-labelled α-synuclein PFFs. The intracellular inclusions formed by the α-synuclein PFFs are visualized by IF.

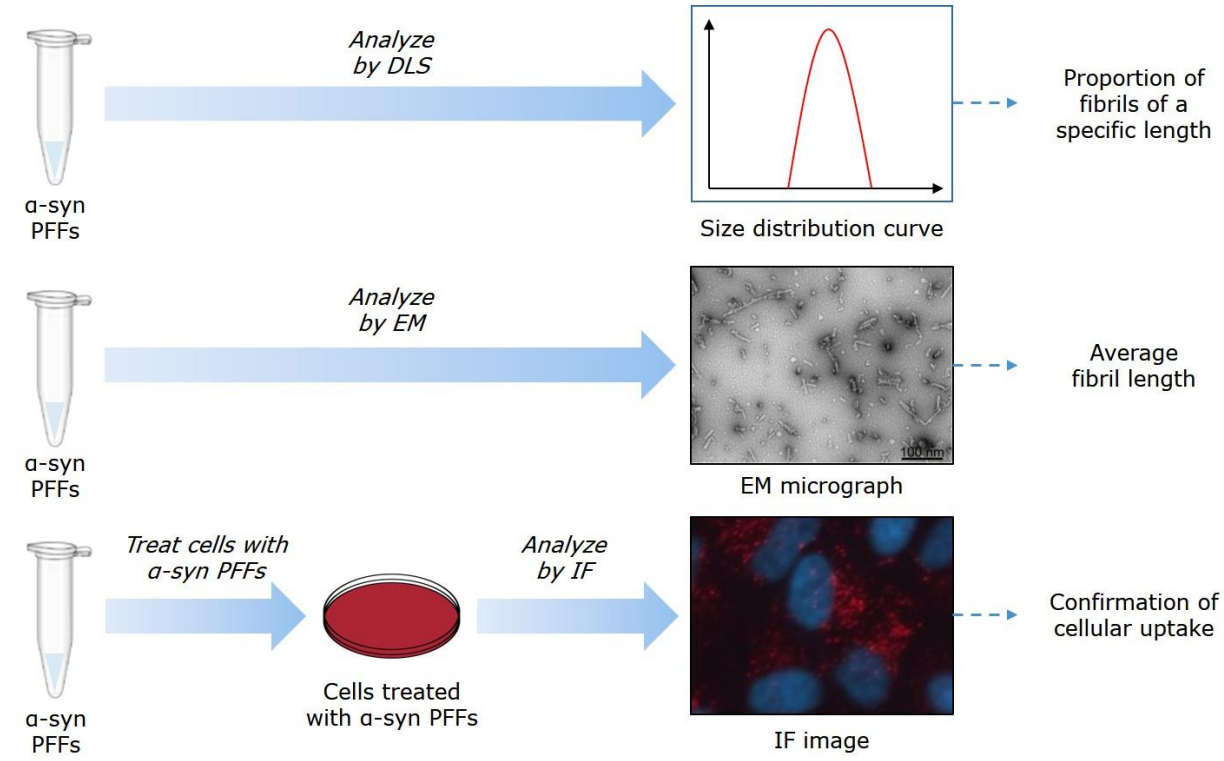


Figure 1. Protocol overview for analyzing α-synuclein PFFs by DLS and EM to determine average fibril length and for analyzing cells treated with α-synuclein PFFs by IF to confirm cellular uptake.

Once validated, α-synuclein PFFs may be used to induce pathology in cell- and animal-based experiments. For in vivo experiments, α-synuclein PFFs must be validated by DLS and EM at minimum. Note that batches of α-synuclein PFFs that pass the quality control analyses described in this protocol are not guaranteed to be pathogenic in vivo.

1.3 Technical and safety considerations

The following information should be read before starting:

- While working with α-synuclein PFFs:
  - Wear appropriate personal protective equipment at all times, including disposable gloves, a laboratory coat, a face mask, and protective goggles.
  - Preparation of PFFs for DLS and EM must be performed in a sterile Class II biosafety (BSLII) cabinet.
  - Avoid inhaling PFFs or getting aerosolized PFFs in eyes.
- After working with α-synuclein PFFs:
  - Clean area with 1% SDS to destroy fibrils, then rinse area with sterile distilled water.
  - Dispose of all tips, tubes, cuvettes, and media into a large empty bottle or a 50-mL tube containing 1% SDS. Store items in 1% SDS for at least 2 hours to disassemble the α-synuclein PFFs.
  - Dispose of gloves and face mask in an autoclavable biohazard bag to be sterilized.
- For further information on the use of SDS to break down α-synuclein PFFs, refer to Bousset et al. 2016. *J. Parkinson Dis*. (6) 143–151.
- Store α-synuclein PFFs at –80°C and thaw at room temperature. Do not store PFFs on ice or at 4 °C, as this causes dissociation and reduces activity.
- Do not vortex α-synuclein PFFs.
- Instructions for using an electron microscope are complex and specific to the microscope model. All users must be trained on the microscope being used.

2 Materials

The material, reagents, and equipment listed in this document can be substituted for those supplied by other manufacturers. However, the performance of the assay may not be the same and may need to be optimized or redeveloped upon significant modifications to the materials and/or methods.

Refer to the product data sheet from the supplier for further details on storage and preparation instructions.

2.1 Labware

Item	Supplier	Catalogue #
Copper grid, carbon-coated, 200 mesh	SPI Supplies	3520C-FA
Coverslips, 12-mm	Fisher	12-545-80
Culture plate, 12-well	Fisher	08-772-29
Dish, Pyrex brand 3140 (190 mm x 100 mm)	Cole-Parmer Scientific Experts	RK-34550-08
Microscope slides	Fisher	12-552-3
Quartz cuvette, ultra low volume	Malvern Panalytical	ZEN2112
Spray bottle	Fisher	01-189-100
Syringe filter, 0.1-µm Whatman Anotop 10	Sigma Aldrich	WHA68091012
Syringe filter, 0.22-µm, nylon	Mandel Scientific	WYV-SFNY013022NC
Tubes, 1.5-mL	Fisher	MCT-175-LC
Tubes, 50-mL	Fisher Scientific	14-959-49A
Tweezers, ultra-fine	Electron Microscopy Sciences (EMS)	78318-3
Whatman® qualitative filter paper, Grade 1, 15-mm, 500 circles	GE Life Sciences	1001-0155

2.2 Reagents

Item	Supplier	Catalogue #	Stock conc.	Working conc.	Storage temp.
DMEM	Wisent Bioproducts	319-015-CL	1x	1x	4°C
Ethanol (EtOH)	Greenfield Global	26456	100%	70%	RT

Item	Supplier	Catalogue #	Stock conc.	Working conc.	Storage temp.
Fetal bovine serum (FBS)	Wisent Bioproducts	080-150	100%	10%	Stock: −80°C Working: 4°C
HeLa cells	ATCC	ATCC® CCL-2™	NA	NA	Liquid nitrogen
Hoechst	ThermoFisher Scientific	H3570	10 mg/mL	5 µg/mL	4°C
Paraformaldehyde (PFA)	Electron Microscopy Sciences (EMS)	30525-89-4	16%	4%	Stock: RT Working: 4°C
Penicillin-streptomycin	Wisent Bioproducts	450-200-EL	100x	1x	Stock: −80°C Working: 4°C
Phosphate buffered saline (PBS)	Wisent Bioproducts	311-012	10x	1x	RT
Sodium dodecyl sulfate (SDS)	Wisent Bioproducts	800-100	20%	1%	RT
Uranyl acetate*	Electron Microscopy Sciences (EMS)	22400-2	NA	2%	4°C

*Light-sensitive reagent. Stock and working aliquots should be covered in aluminum foil to minimize exposure to light.

2.3 Equipment and Software

Item	Supplier	Catalogue #
Tecnai 12 BioTwin 120 kV transmission electron microscope (TEM) or Tecnai G2 Spirit Twin 120 kV Cryo-TEM	FEI	NA
EVOS XL Core cell imaging system	ThermoScientific	EVOS XL Core
Fiji-ImageJ software	ImageJ	NA (download at: https://imagej.nih.gov/ij/)
Malvern Zetasizer Software for Zetasizer Nano S, 7.13	Malvern Panalytical	NA
Matlab R2017b software	Mathworks	NA

Item	Supplier	Catalogue #
Stirrer plate	VWR	97042-706
Zetasizer Nano S	Malvern Panalytical	Zetasizer Nano S

3 Protocol

3.1 Dynamic light scattering

Materials:

- Sonicated unlabelled α-synuclein PFFs
- α-synuclein monomers (optional)
- 1.5-mL tubes
- 1x PBS
- 0.1-µm syringe filter
- Centrifuge
- Zetasizer Nano S system with Malvern Zetasizer software
- Ultra low volume quartz cuvette

Procedure:

1. Pass 10 mL of PBS and through a 0.1-µm syringe filter.
    - The filtered PBS will be the blank for the particle size analyzer.
2. Prepare ≥0.6 mg/mL α-synuclein PFFs by diluting in filtered PBS.
3. Centrifuge α-synuclein PFFs at 13,000 rpm for 5 minutes and then transfer the supernatant to a fresh 1.5-mL tube.
    - Take care not to remove any particles from the bottom of the tube when removing the supernatant.
    - If analyzing α-synuclein monomers, pass at least 60 µL of diluted samples (≥0.6 mg/mL) through a 0.1-µm syringe filter.
4. Analyze the supernatants using the Zetasizer Nano S system. See Appendix 4.2 for instructions to set up and run the measurement SOP using the Malvern Zetasizer software. An example of particle size quantification is shown in Figure 2 and the raw correlation data is shown in Figure 3.
    - The Zetasizer Nano S system must be turned on 20 minutes before use to allow the instrument to thermally equilibrate.
    - The length of the majority of fibrils in a sample of sonicated α-synuclein PFFs should be ≤100 nm.
    - The raw correlation data curve shows the correlation of scattering intensity during time measured (Figure 3). The correlation curve should be smooth and tend to intercept with y axis just below a value of 1 (optimal) or between 0.2 to 0.99 (acceptable). If curve decay starts anywhere below 0.1 and is bumpy at earlier timepoints on the x axis, it indicates that the sample concentration is too low and is not acceptable for the DLS measurement. Other factors that yield a bumpy curve, particularly at later timepoints on the x axis, include

sample sedimentation, polydispersity (Pdi), and aggregation. In these cases, sample preparation should be optimized using methods such as centrifugation or filtration to remove sediments, aggregates, and other large species. For more information on correlation curves, see Appendix 4.2.1.

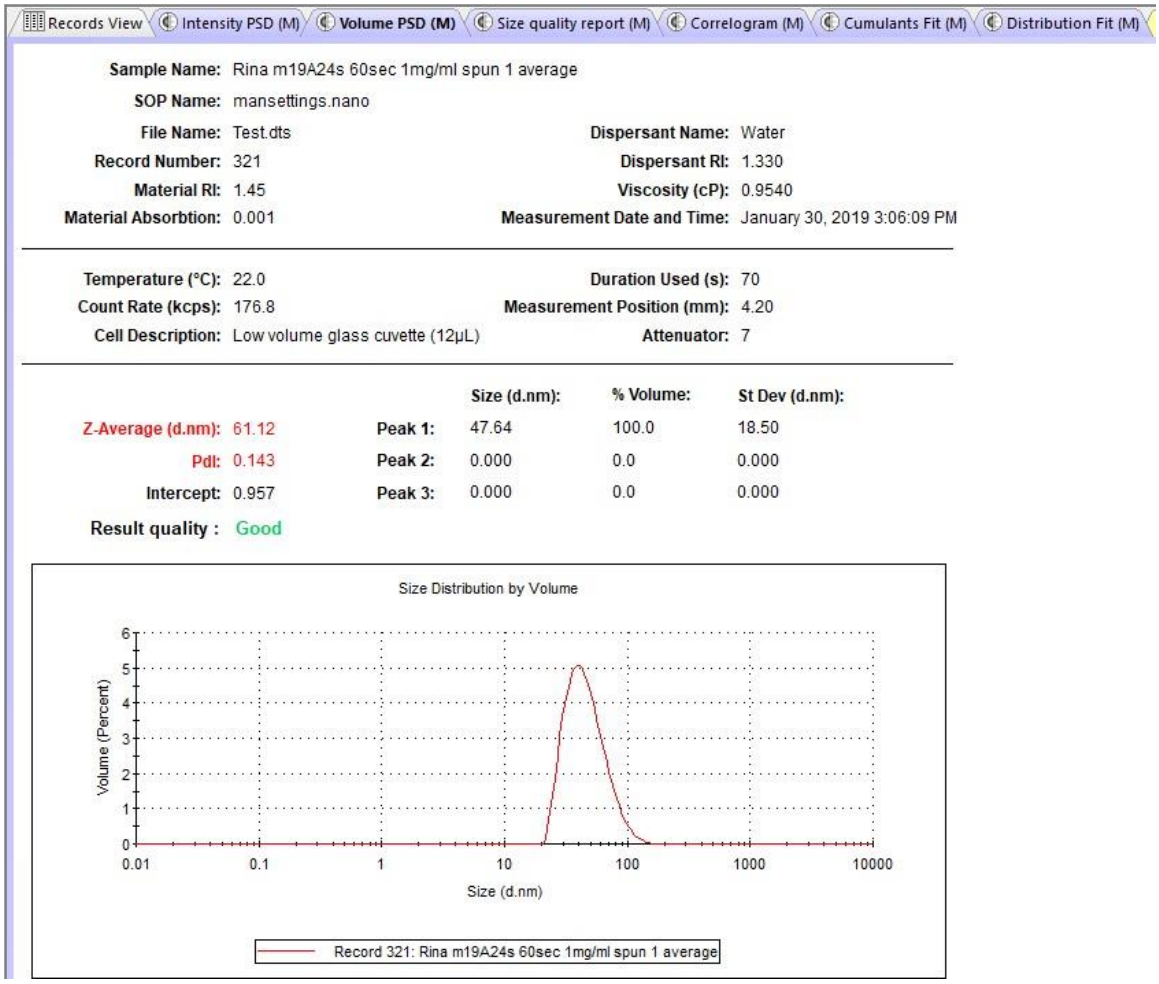


Figure 2. Particle size quantification of α-synuclein PFFs sonicated for 60 seconds (2 x 30 seconds).

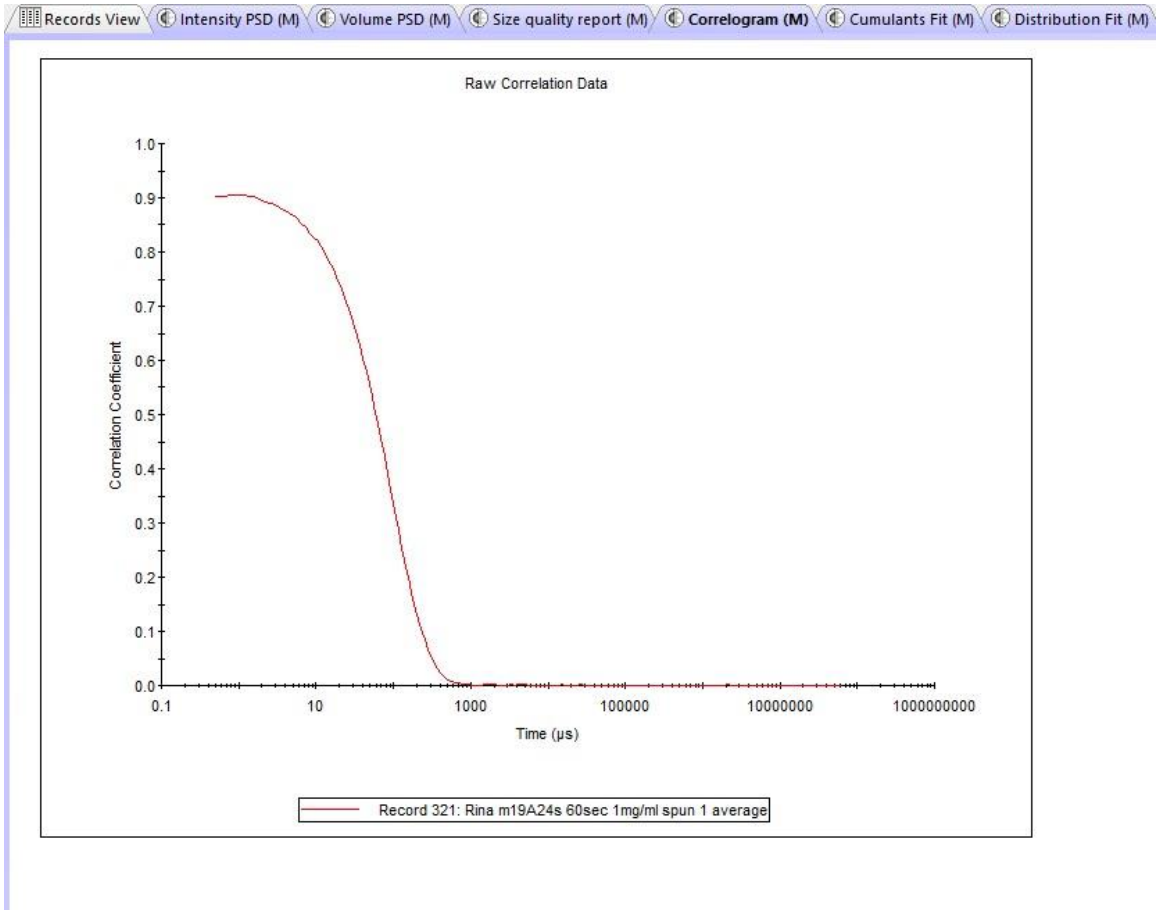


Figure 3. Raw correlation data for particle size quantification of α-synuclein PFFs sonicated for 60 seconds (2 x 30 seconds).

3.2 Electron microscopy

Materials:

- Sonicated unlabelled α-synuclein PFFs
- Non-sonicated unlabelled α-synuclein PFFs (optional)
- 1.5-mL tubes
- 200-mesh carbon-coated copper grid
- Whatman paper
- Ultra-fine tweezers
- Glass dish (190 mm x 100 mm)
- 0.22-µm sterile syringe filter
- 70% EtOH
- 2% uranyl acetate
- 4% PFA
- Centrifuge
- EM
- Software to quantify PFF length (see Appendix 4.1)

Procedure:

1. Clean the biosafety cabinet surface, tweezers, and glass dish with 70% EtOH.
2. To remove impurities from the 4% PFA and 2% uranyl acetate solutions, filter solutions separately with a 0.22-µm syringe filter, or centrifuge solutions at maximum speed for 5 minutes and use the supernatant in steps 5 and 7, respectively.
3. Prepare 20 µM α-synuclein PFFs in ddH_2O.
4. Add 5 µL of 20 µM α-synuclein PFFs to the grid. Wait for 2 minutes, and then remove sample drop from the grid with Whatman paper.
5. Add 5 µL of 4% PFA to the grid. Wait for 1 minute, and then remove the PFA from the grid with Whatman paper.
6. Add 5 µL of ddH_2O to the grid. Wait for 1 minute, and then remove the ddH_2O from the grid with Whatman paper. Repeat this step 3 more times.
7. Add 5 µL of 2% uranyl acetate to the grid. Wait for 1 minute, and then remove the uranyl acetate from the grid with Whatman paper.
8. Cover the grid with the glass dish and allow to dry for at least 30 minutes.
9. Visualize grid on EM to analyze fibril length (see Appendix 4.1 for analysis procedure). Examples of EM micrographs and fibril length quantificaiton data are shown in Figure 4.

    - Average fibril length should be approximately 55 nm after sonication for 30 seconds and ≤50 nm after sonication for 60 seconds.

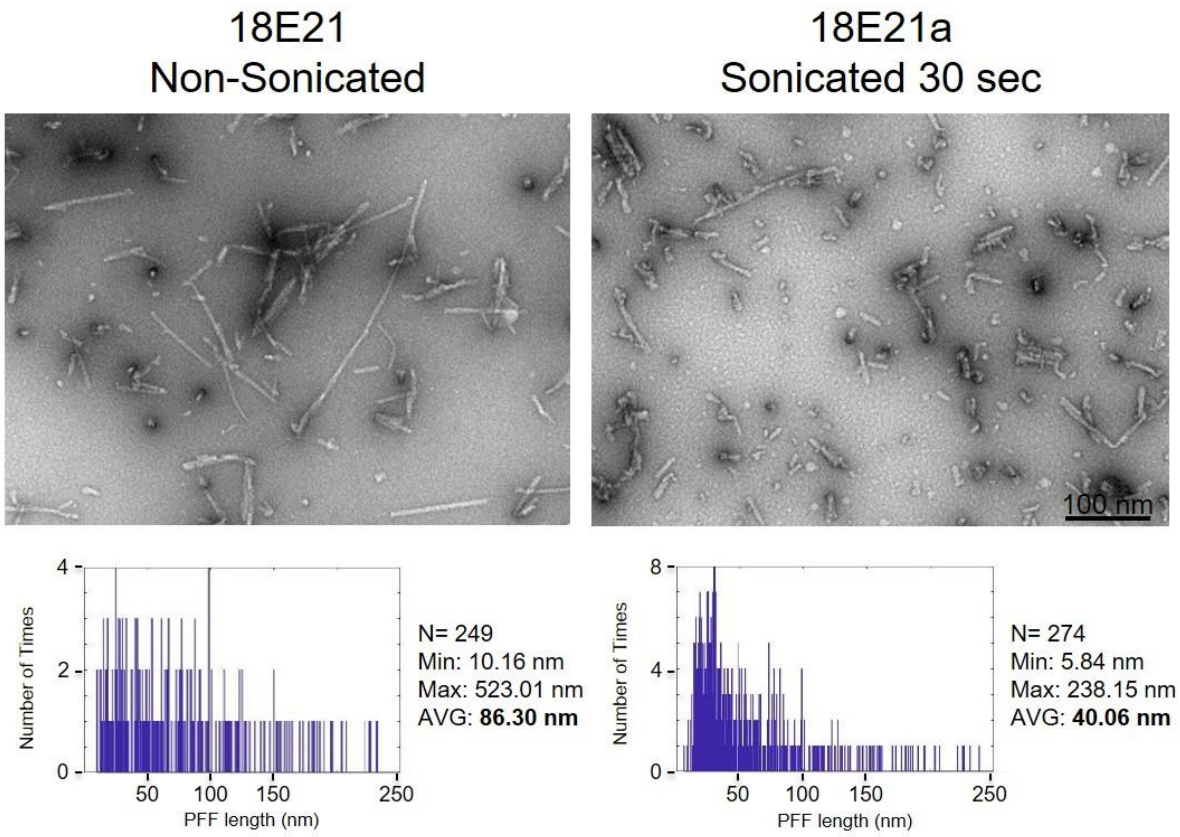


Figure 4. EM microphotographs and fibril length quantification of non-sonicated α-synuclein PFFs and α-synuclein PFFs sonicated for 30 seconds.

3.3 Immunofluorescence

Materials:

- Sonicated α-synuclein PFFs fluorescently labelled with Alexa Fluor™ 488 (PFF-488), Alexa Fluor™ 568 (PFF-568), or Alexa Fluor™ 633 (PFF-633)
- 12-well plate
- Coverslips
- HeLa cells
- Media:

Media	Components
HeLa cell media	• DMEM • 1x Pen-Strep • 10% FBS

- 1x PBS
- 4% PFA
- Hoechst stain (10 mg/mL)
- 37°C/5% CO_2 cell culture incubator
- Microscope slides
- Fluorescence microscope

Procedure:

1. Plate HeLa cells in media at a density of 20,000 cells/1 mL/well on a 12-well plate containing coverslips.
2. The next day, prepare 2 µM α-synuclein PFFs in PBS. Add 10 µL of 2 µM α-synuclein PFFs to each well (final concentration will be 20 nM).
3. After 24 hours, remove media and wash each well with 1 mL PBS.
4. Add 1 mL 4% PFA to each well and incubate at room temperature for 15 minutes.
5. Wash each well twice with 1 mL PBS.
6. Prepare Hoechst stain solution by diluting 10 mg/mL stock 1:2000 in PBS (final concentration is 5 µg/mL). Add 1 mL to each well and incubate at room temperature for 5 minutes.
7. Wash each well three times with 1 mL PBS.
8. Mount coverslips onto slides.

9. Visualize slides on fluorescence microscope using a 10X or 20X objective lens. An example image of fluorescently-labelled α-synuclein PFFs in HeLa cells is shown in Figure 5.

   - Slides can be stored at 4°C protected from light until ready to image.

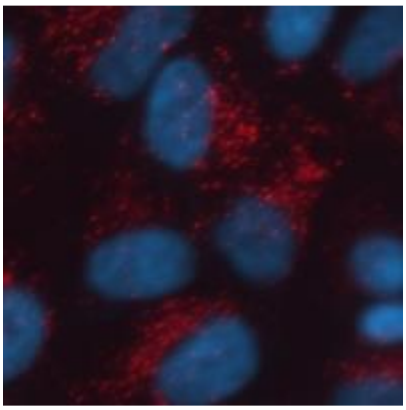


Figure 5. IF of HeLa cells treated for 24 hours with 20 nM α-synuclein PFF-633 Blue: Hoechst; red: PFF-633.

4 Appendix

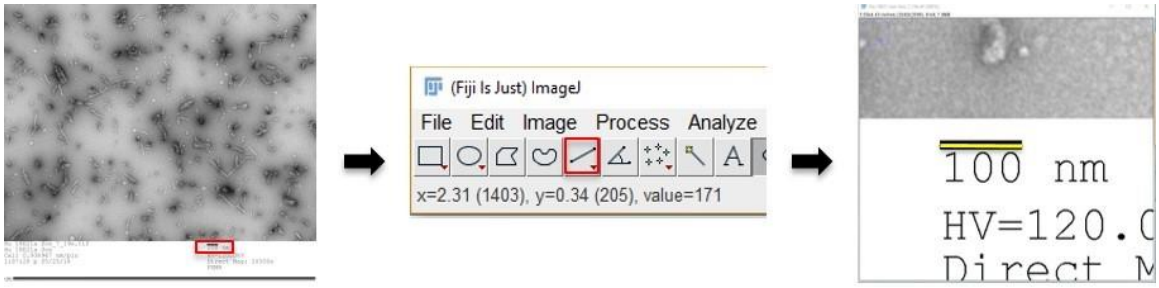
4.1 Quantifying fibril length in EM micrographs

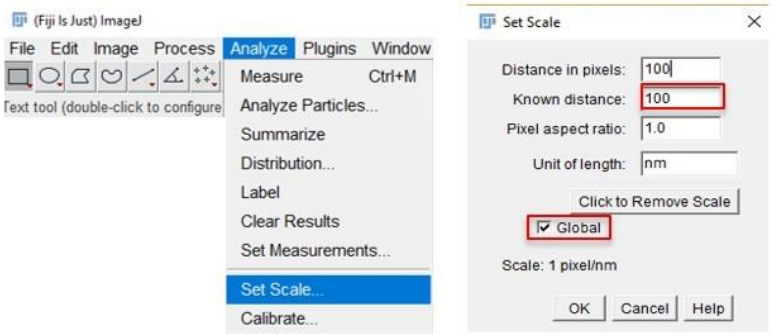
Materials:

- EM micrograph image files
- Fiji-ImageJ software
- Microsoft Excel software
- Matlab R2017b software

1. Open the Fiji-ImageJ software and open image file.
2. Select the **Straight Line** tool and draw a line along the scale bar of the image. From the **Analyze** menu, select **Set Scale…**. Enter the known distance and unit of length of the scale bar (e.g. 100 nm) and check the checkbox for **Global**.

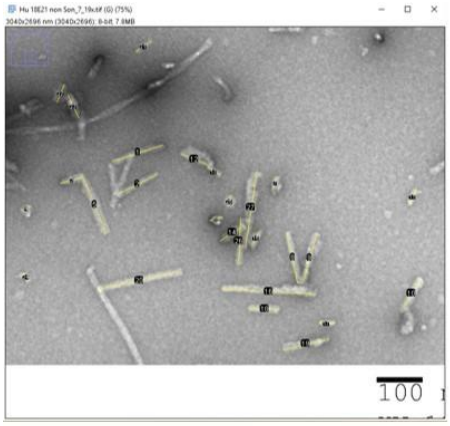
    - The magnification used to acquire the image and the type of microscope defines the scale bar. Therefore, the known distance and unit of length depends on the image scale bar.
    - Setting the scale to Global ensures that measurements for all images are based on the same scale.

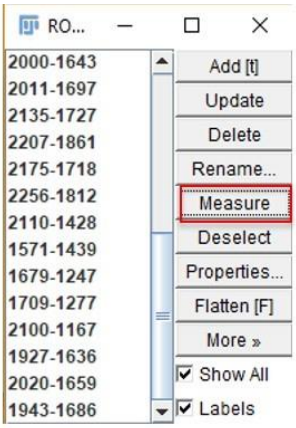


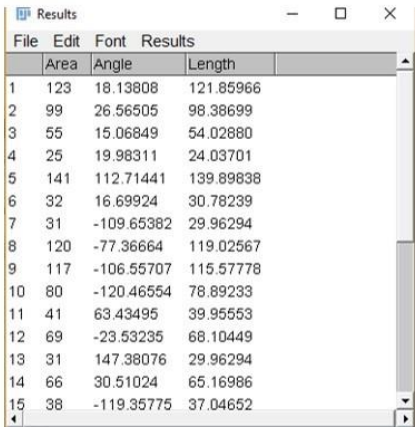


3. Select the **Straight Line** tool to measure the length of all of the fibrils in the image. Repeat process for all images to be analyzed. Open the **ROI manager** by entering CTRL+T. Check the checkboxes for **Show all** and **Labels**.

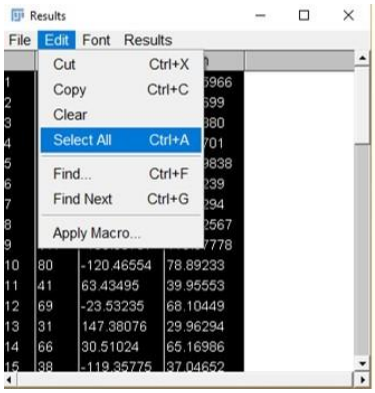
   - Do not measure fibrils that are touching the borders of the image.
   - Measure 100 to 200 fibrils per sample.

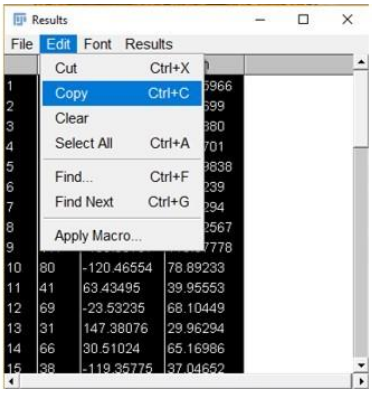


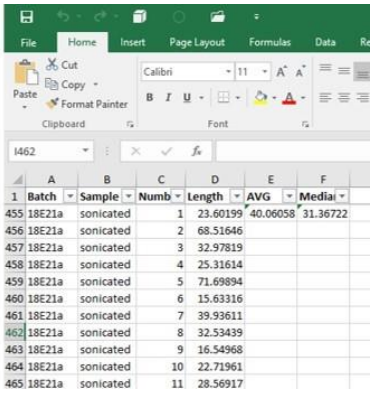




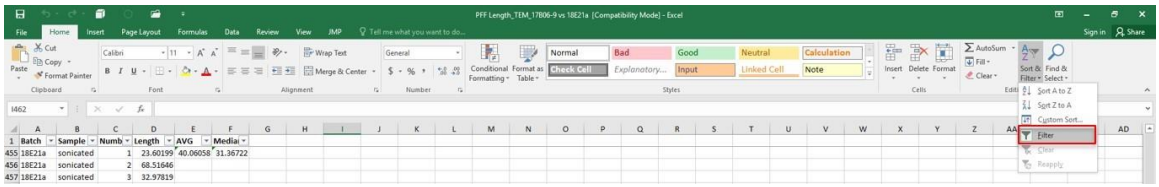
4. In the **Results** window, under the **Edit** menu, select **Select All**. Under the **Edit** menu, select **Copy**. Open a new Excel file and paste the data into the spreadsheet.





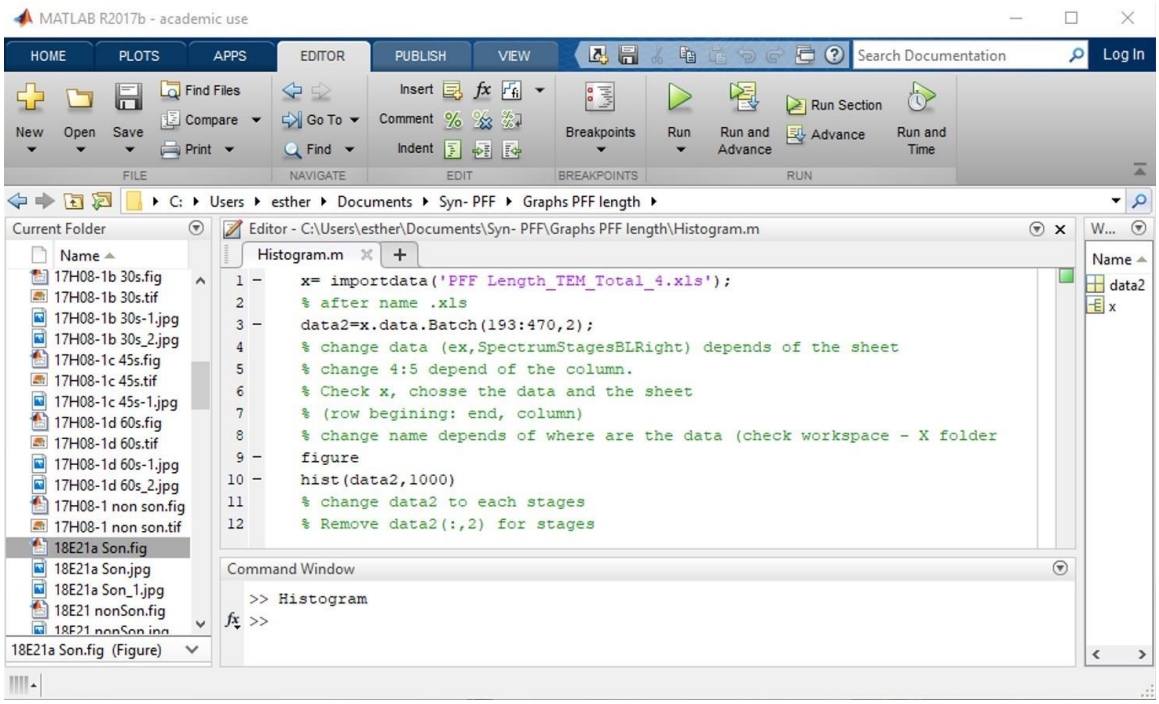


5. In Excel, select **Filter** from the **Sort & Filter** pull-down menu on the **Editing** panel of the **Home** ribbon. Filter the data in the Length column to determine the minimum and maximum fibril length measured. Calculate the average and the median of the data in the Length column.

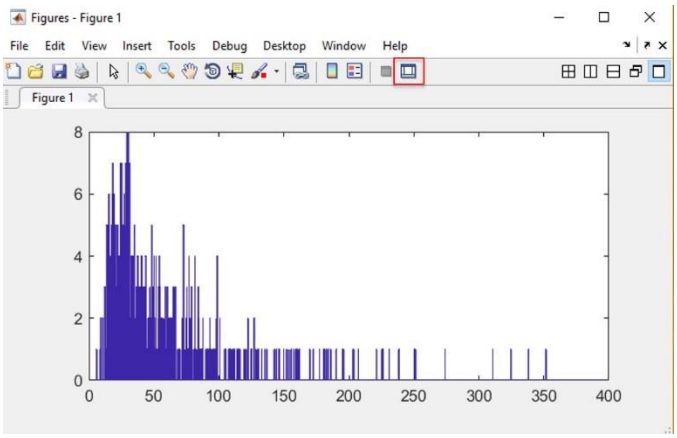


6. Open the Matlab R2017b software. Run the following macro to import the data from the Excel file and generate a histogram of the fibril lengths.

   - Ensure that the file name and extension (.xls or .xlsx) in the macro (purple text) matches the file name and extension of the Excel file containing the data to be imported.



7. In the histogram window, select the **Spot Tool** (indicated by the red box in the image) to modify the x-axis range to 0 to 120 to exclude long fibrils and obtain a better distribution of lengths.



8. Save the histogram as an image file (.tiff or .jpeg).

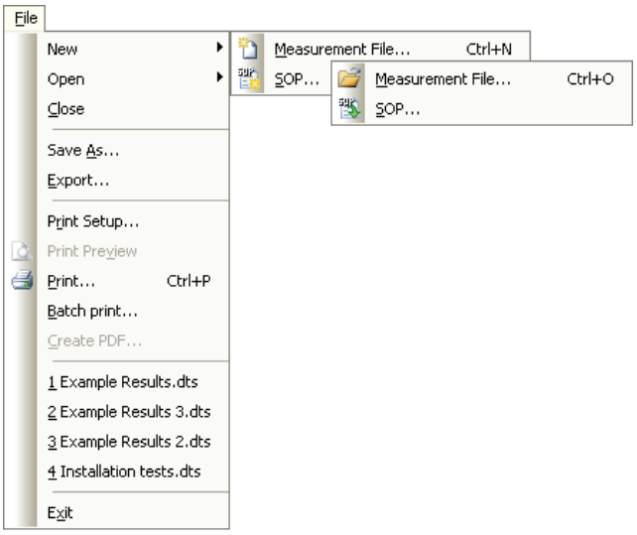
4.2 Instructions for using Malvern Zetasizer software on the Zetasizer Nano S system

1. Turn on the Zetasizer Nano S system and open the Malvern Zetasizer software .

   - The instrument must be turned on 20 minutes before use to allow the instrument to thermally equilibrate.
   - The instrument will beep when turned on, and then beep again once it has completed the initialization routine.
   - The light on the status indicator around the cell access button will turn from red to green once the system is ready for use.

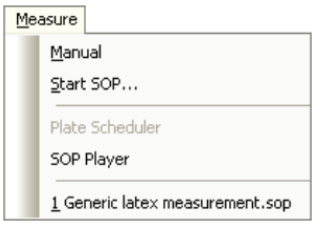
2. In the **Zetasizer** window, under the **File** menu, create a new measurement file by selecting **Measurement File** from the options under **New**.

   - If opening an existing measurement file, select **Measurement File** from the options under **Open**.

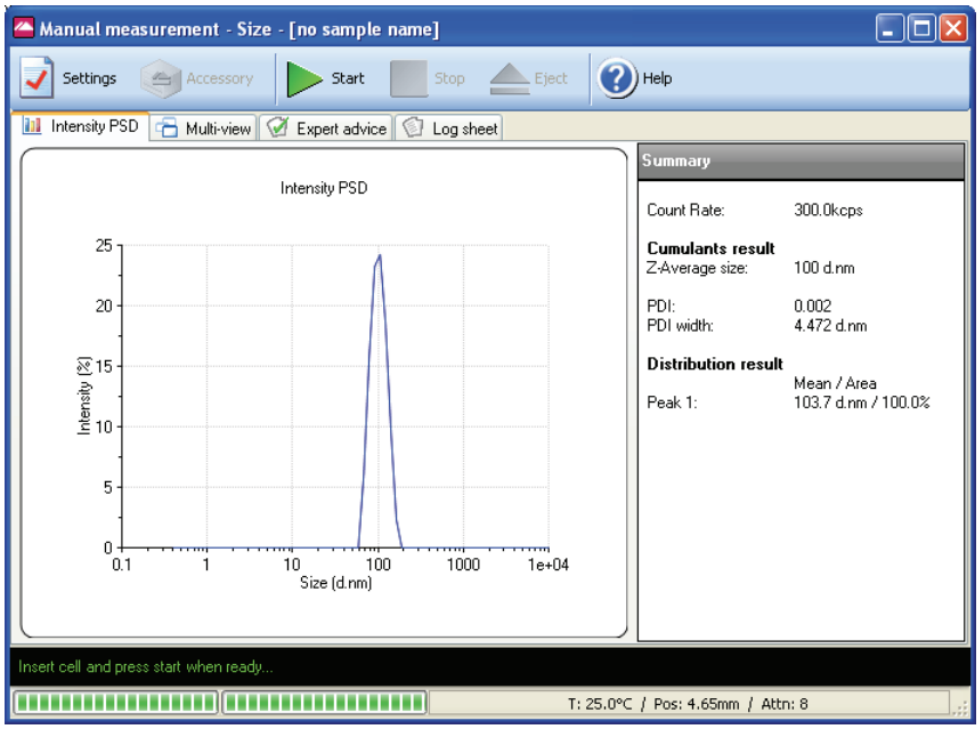


3. In the **Zetasizer** window, under the **Measure** menu, select **Manual** to perform a manual measurement. A **Manual measurement** window will open.

   - If running an existing SOP, select **Start SOP** from the **Measure** menu.

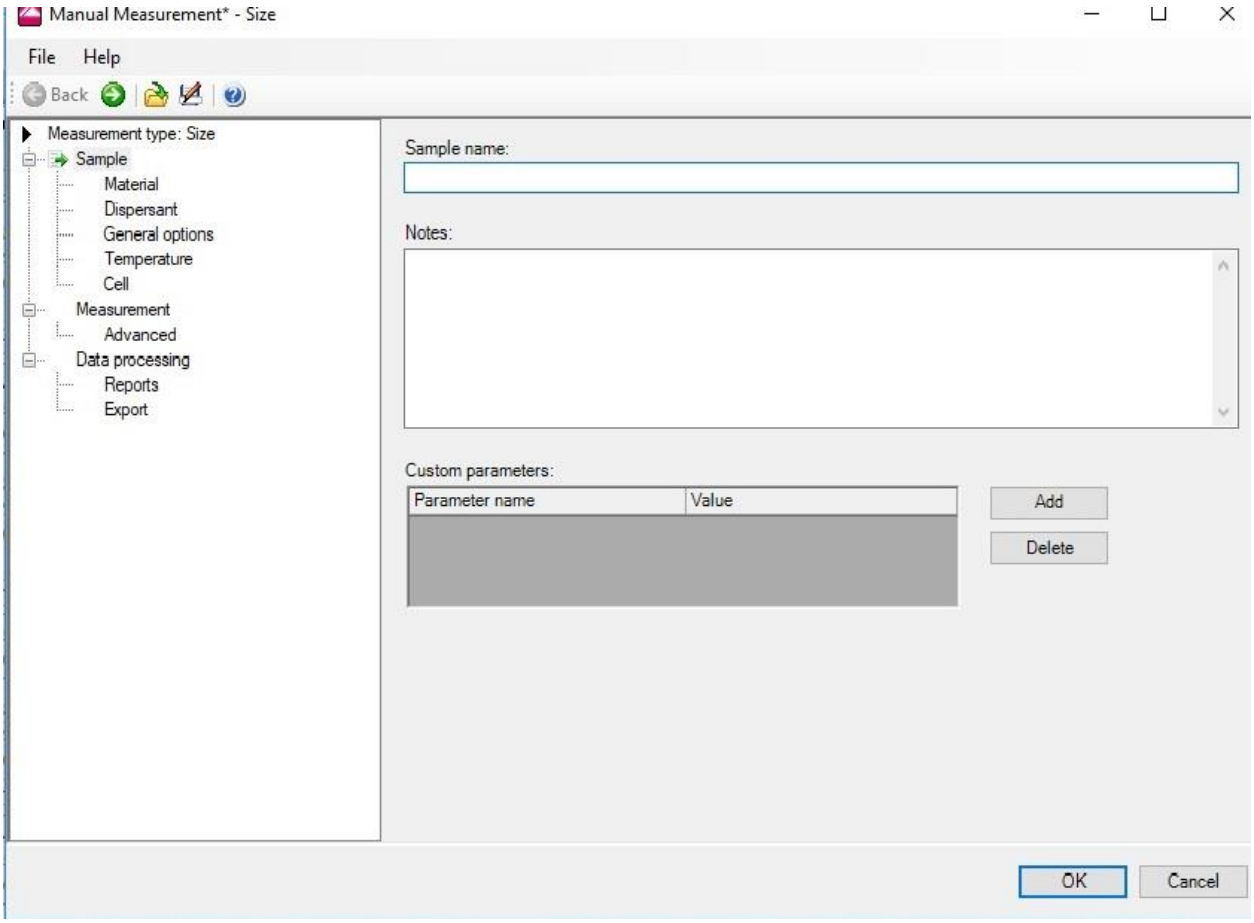


4. In the **Manual measurement** window, select **Settings**. A **Settings** window will open.

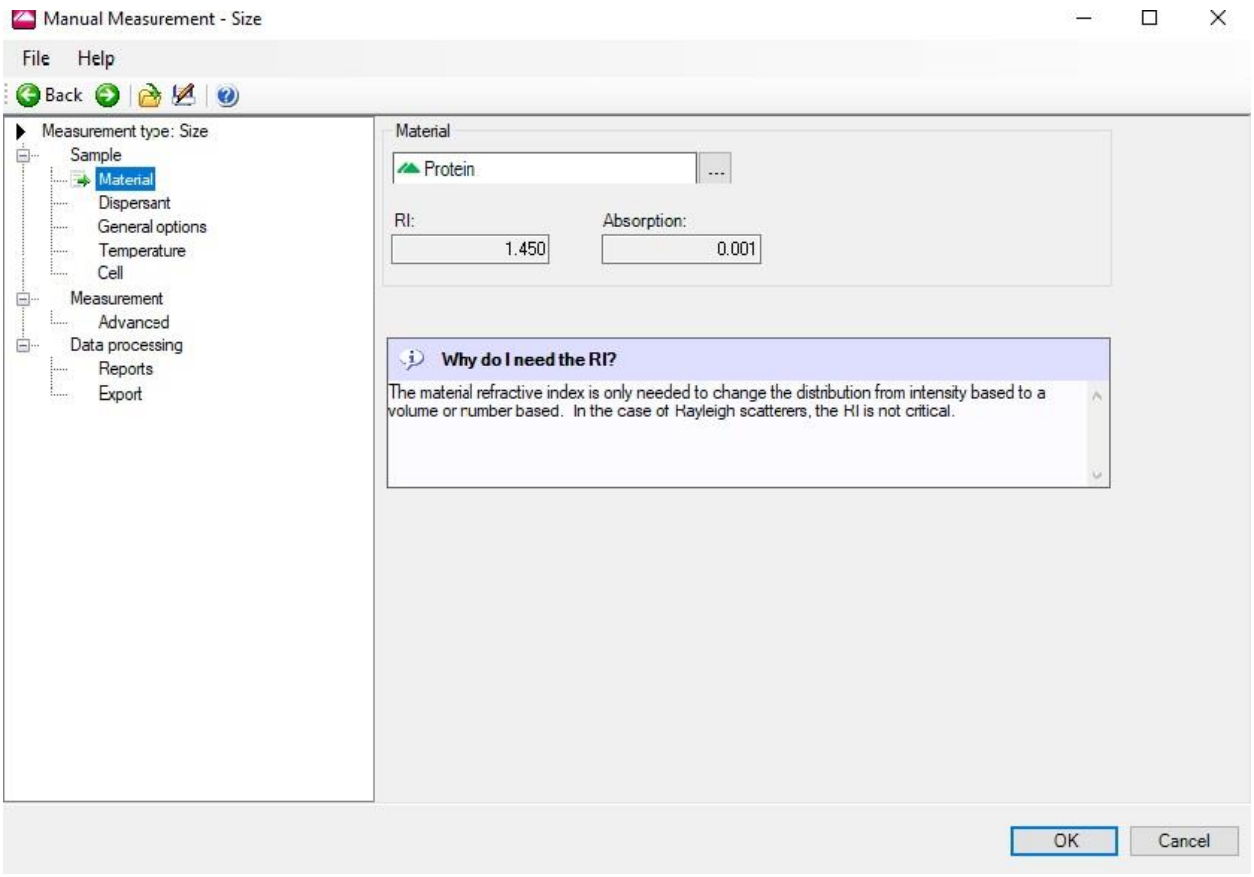


5. In the **Settings** window, input the following parameters only (do not change any other parameters from the default settings).

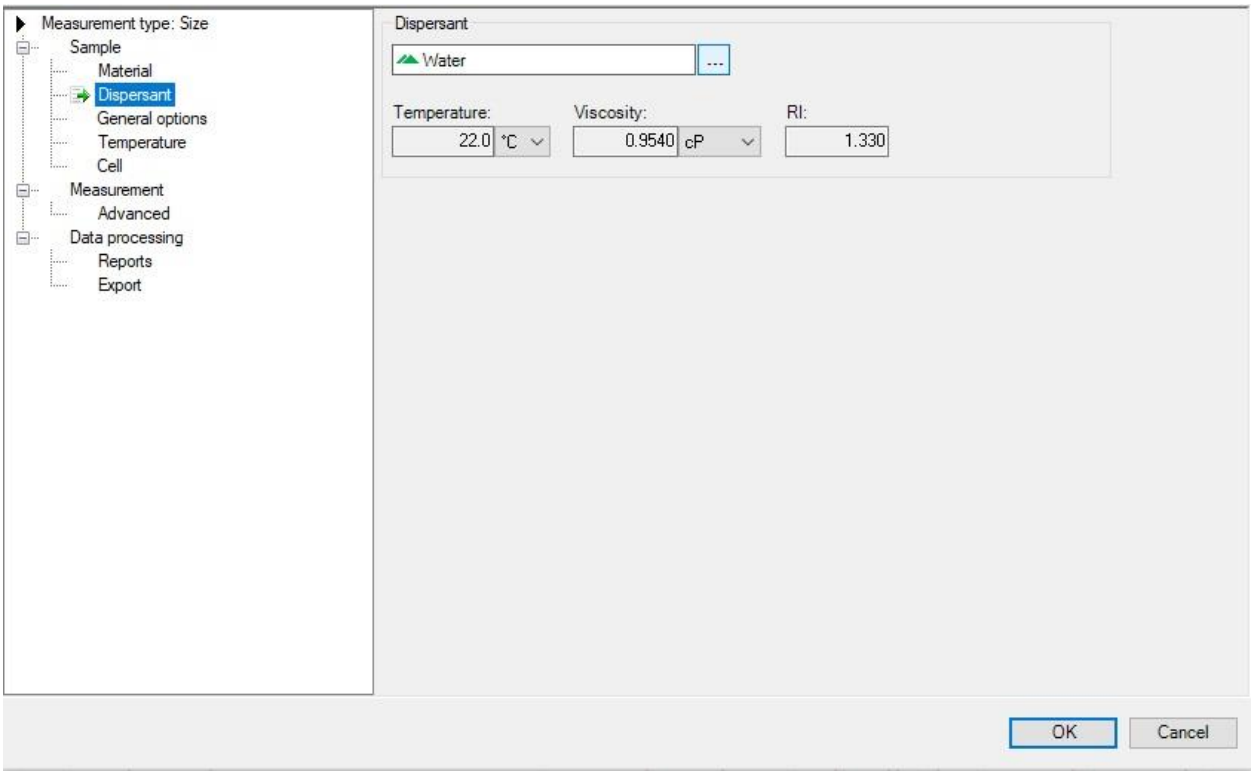
   - In the **Sample** parameters, type "Blank" in the **Sample name** field.

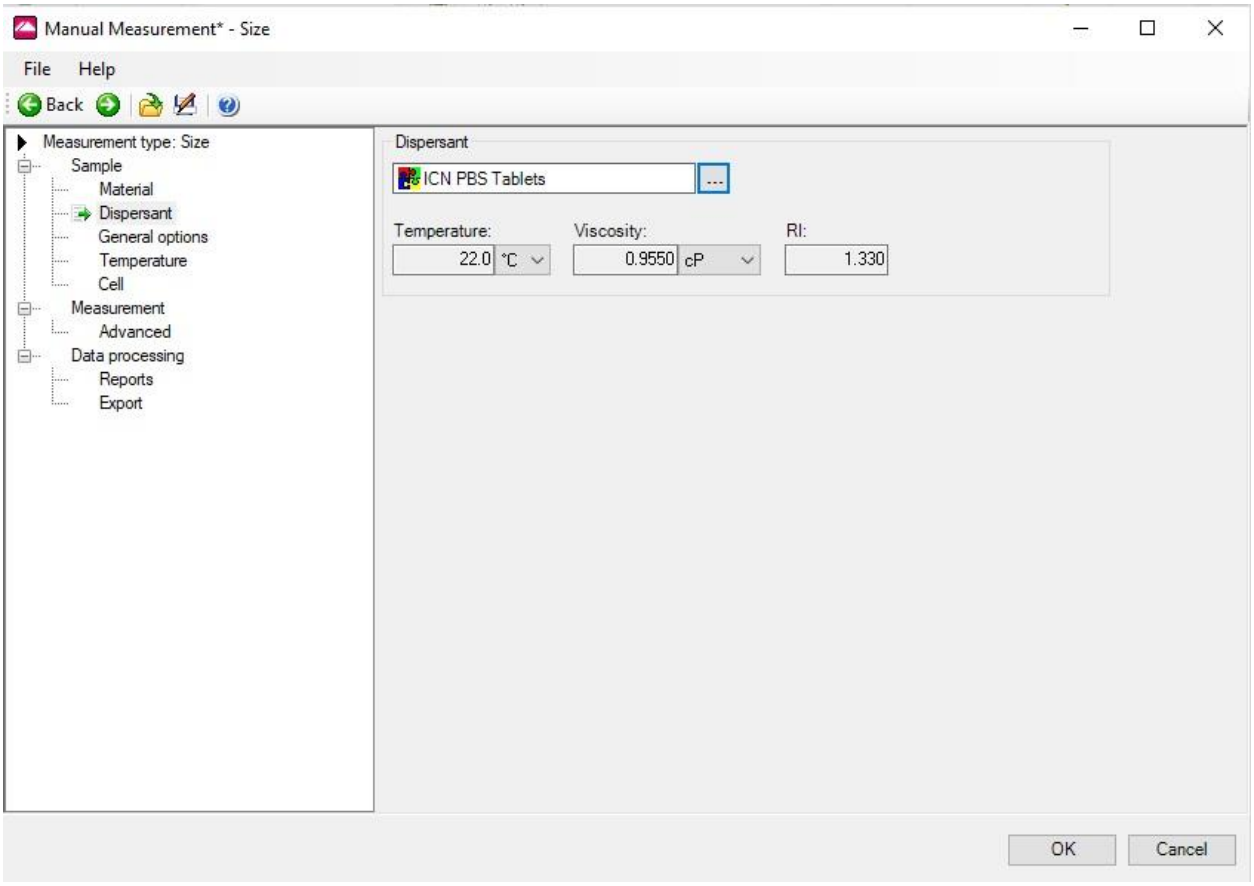


- In the **Material** parameters, select **Protein**.

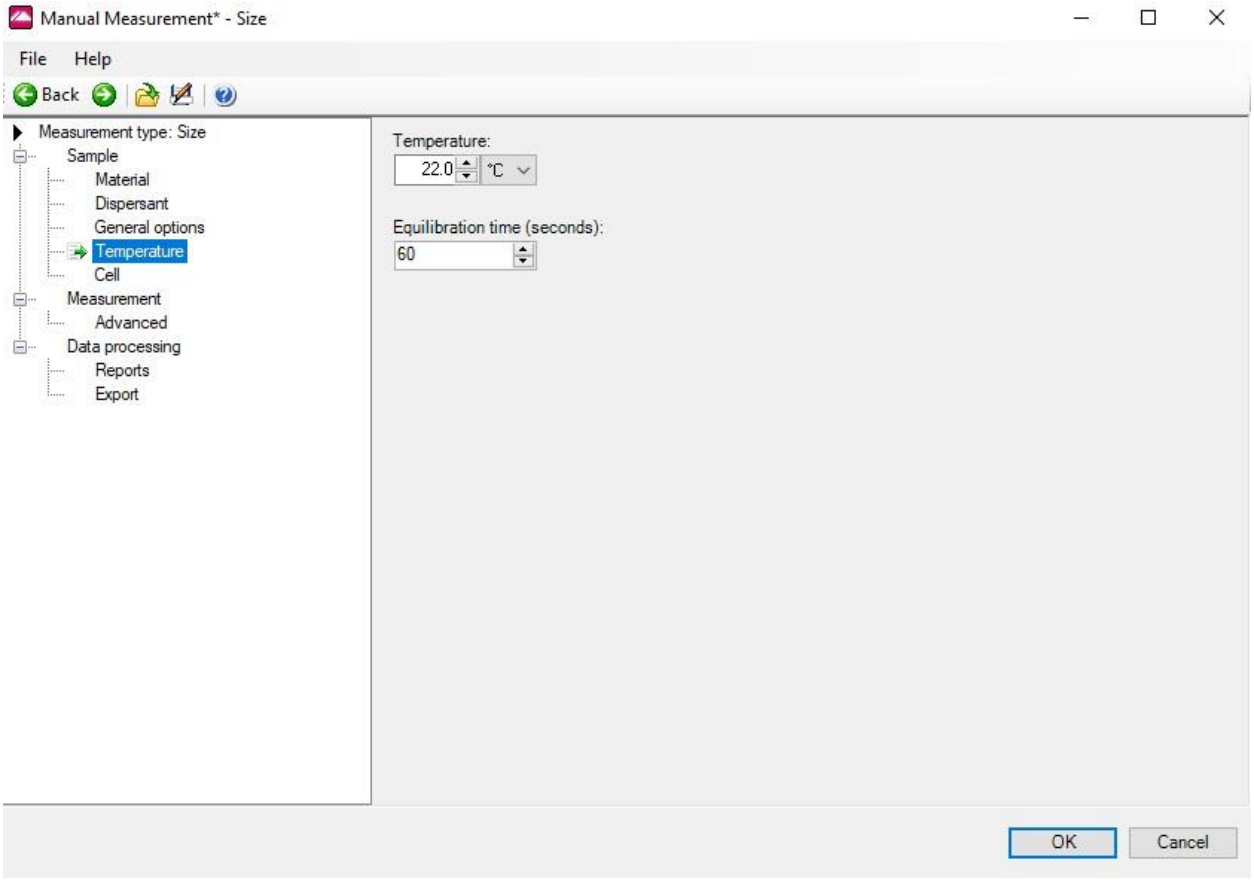


- In the **Solvent** parameters, select **Water** or **ICN PBS Tablets**.

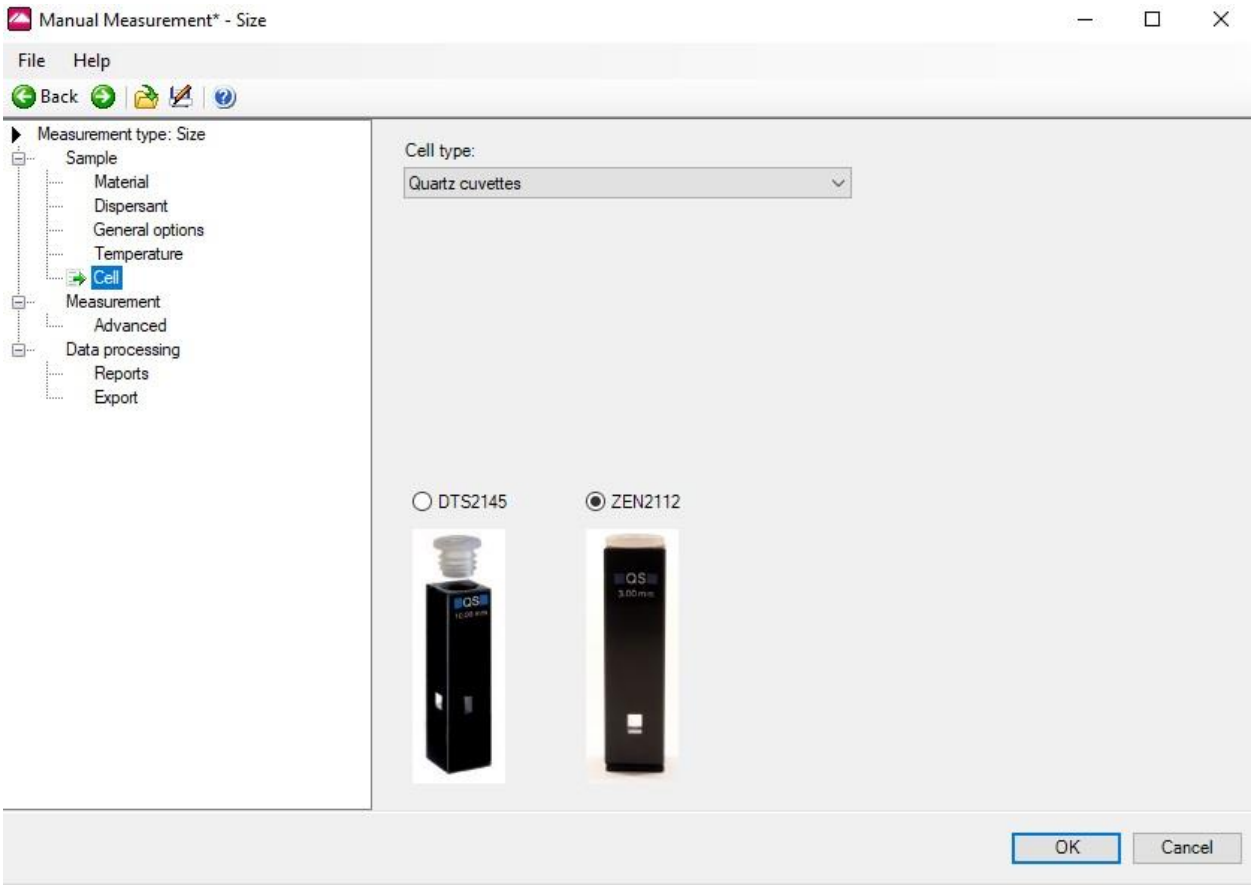



Manual Measurement* - Size
File
Help
Back
Measurement type: Size
Sample
Material
Dispersant
General options
Temperature
Cell
Measurement
Advanced
Data processing
Reports
Export
Dispersant
ICN PBS Tablets
Temperature:
22.0
°C
Viscosity:
0.9550
cP
RI:
1.330
OK
Cancel

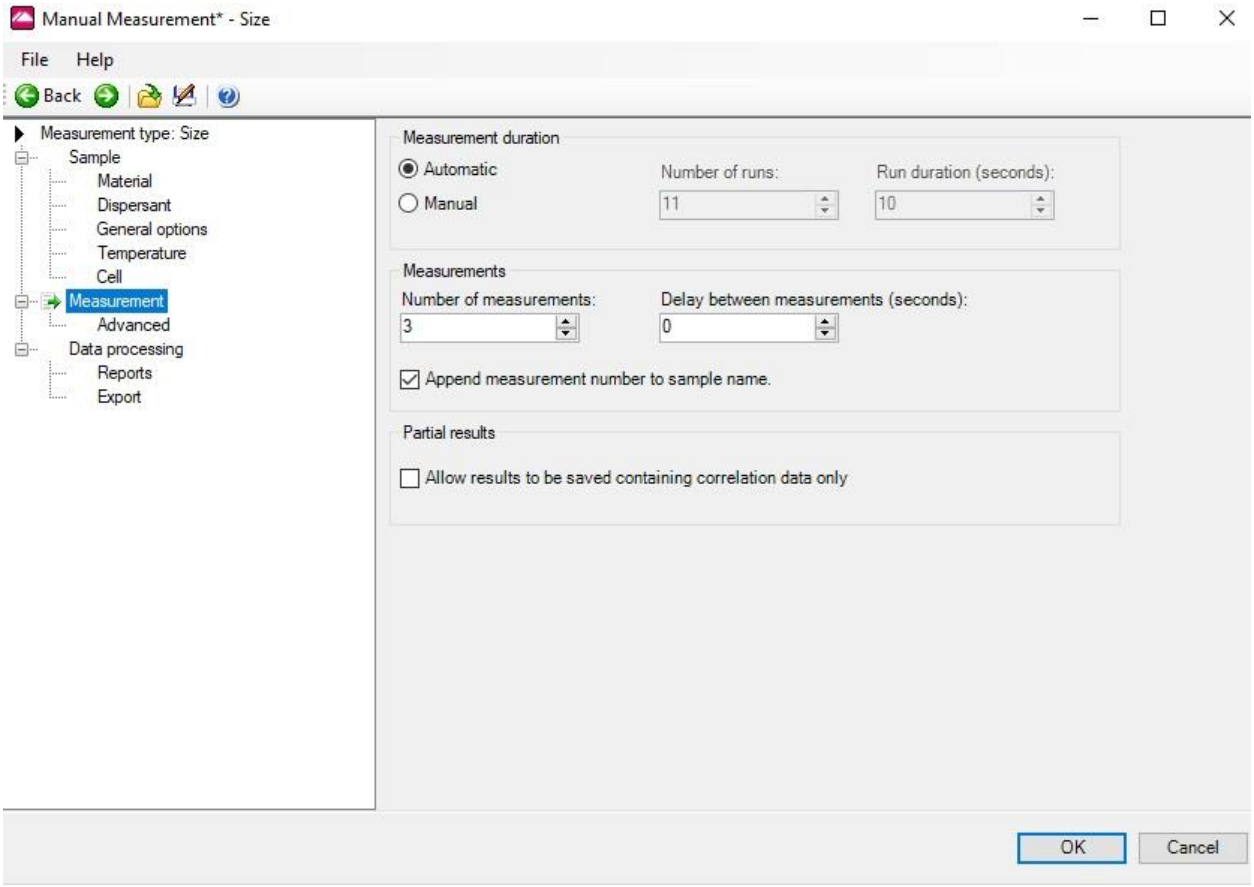
- In the **Temperature** parameters, set the **Temperature** to 22°C and set **Equilibration time (seconds)** to 60.



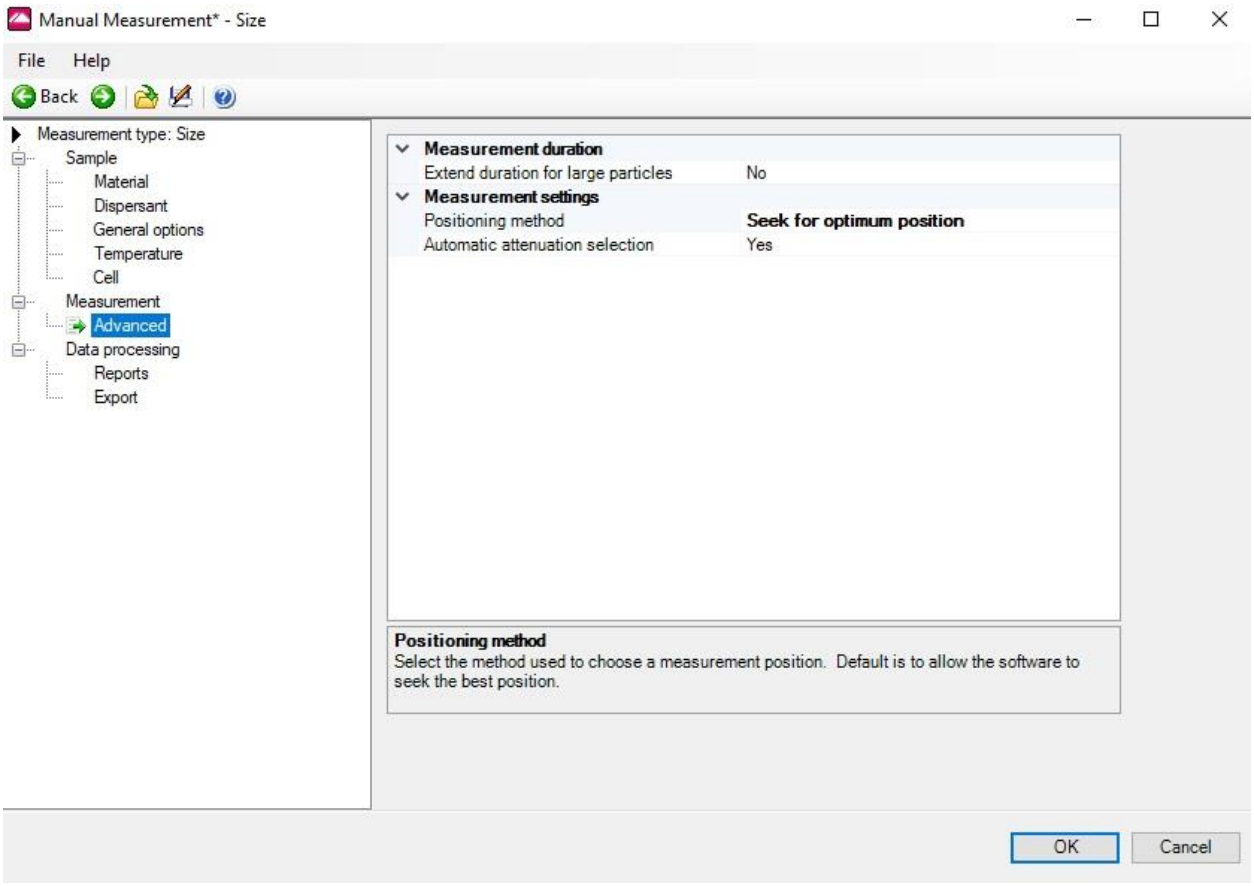
- In the **Cell** parameters, select **ZEN2112**.



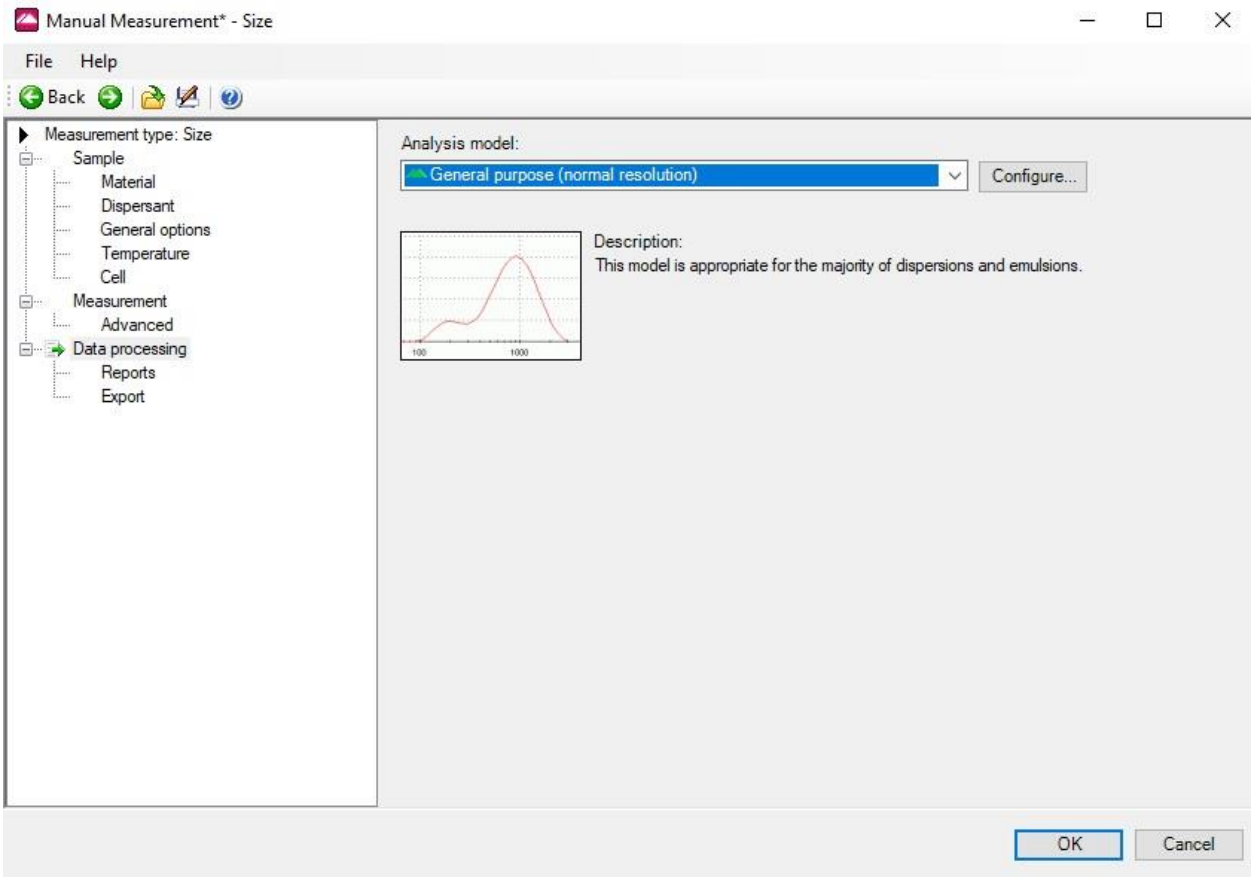
- In the **Measurement** parameters, set the **Number of measurements** to 3 and set the **Delay between measurements** to 0.



- In the **Measurement** parameters, under **Advanced**, keep default parameters.



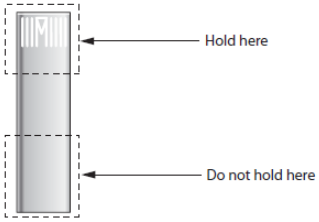
- In the **Data Processing** parameters, select **General purpose (normal resolution)**.



6. Select **OK** to save the settings.

- To create a new SOP with these settings, select **Save** under the **File** menu and then select **Save as SOP**.

7. Add 16 µL of filtered PBS into a clean cuvette and close with cap.
    - Always hold the cuvette from the top.

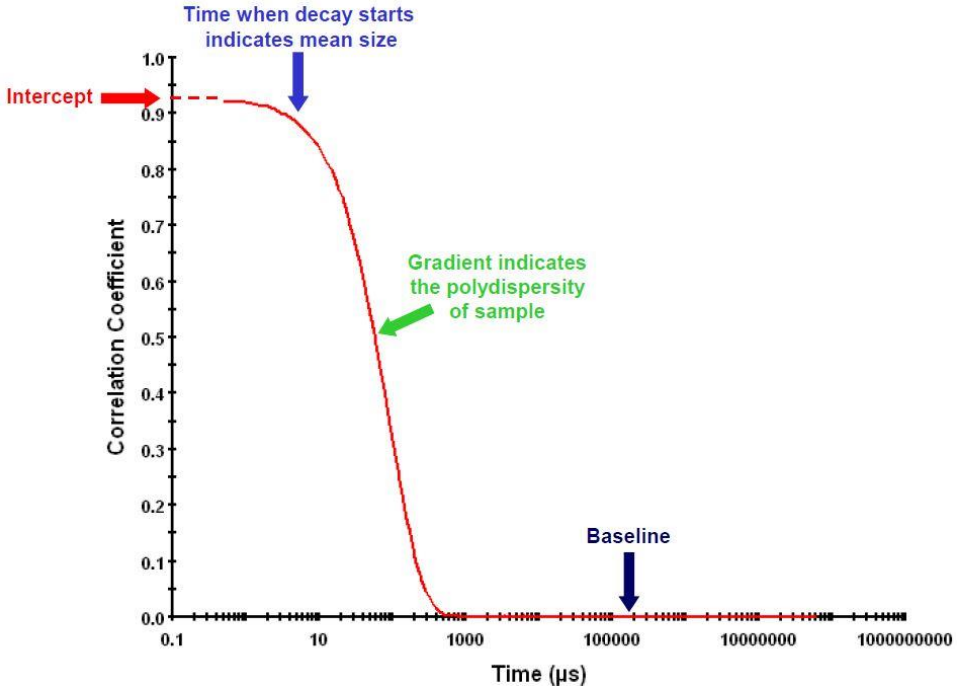


    - Avoid introducing bubbles into the solution when adding it to the cuvette.
8. Place the cuvette containing the PBS into the cell area of the instrument with the label facing you and close the instrument lid.

9. In the **Manual measurement** window, select **Start** . When the measurement is complete, remove the cuvette containing the PBS.
10. In the **Manual Measurement** window, select **Settings** . In the **Sample** parameters, change the text in the **Sample name** field to the sample name. Repeat steps 7 to 10 for each sample.
    - Wash cuvette three times with 500 µL of filtered PBS between samples.
11. Wash cuvette three times with warm ddH_2O water after all samples have been measured.
12. Fill cuvette with 1 mL 1% SDS, cap, and store for 24 hours at room temperature to decontaminate.
13. Turn off the instrument and close the software.

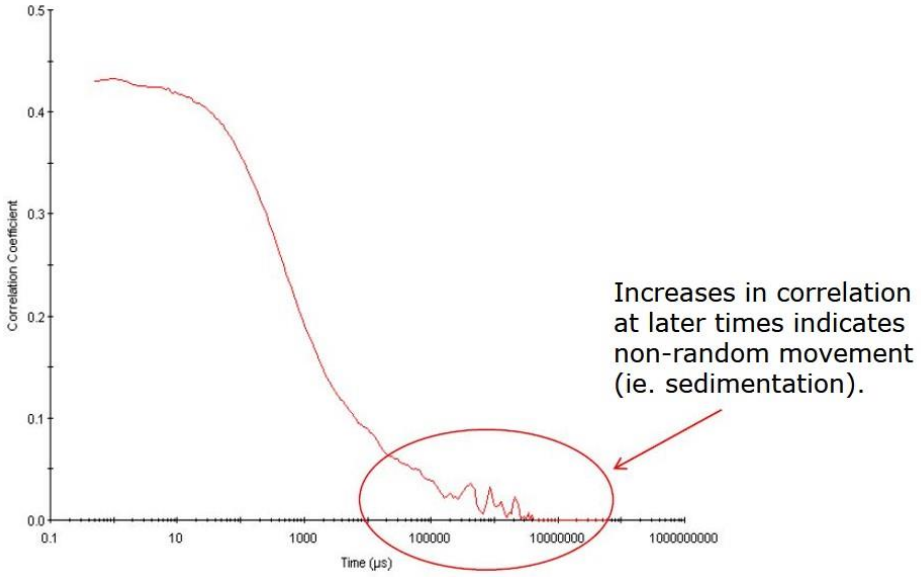
4.2.1 Raw correlation data curve

The raw correlation data curve provides information about the integrity of the sample. The features of the correlation curve are indicated in the image.

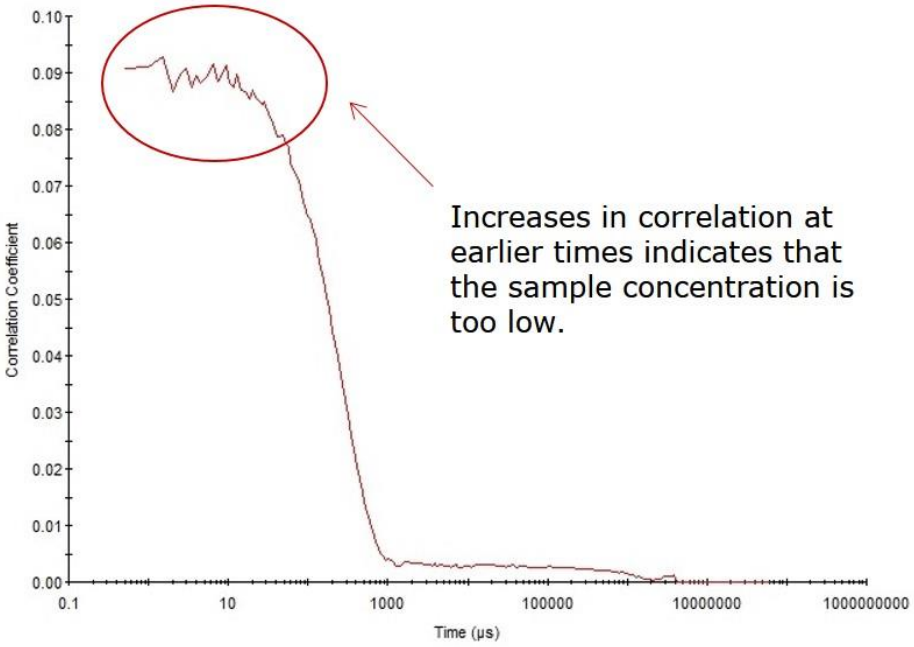


The correlation curve should be smooth. A bumpy curve indicates problems with the sample preparation.

- If there is sedimentation in the sample, the curve will appear bumpy at later timepoints, as shown in the image.



- If the sample concentration is too low, the curve will appear bumpy at earlier timepoints, as shown in the image.



- Similarly, the curve for the blank is bumpy at earlier timepoints, as shown in the image.