Submission ID #: 61741
Scriptwriter Name: Bridget Colvin
Project Page Link: https://www.jove.com/account/file-uploader?src=18831053

Title: Nanoparticle Tracking Analysis of Gold Nanoparticles in Aqueous Media Through an Inter-Laboratory Comparison

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or similar? **N**

2. **Software:** Does the part of your protocol being filmed demonstrate software usage? **Y**

   If **Yes**, we will need you to record using screen recording software to capture the steps. If you use a Mac, QuickTime X also has the ability to record the steps. Please upload all screen captured video files to your project page as soon as reasonably possible.

   Videographer: Screen capture files not provided; please film for video editing reference

3. **Interview statements:** Considering the Covid-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until the videographer steps away (≥6 ft/2 m) and begins filming. The interviewee then removes the mask for line delivery only. When the shot is acquired, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **40**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Sophie Briffa**: This protocol uses nanoparticle tracking analysis to perform accurate, reliable, and reproducible measurements of the hydrodynamic diameters of nanomaterials of interest **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Sophie Briffa**: The method is quick and easy to perform, facilitating measurement of the hydrodynamic spherical equivalent diameter of particles via particle-by-particle real time visual analysis and minimal sample preparation **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Protocol

2. Nanoparticle Tracking Analysis (NTA) System Preparation

2.1. Before performing a measurement, filter the volume of ultrapure water to be used for the analysis through a 0.02-micron syringe filter **[1]** and dilute a sample of 60-nanometer gold colloid dispersion volumetrically by a factor of 50 in the filtered water **[2-TXT]**.

2.1.1. WIDE: Talent filtering water *Videographer: Important step*

2.1.2. Talent diluting dispersion in water, with stock dispersion container visible in frame *Videographer: Important step* **TEXT: *e.g.*, 1 x 10^7 - 1 x 10^9 particles/mL**

2.2. Connect the NTA (N-T-A) instrument, syringe pump, and computer **[1]** and switch on the **[2]** hardware and software **[3]**.

2.2.1. Talent connecting system and/or pump and/or computer NOTE: Use take 1.

2.2.2. Talent switching on hardware and/or software

2.2.3. Added shot: CU of SW indicators turning green when SW initialization is complete

2.3. Remove the laser module from the system **[1]** and use a tissue and compressed air to completely dry the glass surfaces **[2-TXT]** and the LVFC (L-V-F-C) internal channels, tubing, and fluidic ports **[3-TXT]**.

2.3.1. Talent removing module *Videographer: Important step*

2.3.2. Talent cleaning instrument pieces **TEXT: LVFC: low volume flow cell** *Videographer: Important step*

2.3.3. Added shot: Talent drying LVFC **TEXT: Hold air nozzle 15cm from LVFC**

~~2.4. **[1] [2].~~ NOTE: Move 2.6 before 2.4.

~~2.4.1. Talent placing tube into container~~

~~2.4.2. Talent attaching syringe to port~~

2.5. Then flush 900 microliters of liquid through the tubing **[1-TXT]** as quickly as the back pressure allows **[Added 2.5.1]**.

2.5.1. Tubing being flushed **TEXT: Do not remove syringe to prevent siphoning** NOTE: Use shot 2.5.2.

Added shot: 2.5.1 (Talent places end of tube into waste port before flushing)

2.6. Place the LVFC onto the laser module **[1]**, then secure it with the four screws and tightening them diagonally **[1B]**. Attach the outlet tubing to the port on the right side of the cell **[2]**. NOTE: Move 2.6 before 2.4.

2.6.1. Talent placing cell onto module NOTE: Use take 2.

2.6.1B Added shot: Talent securing LVFC with bolts

2.6.2. Talent attaching tubing to port

2.7. Replace the syringe attached to the inlet tubing with a new syringe containing 1 milliliter of filter water **[1]** and connect the inlet tubing to the left port of the flow cell **[2]**.

2.7.1. Talent replacing syringe

2.7.2. Tubing being connected to port

2.8. Then slowly introduce approximately 500 microliters of fluid into the sample chamber, taking care that no air bubbles are introduced during loading **[1]**.

2.8.1. Fluid being introduced to chamber

3. Laser Module Loading and System Check

3.1. To load the laser module, insert the water filled LVFC into the instrument **[1]** and lock the cell in place **[2]**.

3.1.1. WIDE: Talent placing cell into instrument

3.1.2. Talent locking cell in place

3.2. Secure the syringe in the syringe pump cradle **[1]** and click **Start camera** in the software interface to initialize the camera **[2]**.

3.2.1. Talent securing syringe

3.2.2. Talent clicking start camera, with monitor visible in frame

3.3. In the **Hardware** tab, click **Scatter** to move the reference position and set the camera level to 16 **[1]**.

3.3.1. SCREEN: To be provided by Authors: Hardware tab being opened, then Scatter being clicked and camera level being set

3.4. Adjust the focus manually to check the diluent for any particles **[1]** and left click and drag in the main viewing window to adjust the field of view position to check for any particles **[2-TXT]**.

3.4.1. Talent adjusting focus

3.4.2. SCREEN: To be provided by Authors: Field of view being adjusted/checked
TEXT: Repeat cleaning or water filter if >3 particles visible

3.5. Replace the syringe with a syringe full of air only **[1]** and slowly replace the water in the chamber with air **[2]**.

3.5.1. Talent replacing syringe

3.5.2. Air being added to chamber

3.6. Remove the cell from the laser module from the instrument **[1]** and disconnect the pieces of tubing **[2]**.

3.6.1. Talent removing cell

3.6.2. Talent disconnecting tubing

3.7. Clean the optical glass of the laser module and the glass surfaces of the LVFC with water **[1]** and dry with a tissue and compressed air **[2]**.

3.7.1. Talent cleaning optical glass

3.7.2. Talent cleaning LVFC

~~3.8. **[1] [2].**~~

~~3.8.1. Talent drying tubing~~

~~3.8.2. Talent connecting tubing to LVFC on laser module~~

4. Sample Loading and Analysis

4.1. After reassembling the LVFC and laser module and priming the tubing, connect a syringe containing 1 milliliter of the 60-nanometer gold nanoparticle suspension to the Luer port **[1]** and slowly inject 750 microliters of the sample into the LVFC chamber **[2]**.

4.1.1. WIDE: Talent connecting syringe to port NOTE: Action ends at 0:18. *Videographer: Important step*

4.1.2. Talent injecting nanoparticles into chamber *Videographer: Important step*

4.2. After loading the laser module and initializing the camera, click **Scatter** to move the reference focus into position **[1]**, confirming that the focus is set correctly to give a clear image of the particles **[2]**.

4.2.1. SCREEN: To be provided by Authors: Scatter being clicked/reference coming into position NOTE: SCREEN shots not uploaded at postshoot time, author was reminded.

4.2.2. Added shot: Talent adjusting focus

4.3. Check that the field of view is set centrally with respect to the laser beam position and run the **AutoSetup** function to automatically optimize the focus and camera level to ensure that the optimal image quality is achieved **[1]**.

4.3.1. SCREEN: To be provided by Authors: Field of view being checked, then AutoSetup being run

4.4. To analyze the sample, open the **Standard Measurement Standard Operating Procedure** tab, and set the instrument to acquire five, 60-second videos under slow and constant flow **[1-TXT]**.

4.4.1. SCREEN: To be provided by Authors: Tab being opened, then measurement parameters being set **TEXT: Particles should take ≥10 s to cross screen**

4.5. Then set the file name and location for the data and start the run **[1]**.

4.5.1. SCREEN: To be provided by Authors: File name being set, location being set, and run being started

5. Data Analysis

5.1. To analyze the data, drag the slide bar in the **Detection Threshold** tab **[1]** to set the detection threshold so that as many visible particles are detected as possible and tracked. Make sure that no more than 5 blue crosses, indicative of noise, are observed **[2]**.

5.1.1. WIDE: Talent opening Detection tab, with monitor visible in frame

5.1.2. SCREEN: To be provided by Authors: Threshold being set

5.2. To automatically process the particle tracking analysis videos, click **Process** and leave all of the processing parameters on their automatic settings **[1]**.

5.2.1. SCREEN: To be provided by Authors: Process being clicked, then shot of parameters

5.3. Then export the data as a .csv format results file **[1]** and read the mode results and the associated standard deviation from the PDF report **[2]**.

5.3.1. SCREEN: To be provided by Authors: Data being exported

5.3.2. SCREEN: To be provided by Authors: PDF report being scrolled through

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?
2.1., 2.3., 4.1.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?
4.4. Setting an optimal flow speed can be difficult. Before running the measurement, you can test the time in which particles cross the field of view, in order to find the best setting for the pump speed. The optimal speed is when particles cross the field of view in approximately 10 seconds.

Results

6. Results: Representative Nanoparticle Tracking Analyses

6.1. Here round 1 inter-laboratory comparison results obtained using various nanoparticle tracking analysis instrument configurations are shown **[1]**.

6.1.1. LAB MEDIA: Figure 4

6.2. With the exception of Lab 6 **[1]**, the repeatability between the five capture repeats was good **[2]** even though several labs recorded a higher than expected mode size **[3]**.

6.2.1. LAB MEDIA: Figure 4 *Video Editor: please emphasize 6-NS300 data box*
6.2.2. LAB MEDIA: Figure 4 *Video Editor: please emphasize 1-NS300-3-NS300 data boxes*
6.2.3. LAB MEDIA: Figure 4 *Video Editor: please emphasize emphasize 4-LM10, 5-LM20 and 7-LM10 data boxes*

6.3. The nanoparticle tracking analysis result accuracy from round 3 was improved by all of the laboratories implementing the same standard operating procedure and instrument settings **[1]**, with an average mode across all of the labs of 62.02 ± 1.97 nanometers **[2]**.

6.3.1. LAB MEDIA: Figure 5
6.3.2. LAB MEDIA: Figure 5 *Video Editor: please emphasize add/emphasize red dashed line*

6.4. The mean diameter of the particles can be calculated using transmission electron microcopy images as a complementary technique to NTA to validate the particle size, morphology, and aspect ratio **[1]**. In this analysis, the particles appear to be mostly spherical with an average aspect ratio of 1.1 **[2]**.

6.4.1. LAB MEDIA: Supplementary Figure 1
6.4.2. LAB MEDIA: Supplementary Figure 1 *Video Editor: please emphasize/outline at least one rounded particle*

Conclusion

7. Conclusion Interview Statements

7.1. **Sophie Briffa**: It is important to make sure that the LVFC is well-cleaned prior to use, that the sample is appropriately diluted, well-loaded and -focused, and that the SOP is followed **[1]**.

7.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (2.1., 2.3., 2.8., 3.4.)

7.2. **Sophie Briffa**: This method can be used for any other nanoparticle characterization, including exosomes and viruses, to support stability studies and quality-controlled processes **[1]**.

7.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera