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Title: Nanoparticle Tracking Analysis for the Quantification and Size Determination of Extracellular Vesicles

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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 something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, all done**

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?

   ☒ Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, 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? **No**

Current Protocol Length
Number of Steps: 20
Number of Shots: 35

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Nicole Comfort:** NTA results are very prone to operator bias. This protocol demonstrates the effects of altered NTA parameters on obtained results, as a standardized method will help to increase rigor and reproducibility in analyses **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Suggested B role: 4.4*

1.2. **Tessa Bloomquist:** Preparing the sample in a cuvette allows a statistically random sample to be generated for each video, resulting in more reproducible data and the visualization of particles over a wide range of sizes **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Suggested B role: 3.3*

OPTIONAL:

1.3. **Nicole Comfort:** As getting a proper blank in the expected particle concentration range can be difficult, be sure to perform a sequential dilution to identify the ideal dilution factor **[1]**.

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

Introduction of Demonstrator on Camera

1.4. **Nicole Comfort:** Demonstrating the procedure will be Kunheng Cai, a PhD student from Anthony Ferrante's laboratory **[1]**.

1.4.1. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera.

Ethics Title Card

1.5. All work with these samples was performed in compliance with Institutional Animal Care and Use Committee (IACUC) and Institutional Review Board guidelines at Columbia University.

Protocol

2. Cuvette Preparation

2.1. To prepare a cuvette for nanoparticle tracking analysis, cover the workspace with a lint-free material to prevent fibers from entering the cuvettes **[1]**. Wearing gloves, place a cuvette containing a stir bar onto the magnetic cuvette jig **[2]**.
- 2.1.1. WIDE: Establishing shot of talent at the workspace
- 2.1.2. Talent placing the cuvette onto magnetic cuvette jig

2.2. Use a hook tool to place the insert into the cuvette **[1]** with the "notch" of the insert visible at the front of the cuvette **[2].**
- 2.2.1. Talent placing the insert into the cuvette
- 2.2.2. Shot of insert with notch at front of cuvette

2.3. Use a pipette to slowly add 400 to 500 microliters of purified extracellular vesicles into the cuvette through the hole in the insert **[1-TXT]** and mix the sample by gently pipetting without introducing air bubbles **[2].**
- 2.3.1. Talent adding diluted sample to the cuvette **TEXT: See text for EV preparation details**
- 2.3.2. Talent mixing the sample

2.4. Then cap the cuvette, tapping out bubbles as necessary **[1]**, and use a lint-free cloth to wipe the outside surface of the cuvette **[2]**.
- 2.4.1. Talent capping/tapping the cuvette *Videographer: This step is important*
- 2.4.2. Talent wiping the outside faces of cuvette

3. Particle Tracking Instrument Preparation and Blank Nanoparticle Tracking Analysis (NTA)

3.1. To analyze the particle concentration of the diluent, turn on the computer workstation and instrument **[1]** and start the particle tracking analysis program **[2]**.
- 3.1.1. WIDE: Talent turning on computer and/or instrument
- 3.1.2. Talent at computer, opening the software program with monitor visible in frame

3.2. When prompted, click **NTA** (N-T-A) and open the **Recording** tab. Follow the onscreen instructions to fill out all the necessary sample information. For EV (E-V) tracking analysis, set the **Diluent** to PBS. The salinity will auto-populate to 9% **[1-TXT]**.
- 3.2.1. SCREEN: 62447_3.2.1.mp4 0:12-0:39 *Video Editor: speed up the video as necessary* **TEXT: EV: extracellular vesicle**

3.3. To obtain the diluent particle concentration, open the instrument lid **[1]** and remove the protective cap covering where the cuvette will be placed **[2]**. Load the cuvette into the instrument in the correct orientation, with the "notch" of the insert facing the camera **[3]** and replace the cap and instrument lid **[4]**.
3.3.1. Talent opening lid
3.3.2. Talent removing the protective cap covering
3.3.3. Talent placing the cuvette inside the instrument, note orientation of insert *Videographer: This step is important*
3.3.4. Talent replacing the cap cover/instrument lid

3.4. Click the streaming arrow to turn on the camera and click the chevron arrow to expand the Record settings. Adjust the focus until the relatively small particles are clearly visible **[1]**.
3.4.1. SCREEN: 62447_3.4.1.mp4 0:03-0:12

3.5. To set the analysis for small EV quantification, set the **Frame rate** to 30 frames per second, the **Exposure** to 15 milliseconds, the **Stir** time to 5 seconds, the **Wait** time to 3 seconds, the **Blue**, **Green**, and **Red Laser powers** to 210-, 12-, and 8-milliwatts, respectively, the frames per video to 300, and the Gain to 30 decibels **[1]**.
3.5.1. SCREEN: 62447_3.4.1.mp4 0:12-0:31

3.6. Adjust the focus until the relatively small particles are clearly visible. Increasing the Zoom and/or the Gain can help with particle focusing, but if you increase the Gain, remember to set it to 30 decibels prior to recording **[1]**.
3.6.1. SCREEN: 62447_3.5.1.mp4 0:11-0:34

3.7. Once particles are in focus, set the zoom setting to 0.5x to save bandwidth and to prevent lost frames and click **Record** to begin recording the video **[1-TXT]**.
3.7.1. SCREEN: 62447_3.6.1.mp4 0:04-0:35 *Video Editor: speed up the video as necessary* **TEXT: Repeat NTA for particle sample**

4. NTA Data Processing, Display, and Interpretation

4.1. When a prompt appears stating that the videos have been recorded, click **OK** to complete the recording **[1]** and select the **Process** tab. If very large particles were visible in any videos while recording, navigate to the directory of recorded videos and remove the problematic video prior to processing **[2]**
4.1.1. Talent clicking ok, with monitor visible in frame. **NOTE: Not filmed**
4.1.2. SCREEN: 62447_4.1.2.mp4 0:04-0:45

4.2. Check the **Disable auto detection override** box and set the **Feature Diameter** to 30. Click **Process** to initiate video processing and view a live distribution graph **[1-TXT]**.

4.2.1. 4.2.1. SCREEN: 62447_4.2.1.mp4 0:10-0:27 **TEXT: See text for exosome analysis details**

4.3. When the processing is complete, click **OK** and select the **Plot** tab. For EVs, display the Main chart as **LogBinSilica**. Other features of the graph, such as changing the x-axis to set the area for integration for the figure produced, can be customized **[1]**.
4.3.1. SCREEN: 62447_4.3.1.mp4 0:03-0:32

4.4. To create a PDF report of the results, click the **Report** button. The mean, median, mode size, concentration adjusted for the dilution factor, and distribution width will be displayed **[1-TXT]**.
4.4.1. SCREEN: 62447_4.4.1.mp4 0:05-0:45 **TEXT: Subtract blank sample particle concentration from sample particle concentration** *Video Editor: please emphasize mean, median, mode size, concentration, and distribution width when mentioned*

4.5. Then record the instrument settings used to generate the results **[1]**.
4.5.1. Talent at computer, recording instrument settings into lab notebook or similar **NOTE: Not filmed**

5. Cuvette Cleaning

5.1. To clean the cuvettes after analysis, empty the cuvette **[1]** and completely fill the cuvettes 10 to 15 times with de-ionized water **[2]** and 3 times with 70 to 100% ethanol to remove any residual sample **[3]**. *Videographer: This step is important*
5.1.1. WIDE: Talent emptying cuvette
5.1.2. Talent filling cuvette with water
5.1.3. Cuvette being filled with ethanol

5.2. Dry the outside of the cuvettes with a lint free microfiber cloth **[1]** and dry the inside with a compressed air duster **[2]**. *Videographer: This step is important*
5.2.1. Talent cleaning the outside of cuvette with lint free microfiber cloth
5.2.2. Talent cleaning the inside of the cuvette with air duster

5.3. To clean the inserts and stir bars, place the materials in a glass scintillation vial containing 70 to 100% ethanol **[1]** and shake the vial vigorously **[2]**. Then rinse the inserts and stir bars in deionized water with shaking as demonstrated **[3]** and dry them using lint free cloths **[4]**. *Videographer: This step is important*
5.3.1. Talent placing the insert and stir bar in ethanol
5.3.2. Talent shaking vial
5.3.3. Talent placing the insert and stir bar in water
5.3.4. Talent drying the insert and/or stir bar with lint free cloth

5.4. After drying, immediately place all of the cleaned components into storage until the next analysis **[1]**.

5.4.1. Talent placing the clean component in a container

Results

6. Results: Using a Novel NTA Instrument to Estimate EV Size Distribution and Total Particle Concentration

6.1. Before performing an analysis, the instrument calibration was tested using polystyrene beads to ensure the validity of the acquired data **[1]**. As observed, the particle tracking instrument accurately reported the size of the 100-nanometer monodisperse beads **[2]** but only closely reported the size of the 400-nanometer beads **[3]**.
- 6.1.1. LAB MEDIA: Figure 8
- 6.1.2. LAB MEDIA: Figure 8 *Video Editor: please emphasize peak between red lines in Figure 8A*
- 6.1.3. LAB MEDIA: Figure 8 *Video Editor: please emphasize peak between red lines in Figure 8B*

6.2. Therefore, the instrument settings for this protocol were more accurate for smaller particles closer to 100 nanometers in size **[1]**.
- 6.2.1. LAB MEDIA: Figure 8

6.3. Using these settings **[1]**, reported particle concentration scales accordingly with the dilution factor, demonstrating that the instrument can accurately detect the particle concentration at various dilutions with little variability between technical replicates **[2]**.
- 6.3.1. LAB MEDIA: Figure 9
- 6.3.2. LAB MEIDA: Figure 9 *Video Editor: please emphasize the data bars from left to right in succession*

6.4. The optimum dilution for a 4.41 x 10^{10} particles/milliliter mouse tissue-derived EV sample was determined to be between 1000 and 3000 **[1]**.
- 6.4.1. LAB MEIDA: Figure 9 *Video Editor: please emphasize 1500 and 2000 data bars*

6.5. In this analysis, increasing the gain increased the sensitivity of the camera **[1]**, allowing an increase in the visualization of a higher number of smaller particles **[2]**.
- 6.5.1. LAB MEDIA: Figure 10 and Table 5 *Video Editor: please sequentially emphasize images from Figure 10A to 10B*
- 6.5.2. LAB MEDIA: Figure 10 and Table 5 *Video Editor: please emphasize Gain 18, 24, and first 30 data rows*

6.6. Increasing the blue laser power from 70 to 210 milliwatts **[1]**, while keeping the green and red laser powers constant **[2]**, shifted the reported average particle size from 122

to 105 nanometers **[3]** and increased the reported total particle concentration from 1.1 x 10^8 to 1.7 x 10^8 **[4]**.

6.6.1. LAB MEDIA: Table 5 *Video Editor: please emphasize 3rd and 4th data rows*

6.6.2. LAB MEDIA: Table 5 *Video Editor: keeping 3rd and 4th rows emphasized, circle/highlight 12 and 8 values (red and green laser mW) in 3rd and 4th rows*

6.6.3. LAB MEDIA: Table 5 *Video Editor: keeping 3rd and 4th rows emphasized, circle/highlight 122 and 105 values (Average nm values) in 3rd and 4th rows*

6.6.4. LAB MEDIA: Table 5 *Video Editor: keeping 3rd and 4th rows emphasized, circle/highlight 1.10E+08 and 1.70E+08 values (Total Concentration values) in 3rd and 4th rows*

6.7. Increasing the power of the red laser **[1]** increased the reported average particle size from 175 to 246 nanometers **[2]** and decreased the reported total particle concentration **[3]**. Increasing the green laser power **[4]** resulted in a decrease in the reported average particle size **[5]** and an increase in reported total particle concentration **[6]**.

6.7.1. LAB MEDIA: Table 5 *Video Editor: please emphasize 9th and 10th data rows*

6.7.2. LAB MEDIA: Table 5 *Video Editor: keeping 9th and 10th data rows emphasized, circle/highlight 175 and 246 (Average size) in 9th and 10th data rows*

6.7.3. LAB MEDIA: Table 5 *Video Editor: keeping 9th and 10th data rows emphasized, circle/highlight 1.50E+07 and 9.20E+06 (Total concentration) in 9th and 10th data rows*

6.7.4. LAB MEDIA: Table 5 *Video Editor: please emphasize 7th and 8th data rows*

6.7.5. LAB MEDIA: Table 5 *Video Editor: keeping 7th and 8th data rows emphasized, circle/highlight 169 and 148 (Average size) in 7th and 8th data rows*

6.7.6. LAB MEDIA: Table 5 *Video Editor: keeping 7th and 8th data rows emphasized, circle/highlight 2.80E+07 and 4.40E+07 (Total Concentration) in 7th an 8th data rows*

Conclusion

7. Conclusion Interview Statements

7.1. **Kunheng Cai:** Finding the right dilution to place the sample within the optimal detection range can take a few tries for each sample. The cuvette cleaning also requires extra careful handling **[1]**.

7.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B role: 2.3, 5.1 and 5.2*

7.2. **Nicole Comfort:** We recommend applying more than one orthogonal method for each EV particle size and concentration measurement. DLS, resistive pulse sensing, TEM, and western blotting can also be performed to characterize the EVs **[1]**.

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