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TITLE:

Improving Reproducibility to Meet Minimal Information for Studies of Extracellular Vesicles 2018 Guidelines in Nanoparticle Tracking Analysis

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SUMMARY:

Nanoparticle tracking analysis (NTA) is a widely used method to characterize extracellular vesicles. This paper highlights NTA experimental parameters and controls plus a uniform method of analysis and characterization of samples and diluents necessary to supplement the guidelines proposed by MISEV2018 and EV-TRACK for reproducibility between laboratories.

ABSTRACT:

Nanoparticle tracking analysis (NTA) has been one of several characterization methods used for extracellular vesicle (EV) research since 2006. Many consider that NTA instruments and their software packages can be easily utilized following minimal training and that size calibration is feasible in-house. As both NTA acquisition and software analysis constitute EV characterization, they are addressed in Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018). In addition, they have been monitored by Transparent Reporting and Centralizing Knowledge in Extracellular Vesicle Research (EV-TRACK) to improve the robustness of EV experiments (e.g., minimize experimental variation due to uncontrolled factors).

Despite efforts to encourage the reporting of methods and controls, many published research papers fail to report critical settings needed to reproduce the original NTA observations. Few papers report the NTA characterization of negative controls or diluents, evidently assuming that commercially available products, such as phosphate-buffered saline or ultrapure distilled water, are particulate-free. Similarly, positive controls or size standards are seldom reported by researchers to verify particle sizing. The Einstein-Stokes equation incorporates sample viscosity and temperature variables to determine particle displacement. Reporting the stable laser

chamber temperature during the entire sample video collection is, therefore, an essential control measure for accurate replication.

The filtration of samples or diluents is also not routinely reported, and if so, the specifics of the filter (manufacturer, membrane material, pore size) and storage conditions are seldom included. The International Society for Extracellular Vesicle (ISEV)'s minimal standards of acceptable experimental detail should include a well-documented NTA protocol for the characterization of EVs. The following experiment provides evidence that an NTA analysis protocol needs to be established by the individual researcher and included in the methods of publications that use NTA characterization as one of the options to fulfill MISEV2018 requirements for single vesicle characterization.

INTRODUCTION:

Accurate and repeatable analysis of EVs and other nanometer-scaled particles presents numerous challenges across research and industry. Replication of EV research has been difficult, in part, due to the lack of uniformity in reporting necessary parameters associated with data collection. To address these deficiencies, the ISEV proposed industry guidelines as a minimal set of biochemical, biophysical, and functional standards for EV researchers and published them as a position statement, commonly referred to as MISEV2014[1]. The accelerating pace of EV research required an updated guideline, and the "MISEV2018: a position statement of the ISEV"[2] expanded the MISEV2014 guidelines. The MISEV2018 paper included tables, outlines of suggested protocols, and steps to follow to document specific EV-associated characterization. As a further measure to facilitate interpretation and replication of experiments, EV-TRACK was developed as a crowd-sourcing knowledgebase (http://evtrack.org) to enable more transparent reporting of EV biology and the methodology used for published results[3]. Despite these recommendations for standardized reporting of methods, the field continues to suffer regarding replicating and confirming published results.

Fitting with the National Institutes of Health's and National Science Foundation's effort for quality assessment tools, this paper suggests that ISEV requires standardized reporting of methods and details so that data assessment tools might be applied with the goal of replicating results between laboratories. Reporting cell sources, cell culture procedures, and EV isolation methods are important factors to define the qualities of the EV population. Among NTA instruments, factors such as detection settings, the refractive index of carrier fluid, heterogeneous particle populations contributing to polydispersity, lack of standardized reporting requirements, and absent intra- and inter-observer measurement results make NTA comparison between labs difficult or impossible.

In use since 2006, NTA is a popular method for nanoparticle size and concentration determination that is currently used by approximately 80% of EV researchers[4]. The MISEV2018 Guidelines require two forms of single-vesicle analysis, of which NTA is one of the popular options. NTA continues to be in common use for EV characterization due to its wide accessibility, low cost per sample, and its straightforward founding theory (the Stokes-Einstein equation). EV assessment by NTA generates a particle size distribution and concentration estimate using laser light

scattering and Brownian motion analysis, with the lower limit of detection determined by the refractive index of the EV. When using a fluid sample of known viscosity and temperature, the trajectories of the EVs are tracked to determine their mean-square displacement in two dimensions. This allows the particle diffusion coefficient to be calculated and converted into a sphere-equivalent hydrodynamic diameter by a modified Stokes-Einstein equation[5-7]. NTA's particle-to-particle analysis has less interference by agglomerates or larger particles in a heterogeneous population of EVs than other methods of characterization[7]. While a few larger particles have minimal impact on sizing accuracy, the presence of even minute amounts of large, high light-scattering particles results in a notable reduction in the detection of smaller particles due to reduced software EV detection and tracking[8]. As a measurement technique, NTA is generally considered not to be biased toward larger particles or aggregates of particles but can resolve multiple-sized populations through individual particle analysis[9]. Because of the use of light-scattering by particles, one of the limitations of NTA analysis is that any particulate such as dust, plastic, or powder with similar refraction and size attributes compared to EVs cannot be differentiated from actual EVs by this method of characterization.

The NanoSight LM10 (nanoparticle size analyzer) and LM14 (laser module) have been sold since 2006, and although newer models of this instrument have been developed, this particular model is found in many core facilities and is considered a reliable workhorse. Training is needed to properly optimize the NTA settings for high-resolution measurements of size and concentration. The two important settings needed for optimum video recordings are (1) the camera level and (2) the detection threshold. These must be set by the operator based on the sample's characteristics. One of the major constraints of NTA analysis is the recommendation of sample concentrations between 10^7 and 10^9 particles/mL to achieve which sample dilution may be required[10]. Solutions used for dilution, such as phosphate-buffered saline, 0.15 M saline, or ultrapure water, are rarely free of particles less than 220 µm in size, which may affect the NTA measurements. NTA characterization of the solutions used for dilution should be performed at the same camera level and detection threshold as the nanoparticle samples that are being analyzed. The size and concentration of nanoparticles present in diluents used for EV sample dilutions are seldom included in publications involving NTA analysis of EVs.

This protocol uses NTA analysis of synthetic EV-like liposomes evaluated using selected camera levels, detection thresholds, and mechanical filtering of the samples to analyze the systematic effects of camera level, detection threshold, or sample filtration on the NTA dataset. Liposomes were synthesized as described in **Supplemental File S1**. Synthetic liposomes were used in this experiment because of their size uniformity, physical characteristics, and stability in storage at 4 °C. Although actual samples of EVs could have been used, the heterogenicity and stability of EVs during storage may have complicated this study and its interpretation. Similarities in the NTA reports from (A) liposomes and (B) EVs indicate that the systematic effects revealed for liposomes in this paper will likely also apply to EV characterization (**Figure 1**). Together, these findings support the notion that complete reporting of critical software settings and the description of sample processing, such as diluent, dilution, and filtration, impact the reproducibility of NTA data.

The purpose of this paper is to demonstrate that varying the NTA settings (temperature, camera level, and detection threshold) and sample preparation changes the results collected: systematic, significant differences in size and concentration were obtained. As NTA is one of the popular options to fulfill the MISEV2018 characterization specification, these results demonstrate the importance of reporting sample preparation and NTA settings to ensure reproducibility.

(Place **Figure 1** here)

PROTOCOL:

1. General protocol guidelines

1.1. Maintain the microscope on an air table or at a minimum on a vibration-free table. Ensure that extraneous vibrations (e.g., foot tapping on the floor, touching the table, door closures, laboratory traffic) are kept to a minimum.

1.2. Set and maintain the temperature of the laser module at a constant temperature for all video recordings.

NOTE: The temperature chosen was 25 °C because the nanoparticle size analyzer was calibrated at that temperature. Therefore, it is important that all users of the instrument know and use this temperature. Room temperature is not an acceptable setting because it can vary.

1.3. Ensure that all diluents, also called negative controls (e.g., Dulbecco's Phosphate-buffered Saline (DPBS), ultrapure distilled water [DW]), are all NTA-characterized using the same camera level and detection threshold as the nanoparticle samples being measured. Use characterized DPBS for the flushing of the laser module and dilution of samples. Factor contaminants in the negative control DPBS into sample results if their levels are significant.

1.4. Store samples at 4 °C prior to evaluation and never freeze them as this would degrade the liposome sample. Warm samples/standards/diluents to room temperature for 30 min prior to analysis.

NOTE: Sample handling was specific to the material being examined.

1.5 Evaluate sample and standards using **Quick measurement** to establish an appropriate dilution to obtain 10^8 to 10^9 particles/mL (approximately 50 to 100 particles/NTA video screen) determined to be optimal for NTA analyses.

NOTE: The authors have found that particle concentrations at the higher end of this range produce more consistent and reproducible results.

1.6. Fill in all applicable fields within the **Capture** box of the **SOP** tab for both **Quick** and **Standard** measurements, including the dilution and diluent used.

2. Preparation of 50 nm and 100 nm size calibration standards

NOTE: See the **Table of Materials**.

2.1. 50 nm size Transfer Standards diluted 1:5,000

2.1.1. Add 2 µL of the 50 nm standards to 9,998 µL of 0.22 µm-filtered 10 mM potassium chloride (KCl)/0.03% Tween 20 in ultrapure DW into a twice-rinsed 15 mL conical tube. Store the diluted 50 nm standard at 4 °C for up to a year.

2.2. 100 nm size Transfer Standards diluted 1:333

2.2.1. Add 30 µL of the 100 nm standards to 9,970 µL of 0.22 µm-filtered 10 mM KCl/0.03% Tween 20 in ultrapure DW into a twice-rinsed 15 mL conical tube. Store the diluted 100 nm standard at 4 °C for up to a year.

3. Cleaning and assembly of the laser module

3.1. Visually inspect the laser module and flow-cell cover windows for scratches or imperfections.

NOTE: If either glass surface is scratched, imaging may be affected.

3.2. Clean both glass surfaces gently with a good quality lens cleaner and lens paper. Do not use tissue wipes or paper towels on glass surfaces.

3.3. Ensure that the O-ring seal is properly seated in the groove of the flow-cell cover prior to assembly.

3.4. Place the flow-cell cover on the laser module, ensuring the electrical contacts are in the proper orientation. Place the 4 spring-loaded thumbscrews through the flow-cell plate and engage the threads of the laser module but do not tighten individually.

3.5. Putting uniform pressure down on the flow-cell cover, uniformly tighten the thumbscrews in an alternating diagonal manner until snug. Only tighten the thumbscrews until finger-tight. Do not overtighten.

NOTE: Uneven tightening of the thumbscrews can crack the laser module surface. Newer-designed thumbscrews will “bottom out” at the proper pressure, avoiding possible overtightening.

4. Flushing procedure for the laser module prior to and between samples

4.1. Use a newly opened container of DPBS and aliquot into triple-rinsed 15 mL polypropylene tubes. Ensure that products used to flush or dilute samples are NTA-characterized prior to use (see step 1.3).

4.2. Flush two 1 mL tuberculin syringes with slip lock adaptors 3 times with 1 mL of DPBS to remove any particulate residues. Use either port as the input but use it consistently during the experiment. Do not use larger syringes due to the danger of breaking syringe ports from the increased weight and size of the syringe.

4.3. Remove and discard the plunger from the 1st tuberculin syringe and insert it into the remaining port to serve as a voided diluent/sample reservoir.

NOTE: Failure to remove the plunger will cause increased pressure in the sample chamber and leakage around the seal.

4.4. Fill the 2nd syringe with 1 mL of DPBS and attach it to the inlet port of the flow-cell cover.

4.5. Hold the laser module tilted with the outlet syringe port elevated to allow air to be purged from the chamber as the DPBS is injected slowly into the laser module.

4.6. Flush the remaining DPBS from the laser module by injecting 1 mL of air into the inlet port. Repeat flushing 2 more times.

4.7. Empty the laser module as completely as possible after the last flush.

NOTE: Thorough, careful flushing is necessary following NTA analysis of the 50 nm standard, as these particles persist in the laser module. The laser module is now ready to be used.

5. Placement of the laser module on the microscope stage

5.1. Locate the laser module focus alignment guides on the arm of the microscope (**Figure 2**) and align them using the focus knob.

[Place **Figure 2** here]

5.2. Facing the microscope, place the laser module in the grooved stage and gently slide it as far as possible to the right.

NOTE: If done carefully, the alignment and focal spot will be easier to locate between samples.

5.3. Turn on the rocker switch on the laser control box.

6. Focusing and positioning of the laser module

NOTE: This must be performed with fluid in the chamber.

6.1. Load the NTA software (see the **Table of Materials**) from the desktop.

NOTE: An error may appear, "**Temperature H/W not found on COM3**." Simply close and reopen the software to fix it.

6.2. Click **Start Camera** under the **Capture** tab in the upper left corner box. If the camera shuts off automatically after 5 min, simply click **Start Camera** again to restart it.

6.3. In the same tab, adjust **Camera Level** to **14 to 16** to brighten the laser line and simplify particle identification and focus.

6.4. Divert the image from the camera to the eyepieces by moving the top slider on the left side of the headpiece in or out.

6.5. Find the area of increased density, commonly referred to as the **thumbprint**. Center and focus the thumbprint vertically in the field of view.

NOTE: The laser line will be to the left of the thumbprint. Darkening the room may help facilitate locating the thumbprint and focusing on the laser line.

6.6. Center the laser line in the field of view; move the top slider to divert light to the camera as observed on the computer screen.

NOTE: The laser line now visible on the screen is a mirror image of the eyepiece view; left in the eyepieces will be right on the computer screen.

6.7 Adjust the focus to sharpen the image of individual moving particles on the screen with the focus knob.

NOTE: Due to the depth of field, all the particles will not be in focus, which is acceptable. Even particles that are slightly out of focus will be captured by the camera and analyzed by the software.

7. Loading standards/samples/diluent into the laser module for NTA analysis

7.1. Draw up 1 mL of standard/sample/diluent into a rinsed 1 mL tuberculin syringe and attach it to the inlet port of the flow-cell cover. Advance the plunger until fluid is evident in the open syringe attached to the outlet port.

7.2. In the camera view, move to the right of the laser line to an area of a uniform number of particles. If necessary, adjust the vertical orientation to center the horizontal bands of light. Refocus until the highest number of particles are in view. Ensure that for all subsequent

measures, this position is maintained as closely as possible.

7.3. Adjust the **Camera Level** to the point that the **Dark** information symbol flashes intermittently on and off in the top right of the camera view.

NOTE: This helps ensure that a consistent camera level selection is made at the minimum sensitivity level for each data collection series.

NOTE: **Camera Level** cannot be changed during capture.

8. Validation of calibration

NOTE: It is recommended to validate the module calibration using size standards (see section 2) prior to sample analysis. Routine validation is necessary to ensure accurate measurements. In a multiuser laboratory, individual user adjustments of software configuration settings can inadvertently cause inaccurate data collection. For critical data collection, daily validation is a matter of good laboratory practice. The day-to-day reproducibility of validation needs to be included in the reported results. Typically, calibration is set by the technician and is not adjustable by the individual user unless the user has administrator access. This prevents unauthorized reconfiguration by individual users.

8.1. Warm diluted standards (see section 2) to room temperature for 30 min.

8.2. Briefly vortex the standards and then load as described in section 7.

8.3. Perform sample NTA as described in section 10 and record values for subsequent calculation of the coefficient of variation, which should be less than 2% if correctly calibrated.

9. Optimizing sample concentration for NTA

NOTE: The screen should contain between 50 and 100 measurable particles when the camera level and sample concentration are adjusted properly. If there is any question about whether a sample has an appropriate particle number, a **Quick Measurement** can be run on the sample at this point (see steps 9.1 to 9.7). It is used to assess the sample characteristics rapidly prior to longer video captures. The **Quick Measurement** tab is found within the **SOP** tab in the bottom middle box.

9.1. Set **Capture duration** to **30 s**.

9.2. Accept the existing base filename or enter a new filename by clicking the **…** tab for a new storage site for generated data.

9.3. Check the box for **Target temperature** and input the desired temperature.

9.4. Load the sample as previously described (step 7.1) and click **Create and Run Script**.

9.5. Wait for the number of particles per frame to be displayed on the bottom right of the video screen after the completion of the 30 s video. If the number of particles is greater than 100, flush the laser module 3 times (as previously described in step 4.6).

9.6. Dilute the sample to the desired concentration range using the characterized diluent.

9.7. Load the properly diluted sample into the flushed laser module and run the **Quick Measurement** to verify the sample is within the acceptable range.

10. Sample NTA

NOTE: The **Standard Measurement** tab is within the **SOP** tab in the bottom middle box and is used for routine sample analysis (see steps 10.1 to 10.12).

10.1. Set the duration to **30 or 60 seconds** and the number of videos to **5**.

10.2. Accept the existing base filename or enter a new filename by clicking the **…** tab for a new storage site for generated data.

10.3. Check the box for **Target temperature** and input the desired temperature.

10.4. Click **Create Script** to reuse this **Standard Measurement**.

10.5. Once the sample is loaded as described in section 7 and the experiment is ready to run, click **Create and Run Script**.

10.6. Fill out the fields in the **Set Report Details** popup screen with information on the operator, sample description, dilution of the sample, and diluent used.

NOTE: This information will be recorded and printed on the final experimental report.

10.7. When all desired fields have been filled, click **OK** to initiate the script.

NOTE: If **Target temperature** was selected, the heater would stabilize the sample in the laser module to 25 °C for 5 s prior to allowing the script to proceed with the measurement. The diagnostic panel in the lower-left corner of the screen will read HEATER ON and display the temperature of the sample.

10.8. Prior to each video capture, look for a prompt to **Advance** the plunger manually. Inject ~0.05 mL of the sample into the laser chamber and allow the particles to come to “rest” (i.e., not flowing), and then click **OK**.

10.9. Wait for a **Settings Confirmation** box to appear upon the completion of the 5th video capture and for the **Process box** to flash. Set the **Detection Threshold** for processing of the sample by noting the number of blue crosses marking particles on the screen as the frames are advanced manually from the bottom of the video screen. If there are more than 3–4 blue crosses marking particles on each screen as frames are advanced, increase the **Detection Threshold**.

NOTE: The blue crosses on the individual particles are analogous to the "swarm" effect in flow cytometry and should be minimized for optimum accuracy and reproducibility of data collection.

10.10. Click **Settings OK** when the Detection Threshold is acceptable.

10.11. Wait for the videos to be automatically processed and a histogram of results and a dialog box notification of completion to be displayed before clicking **OK**.

10.12. Once the **Export Settings** box appears, save the results by clicking **Export**.

NOTE: All results from videos and analysis will be stored in the destination file defined in step 10.2. This requires a large amount of storage. Monitor and transfer to a secondary storage device as necessary.

11. **Re-analysis of the current sample at different detection thresholds**

NOTE: Immediately following NTA analysis (step 10), the data can be reanalyzed using different Detection Threshold settings. However, **Camera Level** cannot be modified following capture.

11.1. Highlight all 5 of the capture videos listed in the **Current Experiment**.

11.2. Click **Process Selected Files**, and wait for the **Setting Confirmation** box to appear and the **Process box** to flash to change **Detection Threshold**.

11.3. Adjust the **Detection Threshold** to the desired level and click **Setting | OK**.

11.4. Wait for the videos to be automatically processed and a histogram of results and a dialog box notification of completion to be displayed before clicking **OK**.

11.5. Click **Export Settings**. When additional evaluations are performed on the most recent sample, be sure to click the **Export Results** box in the **Current Experiment** as the **Export Results** popup box will not be displayed following the sample re-analysis.

NOTE: As there are no reminders to do this, it can easily be overlooked, and the analysis will be lost. However, the underlying data will remain and can be reanalyzed later.

12. **Analysis of archived files**

NOTE: If previously analyzed experiments have not been saved or additional analysis needs to be done on these samples, the individual files can be reloaded into the NTA software for additional **Detection Threshold** evaluations. **Camera Level** changes cannot be modified following capture.

12.1. Load the NTA software from the desktop.

12.2. Click the **Analysis** tab in the lower middle panel.

12.3. Click **Open Experiment** and navigate to the desired .nano file.

NOTE: The video files associated with the selected .nano experiment must be in the same folder as the main experiment file; otherwise, an error will appear when trying to process the files. The first 6 digits of the filename are the date the experiment was run (**xx-xx-xx**). The last 6 digits of the filename are the time the video was recorded (**xx-xx-xx**) and the individual video identifier. The PDF file of each combined experiment lists the included .nano files for that analysis.

12.4. Click **Process Selected Files** to run the analysis.

12.5. Once the **Process** box flashes to allow changes in **Detection Threshold,** set the threshold to the desired level and click **OK**.

12.6. Wait for the videos to be automatically processed and a histogram of results and a dialog box notification of completion to be displayed before clicking **OK**.

12.7. Click **Export Results**. Be sure to click the **Export Results** box in the **Current Experiment**, as the **Export Results** popup box will not be displayed following the re-analysis.

NOTE: As there are no reminders to do this, it can easily be overlooked, and the analysis will be lost.

13. **Cleaning and disassembly of the laser module**

13.1. Turn off the laser control box.

13.2. Flush the entire sample from the laser module and discard it properly.

13.3. Hold the laser module tilted with the open syringe port elevated to allow air to be purged from the chamber as the DPBS is injected slowly into the laser module and is flushed from the outlet syringe port.

13.4. Flush the remaining DPBS from the laser module and discard.

13.5. Repeat flushing 2 more times and empty the laser module as completely as possible after the last flush.

13.6. Putting uniform pressure down on the flow-cell cover, uniformly loosen thumbscrews in an alternating diagonal manner until disengaged from threads, remove the thumbscrews, and store in the laser module case.

13.7. Remove the flow-cell cover from the laser module.

13.8. Clean both glass surfaces gently with a good quality lens cleaner and lens paper. Do not use tissue paper or paper towels on glass surfaces.

13.9. Visually inspect the laser module and flow-cell cover "windows" for scratches or imperfections following use and report to the supervisor.

13.10. Replace the laser module and flow-cell cover in their cases to prevent damage to glass surfaces during storage.

14. Sample analysis protocol

14.1. Unfiltered samples

14.1.1. Vortex the sample prior to loading and record **5 x 60 s videos** at **Camera Level 12** and **Detection Threshold Level 3** as described above. Reanalyze these videos using **Detection Thresholds 2 and 5**.

14.1.2. Repeat video collections of the same sample at **Camera Levels 13 and 14** and **Detection Threshold Level 3**. Reanalyze these 2 additional videos using **Detection Thresholds 2 and 5.** Repeat the entire process using **5 x 30 s videos** in the **SOP** setting.

14.2. Filtered samples

14.2.1. Vortex the sample and then simultaneously load and filter it (0.22 µm syringe filter) directly into the laser module.

NOTE: The syringe filter was flushed with 2 times the volume of the filter dead space prior to use to remove any resident particulates. The syringe filters (see the **Table of Materials**) had a measured dead space of 0.5 mL and were flushed with 1.0 mL of the sample prior to measurements. Note the filter type in the **SOP** data fields. The filtered sample was processed exactly as described for unfiltered samples in step 14.1.

15. Statistical analysis of NTA results

15.1. For the analysis of main effects or interactions, perform analysis of variance following a

check of ANOVA assumptions (normality, unimodal, homogeneity of variance). Use Kruskal-Wallis one-way ANOVA on ranks in cases of failure of ANOVA assumptions.

15.2. Following the return of significant main effects, use Dunn's method to perform means testing for preplanned comparisons. Consider a p-value of 0.05 to be significant in two-tailed testing.

NOTE: Data files generated here are available from the authors following the completion of a materials transfer agreement.

REPRESENTATIVE RESULTS:

Table 1 contains the results of the NTA videos for the liposome samples (18 filtered and 18 unfiltered) and a representative DPBS diluent. Comparisons across the two groups were completed regardless of the camera level or detection threshold in this paper. Filtered samples had a mean particle diameter of 108.5 nm, a particle mode of 86.2 nm, and a concentration of 7.4×10^8 particles/mL. In contrast, unfiltered samples had a mean particle diameter of 159.1 nm, a particle mode of 105.7 nm, and a concentration of 7.6×10^8 particles/mL. Mean and mode values for the filtered and unfiltered samples, regardless of the camera level or detection threshold, were statistically significant ($p < 0.05$). Differences in concentration between the filtered and unfiltered samples, regardless of the cameral level or detection threshold, were non-significant ($p = 0.86$).

[Place **Table 1** here]

When the liposome sample results were parsed by detection threshold (2, 3, 5) and camera level (12, 13, 14), the results were not significant (**Figure 3**). It should be noted that there were only 3 evaluations (n = 3) at each of these individual levels. This small sample size at each camera level and detection threshold likely contributed to the lack of individual comparisons being significant. However, when detection threshold (2, 3, 5) samples were evaluated regardless of camera level across the filtered and unfiltered samples (n = 3), both the mean size (**Figure 3A**) and mode size (**Figure 3B**) were significantly ($p < 0.05$) different. In contrast, differences between filtered and unfiltered sample concentrations (**Figure 3C)** were not significantly different.

[Place **Figure 3** here]

When the 3 camera levels (12, 13, 14) were evaluated regardless of detection threshold level (n = 3), both the mean size (**Figure 3D**) and mode size (**Figure 3E**) increased in the filtered samples. Sample concentrations showed a tendency to increase as the camera level increased from 12 to 14 (**Figure 3F**). The differences between filtered and unfiltered sample concentrations evaluated at different camera levels were not significantly different.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative NTA reports to compare liposomes to EVs. (**A**) Liposomes: unfiltered sample characterized on NTA on 12 March 2020. (**B**) EVs: unfiltered sample characterized on NTA on 26 August 2021. Abbreviations: NTA = Nanoparticle tracking analysis; EVs = extracellular vesicles.

Figure 2: Laser module focus alignment guide.

Figure 3: Effects of camera level and detection threshold on measured particle size and concentration of filtered and unfiltered samples. Mean (**A**) and Mode (**B**) particle size at combined camera levels 12, 13, 14 as the detection threshold was increased from 2 to 3 to 5 (n = 3), showing a significant decrease in particle sizes of the filtered samples. Particle concentrations (**C**) at combined camera levels 12, 13, 14 as the detection threshold was increased from 2 to 3 to 5) (n = 3), showing a concentration decrease as the detection threshold increased with no significant difference between filtered and unfiltered samples. Mean (**D**) and Mode (**E**) particle size at combined detection thresholds as the camera level increased from 12 to 14 (n = 3), showing a decrease in particle size of the filtered samples. Particle concentrations (**F**) at combined detection thresholds as camera levels increased from 12 to 14 (n = 3), showing a concentration increase as the camera level increases with no significant differences between filtered and unfiltered samples. Abbreviation: DT = detection threshold.

Table 1: Data table of collected values, standard deviation, and percent coefficient of variation for filtered and unfiltered samples. Abbreviations: Cam Lev = camera level; Det Thr = detection threshold; CV = coefficient of variation; St. Dev. = standard deviation; Conc. = concentration.

DISCUSSION:

There are several methods available to estimate the size and concentration of nanoparticles[11]. These include ensemble methods that generate a size estimate from a population, including dynamic light scattering (DLS), centrifugal sedimentation, and single-particle level analysis—electron microscopy, NTA, atomic force microscopy, and tunable resistive pulse sensing. Of these, DLS and NTA are widely used, nondestructive size and concentration measurement methods, based on Brownian movement in an ideal medium. DLS relies on the scattering of light, and the intensity is proportional to the square of the particle volume. Thus, DLS is more sensitive than NTA to the presence of large particles, aggregates, or polydisperse populations.

NTA calculates the diffusion coefficient from the path length of individual particles measured in successive video frames. The main limitation of NTA is the narrow range of particle concentration that it can evaluate compared to DLS and other measurement methods, such that individual particle pathlengths must fall within the diffraction limit of the microscope and the tracking software's capabilities. As DLS and NTA depend on Brownian movement, both can be expected to show good size agreement in monodispersed populations; they diverge when evaluating polydisperse populations and those with aggregates. The latter renders DLS useless and increases the NTA particle size estimate significantly[12]. NTA's best-known limitation is that it requires much lower particle concentration (or greater dilution) than other measurement methods. Despite

this, NTA characterization is popular in nanomaterials research. Because NTA size and concentration estimates depend on a more diluted population, with defined temperature, video capture settings, including recording length, camera level, detection threshold, and sample dilution to be highly reproducible, this paper focuses on the need for reporting these to generate reproducible results.

This paper shows that using a standardized protocol enabled replication of results, and that utilization of positive controls, such as size standards, provides information about the machine's calibration. Furthermore, these results indicated the importance of reporting laser module chamber temperature, camera levels, detection threshold, and filtration (filter type and size). In contrast, laser module chamber temperature, diluent, and dilution factor are equally important for accurate and reproducible results. Although neither MISEV2018 nor EV-TRACK specifically recommends the inclusion of this information, we suggest that the inclusion of these details enables independent confirmation of published results and adds robustness to the experimental design.

Limitations of using latex size calibration standards for NTA calibration in EV analysis are acknowledged and include the known refractive index differences compared to lipid bi-layer nanoparticles of similar size. In this paper, latex beads were used to confirm machine calibration prior to measurements and not to determine the limits of detection. The liposomes have a membrane similar to those of naturally occurring EVs, and the refractive index will be likewise representative of EVs. The size standards, as well as the liposome samples, are monodispersed populations; therefore, their size distribution will follow a Gaussian or log-normal distribution. Natural EVs are polydisperse, and their size distribution will follow a power-law function[13].

Historically, publications using NTA characterization inconsistently report necessary details to duplicate the research results. The ability to reproduce NTA data relies on the ability to duplicate the settings used to capture the original data. Without this information, the reproduction of experimental results using NTA will be extremely difficult. With rigorous adherence to a set protocol and publication of the setting parameters used with the NTA, accurate replication of results can be attained. The following recommendations are made to improve the consistency of nanoparticle characterization of size, concentration, composition, and purity using a nanoparticle size analyzer.

First, always check the calibration of the nanoparticle size analyzer using appropriate size standards, such as latex size standards. This should be done on a regular basis and recorded in the instrument log and prior to the analysis of critical samples. Second, all adjustable parameters, such as laser module chamber temperature, camera levels, and detection thresholds, should be recorded for each sample in the **Sample Log** file, as should the dilutions and diluents used. These parameters should be reported as they are operator-dependent and impact NTA measurements. Third, diluents used for sample dilution need to be characterized for nanoparticle content and reported. The diluents used for individual nanoparticle samples will need to be evaluated using the same camera level and detection threshold settings as those used for the diluted sample. Fourth, syringe filters should be flushed with two times the dead space volume prior to data

collection or sample preparation steps to flush the numerous particulates remaining from the manufacturing process. Fifth, the concentration of the nanoparticles within the sample should be adjusted to within the suggested optimum 1.0×10^8 to 1.0×10^9 per mL.

Acknowledging the above-described limitations in this study, we show that both the size and concentration values obtained by NTA can be affected by NTA parameters, such as camera levels and detection thresholds, and that the size, but not the concentration, can be affected by sample preparation. This drives home the critical importance of reporting these parameters in nanomaterial and EV literature, enabling the production of robust, reproducible literature so that we can systematically investigate the impact of EV source, isolation, and other experimental variables.

ACKNOWLEDGEMENTS:

The work was supported by the state of Kansas to the Midwest Institute for Comparative Stem Cell Biology (MICSCB). OLS received GRA support from the MICSCB. The authors thank Dr. Santosh Aryal for providing the liposomes used in this project and the members of the Weiss and Christenson laboratories for helpful conversations and feedback. Dr. Hong He is thanked for technical support. MLW thanks Betti Goren Weiss for her support and counsel.

DISCLOSURES:

None of the authors have any conflicts of interest.

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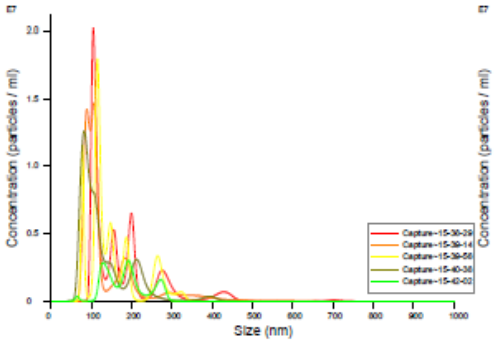
Figure

Figure 1

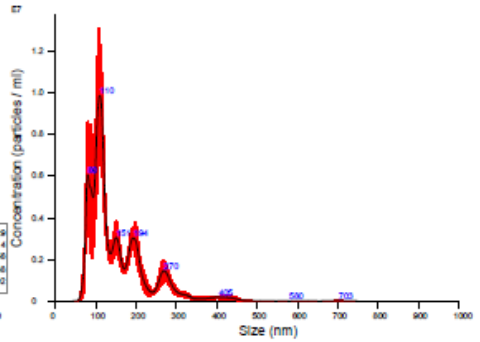
Representive NTA Reports

NANOSIGHT

Capture 2020-03-12 15-37-13



FTLA Concentration / Size graph for Experiment:
Capture 2020-03-12 15-37-13



Averaged FTLA Concentration / Size for Experiment:
Capture 2020-03-12 15-37-13
Error bars indicate + / -1 standard error of the mean

Included Files

Capture 2020-03-12 15-38-29
Capture 2020-03-12 15-39-14
Capture 2020-03-12 15-39-56
Capture 2020-03-12 15-40-38
Capture 2020-03-12 15-42-02

Details

NTA Version:	NTA 3.3 Dev Build 3.3.301
Script Used:	SOP Standard Measurement 03-37-13PM 12~
Time Captured:	15:37:13 12/03/2020
Operator:	Larry Snyder
Pre-treatment:	
Sample Name:	Aryal Lab Liposomes-Sample #4 Unitered
Diluent:	Sample diluted 1:4 with UltraPure DW
Remarks:	

Capture Settings

Camera Type:	sCMOS
Laser Type:	Blue405
Camera Level:	14
Slider Shutter:	1259
Slider Gain:	366
FPS	25.0
Number of Frames:	749
Temperature:	25.0 - 25.0 °C
Viscosity:	(Water) 0.889 - 0.889 cP
Dilution factor:	Dilution not recorded

Analysis Settings

Detect Threshold:	5
Blur Size:	Auto
Max Jump Distance:	Auto: 9.3 - 11.5 pix

Results

Stats: Merged Data

Mean:	151.4 nm
Mode:	109.8 nm
SD:	74.9 nm
D10:	83.5 nm
D50:	121.2 nm
D90:	263.7 nm

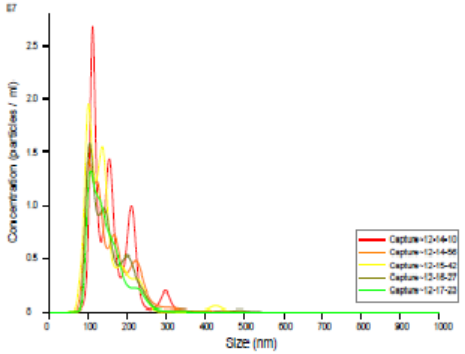
Stats: Mean +/- Standard Error

Mean:	156.0 +/- 10.5 nm
Mode:	120.7 +/- 18.8 nm
SD:	69.0 +/- 6.4 nm
D10:	93.8 +/- 9.2 nm
D50:	134.8 +/- 14.6 nm
D90:	255.5 +/- 12.2 nm
Concentration:	6.76e+08 +/- 1.01e+08 particles/ml
	34.3 +/- 5.2 particles/frame
	37.4 +/- 5.2 centres/frame

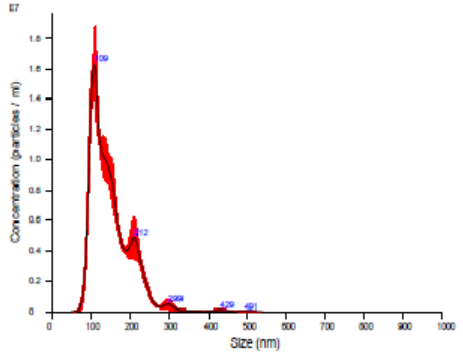
A. Liposome Sample - Unfiltered

NANOSIGHT

Capture 2021-08-26 12-10-44



FTLA Concentration / Size graph for Experiment:
Capture 2021-08-26 12-10-44



Averaged FTLA Concentration / Size for Experiment:
Capture 2021-08-26 12-10-44
Error bars indicate + / -1 standard error of the mean

Included Files

Capture 2021-08-26 12-14-10
Capture 2021-08-26 12-14-56
Capture 2021-08-26 12-15-42
Capture 2021-08-26 12-16-27
Capture 2021-08-26 12-17-23

Details

NTA Version:	NTA 3.3 - Sample Assistant Dev Build 3.3.203
Script Used:	SOP Standard Measurement 12-10-44PM 26Aug2021.txt
Time Captured:	12:10:44 26/08/2021
Operator:	Larry Snyder
Pre-treatment:	
Sample Name:	JF Sample 40
Diluent:	1:20 in DPBS
Remarks:	DPBS used to dilute samples

Capture Settings

Camera Type:	sCMOS
Laser Type:	Blue488
Camera Level:	10
Slider Shutter:	696
Slider Gain:	73
FPS	32.3
Number of Frames:	969
Temperature:	25.0 - 25.0 °C
Viscosity:	(Water) 0.9 cP
Dilution factor:	Dilution not recorded

Analysis Settings

Detect Threshold:	5
Blur Size:	Auto
Max Jump Distance:	Auto: 7.3 - 8.2 pix

Results

Stats: Merged Data

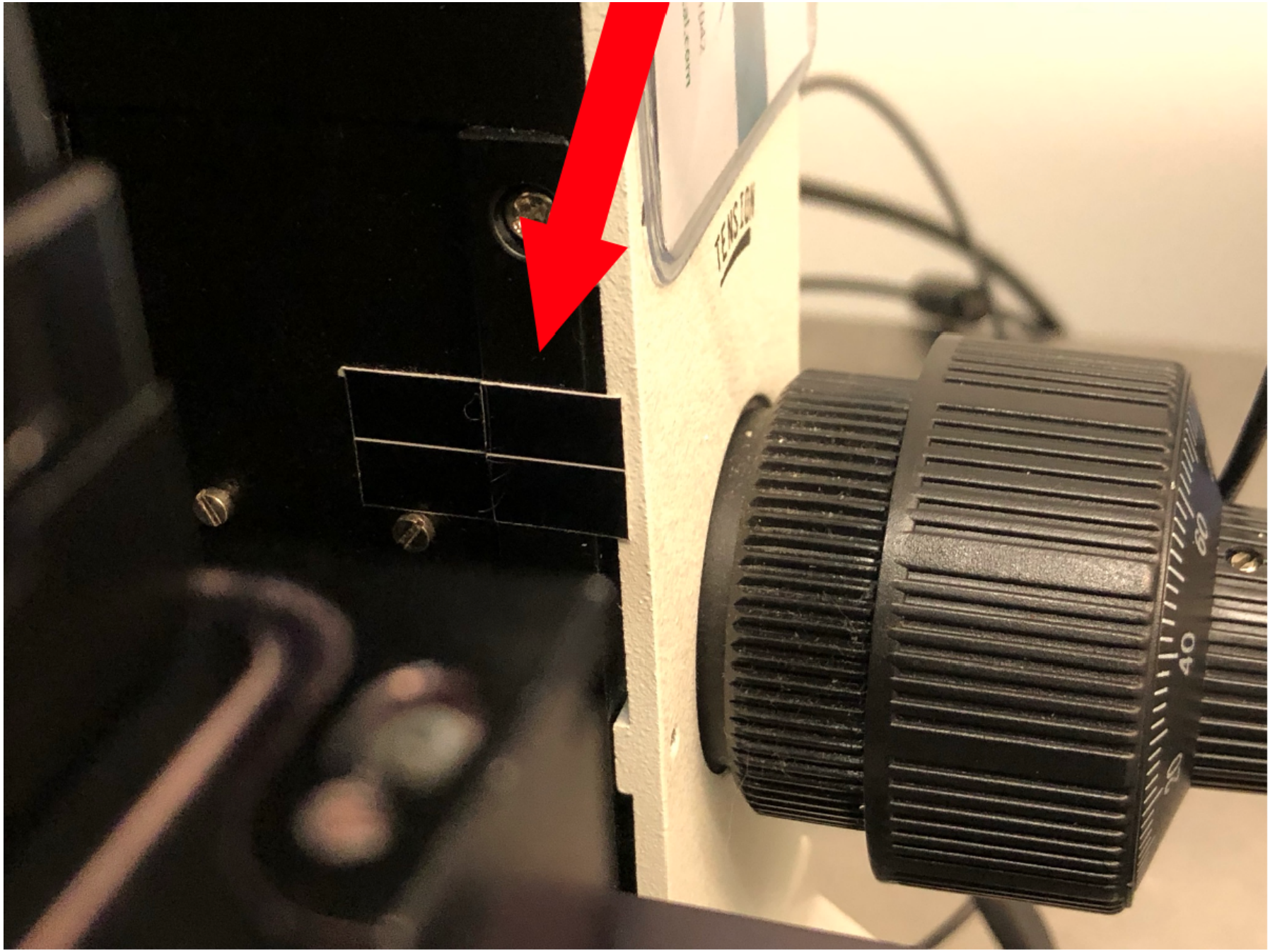
Mean:	149.6 nm
Mode:	108.7 nm
SD:	50.9 nm
D10:	99.1 nm
D50:	137.1 nm
D90:	218.9 nm

Stats: Mean +/- Standard Error

Mean:	149.5 +/- 2.6 nm
Mode:	105.0 +/- 1.8 nm
SD:	49.9 +/- 2.4 nm
D10:	99.5 +/- 2.2 nm
D50:	137.9 +/- 3.0 nm
D90:	216.9 +/- 3.9 nm
Concentration:	1.23e+09 +/- 7.35e+07 particles/ml
	62.5 +/- 3.7 particles/frame
	65.0 +/- 3.8 centres/frame

B. EV Sample - Unfiltered

Figure 2



Figure

Click here to access/download;Figure;Fig. 3 Comp. of Parameters 9.26.pdf

Figure 3

Sample Characteristics Using Varied Measurement Parameters

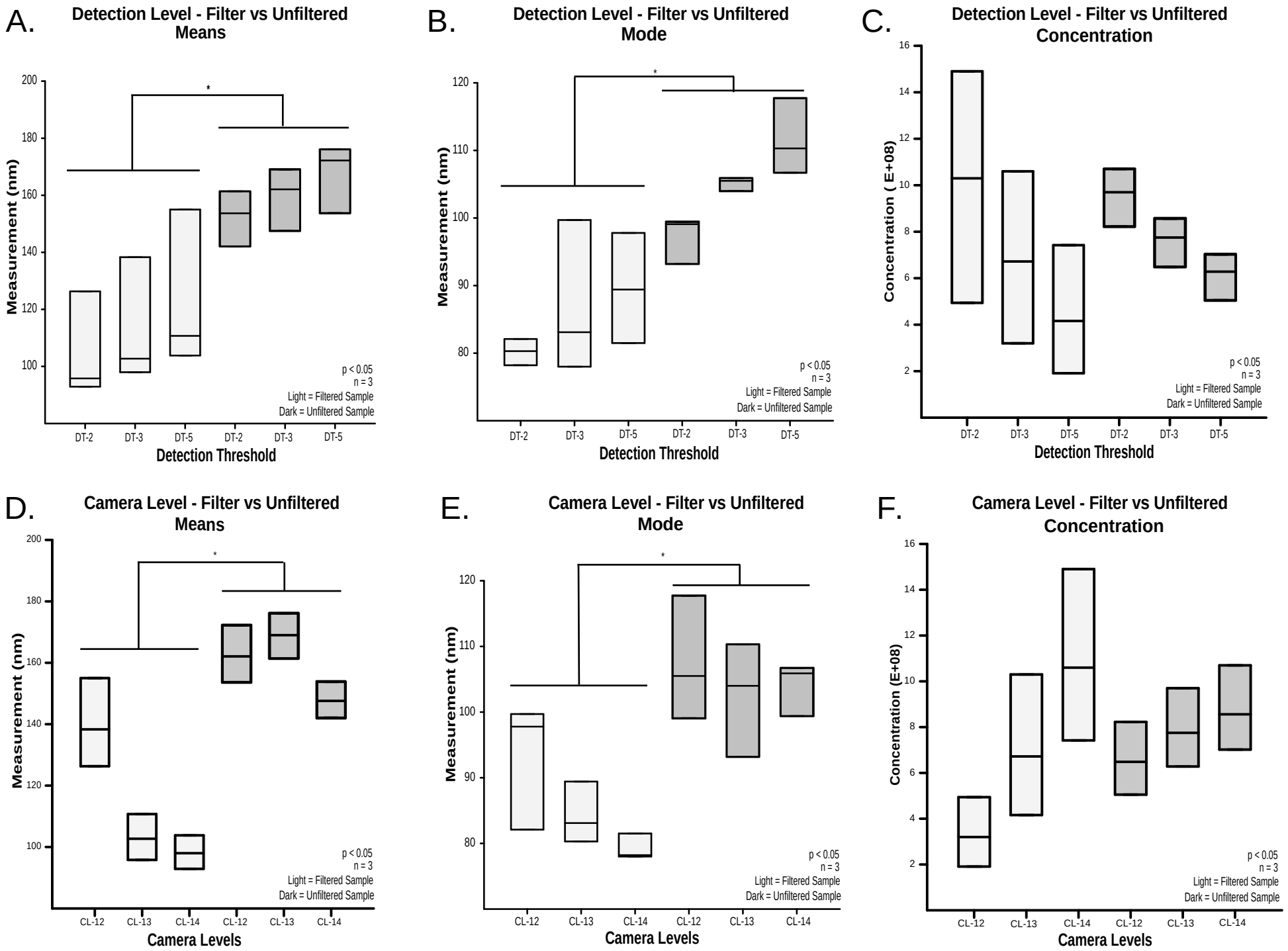
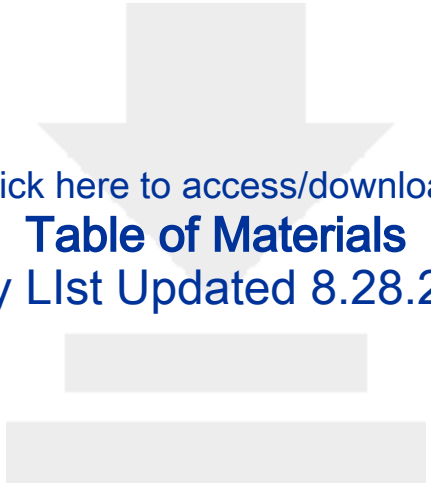


Table 1

Comparisons of Filtered vs Unfiltered Samples

Sample	Cam Lev	Det Thr	Mean		Mean %CV		St. Dev.		Conc. × 10^8		St. Dev. × 10^7	
			Filtered	Unfiltered	Filtered	Unfiltered	Filtered	Unfiltered	Filtered	Unfiltered	Filtered	Unfiltered
Liposome	12	3	138.3	162.1	55.5	47.5	76.7	77	3.2	6.48	1.74	3.16
Liposome	12	2	126.3	153.7	58.7	49.8	74.2	76.5	4.94	8.23	2.74	3.5
Liposome	12	5	155	172.2	51.8	45.6	80.3	78.6	1.91	5.05	1.02	2.9
Liposome	13	3	102.7	169	43.5	51.3	44.7	86.7	6.72	7.75	1.91	1.17
Liposome	13	2	95.8	161.4	43.3	52.7	41.5	85	10.3	9.7	1.61	1.83
Liposome	13	5	110.7	176.1	42.5	47.1	47	83	4.16	6.28	1.83	1.07
Liposome	14	3	98	147.6	42.4	43.8	41.6	64.6	10.6	8.56	2.4	1.66
Liposome	14	2	92.9	142.1	42.6	45.2	39.6	64.3	14.9	10.7	2.54	1.83
Liposome	14	5	103.8	153.8	41.1	42.6	42.7	65.5	7.42	7.02	2.37	1.51
Liposome	12	3	105.6	179.4	22.2	46.7	23.4	83.7	5.2	5.81	1.06	4.28
Liposome	12	2	100.3	170.8	24.3	49.1	24.4	83.8	7.76	7.39	1.61	4.41
Liposome	12	5	112	187.2	20.4	42.9	22.8	80.4	3.27	4.68	0.815	3.93
Liposome	13	3	99.8	153.3	23.4	52.1	23.4	79.8	7.19	7.34	3.37	1.5
Liposome	13	2	94.3	143.6	25.8	53.2	24.3	76.4	10.8	9.53	4.3	2.46
Liposome	13	5	106.8	162	21.3	49.4	22.7	80	4.64	5.76	2.63	1.01
Liposome	14	3	103.4	142.3	31.7	49.6	32.8	70.6	9.91	8.66	3.29	12.5
Liposome	14	2	97.8	136	33.3	51.4	32.6	69.9	13.8	11.5	2.98	15.7
Liposome	14	5	109.5	151.4	31.1	49.5	34	74.9	7.27	6.76	3.42	10.1
Average			108.5	159.1	36.4	48.3	40.5	76.7	7.4	7.6	2.3	4.1



Click here to access/download

Table of Materials

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TITLE:

Improving Reproducibility to Meet Minimal Information for Studies of Extracellular Vesicles 2018 Guidelines in Nanoparticle Tracking Analysis

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SUMMARY:

Nanoparticle tracking analysis (NTA) is a widely used method to characterize extracellular vesicles. ~~For NTA to be reproducible between laboratories, reporting~~ This paper highlights~~of~~ NTA experimental parameters and controls plus a uniform method of analysis and characterization of samples and diluents ~~is~~ necessary~~needed~~ to supplement the guidelines proposed by MISEV2018 and EV-TRACK for reproducibility between laboritories.

ABSTRACT:

Nanoparticle tracking analysis (NTA) has been one of several characterization methods used for extracellular vesicle (EV) research since 2006. Many consider that NTA instruments and their software packages can be easily utilized following minimal training, and that size calibration is feasible in-house. As both NTA acquisition and software analysis constitute EV characterization, they are addressed in Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018) and have been monitored by Transparent Reporting and Centralizing Knowledge in Extracellular Vesicle Research (EV-TRACK) to improve the robustness of EV experiments (e.g., minimize experimental variation due to uncontrolled factors).

Despite efforts to encourage reporting of methods and controls, many published research papers fail to report critical settings needed to reproduce the original NTA observations. Few papers report the NTA characterization of negative controls or diluents, evidently assuming that commercially available products, such as phosphate-buffered saline or ultrapure distilled water, are particulate-free. Similarly, positive controls or size standards are seldom reported by researchers to verify particle sizing. The Einstein-Stokes equation incorporates sample viscosity and temperature variables to determine particle displacement. Reporting of the stable laser

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chamber temperature during the entire sample video collection is therefore an essential control measure that needs to be reported for accurate replication.

Filtration of samples or diluents are also not routinely reported, and if so, the specifics of the filter (manufacturer, membrane material, pore size) and storage conditions are seldom included. The International Society for Extracellular Vesicle (ISEV)'s minimal standards of acceptable experimental detail should include a well-documented NTA protocol for the characterization of EVs. The following experiment provides evidence that an NTA analysis protocol needs to be established by the individual researcher and included in the methods of publications that use NTA characterization as one of the options to fulfill MISEV2018 requirements for single vesicle characterization.

INTRODUCTION:

Accurate and repeatable analysis of EVs and other nanometer-scaled particles presents numerous challenges across research and industry. Replication of EV research has been difficult, in part, due to the lack of uniformity in reporting necessary parameters associated with data collection. To address these deficiencies, the ISEV proposed industry guidelines as a minimal set of biochemical, biophysical, and functional standards for EV researchers and published them as a position statement, commonly referred to as MISEV2014[1]. The accelerating pace of EV research required an updated guideline, and the "MISEV2018: a position statement of the ISEV"[2], expanded the MISEV2014 guidelines. The MISEV2018 paper included tables, outlines of suggested protocols, and steps to follow to document specific EV-associated characterization. As a further measure to facilitate interpretation and replication of experiments, EV-TRACK was developed as a crowd-sourcing knowledgebase (http://evtrack.org) to enable more transparent reporting of EV biology and the methodology used for published results[3]. Despite these recommendations for standardized reporting of methods, the field continues to suffer regarding replicating and confirming published results.

Fitting with National Institutes of Health's and National Science Foundation's effort for quality assessment tools, this paper suggests that ISEV requires standardized reporting of methods and details so that data assessment tools might be applied with the goal of replicating results between laboratories. Reporting cell sources, cell culture procedures, and EV isolation methods are some of the important factors to define the qualities of the EV population. Among NTA instruments, factors such as detection settings, refractive index of carrier fluid, heterogeneous particle populations contributing to polydispersity, lack of standardized reporting requirements, and absent intra- and inter-observer measurement results make NTA comparison between labs difficult or impossible.

In use since 2006, NTA is a popular method for nanoparticle size and concentration determination that is currently used by approximately 80% of EV researchers[4]. The MISEV2018 Guidelines require two forms of single-vesicle analysis of which NTA is one of the popular options. NTA continues to be in common use for EV characterization due to its wide accessibility, low cost per sample, and its straightforward founding theory (the Stokes-Einstein equation). EV assessment by NTA generates a particle size distribution and concentration estimate using laser light

scattering and Brownian motion analysis, with the lower limit of detection determined by the refractive index of the EV. When using a fluid sample of known viscosity and temperature, the trajectories of the EVs are tracked to determine their mean-square displacement in two dimensions. This then allows the particle diffusion coefficient to be calculated and converted into a sphere-equivalent hydrodynamic diameter by a modified Stokes-Einstein equation[5-7]. NTA's particle-to-particle analysis has less interference by agglomerates or larger particles in a heterogeneous population of EVs than other methods of characterization[7]. While a few larger particles have very little impact on sizing accuracy, the presence of even minute amounts of large, high light-scattering particles results in a notable reduction in the detection of smaller particles due to reduced software EV detection and tracking[8]. As a measurement technique, NTA is generally considered to not be biased toward larger particles or aggregates of particles but is able to resolve multiple-sized populations through individual particle analysis[9]. Because of the use of light-scattering by particles, one of the limitations of NTA analysis is that any particulate such as dust, plastic, or powder that has similar refraction and size attributes compared to EVs cannot be differentiated from actual EVs by this method of characterization.

The NanoSight LM10 (nanoparticle size analyzer) and LM14 (laser module) have been sold since 2006, and although newer models of this instrument have been developed, this particular model is found in many core facilities and is considered a reliable workhorse. Training is needed to properly optimize the NTA settings for high-resolution measurements of size and concentration. The two important settings needed for optimum video recordings are (1) the camera level and (2) the detection threshold. These must be set by the operator based on the sample's characteristics. One of the major constraints of NTA analysis is the recommendation of sample concentrations between 10^7 and 10^9 particles/mL, to achieve which sample dilution may be required[10]. Solutions used for dilution, such as phosphate-buffered saline, 0.15 M saline, or ultrapure water, are rarely free of particles less than 220 µm in size and these may affect the NTA measurements. NTA characterization of the solutions used for dilution should be performed at the same camera level and detection threshold as the nanoparticle samples that are being analyzed. The size and concentration of nanoparticles present in diluents used for EV sample dilutions are seldom included in publications involving NTA analysis of EVs.

This protocol uses NTA analysis of synthetic EV-like liposomes evaluated using selected camera levels, detection thresholds, and mechanical filtering of the samples to analyze the systematic effects of camera level, detection threshold, or sample filtration on the NTA dataset. Liposomes were synthesized using the protocol included in **Supplemental File S1**. Synthetic liposomes were used in this experiment because of their size uniformity, physical characteristics, and stability in storage at 4 °C. Although actual samples of EVs could have been used, the heterogenicity and stability of EVs during storage may have complicated this study and its interpretation. Similarities in the NTA reports from (A) liposomes and (B) EVs indicate the systematic effects revealed for liposomes in this paper will likely also apply to EV characterization (**Figure 1**). Together, these findings support the notion that complete reporting of critical software settings and the description of sample processing, such as diluent, dilution, and filtration, impact the reproducibility of NTA data.

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The purpose of this paper is to demonstrate that varying the NTA settings (temperature, camera level, and detection threshold) and sample preparation changes the results collected: systematic, significant differences in size and concentration were obtained. As NTA is one of the popular options to fulfill the MISEV2018 characterization specification, these results demonstrate the importance of reporting sample preparation and of NTA settings to ensure reproducibility.

(Place **Figure 1** here)

PROTOCOL:

1. General protocol guidelines

1.1. Maintain the microscope on an air table or at a minimum on a vibration-free table. Ensure that extraneous vibrations (e.g., foot tapping on the floor, touching the table, door closures, laboratory traffic) are kept to a minimum.

1.2. Set and maintain the temperature of the laser module at a constant temperature for all video recordings.

NOTE: The temperature chosen was 25 °C because the nanoparticle size analyzer was calibrated at that temperature. Therefore, it is important that all users of the instrument know and use this temperature. Room temperature is not an acceptable setting because it can vary.

1.3. Ensure that all diluents, also called negative controls (e.g., Dulbecco's Phosphate-buffered Saline (DPBS), ultrapure distilled water [DW]), are all NTA-characterized using the same camera level and detection threshold as nanoparticle samples being measured. Use characterized DPBS for the flushing of the laser module and dilution of samples. Factor contaminants in the negative control DPBS into sample results if their levels are significant.

1.4. Store samples at 4 °C prior to evaluation and never freeze them as this would degrade the liposome sample. Warm samples/standards/diluents to room temperature for 30 min prior to analysis.

NOTE: Sample handling was specific to the material being examined.

1.5 Evaluate sample and standards using **Quick measurement** to establish an appropriate dilution to obtain 10^8 to 10^9 particles/mL (approximately 50 to 100 particles/NTA video screen) ~~an ~10^8 particles/mL~~ determined to be optimal for NTA analyses. The authors have found that particle concentration at the higher end of this range produce more consistent and reproducible results.

~~1.5.~~

~~1.6.~~1.6.1 Fill in~~Complete~~ all applicable fields~~values~~ within the **Capture** box of the **SOP** tab for both **Quick** and **Standard** measurements, including the dilution and diluent used.

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2. Preparation of 50 nm and 100 nm size calibration standards

NOTE: See the **Table of Materials**.

2.1. 50 nm size Transfer Standards diluted 1:5,000

2.1.1. Add 2 µL of the 50 nm standards to 9,998 µL of 0.22 µm-filtered 10 mM Potassium chloride (KCl)/0.03% Tween 20 in ultrapure DW into a twice-rinsed 15 mL conical tube. Store the diluted 50 nm standard at 4 °C for up to a year.

2.2. 100 nm size Transfer Standards diluted 1:333

2.2.1. Add 30 µL of the 100 nm standards to 9,970 µL of 0.22 µm-filtered 10 mM KCl/0.03% Tween 20 in ultrapure DW into a twice-rinsed 15 mL conical tube. Store the diluted 100 nm standard at 4 °C for up to a year.

3. Cleaning and assembly of the laser module

3.1. Visually inspect the laser module and flow-cell cover windows for scratches or imperfections.

NOTE: If either glass surface is scratched, imaging may be affected.

3.2. Clean both glass surfaces gently with a good quality lens cleaner and lens paper. Do not use tissue wipes or paper towels on glass surfaces.

3.3. Ensure that the O-ring seal is properly seated in the groove of the flow-cell cover prior to assembly.

3.4. Place the flow-cell cover on the laser module ensuring the electrical contacts are in the proper orientation. Place the 4 spring-loaded thumb screws through the flow-cell plate and engage the threads of the laser module but do not tighten individually.

3.5. Putting uniform pressure down on the flow-cell cover, uniformly tighten the thumbscrews in an alternating diagonal manner until snug. Only tighten the thumbscrews until finger-tight. Do not overtighten.

NOTE: Uneven tightening of the thumb screws can crack the laser module surface. Newer designed thumbscrews will "bottom out" at the proper pressure, avoiding possible overtightening.

4. Flushing procedure for the laser module prior to and between samples

Commented [A10]: Are these unhighlighted sections (4, 5, 9, 12 etc) important for preparing for the analysis? If so, please check if you want to highlight important steps to include in the video. However, please remember i) the video must be cohesive, ii) total length of highlighted text must not exceed three pages. To do this, avoid highlighting notes and headings and merge shorter, related, highlighted steps so that a highlighted step contains 2-3 actions but not more than 4 sentences.

Commented [A11R10]: Each of these sections has importance for preparing for the analysis. The highlighted steps now include (1) Cleaning and assembly of the laser module, (2) Flushing procedure for the laser module prior to and between samples, (3) Focusing and positioning of the laser module, (4) Loading samples into the laser module, and (5) Sample NTA data collection.

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4.1. Use a newly opened container of DPBS and aliquot into triple-rinsed 15 mL polypropylene tubes. Ensure that products used to flush or dilute samples are NTA-characterized prior to use (see step 1.3).

4.2. Flush two 1 mL tuberculin syringes with slip lock adaptors 3 times with 1 mL of DPBS to remove any particulate residues. Use either port as the input but use it consistently during the experiment. Do not use larger syringes due to the danger of breaking syringe ports from the increased weight and size of the syringe.

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4.3. Remove and discard the plunger from the 1st tuberculin syringe and insert it into the remaining port to serve as a reservoir of voided diluent/sample.

NOTE: Failure to remove the plunger will cause increased pressure in the sample chamber and leakage around the seal.

4.4. Fill the 2nd syringe with 1 mL of DPBS and attach it to the inlet port of the flow-cell cover.

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4.5. Hold the laser module tilted with the outlet syringe port elevated to allow air to be purged from the chamber as the DPBS is injected slowly into the laser module.

4.6. Flush the remaining DPBS from the laser module by injecting 1 mL of air into the inlet port. Repeat flushing 2 more times.

4.7. Empty the laser module as completely as possible after the last flush.

NOTE: Following NTA analysis of the 50 nm standard, these particles tend to persist in the laser module so thorough, careful flushing is necessary. The laser module is now ready to be used.

5. Placement of the laser module on microscope stage

5.1. Locate the laser module focus alignment guides on the arm of the microscope (**Figure 2**) and align them using the focus knob.

[Place **Figure 2** here]

5.2. Facing the microscope, place the laser module in the grooved stage and gently slide it as far as possible to the right.

NOTE: If done carefully, the alignment and focal spot will be easier to locate between samples.

5.3. Turn on the rocker switch on the laser control box.

6. Focusing and positioning of the laser module

NOTE: This must~~can~~ be performed with fluid in the chamber.

6.1. Load the NTA software (see the **Table of Materials**) from the desktop.

NOTE: An error may appear that the **Temperature H/W not found on COM3**. Simply close and reopen the software to fix it.

6.2. Click **Start Camera** under the **Capture** tab in the upper left corner box. If the camera shuts off automatically after 5 min, simply click **Start Camera** again to restart it.

6.3. In the same tab, adjust **Camera Level** to **14 to 16** to brighten the laser line and make particle identification and focusing easier.

6.4. Divert the image from the camera to the eyepieces by moving the top slider on the left side of the headpiece in or out.

6.5. Find the area of increased density, commonly referred to as the **thumbprint**. Center and focus the thumbprint vertically in the field of view.

NOTE: The laser line will be to the left of the thumbprint. Darkening the room may help facilitate locating the thumbprint and focusing on the laser line.

6.6. Center the laser line in the field of view; then, move the top slider to divert light to the camera as observed on the computer screen.

NOTE: The laser line now visible on the screen is a mirror image of the eyepiece view; left in the eyepieces will be right on the computer screen.

6.7 Adjust the focus to sharpen the image of individual moving particles on the screen with the focus knob.

NOTE: Due to the depth of field, all the particles will not be in focus, which is acceptable. Even particles that are slightly out of focus will be captured by the camera and analyzed by the software.

7. Loading standards/samples/diluent into the laser module for NTA analysis

7.1. Draw up 1 mL of standard/sample/diluent into a rinsed 1 mL tuberculin syringe and attach it to the inlet port of the flow-cell cover. Advance the plunger until fluid is evident in the open syringe attached to the outlet port.

7.2. In the camera view, move to the right of the laser line to an area of a uniform number of particles. If necessary, adjust the vertical orientation to center the horizontal bands of light. Refocus until the highest number of particles are in view. Ensure that for all subsequent measures

this position is maintained as closely as possible.

7.3. Adjust the **Camera Level** to the point that the **Dark** information symbol in the top right of the camera view flashes intermittently on and off. This helps ensure that a consistent camera level selection is made at the minimum level of sensitivity for each series of data collection.

NOTE: **Camera Level** cannot be changed during capture.

8. Validation of calibration

NOTE: It is recommended to validate the module calibration using size standards (see section 2) prior to sample analysis. Routine validation is necessary to ensure accurate measurements. In a multiuser laboratory, individual user adjustments of software configuration settings can inadvertently cause inaccurate data collection. For critical data collection, daily validation is a matter of good laboratory practice. The day-to-day reproducibility of validation needs to be included in the reported results. Typically, calibration is set by the technician and is not adjustable by the individual user unless the user has administrator access. This prevents unauthorized reconfiguration by individual users.

8.1. Warm diluted standards (see section 2) to room temperature for 30 min.

8.2. Briefly vortex the standards and then load as described in section 7.

8.3. Perform sample NTA as described in section 10 and record values for subsequent calculation of the coefficient of variation, which should be less than 2% if correctly calibrated.

9. Optimizing sample concentration for NTA

NOTE: The screen should contain between 5~~2~~0 and 10~~6~~0 measurable particles when the camera level and sample concentration are adjusted properly. If there is any question about whether a sample has an appropriate particle number, a **Quick Measurement** can be run on the sample at this point (See 9.1 to 9.7). It is used to assess the sample characteristics rapidly prior to longer video captures. The **Quick Measurement tab** is found within~~under~~ the **SOP** tab in the bottom middle box.

9.1. Set **Capture duration** to **30 s**.

9.2. Accept the existing base filename or enter a new filename by clicking the **…** tab for a new storage site for generated data.

9.3. Check the box for **Target temperature** and input the desired temperature.

9.4. Load the sample as previously described (step 7.1) and click **Create and Run Script**.

Commented [A12]: How do you do this? Clicking on a button called Quick Measurement or do you type a command? Or are 9.1-9.7 to be performed to run Quick Measurement? Please clarify this.

Commented [A13R12]: The note has been amended to clarify the location of the Quick Measurement tab as well as directing the reader to the specific steps for this measurement.

9.5. Wait for the number of particles per frame to be displayed on the bottom right of the video screen after the completion of the 30 s video. If the number of particles is greater than 1060, flush the laser module 3 times (as previously described in step 4.6).

9.6. Dilute the sample to the desired concentration range using the characterized diluent.

9.7. Load the properly diluted sample into the flushed laser module and run the **Quick Measurement** to verify the sample is within the acceptable range.

10. Sample NTA

NOTE: The **Standard Measurement** tab is within ~~found under~~ the **SOP** tab in the bottom middle box and is used for routine sample analysis (See 10.1 to 10.12).

10.1. Set the duration to **30 or 60 seconds** and the number of videos to **5**.

10.2. Accept the existing base filename or enter a new filename by clicking the **…** tab for a new storage site for generated data.

10.3. Check the box for **Target temperature** and input the desired temperature.

10.4. Click **Create Script** to reuse this **Standard Measurement**.

10.5. Once the sample is loaded as described in section 7 and the experiment is ready to run, click **Create and Run Script**.

10.6. Fill out the fields in the **Set Report Details** popup screen with information on the operator, sample description, dilution of the sample, and diluent used.

NOTE: This information will be recorded and printed on the final experimental report.

10.7. When all desired fields have been filled, click **OK** to initiate the script.

NOTE: If **Target temperature** was selected, the heater will stabilize the sample in the laser module to 25 °C for 5 s prior to allowing the script to proceed with the measurement. The diagnostic panel in the lower-left corner of the screen will read HEATER ON and display the temperature of the sample.

10.8. Prior to each video capture, look for a prompt to **Advance** the plunger manually. Inject ~0.05 mL of the sample into the laser chamber and allow the particles to come to "rest" (i.e., not flowing) and then click **OK**.

10.9. Wait for a **Settings Confirmation** box will appear upon the completion of the 5th video capture and for the **Process box** to flash. Set the **Detection Threshold** for processing of the

Commented [A14]: How do you do this? Clicking on a button called Standard Measurement or do you type a command? Or are 10.1-10.7 to be performed to run Standard Measurement? Please clarify this.

sample by noting the number of blue crosses marking particles on the screen as the frames are advanced manually from the bottom of the video screen. If there are more than 3–4 blue crosses marking particles on each ~~the~~ screen as frames are advanced, increase the **Detection Threshold**. The blue crosses on individual particles are analogous to the "swarm" effect in flow cytometry and should be minimized for optimum accuracy and reproducibility of data collection.

10.10. Click **Settings OK** when the Detection Threshold is acceptable.

10.11. Wait for the videos to be automatically processed and a histogram of results and a dialog box notification of completion to be displayed before clicking **OK**.

10.12. Once the **Export Settings** box appears, save the results by clicking **Export**.

NOTE: All results from videos and analysis will be stored in the destination file defined in step 10.2. This requires a large amount of storage. Monitor and transfer to a secondary storage device as necessary.

11. Re-analysis of the current sample at different detection thresholds

NOTE: Immediately following NTA analysis (step 10), the data can be reanalyzed using different Detection Threshold settings. However, **Camera Level** cannot be modified following capture.

11.1. Highlight all 5 of the capture videos listed in the **Current Experiment**.

11.2. Click **Process Selected Files**, and wait for the **Setting Confirmation** box to appear and the **Process box** to flash to change **Detection Threshold**.

11.3. Adjust the Detection Threshold to the desired level and ~~C~~click **Setting | OK**.

11.4. Wait for the videos to be automatically processed and a histogram of results and a dialog box notification of completion to be displayed before clicking **OK**.

11.5. Click **Export Settings**. When additional evaluations are performed on the most recent sample, be sure to click the **Export Results** box in the **Current Experiment** as the **Export Results** popup box will not be displayed following the sample re-analysis.

NOTE: As there are no reminders to do this, it can easily overlooked and the analysis will be lost. However, the underlying data will remain and can be reanalyzed later.

12. Analysis of archived files

NOTE: If previously analyzed experiments have not been saved or additional analysis needs to be done on these samples, the individual files can be reloaded into the NTA software for additional **Detection Threshold** evaluations. **Camera Level** changes cannot be modified following capture.

Commented [A15]: The effect of different detection and camera levels are shown in Fig. 2, but which setting should be used to obtain a size which is closest to the real size of the liposomes? *The reviewer suggests that optimization of the settings is needed to characterize the sample. This was not the purpose here. As we show, both camera level and detection threshold settings (and sample preparation) affected the results. Clearly, adjusting these machine settings, affects the results obtained. We provide general guidelines for optimizing video capture (degree of screen darkening with camera level or the number of "red crosses" or "blue crosses" with detection threshold) here. Our intent was not to focus on optimization, but on reproducibility of reporting.*

Editor: But in the process of showing how to make your results more reproducible or how to lower reproducibility, is there anything you can add to help users optimize the settings?

Commented [A16R15]: Both the Detection Threshold in 10.9 and the Camera Level in 7.3 have been modified to explain optimization steps to allow more accurate and reproducible results during the video captures.

Commented [A17]: You mean click Setting and then OK?

Commented [A18R17]: Clarification has been made to clarify.

12.1. Load the NTA software from the desktop.

12.2. Click the **Analysis** tab in the lower middle panel.

12.3. Click **Open Experiment** and navigate to the desired .nano file.

NOTE: The video files associated with the selected .nano experiment must be in the same folder as the main experiment file; otherwise, an error will appear when trying to process the files. The first 6 digits of the filename are the date the experiment was run (~~x~~**xx-xx-xx**). The last 6 digits of the filename are the time the video was recorded (**xx-xx-xx**) and the individual video identifier. The PDF file of each combined experiment lists the included .nano files for that analysis.

12.4. Click **Process Selected Files** to run the analysis.

12.5. Once the **Process** box flashes to allow changes in **Detection Threshold,** set the threshold to the desired level and click **OK**.

12.6. Wait for the videos to be automatically processed and a histogram of results and a dialog box notification of completion to be displayed before clicking **OK**.

12.7. Click **Export Results**. B~~When additional evaluations are performed, b~~e sure to click the **Export Results** box in the **Current Experiment** as the **Export Results** popup box will not be displayed following the re-analysis.

NOTE: As there are no reminders to do this, it can easily overlooked, and the analysis will be lost.

13. Cleaning and disassembly of the laser module

13.1. Turn off the laser control box.

13.2. Flush all sample from the laser module and discard properly.

13.3. Hold the laser module tilted with the open syringe port elevated to allow air to be purged from the chamber as the DPBS is injected slowly into the laser module and is flushed from the outlet syringe port.

13.4. Flush the remaining DPBS from the laser module and discard.

13.5. Repeat flushing 2 more times and empty the laser module as completely as possible after the last flush.

13.6. Putting uniform pressure down on the flow-cell cover, uniformly loosen thumbscrews in an alternating diagonal manner until disengaged from threads, remove the thumbscrews, and

Commented [A19]: That's seven.

Commented [A20R19]: Thank you, it should be 6 digits.

Commented [A21]: I brought this in from the earlier step. OK? Or please modify as needed.

Commented [A22R21]: Good idea! Reminders to do this step are important to preserve results.

store in the laser module case.

13.7. Remove the flow-cell cover from the laser module.

13.8. Clean both glass surfaces gently with a good quality lens cleaner and lens paper. Do not use tissue paper or paper towels on glass surfaces.

13.9. Visually inspect the laser module and flow-cell cover "windows" for scratches or imperfections following use and report to the supervisor.

13.10. Replace the laser module and flow-cell cover in their cases to prevent damage to glass surfaces during storage.

14. Sample analysis protocol

14.1. Unfiltered samples

14.1.1. Vortex the sample prior to loading and record **5 x 60 s videos** at **Camera Level 12** and **Detection Threshold Level 3** as described above. Reanalyze these videos using **Detection Thresholds 2 and 5**.

14.1.2. Repeat video collections of the same sample at **Camera Levels 13 and 14** and **Detection Threshold Level 3**. Reanalyze these 2 additional videos using **Detection Thresholds 2 and 5.** Repeat the entire process using **5 x 30 s videos** in the **SOP** setting.

14.2. Filtered samples

14.2.1. Vortex the sample and then simultaneously load and filter it (0.22 µm syringe filter) directly into the laser module.

NOTE: The syringe filter was flushed with 2 times the volume of the filter dead space prior to use to remove any resident particulates. The syringe filters (see the **Table of Materials**) had a measured dead space of 0.5 mL and were flushed with 1.0 mL of sample prior to measurements. Note the filter type in the **SOP** data fields. The filtered sample was processed exactly as described for unfiltered samples in step 14.1.

15. Statistical analysis of NTA results

15.1. For analysis of main effects or interactions, perform analysis of variance following a check of ANOVA assumptions (normality, unmodal, homogeneity of variance). Use Kruskal-Wallis one-way ANOVA on ranks in cases of failure of ANOVA assumptions.

15.2. Following return of significant main effects, use Dunn's method to perform means testing for preplanned comparisons. Consider a p-value of 0.05 to be significant in two-tailed testing.

NOTE: Datafiles generated here are available from the authors following the completion of a materials transfer agreement.

REPRESENTATIVE RESULTS:

Table 1 contains the results of the NTA videos for the liposome samples (18 filtered and 18 unfiltered) and a representative DPBS diluent. Comparisons across the two groups were completed regardless of the camera level or detection threshold in this paper. Filtered samples had a mean particle diameter of 108.5 nm, a particle mode of 86.2 nm, and a concentration of 7.4×10^8 particles/mL. In contrast, unfiltered samples had a mean particle diameter of 159.1 nm, a particle mode of 105.7 nm, and a concentration of 7.6×10^8 particles/mL. Mean and mode values for the filtered and unfiltered samples, regardless of the camera level or detection threshold, were statistically significant ($p < 0.05$). Differences in concentration between the filtered and unfiltered samples, regardless of the cameral level or detection threshold, were non-significant ($p = 0.86$).

[Place **Table 1** here]

When the liposome sample results were parsed by detection threshold (2, 3, 5) and camera level (12, 13, 14), the results were not significant (**Figure 3**). It should be noted that there were only 3 evaluations (n = 3) at each of these individual levels. This small sample size at each camera level and detection threshold likely contributed to the lack of individual comparisons being significant. However, when detection threshold (2, 3, 5) samples were evaluated regardless of camera level across the filtered and unfiltered samples (n = 3), both the mean size (**Figure 3A**) and mode size (**Figure 3B**) were significantly ($p < 0.05$) different. In contrast, differences between filtered and unfiltered sample concentrations (**Figure 3C)** were not significantly different.

[Place **Figure 3** here]

When the 3 camera levels (12, 13, 14) were evaluated regardless of detection threshold level (n = 3), both the mean size (**Figure 3D**) and mode size (**Figure 3E**) increased in the filtered samples. Sample concentrations showed a tendency to increase as the camera level increased from 12 to 14 (**Figure 3F**). The differences between filtered and unfiltered sample concentrations evaluated at different camera levels were not significantly different.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative NTA reports to compare liposomes to EVs. (**A**) Liposomes: unfiltered sample characterized on NTA on 12 March, 2020. (**B**) EVs: unfiltered sample characterized on NTA on 26 August, 2021. Abbreviations: NTA = Nanoparticle tracking analysis; EVs = extracellular vesicles.

Figure 2: Laser module focus alignment guide.

Commented [A23]: The authors should show typical outputs of the software and not just analyzed data (ie. raw histograms of counts). The reviewer indicates that typical outputs should be provided. The individual data files generated here are available for analysis. However, due to the publication limitations , they cannot be published in this paper. Lines 491-492: "Datafiles generated here are available from the authors upon request."

- The authors used liposomes as a model vesicle to demonstrate this technique and assess the impact of certain settings. But it would be more useful to the novice reader if sample data from actual EVs were also shown. *The reviewer indicates that the novice reader would be more informed by reviewing sample data derived from EVs. We agree that sample data from EVs would add something. However, we are dealing with space limitations and adding EV samples would require us to add source, isolation and characterization information which would increase the size of the Ms. If the reviewers can convince the editors to waive the space limitations, we can fulfill this request. Please note that this also would impact our meeting publication deadlines. Sample data from liposomes used in this project and EVs from another project were compared in Figure 2 of the manuscript.*

Editor: You can also upload important information as supplemental files. If you wish to make it available to the reader, assign them as coding files. However, please keep in mind that addition of data previously unavailable to peer reviewers will likely require additional rounds of peer review. Hence, for future reference, include as much data as you feel necessary to convince readers of your point right in the beginning. If you exceed size limits, we will let you know.

Commented [A24R23]: Although the authors agree in principle to the availability of the reader to access to important raw data utilized in the paper, the Excel files generated by the NTA analysis are represented by the experiment and averaged FTLA Concentration / Size Graph in both NTA reports displayed in Figure 1. To the novice reader, the Excel files would provide very little additional information not conveyed by the displayed report. Once again datafiles generated during the experiment are available from the authors upon request.

Commented [A25]: Please explain what you want the reader to take away from this figure and these reports. What do the graphs indicate? How do you distinguish the traces for liposomes vs EVs based on these graphs?

Commented [A26R25]: It had been suggested by one of the reviewers to show representative NTA analysis of EVs in comparison to a representative NTA analysis of liposomes. The similarities of the 2 NTA reports demonstrate the similarities of liposomes to actual EV analysis.

Figure 3: Effects of camera level and detection threshold on measured particle size and concentration of filtered and unfiltered samples. Mean (**A**) and Mode (**B**) particle size at combined camera levels 12, 13, 14 as the detection threshold was increased from 2 to 3 to 5 (n = 3), showing a significant decrease in particle sizes of the filtered samples. Particle concentrations (**C**) at combined camera levels 12, 13, 14 as the detection threshold was increased from 2 to 3 to 5) (n = 3), showing a concentration decrease as the detection threshold increased with no significant difference between filtered and unfiltered samples. Mean (**D**) and Mode (**E**) particle size at combined detection thresholds as the camera level increased from 12 to 14 (n = 3), showing a decrease in particle size of the filtered samples. Particle concentrations (**F**) at combined detection thresholds as camera levels increased from 12 to 14 (n = 3), showing a concentration increase as the camera level increases with no significant differences between filtered and unfiltered samples. Abbreviation: DT = detection threshold.

Table 1: Data table of collected values, standard deviation, and percent coefficient of variation for filtered and unfiltered samples. Abbreviations: Cam Lev = camera level; Det Thr = detection threshold; CV = coefficient of variation; St. Dev. = standard deviation; Conc. = concentration.

DISCUSSION:

There are several methods available to estimate the size and concentration of nanoparticles[11]. These include ensemble methods that generate a size estimate from a population, which include dynamic light scattering (DLS), centrifugal sedimentation, and single-particle level analysis, which includes electron microscopy, NTA, atomic force microscopy, and tunable resistive pulse sensing. Of these, DLS and NTA are widely used, nondestructive size and concentration measurement methods, which are based on Brownian movement in an ideal medium. DLS relies on scattering of light, and the intensity is proportional to the square of the particle volume. Thus, DLS is more sensitive than NTA to the presence of large particles, aggregates, or polydisperse populations.

NTA calculates the diffusion coefficient from the path length of individual particles measured in successive video frames. The main limitation of NTA is the narrow range of particle concentration that it can evaluate compared to DLS and other measurement methods, such that individual particle pathlengths must fall within the diffraction limit of the microscope and within the tracking software's capabilities. As DLS and NTA depend on Brownian movement, both can be expected to show good size agreement in monodispersed populations; they diverge when evaluating polydisperse populations and those with aggregates. The latter renders DLS useless and increases the NTA particle size estimate significantly[12]. NTA's best known limitation is that it requires much lower particle concentration (or greater dilution) than other measurement methods. Despite this, NTA characterization is popular in nanomaterials research. Because NTA size and concentration estimates depend on a more diluted population, with defined temperature, video capture settings, including recording length, camera level, detection threshold, and sample dilution to be highly reproducible, this paper focuses on the need for reporting these to generate reproducible results.

This paper shows that using a standardized protocol enabled replication of results, and that utilization of positive controls, such as size standards, provides information about the machine's

Commented [A27]: Please indicate in the legend that the lighter bars are filtered and darker bars are unfiltered samples.

Commented [A28R27]: Additional legend has been added for the unfiltered samples.

Commented [A29]: Please uppercase letters next to the graphs to indicate the different panels.

Commented [A30R29]: Letters have been added to Figure 3 to correspond to the legend.

Commented [A31]: Do the three bars of each type correspond to the three camera levels (left to right)?

Commented [A32R31]: Graph A, B and C evaluate individual Detection Threshold findings (DT-2, -3, -5) while graph D, E and F evaluate Camera Level findings (CL-12, -13, -14).

Commented [A33]: * Not clear what Aryal liposomes are. This was a reference to a laboratory source of liposomes it was unnecessary and therefore deleted.

Editor: Still there

Commented [A34R33]: My apologies. The names have been corrected to "Liposomes". Thank you.

Commented [A35]: I combined the two tables.

Commented [A36R35]: The Excel table was divided when downloaded. Thank you for combining them.

Commented [A37]: The lower detection limit of NTA is mentioned in the introduction, and it is correctly stated that it depends on the RI. This raises the question: can we use latex beads to determine the detection limit of NTA for EVs? The answer is no, because of the RI mismatch between EVs and latex beads (van der Pol et al. Nano Letters, 2014, Varga et al. Journal of Thrombosis and Haemostasis, 2018*). Here, latex size standards were used to confirm machine calibration prior to measurement of particle size. The RI of the synthetic liposomes used here would mostly likely be similar to that of EVs.*.

This protocol suggests using these beads, which is good in one hand, because many labs do not use any particle standard at all. But on the other hand, the use of latex beads would serve only as a quality control sample in case of the NTA analysis of EVs. For example, 50 nm PS beads can be detected by NTA but 50 nm EVs cannot. This should be at least discussed in the paper. *The reviewer contends that latex beads should be provided as a quality control measure and as confirmation of the calibration of the machine . We agree. Here, we used synthetic liposome particles as a model for EVs. We did not investigate detection limits here..*

Commented [A38R37]: A paragraph was added to express the limitations of the study regarding the reviewer's comments.

calibration. Furthermore, these results indicated the importance of reporting laser module chamber temperature, camera levels, detection threshold, and filtration (filter type and size), while laser module chamber temperature, diluent, and dilution factor are equally important for accurate and reproducible results. Although neither MISEV2018 nor EV-TRACK specifically recommends inclusion of this information, we suggest that inclusion of these details enables independent confirmation of published results and adds robustness to the experimental design.

Limitations of using latex size calibration standards for NTA calibration in EV analysis are acknowledged and include the known refractive index differences when compared to lipid bi-layer nanoparticles of similar size~~[van der Pol, 2014 #1223][van der Pol, 2014 #1223]~~. In this paper the latex beads were used to confirm machine calibration prior to measurements and not to determine the limits of detection. The liposomes have a membrane similar to naturally occurring EVs and the refractive index will be likewise representative of EVs. The size standards, as well as the liposome samples, are monodispersed populations and therefore their size distribution will follow a Gaussian or log-normal distribution. Natural EVs are polydispersed and their size distribution will follow a power-law function[13]. ~~mono~~

Historically, publications using NTA characterization inconsistently report necessary details to duplicate the research results. The ability to reproduce NTA data relies on the ability to duplicate the settings used to capture the original data. Without this information, reproduction of experimental results using NTA will be extremely difficult. With rigorous adherence to a set protocol and publication of the setting parameters used with the NTA, accurate replication of results can be attained. The following recommendations are made to contribute to improving consistency of nanoparticle characterization of size, concentration, composition, and purity using a nanoparticle size analyzer.

First, always check the calibration of the nanoparticle size analyzer using appropriate size standards, such as latex size standards. This should be done on a regular basis and recorded in the instrument log and prior to the analysis of critical samples. Second, all adjustable parameters, such as laser module chamber temperature, camera levels, and detection thresholds, should be recorded for each sample in the **Sample Log** file, as should the dilutions and diluents used. These parameters should be reported as they are operator-dependent and impact NTA measurements. Third, diluents used for sample dilution need to be characterized for nanoparticle content and reported. The diluents used for individual nanoparticle samples will need to be evaluated using the same camera level and detection threshold settings as the ones used for the diluted sample. Fourth, syringe filters should be flushed with two times the dead space volume prior to data collection or sample preparation steps to flush the numerous particulates remaining from the manufacturing process. Fifth, the concentration of the nanoparticles within the sample should be adjusted to within the suggested optimum 1.0×10^8 to 1.0×10^9 per mL.

Acknowledging the above described limitations in this study, we show that both the size and concentration values obtained by NTA can be affected by NTA parameters such as camera levels and detection thresholds, and that the size, but not the concentration, can be affected by sample preparation. This drives home the critical importance of reporting these parameters in

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Commented [A39]: What is the conclusion of these measurements for real EV characterization keeping in mind that the size distribution of EVs and extruded liposomes can be described by completely different functions? *The reviewer asks whether our findings impact EV characterization. We show that both the size and concentrations values obtained by NTA can be affected by NTA parameters such as camera levels and detection thresholds , and that size but not concentration can be affected by sample preparation. This drives home the critical importance of reporting these parameters in nanomaterial and EV literature, and producing a robust reproducible literature so that we can systematically investigate the impact of EV source, isolation and other experimental variables.*

Editor: Consider ending with this clear conclusion (highlighted in your response).

Commented [A40R39]: Thank you for this suggestion that was included in the final paragraph of the paper.

nanomaterial and EV literature enabling the production of a robust, reproducible literature so that we can systematically investigate the impact of EV source, isolation and other experimental variables.

ACKNOWLEDGEMENTS:

The work was supported by state of Kansas to the Midwest Institute for Comparative Stem Cell Biology (MICSCB). OLS received GRA support from the MICSCB. The authors thank Dr. Santosh Aryal for providing the liposomes used in this project and the members of the Weiss and Christenson laboratories for helpful conversations and feedback. Dr. Hong He is thanked for technical support. MLW thanks Betti Goren Weiss for her support and counsel.

DISCLOSURES:

None of the authors have any conflicts of interest.

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Supplemental File #1

Production of Liposome Sample:

- Sample was produced by Dr. Sagar Rayamajhi working in Dr. Santosh Aryal's Lab.
- Liposomes were produced using 900 µg of DSPE-PEG-Succinyl:100 µg of Cholesterol at 1.0 mg/mL in milliQ water.
- The average hydrodynamic size of the liposomes was 180 nm, and the PDI was 0.2 immediately after extrusion using dynamic light scattering on the Malvern ZetaSizer.
- This initial concentration was diluted 1:200 in milliQ water for the final liposome product used in this experiment.