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

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TITLE:

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

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KEYWORDS:

nanoparticle tracking analysis, NTA, standard operating procedures, SOP, interlaboratory comparisons, nanomaterial characterization, gold nanoparticles, hydrodynamic diameter, spherical nanoparticles, Brownian motion, Stokes-Einstein equation

SUMMARY:

The protocol described here aims to measure the hydrodynamic diameter of spherical nanoparticles, more specifically gold nanoparticles, in aqueous media by means of Nanoparticle Tracking Analysis (NTA). The latter involves tracking the movement of particles due to Brownian motion and implementing the Stokes-Einstein equation to obtain the hydrodynamic diameter.

ABSTRACT:
In the field of nanotechnology, analytical characterization plays a vital role in understanding the behavior and toxicity of nanomaterials (NMs). Characterization needs to be thorough and the technique chosen should be well-suited to the property to be determined, the material being analyzed and the medium in which it is present. Furthermore, the instrument operation and methodology need to be well-developed and clearly understood by the user to avoid data collection errors. Any discrepancies in the applied method or procedure can lead to differences and poor reproducibility of obtained data. This paper aims to clarify the method to measure the hydrodynamic diameter of gold nanoparticles by means of Nanoparticle Tracking Analysis (NTA). This study was carried out as an inter-laboratory comparison (ILC) amongst seven different laboratories to validate the standard operating procedure's performance and reproducibility. The results obtained from this ILC study reveal the importance and benefits of detailed standard operating procedures (SOPs), best practice updates, user knowledge, and measurement automation.

INTRODUCTION:
Nanomaterials (NMs) can vary in both physical and chemical characteristics that in turn influence their behavior, stability, and toxicity[1-5]. One of the major difficulties, when developing a thorough understanding of NM properties, hazards, and behaviors, is the ability to obtain reproducible information about physical and chemical nanomaterial characteristics. Examples of such physical properties include particle size and size distribution[6-8]. These are important parameters as they are a key aspect of the European Commission's (EC) definition of the term 'nano'[9].

Achieving precise particle size measurements is also critical for many different industrial and research applications and processes in addition to understanding the fate and toxicity effects of NMs[6,10]. It is important to have well established methods capable of measuring accurately, reliably, and reproducibly the size of NMs. Furthermore, reported information should provide deep understanding of the technique used e.g., indicate the type of size parameter (e.g., actual size or hydrodynamic size) as well as the sample condition e.g., the specific medium in which the NM is present, and for the method to perform reliably in different media. In order to measure size, a number of techniques can be used, including electron microscopy (EM), dynamic light scattering (DLS), single particle inductively coupled plasma mass spectrometry (spICP-MS), differential centrifugal sedimentation (DCS), scanning probe microscopy (SPM), small-angle X-ray scattering (SAXS) and nanoparticle tracking analysis (NTA).

NTA is a relatively new technology which has been well advanced in recent years and has been shown to reliably measure the hydrodynamic diameter of spherical NMs in complex aqueous media such as those with environmental relevance, e.g., freshwater systems. The hydrodynamic diameter is 'the size of a hypothetical hard sphere that diffuses in the same fashion as that of the particle being measured'[11]; in practical terms and in aqueous media this

describes a diameter larger than that of the particle itself, which also includes a layer of molecules (mostly water) held at the surface of the particle by weak electrostatic forces. The hydrodynamic diameter of a particle will vary in different media, getting smaller as the ionic strength of the media in which it is measured gets higher.

An additional important feature of the NTA technique is that it allows the analyst to achieve number-weighted size measurements, which is required in the context of the EC nanomaterial definition. High resolution, particle-by-particle analysis makes this technique less prone to interference caused by agglomerates or larger particles when present in a heterogeneous test sample with a high rate of particle throughput[10,12].

The measurement procedure consists of preparing a suitable suspension of the sample, which often requires sample dilution, followed by video recording of the particles' Brownian motion behavior and video analysis. From the sample chamber, a laser beam is pased, and the suspension particles in the path of the laser beam scatter light leading to their visualization using an optical microscope with a mounted camera. The camera captures a video file of the scattered laser light from the particles moving under Brownian motion. Many particles can be tracked individually to determine their diffusion coefficients and their hydrodynamic diameters can be calculated using the Stokes-Einstein equation: $d = kT/3\pi\eta D$ where d is the hydrodynamic diameter, k is the Boltzmann constant, T is the temperature, η is the viscosity and D is the diffusion coefficient[10]. NTA can also be used to track the aggregation behavior of particles that are generally colloidally unstable (the particles must, however, be colloidally stable over the measurement time scale)[13,14]. If users want to check the system performance this can be easily done by measuring size standard materials as frequently as wanted.

The NTA instrument used is easy to operate with quick analysis time (under 10 min per sample). For high quality measurements with good data repeatability and reproducibility, number of factors should be considered in both sample preparation as well as in instrument operation. If such factors are not carefully considered, measurements on the same material across different laboratories and operators can be subject to unknown or poorly quantified uncertainties. During NP characterization, using best practice in-house developed SOPs does not always guarantee consistency with other laboratories, as shown by Roebben et al. for the DLS technique[15].

In fact, an early (first round) NTA ILC between different laboratories, users and instruments revealed inconsistent results. One of the main issues was with the use of various older legacy instruments which had not had regular services or calibration checks, as well as differences in method interpretation. An NTA ILC study by Hole et al. found that with the absence of shared guidelines on how to use a system and prepare samples, variability across laboratories can be large even for relatively monodispersed samples[16]. This along with the results from the first round of the ILC highlights the need for good instrument maintenance as well as method training and well-developed standard operating procedures (SOPs). The latter act as a powerful tool to describe and document compliance with good practice. If well detailed, standard operating procedures (SOPs) can offer clarity, explanation, understanding, standardization, and quality assurance.

The recommendation for adopting an ILC study is, therefore, ideal for both developing and testing protocols[16]. The ILC exercise acted to validate this specific NTA SOP and hence introduced confidence and clarity into this specific nanomaterial risk assessment method. It involved three rounds. Round 1 analyzed 60 nm gold nanoparticles on each participant's own instruments before training. Round 2 involved analyzing 100 nm latex on the same NTA instrument after training as a simple test in order to determine that the instrument was set up correctly and the users had a good knowledge on how to use the instrument. Round 3 involved the analysis of 60 nm gold nanoparticles on the same NTA instrument after training. Participants in the ILC came from seven different labs, all consortium members of the Horizon 2020 ACEnano project[17].

The aim of this article is to discuss the method and results from a third round of benchmarking for the NTA technology where 60 nm gold NPs were re-analyzed by seven partners following detailed training and SOP development. Comparison and reference to the results obtained in the first round of the ILC will also be made. All analyses from round 3 of ILC were carried out using the same instrument (see **Table of Materials**) of identical configuration supplied with a 405 nm laser and a high sensitivity sCMOS camera. Benchmarking assesses the performance of the technology on samples and hence leads to the development of 'best practice' protocols. Thus, this article also shares and makes the NTA method for the instrument used in this ILC available for the scientific community as it has been harmonized via conducting and evaluating the ILCs according to international standards.

PROTOCOL:

The methodology described here was used for the third round of the inter-laboratory comparisons.

1. Sample Preparation

1.1. Filter water through a 0.02 µm syringe filter. Water filtration is necessary to remove any contamination particles before using it for sample dilution.

1.2. To analyze a freshly prepared sample, dilute a sample of 60 nm gold colloid dispersion volumetrically by a factor of 50 in filtered ultrapure water. The suggested concentration for NTA analysis is $1 \times 10^7 - 1 \times 10^9$ particles per mL.

2. Performing the measurement

2.1. Switching on the system

2.1.1. Switch on the hardware and software. Connect the NTA instrument, syringe pump and the computer. The associated software (see **Table of Materials**) ensures all hardware communications are running and that a live temperature readout is displayed.

2.1.2. Remove the laser module and using a tissue and compressed air completely dry the glass surfaces and the low volume flow cell (LVFC) internal channels, tubing, and fluidic ports.

2.2. Priming the tubing

2.2.1. Rinse the inlet fluidic tubing with ultrapure water to remove any particles and reduce the likelihood of air bubbles that would interfere with measurements. For rinsing, the end of the inlet tubing inside the instrument casing is placed in a waste container.

2.2.2. Insert a 1 mL syringe (without needle) of filtered water into the Luer port and push ~900 µL of liquid through the inlet tubing as fast as the back pressure allows. Leave the syringe containing the remaining liquid attached to prevent any syphoning.

2.3. Syringe pump tubing connection

2.3.1. Assemble the LVFC onto the laser module to create the sample chamber as seen in **Figure 1**. Attach the outlet tubing to the right-hand side port of the LVFC.

NOTE: The inlet and outlet tubing are different in diameters, with the inlet being smaller in diameter than the outlet. Swapping the inlet-outlet tubing connection may cause over pressuring the flow cell and leaking.

[Insert **Figure 1** here]

2.3.2. Disconnect the syringe from the inlet tubing and exchange for a new syringe containing 1 mL of filtered water, ensuring liquid-to-liquid contact. Connect the inlet tubing to the left port of the LVFC. Slowly introduce ~500 µL of fluid into the sample chamber. Take care to ensure no air bubbles are introduced during loading. The final tubing configuration is shown in **Figure 2**.

[Insert **Figure 2** here]

2.4. Laser module loading and system check

2.4.1. Insert the laser module with the water filled LVFC into the instrument and lock into place.

2.4.2. Place the syringe into the syringe pump cradle and secure. Initialize the camera by clicking on **Start Camera** in the software interface. In the **Hardware** tab of the interface, click on **Scatter** to move the reference position.

2.4.3. Set the camera level to 16 and adjust the focus manually to check the diluent for any particles. Adjust the field of view position by left clicking on the main viewing window and using the mouse to drag up and down to check for any particles. If there are more than three particles in the field of view, this implies a problem with the water purity or the cleaning process and, therefore, the cleaning process needs to be repeated or the water needs to be replaced or filtered.

2.4.4. Disconnect the syringe from the inlet tubing and replace it with a syringe full of air only. Slowly introduce the air into the sample chamber to remove the liquid inside. Remove the LVFC from the laser module and disconnect the tubings. Clean the glass surfaces of the LVFC

and optical glass of the laser module with water and dry with a tissue and compressed air. Dry the tubing with compressed air. Reassemble the LVFC onto the laser module and connect the tubing, ready for sample loading.

NOTE: This step is not always required, however, in this case it was added as an extra precaution to further reduce any possible variation.

2.5. Loading sample

2.5.1. Repeat step 2.2.2. Connect a syringe containing 1 mL of the 60 nm gold nanoparticles dispersion made in step 1.1 to the Luer port. Inject 750 µL of the sample into the LVFC via the inlet tubing with the laser module viewed outside the instrument to ensure no bubbles are introduced.

2.5.2. Load the laser module and initialize the camera by clicking on **Start Camera** in the software interface. In the **Hardware** tab of the interface, click **Scatter** to move to the reference focus position, check that this is set correctly to give a clear image of the particles.

2.5.3. Check that the field of view is set centrally with respect to the laser beam position. Adjust accordingly by left clicking on the main viewing window in the software and mouse dragging up and down.

2.5.4. Run the **AutoSetup** function to automatically optimize the focus and camera level ensuring that the optimal image quality is achieved.

NOTE: The automatic camera and focus parameters allow for more consistency amongst the different labs since this is user independent.

2.6 Sample analysis

2.6.1. Create a measurement script in **Standard measurement**, **SOP** tab, to obtain 5 repeat videos of 60 s under slow (particles should be passing across from one side of the screen to the other in approximately 10 s) and constant flow (**Supplementary File 1**).

NOTE: Flow is recommended to ensure a better representation of the overall sample is presented for measurement. Precision and repeatability of concentration measurements are significantly improved when a slow flow is imparted on the sample to ensure that a greater number of new particles flow through the measurement zone and are analyzed during an experiment. The video length depends on the profile distribution and how variable it is over the analysis time. 5 videos of 60 s are considered as a typical measurement duration.

2.6.2. Set the experiment file name and location for the data and start the run. The analysis following the outlined procedure was carried out by the seven laboratories of the Horizon 2020 ACEnano project[17].

3. Data Analysis

NOTE: All data analysis is done within the v 3.4 software (see **Table of Materials**), no additional manual conversions or calculations are used. The particle sizing data is presented in raw form as a histogram distribution and is calculated from the measured change in position of the particle using the Stokes-Einstein equation. The software determines the average distance moved by each particle in the x and y planes. This value allows the particle diffusion coefficient (D) to be determined from which, if the sample temperature T and solvent viscosity η are known, the equivalent spherical hydrodynamic radius, R_H, of the particles can be calculated. The temperature of the sample is automatically recorded by the NTA. The default sample viscosity used by the software is for water and is included in the measurement script shown above, though viscosity can be amended by the user when different sample diluents are used, either before or after the measurement is taken.

3.1. Set the detection threshold (DT) by dragging the slider bar or clicking the **+** and **-** buttons in the software under **Detection Threshold**, which is the analysis parameter for optimal tracking of the visualized particles, between 2 and 20. Ensure that the DT value chosen identify and track as many visible particles as possible (marked automatically as red crosses on the software image screen).

3.1.1. As guidance for setting the detection threshold, the number of identified particles in an image should be in the range of approximately 30-80 where no more than 10 red crosses should correspond to sites not considered to be particles by the observer. There should be no more than 5 blue crosses (indicative of noise) observed.

[Insert **Figure 3** here]

3.2. Automatically process the particle tracking analysis videos by pressing the **Process** button in the software. Leave all the processing parameters on their automatic settings and export the data as a .csv format results file with the full particle size distribution and additional metadata describing the measurement setup. To verify the measurement quality, look at the **Analyze** tab in the software or check the .csv output file for any warnings message or alerts. An example of the PDF results report is shown in **Supplementary File 2.**

3.3. Read the mode results and the associated standard deviation from the PDF report.

NOTE: The mode size results were used to compare the sizes obtained amongst the seven laboratories and are shown and discussed in Section 5.

4. Cleaning and drying

4.1. After use, flush the system thoroughly with clean water to remove all traces of sample from the tubing and optical surfaces.

4.2. Load a syringe of air through the system to empty the tubing and the LVFC. Remove the LVFC from the laser module. Using compressed air, completely dry the glass surfaces, LVFC internal channels, fluidic ports, and tubing. Always leave the NTA instrument clean and dry whenever the system is not in use, with the LVFC removed from the laser module. Turn off and shut down the instrument and the software.

REPRESENTATIVE RESULTS:

The round 1 ILC results using various NTA instrument configurations are shown in **Figure 4**. With the exception of Lab 6, the repeatability between the 5 capture repeats was good but several labs recorded a mode size higher than expected. Lab 6 results showed poor repeatability and a much higher mode size measured. After the investigation, it was found that the systems reporting biggest size variations were either not maintained as recommended or the analysis was affected by inconsistency in sample preparation whereby the dilution step can create variation caused by different pipetting equipment, user operation and technique, and/or measurement set up including the flow cell not being clean, the wrong camera level being used, the image not being focused properly, and setting the analysis Detection Threshold incorrectly.

[Insert **Figure 4** here]

The NTA result accuracy from round 3 was improved by all the laboratories implementing the same SOP and instrument settings. The mode size results obtained for this ILC Round 3 can be seen in **Figure 5**. The average mode across all labs was 62.02 ± 1.97 nm. All measured results from round 3 were more consistent than the first stage results with the results falling well within 10% of the 60.5 nm mean size for the batch as stated by the manufacturer. The coefficient of variation for the gold samples stated by the manufacturer was ≤8%.

[Insert **Figure 5** here]

To verify the particle size as provided by the manufacturer a small number (N=82) of particles were analyzed by Transmission Electron Microscopy (TEM). Approximately 10 µl of the undiluted dispersion was drop cast on a carbon coated Cu TEM grid and dried in air before imaging in an analytical TEM at 200 kV. Images like **Supplementary Figure 1** were taken from areas with minimal particle overlap and analyzed using a semi-automatic image analyses process. An automatic watershed method was applied to separate particles and artefacts of this process were excluded as well as on edge particles[18]. The mean diameter was calculated either as average from the major and minor axis (61 ± 7 nm) or as a conversion from the measured area (62 ± 6 nm) assuming spherical particles. Particles appear to be mostly spherical with an average aspect ratio of 1.1. The TEM results show a slightly higher diameter than the manufacturer value (60.5 nm) but are within the tolerance level. Additionally, there is a very good agreement with the NTA derived value of hydrodynamic diameter.

FIGURE AND TABLE LEGENDS:

Figure 1: Low Volume Flow Cell assembly mounted on laser module.

Figure 2: Low Volume Flow Cell tubing configuration.

Figure 3: Threshold setting observations. A bad (left) and good (right) detection threshold setting observation.

Figure 4: ILC Round 1 Mode size results. Mode size results from all NTA benchmarking partners for Round 1 60 nm gold nanoparticle dispersion carried out on different NTA instruments (as abbreviated in the x axis).

Figure 5: ILC Round 3 Mode size results. Mode size results from all NTA benchmarking partners for 60nm gold ILC round 3 analyzed on the same NTA instrument. The average mode across all labs was 62.02 ± 1.97 nm.

Supplementary Figure 1: TEM image of 60 nm Gold Nanoparticles.

Supplementary File 1: Measurement script.

Supplementary File 2: Example of PDF results report.

DISCUSSION:

The inconsistent results obtained from the Round 1 ILC highlighted the need for instrument health checks for older systems as well as the development of a more detailed SOP, the need for hands-on training and a better understanding of measurement and analysis settings so as to ensure more consistent results across the different labs. In fact, Hole et al. found that the absence of shared guidelines on how to use an NTA system and prepare samples resulted in variability across laboratories even for relatively monodispersed samples[16]. Therefore, all ILC participants attended a training workshop covering the best practices for the system operation and measurement conditions, as well as cleaning and maintenance guidance for the specific NTA instrument. All participants also performed measurements on the same instrument in their own labs for the subsequent ILC rounds. The procedure first involved a round that tested the system locally in each laboratory by running an ILC on latex standard samples (ILC Round 2), before being used by the partners to repeat the gold measurements (ILC Round 3). The aim of measuring these gold samples by means of NTA was to introduce confidence and clarity into nanomaterial risk assessment methods and practices needed to impact nanosafety guidance protocols.

NTA is a technique that can achieve the hydrodynamic spherical equivalent diameter of particles and can be used for particle by particle real-time visual analysis of complex polydispersed systems ranging from 10 nm – 30 nm, to 1 µm – 2 µm in size (depending on the sample properties and instrument configuration). Minimal sample preparation is required. Despite minimal sample preparation, this step is critical for the protocol and great care should be taken when diluting a sample and choosing a diluent. Shape can be a limiting factor with respect to NTA as spherical equivalent size measurements are obtained and non-spherical particles will have a less accurate size value.

For NTA technology, some result variation is always to be expected as only a representative sample is observed from the whole sample. Regardless, all results meet the ISO 19430 standard for particle sizing. The optimal concentration to provide is typically around 10^8 particles/ml within a 30-60 second analysis time. For samples with lower particle concentrations, longer analysis times will be required to ensure reproducible results. For samples containing a concentration of particles greater than 10^9 particles/mL, there is a

greater likelihood of tracking problems and samples will need to be diluted down to a suitable range for NTA measurement.

Overall the results from the 3rd round ILC show good reproducibility of gold nanoparticle measurements with NTA with increased accuracy and repeatability. All NTA measurements were carried out using the automatic camera level and focus settings to adjust the image, as selected by the Auto-Setup feature in the software. The camera level set by the software was very consistent, with a camera level of 10 or 11 being set in all cases showing that as expected, the more automation a process includes the more consistency is achieved. The sizing results were comparable to those obtained by the manufacturer by means of TEM indicating that the results were reproducible, however minimal differences are to be expected from different techniques since TEM does not determine the hydrodynamical diameter. The significant improvement in the consistency of results show the importance and benefits of instrument maintenance, detailed SOPs, best practice updates, user knowledge and applied measurement automation for NTA. In conclusion the ILC validated this specific NTA SOP and hence introduced confidence and clarity into this specific nanomaterial risk assessment method.

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DISCLOSURES:

The author Jo Sullivan, Agnieszka Siupa, Pauline Carnell-Morris and Michele Carboni are employees at Malvern Panalytical Ltd. that manufactures instruments used in this article.

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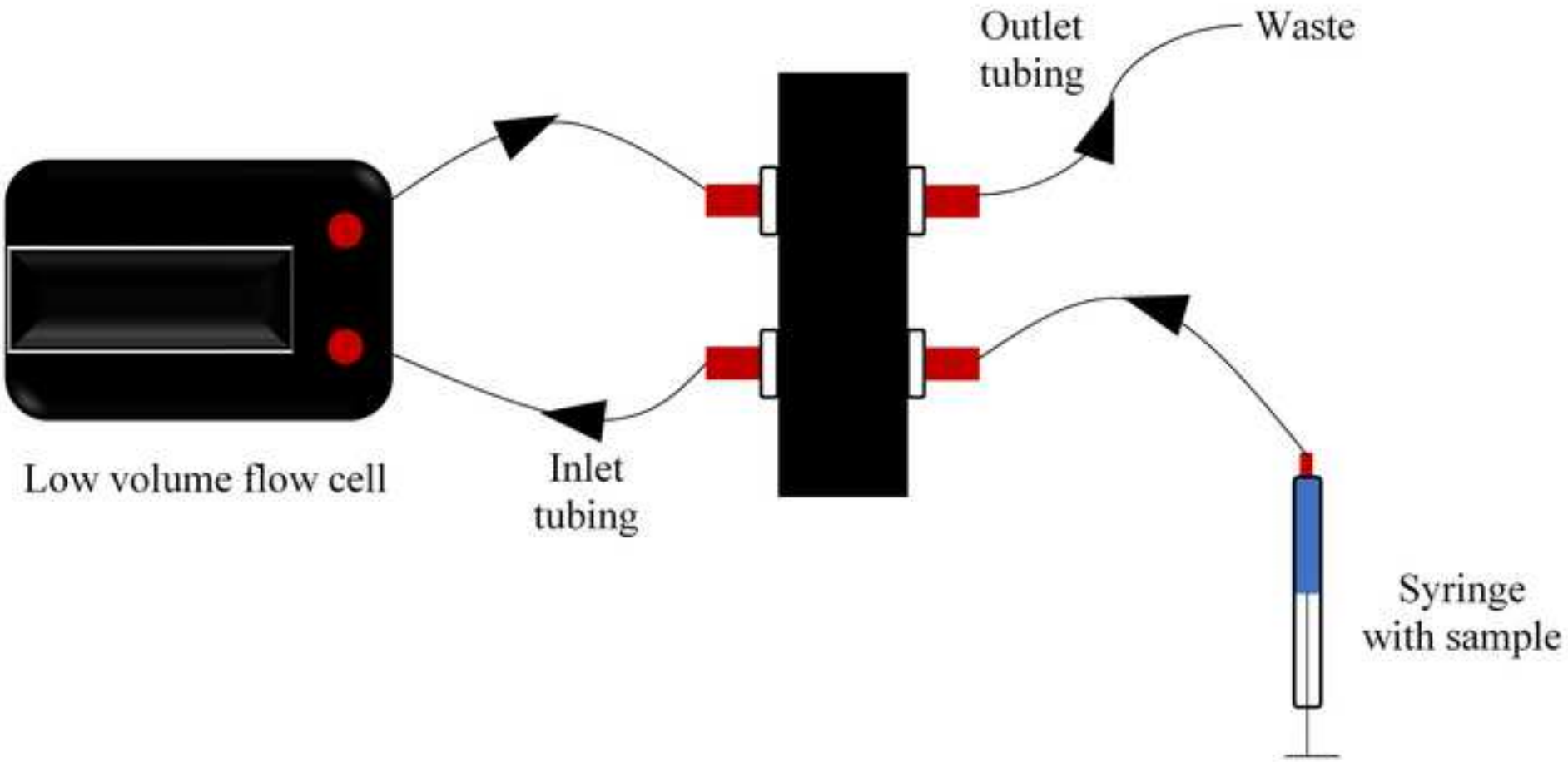
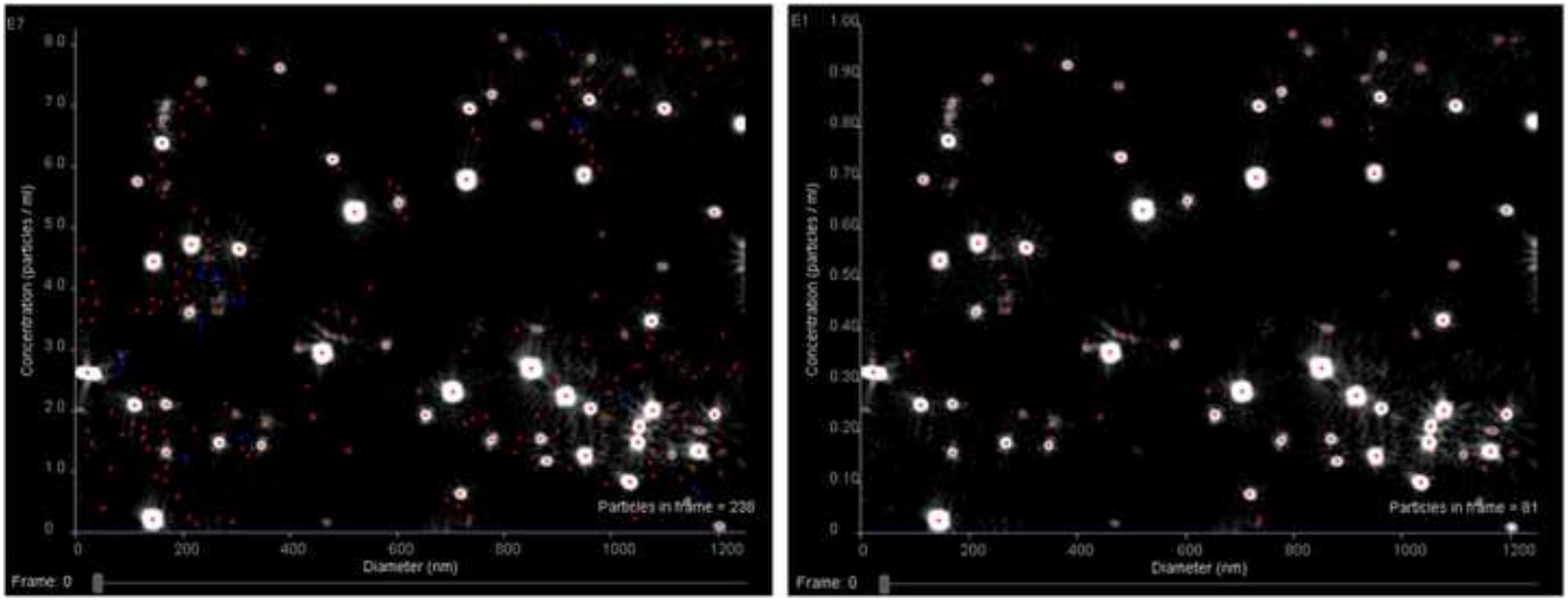
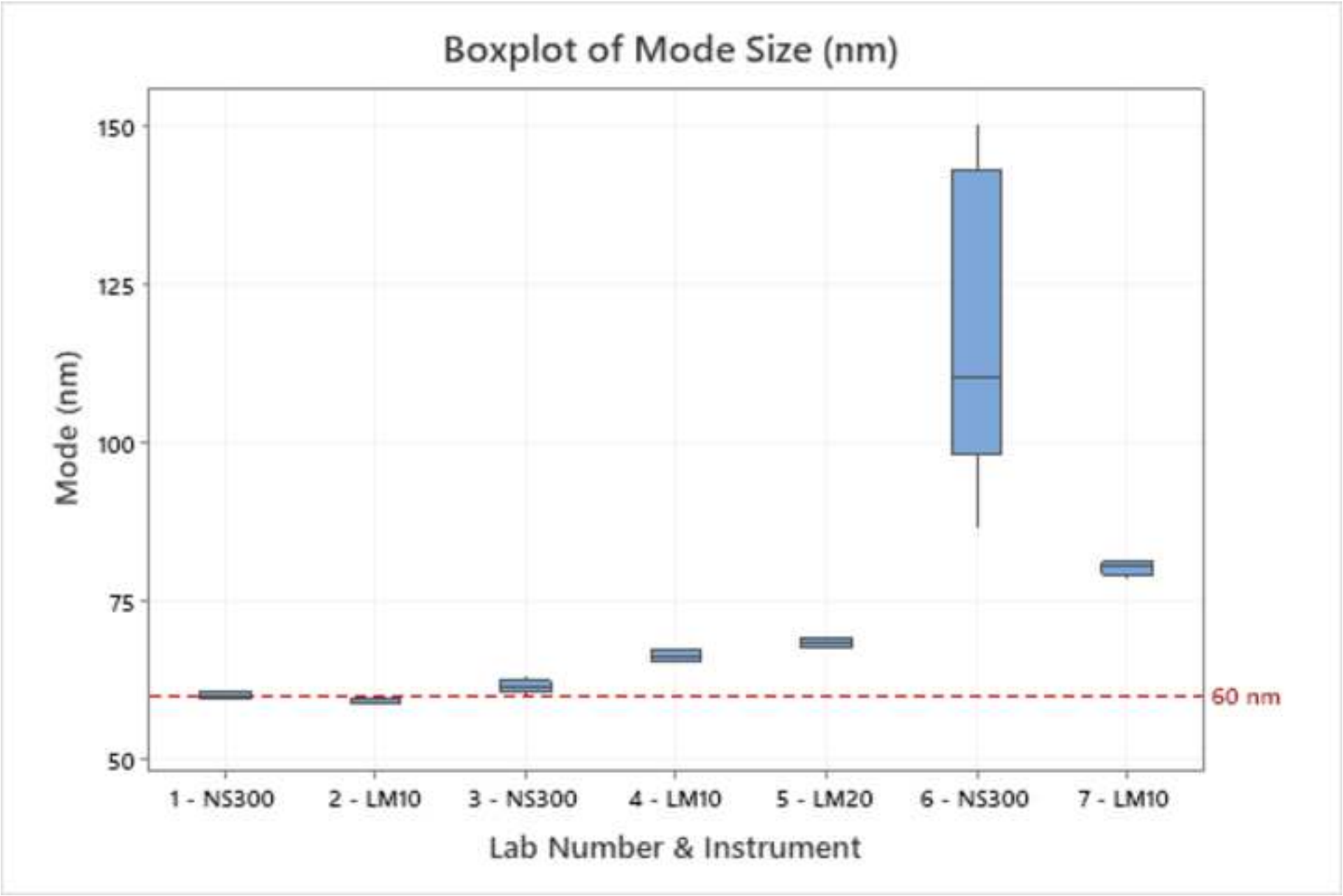
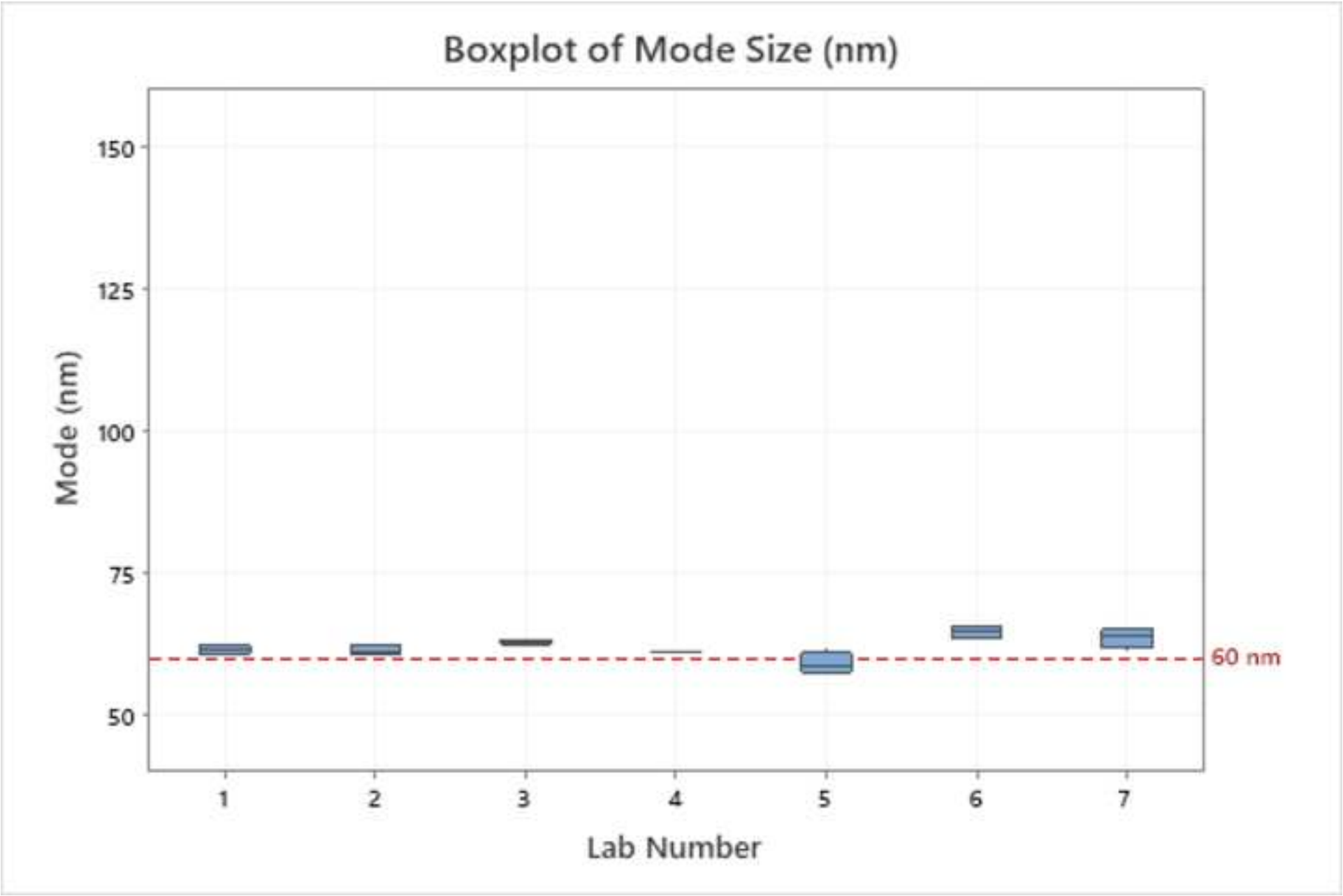








Name of Material/Equipment

60 nm gold colloid dispersion
0.02 µm syringe filter - Whatman Anotop 25 Sterile Syringe Filters
NanoSight
NanoSight NTA Software v3.4
Syringe PP/PE without needle luer slip tip, centered, capacity 1 mL, graduated, 0.01 mL, sterile

Company	Catalog Number	Comments/Description
BBI Solutions OEM Ltd.	Product EM. GC60, Batch number 024650	
Sigma Aldrich	WHA68092102	
Malvern Panalytical Ltd.	NS300	
Malvern Panalytical Ltd.	v3.4	
Sigma Aldrich	Z230723	

Editorial comments:

You will find Editorial comments and Peer-Review comments listed below. Please read this entire email before making edits to your manuscript.
NOTE: Please include a line-by-line response to each of the editorial and reviewer comments in the form of a letter along with the resubmission.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

The manuscript has been proofread to ensure that there are no spelling or grammatical errors.

• Avoid punctuating the title.

The title has been edited slightly to ensure that there is not punctuation.

• **Protocol Language:** The JoVE protocol should be almost entirely composed of numbered short steps (2-3 related actions each) written in the imperative voice/tense (as if you are telling someone how to do the technique, i.e. "Do this", "Measure that" etc.). Any text that cannot be written in the imperative tense may be added as a brief "Note" at the end of the step (please limit notes). Please re-write your ENTIRE protocol section accordingly. Descriptive sections of the protocol can be moved to Representative Results or Discussion. The JoVE protocol should be a set of instructions rather a report of a study. Any reporting should be moved into the representative results.
o Section 2 will need to be edited so that all steps are in the imperative.
o Sections 3-4 will need to be entirely re-written as steps in the imperative voice.

Sections 2-4 have been edited and rewritten as necessary to ensure that they are written in the imperative tense.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

The protocol has been amended accordingly.

• **Protocol Numbering:**

1) Please removing the numbering from the section headings (e.g., introduction, protocol), and start by numbering line 158 as 1.
2) Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary.
3) All steps should be lined up at the left margin with no indentations.
4) Please add a one-line space after each protocol step.

The numbering has been amended accordingly.

• **Protocol Highlight:** After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages, please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

The relevant parts of the protocol have been highlighted.

• **Results:** Please provide a distinct representative results section and a separate discussion section.

A distinct representative results section has been provided.

• **Discussion:**

1) JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

The discussion has been amended and rewritten to ensure all these sections have been included.

2) Merge the conclusions into the discussion.

The conclusion has been merged with the discussion.

• **Figures:** Please remove the embedded figures from the manuscript. Figure legends (including those for supplementary files), however, should remain within the manuscript text, directly below the Representative Results text.

All figures have been removed from the manuscript but captions have been left below the representative results.

• **Figure/Table Legends:** Please expand the legends to adequately describe the figures/tables. Each figure or table must have an accompanying legend including a short title, followed by a short description of each panel and/or a general description.

Done.

• **References:**

1) Move all hyperlinks (e.g., line 141) to the table of materials.

Done.

2) Reference 13 is incomplete.

Reference 13 has been updated.

3) Please make sure that your references comply with JoVE instructions for authors. Citation formatting should appear as follows: (For less than six authors, list all authors. For more than 6 authors, list only the first author then *et al.*): [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. *Source*. **Volume** (Issue), FirstPage – LastPage, (YEAR).]

The JoVE style was downloaded and used.

• **Commercial Language:**JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are NanoSight, NanoSight NS300, Malvern Panalytical NS300

1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

Commercial language has been removed.

2) Fig 1 should be removed.

Figure 1 has been removed.

• **Table of Materials:**

1) Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file.
2) Please sort in alphabetical order.

This has been updated and sorted in alphabetical order.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that

allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Comments from Peer-Reviewers:

Reviewers' comments:

Reviewer #1:

Manuscript Summary: The manuscript provides a detailed experimental description of the procedure that was used to measure 1 single sample in 7 different laboratories

Major Concerns:

The results prove that the described protocol works fine for this specific sample. However, the main question is how the user can know that he is making good measurements on any other sample. From own experience, I know that there are some critical factors, such as the degree of dilution. This was fixed in the protocol by specifying the dilution degree (i.e. 50 times dilution), rather than be proposing a criterium that should be met. In the latter case, the protocol could be applied to other samples, which is not the case now.

It is impossible to define “a” protocol for all samples. Protocol development and sample preparation are significant aspects to successfully using the instrument. The authors have however added further information throughout the paper to guide a user on how they can obtain a good measurement. Details regarding dilution factor, information with respect to shape, checking that the diluent is particle free and figures indicating a good and bad detection threshold setting have all been added.

Whereas the protocol was written out in great detail, I miss important information, such as the max jump distance (as mentioned on line 342), and the minimum number of frames over which a particle can be followed

These have been removed for this specific method as these parameters are not to be set by the user in this protocol. A note has been made in the protocol to leave all the processing parameters on their automatic settings.

The most interesting information would be to know what was different between round 1 (figure 4) and round 3 (figure 5). Line 365-366 only provide very general information, from which nothing can be learned: "not maintained as recommended", "inconsistency in sample preparation", "inconsistency in analysis set up". What do you mean ? What can be going wrong in sample preparation or analysis set up ?

This has been clarified further and now reads the following: ‘After the investigation it was found that the systems reporting biggest size variations were either not maintained as recommended or the analysis was affected by inconsistency in sample preparation whereby the dilution step can create variation caused by different pipetting equipment, user operation and

technique, and/or measurement set up including the flow cell not being clean, the wrong camera level being used, the image not being focussed properly, and setting the analysis Detection Threshold incorrectly.'

Overall, this reads too much as a commercial leaflet: "look how well we can recover the specified size of a sample with our instrument". That is quite weak as take home message.

The authors would like to point out that the main message was that the ILC validated this specific NTA SOP to introduce confidence and clarity into nanomaterial risk assessment methods. Consistency amongst the labs and the collection of data ensured this. This is an important aspect when comparing results from different laboratories particularly in relation to nanomaterial risk assessment. This has been highlighted in more detail in the abstract, introduction and discussion.

Minor Concerns:

*) Figure 1 has no added value and can be removed

This Figure has been removed.

*) Lines 288-301 have no meaning for readers that do not have the same instrument or software; I would delete them

These lines have been deleted.

*) The Stokes -einstein equatuion was already introduced in line 113, and hence lines 313-317 can be removed

These lines have been removed.

*) I do not understand that Detection Threshold discussion at all: line 330 mentions as many visible particles (red crosses) as possible, whereas line 33 specifies less than 10 red crosses (and line 332 30 to 80 particles)

The reviewer is correct to indicate that this is confusing. Therefore in order to clarify this the authors have rewritten as follows:

The DT value chosen should identify and track as many visible particles as possible (marked as red crosses on the software image screen). As guidance for setting the detection threshold, the number of identified particles in an image should be in the range of 30-80 where no more than 10 red crosses should correspond to sites not considered to be particles by the observer. There should be no more than 5 blue crosses (indicative of noise) observed.

*) do not use a second title on top of the figures (for fig.4 and 5)

The second title on top of Figures 4 and 5 has been removed.

Reviewer #2:

Manuscript Summary: Very nice manuscript that sets out how to preform a measurement using the NS300 system which could be of great benefit to new users. There have been some previous ILC studies on NTA, and it might be good to reference back to these. It would be nice to see how this version correlates to previous version (NS300 alone, versuses a mixture of LM10s, NS300, NS500 systems)
This paper shows nicely the expected outcome and repeatability of identical systems (assuming no differences in laser intensity, or alignment)

The authors thank the review for their comments. They particular appreciate the suggestion to make reference to previous ILC studies. The authors have done so referring to Roebben et al and Hole et al. in the introduction. This has strengthened the argument for the importance and need for well-developed SOPs for the characterisation of nanomaterials.

Major Concerns:
No major concerns

Minor Concerns:

It might be nice to specify that the inlet and outlet tubings are different diameters. If these are swapped around does this impact the analysis? or can it damage the flow cell?

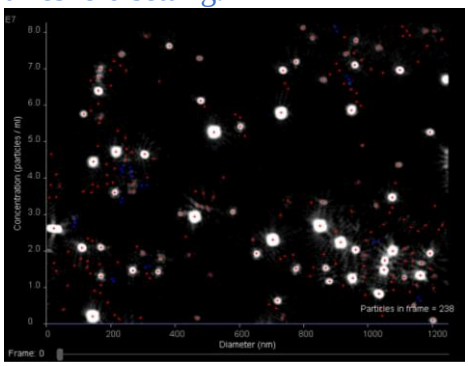
The inlet and outlet tubing are different diameters, with the inlet being smaller in diameter than the outlet. Swapping the inlet-outlet tubing connection may cause in over pressuring the flow cell and leaking. This explanation has been added as a note to section 1.3.3.1.

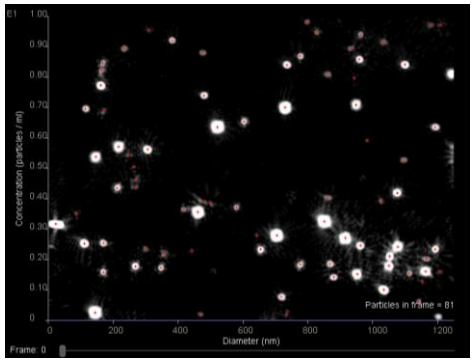
What would be the impact on changing the syringe flow rate, from 25 to say 30 or 5. Is there a load speed that is too fast? perhaps that can be investigated to see what the cuts would be.

It is recommended to set the pump flow speed so that the particles take 10 seconds to travel across the screen as mentioned in section 2.2.6.1. This guidance is the result of previous investigations into what the effect of flow speed on sizing is. Using flow rates faster than this will result in compromising the sizing accuracy of the measurement. The optimal pump speed setting may vary slightly from system to system but a difference of +/-5 units in pump speed will not greatly affect the particle flow speed.

Screen shots of good vs bad focus positions should be included, likewise for selection of detection thresholds.

Since the focus was set automatically, a bad focus will not be achieved. The authors however have included the following screen shots in section 1.4.1 to depict a bad and good detection threshold setting.





It should be pointed out that the results .csv file lists warning about vibration and noise, and that these should be checked to verify results quality.

These warnings also appear in the analysis tab. We added a line "To verify the measurement quality, look at the Analyse tab in the software or check the csv output file for any warnings message or alerts".

Sections 2.2.4.3 runs into 2.2.5.2, and then back to 2.2.4.5 (page 6), this should be amended Checking the assembled unit while filled with clean water is a good idea, but I query the necessity to then disassemble the system, clean it and refill it before sample loading Perhaps this can be clarified, or amended

Yes, while this would not always be needed, for this ILC protocol this step was added as an extra precaution to further reduce any possible variation. This method minimises the risk of any cross contamination of the sample. A note has been added to the relevant section (now section 1.3.4.4 to explain this).

Please comment on the variability of the results for ILC 3, although minor. What is expected to be the main source of error? All systems are considered to be identical (405nm, high sensitivity systems, using autofocus) the difference between lab 4 and 5 for example in figure 5 is striking

For NTA technology, some result variation is always to be expected as a sample rather than the whole sample is seen. Flow helps to reduce this variation however some variation will always be present due to the low number analysis. Regardless, all results meet the ISO 19430 standard for particle sizing.

Reviewer #3:

Manuscript Summary: This paper studies how to use NTA characterize NPs in aqueous media. The authors utilize the technique to conduct a serious experiments and some information has been found. However; this study with the current data lacks the novelty required for publication in Journal of Visualized Experiments.

The authors thank the reviewer for their comments. The aim of this paper was not to have novel data but rather that the ILC validated this specific NTA SOP to introduce confidence and clarity into nanomaterial risk assessment methods. Furthermore to show the vitality of correct instrument set-up and running so as to obtain consistency amongst results. Consistency amongst the labs and the collection of data ensured this. This is an important aspect when comparing results from different laboratories particularly in relation to nanomaterial risk assessment. This has been highlighted in more detail in the abstract, introduction and discussion.

Major Concerns:

(1) The design of the experiment was too simple and the result was obvious.

Although it was a simple measurement we have shown that it is still possible to make errors and not obtain reproducible results. We therefore wanted to highlight that adhering to a

developed method and operating the NS300 system in the recommended way can improve the measurement results.

(2) From my point of view, NTA experimental results strongly depend on the operator and software parameters for which the optimal choices must be empirically determined by the user. Therefore, the screen gain, camera level, detection threshold and focus need to be discussed in Results and Discussion.

This protocol aimed to keep the running of the instrument simple to avoid settings having to be determined by the user and ensure consistency amongst users. In the paper we have emphasised further in section 1.3.5.4 that the automatic camera and focus parameters were used. The DT setting is the only parameter that must be determined by the user – guidance on how to set the DT optimally is included in the paper.

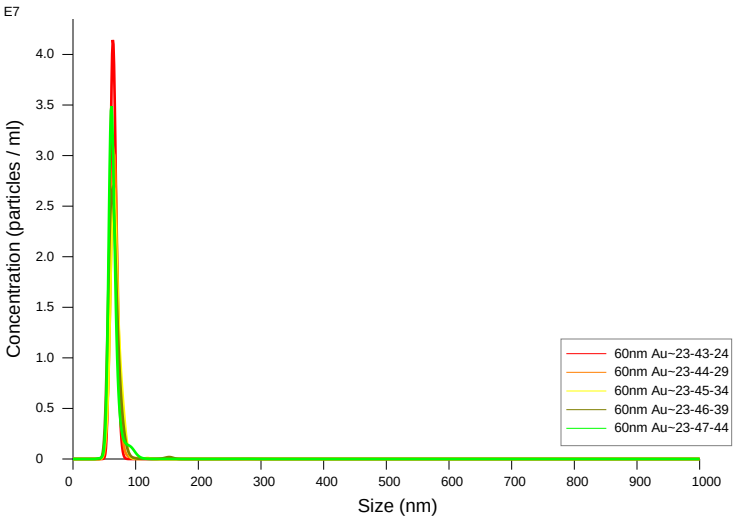
(3) The most important is that this manuscript is more like the NanoSight 300 NTA Software Guide than a scientific paper. Most of the information provided in this manuscript can be found in the NanoSight 300 NTA Software Guide. I would suggest the authors to provide more data or a more informative discussion on their data to make it more suitable for the publication.

Novelty is not a requirement for JoVE publication. The JoVE mission is to increase the reproducibility of science so the focus is on the protocol itself. The importance of this work was to introduce confidence and clarity into nanomaterial risk assessment methods. The discussion has been rewritten to highlight this and include more information regarding the technique's advantages and limitations. Furthermore reference has been made to another NTA ILC which emphasised the need for a standard protocol. Publishing this specific protocol in JoVE would make it widely accessible for the scientific community to access for the visual characterisation of the size and concentration of spherical nanomaterials.

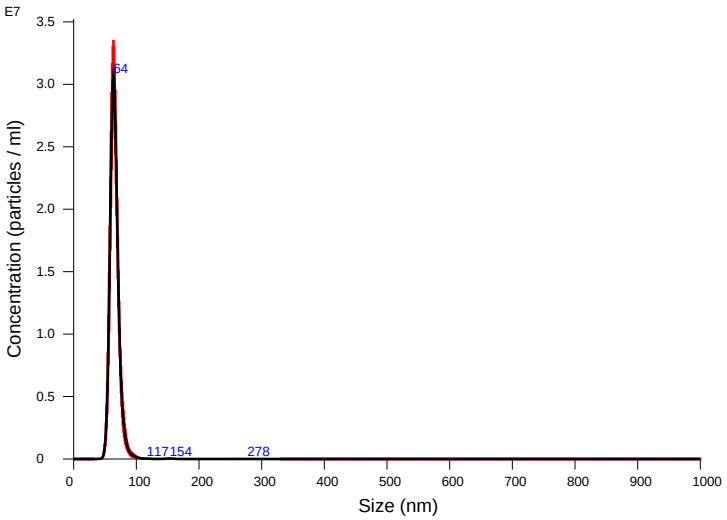
Measurement script:

```
RECORDDILUTION 0
SETVISCOSITY WATER
CAMERASETTINGSMSG
SYRINGELOAD 1000
DELAY 5
SYRINGELOAD 25
DELAY 10
REPEATSTART
CAPTURE 60
DELAY 1
REPEAT 4
SYRINGESTOP
PROCESSSINGLESETTING
EXPORTRESULTS
```

60nm Au_round3_MP_ 2019-04-03 23-42-42



FTLA Concentration / Size graph for Experiment:
60nm Au_round3_MP_ 2019-04-03 23-42-42



Averaged FTLA Concentration / Size for Experiment:
60nm Au_round3_MP_ 2019-04-03 23-42-42
Error bars indicate + / -1 standard error of the mean

Included Files

60nm Au_round3_MP_ 2019-04-03 23-43-24
60nm Au_round3_MP_ 2019-04-03 23-44-29
60nm Au_round3_MP_ 2019-04-03 23-45-34
60nm Au_round3_MP_ 2019-04-03 23-46-39
60nm Au_round3_MP_ 2019-04-03 23-47-44

Details

NTA Version:	NTA 3.4 Build 3.4.003
Script Used:	[Modified] SOP Standard Measurement 11-21-35PM 03~
Time Captured:	23:42:42 03/04/2019
Operator:	
Pre-treatment:	
Sample Name:	
Diluent:	
Remarks:	

Capture Settings

Camera Type:	sCMOS
Laser Type:	Blue405
Camera Level:	11
Slider Shutter:	890
Slider Gain:	125
FPS	25.0
Number of Frames:	1498
Temperature:	23.8 - 24.0 ºC
Viscosity:	(Water) 0.909 - 0.914 cP
Dilution factor:	Dilution not recorded
Syringe Pump Speed:	50

Analysis Settings

Detect Threshold:	5
Blur Size:	Auto
Max Jump Distance:	Auto: 20.0 - 20.8 pix

Results

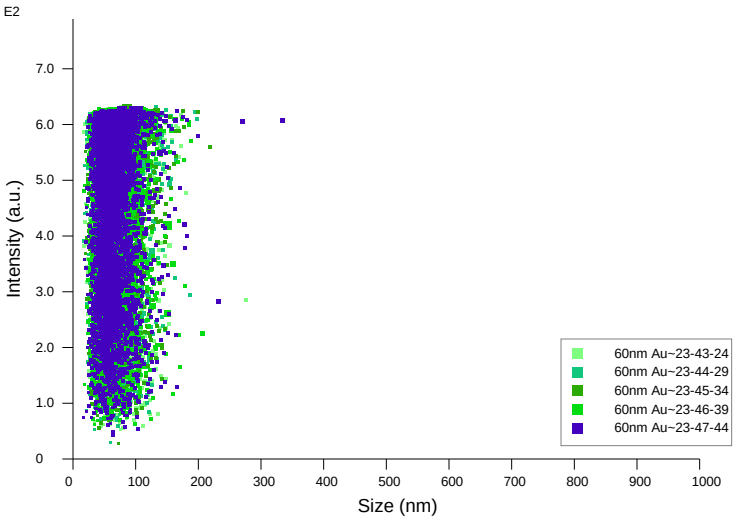
Stats: Merged Data

Mean:	65.6 nm
Mode:	63.5 nm
SD:	8.9 nm
D10:	57.2 nm
D50:	64.5 nm
D90:	74.2 nm

Stats: Mean +/- Standard Error

Mean:	65.6 +/- 0.5 nm
Mode:	63.5 +/- 0.8 nm
SD:	8.6 +/- 1.1 nm
D10:	57.4 +/- 0.6 nm
D50:	64.6 +/- 0.6 nm
D90:	74.3 +/- 1.1 nm
Concentration (Upgrade):	4.68e+08 +/- 1.30e+07 particles/ml
	67.0 +/- 2.0 particles/frame
	59.6 +/- 1.5 centres/frame

60nm Au_round3_MP_ 2019-04-03 23-42-42



Intensity / Size graph for Experiment:
60nm Au_round3_MP_ 2019-04-03 23-42-42

Script Used: (Full Text):

[Modified] SOP Standard Measurement 11-21-35PM 03Apr2019.txt

Supplemental File (Figures, Permissions, etc.)

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Figure S.1.tif