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Corresponding Author:	Nicole Comfort Columbia University Mailman School of Public Health New York, NY UNITED STATES
Corresponding Author's Institution:	Columbia University Mailman School of Public Health
Corresponding Author E-Mail:	nicole.comfort@columbia.edu
Order of Authors:	Nicole Comfort
	Kunheng Cai
	Tessa R. Bloomquist
	Madeleine D. Strait
	Anthony W. Ferrante Jr.
	Andrea A. Baccarelli
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1 **TITLE:**

2 Nanoparticle Tracking Analysis for the Quantification and Size Determination of Extracellular

3 Vesicles

4

5 **AUTHORS AND AFFILIATIONS:**

6 Nicole Comfort[1], Kunheng Cai[2], Tessa R. Bloomquist[1], Madeleine D. Strait[1], Anthony W.

7 Ferrante, Jr.[2], Andrea A. Baccarelli[1]

8

9 [1]Department of Environmental Health Sciences, Columbia University Mailman School of Public

10 Health, New York, NY, USA

11 [2]Department of Medicine, Naomi Berrie Diabetes Center, Columbia University, New York, NY,

12 USA

13

14 Corresponding authors

15 Nicole Comfort (nicole.comfort@columbia.edu)

16 Andrea A. Baccarelli (ab4303@cumc.columbia.edu)

17

18 Email Addresses of Co-Authors:

19 Nicole Comfort (nicole.comfort@columbia.edu)

20 Kunheng Cai (kc3083@cumc.columbia.edu)

21 Tessa R. Bloomquist (tb2715@cumc.columbia.edu)

22 Madeleine D. Strait (mds2247@cumc.columbia.edu)

23 Anthony W. Ferrante, Jr (awf7@cumc.columbia.edu)

24 Andrea A. Baccarelli (ab4303@cumc.columbia.edu)

25

26 **KEYWORDS:**

27 extracellular vesicle, EV, exosome, microvesicle, MV, nanoparticle tracking analysis, NTA,

28 protocol, polydisperse sample, Brownian motion

29

30 **SUMMARY:**

31 We demonstrate how to use a novel nanoparticle tracking analysis instrument to estimate the

32 size distribution and total particle concentration of extracellular vesicles isolated from mouse

33 perigonadal adipose tissue and human plasma.

34

35 **ABSTRACT:**

36 The physiological and pathophysiological roles of extracellular vesicles (EVs) have become

37 increasingly recognized, making the EV field a quickly evolving area of research. There are many

38 different methods for EV isolation, each with distinct advantages and disadvantages that affect

39 the downstream yield and purity of EVs. Thus, characterizing the EV prep isolated from a given

40 source by a chosen method is important for interpretation of downstream results and

41 comparison of results across laboratories. Various methods exist for determining the size and

42 quantity of EVs, which can be altered by disease states or in response to external conditions.

43 Nanoparticle tracking analysis (NTA) is one of the prominent technologies used for high-

44 throughput analysis of individual EVs. Here, we present a detailed protocol for quantification

45 and size determination of EVs isolated from mouse perigonadal adipose tissue and human
46 plasma using a breakthrough technology for NTA representing major advances in the field. The
47 results demonstrate that this method can deliver reproducible and valid total particle
48 concentration and size distribution data for EVs isolated from different sources using different
49 methods, as confirmed by transmission electron microscopy. The adaptation of this instrument
50 for NTA will address the need for standardization in NTA methods to increase rigor and
51 reproducibility in EV research.
52
53 **INTRODUCTION:**
54 Extracellular vesicles (EVs) are small (0.03-2 μm) membrane-bound vesicles secreted by nearly
55 all cell types[1]. They are often referred to as "exosomes," "microvesicles," or "apoptotic bodies"
56 depending on their mechanism of release and size[2]. While it was initially thought that EVs were
57 simply a means of eliminating waste from the cell to maintain homeostasis[3], we now know that
58 they can also participate in intercellular communication via transfer of molecular material -
59 including DNA, RNA (mRNA, microRNA), lipids, and proteins[4,5] - and that they are important
60 regulators of normal physiology as well as pathological processes[1,5–8].
61
62 There are many different methods to isolate and quantify EVs, which have been described
63 elsewhere[9–12]. The isolation protocol used as well as the source of EVs can greatly impact EV
64 yield and purity. Even differential centrifugation, long considered the "gold standard" approach
65 for exosome isolation, can be subject to substantial variability subsequently impacting the EV
66 population obtained and downstream analyses[13]. Thus, the various different methodologies for
67 EV isolation and quantification make it difficult to compare, reproduce, and interpret results of
68 experiments reported in the literature[14]. Furthermore, EV release can be regulated by cellular
69 conditions or various external factors. It has been suggested that EVs play a role in maintaining
70 cellular homeostasis by protecting cells against intracellular stress[15], as several studies have
71 shown that cellular stress stimulates EV secretion. For example, increased EV release has been
72 reported after cellular exposure to hypoxia, endoplasmic reticulum stress, oxidative stress,
73 mechanical stress, cigarette smoke extract, and particulate matter air pollution[16–22]. EV release
74 has also been shown to be modified *in vivo;* mice subjected to a high fat diet or fasting for
75 sixteen hours released more adipocyte EVs[23]. To investigate whether a specific treatment or
76 condition alters EV release, the number of EVs must be accurately determined. Assessment of
77 the EV size distribution may also indicate the predominant subcellular origin of EVs (e.g., fusion
78 of late endosomes/multivesicular bodies with the plasma membrane vs. budding of the plasma
79 membrane)[24]. Thus, there is a need for robust methods to accurately measure the total
80 concentration and the size distribution of the EV prep being studied.
81
82 A rapid and highly sensitive method for the visualization and characterization of EVs in solution
83 is nanoparticle tracking analysis (NTA). A detailed explanation of the principles of this method
84 and comparison with alternative methods for assessment of EV size and concentration have
85 been described previously[25–28]. Briefly, during NTA measurement, EVs are visualized by the light
86 scattered when they are irradiated with a laser beam. The scattered light is focused by a
87 microscope onto a camera which records the particle movement. The NTA software tracks the
88 random thermal motion of each particle, known as Brownian motion, to determine the

89 diffusion coefficient which is used to calculate the size of each particle using the Stokes-Einstein
90 equation. NTA was first applied to measurement of EVs in a biological sample in 2011[25]. Until
91 recently, there were only two mainstream companies offering commercial NTA instruments[29]
92 until the introduction of the ViewSizer 3000 (hereafter referred to as the particle tracking
93 instrument) which uses a combination of novel hardware and software solutions to overcome
94 significant limitations of other NTA techniques.
95
96 The particle tracking instrument characterizes nanoparticles in liquid samples by analyzing their
97 Brownian motion and characterizes larger micron-sized particles by analyzing gravitational
98 settling. This instrument's unique optical system, which includes multispectral illumination with
99 three laser light sources (at 450 nm, 520 nm, and 635 nm), allows researchers to analyze a wide
100 range of particle sizes (e.g., exosomes, microvesicles) simultaneously. A schematic of the
101 instrument setup is shown in **Figure 1**.
102
103 Here, we demonstrate how to perform particle size distribution and concentration
104 measurements of isolated mouse and human EVs using a novel NTA instrument.
105
106 [Place **Figure 1** here].
107
108 **PROTOCOL:**
109 All work with these samples was performed in compliance with Institutional Animal Care and
110 Use Committee and Institutional Review Board guidelines. A schematic overview of the NTA
111 method is depicted in **Figure 2**.
112
113 [Place **Figure 2** here]
114
115 **1. Extracellular vesicle isolation**
116
117 NOTE: Mouse perigonadal adipose tissue EVs were isolated as previously described[23]. Plasma
118 EVs were isolated from 1 mL of human plasma using the following protocol:
119
120 1.1. Collect the plasma and centrifuge at 3,000 x *g* for 15 min to remove cellular debris.
121 Transfer the supernatant to a new tube.
122
123 NOTE: If additional debris remains detectable, centrifuge the supernatant for additional 10 min
124 at 12,000 x *g* and transfer the supernatant to a new tube.
125
126 1.2. Add 67 μL of the exosome isolation reagent per 250 μL of plasma. Mix well by inverting or
127 flicking the tube.
128
129 1.3. Incubate on ice upright for 30 min.
130
131 1.4. Centrifuge the exosome isolation reagent/plasma mixture at 3,000 x *g* for 10 min at 4 ˚C.
132

133 NOTE: Centrifugation may be performed at room temperature or 4 ˚C with similar results, but 4
134 ˚C is preferred. After centrifugation, the EVs may appear as a beige or white pellet at the
135 bottom of the tube.
136
137 1.5. Carefully aspirate off the supernatant. Spin down any residual exosome isolation solution
138 and remove all traces of fluid by aspiration, taking great care not to disturb the precipitated EVs
139 in the pellet.
140
141 1.6. Resuspend the pellet in 200 µL of Buffer B (provided by the manufacturer). Measure and
142 record the sample's protein concentration (for step 2.8) using a spectrophotometer,
143 fluorometer, Bradford assay, or other preferred method.
144
145 **2. Purification of isolated EVs**
146
147 2.1. Add 200 µL of Buffer A (provided by the manufacturer) to re-suspended EVs.
148
149 2.2. Take out the purification column (provided), loosen the screw cap, and snap off the bottom
150 closure. Place the column into a collection tube.
151
152 NOTE: Save the bottom closure for steps 2.7-2.9.
153
154 2.3. Centrifuge at 1,000 x g for 30 s to remove the storage buffer.
155
156 2.4. Discard the flow-through and place the column back into the collection tube.
157
158 2.5. To wash the column, remove the cap and apply 500 µL of Buffer B on top of the resin and
159 centrifuge at 1,000 x g for 30 s. Discard the flow through. Save the cap for steps 2.7-2.9.
160
161 2.6. Repeat steps 2.4-2.5 one more time to wash the column.
162
163 2.7. Plug the bottom of the column with the bottom closure (from step 2.2). Apply 100 µL of
164 Buffer B on top of the resin to prepare it for sample loading.
165
166 2.8. Add the entire content from step 1.6 (or up to volume equivalent of 4 mg of total protein)
167 to the resin. Place the screw cap on the top of the column.
168
169 2.9. Mix at room temperature on a rotating shaker for no more than 5 min.
170
171 **3. Sample elution**
172
173 3.1. Loosen the screw cap and remove the bottom closure, and immediately transfer to a 2 mL
174 microcentrifuge tube.
175

176 NOTE: The sample will start to elute as soon as the bottom closure is removed. Please make
177 sure 2 mL microcentrifuge tubes are ready to receive eluate to minimize sample loss.
178
179 3.2. Centrifuge at 1,000 x *g* for 30 s to obtain purified EVs. Discard the column.
180
181 **4. Sample preparation for nanoparticle tracking analysis**
182
183 4.1. Use a lint-free material like a microfiber cloth to cover the workspace and prevent fibers
184 from entering cuvettes.
185
186 4.2. Wearing gloves, place the cuvette onto the magnetic cuvette jig, then place the stir bar in
187 the cuvette. Always handle the cuvettes with gloves to prevent fingerprints and smudges from
188 appearing on the cuvette's surface.
189
190 4.3. Use the hook tool to place the insert into the cuvette as depicted in **Figure 3**. It is important
191 to note the orientation of the insert for later (Step 5.4).
192
193 [Place **Figure 3** here].
194
195 4.4. Using a pipette, slowly add 400-500 µL of the diluent or diluted sample at room
196 temperature to the cuvette through the hole in the insert. Gently pipette up and down to mix.
197 Avoid introducing air bubbles.
198
199 4.4.1. First prepare a cuvette loaded with the chosen diluent (blank) to measure the particle
200 concentration of the diluent. This should be done before measuring the sample so that the
201 background concentration of the sample can be corrected.
202
203 NOTE: A good blank (phosphate buffered saline [PBS] in this case) will have a concentration <9 x
204 10^6 (depending on the diluent) and will display 1-10 particles per screen in the live view **(Figure
205 4)**. It is recommended that the actual sample particle concentration is at least 3 times the
206 background concentration.
207
208 [Place **Figure 4** here].
209
210 4.4.2. Optionally, before recording the sample prime the cuvette with 400-500 µL of the diluent
211 or diluted sample before measuring. To do so, load 400-500 µL of the diluted sample into the
212 cuvette, discard the solution, and then add 400-500 µL more of the sample for the
213 measurement. This may help with washing out residues within the cuvette.
214
215 4.5. Cap the cuvette and check for bubbles. Tap out bubbles if necessary. Use a lint free cloth to
216 wipe the outside faces of the cuvette.
217
218 **5. Startup procedure of the particle tracking instrument**
219

5.1. Turn on the computer workstation and instrument, wait a few minutes prior to running the first sample and start the program by clicking on the software icon. When prompted, click **NTA** on the screen to conduct Nanoparticle Tracking Analysis. Begin in the **Recording** tab and click on the various software tabs (**Record**, **Process**, **Plot**) to switch between them throughout the protocol. Record the sample (diluent or EV prep) first, then process the recordings, and finally plot the results.

5.2. Follow the instructions on the screen to fill out all the necessary information about the sample. Check that all necessary fields are completed and accurate, i.e., Sample name, Description, sample preparation, Dilution factor [1000], Target temperature (Set to 22 °C), and Diluent: Select diluent from the dropdown menu and use PBS as the diluent for EVs. Selecting PBS from the dropdown menu will auto-populate the salinity to 9%. This information is necessary to determine the dynamic viscosity of the liquid.

NOTE: It can take up to 3 min for the temperature to equilibrate inside the cuvette even if the probe already shows desired temperature (i.e., green dot becomes stable). Sample readouts could vary considerably if target temperature is not set, as sample temperature differences could greatly alter the Brownian motion of particles.

5.3. Open the instrument lid and remove the protective cap covering where the cuvette will be placed.

CAUTION: The particle tracking instrument is certified as a Class 1 laser product (21 CFR Ch. I part 1040), containing lasers that can be dangerous and could cause serious injury such as burns and/or permanent damage to eyesight. To prevent accident or injury, do not remove the instrument cover by unscrewing the bolts from the side. Note that during operation, the laser beams are completely enclosed, posing no threat to users. Also, a magnetic safety interlock is built into the instrument's sample holder to prevent the lasers from operating when the cap of the sample holder is removed.

5.4. Place the cuvette (from Step 4.3) inside the instrument in the correct orientation (see **Figure 5**). Replace the cap over the cuvette and close the instrument lid. Always operate the instrument with the cap in place over the sample holder. Do not disable or attempt to circumvent the safety interlock feature.

[Place **Figure 5** here].

5.5. Turn on the camera by clicking the arrow above **Streaming**.

5.6. Click the chevron arrow to expand the **Record** settings. Set Gain and Laser power to values appropriate for the application. See **Table 1** (and **Supplemental Figure 1**) for parameters used for NTA of small (100 nm) EVs.

263 NOTE: These advanced settings accessed by clicking the chevron arrow may be password-
264 protected. Use the same settings for recording and processing for the diluent (blank) as those
265 used for the subsequent samples.

267 5.7. Adjust the focus until particles are properly focused. Focusing should be done on relatively
268 small particles **(Figure 6)**. For small EV quantification (consistent with exosomes), the following
269 recording settings are recommended: Frame rate: 30 fps, Exposure: 15 ms, Stir time: 5 s, Wait
270 time: 3 s, Laser power - Blue: 210 mW, Green: 12 mW, Red: 8 mW, Frames per video: 300
271 frames, and Gain: 30 dB

273 NOTE: The user can increase the Zoom to 1x and/or increase the Gain to help with focusing.
274 Remember to re-set these parameters to recommended values before recording videos.

276 [Place **Figure 6** here].

278 5.8. Perform a visual quality check to ensure that the sample is properly diluted. If the sample is
279 too concentrated, remove the cuvette from the instrument and dilute the sample sequentially.
280 Repeat until the sample is properly diluted before proceeding to Step 6.

282 NOTE: Do not over-dilute the sample! Always make dilutions sequentially. The ideal blank will
283 display only a few particles on the screen (1-4) as shown in **Figure 4**. Regarding EV samples, a
284 properly diluted sample will have roughly 20-100 particles visible on the screen, with no glow-
285 like or cloudy images in the background (see **Figure 7** as an example). This should result in an
286 optimum particle concentration in the range of 5×10^6 to 2×10^8 particles per mL (not adjusting
287 for dilution factor).

289 [Place **Figure 7** here].

291 **6. Video data acquisition**

293 6.1. (Optional) Set the zoom setting to 0.5x to save bandwidth and prevent lost frames.

295 6.2. Begin recording the videos by clicking on **Record** (see **Table 1** for recommended recording
296 parameters).

298 NOTE: By default, the instrument will stir the sample for 5 s, wait for 3 s, and then record for 10
299 s before repeating this process. Typical measurement time for 50 videos is ~15 min to record
300 and ~13 min to process.

302 6.3. Do not touch the instrument while recording videos. Ensure that the surface of the
303 laboratory bench is not vibrating.

305 NOTE: It is preferred that the instrument is set on an anti-vibration platform or table to reduce
306 any vibration disturbances from nearby equipment that will interfere with the determination of

307 nanoparticle movement. Avoid operating centrifuges, vortex mixers, or other potential
308 vibration-generating devices on the same bench as the particle tracking instrument. Vibrations
309 are easily visible on screen as elongation of usually round particles. If exposed to vigorous
310 vibrations, the instrument may require realignment of optical elements. The instrument was
311 not designed to be serviced or calibrated by the customer; contact the manufacturer for
312 maintenance, service, and calibration.

313

314 6.4. Note any recorded video that has very large particles visible as large, irregular white blobs.
315 Remove these videos from processing in Step 7.3.

316

317 **7. Process acquired data**

318

319 7.1. When a prompt appears stating that videos have been recorded, click **OK** to complete
320 recording. Then select the **Process** tab.

321

322 NOTE: The protocol can be stopped here. Processing of acquired data can be restarted later by
323 moving directly to the **Process** tab after opening the particle tracking instrument's software and
324 specifying the directory where the recorded videos are saved.

325

326 7.2. When analyzing EVs, check the box **Disable auto detection override** and set the Feature
327 Diameter to **30**Click **Process**. (See **Table 2** for processing settings and **Supplemental Figure 2** for
328 processing display). A live distribution graph will display so the user can view the processing in
329 real-time.

330

331 7.2.1. For exosomes, process with Detection Threshold set to the default **Polydisperse Sample.**
332 Process the data with **Detection Threshold Manual** set to 0.8 instead of a standard threshold of
333 0.99 only for solutions with very large differences in particle sizes.

334

335 7.3. If any videos had noticeably very large particles visible (see Step 6.4), navigate to the
336 directory of recorded videos and remove the problematic video. After editing the list of video
337 files, change the number of recorded videos in other user-kept logs.

338

339 7.4. After processing is complete, click **OK.** Then, select the **Plot** tab. For EVs, display the Main
340 chart as **LogBinSilica.**

341

342 NOTE: Here in the **Plot** tab, the user may customize other features of the graph, such as
343 defining the range of the x axis (particle diameter, nm) to set the area for integration for the
344 figure produced.

345

346 **8. Display and interpret results**

347

348 8.1. To create a PDF report, click the **Report** button. The measurement is now complete, and
349 the results may be viewed.

350

351   NOTE: Remember to record and process the blank first so that the background concentration of
352   subsequent samples can be corrected. If this step is forgotten, the blank can be recorded after
353   samples and the sample particle concentration corrected manually.
354
355   8.2. Examine the PDF report which displays the mean, median, and mode size as well as the
356   concentration adjusted for the dilution factor and corrected by subtracting the diluent's particle
357   concentration. The distribution width (standard deviation) is also shown.
358
359   NOTE: There are very few applications where a single value is appropriate and representative.
360   Thus, describing the entire size distribution and reporting the width of the distribution for any
361   sample analyzed is recommended (as shown in **Table 3** for example).
362
363   8.3. Record the following instrument settings used to generate the data which should be stated
364   when reporting results: Diluent, Laser power of each laser [mW], Exposure [ms], Gain [dB],
365   Frame rate [fps], Frames per video, Number of videos recorded, Processing setting (e.g.,
366   LogBinSilica), Integration range [min nm, max nm] (recommended to set min to 50 nm) and
367   Number of particles tracked (desirable to analyze at least ~150 particles per video; minimum
368   3,750 total tracks per sample recommended to avoid artifactual spikes in the particle size
369   distribution and generate statistically significant data).
370
371   **9. Cleaning the cuvettes**
372
373   9.1. Clean cuvettes manually between samples. First, empty the cuvette.
374
375   NOTE: The sample can either be recovered from the cuvette and saved or discarded.
376
377   9.2. Once the cuvette is empty, clean the cuvette by rinsing it 10-15 times with de-ionized (DI)
378   water. Then, rinse 3 times with ethanol (70-100%). When doing this, make sure to completely
379   fill the cuvette with solvent.
380
381   9.3. Dry the outside of the cuvette with a lint free microfiber cloth. Avoid smudges on the
382   surfaces. Air dry the inside of the cuvette or dry using a compressed air duster.
383
384   NOTE: Only lens cleaning paper or lint free microfiber cloth should be used to wipe the optical
385   surfaces of the cuvette, as most paper products contain small wood fibers that may scratch or
386   damage the cuvette's surface.
387
388   9.4. Prepare two glass scintillation vials: one filled with 70-100% ethanol and the other with DI
389   water. Rinse the inserts and stir bars in the ethanol first (then DI water) by placing the
390   insert/stir bar in the appropriate scintillation vial and shaking the vial vigorously. Dry the inserts
391   and stir bars using lint free cloths or compressed air dusters.
392
393   NOTE: The cuvette and insert can also be cleaned using a sonicating water bath. To do so, first
394   ensure the sonicating unit contains enough water (at least 5 cm depth). Then place the cuvette

395   and insert inside a glass beaker (50 mL or larger), fill the beaker with alcohol to the same level
396   as the water bath, place the beaker in the water, and switch on the power. Sonicate for a
397   maximum of 5 min at a time, allowing the machine to rest >5 min between each 5-min burst if
398   longer times are required.
399
400   <mark>9.5. When finished, immediately put all cleaned and dried components away for storage.</mark>
401
402   **REPRESENTATIVE RESULTS:**
403   Before this demonstration, the calibration of the instrument was first tested to ensure the
404   validity of the acquired data by measuring the size distribution of polystyrene bead standards.
405   We tested the size distribution of 100 nm and 400 nm beads using the default recording
406   parameters and the processing settings recommended in this protocol (**Figure 8**).
407
408   For the 100 nm polystyrene bead standard, a concentration of 4.205×10^7 particles/mL was
409   measured. The mean (standard deviation, SD) size was 102 (±17) nm with a coefficient of
410   variation (CV) of 0.16. The D10/D50/D90 values were 76/104/126 nm. This indicates that the
411   particle tracking instrument accurately reported the size of the 100 nm monodisperse beads.
412   For the 400 nm polystyrene bead standard, the concentration was 4.365×10^7 particles/mL. The
413   mean (SD) size was 391 (±47) nm, with a CV of 0.12. The D10/D50/D90 values were
414   150/358/456 nm (**Table 3**). While the reported mean is very close to the true size of the beads,
415   we can see that the instrument settings applied in this protocol are more accurate for the
416   smaller particles ~100 nm. Thus, for particles larger than 400 nm, optimizing the laser
417   parameters is suggested. Researchers should first conduct a method such as transmission
418   electron microscopy (TEM) for an estimate of the resulting EV size distribution from a given
419   source and isolation method prior to NTA.
420
421   A mean concentration of 4.41×10^{10} particles/mL was measured for the mouse tissue EV
422   sample used for this demonstration. This sample was recorded following the parameters
423   summarized in **Table 1** and processed following the parameters outlined in **Table 2**. We
424   demonstrate the impact of various dilution factors in **Figure 9** and report the raw and adjusted
425   particle concentrations in **Table 4.** The optimum dilution for the mouse tissue-derived EVs was
426   between 1,000 to 3,000 whereas for human plasma-derived EVs it was 1,000, indicating that
427   when first testing a given biofluid source or EV isolation method for NTA, serial dilutions should
428   be tested so the optimum dilution for NTA measurement can be identified. We recommend
429   measuring and reporting at least two different dilutions of a sample. We also measured four
430   additional samples at various dilutions on two distinct particle tracking instruments.
431   **Supplemental Figure 3** compares the particle tracking instrument's precision in size
432   measurements compared with another commercially available NTA instrument. **Supplemental**
433   **Table 1** further demonstrates the precision of this instrument's measurements, showing that
434   the particle concentration changed proportionally with the sample dilution, but that the
435   particle size measures did not.
436
437   After processing, the results (along with the instrument/software information, recording and
438   processing settings, and experimental notes) can be saved in a .pdf report. The histogram

439   included on this report can be modified as described in the protocol (section 7.4). The raw
440   results are saved in a .dat file that can be exported. The recorded videos are also saved and can
441   be used for later off-line processing and analysis. However, once videos are recorded, recording
442   settings cannot be changed retrospectively.
443
444   The impact of altering the various laser powers and gain in the recording settings was
445   demonstrated using human plasma derived EVs. This is visualized in Figures 10-12 and the
446   quantitative information based on these images is shown in **Table 5.** Increasing the gain
447   increases the camera's sensitivity, allowing visualization of smaller particles (**Figure 10**)
448   resulting in an increasing total particle concentration with a smaller average particle size (**Table
449   5**). However, increasing the gain above 30 dB (our recommendations) makes the view too
450   grainy, allowing noise to obscure measurement of true particles. At a camera gain of 30 dB, the
451   effect of altering the blue laser power is depicted in **Figure 11**. Increasing the blue laser (450
452   nm, short wavelength) power results in greater resolution to detect smaller particles (i.e. those
453   with a size consistent with exosomes). Holding the green and red laser powers constant at the
454   default settings, increasing the blue laser power from 70 mW to 210 mW shifted the reported
455   average particle size from 122 nm to 105 nm and increased the reported total particle
456   concentration from 1.1×10^8 to 1.7×10^8 (**Table 5**).
457
458   The effect of increasing the green and red laser powers was also demonstrated (**Figure 12**).
459   Increasing the power of the red laser (650 nm, long wavelength) increased the reported
460   average particle size from 175 to 246 nm. The reported total particle concentration decreased,
461   but that is because in this human plasma EV sample we did not have many large particles
462   present, confirmed by TEM negative staining (**Supplemental Figure 4**). Increasing the green
463   laser power resulted in a decrease in the reported average particle size and an increase in
464   reported total particle concentration. Thus, the user can optimize the power of the three lasers
465   to sensitively detect particles of various sizes.
466
467   This protocol is optimized for smaller vesicles (e.g., <400 nm). Researchers interested in
468   microvesicles of larger sizes (e.g., 400-1000 nm) are encouraged to optimize the laser
469   parameters using hollow organosilica beads which have light-scattering properties similar to
470   that of EVs making them a more suitable reference than polystyrene or silica bead standards[30].
471   Yet silica nanoparticles may be the best practical substitute until organosilica standards become
472   more widely available.
473
474   Here, an NTA instrument was used to successfully quantify EVs isolated from mouse tissue by
475   ultracentrifugation and human plasma by using a commercial kit. The effects of altering the
476   NTA parameters were illustrated and show that the parameters in this protocol are optimized
477   for small vesicles. The importance of the dilution step was also displayed and shows that the
478   particle tracking instrument demonstrated here can accurately detect variations in dilution
479   factors. The concentration changed proportionally with multiple dilutions, while the size
480   distribution did not. The resulting data showed little technical variability. Thus, adaptation of
481   this protocol by researchers using this instrument for NTA will increase rigor and reproducibility
482   in the EV research field.

483
484 **FIGURE LEGENDS.**
485
486 **Figure 1: Particle tracking instrument optical system.** The NTA instrument illuminate particles
487 using three lasers with the following wavelengths: 450 nm, 520 nm, 635 nm. Video recording of
488 the scattered light from individual particles is detected and tracked by a digital video camera
489 oriented 90˚ from the cuvette.
490
491 **Figure 2: Overview of NTA method using the particle tracking instrument.** The sample is
492 prepared and inserted into the instrument. The NTA software is opened, recording parameters
493 are adjusted, and the sample is focused. Then, the data is recorded, processed, and displayed.
494
495 **Figure 3: Proper orientation of insert within the quartz cuvette.** The "notch" of the insert
496 should be visible from the front of the cuvette. This should be inserted into the instrument
497 facing the camera.
498
499 **Figure 4: Representative live stream view of a diluent within the proper concentration range**
500 **of a blank.** Dilute EV preps in filtered (0.02 µm or 3 kDa, preferred) PBS. A good blank will
501 display ~1-10 particles per screen in the live view, yielding a concentration within the range 10^5-
502 10^6.
503
504 **Figure 5: Proper orientation of cuvette within particle tracking instrument.** The face of the
505 cuvette (with the "notch" of the insert visible) should face the camera.
506
507 **Figure 6: Representative live stream views showing particle focus.** (**A**) An example live stream
508 view of particles not in focus. Particles have glow-like halo or appear blurry. Adjust focus. (**B**) An
509 example live stream view of particles in proper focus. The smallest particles are in focus.
510 Commence recording.
511
512 **Figure 7: Representative live stream views depicting different particle dilutions.** (**A**) An
513 example live stream view of a sample that is too concentrated. Recording a sample that is too
514 concentrated will yield inaccurate results. (**B**) An example live stream view of a properly diluted
515 sample. There are 60-100 particles visible on screen and recording results in a raw
516 concentration of 5 x 10^6 – 2 x 10^8 particles/mL. (**C**) An example live stream view of a sample that
517 is too dilute. If a sample is this dilute, there will not be enough particles tracked, lowering the
518 sample size and, therefore, the results will be statistically invalid. In this case, increasing the
519 number of videos recorded is recommended.
520
521 **Figure 8: Particle size distributions of monodisperse polystyrene bead standards.** (A) Particle
522 size distribution of 100 nm polystyrene bead standard. (**B**) Particle size distribution of 400 nm
523 polystyrene bead standard. Values are reported in **Table 3**. Inset shows example live stream
524 view of the first measured frame.
525

526   **Figure 9: Raw total particle concentrations by dilution factor for mouse perigonadal adipose**
527   **tissue EVs.** Error bars represent standard deviation. Three measurements were taken for each
528   dilution. Values are reported in **Table 4**.
529
530   **Figure 10: Visual impact of increasing gain.** Representative images of particles measured with
531   default laser settings (Blue = 70 mW, Green = 12 mW, Red = 8 mW) and gain set to (**A**) 18 dB,
532   (**B**) 24 dB - default, and (**C**) 30 dB - recommended.
533
534   **Figure 11: Effect of blue laser on small particles when red and green lasers are set to default**
535   **settings.** (**A**) Blue laser = 70 mW (default). (**B**) Blue laser = 210 mW (recommended). More small
536   particles are visible and in focus when the blue laser power is increased to 210 mW.
537
538   **Figure 12: Visual impact of altered blue, green, and red laser power.** Representative live
539   screen views depict the different particle sizes visualized across different laser settings.
540   Resulting NTA values are reported in **Table 5**.
541
542   **Table 1: Recording parameters for small extracellular vesicles (~100 nm)**.
543
544   **Table 2: Processing parameters for small extracellular vesicles (~100 nm).**
545
546   **Table 3: NTA results of monodisperse polystyrene bead standards.** Both standards were
547   optimally diluted, with raw concentrations for the 100 nm standard and 400 nm standard of
548   4.205 x 10^7 particles/mL and 4.365 x 10^7 particles/mL, respectively. D10 is the point in the size
549   distribution where 10% of the sample is contained, D50 is the point where 50% of the sample is
550   contained (median), and D90 is the point where 90% of the sample is contained. SD: Standard
551   deviation; CV: Coefficient of variation.
552
553   **Table 4: NTA results of mouse perigonadal adipose tissue EVs demonstrate reproducibility of**
554   **NTA measurements.** Raw NTA particle concentrations (particles/mL) measured on the particle
555   tracking instrument and total particle concentration (particles/mL) adjusted for dilution factor
556   of EVs derived from mouse perigonadal adipose tissue at various dilutions.
557
558   **Table 5: NTA results of human plasma derived EVs demonstrate the effect of altering laser**
559   **power and gain on size and particle concentration measurements of human EVs (1:1000**
560   **dilution).** D10 is the point in the size distribution where 10% of the sample is contained, D50 is
561   the point where 50% of the sample is contained (median), and D90 is the point where 90% of
562   the sample is contained. SD: Standard deviation; CV: Coefficient of variation.
563
564   **Supplemental Figure 1: Screenshot of recording panel depicting recommended recording**
565   **parameters.** Access advanced settings to change the camera gain and laser power to
566   recommended settings.
567
568   **Supplemental Figure 2: Screenshot of processing panel depicting recommended processing**
569   **settings.** Ensure "Disable auto detection override" is checked and the Feature Diameter is set to

570   "30."

571

572   **Supplemental Figure 3: Particle size measurements across multiple dilutions: Precision of the**
573   **particle tracking instrument (ViewSizer 3000) vs. comparable commercial NTA instrument**
574   **(NanoSight NS300).** NTA of EVs isolated from mouse perigonadal adipose tissue was performed
575   on two instruments. For each mouse, three dilutions (1:1000, 1:500, 1:100) were measured.
576   Raw values for measures taken on the ViewSizer 3000 (demonstrated in this protocol) are
577   shown in **Supplemental Table 1**. Capture and analysis settings for the NanoSight NS300
578   measures are shown in **Supplemental Table 2**.

579

580   **Supplemental Figure 4: Representative negative-stain transmission electron microscopy**
581   **(TEM) of human plasma-derived EVs.** Grids were imaged with a transmission electron
582   microscope operating at 100 kV accelerating voltage. Images were captured on a 2K x 2K CCD
583   camera. Scale bar reflects the magnification at the camera. Electron micrographs show round
584   vesicles ~25 nm in diameter. 100k magnification, scale bar 200 nm.

585

586   **Supplemental Table 1: ViewSizer 3000 particle size and concentration measurements of**
587   **mouse perigonadal adipose tissue EVs across multiple dilutions.** NTA of EVs isolated from
588   mouse perigonadal adipose tissue was performed. For each mouse, three dilutions (1:1000,
589   1:500, 1:100) were measured. D10 is the point in the size distribution where 10% of the sample
590   is contained, D50 is the point where 50% of the sample is contained (median), and D90 is the
591   point where 90% of the sample is contained. Particle concentrations are background-corrected.

592

593   **Supplemental Table 2: NanoSight NS300 capture and analysis settings.** Settings (using NTA 3.4
594   Build 3.4.003) used to measure mouse perigonadal adipose tissue EV samples reported in
595   **Supplemental Figure 3**.

596

597   **DISCUSSION:**
598   Here, we demonstrate a protocol for NTA of EVs to measure the size distribution of a wide
599   range of particle sizes simultaneously and measure total EV concentration in a polydisperse
600   sample. In this study, mouse perigonadal adipose tissue and human plasma were used as the
601   source of EVs. However, EVs isolated from other tissues or biological fluids such as serum,
602   urine, saliva, breast milk, amniotic fluid, and cell culture supernatant may also be used for NTA.
603   Measurements of polystyrene bead standards ensured that the instrument was properly
604   calibrated and demonstrated that this NTA instrument can correctly measure the size of
605   nanoparticles ≤400 nm. Various dilutions of a mouse tissue EV sample were then tested. As
606   shown in **Figure 9**, the reported particle concentration scales accordingly with the dilution
607   factor, as expected, demonstrating that the instrument can accurately detect the particle
608   concentration at various dilutions with little variability between technical replicates. However,
609   this was when working with particle concentrations within the working range of the instrument
610   (5×10^6 to 2×10^8 particles per mL); appropriately diluting a sample is a critical step in the
611   protocol. Adjusting for the dilution factor yielded roughly the same estimate for the total
612   particle concentration of the sample, with a CV of 15.1 across the four dilutions tested. Also,
613   different measures, repeated on multiple dilutions of the same samples, showed the precision

614   in the particle size distribution assessments, as particle concentrations scaled proportionally
615   with the dilution factor but reported size measures did not.

616

617   EVs in suspension undergo random thermal motion known as Brownian motion, according to
618   which the diffusion of particles in a liquid suspension is inversely proportional to their size. This
619   random motion is modeled by the Stokes-Einstein equation, where the hydrodynamic diameter
620   D of a spherical particle is:

621

623
$$D = \frac{k_B T}{6\pi\eta R}$$

622

624   Where k_B is the Boltzmann constant and R is the particle radius. As one can see from the
625   equation, particle movement in suspension also depends on the temperature (T) and dynamic
626   viscosity (η) of the liquid. During NTA, EVs are irradiated with focused laser beams that pass
627   through the particles in suspension and are detected by the incident illumination coming from
628   the lasers. The light scattered by each individual particle is focused by a microscope onto the
629   image sensor of a high-resolution, light-sensitive charge-coupled device (CCD) camera, which
630   records digital images of the scattered light of the particles (**Figure 1**). The NTA software
631   identifies and tracks the Brownian motion of each individual particle to calculate the diffusion
632   coefficient of each particle. This is used together with the temperature and the viscosity of the
633   liquid containing the particles to calculate particle diameter using the Stokes-Einstein equation.
634   Thus, a single measurement can determine two critical pieces of information: particle size
635   distribution and total particle count of EVs in suspension.

636

637   A comparison of NTA to other techniques for detecting and quantifying EVs has been described
638   elsewhere[31,32]. Compared to other light scattering based methods such as dynamic light
639   scattering (DLS), NTA has higher resolving capabilities and is a particularly powerful method for
640   analyzing particles with a mean diameter of less than 100 nm. Unlike single-particle analytics
641   such as TEM, NTA is not time consuming or technically challenging and can phenotype an entire
642   sample in a relatively low volume at once in a semi-automated manner. While NTA is a
643   powerful characterization technique, it does have limitations. First, NTA is subject to sensitivity
644   limits due to the strong decrease in the intensity of scattered light scaling with particle
645   diameter, causing the scattered light of very small particles to disappear below the background
646   noise[33]. Thus NTA has reduced sensitivity for EVs smaller than ~50 nm[31] and results in an
647   overestimation of EV sizes. Short wavelength lasers are needed to detect the smaller particles
648   of a polydisperse sample due to their light scattering potential. However, this particle tracking
649   instrument offers an improvement to this limitation inherent to nanoparticle tracking
650   technology. Equipped with three variable light sources (450 nm, 520 nm, 635 nm), this system
651   allows for visualization of particles over a wide range of sizes, making this instrument a
652   particularly valuable tool for NTA analyses of polydisperse samples such as a heterogeneous
653   population of EVs. Nonetheless, because most EV preps will be polydisperse in composition and
654   likely contain impurities, researchers should be aware that sample-dependent limits of
655   detection impact the shape of the overall size distribution and therefore exercise caution when
656   interpreting results.

657

658   Another major limitation of NTA is that it measures more than just EVs. The NTA instrument will
659   detect, measure, and count all particles exceeding the detection limit, including protein
660   aggregates, lipoproteins, cellular debris, and contamination caused by diluents. However,
661   particle concentration in the background can be corrected for by measuring the particle
662   concentration of the diluent prior to loading and measuring a sample. We recommend using at
663   minimum 0.02 µm filtered PBS and have found very low particle contamination when using 3
664   kDa filtered PBS. Furthermore, it is possible to extend NTA to measure and analyze
665   fluorescently labeled EVs, overcoming the limitation of non-specific measurement. Commercial
666   kits are available that specifically and efficiently dye the membranes of intact vesicles only,
667   excluding membrane fragments, protein aggregates, and other particles from the NTA
668   measurement. Thus, the data derived from fluorescent NTA more accurately represents the
669   true EV concentration and size distribution. Through selectively tagging EV surface antigens,
670   fluorescent NTA can also measure specific EVs labeled by fluorescently tagged antibodies,
671   allowing for the counting and sizing of antigen-specific, biologically relevant EV subpopulations.
672

673   There has been little work on standardization of NTA protocols, making comparability of results
674   difficult and hindering reproducibility across samples, days, operators, and laboratories. The
675   particle tracking instrument demonstrated in this protocol requires little hands-on time. We
676   have also noted which instrument settings users should report to facilitate reproducibility of
677   generated data. Thus, following the optimized protocol laid forth here should result in
678   standardization and enable comparison of results across laboratories. Another advantage of
679   using this instrument is that the sample is prepared in a cuvette, which allows the instrument to
680   repeatedly stir between videos, ensuring a statistically random sample for each video and
681   yielding a more reproducible result than other commercially available NTA instruments that
682   rely on syringe pumps and flow cells. Also, the multiple lasers allow for detection of particles
683   with varying refractive indices, yielding more robust results. Yet operation of this particle
684   tracking instrument does not require knowledge of particle material properties such as the
685   refractive index, making measurements more reproducible across operators.
686

687   EVs have been identified in nearly all biofluids tested[2,5]. However, effective isolation of specific
688   EVs (e.g., cell-type specific EVs) from different media (e.g., blood, breast milk, cell culture
689   media) presents a difficult challenge. Many different methods for EV isolation have been
690   described[9]. NTA can be performed to compare yields of EVs isolated using different methods
691   and identify variations in the vesicles that arise from different isolation methods. This is
692   important for interpreting downstream results, as currently the function of different EV
693   subpopulations remains unclear. Accurately measuring EV concentration is also important in
694   studies investigating whether a certain treatment, condition, or molecule impacts EV release.
695   For example, our group is interested in the impact of environmental exposures such as
696   particulate matter air pollution on EV content and release. In order to assess whether a
697   condition affects EV release, the number of vesicles has to be accurately quantified. When using
698   EVs as diagnostic biomarkers, the concentration of EVs in the sample could impact the assay
699   results. Thus, a reliable method to determine particle concentration and size distribution is
700   critical.

701
702 Regardless, NTA measurements should be accompanied by other complementary techniques
703 that can measure particles independently of the other particles in the sample, such as TEM.
704 Newer technologies such as microfluidic resistive pulse sensing (MRPS) are also promising for
705 sizing and counting of EVs independent of the polydispersity of the sample. Further biochemical
706 or proteomic analyses of EV samples can be used in addition to the size and concentration data
707 to cluster subsets of EVs into populations of biological significance. In conclusion, measuring
708 valid and reproducible particle concentrations and particle size distributions is important for
709 the EV field. The ViewSizer 3000 achieves accurate and reproducible measurements of total EV
710 concentration and EV size distribution, even for highly polydisperse samples, as demonstrated
711 here. In the future we hope to see further rigorous experimental comparisons of this
712 instrument with other NTA instruments.
713

718

719 **DISCLOSURES:**
720 All authors declared that there are no conflicts of interest.
721

722 **REFERENCES:**
723 1.   Colombo, M., Raposo, G., Théry, C. Biogenesis, secretion, and intercellular interactions
724 of exosomes and other extracellular vesicles. *Annual Review of Cell and Developmental Biology.*
725 **30**, 255–289 (2014).
726 2.   Hessvik, N. P., Llorente, A. Current knowledge on exosome biogenesis and release.
727 *Cellular and molecular life sciences: CMLS.* **75**, 193–208 (2018).
728 3.   Johnstone, R. M., Adam, M., Hammond, J. R., Orr, L., Turbide, C. Vesicle formation
729 during reticulocyte maturation. Association of plasma membrane activities with released
730 vesicles (exosomes). *The Journal of Biological Chemistry.* **262**, 9412–9420 (1987).
731 4.   Théry, C., Ostrowski, M., Segura, E. Membrane vesicles as conveyors of immune
732 responses. *Nature Reviews Immunology.* **9**, 581–593 (2009).
733 5.   Yáñez-Mó, M. et al. Biological properties of extracellular vesicles and their physiological
734 functions. *Journal of Extracellular Vesicles.* **4**, 27066 (2015).
735 6.   Lo Cicero, A., Stahl, P. D., Raposo, G. Extracellular vesicles shuffling intercellular
736 messages: for good or for bad. *Current Opinion in Cell Biology.* **35**, 69–77 (2015).
737 7.   Raposo, G., Stoorvogel, W. Extracellular vesicles: exosomes, microvesicles, and friends.
738 *The Journal of Cell Biology.* **200**, 373–383 (2013).
739 8.   Mathivanan, S., Ji, H., Simpson, R. J. Exosomes: extracellular organelles important in
740 intercellular communication. *Journal of Proteomics.* **73**, 1907–1920 (2010).
741 9.   Zhang, M. et al. Methods and technologies for exosome isolation and characterization.
742 *Small Methods.* **2**, 1800021 (2018).
743 10.   Szatanek, R. et al. The methods of choice for extracellular vesicles (EVs) characterization.
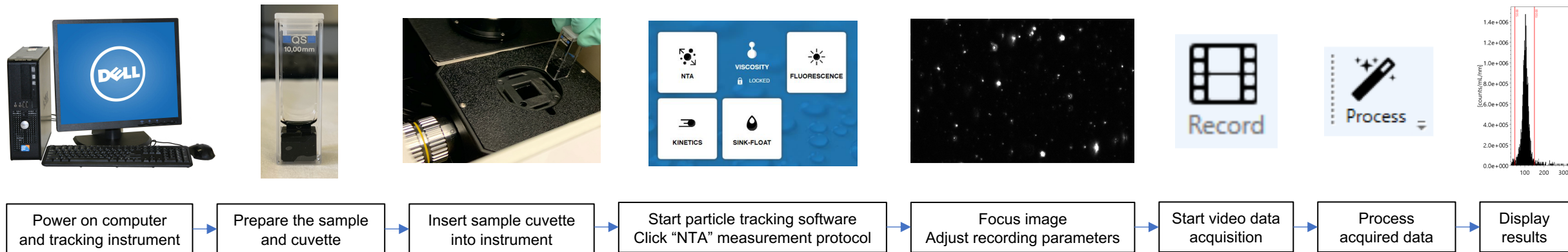744 *International Journal of Molecular Sciences.* **18** (2017).

745 11.   Erdbrügger, U., Lannigan, J. Analytical challenges of extracellular vesicle detection: A
746 comparison of different techniques. *Cytometry. Part A: The Journal of the International Society*
747 *for Analytical Cytology.* **89**, 123–134 (2016).
748 12.   Konoshenko, M. Yu., Lekchnov, E. A., Vlassov, A. V., Laktionov, P. P. Isolation of
749 Extracellular Vesicles: General Methodologies and Latest Trends. *BioMed Research*
750 *International.* **2018**, 1–27 (2018).
751 13.   Cvjetkovic, A., Lötvall, J., Lässer, C. The influence of rotor type and centrifugation time
752 on the yield and purity of extracellular vesicles. *Journal of Extracellular Vesicles.* **3**, (2014).
753 14.   Taylor, D. D., Shah, S. Methods of isolating extracellular vesicles impact down-stream
754 analyses of their cargoes. *Methods (San Diego, California).* **87**, 3–10 (2015).
755 15.   Desdín-Micó, G., Mittelbrunn, M. Role of exosomes in the protection of cellular
756 homeostasis. *Cell Adhesion & Migration.* **11**, 127–134 (2017).
757 16.   Kanemoto, S. et al. Multivesicular body formation enhancement and exosome release
758 during endoplasmic reticulum stress. *Biochemical and Biophysical Research Communications.*
759 **480**, 166–172 (2016).
760 17.   Benedikter, B. J. et al. Cigarette smoke extract induced exosome release is mediated by
761 depletion of exofacial thiols and can be inhibited by thiol-antioxidants. *Free Radical Biology &*
762 *Medicine.* **108**, 334–344 (2017).
763 18.   Saeed-Zidane, M. et al. Cellular and exosome mediated molecular defense mechanism
764 in bovine granulosa cells exposed to oxidative stress. *PloS One.* **12**, e0187569 (2017).
765 19.   Wang, K. et al. Mechanical stress-dependent autophagy component release via
766 extracellular nanovesicles in tumor cells. *ACS Nano.* **13**, 4589–4602 (2019).
767 20.   King, H. W., Michael, M. Z., Gleadle, J. M. Hypoxic enhancement of exosome release by
768 breast cancer cells. *BMC Cancer.* **12**, 421 (2012).
769 21.   Bonzini, M. et al. Short-term particulate matter exposure induces extracellular vesicle
770 release in overweight subjects. *Environment Research.* **155**, 228–234 (2017).
771 22.   Neri, T. et al. Particulate matter induces prothrombotic microparticle shedding by
772 human mononuclear and endothelial cells. *Toxicology In Vitro.* **32**, 333–338 (2016).
773 23.   Flaherty, S. E. et al. A lipase-independent pathway of lipid release and immune
774 modulation by adipocytes. *Science.* **363**, 989–993 (2019).
775 24.   van Niel, G., D'Angelo, G., Raposo, G. Shedding light on the cell biology of extracellular
776 vesicles. *Nature Reviews. Molecular Cell Biology.* **19**, 213–228 (2018).
777 25.   Dragovic, R. A. et al. Sizing and phenotyping of cellular vesicles using Nanoparticle
778 Tracking Analysis. *Nanomedicine: Nanotechnology, Biology, and Medicine.* **7**, 780–788 (2011).
779 26.   Saveyn, H. et al. Accurate particle size distribution determination by nanoparticle
780 tracking analysis based on 2-D Brownian dynamics simulation. *Journal of Colloid and Interface*
781 *Science.* **352**, 593–600 (2010).
782 27.   Van der Meeren, P., Kasinos, M., Saveyn, H. Relevance of two-dimensional Brownian
783 motion dynamics in applying nanoparticle tracking analysis. *Methods in Molecular Biology*
784 *(Clifton, N.J.).* **906**, 525–534 (2012).
785 28.   Filipe, V., Hawe, A., Jiskoot, W. Critical evaluation of Nanoparticle Tracking Analysis
786 (NTA) by NanoSight for the measurement of nanoparticles and protein aggregates.
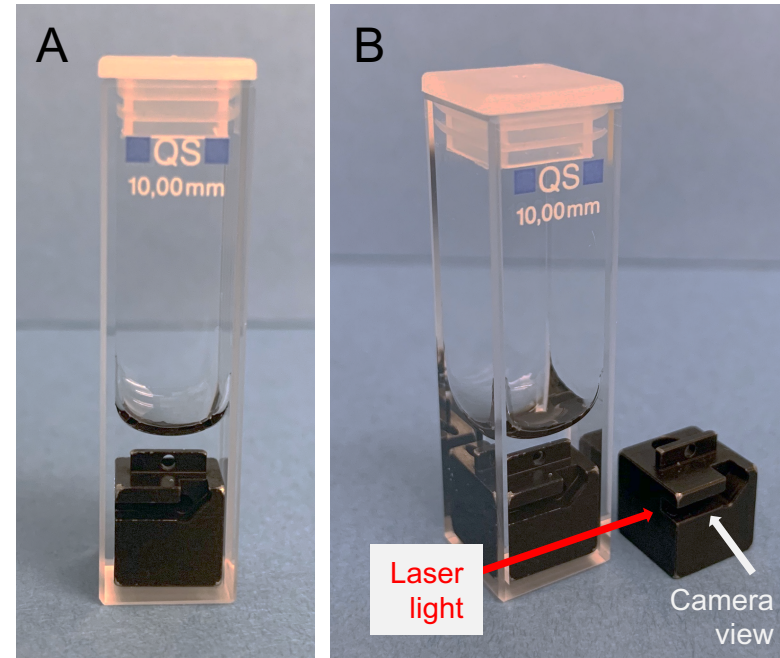787 *Pharmaceutical Research.* **27**, 796–810 (2010).

788   29.   Bachurski, D. et al. Extracellular vesicle measurements with nanoparticle tracking
789   analysis - An accuracy and repeatability comparison between NanoSight NS300 and ZetaView.
790   *Journal of Extracellular Vesicles.* **8**, 1596016 (2019).
791   30.   Varga, Z*. et al.* Hollow organosilica beads as reference particles for optical detection of
792   extracellular vesicles. *Journal of Thrombosis and Haemostasis.* **16**, 1646–1655 (2018).
793   31.   Serrano-Pertierra, E. et al. Extracellular vesicles: Current analytical techniques for
794   detection and quantification. *Biomolecules.* **10**, (2020).
795   32.   Maguire, C. M., Rösslein, M., Wick, P., Prina-Mello, A. Characterisation of particles in
796   solution – a perspective on light scattering and comparative technologies. *Science and
797   Technology of Advanced Materials.* **19**, 732–745 (2018).
798   33.   Bohren, C. F., Huffman, D. R. *Absorption and Scattering of Light by Small Particles*. John
799   Wiley & Sons. (1983).
800
801
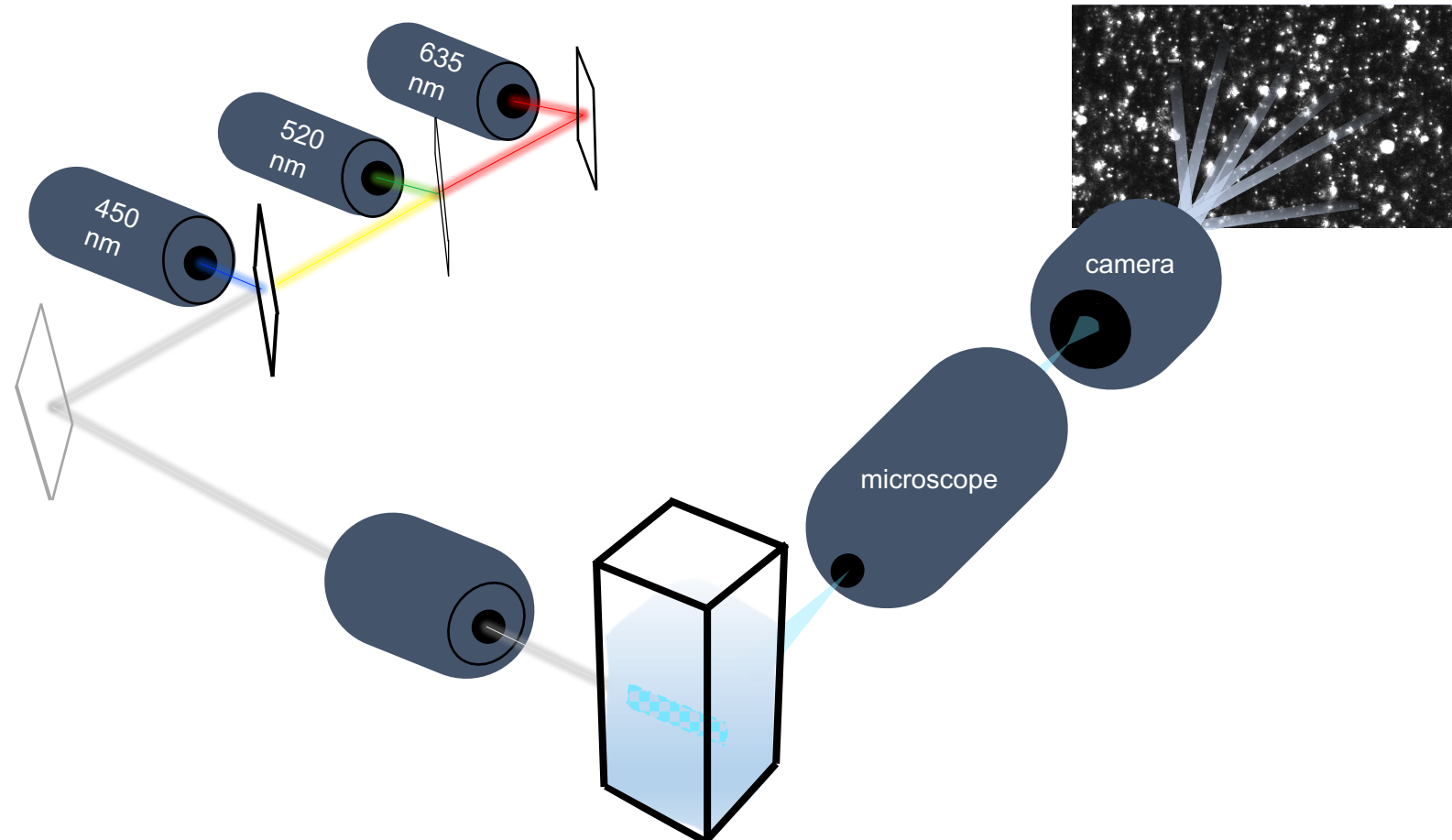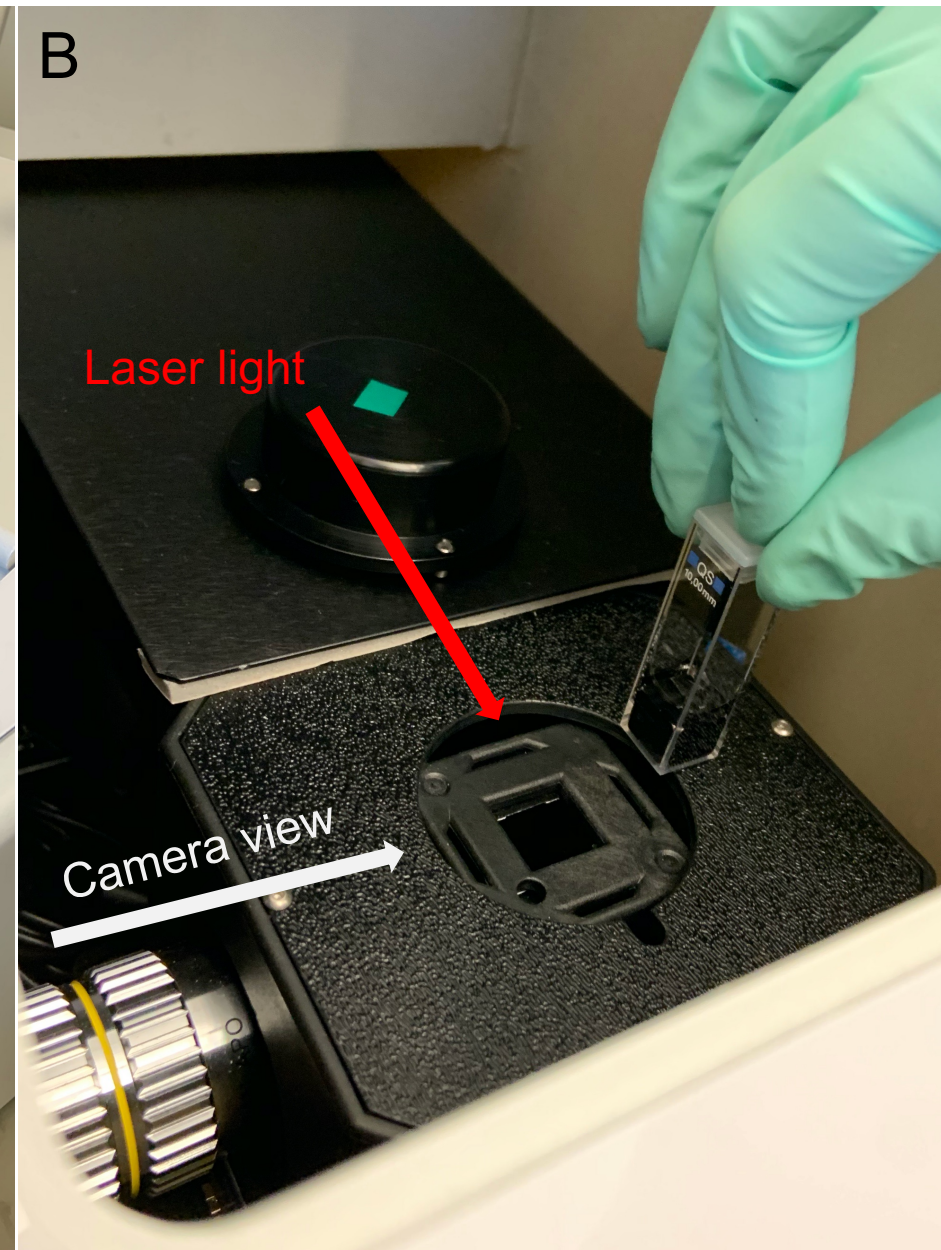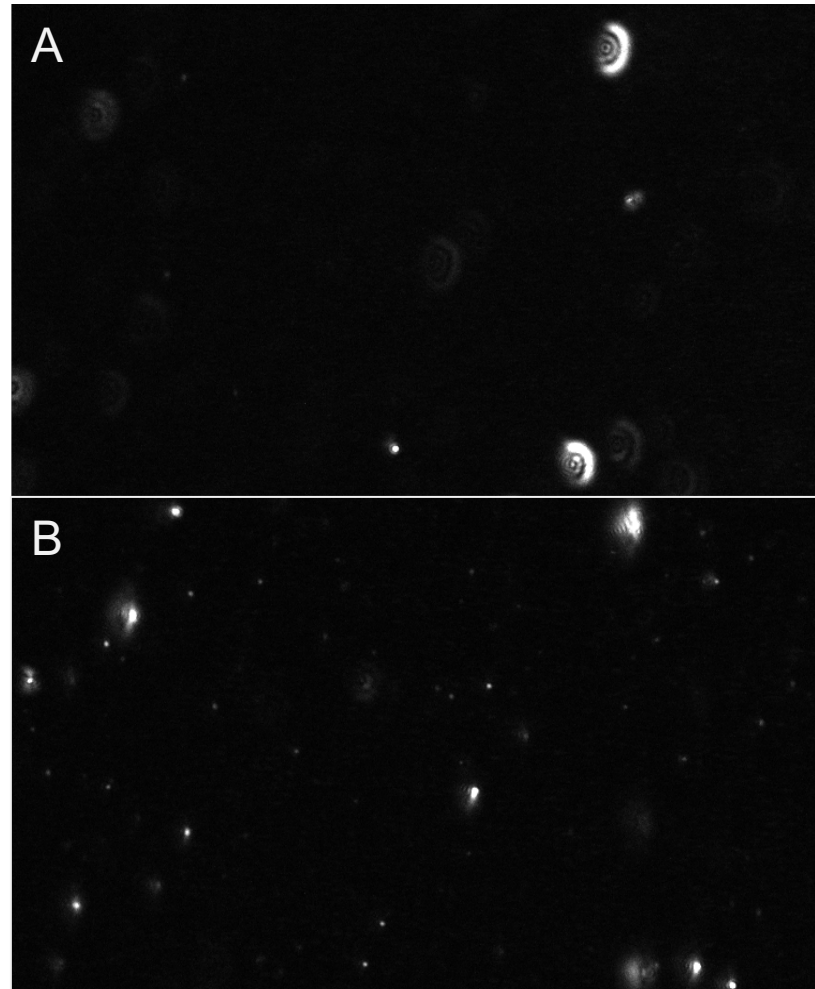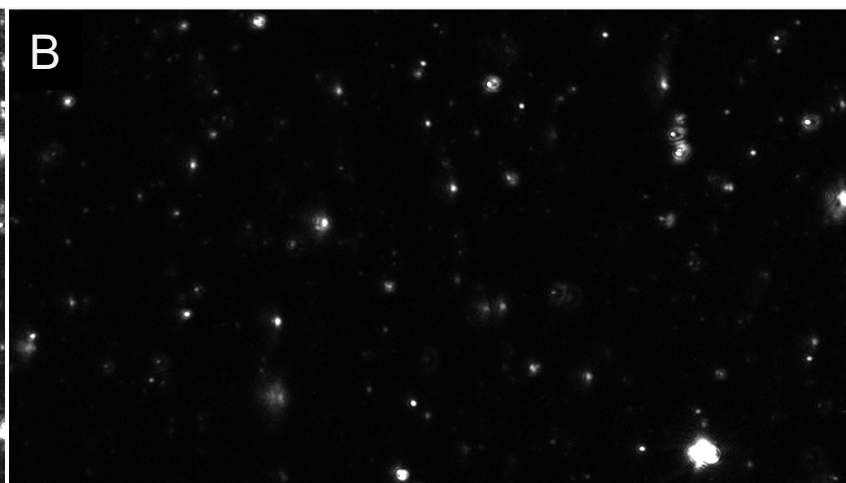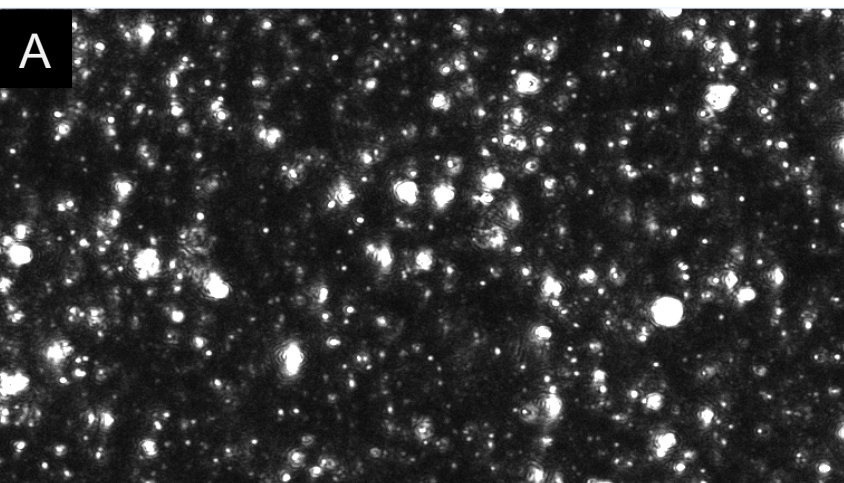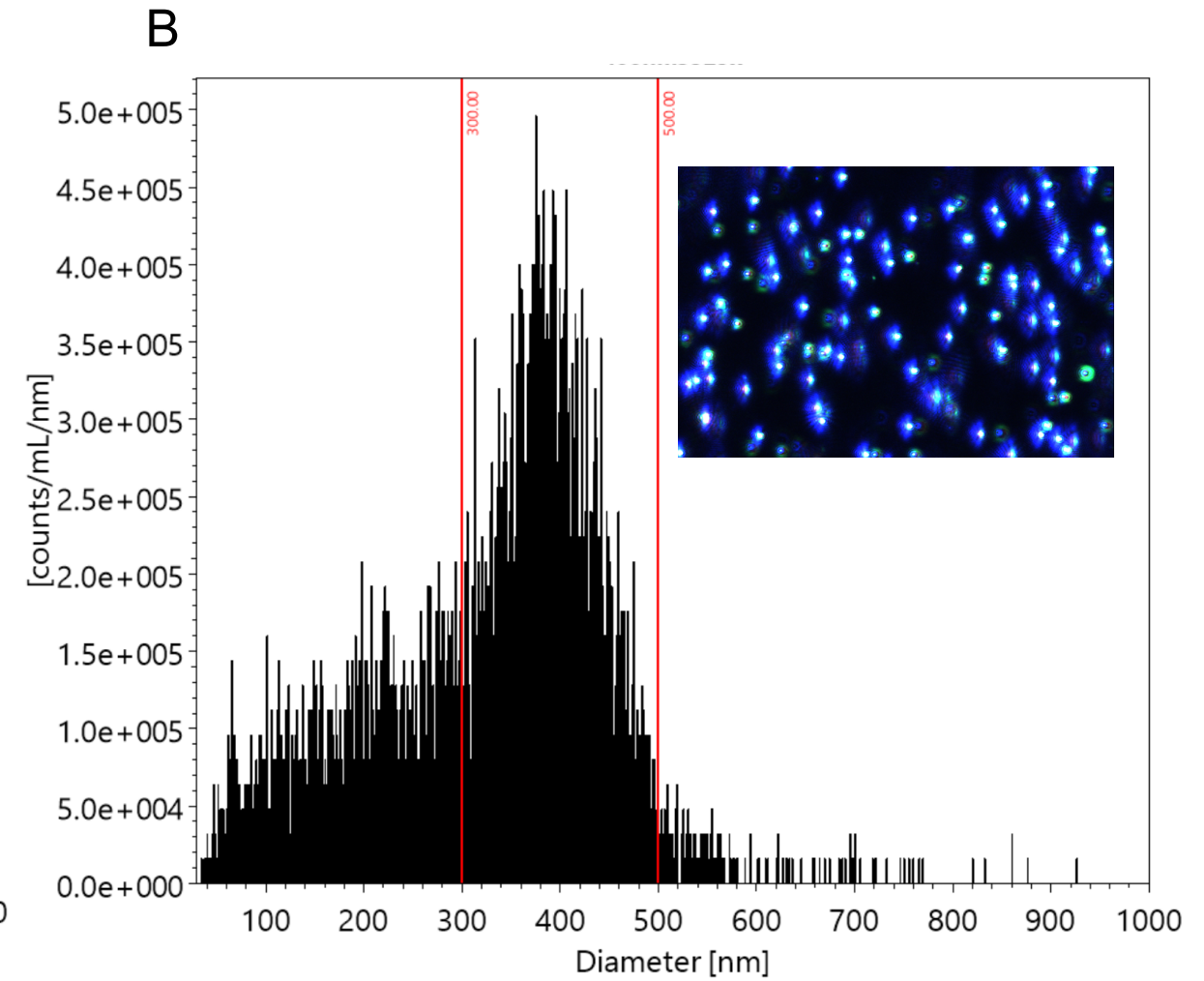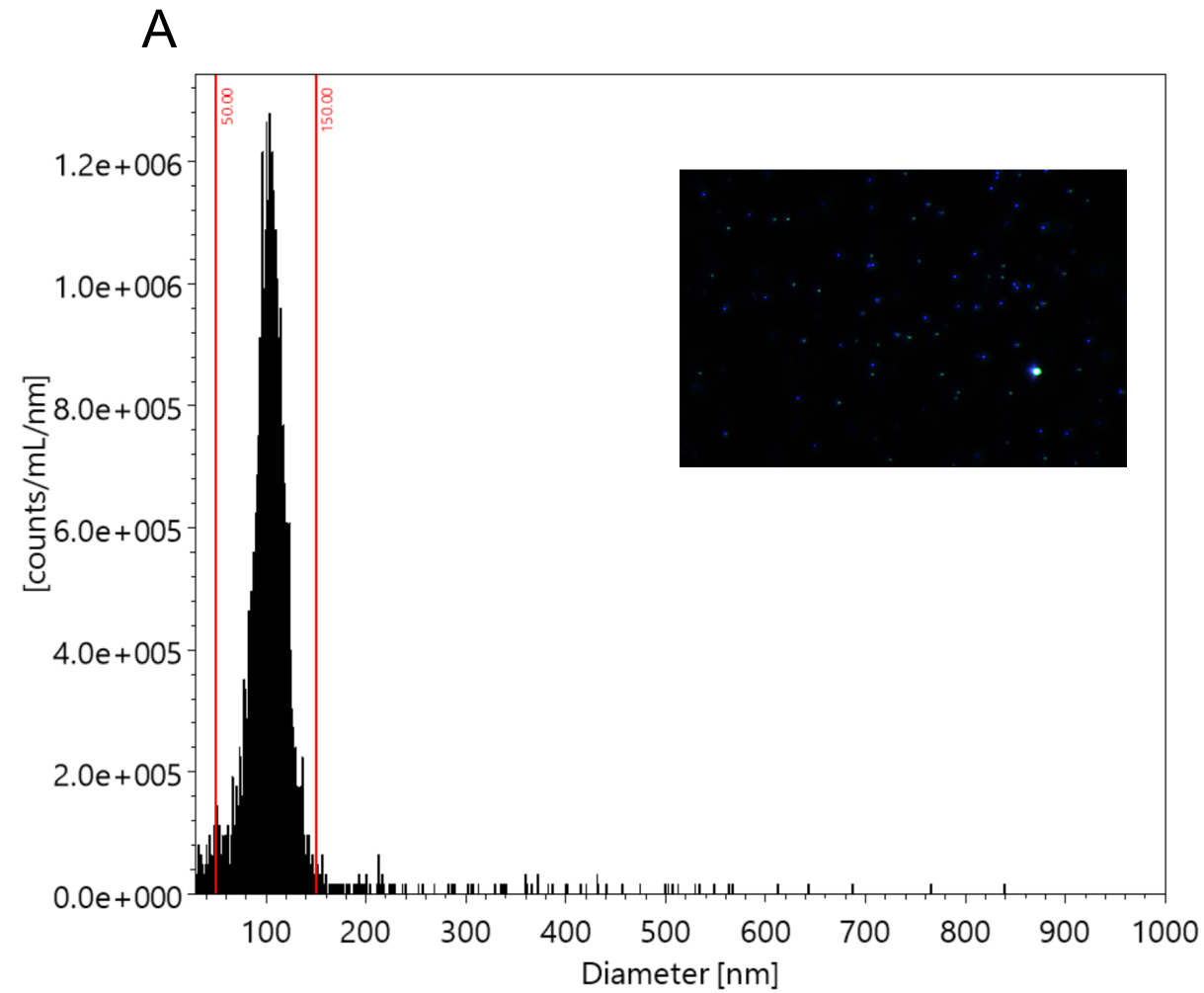
Figure 1

Figure 2 revised

Overview of NTA

| Power on computer and tracking instrument | Prepare the sample and cuvette | Insert sample cuvette into instrument | Start particle tracking software Click "NTA" measurement protocol | Focus image Adjust recording parameters | Start video data acquisition | Process acquired data | Display results |

Figure 3

Figure 4

Figure 5 revised

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10

Figure 11

Figure 12

Figure 12
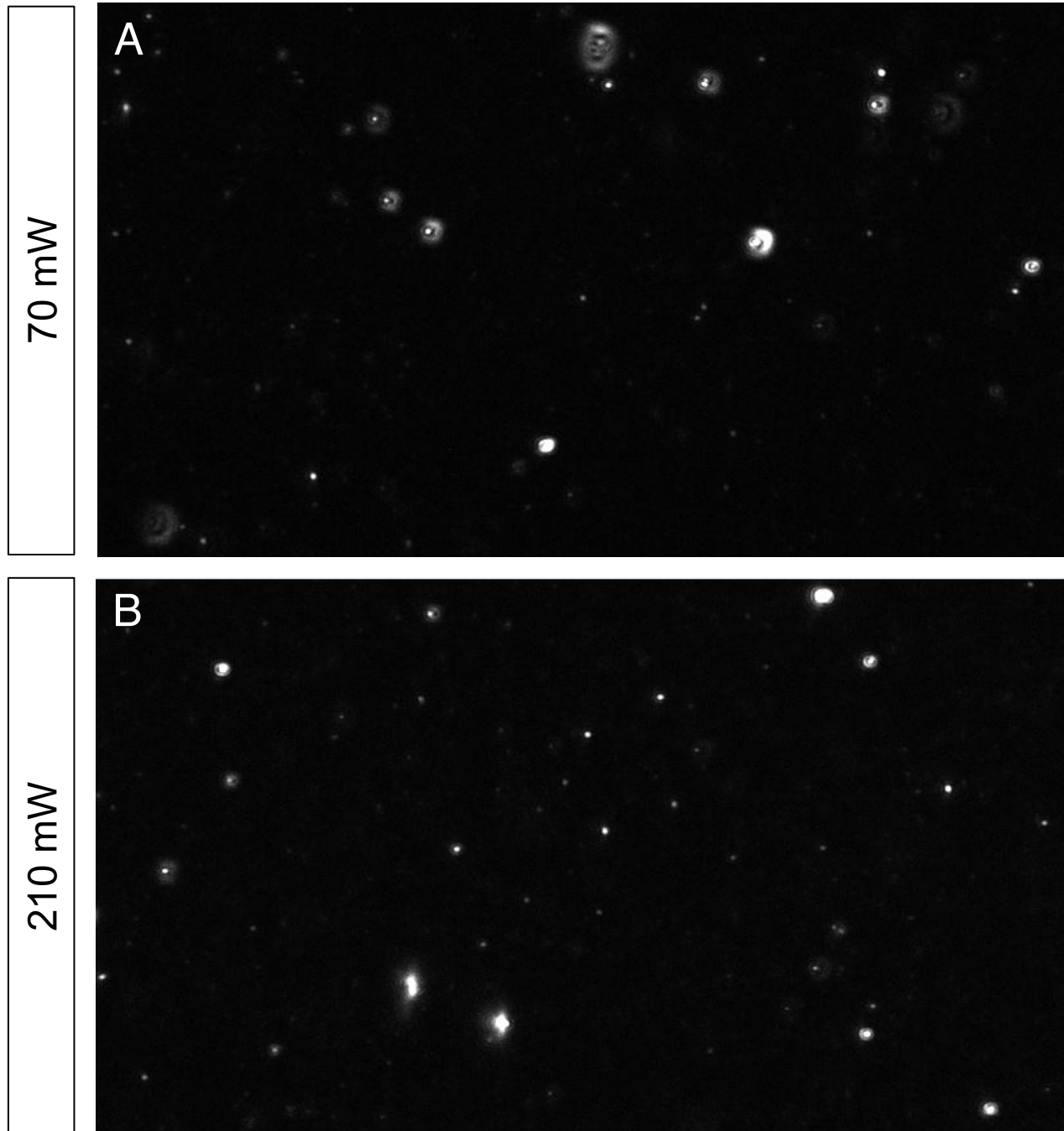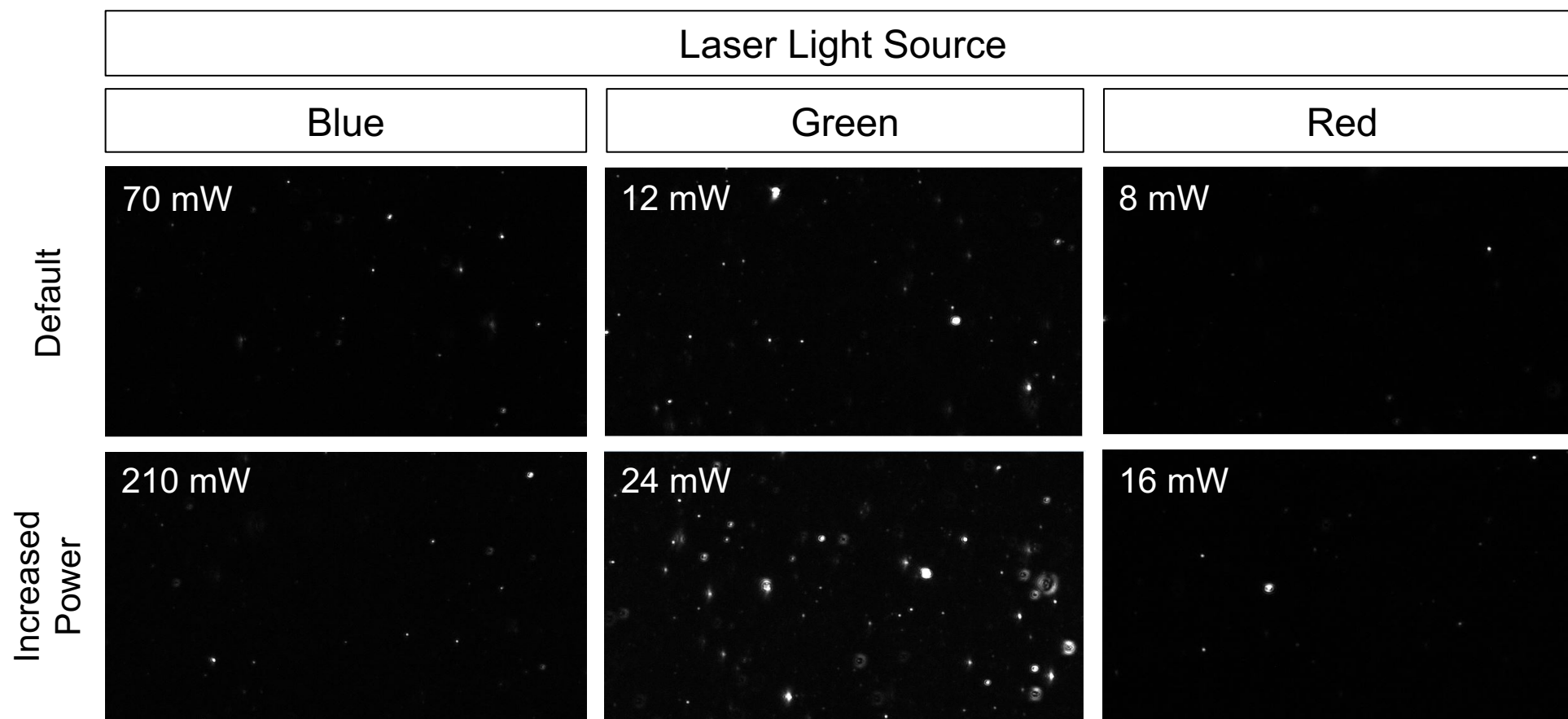
Table 1

Particles	Diameter [nm]	Blue laser [mW]	Green laser [mW]	Red laser [mW]	Camera gain [dB]
Exosomes	100	210	12	8	30

Temperature Regulation	Processing	# Videos
Enabled, set to 22 ℃	Disabled Auto Detection Override	50

Table 2

Process Description	Detection Threshold	Disable Auto Detection	Feature Diameter [px]
Exosomes	Polydisperse Sample	Checked	30

Process Description	Detection Threshold	Disable Auto Detection	Feature Diameter [px]
Exosomes	Polydisperse Sample	Checked	

Table 3

Size of Polystyrene Standard [nm]	Integration range [nm]	Mean size [nm]	SD size [nm]	CV of size	D10 [nm]	D50 [nm]
100	50 to 150	102	17	0.16	76	104
400	300 to 500	391	47	0.12	150	358

Size of Polystyrene	Integration range [nm]	Mean size [nm]	SD size [nm]	CV of size	D10 [nm]	D50 [nm]

D90 [nm]	Total Counts
126	2628
456	2728

Table 4

Dilution Factor	Particle Concentration, particles/mL	Total Particle Concentration, particles/mL
	Raw	Adjusted for Dilution Factor
1000	4.10E+07	4.10E+10
	4.00E+07	4.00E+10
	3.70E+07	3.70E+10
mean (SD)	3.93E+07 (1.70E+06)	3.93E+10 (1.70E+09)
1500	3.60E+07	5.40E+10
	2.60E+07	3.80E+10
	2.90E+07	4.40E+10
mean (SD)	3.03E+07 (4.19E+06)	4.55E+10 (6.60E+09)
2000	2.40E+07	4.80E+10
	2.30E+07	4.60E+10
	2.00E+07	4.00E+10
mean (SD)	2.23E+07 (1.70E+06)	4.46E+10 (3.40E+09)
3000	2.00E+07	6.00E+10
	1.30E+07	3.90E+10
	1.40E+07	4.20E+10
mean (SD)	1.57E+07 (3.09E+06)	4.71E+10 (9.27E+09)

Table 5

Gain [dB]	Blue Laser [mW]	Green Laser [mW]	Red Laser [mW]	Total Concentration, particles/mL (raw)	Average size [nm]*	Standard deviation of size / CV
18	70	12	8	5.90E+07	133	89 / 0.66
24	70	12	8	1.00E+08	118	73 / 0.61
30	210	12	8	1.70E+08	105	57 / 0.54
30	70	12	8	1.10E+08	122	75 / 0.61
30	210	0	0	1.00E+08	116	70 / 0.6
30	70	0	0	7.00E+07	126	79 / 0.63
30	0	12	0	2.80E+07	169	106 / 0.63
30	0	24	0	4.40E+07	148	95 / 0.64
30	0	0	8	1.50E+07	175	129 / 0.74
30	0	0	16	9.20E+06	246	147 / 0.6

*Integrated within 50-1000 nm range

Modal size	D10 / D50 / D90
67	66.57 / 131.08 / 322.70
64	60.21 / 117.93 / 257.08
80	59.83 / 104.74 / 206.07
74	73.17 / 123.09 / 278.70
82	71.33 / 115.63 / 257.65
82	74.81 / 125.16 / 284.23
100	98.28 / 163.39 / 392.50
88	84.24 / 147.69 / 354.15
62	8.81 / 15.32 / 108.93
13	8.55 / 14.93 / 136.75

Name of Material/ Equipment	Company	Catalog Number
1X dPBS	VWR	02-0119-1000
100 nm bead standard	Thermo Scientific	3100A
400 nm bead standard	Thermo Scientific	3400A
Centrifugal Filter Unit	Amicon	UFC901024
Collection tubes, 2 mL	Qiagen	19201
Compressed air duster	DustOff	DPSJB-12
Cuvette insert	HORIBA Scientific	-
Cuvette jig	HORIBA Scientific	-
De-ionized water	VWR	02-0201-1000
Desktop computer with monitor, keyboard, mouse, and all necessary cables	Dell	-
Ethanol (70-100%)	Millipore Sigma	-
ExoQuick ULTRA	System Biosciences	EQULTRA-20A-1
Glass scintillation vials with lids	Thermo Scientific	B780020
"Hook" tool	Excelta	-
Lint-free microfiber cloth	Texwipe	TX629
Microcentrifuge tubes, 2 mL	Eppendorf	22363344
Stir bar	Sp Scienceware	F37119-0005
Suprasil Quartz cuvette with cap	Agilent Technologies	AG1000-0544
ViewSizer 3000	HORIBA Scientific	-

Comments/Description
To dilute samples
To test ViewSizer 3000 calibration
To test ViewSizer 3000 calibration
To filter PBS diluent
For isolation of human plasma extracellular vesicles
To clean cuvettes
Provided with purchase of ViewSizer 3000

To align magnetic stir bar while placing inserts inside cuvette; Provided with purchase of ViewSizer 3000

To clean cuvettes
Provided with purchase of ViewSizer 3000

To clean cuvettes
For isolation of human plasma extracellular vesicles

To clean cuvettes
Provided with purchase of ViewSizer 3000
To clean cuvettes and cover work surface
For isolation of human plasma extracellular vesicles

Initially provided with purchase of ViewSizer 3000

Nanoparticle tracking instrument

COLUMBIA UNIVERSITY | **MAILMAN SCHOOL** of **PUBLIC HEALTH**

Nicole Comfort
Department of Environmental Health Sciences
630 West 168th Street, New York, NY 10032
E-mail: nicole.comfort@columbia.edu

Vidhya Iyer, PhD
Review Editor
Journal of Visualized Experiments

February 16, 2021

Dear Editor:

We thank you for taking the time to review our manuscript JoVE62447, originally titled "Novel nanoparticle tracking analysis technology and method for extracellular vesicle quantification and size determination." We have addressed each of your comments (see below). All line references correspond to the PDF version of "JoVE62447_R1.pdf."

1. We have thoroughly checked the manuscript and there are no spelling or grammar issues that we are aware of.

2. The abbreviations IACUC (line 120), IRB (line 121), PBS (line 239), and CCD (line 747) have been defined before use.

3. We have changed the title from "Novel nanoparticle tracking analysis technology and method for extracellular vesicle quantification and size determination" to "Nanoparticle tracking analysis for the quantification and size determination of extracellular vesicles."

4. 'μm' and 's' units have been corrected with a single space added between the quantity and its unit.

5. We have removed nearly all commercial language from the manuscript and figure/table legends, replacing "Viewsizer 3000" with "particle tracking instrument" where appropriate. We have also removed "ExoQuick ULTRA" and the catalog numbers of the polystyrene beads from the manuscript and added this to the Table of Materials.

   We humbly request to use the term "ViewSizer 3000" (line 87, 839) at least once in the text, without mentioning the company name (which is referenced in the Table of Materials) and thereafter refer to this instrument only as the "particle tracking instrument." There is a paucity of literature and resources using this instrument specifically; Thus, having the term appear in the text of the manuscript will help it appear in literature searches of researchers who are seeking guidance with this particular piece of equipment. By explicitly mentioning the instrument name, we aim to connect current researchers in the field using the ViewSizer 3000 and to build community around its use which will foster the development of best practices. We have seen other *JoVE* articles explicitly mention an instrument name (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4354536/).

6. We have edited parts of the introduction, representative results, and discussion to avoid commercialization of the content. In lines 801-815, rather than make an effort to commercialize the instrument, we attempt to list specific advantages of the instrument/this method for NTA that

would facilitate reproducibility of EV NTA measurements. Additionally, we reiterate to the editorial board that we have no conflicts of interest to disclose and our opinions on the use of this instrument, including advantages compared to similar instruments on the market that we have used, are our own.

7. We have revised the text in the protocol to use the imperative tense in complete sentences wherever possible. Text that contained suggestions were moved to notes.

8. We have edited the protocol section to avoid the use of personal pronouns and additionally have edited the manuscript to minimize the number of personal pronouns used throughout.

9. We have moved the figure legends, table legends, and supplemental figure/table legends to after the representative results section as requested.

10. We have removed all formatting from the tables.

We have also individually responded to reviewers' comments in the subsequent pages:

Dear Reviewer #1:

We thank you for taking the time to review our manuscript. We agree that a successful EV isolation prior to any EV characterization is critical. We therefore have included the isolation of human plasma-derived EVs in the *JoVE* publication (lines 126-214) and will include this aspect of the protocol in the video as well.

However, the inclusion of the protocol for EV isolation from mouse perigonadal adipose tissue would be too lengthy to include. Thus, to comply with article length requirements, we instead reference our article containing the protocol for the reader's benefit and in the future plan to submit a separate *JoVE* manuscript specific to that protocol.

Dear Reviewer #2:

We thank you for taking the time to review our manuscript and for sharing your insight on the need for standardization of methods to improve reproducibility in the EV field. We agree that this manuscript would be more interesting and appeal to a wider audience if fluorescent NTA (fNTA) was demonstrated.

While we have successfully measured fluorescently labelled EVs on the ViewSizer 3000 (mouse adipocyte EVs stained with a commercially available kit), our protocol is still being refined and thus we hesitate to include fNTA in this protocol. fNTA is still a relatively nascent technology and the specificity of commercially available kits to efficiently bind to intact EVs membranes is yet to be demonstrated. While this is outside the scope of this manuscript, in the future, when our protocol for fNTA is optimized, we plan to write another manuscript solely addressing fNTA.

We agree with the reviewer that a thorough examination and understanding of fNTA parameters with some consensus on standardization is needed before labs commence with conducting and reporting results of fNTA analyses, and we thank the reviewer for this suggestion.

Dear Reviewer #3:

We sincerely thank the reviewer for their thorough reading of the manuscript and sharing their substantial expertise in particle tracking analysis.

We have responded to each of your comments, which has greatly improved the manuscript and will educate researchers in the field of extracellular vesicles on principles of particle tracking analysis. Our comments are in red.

MAJOR COMMENTS
1. The authors write: "The intensity of the scattered light allows for detection of particles as low as 10 nm in diameter (50 nm in diameter for a polydisperse sample)". The aforementioned sentences are misleading, because the minimum detectable light scattering signal depends on the particle diameter and refractive index (and shape). Without a statement of the particle refractive index, the specified "measurement range of 10 nm to 15 µm" is not an objective definition of the limit of detection (LOD). Most likely, HORIBA tries to seduce their customers by specifying the LOD of the Viewsizer in terms of smallest detectable diameter of gold nanoparticles, which scatter light substantially more efficient than EVs due to a higher refractive index. Therefore, please omit aforementioned statements or express the LOD in terms of EV diameter by quantifying the signal to noise ratio and apply light scattering theory.

Indeed, we meant to indicate that the instrument's LOD as low as 10 nm would be for measurement of monodisperse gold nanoparticles rather than biological EVs that have a lower refractive index and can now see that this sentence is misleading, especially given that the target audience of this paper is for researchers in the EV field. To address this, we have removed this statement from the text.

2. The authors further write: "The limit of detection (LOD) also depends on sample composition, such that the sensitivity decreases with increasing polydispersity of the sample. For instance, when measuring a mixture, smaller components can be undetected because they are obscured by the scattered light of larger particles which can 'blind the detector.' Because most EV preps will be polydisperse in composition, researchers should be aware of the sample-dependent LODs of NTA that can impact the shape of the overall size distribution and exercise caution when interpreting results. However, the ViewSizer 3000 offers breakthrough improvements to address this limitation inherent to nanoparticle tracking technology.". In line with major comment 1, the quoted statements of the authors remain subjective because the LOD of the ViewSizer is unclear. Please omit aforementioned statements or quantify the LOD of the ViewSizer in terms of EV diameter.

To address both comments 1 and 2, we have omitted statements making claims about the LOD and instead state more generally in the discussion (lines 780-783 of the PDF proof) that the LOD will be sample-dependent. While we are not in a position to make definitive claims about the LOD for this instrument, we wish to make it clear to the reader to consider that a lack of knowledge of the LOD should impact their interpretation of results and that they should exercise caution when reporting results. Other articles in the literature, such as the article you pointed us to (https://onlinelibrary.wiley.com/doi/10.1002/jev2.12052), also note the sample-dependent particle diameter range that can be detected by NTA.

3. Lifesciences face a reproducibility crisis. To prevent the field of EV from generating irreproducible data, please state explicitly which Viewsizer settings users should report to generate data that can be reproduced by colleagues in other labs.

We couldn't agree more and have advised which settings should be reported in section 8.3 of the protocol. We have also highlighted this in the discussion (line 804-805).

4. The authors write: "Increasing the power of the red laser (650 nm, long wavelength) increased the reported average 405 particle size from 175 to 246 nm, as larger particles scatter light in this wavelength very well". This statement is wrong. In this system, the wavelength and thus the scattering cross sections of the particles are static. The power of scattered light is the scattering cross section times the intensity of the incoming light, which is linearly proportional to the power of the red laser. Thus, increasing the power of the red laser increases the light scattered from all particles, small and large, proportionally.

Thank you for pointing out our error and clarifying our understanding. We have corrected this statement.

5. The authors suggest that "Researchers interested in microvesicles of larger sizes (e.g. 400-1000 nm) are encouraged to optimize the laser parameters using a polystyrene or silica bead standard" However, light scattering properties of EVs differ from solid polystyrene and silica beads, because EVs are core-shell particles with a high refractive index shell and a low refractive index core. Therefore, polystyrene and silica beads are not an appropriate

model system to optimize settings of optical measurement techniques. Hollow organosilica beads would for example offer a better alternative to polystyrene and silica beads.

We thank the reviewer for this suggestion and have edited the text accordingly [line 580-583]. We have also added a reference (Varga et al., 2018, reference 30) that compares the light-scattering properties of polystyrene beads, silica beads, and hollow organosilica beads for the reader's knowledge and benefit.

However, given that (1) silica nanoparticles are much more readily available, and (2) they have a refractive index of approximately 1.43-1.46, close to the range of refractive indices reported in the literature for EVs (1.37-1.42), we mention in the text that silica is the best practical substitute for cases when researchers do not have access to hollow organosilica particles (https://onlinelibrary.wiley.com/doi/10.1002/jev2.12052).

This would be more acceptable in this scenario because unlike flow cytometry, the determined particle size is independent of the particle refractive index. The analyzer only uses particle motion, not scattered intensity, to extract particle diameter. The measured particle concentration, however, is a relatively weak function of the refractive index and therefore, while organosilica particles would be preferable to silica and polystyrene given that their refractive index is closer to the refractive index of EVs, silica is still a more suitable substitute than gold nanoparticles.

6. Sizing techniques based on particle tracking analysis (PTA) techniques are known to provide accurate (measurement trueness) results for sizing monodisperse reference particles. However, PTA falls short regarding accuracy of concentration determination and precision (e.g. broadening of the measured size distribution) in general (https://doi.org/10.1002/jev2.12052). Concentration estimates by PTA are rather an order of magnitude guess (https://doi.org/10.1111/jth.12602). Similar to existing commercial PTA techniques, the Viewsizer determines the size of particles by measuring the diffusion coefficient and applying the Stokes-Einstein relation, so I suppose that the aforementioned statements about the limited accuracy and precision also apply to the Viewsizer. Please add quantitative statements about the accuracy and precision in determining the particle diameter and concentration with the Viewsizer, or if not available with a similar PTA method.

Indeed, while this particle tracking instrument is also subject to more limited accuracy and precision with increasing sample polydispersity, it does offer an advantage to comparable NTA instruments because of the multiple light sources, allowing it to detect scattered light intensity produced by differently-sized nanoparticles coexisting in the same sample.

Regarding precision, our measurements of polystyrene bead standards reported a mean (standard deviation) size of 102 (17) nm with a coefficient of variation (CV) of 0.16 for 100 nm beads, compared to a CV of 1.4 on the NanoSight NS300 (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6450530/#CIT0023), showing that at least for a monodisperse sample, the Viewsizer is more precise than a single-laser NTA instrument, with similar accuracy.

We also measured the same four samples (each at three different dilutions) on the Viewsizer 3000 as well as the NanoSight NS300 and found that the Viewsizer reported more precise measures of particle size (mean, d10/d50/d90), as shown in the graphs below. We have included this as a supplemental figure.

Particle size measurements across multiple dilutions: ViewSizer 3000 vs. NanoSight NS300
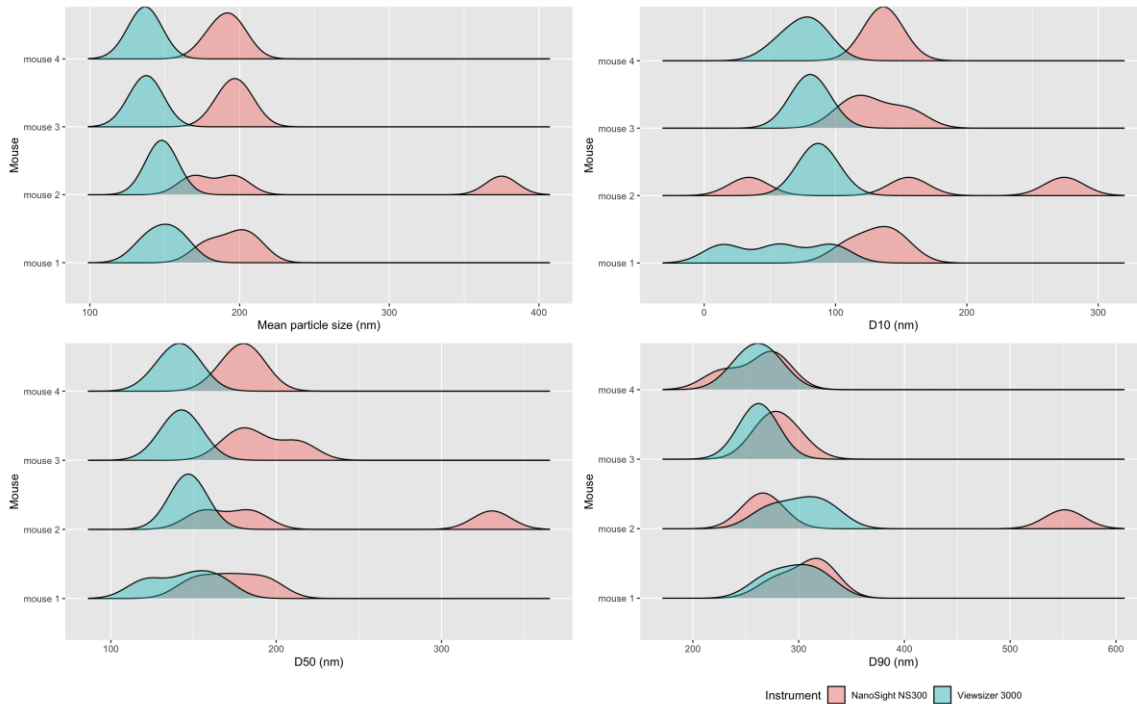
In terms of reported particle concentration, the CVs were significantly smaller for Viewsizer data compared to Nanosight (p=0.035).

Furthermore, our measurements of the same sample at various dilutions (shown in Table 4 and Figure 8) show the Viewsizer's accuracy, because the Viewsizer reported particle concentrations that scaled with the dilution factor as expected, even in a highly polydisperse biological EV sample.

MINOR COMMENTS
1. Many published size distributions of EVs measured by NTA are based on tracking a few hundred particles only, resulting in artefactual spikes in the size distribution. Generally, I recommend people to track at least a few thousand particles to generate a size distribution of particles in biofluids. Although 50 videos of 10 seconds should generate statistically significant data, consider to add a statement about the minimum number of particles to track.

We thank the reviewer for this comment and agree that, though it can be time-consuming, it is much better to base NTA measures on a few thousand particles rather than a few hundred. Our measurements with 25 videos (half of the number we recommend in this protocol) track 4390 total particles on average (4208 counts within range). We have included recommendations on the minimum number of particles to track in section 8.3.10 of the protocol.

We sincerely thank the editor and reviewers for their comments, which have greatly improved the manuscript. We believe that this manuscript is appropriate for publication by the *Journal of Visualized Experiments* and that it will be of great interest to a wide readership.

Sincerely,

Nicole Comfort

Supplemental Fig 1

Instrument ▢ NanoSight NS300 ▢ Viewsizer 3000

200 nm

Supplemental Table 1
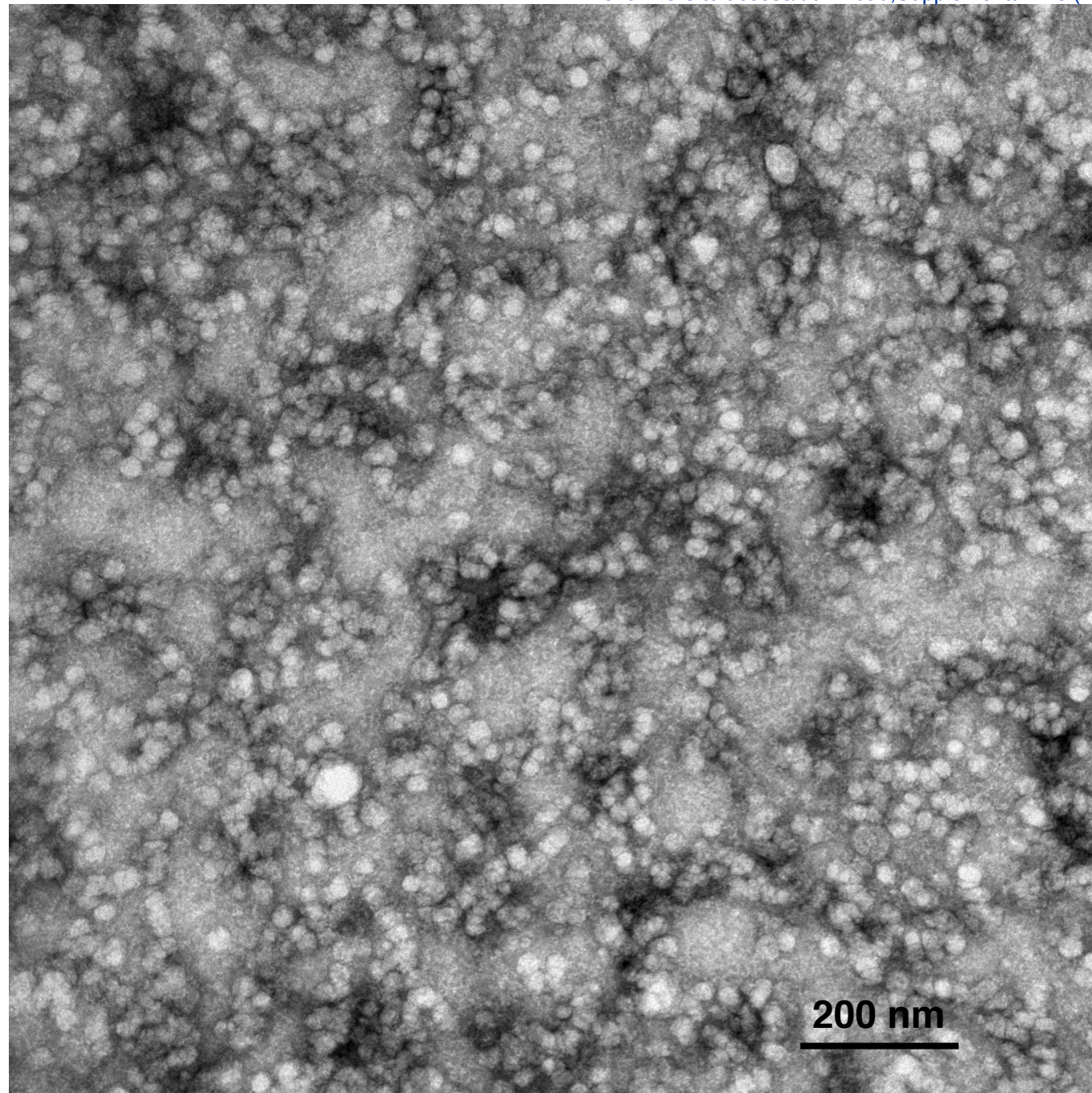
Mouse	Dilution Factor	Mean particle size (nm)	D10 (nm)	D50 (nm)	D90 (nm)
1	1000	137	14.21	121.22	269.47
1	500	150	57.03	146.86	298.13
1	100	161	96.95	164.7	321.6
2	1000	148	82.72	145.95	303.05
2	500	151	84.52	146.75	327.45
2	100	145	93.43	147.78	270.94
3	1000	131	78.45	135.68	261.1
3	500	140	79.11	143.66	266.98
3	100	141	84.78	148.12	258.28
4	1000	131	60.31	132.08	246.07
4	500	139	83.64	143.03	276.03
4	100	140	82.39	147.37	261.77

Particle Concentration, particles/mL

5.70E+07
6.30E+07
1.54E+08
2.30E+07
2.20E+07
1.34E+08
7.70E+07
9.10E+07
2.24E+08
9.40E+07
1.34E+08
2.94E+08

Total Particle Concentration, particles/mL, Adjusted for Dilution Factor

5.70E+10
3.15E+10
1.54E+10
2.30E+10
1.10E+10
1.34E+10
7.70E+10
4.55E+10
2.24E+10
9.40E+10
6.70E+10
2.94E+10

Capture Settings	
Camera Type	sCMOS
Laser Type	Blue488
Camera Level	7
Slider Shutter	121
Slider Gain	24
FPS	25
Syringe Pump Speed	200
Analysis Settings	
Detect Threshold	5
Blur Size	Auto