Chapter 9

Multiple Particle Tracking: A Method for Probing Biologically Relevant Mobility of Bacterial Extracellular Vesicles

Darby Steinman, Robert D. Kirian, and Hannah C. Zierden

Abstract

Bacterial extracellular vesicles (bEVs) are produced by both Gram-negative and Gram-positive bacteria. These biological nanoparticles transport small molecules, nucleic acids, and proteins, enabling communication with both bacterial and mammalian cells. bEVs can evade and disrupt biological barriers, and their lipid membranes protect their cargo from degradation, facilitating long-distance communication in vivo. Furthermore, bacteria are easily manipulated and easily cultured. These combined factors make bEVs an ideal candidate for drug delivery applications. Thus, the study of how bEVs interact with biological barriers is interesting from both a signaling and drug delivery perspective. Here we describe methods for tracking bEV motion in biological matrices ex vivo. We outline methods for growth, isolation, quantification, and labeling, as well as techniques for tracking bEV motion ex vivo and quantifying these data. The methods described here are relevant to bEV communication with host cells as well as drug delivery applications using bEVs.

Key words Bacterial extracellular vesicles (bEVs), Outer membrane vesicles (OMVs), Membrane vesicles (MVs), Bacterial membrane vesicles (bMVs), Multiple particle tracking

1 Introduction

Bacterial cells outnumber mammalian cells in the human body and contribute to various health outcomes [1]. Much research has been done to investigate microbe-host communications with the potential to influence disease, with recent growing interest in communication mediated by bacterial extracellular vesicles (bEVs). bEVs are membrane-bound particles produced by both Gram-positive and Gram-negative bacteria that carry small molecules, nucleic acids, and proteins long distances, enabling communication with host tissues [2, 3]. These biological nanoparticles can penetrate and

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disrupt mucosal and epithelial barriers, facilitating the transport of virulence factors, defense against the host immune system, and horizontal gene transfer. bEVs vary in size from 50 to 500 nm, making them small enough to penetrate biological barriers and distribute widely through the body. Given their targeting capabilities and biocompatibility (specifically for commensal bacteria), bEVs have garnered attention as drug delivery vehicles [4]. Bacteria are relatively easy to grow, enabling large-scale production for therapeutic manufacturing. Furthermore, bacteria lend themselves to manipulations allowing for "pre-loading" of desired cargoes. "Post-loading" techniques have also been employed to develop bEV platforms for delivery of small-molecule drugs and nucleic acids. Despite the attractive qualities of bEVs as drug delivery vehicles, more work needs to be done to characterize and understand bEV interactions within a host environment.

Tracking EVs throughout biological systems has been done at a macroscopic scale—imaging the distribution of labeled particles in vivo [5, 6]. At a microscopic level, attention has been given to cellular uptake of EVs as it relates to signaling [7, 8]. Here, we present details necessary for tracking EV mobility through matrices such as cervicovaginal mucus, sputum, or synthetic mucus. Quantifying the mobility of various bEVs will further the understanding of host-microbe communications that contribute to disease outcomes.

Multiple particle tracking (MPT) has been used in various fields to characterize biological barriers, probe colloid stability, understand cellular mechanisms of movement, and establish the utility of nanoparticle formulations for local drug delivery [9–13]. The methods described in the following sections are intended to facilitate the isolation, labeling, and ex vivo tracking of bEVs in relevant biological matrices (Fig. 1). Probing mobility in relevant biological matrices may prove to be a useful measure in understanding bEV communication in vivo and their potential development as drug delivery vehicles [14, 15].

2 Materials

2.1 Culturing Bacteria

1. Frozen stock of bacteria of interest.
2. Ice.
3. Bunsen burner or ethanol lamp.
4. Agar plates appropriate for bacteria of interest.
5. Inoculating loops.
6. Appropriate media for bacteria of interest (*see* **Note 1**).
7. Vacuum filtration unit (0.22 μm).
8. Sterile tubes (5 mL and 50 mL).

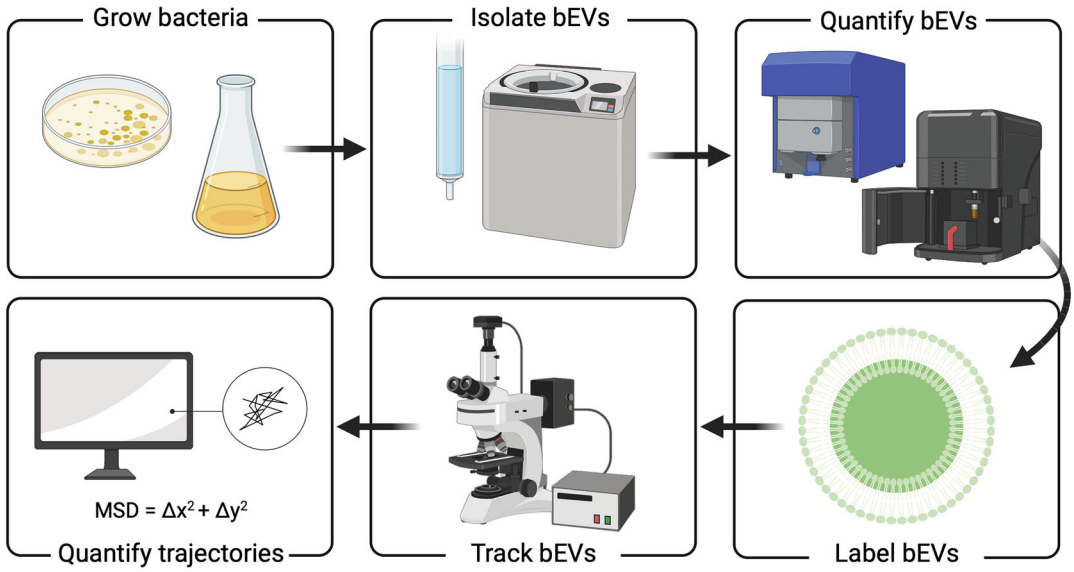

Fig. 1 Overview of method to label bEVs for multiple particle tracking. After culturing bacteria, isolate bEVs using either ultracentrifugation or size exclusion chromatography. Quantify the number of bEVs in the sample prior to labeling bEVs with a commercially available kit. Add labeled particles to a biomatrix and record videos of particle mobility throughout the sample. Quantify particle motion using image analysis software and calculate the effective diffusivity. This workflow allows for the comparison of bEV mobility across matrices and bEV types

9. Incubator with appropriate conditions for bacteria of interest (e.g., temperature, aerobic v. anaerobic, static v. shaking).
10. Spectrometer and appropriate measuring containers (e.g., cuvettes, 96-well plates).

2.2 Isolating bEVs

1. Conditioned media from Subheading 3.1.

2.2.1 Ultracentrifugation

1. Table-top centrifuge equipped with swinging bucket rotor and 50 mL tube adapters.
2. Ultracentrifuge (e.g., Beckman Coulter Optima XPN series, Thermo Scientific Sorvall WX+ series).
3. Swinging bucket rotor (e.g., Beckman Coulter SW-28 Ti, Thermo Scientific AH-650).
4. Ultracentrifuge tubes.
5. Balance.
6. Isolation Buffer: 1× Phosphate buffered saline, filtered at 0.22 μm.
7. Sterile tubes (1.5 mL, *see* **Note 2**).

2.2.2 Size Exclusion Chromatography

1. SEC column (e.g., Izon qEV, biotechne PURE-EVs).
2. Tubes (1.5 or 2.0 mL, *see* **Note 2**).
3. Isolation Buffer: 1× Phosphate buffered saline, filtered at 0.22 μm.
4. Bacteriostatic Buffer: 1× Phosphate buffered saline with 0.05% sodium azide.
5. Cleaning Buffer: 0.5 M NaOH in H_2O.
6. Automated fraction collector (AFC, not required, e.g., Izon AFC V2).

2.3 Quantifying Concentration of bEVs

1. bEVs isolated from Subheading 3.2.
2. 5 mL tubes.
3. NTA Buffer: Distilled H_2O, filtered at 0.22 μm.
4. Rinsing Buffer: Distilled H_2O, filtered at 0.22 μm.
5. Sterile syringe (3 mL or 10 mL, typically).
6. Nanoparticle counting instrument (e.g., Particle Metrix Zeta-View, Malvern NanoSight, Horiba ViewSizer).

2.4 Fluorescently Labeling bEVs with Lipophilic Dyes

1. Isolated and quantified bEVs from Subheading 3.3.
2. Tabletop centrifuge equipped with microtube rotor.
3. Dilution Buffer: 1× Phosphate buffered saline, filtered at 0.22 μm.
4. Sterile tubes (1.5 or 2.0 mL, *see* **Note 2**).
5. Lipophilic dye (e.g., Vybrant, CellVue, IVISense, Cell Linker).
6. Quenching Buffer: 1% Bovine Serum Albumin (BSA) in H_2O.
7. Sucrose Buffer: 20% sucrose in H_2O.
8. Ultracentrifuge (e.g., Beckman Coulter Optima XPN series, Thermo Scientific Sorvall WX+ series).
9. Swinging bucket rotor (e.g., Beckman Coulter SW-28 Ti, Beckman Coulter SW-55 Ti, Thermo Scientific AH-650).
10. Ultracentrifuge tubes.
11. Balance.
12. Amicon® Ultra-4100 kDa molecular weight cut-off microcentrifuge filter units.

2.5 Multiple Particle Tracking

1. Fluorescently labeled bEVs from Subheading 3.4.
2. Electrical tape.
3. Hole puncher.
4. Slide.
5. Biological matrix (e.g., sputum, cervicovaginal mucus, synthetic mucus; *see* **Note 3**).

6. Wiretrol.
7. Pipettes and tips.
8. Super glue.
9. Glass coverslip.
10. Fluorescent microscope (e.g., Zeiss Axiovert, *see* **Note 4**).

2.6 Quantifying Particle Mobility

1. Videos of particle mobility from Subheading 3.5.
2. Hard drive for video storage.
3. Computer equipped with image analysis software (e.g., ImageJ).
4. Multiple particle tracking software (*see* **Note 5**).

3 Methods

3.1 Culturing Bacteria

Culturing should be done aseptically using a flame or biosafety cabinet.

1. From frozen stock of bacteria, use the four-quadrant streaking method to deposit sample on an agar plate (appropriate for bacteria of interest) using an inoculating loop. This plate should be grown for 24–72 h at appropriate conditions (e.g., 37 °C anaerobic culture).
2. After colonies appear on the agar plate (typically 24–72 h), collect colonies with an inoculating loop and use to seed 5 mL of media.
3. Grow 5 mL culture for 24–72 h at appropriate conditions (e.g., 37 °C static anaerobic culture).
4. Use the 5 mL seed culture to inoculate 45 mL of media after 24–72 h. Some bacteria grow best at a particular optical density (OD 600 nm). If desired, check the OD 600 using a spectrometer according to manufacturer instructions. OD 600 values for optimal growth vary between bacterial species. Readings should always be normalized to blank media.
5. Incubate 50 mL cultures at appropriate conditions for 24–72 h.
6. After desired growth, centrifuge cultures using a benchtop centrifuge set to 4 °C. Spin cultures for ~30 min at 4000 rcf. Transfer the supernatant to a new tube. Repeat until no bacteria pellet forms. The resulting supernatant contains bEVs.

3.2 Isolating bEVs

Ultracentrifugation and size exclusion chromatography are two techniques commonly used to isolate extracellular vesicles. Other protocols (density gradient ultracentrifugation, affinity chromatography, anion exchange chromatography, EV precipitation,

ultrafiltration) may improve the recovery and/or purity of isolated EVs, but are not as widely used [16, 17].

3.2.1 Ultracentrifugation

1. Transfer 25–35 mL of conditioned media into ultracentrifuge tubes (*see* **Note 6**), weighing the ultracentrifuge tubes to ensure all are equivalent so the rotor is balanced (*see* **Note 7**).
2. Centrifuge conditioned media for 40 min at 16,000 rcf at 4 °C to pellet residual bacteria and cellular debris.
3. Transfer supernatant from each ultracentrifuge tube to a new ultracentrifuge tube, leaving ~1 mL behind so as not to disturb the debris pellet. As above, weigh the ultracentrifuge tubes to ensure all are equivalent weight.
4. Centrifuge clarified media for 90 min at 129,000 rcf at 4 °C to pellet bEVs.
5. Aspirate the resulting supernatant without disturbing the bEV pellet (leave ~1 mL). Resuspend each bEV pellet with 25–35 mL fresh Isolation Buffer to wash bEVs. Weigh the ultracentrifuge tubes to ensure equivalent weights and a balanced rotor.
6. Centrifuge the bEV pellet resuspended in PBS for 90 min at 129,000 rcf at 4 °C to pellet the bEVs.
7. Aspirate the resultant supernatant without disturbing bEV pellet (leave ~1 mL). Resuspend bEV pellet in the remaining ~1 mL of Isolation Buffer and transfer to microcentrifuge tube. Store at −80 °C until analysis (*see* **Note 8**).

3.2.2 Size Exclusion Chromatography

The methods here describe size exclusion chromatography performed using the IZON Automated Fraction Collector (AFC) and qEV columns.

1. Prepare conditioned media for chromatography to avoid clogging the column. Centrifuge at 12,000 rcf at 4 °C for 45 min to pellet small debris. Transfer supernatant to a new tube for chromatography.
2. Equilibrate qEV column and Isolation Buffer to room temperature. Remove the column Bacteriostatic Buffer (*see* **Note 9**). Load the column into the AFC and flush the column with at least two column volumes of PBS buffer to minimize the effects of Bacteriostatic Buffer on the bEV separation process. The column will stop flowing once all the Isolation Buffer has entered the column.
3. Load the prepared sample onto the column (*see* **Note 10**). As the column begins to flow, the AFC will measure the weight of Isolation Buffer displaced by the sample. The Isolation Buffer will stop flowing once all the samples have entered the column, requiring additional Isolation Buffer to be loaded onto the

column. The AFC will start collecting sample fractions when the total column volume of Isolation Buffer has been displaced. It is often necessary to optimize size exclusion chromatography protocols for each sample type. The final isolated bEV sample is typically a combination of several fractions based on the sizes and ζ-potentials of particles in the fraction (*see* Subheading 3.3). Store sample at −80 °C until analysis (*see* **Note 8**).

4. Clean the column with Cleaning Buffer to remove residual proteins from the resin. The column should be flushed with Isolation Buffer to return the column pH to normal after cleaning and before loading a new sample.
5. **Steps 3** and **4** may be repeated several times using the same column to isolate bEVs from several samples. After isolation, store columns in Bacteriostatic Buffer at 4–8 °C.

3.3 Quantifying Concentration of bEVs

The primary technique utilized for the quantification of bEVs is Nanoparticle Tracking Analysis (NTA) [18–20]. The methods here describe NTA measurements performed using the Particle Matrix ZetaView.

1. Initialize the NTA microscope and software.
2. Flush the automatic pumps connected to the microscope and manually flush the loading cell with particle-free Rinsing Buffer to purge the instrument of any air, which can affect particle analysis.
3. Perform a cell quality check after air has been removed to verify working condition of the cell.
4. Perform an auto-alignment of the microscope. The ZetaView instrument is aligned using 100 nm polystyrene standard beads at a 1:250,000 dilution in NTA Buffer (*see* **Note 11**). Load diluted standard into the NTA microscope to auto-align the microscope. To load, use a bubble-free syringe connected to the cell port. Load at least 2 mL of standard into the cell with a small amount of liquid on the syringe tip to maintain a liquid-liquid interface and to avoid injecting air into the cell. After auto-alignment, flush the cell with particle-free water.
5. Prepare samples for analysis by diluting in NTA Buffer. The minimum concentration of detection for the ZetaView is 5×10^6 particles/mL. When characterizing a sample with an unknown concentration of bEVs, the sample may need to be diluted iteratively for accurate measurements (*see* **Note 12**, Fig. 2a).
6. Load the diluted sample into the cell, as in **step 4**. Measure the concentration of bEVs with appropriate analysis parameters (Fig. 2b). Typically, the instrument analyzes 11 positions within the loaded cell, using light scattering to capture the

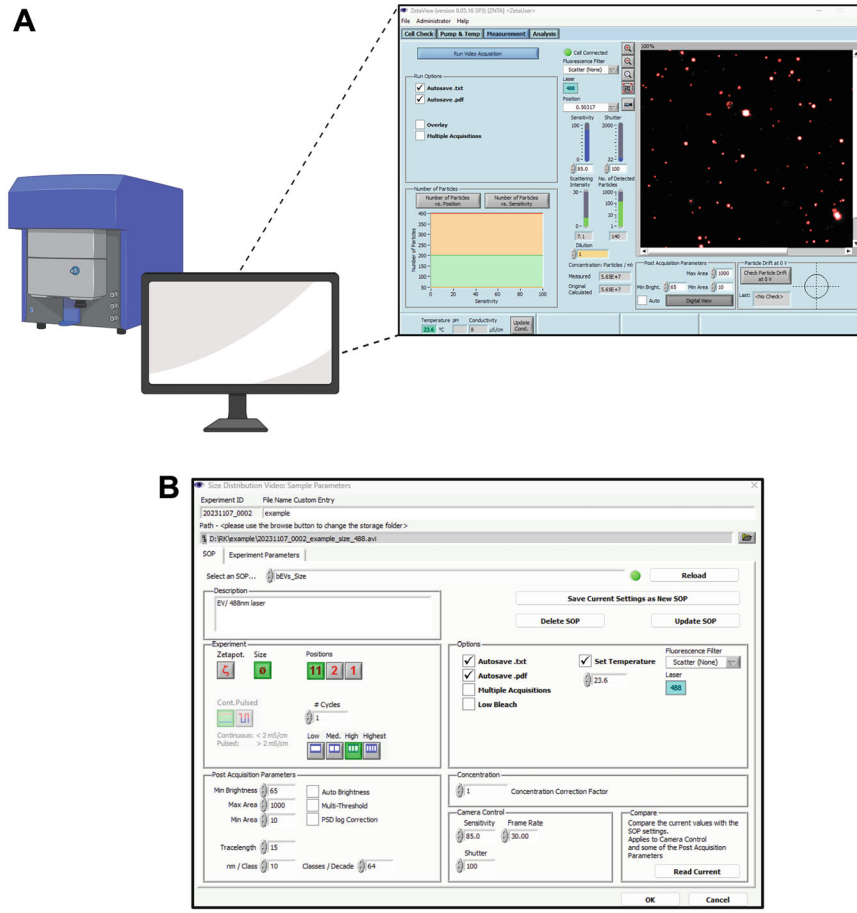

Fig. 2 Parameters used to calculate the concentration of bEVs on a Particle Metrix ZetaView. (**a**) The ZetaView measures particles at a dilution of 100–200 particles/field of view, typically ~10^7 particles/mL. Scattering intensity should be less than 8, indicated by a green bar in the measurement window. This can be tuned by changing the sensitivity or post-acquisition parameters. (**b**) The ZetaView offers control over acquisition and post-acquisition parameters, which may be tuned for specific needs. Settings commonly used in bEV analysis are shown here, for ZetaView Software version 8.05.16 SP3

Brownian motion of individual particles. The number of particles is averaged over the cell and used to calculate the concentration of particles in the sample. The ZetaView also measures size and ζ-potential. These measurements can be useful to confirm the presence of bEVs (and not other small particles, *see* **Note 13**) but these data are not necessary for multiple particle tracking.

7. Clean the instrument with 5–10 mL Rinsing Buffer between samples to prevent cross-contamination.

3.4 Fluorescently Labeling bEVs with Lipophilic Dyes

The methods here describe labeling bEVs using the PKH26 Red Fluorescent Cell Linker Kit (Millipore Sigma, PKH26GL, *see* **Note 14**) [14, 21]. Other commercially available lipophilic dyes may be used according to the manufacturer's instructions. Protein-binding dyes and specific antibodies have also been used to visualize bEVs using similar approaches.

1. Dilute sample to 1×10^{11} particles/mL with Dilution Buffer (*see* **Note 15**).
2. Combine 100 μL of diluted sample with 100 μL of PKH26 dye at 8 μM. Incubate at room temperature for 5 min.
3. Add 100 μL of Quenching Buffer. Incubate at room temperature for 1 min to bind excess dye.
4. Add 2 mL of Sucrose Buffer to an ultracentrifuge tube. Load the 300 μL sample on top of the Sucrose Buffer. Centrifuge for 100,000 rcf for 2 h at 4 °C. The Sucrose Buffer removes impurities from the sample. Impurities may be the same size as bEVs, but with different buoyant density, allowing for separation of pure bEVs. The labeled bEVs will be found in the resulting pellet.
5. Resuspend the bEV pellet in 100 μL of Dilution Buffer. Wash the labeled bEVs using centrifugal filter units. Add resuspended sample to filter and centrifuge at 2000 rcf for 10 min at 4 °C (*see* **Note 16**).
6. Quantify the concentration of the resulting labeled bEV sample (Subheading 3.3).

3.5 Multiple Particle Tracking

Multiple particle tracking (MPT) can assess particle mobility in biological matrices [13, 14]. MPT uses high-resolution fluorescent microscopy to track the movement of individual particles over time as described in the methods below.

1. Assemble 3D well slides using two layers of electrical tape. Apply tape to paper (*see* **Note 17**) and use a standard-sized hole punch to create a well in the tape. Cut tape to a 2 cm × 2 cm square and peel off paper. Apply electrical tape "well" to glass slide.
2. Using a Wiretrol or pipette, apply 20 μL of mucus or other biological matrix to well on slide. Ensure no bubbles in the matrix (*see* **Notes 18** and **19**).
3. Add 1.0–2.0 μL of particles to the well. Labeled samples should be ~10^{10} particles/mL (*see* **Note 20**, Fig. 3). Swirl gently to ensure that particles are well distributed in the matrix.
4. Use superglue to seal a coverslip on the well (*see* **Note 21**).

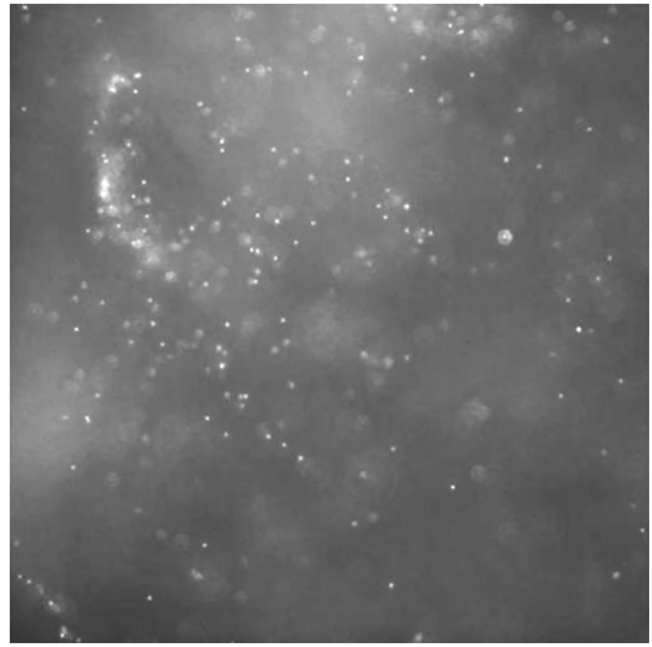

Fig. 3 Representative image of fluorescent particles in human cervicovaginal mucus. Particles should show good contrast with background. There should be ~200 particles per frame, with adequate spacing between particles so the image analysis software can distinguish between particle motion and multiple particles when tracking particle mobility frame-to-frame (*see* **Note 19**)

5. With a fluorescent microscope (e.g., Zeiss Axiovert) equipped with a 100× objective, record 5–8 videos ~20–30 s in length (*see* **Note 22**). Ensure that videos are taken within the sample (i.e., not on the top or bottom of the well—coverslip or slide) and that there is no convective motion in the area. Note the frame rate of the video for particle tracking analysis.

3.6 Quantifying Particle Mobility

Particle tracking software has been used across many fields to understand cellular migration, define microrheological properties of biofluids, and establish nanoparticle stability [9, 10]. Numbers 3–7 below are typically built into software but are described here to give users knowledge of the algorithms used to probe particle mobility in biological matrices. In general, the software will identify particles, establish particle trajectories, calculate the mean squared displacement of each particle, and use the ensemble average mean squared displacement to determine the diffusion coefficient of the particles. For understanding bEV mobility through biological matrices, diffusivity is likely the best numerical output for comparisons. See **Note 5** for tracker options.

1. If necessary, transfer videos from the microscope computer to a computer equipped with image analysis software. Using

ImageJ or another video viewing application, check the videos for convective motion. Make note of any videos or time frames that should not be tracked.

2. Different tracking software may request different user input. Typically, users should load the video or set of videos, and enter the particle size in pixels, the maximum frame-to-frame displacement in pixels, the video frame rate, and the number of frames to track (*see* **Note 23**). Other settings may include the minimum number of consecutive frames for a valid trajectory, and the number of frames a particle may disappear to still consider it the same particle.
3. Load videos and apply a spatial bandpass filter to remove objects much smaller or much larger than the size of the particles in the videos.
4. Resolve the true particles in the video by identifying brightness maxima. This step is typically iterative and relies on accurate user input for the size of particles and frame-to-frame displacement. Use these results to identify particle center, brightness, size, and deviation from circularity. Discard spots that are not identified as particles.
5. Track the particles across the length of the video. This step relies on an accurate user input for maximum displacement between frames. Tracking programs utilize linking algorithms such as those proposed by Crocker and Grier [22], Jaqaman and coworkers [23], and Sergé and coworkers [24]. Once tracking is complete, trajectory data will be output for each particle in the ensemble.
6. To calculate the effective diffusivity of particles in a biological matrix, the mean squared displacement (MSD) must first be determined (Fig. 4). The MSD is calculated as: $\langle \Delta r^2(\tau) \rangle = \langle \Delta x^2 + \Delta y^2 \rangle$, where r is the position of the particle and τ is the lag time of the trajectory. The lag time is an interval over which the particle moves. The changes in position over the coordinates x and y are squared and summed to account for motion in multiple directions. The brackets indicate an average of all squared displacements over the course of the video recording. For a single particle at a shorter lag time, more observations can be averaged together to calculate $\langle \Delta r^2(\tau) \rangle$. Multiple particle tracking allows for multiple particles in the same sample to be averaged together, resulting in the ensemble MSD: $\langle \langle \Delta r^2(\tau) \rangle \rangle$.
7. With the ensemble MSD, the diffusion coefficient of particles through a given biological matrix can be calculated via the relationship: $D_{\text{eff}} = \frac{\langle \Delta r^2(\tau) \rangle}{4\tau}$ or $\langle D_{\text{eff}} \rangle = \frac{\langle \langle \Delta r^2(\tau) \rangle \rangle}{4\tau}$. A histogram of individual particles may be generated using D_{eff}, whereas

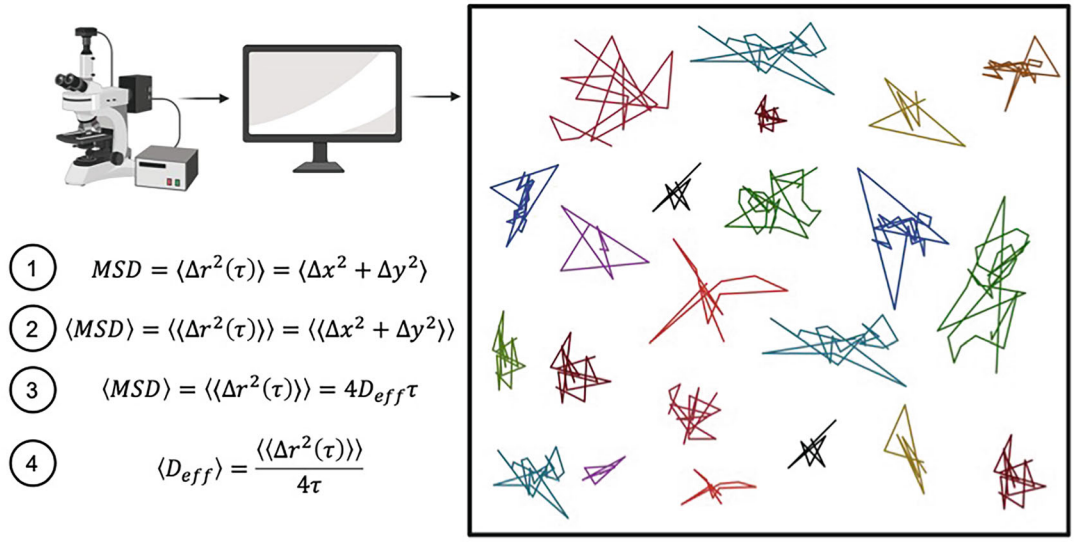

Fig. 4 Calculations used to convert individual particle mobilities into bEV diffusion through a biological sample. (1) The mean squared displacement (MSD) is calculated for each particle by summing the squares of the changes in both the x- and y-directions. (2) The ensemble mean squared displacement is calculated by averaging all of the MSD values for each particle tracked in the video. (3) The MSD is related to the effective diffusivity, (4) which can be calculated at a given lag time, τ

$\langle D_{\text{eff}} \rangle$ provides the average diffusivity for all tracked particles. Effective diffusivity can be used to compare the mobility of different particle types in a given matrix (*see* **Note 24**).

4 Notes

1. Media used for growing bacteria with the intention of collecting bEVs should be cleared of particles by ultracentrifugation at ≥100,000 rcf for 12 h prior to inoculation with bacteria. Centrifuged media should be sterile filtered using a 0.22 μm vacuum filter unit.
2. To prevent particle aggregation and adherence to microtubes, we suggest using protein LoBind tubes or a similar alternative [25].
3. Sample collection may be done in a variety of ways, depending on the biological matrix of interest. Many matrices will require Institutional Review Board approval, or clinical collaborators. Cervicovaginal mucus may be collected using a cervical brush or self-collected by donor using a SoftCup [12, 13]. Sputum may be centrifuged off ventilators. Alternatively, simulated vaginal fluid, hydrogels, or synthetic mucus may be used [14, 26]. Ex vivo particle tracking has been done in tissue slices [27, 28]. For this approach, samples should be ~1 mm thick

and particles injected into the tissue. Imaging setups should include proper environmental conditions to keep the tissue slices alive. In vivo particle tracking is also possible but involves more extensive setup to keep animals immobilized [29].

4. It may be appropriate to track bEVs at a physiologically relevant temperature. This requires a heated microscope enclosure.
5. There are several publicly available tracking programs. mPosTracker and u-track utilize MATLAB, RyTrack is based in IDL, MPTHub is based in Python.
6. Ultracentrifugation tubes should be filled to ≥85% of the tube maximum volume to avoid tube collapse during the ultracentrifugation cycle.
7. Samples of non-conditioned media should undergo the same isolation process as conditioned media to ensure that isolated particles are indeed bEVs produced by bacteria in culture.
8. Despite variable reports on the effects of freeze-thaws on EV concentration and stability, minimizing the number of freeze-thaws may help to preserve EV structure. Experiments should be planned accordingly [30, 31].
9. Commercially available columns are packaged with storage buffer. If planning to reuse a column, storage buffer can be saved and reused at the conclusion of bEV isolations.
10. Commercially available columns vary based on resin packing and column volume, which dictate the volume of the sample to be loaded. Follow the manufacturer's instructions for column usage.
11. When diluting the standard particles, it is recommended to first make a stock of particles diluted to 1:1000, followed by a 1:250 dilution for an accurate dilution.
12. The recommended loading for the ZetaView is 2 mL. However, when determining optimal dilutions, a smaller volume (~0.5 mL) can be loaded to determine if further dilutions are necessary. This helps to conserve low-volume samples. An optimal concentration is ~10^7 particles/mL (~50–200 particles per field of view, shown as a green bar on the "No. of Detected Particles" indicator in the measurement window).
13. Typical bEVs are ~100–300 nm in size and have a negative ζ-potential (surface charge). Transmission electron microscopy can also be used to confirm presence of bEVs in a sample.
14. The color of the labeling dye should be chosen such that the fluorophore does not interfere with autofluorescence in biological samples. Bright, photostable fluorescent dyes are optimal. In general, internal labeling is preferred, as it gives a better representation of particle mobility without modifications.

15. Some labeling kits rely on protein concentration. In this case, a bicinchoninic acid assay (BCA assay) may be used.
16. When using centrifugal filters, particles may become stuck to the filter. We suggest spinning at a relatively low speed and taking the time to carefully pipette particles off the filter after centrifuging to ensure total recovery of labeled bEVs.
17. When applying electrical tape to paper, it is often useful to use a laminated sheet or cardstock to prevent shredding of the paper as the electrical tape "well" is pulled from the paper's surface. If too much paper is left on the adhesive side of the electrical tape, the well may leak or be uneven and prevent proper sealing.
18. It is important that there are no bubbles in the tracking matrix. Bubbles may cause convective flow that will artificially alter mobility measurements.
19. If the sample for tracking is especially precious and/or has low volume, smaller-sized wells can be made to accommodate tracking in smaller volume samples. This may require that the dilution and/or volume of particles added to be adjusted.
20. Particle dilution should be ~10^9 per mL. If particles are too dense, the software will not be able to distinguish between multiple particles and particle motion. If particles are too dilute, quantification of particle motion will be difficult.
21. When sealing the slide, we suggest starting at one corner of the electrical tape well and outlining without lifting the tip of the superglue tube. This helps prevent dripping of the glue onto the sample. Additionally, ensure that the superglue does not leak into the well upon sealing, and that (*see* **Note 18**) there are no bubbles formed upon sealing the slide.
22. It is recommended to take multiple videos from different areas of the well to get a holistic image of how particles move through the biological matrix. This is especially important for heterogeneous samples, such as sputum, where particles may move more freely in one area of the sample as compared to another.
23. When giving input for particle size and maximum jump from frame-to-frame, pixel sizes are typically given as an odd number, allowing for the algorithm to find the center of the particle.
24. Multiple particle tracking can be used to probe sample characteristics, such as viscosity and pore size [10, 12, 13]. Calculating the particle effective diffusivity of bEVs through biological barriers provides a quantitative approach for comparing bEVs from different bacterial sources, with various modifications and understanding bEV mobility as a communication device and as a drug delivery vehicle.

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