Supplementary Materials for

A solution to the biophysical fractionation of extracellular vesicles: Acoustic Nanoscale Separation via Wave-pillar Excitation Resonance (ANSWER)

Jinxin Zhang et al.

Corresponding author: Luke P. Lee, lplee@bwh.harvard.edu; Tony Jun Huang, tony.huang@duke.edu

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Note S1: Brownian force and Pécel number

The Brownian force applied to a nanoparticle is given by

\[ F_B = \zeta \sqrt{\frac{12\pi r_p \mu k_B T}{\Delta t}} \]  

(1)

where \( k_B \) is the Boltzmann constant; \( T \) is the absolute temperature; \( \Delta t \) is the magnitude of the time step; \( \zeta \) is a Gaussian random number with zero mean and unit standard deviation \( (69) \). The Brownian force will encourage the particles to spread from high-density regions to the low-density region. The Pécel number, \( Pe \), can describe the relative importance of drag force and the Brownian force:

\[ Pe = \frac{V_t L}{D} \]  

(2a)

\[ D = \frac{k_B T}{6\pi \mu r_p}, \quad V_t = \frac{3 U_1^2}{8 c_0} \sin \left(4\pi \frac{x}{\lambda}\right) \]  

(2b)

where \( V_t \) is the terminal velocity, \( L \) is the characteristic length, \( D \) is the particle diffusion coefficient, \( r_p \) is the particle radius, \( U_1 \) is the bulk amplitude, \( c_0 \) is the speed of sound in the fluid, \( \lambda \) is the wavelength, \( x \) is the displacement \( (70) \). Here, we set the terminal velocity equal to the maximum streaming velocity \( (v_{2x}) \). The characteristic length was set as 10 \( \mu \)m, the spacing between the channel center and collection region boundary. \( v_{2x} \) can be calculated on the order of \( \mu \)m/s \( (70) \). We chose 5 \( \mu \)m/s here. Other parameters and their values are listed below:

- \( k_B \), the Boltzmann constant: \( 1.38 \times 10^{-23} \)
- \( T \), the absolute temperature: 293 K (20 °C)
- \( \mu \), the fluid viscosity: \( 1.002 \times 10^{-3} \text{ Ns/m}^2 \) (water at 20 °C)

For 100 nm polystyrene particles, the diffusion coefficient can be calculated as:

\[ D_{100nm} = 4.28 \times 10^{-1} \text{ m}^2/\text{s} \]

Choosing 10 \( \mu \)m as the characteristic length, the Pécel number can be calculated as:

\[ Pe_{100nm} \approx 23.36 \]

For 30 nm polystyrene particles, the diffusion coefficient is calculated as:

\[ D_{30nm} = 1.43 \times 10^{-1} \text{ m}^2/\text{s} \]

the corresponding Pécel number is:

\[ Pe_{30nm} \approx 7.0 \]

Similarly, the Pécel number for other particle sizes can be calculated by changing the size parameter (radius \( r_p \)). For sub-100 nm particles, the influence of Brownian force increases when the particle size decreases, but the drag force remains as the dominating force driving the movement of the nanoparticles.
**Note S2: Acoustic simulations**

Numerical simulations were performed using a FEM-based computational modeling software package, COMSOL Multiphysics 5.4, to solve the acoustic field distributions and acoustic streaming present in the fluid channel and analyze the nanoparticle movement under the influence of the acoustic radiation force (55-57).

The flow motion in the channel is governed by the Navier-Stokes equations:

\[
\begin{align*}
\frac{\partial \rho}{\partial t} + \nabla \cdot (\rho \mathbf{v}) &= 0 \\
\rho \frac{\partial \mathbf{v}}{\partial t} + \rho (\mathbf{v} \cdot \nabla) &= -\nabla p + \mu \nabla^2 \mathbf{v} + \left( \mu_b + \frac{1}{3}\mu \right) \nabla(\nabla \cdot \mathbf{v})
\end{align*}
\]

where \( \rho, \mu, \) and \( \mu_B \) represent the density, dynamic viscosity, and bulk viscosity of fluid, respectively; \( p \) and \( \mathbf{v} \) are the fluid velocity and pressure, respectively. By applying perturbation theory, the 1\(^{st}\) order equations governing the acoustic field and 2\(^{nd}\) order equations governing acoustic streaming can be deduced as (66):

\[
\begin{align*}
i\omega \rho_1 + \rho_0 \nabla \cdot (\mathbf{v}_1) &= 0 \\
\rho_0 i\omega \mathbf{v}_1 &= -c_0^2 \nabla \rho_1 + \mu \nabla^2 \mathbf{v}_1 + \left( \frac{1}{3}\mu + \mu_B \right) \nabla(\nabla \cdot \mathbf{v}_1) \\
\rho_0 \nabla \cdot \langle \mathbf{v}_2 \rangle &= -\nabla \cdot \langle \rho_1 \mathbf{v}_1 \rangle \\
-\nabla \langle p_2 \rangle + \mu \nabla^2 \langle \mathbf{v}_2 \rangle + \left( \frac{1}{3}\mu + \mu_B \right) \nabla(\nabla \cdot \langle \mathbf{v}_2 \rangle) &= \rho_0 \langle j\omega \mathbf{v}_1 \rangle + \rho_0 \langle (\mathbf{v}_1 \cdot \nabla) \mathbf{v}_1 \rangle
\end{align*}
\]

In the 1\(^{st}\) order equations (equation (5) and (6)), \( c_0, \rho_0, \mu, \) and \( \mu_B \) are the speed of sound in the fluid, the density, shear viscosity, and bulk viscosity of fluid, respectively; \( \rho_1 \) and \( \mathbf{v}_1 \) are the first order density and velocity. The acoustic pressure can be obtained using \( p_2 = c_0^2 \rho_1 \). In the 2\(^{nd}\) order equations (equation (7) and (8)), the brackets \( \langle \cdot \rangle \) indicates a time average over a harmonic oscillation period; \( p_2 \) is the second order pressure and \( \mathbf{v}_2 \) is the second order velocity. The time-averaged second order velocity is physically the acoustic streaming velocity. In our device, the vibration velocity of the channel floor induced by the standing SAW propagates in the y-direction and can be expressed as

\[
\begin{align*}
v_y &= \varepsilon A \omega \left\{ e^{i(\omega t - k y)} - e^{i(\omega t - k(y_0 - y) - \alpha(y_0 - y))} \right\} \\
v_z &= -i A \omega \left\{ e^{i(\omega t - k y)} - e^{i(\omega t - k(y_0 - y) - \alpha(y_0 - y))} \right\}
\end{align*}
\]

where \( \varepsilon \) is the ratio between longitudinal and transverse vibrations, \( A \) is the transverse vibration amplitude of the substrate, \( \alpha \) is the decay coefficient of the vibration along the direction of the wave propagation, \( k \) is the wave number of the standing SAW, and \( y_0 \) is the width of the fluid domain in y direction. In the COMSOL simulation package, the “Thermoviscous Acoustics” physics module was applied to solve the 1\(^{st}\) order equations. Equation (9) and (10) were applied as the velocity at the bottom of the fluid, as the boundary conditions. The impedance of the PDMS was applied to the sides and top of the channel using the “Impedance” boundary condition. The model was solved using a “Frequency Domain” solver at the resonant frequency of the device. The “Laminar Flow” physics model was applied to solve the 2\(^{nd}\) order equations and solved for a
“Stationary” case. The “No slip” wall condition is applied to all the channel walls. More details of this simulation model can be found here (71).

Note S3: Input power evaluation

The input power can be evaluated as

\[ P = \frac{V_{\text{rms}}^2}{Z} \]

Here \( V_{\text{rms}} \) is the RMS voltage in AC signal, and \( Z \) is the impedance. \( V_{\text{rms}} \) is related to the input voltage \( V_{\text{pp}} \), as shown below,

\[ V_{\text{rms}} = \frac{V_{\text{peak}}}{\sqrt{2}} = \frac{V_{\text{pp}}/2}{\sqrt{2}} = \frac{V_{\text{pp}}}{2\sqrt{2}} \]

Therefore, the input power can be written as:

\[ P = \frac{V_{\text{rms}}^2}{Z} = \frac{V_{\text{pp}}^2}{8Z} \]

The impedance can be measured and then the input power can be evaluated. Here, the impedance of our device is around 450Ω.
Fig. S1. ANSWER device design. (A) Schematic of the ANSWER chip layout. (B) The original image from which the inset of Fig. 1a was obtained, showing the virtual wave-pillar distribution. Scale bar, 100 μm. (C) Time-lapse stacked images showing that 2 μm polystyrene particles are pushed to the two sides of the fluid microchannel due to wave-pillar excitation resonance. The white arrows indicate the flow direction. Scale bar, 40 μm.
Fig. S2. Two-dimensional virtual wave-pillar generation via one pair of IDTs in ANSWER. (A) Numerical calculations show the Gor’kov potential (acoustic pressure) distribution in a pillar shape. (B) Microscope images showing 2 μm polystyrene particle patterns in a “DUKE” shaped channel with different widths ranging from 60 μm to 30 μm under the channel aligned SAW. Because of the interaction between the acoustic waves and the thin PDMS channel, surface acoustic waves can be aligned to generate pressure nodes close to the channel walls even if they are not straight. Scale bar: 100 μm. Channel width for “D”, “U”, “K”, “E” is 60 μm, 50 μm, 40 μm, and 30 μm, respectively.
Fig. S3. Numerical calculation for excitation resonance effect. (A) Numerical simulations showing the relationship between equivalent impedance and the thickness of the PDMS wall. The equivalent impedance is influenced by the resonance of the PDMS. The equivalent impedance $Z_{eq}$ was calculated for increasing PDMS thicknesses in a wider range (from 15 μm to 60 μm). The first valley (I) corresponds to a resonance mode when the PDMS thickness is closest to one wavelength ($\lambda$). When the thickness increases to $1.5\lambda$ and $2.0\lambda$, the $Z_{eq}$ has second (II) and third (III) resonance modes. Therefore, when the PDMS wall thicknesses are in integer multiples of half wavelength, the resonance modes will result in a local minimum value. The $Z_{eq}$ will increase and drop between two resonance modes. (B) Simulation of the excitation resonance frequency of the ANSWER system. The resonance frequency is obtained by calculating the eigenmodes of the system.
**Fig. S4. Modulating the virtual wave-pillar to control the cut-off size.** (A) Illustration of the modulated virtual wave-pillar under increasing acoustic pressure used for separating different sEV subpopulations. $P_a$: acoustic pressure. (B) Numerical results showing dynamic control of the separation cut-off size, achieved by adjusting the acoustic pressure.
Fig. S5. Numerical simulation results showing the acoustic pressure distribution and streaming in different channel planes. (A) In the $z$-$y$ plane, acoustic pressure and streamline distribution at different $x$ slices. Channel width is 40 μm, $0 \leq x \leq 40$ μm. (B) In the $x$-$y$ plane, acoustic pressure and streamline distribution at different $z$ slices. Channel height is 25 μm, $0 \leq z \leq 25$ μm. (C) In the $z$-$x$ plane, acoustic pressure and streamline distribution at one virtual wave-pillar center’s cross-section. The white lines are streamlines. The black arrows point the direction of acoustic radiation force. (D) The acoustic pressure distribution inside the channel most closely resembles a series of concentric cylinders, although the actual shape of the acoustic pressure distribution is far more complex, as shown in (A-C).
Fig. S6. Comparison of the acoustic patterns and acoustic streaming effects in static flow and continuous flow. In static flow (i.e., the flow rate is zero): (A) streamline distribution when acoustic pressure is applied, the streaming is observed in the $y$ direction. (B) 2 μm and (C) 400 nm polystyrene particle distribution when the acoustic field is applied. The movement of 2 μm particles is dominated by the acoustic radiation force, while the movement of 400 nm particles is dominated by the acoustic streaming. These experimental results are in good agreement with the simulation results. In continuous flow: (D) streamline distribution when acoustic pressure is applied, the effect of streaming is removed from the flow in the $y$ direction. (E) 2 μm and (F) 400 nm polystyrene particles distribution when acoustic pressure is applied. In continuous flow, 400 nm particles can be pushed to the two sides via acoustic radiation force due to the absence of acoustic streaming. Scale bar in (B) and (E), 100 μm. Scale bar in (C) and (F), 20 μm.
Fig. S7. Photomicrographs showing the change in 190 nm polystyrene particle distribution as the particles are iteratively separated through the ANSWER device. The 190 nm polystyrene particles were loaded from the center of the channel inlet while sheath flow was loaded from two side inlets. From upper left to the lower right, the particles iterate through around 1050 virtual wave-pillars. As they flow through the chip, the 190 nm particles are moved to two sides at the outlet. Scale bar, 100 μm.
Fig. S8. Photomicrographs showing the changes in 100 nm polystyrene particle distribution as the particles are iteratively separated through the ANSWER device. The 100 nm polystyrene particles were loaded from the center of the channel inlet while sheath flow was loaded from two side inlets. From upper left to the lower right, the particles iterate through around 1050 virtual wave-pillars. As they flow through the chip, the 190 nm particles are moved to two sides at the outlet. Scale bar, 100 μm.
Fig. S9. Photomicrographs showing the changes in 50 nm polystyrene particle distribution as the particles are iteratively separated through the ANSWER device. The 50 nm polystyrene particles were loaded from the center of the channel inlet while sheath flow was loaded from two side inlets. From upper left to the lower right, the particles iterate through around 1050 virtual wave-pillars. As they flow through the chip, the 50 nm particles are moved to two sides at the outlet. Scale bar, 100 μm.
Fig. S10. Photomicrographs showing the changes in 30 nm polystyrene particle distribution as the particles are iteratively separated through the ANSWER device. The 30 nm polystyrene particles were loaded from the center of the channel inlet while sheath flow was loaded from two side inlets. From upper left to the lower right, the particles iterate through around 1050 virtual wave-pillars. The particles were always in the middle of the channel and did not get pushed to the channel sidewalls. Scale bar, 100 μm.
Fig. S11. **Modulated wave-pillar amplitude via frequency tuning.** Numerical computation of the acoustic pressure distribution across the center cross-section of the virtual wave-pillar (inset) under different frequencies centered around the resonance frequency. The right figure shows a zoomed-in view of the virtual pillar pressure distribution (left red box).
Fig. S12. Nanoparticle separation quality as a function of input power. Numerically calculated (A) yield and (B) purity for four different sized particles changes with increased input power. 

\[ \text{Yield} = \frac{\text{collected particles with this size}}{\text{all the particles with this size}} \]

\[ \text{Purity} = \frac{\text{collected particles with this size}}{\text{all the collected particles}} \]

The input power is characterized by the substrate vibration amplitude. Specifically, 1× input power is defined as the power required to generate a substrate vibration amplitude of 0.5 nm. (C) Experimental verification of selectively isolating nanoparticles via adjusting the input power. The purity of different nanoparticle size ranges changes with increased input voltage.
Fig. S13. Size distributions collected from TEM images obtained from three separation results.
Fig. S14. The relationship between input power and nanoparticle separation cut-off size.
| Parameter                        | Symbol  | Value                        |
|---------------------------------|---------|------------------------------|
| Density of the water            | $\rho_{\text{water}}$ | 997 kg/m$^3$                |
| Density of the PBS              | $\rho_{\text{PBS}}$   | 1040 kg/m$^3$               |
| Shear viscosity of the water    | $\mu_{\text{water}}$ | $1 \times 10^{-3}$ Pa $\cdot$ s |
| Shear viscosity of the PBS      | $\mu_{\text{PBS}}$   | $0.9 \times 10^{-3}$ Pa $\cdot$ s |
| Amplitude of substrate vibration| $A_m$   | 0, 0.5, 1, 1.5, 2 nm         |
| Frequency of the SSAW           | $f$     | 50.1, 50.3, 50.5, 50.7, 50.9 MHz |
| Density of polystyrene particles| $\rho_p$ | 1050 kg/m$^3$                |
| Diameter of polystyrene particles| $d_p$  | 30, 50, 100, 190 nm          |
**Movie S1:** Visualization of acoustic virtual wave-pillars via 2 μm polystyrene particle patterns in the continuous flow.

**Movie S2:** Visualization of acoustic streaming influence via 0.4 μm polystyrene particle patterns in the static flow.

**Movie S3:** Removal of the acoustic streaming effect in continuous flow verified via 0.4 μm polystyrene particle patterns.

**Movie S4:** 190 nm polystyrene particle distribution changes as they iterate through the ANSWER device.

**Movie S5:** 100 nm polystyrene particle distribution changes as they iterate through the ANSWER device.

**Movie S6:** 50 nm polystyrene particle distribution changes as they iterate through the ANSWER device.

**Movie S7:** 30 nm polystyrene particle distribution changes as they iterate through the ANSWER device.
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