

Optimizing the quality of acoustophoretic separation by the in-flow mobility-ratio method

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Achieving good acoustic particle or cell separation performance requires skilled operators, who must carefully fine-tune the input parameters (actuation voltages, flow rates, and flow split ratios). Often the fine-tuning is done by time-consuming parameter sweeps, which are tedious and expensive. Here, we present a straightforward model-based approach to determining input parameters that yield optimal separation performance. The optimal parameters are a function of the device performance, the material properties of the separation species (compressibility, density, size), and the properties of the buffer (compressibility and density). The device performance is assessed by a calibration step, while the material properties are combined into the mobility ratio and measured by the in-flow mobility-ratio method, which is introduced in this work. The optimal separation settings are validated by separating green fluorescent 7.8- μm particles from red fluorescent 4.9- μm particles.

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I. INTRODUCTION

Acoustophoresis—the manipulation of micrometer-sized cells and particles by means of acoustic waves—is a promising tool in separation science, biomedical engineering, and cell handling. Acoustophoresis separation has been successful in separating cell mixtures into their sub-components. The technology can, for example, be applied for the separation of leukocyte subpopulations [1], the isolation of circulating tumor cells [2–4], reducing white blood cell background in cancer cell separation products [5], the separation of platelets from whole blood [6], and the separation of neuroblastoma cells from hematopoietic progenitor cell products [7].

The term “separation” denotes separating a mixture of two species into center and side-outlet channels of the separation device. The separation principle is based on the difference in acoustic mobility between the two species. The acoustically induced particle migration speed is proportional to the acoustic mobility ratio. The acoustic mobility ratio is a function of a particle’s compressibility, density, and size. Particles or cells of different acoustic mobility can be separated, while passing through an

acoustic force field, by time-of-flight methods. In the literature good separation parameters (flow rate, actuation voltages, flow-rate split ratios) are usually determined by manually scanning through a range of experimental settings while measuring separation outcome by, for example, flow cytometry [1,2,7–9]. Those approaches are inefficient, time-consuming, and do not allow a novice user to achieve a separation quality comparable to that of an expert operator.

Furthermore, as far as we know, no optimality criterion has ever been defined for an acoustophoretic separation, nor has any group attempted to show that their selected settings were optimal.

Here, we present a complete model-based framework to determine optimal separation parameters. To this end we define an optimality criterion and develop a theory to model the particle trajectories within the separation device. The model is combined with a physical interpretation of the optimality criterion and used to predict optimal settings, which are validated experimentally. Our approach can be applied using a fluorescence microscope only and does not need any flow cytometry measurements to determine the optimal settings. (Note that the flow cytometer results presented in the underlying work were performed for validation purposes.)

As an optimality criterion, we introduce the notion of separation quality (SQ) and show experimentally that the in-flow mobility-ratio method, presented herein, yields optimal separation quality. The separation quality is defined as the minimum between the fractions of species

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collected at their target outlets. The fraction of a species collected at their target outlet is in some works referred to as separation efficiency; see Lee *et al.* [10]. The separation quality is formally given by

$$SQ = \min \left(\frac{\#_{ct}^I}{\#_{sd}^I + \#_{ct}^I}, \frac{\#_{sd}^{II}}{\#_{sd}^{II} + \#_{ct}^{II}} \right), \quad (1)$$

where $\#^I$ and $\#^{II}$ denote the number of species I (high acoustic mobility) and II (low acoustic mobility), respectively, collected at the side (sd) and center (ct) outlets. A perfect separation will yield a separation quality of 100%. A separation quality of 0% indicates a failed separation in which both species/particle types were collected at the same outlet. The separation quality is a symmetric measure, and a maximal (i.e., optimal) separation quality implies that both fractions are equal.

Three free parameters occur in the model of the particle trajectories: the inlet particle positions, the acoustic performance of the device, and the mobility ratio. The particle position at the device inlet can be measured experimentally. The device performance is assessed by a calibration experiment based on focusing micrometer-sized polystyrene reference particles, as presented by Baasch *et al.* [11]. Therefore, the biggest challenge is to determine the particle and buffer properties in an efficient manner.

The close connection between optimal separation conditions and material properties of the separated species is well known in the literature [12–14]. Unfortunately, so far, no solution to measure the relevant material properties in a fast and reliable way has been presented in the literature. Only recently was the notion of mobility ratio and its fundamental relevance for acoustic separations described by Edthofer *et al.* [15]. Therefore, a major challenge in optimized acoustophoresis is the ability to measure the mobility ratio of two species based on a fast reference experiment. Such an experiment needs to be sufficiently simple and robust that it can be applied to small sample volumes as most cells of interest are rare and/or expensive. One approach to determining the mobility ratio, which would be suboptimal, is to compute the respective acoustic mobilities and their ratio based on separate compressibility, density, and size measurements. This suboptimal approach is time-intensive, requires large sample volumes, and requires recombining the parameters, which amplifies uncertainties and decreases accuracy.

This challenge is overcome by combining the material properties of the cells and reference particles (density, compressibility, size) into a single relative parameter (mobility ratio). As the mobility ratio is a relative measure of material properties, no exact knowledge of any of the reference particle or cell properties (density, compressibility, size) is required at any point of the optimal separation procedure. In the in-flow mobility-ratio method

presented here, the reference and target particles are introduced simultaneously in the separation chip. This increases the robustness of the mobility-ratio method with respect to variations in the experimental conditions over time, such as changes in temperature, as well as flow rates and applied voltages. The in-flow mobility-ratio method, demonstrated herein on polystyrene particles, is extremely fast and allows the mobility ratio of one species with respect to a reference particle to be determined in less than 2 h. This opens the door to measuring the mobility ratio and running optimized separation experiments on the same day.

After introducing the underlying theory of in-flow mobility-ratio measurements, we show how the mobility ratio and calibration experiments are performed to determine optimal separation parameters. We prove experimentally that the predicted settings yield optimal separation quality for the separation of fluorescent 4.9- μm - from 7.8- μm -diameter particles. The separation quality is assessed by flow cytometry.

II. METHODS

A. Experimental setup

The microfluidic chip, sketched in Fig. 1, was manufactured by Micronit BV, Enschede, The Netherlands, and previously used to investigate inertial effects in microfluidics [16]. The channels are deep reactive ion etched into silicon and covered by a glass lid. The chip consists of a prefocusing channel (length, width, and height of 2.6 cm $\times$ 300 μm $\times$ 150 μm), a main channel (length $\times$ width $\times$ height of 4.3 cm $\times$ 375 μm $\times$ 150 μm), an inlet flow splitter, and an outlet flow splitter.

Two piezoelectric ceramic transducers (PZTs) drive the acoustic standing waves in the prefocusing and separation channel, respectively. The PZT driving the separation chamber is actuated at a frequency between 1.9 and 2.0 MHz and a peak-to-peak voltage U_{sep} . The prefocusing channel is actuated between 4.8 and 4.9 MHz and a peak-to-peak voltage U_{pref} . The actuation is controlled by a function generator (Tektronix AFG3022B) and amplified by an amplifier constructed in-house. The electric input signals are monitored by an oscilloscope (Tektronix TDS1002).

The flow through the chip is regulated by a pressure system (Festo, VEMA 563303 valve terminal) and flow sensors (Sensirion liquid flow meter SLI 1000). The flow control system is operated via a LABVIEW script developed in-house. The center in-flow can be tuned to push the sample, which enters from the side inlets, further away from the channel center. This results in a larger lateral separation distance, which improves the separation quality; see Fig. 1. In the prefocusing channel the acoustic radiation force focuses the particles in two dimensions. The prefocusing step, achievable either acoustically or hydrodynamically [17], is essential to achieve high separation performance as it forces the particles or cells to enter the main separation

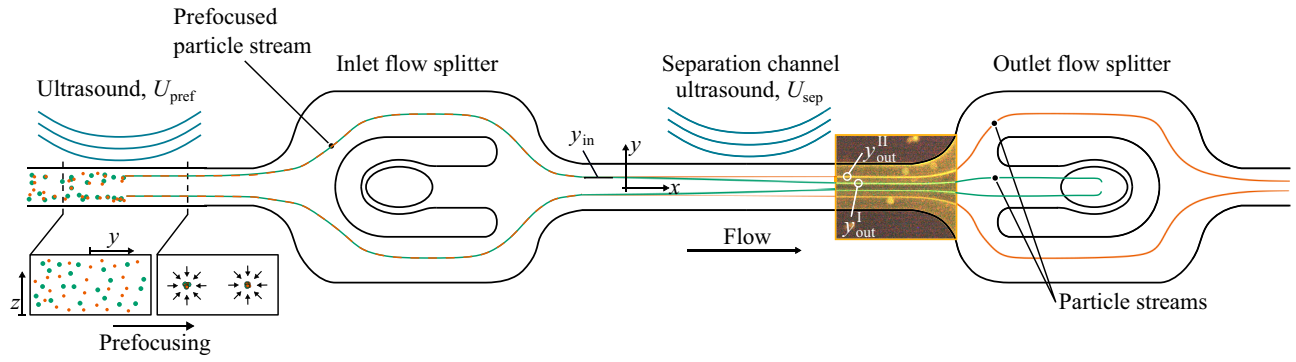


FIG. 1. The microfluidic chip consists of four connected sections: the prefocusing unit, the inlet flow splitter, the separation channel, and the outlet flow splitter. A standing wave can be established in both the prefocusing and separation channel by actuating the prefocusing or separation channel PZT, respectively. Upon entering the prefocusing channel the particles (or cells) are focused into thin bands. The particles (or cells) enter the separation channel at a well-defined lateral position y_{in} . In the separation channel they are pushed towards the center by the acoustic radiation force. Here, the species of high acoustic mobility (I) will be pushed further into the center and separated from the species of low acoustic mobility (II). The lateral positions of species I and II close to the outlet flow splitter are denoted by y_{out}^I and y_{out}^{II} , respectively.

channel as two thin bands at well-defined positions. After the prefocusing channel the particles flow into the separation channel where the acoustic radiation force pushes them towards the pressure nodal line in the center. We focus our analysis on the dynamics of the particles in the separation channel and assume that the prefocusing is always active.

The channel is imaged by a microscope (Olympus SZX16) and a digital microscope camera (Olympus XC10). A broad-spectrum light source (X-Cite 120Q, Lumen Dynamics) provided the lighting of the channel. A Peltier controller (TC0806- R232, CoolTronic) monitored the temperature of the main channel PZT. The particle counts for the separation quality were measured by flow cytometry (FACSCanto II, BD Biosciences, USA).

B. Separation principle and optimality criterion

The acoustophoresis separation chip is sketched in Fig. 1 and consists of a prefocusing channel, an inlet flow splitter, a separation channel, and an outlet flow splitter. This chip design has been extensively used for acoustophoresis separations in the literature [7,9,18–21]. An acoustophoresis separation aims at separating a high-mobility species (I) from a low-mobility species (II). This is done by prefocusing the species to be confined within a thin stream tube, whose geometry we approximate by the center streamline. That way both species enter the separation channel at the same height and lateral position y_{in} . In the main channel, the $\lambda/2$ mode is excited and the species are pushed towards the channel center by acoustic forces. If all parameters are correctly tuned, the high-mobility species exits through the center outlet, while the low-mobility species exits through the side outlet, which concludes the separation. The particle positions close to the outlet flow splitter of the high- and low-mobility species are annotated by y_{out}^I

and y_{out}^{II} , respectively. Due to the symmetry of the device we define, without loss of generality, y_{in} , y_{out}^I , and y_{out}^{II} to be positive numbers and situated in the upper half of the device.

Tuning the separation parameters is, however, challenging and several input parameters need to be determined to run a separation at maximal separation quality: the total flow rate, the separation channel actuation voltage, and the ratio between side and center flow at the inlet and outlet flow splitters. Fortunately, three of the parameters can be determined independently. The total flow rate is determined by the required sample flow rate. The flow rate through the side inlet is given by the need to push the prefocused particle streams sufficiently far from the channel center. The prefocusing voltage is tuned such that good focusing is achieved while keeping the voltage as low as possible to limit heating of the device. Therefore, the optimization problem reduces to finding the optimal flow-rate split ratio at the outlet flow splitter and separation channel voltage U_{sep} .

The underlying idea, sketched in Fig. 2, is to select the actuation voltage such that the maximal gap between the species is achieved at the channel outlet. The outlet splitting ratio is then tuned such that the lateral position y_{stagn} of the stagnation streamline, which splits the flow at the outlet into the fractions that exit via the side and center outlets, is positioned exactly in the middle between the two target species' lateral outlet positions (y_{out}^I and y_{out}^{II}). Again, due to the symmetry of the device, we define, without loss of generality, y_{stagn} to be positive numbers and situated in the upper half of the device. The optimal settings will be computed based on the theory presented herein, the mobility ratio between the species and a calibration particle, and a calibration step. The latter is required to compensate for inherent day-to-day experimental variations.

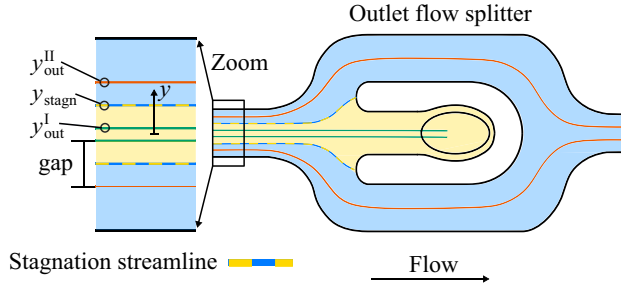


FIG. 2. Sketch of the optimal situation at the outlet flow splitter. The species of high (green) and low (orange) acoustic mobility are located at y_2^I and y_2^{II} , respectively. At optimal conditions the gap g_p between them is maximal and the flow-rate split ratio at the outlet flow splitter is tuned such that the lateral position of the stagnation streamline y_{stagn} is located in the middle between y_2^I and y_2^{II} .

C. Theory and modeling

Although the following analysis assumes acoustic forces, the method and mathematical procedure presented are general and can be applied analogously to any separation procedure in which prefocused particles are separated by a force field orthogonal to the direction of the flow. The motion of a single particle inside the separation chamber is driven by the acoustic radiation force, the acoustic streaming, and the flow along the channel. In the separation channel a one-dimensional acoustic standing wave of amplitude p_a is assumed, formally given by

$$p = p_a \sin(ky) \cos(\omega t), \quad (2)$$

where we introduced the wave number k , the angular frequency ω , and time t .

To derive the motion of the particle, let us first consider the forces acting on the particle. Both the Stokes' drag force $\mathbf{F}_d$ and the acoustic radiation force $\mathbf{F}_a$ act on the particle. According to Newton's second law, we have

$$m\ddot{\mathbf{r}} = \mathbf{F}_d + \mathbf{F}_a, \quad (3)$$

where m and $\ddot{\mathbf{r}}$ denote the mass and the acceleration of the particle, respectively. Baasch *et al.* [22] have shown that the inertial term is negligible in typical acoustofluidics systems. The Stokes drag force is, in our case, given by

$$\mathbf{F}_d = 6\pi\eta a(\mathbf{v}_{\text{str}} + \mathbf{v}_{\text{bg}} - \mathbf{v}_p), \quad (4)$$

where we introduced the streaming velocity $\mathbf{v}_{\text{str}}$, the background hydrodynamic flow field $\mathbf{v}_{\text{bg}}$, the particle velocity $\mathbf{v}_p$, the fluid viscosity η , and the particle radius a . Plugging

the Stokes drag force into the equation of motion yields

$$0 = \mathbf{F}_d + \mathbf{F}_a, \quad (5)$$

$$\mathbf{v}_p = \mathbf{v}_{\text{str}} + \mathbf{v}_{\text{bg}} + \mathbf{v}_{\text{ac}}, \quad \mathbf{v}_{\text{ac}} = \frac{\mathbf{F}_a}{6\pi\eta a}, \quad (6)$$

where contributions to the particle velocity are split up into the contributions due to the hydrodynamic background flow ($\mathbf{v}_{\text{bg}}$), the acoustic streaming ($\mathbf{v}_{\text{str}}$), and the acoustic radiation force, the so-called acoustic migration velocity ($\mathbf{v}_{\text{ac}}$). In our case, the only nonzero component of the acoustic migration velocity ($\mathbf{v}_{\text{ac}}$) is the component along the y -direction (v_{ac}^y).

Note that Eq. (5) shows that without the presence of other fields, the Stokes drag force is always equal to the acoustic radiation force. However, the respective contributions of the acoustic radiation force and the acoustic streaming to the particle velocity are not the same. Therefore Eq. (6), should be referenced to discuss if the motion of a particle is dominated by acoustic streaming or the acoustic radiation force.

For a particle of acoustic contrast factor Φ , density ρ_p , compressibility κ_p , and placed at y_p within the pressure field given by Eq. (2), the acoustically induced radiation force (in the y -direction) is given by [23–25]

$$F_a^y = -4\pi\Phi ka^3 E_{\text{ac}} \sin(2ky), \quad (7)$$

$$E_{\text{ac}} = \frac{p_a^2}{4}\kappa_0, \quad \Phi = \frac{1}{3} \left[\frac{5\bar{\rho} - 2}{2\bar{\rho} + 1} - \bar{\kappa} \right], \quad (8)$$

$$\bar{\kappa} = \frac{\kappa_p}{\kappa_0}, \quad \bar{\rho} = \frac{\rho_p}{\rho_0}, \quad (9)$$

where we introduced the acoustic energy density E_{ac} , the medium density ρ_0 , and the medium compressibility κ_0 .

The corresponding acoustic migration velocity of the particle is then obtained by combining Eqs. (6) and (8):

$$v_{\text{ac}}^y = A m_{\text{ac}} \sin(2ky_p), \quad (10)$$

$$A = -\frac{2}{3} \frac{k}{\eta} E_{\text{ac}}, \quad m_{\text{ac}} = \Phi a^2, \quad (11)$$

where we introduced the acoustic mobility (m_{ac}) of a particle.

The acoustic streaming in a chamber of rectangular cross section of height h and width w is according to Rayleigh [26] given by

$$v_{\text{str}}^y = -\frac{3}{4} \frac{E_{\text{ac}}}{\rho_0 c} \sin(2ky) \left(1 - 12 \frac{(z - h/2)^2}{h^2} \right), \quad (12)$$

$$v_{\text{str}}^z = -\frac{3}{4} \frac{E_{\text{ac}}}{\rho_0 c} kh \cos(2ky) \left(\frac{(z - h/2)^3}{(h/2)^3} - \frac{z - h/2}{(h/2)} \right), \quad (13)$$

where v_{str}^y and v_{str}^z denote the streaming velocities in the y - and z -direction respectively, and c denotes the speed of sound.

If the particle is levitated in the mid-height of the rectangular separation chamber, which is true for the prefocused particle streams, then the inclusion of the acoustic streaming contribution according to Rayleigh [26] is straightforward [27,28]. In this case, the only nonzero component of the streaming velocity $\mathbf{v}_{\text{str}}$ is the component in the y -direction (v_{str}^y) and we have

$$v_p^y = v_{\text{ac}}^y + v_{\text{str}}^y = Ac_{\text{str}}m_{\text{ac}} \sin(2ky_p), \quad (14)$$

$$c_{\text{str}} = 1 + \alpha_{\text{str}}, \quad (15)$$

$$\alpha_{\text{str}} = \frac{9}{16} \left(\frac{\delta}{a} \right)^2 \frac{1}{\Phi} = \frac{9}{16} \frac{\delta^2}{m_{\text{ac}}}, \quad (16)$$

where we introduced the particle velocity along the y -direction v_p^y , the viscous boundary layer thickness $\delta = \sqrt{2\eta/\rho\omega}$, the streaming correction coefficient c_{str} , and the dimensionless streaming contribution number α_{str} . A small or large streaming contribution number α_{str} indicates a motion dominated by the acoustic radiation force or the streaming, respectively.

According to Bruus [29], the hydrodynamic flow in the channel $\mathbf{v}_{\text{bg}}$ has only a component along the channel length (x), which we denote by v_{bg}^x . In a channel of rectangular cross section of width w and height h , with $w > h$, the background flow, and in consequence the particle velocity along the x direction v_p^x , is given by [29]

$$v_p^x = v_{\text{bg}} = Bs(y_p, z_p), \quad (17)$$

$$B = \frac{48Q}{\pi^3(hw)(1 - 0.63(h/w))}, \quad (18)$$

$$s(y_p, z_p) = \sum_{n=\text{odd}}^{\infty} \left(\frac{1}{n^3} \left[1 - \frac{\cosh\left(\frac{n\pi y_p}{h}\right)}{\cosh\left(\frac{n\pi w}{2h}\right)} \right] \right) \sin\left(\frac{n\pi z_p}{h}\right), \quad (19)$$

where Q denotes the total volumetric flow rate ($\text{m}^3 \text{s}^{-1}$). Here the y - and z -coordinates denote the positions along the width w and height h of the channel cross section, respectively. The coordinates x_p , y_p , and z_p denote the particle's x , y , and z positions, respectively. The center of the channel cross section is placed at $y = 0$ and $z = h/2$.

The trajectory of one particle can then be obtained by dividing Eq. (17) by Eq. (14), yielding a separable ordinary differential equation,

$$\frac{v_p^x}{v_p^y} = \frac{dx_p/dt}{dy_p/dt} = \frac{dx_p}{dy_p}. \quad (20)$$

The last term can be expanded to

$$\frac{dx_p}{dy_p} = \frac{B}{Ac_{\text{str}}m_{\text{ac}}} \frac{s(y_p, z_p)}{\sin(2ky_p)}, \quad (21)$$

which can be integrated to

$$\int_{x_{\text{in}}}^{x_{\text{out}}} \frac{Ac_{\text{str}}m_{\text{ac}}}{B} dx = \int_{y_{\text{in}}}^{y_{\text{out}}} \frac{s(y_p, h/2)}{\sin(2ky_p)} dy_p. \quad (22)$$

The integral can be simplified to

$$\frac{Am_{\text{ac}}c_{\text{str}}}{B} \Delta x = \int_{y_{\text{in}}}^{y_{\text{out}}} \frac{s(y_p, h/2)}{\sin(2ky_p)} dy_p = f(y_{\text{in}}, y_{\text{out}}), \quad (23)$$

where we introduced $\Delta x = x_{\text{out}} - x_{\text{in}}$ and the function $f(y_{\text{in}}, y_{\text{out}})$ to simplify the notation. In the following we will assume that the system behaves linearly and that the acoustic energy is proportional to the squared input voltage, $E_{\text{ac}} \propto (U_{\text{sep}})^2$. Equation (23) allows the trajectory, that is, all pairs $(y_{\text{in}}, y_{\text{out}})$, of one particle I collected at some actuation voltage ($U_{\text{sep}}^{\text{I}}$) and total flow rate Q^{I} to be related to the trajectory of another particle II introduced at another total flow rate Q^{II} and PZT actuation voltage $U_{\text{sep}}^{\text{II}}$ via

$$\left(\frac{U_{\text{sep}}^{\text{I}}}{U_{\text{sep}}^{\text{II}}} \right)^2 \left(\frac{c_{\text{str}}^{\text{I}}m_{\text{ac}}^{\text{I}}}{c_{\text{str}}^{\text{II}}m_{\text{ac}}^{\text{II}}} \right) = \left(\frac{Q^{\text{I}}}{Q^{\text{II}}} \right) \frac{f(y_{\text{in}}^{\text{I}}, y_{\text{out}}^{\text{I}})}{f(y_{\text{in}}^{\text{II}}, y_{\text{out}}^{\text{II}})}. \quad (24)$$

We assumed the buffer viscosity η , wave number k and channel geometry (w and h) to be the same for the trajectories of particles I and II. This is the case if particles I and II are manipulated in the same chip and within the same buffer.

Equation (24) is central to our derivations and all further derivations are based on this powerful relationship. We can now combine all material and acoustophysical properties (particle size, contrast factor, boundary layer thickness) into one parameter. We call this parameter the mobility ratio $\text{MR}(\text{I}, \text{II})$ between particle I and particle II and define it as

$$\text{MR}(\text{I}, \text{II}) = \frac{c_{\text{str}}^{\text{I}}m_{\text{ac}}^{\text{I}}}{c_{\text{str}}^{\text{II}}m_{\text{ac}}^{\text{II}}}. \quad (25)$$

The following useful property holds: $\text{MR}(\text{I}, \text{II}) = \text{MR}(\text{I}, \text{III}) \cdot \text{MR}(\text{III}, \text{II})$, where III denotes a third particle type.

When the two particle types are introduced simultaneously, the reference and target total flow rates ($Q^{\text{I}} = Q^{\text{II}} = Q$), as well as applied voltages ($U_{\text{sep}}^{\text{I}} = U_{\text{sep}}^{\text{II}} = U_{\text{sep}}$) are the same. Equation (24) then simplifies to

$$\text{MR}(\text{I}, \text{II}) = \frac{f(y_{\text{in}}^{\text{I}}, y_{\text{out}}^{\text{I}})}{f(y_{\text{in}}^{\text{II}}, y_{\text{out}}^{\text{II}})}. \quad (26)$$

Equation (26) relates the lateral positions of the particle streams collected at two points along the channel to the

particles' material properties and the acoustic streaming. In Eq. (26), we have collected the unknown particle and buffer properties on the left-hand side, while the right-hand side consists of measurable quantities ($y_{\text{in}}^{\text{I}}, y_{\text{in}}^{\text{I}}, y_{\text{in}}^{\text{II}}, y_{\text{out}}^{\text{II}}$). The measurable quantities are sketched in Figs. 1 and 2.

The equation can be further simplified if the initial lateral positions are the same ($y_{\text{in}}^{\text{I}} = y_{\text{in}}^{\text{II}} = y_{\text{in}}$), which is the case for active prefocusing. Further, we can write

$$\text{MR}(\text{I}, \text{II}) = \frac{f(y_{\text{in}}, y_{\text{out}}^{\text{II}} - g_p)}{f(y_{\text{in}}, y_{\text{out}}^{\text{II}})}. \quad (27)$$

Equations (26) and (27) reveal the benefit of the in-flow mobility-ratio method: introducing the reference and target particles into the device simultaneously significantly simplifies Eq. (24) as reference and target particles experience the same flow rate, fluid viscosity, acoustic energy density, channel length, and applied voltage, which therefore cancel out in Eqs. (26) and (27).

The mobility ratio, as defined herein, is a function of viscous boundary layer thickness, contrast factor, and particle sizes. If the streaming contribution number is small, which is the case for most acoustophoresis separations, it reduces to a function of the particle and buffer properties only.

If the streaming number is large, our equations still hold as long as the particles' motion is confined to the mid-height of the device ($z = h/2$). If the streaming number becomes large, and both species' motion is dominated by streaming, the separation becomes impossible; this can be seen by considering the mobility ratio as defined in Eq. (25). In fact, the mobility ratio given by

$$\frac{c_{\text{str}}^{\text{I}} m_{\text{ac}}^{\text{I}}}{c_{\text{str}}^{\text{II}} m_{\text{ac}}^{\text{II}}} = \frac{m_{\text{ac}}^{\text{I}} + \frac{9}{16} \delta^2}{m_{\text{ac}}^{\text{II}} + \frac{9}{16} \delta^2}, \quad (28)$$

tends to 1 for large δ^2 , which indicates that a separation is not feasible. Equation (28) shows that the contribution from acoustic streaming moves the ratio closer towards 1. Therefore, the effect of acoustic streaming always adds difficulty to acoustic separations. Equation (27) shows that the maximal achievable gap distance g_p^{max} , which is related to the maximal achievable separation performance, is only a function of the mobility ratio, channel cross-section dimensions (w and h), and lateral entry position y_{in} .

The in-flow mobility-ratio method aims to measure the parameters ($y_{\text{in}}, y_{\text{out}}^{\text{I}}, y_{\text{out}}^{\text{II}}$) and extract the mobility ratio $\text{MR}(\text{I}, \text{II})$ between species I and II. The optimal separation protocol then uses Eq. (24) in combination with the mobility ratio to determine the optimal input voltage U_{sep} and the optimal position of the stagnation streamline.

Another interesting relationship can be obtained from the following question: if we have two species I and II of known mobility ratio $\text{MR}(\text{I}, \text{II})$ entering the device at the same position y_{in} and we have measured the voltage

$U_{\text{sep}}^{\text{I}}$ needed to position species I at a certain $y_{\text{out}}^{\text{I}} = \alpha$, what should the voltage be to position species II at position $y_{\text{out}}^{\text{II}} = \alpha$? By setting $Q = Q^{\text{I}} = Q^{\text{II}}$, $y_{\text{in}}^{\text{I}} = y_{\text{in}}^{\text{II}} = y_{\text{in}}$, and $y_{\text{out}}^{\text{I}} = y_{\text{out}}^{\text{II}} = \alpha$ we obtain a voltage scaling law for the particle trajectories,

$$\left(\frac{U_{\text{sep}}^{\text{II}}}{U_{\text{sep}}^{\text{I}}} \right)^2 = \text{MR}(\text{I}, \text{II}). \quad (29)$$

When the streaming contribution is negligible it is possible to extract the compressibility of particle I (κ_p^{I}) from the mobility ratio. This requires the density and compressibility of the surrounding fluid (ρ_f, κ_f), the acoustic mobility of particle II, as well as the size (a_1) and density (ρ_p^{I}) of particle I to be known. This approach, is analogous to previous efforts trying to determine the compressibility of cells [13,14,30–34].

D. The in-flow mobility-ratio method

To measure the mobility ratio in flow, a series of images is collected close to the channel outlet while stepwise increasing the actuation voltage on the main-channel piezoelectric transducer; see Fig. 3. The initial position of the streams (y_{in}) is collected at zero separation channel actuation voltage ($U_{\text{sep}} = 0$). This exploits that the particle streams are not displaced in the separation channel and therefore $y_{\text{in}} = y_{\text{out}}^{\text{I}} = y_{\text{out}}^{\text{II}}$. The voltage is then increased in a stepwise manner such that the particle streams are pushed closer to the channel center. Triplicate images of the particle streams are taken at the outlet and averaged for each actuation voltage. At maximal voltage both species will be focused into the channel center. Our experimental data consist of the positions of the both species close to the outlet flow splitter measured based on their fluorescence profiles collected at various voltages. The positions close to the outlet flow splitter of the high- ($y_{\text{out}}^{\text{I}}$) and low-mobility ($y_{\text{out}}^{\text{II}}$) species are depicted in the inset of Fig. 3.

The separation curve in Fig. 4 denotes the graph of experimentally obtained $y_{\text{out}}^{\text{II}}$ data plotted against the corresponding gaps, which are extracted from $y_{\text{out}}^{\text{I}}$ and $y_{\text{out}}^{\text{II}}$. The experimentally obtained acoustic separation curve for red 7.8- μm (R8) and green 5.1- μm (G5) fluorescent polystyrene particles is shown by red crosses in Fig. 4. The background shows the theoretical mobility ratio calculated by Eq. (27). The separation curve in combination with Eq. (27) contains all the information needed to extract the mobility ratio. The influence of measurement errors on the mobility ratio when measuring y_{out} and the gap distance g_p is smallest when the variation of the mobility-ratio function vanishes with respect to small perturbations in those measured parameters, that is, if the gradient of the function is zero. This is approximately achieved by the

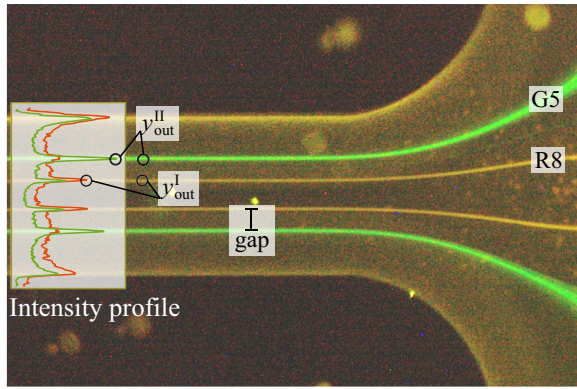


FIG. 3. Fluorescent microscope image taken at the channel outlet. The particles appear as bright beams. The y_{out}^I and y_{out}^{II} positions can be extracted from the fluorescence profile averaged over an x interval, which is shown by the inset.

maximum of the measured y_{out} versus gap curve. The measured mobility ratio for one particle combination is then defined by the maximum of a quadratic fit (white dot) to the measured data points, that is, at the point of largest gap distance. The mobility ratio was measured for 11 different polystyrene particle combinations and good agreement with data from the literature was found. The analysis can be found in Appendix A.

To run the optimization protocol with cells, the mobility ratio of cells with respect to a reference particle needs to be assessed. Cells often present themselves in much

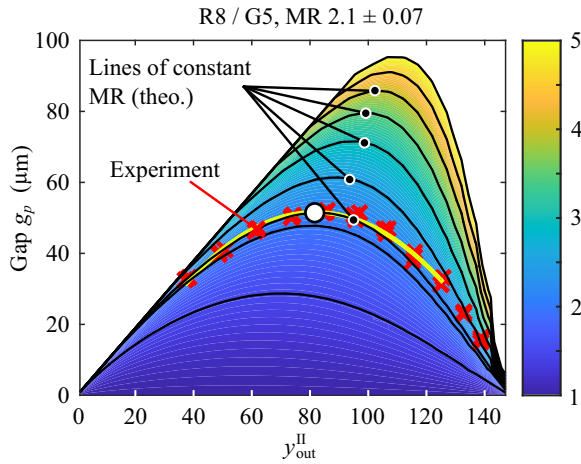


FIG. 4. The mobility ratio (MR) between R8/G5 is shown as a function of y_{out} and the gap distance g_p for a fixed y_{in} . The color bar ranges from 1 (blue) to 5 (yellow). Experiments are represented by red crosses. The mean measurement for the mobility ratio is obtained from the maximum of a quadratic fit to the data. The quadratic fit is shown in yellow. The maximal gap g_p^{max} and the corresponding y_{out}^{II} are represented by the white circle with black outline. Additional explanations can be found in Fig. 1 in the Supplemental Material [35].

wider distributions of material properties as polystyrene particles. This will also be reflected by wider fluorescent beams. In this case, the y_{out} position of the cells should be assessed based on the median of the cell distribution (i.e., fluorescent profile). Measuring the mobility ratio this way and following our procedure will then yield an input voltage U_{sep} placed in the middle of the two 50% separation quality (SQ50) points.

E. Calibration and optimal settings

The calibration is a concise workflow that allows compensating for the daily variations or different devices. For these experiments a calibration particle is used, which can be chosen independently of the species that will be separated. The only requirement is that the mobility ratio between the calibration particle and the target species must be measured as it is needed to compute the optimal separation parameters. The calibration experiments consist of collecting triplicate images of the channel at different settings. More precisely:

- (1) The image collected at the outlet and at zero separation-channel actuation voltage ($U_{sep} = 0$ Vpp).
- (2) Tuning the separation-channel actuation voltage (U_{sep}) to achieve a lateral position (y) of the calibration particle $y_{out}^{cal} \approx \frac{1}{2}y_{in}$, and collecting the image at the outlet.
- (3) Tuning the separation-channel actuation voltage (U_{sep}) such that the particle stream spills into both outlets. This is collected for side-outlet flow-rate ratios of 35%, 60%, and 85%.

The calibration is illustrated in Fig. 5. Calibration step (1) provides the value of $y_{in} = y_{in}^{cal}$ that is used in the subsequent analysis. Calibration step (2) provides y_{out}^{cal} and U_{sep}^{cal} . Calibration step (3) provides the position of the outlet stagnation streamline.

The optimal settings are now deduced from the measured mobility ratios and the calibration by the following procedure:

- (1) Compute the maximal gap g_p^{max} and the corresponding y_{out}^{targII} by maximizing the gap as a function of y_{out}^{targII} in Eq. (25). Here the $MR(targI, targII)$ and y_{in} are input parameters. The optimal positions $y_{out}^{targI} = y_{out}^{targII} - g_p$ follow directly.
- (2) Compute the outlet flow-rate split ratio such that the position of the lateral position of the outlet stagnation line y_{stagn} is $y_{stagn} = y_{out}^{targII} - 1/2g_p$.
- (3) Use Eq. (24) to compute the required actuation voltage that achieves both y_{out}^{targI} and y_{out}^{targII} by Eq. (24). This is achieved by setting $U_{sep}^I = U_{sep}^{cal}$, $y_{in}^I = y_{in}^{cal}$, $y_{out}^I = y_{out}^{cal}$, and further, choosing either $MR(I, II) = MR(cal, targI)$ and $y_{out}^{II} = y_{out}^{targI}$ or $MR(I, II) = MR(cal, targII)$ and $y_{out}^{II} = y_{out}^{targII}$.

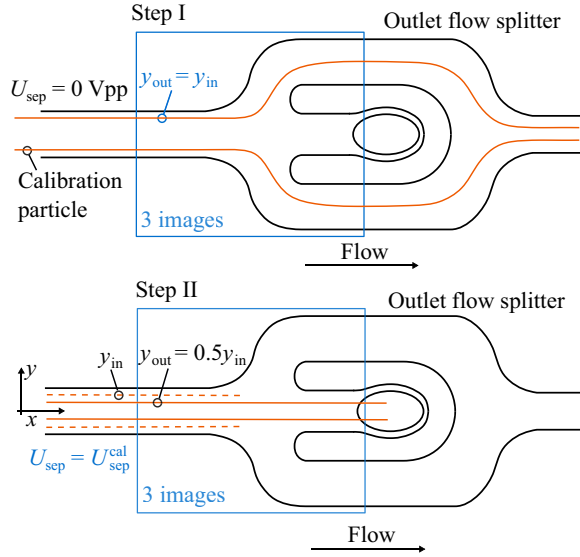


FIG. 5. A calibration particle is introduced into the separation device and three snapshots are collected close to the outlet flow splitter at two different settings. In the first step, the images are collected while the separation channel actuation voltage is turned off ($U_{\text{sep}} = 0$). During the second step the separation channel actuation voltage U_{sep} is tuned such that the lateral position of the calibration particle is focused approximately halfway towards the channel center.

III. OPTIMAL SEPARATION OF POLYSTYRENE PARTICLES

The optimal separation by the mobility-ratio method is validated by performing the separation of red fluorescent 5- μm polystyrene particles (R5, 0.00625% w/v) from green fluorescent 7.8 μm polystyrene particles (G8, 0.0125% w/v).

A prefocusing voltage of $U_{\text{pref}} = 3.52 \text{ Vpp}$, an inlet split ratio of 20% side to 80% center flow, and a total flow rate of 150 $\mu\text{L/min}$ are used for the both the calibration and the separation experiments. The calibration is performed with G8 particles. For the calibration run, an outlet splitting ratio of 50% side flow and 50% center flow is selected, whereas the subsequently derived optimized outlet split ratio is used for the optimized separation experiments.

All subsequent experiments were repeated three times. The data from one experiment are shown in Figs. 6, 7, and 8, and the data of the other two experiments can be found in Figs. 10–15 of the Appendix B. Data points presented in brackets indicate the outcomes of the three individual repetitions.

A. Modeling the trajectories

Equation (24) allows us to compute the particle positions for both particle types (G8 and R5) and various voltages based on one calibration point ($U_{\text{sep}}^{\text{cal}}, y_{\text{out}}^{\text{cal}}$) and the corresponding mobility ratios $\text{MR}(\text{I, cal})$, $\text{MR}(\text{II, cal})$. A

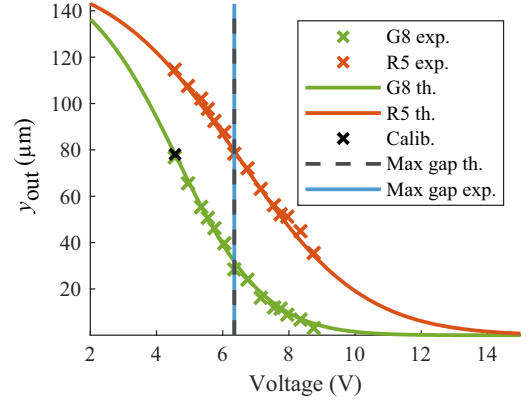


FIG. 6. The position of the particle streams measured at the outlet (crosses) and predicted positions (lines) based on one single calibration data point (black). The difference between predicted and measured positions is below 2 μm on average, which is less than the respective particle radii. The blue and dotted black lines indicate the voltages for the predicted and experimentally achieved gaps, respectively, which were the same.

direct comparison between the theoretical predictions and experimentally obtained positions of the particle streams at the outlet of the separation chamber as a function of the actuation voltage is shown in Fig. 6. The predicted particles positions, obtained by Eq. (24), are shown by the solid lines, while the experimentally obtained particle positions are marked by crosses. The experimental data points are obtained by fluorescence profiles collected close to the separation channel outlet. The black cross denotes data obtained during the calibration experiments, whereas green and red crosses correspond to the experimentally recorded positions of green and red particles, respectively. The target positions of the particle streams at the outlet were predicted with high accuracy. We predicted $y_{\text{out}}^{\text{G8}} = 31.6 \mu\text{m}$ and $y_{\text{out}}^{\text{R5}} = 79.3 \mu\text{m}$ and measured $y_{\text{out}}^{\text{G8}} = 27.25 \mu\text{m}$ and $y_{\text{out}}^{\text{R5}} = 75 \mu\text{m}$. Overall, in this experiment the average deviation from the predicted position was less than 2 μm for both particle types, which is less than their respective radii. In all experiments the average distances from the predicted positions [1.4, 4.5, 6.3] were below the particle diameter of the G8 particles. Therefore, we conclude that our method of calibration and assessing the acoustomechanical material properties of the particles allows their motion within the separation device to be predicted with unprecedented precision.

B. Optimal separation channel voltage

We are now in a position to validate the optimal separation protocol. After the calibration, the predicted optimal settings are $U_{\text{sep}} = 6.36 \text{ Vpp}$ and a flow rate of 68% through the side outlet and 32% through the center outlet. The separation experiments were performed for the optimal settings and 12 additional voltages in the range of 4.55

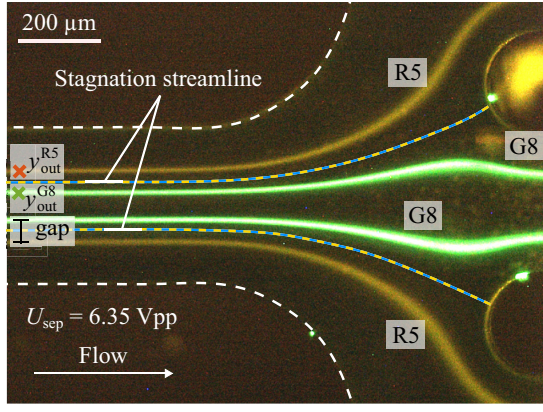


FIG. 7. Snapshot of the particle streams taken at the outlet flow splitter and at the optimal settings ($U_{\text{sep}} = 6.36$ Vpp and 68% of the total flow rate through the side outlet). The achieved gap of $49.9 \mu\text{m}$ differs less than 5% from the theoretical maximum of $47.7 \mu\text{m}$. The blue-yellow dashed line indicates the stagnation streamline measured at $U_{\text{sep}} = 4.84$ Vpp and the optimal flow-rate split ratio. The white dashed line indicates the channel walls.

to 8.75 Vpp. To map the separation quality as a function of the voltage, the separation quality was assessed for every data point by flow cytometry.

A photograph of the channel outlet at the optimal settings is shown in Fig. 7. At the optimized settings a gap distance of $49.9 \mu\text{m}$ is achieved, which differs less than 5% from the predicted maximal gap distance of $47.7 \mu\text{m}$ for this y_{in} and mobility ratio.

The position of the stagnation streamline, indicated by the blue-yellow dashed line, was measured from the photograph taken at $U_{\text{sep}} = 4.84$ Vpp in which the G8 particle streams and the stagnation streamline coincide.

The separation quality as a function of the separation channel actuation voltage is shown in the left panel of Fig. 8, where the experimental data points are represented by blue crosses. To reduce the contribution of experimental noise, the *a posteriori* optimal voltage (solid blue line) was determined by the center of the SQ50 points. The SQ50 points were determined from a linear fit of two to four points on the flanks of largest slope.

The predicted voltages of all three experiments [6.36, 5.6, 5.52] Vpp are within 6.6% of the *a posteriori* determined voltages [6.77, 5.95, 5.91].

C. Optimal outlet flow-rate split ratio

The separation experiment was performed a second time to assess if the protocol is able to predict the optimal outlet flow-rate split ratio. As the experiments were performed on the same day, the same experimental parameters were used: $U_{\text{sep}} = 6.36$ Vpp and a side-outlet flow-rate split ratio of 68%. The separation was performed for the optimal settings and for 12 additional side-outlet flow-rate

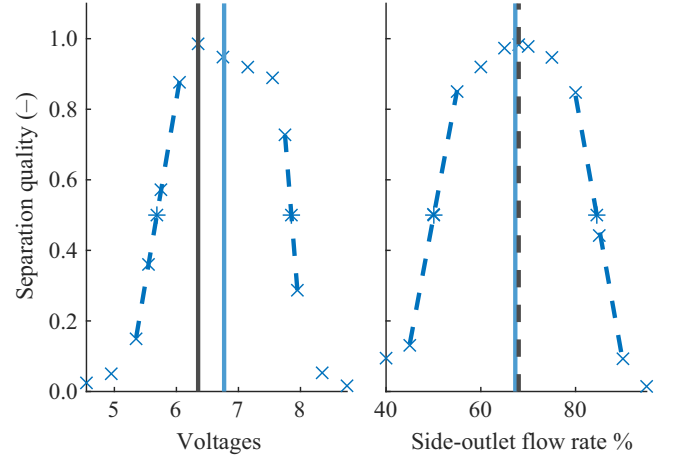


FIG. 8. Separation quality is plotted against the applied voltage (left) and side-outlet flow-rate split ratio (right). Crosses denote experimental data obtained by flow cytometry. (left) The solid blue line is the center of the 50% separation quality (SQ50) points ($U_{\text{sep}} = 6.77$ Vpp), whereas the black solid corresponds to the predicted optimal voltage ($U_{\text{sep}} = 6.36$ Vpp). (right) The separation quality as a function of the side-outlet flow-rate percentage. The predicted optimal side-outlet flow-rate percentage (68%) is indicated by a dashed black line. The center of the SQ50 points is found at a side-outlet flow-rate percentage of 67% and indicated by a solid blue line. Flow cytometry data is shown in the Supplemental Material [35].

ratios between 40% and 95%. The results are summarized in the right panel of Fig. 8. The center of the SQ50 points (see the blue dashed line in Fig. 8) is located at a side-outlet flow-rate percentage of 67%, which proves the optimality of our settings. The repeats showed a similar behavior: overall the predicted optimal side-outlet flow-rate ratios [68%, 63%, 63%] differ less than 2% from the *a posteriori* obtained optimal side-outlet flow-rate ratios [67%, 65%, 61%].

IV. CONCLUSION

A fast, direct workflow to perform optimized acoustic particle or cell separations based on the in-flow mobility-ratio method is derived and validated experimentally.

The method is experimentally proven to be highly accurate as the error on the predicted position is below one particle radius on average. The predicted optimal conditions were validated experimentally by mapping the separation quality for the space of separation channel actuation voltages and outlet split ratios. Our *a priori* determined optimal voltage and outlet split ratio were compared to *a posteriori* averaged measures. The predicted settings are within 6.6% of the *a posteriori* determined optimal settings, which proves the optimality of our predicted settings up to measurement accuracy.

In the future the method will be further expanded. The precise knowledge and control of the particle trajectories within acoustophoresis separation devices can be used for acoustophoresis chromatography (i.e., separating subspecies into fractions of well-defined mobility ratios). The mobility-ratio method can also be used for fast reference experiments to find buffer conditions that optimize separation quality.

ACKNOWLEDGMENTS

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APPENDIX A: MOBILITY OF VARIOUS POLYSTYRENE MICROPARTICLES AND VALIDATION

The mobility ratios of 11 different polystyrene particle combinations were assessed by the in-flow mobility-ratio method. The results are summarized in Fig. 9 and Table I.

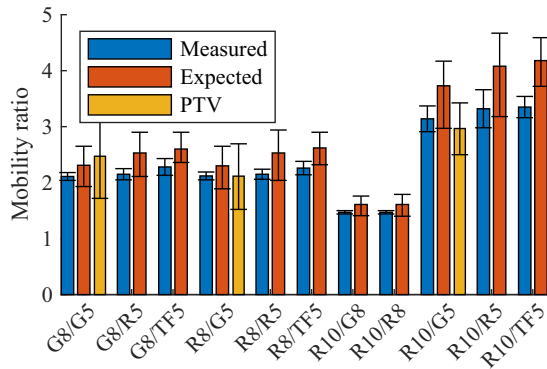


FIG. 9. (a) Comparison of the mobility ratio of different combinations of polystyrene particles suspended in MilliQ obtained by the in-flow mobility-ratio method (measured), Coulter counter measurements (expected), and particle trajectory velocimetry (PTV, Edthofer *et al.* [15]). The nametags are a combination of one or two letters and a number. The letters G, R, TF in the name tags stand for green fluorescent (Microparticles GmbH.), red fluorescent (Microparticles GmbH.), and green fluorescent (ThermoFisher) particles, respectively. The numbers indicate their (approximate) diameter in micrometers. The bar heights and error bars of the expected values are given by the median, 16th and 84th percentiles, respectively. The bar heights and error bar positions for the PTV measurements are the mean and 16th and 84th percentiles, respectively. For the mobility-ratio method the error bars are given by the standard deviation of the data points used in the fit. The mobility-ratio distribution measured by the in-flow mobility-ratio method lies within the measurements by the traditional PTV and the expected values. Shows the measured and expected mobility ratios for 11 different polystyrene particle combinations suspended in MilliQ.

TABLE I. The measured and expected mobility ratios for different combinations of fluorescent polystyrene particles. The expected mobility ratio based on Coulter counter size measurements is shown in the two columns on the right.

combinations (in MilliQ)	fit $\pm$ sd	#pts.	median CoulterCt	percentiles [16%, 84%]
G8 / G5	2.1 ± 0.07	25	2.32	[1.93, 2.65]
G8 / R5	2.15 ± 0.1	26	2.53	[2.11, 2.90]
G8 / TF5	2.28 ± 0.15	26	2.65	[2.36, 2.90]
R8 / G5	2.12 ± 0.07	26	2.30	[1.89, 2.65]
R8 / R5	2.15 ± 0.09	25	2.53	[2.04, 2.94]
R8 / TF5	2.26 ± 0.12	27	2.62	[2.32, 2.90]
R10 / G8	1.47 ± 0.03	21	1.61	[1.41, 1.76]
R10 / R8	1.47 ± 0.03	21	1.61	[1.40, 1.76]
R10 / R5	3.32 ± 0.34	19	4.08	[3.18, 4.67]
R10 / G5	3.14 ± 0.23	19	3.73	[2.97, 4.17]
R10 / TF5	3.35 ± 0.19	20	4.18	[3.72, 4.59]

The nametags of the particles are a combination of their fluorescent color and their (approximate, rounded) diameters in micrometers as given by the manufacturer. Here R stands for red fluorescent and G for green fluorescent particles obtained from Microparticles GmbH. TF stands for green fluorescent particles obtained from ThermoFisher. The particle concentrations were below 0.0025% w/v. The mobility ratio obtained from the in-flow mobility-ratio method (blue bars) is compared to the size-based mobility ratio as measured by the Coulter counter (red bars). The Coulter counter was a Multisizer 3 Coulter counter obtained from Beckman Coulter, Brea, CA, USA. The mobility ratios obtained by particle tracking velocimetry (PTV) were presented by Edthofer *et al.* [15] and are represented by the yellow bars. A particle concentration lower

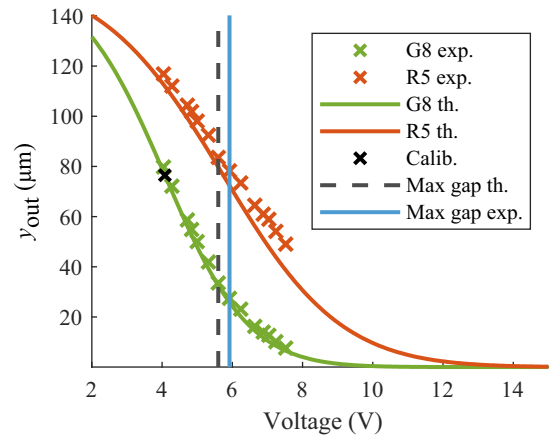


FIG. 10. First repeat of experiment presented in Fig. 6. The position of the particle streams measured at the outlet (crosses) and predicted positions (lines) based on one single calibration data point (black). The blue and dotted black lines indicate the voltages for the predicted and experimentally achieved maximal gaps.

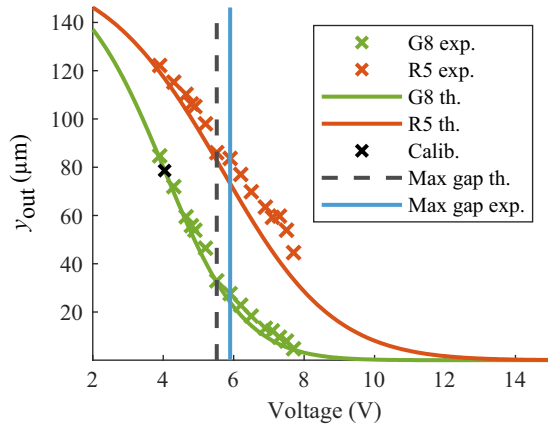


FIG. 11. Second repeat of experiment presented in Fig. 6. The position of the particle streams measured at the outlet (crosses) and predicted positions (lines) based on one single calibration data point (black). The blue and dotted black lines indicate the voltages for the predicted and experimentally achieved maximal gaps.

than 0.0025% w/v, a side flow to total flow-rate ratio of 0.2 and 0.5 at the inlet and outlet, respectively, and a total flow rate of 150 $\mu\text{L}/\text{min}$ were used. The expected mobility ratio is based on particle sizes, measured by a Coulter counter, while assuming that the material properties of the different polystyrene spheres are the same. The bar height of the expected mobility ratio (red) is the median from the mobility-ratio distribution calculated from particle sizes measured by the Coulter counter, while the error bars are the 16th and 84th percentiles. The bar height for the PTV measurements corresponds to the mean value, while the error bars show the 16th and 84th percentiles. The error bars for the in-flow mobility-ratio method measurement

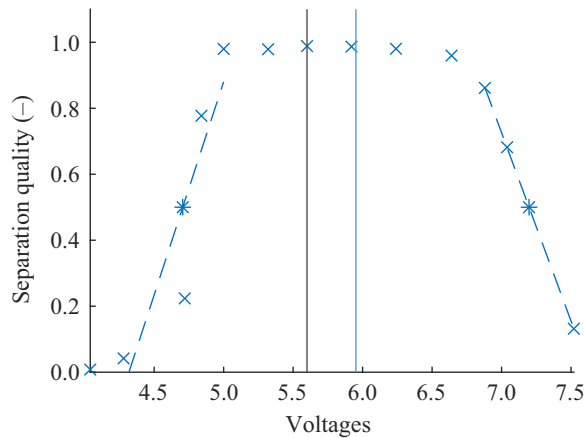


FIG. 12. Separation quality as a function of the voltage, first repeat of the left panel of Fig. 8. Crosses denote experimental data obtained by flow cytometry. The solid blue line is the center of the 50% separation quality points; the solid black line indicates the predicted maximum.

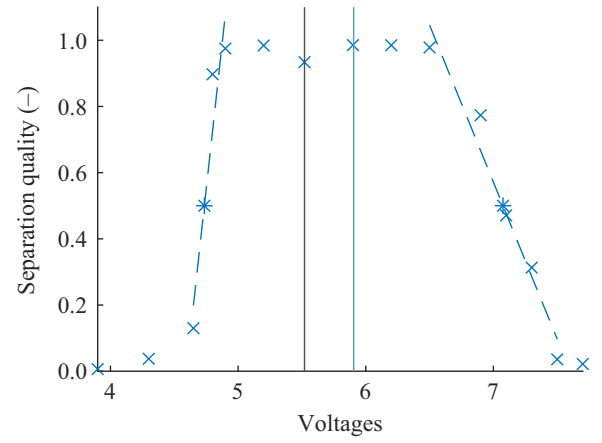


FIG. 13. Separation quality as a function of the voltage, second repeat of the left panel of Fig. 8. Crosses denote experimental data obtained by flow cytometry. The solid blue line is the center of the 50% separation quality points; the solid black line indicates the predicted maximum.

are given by the standard deviation of the data points included in the fit. The height of the bar is the maximum of the quadratic fit. The mobility ratio obtained by the in-flow mobility-ratio method lies well within the error margins as obtained by PTV and the expected values from Coulter counter particle size data. The data obtained by PTV provide much wider error bars with respect to the in-flow mobility-ratio measurement. This is due to the width of the PTV error bars being based on the spread of individual data points, whereas the in-flow mobility-ratio method inherently provides an averaged result. Therefore, we concluded that the in-flow mobility-ratio method provides sufficient accuracy with respect to the standard method (PTV) while being an order of magnitude faster. In comparison, the

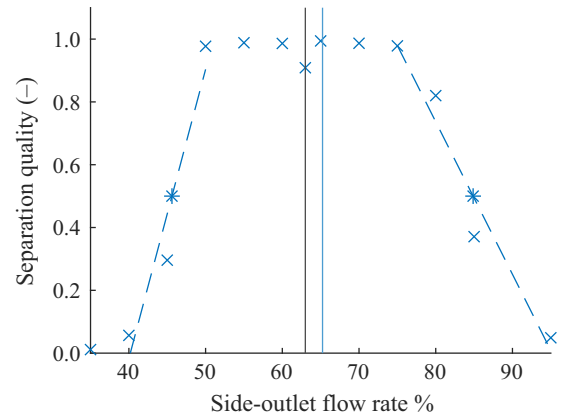


FIG. 14. Separation quality as a function of the side-outlet flow-rate split ratio, first repeat of the right panel of Fig. 8. Crosses denote experimental data obtained by flow cytometry. The solid blue line is the center of the 50% separation quality points; the solid black line indicates the predicted maximum.

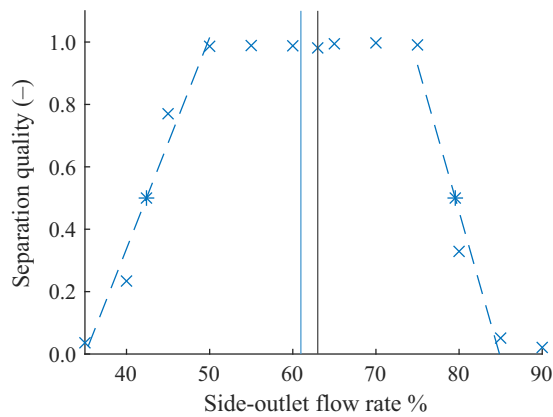


FIG. 15. Separation quality as a function of the side-outlet flow-rate split ratio, second repeat of the right panel of Fig. 8. Crosses denote experimental data obtained by flow cytometry. The solid blue line is the center of the 50% separation quality points; the solid black line indicates the predicted maximum.

study presented in Edthofer *et al.* [15] required several weeks of data collection and processing to measure six mobility ratios, while the 11 mobility ratios presented in Fig. 3a were collected and processed in a few days.

It should be noted that the mobility-ratio validation and Coulter counter measurements presented in this section were performed in 2022. The experiments presented in Sec. III were performed in 2024 and the mobility ratios of the particles used were measured a few days before the experiments to compensate for slow changes in particle properties. The mobility ratios evaluated 2022 and 2024 were within 10% of each other.

APPENDIX B: DATA FOR THE REPEATED EXPERIMENTS

In this section the data of the repeated experiments are summarized in Figs. 10–15.

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