Supplementary Information

S1

Expected Sorting Purity

For most FACS devices, the expected purity can be calculated using Poison statistics and probability theory. The probability of finding x cells within a time interval t is given by the Poison probability function:

$$P(x) = \frac{\mu^x}{x!} e^{-x}$$

and

$$\mu = f \times t$$

where f is the average number of cells per second, and, t ,is the sorting time (time/event) (*e.g.* droplet size or (droplet generation frequency)$^{-1}$ in the conventional FACS). The expected sorting purity for rare event sorting can be simplified as: (see S3 for derivation)

$$Minimum\ expected\ purity = \frac{1}{1 + \mu}$$

For non rare event sorting, the probability of having two or more target cells within the sorting time must be taken into consideration. The higher the starting purity is, the higher probability of having two target cells in one sorting event, thus increasing the sort purity.

The same theory should be applicable for the AFACS with some modifications on the sort time, t_{sort} , and some special cases that can occur.

First, it should be noted that the sort time, t_{sort} , is not the same as acoustic actuation time, t_{burst} , but rather a function of it. t_{sort} should be seen as a measure of how far the sample stream is deflected towards the target outlet during a sort event. To explain this we need to define the variables $t_{critical}$ and $t_{retention}$.

$t_{retention}$ is defined as the retention time for a particle in the "sort zone" of the chip. This is a function of the total flow rate and the particle's position within the microfluidic channel. Due to the acoustic two-dimensional focusing, all particles will be traveling with approximately the same velocity, and hence the retention time can be calculated by dividing the length of the sorting zone with the average velocity of the particles at a given flow rate.

The critical acoustic exposure time, $t_{critical_|}$,is defined as the time a particle must be exposed to the acoustic radiation force (given a specific acoustic radiation force) in order to be deflected sufficiently to be recovered in the target outlet. $t_{critical_|}$ can be found experimentally by continuously actuating the "sort zone" of the microchip at a constant acoustic energy (piezo drive voltage) and step-wise increasing the flow rate until particles are no longer recovered in the target outlet. Once these

criteria are found, $t_{critical} = t_{retention}$. If $t_{critical} < t_{retention}$, no particles will be recovered in the target outlet and this sets the ultimate limit of the system.

The sort time can be calculated by :

$$t_{sort} = t_{burst} + t_{retention} - 2 * t_{critical}$$

This relationship is explained by Supplementary figure 1. Note that the particle concentration is exaggerated in order to better illustrate how long the sample stream stays deflected during a sort event. Also note that the chip is drawn out of scale. Further, the changes in particle velocities downstream of the flow splitter are neglected.
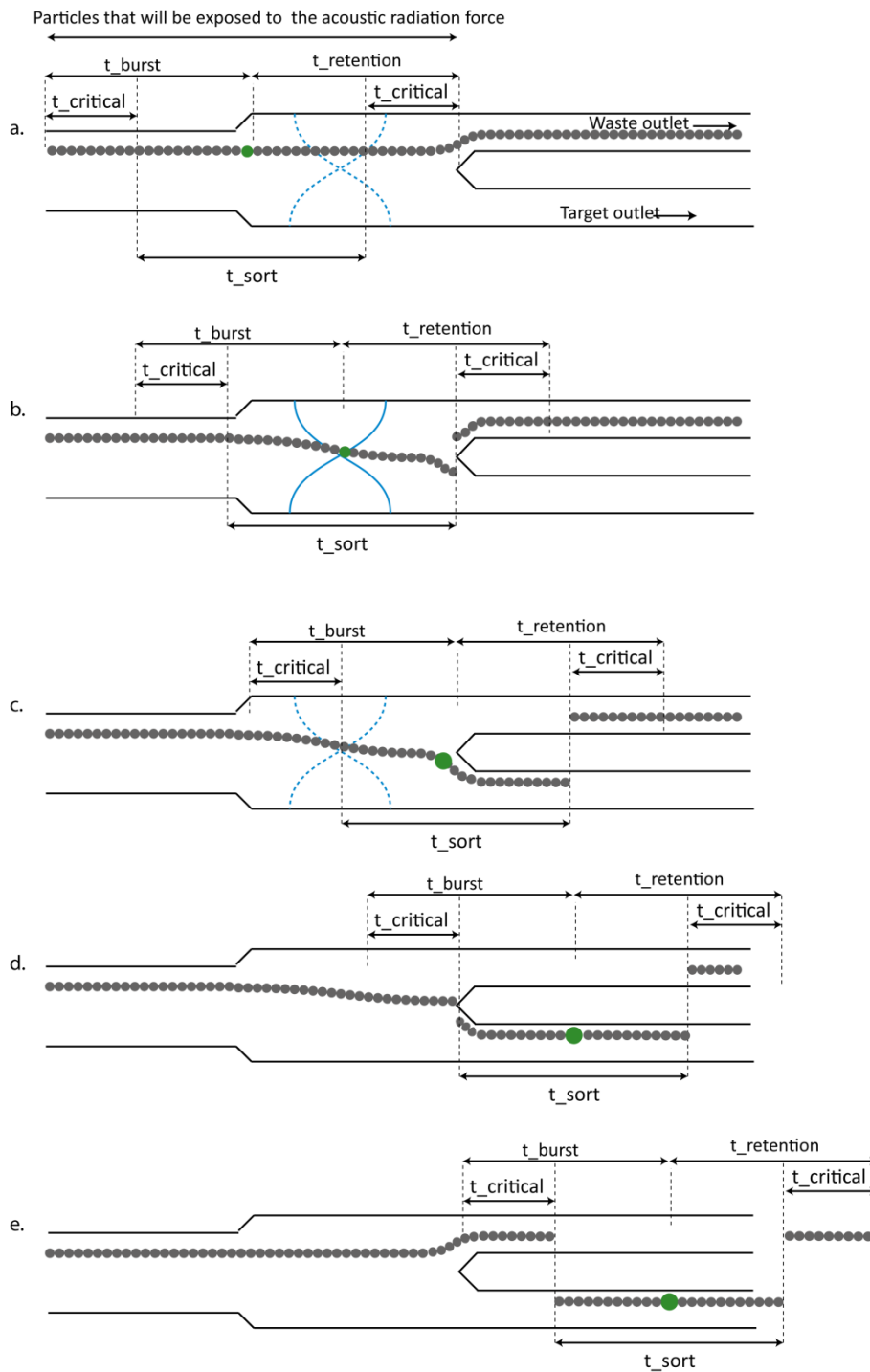
Supplementary figure 1a-e. An illustration of how the sort unit t_{sort} is derived. The dashed blue line indicates that the ultrasound just have been turned on (a) or off (c).

If the retention time of a cell in the acoustic sorting zone, $t_{retention}$, is matched to the ultrasonic burst length, ideally only the cells that triggered the sort event will be exposed to the full duration of the ultrasonic burst. The sort time, t_{sort}, can then potentially be shorter than the ultrasonic burst length, t_{burst}. This is illustrated by the example in Supplementary figure 2.
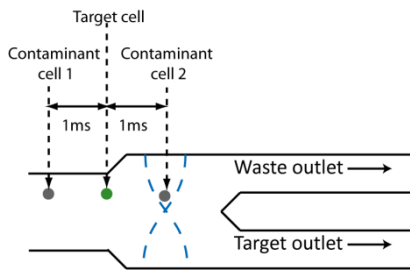
Supplementary figure 2a.
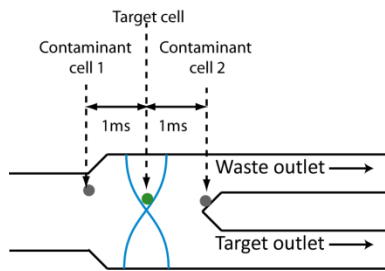$t_{retention}$=2ms
t_{burst}=2ms
$t_{critical}$=1,5ms
t=0ms
The ultrasound is turned on

Acoustic exposure times:
Contaminant cell 1= 0 ms
Contaminant cell 2= 0 ms
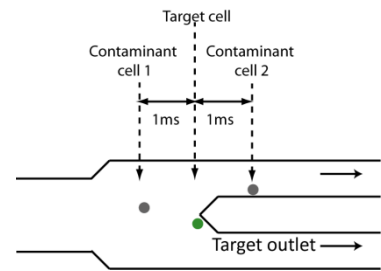Target cell   = 0ms

Supplementary figure 2b.
t=1ms
The ultrasound is active

Acoustic exposure times:
Contaminant cell 1= 0 ms
Contaminant cell 2= 1 ms
Target cell   = 1ms

Supplementary figure 2c.
t=2ms
The ultrasound is turned off. The target cell will be collected in the target outlet. The contaminant cells will be collected in the waste outlet.

Acoustic exposure times:
Contaminant cell 1= 1 ms
Contaminant cell 2= 1 ms
Target cell   = 2ms

If the acoustic energy is set so that $t_{critical}=t_{burst}$, t_{sort} would be very small, resulting in an expected purity close to 100 %, unless cells are clustered. For practical reasons $t_{critical}$ was set to $\approx \frac{1}{2}t_{burst}$, to give margin for differences in size, acoustic contrast factor for different cells, and timing errors. This also gave the effect that $t_{burst} \approx t_{sort}$ in the experiments.

If two triggering events occur within the burst time, everything between the two target particles will be captured into the target outlet, reducing purity. However, the function generator used for the experiments can not be retriggered during an ongoing burst sequence. This limitation decreases recovery but increases purity.

S2.

Particle Velocity Measurements

Measurements of particle velocity in the sorting zone, with standard deviation bars and CVs indicated are presented in supplementary figure 3. The slope of the regression line was used to calculate particle retention time within the sorting zone of the chip
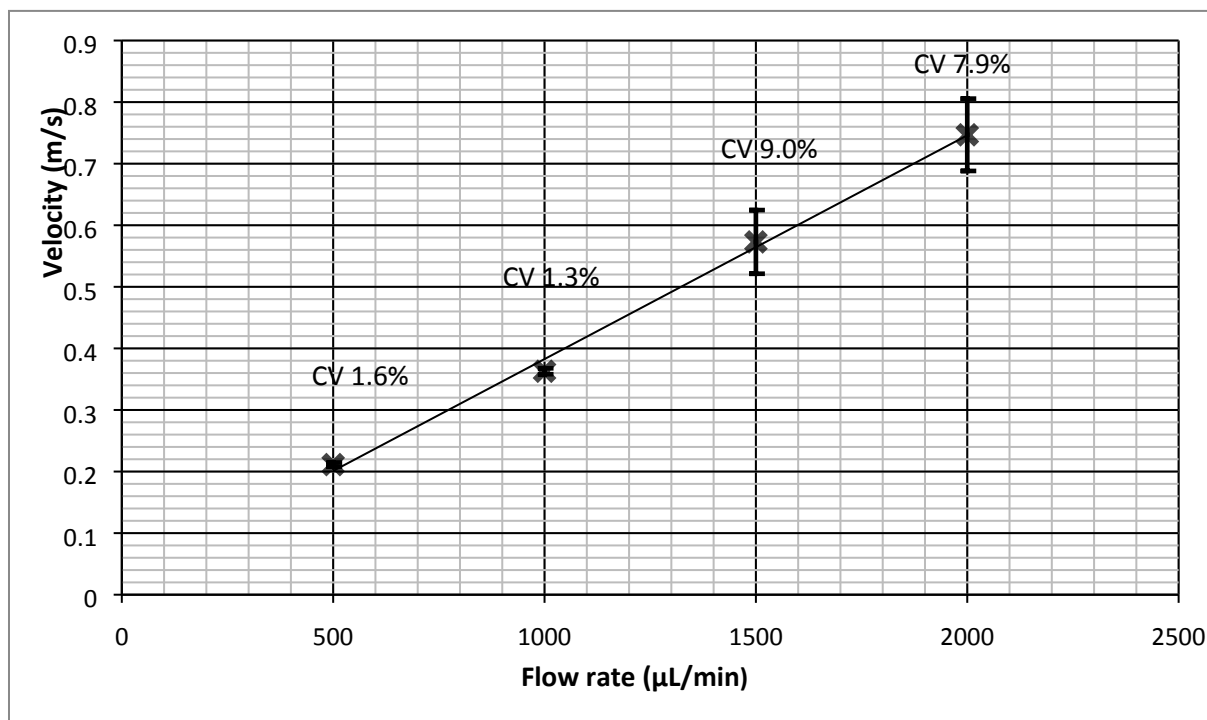
Supplementary figure 3. Particles velocity measurements in the sorting zone as a function of flow rate.

S3.

Derivation of the minimum expected purity:

$$Purity = \frac{number\ of\ targets}{number\ of\ targets + number\ of\ contaminants}$$

Basic assumptions:

- All target cells are sorted accurate with 100 % recovery
- Target cells are very few in relation to the total amount of cells → The likelihood of having two or more target cells within a drop is approximated to zero

$P_d(X)$ = The probability of finding a droplet containing X number of cells.

$$P_d(X) = \frac{\mu^X}{X!}e^{-\mu}$$

Where μ is the expected value of number cells/drop or cells/s*droplet period

$$P_d(X) = \frac{\mu}{x}P(X-1)$$

using the relationship:

$$P_d(X-1) = \frac{\mu^{X-1}}{(X-1)!}e^{-\mu} = \frac{X*\mu^X}{\mu*X!}e^{-\lambda} = \frac{X}{\mu}P_d(X)$$

$P_c(X)$ =The relative distribution of cells loaded into a droplets containing X number of cells, droplets are loaded according to $P_d(X)$. This distribution reflects the probability that a wanted cell is loaded into a droplet with X number of cells. Eg., if a droplet if loaded with 2 cells, there is a 2x probability that this drop will be sorted compared to a droplet containing a single cell.

$$P_c(X) = \frac{X*P_d(X)}{\sum\limits_{0}^{\infty}X*P_d(X)} = P_d(X-1)$$

Using the recursion relationship according above

$$P_c(X) = \frac{X*P_d(X)}{\sum\limits_{0}^{\infty}X*P_d(X)} = \frac{\mu*P_d(X-1)}{\sum\limits_{0}^{\infty}\mu*P_d(X-1)} = \frac{\mu*P_d(X-1)}{\mu*\sum\limits_{0}^{\infty}P_d(X-1)} = \frac{P_d(X-1)}{1} = P_d(X-1)$$

The expected purity from sort:

$$Purity = \frac{1}{1 + 0*P_C(1) + P_C(2) + 2P_C(3) + 3P_C(4)....}$$

$$Purity = \cfrac{1}{1 + \displaystyle\sum_{X=1}^{\infty} (X-1) * P_c(X).} = \cfrac{1}{1 + \displaystyle\sum_{X=0}^{\infty} X * P_c(X+1)}$$

$$\cfrac{1}{1 + \displaystyle\sum_{X=0}^{\infty} X * P_c(X+1).} = \cfrac{1}{1 + \displaystyle\sum_{X=0}^{\infty} X * P_d(X).} = \cfrac{1}{1 + \displaystyle\sum_{X=0}^{\infty} \mu * P_d(X-1).}$$

$$\cfrac{1}{1 + \displaystyle\sum_{X=0}^{\infty} \mu * P_d(X-1).} = \cfrac{1}{1+\mu}$$

S4.

A video showing periodic gating of 10μm polystyrene particles. The sorting zone is actuated for 1ms, and then idles for 9ms, periodically. It should be noted that the flow direction is from right to left.