Electronic Supplementary Material (ESI) for Lab on a Chip.

Virus removal from semen with a pinched-flow fractionation microfluidic chip

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Supplementary Information

A Analysis flow profile CCMVs

To analyze the width of the CCMVs in the broadened segment of the microfluidic chip, a Matlab script was used. In figure 1, an example of the original images and processed images of the intermediate steps are shown. The starting point was the fluorescent image (figure 1B). The images were processed with Gaussian noise removal (low-pass Wiener filter, figure 1C) and the image intensity values were saturated (figure 1D). The image intensity values of a line orthogonal to the outer channel wall (red arrow in figure 1D) were plotted and the width of the fluid flow containing CCMVs was determined.

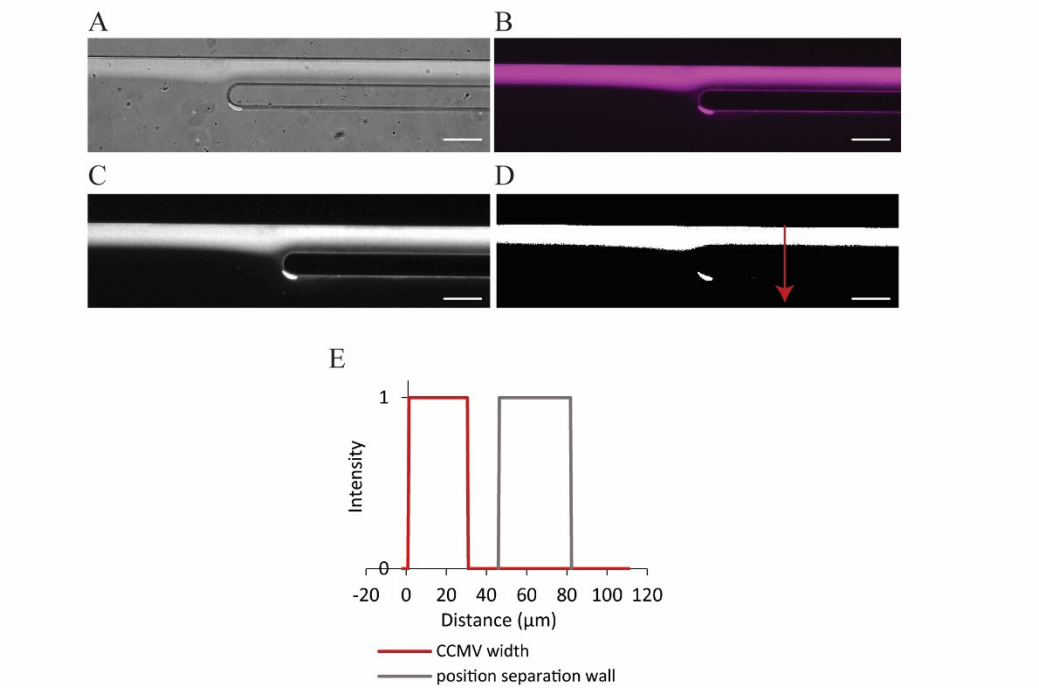


Figure 1: Images analysis to obtain the intensity profile of CCMV in the microfluidic chip: A) Merged brightfield and fluorescent image. B) Fluorescent image. C) Image after Gaussian noise removal (low-pass Wiener filter) D) binary image based on a threshold (red arrow: line of intensity plot) E) Intensity profile of CCMVs in microfluidic chip. (scale bar = 50 µm)

B Calibration Curves Plate Reader

The concentration of CCMVs was determined using a fluorescence imaging plate reader. For relating the fluorescence intensity to a concentration, calibration curves with the applicable sample volumes and CCMV concentrations are needed. CCMVs were diluted in Solusem Bio+ to the desired concentrations and with a linear fit the calibration curves were obtained (figure 2).

In outlet 1, the CCMV concentration was higher than the CCMV concentration in outlet 2. Therefore, two calibration curves were for the expected CCMV range were determined. For a CCMV concentration between 2 - 120 ng/ml ($2.6 \times 10^{11} - 1.6 \times 10^{13}$ CCMVs/ml) the calibration curve shown in figure 2A can be used. For CCMV concentrations of 0.01 ng/ml - 1 ng/ml ($1.3 \times 10^{9} - 1.3 \times 10^{11}$ CCMVs/ml) the calibration curve shown in figure 2B can be used.

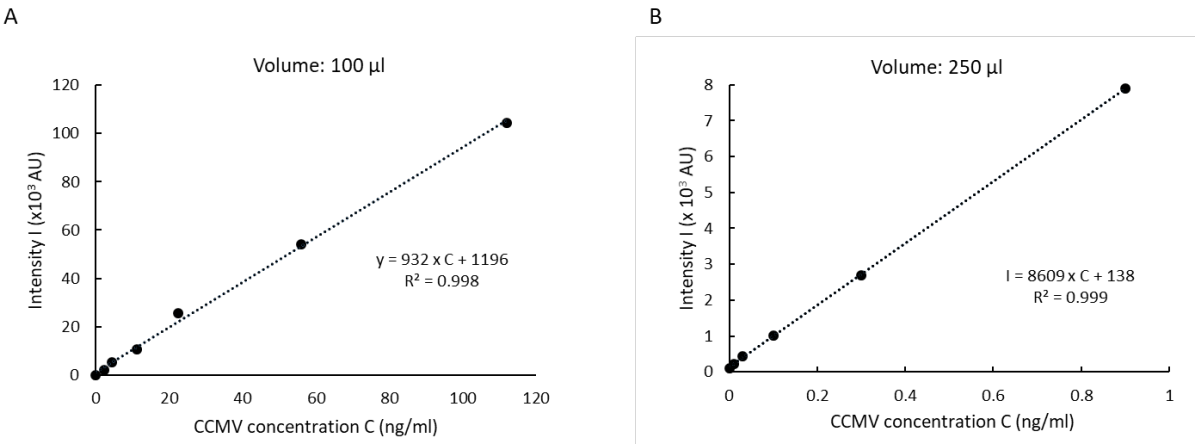


Figure 2: Calibration curves for the determination of the CCMV concentration for volumes of 100 µl (A) and 250 µl (B) with a fluorescence imaging plate reader.

C CCMV characterization with dynamic light scattering (DLS)

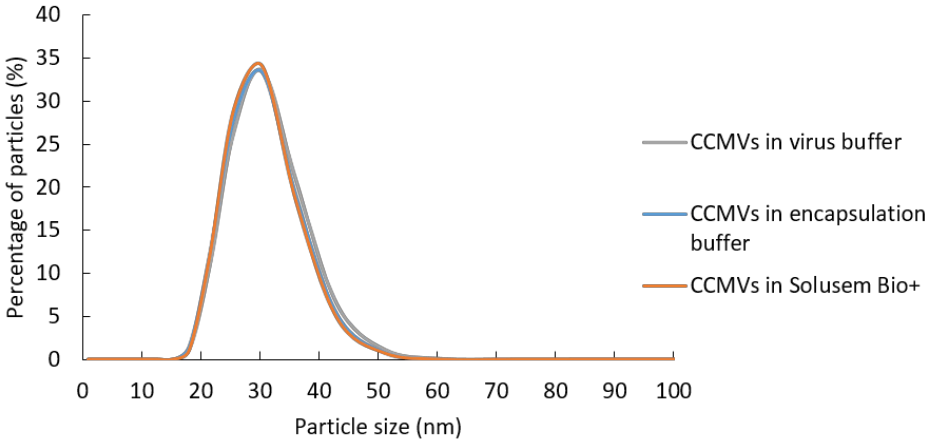


Figure 3: The CCMV size distribution as measured with DLS of CCMVs in virus buffer, encapsulation buffer and Solusem Bio+.

D Chip design optimization

A CCMV spiked semen sample was processed with chip designs I and II to compare the separation quality of both designs. Chip design I had a broadened segment width of 1100 µm, whereas chip design II had a broadened segment width of 2200 µm. With a wider broadened segment width, it is expected that the effluent position of the different sized particles is further apart from each other than for a narrower broadened segment. This effect is thought to improve the separation quality. Because of the enormous size difference between viruses and spermatozoa, it was examined whether the separation quality with chip design I was sufficient. To be able to compare chip design I and II, for both chip designs the flow to outlet 1 (waste) was 2.8% of the total flow. The flow to outlet 1 depends on the channel resistances of both outlets. Therefore, the resistances of outlet 2 were so chosen, that for both chip designs 2.8% of the flow exited at outlet 1. The fluid stream containing the CCMVs was optically investigated during the experiment (figure 4B), whereas the spermatozoa recovery was determined after the separation (figure 4A). With both chip designs, CCMVs were collected in outlet 1, whereas most spermatozoa were collected in outlet 2. The fluid stream containing CCMVs in chip design I (41 µm) was almost twice as wide as the fluid stream in chip design II (23 µm). The spermatozoa recovery was higher in chip design II (72%) compared to the spermatozoa recovery in chip design I (63%). This suggests that the separation with chip design II is more effective than the separation with chip design I.

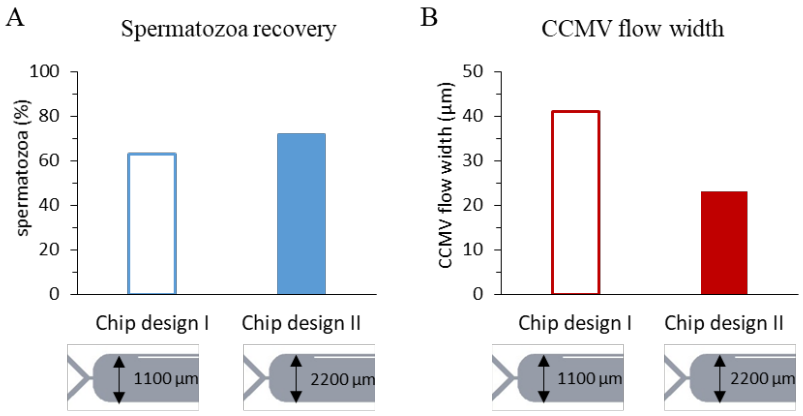


Figure 4: The spermatozoa recovery and CCMV flow width with broadened segment width of 1100 µm (Chip design I) and 2200 µm (Chip design II). The separation quality increases when the broadened segment width is wider. (Outlet 1 volume: 2.8%; sheath pressure 400 mbar; sample pressure: 26 mbar (Chip design I) & 28 mbar (Chip design II); N=1)

E CCMV loss during microfluidic processing

The CCMV loss during microfluidic processing has been investigated. Two sets of experiments were performed to study in which part of the microfluidic setup the CCMVs are lost; (1) the tubing which is connected to the inlets consisting of silicon capillary and Tygon tubing and (2) the setup with the microfluidic PFF chip. Three pressures were applied to induce the sample flow (50, 100, 200 mbar). For the PFF chip, the sample and sheath buffer were the same for this set of experiments. The concentrations before and after the processing are shown in figure 5A and B for the tubing and chip, respectively. In figure 5C the percentage of CCMV loss was determined with respect to the inlet concentration. The CCMV loss in the tubing is between 5 and 10%, whereas CCMV loss approximately 20% after being processed with the PFF chip. This implies that approximately 10% of CCMVs sticks or is absorbed by PDMS. From these measurements, no effect of the applied pressure/flow rate on the CCMV loss is observed.

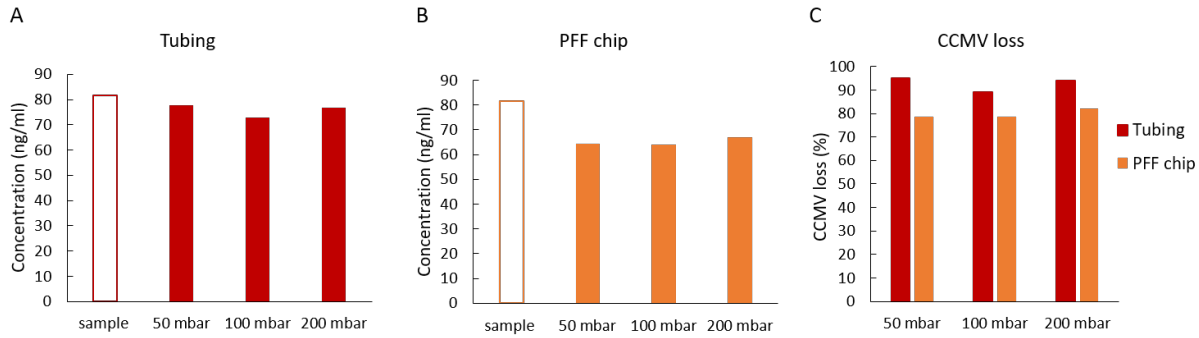


Figure 5: The concentration of CCMV loss during processing with tubing (A) and the microfluidic PFF chip (B) and the percentage of remaining CCMV with respect to the sample (C).

F CCMV and dye adhere to spermatozoa

During the separation experiments with the CCMVs and spermatozoa, the separation has also been visualized with fluorescence microscopy. Thereby it has been observed, that spermatozoa were visible with the FITC filter when CCMVs were spiked to the semen, whereas the spermatozoa were not visible in a sample without CCMVs. Therefore, we have hypothesized that CCMV adhere to spermatozoa. This could downgrade the results of virus separation, because viruses attached to spermatozoa are detected in outlet 2 of the proposed separation technique.

A semen sample with a concentration of $10x10^6$ cells/ml was spiked with CCMV at a concentration of 30 ng/ml. One control sample contained only CCMVs to show that after centrifugation CCMVs free in buffer were eliminated from the sample. Another control sample containing only spermatozoa was used to confirm that spermatozoa are not autofluorescent. The control samples contain CCMVs at a concentration of 30 ng/ml and spermatozoa at a concentration of $10x10^6$ cells/ml. All samples were centrifuged five times at 1500xg for 15 min using the Minispin Plus (Eppendorf, Hamburg, Germany) to eliminate as many free CCMVs in the solution as possible. The samples were re-suspended to a volume of 250 µl and the fluorescence intensity at 488 nm was measured with a fluorescence imaging plate reader. From the fluorescence intensity the concentration was determined with the calibration curve (see Supplementary Information A).

The microscopic images and CCMV concentrations of the CCMV spiked spermatozoa sample and the control samples are shown in figure 6. Spermatozoa imaged with a fluorescence microscope (overlay brightfield and FITC channel) do not show any autofluorescence signal in contrast to spermatozoa which have been spiked with CCMVs (figure 6A and B). To further support this result, the CCMV concentration was determined using a fluorescence plater reader imaging system (figure 6C). The control samples containing only CCMVs or only spermatozoa have both a CCMV concentration below 0.01 ng/ml. Concentrations below 0.01 ng/ml are lower than the calibration curve and the respective intensity value is similar to the background/noise measurement, meaning that these concentrations cannot be determined. From that, we conclude that with the centrifugation steps performed the CCMV concentration below the detection limit and there is no autofluorescence signal from the spermatozoa. The fluorescence signal measured in the CCMV spiked spermatozoa sample corresponds to a CCMV concentration of 0.05 ng/ml, which is according to the calibration curve, above the detection limit. This implies that in a CCMV spiked spermatozoa sample, the CCMVs are not only free in the solution, but also adhere to the spermatozoa.

The CCMVs were labelled with the fluorescence dye ATTO 488 NHS, which can also be the cause of CCMV attachment to spermatozoa. Therefore, it was investigated whether the dye attaches to spermatozoa. A semen sample with a concentration of $10x10^6$ cells/ml was spiked with 10 µg/ml of

ATTO 488 NHS dye. One control sample contained only the dye to show that after centrifugation the dye was eliminated from the sample. Another control sample contained only containing spermatozoa to confirm that spermatozoa are not autofluorescent. The samples contain ATTO 488 dye at a concentration of 10 µg/ml and spermatozoa at a concentration of $10x10^6$ cells/ml. All samples were centrifuged five times at 1500xg for 15 min to eliminate the dye as much as possible. The samples were re-suspended to a volume of 250 µl and the fluorescence intensity at 488 nm was measured with a fluorescence imaging plate reader. From the fluorescence intensity the concentration was determined with a calibration curve (figure 7A).

The concentrations of the dye present in the sample and controls after the centrifugation steps is presented in figure 7B. The control samples consisting of only the dye contains almost no dye after centrifugation, suggesting that the dye was removed by centrifugation. The control sample for the spermatozoa show, that no fluorescence signal is emitted from the spermatozoa. The sample containing both spermatozoa and the dye contains the dye at a concentration 30.6 ng/ml, which is higher than the control groups. This suggests that the dye attaches to spermatozoa.

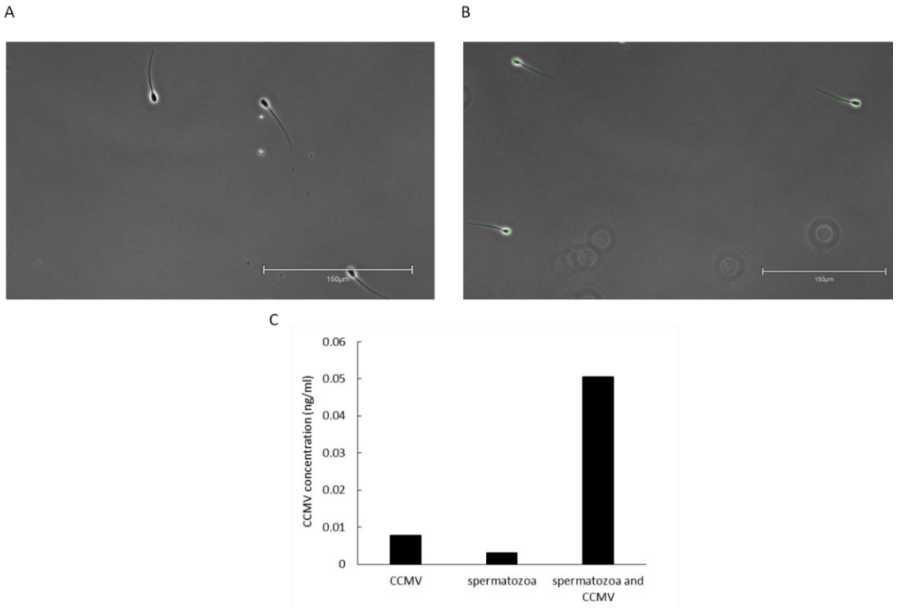


Figure 6: Brightfield and fluorescent image of FITC filter merged of the control sample containing spermatozoa (A) and the centrifuged spermatozoa sample which has been spiked with CCMV (B). C) The measured concentration of CCMV as determined with the fluorescence intensity after several centrifugation steps. CCMV adhere to spermatozoa.

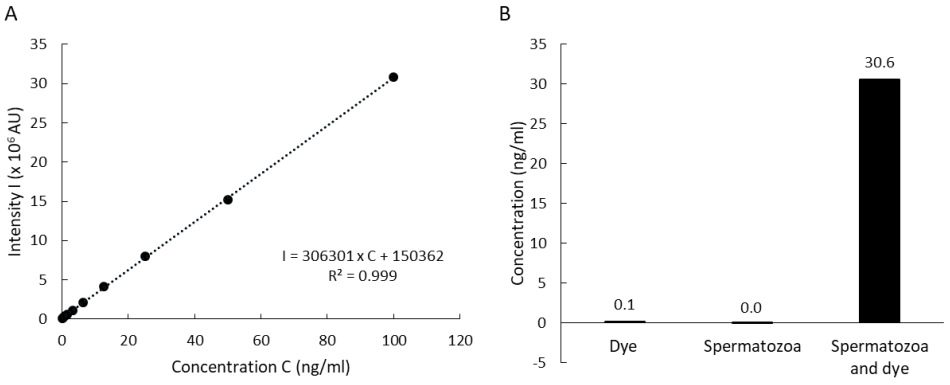
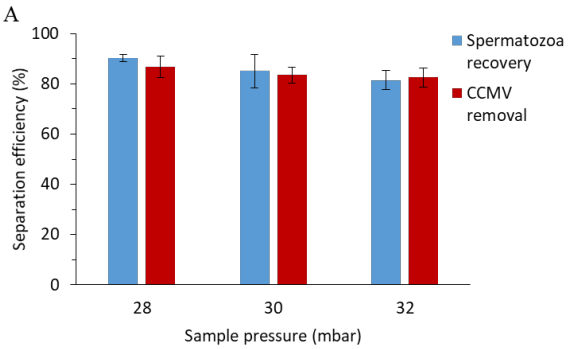


Figure 7: A) Calibration curve of Atto 488 NHS dye in Solusem Bio+. B) The amount of dye present in a spermatozoa sample compared to the control groups. The concentration of dye present in the sample containing spermatozoa and the dye is higher than the control groups, suggesting that the dye attaches to the spermatozoa.

G Separation with chip design I and 2.7% flow removal



B

Sample pressure (mbar)	CCMV flow width (µm)
28	30±1
30	33±3
32	33±1

Figure 8: Separation quality for different sample pressures with chip design II and 2.7% fluid removal ratio A) Spermatozoa recovery and CCMV removal. B) The CCMV flow width from the outer wall in the broadened segment. With increasing sample pressure, the separation quality decreases slightly. The CCMV flow width is below 45 µm, which indicates high CCMV removal. (Error bars=1 SD, N=3, sheath pressure 400 mbar; N=3)

Table 1: Results of independent t-tests

T-tests	p-value		
Sample pressures	Spermatozoa recovery	CCMV removal	CCMV flow width
28 – 30	0.38	0.45	0.28
28 – 32	0.067	0.36	0.14

H Separation of beads (500 nm) from semen

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T. Hamacher, J.T.W. Berendsen, M.L.W.J. Broekhuijse, L.I. Segerink. Separation of viruses from spermatozoa using a microfluidic chip to achieve pinched flow fractionation; NanoBioTech-Montreux Conference; Switzerland, November 18-20, 2019

Separation experiments with polystyrene beads (diameter: 500 nm) were performed in a PFF chip fabricated out of cyclic olefin copolymer (COC) (Micronit, Enschede, the Netherlands). This PFF chips design is similar to chip design I used in the main article; the pinched segment width was 45 µm, the broadened segment width 1100 µm and the separation channel width 45 µm (figure 9).

Spermatozoa were spiked with polystyrene beads with a size of 500 nm. The sample was processed with the COC chip. The number of spermatozoa and beads found in outlet 1 and outlet 2 were manually enumerated using a Neubauer counting chamber. The collection efficiency of spermatozoa was 93%, whereas 98% of the 500 nm beads were depleted (figure 10).

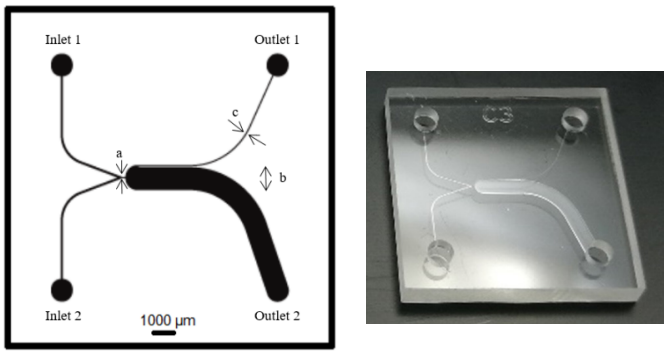

Figure 9: Schematic representation and photograph of the chip. The broadened segment width (b) is 1100 µm and separation channel width (c) is 45 µm.

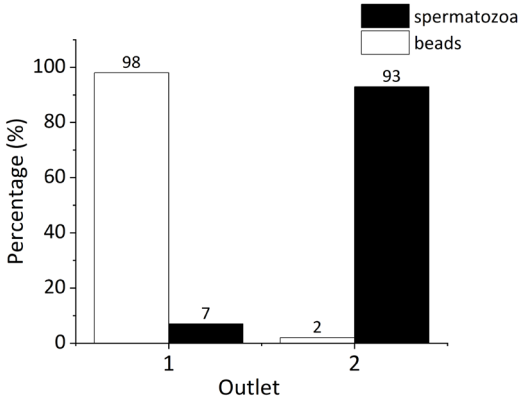

Figure 10: Percentage of particles found in both outlet 1 and 2. Most of the virus-sized 500 nm beads were collected in outlet 1, whereas most of the spermatozoa were collected in outlet 2. Approximately 6000 spermatozoa and 190 000 beads were processed.