Measuring the Sensitivity of a Flow Cytometer using Variation in Laser Power for Extracellular Vesicle research

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Abstract

The sensitivity of the flow cytometer is important for Extracellular vesicle detection, since most EV's are so small that only a fraction gets detected. In this report the sensitivity limit of a modified commercial flow cytometer dedicated to submicrometer measurements is determined. We define the sensitivity limit as the laser power at which the flow cytometer detects only 50% of the polystyrene reference beads. This limit was determined for different sizes of beads by measuring a mixture of 100, 125, 150, 200, and 300 nm beads at 10 different laser powers evenly distributed from 20 to 200 mW with interpolation measurements at 30, 35, and 70 mW. The sensitivity limit was determined to have an upperbound of 20 mW for beads 150 nm and bigger and an underbound of 160 mW for beads 125 nm and smaller.

Keywords: Flow cytometer; Sensitivity; Extracellular Vesicles; Laser power variance

Introduction

Human cells release extracellular vesicles (EV's). EV's are spherical particles with the same phospholipid bilayer as most cells [4]. Bodily fluids normally contain more than 10^{10} EV's per mL [4]. EV's are typically in the range of 30 to 1000 nm [4], making them a lot smaller then the typical eukaryotic cell. In this size range the concentration follows a power law distribution with a negative exponent [4], which emphasizes the importance of the detection of relatively smaller EV's. While the functions of EV's in the human body are, among other things, intercellular communication and waste management, they can also be used as biomarkers for diseases[4]. For example, a higher concentration of EV's in blood plasma is associated with thrombosis and metastatic carcinomas [4]. This is the motivation behind EV analysis.

One of the devices used for EV analysis is a flow cytometer [4]. The flow cytometer uses a sample fluid and a sheath fluid for hydrodynamic focusing [1]. The sample fluid contains the EV's while the sheath fluid is a diluent [1]. By inserting both fluids in a sample stream, with the pressure of the sample fluid higher than the pressure of the sheath fluid, a coaxial flow is created [1]. In this coaxial flow the sample fluid becomes the central core of the stream surrounded by sheath fluid, thereby focusing the sample fluid [1]. Because of this hydrodynamic focusing, a single file of EV's can be created in the sample stream [1], thus the EV's can analysed individually. This analysis is done by shinning a laser beam trough the sample fluid and measuring both the forward scatter (FSC) and the side scatter (SSC) with photomultiplier tubes [1], as shown in figure 1. Using Mie theory a positive correlation between the scatter intensity and the size of the EV is found [3]. This correlation, together with the refractive index of the EV, can relate the scatter intensity to the size of the EV [3]. This is found by measuring reference beads of know size and refractive index [3].

A flow cytometer can accurately analyse around 10,000 EV's/s before swarm detection occurs [2]. Swarm detection is the event that multiple EV's get measured as one EV, because they are simultaneously in the laser beam [4].

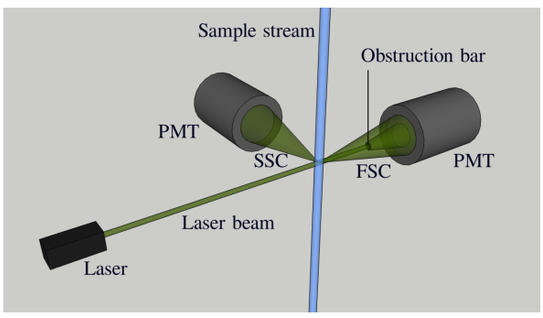

Figure 1: This is a sketch of the scattering in a flow cytometer when an EV from the sample stream comes inside the laser beam. Altough the light will be scattered in all directions, the flow cytometer will only measures forward scatter (FSC) and side scatter (SSC), which is typically at an 90 degree angle with the laser [1]. To not measure the laser beam in the FSC an obstruction bar is used to block the laser [3]. Since the scattered light needs a sensitive detector a photomultiplier tube (PMT) is used [1].

Limiting factors for the size measurements of a flow cytometer are noise and sensitivity [4]. The three factors contributing to the noise generation are optical, electronic and fluidic [4]. To reduce noise, a gate, which is an interval of interest, can be set in the data analysis. This gate is determined

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by a control measurement [4]. But this gate creates a size detection limit for the measurements EV's. Another way to reduce noise is to use lower laser powers. But a size detection limit can also occur by a lack of sensitivity for events with a relatively low scatter intensity, and a lower laser power creates lower scatter intensity. Earlier research has found a size detection limit of 150 to 190 nm for polystyrene beads for flow cytometers dedicated for the measurement of submicrometer particles [4].

For this report, we have measured the sensitivity limit of a modified commercial flow cytometer dedicated to submicrometer measurements. To measure the sensitivity polystyrene reference beads are used instead of EV's, since the size and refractive index of EV's is uncertain [4]. Specifically, we define the sensitivity limit as the laser power where only 50% of the beads are detected. This was found by measuring beads with a diameter of 100, 125, 150, 200 and 300 nm by different laser powers of a coherent sapphire 488 nm laser.

It is important to note that the sensitivity of the flow cytometer is dependant on the scatter of the sample, and thus not only on the size [3]. When applying the results to EV's, which have a different refractive index as polystyrene beads, a conversion calculation using Mie theory should be made [3].

Method

To prevent swarm detection, the initial concentrations, shown in table 1, were diluted with distilled water. The concentrations were diluted to $3.33 \cdot 10^7$ beads/ml to get the desired 10,000 beads/s, since our flow cytometer has a flow rate of 0.3 μL/s. This dilution was done in two steps using multiple pipettes. First, all the concentrations were diluted 1000 fold by combining 999 μL of distilled water with 1 μL of the initial concentration, this dilution was called the halfway dilution. Secondly the halfway dilution was further diluted depending on the initial concentrations of beads to make the final dilution. The exact amount of halfway dilution and distilled water combined is shown in table 1.

SB	IC	HD	DW
100 nm	unknown	20 μL	180 μL
125 nm	$9.3 \cdot 10^{12}$ beads/ml	2 μL	556 μL
150 nm	$5.4 \cdot 10^{12}$ beads/ml	2 μL	322 μL
200 nm	$2.3 \cdot 10^{12}$ beads/ml	5 μL	340 μL
300 nm	$6.7 \cdot 10^{11}$ beads/ml	10 μL	190 μL

Table 1: The second column of this table shows the initial concentrations (IC) for the different sizes of beads (SB). The third and fourth columns show the volumes of halfway distillations (HW) and distilled water (DW) which were combined to make the final dilution.

After the dilution, each different final dilution was measured in the flow cytometer with the laser power at 200 mW. Since the order of magnitude of the measured counts was the same for all sizes except the 100 nm beads, a mixture was made containing 100 μL of the different final dilutions, except for the final dilution of the 100 nm beads, of which only 50 μL was added. This combination was diluted 10 fold because the count rate in the flow cytometer with the laser power at 200 mW with the noise subtracted was above 10,000 beads/s.

10 measurements of the final combination were made in the flow cytometer, with laser power first at 20 mW and increasing in steps of 20 mW until the last measurement at 200 mW was made. 3 extra interpolation measurements at, 30, 35 and 70 mW, were also made. Additionally, 10 control measurements of distilled water were made, with the same laser power as the non-interpolation measurements of the final combination.

When a measurement of the flow cytometer is made, the sample gets added in the sample stream. This process takes about 20 seconds. After the sample is added, the measurement starts. When preforming a measurement, a program will detect when the difference in intensity in the photomultiplier tube gets higher than the threshold of 200 arbitrary units. When this occurs, it saves the height, area, width and time of the fluctuation as an event. All our measurement were made for 50 seconds.

To analyse measurements the program Flowjo is used. Flowjo can create a histogram of the heights of the SSC events, an example of such a histogram is shown in figure 2. This direction of scatter was used, since the SSC is relatively high when the sample is smaller then the wavelength of the laser [4]. The heights of the fluctuations were used, because the maximum intensity occurs when the bead is in the center of the laser beam [1]. Thus the height of the fluctuation measures the total intensity of the scatter of the bead [3]. The peaks in the histogram correspond to the different sizes of beads, because the size is positively correlated with the intensity of the scatter, according to Mie theory [3]. The total events in the peaks can be counted by manually applying a gate around the peak, this number is called the counts of the peak. The noise is mostly in the lower end of the histogram, since stochastic fluctuations tend to have a smaller intensity. This results in some peaks for smaller beads appearing in the noise. To best account for this, the noise counts, found using the corresponding control measurement, are subtracted from the counts in the peaks for the 100, 125 and 150 nm beads measurements. Since no control measurement was made corresponding to the 3 interpolation measurements, the control measurement with the higher intensity where taken.

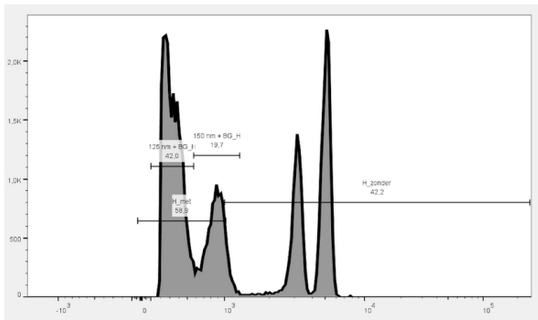

Figure 2: This is a Flowjo generated histogram of the heights of the fluctuations of the measurement taken at 60 mW. The end of the noise area was determined in the corresponding 60 mW control measurement. The two left 200 and 300 nm peaks are outside the noise area while the two right 125 and 150 nm peaks are inside the noise area.

Results

The counts of the peaks depending on laser intensity are plotted in figure 3 and 4. The exact measurements and Flowjo analysis can be found at https://github.com/ChristineSLind/flow-

cytometer-sensitivity-research-rawdata-january-2023.

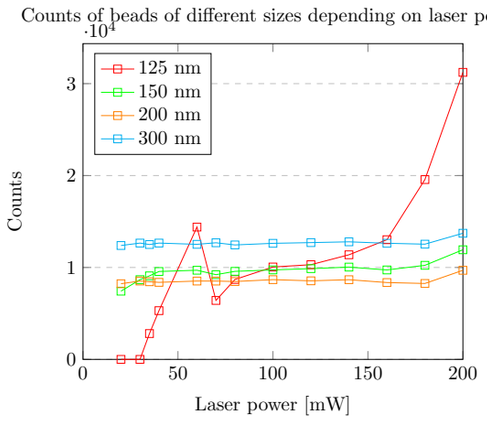

Figure 3: This graph contains the counts of beads of different sizes measured depending on laser power. Each measurement was 50 seconds and the counts were manually gated on the SSC histogram of heights using Flowjo.

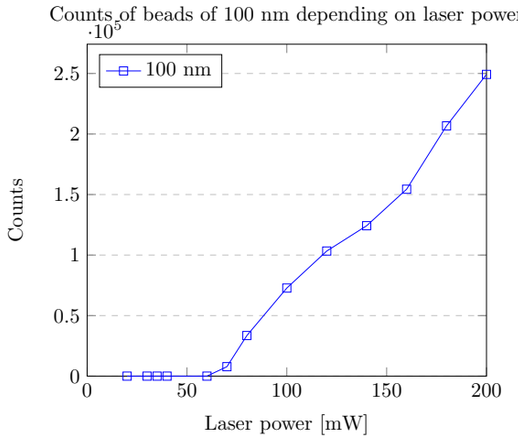

Figure 4: This graph is an addition to figure 3, with 100 nm beads. This case was isolated because of the difference in order of magnitude of the counts.

Since the measurements were all taken for 50 seconds, the results in figures 3 and 4 are proportional to the count rate. Since the sensitivity limit depends only on the proportion of the count rate, a conversion from measured counts to count rate is unnecessary.

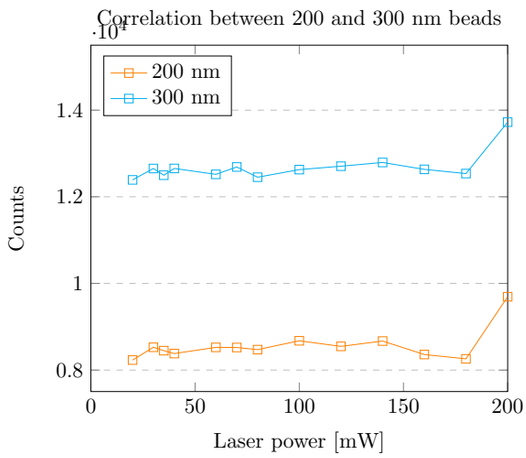

Figure 5: This graph shows the correlation between the measurements with 200 and 300 nm beads.

Discussion

A point of interest in figure 3 are the 200 and 300 nm size beads. Since both are constant, except for relatively small fluctuations. This implies that, for these two sizes, all the beads are measured for all tested laser powers. Thus the sensitivity limit lies below 20 mW.

The trends of 200 and 300 nm beads are correlated, especially around 200 mW, as seen in figure 5. This suggests that the fluctuations in the trends are caused by a variation in the flow rate of the flow cytometer. For this reason, the other bead sizes are scaled compared to measurements of the 200 nm beads in figure 6. There is also a difference of around $4 \cdot 10^3$ counts between the counts of the two sizes of beads. This indicates that the concentrations of the different size beads are not the same. Thus the theoretical concentration of $3.33 \cdot 10^6$ beads/mL for 125, 150, 200 and 300 nm beads was not reached in the final concentration. This can be due to imprecisions introduced when pipetting or inaccuracies in the initial concentrations. Analysis can still be done by assuming that the constant measurements at high laser powers measure the total concentration. Because when all the beads are measured, the amount of measured beads will not increase at higher laser power.

To find the sensitivity limit, the counts are scaled on a relative scale to the counts of the measurement of the laser power at 200 mW, this is done in figure 6.

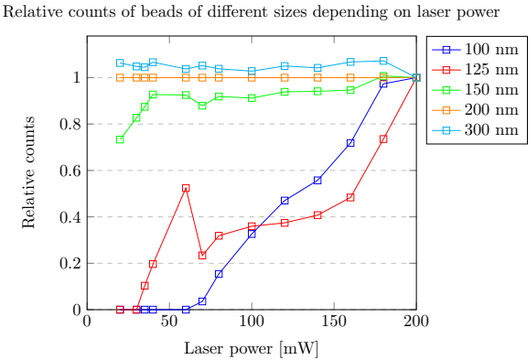

Figure 6: This graph contains the data from figures 3 and 4 scaled to the beads of 200 nm to account for the flow rate variation and then relatively scaled to the value at 200 mW to make the 50% of the sensitivity limit readable.

Figure 6 illustrates that, for 150 nm beads, all beads are detectable when the laser powers exceeds 40 mW. For smaller laser powers, only a portion of the beads get detected, but this proportion stays above 50% for all laser powers above 20 mW. Thus the sensitivity limit of 150 nm beads is below 20 mW.

Although the trends of 100 and 125 nm beads do cross the 50% value in figure 6, there is again not enough data to determine a sensitivity limit. This is because the relative count does not stay constant at relatively high laser powers. Thus it is uncertain, even improbable, that all the beads get detected at 200 mW, and therefore the true relative measurement can not be determined. The lack of sensitivity is not unexpected, since van der Pol also found a size detection limit of 150-190 nm for polystyrene beads using a dedicated flow cytometer [4]. The points where the trends cross the 50% can be used to determine an underbound for the sensitivity limit of the 100 and 125 nm beads. This

should be around 160 mW, since this is the underbound for 125 nm beads, and the sensitivity limit of 100 nm beads should be below that.

There is also an anomaly around 60 mW at the measurement of the 125 nm beads in figure 6. An explanation for this anomaly is 100 nm bead interference. 100 nm beads around 60 mW are not distinguishable from noise. And since this 125 nm measurement is taken with a gate in the noise peak, see also figure 2, it is probable that there are still 100 nm beads detected in the noise which get mistaken for 125 nm beads when the noise counts gets subtracted. The order of magnitude difference in absolute counts between the two bead sizes imply that: a proportionally small amount of 100 nm beads can have a proportionally large effect on the counts of 125 nm beads. This gives explanation for the anomalous peak.

Another, more subtle, anomaly that is indicative for a bigger phenomenon is the dip in the 150 nm beads trend at 70 mW. This is probably caused by a overestimation of the noise for this measurement, since this is an interpolation measurement and thus the 80 mW control measurement was used. Although the anomaly is isolated in this case, the same phenomenon is expected to occur for 30 and 35 mW measurements. This is of interest, since it is the beginning of the downward trend for 150 nm beads. This can indicate that the true downward trend for 150 nm beads is more curved upwards than it appears in figure 6.

Future research into the sensitivity limit of our flow cytometer should be done in the interval of 125 to 150 nm beads. A new study could achieve more data points by preparing a sample with a constant concentration of beads between 125 to 150 nm beads, mixed with a concentration of 300 nm beads. The 300 nm peak could then be used as a reference peak to analyse change in the 125-150 nm plateau with high accuracy using Mie theory [3], when applying varying laser powers.

More general research in the sensitivity limit of flow cytometers could account for a potential interval of only 25 nm when using peak analysis of polystyrene reference beads with concentration determination using the plateau of the bead counts at relatively high laser powers.

Conclusion

Beads with the size of 150 nm and up have an upperbound of 20 mW for the sensitivity limit. Beads with the size of 125 nm and below have an underbound of 160 mW for the sensitivity limit. These results can be converted to EV sizes depending on the refractive index via Mie theory.

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