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Strategies for enumeration of circulating microvesicles on a conventional flow cytometer: Counting beads and scatter parameters

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Abstract

Enumeration of circulating microvesicles (MVs) by conventional flow cytometry is accomplished by the addition of a known amount of counting beads and calculated from the formula: MV/μl = (MV count/bead count) × final bead concentration. We sought to optimize each variable in the equation by determining the best parameters for detecting 'MV count' and examining the effects of different bead preparations and concentrations on the final calculation. Three commercially available bead preparations (TruCount, Flow-Count and CountBright) were tested, and MV detection on a BD FACSCanto was optimized for gating by either forward scatter (FSC) or side scatter (SSC); the results were compared by calculating different subsets of MV on a series of 74 typical patient plasma samples. The relationship between the number of beads added to each test and the number of beads counted by flow cytometry remained linear over a wide range of bead concentrations ($R^2 \geq 0.997$). However, TruCount beads produced the most consistent (concentration variation = 3.8%) calculated numbers of plasma $CD41^+$/Annexin V^+ MV, which were significantly higher from that calculated using either Flow-Count or CountBright ($p < 0.001$). The FACSCanto was able to resolve 0.5 μm beads by FSC and 0.16 μm beads by SSC, but there were significantly more background events using SSC compared with FSC (3113 vs. 470; $p = 0.008$). In general, sample analysis by SSC resulted in significantly higher numbers of MV ($p < 0.0001$) but was well correlated with enumeration by FSC for all MV subtypes ($\rho = 0.62$–0.89, $p < 0.0001$). We conclude that all counting beads provided linear results at concentrations ranging from 6 beads/μl to 100 beads/μl, but TruCount was the most consistent. Using SSC to gate MV events produced high background which negatively affected counting bead enumeration and overall MV calculations. Strategies to reduce SSC background should be employed in order to reliably use this technique.

Keywords

Flow cytometry, absolute counting, microvesicle, microparticles, extracellular vesicles, submicron particles, scatter

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Introduction

Microvesicles (MVs) are a type of extracellular vesicle (EV) that bud off directly from the plasma membrane of activated or dying cells.[1] They are small (<1 μm) membrane-bound particles that can be identified by their cell surface markers, and they circulate throughout the body carrying biological remnants of their cells of origin.[1] MVs are regularly found in body fluids, including human plasma.[2] Their numbers and constitution have been documented to change in times of pathological conditions, and as such, they are considered ideal biomarkers for diagnosis and prognosis of various disorders.[3] However, absolute MV counts vary widely between studies, suggesting that their detection and accurate quantification remains a challenge.

Despite many new 'nano' technologies emerging in recent years, enumeration and analysis of circulating MV by flow cytometry continues to be the method of choice.[4] In spite of its sizing limitations, flow cytometry offers one of the few means to simultaneously detect multiple subsets of MVs and is also the most likely method to be easily adopted for clinical purposes owing to its widespread use in diagnostic pathology. Standardization remains a priority, and there have been numerous reports on how different variables can affect MV measurements. These variables include pre-analytical variables, such as blood collection and timing, needle gauge, anticoagulants, sample transport, centrifugation and storage; testing variables, such as choice of antibody and fluorochrome; and analytical variables, such as brand of flow cytometer, fluorescence compensation approaches, threshold settings and gating strategies.[4–9] To this end, the International Society on Thrombosis and Haemostasis (ISTH) has been instrumental in developing consensus guidelines detailing best practice recommendations for evaluation of circulating MVs by flow cytometry.[7,10,11] More recently, ISTH has collaborated with other key organizations, the International Society of Extracellular Vesicles (ISEV) and the International Society on Advancement of Cytometry, to develop a comprehensive set of methodological guidelines for collection, isolation and measurement of EV using a range of common techniques.[12] In addition, ISEV published 'minimal information for studies of EV' in 2014, providing advice on methods and reporting of EV isolation, characterization and functional studies.[13,14] Such standardization is imperative to reduce the variability within and between methods in order to allow comparison between studies as well as develop diagnostic parameters for routine testing of circulating MVs.

Although the newer generation of flow cytometers can measure volume and thus provide absolute particle counts, more conventional instruments without this capability can still be used successfully for quantitative MV detection.[15–17] An often overlooked aspect of MV enumeration using such classical flow cytometry methods involves the spiking of samples with a known amount of commercially available counting beads to enable calculation of the concentration of MV in the starting material. The general formula for this is:

$$\mathrm{MV}/\mu\mathrm{l} = (\mathrm{MV\ count}/\mathrm{bead\ count}) \times (\mathrm{total\ number\ of\ beads}/\mathrm{test\ volume})$$

Hence, more variables are introduced, which can ultimately affect the final MV result. While all of the variables in the above formula can be manipulated in each experiment, there is limited information on changes of bead concentration and its impact on total MV enumeration. Counting beads are distinct from the small (<1.0 μm) sizing beads available to establish MV gating parameters, and unlike the latter, there are no commercially available counting beads specific for MV enumerations; hence, much larger beads, meant for lymphocyte enumeration, are generally employed. Few manufacturers specify the final analysis volume, leaving the bead concentration up to the individual researcher. Hence, it is unknown whether the relationship remains linear over a wide range of bead concentrations or whether this differs between bead types and sizes.

As for the parameters used to determine the 'MV count', the main testing variable is the actual flow cytometer and gating strategies employed. Enumeration and analysis of such small particles can be performed using forward scatter (FSC) or side scatter (SSC) as the main sizing parameter. The choice is usually determined by the type of instrument being used; with wide angle FSC (1–19°) machines such as Beckman Coulter (Brea, California, USA) generally performing better using FSC, compared with low angle FSC (1–8°) machines such as BD Biosciences (San Jose, California, USA), which typically perform better using SSC. A thorough cross-instrument evaluation has been performed to standardize gating parameters between the different types.[7] However, for those cytometers that perform equally well on either parameter, it is difficult to know which provides more reliable results.

Thus, the aims of this study were to (i) compare different concentrations of commercially available counting beads to establish their limitations and optimal parameters and (ii) determine whether FSC or SSC is a better sizing parameter for enumeration of circulating MVs by flow cytometry on a single BD FACSCanto (BD Biosciences).

Materials and methods

Subjects

A series of 74 platelet-free plasma (PFP) samples from patients with type II diabetes were chosen as representative of a typical patient cohort for analysis. These samples were collected from patients (aged 50–75 years, body mass index (BMI) 25–35 kg/m^2) recruited between 2014 and 2016 from diabetes clinics at the Department of Endocrinology in Austin Health, Melbourne, Victoria, as part of a separate

study on circulating MV levels in type II diabetes. The study followed the guidelines set out within the Australian National Statement on Ethical Conduct in Human Research (2007; Updated May 2015) and was approved by the Austin Health (HREC/12/Austin/63) and Hunter New England Area Human Research Ethics and Governance Committees (SSA/15/HNE/141). All procedures were conducted in accordance with the Helsinki Declaration of 1975, as revised in 2008, and written informed consent was obtained from all participants.

Blood processing

Peripheral blood was collected into 3.2% sodium citrate and processed at room temperature within 2 h of collection. Whole blood was centrifuged at $400\times g$ for 15 min to separate the cellular fraction from the plasma. The latter was carefully removed, transferred to a fresh tube and further centrifuged at $2100\times g$ for 15 min. All but the bottom 500 µl was transferred to a fresh tube and centrifuged again at $2100\times g$ for 15 min to produce PFP. This was aliquoted, stored at −80°C and then thawed at 37°C immediately prior to analysis.

Antibody staining of PFP for MV analysis

Staining of MV was performed as previously described.[11,18] A 10 µl aliquot of PFP was incubated at room temperature for 30 min with various combinations of antibodies conjugated to phycoerythrin (PE), fluorescein isothiocyanate (FITC), allophycocyanin (APC) or PE-cyanine (PE-Cy5): CD41-PE (clone PL2-49, Biocytex, Marseille, France; platelet marker), CD42b-FITC (clone HIP1, BD Pharmingen, San Diego, California, USA; platelet marker), CD235a-APC (clone GA-R2 (HIR2), BD Pharmingen; erythrocyte marker), CD105-PE (clone 1G2, Beckman Coulter; endothelial marker), CD31-PE (clone WM59, BD Pharmingen; against endothelial marker PECAM-1), CD62e PE-Cy5 (clone 68-5H11, BD Pharmingen, against activated endothelial marker E-selectin) and Annexin V-APC (eBioscience, San Diego, California, USA; phosphatidylserine). All assays were diluted to a final volume of 500 µl in phosphate-buffered saline (without Ca^{2+} and Mg^{2+}) or calcium-rich binding buffer (for those stained with Annexin V), with the addition of a known quantity of counting beads and 15 µM D-Phe-Pro-Arg-chloromethylketone (PPAK) to inhibit clumping.

Counting beads

Three different popular brands of counting beads were compared: TruCount (BD Biosciences; size not specified), Flow-Count (Beckman Coulter; 10 µm diameter) and CountBright (Molecular Probes, Eugene, Oregon, USA; 7 µm diameter). Each bead preparation was diluted into the final test volume at the indicated concentration by adding exact quantities based on the individual lot concentration provided by the manufacturer. The plasma source, concentration and acquiring time on the flow cytometer were kept constant. For experiments comparing FSC with SSC, CountBright beads were used at a final concentration of 50 beads/µl.

Analysis of MV by flow cytometry

All flow cytometry analyses were performed on a standard configuration BD FACSCanto (BD Biosciences) equipped with two lasers (488 nm and 640 nm). The FACS flow pressure was set to 3.0 lbf/in^2 and the low flow rate adjusted to a factor of 0.61 (decreased from the original factory settings of 4.5 lbf/in^2 and 0.75, respectively) to improve resolution at smaller sizes. Analysis of MV was performed as previously described[11,18] and according to guidelines established by the ISTH Vascular Biology Scientific Standardization Committee on the standardization of platelet microparticle enumeration by flow cytometry incorporating modifications suggested for the BD FACSCanto (BD Biosciences).[10] The cytometer was calibrated for FSC resolution using Megamix sizing beads (a blend of 2:1:1 of 0.5, 0.9 and 3 µm diameter fluorescent beads) or for SSC resolution using Megamix-Plus SSC (a mixture of 0.16 µm, 0.20 µm, 0.24 µm and 0.5 µm beads) both purchased from Biocytex. Voltages were set at FSC = 570 V and SSC = 390 V for FSC detection or FSC = 350 V and SSC = 631 V for SSC detection. The lower MV detection limits were set according to the manufacturer's instructions, with thresholds of FSC = 200/SSC = 200 employed for FSC enumeration and SSC = 3200 for SSC gating. Fluorescent voltages were set to 654 V for FITC, 485 V for PE, 544 V for PE-Cy5 and 400 V for APC (with the exception of Annexin V-APC detected at 500 V). Counting beads were detected on PerCP-Cy5.5 at 290 V using FSC gating and 549 V using SSC gating. Events were collected for 60 s (bead experiments) or 120 s (patient samples; to enable adequate number of counting bead events[19]) at low flow rate prior to analysis using FACS Diva software (BD Biosciences). The absolute number of MV in each plasma sample was calculated using the formula: MV/µl = (MV count/bead count) × (total # beads/test volume).

Statistical analysis

Data for continuous variables are expressed as mean ± standard deviation or median (interquartile range) where appropriate. Variables that were not normally distributed were analysed using non-parametric tests. Differences in mean levels of multiple normally distributed continuous variables were assessed using one-way analysis of variance (ANOVA) with post hoc Scheffe test for multiple comparisons. Mann–Whitney *U* test and Wilcoxon matched-pair tests were used to detect differences between medians for individual and paired data, respectively. Correlations

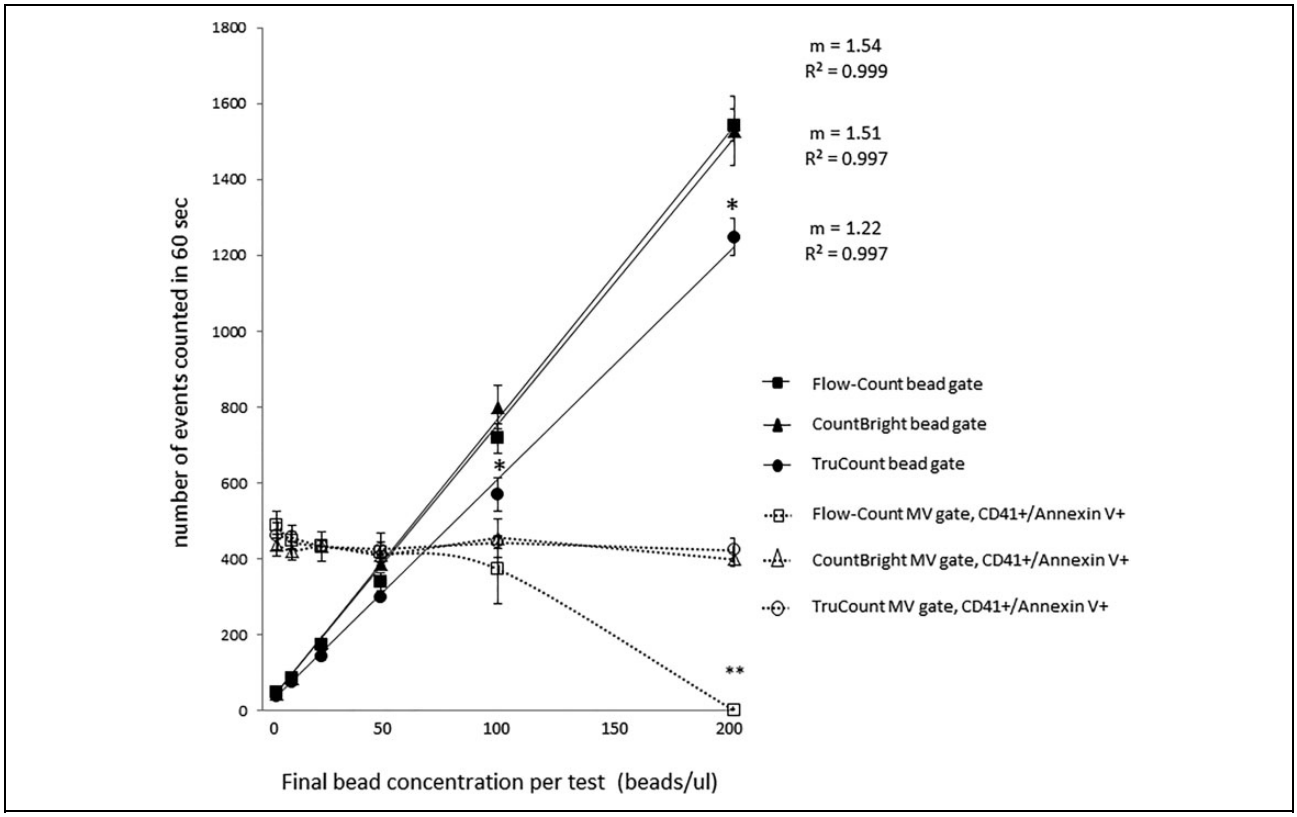

Figure 1. Correlation between the number of bead events counted versus added to each test and MV events counted using different brands of absolute counting beads. Solid lines and filled icons represent bead events, whereas dotted lines and outlined icons represent corresponding raw CD41$^+$/Annexin V$^+$ MV events detected by flow cytometry for each bead dilution. *$p \leq 0.05$ for TruCount versus CountBright and/or Flow-Count bead events; **$p \leq 0.001$ for Flow-Count versus TruCount and/or CountBright MV events.
MV: microvesicle.

between continuous variables were assessed by Pearson's product-moment or Spearman's rho (ρ) where appropriate. All calculations were performed with Statistica v10.0 (StatSoft, Tulsa, Oklahoma, USA) or STATA v11 (StataCorp LLC, College Station, Texas, USA) using two-tailed tests, and p values <0.05 were considered statistically significant.

Results

Comparison of counting beads

The manufacturer's method was followed as closely as possible to prepare a series of identical plasma samples containing varying concentrations of three different brands of counting beads: TruCount (BD Biosciences), Flow-Count (Beckman Coulter) and CountBright (Molecular Probes), respectively. All tests were performed in triplicate.

Figure 1 shows that for all three brands of counting beads tested, the relationship between the number of beads added to each test and the number of beads counted by flow cytometry remained linear over a wide range of bead concentrations (up to 200 beads/μl), as indicated by all correlation coefficients ($R^2 = 0.999, 0.997, 0.997$, respectively), which were close to 1.0. Importantly, these relationships were maintained even at the lowest bead concentrations of 6.26 beads/μl (equivalent to adding just 3125 beads to a 500 μl test). However, the slope (m) of the lines fitted for the CountBright ($m = 1.54$) and Flow-Count ($m = 1.51$) beads was slightly higher than that of the TruCount beads ($m = 1.22$). Thus, the absolute number of TruCount bead events became significantly different from that of the CountBright and Flow-Count at concentrations greater than 50 beads/μl ($p < 0.05$).

We next sought to determine whether the number of raw MV events counted was stable in the presence of different levels of counting beads. As indicated by the dotted lines in Figure 1, the number of CD41$^+$/Annexin V$^+$ raw events was on average 428 $\pm$ 42 and was not significantly different in tubes with added counting beads versus tubes without counting beads (data not shown). This was with the exception of tubes containing 200 beads/μl of Flow-Count beads that derived significantly less MV events than expected ($p < 0.001$), indicating that spiking with high amounts of Flow-Count beads interferes with MV detection.

The numbers of bead and CD41$^+$/Annexin V$^+$ events were then used to calculate the final concentration of CD41$^+$/Annexin V$^+$ MVs in each sample for the three brands of counting beads at different concentrations (Table 1). With the exception of the highest concentration

Table 1. Calculated number of $CD41^+$/Annexin V^+ MVs using different manufacturer's brands of counting beads at different concentrations.

	TruCount™		Flow-Count		CountBright™	
Final bead concentration per μl	Mean ± stdev	%CV	Mean ± stdev	%CV	Mean ± stdev	%CV
200	3397 ± 211	6.2	32 ± 16	50.1	2621 ±49	1.9
100	3912 ± 449	11.5	2591 ± 613	23.7	2841 ± 159	5.6
50	3581 ± 203	5.7	3086 ± 249	8.1	2653 ± 153	5.8
25	3745 ± 204	5.4	3154 ± 244	7.7	3001 ± 171	5.7
12.5	3910 ± 638	16.3	3290 ± 452	13.7	3147 ± 870	27.6
6.26	3693 ± 632	17.1	3167 ± 565	17.8	3051 ± 217	7.1
mean ± stdev	3768 ± 143	10.4	3058 ± 271[a]	14.21[a]	2886 ± 217	9.0
%CV	3.80		8.86[a]		7.50	
p values	0.685		0.417[a]		0.479	
		<0.001			0.724	
Between brands			<0.001			

CV: coefficient of variation; MV: microvesicle; stdev: standard deviation.
[a]Omitting 200 beads/μl results.

of Flow-Count beads (this data point was omitted from the overall analysis), the results were consistent within each bead manufacturer, showing no significant difference between the calculated values at all bead concentrations (within manufacturer ANOVA p values = 0.685, 0.417, 0.479, respectively). TruCount tubes gave the highest overall consistency, with a concentration variation of 3.80% compared to 8.86% and 7.50% for Flow-Count and CountBright, respectively. The number of calculated MV events was highest using TruCount beads (3768 ± 143 MV/μl), and this was significantly different from that calculated using Flow-Count (3058 ± 271 MV/μl, $p < 0.001$) and CountBright (2886 ± 217 MV/μl; $p < 0.001$).

Comparison of FSC versus SSC for gating MV by flow cytometry

In an attempt to optimize the number of raw MV events detected by flow cytometry, we compared two alternate gating strategies on our FACSCanto, one using FSC and the other using SSC as the main sizing parameter. Fluorescent beads of known diameters selected to cover a major part of the theoretical MV size range (0.1–1.0 μm) were used to determine resolution aptitude and establish appropriate MV gates. As the relative position of biological MVs and beads in SSC is different from that in FSC, reference beads of sizes specifically designed for each parameter were used. As shown in Figure 2, the FACSCanto was equally capable of adequately resolving the respective bead mixtures by either FSC or SSC. Threshold parameters were set to exclude as much background as possible, leaving the MV gates set to capture all events below the 0.5 μm bead cloud using FSC and all events between the 0.2 and 0.5 μm bead limits detected using SSC. These MV gates have been shown to be equivalent in order to allow inter-platform comparisons of MV counts.[19]

The respective MV gates were used to detect six different MV subsets in a series of 74 patient plasma samples. The number and type of bead were kept constant (CountBright beads were added to all samples at a final concentration of 50 beads/μl), and events were collected from the same tube for 120 s on each gating parameter. Table 2 presents the number of raw events detected using the respective MV gates as well as the number of CountBright beads counted. These amounts were determined in the absence of added patient plasma at the beginning of each run ($n = 9$) in order to establish the amount of background electronic noise detected using either parameter. This consistently showed significantly more background events in the MV gate using SSC compared to FSC (3113, 2098–23,860 vs. 470, 404–3994; $p = 0.008$), but the number of bead events detected remained equivalent (786, 733–804 vs. 822, 730–865; $p = 0.374$). With the addition of individual plasma samples, events detected in the MV gate were significantly higher using SSC compared to FSC (119,640, 84,320–180,233 vs. 12,476, 7530–27,211; $p < 0.00001$) with an increase that was disproportionate and could not be explained by the initial higher background noise events. In addition, the number of beads counted was significantly reduced in the presence of plasma using SSC (730, 697–771 vs. 822, 730–865 without plasma; $p = 0.008$) and when plasma containing samples were measured on SSC compared to FSC (730, 697–771 vs. 766, 738–793; $p < 0.0001$). In contrast, the number of beads enumerated remained stable when FSC was used to count beads in samples with or without additional plasma (766, 738–793 vs. 786, 733–804; $p = 0.520$). This is further illustrated in Figure 3, which shows a strong negative correlation between the number of CountBright beads counted and the number of events in the MV gate when SSC but not FSC is used as the main gating parameter.

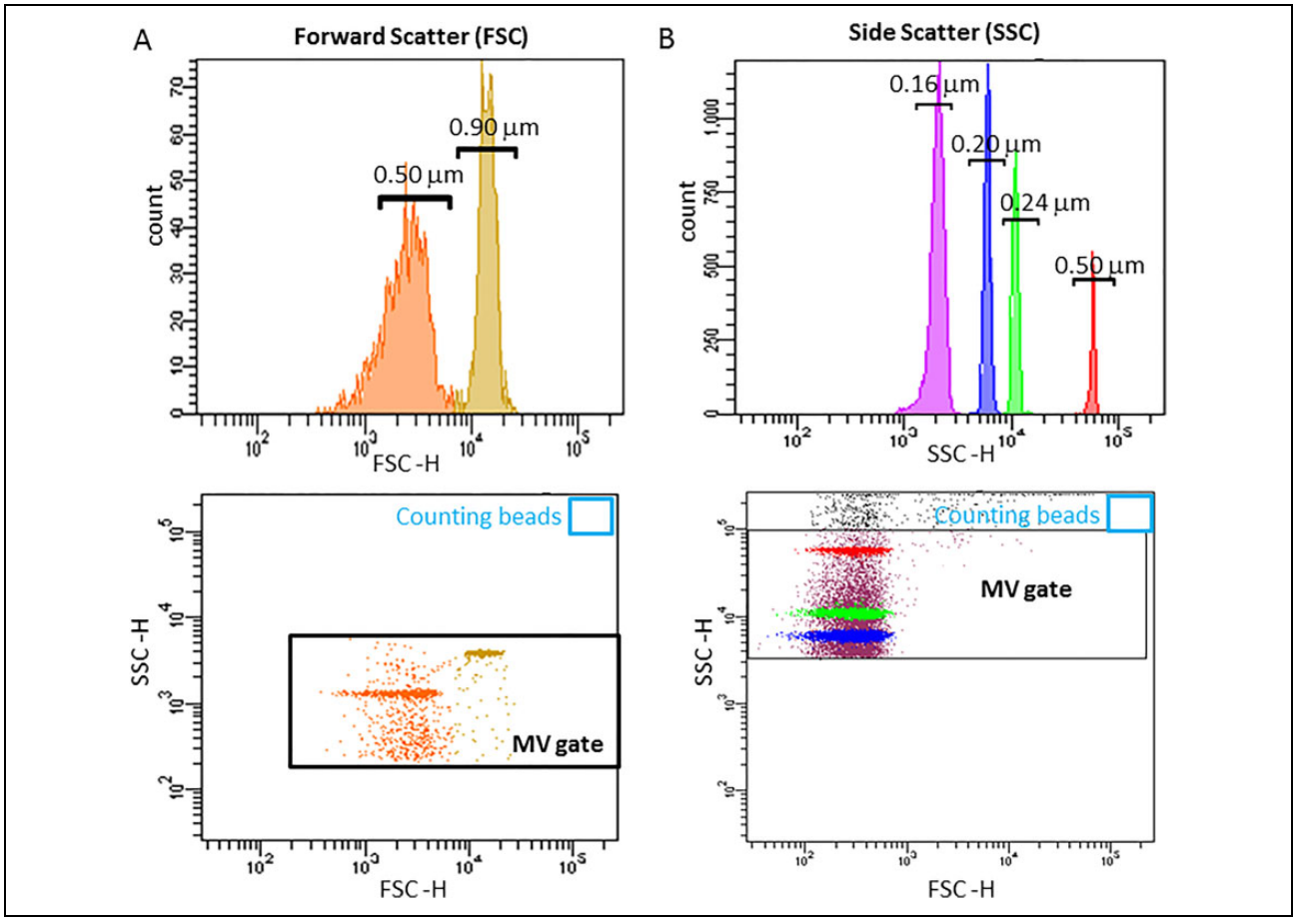

Figure 2. Flow cytometry resolution of sizing beads and MV gate settings using FSC versus SSC as the main size parameter. (a) The histogram in the top panel shows resolution of 0.5 µm (orange) and 0.9 µm (yellow) Megamix beads by FSC. The same beads are depicted in the dot plot below where the 0.9 µm bead cloud is used to set the MV gate. (b) The histogram in the top panel shows resolution of 0.16 µm (pink), 0.20 µm (blue), 0.24 µm (green) and 0.5 µm (red) Megamix-Plus SSC beads using FITC as the main parameter. The same beads are depicted in the dot plot below using SSC as the threshold to eliminate the 0.16 µm (pink) beads and use the 0.5 and 0.2 µm bead clouds to set the MV gate. The gate for capturing the counting beads is depicted in blue on both dot plots.

Table 2. Comparison of raw MV events and beads counted in the presence or absence of plasma using FSC or SSC as the flow cytometry sizing parameter.

	Number of events in MV gate[a]		Number of beads counted[a]		
	Without plasma ($n = 9$)	With plasma ($n = 74$)	Without plasma ($n = 9$)	With plasma ($n = 74$)	p value
FSC	470 (444–656)	12,476 (7530–27,211)	786 (733–804)	766 (738–793)	0.520
SSC	3113 (2497–4727)	119,640 (84,320–180,233)	822 (730–865)	730 (697–771)	0.008
p value	0.008	<0.00001	0.374	<0.00001	

MV: microvesicle.
[a]Values are presented as median (interquartile range).

Not surprisingly, calculation of plasma MV concentrations using data derived from SSC analysis resulted in significantly higher absolute amounts of most MV subsets compared to analysis using FSC ($p < 0.0001$; Figure 4(a)). However, the results for each MV subset were individually well correlated between the two methodologies ($\rho = 0.619$–0.992; $p < 0.0001$; Figure 4(b)).

Discussion

Absolute MV counts vary widely between studies, with circulating platelet MV levels ranging from hundreds to thousands even in control populations.[20–22] Although much attention has been given to the many pre-analytical and methodical variables that can result in such discrepancies,[23] few have addressed the addition of the all-important

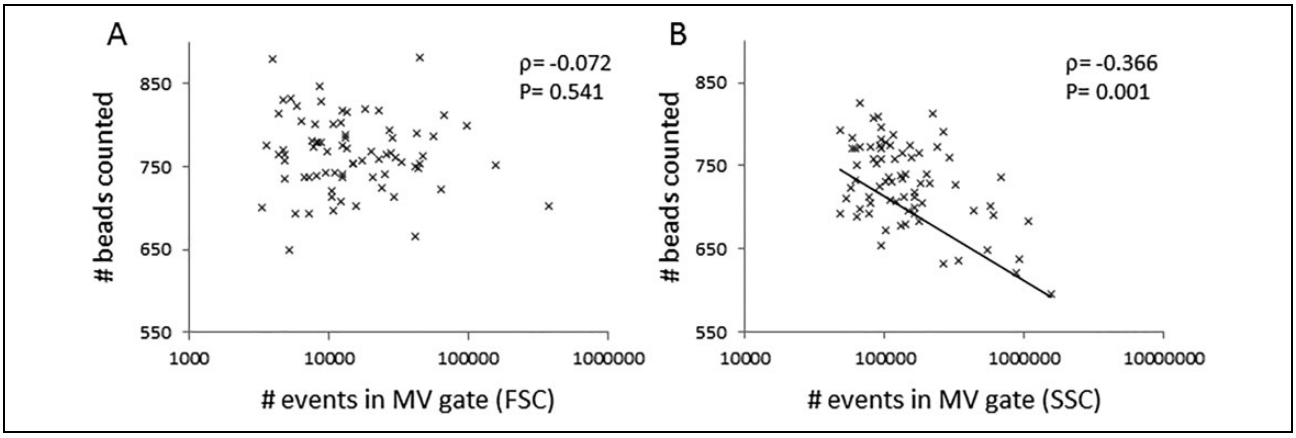

Figure 3. Correlation between the number of beads counted and the number of MV events detected by SSC. Scatterplots show relationship between the number of beads counted and the number of MV events detected in the MV gate when using (a) FSC or (b) SSC as the main detection parameter.

counting beads that enable such calculations. Herein, we compared three different popular brands of fluorescent absolute counting beads: TruCount by BD Biosciences, which is supplied as a lyophilized pellet in individual flow cytometry tubes; Flow-Count (Beckman Coulter) and CountBright (Molecular Probes), both of which are supplied as slurries. All are brightly fluorescent and intended to be used for determining absolute counts of leucocytes in blood. Our results show that although the number of beads counted by the flow cytometer remained linear over a wide range of concentrations for all three brands tested, the TruCount beads gave the most accurate enumerations with the least variation in MV calculated levels. This is perhaps due to the lyophilized format of the beads that may deter clumping and thus minimize the potential for pipetting error that would be more common with slurries. However, the single-use tubes are the most expensive of the three preparations, contain a set amount of beads and require the presence of proteins (such as from plasma or serum) for proper performance, which may limit the utility of such tubes for other, such as purified, MV preparations.

Although all manufacturers specify a certain amount of beads to use per test (equating to 50,000–100,000 beads), only BD provides a recommended final test volume of 520 µl (100 beads/µl) of their TruCount beads, while CountBright manufacturers warn to maintain a final volume of at least 300 µl per test (143 beads/µl). Despite this, both brands performed well at concentrations of up to 200 beads/µl. Of concern was the finding that high levels of Flow-Count beads ($\geq$ 200 beads/µl) significantly interfered with the detection of MV particles. The underlying reasons for inhibition are unclear, and we cannot discount factors other than the beads themselves, such as proprietary stabilizers added by the manufacturer. Nevertheless, our findings are instructive to delineate the concentration parameters where Flow-Count beads can be used to measure MV.

We did not count 1000 bead events as suggested by the manufacturers of Flow-Count and CountBright because our MV enumeration protocol has always been based on that recommended by the ISTH standardization papers. The first of these employed 30 µl of Flow-Count beads in a final volume of 580 µl (approximately 50 beads/µl) and a timed collection of events for 60 s at low flow rate.[10] On our instrument this allows for counts of 350–400 beads, but obviously this varies between laboratories and will be highly dependent on the fluidics pressure of individual machines. We, therefore, have adopted the latest recommendation to increase the collection time to 120 s if the number of beads counted is <500.[19] However, the results presented herein suggest that this will not make any significant difference to the calculated MV results for major populations that are readily detectable (i.e. approximately 400 positive events detected in the MV gate). Perhaps a better guide would be to collect a minimum number of MV events of interest.

Original attempts to standardize flow cytometry analysis of MV using FSC as the main sizing parameter proved that reproducible platelet-derived ($CD41^+$) MV counts could be obtained across many different laboratories worldwide.[10] However, this success was not always shared by laboratories using BD instruments, which demonstrated discrepancies between location of sizing beads and that of biological particles on the FSC parameter, thought to be due to the relatively lower solid angle used to collect FSC signals on these instruments. This could be ameliorated by removing the upper gate limit set by the Megamix beads, a recommendation adopted for the current study.[10] However, the same authors found a more reproducible solution in using SSC as the main sizing parameter, with the use of different sized reference beads for FSC versus SSC being the most critical element for standardization across the different platforms.[7,19]

Our BD instrument produced less variability in MV subset calculations using FSC. Although we found higher

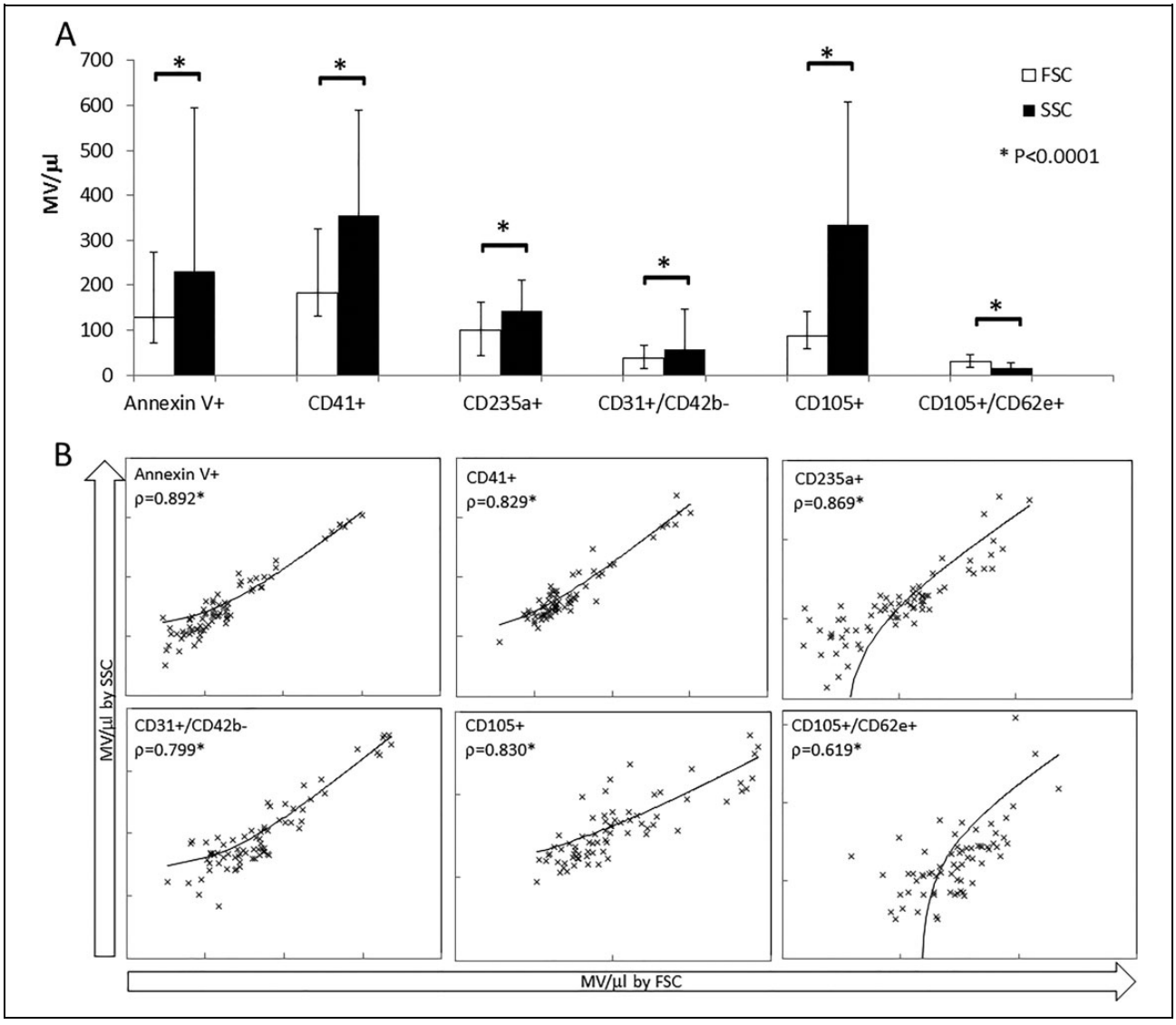

Figure 4. Results of MV subsets calculated from data using FSC or SSC as the main detection parameter. (a) Bar graph illustrating differences in concentrations of MV subsets when calculated from data obtained using FSC versus SSC as the main detection parameter. Bars represent median and interquartile ranges. (b) Correlation of individual MV subset concentrations when calculated from data obtained using FSC versus SSC as the main detection parameter. Scatter graphs are presented on log scales. *$p < 0.0001$.
MV: microvesicle; FSC: forward scatter; SSC: side scatter.

background events detected by the SSC channel compared to FSC, these were not above the recommended background noise threshold ratio of 1.0 (calculated as the number of events per second/maximal number of events per second acceptable by the instrument = [3113/60]/4000 = 0.012 for SSC on our FACSCanto) and consistent, if not better, then most instruments surveyed by Cointe et al.[19] However, the significantly increased number of MV events detected in plasma suggests that SSC may be a more sensitive parameter for small particles, resulting in much higher absolute counts for the majority of MV subsets. This phenomenon was not observed across different instruments in the ISTH multicentre workshop, with similar counts recorded between instruments using SSC or FSC as the preferred sizing parameter.[19] However, the results were highly variable, with standard deviations of up to 50% of the mean for identical samples measured on different instruments. Hence, only a paired study design would be able to address the difference between results measured by different scatter parameters on individual flow cytometers.

The utility of employing polystyrene beads to establish sizing gates for biological material has been the source of much contention. It is well known that polystyrene has a much higher refractive index, resulting in light scattering properties much different from plasma membranes.[24,25] A 400 nm polystyrene microsphere has been shown to produce the same forward light scatter as a 1 µm lipid or cellular vesicle.[24] The Megamix gating strategy originally

established by the Scientific Standardization Committee set the upper size detection limit using 900 nm beads, but this has been estimated in actuality to gate biological vesicles measuring 800–2400 nm in diameter.[25] Such discrepancies have led to proposals by us and others that triggering on fluorescence may provide a much more useful approach.[6,26,27] However, plasma contains many different sizes and shapes of particles, some as small as 30 nm,[28,29] and current technology in flow cytometry remains biased towards detection of only the largest and brightest particles, with many events destined to be lost in the instrument 'noise'.[23] Much more sensitive detection and sizing methods of nanoparticle tracking analysis and/or resistive pulse sensing can provide more accurate measurement of EV concentrations and have confirmed that total plasma EV is highly underestimated by flow cytometry.[28,30,31]

The current study is limited by the few different types of counting beads assayed and the use of a single flow cytometer. Smaller sized counting beads such as the 5.2 μM CytoCount (DAKO, Agilent Pathology Solutions, Santa Clara, California, USA) are becoming more popular and would have made a welcome comparison to the larger Flow-Count and CountBright beads used here. Similarly, all the analyses were done on a single flow cytometer, rendering it the equivalent of a technological 'case study'. It would be of interest to compare our results to other FACSCanto machines as well as other newer instruments with integrated cell counting. Nonetheless, we have highlighted the importance of bead selection, concentration and background minimization for MV analysis by flow cytometry.

Declaration of Conflicting Interests

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