Enhancing Research Output by Combining High Event Rate, Fast Sample Transition Speed, and High-Parameter Flow Cytometry Analysis

Ryan Mendoza, Magda Santos, Elizabeth Dreskin, Veronika Kortisova-Descamps, Richard James Cuthbert
Bio-Rad Laboratories, Inc., 1000 Alfred Nobel Dr., Hercules, CA 94547

Introduction

The ZE5 Cell Analyzer (Bio-Rad Laboratories, Inc., catalog #12004279) is a versatile, high-parameter flow cytometer that has been used to generate data presented in many high-impact publications, covering topics such as antibody development (Momont et al. 2023), cancer (Ng et al. 2023), stem cell therapy (Crees et al. 2023), and molecular biology (DelRosso et al. 2023). However, from these publications alone it is difficult to appreciate how the unique characteristics of the ZE5 Cell Analyzer may have contributed to the quality and quantity of the data collected. Here, we examine three key capabilities of the ZE5 Cell Analyzer that make it the ideal choice for projects that require large amounts of high-quality data. Specifically, we examine its ability to rapidly collect individual samples by maintaining a high event rate. We also demonstrate the speed at which multiple samples can be processed without compromising carryover between samples, as well as how these characteristics can be combined with high-parameter analysis.

The ZE5 Cell Analyzer can be used to obtain data at a rate that is difficult to achieve with other currently available flow cytometers, with no compromise regarding data quality, in both bead- and cell-based assays. Additionally, we reveal how fast sample transition can be combined with high event numbers across a 96-well plate, with minimal carryover but without reducing assay pace. Furthermore, we illustrate how both these concepts can be applied to a functional assay that involves monitoring of cell subpopulations following incubation with a CD20-specific monoclonal antibody. Finally, we describe a complex phenotyping assay using a 27-color panel to measure leukocyte subpopulations and compare a standard throughput to a high-throughput approach in terms of data parity.

Materials and Methods

Flow Cytometry

Unless otherwise stated, all antibody staining was preceded by incubation with Human Seroblock (Bio-Rad, #BUF070B) for 10 min at room temperature. Antibody staining was performed by incubation for 1 hr at room temperature in FACS buffer (Dulbecco's phosphate buffered saline [DPBS], Gibco, #14190144; 2 mM EDTA, Invitrogen, #15575020; 1% bovine serum albumin [BSA], Sigma-Aldrich, #A7284). Following incubation, cells were washed three times, followed by resuspension in FACS buffer. All wash steps were performed by centrifugation at 800 x g. For multicolor experiments, a single-stain control for each included dye was collected for spectral compensation. All data collection was performed using the ZE5 Cell Analyzer, and the data were analyzed using FCS Express 7 (DeNovo Software by Dotmatics).

Bead Assays

A serial dilution of Dragon Green Beads (Bangs Laboratories, Inc.) was prepared. The observed acquisition rate, measured by the ZE5 Cell Analyzer, was compared to the expected acquisition rate, which was calculated using the concentration of beads in the sample and the sample flow rate.

High Event Rate Analysis of PBMCs

Human peripheral blood mononuclear cells (PBMCs) were stained with VivaFix 649/660 Cell Viability Assay (Bio-Rad, #1351118) according to the manufacturer's instructions. The cells were then incubated with the following antibodies at the manufacturer's recommended concentrations: CD3 SBV515 (Mouse Anti-Human CD3 Antibody conjugated to StarBright™ Violet 515 Dye [SBV515], clone OKT3; Bio-Rad, #MCA6146SBV515);

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CD4 SBV440 (Mouse Anti-Human CD4 Antibody conjugated to StarBright Violet 440 [SBV440], clone RPA-T4; Bio-Rad, #MCA1267SBV440); CD8 SBB615 (Mouse Anti-Human CD8 Antibody conjugated to StarBright Blue 615 [SSB615], clone LT8; Bio-Rad, #MCA1226SBB615). For data collection, the cells were resuspended at 5×10^7 cells/ml; three doubling serial dilutions were then prepared to achieve cell concentrations of 5×10^7, 2.5×10^7, 1.25×10^7, and 6.25×10^6 cells/ml, and the cells were analyzed at a rate of 1 µl/sec (n = 5). For data analysis, lymphocytes were identified based on forward and side scatter profiles, doublet exclusion was performed using the side scatter pulse area and height analysis, and dead cells were excluded based on VivaFix 649/660 fluorescence.

Combining a High Event Rate with Fast Sample Transition

Suspensions of stained and unstained Ramos cells (ATCC, #CRL-1596) were loaded into a 96-well plate at a concentration of 14×10^6 cells/ml. Alternating columns of the 96-well plate were loaded as follows: odd-numbered columns were loaded with 100% unlabeled cells and even-numbered columns were loaded with 50% unlabeled cells plus 50% cells labeled with CytoTrack Green (Bio-Rad, #1351203). Analysis was performed starting at row A, column 1 and progressed horizontally to column 12 before moving down one row and restarting at row B, column 1. This pattern was repeated until reaching row H, column 12. All events over the threshold were considered to be cells. To calculate the percent carryover, the number of positive cells observed in odd-numbered columns was compared to the total number of cells measured. This value was then doubled to account for the fact that 50% of the cells in the even columns were stained positively.

High-Speed Analysis of a Functional Assay

Frozen PBMCs were thawed, washed twice in complete RPMI-1640 medium (ATCC, #30-2001) with 1% penicillin-streptomycin solution (Gibco) and 10% fetal bovine serum (FBS; Cytiva, #SH30071.03HI) and then resuspended in complete RPMI-1640 medium at a concentration of 2×10^6 and incubated at 37°C (5% CO_2) overnight. The following day, the cells were passed through a 40-µm filter and washed twice in complete RPMI-1640 medium. The cells were then plated into a 96-well plate at 750,000 cells per well in a volume of 100 µl complete RPMI-1640 medium with Human Anti-CD20 (Rituximab Biosimilar) Antibody (clone 10F381; Bio-Rad, #MCA6091) at concentrations of 100 µg/ml, 25 µg/ml, 6.25 µg/ml, 1.56 µg/ml, 390.06 ng/ml, 97.66 ng/ml, 24.41 ng/ml, and 6.10 ng/ml (n = 5). The cells were incubated at 37°C (5% CO_2) for 4 hr and then washed twice in DPBS.

The cells were stained as described for the high-event rate analysis using the VivaFix 649/660 Cell Viability Assay, followed by incubation with primary antibodies (see Table 1). For data collection, the cells were resuspended in FACS buffer at a concentration of 1×10^7 cells/ml, and 10 µl of each well was collected at a sample rate of 2 µl/sec.

Table 1. Reagents used. Antibodies and the live/dead dye used for high-speed analysis of a functional assay.

Target	ZE5 Cell Analyzer Target Laser: Filter	Fluorophore	Bio-Rad Catalog Number
CD3	405: 700LP	SBV790	MCA6146SBV790
CD4	405: 460/22	SBV440	MCA1267SBV440
CD8	488: 593/52	SBB615	MCA1226SBB615
CD56	488: 525/25	A488	MCA2693A488
CD19	561: 720/60	SBY720	MCA1940SBY720
Live/dead	640: 670/30	VivaFix 649/660	1351118

A488, Alexa Fluor 488; SBB, StarBright Blue; SBV, StarBright Violet; SBY, StarBright Yellow.

High-Speed Analysis of a 27-Color Panel

To demonstrate that high-parameter data can be collected without compromising speed, resolution, or reproducibility, a 27-color panel was developed for immune cell immunophenotyping (Table 2). Major T-cell, B-cell, monocyte, and granulocyte lineages, as well as multiple subsets within these lineages, were clearly identified. For full details of the panel design, staining protocol, and gating strategy, please refer to Bulletin 3597.

Fully stained samples were acquired in standard mode (n = 2) and high-throughput mode (n = 4, Figure 3). Samples collected in high-throughput mode were acquired in approximately 8 sec per sample. All data for the 27-color panel were collected using the ZE5 Cell Analyzer, with a different configuration than the instrument used for all other experiments.

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Table 2. Reagents used. Antibodies and the live/dead dye used for high-speed analysis of a 27-color panel.

Target	ZE5 Cell Analyzer Target Laser: Filter	Fluorophore	Antibody Catalog Number*
HLA DP DQ DR	355: 387/11	SBUV400	MCA477SBUV400
CD20	355: 509/24	SBUV510	MCA1710SBUV510
CD33	355: 577/15	SBUV575	MCA1271SBUV575
Live/dead	355: 615/24	PI	1351101
CD163	355: 670/30	SBUV665	MCA1853SBUV665
CD28	355: 747/33	SBUV740	MCA709SBUV740
CD62L	355: 780LP	SBUV795	MCA1076SBUV795
CD56	405: 420/10	BV421	BioLegend, #318327
CD24	405: 460/22	SBV440	MCA1379SBV440
CD45RA	405: 525/50	SBV515	MCA88SBV515
CD45RO	405: 615/24	SBV610	MCA461SBV610
CD40	405: 670/30	SBV670	MCA1590SBV670
CD2	405: 720/50	SBV710	MCA1194SBV710
CD14	405: 750LP	SBV790	MCA1568SBV790
CD57	488: 525/35	FITC	MCA1305F
CD3	488: 593/52	SBB580	MCA463SBB580
CD11b	488: 692/80	SBB700	MCA551SBB700
HLA ABC	488: 750LP	SBB810	MCA81SBB810
CD10	561: 583/30	SBY575	MCA1556SBY575
CD4	561: 615/24	SBY605	MCA1267SBY605
CD45	561: 670/30	SBY665	MCA87SBY665
CD27	561: 720/60	SBY720	MCA755SBY720
CD38	561: 750/LP	SBY800	MCA1019SBY800
CD16	640: 670/30	A647	MCA5665A647
CD31	640: 720/60	A700	MCA1738A700
CD19	640: 775/50	SBR775	MCA1940SBR775
CD8	640: 800LP	SBR815	MCA1226SBR815

* Antibodies are available from Bio-Rad unless otherwise noted.
A647, Alexa Fluor 647; A700, Alexa Fluor 700; BV, Brilliant Violet; FITC, fluorescein isothiocyanate; PI, propidium iodide; SBB, StarBright Blue; SBR, StarBright Red; SBUV, StarBright UltraViolet; SBV, StarBright Violet; SBY, StarBright Yellow.

Statistics

GraphPad Prism (Dotmatics) was used for all statistical analysis. All box plots show interquartile range (box), mean (line), and extreme values (whiskers). Statistical tests were performed using GraphPad Prism and *P* values lower than 0.05 were considered significant.

Results

High Event Rate Analysis Does Not Negatively Impact Data Resolution

A bead assay was used to examine the capability of the ZE5 Cell Analyzer to process individual samples quickly and whether analysis at a high event rate caused a loss of data resolution. The ZE5 Cell Analyzer maintained a 1:1 ratio between the observed event rate and the expected event at all event rates up to 100,000 events/sec (Figure 1A). For the other flow cytometers tested, the observed event rate began to drop below the expected event rate at approximately 20,000 events/sec (Figure 1A).

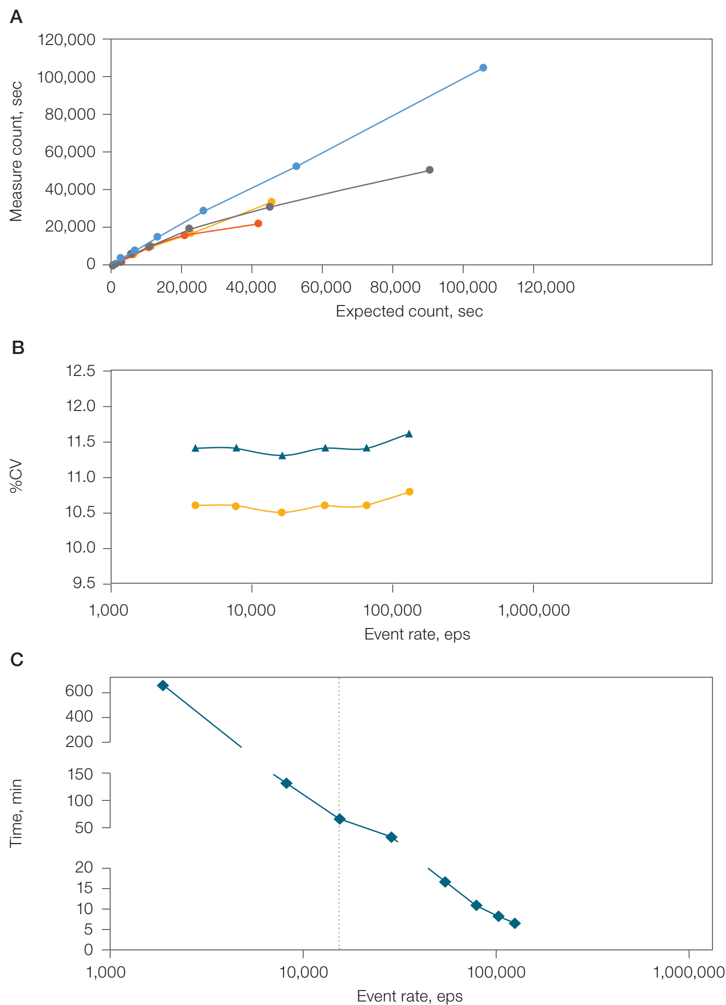

Fig. 1. Acquisition rate demonstration using single samples. A, Serial dilutions of Dragon Green Beads were used to assess the observed versus expected event rate of the ZE5 Cell Analyzer (●) compared to three competitor instruments (●, ●, and ●). **B**, The coefficient of variance (CV) of the fluorescence intensity between 512.5 and 537.5 nm (●) and side scatter (◆) area parameters of a singlet bead population was measured at an event rate of up to 129,000 events/sec (eps). **C**, Acquisition time needed to acquire 40 million events at varying event rates, equivalent to detecting 400 rare events at a frequency of 1:100,000. Data courtesy of Karen Helm, University of Colorado, Denver.

Next, the coefficient of variance (CV) in fluorescence intensity at 512.5 to 537.5 nm and side scatter intensity of Dragon Green Beads at event rates up to 129,000 events/sec was examined. There was no significant change in the data spread due to an increased acquisition rate (Figure 1B). To illustrate the amount of time that can be saved by using a high event rate in rare cell analysis, 40 million events were collected for a single sample; this is equivalent to collecting 400 rare cells at a frequency of 1:100,000. At 20,000 events/sec (equivalent to a data rate achievable with competitor instruments without losing data), acquisition took approximately 50 min; in contrast, acquisition took less than 10 min at 100,000 events/sec (Figure 1C).

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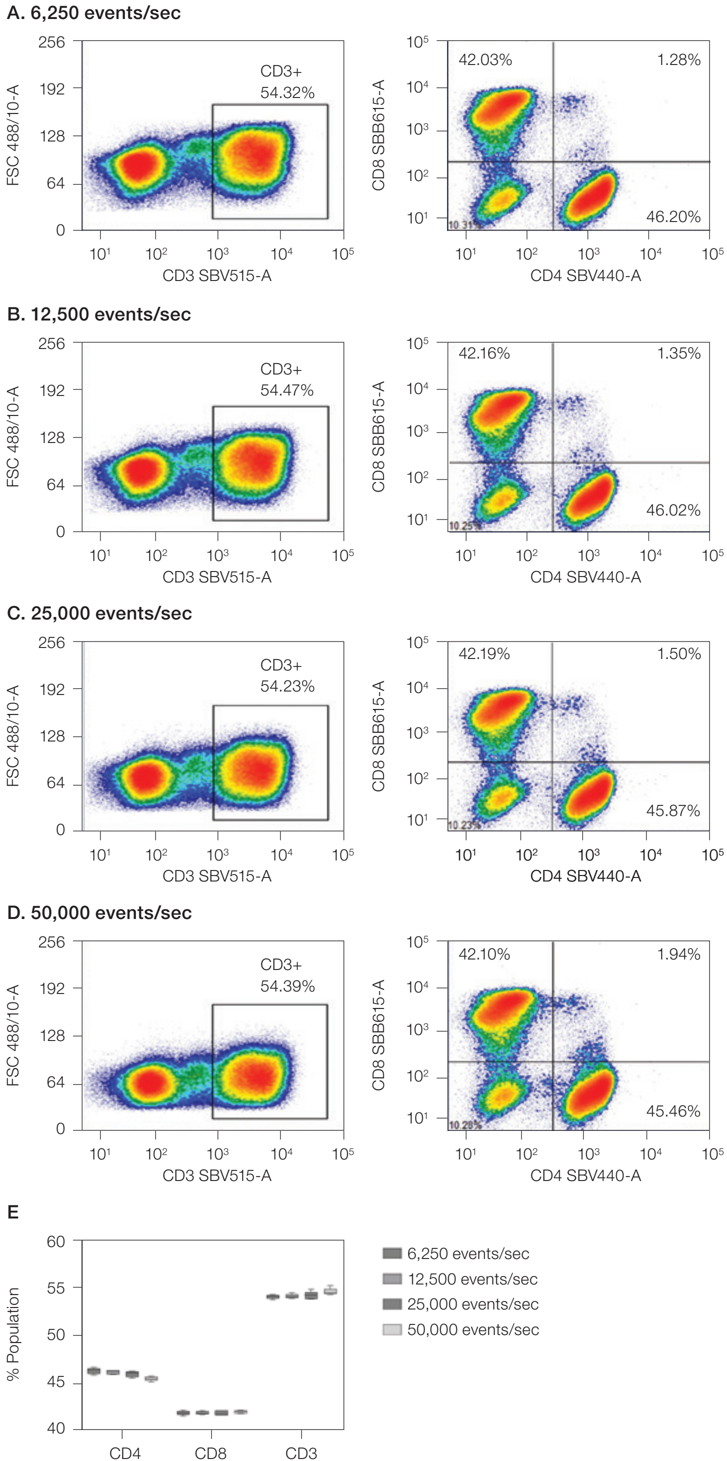

Fig. 2. Data conformity at varying event rates. Following exclusion of debris, dead cells, and doublet events, expression of CD3 was used to identify T cells; CD4 and CD8 expression was subsequently used to distinguish helper and cytotoxic T cells, respectively. Data were acquired at **A**, 6,250 events/sec, **B**, 12,500 events/sec, **C**, 25,000 events/sec, and **D**, 50,000 events/sec. **E**, Box and whisker plot shows cumulatively the proportion of total T cells as a percentage of lymphocytes, CD4+ helper T cells, and CD8+ cytotoxic T cells, n = 5. FSC, forward scatter; SBB, Starbright Blue; SBV, Starbright Violet.

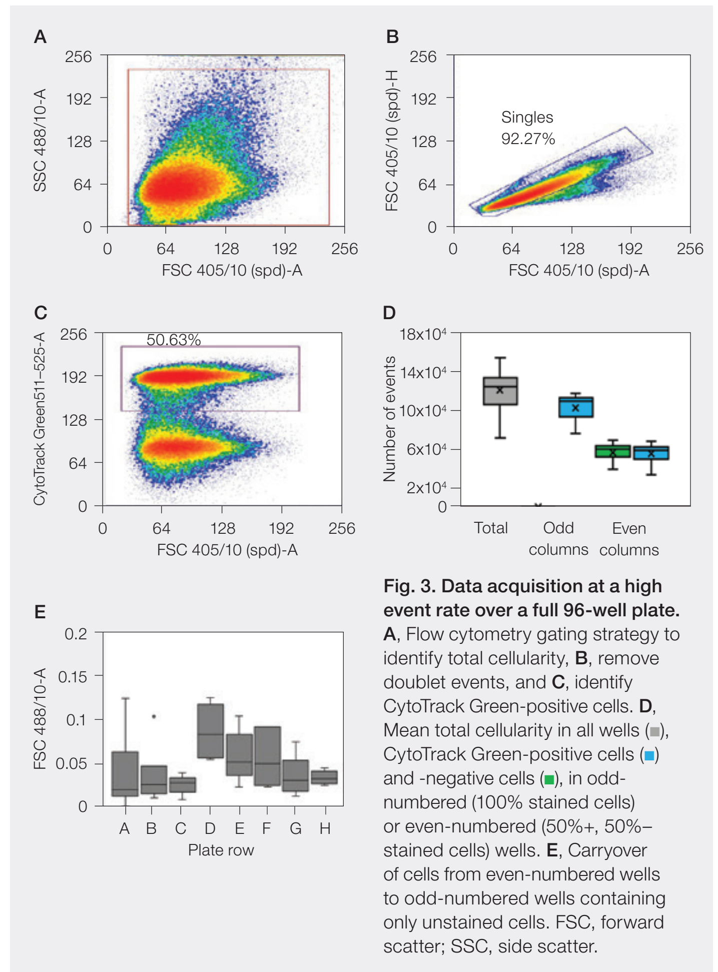

Fig. 3. Data acquisition at a high event rate over a full 96-well plate. **A**, Flow cytometry gating strategy to identify total cellularity, **B**, remove doublet events, and **C**, identify CytoTrack Green-positive cells. **D**, Mean total cellularity in all wells (■), CytoTrack Green-positive cells (■) and -negative cells (■), in odd-numbered (100% stained cells) or even-numbered (50%+, 50%– stained cells) wells. **E**, Carryover of cells from even-numbered wells to odd-numbered wells containing only unstained cells. FSC, forward scatter; SSC, side scatter.

Maintenance of data resolution was confirmed by the analysis of PBMCs using an assay specific for T cells, which were identified based on expression of CD3. Helper and cytotoxic T-cell subpopulations were identified based on expression of CD4 and CD8, respectively (Figure 2A–D). T-cell frequency as a percentage of all viable singlet lymphocytes was comparable at all event rates: 6,250 events/sec (mean = 54.12%; SD = 0.20%), 12,500 events/sec (mean = 54.17%; SD = 0.23%), 25,000 events/sec (mean = 54.24%; SD = 0.41%), and 50,000 events/sec (mean = 54.64%; SD = 0.38%) (all data, n = 5) (Figure 2E). The frequency of helper and cytotoxic T cells measured as a percentage of total T cells showed a similar level of data concordance between event rates (Figure 2E), such that the total mean standard deviation within all groups was 0.31%.

High Event Rate Analysis Can Be Combined with Rapid Sample Transition

The high event rate achievable with the ZE5 Cell Analyzer can be combined with its ability to move between samples rapidly and hence process plates quickly. To demonstrate this, alternating positively and negatively stained Ramos cells in a 96-well plate were analyzed at high speed.

After exclusion of noncellular events (Figure 3A), doublet exclusion was performed (Figure 3B), followed by identification of positive cells (Figure 3C). The total number of cells analyzed in each well

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exceeded the target of 100,000 cells/well (mean = 116,144; SD = 18,280 cells). In even-numbered wells, an accurate 1:1 ratio was confirmed (mean = 50.8%; SD = 0.87%, Figure 3D). The percentage carryover of positive cells measured in odd-numbered wells was remarkably low (mean = 0.045%; SD = 0.033%, Figure 3E). There was no clear correlation between the analysis order and rate of carryover, suggesting effective probe cleaning between the wells (Figure 3E). The total analysis time for the entire plate was <15 min.

Rapid Completion of Functional Assays

To demonstrate how high event rate high-speed analysis can be applied to real-world functional assays, the effect of an anti-CD20–specific monoclonal antibody on PBMCs was assessed. After selection of PBMCs based on forward and side scatter profiles, doublets and dead cells were excluded from the analysis. B cells were selected based on expression of CD19. T cells were identified based on expression of CD3 then further subdivided into helper and cytotoxic T cells based on expression of CD4 and CD8, respectively. CD56 expression was used to identify NK cells from the CD3, CD19 double-negative population (Figure 4A). Incubation with the anti-CD20–specific monoclonal antibody caused a significant drop in the proportion of B cells compared to isotype controls at antibody concentrations of 100 µg/ml, 25 µg/ml, 6.25 µg/ml, 1.56 µg/ml, 390.06 ng/ml, and 97.66 ng/ml, as calculated by two-way ANOVA. Maximal B-cell ablation was observed at an antibody concentration of 390 ng/ml (mean = 92.63%, n = 5) (Figure 4B). However, there was no significant change in any other measured population compared to isotype controls at any antibody concentration tested (Figure 4C). The total number of wells assayed was 81, a mean of 113,408 cells was collected from each well, and the analysis was completed in approximately 10 min.

Samples acquired in each mode revealed a high level of reproducibility across all subpopulations examined (Figure 5). These data confirm the utility of the ZE5 Cell Analyzer for analysis of high-parameter data in high-throughput mode and demonstrate that high-parameter data can be collected at speeds more commonly associated with lower parameter screening assays.

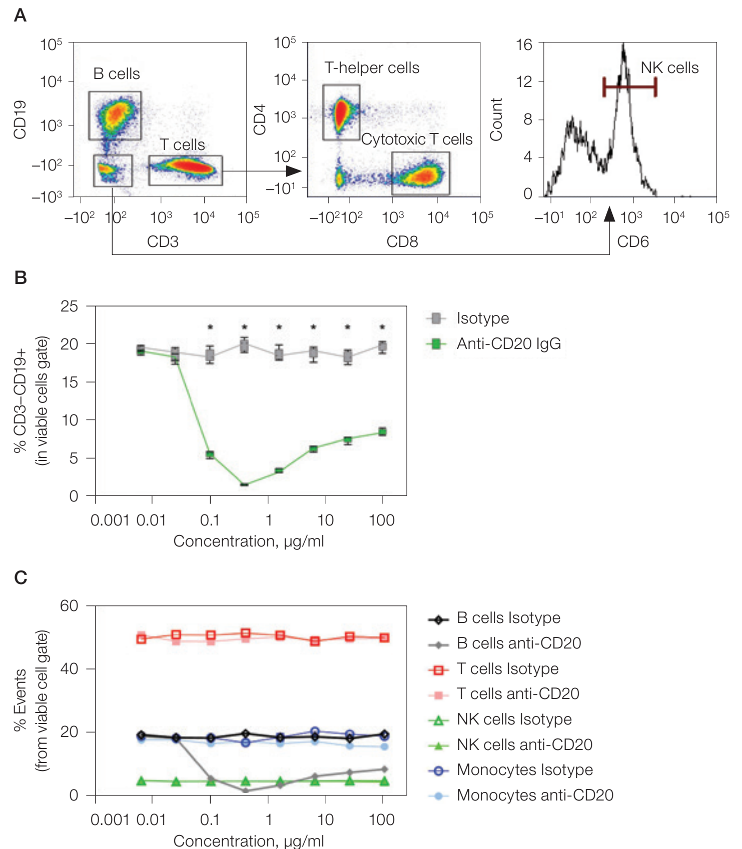

Fig. 4. High-speed functional assay. Immunophenotyping gating strategy — B cells and T cells were identified based on expression of CD19 and CD3, respectively. **A**, T cells were further subdivided based on expression of CD4 and CD8, respectively, and CD56 was used to identify NK cells from the CD3, CD19 double-negative population. **B**, The number of B cells expressed as a proportion of viable cells incubated with varying concentrations of CD20 monoclonal antibody compared to isotype control, n = 5; * denotes $P < 0.05$, as calculated by two-way ANOVA. **C**, All measured cell populations expressed as a proportion of viable cells incubated with varying concentrations of CD20 monoclonal antibody compared to the isotype control; points indicate the mean value, n = 5. Ig, immunoglobulin; NK, natural killer.

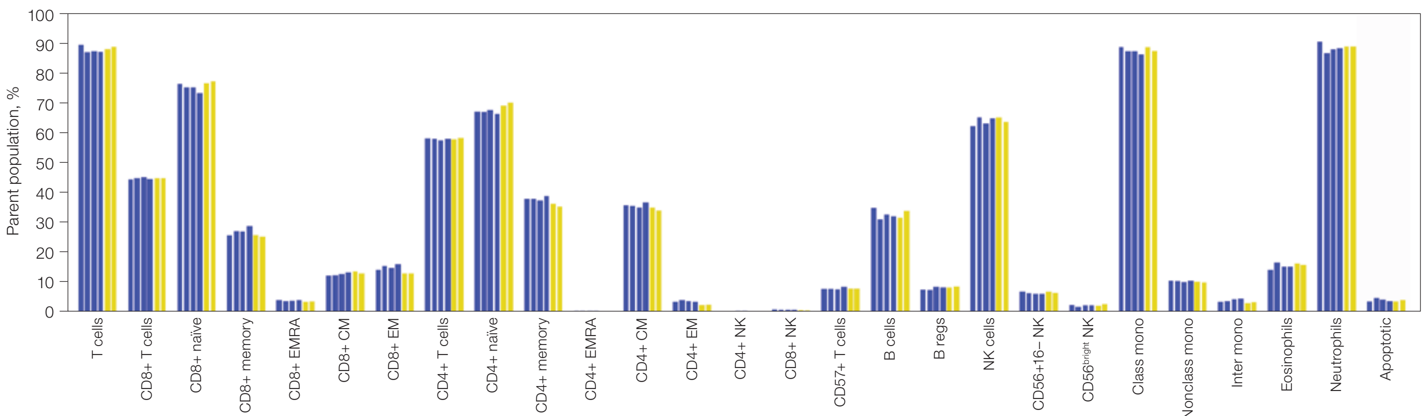

Fig.5. Comparison of data acquired in standard and high-throughput mode on the ZE5 Cell Analyzer. Cell lineages are expressed as a percentage of their parent cell population. Each bar represents an individual replicate collected in high-throughput mode (■) and standard mode (■). B regs, regulatory B cells; Class mono, classical monocytes; CM, central memory T cells; EM, effector memory T cells; EMRA, terminally differentiated effector memory cell re-expressing CD45RA T cells; Inter mono, intermediate monocytes; NK, natural killer cells; NKT, natural killer T cells nonclass mono, nonclassical monocytes.

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Discussion

Three key elements are combined in the ZE5 Cell Analyzer that allow it to collect data faster than other currently available cytometers, including high event rate, fast sample transition, and high multiplexing capabilities. As demonstrated, it can collect up to 100,000 events/sec without losing any data due to electronic aborts. The instrument achieves this without compromising data resolution (Figure 1). We showed that sample flow rates up to at least 50,000 cells/sec are reliably achievable. However, it should be noted that factors including cell size and propensity for aggregation can influence cytometer performance and the need for careful cell preparation is paramount when performing high event rate analysis. We also revealed that this high-speed analysis does not negatively affect data quality (Figure 2), with the potential to save significant amounts of time when collecting data for rare cell populations.

Using the instrument's dedicated high-throughput mode, its ability to analyze data at high event rates can be applied to high-throughput assays requiring many samples to be processed rapidly. This allows a large number of events to be collected from each sample while only spending a few seconds acquiring data from each well. As depicted in Figure 3, we achieved an average cell number in excess of 110,000 cells acquired per well in a 96-well plate, with a total analysis time of less than 15 min. This short analysis time is facilitated by the instrument's ability to load multiple samples into the sample line, separated by an air and water gap, which drastically reduces analysis time by eliminating the need to empty and fill the sample line between each sample. Additionally, the instrument was able to accurately distinguish doublets, leading to a near perfect 1:1 ratio observed between positive and negative cells. This is due to fast electronic data sampling and the high flow rate, which allows doublets to be discriminated easily even at high event rates (Figure 3D). The low number of carried-over cells illustrated in Figure 3E confirms adequate cleaning facilitated by the flying wash collar positioned on the sample probe. Moreover, the lack of a clear correlation between the analysis order and the number of carried-over cells suggests that there is no progressive increase in cells retained in the sample line.

Overall, the high event rate and fast sample transition can be leveraged to perform functional assays rapidly, as demonstrated in Figure 4, clearly indicating that detailed phenotypic assays can be performed rapidly. The extremely high level of reproducibility between replicates shown in Figure 4B–C supports the assertion that this type of assay can be successfully performed with high levels of accuracy. This level of accuracy is facilitated by the high number of events collected from each well. The total analysis time of approximately 10 min to collect data over 81 samples demonstrates that these types of assays can be performed at analysis speeds more commonly associated with dedicated screening instruments. Furthermore, incubation with a CD20-specific antibody caused a decrease in the observed proportion of B cells from a mixed population of PBMCs, with the maximum effect observed at an antibody concentration of 390 ng/ml. However, we found no change in any other measured cell population, suggesting that the biological effect was restricted to B cells. It should be noted that the proportion of B viable cells at antibody concentrations higher than 390 ng/ml was slightly increased, but further investigation is needed to determine the reason for this observation.

Finally, we demonstrated that a high event rate and quick transition time between samples can be combined with the ability to collect up to 27 fluorescent parameters simultaneously. As depicted in Figure 5, we observed a high level of reproducibility between samples regardless of acquisition speed, suggesting no clear difference between data collected in standard versus high-throughput mode. This reinforces the conclusion that phenotyping assays can be performed at high speed without compromising data quality or quantity.

Conclusion

To date, there have been numerous examples of how the capabilities of the ZE5 Cell Analyzer can be leveraged in a diverse range of applications, including in the study of immunogenicity (Siegel et al. 2022), microbiology (Fugaban et al. 2021), and protein trafficking (Coukos et al. 2021), as well as the monitoring of immunotherapy patients (Sanjabi and Lear 2021). The utility of the ZE5 Cell Analyzer stems from its ability to effectively combine a high event rate, fast sample transition speed, and 27 fluorescence–parameter analysis. These attributes are achieved without any deleterious effects, resulting in a synergism that allows virtually any assay to be performed at the speed of a screening instrument. These insights offer potential benefits for laboratories struggling to meet user demand, provide opportunities for researchers to expand the scope of investigations, and reduce lengthy periods of data collection.

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