November 2017

CYTOFLEX

APPLICATION NOTES

EVERY
event matters.

BECKMAN COULTER
Life Sciences

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CytoFLEX Flow Cytometer Platform

Join the Resolution REVOLUTION

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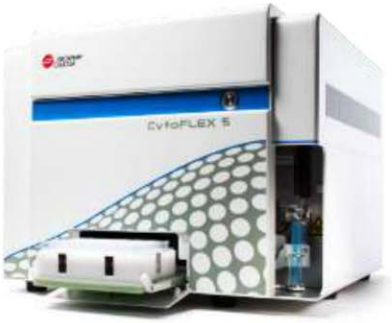
Benchtop Cytometry without Compromises

The CytoFLEX Platform is a revolutionary system presenting optimal excitation and emission, minimizing light loss and maximizing sensitivity. Since its initial unveiling, the compact system with innovative technology borrowed from the telecommunications industry has garnered attention from the flow cytometry community. Since that time, we have continued to expand the platform, creating even more choices for researchers.

We continue to leverage the power of the platform:

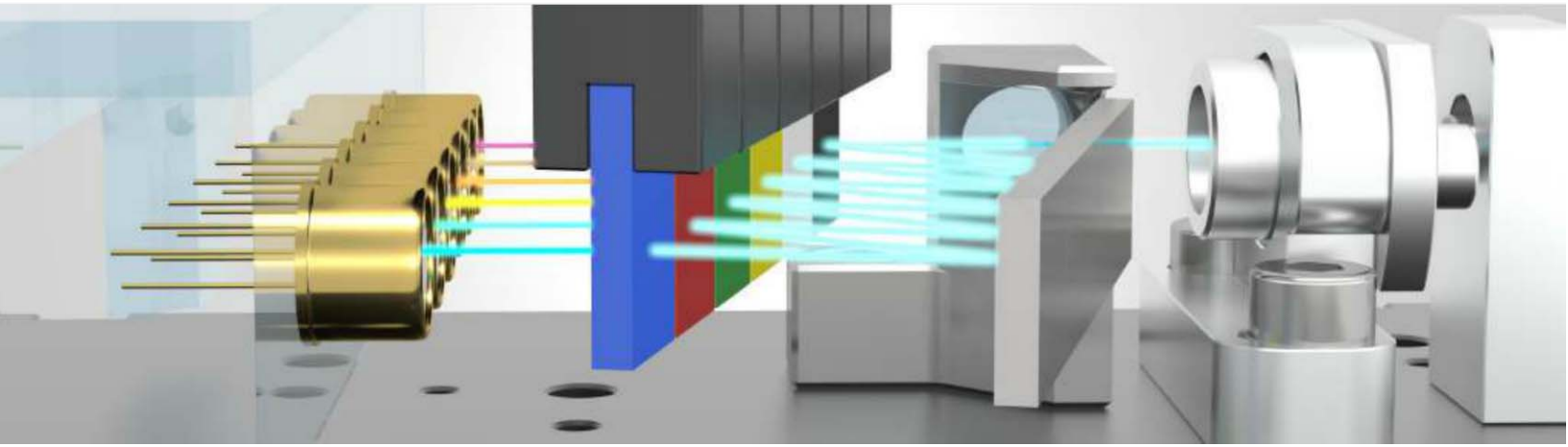
- Exquisite sensitivity
- Small particle analysis in a benchtop analyzer
- Extensive set of repositionable band pass filters
- Flexibility to upgrade by adding additional parameters
- Intuitive software to facilitate multicolor analysis

Up to 3 lasers



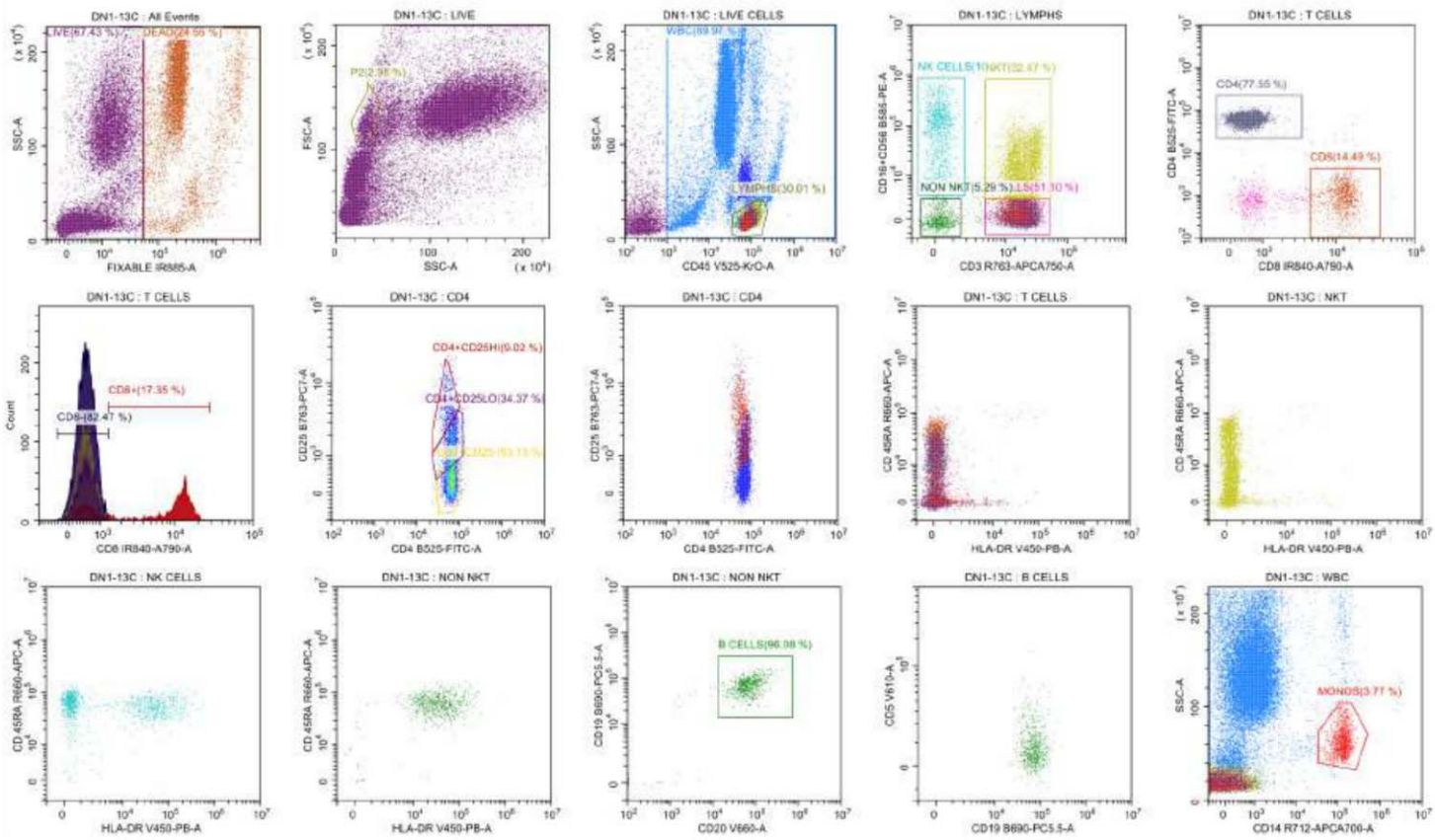
Up to 4 lasers

Up to 6 lasers



Focus on the Science

The CytoFLEX Flow Cytometer provides the performance you need in an easy to use system allowing you to focus on the science, not the instrumentation. The system can be configured for the needs of your laboratory, whether it is routine low complexity analysis, high complexity analysis, or analysis that pushes the boundaries for flow cytometry.



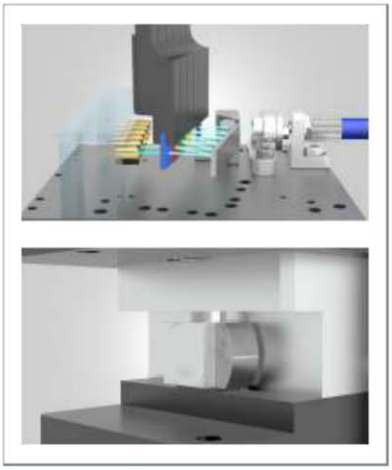
T cell subset analysis of human peripheral blood by 13 color immunophenotyping. Analyses of T cell subsets based on the differential expression of surface molecules related to cell function, differentiation, or activation have evolved. As a result, T cell analysis requires multiple markers to capture the various populations. Using the IR channel to add LIVE/DEAD analysis frees up other channels for typical cell surface marker analysis. In addition, using an IR channel for the relatively bright CD8 marker reduces compensation requirements and preserves sensitivity for dim markers.

"Being within the department of biology, we're able to put a diverse range of samples onto the instrument and to see how it performed. Certainly I'd like to add that it had a very good high resolution. It had a low noise, which meant the signal to background for example was exceptional."

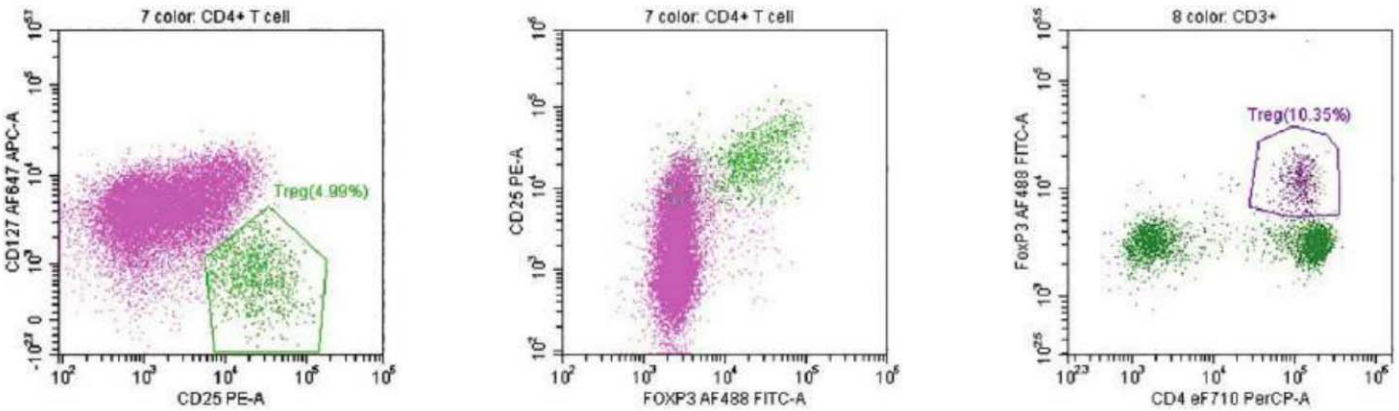
Karen Hogg, PhD, Bioscience Technology Facility, University of York

Harness the Power of Advanced Sensitivity

A unique assembly of technologies contributes to the exquisite sensitivity of the platform. Borrowing technology from the telecommunications industry, the Wavelength Division Multiplexer (WDM) deconstructs and measures multiple wavelengths of light. The WDM relies on fiber optics and band pass filters to separate the light wavelengths. Unlike more traditional instruments, multiple dichroic filters to direct the light path are not required. This makes it much easier to configure the fluorescence channels, but also increases light efficiency as light loss due to refraction is minimized. The WDM utilizes Avalanche Photodiode detectors (APD), versus Photomultiplier tubes (PMT). One hallmark of the photodiode is the high quantum efficiency in excess of 80%, especially for wavelengths greater than 800 nm.



Another component of the system which increases efficiency is the use of integrated optics to focus light onto the flow cell. With conventional analyzers, laser excitation sources are optimized by shaping and focusing light through a series of lenses and filters onto the flow cell. Each of these light interactions is an opportunity for light loss. All of these technologies work together to ensure efficient light management for optimal excitation and emission of fluorochrome-tagged cells, which is critical to performance in the CytoFLEX Platform!

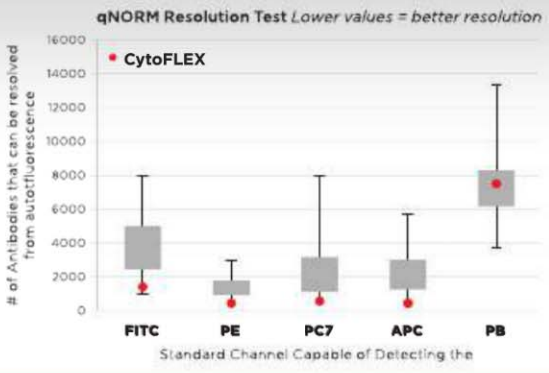


Human PBMCs **Mouse Spleen**

Regulatory T cells characterized by low levels of expression were easily identified.
Rare Regulatory T cell populations FoxP3+ were easily detected without the using a Fluorescence Minus One (FMO) stain.

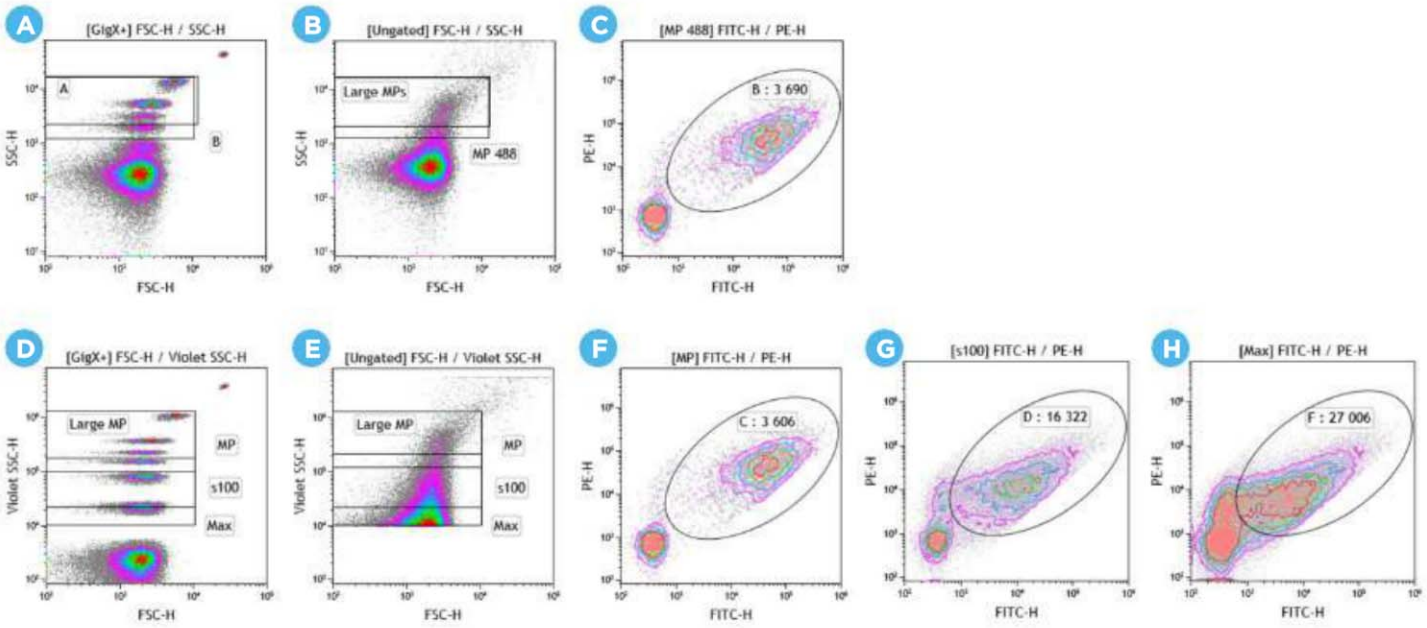
"The CytoFLEX compares very well with all the best instruments out there. It definitely beats every instrument I own in the FITC, PE, PECy7, and APC channels."

Ryan Duggan, UC Flow Core Lab Director



Nanoparticle Detection

The advancement of flow cytometry into nanoparticle scale resolution, makes it possible to ask questions previously left to speculation. Several fundamental capabilities of flow cytometry make this an attractive platform for studying nanoparticles such as extracellular vesicles, the ability to detect large numbers of events, and discrimination of rare events, while simultaneously collecting information on phenotypic expression. The CytoFLEX Flow Cytometer has the resolution to detect particles smaller than 200 nm within a phenotypic context.

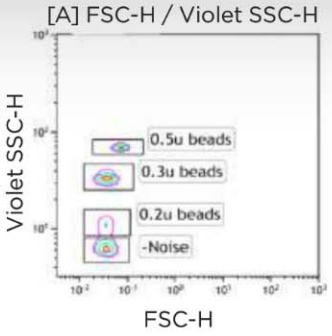


Detection of Cell Line-Derived Microparticles using Blue or Violet Side Scatter. . Instrument settings were standardized using a blend of fluorescent submicron polystyrene beads specially designed to optimize side scatter settings on highly sensitive flow cytometers: Megamix-Plus FSC & Megamix-Plus SSC (BioCytex Refs # 7802 and # 7803 respectively) were mixed V/V to create Gigamix, a SMP-oriented quality control system featuring 7 reference bead sizes of 900, 500, 300, 240, 200, 160, and 100 nm to which was added an additional subset of 75 nm polystyrene beads devoted to challenge nanoparticle analysis capabilities. Gates were set using the enriched Gigamix bead mixture (488 nm side scatter (SSC), panel A and 405 nm violet side scatter (VSSC), panel B) and then applied to the analysis of purified BxPC3 pancreatic cell line-derived microparticles using 488 nm SSC or 405 nm VSSC (panel D and E, respectively). Two classical gates i.e. "Large MP" = 0.6 to 0.22 µm bead-equivalent and (all) "MP" gate = 0.6 to 0.22 µm-eq. were similarly set with the use of reference beads in both the 488 nm and 405 nm SSC channels. Due to increased resolution in the VSSC channel, two additional gates could be defined noted "S100" = 0.6 to 0.1 µm-eq. and "Max" = 0.6 to ~0.08 µm-eq., a newly attained low detection limit located in-between the 75 and 100 nm beads. In dual fluorescence plots relating Annexin-V-FITC vs SBTF1-PE (CD142) labeling, similar number of dual positive events were detected using the standard "MP" gate for both the 488 nm SSC and the 405 nm VSSC (3690, panel C; 3606, panel F, respectively). However, using VSSC, the wider new gates incorporated higher numbers of events, i.e. S100 (13,333 events, panel G), and Max (16,322, panel H) thus opening the door for deeper insight into the "MP iceberg".
Data kindly provided by Philippe Poncelet from BioCytex, a Stago Group Company and Stéphane Robert from VRCM, INSERM S1076, Marseille, F. For complete details see CYTO 2016 poster, Submicron particle analysis and counting is highly favored by the use of side scatter from the violet laser on the CytoFLEX flow cytometer, accessible on Research Gate.

"The CytoFLEX is the first flow cytometer with an acceptable noise range on which we can clearly demonstrate detection of extracellular vesicles down to a size of 150 nm*. The potential to combine small particle analysis with the detection of up to 13 additional fluorescence parameters makes this cytometer an outstanding instrument for extracellular vesicle detection."

Andreas Spittler, MD, Associate Professor for Pathophysiology, Medical University of Vienna, Core Facility Flow Cytometry & Department of Surgery, Research Laboratories

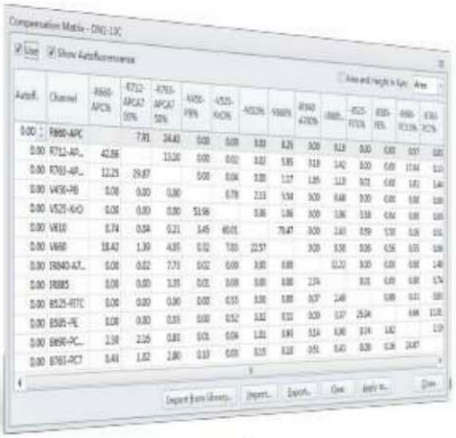
In order to achieve detection smaller than 200 nm, modifications to the method and rigorous control of instrument set up and sample preparation are required. See Set-Up of the CytoFLEX for Extracellular Vesicle Measurement, Andreas Spittler.



Detection Linearity & Compensation

Due to the highly reproducible semiconductor process, the fluorescence intensities measured on the CytoFLEX Platform are linear to the corresponding detector gain settings. Due to the gain linearity of the semiconductor, the compensation matrix obtained at one gain setting can be used for actual experiments at different gain settings. Compensation matrix elements obtained at different gain settings can be mixed together to form a full matrix, the Compensation Library.

1

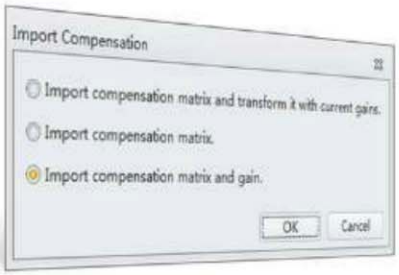


View the compensation matrix to assess the spillover between fluorochromes in an experiment. Save the values to the library or a matrix to be applied to future experiments.

2

The Compensation Library stores previously collected spillover values and the gain used during the acquisition. Use a selection from the library to build a new matrix which can be applied to future experiments.

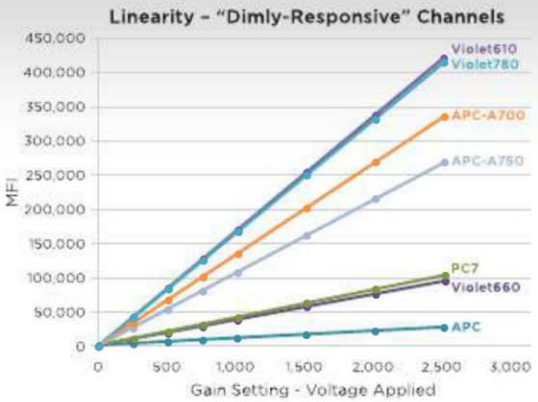
3



When importing the compensation matrix, three options are available.

"Linearity is certainly a great asset of the CytoFLEX. It is truly impressive as all the channels displayed an almost perfect linearity. The minimum R-squared value achieved was 0.9998 which is exceptional."

Loïc Tauzin, Valerie Glutz and Miguel Garcia, Ecole Polytechnique Federale De Lausanne Flow Cytometry Core Facility

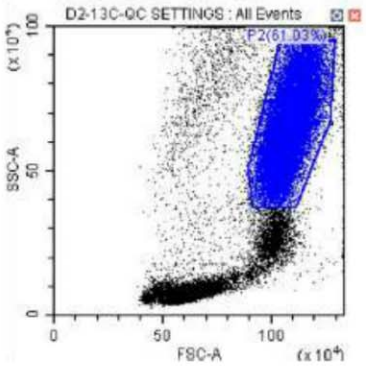


CytExpert Acquisition and Analysis software

Novice to experienced flow cytometrists can learn to operate the system quickly, and can confidently set up experiment based protocols and export publication quality data. The Default installation requires no user login. For multiuser instruments, the User Management installation requires user login and contains features for role management. Electronic Records Management installation provides tools that facilitate compliance with 21 CFR Part 11, Electronic Records and Electronic Signatures. This includes controls for user identification, permissions, electronic signatures, data integrity, operation and experiment logs and audit trails.

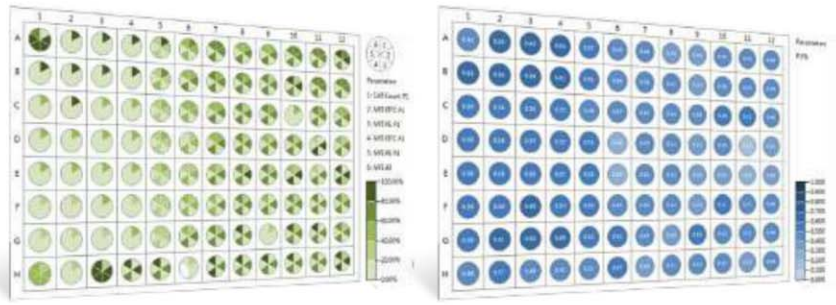
Standardization allows operators to use the QC beads to set target values for different applications and calibrate the gain settings automatically. The Standardization Target Library stores and allows users to retrieve a variety of application target files which are linked to the QC bead lot numbers.

AUTOGATE FUNCTION



Double click the population you want to gate in the 2-D plot.

HEAT MAP FUNCTION



Heat Map analysis function is integrated into the plate loader mode. Import meta-data from .csv or .xlsx file to create a plate. Each experiment can include several heat maps. Maps with up to six parameters are available.

Minimum Computer Requirements

Required processor	4th Gen Intel® Core™ i3 (3 MB Cache, 2.90 GHz) equivalent or above
Required operating system	Windows 7, 8, 10 Professional, 64 bit
Required memory	4 GB RAM or above
Required hard disk space	At least 1 G free space for the disk of the experiment for analysis. Recommend 10 G for data acquisition.
Required display	1920 x 1080 resolution for optimal display
USB Port	USB 2.0 or above for data acquisition

For higher throughput applications, an optional plate loader module can save hands on time.

- Plate Loader option can analyze a 96-well plate in as little as 32 minutes
- Option for integrated Sample Injection Mode Control, switch between single tube and plate acquisition in 5 minutes
- Easy virtual plate layout setup with customizable wash and mix cycles
- Define multiple experiments on a single plate
- Compatible with flat-bottom, U- and V-bottom standard plates

CytoFLEX Flow Cytometer

The CytoFLEX model provides the traditional laser palette and a number of channels to accommodate most basic flow cytometry assay needs.

Blue-Red-Violet (B-R-V) Series

The fully activated instrument includes five fluorescent channels from the 488 nm (Blue) laser, three from the 638 nm (Red) laser, and five from the 405 nm (Violet). The instrument includes 13 band pass filters which can be repositioned as needed. You can activate the number of channels that you need now and add channels later as you research needs grow. See the Configuration Table for a current list of available pre-set configurations.

Band Pass Filters

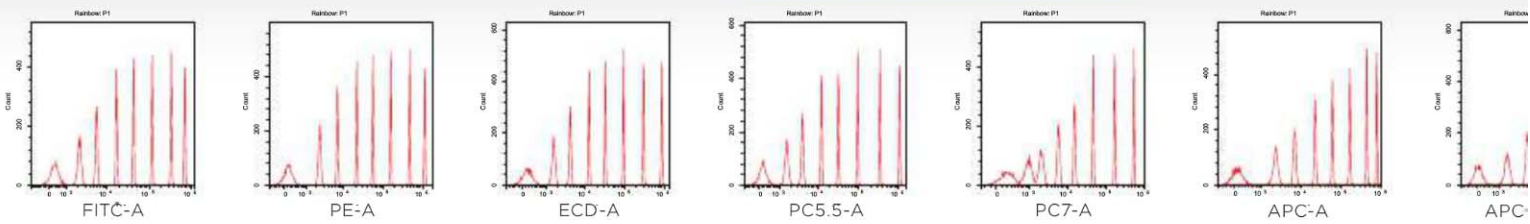
450/45	585/42	660/10 (2)	712/25
525/40 (2)	610/20 (2)	690/50	780/60 (3)

Available Configurations

PART NUMBER	LASERS	FLUORESCENCE CHANNELS	488 NM (BLUE)	638 NM (RED)	405 NM (VIOLET)
B53000	3	13	5	3	5
B53001	3	12	5	3	4
B53002	3	12	4	3	5
B53003	3	11	4	3	4
B53004	3	11	3	3	5
B53006	3	10	4	3	3
B53005	3	10	5	3	2
B53037	2	10	5		5
B53007	3	9	3	3	3
B53008	3	9	4	3	2
B96622	2	8	5	3	
B53009	3	8	3	3	2
C02945	3	8	4	2	2
B53010	3	7	3	2	2
B53011	2	6	3	3	
B53013	2	6	4	2	
B53012	2	6	3		3
C02944	2	6	4		2
C02946	3	6	2	2	2
B53018	1	5	5		
B53014	2	5	3	2	
B53019	1	4	4		
B53015	2	4	3	1	
B53016	2	4	2	2	
B53017	2	4	2		2

BLUE CHANNELS

RED

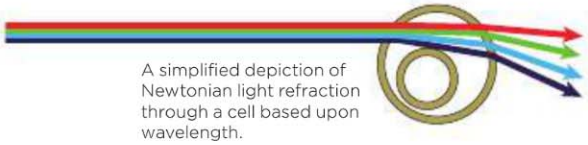


Excellent resolution of 8-speak SPHERO™ Rainbow Calibration Particles.

Bringing Violet Side Scatter to a Benchtop Analyzer

The CytoFLEX Platform features the ability to trigger side scatter of off violet as well as the blue laser. This flexibility increases the range of particles that can be detected and analyzed. The amount of light scattered by any particle is directly proportional to the diameter of the particle and inversely proportional to the wavelength of the light being used to detect it. For this reason, the smaller violet (405 nm) wavelength will result in more orthogonal light scattering at any given particle size than the blue (488 nm) wavelength, and will increase the range of resolution to smaller particles than can be detected by standard side scatter.

Moreover, upon entering a medium of a different refractive index, light waves are refracted by the new medium inversely proportional to the wavelength of the light, with smaller wavelengths having a higher refraction than larger wavelengths. Based upon this physical property, the use of violet light will help to amplify the differences in the refractive indices between the particles and their surrounding media, and in turn increases the ability to detect particles with a lower refractive index, such as exosomes, micro vesicles and silica nanoparticles.

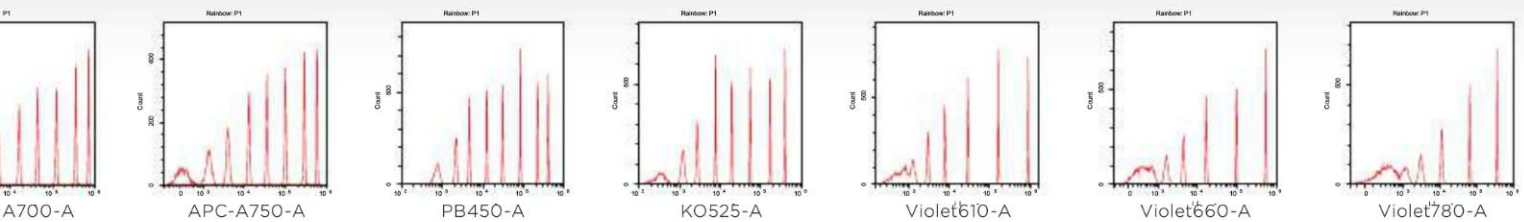


A simplified depiction of Newtonian light refraction through a cell based upon wavelength.

I enjoy the ability to swap out filters-that's a huge advantage of the instrument. I don't have to purchase additional filters, it already comes with all the filters that I would ever need. It also allows me to upgrade the instrument. Currently, I only have 2 lasers and I can upgrade to the violet laser, I can upgrade to a plate loader, I can upgrade to whatever I might need in the future, which is a huge advantage as a core manager.

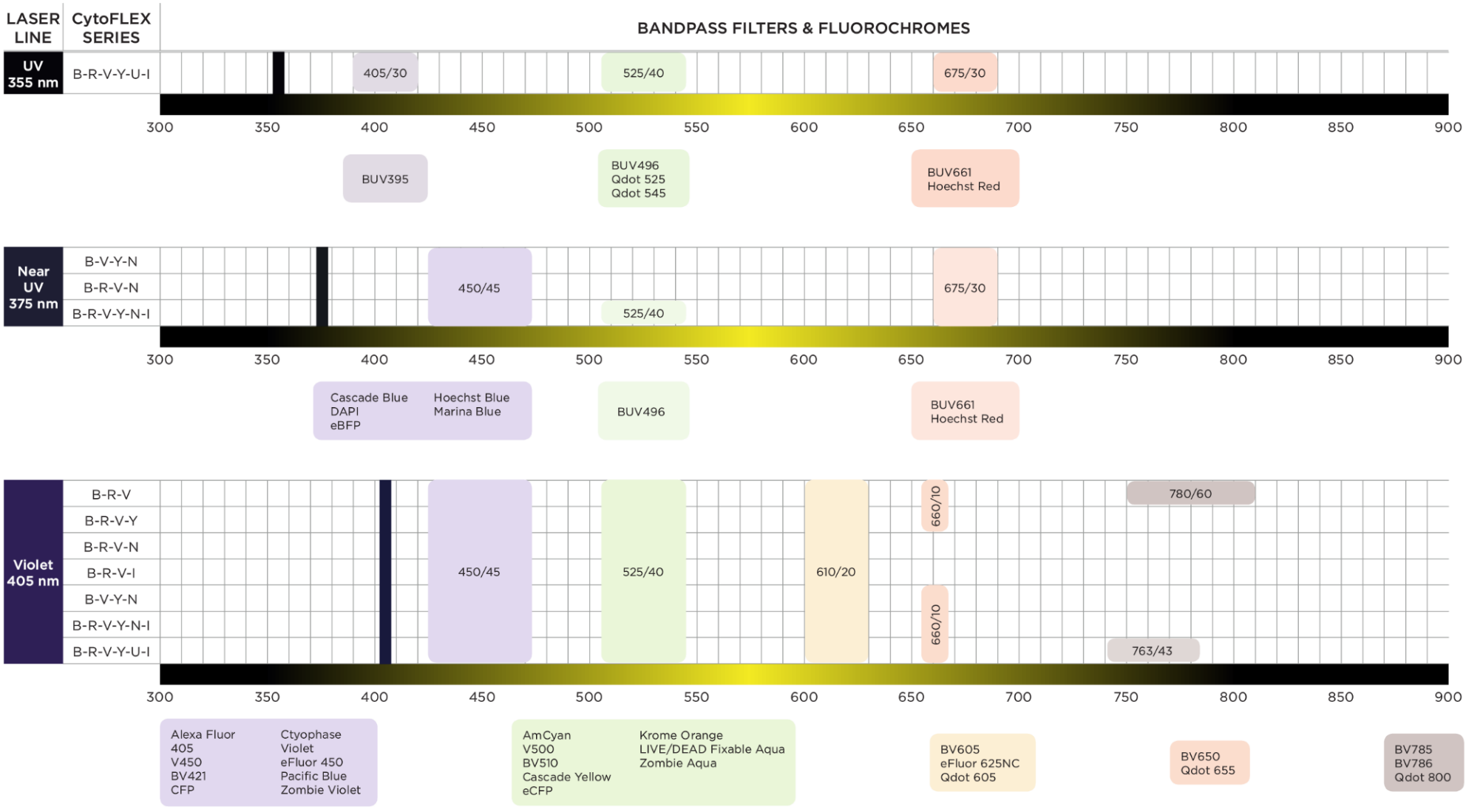
Sarah Schuett
Core Lab Manager
North Carolina State Veterinary College

CHANNELS **VIOLET CHANNELS**



CytoFLEX Platform Fluorochrome Chart

The chart lists the standard* bandpass filters for each channel along with suitable fluorochromes based upon excitation and emission spectra.



Non-standard bandpass filters are also available, see page 18 for a complete listing. These filters can be used to expand the detectable fluorochromes.

CytoFLEX S Flow Cytometer

The CytoFLEX S models bring up to four laser instruments to the research community expanding the fluorochrome palette for special applications.

Blue-Red-Violet-Yellow Green (B-R-V-Y) Series

The fully activated instrument includes two fluorescent channels from the 488 nm (Blue) laser, three from the 638 nm (Red) laser, four from the 405 nm (Violet) laser, and four from the 561 nm (Yellow Green) laser. The instrument includes 13 band pass filters which can be repositioned as needed. You can activate the number of channels that you need now and add channels later as you research needs grow. See the Configuration Table for a current list of available pre-set configurations.

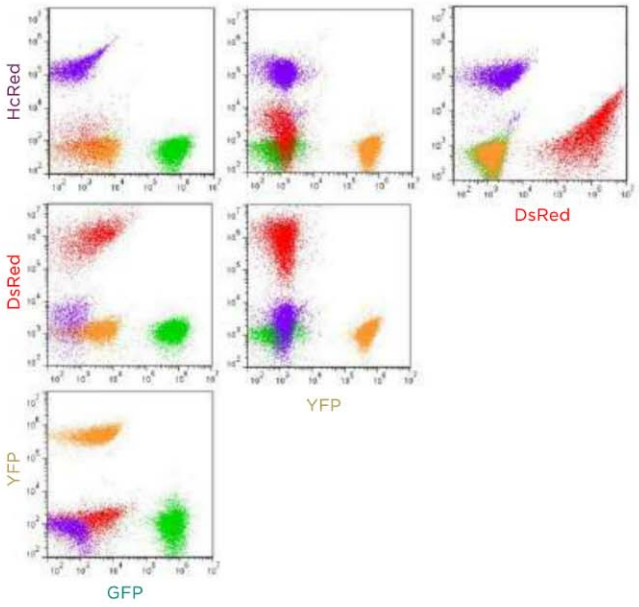
Includes 13 Repositionable Band Pass Filters

450/45	585/42	660/10 (2)	712/25
525/40 (2)	610/20 (2)	690/50 (2)	780/60 (2)

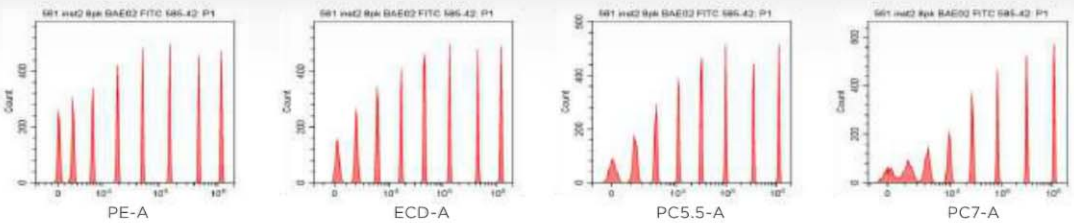
Available Configurations

PART NUMBER	LASERS	FLUORESCENCE CHANNELS	488 NM (BLUE)	638 NM (RED)	405 NM (VIOLET)	561 NM (YELLOW GREEN)
B75408	4	13	2	3	4	4
B96620	3	10	2		4	4
B75811	3	9	2	3		4
B96621	4	9	2	2	2	3
C02948	3	9	2	3	4	
B75812	2	6	2			4
C02947	3	6	2		2	2

The **Yellow Green 561 nm** laser excites RFP and RFP derivatives such as DsRed and HcRed more efficiently than the **488 nm** laser. An additional benefit of spatially separated lasers is increased sensitivity, thus minimizing inter-laser compensation. Therefore, cells expressing GPF, YFP, DsRed, and HcRed, may be analyzed, demonstrating resulting in superior resolution of simultaneously expressed multicolor fluorescent protein signals.



YELLOW GREEN CHANNELS



Excellent resolution of 8-speak SPHERO™ Rainbow Calibration Particles.

Blue-Red-Violet-Near UV (B-R-V-N) Series

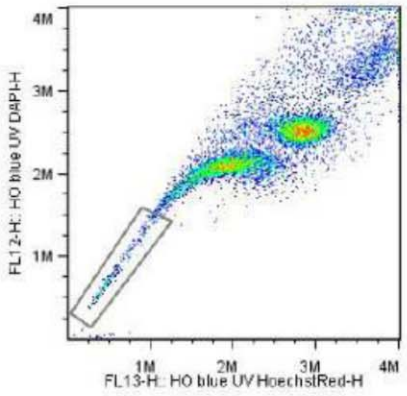
The fully activated instrument includes five channels from the 488 nm (Blue) laser, three from the 638 nm (Red) laser, three from the 405 nm (Violet) laser, and two from the 375 nm (Near UV) laser. The instrument includes 13 band pass filters which can be repositioned as needed. The instrument has the capacity for 15 parameters, including 13 for fluorescence detection. You can activate the number of channels that you need now and add channels later as you research needs grow. See the Configuration Table for a list of available configurations.

Includes 13 Repositionable Band Pass Filters

450/45 (2)	585/42	660/10	690/50	780/60 (2)
525/40 (2)	610/20 (2)	675/30	712/25	

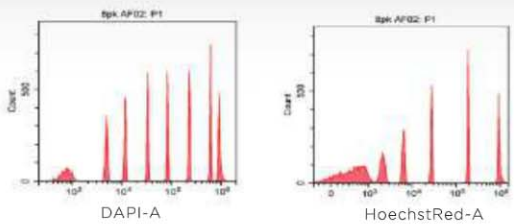
Available Configurations

PART NUMBER	LASERS	FLUORESCENCE CHANNELS	488 NM (BLUE)	638 NM (RED)	405 NM (VIOLET)	375 NM (NEAR UV)
B78557	4	13	**5**	**3**	**3**	**2**
B78559	3	10	**5**	**3**		**2**
B78558	2	6	**4**			**2**



The addition of the **375 nm** laser, in a spatially separated discrete beam spot, enables excellent excitation of Hoescht, DAPI and brilliant UV dyes allowing for use of these dyes without incurring the cost of a true UV laser. Dye Cycle Violet, while useful for performing side population analysis without a true UV laser, requires researchers to compromise on immunophenotyping as it spills over into the FITC and PE channels. Using the **375 nm** laser, researchers can go back to Hoescht for traditional side population analysis. Results are indistinguishable from data collected on much larger traditional instruments.

NEAR UV CHANNELS



Excellent resolution of 8-speak SPHERO™ Rainbow Calibration Particles.

CytoFLEX S Flow Cytometer

Blue-Red-Violet-Infrared (B-R-V-I) Series

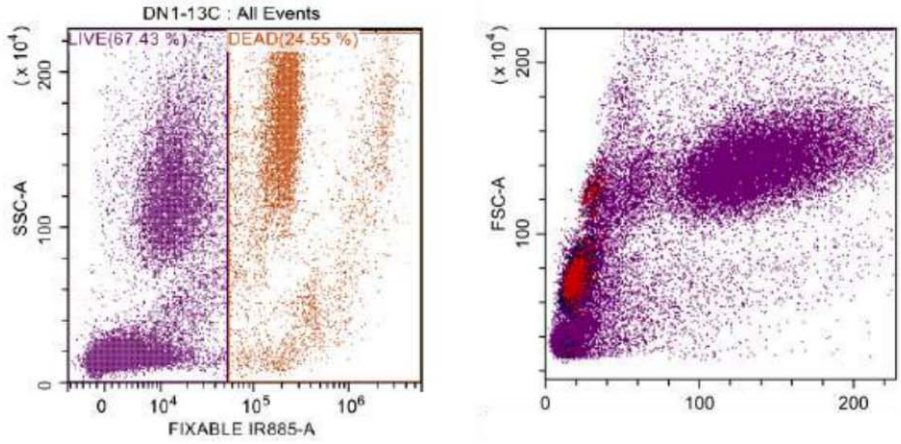
The fully activated instrument includes four fluorescent channels from the 488 nm (Blue) laser, three from the 638 nm (Red) laser, four from the 405 nm (Violet) laser, and two from the 808 nm (Infrared) laser. The instrument includes 13 band pass filters which can be repositioned as needed. You can activate the number of channels that you need now and add channels later as you research needs grow. See the Configuration Table for a current list of available pre-set configurations.

Includes 13 Repositionable Band Pass Filters

450/45	585/42	660/10 (2)	712/25	840/20
525/40 (2)	610/20	690/50	763/43 (2)	885/40

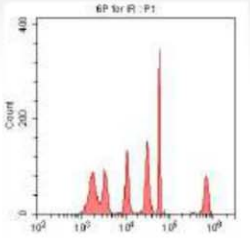
Available Configurations

PART NUMBER	LASERS	FLUORESCENCE CHANNELS	488 NM (BLUE)	638 NM (RED)	405 NM (VIOLET)	808 NM (INFRARED)
C01161	4	13	4	3	4	2
C01160	3	10	4		4	2
C01159	3	9	4	3		2
C01158	2	6	4			2



The addition of the 808 nm laser to the CytoFLEX S series provides additional fluorescent channels not only for use of viability dyes but also bright markers with minimal spectral overlap into traditional channels.

INFRARED CHANNELS



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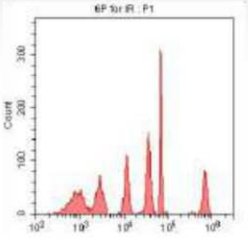


PF840-A

Resolution of SPHERO™ Fluorescent IR Flow Cytometer Particles.

Blue-Violet-Yellow Green-Near UV (B-V-Y-N) Series

The fully activated instrument includes two fluorescent channels from the 488 nm (Blue) laser, four from the 405 nm (Violet) laser, four from the 561 nm (Yellow Green) laser, and two from the 375 nm (Near UV) laser. The instrument includes 12 band pass filters which can be repositioned as needed. You can activate the number of channels that you need now and add channels later as you research needs grow. See the Configuration Table for a current list of available pre-set configurations.

Includes 13 Repositionable Band Pass Filters

450/45 (2)	585/42	660/10	690/50 (2)
525/40 (2)	610/20 (2)	675/30	780/60

Available Configurations

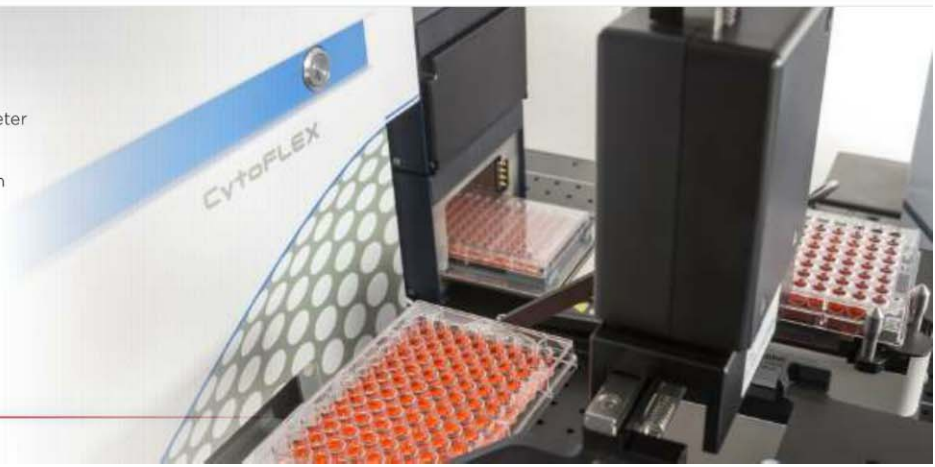
PART NUMBER	LASERS	FLUORESCENCE CHANNELS	488 NM (BLUE)	405 NM (VIOLET)	561 NM (YELLOW GREEN)	375 NM (NEAR UV)
B78560	4	12	2	4	4	2
B96619	3	10	2	4	4	
B78561	3	8	2		4	2
B96618	2	6	2		4	
C02949	2	4	2		2	

For Even Higher Throughput Applications

Gain flexibility in your day by integrating your CytoFLEX Flow Cytometer to the Biomek i-Series Instruments for automated sample processing and data acquisition. Assay plates are transferred with the Biomek gripper directly to the CytoFLEX Flow Cytometer. Sample preparation [well] data, such as sample ID, is correlated with the information collected from the flow cytometer. Automate your complete cellular workflow with one trusted partner.

If you already have an automation solution, the CytExpert is an open platform. Our sales team can assist you in integrating the CytoFLEX Flow Cytometer based upon your workflow requirements.

Visit **biomek.beckman.com** to learn more about the i-Series



CytoFLEX LX Flow Cytometer

The CytoFLEX LX models bring up to six laser instruments to the research community providing up to 21 fluorescent channels.

Blue-Red-Violet-Yellow Green-Near UV-Infrared (B-R-V-Y-N-I) Series

The fully activated instrument includes three fluorescent channels from the 488 nm (Blue) laser, three from the 638 nm (Red) laser, five from the 405 nm (Violet) laser, five from the 561 nm (Yellow) laser, three from the 375 nm (Near UV) laser, and two from the 808 nm (Infrared) laser. The instrument includes 22 band pass filters which can be repositioned as needed. You can activate the number of channels that you need now and add channels later as you research needs grow. See the Configuration Table for a current list of available pre-set configurations.

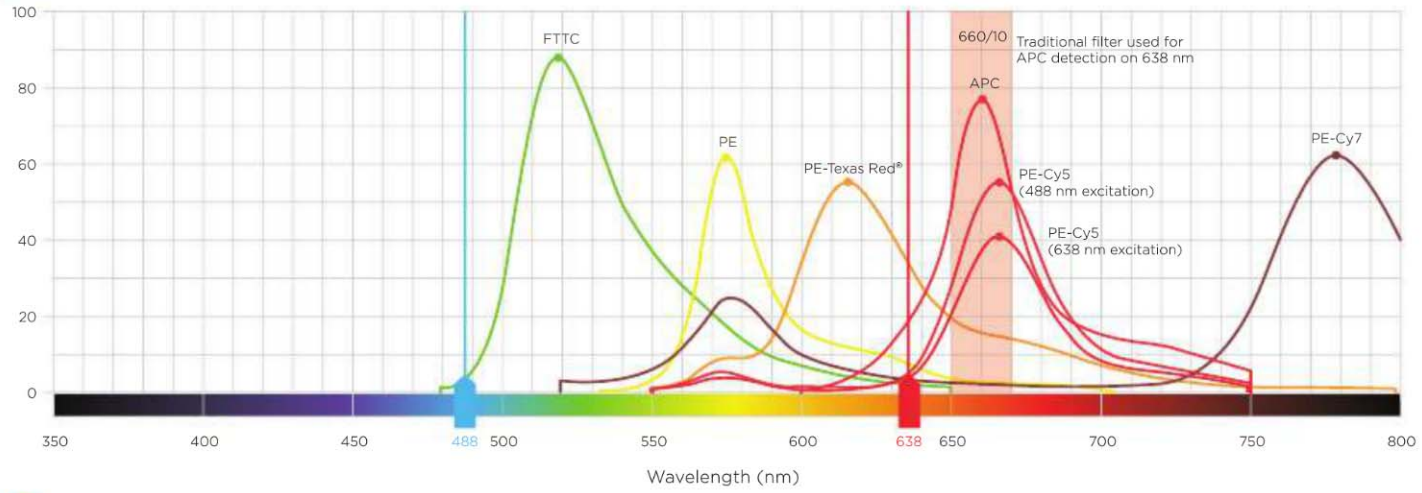
Band Pass Filters

405/10	450/45 (2)	525/40 (3)	585/42	610/20 (3)	660/10 (2)	675/30 (2)
690/50	710/50	712/25	763/43 (3)	840/20	885/40	

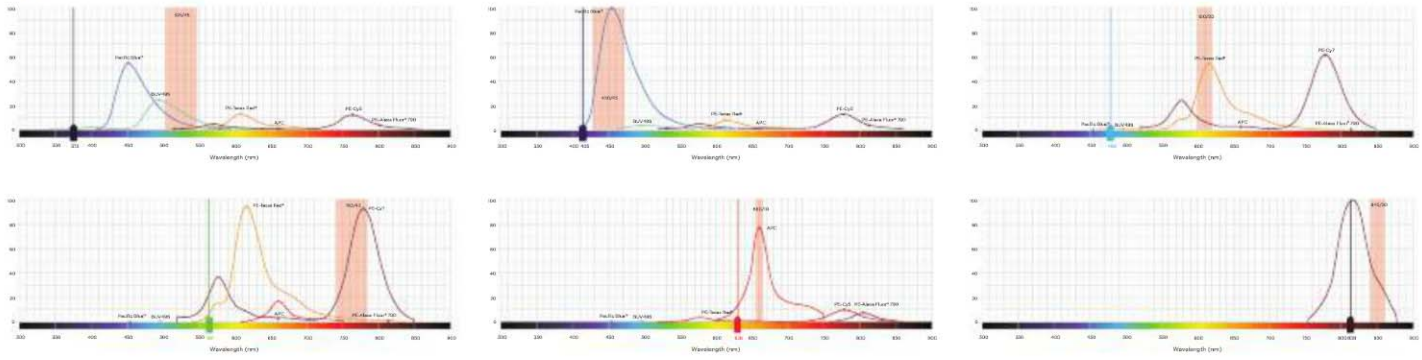
Available Configurations

PART NUMBER	LASERS	FLUORESCENCE CHANNELS	488 NM (BLUE)	638 NM (RED)	405 NM (VIOLET)	561 NM (YELLOW GREEN)	375 NM (NEAR UV)	808 NM (INFRARED)
C00445	6	21	3	3	5	5	3	2
C00446	5	19	3	3	5	5	3	

A



B



Simplify Complex Experiments. Panel A demonstrates spectral overlap of 6 common fluorochromes, FITC , PE , Texas Red, APC, PC5 and APC –Cy7 excited by two lasers, 488 nm and 638 nm. Cross laser excitation of PC-Cy5 into the APC channel is also indicated. In Panel B, expanding the available color palette provides flexibility to optimize panel design for efficient marker detection. The fluorochromes, BUV496, Pacific Blue, PE-Texas Red, PE-Cy7, APC, and AF790, are excited by six different lasers to minimize compensation requirements.

Blue-Red-Violet-Yellow-UV-Infrared (B-R-V-Y-U-I) Series

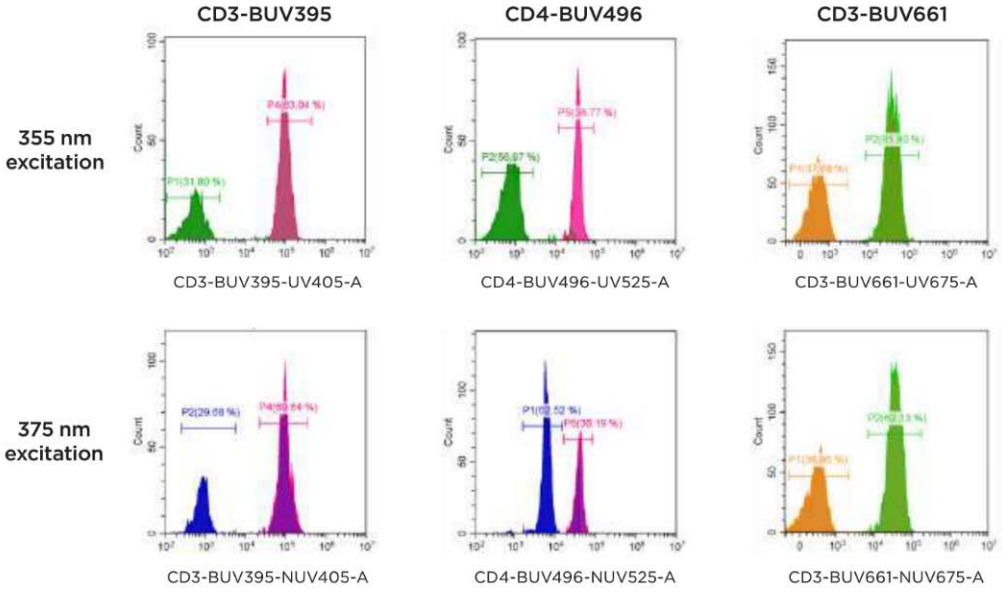
The fully activated instrument includes three fluorescent channels from the 488 nm (Blue) laser, three from the 638 nm (Red) laser, five from the 405 nm (Violet) laser, five from the 561 nm (Yellow Green) laser, three from the 355 nm (UV) laser, and two from the 808 nm (Infrared) laser. The instrument includes 22 band pass filters which can be repositioned as needed. You can activate the number of channels that you need now and add channels later as you research needs grow. See the Configuration Table for a current list of available pre-set configurations.

Band Pass Filters

405/10	405/30	450/45	525/40 (3)	585/42	610/20 (3)	660/10 (2)	675/30 (2)
690/50	710/50	763/43 (3)	712/25	763/43 (3)	840/20	885/40	

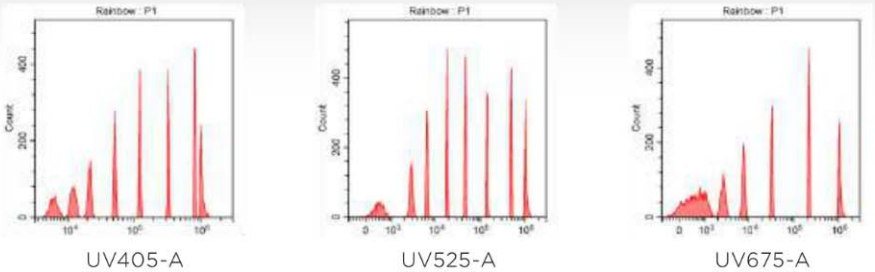
Available Configurations

PART NUMBER	LASERS	FLUORESCENCE CHANNELS	488 NM (BLUE)	638 NM (RED)	405 NM (VIOLET)	561 NM (YELLOW GREEN)	355 NM (UV)	808 NM (INFRARED)
C11186	6	21	3	3	5	5	3	2
C11185	5	19	3	3	5	5	3	
C11183	4	14	3	3	5		3	
C11184	4	14	3		5	3	3	



Comparison of UV versus Near UV excitation sources for Brilliant UV fluorochromes. Whole blood (donor 1, BUV395 and BUV496; donor 2, BUV661) was stained with the indicated reagent and red blood cells removed with VersaLyse lysing solution. Data was collected with a CytoFLEX LX equipped with either a 355 nm UV laser or a 375 nm Near UV laser. The lymphocyte gate (Lymphs) was set based upon forward and side scatter characteristics (not shown). Histograms show fluorescence signals with gates applied on positive and negative staining populations to obtain statistics.

UV CHANNELS



Accessories and Consumables

Start up kits are available to ensure that when your unit arrives you will be ready to start your experiments. We also offer kits and consumables for the routine use and maintenance. Each instrument contains standard band pass filters. We also offer a variety of non-standard filters for specialized applications.

Startup Kits* & Preventive Maintenance Kits

Part Number	Description
B55031	CytoFLEX Startup Reagents (tubes)
C14907	CytoFLEX Startup Reagents (plates)
C14908	CytoFLEX Startup Reagents (IR/tubes)
C14909	CytoFLEX Startup Reagents (IR/plates)

Part Number	Description
C02943	Preventive Maintenance Kit
A04-1-0048	Peristaltic Sample Tubing Replacement Kit
A04-1-0041	Sheath Filter

*Includes daily QC, sheath fluid, FlowClean, Contrad, and sample tubes or plates

Consumables & Miscellaneous Replacement Parts

Part Number	Description
81911	Contrad 70
B53230	CytoFLEX Daily QC Fluorospheres
C06147	CytoFLEX Daily IR QC Fluorospheres
B51503	CytoFLEX Sheath Fluid
A64669	FlowClean Cleaning Agent
609844	Microtiter Plates, 96-well Flat Bottom
609801	Microtiter Plates, 96-well V Bottom
B63213	Plate Loader Sample Probe (with tubing to attach to plate assembly)

Part Number	Description
B71294	Sample Needle, 113 mm (orange bead)
A04-1-0034	Sample Needle, 115 mm (blue bead)
A04-1-0038	Deep Clean Solution Bottle Kits
A04-1-0036	Sheath Bottle Kit
A04-1-0037	Waste Bottle Kit
7547155	10 L Waste Tank
B86549	10 L Waste/Sheath Tanks Wiring Harness

Optional Bandpass Filters

Part Number	Description
A01-1-0048	405/10 nm Bandpass Filter
B99146	405/30 nm Bandpass Filter
A01-1-0049	450/45 nm Bandpass Filter
B90300	450/45 nm Bandpass with OD1 Filter
A01-1-0050	488/8 nm Bandpass Filter
B76128	510/20 nm Bandpass Filter
B90294	510/20 nm Bandpass with OD1 Filter
B76124	515/20 nm Bandpass Filter
A01-1-0051	525/40 nm Bandpass Filter
B90303	525/40 nm Bandpass with OD1 Filter
B76139	550/30 nm Bandpass Filter
B72627	561/6 nm Bandpass Filter
B76121	585/15 nm Bandpass Filter
B71089	585/30 nm Bandpass Filter
A01-1-0052	585/42 nm Bandpass Filter

Part Number	Description
B76117	595/20 nm Bandpass Filter
A01-1-0053	610/20 nm Bandpass Filter
B90297	610/20 nm Bandpass with OD1 Filter
A01-1-0054	638/6 nm Bandpass Filter
A01-1-0055	660/10 nm Bandpass Filter
B78244	675/30 nm Bandpass Filter
A01-1-0056	690/50 nm Bandpass Filter
B71092	710/50 nm Bandpass Filter
A01-1-0057	712/25 nm Bandpass Filter
B78217	740/35 nm Bandpass Filter
B99143	763/43 nm Bandpass Filter
A01-1-0058	780/60 nm Bandpass Filter
B78220	819/44 nm Bandpass Filter
B99144	840/20 nm Bandpass Filter
B99145	885/40 nm Bandpass Filter

DuraClone Antibody Panels

Powered by
DURA Innovations
Dry Unitized Reagent Assays

Beckman Coulter offers expertly designed and optimized pre-formulated antibody panels using our DURA Innovation dry formulation technology. Each panel provides key markers for characterizing the specified cellular population and includes enough reagents for 25 tests. Depending on your CytoFLEX configuration you may extend the panels with additional markers of interest in liquid format.

405 nm		488 nm					638 nm					
450/45	525/40	525/40	585/42	610/20	690/50	780/60	660/10		712/25		780/60	
PB	KrO	FITC	PE	ECD	PC5.5	PC7	APC	AF647	AF700	APC-A700	APC-A750	AF750
DuraClone Immunophenotyping (IM)												
Basic Tube (Part Number B53309)												
-	CD45	CD16	CD56	CD19	-	CD14	CD4	-	CD8	-	CD3	-
B Cell Tube (Part Number B53318)												
IgM	CD45	IgD	CD21	CD19	-	CD27	CD24	-	-	-	CD38	-
T Cell Subsets Tube (Part Number B53328)												
CD57	CD45	CD45RA	CCR7	CD28	PD1	CD27	CD4	-	CD8	-	CD3	-
Dendritic Cells Tube (Part Number B53351)												
HLA-DR	CD45	CD16	Lin**	-	CD1c	CD11c	Clec9A	-	-	CD123	-	-
TCRs Tube (Part Number B53340)												
TCRVδ2	CD45	TCRγδ	TCRαβ	HLA-DR	-	TCRVδ1	CD4	-	CD8	-	CD3	-
Treg Tube (Part Number B53346)												
Helios	CD45	CD45RA	CD25	-	CD39	CD4	-	FoxP3	-	-	CD3	-
Granulocytes Tube (Part Number B88651)												
CD15	CD45	CD294	-	CD16	CD33	CD11b	PD-L1	-	-	Lin***	CD62L	-
Count Tube (Part Number C00162)												
-	-	CD45	Counting Beads	-	7-AAD	-	-	-	-	-	-	-
DuraClone Immune Function (IF)												
T Activation (Part Number B88649)												
CD4	-	IFNγ	TNFα	-	-	IL-2	-	-	CD8	-	-	CD3
T Helper Cell (Part Number C04666)												
IL-17A	-	IFNγ	-	-	-	IL-4	CD4	-	-	-	-	CD3
DuraClone Rare Event (RE)												
CLB Tube (Part Number B80393)												
CD20	CD45	CD81	ROR-1	-	CD79b	CD19	CD5	-	-	-	CD43	-
PC Tube (Part Number B80394)												
CD38	CD45	CD81	CD27	-	CD19	CD200	CD138	-	-	-	CD56	-
ALB Tube (Part Number C00163)												
-	CD45	CD58	-	CD34	CD10	CD19	-	-	-	CD38	CD20	-

** CD3/CD14/CD19/CD20/CD56
*** CD3/14/CD19/CD56

Successfully importing CytoFLEX CytExpert software data into third party software programs

APPLICATION NOTE

Authors: James Tung

Affiliation: Beckman Coulter, Inc. Miami, FL, USA

IN THIS PAPER YOU WILL LEARN

- How to find multiple file export formats
- Which format "fits" best with the third party software used in analysis
- How to display data and parameters collected in cytexpert in third party software

The acquisition and analysis software package for the CytoFLEX research cytometer, CytExpert uses intuitive workflow and novel tools to facilitate complex multicolor flow cytometric applications. CytExpert software offers a wide variety of flexible analysis templates and plotting tools for easy and efficient data analysis and is available to anyone analyzing data from a CytoFLEX without licensing fees.

Intuitive GUI for novice and flow experts

- Experiment-based workspace design
- Linear detector gain control
- Gain-independent real-time compensation
- Unique Compensation Library

Collecting data on the CytoFLEX research flow cytometer using CytExpert software creates FCS 3.0 files. Importing this data into other third-party cytometry analysis software requires setting the preferences of that software to recapitulate the presentation as seen in CytExpert.

This guide provides a brief overview of the steps involved to account for differences seen in some third party analysis software when importing data from CytExpert.

FSC Express Software V4.0

Scaling and Display Preferences

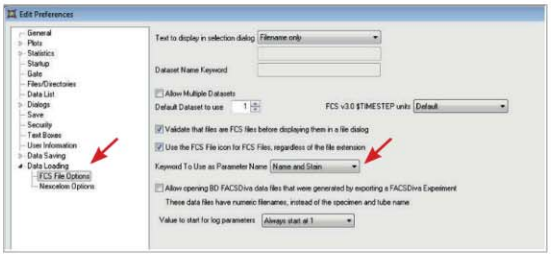
Here we describe how to locate the parameter names in the FCS header and adjust scaling in FCS Express software when importing data from your CytExpert software.

Displaying parameter names:

1. In FCS Express, go to FCS Express icon, select "Edit Preferences"

BECKMAN COULTER Life Sciences

2. In "Edit Preferences" window,
   select "Data Loading",
   then select "FCS File Options".



3. On the right side of the window, select "Name and Stain" for Keyword to Use as Parameter Name.

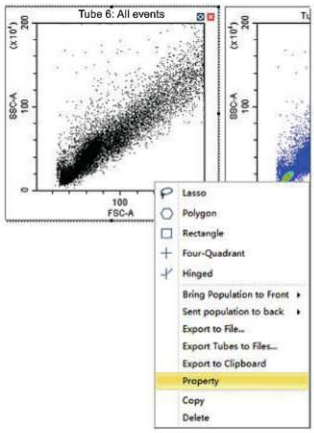
4. Close FCS Express.

5. Restart FCS Express.

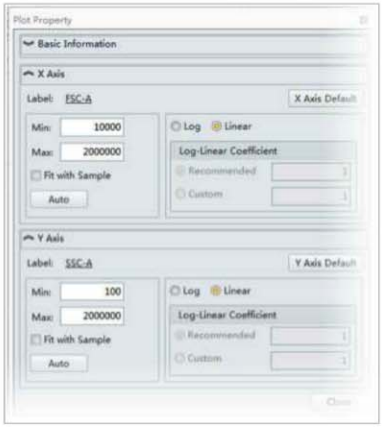
Adjusting scatter scaling in FCS Express V4.0

When viewing data In CytExpert Software:

1. Right click on the plot, select Property.

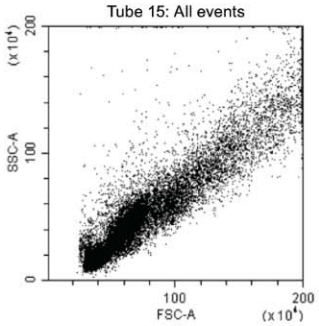


2. Enter minimum (10000) and maximum (2000000) values for FSC and SSC. Alternatively, capture the minimum and maximum values and enter in FCS Express.



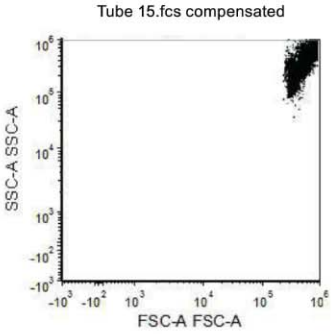
3. Select "Close"

4. The plot appears as here.



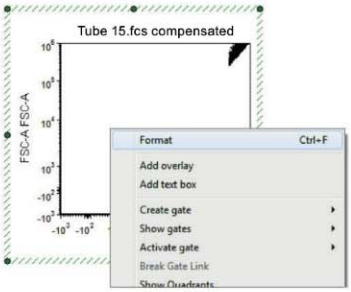
Transferring data files from CytExpert to FCS Express V4.0

When you import data from CytExpert software into FCS Express V4.0 the data will look like this:



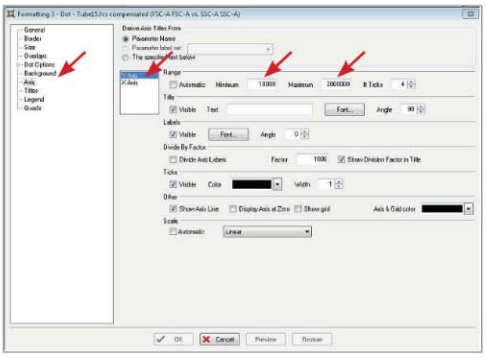
The following steps will allow you to correct this view in FCS Express software V4.0:

1. Right click on the plot and select "format"



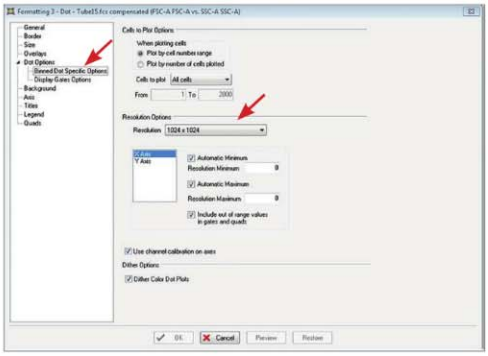
2. Select "Axis" on the left panel.

3. On the right panel, for Y axis:
   a. Enter 10000 for minimum
   b. Enter 2000000 for maximum
   c. On Scale, select Linear from the drop down menu

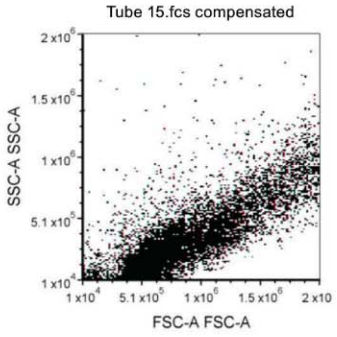


4. Do the same for the X axis.
   a. Enter 10000 for minimum
   b. Enter 2000000 for maximum
   c. On Scale, select Linear from the drop down menu

5. Select "Dot Options" then "Binned Dot Specific Options"
   Select 1024x1024



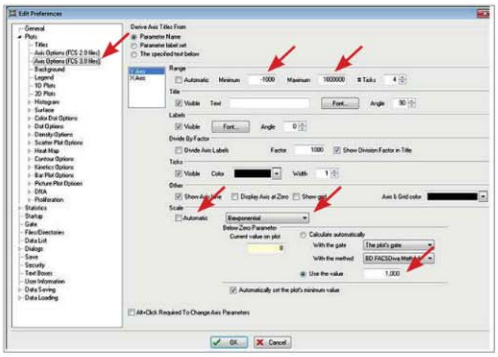
6. Select "ok".
   Now the scatter profile is the same as it did in the CytoFLEX .



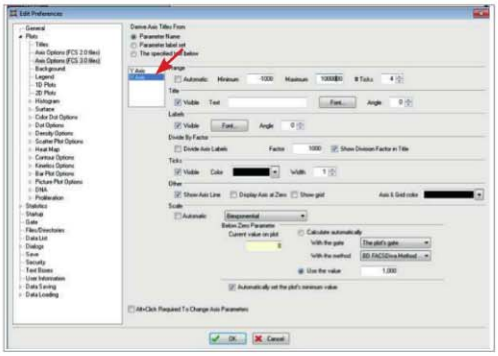
Set default preference for plot view in FSC Express V4.0

1. Select FCSExpress icon,
   then select edit preferences.

2. In the Edit Preferences window,
   Select "Plots"
   then "Axis Options [FCS 3.0 files]".
   On the right panel, input the following for the Y axis:
   a. Minimum -1000
   b. Maximum 1000000
   c. Scale: Select "Biexponential" from the drop down menu and unselect "automatic".
   Select "Use the value" and enter 1000.

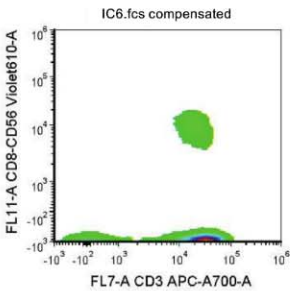


3. Follow the same procedure for the X axis.
   Select ok.

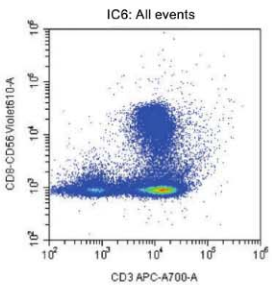


Quit program.
Restart FCS Express.
Import data.

FCS Express default display

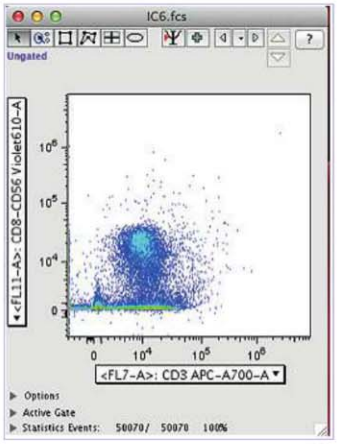


CytExpert default display



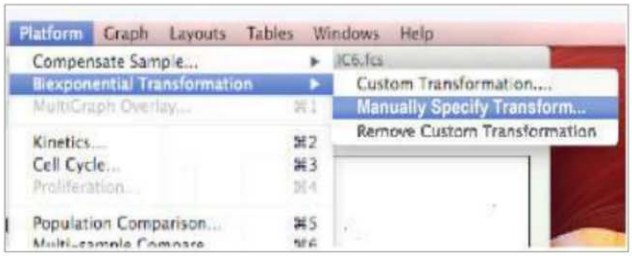
FlowJo V9.7.7

When transporting CytExpert data files into FlowJo V9.7.7 the data appears as here:

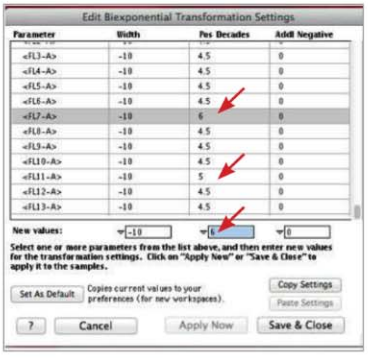


To fix this:

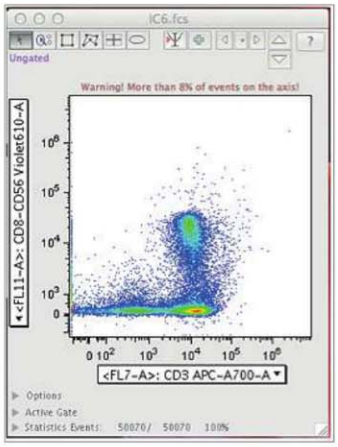
1. Go to Platform
   - Biexponential Transformation
   - Manually specify Transform



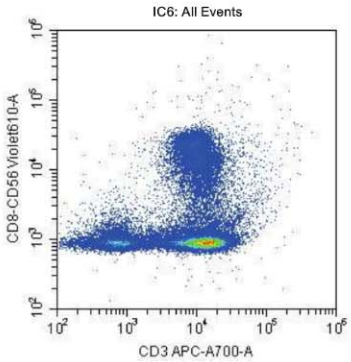
2. In the "Edit Biexponential Transformation Settings" window, enter values that are higher than the default value then press enter, followed by selecting "Apply Now".



3. The new transformation appears in the plot:



This view is now similar to the data as seen in the CytoFLEX CytExpert plot.



Unfortunately, for this version (9.7.7), FlowJo does not allow the user to set the maximum value to be viewed; therefore, the decade display cannot be changed and stayed as a 7-decade plot.

For Research Use Only. Not for use in diagnostic procedures.

FLOW-896FLY04.15-A.

CytoFLEX Research Cytometer Event Rate Settings

TECHNICAL INFORMATION BULLETIN

Digital flow cytometers use optical sensors to collect light signals and convert them into electronic signals to represent the light events occurring in the flow cell of the instrument. The conversion of the light signal into an electronic signal allows for the digital quantification and measurement of the signal event.

The Voltage Pulse

The digital information recorded by a flow cytometer indicates the total light signal collected across the event as a particle moves into and across a laser's path.

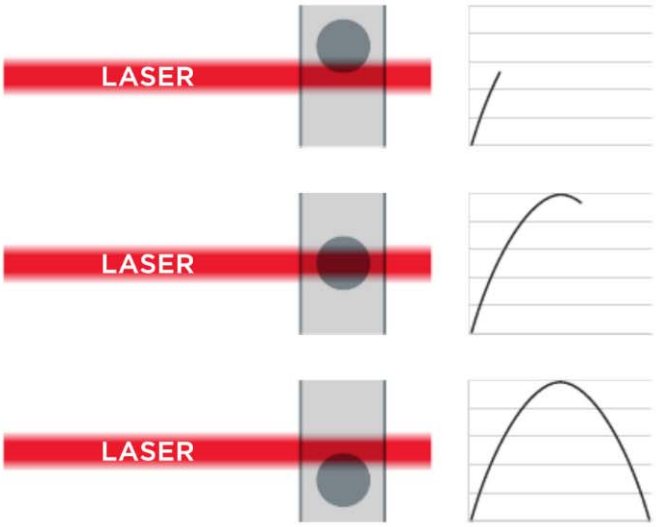


Figure 1

The entire pulse has three characteristics:

Pulse Intensity: The strength of the signal as it passes through the data collection point

Pulse Width: The period of time that it takes for a particle to pass through a data collection/interrogation point.

Pulse Area: The integration of the area under the curve formed from the pulse width and intensity as the particle moves through the collection point.

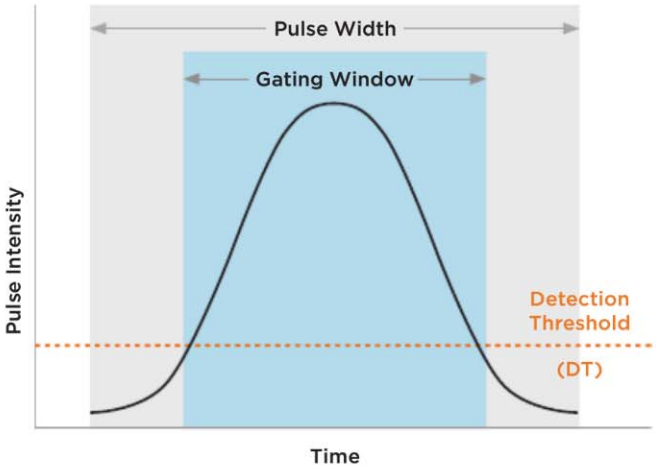


Figure 2

The Pulse Parameters: The Gating Window

In most digital flow cytometers electronic signals are continuously being collected even if no particle is being passed through the collection point. To distinguish background from real signals, an electronic cutoff called the detection threshold is established for each experiment and this determines the signal intensity where the electronic system will begin to record and measure a voltage pulse (Figure 2, orange dotted line, labeled Detection Threshold (DT). The length of time that the machine records the signals above threshold is referred to as the gating window (GW). (Figure 2, and shaded light blue). The electronics within the machine are thus programmed to process any signals that are greater than the DT and occur

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within the timing of the GW, producing an integrated area or signal (Figure 3, shaded blue). Any signals that do not cross the detection threshold or occur within the window gate are not recorded.

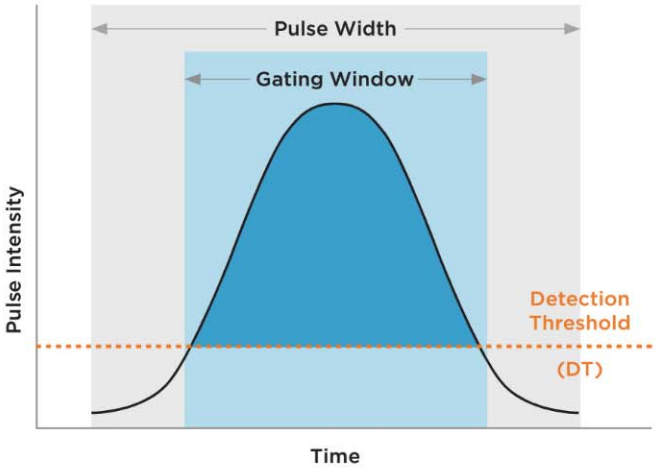

Figure 3

Event Rate Setting

As a consequence of the detection threshold, the entire electronic pulse created by a particle moving across an interrogation point is not always captured. This is because the electronic pulse actually starts before and finishes after the gating window closes. To ensure that the entire signal of an event is captured and integrated, additional time is added to both sides of the gating window. This additional time for the CytoFLEX instrument is termed the **Event Rate** setting (**ER**). (Figure 4, ER, shaded red regions). Thus the signal for an entire pulse is the sum of the GW and ER areas (Figure 4, red + blue shaded regions) and together the values constitute what is often termed the detection window or pulse width of a flow cytometer event (Figure 4).

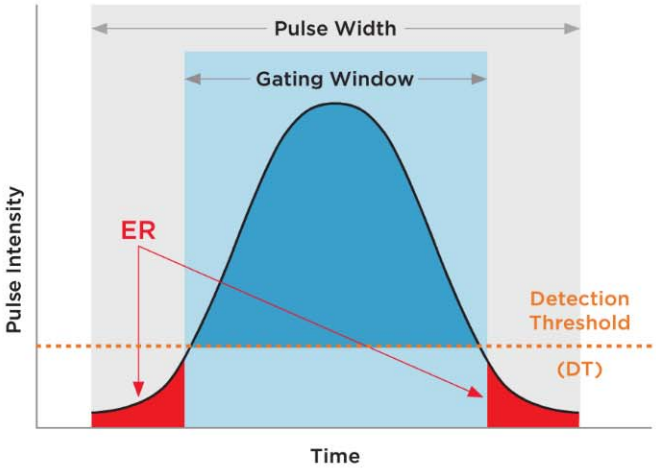

Figure 4

Importance of Event Rate Settings

By adding additional time and capturing the entire pulse, the additional data is often useful for obtaining the greatest fluorescence measurement and scatter measurements between samples, thereby facilitating the highest level of signal discrimination between samples. The proper event rate setting is important for resolution of dim versus negative cells and for resolving DNA peaks in cell cycle studies. Ultimately the highest sample fluorescence measurement and scatter measurement is desired with the lowest noise for all pulses, however, many factors affect the ER value settings and ultimately the resolution of the signal data generated.

Factors Influencing Event Rate Setting Selection

Timing

The event rate setting is an adjustable factor that influences the amount of time during which a pulse from a sample is collected in addition to the gating window (GW) time adjustment. There is a risk that a loss of useful sample information will occur when the event rate setting is too small. In contrast, increasing the event rate setting extends the detection time to allow for a more complete sampling of the signal pulse. However, if the event rate setting is too large, the area of integration stretches and the instrument collects information that really should be considered "noise", leading to a high coefficient of variation (CV) between samples. The overly large event rate setting can lead to a condition called "event overlap" where information from the next event is collected with the current event (Figure 5b). In those cases the cytometer's software automatically determines what is termed an "abort" and eliminates the offending data from the sample population. If the abort rate becomes too high, the value of the data obtained from the population will be adversely affected.

CytoFLEX signal processing uses a proprietary algorithm. Dr. Yong Chen, founder of Xitogen and Chief Technology Officer of Beckman Coulter Life Sciences, describes the Event Rate setting feature found in the CytoFLEX this way, "Flow cytometers are multi-parameter instruments. The operator is required to select one of the parameters, typically the main threshold parameter (DT, figure 2). Based on that, the CytoFLEX digital electronics searches in all parameter space for "peak intensities" or maximum voltages within the specified or system suggested event rate settings and delays. The narrower the event rate setting, the faster the search, therefore the higher system throughput. But narrower ER settings may risk mischaracterization of "peak intensity". For example, due to the inherent statistical fluctuation of particle velocity in a flow system, the true maximum may fall outside of the event rate setting. A wider event rate setting clearly reduces the risk. However, it would increase "abort rate", due to the fact that the digital electronics will be confused if two pulses happen

to fall into the event rate setting of a given parameter". Thus timing of the ER settings is critical for proper and complete pulse width processing.

Threshold Values & Background

Because the threshold value (DT) determines the signal strength at which the software will begin to integrate the sample information there is considerable interplay between the two factors. If the threshold value is set too low, the data collected will be burdened with excessive events and noise which will increase the overall sample CV and lead to excessive electronic aborts because signals cannot be distinguished from one another (Figure 5b). If the threshold is set too high however, then relevant samples expressing weak signals will be ignored and more importantly strong signals will not be completely integrated leading to incorrect data (Figure 5a). Finally, because ER and threshold values essentially govern the extent of sample data collected surrounding the window gate, they have an undeniable interrelationship with one another for a particular experiment, and thus once set they should not be changed during an experiment or data integrity could be compromised.

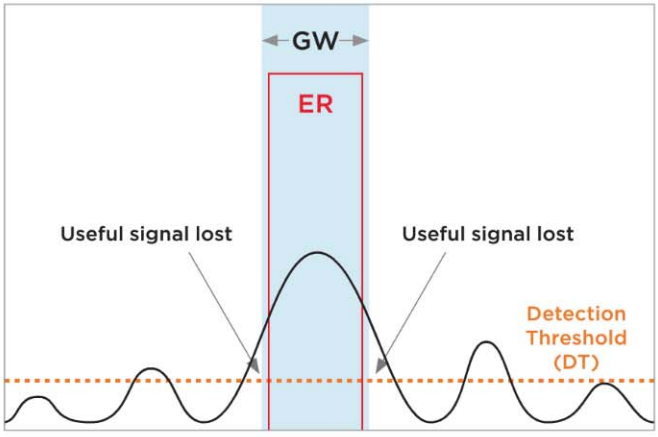

Figure 5a Event Rate (ER) set too short

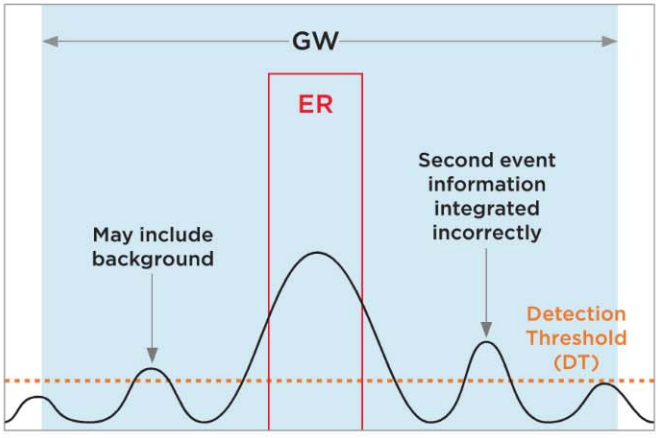

Figure 5b Event Rate (ER) set too long

Laser Delay

In flow cytometers with multiple lasers, by necessity, data from preceding lasers (the first lasers contacting the stream) will be delayed with respect to the last laser once all the data is collected. The actual laser delays settings are typically instrument specific and do not need to be changed once optimized. However, because the event rate setting influences the amount of time data is collected surrounding an event, incorrectly set laser delay values can affect the data obtained surrounding these values.

Incorrectly configured laser delay settings can affect the detection threshold determination such that small events may not appear above the threshold and therefore not recorded. In addition, an ER value that is too short will have the effect of appearing to increase sample CV because it will be "cutting off" too early before a particle has completely passed a specific laser leading to incomplete integration of the pulse. Conversely, an ER value that is too long may assemble data from one or more lasers and thus again produce inaccurate data, and "hiding" incorrect laser delay timings.

Sample Concentration

Because the event rate setting influences the length of time that sample data is collected, it is easy to understand how the concentration of a sample in particular experiment can have an impact upon the event rate setting used to obtain good, reliable, and consistent data with tight CV's and reproducibility. Samples run at a fixed flow rate that are low in concentration will have less of an opportunity to have overlapping signals than samples run at high concentration simply because there is less chance in the detectable events arriving at the detector at the same time (Figure 6, Low Concentration). As such, each detection event will occur separately even with a relatively high (long) ER setting value. If the sample concentration is too high, then even at a low fixed flow rate there is an increased chance of detectable events arriving at the same time and having overlapping pulses. Such overlapping pulses can lead to electronic aborts which results in data loss and incomplete data on the population as a whole, (Figure 6, High Concentration).

Detection Rate (EPS)

The event rate setting also impacts, or is impacted by the detection rate or events per second parameter in a similar fashion to sample concentration primarily because the two are closely related. Typically, the detection rate, or the number of cells detected per second (EPS), is determined by the concentration of cells in a sample, the flow rate, and the capabilities of the machine's electronics to process and manipulate the data stream. Because the ER setting is part of the parameters that set the length of time that a signal is collected and integrated, changes in the number of events per

second can have a great impact upon what ER setting is used, and conversely what ER setting time used, will impact the amount and fidelity of the data collected. In general, the higher the detection rate the shorter the ER settings times should be to maximize the data integrity. This is because too long of an ER setting value will lead to an increase in the frequency of electronic overlaps between pulses (Figure 6), which will lead to aborts. A high abort rate impacts not only amount of data but the quality of the data obtained as often times rare events in particular could be removed as well as otherwise good more common events discarded. Shortening the ER setting value as the detection rate is increased helps to compensate for the necessary "bunching" of the signals and helps ease the occurrence of electronic overlaps that will naturally occur as a consequence of the time between discrete events shortens.

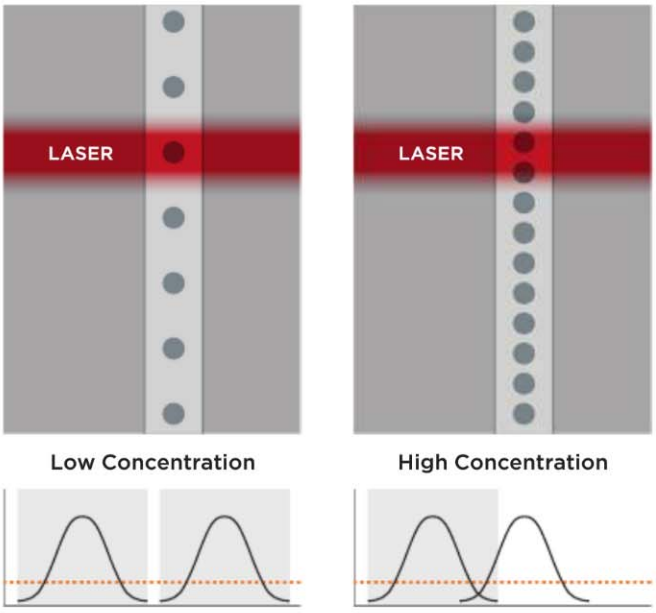

Figure 6 High Concentration

Event Rate Settings Value Summary

As discussed in the proceeding paragraphs, the event rate setting (ER) values, because they impact the amount of time that data is collected and integrated from flow events and can be impacted or make impacts on a wide range of parameters used in everyday flow cytometry, are important parameters to set correctly to maximize not only data signal but data quality from a flow experiment. A summary of the principal consequences surround ER settings values is given in the Table 1.

Table 1

ER SETTING TOO SHORT	ER SETTING JUST RIGHT	ER SETTING TOO LONG
Incomplete integration of a pulse	**Maximal data integration**	Decreased resolution: dim signals are masked by high noise/background events
Lower than necessary signals	**Tight sample CVs**	Backgrounds grow higher, making lower (Poorer) signal to noise ratios
Compounds high threshold problems	**Low background noise**	Potentially false MFI readings because longer than necessary data is integrated
Increased sample CV caused by early cut because of conflict with built in laser delays	**High MFI readings**	Increased incidence of electronic aborts
Lower MFI readings	**Stable readings**	Poor data quality and integrity because of increased abort rate
Lower quality data	**High Quality data**	Hides other incorrect settings such as laser delays because ER masks the data

ER setting value		Bead Concentration 1.56%	3.13%	6.25%	12.50%	25.00%	50.00%	66.67%	100.00%
>10,000 events/sec	Detection Rate	642	1058	2233	4436	8982	16006	20091	28022
	Abort Rate (%)	0.06	0.11	0.22	0.43	0.94	1.89	2.72	5.68
DEFAULT	Detection Rate	636	1082	2074	4141	7990	13216	14298	18228
	Abort Rate (%)	0.39	0.91	1.51	3.17	6.66	12.83	16.71	24.01

The CytoFLEX Flow Cytometry System

The CytoFLEX flow cytometer system is targeted for research users wanting a high quality instrument that has multiple laser capability combined with a level of robustness and ease of use that is unprecedented in the industry at large. Moreover the CytoFLEX is designed to provide researchers with the unmatched usability in the industry at its price point. CytoFLEX is designed to handle both high and low sample volumes and has an extremely broad detection rate (EPS) scale. CytoFLEX can operate from as low as 100 events per second (EPS) to up to 30,000 EPS with simple software and user controls.

With such a large dynamic range one might expect that optimizing the event rate setting would play a critical role in proper data measurement with the CytoFLEX, and this is indeed the case; however the process of setting ER values is made exquisitely simple because of the CytoFLEX's exclusive CytExpert software. The CytExpert software uses specialized integration algorithms for setting ER values thus allowing the CytoFLEX to properly measurement events over a large range of sample concentration and flow rate speeds.

By default, the CytoFLEX ER setting value is optimized for low event rates in the range of 100 events per second to 5000 events per second, and is a reasonably broad time amount that is added simultaneously to both ends of the gating window allowing for complete data integration. However the CytoFLEX also has an advanced special user feature that allows users to optimize event rate settings for rates greater than 10,000 events per second and still maintain robust and quality data set.

The table and graph above demonstrate how event rate (ER) influences the abort rate as the detection rate (EPS) increases. The table shows how, when properly set, the CytExpert's software's ability to properly maintain an acceptable level of low abort rates even when events exceed > 20,000 EPS. The default setting has been optimized for event rates between 100 to 5000 EPS. (Red line in graph, red boxes in table). From

Table 3

	HIGH ER SETTING	LOW ER SETTING
Advantge	1. Integrated pulse area 2. Stable; more room is given for laser delay change 3. Threshold setting change does not affect the area signals too much, unless the setting is too high	1. Low abort rate to achieve higher data acquisition rate 2. Less noises/background are integrated
Disadvantage	1. Increased abort rate 2. More noises/background are integrated	1. Incomplete pulse area integration, especially when the threshold is set high 2. Laser delay change and threshold change effect can be more obvious
Suggested Conditions	1. Normal conditions 2. Data acquisition rate < 10,000 events/second	1. High concentration samples 2. Small particles 3. Very dim signal detection

the analysis one can see that as the detection rate increases with the default event rate setting, the abort rate increases and data integrity compromises. However, by adjusting the event rate value to a shorter value, the new event rate setting allows drastically reduced sample abort rate to an acceptable level of < 3% even at >20,000 EPS. Thus this result demonstrates that with the CytoFLEX users can run at high EPS without compromising event measurement and abort rate.

From similar experiments and extensive testing a table of suggested recommendations for ER value use on the CytoFLEX machine has been compiled in Table 3.

Moreover the CytoFLEX cytometer is not as greatly affected by ER parameters because the CytExpert software integrates signals based on the position of the primary peak and then dynamically searches all the data in the ER around the peak position (after appropriate laser delay adjustment) for peaks in other channels. Integration width in the CytoFLEX, unlike other cytometers, is thus entirely determined by the trigger pulse shape, and therefore independent of "event rate value," thus less sensitive to it. This is why the CytoFLEX can operate over a variety of sample parameters without a great need to continuously adjust event rate setting parameters.

Because CytoFLEX uses a dynamic auto-search algorithm for peak finding and area integration, the system performance is less sensitive to ER settings; therefore there is no need to continuously adjust the ER parameter. The majority of applications will use either of the two primary default settings. It is hoped that this brief discussion surrounding the parameters that influence the event rate settings and how the CytoFLEX innovative solutions simply their impact will help both novice and experienced users appreciate the advantages of the CytoFLEX flow cytometry system and its benefits for their flow cytometry research needs.

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CytoFLEX Flow Cytometers with CytExpert 2.0 Acquisition and Analysis Software

Support for Compliance with 21 CFR Part 11

Introduction

21 CFR Part 11 refers to the section in the Code of Federal Regulations (CFR) that sets forth the United States Food and Drug Administration's (FDA) guidelines on using electronic records and electronic signatures. Chapter 21 covers all regulations pertaining to GCP (Good Clinical Practice), GLP (Good Laboratory Practice) and GMP (Good Manufacturing Practice), relating to the pharmaceutical and healthcare industries. Part 11 covers all FDA regulated issues pertaining to electronic records and electronic signatures.

All computer systems which store data which is used to make Quality decisions or data which will be reported to the FDA must be compliant with 21 CFR 11. The purpose of the law is to define the criteria under which electronic records and electronic signatures are considered trustworthy, reliable, and equivalent to paper records.

The regulation requires organizations to have in place three levels of control, administrative controls such as policies for electronic records, procedural controls such as SOPs for using the system, and technical controls or the functions built into software that ensure the reliability and integrity of electronic records and signatures. Software can be designed to facilitate compliance with 21 CFR 11 technical controls, but it is the user who is responsible for providing policies and procedures to ensure the systems are fully compliant with the regulations.

CytExpert 2.0 has features that were designed to facilitate user compliance with 21 CFR, Part 11, when installed using the Electronic Record Management option. These features were not available in previous versions of the software. The table below references specific sections of the regulation and indicates how CytExpert 2.0 facilitates compliance with that section.

Compliance Support Notes

Section	Requirement	CytoFLEX with CytExpert 2.0 software with Electronic Record Management mode installed
11.10	**Controls for closed systems**	
11.10(a)	Validation of systems to ensure accuracy, reliability, consistent intended performance, and the ability to discern invalid or altered records.	Quality IQ/OQ available to validate performance to specifications upon installation. Instrument QC confirms the instrument is working properly within the specified parameters. Quality control allows you to determine whether your instrument can provide signal strength and precision to specifications. The system can discern tampering of records that are entered in the audit log and indexed database.
11.10(b)	The ability to generate accurate and complete copies of records in both human readable and electronic form suitable for inspection, review, and copying by the agency.	Recorded experiment results and audit trail records can be viewed and exported as readable electronic files (such as PDF) and printed.
11.10(c)	Protection of records to enable their accurate and ready retrieval throughout the records retention period.	The Experiment file and folder are stored in an encrypted format controlled by an indexing database.
11.10(d)	Limiting system access to authorized individuals.	All users need to have a password to login and use the software. The functions and features open to individual users are defined by administrators.

Section	Requirement	CytoFLEX with CytExpert 2.0 software with Electronic Record Management mode installed
11.10	Controls for closed systems (continued)	
11.10(e)	Use of secure, computer-generated, time-stamped audit trails to independently record the date and time of operator entries and actions that create, modify, or delete electronic records. Record changes shall not obscure previously recorded information. Such audit trail documentation shall be retained for a period at least as long as that required for the subject electronic records and shall be available for agency review and copying.	Audit trail records include all key operations of users, such as login, log out, creating experiments, and setting modifications. All changes which are saved in the records that are stored in the indexed database cannot be removed or modified.
11.10(f)	Use of operational system checks to enforce permitted sequencing of steps and events, as appropriate.	All users need to have a password to login and use the software. The functions and features open to individual users are defined by administrators. The system has a software controlled operational daily quality control procedure for system calibration and performance verification.
11.10(g)	Use of authority checks to ensure that only authorized individuals can use the system, electronically sign a record, access the operation or computer system input or output device, alter a record, or perform the operation at hand.	Different levels of authority can be assigned to different roles of users. Administrators can define these based on laboratory policies.
11.10(h)	Use of device (e.g., terminal) checks to determine, as appropriate, the validity of the source of data input or operational instruction.	Rules are set for data input. Invalid data input would be warned and denied.
11.10(i)	Determination that persons who develop, maintain, or use electronic record/electronic signature systems have the education, training, and experience to perform their assigned tasks.	User training is offered by Beckman Coulter. Users who take the training and pass the exam receive verified completion certificates. Laboratory policies and procedures should also been generated to assure users are trained properly.
11.10(j)	The establishment of, and adherence to, written policies that hold individuals accountable and responsible for actions initiated under their electronic signatures, in order to deter record and signature falsification.	Duplicate user IDs are not allowed in the software. Laboratory procedures should enforce not sharing identification information as well as attributing responsibilities for significance of the use of the electronic signatures in the system.
11.10(k)	Use of appropriate controls over systems documentation including: 1. Adequate controls over the distribution of, access to, and use of documentation for system operation and maintenance. 2. Revision and change control procedures to maintain an audit trail that documents time-sequenced development and modification of systems documentation.	System documentation is controlled via ISO compliant ECO (Engineering Change Order) process.
11.3	Controls for Open Systems	
11.30	Controls for Open Systems	N/A The system, CytoFLEX with CytExpert 2.0 software with Electronic Record Management mode, applies to closed systems.
11.50	Signature Manifestations	
11.50(a)	Signed electronic records shall contain information associated with the signing that clearly indicates all of the following: 1. The printed name of the signer; 2. The date and time when the signature was executed; and 3. The meaning (such as review, approval, responsibility, or authorship) associated with the signature.	Each user has a unique user ID. Once a document is signed, the signature contains user ID, date, name, and optional comments. The signature is included in the experiment operation log. Users can find details in the Signature Details window.
11.50(b)	The items identified in paragraphs (a)(1), (a)(2), and (a)(3) of this section shall be subject to the same controls as for electronic records and shall be included as part of any human readable form of the electronic record (such as electronic display or printout)	

Section	Requirement	CytoFLEX with CytExpert 2.0 software with Electronic Record Management mode installed
11.70	**Signature/Record Linking**	
	Electronic signatures and handwritten signatures executed to electronic records shall be linked to their respective electronic records to ensure that the signatures cannot be excised, copied, or otherwise transferred to falsify an electronic record by ordinary means.	Once an experiment is signed, users need to remove the signature before making changes.
11.100	**General Requirements**	
11.100(a)	Each electronic signature shall be unique to one individual and shall not be reused by, or reassigned to, anyone else.	Duplicate user ID is not allowed in the software. A password is required for each user.
11.100(b)	Before an organization establishes, assigns, certifies, or otherwise sanctions an individual`s electronic signature, or any element of such electronic signature, the organization shall verify the identity of the individual.	Only authorized users can create new users for the software. The laboratory should establish the process to define authority to create administrator roles.
11.100(c)	Persons using electronic signatures shall, prior to or at the time of such use, certify to the agency that the electronic signatures in their system, used on or after August 20, 1997, are intended to be the legally binding equivalent of traditional handwritten signatures. 1. The certification shall be submitted in paper form and signed with a traditional handwritten signature, to the Office of Regional Operations (HFC-100), 12420 Parklawn Drive, RM 3007 Rockville, MD 20857. 2. Persons using electronic signatures shall, upon agency request, provide additional certification or testimony that a specific electronic signature is the legally binding equivalent of the signer`s handwritten signature.	Lab procedures should enforce responsibility and accountability for the use of the electronic signatures in the system.
11.200	**Electronic Signature Components and Controls**	
11.200(a)	Electronic signatures that are not based upon biometrics shall: 1. Employ at least two distinct identification components such as an identification code and password. i. When an individual executes a series of signings during a single, continuous period of controlled system access, the first signing shall be executed using all electronic signature components; subsequent signings shall be executed using at least one electronic signature component that is only executable by, and designed to be used only by, the individual. ii. When an individual executes one or more signings not performed during a single, continuous period of controlled system access, each signing shall be executed using all of the electronic signature components. 2. Be used only by their genuine owners; and 3. Be administered and executed to ensure that attempted use of an individual's electronic signature by anyone other than its genuine owner requires collaboration of two or more individuals.	User ID and password are required to login the system. Before signing the records, users are required to enter the password again.
11.200(b)	Electronic signatures based upon biometrics shall be designed to ensure that they cannot be used by anyone other than their genuine owners.	N/A

Section	Requirement	CytoFLEX with CytExpert 2.0 software with Electronic Record Management mode installed
11.300	Controls for Identification Codes/Passwords	
11.300(a)	Maintaining the uniqueness of each combined identification code and password, such that no two individuals have the same combination of identification code and password.	Duplicate user ID is not allowed in the system. Each user needs to have different combination of user ID and password.
11.300(b)	Ensuring that identification code and password issuances are periodically checked, recalled, or revised (e.g., to cover such events as password aging).	Administrators can set the expiration policy for passwords.
11.300(c)	Following loss management procedures to electronically deauthorize lost, stolen, missing, or otherwise potentially compromised tokens, cards, and other devices that bear or generate identification code or password information, and to issue temporary or permanent replacements using suitable, rigorous controls.	Administrators can reset passwords, set account lock policy, or disable accounts.
11.300(d)	Use of transaction safeguards to prevent unauthorized use of passwords and/or identification codes, and to detect and report in an immediate and urgent manner any attempts at their unauthorized use to the system security unit, and, as appropriate, to organizational management.	The system can lock an account after several login attempts with wrong password based on the administrator setting.
11.300(e)	Initial and periodic testing of devices, such as tokens or cards, that bear or generate identification code or password information to ensure that they function properly and have not been altered in an unauthorized manner.	The system has the rules for password settings. Laboratory policy for periodic testing is required.

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Expanding the Useful Spectrum for Flow Cytometry: Increasing the Number of Parameters Available Without Adding Compensation Complexity

Introduction

As our understanding of biological systems increases, so does our knowledge of the complexity of those systems. Flow cytometry with its multi-parametric, high throughput capabilities has enabled the discovery of varied and nuanced cell types in terms of phenotypic markers and biological functions. In the modern marker panel, researchers need a minimum of eight colors to define identity and additional parameters to interrogate function[1]. We can expect that these requirements will continue to grow as cell biology advances.

The visible spectrum places limitations on the number of extrinsic parameters that can be assessed as well as the number of dyes with suitable properties and non-overlapping emission spectra. Expanding the available palette requires exploring options on the periphery of the visible spectrum (Figure 2).

Lower wavelengths, toward the blue and violet end of the spectrum, pose complications from auto-fluorescence. The fluorescence emission arising from endogenous fluorophores is excited by UV/blue wavelengths of light, typically due to structures and compounds in mitochondria and lysosomes[2]. The emission characteristics of auto-fluorescence is similar to fluorescein and PE and will, therefore, interfere with the detection of FITC and GFP fluorescence. Excluding auto-fluorescence isn't always possible and quenching it can affect experimental signals.

Expanding toward the far red end of the spectrum has been hindered due to limitations of photomultiplier tube detectors (PMTs). However, with the introduction of photodiode detectors, with high and stable quantum efficiency has been demonstrated from 400 nm to 1100 nm.

In this whitepaper we demonstrate the biological applications of an Infrared laser in a commercially available flow cytometer, the CytoFLEX S Flow Cytometer, which utilizes avalanche photodiode technology along with innovations in light management to provide a sensitive yet small research flow cytometer.

IR Channel Quantum Efficiency Using Avalanche Photodiode Detectors

Infrared light, of wavelengths from 750 nm to 1000 nm, occupies the edge of the spectrum after the red and extends into the microwave area. Photocathode materials, such as used in PMTs, limit the useful working range of these detectors to approximately 750 nm. In contrast, the Avalanche Photodiode (APD) exhibits good quantum efficiencies up to 1100 nm[3]. APDs improve the detection sensitivity of red and near infrared wavelengths (Figure 1).

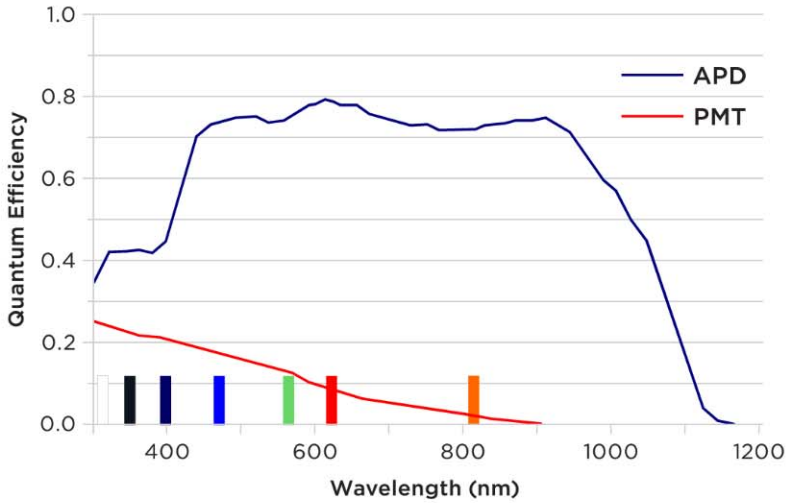


Figure 1. Photomultiplier Tube versus Avalanche Photodiode Quantum Efficiency by Wavelength. The PMT has historically dominated use in flow cytometers. The quantum efficiency drops rapidly for wavelengths above 600 nm. The APD however exhibits stable quantum efficiency up to 1000 nm. The bars on the lower axis indicate the CytoFLEX platform excitation wavelengths at 355 nm, 375 nm, 405 nm, 488 nm, 561 nm, 638 nm, and 808 nm. *Adapted from "A Comparison of Avalanche Photodiode and Photomultiplier Tube Detectors for Flow Cytometry" by Paul Wallace et al, 2008, Proceedings of SPI, Vol 6859.*

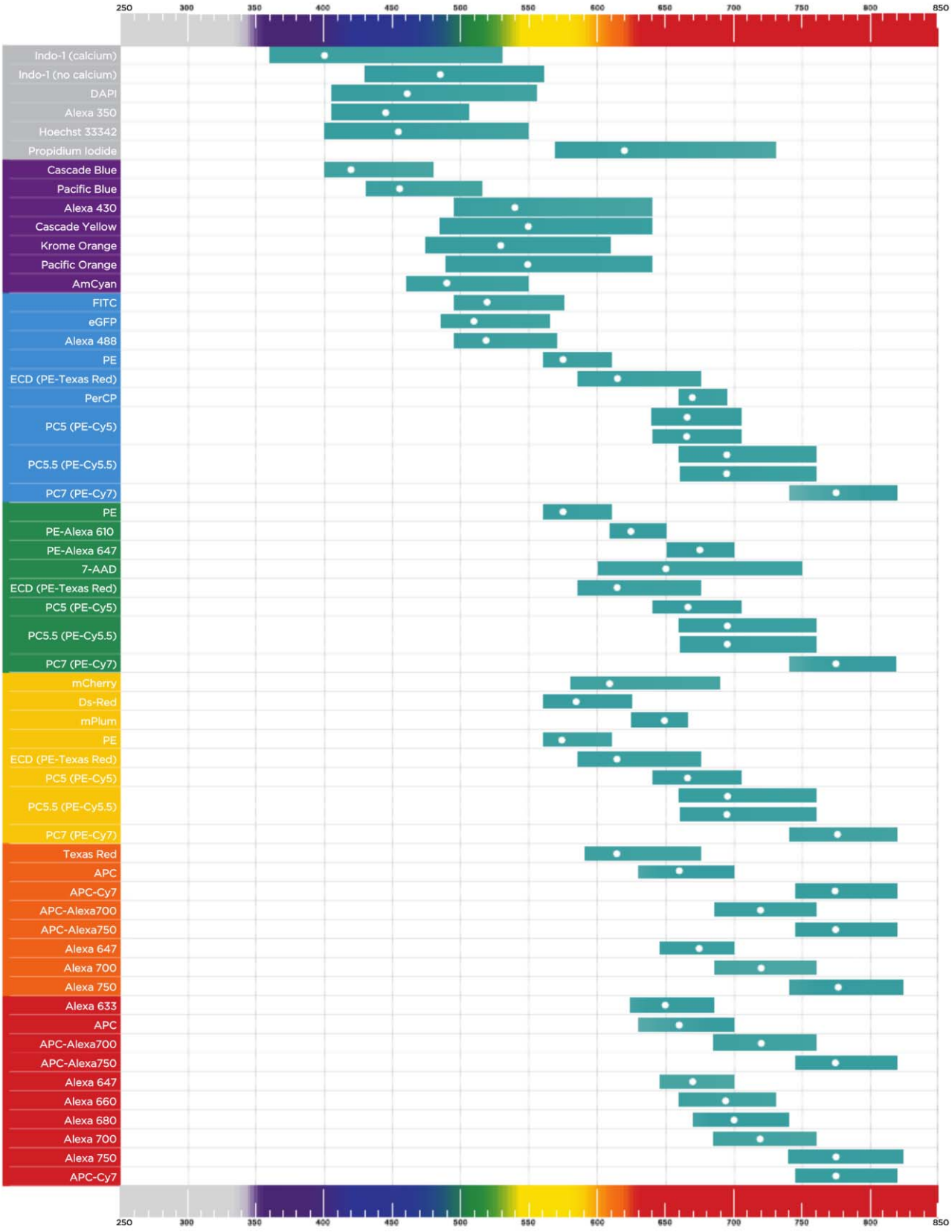


Figure 2. Emission Spectra for Common Fluorochromes. The visible spectrum is 390 to 700 nm and a variety of fluorochromes are available across these wavelengths. Expanding the number of markers that can be interrogated simultaneously requires expanding the useful spectrum at the edges.

First Commercially Available Flow Cytometer Incorporating Infrared Laser and Avalanche Photodiode Detectors

CytoFLEX S Blue-Red-Violet-Infrared (B-R-V-I) Series from Beckman Coulter is the first flow cytometer offering IR laser option as a standard product. The fully activated instrument includes four fluorescent channels from the 488 nm (Blue) laser, three from the 638 nm (Red) laser, four from the 405 nm (Violet) laser, and two from the 808 nm (Infrared) laser. The instrument includes 13 band pass filters which can be repositioned as needed. Instruments with as few as four fluorescent channels activated are available with the ability to activate additional parameters later (Figure 3).

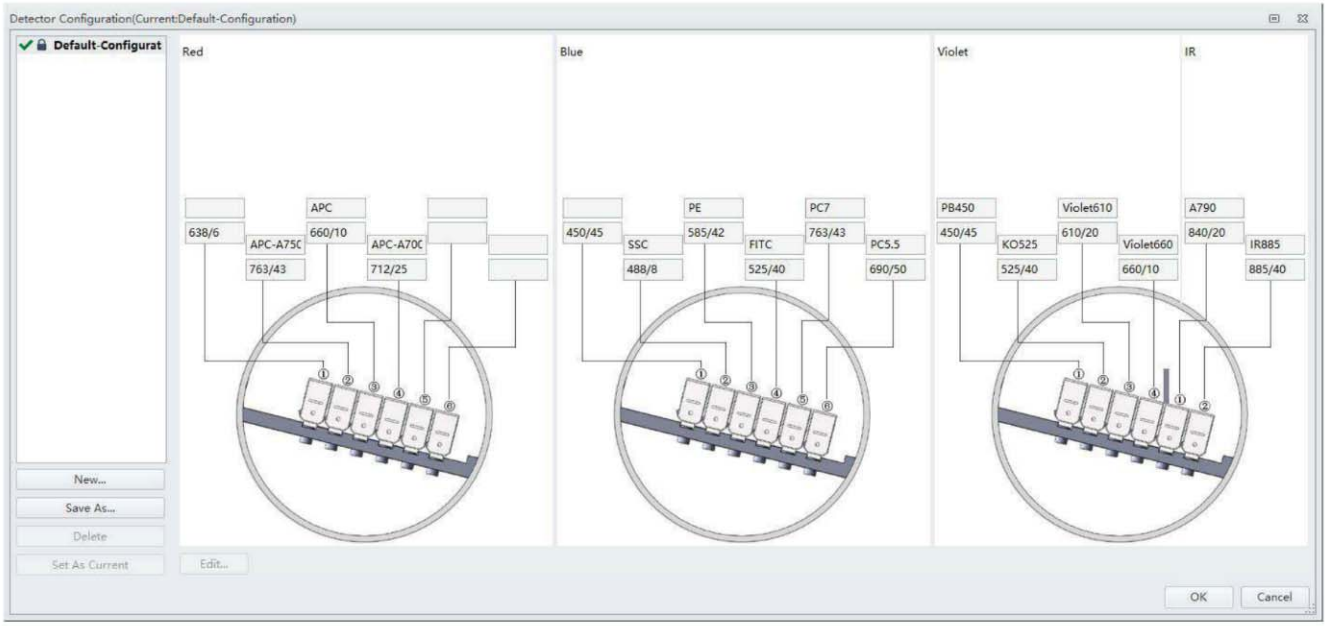


Figure 3. Detector Configuration of the CytoFLEX S Blue-Red-Violet-Infrared Instrument. The instrument includes four fluorescent channels from the Blue (488 nm, 50 mW) solid state laser, three from the Red (638 nm, 50 nW) solid state laser, four from the Violet (405 nm, 80 mW) solid state laser, and two from the Infrared (808 nm, 60 mW) solid state laser.

This instrument is based upon the unique assembly of technologies comprising the CytoFLEX platform, which contributes to the exquisite sensitivity of the instrument. Borrowing technology from the telecommunications industry, the Wavelength Division Multiplexer (WDM) deconstructs and measures multiple wavelengths of light. The WDM relies on fiber optics and band pass filters to separate the light wavelengths. Unlike more traditional instruments, multiple dichroic filters to direct the light path are not required. This makes it much easier to configure the fluorescence channels, but also increases efficiency by minimizing light loss due to refraction. The WDM utilizes Avalanche Photodiode detectors (APDs), versus Photomultiplier tubes (PMTs).

One hallmark of the photodiode is the high quantum efficiency in excess of 80%, especially for wavelengths greater than 800 nm. Integrated optics also increases the efficiency of light movement through the system by focusing light onto the flow cell. With conventional analyzers, laser excitation sources are optimized by shaping and focusing light through a series of lenses and filters onto the flow cell. Each of these light interactions is an opportunity for light loss. All of these technologies work together to ensure efficient light management for optimal excitation and emission of fluorochrome-tagged cells.

This increased quantum efficiency results in decreased measurement variation. Low measurement variation reduces the standard deviation in measurements. The impact of this on resolution can be understood by considering the Staining Index. The staining index characterizes the relative brightness of different fluorochromes under actual experimental conditions. wIt quantifies the variance of the positive and negative population, normalized by the data spread of the negative population. The data spread has two components, one is population or experimental variation and the other is measurement error. The smaller this normalization value, the larger the Staining Index. A large Staining Index indicates that it will be easier to differentiate positive populations from negative populations.

Absence of Laser Cross Talk and Infrared Light Spillover Reduces Compensation in Multicolor Experiments

One advantage of Infrared excitation is to expand the number of colors that can be used for multiparametric analysis. The long wavelengths of the IR laser do not exhibit cross talk into traditional detection channels. Alex Fluor 790 and PromoFlour 840 are two commercially available Infrared excited dyes (Figure 4).

A compensation study was performed to evaluate the spillover of Alexa Fluor 790 (AF790) and PromoFluor 840 (PF840) fluorochromes into other channels and the spillover of other dyes into the two IR channels. The study showed that there is minimal spillover of AF790 and PF840 into the detection channels of other lasers (Figure 5) and that there is minimal spillover of other single color dyes into the IR channels (Figure 6). Altogether, these results confirm that IR excitation and detection can be incorporated into flow cytometry instruments and thus allow users additional options for multi-color design and biological applications.

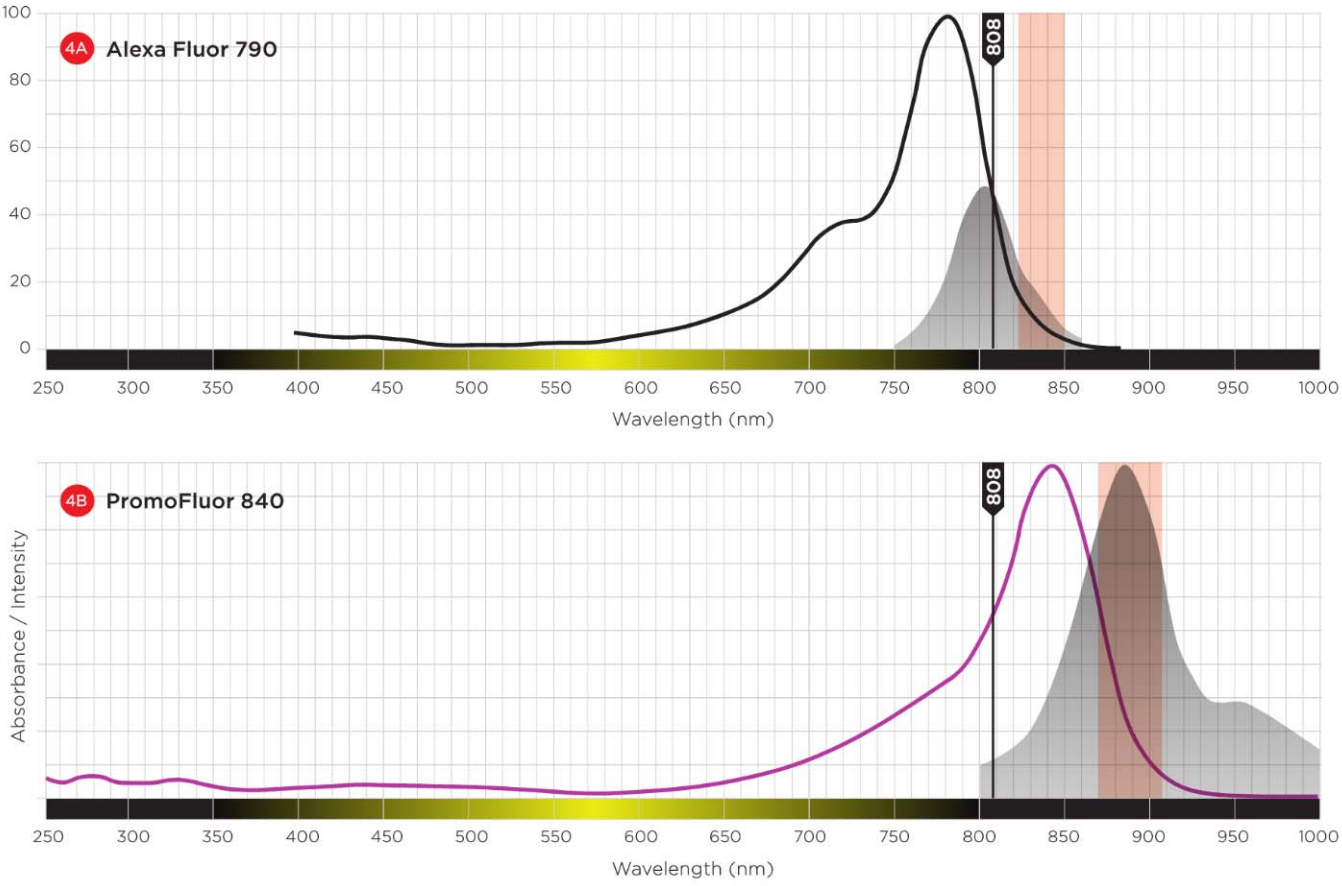
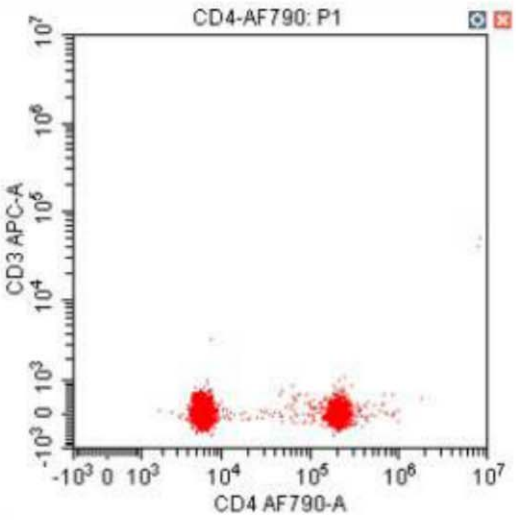
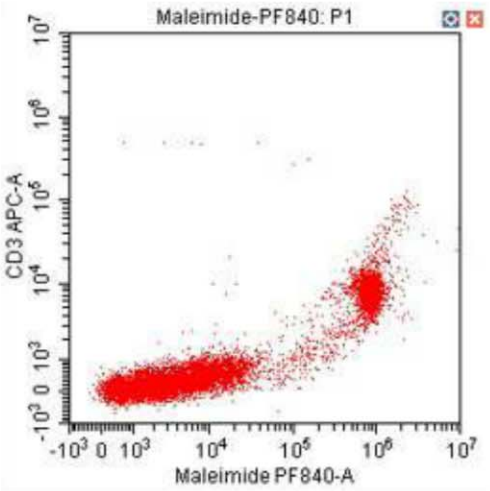
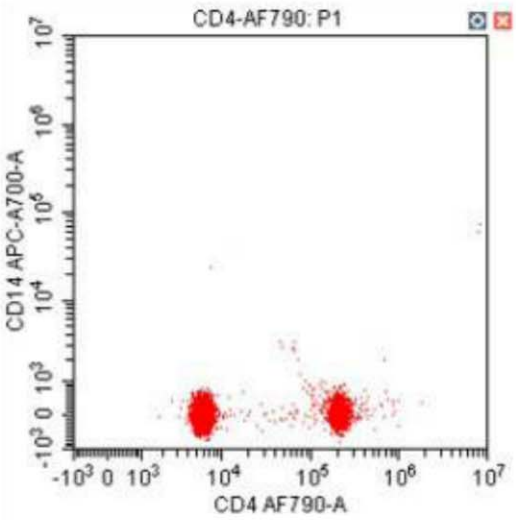
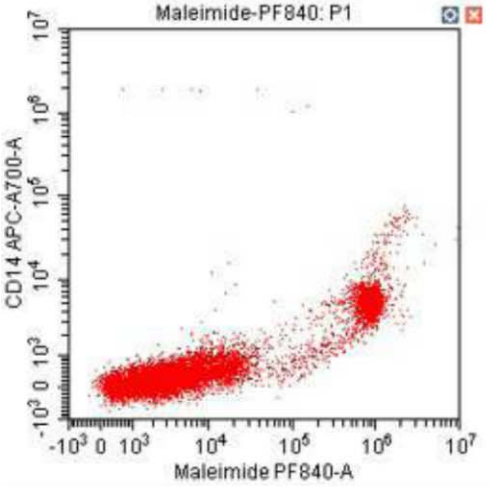
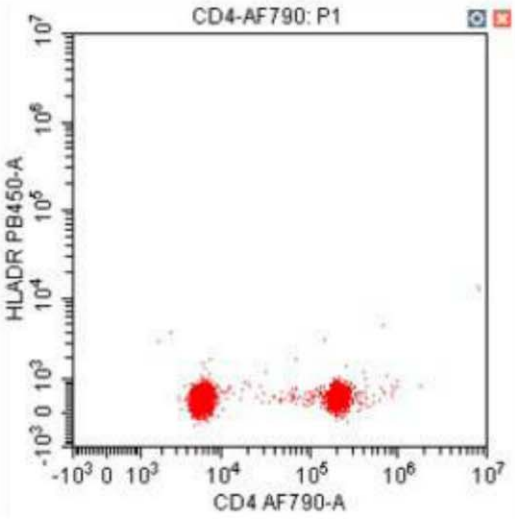
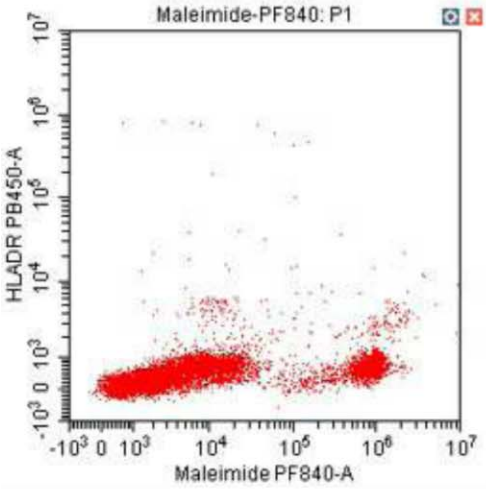
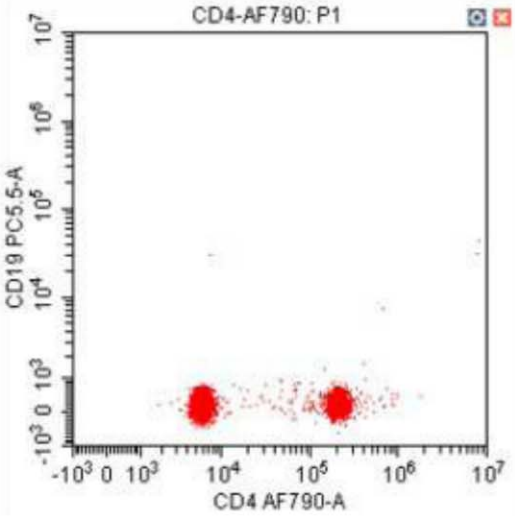
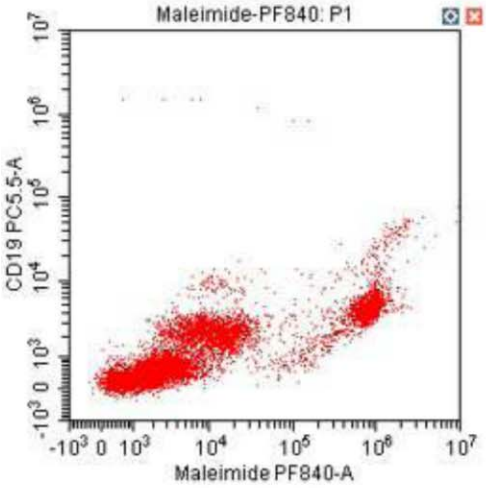
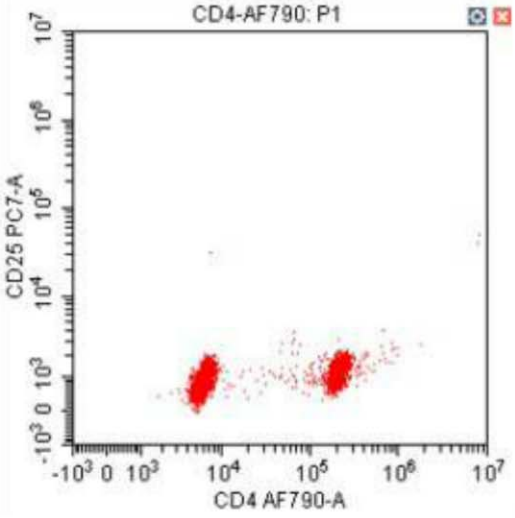
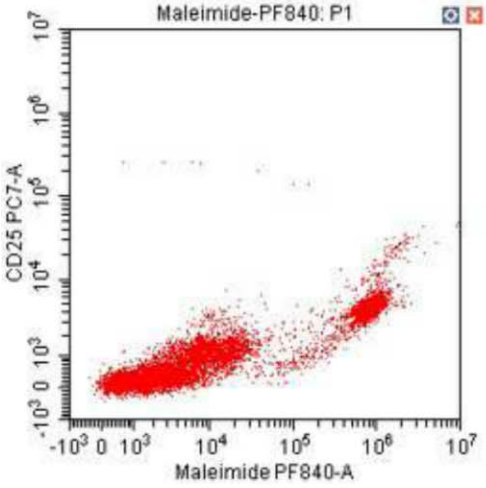
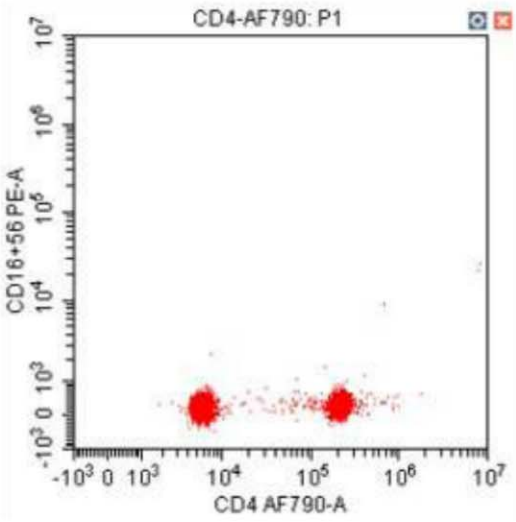
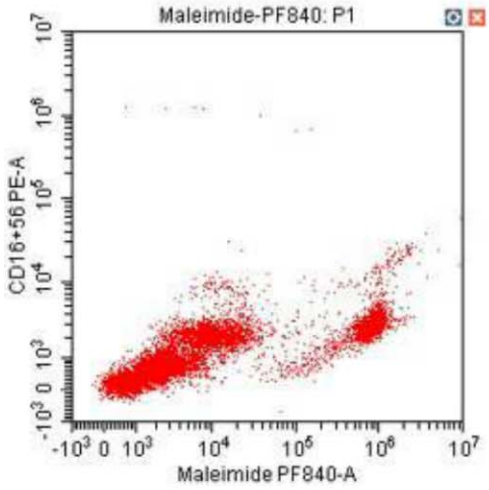
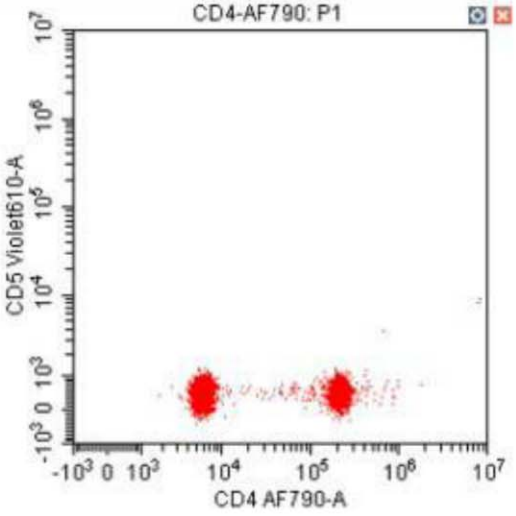
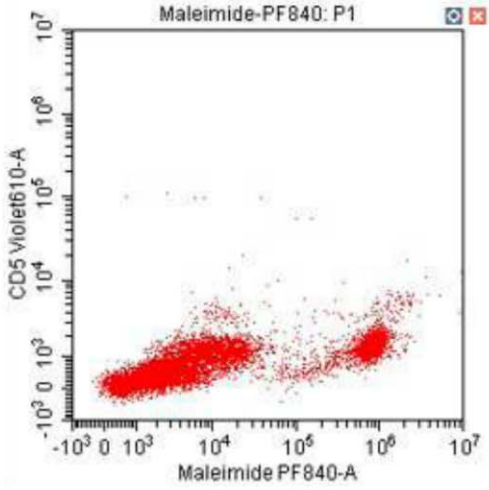
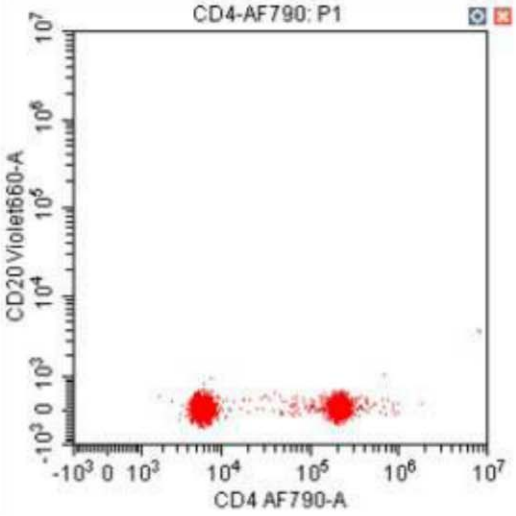
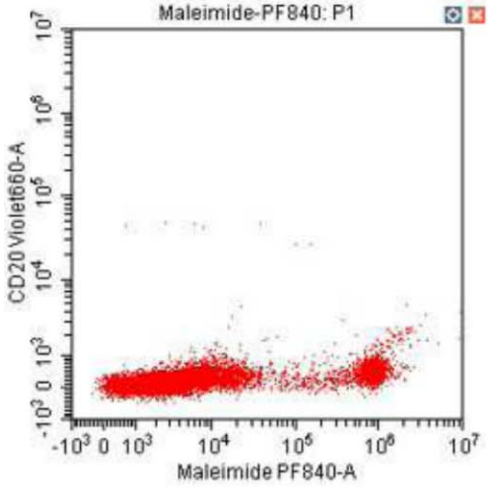


Figure 4: Excitation and Emission spectra for (a) Alexa Fluor 790 and (b) PromoFluor 840. Alexa Fluor has an excitation maximum of 784 nm and an emission maxima of 814 nm. PromoFluor 840 has an excitation maximum of 844 nmand an emission maxima of 884 nm.

Figure 5. IR dye spill over into other detection channels. VersaComp beads (Beckman Coulter, Part Number B22804) were stained with the recommended amount of single color reagents (Table 1, Beckman Coulter) at room temperature, in the dark for 30 minutes. The samples were washed and then resuspended in 1 mL PBS with Fixative Solution (Beckman Coulter, Part Number A07800). The samples were run on the CytoFLEX S Blue-Red-Violet-Infrared Series and analyzed with CytExpert software v1.2. Each single color is evaluated for its spillover into the AF790 IR channel and the PF840 IR channel. Other than reciprocal spillover by the IR dyes, recognizable by the steep diagonal on the dot plot, all other single colors showed minimal spillover into the AF790 and PF840 IR channels.

Channels	AF790 Stained Beads	PF840 Stained Cells
APC		
APC-A700		

Channels	AF790 Stained Beads	PF840 Stained Cells
Pacific Blue		
PC5.5		
PC7		

Channels	AF790 Stained Beads	PF840 Stained Cells
PE		
Violet 610		
Violet 660		

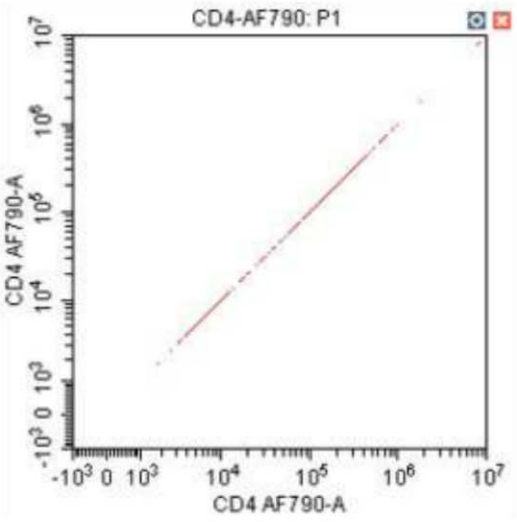
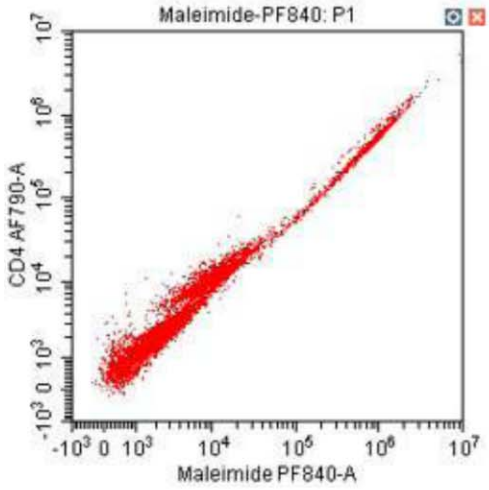
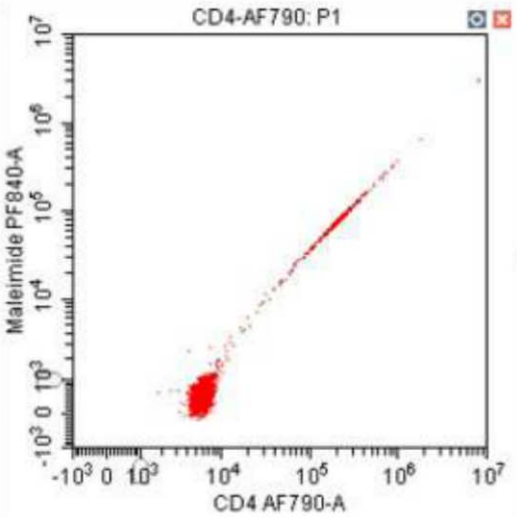
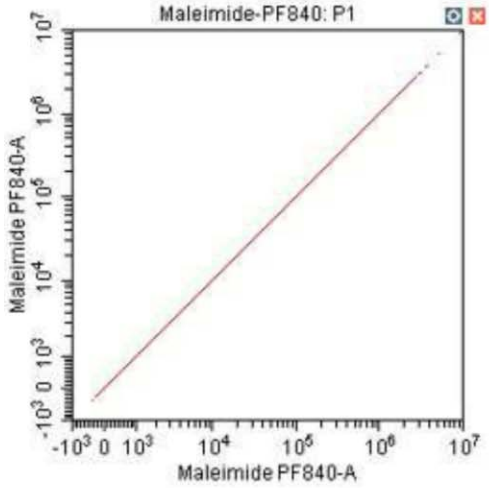
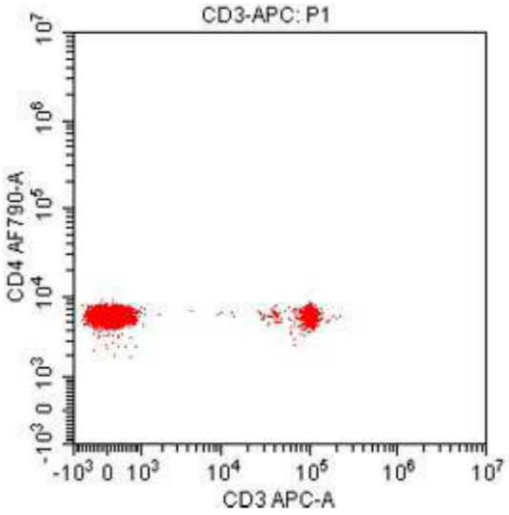
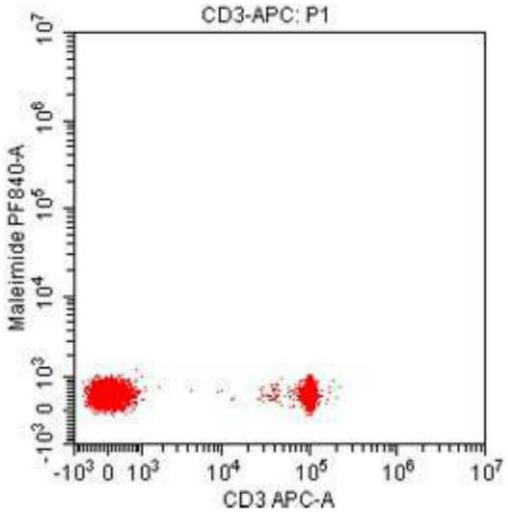
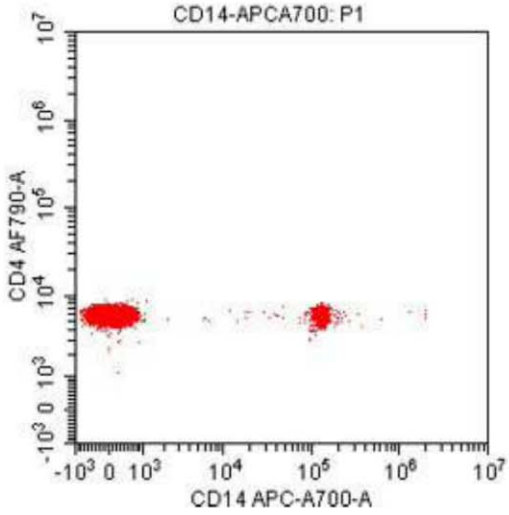
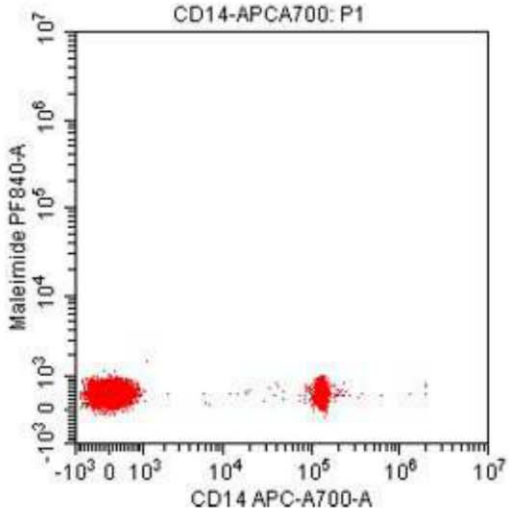
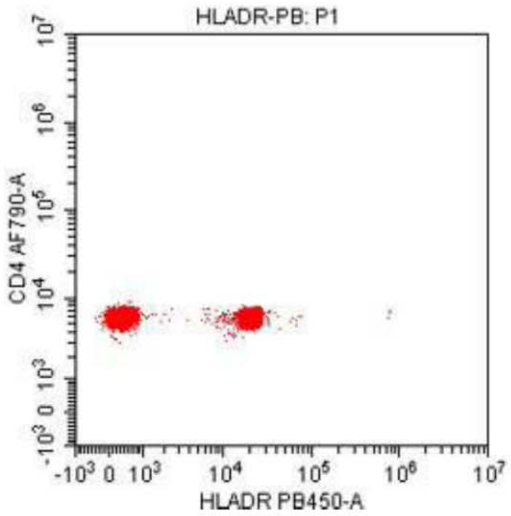
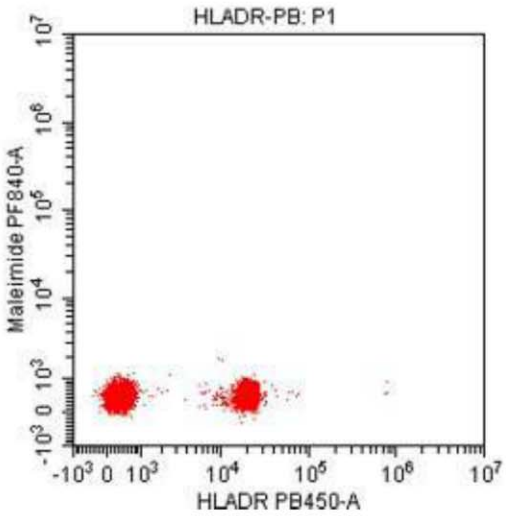
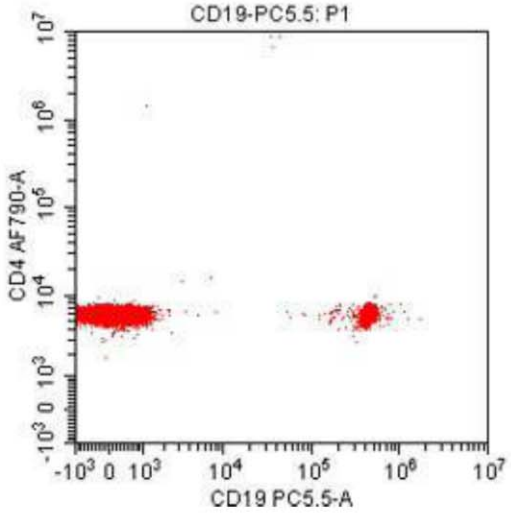
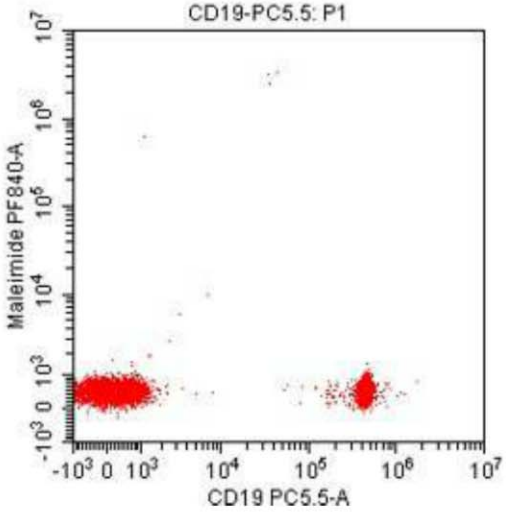
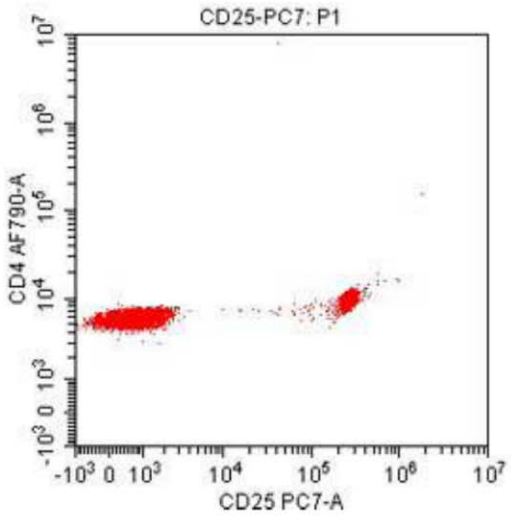
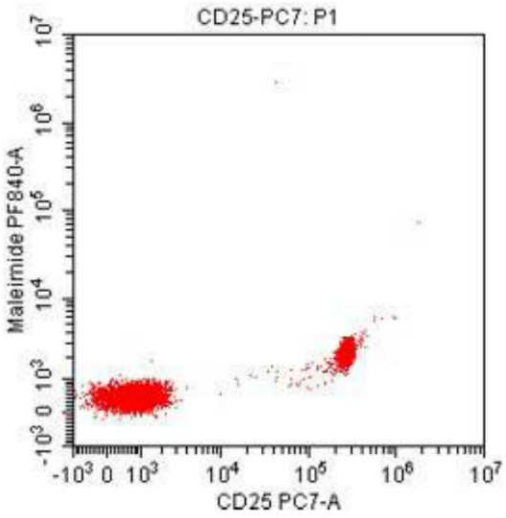
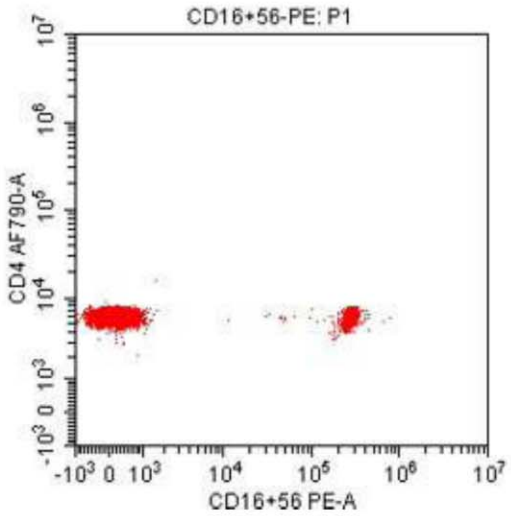
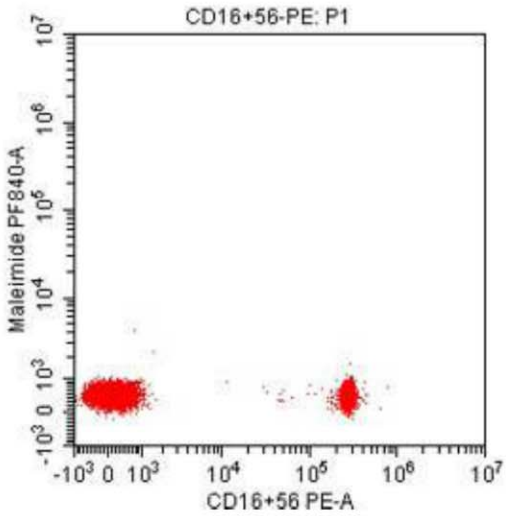
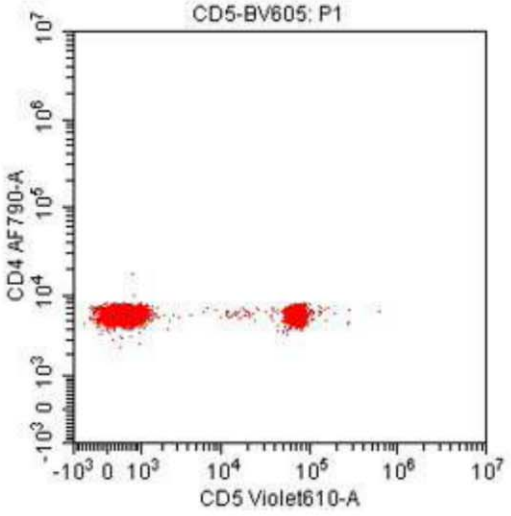
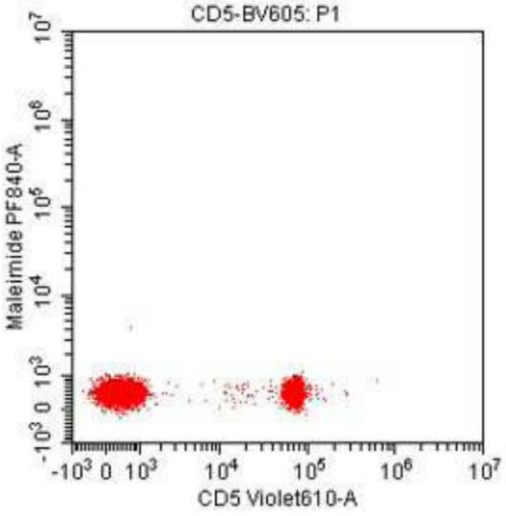
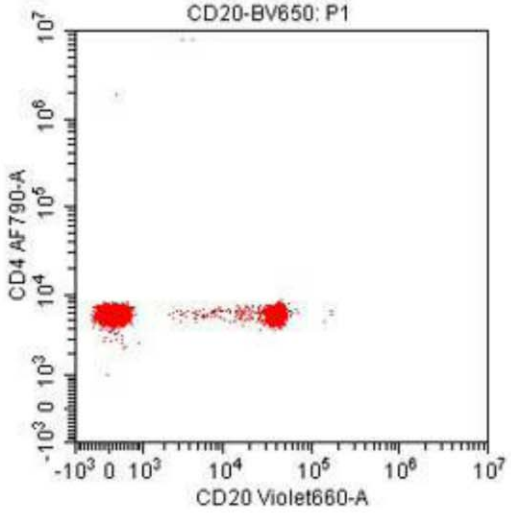
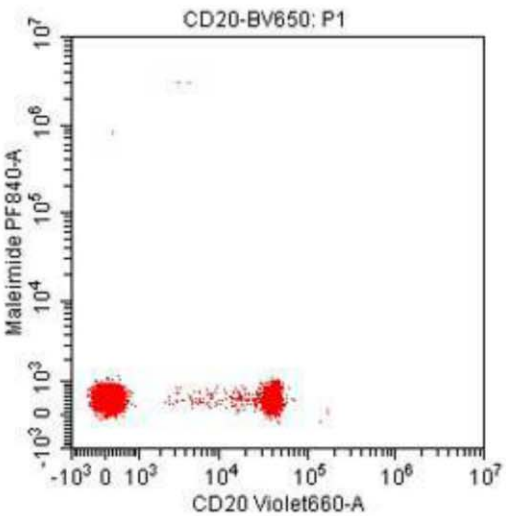
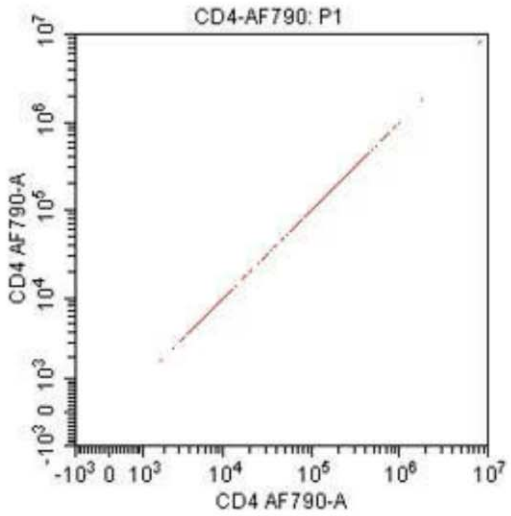
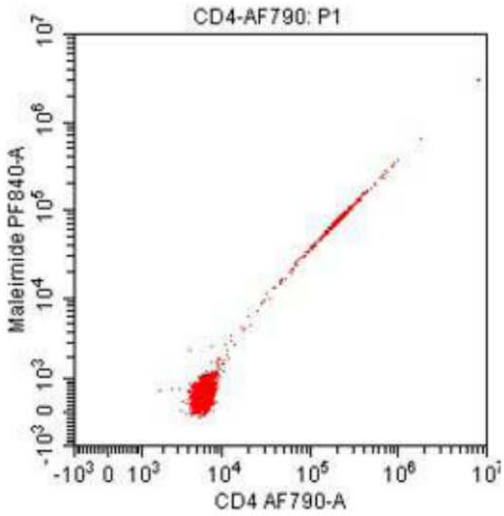
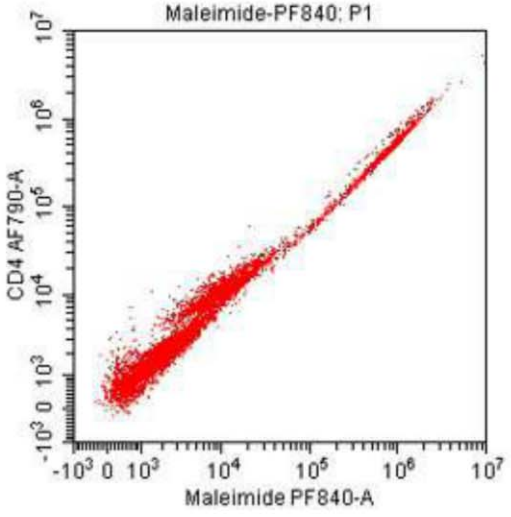
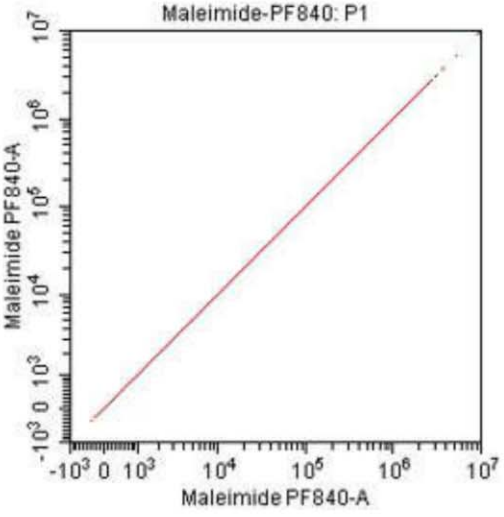
Channels	AF790 Stained Beads	PF840 Stained Cells
AF790		
PF840		

Figure 6. Compensation evaluation of fluorescent dye spillover into the IR channels. AF790-stained VersaComp beads were run on the CytoFLEX S Blue-Red-Violet-Infrared Flow Cytometer (left column). The fluorescence measurement of AF790-stained sample in the AF790 IR channel is compared to all other channels. Very little spillover of AF790 dye is observed in other channels except for the PF840 IR channel. PF840-stained cells was run on the CytoFLEX S Blue-Red-Violet-Infrared Flow Cytometer and the fluorescence measurement in the PF840 channel is compared to all other channels (right column). The PF840 dye does not produce significant spill over into other channels except for the AF790 IR channel.

Fluorochrome	AF790 Channel	PF840 Channel
APC		
APC-A700		

Fluorochrome	AF790 Channel	PF840 Channel
Pacific Blue		
PC5.5		
PC7		

Fluorochrome	AF790 Channel	PF840 Channel
PE		
Violet 610		
Violet 660		

Fluorochrome	AF790 Channel	PF840 Channel
AF790		
PF-840		

Utilizing IR Dyes in Biological Applications Extends Capabilities

The addition of the 808 nm laser to the CytoFLEX S series provides additional fluorescent channels not only for use of viability dyes but also bright markers with minimal spectral overlap into traditional channels.

Lymphocyte Subset Analysis with Live/dead Discrimination

T cells, B cells, and NK cells comprise the lymphocytes and are the mediators of both cellular and humoral immune responses. In healthy individuals, these cells are present in relatively fixed proportions. However, in various diseases the absolute number and proportion will vary. Flow cytometry is the preferred assay for assessing the proportion of cells in these distinct populations. The addition of a parameter to assess cell health and vitality adds an internal sample quality control and allows for dead cells to be removed from the statistical analysis. Dead cells may increase non-specific binding and skew results. A 13 color immunophenotyping panel was designed to detect several major cell populations involved in innate and adaptive immune responses (Figure 7). The gating and population analysis is described in the next paragraph.

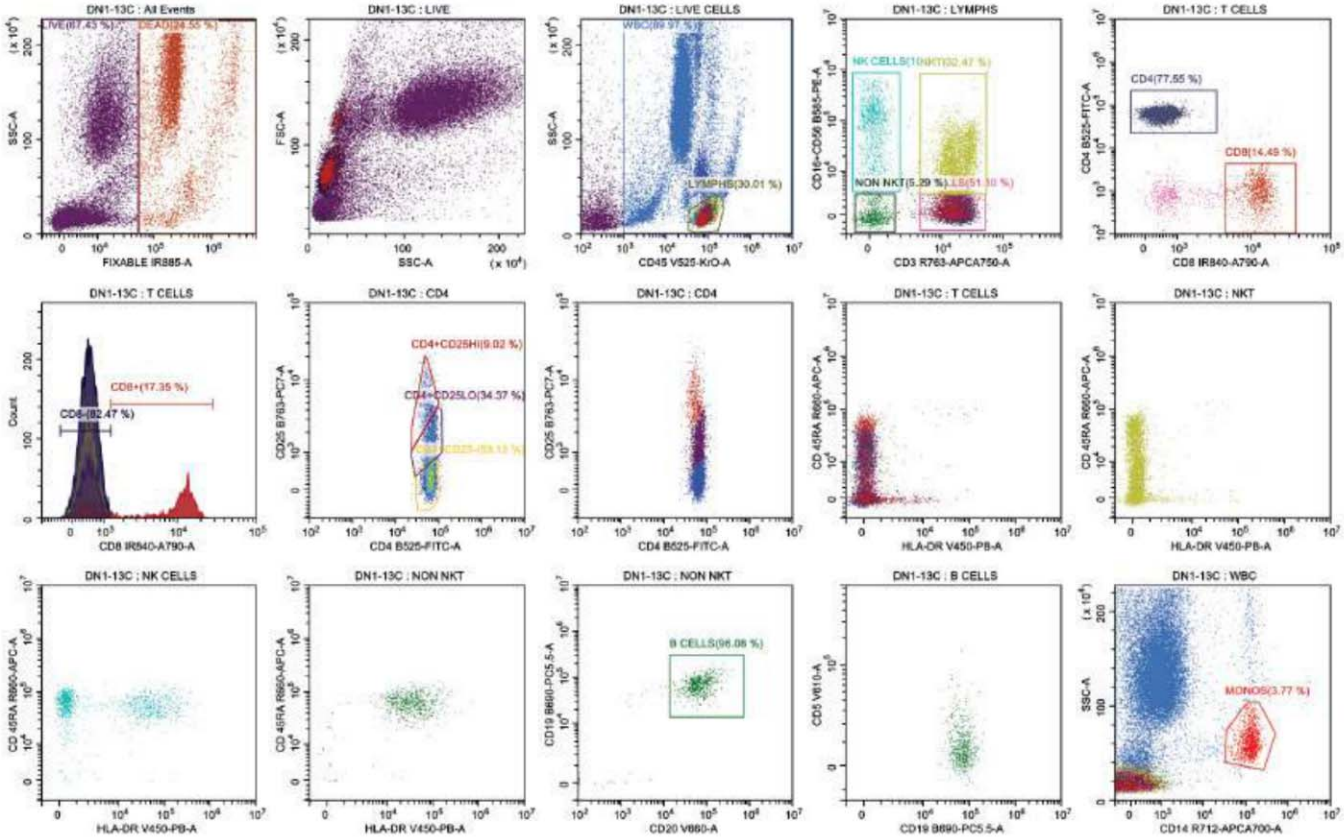


Figure 7. Immunophenotyping of human peripheral blood by 13-color flow cytometry. Blood was drawn from healthy donors by venipuncture into evacuated tubes containing EDTA anticoagulant. The cell surface makers were stained by adding 100 µL of blood to a cocktail of antibodies (Table 1) for 15 minutes in the dark. To lyse the red blood cells, 1 mL of VersaLyse Lysing Solution Ready-for-use (Beckman Coulter Part Number IM3648), vortexed immediately, and then incubated for 10 minutes at room temperature in the dark. After lysing, cells were stained with 2.5 µL of the IR Fixable Dye (PromoKine Catalog Number PK-PF840-3-01) and incubate for 20 minutes at room temperature in the dark. Cells were then fixed with 1 mL of 0.05% of a 1X preparation of Fixative Solution (Beckman Coulter Part Number IM3648) diluted with PBS. This sample was then analyzed on the CytoFLEX S Blue-Red-Violet-Infrared Flow Cytometer using the recommended settings from the Daily QC and applying the compensation matrix (Figure 8).

Compensation Matrix - DN1-13C

Use | Show Autofluorescence | Area and Height in Sync | Area

Autofl.	Channel	-R660-APC%	-R712-APCA700%	-R763-APCA750%	-V450-PB%	-V525-KrO%	-V610%	-V660%	-IR840-A790%	-IR885...	-B525-FITC%	-B585-PE%	-B690-PC5.5%	-B763-PC7%
0.00	R660-APC		7.91	24.43	0.00	0.00	0.03	8.25	0.00	0.19	0.00	0.00	0.57	0.03
0.00	R712-AP...	42.86		13.10	0.00	0.02	0.02	5.95	0.18	1.42	0.00	0.00	17.04	0.13
0.00	R763-AP...	12.25	29.87		0.00	0.04	0.00	1.17	1.85	1.10	0.01	0.00	3.81	5.44
0.00	V450-PB	0.00	0.00	0.00		0.78	2.13	5.54	0.00	0.68	0.00	0.00	0.00	0.00
0.00	V525-KrO	0.00	0.00	0.00	51.96		0.36	1.06	0.00	3.06	3.58	0.04	0.00	0.00
0.00	V610	0.74	0.04	0.21	3.45	60.01		70.47	0.00	2.63	0.59	5.50	0.26	0.51
0.00	V660	18.42	1.39	4.05	0.32	7.83	22.57		0.00	0.50	0.06	0.56	0.55	0.06
0.00	IR840-A7...	0.00	0.02	7.73	0.02	0.00	0.00	0.00		32.22	0.00	0.00	0.00	1.48
0.00	IR885	0.00	0.00	3.35	0.01	0.00	0.00	0.00	2.74		0.01	0.00	0.00	0.74
0.00	B525-FITC	0.00	0.00	0.00	0.00	0.55	0.00	0.00	0.07	2.49		0.88	0.31	0.83
0.00	B585-PE	0.00	0.00	0.03	0.00	0.52	3.32	0.11	0.00	3.37	25.94		6.66	11.81
0.00	B690-PC...	2.30	2.16	0.83	0.01	0.04	1.01	0.93	0.14	0.90	0.74	3.82		2.10
0.00	B763-PC7	0.43	1.02	2.80	0.10	0.03	0.15	0.10	0.51	0.43	0.08	0.36	24.87	

Import from Library... | Import... | Export... | Clear | Apply to... | Close

Figure 8. Compensation Matrix for 13-color Immunophenotyping Analysis. Single color stains were prepared using VersaComp Antibody Capture Beads (Beckman Coulter Part Number B22804) and antibodies (Table 1) and using CytExpert Sofrtware to run in a compensation experiment on the CytoFLEX S Blue-Red-Violet-Infrared Flow Cytometer using the recommended settings from the Daily QC. The resulting compensation matrix shows no values over 70%. Compensation in the adjacent Infrared channels is 32.22%.

Fixable Viability Dye/SSC dot plot was gated on all events and used to differentiate live from dead cells. Using one of the IR channels for the Live/Dead discrimination preserved traditional channels for marker analysis. Side scatter/forward scatter dot plot was gated on live cells. CD45/Side scatter was gated on live cells. This display was used to define the Lymph gate. CD3/CD16 dot plot was gated on Lymphs. This display was used to define NK Cells ($CD3^-$,$CD16^+$), NKT ($CD3^+$,$CD16^+$), Non-NKT ($CD3^-$, $CD16^+$), and T cells ($CD3^+$,$CD16^-$). CD8/CD4 dot plot was gated on T cells. This display was used to define $CD4^+CD8^-$ and $CD4^-CD8^+$ populations. CD8, an abundant marker, was labeled with AF790 in order to prevent potential spillover from this signal into other channels. CD8 histogram for these population indicates approximately 17% of the T cells are CD8 positive. CD4/CD25 dot plot was gated on $CD4^+CD8^-$ cells. This display was used to define $CD25^{low}$, $CD25^{mid}$, and $CD25^{high}$ populations. CD25 expression level correlates with different Treg functions. A pseudocolor plot was used to draw the gates and the resulting colored populations are shown as well. HLA^-DR/CD45RA dot plot was gated on all four defined populations to assess these populations for maturity. CD20/CD19 dot plot was gated on Non NKT cells. This display was used to define the B cell gate. CD19/CD5 dot plot was gated on B cells. Finally, CD14/Side scatter dot plot was used to define the monocyte gate (MONOS).

Table 1. Antibodies Used in Multicolor Staining.

Reagent	Catalog #/ Part Number	Vendor
CD45-Krome Orange	A96416	Beckman Coulter
CD3-APC-Alexa Fluor 750	A66329	Beckman Coulter
CD16-PE	IM1238U	Beckman Coulter
CD56-PE	IM2073U	Beckman Coulter
CD4-FITC	IM0448U	Beckman Coulter
CD8-A790*	IM0102	Beckman Coulter
CD25-PC7	A52882	Beckman Coulter
HLA-DR-Pacific Blue	A74781	Beckman Coulter
CD45RA-APC	B14807	Beckman Coulter
CD20-BV650	563779	BD Bioscience
CD19-PC5.5	A66328	Beckman Coulter
CD5-BV605	563945	BD Bioscience
CD14-APC-Alexa Fluor 700	A99020	Beckman Coulter

*Prepared by conjugating anti-CD8 to AF790 using AF 790 Antibody Labeling kit (Molecular Probes)

Monocyte phagocytosis/adhesion Assay Utilizing Infrared fluorescing Beads

In the immune system, phagocytosis is the primary process by which cells identify, isolate, and destroy invaders by engulfing and digesting them. Several diseases have been linked to defective phagocytic ability. Peripheral blood monocytes are an excellent model system to assess phagocytic function of innate immune cells. This assay demonstrates monitoring phagocytosis in monocytes, identified by the surface marker CD14, utilizing a Jade Green particle, which fluoresces in the near-IR channel (Figure 9).

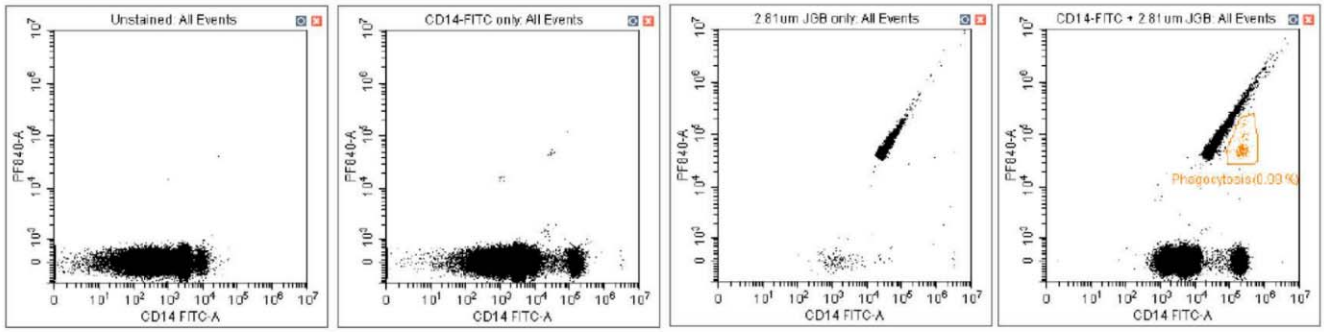


Figure 9. Monocyte Adhesion and Phagocytosis Assay. Samples were prepared in triplicate for unstained, CD14-FITC (Beckman Coulter Part Number B36630) only, and CD14-FITC and SPHERO™ Fluorescent 2.81 µm Jade Green particles (Spherotech Catalog Number FP-3078-2). Whole blood was stained with CD14-FITC for 15 minutes followed by 15 minutes lysis with 1 mL VersaLyse Lysing Solution Ready-for-use (Beckman Coulter Part Number IM3648). The lysed samples were then washed once with 3 mL of 1x PBS and centrifuged for 5 minutes at 400 x g. The supernatant was discarded. The pellet was resuspended in 1 mL of 1 x PBS. After resuspension 500 µL ($5 x10^6$) of Jade Green particles was added. All samples were incubated in 37° C water bath for 60 minutes and then centrifuged for 5 minutes at 400 x g. The supernatant was discarded. The pellet was resuspended in 1 mL of cold 0.02% EDTA in PBS. Samples were analyzed using the CytoFLEX S Blue-Red-Violet-Infrared Flow cytometer. The collected data were analyzed using CytExpert software v 1.2. Monocyte phagocytosis/adhesion function is assessed by the number of CD14 positive events that are also positive in the PF840 channel (right; orange gated).

Conclusion

The introduction of Photodiode detectors expands the accessible electromagnetic spectrum for use in flow cytometry. In contrast to the photomultiplier tube, Avalanche photodiodes exhibit high quantum efficiency in the 800 nm (infrared) range of the spectrum. The CytoFLEX S platform offers the first commercially available flow cytometer incorporating both Avalanche photodiodes and an Infrared laser.

Compensation challenges are reduced as a benefit of using the channels in the infrared range. No spillover from infrared emitting fluorochromes was detected in traditional channels, minimal spillover was detected in adjacent infrared channels only. We demonstrated using two infrared fluorochromes.

To further demonstrate the usefulness, these new channels were used in biological applications. The addition of the 808 nm laser to the CytoFLEX S series provides additional fluorescent channels not only for use of viability dyes but also bright markers with minimal spectral overlap into traditional channels. Alternatively, the Infrared channels could be used for dim markers. The untouched nature of this part of the spectrum, either from the absence of auto-fluorescence or from spillover from other fluorochromes will maximize the sensitivity of detection.

References

1. Mahnke, Y. D., & Roederer, M. (2007). Optimizing a Multi-colour Immunophenotyping Assay. *Clinics in Laboratory Medicine, 27*(3), 469–v. http://doi.org/10.1016/j.cll.2007.05.002

2. Andersson, Baechi, Hoechl and Richter (1998). Autofluorescence of living cells. *Journal of Microscopy*, 191: 1–7. doi:10.1046/j.1365-2818.1998.00347.x

3. William G. Lawrence, Gyula Varadi, Gerald. Entine, Edward Podniesinski, and Paul K. Wallace. (2008). A Comparison of Avalanche Photodiode and Photomultiplier Tube Detectors for Flow Cytometry. *Proc. SPIE 6859, Imaging, Manipulation, and Analysis of Biomolecules, Cells, and Tissues VI*, 68590M (29 February 2008); doi: 10.1117/12.758958

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FLOW-2674WP05.17

VIABILITY ASSESSMENT OF CELL CULTURES USING THE CYTOFLEX

APPLICATION NOTE

The CytoFLEX* benchtop flow cytometer offers a simple and accurate method for determining the percentage of live and dead cells in growing cell cultures when a viability dye is added to the sample. An aliquot of the sample can be taken and run directly on the CytoFLEX to set up the negative gates for the viability dye and to also to determine cell concentration. Once cell concentration is known, the appropriate amount of dye can be added to the sample and run on the instrument in order to determine the viability of the population.

Materials & Methods for viability using 7AAD on the CytoFLEX

- Tissue culture cells grown in appropriate media
- Viability dye

Cell Preparation with 7AAD (stock solution 1mg/mL)

1. If the concentration of the cells are lower than 1 x 10e4 cells/mL, the cells should be concentrated and re-suspended in PBS to a final concentration of ~1e6 cells/mL
2. If the concentration of the cells are at ~ 1e6 cells/mL, the 7AAD can be directly added to the cells in the tissue culture media
3. Use 20 µL 7AAD for every 1mL of cells. NOTE: The concentration of 7AAD may need to be adjusted depending on the cell culture type.
4. Mix and read within 5-10 minutes on the CytoFLEX cytometer

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CytoFLEX Instrument Set-Up

INSTRUCTIONS:

1. Create a new experiment from the menu for the viability test samples.
2. Create two dot plots, one FSC vs SSC and the second 7AAD (PC5.5 channel) vs SSC
3. Set the 7AAD axis to log mode
4. Run the sample at the Low flow rate setting—10ul/minute
5. Set the threshold on the FSC channel.
6. In the Statistics plot add the events/μL parameter
7. Adjust the gain settings for all three parameters with the unstained sample
8. Draw a gate on the entire FSC vs SSC population excluding the debris in the lower left hand corner of the plot
9. Set this gate on the 7AAD vs SSC plot
10. Draw a gate on the negative 7AAD population
11. Run the test sample with 7AAD added
12. Collect 40,000 events total
13. Calculate the viability based on the gating hierarchy for the population in the 7AAD or negative gate

    You can also verify the cell concentration by looking at the events per μL and multiplying it by the total volume in the tube.

RESULTS

Figure 1.

CHO cells were prepared as described and run on the CytoFLEX.
Unstained sample on the left for reference; 7AAD stained on the right.

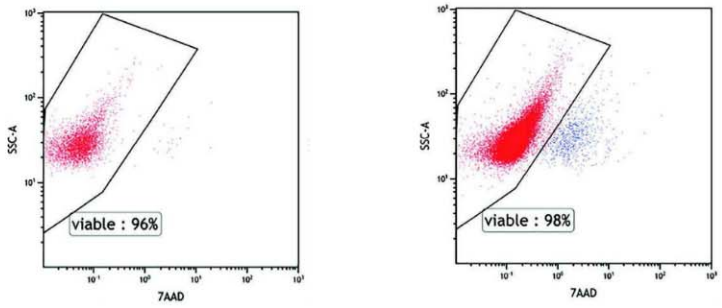


Figure 2.

HeLa cells were prepared as described and run on the CytoFLEX.
Unstained sample on the left for reference; 7AAD stained on the right.

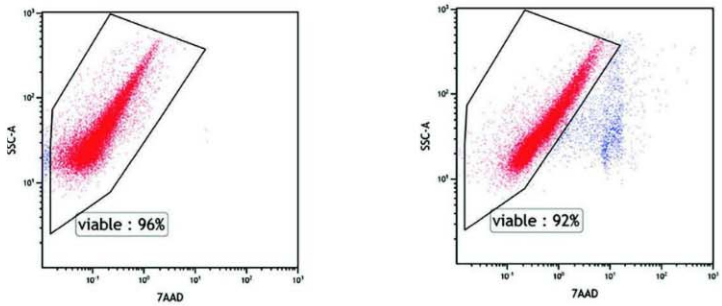


Figure 3.

293T cells were prepared as described and run on the CytoFLEX.
Unstained sample on the left for reference; 7AAD stained on the right.

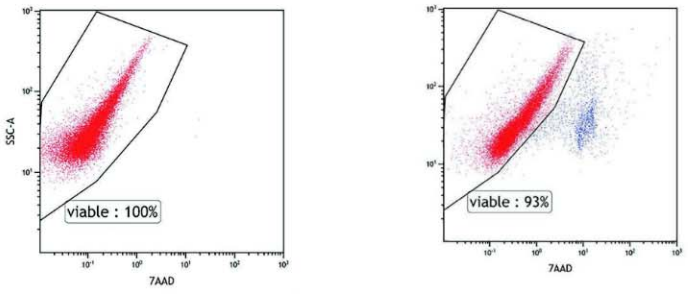
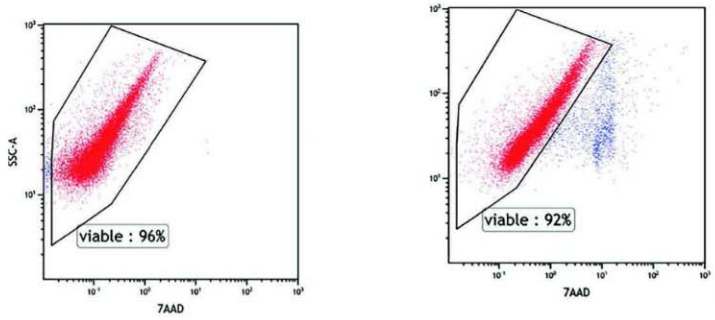


Figure 4.

721 cells were prepared as described and run on the CytoFLEX.
Unstained sample on the left for reference; 7AAD stained on the right.



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FLOW-902FLY04.15-A

Comparing Viability Measurements on Cell Lines Utilizing the CytoFLEX Flow Cytometer

APPLICATION NOTE

Introduction

Discriminating live cells from dead cells is important both for accurate flow cytometric analysis and for determining effects of cytotoxic drugs. Traditional methods for live/dead discrimination (7-AAD and PI) bind DNA and are not compatible with fixation and permeabilization. Zombie dyes act by binding to amine groups in the cytoplasm of dead cells while being excluded from live, membrane-intact cells[1]. Zombie dyes are available in multiple colors and are compatible with most permeabilization protocols. With four lasers and 13 colors, the CytoFlex is compatible with the use of all Zombie dyes and allows for live/dead discrimination along with surface and intracellular staining.

Methods

Cell Lines and Drugs

Cultured P12 and U937 cell lines were grown to confluence. Two million cells/mL were plated into a 24 well tissue culture plate with or without 20 mg/mL of mitomycin-c for 48 hrs at 37°Celsius in complete-RPMI medium + 5% fetal calf serum.

Viability Staining & ViCell Counting

Treated cells were washed in 10 times the volume of PBS, specifically chosen since it lacks protein, at 400g for 5 minutes. An aliquot of each cell line and treatment condition was counted on the ViCell. One million cells from each cell line and treatment condition were stained in 100 µL final volume with either 1 µL of Zombie Yellow, 1 µL of Zombie Aqua, or 20 µL of 7-AAD. All conditions were stained for 30 minutes. Then, all tubes were washed in 3 mL of growth media and spun at 400g for 5 minutes. Cell pellets were resuspended in PBS and acquired on the CytoFLEX.

Laser	405 nm					488 nm					533nm		
Filter		510	575						647				
Dye		Zombie Aqua	Zombie Yellow						7-AAD				

Results

Of the three viability dyes tested, 7-AAD produced the highest signal to noise (S:N) ratio (Figure 1). Each cell line and treatment group showed similar trends in percentage viable cells with the addition of mitomycin-c or Ly-294. Additionally, each viability dye correlated to the results generated by the ViCell (Figure 2). Although the percentages of viable cells differed slightly between the viability dyes tested, it is possible that this is due to the altering mechanisms of actions of the viability dye binding. These data suggest that it is necessary to be consistent in individual experiments with a singular viability dye of choice.

References

1- Perfetto SP, Chattopadhyay PK, Lamoreaux L, Nguyen R, Ambrozak D, Roup RA, Roederer M. Amine-reactive dyes for dead cell discrimination in fixed samples. Curr. Protoc. Cytom. Chapter 9: Unit 9.34, 2010.

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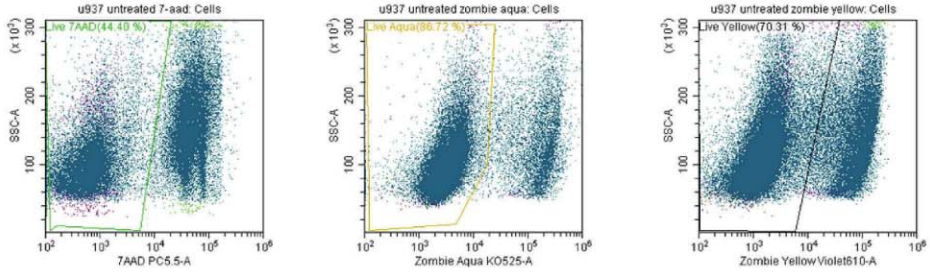
Notes

The results demonstrated in this application sheet represent those generated on the Beckman Coulter CytoFLEX Flow Cytometer. As differences exist in the performance between analyzers, the author cannot guarantee a similar appearance with the use of other flow Cytometers.

Reagent Details

Reagent	Supplier	Catalog No.
7-AAD	Beckman Coulter	A07704
Mitomycin-C	Sigma	M4287
Ly-294	Sigma	L9908
Zombie Yellow	BioLegend	423103
Zombie Aqua	BioLegend	423101

Figure 1. **Viability of U937 Myeloid Cells Measured by Multiple Dyes.**
U937 cells were stained with either 7-AAD (left), Zombie Aqua (middle), or Zombie Yellow (right) and acquired on a CytoFLEX. Ly-293 treatment results not shown.

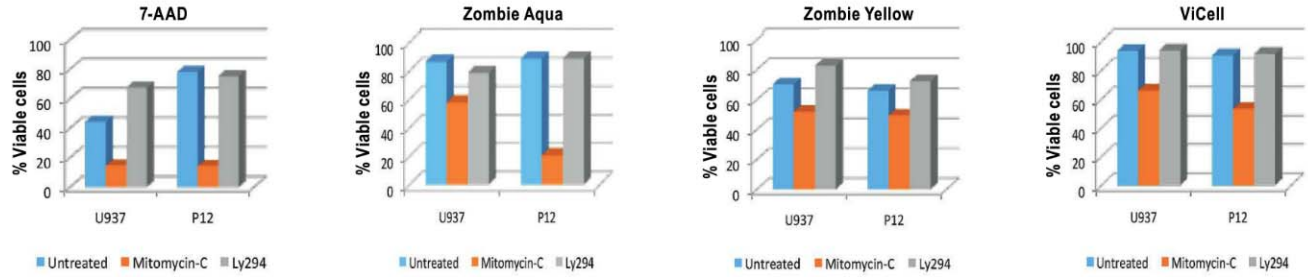


7-AAD
S:N 25.87

Zombie Aqua
S:N 16.93

Zombie Yellow
S:N 21.92

Figure 2. **Viability of U937 and P12 Cell Lines.**
U937 and P12 cell lines were incubated for 48 hours with or without mitomycin-c or Ly294.
After washing, cells were stained with 7-AAD (A), Zombie Aqua (B), Zombie Yellow (C), or counted on the ViCell. Percentage of viable cells from untreated (blue), mitomycin-c treated (orange), or Ly294 treated (gray) is graphed.



Authors

"Comparing Viability Measurements on Cell Lines Utilizing the CytoFLEX Flow Cytometer"
Michael McPherson, Karen Carr, Si-Han Hai
Affiliation: Beckman Coulter

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FLOW-1091APP08.15-A

Measuring Proliferation in Leukemia Cell Lines via Carboxyfluorescein Succinimidyl Ester (CFSE) upon Treatment with Cytostatic Concentrations of Cytosine Arabinoside (Ara-C)

APPLICATION NOTE

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IN THIS PAPER YOU WILL LEARN

- A method to monitor and measure cell proliferation
- Method to measure the activity of a cytostatic compound

Principal of the Technique

Background

Carboxyfluorescein succinimidyl ester (CFSE) is a fluorescent cell staining dye[1], which is cell permeable and covalently couples, via its succinimidyl group, to intracellular molecules. CFSE is retained within cells for a relatively long period of time and once incorporated does not transfer to other cells. CFSE has been utilized as a marker for cell counting and tracing purposes. Due to the fact that CFSE fluorescence decreases with each cell division, as the CFSE is divided between daughter cells, it is generally applied to cell proliferation studies. Given the stability of CFSE in cells it can be used to track 7-8 cell divisions typically. One drawback of using CFSE is that it exhibits very high cytotoxicity; thus careful optimization of the assay under specific circumstances is necessary.

Research Applications: Introduction

Chemotherapeutic treatment of acute myeloid leukemia is based on the standard combination of daunorubicin and cytosine arabinoside (Ara-C). Ara-C is also utilized in the treatment of non-Hodgkin lymphoma. Ara-C works by combining a cytosine base with an arabinose sugar, and thus disrupts DNA synthesis.

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Standard chemotherapy drugs are used at cytotoxic levels, however they are cleared from the body typically within 24 hours. At lower concentrations these drugs are likely to have a cytostatic effect, which would contribute to the effectiveness of the therapeutic regime.

Here we aim to utilize the CFSE proliferation assay to determine the cytostatic effect of sub-toxic concentrations of Ara-C on a number of leukemic cell lines.

Protocol

Standard Procedure

HL-60 and OCI/AML-3 cell lines were maintained at a culture density of $1x10^5$ – $1x10^6$ cell/mL in 10 mL of alpha-MEM medium supplemented with 10 % fetal calf serum (FCS, *v/v*), 100 units of penicillin per ml and 100 µg of streptomycin per mL at 37°C and 5 % CO_2.
The U937 cell line was maintained at a culture density of $1x10^5$ – $1x10^6$ cell/mL in 10 mL of RPMI 1640 medium supplemented with 10 % fetal calf serum (FCS, *v/v*), 100 units of penicillin per mL and 100 µg of streptomycin per mL at 37°C and 5 % CO_2.

1. Cell lines were cultured to a density of $5x10^5$ cells/mL and 2 mL of these cells were collected in 15 mL centrifuge tubes
2. Cells were centrifuged at 400x g for 5 minutes
3. Supernatant medium was removed
4. Cells were washed with 1 mL of PBS
5. Cells were resuspended in 1 mL of PBS containing 0.5 µM CFSE dye (see Note 1)
6. Cells were incubated for 5 minutes at 37°C
7. Cells were centrifuged at 400x g for 5 minutes
8. PBS containing CFSE was removed
9. Cells were washed with 1 mL of PBS
10. Cells were resuspended in culture medium at the concentration of $1x10^5$ cells/mL and a sample was analysed for CFSE fluorescence. This will represent the CFSE uptake of the cells initially.
11. Cells were treated with 20 nM of Ara-C
12. 500 µL of cells are taken for flow at 48 hours for measurement of proliferation *via* CFSE fluorescence decrease.
13. CFSE fluorescence was read on the FITC channel
14. Cells were analysed for fluorescence on a CytoFLEX flow cytometer (Beckman Coulter)
15. Cells were gated on FSC vs. SSC to identify the correct, viable cell population.
16. Gated cells were further gated on FSC-area vs. -height to discriminate singlet cells from doublet cells
17. Analysis of CFSE fluorescence was done using overlay histograms of singlet cells
18. CFSE fluorescence is collected in the standard FITC channel of the CytoFLEX

Materials & Methods

List material required but not supplied Beckman Coulter CytoFLEX , OCI/AML-3 cells, HL-60 cells, U937 cells, Centrifuge, microcentrifuge, Gilson pipetteman (P10, P20, P200, P1000), 15 mL Centrifuge tubes, 1.5 mL microcentrifuge tubes.

Reagents alpha-MEM medium (supplemented with 10 % fetal calf serum (FCS, *v/v*), 100 units of penicillin per ml and 100 µg of streptomycin per mL). RPMI 1640 medium (supplemented with 10 % fetal calf serum (FCS, *v/v*), 100 units of penicillin per mL and 100 µg of streptomycin per mL). CellTrace CFSE Cell Proliferation kit (Life Technologies). Cytosine Arabinoside (Ara-C).

Sample prep

Sample Type (include cell line information if available)	Species	Age of specimen (if available-or time since prep)	Prep Method
OCI/AML3 HL-60 U937	Human Human Human		

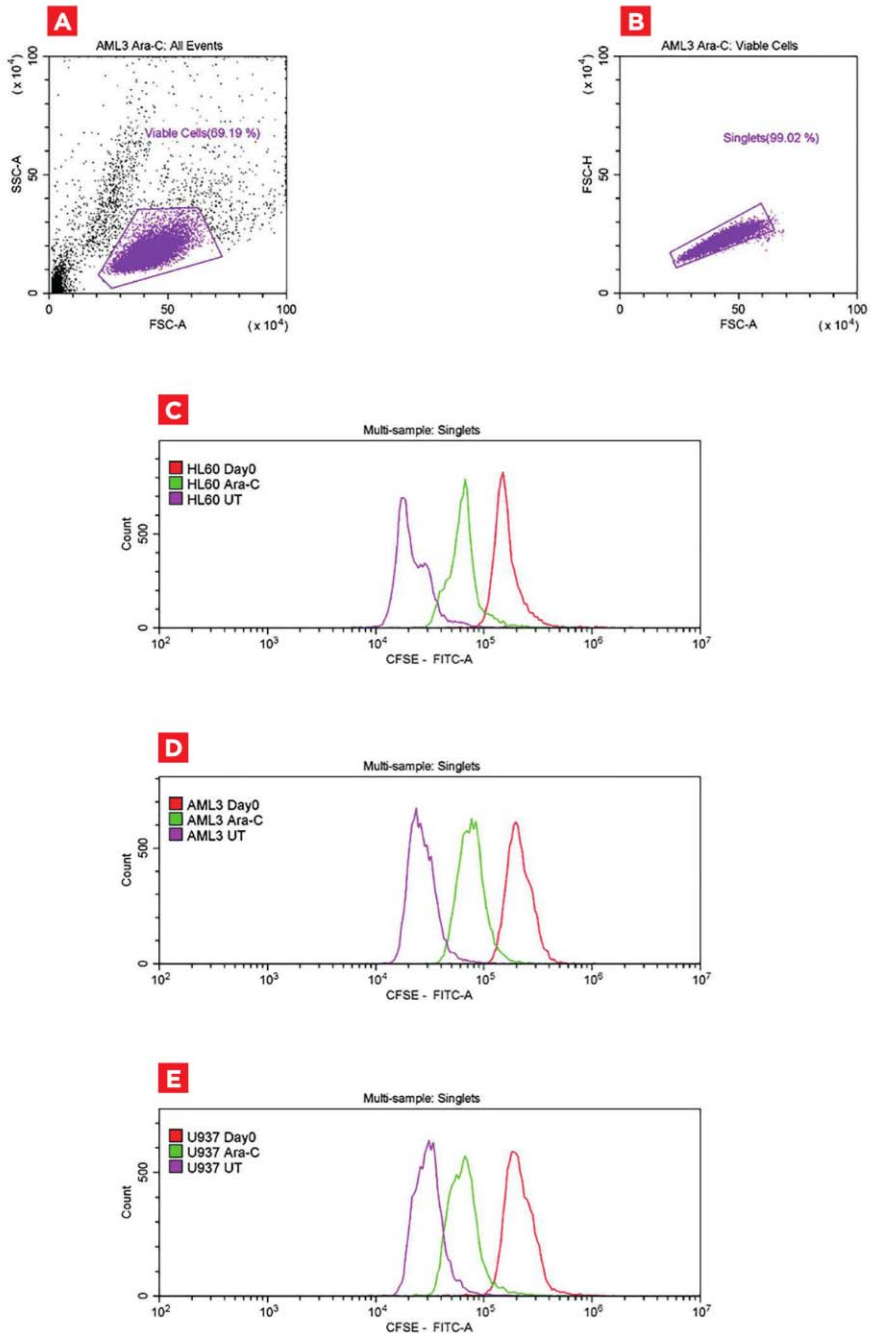

Figure 1: Measuring Proliferation in Leukemia Cell Lines via Carboxyfluorescein succinimidyl ester (CFSE) upon treatment with cytostatic concentrations of Cytosine Arabinoside (Ara-C). (A) Gating strategy for viable cell population on a FSC *vs.* SSC dot-plot, showing OCI/AML-3 cells treated with Ara-C. All samples are gated in a similar manner. (B) Viable cells were further gated to remove possible doublet cells on a dot-plot for FSC-area *vs.* -height. All samples are gated in a similar manner. CFSE fluorescence was analysed on the FITC-A channel in cells immediately post-staining (red histograms), after 48 hours in standard culture (pink histograms) or after 48 hours in Ara-C treatment (green histograms) in (C) HL-60, (D) OCI/AML-3 or (E) U937 cells.

Results

HL-60, OCI/AML-3 and U937 cells were grown for 48 hours in the presence or absence of a 20 nM Ara-C. These cells exhibited effectively no loss in viability, as seen by FSC *vs.* SSC profiles. Thus, this level of Ara-C is determined as sub-toxic (this concurs with previous studies, by our group and others). We next set out to determine if this concentration of Ara-C inhibited cell proliferation. This was done by measuring the reduction of CFSE fluorescence. The greater the reduction in CFSE fluorescence, the more proliferation will have occurred in the culture. CFSE fluorescence was measured in cells immediately subsequent to staining and 48 hours later, with- and without Ara-C treatment.

From the data we see that in all cultures examined, Ara-C treatment resulted in a reduced proliferation than in untreated cultures. Interestingly, all cells proliferated to some extent. So, while Ara-C treatment did affect the rate of proliferation it did not prevent cell growth entirely. The level of CFSE fluorescence decreased from levels after the initially staining even with Ara-C treatment, but not to the same extent as the untreated cells. This could be due to the cells continuing to cycle and divide initially in the presence of Ara-C before the cells arresting eventually.
Thus CFSE staining is a useful tool to rapidly measure the effect of drug treatment on the proliferative capacity of cells in culture.

References

1. Parish CR. 1999. Immunol Cell Biol. 77(6):499-508. Fluorescent dyes for lymphocyte migration and proliferation studies.

Notes

In our optimization of this assay we determined that concentrations greater than 0.5 µM of CFSE and incubation times of greater than 5 minutes resulted in high levels of apoptosis. The staining parameters we used here resulted in the greatest cellular staining with minimal (< 5%) reduction in cell viability.

Reagent Details

Reagent	Supplier	Order Details
CellTrace CFSE Cell Proliferation kit	Life Technologies	C34554
Cytosine Arabinoside (Ara-C)	Sigma Aldrich	C1768

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FLOW-1306APP12.15-A.

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Fully-Automated Cellular Analysis by Flow Cytometry

Summary

- Direct integration of CytoFLEX Flow Cytometer to Biomek i-Series Automated Workstations enables complete automation of sample processing and data acquisition.
  - CytoFLEX, a small footprint bench top analyzer, can collect 15 parameters with high sensitivity.
- Automated the plating, drug treatment, trypsinization, and staining of cells for apoptosis and cytotoxicity analysis
  - Selective tip pipetting enabled serial dilutions and processing of partial plates for time course studies
- Measured dose and time responses for multiple compounds in both suspension and adherent cell lines

Flow cytometry is a widely-used and powerful tool for single-cell analysis – an essential ability for those studying heterogeneous cell populations. However, the need for cells to be in single-cell suspensions can result in challenging sample preparation. This can include trypsinization of adherent cells and/or centrifugation steps to remove staining reagents. Automating these steps can decrease the time at the bench while improving reproducibility by ensuring consistent treatment (i.e. trypsin incubations) across samples. In addition, moving to a plate-based format increases the potential sample throughput.

Here we demonstrate how the Biomek i7 Automated Workstation (Figure 1A) was used to automate the complete cellular workflow for induction and analysis of apoptosis in two cancer lines. The Biomek instrument utilized its HEPA-filtered enclosure to maintain cell sterility during manipulations. In addition, the i-Series instruments enable simple and direct integrations, including the CytoFLEX Flow Cytometer configured with a plate loader (Figure 1B) used here, without the need for additional robotic transports.

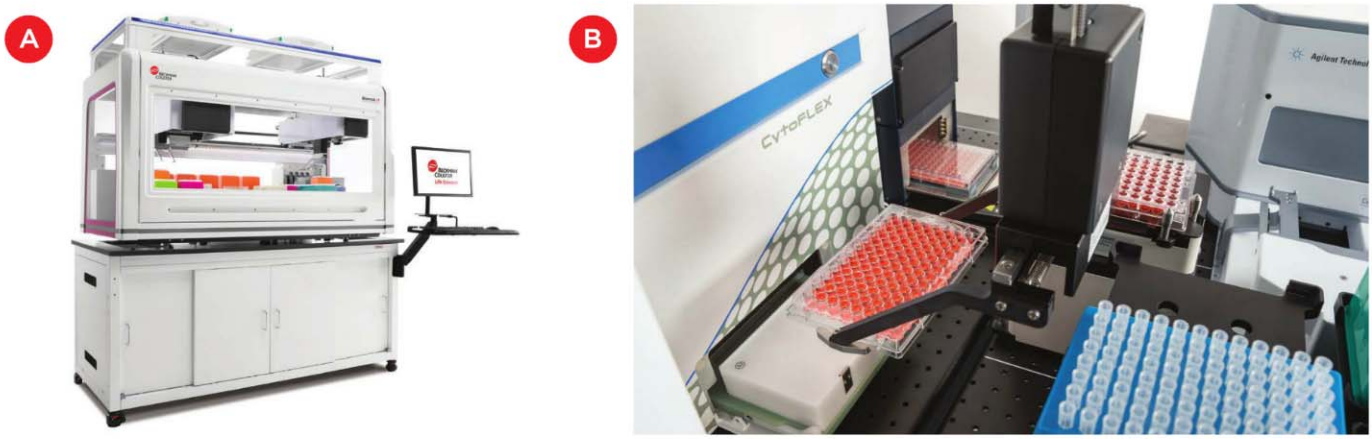

Figure 1. Biomek i7 Automated Workstation with HEPA filters (A) accessing an integrated CytoFLEX Flow Cytometer with plate loader (B).

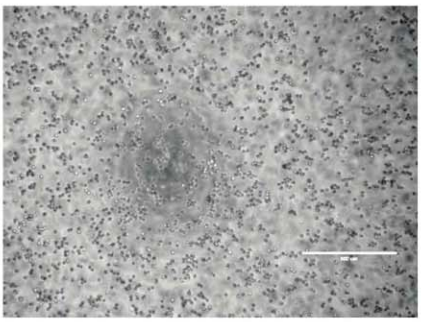

Figure 2. HCT116 cells following automated trypiniszation and resuspension.

We chose human leukemia (Jurkat) and colon carcinoma (HCT116) cell lines to demonstrate the workflows for both suspension and adherent cells. In both cases 25,000 cells were plated in 96-well plates and after 24 hours, the selective tip feature of the multichannel head was used to serially dilute three compounds – staurosporine, camptothecin, and 5-fluorouracil. These apoptosis inducers were added to cells and incubated for 24-72 hours. Prior to staining, the HCT 116 cells were trypsinized, using an on-deck Peltier heating device for incubation and the multichannel head was used for repeated pipetting to create a single-cell suspension (Figure 2). Both cell lines were incubated with CellEvent® Caspase-3/7 Green (Life Technologies) to identify cells undergoing apoptosis and DRAQ7 (Beckman Coulter) to label cells with compromised cellular membranes as a measure of cell death.

Cells were identified using forward and side scatter and apoptosis and cell death stains were measured in the FITC and PC5.5 fluorescence channels respectively. Analysis plots were generated in Kaluza 1.5 software. Figure 3A shows the viable, apoptotic, and dead HCT116 cells for a high, medium, and low dose of 5-fluorouracil treatment at 48 hours, and Figure 3B shows the 48 hour dose response curve and calculated IC50s for all three compounds, illustrating the effectiveness of the automated serial dilutions. Figure 4A shows the progression of Jurkat cells through the cell death pathway over time following a treatment with 78 nM camptothecin. The change in the percentage of cells in each condition is plotted in Figure 4B. This drug time course was made simple by multichannel selective tip pipetting, which enabled the cells to be plated once, the drug dilutions stamped into replicate wells, and one set of wells be harvested per time point.

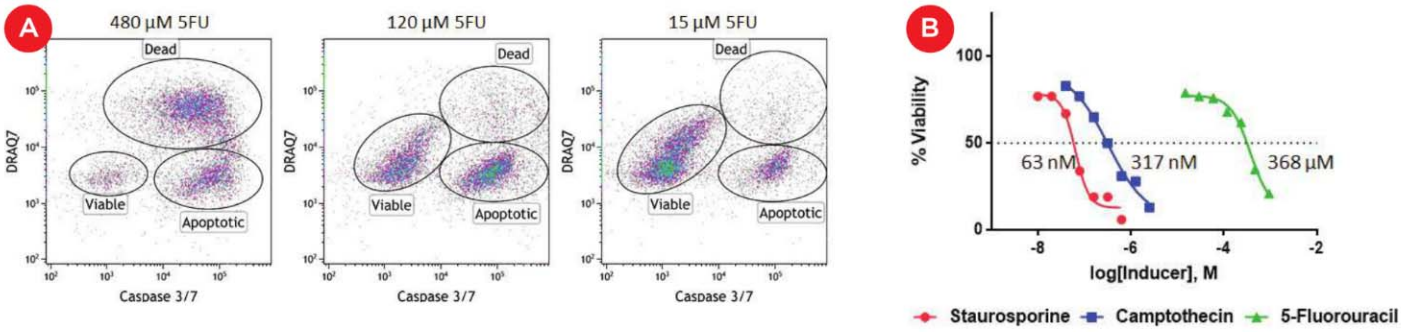

Figure 3. A) Dot plots showing HCT116 cell populations that are viable (unstained), apoptotic (caspase 3/7 positive), or dead (DRAQ7 positive) following 48 hours of 5-fluorouracil treatment. The cytotoxic effects are diminished as concentration decreases indicating effective serial dilutions. B) Dose response curves and IC50 values based on the percentage of viable HCT116 cells following 48 hour treatment with three apoptosis inducers.

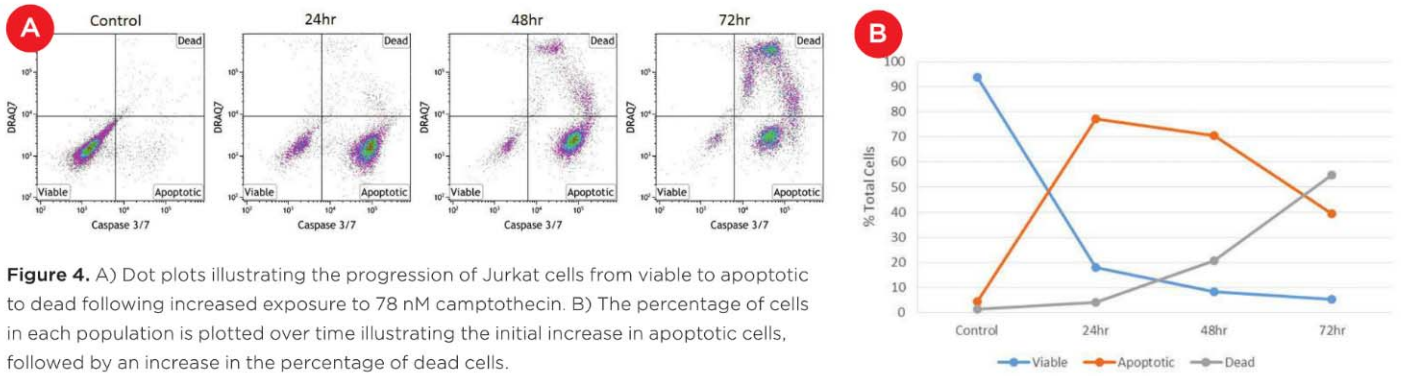

Figure 4. A) Dot plots illustrating the progression of Jurkat cells from viable to apoptotic to dead following increased exposure to 78 nM camptothecin. B) The percentage of cells in each population is plotted over time illustrating the initial increase in apoptotic cells, followed by an increase in the percentage of dead cells.

While the reagents used here did not require washes, the ability to directly integrate microplate centrifuges (Figure 1B), plate washers, and incubators to the i-Series instruments means that antibody-based workflows that discriminate populations in a heterogeneous mixture can also be easily automated. In addition, if samples need to be processed by a tube-based flow cytometer, the Span-8 pipettors can be used to rapidly process samples in tubes or perform a final transfer from plates to tube prior to analysis. Finally, for high throughput applications SAMI EX software can be used to schedule staining and analysis workflows to ensure consistent treatment across plates.

AAG-2348APP01.17

6 Color Panel for Staining Human Peripheral Blood Lymphocytes

APPLICATION NOTE

Introduction

Here we describe immunophenotypic analysis of human peripheral blood lymphocytes utilizing a 6-color antibody panel in a stain/lyse protocol run on the CytoFLEX* Research Flow Cytometer. The markers choosen are commonly used surface CD antigens which can be used to quantify and characterize normal and abnormal lymphocyte populations in peripheral blood.

The CytoFLEX is excellent for immunophenotyping research experiments. The proprietary Wavelength Division Multiplexing (WDM) detection module uses solid-state, high efficiency, low-noise Fiber Array Photodiode Detectors (FAPD), giving exceptional resolution for more precise data and better detection of rare events. This protocol describes the use of the CytoFLEX with a red and blue laser configuration for six-color immunophenotyping analysis.

Materials and tools

1. Human peripheral blood sample drawn in EDTA tube : used within 24 hours of draw
2. Hypotonic Lysing Solution
3. Phosphate Buffered Saline (PBS)
4. Deioniozed water
5. Vortex
6. Centrifuge
7. 12x75mm tubes
8. Pipettes and tips
9. CytoFLEX flow cytometer - red and blue laser configuration

488 nm		
FITC	PE	PERCP
CD3	CD16/CD56	CD45

638 nm		
Cy7	APC	APC-Cy7
CD4	CD19	CD8

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Sample Preparation

1. Add 50µL mixed blood sample to antibody cocktail in a 12 X 75 mm test tube.
2. To prepare cocktail from single color antibody stocks, refer to the package instructions; antibody titration is recommended.
3. Vortex the tube gently to mix.
4. Incubate the sample at room temperature in dark for 15 minutes.
5. Add Lysing Solution per package instructions and vortex gently.
6. Incubate the tube per package instructions in dark.
7. Centrifuge the sample tube at 1500rpm for 5 minutes.
8. Discard the supernatant, add 1mL PBS and vortex gently.
9. Centrifuge the sample tube at 1500rpm for 5 minutes again.
10. Discard the supernatant and add 500µL PBS.
11. Vortex the sample tubes gently. Store tubes at room temperature in dark; perform analysis within 1 hour.

Data Acquisition and Analysis

1. Run the sample at low flow rate setting.
2. Set the threshold on PerCP-Cy5.5 channel to cut off the debris.
3. Adjust the gains and compensation, if needed.
4. Collect 15,000 events.
5. Set the lymphocyte gate on CD45 bright/SSC low population.
6. Use "Fit with Sample" function to show the low signals.

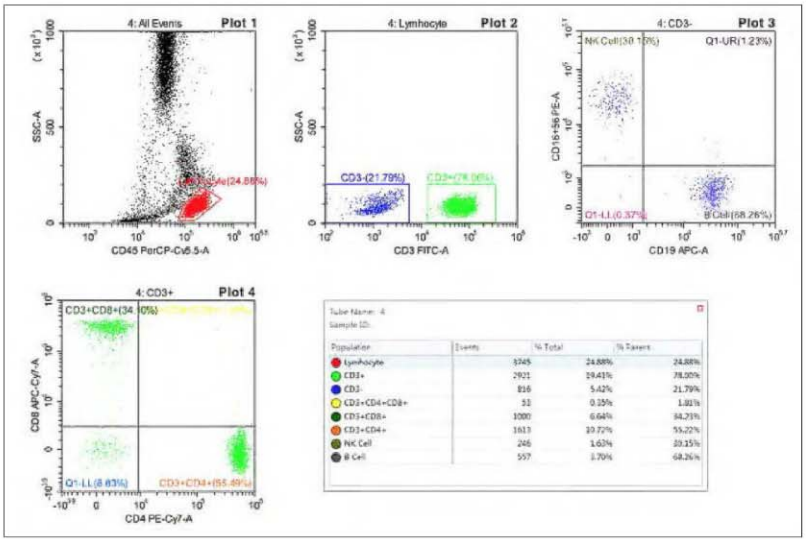

Figure 1: Plot 1 CD45 PerCP Cy5.5 vs SSC showing the positioning of the lymphocyte gate.

Conclusions

Here is a demonstration of a simple six color analysis of human blood peripheral blood lymphocytes using the CytoFlex flow cytometer. There is clear delineation of each of the populations of interest.

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FLOW-856SP03.15-A

T Cell Receptor Characterization of PBMCs Using the CytoFlex

APPLICATION NOTE

Authors : Michael McPherson, Karen Carr, Si-Han Hai.
Affiliation: Beckman Coulter

IN THIS PAPER YOU WILL

- Learn how to stain PBMC for T cell receptor analysis by flow cytometry utilizing DuraClone
- Identify TCR subpopulations using the CytoFLEX flow cytometer and DuraClone

Introduction

T cells play a pivotal role in the function and regulation of the immune system. Mature T cells express both CD3 and a T Cell Receptor (TCR). They can further be divided into two subpopulations based on their type of TCR: αβ T cells (95% of the T cell repertoire) and γδ T cells (5% of the T cell repertoire). TCR αβ T cells are then differentiated into CD4 T cells or CD8 T cells based on their affinity recognition of antigens bound to MHC Class II or MHC Class I, respectively. The largest subset of γδ T cells express the Vδ2 TCR complex. A smaller subset of γδ T cells express the Vδ1 TCR complex. The study of TCR γδ Vδ2 T cells and TCR γδ Vδ1 T cells is important in feto-maternal[1] and allograft tolerance[2]. The understanding of the many T cell subpopulations is important for a variety of normal and pathological studies.

DuraClone IM panels are unitized, dry format reagent cocktails that are room temperature stable. Tube formulations were based on the ONE Study as well as design input from expert flow Cytometry labs for use in clinical research studies. Here, we look at the DuraClone IM TCRs tube with normal PBMCs on the CytoFLEX.

Methods

Peripheral Blood Mononuclear Cell (PBMC) isolation

Ten milliliters of whole blood was diluted to 21 mL with 1X PBS + 3% FCS. Six mL of Ficoll (Sigma:Histopaque 1077 cat # 10771) was added to three, 15 mL conical vials. Seven mLs of diluted blood were added to the Ficoll by addition with a 10 mL pipet at a very steep angle. Diluted blood was then slowly added to over-lay on to the Ficoll. Cells were spun at 1500 RPM for 30 minutes in a swinging bucket centrifuge with the brake turned off. Supernatant was aspirate and samples were pooled and washed in PBS + 5% FCS. Cell pellets were resuspended and counted using the ViCell and diluted to a final concentration of 1 x 10^6 cells/100 µL per tube for staining.

DuraClone Staining Procedure

One million PBMCs were added to a DuraClone IM TCRs tube and then vortexed for 5 seconds. After 20 minutes incubation at room temperature in the dark, the PBMCs were washed with 3 mL of PBS and centrifuged at 400g for 5 minutes. The PBMCs were resuspended in 250 µL of PBS and acquired on the CytoFLEX.

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Laser	405 nm					488 nm					638 nm		
Fluor	Krome Orange	Pacific Blue	V610	V660	V780	FITC	PE	ECD	PC5	PC7	APC	AF700	APC AF750
Marker	CD45	TCR Vδ2				TCR γδ	TCR αβ	HLA-DR		TCR Vδ1	CD4	CD8	CD3
Clone	J.33	IMMU 389				IMMU510	IP26A	Immu-357		R9.12	13B8.2	B9.11	UCHT-1

Results

Rapid TCR Subsetting with the DuraClone IM TCRs Tube

Staining of normal PBMCs with the DuraClone IM TCRs tube depicted here suggests that T cell subsets can be rapidly determined with the CytoFLEX. After approximately 30 minutes, PBMCs were accurately characterized into their appropriate compartments. Using the DuraClone IM TCRs tube is a viable alternative to laborious staining procedures.

References

1- Pregnancy and gamma/delta T cells: taking on the hard questions. Mincheva-Nilsson L. Reprod Biol Endocrinol. 2003 Dec 2; 1:120. Review.

2- Characteristics of Vδ1 (+) and Vδ2 (+) γδ T cell subsets in acute liver allograft rejection. Yu X, Liu Z, Wang Y, Wang H, Zhang M, Sun Y, Su H, Jin L, Wang F, Shi M. Transpl Immunol. 2013 Dec; 29(1- 4):118-22.

Notes

The results demonstrated in this application sheet represent those generated on the Beckman Coulter CytoFLEX Flow Cytometer. As differences exist in the performance between analyzers, the author cannot guarantee a similar appearance with the use of other flow Cytometers.

Reagent Details

Reagent	Supplier	Order Details
DuraClone IM TCRs Tube	Beckman Coulter	B53340
Lymphocyte Separation Medium	Corning	25-072-CI
DPBS, 1X (Dulbecco's Phosphate-Buffered Saline	Corning	21-031-CV

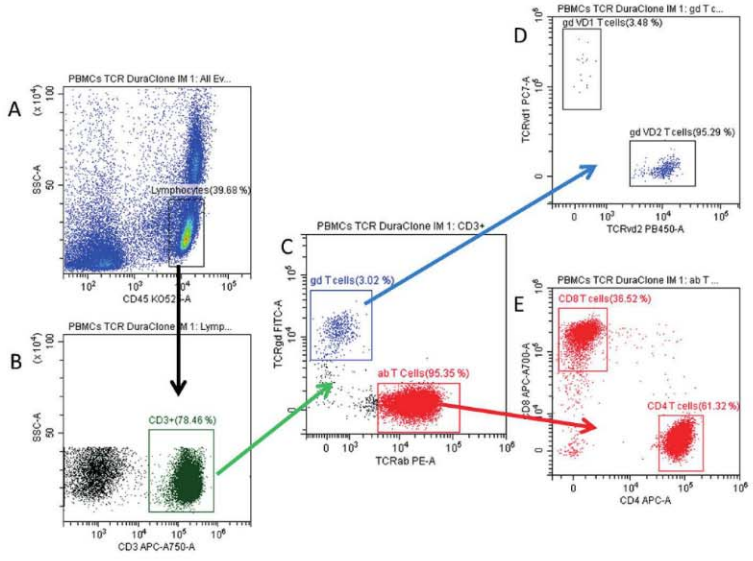

Figure 1. Rapid T Cell Receptor Subsetting with the DuraClone IM TCRs Tube

PBMCs were gated on Lymphocytes by CD45 staining (A). CD3+ lymphocytes (B) were then gated based on expression of TCR γδ or TCR αβ (C). Of the γδ T cells, expression of Vδ1 and Vδ2 was characterized (D). Of the αβ T cells, CD8 and CD4 expression is characterized (E).

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Identification of Circulating Myeloid Cell Populations in NLRP3 Null Mice

TECHNICAL INFORMATION BULLETIN

CytoFLEX Research Cytometer

Matthew Peloquin, Sanford-Burnham Medical Research Institute, Orlando, Florida, USA

Introduction

Surface markers are commonly used to discriminate populations in circulating blood. Using the surface markers Ly6C (hematopoietic cells), Ly6G (granulocyte/neutrophil) and CD115 (MCSF receptor), we are able to describe the circulating cell populations from fresh blood in C57BL/6J wild type control mice and NLRP3 null samples. Due to NLRP3's implication in forming the inflammasome, we hypothesize that there will be a difference in the monocyte to granulocyte ratio. Moreover, we are interested in observing the expression of the CD115 (MCSF receptor) between the control and null groups.

The CytoFLEX flow cytometer is excellent for interrogating different populations circulating in blood. The proprietary Wavelength Division Multiplexing (WDM) detection module uses solid-state, high efficiency, low-noise Fiber Array Photodiode Detectors (FAPD), giving exceptional resolution for more precise data and better detection of rare events. This protocol describes the use of the CytoFLEX flow cytometer with a red and blue laser configuration for analysis.

Materials & Methods

Reagents

Laser	405nm					488nm					638nm		
Fluor	KrO	PB	V610	V660	V780	FITC	PE	ECD	PC5	PC7	APC	APC A700	APC A750
Marker						Ly6G	Ly6C				CD115		

Sample Preparation

1. Aspirate 100-600 µL of mouse blood via cardiac puncture.
2. Place fresh blood into 1.5mL tubes with 40 µL 0.5M EDTA.
3. Lyse red blood cells with 1X hypotonic lysis solution for 5 minutes.
4. Centrifuge tubes at 400 x G for 10 minutes at room temperature.
5. Remove supernatant and resuspend in 500 µL MACS buffer (Miltenyi) or equivalent.
6. Centrifuge cells at 400 x G for 10 minutes at 4 °C.
7. Resuspend in 100 µL MACS buffer with 5% Normal Mouse Serum and 5% Normal Rat Serum.
8. Add antibodies and stains. Incubate for 30 minutes on ice.

Antibody	Quantity
Ly6C - PE	0.5 µL
Ly6G - FITC	1.0 µL
CD115 - PE	2.5 µL

9. Centrifuge at 400 x G for 10 minutes at 4 °C
10. Aspirate supernatant
11. Resuspend in 600 µL MACS buffer
12. Acquire on CytoFLEX flow cytometer

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Data Acquisition on CytoFLEX

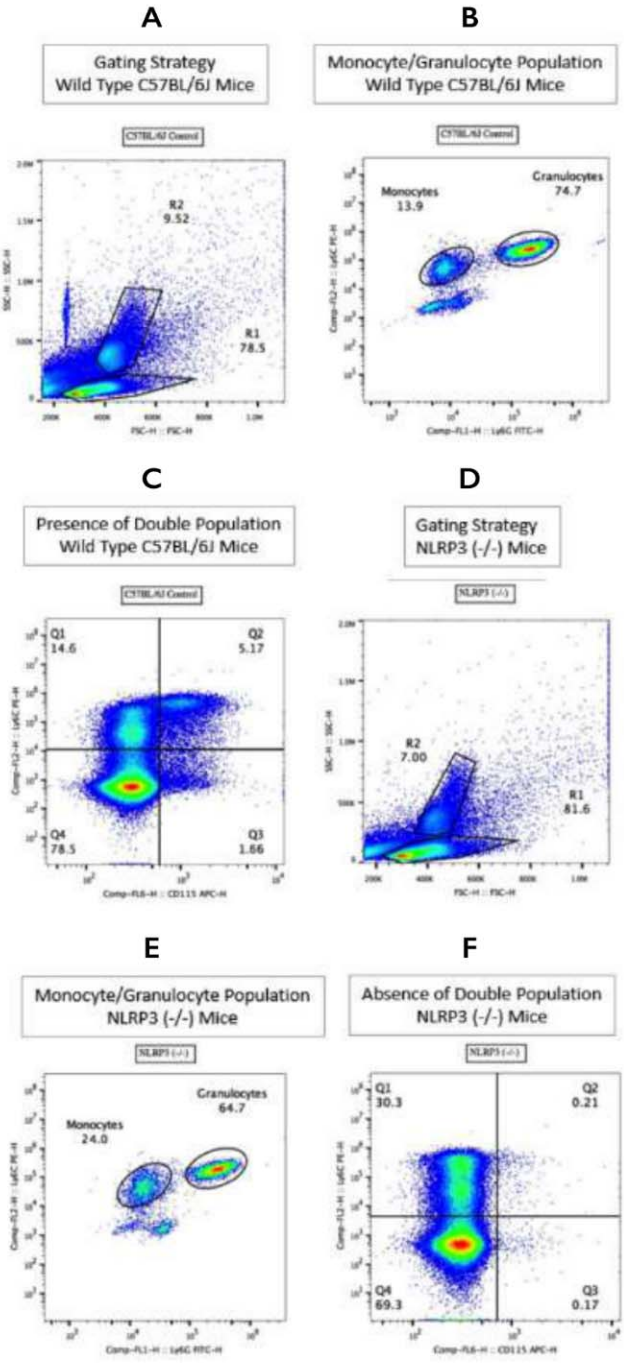
1. Create new experiment
2. Create FSC/SSC, PE/FITC, and PE/APC plot.
3. Import previously established compensation settings for FITC, PE and APC.
4. Create gates for monocytes and granulocytes
5. Run the sample on Fast rate setting.
6. Auto-adjust for scaling.
7. Acquire a minimum of 50,000 events.
8. Save data.

Conclusions

Through the use of surface markers Ly6C and Ly6G, we are able to observe a change in the dynamics of the circulating monocyte and granulocyte population between our wild type controls and the NLRP3 null samples. Furthermore, a double positive population for CD115 (MCSF receptor) and Ly6C observed in the wild type controls is absent in the NLRP3 null samples. This observation was previously not detected on other instrumentation. The CytoFLEX flow cytometer is simple to operate and is a sensitive and reliable method to interrogate the differences in populations circulating in blood.

Data

Plots A and B show the gating strategy. Plots C and D depict the percentage of monocytes and granulocytes in wild type vs. NLRP3 null mice. Plots E and F demonstrate the presence/absence of a double positive population.



For Beckman Coulter's worldwide office locations and phone numbers, please visit "Contact Us" at beckmancoulter.com

FLOW-946FLY08.15-A

Phenotypic characterizing of human Natural Killer (NK) cell populations in peripheral blood

APPLICATION INFORMATION BULLETIN

Author: Christopher A Fraker, Ph.D.,
Affiliation: University of Miami - Miami, Florida

IN THIS PAPER YOU WILL LEARN

The preparation of a 10 color immunophenotyping panel to identify human Natural Killer cells (NK)

The gating strategy to quantify NK population subsets

Introduction

NK cells are part of the innate immune system and implicated in tumor surveillance and killing of virally infected cells. More recently, it has been shown that NK cells also play a role in autoimmune diseases either by regulating the adaptive immune response i.e. T cells or interaction with other regulatory cells, like macrophages, and dendritic cells. We are interested in comparing the NK cell compartment, phenotypically and functionally, of Type 1 diabetic patients to healthy subjects. We are comparing ratios of $CD56^{hi}/CD16^{lo}$ and $CD56^{lo}/CD16+$ NK cells and monitor differential expression of the cell surface receptors including CD8, CD11c, CD38, CD57, and CD117 as well as their expression levels.

Sample Preparation

1. Add 35 mL of fresh human blood (received from blood bank) with 20 mL PBS.
2. Add 15 mL of Ficoll-Paque to 50 mL conical tube and transfer of 35 mL PBS diluted blood.
3. Centrifuge tubes at 400 x G for 30 minutes at room temperature without break.
4. Using a (10-25 mL) pipette or a plastic Pasteur pipette gently aspirate the interphase cells from tubes and transfer into new 50 mL conical tubes.
5. Fill the remaining volume (up to 50 mL) with sterile PBS.
6. Wash. Centrifuge tube at 300 X g for 10 min, room temperature (RT), low brake.
7. Lyse blood red cells (BD cat# 555899, 10 X solutions. Use it at 1X in distilled water. Incubation 15 min RT).
8. Resuspend $1x10^6$ PBMCs in 20 µL staining buffer (PBS with 5% Normal Human Serum).
9. Add antibodies: CD117-BV421 (c-kit), CD7-BV510, CD57-BV605, CD8-BV650, CD14-PE, CD19-PE, CD3-PE, CD66b-PE, CD56-PE-Cy7, CD11c-APC, CD45-AF700, CD38-APC-AF750 (all 5 µL per test), and CD16-FITC (20 µL per test) and incubate for 30 minutes on ice.
10. Centrifuge at 300 x G for 5 minutes at 40C.
11. Aspirate supernatant.
12. Resuspend in 500 µL staining buffer.
13. Acquire on CytoFLEX.

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Laser	405nm					488nm					638nm		
Fluor	Krome Orange	Pacific Blue	V610	V660	V780	FITC	PE	ECD	PC5.5	PC7	APC	APC A700[1]	APC A750[2]
Marker	CD7	c-kit	CD57	CD8		CD16	CD14, CD19, CD3, CD66b			CD56	CD11c	CD45	CD38
Clone	M-T701	104D2	NK-1	SK1		3G8	M5E2 HIB19 UCHT1 G10F5			B159	S-HCL-3	HI30	LS198-4-3

(1) APC-Alexa Fluor® 700 (2) APC-Alexa Fluor® 750

Data Acquisition on CytoFlex

1. Create new compensation.
2. Check each single color control separately and change gain, where applicable.
3. Acquire single color controls (antibody stained VersaComp beads catalog # B22804).
4. Create new experiment.
5. Import previously established compensation settings for BV421, BV510, BV605, BV650, FITC, PE, PE-Cy7, APC, AF700, and APC-AF750.
6. Create following plots: CD45 by SSC, gating on CD45+ cells; FSC by SSC, gating on lymphocytes; FSC-A by FSC-W, gating on single cells; Exclusion by CD7, gating on exclusion-/CD7+ cells; CD16 by CD56, gating on $CD56^{hi}/CD16^{lo}$ and $CD56^{lo}/CD16+$ cells; display for each NK cell subset ($CD56^{hi}/CD16^{lo}$ and $CD56^{lo}/CD16+$) following plots: c-kit by CD38, CD57 by CD11c, and CD57 by CD8.
7. Run the sample on medium.
8. Auto-adjust for scaling.
9. Acquire 250,000-500,000 events.
10. Adjust compensation.
11. Save data.
12. Export to FCS.
13. Analyze in Kaluza.

Conclusions

NK cells are implicated in autoimmune diseases and may also play a role in T1D progression by creating a regulatory environment that favors the destruction of pancreatic beta cells. The current panel was aimed at identifying NK cell subsets that differ phenotypically between healthy subjects and patients suffering from T1D. The final goal is to establish biomarkers that are predictive in the early, pre-onset phase of T1D.

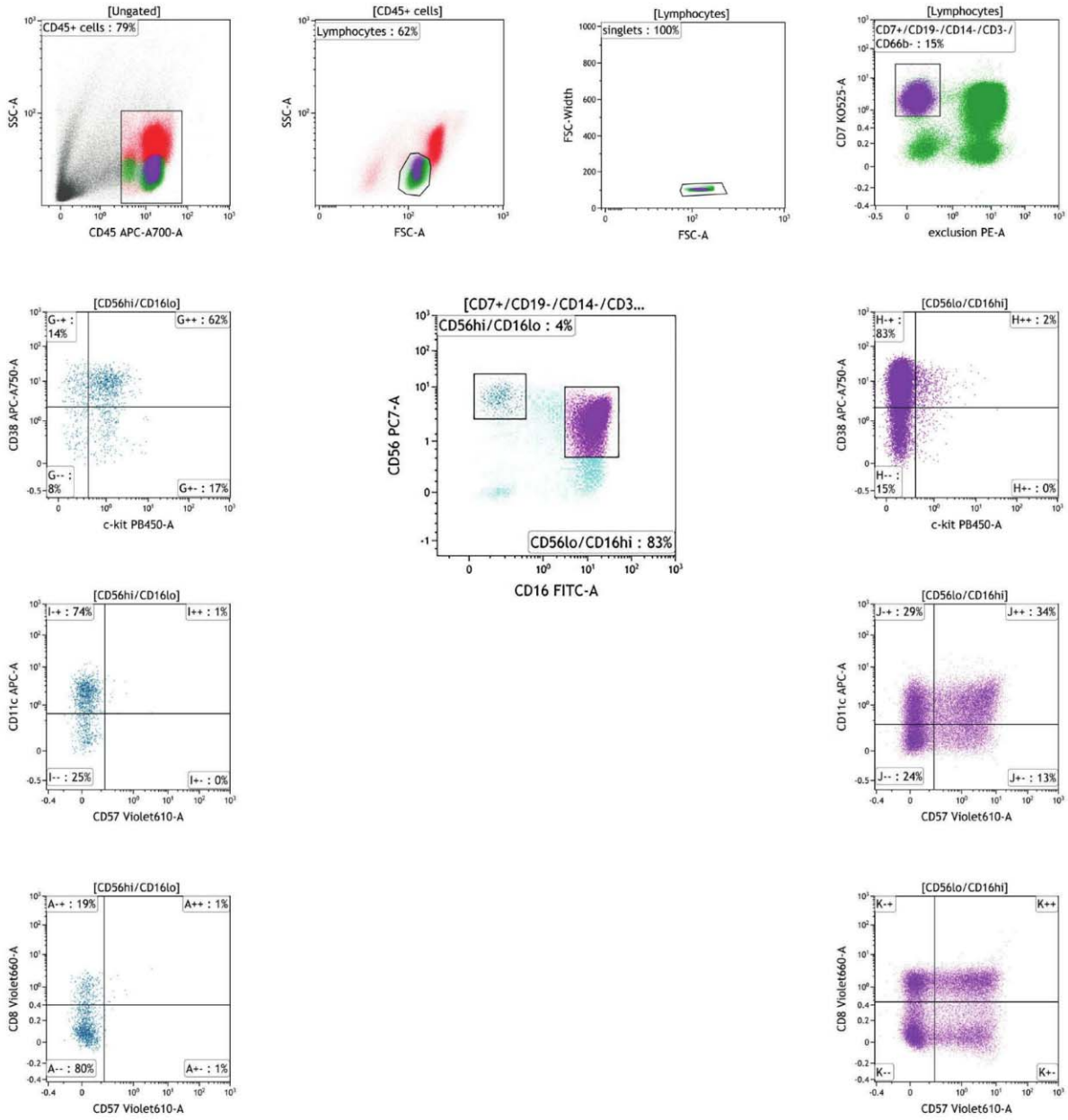

Figure legend:

PBMCs from a healthy donor were stained with CD117-BV421 (c-kit), CD7-BV510, CD57-BV605, CD8-BV650, CD14-PE, CD19-PE, CD3-PE, CD66b-PE, CD56-PE-Cy7, CD11c-APC, CD45-AF700, CD38-APC-AF750, and CD16-FITC. NK cells were defined as CD45+, CD7+, CD19-/CD3-/CD14-/CD66b- (exclusion-). Aggregates were excluded by electronic gating using a FSC-A by FSC-W plot. In humans, two major NK subsets can be identified using the cell surface antigens CD56 and CD16, i.e. $CD56^{hi}$/$CD16^{lo}$ and $CD56^{lo}$/CD16+. $CD56^{hi}$/$CD16^{lo}$ and $CD56^{lo}$/CD16+ NK cells were further subdivided based on the expression of CD57, CD11c, CD8, CD38, and CD117 (c-kit).

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FLOW-999APP06.15-A

Identification of NK subsets in mice

APPLICATION NOTE

Author: Allison Bayer, Ph.D.,
Affiliation: University of Miami - Miami, Florida

IN THIS PAPER YOU WILL LEARN

The preparation of a 10 color immunophenotyping panel to identify mouse Natural Killer cells (NK)

The gating strategy to quantify NK population subsets

Introduction

Human NKs are commonly divided based on their expression of CD56 and CD16, which generally correlates to their activation status. $CD56^{bright}CD16^{neg}$ NKs are abundant in peripheral tissues; they respond to soluble factors by making copious amounts of immunoregulatory cytokines, but acquire cytotoxicity after prolonged activation. In contrast, most circulating human NKs are $CD56^{dim}CD16^{pos}$ and are considered activated; when target cells are recognized they respond by killing the target or producing cytokines. Mouse NKs lack CD56 expression, which has made it difficult to identify functional counterparts to human NKs. However, mouse NKs are commonly identified based on the expression of CD11b and CD27, but these markers focus more on immature versus mature status of mouse NKs. Several reports have shown that mouse NKs express CD127 (IL-7Rα) and may be similar to $CD56^{Bright}CD16^{neg}$ human NKs in that they produce large amount of cytokines and only acquire cytotoxicity function after prolonged activation. The NKp46 (CD335) activation receptor is a common surface marker found on both human and mouse NKs. Together with CD122 (IL-2Rb chain), these markers are used to identify NKs among $CD45^{pos}CD3^{neg}$ cells. There are conflicting reports about NKs in autoimmune models, which could relate to the poor understanding of functional NK subsets in mice.

Sample Preparation

1. Remove mouse spleen.
2. Make single cell suspension. Use 5 mL Hanks per 2 spleens in 35 mM culture dish. Use syringe head to "smash" and transfer suspension to 14mL tube. No more than 2 spleens per tube!! Allow debris to settle to bottom of tube and transfer supernatant to fresh 14 mL tube.
3. Spin cells 5 minutes at 1500 rpm at 4˚C.
4. Pour off supernatant and "flick" pellet to loosen cells.
5. Remove red cells using 2 mL ACK (90% of 0.83% $NHCl_4$ +10% 2.06% Tris pH 7.6-Sterile) per tube. Incubate 1 minute in 37˚C waterbath. Add 8 mL Hanks. Mix by pipetting up and down with 10 mL pipette and allow suspension to settle in the pipette, so that the debris will adhere to the side of the pipette. Slowly place suspension in fresh 14 mL tube leaving the cell debris in the pipette. Repeat steps 3 and 4.
6. Add 10 mL PBS count cells.
7. Stain for fixable live/dead dye for 20 minutes in fridge. Wash with PBS 2X.
8. Surface stain splenocytes for CD45, CD19, CD3. CD335, CD25, CD122, CD127, CD218, and CD117. Incubate 20 minutes in fridge. Wash 2X.

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9. Fix and permeabilize with Foxp3 stain buffer set (e-Bioscience cat. No. 00-5523). For 30 minutes. Wash 2X with Perm buffer.
10. Add antibody for Ki67. Incubate for 20 minutes in fridge. Wash 1X with Perm buffer and 1X with PBS.
11. Acquire on CytoFLEX.

Laser	405nm					488nm					638nm		
Fluor	Krome Orange	Pacific Blue	V610	V660	V780	FITC	PE	ECD	PC5.5	PC7	APC	APC AF700	APC AF750
Marker	CD45	CD218	CD122		CD127	Ki-67	Ckit		CD335		CD19	CD3	Live/Dead
Clone	30-F11	P3TUNYA	TM-b1		SB/199	B56	ACK2		29A1.4		103	17A2	

Data Acquisition on CytoFLEX*

1. Create new experiment
2. Create plots.
3. Run unstained and single color controls for compensation settings
4. Create gates for NK and T-cells
5. Run the sample on fast.
6. Auto-adjust for scaling.
7. Acquire a minimum of 100,000 events.
8. Save data.
9. Export to FSC
10. Analyze in Kaluza

Conclusions

The current panel was aimed at identifying NK subsets with effector and regulatory properties using C57BL/6 mice. We found $CD335^{pos}CD122^{pos}$ cells among $CD45^{pos}CD3^{neg}CD19^{neg}$ cells in spleen. Among bulk NKs, c-Kit^{pos} cells were observed. Unlike T cells where the majority of cells express CD127 (IL-7Rα), only a subset of NK cells express CD127 and among the c-Kit^{pos} NK cells, we found $CD127^{pos}$ and $CD127^{neg}$ cells. The majority of NKs are $CD218^{pos}$ (IL-18R) and about 25% of splenic NKs are proliferating as determined by Ki67 staining.

FLOW-1000APP06.15-A

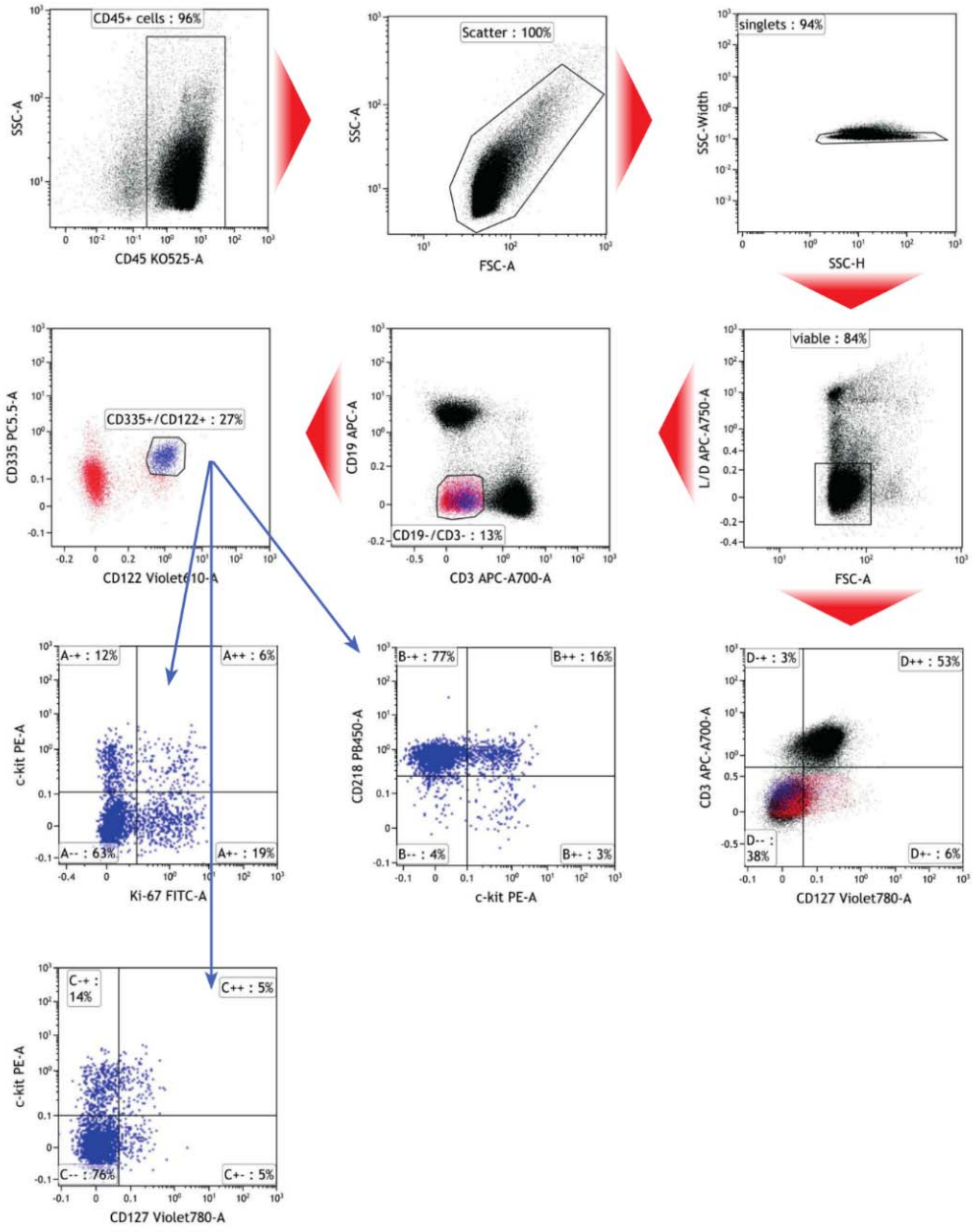

Figure legend: here we show the gating strategy used to identify subpopulaions of mouse NK cells, CD3 CD19 double negative cells which are CD335 CD122 double positive can be further characterized using CD127 and the proliferation markers c-kit and Ki-67.

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Identification of Natural Killer Cells in Rat Placenta Model of Preeclampsia

APPLICATION NOTE

Author: Denise Cornelius , PhD
Affiliation: University of Mississippi Medical Center, Jackson, MS 39216

IN THIS PAPER YOU WILL LEARN

- The preparation of Natural Killer cells from tissue
- The method to measure NK activation

Introduction

Historically, the rat has been the predominant preclinical model system employed by academia and industry alike. An important area of study utilizing the rat is reproduction. A condition affecting a significant percentage of pregnancies, preeclampsia, is a complication defined by hypertension in a woman who had otherwise normal blood pressure. Preeclampsia is often related to chronic inflammation that can endanger the life of both mother and child. Utilizing a pregnant rat model to study the immune response occurring in the placenta can aid our understanding of preeclampsia, with the ultimate goal of developing a therapy to correct the pathophysiology of this serious condition. Here, we characterize the presence of Natural Killer cells in rat placenta.

Materials & Methods

Pregnant mice on day 19 of gestation were euthanized and placentas were harvested. Single cell suspensions were obtained from the placentas. Cells were incubated for 30 minutes at 4°C with or without an antibody against ANK61, a marker of Natural Killer cells. Then both conditions were subsequently washed 3 times by centrifugation at 400 g for 5 minutes and incubated with secondary FITC for 30 minutes at 4°C in the dark. Lastly, these cells were washed 3 times and resuspended in RPMI containing 50% formalin. Cells were analyzed on a CytoFLEX* flow cytometer. The percentage of positive staining cells above the negative control was then analyzed.

BECKMAN COULTER Life Sciences

FLOW-1005APP06.15-A

Reagents

ANK61 mouse-anti rat monoclonal purified antibody / Abcam ab36392.
Goat anti-mouse FITC / Abcam ab6785.

Data Analysis

FSC-A x SSC-A live gate

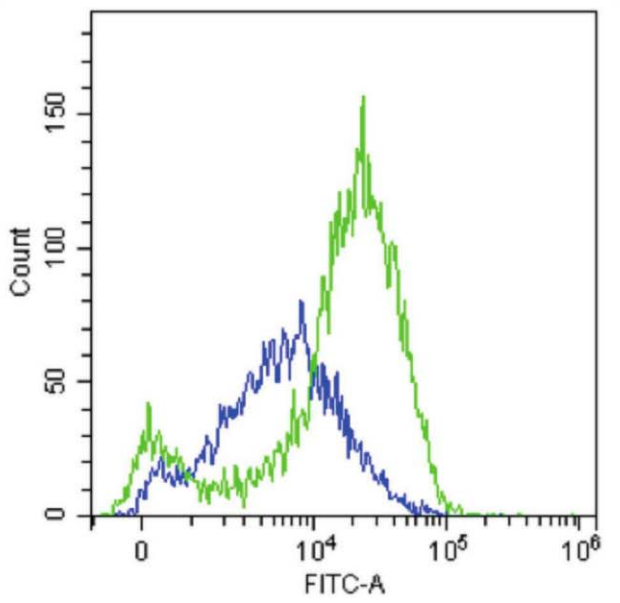

Figure 1. Overlay histogram of secondary goat anti-mouse FITC (blue) and ANK61 mouse-anti rat monoclonal purified antibody followed by goat anti-mouse FITC (green).

Results

Here, we demonstrate an increase in activated NK cells from rat placenta using the CytoFLEX, allowing us to further characterize the immune response occurring in the placenta with the ultimate goal to further understand of the pathophysiology preeclampsia.

Reference

Amaral LM, Cunningham Jr MW, Cornelius DC, LaMarca B. Preeclampsia: long term consequences for vascular health. Vasc Health Risk Manag. 2015 in press.

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Competitive Bone Marrow Transplantation in C57/BL6 Mice

APPLICATION NOTE

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Leonardo Salmena, PhD [1,2]

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2. Princess Margaret Cancer Centre, University Health Network, Toronto, Canada

IN THIS PAPER YOU WILL LEARN

About a method for (mouse) bone marrow transplant based on genetic differences

An associated method to evaluate transplantation results by flow cytometry using the CytoFLEX flow cytometer

Background

Competitive bone marrow transplantation assay measures reconstitution of the blood system adult lineages post-irradiation in transplant recipient mice. The technique hinges on the ability to transplant bone marrow donor cells into a congenic host with normal competitor bone marrow, and is probably the most common and simplest method to determine 'stemness' *in vivo*. In order to distinguish donor from competitor cells upon transplantation, usually competitor mice are congenic and carry the differential B cell antigen originally designated CD45.1/Ly5.1.

Introduction

Hematopoeitic stem cells (HSCs) are cells capable of differentiating into cells of all blood lineages and are essential for maintenance of the blood system. These pluripotent HSCs are capable of self-renewal and regenerate adult blood cells through a hierarchical process generating various multi-potent and lineage-committed intermediate cells.

In order to study the stem niche, researchers must be able to determine the ability of a population of cells to produce adult cell lineages under controlled conditions. A typical strategy employed is to transplant bone marrow, rich in HSCs, into mice whose blood system has been ablated upon sub-lethal irradiation. In these mice both the adult blood cells as well as the stem and progenitor cells will die-off within 2 weeks of irradiation. The transplanted cells will rescue the mice by developing into newly differentiated blood lineages.

HSCs can be subdivided based on their short-term (ST-HSCs), long-term (LT-HSCs) and intermediate-term (IT-HSCS) reconstitution ability [1, 2]. LT-HSCs are determined in most reports by their capacity to generate circulating monocytes, granulocytes, and lymphocytes at time points from 16 to 44 weeks post-transplant. The first analysis of competition by transplanted cells should be done 4-6 weeks post-transplantation.

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Wild-type inbred C57/BL6 mice are typically used in transplant studies given that they carry the *Ptprc*^{b} leukocyte marker (CD45.2/Ly5.2) allele. A congenic strain dubbed C57BL/6-Ly5.1 was developed at the Sloan Kettering Institute by backcrossing SJL mice against wilt-type C57/BL6 mice. These mice carry the *Ptprc*^{b} leukocyte marker (CD45.1/Ly5.1) allele. Using commonly available primary-conjugated antibodies against CD45.1 and CD45.2, researchers can easily identify host and transplanted cells, and determine the contribution of HSCs from either in competition assays. We outline here a simple assay to measure leukocytes in peripheral blood from CD45.2-donor and CD45.1-host mice post-transplantation in irradiated mice.

Procedure

1. On the day of the experiment, one CD45.1 C57/BL6 mouse and one CD45.2 C57/BL6 mouse are sacrificed by preferred method.
2. From these mice, hind limbs are extracted and cleaned.
3. Total bone marrow is flushed from the bone cavity separately with ice cold PBS using a 21 G x 1.5 needle.
4. This is then passed through a 70 µM cell strainer to obtain a single cell suspension.
5. Count the cells under a light microscope using a haemocytometer.
6. Prepare the following mixtures of 2 x 10^{6} cells for transplantation in a total volume of 0.2 mL: (i) CD45.2 and CD45.1 bone marrow cells in a 2:1 ratio, (ii) CD45.1 cells alone and (iii) CD45.2 cells alone
7. Load the 0.2 mL cell suspension into 0.5 mL syringes.
8. The cell suspension is transplanted into irradiated (9.5 Gy) CD45.1 C57/BL6 donor mice by tail vein injection.
9. Following transplantation the donor mice are left to rest for 4 weeks.
10. At 4 weeks post-transplantation, blood is obtained from mice by nicking the tail and collecting approximately 100 µL of peripheral blood into an EDTA lined tube.
11. Collected blood was resuspended in in 10X volume of RBC lysis buffer for 10 minutes (see Note 1).
12. Cells were again resuspended in PBS, with 5 % FBS and kept on ice for 20 minutes
13. Cells were then centrifuged at 400x g for 5 minutes
14. Cells were resuspended in 100 µL staining buffer and incubated at room temperature in the dark for 20 minutes.
15. Cells were centrifuged 400x g for 5 minutes
16. Cells were washed once with PBS
17. Cells were resuspended in 200 µL of PBS
18. Cells were analysed on the Cytoflex flow cytometer.
19. Mouse peripheral blood cells stained CD45.1 FITC and CD45.2 PE were gated for mononuclear cells according to FSC vs. SSC intensity

Materials & Methods

Materials Required:

Centrifuge, microcentrifuge, Gilson pipetteman (P10, P20, P200, P1000), dissection kit, 70 µM Cell Strainer, 21-gauge needles and syringes, 15mL Centrifuge tubes, 1.5 mL microcentrifuge tubes, 2 mL EDTA coated microcentrifuge tubes, scalpel.

Reagents:

1. Red Blood Cell (RBC) lysis buffer: 155 mM Ammonium Chloride (NH_4Cl), 12 mM Sodium Bicarbonate ($NaHCO_3$) and 0.1 mM EDTA were prepared in double distilled H_2O.
2. Phosphate buffered saline (PBS): 8 g NaCl, 0.2 g KCl, 1.44g Na_2HPO_4, 0.24g KH_2PO_4 in 1 L of double distilled H_2O. pH 7.4
3. Staining Buffer: 5 % FBS in PBS. 100 µL staining buffer contains 400 ng CD45.1-FITC and 400ng CD45.2-PE
4. Staining Buffer: 5 % FCS in PBS, made fresh. If not made fresh, an antimicrobial agent such as sodium azide (at a concentration of 0.1 % *v/v*) should be added.

Reagent	Supplier	Order Details
CD45.1-PE	Biolegend	12-0453-81
CD45.2-FITC	Biolegend	11-0454081

Laser	405nm					488nm					638nm		
Fluor	Krome Orange	Pacific Blue	V610	V660	V780	FITC	PE	ECD	PC5.5	PC7	APC	APC AF700	APC AF750
Marker						CD45.2	CD45.1						
Clone						104	A20						

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Figure 1.

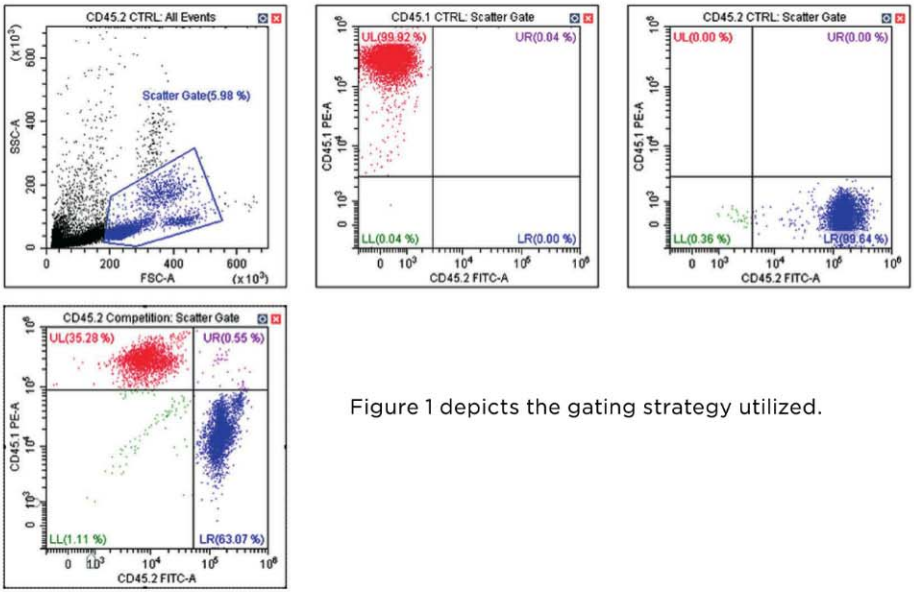

Figure 1 depicts the gating strategy utilized.

Results

Peripheral blood mononuclear cells were gated on the basis of FSC vs. SSC profiles, excluding debris and non-lysed RBCs (Figure 1a). These gated leukocytes were then probed for expression of either the CD45.1 or CD45.2 allele. From the expression of either CD45.1 or CD45.2 we can determine whether a cell originated from the host mouse or from donor bone-marrow. The results show that in mice transplanted whereby CD45.1 bone-marrow cells, as a control, were transplanted back into CD45.1 host mice we only identify CD45.1 cells in the peripheral blood (Figure 1b). The CD45.1 background host mice in which CD45.2 bone marrow cells have been transplanted show a chimeric-blood phenotype, however, at 4 weeks post-transplant (Figure 1c). These mice show an almost 2:1 ratio of CD45.2:CD45.1 leukocytes, as they were transplanted into the mouse originally.

References

1. Li CL, Johnson GR. 1995. Murine hematopoietic stem and progenitor cells: I.Enrichment and biologic characterization. Blood. 1995 Mar 15; 85(6):1472-9.
2. Benveniste P, Frelin C, Janmohamed S, Barbara M, Herrington R, Hyam D, Iscove NN. 2010. Intermediate-term hematopoietic stem cells with extended but time-limited reconstitution potential. Cell Stem Cell. Jan 8;6(1):48-58

Notes

Note 1. It is desirable to remove erythrocytes from spleen mononuclear cell preparations prior to flow cytometry experiments as large numbers of RBCs in the sample can occlude populations of interest. A small number of RBCs remaining in the sample will not prove difficult to gate out however, so partial lysis of RBCs is sufficient and should be optimized depending on the individual experiment being performed.

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Surface staining of mouse splenocytes and peripheral blood cells

APPLICATION NOTE

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IN THIS PAPER YOU WILL

- Learn about multiparametric and mixed population flow cytometry
- Find a brief protocol for preparing mouse splenocytes and peripheral blood for flow cytometry
- Learn gating strategies for mixed population flow cytometry

Background

A major advantage of flow cytometry is the ability to analyze complex mixtures with different cell types. Antigen presentation on the surface allows for the identification of discreet cell types within a mixed sample (e.g. blood) simply *via* staining with antibodies conjugated to various fluorophores. Typically, little or no sample preparation is required for surface marker staining other than singlet cell isolation. Thus, flow cytometry has become the gold standard method for analysis of complex cell mixtures, from blood, spleen, lymph or bone marrow. Here, we describe a method to rapidly identify CD3ε -positive and CD19-positive cells from murine spleen and peripheral-blood samples.

Introduction

Measurement of protein expression *via* flow cytometry offers many advantages in both clinical and research settings. Because it allows for multiparametric measurement, reduced sampling can be achieved. This is especially important in clinical monitoring or diagnosis, whereby sampling is often not trivial (e.g. blood or bone marrow draws). It also allows for separating complex/mixed samples to be separated using specific markers, and subsequent measurement of characteristics of interest. This is of particular interest in research using animal models. Such immunophenotyping assays are essential in order to characterize mouse models rapidly and accurately. Monitoring cell populations in blood is also extremely important when using animal models, as it allows rapid assessment of animal health and phenotypic changes throughout the lifespan of the animal. Here we look at CD3ε and CD19, in both the spleen and the peripheral blood. CD3ε is a member of the T cell receptor complex, essential for antigen recognition and signal propagation. It is a commonly used marker for T-cells. CD19 is a surface marker that couples with the antigen receptor of B lymphocytes and decreases the threshold for antigen receptor dependent stimulation. It is commonly used as a marker for B cells. With these two markers we provide a simple and rapid method to identify two discrete populations in murine splenocytes and blood.

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Standard Procedure

All mouse experiments were performed using male C57/BL6 young-adult mice (10 weeks). Animals were obtained from the Jackson Laboratory (Bar Harbor, MA, USA). The animals were housed in standard on a 12-hour light-dark cycle and at a temperature of 23°C with free access to food and water in groups of 5 mice. All experimental protocols were approved by the research ethics board of this university and were carried out in compliance with the Canadian Council on Animal Care recommendations.

1. C57/BL6 mice were sacrificed at approximately 10 weeks of age.
2. Peripheral blood was taken from heart immediately *post mortem*. Briefly, a 26-gauge needle was inserted into the heart from the sternum. Approximately 500 µL of blood was drawn slowly and transferred to an EDTA coated tube to prevent clotting.
3. Spleens were isolated *post mortem* and placed in PBS on ice.
4. Splenocytes were collected by mashing the spleen through a 70 µM cell strainer utilizing the thumb-piece of a plunger removed from a 1 mL syringe; single cell splenocyte suspensions were collected in 5 mL of ice-cold PBS.
5. Splenocyte preparation was again passed through a 70 µM cell strainer to remove any remaining debris.
6. Cells were then transferred to a 15 mL centrifuge tube on ice.
7. This was then centrifuged at 400x g for 5 minutes.
8. Splenocytes were resuspended in 10 mL RBC lysis buffer and vortexed briefly. The cells were allowed to incubate for 10 minutes (see Note 1).
9. Cells were then centrifuged at 400x g for 5 minutes and supernatant was removed.
10. Cells were again resuspended in PBS, with 5 % FBS and kept on ice for 20 minutes.
11. Cells were then centrifuged at 400x g for 5 minutes.
12. Cells were resuspended in 100 µL staining buffer and incubated at room temperature in the dark for 20 minutes.
13. Cells were centrifuged 400x g for 5 minutes.
14. Cells were washed once with PBS.
15. Cells were resuspended in 200 µL of PBS.
16. Cells were analyzed on the Cytoflex flow cytometer.
17. Mouse spleen cells or peripheral blood cells stained with CD3ε-alexa fluor 488 and CD19-PerCP-Cy5.5 were gated for mononuclear cells (MNCs) according to FSC vs. SSC intensity.
18. Gated cells were further gated on FSC-area vs. FSC-height to discriminate singlet cells from doublet cells.
19. Singlet cells were then plotted on a dot-plot of CD3ε vs. CD19.

Materials & Methods

Material required but not supplied

Microcentrifuge, micro-pipetteman (P10, P20, P200, P1000), dissection kit, 70 µM cell strainer, 26-gauge needles and syringes, 15 mL centrifuge tubes, 1.5 mL microcentrifuge tubes, 2 mL EDTA coated microcentrifuge tubes

Reagents

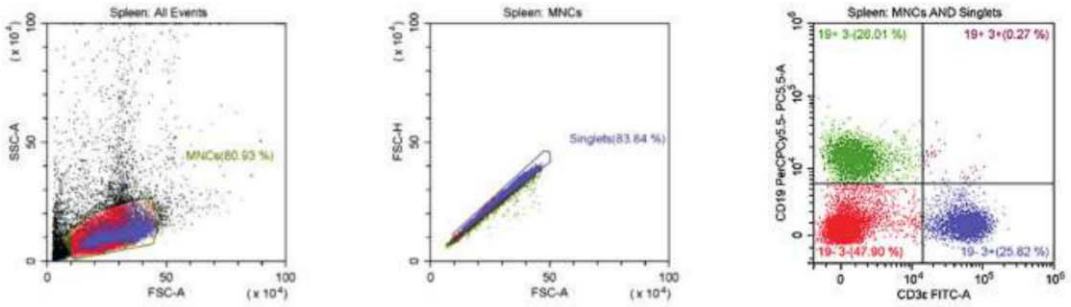
1. Red Blood Cell (RBC) lysis buffer: 155 mM Ammonium Chloride (NH_4Cl), 12 mM Sodium Bicarbonate ($NaHCO_3$) and 0.1 mM EDTA were prepared in double distilled H_2O.
2. Phosphate buffered saline (PBS): 8g NaCl, 0.2g KCl, 1.44 g Na_2HPO_4, 0.24g KH_2PO_4 in 1 L of double distilled H_2O. pH 7.4.
3. Staining Buffer: 5 % FBS in PBS. 100 µL staining buffer contains 400 ng CD19-PerCP-Cy5.5 and 500 ng CD3e-AlexaFluor 488.
4. Staining Buffer: 5 % FCS in PBS, made fresh. If not made fresh, an antimicrobial agent such as sodium azide (at a concentration of 0.1 % *v/v*) should be added.

Laser	405nm					488nm					638nm		
Fluor	Krome Orange	Pacific Blue	V610	V660	V780	Alexa Fluor 488	PE	ECD	PerCP-Cy5.5	PC7	APC	APC AF700	APC AF750
Marker						CD3ε			CD19				
Clone						145-2C11			1D3				

Sample prep

Sample Type	Species	Age of specimen	Prep Method
Mouse splenocytes	C57/BL6	12 weeks	
Mouse Peripheral blood	C57/BL6	12 weeks	

a

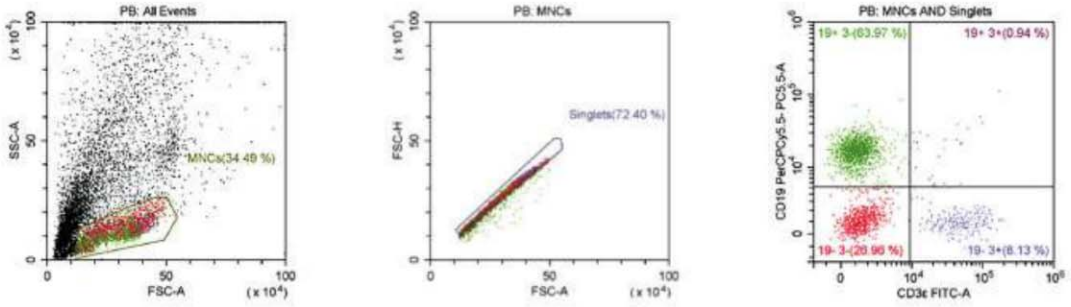


Tube Name: Spleen

Sample ID:

Population	Events	% Total	% Parent
All Events	30170	100.00%	100.00%
MNCs	24418	80.93%	80.93%
Singlets	20473	67.86%	83.84%
MNCs AND Singlets	20473	67.86%	67.86%
19+ 3+	56	0.19%	0.27%
19+ 3-	5325	17.65%	26.01%
19- 3-	9806	32.50%	47.90%
19- 3+	5286	17.52%	25.82%

b



Tube Name: PB

Sample ID:

Population	Events	% Total	% Parent
All Events	11082	100.00%	100.00%
MNCs	3822	34.49%	34.49%
Singlets	2767	24.97%	72.40%
MNCs AND Singlets	2767	24.97%	24.97%
19+ 3+	26	0.23%	0.94%
19+ 3-	1770	15.97%	63.97%
19- 3-	746	6.73%	26.96%
19- 3+	225	2.03%	8.13%

Figure 1 Legend: Surface staining of mouse splenocytes and peripheral blood cells with CD3ε and CD19.

(a) Gating strategy for C57/BL6 murine splenocytes on a FSC *vs.* SSC dot-plot. Viable cells were further gated to remove possible doublet cells on a dot-plot for FSC-area *vs.* -height. Singlet cells were then analyzed for CD3ε and CD19 expression, in the FL1 and FL3 channels respectively.

(b) a) Gating strategy for C57/BL6 murine peripheral blood cells on a FSC *vs.* SSC dot-plot. Viable cells were further gated to remove possible doublet cells on a dot-plot for FSC-area *vs.* -height. Singlet cells were then analyzed for CD3ε and CD19 expression, in the FL1 and FL3 channels respectively.

Results

In both the peripheral blood and spleen samples, cells were gated to exclude debris and the majority of erythrocytes that were not lysed in the RBC lysis step. Typically, not all RBCs will be removed by this lysis but enough will have been removed to allow for efficient gating. Cells were also gated to remove any doublets present. We then examined both the CD3ε and CD19 staining on a dot-plot. Here we see excellent separation of both CD3ε-positive and CD19-positive cells. We see effectively no double-positive cells and thus can easily identify the T- and B-cell populations, with particularly good separation in the spleen sample.
This rapid detection of these two important populations could also be joined with other surface markers to identify more populations such as monocytes, macrophages, dendritic cells etc.

The ability to identify two populations in this way, simply and rapidly, allows for monitoring of these populations. This can be done, of course, with tail bleeds also throughout the life of the mice.

Notes

It is desirable to removed erythrocytes from spleen mononuclear cell preparations prior to flow cytometry experiments as large numbers of RBCs in the sample can occlude populations of interest. A small number of RBCs remaining in the sample will not prove difficult to gate out however, so partial lysis of RBCs is sufficient and should be optimized depending on the individual experiment being performed.

Reagent Details

Reagent	Supplier	Order Details
CD3ε-Alexa Fluor 488	Biolegend	Cat. # 100321
CD19-PerCP-Cy5.5	eBiosciences	Cat. # 45-0193-80

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FLOW-1118APP09.15-A

Advanced analysis of human T cell subsets on the CytoFLEX flow cytometer using a 13 color tube based on DuraClone dry reagent technology

APPLICATION NOTE

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IN THIS PAPER YOU WILL LEARN

- How additional markers allow for increased subpopulation discrimination and biological subsetting
- How easy it is to set up and utilize the compensation library
- How DuraClone dry reagents can simplify your workflow

Principal of the Technique

Summary

Multi-color flow cytometry is a powerful tool to analyze the highly heterogeneous human T cell compartment. Using the 10 color DuraClone IM T Cell Subset dry reagent kit (CD45RA-FITC, CD197-PE, CD28-ECD, CD279-PC5.5, CD27-PC7, CD4-APC, CD8-Alexa-Fluor 700, CD3-APC-Alexa-Fluor 750, CD57-Pacific Blue, CD45-Krome Orange) plus 3 additional liquid antibodies for the violet laser (Brilliant Violet 605 anti-human CD95, Brilliant Violet 650 anti-human CD25, and Brilliant Violet 785 anti-human CD127 antibodies) we defined a 13-color tube which allows for the identification of major peripheral T cell subsets according to classical and more recent characterization criteria, with a minimum of sample preparation effort.

488 Excitation					638 Excitation			405 Excitation				
FITC	PE	ECD	PC5.5	PC7	APC	APC-A700 (1)	APC-A750 (2)	Pacific Blue	Krome Orange	BV (3) 605	BV 650	BV 785
CD45RA	CCR7 (CD197)	CD28	PD1 (CD279)	CD27	CD4	CD8	CD3	CD57	CD45	CD95	CD25	CD127

(1) APC-Alexa Fluor 700 (2) APC-Alexa Fluor 750 (3) Brilliant Violet

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Introduction

T lymphocytes (T cells) form an essential part of the adaptive immune system and are therefore of major interest for the research community. Two recent examples are the work of the international ONE Study (www.onestudy.org) and BIO-DrIM (www.biodrim.eu) consortia, international groups of experts in the field of immune monitoring, funded by the European Commission. Within these approaches, marker and dye selections for flow cytometry have been designed and optimized by expert flow labs to monitor the human immune response [1].

The close cooperation between these groups and Beckman Coulter resulted in the development of the DuraClone IM brand of dry reagents for the analysis of human immune cells. Using the 10 color DuraClone IM T Cell Subsets kit plus 3 additional liquid markers, a 13 color tube has been designed for the phenotypic analysis of major T cell sub-populations in the human peripheral blood.

Protocol

Standard Procedure

After informed consent, 100 µL of human peripheral blood from a healthy donor was added to a DuraClone IM T Cell Subset dry reagent tube (Beckman Coulter), followed by 5 µL each of Brilliant Violet 605 anti-human CD95, Brilliant Violet 650 anti-human CD25, and Brilliant Violet 785 anti-human CD127 antibodies (BioLegend). Cells were mixed for 8 seconds, incubated for 15 minutes at room temperature (RT) in the dark, and red blood cells were lysed by adding 2 mL of VersaLyse Lysing solution plus 50 µL of IOTest 3 Fixative Solution (both from Beckman Coulter). Following incubation (20 min at RT), the suspension was spun down (200 x g, 5 min), the supernatant discarded, and the pellet resuspended in 3 mL 1 x PBS. After an additional centrifugation step (see above), the cell pellet was resuspended in 500 µL 1 x PBS for subsequent analysis on a 13 color / 3 laser CytoFLEX flow cytometry system (Beckman Coulter).

CytoFLEX is the first commercial flow cytometer to utilize fiber array photo diodes (FAPD)s for fluorescence channel detection. The FAPD provides low-noise detection with high quantum efficiency and minimum light loss ensuring high signal to noise ratio and optical resolution especially with small particle measurements and dim fluorescence detection.

References

1. Streitz M. et al. Standardization of whole blood immune phenotype monitoring for clinical trials: panels and methods from the ONE study. Transplantation Research 2013 2:17.
2. Appay V. et al. Phenotype and function of human T lymphocyte subsets: consensus and issues. Cytometry A 2008 73A: 975.
3. Mahnke Y.D. et al. The who's who of T-cell differentiation: Human memory T-cell subsets. Eur. J. Immunol. 2013 43: 2797.

Notes

The results demonstrated in this application sheet represent those generated on the Beckman Coulter CytoFLEX Flow Cytometer with 488 nm / 638 nm / 405 nm laser configuration. As differences exist in the performance between analyzers, the author cannot guarantee a similar appearance with the use of other Flow Cytometers.

Reagent details

Reagent	Supplier	Order Details
DuraClone IM T Cell Subsets Kit	Beckman Coulter	B53328
VersaLyse Lysing Solution	Beckman Coulter	A09777 or* IM3648
IOTest 3 Fixative Solution	Beckman Coulter	A07800 or* IM3515
Brilliant Violet 605 anti-human CD95	BioLegend	305628
Brilliant Violet 650 anti-human CD25	BioLegend	302634
Brilliant Violet 785 anti-human CD127	BioLegend	351330

* Depending on geography.

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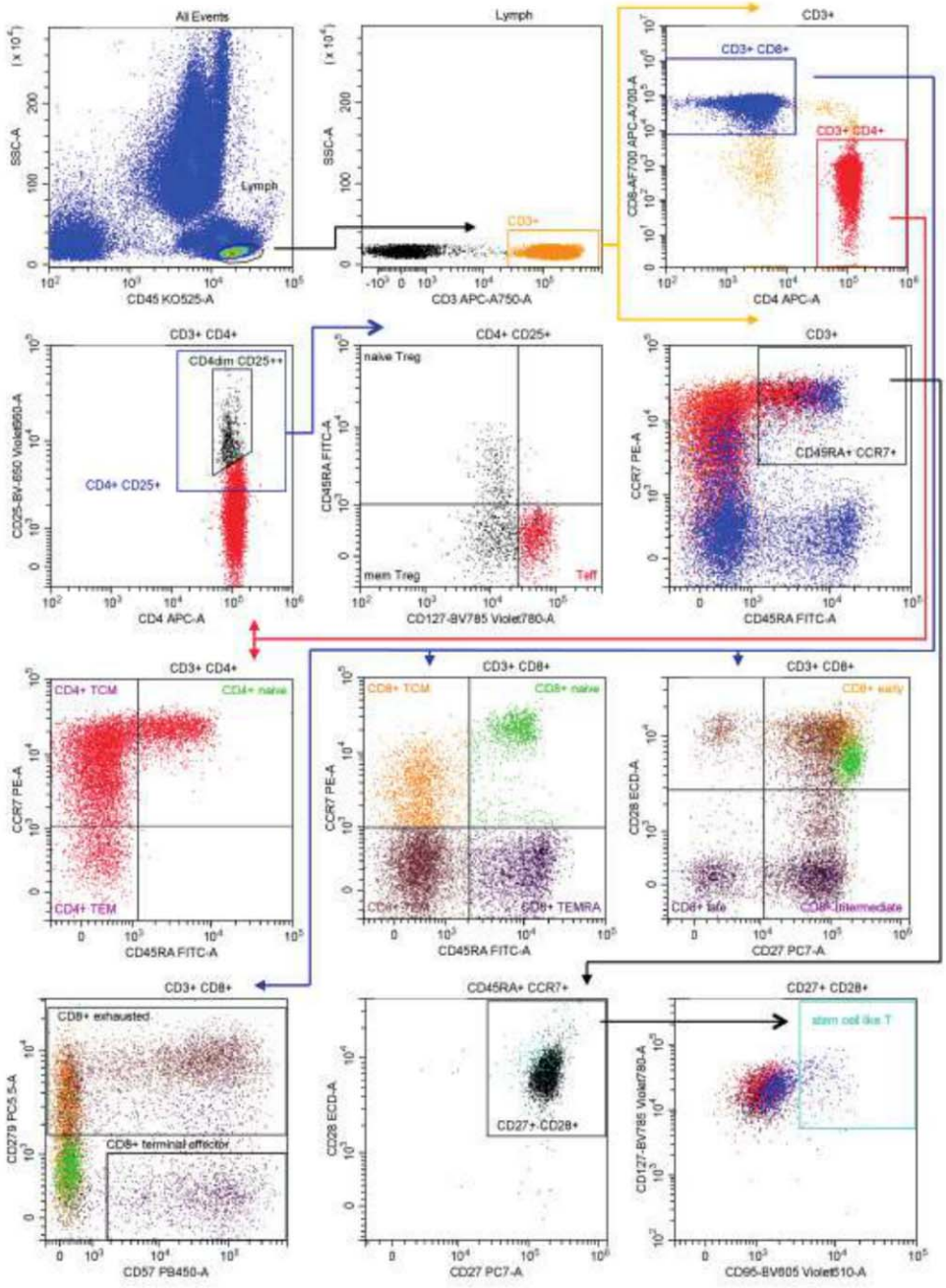
Results



Analyses of T cell subsets based on the differential expression of surface molecules related to cell function, differentiation, or activation have evolved [2]. As a result, T cell analysis requires a multitude of markers to capture the various populations that have been described [3]. The 13 color tube presented here, allows for the identification of most T cell memory subpopulations that can be characterized by surface marker expression patterns. It is suitable for all flow cytometers with a 5-3-5 (488 & 561 nm / 638 nm / 405 nm) optical layout and reduces sample preparation to basically only 4 pipetting steps.

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FLOW-817APP03.15-A.

Flow Cytometric Quantification to Assess Dorsal Endocytosis

APPLICATION NOTE

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IN THIS PAPER YOU WILL LEARN

- How to setup a phagocytosis assay
- How to measure cellular phagocytosis by flow cytometry
- How to set up controls and properly gate a phagocytosis assay using flow cytometry

Background

The accumulation of collagen during fibrosis is caused by excessive production and reduced degradation. To test the ability of fibroblasts to uptake and digest collagen, we used a flow cytometry application that examines the uptake of beads coated with collagen type I under different fibrotic cell culture models.

Principal of the Technique

Collagen coated bead phagocytosis assay to assess collagen phagocytosis

Here, as a proof of principal we used rat embryonic fibroblasts (REF52) to examine phagocytosis of collagen type I coated fluorescent microspheres. Red fluorescent latex beads (maximally excited at 580 nm but excited using the 488 nm laser on the standard CytoFLEX) coated with collagen were incubated with REF52 cells for 10, 30 minutes and 1 hour to assess bead uptake by cells as a measure of collagen phagocytosis, the bead signal was detected in the 660/20 filter.

The use of a flow cytometry approach to quantify cellular phagocytosis is beneficial as it allows for a quick assessment of cellular uptake in a large number of cells and can be applied to other cell types such as macrophages or neutrophils ('professional' phagocytic cells).

Protocol

Cell Line and Cell culture

REF52-WT cells (rat embryonic fibroblasts, W. Topp, Cold Spring Harbor Laboratory) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum and 1 % Penicillin/Streptomycin.
In preparation for the experiment REF52 cells were seeded at a density of $1.5x10^5$ cells per 60 mm dish and cultured overnight.

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Bead Coating with Collagen type I

Stock 1 µm red FluorSphere beads 2% w/v (bead volumes must be individually optimized) were washed three times in PBS supplemented with 0.02 % TX-100. Following the washing step beads were re-suspend in Collagen type I solution (1 mg/mL) and incubated at 37°C for 1.5 h, with frequent agitation. Collagen coated beads were then washed, resuspended in DMEM (serum-free), sonicated and used immediately.

Flow Cytometry for Bead Endocytosis

Beads were added to the cells drop wise and incubated for 10, 30 min and 1 h. Following incubation with collagen coated beads cells were washed twice with PBS, trypsinized with 0.25 % trypsin-EDTA, and collected by centrifugation. Cell pellet was then washed with PBS, resuspend in ice-cold PBS containing 2 % FBS. Samples were then strained into a 5 mL polystyrene round bottom tube with a cell strainer cap (Falcon), samples were then analyzed on CytoFlex (Beckman-Coulter), red laser, 660/20 filter configuration.

Materials & Methods

- DMEM (Gibco-BRL) (supplemented with 10 % FBS + 1 % Penacillin/Streptomycin)
- 0.25 % Trypsin-EDTA (Gibco-BRL)
- 1xPBS (Gibco-BRL)
- 1 µm red FluoroSphere beads, 580/605 (excitation/emission) (Molecular Probes)
- Collagen type I (Sigma)
- 5 mL polystyrene round bottom tube with cells strainer cap (Falcon)

Tissue Culture source:

Sample Type (include cell line information if available)	Species	Age of specimen (if available- or time since prep)	Prep Method
REF52-WT	Rat	Unknown, immortalized cell line	As described in material and methods

Result

The use of a flow cytometry approach for the quantification of collagen I coated bead uptake is a rapid and useful approach to examine collagen clearance in cells undergoing fibrotic differentiation. In combination with other techniques it will provide important information about the mechanism and kinetics of this integral component of wound healing and repair process.

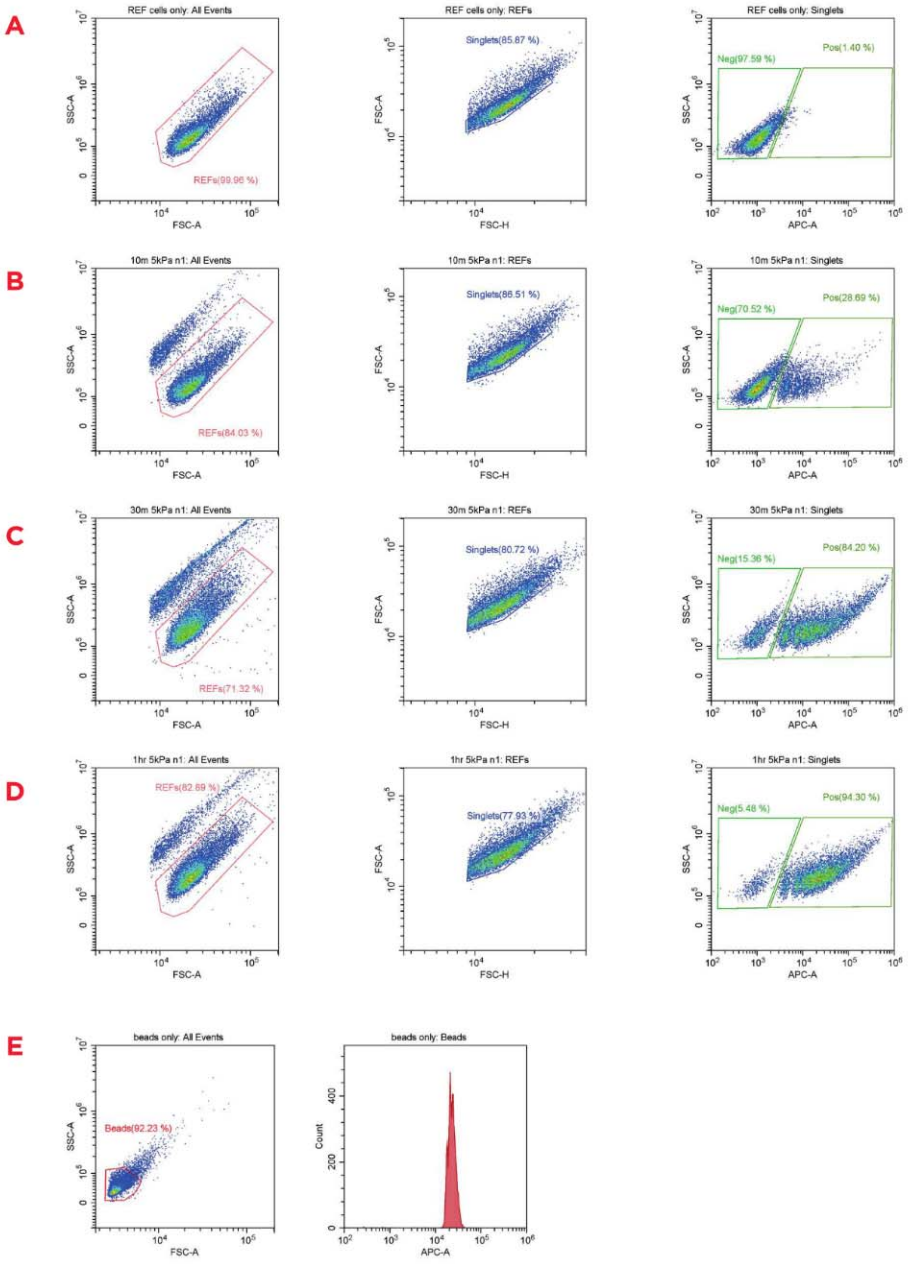

Figure 1: Time course of fluorescent bead phagocytosis by REF52 cells, (beads were coated with Collagen I).

The uptake of fluorescent beads by REF52 cells was identified through hierarchical gating by first selecting the total cell population (SSC-A vs. FSC-A), then by gating on single cell events (FSC-A vs. FSC-H), finally using the (SSC-A vs. APC-C) parameter to separate negative vs. positive population of cells for bead uptake.

A- REF52 cells only, time course:

B- 10min.

C- 30min.

D- 1h.

E- Beads only control.

Phagocytosis of FITC labelled opsonized and non-opsonized *E. coli* bacteria by monocytes and granulocytes in a whole blood assay

APPLICATION NOTE

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IN THIS PAPER YOU WILL

- Learn how to use flow cytometry to evaluate phagocytosis in monocytes and granulocytes.
- Learn optimal E. coli to cell ratios and assay conditions to make data interpretable.
- Learn how easy it is to use the compensation library feature enabled by CytoFLEX flow cytometer fiber array diodes
- Step-by-step instructions for instrument set up for acquiring multiparameter data

Principal of the Technique

Background

Phagocytosis is a process by which cells ingest or engulf particles or other cells. It is an essential part of the innate immune response and therefore necessary for defensive reactions against invasion of the body by foreign antigens. Neutrophilic granulocytes are the first defense line against invading microorganisms. They have a high capacity to engulf bacteria and to destroy these particles by intracellular mechanisms such as oxygen radical production. Circulating monocytes are also capable to ingest bacteria and other particles. In addition to neutrophils monocytes have the possibility to present small peptides of the digested particles on their surface in combination with products of the major histocompatibility complex (MHC).

Neutrophils and monocytes express on their surfaces receptors which make the recognition and the ingestion of with immunoglobulin and/or complement coated particles more effective. However changes of the phagocytic activity can lead to reduced phagocytic capacities and therefore to dramatic clinical situations. For example defects in the expression of complement receptors on granulocytes and/or macrophages can lead to a reduced phagocytic activity and subsequently to recurrent infections in these patients. [1] In patients with long lasting infections the monocyte fraction might be exhausted which also leads to a diminished phagocytic activity.[2] In neonatal children it has been reported that the phagocytic capacity of monocytes is reversely dependent on gestational aging and that the appearance of sepsis correlates with a massively diminished monocyte phagocytic capacity.[3,4] On the other hand at the beginning of infections from time to time an enhancement of phagocytosis can be observed.[5] Therefore the determination of the phagocytic capacities of both, circulating monocytes and neutrophils give a profound insight in the function of the innate immune system.

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Research Applications

Introduction

Flow cytometry is an ideal method to determine the phagocytic activity of neutrophils and monocytes in whole blood. The method is easy to perform and provides results within 90 minutes. The test kit used here has been previously developed without the possibility to additionally stain surface antigens. In the original test kit quenching solution is provided to eliminate the fluorescence signal which is set free from the FITC dye of *E. coli* bacteria which are bound to the cell surface but not ingested into the cell. The staining with antibodies has the big advantage that populations can be much better discriminated instead of gating via the scattergram. In the following protocol non-opsonized and opsonized *E. coli* bacteria were used and compared. *E. coli bacteria* which are opsonized are coated with immunoglobulin and complement. This allows better phagocytic properties than using non-opsonized *E. coli* bacteria. However using non-opsonized particles give the plainest results since these particles have to be first opsonized by the immunoglobulins from the circulating blood before particles can effectively be ingested. When opsonized particles are used the best phagocytic starting conditions are provided to the cells which possibly might not be relevant in the overall context.

Protocol

Standard Procedure

1. Draw heparinized whole blood and process the blood immediately.
2. Precool 500 µL of whole blood on ice for 30 minutes to delimitate the metabolism of the cells.
3. Prepare two flow tubes (75 mm x 12 mm) and put them on ice. Label one with '+' (positive sample) and the other one with '-' (negative control on ice).
4. Enumerate leukocytes per lab method: ViCell, Hemacytometer, or automated instrumentation.
5. Add 100 µL of precooled whole blood to each tube and incubate with 20 µL of opsonized or non-opsonized FITC labeled E. coli bacteria for precise 20 minutes at 37°C (positive sample) and on ice (4°C, negative control).
   Important: The E. coli flask has to be rigorously vortexed before usage. The concentration of E. coli bacteria is calculated for the normal leukocyte count (4,000 – 10,000 leukocytes/µL). Please adjust *E.coli* concentration and/or whole blood when leukocyte count is out of range.
6. After 20 minutes incubation time put the positive sample immediately back on ice.
7. Blood is then stained for 20 minutes on ice with anti-CD45 KrO ,anti-CD14 PC7, anti-CD3 APC and anti-CD56 PE antibodies (all antibodies from Beckman Coulter, Table 1).
8. Red cells are lysed (VersaLyse Lysing Solution, Beckman Coulter)
9. Cells are washed three times with PBS without Ca^{2+}/Mg^{2+} at 350g for 5 minutes (Allegra X-12R Centrifuge, Beckman Coulter) .
10. Cells are immediately analyzed on a CytoFLEX with standard instrument setup and with standard filter configuration.

Table 1

Laser	405nm					488nm					638nm		
Fluor	Krome Orange	Pacific Blue	V610	V660	V780	FITC	PE	ECD	PC5	PC7	APC	APC-A700	APC-A750
Marker	CD45						CD56			CD14	CD3		
Clone	J.33						N901 (HLDA6)			RMO52	UCHT1		

Results

Whole blood was incubated and stained as described above. Cells were immediately analyzed on a CytoFLEX. For opsonized and non-opsonized E. coli phagocytosis the gating strategy was the same which is described as follows. In a first step CD45 positive leukocytes were gated as shown in Figures 1 and 2a; Figures 1 and 2b are then gated on CD45+ events. Subsequent gates were set around the CD14+ monocyte population (CD14+), around the granulocytes (Granulocytes) and around the lymphocytes (Lympho Gate). CD14+ monocytes are then the parent populations for the histograms in Figures 1 and 2c. To demonstrate the phagocytic capacity of these cells, an overlay was created which includes the negative population incubated at 4°C (Fig. 1 and 2d). The same was done with the granulocytes as depicted in Figures 1 and 2e and 1 and 2f, respectively. Subsequently the gated lymphocytes were transferred to a new dot plot (Fig. 1.2g) and gates were set according CD3+ and/or CD56+ cells. CD3+/CD56- are T-lymphocytes, CD3-/CD56+ cells are Natural Killer cells (NK cells) and CD3+/CD56+ cells are cytotoxic T-cells. In Figure 1,2h (CD3+ cells), Figure 1/2i (NK cells) and Figure 1,2k (cytotoxic T-cells) only few positive FITC signals are seen which are either due to phagocytosis and/or due to E. coli bacteria bound on the cell surface. As expected the phagocytosis of non-opsonized particles was much lower than of opsonized particles. This effect displays the necessity of the whole blood to opsonize first the bacteria with immunoglobulins and/or complement before they can effectively ingested.

Conclusion

Flow cytometry is a valuable tool to evaluate the innate immune system; however, there are several critical points to consider. Whole blood phagocytosis only works well when heparin is used as anti-coagulant during blood collection. Precooling of the blood is necessary to get a negative control. Also the exact E. coli incubation time (20 minutes), the exact temperature condition (37°C) and the E. coli to leukocyte ratio are indispensable otherwise data obtained are difficult to interpret or repeat. If all critical points are carefully taken into account the E. coli phagocytosis is a functional assay which delivers excellent results and provides profound insight into the innate immune system.

References

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2. Xiu F, Jeschke MG. Shock. 2013, 40(2):81-8.
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4. Hallwirth U, Pomberger G, Pollak A, Roth E, Spittler A. Pediatr Allergy Immunol. 2004, 15(6):513-6
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Notes

The results demonstrated in this application sheet represent those generated on the Beckman Coulter CytoFLEX Flow Cytometer. As differences exist in the performance between analyzers, the author cannot guarantee a similar appearance with the use of other flow Cytometers.

Reagent Details

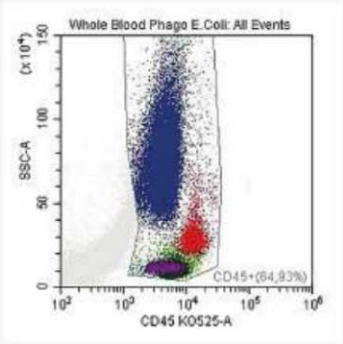
Reagent	Supplier	Order Details
Phagotest	GLYCOTOPE Biotechnology	GLYCOTOPE BIOTECHNOLOGY GmbH Czernyring 22 69115 Heidelberg Germany

Vortex mixer
Water bath for 37°C positive sample incubation
Digital thermometer to control the temperature of the water bath

Figure 1:

Phagocytosis of FITC labelled and **opsonized** *E. coli* bacteria in whole blood

1a



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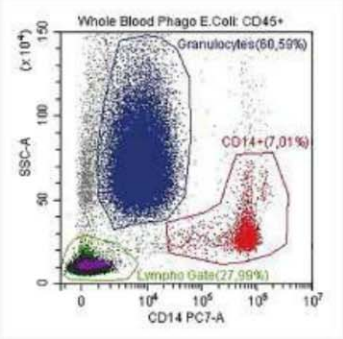

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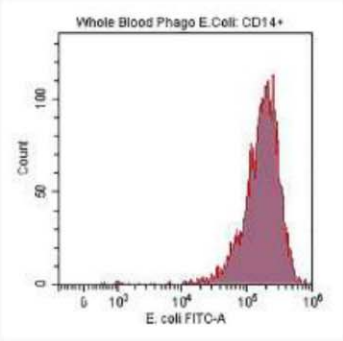

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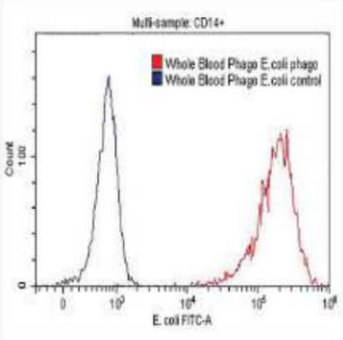
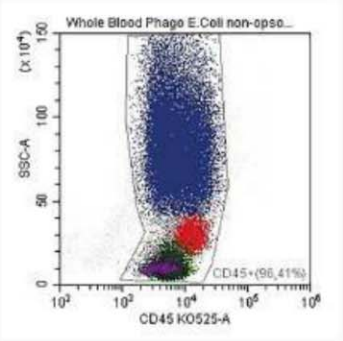


Figure 2:

Phagocytosis of FITC labelled and **non-opsonized** *E. coli* bacteria in whole blood

2a



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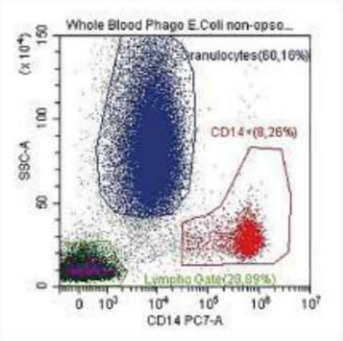

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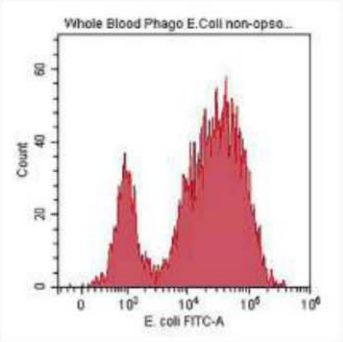

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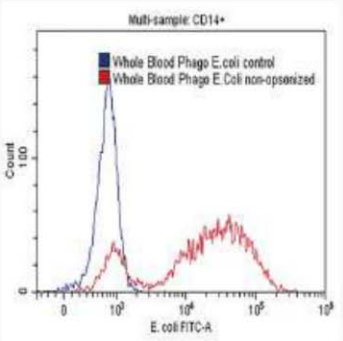
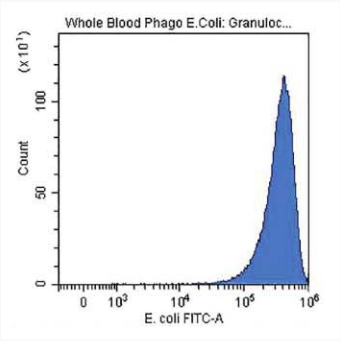


Figure 1:

Phagocytosis of FITC labelled and **opsonized** *E. coli* bacteria in whole blood

1e



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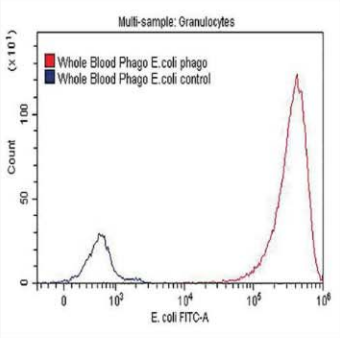

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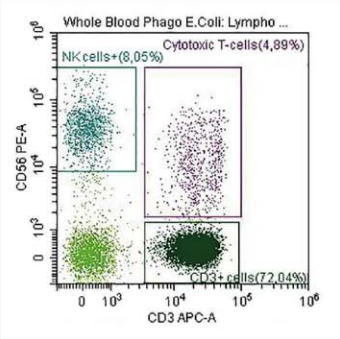

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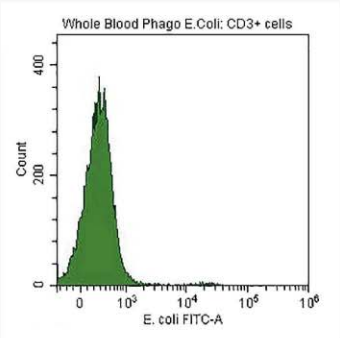
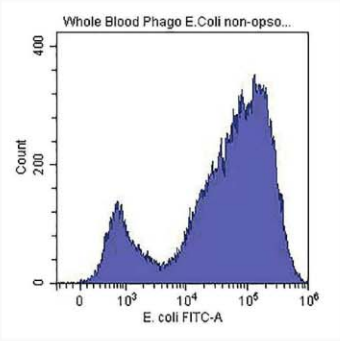


Figure 2:

Phagocytosis of FITC labelled and **non-opsonized** *E. coli* bacteria in whole blood

2e



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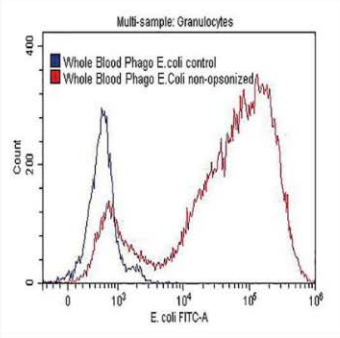

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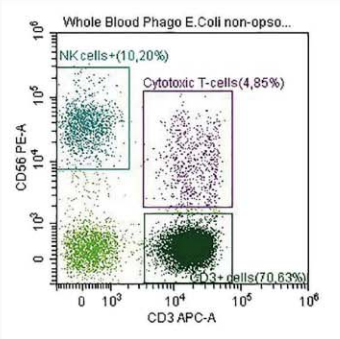

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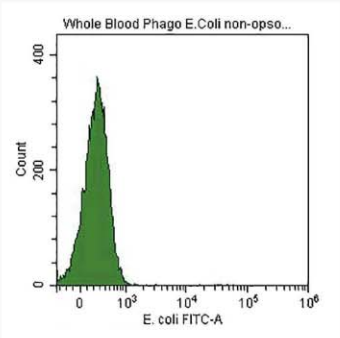
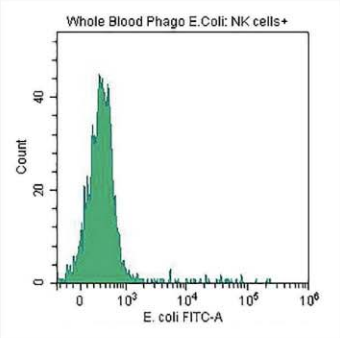


Figure 1:

Phagocytosis of FITC labelled and **opsonized** *E. coli* bacteria in whole blood

1i



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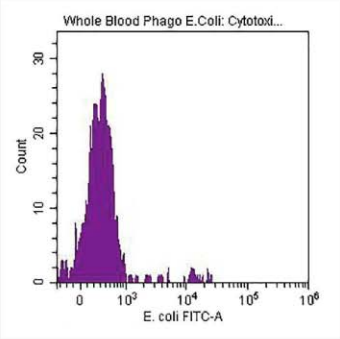
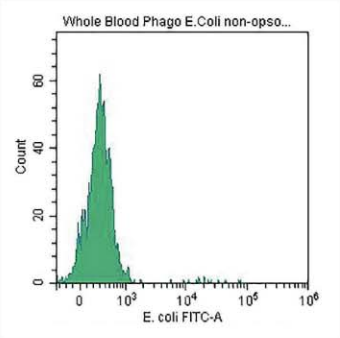


Figure 2:

Phagocytosis of FITC labelled and **non-opsonized** *E. coli* bacteria in whole blood

2i



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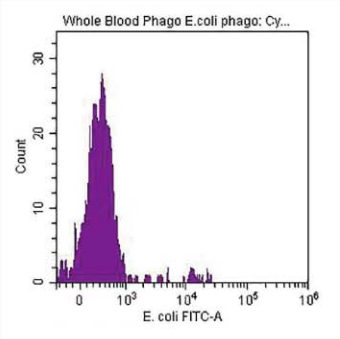

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Detection of Autophagic Vesicle Formation in Leukemia Cell Lines by Flow Cytometry

APPLICATION NOTE

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IN THIS PAPER YOU WILL LEARN

- To be introduced to autophagy
- How to stain autophagic vessicles for measurement by flow cytometry
- A gating strategy to quantify autophagic vessicles

Principal of the Technique

Background:

Autophagy is a cellular phenomenon that controls organelle and protein degradation essential for the regulation of cell survival, growth, development and homeostasis. Initially thought of as merely a mechanism whereby cells dispose of cellular junk, autophagy is now recognized as a mechanism by which the cell removes misfolded and long-lived proteins, damaged organelles, infectious microorganisms but also has diverse roles as an adaptive response to provide nutrients and energy upon activation by stress stimuli, and suggested involvement in cytokinesis. Autophagy is a dynamic process that is typically measured indirectly by the intracellular cleavage of the LC3 protein by western blot. Here we provide a simple and rapid assay to monitor autophagy dynamics in leukemia cells by flow cytometry, via the direct staining of autophagosomes with the fluorescent dye Cyto-ID.

Introduction:

Autophagy has been identified as an important process in cancer, with early studies suggesting impaired autophagy associated with tumorigenesis [1]. The question as to why a cellular process which is known to be essential for cell survival is also tumor suppressive. One potential explanation is that autophagy prevents death from necrosis in apoptosis-deficient cells [2]. Also, cells in which autophagy is impaired accumulate damaged mitochondria, reactive oxygen species (ROS) and protein aggregates that all may result in DNA damage, oncogene activation and tumorigenesis [3].

Thus the interest in autophagy regulation and its contribution to cellular homeostasis is disease is understandable. It is likely that the role of autophagy in individual cancers is extremely context-dependent. Thus being able to monitor autophagy on a single cell basis will allow for greater precision in elucidating the precise signaling dynamics of this fluid process in cells. The autophagic dye monodasylcadaverine (MDC) has previously been used in

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fluorescent microscopy but requires a 365 nm excitation source. The Cyto-ID dye is more convenient, being compatible with flow cytometers equipped with a blue laser (488nm). Cyto-ID has been shown to have excellent specificity for autophagosome staining, with only very weak staining of lysosomes.

Standard Procedure:

OCI/AML-3 cell lines were maintained at a culture density of $1x10^5$ – $1x10^6$ cell/mL in 10 mL of alpha-MEM medium supplemented with 10% fetal calf serum (FCS, *v/v*), 100 units of penicillin per mL and 100 µg of streptomycin per mL at 37°C and 5% CO_2.

The U937 cell line was maintained at a culture density of $1x10^5$ – $1x10^6$ cell/mL in 10mL of RPMI 1640 medium supplemented with 10% fetal calf serum (FCS, *v/v*), 100 units of penicillin per mL and 100 ug of streptomycin per mL at 37°C and 5 % CO_2.

1. Cell lines were cultured to a density of $5x10^5$ cells/mL and 2 mL of these cells were collected in 15 mL centrifuge tubes
2. Positive controls were generated by starving cells in culture medium with 0.5 % FBS for 6 hours, to induce autophagic vesicle formation
3. Cells were centrifuged at 400x g for 5 minutes
4. Supernatant medium was removed
5. Cells were washed with 1 mL of PBS
6. Cells were resuspended in 250 µL of PBS containing 5 % FBS
7. To this, 250 µL of Cyto-ID staining solution was added (see reagent list)
8. Cells were incubated for 60 minutes at 37°C
9. Cells were centrifuged at 400x g for 5 minutes
10. Cells were washed with 1 mL of PBS
11. Cells were resuspended in 250 µL of PBS, and placed on ice
12. Autophagic vesicle quantitation was measured via fluorescence on the FITC channel
13. Cells were analysed for fluorescence on a CytoFLEX flow cytometer (Beckman Coulter)
14. Cells were gated on FSC *vs.* SSC to identify the correct, viable cell population.
15. Gated cells were further gated on FSC-area vs. -height to discriminate singlet cells from doublet cells
16. Analysis of Cyto-ID fluorescence was done using overlay histograms of singlet cells

Reagents:

alpha-MEM medium (supplemented with 10% fetal calf serum (FCS, *v/v*), 100 units of penicillin per mL and 100 µg of streptomycin per mL). RPMI 1640 medium (supplemented with 10% fetal calf serum (FCS, *v/v*), 100 units of penicillin per mL and 100 µg of streptomycin per mL). Cyto-ID staining solution: 1 µL of Cyto-ID detection reagent in 1000 µL of 1X Assay buffer, supplemented with 5% FBS.

Laser	405nm					488nm					638nm		
Fluor	Krome Orange	Pacific Blue	V610	V660	V780	FITC	PE	ECD	PC5.5	PC7	APC	APC AF700	APC AF750
Marker						Cyto-ID							

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Results

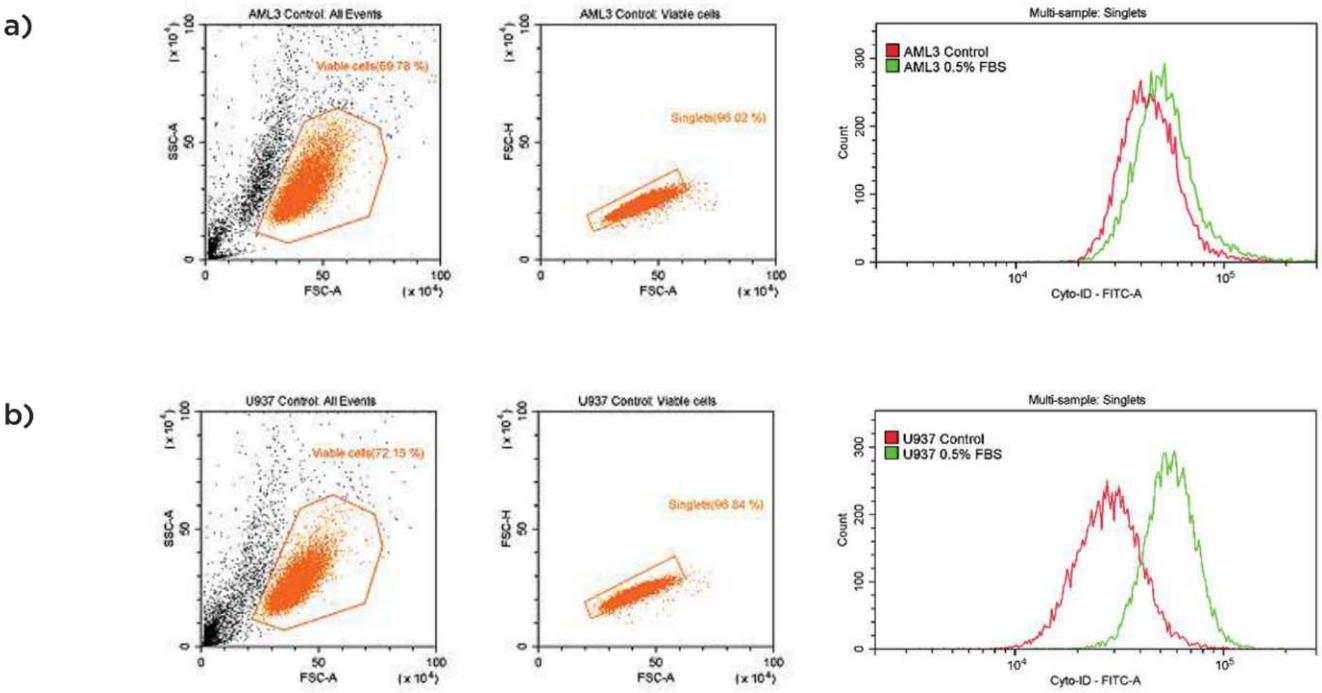

Figure 1: Detection of Autophagic vesicle formation in Leukemia cell lines by Flow Cytometry.

a) Gating strategy for OCI/AML-3 cells viable cell population on a FSC *vs.* SSC dot-plot (upper left). Viable cells were further gated to remove possible doublet cells on a dot-plot for FSC-area *vs.* -height (upper right). Autophagic vesicles were quantified via Cyto-ID fluorescence in the FITC-A channel and plotted as overlayed histograms. Autophagy levels in OCI/AML-3 cells cultured for 6 hours, in the presence of 10 % FBS (red histogram) or 0.5% FBS (green histogram) is shown (bottom).

b) Gating strategy for U937 cells viable cell population on a FSC *vs.* SSC dot-plot (upper left). Viable cells were further gated to remove possible doublet cells on a dot-plot for FSC-area *vs.* -height (upper right). Autophagic vesicles were quantified via Cyto-ID fluorescence in the FITC-A channel and plotted as overlayed histograms. Autophagy levels in U937 cells cultured for 6 hours, in the presence of 10 % FBS (red histogram) or 0.5 % FBS (green histogram) is shown (bottom).

Autophagic vesicle formation is measured by fluorescence in the FITC channel on the Cytoflex. The Cyto-ID reagent has an excitation peak of 463 nm and an emission peak of 534 nm. We used the dye here to examine the effect of starvation on the induction of autophagy in two leukemia cell lines, namely OCI/AML-3 and U937. Cells were starved for 6 hours, by serum withdrawal and compared to control cells grown in full serum. In both cell lines we observed small amounts of apoptosis, as seen by an anti-clockwise shift in cells on the FSC vs. SSC plot. However, the bulk of cells remained in the main population. These cells were gated for singlet cells, FSC-area vs. -height and then plotted for Cyto-ID fluorescence on histogram overlays. From this we can see that starvation in both cell lines increases the fluorescent signal in the FITC channel in the order of a half-log shift to the right. Interestingly, we see that the basal autophagic level of U937 is lower than that of OCI/AML-3. Also, after treatment of both cell lines with serum-depleted medium resulted in a much greater increase in fluorescence in U937 cells. This may be due to their lower baseline levels of autophagic vesicles to begin with. This shows that we can quantify levels of autophagomes with cells, as well as the dynamic increases in these vesicles in cell culture.

FLOW-1111APP09.15-A

References

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3. Mathew R, Kongara S, Beaudoin B, Karp CM, Bray K, Degenhardt K, Chen G, Jin S, White E. Genes Dev. 2007 Jun 1;21(11):1367-81. Autophagy suppresses tumor progression by limiting chromosomal instability.

Reagent Details

Reagent	Supplier	Order Details
Cyto-ID Autophagy Detection Kit	Enzo Life Sciences	ENZ-51031

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Neutrophil Oxidative Burst Assay: A Dihydrorhodamine (DHR) based testing of Chronic Granulomatous Disease (CGD) with CytoFlex Flow Cytometer

APPLICATION NOTE

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2. Beckman Coulter Inc., Miami, FL
3. Stony Brook University School of Medicine, Stony Brook, NY

IN THIS PAPER YOU WILL LEARN

- To be introduced to a method to measure oxidative burst in eukaryotic cells
- Proper controls for an oxidative burst assay
- A method to quantitate oxidative burst by flow cytometry

Principal of the Technique

Background:

Chronic granulomatous disease (CGD) is an inherited disease that leads to recurrent life threatening infections and widespread granulomatous inflammation in tissue [1]. This inherited disorder is caused by the deficiencies in oxidative burst of neutrophils. The neutrophils of individuals with this condition are unable to assemble nicotinamide dinucleotide phosphate (NADPH) oxidase complex which leads to difficulty in forming superoxide anions (O_2^-) and reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2). The lack of proper ROS production leads to deficiencies in phagocytes ability to kill pathogens as well as inflammatory damage to the individual's tissue.

Introduction:

The nitroblue-tetrazolium (NBT) test is the original and most commonly used test to diagnose this condition. However, the dihydrorhodamine (DHR) based flow cytometry test for CGD is more sensitive, less laborious, and is the method of choice. DHR Flow Cytometry test is an indirect detection of the reduced levels of ROS, specifically hydrogen peroxide. In this application note, the ease and robustness of this assay is showcased by using a CytoFLEX Flow Cytometer.

Procedure:

Dihydrorhodamine123 (DHR123) (Molecular Probes, Eugene, OR) (2500 µg/mL in DMSO) is diluted to 15 µg/mL with phosphate buffered saline with azide (PBA)(Ca and Mg free with 2.5 % bovine serum albumin and 0.2 % sodium azide). The phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St Louis, MO) (100 µg/mL in DMSO) is diluted to 300 ng/mL with PBA.

Three tubes are set up for each sample:

1. Blood only
2. Blood + DHR
3. Blood + DHR + PMA

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To all tubes, 100 µL of heparinized blood is diluted 1:10 with PBA. 25 µL DHR123 (375 ng/ml final concentration) is added to tubes 2 and 3. All tubes are incubated in a 37°C water bath for 15 minutes. This allows for the DHR123 to be loaded into the cells. Following incubation, 100 µL of the prepared PMA solution (30 ng/mL final concentration) is added to tube 3 then all tubes are incubated an additional 15 minutes at 37°C. This step allows the neutrophils to undergo the oxidative burst thereby oxidizing the DHR123 to rhodamine which fluoresces when excited by 488 nm laser. After washing and centrifugation, the samples were stained with anti-human CD45 Krome Orange antibody (Beckman Coulter, Miami, FL) according to manufacturer's recommendations and lysed with ammonium chloride (Pharm Lyse, Becton Dickinson, Mountainview, CA) for 10 minutes in the dark, followed by centrifugation, washing, and fixing in 1 % formalin. The samples are then acquired using a CytoFLEX Flow Cytometer (Beckman Coulter, Miami, FL) and subsequently the data was analyzed using CytExpert Software (Beckman Coulter, Miami, FL). Fluorescence is quantitated by mean peak channel fluorescence. Results are expressed as oxidative index of neutrophils which is the ratio of mean fluorescence of PMA to mean fluorescence of unstimulated sample. Oxidative index values over 100 are considered normal, while values below 100 are considered abnormal. Although neutrophils (found in the granulocyte gate of the SSC vs CD45 dotplot) are the cells of interest when studying oxidative burst, we are able to identify and gate internal controls for oxidative burst from the same dotplot: monocytes, which serve as low-level controls and lymphocytes which serve as negative controls, lacking this enzyme system activity. In addition to these gating controls strategies, a blood only control is also run for each assay to discern autoflourescence. The DHR flow cytometry test can detect CGD patients, carriers, and can suggest the genotype of the CGD patients.

Laser	405nm					488nm					638nm		
Fluor	Krome Orange	Pacific Blue	V610	V660	V780	FITC	PE	ECD	PC5.5	PC7	APC	APC AF700	APC AF750
Marker	CD45					DHR							

Results

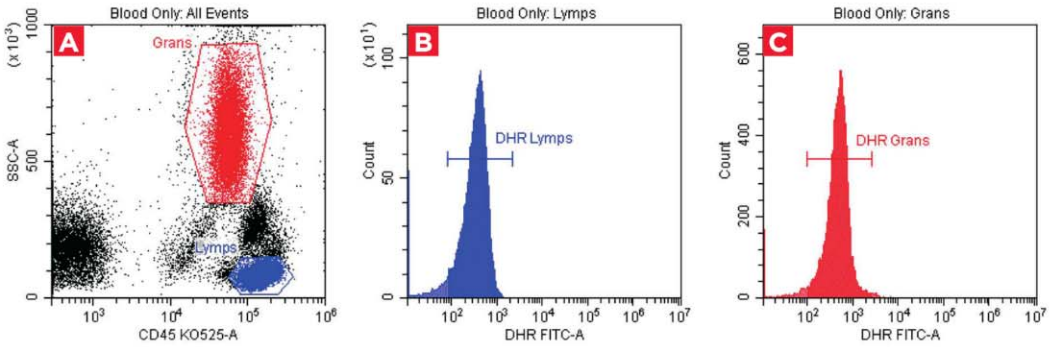
Oxidative burst is the term used to describe the phagocytic response of neutrophils to produce ROS. DHR flow cytometry assay can detect reduced oxidation of dihydrorhodamine, making it a very robust assay to measure the oxidative burst of neutrophils. When oxidized, dihydrorhodamine123 is converted to rhodamine123 which then fluoresces when excited by a 488nm laser. In normal blood, the oxidative burst of neutrophils can be triggered by incubation with PMA. While neutrophils show increased oxidative burst with PMA stimulation, lymphocyte populations lack the oxidative burst components making this population a good internal negative control.

In Figure 1, blood only samples are used as negative control for DHR staining. When we compare the mean fluorescence from this sample with blood + DHR sample, we did not detect any significant increase in either neutrophil gated or lymphocyte gated population. However, the increase in DHR fluorescence is evident in the PMA stimulated sample in the granulocyte population (I), while the lymphocytes show no sign of DHR fluorescence (H) upon PMA stimulation.

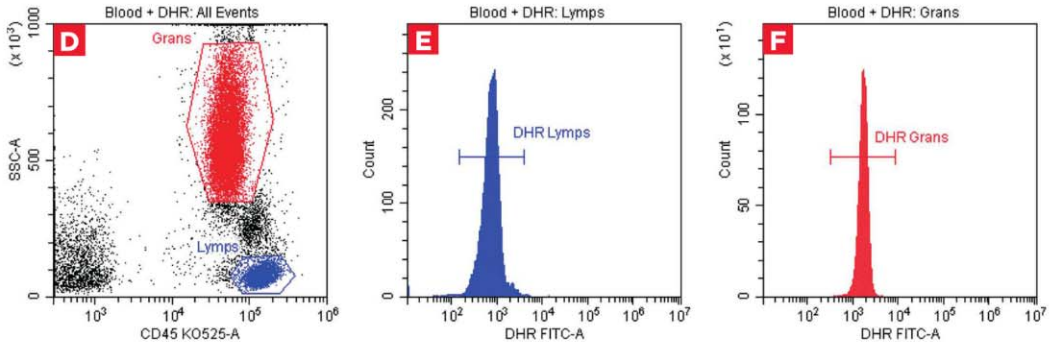
In Figure 2, the oxidative index of neutrophils is calculated by dividing the mean fluorescence of DHR in PMA stimulated sample by unstimulated sample. The result was 367.0, which is above the cutoff for normal functional ratio of 100. The oxidative index of lymphocytes is calculated by dividing the mean fluorescence of DHR in PMA stimulated sample by unstimulated sample. The result was 1.2 which is significantly below 100, which makes this population an adequate internal negative control.

FLOW-1115APP09 15-A

Blood only



Blood + DHR



Blood + DHR + PMA

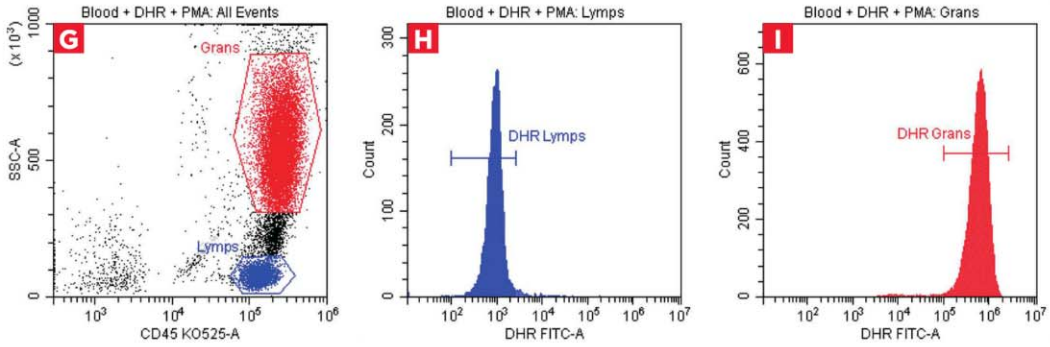


Figure 1:

Figures A-C display the data from Blood alone sample without any stimulants or DHR.

Figures D-F show the data from Blood + DHR sample stained with DHR without any stimulants.

Figures G-I show the data from Blood + DHR + PMA sample stained with DHR and in addition, stimulated with PMA.

In plots A, D, G; the SSC vs. CD45 plot is used to detect the 3 leukocyte populations; lymphocytes, monocytes and granulocytes. Neutrophil and lymphocyte populations are then gated. Lymphocyte gate is used to gate DHR histogram (B, E, H) to detect the DHR fluorescence. Neutrophil gate is used to gate DHR histograms (C, F, I) to detect the DHR fluorescence.

Tube Name: Blood Only

Population	% Total	Mean DHR FITC-A	CV DHR FITC-A
DHR Grans	20.87 %	542.8	55.05 %
DHR Lymps	39.55 %	390.3	47.94 %

Tube Name: Blood + DHR

Population	% Total	Mean DHR FITC-A	CV DHR FITC-A
DHR Grans	55.82 %	1764.3	24.40 %
DHR Lymps	19.01 %	823.8	48.36 %

Tube Name: Blood + DHR + PMA

Population	% Total	Mean DHR FITC-A	CV DHR FITC-A
DHR Grans	62.04 %	647401.1	41.66 %
DHR Lymps	21.41 %	952.7	37.25 %

	DHR Mean PMA stim	DHR Mean PMA unstim	Oxidative Index
Neutrophils	647401.1	1764.3	367.0
Lymphocytes	952.7	823.8	1.2

Figure 2:
The Oxidative Index of Neutrophils is calculated by dividing the mean fluorescence of DHR in PMA stimulated sample by unstimulated sample.

References

1. Golightly, Marc G. ICCS eNewsletter. 2011 Vol II(1)

Notes

The results demonstrated in this application sheet represent those generated on the Beckman Coulter CytoFlex Flow Cytometer. As differences exist in the performance between analyzers, the author cannot guarantee a similar appearance with the use of other flow cytometers.

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FLOW-1115APP09.15-A

The unique peristaltic sample delivery system of the CytoFLEX analyzer enables optimized measurements of transient changes in intracellular calcium in cells following agonist activation

APPLICATION NOTE

Authors:
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Affiliation:
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2 Beckman Coulter Inc., Miami, United States
3 Department of Pathology and Cell Biology, Columbia University Medical Center

IN THIS PAPER YOU WILL LEARN

- How to set up a flow cytometeric assay to measure changes in cellular of calcium
- How to adapt the CytoFLEX flow cytometer to make real-time measurements for cellular assays
- About a rapid screening method of calcium flux by flow cytometry

Principal of the Technique

Generation of fluorescent antibody or genetic labels to identify hormone and neurotransmitter receptor activity can be difficult and time consuming. A useful alternative is recording physiological changes in response to agonist binding its cognate receptor, many of which are G-protein coupled. When an agonist binds a G-protein coupled receptor, it triggers a quick cascade of events that often results in a transient release of calcium from intracellular stores. Alterations in transient intracellular calcium ($[Ca^{2+}]_i$) levels have been used previously in flow cytometry to identify functional receptor expression in cellular subpopulations [2], here we show that peristaltic sample delivery of the new CytoFLEX analyzer is particularly well suited to agonist-based calcium studies. Using the ester based, green fluorescent calcium indicator, Fluo-4 AM (Life Technologies), $[Ca^{2+}]_i$ changes were measured in HEK-293 cells in response to ATP stimulation. Simple plumbing modifications to the CytoFLEX allowed easier access to the sample tube for agonist application; further modifications were made to implement a "stop time" technique. By using response to agonist as our physiological criteria, we have fundamentally enabled receptor identification and conclusively demonstrated its functionality.

Materials and Methods

Calcium indicator and cell loading

5 x 10^6 HEK-293 cells were dissociated and re-suspended in 1.6 mL Dulbecco's phosphate-buffered saline (DPBS) containing 3 µM Fluo-4 AM [1]. The cells were incubated at room temperature in the dark for 20 minutes. Following incubation, cells were spun down and resuspended in 2 mL DPBS. 10 mM ATP was freshly prepared in DPBS and added directly to the cells during the experiment to reach a final concentration of 100 µM.

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CytoFLEX Configuration

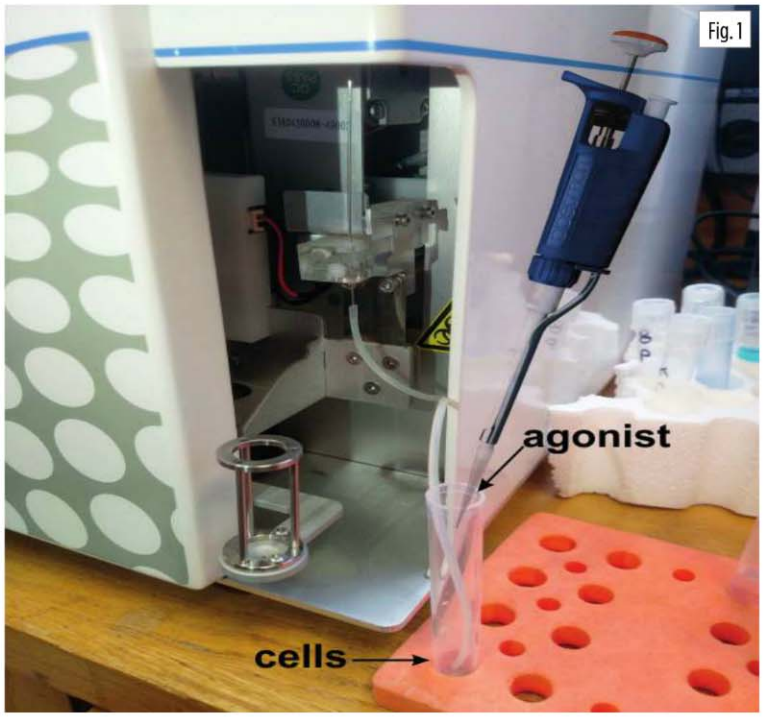
Laser WL	405nm					488nm					638nm		
Detectors	1	2	3	4	5	1	2	3	4	5	1	2	3
Fluorchomes						Fluo-4							

Parts

Vendor	Description	Part No.
Instech Laboratories, Inc.	0.30 mm ID silicone tubing	BTSIL-025
Instech Laboratories, Inc.	3-Way Y connector	SCY25

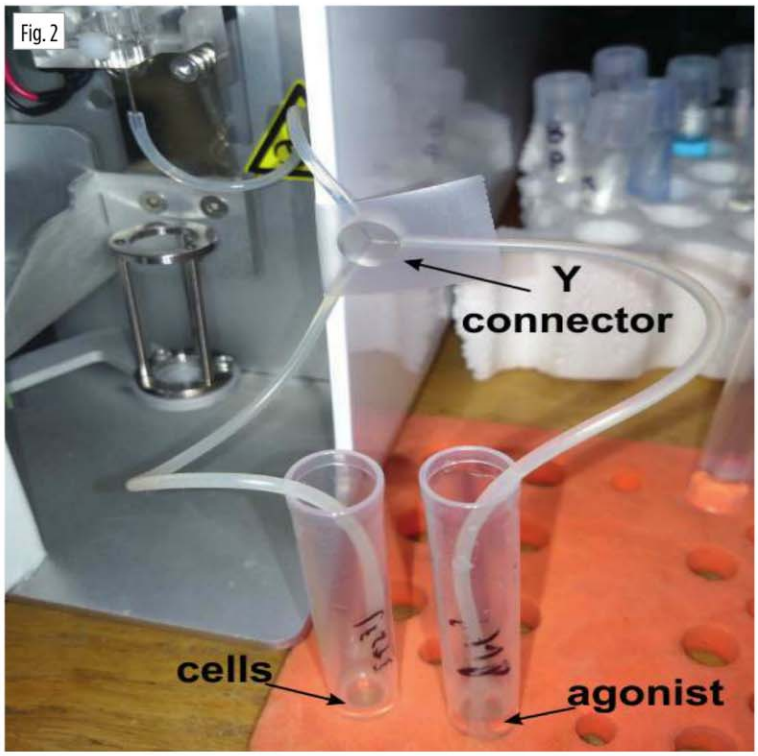
Time course setup

Modifications were made to the CytoFLEX unit configuration in order to be able to detect time sensitive data response. The modification and procedure have not been validated by Beckman Coulter. Howard Hughes Medical Institute, and the Department of Pathology and Cell biology at Columbia University Medical Center have found this to be a viable modification and methodology. A 16 cm long piece of silicon tubing with 0.30 mm internal diameter (ID) was placed onto the end of the CytoFLEX sample pick up probe. This additional tubing permitted easier access to the sample tube enabling agonist addition to the cell suspension during sample analysis. The CytoFLEX sample delivery was set to manual mode in order to implement these modifications. Additionally, this modification permitted the sample tube to be placed in a rack beside the instrument where agonist could be added without disturbing sample flow (Fig. 1).



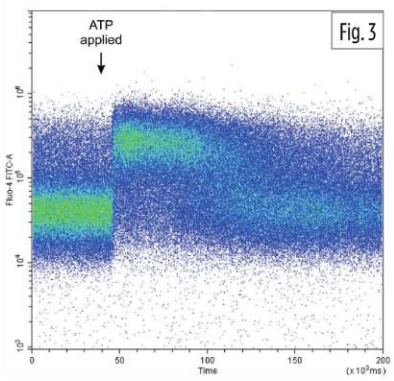
Stop time setup

A 'y' connector was constructed by imbedding 3 different 15 mm lengths of 26 gauge stainless steel tubing into a Plexiglas disk (a similar 'y' connector can be purchased directly from Instech laboratories, Inc.). One of these ports was connected to the sample probe with a 9 cm length of 0.30 mm ID silicon tubing [2]. Two additional pieces of silicon tubing were connected from the other two ports of the 'y' to the cell suspension and the agonist tubes respectively (Fig. 2). This setup created an environment where each analyzed cell had equal exposure time to agonist. The exposure time could be adjusted to capture the peak $[Ca^{2+}]_i$ transient by changing the length of the tubing that goes from the 'y' to the sample probe and by changing the peristaltic pump rate (differential pressure).

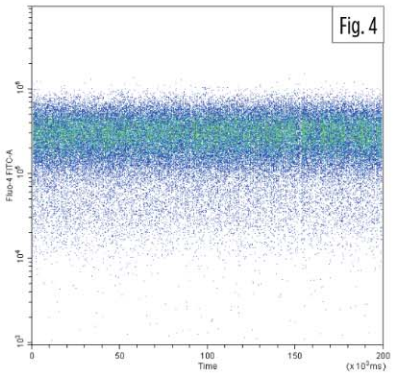


Results

When ATP was applied directly to the HEK-293 cell suspension using the setup in Fig. 1, the transient $[Ca^{2+}]_i$ shift was observed in a plot of time vs. Fluo-4 fluorescence. ATP application response was easily observed as untreated cells continue through the system and treated cells enter the system (Fig.3). The transient $[Ca^{2+}]_i$ shift starts to decrease significantly in approximately 25 seconds and returns to baseline levels over the next 90 seconds. Differential pressure was set to manual mode and set to approximately 75 % of maximum to achieve a short delay between agonist exposure and analysis.



When ATP was applied to the suspension of HEK-293 cells using the 'y' connector to create a fixed exposure time to agonist, virtually all of the detected cells were in the high $[Ca^{2+}]_i$ range (Fig. 4). This optimized "stop time" method allowed cells to maintain their peak $[Ca^{2+}]_i$ change during interrogation due to the cells having equal exposure to ATP. This method increases the sensitivity of detecting and analyzing small sub-populations of cells with the ATP receptor when compared to simply adding the ATP into the tube of cells (Fig. 3).



Discussion

The efficacy of the CytoFLEX peristaltic pump

Our laboratory has been performing $[Ca^{2+}]_i$ assays for many years using flow cytometers with pressurized sample delivery systems. These systems require a break in data collection because the sample pressure has to be stopped to add the agonist. It requires high dexterity to break the seal, add the agonist, and boost sample pressure to get it flowing before ability to detect the response decays. The peristaltic pump on the CytoFLEX maintains a continuous flow of cells and allows direct agonist addition to the open tube, enabling a more complete capture of the time course of receptor activation and deactivation of the cells. In some flow cytometers with large pressurized sample chambers it would be impossible to restart the cell flow quickly enough after agonist addition to detect a response.

Time course response of $[Ca^{2+}]_i$

There is valuable information in the time course of a Ca^{2+} response when investigating receptors on an unknown cell population. The rapidly decaying $[Ca^{2+}]_i$ response after agonist activation is typical of G-protein coupled receptors. A long and sustained $[Ca^{2+}]_i$ increase would be more typical of a receptor activating calcium permeable channels. With some agonists and cells both receptor types may be present and monitored in the data.

Continuous analysis of cells at a set time point of agonist exposure

The "stop time" method that locks agonist exposure time permits the continuous collection of data from cells at their peak $[Ca^{2+}]_i$ change in response to agonist (Figs. 2, 4). This controlled environment supports a more accurate assay for calculating percent of responsive cells and detecting small sub-populations of responsive cells that would normally be undetectable in the time course method.
This difference is illustrated in Figure 5 which displays data collected over a 200 second time course for each sample.

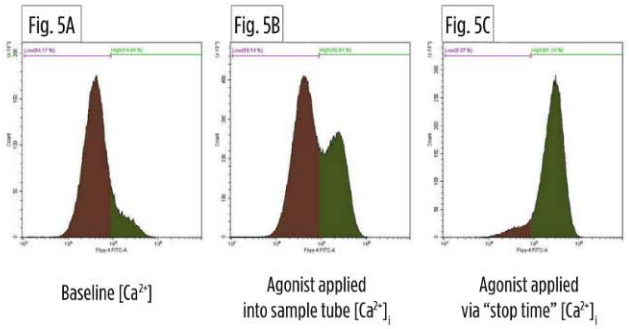


Figure 5A shows a histogram of cell number as a function of fluorescence representing $[Ca^{2+}]_i$ with no ATP added. A small portion of the total cell population has high $[Ca^{2+}]_i$, shown in green. In Figure 5B, ATP was applied as in Figures 1 and 3. There is a clear increase in the population of cells with elevated $[Ca^{2+}]_i$ collected. However, when ATP was applied using the "stop time" method shown in Figures 2 and 4, it becomes clear (Figure 5C) that the majority of cells have elevated $[Ca^{2+}]_i$ and thus are sensitive to ATP. Therefore, the "stop time" method, when optimized for the peak calcium response, is a more accurate test for measuring percent positive cells in a population.

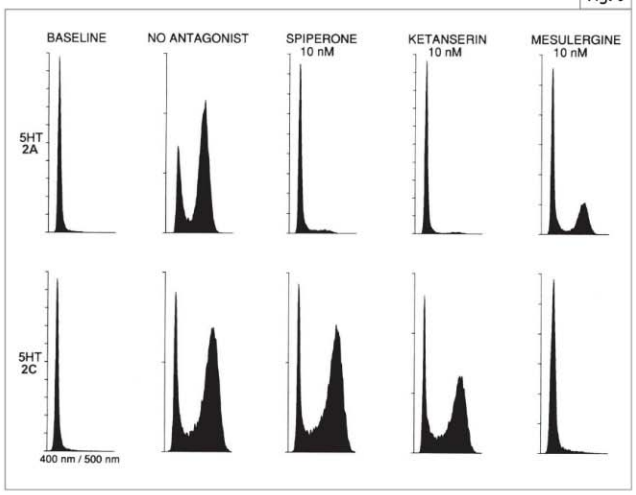
Applications

Identifying receptor subtypes

G-protein coupled receptors often have a variety of subfamilies within each receptor family. These subtypes can be identified by their different pharmacological profiles. CytoFLEX can be used to identify the specific subtypes of G-protein coupled receptor present on cells by testing the cell sample pre-incubated with selective antagonists for specific receptor subtypes. By evaluating which antagonists affect $[Ca^{2+}]_i$ responses to agonist, the receptor subtype can be determined.

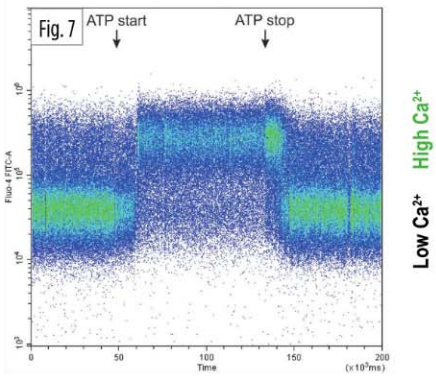
This is demonstrated in Figure 6 showing data previously acquired in this laboratory using the "stop time" method to distinguish the subtype of serotonin receptor transfected into a cell line [3].

Fig. 6



CytoFLEX, a rapid analysis screening tool

It was noted while experimenting with the CytoFLEX and the "stop time" method of ATP activation that we could clamp off the agonist pick up line and quickly see a return to normal $[Ca^{2+}]_i$ levels while the cells continued to run and analysis continued (Fig. 7). The ease of switching sample or agonist tubes while the analysis continues adds flexibility to the system and could be the basis of a fast screening method. Incorporating 96 well plate sampling with the plate loader would add even more efficiency. These simple fluidics changes create a sensitive, efficient and quick, screening assay for functional G-protein coupled receptors on cells.



References

1. Gee KR, Brown KA, Chen WN, Bishop-Stewart J, Gray D, Johnson I (February 2000). «Chemical and physiological characterization of fluo-4 Ca(2+)-indicator dyes». Cell Calcium 27 (2): 97–106.
2. Schieren I, MacDermott A (1988 Nov). "Flow cytometric identification and purification of cells by ligand-induced changes in intracellular calcium". J Neurosci Methods 26(1):35-44.
3. Schieren I, MacDermott AB (1992), Isolation and characterization of transfected cell lines expressing neurotransmitter receptors using a calcium dye and flow cytometry, Wood JN, ed, Neuronal Cell Lines, A practical Approach, Oxford University Press, Oxford. P. 161-179

Modifications described in the document have not been validated by Beckman Coulter Life Sciences.

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FLOW-940APP05.15-A.

APPLICATION NOTE

Flow Cytometric Measurement of CD62P Platelet Activation

Dr Kelly Brown, Jessica Tang, Stephanie Hughes | University of British Columbia, Child and Family Research Institute, Pediatrics Division of Rheumatology

Salima Janmohamed | Beckman Coulter Life Science, Inc.

IN THIS PAPER YOU WILL

- Caveats of platelet collection
- A simple single color measurement of platelet activation

Introduction

This assay uses flow cytometry to measure the expression of P-selectin (CD62P) on the surface of human platelets in whole blood both prior to and following ex vivo activation. The mobilization of CD62P from intracellular granules to the cell surface occurs rapidly in response to activating stimuli; for this assay the stimulus is ADP. Measurement of CD62P by this method may be used to assess platelet integrity and activation state, or simply to identify platelets within a mixed blood cell population or monitor non-specific activation of platelets introduced by a particular experimental method.

Materials - Equipment

- Human whole blood collected in Sodium Citrate Vacutainer (BD # 369714).
  Perform sample preparation as soon as possible after blood collection to avoid ex vivo activation of platelets.
- Anti-human CD62P / P-Selectin Antibody, PE conjugate (Psel.KO2.3, Life Tech #A16339).
- Adenosine diphosphate (ADP) (Chrono-Log Corp #384).
- Dulbecco's Phosphate Buffered Saline (1 x PBS pH 7.4, Gibco #14190-144).
- Polypropylene tubes (Fisher Scientific #: 02-681-200).
- Beckman Coulter CytoFLEX.

Working solutions

1 x PBS sterilized through 0.2 μm filter

CD62P-PE antibody

Prepare:

- 1:32 dilution of antibody from stock:
- 2 μL Ab + 62 μL 1xPBS = 64 μL of 1:32 dilution anti-CD62P-PE antibody

ADP

- Prepare 1 mM stock according to manufacturer's instructions then make a 100 μM working solution.
- 3 μL 1 mM ADP + 27 μL 1xPBS = 30 μL of 100 μM ADP.

Procedure

A. Blood collection

Collect blood aseptically by venipuncture using a 20-gauge needle. Collect the first ~2 mL of blood into any vacutainer; discard this sample as it contains activated platelets. Release tourniquet and collect an additional 3.5 mL of blood into sodium citrate tube; this sample will be used for staining.

B. Activation and Staining of Platelets

1. Prepare the working solutions (see above). Keep at room temperature.
2. Label polypropylene tubes for flow cytometry (12x75 mm) as follows in duplicate:
    1. No Ab, no ADP
    2. CD62P no ADP
    3. CD62P + ADP
3. Add reagents according to Table 1 to the appropriate tubes. Use a fresh tip for each addition and swirl tubes gently to mix.

4. Reagents to add to tubes:

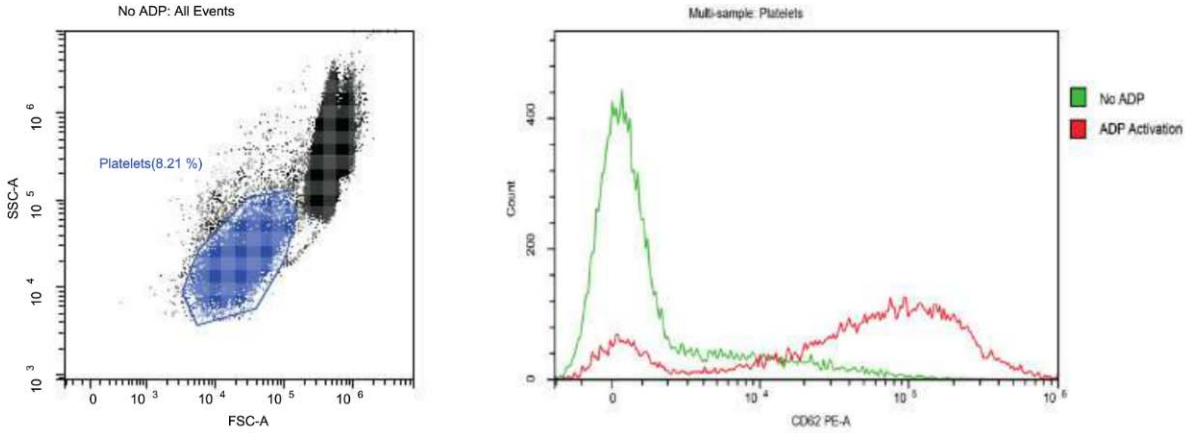
	No Ab, no ADP (tube 1)	CD62P, no ADP (tubes 2)	CD62P + ADP (tubes 3)
PBS	45 µL	34 µL	34 µL
Antibody	-	5 µL	5 µL
ADP	-	-	6 µL

5. Add 5 µL of whole blood to each tube, swirl gently to mix then incubate 30 min at room temperature in the dark.

6. Add 750 µL filtered 1x PBS to each tube.

7. Acquire data as soon as possible.

Results



Overlay of platelets stained with CD62P-PE untreated (green) and treated with ADP (red).

Data Acquisition and Analysis

Initial Hardware Settings

Approximate gains:

- FSC (log scale) - 40
- SSC (log scale) - 90
- FL2 (log scale) – 290

Approximate threshold (dual threshold on both FSC and SSC)

- FSC: 10,800
- SSC: 5200

Tips for Data Acquisition and Analysis

The activated platelet sample was acquired first in order to identify CD62P+ platelets.

A gate was drawn on CD62P+ cells on a CD62P histogram, and the gate was assigned a color.

- This color was used to help locate the platelets on the FSC vs SSC plot, and a tight gate ("platelets") was drawn around the platelets on this plot.
- The CD62P histogram was gated on the "platelet' events.

  Note: The more numerous events in the upper right corner of the FSC vs SSC plot are the leukocytes that will be excluded by this gating.

- The threshold was set such that debris was eliminated without cutting off any platelets.

A media-only control (PBS only) was also acquired to verify the ideal positioning of the threshold to block any background noise.

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FLOW-1911APP08.16

Detecting and counting bacteria with the CytoFLEX research flow cytometer: I-Characterization of a variety of gram-negative bacteria

APPLICATION NOTE

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Affiliation: Beckman Coulter

IN THIS PAPER YOU WILL LEARN

How to set up your CytoFLEX instrument to detect and enumerate bacteria

Principle of the Technique

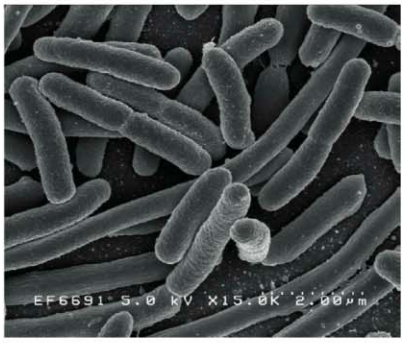
Background

Microbial detection and counting have application in multiple contexts, such as environmental and process monitoring. Flow cytometry offers the potential to identify and count many different species of bacteria in diverse media. With appropriate staining to permit resolution from background, it is possible to count even very low frequencies of bacterial load quite rapidly. Predicate methods can be fast but less accurate (e.g. nephelometry) or very slow by comparison (CFU—colony forming unit—count determination by culture typically takes 48 hours) and may require serial dilutions to encompass a wide range of potential concentrations, compared with a minute or less by FCM (flow cytometry).

Research Applications: Introduction

This is one of three associated notes, looking at a range of different bacterial species, both *coccus* and *bacillus* forms, and examining the capabilities of the CytoFLEX for their detection and enumeration. In this note, four representative gram-stain negative species are compared with negative control samples.

The second note in the series examines a range of gram-positive bacteria and the third note demonstrates count linearity over a wide range (6-log decades) of concentrations and flow rates.



1. E.coli SEM Public Domain
https://upload.wikimedia.org/wikipedia/commons/3/32/EscherichiaColi_NIAID.jpg

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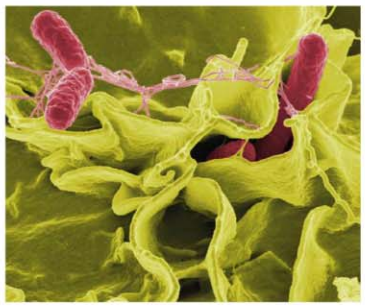
Gram-negative bacteria include many genera that are of economic importance: usually due to their pathogenicity as food contaminants (e.g., *Escherichia coli, Salmonella spp.*) or association with nosocomial disease and infection (e.g., *Acinetobacter baumannii*).



1. Salmonella SEM Public Domain
https://upload.wikimedia.org/wikipedia/commons/thumb/b/b4/SalmonellaNIAID.jpg/1024px-SalmonellaNIAID.jpg

Protocol

Standard Procedure

Cultures of each species of bacterium were assayed by nephelometry (densitometry) and resuspended in PBS to 0.5 McFarland units, or nominally 150 x 10^6 mL^{-1}, identified as [1x]. Aliquots were further diluted with PBS to 1/10x or 1/100x concentration, corresponding to nominal 15 or 1.5 x 10^3 μL^{-1}, respectively, before analysis on the CytoFLEX.

Samples were run unstained or labelled with the nucleic-acid staining SYTO vital dye mixture from the SYTO BC kit [Thermo Fischer Scientific]. Manufacturer's instructions call for use of this dye at 1 µL stock (in DMSO) per 1 mL of sample, but this was found to cause significant secondary staining in practice. Very satisfactory staining was achieved by creating a secondary stock solution at this same rate (1 µL primary stock in 1 mL of PBS) which was used at a rate of 5 µL per 500 µL sample aliquot, or 1 % of recommended concentration.

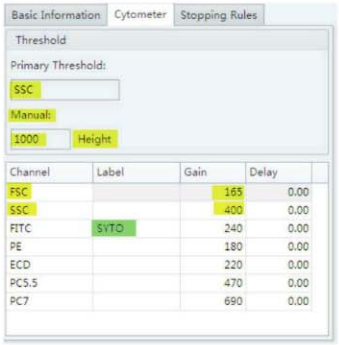
Instrument Configuration

Instrument Configuration	VSSC	Plate or Tube Format
Any: B4-R0-V0 and up	No	Either

Any standard CytoFLEX configuration can be used for this assay, as it only requires the blue laser for forward and side-scatter (FSC & SSC) and green SYTO fluorescence measured in the normal FITC channel.

Acquisition Settings

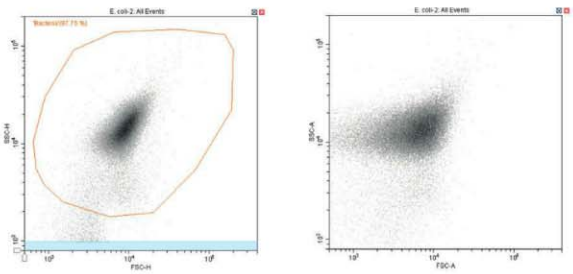
Since bacteria are smaller than most eukaryotic cells typically analyzed by FCM it is necessary to modify the gain settings for FSC (forward scatter) and SSC (side scatter) signals from the CytoFLEX default. Fluorescence gain is within normal range.



Fixed duration acquisitions are preferred over fixed count in order to assay a constant volume, and make any necessary background count subtraction corrections more straightforward.

Initial characterizations were performed at 10 µL per minute (Low) flow rate for 30 seconds, so a fixed volume of 5 µL per sample.

Best resolution of the bacterial populations by scatter was obtained by using height rather than area parameters, and by setting a manual threshold of 1,000 on SSC-H.

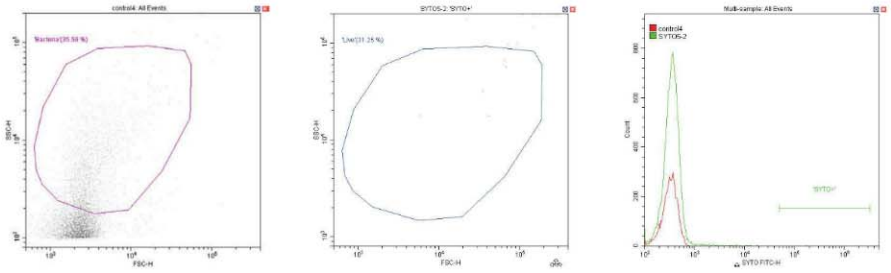


Preferred Log FSC-H vs log SSC-H (Height signals, left) showing manual SSC threshold at 1,000 compared with more typical Log FSC-A vs SSC-A (Area signals, right).

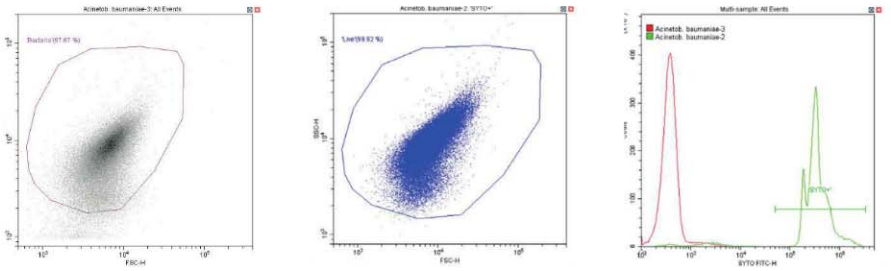
FLOW_1267APP11.15-A

Results

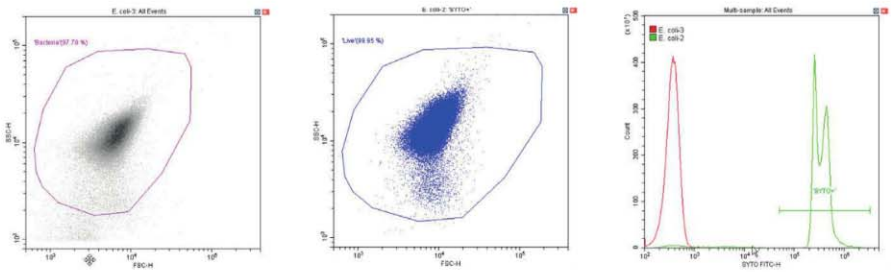
The following series of plots all show an ungated log-log scatter density plot, with a generic 'bacterial' gate (magenta). Except for the controls, this plot displays an unstained aliquot of bacteria.
The second plot is another dual log scatter plot, but this time a dot-plot, which allows color-gating to be shown. A second region is defined here as 'live' (blue) based upon the SYTO-positive population.
The last plot is a single parameter histogram overlay of green (SYTO) fluorescence, comparing unlabeled and labeled aliquots of the bacterium in question, with the 'SYTO+' region used to back-gate the second plot.



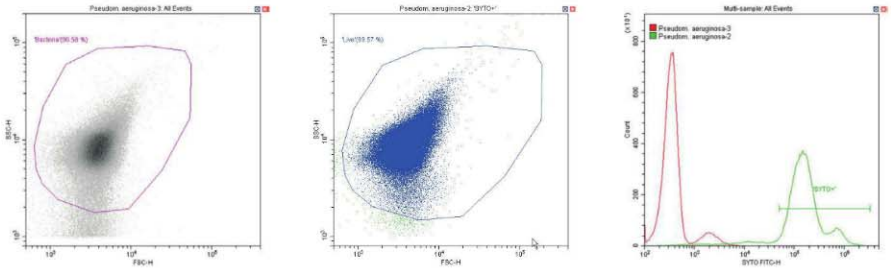
Negative control (left) Sheath only; Negative control (center & right) sheath and SYTO dye at recommended 1 µL/1 mL concentration



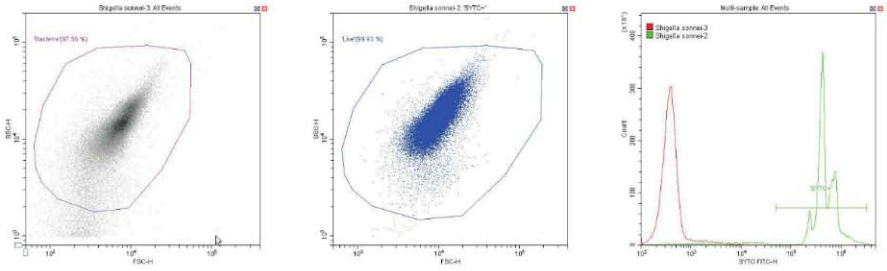
Gram-negative *Acinetobacter baumanii*. Unstained (left) and stained (center)



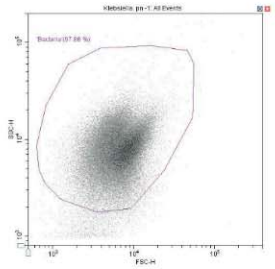
Gram-negative *Escherichia coli*. Unstained (left) and stained (center).



Gram-negative *Pseudomonas aeruginosa*. Unstained (left) and stained (center)



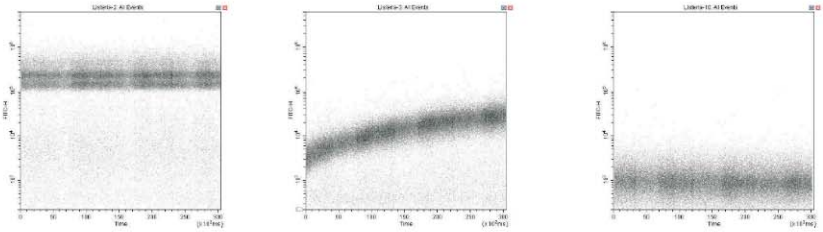
Gram-negative *Shigella sonnei*. Unstained (left) and stained (center)



Gram-negative *Klebsiella spp.*. Unstained

Notes

Even working at a lower SYTO dye concentration (as low as 1 % of recommended), care is still required if a stained sample is followed by an unstained (negative control) sample. This can be monitored using a plot of SYTO fluorescence vs time during sample acquisition. The presumptive mechanism is dye adsorption onto sample tubing and its subsequent release. Running a small volume of dilute (10 %) sodium hypochlorite as a sample and then rinsing with DI controls this issue effectively.



Time-course QC showing SYTO-labelled positive sample (left), negative control showing dramatic time-course labeling due to dye adsorption and release, and correct negative control after cleaning sample-line (right).

The results demonstrated in this application sheet represent those generated on the Beckman Coulter CytoFLEX Flow Cytometer. As differences exist in the performance between analyzers, the author cannot guarantee a similar appearance with the use of other flow Cytometers.

Reagent Details

Reagent	Supplier	Order Details
SYTO BC	Thermo Fisher Scientific	Catalog number: S-34855

https://www.thermofisher.com/order/catalog/product/S34855

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Detecting and counting bacteria with the CytoFLEX research flow cytometer: II-Characterization of a variety of gram-positive bacteria

APPLICATION NOTE

Author: Andrew Lister, DPhil
E-mail: alister@beckman.com
Affiliation: Beckman Coulter

IN THIS PAPER YOU WILL LEARN

How to set up your CytoFLEX instrument to detect and enumerate bacteria

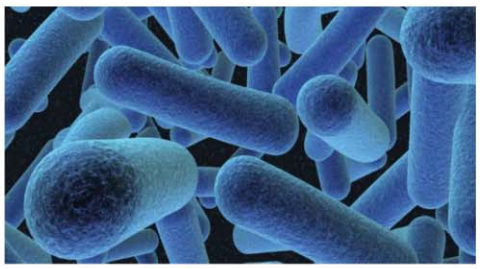
Principle of the Technique

Background

Microbial detection and counting have application in multiple contexts, such as environmental and process monitoring. Flow cytometry offers the potential to identify and count many different species of bacteria in diverse media. With appropriate staining to permit resolution from background, it is possible to count even very low frequencies of bacterial load quite rapidly. Predicate methods can be fast but less accurate (e.g. nephelometry) or very slow by comparison (CFU—colony forming unit—count determination by culture typically takes 48 hours) and may require serial dilutions to encompass a wide range of potential concentrations, compared with a minute or less by FCM (flow cytometry).

Research Applications: Introduction

This is the second of three associated notes, looking at a range of different bacterial species, both *coccus* and *bacillus* forms, and examining the capabilities of the CytoFLEX for their detection and enumeration. In this note, eight representative gram-stain positive species are compared with negative control samples.
The first note in the series examines a range of gram-negative bacteria and the third note demonstrates count linearity over a wide range (6-log decades) of concentrations and flow rates.



Listeria http://www.ambwallpapers.com/listeria-hd-wallpapers/

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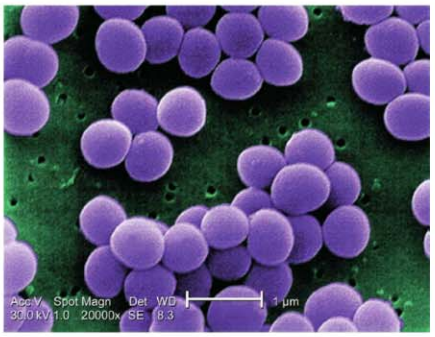
Gram positive bacteria include many genera that are of economic importance: usually due to their pathogenicity as food contaminants (e.g., *Listeria monocytogenes*) or association with disease and infection (e.g., *Staphylococcus spp.* and *Streptococcus spp.*).



1. https://commons.wikimedia.org/wiki/File%3AStaphylococcus_aureus_VISA_2.jpg

Protocol

Standard Procedure

Cultures of each species of bacterium were assayed by nephelometry (densitometry) and resuspended in PBS to 0.5 McFarland units, or nominally 150 x 10^6 mL^{-1}, identified as [1x]. Aliquots were further diluted with PBS to 1/10x or 1/100x concentration, corresponding to nominal 15 or 1.5 x 10^3 μL^{-1}, respectively, before analysis on the CytoFLEX.

Samples were run unstained or labelled with the nucleic-acid staining SYTO vital dye mixture from the SYTO BC kit [Thermo Fischer Scientific]. Manufacturer's instructions call for use of this dye at 1 µL stock (in DMSO) per 1 mL of sample, but this was found to cause significant secondary staining in practice. Very satisfactory staining was achieved by creating a secondary stock solution at this same rate (1 µL primary stock in 1 mL of PBS) which was used at a rate of 5 µL per 500 µL sample aliquot, or 1 % of recommended concentration.

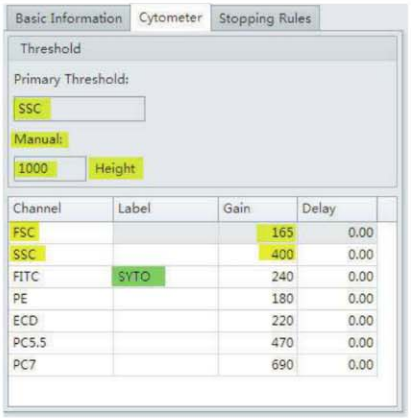
Instrument Configuration

Instrument Configuration	VSSC	Plate or Tube Format
Any: B4-R0-V0 and up	No	Either

Any standard CytoFLEX configuration can be used for this assay, as it only requires the blue laser for forward and side-scatter (FSC & SSC) and green SYTO fluorescence measured in the normal FITC channel.

Acquisition Settings

Since bacteria are smaller than most eukaryotic cells typically analyzed by FCM it is necessary to modify the gain settings for FSC (forward scatter) and SSC (side scatter) signals from the CytoFLEX default. Fluorescence gain is within normal range.



Fixed duration acquisitions are preferred over fixed count in order to assay a constant volume, and make any necessary background count subtraction corrections more straightforward.

Initial characterizations were performed at 10 µL per minute (Low) flow rate for 30 seconds, so a fixed volume of 5 µL per sample.

Results

The following series of plots all show an ungated log-log scatter density plot, with a generic 'bacterial' gate (magenta). Except for the controls, this plot displays an unstained aliquot of bacteria.
The second plot is another dual log scatter plot, but this time a dot-plot, which allows color-gating to be shown. A second region is defined here as 'live' (blue) based upon the SYTO-positive population.
The last plot is a single parameter histogram overlay of green (SYTO) fluorescence, comparing unlabeled and labeled aliquots of the bacterium in question, with the 'SYTO+' region used to back-gate the second plot.

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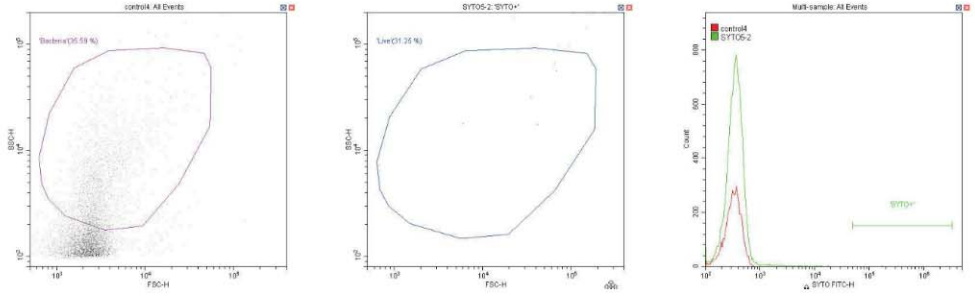
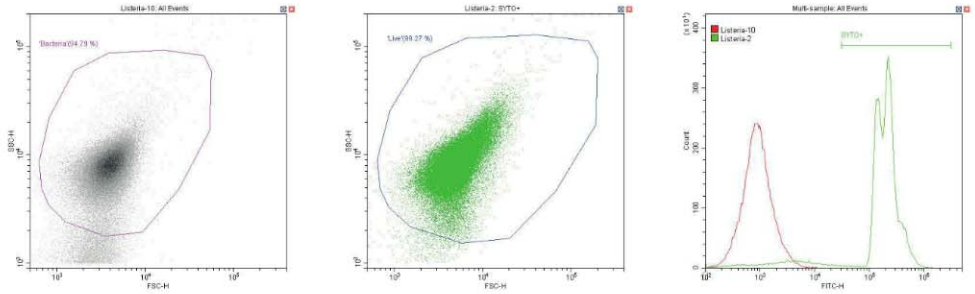
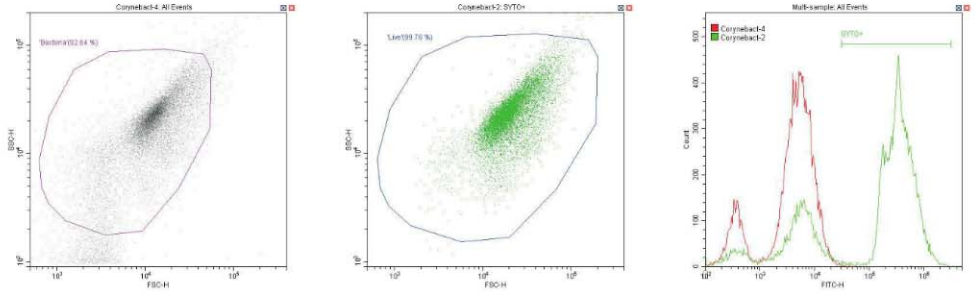


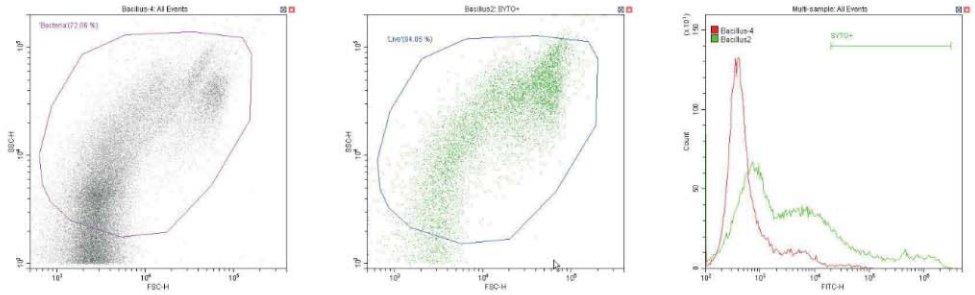
Negative control (left) Sheath only; Negative control (center & right) sheath and SYTO dye at recommended 1 µL/1 mL concentration



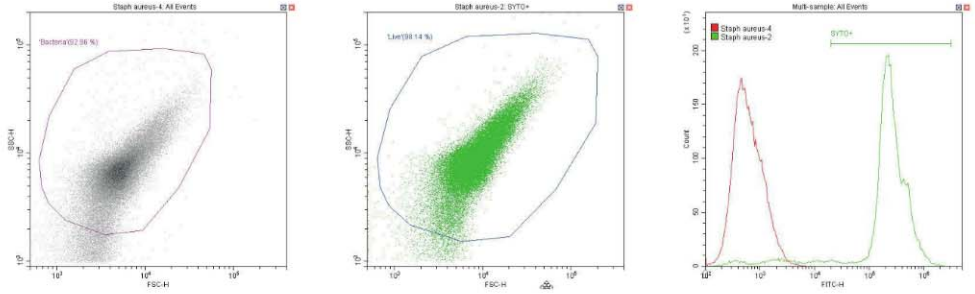
Gram-positive bacillus *Listeria monocytogenes*. Unstained (left) and stained (center)



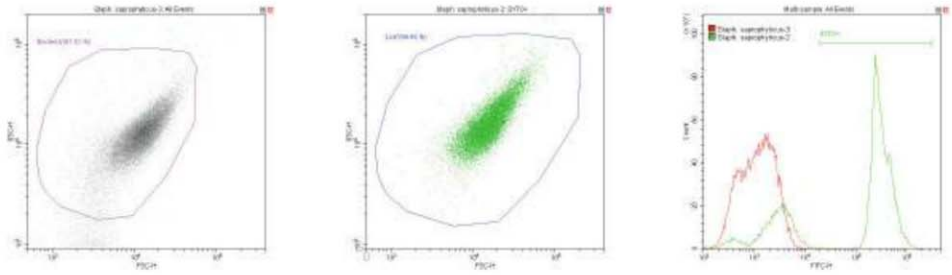
Gram-positive bacillus *Corynebacterium spp*. Unstained (left) and stained (center)



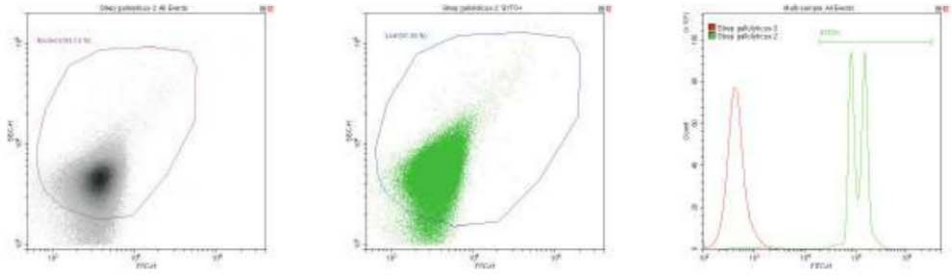
Gram-positive *Bacillus spp*. Unstained (left) and stained (center)



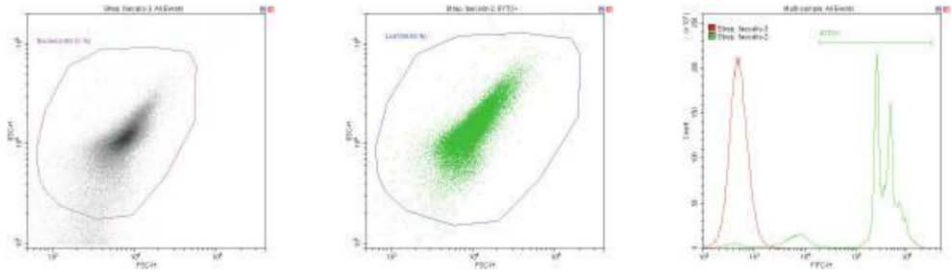
Gram-positive coccus *Staphylococcus aureus*. Unstained (left) and stained (center)



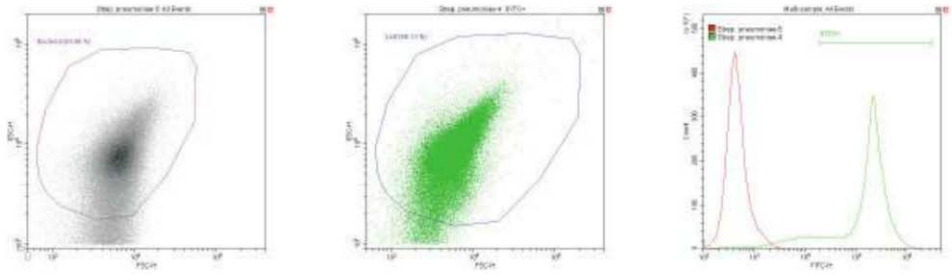
Gram-positive coccus *Staphylococcus saprophyticus*. Unstained (left) and stained (center)



Gram-positive coccus *Streptococcus gallolyticus*. Unstained (left) and stained (center)



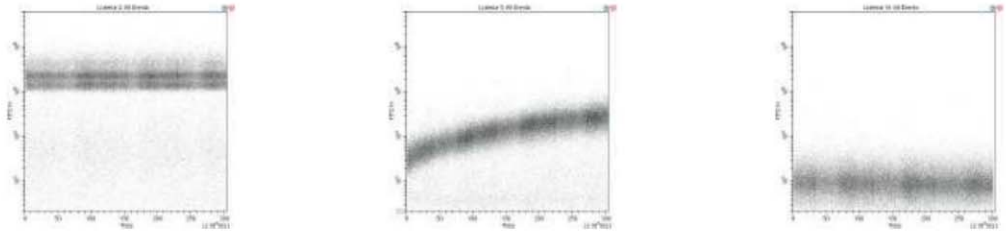
Gram-positive coccus *Streptococcus faecalis*. Unstained (left) and stained (center)



Gram-positive coccus *Streptococcus pneumoniae*. Unstained (left) and stained (center)

Notes

Even working at a lower SYTO dye concentration (as low as 1 % of recommended), care is still required if a stained sample is followed by an unstained (negative control) sample. This can be monitored using a plot of SYTO fluorescence vs time during sample acquisition. The presumptive mechanism is dye adsorption onto sample tubing and its subsequent release. Running a small volume of dilute (10 %) sodium hypochlorite as a sample and then rinsing with DI controls this issue effectively.



Time-course QC showing SYTO-labelled positive sample (left), negative control showing dramatic time-course labeling due to dye adsorption and release, and correct negative control after cleaning sample-line (right).

The results demonstrated in this application sheet represent those generated on the Beckman Coulter CytoFLEX Flow Cytometer. As differences exist in the performance between analyzers, the author cannot guarantee a similar appearance with the use of other flow Cytometers.

Reagent Details

Reagent	Supplier	Order Details
SYTO BC	Thermo Fisher Scientific	Catalog number: S-34855

https://www.thermofisher.com/order/catalog/product/S34855

For Research Use Only. Not for use in diagnostic procedures.

FLOW-1268APP11.15-A.

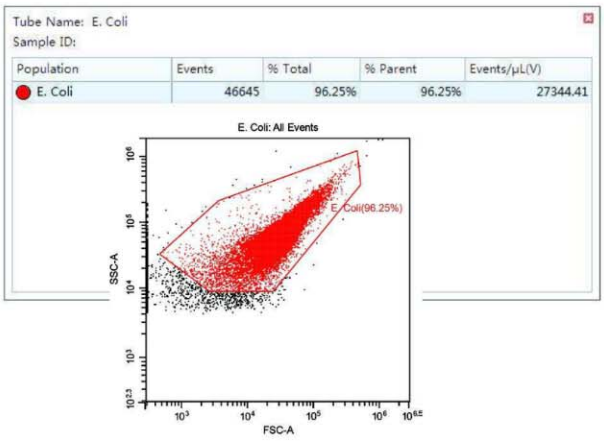
Counting Eschericia coli Using the CytoFLEX Research Flow Cytometer

APPLICATION NOTE

Introduction

Often an enumeration of bacteria is required more quickly than a colony forming unit, CFU, assay can be completed. Here we describe a quick protocol to enumerate bacteria in a sample using the CytoFLEX* fl ow cytometer. The range of resolution can support the identifi cation of bacteria by forward and side scatter parameters and does not require any fluorescent dyes or counting beads to enable detection or enumeration, respectively.

Materials and Tools



E coli sample

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Sample Preparation

1. Use PBS to dilute the E. coli sample if needed.
2. Record the dilution factor.

Data Acquisition and Analysis

1. Create a new experiment.
2. Draw a FSC/SSC plot, both axis are in log mode.
3. Run the diluted sample at Med or Fast rate settings.
4. Set the threshold on SSC channel.
5. Adjust the gains and threshold if needed.
6. Make sure that the abort rate is less than 10%. If not, increase the threshold or dilute the sample again.
7. Acquire at least 10μL sample
8. Create a gate on the E. coli population and check the cell count and concentration calculation in statistics window.
9. Use "Fit with Sample" function to show low signals.

Conclusions

Without using dyes or beads the bacterial sample can be enumerated using fl ow cytometry in a matter of minutes so that your research is not compromised or retarded while awaiting plating data. This is an inexpensive and rapid method for quantifying bacteria in suspension.

For Research Use Only. Not for use in diagnostic procedures.

FLOW-857SP03.15-A

Flow Cytometric Analysis of Bacterial Protein Aggregation During Expression of Foreign Proteins

APPLICATION NOTE

Authors: Jack Coleman [1]
Melis McHenry [2]

E-mail: [1] JColeman@ENZOLifeSciences.com
[2] melis.mchenry@beckman.com

Affiliation:
[1] ENZO Life Sciences, Farmingdale NY
[2] Beckman Coulter Inc, Miami FL

IN THIS PAPER YOU WILL LEARN

How to set up an assay for recombinant protein overexpression and monitor protein production

A simple method for the detection of protein aggregation

Background

Since the advent of recombinant DNA technology, bacteria have been used often to express foreign proteins[1]. Soluble proteins expressed by bacteria can be expressed either in the cytoplasm or they are secreted to the periplasm or outside the cell. In general proteins expressed in the cytoplasm are expressed to higher levels than if they are secreted[1]. Many hydrophobic and highly expressed proteins in the cytoplasm form inclusion bodies of aggregated protein that are difficult to solubilize [1]. Proteins targeted to the periplasm or outside of the cell often do not all get secreted and form insoluble aggregates [1, 2]. Different growth and induction conditions for the expressing bacteria can sometimes allow for soluble expression of a previously insoluble protein [2].

Research Applications

The most common method of aggregate detection in a bacterial culture involves isolating the cells, disrupting them by sonication, separating soluble from insoluble proteins by centrifugation, and finally identifying the location of the protein of interest using polyacrylamide gel electrophoresis. A recently developed dye, Proteostat®, is now available (ENZO Life Sciences ENZ-51035) that specifically stains amyloid type aggregates. The Proteostat dye has been used to stain aggregates formed by overexpressed proteins in bacteria [3]. In this application note, a simple and rapid method to identify aggregation in bacterial cells, showcased using a CytoFlex flow-cytometer, is described.

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Standard Procedure

Prior to staining the bacterial culture for aggregate detection, MG-132, a proteasome inhibitor, is used as a positive control to identify aggresome detection in mammalian cell culture. These results identifying aggresomes in induced mammalian cells are analogous to identifying aggregates in bacteria. ProteoStat Aggresome Detection kit (ENZO Life Sciences, Farmingdale, NY) is used for this assay as per manufacturer's recommendations. Briefly, Jurkat cells were mock-induced with 0.2 % DMSO or induced with 5 µM MG-132 for overnight 18 hours at 37°C. After treatment, cells were fixed and incubated with ProteoStat dye, then acquired using a CytoFlex Flow Cytometer (Beckman Coulter, Miami, FL) without washing. Results are analyzed with CytExpert 1.1 Software (Beckman Coulter, Miami, FL) and presented as histogram overlays.

The bacterial culture for aggregate detection assay is performed as follows: A culture of *Escherichia coli* BL21 with a plasmid (pET151-Klenow) was inoculated and grown overnight with shaking (320 RPM) at 37ºC in Terrific Broth (SIGMA, St. Louis, MO) containing ampicillin. This strain produces DNA Polymerase I Klenow fragment [4] from a T7 RNA polymerase promoter controlled with a lac operator (induced with IPTG). After 24 hour incubation, two flasks containing Terrific Broth and ampicillin were inoculated with one tenth volume of the overnight culture. After 30 minutes of shaking at 37ºC, IPTG (1 mM final concentration) was added to one of the flasks to induce overexpression of DNA Polymerase I, Klenow fragment. Growth was continued for 5 hours with shaking at 37ºC the OD_{550} was about 1. After growth, the cells are placed on ice for 5 minutes. One ml of culture is removed to a Microcentrifuge tube, and the cells are pelleted by centrifugation for one minute at 16,000 x g. The supernatant is removed, and the cells are resuspended in 1 mL 1x Assay Buffer (supplied with Proteostat dye, ENZO Life Sciences, Farmingdale, NY). The cells are again pelleted by centrifugation at 16,000 x g for 1 minute. After removal of the supernatant, the cells are resuspended in 1 mL 1x Assay Buffer containing 10 % Formalin (SIGMA, St. Louis, MO.). The cells remain in the formalin at room temperature for 30 minutes. The fixed cells are again pelleted by centrifugation at 16,000 x g for 1 minute, and washed once with 1x Assay Buffer. After the wash, the cells are resuspended in a minimal volume (50 µL) of 1 x Assay Buffer and 1 ml of permeabilizing solution (0.5 % Triton™ X-100, 3 mM EDTA in 1X Assay Buffer) added. The cells are then mixed by inversion 5-6 times and incubate on ice for 30 minutes. The cells are collected by centrifugation for 1 minute at 16,000 x g. After removing the supernatant, the cells are resuspended in 1 mL 1x Assay Buffer. When the starting OD_{550} was approximately 1, 40 µL of culture is pipetted to a fresh microcentrifuge tube. Staining solution is prepared by adding 1 mL of 1x Assay buffer – 2 µL of Hoechst (included with Proteostat® dye kit ENZO Life Sciences, Farmingdale, NY) and 4 µL Proteostat® dye (if using ENZ 51035-0025, or 1 µL if using from ENZ 51035-K100). 400 µL of the staining solution is added to the cells, stained for 30 minutes at room temperature, followed by centrifugation at 16,000 x g for 1 minute. The cells are resuspended in 1 mL 1x Assay Buffer and subsequently analyzed by using a CytoFlex Flow Cytometer (Beckman Coulter, Miami, FL). The data is analyzed using CytExpert Software (Beckman Coulter Inc, Miami FL). Fluorescence is quantitated by mean peak channel fluorescence.

Note: If higher concentrations of cells are used, the concentration of dye may have to be increased.

The Hoechst dye is used to validate that bacteria are present, and can be used to gate on the bacterial population.

Results

Insoluble aggregates often form in bacterial cells overexpressing non-native proteins for pharmaceutical or therapeutic purposes. The Proteostat® dye is immobilized when it binds the aggregated protein, which causes a significant increase in fluorescence, therefore makes it a simple method to detect aggregates via flow cytometry. Growth of cells under different conditions, such as growth at a different temperature or inducing expression of the protein of interest with different concentrations of the inducer can affect the amount of aggregate formed [3].

Uninduced control and 5 µM MG-132-treated Jurkat cells were used to show the typical results of flow cytometry based analysis of cell populations using the ProteoStat aggresome red detection reagent. After 18 hours treatment, cells were loaded with ProteoStat reagent, and then analyzed without washing by flow cytometry. Results are presented by histogram overlays (Figure 1). Control cells display low fluorescence. In the samples treated with 5 µM MG-132 for 18 hours, the ProteoStat® aggresome red dye (excited with 488nm blue laser and collected with emission filter 610/20 nm) signal increases over 2-fold, indicating that MG-132 induced aggresome formation in Jurkat cells.

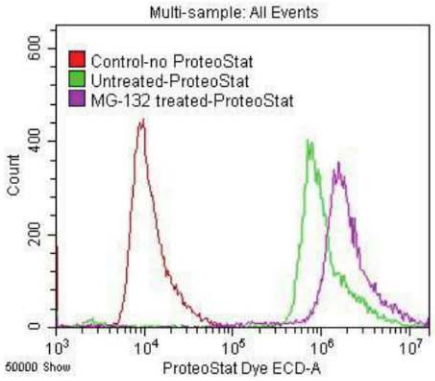

Figure 1. Flow cytometry-based cell aggresome analysis of unstained control, untreated control and MG-132 treated Jurkat cells. MG-132 treated cells show 2-fold signal increase over untreated cells.

The *E.coli* bacterial cells were first identified in a FSC vs SSC plot as shown in Figure 2. The Hoechst dye is used to identify the nucleated cells vs. the debris. The 405nm violet laser is used to excite the Hoechst dye which is then collected with 450/45BP filter. When the samples overlayed in a histogram plot, the Hoechst stained cells are significantly brighter than unstained cells (Figure 3).

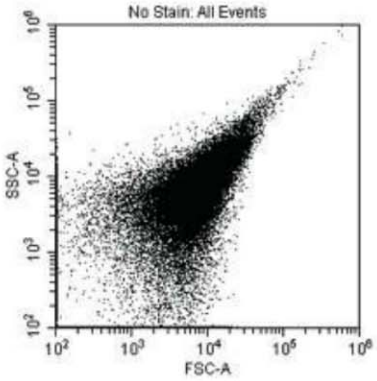

Figure 2. Shows the FSC vs SSC plot for the *E.coli* sample. The plot is in logarithmic scale for both axes.

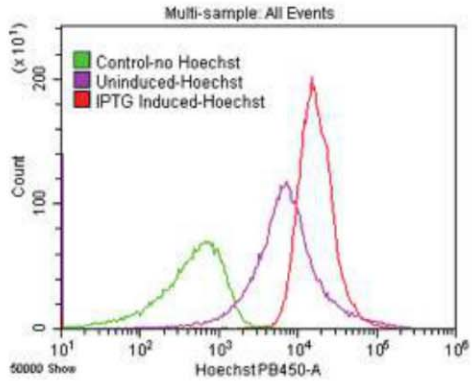

Figure 3. Histogram overlay for Hoechst fluorescence shows the no staining control (green), IPTG uninduced cells (purple) and IPTG induced cells (red).

In figure 4, we show that the induction of bacterial cells BL21/pET151-Klenow with 1 mM IPTG for 5 hours significantly increases the signal from the Proteostat® dye, indicating protein aggregates have formed. There appears to be a small population of cells in the induced cells that do not have aggregates. These cells may no longer be overexpressing the Klenow protein, or may have developed some method to prevent aggregate formation through random mutation.

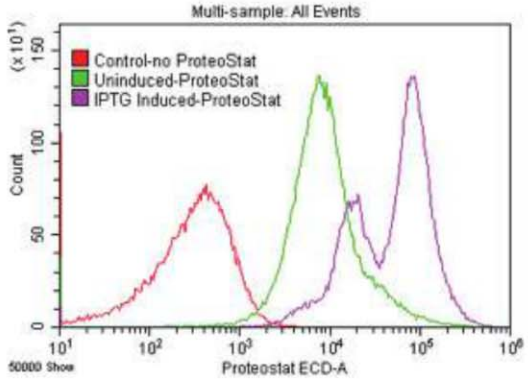

Figure 4. The histogram overlay for ProteoStat fluorescence shows the induction of aggregate formation in E.coli cells induced with IPTG (purple) compared to uninduced cells (green). Unstained control cells are also shown (red).

In figure 5 we see that the mean fluorescence intensity (Proteostat® dye) of the population of IPTG induced cells increases dramatically compared to the uninduced cells.

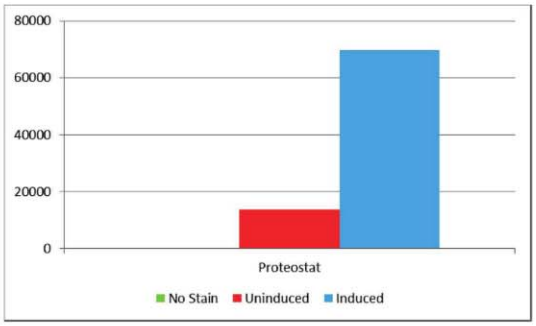

Figure 5. Shows the increase in mean fluorescence intensity in the induced cell population (blue) compared to uninduced (red) cells. The green bars show the signal for unstained control cells.

These results demonstrate that Proteostat® dye using the CytoFlex flow cytometer can be used to screen for methods of growing bacteria to produce soluble protein. Conditions such as different growth media, different temperatures, different lengths of induction, and different amounts of inducer can all be tested in one simple screen for protein aggregate formation.

References

1. Marston, F.A., The purification of eukaryotic polypeptides synthesized in Escherichia coli. Biochem J, 1986. 240(1): p. 1-12.
2. Zhou, Y., et al., Efficient expression, purification and characterization of native human cystatin C in Escherichia coli periplasm. Protein Expr Purif, 2015. 111: p. 18-22.
3. Navarro, S. and S. Ventura, Fluorescent dye ProteoStat to detect and discriminate intracellular amyloid-like aggregates in Escherichia coli. Biotechnol J, 2014. 9(10): p. 1259-66.
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The results demonstrated in this application sheet represent those generated on the Beckman Coulter CytoFlex Flow Cytometer. As differences exist in the performance between analyzers, the author cannot guarantee a similar appearance with the use of other flow cytometers.

Reagent Details

Reagent	Supplier	Order Details
ProteoStat® Aggresome Detection Kit	ENZO Life Sciences	ENZ-51035
Formalin	SIGMA-Aldrich	F8775
Terrific Broth	SIGMA-Aldrich	T5574

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FLOW-1315APP12.15-A.

BECKMAN COULTER
Life Sciences

Flow Cytometric Analysis of auto-fluorescent cells found in the marine demosponge *Clathria prolifera*

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IN THIS PAPER YOU WILL

Learn how flow cytometry can be used to view autofluorescent cells from aquatic samples

Learn about a new type of cells identified in the demosponge, *Clathria prolifera*

Introduction

Sponges are well known for their remarkable capacity for aggregation and reorganization. These phenomena were demonstrated in a classic experiment where sponge tissue is dissociated using fine mesh and observed over time to reaggregate, and in some cases forming a functional miniature sponge [1,2]. A recent study compared seven different sponge species and their reorganization capacities revealing only a few species are capable of forming functional sponges from dissociated tissue, as well as identifying checkpoints in the reaggregation process [3]. *Clathria prolifera* (formerly known as *Microciona prolifera*), a demosponge species found in the east coast of the US, is well known for it's capacity to reaggregate from dissociation and form miniature sponges [2,4].

C. prolifera cells are rich in carotenoids giving them their bright orange color and general auto-fluorescence, excited by the 543nm laser [5]. However, our preliminary observation revealed a new subset of cells that are excited by 405nm laser. Using the CytoFLEX Flow Cytometer, the new 405nm excitable *C. prolifera* cell population was further characterized, namely their size, granularity and population percentage.

Material and Methods

C. prolifera were collected near the Marine Biological Laboratory (MBL), Woods Hole, MA by the Marine Resources Center (MRC). The cell suspension preparation generally followed the methods previously described by Eerkes-Medrano et al. [3]. Sponge tissue was cut into an approximately 5-cm^3 cubes and the flow cytometric analysis performed within an hour of tissue dissociation. The tissue cubes were transferred into a 50mL conical tube filled with 0.22μm-filtered seawater (FSW) and transported to the flow cytometry facility in the Whitman Center, MBL. Calcium- and magnesium-free seawater (CMFSW) was made as previously described [6], in which the tissue cubes were dissociated by using a 20μm mesh. This cell suspension was then transferred to a 1.5mL microcentrifuge tube, which was used for the flow cytometry analysis. The samples were then run on a CytoFLEX Flow Cytometer (Beckman Coulter, Miami, US) with 3 laser 13 color capability. The threshold was set to automatic, and the acquisition was performed in slow mode (10μL/mL).

The images from Figure 1 and 2 were recorded using the Zeiss LSM780 (Oberkochen, Germany), showing excitation with 405nm laser, with a broad emission range peaking at approximately 550nm.

Results

The demosponge *C. prolifera* is able to reaggregate from dissociation and form miniature sponges [2,4]. The carotenoid rich nature of this demosponge gives it the bright orange color [5]. This species is also autoflourescent, which can be excited by the 543nm laser. Our study with this species collected at Woods Hole (Massachusetts, USA) unfolded a new subset of cells excitable by the 405nm laser (Figure 1). The new cells were initially observed under a fluorescent microscope with 405nm laser capability. Excitation using this laser resulted in only a subset of the cells displaying fluorescence in their cytoplasm. These cells seemed to be a specific cell type with a relatively large nucleus and cell size, indicative of the archeocytes (somatic stem cells of the sponge). Further observation under higher magnification has shown that this auto-fluorescence is specifically emitted from numerous vesicles of uniform size packed inside the cells, which display dynamic movement within the cell (Figure 2). Since these cells resemble archeocytes, which are known for their high phagocytic activity, it is possible that these vesicles are lysosomes and the fluorescence comes from a component or the product of the process of digestion.

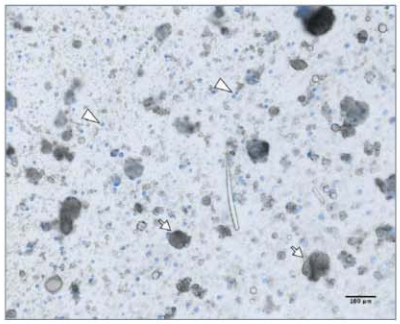

Figure 1. Dissociated *Clathria prolifera* tissue.

Auto-fluorescent cells excited by the 405nm laser (blue). Dissociated *Clathria prolifera* cells in filtered seawater (FSW). Single cells, including the auto-fluorescent cells (arrowheads) immediately start re-aggregating after dissociation, with a few sponge cell aggregates (SCAs) already starting to form (arrows). Note that aggregates are formed from both fluorescent and non-fluorescent cells. Scale bar 100 μm.

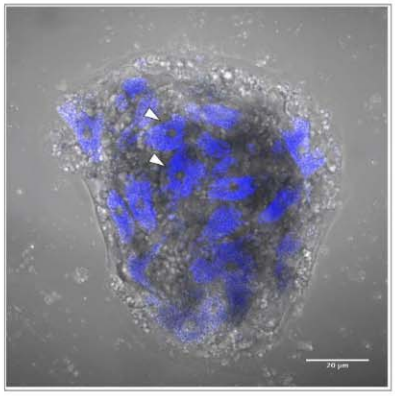

Figure 2. Sponge cell aggregate at 12 hours post-dissociation of *Clathria prolifera* cells.

Auto-fluorescent cells excited by the 405nm laser (blue). Sponge cell aggregate (SCA) at 12 hours post-dissociation of *C. prolifera* cells in filtered seawater (FSW). A subset of the cells in this SCA displays auto-fluorescence in their cytoplasm (arrowheads), which is emitted by the vesicles contained in these cells. Scale bar 20 μm.

Flow cytometric analysis on these fluorescent cells is beneficial in a number of ways. Since not much is known about these cells, analyzing the nature of the fluorescence and quantifying their abundance in the sponge body is essential in characterizing this population. In addition, sorting and isolating these cells will enable further analyses and experimental approaches on these cells, including RNA sequencing to identify key genes involved in the auto-fluorescent capacity of the vesicles in these cells. The technique described in this article shows how using CMFSW (calcium- and magnesium-free sea water) enables flow cytometric analysis on live single cells of a marine sponge that normally undergo rapid reaggregation, a technique which could be applied to other marine organisms. Therefore further investigation with a CytoFLEX Flow Cytometer with 405nm, 488nm and 638nm laser capability was performed to help quantify this new cell population.

The dissociated *C. prolifera* cells were acquired at slow (10μL/mL) flow rate using CytoFLEX Flow Cytometer, and a total of about 40,000 cells were collected. Large granular cells identified by high forward and side scatter were gated to compare these to the major population of non-granular cells, using the FSC and SSC dot plot (Figure 3A). This gated population was then displayed in all 13 parameters as histogram plots to determine which population of cells is autofluorescent (Figure 3B). The histogram plots revealed that the large granular cells were excited by only the 405nm laser, and displayed a well-resolved peak of autofluorescence across the visible spectrum from 450-780nm. The resolution was best with 525nm (KO525) and 610nm (Violet 610) with almost a decade difference between the positive peak and the non-granular cells. It can be concluded that the peak emission of this population is between 525nm and 610nm, consistent with results from the microscopy experiments. Further quantification showed that this population of interest is about 3.87% of all the events acquired (Figure 3A).

The blue laser excited weaker autofluorescence of both the granular and non-granular populations; the autofluorescence was weaker than the violet laser and the populations were not resolved. The autofluorescence of all cells was least excited by the red laser, which would make this a potentially useful channel for immunofluorescence staining or use of vital dyes to label the cells with less interference from autofluorescence.

The next step will be to sort and isolate these cells to enable further analyses and experimental approaches on these cells, including RNA sequencing to identify key genes involved in the auto-fluorescent capacity of the vesicles in these cells.

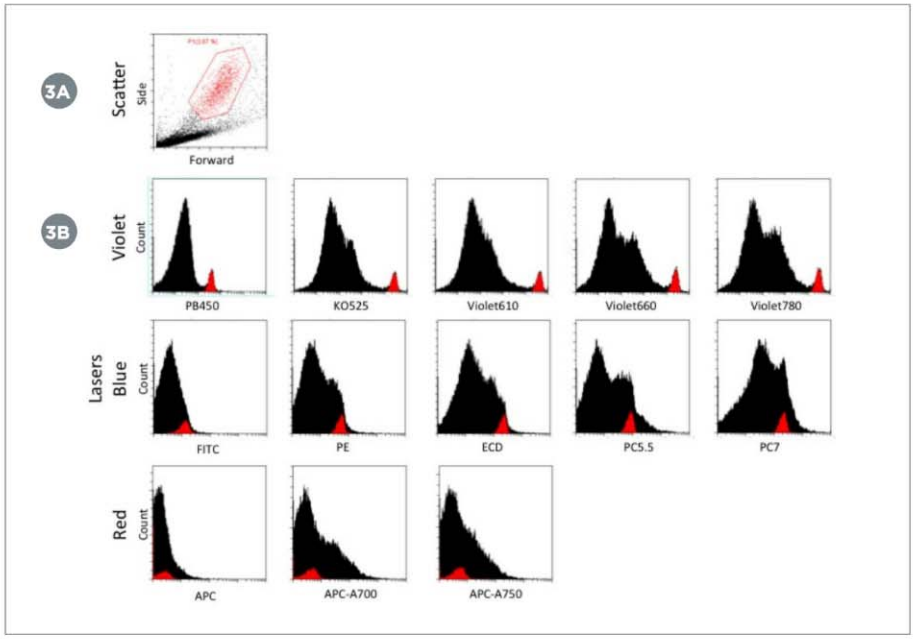

Figure 3. Dissociated *Clathria prolifera* cells acquired at slow (10μL/mL) flow rate, and a total of about 40,000 cells were collected. The subpopulation of large granular cells was gated with a FSC and SSC dot plot. This gated population is displayed in all 13 parameters as histogram plots to observe the resolution of the large granular subpopulation from the rest of the cells. The x-axis range is fixed at 10^2 to 10^6 for all fluorescent parameters.

Discussion

The results shown here demonstrate the identity of a new population of cells in the demosponge, *Clathria prolifera*. These cells are distinct in their ability to be visualized by autofluorescence from the 405 nm laser. Additionally, this work demonstrates the ability of the CytoFLEX flow cytometer to be used in marine/aquatic biology.

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Note:

The results demonstrated in this application sheet represent those generated on the Beckman Coulter CytoFLEX Flow Cytometer. As differences exist in the performance between analyzers, the author cannot guarantee a similar appearance with the use of other flow Cytometers.

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FLOW-1912APP08.16

BECKMAN COULTER
Life Sciences

Flow Cytometric Approach to Probiotic Cell Counting and Analysis

Data provided by Dharlene Tundo, Department of Quality Control, VitaQuest International

IN THIS PAPER YOU WILL

- Compare and contrast culture-based and flow cytometry based workflows for probiotic cell enumeration
- Learn how to set up the flow cytometer for optimal detection of bacterial strains.
- See a detailed staining protocol and gating strategy for enumerating live versus dead probiotic cells.

Introduction

Probiotics were historically defined as substances secreted by one microorganism that promote the growth of another. The history of probiotics goes parallel with the evolution of the human race and can be traced back to the ancient times, nearly 10,000 years ago (1). Extensive research in recent years has exploded our understanding of the 100 trillion gut resident microbial cells contribute to health and disease (2). Indeed, industries devoted to leveraging beneficial organisms to restore balance to the gut microbiota have developed and continue to flourish.

One manufacturer is VitaQuest International, one of the largest custom contract manufacturers of nutritional supplements in the United States. They produce a range of probiotic containing supplements manufactured at large scale. Over twenty different bacterial strains from within the *Lactobacillus* and *Bifidobacterium* species, are handled in the formulation of different products. The complexity of the manufacturing process along with increased demand, and strict criteria for quality, potency, and safety in accordance to FDA regulations necessitates that manufacturers continue to improve production processes that increase productivity and efficiency.

A key method used to ensure product quality is the enumeration of probiotic organisms contained in the product. The traditional method is the Plate Count Method, a culture-based method for ascertaining the number of viable organisms in a sample, see figure 1. This method is laborious, requires specialized equipment for anaerobic incubation, and requires multiple days to develop the results. These characteristics of this assay limit its utility for real time decision making in a large scale production environment.

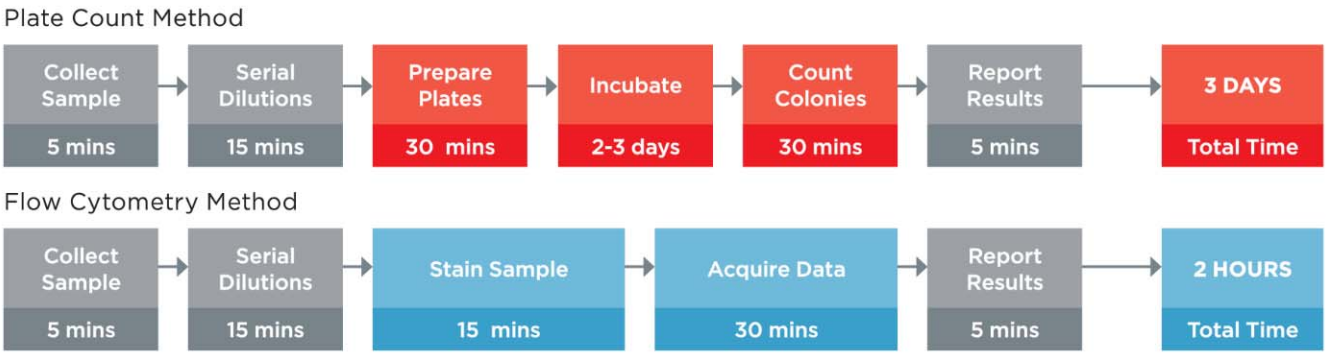

Figure 1. Comparison of the typical workflow for the Plate Count enumeration versus Flow Cytometric analysis. The plate counting method requires more direct time, but also requires the technician to maintain sterile media and plates for the assay. Using flow cytometry, not only are these obsolete, but data on the amount of live and dead organisms in the sample can be fridobtained, allowing the manufacturing process to be optimized. The assay time of 2 hours versus 3 days also allows for in line process improvements to optimize production batches.

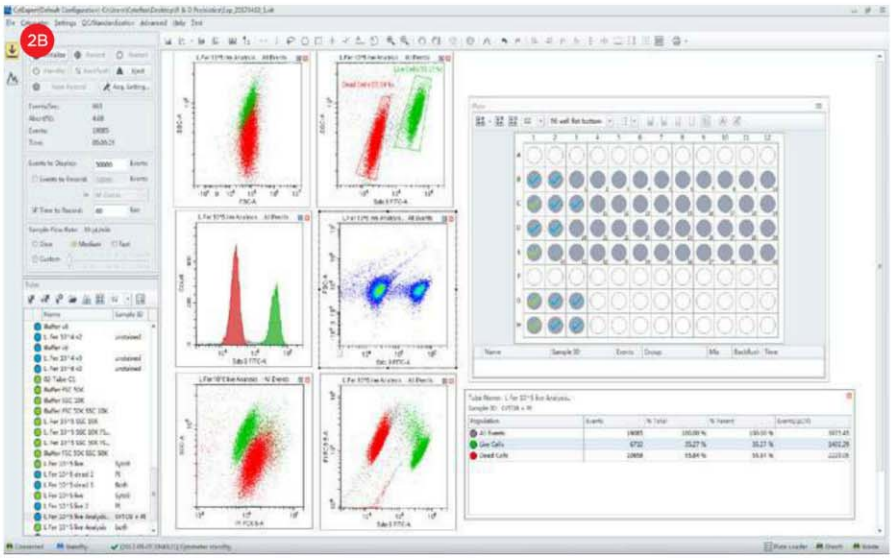

Figure 2. Comparison of the data collection read outs for the Plate Count and Flow Cytometry methods. Panel A is a typical cultured plate from which bacterial colonies are counted. This method requires a technician to accurately discriminate between overlapping or differently sized colonies to obtain counts, typically done in duplicate. Panel B shows the software screen during acquisition using flow cytometry. The instrumentation counts individual bacteria and live versus dead organisms can be differentiated based upon staining characteristics.

In order to decrease the time from sampling to result, VitaQuest embarked on an initiative to improve the enumeration assay by innovating a non-culture-based approach. Flow cytometry offers a fast, reliable method for counting large numbers of events. Advances in the technology result in the ability to reliably detect smaller particles, making it an attractive technology for process development and quality control in the probiotic industry. In addition, the CytoFLEX Flow Cytometry Platform, through an innovative approach to the fluidic system, has the capability to perform absolute counting without the addition of counting beads to the sample.

Materials

- Bacterial cultures, *Lactobacillus fermentum* (150 B/g, expected count based upon plate count method) and *Lactobacillus rhamnosus* (350 B/g, expected count based upon plate count method)
- BD Difco™ Buffered Peptone Water. Catalog Number 218105. Final pH 7.2 ± 0.2. (Prepared according to the manufacturers recommendations and sterilized by autoclaving at 121 °C).
- LIVE/DEAD *BacLight* Viability and Counting kit for Flow Cytometry by Thermo Fisher Scientific, Catalog Number L-34856. The kit includes two nucleic acid stains, green fluorescent SYTO9 and red fluorescent Propidium Iodide and a suspension of microspheres.
- CytoFLEX Flow Cytometer Blue-Red Violet Series B4-R2-V0, Beckman Coulter, Brea, CA)

Tips for Success

- In order to determine the optimal sample dilution for flow cytometric analysis, prepare a range from 1 x 10^5 to 1:10^8.
- The flow cytometer settings will vary depending on the cell size being analyzed and adjustments will be necessary. See protocol and discussion related to optimal settings for *Lactobacillus fermentum*, *rhamnosus* and *B. longum*. It is recommended to try different threshold settings while running the sample to observe the best data resolution at different set points.
- Calibrate the flow rate on the CytoFLEX Flow Cytometer following the directions in the Instructions for Use document. This will ensure that the sample volume is measured correctly to support the absolute counting analysis.

Protocol

1. Weight out 1 gm of the product. Dilute in 9 mL of buffered peptone water, followed by serial dilutions.
   a. *Lactobacillus fermentum* (optimal assay dilution 1:10^5)
   b. *Lactobacillus rhamnosus* (optimal assay dilution 1:10^6)

1. Incubate samples in a 45-47 °C water bath for 10-15 minutes.

2. Aliquot 1 mL of the diluted sample and add the staining reagent:
   a. 96-well Plate: 2 µL of SYTO9 and 2 µL Propidium Iodide
   b. Single Tube: 2 µL of SYTO9 and 2 µL Propidium Iodide

3. Incubate the samples for 15 minutes in the dark.

4. Add 250 µL into the sample well or 1 mL if using single tube mode.

5. Set up the experiment in the flow cytometer.

6. Aliquot 1 mL of the diluted unstained sample to create a control to use for setting the gates based upon forward and side scatter characteristics. Set amplifiers to logarithmic amplification.

7. Set the amplification to position the bacterial population in the middle of the graph. Adjust the threshold level and gain settings to minimize electronic noise.

8. Acquire the data from the sample and apply the gates from unstained.

9. Acquire data from the stained sample and set gates based upon fluorescent staining and calculate population statistics.

10. Calculate total cells by factoring the events/µL multiplied by the dilution factor.

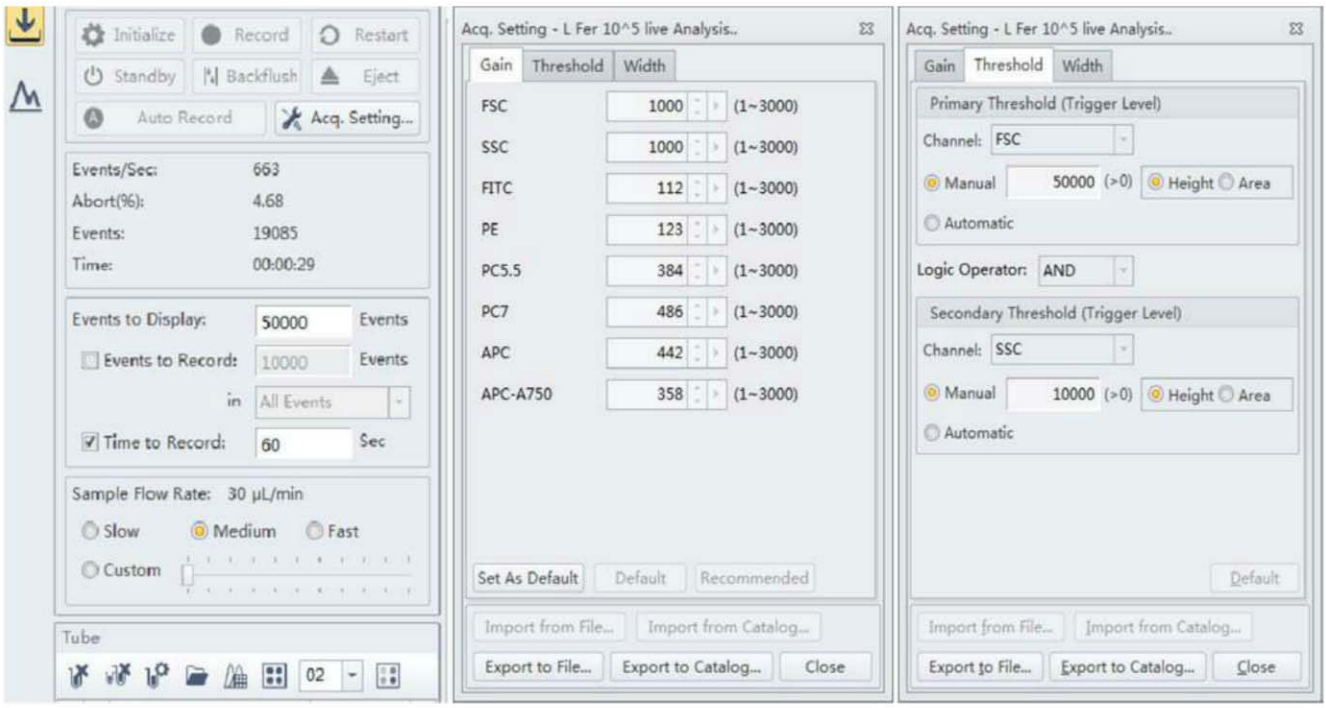

Figure 3. CytoFLEX Instrument and acquisition settings. The best results were observed when the threshold level was set at 50,000 for the forward scatter and 10,000 for the side scatter for these strains of Lactobacillus. The Gain for both forward scatter and side scatter showed good signal when set at 1000 respectively.

Results and Discussion

The data acquired in this study represents the analysis of *Lactobacillus fermentum* serially diluted to a final concentration of 1:10^5, data acquired of the analysis of *Lactobacillus rhamnosus* diluted to a final concentration of 1:10^6 and the data acquired for *Bifidum longum*, final concentration 1:10^5. The LIVE/DEAD kit worked effectively staining both viable and non-viable cells. CytoFLEX provided consistent results with different samples analyzed using the same staining kit, settings were modified accordingly for different bacterial strains (data not shown).

Many of the manufactured probiotic supplements at VitaQuest contain different strains of *Bifido* and *Lactobacillus*; these bacterial cells are very small in size (0.5-1.3 micrometers by 1.0-10.0 micrometers). For *Lactobacillus fermentum* and *rhamnosus* flow cytometric analysis, best results were observed when the threshold level was set at 50,000 for the forward scatter and 10,000 for the side scatter; however these strains were also detected at a threshold level of 5,000 for both side scatter and forward scatter as well. The Gain for both forward scatter and side scatter showed good signal when set at 1000 respectively.

Some events observed outside the gated areas need further analysis, see figure 4.

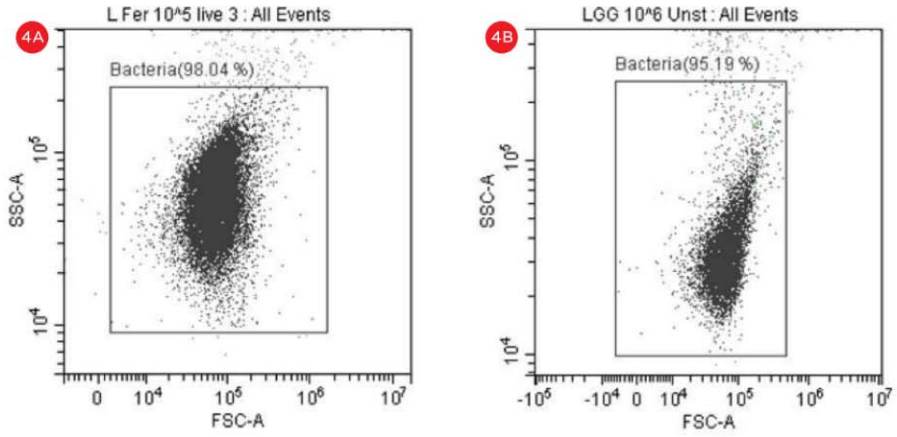

Figure 4. Forward Scatter versus Side Scatter Dot Plot. An unstained suspension of cells were displayed in forward scatter (FSC) vs side scatter (SSC) plot in logarithmic scale and a gate named Bacteria is used to capture the cells of interest. The "bacteria" gate is used to gate the fluorescent plots, panel A, *Lactobacillus fermentum*, panel B *Lactobacillus rhamnosus*.

Traditionally, due to its positive pressure, flow cytometers rely on the addition of a known quantity of beads to the sample in order to calculate the volume of sample acquired; with the pressure differential in the sheath versus the sample quantifying the volume is impossible. In contrast the CytoFLEX Flow Cytometer's fluidics is based upon positive displacement. The volume of sample is a factor of the flow rate and time. The sample uptake rate was calibrated prior to collecting data and by factoring in the final concentration of the sample with the volumetric count of cells/µL(V), the total viable cell count was determined, see Table 1 and 2, *Lactobacillus fermentum*, and *Lactobacillus rhamnosus*, respectively.

Tube Name: L Fer 10^5 live fitc 5K
Sample ID: Live/Dead population of L. fermentum

Population	Events	% Total	% Parent	Events/µL(V)
All Events	62003	100.00 %	100.00 %	6120.38
Live Cells	16213	26.15 %	26.15 %	1600.40
Dead Cells	37525	60.52 %	60.52 %	3704.13

Table 1. Population Statistics. Number of events and percent of total events collected in each gate are calculated for the *Lactobacillus fermentum* cell population.

Using the traditional Plate Count method, the sample of *Lactobacillus fermentum* had a known concentration of 150 B/g. Using our analysis by flow cytometry the cell count was determined by factoring the Events/µL (V) (1600.4) times the dilution factor (10^5). The final number was then multiplied by 1000 to convert the units of µL into mL, resulting in a value of 160 B/g. See Table 1.

Tube Name: LGG 10^6 PI+SYTO
Sample ID: 314970
Volume(µL): 10.0

Population	Events	% Total	% Parent	Events/µL(V)
All Events	10535	100.00 %	100.00 %	1053.56
Bacteria	9911	94.08 %	94.08 %	991.16
Dead	3246	30.81 %	32.75 %	324.62
Live	4226	40.11 %	42.64 %	422.63

Table 2. Cell Population Statistics. Number of events and percent of total events collected in each gate are calculated for *Lactobacillus rhamnosus* cell population.

Using the traditional Plate Count method, the sample of *Lactobacillus rhamnosus* had a known concentration of 350 B/g. Using our analysis by flow cytometry the cell count was determined by factoring the Events/µL (V) (422.63) times the dilution factor (10^6). The final number was then multiplied by 1000 to convert the units of µL into mL, resulting in a value of 423 B/g. See Table 2.

Bifidobacterium and *Lactobacillus* are both *Gram positive*, Lactic Acid (LAB) producing bacteria; and are very small in size (0.5-1.3 micrometers by 1.0-10.0 micrometers). These types of bacteria are the most frequently used in probiotic preparation. *Bifido* and *Lacto* bacteria can behave differently when subjected to environmental stress.

Bifidobacteria is known to be more sensitive to environmental conditions than *Lactobacillus*. Naturally, bacteria are fighting for survival and reproduction; in some cases *Lactobacillus* may inhibit the ability for *Bifidobacterium* to form colonies when a blend of both is cultured together. *Bifidobacterium* may go into a dormant state due to stress induced genes; this means they are still metabolically active but not able to form colonies (VBNC) when using a culture dependent method.

The flow cytometer settings will vary depending on the cell size being analyzed and adjustments will be necessary; during analysis of *B. longum*, best results were observed when the threshold was set at 5,000 for FSC or FITC and 5,000 for SSC compared to the threshold settings used during the analysis of *Lactobacillus*.

Despite the fact that *Lactobacillus* is also detected at the threshold level of 5000, the *Bifido* bacteria did not displayed a good separation when the threshold level was set at 50,000, see Figure 5.

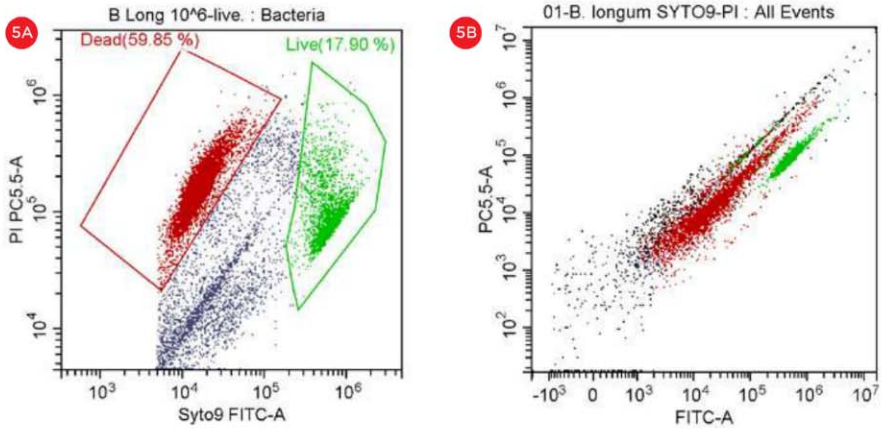

Figure 5. Gating Strategy for Live/Dead *B. longum* Analysis. The SYTO9 staining is shown in a PC5.5 vs SYTO9-FITC dot plot, threshold set at 5,000 (panel A). The events in red are dead and the events in green are viable. The PI staining is shown in a PC5.5 vs FITC dot plot with the threshold set at 50,000 (panel B). Populations of "Live" and Dead" *B. longum* cells are best displayed when settings for the threshold are set at 5,000 (panel A).

Using the traditional Plate Count method, the sample of *B. longum* had a known concentration of 100 B/g. Using the analysis by flow cytometry the cell count was calculated, 150B/g.

Tube Name: B Long 10^6-live.
Sample ID:
Volume(µL): 14.7

Population	Events	% Total	% Parent	Events/µL(V)
All Events	24410	100.00 %	100.00 %	1655.88
Bacteria	12428	50.91 %	50.91 %	843.07
Dead	7438	30.47 %	59.85 %	504.56
Live	2224	9.11 %	17.90 %	150.87

Table 3. Cell Population Statistics. Number of events and percent of total events collected in each gate are calculated for the *B. longum* cell population.

The use of Flow Cytometry for cell count has advantage over the traditional pour-plate method because it provides count for viable cells but also for VBNC cells, see Figures 6 and 7.

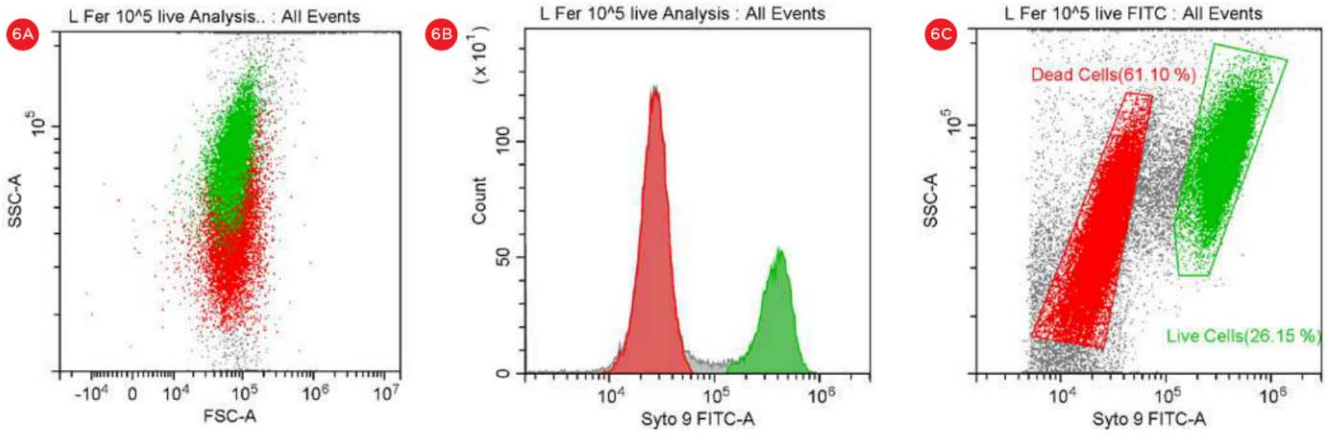

Figure 6. Gating Strategy for Live/Dead *L. fermentum* Analysis. Bacterial population stained with PI and SYTO9 is shown in a SSC vs FSC dot plot (panel A). The number of events collected in each population is shown in a Count vs FITC histogram (panel B). The events in red are dead and the events in green are viable. "Dead" and "Live" gates created to analyze the viable cell count population (panel C).

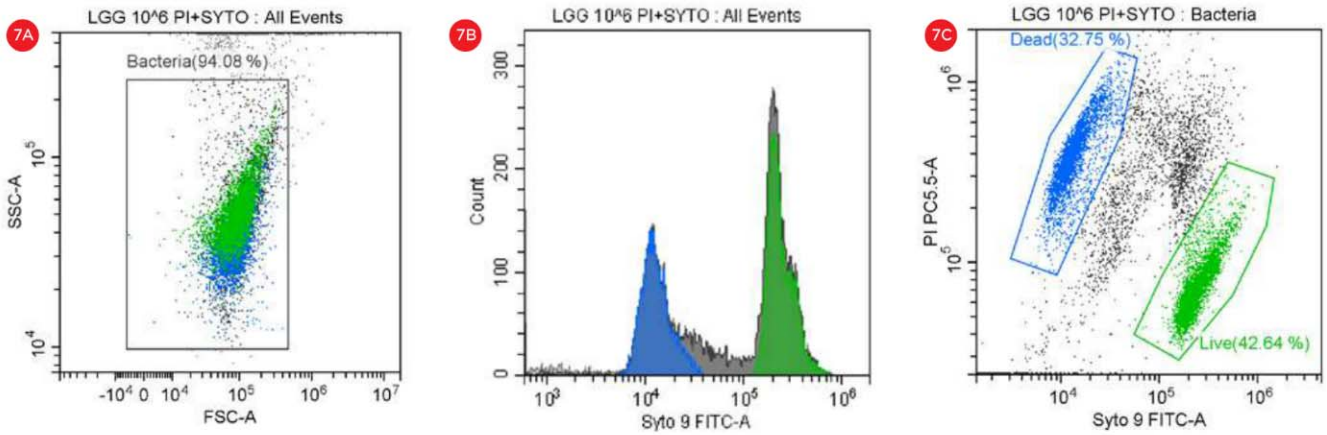

Figure 7. Gating Strategy for Live/Dead *L. rhamnosus* Analysis. The SYTO9 and PI staining is shown in a SSC vs FSC dot plot (panel A). A histogram shows the peaks representing the data collection in the FITC channel (panel B). The events in blue are non-viable and the events in green are viable. The PI staining is shown in a PI-PC5.5 vs SYTO9-FITC dot plot (panel C).

Conclusion

Flow Cytometry is a viable option for a non-culture based method for enumerating cells for probiotics manufacturers. Using the CytoFLEX flow cytometer with its ability to resolve small particles down to 200 nm, we were able to reliably count bacteria as small as 0.5 µm.

The results obtained in this study indicate that flow Cytometry is applicable for probiotic cell count assay; both dyes, SYTO9 and PI were able to identify viable and non-viable cells, providing the analyst additional information on the status of the cultures.

The cell counts obtained with flow cytometry are in good agreement with the plate count method, for *Lactobacillus fermentum, Lactobacillus rhamnosus* and *Bifidum longum*. With the flow cytometry method we anticipate a higher viable cell count than that observed with a Plate Count method. Flow Cytometry counts are typically higher due to the detection of viable, but non-culturable organisms.

The workflow improvements with time to result as low as 2 hours versus 3 days for the traditional method, as well as the additional information provided on viable versus non-viable cells will allow manufacturers to save time and labor as well as provide for new capabilities for in line process testing which can be used to optimize production processes.

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2. Enumeration of probiotic strains: Review of culture-dependent and alternative techniques to quantify viable bacteria. Catherine Davis, Medical Microbiology & Immunology, School of Medicine and Department of Biology, College of Arts & Sciences, Creighton University. 2014. Published by Elsevier B.V. This is an open access article under the CC BY license.

3. Development of a Flow Cytometric Method to Analyze Subpopulations of Bacteria in Probiotic Products and Dairy Starters. Christine J. Bunthof and Tjakko Abee. *Laboratory of Food Microbiology, Department of Agrotechnology and Food Sciences, Wageningen University, 6700 EV Wageningen, The Netherlands,* Received 2 October 2001/Accepted 13 March 2002.

BECKMAN COULTER Life Sciences

FLOW-2747APP06.17

Beckman Coulter CytoFLEX Violet SSC: an Alternative to FSC PMT or Fluorescence in the Detection of Extracellular Vesicles

TECHNICAL INFORMATION BULLETIN

Principle of the Technique

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Introduction

Over the past decade, there has been a rapid growth in studies of secreted membrane vesicles, collectively called extracellular vesicles (EVs). (1) The release of EVs has been reported in the pathologies of cancer (2-5), neurological, hematological (6), cardiovascular (7), autoimmune and rheumatologic diseases (8), and infections such as malaria (9). The study of EVs is gaining increasing interest within both the medical and scientific communities due to the diagnostic and therapeutic possibilities. However, the identification and classification of EVs has been problematic. Although advances in various fields, including microscopy, have addressed some of the preliminary hindrances, flow cytometry remains the dominant approach for the characterization of submicron cell- derived particles. The primary hurdle in analyzing particles at the submicron level has been to accurately represent their size distribution and light scatter profiles. Instrumentation thresholds were originally designed using whole blood as the standard, thereby excluding cellular measurement below 3um. Recently, flow cytometric technology has been developed to distinguish populations spanning the <400nm to 1um range. In this independent study, several of those technologies are evaluated and compared. As most of the hardware adjustments are accomplished by enhancements to the FSC parameter, the study will also evaluate the use of Violet SSC on Beckman Coulter's CytoFLEX as a novel approach to small particle detection. According to Mie theory, it is hypothesized that Violet SSC will give comparable results, as the lower wavelength will allow for detection of smaller particles.

Overview of Methods

Bangs Labs' Dragon Green Beads, Beckman Coulter's PCS controls and Spherotech's SPHERO Nano Fluorescent Particles will be acquired on several different instruments. Each of these instruments utilizes slightly differing hardware features to enhance the instruments ability for small particle detection. 192nm, 520nm and 780nm Dragon Green Beads were obtained from Bangs Laboratories, Inc. Dragon Green is an excellent spectral surrogate for fluorescein (488nm/530nm), and is suitable for use with fluorescein filter sets.

A photon correlation spectroscopy (PCS) latex, 5 x 15 mL, mixed kit was obtained from Beckman Coulter, Inc. 100nm, 200nm, 300nm and 500nm latex beads were chosen. PCS controls are non-labeled and are comparable in scatter profile to Dragon Green Beads.

SPHERO Nano Fluorescent Particles were obtained from Spherotech, Inc. 130nm fluorescent yellow particles are visualized in the fluorescein (488nm/530nm) channel and will be acquired for verification of <200nm detection.

For this flow cytometric assay, particles were chosen at the sizes listed above for later cell tracking. By using beads of differing sizes and fluorescent intensities, one can optimize the flow cytometer for cellular analysis. The bead sizes were chosen to be comparable to the size of the cells being analyzed. Therefore, all voltages, gains, and threshold settings were optimized for the both Dragon Green Beads and PCS controls to develop a relative size distribution matrix. The bead concentrations have been previously determined by serial dilution and subsequent measurement on Beckman Coulter's

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MoFlo Astrios EQ, MoFlo XDP with Propel Labs NanoView attachment, and Gallios flow cytometers in conjunction with manufacturer's specifications.

Instrumentation

MoFlo Astrios EQ

The Beckman Coulter MoFlo Astrios EQ is equipped with two FSC PMT pathways separated by a beam splitter (60/40 split). FSC1 is a direct laser beam pathway, with the FSC2 being directed at an angle from the beam splitter. Seven different and unique masks are provided to optimize particle identification and focus laser light to the photo multiplier tubes (PMTs). Due to this specific design, particles from 200nm to 30um can be identified (together or individually). In addition, The MoFlo Astrios EQ allows for triggering from any of the scatter parameters associated with the seven laser lines.

For this study, the beam splitter is removed from the FSC assembly. This allows the maximum amount of laser light to be detected through the FSC1 detector. In addition, a P1 mask was chosen after testing all the masks (data not shown). For small particle detection, the larger opening on the P1 mask allows for maximum resolution and dynamic range. Finally, 488 SSC was used for triggering. While not a manufacturer's recommended procedure, the Flow Core at Beth Israel Deaconess Medical Center has found this to be a viable and effective methodology.

MoFlo XDP with NanoView module

Propel Labs' NanoView forward scatter detector (FSC) was integrated onto a Beckman Coulter MoFlo XDP cell sorter. The NanoView design has improved the optical and electrical systems over the standard FSC for the purpose of extending the detection range down to < 200nm particles. The new optical system design utilizes a custom aspheric imaging lens that has been optimized to collect the scattered light from the core stream and image it onto a 200 micron pinhole. The collection angles in the forward scatter direction extend up to 18 degrees, which is double the maximum collection angle of a standard MoFlo FSC detector. The pinhole serves to align the system and remove the stray laser light that has not been generated by the particle of interest and greatly reduces the background light that is received at the detector. The NanoView design has further improved the detection system by replacing the photodiode with a much higher sensitivity PMT detector in the FSC path.

For this study, the largest available blocker bar was used (±6.3° - ±12.6° angles blocked as rotated from horizontal to vertical) and set to an approximate angle of 45°. 488 SSC was used as the trigger parameter.

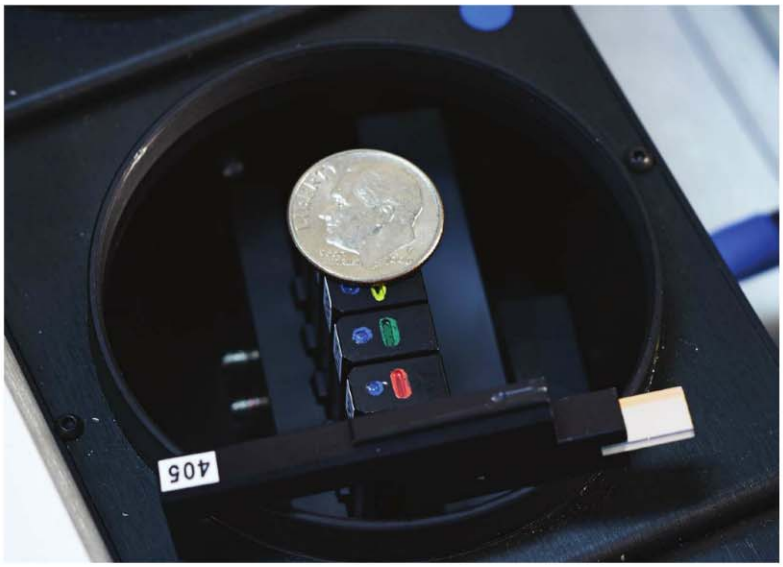
Gallios with Kaluza G

Beckman Coulter Gallios is equipped with an enhanced wide angle scatter setting, listed as submicron on Kaluza G acquisition software. The FS photodiode sensor collects the laser light that is scattered at narrow angles to the axis of the laser beam. The forward angle light is filtered with a 488nm band pass before reaching the FS sensor to generate voltage pulse signals. The FS sensor set at submicron allows for the detection angle to be measured at 9° to 19°. The SS photodiode sensor collects the light that is emitted 90° from the laser excitation point. The emitted light is focused by gel coupling of the flow cell and the light is filtered by a 488nm band pass filter.

CytoFLEX

Beckman Coulter's CytoFLEX is equipped with custom fluidics and the ability to use Violet (405nm) Side Scatter (VSSC) as a trigger parameter. The CytoFLEX has the ability to both trigger off and analyze by VSSC. This is an important feature when considering Mie theory and its effects on small particle detection.

Mie theory predicts that the scattering cross section of a particle, and thus its scattering intensity is dependent on the wavelength of light, the angle of collection, and the size, shape, and refractive index of the particle.



Inside the WDM module, the fluorescence light is divided and tightly focused through a series of band pass filters and integrated optics, on to an array of ultra-low noise silicon photo detectors.

All other factors being equal, using a **shorter illumination wavelength** will result in an increase in scattering cross section, and thus more scattered light.

Therefore, using the VSSC parameter, Dragon Green Beads will be visible and distinct below 500nm as lower wavelengths of laser light allow for smaller particle size detection. Additionally, the CytoFLEX sheath delivery can be easily controlled through the software interface. The intuitive software control allows the user to manually control the sample speed to maximize the amount of laser interrogation at slower uL/min flow rates. Hydrodynamic focusing is also enhanced to limit the ability of particle clustering known as swarming (10).

CytoFLEX Background

The proprietary optical design includes an integrated optics flow cell and photo diode detection system. In addition, all lasers are integrated to present optimal excitation. Emission of light is directed into dedicated fiber optical arrays, minimizing light loss and maximizing sensitivity.

CytoFLEX does not use PMTs – rather, CytoFLEX is the first commercial flow cytometer to utilize photo diodes for fluorescence channel detection. Photo Diodes, are very robust, linear, and sensitive.

The Fiber Array Photo Diode (FAPD) provides low-noise detection with high quantum efficiency and minimum light loss ensuring high signal to noise ratio and optical resolution especially with small particle measurements and dim fluorescence detection. The technology has its origin from the fiber optical communication industry, where the term Wavelength Division Multiplexing or WDM, originated. The CytoFLEX detection module collects the emitted light from each of the laser paths through high-efficiency fiber optic coupling. Each optical fiber delivers emitted laser light by a given excitation laser source, to a wavelength specific WDM detection module. Enhanced detection capability is achieved by using reflective, band-pass only filters to collect light and provide modularity and consistent sensitivity for all channels.

Instrument Optimization

Gating and Analysis

The Dragon Green Bead size distribution protocol, previously established in Research Application Note: Setting up the Beckman Coulter CytoFLEX for detection of Extracellular Vesicles, was applied to assess and measure EVs. Scatter properties were analyzed to determine the most efficient parameters for EV analysis.

QC was performed according to manufacturer's recommendations. All Instrumentation and protocols were configured for small particle detection. While instrumentation differs, the protocol remained consistent throughout. However, due to the MoFlo XDP with NanoView and MoFlo Astrios EQ's alignment system, micrometers on the FSC attachments were adjusted to maximize FSC signaling. The CytoFLEX required a bandpass filter change; no alignment or manual adjustments were performed.

A stock solution of filtered PBS with 0.1% Tween-20 is prepared. 520nm, 780nm and 192nm beads are diluted with the PBS/0.1% Tween-20 solution, to a final concentration of $1.29*10^7$ beads/mL.

Prior to dilution, the stock solutions of the test particles were sonicated to eliminate clumps.

The following samples were run on the Beckman Coulter CytoFLEX for instrument optimization:

1. 780nm Dragon Green Beads
2. 520nm Dragon Green Beads
3. 192nm Dragon Green Beads
4. 520nm/780nm/192nm Dragon Green Beads Mixed

- 780nm Dragon Green Beads were acquired to set the Scatter properties for differentiation between beads and low-end noise. In addition, SSC properties were adjusted to maximize resolution and dynamic range. The largest size is chosen first for ease of particle identification and to prove instrument ability to analyze below 1um.
- 192nm Dragon Green Beads were acquired to test the ability of the instrument to differentiate between the particle and noise. As particle size decreases, instrument Noise populations will begin to overwhelm the Dragon Green Bead's signal. 192nm Dragon Green Beads were used as most instrument manufacturer's specifications quantify lowest detectable level of 300nm.
- 520nm Dragon Beads were acquired for accuracy of separation of 192nm and 780nm beads. This allows for visualization of dynamic range of the instrument.
- Gates were drawn to encompass the three distinct populations.
- Mixed Dragon Beads were acquired to ensure proper gating and maximum separation of bead populations for the determination of a relative sizing distribution matrix. Furthermore, previous analysis has determined the ability of larger particles to mask the existence of their smaller counterparts (data not shown). Therefore, mixed populations verify the ability to separate and distinguish multiple populations.
- Voltages, gains and threshold settings can be adjusted to maximize performance. However, it is strongly suggested that single bead populations be acquired again, if changes are made to settings. Instrument has been optimized; the template and settings are saved for future cellular experiments.
- In addition to the Dragon Green Beads, a sample of the stock PBS solution is acquired for quantification of the background contribution of the PBS (Figure 1).

Results

Both the MoFlo Astrios EQ and MoFlo XDP with NanoView cell sorters were peaked to maximize resolution and separation of populations. The CytoFLEX and Gallios did not require any adjustments to alignment (thresholds, voltages and/or gains were set accordingly).

NOTE: 780nm Dragon Beads were not acquired on the MoFlo Astrios EQ due to the 520nm distribution. For this particular instrument, the resolution and dynamic range were evident by acquiring only the 192nm and 520nm Dragon Green Beads. All three Dragon Green Bead populations were easily separated from background and one another.

Visual inspection of the resolution and dynamic range of the four instruments shows the CytoFLEX be to comparable to the NanoView and MoFlo Astrios EQ, and slightly better dynamic range compared to the Gallios (Figures 2,3).

- Initial results, using Dragon Green Beads, indicate that VSSC is a viable option for the detection of EVs when compared to FSC PMT enhancements.
- Dragon Green Beads fluorescence (488nm/530nm) was used for verification of populations (Figure 4).

To further investigate VSSC as an alternative to the conventional mechanisms of EV detection, PCS control beads of 100nm, 200nm, 300nm and 500nm were evaluated. Results are expected to be similar to Dragon Green Beads. However, PCS controls do not contain a fluorescent marker. Therefore, measurements will be based solely on Scatter detection. Furthermore, due to the minimal change in the dynamic range between 0.19um and 0.52um Dragon Green Beads on the Gallios, it will be excluded from further investigation.
Using the Dragon Green Beads saved protocols on each instrument, the PCS controls were acquired according to the Dragon Beads protocol listed above.

- Clear separation between the Noise and 200nm PCS control was evident in all instruments tested.
- Dynamic range was consistent in all instruments tested.
- Resolution of populations was consistent in all instruments tested (Figures 5,6).

Upon visual inspection and comparison of the Mean Fluorescent Intensities (MFI) of the PCS controls ranging in size from 200nm to 500nm, the CytoFLEX, NanoView, and MoFlo Astrios EQ performed equally well (Figure 7).

Therefore, further evaluation of the CytoFLEX VSSC method of EV detection was done.

As most flow cytometric instrumentation with FSC enhancements have specifications of 200nm as the lowest detectable size, the CytoFLEX VSSC detection option has shown to be a viable option in EV detection. However, past studies have shown the MoFlo Astrios EQ and NanoView to be capable of <200nm for EV detection. Therefore, the CytoFLEX will be tested using both 100nm PCS control and

130nm SPHERO Nano Fluorescent Particle. By using both a fluorescent and nonfluorescent particle, a determination of feasibility of VSSC for <200nm EV detection can be made. First, 100nm PCS control will be acquired to determine if the particles can easily be separated from noise without sacrificing dynamic range (Figure 8).

Using the VSSC detection option on the CytoFLEX allowed for the acquisition of 100nm PCS control particles. However, adjustments in the scaling and threshold were required. 130nm SPHERO Nano Fluorescent Particle and the 192nm Dragon Green Beads were acquired on the CytoFLEX after scaling and threshold were adjusted. The fluorescent characteristics of the two sizing particles were used to separate the two particle populations (Figure 9).

- The two related sizes are difficult to separate using the Scatter parameter alone.
- By using the differing fluorescent intensities, the 130nm and 192nm sizing particles can be differentiated (Figure 10).

PBS Alone

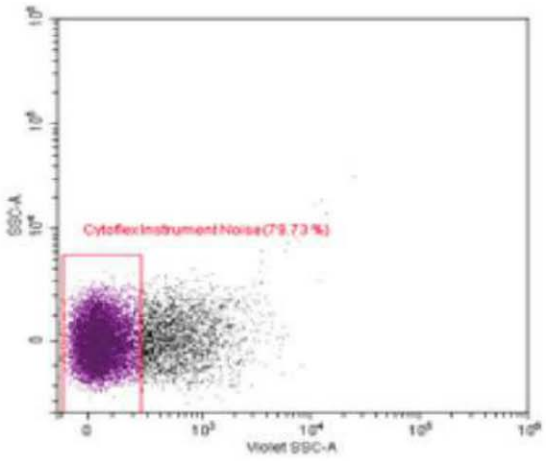


Figure 1. Stock PBS solution for quantification of the background contribution of PBS.

Dragon Green Beads CytoFLEX and Gallios

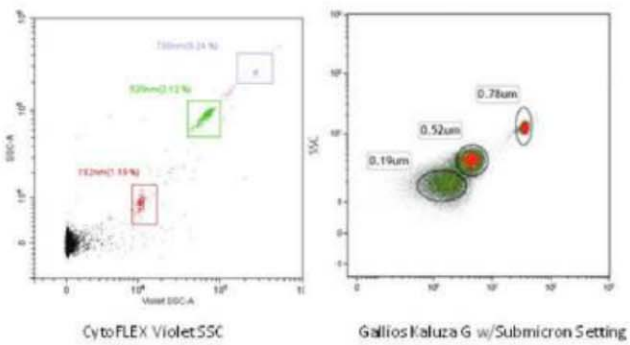
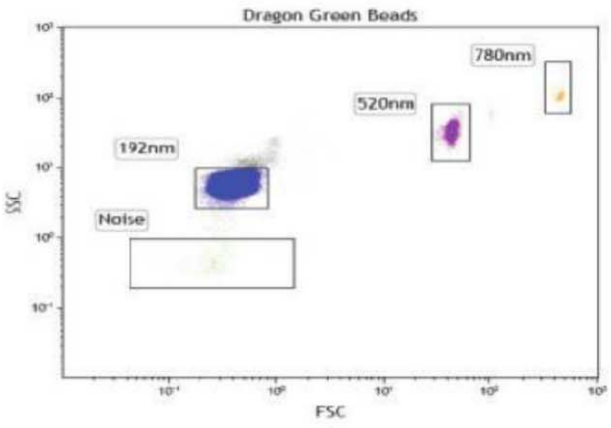
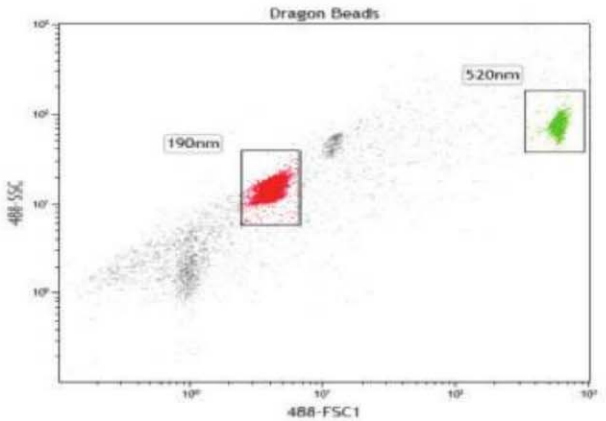


Figure 2 Verification of Dynamic Range by acquisition of 192nm, 520nm and 780nm Dragon Green Beads on CytoFLEX and Gallios.

Mixed Dragon Green Beads NanoView and Astrios EQ



NanoView 488 SSC trigger



Astrios EQ 488 SSC trigger/P1 Mask

Figure 3 Verification of Dynamic Range by acquisition of 192nm, 520nm and 780nm Dragon Green Beads on NanoView and 192nm and 520nm Dragon Green Beads AstriosEQ.

Dragon Green Beads Fluorescent Verification

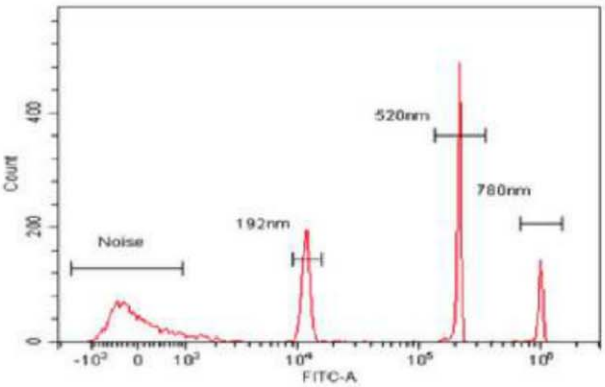


Figure 4 By using the fluorescent characteristics of the Dragon Green Beads, verification of size distribution based on fluorescent intensity can be used for data assurance.

PCS Controls CytoFLEX and AstriosEQ

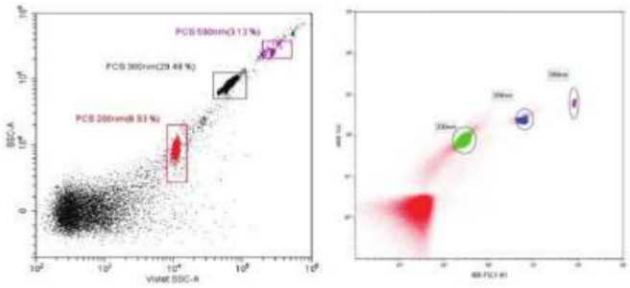


Figure 5 Photon Correlation Spectroscopy Control beads of 200nm, 300nm and 500nm were analyzed on the CytoFLEX and Astrios EQ. The data was used to compare size distribution across platforms using only scatter parameters.

PCS Controls MoFlo XDP w/NanoView

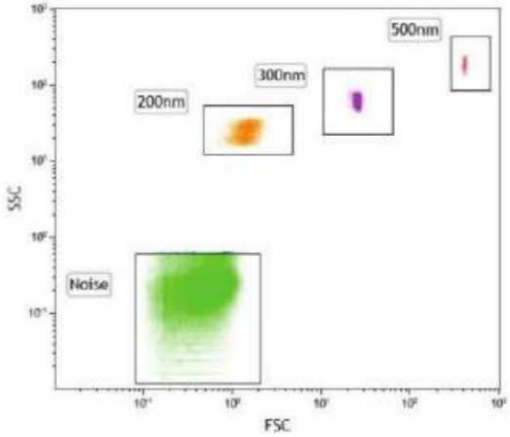


Figure 6 Photon Correlation Spectroscopy Control beads of 200nm, 300nm and 500nm were analyzed on the MoFlo XDP and compared to the Astrios EQ and CytoFLEX. The data was used to compare size distribution across platforms using only scatter parameters.

% Difference of MFI

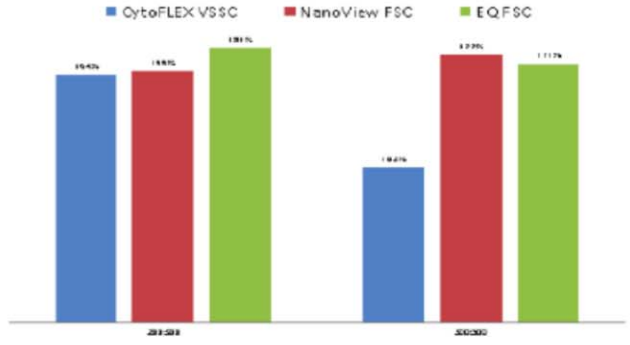


Figure 7 Measurement of Mean Fluorescent Intensity to calculate dynamic range and compare between the CytoFLEX and High End FSC modified Cell Sorters.

CytoFLEX 100nm PCS Control

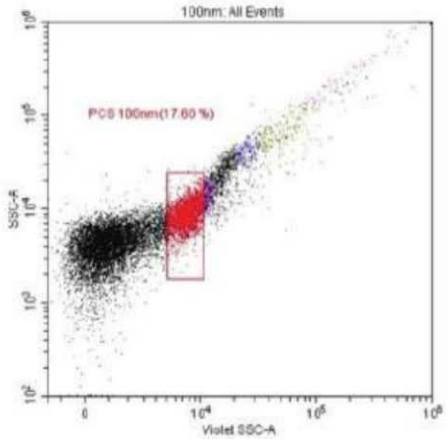


Figure 8 Photon Correlation Spectroscopy Control beads of 100nm in size were analyzed on CytoFLEX to establish a separation between instrument noise and particle.

130nm SPHERO Nano Fluorescent Particles and 192nm Dragon Green Beads

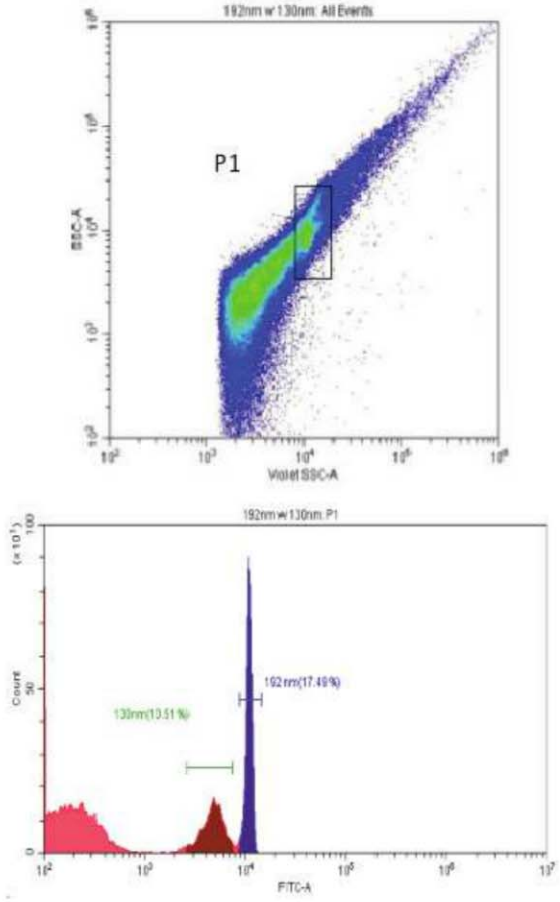


Figure 9 Nano Fluorescent particles and dragon beads 130nm and 192nm respectively were differentiated using a histogram of fluorescence intensities. The Scatter parameter alone was insufficient to separate the two bead samples.

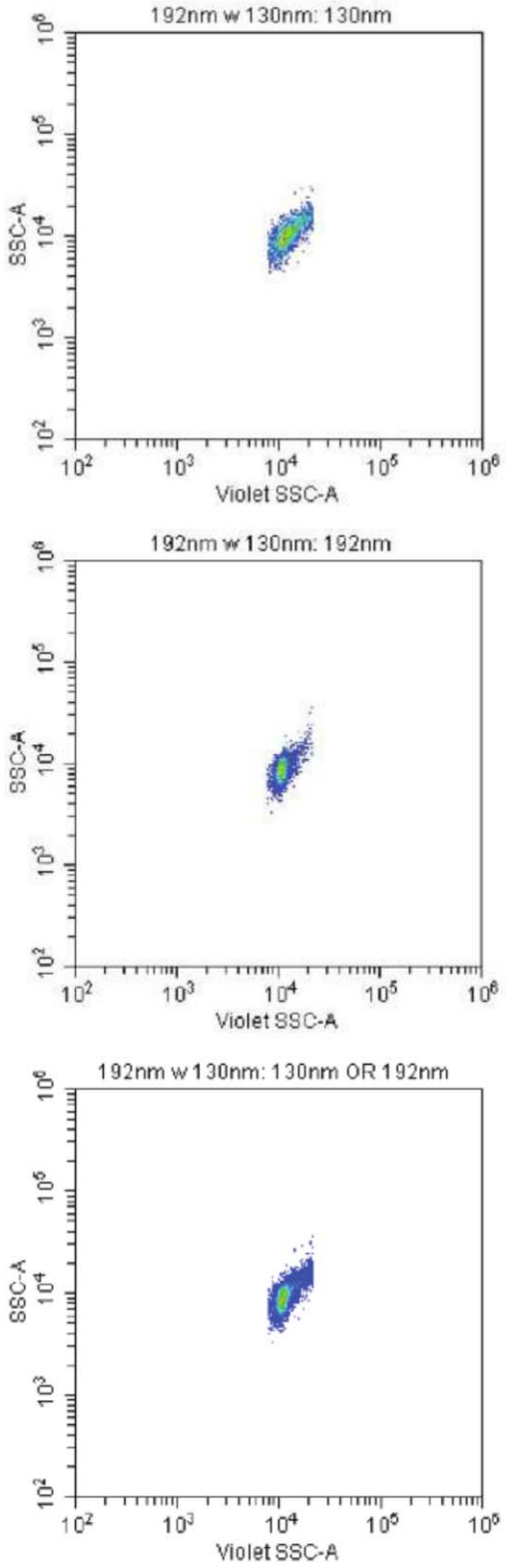


Figure 10 Back gating of the differing fluorescent intensities shows the inability to differentiate the populations via the scatter plots (Gated as listed in Figure 9). The 192nm DGBs mask the 132nm SPHERO Nano Fluorescent Particle.

Conclusion

The Beckman Coulter CytoFLEX's VSSC parameter has proven to be an effective tool for the detection of Extracellular Vesicles based on its ability to detect the different size beads as shown in the data. The ability to set the trigger and threshold using the VSSC parameter, allows the CytoFLEX to be comparable to its larger and more extensive counterparts.

By using methodologies and protocols previously established, the CytoFLEX was compared to three other flow cytometers where EV detection has been possible. Dragon Green Beads, PCS controls and Sphero Nano Fluorescent particles were acquired on a MoFlo Astrios EQ, MoFlo XDP with NanoView attachment, Gallios, and CytoFLEX. The CytoFLEX showed similarities in both resolution and dynamic range. However, there are limitations to the CytoFLEX VSSC detection system. Particles below 200nm can be resolved from the noise background to approximately the 100nm size range. Particles in the size range of 100nm to 200nm are not easily separated from one another on a Scatter Plot. Measuring differences in fluorescent intensity is the best means of separation.

The interest in the identification and detection of submicron particles has increased in recent years. The ability to study them has been hindered by available techniques to measure particles at sizes below 1um. Flow Cytometry has become an important tool in EV research with instrumentation being developed to identify particles at the submicron level. Instrumentation such as cytometers optimized to improve light scattering collection (11,12) and image cytometers (13). However, most equipment designed for the detection of EVs is expensive and complex. Hardware enhancements have focused around the development of the FSC PMT. While the FSC PMT enhancements have proven to enable the flow cytometer to detect particles <200nm in size, the instrumentation is not practical for all lab settings. In this comparison study, it has been shown that the CytoFLEX VSSC is compatible to 488nm SSC and to the results obtained from the AstriosEQ and NanoView enhanced FSC. The ability to resolve and distinguish the populations as effectively as its counterparts, has proven the CytoFLEX Violet SSC to be a viable alternative to the FSC PMT to detect EVs.

Methods based on the following Posters and Technical Papers

- Development of a Flow Cytometric technique for the study of Microparticles; Albert Mairuhu , R. Flaumenhaft, Vasilis Toxavidis, John Tigges
- NanoView: A Novel Approach To Microparticle Cell Sorting; J. Tigges, A, Vandergaw, V. Toxavidis
- Standardization of Flow Cytometry Instrumentation for the Analysis of Microparticles; J. Tigges, K. Groglio, E. Felton, M. Fahlberg, M. Schmelzle, R. Mairuhu, V. Toxavidis
- Microvesicle Detection and Cell Sorting; J. Tigges, V. Toxavidis
- MoFlo AstriosEQ – the New Standard in Forward Scatter and Fluorescence Dynamic Range Performance: a case study on microparticles ; V. Toxavidis, R. Sleiman, T. Reed, J. Tigges

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How to Use Violet Side Scatter to Detect Nanoparticles on the CytoFLEX Flow Cytometer

APPLICATION NOTE

Authors:
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IN THIS PAPER YOU WILL LEARN HOW

- To empirically test the lower threshold for nanoparticle detection on your flow cytometer
- The ability to detect small particles is directly correlated to the wavelength of light used for detection
- The use of violet side scatter on the CytoFLEX enables the detection and resolution of nanoparticles

Background

The detection of sub-micron particles by flow cytometry becomes increasingly difficult as the particle sizes progress smaller than the wavelength of the light being used to detect them. Standard detection of sub-micron particles by forward scatter is problematic because they have a lower scatter signature due to a smaller cross-sectional area, and there is an increasing tendency for isotropic refraction of orthogonally polarized light with decreasing particle sizes (Hielscher et al., 1997). While these properties impair the resolution of sub-micron particles from noise by forward scatter, they actually improve their detection and resolution by side scatter. When utilizing side scatter to detect small particles, a variety of factors can influence their detection, such as the differences between the refractive indices of the particles and their surrounding media, and the internal complexity of the particles being detected. In general, the larger the difference in the refractive indices, the more light will be scattered by the particles; and, the more granular the composition of the particles (i.e., intracellular vesicles, protein aggregates, metal ions, etc.), the more light will be scattered by the subparticle components. In addition, the amount of light scattered by any particle is directly proportional to the diameter of the particle and inversely proportional to the wavelength of the light being used to detect it. This relationship can be seen in the equations for both Mie Theory and Raleigh Light Scattering, which are used for calculating theoretical light scattering by particles either similar in size or much smaller than the wavelength of the light being used to detect them, respectively (Bohren & Huffmann, 2010). For this reason, the smaller violet (405 nm) wavelength will result in more orthogonal light scattering at any given particle size than the blue (488 nm) wavelength, and will increase the range of resolution to smaller particles than can be detected by standard side scatter. Moreover, upon entering a medium of a different refractive index, light waves are refracted by the new medium inversely proportional to the wavelength of the light, with smaller wavelengths having a higher refraction than larger wavelengths. This effect was first discovered by Isaac Newton when he split white light into a rainbow of individual colors using a prism, with red light refracting the least and violet light refracting the most (Figure 1) (Newton, 1704). Based upon this physical property, the use of violet light will help to amplify the differences in the refractive indices between the particles and their surrounding media, and in turn increases the ability to detect particles with a lower refractive index, such as exosomes, microvesicles and silica nanoparticles. The purpose of this paper is to demonstrate how to setup the CytoFLEX flow cytometer to detect small particles by Violet Side Scatter (V-SSC). For practical considerations important to the analysis of small particles by flow cytometry, please refer to recent publications (Nolan, 2015; Poncelet et al., 2015; Arraud et al., 2015).

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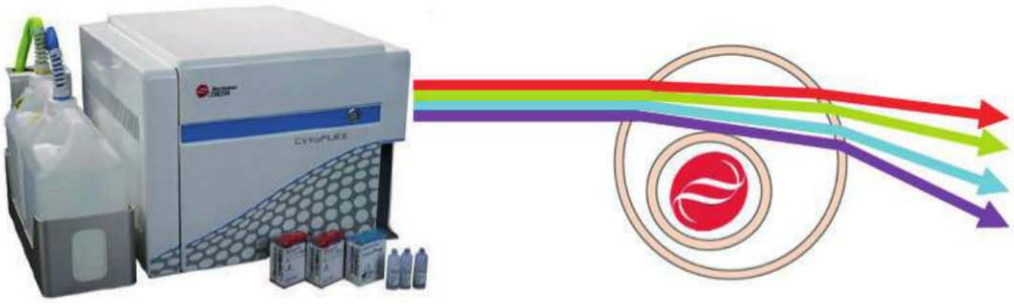

Figure 1. A simplified depiction of Newtonian light refraction through a cell based upon wavelength.

Setting up the CytoFLEX to Detect Violet Side Scatter

Setting up V-SSC detection on the CytoFLEX is easy. In the CytExpert program, simply select the Cytometer tab within the Menu Bar, and then select Detector Configuration. Within the Detection Configuration pop-up window, replace the 450 nm filter with the 405 nm filter, and appropriately label the detector as V-SSC. Once the detector configuration is saved, select that configuration to be the current setting, and physically move the 405 nm filter into the 450 nm slot within the instrument. At this point, you will need to start a new experiment within the CytExpert program for the new detector configuration to apply, and you are ready to go.

Once running, in order to properly separate nanoparticles from background noise using V-SSC, you will need to open up the Acquisition Settings Menu, select V-SSC as the primary trigger (Height is better than Area), and then manually adjust the trigger level until you reach the discrimination threshold between the noise and actual events. This trigger level is usually very consistent with the CytoFLEX at any given laser intensity and PMT voltage, both between experiments and on different days.

Several considerations to keep in mind are that you will need to adjust the range of the V-SSC histogram in order to hone in on the size range of interest since the default chart settings will have the smaller particles pushed up against the y-axis; once within that range, a logarithmic scale will provide for a better distribution of multi-modal particle sizes; and, if the scaling for the Counts axis is selected to "Fit to Sample", it will always re-scale the data to fit to the entire histogram. The latter issue can create confusion particularly if you set the threshold trigger using buffer alone rather than determining it empirically using the smaller particles of interest. In this case, the histogram for the noise will continue to readjust to the maximum regardless of where you place the trigger, and without the particles as a reference, the chosen trigger setting may actually cut off or eliminate the particles of interest when you begin testing. It is best to set the trigger using the smallest detectable particles that you have available, which can clearly be discriminated from the background noise. Using these, you can hone in on the real sample population, and then back the trigger off until you are comfortable with the level of noise that remains (Figure 2). However, if the chosen threshold level is too low, you will have an increased abort rate due to the noise. Ultimately, you should try to keep the abort rate below 5-10 % either by adjusting the threshold, the sample concentration, or the flow rate. Keeping the sample concentration and flow rate low is also important to prevent experimental artifacts due to coincidence (i.e., swarming) (Nolan, 2015; Nolan & Stoner, 2013).

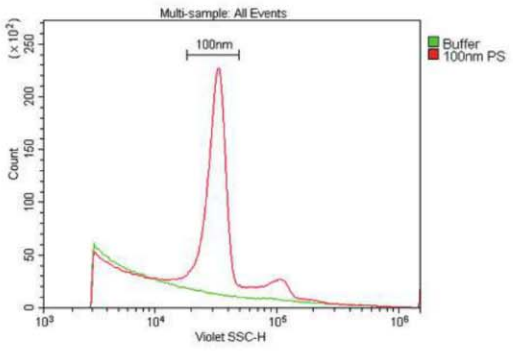

Figure 2. An example of the separation between a real sample population and background noise while setting the V-SSC trigger threshold. This sample is 100 nm polystyrene beads at a 1:100K dilution in 0.02 µm-filtered sheath solution.

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Considerations for Sample Preparation

The analysis of micro- and nanoparticles requires some careful preparation of the samples to be analyzed. First, the buffer that the samples are suspended in should be filtered with an appropriate molecular-weight cutoff in order to eliminate any background debris that may fall within the range of your populations of interest. Second, as is commonly mentioned in protocols and data sheets, the samples should not be mixed in such a vigorous manner as to create air bubbles immediately prior to reading. These bubbles will interfere with data acquisition, as they also refract light and will be detected as events. Third, a lot of small synthetic particles have the tendency to clump together, resulting in the formation of aggregates that may be either inaccurately sized or aborted altogether. In the case of either bubbles or aggregation, sonicating the samples prior to reading can help. After sonication, the samples should be read as quickly as possible because aggregation will begin anew and the sample population(s) will be affected over time (Figure 3). If sonication is not acceptable, such as with biological samples, then simply triturate the samples as well as possible in order to evenly distribute the sample prior to acquisition. Finally, the concentration of the sample should also be titrated in order to determine the optimal working range for acquisition by flow cytometry, as higher concentrations will lead to aggregation and/or swarming (Figure 4).

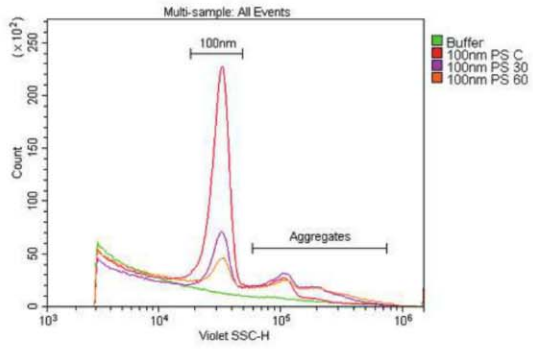

Figure 3. The reduction in signal of a polystyrene nanoparticle population over time. These samples are 100 nm polystyrene beads at a 1:100K dilution in 0.02 µm-filtered sheath solution read at 0, 30, or 60 minutes after sonication.

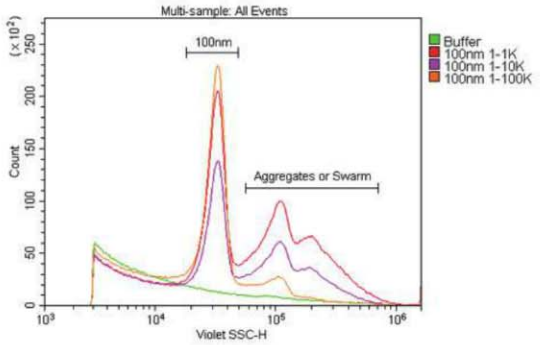

Figure 4. Finding the optimal concentration for the nanoparticle sample. These samples are 100 nm polystyrene beads in 0.02 µm-filtered sheath solution at a dilution of 1:1K, 1:10K or 1:100K from the 1 % stock solution. From these results 1:100K could clearly be seen to provide both the best signal and least aggregation or swarming of the dilutions tested.

Testing the Performance of the CytoFLEX with Nanoparticle Detection by Violet Side Scatter

The objective of the following experiment was to demonstrate the ability of the CytoFLEX to detect nanoparticles smaller than 200 nm by the use of V-SSC.

Instrument and Reagents

Product	Catalog Number	Company
3-Laser CytoFLEX	B53000	Beckman Coulter
CytoFLEX Sheath Fluid	B51503	Beckman Coulter
Whatman Anotop 25 0.02 µm Filters	09-926-13	Thermo Scientific
40 nm Polystyrene Beads NIST	09-980-015	Thermo Scientific
100 nm Polystyrene Beads NIST	09-980-021	Thermo Scientific
200 nm Polystyrene Beads NIST	09-980-024	Thermo Scientific
Multimodal Particle Size Standards	MM-010	Thermo Scientific
94 nm Silica Beads NIST	147020-10	Corpuscular, Inc.
150 nm Silica Beads NIST	147030-10	Corpuscular, Inc.
200 nm Silica Beads NIST	147040-10	Corpuscular, Inc.

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Results

We first compared the sensitivity of nanoparticle detection by V-SSC to standard 488-SSC using Duke Multimodal Particle Size Standards with a mix of 80 nm, 200 nm and 500 nm polystyrene beads. As can be seen in Figure 5A, the V-SSC could easily discriminate the smaller 80 nm and 200 nm particles, while 488-SSC did not begin to discriminate the populations until between 200 nm and 500 nm. Determining the signal-to-noise ratios of the different populations detected by FSC, 488-SSC, or V-SSC, the V-SSC can be seen to perform better than 488-SSC at all measured particle sizes (Figure 5B). By histogram, you can more clearly see the population densities and that the CytoFLEX using V-SSC was easily able to discriminate polystyrene nanoparticles down to 80 nm using the Duke Multimodal Particle Size Standards (Figure 6). Polystyrene particles as small as 40 nm were able to be clearly detected above background, but they were located right on the noise threshold (Figure 7). Since the polystyrene beads have a refraction index of 1.5915 (at 589 nm), we also tested silica nanoparticles with a refraction index of 1.4584 (at 589 nm), closer to the range for exosomes and microsomes, which have a mean refraction index of around 1.426 (Gardiner et al., 2014). As a result, we were able to easily discriminate 150 nm and 200 nm silica nanoparticles, while 94 nm silica nanoparticles were detectable but on the noise threshold (Figure 8).

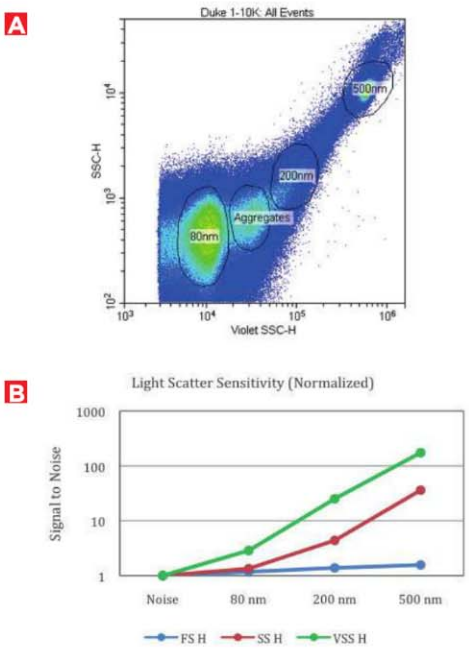

Figure 5. Comparing the sensitivity of nanoparticle detection by V-SSC to 488-SSC.

A) Dot plot showing V-SSC vs. 488-SSC. B) A plot of the signal-to-noise ratios for the different nanoparticles using FSC, 488-SSC, or V-SSC. These are Duke Multimodal Particle Size Standards (80nm, 200 nm and 500 nm) in 0.02 µm-filtered sheath solution at a 1:10K dilution.

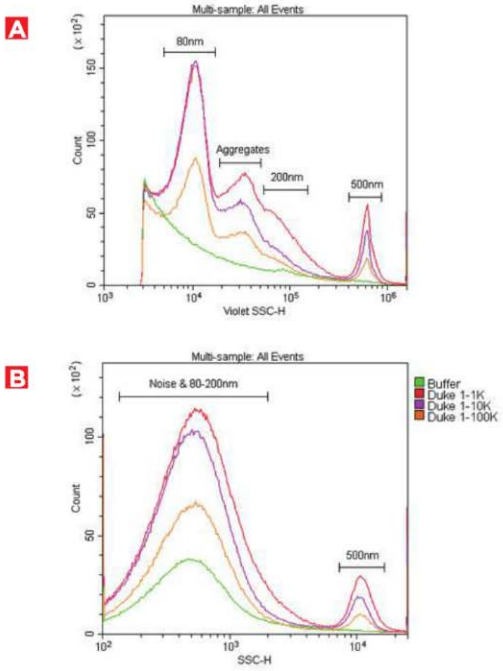

Figure 6. The detection and discrimination of 80 nm, 200 nm and 500 nm polystyrene nanoparticles by V-SSC.

A) V-SSC. B) 488-SSC. These are Duke Multimodal Particle Size Standards (80 nm, 200 nm and 500 nm) in 0.02 µm-filtered sheath solution at a dilution of 1:1K, 1:10K or 1:100K from the 1 % stock solution.

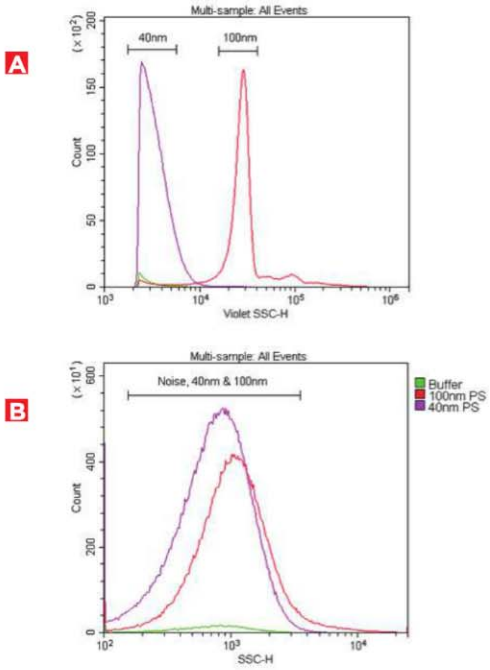

Figure 7. The detection of 40 nm polystyrene nanoparticles by V-SSC.

A) V-SSC. B) 488-SSC. These samples are 40 nm or 100 nm polystyrene beads in 0.02 µm-filtered sheath solution at a dilution of 1:10K or 1:100K, respectively (1 % stock).

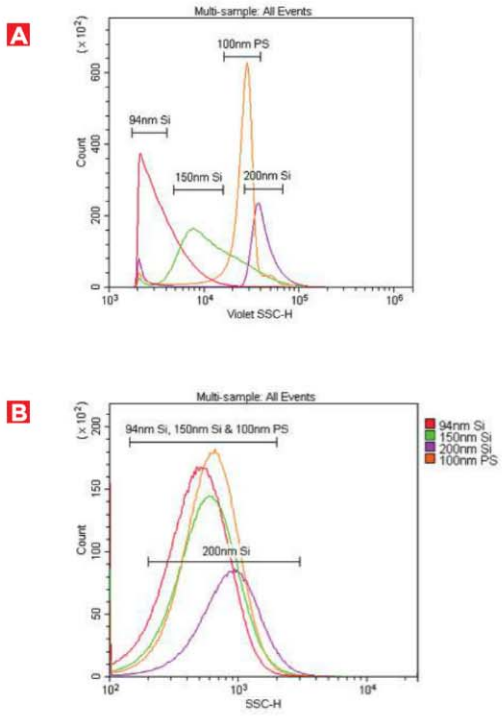

Figure 8. The detection of silica nanoparticles by V-SSC.

A) V-SSC. B) 488-SSC. These samples are 94 nm, 150 nm or 200 nm silica or 100 nm polystyrene nanoparticles in 0.02 µm-filtered PBS. The silica nanoparticles were diluted 1:10K, while the polystyrene nanoparticles were diluted 1:100K, from the 1 % stock solution.

Conclusions

Using V-SSC, the CytoFLEX was able to clearly discriminate 80nm polystyrene and 150 nm silica nanoparticles from background noise. Moreover, the detection and discrimination of nanoparticles by V-SSC was better than 488-SSC for all particle sizes tested. Based upon these findings, we can confidently say that the CytoFLEX can be used for the detection of extracellular vesicles at least as small as 150 nm by V-SSC. It may be possible to use V-SSC to detect even smaller vesicles, as previous research has suggested that the refraction index of extracellular vesicles is inversely proportional to the size of the vesicles, with smaller 50 nm vesicles having a refraction index closer to 1.6 (similar to polystyrene) (Gardiner et al., 2014); however, this will need to be determined empirically.

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Set-Up of the CytoFLEX for Extracellular Vesicle Measurement

APPLICATION NOTE

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IN THIS PAPER YOU WILL LEARN

- How to setup the CytoFLEX for small particle analysis
- How to eliminate background particles found in buffers and reagents to enhance your small particle detection
- How to use control beads to calibrate the CytoFLEX for microparticle detection

Background

The measurement and the characterization of Extracellular Vesicles (EV) have been of growing interest over the last 20 years. Flow cytometry instruments were not the most appropriate way to analyse these particles as the optical resolution of instruments was insufficient to detect particles below 250 nm. However, the Beckman Coulter CytoFLEX now offers the ability to measure EV down to at least 150 nm and allows the detection of their cellular origin using up to 13 fluorescence parameters. Regardless of the technical improvements the set-up of the instrument is still a critical point and several requirements need to be met which are illustrated here.

Introduction

Extracellular vesicles are a heterogeneous cell-derived particle population in a size range between 50 nm to 1,000 nm. There is a growing interest not only from academic research groups to determine EV in several fluids such as cell culture supernatant, in plasma samples or in whole blood but also in clinical research since it has been shown that the measurement of microparticles (MPs)[1] might be of clinical relevance. The methods to identify EV are many and involve high speed centrifugation, Western blotting, proteomics, electron microscopy, imaging methods and flow cytometry. Methods for the detection of EV by flow cytometry have been developed in the last years and special attention has been paid to standardization protocols. Compared with other methods, flow cytometry has the big advantage that EV can be detected as rare events, in high numbers and by antigens on the surface, which characterize their cellular origin.

However, until now flow cytometry technology has had some unfortunate limitations. It was not possible to detect microparticles below 250-300 nm in size in a meaningful manner. This size range does not appear to be very far from the smallest particles of 50 nm in size, however we have to consider that MPs in a size greater than 300 nm are only the "tip of the iceberg" of visible particles and at least as many particles are smaller than 300 nm in size. The importance in clinical research and the technical requirements to detect smaller microparticles was clearly demonstrated in 2013 by Sarlon-Bartoli et al[2]. Using a Beckman Coulter Gallios

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flow cytometer they correlated an increase in plasma levels of leukocyte-derived MPs with unstable plaque in asymptomatic patients with high-grade carotid stenosis. These differences between sample groups were detectable using the Gallios flow cytometer which allowed for better discrimination between noise in an acceptable range and extracellular particles. The CytoFLEX is the first flow cytometer which can detect EVs in a meaningful way down to 150 nm and therefore offers the possibility to detect particles below 300 nm which enhances information. The better resolution of the CytoFLEX can be reached by using the side scatter of the 405 nm laser and by several technical as well as preanalytical preparations. Here we describe how to set-up and standardize the CytoFLEX for particle measurement and discuss some pitfalls which should be avoided to get the best information from EVs detection.

Protocol

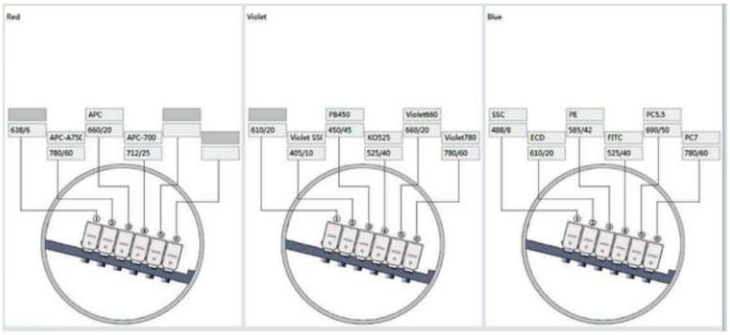
Instrument set-up

Turn on the CytoFLEX and the computer. Proceed with the daily start-up procedure and execute the QC measurement using the *Default Filter Configuration*. Change your filter configuration as follows.

Filter configuration

Change your filter configuration of the Violet laser (405 nm) as shown in Figure 1. The Violet SSC (VSSC) 405/10 channel will now serve as trigger channel and discriminates the noise.

Figure 1. Filter configuration (Trigger signal on VSSC405 nm)



Set-up Dotplots.

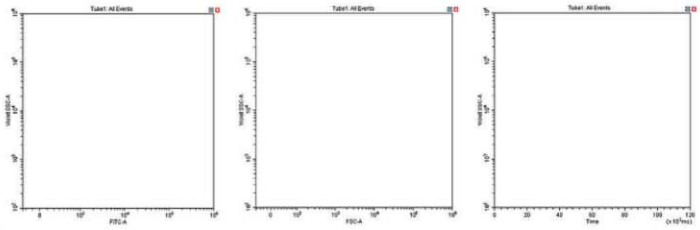
Create 3 Dotplots and 1 Histogram

Dotplot 1: VSSC 405 nm, log–FL1 488nm, log (Figure 2) detects the Gigamix beads (see below) and triggers the noise

Dotplot 2: VSSC 405 nm, log – FSC 488nm, log (Figure 3) determines the region for size

Dotplot 3: Time (120 sec) – VSSC 405 nm, log (Figure 4) follow events during washing steps

Figure 2. Dotplot 1 Figure 3. Dotplot 2 Figure4. Dotplot 3



Prepare additional dotplots and histograms according to your fluorescence staining needs.

Reagents

Prepare the Gigamix solution. The Gigamix solution is a mixture of fluorescent Megamix-Plus SSC and Megamix-Plus FSC beads (BioCytex a Stago group company, Marseille, France) which have different sizes (100, 160, 200, 240, 300, 500, 900 nm) and are recommended for daily standardization for microparticle measurement on the CytoFLEX.

- Vortex the beads for at least 10 seconds each.
- Mix 0.25 mL Megamix-Plus FSC reagent (0.1 µm, 0.3 µm, 0.5 µm and 0.9 µm.) with 0.25 mL Megamix-Plus SSC reagent (0.16 µm, 0.20 µm, 0.24 µm and 0.5 µm.) according to the package instructions provided.

Set-up the Gains and the Threshold

Set the *Threshold* of the trigger signal (VSSC) manually to 2000 and Height Figure 5 Threshold and set the gains of the FSC to -106, VSSC to -61 and FITC to -272 Figure 6 Gains.

Figure 5. Threshold

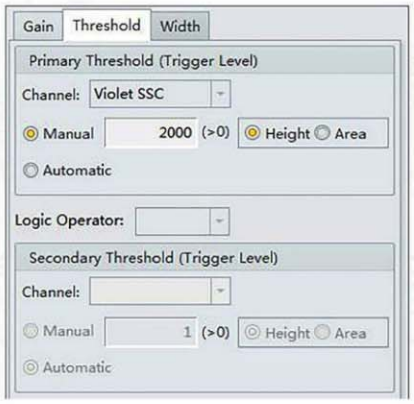
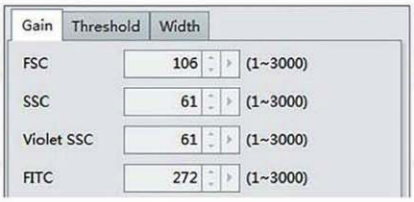


Figure 6. Gains



Clean your sample line with fresh and sterile distilled water for 2 minutes at a flow rate of 60 µL/minute.

- Increase/decrease VSSC gain to an event rate of ~400 events/sec.
- Measure Megamix beads as shown in Dotplot 1 at a flow rate of Slow = 10 µL/minute.
  a) increase/decrease SSC 405 nm gain.
  b) increase/decrease FL1 gain according to Figure (Dotplot 1).
- You should now see a picture as shown below Figure 7 Gigamix beads. If this picture is not seen readjust the gains for FSC, VSSC and FITC until it displays Figure 7.
- Save your sample as "Gigamix".
- For better visibility set 2 regions: the first around the 100 nm beads (blue) and the second around the 900 nm beads (red) as shown in Figure 8.

Figure 7. Gigamix beads ungated

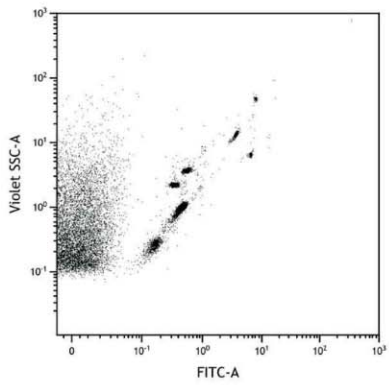
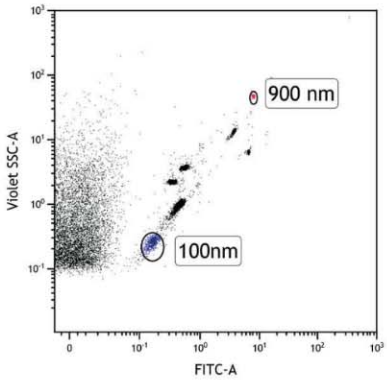
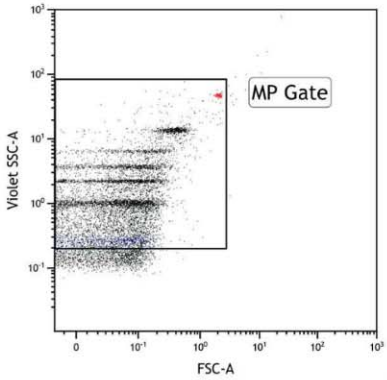


Figure 8. Gigamix beads gated



In Dotplot 2, set a region as shown in Figure 9 around the 900nm and the 100nm bead population. Label this region as MP Gate – in this gate MPs are displayed. The following dotplots and histograms should be gated on the MP gate.

Figure 9. MP Gate setting



After you have measured the Gigamix beads thoroughly rinse the sample line with distilled water for 2 minutes at flow rate *Fast* = 60 µL/minute and watch Dotplot 3 and the Histogram. At the end of the 2 minutes cleaning procedure you should reach an event rate per second which is equal to the first washing step at the beginning of your experiments (~400 events/sec); repeat the washing procedure if it does not return to baseline. Repeat the 2 minutes washing step between each sample measurement!

Sample measurement

Measure your sample at the flow rate *Slow*. Adjust the gains for the other fluorescence parameters according to your staining protocol and your needs.

Examples and pitfalls

Extracellular Vesicle staining

An example from Extracellular Vesicle measurement is shown below (Figure 10) and illustrates detection of Annexin V FITC, CD41 PE and CD63 PE-Cy7 stained particles. Isolation was performed by centrifugation of a platelet concentrate. Particles were subsequently stained and analysed on a CytoFLEX. Figure 10A shows particles in the MP gate. Figure 10B and C demonstrate autofluorescence intensities of particles in the relevant fluorescence channels. Figure 10D shows detection of two Annexin positive populations. The Annexin dim population is indicated as green, expresses CD41 but no or low CD63 and are of a very small in size. The Annexin high population is also CD41 and CD63 double positive and a larger size. (Figure 10E and F)

Figure 10A

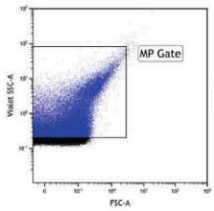


Figure 10B

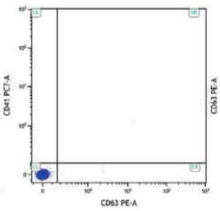


Figure 10C

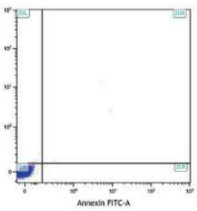


Figure 10D

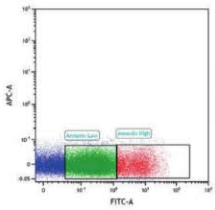


Figure 10E

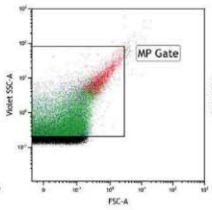
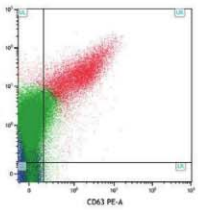


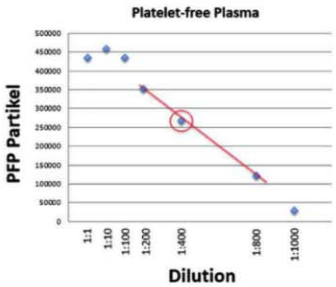
Figure 10F



Swarm detection

When multiple vesicles are simultaneously illuminated by the laser beam and are counted as one larger single cell event, this phenomenon is referred to as swarm detection. As a result the true concentration of EVs is underestimated. To avoid this problem a serial dilution assay has to be performed and the optimal EV concentration which is in the linear range of dilution and EV concentration has to be calculated Figure 11.

Figure 11. Linear range of detection.



Particles from Platelet Free Plasma (PFP) were serially diluted from 1:2 to 1:1000 and measured on the CytoFLEX. The red line indicates the linear range of particle measurement without swarm detection. In the present case a dilution of 1:400 gives the best results.

Sample Media

Each media or buffer in which the sample was diluted was analysed using the Gigamix setting (VSSC Gain: 61, Threshold: 2000, FSC Gain: 106) for 120 seconds at flow rate Slow (10 µL/min)

The following figures show examples of measurements using various samples. NOTE: it is highly critical to clean the sample line between each measurement for at least 2 minutes. Figure 12A shows Gigamix beads measured over time for 2 minutes. Washing after Gigamix (Figure 12B) demonstrates that beads are washed out of the system after about one minute; this varies for different bead populations.

The background noise of distilled water is shown in Figure 12C, even PBS (Figure 12D) slightly increases the background particle noise which is much more elevated when fetal calf serum (FCS, Figure 12E) is added to the sample media. As can be seen in the Figure 12F PBS with 10 % FCS, dots in the red oval indicate the appearance of particles, possibly extracellular events and/or protein aggregates present in the FCS.

Figure 12.

Figure 12A. Gigamix

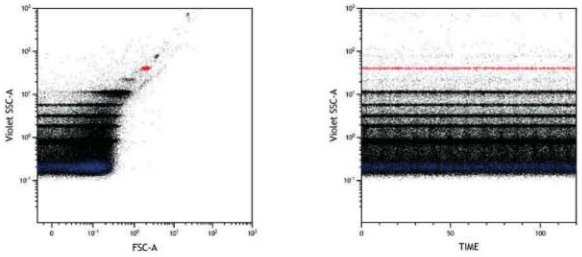


Figure 12B. Washing after Gigamix

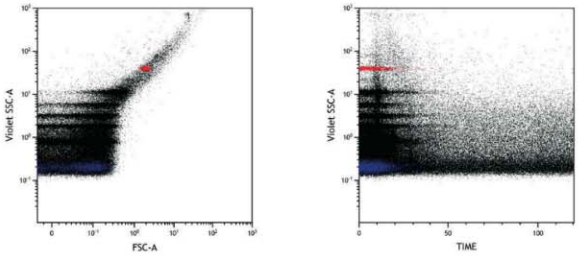


Figure 12C. Distilled water Events/sec: 557

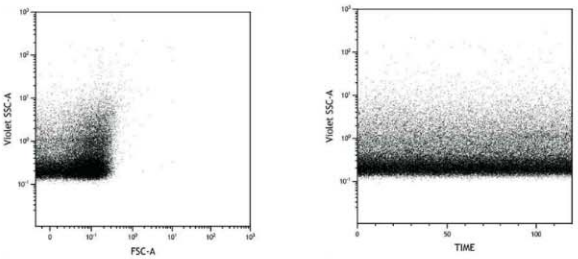


Figure 12D. PBS Events/sec: 533

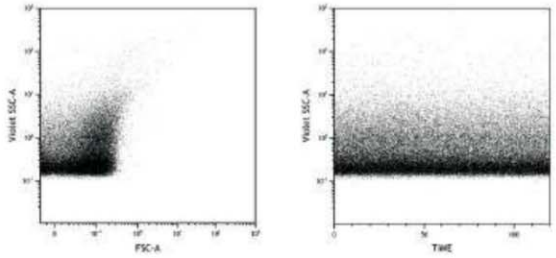


Figure 12E. PBS with 1% FCS Events/sec: 2194

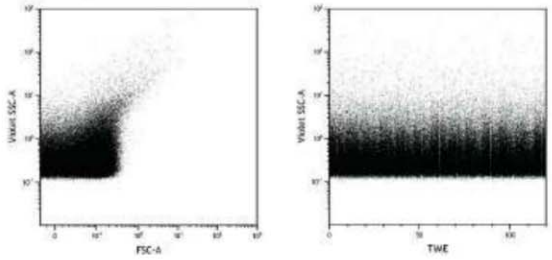
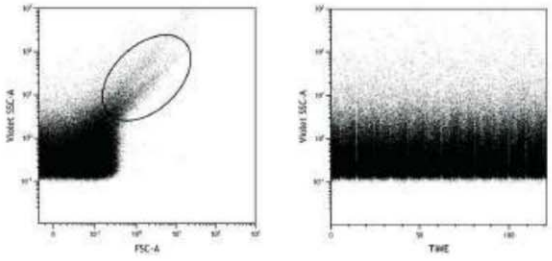


Figure 12F. PBS with 10% FCS Events/sec: 4082



Antibodies and reagents diluted in distilled water

20 µL of monoclonal antibody solution or 1 µL of Annexin V solution with or without centrifugation, were subsequently diluted in 250 µL distilled water and measured for 120 seconds at a flow rate SLOW. As a control, Pentaglobin (IgG, IgM, IgA) solution was diluted in distilled water in a concentration of 1:100. Coloured markers were set for each channel indicating the background of unstained immunoglobulins (Pentaglobín, Figure 13A). Interestingly as can be seen in Figure 13 below, there was a signal for antibody aggregates specific for CD16-FITC, CD14-APC, CD45-Krome Orange and Annexin-FITC; however, the strongest signals were detectable for APC and Krome Orange. Since there was no compensation set, FITC and Krome Orange spillovers could be observed. When antibody cocktails were centrifuged (Figure 13F, Microfuge 22R Centrifuge, Beckman Coulter) prior to staining procedures there was no specific signal detectable.

Figure 13A. Immunoglobulin Solution (Pentaglobin: distilled water = 1:100)

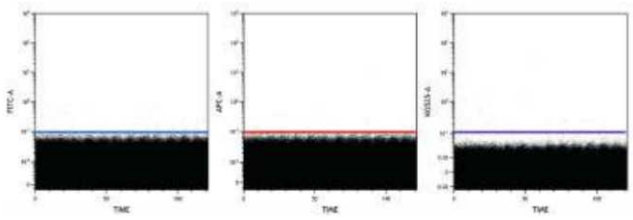


Figure 13B. CD16 FITC

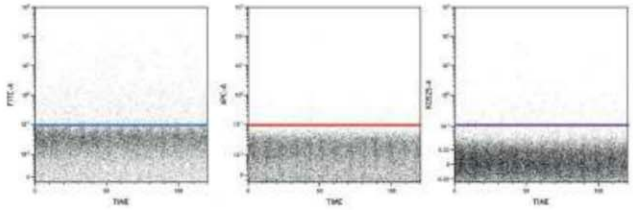


Figure 13C. CD14 APC

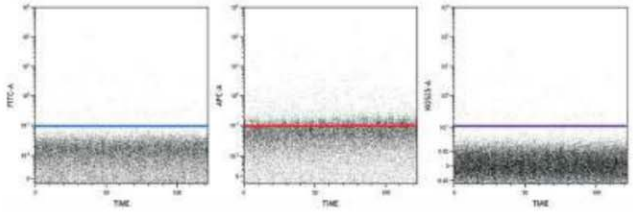


Figure 13D. CD45 Krome Orange

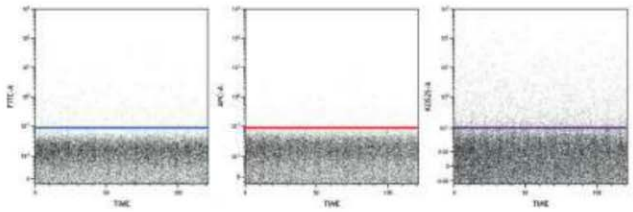


Figure 13E. Annexin V FITC

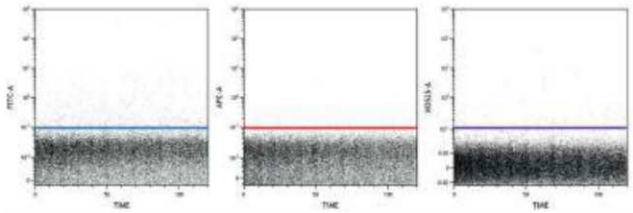
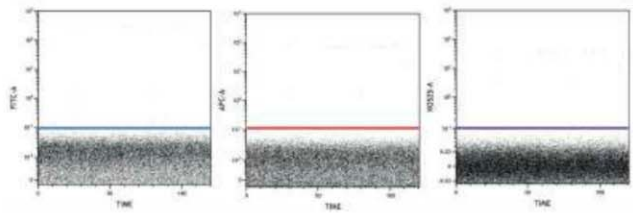


Figure 13F. Antibodies and Annexin V after centrifugation

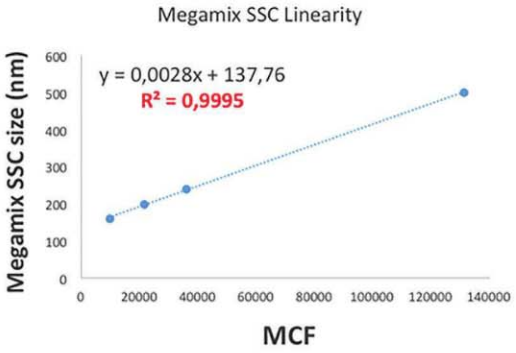


Technical notes

Linearity of Megamix-Plus SSC beads in comparison of size to fluorescence intensity

As shown in Figure 14 there is a strong linearity (R2=0.9995) between the size of the Megamix-Plus SSC beads (100, 160, 200, 500 nm) which are optimized for the SSC light signal. Megamix-Plus SSC beads were measured using the Gigamix protocol described above. For analysis, a marker was set over each single peak. The mean channel fluorescence intensity of each peak was set in relation to the size of the peak and a regression curve analysis was calculated using Excel (Microsoft).

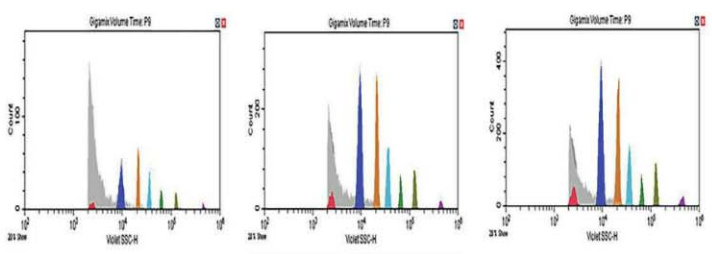
Figure 14. Linearity between size of Megamix-Plus SSC beads and mean channel fluorescence intensity of the measured beads.



Influence of the measured sample flow rate in comparison to the noise

Gigamix beads were diluted 1:5 in distilled water and measured at flow rates of 10, 60 and 120 µL/min. As can be seen in Figure 15 the population of the 100 nm beads is very small and within the range of noise. Increasing the flow rate makes the 100 nm beads more «visible» which clearly indicates that only the beads signal was increased but not the background noise. Also all other peaks are better visible so that it is obvious that by increasing the flow rate the background noise is not increased and remains stable if distilled water is used as diluent.

Figure 15A. 10 µL/min Figure 15B. 60 µL/min Figure 15C. 120µL/min



Summary and discussion

The CytoFLEX is the first flow cytometer with an acceptable noise range on which we can clearly demonstrate detection of extracellular vesicles down to a size of 150 nm. The potential to combine small particle analysis with the detection of up to 13 additional fluorescence parameters makes this cytometer an outstanding instrument for extracellular vesicle detection.

However, the correct measurement is strongly dependent on a series of prerequisites. For example, the staining of extracellular vesicles from whole blood requires blood draw into citrate anticoagulated tubes with special conditions which are described in detail in the literature cited herein. The so-called preanalytic procedures include not only blood drawing procedures but also the handling of the blood – do not shake or mix – otherwise a lot of microparticles are produced and artefacts are measured. Sample preparation is highly dependent on centrifugation steps which are also well described in the literature. Additionally we demonstrate here, again, the selection and preparation of the sample media is important. Measuring samples that contain plasma or measuring cell culture samples which contain FCS give a high background of particles similar to the particles which are intended to be measured.

Staining procedures are also important. Before starting, sample staining dilution steps should be performed to avoid the swarm effect and ensure measurement of single events. Centrifuge the antibody solution(s) before dilution or titration otherwise aggregates can be detected by the flow cytometer. Taken together, these precautions allow for best practice in extracellular vesicle measurement.

While Gigamix beads serve as a good tool to standardize the CytoFLEX on a daily basis for particle measurement, they are in reality polystyrene beads and are very different from biological membranes which ultimately leads to some discrepancy in predicting the size of the extracellular vesicles. The refractory index of beads differs substantially from the refractory index of biological membranes/ particles. The particle size should be only seen in a range of size measurements and the results should be carefully interpretated. The next few years will show whether increasing sensitivities of particle measurement will enhance the knowledge and the biological relevance of extracellular vesicles. The CytoFLEX is a big step forward to nanoparticle detection and offers particle evaluation down to approximately 150 nm.

REAGENTS

Megamix-Plus SSC beads: BioCytex – Reference 7803, 500 µL beads, 50 tests

Megamix-Plus FSC beads: BioCytex – Reference 7802, 500 µL beads, 50 tests

REFERENCES

1. Barteneva NS et al. Circulating microparticles: square the circle. BMC Cell Biol. 2013, 14:23
2. Sarlon-Bartoli G et al. Plasmatic level of leukocyte-derived microparticles is associated with unstable plaque in asymptomatic patients with high-grade carotid stenosis. J Am Coll Cardiol. 2013, 62:1436

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FLOW-861APP0315-A.

Setting up the Beckman Coulter CytoFLEX for Detection of Extracellular Vesicles

TECHNICAL INFORMATION BULLETIN

Vasilis Toxavidis, Virginia Camacho, and John Tigges; Affiliation: BIDMC, CLS 932 – Flow Cytometry Core Facility, 3 Blackfan Circle, Boston, MA 02115

Introduction

There is great interest in both the medical and scientific communities in submicron cell-derived particles termed Extracellular Vesicles (EVs). A great difficulty in this field, however, has been the optimization and standardization of techniques to measure these small particles. Although competing techniques have been developed, flow cytometry is a convenient approach, for life science researchers. The hurdle in analysis has always been the ability to accurately measure the size characteristics of small particles; especially when only considering scatter properties. Flow Cytometry instrumentation was traditionally designed to perform whole blood analysis and, therefore, cellular measurements above 3µm. Particles detected below the 3µm "threshold" were considered to be debris. Due to advances in microscopy and the ability to identify and characterize than 1µm cellular particles, hardware upgrades to the scatter parameters of flow cytometry instrumentation have been developed to micro particle populations from <200nm to 1µm. However, the accuracy of these measurements and the validity of the results are frequently questioned due to insufficient reproducibility. In this study, previous methodologies to define a range for detection of EVs will be explored on Beckman Coulter's new CytoFLEX platform.

Technical Considerations of the CytoFLEX

The patent-pending optical design includes an integrated optics flow cell and photo diode detection system. In addition, all lasers are integrated to present optimal excitation. Emission of light is directed into dedicated fiber optical arrays, minimizing light loss and maximizing sensitivity.

CytoFLEX does not use PMTs – rather, CytoFLEX is the first commercial flow cytometer to utilize photo diodes for fluorescence channel detection. Photo Diodes, are very robust, linear, and sensitive.

The patent-pending Wavelength Division Multiplexing (WDM) detection module uses solid-state, high efficiency, low-noise Fiber Array Photodiode Detectors (FAPD), giving you exceptional resolution for more precise data and better detection of rare events.

The Fiber Array Photo Diode (FAPD) provides low-noise detection with high quantum efficiency and minimum light loss ensuring high signal to noise ratio and optical resolution especially with small particle measurements and dim fluorescence detection. The technology has its origin from the fiber optical communication industry, where the term Wavelength Division Multiplexing or WDM, originated. The CytoFLEX detection module collects the emitted light from each of the laser paths through high-efficiency fiber optic

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coupling. Each optical fiber delivers emitted laser light by a given excitation laser source, to a wavelength specific WDM detection module. Inside the WDM module, the fluorescence light is divided and tightly focused through a series of band pass filters and integrated optics, on to an array of ultra-low noise silicon photo detectors. Enhanced detection capability is achieved by using reflective, band-pass only filters to collect light and provide modularity and consistent sensitivity for all channels.

Materials and Methods

0.19µm, 0.52µm and 0.78µm Dragon Green Beads were obtained from Bangs Laboratories, Inc. Dragon Green is an excellent spectral surrogate for fluorescein (488nm/530nm), and is suitable for use with fluorescein filter sets. Many imaging applications rely on fluorescent microspheres for detection of binding events or signal enhancement. Addressable bead populations may be created with different intensities of fluorescence for the development of multiplexed flow cytometric assays, and small fluorescent spheres can function as reporters for ELISA-type assays. Fluorescent microspheres are also useful for fluid tracing, cell tracking, and phagocytosis studies.

For this project internally labeled Dragon Green fluorescent microspheres were used. Fluorescent microspheres are internally dyed using a solvent swelling/dye - entrapment technique. Internal dyeing produces very bright and stable particles with typically narrow fluorescence CV's. With this strategy, surface groups remain available for conjugating ligands (proteins, antibodies, nucleic acids, etc.) to the surface of the bead, which is important for analyte-detection and immunoassay applications. Internally-dyed beads are also used extensively in imaging applications, as they offer a greater resistance to photobleaching.

For this flow cytometric assay, Dragon Beads were chosen at the sizes listed previously for later cell tracking. By using beads of differing sizes and fluorescent intensities, one can optimize the flow cytometer for cellular analysis. The bead sizes were chosen to be comparable to the size of the cells being analyzed. Therefore, all voltages, gains, and threshold settings were optimized for Dragon Green Beads to develop a relative size distribution matrix. The bead concentrations have been previously determined by serial dilution of the beads and subsequent measurement on Beckman Coulter's MoFlo Astrios EQ, MoFlo XDP with Propel Labs NanoView attachment, and Gallios flow cytometers.

Instrument Optimization

Gating and analysis

The Dragon Bead size distribution protocol was applied to assess the CytoFLEX's ability to measure EVs. Scatter properties will be analyzed to determine the most efficient parameters for EV analysis. The CytoFLEX has the ability to both trigger off and analyze by Violet (405nm) Side Scatter (VSSC). For purposes of this study the VSSC will be used. Based on previous studies, a lack of hardware enhancements to the FSC parameter (a PMT or angle of light adjustment) does not allow for detection of particles below 0.5um (Figure 1).

Using the VSSC parameter, Dragon Green particles will be visible and distinct below 500nm as lower wavelengths of laser light are theorized to allow for smaller particle size detection. Additionally, the CytoFLEX sheath delivery can be easily controlled through the software interface. The intuitive software control allows the user to manually control the sample speed, of particles to maximize the amount of laser interrogation at slower µL/min flow rates. Hydrodynamic focusing is also enhanced to limit the ability of particle clustering. A stock solution of filtered PBS with 0.1% Tween-20 is prepared. 0.52, 0.78, and 0.19um beads are diluted with the PBS/0.1% Tween-20 solution, to a final concentration of $1.29*10^7$ beads/mL.

Prior to dilution, the stock solutions of Dragon Green Beads were sonicated to eliminate clumps.

The following samples were run on the Beckman Coulter CytoFLEX for instrument optimization:

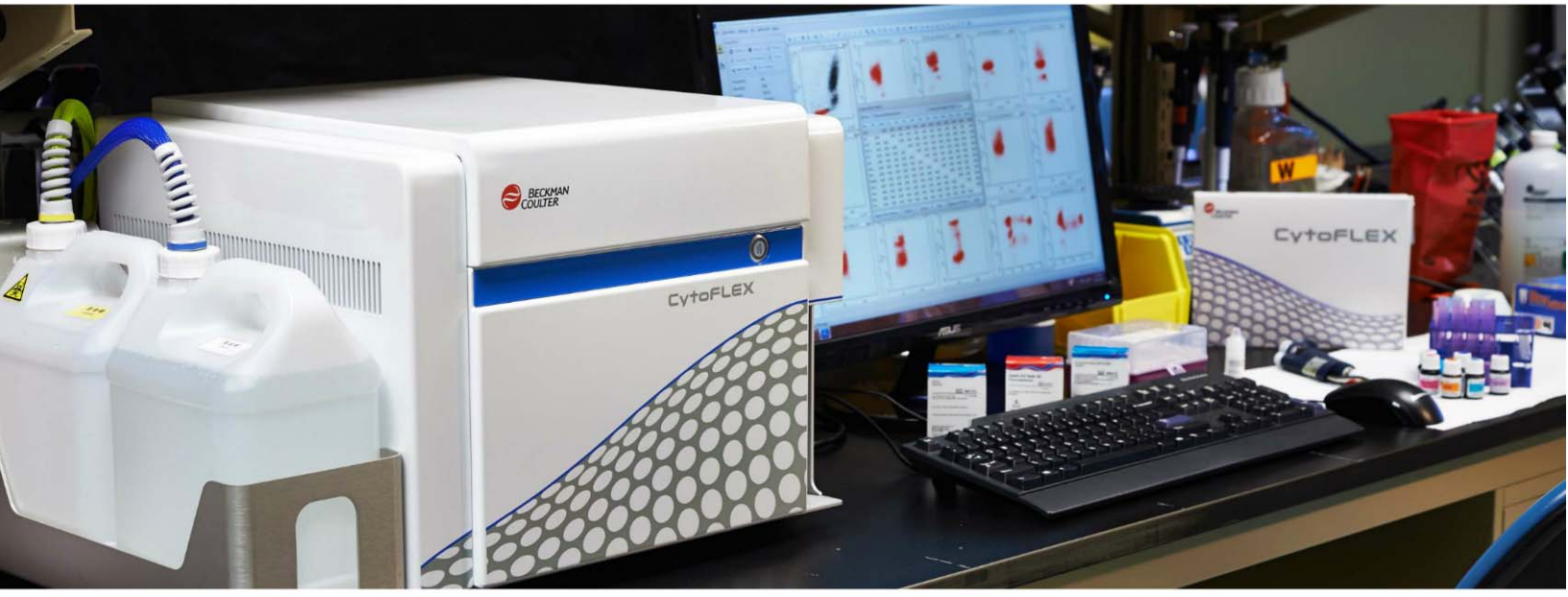
1. 0.78µm Dragon Green Beads
2. 0.52µm Dragon Green Beads
3. 0.19µm Dragon Green Beads
4. 0.52µm/0.78µm/1.01µm Dragon Green Beads Mixed

- 0.78µm Dragon Green Beads were acquired to set the Scatter properties for differentiation between beads and low-end noise. In addition, VSSC properties were adjusted to maximize resolution and dynamic range. The largest size is chosen first for ease of particle identification and to prove instrument ability to analyze below 1µm.
- 0.19µm Dragon Green Beads were acquired to test the ability of the instrument to differentiate between the particle and noise. As particle size decreases, instrument Noise populations will begin to overwhelm the Dragon Green Bead's signal. Also, 0.19µm Dragon Green Beads were used as most instrument manufacturer's specifications quantify lowest detectable level of 0.3µm.
- 0.52µm Dragon Beads were acquired for accuracy of separation of 0.19µm and 0.78µm beads. This allows for visualization of dynamic range of the instrument.
- Gates were drawn to encompass the three distinct populations.
- Mixed Dragon Beads were acquired to ensure proper gating and maximum separation of bead populations for the determination of a relative sizing distribution matrix. Furthermore, previous analysis has determined the ability of larger particles to mask the existence of their smaller counterparts (data not shown). Therefore, mixed populations verify the ability to separate and distinguish multiple populations.
- Gain and threshold settings can be adjusted to maximize performance. However, it is strongly suggested that single bead populations be acquired again for quality assurance purposes.
- Instrument has been optimized and template and settings are saved for future cellular experiments.

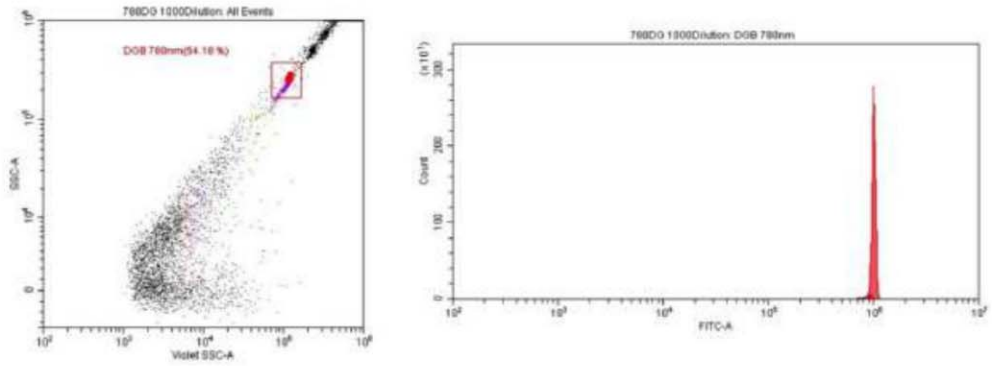
Optimized Settings

- VSSC was set to a Log Area parameter and plotted against Blue (488nm) SSC set to a Log Area Parameter.
- Blue SSC setting was determined to be 350 units on the Gain
- FITC setting was determined to be 370 units on the Gain
- VSSC was placed at the following settings:
  A. Gain units 22
  B. Threshold 2000

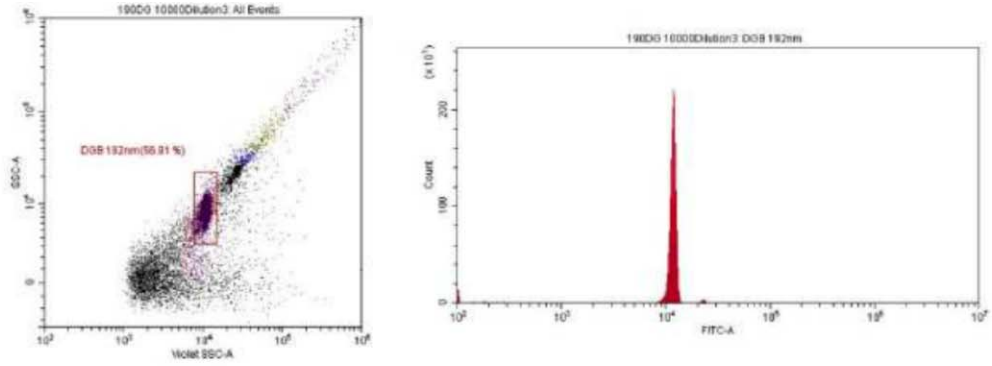
By using the fluorescent characteristics of the Dragon Green Beads, verification of size distribution based on fluorescent intensity can be used for data assurance.
In addition, a sample of the stock PBS solution is acquired for quantification of the background contribution of the PBS. All three populations were easily separated from background and from one another.



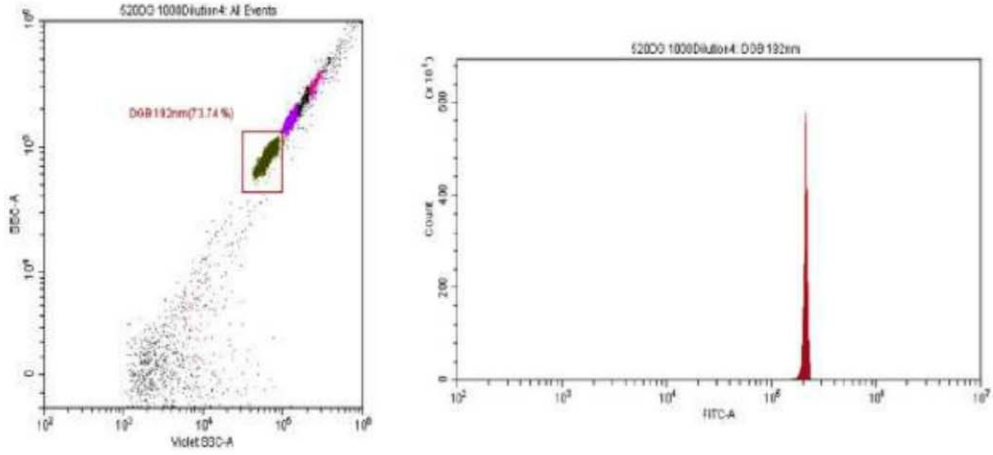
780nm Dragon Green Beads



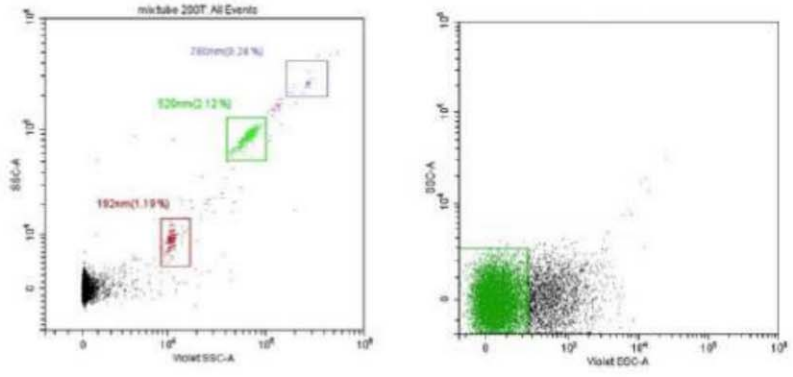
192nm Dragon Green Beads



520nm Dragon Green Beads



Mixed Dragon Green Beads and PBS Alone



Results

All samples were analyzed with either CytExpert* or Kaluza* software. As demonstrated in the previous graphs, the three different size Dragon Beads were identified, gated, and distinguishable from one another and from background. The settings were optimized and saved for later cellular applications. The ability to identify and easily differentiate the Dragon Bead populations based on size has lead to the optimization and standardization of settings for EVs.

Notes

The results demonstrated in this application sheet represent those generated on the Beckman Coulter CytoFLEX Flow Cytometer. As differences exist in the performance between analyzers, the author cannot guarantee a similar appearance with the use of other flow cytometers.

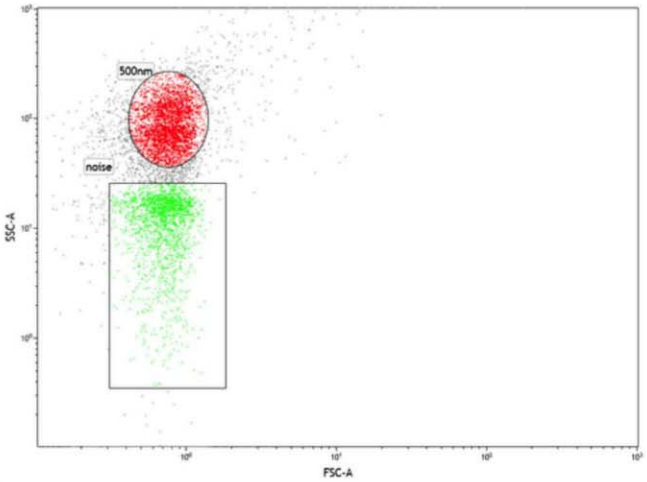


Figure 1: Dynamic range of conventional Flow Cytometry instrumentation. Threshold set on 488nm SSC at the lowest possible setting.

CYTOFLEX						
	192nm Polystyrene	520nm Polystyrene	780 Polystyrene	200nm Latex	300nm Latex	500nm Latex
SSC GAIN 350	8605	85435	263166	8557	87775	270306
VSSC GAIN 22	11183	68552	273086	11120	68816	276329

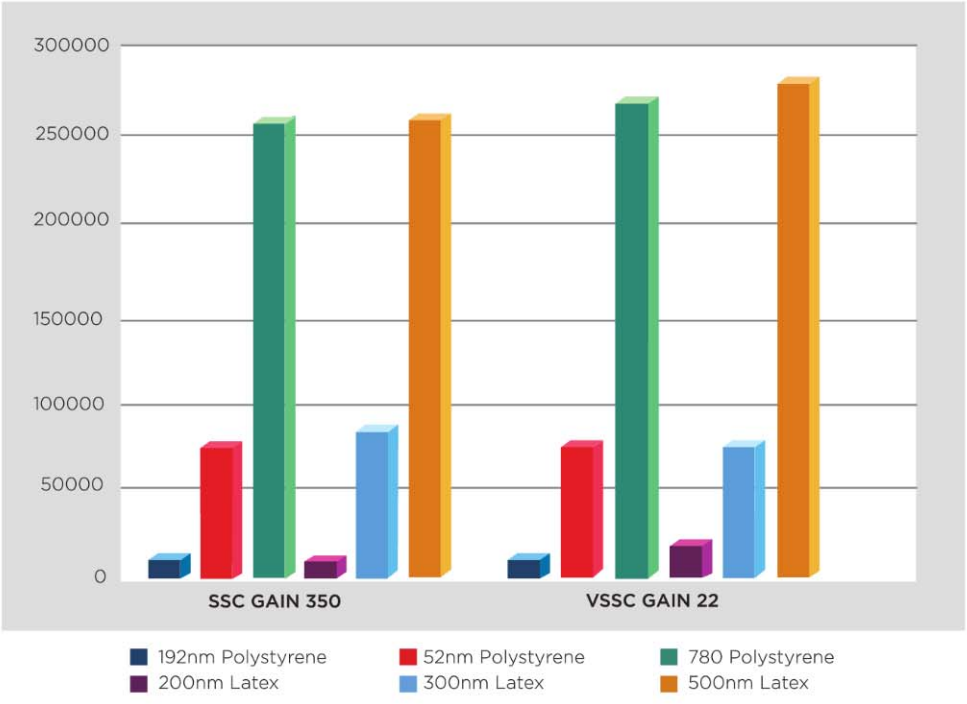


Table shows the differences between SSC and VSSC on the CytoFLEX. The actual MFI are consistent but the Gain values for VSSC are much lower compared to SSC Gain values.

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FLOW-991APP08.15-A

Evaluation of Red Blood Cell Microparticles on the CytoFLEX

APPLICATION NOTE

Author: Albert Donnenberg, PhD
Affiliation: University of Pittsburgh Cancer Center

IN THIS PAPER YOU WILL

- Learn how to prepare microparticles from red blood cells
- Learn how to stain microparticles for analysis by flow cytometry
- Identify RBC microparticles using the CytoFLEX flow cytometer

Introduction

With the understanding that micro- and nanoparticles are mediating many important biological responses, it is critical to be able to analyze these particles. To demonstrate the capabilities of the CytoFLEX to resolve small particles, analysis of Red Blood Cell (RBC) microparticles from human peripheral blood was performed. Three month old human blood samples, stored under standard blood banking conditions, were stained with Annexin V (AnnV). Forward scatter and side scatter revealed RBC microparticles, transition events, as well as, intact red blood cells.

The flow cytometry platforms used for these evaluations are for research use only.

Materials and Methods

1. Randomly selected packed RBC units were obtained from the Central Blood Bank, Pittsburgh, PA
2. Units were nonleukoreduced and preserved in ADSOL solution (standard practice at University of Pittsburgh Medical Center)
3. Quantification of microparticles was performed for each unit of packed RBC
4. 2 µL PE conjugated Glycophorin A was added to 5 µL RBC
5. Incubate 30 minutes at room temperature (RT) in the dark
6. Add 2 µL FITC-Annexin V followed by 500 µL of Annexin V binding buffer, per kit instructions
7. Incubate 30 minutes in dark at RT
8. Analyze by flow cytometry – acquire at least 100,000 events per sample at event rates not exceeding 10,000 eps
9. Use SSC and FSC both set to log scale
10. Microparticles were quantified as a percent of glycophorin A positive events

Full description of methods can be found in Reference 1.

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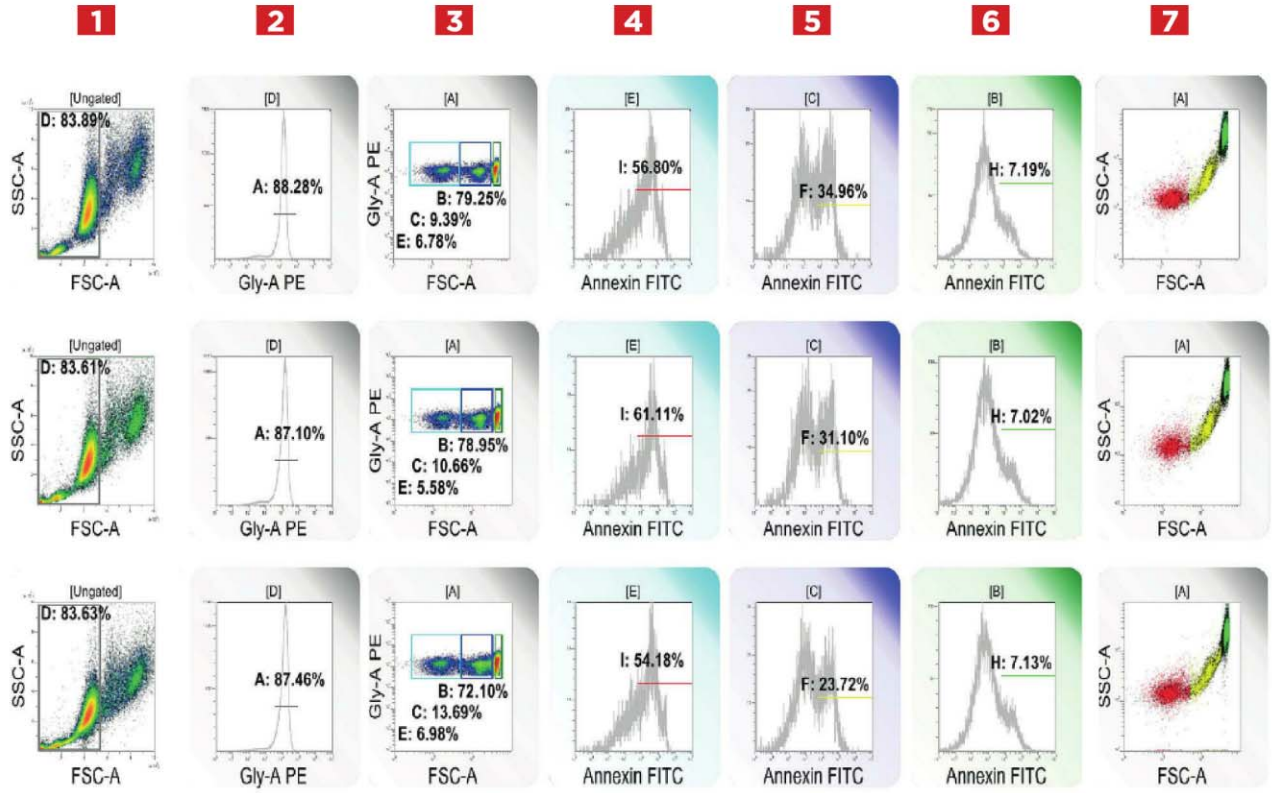
FLOW-1109APP09.15-A

Results

The gating strategy is described shown in Figure 1 utilizing the glycophorin A staining as a method to discern RBC and derivative populations. Samples were prepared in replicate and run to calculate precision of instrument reading and provide statistical validity to the method and data.

Figure 1.



Legend from left to right colums:

- **1** RBC clusters are eliminated
- **2** Glycophorin dim events are eliminated
- **3** Glycophorin+ events are subsetted on the basis of forward light scatter into low, intermediate and high populations
- **4 5 6** Annexin V binding (surface phosphatidyl serine) is detected in low (E, red), intermediate (C, yellow), and high (B, green)
- **7** Pseudocolored events from the annexin V histograms backgated to a dotplot of FSC by SSC displays RBC microparticles (red), transitional events (yellow), and intact RBC (green).

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Table 1

Replicates were performed and inter-replicate precision is shown by CV.

Sample	Total Clean events (A)	FS_Low	FS_Int	FS_Hi	Microparticles Annex P_FS_ Low	Transitional Annex P_ FS_Int	Intact FBCAnnex P_FS_Hi
W084514A	84010	11.2%	1.0%	86.4%	63.0%	18.9%	7.6%
W084514B	83365	11.7%	2.6%	83.7%	59.0%	19.6%	7.4%
W084514C	82680	11.0%	4.4%	81.8%	61.9%	17.5%	7.2%
Mean	83352	11.3%	2.7%	84.0%	61.3%	18.7%	7.4%
SD	665	0.4%	1.7%	2.3%	2.1%	1.1%	0.2%
CV	0.8%	3.2%	64.1%	2.7%	3.4%	5.7%	2.5%
W085315A	84586	9.7%	1.8%	86.2%	55.9%	15.6%	5.2%
W085315B	82371	9.2%	0.4%	88.5%	57.0%	31.0%	5.7%
W085315C	85298	8.6%	1.9%	87.1%	54.8%	15.1%	4.8%
Mean	84085	9.2%	1.4%	87.3%	55.9%	20.6%	5.2%
SD	1526	0.5%	0.9%	1.1%	1.1%	9.0%	0.4%
CV	1.8%	5.8%	62.6%	1.3%	2.0%	43.8%	8.4%
W086114A	79488	6.8%	9.4%	79.3%	56.8%	35.0%	7.2%
W086114B	73871	5.6%	10.7%	79.0%	61.1%	31.1%	7.0%
W086114C	79430	7.0%	13.7%	72.1%	54.2%	23.7%	7.1%
Mean	77596	6.4%	11.2%	76.8%	57.4%	29.9%	7.1%
SD	3226	0.8%	2.2%	4.0%	3.5%	5.7%	0.1%
CV	4.2%	11.7%	19.6%	5.3%	6.1%	19.1%	1.2%

Conclusions

Microparticles from RBC can be detected along with intermediate products and intact RBCs using the CytoFLEX flow cytometer. Additionally, inter-sample replicates showed a high degree of precision as calculated using CV.

Notes

The results demonstrated in this application sheet represent those generated on the Beckman Coulter CytoFLEX Flow Cytometer. As differences exist in the performance between analyzers, the author cannot guarantee a similar appearance with the use of other flow Cytometers.

Reagent Details

Reagent	Supplier	Order Details
Annexin V – FITC kit	BD-Pharmingen	556747
Glycophorin A – PE	BD-Pharmingen	340947

Reference

1. Donadee C, Raat NJH, Kanias T, Tejero J, Lee JS, Kelley EE, Zhao X, Liu C, Reynolds H, Azarov I, Frizzell S, Meyer EM, Donnenberg AD, QU L, Triulzi D, Kim-Shapiro DB, Gladwin MT. Circulatoin. 2011;124:465-476. Nitric Oxide Scavenging by Red Blood Cell Microparticles and Cell-Free Hemoglobin as a Mechanism for the Red Blood Cell Storage Lesion.

BECKMAN COULTER Life Sciences

CytoFLEX
Flow Cytometer
Blue-Red-Violet (B-R-V) Series

OPTICS

EXCITATION OPTICS

The instrument has the capacity for 15 parameters, including 13 for fluorescence detection. The fully activated instrument includes five channels from the 488 nm (Blue) laser, three from the 638 nm (Red) laser, and five from the 405 nm (Violet) laser. Instruments with as few as four fluorescent channels activated are available with the ability to activate additional parameters as needed by purchasing an activation key.

LASER SPECIFICATIONS

Spatially Separated Laser Options (Beam Spot Size: 5 µm x 80 µm)

Laser	Wavelength	Power
Blue	488 nm	50 mW
Red	638 nm	50 mW
Violet	405 nm	80 mW

FLOW CELL

Fixed integrated optics and quartz flow cell design with >1.3 numerical aperture.

Flow Cell dimensions: 420 µm x 180 µm internal diameter

FORWARD SCATTER DETECTION

Proprietary Axial Light Loss (ALL) sensor system using silicon photodiodes with built in 488/8 µm band pass filter.

BAND PASS FILTERS

Includes 13 repositionable filters

450/45	660/10 (2)
525/40 (2)	690/50
585/42	712/25
610/20 (2)	780/60 (3)

FLUORESCENCE AND SIDE SCATTER DETECTION

Fluorescence and side scatter light delivered by fiber optics to Avalanche Photo Diode detector arrays. Proprietary design ensures high performance, high efficiency, low-noise signal detection. Emission profiles are collected using reflective optics and single transmission band pass filters.

VIOLET SIDE SCATTER CONFIGURATION

Option to configure Avalanche Photo Diode detector array to collect side scatter signal from Violet (405 nm) laser. The configured channel (VSSC) can be utilized to better resolve nanoparticles below 200 nm size threshold.

QUALITY CONTROL

CytExpert QC automation pass/fail criteria rCV ≤ 5.0% for the all 13 fluorescent channels.

PERFORMANCE

SCATTER RESOLUTION

Blue (488 nm) Side Scatter Resolution: <300 nm

Violet (405 nm) Side Scatter Resolution (VSSC): <200 nm

Scatter performance is optimized for resolving human lymphocytes, monocytes, and granulocytes as well as nanoparticles.

CARRYOVER

Single Tube Format: < 1.0%

Plate Loader Format: < 0.5%

SENSITIVITY

FITC: <30 molecules of equivalent soluble fluorochrome (MESF-FITC)

PE: <10 molecules of equivalent soluble fluorochrome (MESF-PE)

FLUORESCENCE RESOLUTION

The CytoFLEX Flow Cytometer is capable of achieving 3% rCV with alignment verification particles capable of rCVs <3%.

ELECTRONICS

NOMINAL ACQUISITION RATE

30,000 events per second with 15 parameters

Software capability to modify window extension parameter and to control abort rate during high event rate signal processing

SIGNAL PROCESSING

Fully digital system with 7 decade data display

SIGNAL

Pulse area, height for every channel, width for one selectable channel

FLUIDICS

ULTRA-LOW PRESSURE PERISTALTIC SHEATH AND SAMPLE DELIVERY SYSTEM

Low maintenance system

Sheath Fluid Filter and Sample Pump Tubing can be replaced by the user (no service visit required)

SAMPLE FLOW RATES

Fixed Flow Rates: 10, 30 and 60 µL/min

Custom Flow Rate Control mode from 10 to 240 µL/min in 1 µL increments.

Gravimetric calibration for absolute counts within CytExpert Software.

BECKMAN COULTER Life Sciences

FLUID CAPACITY

Standard 4 L tanks

Optional 10L cubitainers

AUTOMATED MAINTENANCE FUNCTIONS

System Startup, Sample Mixing, Backflush, Prime, Shutdown, Deep Clean

SAMPLE INPUT FORMATS

5 mL (12 x 75 mm) polystyrene and polypropylene tubes

1.5 mL and 2 mL microcentrifuge tubes

PLATE LOADER FORMATS

96-well Standard Flat, U and V bottom plates

DATA MANAGEMENT

SOFTWARE

The CytExpert software is a full-feature software package that controls the instrument's operation, collection of experiment data, and analysis of the results.

Three different installation modes are available, depending on the level of security required.

The Default installation requires no user login.

For multiuser instruments, the User Management installation requires user login and contains features for role management. Electronic Records Management installation provides tools that facilitate compliance with 21 CFR Part 11, Electronic Records and Electronic Signatures.

An API (Application Programming Interface) is available and allows external software to perform operations such as running methods and for basic control of the plate loader.

If desired, export FCS files for offline analysis in Kaluza, FCSExpress, FlowJo, and other platforms.

STANDARDIZATION

Daily QC beads or any other reference material that is relevant for your application may be used as the standardization sample to set target values and calibrate the gain settings automatically.

LANGUAGE

English and Chinese

OPERATING SYSTEM

Windows® 7 Professional 64-bit

Windows® 8 Professional 64-bit

Windows® 10 Professional 64-bit

FCS FORMAT

FCS 3.0

MINIMUM SPECIFICATIONS

CPU: Intel® I3 @ 2.9 GHz 1 Gigabit Ethernet port

RAM: 4 GB 2 USB 3.0 ports

Storage: 256 GB 4 USB 2.0 ports

COMPENSATION

Automatic full matrix compensation

Manual full matrix compensation

Novel Compensation Library: store fluorescent spillover values of dyes to easily determine the correct compensation matrix with new gain settings

Import/export compensation values between experiments

Absolute linear gain amplification enables the use of compensation settings between experiments and sample types

INSTALLATION

DIMENSIONS (W X D X H)

Cytometer (with or without Plate Loader)

42.5 cm x 42.5 cm x 34 cm

16.7 in x 16.7 in x 13.4 in

Tanks and Holder

14 cm x 35.6 cm x 35.6 cm

5.5 in x 14.0 in x 14.0 in

WEIGHT

Cytometer: 23.4 kg / 51.6 lbs

Cytometer with Plate Loader: 28 kgs / 61.7 lbs

POWER SPECIFICATIONS

Voltage: 100–240 V Power: 150 -250 W

OPERATING TEMPERATURE NON-CONDENSING

15-27 °C, 59-80.6 °F

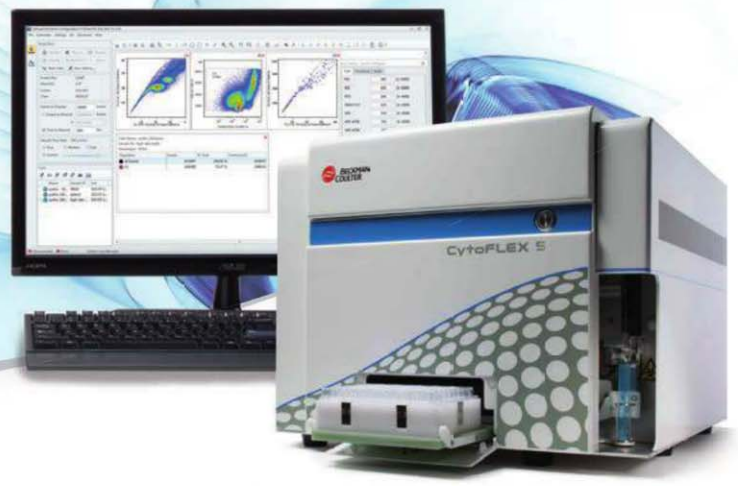
BECKMAN COULTER Life Sciences

For Beckman Coulter's worldwide office locations and phone numbers, please visit "Contact Us" at **beckman.com**

FLOW-2231SPEC01.17

CytoFLEX S
Flow Cytometer

Blue-Red-Violet-Yellow Green
(B-R-V-Y) Series



OPTICS

EXCITATION OPTICS

The instrument has the capacity for 15 parameters, including 13 for fluorescence detection. The fully activated instrument includes two channels from the 488 nm (Blue) laser, three from the 638 nm (Red) laser, four from the 405 nm (Violet) laser, and four from the 561 nm (Yellow Green) laser. Instruments with as few as six fluorescent channels activated are available with the ability to activate additional parameters as needed by purchasing an activation key.

LASER SPECIFICATIONS

Spatially Separated Laser Options (Beam Spot Size: 5 µm x 80 µm)

Laser	Wavelength	Power
Blue	488 nm	50 mW
Red	638 nm	50 mW
Violet	405 nm	80 mW
Yellow Green	561 nm	30 mW

FLOW CELL

Fixed integrated optics and quartz flow cell design with >1.3 numerical aperture.

Flow Cell dimensions: 420 µm x 180 µm internal diameter

FORWARD SCATTER DETECTION

Proprietary Axial Light Loss (ALL) sensor system using silicon photodiodes with built in 488/8 µm band pass filter.

BAND PASS FILTERS

Includes 13 repositionable filters

450/45	660/10 (2)
525/40 (2)	690/50 (2)
585/42	712/25
610/20 (2)	780/60 (2)

FLUORESCENCE AND SIDE SCATTER DETECTION

Fluorescence and side scatter light delivered by fiber optics to Avalanche Photo Diode detector arrays. Proprietary design ensures high performance, high efficiency, low-noise signal detection. Emission profiles are collected using reflective optics and single transmission band pass filters.

VIOLET SIDE SCATTER CONFIGURATION

Option to configure Avalanche Photo Diode detector array to collect side scatter signal from Violet (405 nm) laser. The configured channel (VSSC) can be utilized to better resolve nanoparticles below 200 nm size threshold.

QUALITY CONTROL

CytExpert QC automation pass/fail criteria rCV ≤ 5.0% for the all 13 fluorescent channels.

PERFORMANCE

SCATTER RESOLUTION

Blue (488 nm) Side Scatter Resolution: <300 nm

Violet (405 nm) Side Scatter Resolution (VSSC): <200 nm

Scatter performance is optimized for resolving human lymphocytes, monocytes, and granulocytes as well as nanoparticles.

CARRYOVER

Single Tube Format: < 1.0%

Plate Loader Format: < 0.5%

SENSITIVITY

FITC: <30 molecules of equivalent soluble fluorochrome (MESF-FITC)

PE: <10 molecules of equivalent soluble fluorochrome (MESF-PE)

FLUORESCENCE RESOLUTION

The CytoFLEX Flow Cytometer is capable of achieving 3% rCV with alignment verification particles capable of rCVs <3%.

ELECTRONICS

NOMINAL ACQUISITION RATE

30,000 events per second with 15 parameters

Software capability to modify window extension parameter and to control abort rate during high event rate signal processing

SIGNAL PROCESSING

Fully digital system with 7 decade data display

SIGNAL

Pulse area, height for every channel, width for one selectable channel

FLUIDICS

ULTRA-LOW PRESSURE PERISTALTIC SHEATH AND SAMPLE DELIVERY SYSTEM

Low maintenance system

Sheath Fluid Filter and Sample Pump Tubing can be replaced by the user (no service visit required)

SAMPLE FLOW RATES

Fixed Flow Rates: 10, 30 and 60 µL/min

Custom Flow Rate Control mode from 10 to 240 µL/min in 1 µL increments.

Gravimetric calibration for absolute counts within CytExpert Software.

BECKMAN COULTER Life Sciences

FLUID CAPACITY

Standard 4 L tanks

Optional 10L cubitainers

AUTOMATED MAINTENANCE FUNCTIONS

System Startup, Sample Mixing, Backflush, Prime, Shutdown, Deep Clean

SAMPLE INPUT FORMATS

5 mL (12 x 75 mm) polystyrene and polypropylene tubes

1.5 mL and 2 mL microcentrifuge tubes

PLATE LOADER FORMATS

96-well Standard Flat, U and V bottom plates

DATA MANAGEMENT

SOFTWARE

The CytExpert software is a full-feature software package that controls the instrument's operation, collection of experiment data, and analysis of the results.

Three different installation modes are available, depending on the level of security required.

The Default installation requires no user login.

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STANDARDIZATION

Daily QC beads or any other reference material that is relevant for your application may be used as the standardization sample to set target values and calibrate the gain settings automatically.

LANGUAGE

English and Chinese

OPERATING SYSTEM

Windows® 7 Professional 64-bit

Windows® 8 Professional 64-bit

Windows® 10 Professional 64-bit

FCS FORMAT

FCS 3.0

MINIMUM SPECIFICATIONS

CPU: Intel® I3 @ 2.9 GHz 1 Gigabit Ethernet port

RAM: 4 GB 2 USB 3.0 ports

Storage: 256 GB 4 USB 2.0 ports

COMPENSATION

Automatic full matrix compensation

Manual full matrix compensation

Novel Compensation Library: store fluorescent spillover values of dyes to easily determine the correct compensation matrix with new gain settings

Import/export compensation values between experiments

Absolute linear gain amplification enables the use of compensation settings between experiments and sample types

INSTALLATION

DIMENSIONS (W X D X H)

Cytometer (with or without Plate Loader)

42.5 cm x 42.5 cm x 34 cm

16.7 in x 16.7 in x 13.4 in

Tanks and Holder

14 cm x 35.6 cm x 35.6 cm

5.5 in x 14.0 in x 14.0 in

WEIGHT

Cytometer: 23.4 kg / 51.6 lbs

Cytometer with Plate Loader: 28 kgs / 61.7 lbs

POWER SPECIFICATIONS

Voltage: 100–240 V Power: 150 -250 W

OPERATING TEMPERATURE NON-CONDENSING

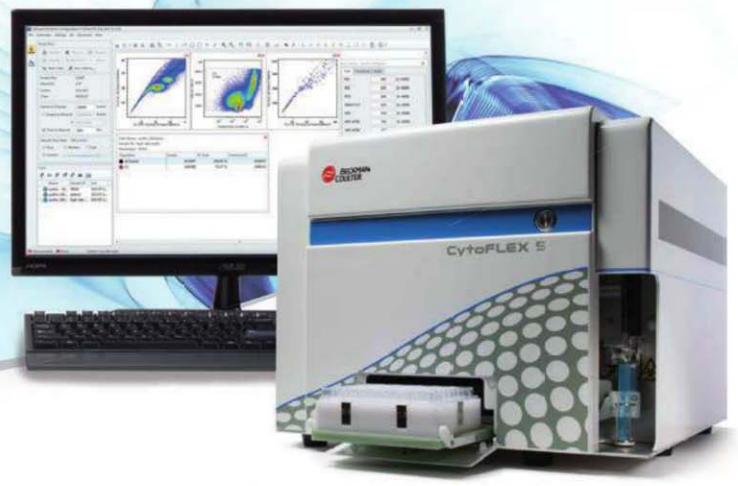
15-27 °C, 59-80.6 °F

BECKMAN COULTER
Life Sciences

For Beckman Coulter's worldwide office locations and phone numbers, please visit "Contact Us" at **beckman.com**

FLOW-2234SPEC01.17

CytoFLEX S
Flow Cytometer
Blue-Red-Violet-Near UV
(B-R-V-N) Series



OPTICS

EXCITATION OPTICS

The instrument has the capacity for 15 parameters, including 13 for fluorescence detection. The fully activated instrument includes five channels from the 488 nm (Blue) laser, three from the 638 nm (Red) laser, three from the 405 nm (Violet) laser, and two from the 375 nm (Near UV) laser. Instruments with as few as six fluorescent channels activated are available with the ability to activate additional parameters as needed by purchasing an activation key.

LASER SPECIFICATIONS

Spatially Separated Laser Options (Beam Spot Size: 5 µm x 80 µm)

Laser	Wavelength	Power
Blue	488 nm	50 mW
Red	638 nm	50 mW
Violet	405 nm	80 mW
Near UV	375 nm	60 mW

FLOW CELL

Fixed integrated optics and quartz flow cell design with >1.3 numerical aperture.

Flow Cell dimensions: 420 µm x 180 µm internal diameter

FORWARD SCATTER DETECTION

Proprietary Axial Light Loss (ALL) sensor system using silicon photodiodes with built in 488/8 µm band pass filter.

BAND PASS FILTERS

Includes 13 repositionable filters

450/45 (2)	675/30
525/40 (2)	690/50
585/42	712/25
610/20 (2)	780/60 (2)
660/10	

FLUORESCENCE AND SIDE SCATTER DETECTION

Fluorescence and side scatter light delivered by fiber optics to Avalanche Photo Diode detector arrays. Proprietary design ensures high performance, high efficiency, low-noise signal detection. Emission profiles are collected using reflective optics and single transmission band pass filters.

VIOLET SIDE SCATTER CONFIGURATION

Option to configure Avalanche Photo Diode detector array to collect side scatter signal from Violet (405 nm) laser. The configured channel (VSSC) can be utilized to better resolve nanoparticles below 200 nm size threshold.

QUALITY CONTROL

For detection channels off of the 405, 488, and 638 nm laser, CytExpert QC automation pass/fail criteria is rCV ≤5.0%. For detection channels off of the 375 nm laser, the criteria is ≤ 7.0%.

PERFORMANCE

SCATTER RESOLUTION

Blue (488 nm) Side Scatter Resolution: <300 nm

Violet (405 nm) Side Scatter Resolution (VSSC): <200 nm

Scatter performance is optimized for resolving human lymphocytes, monocytes, and granulocytes as well as nanoparticles.

CARRYOVER

Single Tube Format: < 1.0%

Plate Loader Format: < 0.5%

SENSITIVITY

FITC: <30 molecules of equivalent soluble fluorochrome (MESF-FITC)

PE: <10 molecules of equivalent soluble fluorochrome (MESF-PE)

FLUORESCENCE RESOLUTION

The CytoFLEX Flow Cytometer is capable of achieving 3% rCV with alignment verification particles capable of rCVs <3%.

ELECTRONICS

NOMINAL ACQUISITION RATE

30,000 events per second with 15 parameters

Software capability to modify window extension parameter and to control abort rate during high event rate signal processing

SIGNAL PROCESSING

Fully digital system with 7 decade data display

SIGNAL

Pulse area, height for every channel, width for one selectable channel

FLUIDICS

ULTRA-LOW PRESSURE PERISTALTIC SHEATH AND SAMPLE DELIVERY SYSTEM

Low maintenance system

Sheath Fluid Filter and Sample Pump Tubing can be replaced by the user (no service visit required)

SAMPLE FLOW RATES

Fixed Flow Rates: 10, 30 and 60 µL/min

Custom Flow Rate Control mode from 10 to 240 µL/min in 1 µL increments.

Gravimetric calibration for absolute counts within CytExpert Software.

BECKMAN COULTER Life Sciences

FLUID CAPACITY

Standard 4 L tanks

Optional 10L cubitainers

AUTOMATED MAINTENANCE FUNCTIONS

System Startup, Sample Mixing, Backflush, Prime, Shutdown, Deep Clean

SAMPLE INPUT FORMATS

5 mL (12 x 75 mm) polystyrene and polypropylene tubes

1.5 mL and 2 mL microcentrifuge tubes

PLATE LOADER FORMATS

96-well Standard Flat, U and V bottom plates

DATA MANAGEMENT

SOFTWARE

The CytExpert software is a full-feature software package that controls the instrument's operation, collection of experiment data, and analysis of the results.

Three different installation modes are available, depending on the level of security required.

The Default installation requires no user login.

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STANDARDIZATION

Daily QC beads or any other reference material that is relevant for your application may be used as the standardization sample to set target values and calibrate the gain settings automatically.

LANGUAGE

English and Chinese

OPERATING SYSTEM

Windows® 7 Professional 64-bit

Windows® 8 Professional 64-bit

Windows® 10 Professional 64-bit

FCS FORMAT

FCS 3.0

MINIMUM SPECIFICATIONS

CPU: Intel® I3 @ 2.9 GHz 1 Gigabit Ethernet port

RAM: 4 GB 2 USB 3.0 ports

Storage: 256 GB 4 USB 2.0 ports

COMPENSATION

Automatic full matrix compensation

Manual full matrix compensation

Novel Compensation Library: store fluorescent spillover values of dyes to easily determine the correct compensation matrix with new gain settings

Import/export compensation values between experiments

Absolute linear gain amplification enables the use of compensation settings between experiments and sample types

INSTALLATION

DIMENSIONS (W X D X H)

Cytometer (with or without Plate Loader)

42.5 cm x 42.5 cm x 34 cm

16.7 in x 16.7 in x 13.4 in

Tanks and Holder

14 cm x 35.6 cm x 35.6 cm

5.5 in x 14.0 in x 14.0 in

WEIGHT

Cytometer: 23.4 kg / 51.6 lbs

Cytometer with Plate Loader: 28 kgs / 61.7 lbs

POWER SPECIFICATIONS

Voltage: 100–240 V Power: 150 -250 W

OPERATING TEMPERATURE NON-CONDENSING

15-27 °C, 59-80.6 °F

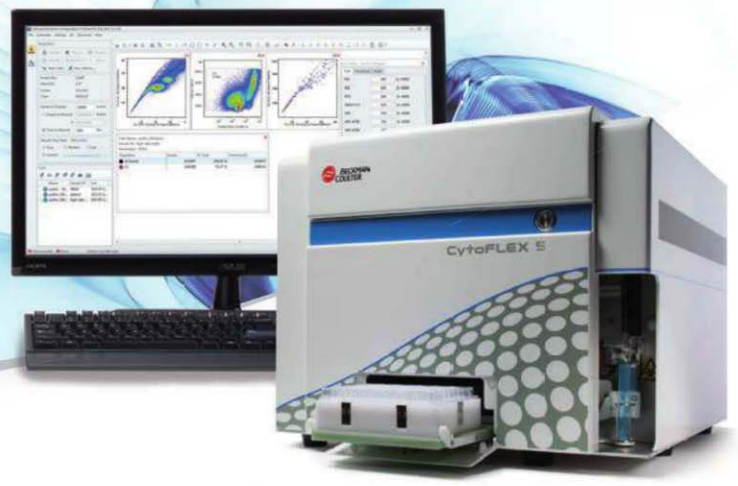
BECKMAN COULTER Life Sciences

For Beckman Coulter's worldwide office locations and phone numbers, please visit "Contact Us" at **beckman.com**

FLOW-2233SPEC01.17

CytoFLEX S
Flow Cytometer

Blue-Red-Violet-Infrared
(B-R-V-I) Series



OPTICS

EXCITATION OPTICS

The instrument has the capacity for 15 parameters, including 13 for fluorescence detection. The fully activated instrument includes four channels from the 488 nm (Blue) laser, three from the 638 nm (Red) laser, four from the 405 nm (Violet) laser, and two from the 808 nm (Infrared) laser. Instruments with as few as six fluorescent channels activated are available with the ability to activate additional parameters as needed by purchasing an activation key.

LASER SPECIFICATIONS

Spatially Separated Laser Options (Beam Spot Size: 5 µm x 80 µm)

Laser	Wavelength	Power
Blue	488 nm	50 mW
Red	638 nm	50 mW
Violet	405 nm	80 mW
Infrared	808 nm	60 mW

FLOW CELL

Fixed integrated optics and quartz flow cell design with >1.3 numerical aperture.

Flow Cell dimensions: 420 µm x 180 µm internal diameter

FORWARD SCATTER DETECTION

Proprietary Axial Light Loss (ALL) sensor system using silicon photodiodes with built in 488/8 µm band pass filter.

BAND PASS FILTERS

Includes 13 repositionable filters

450/45	690/50
525/40 (2)	712/25
585/42	763/43 (2)
610/20	840/20
660/10 (2)	885/40

FLUORESCENCE AND SIDE SCATTER DETECTION

Fluorescence and side scatter light delivered by fiber optics to Avalanche Photo Diode detector arrays. Proprietary design ensures high performance, high efficiency, low-noise signal detection. Emission profiles are collected using reflective optics and single transmission band pass filters.

VIOLET SIDE SCATTER CONFIGURATION

Option to configure Avalanche Photo Diode detector array to collect side scatter signal from Violet (405 nm) laser. The configured channel (VSSC) can be utilized to better resolve nanoparticles below 200 nm size threshold.

QUALITY CONTROL

For detection channels off of the 405, 488, and 638 nm laser, CytExpert QC automation pass/fail criteria is rCV ≤5.0%. For detection channels off of the 808 nm laser, the criteria is ≤ 7.0%.

PERFORMANCE

SCATTER RESOLUTION

Blue (488 nm) Side Scatter Resolution: <300 nm

Violet (405 nm) Side Scatter Resolution (VSSC): <200 nm

Scatter performance is optimized for resolving human lymphocytes, monocytes, and granulocytes as well as nanoparticles.

CARRYOVER

Single Tube Format: < 1.0%

Plate Loader Format: < 0.5%

SENSITIVITY

FITC: <30 molecules of equivalent soluble fluorochrome (MESF-FITC)

PE: <10 molecules of equivalent soluble fluorochrome (MESF-PE)

FLUORESCENCE RESOLUTION

The CytoFLEX Flow Cytometer is capable of achieving 3% rCV with alignment verification particles capable of rCVs <3%.

ELECTRONICS

NOMINAL ACQUISITION RATE

30,000 events per second with 15 parameters

Software capability to modify window extension parameter and to control abort rate during high event rate signal processing

SIGNAL PROCESSING

Fully digital system with 7 decade data display

SIGNAL

Pulse area, height for every channel, width for one selectable channel

FLUIDICS

ULTRA-LOW PRESSURE PERISTALTIC SHEATH AND SAMPLE DELIVERY SYSTEM

Low maintenance system

Sheath Fluid Filter and Sample Pump Tubing can be replaced by the user (no service visit required)

SAMPLE FLOW RATES

Fixed Flow Rates: 10, 30 and 60 µL/min

Custom Flow Rate Control mode from 10 to 240 µL/min in 1 µL increments.

Gravimetric calibration for absolute counts within CytExpert Software.

BECKMAN COULTER Life Sciences

FLUID CAPACITY

Standard 4 L tanks

Optional 10L cubitainers

AUTOMATED MAINTENANCE FUNCTIONS

System Startup, Sample Mixing, Backflush, Prime, Shutdown, Deep Clean

SAMPLE INPUT FORMATS

5 mL (12 x 75 mm) polystyrene and polypropylene tubes

1.5 mL and 2 mL microcentrifuge tubes

PLATE LOADER FORMATS

96-well Standard Flat, U and V bottom plates

DATA MANAGEMENT

SOFTWARE

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OPERATING SYSTEM

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Windows® 8 Professional 64-bit

Windows® 10 Professional 64-bit

FCS FORMAT

FCS 3.0

MINIMUM SPECIFICATIONS

CPU: Intel® I3 @ 2.9 GHz 1 Gigabit Ethernet port

RAM: 4 GB 2 USB 3.0 ports

Storage: 256 GB 4 USB 2.0 ports

COMPENSATION

Automatic full matrix compensation

Manual full matrix compensation

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INSTALLATION

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42.5 cm x 42.5 cm x 34 cm

16.7 in x 16.7 in x 13.4 in

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5.5 in x 14.0 in x 14.0 in

WEIGHT

Cytometer: 23.4 kg / 51.6 lbs

Cytometer with Plate Loader: 28 kgs / 61.7 lbs

POWER SPECIFICATIONS

Voltage: 100–240 V Power: 150 -250 W

OPERATING TEMPERATURE NON-CONDENSING

15-27 °C, 59-80.6 °F

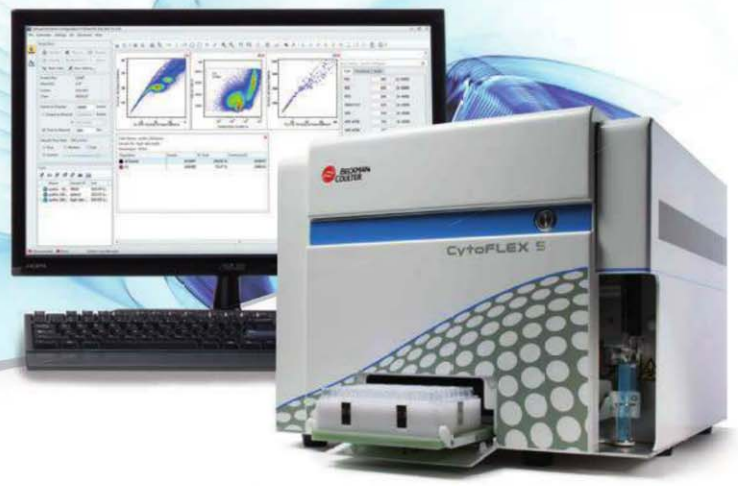
BECKMAN COULTER
Life Sciences

For Beckman Coulter's worldwide office locations and phone numbers, please visit "Contact Us" at **beckman.com**

FLOW-2232SPEC01.17

CytoFLEX S
Flow Cytometer

Blue-Violet-Yellow Green-Near UV
(B-V-Y-N) Series



OPTICS

EXCITATION OPTICS

The instrument has the capacity for 14 parameters, including 12 for fluorescence detection. The fully activated instrument includes two channels from the 488 nm (Blue) laser, four from the 405 nm (Violet) laser, four from the 561 nm (Yellow Green) laser, and two from the 375 nm (Near UV) laser. Instruments with as few as four fluorescent channels activated are available with the ability to activate additional parameters as needed by purchasing an activation key.

LASER SPECIFICATIONS

Spatially Separated Laser Options (Beam Spot Size: 5 µm x 80 µm)

Laser	Wavelength	Power
Blue	488 nm	50 mW
Violet	405 nm	80 mW
Yellow Green	561 nm	30 mW
Near UV	375 nm	60 mW

FLOW CELL

Fixed integrated optics and quartz flow cell design with >1.3 numerical aperture.

Flow Cell dimensions: 420 µm x 180 µm internal diameter

FORWARD SCATTER DETECTION

Proprietary Axial Light Loss (ALL) sensor system using silicon photodiodes with built in 488/8 µm band pass filter.

BAND PASS FILTERS

Includes 13 repositionable filters

450/45 (2)	660/10
525/40 (2)	675/30
585/42	690/50 (2)
610/20 (2)	780/60

FLUORESCENCE AND SIDE SCATTER DETECTION

Fluorescence and side scatter light delivered by fiber optics to Avalanche Photo Diode detector arrays. Proprietary design ensures high performance, high efficiency, low-noise signal detection. Emission profiles are collected using reflective optics and single transmission band pass filters.

VIOLET SIDE SCATTER CONFIGURATION

Option to configure Avalanche Photo Diode detector array to collect side scatter signal from Violet (405 nm) laser. The configured channel (VSSC) can be utilized to better resolve nanoparticles below 200 nm size threshold.

QUALITY CONTROL

For detection channels off of the 405, 488, and 561 nm laser, CytExpert QC automation pass/fail criteria is rCV ≤5.0%. For detection channels off of the 375 nm laser, the criteria is ≤ 7.0%.

PERFORMANCE

SCATTER RESOLUTION

Blue (488 nm) Side Scatter Resolution: <300 nm

Violet (405 nm) Side Scatter Resolution (VSSC): <200 nm

Scatter performance is optimized for resolving human lymphocytes, monocytes, and granulocytes as well as nanoparticles.

CARRYOVER

Single Tube Format: < 1.0%

Plate Loader Format: < 0.5%

SENSITIVITY

FITC: <30 molecules of equivalent soluble fluorochrome (MESF-FITC)

PE: <10 molecules of equivalent soluble fluorochrome (MESF-PE)

FLUORESCENCE RESOLUTION

The CytoFLEX Flow Cytometer is capable of achieving 3% rCV with alignment verification particles capable of rCVs <3%.

ELECTRONICS

NOMINAL ACQUISITION RATE

30,000 events per second with 15 parameters

Software capability to modify window extension parameter and to control abort rate during high event rate signal processing

SIGNAL PROCESSING

Fully digital system with 7 decade data display

SIGNAL

Pulse area, height for every channel, width for one selectable channel

FLUIDICS

ULTRA-LOW PRESSURE PERISTALTIC SHEATH AND SAMPLE DELIVERY SYSTEM

Low maintenance system

Sheath Fluid Filter and Sample Pump Tubing can be replaced by the user (no service visit required)

SAMPLE FLOW RATES

Fixed Flow Rates: 10, 30 and 60 µL/min

Custom Flow Rate Control mode from 10 to 240 µL/min in 1 µL increments.

Gravimetric calibration for absolute counts within CytExpert Software.

BECKMAN COULTER Life Sciences

FLUID CAPACITY

Standard 4 L tanks

Optional 10L cubitainers

AUTOMATED MAINTENANCE FUNCTIONS

System Startup, Sample Mixing, Backflush, Prime, Shutdown, Deep Clean

SAMPLE INPUT FORMATS

5 mL (12 x 75 mm) polystyrene and polypropylene tubes

1.5 mL and 2 mL microcentrifuge tubes

PLATE LOADER FORMATS

96-well Standard Flat, U and V bottom plates

DATA MANAGEMENT

SOFTWARE

The CytExpert software is a full-feature software package that controls the instrument's operation, collection of experiment data, and analysis of the results.

Three different installation modes are available, depending on the level of security required.

The Default installation requires no user login.

For multiuser instruments, the User Management installation requires user login and contains features for role management. Electronic Records Management installation provides tools that facilitate compliance with 21 CFR Part 11, Electronic Records and Electronic Signatures.

An API (Application Programming Interface) is available and allows external software to perform operations such as running methods and for basic control of the plate loader.

If desired, export FCS files for offline analysis in Kaluza, FCSExpress, FlowJo, and other platforms.

STANDARDIZATION

Daily QC beads or any other reference material that is relevant for your application may be used as the standardization sample to set target values and calibrate the gain settings automatically.

LANGUAGE

English and Chinese

OPERATING SYSTEM

Windows® 7 Professional 64-bit

Windows® 8 Professional 64-bit

Windows® 10 Professional 64-bit

FCS FORMAT

FCS 3.0

MINIMUM SPECIFICATIONS

CPU: Intel® I3 @ 2.9 GHz 1 Gigabit Ethernet port

RAM: 4 GB 2 USB 3.0 ports

Storage: 256 GB 4 USB 2.0 ports

COMPENSATION

Automatic full matrix compensation

Manual full matrix compensation

Novel Compensation Library: store fluorescent spillover values of dyes to easily determine the correct compensation matrix with new gain settings

Import/export compensation values between experiments

Absolute linear gain amplification enables the use of compensation settings between experiments and sample types

INSTALLATION

DIMENSIONS (W X D X H)

Cytometer (with or without Plate Loader)

42.5 cm x 42.5 cm x 34 cm

16.7 in x 16.7 in x 13.4 in

Tanks and Holder

14 cm x 35.6 cm x 35.6 cm

5.5 in x 14.0 in x 14.0 in

WEIGHT

Cytometer: 23.4 kg / 51.6 lbs

Cytometer with Plate Loader: 28 kgs / 61.7 lbs

POWER SPECIFICATIONS

Voltage: 100–240 V Power: 150 -250 W

OPERATING TEMPERATURE NON-CONDENSING

15-27 °C, 59-80.6 °F

BECKMAN COULTER Life Sciences

For Beckman Coulter's worldwide office locations and phone numbers, please visit "Contact Us" at **beckman.com**

FLOW-2235SPEC01.17

CytoFLEX LX
Flow Cytometer
Blue-Red-Violet-Yellow Green-Near UV-Infrared
(B-R-V-Y-N-I) Series

OPTICS

EXCITATION OPTICS

The instrument has the capacity for 23 parameters, including 21 for fluorescence detection. The fully activated instrument includes three channels from the 488 nm (Blue) laser, three from the 638 nm (Red) laser, five from the 405 nm (Violet) laser, five from the 561 nm (Yellow Green), three from the 375 nm (Near UV), and two from the 808 nm (Infrared) laser.

LASER SPECIFICATIONS

Spatially Separated Laser Options (Beam Spot Size: 5 µm x 80 µm)

Laser	Wavelength	Power
Blue	488 nm	50 mW
Red	638 nm	50 mW
Violet	405 nm	80 mW
Yellow Green	561 nm	30 mW
Infrared	808 nm	60 mW
Near UV	375 nm	60 mW

FLOW CELL

Fixed integrated optics and quartz flow cell design with >1.3 numerical aperture.

Flow Cell dimensions: 420 µm x 180 µm internal diameter

FORWARD SCATTER DETECTION

Proprietary Axial Light Loss (ALL) sensor system using silicon photodiodes with built in 488/8 µm band pass filter.

BAND PASS FILTERS

Includes 21 repositionable filters

450/45 (2)	690/50
525/40 (3)	710/50
585/42	712/25
610/20 (3)	763/43 (3)
660/10 (2)	840/20
675/30 (2)	885/40

FLUORESCENCE AND SIDE SCATTER DETECTION

Fluorescence and side scatter light delivered by fiber optics to Avalanche Photo Diode detector arrays. Proprietary design ensures high performance, high efficiency, low-noise signal detection. Emission profiles are collected using reflective optics and single transmission band pass filters.

VIOLET SIDE SCATTER CONFIGURATION

Option to configure Avalanche Photo Diode detector array to collect side scatter signal from Violet (405 nm) laser. The configured channel (VSSC) can be utilized to better resolve nanoparticles below 200 nm size threshold.

QUALITY CONTROL

For detection channel off of the 405, 488, 561, and 638 nm laser, CytExpert QC automation pass/fail criteria is rCV ≤5.0%. For detection channels off of the 375 nm and 808 nm laser, the criteria is ≤ 7.0%.

PERFORMANCE

SCATTER RESOLUTION

Blue (488 nm) Side Scatter Resolution: <300 nm

Violet (405 nm) Side Scatter Resolution (VSSC): <200 nm

Scatter performance is optimized for resolving human lymphocytes, monocytes, and granulocytes as well as nanoparticles.

CARRYOVER

Single Tube Format: < 1.0%

Plate Loader Format: < 0.5%

SENSITIVITY

FITC: <30 molecules of equivalent soluble fluorochrome (MESF-FITC)

PE: <10 molecules of equivalent soluble fluorochrome (MESF-PE)

FLUORESCENCE RESOLUTION

The CytoFLEX Flow Cytometer is capable of achieving 3% rCV with alignment verification particles capable of rCVs <3%.

ELECTRONICS

NOMINAL ACQUISITION RATE

30,000 events per second with 15 parameters

Software capability to modify window extension parameter and to control abort rate during high event rate signal processing

SIGNAL PROCESSING

Fully digital system with 7 decade data display

SIGNAL

Pulse area, height for every channel, width for one selectable channel

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FLUIDICS

ULTRA-LOW PRESSURE PERISTALTIC SHEATH AND SAMPLE DELIVERY SYSTEM

Low maintenance system

Sheath Fluid Filter and Sample Pump Tubing can be replaced by the user (no service visit required)

SAMPLE FLOW RATES

Fixed Flow Rates: 10, 30 and 60 µL/min

Custom Flow Rate Control mode from 10 to 240 µL/min in 1 µL increments.

Gravimetric calibration for absolute counts within CytExpert Software.

FLUID CAPACITY

Standard 4 L tanks

Optional 10L cubitainers

AUTOMATED MAINTENANCE FUNCTIONS

System Startup, Sample Mixing, Backflush, Prime, Shutdown, Deep Clean

SAMPLE INPUT FORMATS

5 mL (12 x 75 mm) polystyrene and polypropylene tubes

1.5 mL and 2 mL microcentrifuge tubes

PLATE LOADER FORMATS

96-well Standard Flat, U and V bottom plates

DATA MANAGEMENT

SOFTWARE

The CytExpert software is a full-feature software package that controls the instrument's operation, collection of experiment data, and analysis of the results.

Three different installation modes are available, depending on the level of security required.

The Default installation requires no user login.

For multiuser instruments, the User Management installation requires user login and contains features for role management. Electronic Records Management installation provides tools that facilitate compliance with 21 CFR Part 11, Electronic Records and Electronic Signatures.

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If desired, export FCS files for offline analysis in Kaluza, FCSExpress, FlowJo, and other platforms.

STANDARDIZATION

Daily QC beads or any other reference material that is relevant for your application may be used as the standardization sample to set target values and calibrate the gain settings automatically.

LANGUAGE

English and Chinese

OPERATING SYSTEM

Windows® 7 Professional 64-bit

Windows® 8 Professional 64-bit

Windows® 10 Professional 64-bit

FCS FORMAT

FCS 3.0

MINIMUM SPECIFICATIONS

CPU: Intel Core i7, up to 3.9 GHz

RAM: 8 GB, 4 USB 3.0 ports

Storage: Two 1 TB drives

Ethernet: Integrated 10M/100M/1000M GB

COMPENSATION

Automatic full matrix compensation

Manual full matrix compensation

Novel Compensation Library: store fluorescent spillover values of dyes to easily determine the correct compensation matrix with new gain settings

Import/export compensation values between experiments

Absolute linear gain amplification enables the use of compensation settings between experiments and sample types

INSTALLATION

DIMENSIONS (W X D X H)

Cytometer (with or without Plate Loader)

60.5 cm x 73.3 cm x 45.1 cm

23.8 in x 28.9 in x 17.8 in

Tanks and Holder

25 cm x 25 cm x 25 cm

9.9 in x 9.9 in x 9.9 in

WEIGHT

Cytometer: 79 kg / 174.2 lbs

Cytometer with Plate Loader: 83.6 kgs / 184.3 lbs

POWER SPECIFICATIONS

Voltage: 100–240 V Power: 150 -250 W

OPERATING TEMPERATURE NON-CONDENSING

15-27 °C, 59-80.6 °F

BECKMAN COULTER
Life Sciences

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FLOW-2429SPEC03.17

CytoFLEX LX
Flow Cytometer
Blue-Red-Violet-Yellow Green-UV-Infrared
(B-R-V-Y-U-I) Series

OPTICS

EXCITATION OPTICS

The instrument has the capacity for 23 parameters, including 21 for fluorescence detection. The fully activated instrument includes three channels from the 488 nm (Blue) laser, three from the 638 nm (Red) laser, five from the 405 nm (Violet) laser, five from the 561 nm (Yellow Green), three from the 355 nm (UV), and two from the 808 nm (Infrared) laser.

LASER SPECIFICATIONS

Spatially Separated Laser Options (Beam Spot Size: 5 µm x 80 µm)

Laser	Wavelength	Power
Blue	488 nm	50 mW
Red	638 nm	50 mW
Violet	405 nm	80 mW
Yellow Green	561 nm	30 mW
Infrared	808 nm	60 mW
UV	355 nm	20 mW

FLOW CELL

Fixed integrated optics and quartz flow cell design with >1.3 numerical aperture.

Flow Cell dimensions: 420 µm x 180 µm internal diameter

FORWARD SCATTER DETECTION

Proprietary Axial Light Loss (ALL) sensor system using silicon photodiodes with built in 488/8 µm band pass filter.

BAND PASS FILTERS

Includes 22 repositionable filters

405/10	675/30 (2)
405/30	690/50
450/45	710/50
525/40 (3)	712/25
585/42	763/43 (3)
610/20 (3)	840/20
660/10 (2)	885/40

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Fluorescence and side scatter light delivered by fiber optics to Avalanche Photo Diode detector arrays. Proprietary design ensures high performance, high efficiency, low-noise signal detection. Emission profiles are collected using reflective optics and single transmission band pass filters.

VIOLET SIDE SCATTER CONFIGURATION

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QUALITY CONTROL

For detection channel off of the 405, 488, 561, and 638 nm laser, CytExpert QC automation pass/fail criteria is rCV ≤5.0%. For detection channels off of the 355 nm and 808 nm laser, the criteria is ≤ 7.0%.

PERFORMANCE

SCATTER RESOLUTION

Blue (488 nm) Side Scatter Resolution: <300 nm

Violet (405 nm) Side Scatter Resolution (VSSC): <200 nm

Scatter performance is optimized for resolving human lymphocytes, monocytes, and granulocytes as well as nanoparticles.

CARRYOVER

Single Tube Format: ≤ 1.0%

Plate Loader Format: < 0.5%

SENSITIVITY

FITC: <30 molecules of equivalent soluble fluorochrome (MESF-FITC) from the 488 nm laser.

PE: <10 molecules of equivalent soluble fluorochrome (MESF-PE) from the 488 nm laser.

FLUORESCENCE RESOLUTION

The CytoFLEX Flow Cytometer is capable of achieving <3% rCV with alignment verification particles capable of rCVs <3%.

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NOMINAL ACQUISITION RATE

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Fully digital system with 7 decade data display

SIGNAL

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FLUIDICS

Ultra-low pressure peristaltic sheath and sample delivery system

Sheath Fluid Filter and Sample Pump Tubing can be replaced by the user (no service visit required)

SAMPLE FLOW RATES

Fixed Flow Rates: 10, 30 and 60 µL/min

Custom Flow Rate Control mode from 10 to 240 µL/min in 1 µL increments.

FLUID CAPACITY

10L cubitainers

AUTOMATED MAINTENANCE FUNCTIONS

System Startup, Sample Mixing, Backflush, Prime, Shutdown, Deep Clean

SAMPLE INPUT FORMATS

5 mL (12 x 75 mm) polystyrene and polypropylene tubes

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Windows® 8 Professional 64-bit

Windows® 10 Professional 64-bit

FCS FORMAT

FCS 3.0

MINIMUM SPECIFICATIONS

CPU: Intel Core i7, up to 3.9 GHz

RAM: 8 GB, 5 USB 2.0 and above ports

Storage: 1 TB drive in RAID1

Ethernet: Integrated 10M/100M/1000M GB

COMPENSATION

Automatic full matrix compensation

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Tanks and Holder

25 cm x 25 cm x 25 cm

WEIGHT

Cytometer: 79 kg

Cytometer with Plate Loader: 83.6 kg

POWER SPECIFICATIONS

Voltage: 100–240 V Power: 150 -250 W

OPERATING TEMPERATURE NON-CONDENSING

15-30 °C

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FLOW-2870SPEC08.17