

Claudio Ortolani

Flow Cytometry Today

Everything You Need to Know about
Flow Cytometry

 Springer

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*To the memory of
Howard Maurice Shapiro
"Il miglior fabbro."*

Contents

1	General Principles	1
1.1	Flow Cytometers General Layout	3
1.2	Flow Cytometers Features	4
1.3	Parameters and Signals	5
1.4	Time	6
	References	7
2	Signals: Scattering	11
2.1	Forward Scatter (FSC)	14
2.2	Side Scatter (SSC)	15
2.3	Raman Scattering	16
2.4	Depolarized Scatter	18
	References	19
3	Signals: Fluorescence, Phosphorescence, Impedance, Extinction	23
3.1	Fluorescence	23
3.1.1	Depolarized Fluorescence	25
3.1.2	Autofluorescence	25
3.2	Phosphorescence	28
3.3	Impedance	28
3.4	Axial Extinction	30
	References	31
4	Fluidics	37
4.1	Overview on Fluids	37
4.1.1	Laminar Flow and Turbulent Flow	38
4.1.2	Hydrodynamic Focusing	39
4.2	Cytometer Fluidics	40
4.2.1	Sheath and Core	40
4.2.2	Flow System Components	41
4.2.3	Flow Rate Control	42

4.2.4	Sample Injection	43
4.2.5	Event-Light Interaction: The Interrogation Point	47
	References	50
5	Light Sources	53
5.1	Arc Lamps	54
5.2	Lasers	55
5.2.1	Gas Lasers	58
5.2.2	Solid-State Lasers (SSLs)	62
5.2.3	Liquid State Lasers (Dye Lasers)	72
5.3	Light Emitting Diodes (LEDs)	74
	References	74
6	Optical Benches	79
6.1	From the Light Source(s) to the Interrogation Point(s)	79
6.2	From the Interrogation Point(s) to the Detector(s)	81
6.3	Optical Bench Components	82
6.3.1	Absorption Filters	83
6.3.2	Interference Filters	83
6.3.3	Neutral Density Filters	86
6.3.4	Polarizing Filters	87
6.3.5	Beam Splitters	87
6.3.6	Wavelength Division Multiplexing (WDM)	88
6.3.7	Prisms, Gratings, Coarse WDM (CWDM)	88
6.4	Optical Bench Layouts	88
6.4.1	Transmission Benches	89
6.4.2	Reflection Benches	90
6.4.3	Multilaser Benches	90
6.4.4	Special Solutions	94
	References	95
7	Detectors and Electronics	97
7.1	Photodetectors	97
7.1.1	Photodiodes (PDs)	97
7.1.2	Avalanche Photodiodes (APDs)	98
7.1.3	Photomultipliers (PMTs)	99
7.1.4	Multi-anode Photomultipliers	101
7.1.5	Silicon Photomultipliers (SiPMs)	101
7.1.6	Charged-Coupled Devices (CCDs)	102
7.1.7	Trans-impedance Amplifiers (TIAs)	102
7.2	Circuitry	102
7.2.1	Analog Model	104
7.2.2	Digital Model	113
7.2.3	Hybrid Model	117
	References	117

8	Signal Analysis	121
8.1	The Background	121
8.1.1	Instrumental Background (B_{CAL})	122
8.1.2	Experimental Background (B_{SOS})	122
8.2	The Pulse	122
8.2.1	Pulse Analysis in Analog Systems	125
8.2.2	Pulse Analysis in Digital Systems	126
8.2.3	Practical Applications of Pulse Analysis	129
8.3	Dynamic Range of the Signal	131
8.3.1	Effective Resolution	132
8.3.2	Picket Fence Phenomenon	133
	References	134
9	The Cytometric File	137
9.1	FCS Format	138
9.2	Segments	139
9.2.1	Header Segment	140
9.2.2	Text Segment	140
9.2.3	Data Segment	141
9.2.4	Analysis Segment	142
9.2.5	Optional Segments	142
9.3	Keywords	143
9.3.1	Standard Keywords	144
9.3.2	Non-standard Keywords	147
9.3.3	Relationships Between Keywords and Compensation Procedures	147
	References	148
10	Data Transformation	151
10.1	Logarithmic Transformation	151
10.2	Log-Like Transformations	152
10.3	Polynomial Transformation	154
	References	155
11	Data Representation	157
11.1	Histogram	157
11.1.1	Histograms of Lin Amplified Data	158
11.1.2	Histograms of Log Amplified/Transformed Data	159
11.1.3	Histograms of Log-Like Transformed Data	162
11.2	Cytograms	162
11.2.1	Representation by Dots (Dot Plot)	163
11.2.2	Representation by Contours (Contour Plot)	165
11.2.3	Representation by False Colors or Gray Tones	167
11.2.4	Pseudo-Three-Dimensional Representation	168
11.2.5	Three-Dimensional Representation	169
	References	170

12	Data Analysis	171
12.1	Immunofluorescence Measurements	171
12.1.1	The Vexed Question of the Negative Control	171
12.1.2	Histograms	173
12.1.3	Cytograms	176
12.1.4	Weak Positivity in Immunofluorescence	177
12.2	DNA Content Measurements	182
12.2.1	DNA Content versus BrdU Incorporation	185
12.3	Concept of Gate and Concept of Region	185
12.3.1	Combined (Boolean) Use of Regions and Gates	187
12.4	Advanced Tools and Future Perspectives	195
12.4.1	Pre-processing Programs	197
12.4.2	Data Processing Programs	198
	References	200
13	Standards, Setup, Calibration, and Control Techniques	205
13.1	Standards in Flow Cytometry	206
13.1.1	Natural Standards	206
13.1.2	Artificial Standards	207
13.1.3	Standard Use in Quality Procedures	217
13.2	Optical Bench Setup	218
13.3	Photodetectors' Setup	220
13.3.1	SDeN (Electronic Noise Standard Deviation)	220
13.3.2	PMT Setup	221
13.3.3	APD Setup	223
13.4	Calibration	223
13.4.1	Calibration in ERF	225
13.4.2	Calibration in MESF	225
13.4.3	Calibration in ABC	225
13.4.4	Calibration in FLU	226
13.4.5	Calibration in Nanometers	227
13.5	Instrument Performance and Its Control	229
13.5.1	Linearity	229
13.5.2	Accuracy	229
13.5.3	Resolution	231
13.5.4	Sensitivity	232
13.5.5	Limits of Blank (LOB), Detection (LOD), and Quantification (LOQ)	236
13.5.6	Precision	240
13.5.7	Specificity	241
	References	241
14	Fluorochromes: Overview	247
14.1	Spectral Behavior of Fluorescent Molecules	249
14.2	Relationships with the Environment	253

14.2.1	Spectral Effects	253
14.2.2	Other Effects	254
14.3	Accessory Groups	256
	References	257
15	Fluorochromes Suitable for Antibody Conjugation	259
15.1	Large Protein Molecules	259
15.1.1	Phycobiliproteins	260
15.1.2	Peridinin–Chlorophyll–Protein (PerCP)	265
15.1.3	AmCyan and AmCyan 100	267
15.2	Small Organic Molecules	268
15.2.1	Pyrene Derivatives	268
15.2.2	Pyrydil–Oxazole Derivatives	269
15.2.3	Coumarin Derivatives	270
15.2.4	Xanthene Derivatives	271
15.2.5	Cyanines	278
15.2.6	Proprietary Molecules	283
15.3	π -Conjugated Organic Polymers (Brilliant Violet and Others)	288
15.3.1	Organic Polymers-Based Tandems	289
15.3.2	Narrow-Band Emissive Chromoforic Polymer Dots (Pdots)	290
15.4	Nanocrystals	291
15.4.1	Quantum Dots (Qdots)	291
15.4.2	Upconverting Nanoparticles (UCNPs)	293
15.5	Tandem Fluorochromes	293
15.5.1	UV-Excited Tandem	297
15.5.2	Violet-Excited Tandem	300
15.5.3	Blue-Excited Tandems	301
15.5.4	Green–Yellow-Excited Tandems	306
15.5.5	Red-Excited Tandems	307
15.6	New Solutions	308
15.6.1	Nanoparticle-Encapsulated Fluorophores	308
15.6.2	Backbone-Linked Fluorophores (Kiravia, NovaFluor)	309
	References	311
16	Fluorochromes That Bind Nucleic Acids	325
16.1	Triaryl Derivatives	329
16.2	Stylbene Derivatives	330
16.3	Phenylindole Derivatives (DAPI, DIPI)	330
16.4	Bisbenzimidazole Derivatives	333
16.4.1	HO33258	334
16.4.2	HO33342	335
16.4.3	HO34580	338
16.4.4	Dyecycle Violet (DCV)	338

16.5	Phenanthridine Derivatives	339
16.5.1	Propidium Iodide (PI)	339
16.5.2	Ethidium Bromide	344
16.5.3	Benzophenanthridine Alkaloids	344
16.6	Cyanines	344
16.6.1	TOTO Series Molecules	345
16.6.2	TO-PRO Series Molecules	347
16.6.3	SYTOX® Series Molecules	349
16.6.4	SYTO® Series Molecules	350
16.6.5	SYBR Series Molecules	351
16.7	Aromatic Molecules, Heterocyclic	352
16.7.1	Acridines	353
16.7.2	Oxazines	355
16.7.3	Thiazole Orange (TO) and Its Analogs (DETC)	355
16.7.4	7-Amino-Actinomycin D	357
16.7.5	Pyronin Y	358
16.7.6	LDS-751	359
16.7.7	Berberine (BRB)	361
16.8	Aromatic Molecules, Non-heterocyclic	361
16.8.1	Tricyclic Antibiotics	361
16.8.2	Anthraquinones	362
	References	364
17	Fluorochromes for the Study of the Cell Features	381
17.1	Protein Content	382
17.2	Nucleic Acid Content and Chromatin Organization	384
17.3	Cell Viability	384
17.3.1	DNA Impermeant Probes	385
17.3.2	Amine Reactive Dyes	385
17.3.3	Fluorescein Derivatives	387
17.3.4	Calcofluor White	389
17.3.5	Trypan Blue	389
17.4	Membrane Potential	390
17.4.1	Carbocyanines	391
17.4.2	Oxonol Derivatives	394
17.4.3	Xanthene Derivatives	394
17.5	Mitochondrial Membrane Potential	395
17.5.1	JC-1	395
17.6	Mitochondrial Mass	397
17.6.1	Nonyl Acridine Orange (NAO)	397
17.6.2	Mitotracker Molecules	397
17.6.3	Mitofluor Molecules	399
17.7	Intracellular pH	399
17.7.1	DCH	400
17.7.2	Bcecf-Acetoxymethyl Ester	400

17.7.3	SNARF-1 Acetoxymethyl Ester	401
17.8	Lysosomal Mass and Lysosomal pH	402
17.8.1	Lysotracker® and Lysohunt Molecules	402
17.8.2	Lysosensor™ Molecules	403
17.8.3	Acridine Orange (AO)	404
17.9	Free Radicals (Oxidative Burst)	404
17.9.1	Dihydroethidium (DHE)	406
17.9.2	Dichlorofluorescein Diacetate (DCF-DA)	406
17.9.3	Dihydrorhodamine 123 (DHR123)	406
17.9.4	Tetrazolium Derivatives	406
17.10	Calcium Content	407
17.10.1	INDO-1 Acetoxymethyl Ester	408
17.10.2	Xanthene Derivatives (Fluo Molecules and Others)	409
17.10.3	Fura Molecules	410
17.10.4	BTC	412
17.11	Sodium Content	413
17.11.1	SBFI	413
17.11.2	Sodium Green	413
17.12	Potassium Content	414
17.12.1	PBFI	414
17.12.2	BCECF	415
17.13	Chloride Content	415
17.14	Magnesium Content	415
17.15	Glutathione Content	416
17.16	Heavy Metals Content	416
17.17	Cell Proliferation	417
17.17.1	Fluorescein Esters (CFSE)	418
17.17.2	Lipophilic Carbocyanines	418
17.17.3	CellVue® Series Molecules	420
17.18	Multidrug Resistance	421
17.18.1	Rhodamine 123 (RH123)	422
17.18.2	Calcein Acetoxymethyl Ester	422
17.19	Membrane Fluidity	423
17.20	Lipid Content	424
17.20.1	NILE RED	424
17.20.2	Bodipy and Its Derivatives	425
17.21	Lipid Peroxidation	426
17.21.1	Cis-Parinaric Acid	426
17.21.2	C11-BODIPY581/591	426
17.22	Endoplasmic Reticulum (ER) Labeling	427
	References	427
18	Fluorescent Proteins	445
18.1	Fluorescent Proteins and Flow Cytometry	447
18.1.1	Green Fluorescent Proteins (GFPs)	448

18.1.2	Blue Fluorescent Proteins (BFPs)	449
18.1.3	Cyan Fluorescent Proteins (CFPs)	450
18.1.4	Yellow Fluorescent Proteins (YFPs)	450
18.1.5	Orange Fluorescent Proteins (OFPs)	450
18.1.6	Red Fluorescent Proteins (RFP)	451
18.1.7	Infrared Fluorescent Proteins (iRFPs)	452
	References	453
19	Spillover and Compensation	457
19.1	Spillover	457
19.1.1	Intra-Laser Spillover	457
19.1.2	Inter-Laser Spillover	462
19.1.3	Spillover Matrix and Compensation Matrix	463
19.2	Compensation	464
19.2.1	Paradoxical Effects	466
19.2.2	Negative Values and their Management	469
19.2.3	Compensation by Hardware	470
19.2.4	Compensation by Software	471
	References	472
20	Artifacts	475
20.1	Escapees	475
20.2	Debris	477
20.3	Other Artifacts	477
20.3.1	Due to Anticoagulants	477
20.3.2	Due to Fluorochromes	478
20.3.3	Due to Serum Factors	480
20.3.4	Anecdotal Reports	481
	References	482
21	Cell Sorting	485
21.1	Fluid Switching Sorters	485
21.1.1	Pros and Cons of Fluid Switching Sorters	486
21.2	Electrostatic Sorters	486
21.2.1	Sorting Procedures	488
21.2.2	Sorting Modalities	490
21.2.3	High-Pressure Systems	494
21.3	Pneumatic Sorters	494
	References	495
22	Non-Conventional Flow Cytometry	497
22.1	Imaging Flow Cytometry	497
22.1.1	CCD-Based Imaging Flow Cytometry	498
22.1.2	PMT-Based Imaging Flow Cytometry	500
22.2	Spectral Flow Cytometry (SFC)	501
22.2.1	Optical Bench in Spectral Flow Cytometry	502

22.2.2	Spectral Unmixing in Spectral Flow Cytometry	502
22.2.3	Format Issues in Spectral Flow Cytometry	503
22.2.4	PROs and CONs in Spectral Flow Cytometry	503
22.3	Mass Cytometry (MC)	504
22.3.1	Format Issues in Mass Cytometry	505
22.3.2	PROs and CONs in Mass Cytometry	506
22.4	Lifetime Cytometry	507
22.5	Raman Cytometry	509
22.6	Microfluidic Devices (Labs on Chips)	510
22.6.1	Hydraulic Issues in Microfluidic Devices	511
22.6.2	Sorting Procedures in Microfluidic Devices	512
	References	513
23	Statistics: A Cytometric Point of View	523
23.1	Concept of Distribution	523
23.1.1	Log-Normal Distribution	523
23.1.2	Normal (Gaussian) Distribution	524
23.1.3	Poisson Distribution	524
23.2	Location Measurements	525
23.2.1	Mean ($\bar{A}_m$)	526
23.2.2	Geometric Mean ($\bar{g}_M$)	526
23.2.3	Truncated Mean	526
23.2.4	Mode (v_0)	527
23.2.5	Median ($\bar{M}_e$)	527
23.3	Spread Measurements	527
23.3.1	Variance (Var)	528
23.3.2	Standard Deviation (SD)	528
23.3.3	Median Absolute Deviation (MAD)	528
23.3.4	Robust Standard Deviation (rSD)	529
23.3.5	Coefficient of Variation (CV)	529
23.3.6	Robust Coefficient of Variation (rCV)	530
	References	530
	Correction to: Fluorochromes That Bind Nucleic Acids	C1
	Index	533

Chapter 22

Non-Conventional Flow Cytometry

Other flow techniques than Conventional Flow Cytometry have appeared on the cytometry stage in recent years, primarily differing on the exploited signals. These “non-conventional” flow techniques rely on different technological solutions, some of which are scarcely represented in laboratories worldwide or not even commercially available, while others are thriving and even rapidly expanding. The most relevant “non-conventional” flow techniques encompass (1) Imaging Flow Cytometry (IFC), (2) Spectral Flow Cytometry (SFC), (3) Mass Cytometry (MC), (4) Lifetime Cytometry, and (5) Raman Cytometry. All of them are covered in this chapter for completeness’ sake; a final section is devoted to the so-called microfluidic devices or “labs-on-chips,” representing a turbulent evolutive branch of Flow Cytometry.

22.1 Imaging Flow Cytometry

Like in Conventional Flow Cytometry, in Imaging Flow Cytometry (IFC), the events in suspension are moved in a fluid to intercept one or more light lines. The result, or one of the results of this interaction, is creating a digitized image associated with each analyzed event. Depending on the models, the implemented technology can rely on CCDs, high-speed brightfield cameras, or PMTs. The instruments commercially available are very different from each other, both in performance and structural layout, and encompass:

1. Two CCD-based Imaging Flow Cytometers (FlowSight® and Image Stream®X MKII, marketed by DiaSorin), which can detect up to two brightfield images, two darkfield images, and eight fluorescence-related images, depending on the model.
2. A conventional analyzer (Attune CytPix Flow Cytometer, marketed by Thermo Fisher Scientific), which besides pulse analysis, can also capture high-resolution brightfield images thanks to a high-speed brightfield camera.

3. A module based on fluorescence imaging using radiofrequency-tagged emission (FIRE, also known as BD CellView™ Image Technology, marketed by Becton Dickinson), which can transform a PMT-based conventional cell sorter (e.g., the BD FACSMelody™ system) into an image-enabled cell sorter (ICS) capable of detecting axial extinction, FSC, SSC, and four fluorescence signals.

22.1.1 CCD-Based Imaging Flow Cytometry

In the CCD-based Imaging Flow Cytometry (IFC), the image of each analyzed event is detected by a CCD sensor and resolved in a pixel matrix (George et al. 2004; Basiji 2005; Basiji et al. 2007). Every pixel intensity value is digitalized and stored in an array of data associated with each event (Fig. 22.1). These actions allow the achievement of four primary goals, i.e., (1) to retrieve and directly observe the image of the event and the topographical distribution of the signals, (2) to evaluate the event shape, calculating all the parameters made available by the image analysis algorithms, like shape, perimeter, and elongatedness, (3) to evaluate the texture of the image, understood as the signal modulation in the event's context, and (4) to calculate for each event the total signal strength, obtained by summing all the pixels

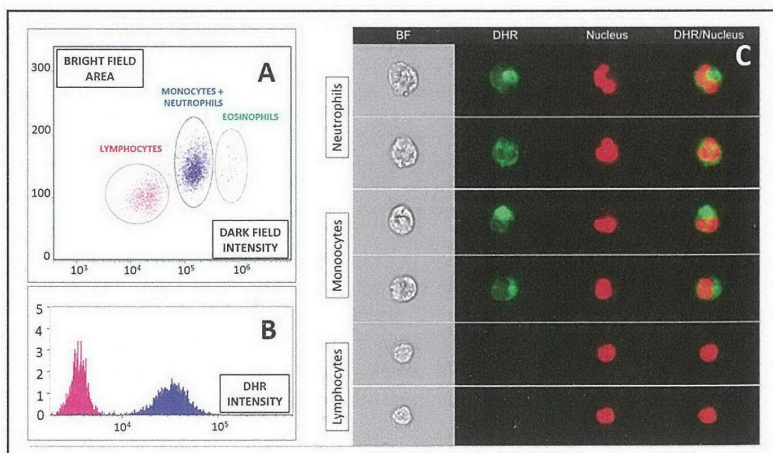


Fig. 22.1 The figure shows the result of a flow image analysis of a sample of peripheral blood carried out for physical parameters (darkfield intensity vs. brightfield area), DNA content (Propidium iodide), and oxidative activity (Dihydrorhodamine). The dot plot representation (panel A) allows the distinction between the main cell types and the oxidative activity evidenced by the transformation of the non-fluorescent Dihydrorhodamine into the fluorescent Rhodamine 123 (panel B). On the right, the gallery (panel C) demonstrates the image features of the five selected elements, including the bright field image, the rhodamine 123 emission, the shape of the nucleus, and the combination of the two last parameters

intensities. It is hardly necessary to observe that the total signal strength is equivalent to the peak value obtained by Conventional Flow Cytometry; it follows that the results of an IMC analysis can also be stored in FCS format, as far as it pertains to the total signal strength values.

22.1.1.1 Optical Bench in CCD Based Imaging Flow Cytometry

Even though the details can vary depending on the models, in CCD-based IFC, the optical bench spectrally resolves each event's signals through a dichroic filter stack. It leads them to different sections of the CCD spectrally selected by an array of band-pass filters allowing the separate transmission of adjacent sections of the spectrum. In this way, an event generates several spectrally different images, each filtered by a different band-pass interpolated before the detector; the integration of these images allows the topographical recognition of all the signals in the event's context. In 12-channel commercial instruments, the twofold optical bench creates at once the following images, i.e.,

1. Two brightfield images (one for each bench) elicited by an array of colored LED lights.
2. Two darkfield images (one for each bench), conceptually equivalent to the side scatter, elicited by a 785 nm laser and collected through a 740–800 nm band-pass.
3. Up to eight fluorescence-related images (four for each bench), which can be increased to ten if you exploit the 785 nm line to elicit fluorescence instead of scattering signals.

22.1.1.2 Compensation Issues in CCD-Based Imaging Flow Cytometry

In CCD-based IFC, compensation procedures are not exceedingly far from Conventional Flow Cytometry; still, significant differences exist. Given that this technique records each event image as a pixel matrix, the compensation procedure applies the compensation matrix to each pixel of each image related to each analyzed event (Ortyn et al. 2006).

22.1.1.3 Format Issues in CCD-Based Imaging Flow Cytometry

The result of an analysis performed by CCD-based IFC is usually saved by the acquisition applicative (InSpire) in a file in rif (raw image file) format, containing the raw data (pixel intensity data and uncorrected image data) and the instrument set-up data. The rif file is subsequently analyzed by an image analysis program (Ideas) which in turn creates daf (data analysis file) and cif (compensated image file) format files. Daf files contain the calculated feature values, the graphs, and the statistics, while cif files contain the compensated data.

The result of an analysis performed by Imaging Flow Cytometry (IFC) can also be saved as an FCS format file; this file is unsuitable for being re-analyzed by the Ideas software, having lost the data needed for image analysis.

22.1.1.4 Pros and Cons in CCD-Based Imaging Flow Cytometry

One of the direct advantages of taking events' digitalized images is the capability of performing their morphometric analysis, i.e., the evaluation of parameters other than photometric, such as cell area, perimeter, aspect ratio, texture, spot counts, cell centroid, gradient intensity, spatial frequency, and others (George et al. 2004).

As extensively said before, the second great asset of CCD-based IFC is the possibility of seeing the morphology of the analyzed events and the co-localization of the probes adopted in the analysis. In other words, and as an example, it is possible to decide if a target is associated with the membrane, the nucleus, or other structures dwelling in the cytoplasm. This capability assumes the utmost importance in studying a series of biological phenomena, including apoptosis, autophagy, cytotoxicity, phagocytosis, trogocytosis, and NETosis.

The main drawback of CCD-based IFC is its low speed, which does not usually exceed 1000 events/sec (Mikami et al. 2020). Another drawback is its exquisitely analytical nature, which does not allow separating the events of interest.

22.1.2 PMT-Based Imaging Flow Cytometry

A PMT-based image-enabled cell sorter (ICS) is a cell sorter displaying a conventional architecture in which, besides the other conventional laser lines, an optical module based on radiofrequency-tagged emission (FIRE) is implemented (Schraivogel et al. 2022). The FIRE module, also known as BD CellView™ Image Technology, consists of a blue laser whose emission is modulated by a couple of radiofrequency-driven acousto-optic deflectors (AOD) into an array of roughly 100 beamlets, each carrying a sinusoidal modulation at a different frequency (Diebold et al. 2013). The array of spots interacts with the event traversing the interrogation point, and each parameter under analysis gives out a pulse whose shape displays a series of modulations depending on the radio-frequency modulations applied by the AODs to the beamlets (Schraivogel et al. 2022).

Each pulse, transduced by a conventional PMT, is sampled at a very high frequency (180 MHz), digitized by a 16 bit ADC, and pipelined into an array of digital signal processors that compute its A, H, W, and time-to-peak values, and manage the Fourier transform-based extraction of the morphology-related details.

For each event, all these data are compensated and assembled in an information bulk called "event packet" (EP) and fed to further FPGA responsible for the sorting decisions, based both on traditional pulse components and on extracted image

parameters (i.e., eccentricity, max intensity, size, radial moment, the correlation between two imaging channels, and delta center of mass) (Schraivogel et al. 2022).

The short time (not exceeding 400 μ sec) required by the whole operation allows for sorting procedures and makes possible an event rate of close to 15.000 events per second (Schraivogel et al. 2022).

22.1.2.1 Compensation and Format Issues in PMT-Based Imaging Flow Cytometry

In PMT-based IFC, the compensation procedures rely on traditional linear unmixing (Schraivogel et al. 2022).

The final output of the analytical run consists of (1) image data encompassing a multi-plane floating-point TIFF file, (2) a conventional data set in FCS 3.1 standard, and (3) an event table including scatter, intensity, and image-derived parameter values. Non-FCS format values can be re-analyzed outside the native program thanks to plug-ins made on purpose (Schraivogel et al. 2022).

22.2 Spectral Flow Cytometry (SFC)

Spectral Flow Cytometry (SFC) has long been the subject of research and development (Wade et al. 1979; Gauci et al. 1996; Robinson 2004; Leavesley et al. 2005; Robinson et al. 2004, 2005a, 2005b, 2007; Grégori et al. 2012; Nolan and Condello 2013). Still, its full realization has been long hampered by a series of issues related to the inadequacy of the technologies needed to realize it.

The term “Spectral Flow Cytometry” defines a variant of Flow Cytometry that relies on a new way of detecting fluorescent light signals since it does not detect the emission peak of each fluorochrome like in Conventional Flow Cytometry, but its total emission over the whole visible range of the spectrum. It follows that in Spectral Flow Cytometry, it is possible to distinguish between the signals of fluorochromes sharing the same emission peak, provided their emissions differ in another region of the spectrum.

Spectral Flow Cytometers and Sorters are currently marketed by Cytex Biosciences, Sony, and Thermo Fisher Scientific. A module for spectrally enhanced cytometric analysis has been recently made available by Becton Dickinson for their instrument FACSymphony™ A5 SE (for further information on this topic, see Sect. 6.4.4.3).

22.2.1 Optical Bench in Spectral Flow Cytometry

In a spectral cytometer, the light signal generated in the interrogation point is resolved along the entire spectrum by a device consisting of an array of prisms, gratings, or coarse WDM (CWDM) demultiplexers (Cytek Biosciences 2019) (for further information on this topic, see Sect. 6.3.7). The resulting signals are detected by a single multichannel sensor, which may consist of a CCD camera, a multianode (multichannel) PMT, or an array of APDs (Grégori et al. 2012; Nolan and Condello 2013; Feher et al. 2016; Cytek Biosciences 2019) (for further information on this topic, see Sect. 7.1.4). More complex models rely on multiple lasers; in that case, lasers are separated in space, and the signals evoked by each rely on a separate optical pathway, encompassing a dispersion optic and a multichannel sensor. The number of channels in the multichannel sensors depends on the laser eliciting the signals and decreases as the laser emission shifts towards red; it follows that the total number of channels available in a given instrument varies according to the characteristics of the optical bench.

22.2.2 Spectral Unmixing in Spectral Flow Cytometry

In Spectral Flow Cytometry (SF), spillover management has been considered not conceptually different from Conventional Flow Cytometry (Roederer 2019). Still, compensation procedures differ in that the inappropriate signals are not subtracted from the detected peak of the signal to be compensated. Instead, the signal relative to each parameter is computationally reconstructed through a procedure known as “spectral unmixing” by subtracting the inappropriate signals channel by channel for the entire spectral range. From a computational point of view, this procedure can rely on different mathematical models, including least-squares methods (LSM), multivariate curve resolution (MCR), principal component analysis (PCA), and probabilistic spectrum analysis (PSA), or a combination of them. The choice of the model can depend on different factors, among which are the availability of negative controls (i.e., the knowledge of the single components’ spectra) and the linearity of the light dispersion (Nolan and Condello 2013; Futamura et al. 2015; Feher et al. 2016; Niewold et al. 2020). One of the most successful computational models is the least-squares method (LSM), which carries out a linear unmixing procedure based on the assumption that the different detectors’ noise level displays the same statistical behavior (Nolan and Condello 2013).

It is noteworthy that spectral unmixing can also subtract an autofluorescence signal, provided that such a control is available (Nolan and Condello 2013; Novo 2022). It follows that spectral Flow Cytometry can brilliantly resolve a dim positivity in the context of a highly autofluorescent population, as often occurs in the analysis of cell lines or special samples like the bronchoalveolar lavage fluid (BALF).

22.2.3 Format Issues in Spectral Flow Cytometry

In Conventional Flow Cytometry, the quantitative expression of a single parameter is stored in the data set as the peak value detected by the detector devoted to its measurement. Still, in Spectral Flow Cytometry (SFC), all the available detectors measure the parameter expression along the whole spectrum. It follows that the quantitative expression of a single parameter could be stored as a string of different quantitative values, each of them related to the channel managed by the detector responsible for that definite spectral span (Watson et al. 2008). From a theoretical point of view, this approach is still compliant with the FCS format but generates files of cumbersome and unmanageable dimensions and breaks the continuity of the measured spectrum, particularly when the measurement is performed by a CCD (Nolan and Condello 2013).

Since raw data produced by spectral cytometers can also be stored as spectra in ASCII format (Nolan et al. 2013), a solution has been devised in which a zipped archive encompasses a file in ASCII format containing the streamed data together with a related FCS format-compliant file (Nolan et al. 2012). In reality, the files produced by commercial spectral cytometers are stored in proprietary formats specifically designed by the manufacturers, which can still be translated into the FCS format and read by third-party applications.

By computational means, it is possible to apply virtual spectral filters to the spectra generated by each event to analyze the signals relating to defined wavelength ranges. This approach recreates the situation existing in conventional cytometers, in which the spectral ranges are defined by band-pass, and allows the performance of traditional ratio and compensation procedures (Nolan et al. 2013). Moreover, this approach is fully compliant with the FCS 3 format since it reproduces the “one fluorochrome-one detector” mantra of Conventional Flow Cytometry and assigns only one value (the peak value) to each event; nevertheless, even though technically possible, it nullifies all the advantages connected to the spectral measurement. Still, switching between this approach and the full spectral one allows a comparison between conventional compensation and spectral unmixing algorithms on the same data set (Grégori et al. 2012). In this regard, it is interesting to note that spectral unmixing and compensation procedures produce substantially similar results (Niewold et al. 2020).

22.2.4 PROs and CONs in Spectral Flow Cytometry

Spectral Flow Cytometry (SFC) can simultaneously manage fluorochromes with the same emission peak, provided their emissions differ in other spectrum regions. This capability allows the simultaneous use of more fluorochromes than conventional flow cytometry techniques; actually, the highest number of extrinsic parameters

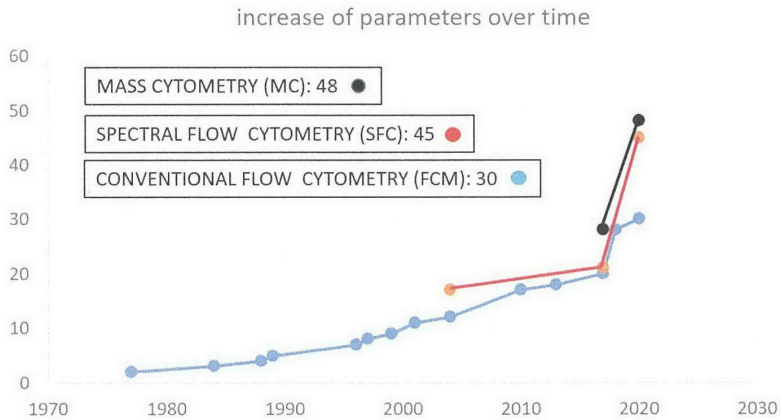


Fig. 22.2 Increase over time of total parameters detected in Flow and Mass Cytometry according to the literature at the publication of this book. Of course, the data are provisional and likely to increase shortly

measured by SFC so far is 43 (Sahir et al. 2020), against 28 measured by FCM (Liechti and Roederer 2019a, 2019b) (Fig. 22.2).

Another advantage of Spectral Flow Cytometry (SFC) is getting rid of the autofluorescence signal by computational spectral unmixing techniques (Novo 2022). Finally, it should be noted that the circuitry at the basis of the spectral flow cytometry allows fast multiparametric spectral-based cell sorting.

Regarding the technique’s sensitivity, there is a non-peer-reviewed debate on the Internet which objects that, compared to Conventional Flow Cytometry, the greater number of detectors available in Spectral Flow Cytometry would be susceptible to decreasing the amount of light detected by each detector with an increase of the noise and a consequent increase in the signal to noise ratio. This theoretical claim has been refuted because the total Poisson noise would be the same in both cases (Novo 2019). Moreover, it has also been observed that the total spectral range of the optical filter system used in Spectral Flow Cytometry is larger than in Conventional Cytometry; consequently, it should detect more photons under the same experimental conditions, allowing “more accurate measurements and hence better data” (Novo 2022).

22.3 Mass Cytometry (MC)

Mass Cytometry (MC) differs from fluorescence-based Flow Cytometry because of the nature of the detected signal. Instead of fluorochromes, in Mass Cytometry, Mabs are conjugated with metal isotopes whose mass is detected by time-of-flight

inductively coupled plasma mass spectrometry (TOF-ICPMS) (Bandura et al. 2009; Ornatsky et al. 2010; Bjornson et al. 2013).

The detected isotopes, absent in biological materials, include the Lanthanides Cerium (140Ce, exploited for the verification of the plasma state (Ornatsky et al. 2010)), Praseodymium (141Pr), Neodymium (142Nd, 143Nd, 144Nd, 145Nd, 146Nd, 148Nd, 150Nd), Samarium (147Sm, 149Sm, 152Sm, 154Sm), Europium (151Eu, 153Eu), Gadolinium (155Gd, 156Gd, 158Gd, 160Gd), Terbium (159 Tb), Dysprosium (161Dy, 162Dy, 163Dy, 164Dy), Holmium (165Ho), Erbium (166Er, 167Er, 168Er, 170Er), Thulium (169Tm), Ytterbium (171Yb, 172Yb, 173Yb, 174Yb, 176Yb), and Lutetium (175Lu) (Dusoswa et al. 2019). Other metals exploited in tagging are Palladium (102Pd, 104Pd, 105Pd, 106Pd, 108Pd, 110Pd, mainly used in barcoding procedures), Platinum (194Pt, mainly used as Cisplatin in viability assessment), Rhodium (mainly used coupled with an intercalating molecule for DNA detection), and Iridium (191Ir and 193Ir, also mainly used coupled with an intercalating molecule for DNA detection/dead cells identification). Finally, further exploited metals are Indium (113In and 115In) (Grenier et al. 2020) and Yttrium (Han et al. 2018), to which Bismuth, Cadmium, Hafnium, Iodine, Niobium, Osmium, Ruthenium, Silver, Tantalum, Tellurium, Tin, and Zirconium have been anecdotally added (Han et al. 2018; Dusoswa et al. 2019; Devine et al. 2021).

Another significant difference with Conventional Flow Cytometry is the absence of a hydraulic component. Instead of being transported by the flow, the suspension containing the tagged events is nebulized, prewarmed, and injected into an inductively coupled plasma (ICP) torch that ionizes each event. The resulting ion clouds are (1) sampled by a three-aperture plasma-vacuum interface, (2) deflected by a dc quadrupole which leaves photons and neutral elements undeflected, and (3) led to the time-of-flight (TOF) analyzer by a radiofrequency quadrupole ion guide which gets rid of low-mass ions deriving from organic moieties (Bandura et al. 2009; Ornatsky et al. 2010; Bendall et al. 2011; Olsen et al. 2019). Every 13 μ s, a push-out plate pushes the ions into a TOF analyzer, determining the ions' mass according to their time of flight (Lee and Rahman 2019). At the end of the procedure, the values of each event's spectra are digitalized and recorded.

The very narrow band of the signals detected by Mass Cytometry allows reaching two very important intertwined goals, i.e., a very high number of parameters to be analyzed simultaneously and the virtual absence of spillover between different signals.

Cytometers based on mass detection are currently produced and commercialized by Fluidigm (<https://www.fluidigm.com/>).

22.3.1 *Format Issues in Mass Cytometry*

In Mass Cytometry, data are recorded in an integrated mass data (IMD) format file, which contains, for every channel, the ion counts related to each ion cloud shoved every 13 microseconds towards the TOF. An IMD format file is usually very heavy

(even some tenths of Gigabytes), and it is usually converted into an FCS file (Newell et al. 2012; Lee and Rahman 2019; Olsen et al. 2019), which contains an integrated value for every event instead, and can be read by the common analysis programs. Note that in the transition from IMD to FCS format, only are translated the clouds whose signals comply with the requisites preset by the operator, while the other clouds are not. It follows that the content of the translated FCS file depends on the criteria set by the operator, and it is good practice, when possible, not to rid of the IMD file, which can be converted again according to different criteria.

22.3.2 PROs and CONs in Mass Cytometry

22.3.2.1 Pros

The main asset of Mass Cytometry is its multiparametricity. From a theoretical point of view, up to 135 parameters can be simultaneously detected (Han et al. 2017); nevertheless, the relative scarcity of available metal tags prevents reaching theoretic proficiency (Schulz et al. 2017). At the time of publication of this book, the highest number of signals simultaneously analyzed with this method was 48 (Rodriguez et al. 2020); in this regard, it should be remembered that the largest number of parameters currently analyzed with Fluorescence Cytometry techniques (Spectral Flow Cytometry) was 45, encompassing two scatter and 43 fluorescence signals related to antigen expression (Sahir et al. 2020) (Fig. 22.2).

The second most important advantage is the elimination of spillover. Since the isotopes' atomic weights are unequivocally separated, no compensation should be needed in Mass Cytometry. Nevertheless, despite these premises, spillover issues are not entirely eliminated since some crosstalk can still exist due to variations in abundance sensitivity, unsatisfactory isotope purity, oxide formation adding to the tag the 16 mass units of the Oxygen atom (^{16}O) (Olsen et al. 2019), and other confounding signals (Leelatian et al. 2015; Lu et al. 2015; Keller et al. 2016; Rahman et al. 2018). Computational procedures have been published to overcome this problem (Chevrier et al. 2018; Miao et al. 2020). Finally, the absence of autofluorescence drastically reduces the instrumental background.

22.3.2.2 Cons

One of the main limitations of Mass Cytometry is the missed detection of the light scattering signals. In Mass Cytometry, the cell is recognized as such based on three main criteria, i.e.:

1. The presence of DNA, detected thanks to intercalating molecules conjugated with Platinum and Iridium (Fienberg et al. 2012) or with Iridium and Rhodium (Ornatsky et al. 2010); the use of Iridium and Rhodium allows the recognition

- of nucleated events (Iridium +) and the distinction between living (Iridium + Rhodium -) and dead cells (Iridium + Rhodium +).
2. The value of a parameter called “cell length” or “event length,” which does not correlate with the size of the event but is related to the ion cloud size and the overall metal ion content (Stern et al. 2017).
 3. The value of a parameter called “lower convolution threshold,” i.e., the total signal intensity across all acquisition channels, which should exceed a preset threshold (Olsen et al. 2019).

In Conventional Flow Cytometry, the term “TOF” (time of flight) corresponds to the width of the pulse and is somehow proportional to the size of the event, but in Mass Cytometry, it does not provide any information on the physical parameters since it defines the time that the ions take to travel from the torch to the detector (Tanner et al. 2013). Anyway, there are some attempts to detect parameters co-varying with cell size; these consist of the detection of wheat germ agglutinin (WGA) or Osmium tetroxide (OsO_4) (Stern et al. 2017). WGA staining intensity correlates well with both cell size and FSC and, in Mass Cytometry, can be detected through staining the cells with AF488-conjugated WGA followed by incubation with a Holmium-conjugated anti AF488 antibody. Osmium tetroxide, widely used in electron microscopy sample fixation, binds proportionally to plasma membrane lipids and can be detected as such because of its atomic weight. Another attempt was made to correlate SSC with the expression of VAMP-7 in hematological samples (Tsai et al. 2020). Other significant disadvantages of Mass Cytometry are:

1. The definitive unfeasibility of sorting procedures due to the ionization of the events.
2. The inability to detect cellular function usually detected by fluorescent probes, except for anecdotal reports concerning (1) a proliferation test allowed by the use of an anti-FITC CFSE-crossreacting ^{172}Yb -conjugated Mab (Good et al. 2019) and (2) alternative methods of viability determination based on Hafnium, Niobium, Platinum, and Zirconium (Devine et al. 2021).
3. The inability to perform the autofluorescence-based analysis of phytoplankton.
4. The inability to detect Fluorescent Proteins, except for a couple of anecdotal reports tracing GFP thanks to a GFP-specific ^{139}La - (Ajami et al. 2018) or ^{169}Tm -conjugated Mab (Chen et al. 2020).
5. The low speed of analysis, which currently stands at around 1000 events per second (Bendall et al. 2012) against 30.000 and more attainable in Conventional Fluorescence Cytometry.

22.4 Lifetime Cytometry

The Fluorescence Lifetime (τ) is the time elapsing between the excitation of a fluorochrome and the emission of fluorescence, i.e., the time an excited fluorochrome spends before coming back to the fundamental state. The Fluorescence

Lifetime generally varies from 100 picoseconds to 15 nanoseconds, depending on the structural characteristics of the molecule (Lakowicz 2006). Consequently, it may happen that two different molecules, characterized by the same emission peak, have different lifetimes and can be mutually distinguished based on this parameter. Moreover, modulation of fluorescence lifetime can be induced in the same molecule by variously modifying the molecule or its environment; consequently, the study of this parameter has been exploited in the label-free evaluation of a series of conditions, including the status of residual chromatin in apoptotic cells (Sailer et al. 1998b), the type of nucleic acid hosting an intercalating probe (Sailer et al. 1998a; Cui et al. 2003), the cell pH (Islam et al. 2013), bacterial phagocytosis (Li et al. 2017), leukemic blast recognition (Lee et al. 2018), integrin activation (Sambrano et al. 2018), apoptosis (Alturkistany et al. 2019; Nichani et al. 2020), FRET phenomena (Sailer et al. 1997a, 1997b; Steinkamp et al. 1999, 2000; Nedbal et al. 2015), and others (Sailer et al. 1996, 1997b, 1997a, 1998a, 1998b; Keij et al. 1999; Steinkamp 2001; Cui et al. 2003; Gohar et al. 2013; Sands et al. 2014).

The technologies available for measuring this parameter belong to two different groups, i.e., frequency-resolved techniques and time-resolved techniques (Bitton et al. 2021).

The frequency-resolved techniques rely on an excitation sinusoidally modulated in intensity at a high frequency; given that the emission will also be modulated in this case, these techniques measure the phase shift between the two signals, which is directly proportional to the fluorescence lifetime (Steinkamp et al. 1999). Another ingenious frequency-resolved technique evaluates the phase shift between the fluorescence signal and the side scatter signal (Houston et al. 2010).

The time-resolved techniques rely on serial measurements of the fluorescence emission resulting from a very short pulsed excitation; these techniques encompass different methods, including time-correlated single-photon counting (TCSPC), time gating, and pulse sampling, also known as direct waveform recording. A particular lifetime cytometry exploiting a technology belonging to the latter group is the Fluorescence-lifetime Excitation Cytometry by Kinetic Dithering (FLECKD). In FLECKD, the exciting laser oscillates (dithers) transversely to the events' direction, delivering a series of very brief excitation pulses lasting about 25 ns. The fluorescence lifetime can be devised thanks to multiple detectors evaluating the gap between the appearance of the side scatter signals and the fluorescence-related signals, whose serial sampling allows detection of the decay curves, which can be further deconvoluted computationally (Li et al. 2014; Vacca and Houston 2015).

The fluorescence lifetime-based distinction of the emission of multiple fluorochromes sharing the same emission peak makes feasible three particularly advantageous objectives:

1. The possibility of increasing the number of probes to be exploited simultaneously in a multiparametric analysis.
2. The elimination of the spillover phenomenon, given that the emissions are mainly resolved based on fluorescence lifetime and not wavelength.

3. The elimination of autofluorescence, whose signal is also resolved based on its lifetime and can be selectively subtracted.

The principles of lifetime cytometry have also been experimentally applied to cell sorting (Houston et al. 2010). The existing fluorescence lifetime-based cytometers are exquisitely analytical instruments, but an experimental project has considered an instrument with sorting capabilities equipped with SiPMs (Rocca et al. 2016).

A fluorescence lifetime-based flow cytometer is currently commercially available from Kinetic River Corporation (<https://www.kineticriver.com/>).

22.5 Raman Cytometry

Besides Rayleigh scattering, a molecule hit by a monochromatic incident light emits another scattering signal known as Raman scattering (for further information on this topic, see Sect. 2.3). Raman scattering displays different peaks whose wavelengths differ from incident light and depend on the molecule's vibrational modes, which, in turn, depend on several features, among which the presence of double bonds. The Raman scattering behaves as the spectral signature of a molecule, and its detection can be exploited in an analytical technique known as "Raman spectroscopy," which can be applied to Flow Cytometry (Watson et al. 2008; Nolan and Sebba 2011; Nolan et al. 2012). In a way similar to Spectral Fluorescence Cytometry, in Raman Cytometry, the scattering is spectrally resolved by a prism, a grating, or an equivalent device and led to a detector consisting of a multi-anode PMT or a high-density array detector such as a CCD.

A condition that hampers the detection of Raman scattering in Flow Cytometry is the very low intensity of the signal. A series of technical solutions can overcome this problem, allowing the molecules under analysis to adhere to a metal nanoparticle surface that amplifies the signal. This phenomenon is known as surface-enhanced Raman scattering (SERS) (Kerker 1983).

Raman cytometry can be theoretically exploited according to two different approaches. In the first approach, the technique analyzes label-free cells to gather information about their intrinsic parameters (metabolic features), et cetera; in the second, the technique analyzes cells labeled with Raman scattering probes, whose signal has been amplified by nanoparticles.

SERS-tagged antibodies and ligands have been increasingly used to detect cell surface markers or intracellular antigens (Pilot et al. 2019). In this particular case, the Raman scattering allows the exploration of extrinsic parameters, similarly to what happens with the conventional scattering elicited by antibodies conjugated with colloidal gold (Bohmer and King 1984a, b). No Raman scattering-based flow cytometers are commercially available; experimental devices have been devised based on a microfluidic architecture (Lau et al. 2008; Zhang et al. 2015).

22.6 Microfluidic Devices (Labs on Chips)

In-depth treatment of Microfluidics Devices is beyond the scope of this book, and further information on the subject can be obtained from the consultation of a series of reviews and monographs to which we refer (Hughes and Hoettges 2010; Yu et al. 2014; Warkiani et al. 2015; Tang et al. 2019; Witek et al. 2020). Nevertheless, this article will cover the subject briefly for completeness' sake.

Microfluidics Devices are a heterogeneous family of small flow cytometers, relying on different technological solutions. They are generally very small in size, with a fluidic system featuring conduits with a diameter lesser than 1 mm, and are often collectively referred to as "lab on chips".

Microfluidics Devices are currently built at a reasonable low cost by engraving the hydraulic circuit with computer-assisted design (CAD) techniques, printing a mask with photolithographic techniques, and embedding it in a chip made of glass, silicone, or other polymers (Huh et al. 2005; Chinn 2010; Wolfe et al. 2010). They can display a series of different capabilities, including:

1. Management of very small samples (tens of nanoliters).
2. System integration (for example, labeling and detection on the same chip) (Shields et al. 2017; Witek et al. 2020).
3. Modularity by docking to more complex instrumentation (Watanabe et al. 2014a; Futamura et al. 2015).
4. The exploitation of signals other than Lorenz-Mie scattering and fluorescence, i.e., axial extinction, impedance by Coulter effect, impedance by electrorotation (Mansor and Ahmad 2015), dielectrophoretic (DEP) crossover frequency (Cheung et al. 2010), and direct dielectric features (Gawad et al. 2001; Holmes and Gawad 2010; Sun and Morgan 2010).
5. Sorting capabilities (Witek et al. 2020).

Moreover, their low cost makes them disposable, a highly desirable feature in managing infectious materials.

Microfluidic Devices have been designed for many different purposes, and samples have been built dedicated to a series of tasks, among which (1) bacterial detection and analysis (Bernabini et al. 2011), (2) bacterial counts (Bernabini et al. 2011), (3) bacterial counts in milk (Fernandes et al. 2014), (4) circulating tumor cells counts, enrichment and phenotyping (Teo et al. 2017; Watanabe et al. 2018; Abdulla et al. 2020; Xu et al. 2020), (5) detection and isolation of circulating myelomatous plasma cells (Qasaimieh et al. 2017), (6) evaluation of CD64 expression on neutrophils (Hassan et al. 2017), (7) exosomes analysis (Kanwar et al. 2014), (8) identification of leukemic blasts (Lee et al. 2018), (9) determination of RBC volume and Hb content (Schonbrun et al. 2014), (10) sorting of motile spermatozoa (Schuster et al. 2003), (11) evaluation of cell density (Grover et al. 2011) (12) evaluation of cytoplasmic viscosity (Wang et al. 2020), (13) evaluation of membrane potential (Farinas et al. 2001), and (14) human white blood cell (WBC) counts (Peng et al. 2021).

Microfluidic Devices are still largely experimental and not commercially available (Volpatti and Yetisen 2014) except for isolated cases, which include (Shields et al. 2017):

1. Disposable special microfluidic sorting cartridges to be docked to a dedicated conventional platform, including:
 - (a) The SH800 Cell Sorter marketed by Sony (<https://www.sonybiotechnology.com/>).
 - (b) The Wolf[®] Sorter marketed by Biosystems (<https://il-biosystems.com/de-en/>).
 - (c) The MACSQuant[®] Tyto[®] Sorter, marketed by Miltenyi (Beuk et al. 2018).
 - (d) The On-chip Sort, marketed by On-chip Biotechnologies.
2. CellSearch[®] (Watanabe et al. 2014b; Hardingham et al. 2015; Watanabe et al. 2018) and DEPArray[™] (Di Trapani et al. 2018) microdevices, marketed by Menarini for the identification and separation of circulating tumor cells (CTC) (<https://siliconbiosystem.com/>).
3. ZellSafe[™] Chip microdevice (Teo et al. 2017), marketed by Canopy Biosciences (<https://canopybiosciences.com/>) for CTC characterization.

22.6.1 Hydraulic Issues in Microfluidic Devices

The most important feature of this class of fixtures is the conditions in which the flow occurs. Due to miniaturization, the laws of hydraulics, while keeping to be respected, take on different importance because inertial effects become negligible, and viscosity, density, and surface tension take over (Holmes and Gawad 2010). It follows that the Reynolds number collapses, and the flow keeps being laminar in virtually all situations; consequently, the displacements of solvents, solutes, and particles can only occur by diffusion.

Nonetheless, even the microfluidic devices have to face a couple of the greater cytometers' most important hydraulic issues, i.e., (1) the regulation of the fluid progression in the conduits and, except for the sheathless models, (2) the focalization of the sample in the center of the conduit.

Besides the classical application of a pressure gradient (hydrodynamic flow), the progression of the fluid in the conduits is often entrusted to an electric field, which generates an electro-osmotic flow due to the migration of the fluid's electrolytes (Slater et al. 2010; Shields et al. 2015). The fluid profile, usually parabolic if pressure-driven, in electro-osmotic-driven flow displays a flat head that is very useful to minimize the effect of the event's position on signal generation (Slater et al. 2010).

The focalization of the sample in the center of the conduit can be obtained by 3D hydrodynamic thanks to the injection of the sample flow in the center of a wider orthogonally driven sheath flow (Golden et al. 2012; Daniele et al. 2015; Testa et al.

2015; Zhao and You 2016), but in sheath-less fluidics, other technical solutions are needed, like acoustic focusing (Piyasena et al. 2012; Wang et al. 2021), dielectrophoretic focusing (Yu et al. 2005), inertial focusing (Gou et al. 2018), and magnetic focusing (Zeng et al. 2012).

22.6.2 Sorting Procedures in Microfluidic Devices

Microfluidic devices can often sort events, relying on many different technologies. Due to their particular structural features, the events of interest can not be sorted by stream-in-air electrostatic sorting; their separation is made possible by a series of different separation methods divided into active and passive separation.

Active separation is conceptually similar to “traditional” sorting since it requires the preliminary staining of the population of interest and its subsequent diversion from the main flow. In microfluidic devices, the separation can be achieved by a series of different methods, including:

1. Immunocapture (Mab attached to the conduit/microchannel surface) (Sheng et al. 2012).
2. Immunoseparation by magnetophoresis (through magnetic-bead-conjugated-Mabs adhering to the cells) (Plouffe et al. 2012) and other magnetic-based techniques (Magnetic, Magnetotactic, and Magnetoresistance Cell Sorting (Zborowski and Chalmers 2011; Witek et al. 2020)).
3. Microvalve sorting (Piyasena and Graves 2014).
4. Electrokinetic switching (Fu et al. 2004).
5. Spark-generated microbubble cavitation (Zhao and You 2018).

Three microfluidic active separation-based cell sorters are commercially available that rely on disposable micro-cartridges to be docked to a more or less conventional platform (for further information on this topic, see Sect. 21.1).

Passive separation is of particular interest because it is label-free. In passive separation, events separate, so to speak, by themselves, i.e., only based on the interaction they establish with the conduit walls and with the microstructures possibly contained in it. These relationships can require direct contact, as in mechanical filtration (Tan et al. 2009; Zheng et al. 2011; Qin et al. 2015; Sarioglu et al. 2015; Hvichia et al. 2016; Kim et al. 2017), but in other cases can exploit the event deformability without requiring direct contact. The contactless passive separation techniques are called hydrodynamic sorting and include

1. Inertial microfluidics (Sun et al. 2012b; Sollier et al. 2014; Abdulla et al. 2018)
2. Pinched flow fractionation (Yamada et al. 2004; Lu and Xuan 2015)
3. Deterministic lateral displacement (DLD) (Huang et al. 2004; Loutharback et al. 2012; Salafi et al. 2019; Witek et al. 2020)
4. Deterministic cell rolling (Choi et al. 2012)
5. Lift-force cell sorting (Witek et al. 2020)

Some hydrodynamic sorting techniques may require the application of an external force, consisting of an electric field or acoustic wave; the most frequent separation techniques belonging to this group are:

1. Dielectrophoresis (DEP) (Kang et al. 2006; Sun et al. 2012a)
2. Electrorotation (ROT) (Mansor and Ahmad 2015)
3. Acoustophoresis (Warkiani et al. 2015; Yang and Soh 2012; Li et al. 2015; Ding et al. 2013; Olm et al. 2021)
4. Electrokinetic switching (Fu et al. 2004)

Hybrid devices exploiting both active and passive sorting techniques have been devised (Yan et al. 2017). Moreover, microdevices are known for combining microfluidic architecture with non-conventional microscopic techniques, such as a lab-on-chip relying on fluorescence lifetime imaging microscopy (FLIM) devised to identify leukemic blasts (Lee et al. 2018). Other, much simpler, require microscopic observation as a final step (Hassan et al. 2017). Further information on this topic can be found in the excellent review by Tang and colleagues (Tang et al. 2019).

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