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Chapter

Particle and Cell Separation

J. Paul Robinson

Abstract

Many processors are available for separating particles and/or cells, but few can match the capacity of flow cytometry – in particular the sorting component. Several aspects unique to cell sorting give it such power. First, particles can be separated based on size, complexity, fluorescence, or any combination of these parameters. Second, it is entirely possible to separate particles under sterile conditions, making this technology very advantageous for selecting cells for culture. Third, when this sterile environment is combined with a highly controlled safety system, it is possible to safely sort and separate highly pathogenic organisms or even cells containing such pathogens. The very latest instruments available add even more power by introducing the ability to sort cells based on spectral unmixing. This last option requires incredible computer power and very-high-speed processing, since the sort decision is based on computational algorithms derived from the spectral mixture being analyzed.

Keywords: flow cytometry, sorting, single-cell analysis, fluorescence, light scatter, multiparameter analysis

1. Introduction

Available for over 50 years, cell sorting is now a technology undergoing significant change as new approaches emerge. The first effective cell sorter that demonstrated an ability to sort a significant number of particles or cells was developed by Mack Fulwyler in 1965 [1]. The foundation of the technology was the ultimate result of a publication by a pathologist named Lushbaugh that purported to identify multiple populations of red blood cells based on measurement of Coulter volume [2]. Fulwyler determined from an engineering perspective that Lushbaugh had incorrectly used the Coulter counter and set out to prove that red blood cells were not separate populations by designing an instrument that could physically separate individual populations. A few years later, Len Herzenberg expanded and developed the technology [3, 4] primarily for the immunology community as a fundamental tool in fluorescence analysis of cells [5–8].

Since those early days, sorting technology has been used in every aspect of science. Early practical applications of flow cytometry were for analysis of sperm [9], determination of fertility potential [10], and separating female and male sperm for breeding purposes [11]. Sorting was subsequently used for determination of DNA content [12], separation of human chromosomes [13], separation of live cells from dead ones [14], sorting of hemopoietic stem cells [15], and isolation of cloned isotype switch variants by fluorescence cell sorting [16]. All these were in the ten years following release of the first commercial instrument. Since that time, every conceivable particle and/or cell type has been physically sorted by flow, making it one of the most important scientific technologies in our armory.

2. The evolution of cell sorting

Cell sorting is the most efficient method currently available for creating single-cell populations. An advantage of flow cytometry is that this process can be achieved without damaging the cells to any significant extent, and in a sterile environment to boot.

2.1 Coulter volume–based sorting

The original device developed by Fulwyler was based on sorting by Coulter volume, as illustrated in **Figure 1**. The concept as originally defined was that cells of different volumes would be separable because the impedance varied with cell volume. This method of sorting provides only a single parameter, Coulter volume, and thus does not require a light source as later flow-cytometry technologies did. Interestingly, Fulwyler's techniques, while they created the stimulus for an entire industry, did not become integrated into the field of cell separation for almost 40 years, when microfluidic systems saw huge advantages in impedance-based separation (discussed below).

2.2 Principles of piezo-based cell sorting

The most common approach for current cell sorts is the use of piezo crystals to general a high-frequency vibration within the sorting chamber. Because the entire chamber vibrates, the stream emanating from the nozzle breaks up into droplets. These droplets contain the item of interest for sorting – of course it is also possible that the droplet fails to contain the particle or contains more than one. Separating a particular droplet from the rest of the stream results in the sorting process for any particle or cell that is within that droplet. **Figure 2** shows an overview of the process.
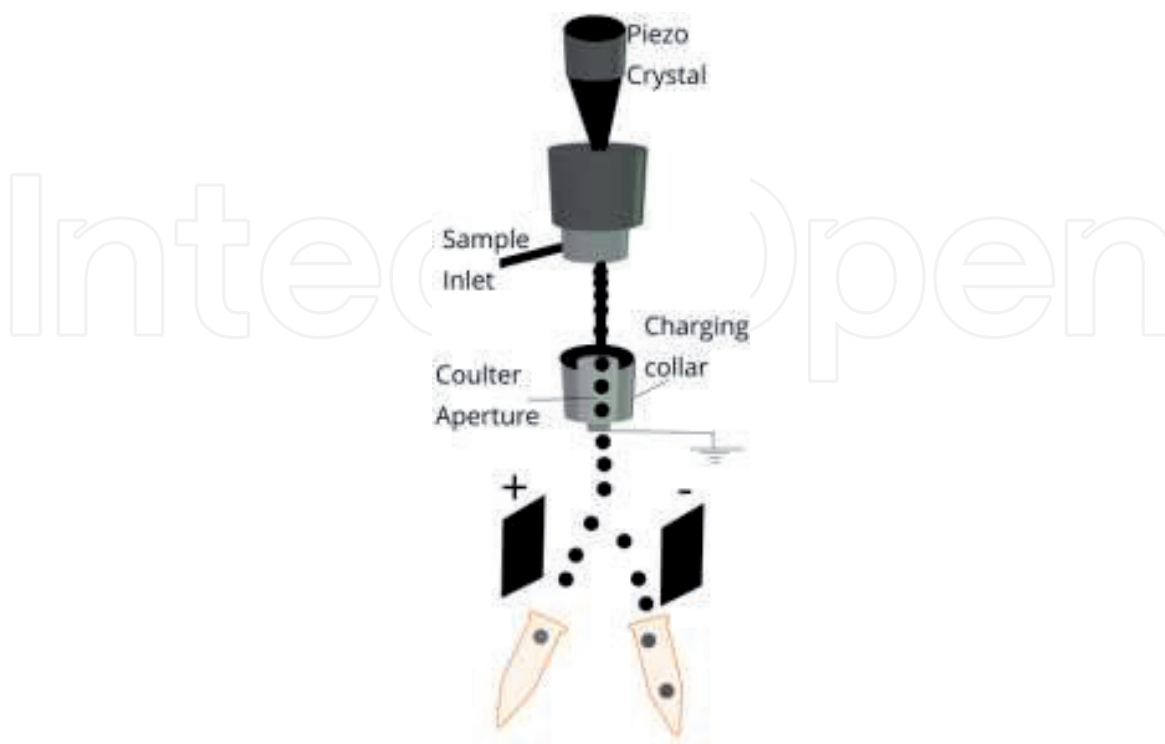

Figure 1.
Sketch of the original Fulwyler impedance-based cell sorter. This instrument had a single channel of data – Impedance. Reproduced with permission: J. Paul Robinson - Purdue University Cytometry Laboratories.
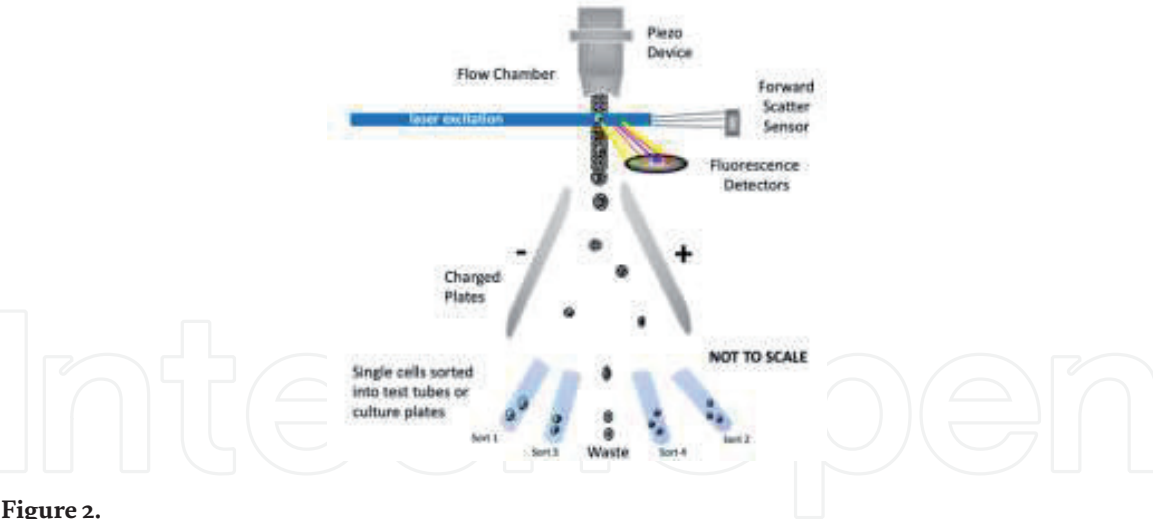
Figure 2.
Overview of the typical setup for a piezo-based cell or particle sorter. Shown at the top is a piezo device that causes the entire chamber to vibrate at high frequencies. This results in the stream's breaking into single droplets, each of which can be individually charged and then pulled toward a charged plate. Reproduced with permission: J. Paul Robinson - Purdue University Cytometry Laboratories.
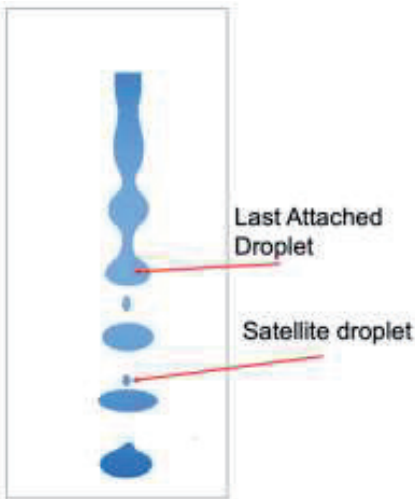

Figure 3.
The stream breaks up at a rate that is determined by the frequency of the piezo vibration. The last attached droplet is very important, as this is the point at which the computer determines exactly when to put a charge on the stream. As the droplet leaves the stream, it carries the same charge as was placed on the stream for that droplet. Thus, this droplet can then be manipulated to one side or other of the deposition area under the stream.

The sort stream itself has some important components that must be appreciated in order to understand how the sorting mechanism operates. The stream is generally a salt solution that can carry a charge. As can be seen in **Figure 3**, when the piezo crystal is activated the stream breaks up into droplets at a rate determined by the frequency of vibration. While these droplets can contain the particle of interest, there are also sub-droplets, termed satellite droplets, that break away from the main drops, as noted in **Figure 3**. As a rule, the higher the frequency of vibration, the smaller the droplet size and consequently the smaller the particle or cell that can be sorted. Other factors to be considered include the sorting pressure, which can be very high and can cause cellular damage.

3. Fluorescence-based cell sorting

The development of single-cell sorting as a new technology had little impact until the integration of fluorescence detection and laser-based excitation. These

achievements emerged from the laboratory of Len and Lee Herzenberg at Stanford University as the process of fluorescence-based cell sorting that they termed fluorescence-activated cell sorting, or "FACS." This term was subsequently trade-marked by Becton Dickinson, who first commercialized the Herzenberg technology. The early work from this laboratory used an intracellular fluorochrome (fluorescein diacetate) to label cells with very bright fluorescence that was excited by a mercury-arc light source; signals were detected by a single photomultiplier tube (PMT) [4]. Thus began decades of development from this laboratory: first an integrated laser excitation source [3], followed by multiple lasers and scatter detectors [17], advanced light-scatter analysis [18], and ultimately the first multiple fluorescence detection using two PMTs [8], the last of which (as we know) expanded over the next three decades to the current demonstration of 40 colors [19]. The Herzenbergs were without doubt the most significant team in the field of flow-cytometry cell sorting, and their developments over four decades created the foundation for an entire field of fluorescence-based cell sorting as well as core applications of fluorescence-based analysis. Len Herzenberg was awarded the Kyoto Prize in 2006 in honor of his accomplishments.

4. Magnetic particle sorting

Cell sorting using magnetic beads is a fast and efficient technology for separating cells without the need for the sort of high technology incorporated into flow cytometry. The huge advantage is the speed of separation and the fact that very large volumes of sample can be processed. The technology was developed by Miltenyi [20] in 1990 and is well embedded within current separation techniques today. In quick succession, the technology was used to separate pituitary cells [21], endothelial cells [22], B cells [23], eosinophils [24], proviral DNA [25], CD34+ cells [26], T cells [27], and bacteria [28]. The use of magnetic beads for sorting is demonstrated in **Figure 4**: a typical setup shows how magnetic beads attached to
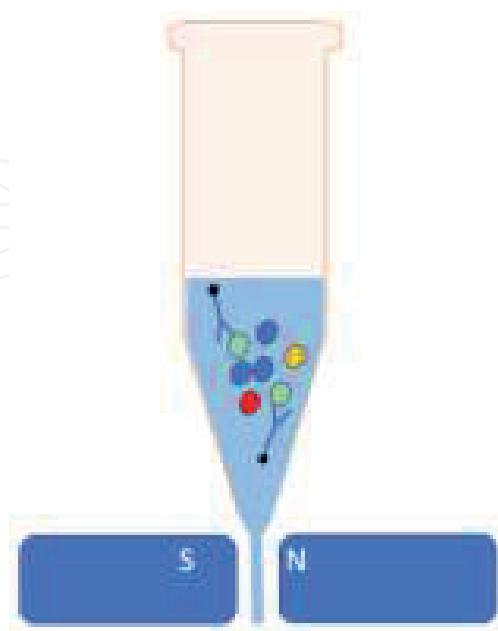

Figure 4.
A typical setup for magnetic sorting. Samples in the upper sample container can be separated through the addition of magnetic beads, typically labeled with an antibody to the target cell type. When the samples are passed by a powerful magnet, the magnetic beads and their targets are retained and thus purified. This technique can also be applied in large culture dishes such as 96- or 384-well plates.

antibodies are able to selectively separate cells or particles, as these are captured by the magnetic field and remain in the container while all other cells or particles unattached to the beads are removed.

The advantages of this technology are the very high speed of separation and the applicability to large volumes of sample. The disadvantage is that the selected population is still attached to a bead and an additional operation is required to separate the bead from the cell. However, this technique is very effective in isolation of rare cells when an appropriate marker is available. Another advantage of magnetic-bead separation is that if the beads are of different size or can be marked with dyes of different intensities, it is then possible to perform multiplexed assays to target multiple analytes simultaneously. The disadvantage of this technique is the fact that you either use negative selection, i.e., you target everything except the phenotype you want, or must use a magnetic bead attached to your selected phenotype.

5. Microfluidic chip–based sorting

The fundamental principle of microfluidic sorting is the use of narrow-bore tubes through which particles flow. Depending on the type of chip, it is possible to use a variety of methods to move cells of interest to flow toward one output or another, as shown in **Figure 5**. With the advent of new manufacturing technology over the past couple of decades, many systems utilize microfluidic chip–based sorting effectively. The advantage is that very small volumes, even less than a microliter, can be processed in complex lab-on-chip environments in which reaction sequences can be integrated within the chips. Many types of microfluidic chips have been developed that can physically separate cells, particles, and droplets at very high rates, making these lab-on-a-chip approaches very popular.

6. The limits of cell sorting

Cell-sorting technology is by no means a single entity or technology. There are several commercial instruments on the market, each with its advantages and disadvantages. However, some basic elements of sorting are fundamental to all instruments. For example, the goal of sorting differs depending on the sample being sorted. A sample containing desired rare cells to be isolated creates a significant problem that must be approached differently from a sample containing 20% of the desired phenotype. In the latter case, the sorting speed may not be a

Figure 5.
A generic microfluidic channel with 2 outputs. The process for selecting one or another population depends on the nature of the chip. Several different techniques have been demonstrated.

significant issue at all. However, if the sample contains 0.01% or less of desired cells, then many tens or hundreds of thousands of cells must be evaluated before even a few desired cells can be collected. It has not been uncommon for cell sorters to be running 10-15 hours to obtain sufficient cells of rare populations in order to perform a desired experiment. Instrument designers were naturally driven to create instruments that could operate at far higher sampling rates than previously; an entire generation of high-speed sorters was designed, starting with the MoFlo that emerged in the late 1990s and was without doubt the most significant commercial sorter available.

Sorters can be programmed to increase yields, for example where maximum recovery of desired cells is established. This may mean that undesirable cells contaminate the sorted sample, but the operator is prepared to accept this in return for maximum recovery. An alternative is sorting for purity, where no undesirable cells are acceptable. However, in this mode, it is likely that the operator will lose some desirable cells.

Another consideration has been instrument dead-time – the time between calculations of the sorting algorithm during which the instrument cannot make a decision. In such cases, cells will be lost. Cell sorting is a complex process that ultimately depends upon the frequency of coincident events that occur at any stage of the sorting process. The process is determined by Poisson statistics ultimately based on the probability of observing a fixed number of events within a fixed time where we know the probability of an event's occurring [29]. We can thus define the probability of a particle's being in a droplet or being lost to coincidence, for example. Such calculations in essence determine the efficiency of instrument sorting capacity. Many factors impact these decisions, such as the type of sort (purity or recovery) that one desires and the limits that one allows for errors.

The maximum sort speed is based on several factors, one of which is the electronics cycle time. Given that normal analysis time is in the range of 3-5 μs, this translates to a range around 250,000 events per second. However, achieving these rates would require enormous pressures that would be counterproductive to the use of most cellular materials, so the sample pressure may well be one of the limiting factors in cell sorting.

The fundamental process for droplet formation was defined over 150 years ago by Lord Rayleigh [30] in his treatise on acoustics. Essentially, based on Rayleigh criteria, a cylinder of liquid with a diameter D will break into drops spaced by $\lambda = \pi D$. While the goal of this chapter is not to discuss the fluid dynamics in detail, it is sufficient to note that the relationship between nozzle diameter, pressure, and frequency defines the characteristics of a particular system. These details are well described by van den Engh [31].

7. The advantages of cell sorting

While numerous techniques exist for establishing bulk cell populations, based on viscosity or by using magnetic beads, for example, there are few methods whereby absolute purity can be created from mixed populations, and even fewer where this can be achieved at the single-cell level. Cell sorting in flow cytometry can be achieved by use of a targeting fluorescent-conjugated antibody tag to extract the targeted cell type. The primary advantage of flow-cytometry sorting is clearly the ability to separate individual cells from complex mixtures given that one has access to appropriate targeting antibodies. Cell sorting is the most efficient method currently available for creating single-cell populations from complex mixtures. One advantage of flow cytometry is that this process can be

achieved without damaging the cells to any significant extent, in either a normal or a sterile environment. The ability of flow cytometry to sort under sterile conditions allows the sorting of cells of interest for cloning, frequently into 96-well plates for subcloning cells of interest [32–34].

Some cell-sorting applications do not need complexity of signals, but instead accuracy and purity based on very few parameters. One such application is sperm sorting, which has become an area of significant economic impact in flow cytometry. From the earliest concepts of evaluating X and Y chromosome–bearing sperm from domestic animals [35] to the effective use of cell sorters to electronically separate animal male and female sperm based on DNA content [11, 36, 37] for subsequent sperm sorting, flow cytometry has been the only really successful technology. Indeed, flow cytometry has also been extensively used for sorting human sperm [38], with extensive studies showing that the presence of Hoechst dye combined with UV excitation does not damage human sperm [39]. This approach was extended to sorting human sperm for in-vitro fertilization [40], a procedure that was allowed for several years by the Federal Drug Administration (FDA) but was eventually withdrawn, mostly for ethical reasons, a few years ago. What has persisted, however, with regard to sperm analysis, is the well-defined analytical assays for determination of sperm quality [41–44].

A clear advantage of flow cytometry–based cell sorting is the ability to separate a unique signature in a highly heterogeneous environment. With significant advances in recent years in availability of and options in monoclonal antibodies, almost every cell type possible can be targeted. The vast increase in the number of available fluorochromes has enabled the near effortless separation of complex mixtures.

8. Next-generation cell sorting

To move to the next generation of cell sorting, an instrument must have capacity for multiple lasers, a large number of detectors, and ability to sort under multiple algorithmic processes. Ideally a next-generation sorter has the ability to utilize spectral unmixing as well. The principle of spectral flow cytometry was developed some years ago by our laboratory [45–49]; however, it was restricted to analysis instruments. More recently, a commercially available instrument called "Bigfoot," a sophisticated and advanced sorter developed by Propel and now owned by ThermoFisher, is capable of real-time spectral unmixing for sort-decision making, which will be discussed later.

Over the past several years several companies have developed sorters with impressive automation capabilities. One example is the Sony SH800 cell sorter. In developing this sorter, Sony clearly identified one of the classic failures of sorter manufactures – lack of automation of instrument setup and calibration. As stated in the instrument brochure, "All setup steps, including optical alignment, droplet formation, side stream calibration, and delay time adjustment, are automated using Sony-developed CoreFinder technology™. This completely eliminates all the complicated setup work required with conventional cell sorters." This implementation by Sony is crucial; previous sorter manufacturers had to a large extent ignored this task because they assumed that all their sorters required highly skilled technologists to perform critical alignments. Earlier instruments had established components that assisted calibration and setup. For example, in the early 1990s, Coulter Corporation (now Beckman Coulter (BC)) integrated a video camera and screen to view the droplet breakoff. Interestingly, the Coulter system showed a screen with a horizontal stream image. Becton Dickinson (BD) also installed a camera in the next iteration of *their* sorter but made the screen vertical in the same path as the operator

would view the stream using the previous high-technology approach (a telescope). Such iterations perpetuated iterative updates with useful but not really transformational features for each version of sorter instrument. Sony therefore broke the mold and moved to a fully automated setup that was faster and more accurate than even a highly trained technician could achieve.

Another important development implemented by Sony was the use of a disposable sorting "chamber." It was in fact a sorting chip and is shown in **Figure 6**. Integrating a disposable chip into a cell sorter required many innovative modifications to the sorting design that even changed some time-honored expectations of how a sorter should operate. The integration of Sony's outstanding mechanics in the x-y-z stage for plate sorting was also innovative in implementation. This allowed for index sorting that gave the user confidence regarding the precision of sort decisions. While Sony had not been previously competing in the cell-sorter market, they focused heavily on features that promoted automation in setup and operation and as such drove competitive products to accommodate these features, resulting in significant advantages to the user base in the cytometry field.

Cell sorting can be achieved using most particles or cell types. As noted earlier, sorting can be achieved directly into test tubes, 96-well plates, or for that matter virtually any type of plate. In addition, we have used cell sorting for bacterial sorts directly onto petri dishes as a single bacterium or in multiples (**Figure 7**).

The recent debut of the top-level Bigfoot cell sorter again broke many previously accepted norms for sorting instruments. Apart from the name, which as a previous Everest summiter (May 23, 2009) I will happily ignore, the Bigfoot is an almost fully automated 60-channel, 9-laser sorter. One of the most pleasant features is almost silent operation. Researchers who sorted for decades on other instruments became used to noisy pressure and vacuum pumps running almost continuously. The Bigfoot is pleasantly silent. In addition, the sorter has what I would classify as the safest operating environment of all sorters. While integration of sorters within class-II safety cabinets began in the early 2000s and was copied across the industry, the Bigfoot chose a more effective route. The engineers integrated a custom-designed class-II safety chamber as part of the instrument itself; within this chamber is another chamber for the actual sorting. There is little doubt that this sorter can safely accommodate pathogens or human samples.

Bigfoot embodies multiple features that I consider entirely sensible and that will no doubt be copied across the industry. Remote control of the instrument is effective
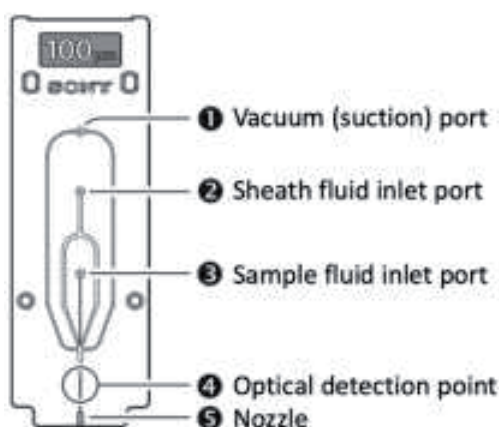

Figure 6.
Sony's innovative sorting chip introduced into their cell-sorter line was mass produced and could easily be replaced. (figure modified from Sony website).
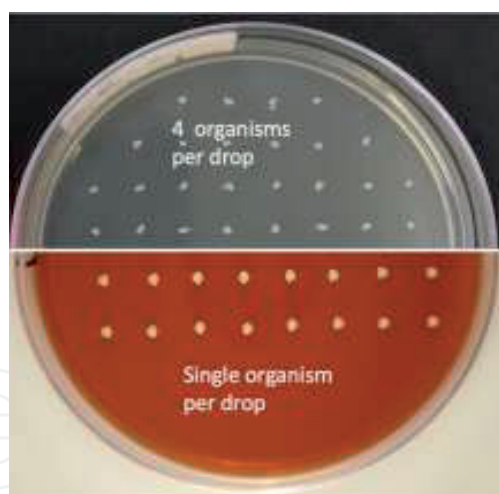
Figure 7.
Single organisms can be sorted directly onto petri dishes. On the top, organisms were sorted at four organisms per "well" location, and on the bottom a single organism was plated in a 96-well format.

and well managed, with direct support from company engineers available in minutes. The implementation of bead standards within a cooled vessel allows the instrument to proceed through setup and calibration without any user input except initiation of the software process. Combining this feature with remote control allows a user to literally prepare the instrument for sorting, even prior to entering the laboratory, a time-saving feature that I find valuable and practical. Similarly, the system comes standard with six sample stations that a user can fill and then proceed with virtually automated sorting without having to physically enter the sorting environment. For sorting of pathogenic samples, or samples with potential infectious agents, this feature is both innovative and a really sensible and practical advantage to the user.

The other major feature of this instrument is its capability for performing spectral unmixing and making sort decisions on those calculated curves. This is the first instrument capable of spectral sorting and it opens up an entirely new area of usage owing to the increase in power of spectral flow cytometry. In the years since it was first developed by our group [45, 46, 50], spectral flow cytometry has come to dominate the interest of immunologists and cytometry-related scientists. One reason is that spectral cytometry captures all the information available from multiple detectors and can resolve spectra that are very similar, but that emanate from different chemical species [51]. This discrimination allows a greater range of chromophores to be integrated into a highly complex panel. An example of this is the recent demonstration of 40-color phenotyping [19]. For decades there has been a rivalry to increase the number of simultaneous fluorophores in flow cytometry, which began with a single fluorophore and now is at 40. The impact of spectral cytometry is certain to pay dividends as the need to physically sort spectrally defined populations becomes evident.

While spectral analysis has been implemented for the past several years, the ability to sort based on a specific spectrally defined population has not been possible. With the implementation of Bigfoot's spectral sorting capacity (see **Figure 8**), physical sorting based on real-time spectral-unmixing algorithms at rates approaching 70,000 cells/second is now possible.

Flow cytometry sorting has changed significantly in recent years after many decades of iterative changes. In the 1980s and early 1990s, almost all users were focused on cell sorters as the core support instruments in their laboratories. This changed with the rapid grown of small analyzers, which became the primary instrument for flow cytometry, being efficient as well as cheaper and easier to manage than cell sorters. Ironically, the growth of the analyzer market and the excellence of competitive technologies resulted in a resurgence of demand for cell sorters with highly advanced features.
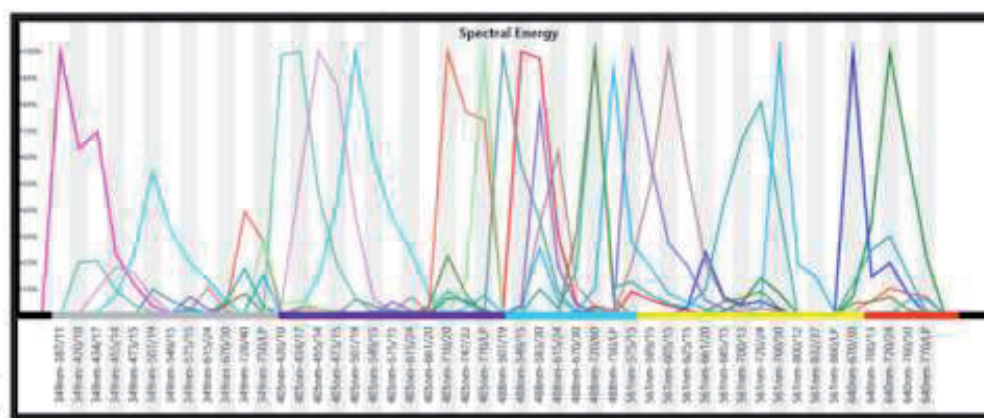
Figure 8.
An example of Bigfoot's spectral capability, which can be implemented within its sorting algorithms to perform sorting based on spectral separation.

9. Summary

Cell-sorting technologies have advanced over the 55 years since single-cell separation techniques were first invented. Of the multiple tools in the cell-separation toolkit, the most effective so far has been flow cytometry based on fluorescence detection. The combination of multiparameter scatter signals from laser excitation and fluorescence emission from multiple detectors provides a unique multiparameter analysis capability, which together with sorting algorithms gives flow-cytometry sorters unique capabilities in separating even very rare cell populations with excellent purity. In the first two decades of the emergence of flow cytometry, cell sorting was the predominant implementation. However, within the last couple of decades the predominant impact in the field has been analytical instruments, not sorters. In fact, these analytical instruments have become so advanced that they have expanded the field significantly as very high levels of complexity have driven tremendous immunological advances. What has resulted from this is a new demand for cell sorters with equal levels of complexity, with the unfortunate large increase in cost, but the advantage of vastly increase automation. If there are any drawbacks to this direction, it is the cost of instruments that can now exceed $1 M each. These instruments are most likely going to be in core laboratories that service multiple clients, since the average laboratory or even small companies cannot afford this level of expenditure. Regardless of these issues, piezo-based sorting is still the fastest and most efficient technique available for the sorting of any complex mixture.

Integration of a variety of detection approaches has enable a vast range of microfluidic systems – typically lab-on-a-chip technologies. These lab-on-chip approaches are heavily focused on diagnostic assays, which are likely to become a much larger segment of the cell-sorting market. The emergence of spectral unmixing using multi-array detectors has expanded both detection and sorting technology, largely owing to the high-speed FPGA-based electronics necessary for the current high-complexity sorting instruments.

Acknowledgements

Conflict of interest

The author declares no conflict of interest.

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References

[1] Fulwyler MJ. Electronic separation of biological cells by volume. Science 1965;150:910-911.

[2] Lushbaugh CC, Hale DB. Electronic measurement of cellular Volume. VII. Biologic evidence for two volumetrically distinct subpopulations of red blood cells. *Annual Report Biological and MedicalResearch Group (H-4) of the Health Division, July 1962 through June 1963.* Los Alamos Scientific Laboratory of the University of California; 1963:270-278.

[3] Bonner WA, Hulett HR, Sweet RG, Herzenberg LA. Fluorescence activated cell sorting. Review of Scientific Instruments 1972;43(3):404-409.

[4] Hulett HR, Bonner WA, Barrett J, Herzenberg LA. Cell sorting: automated separation of mammalian cells as a function of intracellular fluorescence. Science 1969;166(3906):747-749.

[5] Herzenberg LA, Black SJ, Loken MR, Okumura K, van der Loo W, Osborne BA, et al. Surface markers and functional relationships of cells involved in murine B-lymphocyte differentiation. Cold Spring Harbor Symposia on Quantitative Biology 1977;41 Pt 1:33-45.

[6] Herzenberg LA, Sweet RG, Herzenberg L. Fluorescence-activated cell sorting. Scientific American 1976;234:108-117.

[7] Loken MR, Parks DR, Herzenberg LA. Identification of cell asymmetry and orientation by light scattering. Journal of Histochemistry and Cytochemistry 1977;25:790-795.

[8] Loken MR, Parks DR, Herzenberg LA. Two-color immunofluorescence using a fluorescence-activated cell sorter. Journal of Histochemistry and Cytochemistry 1977;25:899-907.

[9] Gledhill BL, Lake S, Steinmetz LL, Gray JW, Crawford JR, Dean PN, et al. Flow microfluorometric analysis of sperm DNA content: effect of cell shape on the fluorescence distribution. Journal of Cellular Physiology 1976;87:367-375.

[10] Evenson DP, Darzynkiewicz Z, Melamed MR. Relation of mammalian sperm chromatin heterogeneity to fertility. Science 1980;210:1131-1133.

[11] Johnson LA, Flook JP, Look MV. Flow cytometry of X and Y chromosome-bearing sperm for DNA using an improved preparation method and staining with Hoechst 33342. Gamete Research 1987;17(3):203-212.

[12] Arndt-Jovin DJ, Jovin TM. Analysis and sorting of living cells according to deoxyribonucleic acid content. Journal of Histochemistry and Cytochemistry 1977;25:585-589.

[13] Carrano AV, Gray JW, Langlois RG, Burkhart-Schultz KJ, Van Dilla MA. Measurement and purification of human chromosomes by flow cytometry and sorting. Proceedings of the National Academy of Sciences 1979;76:1382-1384.

[14] Green DK, Malloy P, Steel M. The recovery of living cells by flow sorting machine. Acta Pathologica et Microbiologica Scandinavica Supplementum 1981;274:103-106.

[15] Visser JWM, Bol SJL, van den Engh G. Characterization and enrichment of murine hemopoietic stem cells by fluorescence activated cell sorting. Experimental Hematology 1981;9:644-655.

[16] Dangl JL, Parks DR, Oi VT, Herzenberg LA. Rapid isolation of cloned isotype switch variants using fluorescence activated cell sorting. Cytometry 1982;2(6):395-401.

[17] Hulett HR, Bonner WA, Sweet RG, Herzenberg LA. Development and application of a rapid cell sorter. Clinical Chemistry 1973;19(8):813-816.

[18] Loken MR, Sweet RG, Herzenberg LA. Cell discrimination by multiangle light scattering. Journal of Histochemistry and Cytochemistry 1976;24:284-291.

[19] Park LM, Lannigan J, Jaimes MC. OMIP-069: Forty-color full spectrum flow cytometry panel for deep Immunophenotyping of major cell subsets in human peripheral blood. Cytometry Part A 2020;n/a(n/a).

[20] Miltenyi S, MÅller W, Weichel W, Radbruch A. High gradient magnetic cell separation with MACS. Cytometry 1990;11:231-238.

[21] Wynick D, Bloom SR. Magnetic bead separation of anterior pituitary cells. Neuroendocrinology 1990; 52:560-565.

[22] Hewett PW, Murray JC. Human microvessel endothelial cells: isolation, culture and characterization. In Vitro Cell Dev.Biol.Anim 1993;29A(11): 823-830.

[23] McKnight ME, Mukerjee S, Nasoff M, Gaskins C, Glassy MC. Isotype-directed enrichment of B cells by magnetic beads in the generation of immunoreactive human monoclonal antibodies. Hybridoma 1996;15(4): 255-261.

[24] Sedgwick JB, Shikama Y, Nagata M, Brener K, Busse WW. Effect of isolation protocol on eosinophil function: Percoll gradients versus immunomagnetic beads. J.Immunol.Methods 1996;198(1): 15-24.

[25] Dorenbaum A, Venkateswaran KS, Yang G, Comeau AM, Wara D, Vyas GN. Transmission of HIV-1 in infants born to seropositive mothers: PCR-amplified proviral DNA detected by flow cytometric analysis of immunoreactive beads. J.Acquir.Immune.Defic.Syndr. Hum.Retrovirol. 1997;15(1):35-42.

[26] Kawano Y, Takaue Y, Law P, Watanabe T, Abe T, Okamoto Y, et al. Clinically applicable bulk isolation of blood CD34+ cells for autografting in children. Bone Marrow Transplant. 1998;22(10):1011-1017.

[27] Maeda S, Miyawaki T, Nakanishi T, Takigawa M, Shimada M. Peripheral type benzodiazepine receptor in T lymphocyte rich preparation. Life Sciences 1998;63(16):1423-1430.

[28] Porter J, Robinson J, Pickup R, Edwards C. An evaluation of lectin-mediated magnetic bead cell sorting for the targeted separation of enteric bacteria. J.Appl.Microbiol. 1998;84: 722-732.

[29] Taylor JR. An introduction to Error Analysis. Sausilito, CA: University Science Books; 10997.

[30] Raleigh JWS. Theory of Sound. London, Reprinted 1945, Dover, New York: mCMillan; 1877.

[31] van den Engh G. High Speed Cell Sorting. In: Durack DaR, J. P., editor Emerging Tools for SIngle Cell Analysis. New York: Wiley-Liss; 2000, p. 2148.

[32] Lei L, Tran K, Wang Y, Steinhardt JJ, Xiao Y, Chiang C-I, et al. Antigen-Specific Single B Cell Sorting and Monoclonal Antibody Cloning in Guinea Pigs. Frontiers in microbiology 2019;10:672.

[33] Smith MJ, Packard TA, O'Neill SK, Hinman RM, Rihanek M, Gottlieb PA, et al. Detection and Enrichment of Rare Antigen-specific B Cells for Analysis of Phenotype and Function. Journal of Visualized Experiments : JoVE 2017(120):55382.

[34] Zhou Y, Liu Z, Wang Z, Zhang Q, Mayer CT, Schoofs T, et al. Single-Cell Sorting of HBsAg-Binding Memory B Cells from Human Peripheral Blood Mononuclear Cells and Antibody Cloning. STAR Protocols 2020;1(3): 100129.

[35] Garner DL, Gledhill BL, Pinkel D, Lake S, Stephenson D, Van Dilla MA, et al. Quantification of the X- and Y-chromosome-bearing spermatozoa of domestic animals by flow cytometry. Biology of Reproduction 1983;28: 312-321.

[36] Johnson LA. Separation of X and Y chromosome-bearing sperm on the basis of flow cytometric analysis for DNA. No Journal Name 1992.

[37] Johnson LA. Sex preselection by flow cytometric separation of X and Y chromosome-bearing sperm based on DNA difference: a review. Reprod.Fertil. Dev. 1995;7(4):893-903.

[38] Auger J, Leonce S, Jouannet P, Ronot X. Flow cytometric sorting of living, highly motile human spermatozoa based on evaluation of their mitochondrial activity. Journal of Histochemistry and Cytochemistry 1993;41(8):1247-1251.

[39] Catt SL, Sakkas D, Bizzaro D, Bianchi PG, Maxwell WM, Evans G. Hoechst staining and exposure to UV laser during flow cytometric sorting does not affect the frequency of detected endogenous DNA nicks in abnormal and normal human spermatozoa. Mol.Hum.Reprod. 1997;3(9):821-825.

[40] Vidal F, Fugger EF, Blanco J, Keyvanfar K, Catala V, Norton M, et al. Efficiency of MicroSort flow cytometry for producing sperm populations enriched in X- or Y-chromosome haplotypes: a blind trial assessed by double and triple colour fluorescent

in-situ hybridization. Human Reproduction 1998;13(2):308-312.

[41] Aravindan GR, Bjordahl J, Jost LK, Evenson DP. Susceptibility of human sperm to in situ DNA denaturation is strongly correlated with DNA strand breaks identified by single-cell electrophoresis. Experimental Cell Research 1997;236(1):231-237.

[42] Evenson DP, Darzynkiewicz Z, Melamed MR. Comparison of human and mouse sperm chromatin structure by flow cytometry. Chromosoma 1980;78:225-238.

[43] Evenson DP, Darzynkiewicz Z, Melamed MR. Simultaneous measurement by flow cytometry of sperm cell viability and mitochondrial membrane potential related to cell motility. Journal of Histochemistry and Cytochemistry 1982;30:279-280.

[44] Spano M, Evenson DP. Flow cytometric analysis for reproductive biology. Biology of the Cell 1993;78(1-2):53-62.

[45] Grégori G, Patsekin V, Rajwa B, Jones J, Ragheb K, Holdman C, et al. Hyperspectral cytometry at the single-cell level using a 32-channel photodetector. Cytometry Part A 2012;81A(1):35-44.

[46] Robinson JP. Multispectral cytometry: The next generation. Biophotonics International 2004;October, 2004 p36-40.

[47] Robinson JP, Grégori G, Rajwa B, Jones J, Patsekin V. Multispectral detector and analysis system. In: USPTO, ed. USA: Purdue University; 2007.

[48] Robinson JP, Patsekin V, Grégori G, Rajwa B, Jones J. Multispectral flow cytometry: Next generation tools for automated classification. Microsc. Microanal. 2005;11(S02):2-3.

[49] Robinson JP, Rajwa B, Grégori G, Jones J, Patsekin V. Multispectral cytometry of single bio-particles using a 32-channel detector. In: Dinh TV, Grundfest WS, Benaron DA, Cohn GE, eds. 5692. Bellingham, WA: SPIE; 2005:359-65.

[50] Robinson JP, Rajwa B, Grégori G, Jones J, Patsekin V. Collection hardware for high speed multispectral single particle analysis Cytometry 2004;59A:12-.

[51] Robinson JP. Spectral flow cytometry—Quo vadimus? Cytometry Part A 2019;95(8):823-824.