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Background: Cell sorting has a history dating back approximately 40 years. The main limitation has been that, although flow cytometry is a science, cell sorting has been an art during most of this time. Recent advances in assisting technologies have helped to decrease the amount of expertise necessary to perform sorting.
Methods: Droplet-based sorting is based on a controlled disturbance of a jet stream dependent on surface tension. Sorting yield and purity are highly dependent on stable jet break-off position. System pressures and orifice diameters dictate the number of droplets per second, which is the sort rate limiting step because modern electronics can more than handle the higher cell signal processing rates.
Results: Cell sorting still requires considerable expertise. Complex multicolor sorting also requires new and more sophisticated sort decisions, especially when cell subpopulations are rare and need to be extracted from background. High-speed sorting continues to pose major problems in terms of biosafety due to the aerosols generated.
Conclusions: Cell sorting has become more stable and predictable and requires less expertise to operate. However, the problems of aerosol containment continue to make droplet-based cell sorting problematical. Fluid physics and cell viability restraints pose practical limits for high-speed sorting that have almost been reached. Over the next 5 years there may be advances in fluidic switching sorting in lab-on-a-chip microfluidic systems that could not only solve the aerosol and viability problems but also make ultra high-speed sorting possible and practical through massively parallel and exponential staging microfluidic architectures. © 2005 International Society for Analytical Cytology

Key terms: cell sorting; ultra high speed; flow cytometry

It has been more than 40 years since Mack Fulwyler (1) and others (2–5) demonstrated the first successful droplet-based cell sorting adapting ink-jet printing technology (6). Fulwyler, who was then a physicist at Los Alamos National Laboratory, used piezo-electric crystal based ink-jet printed developed by Richard Sweet to sort cells on the basis of their Coulter volume signals. This quickly was followed at Los Alamos by the use of light scattered in the near forward direction and at orthogonal angles ("side scatter"), which showed the utility of these signals for identifying and sorting lymphocytes, monocytes, and granulocytes from blood. Sweet then worked with Leonard Herzenberg at Stanford University to develop the first cell sorter based on fluorescence signals ("FACS," or fluorescence activated cell sorting). The use of fluorescence greatly widened the practical utility of cell sorting by making use of fluorescently labeled antibodies. This use then skyrocketed with the invention of monoclonal antibodies and its rapid commercialization. Suddenly the number of possible cellular markers and probes was in the thousands.

By any measure flow cytometry during this period has been extremely successful. Cell sorting has also been successful but has been plagued by at least three limitations. First, conventional sorting speeds (5,000 to 20,000 cells/s) are still too slow for many applications. Second, sorting has been much more of an "art" requiring a highly skilled cell sorter technician. Historically, sorting has not been for beginners. Third, in this age of droplet-based sorting of live cells that are potentially infectious, there has been a problem with this type of droplet sorting, which by its nature generates aerosols. Although a growing number of cell sorters are being placed in BSL-3 biocontainment facilities, this is not a very satisfactory solution to the problem. Perhaps it is time to fundamentally revisit the problem and develop alternative ways to sort cells without droplet-generating aerosols.

In recent years "high-speed sorting" in commercial instrumentation has reached much more practical rates of at least 40,000 cells/s (7). This new generation of cell sorters has also become easier (but still not easy) to operate.

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However, the aerosol generation from these high-speed sorters is even worse. Although aerosol containment modules have been added to many of these units, containment of potentially infectious material remains a problem. Further, sterility to the level of practical isolation of cells for human transplantation continues to be a problem.

"Ultra high-speed sorting" is a new realm of cell sorting that requires new thinking beyond droplet-based or conventional fluidic lab-on-a-chip sorting technologies. This will require implementations of massively parallel and possibly "exponential staging" cell sorting architectures (8). More importantly, these new branching tree architecture microfluidic designs allow for the continuous resorting of cells from the previous stage. My colleagues and I previously showed that one of the bottlenecks of ultra high-speed sorting is processing the information (9). Digital signal processing (DSP) complicates that process by providing for complete pulse shape signatures or requiring a DSP analysis of the waveform that, although fast, still requires some time. However, if such branching tree sort architectures are successful, they could produce overall cell sorting speeds in excess of 1 million cells per second. The technologic hurdles are considerable, but the promise is exciting.

MATERIALS AND METHODS

Fluidic Switching Sorting

Fluidic switching technology has existed since the 1960s. Fulwyler, while at Los Alamos National Laboratory, implemented droplet-based cell sorting based on a combination of Coulter volume and light scatter signals from cells (1). Bonner et al. (4) pioneered the use of fluorescence-based signals that could take advantage of the tremendous growth in possible molecular probes brought on by the invention of monoclonal antibodies during the 1970s. Some of the original attempts to sort cells based on fluidic switching were done by Kamensky and Melamed (2) before the use of droplet-based sorting. The switching speeds were not thought to be fast enough to be practical. Switching times were on the order of 10 ms. Only recently has there been a revived interest in closed, fluidic switching cell sorting. This new interest has been driven by two forces. First are the hazards of droplet-based sorting for processing human samples and particularly infectious material. Second, due to advances in micro- and nanofabrication techniques, there is a renewed interest in sorting cells by microfluidic lab-on-a-chip flow cytometers/sorters (10), which, although presently slow, could possibly be made to operate at high speeds by using massively parallel fluidic architectures currently possible using new nano-/microfabrication techniques.

Droplet-Based Sorting

Droplet-based sorting quickly showed a capability of generating reasonably stable jets with 30,000 to 50,000 droplets/s. This allowed sorting rates of 5,000 to 10,000 cells/s, which at the time was fast enough for most applications. Higher droplet rates require increasing the pressure or decreasing the orifice size of the jet nozzle that creates the droplets. Due to clogging problems, a practical lower limit in orifice size seems to be about 50 μm. System pressure limits are governed partly by the structural integrity of the apparatus, but more by the structural integrity of the live cells flowing though it. The combination of orifice diameter and system pressure dictates practical limits of about 150,000 droplets/s. Assuming Poisson arrival statistics, as is the case for undamaged cells (9,11), the practical upper limit for sorting rates through a single nozzle is about 100,000 cells/s. To go to ultra high-speed sorting rates (e.g., >500,000 cells/s) will not be attainable with incremental advances of the present design but will require a fundamentally different sorting method to be used. Anti-coincidence circuitry can decrease the incidence of nearby contaminating cells in a given droplet, but this comes at the usual tradeoffs between yield and purity. However, much more sophisticated "anti-coincidence" decisions can be made to improve yield without sacrificing as much purity (12,13). One droplet per sorting unit gives the minimal queue length and, hence, the minimal condition of coincidence, but one droplet-sorting unit requires considerable instrument stability during the sort experiment. When cells are very close to each other in the stream, a cell can disturb the break-off point. It can also allow a variable amount of charge to be left on the break-off droplet if it lies in the neck region of the droplet, as described previously (14). This leads to temporary fanning of the sort stream. To maintain higher stability at ultra high-speed sorting rates, we always sort the desired droplets that contain cells straight ahead without using any charge, and deflect all undesired cells that can still be eliminated even if they are in a slightly fanned stream. The variations in stream velocity due to pressure variations do not always correspond to a change in sort delay timing, even if the apparent droplet break-off position changes (15). Demanding even greater stability of the fundamentally nonlinear turbulent flow condition of droplet formation is probably not realistic.

Basic Sort Decisions

Initially, sort decisions involved simple rectilinear boundaries on at least one parameter. The sort decision logic was sufficiently simple that it could be implemented with simple analog comparators and Boolean logic circuitry. Specific biological applications drove the field to develop non-rectilinear boundaries using "bit maps." These sort regions of arbitrary shape in two dimensions could then be combined in a Boolean logic with other two-dimensional bit maps to create "gates" that represented more complex sort decisions. These simple sort logic structures were adequate for three- or four-color fluorescence of rare cells (e.g., 1% subpopulations) but were and remain inadequate for ultra-rare cell subpopulations, where picking these cells out of a high background of nonspecific fluorescence is the challenge rather than just raw cell processing speed. The utility of such advanced sort decision architectures was demonstrated in a number of applications in the 1990s whereby the feasibility of multivariate statistical sort decisions, including

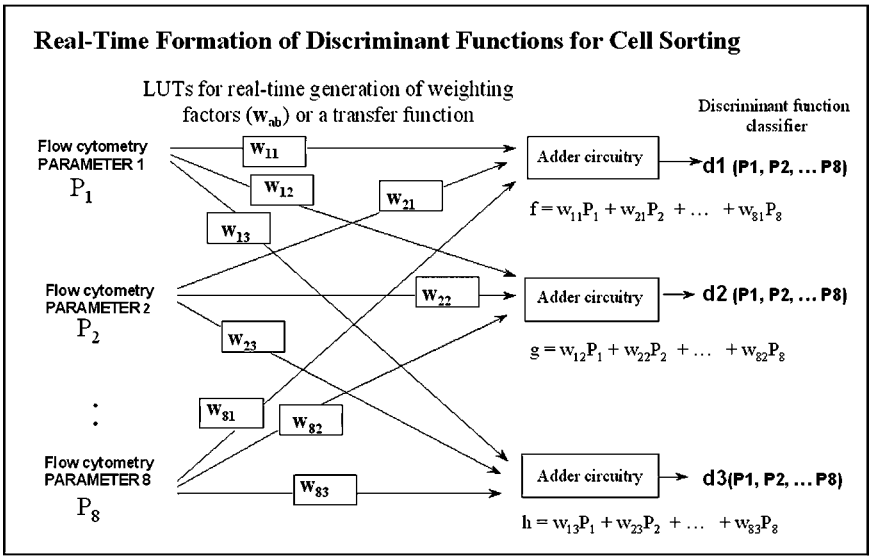

FIG. 1. Theoretical architecture of an eight-parameter high-speed cell sorter capable of making real-time multivariate statistical sort decisions (in this case, the first three discriminant functions d1, d2, and d3). This architecture is similar to that of a single-layer neural network in which the weighting coefficients can be used in a wide variety of ways to represent not only neural networks but also complex multivariate statistical functions such as principal components, discriminant functions, and logistic regression functions. Even nonlinear functions can be approximated using generalized additive functions.

costs of misclassification, was demonstrated (16). To perform these multivariate statistical decisions in real time requires at least a single layer neural network architecture (which is now much simpler to create using DSP technology) and lookup tables for more rapid signal processing to map input signals to more complex mathematical functions (Fig. 1). We have already implemented this neural network architecture in our full-size high-speed sorter and are now constructing a microfluidic high-speed sorter that will implement these features more compactly using DSP chips.

"Biological Need" for High-Speed Cell Sorting

As with all technologies, the possibility of high-speed cell sorting existed long before there was a perceived "biological need" involving specific biological applications. Only when there was sufficient interest in analysis of rare cells ("rare-event analysis") (13) was there enough driving force to create commercially available sorters in the mid-1990s, although such high-speed sorters existed in research laboratories for more than a decade before that commercial introduction.

Development of High-Speed Droplet-Based Cell Sorting

High-speed flow cytometry and cell sorting were developed in laboratories at the Lawrence Livermore National Laboratory to sort human chromosomes for the predecessor to the Human Genome Project (17) and at the University of Rochester to sort rare human fetal cells from maternal blood and human stem/progenitor cells (18). Once the need was strongly expressed by the biology community, a new company, Cytomation, Inc., rapidly stepped in to fill a need (based on the patents of van den Engh) not perceived to be a sufficient market by the existing companies, and newer companies such as Cytopeia, Inc. (based on the patented technologies of van den Engh) are now offering a new generation of high-speed cell sorters that reach cell sorting speeds of 50,000 to 70,000 cells/s. A new generation of ultra high-speed, microfluidic cell sorters is currently being developed by Cytomics Technologies, Inc. (based on the patented technologies of Leary), which may have speeds in excess of 1,000,000 cells/s.

Methods for High-Throughput Flow Cytometry/Cell Sorting

Over the past 20 years our laboratory has developed a large number of instrument hardware and software technologies and methodologies for dealing with the high-throughput processing of cells and other objects (9,19–21) and a wide variety of cell labeling and manipulation techniques for rare-event analysis, namely the analysis and sorting of cell subpopulations with frequencies less than 0.1% (12,13). More recently, we have extended these procedures to the realm of "ultra-rare" cells, namely those cell subpopulations of frequencies less than 0.001% (12,16,22). To accomplish this level of high-throughput cell processing, we have not only developed a very high-speed flow cytometers/cell sorter but also given it a sophisticated hardware/software, two-stage cell classification, and a sorting system capable of using real-time multivariate statistical classifications, including measures of the probability of correct cell classification (also referred to sometimes as "penalties of misclassification") in several patents (23,24). Going faster alone does not do the job if one merely generates huge numbers of false-positives. Ultra high-speed sorting has to be faster and smarter. Rare cell sampling statistics is another important issue for ultra high-speed sorting that we have studied at some length and for which we have developed new rare-cell sampling statistical techniques (25).

The result of these efforts has been the development of an ultra high-throughput cell sorter (Fig. 2) that we have been adapting to a number of projects including (a) minimal residual disease monitoring, including single cell DNA sequencing of rare sorted metastatic breast cancer cells,

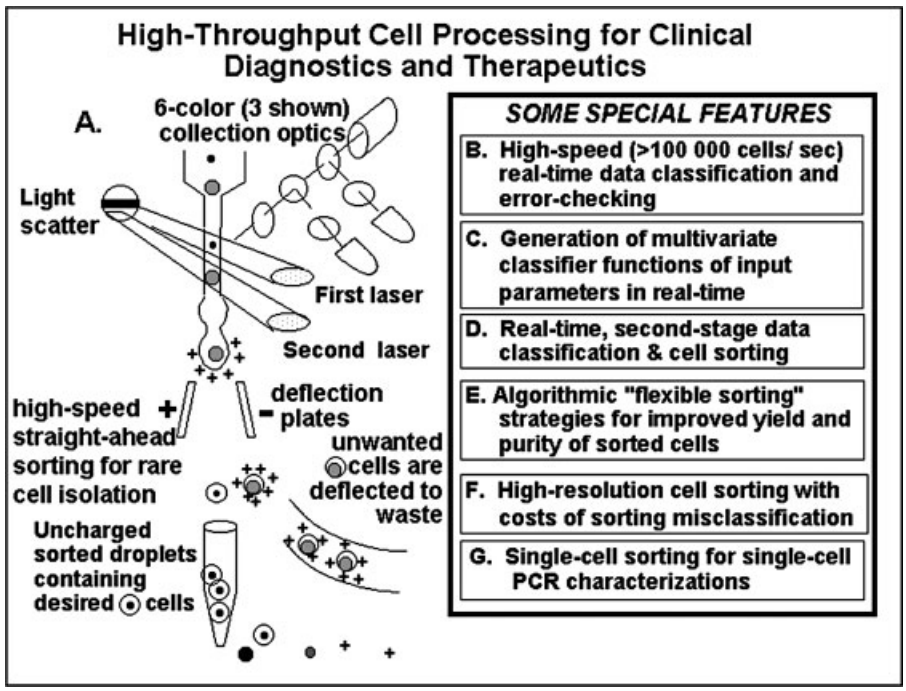

FIG. 2. Overview of a six-color high-speed flow cytometer with a single-layer neural network architecture that allows real-time classification/sort decisions on the basis of multivariate statistical functions. For high recovery of cells sorted at high speeds and for efficient single-cell recovery, we often sort cells of interest straight ahead, achieving sort recoveries greater than 95% even at the single-cell level at rare cell frequencies as low as 10^{-6}. The multivariate statistical functions calculated in real time provide for statistically based sort boundaries and a measure of the degree of misclassification.

(b) autologous stem cell transplantation with simultaneous tumor purging, and (c) high-throughput screening of bead-based combinatorial chemistry libraries.

Several engineering design features of ultra high-speed cell sorters have been previously discussed (12). Interestingly, cell sorters are inherently much more efficient at higher cell processing speeds as long as the instrument dead time, the period that it takes for an instrument to process one cell and be ready to measure the next cell, can be kept very low. A simple example of this is shown in Figure 2. Enrichment sorting has been implemented in many modern cell sorters. It provides a way to decrease the overall sort time by almost a factor of 10 if cells are first enriched and then resorted to desired purity. Instrument dead time is a critical issue in ultra high-speed cell sorting. If cells pass through the sorter during periods of instrument dead time, the sorter does not even know they are present. This means that anti-coincidence circuitry is never engaged. The trend toward complete DSP, although providing many advantages, may actually slow down the sorting function because it takes time to perform analyses of digital waveforms. An ultra high-speed cell sorter should probably have an instrument dead time shorter than 2 μs to be able to sort at rates in excess of 100,000 cells/s (Fig. 3). The dead-time first stage of our two-stage cell processing system is shorter than 2 μs as compared with typical dead times of 10 to 15 μs on most commercial cell sorters and approximately 5 μs on the fastest commercial cell sorter currently available. The first-stage classification allows information to be excluded from the second stage, which otherwise would need to make sophisticated analyses on much larger information flows. A detailed discussion of the importance of instrument dead time from the viewpoint of queuing theory is beyond the scope of this paper but has been clearly and simply discussed in two book chapters (12,13). A very simple illustration that makes the point of enrichment sorting obvious is shown in Figure 4. The present instrument does two-stage information processing for the sort decisions, doing a "rough cut" decision in the first stage and a very sophisticated decision based on multivariate statistical classifications in the second stage. Unfortunately, the physical sorting of cells is one stage. However, a multistage microfluidic high-speed sorter is currently being prototyped in our laboratory that will permit physical sorting and resorting of cells through multiple cell sorters. Such

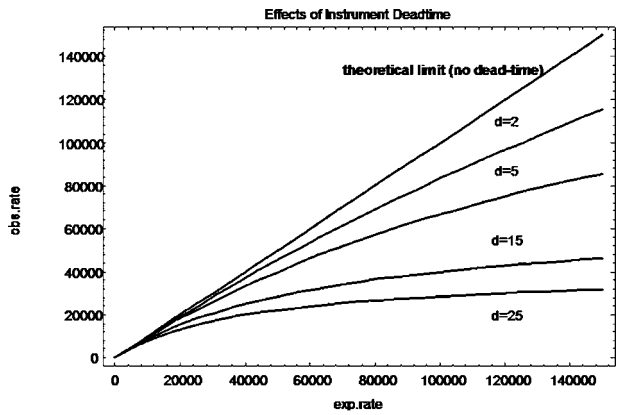

FIG. 3. Observed versus expected numbers of cells parameterized according to instrument dead time as predicted by queuing theory modeling using Mathematica (Wolfram Research, Inc., Champagne, IL). Early commercial flow cytometers had dead times in the range of 15 to 20 ms, whereas more recent commercial higher-speed flow cytometers have dead times of 5 to 10 ms. Some experimental flow cytometers have had dead times of 2 to 3 ms for longer than 10 years.

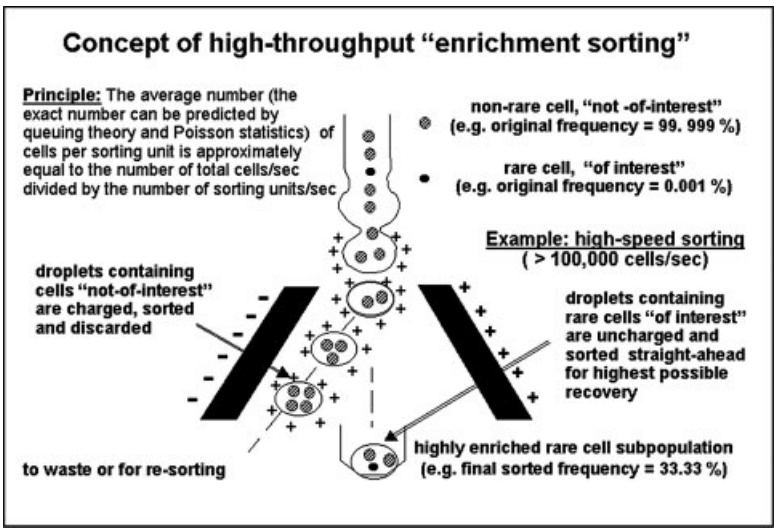

FIG. 4. Cell sorters are much more efficient at very high-throughput speeds in terms of the enrichment factor from the original rare cell concentration to the final sorted rare cell concentration. In this concept, a cell of original frequency of 10^{-5} (1 rare cell per 100,000 total cells) can be sorted at 100,000 cells/s with a sorter capable of generating 33,000 droplets/s. A high-speed first-pass sort enrichment of more than 30,000-fold, based on up to three rare parameters and five additional total parameters, can be attained. Sorted cells can then be resorted to any desired purity based on the quality of the selection probes.

an arrangement provides something more than simple parallel processing. The process appears to stage exponentially (data not shown).

We Are Rapidly Reaching the Practical Speed Limits for Droplet-Based Sorting

As pointed out in several reports (26), we are fast approaching the practical limits of droplet-based sorting in terms of the constraints of the fluid physics and cell viability. The problems of explosive decompression of cells were amply pointed out during the development of the high-speed chromosome sorter (17). Most cells do not survive explosive decompression pressures greater than 3 atm above ambient pressure (approximately 60 psi). Although shear forces in the flow cell do not appear to be causing major problems to the cells, the gravitational forces encountered by cells traveling at 25 to 30 m/s and coming to a sudden stop are major problems for most cells.

Is There a Need for Ultra High-Speed Sorting?

Current commercial high-speed flow cytometers/cell sorters are adequate (with many hours of diligent sorting!) for many applications, but there are at least two new application areas that demand new "ultra high-speed cell sorting" technologies. The number of these ultra high-speed sorting applications will grow in the years ahead. However, as was the case for the perceived need for "high-speed cell sorting," it will probably be 3 to 5 years before the biological need for ultra high-speed sorting technology is enough to drive a commercial market. However, this market will be very different from existing ones and will not represent a simple extension of high-speed sorting to ultra high-speed sorting. High-speed sorting by droplets is reaching limits dictated by the physics of fluid flow and droplet-generation requirements. New ways of approaching these much higher (factor of 10 times higher than current "high-speed" sorting) speeds need to be developed.

Rare Cell Sorting for Genomics and Proteomics ("Cytomics")

The sorting of cell subpopulations for subsequent gene expression microarray analyses pushes the limits of current sorting technology. Because the gene expression profile (GEP) of heterogeneous cell populations will reflect the weighted average of all cell subpopulations present according to their relative frequencies, cells must be sorted to sufficient purity to allow for meaningful results. In addition, to avoid the distortions of the GEP caused by so-called linear amplification technologies (that are never linear!), the number of sorted cells per gene chip needs to be on the order of 100,000 cells to be realistically analyzed. To obain meaningful results for even "moderately expressing" genes requires sort purities greater than 70%. Low expressing genes require sort purities greater than 90% and more typically greater than 95% purity (27). If one is trying to purify cell subpopulations with less than 10% purity, this requires a minimum cell sorting rate faster than 100,000 cells/s. For cell subpopulations with less than 1%, e.g., stem/progenitor cells, a more realistic sorting rate would be in excess of 500,000 cells/s. Importantly, it should be remembered that the GEPs of these live cells are rapidly changing, so sort experiment times of 4 to 10 h are not possible without considerable degradation of the mRNA of these cells. Brute-force sorting for many hours is simply not an option with live cells because the cells themselves are changing faster than this time interval. Stating an obvious but frequently ignored scientific logic, the measuring process must always be much faster than the rate of change of the objects being analyzed!

Proteins tend to be a little more stable in terms of half-life. Subsequent mass spectrometry of proteins from sorted cells probably requires on the order of 10^6 to 10^7 cells, depending on the type and sensitivity of the instrumentation. Hence, sorting a 10% cell subpopulation would require analysis of 10^7 to 10^8 total cells, assuming no cell losses. This is doable with current high-speed cell sorters. In contrast, if only 1% of the cells are sorted, we

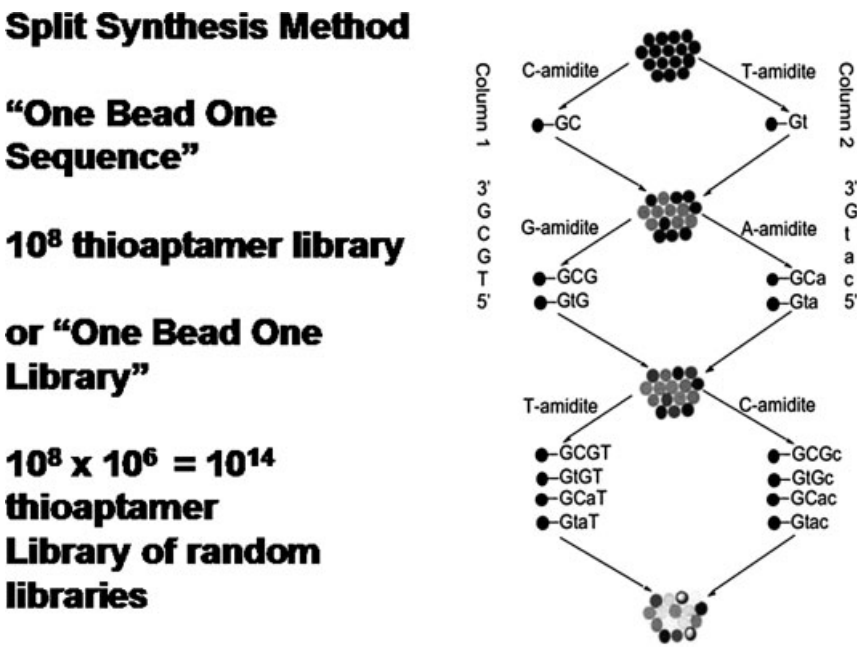

FIG. 5. General flow diagram for the production of bead-based thioaptamer libraries. After splitting and pooling through three cycles, the eight possible tetra-oligonucleotides are represented on separate beads with a unique sequence on each bead.

start to get into the realm where higher rates of cell sorting are needed.

Bead-Based Combinatorial Chemistry Libraries

High-throughput screening of cell libraries has been previously accomplished (28,29). An application that truly demands much higher analysis and sorting speeds is the screening of bead-based combinatorial oligonucleotide chemistry libraries built by split synthesis methods (Fig. 5). Application of high-speed flow cytometry and sorting to bead-based combinatorial chemistry libraries has been demonstrated in two recent papers (30,31).

Flow cytometry and sorting are very useful technologies for finding sequences of interest, but these libraries easily reach the size of 10^9 and possibly 10^{14}. Libraries of 10^9 are doable with conventional high-speed sorting at rates of approximately 80,000 to 100,000 beads/s, but even 10^{10} poses a realistic barrier. Larger libraries will require invention of new techniques for analyzing "libraries of libraries" (currently in development) that will reduce the process to screening perhaps libraries of 10^9 that each carry 10^5 possible sequences. Even analysis speeds of 100,000 beads/s are far too slow. Although the chemistry of bead-based libraries is more stable than the mRNA of live cells, it is very difficult and somewhat impractical to perform high-

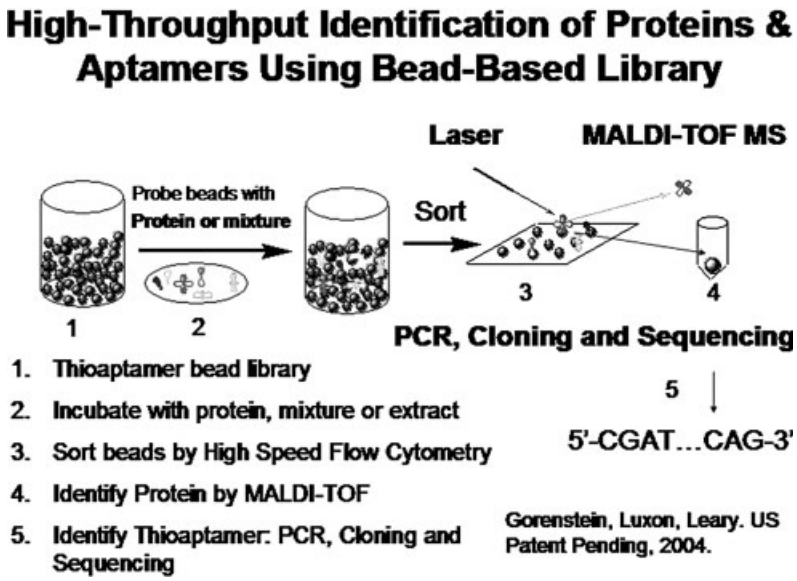

FIG. 6. Schematic for the high-throughput identification and separation of combinatorial libraries of thioaptamers and subsequent proteomic analysis by MALDI-TOF. Sequencing can be performed at the single-bead level.

speed sorting experiments of 10 h or longer. To find consensus sequences requires the sampling of an adequate number of beads that then must be individually sequenced and compared. An example of how this is done in practice is shown in Figure 6.

Biohazard Problem of Droplet-Based Sorting Aerosols

Probably more than any other factor, the biohazards produced by droplet-based sorters have limited their use on live human cells and any infectious agents. This has been a very significant limitation. Most sorting facilities have wisely put into place policies to protect personnel from the biohazards generated from aerosols created from these droplet-based sorters. The seriousness of this problem was not realized in the days before the discovery of human immunodeficiency virus and acquired immunodeficiency syndrome and the perhaps even more dangerous problem of transmission of hepatitis C virus. The current policy of "universal precautions" wisely assumes that all human material must be treated as if it may contain dangerous viruses. These rules are less commonly applied to cell lines that might contain endogenous viruses (or even may have been created through use of viruses such as Epstein-Barr virus). Given the increasing frequency of animal to human virus transfers (e.g., Severe Acute Respiratory Syndrome (SARS)), probably the sorting of animal cells with potential airborne viruses should be more carefully scrutinized. There are now many excellent guidelines as to how sorting of potentially infectious material should be accomplished (32–34). Despite the addition of containment boxes on cell sorters, the problem remains. The growing solution is to simply recognize that aerosols will be generated, the sorter needs to be placed in a certified BSL-3 facility with proper air handling to contain the aerosols in the room, and all personnel must be properly protected with BSL-3 protection gear (lab coats, gloves, masks, eye protection, etc.). This is the proper but a pretty drastic and expensive solution.

Need for High-Speed, Closed System Cell Sorting

Based on the preceding discussion, there is a very real need for the development of "closed system" cell sorters that probably will not be based on droplets. Current closed system sorters based on microfluidics and fluidic switching technologies are very slow. Most of these are still in research laboratories, although some systems are starting to be introduced to the marketplace. These systems, if properly designed, could be put inside biohazard hoods, which would be a much more practical and less expensive alternative than placing droplet-based cell sorters inside BSL-3 space.

RESULTS

Although there are numerous examples of high-speed cell sorting in the literature, for purposes of illustration two cases are presented that demonstrate the power and utility of high-speed cell sorting.

Sorting of Very Rare Cells With DNA Mutations

This is an application of rare cell sorting that requires high-speed or ultra high-speed analysis and moderate speed, but very high precision, single-cell sorting. The problem is to look at a statistically meaningful number of cells to find the mutant cell and then have enough power in the sort decision to precisely grab that cell free from a large background. In this example, a rare (10^{-6} frequency) tumor cell bearing a mutation in a tumor suppressor gene is analyzed and carefully sorted to a single-cell level (Fig. 7). The DNA from the sorted single cell is then amplified with polymerase chain reaction (PCR), cloned into a vector, further grown, and then DNA sequenced. The result is the detection of a single base-pair mutation in the PTEN tumor suppressor gene thought to be important in the outcome of breast cancer (Fig. 8) (35).

Detection of mutations in breast cancer tumor suppressor genes by high-throughput flow cytometry, single-cell sorting, and single-cell sequencing. An example of rare tumor cell detection (9) used this methodology. A rare tumor cell clone was mixed with a human T-cell line at a frequency of 10^{-6}. A defined cell mixture of CEM/C7 (human T cells) and MCF-7 cells (ATCC no. HTB-22, name MCF-7, human breast carcinoma cells) was constructed to yield a frequency of 10^{-6} tumor cells for flow cytometry and cell sorting. The cells were then analyzed on the basis of cytokeratin-positive fluorescence and for negative labeling with CD45. We then tested for sort recovery and purity in the case of a defined cell mixture of MCF-7 and CEM/C7 cells. We used DNA HLA-DQalpha typing of the MCF-7 cells (1.2 and 4) and the CEM/C7 cells (1.2 and 1.3) to see our efficiency of successful selection of tumor cells. The primers and oligo dot-blot probes used in these studies were done as previously described (35). Because the amount of PCR-amplified DNA from a single cell is too small to be seen by conventional ethidium bromide staining of the PCR products on a gel, enzymatic amplification techniques must be used. One such technique we have used is enhanced chemiluminescence (ECL; Amersham Life Science, catalog no. RPN 3021) whereby Southern blotting is performed with enzymatically labeled complementary sequence oligonucleotide ("oligo") probes. An enzymatic reaction gives rise to chemiluminescence that can then be detected on x-ray film, but without the hazards and disposal problems of radioactive probes. ECL provides an enzymatic amplification factor of approximately 1,000 times, thus permitting detection of PCR products from single cells (35).

Single-cell DNA sequencing results from TA cloning. As we described previously (22,35), DNA sequencing of sorted single tumor cells can be performed. We sorted single cells with several mutations, including the BT-549 cells that have a single base-pair mutation in the so-called PTEN gene, a tumor suppressor gene that has been previously described (36). A clone of BT-549 cells was analyzed by flow cytometry and sorted into single PCR tubes for subsequent analysis by PCR and TA cloning. The DNA from the PTEN gene region of sorted single cells was

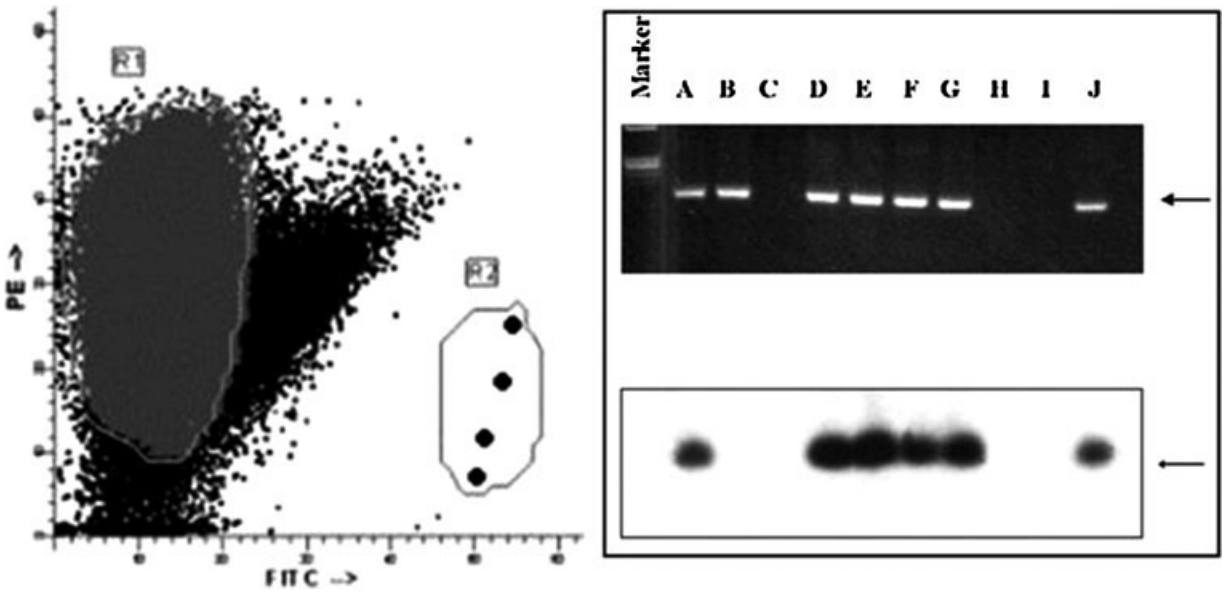

FIG. 7. Flow cytometric results from a defined cell mixture of 10^{-5} frequency MCF-7 cells in a major population of human CEM/C7 T cells. Cells were labeled with a phycoerythrin (PE)-conjugated anti-CD45 antibody and a fluorescein isothiocyanate (FITC)-conjugated anti-cytokeratin antibody. A small subpopulation of rare MCF-7 cells was detected in region R2 in an aliquot of the sample. Cells in this region were then sorted at the single-cell level for subsequent PCR analysis, TA cloning, and DNA sequencing. The four tumor cells, shown in this aliquot of cell sample, have been highlighted as dark enlarged circles in the flow cytometric distribution for easier viewing. The right-hand panel shows ECL detection of PCR-amplified sequences from sorted, rare, single tumor cells as shown in left-hand panel. The top subpanel shows nested PCR product on 2% agarose gel stained with ethidium bromide. The lower subpanel shows the result of ECL Southern blotting with HLA DQα type 4 probe. The result indicated that the overall sorting efficiency was seven recovered single rare cells out of 10 (a fairly typical recovery over many experiments), and the Southern blot revealed that six of those seven sorted cells were MCF-7 cells (lanes A, D, E, F, G, and J), showing that the sort classification was fairly accurate.

amplified by PCR and cloned into a TA cloning vector. The vector was then amplified by growth in host *Escherichia coli* bacterial cells, and the plasmid DNA was isolated and purified. This purified plasmid DNA containing the PTEN gene sequences from sorted single cells was then analyzed on a DNA sequencing instrument. A typical DNA sequencing result from a single sorted BT-549 cell is shown in Figure 8. Cells without the mutated sequence show normal PTEN gene sequences (data not shown). An alignment analysis of this sequence data reveals a single base-pair deletion at nucleotide 61 of exon 8 of the PTEN gene, which is indicative of the PTEN mutation present in these BT-549 cells.

Sorting of Bead-Based Combinatorial Libraries for Drug Discovery

One application requiring high-throughput analysis and sorting at the single-bead level is that of combinatorial chemistry library screening. In this application we are

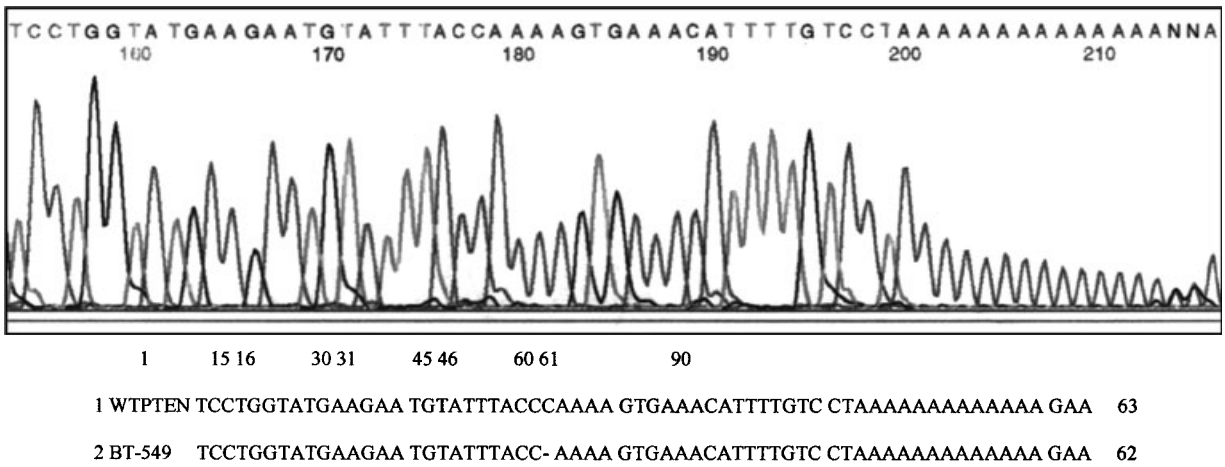

FIG. 8. DNA sequencing of the PTEN gene region from a single sorted cell (top) and alignment (bottom). The alignment shows that the PTEN mutation consists of a missing single base pair at position 61. Results show successful detection of a single base-pair mutation in a single sorted cell.

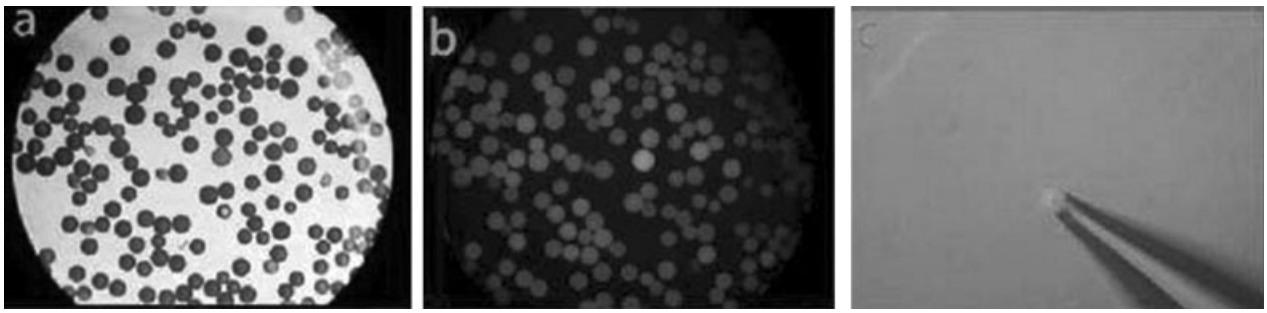

FIG. 9. **a:** Brightfield microscopy of a thioaptamer bead library generated by combinatorial chemistry using split synthesis method, as described earlier. **b:** Fluorescence microscopy of the same field of beads. **c:** Manual micromanipulation originally used to select beads to test feasibility of overall method (31).

screening large thioaptamer libraries. Thioaptamers have shape-recognition properties similar to that those of monoclonal antibodies. The difference is that one can screen 100 million monoclonal antibody equivalents in an afternoon. This has considerable implications for drug discovery. An example of a bead-based thioaptamer library is shown in Figure 9. Originally the beads for this application were roughly 80 μm in diameter, which is much too large for high-speed sorting. We subsequently decreased the size of the beads to 20 μm, which permitted relatively high-speed analysis and single-bead sorting, with DNA sequencing of thioaptamer sequences after PCR amplification.

Smaller (20 μm) bead libraries containing approximately 1.6×10^7 unique 52-mer thioaptamer sequences (with a 22-mer variable region specific to each protein) were screened by high-throughput flow cytometry for their capability to bind specifically to either the p50 or the p65 subunits of transcription factor nuclear factor-κB. To validate the assay we analyzed NF-κB bound to subsets

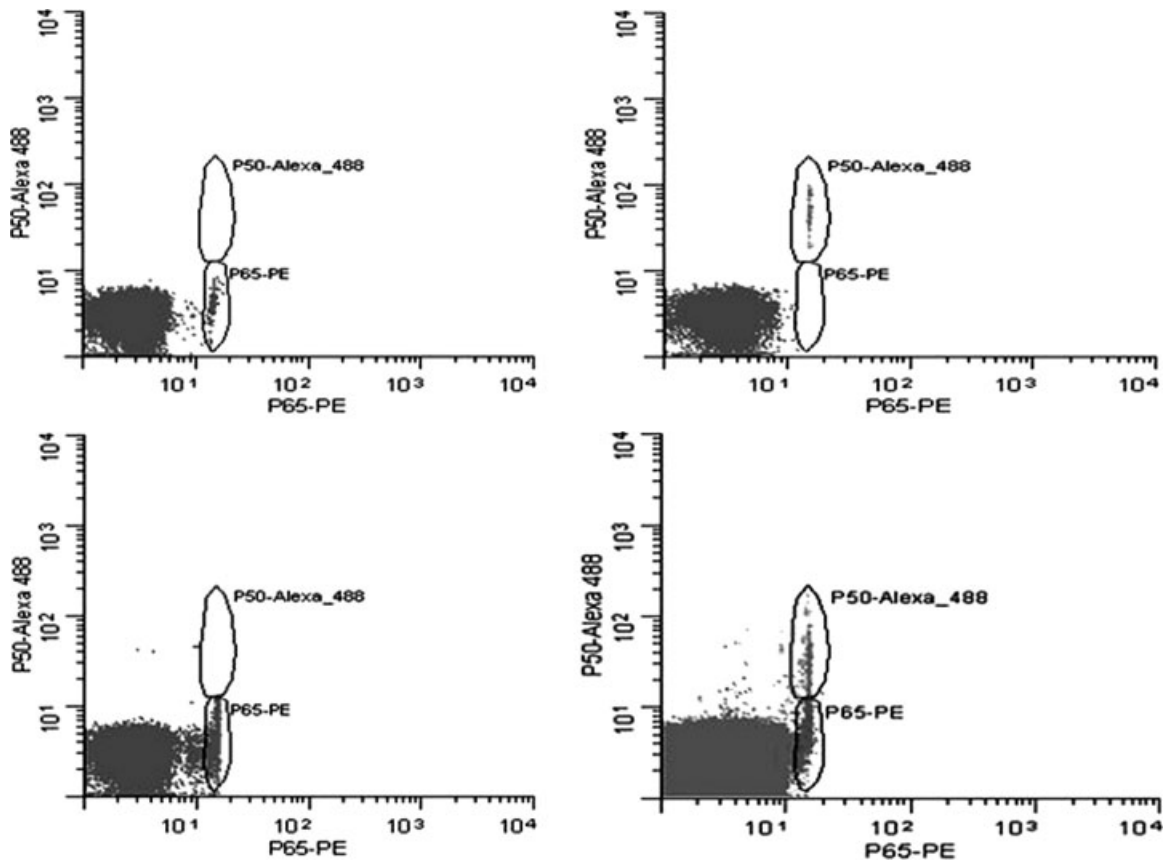

FIG. 10. Flow cytometric analysis of NF-κB p50 and p65 proteins bound to 20-μm beads containing a library of thioaptamer sequences. Alexa-488 conjugated antibody against p50 and phycoerythrin (PE)-conjugated antibody against p65 were then used to bind to these proteins to show whether the thioaptamers could correctly discriminate between these two proteins.

of the beads with various avidities, corresponding to the ability of the thioaptamers to recognize these proteins. As seen in Figure 10 the thioaptamers on a subset of beads were able to recognize and discriminate between these two subunits of NF-κB.

DISCUSSION

However, despite the problems and limitations of sorting, I see a bright future for cell sorting, perhaps by approaching the general problem a little differently. To date cell sorters have been large, complex devices that are much too large to be placed in a standard biohazard hood. Moreover, those large systems that some vendors have tried to surround with a biohazard containment cabinet still generate aerosols. Interestingly, the answer to these problems may lie in a revisiting of some of the early days of cell sorting when people were still trying fluidic switching technology in closed systems. The limitation was thought to be the switching speeds of these switches. However, if these systems are constructed to be massively parallel, it may be possible to construct a closed system for cell sorting that by being parallel, rather than serial, in nature can accomplish ultra high-speed sorting rates. By using modern microfabrication techniques and constructing these in a modular fraction, the cell-handling portion could go inside a biohazard hood with the remaining electronics and computers outside on a laptop computer.

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This article was written to honor the memory of Dr. Mack Fulwyler. His fundamental scientific work and enthusiasm for cell sorting (and life!) inspired a generation of flow cytometrists, including this author.

LITERATURE CITED

1. Fulwyler MJ. Electronic separation of biological cells by volume. Science 1965;150:910–911.
2. Kamentsky LA, Melamed MR. Spectrophotometric cell sorter. Science 1967;156:1364–1365.
3. Dittrich W, Gohde W. [Impulse fluorometry of single cells in suspension]. Z Naturforsch 1969;24:360–361.
4. Bonner WA, Hulett HR, Sweet RG, Herzenberg LA. Fluorescence activated cell sorting. Rev Sci Instr 1972;43:404–409.
5. Herzenberg LA, Sweet RG, Herzenberg LA. Fluorescence-activated cell sorting. Sci Am 1976;234:108–117.
6. Sweet RG. High frequency recording with electrostatically deflected ink jets. Rev Sci Instr 1965;36:131–136.
7. Ashcroft RG, Lopez PA. Commercial high speed machines open new opportunities in high throughput flow cytometry (HTFC). J Immunol Methods 2000;243:13–24.
8. Leary JF, Frederickson CJ. Flow sorting system and methods regarding same (high-speed microfluidics cell sorter). US patent application 20030153085. Filed 10 January 2003.
9. Leary JF, McLaughlin SR, Reece LN, Rosenblatt JI, Hokanson JA. Real-time multivariate statistical classification of cells for flow cytometry and cell sorting: a data mining application for stem cell isolation and tumor purging. SPIE Proc 1999;3604:158–169.
10. Fu AY, Spence C, Scherer A, Arnold FH, Quake SR. A microfabricated fluorescence-activated cell sorter [see comment]. Nat Biotechnol 1999;17:1109–1111.
11. Lindmo T, Fundingsrud K. Measurements of the distribution of time intervals between cell passages in flow cytometry as a method for the evaluation of sample preparation procedures. Cytometry 1981;2:151–154.
12. Leary JF. Rare event detection and sorting of rare cells. In: Durack G, Robinson JP, editors. Emerging tools for cell analysis: advances in optical measurement technology. New York: Wiley-Liss; 2000. p 49–72.
13. Leary JF. Strategies for rare cell detection and isolation. In: Darzynkiewicz Z, Crissman HA, editors. Methods in cell biology: flow cytometry. Volume 42. New York: Academic Press; 1994. p 331–358.
14. Stovel RT. The influence of particles on jet breakoff. J Histochem Cytochem 1977;25:813–820.
15. Petersen TW, van den Engh G. Stability of the breakoff point in a high-speed cell sorter. Cytometry 2003;56A:63–70.
16. Hokanson JA, Rosenblatt JI, Leary JF. Some theoretical and practical considerations for multivariate statistical cell classification useful in autologous stem cell transplantation and tumor cell purging. Cytometry 1999;36:60–70.
17. Gray JW, Dean PN, Fuscoe JC, Peters DC, Trask BJ, van den Engh GJ, Van Dilla MA. High-speed chromosome sorting. Science 1987;238:323–329.
18. Leary JF, Schmidt D, Gram JG, McLaughlin SR, DallaTorre C, Burde S. High-speed flow cytometric analysis and sorting of human fetal cells from maternal blood for molecular characterization. In: Simpson JL, Elias S, editors. Fetal cells in maternal blood: prospects for non-invasive prenatal diagnosis. Volume 731. New York: Annals of the New York Academy of Sciences; 1994. p 138–141.
19. Cupp JE, Leary JF, Cernichiari E, Wood JC, Doherty RA. Rare-event analysis methods for detection of fetal red blood cells in maternal blood. Cytometry 1984;5:138–144.
20. Leary JF, Hokanson JA, McLaughlin SR. High speed cell classification systems for real-time data classification and cell sorting. SPIE Proc 1997;2982:342–352.
21. Leary JF, McLaughlin SR, Hokanson JA, Rosenblatt JI. High speed real-time data classification and cell sorting using discriminant functions and probabilities of misclassification for stem cell enrichment and tumor purging. SPIE Proc 1998;3260:274–281.
22. Leary JF, Reece LM. Application of advanced cytometric and molecular technologies to minimal residual disease monitoring. SPIE Proc 2000;3913:36–44.
23. Leary JF, Corio MA, McLaughlin SR. System for high-speed measurement and sorting of particles. US patent 5,204,884; 1993.
24. Leary JF, Corio MA, McLaughlin SR. System for high-speed measurement and sorting of particles. US patent 5,804,143; 1998.
25. Rosenblatt JI, Hokanson JA, McLaughlin SR, Leary JF. Theoretical basis for sampling statistics useful for detecting and isolating rare cells using flow cytometry and cell sorting. Cytometry 1997;27:233–238.
26. Ibrahim SF, van den Engh G. High-speed cell sorting: fundamentals and recent advances. Curr Opin Biotechnol 2003;14:5–12.
27. Szaniszlo P, Wang N, Sinha M, Reece LM, Van Hook JW, Luxon BA, Leary JF. Getting the right cells to the array: gene expression microarray analysis of cell mixtures and sorted cells. Cytometry 2004;59A:191–202.
28. Chen W, Georgiou G. Cell surface display of heterologous proteins; from high throughput screening to environmental applications. Biotechnol Bioeng 2002;79:496–503.
29. Daugherty PS, Iverson BL, Georgiou G. Flow cytometric screening of cell-based libraries. J Immunol Methods 2000;243:211–227.
30. Yang X, Bassett SE, Li X, Luxon BA, Herzog NK, Shope RE, Aronson J, Prow TW, Leary JF, Kirby R, et al. Construction and selection of bead-bound combinatorial oligonucleoside phosphorothioate and phosphorodithioate aptamer libraries designed for rapid PCR-based sequencing. Nucleic Acids Res 2002;30:e132.
31. Yang X, Li X, Prow TW, Reece LM, Bassett SE, Luxon BA, Herzog NK, Aronson J, Shope RE, Leary JF, et al. Immunofluorescence assay and flow-cytometry selection of bead-bound aptamers. Nucleic Acids Res 2003;31:e54.
32. Schmid I, Nicholson JK, Giorgi JV, Janossy G, Kunkl A, Lopez PA, Perfetto S, Seamer LC, Dean PN. Biosafety guidelines for sorting of unfixed cells. Cytometry 1997;28:99–117.
33. Schmid I, Kunkl A, Nicholson JK. Biosafety considerations for flow cytometric analysis of human immunodeficiency virus–infected samples. Cytometry 1999;38:195–200.
34. Schmid I, Roederer M, Koup R, Ambrozak D, Perfetto SP. Biohazard sorting. In: Darzynkiewicz Z, Roederer M, Tanke H, editors. Methods in cell biology. Volume 75. New York: Elsevier; 2004. p 221–240.
35. Leary JF, He F, Reece LM. Detection and isolation of single tumor cells containing mutated DNA sequences. SPIE Proceedings in Systems and Technologies for Clinical Diagnostics and Drug Discovery II. SPIE Proc 1999;3603:93–101.
36. Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, Miliaresis C, Rodgers L, McCombie R, et al. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer [see comment]. Science 1997;275:1943–1947.