Overview of Flow Cytometry and Microbiology

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Although in recent years flow cytometry has become commonplace in hematology and immunology laboratories, application of the technology to microbiology remains largely unrealized. This overview presents the historical background, discusses applications in various areas of the field, and speculates on the directions of future developments. The availability of high-quality methods should be a prime factor in convincing microbiologists that flow cytometry may have certain advantages over traditional methods and that it does indeed have much to contribute to microbiology. © 2018 by John Wiley & Sons, Inc.

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In recent years, flow cytometry has become a relatively common, everyday technique in immunology and hematology laboratories. In almost any situation where it is necessary to phenotype a cell population, identify an antigen of interest, or determine the cell cycle status of a population, the time and effort required to develop a flow cytometric approach will be richly justified. Flow cytometry is widely accepted as a mature technology, and the applications as necessary and even desirable. For a long time, however, the application of flow cytometry to microbiology—although in theory inviting—has in practice remained an idea whose time has never quite come. In light of recent technological developments, perhaps that trend is changing, as there are now so many flow cytometers available and many new small benchtop instruments that are so easy to use, we are seeing a resurgence of microbial flow cytometry.

HISTORICAL DEVELOPMENT

The application of flow cytometry to the analysis of microbial systems has been a slow and difficult process. Early attempts in the 1970s showed promise (Hercher, Mueller, & Shapiro, 1979; Hutter and Eipel, 1979), with initial possibilities that appeared almost too good to be true—for example, rapid identification, rapid determination of antibiotic resistance, rapid enumeration, and an ability to provide quantitative information in a field that otherwise essentially lacked high technology. The 1980s saw the implementation of several innovations in system design along with an enhanced understanding of the nature of small-particle light scatter (Salzman, Griffith, & Gregg, 1982), both of which facilitated the detection of small particles (Steen, 1986). It was freely predicted that flow cytometry would rapidly develop into a clinically applicable technique (Boye, Steen, & Skarstad, 1983; Ingram, Cleary, Price, Price, & Castro, 1982).

Although flow cytometry gained almost immediate acceptance in the hospital pathology and immunology environment, microbiology laboratories were essentially oblivious to the technology, for a number of reasons. One immediate stumbling block was cell size. As discussed by Shapiro (2003), the difference in size and volume between microbial and mammalian cells is enormous (see Table 1). Because of the lack of interest from microbiologists and the difficulty of the engineering concepts, flow cytometry instruments were not designed to measure microorganisms, but rather

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Table 1 Relative Size Ratios for Bacteria, Yeast, and Eukaryotes

Measurement	Bacteria	Yeast	Eukaryote
Diameter	0.5-5	3-5	10-30
Surface area	3-12	30-75	300-3000
Volume	0.3-3	20-125	500-1500
Dry cell mass	1	10	300-3000

cells in the range of 5 to 15 μm. In practice, the measurement of smaller particles, while possible, often required modifications to the instrument or a greater understanding of and interest in the technological aspects of cytometry than was generally possessed by those with any expertise in microbiology. For reasons other than microbiology, many scientists are interested in small particles (see *UNIT 13.14*, Nolan, 2015), which are often smaller than most microbes. This has driven a stronger interest in measuring bacteria, for example, that are far easier to measure than extravesicles (but still not comparatively very easy to measure like blood cells).

A second problem was the notion that the fluorescent dyes used in flow cytometry were better understood in mammalian systems and relatively poorly understood in microbial systems. A great deal of effort has been put into developing fluorescent probes appropriate to the biochemical characteristics of mammalian cells. Unfortunately, this focus on mammalian systems resulted in a general failure to link microbiologists with those interested in fluorescence measurement, and hence the philosophy negatively affected the widespread acceptance of the technology.

A third factor was the cost of instrumentation. Microbiology has never been considered a high-technology field, and few microbiology laboratories would normally consider making the enormous outlays on technology that are considered reasonable in a pathology laboratory. The cost of flow cytometry instrumentation was generally felt to be prohibitive; so were the costs per operation, when compared to the few cents per test for a bacterial identification using traditional techniques. For all these reasons, flow cytometry technology has heretofore interested only research microbiologists, and has not had any substantial impact on the field of microbiology as a whole (which includes environmental, public health, medical, food science, industrial, and military applications).

APPLICATIONS

Environmental Microbiology

Marine and environmental microbiologists have been among the first to recognize the potential of flow cytometry (Amann et al., 1990; Allman, Manchee, & Lloyd, 2013; Cunningham, 1993; Edwards et al., 1993; Tarran & Burkill, 1993; Troussellier, Courties, & Vaquer, 1993). Environmental microbiology brings with it certain problems for which flow cytometry can to some extent provide solutions. Perhaps the most significant of these is the issue of culturability of organisms. There is a traditional microbiological viewpoint that an organism must be culturable in order to be designated "viable." However, an enormous number of organisms remain unstudied, unclassified, even undiscovered, because their specific culture conditions are not known. Currently a fierce debate rages within the microbiology community concerning the existence of organisms in a state termed viable but not culturable (VBNC). The controversy highlights the fact that many of the organisms that exist cannot be cultured, and therefore cannot be identified (Bogosian, 1998).

The use of flow cytometry as a microbiological tool casts new light on the controversy: with this technique, it is not necessary to culture an organism to determine its viability, at least in terms of metabolic state. Using vital fluorescent dyes, it is possible to identify populations of organisms that, although they may still be unculturable, are at least definitely "not dead." Work on this complex issue has opened a fertile area for creating new detection methods by linking up-to-date molecular techniques with rapid analytical technologies such as flow cytometry. Molecular tools can be used to create new microbial probes that can easily be converted to fluorescent conjugates suitable for flow cytometry.

Bioterrorism and Detection of Biological Warfare Agents

With the development of advanced genetic engineering technologies, it is possible to develop microorganisms that can produce as much as 100 times more pathogen or toxin per cell than that which is produced by naturally occurring strains. Such enhanced pathogens can be significant weapons in the hands of bioterrorists. Bioterrorism agents can be classified into several groups: bacteria, viruses, rickettsiae (which have characteristics common to both bacteria and viruses, but like the latter will grow only within

other cells), chlamydia (which are obligate intracellular parasites incapable of generating their own energy sources), fungi (and importantly fungal spores), and finally toxins. Each of these agents presents a significantly different problem in the area of detection. While many current biowarfare detection kits depend on antibodies reacting with the antigenic surface coatings of pathogenic bacteria or viruses, immunologically based detection mechanisms have advantages and disadvantages.

Detection systems related to flow cytometry

Flow cytometry can provide rapid, accurate, and quantitative information about airborne and waterborne pathogens and perhaps even toxins. One area where these characteristics may be of considerable utility is the detection of biological warfare agents. Culture systems that require several hours to identify microorganisms are of little value to front-line soldiers or civilian populations faced with possible biological assault. Flow cytometry has the advantage of being able to differentiate between nonbiological and biological particles, and, perhaps even more importantly, determine whether or not any organisms that are found are alive. Although molecular tools are often considered to be superior because tests can usually be done in batches, and because of the perception that they are more accurate, it is not clear that this is always the case. For example, consider the situation of a possible biological weapon. A sample is collected that may contain biological agents. Using molecular techniques, an organism or a spore is determined to be present, but there is no way to tell if it is alive or viable. In contrast, viability can be determined for many organisms in a relatively short time using flow cytometry. In addition, flow cytometry can differentiate very quickly between biological and nonbiological samples—a task that is considerably more difficult using molecular tools, since a negative answer may be somewhat less convincing and less conclusive. Unfortunately, after a major drive in the late 1990s to develop new instrumentation and fluorescent indicators for microbiology, it appears this effort has again fallen short. It is conceivable that in rapid-detection scenarios, flow cytometry will fail to gain acceptance until a future crisis emphasizes once again how powerful this technology is for identifying small particles in suspension. Regardless, the recent short burst of activity has raised the level of interest and knowledge concerning the application of flow cytometry to microbial systems.

One new opportunity is offered by multispectral technologies with the capability of using advanced mathematically based classification systems. Although these technologies are only just emerging, it is predictable that such developments will impact both the speed and accuracy of flow cytometry–based technologies. A number of technologies were recently discussed as "next-generation" detection possibilities, but it is clear that at present detection solutions contain more proposal than reality (Spencer & Lightfooty, 2001).

Food Microbiology

Perhaps one of the most useful potential applications of flow cytometry is in food microbiology. Foods are easily reduced to liquid form, the natural sample state for flow cytometry. The difficulty lies in the need to remove the perhaps 99.9999% of the particles present that are normal, and irrelevant to the measurement, before one can observe the 1 in 10^6 that is a living organism, and potentially requiring of further study. Thus, enrichment of the microbial population is often still necessary. It is also necessary to be able to identify the organism of interest—for example, *E. coli* O157:H7 in juices or foods—among the many other microbes that may be present; identifying a particular organism or strain requires specific monoclonal antibodies (see also *UNIT 11.6*, Raybourne, 2001). Routine use of flow cytometry technology may not be feasible given the lack of easy-to-use flow-based protocols, but in epidemic situations, its use should be considered a real possibility. Of course, the development of protocols for sorting bacteria, such as those in *UNIT 11.4* (Hawkins, 2001), will help to change this state of affairs.

General Applications

In recent years, Lyme disease has become more prevalent (or perhaps detecting it has become easier). *UNIT 11.5* (Callister et al., 2004) provides an elegant method of detecting Borrellacidal antbodies, which are known to be lethal to the spirochete *Borrelia burgdorferi*, the causative agent of Lyme disease. This unit outlines the methods for testing for serum antibodies. This test does require the use of live *B.burgdorferi* and so it requires significant attention to technician safety. Further, as the unit notes, serum must be filtered to remove any microbials present before a titer can be run. An additional issue that must be

taken into consideration is the protocol uses Acridine Orange that is known to cause contamination problems with some instrument plastic delivery lines. Acridine Orange is well known to bind to plastic and can raise background levels on subsequent tests so careful cleaning of the instrument is required after running such an assay.

Another, related application might be the detection of *Cryptosporidium* or *Giardia* in water supplies. *Cryptosporidium* in normally safe drinking systems (in the state of Wisconsin, USA, and the city of Sydney, Australia) have brought it to the attention of microbiologists that there is some confusion and lack of understanding even among experts. In Sydney, for example, *Cryptosporidium* was detected repeatedly over a period of several months in the normally safe water supply. No clear foci of infection have been identified, and few if any cases of cryptosporidiosis have been clinically identified. Even with expert flow cytometry available, no persuasive evidence has been provided that the organisms can be reproducibly identified. The problems are many: the lack of good antibodies for identifying *Cryptosporidium*, the very small numbers of organisms, and the difficulty routinely experienced in identifying these particular organisms absolutely.

There are many techniques for detection of pathogens in food or drinks and most are based on isolation, concentration and subsequent culture and identification by traditional means. It goes without saying, but we will say it, that working with pathogens like *E.coli* O157:H7 requires appropriate microbial laboratory facilities and attention to personnel safety must be a priority. That being said, *UNIT 11.6* (Raybourne, 2001) provides a protocol for detection of *E.coli* 0157:H7 by flow cytometry. Typically, the protocol does require an enrichment step as well as careful calibration of the assay to determine the minimum detectable limits using the flow cytometer available to the user. It is clear that each instrument will have a different detectable limit and so following all steps of this protocol is important. The protocol provides additional methods for sorting of fluorescenct *E.coli* 0157:H7. It is imperative that prior to attempting this, the sorter must be tested to ensure that the sorter does not contaminate the air (or the user!). Sorting of pathogens requires a great deal of careful preparation, safety equipment, and ensuring that other staff are not present in the vicinity of the sorter. Consulting *UNIT 3.5* (Oberyszyn, 2002), as well as *UNIT 3.6* (Schmid et al., 2007) would be good places to start before attempting to sort pathogenic organisms.

While discussing dangerous organisms, *UNIT 11.7* (Schell et al., 2004) provides an excellent approach to determination of *Mycobacterium tuberculosis* susceptibility using flow cytometry. Again, the assumption made in this unit is the ability of the user to safely culture and use live pathogens in the laboratory. Appropriate safety conditions must be met prior to attempting this protocol. Working with microbials requires a different approach to flow cytometry than working with blood. The reason for this is that all of the "particles" being evaluated are in the typical "noise" area of most flow cytometers. For example, sheath fluid must be filtered and all tubing thoroughly cleansed if one is to successfully run samples containing microorganisms. Since the purpose of this protocol is to determine susceptibility, it does take some time. Results can be obtained after 24 hr, which is several days sooner than what a traditional assay will provide.

UNIT 11.8 (Mason et al., 2001) provides a general outline of susceptibility testing of most organisms of interest in a very short time period, which is the key advantage of using flow cytometry for such an assay. This protocol uses commonly available dyes such as $DIBAC_4(3)$ or SYBR Green and is a highly generic protocol applicable to virtually any bacterial species of interest on virtually any flow cytometer, since the excitation required is 488 nm. Another unit that describes susceptibility is *UNIT 11.14* (Nuñez, et al., 2001), which focuses on the parasite *Leishamia infantum*. The cytometric approach permits one to detect, differentiate, and quantify cellular changes in these parasites resulting from drug treatment.

UNIT 11.9 (Robertson and Button, 2001) provides a technical approach to the determination of microbial biomass. This determination is not a straightforward calculation and this unit outlines the process with great clarity. The relationship between light scatter and biomass is very complex and the user is warned to be sure you have your "mathematical hat" on when approaching this determination. However, the key point of this protocol is that flow cytometry provides a rich array of data from which to calculate many parameters, including biomass. While the protocol was written for the use of a UV excitation source (353 nm), it is possible to perform this assay using DAPI

excited at 405 nm (a common laser line on recent cytometers).

UNITS 11.10 (Lloyd, 2001) and *11.13* (Fortuna et al., 2001) provide a detailed overview of analysis of yeasts by flow cytometry. Again, these protocols can be successfully achieved using a common flow cytometer with a 488-nm laser. *UNIT 11.10* (Lloyd, 2001) provides a number of protocols related to cell cycle analysis (Basic Protocol 1), Determination of Viability (Basic Protocol 2), evaluation of the mitochondrial (respiratory) function of yeasts (Basic Protocol 3), and finally determination of β-Galactosidase Activity In Vivo. *UNIT 11.13* (Fortuna et al., 2001) focuses on measurement of cell cycle using SYBR green dye and this is a particularly easy and straightforward protocol to follow.

There are several units on phytoplankton, viruses, and most other organisms that can be found in marine environments. *UNITS 11.11* (Marie et al., 2001a), *11.12* (Marie et al., 2001b), *11.15* (Grégori et al., 2003), and *11.19* (Gerashchenko et al., 2010) provide a comprehensive coverage of the evaluation and analysis of marine microorganisms. Each of these units provides detailed protocols for each type of microorganism. Complex mixtures of organisms can be analyzed by use of a variety of DNA/RNA dyes such as TOTO, YOYO, TO-PRO, YO-PRO and a variety of other charming nucleic acid dyes. Further differentiation of microorganisms by size, combined with different nucleic acid content and other dye labeling properties provides a very rich amount of analytical data. Some organisms must be stained to differentiate them and others can be analyzed by their autofluorescence properties. *UNIT 11.19* (Gerashchenko et al., 2010) focuses on algae and biomass determination and provides detailed protocols in how to evaluate the life cycle, cell number and biomass.

One of the few units focusing on functional parameters of microorganisms is *UNIT 11.16* (Herrera et al., 2003), which provides detailed protocols on the measurement of oxidative stress in genetically engineered *E.coli* strains. This unit provides two very detailed protocols for the measurement of intracellular superoxide (Basic Protocol 1) and intracellular peroxides (Basic Protocol 2). The protocols are not particularly complex to perform but the protocol provides detailed instructions on how to manage the many different activating molecules that can be used for stimulation of the oxidative process, as well as the careful controls required. The second unit focusing on microbial functional determination is *UNIT 11.18* (Duhamel et al., 2009), which provides details on detection of extracellular phosphatase activity of heterotrophic prokaryotes using flow cytometry.

UNIT 11.17 (Harkins & Harrigan, 2004) is a highly detailed review of how to analyze the most common of bacteria that are of critical importance from a pathogenicity perspective. These include *E.coli* 0157:H7, *Salmonella* Sp, *Listeria* Sp and *Campylobacter* Sp, *Staphylococcus aureus*, *Pseudomonas aeruginosa*; all of which are found in common food contamination, or are important in areas such as pharmaceutical manufacturing or production of commonly used sterile reagents. A very valuable feature of these protocols is a detailed table that outlines successful methods for labeling all of the above organisms with fluorescently conjugated antibodies. This table alone will save time for anyone starting to work in the bacterial domain. There are protocols for both direct and indirect microbial labeling included. Finally, a protocol is included to perform *in situ* hybridization of rRNA.

The final three units in this chapter focus on very different areas. *UNIT 11.20* (Shapiro et al., 2013) discusses the potential for flow cytometry to analyze malarial parasites. This unit provides a highly detailed commentary in addition to providing protocols. It is by far the most detailed manuscript currently available that discusses the issues of managing the huge burden of malaria worldwide, and is worthy of consideration as a valuable commentary on the problem even if the area of malarial detection is not something of direct interest to the user's research field. *UNIT 11.21* (Schmidt et al., 2016) discusses the pitfalls of sorting live parasitic nematode eggs using relatively large 200 μm sorting nozzles and provides very detailed instruction on the difficult problem of sorting large particles. *UNIT 11.22* (Smirnov et al., 2017) is the most recent unit in this chapter and focuses on high throughput particle uptake using imaging flow cytometry. Over the years, there have been many protocols that describe phagocytosis and this adds to that count, but this protocol distinguishes itself by combining traditional flow histograms with image analysis, providing some unique analytical capacity.

FUTURE DEVELOPMENT

Molecular techniques may hold promise for flow microbiology. Flow identification and sorting using fluorescence in situ hybridization (FISH) techniques offer great potential.

Amann (1995) has demonstrated a clearly effective application of 16S rRNA probes in microbial ecology. More recently, Wallner, Erhart, and Amann (1995) were able to use this approach to separate labeled subpopulations, which were subsequently used as templates for PCR amplification of the 16S rRNA gene. Application of such combinations of flow and molecular techniques to highly mixed populations such as those in sludge or soil provides unique solutions only achievable by a combination of flow and molecular techniques.

Among the real issues that will affect the use of flow cytometry technology in microbiology, the first and foremost is bound to be cost. The perception exists in clinical microbiology at least, that flow cytometry and similar technologies will never be able to perform as cost effectively as traditional culture methods. "Pennies a test" is an expression frequently quoted in clinical laboratories; this is certainly difficult to achieve when the expenditure of $100,000 or more for a flow cytometer must be factored in. The second real problem is the lack of knowledge and understanding about the capabilities of flow cytometry on the part of clinical microbiologists who have been told repeatedly for the past 10 years that the time for flow cytometry in microbiology is imminent. It is also true that microbial diversity and the unique ability of organisms to alter their antigenic expression make it difficult to use antibodies as effectively as in human immunophenotyping. It is clear, however, that new alternatives using bead technologies and cheaper, easier-to-use instruments will make flow cytometry more attractive and perhaps even less expensive than the current techniques it can replace. Bead technologies promise to bring major changes to flow cytometry and microbiology. The ability to create these small "identification" laboratories within test tubes may be a powerful addition to the tools flow cytometry can offer the microbiologist.

Ever since *Current Protocols in Cytometry* was in the planning stages, coverage of flow cytometry and microbiology has been under continued discussion. The editors now believe that bringing specific methods and ideas to the bench scientist in accurate, reproducible, and detailed format can foster a real and measurable growth in application of flow cytometry to microbiology. The availability of high-quality methods should encourage experiments by many people who might have previously felt the techniques were too complex and the instrumentation too difficult to manage. The provision of both commentary and protocol units will, we hope, be a self-fulfilling prophecy in the long-awaited progression of flow cytometry into the field of microbiology. Those who have for years been developing expertise at the interface between the two fields should now be encouraged to share this hard-earned knowledge and provide all the assistance they can to colleagues who wish to implement the sometimes difficult assay systems in flow cytometry. By combining all the above developments, together with a greater desire of microbiologists to encompass new technologies, it is possible that flow cytometry and microbiology will finally complement each other.

We are now in a different era in flow cytometry and microbiology. As noted earlier, low cost instruments abound, and in addition, most new instruments have the capacity to use microtiter plates making large assays more manageable and semi-automated at the least. We will start to see more microbial assays particularly focused on the clinical environment. This has been an area that has been the last bastion of flow cytometry. Predictions of its readiness to accommodate flow cytometry have been made every decade since the 1980s. Let us hope this is the decade it really comes into being.

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