

MiniReview

Flow cytometry for microbial sensing in environmental sustainability applications: current status and future prospects

Cyndee Gruden ^{a,b}, Steven Skerlos ^{b,c}, Peter Adriaens ^{a,b,*,1}

^a Department of Civil Engineering, University of Toledo, Toledo, OH 43606, USA

^b Department of Civil and Environmental Engineering, University of Michigan, Ann Arbor, MI 48109, USA

^c Department of Mechanical Engineering, University of Michigan, Ann Arbor, MI 48109, USA

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Abstract

Practical and accurate microbial assessment of environmental systems is predicated on the detection and quantification of various microbial parameters in complex matrices. Traditional growth-based assays, considered to be both slow and biased, are increasingly being replaced by optical detection methods such as flow cytometry. Flow cytometry (FCM) offers high-speed multi-parametric data acquisition, compatibility with current molecular-based microbial detection technologies, and is a proven technology platform. The unique technical properties of flow cytometry have allowed the discrimination of bacteria based on nucleic acid staining, microbial identification based on genomic and immunologic characteristics, and determination of cell viability. For this technology to achieve the ultimate goal of monitoring the microbial ecology of distributed systems, it will be necessary to develop a fully functional, low cost, and networkable microsystem platform capable of rapid detection of multiple species of microorganisms simultaneously under realistic environmental conditions. One such microsystem, miniaturized and integrated in accordance with recent advances in micro-electro-mechanical systems technology, is named the Micro Integrated Flow Cytometer. This manuscript is a minireview of the current status and future prospects for environmental application of flow cytometry in general, and micro-flow cytometry in particular.

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1. Introduction

Recent high profile microbial contamination events coupled with a national security-driven focus on bioterrorism prevention have prompted extensive interdisciplinary research toward the development of rapid and accurate detection methods for specified target microorganisms in a variety of environmental (infrastructure) applications [1–4]. Whereas the microbial contaminant sources tend to be varied in scope and composition, the lack of rapid quantitative screening tools adaptable to

the myriad of environmental matrices under consideration precludes high throughput information gathering on the spatial distribution of potential microbial contaminants. This limits the effectiveness of strategies aimed at mitigating the impact of microbial contamination events on public health. Whether intentional, or as the by-product of urbanization, microbial contaminants pose proven and potential risks to public health and demand increasingly stringent monitoring [5,6]. Similarly, the control of microorganisms in bioremediation applications suffers from spatial and temporal uncertainties with respect to cause and effect.

The highly heterogenous nature of microbial occurrence and activity or viability in natural and engineered systems indicates a need for distributed microbial sensing capabilities, which will be highly dependent on the near-term technological capabilities for on-line

* Corresponding author. Tel.: +1-734-763-8032; fax: +1-734-763-2275.

E-mail address: adriaens@umich.edu (P. Adriaens).

¹ Current address: Department of Mechanical Engineering, University of Michigan, Ann Arbor, MI 48109, USA.

microbial detection, quantification, and analysis. Distributed microbial sensing networks are relevant in environmental applications such as groundwater and surface water monitoring, early detection of potential drinking water contamination, and optimization of microbially impacted industrial systems, through the implementation of engineered controls. The necessity for intervention in, and manipulation of, these highly complex microbial ecosystems, requires the accurate detection and quantification of microbial parameters as a function of matrix variables describing the relevant physical–chemical environments. An often cited example is the recent urbanization trend, which has resulted in an increase of impervious surfaces, thereby impacting the quality of water that recharges aquifers and discharges to streams, lakes and wetlands and, ultimately, is recycled for potable use. A distributed microbial (and chemical) sensing network implemented within this framework would provide an intelligent infrastructure for decision support systems on land-use, and water treatment optimization by providing valuable data including identification, quantification, and spatial distribution of microbial (and viral) contamination (e.g. [7]). Moreover, when coupled to distributed computational groundwater models incorporating geographic features, information would be gained on local and regional impending threats as the result of microbial and chemical transport. More relevant to infrastructure systems, such as metalworking and drinking water applications, Fig. 1

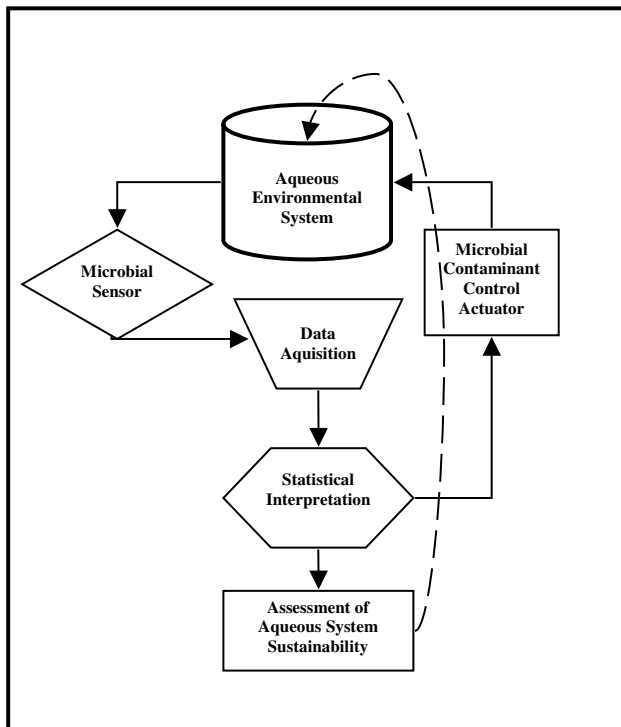


Fig. 1. Control system paradigm for sustainable aqueous environmental system.

illustrates the control system paradigm for engineering sustainable aqueous systems through microbial contamination control. The interpreted data could then be utilized to adapt design alternatives and to optimize treatment technologies as a function of the source quality and end-use of the aqueous system (e.g. [8]).

Solutions to the technological challenges of achieving automated, low-cost, and robust detection of microbial parameters of interest in field applications are currently under development in numerous research laboratories world-wide [3,9,10]. The current technology platforms indicate an emphasis on innovations in micro-electro-mechanical systems (MEMS) and improvements in the environmental application of molecular tools as the main technological drivers for the advancement of this research. The projected product of these efforts will be the development of faster, better, and cheaper microbial detection technologies that can be applied more frequently and in more locations. Moreover, the quantitative analysis of the microbial attributes (i.e., characteristics) of a targeted system will permit statistical risk-based decision-making. This manuscript will review the state-of-the art of flow cytometry as a technology for environmental microbial characterization, and its potential future use as a miniaturized platform for sustainable systems engineering through distributed microbial sensing.

2. Detection method for distributed microbial sensing

State-of-the-art technologies for microbial sensing include optical, electrochemical, impedance, piezoelectric, acoustic, and electrochemical biosensors. For achieving acceptable specificity and for application in the environment, most microbial detection methods necessitate an amplification or enrichment step, however, the performance characteristics (e.g., detection limit, setup time, adaptability, matrix interferences) of these approaches vary. A comparison of various attributes of selected technology platforms (Table 1), indicates that their applicability will be dependent on the selection criteria imposed by the system under consideration [2,9–11].

2.1. Fundamental approaches for microbial sensing

Although plating techniques continue to be the most adaptable method available for microbial detection, it is well known that these methods are slow and labor intensive (1–3 days), and they underestimate numbers and variability due to biases introduced by selected incubation conditions, the occurrence of isovars, and physical factors which reduce culturability [12–14]. Alternative methods, including piezoelectric, acoustic, impedance, bioluminescent, and certain optical sensors, are consid-

Table 1
Comparison of selected technology platform attributes for microbial sensing

Methodology	Detection limit ^a (cfu/mL or cfu/g)	Setup time	Adaptability	Matrix interference
Plating techniques	1	1–3 days	Excellent	Low
Bioluminescence	10 ³ –10 ⁴	1/2 h	Low	Medium
Piezoelectric	10 ⁶	5 h	Good	High
Impedance	1–10 ⁵	6–24 h	Moderate/good	Medium
Flow cytometry	10 ² –10 ³	1/2 h	Good	Medium
Acoustic	5 × 10 ⁴ –10 ⁶	3 h	Moderate	High
Electrochemical	10 ³	1/2–2 h	Low	Low

^a Microorganism-specific in some instances.

ered direct detection methods because they allow label-free monitoring of cells, based on direct measurement of physical phenomena occurring during the biochemical reactions on a transducer surface. Piezoelectric sensors, used for wide-ranging applications, are coated with a selectively binding substance (i.e., antibody specific to bacteria) and are subsequently introduced to samples purported to contain target bacteria. Changes in mass of the crystal and resonance frequency of oscillation are indicators for microbial detection. Similarly, acoustic methods involve coating an acoustic wave sensor with bacteria (or antibodies for bacteria). Either method exhibits limitations due to lack of sensitivity (unless enrichment is allowed, which substantially lengthens assay time), and both methods demonstrate a high potential for matrix interferences. Matrix interferences similarly affect electrical impedance methods. Since impedimetry is based on an inverse relationship between microbial metabolism and impedance, this technique requires large numbers of viable cells. Finally, although bioluminescent methods perform well in complex matrices, these methods require relatively high numbers of metabolically active bacteria (10³–10⁴/mL) to be effective [10].

Indirect methods rely upon microbial metabolism (electrochemical sensors) or fluorescent labels (flow cytometry). Electrochemical assays have fewer interference issues (i.e., particulate matter) since their sensors are not optically based, however the amount of time required for diffusion to the electrode interface is rate-limiting. In addition, adaptability can be problematic since there can be variations in signals produced by different strains of bacteria [10]. An assessment of the detection method characteristics found in Table 1 indicates that flow cytometry provides the best balance of microbial sensitivity, speed, adaptability, and robustness in complex matrices [11].

2.2. Technical aspects of flow cytometry

Flow cytometry is the measurement of physico-chemical characteristics of cells as they flow through an observation channel. This technique has been widely used for biomedical, biotechnology, and environmental

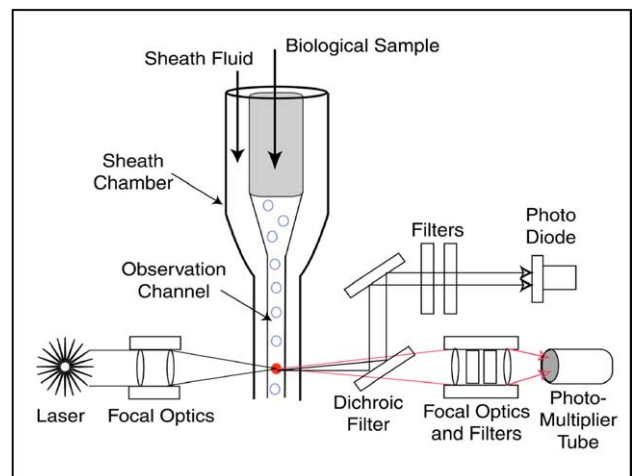


Fig. 2. Conceptual illustration of flow cytometry.

microbiology research. A single-file flow of microbes inside the observation channel (Fig. 2) is achieved by injection of the sample into a coaxial fluid stream where the mixture is focused using a sheath flow of aqueous solution. A laser beam(s) is directed at the observation point and the resulting light scatter and emitted fluorescence is detected with a photosensor (photomultiplier tubes). Most commercially available flow cytometers have a primary laser (argon-ion laser fixed at 488 nm), although green (532 nm) and red (635 nm) diode laser excitation are becoming more common. Several parameters of detection are monitored: low angle or forward light scatter (FSC), right angle light scatter or side scatter (SSC), and several fluorescence detection channels with wavelength ranges defined by selected short and long pass filters.

While scatter information is related to particle size and cellular characteristics, the key to flow cytometry is that specific fluorescent probes can be added to the sample such that fluorescence only occurs when microbes are present. Thus, a flow cytometer can distinguish between ordinary particles and microbial cells. By designing fluorescent tags that are specific to DNA sequences or descriptive proteins, the flow cytometer can also distinguish between microbial species. The

Table 2
Molecular methods for microbial sensing compatible with flow cytometry

Assay type	Criteria	Specific examples	Reference
Non-specific	Nucleic acid stains	<i>Picogreen</i> , <i>SYBR-Green</i>	[11,13,25]
	Cytoplasm stains	<i>Rhodamine</i>	
Specific	Antigen-based	<i>Immunoassays</i> , <i>fatty acid signatures</i>	[1–3,5,8–9,25]
	Nucleic acid-based	<i>DNA/RNA probes</i> , <i>FISH PCR</i> , <i>sequencing</i> , <i>ribotyping</i>	
Cell functioning	Membrane integrity	<i>Propidium iodide</i>	[11,12,14,25,31,32,35]
	Enzyme activity	<i>Tetrazolium salts</i> , <i>fluorogenic enzyme assays</i> , <i>esterase assays</i>	
	Membrane activity	<i>Rhodamine</i> , <i>Bis-oxonol</i>	

fluorescent emission from these cells is separated from the scattered light using a filter or dichroic mirror and is collected by a high-sensitivity, low-noise photosensor (photomultiplier tube). The nature of the fluorescent signal is related to microbial species, concentration, and physical characteristics of the system.

2.3. Molecular methods for microbial detection compatible with FCM

Although FCM was initially developed to study mammalian cells that are three orders of magnitude larger than bacteria, it has effectively been used for non-specific bacterial discrimination on the basis of nucleic acid staining, specific identification based on genomic and immunological characteristics, and characterization of basic cell functions (Table 2). Since FCM includes a fluorescence-based detection technology, fluorescent labeling is the focus of this section. The selection of the appropriate fluorescent labeling technology may vary depending upon the preferred characteristic biological target (i.e., metabolic enzyme, nucleic acid).

2.3.1. Non-specific detection

The proven high-affinity blue DNA specific stains (e.g., 4',6-diamidino-2-phenylindole) typically employed in fluorescent microscopy are not compatible with typical benchtop FCMs, because the primary laser excitation is 488 nm. However, several blue-excitable stains with varying quantum yields and nucleic acid binding affinities have been developed (Molecular Probes, Eugene, OR) for such applications (e.g., Picogreen), as the stains are excitable by the 488nm cooled argon laser typically incorporated into most modern bench-top flow cytometers. Several of these dyes (SYBR and SYTO families) have been compared for application in natural waters with varying biogeochemistry [15]. SYBR-II and SYTO-9 were deemed most appropriate for bacterial enumeration in non-saline natural waters while SYBR-II proved most effective in seawater samples [15]. Among the increasing number of FCM-compatible stains available, high affinity green nucleic acid dyes such as Picogreen, achieved the best separation of the community using direct count methods in marine, soil, and sediment-derived samples [16–18]. In particularly

complex matrices (e.g., sediments), sample fixation caused the fluorescent signal to quench, thereby hindering microbial differentiation [17].

2.3.2. Specific detection

Distributed microbial sensing systems require a sensor that has the specificity to identify target bacteria in a multi-organism matrix [10]. Microorganisms can be identified using flow cytometry in mixed populations due to advances in immunology and molecular biology. Fluorescent markers have been designed to label characteristic antigens and genomic material (e.g., nucleic acids). Antigen-based methods target organism-specific signatures (e.g., proteins, fatty acid composition), while nucleic acid-based protocols rely upon genomic material (e.g., DNA, rRNA).

Methods based on the detection of antibodies against specific microbial antigens are characterized by their simplicity, rapid response, and financial viability [10]. Antibody-based FCM is commonly used to detect *Cryptosporidium* oocysts and *Giardia* cysts in treated and untreated drinking water [19]. Infectious rotaviruses have been detected in source water by indirect immunofluorescence and flow cytometry [20]. The application of antibody-based FCM detection of specific cells in environmental applications has been previously reviewed [21,22]. Problems associated with antibody-based tests include variable sensitivity and cross-reactivity that can be exacerbated in complex matrices. In addition, epitopes expressed in pure cultures often differ significantly from those developed under environmental conditions presenting problems with method development [23].

Such problems have prompted the investigation of molecular-based approaches that target nucleic acid sequences of bacteria and can rapidly identify different species under environmental conditions. In the last decade, many 16S and 23S rRNA-targeted DNA probes have been designed to detect various phylogenetic subgroups or species. Multiple species can be detected in one sample by selection of unique fluorochromes for species-specific probes. The application of comparative rRNA sequencing eliminates culturability problems, and permits the direct study of microbial ecology in natural and engineered systems [24,25]. rRNA probes tend to be

more effective in environmental applications since there are numerous copies of rRNA within cells, which increases the likelihood of detection. Fluorescent in situ hybridization (FISH) allows for the identification and quantification of single cells based on whole-cell hybridizations with fluorescent oligonucleotide probes to rRNA [26]. In natural environments, where microbial activity (and rRNA production) is limited, nucleic acid amplification may be required for signal detection [27].

Polymerase chain reaction (PCR)-based methods have the advantage of amplification and subsequent detection of bacteria without relying on culturing. PCR methods coupled with fluorescence-based detection, are capable of near-real-time monitoring of bacteria in environmental samples [28,29]. The main advantage of PCR is that it allows the tracking of several different genes concurrently, allowing a greater level of certainty in microbial detection. Concentration steps may be required to increase the sensitivity of PCR-based methods to acceptable ranges. In turn, sample concentration tends to exacerbate interference issues by simultaneously increasing the inhibitory compounds found in environmental samples (e.g., humics) and, hence, requires extensive sample preparation [2]. In addition, preferential amplification of some genes and the generation of artificial sequences are potential issues that can be minimized by optimizing PCR conditions (e.g., “hot starts”, alkali denaturation, and inclusion of co-solvents) [30,31].

Many nucleic acid-based molecular typing methods (e.g., terminal restriction fragment length polymorphism or T-RFLP), which typically follow amplification (PCR), involve cutting sample-extracted DNA with selected restriction enzymes. The resulting characteristic fragments may be fluorescently labeled and analyzed by flow cytometry. Other methods such as denaturing gradient gel electrophoresis (DGGE) rely on the melting properties of PCR products. Although these methods are discriminatory and reproducible, many of them are technically demanding, time consuming, and may require expensive equipment. In addition, some of the methods, such as ribotyping, are limited by the need for extensive culture-based libraries [2].

2.3.3. Classification of cell functioning

Flow cytometry can be used applied to determine the physiological state and function of cells. For example, flow cytometry has routinely been applied to detect green fluorescent protein (GFP) used as a reporter for a variety of biological functions of proteins (e.g., gene induction) in mammalian cells [32–34]. Numerous stains have been developed for the assessment of cell function including reproductive ability, metabolic activity, membrane activity, and membrane potential. These methods, which are rapid (typically less than 1-h of incubation), have been employed in conjunction with non-

specific stains to distinguish viable, but non-culturable, microorganisms that would be overlooked by traditional, growth-based assays. Several of these probes are compatible with flow cytometry including: (i) membrane activity stains (e.g., Rhodamine 123 or Rh123), (ii) membrane integrity stains (e.g., propidium iodide or PI), and (iii) enzyme activity stains (e.g., 5-cyano-2,3-ditolyl tetrazolium chloride or CTC) [13,35]. Stains that reveal attributes of cell physiology are typically employed in conjunction with non-specific stains. Simultaneous application of multiple stains can provide detailed information about cell physiology and can permit differentiation of vital (demonstrates metabolic activity), viable (demonstrates membrane integrity), and dead cells [36].

Considering the limitations of metabolic activity measurements and the crucial importance of membrane integrity, a combination of total cell staining with membrane integrity and immunofluorescence provides a basic combination for a rapid detection method [27]. Dual-staining techniques have been applied to sediment matrices to quantitatively resolve total microbial communities and sub-populations as a function of sediment geochemistry and ambient hydrogen concentrations [16] or in mesophilic anaerobic digesters [37]. In addition, multi-staining was used to phenotypically monitor and quantitatively evaluate complex microbial communities over time during the biodegradation of naphthalene using multivariate flow cytometric analysis [12]. Interpreting viability data may be complicated because appropriate controls and validation methods have yet to be developed for mixed populations to address complicating issues such as dye extrusion, dye retention in vacuoles despite cell permeabilization, and differential uptake/exclusion of selected stains [13,27].

3. Environmental application of FCM

With very few exceptions, the microbial characterization of environmental matrices is based on either membrane filtration (aqueous samples) or cell separation (solids samples) followed by culturing and phylogenetic or functional analysis. Current predominant microbial detection methods have major disadvantages including long turn-around time, inherent species bias, and insufficient evidence to support method reliability in complex media. Despite the speed, sensitivity, and reproducibility, documented FCM applications in complex environmental matrices are virtually nonexistent. Environmental and clinical matrices pose the challenge of a broad range of biogeochemical conditions (ionic strength, pH, particulate matter), which may impact label specificity, fluorescent response, and method sensitivity.

3.1. Specific challenges to environmental FCM applications

Environmental matrices pose unique challenges to optical detection methods such as FCM due to their inherent complexity. Sample complexity is determined by the combination of physical and chemical characteristics of an environmental matrix. Environmental systems represent a broad range of biogeochemical conditions (e.g., ionic strength, pH, particulate matter). In addition, bacteria often colonize available surfaces, resulting in the formation of biofilms in the environment. During environmental flow cytometry, the limitations on fluorescent-based assays may be dependent on the presence or absence of background fluorescence, on the heterogeneity of the sample, on the fluorescence distribution, on the signal-to-noise ratio, on adequate cell recovery, and on efficient hybridization between the probe and the target biological molecule [38,39]. High levels of autofluorescence and non-specific dye-binding affects the application of fluorescent labeling methodologies in marine and continental waters due to phototrophic pigments and some organic compounds [22].

In addition to background fluorescence, the abundance of non-living bacterial sized particles in aqueous and subsurface microbial samples impart obvious challenges to optical detection methods [40,41]. Physical affinity (e.g., sorption, sequestration) of selected fluorochromes to abiotic particles will dominate their efficacy and use in certain environmental matrices. Particulate matter in natural waters includes humic substances that are heterogeneous, of moderate molecular weight, and characterized by organic acids of biological origin. Humics are ubiquitous due to their diverse origins and pathways of formation as well as their recalcitrance to geochemical and microbial degradation [42]. Also, humics typically interfere with nucleic acid-based microbial detection methods because they are PCR inhibitors.

Finally, differences in the biogeochemistry of freshwater and marine (or estuarine) environments substantially affect microbial diversity and activity, as well as method applicability due to interferences from ionic strength with probe hybridization and DNA/RNA recovery. Moreover, since DNA extraction is dependent on effective lysing of the cells, the community complexity may result in a differentiation of DNA recovery (e.g., gram-positive vs. gram-negative bacteria). Other variations in microbial composition may be attributed to nutrient availability. In oligotrophic environments, the method sensitivity can be limited due to negligible bacterial numbers and an associated decrease in metabolic activity. Observed variations in microbial composition may require site-specific sample preparation methodologies for environmental FCM applications [16].

High levels of autofluorescence and non-specific dye-binding as well as high particulate and solid phase content, common characteristics of natural waters and sediments, have thus far hindered the efficient application of flow cytometry to environmental samples [21,22]. The level of analytical interference in a sample is typically directly proportional to the sample complexity (i.e., sediment samples introduce more analytical interference than surface waters). However, the unique challenges posed by complex matrices may be overcome with recent advances in molecular methods for microbial detection, site-specific sample preparation methodologies, and appropriate experimental controls [15–20,28].

3.2. Recent advances enabling environmental application of FCM

3.2.1. Advances in specific detection methods

Recent advances have been made in increasing the specificity of nucleic acid (NA)-based fluorescent probes by using peptide nucleic acids (PNAs). PNA consists of a non-charged polyamide backbone to which the different nucleotide bases are attached, which allows for faster hybridization kinetics and more stable PNA/NA hybrids even at low salt concentrations. PNAs, which can be readily conjugated to a wide range of fluorochromes using standard chemistries, have been successfully used for both enumeration and identification of microorganisms in growth indicator tube cultures and complex environmental or clinical samples [43–47].

Further recent improvements include the use of molecular beacons [48]. A molecular beacon consists of a specific-probe sequence flanked by two complementary sequences, which allow formation of a stem-loop structure when the molecular beacon is free in solution. They can be used to detect specific DNA or RNA sequences in an aqueous solution or in intact cells, eliminating the prerequisite of immobilizing either the target nucleic acid or the probe as required in traditional hybridization assays. The combination of PNA probes with molecular beacons is potentially extremely sensitive and accurate detection and enumeration technique, compatible with flow cytometry [46].

3.2.2. Advances in overcoming environmental interference

Optical interference from particulate matter has previously been minimized with optimum stain selection and application protocols, modifications to sample preparation, and sample-specific calibration of the FCM. The selection of high affinity green nucleic acid dyes and optimization of incubation conditions effectively achieved separation of microbial communities in sediment and soil samples [49–51]. Environmental sample preparation for FCM analysis can involve repeated cell pelleting and resuspension to minimize ma-

trix effects and subsequent addition of surfactant (e.g., Tween-80) to minimize cell aggregation as reported in standard molecular biology protocols. Required sample preparation may also be linked to the characteristics of the target microorganism. Cell wall characteristics might dictate preparation steps such as surfactant (Tween-80) or solvent addition for improved fluorescent labeling efficiency [52]. FCM analysis protocols for microbial detection in environmental samples have included the analysis of control samples to define appropriate sample-specific gating protocols [50]. Site-specific protocols may need to be developed including optimum incubation conditions for each of the selected fluorescent stains, microbial elution/enrichment methods, and appropriate FCM operating parameters.

3.2.3. Advances in flow cytometry

Lastly, the application of cell sorting to microbial ecology and quantification of protozoa and viruses, which are the main causative agents of waterborne pathogenic diseases [53], is still under development [27]. The resolution of flow cytometry in enumerating protozoa is limited mainly because of their environmental concentrations, which require a preconcentration or pre-enrichment phase unless in situ hybridization with specific fluorescent oligonucleotide probes can be implemented such as in multi-color flow cytometric applications [54]. Many difficulties are encountered in the enumeration, infectivity determination and classification of viruses in aquatic samples, but recently a flow cytometric approach using SYBR Green I indicated that promising progress has been made for this group of environmentally active components [55]. An overview of the current state-of-the-art in environmental flow cytometry is presented in Table 3, which shows the applicability in a range of complex environmental matrices

using a number of target analytes. This table clearly shows the promise of this detection platform for both total bacterial enumeration, target pathogen detection, and in some cases even viability/activity assessment, though it should be noted that the main advances have been made in surface water, drinking water, and wastewater-related applications. Each of these matrices offers an opportunity for automation, on-line microbial detection, and distributed microbial sensing applications, whether for process control or better (more accurate, more sensitive) field characterization, using miniaturized FCM platforms.

4. Future prospects: flow cytometers of lesser size and cost

Although conventional flow cytometers have exceptional high-speed analytical and physicochemical characterization capabilities, they are costly, large, and mechanically complex. This prohibits the widespread use of flow cytometry outside of the laboratory for the detection of bioterrorism agents, waterborne pathogens, and other human disease agents. Section 4.1 describes previous research and existing commercial systems which intend to reduce the size and cost of flow cytometers, so as to move such instruments closer to the field. To allow for distributed and widespread quantitative identification of microorganisms in the environment, a technology platform based on the integration of flow cytometry principles with state-of-the-art micromachining approaches, currently under development at the University of Michigan, will be illustrated [46,69,70]. For the prototype microsystem, only the basic functions necessary for microbial detection and quantification have been maintained. As these functions have been miniaturized and integrated in accordance

Table 3
Current environmental applications of flow cytometry and prospects for micro-integrated flow cytometry

Medium	Target	Reference	Prospects for MIFC
Drinking water	<i>Cryptosporidium</i> , <i>Giardia</i> ; human rotaviruses	[19,20]	On-line sensing in distribution systems
Wastewater, sewage, activated sludge	Total and target bacteria; activated sludge viability	[56–59]	On-line detection and quantification; process control
Surface water	<i>Escherichia coli</i> ; viruses; marine bacterioplankton; bacterial production	[50,55,56,60,61]	Microbial surveys, watershed mapping, rapid microbial assessment during sediment resuspension events
Groundwater	<i>Legionella</i> ; <i>Cryptosporidium</i> ; total bacteria	[62–64]	Dynamic watershed mapping of microbial contami- nation; Bioremediation and microbial transport applications; Incorporation with seepage meters for groundwater-surface water characterization
Soils	<i>Flavobacterium</i> total bacteria; viability	[65,66]	Bioremediation monitoring; Evaluation of agricultural practices (e.g., sludge amendments)
Sediments	Total bacterial enumeration; bacterial activity; microalgae	[16,67]	Sediment pore water analysis during site characterization
Metalworking fluids	Total bulk fluid microorganisms; <i>Mycobacterium</i> spp.; viability	[46,68]	Tool-specific applications; Aerosol mist characterization; Evaluation of current microbial management strategies

with recent advances in micro-electro-mechanical systems (MEMS) technology, the instrument is named the Micro Integrated Flow Cytometer (MIFC). Section 4.2 describes on-going research related to the MIFC, which involves the design and characterization of a microfluidic observation channel capable of performing flow cytometry measurements in conjunction with compact and economical solid-state lasers and photodetectors.

4.1. Previous research and existing commercial systems

4.1.1. Commercialized systems

FCM has been the focus of a significant amount of research aimed toward miniaturization. Despite these efforts, the smallest fully functional flow cytometers commercially available (e.g., Geneq Microcyte FCM and Guava PCA) are larger than 0.2 m³, weigh over 4.5 kg, and cost over \$40K. As discussed below, MEMS-based FCM has the potential to bring this cost down by two orders of magnitude. This would render FCM far more useful for applications ranging from bioterrorism to microbial detection in metalworking fluids and drinking water infrastructure.

4.1.2. MEMS-based FCM research: hydrodynamic focusing

Prior to the MIFC, several research efforts had been undertaken to develop microfluidic hydrodynamic focusing channels as a first step toward a MEMS-based flow cytometer. Altendorf et al. [71] etched a V-shaped channel in a silicon wafer using an anisotropic wet etch, and bonded the wafer with a pyrex glass substrate to fabricate a microchannel which could be utilized for flow cytometry [56]. Sobek et al. [72] demonstrated a hexagonal silicon-based flow chamber with a built-in waveguide for optical measurement, which was fabricated by bonding together two anisotropic etched silicon wafers. Miyake et al. [73,74] fabricated a 3-D micromachined flow chamber consisting of three stainless steel (SUS 304, 100 µm thick) plates sealed with two glass covers. Each of these designs featured inherent cost and manufacturing challenges resulting from the materials selected for processing (i.e., silicon, glass, and metal). These materials, and associated designs, also posed difficulties for tight integration of optical components at a scale suitable for microFCM.

4.1.3. Optical detection in FCM

Typically photomultiplier tubes (PMTs), and occasionally avalanche photodiodes (APDs), are used for fluorescence detection in FCM. These detectors are utilized due to their high sensitivity, high internal gain (>10⁶), and fast response (10⁻⁷–10⁻⁹ s) [69,75,76]. However, PMTs are bulky, fragile, consume large amounts of power, and are generally sensitive to environmental conditions. APDs are also somewhat bulky

and require high-power, resulting from their need for active thermal management to maintain high *S/N*. Although both PMT and APD photodetectors yield a large *S/N* due to their internal gain, optical systems incorporating these devices into flow cytometers are costly and difficult to miniaturize.

4.1.4. MEMS-based flow cytometry: need for future research

The discussion above indicates that there were a number of technical issues that needed to be resolved in order to achieve fluorescence detection and quantification in a miniaturized and low-cost sensor (<\$1000) such as the MIFC. First, a microfluidic observation channel had to be developed with: (1) adequate hydrodynamic focusing and (2) integrated optical excitation and fluorescence detection. This observation channel also needed to be designed to minimize channel clogging while maintaining low cost so that the channel could be disposed of if necessary. Second, a method of applying silicon-based PIN photodiodes to detect single-cell fluorescence (in place of PMTs and APDs) had to be developed. Third, a means of self-aligning the optical system, without complicated alignment procedures, needed to be developed. Fourth, a means for integrating multiple colors and channels without significantly increasing cost or size was required. And finally, a detection approach which permits high fidelity measurements and advanced signal processing was necessary. The MIFC discussed in the next section has accomplished each of these tasks.

4.2. Micro-integrated flow cytometer

4.2.1. MIFC observation channel

Soft lithography is a non-photolithographic micro-fabrication technique that is based upon self-assembly and replica molding applied to polydimethyl siloxane (PDMS, a.k.a. silicone rubber). Soft lithography has been used to fabricate various microfluidic devices [69,77–80], because PDMS is inexpensive, easily molded, mechanically robust, disposable, chemically inert, non-toxic, optically transparent (well into the UV), and its surface properties can be easily modified by chemical treatment. All of these material properties are highly desirable for developing miniaturized bio-analysis sensors based on optical detection.

The disposable observation channel for the MIFC was developed using PDMS as illustrated in Fig. 3. Design parameters for the channel such as lengths, angles, and volumes were determined using microfluidic models of the channel that were constructed within the computational fluid dynamics (CFD) software Fluent 5.5 (Fluent Inc., NH). The final dimensions and operating parameters for the channel were selected such that the sample fluid is well focused with a constant velocity

in the center of the channel. CFD modeling and experimental results were found to be in excellent agreement (deviations <5%).

4.2.2. MIFC optical sub-system

As illustrated in Figs. 3 and 4, the observation channel was designed with integrated microgrooves that permit the physical registration of optical fibers that serve as waveguides for laser excitation and fluorescence detection. The optical fiber arrangement is designed to permit simultaneous multi-color excitation of sample particles, at a single interrogation point in the observation channel, using multiple angles of excitation and detection. In addition, by embossing grooves that serve as fiber receptacles directly into the PDMS observation cell, the optical fibers can be fit tightly in the system without separate alignment steps during assembly.

Fig. 4 illustrates the multiple light impingement and collection angles possible in the MIFC. The MIFC can therefore be reconfigured to collect information from any or all angles simultaneously as desired in a given application. The multiple angles can be used: (1) to increase signal-to-noise ratio (S/N) by performing two independent fluorescence measurements simultaneously

and (2) to permit multiple cytometric measurements to occur at a single interrogation point. Such possibilities do not exist in conventional flow cytometers. For instance, it is possible in the MIFC to have two different excitation sources modulated out of phase at 180° (100 kHz) to achieve non-interfering multi-color excitation at a single interrogation point [69].

Experiments with the MIFC have also demonstrated the feasibility of developing a high S/N single-cell fluorescence detection system based exclusively on silicon-based PIN photodiodes coupled with the lock-in amplification technique. While common in other applications, the lock-in amplification approach had not been previously discussed in the literature for flow cytometry (single-cell fluorescence detection). The discovery was important because the lock-in circuit can be fully integrated on a silicon microchip along with PIN photodiodes at a size and cost two orders of magnitude less than PMT or APD photodiode configurations.

Using PIN photodiodes in conjunction with the multi-angle design illustrated in Fig. 4, it has also been proven that the MIFC provides consistent color differentiation that allows the instrument to achieve spectrometer capabilities not possible on a conventional flow

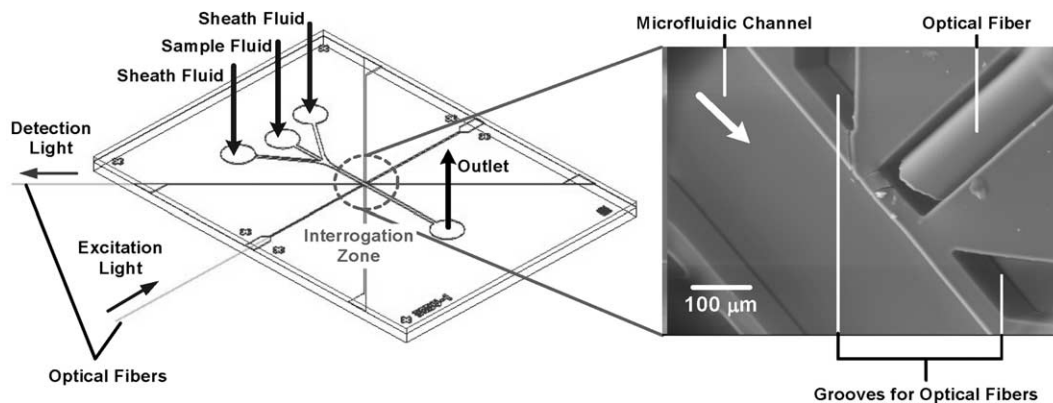


Fig. 3. Schematic of PDMS microfluidic observation channel with integrated optical.

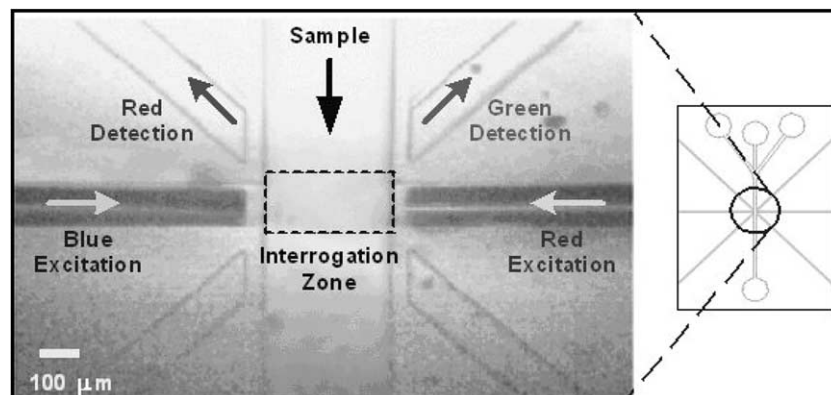


Fig. 4. The arrangement of optical fibers for two-color excitation/Detection in the MIFC.

cytometer. Specifically, experimental results have shown that the relative S/N between various detection angles is sensitive to the wavelength of fluorescence emission of sample particles (data not shown). Consequently, by taking advantage of simultaneous multiple-angle detection, a micro flow cytometer with spectrometer characteristics is possible. This occurs due to a highly wavelength-dependent optical path bending process. Based on these results, it will be possible to incorporate basic spectrometry capabilities into the MIFC at low cost.

4.3. Current status of MIFC

The ultimate goal for the MIFC is to reduce the size and cost of flow cytometry technology applied for basic environmental field analysis by two orders of magnitude. The main challenges for this technology require research advances in the areas of automated microbial sample preparation, refining the optical system, and data analysis as they relate to the development of the MIFC. The technology development is currently in the pre-prototype stages, and the impact of miniaturization is being validated using pure cultures of bacteria and protozoa as target screening organisms. Optical detection of the former is realized using general DNA stains, and fluorochrome-labeled antibodies and PNA molecular beacons [46,70].

5. Concluding remarks

Direct optical detection methods such as flow cytometry (FCM), which employ fluorescent stains specific to biological molecules (e.g., DNA stains), are being employed for microbial characterization due to increasing awareness of problems associated with traditional growth-dependent analyses. FCM is advantageous because samples stained with multiple fluorochromes (e.g., non-specific and viability) undergo subsequent multiparametric data acquisition and analysis. This allows for the rapid assessment of microbial heterogeneity and for understanding the functional differences revealed by each stain in practical applications [12,13,27].

Miniaturized, low-cost flow cytometers are being developed for in-situ applications toward the development of distributed microbial sensing (DMS) networks. This application places a number of constraints and demands on microbial sensing technology: low cost, large span, low maintenance, automated, compact, fast, versatile, artificially intelligent, and networkable. The distributed networks must be appropriately established in terms of maximal coverage with minimal cost. Given technology on the near-term horizon, meeting these constraints will require a tiered detection approach in-

volving a rapid, non-specific screening which would trigger a more expensive process that involves specific target identification.

Specific applications that could benefit from DMS include ecological and metabolic mapping to help select aquifer or sediment bioremediation units, and to assess water quality parameters for reclamation and reuse. Despite the prevalence of microbial and chemical indicators suggesting that natural bioattenuation of contaminants is abundant in the subsurface, these laboratory-based causal relationships cannot be easily transferred to the field, due to the laborious procedures involved in (phenotypical and functional) microbial characterization. DMS may provide a quantitative interpretation of target microbial responses to natural or engineered perturbations in the subsurface geochemistry.

The convergence of recent advances in the development of miniaturized sensors, data acquisition systems, and communication technologies is the driver toward the development of elaborate sensor networks. These products of interdisciplinary efforts may be integrated into complex environments under hostile conditions and into existing engineering infrastructure. These networks will be based upon sensor technology, such as the MIFC, that is low cost, automated, compact, fast, and versatile. Sensor development must incorporate networkability as well as adaptability to projected advances in adjacent technologies. Modern data acquisition and communication systems will eliminate the impediments of time and distance by providing rapid access to information, which will be interpreted for risk-based decision-making [7].

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