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REVIEW ARTICLE

High-throughput Fluorescence Detections in Microfluidic Systems

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High-throughput detection is always attractive for researchers in developing sensing techniques. Recently, MEMS techniques have become well-established to produce miniaturized microfluidic devices with low-cost and small size. However, large-scale conventional off-chip detection approaches may limit the throughput of the detection systems such that the benefits come with the miniaturization are hindered. This review gives a brief overview for the development of high-throughput detection techniques in microfluidic systems, especially for the major optically fluorescence detections. Fundamental fluorescence techniques and two microfluidic systems including the micro capillary electrophoresis and the micro flow cytometer are introduced in this review. Various approaches for developing high-throughput and parallel fluorescence detection in microfluidic system are reviewed. A state-of-the-art diascopic illumination system for multispectral analysis in microfluidic systems is also included. Even though great progress for high-throughput bio-analysis has been made during the past decades, there is plenty of room for further improving the performance of existing detection techniques.

Key Words: diascopic illumination; fluorescence technique; high-throughput detection; micro capillary electrophoresis; micro flow cytometry

Background

MEMS (micro-electro-mechanical-system) technology has introduced many interesting possibilities for physical, chemical and biomedical applications in the past decades.[1] A number of advantages came with miniaturizing the bio-analytical chip devices including less sample and reagent consumption, higher spatial resolution, faster detection speed, lower driving power and better portability. Integrated microsystems for chemical and biological analysis, also known as the Micro total-analysis-systems (μ-TAS), has attracted a lot of attention for researchers in the bio-analytical fields.[2–4] Therefore, much literature regarding the electrical, optical and chemical detection techniques within microfluidic channels has been reported. Over the developed detection methods, optical detection approach, especially for the fluorescence techniques, is the most promising for detecting bio-samples in microfluidic channels because this method has the advantages of low detection limit, high stability, good spatial resolution and excellent discrimination capability. Typically, the fluorescent analysis is a crucial technique applied to bio-analytical applications for detecting and diagnosing DNA, RNA, peptide, protein, organelle and even living cells. However, delicate optical components as well as a relatively

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large space are required for building an optical detection system for bio-analytical applications. The benefits that came with the miniaturization may be hindered with these drawbacks. In this regard, there is an emerging need to develop an optical detection technique using fewer optical components but which has benefits for bio-detection.

Laser-induced-fluorescence (LIF) detection technique is the most popular method for detecting fluorescence-labeled bio-samples in imaging, capillary electrophoresis (CE), and flow cytometer systems. Usually, specific filter sets are required to block the incident excitation light, retaining the emitted fluorescent light from fluorescent probes to transmit. However, the selection of the filter sets is usually constrained by the selected fluorescent probes, which have specific excitation and emission spectra. In the conventional microfluidic system, detection of a single fluorescence is an established technique in capillary electrophoresis and cytometry applications. However, the throughput of the conventional fluorescence detection cannot meet the requirements for modern bio-analysis applications. Thus, there is a great need for high-throughput fluorescence detection that simplifies the control system and reduces the number of optical components. Multicolor labeling/detection approach uses two or more probes to simultaneously monitor various biochemical signals. This technique has been wildly used in application including fluorescence microscopy,[5,6] fluorescence *in situ* hybridization,[7,8] DNA sequencing[9] and flow cytometry.[10] In order to simultaneously observe various fluorophores in a single device, multiple filter sets are usually required to exclude excitation and scattered light from bio-sample detection, leaving only emitted fluorescence. Nevertheless, a multicolor detection system is usually bulky, expensive and needs a specialist to operate. Thanks to the rapid development of optic technologies, various multicolor detection schemes have been developed.

In this review, fluorescence techniques including stoke shift, light filtering and fluorescence excitation are briefly introduced. Two major microfluidic components of the micro capillary electrophoresis and the micro flow cytometer are adopted to demonstrate the high-throughput fluorescence detections in microfluidic systems. Finally, a novel diascopic illumination configuration is presented for parallel detecting fluorescence samples in a low-cost and rapid approach.

Fluorescence Techniques

Fluorescence is a physical property whereby special atoms or molecules absorb lights at a particular

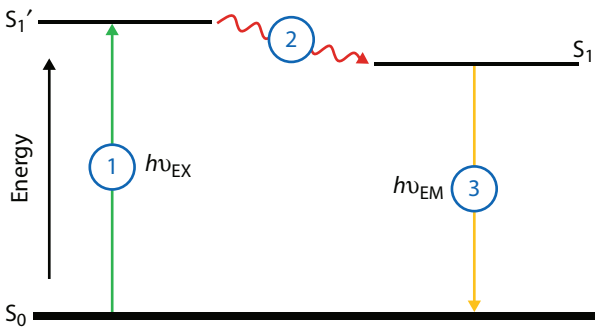

Figure 1 Jablonski diagram illustrating the process involved in the creation of an excited electronic singlet state by optical absorption and subsequent emission of fluorescence molecules.

wavelength and then subsequently emit lights with longer wavelength. The fluorescence process is governed by three important stages, excitation, excited-state lifetime, and fluorescence emission. The time interval from fluorescence excitation to emission is approximately 10^{-9} seconds such that fluorescence is a nice indicator for real-time observations. Figure 1 shows a simple electronic-state diagram corresponding to the fluorescence process (known as the Jablonksi diagram).[11,12] In the excitation stage, a photon of energy is introduced and then absorbed by the fluorophore. In general, an external energy source such as the incandescent lamp, UV light or laser is usually adopted for this application. The vibrational relaxation pushes the excited fluorophore to a lower energy level (S_1^l) in picoseconds. In the excited-state lifetime stage, the excited molecule stays in the lowest excited singlet state (S_1^l) for nanoseconds before finally relaxing to its initial state. Meanwhile, the fluorophore undergoes conformational changes and the releases its energy to other molecules or local environments. The last stage for the fluorescence process is called the fluorescence emission stage. A photon of energy is emitted, returning the fluorophore to its ground state S_0. Due to energy dissipation during the excited-state lifetime, the energy of this photon is even lower. Therefore, an emitted light exhibits a longer wavelength than the excitation photon energy ($h\nu_{EX}$) originates. The energy difference or the wavelength shift represented by ($h\nu_{EX} - h\nu_{EM}$) is called the Stokes shift. The value of Stokes shift is critical for fluorescent measurements because this value presents how good the emitted signals can be isolated from excitation lights.

The fluorescence process is repeated excitement and emission with the existence of the excitation sources until the fluorescent molecule is irreversibly destroyed in the excited state (known as photobleaching). The fact is that a single fluorophore can repeatedly generate detectable emitted photons

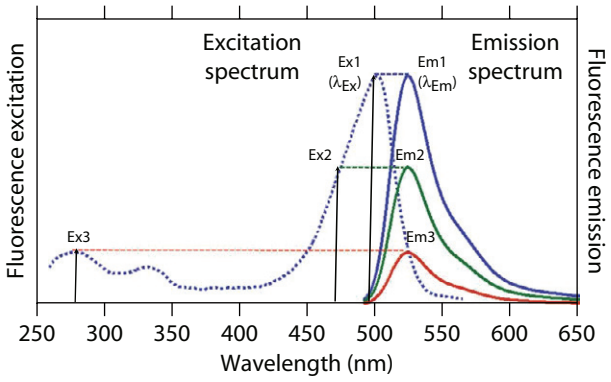

Figure 2 Excitation of a fluorophore (2′,7′-dichorofluorescesin) at various wavelengths (Ex1, Ex2, Ex3) does not influence the emission wavelength profiles but the emission intensities (Em1, Em2, Em3).

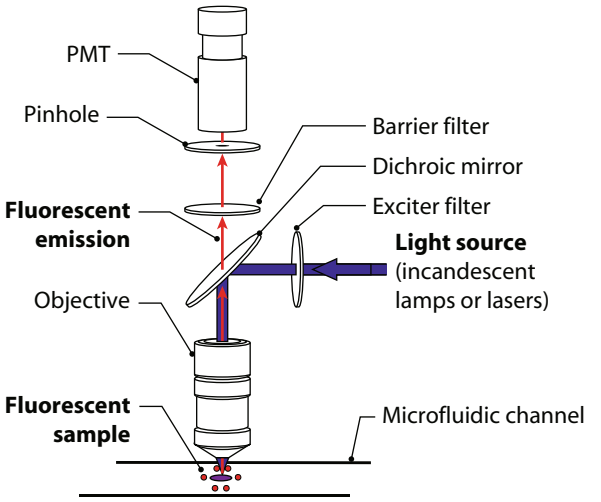

Figure 3 Basic epi-fluorescence configuration for high sensitivity detection in microfluidic systems.

thousands of times. The discrete electronic transitions of and in Figure 1 are usually presented in broad energy spectra called the fluorescence excitation spectrum (or called absorption spectrum) and fluorescence emission spectrum, respectively. As shown in Figure 2, the bandwidths of these emission spectra are important factors that determines two or more different fluorophores can be simultaneously detected or not. In addition, the emission intensity is proportional to the amplitude of the effective excitation wavelength.

In order to excite and detect the fluorescence without interfering the excitation and emission spectrum of the fluorescein, optical spatial filters are essential for fluorescence detections. The wavelength filter is of critical importance since it significantly reduces the intensity of the excitation light. The less intensity of the excitation light reaches the detector, the weaker emitted fluorescence intensity can be detected. This is challenging since the intensity of the excitation light is typically higher than the intensity of the fluorescent signal in several orders. In general, a filter set for fluorescence detection is composed of three optical filters of an excitation filter, a dichroic mirror and an emission filter. Recent progresses in light filtering technology have yielded several sophisticated devices that feature superior performance compared to the conventional absorption or interference filters. Both liquid crystal and acousto-optical devices have been developed to execute wavelength-selection by an electrical control system, called a tunable filter. A typical wavelength-selective liquid crystal tunable filter is constructed by stacking various filters consist of liquid crystal layers and linear polarizer.[13,14] Alternatively, an acousto-optical tunable filter composed of a crystal was also reported for filtering lights with different wavelength. In that approach, radio frequency acoustic waves are used to separate the single wavelength light out of a broadband light source.[15,16] Various applications including spectral image, multi-color and multi-fluorescent detections can be achieved with these filtering techniques.

Fluorescence Detection in Microfluidic Systems

To achieve high-resolution fluorescent detection in a microfluidic system, a filter combination including an exciter filter, a dichroic mirror and a barrier filter is adopted to select specific wavelengths in a typical epi-fluorescence microscopy. Figure 3 presents the basic setup for an epi-fluorescence detection scheme. Exciter filters permit only selected wavelengths from the illuminator (incandescent lamps or lasers) to pass through the filter toward the specimen. Barrier filters are filters which are designed to suppress or block the excitation wavelengths and permit only selected emission wavelengths to enter the detector. Dichroic mirrors are specialized filters which are designed to efficiently reflect excitation wavelengths and pass emission wavelengths. Figure 4 shows the transmission spectra of each filter for an epi-fluorescence microscope. LIF technique is the most preferred detection method for microfluidic systems because of its high sensitivity. Carefully adjusting the optical system to optimize various parameters such as pinhole size, channel depth, excitation efficiency and laser spot size for this epi-fluorescence system is capable of achieving the detectable concentration at as low as 300 fM fluorescein.[17] A detection limit of 9 pM of Cy-5 was reported using the same setup but a diode laser as the excitation light source,

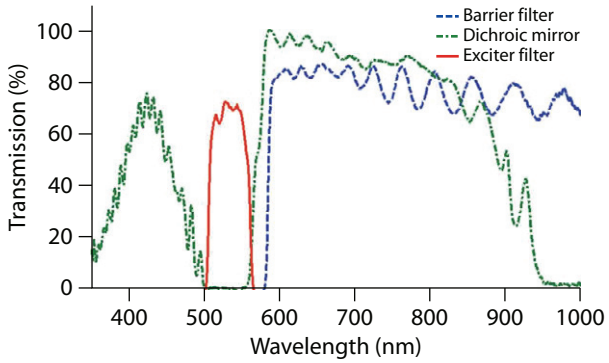

Figure 4 The transmission profiles for a typical fluorescence filter combination (G-2A, Nikon, Japan) used in epi-fluorescence configuration. The solid line (red) corresponds to the exciter filter, the center line (green) is the dichroic mirror and the dashed line (blue) is the barrier filter.

corresponding to the detectable concentration of 900 molecules in 1.6 pL of sample volume.[18] The ultrasensitive LIF scheme was also applied to other studies.[19,20] For achieving single molecule detection in fluorescence and bio-samples.

Recently, micro-CE chips fabricated using micromachining techniques have brought a number of advantages including higher separation efficiency, miniaturization, less sample and reagent consumption, and a higher detection limit. Fluorescence detection is a common scheme used in CE system for detecting samples with native fluorescence or modified with fluorescent probes. This detection scheme offers high sensitivity and good selectivity for analyzing the labeled samples, especially for using laser as the excitation light source. In this regard, LIF detection scheme has been considered as one of the best optical systems for CE detection. It has been used in CE systems with detection limits as low as $10^{-19}-10^{-20}$ M. The sensitivity of the technique is attributed to the high intensity of the excitation light and the ability to accurately focus the light in the capillary tube.[21]

Alternatively, micro flow cytometry is a powerful tool in biological research and clinical diagnostics. This microfluidic system is adopted to interrogate individual particles or cells with various physical or biochemical parameters in a rapid and quantitative way. Over the detection principle for this system, optical detection is the most common and well-established method which is capable of interrogating, detecting, classifying, and identifying the cells or particles in large-scale systems. Flow cytometers detect the light-scattering and florescence emitted from the particles/cells such that the optical system is more complicated when compared with the microfluidic CE systems. Scattered light exhibits strong dependence on size, geometry, surface characteristics and also on the effective refractive index of the particles/cells. Fluorescence criteria provide an invasive technique for analyzing cells labeled with various fluorescent markers. There are two detection schemes adopted in a flow cytometry system, including the forward scatter (FSC) approach and the side scatter (SSC) approach. The FSC is to detect light scattered within a few degrees (usually less than 10°) near the direction of the incident beam. The information obtained from FSC measurement is related to the size and the shape of passing objects. FSC has been used to detect airborne droplets[22,23] and polystyrene microspheres in flow cytometry.[24] Furthermore, Venkatapathi et al[25,26] demonstrated an alternative angular light scatter model which used statistical approach to detect transparent biological cells in a microfluidic device. On the other hand, SSC detection scheme measures the lights deflected from large angle directions (usually at 90° direction). The information from SSC corresponds to the nonhomogeneities inside the cells or to the surface characteristics of a cell. In general, FSC configuration is much more promising for integrating with microfluidic chips than SSC configuration, since the light information for FSC scheme is more easily detected than the SSC scheme is.

Multiple Bio-samples Detection in Microfluidic Systems

Biological detections often require the labeling of bio-samples with multiple fluorophores for correlation analysis to the properties of the labeled objects. However, it is difficult to find fluorescent dyes that have similar excitation wavelength but have separated emission spectra. It is impossible to quantify multiple fluorescence parameters using a single excitation (wavelength) source. Therefore, the methods for simultaneously detecting multiple lanes, multiple wavelengths and multiple sensing parameters were developed to realize multiple biodetections and increase the throughput of biosensing. High-throughput optical detections for micro CE and micro flow cytometer are described below.

High-throughput detection in micro CE systems

In general, the conventional slab gel electrophoresis is a kind of high throughput detection since various bio-samples are simultaneously separated and analyzed in their corresponding lanes within a single test run. The detection for slab gel electrophoresis is quite parallel. However, slab gel electrophoresis systems exhibit various drawbacks including smearing,

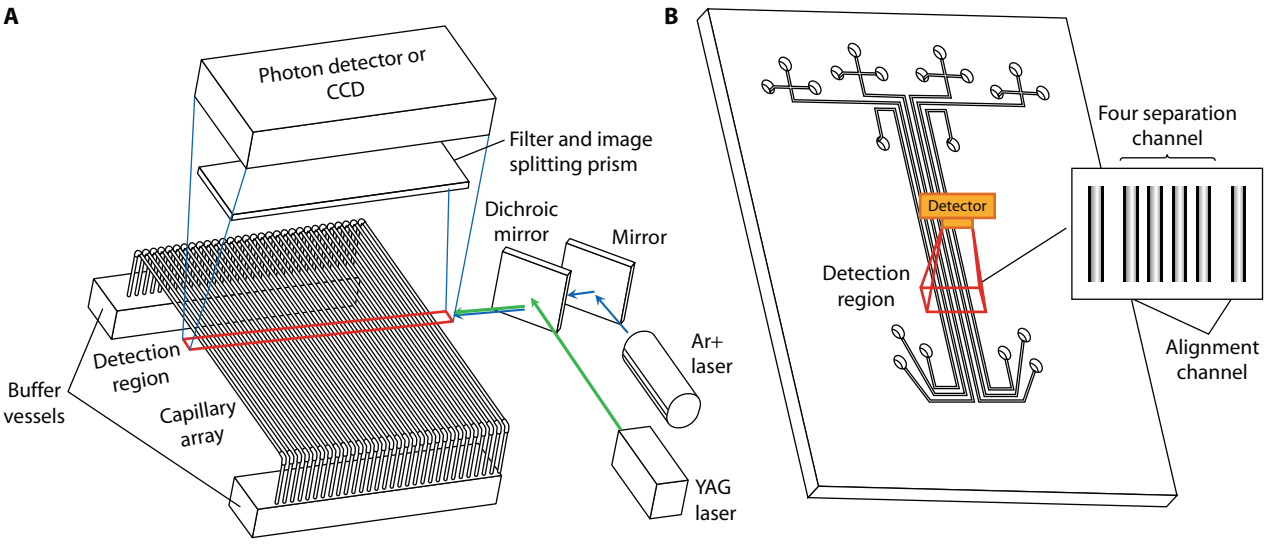

Figure 5 The different multplexed or multchannel detection schemes: (A) schematic view of the multicolor detecting capillary-array system; (B) four-channel microchip design with two alignment channels.

long separation time and low plate number. In contrast, capillary electrophoresis systems have advantages that slab gel electrophoresis does not have. Yet it is difficult to achieve this high-throughput experimental apparatus in a standard CE system with a single separation channel. Therefore, a cyclic injection/separation procedure is the most commonly adopted method to realize the multiple samples analysis in a CE system.[27] The repeatability with regard to the sample migration time of a conventional CE system could be also problematic while determining samples with similar CE properties.[28,29] The sensing variations from multiple testing runs must be well controlled to obtain satisfactory CE test results. However, this work is challenging for practical operations. In this regards, there is an emerging need to develop a high-throughput CE system with a parallel detection capability for simultaneously detecting multiple samples in a single run. To realize this goal, multi-lanes CE systems[30] and multi-wavelengths detection[31,32] approaches have been reported in recent years.

A multi-lanes or a multiplexed CE system (Figure 5) is mainly composed of a parallel capillary tube array that can perform parallel detection similar to slab-gel electrophoresis in a miniaturized system. A laser beam was aligned transversely to the parallel capillary array in order to simultaneously excite the fluorescent labeled analytes in the channels. The emitted fluorescence was then filtered with a spatial filter set and detected using a cooled charged-coupled device (CCD) system.[33–35] These methods improve the throughput significantly in comparison with the conventional single-tube CE system. Furthermore, this approach partly eliminates the repeatability problem mentioned above. However, since the incident laser beam has to pass through all the capillaries, the attenuating light intensity of the laser beam in each capillary tube is an issue for quantitative measurements. The alignment of the capillary array with the laser beam is also delicate and the laser reflection from the capillary wall may generate considerable cross-talk noises. Alternatively, a fast-scanning laser beam and a planar laser beam generated using concave and cylindrical lenses were adopted to excite the samples in parallel channels from the out-of-plane direction such that the time required for laser alignment could be reduced.[36,37] In addition, a microfluidic chip configuration was used to further miniaturize the multiplex CE system. These detection schemes provided an effective method to increase the throughput and also reduce the drawbacks of a single-lane CE system. Nevertheless, a complex electric circuit to drive the samples in individual channels and a delicate laser scanning system are required to achieve this high-throughput or multiple bio-samples detection. Furthermore, the variations of the operating conditions in these individual channels, including the buffer matrix, temperature distribution, and the surface properties of the channel wall, may also influence the detection results.

In order to overcome the inherent drawbacks of the multi-lanes CE system and to achieve a high-throughput CE system, single channel detections with a multi-wavelength illumination for multiple samples excitation have been developed (Figure 6). Various fluorescence-labeled samples in a single channel were excited using light sources with different wavelengths. The emitted light was filtered with the corresponding filter set and finally detected using several PMT modules. For example, Iyer et al[38]

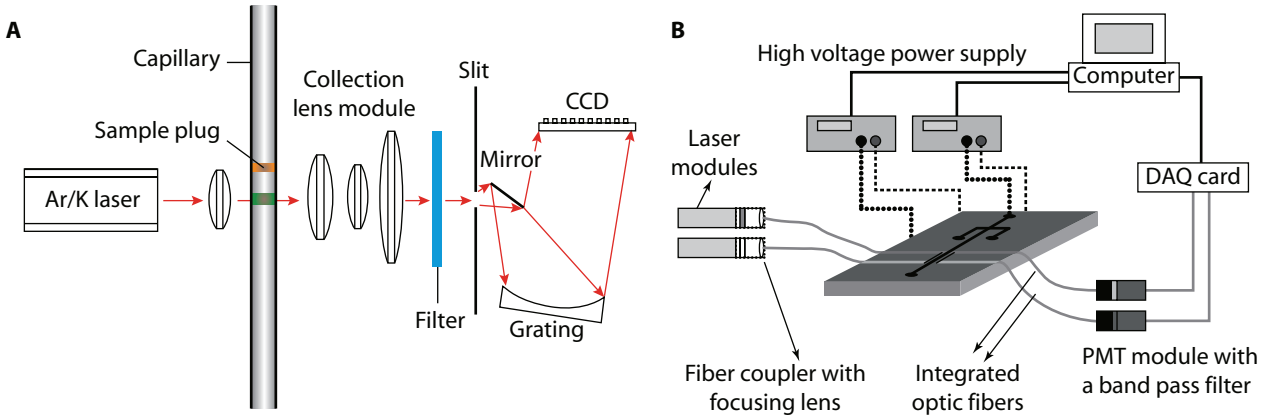

Figure 6 (A) Schematic of the wavelength-resolved laser-induced fluorescence detection system. (B) Schematic representation of experimental setup.[39] Note that detection light sources with different wavelengths are transmitted through the two paired buried optic fibers.

used two different wavelength lasers, argon ion laser (488 nm) and Nd:YAG diode pumped laser (532 nm), to excite fluorescein isothiocyanate and Rhodamine fluorescence, respectively. Two PMT (photo multiplier tube) modules with corresponding interference filter sets were used as the detector for collecting the emitted fluorescence.[38] Alternatively, a miniaturized two-color CE system fabricated with two-pairs of parallel embedded optic fibers was reported for high throughput bio-analytical detection. The excitation and emission lights were guided into/out of the microchip device via the corresponding optic fibers.[39] Although these configurations, which use multi-laser modulated excitation, photon detectors, and filter sets, provide a reliable and straight forward solution concept for increasing the throughput in the CE system, the cost of this system will increase with the increase in the number of analytes tagged with different fluorescent species. Instead of using several optical detectors for simultaneously monitoring multiple wavelengths, a cooled CCD equipped with a spectrograph or a grating was adopted for simultaneous multiple wavelength measurements.[40–44] This detection scheme was commonly referred to as "wavelength-resolved" detection, which can provide the information-rich spectra, such as migration speed, absorption spectrum, and emission spectrum for each fluorescently-labeled sample. However, these systems also rely on several filter sets to select the light of the target wavelength. To overcome this drawback, James et al[45] proposed a multicolor detection scheme based on an acousto-optic tunable filter and a PMT module to simplify the optical system configuration. Nevertheless, the wavelength resolution of this system is lower than that of the spectrograph system. In addition, multi-color detection with a novel frequency modulation of the excitation lights with different wavelengths was also developed. Color separation based on Fourier analysis was used to mathematically filter the nonmodulated noise, leaving the modulated laser-induced signals.[46]

Multi-color detection in micro flow cytometers

Flow cytometry has a wide range of applications, from immunophenotyping,[47,48] to gene diagnosis,[49,50] to bacteria analysis[51–53] and clinical hematology diagnosis.[54–56] Typically, these applications often require the labeling of cells with multiple fluorophores for correlated analysis of cellular characteristics and properties. Early cytometer systems only measured three parameters such as one fluorescence signal and two scattered-light signals (FSC and SSC) using a monochromatic laser source such that the throughput of conventional detection schemes is relatively slow. Therefore, it is essential to develop flow cytometer systems capable of multi-parameter measurement for cell identification and for cell functional and biochemical studies.

To realize this goal, researchers have developed discrete polychromatic detection systems[12] and spectral analysis system[57] in recent decades. For example, a planar flow-cytometer chip integrated with embedded parallel optical fibers[58] or waveguides[59] on glass and polymer substrates has been reported for detecting FSC. In chip-based SSC detections, the orientation must be orthogonal to the incident beam using an out-of-plane configuration for collecting signals.[60] However, only the intensities of SSC are used for data acquisition in most cytometer applications. Intrigued by this issue, Singh et al[61–63] developed an integrated microfluidic planar optical waveguide system for measuring 2D side-scatter patterns from single particles/cells. The ability to obtain and analyze the 2D side-scatter patterns can provide rich information and is potentially important

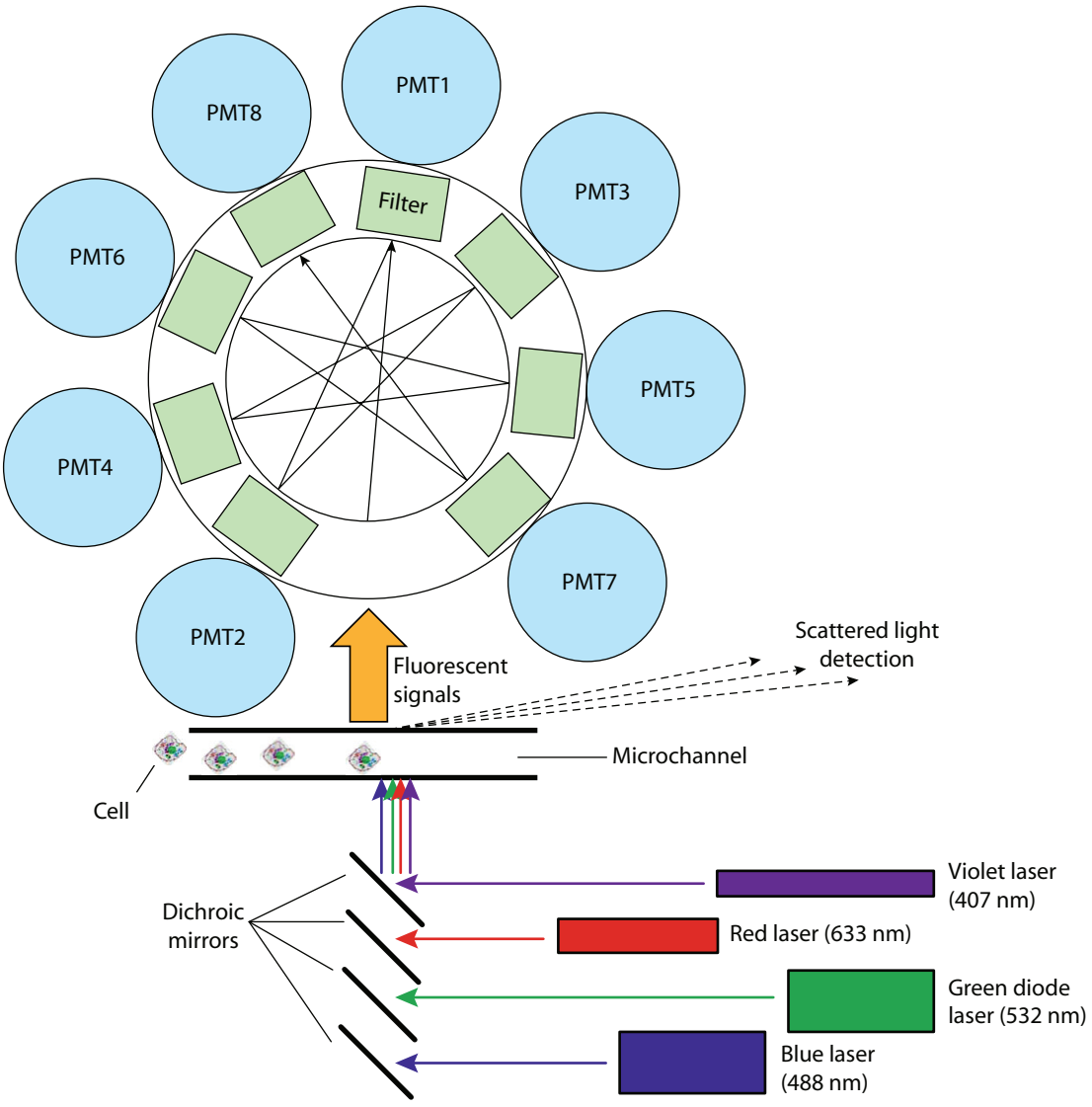

Figure 7 A delicate flow cytometer system for parallel detecting 19 optical parameters. A graphical representation of this assembly showing the light paths of the designed system. The instrument was equipped with three diode-pumped solid-state lasers.

for cellular analysis. Although light scattering detection can provide information on scattering-related properties, such as size and geometry, it is nonspecific for classifying cells and particles with close geometrical properties. Fluorescence provides another detection parameter for specific cell targets labeled with different fluorescent probes. A single flow instrument that employed multiple illumination regions which are supplied from lasers to excite and measure fluorescence, light scatter, and absorption has previously been reported for cell or particle classification.[64,65]

In order to discriminate functionally distinct subsets of heterogeneous cells such as leukocytes with different anti-expression, it is beneficial for multiparametric analysis to detect more colors of fluorescence signals. Several light sources, filter sets and photon detectors were used for this purpose in past research.[10,53,65–67] For example, Stiphen et al[10] utilized four laser sources, eight PMT modules and several filter sets to distinguish multiple fluorescence with different colors corresponding to the excitation light. The experimental setup for this detection scheme is shown in Figure 7. The detection method can simultaneously obtain 19 parameters for detecting multiple fluorescence and quantum dots (QDs) labeled samples. Alternatively, instead of the bench-top cytometer system, similar detection schemes have been realized using several optical fiber couples buried in micro-flow cytometer chips for transmitting the excitation light and collecting the fluorescent signals.[52,68–70] For example, Golden et al[68] used two fiber couples, two laser modules, two band-pass filters and one low-pass filter

to create a four parametric detection on a microflow cytometer chip, including three fluorescence signals and one scatter signal. All of these configurations are very efficient in fluorescent detection for the multi-parametric approach. However, delicate optical components, various optical filters and photo-detectors such as PMTs make these systems relatively bulky and expensive when increasing the detection parameters. In addition, the fluorescent signal cannot be infinitely distinguished by the corresponding narrow-band pass filters due to the spectral overlapping between the various fluorescent probes. Therefore, spectral detection is another solution for detecting multiple fluorescent signals. The spectra can sufficiently provide rich information for identifying and discriminating the fluorescence probes of different colors. In addition, the fluorescent signal cannot be infinitely distinct by the corresponding narrow-band pass filters due to the spectral overlapping between all of the fluorophores with different colors. Therefore, the spectral detection concept is an alternative solution for detecting the fluorescent signals. The rich-information spectra can sufficiently provide the various parameters for discriminating and identifying the fluorescence.

In spectral detection schemes, photo detectors (i.e., CCD or PMT) equipped with grating[71,72] or tunable filters[57,73] instead of multiple photo detectors were adopted to achieve wavelength measurements for the multi-color approach. For example, Goddard et al[74] utilized a diffraction grating to disperse the collected fluorescence from cells or microspheres passing through the interrogation region and a CCD image sensor to analyze the spectral distribution. For detecting a broad fluorescence spectra and simplifying the instrument setup, spectral detection schemes usually adopted a single monochromatic light source, such as an argon laser at UV wavelength (488 nm), to excite the fluorescence-labeled samples. However, the available fluorescent probes used in these detection schemes are constrained due to the wavelength limitation of the monochromic excitation. Another labeling method, QDs labeling[75,76] was reported to replace the conventional organic fluorophores for the purpose of overcoming the monochromatic excitation issue in spectral detection because all the QDs reagents have the same excitation region at UV. Alternatively, Watson et al[77] adopted Raman spectral detection instead of a spectrograph and CCD detector to increase the multi-parameter analysis capabilities of the flow cytometer. Raman flow cytometry opened up another solution for multi-parameter and multiplexed measurements of cells and particles using a single detector and light source. Although these configurations provide an effective measurement for increasing the detection parameters in a cytometer system, the systems also rely on using a more complicated sample treatment as a labeling technique than conventional organic fluorescent probes.

Dark-field Illumination for High-throughput Detection

Dark-field illumination in microscopy is used to detect the scattered light in order to monitor sample contour or detailed structure of particles (Figure 8). The direct light (zero-order light) is excluded from the formation of the image by illuminating the specimen with a hollow cone of light of such obliquity that all the light falls outside the acceptance angle of the objectives. In general, dark-field illumination can be created an opaque stop on light pathways through a simple refractive condenser.[78,79] This approach is easy to achieve and the diameter of the opaque disk can be adjusted in order to obtain suitable inner numerical aperture in dark-field detection. In advanced techniques of dark-field formation, a specularly reflective condenser, such as parabolic or cardioid reflecting surface, replaces a refractive condenser to achieve high numerical aperture and to reduce the chromatic aberration in dark-field illumination. In order to retain the most illumination from the light source, axicon lenses have been used to create dark-field illumination rather than the opaque stop in this approach.[80–82] All of these mentioned dark-field configurations can create a hollow-cone illumination for revealing edges and boundaries of samples by scattered light. An alternative scheme also obtains this effect by using the

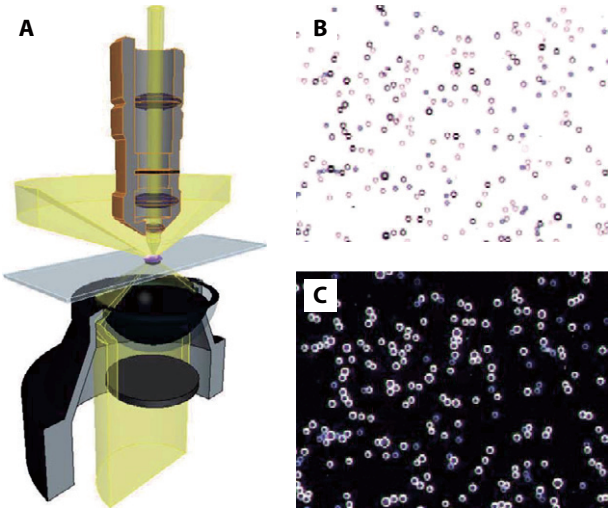

Figure 8 (A) The illustration of a typical dark-field illumination scheme. Images captured using the (B) bright-field and (C) dark-field illumination schemes. Note that the sample was composed of micro-beads labeled with different fluorescence.

polarization technique to detect only the edge-diffracted wave.[83,84] The principle, however, is the same as in all dark-field configurations in the sense of forming an image where no direct light is present.

In previous investigations, dark-field techniques have provided a solution to detect the motion of particles near a surface for monitoring a variety of physical, chemical, and biological process.[85] In biological applications, it is used in tracking the motion of much smaller particles attached to a molecule by detecting light scattering from microbeads or nanoparticles.[86,87] Because a major drawback of fluorescence labeling for monitoring bio-samples is the limited observation time due to photobleaching, the micrometer-sized beads with nanometer precision offer greater photostability and no blinking. The size of such beads, however, might influence the behavior of the attached biomolecules and limit the time resolution due to their viscous drag. Therefore, metal nanoparticles offer a labeling technique as an alternative to fluorescent dyes or quantum dots. Metal nanoparticles show strong light scattering at the plasmon resonance wavelength due to the collective oscillation of their conduction electrons. This light scattering is orders of magnitude greater than that of a nonmetallic object of the same size. For this reason, previous research[88–90] has developed dark-field technique combined with a spectroscopy image for detecting and measuring the surface plasmon effect in metal nanoparticles.

In dark-field applications, only a few studies mention the capacity for fluorescence detection and excitation.[81] Roulet et al[91–93] developed a similar method of dark-field technique that integrated microfluidic and micro-optical elements for fluorescence detection. These integrated systems focus an excitation beam into the detection volume (μL or even sub-μL scale) and collect the emitted light from fluorescent molecules. These studies present the ray tracing simulation, fabrication, and measurement of different illumination systems (Figure 9). Although their measurements show that an adroit placement and combination of microfabricated lenses and stops can increase the separation between the excitation light and fluorescence light, the curvature of the microlens and stop positions were not optimized to achieve a practical measurement for biological application. Recently, the current author reported a novel method regarding a wavelength-resolved fluorescence detection scheme for high-throughput analysis of bio-samples in a micro-CE chip. Figure 10 presents the schematic setup for the system. Instead of using the conventional LIF microscope equipped with delicate spatial filters and complex control systems, this study adopts a hollow cone illumination generated using a dark-field condenser for exciting fluorescence in the microchannel and an UV-VIS-NIR spectrometer for detecting the emission signals. The proposed system is simple and economical since no sophisticated optical filter sets and laser sources are required for the detection purpose. However, the detection of the developed system is limited by the value of numerical aperture which comes with the commercial dark-field condenser. In order to enhance the detection performance of the diascopic system, the same group adapted an objective with a high numerical aperture value and stop-films with various patterns to create an objective-type dark-field condenser to replace commercial condensers.[94] That method created the excitation spot of smaller size for reducing the background noise caused by the excited light. This study optimizes the performance of this configuration to the point where it has a higher

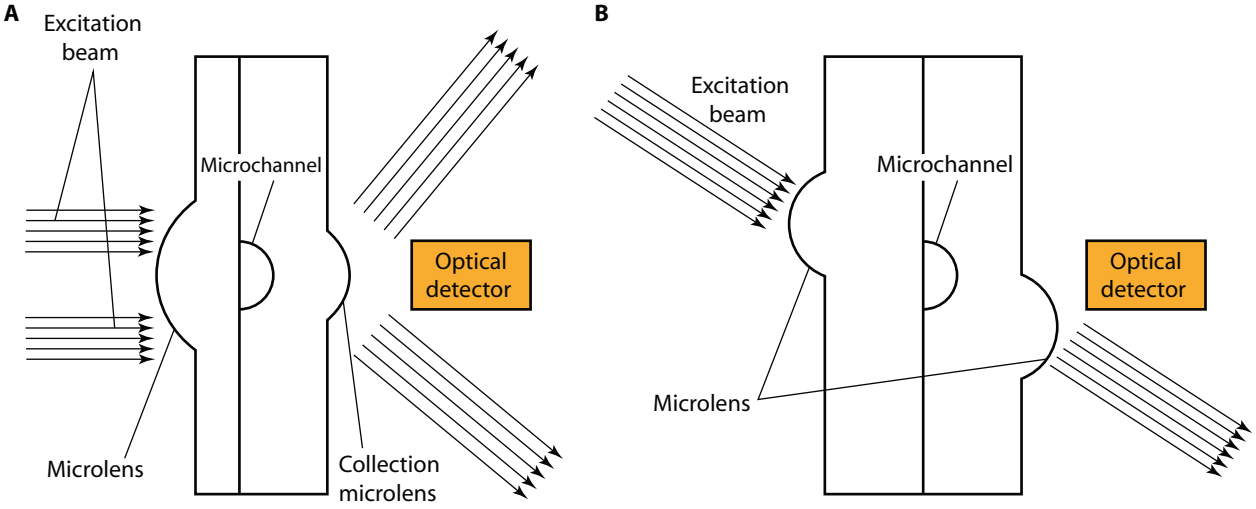

Figure 9 Two-dimensional ray-trace simulations of (A) beam-splitting illumination with two layers of microlenses, (B) an off-axis illumination. Both are based on dark-field principle for fluorescence detection.

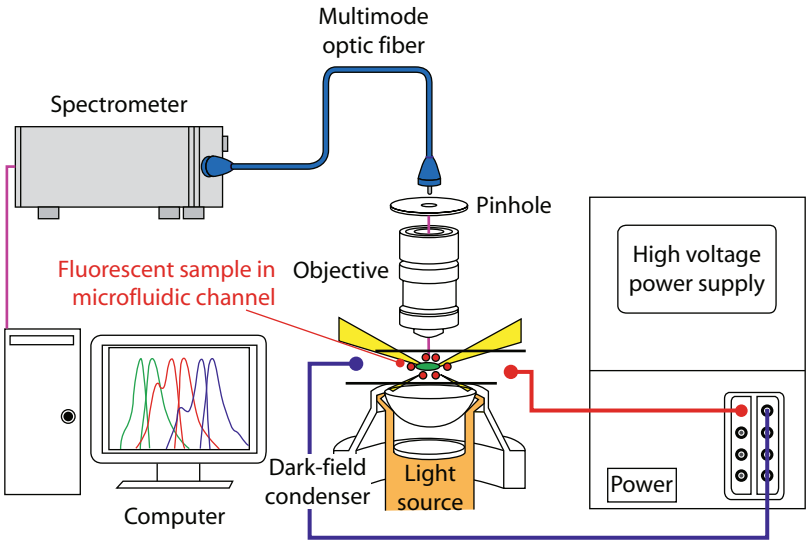

Figure 10 A schematic presentation of the experimental setup for the multi-wavelength fluorescence detection scheme for high-throughput microfluidic applications.[96]

sensitivity than the commercial dark-field effect. The sensitivity performance of the objective-type dark-field system is more than 100-fold greater than the system using a commercial dark-field condenser. Recently, similar optical configuration has been successfully used for high-throughput microfluidic cytometer.[95] A mixed sample composed of four various kind of label and nonlabeled particles was successfully discriminated with the system.

Conclusions

Increasing the detection throughput for microfluidic systems is always challenging and interesting especially for microfluidic systems. The limited chip space and small channel dimension make the integration of various optical components difficult. Thanks to the rapid development of modern optics, a number of high performance optical components such as tunable lasers, tunable filters, ultra-high numerical aperture objectives and many other advanced microscope systems have been commercialized. With the efforts of the researchers in multidiscipline fields, microfluidics systems have the potential to transform high-throughput biochemical analysis in the way like the progress in semiconductor industry, making detection systems that are smaller, more integrated, less expensive, and a lot faster in the near future.

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