Single-Molecule Detection and Probe Strategies for Rapid and Ultrasensitive Genomic Detection

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Abstract: This paper reviews the current state-of-the-art development of single-molecule detection (SMD)-based methods for ultrasensitive and specific analysis of genomic sequences. We first discuss several newly devised single fluorescent probe strategies that allow separation-free detection of low-abundance DNA sequences, such as quantum dot (QD)-mediated fluorescence resonance energy transfer (FRET) technology and dual-color fluorescence coincidence and colocalization analysis. Various schemes toward single DNA sizing and sequencing in solutions or on surfaces are also reviewed. In the end, we summarize the different microfluidic approaches developed for use with SMD to facilitate rapid, low-volume and quantitative analysis, such as electrokinetic and hydrodynamic single-molecule manipulation techniques.

Key Words: Single molecule detection, Quantum dot, Dual-color fluorescence coincidence, Fluorescence resonance energy transfer, Nucleic acid detection, Microfluidics.

INTRODUCTION

Genomic detection, including sequence-specific nucleic acid detection and genetic point mutation detection, has become one of the central themes for modern medical diagnostics and therapeutics. For example, sequence-specific nucleic acid detection is important in pathogen detection and gene expression analysis, while detection of point mutations that serve as genetic biomarkers is useful in diagnosis of diseases such as sickle cell anemia and certain cancers. Conventional genomic (DNA/RNA) detection methods predominantly rely on polymerase chain reaction (PCR) to achieve high sensitivity and thereby suffer from the PCR-associated complications, such as false positive results due to contamination, long setup and analysis times, and high costs. Besides, PCR is not an ideal method for quantitative analysis due to its nature of nonlinear amplification in thermal cycles and the fact that the amplification level is sensitive to the reaction conditions and target sequences. To circumvent these issues, recently there is an increasing interest in using single-molecule detection (SMD) for genomic detection. The driving force not only comes from its ultrahigh sensitivity that can allow the detection of low-abundance nucleic acids without the need of amplification but also from its potential in achieving high-accuracy quantification of rare targets *via* single-molecule sorting.

Over the past two decades a variety of optical SMD techniques (in solutions or on a solid surface) have been developed [1-9], greatly advancing fundamental bio-chemical and bio-physical studies. In-solution SMD is mainly based on the use of confocal fluorescence spectroscopy, which detects the emission from a single fluorescent molecule when it traverses through a femtoliter-sized detection volume. As shown in Fig. (**1**), such a small detection volume is defined by the convolution of the illumination volume given by a high-numerical-aperture objective (typically N.A. 1.2) and the collection efficiency function defined by a confocal pinhole (typical 50-100 μm) and the objective. This small detection volume significantly reduces the background noise originating from spurious fluorescence of impurities and Raman scattering of solvent molecules, so that the high signal-to-noise ratio (SNR) and thereby high sensitivity is achieved. When measuring low-concentration targets (< 1 nM), the detected fluorescence signals become digital because the molecular occupancy (the average number of molecules residing in the detection volume) is smaller than unity. Fluorescence bursts are detected only when single fluorescent molecules pass through the detection volume. Since the SNR of the single-molecule fluorescence bursts does not decrease with decreasing concentrations of the molecules under investigation, SMD provides an ideal platform for analysis of low-concentration targets. In the past, SMD techniques have been largely used for the fundamental study in molecular interactions [10-12]. Recently, by incorporating new probes or probe strategies, such as fluorescence resonance energy transfer (FRET)-based probes [13-15] and dual-color fluorescence coincidence analysis [16-20], SMD has been applied to sequence-specific detection of DNA/RNA targets in solutions.

Another unique advantage of applying spectroscopic SMD to genomic detection lies in the fact that a greatly-reduced volume of sample can be used for detection without compromising the assay sensitivity. For quantitative analysis, the femtoliter detection volume in spectroscopic SMD renders the use of sample volume as little as 1 nanoliter sufficient to achieve measurements with high statistical confidence (in such case it would allow ~ 10^6 sampling events). The small detection volume of SMD, however, demands an efficient scheme for transporting molecules through the detection volume to improve the mass detection efficiency.

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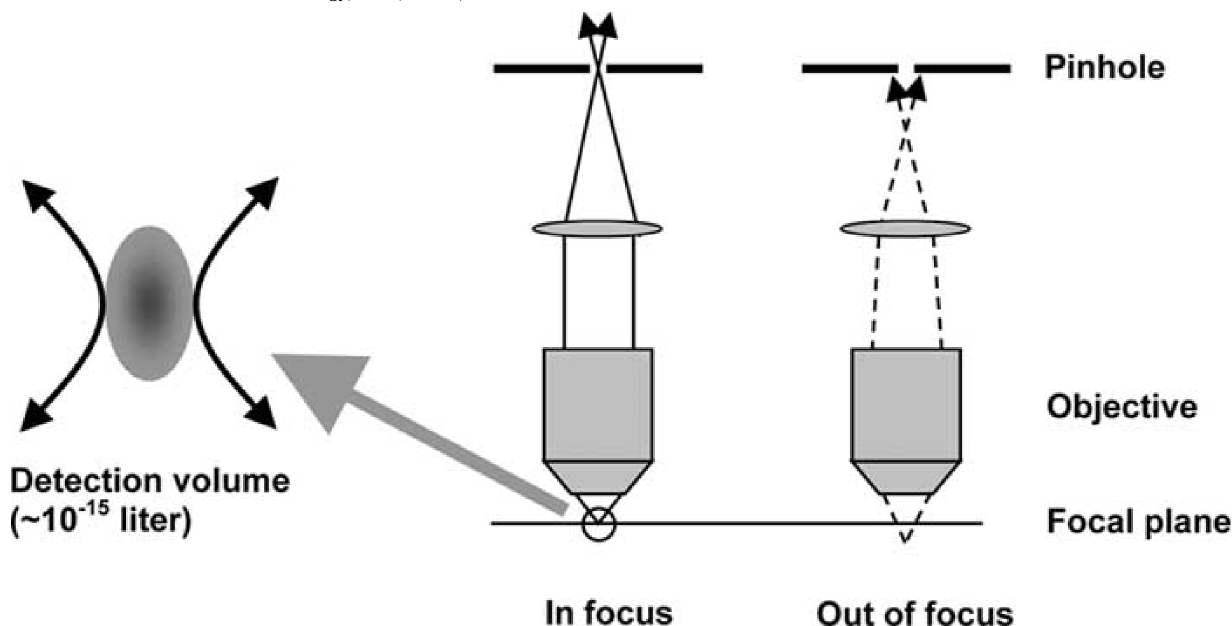

Fig. (1). A conceptual diagram of confocal optics.

Recent advances in micro- or nanofluidics technologies have facilitated the controlled transport of minute amounts of fluids as well as the precise manipulation of biomolecules using electrokinetic [14, 21-24] or hydrodynamic forces [25, 26]. With the high detection sensitivity of SMD and the high mass detection efficiency enabled by the use of microfluidic devices, the integrative microfluidic-SMD approach makes possible the accurate and quantitative analysis of nucleic acids at low abundance [13, 21, 27].

High-accuracy quantification of target molecules at extremely low concentrations (e.g. picomolar or lower) can still be achieved with SMD by either extending the measurement time or by increasing the flow speed to allow a larger sample volume to be interrogated [21, 28-30]. Although accelerating the flow speed is preferable due to the interest of rapid analysis, this approach reduces the transit time of molecule passing through the detection volume, thereby leading to a decreased SNR of single-molecule bursts. The incorporation of the newly developed nanoscale, high-performance fluorescent tags such as semiconductor quantum dots (QDs) may enable high-SNR SMD in a high-speed flow. Compared to conventional organic fluorophores such as Rhodamine 6G, QDs are 20 times as bright and 100 times as stable against photobleaching [31]. These great photophysical properties have also allowed the detection of single DNA molecules by simply using standard microscopy [32, 33] instead of sophisticated confocal optics. In the near future, the combination of nanotechnology and single-molecule detection is expected to lead to a new ultrasensitive, cost-effective genetic detection platform amenable to routine tests in laboratories and clinical settings.

SINGLE-MOLECULE DETECTION OF SPECIFIC NUCLEIC ACIDS

FRET-Based Methods

Based on fluorescence resonance energy transfer (FRET), a variety of molecular probes, such as Taqman [34] and molecular beacons [35], have been developed for the detection of nucleic acids of specific sequences [15, 34-37]. FRET is a non-radiative spectroscopic process in which the energy of an excited donor transfers to its nearby acceptor, which occurs at a length scale typically less than 10 nm. The energy transfer efficiency (E) inversely depends on the sixth-power of the separation distance (r) of a donor and an acceptor:

$$E = \frac{R_0^{\ 6}}{R_0^{\ 6} + r^6} \qquad (\mathbf{1})$$

where the Förster distance (R_0) is the donor-acceptor distance at which the energy transfer efficiency is 50%. The interaction between targets and FRET-based probes leads to a change in molecular conformation and thereby a change in inter-molecular distance between the donor and acceptor. As a result the presence of the targets can be determined by monitoring the change in energy transfer efficiency (e.g. the increase or decrease in acceptor emission). The biggest advantage of FRET-based probes is that they enable a homogeneous detection scheme (without the need of separation of unbound probes) and therefore can be incorporated with SMD for DNA/RNA detection in solutions.

Molecular beacons, which are oligonucleotide probes with stem-loop structures that become fluorescent upon hybridization to their complementary sequences (Fig. (**2**)), have recently been incorporated with SMD for the detection of nucleic acids [21, 29, 38] and point mutations [14]. We have demonstrated a dual-color SMD assay for comparative quantification of specific nucleic acids using molecular beacons (Fig. (**2**)) [29]. Two different color molecular beacons were used to perform a comparative hybridization assay for simultaneous quantification of both target and control strands.

Dual-color fluorescence analysis is a technique commonly used in comparative quantification for gene expression analysis. Detections based on dual-color fluorescence

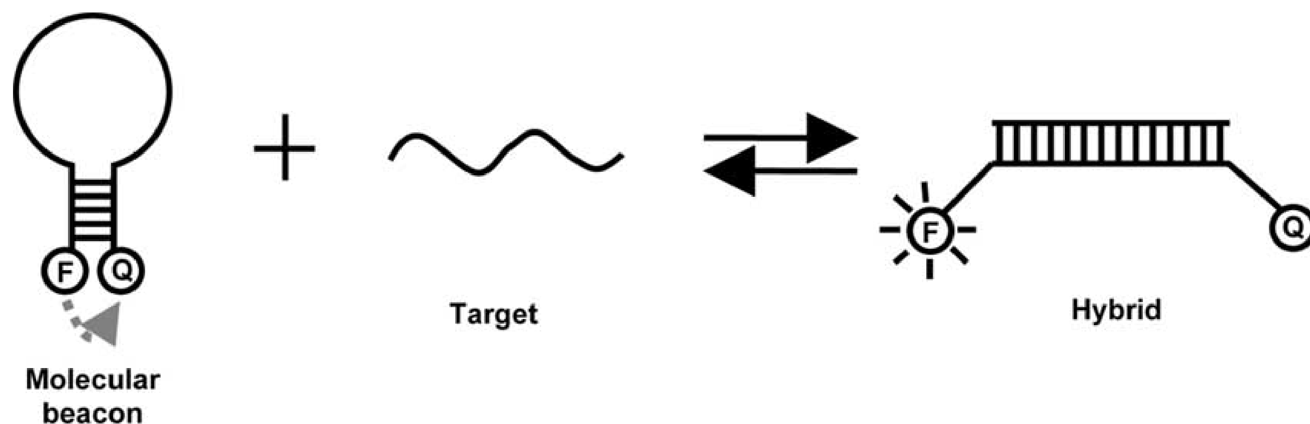

Fig. (2). Structure and operation of the molecular beacon. A molecular beacon in its hairpin conformation is non-fluorescent because the quencher and the fluorophore are in close proximity. Molecular beacon undergoes a conformational change once it hybridizes with a target strand by which the fluorophore is separated from the quencher and the fluorescence is restored.

analysis (e.g. dual-color microarray [39]), however, are often complicated by the disparity of the photophysical or photochemical properties between different fluorophores. In addition, when analyzing low-concentration targets, the quantification accuracy is limited by the reduced SNR and the relatively large variations in signal levels. Dual-color fluorescence coincidence-based SMD method, on the contrary, obviates such technical complications since both target and control samples are quantified by counting the discrete single-molecule fluorescence bursts in different wavelengths, which retain high SNR despite the decrease in target concentration. As a result, this method has allowed comparative quantification of low-concentration (picomolar or lower) nucleic acid targets and is able to discriminate as small as 2-fold difference in target quantity at such concentration levels [29].

Quantum Dot-Based FRET Assay

Our group has recently developed an inorganic/organic hybrid FRET nanosensor that detects DNA based on probe-target hybridization and FRET using QD (donor)/fluorophore (acceptor) as an energy transfer pair [13]. The aforementioned unique properties of QDs such as size-tunable photoluminescence spectra, broad absorption and narrow emission spectra, and high quantum yields make them greater candidates to serve as energy donors, as opposed to organic fluorophores, in a FRET system. By using QD as a donor, the issues commonly associated with organic fluorophore-based FRET systems, such as spectral cross-talk and direct acceptor excitation, can be overcome. As shown in Fig. (**3**), each DNA nanosensor is comprised of two target-specific oligonucleotide probes. One is a reporter probe labeled with an organic fluorophore (Cy5, emission peak at 670nm) and the other is a biotinylated capture probe. A streptavidin-conjugated QD (emission peak at 605nm) serves as both a target concentrator and a FRET donor. When a target DNA strand is present in solution, it is sandwiched by the two probes and the resulting hybrid is captured by a QD through biotin/streptavidin binding. QD functions as a concentrator that captures multiple probe-target hybrids within a nanoscale domain, resulting in strong fluorescence emission from the Cy5 *via* resonance energy transfer on illumination of the QD (Fig. (**3**) panel B). Detection of acceptor emission therefore indicates the presence of targets.

Performance of the QD-FRET nanosensor has been evaluated by comparing the sensing responsitivity, which is defined as the ratio between the counts of fluorescence bursts detected after and before addition of targets, between the nanosensor and molecular beacons. The nanosensors showed much higher sensing responsitivity than did molecular beacons at almost every target concentration tested [13]. The unoptimized sensitivity of the QD-FRET nanosensor is 4.8 fM, which is 100-fold more sensitive than conventional FRET-based molecular beacons in DNA detection, as monitored by confocal SMD spectroscopy [13].

Fluorescence Correlation Spectroscopy (FCS)

In the last decade, several assay methods for nucleic acid detection based on fluorescence correlation spectroscopy (FCS) have been reported [16, 18, 38, 40-49]. The principle of FCS lies in temporal and spatial fluorescence fluctuation due to molecules diffusing in and out of the small detection volume. The characteristic diffusion time constant ($_d$) is typically determined through autocorrelation analysis of fluorescence signals, followed by its fitting to the autocorrelation function *G( )* derived based on a 3D diffusion model [41]:

$$G(\ ) = \frac{1}{N(1+\frac{}{_d})(1+(\frac{_0}{z_0})^2\ \frac{}{_d})^{1/2}} \qquad (\mathbf{2})$$

where is the lag time; $_0$ and z_0 are half axes of the detection volume. When coupled with the use of a target-specific fluorescent probe, FCS can be used for biomolecule detection by monitoring the change in diffusivity caused by target-probe binding. Rigler and co-workers have combined FCS with a confocal setup for DNA detection and sorting [41]. This technique of one-color autocorrelation analysis has also been used for the study of DNA conformational fluctuation [42, 43] and DNA hybridization kinetics [44]. One complication of one-color FCS analysis is that it is difficult to differentiate the binding and non-binding events that

involve only small changes in diffusion time constants [19]. Therefore, one-color FCS is limited to the analysis of the targets that are substantially larger than the fluorescent probes. Such limitation is overcome by dual-color FCS analysis as it characterizes molecular binding from the change in cross-correlation functions rather than in the diffusion properties. In addition, simpler mathematical evaluation and shorter readout time can also be achieved [45]. Dual-color FCS [41, 46] has been demonstrated for the detection of amplified DNA target sequences in multiplexed polymerase chain reactions (PCR) [47], and mRNA [16, 18, 48, 49]. The typical concentration range of FCS measurements is around 1~100 nM and an average of one to hundreds of fluorescent molecules are under interrogation within the detection volume at any time.

Like other fluorescence-based detection methods, FCS is limited by the background fluorescence of the unbound fluorescent probes that are in large excess to the targets. Recently, several schemes for fluorescence background reduction based on FRET have been reported. The use of hairpin molecular beacon probes instead of linear probes in dual-color FCS has been shown to decrease of background by a factor of 40 to 100 [38]. The significant reduction in background, as a result, makes dual-color FCS amenable to the analysis of low-abundance, unamplified targets.

Single-Molecule Fluorescence Burst Coincidence Detection

Detection of single DNA molecules has also been achieved based on the coincidence analysis of discrete single-probe fluorescence bursts [17]. As shown in Fig. (**4**) panel A, the coincidence detection method uses two probes, each labeled with a different fluorescent tag, to bind a specific target. Emission of the two different fluorescent tags is then detected by dual-color confocal spectroscopy (Fig. (**3**) panel C). Single-molecule fluorescence coincidence detection is typically performed at a concentration level of subnanomolar or lower. At this concentration level, the average number of probe molecules residing in the confocal detection volume (~ fL), i.e. the molecular occupancy, is smaller than unity at any time. The discrete fluorescence bursts are then detected as individual probes or hybrids flow through the detection volume (Fig. (**4**) panel B and C). When targets are not present in the solution and thus the two probes move independently, the digital single-probe fluorescence bursts, detected in the two separate emission channels, are not correlated (Fig. (**4**) panel B). On the other hand, when mixed with a sample containing the specific targets, the two probes bind to a target, forming a doubly-bound molecular hybrid. Simultaneous fluorescence bursts can be seen in the two emission channels as the individual doubly-bound hybrids flow through the detection volume (Fig. (**4**) panel C). The probability of having more than one unbound probe simultaneously present in the detection volume due to stochastic events can be estimated using Poisson statistics:

$$P_x = \frac{\exp(-\ \ )\ \ ^x}{x!} \qquad (3)$$

where P_x stands for the probability of finding exactly x molecules in the focal volume at a given time.   is the average number of molecules in the detection volume. At a probe

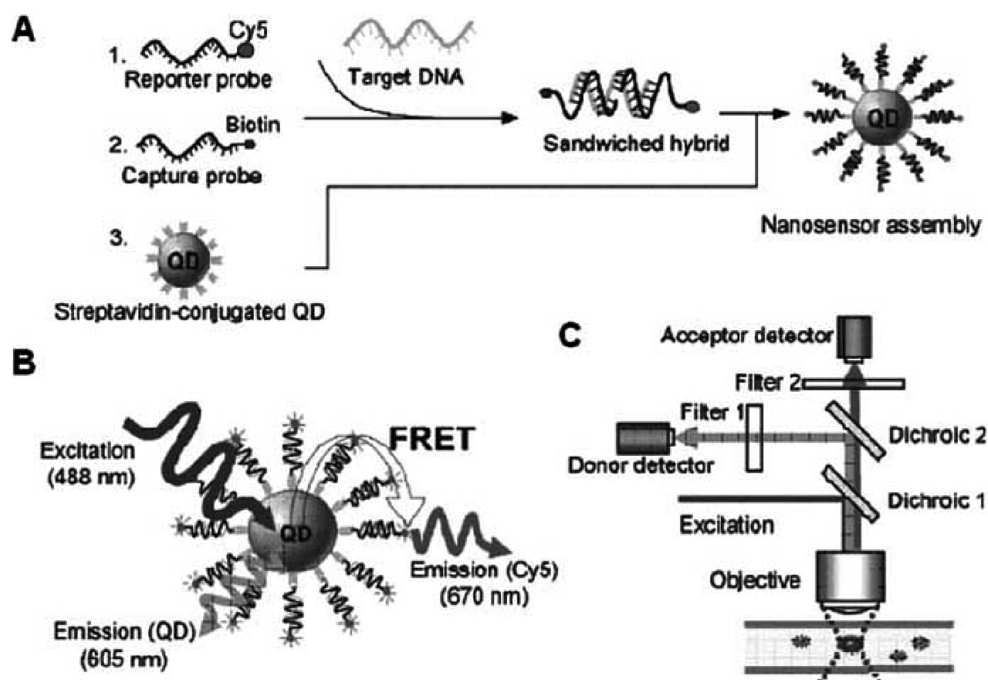

Fig. (3). Schematic of single QD-FRET DNA nanosensors. The conceptual scheme showing the formation of a nanosensor assembly in the presence of targets (panel A). Fluorescence emission from Cy5 acceptors on illumination of QD donor due to FRET between Cy5 and a QD in a nanosensor assembly (panel B). A custom-made confocal spectroscopic setup was used for single-QD detection (panel C). Copyright 2005 Nature Publishing Group [13] .

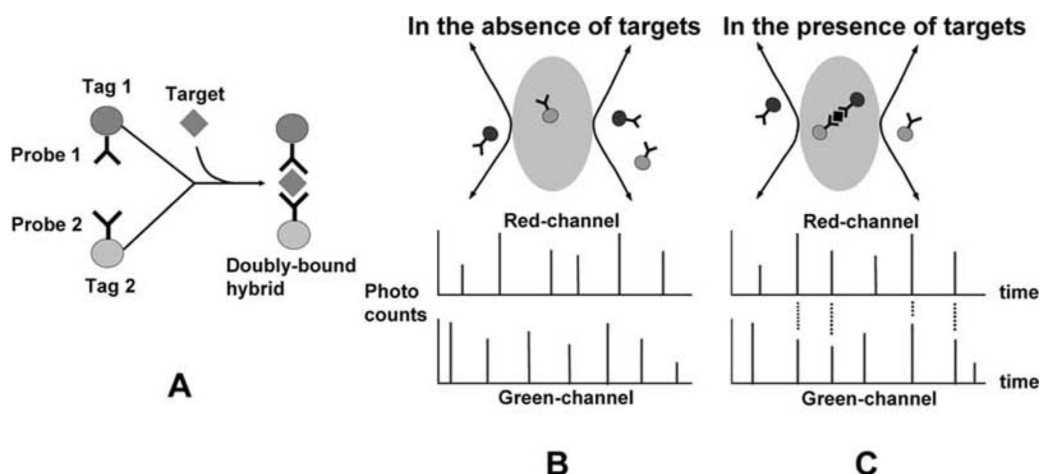

Fig. (4). Conceptual schematic of dual-color coincidence analysis. Two fluorescently labeled probes are designed to bind a target strand (panel A). The fluorescence bursts from two emission channels do not correlate with each other in the absence of targets (panel B). (c) Coincident fluorescence bursts from the both detection channels are detected, indicating the formation of doubly-bound hybrids and thereby the presence of targets.

concentration level of 1 pM, is ~ 10^{-4}. Calculation shows the probability of finding one (P_1) and two (P_2) molecules in the detection volume is 10^{-4} and 5.0×10^{-9}, respectively. P_2/P_1 is thus 5.0×10^{-5}, implying a low probability of having false coincidences due to stochastic events that two different color, unbound probes are simultaneously present in the detection volume. Therefore, on one hand there is no need to separate the unbound probes from the hybrids due to the low false coincidence probability; on the other hand, the requirement for two probes in coincidence detection increases the detection specificity. The detection specificity can be further improved by using peptide nucleic acid (PNA) probes. PNA are DNA mimics with a pseudo-peptide backbone and it has strong affinity to bind DNA targets. Combining PNA probes with a coincidence detection scheme, Castro and coworkers demonstrated SMD of specific DNA sequences in unamplified genomic DNA samples [5, 17, 28]. Li and coworkers incorporated a dual-laser excitation confocal setup and a doubly-covalently-labeled DNA molecules to investigate the detection sensitivity of coincidence detection in a large excess of singly-covalently-labeled DNA molecules [19]. Using dual-laser excitation and having subfemtoliter-sized detection volume, the cross-talk and background noise were further reduced. Ultrasensitive coincidence detection of doubly-covalently-labeled DNA molecules down to 50 fM in the presence of a 1000-fold excess of singly-covalently-labeled DNA molecules was demonstrated. Coincidence detection in combination with two additional quencher-labeled oligonucleotides that quenched the unbound probe fluorescence was also reported [16]. Detections of target DNA sequences at concentrations as low as 100 fM in the presence of a ~ 5000-fold excess of labeled hybridization probes were shown.

Quantum Dot-Based Dual-color Coincidence Assay

An important factor in dual-color coincidence detection is that the emission spectra of the two fluorescent tags must have minimum overlap in order to prevent cross-talk-induced false coincidences. Organic fluorophores typically have small (~ 20-30 nm) Stokes' shifts. When two fluorophores that can be excited by a single-wavelength laser are selected for fluorescent labeling, they have very close peak emission wavelengths, thereby resulting in substantial spectral cross-talk. The complication of cross-talk can be overcome by using dual-excitation confocal spectroscopy as it allows use of two fluorophores with distinct excitation and emission spectra. However, to obtain good coincidence detection results, a highly chromatic aberration-corrected objective for photon collection and an effort in optical alignment are necessary in order to achieve good overlap between the illumination volumes of the two excitation lasers [46].

Using QDs as fluorescent tags, we have demonstrated the dual-color coincidence detection of DNA targets with only single-laser excitation. As aforementioned, QDs have great photophysical properties such as size-tunable spectra, broad absorption and narrow emission spectra, and large Stokes' shifts. Consequently, multiple QDs with distinct emission wavelengths can be excited by a single laser source, facilitating the multiplexed detection. Fig. (**5**) shows an example of such a multiplexed detection using three different QDs (peak emission wavelengths at 525 nm, 605 nm, and 705 nm, respectively). Three different kinds of DNA targets were simultaneously identified using a combinatorial dual-color coincidence detection and analysis scheme and a single-laser excitation confocal spectroscope [20].

Nanoparticle-Based Single-Molecule Colocalization Analysis

The use of QDs as fluorescent tags has also allowed the high SNR measurements of single DNA molecules using a CCD-coupled, wide-field microscope instead of a sophisticated confocal optical setup [32, 33]. Our group has demonstrated the detection of single-molecule hybridization using oligonucleotide-conjugated QD nanoprobes [33]. Two different-color QD nanoprobes are designed to bind in juxtaposition to the same target DNA and to form a sandwiched nanoassembly. When measuring with a fluorescence microscope, the nanoassembly is imaged with a combined color due to colocalization of the two linked QD nanoprobes (Fig.

(**6**) panel A). Consequently, the presence of targets can be determined though colorimetric measurements of the QD nanoprobes.

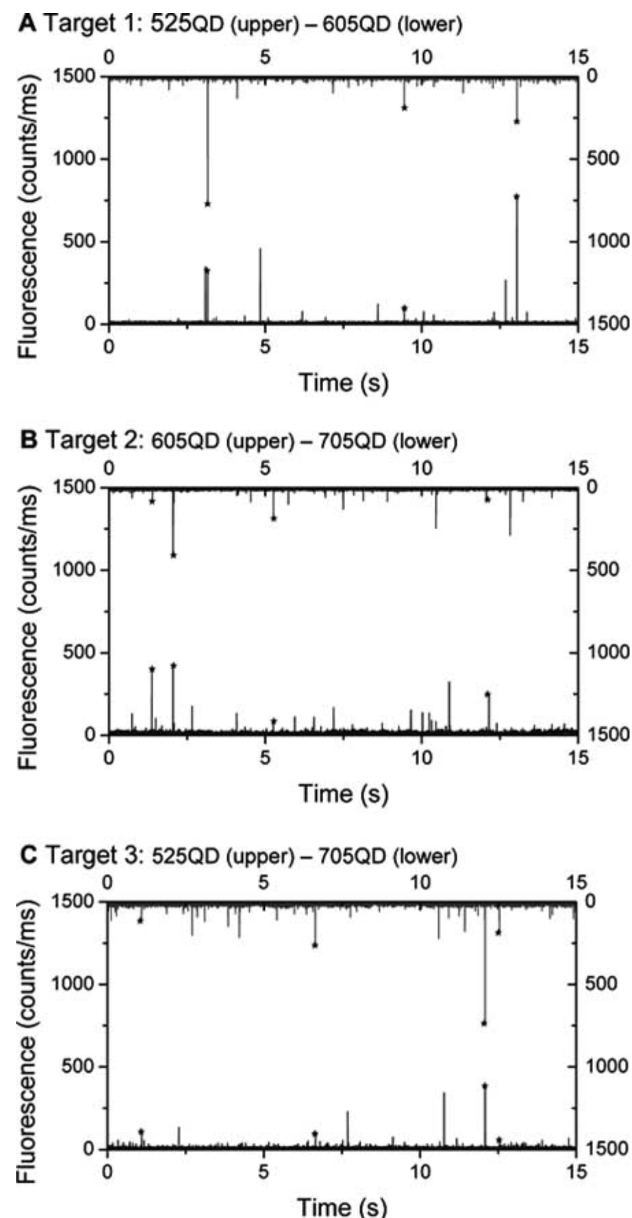

Fig. (5). Demonstration of QD dual-color coincidence assay in multiplexed detection of three DNA targets. The combinatorial coincident signals in panel A, panel B, and panel C indicate the presence of target 1, 2, and 3, respectively. Asterisks (*) denote the coincident signals. Copyright 2005 Elsevier [20].

In a DNA detection experiment, a pair of green (525QD) and red (605QD) QD nanoprobes was used. Fluorescent spots in combined pseudo orange color, which indicated the QD-DNA nanoassemblies, were observed in the presence of DNA targets (Fig. (**6**) panel C, image I). Colocalization of the QD nanoprobes was also verified by analyzing the 3-D contour of the fluorescent signals in the two separate color panels (Fig. (**6**) panel D). Specificity of this method was validated using a negative control sample comprised of genomic DNA from *Escherichia coli*. As shown in Fig. (**6**) panel C, image II, colocalization (orange color) was rarely observed throughout the entire field of view in the negative control experiment, demonstrating high specificity and low background noise with this method. With this scheme, n different QDs can each be used for conjugation of (n - 1) different oligonucleotide probes to prepare n(n - 1) QD nanoprobes capable of simultaneously detecting 1/2n(n - 1) targets (Fig. (**6**) panel B). As proof of concept, multiplexed detection of three targets sequences derived from *B. anthracis*-related genes has been demonstrated with high sensitivity and specificity [33].

SIZING AND SEQUENCING OF SINGLE DNA MOLECULES

In the past years, several ideas for single molecule-based DNA sizing/sequencing have been proposed [24, 50-55]. Contrary to the DNA sequencing schemes based on the Sanger method, which only allows short DNA fragments up to 1,000 bp to be sequenced, single molecules-based sizing/sequencing methods promise to sequence DNA of tens of thousands base pairs in length [54, 55]. The schemes of confocal spectroscopy-based single DNA-molecule sequencing in a continuous flow format have been proposed [54, 55]. The method starts with the polymerization of a target DNA strand with distinctly fluorophore-labeled nucleotides. The resulting labeled strand is anchored in a flow system. Exonuclease is then added to the solution to sequentially cleave nucleotides from the free end of the strand. The sequence information is obtained based on the detection and identification of single fluorescent nucleotides as they flow downstream and sequentially pass through the detection volume. Three crucial problems associated with this single-molecule sequencing scheme, including low detection efficiency, nonspecific adsorption of single nucleotides to the channel walls, and the unwanted electroosmotic flow have been improved or prevented by using tapered microchannels, by chemical modification of the channel walls, and by the addition of detergents [54]. A processive digestion of DNA strands was also verified using a diluted solution of Exonuclease I [55]. The sequencing rate is only determined by the cleavage rate of exonuclease enzyme, which potentially can be several hundreds of bases per second. Among four nucleotides, only two need to be labeled and still provide a full, four-base DNA sequence [55]. This is easier to polymerize and digest a partially labeled DNA strand and also simplifies the fluorescence detection scheme [55]. Alternative to the emission spectra, the fluorescence decay time is also used as a means to identify the fluorescent label on a single-molecule basis [54]. Another single DNA molecule sequencing scheme is the so-called sequencing-by-synthesis method [56]. The idea is to image the sequence information in a single DNA template as its complementary strand is synthesized. The fluorescence signals are measured in sequence and then nulled by photobleaching. The noise is greatly reduced by using a combination of evanescent wave microscopy and single-pair FRET.

MANIPULATION AND FOCUSING OF SINGLE MOLECULES

The extremely small detection volume associated with confocal SMD spectroscopy renders it a highly sensitive

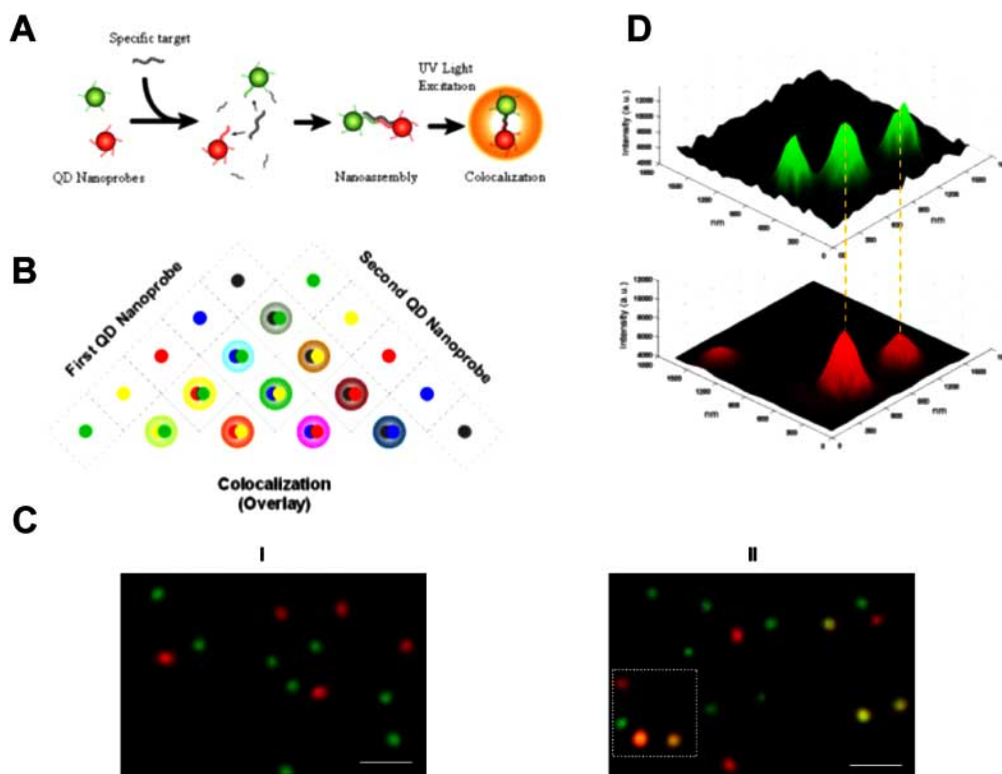

Fig. (6). QD nanoprobes prepared by surface-functionalizing QDs with target-specific oligonucleotide probes. Two target-specific QD nanoprobes with different emission wavelengths sandwich a target, forming a nanoassembly (panel A). The nanoassembly is detected as a blended color (orange) due to the colocalization of both QD nanoprobes (panel B). Multiplexed detection can be achieved with various combined color of colocalized nanoassemblies. Fluorescent images I (panel C) is the control sample where target is absent but containing genomic DNA from *E. coli*. Fluorescent images II (panel C) correlates the sample in which target DNA of interest is present. Bar dimension is 1 μm. 3-D intensity contour from the range of interest of images II (where squared) demonstrates the colocalization occurs with presence of target (panel D). Copyright 2005 American Chemical Society [33].

method for biological detection because of the maximized SNR [57]. However, only a small fraction of sample is typically interrogated in SMD analysis, which in turn raises the ultimate detection limit (i.e. mass detection limit). In order to realistically apply SMD in detecting rare or limited samples, the use of a delicate mechanism or device to transport the sample and guide it through the small SMD detection region is required. A number of techniques have been proposed to enhance the mass detection efficiency in SMD: using nanometer-sized fluidic channels [1, 50, 54, 58], using hydrodynamic focusing [25, 26], and using electrokinetic focusing such as electric-current focusing [21, 24, 52].

We have developed a microfluidic device capable of manipulating single DNA molecules (Fig. (**7**)) and used it to facilitate the quantitative detection of low-abundance DNA using SMD [21]. Transporting and focusing molecules through a laser-focused detection region of a SMD spectroscope was implemented by an electrode-embedded microfluidic channel (Fig. (**7**) panel A). A sample solution was introduced at the inlet of a microchannel and then driven through using hydrodynamic pumping. When fluorescent target molecules entered the electrode region, their movements were governed by the electrical-field induced electrokinetic forces, which steered them toward the region of minimal energy located at the center of the middle electrode. The focused laser beam of a confocal fluorescence spectroscope was positioned at the downstream end of the energy minimum region, wherein fluorescence bursts emitted from individual molecules were detected. The amount of target molecules was quantified according to the number of the counted single-molecule fluorescence events. As shown in Fig. (**7**) panel C, we applied ac and dc electric fields to the electrodes so that the induced electrophoretic (EP) and dielectrophoretic (DEP) forces effectively brought DNA molecules (T2 DNA) to the centre of the microchannel (a real-time video showing the molecular focusing process can be accessed through reference [21] on the American Chemical Society website). With properly applied electric fields, we have demonstrated this electro-molecular manipulation technique effective in manipulating a variety of bioparticles of sizes ranging from micro- (e.g. T2 DNA, DNA, and 1 μm-diameter latex sphere) to nanoscales (e.g. 25-mer molecular beacons (Fig. (**7**) panel D). The electro-molecular focusing device has enabled rapid, accurate, and quantitative detection of low-abundance DNA, allowing measurements of DNA of 0.7 nM with 99-percent accuracy under 1 second [21].

CONCLUSION

The incorporation of the molecular probe strategies such FRET-based and dual-color coincidence detection-based approaches into SMD has enabled ultrasensitive genomic detection in a homogenous, separation-free format. Recently,

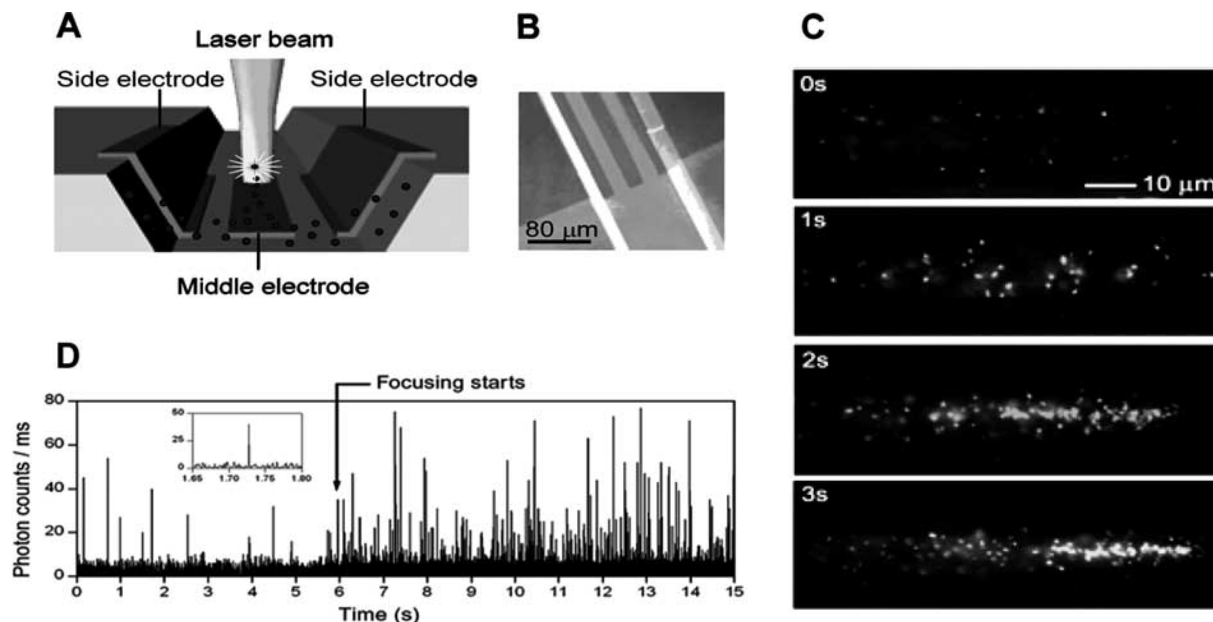

Fig. (7). Manipulation of single DNA molecules. Schematic (panel A) and a SEM picture (panel B) of the molecular manipulation chip are shown. Time-serial epifluorescence images illustrate how flowing DNA molecules gradually focus on the centre of the downstream micro-channel after a potential is applied (panel C). Trace moving molecular beacon-target hybrids in a microchannel before and after introduction of an electric potential, causing a significant increase in fluorescence burst count rate (panel D). Copyright 2005 American Chemical Society [21].

new probes developed based on the use of nanomaterials such as QD-FRET nanoprobes, which display an extremely low level of background fluorescence unmatched by the conventional molecular probes, have improved the detection limits of such SMD assays as a large excess probes can be used to enhance binding kinetics without increasing the background noise. The unique features of QDs such as tunable emission spectra, narrow emission linewidths, and high brightness and photostability have allowed high SNR, multiplexed SMD simply using simple, standard microcopy. The rapid advances in microfabrication and microfluidics technologies have made possible the SMD analysis in a rapid, quantitative, and high-throughput format. Clearly, the future development of a bioanalytical platform based on integration in SMD, nanotechnology and microfluidics promises to tackle the critical technological challenges in genomic analysis such as amplification-free detection of rare DNA targets and genetic defects, accurate quantification of low-abundance targets, and differentiation of minute difference in molecular quantity (for example, the change of DNA copy numbers within cells).

ABBREVIATIONS

CEF = Collection efficiency function

PCR = Polymerase chain reaction

SMD = Single-molecule detection

PNA = Peptide nucleic acid

CCD = Charge-coupled device

FCS = Fluorescence correlation spectroscopy

QD = Quantum dot

SNR = Signal-to-noise ratio

MB = Molecular beacon

N.A. = Numeric Aperture

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