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High-density, microsphere-based fiber optic DNA microarrays

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Abstract

A high-density fiber optic DNA microarray has been developed consisting of oligonucleotide-functionalized, 3.1-μm-diameter microspheres randomly distributed on the etched face of an imaging fiber bundle. The fiber bundles are comprised of 6000–50 000 fused optical fibers and each fiber terminates with an etched well. The microwell array is capable of housing complementary-sized microspheres, each containing thousands of copies of a unique oligonucleotide probe sequence. The array fabrication process results in random microsphere placement. Determining the position of microspheres in the random array requires an optical encoding scheme. This array platform provides many advantages over other array formats. The microsphere-stock suspension concentration added to the etched fiber can be controlled to provide inherent sensor redundancy. Examining identical microspheres has a beneficial effect on the signal-to-noise ratio. As other sequences of interest are discovered, new microsphere sensing elements can be added to existing microsphere pools and new arrays can be fabricated incorporating the new sequences without altering the existing detection capabilities. These microarrays contain the smallest feature sizes (3 μm) of any DNA array, allowing interrogation of extremely small sample volumes. Reducing the feature size results in higher local target molecule concentrations, creating rapid and highly sensitive assays. The microsphere array platform is also flexible in its applications; research has included DNA–protein interaction profiles, microbial strain differentiation, and non-labeled target interrogation with molecular beacons. Fiber optic microsphere-based DNA microarrays have a simple fabrication protocol enabling their expansion into other applications, such as single cell-based assays.

Keywords: DNA; Microarray; Microsphere; Fiber optic

1. Introduction

Optical fibers consist of an inner core surrounded by a clad material of lower refractive index. The differences in refractive index cause total internal reflection of light. A fiber optic bundle consists of thousands of individual fibers fused together such that each fiber retains its ability to transmit light independently of its neighbors (Fig. 1). By selectively etching the fiber core, an array of microwells is formed (Pantano and Walt, 1996). These microwells may be filled with oligonucleotide-functionalized microspheres. The array dimensions can be tailored to suit any size of oligonucleotide-functionalized microsphere. The well diameters are equal to those of the fiber cores, and the depths are dependent on the etchant concentration, the exposure time, and the fiber composition. Because each microsphere is optically wired to a fiber, the specific interactions on each microsphere surface can be independently monitored. Fiber optic microsphere-based DNA detection is a viable alternative to other high throughput microarray methods. Microarrays employ thousands of single-stranded oligonucleotide sequences immobilized to discrete sensing regions on a solid substrate.(Fodor et al., 1991; Schena et al., 1995, 1996) The tethered sequences, or probes, hybridize with their complementary sequences, or target molecules, which are detected in the hybridization solution. Thousands of discrete sensing regions are patterned on a solid substrate so that each different sensing region simultaneously detects a unique target sequence that may be related to a disease state or expression profile. The fiber optic array platform uses probe sequences coupled to the microspheres as the oligonucleotide sensing regions (Epstein et al., 2002;

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Ferguson et al., 2000; Michael et al., 1998; Walt, 2000). The desired oligonucleotide sequences are attached to individual microspheres and added to the etched wells on the fiber optic bundle face.

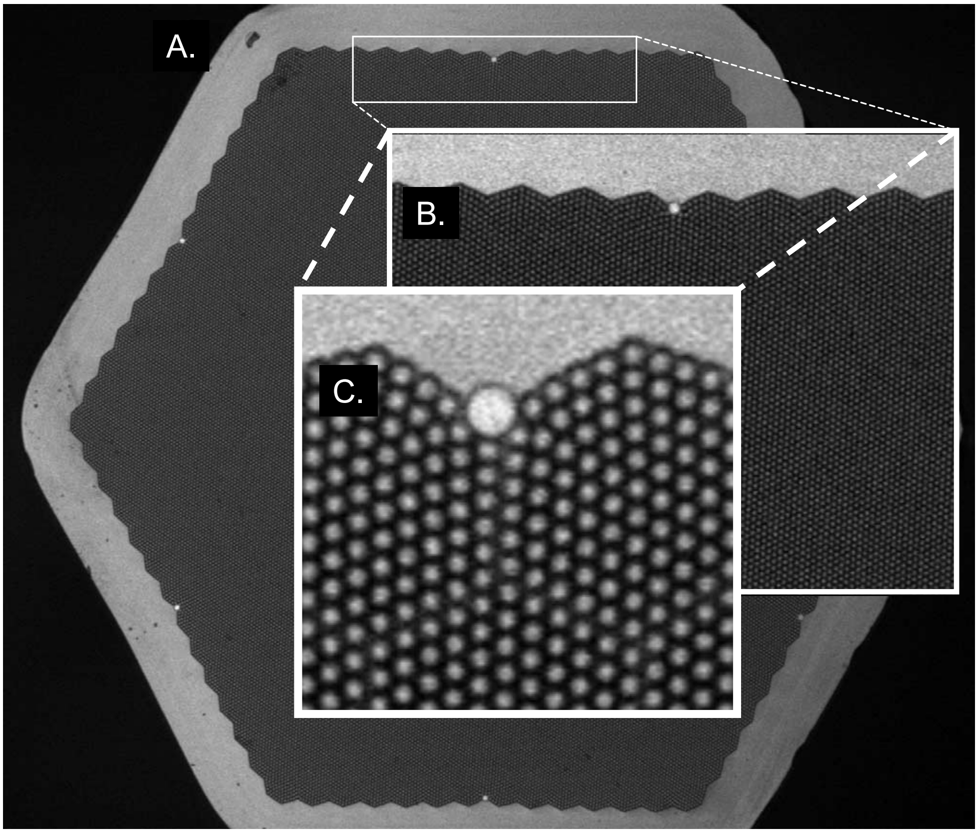

Fig. 1. White light images of a 1 mm diameter fiber bundle. (A) The entire hexagonally packed bundle. (B,C) Higher magnifications of the fiber bundle illustrating the miniature feature sizes (3 μm) and close-packed arrangement. The large circle is a positional marker built into the array.

The detection scheme combines the intrinsic recognition abilities of nucleic acids with fluorescence-based detection methods. Fluorescence-based assays are more desirable than traditional radiolabeled methods due to their increased safety and experimental versatility. Fluorescence can be incorporated into microarray assays by fluorescent intercalating dyes, fluorescently-labeled targets, or label-less methods employing fluorescence resonance energy transfer (FRET). Fluorescence-based assays enable the measurement of multiple wavelengths independently and simultaneously. The use of multiple fluorophores enables parallel interrogation schemes.

Parallel analysis can be further realized with microarrays that incorporate numerous sensing elements. The microspheres are fabricated in a batch process, where 10^9 identical microsensors can be made simultaneously in 1 ml, and used to make a microsphere stock for multiple arrays. The placement of different microsphere types on the etched fiber face is random, so the exact position of each microsphere must be determined after assembly of the array. An optical decoding scheme was developed whereby each microsphere-type was impregnated with a fluorescent dye or combination of dyes, creating a dye ‘bar code’ that can be used to locate and identify the microsensors in an array (Ferguson et al., 2000; Michael et al., 1998; Walt, 2000).

The fiber-optic microsphere-based platform provides a number of advantages over other array-based methods (Epstein et al., 2002; Ferguson et al., 2000; Walt, 2000). This platform provides a high-density array with the smallest individual feature sizes available. A higher packing density corresponds to more sensing elements per array, enabling simultaneous measurements and higher throughput. The reduced array size also enables smaller volumes to be interrogated, and since many array interactions are diffusion dependent, more rapid responses are possible with reduced volumes, further increasing throughput. The miniature feature sizes also provide a detection advantage. The same number of target molecules confined to a smaller volume yields a higher local concentration. For example, a few hundred target molecules limited to one 3 micron well of 30 fl volume corresponds to nanomolar concentrations. Nanomolar concentrations of fluorophores are readily detectable (Epstein et al., 2002; Ferguson et al., 2000). In addition, the platform allows for multiple assays with one array while most other array-based systems are single use. We have demonstrated the use of these microsphere-based arrays over 100 times with less than 8% S.D. (Ferguson et al., 2000). Reusable arrays have an impact on overall assay cost and preparation time. Furthermore, they eliminate issues regarding array-to-array reproducibility. This platform also allows flexibility in design as research needs evolve. A microsphere sensor pool used to fabricate an existing array can be combined with additional microsphere probes to fabricate a new array with additional capabilities. Because of the random assembly process, these arrays are fabricated containing multiple copies of each microsphere type. Such redundancy reduces the occurrence of false positives and false negatives. Identical sensors provide consensus-based analysis, where individual responses from redundant counterparts in the array must agree. The fiber optic array platform is also flexible in its ability to incorporate different nucleic acid detection schemes, such as FRET-based molecular beacon assays (Steemers et al., 2000) and aptamers (Lee and Walt, 2000).

2. Instrumentation

Fiber optic microarrays are monitored with a custom-built modified Olympus epifluorescence microscope (Fig. 2) (Epstein et al., 2002; Ferguson et al., 2000). A xenon arc lamp is used for sample excitation. The system is equipped with excitation and emission filter wheels, a dichroic housing, and a charge-coupled device (CCD) camera (Hamamatsu, Bridgewater, NJ). The entire instrument is computer controlled, run by IPLab software (Scanalytics, Fairfax, VA).

The white light source excites indicators localized on the distal array face; the fluorescent emissions are recorded by a CCD camera. The fluorescence intensity is proportional to the extent of hybridization at each probe position. The camera is equipped with an internal chip that provides megapixel resolution (1280 × 1024). This megapixel chip is able to resolve an array's miniature feature sizes (3 μm), and provides multiple pixels for each optical channel in the fiber bundle. Standard array scanners typically have a resolution of 5–10 μm, and would not be suitable for this system. This type of camera also provides a high quantum efficiency and low dark current, which provides an optimal signal-to-noise enhancement. While CCD cameras generally provide less signal amplification compared to a PMT, the extremely low dark current enables longer scan times without significant increase in noise. By employing a white light source as opposed to more complicated and expensive laser systems, we limit photobleaching associated with longer scan times and intense (laser) excitation sources without sacrificing detection limits. A white light excitation system also provides excitation wavelengths over the entire visible region, enabling a more extensive range of fluorescent dyes.

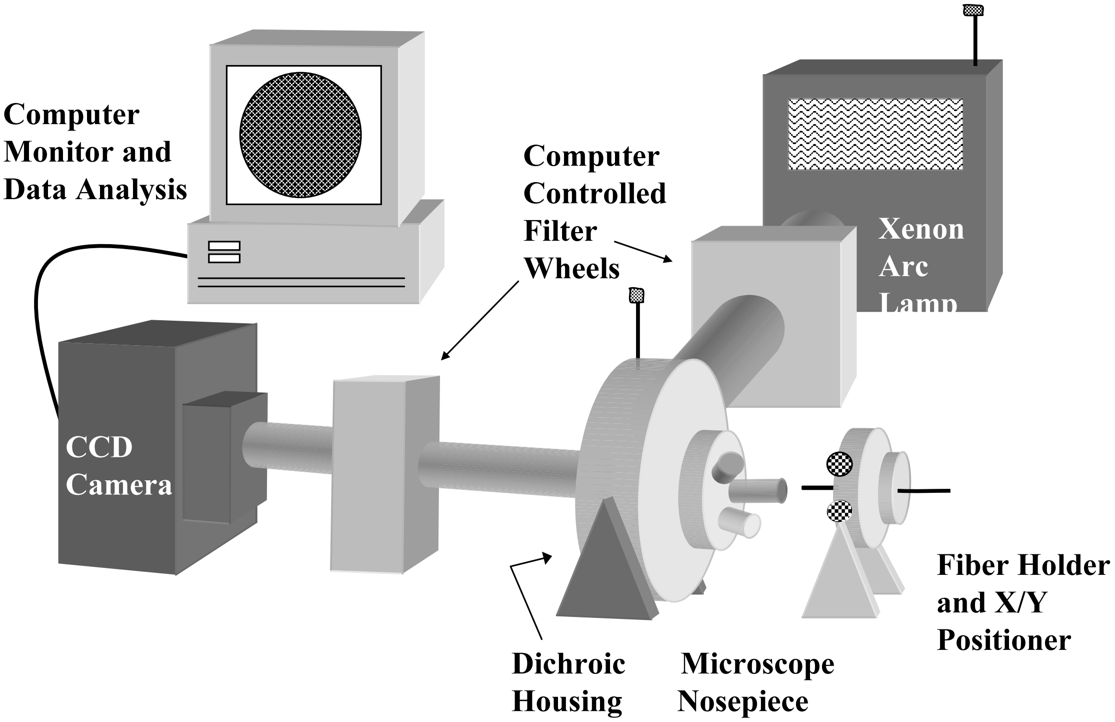

Fig. 2. Schematic diagram of the custom-built epifluorescence imaging system.

3. Fiber optic DNA biosensors

A general array protocol entails immobilizing a probe sequence that can hybridize to its fluorescently-labeled complementary target. The fluorescent tag is commonly incorporated into the target molecules, via the polymerase chain reaction (PCR) (Mullis, 1994; Saiki et al., 1986). This primer labeling method is convenient when amplification is needed for detection, or when it is used to construct a cDNA library from a genomic RNA pool via reverse transcription.

Prior to microsphere-based arrays, the first generation of DNA arrays in our laboratory explored oligonucleotide probe sequences photopolymerized onto fiber bundle surfaces (Healey et al., 1997) or coupled directly to fiber cores (Ferguson et al., 1996). Many advantages of the microsphere-based arrays were first determined with these formats. The initial photopolymerized fiber optic sensor arrays were capable of single nucleotide polymorphism (SNP) detection and sampled extremely small (submicroliter) volumes. The arrays were regenerable over three assays with reproducible experimental results and provided a rapid assay time with high specificity (Healey et al., 1997). The array's miniature features and small sample volumes addressed the slow nucleic acid hybridization kinetics associated with immobilized probe arrays (Ferguson et al., 1996). The derivatized fiber core experiments were able to detect unlabeled (non-fluorescent) target solutions by competitive hybridization with fluorescent target samples. In this method, fluorescent synthetic target complements were synthesized and initially hybridized to the array to saturate the array probe elements. The unlabeled target solution was then hybridized to the same array, competing with the prehybridized synthetic targets. The presence of unlabeled target was determined by a fluorescence decrease caused by displacement of the fluorescent synthetic target by the unlabeled species. This procedure eliminates the need to incorporate fluorescence into the target and allows quantitative measurements to be performed. These fiber optic platforms were the basis for microsphere array designs that improved array fabrication and allowed extremely high-density sensor placement.

3.1. Microsphere-based fiber optic DNA arrays

The second generation of fiber optic arrays incorporated microspheres into its sensing element design (Epstein et al., 2002; Ferguson et al., 2000; Walt, 2000). The microspheres were localized into the etched wells of a fiber optic imaging bundle. Microsphere-based sensing elements provided the experimenter more control over the array design. The number of identical microspheres present in an array influences the assay parameters (Epstein et al., 2002). Greater numbers of identical microspheres provide a signal averaging benefit. The signal-to-noise ratio increases by the square root of the number of identical sensors examined. In contrast, fewer identical microspheres present in an array offers a concentration advantage. Assuming that hybridization goes to completion, the same number of target molecules hybridized to fewer numbers of microspheres, yields more target molecules per microsphere and results in an increased signal. These opposing factors enable researchers to customize arrays to suit the specific experimental requirements. Using this methodology, we have been able to detect zeptomolar concentrations of DNA target molecules (10^{-21} mol, $\sim$600 total target molecules) (Epstein et al., 2002). Multiplexed arrays were fabricated to monitor specific hybridizations in real time, enabling quantitative analysis of unknown target concentrations (Ferguson et al., 2000).

3.1.1. Microsphere-based arrays for genomic analysis

Typical microarray analysis involves comparison of two varied physiological states; for example, cancerous tissue versus non-cancerous tissue. These assays hybridize two representative cDNA libraries simultaneously, employing two different dyes to represent each state. In this fashion, microspheres that show higher signal for one dye relate directly to greater occurrences of the specific gene transcript. This experimental method leads to better understanding of molecular events underlying the specific cellular phenotype.

Fiber optic microsphere-based arrays have been applied to genomic analysis (Yeakley et al., 2002). A single RNA transcript can undergo changes that allow it to be represented in numerous different forms.(Graveley, 2001; Zhang et al., 1994) These interrelated forms can be the result of sequence changes due to post-translational mRNA processing or gene splicing (Dance et al., 2002). These isoforms have been associated with potential developmental factors (Anantharaman et al., 2002; Lopez, 1998), including rapid growth factors associated with cancer. Research was performed with microsphere-based arrays interrogating RNA isoforms for widespread mRNA splicing of single genes. This array format enabled large-scale genomic analysis without previous purification or cDNA synthesis (Yeakley et al., 2002). Furthermore, this platform could more readily address similar sequences or minor differences due to insertions/deletions. The RNA isoforms were discriminated from only a few cells ($\sim$10 pg of total cellular RNA). Fiber optic microsphere-based gene arrays demonstrated allelic discrimination or identification of RNA isoforms originating from a single gene. Employing microspheres containing addressable sequences for decoding, a single series of microsphere arrays can serve as a universal platform for multiple genomic applications.

3.1.2. Molecular beacon and aptamer fiber optic DNA arrays

A third generation of microsphere-based arrays employs a different approach by changing the nucleic acid probe design (Lee and Walt, 2000; Steemers et al., 2000). Detection using molecular beacons (Steemers et al., 2000) and aptamers (Lee and Walt, 2000) have provided another level of nucleic acid interactions not established with other platforms. Microspheres continue to be the vehicle for loading of sensing elements to the array, but the nucleic acid recognition method has been modified.

Molecular beacon (Steemers et al., 2000; Tyagi and Kramer, 1996) methods were adapted to microarray analysis for label-less detection of gene sequences relevant to specific disease states (Steemers et al., 2000; Tyagi and Kramer, 1996). Molecular beacons use FRET methods, where the molecular beacon probe sequences contain a reporter fluorophore, which is quenched by an adjoining quenching molecule (Fig. 3). The quenching molecule can be either a non-fluorescent species that non-radiatively captures the reporter fluorophore's energy, or another fluorophore with an excitation wavelength overlapping the emission wavelength of the reporter fluorophore. In the absence of target molecules, the quencher and reporter fluorophore are close to one another due to the self-complementary DNA sequences, or stem structures present in the molecular beacon molecule. When proximal, the fluorescence energy is transferred from the molecular beacon's reporter fluorophore, and a low signal is measured. The probe sequence connecting the two self-complementary stems is termed a loop sequence. Interactions between the molecular beacon's loop sequence and the correct target molecule cause a conformational change in the molecular beacon's structure, spatially separating the two stems as well as the reporter fluorophore and quencher. FRET signal quenching is a distance dependent process, so this spatial separation allows the molecular beacon's reporter to fluoresce. The appearance of signal corresponds to complementary target binding to the molecular beacon. Fluorescein and 4-(4-dimethylaminophenylazo) benzoic acid could be used as a universal fluorophore and quencher respectively for every probe. Assays were reproduced over 50 × on the same array and hybridized to unlabeled PCR products. The PCR samples interrogated sequences containing known closely related cystic fibrosis sequence mutations (Steemers et al., 2000).

Aptamers are another type of nucleic acid probe element that has been used with the microsphere-based array platform (Lee and Walt, 2000). Aptamers are nucleic acid sequences isolated from a single-stranded oligonucleotide combinatorial pool based on successive rounds of interaction with a specific target (Ellington and Szostak, 1992; Jhaveri et al., 2000; Tuerk and Gold,

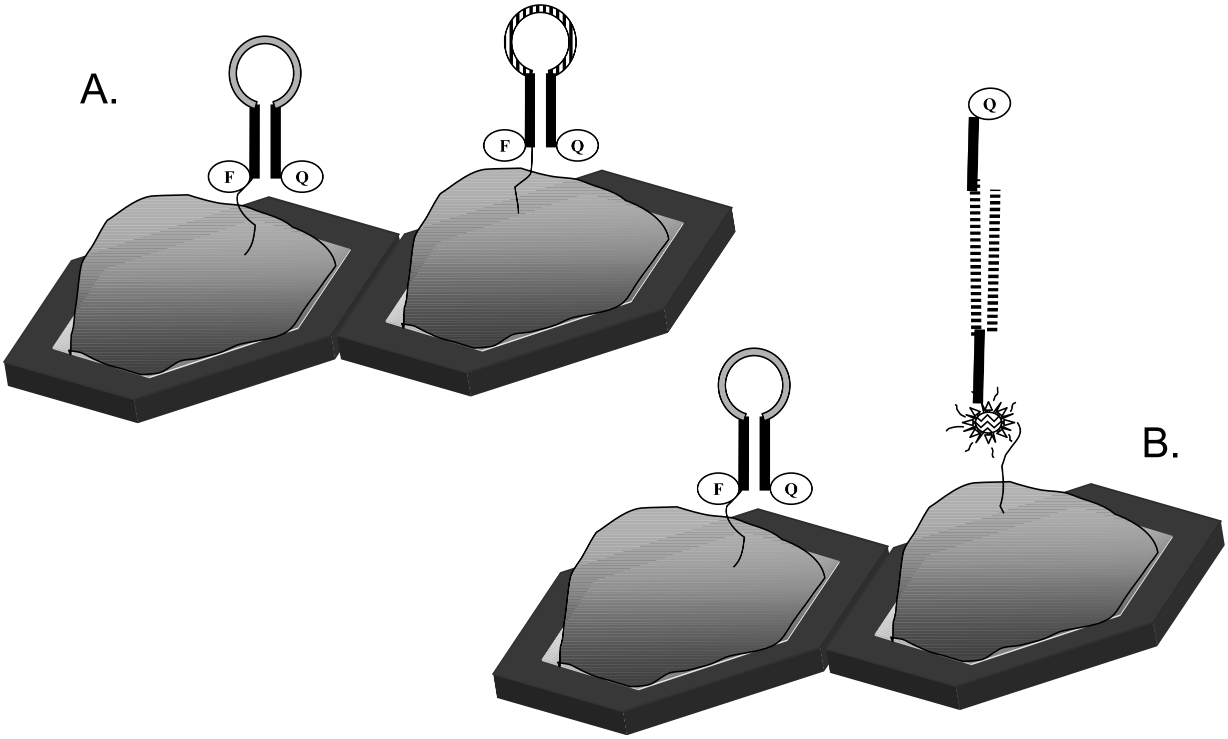

Fig. 3. Schematic diagram of molecular beacon probes attached to microspheres in microwells. (A) Each microsphere is associated with a different loop sequence, such that (B) in the presence of target sequences, the correct stem-loop structure will unfold, spatially separating the fluorophore and quencher to generate a signal.

1990). Nucleic acid sequences that demonstrate favorable binding interactions are subjected to a subsequent series of isolation and amplification steps. After each amplification stage, the nucleic acid aptamers are reexposed to the target. The overall process has been termed systematic evolution of ligands by exponential enrichment, or SELEX. The multiple SELEX cycles yield nucleic acid sequences with specific affinity for a known ligand based on the sequence's three-dimensional structure. These sequences are easily prepared and isolated and have binding affinities similar to antibodies. Sequences engineered with specific interactions for the identification of thrombin, an important biomolecule in blood clotting, were incorporated onto microspheres and used for rapid, regenerable detection assays (Lee and Walt, 2000).

4. Conclusions

The fiber optic microsphere-based biosensor is a versatile platform. The platform has micro-scale features and an overall array size that enables rapid analysis and extremely low detection limits. Redundant detection elements in the array increase the signal-to-noise ratio, and avoid the potential for false positive and false negative results. Microsphere-based arrays are reusable, and are easy to fabricate. The fiber optic platform has also been applied to other applications including artificial olfaction and cell-based array sensing.

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