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Time-resolved Förster-resonance-energy-transfer DNA assay on an active CMOS microarray

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ABSTRACT

We present an active oligonucleotide microarray platform for time-resolved Förster-resonance-energy-transfer (TR-FRET) assays. In these assays, immobilized probe is labeled with a donor fluorophore and analyte target is labeled with a fluorescence quencher. Changes in the fluorescence decay lifetime of the donor are measured to determine the extent of hybridization. In this work, we demonstrate that TR-FRET assays have reduced sensitivity to variances in probe surface density compared with standard fluorescence-based microarray assays. Use of an active array substrate, fabricated in a standard complementary metal-oxide-semiconductor (CMOS) process, provides the additional benefits of reduced system complexity and cost. The array consists of 4096 independent single-photon avalanche diode (SPAD) pixel sites and features on-chip time-to-digital conversion. We demonstrate the functionality of our system by measuring a DNA target concentration series using TR-FRET with semiconductor quantum dot donors.

1. Introduction

DNA microarrays have risen to prominence in genomics with a breadth of applications ranging from polymorphism and mutation detection (Peeters and Van der Spek, 2005) to forensic analysis (Divne and Allen, 2005). In epidemiology, they have been used to identify pathogens (Wilson et al., 2002), to characterize benzene poisoning (Forrest et al., 2005), and for genotyping virus strains (Song et al., 2006). They can be used to evaluate the progression of many diseases, including leukemia (Schroers et al., 2005) and ovarian cancer (Motamed-Khorasani et al., 2007). They are also a valuable tool in pharmacogenetics, for example, to screen chemotherapy drugs for efficacy and toxicity (Watters and McLeod, 2003) and for psychotropic drug development (Murphy, 2006).

In many regards, microarray technology has matured greatly since its introduction (Hoheisel, 2006). Current technologies allow up to one-million-site multiplexing and accurate single nucleotide polymorphism (SNP) detection (Chittur, 2004). Probe spot diameters are as small as 11 μm (Kawasaki, 2006). These achievements have been made possible through vast improvements in parallelism, throughput, and sensitivity resulting from advances in probe design, surface chemistry, probe deposition techniques, and statistical data analysis (Heller, 2002). Nevertheless, challenges remain with current microarray technologies.

One persistent issue is spot variability (Draghici et al., 2006), primarily determined by variance in probe immobilization (Auburn et al., 2005), which is affected by spotting time, temperature, humidity, spotting and hybridization solution composition, and inconsistencies in the microarray substrate surface itself (Mary-Huard et al., 2004). Replication is generally employed to enable statistical averaging of this variability but this reduces throughput (Ramakrishnan et al., 2002), and quantitative analysis is still restricted to relative rather than absolute concentrations of analyte (Hoheisel, 2006).

At the same time, the basic techniques of immobilizing probe on a passive glass substrate, hybridizing with fluorophore-labeled-target, and imaging in a laboratory-scale microarray scanner have remained fundamentally the same. This limits microarray applications to research environments due to the size and expense of detection hardware (Heller, 2002).

In a standard oligonucleotide microarray expression assay, known DNA probe sequences are bound to sites on a functionalized substrate and exposed to target analyte molecules modified with fluorescent labels. The microarray is then illuminated and the relative intensity of the fluorophore emission at each site is measured. This emission intensity is correlated to the quantity of bound target at each site and can be used to estimate the relative concentrations

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of target molecules complementary to each probe sequence (Heller, 2002).

A donor-only time-resolved Förster-resonance-energy-transfer (TR-FRET) oligonucleotide microarray assay differs from a conventional assay in that the fluorescent label is attached to the immobilized probe instead of the analyte target and its lifetime, rather than its intensity, is measured. To perform the assay, a "donor" fluorophore is attached to one end of an immobilized DNA probe molecule and a compatible fluorescence quencher is attached to the complementary end of each target molecule. Upon hybridization, a FRET interaction between the donor and quencher is induced, leading to a reduction in the donor fluorescence lifetime. The change in average donor lifetime can be used to determine the complementary target concentration for each probe sequence (Cardullo et al., 1988).

Whereas in a standard assay the signal is dependent on the number of bound target molecules, the signal in a TR-FRET assay is determined by the fraction of probe molecules associated with bound target. This leads to reduced signal variability with probe surface coverage. In addition, in a TR-FRET assay, unbound target in the hybridization solution does not affect the signal, obviating the need for rinsing before measurement.

Time-resolved fluorescence detection approaches applicable to TR-FRET assays have been developed for fluorescence lifetime imaging microscopy (FLIM) applications. Time-domain FLIM systems employ pulsed laser sources to stimulate fluorophores and one of two approaches for the detection of the induced fluorescence.

In the first approach, either a charge-coupled device (CCD) or complementary metal-oxide-semiconductor (CMOS) photodiode-based imager is employed. Gating is usually effected by an intensifier which precedes the imager (Wang et al., 1991; Dowling et al., 1997; Nicholas and Barry, 2006). By comparing the intensity integrated over time windows with varying offsets, the fluorescence decay function can be calculated. Complex multiexponential decay functions require repeated measurements, increasing overall measurement times (Sharman et al., 1999; De Grauw and Gerristen, 2001).

The second detection approach in FLIM is time-correlated single-photon counting (TCSPC), implemented by repeatedly stimulating a fluorophore and recording the time until the first emitted photon is detected by a sensor. A histogram of the number of photons arriving per time bin is constructed and as long as the probability of photon detection for each measurement is sufficiently low (no greater than one photon per approximately one hundred measurements), the histogram will correlate with the intensity decay profile of the fluorophore (Harris and Selinger, 1979). A TCSPC system requires a high-gain sensor capable of single-photon detection, usually a photomultiplier tube (PMT), and a method of accurately measuring time intervals. PMTs employ high electric fields and secondary electron emission effects to generate picoampere currents from incident photons. They are bulky, expensive, and not easily formed into arrays. As a result, laser scanning is necessary, complicating the optical setup and leading to a measurement time that scales linearly with imaged area (Becker et al., 2004).

An alternative to the PMT is the single-photon avalanche diode (SPAD), a solid-state device that converts arriving photons into measurable current pulses through avalanche breakdown of a semiconductor junction. Recently, SPADs have been fabricated in standard CMOS technologies (Rochas et al., 2002) enabling active two-dimensional arrays integrated with time-to-digital conversion circuits, eliminating the need for laser scanning and greatly reducing the complexity and cost of detector systems.

In this paper, we present a demonstration system that utilizes a custom SPAD-based CMOS imager as an active substrate for a TR-FRET assay measuring hybridization through fluorescence lifetime changes. The active CMOS microarray substrate, in which probe is immobilized directly on or above an array of detectors, replaces the traditional confocal scanner, allowing for an integrated, portable detection platform that incorporates data conversion and data processing on chip. Because we have designed a custom chip for this application, our design differs from previous work (Lamture et al., 1994; Vo-Dinh et al., 1999; Mallard et al., 2005) in which conventional imager chips were employed as active platforms. The unique capabilities of this chip for time-gated, time-resolved measurement enable TR-FRET applications. On-chip TCSPC with SPADs also allows for higher sensitivities than traditional imagers based on integrating photocurrents. The use of TR-FRET allows for the direct quantification of the fraction of bound target, reducing susceptibility to probe coverage variation.

2. Experimental

2.1. The CMOS active microarray

We have developed a 64-by-64 array of active SPAD sensor "pixels" with on-chip time-to-digital conversion and supporting circuitry. The details of the chip are described in Schwartz et al. (2007). The array is capable both of TR-FRET measurement using TCSPC and standard intensity-based measurement, which can be accomplished by computing the average photon arrival probability in a fixed time window after stimulation of the fluorophore. Used as an active array platform, probes are spotted directly on or above the surface of the chip. To facilitate this, the chip is assembled in a ball-grid-array package and encapsulated with a combination of Hysol FP4450 and Hysol FP4451 epoxies (Henkel KGaA, Düsseldorf, Germany), protecting the bonding wires from hybridization buffers and other solutions while keeping the sensor array of the chip exposed. Photographs of the packaged array appear in Fig. 1A.

The array is fabricated in a commercial 0.35-μm CMOS process and measures 4 mm × 4 mm. The active area of each SPAD sensor is 15 μm^2 and the pixel pitch is 40 μm. Accurate timing is maintained with a delay-locked-loop-stabilized multiphase clock. Each of the 64 columns of the array has an individual time-to-digital converter (TDC) which latches the arrival-time information of photons within that column of the array. The TDCs have a timing resolution of 350 ps. The noise floor is determined by an average dark count rate of 1059 Hz, corresponding to approximately two to four photons per one thousand measurements.

2.2. Lifetime measurement technique

Fluorescence lifetimes are measured using our array synchronized to a PiLAS 406-nm gain-switched diode laser (Advanced Laser Diode Systems, Berlin, Germany) with a DG-535 delay generator (Stanford Research Systems, Sunnyvale, CA, USA) and triggered at 5 kHz. The laser pulse has a full duration at half maximum of approximately 36 ps, and the average pulse energy is 40 pJ, corresponding to 8.2×10^7 photons. The beam is focused onto an area of approximately 4 mm^2, giving a photon flux density of 2×10^9 photons cm^{-2}.

Fig. 1B shows a simplified diagram of the measurement setup. A sample of analyte, such as DNA, could be immobilized directly on the chip surface. Instead, to ease surface preparation, we immobilize the sample on a glass slide which is carefully scored and broken to fit within the donut epoxy surrounding the array (see Fig. 1A) and then inverted over the sensor site, bringing the sample into contact with the chip surface and minimizing optical losses. As the measurement is time-resolved and the laser impulse is