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Label-less fluorescence-based method to detect hybridization with applications to DNA micro-array

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Abstract

By coupling scattered light from DNA to excite fluorescence in a polymer, we describe a quantitative, label-free assay for DNA hybridization detection. Since light scattering is intrinsically proportional to number of molecules, the change in (scattering coupled) fluorescence is highly linear with respect to percent binding of single stranded DNA (ssDNA) target with the immobilized ssDNA probes. The coupling is achieved by immobilizing ssDNA on a fluorescent polymer film at optimum thickness in nanoscale. The fluorescence from the underlining polymer increases due to proportionate increase in scattering from double stranded DNA (dsDNA) (i.e., probe–target binding) compared to ssDNA (i.e., probe). Because the scattering is proportional to fourth power of refractive index, the detection of binding is an order of magnitude more sensitive compared to other label-free optical methods, such as, reflectivity, interference, ellipsometry and surface-plasmon resonance. Remarkably, polystyrene film of optimum thickness 30 nm is the best fluorescent agent since its excitation wavelength matches (within 5 nm) with wavelength for the maximum refractive index difference between ssDNA and dsDNA. A quantitative model (with no fitting parameters) explains the observations. Potential dynamic range is 1 in 10^4 at signal-to-noise ratio of 3:1.

Keywords: DNA chip; Micro-array; Label-free; Label-less; DNA scattering; Biosensors

1. Introduction

Rapid, inexpensive gene sequencing and mutation analysis is the corner stone of future medicine with the potential to diagnose disease before clinical signs, evaluate the efficacy of an experimental drugs within months compared to years and personalize medicine. Micro-array technology (Fodor et al., 1991; Lockhart and Winzeler, 2000) has emerged as the best approach for gene sequencing and mutation analysis at high throughput and low cost. Although DNA micro-arrays are now widely used in laboratories (Bammler et al., 2005; Miklos and Maleszka, 2004) and instrumentation has been commercialized, there are certain areas which would improve the accuracy and reliability of the micro-array data. These include clinical sample preparation, array processing and hardware detection (Ji and Davis, 2006). Some of the challenges in the technique to measure binding are: (1) *Label interference*: In micro-array technology, the targets are labeled with fluorophore at several bases during the PCR process (Naef and Magnasco, 2003). The labels are known to interfere with the hybridization process as manifested by change in dehybridization or melting temperature, T_m (Haralambidis et al., 1987; Randolph and Waggoner, 1997). Because of this interference from the labels, on a typical DNA chip such as, human HG-U95A chip with 400 K spots, on average, 30% of probe-pairs have the differential-spot brighter than the redundant-spot (Naef et al., 2002). (2) *Background from non-specific binding*: This is one of the major challenges. The data (made available by Affymetrix Inc. on their web site, www.netaffx.com) on a 'calibration' chip, Human HG-U95A Latin Square with 40,000 spots (targeting 14 genes with over 14 probe set each) exposed to targets of known sequence and at 14 different concentrations (ranging from 0 to 1024 pM) was analyzed to show that: (a) the non-specific binding is significantly higher on spots where the probe–target duplex has higher melting temperature (i.e., lower free energy) (Held et al., 2003) and (b) the non-specific binding on differential

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spot can be larger than redundant-spot if the mismatch base is a G or a C, while opposite occurs for A or T (Hekstra et al., 2003). Because, all the PCR products have high number of fluorophores per molecule, the non-specifically attached molecules contribute significantly to the (fluorescence) signal (Hekstra et al., 2003). Because the estimated variation in background is over 8-fold due to difference in probe sequences (Held et al., 2003), the assumption of a constant average background (from non-specific binding) that is estimated by averaging the fluorescence over (the low brightness) differential-spots (Li and Wong, 2001; Naef et al., 2003; Sasik et al., 2002) is not valid. (3) *Non-linear device response*: Because the kinetics of hybridization of the target to the probe can be modeled as a Langmuir adsorption process (Nelson et al., 2001; Okahata et al., 1998; Peterson et al., 2002) and its modification (Vainrub and Pettitt, 2004), the relative concentrations of the target cDNA oligomers can be calculated from the number of hybridizations on the spot. To estimate the number of hybridization on a spot, it is assumed that the brightness of the spot (i.e., fluorescence intensity) is linearly proportional to the number of targets on the spot (i.e., the device response, defined as signal versus number of hybridization, is linear). However, in a chip with 6400 spots of (exactly) known number of Cy3 and Cy5 labeled cDNA (per spot) showed that the device response was highly non-linear over a 128-fold range of cDNA concentration (Ramdas et al., 2001). The non-linearity is attributed to quenching effects that occur because of the (well known effect of) reduction in extinction coefficient at high labeling densities (Randolph and Waggoner, 1997; Mujumdar et al., 1993). Furthermore, non-linearity can also occur because the number of fluorophore labels on the target has a distribution due to the random nature of polymerization during the PCR synthesis (Jacobs et al., 1999). (4) *Background correction* (*Optical*): Because the difference in wavelength between the excitation and emission light for most labels used in micro-array is <20 nm (http://www.idtdna.com/Catalog/Modifications/Modifications.aspx?catid=3), some light from the excitation beam due to scattering and reflection will contribute to measured intensity at the detector as an optical background. Because the fluorescence is at most 0.5% of the excitation intensity, the optical background from scattering can be very significant. A more critical but not reported difficulty (and also the central "working" principle of this report) is that, because there is a large difference in refractive index between ssDNA and dsDNA (Elhadj et al., 2004) this optical background due to scattering will depend on the amount of hybridization on a spot.

In this report we describe a label-free fluorescence method, proof of principle, which will address the four issues mentioned above. The interference effects will be avoided by generating the fluorescence contrast without labeling the target or the probe. The device response is expected to be linear because the change in fluorescence on binding is generated due to change in light scattering that is a colligative property (i.e., the change in scattering is proportional to number of hybridization on a spot). The background due to non-specific binding is negligible compared to the signal because the scattering from the non-specifically absorbed target is significantly less compared to hybridized target. The optical background is significantly reduced because the difference in excitation and emission background is 65 nm compared to <20 nm for conventional fluorescent labels used in micro-array. In this report the goal is to demonstrate the principle of the novel device rather than its extension to a DNA micro-array which is realizable because of it compatibility with current micro-array method in terms of chip-format. Thus, the study will discuss the characteristics and sensitivity of the detection method on a single spot rather than multiple spots in an array. Furthermore, to avoid the complexities from the biochemistry of the sample preparation, such as, functionalization of the surface for probe immobilization, and dependence of sequence on probe–target hybridization kinetics, the sensitivity will be defined in terms of percentage of probes on the surface that will hybridize to produce a detectable signal above the thermal and instrumentation noise and background from non-specific binding and instrumentation.

The method is based on transduction arising from two processes—scattering and fluorescence. Rayleigh scattering (Kocinski and Wojtczak, 1978) has been used to determine the conformation of DNA and polymers in solution (Fishman and Patterson, 1996). Rayleigh scattering shows an inverse fourth power dependence on wavelength and is proportional to the fourth power of the refractive index (discussed later in terms of Eqs. (1) and (2)). Because DNA has a strong absorption at ∼260 nm (i.e., Lorentz oscillator), the difference in refractive index at this wavelength is large (Elhadj et al., 2004). Therefore, when ssDNA hybridized to form dsDNA, the scattering cross-section of the molecule will change significantly due to fourth power dependence. By coupling the change in light scattering to the underlying polymer thin film tuned to fluorescence-excitation close to 260 nm, we have been able to fabricate a novel assay for detection of DNA hybridization. The method is compatible with micro-array platform where probe ssDNA molecules are tethered to the fluorescent polymer thin film deposited on a highly reflecting Si substrate. As the ssDNA probe specifically binds to the complimentary target ssDNA to form dsDNA, the fluorescence from the underlining polymer increases due to larger scattering from the latter. Fortuitously, polystyrene is ideal because its excitation wavelength is 265 nm. The best coupling of scattered light to the polystyrene film is obtained when the thickness of the polymer film is in the nanometer scale (∼30 nm).

Light scattering has been used as a method for DNA detection. However in all the methods reported, except for physical properties of DNA, such as molecular size and shape (Harpst and Dawson, 1989), intrinsic scattering of DNA has not been used to detect hybridization. For example, scattering from nanoparticle as labels on probe ssDNA have been shown to detect hybridization in solution where the probe is tethered on the particles which tend to from a network as it hybridizes with the target ssDNA leading to increased scattering (Du et al., 2006). In another experiment, 200 nm Se nanoparticles label on target ssDNA is used to detect hybridization by observing scattering from the particles by an evanescent wave coupled to the surface covered with a monolayer of immobilized probe ssDNA (Stimpson et al., 1995). Several promising optical methods, such as surface