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Direct detection of nucleic acid hybridization on the surface of a charge coupled device

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ABSTRACT

A method is described for the detection of DNA hybrids formed on a solid support, based upon the pairing of oligonucleotide chemistry and the technologies of electronic microdevice design. Surface matrices have been created in which oligonucleotide probes are covalently linked to a thin SiO_2 film. ^{32}P labeled target nucleic acid is then hybridized to this probe matrix under conditions of high stringency. The salient feature of the method is that to achieve the highest possible collection efficiency, the hybridization matrix is placed directly on the surface of a charge coupled device (CCD), which is used to detect ^{32}P decay from hybridized target molecules (1, Eggers, M.D., Hogan, M.E., Reich, R.K., Lamture, J.B., Beattie, K.L., Hollis, M.A., Ehrilich, D.J., Kosicki, B.B., Shumaker, J.M., Varma, R.S., Burke, B.E., Murphy, A., and Rathman, D.D., (1993), Advances in DNA Sequencing Technology, Proc. SPIE, 1891, 13–26). Two implementations of the technology have been employed. The first involves direct attachment of the matrix to the surface of a CCD. The second involves attachment of the matrix to a disposible SiO_2 coated chip, which is then placed face to face upon the CCD surface. As can be predicted from this favorable collection geometry and the known characteristics of a CCD, it is found that as measured by the time required to obtain equivalent signal to noise ratios, ^{32}P detection speed by the direct CCD approach is at least 10 fold greater than can be obtained with a commercial gas phase array detector, and at least 100 fold greater than when X-ray film is used for ^{32}P detection. Thus, it is shown that excellent quality hybridization signals can be obtained from a standard hybridization reaction, after only 1 second of CCD data acquisition.

INTRODUCTION

Hybridization of target nucleic acids to immobilized nucleic acid probes has been developed into a family of technologies (5,6). Recently, the possibility has also been discussed that nucleic acid sequence might be deduced by hybridization of an unknown target segment to a matrix of oligonucleotide probes (1,7–12). A similar technology has also been proposed for the analysis of mutational damage implicated in genetic diseases (13,14).

Implementation of these hybridization techniques has always been limited by the ability to detect very small amounts of bound nucleic acid target in a practical time scale, while retaining good selectivity with respect to base pairing of a surface-bound probe. Clearly, the most sensitive current methodology involves detection of radionuclide-tagged nucleic acid (1–14) by film emulsions, or by more sensitive methods such as the phosphorimager or gas phase array detector. Luminescence (15) or chemiluminescence (16,17,18) methods have also become practical alternatives.

Here, we explore an alternative approach for very high-sensitivity detection of radiolabelled nucleic acid. It has been known for some time that in addition to high sensitivity for photon detection, a charge coupled device (CCD) can be designed with almost perfect sensitivity for detection of high energy beta particles (1). Secondly, if a hybridization matrix were to be placed directly upon the surface of the CCD microdevice, roughly 1/2 of the total beta emission would be collected by the CCD array, thereby resulting in high collection efficiency as well (1). Therefore, we have reasoned that a direct CCD method offers the possibility of sensitive, quantitative, high speed detection of nucleic acid hybrids. Also, since CCDs typically comprise 100,000 to 200,000 individual detection elements per cm^2, which can be read into auxiliary computer hardware in less than a second, the methodology offers the capacity for efficient analysis of very dense arrays of bound nucleic acid probe and will be of a great practical value both in research and clinical diagnostics.

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MATERIALS AND METHODS

Oligonucleotides were custom synthesized by the betacyanoethyl phosphoramidite method and reverse phase HPLC purified by GenoSys Inc. 3'-Amino modified probes were synthesized by employing propylamine modified CPG (Cruachem). The epoxysilanizing reagent, 3-glycidoxypropyl- trimethoxysilane, and analytical grade diisopropylethylamine, xylene and potassium hydroxide were purchased from Aldrich and used without additional purification. Other solvents were from J.T. Baker. The reaction vessels were 10 mL glass vials with stoppers, usually presilanized with dimethydichlorosilane/triethylamine.

Hybridization matrices were formed directly by covalent coupling of probe upon the SiO_2 coating at the surface of a CCD. Alternatively, matrices were formed upon chips comprising a thin layer of SiO_2 coated on 1cm×1cm silicon wafers. The CCD detector described here is a 1cm×1cm 420×420 pixel frame-transfer array device, designed and fabricated at MIT Lincoln labs. Simple SiO_2 coated silicon wafers were also prepared at Lincoln labs. [γ-^{32}P]ATP (6000 Ci/mMol) was purchased from DuPont, Nen. T4-kinase and the buffers were from Boehringer Mannheim.

Probe immobilization on the CCD surface

Oligonucleotide probes were attached to the SiO_2 coated wafer surface by secondary amine formation between an epoxide monolayer and the 3'amine linker which had been synthesized onto the oligonucleotide. In one implimentation, the SiO_2 coating is deposited directly upon the CCD and hybridization is performed directly upon the CCD device. In a second implimentation, a disposible SiO_2 wafer is employed, which is then placed face to face upon the CCD, subsequent to hybridization and washing. Attachement chemistry is the same in both instances.

Epoxide modification. SiO_2 coated silicon chips were cleaned by sonication (Branson-2200) for 12 minutes in each of hexane, acetone and ethanol, dried at 80°C for 5 minutes and epoxysilanized (8,20), as described by Southern et.al. at 80°C overnight, using 25% 3'-glycidoxy propyltrimethoxysilane in dry xylene containing a catalytic amount of diisopropylethylamine. The chips were then washed thoroughly with ethylacetate and used immediately.

Probe coupling. The 3' amine modified oligonucleotide probe was applied as a 5μL microdroplet to the epoxysilanized surface of the chips and incubated at 37°C for 6 hours in air regulated at 100% relative humidity. Concentration was varied from 5−50 μM in 0.1M KOH. Finally the droplets were allowed to go to dryness in the air at 37°C. Unreacted probe was then removed by washing with H_2O at 50°C for 15 minutes with constant shaking. Chip-bound hybridization matrices were then air dried.

Radiochemical determination of nucleic acid surface density

Covalent coupling of amine modified probes to the surface of a silicon chip, or subsequent binding of complementary targets to these probes was obtained by labelling of oligonucleotides with ^{32}P at their 5' terminus. Labelling was performed with [γ-^{32}P]ATP and T4 polynucleotide kinase, followed by deproteinizization by phenol extraction and desalting by Sephadex G-15 chromatography. Specific activity of the purified oligonucleotides was determined by scintillation counting and absorbance spectroscopy. Probe density tests employed a 3'-propylamine modified 36-mer: 5'-TTGTGGTGGTGGTGTG-GTGGTGGGGTTGGGTGGTGG-3'A or its counterpart without a terminal amine. Radiochemical density on surfaces was measured with a Betascope 603 gas phase array analyzer (Betagen, Inc.). Raw data, comprising decays/minute/cm^2 were converted to molecules/mm^2, by inclusion of the specific activity.

CCD analysis of nucleic acid surface density

Data were accumulated by direct CCD readout into standard storage and processing hardware (Pulse Instruments 4800a Data generator, LeCroy Waveform Recorder, and a MicroVax 3100 workstation with IDL image processing software). Data were signal averaged over 0.5 second. Two 0.5 second data sets were subtracted to form the image, yielding an overall accumulation time of 1 second. This subtraction process removes the average parasitic signal accumulated from the CCD dark current.

Nucleic acid hybridization on a SiO_2 film

For both radiochemical and CCD detection, target oligonucleotide solutions were prepared at 10^{-7}M in the standard hybridization buffer (3M TMAC, 60mM Tris, 6mM EDTA and 0.03% SDS, pH 7.8), at a volume activity of approximately one million CPM/μL. Chips with attached probes were prehybridized for 2 hours at 4°C, with constant mild shaking, in the standard hybridization solvent plus 5×Denhardt solution, minus added nucleic acid. After prehybridization, the solvent was decanted, target solutions applied to the chip surface array and incubated for 2 hours at 4°C. The chips were then washed with the hybridization solvent at 4°C for 15 minutes, followed by air drying then detection by either film, the Betascope or CCD methods.

RESULTS AND DISCUSSION

Surface chemistry

Short oligonucleotides are useful as immobilized hybridization probes, especially for detection of single base sequence changes in a target nucleic acid (19), as would result during analysis of point mutation. For studies with the CCD detector, we have found

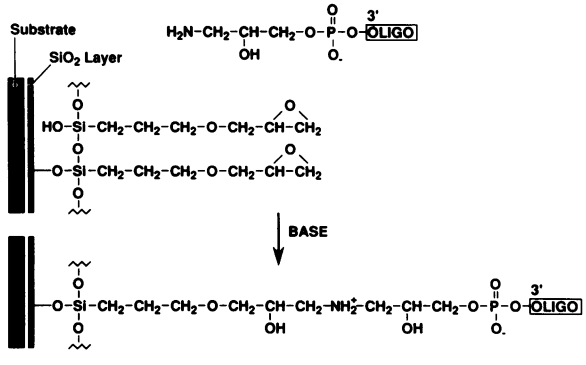

Figure 1. Probe attachment to SiO_2 surface. 3'-Amino modified probes were attached to a thin SiO_2 film on the surface of a silicon chip. Attachment occurs by secondary amine formation between an epoxysilane monolayer and the 3'amino linkage.

it useful to create such hybridization matrices upon a SiO_2 film which had been deposited on the surface of a thin, disposable silicon wafer (chip). Subsequent to target hybridization, the chip-bound matrix is dried and placed directly, face to face, upon the surface of the CCD. Alternatively, the CCD is modified directly and, thus, is dedicated to analysis of a single probe matrix.

To immobilize probes upon the SiO_2 coating, a uniform epoxide layer is linked to the film surface, employing an epoxysilane reagent and standard SiO_2 modification chemistry (8,20). As seen in Figure 1, amine modified oligonucleotide probes are then linked to the SiO_2 surface by means of secondary amine formation with the epoxide ring (21,22), most likely at the alpha carbon atom (23,24). The resulting linkage provides 17 rotatable bonds of separation between the 3′ base of the oligonucleotide and the SiO_2 surface. In order to ensure complete amine deprotonation and to minimize secondary structure formation during coupling, the reaction is performed in 0.1M KOH (25,26,27) and incubated at 37°C for 6 hours.

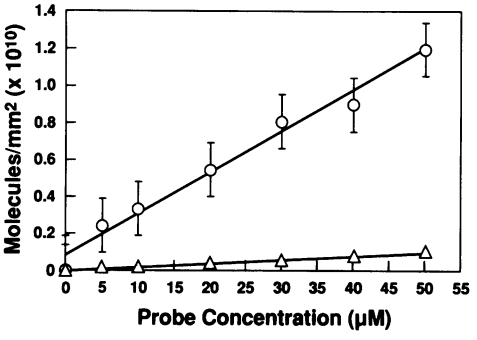

Figure 2. Covalent binding of oligonucleotides probes to a SiO_2 surface. The concentration dependence of probe coupling to the epoxysilane modified SiO_2 film was obtained by radiochemical methods with the [γ-^{32}P]ATP labelled 36-mer test oligonucleotide described in Materials and Methods. The radiochemical density of covalently linked probe was obtained by analysis on a gas phase array analyzer (Betagen, Inc.). Top: coupling of an amine-modified 36-mer probe. Bottom: coupling of 36-mer probe lacking a terminal amine.

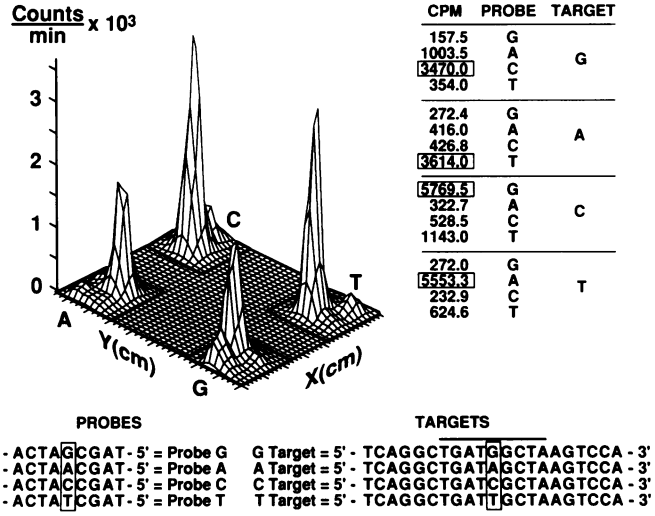

Figure 3. Hybridization selectivity on SiO_2 films. The hybridization field displayed in Part A has 16 elements. Four probes (as defined at the bottom) were attached by the chemistry described in Materials and Methods to form four identical quartets. One of the four 21 base targets, labelled with [γ-^{32}P]ATP, was hybridized to each quartet then washed at 4°C in hybridization solution. Arrays have been formed so that the innermost element of each quartet constitutes the correctly matched probe-target pair. Data were obtained after 30 minutes of data acquisition on a gas phase array detector. *Left*: The data have been presented in a 3 dimensional format. *Right*: The total radiochemical signal within each of the 16 quadrants is summed. As seen, a minimum discrimination factor of 3–10 was routinely obtained between specific and mismatched pairs. *Bottom:* The sequence of the four targets and four probes used in the study.

The probe surface density which can be achieved by this chemistry on the SiO_2 film has been analyzed with a gas phase array detector, employing a probe labeled with [γ-^{32}P]ATP at its 5′ terminus. As expected, the chemistry appears to be first order in probe concentration (Figure 2, upper curve), reproducibly yielding a surface density of 1×10^{10} molecules/mm^2 on the SiO_2 surface, at an added probe concentration of 50μM. This has been chosen as the standard probe coupling concentration for these studies. Identical experiments, employing probe which lacked a terminal amine displayed a 10-fold lower coupling rate (Figure 2, lower curve), confirm that amine modified probes have been coupled to the SiO_2 surface by secondary amine formation through the 3′ amino terminus, as proposed in Figure 1.

Hybridization detection: gas phase array detector

To confirm the selectivity of duplex formation upon a SiO_2 film, a matrix of four 9-mer probes was chosen, differing by only one base at their center (Figure 3, bottom). This probe matrix was then hybridized to a series of ^{32}P-labeled 21 base single stranded DNA targets which possess, at their center, a 9 base sequence which is complementary to each of the four 9-mer probes.

After attaching probes to the chip surface, hybridization to the solution state target DNA was performed using 3M tetramethyl ammonium chloride (TMAC) at 4°C, which is known to obviate the base composition dependence of duplex association and dissociation rates (8,28). After a 2 hour hybridization reaction, then washing at 4°C with 3M TMAC, hybrid formation upon the SiO_2 surface was analyzed by the gas phase array detector. Under these standard conditions, adequate signal was obtained after a 30 minute accumulation time (Figure 3, top), which is known to be about 10–20 times faster than the time required to obtain equivalent signal by analysis with enhanced x-ray film at −80°C (29,30,31).

Analysis of the sequence dependence of duplex hybrid formation (Figure 3) suggests that the probe coupling and target hybridization chemistries give rise to the expected one base pair mismatch selectivity. Relative to singly mismatched elements, perfect 9-mer associations (the innermost elements of the quadrants in Figure 3) display a 3–10 fold enhancement in hybridization rate, which is equal to the best discrimination that can generally be achieved in solution.

Hybridization detection: direct CCD readout

Finally, signals derived from duplex formation upon the SiO_2 film were analyzed by direct detection of the hybridization matrix with the CCD. The CCD detector described here is a 420×420 pixel frame-transfer array device which generates on average approximately $9\times10^{+4}$ electrons per impingent 1.7MeV ^{32}P beta particle. As such, it operates at room temperature as a quantum device capable of detecting single ^{32}P decay events. Due to reduction of dark current, that already good signal to noise

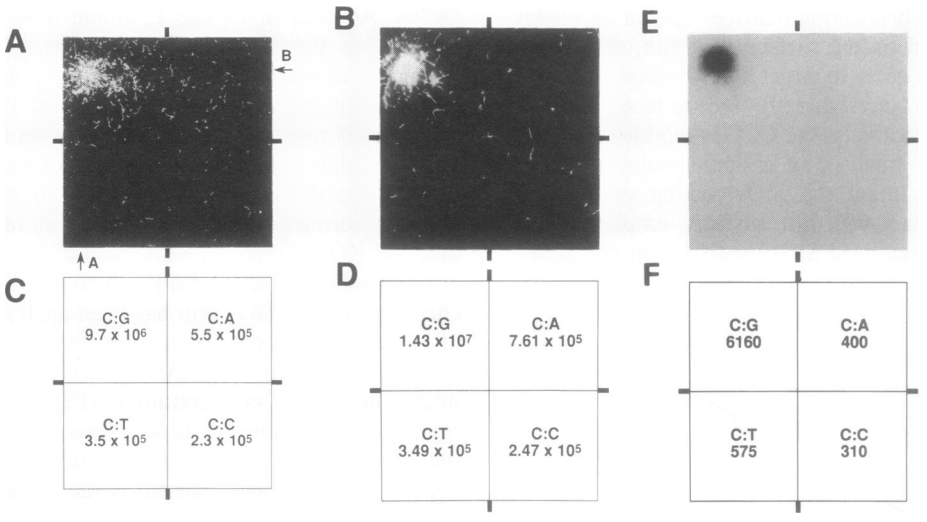

Figure 4. Hybridization detected by direct CCD imaging. The 9-mer probes described in Figure 3 were attached as a 2×2 matrix to the surface of a SiO_2 coated silicon chip. This matrix was then hybridized to target C under the conditions described in Figure 3. The SiO_2 coated silicon chip is then placed, face to face, on the CCD surface in such a way that the matrix carrying the duplexes is in direct contact with the CCD microdevice. **A** and **B**: CCD analysis of the hybridization matrix at room temperature and −40°C respectively. The two arrows in 4a indicate the direction of cross-section as described in figure-5. **C** and **D**: Summed CCD signal intensity within the four elements of the matrix. The total electron signal derived from a quadrant within the hybridization matrix was derived by summing over all pixels. The standard deviation associated with each quadrant shows that an adequate signal to noise ratio (approximately 13) has been obtained during the one second of total accumulation comprising the data set. **E**: Secondary analysis of the matrix with the gas phase detector. The hybridization matrix in part 4a, was reanalyzed with the Betascope, employing a 30 minute acquisition time at room temperature. **F**: Summed signal intensity within the matrix, as detected by the gas phase array detector. The radiochemical decay signal from the Betascope has been summed over each quadrant and is presented as total counts per minute.

characteristic is additionally improved when the CCD chip is operated at −40°C.

Figure 4 displays a quartet of hybridization reactions which were performed upon a disposible 1cm×1cm SiO_2 coated wafer. Subsequent to washing and drying, the wafer was placed face to face upon the surface of the 1cm×1cm CCD detector. Identical detection sensitivity was obtained by direct coupling of the array to the SiO2 coating of the CCD (data not shown). Coupling and hybridization conditions were as defined in Materials and Methods. Detection by the CCD array was performed at either room temperature (4a) or at −40°C (4b). It is found that adequate signal to noise was obtained at either detection temperature, subsequent to only 1 second of total data acquisition.

Figure 4c and 4d show the total integrated electron signal, as read from the CCD at each of the four elements of the hybridization matrix at room temperature or −40°C respectively. For each of the four, summation has been performed over all pixels within a quadrant. As seen, the total signal derived from the quadrant where an exact match had occurred (C:G, 4a, upper left) is at least 11 fold greater than seen at the 3 quadrants with a single mismatch, at either room temperature or −40°C. Very similar results were obtained when the hybridization matrix were analysed with the gas phase array detector (Figures 4e & 4f).

Visual inspection of the CCD image (Figure 4a/4b) or analysis of crossections through the image (Figure 5), suggest that target association is uniform within the 3 quadrants of the matrix in which mismatches might occur, even though the site of probe attachment is smaller than each quadrant. Therefore, much of the signal ascribed to mismatches is not due to nucleic acid-nucleic acid interaction and may instead reflect target binding to surface elements other than probe. If so, the data presented define a

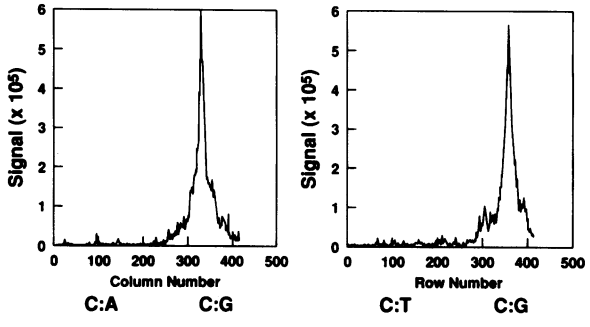

Figure 5. Signal Crossections through the 2 dimensional CCD data set. In order to better assess the distribution of signal derived from the hybridization matrix, the electron signal from individual CCD pixels (the one second, −40°C data set) has been displayed as a function of position. *Left*, a horizonal crossection beginning at site **B**, as defined in Figure 4a. *Right*, a vertical crossection beginning at site **A**, as described in Figure 4a. Symbols beneath the X axes identify the region of probe attachment in the image crossections.

conservative estimate of the specificity of nucleic acid base pairing on SiO_2 films at the surface of the CCD.

The efficiency of ^{32}P detection for the gas phase array detector has been estimated to be 0.1529, while that for X-ray film with intensifying screens at −80°C is near to 0.00130. CCDs are known to display efficiencies in the 0.9 to 1.0 range, with negligible thermal noise, relative to the signal from a single high energy beta particle (1).

The enhancement in the time of analysis of ^{32}P detection sensitivity presented in this study is consistent with the nearly

perfect signal to noise characteristics of the direct CCD approach for detection of ^{32}P-tagged nucleic acids.

CONCLUSIONS

By monitoring nucleic acid hybridization directly on the surface of a CCD device, it is shown that, as assessed by the time required to achieve comparable signal to noise, the required for DNA hybridization detection by radiochemical methods can be increased approximately 10 fold, relative to the most sensitive alternative technology (gas phase array detector) and greater than 100-fold greater than X-ray film. It is shown that this speed can be obtained on SiO_2 coated chips without sacrificing the intrinsic base mismatch specificity of duplex formation.

In parallel, the disciplines of nucleic acid chemistry and microelectronic device design have grown into sophisticated technologies. Therefore, although the device described in this paper is crude, we believe that the two technologies upon which the device is based provide sufficient latitude that a families of fast, very high sensitivity electronic devices can now be developed for routine detection of nucleic acid hybridization.

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