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Real-time detection of DNA hybridization and melting on oligonucleotide arrays by using optical wave guides

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ABSTRACT **The challenge of the Human Genome Project is to increase the rate of DNA sequence acquisition by two orders of magnitude to complete sequencing of the human genome by the year 2000. The present work describes a rapid detection method using a two-dimensional optical wave guide that allows measurement of real-time binding or melting of a light-scattering label on a DNA array. A particulate label on the target DNA acts as a light-scattering source when illuminated by the evanescent wave of the wave guide and only the label bound to the surface generates a signal. Imaging/visual examination of the scattered light permits interrogation of the entire array simultaneously. Hybridization specificity is equivalent to that obtained with a conventional system using autoradiography. Wave guide melting curves are consistent with those obtained in the liquid phase and single-base discrimination is facile. Dilution experiments showed an apparent lower limit of detection at 0.4 nM oligonucleotide. This performance is comparable to the best currently known fluorescence-based systems. In addition, wave guide detection allows manipulation of hybridization stringency during detection and thereby reduces DNA chip complexity. It is anticipated that this methodology will provide a powerful tool for diagnostic applications that require rapid cost-effective detection of variations from known sequences.**

Sequencing by hybridization (SBH) is a revolutionary technique for the generation of nucleic acid sequence information (1–6). A single hybridization experiment allows examination of a large number of different sites on a DNA molecule. Diagnosis of several human genetic conditions such as Duchenne muscular dystrophy (7, 8) or cystic fibrosis (9) will require the resolving power of an SBH type system to determine the mutation associated with the disease state in a cost-effective manner. One case in point is cystic fibrosis where >300 mutations have been identified (10).

SBH uses a large number of oligonucleotides immobilized in a high-density two-dimensional array and is particularly suited to multiplex applications. Such a device has been called a "DNA chip" analogous to the high-density circuits produced by the electronics industry (11, 12). A sample of unknown DNA is applied to the chip and the pattern of hybridization is determined and analyzed to obtain sequence information (13, 14).

Most of the DNA hybridization detection methods employed thus far use radioactive (15–20), enzyme-based chemiluminescent (21), or fluorescent (22) labels. Detection and measurement can be accomplished with phosphor systems (23) or, for the latter two labels, with charge-coupled device (CCD) cameras (24), cooled CCD cameras, image intensifiers coupled to CCD cameras, or a photomultiplier tube coupled with mechanical raster scanning (12, 25). In a typical SBH experiment, a labeled DNA sample is applied to the chip to allow hybridization. Excess label/DNA may be washed from the chip surface to minimize background. By use of a confocal microscope, the measurement of the fluorescent light is confined to the surface of the chip and the washing step is not required (26). Because the amount of fluorescent label on the surface of a chip is quite low, the time required to scan the array is on the order of 1 min. Such integration times are also typical for cooled CCD camera systems. Much higher DNA densities can be achieved by using a gel matrix and in this case the fluorescent signal can be read at standard speed with a low-sensitivity CCD camera (24). However, the gel system affects the kinetics of hybridization/melting through multiple binding events in the three-dimensional matrix of immobilized DNA and requires a washing step (27).

Melting curves could provide an additional dimension to the system and allow differentiation of closely related sequences, a concern in implementation of SBH technology (28). The ability to change temperature and monitor the chip hybridization patterns would also be useful in cases where there is a wide variation in GC content and may obviate the need for agents like tetramethylammonium chloride (17). However, if 1 min is required to read/wash a DNA chip, then a high-resolution melting curve from 30 to 70°C would require 40 min; i.e., measurement is rate limiting. Removal of background signal would require some sort of washing system to eliminate the label as it dissociates from the capture site.

The present report suggests the use of a two-dimensional optical wave guide and light scattering labels to detect hybridization patterns. While generation of scattering signals using an optical wave guide is not new (29), to our knowledge, the use of a wave guide with an array of binding sites has not been reported. The evanescent wave created by the wave guide is used to scatter light from a particulate label adsorbed at multiple DNA capture zones placed on the wave guide surface. Since an evanescent wave only extends a few hundred nanometers from the wave guide surface (30), the unbound/dissociated label does not scatter light and a wash step is not required. The signal intensity is sufficient to allow measurement of the surface binding and desorption of the light scattering label can be studied in real time; i.e., detection is not rate limiting. The hybridization pattern on the chip can be evaluated visually or acquired for quantitative analysis by using a standard CCD camera with an 8-bit video frame grabber in 1/30 of a second.

MATERIALS AND METHODS

Chips. DNA chips for radioactive detection were prepared by washing glass microscope slides with Ivory soap (Procter & Gamble), rinsing with water, heating at 80°C with concentrated sulfuric or nitric acid for at least 30 min, rinsing, and storing

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Abbreviations: SBH, sequencing by hybridization; CCD, charge-coupled device.

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in deionized water. Slides were then treated with 1% glycidoxypropyl silane (Aldrich) in 95% ethanol (pH adjusted to 5.5 with sulfuric acid) for 1 h. Excess silane was removed by dipping the slide in 95% ethanol for 1 min, and the slide was dried by baking at 150°C for 20 min. Silane-treated slides were cooled and stored in an argon-filled desiccator until use. The 3′-amino linked oligonucleotides (Table 1) from Synthecell were diluted to 50 μg/ml in PBS (10 mM sodium phosphate, pH 7.4/120 mM NaCl/2.7 mM KCl; Sigma), placed as 1-μl drops on the slides, and allowed to dry. After at least 1 h, the slides were rinsed and stored in TE (TE = 10 mM Tris·HCl, pH 7.5/0.5 mM EDTA). DNA density was 30–300 molecules per μm^2 as determined by autoradiography with oligonucleotides of known specific radioactivity.

DNA chips for wave guide detection were constructed by using presynthesized 3′-amine-labeled oligonucleotides obtained from Synthecell or Genosys. Glass substrates (no. 2 microscope cover slides, Corning, or equivalent) were cleaned by soaking in 2 M NaOH for 1 h followed by rinsing with HPLC grade water (Fisher). The glass was protein-coated by application of 0.05% casein (Abbott) for 1 min. The casein solution was flushed from the surface by using a wash bottle. The 3′-amine oligonucleotides were rehydrated with 50 μl of water (Table 1) and then diluted 1:20 into PBS for spotting onto the casein-coated slides. The oligonucleotide solutions were applied to the slide with a pipette in a 0.5-μl spot or by dipping the flat end of a drill blank "pin" (HSS 67, Hayden Twist Drill, Warren, MI) into the DNA solution and touching it to the slide surface. The latter procedure was automated by using an X-Y-Z table (Asymtek). After drying, the excess DNA was washed from the chip. In some cases a second glass slide was fixed to the wave guide to form a channel that holds the sample or conjugate solutions (Fig. 1*A*).

Selenium Colloid and Conjugate. Selenium colloid was produced by addition of 4 ml of 1% sodium ascorbate (Sigma) to 200 ml of boiling water followed by addition and rapid mixing of 2 ml of 1% selenium dioxide (Aldrich) (32). The colloid used in these studies had a particle size of ≈0.2 μm, a pH of 5.4, an OD_{546} of 32, and an absorption maximum at 546 nm. Selenium-antibody conjugate was prepared fresh before use by mixing of 2.5 μl of anti-biotin (polyclonal rabbit anti-biotin, 1.13 mg/ml in PBS) to 1 ml of selenium colloid followed by addition of 30 μl of 20% (wt/vol) bovine serum albumin (Sigma).

Hybridization and Staining for Wave Guide. Solutions of 3′-biotinylated DNA (Table 1) were diluted 1:1000 into 1% casein/10 mM Tris·HCl, pH 7.8/15.4 mM NaCl and applied to the surface of the chip for 5 min at room temperature (23–25°C). The DNA solution was drained off and, without allowing the surface to dry, a solution of anti-biotin selenium

Table 1. DNA sequences for chip construction and hybridization experiments

DNA no.	Sequence	Concentration, μM
	Immobilized on the chip	
1	5′-TATCATCTTTGGTGT-3′-NH_2 (ΔF508WT)	139
2	5′-AATATCATTGGTGTT-3′-NH_2 (ΔF508)	169
3	5′-AGTGGAGGTCAACGA-3′-NH_2 (G551D WT)	154
4	5′-AGTGGAGATCAACGA-3′-NH_2 (G551D)	282
5	5′-AGGTCAACGAGCAAG-3′-NH_2 (R553X WT)	127
6	5′-AGGTCAATGAGCAAG-3′-NH_2 (R553X)	139
7	5′-TGGAGATCAATGAGC-3′-NH_2 (G551D + R553X)	31
8	5′-TGGAGATCAACGAGC-3′-NH_2 (G551D + R553X WT)	18
9	5′-TGGAGGTCAATGAGC-3′-NH_2 (G551D WT + R553X)	74
	Complementary sequences for radioactive detection	
11	5′-ACACCAAAGATGATA-3′	112
12	5′-AACACCAATGATATT-3′	119
13	5′-TCGTTGACCTCCACT-3′	219
14	5′-TCGTTGATCTCCACT-3′	214
15	5′-CTTGCTCGTTGACCT-3′	226
16	5′-CTTGCTCATTGACCT-3′	200
17	5′-GCTCATTGATCTCCA-3′	199
18	5′-GCTCGTTGATCTCCA-3′	60
19	5′-GCTCATTGACCTCCA-3′	167
	Biotinylated complementary sequences	
21B	5′-ACACCAAAGATGATA-3′-biotin	356
22B	5′-AACACCAATGATATT-3′-biotin	208
23B	5′-TCGTTGACCTCCACT-3′-biotin	396
24B	5′-TCGTTGATCTCCACT-3′-biotin	473
25B	5′-CTTGCTCGTTGACCT-3′-biotin	473
26B	5′-CTTGCTCATTGACCT-3′-biotin	459
27B	5′-GCTCATTGATCTCCA-3′-biotin	151
28B	5′-GCTCGTTGATCTCCA-3′-biotin	259
29B	5′-GCTCATTGACCTCCA-3′-biotin	225

Sequence identification number, sequence, and DNA concentrations obtained from the vendor are shown. DNA chips were constructed with 15-mer oligonucleotides to detect three mutations involved in cystic fibrosis. Note sequences 1 and 2 have little in common with sequences 3–9. Three human genetic mutations involved in cystic fibrosis are indicated in parentheses by standard notation. ΔF508 indicates a 3-bp deletion that results in removal of Phe-508 of the cystic fibrosis transmembrane conductance regulator polypeptide (31). The G → A change at codon 551 results in a change from Gly to Asp (G551D) and the C → T change results in a stop codon in place of the normal codon for Arg (R553X) (16). WT indicates the wild-type or normal sequence at each position. For each single mutation, the wild-type sequence was also present on the chip. In addition, sequence 7 contained a double mutation. While not physiologically relevant, the double mutation was included to evaluate chip performance. Complementary oligonucleotides 11–19 for ^{32}P-end-labeling and oligonucleotides 21B–29B with 3′-biotin labels for wave guide detection were synthesized.

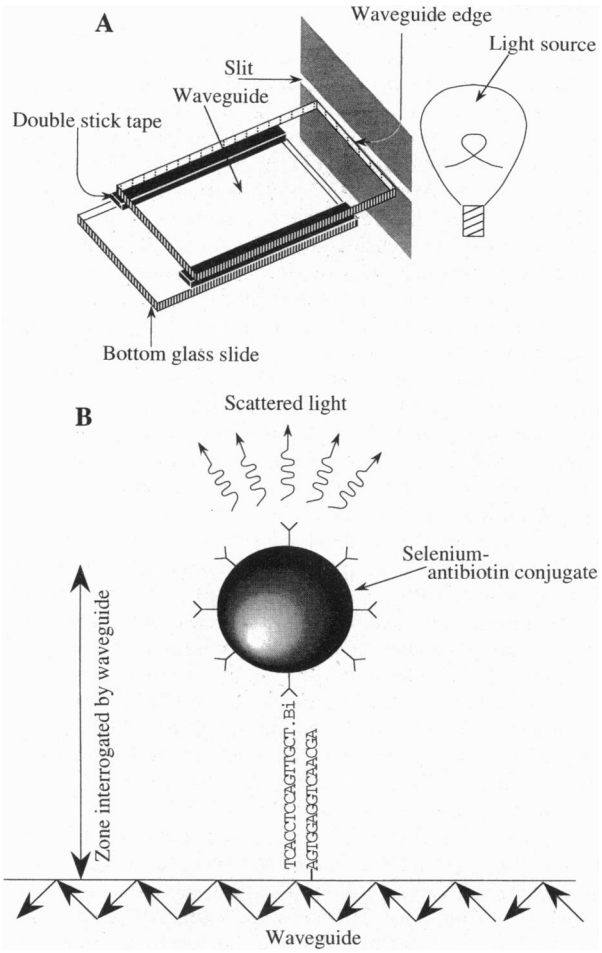

FIG. 1. (*A*) Wave guide glass slide with a bottom glass slide joined with double-stick tape to form a channel 75 μm thick and 2.54 cm wide. The DNA capture zones are placed on the upper wave guide slide. The DNA solutions or the anti-biotin conjugate are introduced into channel by capillary action. Light is injected into the edge of the wave guide and propagates by total internal reflection [zig-zag pattern (*B*), not drawn to scale!]. An evanescent wave is created on the wave guide, and light is scattered when particulate label binds to the surface. The light scattering signal is viewed/imaged from above.

conjugate was applied for 1–5 min. For the channel, the DNA solution is introduced by capillary action. The conjugate solution was then applied as a pool at one end of the channel, a paper towel was placed at the opposite end, and the conjugate solution replaced the DNA solution. When a single slide was used, 50 μl of DNA solution was applied to the chip, incubated 5 min, drained, and rinsed with PBS, and a drop of conjugate solution was applied to stain. In this case, the chips were washed and then stored in PBS in a Petri dish before imaging by wave guide illumination.

Hybridization for Autoradiography. Oligonucleotides were end-labeled with ^{32}P by reacting 0.5 μg of DNA with 5 μl of 10× New England Biolabs kinase buffer, 30 μCi of [^{32}P]ATP (3000 mCi/mmol; 1 Ci = 37 GBq; Amersham), and 20 units of New England Biolabs T4 polynucleotide kinase in a total volume of 50 μl for 30–60 min at 37°C. Hybridizations were carried out in seal-a-meal pouches with 2.5 ml of 6× SSC/0.5% SDS (6× SSC = 900 mM NaCl/100 mM sodium citrate, pH 7.0) for 16 h. The slides were washed four times with 6× SSC, dried, and exposed to Kodak XAR5 film for 1–2 h.

Wave Guide Detection. Light from a 150-W lamp (Dolan Jenner Fiberlite series 180) was injected into the end of the wave guide by use of a slit (Fig. 1*A*). Light scattering from the selenium conjugate adsorbed on the wave guide chips was observed visually and was also recorded by using a Cohu CCD camera (Cohu model 4815) operating at 30 frames per sec. Output from the camera was either fed into a video cassette recorder for later analysis or directly into an 8-bit frame grabber (Imaging Technology, PC Vision plus). The single digitized images were analyzed by using the software IMAGE MEASURE (Phoenix Technology, Seattle), IMAGEPRO PLUS (Media Cybernetics, Silver Spring, MD), or NIH IMAGE (obtained from the FTP Internet site zippy.nimh.nih.gov/pub/nih-image).

For the wave guide melting studies, an aluminum heating block, 1.5 × 1.5 × 0.25 inches (1 inch = 2.54 cm), containing two heating elements and a thermocouple was placed beneath the wave guide (Watlow 965 demonstration unit, Winona, MN).

Liquid Melting Studies. Melting temperatures in liquid were measured with a Hewlett–Packard model 8452A spectrophotometer. The single-stranded DNA was diluted to an A_{260} of 0.05–0.1 in 10 mM Tris·HCl (pH 7.8) with 1 M NaCl or 0.15 M NaCl (0.5–1 μM DNA).

RESULTS

Fig. 1*B* shows the basic principle involved in signal generation with the wave guide DNA chip. Light is injected into the end of the wave guide and propagates by total internal reflection thereby creating a uniform two-dimensional evanescent wave. The evanescent wave extends 100–300 nm into the solution above the wave guide. Thus, only the selenium particles that are in close proximity to the wave guide surface scatter light. Selenium particles are concentrated on the wave guide surface via DNA hybridization and the subsequent binding of the conjugate to biotin. Signals appear as bright spots on a darker background wherever DNA is hybridized. Experiments with 70-nm gold particles produced scattering signals that were much less intense than the 200-nm selenium particles.

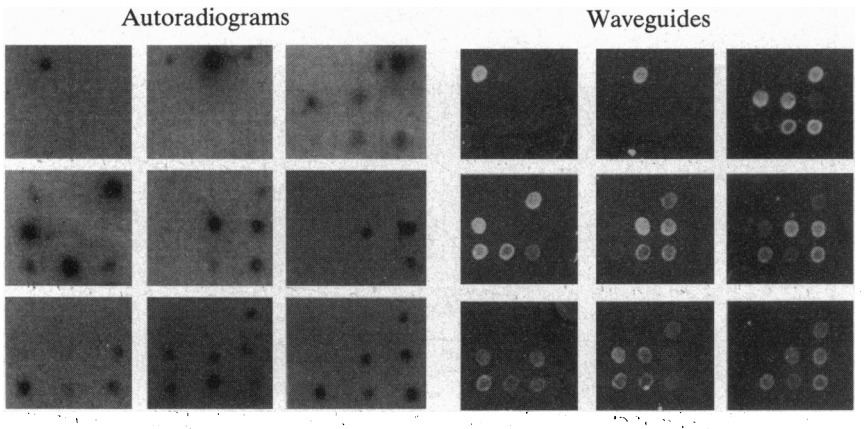

FIG. 2. Comparison of hybridization patterns obtained by optical wave guide and autoradiography. Sequences 1–9 were immobilized to form 3 × 3 DNA chips so that row 1 had sequences 1–3, row 2 had sequences 4–6, and row 3 had sequences 7–9. The chips were incubated with each of the nine complementary oligonucleotides. (*Left*) Results from ^{32}P-labeled sequences 11–19. A dark zone on a light background indicates hybridization. (*Right*) The wave guide results with oligonucleotides 21B–29B. The chips are arranged as in Table 2. The wave guide signal is a bright zone on a dark background. In this case, the wave guide chips did not have a second glass slide to form a channel and, hence, were washed with PBS and submerged in a Petri dish containing PBS for imaging.

Table 2. Theoretical melting temperatures computed for the various combinations of DNA sequences

Seq. 11 or 21B			Seq. 12 or 22B			Seq. 13 or 23B		
50	27	<0	24	47	<0	<0	<0	58
<0	<0	<0	<0	<0	<0	52	35	25
<0	<0	<0	<0	<0	<0	37	44	44
Seq. 14 or 24B			**Seq. 15 or 25B**			**Seq. 16 or 26B**		
<0	<0	49	<0	<0	35	<0	<0	20
56	20	10	25	58	52	10	49	56
41	49	33	33	41	41	38	29	46
Seq. 17 or 27B			**Seq. 18 or 28B**			**Seq. 19 or 29B**		
<0	<0	30	<0	<0	41	<0	<0	41
38	26	34	49	38	29	33	38	46
56	49	49	52	58	45	52	45	58

Theoretical melting temperatures (T_m, °C) were computed for hybridization products formed between sequences 11 and 19 or 21B and 29B and each of the nine immobilized sequences on the chip. The melting temperatures were calculated by using the equation: $T_m = 81.5 + 16.6 \log[Na] + 0.41(\%\,GC) - 675/(length) - (\%\,mismatch)$, with [Na] = 1. The computed melting temperatures are spatially arranged to correspond with the location of DNA capture zones displayed in Fig. 2.

The DNA sequences 1–9 were used to form 3 × 3 arrays of capture sites. Each of the complementary sequences were incubated with a chip and the hybridization patterns were detected by using autoradiography or the optical wave guide (Fig. 2). The patterns of hybridization were similar for the two detection methods. In most cases the wave guide signals were visible as soon as the selenium conjugate solution covered the chip. Incubation of conjugate for periods longer than ≈5 min did not result in an appreciable increase in signal intensity at any of the spots.

Only DNA complementary to zones 1 and 2 gave simple hybridization patterns. The other more complicated patterns for hybridizations 23–29 are consistent with the sequence homologies and each pattern is indicative of the particular target. By using the equation described in the program OLIGO 4.0 (National Biosciences, Plymouth, MN), the theoretical melting temperatures (33) were computed and are displayed in Table 2. There is a qualitative correlation between the theoretical melting temperatures (Table 2) and the hybridization patterns (Fig. 2). Apparently, pattern analysis of chips with multiple probes of different effective lengths, sequences, and therefore, different hybridization constants is a method of resolving targets that differ by one or two bases.

Hybridization under low-stringency conditions does not allow for discrimination of single-base mutations when analysis is limited to the wild-type and mutant probes; compare zones 3 and 4 when hybridized with sequence 23B or 24B and zones 5 and 6 when hybridized with sequence 25B or 26B. The single-base change in the middle of a 15-mer results in similar binding to both the exact matching and the mismatched zones when hybridization is carried out at room temperature.

Fig. 3 shows the results of an experiment in which a single-base change can be resolved with a two-zone chip in ≈5 min via melting. DNA chips with two spots, sequences 3 and 4, were hybridized with sequence 23B or 24B, so that in each case an exact match and a single-base mismatch was present. The wave guide scattering signals appeared in a matter of seconds after application of the selenium conjugate and stopped increasing after 2–3 min. Some of the initial increase can be seen in the data for sequence 24B (Fig. 3) as heating was initiated before signal development stopped. Since DNA was already hybridized, the initial increase in light scattering was due to the binding of selenium conjugate to the biotinylated DNA captured on the wave guide surface. As the DNA duplex dissociates, the attached selenium conjugate diffuses out of the evanescent wave and the scattering signal decreases. In each experiment, the exact matching zone (i.e., zone 3 for 23B and zone 4 for 24B) displayed the higher melting temperature and was the last to disappear. Our

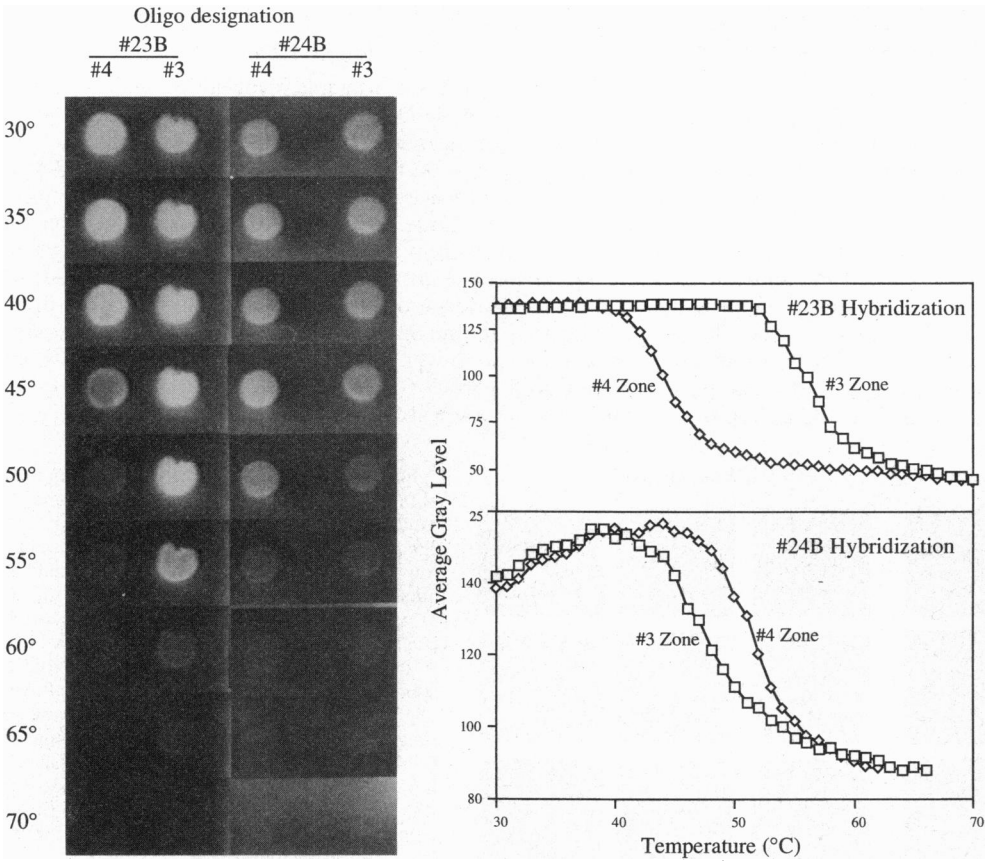

FIG. 3. (*Right*) Chips with two capture zones, sequences 4 on left and 3 on right, were incubated with sequences 23B or 24B as indicated. Selenium conjugate was then introduced into the channel and the wave guide signal was allowed to develop for 2–3 min. (*Left*) The temperature was increased and the signal intensity at each spot was measured at 1°C increments to produce the DNA chip melting curves. Images of the chip were captured at 5°C increments and are displayed next to the temperature (in °C). The selenium conjugate remained in the channel throughout the experiment.

Table 3. Effect of a single-base mismatch on melting temperature

Sequences in duplex formed	Melting temperature, °C: Liquid 1 M NaCl	Liquid 15.4 mM NaCl	Wave guide chip
3–13/23B	68	47	57
4–13/23B	54	34	45
3–14/24B	59	37	48
4–14/24B	66	43	52

Melting temperatures were measured in liquid by using UV adsorption and on the wave guide by using selenium anti-biotin label. Sequences 13 and 14 were used in the liquid melting experiments and the biotinylated analogs 23B and 24B were used for the wave guide melting experiments. The concentration of Na^+ in the wave guide experiment is >15 mM due to the presence of Na present in the casein; the final Na concentration is not known.

interpretation of this data is that the DNA duplex is the most thermal-labile noncovalent interaction in the system.

The melting temperature was estimated as the midpoint between the high and low points of the transition. Table 3 shows the chip results compared with the analogous experiments in liquid at two ionic strengths. The chip melting temperatures correlate with the liquid melting temperatures for 15.4 mM and 1 M NaCl conditions, albeit ≈10°C different in each case. Both the chip and liquid melting curves show a greater difference in the effect of the mismatch for the 23B hybridization than for the 24B hybridization as expected from the relatively stable mismatch.

Sensitivity of the wave guide system was estimated by incubation of sequence 23B with a DNA chip containing two sequence 3 spots and two sequence 1 spots in a cross-diagonal pattern with 0, 0.4, 4.0, and 40 nM DNA. At 0.4 nM DNA, a signal could just be seen/detected at zone 3. Except at the highest concentration, no measurable signals at the sequence 1 capture zones were observed and the zero concentration of sequence 23B was not detectable at zone 3. Digital analysis gave an average signal minus background of 7 gray levels for the 0.4 nM spot compared to ±2 gray levels for background variation in the zero concentration. Thus, 0.4 nM is an approximate limit of detection (defined by a signal >2 standard deviations above the mean background signal). It is likely that sensitivity could be increased by increasing the conjugate concentration.

DISCUSSION

The fluorescent oligonucleotide concentration that was reported in the evaluation of the Affymetrix photolithographically produced DNA chips, with confocal microscopy was 10 nM (12). A wave guide signal sufficient for single-base discrimination has been generated between 4 and 40 nM DNA and is, therefore, comparable to a fluorescence signal system. The pin/spotting method did not create a sufficiently uniform capture zone for further quantitative analysis.

The use of the optical wave guide readout has significant advantages over fluorescence readout with confocal microscopy (12), which is regarded as the current "gold standard." The readout is essentially instantaneous with real-time video imaging, as opposed to the requirement for a time-consuming scanning procedure; backgrounds are minimal as the wave guide only interrogates a boundary layer for signal generation (Fig. 1*B*), and paradoxically, high concentrations of unbound colloid have a high absorbency and, hence, actually suppress background scattering from the solution phase.

Although the initial signal develops for 2–3 min before showing saturation, it is not reasonable to assume the dissociation reaction will occur with the same kinetics. Signal generation is dominated by the diffusion of selenium conjugate to the adsorbing surface from the bulk solution (≈20 μm). In contrast, signal reduction via melting is limited only by the time required for the selenium to diffuse out of the 200- to 300-μm evanescent wave. This process has been accelerated by using forced-air heating and melting for an entire array accomplished in a few seconds with single-base discrimination maintained (D.I.S., unpublished results).

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