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DNA Computing on Surfaces: Encoding Information at the Single Base Level

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ABSTRACT

The feasibility of encoding a bit (0 or 1) of information for DNA-based computations at the single nucleotide level is evaluated, particularly with regard to the efficiency and specificity of hybridization discrimination. Hybridization experiments are performed on addressed arrays of 32 (2^5) distinct oligonucleotides immobilized on chemically modified glass and gold surfaces with information encoded in a binary (base 2) format. Similar results are obtained on both glass and gold surfaces and the results are generally consistent with thermodynamic calculations of matched and mismatched duplex stabilities. It is found that under the conditions required to obtain single nucleotide specificity in the hybridization process, hybridization efficiency is low, compromising the utility of single nucleotide encoding for DNA computing applications in the absence of some additional mechanism for increasing specificity. Several methods are suggested to provide such increased discrimination.

Key words: DNA computing, hybridization, single base, secondary structure.

INTRODUCTION

THE FIELD OF DNA COMPUTING was initiated in 1994 with a seminal paper by Adleman (Adleman, 1994), in which it was proposed that tools of molecular biology could be used to solve computational problems. A modest proof-of-principle was performed by solving a tiny instance of the Hamiltonian Path problem using a test tube-based approach. Although this experimental implementation of DNA computing was an important demonstration, the test tube methodology employed is not well-suited for scale-up to large combinatorial problems, involving as it does necessarily inefficient transfer and handling steps performed on a large scale, with macroscopic volumes and amounts of material.

We have described in the adjoining paper (Smith *et al.*, 1998), an alternative implementation of DNA computing, in which complex combinatorial mixtures of polynucleotides representing a family of possible solutions to a multivariable problem are immobilized and manipulated while attached to supports (Liu *et al.*, 1997; Cai *et al.*, 1997; Smith *et al.*, 1998). In one simple implementation of this approach, members of the set containing desired subsequences are MARKED by hybridization with their perfectly matched complements, and the remaining single-stranded molecules which were not MARKED are then DESTROYED using a single-strand specific exonuclease. After several cycles of such MARK and DESTROY operations, the sequences of the molecules remaining on the surface may be determined, yielding solutions to the computational problem under investigation. One critical aspect of this approach concerns the manner in which information is encoded

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within the DNA molecules. There are many possibilities, ranging from utilizing the full base 4 (A, C, G, T) information carrying capacity of DNA at the single nucleotide level, to encoding a single bit of information in a DNA 20mer as shown by Adleman (1994). In general, the more densely information can be stored within the DNA molecules, the greater the computational power accessible for a given mass of DNA. However, this benefit must be weighed against the increased difficulty of specifically targeting members of a complex combinatorial mixture which differ only slightly in their sequence from other members of the mixture.

In this paper the feasibility of encoding information at the single nucleotide level is explored, particularly with regard to the degree of hybridization discrimination and efficiency obtainable. Hybridization experiments were performed on addressed arrays of 32 oligonucleotides encoding information in a base 2 (A or T, G or C) format in which the G/C content was held constant to reduce duplex stability variations within the set. Both gold and glass surfaces were employed with similar results. A key finding is that although "single-base" discrimination may be achieved in this system, it is at the cost of a severe decrease in the efficiency of hybridization to perfectly matched sequences. The results are generally consistent with calculations of perfectly matched and mismatched duplex stabilities. For DNA computing with single-base encoding of information to be feasible, it will be necessary to increase the discrimination beyond that available by DNA hybridization alone. The results obtained suggest several ways in which this might be accomplished.

MATERIALS AND METHODS

Oligonucleotides

Two classes of oligonucleotides were synthesized for these studies: a) "probe" oligonucleotides which were subsequently coupled to the surface by means of amino groups introduced at the 5′ terminus during their synthesis, and b) "target" oligonucleotides which were complementary to the "probes" and possessed a fluorescein group at the 5′ terminus, also introduced during synthesis. The sequences of these molecules are as follows:

a) A set of 32 distinct DNA molecules (probes), each with an overall length of 30 nucleotides (nt), consisting of 15 Ts as a spacer at the 5′ end (Guo *et al.*, 1994) and a 15 nt hybridization sequence at the 3′ end. Their sequences are 5′ NH_2–$(T)_{15}$–AGGAWWSSSGGTTAT 3′ where W represents T or A and S represents C or G. The 5′ amino group was introduced using the reagent N-trifluoroacetyl-6-aminohexyl-2-cyanoethyl N′, N′-diisopropyl-phosphoramidite (Applied Biosystems Division of Perkin-Elmer, Inc., Foster City, CA, (ABI)). Each of these 32 molecules was synthesized and HPLC purified separately.

b) A set of complementary oligonucleotides (targets), each of which was fluorescein-labeled using 6-FAM phosphoramidite (ABI). Their sequences are 5′ FAM–ATAACCSSSWWTCCT 3′. A combinatorial mixture consisting of all $2^5 = 32$ complementary sequences was synthesized by employing both the C and G nucleoside phosphoramidites at positions labeled with S, and both the T and A nucleoside phosphoramidites at positions labeled with W. One unique target was also synthesized, with the sequence 5′ FAM–ATAACCGGCATTCCT.

All oligonucleotides were synthesized by the University of Wisconsin Biotechnology Center on an ABI 308B or 391 DNA synthesizer. The oligonucleotides were purified by reversed-phase high performance liquid chromatography, and their concentrations were determined by UV spectrophotometry at 260 nm (Smith *et al.*, 1987).

Preparation of oligonucleotide arrays on glass supports

Glass slides activated with 1,4 phenylenediisothiocyanate were prepared as previously described (Guo *et al.*, 1994). Amino-oligonucleotides purified by HPLC were dried on a Savant rotary evaporator and redissolved in reverse osmosis deionized water (18 MΩ-cm purity, henceforth dH_2O) to yield 0.2 mM solutions. 1 μl aliquots were applied directly to the activated microscope slide using a Hamilton Microlab 2200 robot (Hamilton Co., Inc., Reno, NV) to yield the desired pattern. This resulted in spots that were approximately 2 mm in diameter. Slides were incubated for 2 h at 37°C in a covered Petri dish containing a small amount of water, removed, washed once with 1% NH_4OH for 5 min, twice with dH_2O for 5 min and dried under a stream of nitrogen at room temperature. The slides were then ready for use in hybridization experiments.

Preparation of oligonucleotide arrays on gold supports

Thin gold films (~50 nm) were vapor-deposited onto glass microscope cover slips (No. 2, 18 × 18 mm) that had been silanized with (3-mercaptopropyl) trimethoxysilane (Aldrich Chemical Co., Milwaukee, WI) as described previously (Hanken and Corn, 1995; Frey *et al.*, 1993). The coated cover slip was immersed in a 1 mM ethanolic 11-mercaptoundecanoic acid (MUA, Aldrich) solution for at least 18 h, rinsed with absolute ethanol (Pharmco, Brookfield, CT) followed by water, then dried with a stream of nitrogen. A monolayer of poly-L-lysine was then adsorbed as described previously (Jordan *et al.*, 1994). The cover slips were subsequently derivatized with a 15 mM solution of 1,4 phenylenediisothiocyanate (Aldrich) in 90% DMF (Aldrich), 10% pyridine (Aldrich) for 2 h at room temperature. The cover slip was washed for 5 min in DMF followed by an ethanol rinse and dried with a stream of nitrogen. One-μl droplets of 0.2 mM amino-oligonucleotide solutions were applied to the derivatized gold surface, using the Hamilton Microlab 2200 robot and the same pattern as on glass. Cover slips were incubated over water at 37°C in a covered Petri dish for 2 h, removed, washed once with 1% NH_4OH for 2 min, washed twice with water for 5 min each, and dried under a stream of nitrogen. The surfaces were now ready for use in hybridization experiments.

Hybridization on glass and gold supports

Three hundred μl of either 2 μM (total strand concentration) of the fluorescently labeled combinatorial set of 32 DNA targets or 2 μM of the individual DNA target in hybridization solution (5xSSPE/0.5%SDS; here 1xSSPE is 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, adjusted to pH 7.4 with NaOH) was applied to each glass or gold surface, which was then incubated for 30 min in a 37°C oven in a closed Petri dish over water. Two 5 min washes were performed at 37°C with 2xSSPE/0.2%SDS wash buffer, followed by a 10 min wash at 43°C, and a 10 min wash at 50°C.

Fluorescence detection

After hybridization and washing, 160 μl of 2xSSPE/0.2%SDS wash buffer was placed on a glass tray onto which the glass slide or gold-coated cover slip was placed face down, providing an aqueous environment for fluorescence scanning. They were then scanned using a Molecular Dynamics FluorImager 575 (Guo *et al.*, 1994). Quantitation was done using Molecular Dynamics ImageQuaNT Version 4.1 software.

Thermodynamics calculations

Perfectly matched duplex stabilities were calculated as described by Breslauer (Breslauer *et al.*, 1986), using a program written in C++. Rough estimates were made of the stabilities of mismatched duplexes using the Breslauer nearest neighbor parameters with the modification that free energy and enthalpy contributions from mismatched nearest neighbor pairs were set to zero. This methodology is denoted as "Method 1" in the following text.

Estimates of the stabilities of mismatched duplexes were also made using a set of parameters suggested by Wetmur (1996), which predicts stability based upon the type of mismatch. In this method, the duplex stability was first calculated as if the mismatch had no effect, after which the thermodynamics were corrected by adding the appropriate destabilization factor(s) depending upon the identity of the mismatch(es). This calculation includes the contribution of the 5′ dangling end (Wetmur, 1996), but ignores the influence of the 5′ fluorescein label which would lead to a slightly higher T_m (Morrison and Stols, 1993). This methodology is denoted as "Method 2" in the following text. Method 1 and Method 2 as described here were also used to predict the duplex stabilities in the paper "Demonstration of a Word Design Strategy for DNA Computing on Surfaces" (Frutos *et al.*, 1997). Predicted T_ms were calculated based upon equation (2a) in Wetmur (1991).

Secondary structure prediction

Secondary structure folding predictions were performed using the Genetics Computer Group "STEM-LOOP" program (Wisconsin Package Version 8.0, Genetics Computer Group, Inc., Madison, Wisconsin).

UV absorbance melting curve measurements

Absorbance vs. temperature melting curves were measured at 260 nm on a Hewlett-Packard (Palo Alto, CA) 8452A UV spectrophotometer equipped with a HP89090A Peltier block. A ramp rate of 1°C/min was used and melting curves were measured in 2xSSPE buffer, pH 7.4, at a strand concentration of 1.2 μM. The

melting temperature (T_m) was determined as the maximum of the first derivative. T_m data are estimated to be accurate within $\pm 1.5°C$.

RESULTS AND DISCUSSION

The goal of this study was to analyze the efficiency and discrimination of surface hybridization when information is encoded at the single nucleotide level for DNA computing applications. A set of 32 oligonucleotides corresponding to the members of a five-binary-variable set of DNA sequences was employed. The sequences chosen for this study are variations upon oligonucleotide sequences utilized in an earlier study of polymorphisms within the human tyrosinase gene (Guo *et al.*, 1994). Both planar glass and gold surfaces were employed as substrates, and the surface attachment chemistries employed were based upon previously described work (Guo *et al.*, 1994; Jordan *et al.*, 1994). Figure 1 illustrates how the set was used in hybridization experiments. The left duplex in Figure 1 illustrates the hybridization of a specific fluorescently tagged oligonucleotide to a specific complementary strand immobilized on the surface, and the right duplex illustrates the hybridization of a 32-fold degenerate (all 2^5 possibilities) mixture of fluorescently tagged oligonucleotides to the same surface-bound oligonucleotide. These two hybridization formats were employed for the experimental work in this study. All experiments reported here were performed on an array of 32 individual oligonucleotides immobilized on the surface.

Hybridization of a combinatorial set of 32 DNA molecules

Figure 2 shows the results obtained when a combinatorial mixture of 32 fluorescently tagged DNA targets is hybridized to an array of their 32 complements immobilized on a glass surface. The sequences of the 5 nt variable regions are shown in panel A, and the pattern of fluorescence binding in panel B.

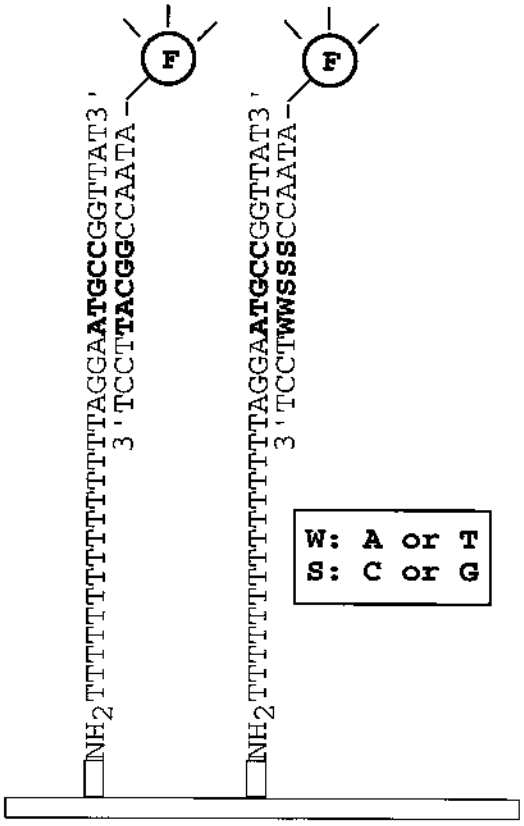

FIG. 1. Illustration of the hybridization between a set of 32 distinct immobilized oligonucleotides and a specific fluorescently tagged oligonucleotide or a 32-fold degenerate mixture of fluorescently tagged oligonucleotides.

A

TTGGG	TTGGC	TAGGG	TAGGC	AAGGG	AAGGC
TTGCG	TTGCC	TAGCG	TAGCC	AAGCG	AAGCC
TTCGG	TTCGC	TACGG	TACGC	AACGG	AACGC
TTCCG	TTCCC	TACCG	TACCC	AACCG	AACCC
ATGGG	ATGGC	ATCCG	ATCCC	ATCGG	ATCGC
ATGCG	ATGCC				

B

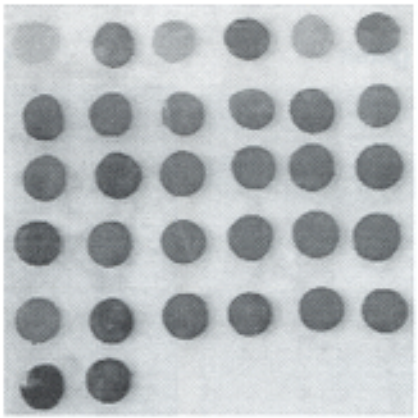

FIG. 2. Results obtained when a combinatorial mixture of 32 fluorescently tagged 15mer DNA targets is hybridized to an array of their 32 complements immobilized on a glass surface. Panel A shows the sequences of the 5 nucleotide variable regions. Panel B shows the hybridization pattern obtained after washing at 37°C as described in Materials and Methods.

It may be noted that as the hybridization and washing conditions employed in this experiment were not very stringent, it is likely that there are contributions to the observed fluorescence intensities from binding of both perfectly matched and mismatched complements to the same spot. Several observations can nonetheless be made. First, under the conditions employed, all members of the addressed array show significant fluorescence binding, demonstrating the accessibility of the surface-bound oligonucleotides to hybridization. The degree of binding is readily quantified by fluorescence scanning. Second, the spots in [1, 1], [3, 1], [5, 1] and [1, 5] (the first number indicates the column and the second number represents the row), show significantly reduced fluorescence intensities compared to other members of the set. Examination of the sequences of these spots showed that with G-T mismatches, which are known to be among the most stable mismatches formed (Aboul-ela *et al.*, 1985; Werntges *et al.*, 1986; Ikuta *et al.*, 1987), these oligonucleotide sequences can form a hairpin structure as shown below. This hairpin involves three adjacent Gs in the variable region (see panel A) that are contiguous with two adjacent Gs in the non-variable region.

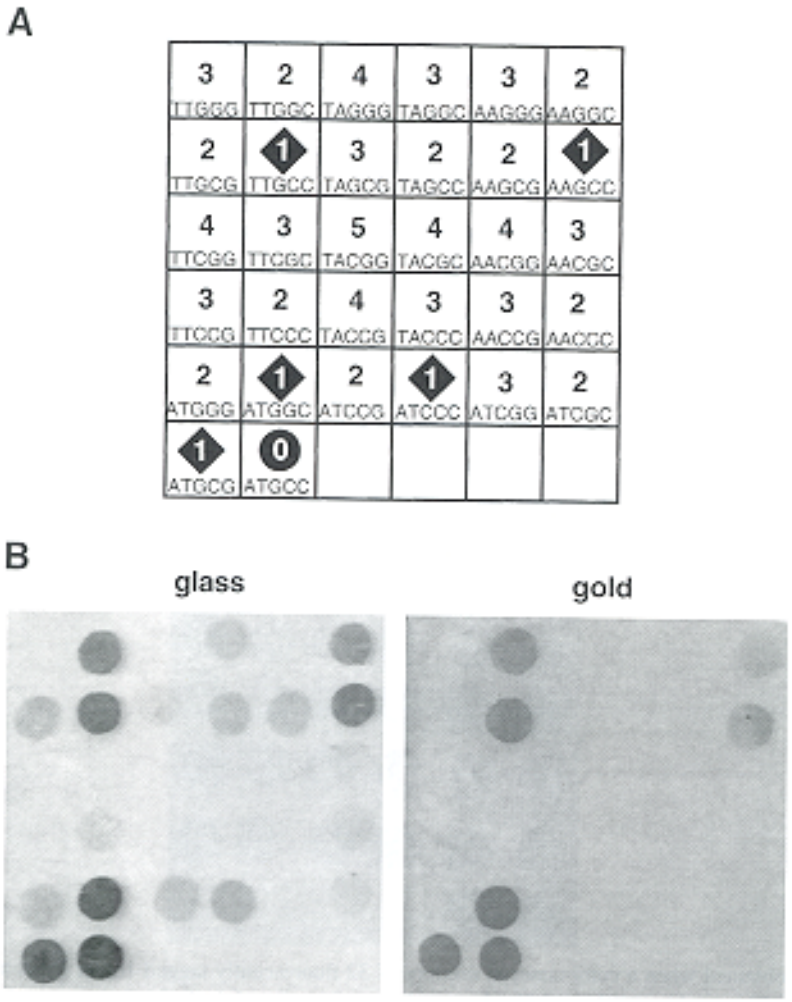

FIG. 3. Results obtained when arrays of 32 oligonucleotides immobilized on both glass and gold surfaces are hybridized to a single fluorescently tagged oligonucleotide complement, perfectly complementary to the sequence at [2, 6]. Panel A shows the number of mismatches obtained along with the sequences of the five variable bases for each member of the array, and Panel B shows the hybridization pattern obtained after washing at 37°C as described in Materials and Methods.

Experimental evidence supporting the existence of this hypothesized hairpin structure was obtained in experiments (not shown) in which *E. coli* Exonuclease I, a single-strand specific 3′⇒5′ exonuclease, was incubated with the surface-immobilized array. It was found that the DNA molecules with the sequences pointed out above were resistant to exonuclease digestion, in contrast to other members of the array which were susceptible to digestion. These results support the hypothesis that these sequences form stable hairpin structures under the conditions employed and that these structures interfere significantly with DNA hybridization. The ubiquitous nature of such stable secondary structures in DNA has been noted in recent publications (Brow *et al.*, 1996; Milner *et al.*, 1997). Their occurrence in this study highlights the importance of careful design and testing of the DNA sequences employed for DNA computing applications to eliminate interfering secondary structures. It may be noted that the above secondary structures were not detected using the commercial folding program STEMLOOP; improved methods for the identification of secondary structures would be most useful. Experimental methods for the detection of secondary structures such as the exonuclease digestion employed here may also prove useful. Sequences containing multiple Gs are known to form a variety of intra and intermolecular structures and might well be avoided (Breslauer *et al.*, 1986; Williamson *et al.*, 1989; Aboul-ela *et al.*, 1992; Olivas and Maher, 1995). The 15mer poly dT spacer employed in the present study also contributed to formation of this hairpin, indicating the need to consider the spacer structure in sequence design.

The fluorescence intensity for each member of the array was quantified, and the values of $-\Delta G^{\circ}_{298}$, $-\Delta H^{\circ}_{298}$, and T_m were calculated for the perfectly matched duplexes using Method 1, as described in Materials and Methods (Breslauer *et al.*, 1986). Omitting the four anomalous spots discussed above, the measured fluorescence

TABLE 1. CALCULATED THERMODYNAMIC VALUES AND EXPERIMENTAL FLUORESCENCE INTENSITY VALUES FOR DUPLEXES FORMED BETWEEN 32 SURFACE-IMMOBILIZED DNA MOLECULES AND AN INDIVIDUAL FLUORESCENTLY TAGGED OLIGONUCLEOTIDE COMPLEMENT AS SHOWN IN FIGURE 3

Probe sequences	*No. of bms*	*Mismatches*	$-\Delta G^{\circ}_{298}$ (kcal/mol) *Method 1*	$-\Delta G^{\circ}_{298}$ (kcal/mol) *Method 2*	T_m (°C) *Method 1*	T_m (°C) *Method 2*	*rfu on glass*	*rfu on gold*
ATGCC	0	—	25.1	24.5	63.6	57.7	4754	738
ATGGC	1	G:G	18.9	21.6	51.2	51.5	3862	632
ATGCG	1	G:G	18.4	21.6	49.6	51.5	4150	583
AAGCC	1	A:A	21.7	20.8	58.2	49.7	1665	276
ATCCC	1	C:C	20.1	20.6	53.6	49.3	358	32
TTGCC	1	T:T	21.7	20.5	59.6	49.2	2179	466
ATCGC	2	CG:CG	17.0	18.3	46.1	44.1	41	—
ATGGG	2	GG:GG	15.3	18.2	41.5	43.5	267	—
AAGGC	2	AxG:AxG	15.5	17.9	43.0	43.0	843	110
AAGCG	2	AxxG:AxxG	15.0	17.9	41.2	43.0	148	—
ATCCG	2	CxG:CxG	13.4	17.7	35.4	42.6	150	32
TTGGC	2	TxxG:TxxG	15.5	17.6	44.0	42.4	1386	317
TTGCG	2	TxxxG:TxxxG	15.0	17.6	42.1	42.4	302	—
AACCC	2	AC:AC	18.6	16.9	51.3	40.6	40	—
TTCCC	2	TxC:TxC	16.7	16.6	47.2	40.0	53	—
TAGCC	2	TA:TA	19.8	16.3	55.0	39.3	182	—
ATCGG	3	—	13.4	14.8	35.4	35.4	—	—
AACGC	3	ACG:ACG	15.5	14.6	43.0	34.9	20	—
AAGGG	3	—	11.9	14.5	30.8	34.3	—	—
TTCGC	3	—	13.6	14.3	37.6	34.2	—	—
TTGGG	3	—	11.9	14.2	31.4	33.6	—	—
AACCG	3	—	11.9	14.0	30.8	33.3	—	—
TTCCG	3	—	10.0	13.7	23.4	32.6	—	—
TAGGC	3	TAxG:TAxG	13.6	13.4	37.6	31.9	74	—
TAGCG	3	TAxxG:TAxxG	13.1	13.4	35.6	31.9	36	—
TACCC	3	—	16.7	12.4	47.2	29.2	—	—
AACGG	4	—	11.9	11.1	30.8	25.5	—	—
TTCGG	4	—	10.0	10.8	23.4	24.7	—	—
TACGC	4	—	13.6	10.1	37.6	22.6	—	—
TAGGG	4	—	10.0	10.0	23.4	22.2	—	—
TACCG	4	—	10.0	9.5	23.4	20.9	—	—
TACGG	5	—	10.0	6.6	23.4	12.0	—	—

intensities ranged from 1969 to 5820 relative fluorescence units, with an average value of 3269 and a standard deviation of 920, showing reasonably consistent hybridization behavior within the set. Solution T_ms measured for two selected perfectly matched duplexes from this set (ATGCC and TACGC) were 62.8°C and 63.0°C respectively, in reasonable agreement with the calculated values of 63.6°C and 60.7°C.

Hybridization of a selected individual complement to arrays of 32 DNA molecules attached to either a glass or a gold surface

Figure 3 shows the results obtained when the surface-immobilized arrays on both glass and gold are hybridized to a single fluorescently tagged oligonucleotide complement. Panel A shows the number of mismatched bases along with the sequences of the five variable bases for each member of the array. The number of mismatches ranges from zero (perfect match) for the oligonucleotide at [2, 6], to all five bases for the oligonucleotide at [3, 3]. There is one perfectly matched duplex, five single base mismatched duplexes (1bm), ten 2bm, ten 3bm, five 4bm and one 5bm duplexes (for a total of 32 possible combinations). Note that the mismatches are all of the self-self type (i.e., A·A, C·C, G·G or T·T) because of the encoding strategy. The hybridization results shown in Panel B were obtained under relatively low stringency conditions (37°C in 2xSSPE/0.2%SDS wash buffer), as is evident from the substantial degree of hybridization observed to mismatched members of

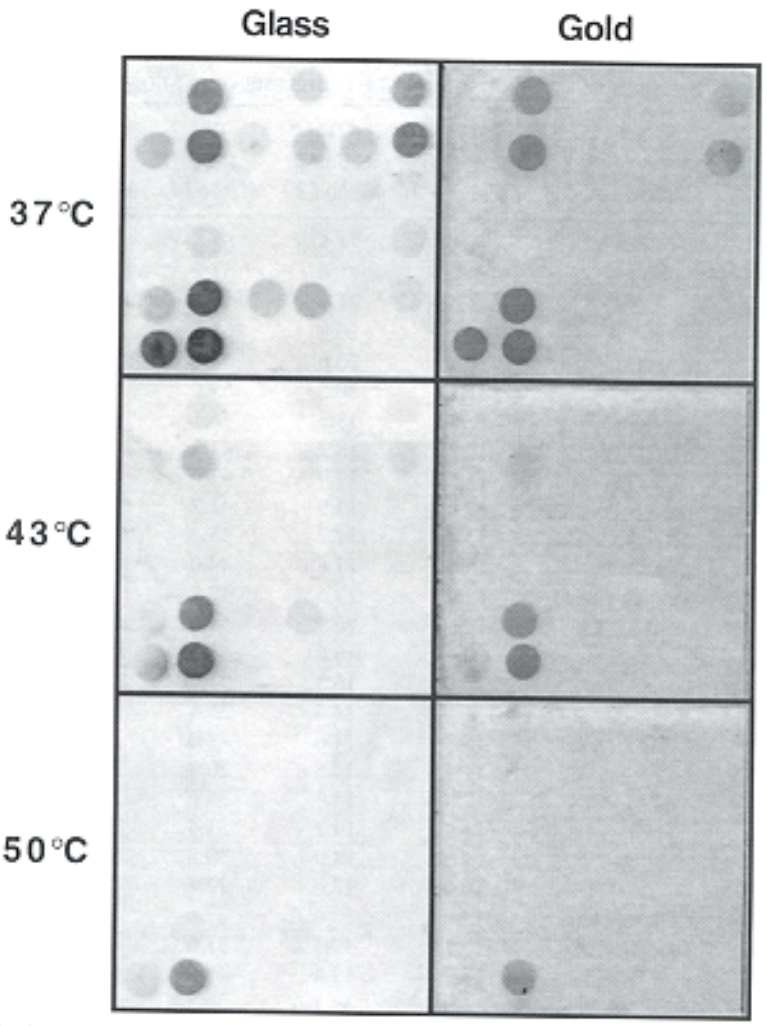

FIG. 4. Results obtained when the hybridized surfaces from Figure 3 were washed under conditions of increasing stringency (temperature). The top two panels are identical to the data in Figure 3 Panel B.

the array. Similar results are obtained on both glass and gold, with the difference that in this experiment the fluorescence intensities obtained on the gold surface were approximately 6 fold lower than those obtained on glass, possibly due to fluorescence quenching from the gold surface (Chance *et al.*, 1978).

The hybridization stabilities for these mismatched duplexes were calculated by two alternative approaches (see Materials and Methods), with the results shown in Table 1. Both approaches are in reasonable qualitative agreement with the experimental results, in that the calculated stabilities correlate roughly with the observed fluorescence intensities. Method 1 takes into account the arrangement of mismatched pairs, but not their identities; in contrast, Method 2 considers their identities but not their arrangement. A more accurate estimation of mismatch hybridization thermodynamics would include consideration of longer range interactions such as the formation of stems and loops (Frutos *et al.*, 1997).

In Figure 4 the results of experiments in which the stringency of hybridization is gradually increased to yield single-base hybridization specificity are presented. At relatively low stringency conditions (37°C in 2xSSPE/0.2%SDS wash buffer), the fluorescently tagged complement hybridized to both matched and mismatched complements immobilized on the surfaces; under higher stringency conditions (43°C in 2xSSPE/0.2% SDS wash buffer, then 50°C), the dissociation rate of mismatched strands is more rapid than that of matched strands (Ikuta *et al.*, 1987). This leads after some time to apparent "single-base" discrimination, as the perfect matches are present at substantially higher levels than are the mismatches. Although this is useful for many applications, quantitative analysis of the results shows clearly that this degree of hybridization specificity is obtained at substantial cost: the fluorescence intensity obtained from the perfectly matched oligonucleotide under the 50°C high stringency conditions is approximately 20–30% of that obtained under the low stringency 37°C conditions. That is, 70–80% of the perfectly matched surface-bound oligonucleotides

are in a single-stranded form rather than the desired double-stranded form. This low efficiency of hybridization under the conditions necessary for single-base specificity presents problems for the DNA computing strategy, where significant numbers of hybridization operations must be performed in successive rounds of querying of the combinatorial mixture (Smith *et al.*, 1998).

There are several ways in which this lack of specificity might be addressed; possible approaches include a) employing an enzymatic recognition process sensitive to mismatches, such as MutS (Wagner *et al.*, 1995), T4 endonuclease VII (Youil *et al.*, 1996), or even a single restriction endonuclease cleavage (Kim *et al.*, 1996); b) utilizing artificial mismatch hybridization to increase discrimination (Guo *et al.*, 1997); c) utilizing peptide nucleic acids which have been shown to provide superior hybridization discrimination (Egholm *et al.*, 1992; Egholm *et al.*, 1992); or d) changing the encoding scheme to utilize multiple bases rather than single base. This latter approach is explored elsewhere in considerable detail, where a four-base encoding scheme is shown to greatly increase hybridization discrimination and appears to be suitable for DNA computing applications (Frutos *et al.*, 1997).

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