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Published on Web 06/17/2002

Structure-Specific DNA Cleavage on Surfaces

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Received August 31, 2001

Abstract: The structure-specific invasive cleavage reaction is a useful means for sensitive and specific detection of single nucleotide polymorphisms, or SNPs, directly from genomic DNA without a need for prior target amplification. A new approach integrating this invasive cleavage assay and surface DNA array technology has been developed for potentially large-scale SNP scoring in a parallel format. Two surface invasive cleavage reaction strategies were designed and implemented for a model SNP system in codon 158 of the human ApoE gene. The upstream oligonucleotide, which is required for the invasive cleavage reaction, is either co-immobilized on the surface along with the probe oligonucleotide or alternatively added in solution. The ability of this approach to unambiguously discriminate a single base difference was demonstrated using PCR-amplified human genomic DNA. A theoretical model relating the surface fluorescence intensity to the progress of the invasive cleavage reaction was developed and agreed well with experimental results.

Introduction

Single nucleotide polymorphisms (SNPs) are the most abundant and stable type of variations found in the human genome, with an estimated frequency of one polymorphic nucleotide per kilobase.[1] This property gives them utility as genetic markers in linkage and association studies aimed at identifying and characterizing genes involved in biological function and human disease.[2−5] However, estimates suggest that for such studies to be successful in the analysis of common disease genes, it may be necessary to type several hundred thousand SNPs in hundreds or thousands of individuals.[6] This has presented a tremendous challenge and obstacle to the performance of such studies.

Recent work has described an invasive cleavage reaction for SNP scoring which has a number of desirable features, including the ability to directly analyze genomic DNA, high accuracy, robustness, and an isothermal homogeneous format.[7,8] The reaction is based upon cleavage of a unique secondary structure formed between two adjacent oligonucleotides, one referred to as the “upstream” oligonucleotide and the other as the “probe” oligonucleotide, hybridized to a target DNA sequence (Figure 1). The nucleotide at the 3′ end of the upstream oligonucleotide is designed to overlap at least one base into the downstream duplex formed by the probe and the target strand. The unpaired region on the 5′ end of the probe, or “flap”, along with an immediate downstream paired nucleotide can then be removed by a class of structure-specific 5′-nucleases.[9] Absolute complementarity between the probe and the target sequence at the position of overlap is required for efficient enzymatic cleavage, which provides a cleavage rate at least 300 times higher than that for a noncomplementary substrate.[10] This huge difference in cleavage rate is the basis for the discrimination of single base differences in the target DNA strand. The use of a

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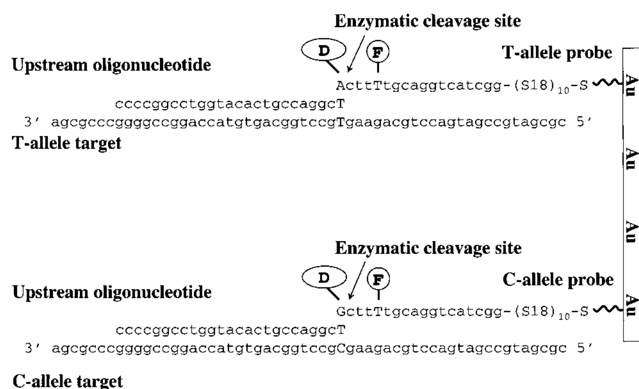

Figure 1. Two sets of oligonucleotides are required for typing codon 158 of the human ApoE gene using the invasive cleavage reaction. The bases at the polymorphic site are T and C. In either reaction set, the 3′ terminal nucleotide of the upstream oligonucleotide overlaps (or invades) the first base pair of the downstream probe-target duplex (A−T for the T-allele target and G−C for the C-allele target). The 5′-nuclease specifically cleaves the probes at the positions marked by the arrows. Cleavage separates the dabcyl−fluorescein FRET pair (denoted D and F) and disables the quenching action of the dabcyl. In the first invasive cleavage reaction strategy, only the probe oligonucleotide is attached to the surface, whereas in the second invasive cleavage reaction strategy, both the 3′ end of the probe oligonucleotide and the 5′ end of the upstream oligonucleotide are immobilized. The merits of each reaction strategy are detailed in the text. Oligonucleotides attached to the surface were previously modified with 10 spacer phosphoramidite 18s, $(S18)_{10}$, and free thiols. The free thiols of the oligonucleotide reacted with maleimide groups on modified gold surfaces.

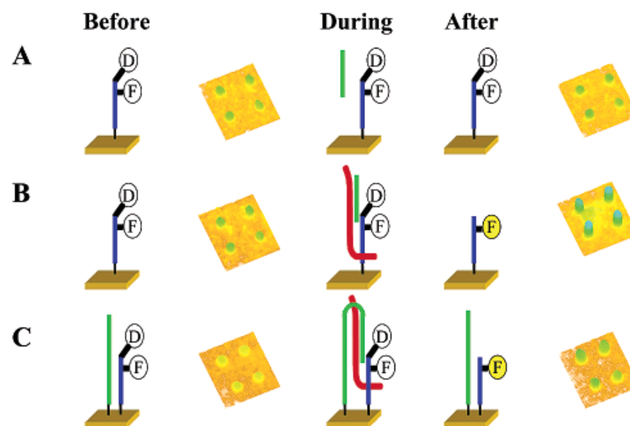

Figure 2. Fluorescence images and schematics of both surface invasive cleavage reaction strategies (probe − blue line, upstream oligonucleotide − green line, target − red line). (A) Strategy 1 control − The probe oligonucleotide is attached to the surface, and the upstream oligonucleotide is added in solution. No target was added to this reaction, and thus no significant signal increase was observed. (B) Strategy 1 reaction − The probe oligonucleotide is attached to the surface, and the upstream oligonucleotide is added in solution. Upon addition of 50 pM target (T-allele) and incubation at 54.5 °C for 24 h, the fluorescence intensity increased, on average, by a factor of 3.5. (C) Strategy 2 reaction − Both the probe and the upstream oligonucleotides are attached to the surface. Upon addition of 50 pM target (T-allele) and incubation at 54.5 °C for 24 h, the fluorescence intensity increased, on average, by a factor of 2.3. The control experiment for the co-immobilized surface generated similar results to the control experiment described in (A).

thermostable 5′-nuclease allows the reaction to be performed near the melting temperature (T_m) of the hybridization region between the probe and target strand, so that with excess probe oligonucleotide present, a cleaved probe will quickly be replaced by an uncleaved one. The probe oligonucleotides exchange on and off the target strand for a reaction run near the T_m, which results in a linear accumulation of cleavage product with respect to both time and target strand concentration. Under optimal operating conditions, approximately 3000 cleaved probes can be generated per target molecule in 90 min.[7] Unlike the target amplification employed in most current SNP scoring technologies, the signal-amplification format of this assay eliminates carryover contamination which can occur in PCR.[11−14] The combination of sequence-specific probe hybridization and structure-specific enzymatic cleavage imparts a high degree of specificity to the reaction, sufficient for the robust detection of a single nucleotide change directly from nanogram amounts of genomic DNA in a serial two-step invasive cleavage reaction.[8] This assay is in routine use today for clinical SNP screening.[15,16]

The invasive cleavage reaction has been adapted to a variety of different formats, including the use of mass spectrometric[17] and microparticle-based[18] detection of the cleavage products. One convenient way to monitor the reaction in a homogeneous format is using a fluorescence resonance energy transfer (FRET) mechanism, where the energy emitted by a donor fluorophore is transferred to a nearby acceptor dye, and dissipates as heat, rather than being emitted as fluorescence.[19] During the reaction, cleavage physically separates the donor fluorophore from the acceptor dye on the probe, eliminating the dye-quenching and generating a fluorescence signal.

A powerful approach to the parallel analysis of SNPs would be to implement the invasive cleavage reaction in a surface array format. By preparing DNA arrays on surfaces where each element of the array contains a particular SNP-specific probe, addition of a single sample of human genomic DNA to the surface would lead to formation of the invasive cleavage structure at every site on the surface which corresponded to a SNP allele in the genome being analyzed. The invasive cleavage reaction would give rise to an increase in fluorescence at that element of the array, indicating the presence of the corresponding SNP allele in the target DNA. In essence, the use of the planar surface format parallelizes the invasive cleavage reaction, so that each different SNP allele in the genome is queried in parallel by the corresponding site on the surface. A DNA array containing 1 million such features would permit the analysis of 500 000 biallelic SNPs in a single step.

The upstream oligonucleotide, which is also required for the invasive cleavage reaction, could either be added in solution or alternatively co-immobilized on the surface along with the probe, which we have shown in previous work to be effective in solid-phase invasive cleavage reactions[18] (Figure 2). The latter strategy obviates the issues associated with having many different upstream oligonucleotides interacting in solution in a multiplexed format.

In this report, we describe proof-of-concept experiments demonstrating the feasibility of performing such invasive

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cleavage reactions on planar substrates, using either synthetic oligonucleotides or a PCR amplicon as a target. A polymorphism in codon 158 of the human ApoE gene, which plays a key role in the transport and metabolism of plasma cholesterol and triglycerides,[20] was employed as a model system. The surface cleavage reaction was studied by measuring the surface fluorescence intensity as a function of probe cleavage, and a theoretical model was developed relating these two parameters. Variables affecting the rate of the surface invasive cleavage reaction were examined.

Experimental Section

Sequence Design. A polymorphic site in codon 158 of the human ApoE gene was used as a model system to test the surface invasive cleavage reaction. A pair of probe oligonucleotides, differing only at the polymorphic nucleotide (T-allele probe and C-allele probe), one upstream oligonucleotide, and two synthetic targets (T-allele and C-allele) were designed to meet the normal requirements for an invasive cleavage reaction (Figure 1). A dabcyl−fluorescein FRET pair is incorporated in the probe oligonucleotide sequence with dabcyl, the quencher, at the 5′ end. The 3′ end of the molecule contains a free thiol group for covalent coupling to a maleimide group present on the surface followed by a series of 10 18-atom spacer moieties, providing a total spacer length of 240 Å. The use of such a spacer region between an oligonucleotide and a surface is often critical to obtaining good performance in surface hybridization.[21]

Oligonucleotide Synthesis. All unmodified oligonucleotides, including the upstream oligonucleotide, target strands (Figure 1), and PCR primers (see the following section), were obtained PAGE purified from Integrated DNA Technologies (Coralville, IA). The surface-bound FRET probe oligonucleotides and upstream oligonucleotide (Figure 1) were obtained from Third Wave Technologies (Madison, WI). The surface-bound cleaved probe (5′-CTT-(fluorescein-dT)-TGCAGGT-CATCGG (spacer phosphoramidite 18)$_{10}$-SH-3′) was synthesized at the University of Wisconsin Biotechnology Center (Madison, WI). The 5′ dabcyl phosphoramidite, fluorescein-dT, spacer phosphoramidite 18, and 3′-thiol modifier C3 S−S CPG500 used in the synthesis were all purchased from Glen Research (Sterling, VA). Prior to purification, both 3′ and 5′ thiol-modified oligonucleotides were deprotected as outlined by Glen Research Corp.[22] The oligonucleotides containing free thiol groups were then purified by reverse-phase binary gradient elution HPLC (Shimadzu SCL-6A), and stored under an inert atmosphere. Oligonucleotide concentrations were determined by measuring absorption at 260 nm with an HP8453 UV−vis spectrophotometer.

DNA Surface Attachment Chemistry. The thiol-modified oligonucleotides were immobilized on gold thin films via a four-step chemical modification described elsewhere.[23,24] In brief, a self-assembled monolayer of the alkanethiol, 11-mercaptoundecanoic acid (MUA) (Aldrich), was formed on a gold-coated glass substrate (Evaporated Metal Films, NY), followed by electrostatic adsorption of a poly-L-lysine (PL) (Sigma) monolayer through the carboxylic acid groups of MUA and the amine groups of PL. Free amine groups on PL not involved in the electrostatic interaction with the acid-terminated surface were then reacted with the heterobifunctional linker sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SSMCC) (Pierce), creating a thiol-reactive, maleimide-terminated surface that can covalently interact with thiol-modified DNA strands. For the probe-only immobilization strategy, 0.5 μL of 0.8 mM thiol-modified probe oligonucleotides was deposited at discrete locations on this maleimide-terminated surface. For the co-immobilization strategy, 0.5 μL aliquots containing both 0.4 mM thiol-modified probe oligonucleotide and 0.4 mM thiol-modified upstream oligonucleotide were mixed first and deposited. The surface attachment reaction was permitted to occur for approximately 20 h in a humid chamber to prevent evaporation. Afterward, the surface was rinsed with distilled water and soaked in 10 mM 4-morpholinepropanesulfonic acid (MOPS)/7.5 mM $MgCl_2$ (pH 7.5), the invasive cleavage reaction buffer, at 60 °C for 3 h to remove nonspecifically bound DNA.[23]

An alternative method of DNA attachment was used for the allelic discrimination experiments using PCR-amplified target DNA. Thiol-modified probe oligonucleotides were linked via SSMCC to an amine-terminated alkanethiol 11-mercaptoundecylamine (MUAM) (Dojindo Laboratories, Japan)-modified gold substrate. The covalent bonds between the layers of the chemical linkers created a more stable surface.[25]

The Surface Invasive Cleavage Reaction. The 200 μL reaction solution contained 10 mM MOPS (pH 7.5), 7.5 mM $MgCl_2$, 0.25 μM upstream oligonucleotide in the case of probe-only immobilization strategy, 1000 ng of Afu FEN 1 (commercially available in the Factor V Leiden RUO Kit from Third Wave Technologies, Madison, WI), and 50 pM-5 nM synthetic target DNA or single-stranded PCR product (see below). The gold surfaces were fully covered by the 200 μL reaction mix, and incubated at a temperature between 52 and 61 °C for up to 24 h in a humid chamber. The surface fluorescence was measured with a FluorImager 575 (Molecular Dynamics, Sunnyvale, CA) both before and after the reaction.

DNA Amplification, Strand Separation, and Quantification. A set of PCR primers, 5′-biotin-ACAGAATTCGCCCCGGCCTGGTA-CACTGCCA-3′ and 5′-TCCAAGGAGCTGCAGGCGGCGCA-3′, yielded a 228 nucleotide (nt) fragment containing codon 158 of the human ApoE gene. The 25 μL amplification reaction mixture contained 10% DMSO (Sigma), 1x PCR buffer, 2 mM $MgCl_2$, 200 μM each dATP, dCTP, dTTP, and dGTP, 2.5 U of AmpliTaq DNA polymerase (Applied Biosystems, CA), 1 μM each primer, and 100 ng of genomic DNA sample (provided by Third Wave Technologies). The PCR reactions were performed on a PTC-200 Peltier Thermal Cycler (MJ Research, Waltham, MA) using the following program: denaturation at 94 °C for 2 min, 40 PCR cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s, and extension at 72 °C for 45 s, with the final cycle extension running for 10 min.

The PCR mixture was purified using the High Pure PCR Product Purification Kit (Roche Molecular Biochemicals). Strand separation of the PCR product was accomplished using streptavidin-coated magnetic beads (Dynabeads M-280, Dynal, Great Neck, NY). One milligram of beads were prewashed with PBS, pH 7.4 (GIBCO BRL, Grand Island, NY), containing 0.1% bovine serum albumin (Sigma), and 1x B&W buffer (5 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1.0 mM NaCl). One hundred microliters of biotinylated PCR product was added to the streptavidin beads along with 100 μL of 2x B&W buffer. The mixture was incubated at room temperature for 15 min with frequent shaking. The beads were then washed twice in 1x B&W buffer before addition of 100 μL of 0.1 N NaOH to separate the double-stranded PCR product bound to the beads. The denaturation reaction was kept at room temperature for 10 min. The supernatant containing the nonbiotinylated DNA strand was collected and then neutralized with 10 μL of 1 M HCl. The single-stranded DNA was purified with Microcon 50 (Amicon, Beverly, MA) to remove EDTA and excess salt before use in the invasive cleavage reaction.

To quantify the amount of PCR amplicon used in the reaction, the primer corresponding to the final single-stranded PCR product was

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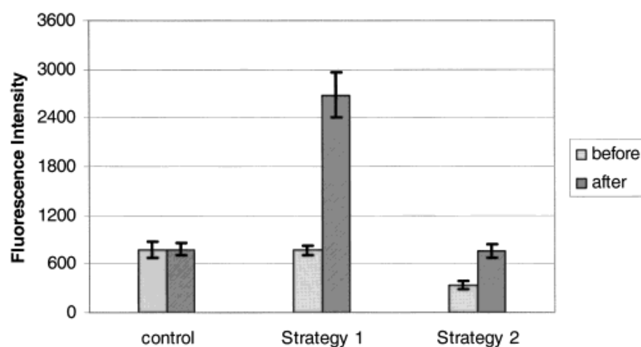

Figure 3. This histogram details the fluorescence intensity changes of the surface invasive cleavage reactions shown in Figure 2, where the gray columns represent the fluorescence intensities before the reaction, and the shaded ones represent the intensities after the reaction (24 h). In strategy 1, only the probe oligonucleotide is attached to the surface, whereas in strategy 2, both the probe and the upstream oligonucleotides are attached to the surface. Four spots (~150 pixels total) were statistically analyzed for each strategy. The error bars represent the standard deviation of those pixels.

modified with fluorescein as 5′-fluorescein-TCCAAGGAGCTGCAG-GCGGCGCA-3′. Following the strand separation described above, the fluorescein-tagged single-stranded PCR product was collected, and the fluorescence emission of the sample at 520 nm (excitation wavelength of 497 nm) was measured with a Hitachi F-4500 fluorescence spectrophotometer. The amount of this unknown sample was estimated to be approximately 0.5 pmol by reference to a standard curve prepared from a series of known fluorescent samples.

Simulation of the Progress of the Surface Cleavage Reaction. To relate the observed changes in surface fluorescence intensity to the progress of the surface invasive cleavage reaction, two oligonucleotides, the surface-bound FRET probe oligonucleotide (containing both fluorescein and dabcyl, and hence quenched) and the cleaved probe oligonucleotide (containing only fluorescein, and hence unquenched), were mixed in ratios of 5:0, 4:1, 3:2, 2:3, 1:4, and 0:5, corresponding to probe cleavage fractions of 0, 20, 40, 60, 80, and 100%, before deposition onto the surface in quadruplicate. The total concentration of the two oligonucleotides in the mixture was kept at 0.8 mM, consistent with the conditions under which the surface invasive cleavage reaction was performed in a probe-only immobilization format. The fluorescence intensity of each spot on the surface was measured with the FluorImager 575. A plot of the signal increase as a function of the probe cleavage fraction was made using the average fluorescence intensity of each quadruplicate sample.

Results and Discussion

Two Surface Invasive Cleavage Reaction Strategies. Figures 2 and 3 show the results of experiments in which the invasive cleavage reaction was performed on oligonucleotide-immobilized planar gold substrates using 50 pM synthetic T-allele target and a 24 h reaction time. The T-allele probe, along with upstream oligonucleotide in a 1:1 molar ratio for the case of co-immobilization, was coupled to the 18 × 18 mm gold surface to form a 2 × 2 array of 2 mm diameter spots. In panel B, where only the probe oligonucleotide was immobilized on the surface but the upstream oligonucleotide was added in solution with a target strand, the fluorescence intensity increased by a factor of 3.5 (average of the four spots) after the invasive cleavage reaction. In panel C, where both the probe and the upstream oligonucleotides were immobilized on the surface and only the target strand was added in solution, a 2.3 times increase in fluorescence intensity was produced. In both cases, a significant increase in fluorescence signal was observed in the presence of the target molecules, but was not observed in the control experiment with no added target (panel A), demonstrating the target-dependent specificity of the reaction.

The fundamental source of the initial intensity of the probe oligonucleotide is residual fluorescence from incompletely quenched fluorophore donor. After subtraction of the background signal from the surface, the initial fluorescence signal on the co-immobilized surface (332 RFU) is lower than that on the probe-only immobilized surface (772 RFU) (see Figure 3). On the basis of the assumption that the total oligonucleotide surface density under the experimental conditions employed remains constant, this lower initial fluorescence signal can be attributed to the 2-fold lower surface probe density resulting from the dilution of the co-immobilized upstream oligonucleotides at a 1:1 molar ratio. This lower surface probe density on the co-immobilized surface also yielded considerably less signal increase (430 RFU vs 1910 RFU on the probe-only immobilized surface, see Figure 3) as the signal generation in the invasive cleavage reaction is directly associated with the amount of probe oligonucleotide.[10]

Although the co-immobilization strategy yields a lower increase in fluorescence signal intensity, it may nonetheless prove to be a more practical format for large-scale genotyping on DNA arrays. There are several reasons for this: first, it would likely be problematic to have a sufficient concentration of hundreds of thousands of different upstream oligonucleotides in solution at one time; second, it is likely that interactions between these strands would occur, which would compromise their ability to function in the surface cleavage reaction; third, it would introduce the issue of having to synthesize, quality control, and dispense hundreds of thousands of individual chemical reagents, whereas if the DNA molecules were all synthesized in situ on the support, as is done in existing oligonucleotide array fabrication, these issues do not exist. Finally, having the upstream oligonucleotide already in close proximity to its companion probe oligonucleotide on the support may provide advantages in the formation of the necessary quaternary complex required for the invasive cleavage reaction.

SNP Analysis Using the Surface Invasive Cleavage Reaction. Figure 4 shows the results obtained using surfaces to which both the C-allele probe and the T-allele probe were attached, and the target employed was a single-stranded PCR amplicon from the human ApoE gene generated from a human genomic DNA sample. Although it is simpler to prepare double-stranded PCR products than single-stranded, hybridization of the double-stranded molecule to the surface will necessarily suffer from competition of the complementary strand with the surface-bound probe oligonucleotide. Our previous work showed that the surface hybridization efficiency is substantially higher with the single-stranded than with the double-stranded product.[21] Therefore, single-stranded PCR amplicon was used exclusively in our surface invasive cleavage reaction experiments. Control experiments were performed with synthetic targets corresponding to either the C-allele, the T-allele, or a 1:1 mixture of both, representing a heterozygous genotype. In each case, the appropriate results were observed. The homozygous T-allele or C-allele targets yielded a fluorescence signal increase only for the corresponding probe oligonucleotide, T-allele probe or C-allele probe, respectively, whereas the mock heterozygous sample generated similar signal increases for both of the probe oligonucleotides. The PCR amplicon target resulted in increased

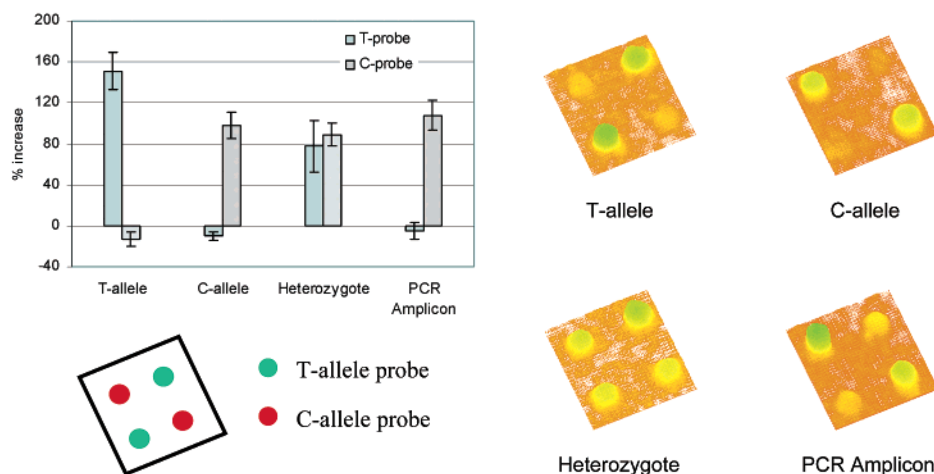

Figure 4. Synthetic target and PCR amplicons of codon 158 of the human ApoE gene were genotyped using the first surface invasive cleavage reaction strategy (probe immobilized). Three experiments were performed with 1 pmol total of synthetic target in combinations representing the three possible SNP genotypes. One experiment was performed using approximately 0.5 pmol of a single-stranded PCR amplicon. Postreaction fluorescence images of each surface are shown above. The percentage signal change for each sample is shown in the corresponding histogram. Relative (rather than absolute) fluorescence intensity is used because of some minor variability in the amount of probe at each spot of the array. Two spots (∼75 pixels total) for each sample were statistically analyzed for each time point. The error bars represent the standard deviation of those pixels.

surface fluorescence only for the C-allele probe, indicating a homozygous C-allele genotype for the individual in question. This result is consistent with the result obtained using a standard solution invasive cleavage reaction. These results demonstrate the formation of the invasive cleavage structure on the surface and its specific recognition and cleavage by the Afu FEN enzyme, with single nucleotide specificity. In addition, the ability to employ a PCR amplicon as a target demonstrates the feasibility of SNP genotyping on surfaces from genomic DNA samples.

It may be noted that the signal increase is not uniform for the two probe oligonucleotides under the same reaction conditions. It has been shown in a theoretical analysis of the solution-phase invasive cleavage reaction that the exchange of the probe oligonucleotide on and off the target strand is the rate-limiting kinetic step of the reaction.[10] The generation of multiple cleavage events per target molecule is achieved by operating the reaction near the T_m of the probe-target duplex, where the cleaved probe is readily melted off from the target strand, and replaced by an uncleaved one, starting another cycle of cleavage. Therefore, for a given set of upstream, probe, and target oligonucleotides, reaction buffer conditions, and enzyme concentration, there is an optimum temperature for maximum signal amplification. A higher temperature would result in unstable hybridization between the probe and the target, and a lower temperature would inhibit the cycling of the probe oligonucleotide, resulting in a lower amount of cleavage. The two probe oligonucleotides used in the experiments have different T_{ms} because of the sequence difference at the polymorphic site (T vs C). Therefore, one likely reason for the observed difference in signal generation for the two probe oligonucleotides on the surface is the difference in their T_{ms}. This difference can be minimized, if desired, by varying the length and/or composition of the probe oligonucleotides to yield similar T_{ms}. A preliminary investigation of this temperature issue for the surface invasive cleavage reaction on the probe-only surfaces will be discussed below. Another possible reason for this difference in signal generation is variability in the surface density of the two probe oligonucleotides resulting from differences in the self-assembled monolayer, the layers of the chemical linkers, and/or the coupling efficiency of the probes on each gold slide. Such surface variability is not expected to be large, as all the surfaces and oligonucleotides employed were prepared at the same time and under similar conditions.

Optimum Reaction Temperature. The effect of reaction temperature on the surface invasive cleavage reactions was investigated on surface arrays of T-allele probes using a 5 nM synthetic T-allele target with a reaction time of 3 h. Varying the temperature from 52 to 61 °C showed that the greatest increase in fluorescence intensity occurred at approximately 54 °C. Interestingly, this optimum temperature of 54 °C at which the surface invasive cleavage reaction proceeds at a maximum rate is significantly different than the optimum temperature of 60 °C observed in solution experiments with the same sequences (J.G.H., unpublished results). A similar decrease in the optimum temperature was observed with oligonucleotides immobilized on latex microparticles.[18] As discussed above, the optimum temperature in the solution-phase invasive cleavage reactions is near the melting temperature (T_m) of the probe-target duplex structure.[10] The reduced optimum temperature observed on surfaces, therefore, might indicate a lower T_m for the surface hybridization than for the corresponding solution hybridization. The T_m is known to depend strongly upon the concentration of the DNA strands,[26] and in the case of the surface invasive cleavage experiments, the "concentration" of the surface-immobilized probe is quite low, limited by the amount of surface area available and the surface density of the oligonucleotides of $\sim 5 \times 10^{12}$ molecules/cm.[23−25] The total amount of probe oligonucleotide available in the four 2 mm diameter spots on the surface is approximately 1 pmol. However, the two-dimensional surface system makes the definition of DNA "concentration" complicated as the attached probe oligonucleotides are no longer uniformly distributed as they are in a three-dimensional solution. A very simplistic approach to this problem would be to neglect this surface effect, and calculate "effective" concentrations as if the probe oligonucleotides were uniformly

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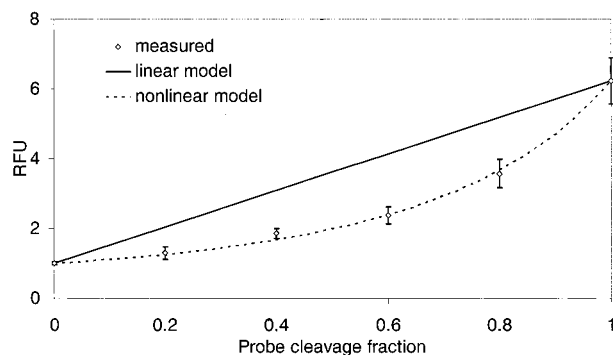

Figure 5. The average surface fluorescence intensity changes as a function of probe cleavage fraction. A series of samples were prepared to simulate different stages in the progress of the surface invasive cleavage reaction (see Experimental Section). The data points shown are the average of the quadruplet of each sample, and the error bars represent the standard deviation of the measured intensities from the ~80 pixels in each quadruplet. The straight line connecting the 0 and 100% points is a model based on the assumption of intramolecular only energy transfer with constant FRET efficiency. The curved dashed line is a model which takes into account both intramolecular and intermolecular FRET. This second model, which appears to accurately describe the data, is described fully in the text.

dispersed in the entire experimental solution volume. For the 200 μL volume employed here, this yields an "effective" probe concentration of 5 nM, as compared to the typical probe concentration employed in solution-phase invasive cleavage reactions of 500 nM. Using the nearest-neighbor model,[27,28] we performed estimates of the expected difference in T_m for the solution and surface experiments, based upon these differences in probe "concentration" in the two experiments, which yield a predicted 7.5 °C lower T_m for the surface cleavage reaction than that for the solution reaction. Solution-phase temperature titration experiments using the lower probe concentration, 5 nM, also generated a 6.6 °C lower optimum temperature (data not shown). Both of these results are comparable to the observed decrease of 6 °C on the surface. Thus, one likely explanation for the observed difference in optimum temperature is that it is a direct consequence of the relatively low numbers of probe molecules participating in the reaction in the surface experiments. Other possible explanations include electrostatic effects of the surface upon DNA or enzyme binding,[29] and steric or other effects of the surface upon the kinetics of the DNA hybridization or enzymatic cleavage reactions. Further work will be required to delineate the relative importance of these and other factors.

Theory of Surface FRET. To study the underlying mechanism of the surface cleavage reaction, it is essential to be able to determine the fraction of probe molecules that are cleaved on the surface under a given set of conditions. The most straightforward approach to obtain such information is to monitor the changes in surface fluorescence intensity during the course of the cleavage reaction; this requires, however, that the relationship between surface fluorescence intensity and the fraction of cleaved probes on the surface be known. To evaluate this relationship, surfaces were prepared with varying proportions of cleaved and uncleaved probe oligonucleotides (see Experimental Section), and the surface fluorescence intensity was measured for each sample (Figure 5).

A very useful and important parameter in describing FRET on surfaces is the energy transfer efficiency, E, which can be readily obtained from the data of Figure 5. As it is in solution, E is defined as[30]

$$E = 1 - \frac{I_{FQ}}{I_F} \quad (1)$$

Here, I_{FQ} and I_F denote the fluorescence intensities of the quenched and nonquenched probes, respectively. The fluorescence intensities shown in Figure 5 were normalized to the intensity measured at a probe cleavage fraction of 0, giving a value for $I_{FQ} = 1.0$. The fluorescence intensity at a probe cleavage fraction of 1 (complete probe cleavage) provides the other limit, corresponding to $I_F = 6.23$. Using these two values, we readily calculated E to have a value of 0.84. Interestingly, this surface efficiency is lower than that observed in solution with the same probe oligonucleotides ($E_{solution} \approx 0.91$, data not shown). A possible explanation for this less efficient FRET process on the surface is that the attachment of the oligonucleotide onto the surface restricts its conformational flexibility and that this reduced flexibility reduces the efficiency of the dipole–dipole interaction between the dye and quencher molecules that mediate the FRET process.

An interesting aspect of the experimental results shown in Figure 5 is the nonlinear relationship between the surface fluorescence intensity and the fraction of cleaved probe. If one assumes that the FRET process is restricted to interactions between the fluorescence donor and acceptor on the same probe and that the corresponding energy transfer efficiency is a constant during the reaction, the fluorescence intensity is expected to be a linear function of the probe cleavage fraction, given by the sum of the fluorescence contributions of the two populations of molecules on the surface, as follows:

$$\begin{aligned} I(x) &= xI_F + (1-x)I_{FQ} \\ &= x \times 6.23 + (1-x) \times 1 \\ &= 5.23x + 1 \end{aligned} \quad (2)$$

where x corresponds to the probe cleavage fraction, and $I(x)$ is the total fluorescence intensity observed.

However, as shown in Figure 5, at each measured probe cleavage fraction between 0 and 1, the observed fluorescence intensity is lower than that predicted by this linear relationship. To explain this behavior, we hypothesized that in addition to the intramolecular energy transfer process, there might be fluorescence quenching effects occurring between adjacent probe oligonucleotides on the surface. From the surface density of approximately 5×10^{12} molecules/cm^2, the average distance between two adjacent probe molecules may be estimated to be around 50 Å. As the energy transfer efficiency of FRET is known to be inversely proportional to the sixth power of the distance between the donor and acceptor dye molecules, and is generally effective within the range of 10–100 Å,[31–34] this

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mechanism seemed to be a likely possibility. The prediction of this hypothesized mechanism is qualitatively in accord with the observations; at low probe cleavage fractions with a large number of dabcyl quenchers on the surface, the emission from fluorescein on the cleaved probe is substantially suppressed by the intermolecular quenching effect. However, this effect becomes less significant as the probe cleavage fraction increases, because the density of quenchers on the surface decreases, and thus the intermolecular quenching process becomes less efficient. Therefore, a greater increase in fluorescence signal is observed at higher cleavage fractions than would be predicted by the linear model.

To provide a quantitative description of this hypothesis, we developed a simple mathematical model that relates the steady-state fluorescence signal measured from the surface to the progress of the invasive cleavage reaction. In establishing this model, the goal was to achieve a high level of fitness using a minimum number of assumptions. For convenience, the oligonucleotides attached on the surface are assumed to be assembled in a hexagonal close packed monolayer with a spacing of approximately 50 Å. As discussed before, we divide the signal generated from the surface into two parts corresponding to the state of the probe molecule. The signal from intact probe molecules having both fluorophore and quencher is denoted I_{FQ}, and the signal from cleaved probe molecules with only fluorophore is I_F.

$$I(x) = xI_F(x) + (1 - x)I_{FQ} \quad (3)$$

Furthermore, because of the proximity of quencher and fluorophore on intact probe molecules, intramolecular quenching is the dominant form of energy transfer. Interactions between fluorophores on intact probes with other molecules are therefore ignored, and the total contribution to the measured intensity of the intact probes is taken to be simply proportional to the fraction of uncleaved probes $(1 - x)$. The contribution to the measured intensity from cleaved probe molecules, $I_F(x)$, requires a more detailed explanation, which is provided in the derivation below.

We begin by looking at the probability that any fluorophore is excited at time t, $P_F(t)$. The time derivative has the following form:

$$\dot{P}_F(t) = -\frac{1}{\tau}P_F(t) - \sum_i k_i P_F(t) + c \quad (4)$$

This equation takes into account the fluorescence decay $-1/\tau P_F(t)$, where τ is the fluorescence lifetime, quenching/energy transfer $-\sum_i k_i P_F(t)$, where k_i is the rate constant for quenching by the ith quencher, and steady-state pumping c.

At steady state, $\dot{P}_F(t) = 0$, making

$$P_F\left(\frac{1}{\tau} + \sum_i k_i\right) = c \quad (5)$$

Averaging over all possible configurations of quenchers gives

$$\langle P_F \sum_i k_i \rangle \approx \langle P_F \rangle \langle \sum_i k_i \rangle \quad (6)$$

and therefore

$$\langle P_F \rangle \left(\frac{1}{\tau} + \langle \sum_i k_i \rangle\right) = c \quad (7)$$

or upon rearrangement

$$\langle P_F \rangle = \frac{c}{\frac{1}{\tau} + \langle \sum_i k_i \rangle} = \frac{c\tau}{1 + \tau \langle \sum_i k_i \rangle} \quad (8)$$

For probes on a hexagonal lattice

$$\tau \langle \sum_i k_i \rangle = 6\tau \langle k_i \rangle = 6\tau \frac{1}{\tau}\left(\frac{R_0}{l}\right)^6 (1 - x) \equiv a(1 - x) \quad (9)$$

where $a = 6(R_0/l)^6$, R_0 is the Förster radius, and l is the intermolecular spacing.

For a hexagonal lattice

$$l = \sqrt{\frac{2}{\sqrt{3}\rho}} \quad (10)$$

where ρ is the surface density.

Because $I_F(x) \propto \langle P_F \rangle$, $I_F(1) \equiv I_F$, and $\langle \sum_i k_i \rangle = 0$ when there are no quenchers, then

$$I_F(x) = \frac{I_F}{1 + \tau \langle \sum_i k_i \rangle} \quad (11)$$

By making all substitutions into our original equation, then

$$I(x) = \frac{xI_F}{1 + a(1 - x)} + (1 - x)I_{FQ} \quad (12)$$

The next task is to test the fitness of this model with the signal measured from the simulated surfaces. As discussed previously, the normalized fluorescence intensities generated a value for $I_{FQ} = 1.0$ and $I_F = 6.23$. The model is then fitted to the data with a single adjustable parameter "a". With this set of data, "a" was found to be 2.18 (Figure 5). The last remaining question is whether this is a reasonable value. Under our experimental conditions, the surface probe density, ρ, is estimated to be 5×10^{12} molecules/cm^2 providing a value for l of 50 Å. Using this value of l together with $a = 2.18$ yields a value for R_0 of 42 Å for our system, which falls nicely in the typical range of Förster radii (10−100 Å).[33,34]

Conclusion

In the present work, it is demonstrated that a structure-specific invasive cleavage reaction can be performed on planar substrates with single nucleotide specificity, permitting the identification of SNP genotypes in a surface array format. In this study, the target species employed were either synthetic oligonucleotides or a PCR amplicon, at concentrations of 50 pM (Figures 2−4). The eventual goal of the work is the determination of SNP genotypes directly from genomic DNA samples, which will require a further reduction in the target DNA concentration to

5 pM or less. Future work will focus on increasing the detection sensitivity of the surface invasive cleavage reaction to permit such direct analysis. A theoretical model for the surface FRET process was developed to relate the observed surface fluorescence intensity to the progress of the invasive cleavage reaction. This model was consistent with the experimental results, indicating that both intramolecular and intermolecular energy transfer processes are occurring between dye and quencher molecules on the surfaces.

Acknowledgment. We wish to thank Mr. Brian Aizenstein for technical assistance. This work was supported by Grant R01HG02298 from the National Institutes of Health.

JA012082C