Single base mismatch detection by microsecond voltage pulses

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Abstract
A single square voltage pulse applied to metal electrodes underneath a silicon dioxide film upon which DNA probes are immobilized allows the discrimination of DNA targets with a single base mismatch during hybridization. Pulse duration, magnitude and slew rate of the voltage pulse are all key factors controlling the rates of electric field assisted hybridization. Although pulses with 1 V, lasting less than 1 ms and with a rise/fall times of 4.5 ns led to maximum hybridization of fully complementary strands, lack of stringency did not allow the discrimination of single base mismatches. However, by choosing pulse conditions that are slightly off the optimum, the selectivity for discriminating single base mismatches could be improved up to a factor $\sim 5$ when the mismatch was in the middle of the strand and up to $\sim 1.5$ when the mismatch was on the 5' end and. These results demonstrate that hybridization with the appropriate electric field pulse provides a new, site-specific, approach to the discrimination of single nucleotide polymorphisms in the sub-millisecond time scale, for addressable DNA microarrays.

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Keywords: Single-base mismatch; Voltage pulse; DNA chip; DNA microarrays; SNPs

1. Introduction
DNA chips are powerful devices that combine the speci-
ficity and selectivity of biological molecules with massively parallel analytical capabilities. These biochips are revolu-
tionizing the acquisition and analysis of genetic information (Southern et al., 1999; Ramsay, 1998; Pease et al., 1994; Marshall and Hodgson, 1998; Lipshutz et al., 1999; Wang, 2000). In microarray technology, DNA hybridization with immobilized single-strand DNA probes occurs in parallel at numerous specific sites. Examples of current applications of DNA chips include pathogen identification, gene expression profiling, drug discovery and pharmacology (Stears et al., 2003).

A single nucleotide polymorphism (SNP) is a common type of genetic variation, which appears on average every 1000 bases in the human genome (Tawa and Knoll, 2004; Schmalzing et al., 2000). The importance of SNPs in the identification of human diseases (e.g., cancer), and prediction of drug efficacy or adverse drug reactions (Burmeister et al., 2004) has increased the demand for high throughput, reliable and affordable technologies for analysis of these point mutations. There are several solution-based methods for identifying SNPs (Nollau and Wagener, 1997), including identification of single-strand conformation polymorphism (SSCP) (Orita et al., 1989; Bernat et al., 2002), and denaturing gradient gel electrophoresis (Myers et al., 1985). DNA microarrays have also been used to screen and identify SNPs (Hacia, 1999). In conventional hybridization assays single-base-pair discrimination is achieved by adjusting the stringency of hybridization conditions (e.g., temperature, ionic strength, or formamide concentration). In DNA microarrays all the sequences on the array are exposed to the same conditions and hence the capture probes must have similar melting temperatures to achieve the same levels of hybridization stringency (Heller et al., 2000).

Nanogen has proposed the use of an electric field as a stringency condition to detect single base mismatches in DNA hybrids (Edman et al., 1997). This discrimination is achieved by
reverting the potential with the application of a 5 Hz square pulse for 20 s (Tu et al., 2000; Gilles et al., 1999; Heller et al., 2000). The intensity of the applied E-field is increased so that it surpasses the binding energy between non-complementary DNA base pairs, which depends on the melting temperature (Tm) of the sequence. The E-field is tuned to reach the energy that corresponds to the melting temperature of the double-strand DNA with non-complementary base pairs. Under these conditions the base pairs that are non-complementary will leave the chip surface and the loss of the fluorescence on these pixels indicates the mismatched sequences.

Previously, we have reported the use of a single square voltage pulse to enhance by 7–9 orders of magnitude the rate of covalent immobilization and hybridization of single stranded DNA probes on a chemically functionalized SiO2 surface (Fixe et al., 2002, 2003). The voltage pulse was applied to integrated metal electrodes incorporated below the functionalized thin film surface. Pulses below 1 V, lasting less than 1 ms, are sufficient to reach the same level of DNA covalent bonding and hybridization as that achieved with more than 3 h in the passive reaction conditions. These results suggest that the electronic addressing of pixel elements can be used, coupled with microfabrication techniques, to (1) produce high-density DNA chips by selective and covalent immobilization of a large number of different probe molecules at specific pixels in a matrix (E-immobilization) and (2) increase the speed of biological data acquisition in both research and clinical applications (E-hybridization).

In the present work, electric field-assisted (E-assisted) DNA hybridization conditions were used to resolve match and mismatch probe-target duplexes, without modifying the other hybridization conditions. The single voltage pulses used for SNP detection are in the micro and millisecond time scale, with voltages ranging from 1 to 5 V and rise/fall times between 5 ns and 1 μs. The results described here show that E-assisted hybridization provides a new method for SNP genotyping in a DNA microarray at pixel-level. The E-assisted hybridization concept presented has the potential for simultaneously detecting thousands of SNPs in a single DNA chip platform, with stringency conditions controlled at each pixel by the locally applied electric field.

### 2. Methods

#### 2.1. Chemicals

All chemicals and solvents are purchased from Sigma–Aldrich or Merck, unless stated otherwise, and used without additional purification. The end-modified DNA oligonucleotides used as probes and targets are from Thermo Hybaid. The hetero-bifunctional cross-linker sulpho succinimidyl 6-maleimidylhexanoate (sulfo-EMCS) is from Pierce.

#### 2.2. Pixel fabrication

The prototype DNA chip is fabricated by first evaporating 300 nm of Al on a flexible polyimide (PI, GoodFellow) substrate through a metallic shadow mask. This mask defines two Al lines (2 mm wide)—a central straight voltage line and a U-shaped ground line which surrounds the voltage line (2 mm gap) (Fig. 1A) (Fixe et al., 2004a,b). Next, 200 nm layers of aluminum oxide (Al2O3) and SiO2 were sequentially deposited all over the chip by magnetron sputtering and plasma-enhanced chemical vapor deposition, respectively. All films were deposited at temperatures ≤150°C. The insulating Al2O3 layer prevents any unwanted electrochemical reactions with the Al electrodes during application of the voltage.

#### 2.3. Surface functionalization

The SiO2 thin-film was functionalized by a silanization and cross-linking procedure before covalent immobilization of DNA probes (described elsewhere Fixe et al., 2002, 2003). Briefly, the SiO2 layer was first cleaned with cholic acid (12 h at room temperature). After this cleaning, the surfaces were silanized with 3-aminopropyltriethoxysilane (APTES) 2% (v/v) in acetone, for 2 h at room temperature. This process results in a surface covered with reactive primary amines (–NH2). The NH2 groups were further functionalized by covalently attaching the hetero-bifunctional cross-linker, sulpho succinimidyl 6-maleimidylhexanoate (sulfo-EMCS), for 2 h at room temperature. Since the SiO2 is not patterned, DNA immobilization/hybridization is expected to occur over the entire surface of the chip affected by the field.
Table 1

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence (5'-3')-end</th>
<th>5'-end</th>
<th>3'-end</th>
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</thead>
<tbody>
<tr>
<td>ss-DNA-SH</td>
<td>CCC AGG AAT GCC CCA C SH NH</td>
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<td></td>
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Positive control

<table>
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<tr>
<th>Name</th>
<th>Sequence (5'-3')-end</th>
<th>5'-end</th>
<th>3'-end</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTG GCT GGG TCC TGG G FITC</td>
<td>–</td>
<td>–</td>
<td>–</td>
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</table>

Negative control

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'-3')-end</th>
<th>5'-end</th>
<th>3'-end</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACA AAC CCA TGC CGG CT</td>
<td>FITC –</td>
<td>–</td>
<td>–</td>
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</table>

30% mismatch

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<th>3'-end</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGG AAG GAT TTA GGG G</td>
<td>FITC –</td>
<td>–</td>
<td>–</td>
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50% mismatch

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</tr>
</thead>
<tbody>
<tr>
<td>GGG AAG GAT TTA GGG G</td>
<td>FITC –</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

SNP

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5'-3')-end</th>
<th>5'-end</th>
<th>3'-end</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTG GCT GGG TCC TGG G</td>
<td>FITC –</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

SNP 3'-end

<table>
<thead>
<tr>
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<th>5'-end</th>
<th>3'-end</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTG GCT GGG TCC TGG G</td>
<td>FITC –</td>
<td>–</td>
<td>–</td>
</tr>
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</table>

SNP 5'-end

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<thead>
<tr>
<th>Name</th>
<th>Sequence (5'-3')-end</th>
<th>5'-end</th>
<th>3'-end</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGG GCT GGG CAT TCC TGG G</td>
<td>FITC –</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

2.5. E-assisted hybridization

The chip structure with passively immobilized DNA probes was placed under an O-ring-sealed cuvette containing a solution of FITC-labeled DNA targets (Fixe et al., 2004b). A square voltage pulse was applied to the central metallic line with an HP 8112A 50 MHz pulse generator, with the U-shaped electrode grounded. The chip was then washed and dried as described above.

2.6. Signal detection

The DNA target molecules were labeled on one end with FITC for fluorescence microscopy detection ($\lambda_{exc} = 490$ nm and $\lambda_{em} = 520$ nm). Fluorescence images were digitized with an Olympus CV40 and analyzed with commercial image processing software. Images from different regions of the sample are taken after each different hybridization experiment.

3. Results and discussion

We have previously reported that the use of a single sub-nanosecond square voltage pulse increases the rate of covalent DNA immobilization and hybridization of single stranded oligonucleotides on a chemically functionalized thin film SiO$_2$ surface by 7–9 orders of magnitude (Fixe et al., 2004a). The hybridization results are summarized in Fig. 2 and show the dependence of E-assisted DNA hybridization with the duration of the time pulse ($t_p$) (Fig. 2A), with the amplitude of the voltage pulse ($V$) (Fig. 2B) and with the rise/fall time of the pulse ($t_{r/f}$) (Fig. 2C). Fig. 2A shows that the kinetics of hybridization is increased by around 7–9 orders of magnitude compared to passive hybridization. A single voltage pulse with 10 μs duration and +1 V amplitude results in a DNA hybridization level of approximately 3.7 pmol/cm$^2$, similar to the maximum achieved under passive conditions after several hours. Fig. 2B shows that increasing voltage up to a maximum of +3 V results in increased levels of hybridization. Finally, Fig. 2C shows that the maximum levels of hybridization are achieved for the shortest rise/fall times of the pulse (4.5 ns). A discussion of a physical interpretation of these results is published elsewhere (Fixe et al., 2004a). In summary, the mechanism of enhanced immobilization and hybridization by these E-field pulses is neither electrostatic attraction of charged DNA, nor dielectric pressure. At least a
monolayer of ssDNA adsorbs to the functionalized surface before the pulse is applied. The electric field penetrates the solution and speeds DNA reactions at the surface, by desorbing the DNA from the functionalized surface. Reorienting the DNA reduces steric barriers to reactions. The desorption is stimulated by a rapidly rising electric field, when this rise is shorter than the dielectric relaxation time of the solution, but immobilization and hybridization then requires an additional 10–100 ms.

3.1. E-assisted detection of mismatches

From these results, a set of optimum pulse conditions (gray rectangles in Fig. 2) was chosen for the first studies of SNP detection by E-field assisted hybridization. The conditions selected were a pulse with 1 ms time duration, amplitude of +1 V and rise/fall times of 4.5 ms. Fig. 3 shows the fluorescence microscopy results obtained when passive and E-assisted hybridization of DNA targets with different degrees of mismatches was promoted on the test chip. The results obtained with the E-assisted hybridization are similar to those obtained with the passive reaction after 16 h, for each of the different degrees of target mismatch studied. The observed decrease in fluorescence signal after hybridization as the number of complementary bases is reduced, is in agreement with the results described in literature (Benters et al., 2002). The target molecule with a 30% base-pair mismatch also has 10% fewer GC bases compared to the fully complementary target. These two features lead to a 60% decrease in the level of hybridization. In the case of a 50% mismatch, the low hybridization signal detected (17%) can be attributed to the high degree of mismatch as well as to the fact that the probability of a DNA target hybridizing with an immobilized probe is comparable to that of its binding with another strand of DNA target present in the solution (in both situations the double strand can be established by seven (G-C) and three (A-T) bonds). The presence of fluorescence on the surfaces incubated with the full or partially complementary DNA target and the absence of fluorescence in the case of non-complementary DNA (100% mismatch) clearly indicates that the measured fluorescence signal originates from targets that have hybridized with immobilized probes and not from non-specific adsorption on the chip surface. Under both passive and E-assisted conditions, it was not possible to distinguish between the hybridization levels with a fully complementary target and one that had one base pair mismatch (Fig. 3). This lack of selectivity is consistent with the observation that it is not possible to discriminate a single base mismatch in the 19 nucleotides of the target without using stringency conditions (e.g., by using formamide in the hybridization media and/or in the washing solutions or profiling the temperature) (Benters et al., 2002; Guo et al., 1994).

The results in Fig. 3 show that, if E-assisted hybridization is to be used for the discrimination of single base modifications, voltage pulse parameters must be chosen to be off the optimum conditions (defined as those that give maximum hybridization). Consequently, pulse conditions that result in hybridization levels for fully complementary strands of around 60% of the maximum were chosen (shown by the arrows in Figs. 2A–C). The optimum and three sets of off-optimum conditions were tested: (0) optimum conditions: $t_p = 1$ ms, $V = 1$ V, $t_r = 4.5$ ms; (1) off-optimum pulse width: $t_p = 10$ μs, $V = 1$ V, $t_r = 4.5$ ms; (2) off-optimum voltage: $t_p = 1$ ms, $V = 5$ V, $t_r = 4.5$ ms; (3) off-optimum rise time: $t_p = 1$ ms, $V = 1$ V, $t_r = 1$ μs.
3.2. E-assisted SNP discrimination

Fig. 4A shows that when E-assisted hybridization is carried out at any of the above “off-optimum” pulse conditions, a significant discrimination is obtained in the hybridization levels between fully complementary targets and targets with a single base mismatch. The use of the electric field pulse to discriminate a single base mismatch is more effective when the mismatch occurs in the middle of the target strand than when the mismatch is at either of the two ends. Clearly, the strongest discrimination occurs when the mismatch is located in the middle of the strand (factor of ~5 decrease in hybridization) and the weakest occurs when the mismatch is placed on the 5′-end (factor of ~1.5 decrease in hybridization). When no electric field pulse (passive condition) or when an electric field pulse with conditions that give maximum hybridization levels are used, there is no discrimination between fully complementary targets and those which carry a single base mismatch. It is concluded that it is the “off-optimum” electric field pulse that provides the stringency conditions that enable the discrimination of the single base mismatch.

According to the hypothesized mechanism of enhanced E-assisted immobilization and hybridization, at least one monolayer of ssDNA adsorbs to the functionalized surface before the pulse is applied (Fixe et al., 2004a). The electric field then penetrates the solution and speeds DNA reactions at the surface, possibly by desorbing and reorienting the DNA. The pulse duration, rise and fall times, and amplitude control the extent of hybridization in the short-time scale. The use of voltage pulses optimized for the maximum hybridization of fully complementary DNA targets does not offer enough stringency to enable the discrimination of corresponding SNPs. On the other hand, off-optimum voltage pulse conditions chosen to give hybridization yield of ~60% with respect to the maximum, clearly show a stringency level that enables the distinction between the fully complementary target and their SNPs. It is well described in literature that single nucleotides can significantly change the 3D conformation of single strand DNA (Bernat et al., 2002). These different conformations present in the SNP targets appear to show a stronger dependence on the pulse conditions (pulse duration, rise and fall times, and amplitude) than the fully complementary strand, possibly because the pulse parameter space available to achieve high hybridization yield correlates with the strength of the hybridization bond between DNA strands.

The ability of this methodology to detect single-base mismatches generally, irrespective of sequence mismatches (e.g. G=A, C=A), highlights the power and the novelty of this method for mutational assays as well as in tests of genetic variability (SNPs).

3.3. Comparison with other E-assisted SNP stringency conditions

The Nanogen technique for mismatch detection uses an electric field with a negative polarity, which is applied in a quasi-dc mode to the electrodes after hybridization to discriminate single-base mismatches. The mechanism proposed by Nanogen (Edman et al., 1997) for the observed SNP electric field discrimination is based on the reduction of hybrid stability by a combination of electrochemically induced local pH changes and the electric field repulsion. The field effect should be a consequence of selectively raising the energy of re-hybridization. In the Nanogen case, 0.6 μA current levels are applied during 0.1 s several times, which will involve significant electrochemical reactions at the electrodes.

In this work, a single fast voltage pulse is applied to the electrodes during hybridization to resolve single base mismatches by their lower level of hybridization when compared to full matches. The technique described in this work is different from that of Nanogen in its basic mechanisms, as described above. In summary, Nanogen’s technique is a post-hybridization technique, while the technique described in this work uses the electric field pulse during hybridization to control the level of hybridization. The polarity of the voltage does not make any difference because the mechanism is not related to the charge of the DNA strand (i.e., it is not electrophoretically driven), whereas in the Nanogen technique, a negative polarity must be used for selectivity. The use of single 10 μs wide pulses should not cause any electrochemically-induced change in local environment and thus is not responsible for the observed selectivity. The fast rise time of the voltage pulse is critical for the electronic addressing technique described here. Finally, by adjusting the voltage pulse parameters, it is possible to achieve a higher level of hybridization when the target is fully complementary than when it has a single base mismatch. It is this difference in hybridization level that allows us to distinguish single-base mismatches during the E-assisted hybridization process.

Fig. 4. E-assisted and passive DNA hybridization with fully complementary DNA target. DNA target with one mismatch on the middle (SNP), on the 5′-end (SNP 5′-end) or on the 3′-end (SNP 3′-end) of the strand. Fluorescence signal is normalized to the saturated passive level. The E-assisted hybridization was performed using four different pulse conditions: (0) t_r = 1 ms, V_r = 1 V, t_p = 4.5 ms; (1) t_r = 10 ms, V_r = 1 V, t_p = 4.5 ms; (2) t_p = 1 ms, V_r = 5 V, t_p = 4.5 ms; (3) t_p = 1 ms, V_r = 1 V, t_p = 1 ms.
4. Conclusions

DNA microarray-based analysis of SNPs is important for the early diagnosis of genetic diseases and for the correlation of genetic variations and individual phenotypes. A new strategy for detecting on-chip single-base mismatches in hybridized duplexes between covalently immobilized DNA probes and ssDNA targets was developed based on the use of a single square voltage pulse that simultaneously increases the rate of hybridization and allows the discrimination of SNPs without stringent hybridization conditions. Pulses between 1 and 5 V, with duration below 1 ms, and with rise/fall times smaller than 1 μs were sufficient to achieve site-specific, on-chip, match and mismatch duplexes. Different single-base mismatches at different positions of the DNA target were successfully discriminated with signal ratios (selectivities) between the perfect match and the mismatched strands from 1.8 to 5.2. Further optimization should be possible by testing different pulse conditions.

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