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TECHNICAL NOTE

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Multiplexed SNP genotyping using nanobarcode particle technology

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Abstract Single-nucleotide polymorphisms (SNP) are the most common form of sequence variation in the human genome. Large-scale studies demand high-throughput SNP genotyping platforms. Here we demonstrate the potential of encoded nanowires for use in a particles-based universal array for high-throughput SNP genotyping. The particles are encoded sub-micron metallic nanorods manufactured by electroplating inert metals such as gold and silver into templates and releasing the resulting striped nanoparticles. The power of this technology is that the particles are intrinsically encoded by virtue of the different reflectivity of adjacent metal stripes, enabling the generation of many thousands of unique encoded substrates. Using SNP found within the cytochrome P450 gene family, and a universal short oligonucleotide ligation strategy, we have demonstrated the simultaneous genotyping of 15 SNP; a format requiring discrimination of 30 encoded nanowires (one per allele). To demonstrate applicability to real-world applications, 160 genotypes were determined from multiplex PCR products from 20 genomic DNA samples.

Keywords SNP genotyping · Nanowires · Cytochrome P450 · Encoded particles · Nanotechnology

Introduction

After completion of the sequencing of the human genome [1, 2], attention has turned to discovering and measuring genetic variability. The most common form of human genetic variability is the single nucleotide polymorphism (SNP). The central repository for SNP (dbSNP) contains over 1.8 million SNP with genotype information [3]. SNP detection technology falls into two categories, SNP discovery [4] and SNP genotyping. The availability of large SNP databases has driven the development of inexpensive, robust, and scalable SNP genotyping tools. In this paper we describe a novel particle-based genotyping assay.

Many technologies are used for genotyping. Each has its strengths and weaknesses in different types of analysis, and all have been the subject of extensive reviews [5–7]. We will focus here on discussion of particle-based genotyping technology. The most widespread encoded particle based technology available commercially is that from Luminex Corporation. Luminex Corporation uses polystyrene beads that are labeled internally with precise ratios of two fluorescent dyes, to generate 100 "spectral addresses". By coupling a unique assay to a unique spectral address, multiplexing is facilitated. A third fluorophore is used to determine the assay result, and the beads are analyzed using flow cytometric analysis (Luminex xMAP system). In addition, a DNA sequence, termed a "ZipCode" is included in the capture probe, and is complementary to a sequence on a unique fluorescent microsphere. After the genotyping reaction (of which a variety have been utilized) the microspheres are added to the reaction and analysis is performed by flow cytometry. A variety of genotyping schemes have been coupled with this method. Single-base extension was reported by Chen et al. [8], who performed a 52-plex reaction, and by Taylor et al. [9], who performed a 20-plex SNP reaction for 633 patients. Allele-specific primer extension has been reported by Ye et al. [10] in a 15 multiplexed SNP panel across 96 patients. The standard SNP-specific long oligonucleotide-based ligation assay strategy was first introduced by Landegren et al. [11] and has been successfully used in genotyping formats, including the Luminex platform in which Iannone et al. [12] genotyped seven DNA samples for nine SNP markers. Although the Luminex platform is accurate, it is limited to multiplexing of a maximum of approximately 100 reactions and requires a dedicated analysis system.

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A similar flow-based instrumentation approach has been demonstrated using quantum dot encoded beads [13]; this approach also uses hybridization for SNP analysis. Another bead-based approach based on the Illumina platform uses *randomly* assembled arrays of beads. Ultra-high multiplexed genotyping assays are performed on the beads, followed by decoding of the bead by use of DNA hybridization after the fact [14, 15]. Although this is a very powerful technology, it is cost-effective for ultra-high-throughput users only, because of the cost of the instrumentation, infrastructure, and oligonucleotides necessary to perform an assay. Finally, novel encoded particles are also reported to be in development for SNP-based technology [16, 17].

In this paper we report the application of a universal, short oligonucleotide-based ligation strategy [18] for SNP genotyping with nanobarcodes particles. Nanobarcodes particles are encoded sub-micron metallic nanowires [19]. The nanowires are manufactured by electroplating inert metals such as gold and silver into templates and then releasing the resulting striped nanoparticles [20]. The power of this technology is that the particles are intrinsically encoded by virtue of the different reflectivity of adjacent metal stripes, enabling the generation of many thousands of unique encoded substrates [21]. For example, the number of combinations for a 9-μm-long particle with eleven stripes composed of only two metals, gold and silver (as used in this work), is 1056.

We chose to analyze SNP within the cytochrome P450 family for two reasons. First, they are known to be important in drug metabolism [22, 23] and cancer risk susceptibility [24] and are therefore of interest to the pharmaceutical and biotechnology industries. Second, their close homology with one another [25] makes the system a non-trivial one to study, because it challenges cross-hybridization and multiplexed amplification. Thus, this system is a good test case for any new genotyping scheme [13, 26].

Material and methods

Human genomic DNA sample

Human genomic DNA samples were purchased from Coriell Cell Repositories (Camden, NJ, USA). The 20 DNA samples were selected to represent the ethnic origins Caucasian (10), African American (4), Chinese Asian (3), and Japanese Asian (3). There were 9 males and 11 females ranging in age from 20 to 73 years.

Multiplex PCR amplification

Samples were amplified using a two-step PCR approach. The first step was the same for both single and for multiplex PCR schemes—use of a GeneAmp Gold Hot Start PCR kit (PE Biosystems, Foster City, CA, USA) with 100 ng human genomic DNA and a mix of all eight pairs of primers (0.03 μmol each). Second-step PCR was performed in 100 μL reactions, using 1 μL of 20-fold diluted first-step PCR product. For each amplicon in this step one of the two primers was 5′ phosphorylated. One specific pair of primers (0.3 μmol each) and 5 U Qiagen AmpliTaq (Qiagen, Valencia, CA, USA) were used for the single-plex PCR, and a mix of all eight pairs of primers (0.3 μmol each) and the multiplex PCR kit from Qiagen were used for the multiplex PCR.

PCR products, premixed with sodium acetate (5% final concentration), were purified using Qiagen QIA Quick Mini columns and were eluted in 30 μL water. For the single-plex PCR, the DNA concentration was obtained by electrophoresis of 2 μL of the purified PCR product with 2 μL of the low-molecular-weight DNA mass ladder (Invitrogen, Carlsbad, CA, USA) on a 1% (*w*/*v*) agarose gel. A total of 28 μL double-stranded PCR product (1–10 μg DNA) was digested using Callida's premade mix with an optimized amount of lambda exonuclease (Epicenter Technologies, Omaha, NE, USA) at room temperature for 15–30 min. This digested the phosphorylated PCR strand, leaving the unphosphorylated single strand intact. The enzyme was then inactivated at 95°C for 5 min. All PCR products were gel-sequenced by SequeTech (Mountain View, CA, USA) to confirm genotyping data. It should be noted that in cases of discrepancy, including SNP 19 cell lines NA01850, NA03433, NA03725, and NA08729, only five clones were sequenced to confirm gel sequencing results, which may be insufficient to detect the second allele in these samples.

Nanowire synthesis

Synthesis of the nanowires has been described elsewhere [20, 21]. Briefly, alternating layers of gold and silver are electroplated into the pores of an alumina template, resulting in the formation of striped nanowires. After synthesis of the nanowires the alumina template is removed by use of a strong base. The particles are subsequently coated in mercaptoundecanoic acid (MUA), enabling formation of a carboxyl terminated self-assembled monolayer (SAM) on the particle surface.

Single-nucleotide polymorphism probe design

The cytochrome P450, NAT2, and GSTP genes were chosen for analysis. A total of 20 SNP were selected from the genes CYP1A1, CYP1A2, CYP2C8, CYP2C18, CYP 2C19, CYP2D6, CYP3A4, CYP3A5, GSTP1, and NAT2 (Table 1). For each SNP, two attaching 6-mer probes (one for each allele) and a labeled 5-mer probe were selected from Callida's validated universal libraries of all 6-mers and all 5-mers. The attaching probes have six informative and one, two, or three degenerate bases, and contain a 5′ amino group that enables covalent coupling to the derivatized nanowires (5′NH_2-C_{18}-C_{18}-N1to3-B63′). The 3′ end base of the attaching probe corresponds to the SNP position. The 5′ phosphorylated labeled probes have five informative and one or two degenerate bases, and con-

Table 1 Allele-specific oligonucleotides used for SNP genotyping

Nanoplex Name	Gene	Accession #	Position	wt	mut	Wildtype FP	Mutant FP	Common LP
N1	CYP1A1-A4889G	X02612	6819	G	A	agaccg	agaccA	ttgcc
N2	CYP1A1-T6235C	D12525	360	T	C	tggagt	tggagC	gcact
N3	CYP1A2-CINTRONA	M31664	2640	C	A	tgggcc	tgggcA	cagga
N4	CYP2C18-T479C	L16869	792	T	C	agcact	agcacC	taggg
N5	CYP2C19-G681A	M61854	686	G	A	ttcccg	ttcccA	ggaac
N6	CYP2C8-C1196T	Y00498	1238	A	G	tgacaa	tgacaG	agaat
N7	CYP2C8-C1479T	Y00498	1539	T	C	gctgct	gctgcC	gatct
N8	CYP2C8-G416A	Y00498	458	G	A	gaagag	gaagaA	gagca
N9	CYP2D6-C188T	M33388	1719	C	T	gctacc	gctacT	cacca
N10	CYP2D6-C2938T	M33388	4469	C	T	acctgc	acctgT	gcata
N11	CYP2D6-G1749C	M33388	3280	G	T	tccgtg	tccgtT	tccac
N12	CYP2D6-G1934A	M33388	3465	G	A	ccccag	ccccaA	gacgc
N13	CYP2D6-G4268C	M33388	5799	G	C	ggtgag	ggtgaC	cccat
N14	CYP3A4-A816G	D11131	816	A	G	gggcaa	gggcaG	gagag
N15	CYP3A5-T(-369)G	AF280107	145601	T	G	gggctt	gggctG	gcaag
N16	GSTP1-A313G	M24485	2630	T	C	acatct	acatcC	ccctc
N17	NAT2-C282T	U53473	282	C	T	ttttac	ttttaT	atccc
N18	NAT2-C481T	U53473	481	C	T	ggtacc	ggtacT	tggac
N19	NAT2-G590A	U53473	590	G	A	acctcg	acctcA	aacaa
N20	NAT2-T341C	U53473	341	T	C	gaccat	gaccaC	tgacg

Key: FP=fixed probe; LP=labeled probe; wt=wild type (A allele) ; mut=mutant (B allele)

tained a 3′ fluorescent dye, TAMRA (5′P-B5-N1to2-TAM RA3′). All probes were purchased from Biosearch Technologies (Novato, CA, USA).

1-Ethyl-3-(3- dimethylaminopropyl) carbodiimide HCl (EDC) chemistry was used to attach the primary amine groups on the oligonucleotide fixed probe to the carboxyl groups on the nanowires. Approximately $5x10^7$ nanowires in 50 μL 10 mmol L^{-1} MUA were washed in 50 mmol L^{-1} of the buffer 2-(*N*-morpholino) ethanesulfonic acid (MES buffer) at pH 4.5. The particles were resuspended in 170 μL 50 mmol L^{-1} MES (pH 4.5) containing 150 pmol of the appropriate fixed probe oligonucleotide. EDC (20%, 15 μL) was added and the reaction mixture was mixed well and incubated for 1 h at 4°C. The reaction was gently agitated by rotating. After 1 h the particles were washed four times with 500 μL 10 mmol L^{-1} PBS to remove the unconjugated oligonucleotide, using centrifugation. The particles were resuspended in 50 μL PBS buffer.

Universal short-oligonucleotide-based ligation assay using nanowires

The target, at a concentration of 1 pmol in 10 μL (either synthetic oligonucleotide target or single-stranded PCR product), was denatured by boiling for 2 min and snap cooled on ice. This was mixed with 33.5 μL Callida's premade 3X ligation buffer containing ligase from Epicenter Technologies (Madison, WI, USA), 1.5 μL labeled probe (5 pmol μL^{-1}), 5×10^6 fixed probe conjugated particles in 5 μL, and diluted to a total volume of 50 μL. The reaction was left to proceed at 25°C for 1.5 h. After the ligation step, high stringency washes were performed to remove unligated and mismatched labeled probe, using the conditions: 1X SSC for 5 min at room temperature then 0.1X SSC for 8 min at 55°C.

Data collection and analysis

Data collection was performed with a Zeiss Axiovert 100 microscope fitted with a Prior H107 stage, Sutter Instruments 300W Xe lamp with liquid light guide, Physik Instrumente 400 micron travel objective positioner, and Photometrics CoolSnapHQ camera. Images were acquired with a 63X, 1.4NA objective. The microscope and all components were controlled by a proprietary software package that performs intra and inter-well moves, automatically focuses at each new position, acquires a brightfield image of the particles at 405 nm (to image particle reflectance), and then acquires the corresponding fluorescence (TAMRA) image. The average time for image pair acquisition including stage move, filter selection, and focus was 2 s. The brightfield and fluorescence image pairs were analyzed by NBSee Software, a proprietary image-analysis software package that identifies the nanowires and quantifies their associated fluorescence. Results were stored in a local database for final analysis and SNP scoring.

Analysis begins with examination of the mean pixel fluorescence value over each nanowire particle in the fluorescent image, followed by background subtraction and a calculation of the median of fluorescent values across nanowires of the same striping pattern. Allele classification is performed by a simple ratio method. If the ratio of the

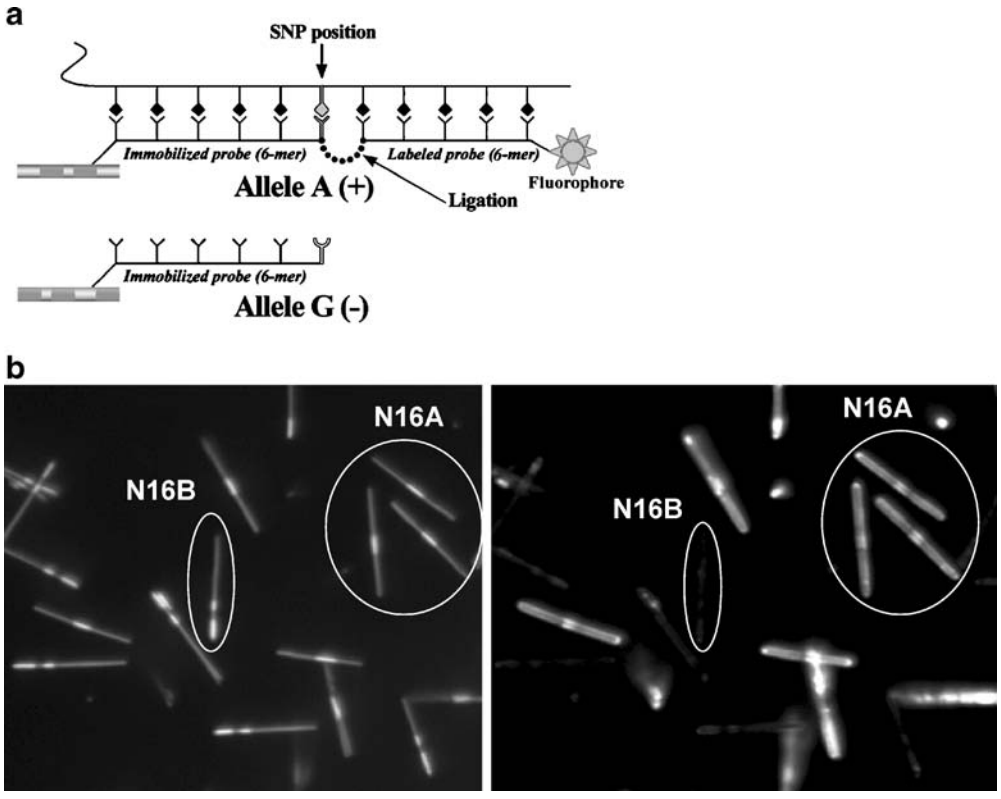

Fig. 1 **a** Schematic diagram of work-flow for nanowire genotyping assay. Each nanowire library member is conjugated to a different oligonucleotides fixed probe. Fluorescently labeled oligonucleotides (labeled probes) and PCR target are added to the reaction. The fixed probe and labeled probe hybridize to the PCR target, and ligation of the fixed and labeled probe occurs if the SNP sequence is complementary to both probes. In this figure allele A is a perfect full-match to the PCR sequence and, therefore, the labeled probe and fixed probe are ligated. Allele G is mismatched and is not ligated. Both nanowires are images and analyzed to determine which probe is complementary to the PCR sequence. **b** Representative experimental image. *Left image*=reflectance image showing two populations of nanowires, each conjugated to a different oligonucleotide probe (N16A=00000110000 ; N16B=00000001011). *Right image*=fluorescence image. On addition of PCR product of SNP N16 from genomic DNA sample NA10356, only nanowire 00000110000 gives fluorescent signal, indicating that this genomic DNA sample is homozygous for allele A (wild-type)

median fluorescence intensity (MFI) between nanowire-associated alleles is greater than 2, the SNP call is homozygous, AA or BB. Heterozygotes (AB) are called if the ratio was between 1/2 and 2. For example, when an allele target was added to the SNP N19 (from gene NAT2) reaction, only the nanowire with the A allele probe was fluorescent (MFI=603), compared with a lower signal for the B allele (MFI=133). When both targets were added to the reaction, both nanowires were fluorescent (A allele= 752 MFI, B allele=843 MFI).

A fixed threshold ratio of 2.0 was chosen by misclassification analysis. Individual synthetic SNP targets for each allele were added to solutions of fixed and labeled probes and fluorescence ratios for each SNP allele pair were computed. Because the allelic targets are known, we can compare different threshold ratios with the misclassification percentage over a set of SNP pairs. In one experiment we had a 320 median fluorescent ratio pairs over 13 different SNP, including replicates. We calculated the percentage misclassification spanning threshold ratios from 1.5 to 2.5. The results are plotted in Fig. 1. Note, the minimum percentage misclassification corresponded to an A/B (or B/A) median intensity fluorescent ratio of 2.0.

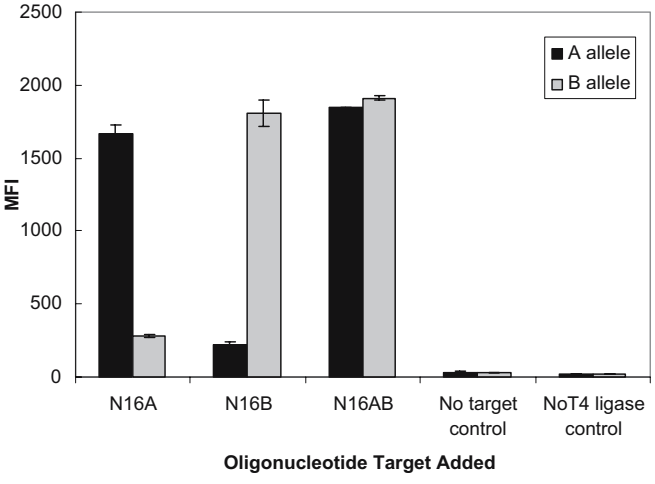

Fig. 2 Results from 2-plex SNP genotyping assay (SNP N16) performed in duplicate (average of two experiments). *Black bars* represent the signal on nanowires conjugated with fixed probe complementary to wild type allele (target SNP N16A) and *grey bars* represent the signal on nanowires conjugated with fixed probe complementary to mutant allele (target SNP N16B)

Results

Demonstration of short-oligonucleotide-based ligation assay using nanowires

The workflow of the short-oligonucleotide-based ligation assay (Fig. 2a) is as follows. Two attaching 6-mer oligonucleotide probes (referred to as "fixed probes") were selected such that the 3′ end of the oligonucleotide is complementary to each of the two alleles being interrogated. The 5′ end contains an amino group, such that it can be conjugated to the nanowires. Each fixed probe is attached to a uniquely striped (encoded) nanowire. Therefore, in each reaction there are two populations of striped particles for each SNP being interrogated. A "labeled probe" is selected that is complementary to the sequence immedi-

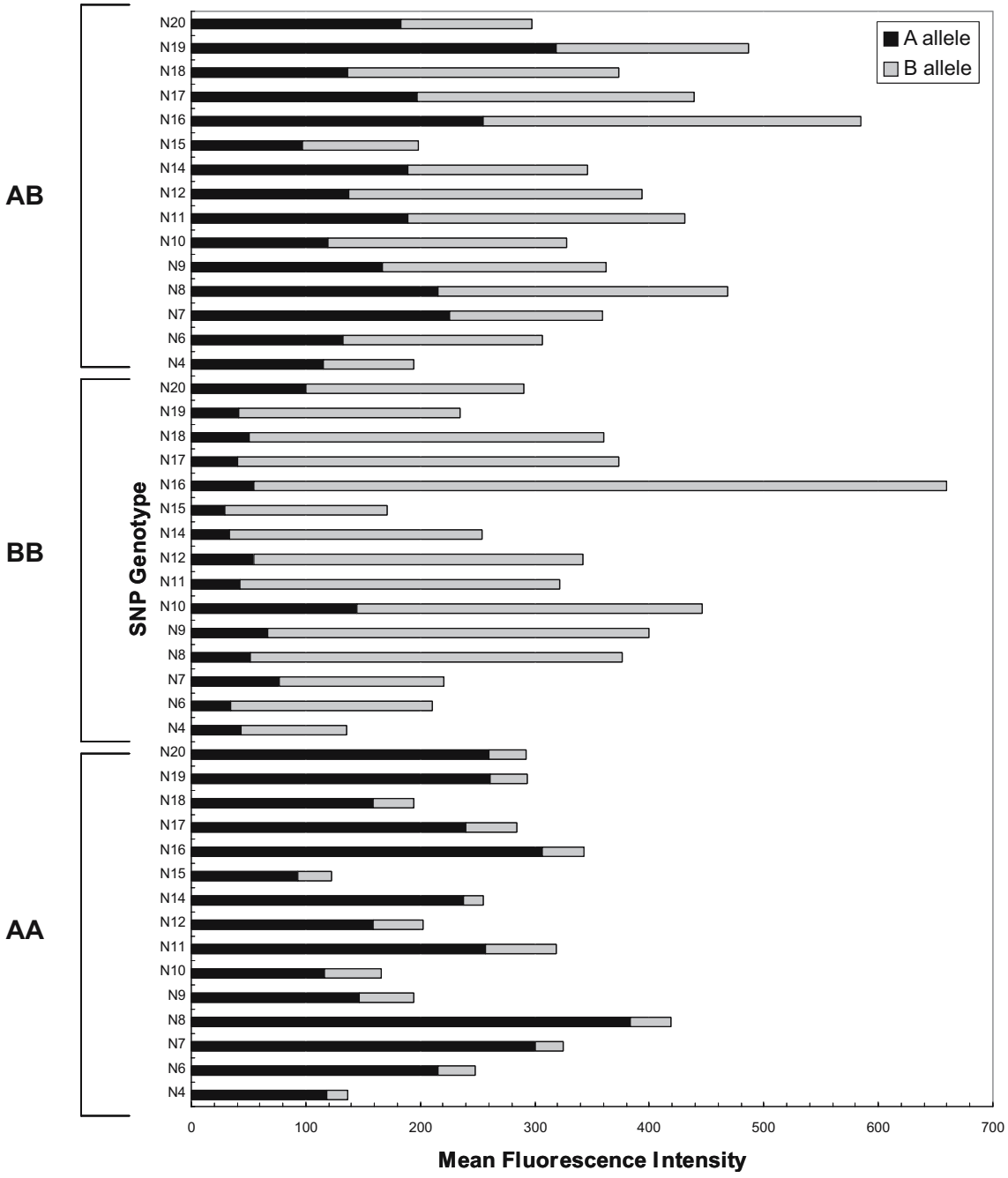

Fig. 3 Bar graph showing 15-plex SNP genotyping assays (N4, N6, N7, N8, N9, N10, N11, N12, N14, N15, N16, N17, N18, N19, and N20) corresponding to 30 nanowire library. *Black bars* represent the signal on nanowires conjugated with fixed probe complementary to wild type allele (A) and *grey bars* represent the signal on nanowires conjugated with fixed probe complementary to mutant allele B). Three reactions were performed in which only allele A target was added (AA), allele B target was added (BB), or both allele A and allele B were added (AB)

ately following the SNP. This probe contains a 3′ fluorophore. The two populations of nanowires conjugated to fixed probes are mixed with the labeled probes and the target material (either synthetic oligonucleotides or single-stranded PCR product). If the fixed and labeled probe successfully hybridize with the target as a template, the two sequences will be ligated by addition of a T4 ligase present in all mixtures. The result is that, after a wash step, one population of nanowires has a fluorescence dye conjugated to the surface, and appears fluorescent. Analysis follows. A representative image is shown in Fig. 2b.

As described above, we selected 20 SNP from the P450, GSTP1, and NAT2 genes. Corresponding fixed probes, labeled probes, and synthetic targets were selected. Their sequences are detailed in Table 1.

Initial work focused on demonstrating proof of concept, using synthetic oligonucleotide target in a one SNP, two nanowire experiment. This is shown in Fig. 3, in which a single SNP (SNP N16 from the GSTP1 gene) is interrogated in a two-plex reaction. Each nanowire is conjugated to either the wild type fixed probe or the mutant fixed probe, using procedures described above, and the two nanowire populations are mixed together. When synthetic target complementary to wild type SNP is added to the reaction only the corresponding nanowire population is fluorescent. When both synthetic targets are added to simulate a heterozygote, both nanowire populations are fluorescent. Two controls were performed, one in which there was no target and one in which there are both targets but no ligase. In both cases there is negligible background signal. The experiment was performed in duplicate, the results averaged and good reproducibility was observed. The same experiment was performed for each of 20 SNP, again using synthetic oligonucleotide targets, and the results confirmed that each probe has a sequence-specific reaction. In the control, in which the T4 ligation buffer and the synthetic DNA targets were eliminated, both had minimal median intensity. The sensitivity of the assay in a one-plex format was found to be between 2 nmol L^{-1} and 5 nmol L^{-1} of target, equating to approximately 0.1 pmol material.

Development of a 30-plex assay for 15 SNP, using synthetic DNA targets

After optimization of the probes and conditions within a 2-plex assay we progressed to a 30-plex assay, using synthetic oligonucteotide targets. We conjugated 30 fixed probes corresponding to each allele for 15 SNP to 30 different nanowire populations using the methods described above. For each pair of SNP we carefully selected the two striping patterns to give the greatest possibility of distinguishing each from the other. This was done empirically by imaging sets of single-population nanowires and measuring the percentage of miscalls to opposing types, establishing what is a termed a "confusion" matrix. Those pairs with the minimum value in this table, i.e. minimal confusion, were chosen as allelic SNP pairs. To one aliquot of the nanowire mixtures we spiked 15 synthetic target oligonucleotides corresponding to the "A" allele for each SNP (to mimic a homozygote A). To a second aliquot we spiked 15 synthetic target oligonucleotides corresponding to the "B" allele for each SNP (to mimic a homozygote B). To a third aliquot we spiked in all A and B synthetic target to mimic a heterozygote. The results are shown in Fig. 4. The data represent median fluorescent intensities across nanowires of each type, or SNP allele. The results showed that the 15 target "A" allele or 15 target "B" allele and the combination of "A" and "B" allele are clearly discriminated.

Multiplexed SNP genotyping for human genomic DNA

Having demonstrated proof of principle with synthetic oligonucleotide targets, the assay was optimized for use with PCR products ("real-world samples"). Initially con-

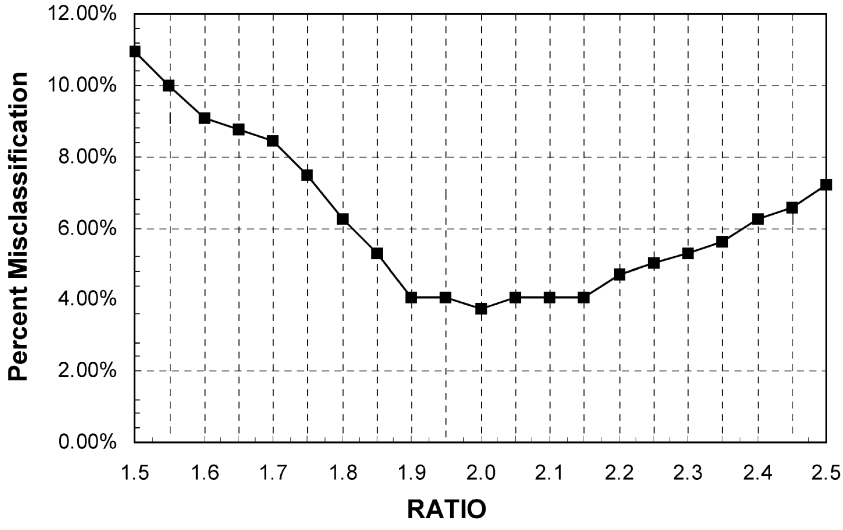

Fig. 4 Misclassification versus the ratio of median fluorescent intensity on nanowires corresponding to allelic pairs. The experiment used 320 known synthetic allelic pairs with replicates

Table 2 Summary of genotyping results from 8-plex multiplexed PCR product, in 16 NBC experiment. Results show comparison of gel sequencing (Gel Seq) to nanowires (NBC) assay for each of 20 genomic DNA samples

Coriell DNA	SNP N3		SNP N6		SNP N10		SNP N14		SNP N16		SNP N17		SNP N18		SNP N19	
Cell Line ID	Gel Seq	NBC	Gel Seq	NBC	Gel Seq	NBC	Gel Seq	NBC	Gel Seq	NBC	Gel Seq	NBC	Gel Seq	NBC	Gel Seq	NBC
NA01850	a	a	a	a	c/t	c/t	g	g	t	t/c	c/t	c/t	c	c	g	g
NA03433	a	a	a	a	c	c	a	a	t	t	t	t	c	c	g/a	g
NA03725	c/a	c	a	a	c/t	c/t	a/g	a/g	t	t/c	c/t	c/t	c/t	c/t	g	g
NA07513	c/a	c/a	a	a	t	t	a	a	t	t	t	t	c	c	a	a
NA07524	c/a	c/a	a	a	c	c	a	a	t	t	t	t	c	c	a	a
NA08729	c	c	a	a	ct	c/t	a	a	t	t	c/t	c/t	c/t	c/t	g/a	g
NA08786	c/a	c/a	a/g	a/g	c/t	c/t	a	a	t	t	c	c	c/t	c/t	g	g
NA09549	a	a	a	a	c	c	a	a	t	t/c	c	c	c/t	c/t	g	g
NA09618	a	a	a	a	c	c	a/g	a/g	t	t	c	c	c/t	c/t	g	g
NA10356	a	a	a	A	c	c	a	a	t	t	c/t	c/t	c/t	c/t	g/a	g/a
NA10384	c/a	c/a	a/g	a/g	c/t	c/t	a	a	t	t	c	c	c	c	g	g
NA10432	c/a	c/a	a/g	a/g	c/t	c/t	a	a/g	t	t	c/t	c/t	c/t	c/t	g/a	g/a
NA10647	a	a	a/g	a/g	c	c	a	a	t	t	c/t	c/t	c/t	c/t	g/a	g
NA11322	a	a	a	a	c	c	a	a	t	t	t	t	c	c	a	a
NA11324	c/a	c/a	a	a	c	c	a	a	t	t	c	c	c	c	g	g
NA11589	a	a	a	a	c	c	a	a	t	t	c	c	c	c	g	g
NA11590	a	a	a	a	c	c	a	a	t	t	c	c	c	c	g	g
NA13787	c/a	c/a	a	a	c	c	a	a	t	t	t	t	c	c	a	a
NA14892	c/a	c/a	a	a	c/t	c/t	g	g	t	t	t	t	c	c	g/a	g/a
NA14893	c	c	a	a	c/t	c/t	g	g	t	t	c	c	c/t	c/t	g	g
SNP	C-A		A-G		C-T		A-G		T-C		C-T		C-T		G-A	

ditions were optimized using a single PCR amplicon corresponding to N6, a SNP found in CYP2C8. It was determined that in a two-nanowire, one-SNP experiment; 4 μL of a 100 μL PCR reaction using 100 ng of genomic DNA (cell line NA10356) gave ample discrimination. Next, eight individually amplified PCR products were pooled at equal concentrations to simulate a multiplexed PCR product. This was done to ensure equal concentrations of products, because quantification of multiplex PCR reactions is problematic. The eight SNP selected were N3, N6, N10, N14, N16, N17, N18, and N19, in a 16-plex NBC-probe reaction. The experiment was performed in two cell lines (NA10356 and NA14893). The results showed that each of the SNP could be easily identified simultaneously by its sequence-specific probe (data not shown). For the 16 data points collected (two cell lines, eight SNP) all SNP could be called with a 100% concordance to gel sequencing.

To demonstrate the robustness of the assay, the eight SNP selected above were amplified in a multiplexed PCR

Table 3 Genotyping results from 30 nanowire, 15 SNP assay with 8-plex PCR genomic DNA, using cell line NA14893. Multiplexed PCR reaction contains N3, N6, N10, N14, N16, N17, N18, N19 amplicons only

SNP	Allele	PCR Product	A Allele MFI	B Allele MFI	Nanowires SNP call	Gel Seq SNP call
N3	C/A	Yes	684	20	C	C
N6	A/G	Yes	511	127	A	A
N10	C/T	Yes	370	591	C/T	C/T
N14	A/G	Yes	31	269	G	G
N16	T/C	Yes	381	40	T	T
N17	C/T	Yes	450	44	C	C
N18	C/T	Yes	670	764	C/T	C/T
N19	G/A	Yes	299	27	G	G
N2	T/C	No	17	23		
N4	T/C	No	19	10		
N7	T/C	No	32	25		
N8	G/A	No	29	43		
N12	G/A	No	27	19		
N13	G/C	No	16	22		
N15	T/G	No	91	10		

reaction (N3, N6, N10, N14, N16, N17, N18, and N19), in all 20 cell lines. The SNP genotyping results are summarized in Table 2. These results show that each pair of SNP probes discriminated between homozygous and heterozygous SNP genoptypes from all 20 individuals' DNA samples tested. Of 160 genotypes, 155 were called correctly using gel sequencing as a reference. Three genotypes from SNP N16 (cell line NA01850, NA03725, NA09549) were incorrectly called, because of very low signal (essentially no-calls). Upon gel sequencing a mutation within the fixed probe position was discovered, which was deemed responsible for the poor hybridization, and thus resulted in low fluorescence signal in the alleles of the SNP. Two genotypes (one in SNP N3 and one in SNP N14) did not agree with gel sequencing. In both cases the correct alleles gave high signals, but using the unsophisticated approach of using a call-ratio of ±2, led us to miscall them. Further work is in progress to develop more sophisticated data-analysis tools (see Discussion section). Finally, two genotypes from SNP 19 (NA03433, NA08729) gave incorrect calls.

In addition, to further demonstrate the real-world application of the assay, we repeated the experiment with 30 nanowires corresponding to 15 SNP, and genotyped the 8-plex PCR amplicons from one cell line (NA14893) (Table 3). In this case, data show there is 100% concordance with gel sequencing, in agreement with Table 2, and minimal signal from non-specific binding.

Discussion

Although there are different designs of SNP genotyping experiment (for example, many patients versus many SNP) for which different technologies are appropriate, the common key requirements for any SNP genotyping technology is that it should be cost-effective, highly accurate, and amenable to multiplexing [27]. Nanowire technology has several key features which make it attractive as a genotyping platform.

(1) The multiplexing capability is large; the current eleven-stripe format, as used in this paper, allows for 1056 unique particle types. Thirteen-stripe particles would enable a library size of 4160 unique particles. Because the format of the assay here utilizes a 6-mer fixed probe, of which there are 4096 combinations, expansion of this assay using a 13-stripe particle format would result in a universal genotyping platform. In the current assay format we utilize only a single fluorescent dye as a reporter. Doubling of the multiplexing capability is, however, easily achievable by assigning each allele a different fluorescent dye, such that each SNP can be interrogated by one nanowire, not two. This is an option not open to other fluorescent particle-based systems.
(2) The data collected in this, albeit small, study are highly accurate.
(3) The system is very flexible, enabling additional SNP to be added into an existing master mix. For example, as discussed above we identified three genotypes in SNP 16 that were incorrectly called during the course of the experiment. On sequencing of the PCR products, mutations within the fixed probe sequences were identified. The problem can be rectified quite simply by adding an additional pair of fixed probes (selected from the validated universal set) matching this mutant into the existing mix. This is less straightforward in an arrayed format.
(4) In house we have developed high-throughput screening capabilities, using standard inverted microscope equipment.
(5) We believe that compared with a two-dimensional microarray, suspended nanoparticles may have better reaction kinetics, minimizing the problems of microarrays related to slow diffusion at the solid and liquid interface [28]. This property enables us to complete the reaction and obtain results, assuming one starts with conjugated particles, in less than 2 h.

One shortcoming of solution arrays is often thought to be settling of particles before the reaction is complete [28]. We circumvent this issue by performing the assay in a high concentration of PEG (10%) to increase the viscosity of the solution, and by rotating the reaction during the course of the assay. We can also take advantage of the settling behavior to perform physical separations such as those needed to remove excess target, by using gravity and centrifugation to pellet the particles before removing the supernatant. Currently a limitation of this assay is the amount of DNA required per assay. We currently require approximately 6 pg genomic DNA per SNP. This is greater than other assay formats, and so sample preparation is currently a limiting step. Although the nanowire technology can clearly achieve higher levels of multiplexing than demonstrated here, our current sensitivity means that multiplex PCR amplification remains a bottleneck, because of the inability of highly multiplexed PCR reactions to generate sufficient quantity of amplicons required for our assay. Several laboratories report the pooling of PCR target to obtain a highly multiplexed level of SNP genotyping [29]; this is not, however, an optimum approach. There are many approaches to increasing PCR yield in a multiplexed format, including the use of universal primers [30], the use of ligated primers [31], or whole genome amplification approaches [32]. We are continuing to develop this platform to achieve better sensitivity. In summary, we have demonstrated the use of encoded nanowires as a platform for genotyping.

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