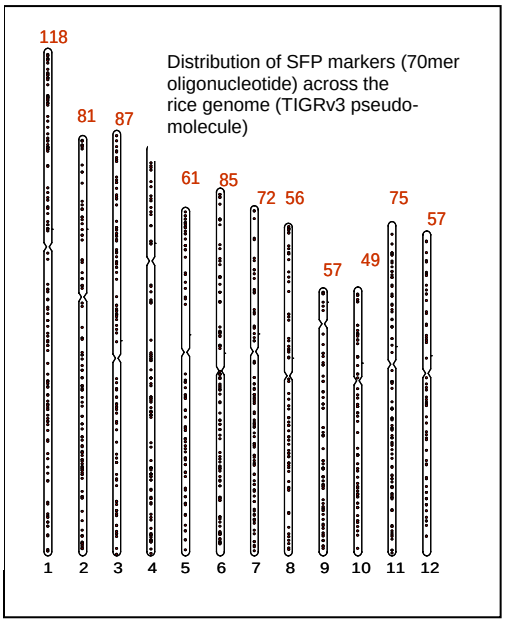
Microarray-based genotyping of Single Feature Polymorphism (SFP)

Introduction

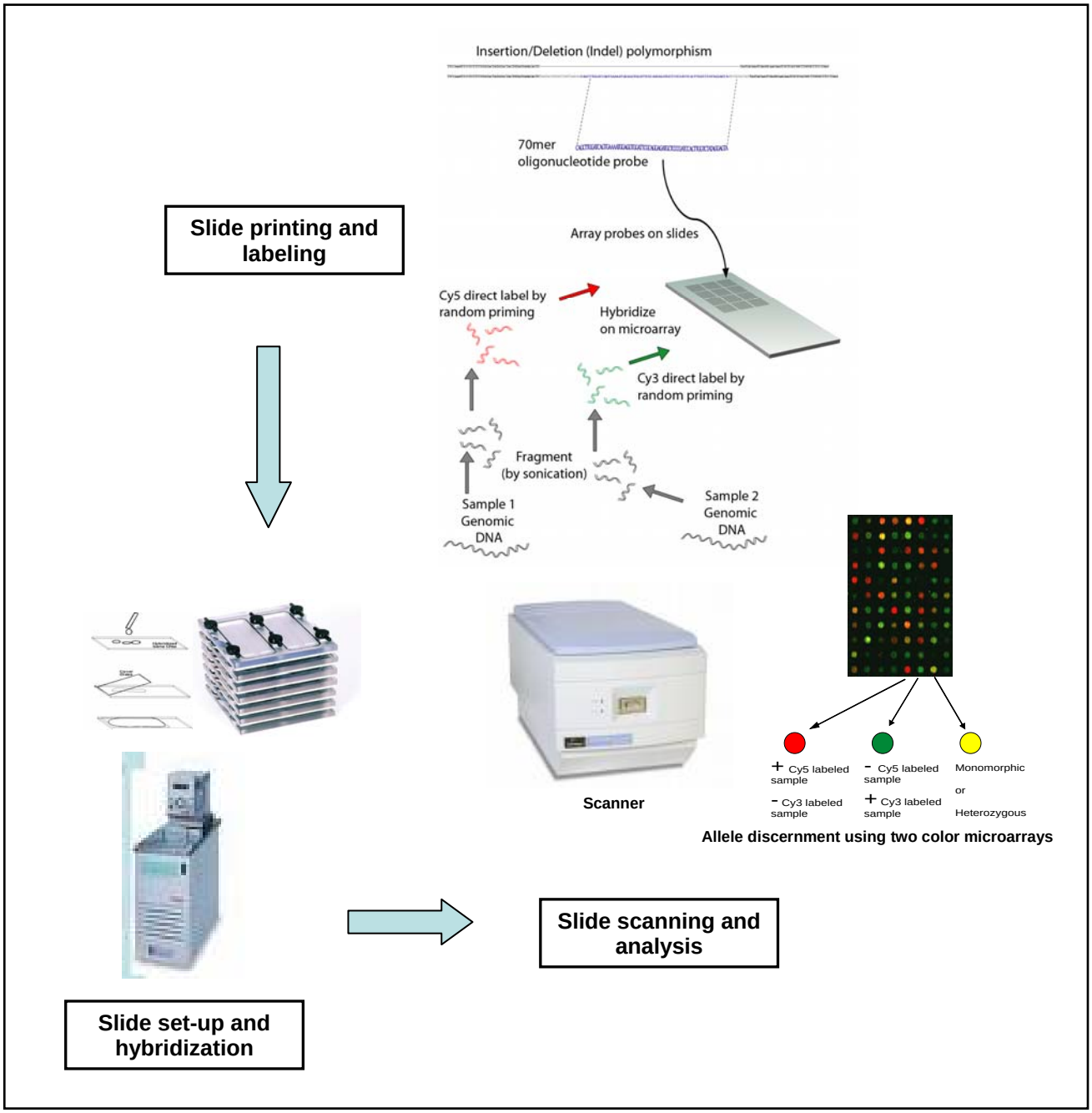
Microarray-based genotyping provides the means to simultaneously screen hundreds to thousands of markers per individual. This technology is particularly suited for applications requiring whole-genome coverage, and the relatively low cost of this assay makes it possible to implement this genotyping strategy using large populations. Along with foreground selection for the target traits, high-resolution whole-genome selection will provide a greater capacity for background selection to retain the positive attributes of popular varieties in backcrossing programs. Obtaining graphical genotypes of individuals will facilitate pyramiding of desirable alleles at multiple loci, and will shorten the time needed for the development of new varieties.

Indel polymorphisms, also known as single feature polymorphisms (SFPs), are particularly amenable to microarray-based genotyping. These assays are done by labeling genomic DNA (target) and hybridizing to arrayed oligonucleotide probes that are complementary to indel loci. The SFPs can be discovered through sequence alignments or by hybridization of genomic DNA with whole genome microarrays. Each SFP is scored by the presence or absence of a hybridization signal with its corresponding oligonucleotide probe on the array. Both spotted oligonucleotides (Barrett *et al.*, 2004) and Affymetrix-type arrays (Borevitz *et al.*, 2003) have been used in these assays. For genotyping large populations, the cost per individual is more critical than the cost per data point. Spotted oligonucleotide microarrays have the potential to provide low cost genotyping platforms (Stickney *et al.*, 2002). The availability of genomic sequence from multiple accessions presents opportunities for the design of spotted long oligonucleotide microarrays for low cost/high density genotyping of rice.

The SFP genotyping slide was developed through a collaboration between D. Galbraith, University of Arizona and H. Leung, IRRI. By aligning the publicly available genomic sequences of the Nipponbare and 9311 cultivars representing the *japonica* and *indica* sub-species of rice, they have identified 1,264 SFPs suitable for probe design. The SFPs are evenly distributed over the whole genome (median distance between markers 128 kb, Figure 1). Results showed that these probes yielded conservatively 30-50% polymorphism between a pair of rice lines (lowest between *japonica* types). Thus a single contrast produces around 400 well-spaced, polymorphic gene-based markers for any pair of unrelated parental lines. One advantage of the DNA hybridization based genotyping procedure is that it can be used for quantitative genotyping of pooled samples.



880 markers designed, 234 kb median distance between markers



Workflow of SFP genotyping: Slide printing, labeling, hybridization, scanning and analysis

I. Genomic DNA labeling with Cy3 and Cy5 fluorescent dyes

A. Setting-up digests using *Dpn II* restriction enzyme:

Reagents	**Final concentration**	**Volume**	
		Sample 1	**Sample 2**
Genomic DNA	4μg	____μl	____μl
Dpn II buffer		4.0 μl	
Dpn II enzyme (10u/μl)	20 U	2.0μl	
Sterile DW		15 μl - ___ μl DNA	15 μl - ___ μl DNA
Total		**21 μl**	

Quick spin the reaction. Place in a PCR machine programmed @ 37°C for 2 hrs to proceed digestion.

Inactivate reaction @ 65°C for 20 min (programmed at the PCR machine)

(Optional) Confirm digestion by running 1ul of the digest in 0.7 % agarose gel in 1XTAE. Mupid gel.

1. Clean digest by adding 42 μl (2X volume) of cold absolute ethanol to each tube
2. Incubate @-80ºC for 1 hr
3. Centrifuge for 30 min at 0ºC for 12500 rpm
4. Remove supernatant
5. Wash with 750-1000 μl 95% ETOH @ RT. Invert tube.
6. Centrifuge for 10 min @ 4ºC at for 12500 rpm
7. Air dry completely. Use a concentrator or speed vac if available.
8. Add 21 μl SDW. Allow pellet to dissolve for 10 -15 min.

B. Labeling with Cy3 & Cy5 fluorescent dyes (BioPrime Array CGH Genomic labeling system, 30 reactions; Cat# 18095-012, INVITROGEN)

	Cyanine 3 (Control) Sample _____	**Cyanine 5 (Treatment)** Sample _____
Digested genomic DNA	21 μl	21 μl
2.5X Random primers (random octamers) solution	20 μl	20 μl
Incubate @ 95C (programmed in a PCR machine) for 5 min. Immediately cool on ice for another 5 min. Add the following on ice:		
10X dUTP Nucleotide mix	5 μl	5 μl
Add	**1**μl of cyanine 3-dUTP	**1** μl of cyanine 5-dUTP
Exo-klenow Fragment	**1** μl	**1** μl
Mix gently and spin down. **Incubate at 37^{o} C for 2 hrs.**		
Add Stop buffer	5 μl	5 μl

C. Purification of labeled targets using NucleoSpin Extract II (Machery-Nagel, Cat# 740 609 250, 250 preps)

1. Mix the 2 tubes (Cy3 & Cy5), violet color will appear
2. Add 220 μl Buffer NT
3. Load sample into the column-tube set up
4. Spin at 11,000 x g for 1 min
5. Discard flow through, and place the column back to the tube
6. Add 600 μl NT3
7. Centrifuge at 11,000 x g (12000 rpm) for 1 min. Discard flow through.
8. Dry silica membrane by spinning at 11,000 x g (12000 rpm) for 2 min
9. Place the column into a fresh 1.5 ml amber tube and add 40ul SDW.
10. Incubate for 1 min
11. Centrifuge for 1 min at 11,000 x g (12000 rpm)
12. Prepare for hybridization

II. Pre-processing of SFP slides before hybridization

1. Rehydration & fixing: *(This can be performed well in advance before hybridization. The slides can be stored at RT for several months.)*
   a. Rehydrate the spots by placing the slides over 50 ºC water bath for 10 sec, and snap dry over 65 ºC heating block for 5 secs. Repeat 4-5 times.
   b. Fix the spots by UV cross-linking set at 600mJ.
2. Washing: (The following steps can be done at the same time while performing labeling of samples)
   a. Using 50ml sterile falcon tubes, wash slides in sterile distilled water. Soak in 1% BSA solution (in 6.67X SSC) for at least 2 hrs in a water bath set @ 37ºC.
   b. Wash in 1% SDS for 5min @ RT.
   c. Wash in 5 changes of sterile distilled water for 5 min.
   d. Spin dry at 2000rpm for 2 min @ RT.
   e. Slides are ready for hybridization.

III. Slide Hybridization

1. To prepare hybridization solution, add the following in 0.5 ml amber tube

40.0 μl	Purified labeled targets (Cy3 & Cy5)
3.0 μl	Herring sperm DNA (10ug/ul)
10.0 μl	2X MES buffer

53.0 μl	Total

2. Denature hybridization solution at 99ºC for 5 min in a PCR machine. Prepare the hybridization chamber as illustrated in the schematic diagram. Hybridize overnight in a water bath set @ 50ºC.

III. Washing slide after hybridization

1. Prepare washes and wash slide for 5 min each as follows:
   - 6X SSPE, 0.005% SDS @ 50ºC, (incubate the wash solution in a water bath before hand @ 50ºC);
   - 0.06X SSPE @ RT;
   - 0.02X SSPE @ RT
2. Spin down slide using a swinging bucket centrifuge @ 2000 rpm, RT for 1 min to dry slide. Scan.

SFP genotyping cost estimate

Items/reagents	Price/unit ($)	Remarks	Cost per hybridization ($)
BIO Prime Array CGH Genomic labeling system (30 reactions)	311.00	good for 15 dual labeling reactions	20.73
CyDye Cy5-dUTP, 25 nmole	329.00	good for 25 labeling reactions	13.16
CyDye Cy3-dUTP, 25 nmole	318.00	good for 25 labeling reactions	12.72
Dpn II restriction endonuclease enzyme (1000 units)	63.00	good for 100 digests (needs 2 digestion set-up per hyb	1.26
MN Nucleo-Spin Extract II, 250 preps	450.00	For cleaning labeled target	1.8
SFP slide from Galbraith's lab	11.00	Contains 1,260 indels	11.00
		Total	**60.67**
		Cost per data point	***0.048***

Note: Optimization of protocol is underway to reduce cost per slide hybridization. We will replace labeling kit with home made reagents. Target is to cut cost by half.