

**Real-time fluorescent image analysis of DNA spot hybridization kinetics  
to assess microarray spot heterogeneity**

*Supporting Information*

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**Data Presented:**

**Material and Methods:**

- Table S-1: Oligonucleotide sequences and terminal modifications used in this study.
- Detailed explanations for printing and post-printing methodologies; real time hybridization and FRET studies with both confocal microscopy and fluorescence spectrophotometry.

**Results and discussion:**

- Figure S-1: Schematics of real-time hybridization and FRET analysis of DNA duplexes in different probe-target dye orientations.
- Figure S-2: Confocal measurements of duplex formation with target on immobilized probe DNA spots: reduction in Cy3-DNA probe fluorescence emission intensities irrespective of target DNA with or without Cy5 dye label.
- Figure S-3: Plot of real-time DNA spot hybridization kinetics.
- Figure S-4: Plot of FRET in solution compared to controls of individual excitation and emission spectra for probe DNA (5'-Cy3 oligo1) and target DNA (5'-Cy5 oligo2) and (3'-Oligo2-Cy5).
- Figure S-5: Fluorescence scanner images for spot Cy3 fluorescence emission intensity before real time hybridization analysis with confocal and after completion of target hybridization: (Cy3) at 535nm and (Cy5) 635nm, respectively.
- Figure S-6. Increase in time to crossover of Cy3-Cy5 emission intensities, and time-to-signal saturation in single printed spots with increasing printed DNA probe concentration observed with real-time DNA single spot hybridization assays.
- Figure S-7. Plot of kinetics of DNA hybridization (Cy5 dye target vs. time in seconds).
- Figure S-8. Schematic of DNA microarray FRET assay using dye-labeled DNA with different dye orientations in duplexes of surface-bound single spot assays.
- Figure S-9. Fluorescence emission intensities of Cy3 probe and Cy5 target as a function of depth in single hybridized (bleached vs. unbleached) microspots using step-wise confocal z-slices of 0.5  $\mu\text{m}$ .

## Materials and Methods:

**Table S-1.** Oligonucleotide sequences and terminal modifications

DNA	identifier	5'-modification	sequence	3'-modification
probe	Oligo1-NH <sub>2</sub>	--	CTGAACGGTAGCATCTTGAC	--
probe	Cy3-Oligo1-NH <sub>2</sub>	Cy3-C <sub>6</sub> -	CTGAACGGTAGCATCTTGAC	C6-NH <sub>2</sub>
target	Oligo2	--	GTCAAGATGCTACCGTTCAG	--
target	Cy5-Oligo2	Cy5-C <sub>6</sub> -	GTCAAGATGCTACCGTTCAG	--
target	Oligo2-Cy5		GTCAAGATGCTACCGTTCAG	Cy5-C <sub>6</sub> -

**Fabrication of DNA probe microspots with contact printing:** Multiple Microarray Format SpoCLE Generator version 1.1.02 software (ArrayIt) was used to print microarrays on commercial microarray polymer-coated slides (Slide-H, Schott-Nexterion). SMP4 pins were used to spot 1.1 nanoliters of DNA solution, yielding probe spots with dried diameters of approximately 135 microns and spot-to-spot spacing of 160 microns. DNA spots were printed with optimized pin wash and dry cycles to prevent pin carryover of DNA probe solution between prints. Spots were printed in replicates of 5 followed by a wash (wash buffer of 20% ethanol in water with 0.01% Tween 20) and dry cycle. Humidity during printing was approximately 60%. Printed slides were incubated overnight (>12 h) at room temperature under 75% relative humidity controlled by a saturated saltwater bath. Prior to starting a print run, the print head was calibrated according to manufacturer's recommended procedure with SNS4 solid calibrating pins for 135-micron diameter spots.

**Post-print array slide blocking:** Printed slides were treated with a blocking solution to avoid non-specific DNA surface absorption in a stepwise protocol similar to recommendations from the manufacturer. Printed slides were rinsed in print buffer and then immersed in a blocking solution (50 mM ethanolamine in 0.1 M Tris, pH 9.0) at 50°C for 30 min to consume residual amine-reactive surface groups. Following blocking, slides were rinsed thoroughly with ultrapure water 3 times, blown dry with nitrogen and scanned in a fluorescence scanner to locate and record spot intensity and shape. Slides were then stored in glass slide containers filled with nitrogen and sealed in parafilm until analyzed with confocal microscopy.

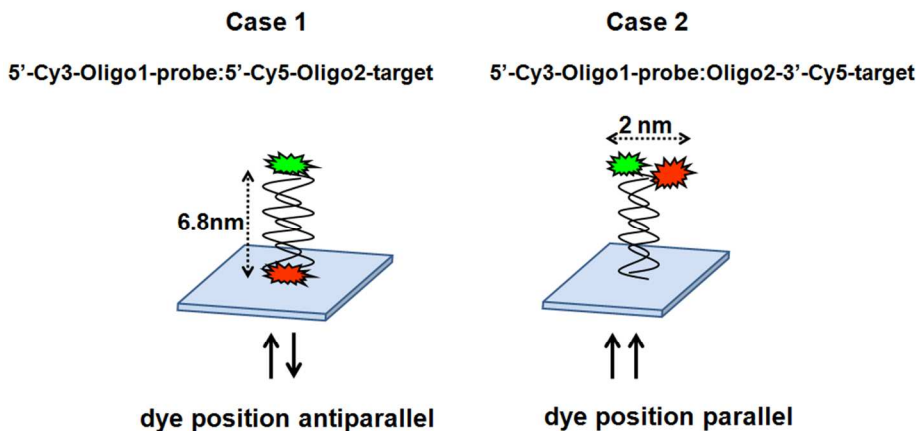
**Real-time hybridization and FRET with confocal microscopy:** Real-time hybridization and FRET was performed similarly to well-established protocols for microarrays. Complementary target DNA (~20  $\mu$ l volume) was added over single probe spots for real-time *in situ* hybridization studies. A transparent silicone gasket with adhesive on both sides (Grace Biolabs, specific for imaging) was placed around the probe array to contain the target DNA solution for hybridization. The slide was maintained at 60% humidity and 23°C to limit evaporation of small target solution volumes (consistent with most protocols). Sequential excitation/emission of Cy3 and Cy5 in the same spot location was measured during the time-lapse for real-time hybridization. Optimization of the signal intensity and array imaging in buffer was established using control spots prior to real-time detection with hybridization buffer on different spots.

Real-time 3D images of hybridization of Cy5-labeled DNA targets in hybridization buffer with immobilized Cy3-labeled DNA probes were collected. A single centrally located probe array spot was used to adjust the focus and Cy3 signal intensity. A 30-min time lapse was then recorded both Cy3 and Cy5 at single spot images every 15 seconds. Experiments were performed with various DNA probe concentrations (40 $\mu$ M,

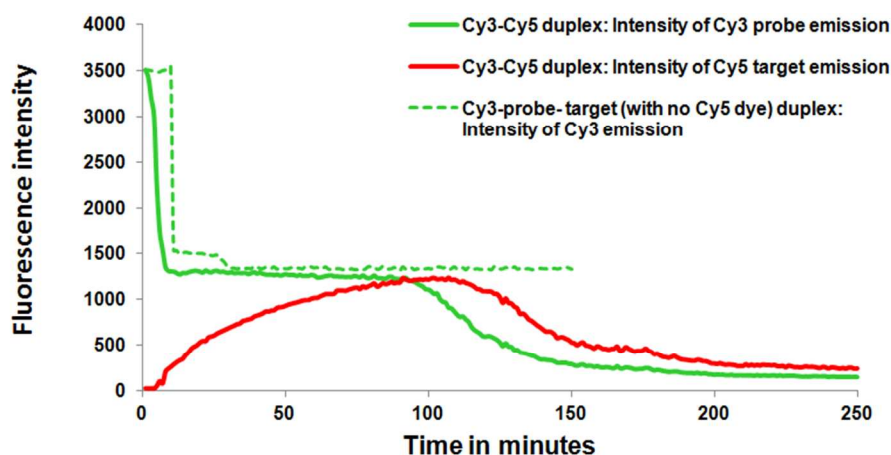
20 $\mu$ M and 10 $\mu$ M) and varying amounts of printed Cy3-DNA probe fractions (100% 75%, 50%, 25% and 10%). Real-time hybridization kinetics for single printed spots were recorded for two different cases of probe-target relative dye orientations: Cy3-probe dye-up: Cy5-target dye-down (i.e., antiparallel dyes, case 1), and both probe and target dye-up (i.e., adjacent dyes, case 2) as illustrated in Figure 1. Hybridization of unlabeled probe (no Cy3 dye) with target was not performed due to the difficulty in finding/visualizing and focusing on unlabeled printed DNA spots after addition of bulk Cy5-target.

**DNA duplex-induced Cy3-Cy5 FRET in solution:** This experiment was performed using varied total DNA and dye-labeled DNA fractional concentrations in hybridization buffer at room temperature (23 $^{\circ}$ C). Initially Cy3 and Cy5 excitation and emission spectra were recorded separately to obtain the  $\lambda_{ex,max}$  and  $\lambda_{em,max}$  for Cy3-DNA probe with fluorophore at the 5' position and Cy5-DNA target having fluorophores at either 3' or 5' positions. FRET experiments were then performed for these same configurations. In cuvettes containing Cy3-DNA, Cy5-DNA was added with optical excitation set to 547 nm, and emission was recorded from 555-750nm over time. Optical excitation was then changed to 645 nm, and emission was recorded from 655-750nm. FRET was assessed by observing shifts in the wavelength or changes in the wavelength intensity of acceptor (Cy5) by energy transfer from the donor (Cy3).

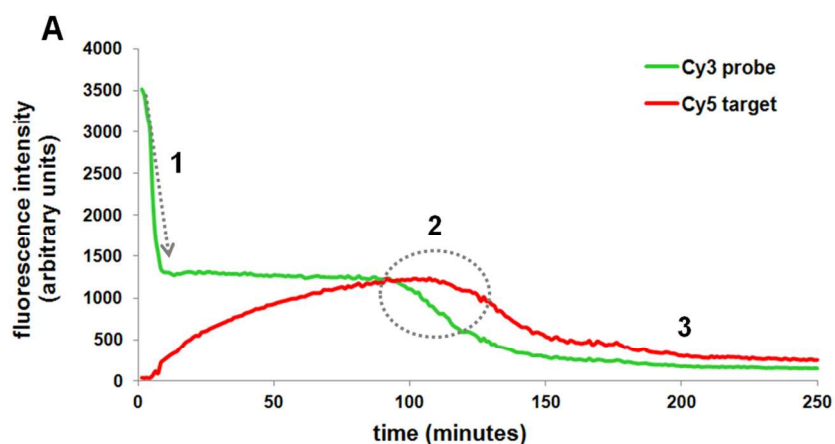
## Results and Discussion:



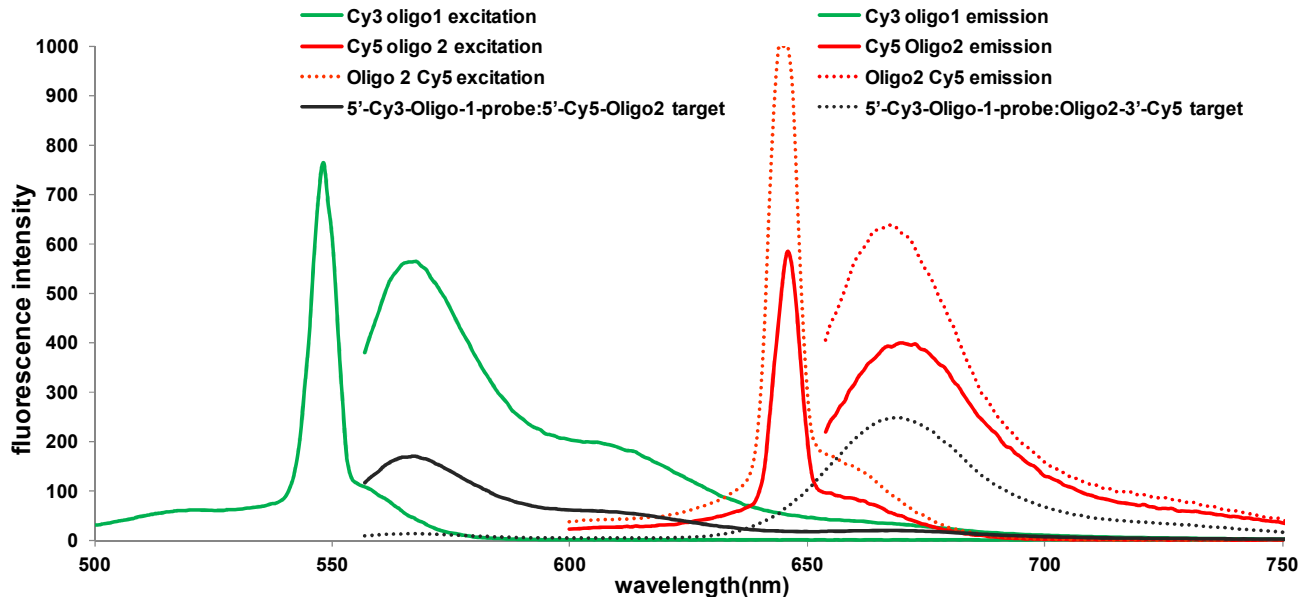
**Figure S-1.** Schematics for real-time hybridization and FRET analysis of DNA duplexes in different probe-target dye orientations. **Case 1:** Cy3 and Cy5 are oriented antiparallel after DNA duplex formation. **Case 2:** both dyes are located at the same duplex end after duplex formation.



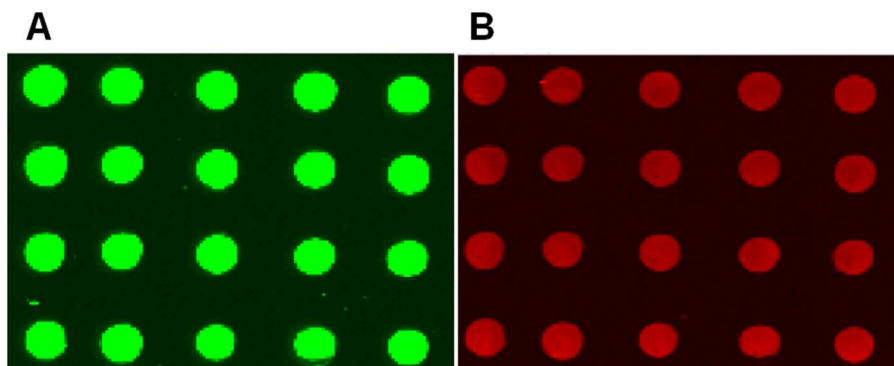
**Figure S-2.** Integrated Cy3 fluorescence emission intensity from a single printed Cy3-DNA probe spot after addition of target DNA in hybridization buffer both with (green solid line) and without (green dotted line) Cy5 dye labeled to target DNA. Resulting Cy5-target DNA emission signal is shown as red solid line. Addition of complementary target with no fluorophore and with Cy5 fluorophore in separate experiments both exhibit rapid decreases in probe Cy3 emission intensity with duplex formation in the spot.



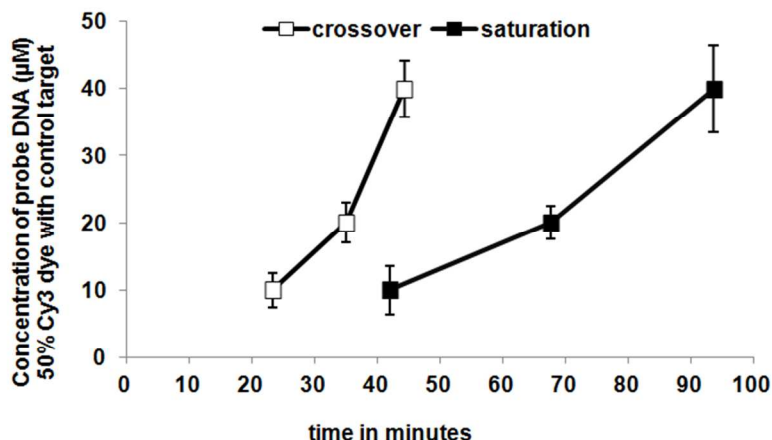
**Figure S-3.** Real-time DNA hybridization kinetics indicating 1) a rapid decrease in Cy3 intensity due to duplex formation (rapid hybridization of initially readily accessible probes, lack of coulombic blockade), 2) increase in Cy5 intensity and simultaneous decrease in Cy3 from FRET, and 3) decrease in both Cy3 and Cy5 intensities from duplex formation, and relatively constant signal intensity over time due to saturation/non-availability of probes. Circle represents crossover point.



**Figure S-4.** Fluorescence spectra from solution phase Cy3 probe and Cy5 target DNA oligomers used for FRET analysis: Cy3-oligo1 excitation and emission spectra (green), Cy5-Oligo2 excitation and emission spectra and Oligo 2 Cy5 excitation and emission spectra (red) are also shown for both antiparallel and parallel duplex combinations of Cy3 and Cy5 dyes (black). Experiments were performed with 40 $\mu$ M 100% dye-labeled probes and targets in print buffer and hybridization buffer, respectively at 23°C. Using Cy3 excitation/emission at 547/569nm (5/10 nm slit width) and Cy5 excitation/emission 644/655nm, FRET was measured by exciting at 547nm and recording emission spectra from 557 to 750nm, and similarly exciting Cy5 at 644nm and measuring emission from 655-750nm.

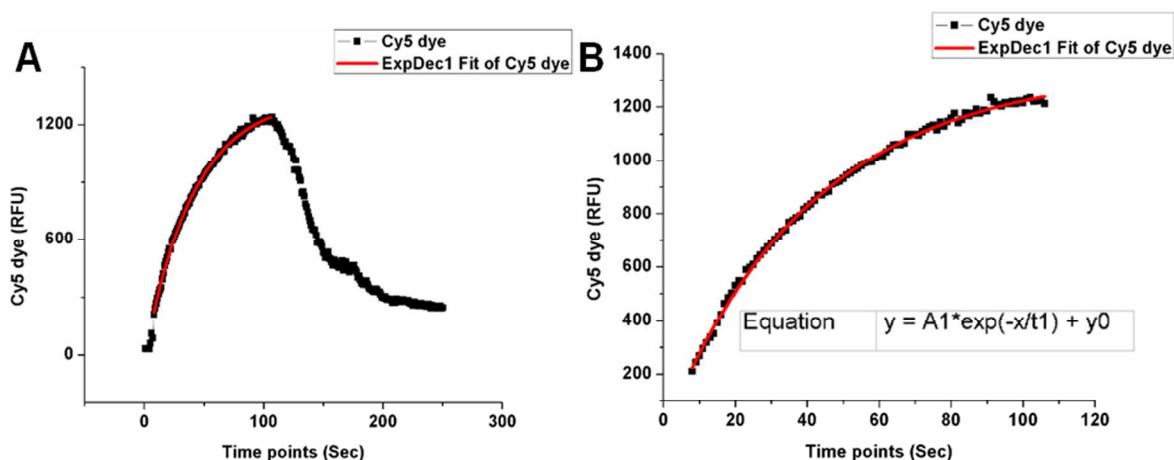


**Figure S-5.** Fluorescence scanner images of DNA spots immobilized on commercial polymer-coated arraying slide before and after real time hybridization analysis of individual microspots. A) Probe Cy3 fluorescence intensity at 535nm; B) The same set of spots after hybridization with complementary Cy5-target DNA scanned at 635nm.

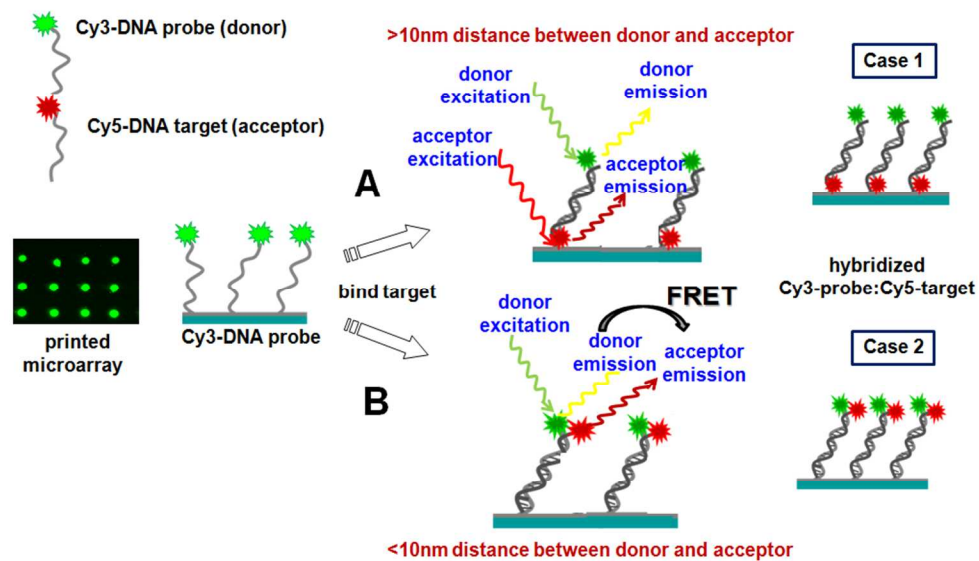


**Figure S-6.** Increase in time to crossover of Cy3-Cy5 emission intensities, and time to signal saturation in single printed spots with increasing printed DNA probe concentration.

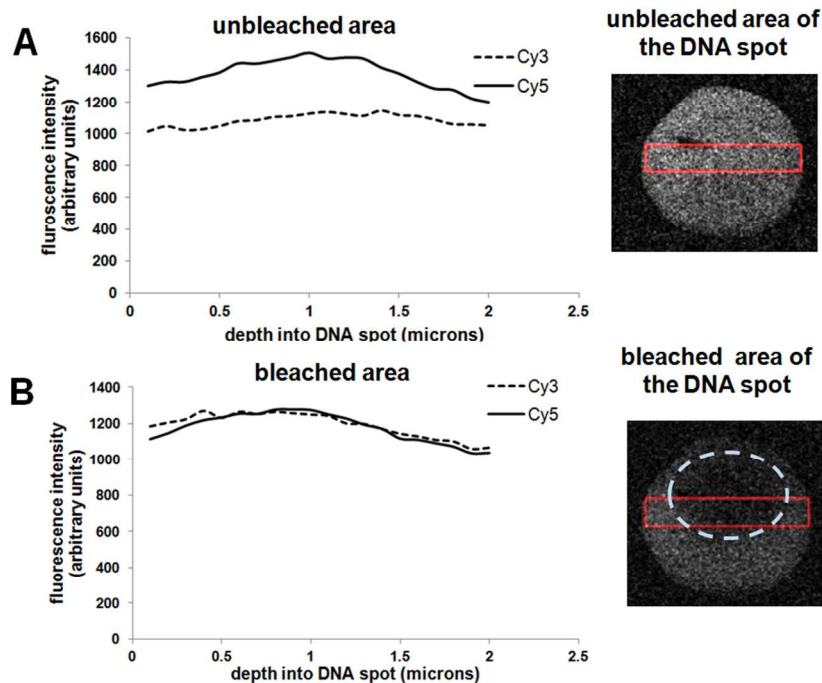
Increase in both crossover time points with increasing printed probe DNA concentration (spot density) was observed (Figure S-6), reflecting reduced hybridization kinetics with increased probe density. Increasing printed DNA density increases both probe-based electrostatic barriers and steric hindrance, impacting the incoming target in encountering probe partners and slowing equilibrium and saturation time points.



**Figure S-7.** A) DNA hybridization kinetics: plot of Cy5 intensity (change in concentration of bound target) vs. time (for case-1 antiparallel hybridization). B) Curve fit of data in (A) red line showing first order kinetics with an on-rate constant of  $k_1 = 0.0243 \text{sec}^{-1}$ .



**Figure S-8.** Schematic of DNA microarray FRET assay designed with labeled-DNA dye with different dye orientations in duplex of surface-bound single spot assays. A) Case 1: no FRET anticipated from antiparallel duplex distances between Cy3/Cy5 positions; B) FRET anticipated with Cy3-Cy5 fluorophores in close proximity with parallel pairing of the duplex in printed spots.



**Figure S-9.** Fluorescence emission intensities of Cy3 probe and Cy5 target as a function of depth in single hybridized microspots on surfaces using confocal z-slices of 0.5  $\mu\text{m}$  each for: A) unbleached areas, and B) bleached areas (dotted oval) of the same single DNA spot. Figure A shows the intensity in unbleached area similar to the real-time hybridization measurements with Cy5 intensity greater than Cy3 (FRET) whereas B indicates the decrease in Cy5 intensity compared to Cy3. Additionally Cy5/Cy3 intensities (solid rectangle) measured at different Z slices indicate the probes and target in different layers of the dried DNA spot.