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Key Note Lecture II

SURFACE ANALYSIS OF DNA AND PROTEINS IMMOBILIZED IN MICRO-ARRAYING DEVICES

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INTRODUCTION: Surface-immobilized microarrays of specific capture reagents are actively studied and marketed for bioassay and drug discovery applications.[1,2] Microarrays of antibodies, nucleic acids, and antigens all encounter problems with prolonged bioactivity and desired capture sensitivity in immobilized formats. Surface chemistry enhances high target capture activity with low non-specific binding. Some microarray surfaces exhibit shelf-life problems from intrinsic hydrolysis of amine-reactive coupling chemistry (active esters, aldehydes) even under protective conditions. Due to the popularity of exploiting amine nucleophiles in biomolecules (e.g., lysines in proteins, derivatized amines in oligonucleotides) for surface coupling, many commercial arraying formats utilize 'amine-reactive' chemistries.[3] Loss of amine-coupling reactivity produces poor reliability, poor signal and sensitivity, high noise and high variability. We have focused on the reactivity of microarray surfaces to both DNA probes and hybridization targets to understand variability and quantitation problems in assay reporting. Due to DNA immobilization variations, we have analyzed the process of regenerating reactive commercial array surfaces targeting amine-reactive nucleic acids or proteins in situ using N-hydroxysuccinimide (NHS) to re-activate amine reactivity, improving both functionalization of the commercial surfaces and immobilization of amine-terminated probes above original capacity. Such methods also offer a possible route to probe binding standardization, and to further understanding quantitative binding issues between immobilized probes and target hybridization.

METHODS: Oligonucleotide selection. Four different synthetic DNA oligonucleotides (MWG Biotech, high purity salt-free) were used: oligonucleotide 5'-CTGAACGGTAGCATCTTGAC (probe) forms a stable duplex with its complementary pair at room temperature with minimal interference due to self-complementarity or secondary structure.[4,5] A 15-T spacer was added to the 3' end of this DNA probe.[6] The complementary 20-base sequence 3'-GACTTGCCATCGTAGAACTG is the target to this probe. Terminal hexylamine-functionalized NH_2-probe-Cy3 and probe-Cy3 molecules were used to study specific surface chemical immobilization vs. non-specific binding (NSB) using fluorescence detection. Terminal hexylamine-functionalized NH_2-probe and target-Cy3 molecules were used for hybridization assays on array surfaces.

DNA probe printing on microarray slides. Commercial polymer-coated amine-reactive slides from Amersham (Codelink™) and Accelr8 Technologies (Optarray™) were purchased and stored per each vendor's recommendations. DNA oligonucleotides were spotted onto microarray slides (pin spotter, spot volumes ~0.7 nL, replicates at 20, 10, 5 and 1 μM DNA in 150mM PBS pH 8.5, with 0.001% Tween20 and 0.001% sarcosine; humidity was 50%), providing dried spots approximately 70-100 μm diameter. Each slide vendor's printing specifications were closely followed for handling, printing, buffers and rinsing. Immobilization was attempted by incubating printed slides overnight, room temperature at 75% humidity.

Microarray surface NHS regeneration. Commercial slides taken directly from vendor storage boxes and printed immediately were denoted "fresh" slides. Other slides were deactivated to amine-reactivity by 10mM NaOH for 0.5 h ("deactivated" slides), or surface 'blocked' using 50mM ethanolamine for 0.5h to quench NHS groups ("blocked slides"). Microarray slides were kept at room temperature in sealed vendor-supplied storage bags for a month and denoted "aged" slides. NHS reactive surface regeneration was then performed on "deactivated" slides, re-named "regenerated" slides post-treatment. Regeneration included full immersion of deactivated slides in 0.5M N-hydroxysuccinimide (NHS) and 0.5M DCC in dry DMF for 3 h at -20°C, then left overnight at +4°C.[7] Slides were rinsed with dry DMF and dried with nitrogen for printing.

Post-print substrate treatment. Residual NHS reactive groups post-printing were blocked using 50mM ethanolamine according to slide manufacturer's specifications. Slides were then rinsed with deionized water, then with 4X saline sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS, 50°C) for 30 min., and dried with N_2. Target hybridization was performed with commercial coverslips at room temperature (100% humidity, 4 h) in 5X SSC containing 1μM DNA target. The solution ratio of Cy3-labeled DNA to non-labeled DNA was 1:20. Slides were rinsed with 4xSSC then with 2xSSC/0.1%SDS for 5 min twice, then 0.2xSSC and 0.1xSSC each for 1 min, and finally blown dry with nitrogen.

Microarray scanning, fluorescence detection and array image processing. Microarray slides were scanned using a Packard BioChip Imager (Cy3 channel). Laser power and PMT sensitivity were set

at 70% and 80%, respectively, for probe immobilization scans, and 90% and 100%, respectively, for hybridization scans. Resolution was set to 10 µm. Scanned fluorescence images were processed with ScanAlyze™ software written by Dr. Michael Eisen (University California-Berkeley, http://rana.lbl.gov/EisenSoftware.htm).

RESULTS: Surface regeneration of NHS chemistry on aged microarray slides. Imaging of the efficiency of printed probe immobilization, shown in Figure 1 below, reveals that surface regeneration of 'aged' slides can produce reactivity to amine-modified oligoDNA similar to that from 'fresh' slides. NHS reactivity is lost in storage (data not shown), reducing intrinsic DNA amine reactivity substantially. After surface NHS regeneration, the same slide offered DNA immobilization efficiency equivalent to the original fresh slide. Hybridization yields are also improved by the NHS regeneration treatment as shown in Figure 2.

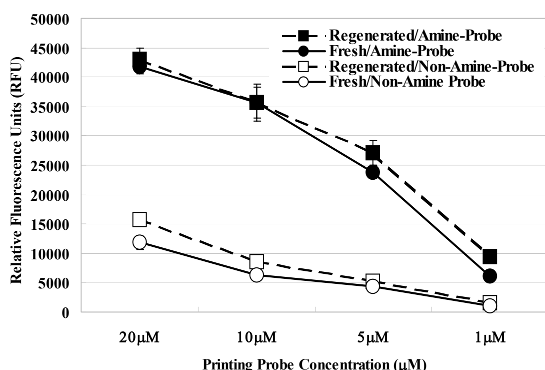

Fig. 1: Fluorescence image quantification for probe printing and immobilization onto commercial reactive polymer-coated microarray substrates under treatments and probe reactivity conditions. RFU data obtained from slide scans show changes in spot pixel average intensities as a function of probe print concentration and slide treatment conditions for regenerated compared to fresh array substrates.

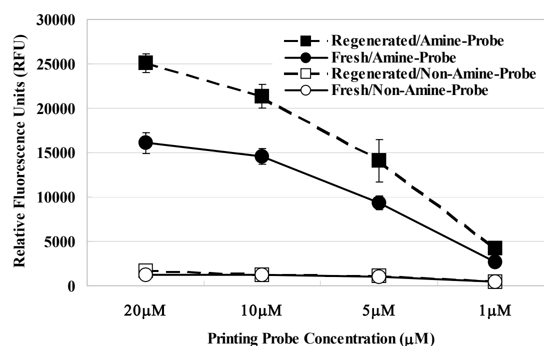

Fig. 2: Fluorescence image quantification for Cy3-labeled complementary target DNA hybridization to oligoDNA probes printed onto commercial reactive polymer-coated microarray substrates under substrate treatments and probe reactivity conditions. RFU data obtained from slide scans show changes in spot pixel average hybridization intensities as a function of probe print concentration and slide treatment conditions for regenerated compared to fresh array substrates.

DISCUSSION & CONCLUSIONS: The NHS regeneration process allows microarray substrates adversely affected by NHS hydrolysis and compromised reactivity to be restored to full or even improved amine-coupling functionality. Regenerated polymer slides subject to hybridization assay perform similarly to fresh NHS-derivatized amine-reactive slides from each vendor, providing enhanced reliability, a standardization 'set-point' for immobilization reactions, and high reactivity for standard microarray printing.

REFERENCES: [1]Ramsay (1998) G. *Nat. Biotechnol.* **16:**40 44; [2]Speed, Y. H. Y. T. (2002) *Nature Rev. Gen.* **3:**579-588; [3]Hermanson, G. T. (1996) *Bioconjugate Techniques*; Elsevier, NY. [4]L.T. Mazzola, C. W. F., S. P. A. Fodor, C. Mosher, R. Lartius, E. Henderson (1999) *Biophys. J.* **76**:2922-2933; [5]J.E. Forman, I. D. W., D. Stern, R.P. Rava, M.O. Trulson (1998) *ACS Symp. Ser.* **682**:206-228; [6]U.J. Krull, L. H. (1999) *Can. J. Anal. Sci. Spectrosc.* **44**:61-70; [7]Kopecek, J. (1977) *Makromol. Chem.* **178**:2169-2183.

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