Surface vs Solution Hybridization: Effects of Salt, Temperature, and Probe Type

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S1. Experimental Methods.

S1.1. Materials. Monosodium and disodium phosphate (Sigma-Aldrich, St. Louis, MO), sodium borohydride ($NaBH_4$) and Tween 20 (Fisher Scientific, Pittsburgh, PA) were used as received. SuperAldehyde 2 slides (Arrayit, Sunnyvale, CA) were stored at room temperature in vendor-sealed packaging. Morpholino (MO) probes (Gene Tools, Philomath, OR) and DNA probes (Integrated DNA Technologies, Coralville, IA) were stored at -20 ºC. Table S1 lists the 25mer probe sequences used. Complementary 25mer DNA targets (Integrated DNA Technologies) were modified with a fluorescent Cy5 label at the 3' end. Strand concentrations were confirmed spectrophotometrically before each use.

Table S1. Probe sequences.[a]

#	**Sequence**
1	5'-NH_2-GTA GCT AAT GAT GTG GCA TCG GTT G
2	5'-NH_2-CTC TCG GCA TCC ATC AAG AAT ACC T
3	5'-NH_2-ACA CAA GTT GTA ACA GCC GGA CAA A
4	5'-NH_2-TAT CAA ACT CAT GGT CGT CTG GTA C
5	5'-NH_2-GAC ACA TCT GTC GGC CAT ACC ACT T
6	5'-NH_2-CAC CGG GAC TGC CAT TCT CTA TAT C
7	5'-NH_2-CGA TCC GGC GAA TCA TCT TAA ACG C

[a] Sequences 1-4 are from *Legionella pneumophila*; sequences 5-7 are from *Escherichia coli*.

S1.2. Solution Melting Measurements. Probe and its complementary target sequence were combined in stoichiometric proportion to a total strand concentration of 1 μmol L^{-1} in pH 7.0 sodium phosphate buffer. Measurements were performed on a Cary 50 UV-Vis spectrophotometer equipped with a Quantum Northwest (Liberty Lake, WA) temperature-controlled cuvette holder. Absorbance was monitored at 260 nm. Melting scans were performed from 20 ºC to 80 ºC using a ramp rate of 0.2 ^oC min^{-1}, with a 10 min hold time at both ends. A cooling ramp was followed by a heating ramp, with absorbance collected every 0.05 ^oC. Equilibrium nature of the data was assessed from superposition of cooling and heating traces. Cooling data were used for derivation of ΔH^o and ΔS^o, performed by fitting experimental melting transitions to a two-state model as described previously.[1] The slope of the high temperature baseline was constrained to be zero; in the absence of this constraint, the baseline tended to negative slopes in attempt to capture sharp curvature as melting was driven to completion in the high

temperature limit. The difference between values derived with and without the constraint of zero slope was up to 10 % for ΔH^{o} and ΔS^{o}, and up to 3 % for ΔG^{o}. The lower variability in $\Delta G^{o} = \Delta H^{o} - T\Delta S^{o}$ reflects cancellation of enthalpic and entropic uncertainties that arise from correlations between ΔH^{o} and ΔS^{o} during fitting.

S1.3. Microarray Fabrication. Morpholino and DNA probes were deposited onto aldehyde slides using an XactII™ contact printing system and 300 μm Xtend microarray pins (LabNext, West New York, NJ). Spotting was performed from 10 μmol L^{-1} solution of MO or DNA probe in 0.1 mol L^{-1} pH 9.0 sodium phosphate buffer. These conditions resulted in MO and DNA spots measuring 300 μm and 200 μm in diameter, respectively. After printing, slides were kept for 22 hours at 25 ˚C and a humidity of 30% or less. Next, slides were washed first for 5 min with an $NaBH_4$ solution (38 ml 0.1 mol L^{-1} pH 7.0 sodium phosphate buffer, 12 ml absolute ethanol, 0.14 g $NaBH_4$) and second for 5 min with a Tween 20 surfactant solution (0.05% w/v Tween 20 in deionized water), as previously described.[2] The $NaBH_4$ wash stabilizes probe attachment by reducing the imine bonds between probe amines and surface aldehydes, while the Tween 20 wash removes non-specifically adsorbed probes. After a final 2 min wash in deionized water, slides were dried under a nitrogen stream and stored in a slide box at room temperature until use. The printed geometry allowed 36 spots to be imaged simultaneously; therefore, two spots of each of the seven MO and seven DNA probes (total of 28 spots) were monitored in parallel. Experiments were performed in duplicate.

For studying the impact of probe-surface interactions on hybridization, modified protocols were used to produce two alternate surface chemistries. The first of these protocols was selected to produce a more hydrophilic surface. After printing probes on aldehyde slides as above but before the $NaBH_4$ wash, the slides were immersed for 5 minutes in 10 mmol L^{-1} solution of tris(hydroxymethyl) aminomethane (THAM, Sigma-Aldrich) in dimethyl sulfoxide.[3] After modification with THAM, the slides were processed with $NaBH_4$ and Tween 20 washes, as above. The contact angle of deionized water on the slides after the THAM treatment was $36.2^{o} \pm 2.7^{o}$, compared to $51.3^{o} \pm 2.4^{o}$ for the conventional preparation. The increased hydrophilicity is attributed to modification of residual aldehyde groups with the THAM tridentate hydroxyl groups, noting that more than one addition product may be present.[3a] The second protocol was selected to introduce phenyl groups to the solid support, in expectation of interactions with nucleic bases that would affect probe affinity toward target molecules. Commercial aminopropylsilane slides (EMS Corp., Hatfield, PA) were first immersed for 17 hours in 10 mmol L^{-1} solution of *p*-phenylene diisothiocyanate (PDITC) in dimethylformamide (DMF).[4] This step modified the slide amino groups with a phenylene isothiocyanate moiety for subsequent immobilization of MO and DNA probes. After washing of PDITC-modified slides in DMF for 2 minutes and drying under a nitrogen stream, printing of MO and DNA probes was performed as for aldehyde slides. After printing, the slides were immediately washed for 5 minutes with the 0.05% w/v Tween 20 solution and then for 2 minutes with deionized water. Lastly, the slides were dried under a nitrogen stream and stored in a slide box at room temperature until use (see Figure S4 for melting transitions on the THAM and PDITC-modified slides).

S1.4. Determination of Probe Coverages. MO and DNA probe coverages were estimated as follows. The volume of droplets spotted by the Xtend microarray pin was determined using a high-speed imaging system consisting of a telemetric lens (Edmund Optics #63-743) and a high speed camera (PixeLINK PL-B741U). Droplet deposition was imaged at 100 frames per second to capture droplets immediately after printing, minimizing evaporation losses. These measurements yielded a volume of 3.9 nL and 3.2 nL for MO and DNA spotting, respectively. Various probe concentrations (1 to 10 μmol L^{-1}, sequence 5'-NH_2-TAGCTAATGATGTGGCATCGGTTGC-fluorescein) were printed and the droplets allowed to evaporate to dryness, but no washing was performed so as to retain all of the printed probes. By using fluorescein-labeled probes, the printed area was visualized. These

measurements enabled the surface coverage of probes to be calculated from the droplet volume, known probe concentration, and spot area, and moreover to correlate the coverage to fluorescence intensity and thus establish a calibration. Next, the fluorescence of spots printed using 10 μmol L^{-1} concentration was measured after the standard washing protocol, and through the calibration the coverage of remaining probes was determined. Two independent measurements resulted in coverages of (2.3 ± 0.3) × 10^{12} cm^{-2} for MO probes and (2.9 ± 0.8) × 10^{12} cm^{-2} for DNA probes.

S1.5. Surface Melting Measurements. Microarray slides were assembled into a flow cell by sealing them against a temperature-controlled metal block with one inlet and one outlet port, using a gasket. The flow cell (internal volume: 600 μL) was filled with hybridization solutions containing mixtures of single-stranded, Cy5-labeled DNA targets complementary to probes 1-6 (Table S1), with each sequence dissolved at a concentration of 0.1 μmol L^{-1} in pH 7.0 phosphate buffer. Used buffer concentrations included 0.012, 0.021, 0.037, and 0.11 mol L^{-1}, with concentrations expressed in molarity of phosphate groups. Excitation light from a 640 nm laser (Laserglow Technologies, Toronto ON, Canada) was coupled through the edge of the slide in a total-internal-reflection fluorescence (TIRF) geometry using a commercial TIRF stage (TIRF Technologies, Cary, NC). In this setup the slide doubles as a waveguide. Fluorescent emission from target Cy5 labels within the evanescent field at the slide/solution interface was collected by a microscope objective under 4X magnification, passed through a 650-690 nm bandpass filter to reduce background, and detected by an EMCCD Ixon DU-885K camera (Andor Technology, South Windsor, CT) using a 1 sec exposure. EM gain was used in 0.012 and 0.021 mol L^{-1} buffer, where a gain between 5 and 50 was used to amplify the weaker signals in this low ionic strength limit. Gains were kept sufficiently low to avoid saturation of the camera. Figure S1 shows TIRF images at several temperatures.

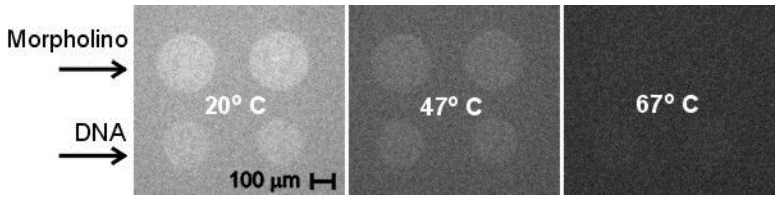

Figure S1. TIRF images of morpholino and DNA spots at several temperatures.

Temperature control was provided by a home-built thermoelectric control module. After pre-heating the TIRF flow cell to 80 ºC, hybridization buffer with targets was injected with a syringe and allowed to equilibrate at 80 ºC for 10 min. A first temperature ramp from 80 ºC down to 20 ºC was followed by heating back to 80 ºC, using a ramp rate of 0.3 ^{o}C min^{-1} and a 10 min hold time at both ends. TIRF images were taken every 2 ^{o}C. Image analysis was performed with Andor SOLIS software using a custom Andor Basic program. The location of spots was acquired directly from the image. For each spot, intensity was calculated by subtracting the averaged local background intensity from the corresponding averaged spot intensity, as described previously.[2]

Because dye quantum yields typically depend on temperature, a correction was needed to normalize for any variation of Cy5 emission with temperature. Prior reports indicated that Cy5 emission is temperature-dependent[5] following an exponential decay [6]

$$I_T = I_{T_0} \exp(-d(T - T_0))$$

where I_T and I_{T0} are emitted intensities at temperatures T and T_0, and d is the decay rate. Since the decay rate under surface conditions is not known, corrections were instead applied by adjusting d so as to realize a horizontal baseline at low temperature, assumed to correspond to hybridization at saturation. This criterion led to an average d of 0.015 ± 0.006. Higher values of d, such as d = 0.027 reported for Cy5 in solution,[6] overcompensated the Cy5 temperature-dependence as illustrated in Figure S2. Corrected data were fit to a two-state model to derive ΔH^o and ΔS^o, using horizontal baselines at both low and high temperatures. Figure S3 shows examples of fitted experimental data for both MO and DNA probes.

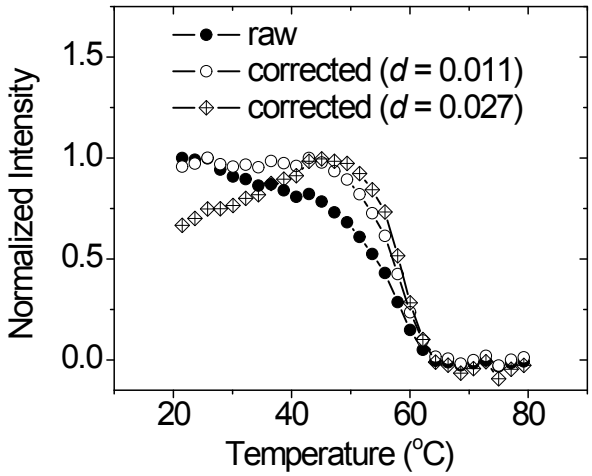

Figure S2. DNA-DNA surface melting curves illustrating raw and temperature-corrected data. Buffer: 0.037 mol L^{-1} pH 7.0 sodium phosphate; sequence: #6.

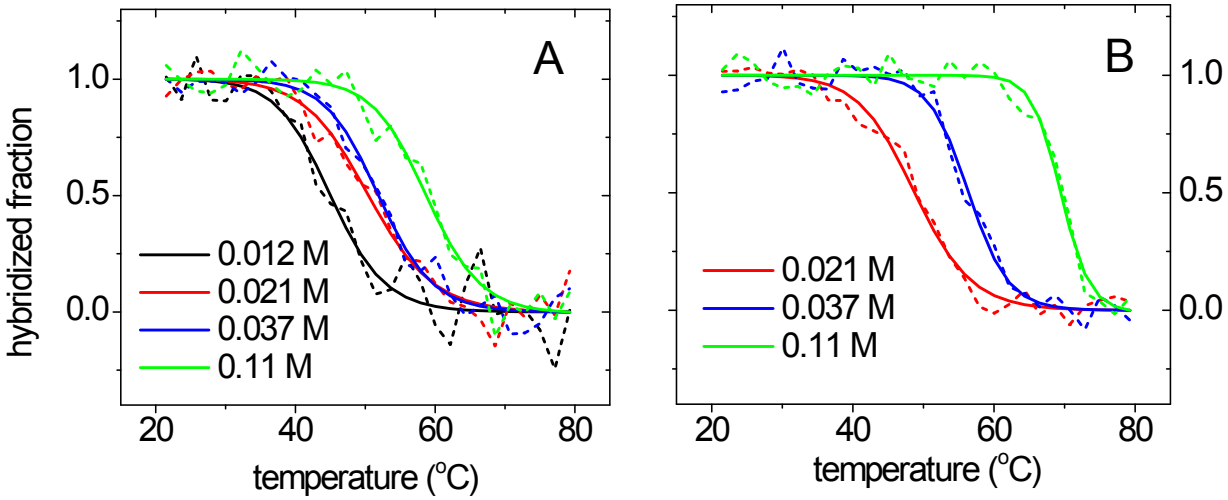

Figure S3. Examples of two-state model fits (solid lines) to experimental melting data (dashed lines) for **(A)** MO and **(B)** DNA probes. Sequence: #1.

Probe sequence #7 did not have a corresponding target sequence in solution and thus served as a control for nonspecific adsorption. Target signals could not be discerned from these spots over the range of investigated conditions for either MO or DNA probes, consistent with assignment of signals

from other spots, whose complementary targets were present, to sequence-specific hybridization.

S2. Hybridization Data.

S2.1. Solution Hybridization. Table S2 presents enthalpy ΔH^o and entropy ΔS^o derived from fitting of solution melting transitions, as well as the free energy ΔG^o evaluated at 55 °C. Each of the six DNA and six MO probe sequences was separately hybridized with complementary strands (1 μmol L^{-1} total strand concentration) in pH 7.0 sodium phosphate buffers of three different strengths: 0.012, 0.037, and 0.11 mol L^{-1} phosphate concentration.

Table S2. Results: Solution hybridization.

	ΔH^o (kcal mol^{-1})			ΔS^o (kcal mol^{-1} K^{-1})			ΔG^o_{55C}(kcal mol^{-1})		
	buffer (mol L^{-1})			buffer (mol L^{-1})			buffer (mol L^{-1})		
Probe	***0.012***	***0.037***	***0.11***	***0.012***	***0.037***	***0.11***	***0.012***	***0.037***	***0.11***
1 MO	-94.5	-90.7	-91.7	-0.247	-0.237	-0.240	-13.3	-13.0	-12.9
1 DNA	-159	-181	-179	-0.462	-0.513	-0.497	-7.78	-12.2	-15.8
2 MO	-105	-108	-109	-0.281	-0.289	-0.293	-13.1	-13.1	-13.0
2 DNA	-141	-171	-163	-0.404	-0.483	-0.448	-8.14	-12.3	-15.7
3 MO	-86.4	-80.5	-80.9	-0.225	-0.208	-0.210	-12.5	-12.3	-12.1
3 DNA	-159	-164	-170	-0.457	-0.458	-0.467	-8.62	-13.6	-16.5
4 MO	-83.0	-86.3	-85.9	-0.214	-0.224	-0.223	-12.7	-12.7	-12.6
4 DNA	-164	-179	-178	-0.475	-0.507	-0.493	-8.02	-12.4	-16.4
5 MO	-90.6	-108	-97.9	-0.236	-0.287	-0.258	-13.2	-13.9	-13.3
5 DNA	-169	-177	-181	-0.483	-0.497	-0.495	-10.7	-14.3	-18.0
6 MO	-89.1	-94.5	-94.9	-0.231	-0.247	-0.248	-13.4	-13.5	-13.4
6 DNA	-169	-164	-152	-0.489	-0.462	-0.416	-8.44	-12.4	-15.5

S2.2. Surface Hybridization. Table S3 lists enthalpies and entropies of hybridization from fitting of surface melting transitions, together with the free energy ΔG^o evaluated at 55 °C. Slides printed with the six DNA and six MO probe sequences were hybridized to a mixture of the six complementary targets, each dissolved at 0.1 μmol L^{-1} concentration in pH 7.0 sodium phosphate buffers of various strengths. MO probes yielded melting transitions under nearly all conditions studied, including in the lowest ionic strength 0.012 mol L^{-1} buffer. For DNA probes, one or more sequences exhibited transitions in 0.021, 0.037 and 0.11 mol L^{-1} buffers. However, a number of DNA probe transitions were too weak for analysis in 0.021 mol L^{-1} buffer, while three of six DNA probe transitions in 0.11 mol L^{-1} buffer occurred at too high a temperature to be fully captured within the accessible temperature range (up to 80 °C). The uncertainties in Table S3 are from measurements on two independently prepared slides. Due to correlation between ΔH^o and ΔS^o during fitting the uncertainties on these parameters are typically significant. Since such correlation tends to cancel out in calculating $\Delta G^o = \Delta H^o - T\Delta S^o$, relative uncertainties associated with ΔG^o are lower than those for ΔH^o and ΔS^o. The significant ionic strength dependence of ΔH^o and ΔS^o for DNA-DNA surface hybridization (and to a lesser degree

DNA-DNA solution hybridization, Table S2) is most likely a consequence of imperfect formation of base pairs between two like-charged DNA strands at lower ionic strengths.

Table S3. Results: Surface hybridization.

	ΔH^o (kcal mol^{-1})				ΔS^o (cal mol^{-1} K^{-1})			
	buffer (mol L^{-1})				buffer (mol L^{-1})			
Probe	***0.012***	***0.021***	***0.037***	***0.11***	***0.012***	***0.021***	***0.037***	***0.11***
#1 MO	-50±2	-38	-47±1	-53±7	-120±10	-83	-110±30	-130±20
#1 DNA	--	-46	-90±12	-130±30	--	-110	-240±40	-360±80
#2 MO	-84±20	-58	-52±2	-56±15	-230±70	-150	-130±10	-140±40
#2 DNA	--	--	-82±18	-130±40	--	--	-220±50	-360±120
#3 MO	--	--	-45±8	-53±22	--	--	-110±30	-130±70
#3 DNA	--	--	-89±11	--	--	--	-240±30	--
#4 MO	-55.4±0.3	-54	-49±10	-51±1	-140±0	-133	-120±30	-120±0
#4 DNA	--	--	-120±2	-150±20	--	--	-330±10	-400±60
#5 MO	-40±1	-51	-48±7	-47±3	-92±2	-130	-120±20	-110±10
#5 DNA	--	--	-100±30	--	--	--	-280±100	--
#6 MO	-45±9	-38	-62±2	-41±7	-110±30	-86	-160±10	-92±23
#6 DNA	--	-59	-81±8	--	--	-150	-210±30	--

	ΔG^o_{55C} (kcal mol^{-1})			
	buffer (mol L^{-1})			
Probe	***0.012***	***0.021***	***0.037***	***0.11***
#1 MO	-9.1±0.3	-10.1	-10.1±0.1	-11.0±0.2
#1 DNA	--	-9.6	-10.9±0.1	-15.9±0.7
#2 MO	-7.6±1.0	-10.2	-9.6±0.0	-10.3±0.1
#2 DNA	--	--	-10.9±0.0	-16.1±1.4
#3 MO	--	--	-9.5±0.1	-10.2±0.3
#3 DNA	--	--	-11.6±0.3	--
#4 MO	-9.0±0.0	-10.3	-10.0±0.2	-10.8±0.3
#4 DNA	--	--	-11.6±0.0	-16.8±0.6
#5 MO	-9.9±0.1	-9.6	-9.7±0.3	-10.7±0.0
#5 DNA	--	--	-12.8±0.6	--
#6 MO	-8.9±0.5	-9.8	-10.0±0.0	-10.6±0.0
#6 DNA	--	-9.6	-11.0±0.1	--

S2.3. Effects of Surface Chemistry. Figures S4.A and S4.B show melting transitions for MO and DNA probes measured on slides modified with THAM (Figure S4.A) and PDITC (Figure S4.B), prepared as described in section S1.3. Measurements were performed using sequence #4 dissolved at

0.1 µmol L^{-1} in 0.037 mol L^{-1} phosphate buffer, pH 7.0. Figure S4.C compares the $\Delta G^{o}_{55C}/RT$ values derived from these experiments with those for the aldehyde slides. These results show that, for the three types of slides considered, surface chemistry did not strongly affect hybridization.

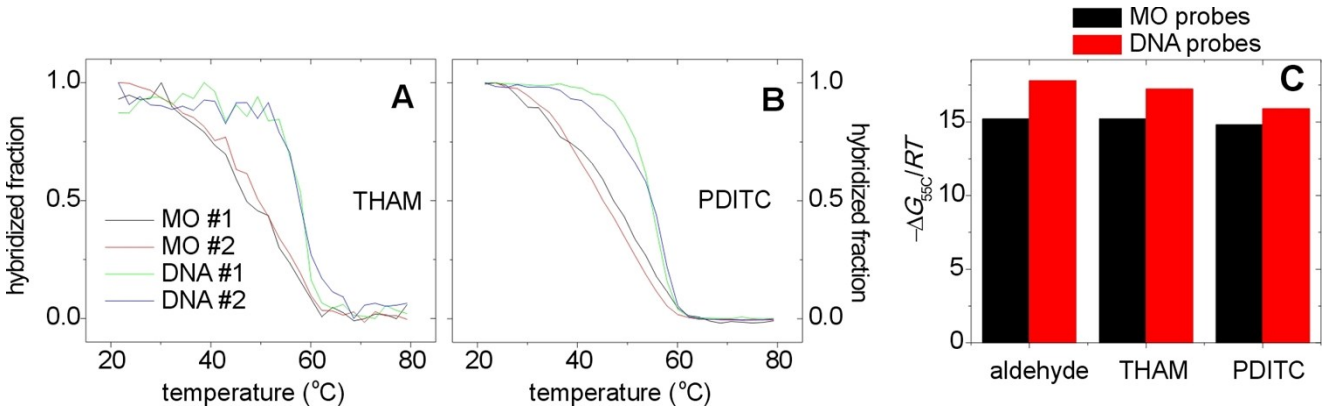

Figure S4. Melting transitions for MO and DNA probes (sequence #4), measured on two independently prepared slides modified with the **(A)** THAM or **(B)** PDITC chemistry, as per section S1.3. **(C)** Resultant free energies of surface hybridization for MO (black) and DNA (red) probes. Buffer: 0.037 mol L^{-1} phosphate buffer, pH 7.0.

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