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TRANSCRIPTIONAL PROFILING USING THE FLOWTHROUGH GENOSENSOR

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Abstract

A Cooperative Research and Development Agreement (CRADA) between Lockheed Martin Energy Research Corporation (Contractor) and Gene Logic, Inc., (Participant) was carried out to evaluate the technical feasibility study of the application of the flowthrough genosensor for gene expression (transcriptional) profiling, over the current industry practice of using flat surface hybridization arrays to monitor the relative abundance of individual mRNA species in a cell. Various parameters, including substrate preparation, flow rates, hybridization conditions and sample concentrations, were evaluated on the flowthrough genosensor. The superiority of the flowthrough genosensor, in terms of hybridization rate and sensitivity were established.

1. Objectives

Various parameters that influence DNA hybridization on flowthrough DNA chips were investigated as well as parameters influencing hybridization detection as listed below.

- Investigate the effects of flow rate on hybridization and washing using flow through glass channel chips.
- Investigate the detection sensitivity at a target flow rate of 0.05 ml/min for hybridization and 0.25 ml/min for washing using target amounts of 10, 50 and 1000 fmol.
- Investigate the contributions to the nonuniform hybridization signal.
- Investigate the effect of ionic strength on hybridization.
- Compare the results of hybridization between flat and channel glass chips.
- Investigate the uniformity of signal detection by using the same probe spotted in different locations of a 6X6 array.
- Investigate prewashing procedures by varying the ionic strength during the first wash and compare the fluorescence detection sensitivity for probes immobilized on non-treated and treated glass chips provided by Matt. Specifically, two types of probes were used: (1) Probes with 3'-propanolamine derivatized oligonucleotides attached to non-treated channel glass; (2) GMBS linked probes spotted on the MPTS channel glass.
- Investigate target recirculation for increased hybridization sensitivity.

2. Experimental Procedures

General Procedures

• Chip cleaning: The glass channel chips were cleaned by four successive 15-min sonication steps in 1N HNO_3, Acetone, EtOH and dH_2O, respectively. The chips were then dried in a vacuum desiccator overnight.

• Array Design and probe attachment: Generally, a 6 x 6 array (0.5 mm interval) of probes were prepared in a microtiter plate and spotted using the modified Hamilton Robot. All probes contain the 3'-propanolamine derivatization.

• Prewash: Prior to hybridization, the chips were washed using 5 ml of 5X SSPE buffer to remove loosely bound probes and other materials. This was done in the chip holder using the syringe pump. No other "blocking" was performed.

• Hybridization: Generally, a 0.5 ml solution of target was injected using the HPLC-style injection valve. This was done under different flow rates by programming the syringe pump. A total volume of 2 mls was flowed at this rate to ensure passage of the sample plug through the chip.

• Washing: A final washing step was then performed using either buffer or deionized water

• Detection: The fluorescence signal of the labeled target was monitored using the microscope imaging system. The pixel intensities of the regions of interest were recorded and analyzed. The

relative pixel intensity (RPI) of DNA hybridization was recorded as:
RPI = (Signal -Background) / (Background)

Flow Rate Evaluation

The array design is shown in Table 1 below. The array contains two identical sets of the 14 B-actin probes complementary to GLA-23 and three T7 probes. 10 nl of a 20 µM concentration of probe was spotted for the GLA probes and 10, 20 and 40 µM concentrations of T7 probe was spotted.The flow rates primarily used were: 0.05, 0.15 and 0.5 and 0.01 ml/min. An initial "hybridization" phase, involving the injection of a 2 ml volume that included the 0.5 ml target solution (50 nM GLA-23TF, 50 nM TMR-T7), was followed by a 5 ml "washing" step. Both steps were conducted at the same flow rate and images were collected at time points corresponding to 0.1 ml volume increments. Pixel values corresponding to the hybridization spot and a neighboring "background" region were recorded. Each flow rate experiment was repeated two or three times using a fresh array.

Table 1. The 6 x 6 array design

	1	**2**	**3**	**4**	**5**	**6**
A	FITC Dye	T7(10 nM)	T7(20 nM)	T7(40 nM)	FITC Dye	FITC Dye
B	GLA-2	GLA-4	GLA-6	GLA-8	GLA-9	GLA-11
C	GLA-12	GLA-13	GLA-14	GLA-16	GLA-17	GLA-18
D	GLA-20	GLA-21	GLA-2	GLA-4	GLA-6	GLA-8
E	GLA-9	GLA-11	GLA-12	GLA-13	GLA-14	GLA-16
F	FITC Dye	dH_2O	GLA-17	GLA-18	GLA-20	GLA-21

Sensitivity Evaluation

Target amounts of 10, 50 and 1000 fmol in a 0.5 ml volume were used to assess the detection sensitivity. The flow rate for these experiments were 0.05 ml/min for hybridization and 0.25 ml/min for washing.

Ionic Strength

Two different buffer concentrations, 1XSSPE and 5XSSPE, were used for hybridization at 0.05 ml/min flow rate and 50 nM target concentrations.

Flat glass chips

Non-gridded flat glass microscope slides were used for hybridization by first cutting to a 12X12 mm size. Both flow and non-flow hybridization experiments were conducted. The flow hybridization experiments were done at a flow rate of 0.05 ml/min and a 50 nM target concentration. The non-flow hybridization experiments were performed overnight (15 hours), using a volume of 0.5 mls and a concentration of 50 nM.

Chip uniformity experiments

A 6X6 array was spotted as shown in Table 2. The concentrations for GLA-6, GLA-17 and T-7 were 10 µM. As shown in Table 1, GLA-6 and T-7 were distributed throughout the chip with the spacing between spots set at 0.5 mm. Different target concentrations were used for hybridization and were either 2 nM, 5 nM, 10 nM or 50 nM. Prewashing consisted of flowing 0.5 ml 5XSSPE at 0.5 ml/min. The hybridization step used 2 ml 5XSSPE at 0.05 ml/min, which contained the 0.5 ml target solution, and was followed with a washing step that used 5 ml 5XSSPE at a flow rate of 0.25 ml/min.

Table 2. Array design for chip uniformity studies

	1	2	3	4	5	6
A	Rhoda-mine	A-6	T7	A-6	A-6	Rhoda-mine
B	A-6	T7	A-6	A-6	T-7	A-6
C	T7	A-6	A-6	T-7	A-6	T-7
D	A-6	T-7	A-6	A-6	A-17	A-6
E	Rhoda-mine	A-6	T-7	A-6	A-6	A-6
F	Rhoda-mine	A-6	A-6	T-7	A-6	A-6

Detection Uniformity

Flow Rate

Of the various probes, the data analysis concentrated on probe GLA-17. After a 2-ml "hybridization" volume, that was injected at either 0.10 or 0.25 ml/min, the washing phase was performed at 0.25 ml/min. Note that only 0.5 ml target solution was used in the hybridization phase. At all flow rates, the maximum intensity values for both the signal and background occur during the injection of the sample. There is ~250 µl of dead volume that precedes the 0.5 ml sample plug. As the sample plug passes through the chip, the intensities peak. After ~0.8 mls of flow, the intensities decrease and the majority of the nonspecific signal is gone after ~2 ml of flow. These trends are independent of flow rate. However, the specific hybridization signal that is obtained is dependent on flow rate. The slowest flow rate yields the highest specific signal. Little distinction is observed when comparing the two faster flow rates. Approximately twice the specific signal can be obtained when using 0.05 ml/min as compared to 0.15 or 0.5 ml/min. This can be understood as the hybridization reaction is dependent on the concentration of the target and the time given to react (among other things). The target concentration is the same for all flow rates, however the time varies from 1 min to 10 min. The background signal during this time period should only depend on the target concentration. However, after passage of the sample plug, the amount of specific complex will primarily depend on time, whereas the nonspecific signal will depend on the wash volume. The effect of these steps are seen by comparing the slopes of the signal (after subtracting background) with volume during flow of the sample plug (0.3 - 0.7 ml) and after (0.9 - 1.5 ml). The best "on" rates are observed with the slowest flow while washing appears independent of flow. This leads to the conclusion that a hybrid flow rate may be optimal where a slow flow rate is used during passage of the sample plug and is followed by a rapid washing step. Under the conditions evaluated, only two to three milliliters are necessary for the entire experiment. Using the slowest flow rate for hybridization and the fastest flow rate for washing this would correspond to a ~20 minute experiment for a 3 ml volume. However, the effect of flow rate with different target concentrations will need to be determined. The standard deviation, from experiment to experiment, appears to be about 10%. However, when evaluating probe GLA-17 in position 1 (near the edge of the chip) versus position 2 (near the center of the chip) there is a large, reproducible difference. This may be attributable to a variety of factors including chip construction, sample differences, fluid flow differences, and the detection system.

The RPIs of the A-17 hybridization spot, at a flow rate of 0.01 ml/min, are almost double the signal obtained at a flow rate of 0.05 ml/min. The experiments at the 0.01 ml/min are the result of two experiments, while hybridization at the other flow rates are the result of 3 experiments.

Clearly, the hybridization signal increases with the decreased flow rate. The limiting case would be a static hybridization experiment and preliminary experiments show that the signal is decreased relative to the case of slow flow. Apparently, the increased hybridization time, due to the lower flow rates, leads to greater hybridization signal. The RPI of GLA-17 hybridization reaches a maximum of ~1.9 after an injection volume of 1.8 ml for the 0.01 ml/min flow rate. This corresponds to a total time of about 3 hours.

Washing

Two types of washing reagents, 5X SSPE and deionized water, were used to investigate the bound mechanisms between target and the glass surface or probe. Deionized water as a washing reagent more rapidly decreased the pixel intensity than the buffer. This is probably due to dissociation of the target-probe hybrid in the low ionic strength. This is expected because of melting. However, after injecting 20-ml of buffer, the remaining relative pixel intensity is still strong enough to be observed. The specific hybridization signal disappeared after flowing 35-ml of washing reagents (5-ml buffer and 30-ml deionized water).

Chip Reusability

Initial investigations into chip reusability were made. After initial hybridization and washing, a second target injection was made. The RPI of the second hybridization is lower than that of the first hybridization, though the actual pixel intensity appears similar.

Sensitivity

The flow rate for these experiments are 0.05 ml/min. The synthetic T7 primer, labeled with TAMRA, can be detected at a level of 50 fmol. The detection sensitivity of the fluorescein labeled GLA-23 to the B-actin probe GLA-17 was found to be 0.5 pmol. The differences in the sensitivity limits could be attributed to several factors. Both signals may be increased at a slower flow rate (i.e., 0.01 ml/min) and chip configuration that decreased the dead area (only 9 mm^2 are imaged of the total 49 mm^2 and less than 1 mm^2 is used for the hybridization spot) The microscope/ccd detection system is more sensitive in the TAMRA area of the spectrum than the fluorescein area of the spectrum. The B-actin target may contain secondary structures that prevent effective hybridization and the B-actin target is distributed to more potential probe spots (28) than the T7 target (3).

Detection uniformity

The genosensor chip was rotated in its holder to assess the effects of location and flow on genosensor hybridization as different hybridization signal intensities have been observed for probes immobilized in different chip locations. Rotating the chip will help identify whether the origin of this is due to different detection sensitivities, different flow profiles, or physical differences in the spotted sample. Clear differences are observed on the hybridization of GLA-12 before and after rotating the chip. The intensity of this and other spots tentatively indicate that the primary influence is a nonuniform distribution of the target molecule. This is supported by altering the inlet and outlet ports for solution flow. For example, closing the left bottom inlet affects the signal intensity more than closing the right bottom inlet. The holder was originally designed to flow from the top center inlet, through the chip and out the two bottom, side outlets. However, preliminary experiments with flow going from the top to bottom gave generally lower signal.

Ionic strength

The hybridization signal of GLA-17 using 5XSSPE and 1XSSPE was assessed. Greater signal for this particular probe is observed with the higher ionic strength buffer as would be expected due to the greater stabilizing effect of the higher salt. However, this trend is not observed with all probes. Though no signal is as strong as GLA-17, at the higher salt concentration, many are quite similar. In fact, probe A-11 only appears at the lower salt concentration, while A-16 and A-21 appear higher. This would be expected if the higher salt concentration is stabilizing some structure in the target that prevents hybridization to the probe.

Flat glass chips

The RPI comparison of hybridization performed using channel glass chips and flat glass chips was performed. The RPI is ~5 fold lower using flat glass chips under the same conditions of target concentration and flow. The reproducibility of the flat glass hybridization experiments were poor and the value above represents the best result. In general, only weak images were obtained.

Chip uniformity

22 identical probes, GLA-6, were spotted in different locations of a channel-glass chip. (The T-7 probes also faintly appear as the filter does not cut off the long wavelength emission.) The quantitative results are shown in Table 3. Signal, background and relative pixel intensities vary with location. The greatest pixel values appear in the center area of the array. Lower values appear at the bottom of the array (row F in Table 1). The raw values vary as much as a factor of two with the relative values (RPI) ranging from 0.39 (at the top-right) to 0.10 (at the bottom).

Hybridization results for the 9 identical T-7 probes, spotted at different locations of the same channel-glass chip, are similar. Signal, background and relative pixel intensities of the T-7 hybridization at different location are also shown Table 3. The maximum raw signal is 711 (at the right), while the minimum is 450 (at the bottom). Maximum background is 269 (in the center), while the minimum is 192 (at the bottom). The maximum RPI of T-7 hybridization is 1.975 (at the right), while the minimum RPI is 1.344 (at the bottom). These values are five and thirteen fold

higher than the values for the B-actin probes, respectively. This great increase in relative intensity is probably due to the more efficient hybridization of the T-7 and the lower background in the "red" region.

The standard deviations (Table 3) also indicate the range of signal that is obtained as a function of position. This is on the order of 15% for both the signal and the background. Clearly there is an inhomogeneity in the obtained signal. The present experiments do not offer much insight into the origin, though they do quantitate the variance. The most likely culprit is an uneven flow. The imaged area is only a fraction of the area available for flow, so this inhomogeneity may be even larger when evaluating the full 7X7 mm area. The "flatness" of the field will need to be evaluated to correct for distortions induced by the lenses and lamp source.

Table 3. Chip uniformity test

GLA-6						T7					
Signal		Background		RPI		Signal		Background		RPI	
Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
233	36.5	186	28.8	0.25	0.08	589	80.3	227	24.3	1.59	0.21

Prewashing using solutions of different salt concentration

Chips, with attached 3'-propanolamine derivatized oligonucleotides, were washed with different ionic strength buffers, prior to hybridization, to determine if washing had an effect on the hybridization signal. The RPI of GLA-17 hybridization, when using 60 ml deionized water at 0.5 ml/min, is only half of that when using 1xSSPE flowing at the same rate. Similar RPI values were obtained when washing with either 60 ml of 1xSSPE or 20 ml of 5xSSPE at a flow rate of 0.5 ml/min. These experimental conditions were only evaluated once each, however, it appears that probe is lost when washing with the low ionic strength water. This experiment will need to be repeated to confirm this finding. Another set of experiments tested the GMBS linked probes spotted on the MPTS channel glass sent by Matt Torres. Prewashing using deionized water or 5xSSPE led to similar hybridization RPIs. The attachment mechanism between these two methods certainly appears different.

Additional experiments with the GMBS linked probes on the MPTS channel glass have been performed to compare this attachment chemistry to the propanol amine method. All chips were provided by Matt. Three chips of each derivatization method were used under identical working conditions (flow rates, concentrations and volume of target, temperature and buffer) The hybridization results from chips with the 3'-propanolamine did not appear as good as those with GMBS linked probes spotted on the MPTS channel glass. For example, hybridization using 10nM GLA-23 FT on non-treated chips yielded weaker signals for 3'-propanolamine derivatized oligonucleotides probes, while clearer signals were observed for hybridization with the GMBS linked probes spotted on the MPTS channel glass. These results need to be quantitated and are still considered preliminary.

Recirculation of target solutions

Experiments were conducted to try to improve the hybridization sensitivity by recirculating the target solution. This was accomplished by reversing the flow direction after passage of the sample through the chip. Initial experiments uncovered technical problems in that the volume injected and withdrawn did not appear to be conserved. After refinement of the procedure, it was found that a higher detection sensitivity can be obtained with recirculation at high flow rates. Hybridization involving only 10 fmoles of target was detectable by injecting and withdrawing the sample five times at a flow rate of 0.25 ml/min. This appears promising for rapid, sensitive detection.