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PERFORMANCE CHARACTERISTICS OF 65-MER OLIGONUCLEOTIDE MICROARRAYS

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Abstract

Microarray fabrication using pre-synthesized long oligonucleotide is becoming increasingly important, but a study of large-scale array productions is not published yet. We addressed the issue of fabricating oligonucleotide microarrays by spotting commercial, pre-synthesized 65-mers with 5' amines representing 7500 murine genes. Amine-modified oligonucleotides were immobilized on glass slides having aldehyde groups via transient Schiff base formation followed by reduction to produce a covalent conjugate. When RNA derived from the same source was used for Cy3 and Cy5 labeling and hybridized to the same array, signal intensities spanning three orders of magnitude were observed, and the coefficient of variation between the two channels for all spots was 8–10%. To ascertain the reproducibility of ratio determination of these arrays, two triplicate hybridizations (with fluorochrome reversal) comparing RNAs from a fibroblast (NIH3T3) and a breast cancer (JC) cell line were carried out. The 95% confidence interval for all spots in the six hybridizations was 0.60 – 1.66. This level of reproducibility allows use of the full range of pattern finding and discriminant analysis typically applied to cDNA microarrays. Further comparative testing was carried out with oligonucleotide microarrays, cDNA microarrays and RT-PCR assays to examine the comparability of results across these different methodologies.

INTRODUCTION

The technology developed to produce dense arrays of poly- or oligonucleotides to carry out sequence analysis [1,2] was quickly recognized as an ideal way to carry out the previously described experimental approach of using many immobilized cDNAs to detect specific patterns of gene expression [3,4]. Prototypic versions of microarray chips using cDNAs and short oligonucleotides were quickly produced [5,6]. Since these first demonstrations, arrays have been successfully used to examine many aspects of gene expression, and considerable effort has been expended to improve the production and performance of microarrays.

Microarrays are constructed by placing thousands of DNA species on solid substrates like silicon wafers or coated microscope slides. If long polynucleotides are used, they are usually the products of selective amplification of cloned cDNAs by polymerase chain reaction, printed

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by contact or jet methods. The shorter 20 to 65-mer probes for oligonucleotide arrays are usually synthesized *in situ* on the surface of the matrix using a combinatorial synthesis methods based on photolithography or jet technology [2,7]. Oligonucleotide arrays have many potential advantages. Highly accurate, stringent hybridization using oligonucleotides would, in principle, allow differential detection of closely related sequences and splice variants, a significant improvement over cDNA microarrays. Oligonucleotide arrays could also remove the labor intensive, time consuming and error-prone handling of cDNA resources and PCR amplification required for cDNA arrays. Realizing these benefits has been somewhat difficult, as the length of the immobilized oligonucleotide is both critical and expensive. Signal intensity in short surface-bound oligonucleotide arrays appears to be reduced by steric interference from the surface and from neighboring chains [8]. This defect can reportedly be overcome by using 50 – 60-mers [7,9]. Unfortunately, the current, *in-situ*, oligonucleotide chip production technologies have economic limitations in oligonucleotide length because the combinatorial synthesis process has an intrinsic efficiency problem and the jet method of synthesis consumes expensive intermediates.

As a result, there is currently intense interest in developing chip production methods in which pre-synthesized oligonucleotides would be printed. This approach would provide the benefits of using long oligonucleotides with high sequence specificity, producing these oligonucleotides by existing, inexpensive commercial methods and retaining the design flexibility of printing systems. Many methods of immobilizing oligonucleotides to glass surfaces have been described, however no characterization of the measurement performance of a large-scale array produced by spotting longer oligonucleotides has been provided. Here, we describe expression profiling results for a spotted 65-mer oligonucleotide microarray representing 7500 mouse genes.

MATERIALS AND METHODS

Slide modification

The slides were modified to generate aldehyde groups on their surface to which oligonucleotides could bind covalently through their amino group [10]. Twenty-five microscope slides (Becton Dickinson, Franklin Lake, NJ) were placed in a plastic rack and pretreated with 400mL of 1N NaOH overnight. After washing with water, the slides were reacted with 200mL of 8% (v/v) GPTMS (Aldrich, Milwaukee, WI) solution in 0.1 M sodium acetate buffer (pH 5.2) at 93°C for 24hr without shaking. The GPTMS solution was allowed to hydrolyze for 30 min before adding to the slides. The slides were then rinsed with water and ethanol, and dried under vacuum. To convert epoxy groups into diols, the slides were treated with 10 mM sulfuric acid at 93°C for 1h followed by washing with water and ethanol, and drying under vacuum. One day before printing, diols were cleaved with 20mM sodium metaperiodate (Sigma, St. Louis, MO) in 0.1 M sodium acetate buffer, pH 5.2 for 2 h to yield aldehyde groups. After rinsing with water and ethanol, the slides were dried under vacuum.

Microarray preparation

The 7524 mouse oligonucleotides (65-mers) with 5'-C₆ amino modifier were purchased from Compugen (Jamesburg, NJ). These represent 7445 unique genes. The oligonucleotides were diluted to a final concentration of 10 μ M in 3x standard saline citrate (SSC) and spotted on the aldehyde group derivatized slides. The printed slides were dried under vacuum overnight and processed following Schena *et al.*'s method [11] with modifications. The slides were washed twice in 0.2% SDS and twice in water for 2 min. After drying, they were reacted with a solution of sodium cyanoborohydride (NaCNBH₃, 20 mg/mL in 0.05M PBS, pH 8.0, Aldrich) for 30 min to reduce Schiff bases formed between aldehyde and amino groups. The remaining aldehyde groups were then reduced by treating with sodium borohydride solution (0.88 g of

NaBH₄ in 265 mL of PBS, pH 7.6 and 85 mL of ethanol) for 5 min. As a final step, the slides were washed with 0.2 % SDS three times and with water twice. After drying, they were stored at room temperature until use. Microarray spotting was performed at the NHGRI microarray core facility.

cDNA Microarrays

Ink-jet printed mouse cDNA microarrays were purchased from Agilent Technologies (Palo Alto, CA). The microarrays representing 8737 unique clones were prepared by ink-jet technology. Pen spotted cDNA arrays containing the set of BMAP ESTs were prepared at NIMH/NIH.

RNA extraction, sample labeling and hybridization

Total RNA was extracted from mouse NIH3T3 and JC cell lines. RNA extraction, sample labeling, hybridization and image analysis were performed essentially as previously described [12]. Cy3- or Cy5-labeled cDNAs were prepared by reverse transcription from 150–200 µg of total RNA and purified by four cycles of centrifugal filtration using Microcon 30 (Millipore, Bedford, MA) diafilters. Forty to fifty µg of each labeled cDNA was mixed with 2X hybridization buffer (Agilent Technologies PN G2558A) to a final volume of 27 µL, and incubated at 98°C for 2 min and at 4°C for 10 s. It was then hybridized at 65°C for 16 h under a 24 × 40 mm cover slip (Corning Inc., Big Flats, NY PN 2935 244) that was sonicated for 5 min in H₂O prior to use. The slides were washed for 2 min each in 0.5X SSC/0.01% SDS and 0.06X SSC.

Fluorescence scanning and image analysis was performed with DNA Microarray scanner (Agilent Technologies PN G2565BA) and DeArray software as described previously [13].

Quantitative PCR

Validation of expression ratios was done by RT-PCR for 15 selected genes using the LightCycler instrument (Roche Diagnostics, Indianapolis, IN). Total RNA (400 ng) was subjected to RT-PCR with SYBR Green I as a detection system (Roche PN 3 064 760).

A commercial control target dilution series and primers were used for calibration (Roche PN 2 158 841). The threshold cycle method as implemented in the manufacturer's software was used to measure the relative amount of target sequence in each sample. The PCR products were then analyzed by electrophoresis in a 2% agarose gel to verify the presence of a single, appropriately sized band.

RESULTS AND DISCUSSION

Slide modification

Organofunctional silanes have been widely used as coupling agents in diverse applications such as protective layers on organic polymers [14], anticorrosion coating [15] and adhesion promoters [16]. They are also commonly used cross-linkers in biotechnology. Many studies have been reported on the use of these materials for immobilization of enzymes [17,18], antibodies [19,20] and DNA [21,22]. For coupling, organofunctional alkyltrialkoxysilanes are the most successfully used silanes. Hydrolysis of these silanes yields silanols (SiOH), and the resulting hydrolyzed molecules form siloxane bonds with each other or with surface silanol groups through condensation.

(R': a short hydrocarbon chain with an organic functional group, R: an alkyl group)

Because each monomer has three alkoxide groups, their condensation can lead to large cross-linked polymers causing surface roughness. These large polymers can be very problematic in applications requiring very high surface homogeneity. In fields like photonics, chemical vapor deposition is necessary to avoid uneven surfaces. Biological labs typically do not have access to such sophisticated vapor deposition systems. However, simple coating by immersion does not have an adverse effect on standard biological analyses using fluorescence or radioisotope [23]. Indeed, heavy surface depositions may be advantageous for increasing the density of reactive groups for subsequent modification.

Aldehyde groups are derived from the silanes deposited on the slide surface as shown in Fig. 1A. Higher concentrations of 3-glycidypropyltrimethoxysilane (GPTMS) in the coating solutions gave an increased amount of silane bonding leading to greater oligonucleotide conjugation as judged by relative signal intensities (~3 fold increase) after hybridization with labeled cDNA. Coating concentrations of GPTMS higher than 8% produced an irregular, foggy coating and higher background noise, therefore an 8% solution was used. After silanization, the slides were cured under vacuum overnight at room temperature. Curing was essential, but extended curing periods did not improve signal intensities.

The slides used in this study were cleaned with 1 N NaOH overnight to activate the hydrophilic surface. There are a large number of cleaning methods available for this purpose [24], but no consensus has been reached as to the most effective method. To increase silane coverage on the surface, several pretreatment methods were initially performed by cleaning slides with either higher concentrations of bases and acids, or with combinations of organic solvents, acids and bases. These harsh cleaning methods did not lead to higher silane coverage, and in some cases, elevated background intensities were observed. Silanization immediately after the cleaning step was necessary to lower background. Substantially increased background noise (>2 fold) results when slides are silanized after exposure to the atmosphere for several hours following cleaning. Bipolar organic molecules in the air can reportedly strongly adsorb to glass surfaces due to the ionic characteristics of the SiOH groups that are exposed after cleaning [25]. These contaminant materials are thought to react with fluorescent dyes resulting in higher background intensity.

Post-printing process

The dehydration reaction between amino and aldehyde groups leads to formation of a Schiff base (Fig. 1B). The Schiff base is an unstable intermediate that can be hydrolyzed easily, and it must therefore be reduced to obtain irreversible immobilization. This step is critical for achieving good hybridization signals using oligonucleotide microarrays. Signal intensities could be increased by more than ten-fold by optimizing reduction conditions in our experiment. We evaluated the dependence of immobilization efficiency on NaCNBH₃ concentrations, buffer pH and reaction time. Immobilization was enhanced with increasing cyanoborohydride concentrations and reaction time at pH 8.0. More aggressive reduction also increased background levels. A 30-min incubation at a concentration of 20 mg/mL NaCNBH₃ was found to enhance target intensities significantly without increasing background noise. At pH 7.6, even

a 15-min incubation time increased background fluorescence significantly. Further systematic testing of reduction conditions might yield a more optimal process.

The remaining, unreacted aldehyde groups could interact with labeled cDNA during hybridization and give elevated background levels and should therefore be reduced to non-reactive primary alcohols before hybridization. Experimentation with these reduction conditions demonstrated that the pH of the NaBH₄ solution has a great effect on background fluorescence. We conducted the reaction at pH's of 7.2, 7.6 and 8.0, and found substantially increased background fluorescence (2.5–3 fold) with the pH 7.2 solution. To confirm that the background resulted from the lowered pH, we incubated the slides originally treated with pH 7.2 solution in a pH 7.6 NaBH₄ solution and could reduce the background levels to approximately 300 – 500 arbitrary fluorescence units (AFU), nearly the same as the 300 – 400 AFU seen for clean glass slides. Reaction in pH 7.6 and 8.0 solutions produced equivalent results.

Reliability of microarrays

We conducted hybridizations using Cy3 and Cy5 labeled cDNA, both prepared from RNA from an NIH3T3 (mouse fibroblast) cell line. Fig. 2A shows a typical log-log scatter plot of calibrated Cy3 and Cy5 fluorescence signals from all spots. As expected, all genes showed a Cy3/Cy5 ratio close to 1 lying along the 45° diagonal line ($r = 0.99$). We performed several hybridizations, and each showed a dynamic range of intensities greater than three orders of magnitude. The average coefficient of variance (CV) of pairs of intensities from the two normalized channels for all spots across the entire signal range was 8 – 10%. The histogram in Fig. 2B shows that the distribution of the $\log_2$ values of average ratios from three hybridizations for the 5347 spots that have the highest measurement quality on the three arrays in terms of fluorescent intensity, target area, background flatness and signal intensity consistency [26]. The average standard deviation of the $\log_2(\text{ratio})$ for those spots was 0.17, an average fold difference of 1.125. These results demonstrate the uniform hybridization and high reproducibility of this microarray system.

To examine the reproducibility of differential gene expression patterns, two sets of total RNA from the two different cell lines, JC and NIH3T3) were labeled with Cy3 or Cy5 and co-hybridized. In contrast to the homotypic hybridization, significant differences in the expression levels of genes from these very different cell types were detected ($r = 0.82$, Fig. 3A). The reproducibility of results was also evaluated in fluorescent dye reversal experiments. Hybridizations were performed on three different days using three separately labeled batches of cDNA in two sets of triplicates. RNA from the NIH3T3 cell line was labeled with Cy3 and RNA from the JC cell line with Cy5 in the first set. In the second triplicate, the labeling was reversed. The values of average $\log_2(\text{JC/NIH3T3})$ from the first set were compared with the average $\log_2(\text{JC/NIH3T3})$ from the first set (Fig. 3B). Most of spots lie close to the 45° diagonal line, which shows that no systematic bias is introduced by the reciprocal labeling. However in some cases, target signals from highly expressed genes saturated the detector and were found to distort ratios. Even though this distortion degraded the precision of the system, an average standard deviation of 0.35 for all spots ($n=7812$), regardless of measurement quality, was obtained from the $\log_2(\text{ratio})$ values (Fig. 3C). A 95% confidence interval was estimated for the data from all spots, using the median variance as an estimate of sigma as previously described [27], yielding an interval of 0.60 – 1.66. A narrower interval of 0.70 – 1.44 results from an estimate based only on the 3797 spots having the highest technical measurement quality.

Oligonucleotide concentration

To investigate the impact of oligonucleotide concentrations on target signals, we printed 288 oligonucleotides at two concentrations, 10 and 25 μM , on the same slides with all other oligonucleotides printed at 10 μM . The average $\log_{10}$ (fluorescence intensity) values from six hybridizations was compared for 165 genes which have highest measurement quality at both concentrations in all 6 experiments (Fig. 4A). When these measurements are considered in aggregate, a t-test (paired, two-tailed) shows no significant difference in the means at $\alpha=0.01$. Neither is skewing of the intensities observed when the distribution of t-values for each of the 165 genes is plotted (Fig. 4B). This result indicates that the oligonucleotide concentration of 10 μM is high enough to effectively saturate the oligonucleotide binding capacity of the slide surface under the conditions used in our experiment.

Comparison of expression ratios between oligonucleotide and cDNA microarrays

The comparability of measurements made with different types of microarrays is of considerable importance for cross-comparisons of data produced in different laboratories using different arrays. The oligonucleotides on these arrays are made from representations near the 3' ends of genes and hybridizations can be carried out under the same conditions used for cDNA microarrays, making it reasonable to compare measurement results across these devices. Hybridizations were done with two kinds of cDNA microarrays, pen printed ($n=6$) and ink-jet printed ($n=2$). The three types of microarrays have 1,914 genes in common. A comparison of the average ratios determined on each array type across the various platforms was carried out (Table 1). Ratio agreement was high, with more than half of all measurements between the common genes being within 1.6 fold of each other (our estimate of the indeterminacy of the oligonucleotide ratio measurements) in all of the comparisons. Where the ratio disagreements were greater than 1.6 fold, two different assessments of agreement can be used. Agreement on the direction of change can be unambiguously assessed when both ratios of the pair are sufficiently far from ratio=1 (< 0.6 or > 1.6) that the 1.6 fold indeterminacy cannot shift the ratio from greater than one to less than one or vice versa. For the genes where this was true, the ratios agreed on the direction of change more than 85% of the time. On the other hand, if one of the ratios in the pair is close to 1 and vulnerable to direction shift by the measurement indeterminacy, directionality comparison of ratio change would be inappropriate. In this case, a measure of the level of increase in disagreement was used. For these genes, the average fold differences in the oligo to cDNA comparisons represented less than a 50% increase above 1.6 fold change. While the ratio agreement between the cDNA measurements was greater than between the cDNA and oligo measurements, it was not strikingly so.

Comparison of expression ratios between arrays and RT-PCR

In examining the numerous cases of validation of microarray results by other measurement methods in the literature one can find examples of both systematic and sporadic forms of measurement variance. To explore the validation properties of oligonucleotide-based abundance measurements, we selected 15 genes that showed concordant results between the two types of cDNA microarrays, but a mixture of concordant and discordant results between cDNA and oligonucleotide microarrays. Assays of the abundance of transcripts for these genes in the original total RNA pools were then carried out by RT-PCR, using primers shown in Web supplementary Table 1. A constant input amount of 400 nanograms of total RNA was used in the RT-PCR of the two RNA samples. A crude estimate of the number of RNA transcripts present at the start of the PCR reaction for each gene assayed was extrapolated from a dilution series of a commercial cytokine control RNA run at the same time. The ratios expected for these genes on the basis of the RT-PCR results, the observed ratios from each array assay, the estimated transcripts per 0.4 μg of total RNA and the distance of the arrayed oligonucleotide from the 3' end of the gene are shown in Table 2.

The variances in abundance estimation seen in Table 2 seem similar to several previously observed types of variance, position-based detection sensitivity and ratio compression. Testing in early microarray studies demonstrated that location of the detector near the 3' end of the gene improves detection, presumably due to the formation of short 3' reverse transcripts in the typical poly-dT primed reaction used to generate labeled transcripts [28]. If the immobilized DNA is chosen from a region of the gene sufficiently far from the 3' end, then reverse transcription will fail to produce a reverse transcript long enough to hybridize to that detector. For those ten genes where the immobilized oligonucleotide representing the gene was within 600 bases of the 3' end of the gene, the oligonucleotide ratio is in agreement with the direction of the RT-PCR estimated ratio for all genes except *Nras*. For those genes where the representative oligonucleotide is further than 600 bases from the 3' end, agreement with RT-PCR is more variable. Mass-spectrographic testing by the manufacturer of the *Nras* oligonucleotide indicated that it was not of the expected molecular weight and thus probably not the expected sequence. In those cases where oligonucleotide design criteria drive the choice of sequence far from the 3' end of a transcript, recourse to random priming of the input RNA may provide a way to improve ratio estimates.

A further common observation in microarray studies is that when large differences in transcripts are measured by both microarrays and another method, the microarray measurement tends to produce a smaller estimate of the extent of difference relative to other method. This compression of ratios was observed relative to RT-PCR in this study. For most of the genes, RT-PCR produced higher estimates of differences in transcript abundance than the array methods. This difference was least for the most highly abundant genes (10^7 transcripts/ $0.4\ \mu\text{g}$ total RNA), suggesting that microarray ratio compression may be greater for less abundant genes. The reason for such compression is not understood, however the efficiency of hybrid formation between a nucleic acid species from a sample and its complementary detector is far more favorable when both species are in solution, as is the case in RT-PCR assays. The data also suggests that the extent of ratio compression varies among the different array types, making it likely that a potentially profitable avenue for improvement of ratio estimates with oligonucleotide arrays may lie in optimization of the amount and spacing of oligonucleotides bound to the array. In the preliminary optimization experiments used to choose the slide production conditions for these experiments, it was observed that increasing the amount of oligonucleotide immobilized on the slides exacerbated ratio underestimation, even though signal intensities were increased.

The high reproducibility of ratio measurement that can be achieved with printed oligonucleotide slides combined with their agreement with RT-PCR at the level of direction of ratio change are sufficient to allow this kind of array to be used confidently for the detection of patterns of expression change. As the oligonucleotide manufacturers supply the position of the representative oligonucleotide along the gene, those oligonucleotides in commercial sets likely to produce more variable measurements due to position can be flagged so that their estimates can be interpreted with appropriate caution.

There is increasing interest in fabrication method for long oligonucleotide microarray by printing. However, there was no systematic and large-scale study performed on this issue. In this paper we investigated microarray production methods by printing pre-synthesized 65-mer on aldehyde group functionalized slide. Signal intensities and measurement reproducibility were comparable to cDNA microarray results. Furthermore, the gene expression ratio data were compared with cDNA microarrays and RT-PCR experiment results. A varying degree of concordant and discordant results were obtained from the platform comparison. In a companion paper [29], we sought to characterize microarray performance and investigate the cause of discordance with RT-PCR data

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References

1. Southern EM, Maskos U. Parallel synthesis and analysis of large numbers of related chemical compounds: applications to oligonucleotides. *J Biotechnol* 1994;35:217–227. [PubMed: 7765059]
2. Pease AC, Solas D, Sullivan EJ, Cronin MT, Holmes CP, Fodor SPA. Light-generated oligonucleotide arrays for rapid DNA sequence analysis. *Proc Natl Acad Sci USA* 1994;91:5022–5026. [PubMed: 8197176]
3. Augenlicht LH, Taylor J, Anderson L, Lipkin M. Patterns of gene expression that characterize the colonic mucosa in patients at genetic risk for colonic cancer. *Proc Natl Acad Sci USA* 1991;88:3286–3289. [PubMed: 1849648]
4. Gress TM, Hoheisel JD, Lennon GG, Zehetner G, Lehrach H. Hybridization fingerprinting of high-density cDNA-library arrays with cDNA pools derived from whole tissues. *Mamm Genome* 1992;3:609–619. [PubMed: 1450511]
5. Schena M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 1995;270:467–470. [PubMed: 7569999]
6. Lockhart DJ, Dong H, Byrne MC, Follettie MT, Gallo MV, Chee MS, Mittmann M, Wang C, Kobayashi M, Horton H, Brown E. Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nat Biotechnol* 1996;14:1675–1680. [PubMed: 9634850]
7. Hughes TR, Mao M, Jones AR, Burchard J, Marton MJ, Shannon KW, Lefkowitz SM, Ziman M, Schelter JM, Meyer MR, Kobayashi S, Davis C, Dai H, He YD, Stephanians SB, Cavet G, Walker WL, West A, Coffey E, Shoemaker DD, Stoughton R, Blanchard AP, Friend SH, Linsley PS. Expression profiling using microarrays fabricated by an ink-jet oligonucleotide synthesizer. *Nat Biotechnol* 2001;19:342–347. [PubMed: 11283592]
8. Shchepinov MS, Case-Green SC, Southern EM. Steric factors influencing hybridisation of nucleic acids to oligonucleotide arrays. *Nucleic Acids Res* 1997;25:1155–1161. [PubMed: 9092624]
9. Kane MD, Jatkoe TA, Stumpf CR, Lu J, Thomas JD, Madore SJ. Assessment of the sensitivity and specificity of oligonucleotide (50mer) microarrays. *Nucleic Acids Res* 2000;28:4552–4557. [PubMed: 11071945]
10. de Frutos M, Paliwal SK, Regnier FE. Liquid chromatography based enzyme-amplified immunological assays in fused-silica capillaries at the zeptomole level. *Anal Chem* 1993;65:2159–2163. [PubMed: 8372972]
11. Schena M, Shalon D, Heller R, Chai A, Brown PO, Davis RW. Parallel human genome analysis: microarray-based expression monitoring of 1000 genes. *Proc Natl Acad Sci USA* 1996;93:10614–10619. [PubMed: 8855227]
12. Jiang, Y.; Leuders, J.; Glatfelter, A.; Gooden, C.; Bittner, M. *Current Protocols in Molecular Biology*. Ausubel, FM., et al., editors. Vol. 4. John Wiley & Sons; Hoboken, NJ: 2000. p. 22.23.21–22.23.26.
13. Chen Y, Kamat V, Dougherty E, Bittner M. Ratio-based decisions and the quantitative analysis of cDNA microarray images. *J Biomed Optics* 1997;2:364–374.
14. Hoebbel D, Nacken M, Schmidt H. On the existence and hydrolytic stability of titanoxane bonds in the system: glycidoxypolytrimethoxysilane-water-titaniumtetraethoxide. *J Sol-Gel Sci Techn* 1998;13:37–43.
15. Schmidt H. Multifunctional inorganic-organic composite sol-gel coatings for glass surfaces. *J Non-Cryst Solids* 1994;178:302–312.
16. Daniels MW, Francis LF. Silane adsorption behavior, microstructure, and properties of glycidoxypolytrimethoxysilane-modified colloidal silica coatings. *J Colloid Interface Sci* 1998;205:191–200. [PubMed: 9710513]
17. Weetall, HH. *Methods in Enzymology*. Mosbach, K., editor. Vol. 44. Academic Press; San Diego: 1976. p. 34–148.

18. Ennéus J, Gorton L. Comparison between different inorganic supports for the immobilization of amyloglucosidase and -amylase to be used in enzyme reactors in flow-injection systems. *Anal Chim Acta* 1993;276:303–318.
19. Flounders AW, Brandon DL, Bates AH. Immobilization of thiabendazole-specific monoclonal antibodies to silicon substrates via aqueous silanization. *Appl Biochem Biotechnol* 1995;50:265–284.
20. Williams RA, Blanch HW. Covalent immobilization of protein monolayers for biosensor applications. *Biosensors & Bioelectronics* 1994;9:159–167. [PubMed: 8018317]
21. Rogers Y, Jiang-Baucom P, Huang Z, Bogdanov V, Anderson S, Boyce-Jacino MT. Immobilization of oligonucleotides onto a glass support via disulfide bonds: a method for preparation of DNA microarrays. *Anal Biochem* 1999;266:23–30. [PubMed: 9887210]
22. Podyminogin MA, Lukhtanov EA, Reed MW. Attachment of benzaldehyde-modified oligodeoxynucleotide probes to semicarbazide-coated glass. *Nucleic Acids Res* 2001;29:5090–5098. [PubMed: 11812841]
23. Möller R, Csáki A, Köhler JM, Fritzsche W. DNA probes on chip surfaces studied by scanning force microscopy using specific binding of colloidal gold. *Nucleic Acids Res* 2000;28:e91. [PubMed: 11024193]
24. Cras JJ, Rowe-Taitt CA, Nivens DA, Ligler FS. Comparison of chemical cleaning methods of glass in preparation for silanization. *Biosensors & Bioelectronics* 1999;14:683–688.
25. Schmidt H. Multifunctional inorganic-organic composite sol-gel coatings for glass surfaces. *J Non-Cryst Solids* 1994;178:302–312.
26. Chen Y, Kamat V, Dougherty ER, Bittner ML, Meltzer PS, Trent JM. Ratio statistics of gene expression levels and applications to microarray data analysis. *Bioinformatics* 2002;18:1207–1215.
27. Bittner M, Meltzer P, Chen Y, Jiang Y, Seftor E, Hendrix M, Radmacher M, Simon R, Yakhini Z, Ben-Dor A, Sampas N, Dougherty E, Wang E, Marlncola F, Gooden C, Lueders J, Glatfelter A, Pollock P, Carpten J, Gillanders E, Leja D, Dietrich K, Beaudry C, Berens M, Alberts D, Sondak V, Hayward N, Trent J. Molecular classification of cutaneous malignant melanoma by gene expression profiling. *Nature* 2000;406:536–540. [PubMed: 10952317]
28. DeRisi J, Penland L, Brown PO, Bittner ML, Meltzer PS, Ray M, Chen Y, Su YA, Trent JM. Use of a cDNA microarray to analyze gene expression patterns in human cancer. *Nat Genetics* 1996;14:457–460. [PubMed: 8944026]
29. Lee M, Trent J, Bittner M. Optimization of oligonucleotide microarray fabricated by spotting 65-mer. *Anal Biochem.* submitted

Fig. 1.

Glass slide derivatization and oligonucleotide attachment. (A) The slide is first silanized with 3-glycidypropyltrimethoxysilane, and the epoxy rings are then opened to yield diols under acidic conditions. The cleavage of diols leads to the formation of aldehyde groups. (B) Attachment of 5' end amino-modified oligonucleotides onto an aldehyde group activated slide surface via Schiff base formation. The Schiff base is reduced by sodium cyanoborohydride to give a stable alkylamine bond and remaining aldehyde groups are also reduced by sodium borohydride to lower background signals.

Fig. 2. Homotypic hybridization results. (A) Typical scatter plot of the normalized Cy3 and Cy5 fluorescence intensities from an NIH3T3:NIH3T3 hybridization (single experiment). (B) Histogram showing the distribution of average $\log_2(\text{ratio})$ from three hybridizations for 5347 spots which have highest measurement quality in all 3 experiments.

Fig. 3. Heterotypic hybridization results. (A) Normalized signal intensities are plotted (single experiment). (B) Average $\log_2(\text{ratio})$ from three NIH3T3:JC hybridizations versus average $\log_2(\text{inverted ratio})$ from three JC:NIH3T3 hybridizations. Plotted are 3797 spots that have highest measurement quality in all 6 experiments. (C) The distribution of the standard deviations of the $\log_2(\text{ratio})$ values for all spots ($n=7812$) in the dye swap experiments.

Fig. 4.

Dependency of signal intensity on concentration of oligonucleotide printed. (A) Average log₁₀ (fluorescence signal) from six hybridizations is plotted for 165 genes which are spotted in two concentrations, 10 μ M (filled triangles) and 25 μ M (open squares). (B) Histogram showing the distribution of t-statistics applied to two fluorescence intensities for each gene. At $\alpha=0.05$, the null hypothesis, that the sample populations are the same would be rejected for a t-statistic value greater than 2.23 (dotted lines).

Table 1

Ratio agreement between microarray measurements (n=1914).

Comparison [average fold difference]	Ratio Difference $\leq$ 1.6 fold	Ratio Difference > 1.6 fold		
		0.6 < One Ratio < 1.6 (average fold difference)	Both Ratios <0.6 or > 1.6	
			Concordant Direction	Discordant Direction
Oligo vs cDNA (jet) [1.75]	1093	413 (2.38)	349	59
Oligo vs cDNA (pen) [1.60]	1194	390 (2.29)	293	37
cDNA (jet) vs cDNA (pen) [1.50]	1338	255 (2.62)	247	24

Table 2

Comparison between gene expression ratios obtained by RT-PCR and microarrays (3T3 versus JC). Transcript numbers in samples were estimated from cytokine RNA copies of a control kit.

Gene	Transcript Number in JC	Transcript Number in 3T3	cDNA Microarray Ratio			Oligonucleotide distance from the poly-A tail (bp)	
			RT-PCR Ratio	Agilent	BMAP		
Cox7c	1.2×10^7	9.8×10^6	0.79 ± 0.20	0.95 ± 0.08	0.94 ± 0.05	0.98 ± 0.12	257
Igfbp6	9.7×10^5	6.6×10^6	6.78 ± 0.61	8.74 ± 0.82	7.33 ± 0.66	9.84 ± 0.40	271
Nras	5.4×10^4	2.6×10^4	0.48 ± 0.04	0.93 ± 0.02	0.92 ± 0.04	0.042 ± 0.008	277
Ifit3	4.4×10^6	2.1×10^4	0.0048 ± 0.0007	0.013	0.041 ± 0.010	0.088 ± 0.057	302
Cstb	9.7×10^6	5.6×10^6	0.58 ± 0.05	1.09 ± 0.13	0.93 ± 0.03	0.68 ± 0.08	318
Rpl36	8.2×10^6	2.0×10^7	2.50 ± 0.33	4.41 ± 0.05	3.42 ± 0.47	4.40 ± 1.55	318
Thbs2	35*	7.4×10^3	>200	44.28 ± 10.48	24.67 ± 7.77	17.75 ± 4.57	393
Cib1	7.1×10^5	3.8×10^5	0.63 ± 0.14	0.81 ± 0.07	n.a.	1.05 ± 0.10	431
Eno3	4.4×10^6	9.7×10^4	0.021 ± 0.005	0.087 ± 0.002	0.11 ± 0.02	0.13 ± 0.05	517
Pctk1	1.8×10^6	9.1×10^5	0.48 ± 0.12	1.16 ± 0.03	0.95 ± 0.04	0.64 ± 0.15	551
Sgcb	6.2×10^5	5.8×10^5	0.94 ± 0.05	1.19 ± 0.06	1.38 ± 0.12	0.42 ± 0.44	673
Tgm2	1.9×10^6	8.9×10^4	0.048 ± 0.006	0.23 ± 0.31	0.21 ± 0.08	0.80 ± 0.38	721
Gsta4	1.1×10^7	9.1×10^5	0.10 ± 0.01	0.22 ± 0.02	0.23 ± 0.03	0.66 ± 0.09	856
MD3	1.2×10^4	7.2×10^5	59.5 ± 11.95	27.97 ± 31.76	16.59 ± 4.46	5.87 ± 0.74	891
Pex5	3.9×10^5	6.6×10^5	1.68 ± 0.12	2.81 ± 0.24	2.07 ± 0.14	0.70 ± 0.07	1358

* The amount of Thbs2 transcript in JC was very low in $0.4 \mu\text{g}$ of total RNA and was not detected accurately by RT-PCR.