Development of chemically modified glass surfaces and methods for microarray technologies

PhD Thesis

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2004

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CONTENTS

PUBLICATIONS DIRECTLY RELATED TO THE THESIS

1. **László Hackler Jr.**, György Dormán, Zoltán Kele, László Ürge, Ferenc Darvas and László G. Puskás. (2003) Development of chemicaly modified glass surfaces for nucleic acid, protein and small molecule microarrays. *Mol. Divers.* 7, 25-36.

2. László G. Puskás, Ágnes Zvara, **László Hackler Jr.**, and Paul Van Hummelen (2002) RNA amplification results in reproducible microarray data with slight ratio bias. *Biotechniques* 32, 1330-1340.

3. László G. Puskás, Ágnes Zvara, **László Hackler Jr.**, Tamás Micsik and Paul van Hummelen (2002) Production of Bulk Amounts of Universal RNA for DNA-microarrays. *Biotechniques* 33, 898-904.

4. Ágnes Zvara, **László Hackler Jr.,** Zsolt B. Nagy, Tamás Micsik and László G. Puskás (2002) New molecular methods for classification, diagnosis and therapy prediction of hematological malignancies. *Pathol. Oncol. Res.* 8, 231-240.

PUBLICATIONS INDIRECTLY RELATED TO THE THESIS

1. László G. Puskás, Zsolt B. Nagy, Zoltán Giricz, Annamária Ónody,Csaba Csonka, Klára Kitajka, **László Hackler Jr.**, Ágnes Zvara, Péter Ferdinándy (2004) Cholesterol diet-induced hyperlipidemia influences gene expression pattern of rat hearts: a DNA microarray study. *FEBS Letters*, 562, 99-104.

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6. Klára Kitajka, László G. Puskás, Ágnes Zvara, **László Hackler, Jr.**, Gwendolyn. Barceló-Coblijn, Y. K. Yeo, and Tibor. Farkas (2002) The role of n-3 polyunsaturated fatty acids in brain: Modulation of rat brain gene expression by dietary n-3 fatty acids. *Proc. Natl. Acad. Sci. USA* 99, 2619-2624.

7. László G. Puskás, **László Hackler Jr.**, Györgyi Kovács, Zoltán Kupihár, Ágnes Zvara, Tamás Micsik and Paul van Hummelen. (2002) Recovery of Cyanine-dye Nucleotide Triphosphates. *Anal. Biochem.* 305, 279-281.

PATENTS

1. ID no.: P0201091 **Submitted:** 2002. 03. 29**Title:** New activated surfaces and method for immobilizing combinatorial compounds and compound libraries. (Új aktív hordozó anyag és eljárás kombinatorikus vegyületek vagy vegyület-könyvtárak felületi immobilizálására)

2. ID no.: P0104423 **Submitted:** 2001. 10. 20 **Title:** Activated solid surfaces, fabrication and application. (Reaktív szilárd hordozók, elõállításuk és alakalmazásuk.)

AIMS

The thesis deals with three aspects of microarray technology: first is to create the bedrock of the technique, develop suitable solid supports for immobilization, second is to investigate and validate different labeling techniques to produce labeled samples for gene expression analyses, and third is to minimize experimental variations through the application of indirect comparison of biological samples in DNA microarray analyses.

Surface developments

Our goal was to develop diverse linker systems capable of anchoring different biological samples, especially DNA and drug-like small molecules and proteins. The developed surfaces were to be applied in several microarray applications after characterization of the anchoring parameters and investigation of the mechanism of sample binding.

Investigation of labeling techniques

Microarray expression analysis demands large amounts of RNA that is often not available. To overcome this problem RNA amplification techniques have been developed, but limited data were available regarding reproducibility and maintaining original transcript ratios. The goal of our work was to optimize and validate two amplification techniques: a modified *in vitro* transcription (IVT) for linear amplification of 3 micrograms of total-RNA, and a SMART PCR-based technique for exponential amplification of 50 nanograms of total-RNA. To determine biases in transcript ratios we compared expression profiles in mouse testis versus spleen between the two amplification methods and a standard labeling protocol by using microarrays containing 4596 cDNAs spotted in duplicate.

Technical developments

In DNA microarray technology repeatability and reliability are very important to compare multiple RNA samples from different experiments. The application of common or universal RNA as a standard control equalizes the differences in hybridization parameters and array variations. For this purpose high-quality reference RNA is necessary in bulk amounts. Our goal was to develop a novel approach to get milligrams of sense or anti-sense RNA starting from micrograms of pooled total RNA from different cell lines, tissues or organisms.

INTRODUCTION

The idea of deposition and immobilization of thousands of samples in an array format has led to the development of several microarray techniques utilizing different biological samples like cDNA, pre-synthesized oligodeoxyribonucleotides, proteins, tissues (52) and small molecules from combinatorial chemical libraries. Immobilized DNA molecules, either as double-stranded segments (24, 47) or as short synthetic oligodeoxyribonucleotides (20), have been utilized for gene expression monitoring (38, 43, 61), DNA-sequencing (42, 44), disease screening (10, 11, 63), diagnostics and genome analysis (7, 18). Microarrays containing DNA molecules can be divided into two groups: cDNA microarrays and oligonucleotide microarrays (oligo-arrays). On the surface of a cDNA microarray double stranded cDNA molecules - from PCR amplification - are the immobilized samples, while oligo arrays utilize pre-synthesized or in situ synthesized oligonucleotides (22, 32, 41). Protein arrays containing numerous different entities are efficient tools in applications like antibody profiling, serum screening and ultimately in drug discovery (34, 40, 60). Newly emerging platform of microarrays are chemical microarrays. These arrays contain thousands of drug-like small molecules from parallel combinatorial synthesis and enable the identification of new and screening of existing lead molecules (28, 33, 35, 60). All mentioned techniques demand permanent and efficient anchoring of the samples. A great number of attachment methods have been patented or published, which vary widely in chemical mechanism, chemical structure of linker system, ease of use, attachment stability and cost. Biomolecules can be attached to solid surfaces covalently or non covalently. Non-covalent anchoring methods utilize the electrostatic interactions between the surface and the biomolecules through secondary chemical bonds (e.g. ionic interactions and hydrogen bonds). Standard methods for covalent attachment of oligonucleotides onto solid surfaces include reactive aldehyde-, thiol- (15, 25, 49), or epoxy-groups, where chemically modified nucleic acids are needed for their attachment. A number of different substances have been tested as the solid support for attachment of nucleic acids (48, 50), but glass slides are generally favoured for DNA and oligonucleotide arrays (5, 41).

The most important and most informative application of DNA-microarrays is the parallel study of gene expression from different biological samples that focuses on the functionally active parts of the genome. DNA microarrays with sets of DNA fragments on their surface can be used to obtain a molecular fingerprint of gene expression of cells (2, 3, 6, 31, 39).

DNA-microarray technology provides a rapid and comprehensive approach to simultaneously monitor the expression levels of thousands of genes between diverse biological samples in a comparative way. The arrays are constructed of thousands of DNA fragments, which can be collections of short or long oligonucleotides (20-70 nucleotides) or cDNAs of variable length (200-2000 base pairs). Oligonucleotide microarrays are also suitable for the simultaneous detection of several thousands of single nucleotide polymorphisms (SNPs) in an amplified genomic DNA sample (20, 58). This has a great significance in searching for polymorphic loci, or in detection of single or multiple mutations in medical samples.

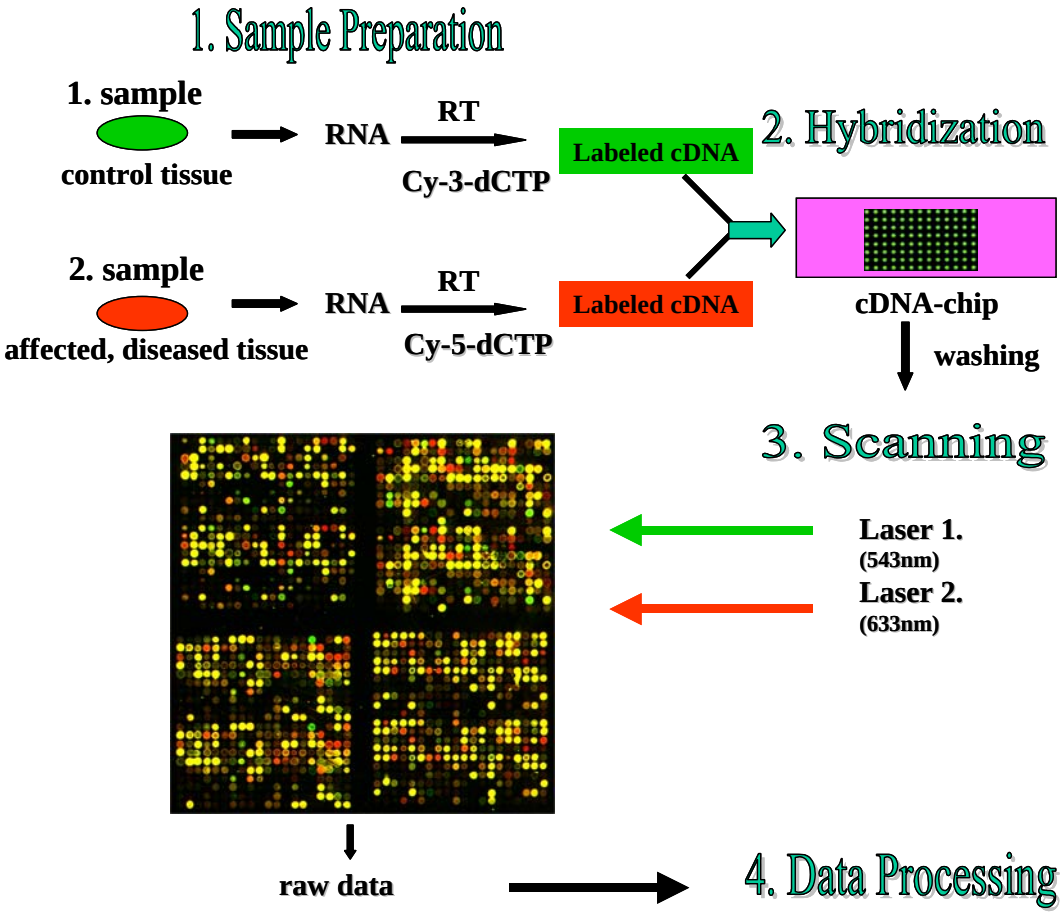

Figure 1. Application of cDNA microarrays to investigate gene expression changes

DNA microarray technology has enabled large numbers of genes, from specific cell populations, to be studied in a single experiment. The investigation is based on the relative quantification of mRNA species, which are produced from active genes during transcription. Gene expression analysis with microarray technology is a reverse blotting technique, where mRNA populations gained from diverse biological samples (tissues or cell cultures) are

converted into fluorescently labeled cDNA during reverse transcription in the presence of fluorescence dye (e.g. Cy3 or Cy5) labeled nucleotides.

Using a co-hybridization strategy, with Cy3-labeled cDNA from the test sample and Cy5-labeled cDNA from the control sample, the relative intensity ratio on the microarray can be determined and the gene expression pattern can be analysed (Figure 1).

This direct comparative hybridization method allows a quantitative comparison of the relative abundance of individual sequences, although experimental variation introduced by uneven incorporation of labels, differences in hybridization, washing and reading often occurs (22, 36). It can cause discrepancies in relative and accurate comparisons of separate experimental results, especially when they are performed by different research laboratories or at different times. While kits are available to optimize labeling steps, there is no consensus even in protocol to apply by different groups. The most reliable approach to overcome these problems is the indirect comparison of signal intensities. In these kinds of experiments each mRNA sample is compared to a reference mRNA pool composed of an equal mixture of all experimental RNA targets. The importance of this approach was first suggested by Eisen and Brown (13) and used in comparison of different cancer cell lines (51) and different non-Hodgkin's lymphomas (1). The application of a common reference RNA pool in each cDNA microarray experiment allows the relative gene expression in multiple samples to be analyzed. It is very difficult to obtain reliable reference RNA not only because of the need of its complexity but also because of the large quantity. Today, universal reference RNA is commercially available but it is expensive for routine use, and only certain organism specific reference RNA is available like human, mouse, rat. To obtain reference RNA from pooled, high-quality RNA, RNA isolation should be performed from cell lines representing different tissues, or from different tissue samples. However, this is costly and time consuming if one wants to obtain milligrams of highly purified RNA.

We developed a novel amplification technique based on PCR amplification and a modified version of an *in vitro* amplification (http://www.microarrays.org/pdfs/ModifiedEberwine.pdf) to obtain high-quality reference RNA in bulk amounts starting from micrograms of mixed total RNA. Both antisense and sense amplified RNA can be synthesized with this method and can be used as standards in diverse microarray studies.

In microarray analysis the quantity of the starting mRNA depends on the type of tissue (e.g. liver or brain tissues). The amount of RNA is strongly limited in those cases, where the amount of the sample tissue is small, for example in cases of biological sample obtained with biopsies or other operative methods, or in experimental systems where 1000–50000 cells are

the objects of the investigation. In these cases, amplification of the RNA sample or the signal is necessary (5, 16, 17, 27). During the amplification steps, however, it is very important to keep the quantitative ratios presented in the original mRNA population. The proper use of exponential (PCR) and linear (in vitro transcription, IVT) amplification can solve this problem.

With DNA microarray technology, changes in cells generated by various effects (e.g. pharmaceutical treatment, pathological processes) can be followed (45), new biochemical markers and genes responsible for pathological phenotype can be discovered (53), drug effects can be followed (19, 57) and the treatment can be optimized. The differences in gene expression of the treated and untreated cells or tissues provide information about the regulation of the enzymatic pathways influenced by drugs, about the enzymes, transporters playing a role in drug resistance. Identification of gene expression patterns may provide vital information for the understanding of the pathological processes and may contribute to diagnostic decisions and therapies tailored to the individual patient.

The thesis is addressing challenges and questions from three areas of microarray technology and is divided into three sections. The first section is dealing with the development of different solid supports. The developed supports are tested in several immobilization experiments with different biological samples. Optimal parameters of anchoring are determined, and the mechanism of anchoring is also studied. The second section investigates available and a new RNA labeling method described by us. Methods are compared to each other by means of reproducibility and applicability depending on the amount of available RNA. The third section provides an affordable way to produce large amounts of sense or antisense reference RNA to enable the investigator to use indirect comparison of biological samples.

MATERIALS AND METHODS

Developing the reactive surfaces

The reactive surfaces were obtained in multiple reaction steps. The final reaction step differs in case of producing either acryl or epoxy derivatized surfaces. Epoxy functions were introduced with two different reagents.

Support media

As starting material, commercially available non-derivatized microscope slides were used (Spektrum 3D, Debrecen, Hungary). Reactions were carried out in polypropylene or glass chambers. For comparison Superaldehyde (ArrayIt, Telechem) aldehyde (Genetix), Epoxy silane (Erie Scientific Company), and poly-L-lysine slides (Erie Scientific Company) were used.

Dendrimer-like linker systems

The non-derivatized glass slides were etched in 10% NaOH for 24 h, then washed with water. After the alkaline treatment the slides were neutralized with 1% HCl for 1 minute, and washed again with water then dried. The activated glass slides were treated with 3% methacryloxypropyl-trimethoxysilane (ICN Biomedicals Inc.) solution in 95% methanol for 2 h. Afterwards the slides were washed with methanol, then water, dried and baked at 105 ºC for 15 min. The acryl silanized slides were incubated for 48 h in 70 ml solution of 0.033 M tetraethylenepentamine in dimethylformamide (DMF). Afterwards the slides were washed with DMF, methanol and dried. For preparing acrylic surface the slides were treated with 70 ml solution of 0.128 M acryloyl-chloride and 0.128 M diisopropylethyl-amine (ICN. Biomedicals Inc.) in anhydrous dicloroethane (ALFA) for 2 h, then washed with dichloroethane and dried. For preparing hydrophobic epoxy surface slides were treated with 70 ml solution of 0.43 M epichlorohydrin and 0.17 M pyridine in chloroform, then washed with chloroform and dried. For preparing hydrophylic epoxy surface slides were treated with 70 ml solution of 0.43 M 1,4-butanediol diglycidyl ether in the presence of 0.2 g NaOH in ethanol, then washed with ethanol and dried.

Linker systems based on triamino-silanized surface

The non-derivatized glass slides were etched in 10% NaOH for 24 h, then washed with water, neutralized with 1% HCl, and washed again with water then dried. The activated glass slides were treated with 3% 3-[2-(2-Aminoethylamino)ethylamino)propyl-trimethoxysilane (Fluka) solution in 95% methanol for 2 h. Afterwards the slides were washed with methanol, then water, dried and baked at 105 ˚C for 15 min. For preparing acrylic surface the silanized slides were incubated for 2 h with 70 ml solution of 0.43 M acryloyl-chloride (Fluka) and 0.43 M diisopropylethyl-amine (ICN. Biomedicals Inc.) in dichloroethane. Afterwards the slides were washed with DMF, methanol and dried. For preparing hydrophobic epoxy surface the silanized slides were incubated for 2 h with 70 ml solution of 0.43 M epichlorohydrin (Fluka) and 0.17 M pyridine (Fluka) in chloroform (Molar). Afterwards the slides were washed with chloroform and dried. For preparing hydrophylic epoxy surface the silanized slides were incubated for 2 h with 70 ml solution of 0.43 M 1,4-butanediol diglycidyl ether (Fluka) in the presence of 0.2 g NaOH in ethanol (Molar). Afterwards the slides were washed with chloroform and dried.

Spotting onto activated surface

Spotting was performed using a pin-tool based spotting robot MicroGrid Total Array System (BioRobotics, UK) or pipetting manually. When using automated spotting 1-10 nl was introduced to the surface using splitted or solid pins, respectively. When applied manually 1 or 0,5 µl of solution was introduced to the surface. After spotting the slides were incubated in a humid chamber for 2 hours. In comparison experiments the commercially available slides were treated following the manufacturers instructions. The slides were stored at room temperature in the dark.

The pH characteristics of immobilization to the novel acryl derivatized surface

The optimal parameters of immobilization were determined using fluorescently labeled 5`-Cy5 oligonucleotides as subjects of anchoring. Spotting was carried out manually in 0.5 µl aliquots. Two dilutions of oligonucleotide solution, 0.25 µM and 0.062 µM respectively were introduced to the surface at seven pH values (pH 4, pH 5, pH 6, pH 7, pH 8, pH 9, pH 10) in 0.1 M sodium-phosphate buffer. The slides were incubated for 2 hours in a humid chamber and UV irradiated with a UV Crosslinker (700 mJ, Ultra Lum), then washed with deionized

water extensively and dried. The slides were scanned with a ScanArray Lite (GSI Lumonics, Billerica, USA) microarray scanner at 633 nm.

Mutation detection by hybridization

Oligonucleotides having 0, 1, 2, or 3 nucleotide sequence alterations in middle positions, were arrayed on the surfaces at 50 μM concentration. Arrays were hybridized with complementary Cy5-labeled oligonucleotide (0.1 μM) for 2 hours then washed, dried and scanned (see above). The applied oligonucleotide sequences are presented in Table 1.

Hybridization detection of PCR products

Different species-specific, random sequenced (negative control) and Cy5-labeled control oligonucleotides (Table 1) were printed on the acryl derivatized dendrimer like surface and on a commercially available aldehyde coated support (ArrayIt) using solid pins and BioRobotics spotter. Specific PCR products were hybridized for 2 hours to the arrays in 20 μl Huntsman hybridization buffer (50% formamide, 5x SSC, 0.1% SDS, 100 μg/ml salmon sperm DNA). The arrays were washed, dried and scanned as described above. Sequences for human cytomegalovirus (HCMV) were designed according to HCMV major immediate-early protein (IE) gene, (Accession number: M21295). PCRs were carried out by using an unmodified forward and a Cy5-end-labeled reverse primer (Table 1) with the following protocol: 360 sec heat start, 45 cycles of denaturation at 95^{o}C for 30 sec, annealing at 58^{o}C for 30 sec and extension at 72^{o}C for 40 sec. PCR products were analyzed with agarose gel-electrophoresis and ethidium-bromide staining. PCR products were denatured with incubation at 95^{o}C for 6 minutes before applying onto the arrays. Both the acrylic-based and triamino-based chemically modified surfaces were tested in hybridization experiments.

Protein immobilization

1 μl of 0.5 mg/ml Alexa Fluor 546 labeled streptavidin (Molecular Probes, Leiden, The Netherlands) solution in pH 10 phosphate buffer was introduced to the surfaces. The same protein solution without pH buffer was also spotted. Slides were incubated for 30 minutes in a humid chamber and washed with water then 1x SSC, 0.15% SDS for 5 minutes. Slides were scanned at 543 nm after each washing step.

Mimicking surface immobilization

In order to determine the way biomolecules are immobilized on the acryl derivatized, surface a model experiment was designed. Saturated aqueous solutions of nucleosides (cytidine, adenosine, guanosine, thymidine) in 10 X excess were incubated with acrylamide at 37 ˚C. After 2 hours the reaction mix was UV irradiated with 2 x 700 mJ in a UV crosslinker (Ultra Lum), and analyzed by reverse phase HPLC and mass spectrometry.

HPLC analysis.

HPLC was performed on a HP1100 instrument with the following conditions: Lichrospher RP select B column, 60 Å, 250 x 4 mm (Merck, Budapest, Hungary); detection at 260 nm; flow rate, 1 ml/min; eluent A, 0.1 M aq. TEAC (pH 7.0); eluent B, 0.1 M aq. TEAC (pH 7.0): acetonitrile 1:4 gradient, 0-30 % B in A in 30 min.

Mass Spectrometry

For mass spectrometric measurements a Finnigan TSQ 7000 tandem mass spectrometer equipped with a microelectrospray ion source was used with the previously described protocol (26).

Small molecule microarrays

Anchoring

Biotin and benzamidine with and without linker arm were printed on the developed surfaces at 6 concentrations (20 mM, 4 mM, 2 mM, 0.8 mM, 0.2 mM, and 0.1 mM) to determine optimal immobilization concentration. Test chemical arrays were prepared containing 140 molecules from combinatorial, parallel synthesis. In order to achieve comparable immobilization among the different molecules each species carried a spacer-arm with an amino functional group. The molecules were printed manually and with a printing robot. Each molecule is arrayed from a 20 mM solution in DMF.

Among the immobilized molecules were biotin and benzamidine that are substrates of streptavidin and trypsin, respectively. Both the acrylic-based and triamino-based chemically modified surfaces were tested in anchoring small chemical compounds.

Affinity experiments

For affinity expriments Alexa Fluor 555-labeled streptavidin (Molecular Probes, Leiden, The Netherlands) and Alexa Fluor 647-labeled trypsin were used.

Labeling of trypsin

30 µl of 1 µg/µl Alexa Fluor 647 carboxylic acid succinimidyl ester (Molecular Probes, Leiden, The Netherlands) solution in DMF containing 1 % diisopropylethyl-amine and 6 µl of 10 µg/µl trypsin solution containing 0.5% TFA were incubated at room temperature for 1 hour. To the reaction mixture 100 µl 20 mM Tris pH 7.0 was added. The mixture was loaded onto a Millipore centrifuge column (3000 and 10000 Da cutoff) and centrifuged for 20 minutes at 10.000 rpm. Afterwards 200 µl 20 mM Tris pH 7.0 was added and the column was centrifuged again for 20 minutes at 10.000 rpm. The process was repeated until the overflow was colorless. The labeled trypsin was redissolved in 200 µl PBS.

Development and scanning of microarrays

Printed microarrays were blocked with 1X SSC, 0.2% SDS, and 1% BSA solution for 30 min at 42 ˚C, then washed thoroughly with deionized water and dried. The spots were developed with fluorescently labeled trypsin or streptavidin (app. 0.28 µg/µl) under glass coverslips for 2 hours at room temperature in a humid chamber in a total volume of 20 µl. Afterwards the coverslips were removed in PBS solution then the slides were washed with PBS, 1x SSC and 1x SSC and 0.1% SDS for 5 min each. Finally the slides were rinsed with deionized water and dried.

The slides were scanned with a ScanArray Lite (GSI Lumonics, Billerica, USA) microarray scanner at 543 nm for Alexa Fluor 555 and at 633 nm for Alexa Fluor 647 labeling.

Construction of Microarrays

In labeling comparison experiments mouse VIB (Leuven, Belgium) slides were used. The slides comprised of a total of 4596 PCR amplified cDNA fragments from sequenced verified IMAGE clones (Mouse Gem I, Incyte, USA).

PCR amplified cDNA fragments were spotted in duplicate on aminoalkyl-silane treated microscope slides (Silane-PrepTM Slides, Sigma) using a MicroGrid Total Array System printer (BioRobotics, Cambridge, UK). The cDNA clones were obtained from mixed libraries, cloned in pBluescript SK II (-) (New England Biolabs, Hertfordshire, England) or pGEM

(Promega) plasmids with standard cloning techniques. The cDNA inserts were amplified with vector specific primers, purified with MultiScreen-PCR plate (Millipore), resuspended in 50% dimethylsulfoxide/water and loaded into a 384-well format microtiter plate. To test produced reference RNA alfalfa, rat and human microarrays were manufactured in this fashion. Alfalfa arrays represented 1600 genes, while rat and human arrays comprised of 3200 PCR fragments spotted in duplicate.

Slides were blocked just before hybridization in 3.5%SSC, 0.2%SDS, 1%BSA for 10 minutes at 60^{o}C.

RNA samples

Comparison experiments

To avoid variability due to RNA preparation, we used highly purified commercially available poly(A) and total RNA from Clonetech (Becton Dickinson, Belgium) and Ambion (USA). All experiments were done with RNA from mouse adult spleen and mouse adult testis.

Direct labeling of RNA during reverse transcription, (STD)

First-strand cDNA probes were generated by reverse transcription of 2 µg poly(A) RNA using an anchored oligo-dT (d-T_{25}-dA/C/G) primer (0.4µM; Genset, France), 0.1 mM d(G/T/A)TPs, 0.05 mM dCTP (Amersham Pharmacia Biotech, UK), 0.05 mM Cy3-dCTP or Cy5-dCTP (Amersham Pharmacia Biotech, UK) 1x first strand buffer, 10 mM DTT and 200 Units of SuperScript II (Life Technologies) in 20 µl total volume. The RNA and primers were denatured at 75^{o}C for 5 minutes and cooled on ice before adding the other reaction components. After 2 hours incubation at 42^{o}C, mRNA was hydrolyzed in 250 mM NaOH for 15 minutes at 37^{o}C. The sample was neutralized with 10 µl of 2 M MOPS and purified using Qiaquick columns (Qiagen).

Linear amplification by in vitro transcription, (IVT)

Antisense RNA amplification was performed using a modified protocol of *in vitro* transcription as published by Barry (5) and Eberwine (46, 55). For the first strand cDNA synthesis, 3 µg of total RNA was mixed with 2 µg of a HPLC-purified anchored oligo-dT + T7 promoter (5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-T_{24}(ACG)-3`) (Eurogentec, Belgium) 40 units of RNAseOUT (Life Technologies, Belgium)

and 0.9M D(+)trehalose (Sigma Belgium) in a total volume of 11µl, and heated to 75^{o}C for 5 minutes. The following was added to this mixture to give a final 20 µl total volume: 4 µl 5x first strand buffer (Life Technologies, Belgium), 2 µl 0.1 M DTT, 1 µl 10 mM dNTP mix, 1 µl 1.7 M D(+)trehalose (Sigma Belgium) and 1 µl, 200 Units of SuperScript II (Life Technologies). The sample was then incubated in a Biometra-UnoII thermocycler at 37^{o}C for 5 minutes, 45^{o}C for 10 minutes, 10 cycles at 60^{o}C for 2 minutes and at 55^{o}C for 2 minutes. To the first strand reaction mix, 103.8 µl water, 33.4 µl 5x second strand synthesis buffer (Life Technologies), 3.4 µl 10 mM dNTP mix, 1 µl of 10U/µl *E.coli* DNA ligase (Life Technologies), 4 µl 10 U/µl *E.coli* DNA Polymerase I Life Technologies,) and 1 µl 2U/µl *E.coli* RNAse H (Life Technologies,) was added, and incubated at 16^{o}C for 2 hours. The synthesized double-stranded cDNA was purified with a Qiaquick kit. Antisense RNA was synthesized in total volume of 20 µl using a AmpliScribe T7 high yield transcription kit (Epicentre Technologies, USA) according to the manufacturer's instructions. The RNA was purified with a RNeasy purification kit (Qiagen). From this a total of 3 µg of amplified RNA (aRNA) was labeled and purified using random nonamers in the above standard protocol (Genset, France).

Exponential amplification by SMART cDNA synthesis and PCR, (SMART)

SMART method is based on intrinsic terminal transferase activity of a point mutant Rnase H-reverse transcriptase. When the RT reaches the end of mRNA template, it automatically adds a stretch of cytosine-rich sequence to the end of newly synthesized strand of cDNA. An oligonucleotide having guanosine strech at its 3'-end could hybridize to the cytosine stretch, creating an extended template for the RT. cDNA synthesis continues to the end of this oligomer. Second-strand cDNA synthesis is then easily accomplished using PCR with the guanosine extended primer and an oligo(dT) primer.

50 ng of total RNA was reverse transcribed in 20 µl according to the manufactures protocol of the SMART cDNA synthesis kit (Clontech, Becton Dickinson) with an anchored poly(dT) primer ("CDS") and a SMART II primer (Clontech, Becton Dickinson). 3 µl of the RT reaction was added to 100 µl PCR mix (1x Advantage 2 PCR buffer, 0.2 mM dNTPs, 30 nM PCR primer, 1x Advantage 2 Polymerase mix containing 40 pmoles CDS and SMART PCR primers. Amplification was done in a Biometra-UnoII thermocycler at 95^{o}C for 1 minutes; 17-25 cycles at 95^{o}C for 25 seconds, at 65^{o}C for 40 seconds, and at 68^{o}C for 6 minutes. To determine the optimal number of PCR cycles, 6 µl aliquots were removed from the reaction every second cycle after 17 cycles, and analyzed on a 1.5 % agarose gel. After optimization

we performed two 100 µl PCRs using the same parameters and optimal number of cycles to get amplified cDNA. After purification with Qiaquick purification kit, the products were labeled in two 60 µl PCRs using asymmetric amplification containing 1x Advantage 2 PCR buffer, 0.2 mM d(A/T/G)TPs, 0.04 mM dCTP, 0.05 mM Cy3-dCTP, or Cy5-dCTP, 60 nM dT_{25}-(A/C/G) primer, 1x Advantage 2 Polymerase mix in a Biometra-UnoII thermocycler at 95°C for 1 minutes; 10 cycles of 95°C for 25 seconds, 50°C for 40 seconds, 62°C for 3 minutes and 68°C for 5 minutes. The amplified and labeled probes were purified using Qiaquick columns. Usually sufficient labeled probe was obtained for two experiments.

Reference RNA production

RNA extraction, RNA pools

For production of rat reference RNA, commercially available total RNA were pooled. 5 µg RNA from each of the following tissues were mixed: liver, brain, thymus, heart, lung, spleen, testicle, ovary, and kidney. 35 µg of rat embryo total RNA was added to this mixture. All of the RNA were purchased from Ambion. For human reference RNA 60 µg Universal Human Reference RNA (pooled from 10 different cell lines representing different tissues, Stratagene) was mixed with 5 µg human heart total RNA (Ambion), 5 µg human fetal brain total RNA (Clontech, Palo Alto, CA), 5 µg human thyroid total RNA and 5 µg total RNA from human carotid tissue. Total RNA from thyroid and carotid tissue were purified with NucleoSpin® RNA II extraction kit (Macherey-Nagel, Düren, Germany). For alfalfa reference RNA 14 µg of total RNA from the following tissues were pooled: flower, leaf, root, seed, germ. This RNA was mixed with 10 µg of total RNA prepared from protoplasts. For RNA purification FastRNA® kit (BIO 101, Vista, CA) was used. The quality of the extracted RNA was assessed by gel electrophoresis, as well as by OD_{260}/OD_{280} ratios.

Reference cDNA synthesis

33 µl pooled total RNA (80 µg) from different organisms was reverse transcribed in a total volume of 60 µl. For synthesis of antisense reference RNA, total RNA was mixed with 3 µl (100 pmole/µl) $T7T_{25}V$ and 3 µl (100 pmole/µl) FOR primers, and 3 µl (20 Unit/µl) RNAsin (Fermentas, Vilnius, Lithuania), heated to 75°C for 5 min and cooled on ice. To this mixture, 12 µl 5x first strand buffer (Fermentas), 3 µl 10 mM dNTP mix and 3 µl, 200 Units/µl of RNAse H (-) point mutant M-MLV reverse transcriptase (Fermentas) were added, and incubated at 42°C for 2 hours. For production of sense reference RNA the same protocol is

used for cDNA synthesis, but $REVT_{25}V$ and T7FOR primers were used instead of $T7T_{25}V$ and FOR primers. After cDNA synthesis, 240 µl TE (10 mM Tris-HCl, 0.2 mM EDTA pH 7.5) was added.

Exponential amplification of reference cDNA

0.5 µl from the first strand cDNA synthesis reaction mix was amplified in a total volume of 100 µl PCR with $REVT_{25}V$ and T7FOR, or REVT and T7FOR primers in case of sense reference RNA production, or $T7T_{25}V$ and FOR, or T7T and FOR primer pairs in case of antisense amplified RNA production. The reaction mix contained 50 nM final concentration of each primers, 1X PCR buffer (Amersham-Pharmacia Biotech.), 4 U Taq polymerase (Amersham-Pharmacia Biotech.) and 200 µM dNTP. Amplification was done under the following conditions: initial denaturation at 95^{o}C for 1 min; 22 cycles of denaturation at 95^{o}C for 35 sec, annealing at 58^{o}C for 40 sec, and extension at 68^{o}C for 4 min. PCR products were purified with a PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, but eluted twice with 40 µl of 1/5 Elution buffer. The concentration of the eluted, double-stranded DNA was determined by UV absorbance measurement at 260 nm. The usual yield was approximately 2 µg DNA from 100 µl PCR. 40 PCRs were done from each cDNA synthesis reaction mix from the different pooled samples. The purified PCR products were pooled, vacuum concentrated, dissolved in DEPC treated water to have final concentration of 500 ng/µl.

Production of reference RNA using in vitro transcription

20 µg of PCR amplified cDNA template was transcribed in a total volume of 200 µl using Ribomax Large Scale RNA Production System (Promega) according to the manufacturer's instructions. The RNA was diluted with 0,3 ml DEP-treated water and purified using 15 columns from the RNeasy purification kit (Qiagen), and measured spectroscopically. The usual yield was 1.5-2 mg of amplified RNA which corresponds to approximatelly 150-200 x amplification of the PCR amplified DNA. For long term storage RNA was aliquoted (aliqouts contained 30 µg of amplified RNA) in the presence of RNAsin (final concentration of 0.1 Unit/µl), dried using speed vacuum and stored at –80 ^{o}C.

Probe preparation and labeling

2.5 µg of amplified reference RNA was labeled with Cy3 during reverse transcription using 0.4 µM random nonamer as primer, 0.1 mM d(G/T/A)TPs, 0.05 mM dCTP (Amersham

Pharmacia Biotech, UK), 20 Units of RNAsin (Fermentas), 1x first strand buffer, 200 Units of RNAse H (-) point mutant M-MLV reverse transcriptase (Fermentas), and 0.05 mM Cy3-dCTP (Amersham Pharmacia) in 20 µl total volume. The RNA, primer and RNAsin were denatured at 75°C for 5 min and cooled on ice before adding the remaining reaction components. After 2 hours of incubation at 37 °C, the heteroduplexes were denaturated and the mRNA was hydrolyzed with NaOH (250 mM final concentration) for 15 min at 37°C and neutralized with 10 µl of 2 M MOPS (pH 6.0). The labeled cDNA was purified with a PCR purification kit (Qiagen) according to the manufacturer's instructions, but eluted twice with 40 µl of 1/2 Elution buffer. The probes were concentrated by speed vacuum and dissolved in 20 µl hybridization buffer (50 % formamide, 5x SSC, 0.1 % SDS, 100 µg/ml salmon sperm DNA).

Array hybridization and post-hybridization processes

In labeling comparison experiments the probes were resuspended in 30µl hybridization solution (50 % formamide, 5x SSC, 0.1 % SDS, 100 µg/ml salmon sperm DNA). To block hybridizations of polyA/T tails of the cDNA on the arrays (poly-dA for STD and SMART, or poly-dT for the IVT hybridizations) 1 mg/ml mouse COT DNA (Life Technologies) was added to the mixture.

For prehybridization of labeled reference cDNA, 2 µl of 1 mg/ml Lambda DNA (Fermentas) and 2 µl of 1 mg/ml salamon sperm DNA (Fermentas) were added. All probe mixtures were incubated for 30 min at 42 °C after denaturation by heating for 5 min at 80 °C. Samples were loaded onto blocked arrays under 24 mm x 32 mm glass cover slips (Menzel-Glaser, Germany). To the edges of the coverslips DPX Mountant (Fluka) was poured in order to prevent evaporation. Slides were incubated at 42°C for 18 hours in a humid hybridization chamber. After hybridization the mountant was removed. Post-hybridization washing were performed for 10 minutes at 56°C in 1xSSC, 0.1% SDS, two times for 10 minutes at 56°C in 0.1xSSC, 0.1% SDS and for 2 minutes at 37°C in 0.1xSSC.

Scanning and data analysis

Arrays were scanned at 543 nm and 633 nm with 10 µm resolution using a ScanArray Lite (GSI Lumonics, Billerica, USA) microarray scanner.

The resulting images were quantified by using the software program SCANALYZE2 (12). Each spot was defined by manual positioning of grid circles over the image. The average pixel intensity and the local background of each spot were determined. Genes were labeled ON or OFF according to a predetermined intensity threshold. The threshold was set at 1.5 times of the local background intensity. This cut off level was determined from the "CH1GTB2" values (obtained from the ScanAlyze2 software, Eisen, M., 1999, Stanfrod Univ., Stanford, CA, Ver. 2.32., www.microarrays.org/software.html), which corresponds to fraction of pixels in the spot greater than 1.5 times the background. Only if the mean spot intensity was greater than this threshold a spot was considered significantly above background. A measure (MRAT, denotes the median of the set of background-corrected single pixel intensity ratios of the two fluorescence channels for all pixels within the spot) was determined (14). This average expression ratio for all genes on the array was normalized to 1.0. Those results were excluded where the replicate spots from a different site of the same array or results from replicate experiments were significantly different. Data analysis and visualization of scatter images were performed with Microsoft EXCEL software.

Single-gene RT-PCR and Southern-blotting

Semi-quantitative RT-PCR was performed similarly as described in Huntsman et al. 1998 (24). Briefly, 100 ng mouse spleen and testis mRNA was reverse transcribed using specific primer complementary to the 3'-end of the coding region of the desired gene with the Superscript II system in total volume of 30 µl (Life Technologies). Genespecific primers used for the amplification of the desired clones with 0.5 µl RT mix as template in total volume of 50 µl. To avoid overamplification, the optimal number of PCR cycles was determined by agarose gelelectrophoresis and ethidium bromide staining (18-26 cycles were performed according to the abundance of the appropriate mRNA). PCR products were separated with electrophoresis using 2 % agarose gel and transferred onto nylon membranes (Amersham Pharmacia Biotech, UK) by cappliary action. ^{32}P-labeled probes were synthesized with PCR using plasmids having the corresponding cDNA insert that were spotted on the array with M13 forward and reverse primers in 100 µl of total volume, containing; 1X PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.5), 0.1% Triton X-100), 1.5 mM $MgCl_2$, 125 µM each dNTP, 5 µCi [α-^{32}P]dCTP (3000 Ci/mmol), 50 pmol each primer, and 3 U Platinum Taq polymerase (Life Technologies). Labeled PCR fragments were purified with Qiaquick, resuspended in hybridization buffer, denatured and applied onto the membrane.

Hybridizations and washing steps were done using standard protocols (14). Membranes were autoradiographed using phosphorimager (Molecular Dynamics) and intensity ratios were calculated with ImageQuant 4.2 software. ^{32}P-labeled products were normalized to amplified levels of the ubiquitously expressed glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

RESULTS AND DISCUSSION

Surface chemistry

In microarray technology several problems and challenges are to be solved and faced, especially when one is starting from scratch. Array technology started from macroarrays (reverse dot blots) where tens or hundreds of biological samples were deposited on the surface of a membrane, and evolved into microarray technology where tens of thousands of diverse samples are immobilized in different ways onto diverse surfaces. Several methods are available to permanently attach molecules onto a surface. The mode of attachment depends on the physical and chemical properties of the surface applied and the molecules to be immobilised. The interaction between a surface and a molecule is either physical (absorption) or chemical (secondary or primary bonding). The nature of interaction determines the strength of immobilization in most cases. Chemical immobilization through covalent bonds is certainly the most stable way of anchoring. In order to couple molecules through chemical bonding to a surface, either the surface, the molecules or both have to be activated (derivatised). Numerous examples are described for either method in the literature (49, 54, 62). We have chosen surface modification to achieve permanent immobilization.

6 different chemical surfaces having a 3D-like linker system for biomolecule immobilization are described in the thesis. The surfaces were compared to previously described immobilization strategies.

The surfaces can be sorted into two groups according to their structure.

First group includes the dendrimer-like structures. The multiplication of active sites is achieved through the incubation with TEPA. This reaction step theoretically increases the number of active sites by a factor of four (Figure 2A). The second group includes the triamino-based surfaces. The increase of active sites is achieved through the application of 3-[2-(2-Aminoethylamino)ethylamino)propyl-trimethoxysilane, which introduces three amine functions. In this case the multiplying factor is three (Figure 2B).

The created branching structures also provide good accessibility of the immobilized molecules in hybridization and affinity experiments.

Optimal binding parameters were determined with the anchoring of labeled oligonucleotides. The application of labeled oligonucleotides provides the ease of direct detection and determination of efficiency of anchoring. Immobilization occurred at all investigated pH values, but more effective anchoring was carried out at higher pH values, revealed by harsh

(more stringent) washing conditions. The optimal pH value for immobilization was pH10 (Figure 3). In all later experiments the pH was adjusted to 10.

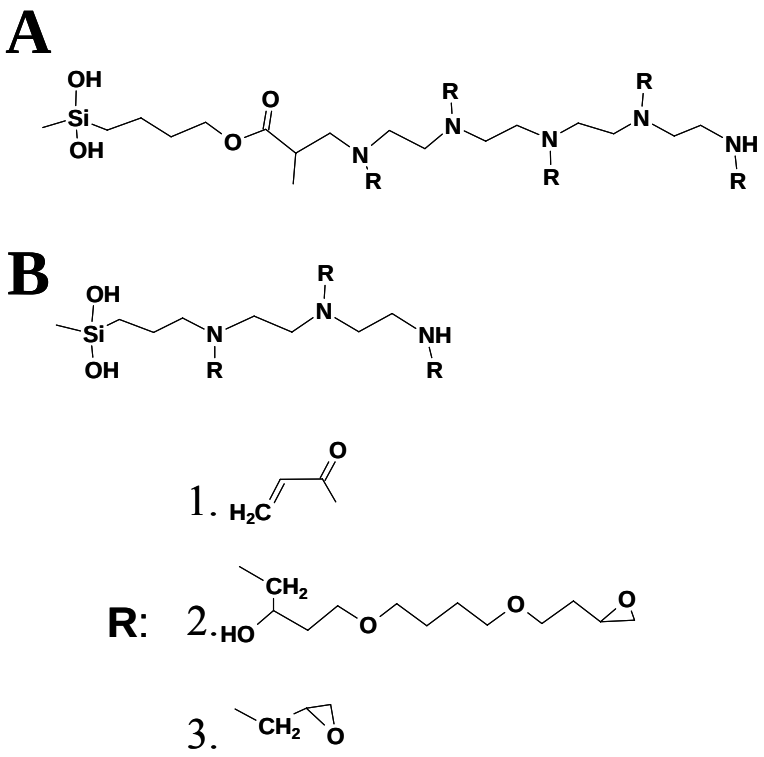

Figure 2. Chemical structure of the developed solid supports and the active linker arms (A: dendrimer like linker system. B: triamino based linker system).

Advantage of the system is that processing of the microarrays includes only temperature-controlled incubation, after printing. Reductive coupling step is not necessary, as it is when using e.g. carbonyl-activated surfaces (15). Reduction may interact with reduction-sensitive functional groups linked to nucleic acids (different fluorescent dyes linked to one or both ends of the nucleic acid). Therefore, oligonucleotides having fluorophores at either or both ends can be easily attached using our protocol.

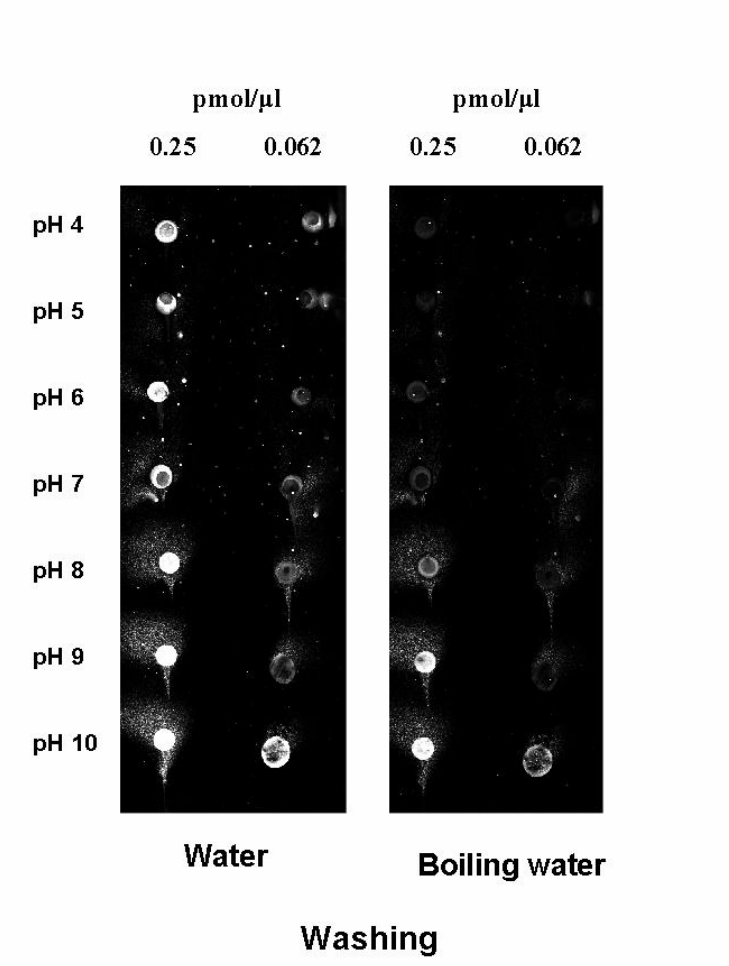

Figure 3. pH characteristics of immobilization to the acrylic surface

Oligonucleotide hybridization and the mechanism of anchoring

The hybridization properties of the surfaces were asessed and compared to commercially available solid supports. 17 and 22 nucleotides long oligonucleotides were spotted. The longer oligonucleotides carried 0, 1, 2 and 3 nucleotide alterations in middle positions (AREV, AREV-M1, AREV-M2, AREV-M3). Shorter oligonucleotides carried 0, G→C, G→A, G→T and 2 alterations in middle positions (AR17, AR17-MC, AR17-MA, AR17-MT, AR17-M2). Complementary Cy5-labeled oligonucleotide was hybridized to the arrays. All arrays showed high hybridization signals.

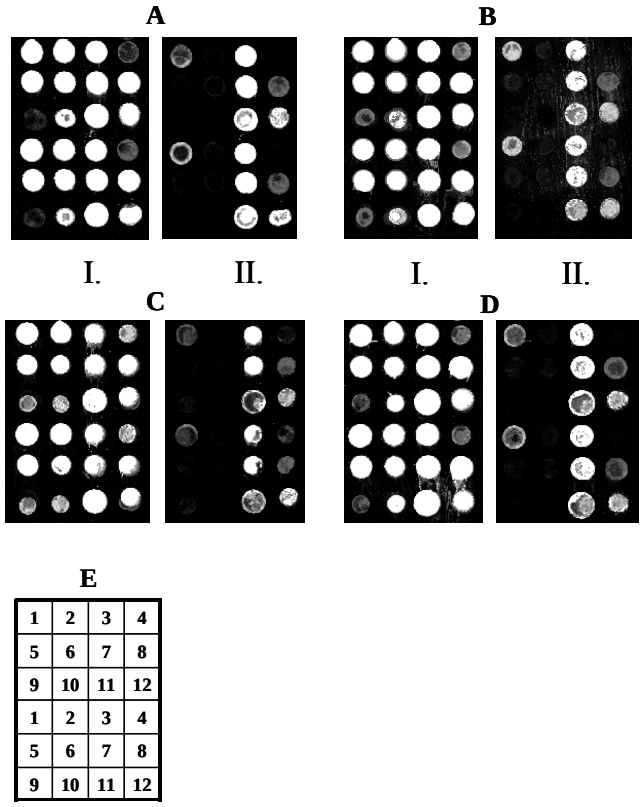

Figure 4. Fluorescently labeled oligonucleotide hybridized to arrays with oligonucleotides having perfect match and altered sequences. A: Dendrimer surface with acryl functions, B, C, D: Triamino surface with hydrofobic epoxy, acryl and hydrophilic epoxy functions, respectively. E: layout of the printed oligonucleotides. 1.: AR17, 2.: AR17-MT, 3.: 3'A-AREV, 4.: Cy5-REV, 5.: AR17-MA, 6.: AR17-MC, 7.: AREV-M1, 8.: AREV-M3, 9.: Cy5-REV, 10.: AR17-M2, 11.: AREV-M2 12: Cy5-AREV.
I.: Wash 1. (1X SSC for 5 min), II.: Wash 2. (0.1x SSC for 5 min)

The longer oligonucleotides had amino modification at their 3`-end. The shorter, native oligonucleotides gave high intensity and reproducible signal and could differentiate between perfect match and altered sequences, while longer amino modified oligonuceotides were unable to differentiate between perfect match and 1 alteration sequences, only between oligonucleotides with perfect match and 2 alteration sequences (Figure 4). Spot size varied between supports depending on hydrophylic character. Among the developed supports the dendrimer like, acryl-derivatized support performed slightly better than the others. Using this support the highest intensity differencies could be obtained - by hybridizing a labeled oligonucleotide without mismatch - between an altered sequence bearing one base mismatch in the middle position and an attached complementary sequence.

The binding efficiency of chemically modified and unmodified oligonucleotides was comparable. Applying unmodified oligonucleotides results in greater cost efficiency in microarray production.

From these findings we concluded that 3`- or 5`-end modifications in oligonucleotides are not the only sites for immobilization. To determine the mechanism of anchoring, a model reaction was designed. Nucleosides (cytidine, adenosine, guanosine, thymidine) were incubated with acrylamide at 37 ˚C in aqueous solution. After 2 hours the reaction mixtures were analyzed by HPLC and mass spectrometry. In the HPLC chromatograms a new peak represented the product of the conjugation. To determine the properties of the conjugate the fraction containing the reaction product was analyzed by mass spectrometry (Figure 5).

All four bases reacted with acrylamide and the products were represented by a new peak on each spectrum with an m/z value corresponding to the mass of the acrylamide-nucleoside adduct. To evaluate the relationship between acrylamide and the nucleosides secondary (collision) spectras were taken (data not shown). In the spectras two new major peaks appeared. One corresponds to the sugar part of the nucleoside, the other to the acrylamide nucleobase adduct. Secondary spectras of the reaction products proved the acrylamide and the nucleosides formed covalent bonds.

From these findings we concluded that the immobilization occurs via the bases of the unmodified oligonucleotides in alkaline pH, supposedly through amino functions.

Oligo name	**Sequence**	**Description** *[length]*
AREV-M1	5’-CCTGTGT**T**AAATTGTTATCCGC-NH_2-3’	1 alteration [22]
AREV-M2	5’-CCTGT**T**TGAAATT**T**TTATCCGC-NH_2-3’	2 alterations [22]
AREV-M3	5’-CCTGT**T**TGAAATT**T**TTATCC**T**C-NH_2-3’	3 alterations [22]
3’A-AREV	5’-CCTGTGTGAAATTGTTATCCGC-NH_2-3’	Perfect match [22]
5’A-AREV	5’-NH_2-CCTGTGTGAAATTGTTATCCGC-3’	Perfect match [22]
AREV	5’-CCTGTGTGAAATTGTTATCCGC-3’	Perfect match [22]
Cy5-AREV	5’-Cy5-CCTGTGTGAAATTGTTATCCGC-3’	Perfect match [22]
AR17	5’-GTGAAATTGTTATCCGC-3’	Perfect match [17]
AR17-MC	5’-GTGAAATT**C**TTATCCGC-3’	1 alteration G -> C [17]
AR17-MA	5’-GTGAAATT**A**TTATCCGC-3’	1 alteration G -> A [17]
AR17-MT	5’-GTGAAATT**T**TTATCCGC-3’	1 alteration G -> T [17]
AR17-M2	5’-GT**T**AAATT**T**TTATCCGC-3’	2 alterations [17]
Cy5-REV	5’-GCGGATAACAATTTCACACAGG -3’	Complementary [22]
R2	5’- NH_2-GATCGATTAAGTTCCTCGTTCGC-3’	Random [23]
M1	5’- NH_2-GGCGCCTTTAATATGATGGGAGGA-3’	CMV probe1 [24]
M2	5’- NH_2-CCTTTCGAGGAGATGAA-3’	CMV probe2 [17]
MPF	5’-CGGCATAGAATCAAGGAGCACATGC-3’	CMV forward [24]
MPR	5’-Cy5-AAGGCTGAGTTCTTGGTAAAGAAC-3’	Cy5-CMV reverse [24]

Note. Nucleotides changed in altered sequences are denoted as bold underline characters.

Table 1. Oligonucleotides used in anchoring study

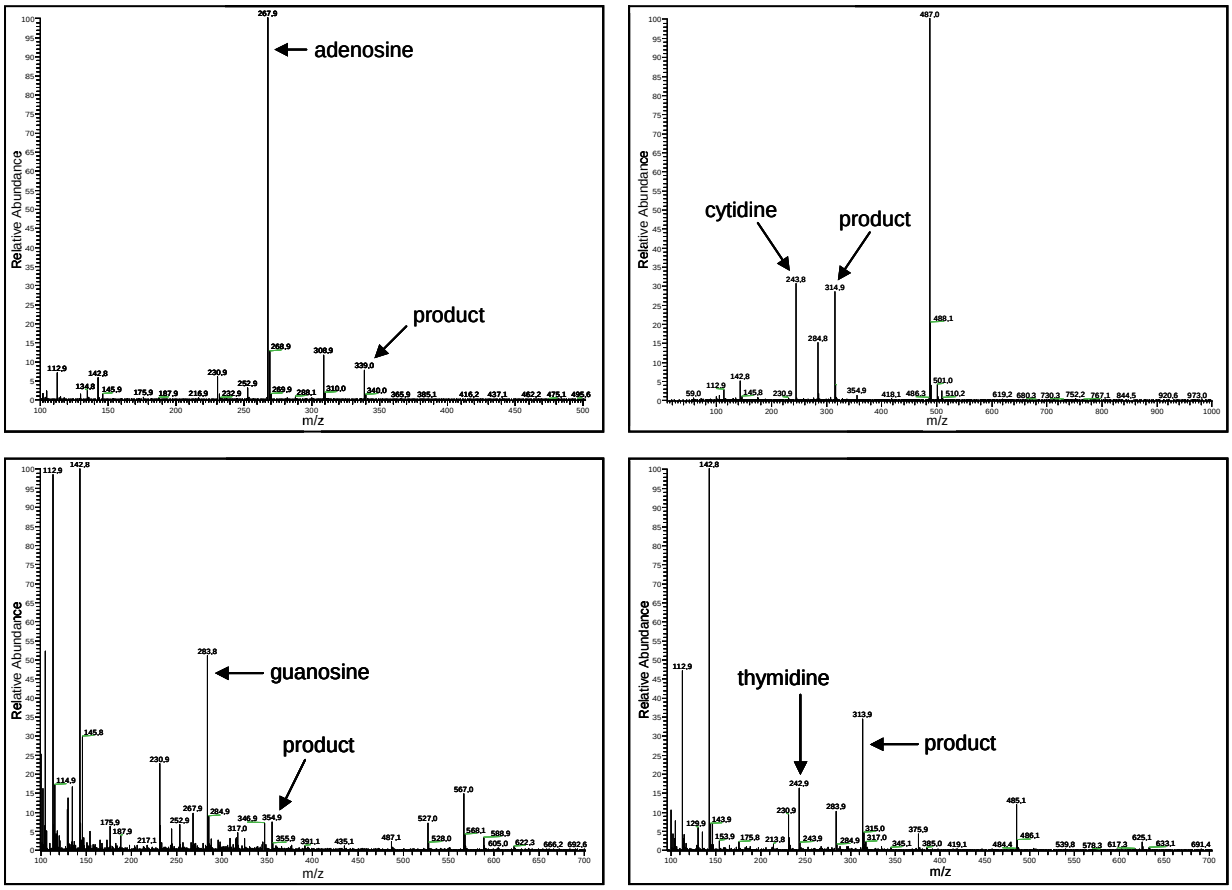

Figure 5. Mass spectras of the samples from four test reactions to evaluate immobilization. Product represents nucleoside-acrylamide adduct.

Detection of PCR products with immobilized oligonucleotides

To test the applicability of the developed solid supports as potential diagnostic tools in clinical molecular biology we studied the attached oligonucleotide - PCR product interactions. Sense (complementary to labeled reverse strand of the amplicon) HCMV-specific oligonucleotides with different length (17 and 24-mers), random sequences (negative control) and Cy5-labeled control oligonucleotides (Table 1) were printed on the acryl derivatized dendrimer-like surface and on a commercially available aldehyde coated support. After hybridization, washing and scanning the slides signal intensities were compared. Higher intensities could be obtained by using the dendrimer-like surface than using the simpler aldehyde-coated support (data not shown). Moreover, the spotted control oligonucleotides bearing fluorescent dyes could be visualized in only the acryl-surface, while no signal could be detected in the case of the aldehyde-coated slide, because of the reduction step after

immobilization. Surfaces based on triamino-silane starting linkers gave smaller signals then acrylic support, however exhibited higher intensities than the aldehyde-coated support.

Protein immobilization

DNA microarrays are efficient tools of describing biological systems at the level of transcription. The method detects mRNA abundance in a system at a certain time point. Although transcript levels are informative they do not always correlate with protein concentration, and posttranslational modifications, which may define functionality of a protein, are invisible for this technique. Functional protein microarrays seem to overcome this problem (21, 37). Similarly to DNA arrays, protein arrays comprise of immobilized proteins on solid surfaces.

Because all proteins have numerous free amino groups on their side chains and the developed linker systems are capable to anchor molecules with free amino functional groups, a simple experiment was carried out to test protein immobilization. The developed surfaces anchored the applied fluorescent-labeled protein at both applied pH values (pH 7, pH 10), but according to previous findings the binding was more effective at higher pH.

Small molecule arrays

Similarly to protein arrays, small molecule (or chemical) microarrays are efficient tools to investigate the proteome. From different approaches several techniques evolved in the field of investigation of protein-protein, protein-DNA and protein-small molecule interactions (33, 30, 37, 59). The latter interactions can be followed by small molecule microarrays, where tens of thousands of compounds e.g. from parallel combinatorial synthesis are immobilized on the surface of a microscope slide. The technique delivers the possibility of high throughput screening of combinatorial molecule libraries for possible lead molecules in drug discovery.

To optimize parameters of small molecule-protein interactions on solid surfaces, two common conjugate system, the biotin-streptavidin and benzamidine-trypsin pairs were selected and tested on all the chemically modified slides described before. Biotin and benzamidine were printed at 6 different concentrations on the surfaces. All slides had low background and spots corresponding to all 6 applied concentrations gave specific signals. Although spots corresponding to 20 mM and 4 mM concentrations had similar, high fluorescent intensity, the concentration for further experiments were set to 20 mM (Figure 6A). Using the developed

supports we created a test chemical array containing 140 molecules from combinatorial, parallel synthesis. The multiply-tethered, small molecules represent a diverse subset of a 240,000-member discovery library composed of pharmaceutically relevant structures (8, 9).

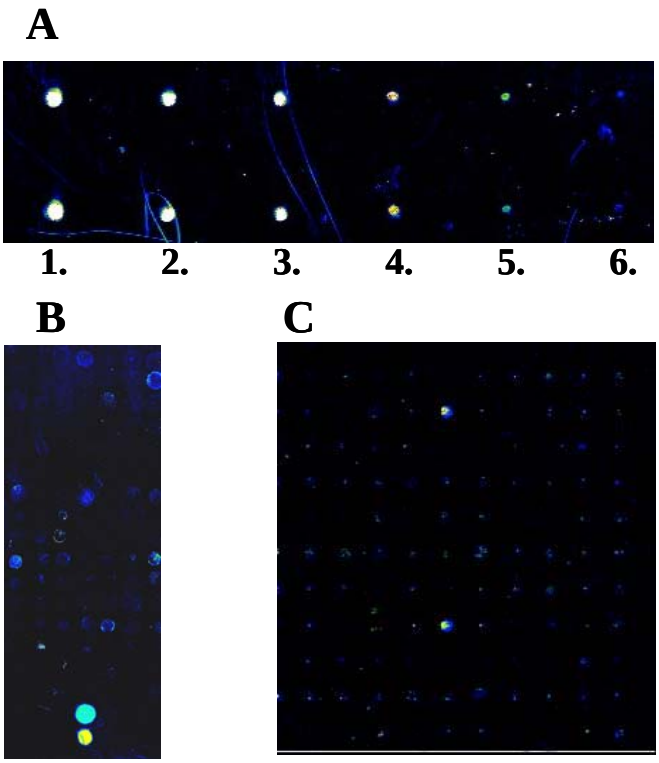

Figure 6. Small molecule arrays

A: Benzamidine was spotted onto the array at different concentrations (1. 10 mM; 2. 2 mM; 3. 1 mM; 4. 0.4 mM; 5. 0.1 mM; 6. 0.05 mM - meaning 1. 10 pmol; 2. 2 pmol; 3. 1 pmol; 4. 0.4 pmol; 5. 0.1 pmol; 6. 50 fmol amounts in the spotted area) and the array was incubated with Alexa Fluor 647-labeled trypsine and visualized after scanning.

B: Manually printed test array containing 140 small molecules. The array was developed with Alexa Fluor 555-labeled streptavidin. Illuminated spots represent anchored biotin.

C: Test array containing 140 small molecules printed with printing robot. The array was developed with Alexa Fluor 647-labeled trypsin. Illuminated spots represent anchored benzamidine.

In order to achieve comparable immobilization among the different molecules each species carried an amino functional group active toward the derivatized surfaces. The amino groups were situated on a linker arm that were to provide greater accessibility once anchored to the surface. Among the immobilized molecules were biotin and benzamidine that are substrates of streptavidin and trypsin, respectively. For affinity expriments Alexa Fluor 555-labeled

streptavidin and Alexa Fluor 647-labeled trypsin were used. Results show that arrays developed by the triamino-based chemistry showed low background after small chemical compounds were printed manually or with a printing robot after incubation with interacting fluorescent labeled proteins. The anchored molecules were accessible for interaction and protein specific signals were obtained (Figure 6B, 6C).

Comparison of labeling methods

The accuracy and sensitivity of DNA microarray analysis is a very important consideration in monitoring the transcriptome. Indeed, small changes in expression levels could be of great value in studying biochemical or other biological functions. Another challenge of microarray gene-expression analysis is often the limiting amount of RNA that can be obtained from small or limited biological samples. Thus we optimized two sample amplification techniques: a linear amplification based (IVT), and an exponential amplification based (SMART) method. The major adaptation for the IVT-linear amplification was the higher temperature during the first strand reverse transcription. This could be accomplished by adding trehalose to prevent the denaturation of Superscript II enzyme. This modification resulted in higher yields of first strand cDNA, possibly by eliminating secondary structure of the template. A typical yield of 10-20 µg amplified RNA was obtained starting from 3µg of total RNA, which corresponded to >200x amplification. Another critical component in the protocol was the purity of the oligo-dT-T7 primer. Only highly purified primer resulted in appropriate yields of cDNA, and we found it advisable to compare primers from different companies. For exponential amplification, we adapted the PCR amplification and labeling procedure from the SMART^{TM} PCR cDNA synthesis kit produced by Clontech. This technique does not use random primers and omits the ligation of sequences after second strand cDNA synthesis. Therefore, the reproducibility of the amplification was expected to be better than other PCR amplification approaches where contamination of genomic DNA, or poly(A)$^-$ RNA can be amplified as well, thus leading to nonspecific hybridization. After PCR amplification of the cDNA, the probe was generated by asymmetric PCR resulting in only one labeled strand. This leads to a considerable enhancement of the signal. The optimal number of PCR cycles had to be determined in every case. More than 10 % of the spots exhibited irreproducible intensity data (more than 2.5-fold differences between intensities from duplicate experiments) due to the negative effect of overcycling. We used 50 ng of total RNA as starting material, and the approximately 2000x amplification rate of SMART resulted in sufficient labeled probe for two hybridization experiments.

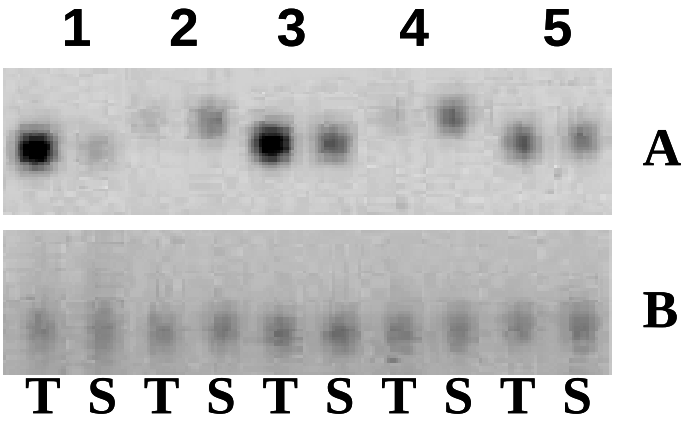

Figure 7. Autoradiograph of 12 genes analyzed by semiquantitative RT-PCR between testis (T) and spleen (S). Five of these genes showed inconsistent or contradictory expression ratios among the methods tested. Lane 1: AA049537, lane 2: AA050371, lane 3: AA058055, lane 4: AA175441, lane 5: W13718. As a control the expression of GAPDH gene is shown.

In this work we mainly focused on two questions: "Are amplification methods reproducible?" and "Do amplification methods introduce biases in relative transcript levels?". For the first question we compared SpleenCy5/SpleenCy3 ratio distributions between each method (Figure 8) and compared TestisCy5/SpleenCy3 replicate experiments within each method (Figure 9). For the second question, we compared directly the TestisCy5/SpleenCy3 of the amplification methods with the standard labeling (Figure 10, 11). In addition, 12 genes having ratios that were inconsistent or contradictory among the three methods were verified via quantitative RT-PCR (Figure 7).

Reproducibility was very high for STD and IVT but slightly lower for SMART. For SpleenCy5/SpleenCy3 hybridizations over 99% of the spots showed Cy5/Cy3 ratio that were within a 1.5-fold variation from the expected mean; for IVT this was 96% and for SMART 84%. Using standard and IVT protocols, around 0.3% of the clones have a ratio greater than 2 or less than 0.5, while in the case of SMART up to 1.5% of these outliers were observed.

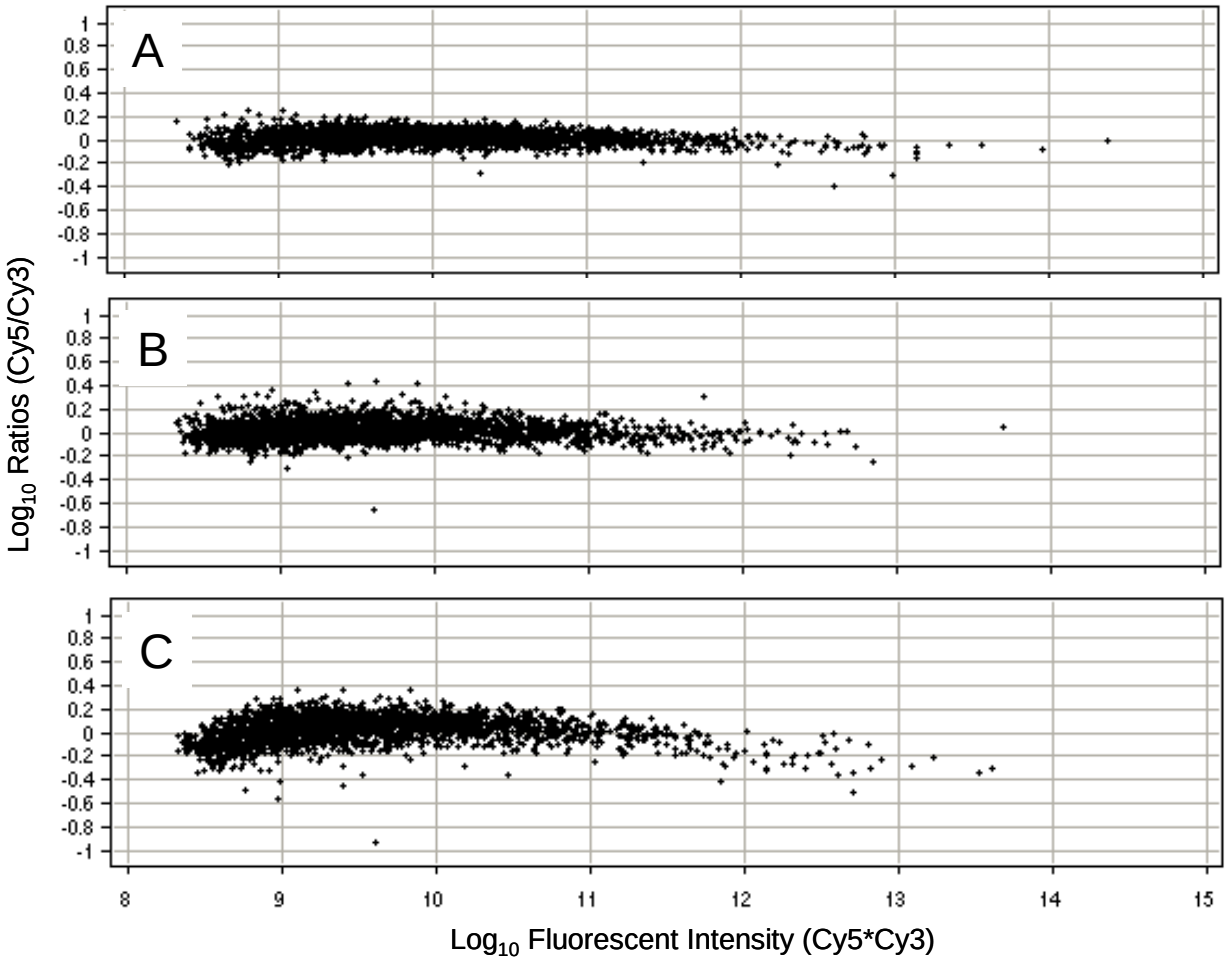

Figure 8. Reproducibility of spleen versus spleen microarray analysis performed by three different labeling methods. For each method, mouse spleen RNA was labeled with Cy5 and Cy3, separately, and hybridized together. The figure shows scatter plots of $\log_{10}$(Cy5/Cy3) ratios of all 9192 spots (2 replicate spots per gene) against $\log_{10}$(intensity of Cy3*Cy5). (A) Standard labeling starting from 2 µg poly(A) RNA; (B) IVT starting from 3 µg total RNA ; (C) SMART starting from 50 ng total RNA. Only ratios that had fluorescent signals significantly above background were considered for spots. No further selections were made

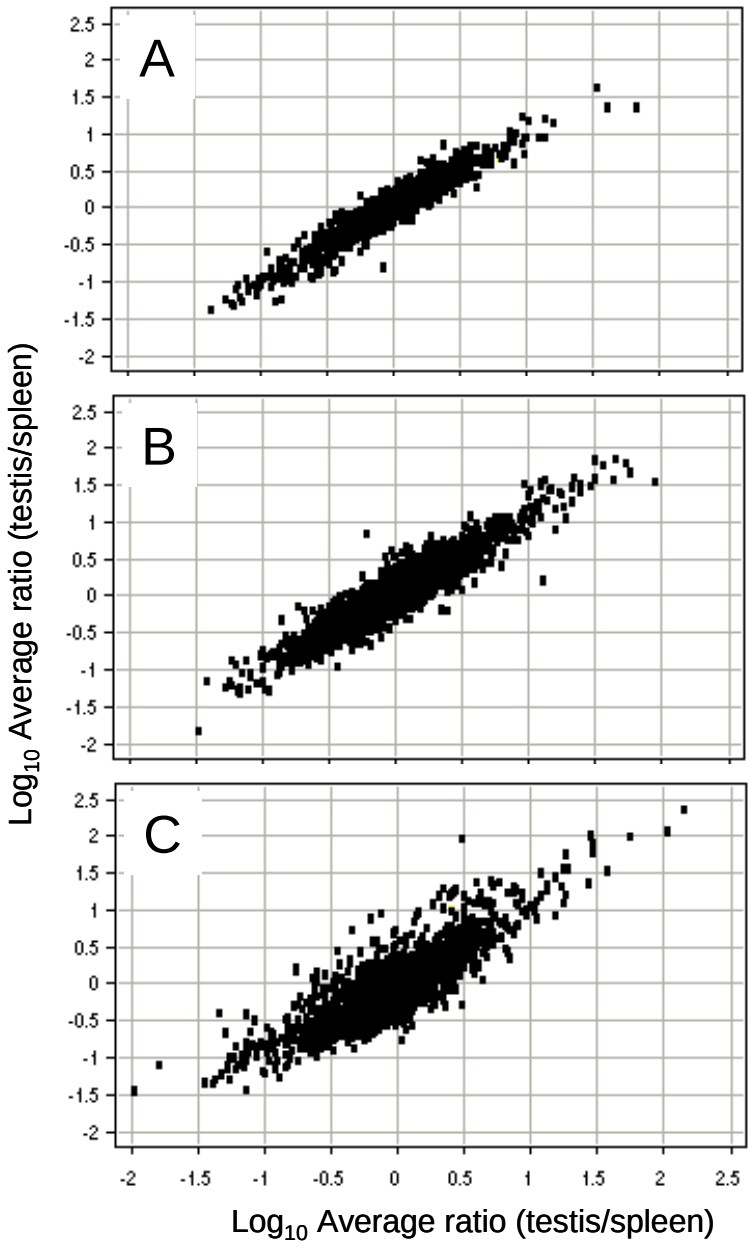

Figure 9. Reproducibility and preservation of the differential expression levels of testis versus spleen between replicate experiments for each labeling method. The figures show scatter plots of $\log_{10}$ average ratio (Testis/Spleen) against the same results of an independent replicate experiment within each labeling method. (A) Standard labeling (STD1 and STD2); (B) linear sample amplification (IVT1 and IVT2); (C) exponential sample amplification by SMART-PCR (SMART1 and SMART2). The only average values considered were those for which both replicate spots had fluorescent signals significantly above background and also had a less than 2 fold difference from one another. See table 2 for the number of genes used in this comparison.

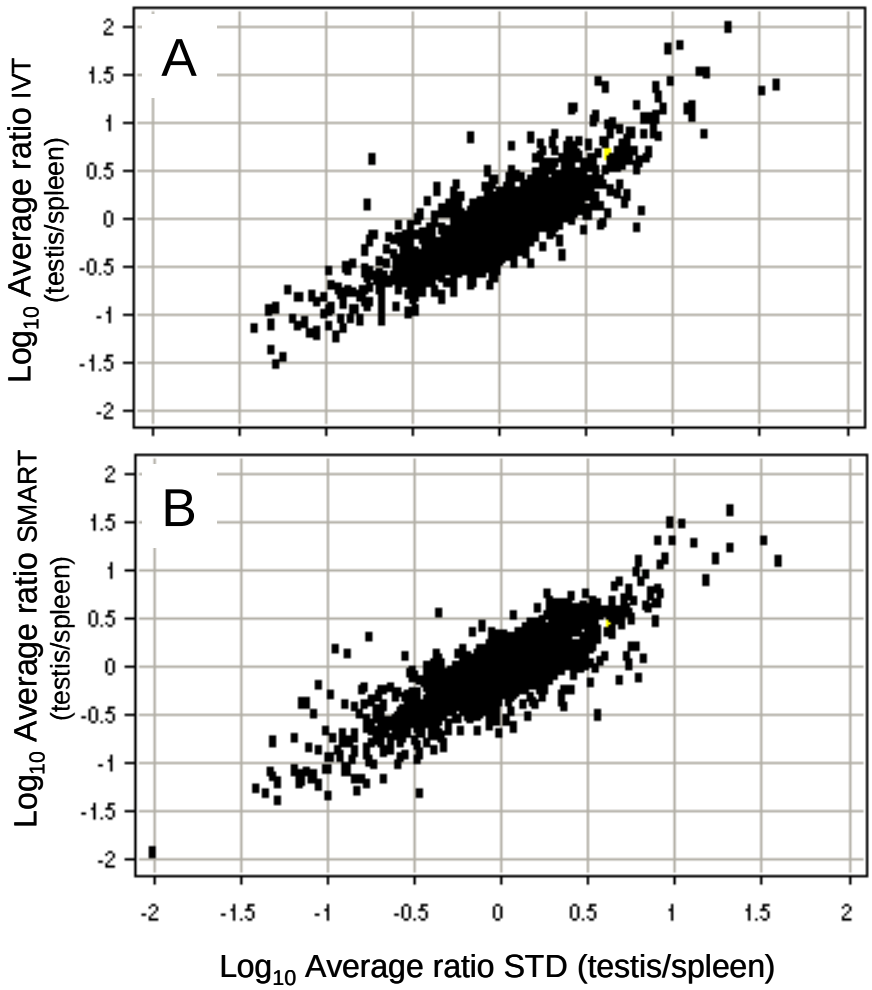

Figure 10. Reproducibility and preservation of the differential expression levels of testis versus spleen among labeling methods. The figures show scattergrams of $\log_{10}$ average ratio (Testis/Spleen) from STD1 labeling versus IVT1 (A) and versus SMART1 (B). The only average values considered were those for which both replicate spots had fluorescent signals significantly above background and also had a less than 2 fold difference from one another.

This could also be observed in spleen/testis hybridizations. These hybridizations were repeated for each method starting from labeling a different batch of RNA extracts from Clontech. The correlation coefficients for Cy5/Cy3 ratios between the repeats were high for all three methods (Table 2). However, using a Paired t-test, SMART replicates were statistically significantly different. Overall, SMART scored less on these comparisons as STD and IVT and was highly dependent on a careful optimization of the number of PCR cycles in each experiment.

Although reproducibility was high within each method, direct comparison between labeling strategies did reveal different testis/spleen ratios and different absolute fluorescent intensities for some genes. Comparing testis/spleen ratios of the amplification methods to STD again showed lower correlation coefficients than between repeats within each method (Table 2). The correlation coefficients between the methods ranged between 0.77 and 0.85 for IVT-STD and between 0.70-0.84 for SMART-STD. Correlation coefficients between IVT and STD were not statistically significant but they were between SMART and STD depending on the repeat. Focusing on the >2-fold differentially expressed genes, IVT and SMART, respectively, did not detect 20 and 23 % of the total number of differentially expressed genes measured by STD (Figure 11).

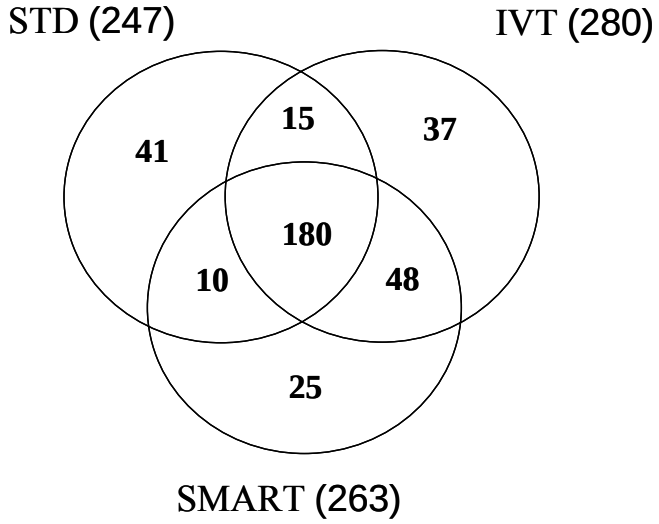

Figure 11. Venn Diagram of differentially expressed genes compared among standard labeling (STD), linear amplification (IVT) and exponential amplification (SMART). The total number of differentially expressed genes per method is given between brackets. A two-fold threshold allowing a 10% window determined the differentially expressed genes.

Otherwise, there were 16 to 33 more differentially expressed genes determined by the amplification methods. Another important observation was that absolute fluorescent intensities were different among the 3 different methods. This difference in absolute fluorescence was consistent among the repeat experiments and therefore likely to be dependent on the method used. However, it did not immediately affect the expression ratios as long as the same amplification method was used for all samples tested. Interestingly, also the

fraction of spots with fluorescent signals significantly above background was different among the various methods, being 12 to 16% higher for IVT and SMART.

Comparison*	Mean Difference	Number of Genes**	t-value	*p*-value	Correlation coefficient***
STD1-STD2	-0.065	1194	-1.360	0.174	0.95
IVT1-IVT2	0.620	1934	0.772	0.440	0.93
SMART1-SMART2	-0.361	1740	-3.771	0.001	0.85
STD1-IVT1	0.010	1253	0.111	0.911	0.84
STD2-IVT1	-0.001	1088	-0.005	0.996	0.85
STD1-IVT2	-0.172	1366	-1.296	0.195	0.78
STD2-IVT2	-0.167	1192	-1.414	0.157	0.77
Average STD-Average IVT#	-0.097	965	-1.097	0.273	0.83
STD1-SMART1	0.199	1243	4.436	0.0001	0.84
STD2-SMART1	0.262	1078	4.292	0.0001	0.81
STD1-SMART2	-0.245	1348	-1.761	0.078	0.70
STD2-SMART2	-0.270	1175	-1.636	0.102	0.70
Average STD-Average SMART#	-0.035	940	-0.376	0.707	0.79

* "1" and "2" stand for first and replicate experiment
** number of genes that gave significant fluorescent signals above background in both datasets compared
*** correlation coefficients were calculated after log10 transformation of the data
# comparison was performed on average expression ratios from replicate experiments

Table 2. 2-tailed Paired *t*-test of Testis/Spleen ratios within and between labeling methods

Because the standard labeling method was not necessarily the best method to conserve initial transcript ratios, a set of genes that did not show consistent testis/spleen transcript ratios among the three methods was selected for confirmation by quantitative RT-PCR (Table 3). The results could be grouped by a) correctly classified differential expression (ratio of microarray data were in agreement with quantitative RT-PCR), b) miss-classified differential expression, and c) missing data (no fluorescent signals above background). Two clones did not result in RT-PCR products, probably due to sub-optimal primers. The results showed that only 2 out of 12 genes were wrongly classified by STD, while in IVT repeats: 3 to 4, and in

SMART repeats: 4 to 5 genes were misclassified. Interestingly, after IVT amplification less missing data were observed than the two other methods, which may mean that amplifying the starting material may increase the sensitivity of the assay.

Genbank_Acc	Q-RT PCR*	STD1	STD2	IVT1	IVT2	SMART1	SMART2
AA049537	6.55	**7.14**	**6.25**	**3.57**	2.00	-1.25	
AA058055	3.23	**5.26**	**2.94**	-3.85	-7.69		-5.26
W82946*				-16.67	-14.29	-7.69	-20.00
AA050371	-2.94	**-2.70**	**-2.44**	**-12.50**	**-11.11**		**-6.67**
AA118976	-2.38	**-3.70**	**-8.33**	-1.61	1.56	3.45	2.04
W36382	5.15	1.06	1.15	**4.35**	**9.09**	1.61	**3.45**
W47799*		100.00			50.00	90.90	30.30
AA175329	-20.00	**-11.11**		**-50.00**	**-50.00**	**-33.33**	**-20.00**
AA175441	-12.50			**-10.00**	**-12.50**	**-5.56**	**-7.69**
W13718	1.59	-6.25	-4.35	**1.32**	**1.41**	**1.43**	2.22
AA124396	1.85	**1.03**	**1.10**	4.55	4.35	3.03	5.03
AA011839	-4.17			**-11.11**	**-10.00**	**-11.11**	**-9.09**
Correctly classified (bold)		**6**	**5**	**7**	**6**	**4**	**5**
Wrongly classified		**2**	**2**	**3**	**4**	**4**	**4**
No Data	**2**	**3**	**5**	**1**	**0**	**2**	**1**

Negative values are underexpressed ratios.

*In the case of W47799 and W82946 no amplification products were obtained either from spleen, or from testis RNA by quantitative-RT PCR.

Table 3. Comparison of 12 differentially expressed genes (mouse testis / mouse spleen), that showed inconsistent or contradictory expression ratios among labeling methods, between microarray analysis and semi-quantitative-RT PCR.

Reference RNA

The application of common reference RNA provides an internal control and thus normalizes differences in hybridization parameters and array variations. The reference RNA should represent a mixture of different gene products with the highest possible complexity. The reference RNA, produced by our method corresponds to poly(A)+ sequences and can be prepared in bulk amounts sufficient for thousands of experiments at low cost**.**

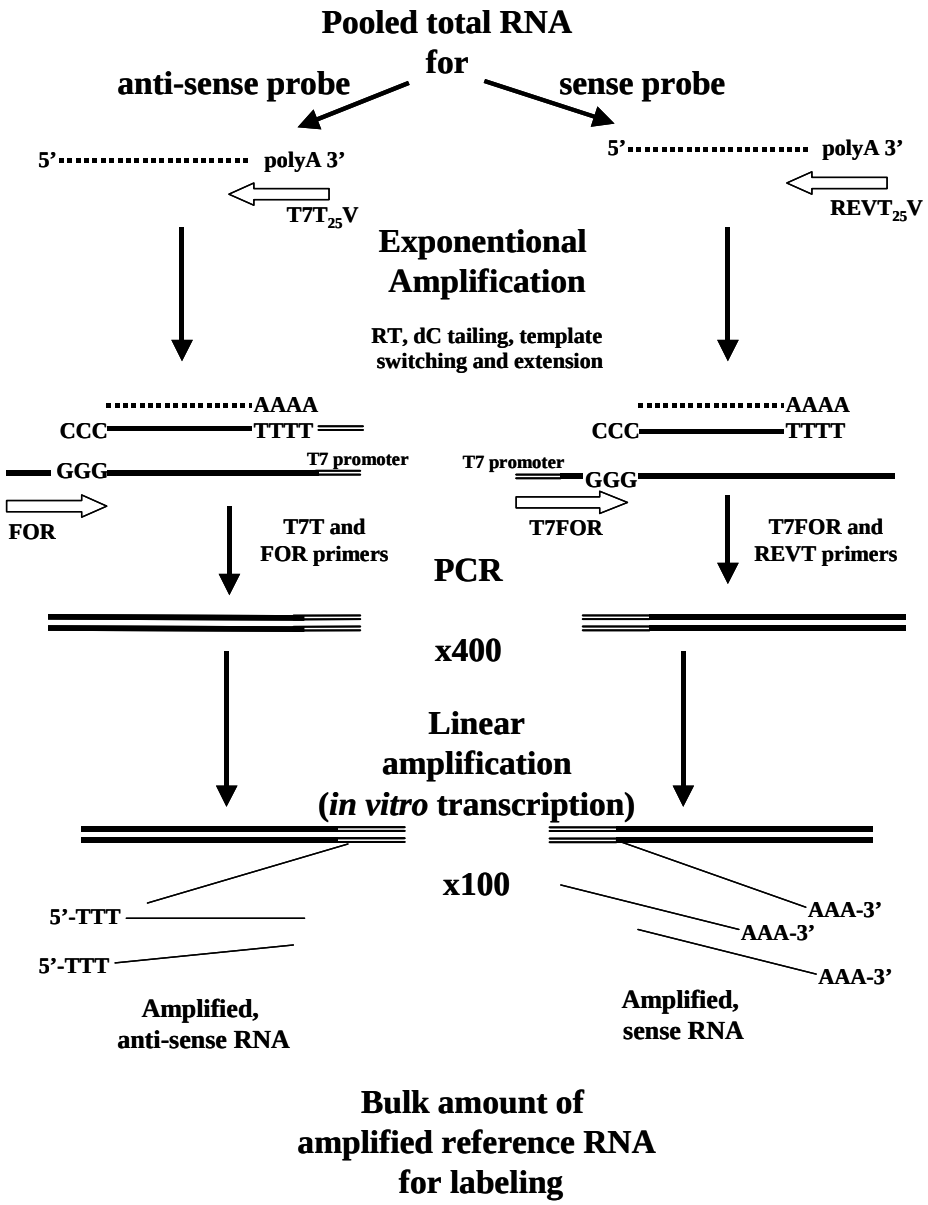

Figure 12. Bulk amount of sense and anti-sense reference RNA can be obtained with combinations of exponential (PCR) and linear (IVT) amplification methods.

Mixtures of total RNA isolated from different tissues deriving from different organisms, including human, rat and alfalfa were prepared. In case of human total RNA pool, commercially available Universal Human Reference RNA was mixed with additional total RNA isolated from different tissues in order to increase the complexity of the mixture. In case of the other samples purified or commercially available total RNA were mixed. We used these mixtures as starting material for amplification to obtain labeled sense or antisense reference cDNA.

To obtain bulk amount of reference RNA starting from 80 μg of pooled total RNA, a double amplification technique was applied. The summary of the method can be seen in Figure 12. At first total RNA pools from different organisms were reverse transcribed using a modified SMART cDNA synthesis technique (Clontech). For production of antisense probe, $T7T_{25}V$ and FOR primers were added to the reverse transcription mixture, while in case of sense probe preparation $REVT_{25}V$ and T7FOR primers were used. T7 denotes for the T7 RNA polymerase promoter sequence at the 5'-end of the primers. All the oligonucleotides used in this study were HPLC purified (for sequences see Table 4).

Name	Sequence
$T7T_{25}V$	5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG$(T)_{25}$V-3'
$REVT_{25}V$	5'-TGTCTGCAGTGGTAACAACGCAGAGTACG$(T)_{25}$V-3'
T7T	5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGGTTT-3'
REVT	5'-TGTCTGCAGTGGTAACAACGCAGAGTACGTTT-3'
T7FOR	5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGGG-3'
FOR	5'-TGTCTGCAGTGGTAACAACGCAGAGTACGCGGG-3'

Table 4. Oligodeoxynucleotides used in reference RNA study

After PCR amplification of the cDNA pool, double-stranded DNA mixture was generated having T7 promoter sequence at 5'- or 3'-end relative to the orientation of the original RNA. With this exponential amplification we could achieve an approximate 400-fold improvement in yield of the starting nucleic acid.

The quality of the amplified cDNA was evaluated by gel electrophoresis (Fig 13). The image of the agarose gel shows continuous smears between the 100 and 1000 base pair region, meaning the RNA pool was successfully amplified and no specific sequences were multiplied during PCR.

After PCR, a second amplification, an *in vitro* transcription was carried out. From DNA template having T7 promoter at the 3'-end (relative position to the poly(A) tail of the mRNA), antisense RNA was produced which could be labeled by incorporation of Cy5- or Cy3-labeled dUTP or dCTP using random oligonucleotides as primers in a reverse transcription reaction. If T7 promoter lied at the other side, sense RNA was produced which could be primed both with random and oligo(dT) oligonucleotides. From alfalfa and rat antisense RNA was produced. From human both sense and antisense RNA was generated. In all cases the average yield of the double amplification was approximately 1.5-2 mg of RNA starting from 1/12 of the PCR amplified templates.

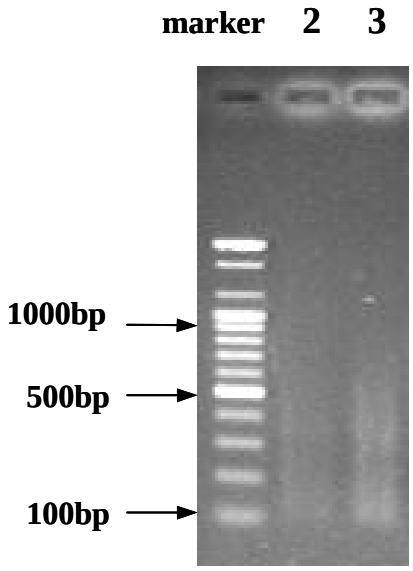

Figure 13. Gel electrophoresis of ampflified cDNA samples produced from human (**2**) and rat (**3**) reference RNA.

To test the quality of the reference RNA we synthesized Cy3 fluorescent-labeled cDNA samples from all the organisms and hybridized them to species-specific microarrays. Alfalfa, rat and human antisense RNA and human sense RNA were converted into labeled samples. Human and rat reference samples were hybridized onto DNA microarrays containing 6400 spots (3200 different genes in duplicate) the alfalfa samples were tested on an array containing 1600 genes. Representative images of alfalfa, rat and human arrays were prepared,

and the percentage of spots with signals that were significantly above background were 90%, 92% and 91% for alfalfa, rat and human, respectively. All the arrays showed high signals in almost all the spots, which confirmed the high complexity and good applicability of the obtained reference probes. Thus, amplified reference RNA could serve as reliable standard could help to create normalized expression databases and delivers the possibility for inter-laboratory comparisons.

CONCLUSIONS

Microarray technology evolved into a diverse investigation technique utilizing several species of molecules and biological samples like DNA, proteins, even cells or tissue. All fields of microarray technology share a requirement that is to immobilize entities on a solid surface. It seemed to be a logical perspective to develop a general method to immobilize all sorts of samples or a spectrum of solid surfaces that are compatible with different applications.

In the thesis diverse chemical modifications of glass surfaces was described based on two different approaches to achieve reactive 3D-like branching structures capable of anchoring nucleic acids, proteins and small chemical molecules. The developed surfaces were compared to previously described immobilization strategies. Our system performed well in oligonucleotide, protein and small molecule anchoring experiments.

The developed surfaces utilize either the reactive properties of epoxy or acrylic functions. Epoxy functions are introduced with two methods. One way is to incubate with epichlorohydrin. To create a more hydrophylic surface, epoxy functions are incorporated via a bifunctional linker, 1,4-butanediol diglycidyl ether. The latter method provides a longer linker arm holding the active functions. The binding capacity of the surface is enhanced through multiplying the active sites by creating a branching structure on the surface.

The increased capacity of the supports were confirmed by comparing the developed surfaces with commercially available, chemically modified glass slides in attaching oligonucleotides in hybridization studies. Higher signals could be obtained using surfaces with the branching structure than in the case of commercially available aldehyde-coated surfaces. The increased signals could be the results of higher sample density in the spotted feature and better accessibility of the probes to the immobilzed samples.

Furthermore, the methods described in the thesis provide the possibility to modulate the hydrophylic/hydrophobic properties of the surfaces, which can have an effect on specificity in experiments where protein-protein or protein-small molecule interactions are investigated (protein arrays and chemical arrays).

From HPLC and mass spectrometry experiments we concluded that the immobilization occurs via the bases of the unmodified oligonucleotides in alkaline pH, supposedly through amino functions. Hence all four bases could react with the acrylic groups at alkaline pH, therefore attachment of oligonucleotides does not depend on their base contents. The tested surfaces offer the capability to permanently immobilize several unmodified biomolecule species in an array format at high density using high-precision robotic spotters. Applying unmodified

biomolecules (e.g. oligonucleotides) results in greater cost efficiency in microarray production. The generated microarrays can be applied in DNA-microarray, protein-microarray and chemical-microarray studies.

In the field of microarray technology technical developments may add more parameters to an already multivariable equation. These parameters are to be thoroughly investigated in order to produce reliable results. A way to test reliability is through the reproducibility of an experiment. Several methods are available to produce applicable sample from even limited amounts of staring material in DNA microarray experiments. One has to bear in mind not only the advantages but also the possible drawbacks of an applied method.

Our results showed that amplifying RNA starting material could produce reproducible microarray data, but induce slight distortions of the initial transcript levels. However, for IVT the percentage of potentially miss-interpreted gene expression levels are negligible, and more data points above background level could be observed. Therefore we suggest that direct labeling is preferred when micrograms of poly(A) RNA are available and that IVT can be considered as a good alternative if only a few micrograms of total RNA can be obtained or when large-scale extractions may negatively affect the RNA quality. SMART-amplification can be used when the RNA is really limiting, e.g. cells obtained after cell sorting or laser capture microscopy. However, using different labeling or amplification strategies within one experiment should be avoided at all costs to retain high reproducibility between samples. Nevertheless, it is clear that the sooner robust methods that can label small amounts of RNA without amplification become available the better.

Finally, we have developed a novel method to produce bulk amounts of reference RNA for the indirect comparison of microarray experiments. Although the use of reference RNA adds an extra step when comparing one experiment to another, it also decreases the variance due to different experiment conditions, and enables inter-laboratory comparisons.

ACKNOWLEDGEMENT

I would like to thank Dr. László Puskás for the opportunity to work in his group. I would also like to thank him for introducing me the methods and techniques of molecular biology and the ideas, help and instructions he provided during my work. I am thankful for the staff of the Laboratory of Functional Genomics for the creative atmosphere of the lab.

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