

**Development of chemically modified glass surfaces and methods
for microarray technologies**

Summary of PhD Thesis

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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 and small molecules from combinatorial chemical libraries. Immobilized DNA molecules, either as double-stranded segments or as short synthetic oligodeoxyribonucleotides have been utilized for gene expression monitoring, DNA-sequencing, disease screening diagnostics and genome analysis. 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. Protein arrays containing numerous different entities are efficient tools in applications like antibody profiling, serum screening and ultimately in drug discovery. 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. All mentioned techniques demand permanent and efficient anchoring of the samples.

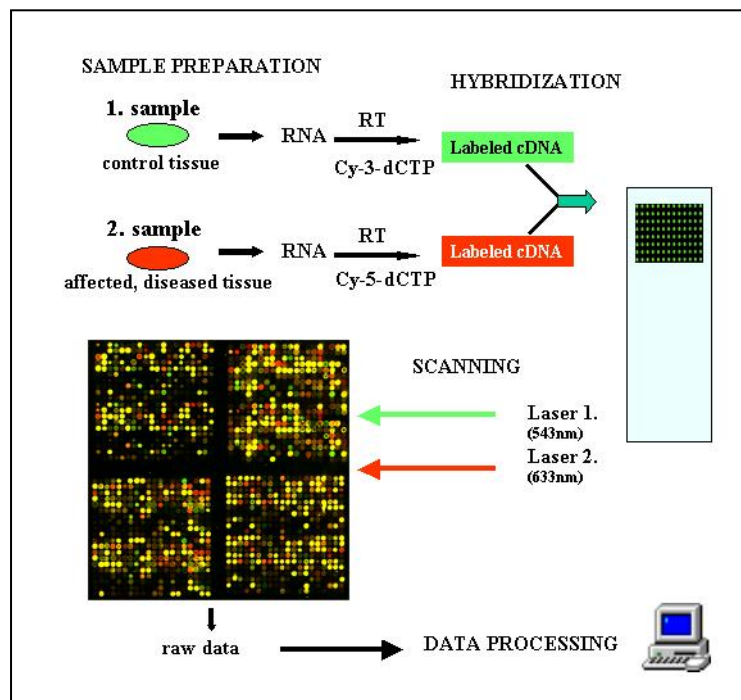


Figure 1. Flowchart of DNA microarray analysis

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 (Figure 1). DNA microarrays with sets of DNA fragments on their surface can be used to obtain a molecular fingerprint of gene expression of cells.

DNA-microarray technology provides a rapid and comprehensive approach to simultaneously monitor the expression levels of thousands of genes – through the measurement of relative mRNA abundance - between diverse biological samples in a comparative way.

The 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. 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. 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.

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. 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.

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.

METHODS

Developing the reactive surfaces

As starting material non-derivatized microscope slides were used. 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.

Construction of microarrays

All samples (either DNA, proteins or small molecules) were delivered to the surface of the microscope slides using a pin-tool based spotting robot MicroGrid Total Array System (BioRobotics, UK) or by pipetting manually.

Investigation of anchoring

Samples prepared in a model reaction investigating the mechanism of immobilization on a surface were analyzed by HPLC and mass spectrometry measurements.

Fluorescent labeling reactions

Before hybridization, DNA samples were labeled by incorporation of fluorescently labeled nucleotides during reverse transcription.

Protein samples were labeled with fluorescently labeled carboxylic acid succinimidyl ester.

Scanning of microarrays

Hybridized microarrays were scanned with a ScanArray Lite (GSI Lumonics, Billerica, USA) microarray scanner at 543 nm for Alexa Fluor 555 or Cy3 and at 633 nm for Alexa Fluor 647 or Cy5 labeling.

Single-gene RT-PCR and Southern-blotting

In comparison analysis a Southern blot experiment of 12 selected genes were carried out to evaluate the results obtained from microarray techniques with different labeling strategies.

RESULTS

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 molecules (Figure 2). The developed surfaces were compared to previously described immobilization strategies. Our system performed well in oligonucleotide, protein and small molecule anchoring experiments.

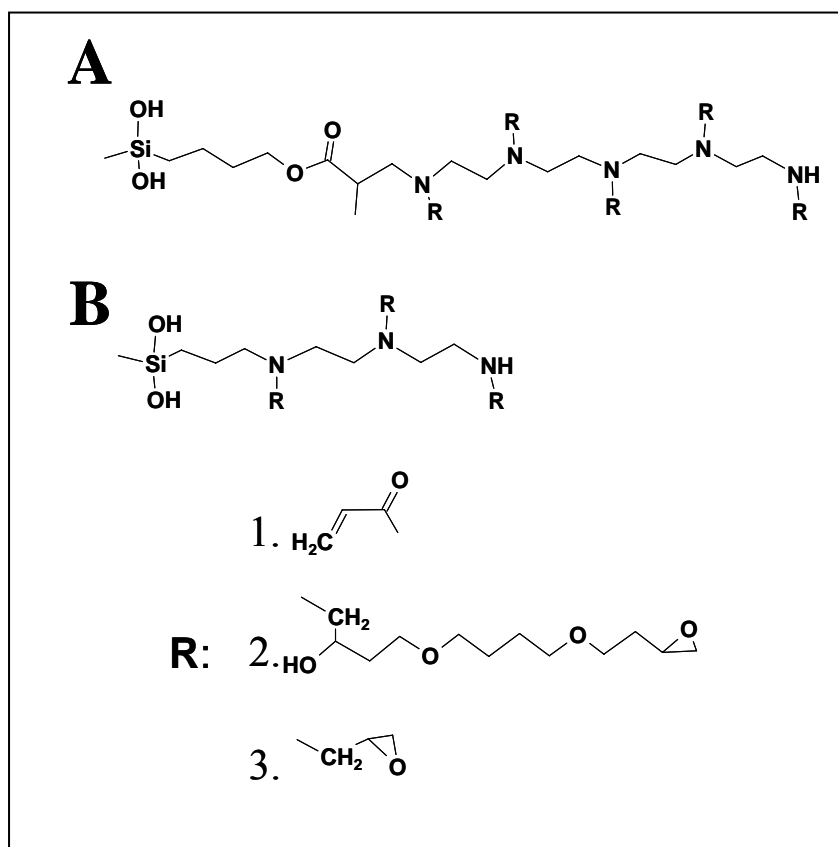


Figure 2. Chemical structure of the developed solid supports and the active linker arms (A: dendrimer like linker system. B: triamino based linker system)

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 was confirmed by comparing the developed surfaces with commercially available, chemically modified glass slides 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 immobilized 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, 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 starting 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 (Figure 3). However, for *in vitro* transcription (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.

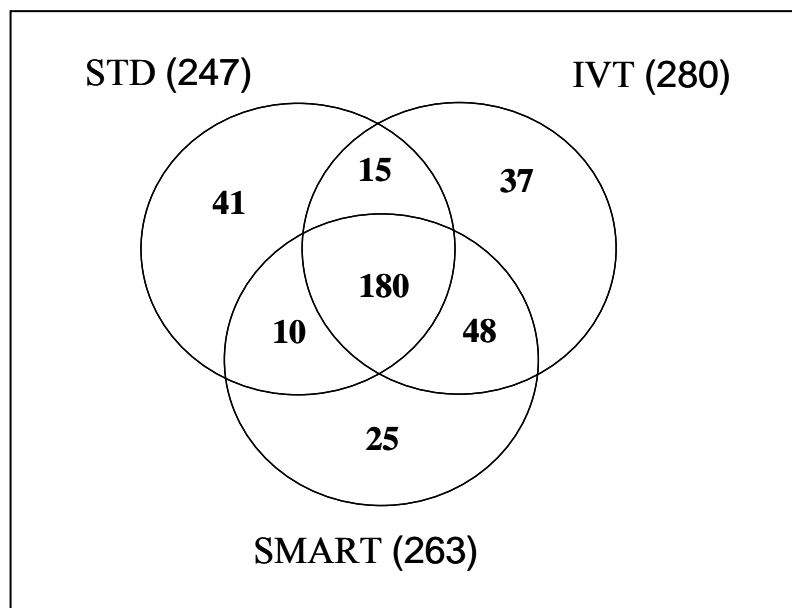


Figure 3. Venn Diagram of differentially expressed genes compared among standard labeling (STD), linear amplification (IVT) and exponential amplification (SMART). The total number of differentially expressed genes per method is given between brackets. A two-fold threshold allowing a 10% window determined the differentially expressed genes.

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.

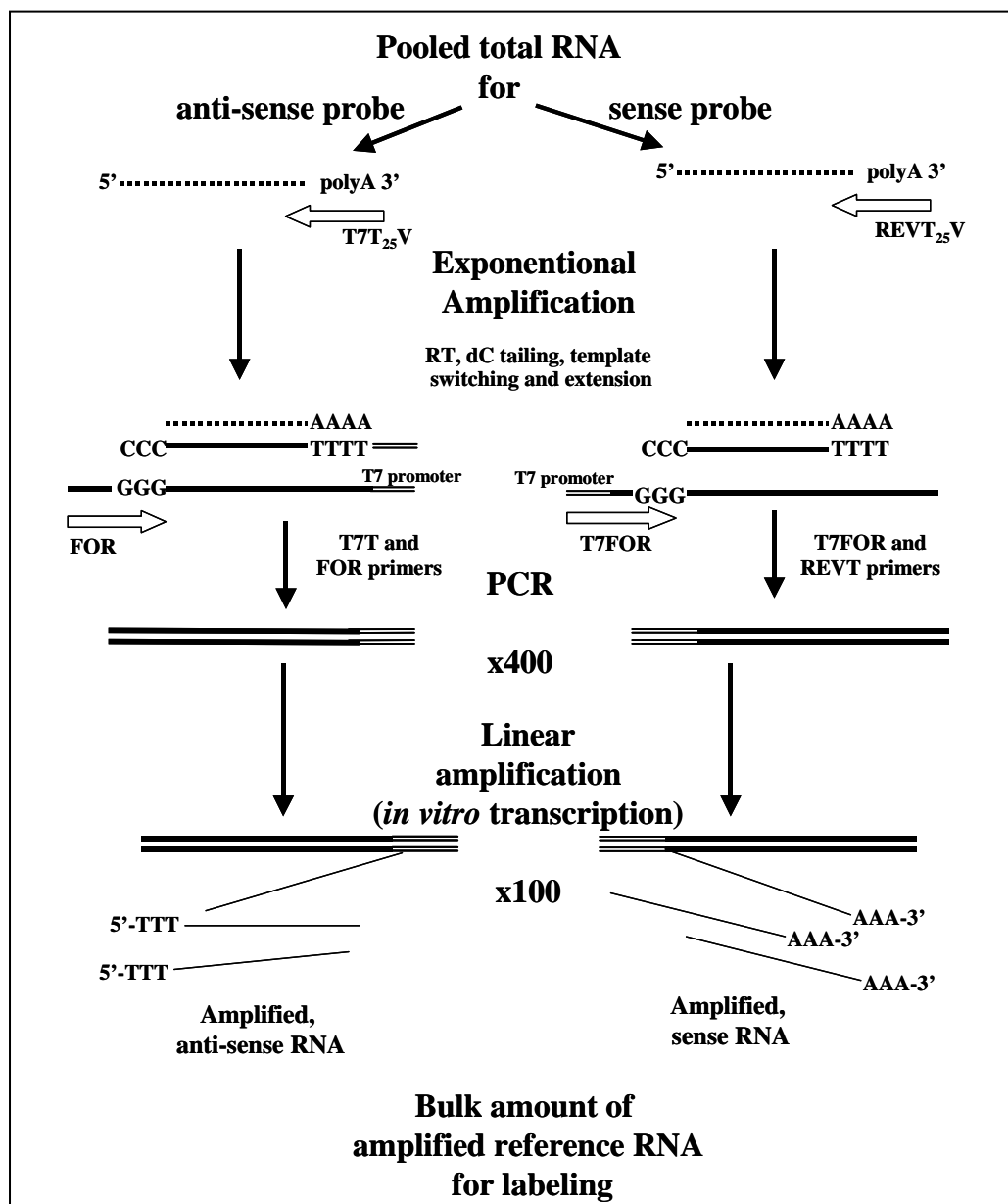


Figure 4. Bulk amount of sense and anti-sense reference RNA can be obtained with combinations of exponential (PCR) and linear (IVT) amplification methods.

PUBLICATIONS DIRECTLY RELATED TO THE THESIS

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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.
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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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4. Gwendolyn Barcelo-Coblijn, Endre Hőgyes, Klára Kitajka, László G. Puskás, Ágnes Zvara, **László Hackler Jr.**, Csaba Nyakas, Zsuzsa Penke and Tibor Farkas (2003) Modification by docosahexaenoic acid of age-induced alterations in gene expression and molecular composition of rat brain phospholipids. *Proc. Natl. Acad. Sci. USA* 100, 11321-11326.

5. Annamária Ónody, Ágnes Zvara, **László Hackler Jr.**, László Vígh, Péter Ferdinandy and László G. Puskás (2002) Effect of classic preconditioning on the gene expression pattern of rat hearts: a DNA microarray study. *FEBS Letters* 536, 35-40.
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.
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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, előállításuk és alkalmazásuk.)