Neural Degeneration and Repair

Gene Expression Profiling, Proteomics, and Systems Biology

Edited by
Hans Werner Müller

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Cover illustration
The cover picture shows an immunofluorescence image of sensory neurons (red) in a dorsal root ganglion expressing gastric inhibitory polypeptide (green). The neurons respond to peripheral nerve injury by altered gene expression.
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Preface

During the past ten years, since the introduction of microarray technology, neuroscientists became aware that highly complex gene expression networks are activated in traumatic nervous system injuries and devastating neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD). The classical approach of studying single or small sets of genes or proteins has been quite disappointing to dissect the intricate network of molecular reactions and failed to provide a more complete picture of the pathophysiological and degenerative events. Despite a great deal of initial skepticism, the advent of microarray technology that allows the study of changes in gene expression at a (near) genome wide scale has opened a new avenue for a molecular systems approach to neural degeneration and repair. As a step further, proteome analysis, the ''analysis of the entire PROTEin complement expressed by a genOME'', involves the simultaneous separation, identification and/or quantification of hundreds or thousands of proteins from a single tissue sample.

There is a desperate need for a critical overview of the present state of research in this field that has reached a certain state of maturation and acceptance and is at the edge to be widely and routinely used among neuroscientists all over the world. Thus, it appears to be timely to describe the potential as well as the limitations of the application of these recent technologies to highly relevant neurological disorders such as trauma, neurodegenerative disorders and neural tumors and their transition into clinical applications (diagnosis, prevention, therapy).

This book highlights the state-of-the-art application of microarrays and proteomics in systems neurobiology and translational neuroscience from genome research to clinical application with particular emphasis on peripheral (PNS) and central nervous system (CNS) injury and repair, neuropathic pain, ageing and neurodegenerative diseases such as AD, PD, and neurooncology. Microarray technology will not only be critically compared with previously existing high-throughput gene expression technologies. As outlined in the following chapters, the genomic and proteomic technologies have proven a tremendous impact on elucidating genetic networks and molecular pathways underlying successful axonal plasticity, regeneration and retrograde axonal signaling in the damaged PNS and CNS. Moreover, the potential of pharmacogenomics for future applications in personalized therapies, the

development of new quantitative proteomics technologies (SILAC, ICAT, iTRAC), and the impact of novel redox proteomics approaches to analyze protein modifications due to oxidative damage in nerve cell cultures and animal models of AD are presented. On the other hand, gene expression profiling of gliomas has opened entirely new perspectives on tumor classification and deciphering tumor heterogeneity, pathway-associated expression signatures, and lineage-specific molecular signatures to localize the origin of a glial tumor.

We hope that this book provides useful information for a wide range of basic researchers and biomedical and clinical scientists from the level of neurobiology/medical students, postdocs to advanced specialists in academics as well as industry.

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Düsseldorf, January 2008 *Hans Werner Müller*

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1
Microarrays in Systems Neurobiology and Translational Neuroscience – From Genome Research to Clinical Applications

Jeremy A. Miller and Daniel H. Geschwind

1.1
Introduction

Although microarray technology was introduced just 10 years ago, over 20 000 articles have been published using this technology as of 2006, covering areas ranging from soil ecology and yeast genomics to cancer and neurological disorders. In neuroscience, much of this represents publications since 2000, showing a remarkable trajectory as well as reflecting early skepticism that has now given way to acceptance and appreciation (Figure 1.1). Entire transcriptomes can now be assayed on a single chip at a reasonable cost, and technologies are becoming cheaper and more accurate day by day. In basic neuroscience research, microarrays have been used to assess gene expression differences across mouse strains [1], brain areas [2], cell types [3–5], and brain tumor strains [6]. They have also been used to identify genes that play an important role in neural stem cell biology [7,8], mouse models of neurodevelopmental disorders [9], and postmortem assessments of many neurodegenerative diseases such as Alzheimer's disease (AD) [2,10–15], Parkinson's disease (PD) [16], Huntington's disease (HD) [17,18], amyotrophic lateral sclerosis (ALS) [19,20], and schizophrenia [21]. In addition to providing a useful tool for basic neuroscience research, microarrays hold significant promise clinically as patient classifiers in acute and chronic neurological diseases [22].

This chapter summarizes the current state of microarray technology, presenting several clinical applications. In the next section, gene expression technologies leading up to microarray technologies are presented along with alternative high-throughput techniques. Section 1.3 provides a primer on how to design and implement a successful microarray experiment and presents challenges to the field and analytic methods that have been developed to get the most out of expression data. The last section summarizes recent microarray experiments in the field of neuroscience, highlighting key, representative papers documenting state-of-the-art experimental design, clinical uses in brain cancer, and the use of peripheral blood as a substitute for brain tissue in various neuropsychiatric conditions. Finally, genomic DNA microarrays are briefly discussed, along with speculation on the future of clinical microarray applications.

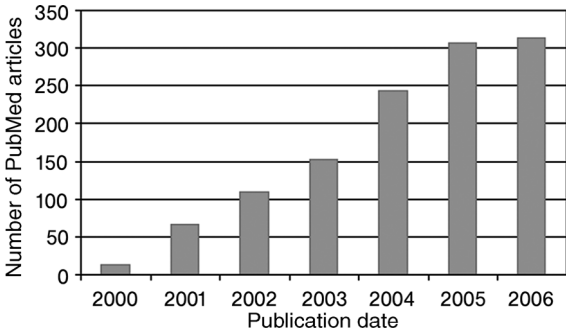

Figure 1.1 Acceptance and use of microarrays in the twenty-first century. Since the year 2000, publications on microarrays have gained popularity in the area of neurosciences, indicating their more widespread acceptance and use as a viable tool. The *X*-axis indicates publication year, while the *Y*-axis indicates number of publications turning up in PubMed searches for ''microarray'' and ''brain''.

1.2
Gene Expression Before Microarrays

Since the discovery of DNA in the early 1900s and the subsequent discovery of RNA as the substrate for protein synthesis, gene expression assays have become an essential component of disease research. Gene expression approaches initially took a gene-centric view. A scientist would hypothesize a relationship between a gene and a phenotype, and then test this hypothesis using methods such as Northern blot and *in situ* hybridization. In Northern blot analysis, mRNA is denatured and separated by weight on a gel using agarose gel electrophoresis, transferred onto a membrane, and hybridized with complementary labeled probes [23]. Thus, gene expression correlates with intensity of the labeling. *In situ* hybridization, on the contrary, involves directly applying labeled probes to the tissues of interest to determine where the mRNA is expressed *in situ* [24]. Although still important for studying single genes, these high-resolution techniques are at a disadvantage with regard to the throughput now available using techniques such as RT-PCR, serial analysis of gene expression (SAGE), differential display, and microarrays. As is the case in complex, dynamic tissues such as the brain and nervous system, there is often a trade-off between scale and resolution [25].

1.2.1
High-Throughput Gene Expression Techniques

A paradigm shift occurred in the early 1990s, as technology improved and knowledge of the genome became widely accessible. This challenged scientists to move from a gene-by-gene study to develop methods that took into consideration the entire

system of gene expression, moving from the unimolecular to the systems level [26]. One of the earliest methods using high-throughput techniques to identify a large number of genes differentially expressed between two tissues or conditions was differential-display reverse-transcription polymerase chain reaction (DDPCR) [27]. In DDPCR, the 5′ end of mRNA is bound to anchor primers and reverse transcribed. A subset of this cDNA is then PCR amplified near its 3′ end using short arbitrary primers. The resulting amplified cDNAs from two samples are run side by side on a gel, and any differentially displayed bands of interest can be excised from the gel, reamplified, cloned, and sequenced. This method is relatively inexpensive and can test gene expression of all transcripts amplified simultaneously; however, every interesting band has to be sequenced individually, and the completion of the human genome project has rendered such time-consuming sequencing unnecessary.

Representational difference analysis (RDA) represents a more elegant genome-wide subtraction method that, unlike DDPCR, does not require sifting through an entire gel of genes to find some that are different [7,28,29]. In RDA, populations of mRNA from two separate tissues are transcribed into cDNA, digested using restriction enzymes, converted into primers, and PCR amplified. These populations are then cross-hybridized by combining an excess of one population (driver) and using that to remove identical transcripts from the less concentrated population (tester). By iteratively performing this process with each population as the driver and the tester, and then shotgun cloning the subtraction products, libraries for genes enriched in each tissue can be created. We have used this method coupled to microarray screening, which provides a powerful approach to screening genetic subtractions [7,28].

1.2.2 Contemporaneous Alternatives to Microarrays

SAGE [30] is one of the several high-throughput sequencing methods that provide a powerful technique for high-resolution assessment of gene expression in a relatively small number of samples. In SAGE, cDNA is positionally anchored using restriction digestion, and short nucleotide chains around 14 base pair (bp) are removed from specific positions in each molecule, serving as tags, concatenated together into polymers of such tags, many multiples of which can be processed in a single sequencing run. Thus, small tags of each gene are present in proportion to their abundance in the starting mRNA and can be counted by efficient sequencing and bioinformatic identification of the gene from which they originate. The resolution in SAGE is limited only by the cost and time of sequencing, but it typically requires about 2000 sequencing reactions for each SAGE library to identify 50 000 tags. However, often one needs to sequence 1 million or more tags to identify low-abundance species in a complex tissue such as the CNS. To compare two tissues, several such libraries need to be prepared from each tissue, making this a high-resolution but low-throughput approach (relative to sample numbers that can be studied). In theory, this technique is sensitive enough to find any mRNA species and

has the advantage over differential display, as each sequencing run determines multiple mRNA species. In practice, however, this technique is too expensive and time consuming for massive parallelization and clinical use.

Massively parallel signature sequencing (MPSS) determines mRNA counts using a principle similar to SAGE [31]. In MPSS, fluorescently labeled cDNAs from the input sample are hybridized to a microbead cDNA library, and hybridized beads are fluorescently sorted and placed on a 2D grid. All beads are then simultaneously decoded and digested 4 bp at a time by binding unique adaptors, which can be read using a charge-coupled device (CCD). MPSS has all the advantages of SAGE and can read many more mRNA species for similar time investment (~250 000), but it requires special equipment and is expensive. Thus, for most, it remains primarily a research tool for in-depth investigation of a few specific samples of interest, although the recent advent of new sequencing technologies will significantly decrease the price of these clone and count techniques.

1.2.3 Microarray Technologies

Microarrays balance sensitivity and throughput to allow efficient study of about 10 000 detected mRNA species in parallel in a large number of samples. This may not allow the maximum depth possible as with MPSS or SAGE but has the advantage of high scalability. The first high-throughput gene expression study, published in 1987, was carried out by Augenlicht *et al.* who used a nylon membrane containing 4000 cDNA sequences to examine gene expression changes in colon cancer [32]. Once solid substrates replaced nylon in the 1990s, this method provided a relatively cheap, quick, and reproducible way for high-throughput gene expression analysis. Owing to the abundant clinical and research applications of this technology, many groups and companies have created their own microarray platforms. Although an entire chapter could be devoted to describing the similarities and differences of these platforms (see [33]), there are two general categories of microarrays: one-color arrays and two-color arrays.

1.2.3.1 One-Color Oligonucleotide Arrays

One-color oligonucleotide (oligo) arrays (or chips) marked the first of the commercial microarray technologies [34–38] and were released by Affymetrix in 1996. These arrays required the development of two novel methodologies. Light directed chemical synthesis allows for the direct application of hundreds of thousands of nucleotides to specific positions on the chip at once, bypassing the need for PCR-amplified cDNA probes. By masking all array positions not associated with the applied nucleotide and repeating this chemical coupling for each nucleotide using multiple masks, gene-specific oligo probes, 25 bp in length, are synthetically created. After synthesis of the array, laser fluorescence microscopy can detect hybridization of fluorescently labeled cDNA (target). Expression values for each gene can then be deduced by averaging over multiple probes and using mismatch probes (where the 13th bp has been purposely changed) to account for nonspecific binding.

1.2.3.2 Two-Color Arrays

Contemporaneous with the development of oligonucleotide arrays, a separate yet equally powerful method for running massively parallel gene expression experiments was created [39–43] in which thousands of cDNA probes between 0.2 and 2.5 kb in length were PCR amplified and printed onto poly-L-lysine-coated microscope glass slides using one of two printing techniques. In mechanical microspotting (or passive dispensing) – currently the more popular method – the target is loaded into a dispensing pin using capillary action and placed onto the cDNA microarray by directly contacting the slide. Drop-on-demand (or inkjet) printers use pins with piezoelectric fittings to drop a precise amount of the target onto the slide using an electrical current, without actually having the pins contact the slide. Once synthesized, these cDNA arrays, unlike their one-color counterparts, detect the differential expression between two reference samples, each of which is labeled with separate dyes (typically Cy3-dUTP and Cy5-dUTP). Hybridization fluorescence signals from each dye are detected separately with a dual-wavelength laser scanner and combined into a single pseudocolor image using computer software. Recently, most two-color platforms have shifted from cDNA probes to longer oligonucleotides (30–60 bp), as oligos are generally more customizable, potentially more target specific, and less difficult to amplify and purify than cDNAs. The Agilent platform is an example of a commercial two-color platform based on oligonucleotides [44].

1.2.3.3 Bead-Based Arrays

Most current microarray systems, whether one-color (Affymetrix) or two-color (Agilent), are based on oligos attached to a solid substrate, each with a known address. Illumina universal bead arrays [45,46], however, consist of densely packed wells, ~3 μm in diameter, which are randomly filled with beads containing 75 bp chimeric oligos. These wells are etched either into bundles of fiber-optic strands or onto specialized chips. Each array has an average coverage of ~30 beads per feature, with the exact number variable due to the random filling of wells. For each bead, oligonucleotides consist of a 25 bp bead identifier followed by a 50 bp gene-specific probe, and ~700 000 such oligos are attached to each bead. Bead types are decoded by repeated hybridization (and subsequent dehybridization) of fluorescently labeled cDNA sequences complementary to the bead identifiers. Fluorophores are chosen such that each bead has a unique sequence of fluorescent signals (e.g., red-green-none-red-red-none-green-red after eight hybridizations). After decoding, cRNA from one sample is fluorescently labeled and scanned, and the absolute abundance of transcript is determined by averaging the intensities of each bead containing that transcript.

1.3 Designing and Implementing a Microarray Experiment – From Start to Finish

Many articles and guides on the basic design of microarray experiments in the field of neuroscience are available [47–50]. Here, we highlight some of the key issues, starting with the basics.

1.3.1 Choosing the Proper Microarray Platform

Given optimal conditions, all microarray platforms work very well; however, conditions are never optimal, and issues such as experimental assay, local expertise, cost, and gene coverage all play a role in platform selection. A two-color design is most suited for comparative assays, for example, if the experimental goal is to compare multiple tissues from a single subject (tumor versus normal tissue, cerebellum versus cortex, etc.). However, experiments seeking to correlate gene expression with phenotype (such as aging) in a single tissue tend to use one-color arrays; although two-color arrays can be used, by comparing each sample with the same reference sample [41,51]. This choice should be dictated by the statistical design of the analysis, so as to allow optimal power to detect the desired changes.

Another issue to consider is cost versus reproducibility. Laboratory-made spotted oligo arrays cost significantly less than factory-born arrays, whether one-color or two-color, but require more effort to make. All microarrays are prone to batch effects, which can be removed by proper normalization [52,53], but may be more significant in homemade arrays. Thus, in a research-based experiment, custom arrays may be appropriate, whereas biomarker assays would more likely require factory-made arrays since thousands of identical arrays will eventually have to be made quickly. Then, local expertise has also to be taken into account. If all of the current lab personnel were trained using a specific kind of array, then the continued use of those arrays would decrease both experimental time and error. One more advantage of homemade arrays is that they are not vulnerable to changes in designs of manufacturers during the course of a series of experiments, as has been the case with every commercial platform so far.

The final issue to consider when choosing an array platform is customizability versus scalability. Both homemade and Agilent two-color arrays allow for the quick and cheap creation of arrays containing any target of interest. For example, if an experimenter aims to test the expression of multiple splice variants of a gene or to make a biomarker assay for testing the expression of 100 specific genes, such arrays would be appropriate. Nimblegen, which uses a mirror-based masking system, has the maximum synthetic flexibility and offers custom arrays on a commercial platform [54]. A wide variety of configurations are available, but the cost is far higher than homemade spotted arrays. Spotted array technologies do not lag far behind, however, as just about every microarray platform currently has an array to test the expression of every known human transcript. All of these factors have to be taken into account while choosing a proper platform for the experiment at hand.

1.3.2 Preparing the Tissue for Hybridization

After selecting the microarray platform, tissue must be acquired and prepared in such a way to avoid inducing unwanted changes in gene expression. An experiment

using postmortem tissue must carefully control for gender, ethnicity, and cause of death to avoid outlier arrays [22,47,55]. Once tissue is acquired it must be properly cared for, as excessive postmortem interval, changes in pH or temperature, or improper tissue handling at any stage can lead to RNA degradation [50]. Nonlinear or excessive PCR can selectively amplify smaller segments of cDNA, leading to increased variability, while improper tissue preservation can make a sample completely unusable. Generally, an experiment should include duplicate spots or arrays to quantitatively assess variability due to human error or choice of array platform, thus, effectively determining the sensitivity of the experiment [50].

The precise steps to be taken between tissue acquisition and target hybridization depend highly on the experiment at hand and generally involve the use of a series of well-characterized procedures and commercially available kits. Generally, mRNA or total RNA samples are extracted from the tissue or cells of interest and converted into their cDNA sequences. In the case of genomic DNA assays, DNA is cut into manageable sizes. When necessary, the cDNA is then amplified. Finally, each sample is fluorescently labeled using one or two dyes, as required.

1.3.3
Single-Cell Assays and Tissue Heterogeneity

Under ideal conditions, microarrays can detect mRNA in relative abundances as low as one part per 500 000 [39,43], allowing for a resolution of 3–10 copies per cell in simple tissues and cell lines. In practice, however, while these species may be detected, their detection may not be reliable enough to ascertain differential expression; so it is safer to assume reliable detection at the 1/100 000 level. In the nervous system and other complex tissues consisting of multiple cell types with uniquely expressed transcripts, resolution of cell type specific species is hampered [50]. To increase resolution, therefore, many microarray experiments now use single-cell assays to filter individual cell types of interest from heterogeneous tissue before assessing changes in gene expression [25], although this also has its costs.

Many cell purification assays, including flow cytometry, microaspiration, and laser-capture microdissection (LCM), have arisen to combat tissue heterogeneity in different situations. Flow cytometry allows thousands of cells per second to be counted, examined, and separated based on any of a number of characteristics of the cells [56]. This method is generally used to quickly obtain large quantities of a single cell type. Several studies have recently demonstrated the use of automated flow sorting [3,4] for purifying neurons from developing and adult brains [4]. In addition, fluorescence can also be applied manually [5], although it is more tedious. The use of automated sorting allows for large-scale purification of thousands of neurons in a single sort. Cells can be labeled by tracer injection [3] or in genetically modified mice [4], which are now available in many forms via the GENSAT project [57]. Microaspiration, on the contrary, involves patching onto individual cells and removing them one at a time [58]. This process is much more painstaking than flow cytometry and provides many fewer cells; however, it can provide much more accurate