Opinion

Exploiting microarrays to reveal differential gene expression in the nervous system

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Published: 29 January 2003

Genome Biology 2003, 4:105

The electronic version of this article is the complete one and can be found online at http://genomebiology.com/2003/4/2/105
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Abstract

Microarrays have been used in a wide variety of experimental systems, but realizing their full potential is contingent on sophisticated and rigorous experimental design and data analysis. This article highlights what is needed to get the most out of microarrays in terms of accurately and effectively revealing differential gene expression and regulation in the nervous system.

The hypothesis-driven, single-gene analytic approach has dominated molecular neurobiology research and has been very successful. In grant submissions, the US National Institutes of Health demand succinctly defined, closed hypotheses based on expected outcomes. Where does this leave experiments utilizing high-density microarrays, which do not fit this mold? The fact that microarray investigators do not know which genes or pathways will be discovered by their experiments is precisely the strength of microarrays. The ability to examine the expression profile of potentially the entire genome at once, without preconceptions, offers the possibility of completely novel and unexpected insights into entities as complex as the nervous system. But before these insights can be made, the practical problems of experimental design, data analysis, verification, and interpretation need to be addressed.

Optimizing experimental models and design

The full potential of microarrays will be realized only when the questions that are asked about a system are sophisticated, rather than seeking simple changes or differences in expression profiles. We need to avoid experiments that generate lists of genes, suitable only for archiving and future data screening. In addition, technical aspects of any experiment require attention; the speed of tissue removal to prevent RNA degradation, extraction of high quality RNA, optimal reverse transcription, amplification, labeling and hybridization with arrays and the use of quality control measures at each step are all critical. The suitability of different array formats also needs to be considered in terms of representation, variability, and sensitivity. Before considering data analysis and verification directly, we will discuss several of the microarray studies of neurobiological interest that have been completed to date. In particular, we focus on those aspects of model choice and experimental design that allow the conclusions of greatest biological importance to be drawn.

Carefully designed microarray studies have already begun to offer new forms of biological insight. To illustrate, microarrays have been used not only to validate putative drug targets but also to identify the cellular consequences of drug treatment and to differentiate target-specific drug effects from non-specific effects. For example, a comparison has been made between wild-type yeast treated with FK506, a putative axon-regeneration-promoting drug [1] that is better known for its use as an immunosuppressive agent, and untreated yeast lacking the gene for calcineurin, the drug target [2]. By identifying genes that were regulated in the drug-treated yeast lacking the drug’s therapeutic target, the investigators were able to describe genes representing the drug’s transcriptional side-effect profile. Yeast studies have also pioneered the use of arrays to identify cis-acting control elements that regulate the transcription of many co-regulated
genes [3], a phenomenon that may be important in both neuronal development and the response of neurons to injury.

Another promising neurobiological use of microarrays has been in studying tumors of the central nervous system: here arrays have been used not only for diagnosis but also as a prognostic tool. Pomeroy and colleagues [4] collected a microarray dataset from 99 embryonic tumor samples associated with known diagnoses and patient survival outcomes. They were able to distinguish the different histopathological classes of medulloblastoma on the basis of expression profiles. In addition, they derived subsets of 21 or fewer genes whose combined expression levels could classify the medulloblastoma samples according to survival versus treatment failures. There were no known prognostic markers for medulloblastoma prior to this study.

A variety of experimental models in neuroscience have now been investigated using microarrays. For example, arrays have been used to study differences in the expression profiles of two inbred strains of mice with different levels of susceptibility to seizure [5]. This study found that in a strain of mice resistant to seizure-induced hippocampal cell death (C57BL/6) the expression of many more genes was induced in the hippocampus by seizure than in a strain of mice susceptible to post-seizure cell death (129Sv/Ev). Other array studies have examined the effects of diet and aging on the mouse brain [6,7], and the effects of environmental influences, such as exposure to an enriched environment [8]. Arrays have also been used to examine gene-expression changes in models of Parkinson’s disease [9], in patients with Alzheimer’s disease [10], and in schizophrenia [11]. Although these studies are so far purely descriptive or observational, they have uncovered novel genes or suggested novel mechanisms, the significance of which now needs to be explored further.

The ability to use microarrays to view changes in expression induced by null mutations or gene knockouts is also of interest. A gene-expression study of the pons and cerebellum has been investigating using microarrays. For example, arrays have been used to study differences in the expression profiles of two inbred strains of mice with different levels of susceptibility to seizure [5]. This study found that in a strain of mice resistant to seizure-induced hippocampal cell death (C57BL/6) the expression of many more genes was induced in the hippocampus by seizure than in a strain of mice susceptible to post-seizure cell death (129Sv/Ev). Other array studies have examined the effects of diet and aging on the mouse brain [6,7], and the effects of environmental influences, such as exposure to an enriched environment [8]. Arrays have also been used to examine gene-expression changes in models of Parkinson’s disease [9], in patients with Alzheimer’s disease [10], and in schizophrenia [11]. Although these studies are so far purely descriptive or observational, they have uncovered novel genes or suggested novel mechanisms, the significance of which now needs to be explored further.

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Complex tissues and diverse cell populations
Heterogeneity of the tissue being examined is a major concern when using arrays to analyze the nervous system. Zirlinger et al. [14] have identified region-specific gene expression within the brains of mice using microarrays. In addition, they observed that some of the genes they identified could be grouped into three spatial expression patterns, each consisting of expression within a different subset of the amygdaloid subnuclei. Other genes, however, seemed to exhibit expression patterns that did not conform to neuroanatomical subnuclei. Lockhart and Barlow [15] have used microarrays to compare gene-expression profiles in six specific regions of the brain (amygdala, cerebellum, cortex, entorhinal cortex, hippocampus and midbrain) in human and mouse tissue; they describe 23 genes that are specific to the cerebellum, giving this region the highest number of unique genes among the regions they studied.

Averaging expression levels of an entire region of tissue, such as the brain or even a specific brain area or nucleus, will clearly minimize or even conceal large expression changes that occur in small subpopulations of cells. There are promising techniques for overcoming this obstacle, however: laser capture to microdissect single cells [16], fluorescence-activated cell sorting (FACS) [17], and cDNA synthesis from single cells [18,19]. A key issue for all these techniques is that the isolated RNA needs to be faithfully amplified. New approaches have made it possible to start with a limited number of cells and still obtain sufficient material for successful microarray experiments. For example, Affymetrix has developed a double in vitro transcription protocol that allows array hybridization to be based on as little as 50 ng of total RNA from the starting material [20]. These amplification methods will be particularly useful for studies of the development of the nervous system, where region-specific tissue amounts are very limited [13]. Recently, Yamagata and colleagues [21] have successfully applied single-cell RT-PCR to retinal ganglion cells, in an effort to identify those genes involved in regulating topographic mapping in the chick retinotectal projection. They were able to identify Sidekick-1 and Sidekick-2, adhesion molecules that appear to have a role in lamina-specific target recognition. The applicability of this technology to microarray studies remains to be explored, however.

Experimental replication and modes of data analysis
The analysis of data from microarrays is non-trivial. Many problems may arise in developing a means for ranking
genes, which is typically based on the degree of difference between two experimental conditions. Regardless of the particular measure of differential expression that the researcher settles on, it is now clear that methods based on replicate independent arrays are necessary for accurate gene identification (Figure 1). Even apart from variability in technique, most neurobiological experiments face such inter-individual and inter-group biological variability that it is unlikely that a single array comparison will ever provide a reliable estimate of gene-expression levels, even for pilot studies. As with any other experiment, multiple independent trials are necessary if we are to obtain accurate data.

The next challenge is to determine how many of the genes estimated from the array expression data are truly regulated. Hundreds, or thousands, of genes are examined in microarray experiments. A single null hypothesis (for example, the absence of regulation) is generally rejected when the probability of false rejection is 1/20. When hundreds of hypotheses are tested in the same experiment, multiple significant test

**Figure 1**
Filtering array data. It is essential to remove as many false positives as possible at the earliest stages of analysis. Random errors occurring in oligonucleotide array data were assessed by comparing three biologically independent control arrays to three other biologically independent control arrays. Genes with a \( t \) test \( p < 0.05 \) in this comparison are plotted (a). Comparison of these results with the frequency of genes differing at \( p < 0.05 \) between control and experimental triplicate arrays (b) were used to define a set of criteria based on present/absent, fold change, \( t \) test \( p \) value, and a signal threshold that minimized the estimated random error [31].
results will be obtained. If each test was performed with a threshold for a positive result of 0.05, then of the test results identified as positives, it is likely that a subset of these will be falsely assigned. Several different statistical corrections are available to limit the accumulation of false positives, most conservatively by dividing the \( p \) value threshold by the number of hypothesis tests (Bonferroni correction). This concern will be of the greatest importance when the population under study contains rare true positives, as might occur when comparing wild-type animals to mutants that have very few transcriptional differences. Conversely, false positives will be less of a problem when there is a high prior probability of true positives. Recently, methods have been developed to limit the overall rate of falsely identified genes with the specific case of microarray experiments in mind [22]. This approach involves mathematically controlling the overall proportion of false positives in the dataset, as opposed to controlling the probability that any given test result is a false positive. But the conservative nature of most available statistical methods means that researchers often find that even well-characterized genes with distinct differential expression patterns fall short of statistical significance within array data sets. Thus, it might be appropriate to consider the values of test statistics as a means for weighting genes according to their reliability or promise for further study, not as definitive rule-in or rule-out thresholds. Researchers may also be able to define empirical statistical thresholds that are useful in their specific experimental systems by performing appropriate control experiments (Figure 1).

Microarray experiments offer the possibility of characterizing the expression of large groups of genes simultaneously using cluster analysis [23]. Briefly, clustering algorithms may be divided into supervised and unsupervised methods. Unsupervised methods are designed to group similar expression patterns in a dataset without referring to any outside information. The hierarchical, k-means, and self-organizing map clustering algorithms fall into this category, and they assist the researcher by identifying groups of co-regulated genes. In contrast, supervised methods use outside information about the experimental condition to shape the derivation of a model from the dataset. For example, a k-nearest neighbors algorithm was used by Pomeroy et al. [4] to derive a set of genes that predict medulloblastoma treatment outcome. In addition to being useful for the researcher in thinking about the large datasets that result from microarray experiments and in shaping future hypotheses, the groups of genes resulting from either supervised or unsupervised clustering approaches can be used as a platform for further studies of signaling systems or metabolic pathways [24].

Grouping genes into functional categories on the basis of data from other experimental systems [25] provides a useful heuristic tool for analyzing microarray data. Websites have been set up to assist functional categorization, notably the KEGG database [26]. Grouping can be used either alone or in combination with cluster analysis to identify genes of related function that exhibit similar expression patterns over time (Figure 2) [3]. But this type of analysis has potential pitfalls when applied to the study of the nervous system: genes identified in other systems in the body frequently have entirely different functions in the nervous system. For

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**Figure 2**
Candidate gene identification. Schematic of a protocol that may be used to select a few genes that obey a defined set of criteria. See text for further details.
example, the platelet-activating factor signaling system, involved in platelet aggregation and blood clotting, and the major histocompatibility (MHC) class I genes involved in cell-mediated immunity, are both involved in developmental patterning of the brain [27,28].

Experimental validation
Once the appropriate mathematical tools have been used to identify genes likely to be regulated in the manner of interest, the next step is biological characterization of these genes. The first stage of this process is validation, which requires measuring the expression of specific genes of interest using independent methods and independent RNA samples. There are important reasons why a gene identified on an array should be validated by another method. For oligonucleotide arrays, the labeled RNA used for hybridization to the chip is not the original RNA population but an amplified copy whose production requires multiple enzymatic steps, each of which may bias array results. Cross-hybridization due to sequence homology may lead to erroneous results, especially when using cDNA arrays to examine genes that have many closely related family members. If all things were equal, then different probe sets (or cDNAs) representing the same gene should behave identically on the same array, but this is often not the case [29]. To illustrate this problem, in a study of amygdala-enriched genes, the authors were able to validate expression of only approximately 60% of the transcripts identified by the microarray analysis [14].

Several possibilities are available for confirming expression patterns that are shared by multiple genes. Real-time PCR allows the expression profiles of many genes to be plotted across many RNA samples [30]. We have found that slot dot blotting of RNA is a successful alternative option: placing RNA directly on a filter allows the use of relatively low amounts of RNA per sample (1 µg of total RNA) and reasonable scale production of filters (10-15 per hour) [31]. Visualizing changes in mRNA expression using in situ hybridization allows the cellular localization of regulated genes to be identified. This is critical for the nervous system, where it is the specific pattern of circuitry that is the vital information [14,32]. Finally, it should be stressed that interpretations of microarray results rely only on mRNA signals. Changes in intracellular distribution of proteins, post-transcriptional modifications, receptor sensitization and desensitization, protein dimerizations, and other protein-protein interactions are beyond the scope of microarray analysis. Substantial changes in protein levels can occur in neurons without any detectable change in mRNA, indicating that translation rate may be important in determining the amounts of some proteins [33].

We are about to face a massive expansion of microarray studies of the nervous system [34]. But realizing the full potential of this technology requires a clear understanding of the technical, bioinformatic and scientific issues. With attention to design, quality control and methods of analysis, the potential rewards are great.

Acknowledgements
The authors’ work is supported by NIH NS3825, HD38533 NS39518 (CJW). C.D.M. is supported by NINDS grant NS45459.

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