

# Subtraction-coupled Custom Microarray Analysis for Gene Discovery and Gene Expression Studies in the CNS

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## Abstract

The revolution in our knowledge about the genomes of organisms gives rise to the question, what do we do with this information? The development of techniques allowing high throughput analysis of RNA and protein expression, such as cDNA microarrays, provide for genome-wide analysis of gene expression. These analyses will help bridge the gap between systems and molecular neuroscience. This review discusses the advantages of using a subtractive hybridization technique, such as a representational difference analysis, to generate a custom cDNA microarray enriched for genes relevant to investigating complex, heterogeneous tissues such as those involved in the chemical senses. Real and hypothetical examples of these experiments are discussed. Benefits of this approach over traditional microarray techniques include having a more relevant clone set, the potential for gene discovery and the creation of a new tool to investigate similar systems. Potential pitfalls may include PCR artifacts and the need for sequencing. However, these disadvantages can be overcome so that the coupling of subtraction techniques to microarray screening can be a fruitful approach to a variety of experimental systems.

## Introduction

DNA microarrays are a powerful technique for the simultaneous measurement of the expression of thousands of genes. Such studies can quickly yield a genome-wide description of RNA levels in a given cell or tissue at a given point in time, or a genetic characterization of a tissue's response to experimental manipulation. Information gleaned from these studies can generate working hypotheses for molecular pathways essential to a given biological process, or potential drug targets for therapies. The utility of the large volumes of data generated, however, depends upon proper experimental design at many levels. An important choice often not given due consideration is which microarray or clone set one should use. Frequently, this choice is made based primarily on convenience and availability, rather than focusing on assaying a set of genes important to the process at hand. This review discusses the advantages of using a subtractive hybridization technique, such as a representational difference analysis (RDA), to generate a custom microarray enriched for genes relevant to the experimental process being investigated. We will begin with a hypothetical example and then discuss a specific implementation of the technique to investigate neural stem cells (Geschwind *et al.*, 2001). RDA-coupled microarrays provide unique, focused arrays that allow for novel gene

discovery and that become a re-useable tool for the analysis of similar systems.

## A hypothetical example: the motivations for RDA-coupled microarrays

In the mid-1830s, Müller developed the doctrine of specific nerve energies: 'the same stimulus, for example, electricity, may act simultaneously on all the organs of sense—all are sensible to its action; but the nerve of each sense is affected in a different way—becomes the seat of a different sensation' (Müller and Baly, 1838). In other words, any signal carried by a nerve of a specific sense will be interpreted as a stimulus of that sense. Thus, mechanical stimulation of the retina, for example by rubbing the eyeballs, will result in a perceived visual experience of seeing stars. Likewise, although electrical activity is similar across modalities, it always results in modality-specific sensations. This simple theory is the basis of our modern understanding of the working of the senses. Even mechanical or electrical stimulation of a spot on the somatosensory cortex will result in perceived sensations arriving from the appropriate region of the body (Penfield, 1959). Thus, a major question in sensation research concerns what is inherently particular about one primary sensory cortex that allows for signals

arriving there to be interpreted as they are. One hypothesis is that differences in gene expression, which reflect or underlie differences in neuronal identity and circuitry, contribute to Müller's doctrine. If that is so, then we should be able to find genes that are differentially expressed between the different sensory cortices. In this case, our system of interest is olfaction and, hence, we wish to uncover genes that define the olfactory cortex, that distinguish it from other sensory cortices. Hypothetically, one approach that could be taken would be to perform a microarray experiment comparing the gene expression in the olfactory cortex to primary sensory cortices from the other senses to identify genes enriched in the olfactory cortex.

### A brief introduction to microarrays

Microarrays are solid surfaces such as glass slides, on which small quantities of cDNAs or oligonucleotides complementary for thousands of genes have been deposited in ordered arrays, with each spot or group of spots representing one gene. These ordered arrays can be used for the simultaneous measurement of the expression of thousands of genes from a given tissue by hybridizing to the slide a radioactively or fluorescently labeled probe derived from mRNA. A common method involves synthesizing and labeling cDNA from two tissues with different fluorescent dyes and hybridizing both probes onto the same array. This allows for a direct comparison of gene expression in the two tissues, for example the olfactory cortex versus a pooled RNA from the other primary sensory cortices. The advantage of a two-color system over a radioactive or a one-color system is that it controls for irregularities in spotting of the slide and allows the direct comparison of two tissues since they are co-hybridized onto the same spots. Such microarrays have been used successfully for such varied tasks as studying development in *Drosophila*, profiling of tumors, and genetic characterization of neural progenitors (Dhanasekaran *et al.*, 2001; Furlong *et al.*, 2001; Geschwind *et al.*, 2001). A more general review of microarray applications in neuroscience can be found in Luo and Geschwind (Luo and Geschwind, 2001).

### Commercial versus custom arrays

The primary advantages of commercial arrays are their wide availability and general applicability. Most have been tested in a variety of systems and are not biased towards one particular system. However, there are two main disadvantages to using a prefabricated array. In our example involving the olfactory cortex, the tissues of interest are neural; however, a prefabricated array may contain many genes specific to non-neural tissue. For most 10 000–15 000 gene arrays, only 25–60% of the spots in the array show a measurable hybridization using neural tissue, suggesting that a significant proportion of genes being assayed are not relevant to the experiment. Also, until whole genome arrays become available, it is quite likely that the given array used

in an experiment will be lacking genes that may be essential for the process being studied. Prefabricated arrays will be biased towards more common transcripts, as rare transcripts are less likely to be present in databases and available for arraying. Therefore, experiments with prefabricated arrays may provide only an incomplete picture of the genes behind the process being studied, with many key genes being excluded from the analysis and many irrelevant genes being included.

### Custom arrays include more relevant genes

One option is to generate a cDNA library from the tissue being studied, for example the olfactory cortex, and to print an array from that library. This custom array will include almost all of the genes expressed in the tissue of interest, filling the holes that would be left by a prefabricated array. However, it would also include many genes expressed in the tissue, but not truly of interest to the question being addressed. For example, this library would likely include many clones for genes commonly expressed in all cells, such as basic metabolic and 'housekeeping' genes. This means that, as with the prefabricated array, an array made from a cDNA library would include a significant proportion of genes not relevant to the experimental question. Furthermore, an array printed from a cDNA library has one large disadvantage relative to a prefabricated array—the identity of the clones is unknown without sequencing. One does not want to spend time and effort sequencing an entire library of genes, many of which are not relevant. What is needed is a way in which to focus this library on the condition of interest, to narrow the breadth of the genes assayed so that a more limited number of highly relevant genes is measured.

### Custom arrays can be generated from the products of subtractions to enrich for genes of interest

One solution is found in the RDA technique (Lisitsyn and Wigler, 1993; Hubank and Schatz, 1994). RDA is one of a family of techniques, such as suppressive subtractive hybridization (Diatchenko *et al.*, 1996), that couple hybridization-based deselection of common cDNAs to differential PCR amplification to enrich for differentially expressed transcripts from two populations of nucleic acids. Typically, two populations of mRNA are separately transcribed into cDNA, restriction digested and ligated to primers for PCR amplification. The two populations are then mixed and put through iterative rounds of amplification and subtraction of cross-hybridizing products. In each round, an excess of one population, called the driver, is used to remove identical transcripts from the second, less concentrated population, called the tester. This results in a sequential enrichment for clones more abundant in the tester population than the driver population. The products are then shotgun cloned or size-selected and cloned into a bacterial vector to create a cDNA library. Normally, two libraries are created, with the roles of tester and driver reversed, creating, in our example,

one library enriched for genes prevalent in the olfactory cortex and one library enriched for genes prevalent in other primary cortices. There is an implicit trade-off between the degree of subtraction and the complexity of the subtracted mixture. The more rounds of subtraction performed, the higher the differential expression, but the fewer the number of unique products. The fewer the number of rounds, the more unique species identified, but a larger number of false positives (i.e. equally expressed clones remaining in the subtracted library). One can make the best of this situation by empirically determining the optimal number of rounds to maximize differential expression, while detecting the highest number of differentially expressed clones.

#### **Subtraction alone may miss subtle differences in complex tissues**

When RDA is done alone, three to four rounds of subtraction are conducted and a handful of differentially expressed clones are identified. To detect a larger number of differentially expressed genes, or perhaps more subtle differences, one can perform fewer rounds of subtraction and assay the surviving clones using microarrays. The two libraries resulting from our hypothetical subtraction are printed onto a glass slide or filter, resulting in a microarray that contains exclusively genes present in the olfactory cortex and other sensory cortices, and biased towards those that are differentially expressed. Following differential hybridization experiments, the spots with the greatest difference in signal intensity are sequenced and identified. This offers several advantages over the use of prefabricated arrays. In addition to the previously mentioned advantages of having a more focused array, this methodology allows for the inclusion of novel genes on the array, thus leading to gene discovery. This ability to include novel or unknown transcripts is one of the greatest strengths of RDA-coupled microarrays. Finally, the array created becomes a tool to study similar systems. Does the olfactory bulb also express some of these olfactory cortex specific genes? Once the RDA-coupled microarray has been designed, it can be made in quantity and used in a variety of related experiments.

#### **Proof of principle for RDA-coupled microarrays**

It is evident that RDA-coupled microarrays provide an elegant manner in which to begin a comprehensive study of the most important genetic differences between two complex conditions or tissues, such as the differences that may underlie Müller's doctrine of specific nerve energies. But is such an approach feasible? Since the introduction of this combination of techniques to investigate the differences between malignant and non-malignant sarcomas (Welford *et al.*, 1998), several laboratories have used just such an approach successfully, including Boeuf's comparison of brown and white preadipocytes, and our laboratory's characterization of neural stem cells (Boeuf *et al.*, 2001; Geschwind *et al.*, 2001). However, several important ques-

tions arise when performing these sorts of studies. First, how should one analyze these arrays—how does one select which clones to sequence, i.e. what constitutes a hit on a microarray? What sorts of analyses are appropriate to these sorts of arrays? How much sequencing should one do? Are there methods to limit the sequencing of redundant clones? Second, what are the potential pitfalls—what is now known about RDA from doing these experiments? Are the most common clones in each library the most differentially expressed? What sort of artifacts may arise from using this system? Finally, and most importantly, do RDA-coupled microarrays fulfill their promises—do they provide a more focused array? Do they allow for the discovery of novel genes? Do they provide a tool for the study of similar systems?

Following the publication of our study of neural progenitors (Geschwind *et al.*, 2001), we had the opportunity to sequence all clones in one of our libraries of RDA products. This has allowed us retrospectively to assess the quality of our procedures and offer insight into the benefits and idiosyncrasies of RDA-coupled microarrays. Here, we briefly describe the experiment and discuss the lessons learned from the subsequent sequencing to answer the questions posed.

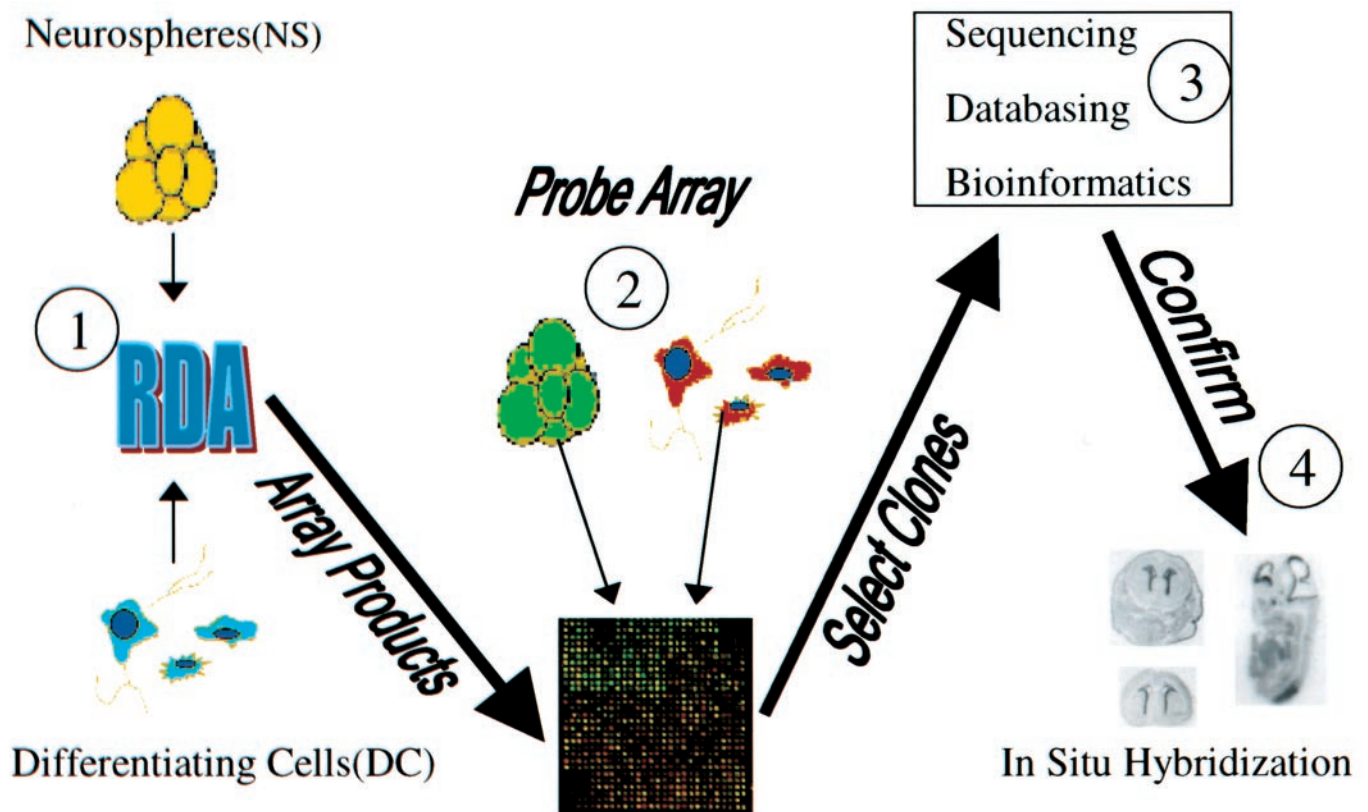
#### **RDA and microarrays for the study of neural progenitors**

Neural stem cells are pluripotent, self-renewing cells that may have the potential to give rise to the three major cell types of the brain: neurons, astrocytes and oligodendrocytes. Essential to the normal development of the brain, neural stem cells are thought to continue to produce progeny throughout the lifespan to replace lost cells, notably in the olfactory bulbs. As they could potentially prove a renewable resource to replace lost or damaged tissue associated with neurodegenerative disorders such as Parkinson's disease, some spinocerebellar ataxias, and Alzheimer's disease, it is of great interest to understand the genes responsible for maintaining these cells in an undifferentiated state and the genes responsible for commitment to specific lineages (Gage, 2000). To discover genes that are enriched in neural stem cell populations, we performed RDA followed by a cDNA microarray experiment (Geschwind *et al.*, 2001), as illustrated in Figure 1. The experiment was undoubtedly successful in that it has yielded a long list of candidate genes important in the maintenance and development of neural stem cells. Here, we will present some of the conclusions from a further in-depth analysis of the technique itself.

#### **How should one select which clones to sequence?**

##### **Effectiveness of selection criteria**

To select clones for further sequencing and analysis, we required that the ratio in the mean difference in signal intensity be at least 1.5 and that the ratio ranked in the top 16% in at least two of three replicates. By requiring expres-



**Figure 1** (1) We collected RNA from neurospheres (NS) and from sister cultures allowed to differentiate for 24 h under mitogen withdrawal (differentiated cells or DC). Two rounds of RDA were performed to create two libraries, one enriched for genes present in NS and the other enriched for genes present in DC. RDA is ideal for this sort of application—the comparison of two heterogeneous populations—because it allows for the subtraction of common transcripts, leaving, in our example, those transcripts that are present in the cell type that is unique to the NS condition: the neural stem cell. (2) Two thousand two hundred and eight clones from the NS library and 960 clones from the DC library were printed onto a microarray, which was subsequently probed with cy3 (red) and cy5 (green) labeled cDNA from the two populations. (3) Spots on the array that demonstrated the greatest difference in gene expression were sequenced and identities were determined using NCBI's BLAST algorithm (Altschul *et al.*, 1997), against the nr, EST and Unigene databases. Early sequencing revealed a high degree of redundancy in the clones spotted on the array, consistent with what others have found using this technique (Welford *et al.*, 1998). To eliminate the need for sequencing many redundant spots, we performed a hybridization experiment—cy3 and cy5 labeled RNA from the already sequenced clones was hybridized to the array. Spots with raw signal intensity above empirically determined values were eliminated from the list to be sequenced. From among the remaining spots, those that yielded a 1.5-fold greater expression in two of the three replicates were sequenced. Northern blots were used to confirm the differences in gene expression for 20 of the up-regulated clones. Consistent with other studies on the reliability of microarrays, all Northern blots confirmed microarray results qualitatively, though microarrays tend to underestimate the magnitude of the difference relative to Northern blots (Taniguchi *et al.*, 2001). (4) Finally, *in situ* hybridizations were performed to analyze the locations of gene expression in developing mouse brain. Many, but not all, genes examined showed strong expression in the ventricular zone, appropriate for genes expressed in neural stem cells.

sion in the top 16% of all clones in two of three replicates, we eliminated clones that had high mean ratios based solely on one spuriously high ratio skewing the mean, thereby emphasizing those clones that yielded consistently high ratios. These criteria were empirically determined to yield an amenable number of clones for follow up with *in situ* hybridizations and Northern blots. The libraries used to create the microarray provide a readily available source of probes to use for these confirmatory studies, demonstrating another advantage of a custom array. Two major conclusions can be drawn from this: first, if one is planning to follow up the microarray with confirmation with a separate technique it is better to use more inclusive criteria and, second, criteria based on consistent expression across replicates are fairly effective at eliminating false positives.

#### **RDA libraries are highly redundant. Systematic sequencing of the library results in diminishing returns, but allows for identification of a greater number of rare transcripts**

Large-scale sequencing of a library can require significant time and resources, and RDA libraries are highly redundant. Clones with identical sequence can be grouped into contigs. While half of the contigs were discovered in the first five plates (480 clones) sequenced during a systematic sequencing of the library, it is surprising and interesting to note that 10–20% of subsequently sequenced clones represented new contigs in each of the additional 18 plates. Thus, sequencing continued to be fruitful throughout the library. Therefore, how much sequencing to do becomes a choice between the desire to identify rare transcripts and the time and effort involved in sequencing. If time and resources



are limited, it is best to allow the microarray to decide which clones are a priority to sequence by selecting the most differentially expressed for further analysis.

#### **A hybridization experiment can reduce the amount of sequencing necessary, but at a cost**

The goal of this experiment, a 'repeating clones' experiment (Welford *et al.*, 1998; Geschwind *et al.*, 2001), was to reduce the amount of sequencing necessary by eliminating spots on the array representing clones that had already been sequenced. Thirty-five of the differentially expressed clones in the first two sequenced plates were re-hybridized onto the array to identify genes already sequenced. While 85% of the clones were correctly classified, there were four rare transcripts, up-regulated in the NS library relative to the DC, that were missed because they gave spurious hybridization signals. Since this was effectively a one-dye experiment, this result could be due to differences in DNA concentration across spots. This stresses the importance of using replication, even for this simple experiment, and the need to control for variability in concentration of DNA. A second probe, based on the PCR primer common to each clone, could be labeled with another dye, allowing a ratio of probe to DNA to be calculated for each spot. When deciding to use this approach, one must think in terms of a cost-benefit analysis. Redundant sequencing will be reduced, but some rare transcripts may also be missed.

#### **What is now known about RDA from these experiments?**

##### **Are the most abundant clones the most differentially expressed?**

In contrast to previous work (Welford *et al.*, 1998), we did not find a significant positive correlation between the number of clones in a contig and the relative expression of that clone. While it is clear that some of the contigs with strong differential expression also have a great number of clones, it is also clear that some contigs with no differential expression have many clones. This suggests that there are perhaps two factors influencing the number of clones representing a contig in the library. One would be the enrichment resulting from the RDA subtraction and the other would be the prevalence of the sequence in the starting populations, with sequences that are extremely common surviving the mild subtraction of a two-round RDA. Another important implication is that using the abundance of transcript in the library as a gauge of relative expression, as is done with SAGE, could lead to an overestimate of the biological importance of certain housekeeping transcripts in the experimental system.

##### **PCR hybrids may create RDA artifacts**

Recently, some authors have noted that RDA is vulnerable to certain types of PCR artifacts (Hansen-Hagge *et al.*,

2001). Notably, common repeat elements in a sequence may result in partial hybridizations that will survive the subtraction of RDA and be amplified along with genuinely differentially expressed products. These products can be detected by their partial sequence homology to a common element, and partial novel sequence. Hansen-Hagge *et al.* have suggested a novel ligation-mediated subtraction (LiMeS), which may resolve these issues of amplification of PCR hybrids by introducing a ligation step that necessitates full rather than partial hybrids prior to amplification (Hansen-Hagge *et al.*, 2001). To our knowledge, this technique has not yet been tested for the generation of a microarray, but may represent the next logical improvement on RDA-coupled microarrays.

#### **Do RDA-coupled microarrays fulfill their promises?**

##### **RDA-coupled microarrays create a more focused array**

As stated above, in the typical microarray experiment only 25–60% of the spots show measurable hybridization. Using this RDA-coupled microarray, >90% of the spots showed consistent hybridization, demonstrating that a greater majority of the genes on the array were sufficiently expressed in the system of interest. Thus, most of the genes on the array are relevant to the experiment and many of them are low-abundance species, demonstrating the utility of this approach.

##### **RDA-coupled microarrays facilitate gene discovery**

Of the 455 contigs discovered through sequencing, 209 mapped clearly to Unigene clusters. Ignoring 22 contigs that may represent PCR hybrids, that leaves 49 that have only partial homology to Unigene clusters and 70 that did not map onto Unigene clusters. These novel genes would not have been present on a prefabricated array. Most importantly, six of the seven of these genes that we have followed up thus far appear to be strongly expressed in the ventricular zone of the developing mouse brain, suggesting a role for these novel genes in neural stem cells on their immediate progeny.

##### **RDA-coupled microarrays provide a tool to study similar systems**

In creating a custom microarray, one also creates a tool that can be shared among researchers to investigate related systems to discover common patterns of gene expression. In a collaborative experiment (Terksikh *et al.*, 2001) gene expression in hematopoietic stem cells was compared to whole bone marrow using the NS/DC array. By comparing the lists of genes generated by this experiment and the genetic analysis of neural progenitors, Terksikh *et al.* could create a short list of genes common to two different populations of stem cells. Continuation of this approach could lead to the definition of a core set of genes expressed in stem

cells of all tissues of the body. Thus the RDA-coupled microarrays utility may expand beyond the experiment for which it was initially generated. Certainly, this array contains a large number of genes relevant to neural development in many systems and experimental paradigms. For example, this array could also be amenable to the study of developing cells of the rostral migratory stream, to examine the changes in their suite of gene expression as they mature into olfactory bulb neurons (Rousselot *et al.*, 1995).

## Conclusion

We have reviewed the use of RDA-coupled microarrays for the creation of custom microarrays and discussed the benefits, such as the improved focus of the array, the inclusion of novel transcripts and the creation of a tool to investigate similar systems, as well as the disadvantages, such as RDA artifacts and the need for sequencing. However, these disadvantages can be overcome and we believe that the coupling of subtraction techniques to microarray screening will be a fruitful approach in a variety of experimental systems.

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