

Basic science for the practicing physician: gene expression microarrays

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Objective: To provide a general overview of gene expression microarray technology and its relevance to physicians practicing allergy/immunology.

Data Sources: The PubMed interface to MEDLINE was searched for primary and review articles on gene expression microarrays. Specific articles on clinical applications of microarrays were retrieved, along with articles on use of microarrays in models of allergy, asthma, and immunologic diseases.

Study Selection: The author's knowledge of the field was used to include sources of information other than those obtained through the MEDLINE search.

Results: A synopsis of gene expression microarray technology, with emphasis on the relevance to allergy, asthma, and immunology, is presented.

Conclusions: Gene expression microarray technology allows investigators to measure gene expression across the genome. This has allowed researchers to improve our understanding of immunologic mechanisms in disease models. Initially used solely as a research tool, microarray-based clinical tests are now available, and many more are in development. Use of microarrays in allergy, asthma, and immunology will support the development of novel diagnostic tests for the physician and facilitate exploration of the basic mechanisms underlying allergic and immunologic diseases.

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INTRODUCTION

The development of genome sequencing technologies and the resulting sequences of human and other genomes has, in essence, built a catalog of all the genes within a given organism. Once this information was known, the limitations of measuring expression of only a handful of genes became clear. At the time, however, no technology was available that would allow an investigator to measure all of the genes (or a significant subset of genes) within a sample. The emergence of gene expression microarray technology in the mid-1990s has enabled genome-wide measurement of gene expression in a single experiment. This in turn has allowed for significant advances in our understanding of gene expression, regulation, and function and continues to serve as an important tool in basic science research. As the technology has matured, it has begun to move into clinical applications. This article seeks to provide a synopsis of this important technology for the practicing physician and of its current and potential relevance in both research and clinical medicine, focusing on the impact in allergy and immunology.

The PubMed interface to MEDLINE was searched for primary and review articles on gene expression microarrays. Specific articles on clinical applications of microarrays were retrieved, along with articles on use of microarrays in models of allergy, asthma, and immunologic diseases. The author's knowledge of the field was used to include sources of information other than those obtained through the MEDLINE search.

HISTORY AND TECHNOLOGY OVERVIEW

Gene expression microarrays emerge from the integration of 2 separate families of technologies: the first for nucleic acid detection and the second for the development of multiplex solid-phase assays. The technique for detection of specific nucleic acid sequences by hybridization of labeled nucleic acid sequences to known sequences immobilized on a solid support was developed by Edwin Southern in the 1970s.¹ Initially used for specific DNA sequence detection as the Southern blot, the technique was adapted for messenger RNA (mRNA) detection² and subsequently named the Northern blot. Although this use of a solid support was novel in its use for the detection of nucleic acids, it had been used previously in radioimmunoassays³ and was critical in the development of the enzyme-linked immunosorbent assay (ELISA).⁴ Whereas ELISA uses the solid phase to immobilize the target protein (Fig 1a), the Southern (and Northern) blot uses the solid phase to immobilize the target nucleic acid (Fig 1b). In each case, reagent is then added to specifically detect and quantify

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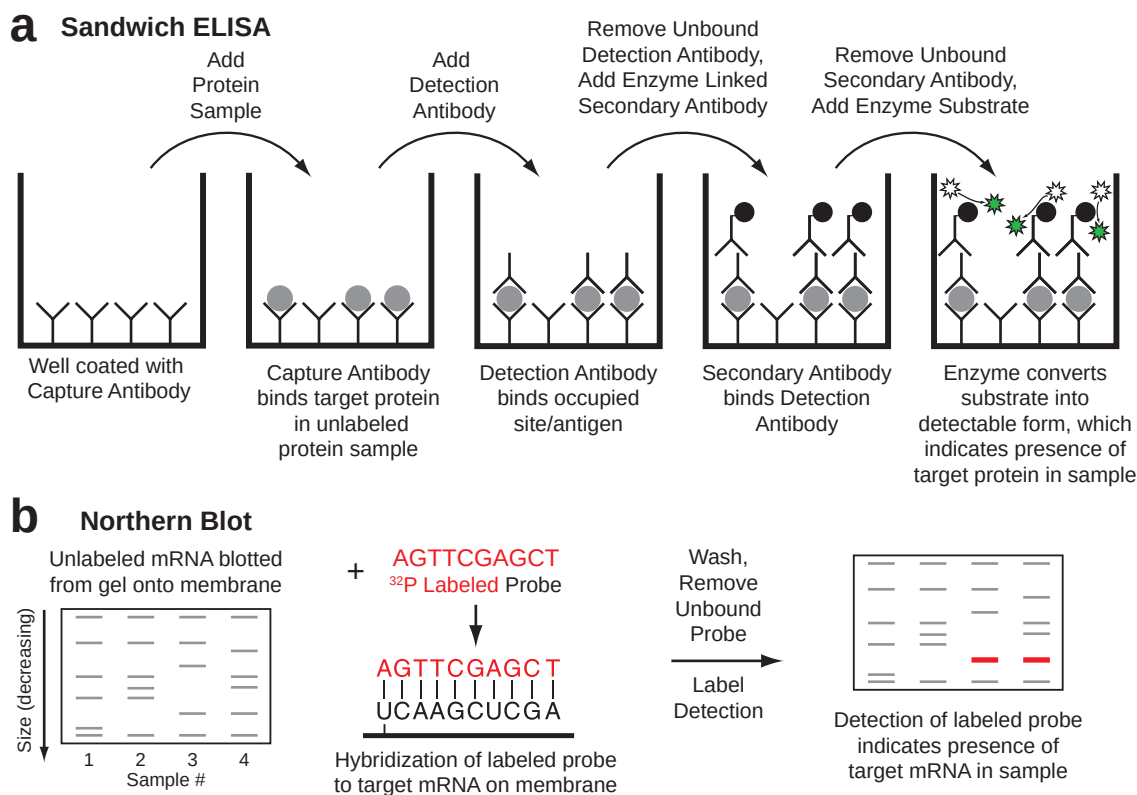


Figure 1. Precursors to the gene expression microarray. The enzyme-linked immunosorbent assay (ELISA) (a) represented the first use of a solid phase to immobilize proteins for detection and combined this with nonradioactive, enzymatic substrate-based detection of proteins by specific antibody. The Northern blot, a modified Southern blot for messenger RNA (mRNA) detection (b), represented the first technology for specific mRNA detection, adapting the use of the solid phase to immobilize the mRNA sample rather than the protein sample. Each band denotes an individual mRNA species, separated by gel electrophoresis before blotting onto the membrane. A labeled sequence (probe), specifically complementary to a portion of the mRNA of interest (target), is added to the unlabeled sample mRNA on the gel. If the target is present, the probe will hybridize. Once excess probe is removed, any label detected indicates binding of probe to target and thus the presence of the target.

the molecule of interest. Modifications of both techniques were developed that place the detection reagent (antibody or nucleic acid probe) on the solid support and leave the analyte in the liquid phase (eg, reverse Northern blot, dot blot).^{3,5}

The next significant technological developments occurred in the 1980s, when Roger Ekins and colleagues developed the ambient analyte immunoassay.^{6,7} Ekins et al describe a system for detecting multiple analytes via the use of microspots. Each microspot would contain a capture antibody to bind the analyte if present. A second antibody would be used to detect the presence of bound sensor antibody. If labeled fluorescently with different fluorophores, the ratio of fluorescence intensity between bound and unbound sensor antibody could be determined by laser scanning confocal microscopy, and with appropriate standards, this could be used to precisely quantify the amount of a given analyte (Fig 2a). Thus, Ekins et al, although conceptualizing the technique for the detection of protein analytes, had in essence described the microarray—the microspots, the bound detection reagent, and the

fluorescent readout—all features of gene expression microarray technology.^{6,8}

As this concept was becoming mature through the work of Ekins and others, genome sequencing projects were simultaneously being started in multiple organisms. The gene expression microarray emerged as a direct consequence of the multiple whole-genome sequencing projects started in the 1990s. As the amount of known genes increased exponentially, the need arose for a technique that would allow for the detection and quantification of multiple targets with a single assay and was answered by the development of the gene expression microarray.

However, the development of the gene expression microarray required several new technologies and information resources. First, the use of impermeable support materials allowed for the use of smaller amounts of probe material and resolved issues arising from diffusion kinetics.⁹ Second, probes needed to be selected for each target. This information came from the sequencing projects, initially as largely unan-

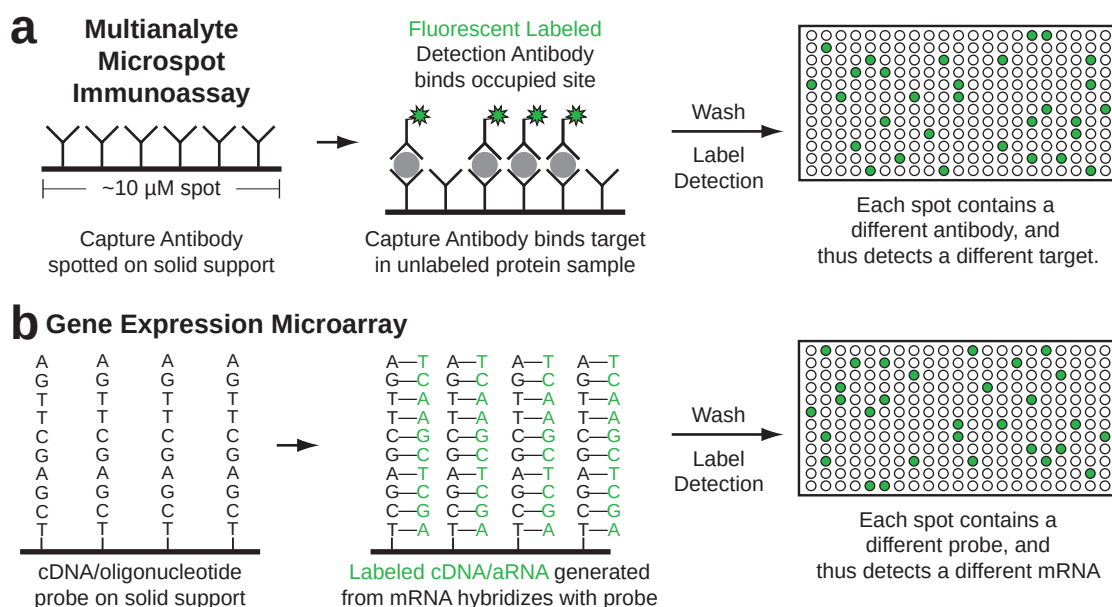


Figure 2. Parallels between multianalyte microspot immunoassay and gene expression microarrays. As molecular biology techniques leading to genome sequencing emerged, Ekins and colleagues^{6–8} developed the concept of a multianalyte microspot immunoassay, capable of looking for multiple targets in one sample (a) but fundamentally derived from the immunodetection methods of the enzyme-linked immunosorbent assay. The gene expression microarray (b) emerges as a combination of the basic molecular biology of the Northern blot and the high-throughput, multiple target, microspot assay envisioned by Ekins et al. Although technology varies from vendor to vendor (Table 1), panel b outlines the fundamental workings of any gene expression microarray. cDNA indicates complementary DNA; aRNA, antisense RNA; and mRNA, messenger RNA.

notated complementary DNA (cDNA) libraries and over time as the annotated sequences of each gene and each mRNA transcript. Third, probes needed to be synthesized, either directly on the array slide^{10,11} or conventionally,¹² and subsequently attached to the glass substrate.¹³ Fourth, systems for detection of sample material bound to probe needed to be developed. In this instance, microarray developers were able to take advantage of advances in labeling techniques for nucleic acids¹⁴ and laser scanning confocal microscopy,¹⁵ each developed for other purposes. This led to methods for the detection of small amounts of labeled nucleic acid bound to complementary probes on small spots via confocal or nonconfocal laser scanning techniques. Fifth, image analysis techniques^{16,17} and data analysis techniques¹⁸ were developed and continue to evolve. The statistical analyses for microarray experiments represent a significant challenge given the large number of variables, which has led to multiple approaches for microarray data analysis.

Ultimately, although different approaches have been taken in the design of probes, the synthesis, the substrate, the label, and detection (Table 1, Fig 3), all gene expression microarrays rely on hybridization of labeled nucleic acid (labeled mRNA or cDNA) in the sample to complementary probes on the microarray. Each spot ideally detects only 1 sequence and, if the oligonucleotides are selected correctly, only 1 gene or transcript, allowing for the detection and quantification of each assayed transcript from a single sample (Fig 2b). More

recently, thanks to improvements in miniaturization, microarrays are available that detect the expression of not only 1 transcript but each individual exon within a transcript, allowing for the analysis of splicing variants.¹⁹

Initial microarray experiments used arrays with relatively few features, did not cover the genome, and used crude statistical methods for analysis, most often simple fold-change of gene expression between nonreplicated samples.^{13,20} However, over time, the field has matured significantly because of several factors. Array coverage of genomes is now essentially complete for mouse, human, rat, and several other organisms (Table 1). Microarray experiment design itself has become a field of research, and the modern experiment is designed with biological replication,^{16,21} with continuously improving normalization^{16,22} and statistical data analysis methods.^{16,18,23} Finally, although there are differences in technology among platforms, recently published studies from the MicroArray Quality Consortium suggest that results are highly consistent among platforms²⁴ and consistent with quantitative assays for gene expression.²⁵

BASIC SCIENCE APPLICATIONS

Immunologic targets have been evaluated with microarrays since the dawn of the technology. After their initial publication of gene expression microarrays in *Arabidopsis thaliana*,¹³ the first microarray study of human cells was published, using the Jurkat T-cell line to evaluate gene

Table 1. Comparison of Gene Expression Microarray Platforms^a

Platform Manufacturer	Spotted	GeneChip Affymetrix	BeadChip Illumina	NimbleChip NimbleGen (Roche)
Probes	In house; various (eg, Agilent, ABI, Eppendorf) cDNA/Oligo, 30–70 bp	25 bp oligo, with positive match and mismatch probes	50 bp oligo, with 23- to 29-bp address sequence	24–70 bp oligo, usually 60 bp
Manufacturing Process	Mechanical spotting onto glass slides, plastic slides, or nylon matrices	Probes are synthesized directly on quartz wafers using photolithographic masks	Each bead has a specific probe attached. Beads self-assemble on a microwell planar silica slide	Probes are synthesized directly on glass slides using maskless photolithography via digital micromirror device
Array Capacity (maximum No. of probes)	50–57,000	1.4 million	1.6 million	2.1 million
Sample and labeling	cDNA or aRNA, 1- or 2-color fluorescent (i.e. Cy3/Cy5), chemiluminescent, radionuclide (eg, ³³ P, ³² P)	Biotin labeled aRNA, detected with phycoerythrin-streptavidin-antibody complex	Biotin labeled aRNA, detected with streptavidin-Cy3	Biotin-labeled aRNA, detected with streptavidin-Cy3
Detection	Phosphor imager, laser scanner	Confocal laser scanner	Confocal laser scanner	Nonconfocal laser scanner
Starting amount of RNA needed	Varies	1–15 µg with 1 round of IVT; 10–100 ng with 2 rounds of IVT	50–500 ng with 1 round of IVT	10 µg
Organisms	Any	24 catalog plant and animal arrays, exon arrays (human/mouse), custom available	Human, mouse, and rat, custom available	100 catalog arrays, including human, mouse, and rat, custom available

Abbreviations: aRNA, antisense RNA; cDNA, complementary DNA transcribed from RNA; Cy3, fluorescent Cyanine dye 3 (excitation, 550 nm; emission, 570 nm); Cy5, fluorescent Cyanine dye 5 (excitation, 649 nm; emission, 670 nm); IVT, in vitro transcription (process by which a small amount of mRNA is transcribed into cDNA, then amplified into a larger amount of aRNA. Thus, less input mRNA is needed for microarray analysis¹⁴).

^a Data are from manufacturers' published information and literature review.

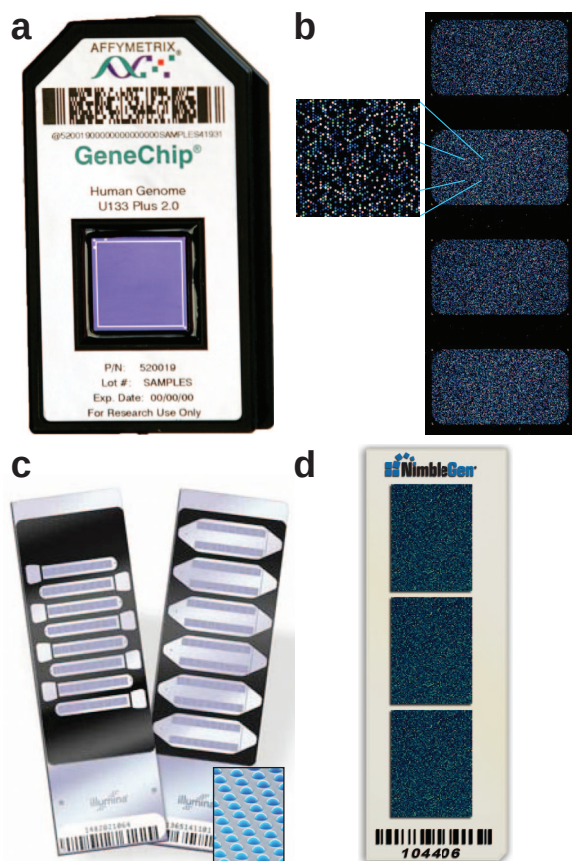


Figure 3. Examples of current microarrays. a, Affymetrix Human U133 Plus 2.0 Array contains 1.3 million features and assays 47,000 targets. b, Agilent G4112F Human Genome Oligo Microarray contains 4 arrays each with 45,015 features and assays 43,376 targets in each of 4 samples (inset: individual spots under magnification). c, Illumina HumanRef-8 v2 (left) and Human-6 v2 (right). HumanRef-8 v2 contains 8 arrays each with 900,000 beads and assays more than 22,000 targets for each of 8 samples. Human-6 v2 contains 6 arrays each with 1.8 million beads and assays for more than 48,000 targets for each of 6 samples. Bead arrays are composed of randomly arranged beads (inset) each containing a probe for a target and an address allowing software to identify which probe is on a given bead. d, Nimblegen HD2 Array contains 2.1 million features; applications in gene expression are forthcoming. Nimblegen's human gene expression array contains 385,000 features (HG18 60mer expr) and assays 47,633 targets with 8 probes per target. (All images were obtained from the individual manufacturers Web sites, product literature, and/or media relations.)

expression under heat shock, phorbol-12-myristate-13-acetate (PMA), and control conditions.²⁰ The microarray has continued to be a useful tool for the exploration of cell signaling and function throughout the immune system since that time.

Since those early studies, microarrays have been leveraged extensively in basic immunology. Many groups have used microarrays to assay isolated cellular response to immune stimuli, whereas others have focused on the *in vitro* effects of transcription factors, chemokines, and cytokines. This ap-

proach has led to a greater understanding of the genes induced by interferon,²⁶ including the discovery of novel interferon stimulated genes, and the specific response to virus orchestrated by interferon.²⁷ Studies of STAT1 transcriptional targets²⁸ and STAT1 function in epithelial host response²⁹ have led to new insight into STAT function and relevance. Other work in antiviral immunity examines the effect of human immunodeficiency virus on immune cells³⁰ and the role of CCL5-CCR5 interactions in antiviral host defense.³¹ Explorations of host-pathogen interactions include analysis of antigen-presenting cell³² and epithelial cell³³ gene expression in response to bacterial pathogens and/or proteins.

Other work has focused on gene expression in disease models. For the purposes of this review, I focus on asthma, but work has also been performed in models of many other diseases. Both the allergic sensitization/challenge and virus-induced mouse models of asthma have been explored using microarray analysis.^{34,35} Other groups have used microarrays to study interleukin 13 (IL-13), a cytokine implicated in asthma pathogenesis.³⁶ From this work have come insights into the roles of many potential asthma mediators, including arginase, IL-13/STAT6, Clca3/Gob5, C5, and many others.^{34,37} Results from these studies have led, in part, to work on multiple potential therapeutics for human asthma, including treatments directed against IL-13³⁸ and arginase.³⁹

The work on disease pathobiology *in vitro* and in animal models discussed herein has led to extensive microarray studies of gene expression in human patients. Studies in asthma initially examined the difference between asthmatic and nonasthmatic airway tissue.⁴⁰ However, more recent work has focused on the response to therapy. A recent study of patients with asthma treated with corticosteroids disclosed genes that respond to therapy in the context of asthma.⁴¹ Another recent study evaluates gene expression in peripheral blood mononuclear cells from patients with asthma in an effort to develop predictors for response to corticosteroids.⁴² Thus, pharmacogenomics of asthma, in terms of understanding the variation among patients in response to asthma therapy, has and continues to be studied with microarrays. Outside asthma, work has been performed in evaluating gene expression in patients with atopic dermatitis,⁴³ allergic rhinitis,⁴⁴ and primary immunodeficiencies.⁴⁵ These studies have allowed investigators to discover new potential therapeutic targets and new disease markers, which will ultimately lead to novel therapeutics and diagnostics.

Clinical Applications

As indicated herein, the gene expression microarray is an enabling tool for research scientists. However, clinical applications are only now beginning to emerge. To examine the potential of gene expression microarray-based therapeutics, I discuss the MammaPrint from Agendia because it is the first Food and Drug Administration (FDA)-approved gene expression microarray diagnostic test.⁴⁶ The MammaPrint's story^{47,48} begins with a whole-genome gene expression study of breast tumors performed at the Netherlands Cancer Institute

in Amsterdam from 78 node-negative breast cancers. This profile of noninvasive tumors was then compared with a larger cohort of both node-positive and node-negative cancers and yielded a 70-gene signature that was found to predict both time to distant metastasis and overall survival in breast cancer. Further, the 70-gene signature was better at assessing the risk of metastasis more accurately than clinical risk evaluation tools currently in use in oncology. The success of this gene signature in predicting risk for metastasis and overall survival led to the development of a custom-made, spotted oligonucleotide array that measures expression of the 70 genes, the MammaPrint.⁴⁷

The approval by the FDA of the MammaPrint is notable in several ways. First, the FDA has not generally had oversight over or regulated laboratory tests the way it does drugs or devices. The FDA approved the MammaPrint and simultaneously began exploring the need for greater oversight of laboratory tests, especially multigene tests like the MammaPrint and another tumor gene expression assay, the OncoType DX, which has not yet been approved.⁴⁶ Second, it is the first conversion of the gene expression microarray from research tool to clinical diagnostic and portends the future development of similar tests for other diseases.

Although the data for the MammaPrint were adequate to obtain FDA approval and have been validated by groups outside the Netherlands,^{47,48} there is still concern about using the test as a guide for therapeutic decision making in clinical oncology. This has led to the MINDACT (Microarray In Node negative Disease may Avoid ChemoTherapy) trial. In this international, prospective, randomized, multicenter trial, the 70-gene signature classifier will be compared with clinicopathologic criteria for determining whether a patient with node-negative breast cancer should receive adjuvant chemotherapy.⁴⁸ Not only will the trial validate the initial 70-gene signature, but it will also be used to evaluate markers of treatment response; in essence, the samples and data collected will serve to refine the MammaPrint and/or will lead to the development of other microarray-based tests for breast cancer.

CHALLENGES AND CONSIDERATIONS

Although the ability to assay gene expression in a whole-genome context allows many opportunities, use of the gene expression microarray and interpretation of microarray data carry several caveats. Initially, the major concern regarding gene expression microarrays was the question of accuracy: could microarrays perform as well as “gold standard” methods for quantifying gene expression (ie, quantitative reverse-transcriptase polymerase chain reaction). These concerns have largely been addressed with ongoing improvements in microarray technology, analysis software, and the accuracy of the microarray as supported by the MAQC study.^{24,25} However, as those concerns have been addressed, new concerns have emerged.

One major challenge in gene expression microarray analysis involves issues of multiple comparisons. The term *mul-*

tiple comparisons refers to the statistical issues that arise when several hypotheses are tested simultaneously on a given sample. If significance testing via the *t* test is performed without considering the effects of multiple comparisons, false-positive discoveries occur by chance alone, leading to genes being reported as statistically significantly differentially expressed, when this is not the case. Early microarray analyses using simple filtering followed by *t* test comparison between samples or conditions led to significant percentages of false discovery. Many contemporary microarray analysis methods^{18,23} take this into account, controlling for the expected number of false-positive results in a given experiment (the false discovery rate¹⁸). Reducing the reported list of differentially expressed genes in this fashion yields gene lists that are more consistent with other measures of gene expression and is a critical analytical consideration when selecting an analysis approach to microarray data.

Beyond the issues of statistical methods, the greatest challenge for microarray analysis in human diseases is the difficulty in sorting out the processes involved in a disease or pathobiology of interest when so many genes are differentially expressed. To a great extent, this difficulty reflects the large number of new genes (and in this moment, new splice variants and the expanding classes of noncoding RNAs) discovered by the ongoing sequencing of genomes and transcripts. Thus, once we have found genes differentially expressed, the list invariably contains genes for which function has never been explored experimentally or for which new functions are later found, sometimes contradicting prior information. Complicating this is the lack of information on molecular interactions; gene expression microarrays can only measure gene transcription, not translation, and certainly not the complex regulatory pathways and interactions leading to the array result, a snapshot of one sample at one moment. This leads to the current, somewhat frustrating state of gene expression microarray data in asthma, where although many differentially expressed genes have been found and verified,^{34–42} potential biomarkers and/or therapeutic targets are still being validated, evaluated, and explored, with no clinical diagnostic or therapeutic benefit to date. However, as data continue to be generated, investigators continue to improve their analytical approaches and experimental designs. An entire field of biology (Systems Biology) has emerged, with the goal of unraveling the complex networks of cells and signals underlying all biological processes. This ongoing exploration should lead to better tools to help us interpret gene expression microarray data and will ultimately allow us to leverage this technology in the diagnosis and treatment of diseases.

A GLIMPSE INTO THE FUTURE

The gene expression microarray has already proven useful in the study of allergy, asthma, and immunology, but to date the utility of this technology has been largely limited to basic science investigations. The next phase of the gene expression microarray's story will involve the development of clinical

tests. One area of clear need in the asthma field is in the prediction of patient response to therapy. Although asthma therapy has advanced greatly, largely because of the use of inhaled corticosteroids, we are still limited in our ability to predict which patients will respond to one agent or the other. This is illustrated in the results of a National Heart, Lung, and Blood Institute Childhood Asthma Research and Education Network trial, which compared inhaled fluticasone to montelukast in a 18-week, crossover design.^{49,50} Although fluticasone monotherapy was superior to montelukast monotherapy in terms of clinical outcomes and spirometry,⁴⁹ there was significant variance in response to therapy, with some patients responding to both therapies, fluticasone monotherapy alone, or montelukast monotherapy alone and most responding to neither therapy.⁵⁰ If a gene expression signature could be determined for different patterns of therapeutic response to asthma (or different disease phenotypes encompassed in the clinical diagnosis of asthma), we could potentially avoid morbidity from uncontrolled disease because of the wrong choice of controller therapy and also prevent overmedication with multiple agents; we would be able to pick the agent most likely to work for that particular patient. Similar tests can easily be envisioned for other diseases, and thus, the gene expression microarray appears poised to enable the march toward personalized medicine.

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