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Application Notes

Microarray Applications in Infectious Disease

Modern biology and genomic sciences are rooted in infectious disease research. One of the earliest investigations into the biology of pneumococci led to the landmark 1944 discovery that genes are made of DNA and not protein. A half century later, inexpensive, reliable, and automated DNA sequencing methods have allowed scientists to sequence the complete genomes of nearly 2,000 different bacteria and viruses, as well as the genomes of multiple host organisms, including humans. In the wake of this flood of information, we are now faced with the far more daunting task of determining how the knowledge of billions of nucleotide bases can be put to practical use to understand disease and ultimately improve the human condition.

Microarrays opened up an entire new world to researchers[1-3]. No longer is one restricted to studying a unique single aspect of the host/pathogen relationship; instead one can explore a genome-wide view of this complex interaction. This comprehensive and hypothesis-free analysis method is helping scientists discover and understand disease pathways, and ultimately develop better methods of detection, treatment, and prevention (Figure 1).

Data generated from whole-genome microarray studies are richer and deeper than ever before. Data from a single array experiment – whether gene expression or DNA analysis – can often be used for a number of different studies that otherwise would have required the compilation of data from numerous independent experiments. For instance, the same expression data from an infected host could be used to understand the mechanism of virulence, and might also be used to identify a unique host-response signature for pathogen and disease identification purposes. Moreover, arrays are being designed to simultaneously monitor whole-genome host and pathogen gene expression, providing a complete view of the progression of an infectious disease state—how a pathogen responds to its host and the host to its pathogen. The flexibility of the Affymetrix GeneChip® microarray analysis allows a single array and a single experiment to encompass different types of infectious disease studies.

The most recent generation of microarrays allows scientists to readily perform DNA sequence analysis, often providing the ability to sequence complete genomes in a single experiment. Whereas conventional sequencing methods typically require extensive resources (time, cost, and labor), the individual scientists can now use GeneChip CustomSeq® Arrays, for example, to resequence up to 300 kb of a genome within 48 hours with minimal amplification of the genomic target. By comparing sequences from different strains, scientists can now identify important genetic variations, leading to improved typing systems, vaccine candidates, and pathogen detection and identification methods. This application note describes the impact of GeneChip resequencing, genotyping, and expression arrays in all areas of infectious disease research, from understanding pathogenesis to developing better therapeutics and diagnostics (Table 1).

Figure 1: Microarray applications in infectious disease.

Pathogenesis | Host susceptibility | Drug response | Vaccine development | Pathogen identification

Table 1: Affymetrix® GeneChip® microarrays exploring sequence variation, as well as gene expression, have been used in multiple aspects of infectious disease research.

Study Goal	Application	GeneChip® CustomSeq® Resequencing Array	GeneChip® Mapping 100K Array or 500K Array Set	Whole-Genome Expression Arrays
Pathogenesis	· Identification of virulence factors · Immune evasion mechanisms	Pathogen Pathogen	— —	Pathogen Host, Pathogen
Susceptibility	· Host susceptibility to pathogen	Host	Host	Host
Drug Response	· Host response to drug · Pathogen response to drug	Host Pathogen	Host —	Host Pathogen
Vaccine Development	· Antigen discovery	Pathogen	Host	Pathogen
Pathogen Identification	· Host response signature · Pathogen sequence analysis	— Pathogen	Host —	Host Pathogen (CGH)

Host-Pathogen Interactions

HOST IMPACT ON PATHOGEN EXPRESSION

Relatively simple pathogenic microorganisms, like bacteria and viruses, often employ complex mechanisms of virulence developed over millions of years of evolution, which have resulted in a variety of diverse ways for pathogens to successfully infect their host by subverting host defense pathways. While scientists have typically focused on small subsets of genes as suspected virulence factors, the emergence of microarrays has enabled microbiologists to explore genome-wide expression, uncovering virulence pathways consisting of many genes that may have been previously unknown. This technique has proven particularly useful when analyzing a pathogen's response to its host environment. For example, Wolfgang *et al*. recently used GeneChip *Pseudomonas aeruginosa* Genome Arrays to study the interaction between *P. aeruginosa* and the airway liquids from chronically infected cystic fibrosis (CF) patients[4]. By examining genome-wide expression, the group identified genes that exhibited a statistically significant change in expression as a result of that environment. They noticed that a majority of the repressed genes encoded proteins relating to flagellar biosynthesis, and when they examined the bacteria by electron microscopy, they found that surface flagella were indeed reduced. Flagella are highly immunogenic and repression is a way for the bacteria to avoid detection by host defense mechanisms, allowing the bacteria to successfully establish infection in immunocompromised CF patients. By understanding the virulence factors and toxins elaborated by *P. aeruginosa* and other pathogens, researchers are able to identify disease mechanism pathways for potential treatment.

PATHOGEN IMPACT ON HOST EXPRESSION

However, a complete understanding of infectious disease requires scientists to examine both the virulence factors expressed by the microbe, as well as the host response mechanisms and host pathways that are subverted by that microbe. To this end, scientists have used GeneChip microarrays to understand virulence by monitoring changes in host gene expression following challenge with a microbe[5-9] or with purified virulence factors. Izmailova and colleagues used human GeneChip expression microarrays to study the effects of HIV-1 and its Tat protein (a major HIV virulence factor) on immature dendritic cells, which are among the first cells to be infected by retroviruses[8]. Once again, by examining genome-wide expression, the researchers were able to identify induction of a complete interferon pathway. Chemokines are among the molecules induced by this pathway, which in turn recruit macrophages and T cells, which are the ultimate targets of the virus and thus facilitate the expansion of the viral infection. Based on these studies, designing therapies against the Tat protein or against the members of the interferon pathway may produce the combined benefit of limiting viral transcription while reducing the expansion of viral infection into uninfected cell types.

While most infectious disease microarray research has focused on gene expression studies, new microarray tools for DNA sequence analysis are now available, which allow scientists to begin looking at the biology of infectious disease in another genomic light. Microarrays designed for custom genotyping enable researchers to explore sequence variation between pathogenic strains at single nucleotide resolution. The ability to quickly resequence a genome in a single experiment dramatically reduces the expense, time, and labor that would have been required using traditional sequencing methods and enables scientists to more readily determine how genetic differences are manifest in disease outcomes. For example, in the face of the SARS outbreak of 2003, Affymetrix, NIAID and TIGR collaborated to develop a microarray to resequence the complete genome of the SARS virus. The Centers for Disease Control and Prevention is also using Affymetrix resequencing arrays to identify and catalog hundreds of different Variola major (smallpox) strains. By sequencing more isolates, scientists can more easily relate pathogen subtypes to patient outcome and develop a better understanding of which subtypes are, for example, the most virulent. Clinicians can also use this information for diagnostic purposes by identifying the specific strain responsible for disease (see Pathogen ID below). These tools are also useful for epidemiologists, who can study how a pathogen is evolving over time while surveying its spread into

different geographies and populations. Monitoring the full genome over the time of an epidemic, for example, allows the tracking of multifactorial characteristics, such as drug resistance, that are crucial for the treatment of these diseases.

Pathogen Detection and Identification

Perhaps the most direct application of microarray technology for infectious disease is the ability to quickly identify an infecting pathogen. Because pathogens have distinct genetic compositions and microarrays are able to examine all gene sequences, the array is an ideal tool for this application.

Wilson *et al*., for example, developed a Multi-Pathogen Identification (MPID) microarray that identifies eighteen pathogenic prokaryotes, eukaryotes, and viruses. In this study[10], researchers amplified unique regions of DNA from each microorganism, and then used the microarray to detect the presence or absence of pathogen-specific DNA sequences. In some cases, the limit of detection was found to be as little as 10 femptograms of pathogenic DNA, much below the detection limits of other existing technologies. Microarrays have also been used in comparative genome hybridization studies to distinguish different strains of *P. aeruginosa*[11] and *Mycobacterium tuberculosis*[12], and have even been used to examine 16S ribosomal DNA sequences for the identification of pathogens, such as *M. tuberculosis* to the species level[13], and to characterize mutations in the *rpo*B gene that confer rifampin resistance in *M. tuberculosis*[14].

Strain Typing/Epidemiology

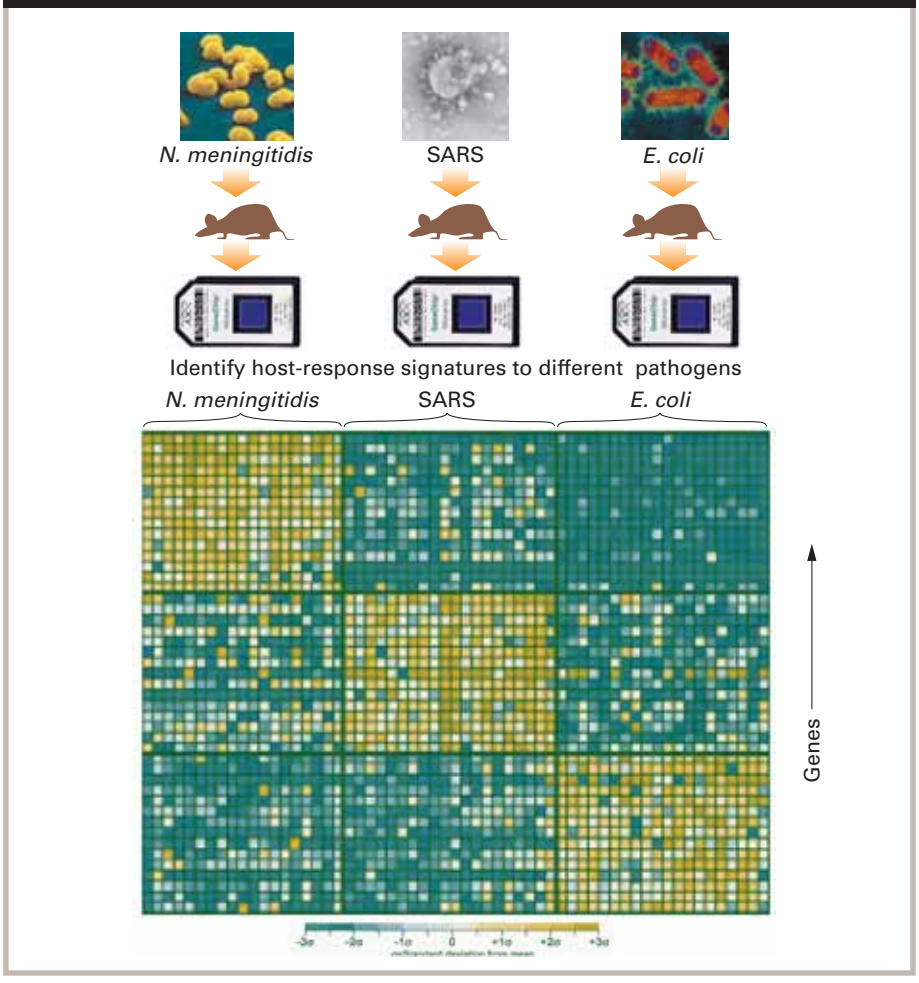
In addition to detection and identification of pathogens, microarrays are ideal for characterizing genetic differences between isolates of the same species to the strain level using a resequencing approach.

GREATER ACCURACY AND DISCRIMINATION

Recently, scientists at the United Kingdom's Health Protection Agency (HPA) used resequencing microarrays containing genomic sequences that vary between different meningitis subtypes to classify different isolates of *Neisseria meningitidis*[15]. Using the resequencing array, the scientists were able to correctly classify 45 samples that were previously identified by traditional methods, but more importantly, they were able to classify 12 previously unclassifiable samples into existing meningitis serotypes. Traditionally, the HPA has classified meningitis by using immunoassays to identify serotypes in combination with capillary sequencing to identify sub-serotypes. In addition to being more accurate than the traditional serotyping methods, resequencing microarrays provide results in just 48 hours, much faster than traditional methods. The meningitis resequencing array can now be used to quickly identify new meningitis strains, as well as for epidemiological studies and vaccine research.

In a different study, the ability of a GeneChip array to accurately discriminate 21 oxacillin-resistant isolates of *Staphylococcus aureus* was compared to that of ribotyping and pulsed-field gel electrophoresis (PFGE), both standard molecular epidemiological methods[16]. Although strain clustering was similar among the three methods, the GeneChip microarray results provided a higher level of discrimination, especially on such genetic elements as virulence factors, antimicrobial resistance determinants, and *agr* type.

Figure 2: Host response signatures for pathogen identification. In this animal model, mice are infected with different pathogens and whole-genome expression is measured by microarray analysis, resulting in a specific host-response signature. This type of study can identify specific patterns for microarray diagnostic applications.



GeneChip arrays have also been applied to strain typing of biothreat agents, which could be useful for discerning engineered from naturally-occurring pathogens and for the determination of forensic attribution. For instance, Zwick and colleagues used a custom resequencing array to analyze a panel of 56 *Bacillus anthracis* strains[17]. The total time to generate results was much faster with the array as compared to conventional sequencing methods and the results were comparable, demonstrating that the arrays represent a rapid and cost-effective means of generating sequence data for strain typing purposes.

EXPRESSION PROFILE SIGNATURES FOR PATHOGEN IDENTIFICATION

Researchers are also using GeneChip expression arrays to identify host expression profiles, or signatures, which can be used to identify the pathogenic agent. While the genes making up these signatures may be important to disease mechanism, identifying gene function is not critically important for this type of predictive application. Much like the way expression profiles and sets of predictor genes have been used to classify different types of cancer, profiles can also be used to classify different kinds of infectious disease (Figure 2). For example, when comparing *Chlamydophila pneumoniae*, *Chlamydia trachomatis*, and intracellular *Salmonella typhimurium*, Hess *et al*. found distinct host-response expression profiles[18]. Genus- or group-specific transcriptional response patterns likely contribute to the different pathologies of each disease, but could also be used to characterize disease via host response signatures.

In another example, Nau and coworkers examined the whole-genome expression from macrophages that had been exposed to Gram-negative or Gram-positive bacteria. These two types of bacteria represent the two divisions of prokaryotes, and contain, among other variations, different cell wall structures. By analyzing macrophage expression, the researchers found a distinct host response expression signature for both types of bacteria, with the Gram-negative bacteria gene expression changes encompassed by those induced in Gram-positive bacteria. This distinct response may provide the basis to diagnose clinical Gram-negative infections[19].

Disease Susceptibility

Why is one person susceptible to an infection while another one is not? Surely, environmental exposure plays a crucial role, but the genetics of the individual contribute as well. By using microarrays for DNA sequence analysis, one could rapidly identify mutations in "susceptibility genes" that influence an individual's risk of acquiring disease. For instance, mutations in the CKR5 human co-receptor for HIV have been demonstrated to endow resistance to viral infection[20]. Researchers anticipate using resequencing microarray technology to quickly screen for these mutations in large populations and understand an individual's predisposition to the disease. Additional "susceptibility genes" such as the HIV receptor CCR2[21] can readily be included on the array and sequenced in the same single assay. Because scientists are able to select any portion of the genome, including long regions of contiguous sequence or different segments of genes for resequencing, a full understanding of all the major genetic contributions to susceptibility and its monitoring is possible.

While researchers often use microarrays to examine specific genes, high-density microarrays allow scientists to look at genetic variation across the complete genome. The GeneChip Mapping 500K Array Set, for example, is capable of genotyping over 500,000 single nucleotide polymorphisms (SNPs) and is the latest in a family of products designed for previously unaffordable or unattainable whole-genome association studies. These high-density genome scans have typically been performed to understand the genetics of complex diseases and drug response, but the same types of studies can be designed to understand infectious disease susceptibility. By examining large groups of equally exposed individuals – some who develop disease, and others who do not – scientists will be able to identify genes associated with resistance or predisposition towards disease.

Drug Response

GENOTYPIC VARIATION IN THE PATHOGEN

Antibiotic resistance represents a serious threat to the effectiveness of traditional antibiotics. DNA sequencing microarrays allow researchers to look for antibiotic resistance at a level of detail not previously possible. There are literally dozens of genes and mutations that encode various forms of antibiotic-resistance. For example *mec*A encodes methicillin resistance[22], *tet*A encodes tetracycline resistance, *bla* encodes penicillin resistance, and mutations in the *rpo*B genes confer rifampicin resistance. With conventional methods, scientists can only examine one gene at a time, and many of these assays do not provide the type of DNA sequence information that is required to identify single nucleotide mutations responsible for resistance to drugs like rifampicin[23]. By using resequencing microarrays, researchers can not only identify the presence of multiple resistance genes in a single experiment, but can also generate the sequence information that can identify specific antibiotic-resistant mutations. In what has become one of the largest threats to public health of this century, new mechanisms for antibiotic resistance continue to emerge, and with that, resequencing microarray technology is well positioned to help scientists generate a more complete understanding of resistance mechanisms.

GENOTYPIC VARIATION IN THE HOST

Antibiotic toxicity to humans is significant in limiting the amount of drugs which can be administered to a patient[24]. Arrays are now being used to catalog individual genetic variations in drug-metabolism genes. For instance, out of 143 *Helicobacter pylori* infected patients, 50 failed one week of triple therapy; all 50 who remained infected were homozygous or heterozygous for the extensive metabolizer CYP2C19 genotype[25], resulting in rapid clearance of the antibiotic from their system. Knowledge of these variations prior to treatment can help a physician select the best drug and set the right dose for a patient sooner, as well as avoid drugs that may cause the patient to suffer adverse reactions.

Studies to identify genes associated with drug response, efficacy, and toxicity may become the most promising application for whole-genome DNA analysis. Tools like the Mapping 500K Array Set allow scientists to readily genotype populations of responders vs. non-responders to a given drug for phenotypes including efficacy and toxicity, and scientists hope to elucidate the genes contributing to these phenotypes. In late-stage clinical trials, microarray genotype analysis could be used to stratify patient populations to eliminate poor or toxic responders from key Phase III trials, ensuring optimal effectiveness, reducing the size and cost of the trials through clearer statistical differentiation between drug and placebo, and improving the odds for drug approval. Once a drug is on the market, patient stratification could be used to accelerate drug expansion into new indications through faster, smaller, more definitive Phase IV trials or to establish medical superiority of a late-to-market drug relative to entrenched competitors in an important class of patients. Genotype information will also fuel future research. By better understanding the genetic mechanisms of drug response in patients, researchers will have made significant progress on finding the next generation drug.

Just as humans respond to drugs in different ways, so do pathogens, and researchers are using microarrays to understand this variable microbial response as well. For example, Utaida and colleagues found that treatment of *S. aureus* with antibiotics, such as oxacillin, D-cycloserine or bacitracin, resulted in the induction of a number of biological host- and stress-response pathways in an attempt by the organism to defend itself against the antibacterials[26]. In the case of Pseudomonads, researchers have found that treatment of *P. aeruginosa* biofilms with sub-inhibitory concentrations of a beta-lactam antibiotic imipenem actually resulted in increased biofilm volume, not a decrease. The expression profile showed that the bacteria were countering the antibiotic with expression of the *amp*C gene, which codes for chromosomal beta-lactamase. Whole-genome expression analysis also helped these scientists determine that alginate biosynthesis pathways were induced following exposure to imipenem; alginate production has long been correlated with impaired lung function in CF patients[27]. By examining genome-wide expression, researchers can not only identify pathways of drug resistance, but can also identify potential hazardous side effects resulting from an inadequately treated infection.

Vaccine Development

One of the primary goals of infectious disease research is to prevent infection, by developing a vaccine that protects an individual from the disease in the first place. Historically, finding the right vaccine candidate has been a challenge since researchers typically focused on major surface proteins for potential vaccine candidates, while critical minor proteins would often escape discovery. Using GeneChip arrays, researchers can examine transcriptional activity of all genes of a pathogenic microorganism under *in vivo* conditions, allowing the identification of even rarely expressed, but potentially important, genes.

DISCOVERING VACCINE TARGETS

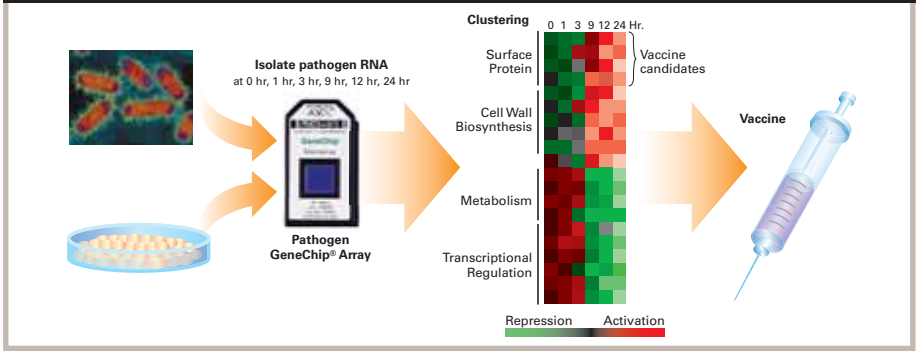
For example, scientists at the University of Wurzburg, Germany analyzed the genome-wide expression of *N. meningitidis* (meningococcus) to find surface proteins that were induced under *in vivo* conditions[28]. They analyzed gene expression during different stages of infection in *N. meningitidis* serogroup B, one of the serogroups that is responsible for the majority of meningococcal disease in industrialized countries. They were able to identify group-specific antigenic determinants, which served as a basis for rational, protein-based vaccine design against bacterial meningitis.

Microarrays not only provide scientists with a quantitatively larger list of potential vaccine candidates, but expression profiles also provide a richer type of information that allows scientists to more quickly identify successful vaccine candidates (Figure 3). For example, scientists studying malaria have used a whole-genome GeneChip array to understand global gene activity during the different stages of the *Plasmodium falciparum* parasite life cycle that transfers from vector to host and back to vector[29]. This group found that most genes used in current vaccine trials are expressed at the same time and map to the same cluster. By looking at the uncharacterized genes from that "vaccine cluster," the scientists anticipate finding additional vaccine candidates. They are now using follow-up approaches to confirm these potential targets as items of genuine vaccine interest.

ELUCIDATING IMMUNOSURVEILLANCE ESCAPE MECHANISMS

This same Malaria research group has also used GeneChip arrays to identify genetic variation across the genome of *P. falciparum*[30]. Often, mutated genes are selected because they are critically important to the microbe and the genetic change allows the

Figure 3: Whole-genome expression profiling allows researchers to identify antigenic candidates for rational vaccine design. By analyzing which genes are expressed by the pathogen over time, researchers identify genes crucial for infection. Ontology maps can be created and vaccine candidates identified.



microbe to avoid host defense mechanisms. Proteins encoded by these genes represent rational and plausible vaccine candidates. This group used the array to scan for single nucleotide polymorphisms on *P. falciparum* chromosome 2. They found most of the variation in the subtelomeric 100 kb regions at each end of the chromosome, and in known antigenic determinants and proteins associated with the cell membrane. A number of uncharacterized genes were identified in these regions as well, representing potential gene candidates. The same way that vaccine candidates cluster together in gene expression studies, gene variation also clusters, shown in studies like this one. Understanding the genetic differences between different strains and isolates of a pathogen allows researchers to identify antigenic determinants that might otherwise have been missed.

The Way Ahead™

The flexibility and high-throughput nature of current microarray technology offers unprecedented opportunities for infectious disease research. This technology has placed a completely new set of tools into the microbiologists' arsenal, and has created a novel method of experimental design. Whole-genome analysis studies promise to rapidly accelerate our understanding of the host-pathogen interaction, and to allow major changes in clinical approaches to infectious disease detection, treatment, and prevention, not only in humans, but animals and plants as well.

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