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Microarrays in veterinary diagnostics

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Abstract

Microarrays have numerous applications in the clinical setting, and these uses are not confined to the study of common human diseases. Indeed, the high-throughput technology affects clinical diagnostics in a variety of contexts, and this is reflected in the increasing use of microarray-based tools in the development of diagnostic and prognostic tests and in the identification of novel therapeutic targets. While much of the value of microarray-based experimentation has been derived from the study of human disease, there is equivalent potential for its role in veterinary medicine. Even though the resources devoted to the study of animal molecular diagnostics may be less than those available for human research, there is nonetheless a growing appreciation of the value of genome-wide information as it applies to animal disease. Therefore, this review focuses on the basics of microarray experimentation, and how this technology lends itself to a variety of diagnostic approaches in veterinary medicine.

Keywords: microarrays; diagnostics; DNA; molecular markers

Microarray basics

Microarray technology has been reviewed extensively in the recent literature (Duggan *et al*., 1999; Freeman *et al*., 2000; Southern, 2001; Chuaqui *et al*., 2002; Chung *et al*., 2002; Churchill, 2002; Hardiman, 2002; Holloway *et al*., 2002; Petricoin *et al*., 2002; Slonim, 2002). Briefly, arrays can be of two major types. Two-color arrays typically make use of contact-printed chips carrying covalently attached cDNAs or long (50–70 bases) oligonucleotides, known collectively as probes. An array may carry tens of thousands of probe species, each spotted to an area of approximately 100 μm. In the case of two-color array systems, comparisons are made on a single chip between competitively hybridized populations of labeled molecules, known as targets. These targets can be generated from RNA derived from experimental and reference samples and labeled with different fluorophores. In this case, the relative amount of hybridization of each of the fluors at any spot on the microarray is related to the numbers of cDNA molecules complementary to that probe generated within each population, which, in turn, is related to the transcriptional activity of that gene in the sample. Alternatively, cDNA or long oligonucleotide arrays can be used to assess particular genomic parameters of an organism relative to a control, in which case the labeled target population is derived from genomic DNA. In either case, because of the difficulty in assessing copy numbers of probes at each spot and the variability of hybridization kinetics across many thousands of sequences, these types of arrays are used to measure the relative hybridization of targets derived from reference and control populations.

An alternative approach is offered through the use of Affymetrix arrays, which are based on a short (25 bases) oligonucleotide format manufactured by a photolithography procedure. The single-stranded probes are generated *in situ*, and each spot is made up of a known number of identical sequences in an area less than 20 μm. On these arrays, each gene is represented by several probes, providing greater redundancy than is

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common on contact printed arrays. Corresponding to each perfect probe is a probe with a single internal mismatch. Assessment of the relative binding of biotin-labeled sample to all perfect match probes compared with mismatch probes provides information about the expression level of each gene. In these experiments, control and experimental samples are hybridized to separate chips, and comparisons are made across multiple arrays. As for contact printed arrays, target populations can be derived from either RNA or DNA of the organism of interest, depending on the question being addressed.

While the details of experimental protocol differ somewhat between the two types of array, the general underlying phenomenon is similar in that both rely on the hybridization of single-stranded, labeled nucleic acid to a series of immobilized single-stranded DNA probes, each of which represents a particular gene. The issues surrounding experimental design, data acquisition, data normalization and data management are well covered in the current literature (Eisen *et al*., 1998; Duggan *et al*., 1999; Brazma and Vilo, 2000; Burke, 2000; Emmert-Buck *et al*., 2000; Freeman *et al*., 2000; Hegde *et al*., 2000; Kane *et al*., 2000; Lockhart and Winzeler, 2000; Hughes *et al*., 2001; Kerr and Churchill, 2001; Siedow, 2001; Bakay *et al*., 2002; Bilban *et al*., 2002; Brody *et al*., 2002; Dettling and Buhlmann, 2002; Lee and Whitmore, 2002; Hardiman, 2002; Murphy, 2002; Quackenbush, 2002; Yang and Speed, 2002; Chen *et al*., 2003; Draghici *et al*., 2003; Smyth *et al*., 2003) and will not be addressed here. However, the very large literature related to these issues underscores the idea that decisions regarding experimental design, sample type, array type, sample preparation and data analysis are critical in determining what types of questions can be answered by a particular approach. It is this versatility of the microarray platform that makes the technology of such broad potential use in molecular diagnostics.

Microarrays in clinical diagnostics

Several major categories of diagnostic question can be addressed using microarrays as a starting point. These include scanning for the presence of a mutation in a specific disease gene, scanning for expression profiles that differentiate molecular subcategories of disease with clinical relevance, scanning for the large-scale changes in DNA copy number that can accompany some acquired diseases, the identification of markers associated with disease, and the identification of pathogens underlying a particular disorder. In some of these cases, microarrays represent only a starting point, and would probably would not be part of a final diagnostic test. In this context, microarrays in diagnostics really represent a useful tool for the development of novel tests that would probably rely on a more focused and easily controlled technology in the clinical setting.

Scanning for mutations in a known gene

The use of microarrays for rapid sequencing or scanning of entire genes requires the generation of arrays on which have been tiled short oligonucleotides that span the coding sequence of the gene or genes of interest. Arrays can be customized to scan as little or as much of the coding, regulatory and intronic sequences of a gene as necessary. Two major methods for mutation scanning currently exist. The first relies on allele-specific hybridization of the amplified gene from the sample to an array with oligonucleotides that span the mutation site (Saiki *et al*., 1989). If the array carries not only oligonucleotides based on the wild-type sequence but also oligonucleotides with the middle base changed to represent all possible variants at that position, the pattern of hybridization across the array reveals the sequence present in the experimental sample. The second relies on primer extension assays, during which hybridization of fragmented genomic DNA from the region of interest to the gene specific chip is followed by extension of the double-stranded hybridized product by a single base. The differential labeling of each of the four dideoxynucleotides in the extension reaction provides the mechanism by which base calls can be made (Chen *et al*., 2000; Landi *et al*., 2003).

As in humans, animal populations are subject to hereditary diseases that can be called single-gene disorders (Venta *et al*., 2000; Haskins *et al*., 2002; Abramson *et al*., 2003; Drogemuller *et al*., 2003; Shimatsu *et al*., 2003; Sieb *et al*., 2003; Takeda and Sugimoto, 2003). Although not currently in use, the application of microarray technology to rapid mutation scanning of entire genes is feasible in situations where demand makes it economical, such as breeding programs. Currently in veterinary medicine, direct mutation testing is offered for a variety of single-gene disorders in dogs, horses and some livestock species (for relevant web sites see Meyers-Wallen, 2001). The application of the microarray platform to existing tests is a possibility in the future in situations where either a rapid turn-round is desired, or where large genes are being screened for unknown mutations.

Scanning for diagnostic or prognostic expression profiles or molecular markers of disease

The use of microarray technology for the identification of either molecular subtypes of disease or for the identification of novel combinations of molecular markers represents the bulk of published microarray studies to date. Generally, the idea that complicated outputs from raw microarray runs can or should become diagnostic tests in themselves is not accepted (Snijders *et al*., 2000; Gabrielson *et al*., 2001; Wildsmith and Elcock, 2001; Firestein and Pisetsky, 2002; Petricoin *et al*., 2002;

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Stremmel *et al*., 2002; Zhang *et al*., 2003). Rather, data derived from such microarray-based work is meant to provide direction for the identification of novel pathways or genes that can serve as markers of a particular disease type or state. In studies of human disease, expression profiling patterns of clinically important subtypes of cancers has provided evidence that such large-scale genomic surveys can provide information that can gradually become part of a simpler diagnostic tool. For example, data from microarray-based surveys of hundreds of samples assessed for the expression of tens of thousands of genes have been successfully reduced to manageable numbers following appropriate statistical interpretation of the data and clustering of the original samples into clinically relevant subgroups (Alizadeh *et al*., 2000; Perou *et al*., 2000; Brenton *et al*., 2001; Dhanasekaran *et al*., 2001; Gordon *et al*., 2002; Hedenfalk *et al*., 2001; Lakhani *et al*., 2001; Rajeevan *et al*., 2001; Jenssen *et al*., 2002; Mariadason *et al*., 2002; Shipp *et al*., 2002; van de Vijver *et al*., 2002; van 't Veer *et al*., 2002; Hedenfalk *et al*., 2003; Ramaswamy *et al*., 2003). The reduced data sets focus on tens rather than thousands of genes, and allow the development of novel diagnostic or prognostic tests on an alternative platform, avoiding the noise and variability of a typical microarray experiment.

Efforts in animal sciences to date have been geared towards the development of useful resources for such large-scale studies, in the form of increased sequencing to identify genes, and in the development of whole genome arrays (Burton *et al*., 2001; Liu *et al*., 2001; Yao *et al*., 2001; Band *et al*., 2002; Coussens and Nobis, 2002; Yao *et al*., 2002; Suchyta *et al*., 2003). Currently, these resources are being used primarily to increase understanding of the basic biology of important pathways in order to develop novel molecular markers that can act either as diagnostic markers or novel targets for therapy. For instance, efforts have focused on understanding the molecular basis of bovine immunobiology (Burton *et al*., 2001; Yao *et al*., 2001; Coussens *et al*., 2002), with the goal of identifying candidate genes important in this process. Future directions may include the incorporation of data derived from these studies into simple tests that provide disease information about an animal and allow individualization of treatment.

Genome-wide copy number changes correlated with acquired disease

Microarrays can also be useful in the detection and cataloging of the large-scale genomic changes that often accompany acquired diseases such as cancer. In humans, many somatic cancers are accompanied by predictable changes in DNA organization, including regions of chromosomal losses and/or gains (Pollack *et al*., 1999; Forozan *et al*., 2000; Pollack *et al*., 2002; Seo *et al*., 2003). In some cases, these changes can be used as molecular confirmation of diagnoses; in others the changes are associated with particular outcomes. Therefore, strategies to rapidly and accurately identify such changes have evolved, and are generally focused on technologies such as fluorescence *in situ* hybridization (FISH), which is suitable for tracking single important changes. However, as our understanding of the etiology of diseases grows, it may become beneficial to have an appreciation of global imbalances over the entire genome, and to correlate multiple changes with outcome or diagnosis. Comparative genomic hybridization technologies can be adapted to a microarray format for such testing (Carter *et al*., 2002). In this context, the hybridization is between the single-stranded DNA probe on the array and labeled fragmented genomic DNA targets from the animal of interest. Tracking the relative changes in copy number between the experimental and control sample over regions of chromosomes provides genome-wide information regarding chromosomal gains or losses.

Although this technology has yet to find application in animal science, its precursor, FISH-based CGH hybridization, has been used successfully to study chromosomal imbalances in a canine glial tumor cell line (Dunn *et al*., 2000) and in canine lymphoma (Thomas *et al*., 2003), suggesting that the importance of identifying consistent genomic imbalances associated with animal tumors is of interest in the diagnostic setting.

Microarray technology to assist in breeding decisions

Microarray experimentation may eventually have utility in decision-making in animal breeding programs. Quite apart from gene expression profiling to determine fingerprints of animals with desirable characteristics, direct genotyping of DNA for variants associated with genes that produce desirable traits will be of interest. Single-nucleotide polymorphisms (SNPS) are variant bases found scattered throughout the genome of all animals (Marth *et al*., 1999; Gut, 2001; Judson and Stephens, 2001; Landi *et al*., 2003; Reich *et al*., 2003). They are generally biallelic, stable, and more or less equally scattered throughout the entire genome, and therefore represent a convenient way to tag chromosomal regions. While the average spacing of these polymorphisms remains to be determined, a working estimate of one SNP for every 1000 bases of DNA in humans is generally accepted, and there is some indication that the density may be higher in non-human genomes (Vignal *et al*., 2002). SNPs generally fall into two categories: coding SNPs are found within coding or regulatory regions of genes, while non-coding SNPs are found in potentially non-critical regions of the genome. Therefore, SNPs may directly cause changes in the function of a gene product which may be associated with a particular outcome, or

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may simply be associated with such changes by virtue of linkage disequilibrium. In this case, the association of a variant with a phenotype is a result of very tight linkage between the SNP and the gene underlying the phenotype, ensuring that they almost always segregate together. Whether causative or not, SNPs can mark particular genes of interest, and when they do they represent a simple tag by which animal chromosomes can be assessed.

Development of dense SNP maps for animals lags behind the development of a similar map for humans, but is under way (O'Brien *et al*., 1999; Heaton *et al*., 2001; Vignal *et al*., 2002; Brooks *et al*., 2003; Emara and Kim, 2003; Kirkness *et al*., 2003). The use of these resources in animal breeding will be through animal genotyping to avoid particular phenotypes associated with disease or other outcomes known to be linked to a particular marker. The use of microarray technology for this purpose will allow the large-scale screening of many hundreds of such markers in a single experiment, allowing selection based on multiple traits.

Microarray-based detection and genotyping of pathogens

Perhaps the most widely used application of microarrays in animal medicine is the rapid and accurate detection of a range of animal pathogens, including bacteria, fungi and viruses. The underlying principles for the application of microarrays are similar to the those involved in mutation scanning for specific genes, in that arrays are created that carry short oligonucleotides corresponding to unique sequences capable of differentiating one species of pathogen from another (Vernet, 2002; Wang *et al*., 2002a). Because of the widespread availability of sequencing information from complete genomes of an increasing number of pathogens, this approach is becoming more viable and is likely to see widespread use in animal medicine (Sellon, 2003). The use of antigenic determinants and/or virulence factor genes allows multiple probes for each pathogen to be spotted onto the same chip, and the pattern of hybridization of labeled DNA derived from the host animal is then used to determine the identity of the infectious agent or agents (Volokhov *et al*., 2002; Wang *et al*., 2002a, b; Booth *et al*., 2003; Borucki *et al*., 2003).

A related application is the use of expression profiling microarray technology to determine the presence of a particular pathogen based on perturbations to the host cells (Coussens *et al*., 2002; Berthier *et al*., 2003). In these cases, the pathogen itself is not detected, but the results of the infection can be monitored, and this can lead to a rapid diagnostic test. In addition, expression profiling of infected animals leads to increased understanding of the biology of a particular host–pathogen interaction. Increased understanding of the basic biological pathways perturbed in a host allows the identification of key genes that could represent targets in therapeutic approaches and in the development of novel vaccines (Chizhikov *et al*., 2001; Fitzgerald *et al*., 2001; Knox *et al*., 2001; Chizhikov *et al*., 2002; Lorenz, 2002).

Conclusions

Microarray technology burst onto the scene almost a decade ago, and the subsequent increase in applications has potentially increased its use in clinical diagnostics in human and animal medicine. While there remain many considerations regarding experimental design, array fabrication, sample preparation, variability between experiments, data analysis and the interpretation of data, the positive message is that microarray technology, carefully applied and controlled, has much to offer the world of clinical diagnostics. While much of the potential has yet to be realized, the technology is becoming increasingly robust and appropriate for studies in animal disease diagnostics.

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