Aquaculture Genome Technologies
Zhanjiang (John) Liu

Chapter 21
Microarray Fundamentals: Basic Principles and Application in Aquaculture

Eric Peatman and Zhanjiang Liu

Researchers have long harnessed the basic molecular principle of nucleic acid hybridization to study the expression patterns of cell transcripts. Transcript studies allow a valuable assessment of the genetic response to environmental changes (i.e., infection, temperature, feeding rates). Incremental progress over the last 2 decades has been made from radioactively labeled probing of one gene to tens of genes to nylon-filter-based macroarrays containing hundreds of genes. In early years, progress in transcript detection techniques largely corresponded to strides in gene sequencing and discovery. However, as gene sequencing grew exponentially in the early 1990s and genomic approaches revolutionized molecular biology, a similarly radical leap forward was needed to bring transcript studies into the "–omics" era. Microarray technology provided such a boost by combining simple nucleic acid hybridization with high-density spotting robots, fluorescence-based signal detection, high-resolution laser scanners, and bioinformatic tools, allowing simultaneous expression analysis of thousands of genes (Schena et al. 1995). *In situ* oligonucleotide synthesis through photolithography (Fodor et al. 1991) as developed by Affymetrix, offered even greater gene densities than spotted arrays, albeit at a much higher cost. A decade of refinements of both spotted and *in situ* microarray technologies have resulted in further capacity increases and widened array applications without altering the fundamentals of either approach. Microarray technology is now widely accessible in biomedical and agricultural genetics research. Only within the last several years, however, have researchers in aquaculture species generated sufficient expressed sequence tags (EST) to justify using transcriptomic approaches for expression analysis. Furthermore, the high cost of microarray technology has hindered its implementation in underfunded species groups. Of the aquaculture species cultured most prevalently around the world, the majority still lacks microarray resources. A current review of microarray technology, therefore, from an aquaculture perspective should be helpful for the many species groups only now beginning to harness the potential of genomic approaches to research. In this chapter, we will review the basic principles of microarrays, present and compare the two prevalent array technologies, and discuss important factors to consider before beginning microarray research. Because of the complicated and ever-changing nature of microarray research, it is beyond the scope of this chapter to provide detailed protocols or exhaustive lists of microarray techniques and tools. Readers looking for more technical details after reading this chapter are referred to Chapter 22 concerning applications of microarrays in aquaculture and to the cited references of this chapter and Chapter 22.

Principles of Microarrays

Although microarrays use several recent technological innovations, they are, at their core, simply a high-density dot blot. In both spotted microarrays and *in situ* arrays, DNA is anchored or spotted onto a surface and then probed with labeled molecules. Hybridization and subsequent signal detection depends on the presence of complementary nucleotide sequences between the probes and the spotted sample. Microarrays achieve higher gene feature densities and, therefore, greater power for expression analysis by applying new tools to this old process. High-density spotting robots and photolithography allow each feature to be placed accurately within nanometers of the next feature on a glass slide, clearly an impossible task with the human hand. Furthermore, fluorescence-based probe labeling provides a cleaner and clearer signal than the radiation traditionally used in blotting. Finally, laser scanners facilitate the resolution of such tremendous feature densities and provide accurate fluorescent signal quantification. With an understanding of the basic principles of microarrays, we can now examine the two prevalent microarray platforms: the spotted arrays and the *in situ* arrays.

Spotted Array Design and Construction

There are two primary approaches to microarrays, differing in both their construction and their sample labeling. Spotted arrays are constructed by spotting long oligos or complementary DNAs (cDNA) using a printing robot, whereas *in situ* arrays are constructed by synthesizing short oligos directly onto the slide by photolithography. The terms spotted array and *in situ* array will be used throughout this chapter to refer to these two approaches.

Spotted array technology encapsulates the printing of either PCR products or long oligos (60–70 mers). Traditionally referred to as cDNA arrays, spotted arrays are today just as likely to be long oligos, as the cost of synthesizing oligos continues to decline, and because the parallel polymerase chain reaction (PCR) required to prepare for cDNA arrays is labor-intensive, costly, and requires having clones on hand. While these cDNA-associated difficulties can be overcome through hard work and collaboration among members of a species group, the printing of long oligos offers advantages in start-up time, the purity of commercial oligo synthesis, easier clone tracking, and the ability to use all available sequences in public genetic databases for array construction. Regardless of whether cDNA or synthesized oligos are used, most steps in array construction are similar for all spotted arrays (Figure 21.1).

Many research groups have combined EST sequencing projects with microarray construction, a logical, time-saving approach given that many of the steps of EST analysis are also needed for probe selection. Clustering of ESTs by sequence similarity allows an assessment of the number of unique sequences available for array construction and, in the case of repeated sequences, allows one to choose the best clone for the array. A unique gene (Unigene) list is a starting place for picking the sequences that will constitute the microarray. Depending on array design and experimental goals, clones can be picked to bias them toward the 3′ end, ensure complete inserts, maximize unique sequence stretches, and/or maximize genes (or features) included

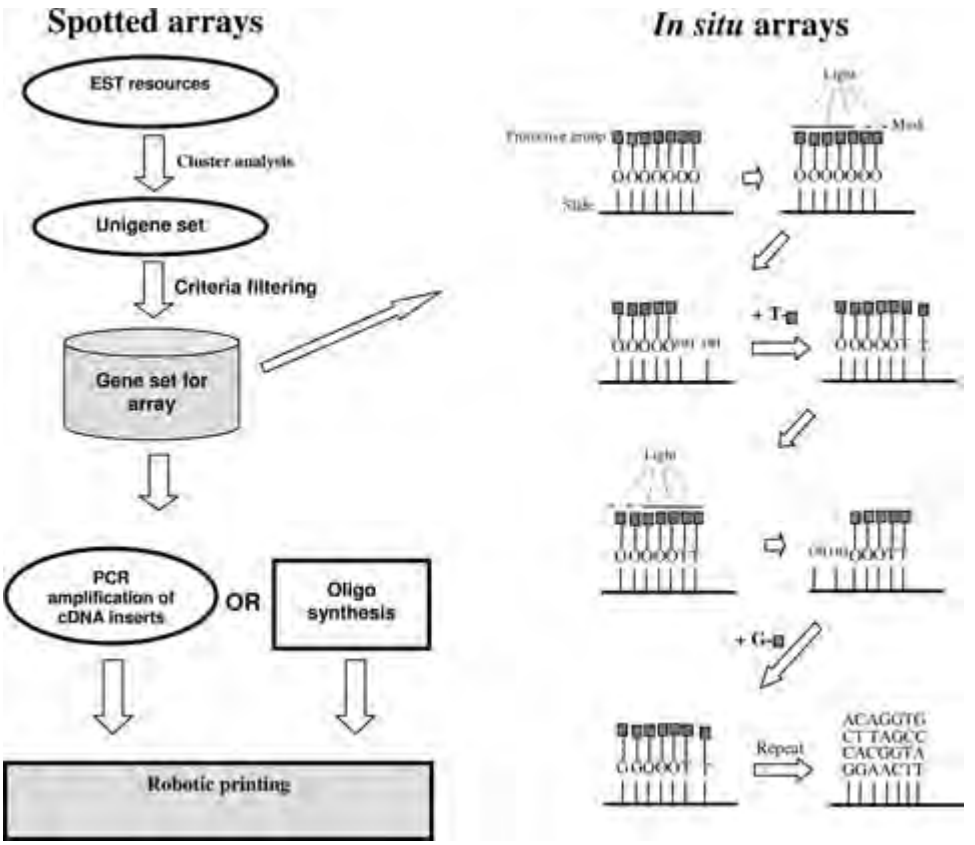

Figure 21.1. Microarray design and construction for spotted arrays and *in situ* arrays. Array design, deciding which genes to include on the array, is similar for both platforms. Construction of the physical microarray differs between robotic printing (spotted arrays) and photolithography (*in situ*). For photolithography, oligonucleotides are synthesized directly on the surface of the array, one base at a time. Unique physical lithographic masks are created for each array design, to either block or allow light to reach the slide. In the places the mask does not cover, light deprotects, or converts the protective group to a hydroxyl group, allowing the binding of single oligo at that specific site by its phosphate group. This oligo also bears a protective group that must be deprotected before an additional oligo can be coupled to it. Through repeated cycles of deprotection and coupling, 25-mer oligos are synthesized directly on the slide.

on the slide. Good examples of spotted microarray design considerations can be found in the literature, and the specific approach chosen by each group may differ. Interested readers are referred to Chapter 22, and to Whitfield and others' (2002) EST sequencing and microarray research on the honey bee using spotted cDNAs; Rise and others (2004a) and von Schalburg and others (2005a) describe considerations taken in construction of salmonid spotted cDNA arrays; and Zhao and others (2005) report validation of a porcine spotted oligo array. Operon Biotechnologies (http:// www.operon.com/) is a leading provider of sets of synthetic oligos for microarray spotting, and their Web site provides an excellent resource for criteria used in gene selection and long oligo design. Additionally, the Institute for Genomic

Research (TIGR), well known for its EST indices, provides 70 mer oligo predictions for genes in each of its indices that have been used by some groups (Zhao et al. 2005). Researchers also should decide in the design phase their array layout, feature duplication, and controls to be spotted on the slide (Whitfield et al. 2002, Smyth et al. 2005).

The clone set selected for inclusion in the microarray must now be amplified by PCR (in the case of cDNA arrays), or probes based on their sequences synthesized (long oligos). For large array designs, this is best done using robotic handling to avoid mistakes and to simplify clone tracking throughout the process. PCR products, after purification, or long oligos are usually rearrayed into 384 well plates in preparation for printing.

A variety of microarray slides are available for printing, most are poly-L-lysine and amino silane-coated. See Hessner and others (2004) for surface-chemistry comparisons. Telechem (http://www.arrayit.com/Products/Substrates/) and Erie Scientific (http://www.eriemicroarray.com/index.aspx) are leading providers of microarray slides. The actual robotic printing of microarrays is increasingly being outsourced to large university core labs or private companies, which now have years of experience in the field. For groups that anticipate printing multiple array designs and batches and want increased printing flexibility, purchasing a spotting robot may be a good choice. Perkin Elmer (http://las.perkinelmer.com/) and Genomic Solutions (http://www.genomicsolutions.com) offer popular printing systems.

In Situ Array Design and Construction

In situ array technology relies on photolithography for microarray construction (Lipshutz et al. 1999), a technique often used in computer chip fabrication. In contrast to spotting nucleotide products on the slide surface, oligonucleotides are synthesized directly on the surface of the array, one base at a time. To achieve sufficient feature densities, unique physical lithographic masks are created for each array design, to either block or allow light to reach the slide (Figure 21.1). In the places the mask does not cover, light deprotects, or converts a special protective group to a hydroxyl group. This allows the binding of single oligo at that specific site by its phosphate group. This oligo also bears a protective group that must be deprotected before an additional oligo can be coupled to it. Through repeated cycles of deprotection and coupling, 25-mer oligos are synthesized directly on the slide at densities currently as high as 1.3 million features per array. Affymetrix (http://www.affymetrix.com/) is recognized as the developer and industry leader for *in situ* arrays. Although their technology made genome-wide arrays a reality for model species and continues to expand the horizons of microarray research in biomedical fields, the technology has been prohibitively expensive for the smaller species groups including aquaculture species. Nimblegen Systems (http://www.nimblegen.com) has recently developed a "maskless" version of the Affymetrix technology that uses digital mirrors to achieve the same effect (Nuwaysir et al. 2002) at a significantly lower start-up cost, making *in situ* arrays now a feasible choice for aquaculture genomic research.

The majority of array design considerations for *in situ* arrays overlap with those of spotted arrays. EST analysis, clustering, quality control, and probe selection are still necessary steps to arrive at the set of genes that will be synthesized on the array. The higher feature density allowed with *in situ* arrays means that more genes, duplicates,

and/or controls may be included on the array, if desired. Because the per array cost is significantly higher for *in situ* arrays and project flexibility considerably less than for spotted arrays, researchers usually attempt to maximize the information that can be gained from each slide. Usually, desired sequences for the array are sent electronically to the company, which then carries out oligo probe selection (23–25 mers) and designs the array layout. Both Affymetrix and Nimblegen use a perfect match (PM) and mismatch (MM) system that accounts for the majority of the features on *in situ* arrays. Mismatch probes, as their name suggests, contain one or more mismatched nucleotides in the PM probe sequence and are used to detect and screen out false background fluorescence resulting from nonspecific cross-hybridization. Commonly, 10 PM and 10 MM probes are synthesized for each gene included on the array, and are believed to significantly increase the accuracy and sensitivity of gene expression detection. See Chen and others (2005), Han and others (2004), and Irizarry and others (2003) for more information on PM and MM probe theory.

Experimental Sample Labeling and Hybridization

Spotted and *in situ* microarrays differ not only in their array construction but also in the procedures used to label and hybridize experimental samples (probes in the traditional sense) to them (Figure 21.2). Both array platforms require that you start with RNA sources. The RNA is extracted from the samples of interest. For the sake of simplicity, we will use the example of a control sample and a treatment sample. Each RNA sample is reverse transcribed to cDNA, after quantification and quality checking by spectrophotometer measurement and agarose gel electrophoresis. From this step, differences in the procedure arise between the two microarray platforms. We will follow the spotted array protocol first, before returning to the *in situ* procedure.

The cDNA samples for spotted arrays are labeled with two different fluorescent dyes, Cy3 and Cy5, which fluoresce "green" and "red," respectively, under two different wavelengths of light (633 nanometer [nm] and 543 nm). The control sample is labeled with one dye and the treatment sample with the other. Dye assignments should be swapped in replicates to avoid dye-associated bias of hybridization (Churchill 2002). Dye labeling is most commonly done either directly or through indirect aminoallyl labeling. See Manduchi and others (2002) and Badiee and others (2003) for a comparison of labeling methods. The two labeled samples are hybridized simultaneously in equal amounts to the same array for 16–20 hours. The hybridized array, after washing to remove unhybridized probes, is scanned under a laser scanner (e.g., Molecular Devices/Axon Instruments' Axon 4000B) at both fluorescent wavelengths (or channels) for the two dyes. A digital image is acquired for both channels, and, by overlaying the two images, a fluorescent signal ratio for each array feature is obtained. This fluorescent signal ratio indicates gene expression levels. Using the Cy3/Cy5 labeling system, yellow spots indicate approximately equal levels of mRNA from both the control and treatment samples (equal signals from the green Cy3 and the red Cy5). Features that appear red or green have hybridized a majority of mRNA from only one sample. Fluorescent intensity data for each feature are recorded, and the scanned image and data can be linked back to gene feature identities through programs such as Molecular Devices/Axon Instruments' GenePix Pro software. Background subtraction and

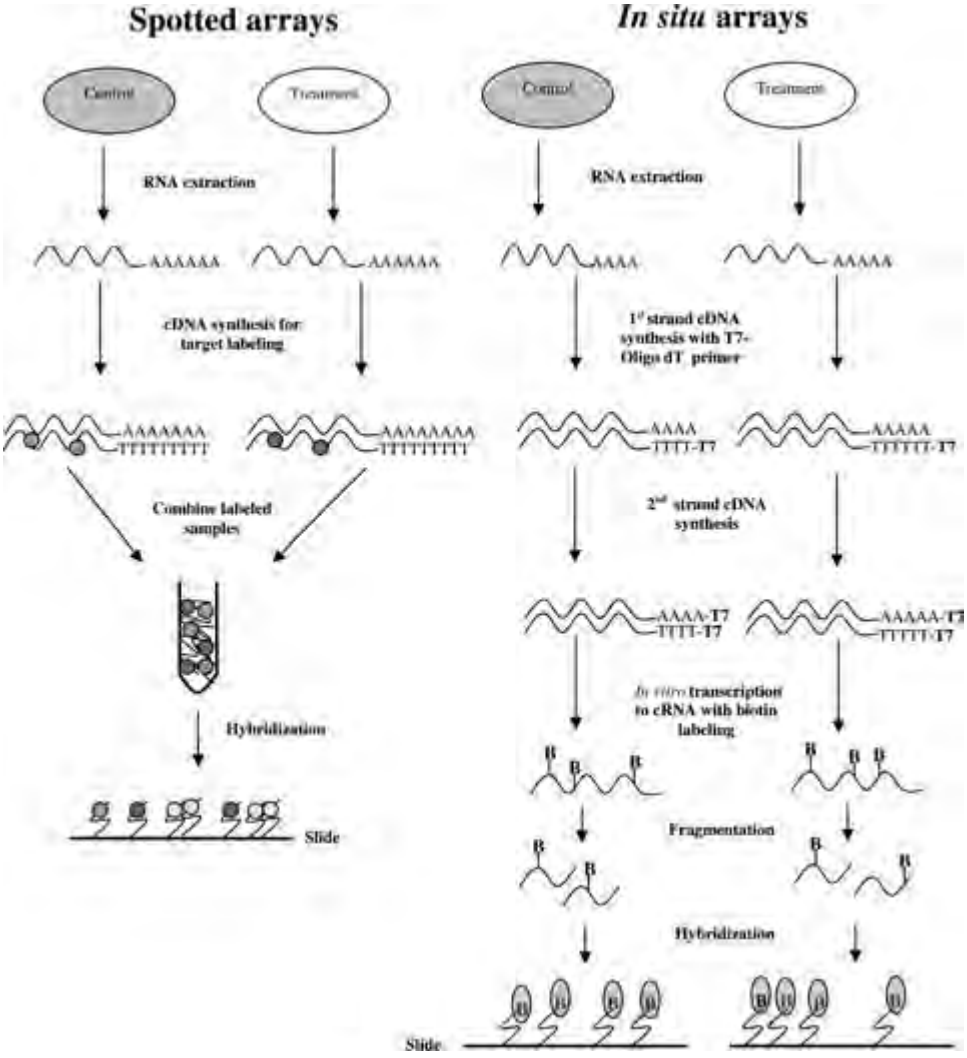

Figure 21.2. Experimental sample labeling and hybridization for spotted and *in situ* microarrays. In both cases, RNA is extracted from control and treatment samples. For spotted arrays, these samples are reverse-transcribed and labeled with two fluorescent dyes, Cy3 and Cy5, as described in the text. These two samples are then mixed equally and hybridized to a single microarray slide. Where the "green" and "red" probes hybridize to the same spot, yellow is seen. In the case of *in situ* arrays, the RNA samples are reverse transcribed using a T7 promoter oligo-dT primer. The resulting cDNA is converted to a double-stranded template by a second strand synthesis reaction, and these double-stranded cDNA samples are converted by *in vitro* transcription to biotin-labeled (B) cRNA using a T7 RNA polymerase. The cRNA from each sample is fragmented and hybridized to its own slide. Streptavidin-phycoerithryn is added as the fluorescent dye for both the control and treatment samples.

normalization are customarily carried out at this point, followed by microarray analysis and validation of genes determined to be significantly differentially expressed after treatment. For more on microarray analysis and validation for both platforms, refer to Chapter 22 in this book as well as numerous excellent research papers and reviews in

Table 21.1. A comparison of several important aspects of *in situ* and spotted array platforms. *Cost/slide can vary significantly from these figures depending on design, quantities ordered, core facility discounts, etc.

	In situ arrays	Spotted arrays
Starting material	DNA sequences	DNA sequences or cDNA
Array fabrication	*In situ* synthesis by photolithography	Robotic spotting
Features	>400,000	<50,000
Spot quality	High	Variable
Oligo length	23–25mer	Usually 70mer
Labeling	Single dye label, e.g., biotin-streptavidin-phycoerithryn	Two dye label—Cy3, Cy5
Cost/slide	>500*	<100*
Probe/slide	One	Two
Dye swapping?	No	Yes
Controls	PM/MM, ±	Duplicates, ±
Providers	Affymetrix, Nimblegen, etc.	Species groups, core facilities, biotech

the literature (e.g., Leung and Cavalieri et al. 2003, Walsh and Henderson 2004, D'Ambrosio et al. 2005).

We return now to *in situ* arrays in order to contrast the labeling and hybridization of their samples with that of spotted arrays. The original RNA samples are reverse transcribed using a T7 promoter oligo-dT primer. The resulting cDNA is converted to a double-stranded template by a second strand synthesis reaction. After purification, these double-stranded cDNA samples (again control and treatment) are converted by *in vitro* transcription to biotin-labeled cRNA using a T7 RNA polymerase. The cRNA from each sample is fragmented and hybridized to its own slide (note: no mixing of samples). Streptavidin-phycoerithryn is added as the fluorescent dye for both the control and treatment samples (Figure 21.2). To clarify, each biological sample for *in situ* arrays is hybridized to a *different* slide and labeled with a *single* dye. Differential expression is measured by comparing the fluorescent intensity measurement of a given gene on the control slide with a separate measurement for the same gene from the treatment slide. Labeling reactions and hybridizations of *in situ* arrays are commonly carried out by the array provider or core lab.

Table 21.1 provides a side-by-side comparison of spotted and *in situ* arrays, summarizing the advantages and drawbacks of each platform as reviewed above. Several groups have experimentally compared the precision and accuracy of the two platforms using the same biological samples. Their studies may prove helpful to those considering which system to implement in their own research. See Woo and others (2004), Yauk and others (2004), and Meijer and others (2005).

Microarray Research in Aquaculture Species

Microarray research has advanced dramatically in recent years in aquaculture species. However, the field is still in its infancy, and distribution of resources remains uneven.

Table 21.2. Some examples of microarray studies in aquaculture species and their pathogens.

Species	Common name	References
Cyprinus carpio	Common carp	Gracey et al. 2004
Ictalurus punctatus	Channel catfish	Ju et al. 2002
Ameiurus catus	White catfish	Kocabas et al. 2004
Paralichthys olivaceus	Japanese flounder	Kurobe et al. 2005; Byon et al. 2005, 2006
Platichthys flesus	European flounder	Williams et al. 2003
Salmo salar	Atlantic salmon	Morrison et al. 2006; Martin et al. 2006; Jordal et al. 2005; von Schalburg et al. 2005a; Aubin-Horth et al. 2005; Ewart et al. 2005; Rise et al. 2004a, 2004b
Oncorhynchus mykiss	Rainbow trout	Purcell et al. 2006; MacKenzie et al. 2006; von Schalburg et al. 2006, 2005b; Tilton et al. 2005; Krasnov et al. 2005a, 2005b, 2005c; Vornanen et al. 2005; Koskinen et al. 2004a, 2004b
Oncorhynchus keta	Chum salmon	Moriya et al. 2004
Astatotilapia burtoni	African cichlid	Renn et al. 2004
WSSV and *Penaeus* sp.	White spot syndrome virus and shrimps	Lan et al. 2006, Marks et al. 2005, Tsai et al. 2004, Dhar et al. 2003, Khadijah et al. 2003
Sparus auratus	Gilthead seabream	Sarropoulou et al. 2005
Aeromonas salmonicida	Furunculosis	Nash et al. 2006
Crassostrea sp.	Oyster	Not published yet

Concerted efforts by researchers working on salmonid species has resulted in the generation of several arrays that are now available to the general research community (see Chapter 22). These arrays have been rapidly integrated into salmonid research, as seen in Table 21.2. The largest salmonid microarray generated to date contains 16,006 cDNAs with 13,421 coming from Atlantic salmon and 2,576 from rainbow trout (von Schalburg et al. 2005a). Table 21.2 lists additional microarray studies conducted on aquaculture species or aquaculture-associated pathogens. With the exception of salmonids, other microarray studies have, for the most part, been small-scale, noncollaborative efforts. A forthcoming microarray for the oyster should also be widely distributed. To date, most published microarray studies have used PCR-amplified spotted cDNA clones to fabricate the array. However, as microarray research typically takes several years from its inception to reach publication, the recent trends toward spotted oligos and *in situ* microarrays may not be reflected in the aquaculture literature for several years. A well-designed microarray can be a valuable asset to an aquaculture species group, especially if the cost per slide can be minimized to the extent that researchers can integrate transcriptomic approaches into their already established research. Microarray studies are most successful when they are just one of several approaches used to answer biological questions. For example, salmonid researchers have implemented array technology in their study of reproductive development, toxicology, physiology, and repeat structures (von Schalburg et al. 2006, Tilton et al. 2005, Vornanen et al. 2005, Krasnov et al. 2005a).

Future Directions of Microarray Research

Due to low funding levels and a relatively small research community, aquaculture genomics stands today where the model species did almost a decade ago. In the same way, microarray research in aquaculture species is only in its infancy. Like researchers of humans and mice 10 years ago, we are currently using microarrays to accelerate gene expression analysis under varied experimental conditions, to reveal novel functions in genes, and to discover possible gene interactions and networking through cluster analysis. To find future directions for microarray research in aquaculture species, we need only to observe microarray studies in model species today. The future looks especially promising for using microarrays for single nucleotide polymorphisms (SNP) analysis and quantitative trait loci (QTL) mapping to make tangible progress toward widespread marker-assisted selection (MAS) in aquaculture (see Chapter 12, and also Walsh and Henderson (2004) and Li and Burmeister (2005) for reviews). In particular, merging positional candidate genes with expression candidate genes from microarray information may reveal QTL genes responsible for important performance traits (see Drake et al. 2006). Microarrays have, furthermore, evolved to allow studies of metabolomics and proteomics that will be important in development of fish vaccines (Cretich et al. 2006). A Veterinary Immune Reagent Network has already been established in the U.S. toward development of a set of antibodies for use in agricultural research including aquaculture (http://www.avma.org/onlnews/ javma/jun06/060615b.asp). Microarrays are also being used in livestock disease diagnostics, a use easily adapted for detection of outbreaks of aquaculture pathogens (Schmitt and Henderson 2005, Baxi et al. 2006). Much of the groundwork for practical microarray research has already been laid. It is up to the aquaculture community to exploit and adapt these advances for the advantage of their respective species.

Considerations When Starting Microarray Research

The aquaculture researcher is faced with several important considerations when starting microarray research. Decisions made in the early planning phases may critically impact research for years to come, and, therefore, should be made carefully. Three broad, interrelated areas of consideration should be addressed. First, the investigators should decide why and how they plan on using microarrays in their research. Approaches will differ based on whether the investigator wants to answer a single biological question or use microarrays in several different experiments over an extended period of time. Smaller "subset" arrays of just immune-related genes or developmental genes may be more appropriate for some research. Will the arrays be used in hypothesis-driven experiments or as hypothesis-generating experiments (candidate gene fishing), or both? Second, the investigator must assess the resources available for constructing a microarray including both financial and genomic resources. While it is simple to make a decision based on financial limitations, many questions should be answered with regard to the availability of genomic resources. Some questions to ask follow: How many ESTs are available for the species of interest, and will this number increase significantly in the near future? If a spotted cDNA approach is taken to

array fabrication, are the clones available for amplification and printing? Is sequence information publicly available to allow construction of a spotted oligo array or an *in situ* array? How many researchers are interested in an array for this species? Can a collaborative, cost-sharing agreement be reached, or will a single lab have to pay all costs? What are the current costs per slide for the different platforms? Will per array costs be low enough to use the microarray as part of regular research, or is this a "one-shot" approach? Can a previously constructed array be used or adapted from a different species, a cross-species approach (Renn et al. 2004)? Third, the investigator must give intensive consideration to experimental design; this design must be statistically rigorous and stand up to scientific scrutiny (Churchill 2002, Dobbin and Simon 2002, Simon et al. 2002, Shih et al. 2004). Some design considerations include the levels of biological replication and technical replication needed to allow vigorous statistical analysis. Furthermore, the design should maximize existing resources and the ability to answer biologically meaningful questions (Chen et al. 2004). The design should also meet the standards of the Microarray Gene Expression Data Society (MGED). Compliance with their standard Minimum Information About a Microarray Experiment (MIAME) is required for publication in most journals (http://www.mged.org/Workgroups/MIAME/miame.html). Researchers should plan beyond the initial microarray experiment to likely follow up experiments to avoid generating only a list of gene names. Adequate consideration of each of these areas before starting work will help to ensure that the constructed microarrays can be used as a powerful genomic tool for long-term research on aquaculture species.

Researchers of aquaculture species may benefit from the several points of consensus that have only recently emerged after more than a decade of debate over microarray experimental design, inference, and validation. The mountain of literature describing new methods of microarray design and analysis has continued to grow rapidly, overwhelming the ability of the average scientist to keep up. For example, the journal *Bioinformatics* alone has published more than 500 papers to date on microarray analysis, many proposing novel algorithms. Therefore, the emergence of consensus points (as outlined by Allison et al. 2006) should provide some welcome and much-needed clarity to researchers beginning microarray projects. Of greatest impact to investigators is the emerging consensus that "pooling biological samples can be useful" for microarray analysis (Allison et al. 2006). Real-world financial and RNA sample constraints often make pooling a necessity for microarray research in underfunded agricultural species, but pooling may also have the added benefit of reducing variability among arrays so that broad, global expression changes can be meaningfully assessed. However, multiple pools must be used to estimate variance for inference testing. Three technical replicates of a single RNA pool is not statistically equivalent to three distinct RNA pools each hybridized to a separate array. A second consensus point likely to influence many microarray researchers is that false-discovery rate (FDR) is a better scale with which to quantify confidence than the standard p-value (Benjamini and Hochberg 1995, Pawitan et al. 2005, Allison et al. 2006). The false-discovery rate is the expected proportion of false positives among the results declared significant and is better suited to testing the tens of thousands of individual hypotheses associated with microarrays than is a too lenient p-value or the too conservative Bonferroni correction. Inferences based on expression fold change and FDR as implemented in microarray analysis programs such as Significance Analysis of Microarrays (SAM) (Tusher et al. 2001) allow users to decide the level of acceptable

Type I error suitable for their needs when evaluating differentially expressed gene lists. By adapting these, and other, newly emerged points of consensus into their research, aquaculture researchers can avoid some of the pitfalls and controversies that have hindered past microarray projects.

Acknowledgments

Research in our laboratory is supported by grants from the USDA NRI Animal Genome and Genetic Mechanisms Program, the USDA NRI Basic Genome Reagents and Tools Program, the Mississippi-Alabama Sea Grant Consortium, the Alabama Department of Conservation, the USAID, and the BARD.

References

Allison DB, X Cui, GP Page, and M Sabripour. 2006. Microarray data analysis: from disarray to consolidation and consensus. Nat Rev Genet, 7, pp. 55–65.

Aubin-Horth N, CR Landry, BH Letcher, and HA Hofmann. 2005. Alternative life histories shape brain gene expression profiles in males of the same population. Proc Biol Sci, 272, pp. 1655–1662.

Badiee A, HG Eiken, VM Steen, and R Lovlie. 2003. Evaluation of five different cDNA labeling methods for microarrays using spike controls. BMC Biotechnol, 3, p. 23.

Baxi MK, S Baxi, A Clavijo, KM Burton, and D Deregt. 2006. Microarray-based detection and typing of foot-and-mouth disease virus. Vet J, 172, pp. 473–481.

Benjamini Y and Y Hochberg. 1995. Controlling the false discovery rate—a practical and powerful approach to multiple testing. J R Stat Soc Ser B, 57, pp. 289–300.

Byon JY, T Ohira, I Hirono, and T Aoki. 2005. Use of a cDNA microarray to study immunity against viral hemorrhagic septicemia (VHS) in Japanese flounder (*Paralichthys olivaceus*) following DNA vaccination. Fish Shellfish Immunol, 18, pp. 135–147.

Byon JY, T Ohira, I Hirono, and T Aoki. 2006. Comparative immune responses in Japanese flounder, *Paralichthys olivaceus* after vaccination with viral hemorrhagic septicemia virus (VHSV) recombinant glycoprotein and DNA vaccine using a microarray analysis. Vaccine, 24, pp. 921–930.

Chen DT, JJ Chen, and SJ Soong. 2005. Probe rank approaches for gene selection in oligonucleotid arrays with a small number of replicates. Bioinformatics, 21, pp. 2861–2866.

Chen YA, DJ McKillen, S Wu, MJ Jenny, R Chapman, PS Gross, GW Warr, and JS Almeida. 2004. Optimal cDNA microarray design using expressed sequence tags for organisms with limited genomic information. BMC Bioinformatics, 5, p. 191.

Churchill GA. 2002. Fundamentals of experimental design for cDNA microarrays. Nat Genet, 32 Suppl, pp. 490–495.

Cretich M, F Damin, G Pirri, and M Chiari. 2006. Protein and peptide arrays: Recent trends and new directions. Biomol Eng, 23, pp. 77–88.

D'Ambrosio C, L Gatta, and S Bonini. 2005. The future of microarray technology: networking the genome search. Allergy, 60, pp. 1219–1226.

Dhar AK, A Dettori, MM Roux, KR Klimpel, and B Read. 2003. Identification of differentially expressed genes in shrimp (*Penaeus stylirostris*) infected with White spot syndrome virus by cDNA microarrays. Arch Virol, 148, pp. 2381–2396.

Dobbin K and R Simon. 2002. Comparison of microarray designs for class comparison and class discovery. Bioinformatics, 18, pp. 1438–1445.

Drake TA, EE Schadt, and AJ Lusis. 2006. Integrating genetic and gene expression data: application to cardiovascular and metabolic traits in mice. Mamm Genome, 17, pp. 466–479.

Ewart KV, JC Belanger, J Williams, T Karakach, S Penny, SC Tsoi, RC Richards, and SE Douglas. 2005. Identification of genes differentially expressed in Atlantic salmon (*Salmo salar*) in response to infection by *Aeromonas salmonicida* using cDNA microarray technology. Dev Comp Immunol, 29, pp. 333–347.

Fodor SP, JL Read, MC Pirrung, L Stryer, AT Lu, and D Solas. 1991. Light-directed, spatially addressable parallel chemical synthesis. Science, 251, pp. 767–773.

Gracey AY, EJ Fraser, W Li, Y Fang, RR Taylor, J Rogers, A Brass, and AR Cossins. 2004. Coping with cold: An integrative, multitissue analysis of the transcriptome of a poikilothermic vertebrate. Proc Natl Acad Sci USA, 101, pp. 16970–16975.

Han ES, Y Wu, R McCarter, JF Nelson, A Richardson, and SG Hilsenbeck. 2004. Reproducibility, sources of variability, pooling, and sample size: important considerations for the design of high-density oligonucleotide array experiments. J Gerontol A Biol Sci Med Sci, 59, pp. 306–315.

Hessner MJ, L Meyer, J Tackes, S Muheisen, and X Wang. 2004. Immobilized probe and glass surface chemistry as variables in microarray fabrication. BMC Genomics, 5, p. 53.

Irizarry RA, B Hobbs, F Collin, YD Beazer-Barclay, KJ Antonellis, U Scherf, and TP Speed. 2003. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics, 4, pp. 249–264.

Jordal AE, BE Torstensen, S Tsoi, DR Tocher, SP Lall, and SE Douglas. 2005. Dietary rapeseed oil affects the expression of genes involved in hepatic lipid metabolism in Atlantic salmon (*Salmo salar* L.). J Nutr, 135, pp. 2355–2361.

Ju Z, RA Dunham, and Z Liu. 2002. Differential gene expression in the brain of channel catfish (*Ictalurus punctatus*) in response to cold acclimation. Mol Genet Genomics, 268, pp. 87–95.

Khadijah S, SY Neo, MS Hossain, LD Miller, S Mathavan, and J Kwang. 2003. Identification of white spot syndrome virus latency-related genes in specific-pathogen-free shrimps by use of a microarray. J Virol, 77, pp. 10162–10167.

Kocabas A, R Dunham, and ZJ Liu. 2004. Alterations in gene expression in the brain of white catfish (*Ameirus catus*) in response to cold acclimation. Marine Biotechnology, 6, pp. S431–S438.

Koskinen H, A Krasnov, C Rexroad, Y Gorodilov, S Afanasyev, and H Molsa. 2004a. The 14-3-3 proteins in the teleost fish rainbow trout (*Oncorhynchus mykiss*). J Exp Biol, 207, pp. 3361–3368.

Koskinen H, P Pehkonen, E Vehniainen, A Krasnov, C Rexroad, S Afanasyev, H Molsa, and A Oikari. 2004b. Response of rainbow trout transcriptome to model chemical contaminants. Biochem Biophys Res Commun, 320, pp. 745–753.

Krasnov A, H Koskinen, S Afanasyev, and H Molsa. 2005a. Transcribed Tc1-like transposons in salmonid fish. BMC Genomics, 6, p. 107.

Krasnov A, H Koskinen, P Pehkonen, and CE Rexroad III, S Afanasyev, and H Molsa. 2005b. Gene expression in the brain and kidney of rainbow trout in response to handling stress. BMC Genomics, 6, p. 3.

Krasnov A, H Koskinen, C Rexroad, S Afanasyev, H Molsa, and A Oikari. 2005c. Transcriptome responses to carbon tetrachloride and pyrene in the kidney and liver of juvenile rainbow trout (*Oncorhynchus mykiss*). Aquat Toxicol, 74, pp. 70–81.

Kurobe T, M Yasuike, T Kimura, I Hirono, and T Aoki. 2005. Expression profiling of immune-related genes from Japanese flounder *Paralichthys olivaceus* kidney cells using cDNA microarrays. Dev Comp Immunol, 29, pp. 515–523.

Lan Y, X Xu, F Yang, and X Zhang. 2006. Transcriptional profile of shrimp white spot syndrome virus (WSSV) genes with DNA microarray. Arch Virol, 151, pp. 1723–1733.

Leung YF and D Cavalieri. 2003. Fundamentals of cDNA microarray data analysis. Trends Genet, 19, pp. 649–659.

Li J and M Burmeister. 2005. Genetical genomics: combining genetics with gene expression analysis. Hum Mol Genet, 2, pp. R163–R169.

Lipshutz RJ, SP Fodor, TR Gingeras, and DJ Lockhart. 1999. High density synthetic oligonucleotide arrays. Nat Genet, 21, pp. 20–24.

MacKenzie S, D Iliev, C Liarte, H Koskinen, JV Planas, FW Goetz, H Molsa, A Krasnov, and L Tort. 2006. Transcriptional analysis of LPS-stimulated activation of trout (*Oncorhynchus mykiss*) monocyte/macrophage cells in primary culture treated with cortisol. Mol Immunol, 43, pp. 1340–1348.

Manduchi E, LM Scearce, JE Brestelli, GR Grant, KH Kaestner, and CJ Stoeckert Jr. 2002. Comparison of different labeling methods for two-channel high-density microarray experiments. Physiol Genomics, 10, pp. 169–179.

Marks H, O Vorst, AM van Houwelingen, MC van Hulten, and JM Vlak. 2005. Gene-expression profiling of White spot syndrome virus in vivo. J Gen Virol, 86, pp. 2081–2100.

Martin SA, SC Blaney, DF Houlihan, and CJ Secombes. 2006. Transcriptome response following administration of a live bacterial vaccine in Atlantic salmon (*Salmo salar*). Mol Immunol, 43, pp. 1900–1911.

Meijer AH, FJ Verbeek, E Salas-Vidal, M Corredor-Adamez, J Bussman, AM van der Sar, GW Otto, R Geisler, and HP Spaink. 2005. Transcriptome profiling of adult zebrafish at the late stage of chronic tuberculosis due to *Mycobacterium marinum* infection. Mol Immunol, 42, pp. 1185–1203.

Moriya S, S Urawa, O Suzuki, A Urano, and S Abe. 2004. DNA microarray for rapid detection of mitochondrial DNA haplotypes of chum salmon. Mar Biotechnol (NY), 6, pp. 430–434.

Morrison RN, GA Cooper, BF Koop, ML Rise, AR Bridle, MB Adams, and BF Nowak. 2006. Transcriptome profiling the gills of amoebic gill disease (AGD)-affected Atlantic salmon (*Salmo salar* L.)—A role for tumor suppressor p53 in AGD-pathogenesis? Physiol Genomics, 26, pp. 15–34.

Nash JH, WA Findlay, CC Luebbert, OL Mykytczuk, SJ Foote, EN Taboada, CD Carrillo, JM Boyd, DJ Colquhoun, ME Reith, and LL Brown. 2006. Comparative genomics profiling of clinical isolates of Aeromonas salmonicida using DNA microarrays. BMC Genomics, 7, p. 43.

Nuwaysir EF, W Huang, TJ Albert, J Singh, K Nuwaysir, A Pitas, T Richmond, T Gorski, JP Berg, J Ballin, M McCormick, J Norton, T Pollock, T Sumwalt, L Butcher, D Porter, M Molla, C Hall, F Blattner, MR Sussman, RL Wallace, F Cerrina, and RD Green. 2002. Gene expression analysis using oligonucleotide arrays produced by maskless photolithography. Genome Res, 12, pp. 1749–1755.

Pawitan Y, S Michiels, S Koscielny, A Gusnanto, and A Ploner. 2005. False discovery rate, sensitivity and sample size for microarray studies. Bioinformatics, 21, pp. 3017–3024.

Purcell MK, KM Nichols, JR Winton, G Kurath, GH Thorgaard, P Wheeler, JD Hansen, RP Herwig, and LK Park. 2006. Comprehensive gene expression profiling following DNA vaccination of rainbow trout against infectious hematopoietic necrosis virus. Mol Immunol, 43, pp. 2089–2106.

Renn SC, N Aubin-Horth, and HA Hofmann. 2004. Biologically meaningful expression profiling across species using heterologous hybridization to a cDNA microarray. BMC Genomics, 5, p. 42.

Rise ML, SR Jones, GD Brown, KR von Schalburg, WS Davidson, and BF Koop. 2004b. Microarray analyses identify molecular biomarkers of Atlantic salmon macrophage and hematopoietic kidney response to *Piscirickettsia salmonis* infection. Physiol Genomics, 20, pp. 21–35.

Rise ML, KR von Schalburg, GD Brown, MA Mawer, RH Devlin, N Kuipers, M Busby, M Beetz-Sargent, R Alberto, AR Gibbs, P Hunt, R Shukin, JA Zeznik, C Nelson, SR Jones, DE Smailus, SJ Jones, JE Schein, MA Marra, YS Butterfield, JM Stott, SH Ng, WS Davidson, and BF Koop. 2004a. Development and application of a salmonid EST database and cDNA

microarray: data mining and interspecific hybridization characteristics. Genome Res, 14, pp. 478–490.

Sarropoulou E, G Kotoulas, DM Power, and R Geisler. 2005. Gene expression profiling of gilthead sea bream during early development and detection of stress-related genes by the application of cDNA microarray technology. Physiol Genomics, 23, pp. 182–191.

Schena M, D Shalon, RW Davis, and PO Brown. 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science, 270, pp. 467–470.

Schmitt B and L Henderson. 2005. Diagnostic tools for animal diseases. Rev Sci Tech., 24, pp. 243–250.

Shih JH, AM Michalowska, K Dobbin, Y Ye, TH Qiu, and JE Green. 2004. Effects of pooling mRNA in microarray class comparisons. Bioinformatics, 20, pp. 3318–3325.

Simon R, MD Radmacher, and K Dobbin. 2002. Design of studies using DNA microarrays. Genet Epidemiol, 23, pp. 21–36.

Smyth GK, J Michaud, and HS Scott. 2005. Use of within-array replicate spots for assessing differential expression in microarray experiments. Bioinformatics, 21, pp. 2067–2075.

Tilton SC, LG Gerwick, JD Hendricks, CS Rosato, G Corley-Smith, SA Givan, GS Bailey, CJ Bayne, and DE Williams. 2005. Use of a rainbow trout oligonucleotide microarray to determine transcriptional patterns in aflatoxin B1-induced hepatocellular carcinoma compared to adjacent liver. Toxicol Sci, 88, pp. 319–330.

Tsai JM, HC Wang, JH Leu, HH Hsiao, AH Wang, GH Kou, and CF Lo. 2004. Genomic and proteomic analysis of thirty-nine structural proteins of shrimp white spot syndrome virus. J Virol, 78, pp. 11360–11370.

Tusher VG, R Tibshirani, and G Chu. 2001. Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci USA, 98, pp. 5116–5121.

von Schalburg KR, SP McCarthy, ML Rise, JC Hutson, WS Davidson, and BF Koop. 2006. Expression of morphogenic genes in mature ovarian and testicular tissues: potential stem-cell niche markers and patterning factors. Mol Reprod Dev, 73, pp. 142–152.

von Schalburg KR, ML Rise, GD Brown, WS Davidson, and BF Koop. 2005b. A comprehensive survey of the genes involved in maturation and development of the rainbow trout ovary. Biol Reprod, 72, pp. 687–699.

von Schalburg KR, ML Rise, GA Cooper, GD Brown, AR Gibbs, CC Nelson, WS Davidson, and BF Koop. 2005a. Fish and chips: various methodologies demonstrate utility of a 16,006-gene salmonid microarray. BMC Genomics, 6, p. 126.

Vornanen M, M Hassinen, H Koskinen, and A Krasnov. 2005. Steady-state effects of temperature acclimation on the transcriptome of the rainbow trout heart. Am J Physiol Regul Integr Comp Physiol, 289, pp. R1177–1184.

Walsh B and D Henderson. 2004. Microarrays and beyond: what potential do current and future genomics tools have for breeders? J Anim Sci E-Suppl, pp. E292–299.

Whitfield CW, MR Band, MF Bonaldo, CG Kumar, L Liu, JR Pardinas, HM Robertson, MB Soares, and GE Robinson. 2002. Annotated expressed sequence tags and cDNA microarrays for studies of brain and behavior in the honey bee. Genome Res, 12, pp. 555–566.

Williams TD, K Gensberg, SD Minchin, and JK Chipman. 2003. A DNA expression array to detect toxic stress response in European flounder (*Platichthys flesus*). Aquat Toxicol, 65, pp. 141–157.

Woo Y, J Affourtit, S Daigle, A Viale, K Johnson, J Naggert, and G Churchill. 2004. A comparison of cDNA, oligonucleotide, and Affymetrix GeneChip gene expression microarray platforms. J Biomol Tech, 15, pp. 276–284.

Yauk CL, ML Berndt, A Williams, and GR Douglas. 2004. Comprehensive comparison of six microarray technologies. Nucleic Acids Res, 32, p. e124.

Zhao SH, J Recknor, JK Lunney, D Nettleton, D Kuhar, S Orley, and CK Tuggle. 2005. Validation of a first-generation long-oligonucleotide microarray for transcriptional profiling in the pig. Genomics, 86, pp. 618–625.