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A Review on DNA Microarrays: A Novel Tool for Identification and Exploitation of Fish Conservation in Aquaculture

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Abstract: DNA-based identification methods offer an analytically powerful addition or even an alternative. In this study, a DNA microarray has been developed to be able to investigate its potential as a tool for the identification of fish species. Microarray technology has arguably caught the attention of the worldwide life science community and is now systematically supporting major discoveries in many fields of study. The majority of the initial technical challenges of conducting experiments are being resolved, only to be replaced with new informatics hurdles, including statistical analysis, data visualization, interpretation and storage. Best practice in aquaculture requires a full understanding of the genomic controls and transcriptional profiles of cultured fish species. Improvements in aquaculture can be made by regulation of the expression of functional genes. Microarray technology is a powerful tool for rapid screening of genes or transcriptional profiles in a particular fish or for a particular economic character; for example, genes that are related to growth and disease control in the fish. This review provides a brief introduction to microarray technology and its applications, together with a discussion of the achievements in fish biology that have resulted from this technology.

Key words: Bioinformatics · Microarray · CDNA · Teleost Fish · Aquaculture

INTRODUCTION

The historical discovery of the DNA in the last century, research on gene structures, gene discovery and functions has accelerated. The traditional approaches to molecular studies of a given effect by analyzing a single gene or a few genes at a time are important for investigation of gene function and regulation. However, the biological characteristics of a given cell or tissue or even those of an organism, may be controlled by many molecular events and involve hundreds or thousands of genes [1, 2]. The eminence of DNA microarray technology [3] is the aptitude to be used to simultaneously monitor and study the expression levels of thousands of genes, their functions and classifying genes or samples that perform in a parallel or synchronized manner during imperative biological processes. Functional genomics can be better implicit when the veiled patterns in gene expression data is elucidated, however, it is very challenging to comprehend and construe this due to the complexity of biological networks and large number of genes. To perceive and identify appealing patterns of expression across multiple genes and experiments, reveal natural structures and compress high-dimensional array data clustering must be ascertained to allow easier management of data set. This data reduction method is a simple tool yet powerful method of organizing genes based on their interdependence behaving similarly over the different conditions in different mutants and properties into a set of disjoint groups based on specific features so that the underlying structures can be acknowledged and explored [4].

Recently, a number of studies have used microarray technology to determine gene profiles in different fish species [5]. For example, a microarray consisting of 4512 complementary DNAs (cDNAs) was constructed to investigate the adaptive molecular responses of zebra fish to hypoxia during development [6, 7]. Linney *et al.* [8] analyzed 15512 unique transcripts from wild-type *Danio rerio* and found that 23 muscle specific genes

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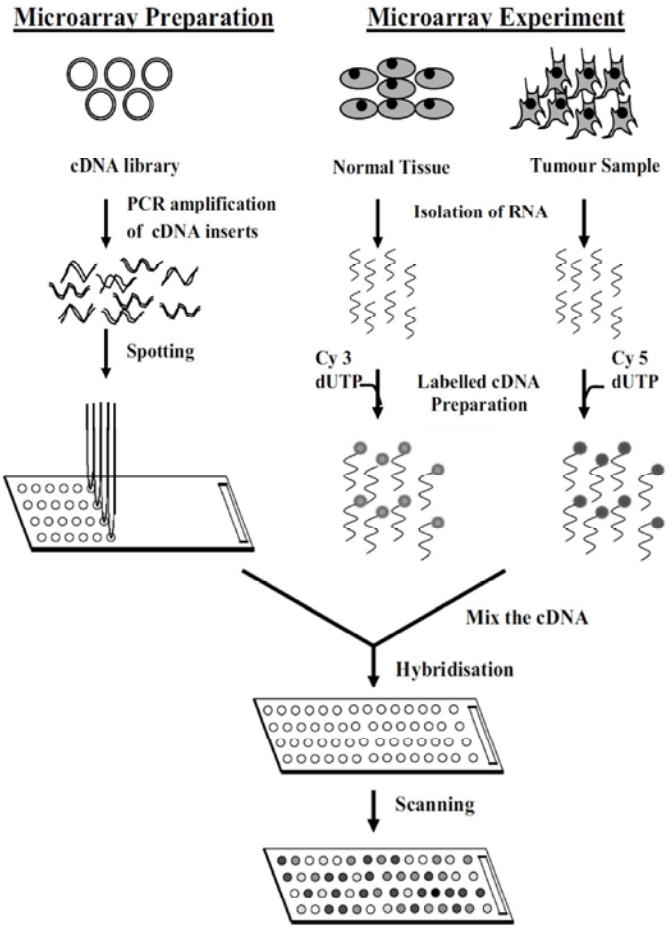

Fig. 1: Preparation of microarrays and microarray hybridization [24]

were up-regulated as part of somite development. An Affymetrix microarray was also used to screen the genes that are involved in regeneration of the caudal fin [9]. Microarray analyses have been used to search for immune-related genes that are expressed following DNA vaccination or infection by fish pathogens [10-13] and to study environmental influences on gene expression [14-16]. Obviously, the use of DNA microarray technology in fish biology and aquaculture may have great significance and may be applied to discovery of novel genes, gene expression profiling from fish species and identification of the genomic responses to environmental stimulation in aquaculture.

Principle of DNA Microarray Technology: The principle of a microarray experiment, as opposed to the classical northern-blotting analysis, is that mRNA from a given cell line or tissue is used to generate a labeled sample, sometimes termed the 'target', which is hybridized in parallel to a large number of DNA sequences, immobilized on a solid surface in an ordered array [17, 18]. Tens of thousands of transcript species can be detected and quantified simultaneously. During recent years, DNA microarray technology has been advancing rapidly. The development of more powerful robots for arraying, new surface technology for glass slides and new labeling protocols and dyes, together with increasing genome-sequence information for different organisms, including humans will enable us to extend the quality and complexity of microarray experiments.

Array Platforms: Although many different microarray systems have been developed by academic groups and commercial suppliers, the most commonly used systems today can be divided into two groups, according to the arrayed material: complementary DNA (cDNA) and oligonucleotide microarrays are shown in Figure 1. The arrayed material has generally been termed the probe since it is equivalent to the probe used in a northern blot analysis. Probes for cDNA arrays are usually products of

the polymerase chain reaction (PCR) generated from cDNA libraries or clone collections, using gene-specific primers and are printed onto glass slides or nylon membranes as spots at defined locations. Spots are typically 100-300 µm in size and are spaced about the same distance apart. Using this technique, arrays consisting of more than 30,000 cDNAs can be fitted onto the surface of a conventional microscope slide. For oligonucleotide arrays, short 20-25mers are synthesized in situ, either by photolithography onto silicon wafers (high-density-oligonucleotide arrays from Affymetrix [19, 20], http:// www.affymetrix.com) or by ink-jet technology (developed by Rosetta Inpharmatics, http://www.rii.com and licensed to Agilent Technologies). Alternatively, presynthesized oligonucleotides can be printed onto glass slides. Synthetic oligonucleotides offer the advantage that because sequence information alone is sufficient to generate the DNA to be arrayed, no time-consuming handling of cDNA resources is required. Also, probes can be designed to represent the most unique part of a given transcript, making the detection of closely related genes or splice variants possible. Although short oligonucleotides may result in less specific hybridization and reduced sensitivity, the arraying of presynthesized longer oligonucleotides (50–100mers) has recently been developed to counteract these disadvantages. However, the high cost of commercially available, *in situ*-synthesized oligonucleotide arrays can make them inaccessible for academic laboratories and purchase of large numbers of long oligonucleotides also incurs significant cost.

Spotted arrays allow a greater degree of flexibility in the choice of arrayed elements, particularly for the preparation of smaller, customized arrays for specific investigations. As a result, cDNA gridded arrays have so far been the technique most frequently used in academic labs (http://cmgm.stanford.edu/pbrown/mguide/index.html and http://www.nhgri.nih.gov/DIR /LCG/ 15K/ HTML for information about cDNA array technology). In addition, arraying of unsequenced clones from cDNA libraries can be useful for gene discovery. However, with prices for oligonucleotide synthesis falling all the time, spotted long- ligonucleotide arrays could be a viable alternative for the future.

Target Preparation: Another important difference between *in situ* synthesized, high-density oligonucleotide arrays (Affymetrix) and spotted arrays lies in target preparation are shown in figure 1. In both cases, mRNA from cells or tissue is extracted, converted to DNA and labeled, hybridized to the DNA elements on the array surface of the array and detected by phospho-imaging or fluorescence scanning. The high reproducibility of *in situ* synthesis of oligonucleotide chips allows accurate comparison of signals generated by samples hybridized to separate arrays [21]. In the case of spotted arrays, the process of gridding is not accurate enough to allow comparison between different arrays. The use of different fluorescent dyes (Cy3 and Cy5) allows mRNAs from two different cell populations or tissues to be labeled in different colors, mixed and hybridized to the same array, which results in competitive binding of the target to the arrayed sequences. After hybridization and washing, the slide is scanned using two different wavelengths, corresponding to the dyes used and the intensity of the same spot in both channels is compared. This results in a measurement of the ratio of transcript levels for each gene represented on the array.

As array technology has advanced, more sensitive and quantitative methods for target preparation have had to be developed. In cases in which the quantity of RNA is not limited, incorporation of nucleotides coupled to fluorescent dyes during synthesis of the first strand of cDNA is the method of choice, as it provides the most linear relationship between starting material and labeled product. However, most protocols require between 25–100 µg total RNA, which is often not readily available in studies using primary cells or tissues. Various procedures have been developed to increase sensitivity and reduce the amount of RNA required. One strategy is target amplification by *in vitro* transcription, whereby up to 50 µg of labeled cRNA can be produced from 1 µg of mRNA. In addition, several rounds of *in vitro* transcription can be combined with cDNA synthesis to enhance the amplification even further. Using these protocols, it is even possible to profile the transcripts of a single cell [22]. Another strategy is post-hybridization amplification using labeled antibodies or molecules carrying large numbers of fluorophors [23]. Several studies have used target-amplification techniques to compare the expression profiles of defined cell populations extracted from tissue sections by laser-capture micro dissection.

However, suitable controls are required to ensure that amplification has not introduced significant experimental bias into the target preparation. This problem has been particularly evident in the expression profiling of tumor samples. In the case of solid tumors, obtaining pure populations of tumor cells for microarray analysis would require microdissection. However, a study using grossly dissected breast-cancer specimen has demonstrated a

way to circumvent the problem of sample heterogeneity. Expression profiles from whole solid tumors can be compared to profiles from potential untransformed infiltrating cell types, such as lymphocytes or endothelial cells, to identify a subset of genes with expression patterns that are specific to the tumor cells.

Data Analysis, Reproducibility and Validation: The data of a microarray experiment typically constitute a long list of measurements of spot intensities and intensity ratios, generated either by pair wise comparison of two samples or by comparing several samples to a common control. Replication has been shown to reduce markedly the number of potential false positive results, but may be difficult because of high cost or limitation of the amount of sample. However, as the efficiency of incorporation of nucleotides labeled with different fluorescent dyes during target sample preparation may not be equal, reciprocal labeling with swapped colors is recommended. Several researchers have found that variability of microarray results can be significant, especially for genes with low expression levels and replication is needed to establish a high degree of confidence in the data. Another problem arises from the fact that data about genes that are found to be unchanged have to be treated with particular care. It is very possible that certain DNA elements on the array simply fail to detect the right transcript species, as a result of cross-hybridization or adverse secondary structure. Verification of a subset of results by alternative techniques such as northern hybridization, RNase protection or PCR with reverse transcription, in particular 'real-time' RT-PCR, can help to establish an estimate of the variability of a given experimental system. More experience in using microarray technology, particularly concerning the choice of DNA sequences to be arrayed, will improve confidence in the reliability of the data. Because of the complexity of the data sets generated by microarray experiments, the use of data analysis software is essential. Several data analysis tools have been developed by commercial suppliers such as Gene Spring from Silicon Genetics (http://www.sigenetics.com/) and others are available from public sources (http://genome-www4.stanford.edu/MicroArray/SMD/ restech.html for an overview).

Challenges after Analysis: After several microarray analyses, it quickly becomes obvious that the rate-limiting step in functional genomics experiments is neither the handling of the biological samples nor the actual analysis. The information might not yet be available for genes that have been found to be significant, even though these genes might have been measured on microarrays for years. This complicates the interpretation of results. The official gene name, predicted protein domains or gene-ontology 50 classifications might become available as early as tomorrow, or as late as decades from now. There are further post-analysis challenges. Occasionally, microarray probes are designed against chromosomal regions instead of expressed products and when these probe sets are positive in an analysis, it is usually not clear which genes are being detected. The probe sets are incorrectly designed against the wrong strand or wrong species. Oligonucleotide sequences that were once thought to be unique for a particular gene might not remain unique as more genomic data are collected. Finally, the spotted cDNA arrays, particularly those for which the probe sequences have not been validated, the findings might be incorrect. This means that one is never done analyzing a set of microarray data. The infrastructure has to be developed to re-investigate constantly genes and gene information from microarray analyses performed in the past.

Application and Progress of Microarray Technology in Fish Biology

Gene Expression Profiling and Discovery: The screening of global gene expression profiles and discovery of differential gene expression have been used for identification of genes in tissue-specific or development specific profiling in various fish species. Zebra fish has provided a model for the application of microarray technology. By construction of a zebra fish cDNA microarray, gene expression patterns and profiling in zebra fish during development have been well defined. This work has revealed that some functional genes, such as those for actin, myosin heavy and light chains and parvalbumin, are expressed in close correlation with the muscle development and growth of the fish [6]. Linney *et al*. [8] analyzed 15512 unique transcripts from wild-type *Danio rerio* and detected 420 up-regulated and 386 down-regulated genes during development at 12 hours past fertilization (hpf). Microarray technology is also a functional tool for investigating tissue or organ development of fish at the transcriptional level. For example, Nishidate *et al.* [25] constructed a cDNA microarray and identified 140 transcripts that were up-regulated during regeneration of fins in the medaka fish. Katogi *et al*. [26] identified six candidate genes that were associated with blastema formation. During the development of the retinal pigment epithelium (RPE) in

zebra fish, 8810 genes were significantly expressed in the RPE at 52 hpf, of which 1443 potentially had biologically meaningful expression levels. In addition, heart regeneration in zebra fish is associated with sequential up-regulation of genes that are associated with wound healing and growth factors, which suggest that the plate-derived growth factor (PDGF) signaling is required [27].

Identification of Immunity or Disease Resistance Related Genes: Bacterial and viral infections are associated with important diseases of fish. Analysis of the expression pattern of immunity-related genes not only provides insight into the molecular mechanisms underlying the fish immune system, but also assists in the development of effective vaccines for use in aquacultural practice [28]. Microarray technology has made it possible to clarify the immune system in detail and several pioneer works have applied the techniques to this system [29-32]. For example, the *Mycobacterium marnum*-zebra fish infection model has been used for analysis of a host transcriptome response to mycobacterial infection. Three types of array were used, namely, the MWG and Sigma zebra fish oligonucleotide arrays and the Affymetrix zebra fish chip and it was found that 66 unregulated and 93 down-regulated genes consistently showed an altered expression in each of the three microarrays [33]. Red sea bream iridovirus (RSIV) is an infectious pathogen that is responsible for causing serious diseases in aquatic animals, including fish and the RSIV disease causes mass mortalities and huge economic loss in aquaculture. Lua *et al*. [13] developed a DNA microarray for RSIV to monitor the temporal kinetic transcription program of the viral genes during *in vitro* infection. They demonstrated that the major capsid protein (MCP) is a structural protein that comprises up to 45% of the total protein expression and the proteins are assembled into RSIV virions about 2 days after infection. Byon *et al*. [10] constructed a cDNA chip that contains approximately 900 different cDNA clones, including more than 200 immune-related genes. These researchers immunized juvenile Japanese flounder with a recombinant plasmid expressing the G-protein of viral haemorrhagic septicaemia virus (VHSV) and analyzed gene expression with cDNA microarrays. Using this method, the genes that are responsible for a strong protective non-specific immune response and a specific immune response were elucidated (Byon *et al*., 2005, 2006). Tsoi *et al*. [29] used a human cDNA microarray to identify differentially expressed genes in Atlantic salmon liver during infection with *Aeromonas salmonician*. These examples show that the use of microarray technology is an efficient technique for screening and identification of genes that are related to the immune system.

Identification of Genes That Is Responsive to Environmental Variation: Fish offers important advantages for defining the organism-environment interface and the genomic responses to natural stressors. Microarray technology serves as a valuable tool in toxicogenomics, with which environmental adaptation in fish has been, evaluated [7, 34-37]. Brown *et al*. [38] have developed a microarray for transcriptomic analysis of chemical responses in populations of *Gasterosteus aculeatus* under laboratory and field conditions and a full set of genes that are responsive to pollutants has been identified. Finne *et al*. [39] applied a cDNA microarray platform in ecotoxicological screening of single chemicals and environmental samples that are relevant to the aquatic environment. The work was performed to validate biomarker gene responses of *in vitro* cultured rainbow trout (*Oncorhynchus mykiss*) hepatocytes that were exposed to model chemicals and to investigate the effects of mixture toxicity in a synthetic mixture. Chemicals used for 24-h exposure to single chemicals and mixtures were 10 nmol/L 17alpha-ethinylestradiol (EE2), 0.75 nmol/L 2,3,7,8-tetrachloro di-benzodioxin (TCDD), 100 mol/L paraquat (PQ) and 0.75 mol/L 4- nitroquinoline-1-oxide (NQO). The results revealed that exposure to this mixture led to an average loss of approximately 60% of the transcriptomic signature found during exposure to a single chemical. Larkin *et al.* [40] used microarray techniques for detection and distribution of estrogenic compounds in sheep shead minnow and several estrogen-responsive genes were identified. A similar investigation led to the identification of a transcriptional fingerprint of estrogen exposure in live rainbow trout [41, 42]. Hypoxia is important in both biomedical and environmental contexts and requires rapid adaptations in metabolism. Fish live and survive in environments with low and variable levels of oxygen and the survival mechanisms induced in response to environmental variation have been studied. During this response, several changes may occur in physiology, genomics and gene expression [7, 43, 35]. Ton *et al*. [7] used a zebra fish cDNA microarray to examine the expression of more than 4500 genes in zebra fish embryos that were exposed to 24 h of hypoxia during development. The work revealed that hypoxia resulted in changes in the gene expression profile of the zebra fish embryos and that these changes could be reversed by exposure to a normoxic (20.8% O2) environment. Ju *et al*. [35] developed

a microarray containing 8046 medaka unigenes and measured gene expression changes in the brain, gills and liver of fish exposed to hypoxia. They found that 501 genes in the brain, 442 in the gills and 715 in the liver were differentially expressed in medaka exposed to hypoxia. Two biological pathways, namely, ubiquitin-proteasome and phosphatidylinositol signaling, were significantly dysregulated in medaka upon exposure to hypoxia. In long-term adaptive responses to hypoxia in adult zebra fish, use of the microarray identified 367 differentially expressed genes, of which 117 showed hypoxia-induced and 250 hypoxia-reduced expressions. A novel adaptive mechanism to hypoxia, i.e., the induction of genes for lysosomal lipid trafficking and degradation, was suggested as a result of these findings [43]. In addition, a change in water temperature is another factor that affects fish growth and physiology and exposure to different water temperatures may cause changes in the pattern of gene expression [44-46].

Study of Genes in Other Organisms, or Heterogeneous Microarray Hybridization: With the limited availability of custom-made and commercially produced cDNA microarrays for fish, it is advantageous to exploit the possibility of hetero-hybridization of one array to other species. Cohen *et al*. [47] utilized a cDNA microarray for European flounder (*P. flesus*) containing 11060 clones to cross-hybridize with genes of several other fish species. The work revealed that cross species cDNA microarray hybridization in fish, at a suborder level and closer, is a useful tool for gene expression profiling. Renn *et al*. [48] applied a cDNA microarray from an African cichlid fish, *Astatotilapia burtoni*, to analyze the transcription profile of eight different fish species. Ju *et al*. [35] used Japanese medaka (*Oryzias latipes*) cDNAs to screen several related human genes and Lam *et al*. [16] used a zebra fish cDNA microarray to screen for genes that are involved in human liver cancer, thus providing additional confidence in the use of the zebra fish cDNA microarray. Such applications indicate that use of the zebra fish cDNA microarray is a valid way to examine other fish (and mammalian) species. Because cDNA microarrays are not available for the Chinese mandarin fish and the silver carp, the Affymetrix zebra fish cDNA microarray has been used in our laboratory. Although the Chinese mandarin fish is far removed from the zebra fish in an evolutionary sense, the results of this research showed that the zebra fish cDNA microarray is suitable for analysis of mRNA populations in the Chinese mandarin fish. The cDNAs from both Chinese mandarin fish and silver carp hybridized successfully to the Affymetrix Zebra fish Genchip. Three hundred seventy-five genes were identified in the muscle tissues of both the Chinese mandarin fish and the silver carp. The number of genes identified in the muscle tissue of each fish appears to be a reasonable representation of the transcription profile for a single muscle tissue. The microarray data were also validated by quantitative real-time PCR. However, the possibility cannot be excluded that certain genes in both fish species were not detected.

CONCLUSION

The use of DNA microarrays to explore gene expression on a global level is a rapidly evolving technology that seems set to become more powerful tool for analysis of an organism's genome or transcriptome by measuring the expression levels of thousands of genes simultaneously in a particular cell or tissue. The biochemistry of the microarrays is proving very useful and it seems likely that the significant advances in the next few years will come in the interpretation of the data sets generated. At present, it seems that we may only be scratching the surface when it comes to extracting useful information from these large quantities of data. Its applications to fish biology and aquaculture exist on two basic platforms: commercially produced high-density arrays, such as the Affymetrix Zebra fish Genchip and customized cDNA microarrays using glass slides or nylon membranes spotted with PCR amplified cDNA fragments or synthetic oligonucleotides based on ESTs. With these platforms, great progress has been achieved in the understanding of fish genomics and molecular biology [49-51]. The application of DNA microarrays to fish in India is still at an early stage. There are many commercially important fishes, which make a great contribution to the aquaculture of the country. However, their genomics, transcriptional profiles and functional genes are need more investigations. Fish scientists and biologists should work together to exploit microarray technology and bioinformatics in research of the genomics of commercially important fish in order to have a better understanding of the biology of fish and make further achievement in aquaculture. Recent work in the field indicates that this is already beginning to occur and that microarray technology is set to contribute much to the post-genomic future of biology.

Finally, the use of microarrays in basic and applied research in drug discovery is only going to increase, but as these data sets grow in size, it is important to recognize that untapped information and potential discoveries might still be present in existing data sets. It should be clear that any set of microarray measurements could be analyzed and re-analyzed in many different ways. In the application of functional genomics to drug discovery, to extract the most information from microarrays, an open mind always needs to be kept with regard to the choices of analytical methods, using supervised and unsupervised techniques and methods yet to come.

Future Perspective: The application of gene expression profiling or genome typing to obtain information about individual species within a natural community would prove invaluable for ecology and for systematic alike. Assuming that appropriate hybridization stringencies are employed and given sufficient fish diversity within the population of interest, there is no theoretical reason for this approach to fail. Microarrays can also be used to gain clues to gene function through looking at knockout mutants, particularly of predicted regulatory genes. Each mutant was tagged with a unique oligonucleotide sequence (a molecular barcode) that was detected by hybridization to a custom-built microarray to determine growth conditions when certain mutants were unable to grow. This methodology combined with a massive parallel analysis of mapped mutants offers a rapid route to determining the function of the genes found in every genome. We are moving from the period of genomics towards the post-genomic future and we are entering what is arguably the most exciting period in the history of microbiology. At last we have the potential to ask questions at a relevant scale, that of the whole genome and hence the whole organism. We are optimistic that swift progress will be made as we learn to implement microarray technology more effectively and we look forward to the time when innovative ideas can be tested extremely quickly.

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