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Eco-toxicogenomics Research with Fish

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Abstract

There are some critical drawbacks in the use of biomarkers for a global assessment of the toxicological impacts many chemicals and environmental pollutants have, primarily due to an individual biomarker's specificity for an explicit chemical or toxicant. In other words, the biomarker-based assessment methodology used to analyze toxicological effects lacks a high-throughput capability. Therefore, eco-toxicogenomics, or the study of toxicogenomics with organisms present within a given environmental locale, has recently been introduced with the advent of the so-called "-omics" era, which began with the creation of microarray technologies. Fish are comparable with humans in their toxicological responses and thus data from toxicogenomic studies performed with fish could be applied, with appropriate tools and implementation protocols, to the evaluation of environments where human or animal health is of concern. At present, there have been very active research streams for developing expression sequence tag (EST) databases (DBs) for zebra fish and rainbow trout. Even though few reports involve toxicogenomic studies with fish, a few groups have successfully fabricated and used cDNA microarrays or oligo DNA chips when studying the toxicological impacts of hypoxia or some toxicants with fish. Furthermore, it is strongly believed that this technology can also be implemented with non-model fish. With the standardization of DNA microarray technologies and ample progress in bioinformatics and proteomic technologies, data obtained from DNA microarray technologies offer not only multiple biomarker assays or an analysis of gene expression profiles, but also a means of elucidating gene networking, gene-gene relations, chemical-gene interactions, and chemical-chemical relationships. Accordingly, the ultimate target of eco-toxicogenomics should be to predict and map the pathways of stress propagation within an organism and to analyze stress networking.

Keywords: fish, toxicogenomics

Ecotoxicogenomic Research

Environmental contaminants and wastes or byproducts from industrial activities after industrialization started have been ceaselessly abandoned into environment. Most of them including xenobiotics cause serious damages to ecosystems or lead to toxic and hazardous impacts to human as well as environment. More than 70,000 different chemicals have been known to be commercially available at present. It has been reported that more than 2-4 million dollars are cost for instrumental analysis for one specific chemical, and even it was known to need a few years to complete analysis of a chemical in a specific sample[1]. Furthermore, environmental pollutants are seldom to be a single component, but various physico-chemical compositions. Thus, it would not be possible to analyze all the chemical compositions even though instrumental analysis for a single component would be realized. It is, therefore, unlikely that environmental hazardous effects or toxicological impacts of such samples could be predicted or estimated based upon instrumental analysis.

Bioassay or biomonitoring and hazardous assessment would be alternative measures for evaluation of hazardous effects or toxicological impacts based upon lethality of an organism, behavior changes, feeding responses, hatching rate, and so on for the protection of environmental pollutions. In addition, considering the facts that environmental pollutants are usually transported and accumulated to aquatic ecosystem via natural environmental circulation, the assessment and monitoring of water pollution is a vital issue and important for protection of environment.

In the light of this view, the use of fish, vertebrate organisms living in aquatic environment, is a logical selection for environmental biomonitoring. The fish, as high-level consumers, would be influenced directly by water pollution, and so it should be a good model organism[2]. In addition, it is also speculated that the lethality-based general toxic impacts of water pollutants on fish can be compared with that of human because fish is vertebrate and show similar physiology to human beings.

Meanwhile, there are some limitations in analysis of chronic and global molecular level impacts from the use of fish as toxicity bioassay reflectors for water pollution. In tracking and detecting of the chronic effects in molecular and cellular levels such as endocrine disrupting effects or accumulation of carcinogenic materials in tissues, any clues or epitope would not come out from a lethality-based approach using fish. For detection of toxicological impacts in molecular and cellular levels, therefore, the use of biomarkers has been introduced by measuring expression of a specific gene or activity of a specific enzyme as indication of the response of fish[3-7]. However, there are still limitations in the use of biomarkers for the analysis of toxicological impacts, especially in terms of understanding of global toxicological impacts and the mode of toxic actions in molecular and cellular levels. The biomarker-based method, in addition, lacks simultaneous analysis of additional impacts, even if the biomarker is usually very specific to a certain toxic impact. These limitations naturally lead to the introduction of DNA microarray technologies into the field of environmental toxicology as soon as the DNA microarray was introduced for genomics research in the world. The (eco)-toxicogenomics research, as a result, has started with DNA microarray high-throughput technologies and is expected to influence on toxicology as well as environmental toxicology profoundly.

Since Brown PO research group published the DNA microarray technology in Science for the first time in 1995, this technology has been implemented within many different scientific and engineering fields[8]. Especially, with its introduction to toxicology field so-called "toxicogenomics" research field opened, i.e., toxicogenomics is just simple combination of toxic (from toxicology) plus genomics. In 1999, Afshari mentioned the direction and content of toxicogenomic research in Cancer Research[9]. This has been also posted at NIEHS as the following[10]:

- Characterize potential mechanisms of action of environmental contaminants through the identification of gene expression networks.
- Identify modes of action for previously uncharacterized toxicants based on correlations with the molecular signatures of toxicants with well-characterized etiologies.
- Assess toxicant-induced gene expression as a biomarker of chemical exposure
- Ascertain overlapping patterns of gene expression in animal species exposed to specific toxicants
- Use microarray information to extrapolate effects of toxicants from one animal species to another
- Characterize the biological effects of complex chemical mixtures
- Examine the effects of chronic vs. acute exposure to chemicals
- Characterize genetic polymorphisms in populations and assess the importance of genetic polymorphisms in modifying individual susceptibilities to a contaminant or toxicant

It is anticipated that toxicogenomic research will assist bioassay- and biomarker-based analysis methodologies and aid in both identifying a toxicant's mode of action and understanding the global impacts and effects toxic materials have at the molecular and cellular levels.

Although the precise definition of eco-toxicogenomics includes toxicogenomic studies with any organism in their natural environment, the selection of fish as a model for toxicogenomic research is logical since fish are vertebrates living in aquatic environments, which are a central part of many ecosystems. In addition, owing to known similarities between physiologies of humans and fish, research performed with fish may be extrapolated so as to provide some clues or epitopes for predicting the toxicological impact of an exposure to humans. For the remainder of this review, therefore, the terms "eco-toxicogenomics" is used to indicate "toxicogenomic research performed with fish".

Fish DNA Microarrays for Eco-toxicogenomic Research

DNA microarrays are classified in two groups, depending on the type of DNA spotted within the array-oligonucleotides or cDNA. The oligonucleotide array uses 20-70 base pairs sized oligonucleotides while the cDNA arrays are spotted with PCR amplicons[11]. In situations that information about the gene-

Table 1. Three Commercially Available Zebra Fish Oligo Microarrays and Their Comparison

	Affymetrix	MWG	Sigma
Characteristics of the zebrafish microarray			
Product name	GeneChip	Zebrafish 14k	Sigma-Compugen XEB384
Oligo length	25	50	65
# Oligo	15502 (× 16)	14067	16399
#Oligo with UniGene assignment	14953	8669	12662
# Unique UniGene cluster	12525	5416	11166

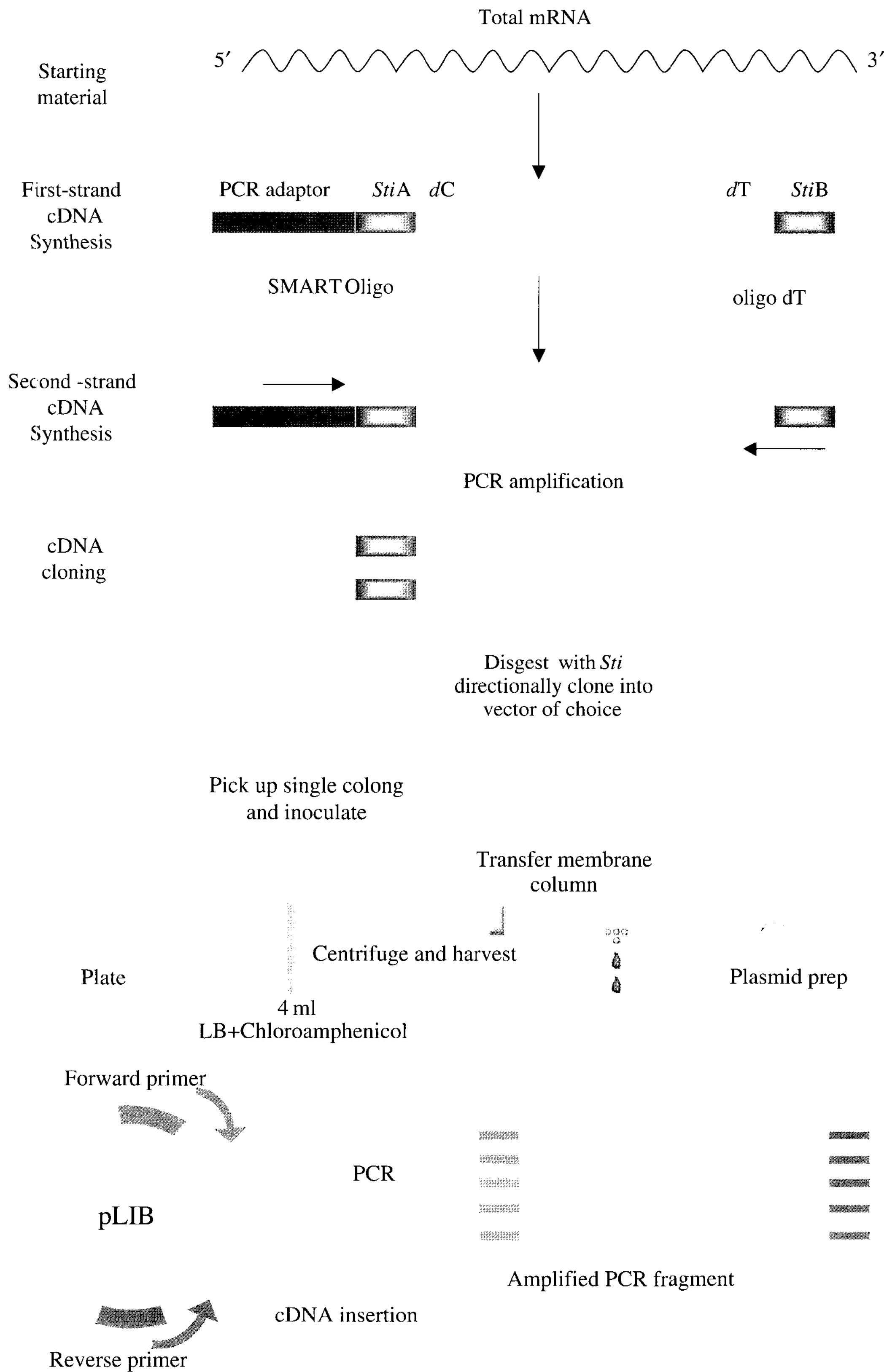

Fig. 1. Construction of cDNA Micrarrays Using a Random Colony Pick Up Method

tic sequences are not available, cDNA libraries are initially constructed using the cellular RNA and then the PCR amplicons are obtained from cDNA libraries[12-13]. In this case, analysis of gene sequences and their functions may be needed to positively select the PCR amplicons of interest. Although both the PCR amplicon-based array and oligonucleotide-based array can be implemented where the genome sequence or specific genetic information is available, the primary merit of the PCR amplicon array over the oligonucleotide array is its cost effectiveness, but at the price of specificity[11].

The most frequently used model fish is the zebra fish (*Danio rerio*), while cDNA microarrays have been fabricated using its genomic sequence. Furthermore, there are commercially available oligonucleotide sets for the whole zebra fish genome and, thus, customized oligonucleotide arrays can be fabricated[14-16]. One such an example is a cDNA microarray that was fabricated with the cDNAs obtained from a 3 day old embryonic heart, an adult heart, and the adult skeletal muscle of zebra fish[17]. There are also a few commercially available oligonucleotide microarrays, including Compugen Zebra fish Oligo-Library™ (Sigma), and other from MWG Biotech. and Affymetrix Co., as shown in Table 1[16].

In addition to the zebra fish, rainbow trout, a lineage of salmonid, is often used as a target model in studies and EST information for this fish is continuously updated and released[18]. Rexroad *et al.* (2003) reported on a cDNA microarray that was fabricated after random cDNAs were secured from six different tissues with a suppression subtractive hybridization (SSH) method. These cDNAs were used alongside other known cDNAs that were selected from among various functional genes (stress and defense response, regulation of cell cycle, signal transduction, chaperon activity and apoptosis)[19].

For Atlantic salmon (*Salmo salar*), Douglas *et al.* fabricated cDNA microarrays using 4000 genes related primarily with immunity from the ESTs of the liver, spleen and head kidney[20]. Furthermore, a few more model fish that have been targets for DNA microarray construction, including *Gillichthys mirabilis, Platichthys flesus, Paralichthys olivaceus,* and *Austrofundulus limnaeus.*

The first fish DNA microarray was constructed using sequences from *Gillichthys mirabilis*. Gracey *et al.* employed both SSH and random cDNA methods to construct the cDNA microarrays, which included 3840 clones from the liver, 1152 from the skeletal muscle and 384 from the brain tissues[12]. Likewise, a cDNA microarray constructed for the European flounder (*Platichthys flesus*) contained 110 cDNAs which were PCR amplified with degenerate primers from the liver and ovaries after selecting genes from the literature and 22 additional genes picked up with the SSH method[21].

In addition, a recent study reported that a cDNA microarray was fabricated using 228 immune-related

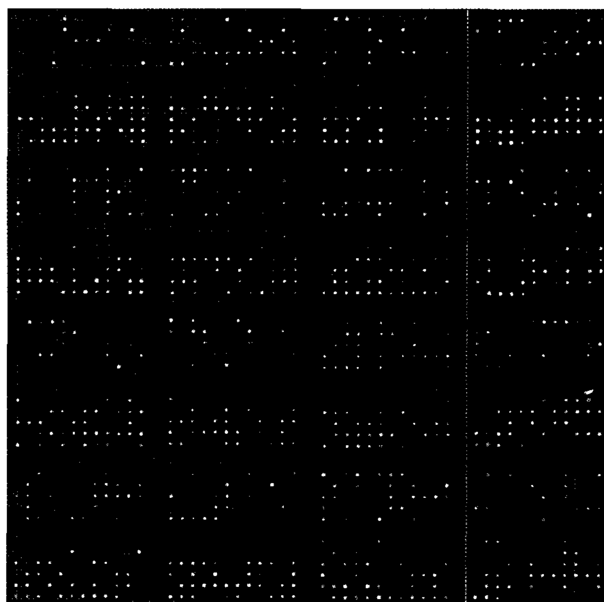

Fig. 2. Scan Image of cDNA Microarray for Japanese Medaka Fish

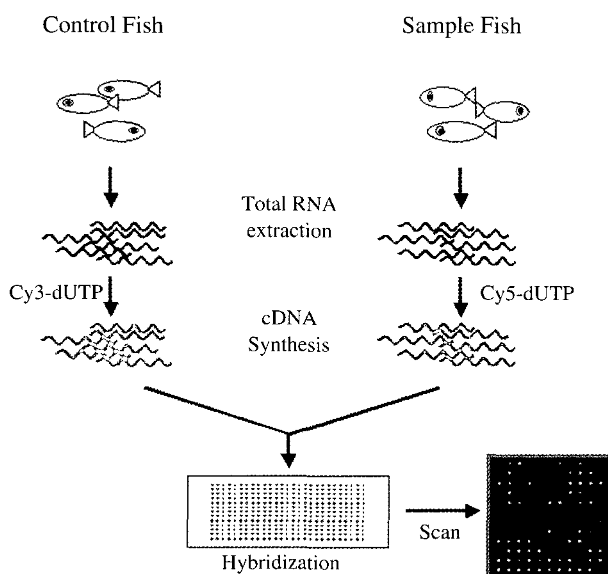

Fig. 3. A Cartoon Showing Steps for DNA Microarray Experiment

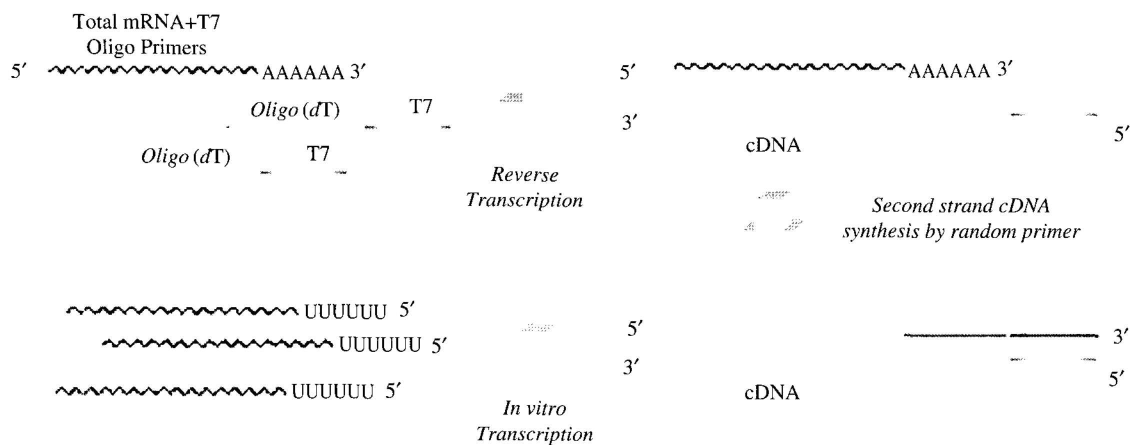

Fig. 4. RNA Amplification Using *In Vitro* Transcription (Adapted from ref. 25)

genes and an additional 551 unknown genes from the Japanese flounder, *Paralichthys olivaceus*[22].

There have been a couple of reports of cDNA microarrays that have been fabricated using ESTs from embryos for developmental genetics studies[23] or the use of some ESTs related with fin regeneration and blastema formation[24] for Japanese medaka fish. Furthermore, we have constructed a cDNA microarray containing a total of 2000 unknown cDNAs obtained from the liver of Japanese medaka using a random colony selection method (Fig. 1) alongside 66 functional genes that were selected from the literature (manuscript in preparation, 2005) (Fig. 2). However, to the best of our knowledge, no reports on the development of oligonucleotide DNA microarrays or oligo sets for Japanese medaka have been published, likely due to an incomplete knowledge of the entire genome sequence.

Eco-toxicogenomic Analysis with Fish DNA Microarray Bioinformatics

The basic principle of a DNA microarray analysis is to compare the dye intensities of both the control and the sample after the dyes are incorporated within cDNAs during the reverse transcription step and the cDNAs have been hybridized on to the slide (Fig. 3). Because there are ESTs spotted on the slide (DNA chip), a high-throughput analysis of the ESTs is possible. In situations where the RNA is limited, a linear RNA amplification method (Fig. 4) can also be used[25].

Primary applications of the DNA microarray data results include 1) the finding of specific biomarker genes that highly up-regulated under a specific condition, and 2) identification of unknown toxicants using gene expression "fingerprints", as shown in Fig. 5[26]. Furthermore, the DNA microarray results can be mined and managed using currently available bioinformatics tools. One such an example is clustering analysis, in which the relationships among genes are presented as a tree whose branch lengths reflect the degree of similarity between the genes, as evaluated by a pair-wise similarity function[27] (Fig. 6). This tool can be used to suggest an epitope showing gene to gene relations. In addition, Butt and Kohane[28] were able to show more complicated intergenic relationships using relevance networks based on an opposite algorithm, while K-mean clustering and Self-Organizing Maps (SOM) have been used to derive specific gene clusters from the analysis[29].

With regards the data analysis, and bioinformatics, of DNA microarray results for fish, Gracey *et al.*[12] reported DNA microarray study for the first time that a time-course analysis has been conducted for hypoxia-induced gene expression for euryoxic fish (*Gillichthys mirabilis*). Because the DNA microarray they used contained random unknown genes (obtained from random colony selection method), the cDNA sequences were determined for differentially expressed genes and then the groups of genes were hierarchically clustered. Northern blot analysis was also used to validate the expression of certain genes. Their findings indicated that hypoxic stimulation caused a reorganization in the cellular ATP-generation pathways. Furthermore, it was also found that some pathways that quickly spent energy were shut down in the skeletal muscles, resulting in anaerobic ATP production, and gluconeogenesis in the liver. It should be noted that this study was undertaken with a non-ideal animal model (defined here as organism with incomplete genetic information), demonstrating the versatility of random cDNA microarrays.

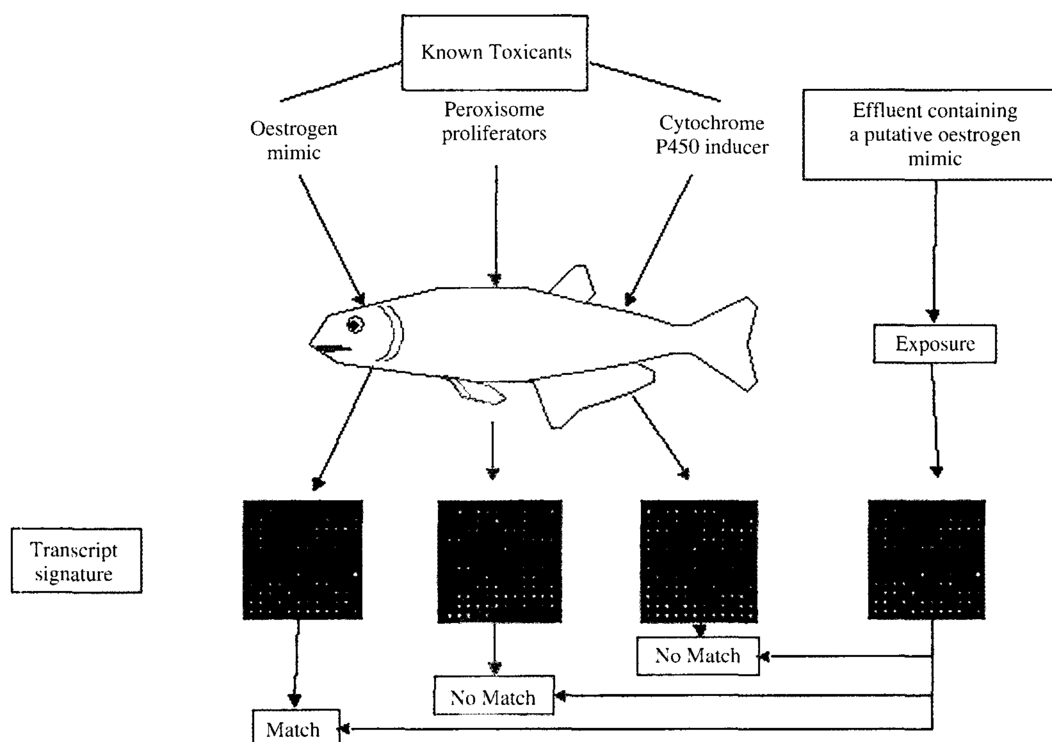

Fig. 5. A Simplistic Schematic Illustration of the Application of Gene Expression Signatures to Identify the Potential Mechanism of Action of an Unknown Environmental Contaminant (Adapted from ref. 26)

Generally speaking, specific cDNAs cannot be obtained from any organisms without genetic information and so cDNA libraries should be constructed after reverse transcription. However, one critical drawback of this method is the redundancy of unknown cDNAs. From studies such as the one shown above, it is clear that this is not a serious issue and that vast amount of genetic information can be obtained from randomly selecting clones. Consequently, the use of random selection is useful in studies employing non-model fish.

Williams *et al.*[21] also studied the toxic effects of control and test sites for the European flounder, another non-model organism fish. The cDNA microarray was fabricated after PCR amplification using degenerate primers[30] from a cDNA pool. After being sequenced, the PCR fragments were then compared and identified from BLASTN and BLASTX analysis. Real-time PCR analysis was used to confirm the gene expression levels for specific genes from among the up-regulated genes. This study showed that cDNA microarrays can be implemented with non-model fish directly. In addition, it indicates that the use of degenerate primers solves the issue related with redundancy of random cDNA microarrays. By adopting this approach with degenerate primers, functional cDNA microarrays containing only selected genes can be developed for non-model fish in which complete genetic information is not available. This should be a quite meaningful for specific researchers, considering the fact that the sequencing of many fish species genomes is still not a subject of interest to many.

Koskinen *et al.*[31] reported that 1380 non-redundant genes obtained from subtracted EST libraries and a single normalized cDNA library were used to fabricate cDNA microarrays for rainbow trout. Yolk-sac fry of the fish was used for analysis after exposure to 4 toxicants (beta-naphthoflavone, cadmium, carbon tetrachloride, pyrene) and hierarchical gene clustering analysis was conducted for each chemical dose (Fig. 7). This study is a typical example of the application of fish cDNA microarray technologies to environmental toxicology. The outcome of this study was the identification of a few genes as biomarker candidates, since they were highly up-regulated for each chemical. To further support their selection as biomarkers, the expression levels of each gene were determined using quantitative PCR (qPCR). Finally, they discussed the possible molecular toxic mech-

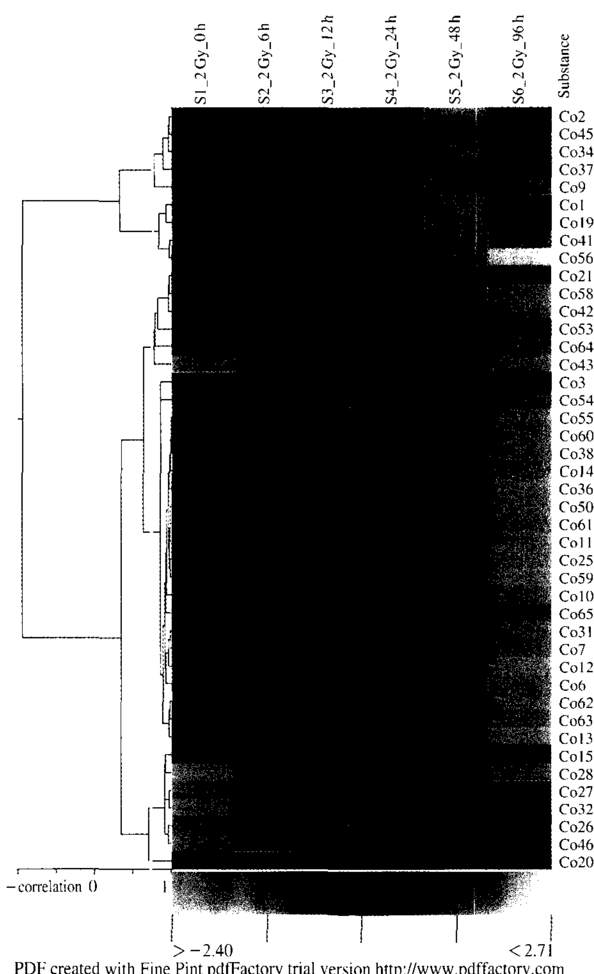

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Fig. 6. Hierarchical Clustering Image of Expression Profile

anisms in an effort to elucidate the mode of toxic action. Their results strongly demonstrate that this type of cDNA microarray analysis protocol can be used as a systematic evaluation tool to categorize and identify the mode of toxicity for a variety of different chemicals that are being released into environment everyday. However, to make such a system practical, a standardized gene expression profile DB is needed so researchers can compare their results with those of well known compounds and stimuli. Furthermore, hierarchical clustering analysis of the cDNA microarray results for an unknown toxic material could be performed and then compared with the results in the DB. Such a system would aid in the identification of toxic mechanisms of the unknown chemicals and even the categorization of the compounds according to their mode of toxicity.

Another study that used a DNA microarray to study an infection model based on host transcriptome

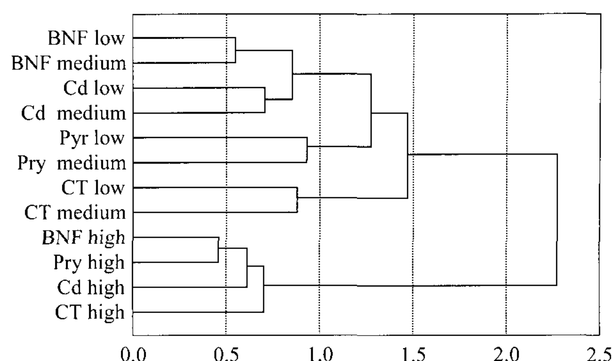

Fig. 7. Response of Rainbow Trout Fry to Betanaphthoflavone (BNF), Cadmium (Cd), Carbon Tetrachloride (CT), and Pyrene (Pyr) at Different Levels (Adpopted from ref. 31)

analysis looked at the progression of a *Mycobacterium marinum* infection on zebra fish[16]. In this study, two different oligonucleotide sets and commercialized Affymetrix GeneChips were compared to provide information about a cost effective selection of gene chips. In addition, lots of gene expression profiles from high-density DNA microarrays as well as the comparison studies for human tuberculosis were found to be possible from the analysis of tanscriptome profiling of this *M. marinum*-zebra fish infection model.

Concluding Remarks

Eco-toxicogenomic research, *i.e.*, toxicogenomic research done with fish or other organisms in their natural environment, is a promising field since this approach is capable of overcoming the limits that present technologies, including bioassay or biomarker-based approaches, have. DNA microarrays, a core technology for eco-toxicogenomic research, offers a broad glimpse of an organisms response to environmental stimuli, such as information about genes that are both up and down regulated, genes that may be later used as specific biomarker candidates. Furthermore, the gene expression profiling can be used for signature pattern analysis via a comparison with known toxic chemicals present within various DBs. This primary result in the least can lead to the development of a platform tool using multiple biomarkers and the identification of unknown toxicants from their expression signature analysis. With the introduction of DNA microarray bioinformatics and proteomics tools it is anticipated that eco-toxicogenomics research will provide answers for ecotoxicological issues and questions within a complete framework. In addition, this standard protocol in ecotoxicogenomics research would also be valid for non-

model organisms because, as discussed before, specific cDNA microarrays can be fabricated by using a random colony selection method or degenerate primers for the genomes of organisms that have not been fully sequenced or have incomplete genetic information. Therefore, eco-toxicogenomic research is beneficial to researchers studying various environmental sites with both model and non-model organisms.

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