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DNA Microarray Data Analysis and Mining: Affymetrix Software Package and In-House Complementary Packages

Lizhe Xu
University of New Orleans

DNA MICROARRAY DATA ANALYSIS AND MINING

AFFYMETRIX SOFTWARE PACKAGE AND IN-HOUSE COMPLEMENTARY PROGRAMS

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Lizhe Xu

B. S., Ocean University of China, 1986
M.S., Université d'Aix-Marseille II, Marseille, France, 1989
Ph. D., Université Pierre et Marie Curie, Paris, France, 1994

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Abstract

Data management and analysis represent a major challenge for microarray studies. In this study, Affymetrix software was used to analyze an HIV-infection data. The microarray analysis shows remarkably different results when using different parameters provided by the software. This highlights the fact that a standardized analysis tool, incorporating biological information about the genes is needed in order to better interpret the microarray study. To address the data management problem, in-house programs, including scripts and a database, were designed. The in-house programs were also used to overcome problems and inconveniences discovered during the data analysis, including management of the gene lists. The database provides rapid connection to many online public databases, as well as the integration of the original microarray data, relevant publications and other useful information. The in-house programs allow investigators to process and analyze the full Affymetrix microarray data in a speedy manner.

Introduction

Biological Background

The cell is the basic building block of a free-living organism. No matter how simple or how sophisticated the organism is, every cell contains the complete organism's hereditary information which is comprised of a set of genes. For a given species with only a few exceptions, each of its cells contains identical genes, and the whole set of genes forms the genome of that species. The number of genes varies from hundreds to tens of thousands depending on the organism. In the human genome, the latest estimates from gene-prediction programs suggest that there might be 24,500 or fewer protein-coding genes [2]. The mystery of life in a living cell resides in the function of its genes and their products. For a multiple-cell organism, cells have a variety of functions. The biological difference between the cells is achieved by an "on/off toggle" to control which genes are expressed in a cell and a "volume control" to manage the level of expression of particular genes as necessary [3]. The number and the level of these "turned-on" genes in a cell form the so-called gene expression profile, which determines the biological properties of that cell.

In humans, all the genetic information is contained within a set of deoxyribonucleic acid (DNA) molecules: the genome of human, which is arranged into 23 chromosome pairs. Gene

expression is usually composed of two separate steps: transcription and translation (see Fig. 1). The former denotes transcribing the genetic information contained in a gene into messenger RNA (mRNA, ribonucleic acid) molecules. The total information (qualitative and quantitative) of mRNA generated from a given genome is called the Transcriptome. Translation is

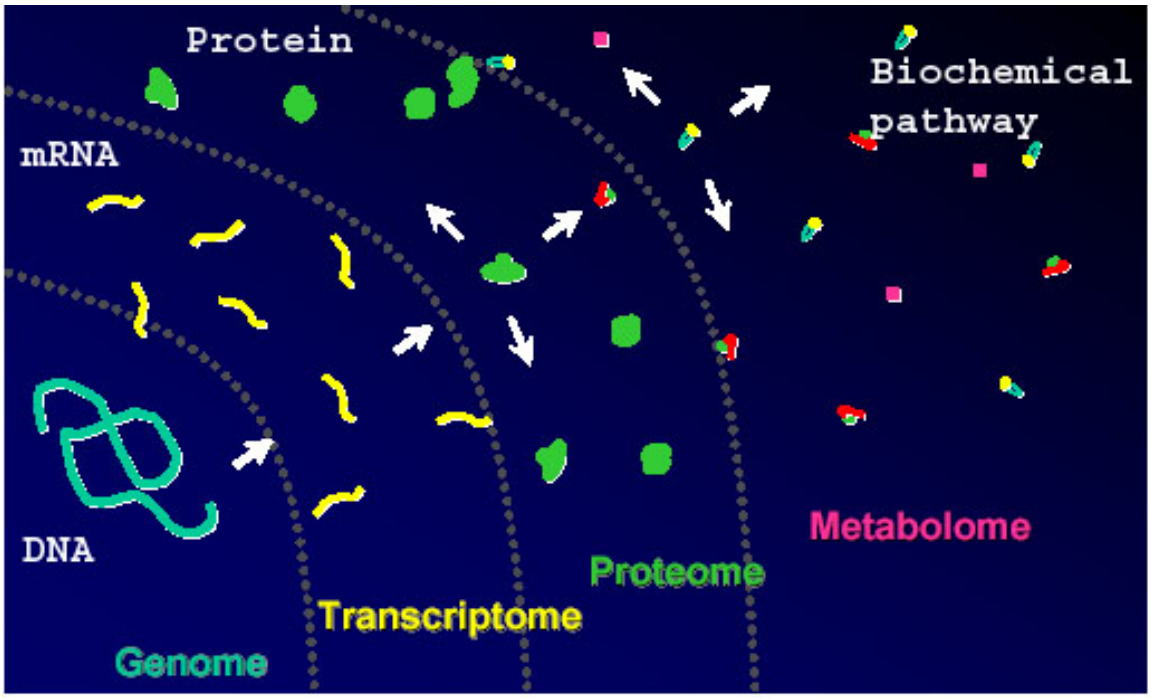


Figure 1. The four corner stones of System Biology, (System Biology is an emergent field that aims at system-level understanding of biological systems [1]. The picture is modified from the presentation slide of Affymetrix microarray data analysis training workshop, San Diego, CA 2003).

converting the coding information in the transcriptome into the corresponding proteins, which in turn perform most of the critical functions of cells. The total information about the proteins and their functions are called Proteome and Metabolome respectively (see Fig. 1). The integration of these four layers (genome, transcriptome, proteome and metablolome) of studies, termed 'Systems Biology,' can tackle the complexity of biological systems by gathering and incorporating all the available information into one comprehensive model [4]. As a result, the study of gene expression can be conducted at two different levels: mRNA and protein.

Traditional methods in molecular biology generally work on a "one gene or few genes in one experiment" basis. This means that we can only study a small part of the cellular functions at a time, and it is very difficult to obtain the whole gene expression profile of the cell. However, it is obvious that the entire gene expression information in a cell is needed to better understand its function, since thousands of genes (usually just a fraction of human genome) inside the cell are working in a complicated and orchestrated way to support the organism's biological function and to make the cell perform its normal role. In order to better understand the extreme complexity of living system, a more powerful research tool is required for cellular gene expression studies.

The DNA microarray, also known as genome chip, biochip, DNA chip, gene array and GeneChip® (a registered trademark owned by Affymetrix, Inc, CA) is an approach provided to accomplish this quest. DNA microarray is currently one of the fastest developing tools in the biological sciences. This technology promises to monitor a specimen's entire genome on a single chip in a single experiment. As a result, investigators can have a more precise and complete knowledge of the interactions among the thousands of genes expressed in a cell simultaneously.

DNA Microarrays

Each DNA molecule is made up of four different nucleotide bases, [adenine (A), thymine (T), guanine (G), and cytosine (C)], that are linked end to end. The order of the 4 bases (A, G, C, T) determines the contents of the genetic information of DNA, either directly (the sequences of the bases encodes the genes) or indirectly (the fragment of the DNA can play a regulatory role). In general, two DNA molecules can form a very stable structure through the complementarity of their bases, the famous "Double Helix". That is, adenine being the complement of and always pairing with thymine, and guanine being the complement of cytosine. This natural base-pairing creates and stabilizes the double helix DNA structure. When many single stranded DNA

sequences mix together and one finds its complement, such as, the sequence A-G-C-T-T-G-G and its complementary sequence T-C-G-A-A-C-C, the two sequences will lock together by base-pairing. In molecular biology, this is called hybridization. It is complementary base-pairing or hybridization that forms the foundation of the DNA microarray. RNA molecule follows the same basic rules of base-pairing as DNA, but with a substitution of uracil (U) for T. The base pair for RNA is A and U or G and C. One RNA molecule can also hybridize with a DNA molecule based on the base-pairing rule.

In The American Heritage Dictionary, "array" is defined as "to place in an orderly arrangement". DNA microarrays are small, solid supports onto which the sequences from selected thousands of different genes are attached at fixed locations [3]. The solid supports can be nylon membranes, glass microscope slides or silicon chips. Genes are printed (similar mechanics to an ink-jet printer), spotted by high-speed and precision robotics, or synthesized directly onto the support (see Fig. 2). These immobilized genes are used to capture the test DNA samples based on base-pairing rules. According to the nomenclature recommended by B. Phimister of Nature Genetics, these immobilized sequences are called "probes" and those sequences captured by probe are the "target" [5]. The probes in a microarray can be DNA, cDNA (DNA copied from RNA) or synthetic oligonucleotides. Usually, probes of DNA or cDNA can be 500 to 5000 bases long, whereas the size of an oligonucleotide microarray (Oligo-Array) probe is only 20 to 80 bases long. Due to the small size of an oligonucleotide probe, these arrays can hold more gene probes per unit space. As a result, the Oligo-Array is a high density microarray (up to tens of thousands of probes in one microarray) compared to DNA or cDNA arrays.

DNA microarray permits the study of cellular gene expression at the transcription level. In other words, the microarray can detect the existence and measure the quantity of cellular mRNA. To achieve the qualitative and quantitative analysis, the sample of cells must be pre-treated according to the following steps:

1) RNA extraction. The first step is to isolate RNA from cells by eliminating all other cellular components. Depending on the experimental design and protocol, the isolated RNA can be further purified to get the only mRNA prior to the second step.

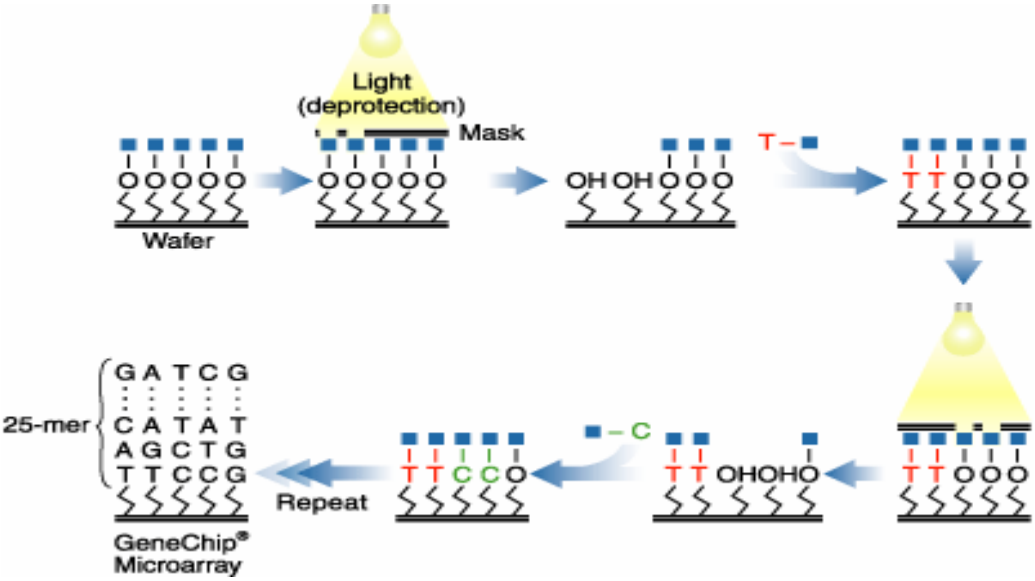


Figure 2. Affymetrix Manufacturing Technology, Affymetrix uses a unique combination of photolithography and combinatorial chemistry to manufacture GeneChip® Arrays (from Affymetrix.com)

2) Fluorescent labeling of the target RNA or mRNA. This allows detection and quantification of gene expressions by measuring the hybridization signals under fluorescent microscopy/scanner with laser excitation.

The labeling process can be simply one step of reverse transcription of RNA to cDNA, during which the cDNA are labeled, or it may be composed of multiple reactions. In the Affymetrix microarray for example, there are two sub-steps in this process: reverse transcription of RNA to cDNA without labeling, then followed by *in vitro* transcription of cDNA to cRNA labeled with biotin (Affymetrix manual, CA). It is not the intention of this thesis to cover the sample preparation of DNA microarray. However, based on how the samples are labeled, DNA microarray can be separated into two groups: single-color or two-color microarray. In the two-color system, two samples to be compared (for example, disease versus healthy, or non-treatment sample versus treatment sample, etc) are labeled individually with different fluorescent dyes, and then hybridized to one microarray. The same gene in the two samples will compete with each other to hybridize with the corresponding probe on the array. The relative expression levels of genes in one sample are determined by the signal densities captured in the corresponding fluorescent channel. The ratio of the signals in two channels of a given probe represents the difference of the corresponding gene in the two samples under study. In the one-color microarray, only one labeled sample can be used to hybridize with one microarray. After the image capture, the relative abundance of transcripts in the sample is obtained by image processing software which contains the algorithms for spot identification, local background determination and background-subtracted hybridization signal density calculation [6]. Comparisons between groups are then made on separate chips, thus requiring greater standardization and normalization. Oligo-Arrays usually use a single-color labeling system. Affymetrix only produces Oligo-Arrays and as a result, its sample preparation uses single color method. Affymetrix's software package is dedicated to data analysis of one-color microarrays.

As mentioned previously, the study of gene expression can be conducted at two different

levels: mRNAs and proteins. DNA microarray provides the research tool to scientists at the mRNA level. Another technology called the protein chip, is the approach to investigate at the protein level. The protein chip is another rapidly developing research technology. For example, a recently finished strategic report conducted by BioPerspectives (www.biotechinsights.com) estimates that the sale of protein chips will be increased from $76 million in 2001 to $700 million in 2006 [7]. The protein chip shares a similar technique as the DNA chip, but instead of DNA probes, it uses proteins or peptides immobilized on a surface to capture other proteins. Through the protein-protein or protein-ligand interaction, protein chips realize the analysis of thousands of proteins expressed in a cell in parallel. Although the topic of the protein array is out of the scope of this thesis, it is worth noting that the protein microarray faces the same challenges in its data analysis and data mining as the DNA microarray. In other words, a better data analysis and data mining strategy for DNA microarray will certainly benefit the protein microarray and vice versa.

Benefiting from the powerful microarray technology, scientists can now determine simultaneously the relative expression levels of all the genes represented in the array from only one experiment. Microarrays are currently available that claim to probe most, if not all, genes in the human genome. In addition to gene expression analysis, the microarray technology has been widely used in many other fields, such as, gene discovery, sequence identification, disease diagnosis, drug discovery, drug evaluation, and toxicological research.

Microarray Data Analysis and Mining

Challenge of Microarray Data Analysis

The ability of the microarray to address thousands of genes at a time is its strength, as well as its weakness: the data analysis and data mining are problematic. Since one microarray experiment can generate tens of thousands of data points representing the expression levels of the genes it probes, it is impossible to manipulate and analyze these data manually. Microarray data analysis requires

1) carefully designed computational tools to manage the data, including but not limited to data storage, gene annotations, probe and/or gene sequences, biochemical pathway and a variety of other biological knowledge about the genes immobilized on the array;

2) robust statistical and biological analysis methods (requiring computer support for semi- or fully automatic performance) to turn the numerical data of a gene in the array into a biologically meaningful interpretation.

From the initial cells to the final data, there are many intermediate preparation steps for a gene expression study, which can impart uncertainties, called technical variances, to the result. Because of technical variances, the results of microarray studies from the same cohort of cells can differ from one experiment to the next. As the averages of signal among replicates typically have less variability than their individual component does, replicates are required for any

microarray study. Moreover, replicate arrays allow the use of formal statistical methods for the downstream data analysis. So, there are generally at least two arrays (duplicate) for one experiment. Meanwhile, microarray studies are generally used to determine differential gene expression between identical cells subjected to different stimuli or between different cellular phenotypes or developmental stages. These kinds of differences generated from the initial samples are called biological variances. One set of data from a single array only gives gene expression information at a given moment. It is impossible to get a difference in mRNA expression levels from a single array. At least one other array from a different time point or representing a different treatment condition is required to evaluate the expression changes. For a simple microarray study, there are at least two experimental conditions with replicates for each condition (meaning at least 2 *x* 2 microarrays per study). Moreover, a living cell has the ability to rapidly respond to surrounding environmental or internal changes (for example different stage of cellular life or development cycle). The expression of cellular genes is changing dynamically and continuously over time. A simply array study with only two conditions cannot catch the changing details of gene expression, which can be important for a given study. A proper and careful experimental design can solve this problem by multiple sampling over time. Sampling over a relative long period of time is called "time course study", which is a common design used in microarray studies.

It is worth noting that there are two kinds of replicates in gene expression studies. The one starting from the initial point of the microarray experiment is called a biological repeat. If the same sample preparation is used to hybridize with different arrays, this kind of replicate is called a technical repeat. For example, the relationship of A - B and A – C is considered a biological repeat and that of B - C is a technical repeat (see Fig. 3). The technical repeat is only

relevant to display the technical variances of the steps it covers. Only the biological repeat can reveal both the biological variances and the technical variances of the whole experiment. The biological repeat also gives more analysis power to the downstream statistical tools. This is why biological repeats are preferred in microarray studies rather than technical repeats.

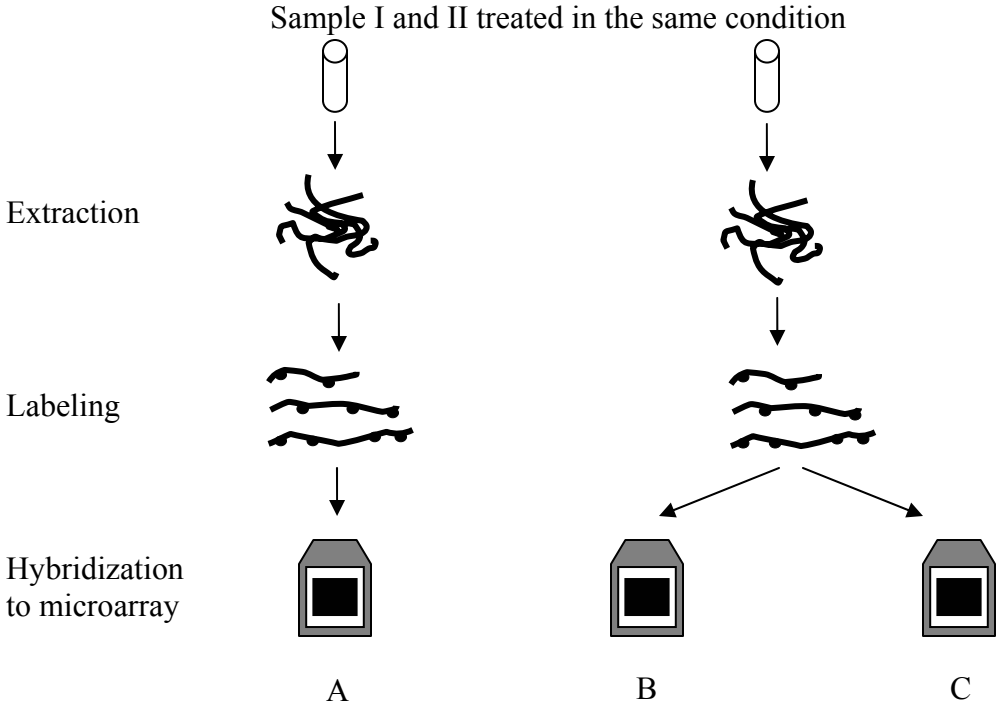


Figure 3. Two repeat schemas in microarray studies: Biological repeat (A and B, A and C) versus technical repeat (B and C).

With multiple microarrays in one study, we must compare one with another (between replicates and between conditions) to reveal gene expression changes. In order to compare data from two arrays, a mathematical technique called normalization must be applied to the data of each array. The aim of the normalization is to minimize discrepancies due to the technical variables including but not limited to sample preparation, RNA quantification, hybridization conditions, or image capture. For example, if the scanning time for two arrays is slightly different during the image capture step, the array scanned for a longer time will have overall

signal density higher than the array scanned for a shorter time. It is obvious that incorrect results will be obtained if the two are compared without normalization. Depending on the experimental design, there are several useful techniques for normalization. For a single color array, the most commonly used method is to divide the signal of each gene by the mean or median of total measured intensity in its array. The reason is that the mean or median of an array is an index of relative intensity (or baseline) of the given array. An alternative method is to use the mean or median of selected housekeeping (HK) genes in the array to serve as the index of relative intensity. The assumption underlying this method is that the housekeeping genes are a set of predefined genes required for fundamental cellular processes in a wide range of cell types and tissues and thus whose expression should be constant across the conditions of microarray studies [8]. Besides these two methods, there are several others such as using spiked positive controls for normalization baseline and intensity-dependent normalization[6, 8]. Normalization is the first step of microarray data analysis after image processing. This starting point fixes the tone of the following analysis and more or less determines the final output of the analysis.

Following normalization, data are analyzed to identify genes that are differentially expressed between the experimental conditions. Generally, the majority of genes presented on a microarray are invariant across the conditions under study, except in some customized arrays which contain only the genes of interest that are likely to vary. The first step of analysis is to filter out these invariants. Besides some user-defined filters, for example a requirement of minimum value on normalized signal, statistic tests are the most useful tools in this step. Depending upon the distribution of the data, users can apply either parametric or non-parametric statistical tests. With hundreds to thousands of genes in an array, the whole data set after log transformation can be generally treated as a normal distribution. As a result, a t-test can be used

for two-condition experiments and ANOVA (Analysis of Variance, 1-way or 2-way) for multiple-condition (time course) studies. During these statistic tests, user-defined cutoff values allow the scientists to tune the analysis stringency to achieve the desired balance of sensitivity and specificity. This fact results in a certain amount of flexibility (and arbitrariness) when interpreting the microarray data. The final list of "genes whose expression is altered" generated from a gene expression study may change as the analysis parameters or cutoff values are modified. Moreover, since the number of tests greatly exceeds the number of samples (tens of thousands of probes per sample for an array), microarray data analysis really pushes the standard statistical methods for multiple comparison to the limit of their utility [4]. As an unusual statistical case, the survival list passed through the traditional statistical tests will contain a considerable amount of false positive genes (type I error: invariant genes being selected by error). To mitigate the type I error in microarray analysis, several multiple test corrections (MTC) have been proposed, for example: Bonferroni correction; Bonferroni Step-down [9], Westfall-Young permutation [10] and Benjamini and Hochberg's False Discovery Rate (FDR) [11]. The order of these methods represents their stringency, with Bonferroni correction being the most conservative method. It gives the least false positive genes among the four methods but can filter out some true variant genes (type II error: variant genes being dropped by error). In contrast FDR generates the least type II error but has more type I error. The choice of MTC method is a study-specific decision in microarray data analysis. Therefore, genes with biologically relevant expression changes may not be effectively captured with statistical tests. This continues to be an active area of statistical research. This is why non-statistical approaches must be used in conjunction with statistical methods to interpret and validate the biological importance of the data.

The next step is to classify the genes that are statistically significant into different groups based on their expression patterns. This can be realized by several analysis and visualization tools, including, but not limited to, SOM (Self Organizing Map), hierarchic clustering (K-means, Gene tree, condition tree etc), and PCA (principal component analysis) [12-15].

The last step in the analysis of gene expression data is the biological interpretation of the results, where expression profiles contribute to the functional genomics characterization of the biological system under investigation [4]. Gene expression changes are controlled through highly complex, non-linear interactions between proteins, DNA, RNA, and a variety of metabolites. To find the functional relevance of expression data requires gathering and organizing a variety of additional bioinformatics associated with the sequences that show significant changes. It also involves correlating expression results with other types of data that can gathered as part of the experiment, such as, genomic, proteomic, or metabolomic data (see Fig. 1) [4]. The challenges of biological interpretation and the few tools available have made this step the bottleneck in microarray data analysis. One fundamental difficulty is the requirement for human review and understanding of complex types of data, scattered across a variety of sources, including online data bases and journal publications. While most investigators rely largely on 'manual' interpretation of results, through the review of functional annotations, pathway information, and associated literatures, there are efforts to develop tools that would truly automate some of the biological interpretation tasks, such as knowledge mining tools and gene network modeling and prediction [4]. In summary, the analysis steps mentioned above form the simplified pathway for the microarray data analysis from one gene expression study.

There are several software products available specifically designed for microarray analysis: from freely-downloadable to commercially licensed programs, such as TIGR (The

Institute for Genomic Research) MeV (multiExperiment Viewer v2.1 [16], free), D-Chip (free) [17, 18] and RMA (Robust Multi-array Analysis, free) [19, 20], Affymetrix's DMT (Data Mining Tool) and Silicon Genetics' GeneSpring [21] etc. Although the license of a commercial program can cost tens of thousands of dollars for a limited time of usage, the different software products and even the different settings within the same software product can greatly affect the analysis results and conclusion [15]. As a result, direct techniques of biological validation techniques, such as real time RT-PCR or analysis of specific proteins, are needed to confirm the final results by directly measuring the mRNA or protein quantities. But, these manual laboratory methods are time consuming and can only be applied to a small subset of the genes identified by microarray studies.

As cited above, biological context is needed during this process to help achieve the final results. However, since the high throughput DNA sequencing technology is advanced, vast amounts of sequence information have been generated from difference species. For example, the human genome project was originally planned to be a 15-year project, but completed 2 years ahead of its schedule. During the 13 research years, 3 billion nucleotide base pairs were sequenced, from which about 25,000 genes (this number is still changing depending on the new prediction tools) have been identified. Among these tens of thousands of genes, only a small number have more or less related genomic, proteomic, or metabolomic information and most of them just have a gene name assigned [22]. In addition, some microarrays use expressed sequence tags (EST: A short strand of DNA that is a part of a cDNA molecule and can act as an identifier for locating and mapping genes) as probes, in which case there may be no information other than sequence available. This means that a majority of genes in a microarray could have no biological information, other than sequence, to help the data analysis. This makes microarray

data analysis even more challenging. Indeed, the lack of information for genes creates another application field for microarray study besides gene expression analysis. That is to determine the function of genes, and even to identify new genes by comparing their expression profiles with well-characterized genes (that is why the EST is used as probe in microarrays).

With all the problems accumulated from the various steps presented above, the most challenging part of microarray data analysis is to compare two or more similar studies, especially ones coming from different laboratories. Since the microarray technology was developed independently from multiple sources, different microarray techniques are available in the market vis-à-vis the number, type, sequence of probes, solid supports, sample preparations, and labeling systems. Similar studies performed in different laboratories can use different microarray techniques, different programs or different settings of the same program for data analysis. The results can be widely variable, so in most cases it is problematic, if not impossible to compare the final results between two studies. To facilitate the comparison and sharing of microarray data, the international Microarray Gene Expression Data (MGED) Society drafted the requirement of MIAME (minimal information about a microarray experiment) [23]. MIAME is not a strict rulebook for microarray experiments, but provides a set of guidelines. It aims to unambiguously interpret microarray data and to allow sharing and interpretation of raw data between different studies [23]. The data sharing allows other investigators to assess and validate the quality of data, further analyze, and mine the data beyond that which might be presented in an original study [15]. Additionally, it facilitates the development of more powerful and comprehensive software for analysis by providing real data for testing. Moreover, as there are many variations involved in microarray studies, accumulation and sharing of data from many studies makes it possible to reduce non-biological variation and to reveal the true biological gene expression profile. Finally,

thousands of microarray studies from different types of cells and tissues, at different stages of the life cycle or in different conditions, will construct organism-level gene expression profiles which show the dynamical changes cross time and conditions. With these precious gene expression pictures in hand, scientists will be able to finally discover the mechanisms of life.

Affymetrix Microarray System

Affymetrix Inc (CA) is one of the earliest, most successful companies to develop microarray technologies. To date, there are about 1400 scientific publications related to Affymetrix's GeneChip® [24]. The Affymetrix GeneChip®s are high density Oligo-arrays (with length around 25), in which the probes are synthesized directly onto the supports (see picture 3). In order to increase the sensitivity and specificity of the test, there are a set of 11-16 different probe pairs for each gene on the chip, which cover different areas of the given gene. A probe pair is composed of one perfect match (PM) probe and one mismatch probe (MM). The PM probe has the complimentary sequence to the gene of interest and the signal of the PM represents the specific hybridization. The MM probe has the same sequence as the PM, except for a homomeric base change (A - T, or G – C) at the middle of the sequence (at the 13^{th} position). The signal of the MM represents non-specific hybridization. The detail of the usage of the PM and MM can be found in the following references [4, 14, 25-27]. Affymetrix's gene expression analysis arrays cover the genomes of *Arabidopsis*; *P. aeruginosa*; *E. Coli*; yeast; *C. elegans*; rat; mouse and human. Among several different chip sets of human genome, the U133 AB chip set is the newest chip and at the time of this study, contained the highest number of genes available. The U133 AB chip set is composed of 2 chips, named U133A and U133B. The U133A chip contains 22283 known human genes (many of them having unknown biological functions). The U133B chip has 22645 human transcripts, the vast majority representing EST and the remainder being control

genes. The data used in this thesis was generated from this chip set in an HIV (human immunodeficiency virus) infection study (funded by LA Board of Regents grant LEQSF (2002-05)-DR-B-06, led by Dr Seth Pincus, Director of the Research Institute for Children). The details of the HIV infection microarray experiments will be presented in the following section.

In addition to GeneChip®, Affymetrix also provides a software package, including Microarray Suite (MAS) 5.0 [26], MicroDB [27], Data Mining Tool (DMT) 3.0 [14] and even an online analysis tool called Affymetrix® NetAffx™ Analysis Center. These programs and tools cover the entire gene expression experiment, from the initial chip hybridization instrument control to final biological interpretation (see Fig. 4). During the analysis of HIV infection study, I used all Affymetrix's analysis tools mentioned above as well as several other programs such as Silicon Genetics: Gene Spring [21] and Tigrs MeV [16]. The different parametric settings (including normalization methods) of these software tools have been tested and significant differences were obtained from different programs and different settings of the same program [28]. The results, details of the analysis process, and the different settings of parameters will be published elsewhere and not covered here [28]. In this thesis, I will only present a small part of the data analysis study, which starts from the MAS and DMT analysis and ends with use of several in-house programs, including scripts and a customized database. Both the scripts and database have been created to overcome weakness of the Affymetrix program and to help the investigators conduct the analysis in a fast and easy manner, as will be shown in later sections.

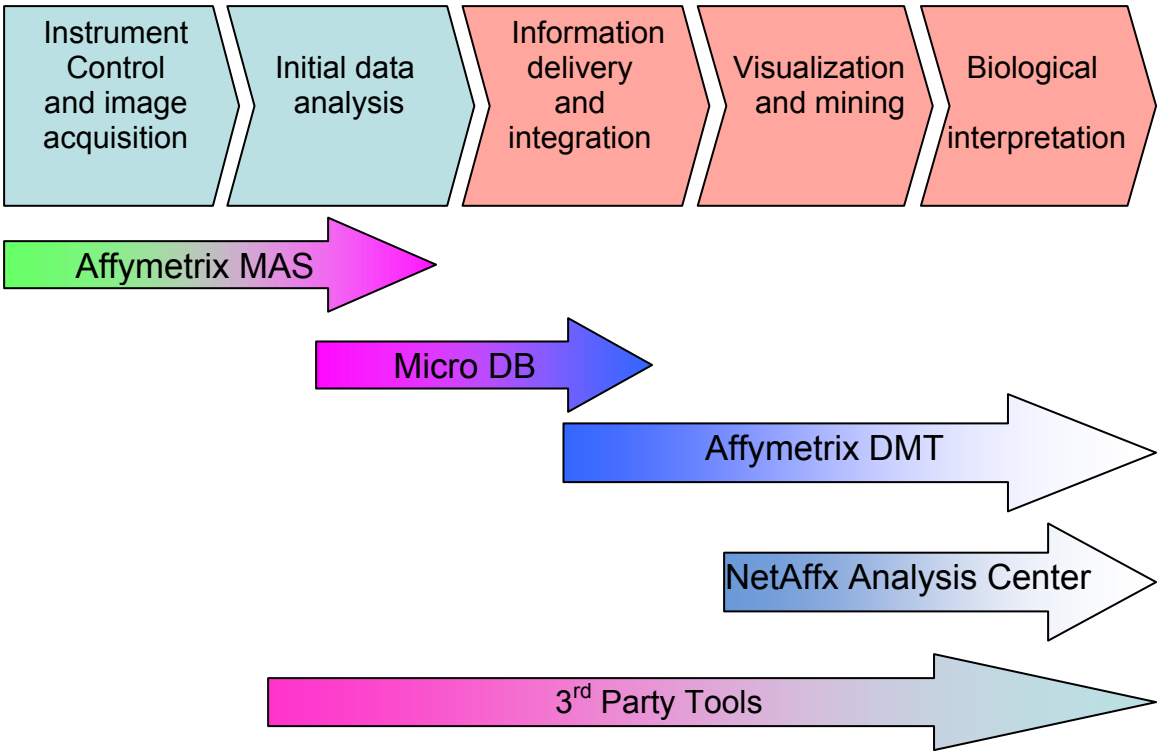


Figure 4. Affymetrix gene expression analysis software package

HIV Infection Study

The HIV infection experiment was performed at the Research Institute for Children (RIC) and was composed of two parts: chronic infection study and acute infection study. The first was a simple study of gene expression, with only two conditions: persistently infected cells versus non-infected cells. The uninfected parental cell line, designated H9, is a clonal derivative of the Hut 78 cell line, isolated from human cutaneous T CD4+ lymphocyte. It was selected for permissiveness for HIV-1 replication [29]. H9 cells were infected with the molecularly cloned HIV NL4-3 [30]to obtain the persistently infected cell line H9/NL4-3 [31]. The chronic study was performed by directly comparing the microarray data between H9/NL4-3 cells and H9 cells. The acute infection study, although using the same two cell lines, is more complicated than the

previous one. It was designed as a time course study by using H9/NL4-3 cells to infect H9 cells and evaluating the gene expression changes in H9 cells over time. H9 cells at their mid-log phase of growth were mixed with H9/NL4-3 cells at ratio of 50 to 1 (H9 : H9/NL4-3) and one tenth of the mixed volume (equals to one tenth of the mixed cells) was removed immediately after the initial mixing and at various time afterwards. Cells were washed X 2i in phosphate buffered saline, resuspended in RNALater, and stored at -20^0 until ready for use. RNA was extracted using TRIzol®,a reagent which can disrupt cells and dissolve cellular components, while maintaining the integrity of the RNA [32]. in addition to the initial sample, other samples were taken at 3 H, 12 H, 24 H, 3 days (D), 5 D and 8 D after the time zero (see Fig. 5).

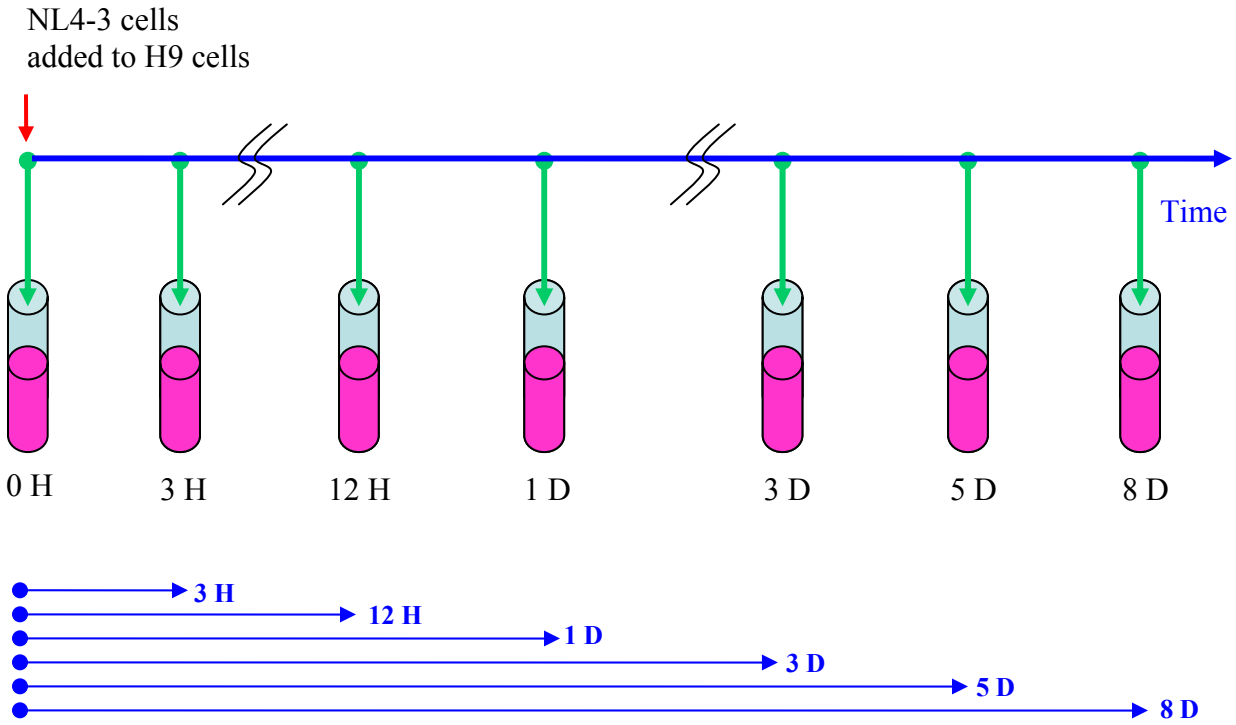


Figure 5, Sampling schema of the time course study. H: hour, D: day

The experiments from RNA extraction to hybridization followed exactly the Affymetrix protocol [25] and the whole process is illustrated in 6. Thanks to the investigators (see my

Acknowledgement on page 2), I started my analysis without worrying about the preparation steps and took charge of the study just after the step of "Scan" (see Fig. 6).

For the reason I cited previously, both chronic and acute infection studies were repeated three times independently from cell culture and cell infection to final hybridization (biological triplicates). As a result, I obtained a total of 54 image files obtained by scanning the hybridized chips. Each file had a size of 43 MB. They are from the U133A and U133B chip set, with each set having two samples of chronic infection and seven samples of time course study for triplicates per sample [2x (2+7) *x* 3 = 54].

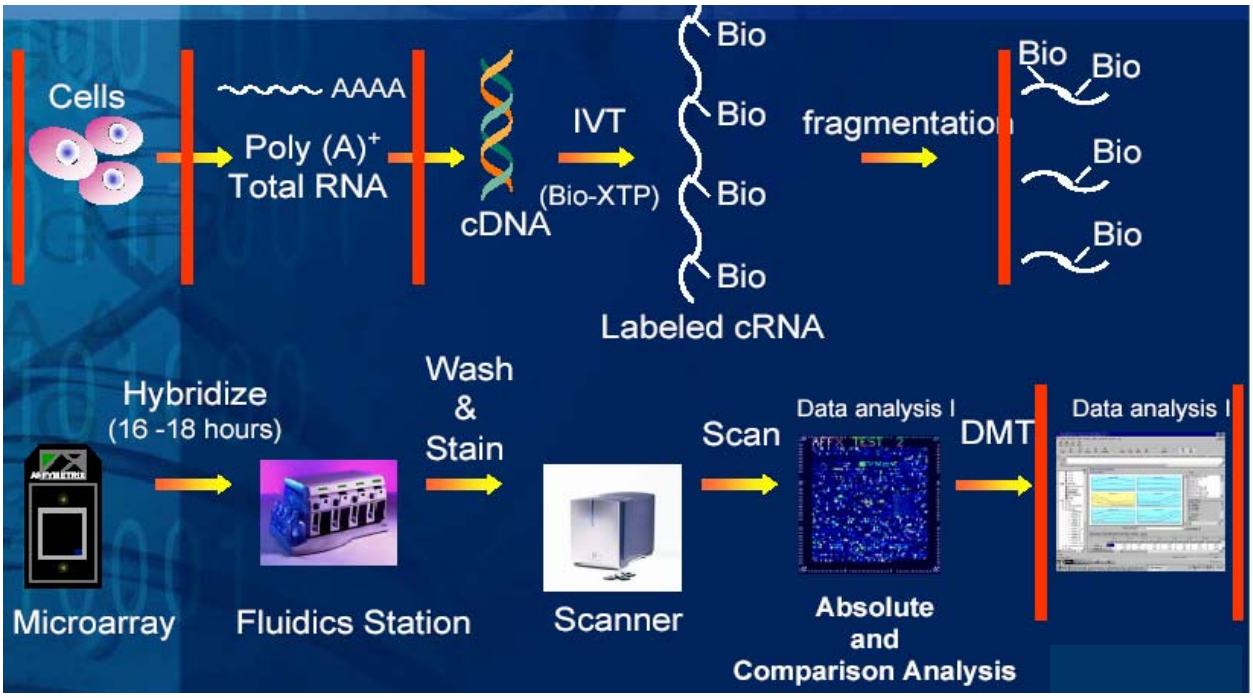


Figure 6, Affymetric microarray study process (coming from [4]).

Data Analysis Schema with Affymetrix's Programs

1) MAS (Microarray Suite 5.0) . The first step of the data analysis was to use the image processing algorithm in MAS to analyze the received image files. MAS evaluated the whole set of pixels of a probe and calculated a single density value assigned to that probe. At the end, a

".cel" file was generated from each image file. The next step is called absolute data analysis or single array data analysis (see Fig. 7). The resulted ".chp" files contains both quantitative and qualitative measurements for each probe set by incorporating the density value of its 11-16 primer pairs from the ".cel" file. The quantitative measurement shows the absolute signal value of each probe set (obtained by evaluating all its probe pairs). The qualitative measurement is given as a form of detection call with an associated p-value for confidence: *P* (Present, gene expression above the detection threshold), *M* (Marginal, at the limit of detection threshold) and *A* (Absent, below the detection threshold). The two measurements were calculated using different algorithms of detection and they were independent from each other (for details please refer to Appendix C of AMS manual [26]). The following step is the comparative analysis to compare two conditions: experiment versus control (or baseline) (see Fig. 7). Just like in the single array analysis, MAS uses different algorithms to generate both quantitative and qualitative measurements. The quantitative measurement gave the signal log ratio (SLR, log base of 2) of the two experimental conditions (experiment divided by control). For example, if SLR for one gene is 1, it means that the expression of this gene is two fold higher in the experiment than that in the control (up-regulated gene) since $\log_2 2 = 1$. If SLR is -1, it means that the expression of this gene in the experiment is half of that in the baseline ($\log_2 0.5 = -1$, down-regulated gene). The qualitative measurement gave the following calls with an associated p-value: *I* (Increase, the gene expression is increased in the experiment compared to control); *MI* (marginal increase); *NC* (no change); *MD* (marginal decrease) and *D* (decrease) (see Fig. 7). Both measurements are included in the comparison analysis ".chp" file, the output of the analysis. If we start with 6 image files of microarrays (two conditions and triplicates for each condition), we will get a total of 21 new files at the end of MAS analysis, including 6 ".cel" files, and 6 single array analysis

".chp" files and 9 comparison analysis ".chp" files.

2) MicroDB. This software is used to "publish" (term used by the MicroDB) [27] the ".chp" files to a database in order to allow the DMT program to access them and perform statistical and clustering analysis. Affymetrix's MicroDB can only open one data base at a time, and each data base can hold maximum 128 ".chp" files [27].

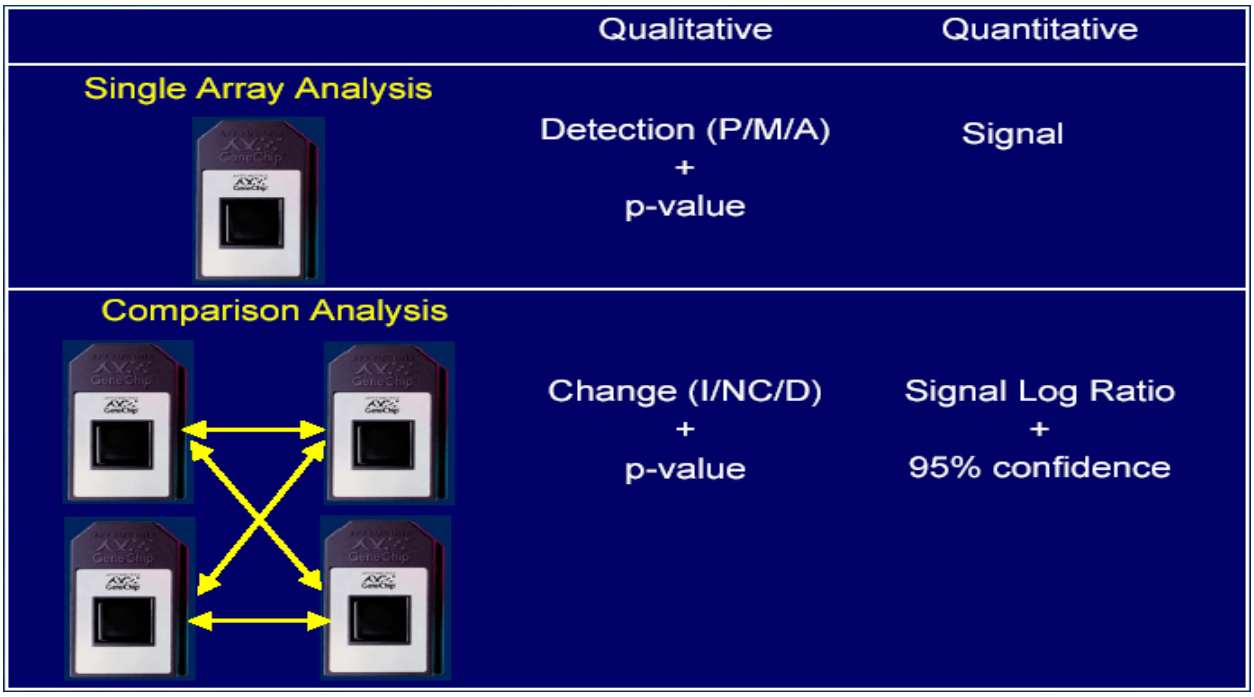


Figure 7, MAS data analysis output formats. For a comparison analysis of duplicate per condition study, there are total 4 files as an analysis result. P: present; M: marginal; A: absent; I: increase; NC: no change; D: decrease [4].

3) DMT (Data Mining Tool). In the MAS step, the data of two conditions were compared and the detection call of *I* or *NC* or *D* was generated for each gene, however, no genes were filtered out at that step. The analysis with DMT will reduce the complexity of data by filtering out the majority of genes, keeping only the ones with statistically significant expression changes. The DMT, just like the MAS, does not have the ANOVA (Analysis of variance) model implemented and has to do pair-wise analysis for the data in a time course study (see Fig. 8).

Once a given database, created by MicroDB, is selected in DMT parameter setting window, DMT will have accessibility only to the ".chp" files in that data base. In other words, DMT can access only one data base at a time. The genes with statistically significant expression changes can be separated into two groups: up-regulated genes (expression increased in experiment or decreased in baseline) and down-regulated genes (expression decreased in experiment or increased in baseline). The analysis with DMT for data of two conditions has to be performed twice in order to discover both up-regulated and down-regulated genes (simplified as up genes and down genes afterwards). Both searches were realized with four tests based on the qualitative and quantitative measurements of the absolute and comparison analysis.

The first test (T1) was done by using the detection call of a single array analysis to avoid genes with *A-A* detection calls (absent in both experimental and –baseline conditions). When looking for up-regulated genes, it is obvious that the genes must be present (*P)* in the experimental condition, no matter what calls they have in the baseline (up-regulated gene could be *P-X* but not *M(marginal)-X*, or *A-X*, where *X* is *P* or *M* or *A* in control condition, in other words, a gene with *M* or *A* call in the experiment can not be an up-regulated gene). Using similar reasoning, only genes having *P* call in the baseline can be the candidates for down-regulated genes with a pattern of *X-P*, where *X* is *P* or *M* or *A* in the experimental condition.

The second test (T2) was a statistical test based on the density signals of the single array analysis files. DMT provides two algorithms: a parametric method, the t-test and a nonparametric method, the Mann-Whitney test, to compare two experimental conditions. Both tests give each gene a p-value and change direction call (see Fig. 9).

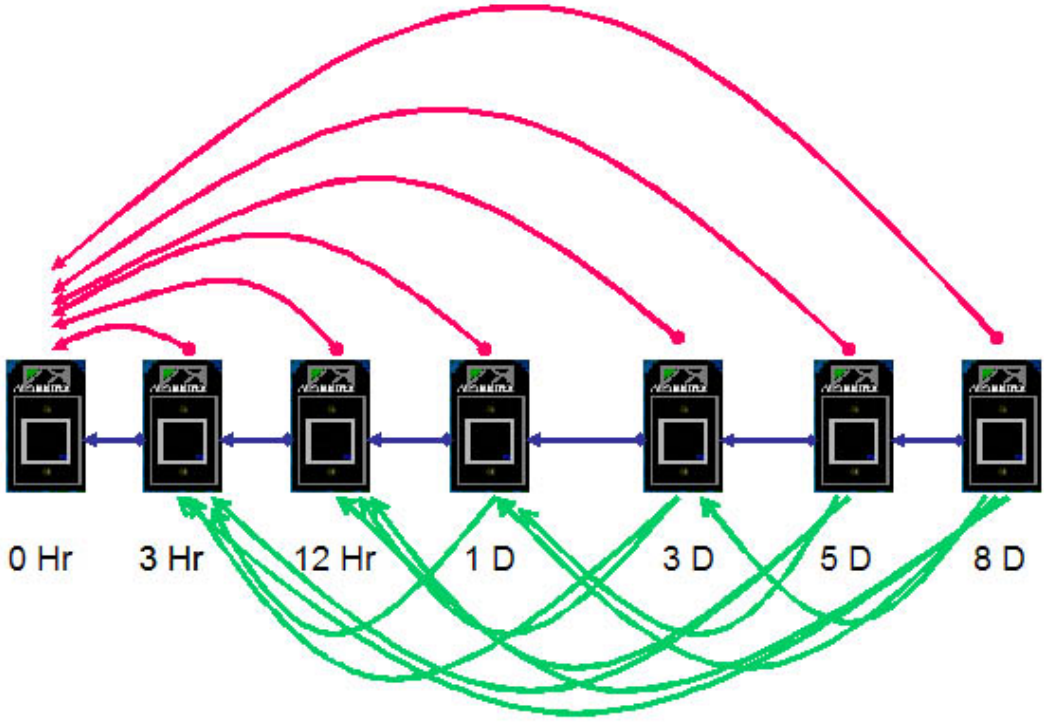


Figure 8, Time course study comparison analysis schema. The red lines represent the comparison versus time 0, the blue lines represent that between adjacent time points and the green ones represent other pair-wise comparisons. Totally, there are 20 comparison analyses for the time course study. For each line, the start point indicates the experimental chip and the arrow points to the control (or baseline) array.

The third test (T3) was performed based on the detection call in the comparison analysis files. The general rule is choosing genes with *I* and/or *MI* detection calls when looking for up-regulated genes and genes with *D* and/or *MD* calls for down-regulated genes.

The fourth test (T4) was a fold-change-selection filter. Based upon the minimum fold changes selected by the investigators, a different cutoff value will be determined for SLR. For example, if at least two fold change genes are the targets, the genes passed through this filter will have SLRs not smaller than 1 (up genes) or not greater than -1 (down genes).

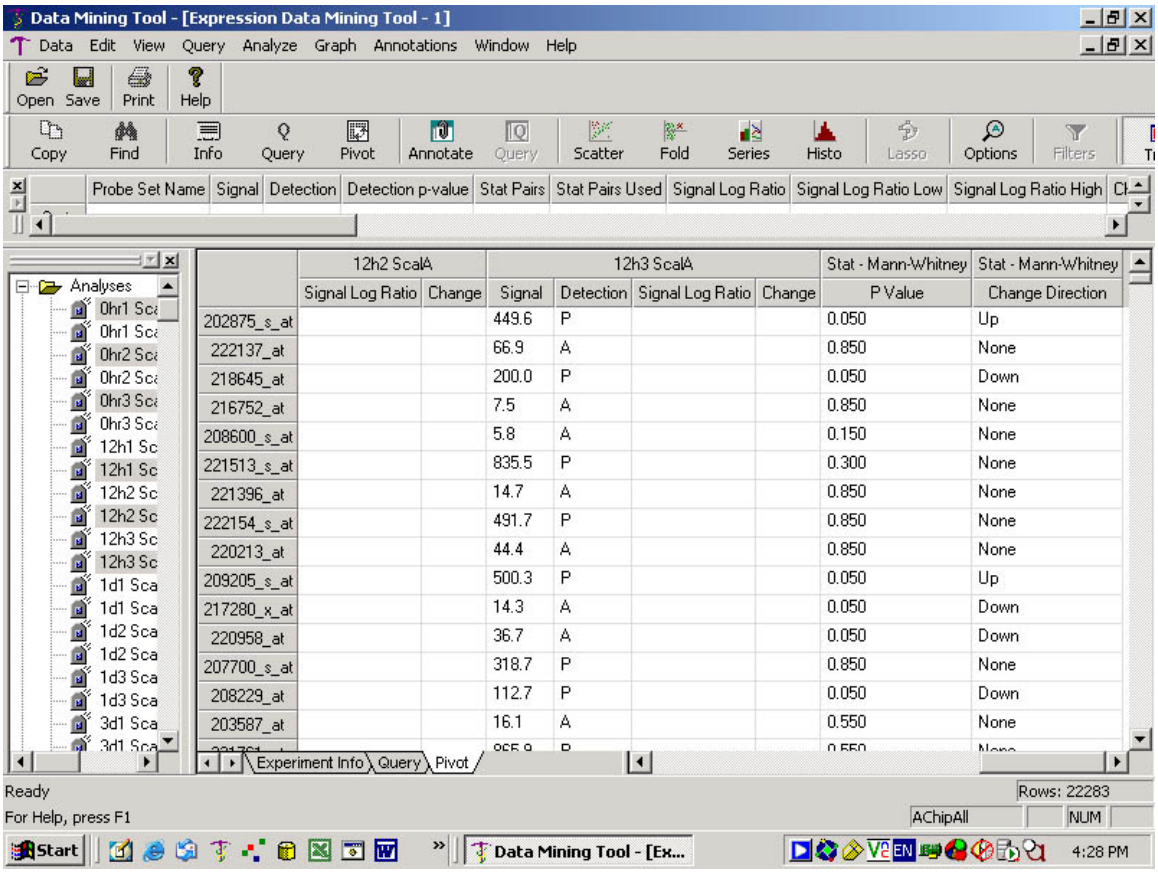


Figure 9, Output of the Mann-Whitney test in DMT. The test gives both the p-value and the change direction call (up, none and down).

In a summary, T1 is based on detection call of individual array (*P-M-A*). T2 is a statistic test base comparing signal densities and variation of individual genes. T3 is based on detection call of comparison analysis (*I-NC-D*). T4 is a filter on fold change by using the SLR in comparison files. The combination of these four tests will give the genes with statistically significant changes from DMT. As we have seen, to compare two conditions, all the corresponding single array analysis and comparison analysis ".chp" files are needed (in the HIV study, 6 single array analysis files and 9 comparison analysis files for any two given conditions), so all the files have to be added into the same database to allow DMT access to the data at the same time.

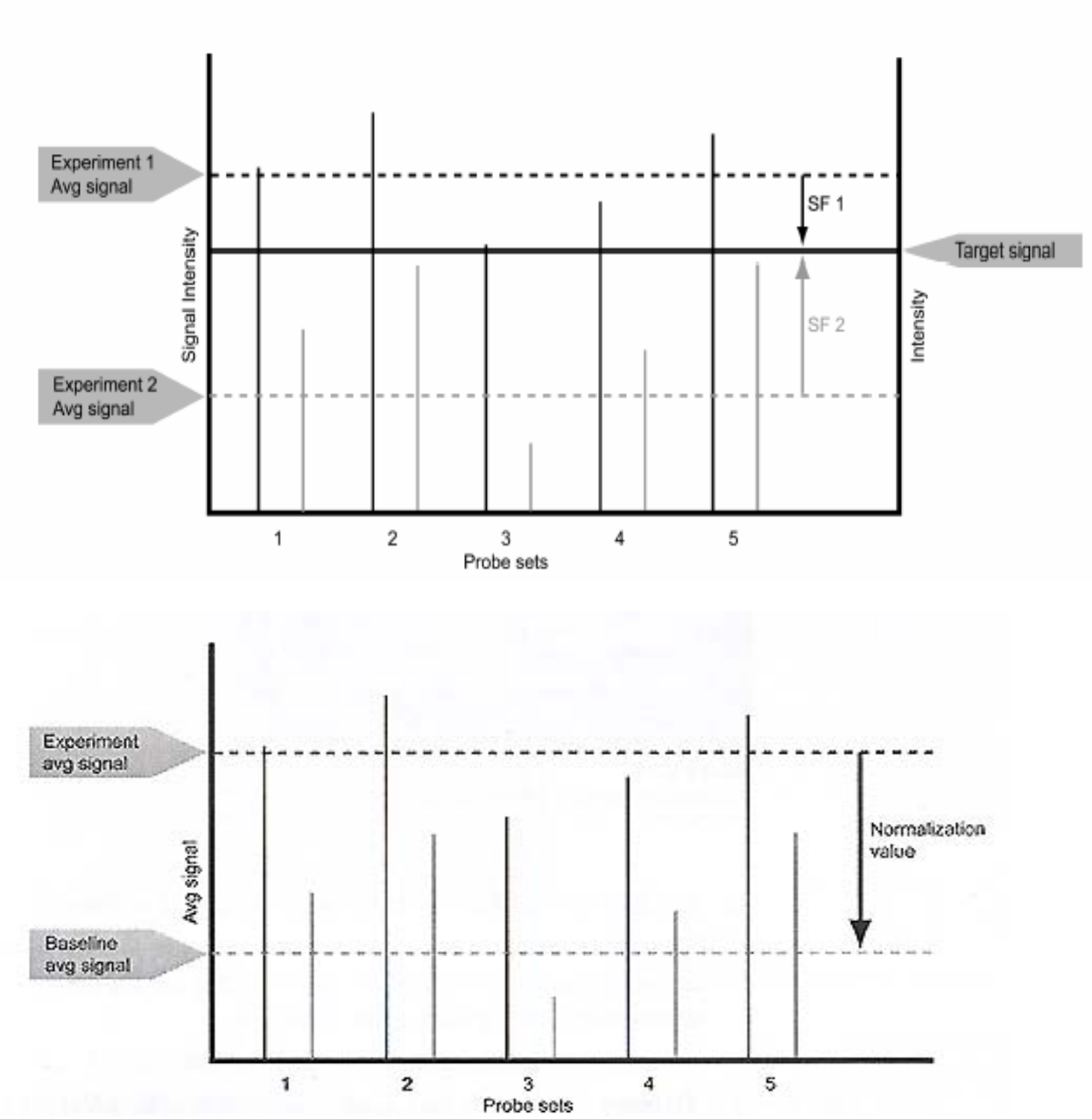


Figure 10. The difference between MAS Scaling and Normalization.

Parameter Settings of Affymetrix's Programs

Prior to single array or comparison analysis in MAS, there is an important parameter to set: normalization, which is essential for comparing different array signals. MAS provides two forms of normalization: Scaling and Normalization. Scaling and Normalization are mathematical techniques applied to the data from several different arrays of the same type to minimize

discrepancies due to technical variables [26].They will bring the average signal of the genes in two arrays to the same level (target signal) before the comparison can be performed. The target signal can be determined in two ways, either by users' selection or using the average signal of one of the two arrays that are being compared (usually using the baseline array). If the target signal is selected by users, the signal of each gene in both arrays will be normalized in a way that the final average signal of modified data will equal to selected value. This is the Scaling method. If the average signal of the baseline is selected as the target value, only the data in the experimental array will be normalized. As a result, its new average signal will be equal to that of the baseline. This is the Normalization method (see Fig. 10). Both methods will use the following formula to normalize the raw data.

normalized data of a given gene = its raw data *x* Scale Factor (SF)

where SF = Target signal / average signal of the array

Since the baseline of our pair-wise comparison was changed during the time course study, the Normalization will cause problems for downstream analysis between these pair-wise comparisons. So the Scaling method was chosen to normalize data in the HIV study and the default value of MAS: 500 was used as target signal. As previously mentioned, there are two methods to calculate the average signal of the array one based on all genes in the chip and the other based only on some pre-selected HK genes. MAS supports both methods and Affymetrix provides two groups of 100 HK genes for U133A and U133B separately. According to Affymetrix's experiments, the expression levels of these HK genes are generally constant across different cell types and tissues. For the HIV study, both methods (named S-All and S-HK) for

Scaling were used, because (1) there is a debate among the microarray users about which method is better; (2) direct visualization of our data (plot of the raw data of the 100 HK genes or all genes) does not favorite either one; and (3) we want to compare the results of these two settings.

In DMT, the parameter setting for the four tests are as follows: (1) T1. Since there are 3 detection calls for each gene in every condition (triplicate), genes having at least two *P* calls were selected in the experimental condition for up genes and at least two *P* calls in the baseline for down genes. (2) T2. Since the data do not have symmetric distributions (mean and median is quite different) and there is no log transformation available in DMT, Mann-Whitney test was chosen with a cut-off p-value of 0.05 (triplicates of experiment versus triplicates of baseline). Then genes with "Up" or "down" direction call were selected for up or down genes respectively. (3) T3. There were nine change direction calls for each gene between two conditions. Genes with at least five *I* or *MI* detection calls were picked up for up genes and at least five *D* or *MD* calls for down genes. (4) T4. There were nine SLRs between the two conditions for each gene. Both the median and the mean can be calculated and the median was used for the analysis to minimize the effect of outliers. The cutoff value for SLR is another hot topic debated among the microarray users. In the early microarray studies, an arbitrary number of 1 (equals 2 fold change) was selected by Affymetrix scientists, and this cutoff became the conventional one. However, more and more scientists are asking if the requirement of 2 fold changes is too harsh and may cause some important genes to be missed. Especially for a time course study, the samples in early stages such as 3 hours or 12 hours could have few 2 fold changes genes since the time frame is too short for the variant genes to build up to the two-fold change level. Based on these arguments, many scientists started to choose different value for SLR cutoff depending on their experimental conditions. Since in the HIV studies both chronic and acute infection samples were

available, and we would like to see how different the results can be when selecting different SLR,two cutoff values and a combination of the two values were selected for time course study. These values are 0.5 and 1 for SLR, equals 1.4 and 2 fold changes respectively, as well as the combination of 0.5 for samples not longer than 1 day (including 3h, 12h and 24 h in our case) and 1 for samples old than 1 day (including 72 h, 120 h and 192 h in our case). Meanwhile three SLR cutoff values: 0.5, 1 and 2 (4 fold) were used for the chronic study.

Analysis Results

The data analysis was performed on a Compaq Evo D510 CMT computer powered with Pentium 4 processor at 2.53 GHz and 512 MB memory. All three of Affymetrix's programs were installed on this computer under the window 2000 operating system. The comparison of the two normalization methods, three SLR cutoff values, total six analysis settings (one analysis setting is one SLR cutoff value combined with one normalization method) were completed only with data of U133A chip. Based on discussions among the investigators, one analysis condition was chosen and the analysis of U133B was completed under that condition [28]).

In MAS, from the initial 54 image files (43 MB for each), a total of 54 ".cel" files were generated (12 MB for each). Since analysis was performed with S-All, S-HK, and an extra one without normalization (some third part programs require the import of Affymetrix's non-normalized data, see Fig. 4), three single array ".chp" files were generated per each ".cel" file, and a total of 162 single array ".chp" files (3 normalizations *x* 54) were generated, 14 MB each.

The comparison analysis was simple for the chronic study, where the arrays generated with H9/NL4-3 were directly compared to those of H9. With triplicates for each condition, 9 comparison ".chp" files per normalization method and per chip set were obtained for the chronic study (3 *x* 3 see Fig. 7),15 MB each file. As a result, there were 36 comparison ".chp" files for the chronic study (9 files *x* 2 normalizations *x* 2 chip sets).

Table 1. Affymetrix software analysis results for chronic study (U133A only).

(a) The number of the common genes (in bold) between two compared analysis settings.

			S-All**		
			SLR=0.5	1	2
		Num of Gene	1377	628	178
S-HK*	SLR=0.5	1396	**1196**	**606**	**178**
	1	670	**644**	**555**	**178**
	2	179	**179**	**179**	**168**

(b) The percentage difference between two compared analysis settings.

			S-All**		
			SLR=0.5	1	2
		Num of Gene	1377	628	178
S-HK*	SLR=0.5	1396	**24%**	57%	87%
	1	670	54%	**25%**	73%
	2	179	87%	71%	**11%**

* S-HK: Scaling on 100 house keeping genes on Chip U133A provided by Affymetrix
** S-All: Scaling on all genes of the U133A chip.
The percentage difference is defined as the number of differences between the union and intersection of two compared lists, divided by the number of the union list. For example, there are α genes in list *X*, β genes in list *Y* and δgene in common when comparing *X* and *Y*, the difference between *X* and Y is $(\alpha+\beta-2\delta) \div (\alpha+\beta-\delta)$.

For the time course study, the comparison schema was very complicated. In order to catch all genes with expression changes reaching the cutoff value between any two time points, 20 pair-wise comparisons were performed individually (see Fig. 8, including 6 versus time zero, 6 between adjacent time points and 10 for other conditions). So 720 comparison ".chp" files (2 normalizations *x* 20 pair-wise comparisons *x* 9 comparison analysis files *x* 2 chips sets) were produced by MAS for the time course study.

Table 2. Affymetrix software analysis results for time course study (U133A only). The results are the combination of all time points pair-wise comparison.

(a) The number of the common genes (in bold) between two compared analysis settings.

			S-All		
			SLR=1	0.5 &1	0.5
		Num of Gene	948	1756	4177
S-HG	SLR=1	1259	**748**	**872**	**1213**
	0.5 & 1	1959	**790**	**1441**	**1843**
	0.5	4155	**902**	**1616**	**3437**

(b) The percentage difference between two compared analysis settings.

			S-All		
			SLR=1	0.5 &1	0.5
		Num of Gene	948	1756	4177
S-HG	SLR=1	1259	**49%**	*59%*	71%
	0.5 & 1	1959	63%	**37%**	57%
	0.5	4155	79%	62%	**30%**

S-HK: Scaling on 100 house keeping genes on Chip U133A provided by Affymetrix
S-All: Scaling on all genes of the U133A chip.
The percentage difference is defined as the number of differences between the union and intersection of two compared lists, divided by the number of the union list. For example, there are α genes in list *X*, β genes in list *Y* and δgene in common when comparing *X* and *Y*, the difference between *X* and Y is $(\alpha+\beta-2\delta) \div (\alpha+\beta-\delta)$.

There were a total of 918 ".chp" files (162 +36 +720) generated for the HIV infection study at the end of MAS. Without counting the space occupied by the 54 ".cel" files and the 54 non-normalized ".chp" files (generated specifically for 3rd part software), the storage of the files was increased by 13 GB (15 MB *x* 864 files), while initially it was only 2.3 G (43 MB *x* 54 image files). In addition, the comparison analysis for time course data was the most time-consuming and error-prone step in the study presented in this thesis, since manual selection of

these 756 pair of files (total 1512 files) and assignments of a unique name for each of 756 output files had to be conducted one by one before the DMT could start the analysis.

In MicroDB, because of the limited number of the files (128) which can be held in a single database, several databases had to be created for each normalization method and each chip set, to cover its 216 ".chp" files (864 divided by 4). Three databases were created for chipA set and there were more than 300 ".chp" files total. This is because single array analysis ".chp" files had to be duplicated and put into different databases. So even more disk space was required to store the data.

In DMT, there were two approaches to do the entire analysis with four tests. The first approach is applying the following test only on the result of the previous tests (in a pipeline manner). In other words, the input gene of the following test is the output of the previous one. However, this approach is very slow on our computer compared to the following method (the possible reason for this slowness will be given in the following sections). The second approach is to apply each test independently on all of the genes in the chip under study (in a parallel manner). After saving them, the four resulting gene lists were exported from DMT and the intersection of the four lists was generated with a program written by me (Perl Script I, see page 59). Both approaches were performed for U133A chip to double verify the final gene lists (up and down genes) for each pair-wise comparison.

After analysis with DMT, there were two gene lists (up and down genes) for chronic study and forty gene lists for time course study (20 pair-wise comparisons) for each analysis setting. There were a total of 252 gene lists for U133A chip only (42 *x* 3 cutoff values *x* 2 normalization methods) with DMT analysis (U133B had less, since it had been completely analyzed only in one analysis setting). For a given analysis setting, the union of the two gene

lists from chronic study and the union of the forty gene lists from time course study were the final results for the two studies respectively.

Table 3. Affymetrix software analysis results for up and down genes in time course study (U133A only). The results are the combination of all time points pair-wise comparison.

(a) The number of the common genes (in bold) between two compared analysis settings.

				S-All					
				Up			Down		
				1	0.5 & 1	0.5	1	0.5 &1	0.5
			Num of Gene	450	931	2091	612	1161	2810
S-HG	Up	SLR=1	285	**259**	**267**	**279**			
		0.5 & 1	626	**286**	**573**	**594**			
		0.5	1418	**382**	**701**	**1261**			
	Down	SLR=1	1050				**537**	**622**	**1002**
		0.5 & 1	1625				**552**	**1047**	**1452**
		0.5	3364				**592**	**1101**	**2611**

(b) The percentage difference between two compared analysis settings.

				S-All					
				Up			Down		
				1	0.5 & 1	0.5	1	0.5 &1	0.5
			Num of Gene	450	931	2091	612	1161	2810
S-HG	Up	SLR=1	285	**46%**	72%	87%			
		0.5 & 1	626	64%	**42%**	72%			
		0.5	1418	74%	57%	**44%**			
	Down	SLR=1	1050				**52%**	61%	65%
		0.5 & 1	1625				67%	**40%**	51%
		0.5	3364				83%	68%	**27%**

S-HK: Scaling on 100 house keeping genes on Chip U133A provided by Affymetrix
S-All: Scaling on all genes of the U133A chip.
The percentage difference is defined as the number of differences between the union and intersection of two compared lists, divided by the number of the union list. For example, there are α genes in list *X*, β genes in list *Y* and δgene in common when comparing *X* and *Y*, the difference between *X* and Y is $(\alpha+\beta-2\delta) \div (\alpha+\beta-\delta)$.

The final results of the different analysis settings are given in tables 1 to 3. For the chronic study, the difference of the number of genes is 1 to 52 between S-All and S-HK (see Table 1a), while the real difference is larger than that. For example, with SLR = ± 2 there are 178 and 179 for S-All and S-HK respectively. By comparing these genes one by one, 168 common genes were found between two lists, so there are 10 and 11 unique genes for S-All and S-HK respectively. The real difference between S-All and S-HK with SLR = ± 2 is 21 genes instead of 1, which count for 11 % difference (21/189, where the percentage difference is defined as the number of genes that are unique to only one of the lists, divided by the total number of the genes in both lists). Considering different SLR cutoff values, the difference between S-All and S-HK varies from 11% to 25% for the chronic study (see Table 1b).

For the time course study, the difference between S-All and S-HK is higher than that in chronic study, which varies from 29% to 49% (see Table 2b). If we break the list to the up and down genes separately, the situation is even worse with difference up to 45% for up genes and 52% for down genes (see Table 3b). In comparing two normalization methods, S-HK picked up 311 (1259 - 948) more genes than S-All with SLR = ± 1, while the number of unique genes picked by S-HK is 511 (1259 – 748). However with SLR = ± 0.5, S-All caught 22 (4177-4155) more genes than S-HK while the number of unique genes is 740 (4177-3437) (see Table 2a). It seems that the S-HK emphasize its selection on down regulated genes since for each given SLR cutoff value there are always more down genes but less up genes in S-HK than that in S-All (see table 3a). We don't know if these results are real or bias introduced by S-HK (at least it cannot be answered in this thesis). From these differences only, we really cannot answer which normalization method is better in Affymetrix's software. Generally other algorithms or software and/or biological information are needed to help reaching the decision.

When looking at different SLR cutoff values in chronic study (see Table 1b), there is the least difference (11%) between S-All and A-HK for 4 fold change genes (SLR = ± 2). However, the highest difference between two Scaling methods is not coming from 1.4 fold change genes (SLR = ± 0.5, difference 24%) as we maybe thought. Instead, it comes from the 2-fold change genes (SLR = ± 1, difference 25%). This means that in our chronic data set, there are slightly more genes around two-fold change cutoff line than that around 1.4-fold cutoff line because little changes on signal value (caused by the two different scaling methods) will change the positions of more genes versus the cutoff line at 2-fold than at 1.4-fold. While in time course study, the differences between two scaling methods are increased (30%, 37%, 49% see Table 3b) when SLR cutoff value goes down (2-fold, 2-fold at later time and 1.4-fold at early time, 1.4-fold). These results show more genes around cutoff line of 1.4-fold change than that around 2-fold. This is because the infection by HIV caused the genes in H9 cells to start changing their expression status in the time course study. For both up and down genes, their expression levels were in a phase with continuous change and the changing folds were being built up slowly. Without counting the effect of vibrating genes (whose expressions switch between up and down directions frequently), only the truly up or down genes in their transit stages will naturally make the distribution: more genes around cutoff line at 1.4-fold than at combination of 1.4-fold and 2-fold, much more than at 2-fold. While in the chronic study, the expression profile in H9/NL4-3 was in a stable condition, possibly explaining why there could be fewer genes around 1.4-fold changes than at 2-fold in chronic study.

In summary, the results of this study confirm the conclusion made by other investigators: the different programs (data not shown here) or different settings in the same software can greatly affect the results of the array analysis [15]. This highlights the importance of developing

a standard and universal analysis tool for microarray studies. These results also enhance the fact that other information about genes, such as related genomic, proteomic, or metabolomic data are necessary to confirm and validate the results of gene expression studies.

Since DMT does not provide the MTC (multiple Test Correction), the intersection of S-All and S-HK were selected to reduce the type I error. As the number of genes is too many to handle in the downstream analysis for small SLR cutoff, the genes with 2-fold changes were selected to continue the analysis. The results of further analysis are out of the scope of this thesis, several screen shots of some pattern analysis results are presented here to give a flavor of the downstream analysis (see Fig. 11 and 12).

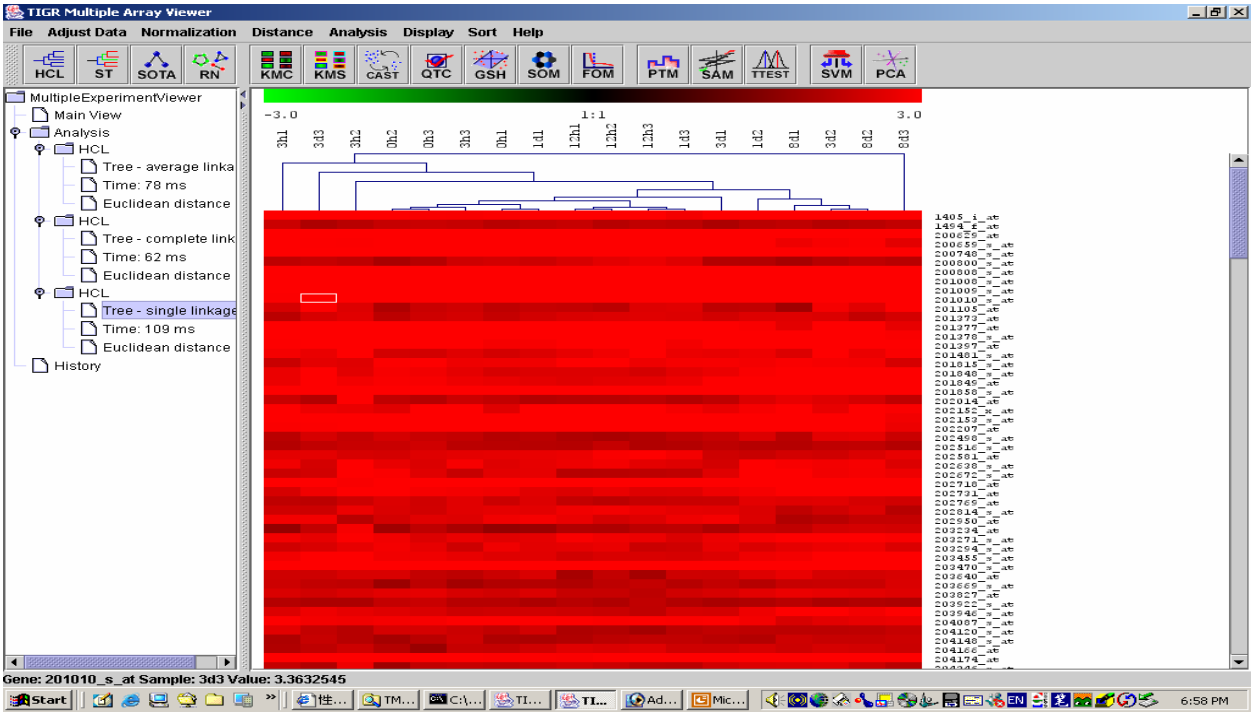


Figure 11. Screen shot of HCL of experiments in Tiger MeV. HCL represents Hierarchical clustering.

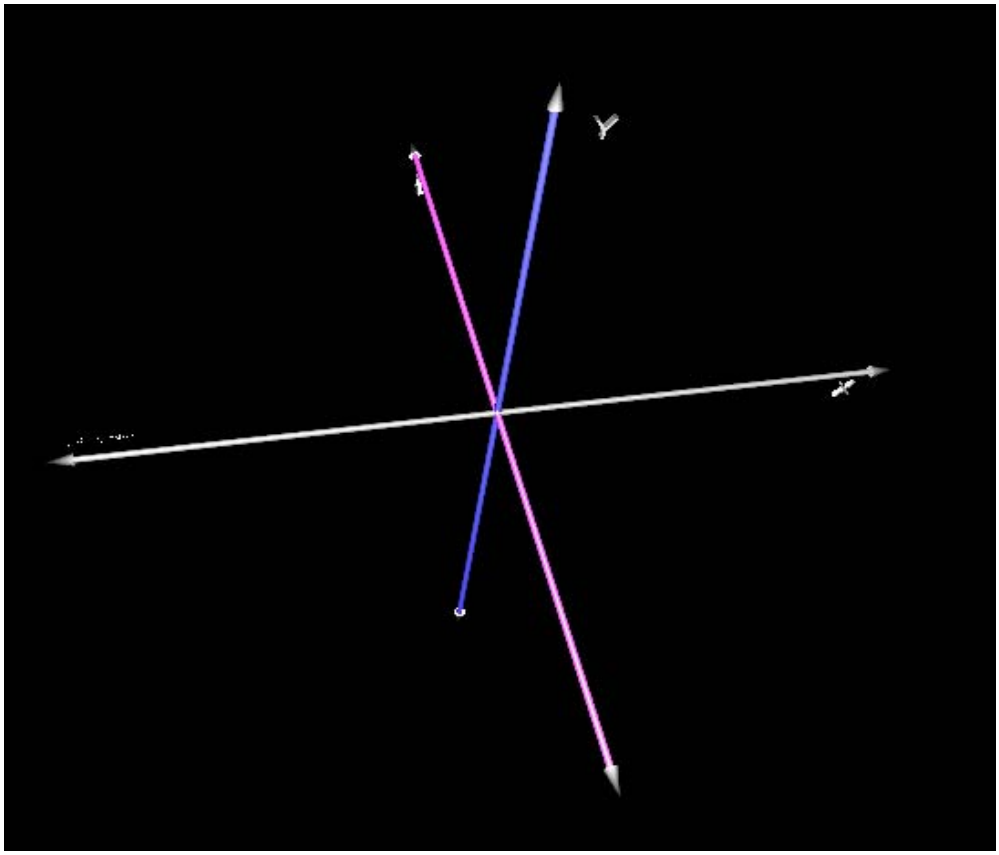


Figure 12. Screen shot of PCA analysis in Tiger MeV. PCA represents principal component analysis.

Shortcoming of DMT and Our Unique Solutions

1) Slowness of DMT for Managing Gene Lists

During the data analysis, the management of gene lists was very slow inside DMT. Whether analyzing the data in the pipeline manner with the four tests, or merging and intersecting gene lists, the computer took a long time to respond (up to one minute for merging or intersecting two gene lists). The possible explanation is that the very large data sets slowed the computational analyses. In addition, DMT can only combine or intersect two lists at one time. The required time was increased tremendously when working on several gene lists (not counting the time to name each new generated list). To solve this problem and increase efficiency, gene lists were exported from DMT (the final results of DMT analysis had to be exported anyway)

and Perl Script was developed and used to unify or intersect them.

2) Perl Scripts

For this data analysis study, three Perl scripts have been written to answer different questions. The first script, called *Parallel-Analysis.pl* (see page 59) was specially developed to do the parallel analysis with DMT. The script must be started from the command line since arguments are needed. Once it starts running, the script will ask for the file names of the probe lists one by one, and the user has to input the name of each gene list file followed by return (enter key). When all the files have been entered, hit return again and the script will complete the calculation and generate the list of common genes (intersection) among the lists typed in.

The results of the Script are produced also in a pipeline manner. The software starts to intersect the genes in the first two files, then the same procedure will be repeated by intersecting the new list with the following file until all inputted files have been scanned. For each iteration the script will output the number of genes in the common list on the screen and in the meantime save the list on the hard disk in the same folder as the script. The script has three requirements for the input files: (1) being in the same folder as the script, (2) being saved as plain text form (file extension is .txt) and (3) being entered without the extension (without .txt).

The second script, called IS-UN-GL.pl (for Intersection and Union of Gene Lists, page 63) was developed for pulling together the gene lists of pair-wise comparison to generate the final result for time course study. It was built on the basis of the first script, so it has the same requirements as the first one and should be lunched in the same way. It generates both the union lists and intersection lists of genes among the input files. Its output will show the number of genes in the union and common lists in each step, at the same time, the intermediate and final results will be saved on the hard disk.

These two scripts have been designed to run with at least two arguments (input files). When the user enters only one file name, the scripts will show an instruction and allow the user to start over. The names of the saved output files are very instructive and it is easy for users to know the contents (see Fig. 13). In addition, both scripts will create a log file containing all the on-screen output in the same folder as the given script.

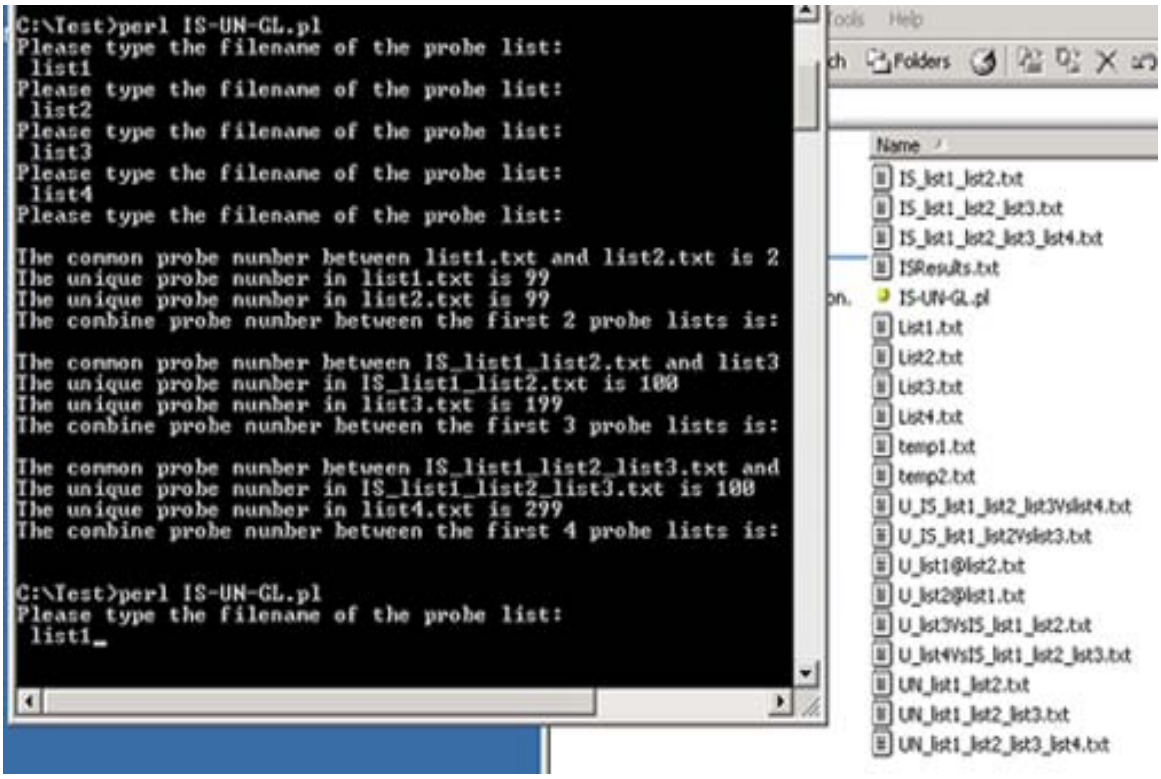


Figure 13. Screen shot of the Perl Script II IS-UN-GL.pl. The command line window (left) shows how to start it and shows the input argument and the on-screen displayed results (the result was cut in order to show the right side results). The right side shows the contents of the folder where the script and initial gene lists are located after the running of the script. The files starting with UN stand for Union lists, ones with IS stand for intersection lists and ones with U stand for unique lists. The final results of the initial 4 gene lists are UN_list1_list2_list3_list4.txt for union list and IS_list1_list2_list3_list4.txt for intersection list.

The third Perl script, called ListFinder.pl (page 79) was developed under the requirement of investigators. When the final results from Chip U133A set for the time course study were

delivered, investigators who will explore the biological function of these genes asked a question: if a biologically interesting gene is found in the final list, how can one know in which pair-wise comparison (or between which two time points) this gene was picked up and which direction it had (up or down). The question could be solved in different ways:

(1) Accessing the original data in ".chp" files.

The problem is that the ".chp" files can only be opened by Affymetrix software. However, not only is the searching time quite long, but the accessibility is limited by the licensing agreement of the software. We have only one license for the package and the programs were installed at the Compaq computer. At that time, this computer was occupied in analyzing the data from U133B set.

(2) Exporting the data from ".chp" files to the spread sheet form and searching in the spread sheet.

The potential problem is that the file is quite large and the investigators have to go through the data for all the time points and manually pick up the interesting pair.

(3) Creating another short program to automatically find the results.

This solution was selected because the individual lists of up and down gene for each pair-wise comparison were already exported from DMT and each file's name included the information about the two time points compared. Another Perl script was created and put into the same fold of these individual gene lists. To simplify the script, a free software called printFolder was downloaded [33], which can catalog all the files in a selected folder and save their names into a text file. The individual gene lists were cataloged with printFolder and the saved file was re-named as fileLists.txt. This file name was coded directly into the script and in this way, the

only input needed for the script is the Affymetrix probe set name of the interesting gene. The script will output a list of file names which contains the given gene both on the screen and on the disk. From the name of these individual gene list files, the investigators can get the information about the interesting gene: which pair-wise comparison it was selected for and what direction of change it had at the given time points.

After the discovery of the printFold software, Script II was modified to allow the gene lists being inputted from the text file (generated by printFold) instead of through command line (see Script IIb, page 79). As a result, the script IIb will run without arguments. It is not only fast but also error-free especially when working on many lists at a time (the biggest number in this study was 40 gene lists when combining up and down genes of 20 pair-wise comparisons). Another advantage of Script I, IIa and IIb over DMT is that the names of all lists (intermediate and final) generated by scripts are coded into the scripts and manually naming is not needed. In addition, the names of the new files are very instructive once you understand the naming rules (see Fig. 13).

3) Problems for Incorporating Biological Information

In the final results (both for chronic and time course studies) of the U133A set, more than half of the genes are not associated with any biological functions. The investigators had to look at these genes one by one and try to manually assign some functions to them by comparing their sequences with other well-characterized genes. These manually assigned functions, which we refer to as “in-house” definitions of genes, were initially listed in a spread sheet file. However, it was very inconvenient to browse and utilize this information duringthe biological interpretation of the information on the spreadsheet..

As previously mentioned, the incorporation of genomic, proteomic, or metabolomic

information is essential for microarray data analysis. Many very useful online databases provide these kinds of data to help investigators deal with the microarray data. These public databases include but are not limited to UniGene (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene), Ensembl (www.ensembl.org), LocusLink (www.ncbi.nlm.nih.gov/LocusLink/), Swiss-Prot (us.expasy.org/sprot/), EC DB (www.chem.qmul.ac.uk/iubmb/enzyme/search.html), Gene Ontology (www.geneontology.org/), OMIM (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM&cmd=Limits), UCSC Genome Mapping DB (genome.ucsc.edu) , Incyte Proteome DB (www.incyte.com/sequence/proteome/index.shtml), Scop (scop.mrc-lmb.cam.ac.uk/scop/search.cgi?), Blocks (blocks.fhcrc.org/blocks-bin/getblock.sh), GenMapp (www.genmapp.org/default.asp), InterPro (www.ebi.ac.uk/interpro/), KEGG DB (www.genome.ad.jp/kegg/kegg2.html), Pfam (pfam.wustl.edu/textsearch.shtml), TMM (www.cbs.dtu.dk/services/TMHMM-2.0/), Blast (www.ncbi.nlm.nih.gov/BLAST/).

In addition, the related publications are another useful source to help the interpretation of the analysis results. Unfortunately, Affymetrix's three programs cannot manage this information nor incorporate them, and they cannot directly access these online databases either. Affymetrix does provide an online analysis center -- NetAffx, which can directly connect to all the databases cited above. However, it still cannot manage the information collected by investigators, such as, in-house definition and related publications. In addition, there were some inconveniences in using NetAffx. First, the query results presented by NetAffx were hard to manage, because as a spread sheet with a lot information, the results had to be broken into several windows horizontally and/or vertically (had to slide the horizontal bar and/or flip several pages to find the useful information). Second, access to NetAffx is protected by a password (free to any one). Inactivity for a short time in Affymetrix's web page resulted in the user being automatically logged out and losing all the information just collected. Often the user had to log in several times

and flip several pages each time to reach the correct screen for an analysis of one gene.

4) In-house Database Design and Implementation

To overcome the problems of incorporating useful information and of accessing the data (limited by the software license and time out of NetAffx), we designed our own database (referred to as "in house" database). The database was designed to hold the original data, to be able to directly connect to public databases, and to have the ability to incorporate useful information selected by the investigators. Since the investigators use Apple computers but the developing environment is window based, FileMaker Developer 6 (FileMaker Inc, CA) was selected to implement the design. The FileMaker is cross-platform software which can run on both Apples and PCs. With the developer tool of FileMaker, the completed database can be transferred into standalone license-free software.

The structure of the database is illustrated in the Figure 14. It is composed of 9 files: MainMenu, Probe-Set, Data-Set, SF [what is SF], DataImport, Publications, LineItem, Location and GenMapp. MainMenu is the starting point of the database. Through Mainmenu the user can open thye other 8 files. Location contains the information about the probe set's chromosomal location and it will display the probes in an ordered list according to their location. GenMapp displays pathway information from the public database GenMapp. Publications will hold relevant research articles and their contents selected by the investigators. LineItem is a "bridge" file allowing Probe-Set to display some information from Publications and it establishes a link between these two files in a many-to-many relationship. DataImport is the entry point for loading gene lists and other information, such as in-house definition, into the database. After importing a gene list, DataImport can automatically make both Probe-Set and Data-Set files only show the records of the genes in the given list (realized by scripts).

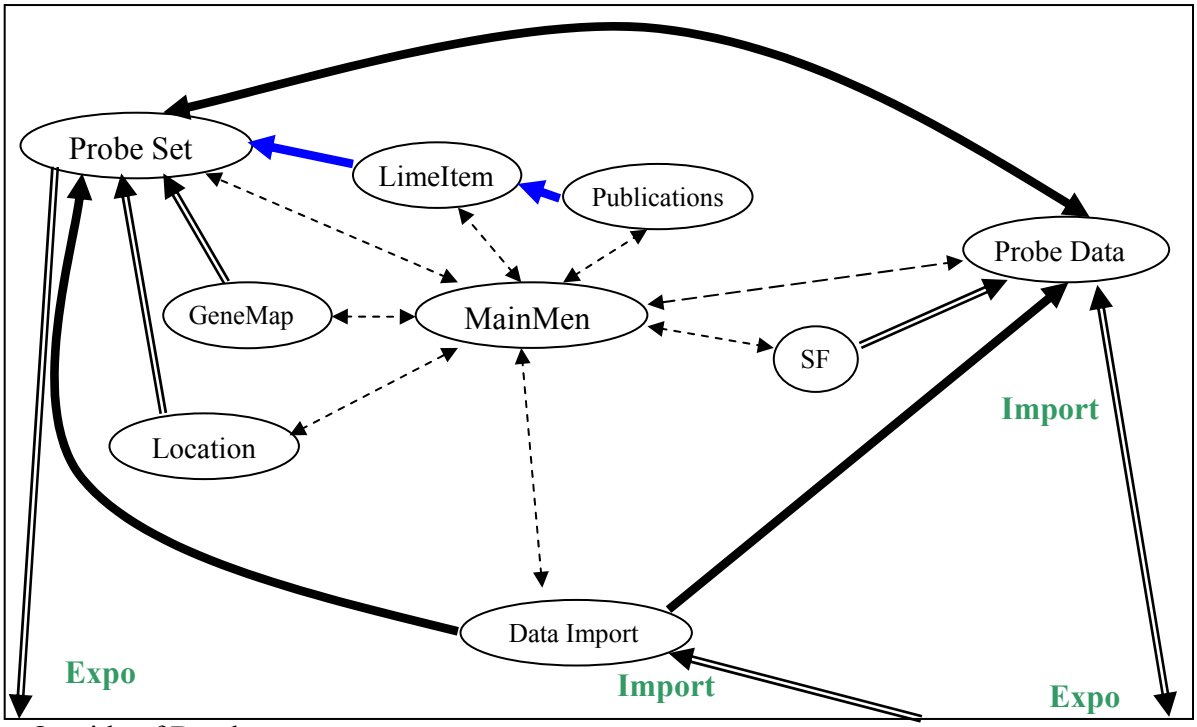


Figure 14. The Structure of the in-House Database. The dotted line indicates that the files can open each other through the counter part. The double line, besides the function of the dotted line, indicates that one file provides information or data to another file (pointed by the arrow). The solid and thick lines provide functions of the dotted lines and double lines. In addition, it indicates that the file accepts the data or information provided by another file and displays them in its own file. The blue line means that the information provided by Publications and displayed in Probe-Set is transferred through the “bridge” file LineItem.

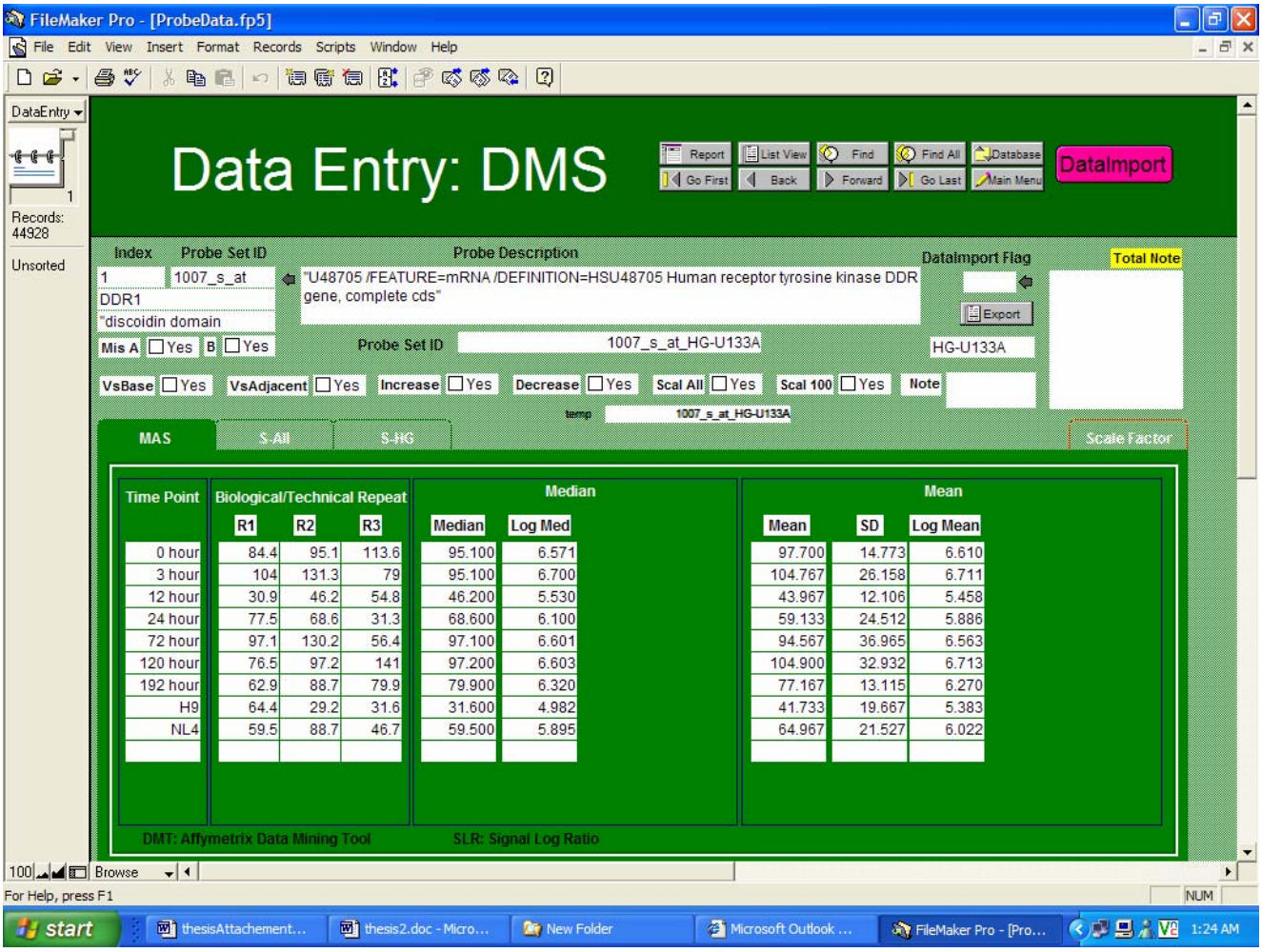
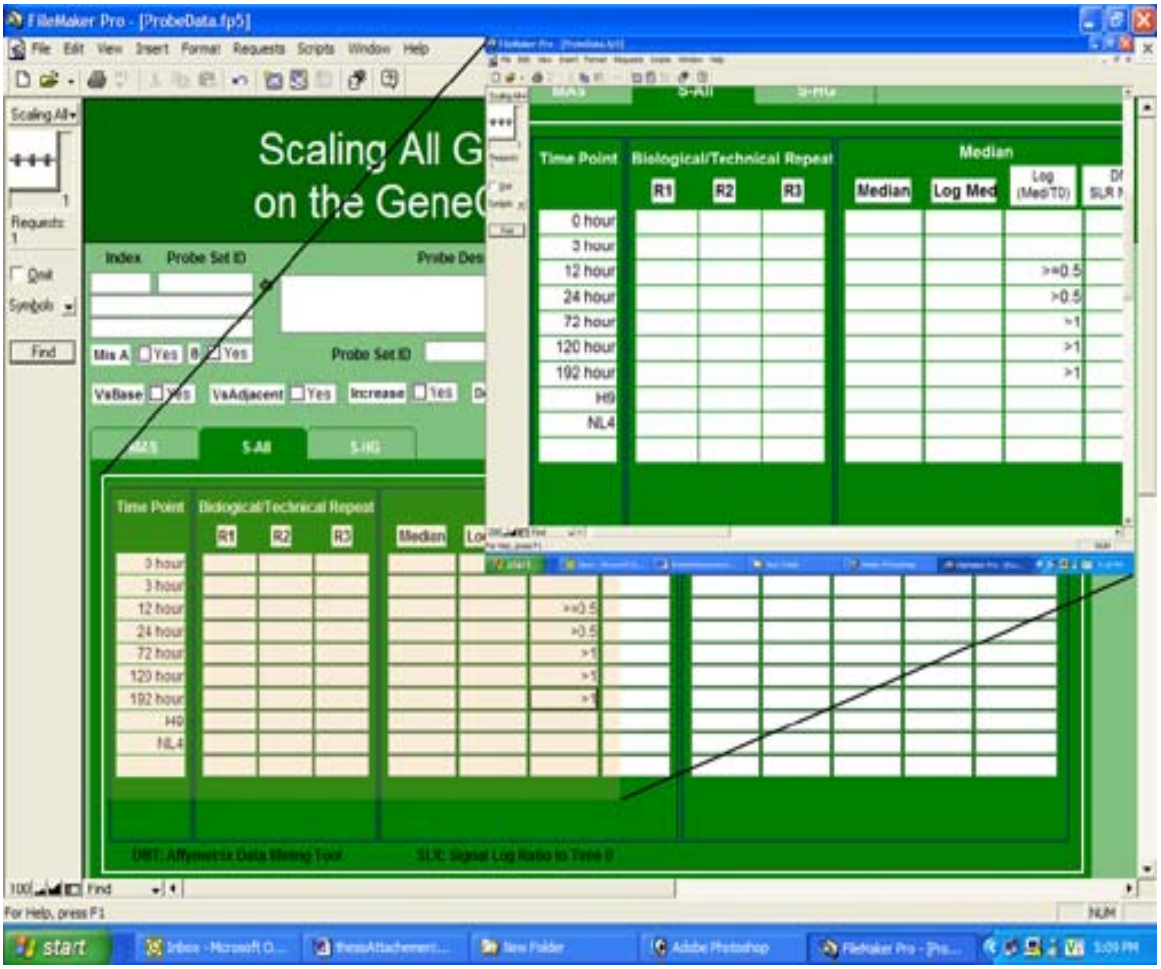


Figure 15. Screen shot of Probe Data. The layout contains the original data. It has 44928 records (indicated by red circle) and each record represents one probe set and shows the density value of the probe set in all experimental conditions.

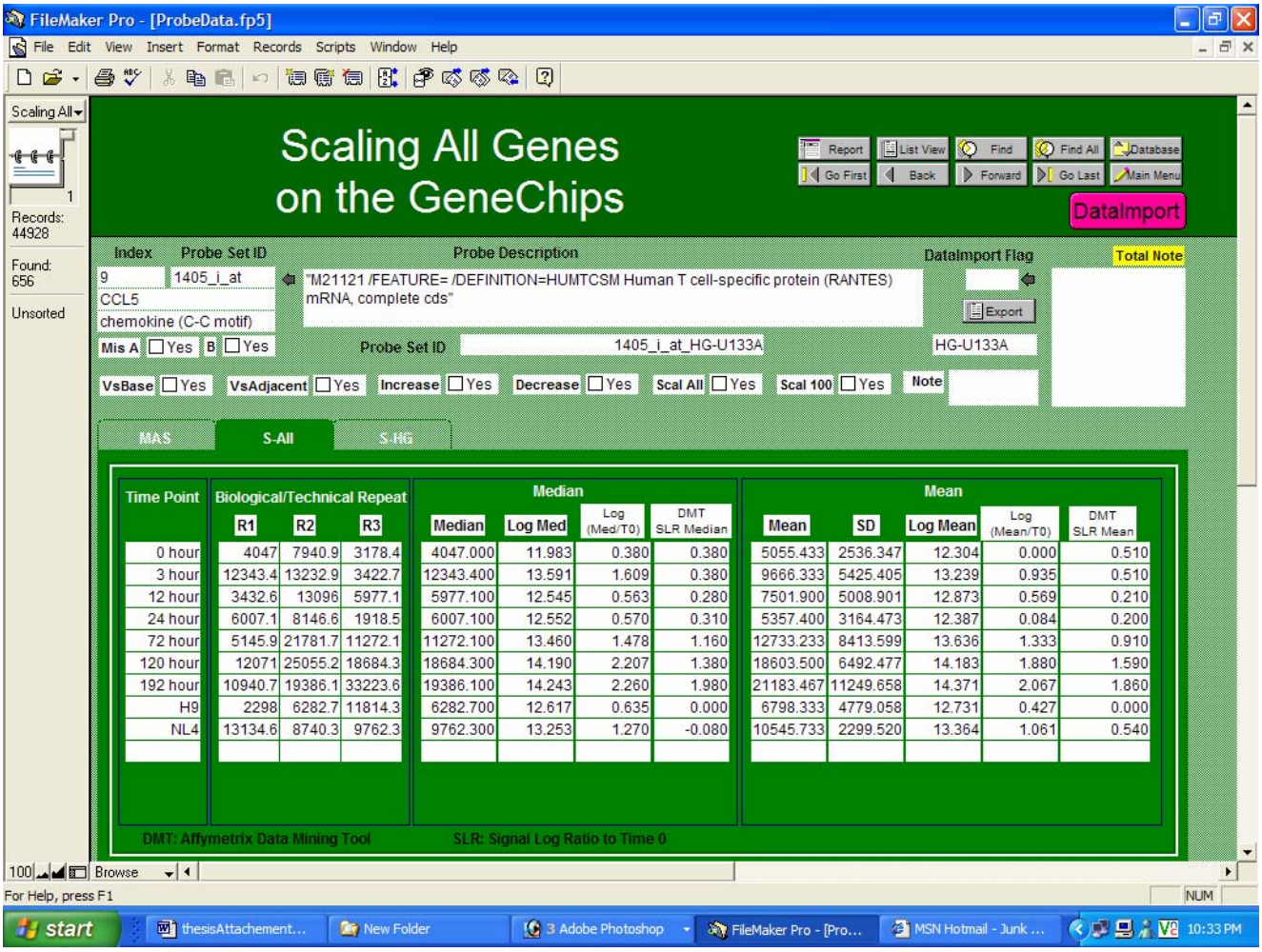
SF contains the value of scaling factor for each array in two scaling methods (S-All and S-HK). Data-Set holds original data of each chip in the study (see Fig. 15). It displays each probe set of the U133 A and B chips as a record with its description. Since there is a total of 44928 probe set in U133A and B (there are only 44760 unique probe sets because some probes exist in both chips), Data-Set has 44928 records in order to hold 44928 data sets. After importing the raw data, Data-Set utilizes scaling factors saved in SF to calculate the normalization value for S-All and S-HK. It also automatically displays the mean, median, log mean, log median and standard

deviation for each gene in each experimental condition and each normalization method. All these values can be easily exported for a selected group of genes as a tab-separate text file to be used by a 3rd party software. Both SF and Data-Set were designed to hold 10 experimental conditions with triplicates for each condition. In total, they can hold 30 chips' data. Since each record in Probe-Data file contains data of all experimental conditions of a given probe set, a gene expression pattern search in the layout of normalized data (layout S-All or S-HK) can be performed by entering required expression value in multiple fields of conditions. This pattern search function (see Fig. 16), not provided by any of the Affymetrix's software is a very useful tool for microarray data analysis, especially for gene discovery.

Probe-Set is the major file in the database, which is built on the basis of the Affymetrix's annotations for U133 chip set. It displays each probe set as a single record and all available information provided by Affymetrix's annotation for the probe are included in that record. Probe-Set has a total of 44928 records and through the internal link with Data-Set, it displays the normalized data (S-All) of all experimental conditions in the record window of that gene (see Fig. 17). For a gene *X*, all its related information has been divided into several layouts in its record. For example, the GO layout contains the Gene Ontology information of *X*, the References layout has direct link with the Publications file and holds the specific published information of *X*, and the InhouseDef layout keeps all functions of *X* assigned by the investigators. There is also a layout of Promoter, which will be filled-in with related information regarding the genetic structure of the promoter region responsible for controlling gene expression in a separate study currently carried out at the RIC.


FileMaker Pro - [ProbeData.fp5]
File Edit View Insert Format Requests Scripts Window Help
Scaling All G
on the Gene
Index
Probe Set ID
Probe Set ID
VsBase
VsAdjacent
Increase
Time Point
Biological/Technical Repeat
R1
R2
R3
Median
Log Med
0 hour
3 hour
12 hour
24 hour
72 hour
120 hour
192 hour
H9
NL4
DRT: Affymetrix Data Mining Tool
SLR: Signal Log Ratio to Time 0
For Help, press F1
start

(a)



(b)

Figure 16. The example of pattern search in Probe-Data. (a) The find-mode window shows the condition of the expression pattern we want to find. In this example, we want to find the genes that have at least 1.4-fold up at 12 hour, more than 1.4-fold at 24 hour and more than 2-fold thereafter but we don't care about the level at 3 hour (comparing to time 0, since the fields that we typed the numbers in are the SLR based on time 0). (b) The results of the pattern search of the condition given in (a). There are a total of 656 probe sets that the expression pattern queried.

In order to prevent accidental modification of pre-load annotations in Probe-Set, a button function was created for each important data field. As a result, when clicking on these fields, Probe-Set will display a find-mode layout. In addition, a power search was implemented in Probe-Set to allow a multi-field search with a single entry of terms (see Fig. 18). Probe-Set

provides a single interface through which our investigators can search multiple databases simultaneously. Besides those public databases cited above, Probe-Set can directly access several more public databases which were not linked by NetAffx, such as, Human Promoter Database (zlab.bu.edu/~mfrith/HPD.html), Whitehead Institute GeneCruiser (www-genome.wi.mit.edu/cancer/genecruiser/src/AffyQuery.jsp), Eukaryotic Promoter Database (www.epd.isb-sib.ch/), Transcription Factor DB (transfac.gbf.de/TRANSFAC/).

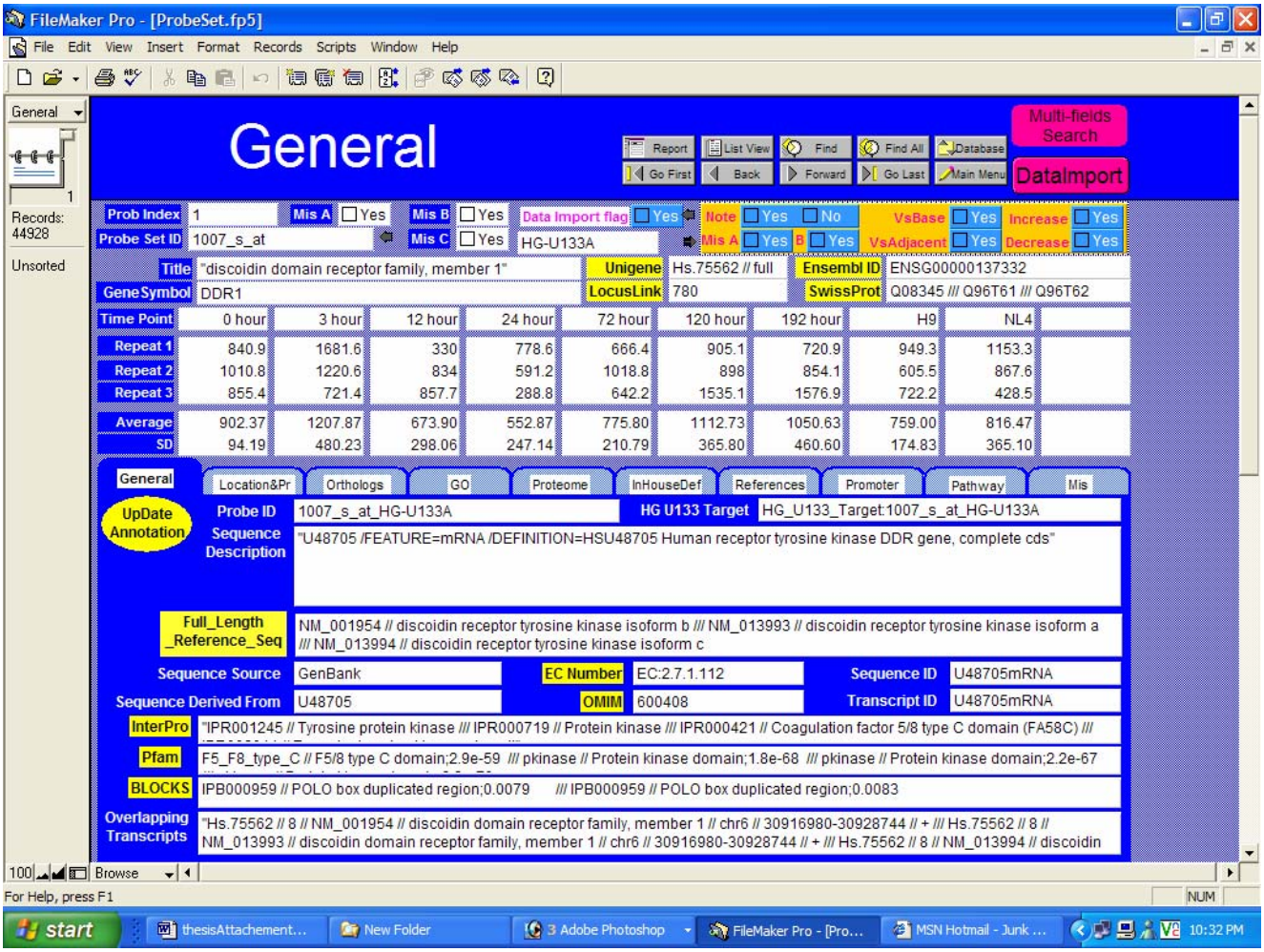


Figure 17. Screen shot of the General Layout of Probe-Set.

Of course, the database also can connect to NetAffx by a simply click. It is independent from NetAffx but covers most functions provided by NetAffx. For data from studies performed by other groups, the database handles it in three different ways. If the chip used is U133 set and

original data is available, we can import them into the database, and analyze and mine them with our data together. If the gene lists are available, we can import them and use them as a comparison list. If only a publication is available, we can extract the useful data and stock them in Publications file. This information can be shown in the Probe-Set file through the internal link and can be used for the data analysis.

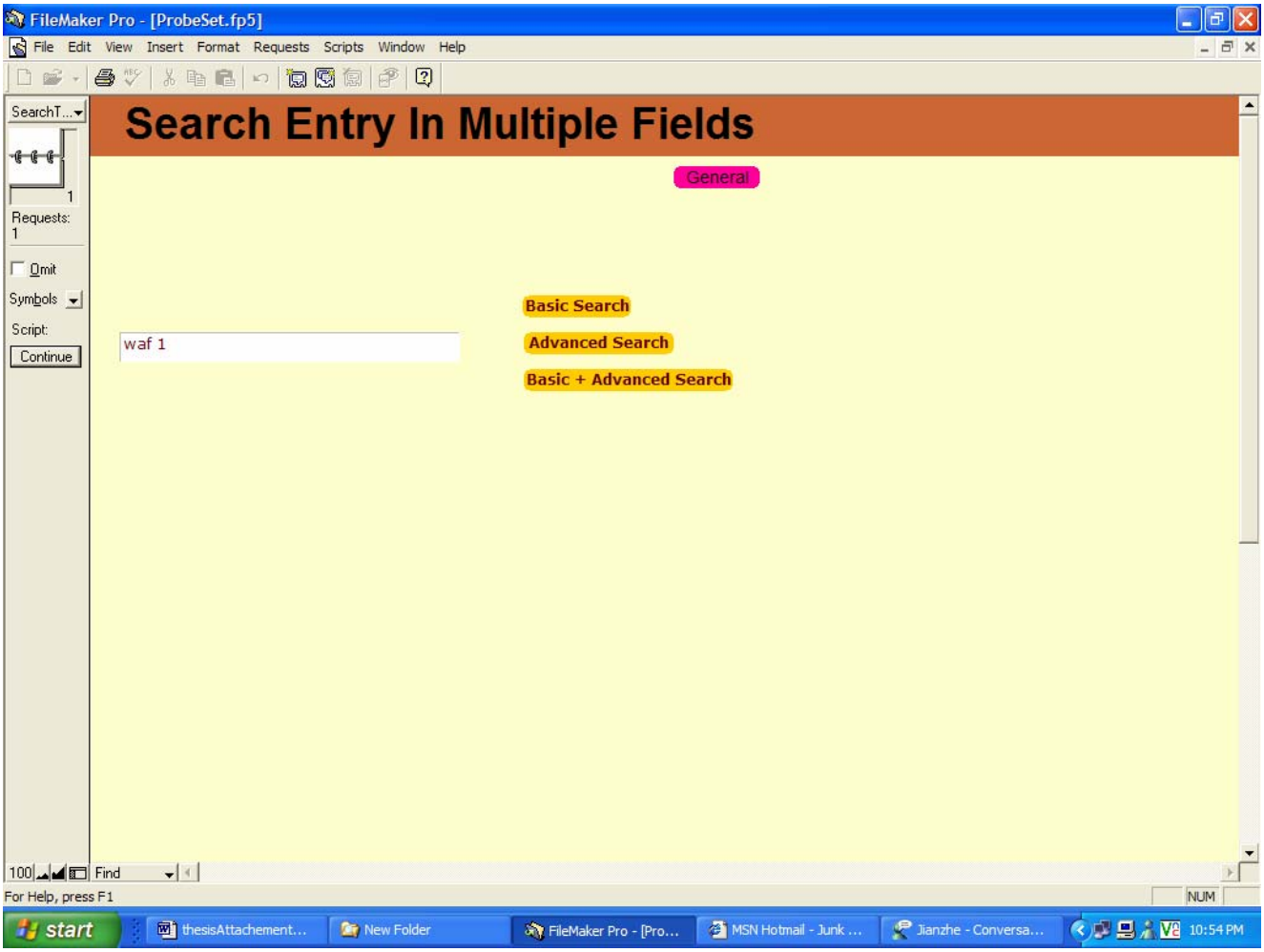


Figure 18. Screen shot of the layout of Search Entry in Multiple Fields in Probe-set.

As a whole, the database is composed of 9 files, which contains a total of 42 layouts, 682 fields and around 150 scripts (see Fig. 19). By using the click buttons powered by scripts in each window, investigators can quickly switch between different layouts and different files to catch

the useful information. Besides the layouts of record (probe set) per window, files (such as, Probe-Set, Probe-Data and Publications) and layouts (such as the different layouts in Probe-Set) have their own list view layout, where the selected probe sets are displayed together in a spread sheet form (see Fig. 20). This form allows investigators to quickly and easily spot an interesting gene among the selected group. By just clicking on its ID in the corresponding row, the investigators can go back to its individual record layout.

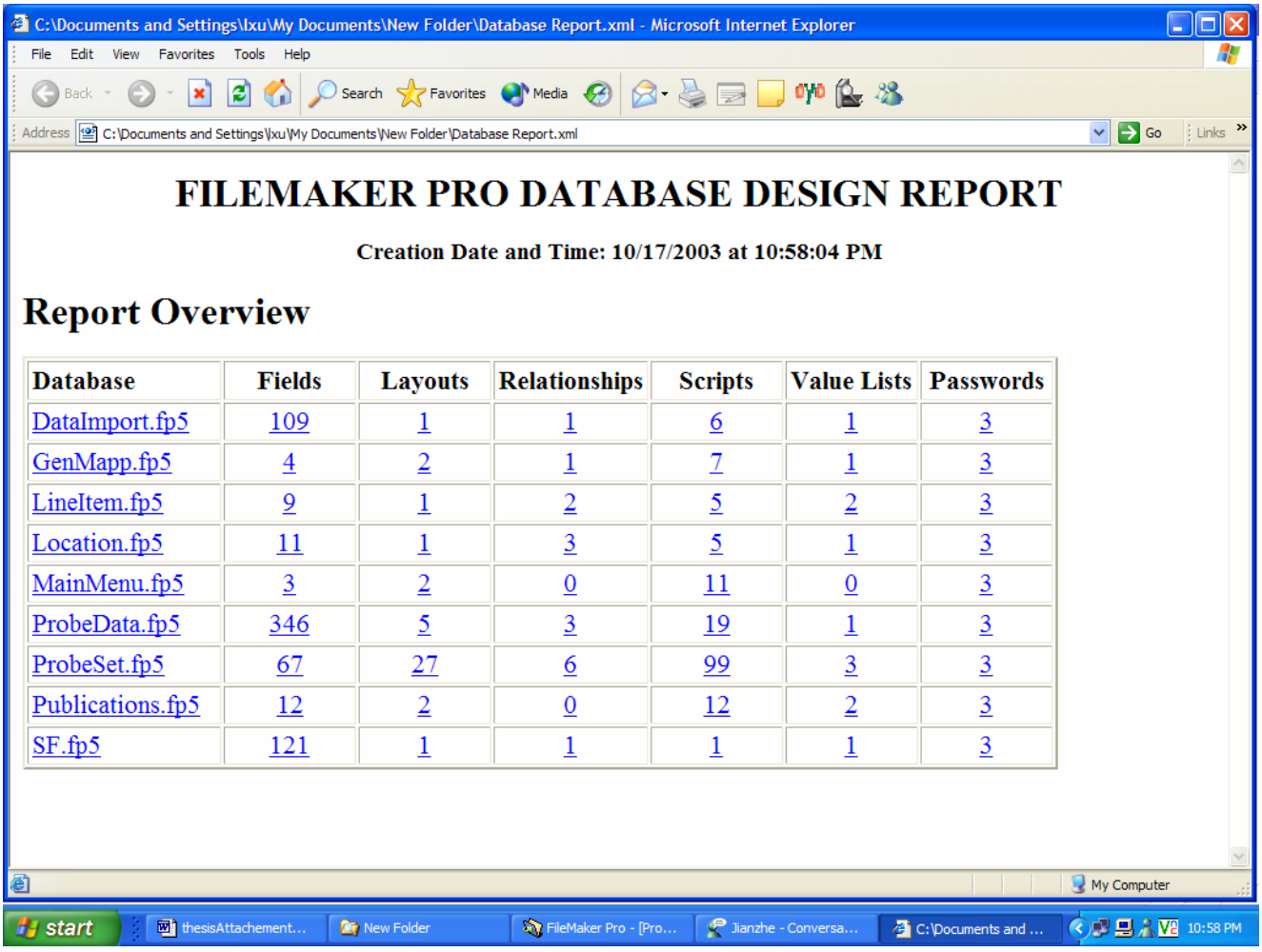


Figure 19. Screen shot of the design report of the in-house database. This report was generated by FileMaker Pro Developer 6.

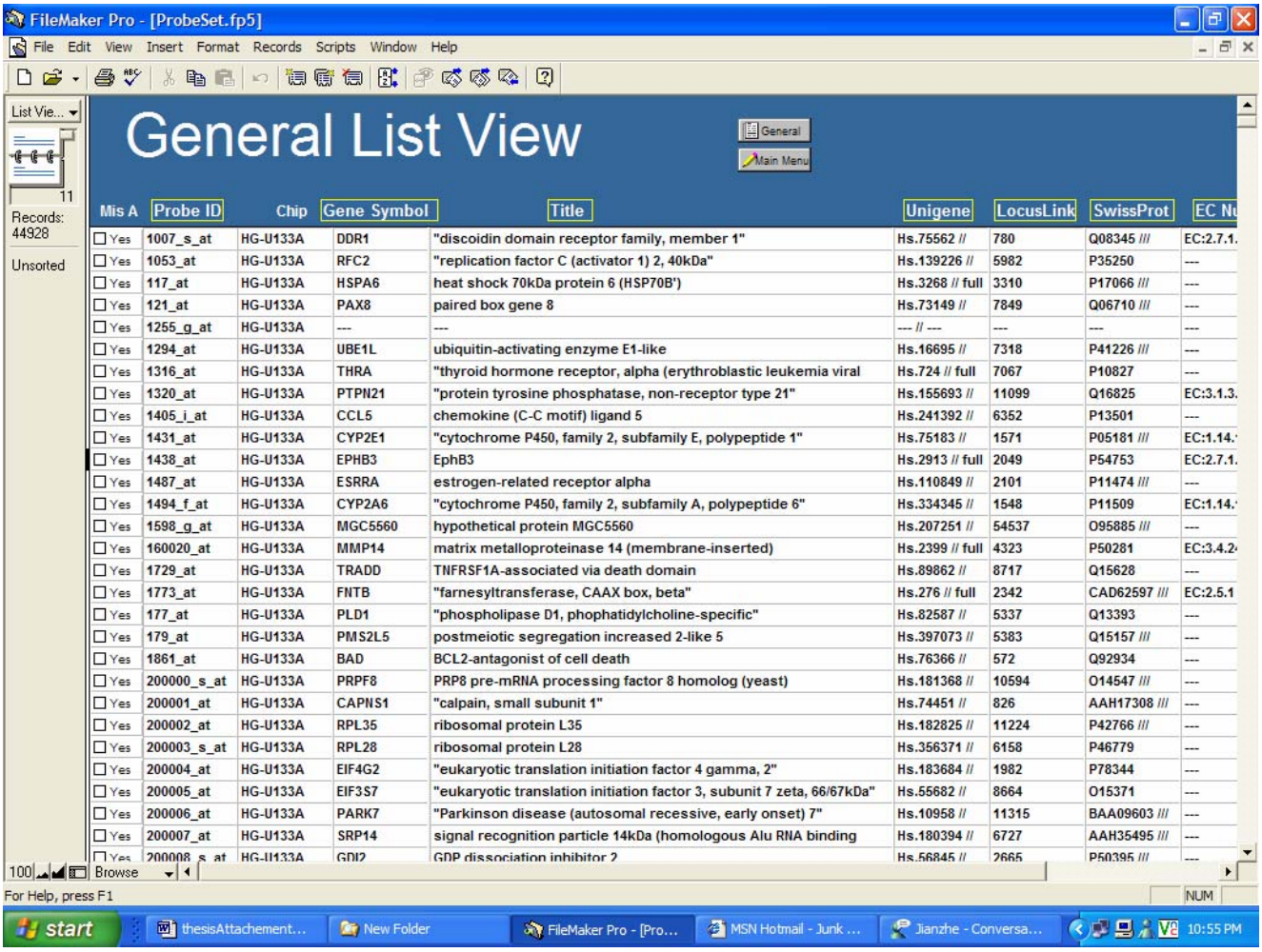


Figure 20. Screen shot of the layout of General List View in Probe-Set. This layout shows multiple records in one window under spread sheet form. Almost all the layouts in Probe-set and several layouts in other files have their own list view layout. With this form, it becomes easy and rapid for the investigators to choose several genes among many ones.

The database has a setting for multiple passwords and can assign them into different privilege groups. As a result, the administrator of the database can easily grant different levels of data access and the permission of modifications to different users. The total size of the database after importing data from all chips in HIV study is only 266 MB, including 54 chips with two Scaling methods, which is significantly smaller than 13 GB of the flat ".chp" files. As it is a freestanding software and can be run on Apple and PC, our investigators can use it anywhere. In addition, the database is potentially networkable and can be adapted to other types of array. The

database physically frees the investigator from the restriction of license limit of Affymetrix software (both AMS and DMT). By using it, our investigators can process and analyze full Affymetrix microarray data in a speedy and easy manner.

Conclusion

The DNA microarray is a very powerful and advanced molecular biological tool. It allows scientists to capture the expression profile of thousands of genes simultaneously. As a technology, the experimental process of a microarray study is already mature with several commercialized kits and protocols available. However, the data analysis is a bottleneck for microarray studies and restrictive in its usage. For this thesis, the microarray data were generated from a real HIV infection study with one of the most popular microarray systems: Affymetrix Human U133 chip set. To simplify the analysis, we chose the Affymetrix software package to analyze the data with the expectation that the algorithms provided in Affymetrix software will fit well to its own microarray data. By analyzing the data with six different settings: two normalization methods (called Scaling for Affymetrix) combined with 3 cutoff values for fold changes, the final results varied significantly from setting to setting. For a given fold change value, the difference between normalizations (Scaling) can reach 49%. When looking at the difference from the number of genes, there are as many as 510 genes picked up by one scaling method but dropped by another, while the number of the common genes between them are only 748 (see Table 2a). As both microarray and software come from the same (widely accepted) commercial company, the results have clearly shown that different programs (results not shown here) or different settings in the same software can greatly affect results of the array analysis [15]. In order to fully benefit from the microarray study, a standard analysis method for microarray data must be quickly developed. The result particularly highlight that biological information,

such as related genomic, proteomic, and metabolomic data, as well as relevant publications, must be incorporated into the process of the microarray data analysis. To address the information integration problem and also to ease analysis and mine our data, an in-house database was created during this study. The database is dedicated to incorporate all available information about the genes under study. It can hold entire data from 30 chips of U133 set which contains 44928 data points, and it provides a well organized structure for holding specific information selected by investigators, from publications to in-house definitions. Meanwhile, it allows the investigator to quickly and easily apply these collections of knowledge into the microarray analysis. In addition, it can directly connect to more than twenty public databases which further enhance its ability to analyze the data. As a free-standing, cross platform (PC and Mac) software, the in-house database allows investigators to better access, analyze and mine data. Although, it is a good supporting tool for our study, a program which can combine the properties of our in-house database and a standard and universal algorithm for analysis will be a better choice in order to fully explore the powerfulness of the microarray technology. In conclusion, a better and more powerful tool with the ability to analyze and mine data needs to be developed for microarray data analysis. This can be realized only by a wide range of cooperative efforts between scientists in Computer Science, Statistics, Biology and Bioinformatics.

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Appendix: Perl Scripts:

Script I. Parallel-Analysis.pl, the tool for parallel DMT analysis.

```
#!/usr/bin/perl

# Parallel-Analysis.pl
# This script will take two or more gene lists and find the intersection genes among the
# input gene lists. It saves the final and internal result on disk. Meantime, it
# also save the unique gene list for each input file for the pair-wise comparison
# between the input files (in the order of input)

use strict;
use warnings;

my @probeLists = ();
my $fileNumber = @probeLists;
my $flag =0;

while($flag < 1) {
	if ($fileNumber > 0) {
		# clean the old record
		print "Now start from the beginning again.\n";
		$fileNumber = 0;
	}
	# Ask user to input the filename of the file containing gene list
	do {
		print "Please type the filename of the gene list:\n ";
		my $temp= <STDIN>;
		chomp $temp;
		$probeLists[$fileNumber] = $temp;
		++$fileNumber;
	} until ($probeLists[$fileNumber-1] =~ /^\s*$/);

	if ($fileNumber <=2) {
		print "The program needs at least two files to find the common genes between
	them.\n";
	}
	else {
```

```
                $flag = 1;
        }
}

# delete the last enter which has no file name
pop (@probeLists);
--$fileNumber;

# find the common part between the first two probe lists
my (@resultCM) = common($probeLists[0], $probeLists[1], 1);

# continue to find the common part in the following probe lists
for (my $i=2; $i< $fileNumber; $i++) {
        (@resultCM) = common($resultCM[0], $probeLists[$i], $i);
}
exit;

sub common {
        my ($probe1, $probe2, $index) = @_;
        my $motif;
        my @shareList= ();
        my @probe1unique= ();
        my @probe2unique = ();
        my @outputFiles = ();

        # construct the name of the output files
        if ($index ==1){
                @outputFiles = ("IS_".$probe1."_".$probe2, "U_".$probe1."Vs".$probe2,
        "U_".$probe2."Vs".$probe1);
        }
        else {
                @outputFiles = ($probe1."_".$probe2, "U_".$probe1."Vs".$probe2,
        "U_".$probe2."Vs".$probe1);
        }

        # add the file extension to the file name
        $probe1=$probe1.".txt";
        $probe2=$probe2.".txt";
        # open the files, or exit
        unless ( open(PROBEFILE1, $probe1) ) {
                print "Cannot open file \"$probe1\"\n\n";
                exit;
        }

        unless ( open(PROBEFILE2, $probe2) ) {
                print "Cannot open file \"$probe2\"\n\n";
```

```
	exit;
}

# Read the gene name from the file, and store it
# into the array variable @probe 1 or 2
my @probe1 = <PROBEFILE1>;
my @probe2 = <PROBEFILE2>;

# Close the file - we've read all the data into @probe 1 or 2 now.
close PROBEFILE1;
close PROBEFILE2;

my $j =0;
# Now starting search for common probe number, list and unique probe list for probe1
foreach $motif (@probe1){
	$j = 0;
	chomp $motif;
	foreach (@probe2) {
		chomp $_;
		if ($_ eq $motif) {
			push (@shareList, $motif);
			$j=1;
			last;
		}
	}
	if ($j == 0) {
		push (@probe1unique, $motif);
	}
}

# Finding the unique probe list for probe2
foreach $motif (@probe2) {
	$j = 0;
	chomp $motif;
	foreach (@shareList) {
		chomp $_;
		if ($_ eq $motif) {
			$j = 1;
			last;
		}
	}
	if ($j == 0) {
		push (@probe2unique, $motif);
	}
}
```

```
	# output the results
	print "The common probe number between ", $probe1, " and ", $probe2, " is ", scalar
	@shareList, "\n";
#	print "The common probe is:\n", "@shareList\n\n";
	print "The unique probe number in ", $probe1, " is ", @probe1-@shareList, "\n";
#	print "The probe1 unique probe is:\n", "@probe1unique\n\n";
	print "The unique probe number in ", $probe2, " is ", @probe2-@shareList, "\n";
#	print "The probe2 unique probe is:\n", "@probe2unique\n\n";

	my $logfile = "Log".$index.".txt";
	my $outputfile1 = $outputFiles[0].".txt";
	my $outputfile2 = $outputFiles[1].".txt";
	my $outputfile3 = $outputFiles[2].".txt";
	open (outPutFile, ">$logfile");
	open (outPutFile1, ">$outputfile1");
	open (outPutFile2, ">$outputfile2");
	open (outPutFile3, ">$outputfile3");
	print outPutFile "The common probe number between ", $probe1, " and ", $probe2, " is ",
	scalar @shareList, "\n";
	foreach (@shareList){
		print outPutFile;
		print outPutFile "\n";
		print outPutFile1;
		unless ( /$shareList[@shareList-1]/) {
			print outPutFile1 "\n";
		}
	}
	print outPutFile "\n\n";
	print outPutFile "The unique probe number in ", $probe1, " is ", @probe1-@shareList,
	"\n";
	foreach (@probe1unique){
		print outPutFile;
		print outPutFile "\n";
		print outPutFile2;
		unless ( /$probe1unique[@probe1unique-1]/) {
			print outPutFile2 "\n";
		}
	}
	print outPutFile "\n\n";
	print outPutFile "The unique probe number in ", $probe2, " is ", @probe2-@shareList,
	"\n";
	foreach (@probe2unique){
		print outPutFile;
		print outPutFile "\n";
		print outPutFile3;
		unless ( /$probe2unique[@probe2unique-1]/) {
```

```
                print outPutFile3 "\n";
            }
        }
        close (outPutFile);
        close (outPutFile1);
        close (outPutFile2);
        close (outPutFile3);
        return @outputFiles;
}
```

Scrip II. IS-UN-GL.pl, the modification of Script I, which will do the union as well as intersection of selected gene lists.

A) Inputting gene lists' name in command line.

```
#!/usr/bin/perl

# IS-UN-GL.pl

# This script will take two or more gene lists and find the intersection and union genes among the
# input gene lists. It saves the final and internal result on disk. Meantime, it
# also save the unique gene list for each input file for the pair-wise comparison
# between the input files (in the order of input)

use strict;
use warnings;

my @probeLists = ();
my $fileNumber = @probeLists;
my $flag =0;

while($flag < 3) {
        if ($fileNumber > 0) {
                # clean the old record
                print "Now start from the beginning again.\n";
                $fileNumber = 0;
        }

        # Ask the user for the filename of the file containing probe list
        do {
                print "Please type the filename of the probe list:\n ";
                my $temp= <STDIN>;
                chomp $temp;
                $probeLists[$fileNumber] = $temp;
                ++$fileNumber;
```

```
	} until ($probeLists[$fileNumber-1] =~ /^\s*$/);

	if ($fileNumber <=2) {
		print "The program needs at least two files to combine them and find the common
parts between them.\n";
		$flag += 1;
	}
	else {
		$flag = 4;
	}
}

if ($flag ==3) {
	print "Since you only input one file for three times, the program terminates now.";
	exit;
}

# delete the last enter which has no file name
pop (@probeLists);
--$fileNumber;

# creat the output file
my $outputfile = "Log.txt";
open (outPutFile, ">$outputfile");

# find the intersection of the first two probe lists
my (@resultCM) = common($probeLists[0], $probeLists[1], 1);

# Combine the first two probe lists by joining the intersection
# and the unique gene lists of the first two probe lists.
my ($resultCB) = combine (@resultCM, 1);

for (my $i=2; $i< $fileNumber; $i++) {
	# continue to intersect following probe lists
	(@resultCM) = common($resultCM[0], $probeLists[$i], $i);

	# continue to unit probe lists
	my (@temp) = commonInternal ($resultCB, $probeLists[$i]);
	($resultCB) = combine(@temp, $i);
}

#close the output file
close (outPutFile);

exit;
```

```
sub common {
	my ($probe1, $probe2, $index) = @_;
	my $motif;
	my @shareList= ();
	my @probe1unique= ();
	my @probe2unique = ();
	my @outputFiles = ();
	if ($index ==1){
		@outputFiles = ("IS_".$probe1."_".$probe2, "U_".$probe1."@".$probe2,
"U_".$probe2."@".$probe1);
	}
	else {
		@outputFiles = ($probe1."_".$probe2, "U_".$probe1."@".$probe2,
"U_".$probe2."@".$probe1);
	}

	# add the file extension to the file name
	$probe1=$probe1.".txt";
	$probe2=$probe2.".txt";

	# open the files, or exit
	unless ( open(PROBEFILE1, $probe1) ) {
		print "Cannot open file \"$probe1\"\n\n";
		exit;
	}

	unless ( open(PROBEFILE2, $probe2) ) {
		print "Cannot open file \"$probe2\"\n\n";
		exit;
	}

	# Read the data from the file, and store it
	# into the array variable @probe 1 or 2
	my @probe1 = <PROBEFILE1>;
	my @probe2 = <PROBEFILE2>;

	# Close the file - we've read all the data into @probe 1 or 2 now.
	close PROBEFILE1;
	close PROBEFILE2;

	my $j =0;
	# Now starting search for common probe number, list and unique probe list for probe1
	foreach $motif (@probe1){
		$j = 0;
		chomp $motif;
		foreach (@probe2) {
```

```
                chomp $_;
                if ($_ eq $motif) {
                        push (@shareList, $motif);
                        $j=1;
                        last;
                }
        }
        if ($j == 0) {
                push (@probe1unique, $motif);
        }
}

# Finding the unique probe list for probe2
foreach $motif (@probe2) {
        $j = 0;
        chomp $motif;
        foreach (@shareList) {
                chomp $_;
                if ($_ eq $motif) {
                        $j = 1;
                        last;
                }
        }
        if ($j == 0) {
                push (@probe2unique, $motif);
        }
}

# output the results
print "The common probe number between ", $probe1, " and ", $probe2, " is ", scalar
@shareList, "\n";
#       print "The common probe is:\n", "@shareList\n\n";
print "The unique probe number in ", $probe1, " is ", @probe1-@shareList, "\n";
#       print "The probe1 unique probe is:\n", "@probe1unique\n\n";
print "The unique probe number in ", $probe2, " is ", @probe2-@shareList, "\n";
#       print "The probe2 unique probe is:\n", "@probe2unique\n\n";

my $outputfile1 = $outputFiles[0].".txt";
my $outputfile2 = $outputFiles[1].".txt";
my $outputfile3 = $outputFiles[2].".txt";

open (outPutFile1, ">$outputfile1");
open (outPutFile2, ">$outputfile2");
open (outPutFile3, ">$outputfile3");
print outPutFile "The common probe number between ", $probe1, " and ", $probe2, " is ",
scalar @shareList, "\n";
```

```
	foreach (@shareList){
#		print outPutFile;
#		print outPutFile "\n";
		print outPutFile1;
		unless ( /$shareList[@shareList-1]/) {
			print outPutFile1 "\n";
		}
	}
#	print outPutFile "\n\n";

	print outPutFile "The unique probe number in ", $probe1, " is ", @probe1-@shareList,
"\n";
	foreach (@probe1unique){
#		print outPutFile;
#		print outPutFile "\n";
		print outPutFile2;
		unless ( /$probe1unique[@probe1unique-1]/) {
			print outPutFile2 "\n";
		}
	}
#	print outPutFile "\n\n";

	print outPutFile "The unique probe number in ", $probe2, " is ", @probe2-@shareList,
"\n";
	foreach (@probe2unique){
#		print outPutFile;
#		print outPutFile "\n";
		print outPutFile3;
		unless ( /$probe2unique[@probe2unique-1]/) {
			print outPutFile3 "\n";
		}
	}

	close (outPutFile1);
	close (outPutFile2);
	close (outPutFile3);

	return @outputFiles;
}

sub combine {
	my ($probe1, $probe2, $probe3, $index) = @_;
	my $motif;
	my @combineList= ();
	my $probet1=$probe1.".txt";
	my $probet2=$probe2.".txt";
```

```
my $probet3=$probe3.".txt";

# open the files, or exit
unless ( open(PROBEFILE1, $probet1) ) {
        print "Cannot open file \"$probet1\"\n\n";
        exit;
}

unless ( open(PROBEFILE2, $probet2) ) {
        print "Cannot open file \"$probet2\"\n\n";
        exit;
}
unless ( open(PROBEFILE3, $probet3) ) {
        print "Cannot open file \"$probet3\"\n\n";
        exit;
}

my @probe1 = <PROBEFILE1>;
my @probe2 = <PROBEFILE2>;
my @probe3 = <PROBEFILE3>;

close PROBEFILE1;
close PROBEFILE2;
close PROBEFILE3;


#combine the three probe list
@combineList = (@probe1,@probe2,@probe3);

# output the results
print "The combine probe number between the first ", $index+1, " probe lists is: ";
print scalar @combineList, "\n\n";
#print "The combine probe is:\n", "@combineList\n\n";

# save the result on file
if ($index ==1){
        $motif = 'CB_'.substr($probe1, 3,,);
}
else{
        $motif = $probe1;
}

my $outputfileB = $motif.".txt";
open (outPutFileB, ">$outputfileB");
print outPutFile "The combine probe number is ", scalar @combineList, "\n";
foreach (@combineList){
```

```
                chomp $_;
                print outPutFileB;
                unless ( /$combineList[@combineList-1]/) {
                        print outPutFileB "\n";
                }
        }

        close (outPutFileB);

        return $motif;
}

sub commonInternal {
        my ($probe1, $probe2, $index) = @_;
        my $motif;
        my @shareList= ();
        my @probe1unique= ();
        my @probe2unique = ();
        my @outputFiles = ();
        @outputFiles = ($probe1."_".$probe2, "temp1", "temp2");
        $probe1=$probe1.".txt";
        $probe2=$probe2.".txt";

        # open the files, or exit
        unless ( open(PROBEFILE1, $probe1) ) {
                print "Cannot open file \"$probe1\"\n\n";
                exit;
        }

        unless ( open(PROBEFILE2, $probe2) ) {
                print "Cannot open file \"$probe2\"\n\n";
                exit;
        }

        # Read the data from the file, and store it
        # into the array variable @probe 1 or 2
        my @probe1 = <PROBEFILE1>;
        my @probe2 = <PROBEFILE2>;

        # Close the file - we've read all the data into @probe 1 or 2 now.
        close PROBEFILE1;
        close PROBEFILE2;

        my $j =0;
        # Now starting search for common probe number, list and unique probe list for probe1
        foreach $motif (@probe1){
```

```
	$j = 0;
	chomp $motif;
	foreach (@probe2) {
		chomp $_;
		if ($_ eq $motif) {
			push (@shareList, $motif);
			$j=1;
			last;
		}
	}
	if ($j == 0) {
		push (@probe1unique, $motif);
	}
}

# Finding the unique probe list for probe2
foreach $motif (@probe2) {
	$j = 0;
	chomp $motif;
	foreach (@shareList) {
		chomp $_;
		if ($_ eq $motif) {
			$j = 1;
			last;
		}
	}
	if ($j == 0) {
		push (@probe2unique, $motif);
	}
}

# output the results

my $outputfile1 = $outputFiles[0].".txt";
my $outputfile2 = $outputFiles[1].".txt";
my $outputfile3 = $outputFiles[2].".txt";

open (outPutFile1, ">$outputfile1");
open (outPutFile2, ">$outputfile2");
open (outPutFile3, ">$outputfile3");

foreach (@shareList){
	print outPutFile1;
	unless ( /$shareList[@shareList-1]/) {
		print outPutFile1 "\n";
	}
```

```
	}

	foreach (@probe1unique){
		print outPutFile2;
		unless ( /$probe1unique[@probe1unique-1]/) {
			print outPutFile2 "\n";
		}
	}

	foreach (@probe2unique){
		print outPutFile3;
		unless ( /$probe2unique[@probe2unique-1]/) {
			print outPutFile3 "\n";
		}
	}

	close (outPutFile1);
	close (outPutFile2);
	close (outPutFile3);

	return @outputFiles;
}
```

B) No requirement for Inputting gene lists' name in command line.

```
#!/usr/bin/perl

# IS-UN-GL-printFolder.pl

# This script will intersect and unit genes among the given gene lists
# It saves the final and internal result on disk. Meantime, it also save
# the unique gene list for each input file for the pair-wise comparison
# between the input files (in the order of input)

# Requirements:
# input gene lists must be in the same fold of this script
# Use printFolder software to catalog the gene lists without the extension
# and save the list in the same fold using the default name.
# then open the text file to delete all unnecessary files, such as this script
# name and the fold name and save the modified file

use strict;
use warnings;

my $fileList = "FileLists.txt";
```

```
# open the input file or exit
unless ( open(FILE, $fileList) ) {
	print "Cannot open file \"$fileList\"\n\n";
	exit;
}

# Read the data from the input file, and store it
# into the array variable @ProbeList and close the input file
my @probeLists = <FILE>;
close (FILE);

my $fileNumber = @probeLists;

if ($fileNumber == 1) {
	print "Since there is only one file in the list, the program terminates now.";
	exit;
}

# creat the output file
my $outputfile = "Log.txt";
open (outPutFile, ">$outputfile");

# find the intersection of the first two probe lists
my (@resultCM) = common($probeLists[0], $probeLists[1], 1);

# Combine the first two probe lists by joining the intersection
# and the unique gene lists of the first two probe lists.
my ($resultCB) = combine (@resultCM, 1);

for (my $i=2; $i< $fileNumber; $i++) {
	# continue to intersect following probe lists
	(@resultCM) = common($resultCM[0], $probeLists[$i], $i);

	# continue to unit probe lists
	my (@temp) = commonInternal ($resultCB, $probeLists[$i]);
	($resultCB) = combine(@temp, $i);
}

#close the output file
close (outPutFile);

exit;

sub common {
	my ($probe1, $probe2, $index) = @_;
```

```
my $motif;
my @shareList= ();
my @probe1unique= ();
my @probe2unique = ();
my @outputFiles = ();
chomp $probe1;
chomp $probe2;
if ($index ==1){
        @outputFiles = ("IS_".$probe1."_".$probe2, "U_".$probe1."@".$probe2,
"U_".$probe2."@".$probe1);
}
else {
        @outputFiles = ($probe1."_".$probe2, "U_".$probe1."@".$probe2,
"U_".$probe2."@".$probe1);
}

# add the file extension to the file name

$probe1=$probe1.".txt";
$probe2=$probe2.".txt";

# open the files, or exit
unless ( open(PROBEFILE1, $probe1) ) {
        print "Cannot open file \"$probe1\"\n\n";
        exit;
}

unless ( open(PROBEFILE2, $probe2) ) {
        print "Cannot open file \"$probe2\"\n\n";
        exit;
}

# Read the data from the file, and store it
# into the array variable @probe 1 or 2
my @probe1 = <PROBEFILE1>;
my @probe2 = <PROBEFILE2>;

# Close the file - we've read all the data into @probe 1 or 2 now.
close PROBEFILE1;
close PROBEFILE2;

my $j =0;
# Now starting search for common probe number, list and unique probe list for probe1
foreach $motif (@probe1){
        $j = 0;
        chomp $motif;
```

```
		foreach (@probe2) {
			chomp $_;
			if ($_ eq $motif) {
				push (@shareList, $motif);
				$j=1;
				last;
			}
		}
		if ($j == 0) {
			push (@probe1unique, $motif);
		}
	}

	# Finding the unique probe list for probe2
	foreach $motif (@probe2) {
		$j = 0;
		chomp $motif;
		foreach (@shareList) {
			chomp $_;
			if ($_ eq $motif) {
				$j = 1;
				last;
			}
		}
		if ($j == 0) {
			push (@probe2unique, $motif);
		}
	}

	# output the results
	print "The common probe number between ", $probe1, " and ", $probe2, " is ", scalar
	@shareList, "\n";
#	print "The common probe is:\n", "@shareList\n\n";
	print "The unique probe number in ", $probe1, " is ", @probe1-@shareList, "\n";
#	print "The probe1 unique probe is:\n", "@probe1unique\n\n";
	print "The unique probe number in ", $probe2, " is ", @probe2-@shareList, "\n";
#	print "The probe2 unique probe is:\n", "@probe2unique\n\n";

	my $outputfile1 = $outputFiles[0].".txt";
	my $outputfile2 = $outputFiles[1].".txt";
	my $outputfile3 = $outputFiles[2].".txt";

	open (outPutFile1, ">$outputfile1");
	open (outPutFile2, ">$outputfile2");
	open (outPutFile3, ">$outputfile3");
```

```
	print outPutFile "The common probe number between ", $probe1, " and ", $probe2, " is ",
	scalar @shareList, "\n";
	foreach (@shareList){
#		print outPutFile;
#		print outPutFile "\n";
		print outPutFile1;
		unless ( /$shareList[@shareList-1]/) {
			print outPutFile1 "\n";
		}
	}
#	print outPutFile "\n\n";

	print outPutFile "The unique probe number in ", $probe1, " is ", @probe1-@shareList,
	"\n";
	foreach (@probe1unique){
#		print outPutFile;
#		print outPutFile "\n";
		print outPutFile2;
		unless ( /$probe1unique[@probe1unique-1]/) {
			print outPutFile2 "\n";
		}
	}
#	print outPutFile "\n\n";

	print outPutFile "The unique probe number in ", $probe2, " is ", @probe2-@shareList,
	"\n";
	foreach (@probe2unique){
#		print outPutFile;
#		print outPutFile "\n";
		print outPutFile3;
		unless ( /$probe2unique[@probe2unique-1]/) {
			print outPutFile3 "\n";
		}
	}

	close (outPutFile1);
	close (outPutFile2);
	close (outPutFile3);

	return @outputFiles;
}

sub combine {
	my ($probe1, $probe2, $probe3, $index) = @_;
	my $motif;
	my @combineList= ();
```

```
chomp $probe1;
chomp $probe2;
chomp $probe3;

my $probet1=$probe1.".txt";
my $probet2=$probe2.".txt";
my $probet3=$probe3.".txt";

# open the files, or exit
unless ( open(PROBEFILE1, $probet1) ) {
        print "Cannot open file \"$probet1\"\n\n";
        exit;
}

unless ( open(PROBEFILE2, $probet2) ) {
        print "Cannot open file \"$probet2\"\n\n";
        exit;
}
unless ( open(PROBEFILE3, $probet3) ) {
        print "Cannot open file \"$probet3\"\n\n";
        exit;
}

my @probe1 = <PROBEFILE1>;
my @probe2 = <PROBEFILE2>;
my @probe3 = <PROBEFILE3>;

close PROBEFILE1;
close PROBEFILE2;
close PROBEFILE3;


#combine the three probe list
@combineList = (@probe1,@probe2,@probe3);

# output the results
print "The combine probe number between the first ", $index+1, " probe lists is: ";
print scalar @combineList, "\n\n";
#print "The combine probe is:\n", "@combineList\n\n";

# save the result on file
if ($index ==1){
        $motif = 'CB_'.substr($probe1, 3,,);
}
else{
        $motif = $probe1;
```

```
	}

	my $outputfileB = $motif.".txt";
	open (outPutFileB, ">$outputfileB");
	print outPutFile "The combine probe number is ", scalar @combineList, "\n";
	foreach (@combineList){
		chomp $_;
		print outPutFileB;
		unless ( /$combineList[@combineList-1]/) {
			print outPutFileB "\n";
		}
	}

	close (outPutFileB);

	return $motif;
}

sub commonInternal {
	my ($probe1, $probe2, $index) = @_;
	my $motif;
	my @shareList= ();
	my @probe1unique= ();
	my @probe2unique = ();
	my @outputFiles = ();

	chomp $probe1;
	chomp $probe2;

	@outputFiles = ($probe1."_".$probe2, "temp1", "temp2");
	$probe1=$probe1.".txt";
	$probe2=$probe2.".txt";

	# open the files, or exit
	unless ( open(PROBEFILE1, $probe1) ) {
		print "Cannot open file \"$probe1\"\n\n";
		exit;
	}

	unless ( open(PROBEFILE2, $probe2) ) {
		print "Cannot open file \"$probe2\"\n\n";
		exit;
	}

	# Read the data from the file, and store it
	# into the array variable @probe 1 or 2
```

```
my @probe1 = <PROBEFILE1>;
my @probe2 = <PROBEFILE2>;

# Close the file - we've read all the data into @probe 1 or 2 now.
close PROBEFILE1;
close PROBEFILE2;

my $j =0;
# Now starting search for common probe number, list and unique probe list for probe1
foreach $motif (@probe1){
	$j = 0;
	chomp $motif;
	foreach (@probe2) {
		chomp $_;
		if ($_ eq $motif) {
			push (@shareList, $motif);
			$j=1;
			last;
		}
	}
	if ($j == 0) {
		push (@probe1unique, $motif);
	}
}

# Finding the unique probe list for probe2
foreach $motif (@probe2) {
	$j = 0;
	chomp $motif;
	foreach (@shareList) {
		chomp $_;
		if ($_ eq $motif) {
			$j = 1;
			last;
		}
	}
	if ($j == 0) {
		push (@probe2unique, $motif);
	}
}

# output the results

my $outputfile1 = $outputFiles[0].".txt";
my $outputfile2 = $outputFiles[1].".txt";
my $outputfile3 = $outputFiles[2].".txt";
```

```
	open (outPutFile1, ">$outputfile1");
	open (outPutFile2, ">$outputfile2");
	open (outPutFile3, ">$outputfile3");

	foreach (@shareList){
		print outPutFile1;
		unless ( /$shareList[@shareList-1]/) {
			print outPutFile1 "\n";
		}
	}

	foreach (@probe1unique){
		print outPutFile2;
		unless ( /$probe1unique[@probe1unique-1]/) {
			print outPutFile2 "\n";
		}
	}

	foreach (@probe2unique){
		print outPutFile3;
		unless ( /$probe2unique[@probe2unique-1]/) {
			print outPutFile3 "\n";
		}
	}

	close (outPutFile1);
	close (outPutFile2);
	close (outPutFile3);

	return @outputFiles;
}
```

Script III. ListFinder.pl, look for particular genes among different gene lists.

```
#!/usr/bin/perl

# ListFinder.pl
# Taking a name of gene and give the file names which contains the gene
# Requirements:
# input gene lists must be in the same fold of this script
# Use printFolder software to catalog the gene lists without the extension
# and save the list in the same fold using the default name.
# then open the text file to delete all unnecessary files, such as this script
# name and the fold name and save the modified file
```

```
use strict;
use warnings;

my $fileList = "FileLists.txt";

# The array holds the results of this program
my @results= ();

# open the input file or exit
unless ( open(FILE, $fileList) ) {
	print "Cannot open file \"$fileList\"\n\n";
	exit;
}

# Read the data from the input file, and store it
# into the array variable @FileList and close the input file
my @FileList = <FILE>;
close (FILE);

my $fileNumber = @FileList;

if ($fileNumber == 1) {
	print "Since there is only one file in the list, the program terminates now.";
	exit;
}

# Ask the user for the probe name
print "Please type the probe name:\n ";
my $motif= <STDIN>;
chomp $motif;

# creat the output file
my $outputfile = "Results.txt";
open (outPutFile, ">$outputfile");

my $temp;
my @temp;

foreach $temp (@FileList) {
	@temp=();

	chomp $temp;
	$temp = $temp.".txt";
	#open each file which is listed in the input file
	unless ( open(FILELIST, $temp) ) {
```

```
                print "Cannot open file \"$temp\"\n\n";
                exit;
        }

        # Read the data from the individual file, and store the probe lists
        # into the array variable @temp
        @temp=  <FILELIST>;
        close (FILELIST);

        # find the given probe among the probe lists
        foreach (@temp) {
                chomp $_;
                if (/$motif/) {
                        # put the individual file name into the result array
                        push (@results, $temp);
                }
        }
}

#print the results on the screen and the output file
print "The following files contains the given probe \"$motif\":\n";
print outPutFile "The following files contains the given probe \"$motif\":\n";

foreach (@results) {
        print;
        print "\n";
        print outPutFile;
        print outPutFile "\n";
}

#close the output file
close (outPutFile);

exit;
```

Vita

Lizhe Xu received his B. S. of Marine Biology from Ocean University of China in 1986, and M.S. of Marine Biochemistry from Université d'Aix-Marseille II, Marseille, France in 1989, his Ph. D. of Virology from Université Pierre et Marie Curie, Paris, France in 1994 and M.S. of computer science from University of New Orleans in 2003.