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Tool for the identification of differentially expressed genes using a user-defined threshold

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Tool for the Identification of Differentially Expressed Genes Using a User-Defined Threshold

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Submitted in partial fulfillment of the requirements for the Master of Science degree in Bioinformatics at the Rochester Institute of Technology.

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Abstract

Microarray and 2D gel experiments are used for the large scale measurement, and comparison of gene expression. Since these experiments generate large and complex amounts of data, a great challenge the researcher faces is trying to find ways to analyze this data. This paper focuses on the tool DiffExpress, which was designed to make the gene expression analysis process easier. One of the main features of DiffExpress is the user defined threshold which allows users to set their personal restriction of the expression change at which genes are differentially expressed. DiffExpress also makes use of graphs such as the Scatter Plot, Box and Whisker Plot and Volcano Plot for easier visualization of data.

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1 INTRODUCTION

1.1 Genes and Proteins

The gene, often defined as the basic unit of heredity, is a segment of DNA which codes for a protein or RNA molecule. Cells in the body contain identical genes, but in each cell, not all of these genes are expressed. At any given time, a gene in one cell may be active while in another cell this same gene may be inactive. The type of cell and the cell's environmental conditions are some factors that may determine which genes are expressed.

The protein is the product of gene expression. It is one or more polypeptides folded into a specific 3-dimensional conformation. A protein's function is dependent on its specific 3-dimensional conformation which in turn is dependent on the sequence of its amino acids. There are tens of thousands of proteins in the body, each with a specific function and structure. It is the protein (not the gene) that carries out most of the work necessary for the cell to function normally.[1]

1.2 Gene Expression: Genome to Proteome

Gene expression (also known as protein expression) is the process by which a gene is turned on, and its information is used in RNA production (RNAs other than mRNA which are a product of transcription) or protein production (transcription followed by translation). A gene is said to be expressed when its mRNA or protein are detected. When looking at gene expression, we want to identify which genes are expressed and the amount of expression.

Transcription (Figure 1) is the process by which a strand of DNA is used as a template to produce an RNA strand known as the primary transcript (pre-mRNA). RNA processing occurs and modifies the primary transcript, creating a mature mRNA. In translation the mature mRNA is used to produce a polypeptide.

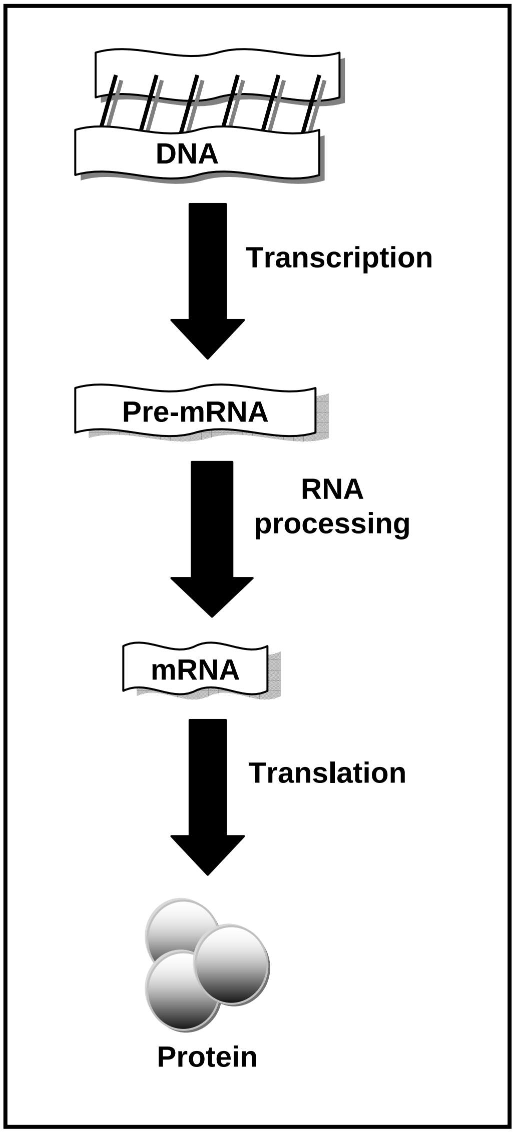


Figure 1: Steps in Gene Expression and its Regulation

Cells are able to regulate gene expression by adjusting the rate of gene transcription and translation, hence determining which genes are being expressed and the quantity. Alterations in this cell regulation mechanism can cause over or under expression of genes, causing diseases or other damage. Regulation of gene expression usually occurs at the level of transcription. This involves the binding of transcription factors to the promoters and enhancers of genes, helping to activate or inactivate these genes. Gene expression may also be regulated at the level of translation, although it does not occur as much as regulation at the level of transcription. RNA interference, riboswitches and proteins are some of the factors of gene expression regulation.[2]

1.3 mRNA and Protein Expression Levels

Gene expression analysis involves the measurement and analysis of gene expression, using mRNA expression levels and/or protein expression levels in a sample. It is easier to measure mRNA expression, but it is believed that measuring the variation in protein expression patterns is more accurate with respect to the analysis of gene expression.

Microarray analysis allows researchers to determine which genes in a sample are activated.[3] In a sample, only active genes produce mRNA, so based on the mRNA present a gene expression profile can be constructed to obtain a map (list) of the genes that are active or inactive in the sample.[3] mRNA levels can give a lot of information about the state of the cell and its gene activity. Up-regulation and down-regulation of mRNA is believed to be associated with functional changes in the cell. This is true in some cases, but it is usually the proteins that affect most of the cell's processes.

Protein expression analysis is a collection of techniques that researchers use to determine which proteins are being produced in a sample and are functional. A protein expression profile can be constructed to obtain a map (list) of all the proteins that are present in a sample at a given time.[1] Because of the many stages between mRNA expression and protein expression, there is not always a strong correlation between mRNA and protein expression. A large quantity of mRNA may be produced, yet the protein produced may not display any over-expression.[1]

1.4 DNA Microarrays

The DNA microarray is used for the simultaneous measurement and examination of thousands of mRNA expression levels (level of transcription) in a sample. The microarray is simply a microscope slide, nylon membrane or silicon chip upon which thousands of genes (DNA targets) are spotted, printed or synthesized.[4]

DNA microarray technology takes advantage of the fact that mRNA molecules hybridize to their complementary DNA sequence.[4] Target DNA is immobilized to a solid support to create the microarray. Researchers use the location of the each spot on the microarray to identify a specific gene, therefore it is imperative that these targets are immobilized to the array in an orderly fashion.[4] mRNA is isolated from samples and reverse transcribed into cDNA which is labeled and used as a probe. These probes are incubated with the microarray and bind to their complementary target DNA. By measuring the amount of mRNA adhered to each microarray spot, the expression level each gene can be obtained.

DNA microarrays are commonly used for comparing gene expression in different cell populations. For example, the use of microarrays for the comparison of healthy cells/tissues versus diseased cells/tissues in order to discover which genes may be the potential cause of the disease.

Another widely used application is in the examination of the effects of experimental conditions (e.g. drug response or time-course studies) by measuring and detecting the changes in gene expression levels of a sample under different conditions.

Two predominantly used types of microarrays are cDNA (complementary DNA) arrays and oligonucleotide arrays. cDNA microarrays produce a ratio of red (cy5) channel to green (cy3) channel for each spot. The ratio is indicative of the relative expression change for each gene under two different experimental conditions, and may be raw or log-transformed. Unlike cDNA microarrays, oligonucleotide microarrays do not produce ratios, but instead produce an absolute intensity for each spot.

1.5 cDNA Microarrays

cDNA microarrays use DNA fragments which are 500 to 1500 base pairs long, and can be used to measure the change in expression between two different samples, for example a sample taken from healthy tissue and a second sample taken from diseased tissue. Figure 2 illustrates an example of the basic method for conducting an experiment using a cDNA microarray. The fundamental steps in this method are as follows [6]:

1. DNA fragments (also known as probes) are spotted and immobilized onto the microarray (usually a glass slide).

2. mRNA from two cell samples (usually a control cell and an experimental cell) in question is extracted and reverse-transcribed into cDNA .

3. To differentiate between the two samples, the cDNA from each sample is labeled with either a red (Cy5) or green (Cy3) fluorescent dye. For example the control sample's cDNA may be labeled with Cy5 while the experimental sample's cDNA is labeled with Cy3, or vice versa.

4. The two pools of fluorescently labeled cDNA are combined in equal amounts and applied to the microarray.

5. The labeled cDNA from each sample compete to hybridize to its complementary DNA fragment on the microarray. The sample that contains more of an mRNA transcript for a specific gene will have a better chance of hybridizing to that gene.

6. The microarray is washed to eliminate any cDNA that was not hybridized.

7. A digital image (e.g. Figure 4) is created of the red and green signals and computer software is used to calculate the red to green fluorescence ratio for each spot. The signals from a spot indicate the relative abundance of the corresponding mRNA in the two cell populations. For example, for a given gene, if the control sample was labeled with Cy3 and it contains more mRNA transcript than the experimental sample labeled with Cy5, then the probe (spot) on the array will be green. If, on the other hand, the experimental sample's mRNA content exceeds that of the control sample's mRNA content, then the probe will fluoresce red. If both the samples have the same amount of mRNA (hybridize equally to the target DNA), the dyes cancel each other

and the probe will fluoresce yellow. If nothing has hybridized to the spot, then there will be no signal and the probe will be black.[7]

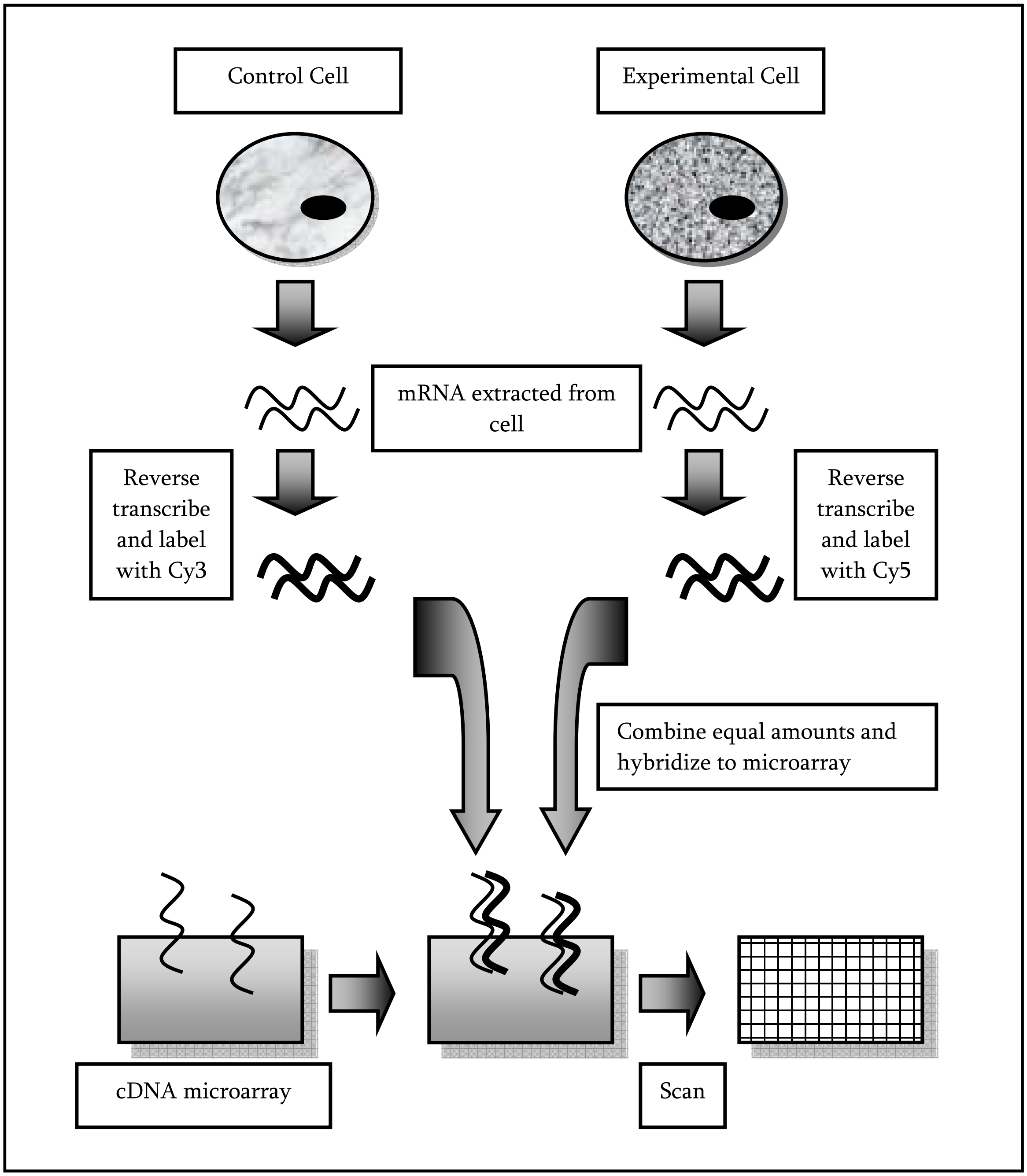

Control Cell
Experimental Cell
mRNA extracted from cell
Reverse transcribe and label with Cy3
Reverse transcribe and label with Cy5
Combine equal amounts and hybridize to microarray
cDNA microarray
Scan

Figure 2: cDNA Microarray Experiment Example

	Experiment 1	Experiment 2	Experiment 3
Gene A	Red	Green	Red
Gene B	Black	Red	Yellow
Gene C	Yellow	Black	Green

Figure 3: Simplified example of some cDNA signals in a digital image.
Red indicates that Cy5 > Cy3, Green indicates that Cy3 > Cy5, Yellow indicates that Cy5 = Cy3 and Black indicates that no hybridization of the probe to the target occurred.

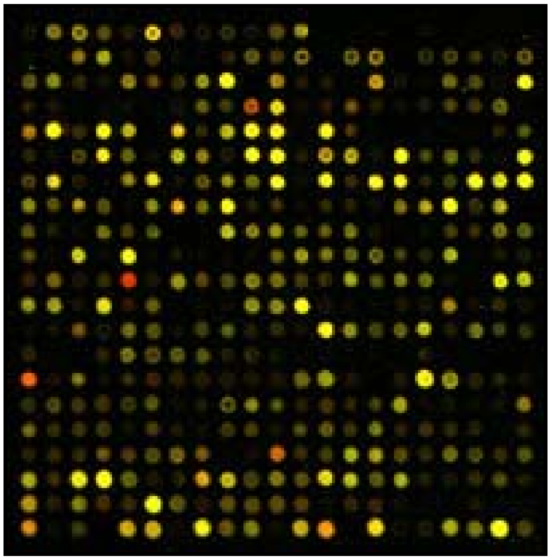


Figure 4: Actual Representation of the colors of a microarray
(http://www.liv.ac.uk/researchintelligence/issue23/geneactivity.html)

1.6 Oligonucleotide Arrays

Unlike cDNA microarrays, oligonucleotide arrays use short 25 base-pair DNA fragments as their probes and only one sample is hybridized to the array. This type of array can be used to measure the RNA content in a sample, or it can be used to compare two different samples (these samples must be hybridized on separate arrays). Figure 5 illustrates an example of a typical oligonucleotide array experiment. The basic steps in this method are as follows [6]:

1. UV masks and photo-activated chemistry are used in cooperation to immobilize the DNA oligonucleotides on the microarray.

2. mRNA is extracted from the sample and reversed transcribed into cDNA.

3. cDNA is transcribed into cRNA and labeled (usually with biotin).

4. The labeled cRNA is fragmented and hybridized to the oligonucleotide array.

5. The array is washed to remove any excess cRNA, and stained to visualize the amount of hybridization.

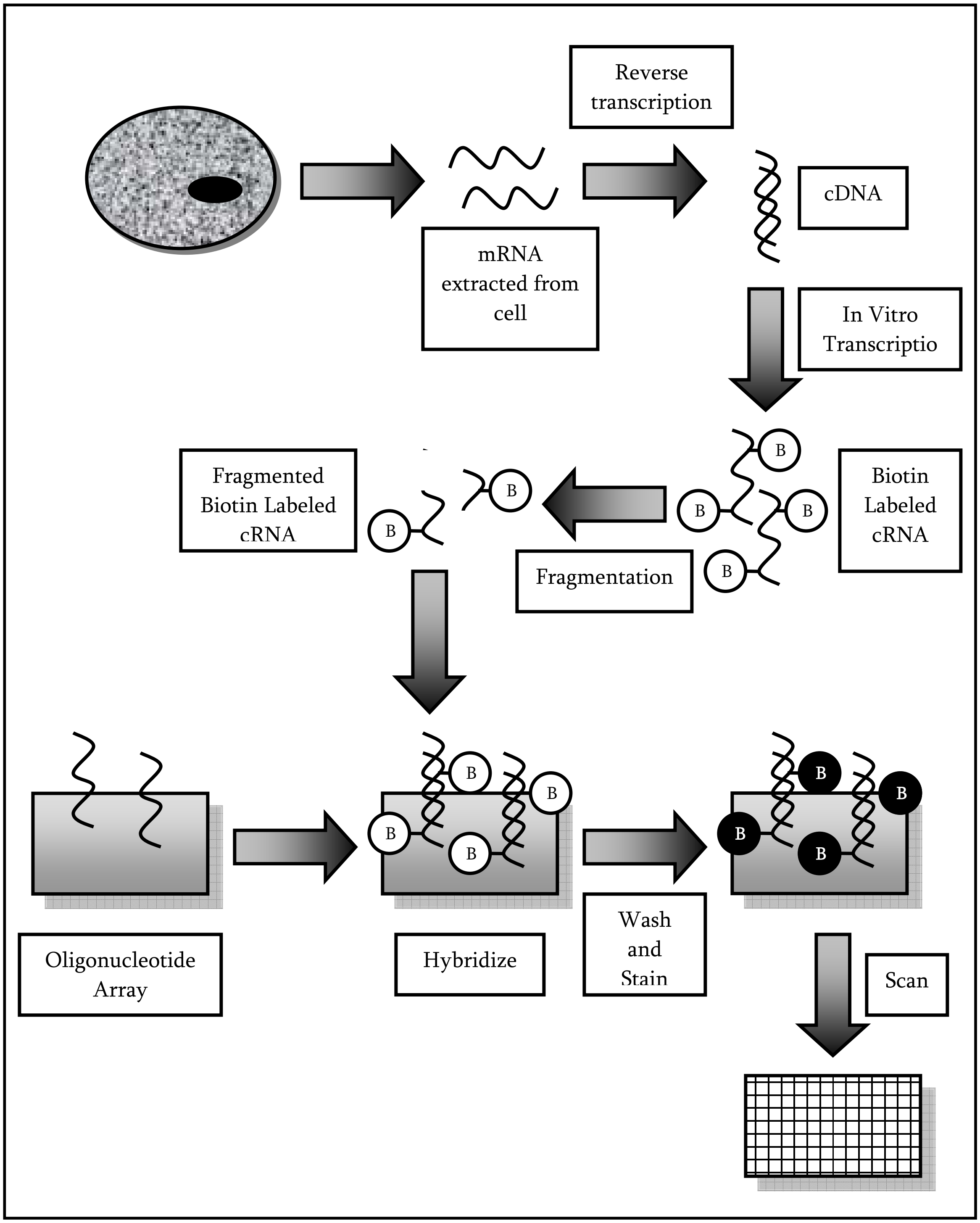


Figure 5: Oligonucleotide Array Experiment Example

1.7 2D Gels and Mass Spectrometry

Like DNA microarrays, 2D gels and mass spectrometry are high-throughput techniques, but unlike DNA microarrays, 2D gels and mass spectrometry are associated with proteins and not mRNA. Measuring protein expression is different from mRNA expression measurement. Global changes in protein expression can be detected by the use of 2D gels and mass spectrometry. 2D gels are used to separate proteins in a sample while mass spectrometry is used for the large-scale protein identification and the measurement of expression of the proteins in the sample (and find differences in protein expression between two of more samples).[1] In order to find proteins that are differentially expressed, data from different samples or from one sample under different conditions are compared. Given two or more samples, comparing their mass spectra can provide information about variations in the protein expression level patterns between them. There is software available that provides features for spot detection, spot quantification and comparison of multiple gels.

1.8 Expression Data Analysis

Table 1 shows a very basic layout of an expression data matrix. An expression matrix is a table of expression levels in the case of oligonucleotide microarray data, or expression level ratios in the case of cDNA microarray data. Actual results usually contain thousands of gene/ spot id data. The columns represent samples and experiments while the rows represent genes corresponding to an mRNA or protein. Each cell (bordered by the bold shaded squares in Table 1) gives the expression level (oligonucleotide microarrays) or ratio of expression levels between two samples (cDNA microarrays) of a gene or protein (based on either mRNA or protein expression levels) to its corresponding sample. For example, Expression Level C2 would either be the mRNA level or protein level of gene C in sample 2.

		Samples		
		1	2	3
Gene	A	Expression Level A1	Expression Level A2	Expression Level A3
	B	Expression Level B1	Expression Level B2	Expression Level B3
	C	Expression Level C1	Expression Level C2	Expression Level C3

Table 1: Simplified Example of an Expression Data Matrix

If we want to determine how similar or dissimilar the expressions of two genes are, we compare the rows (expression profiles). Co-regulation of the genes can also be determined, i.e. if gene A is up-regulated (or down-regulated), are genes B or C also up-regulated (or down-regulated).

If we want to determine if samples are related or unrelated, we compare the columns. If they are unrelated, genes that cause this dissimilarity can be found through the comparison. Alterations in gene expression can occur from drugs, changes in the environment, etc. Researchers may want to examine differences in expression between sample 1 and 2 given that (determine which genes change significantly across the samples):

1. Sample 1 and sample 2 are taken from the same sample under different experimental conditions (drug response experiment e.g. drug treatment, radiation). For example in drug response experiments, sample 1 may be a cell treated with specific drug while sample 2 may be the same cell treated with a different drug (Figure 6). One drug may cause some changes in the expression of certain genes of the cell while the other drug may alter the expression of completely different genes. Comparison of these two samples will then provide insight into which drug could possibly be successful in treating a specific condition.

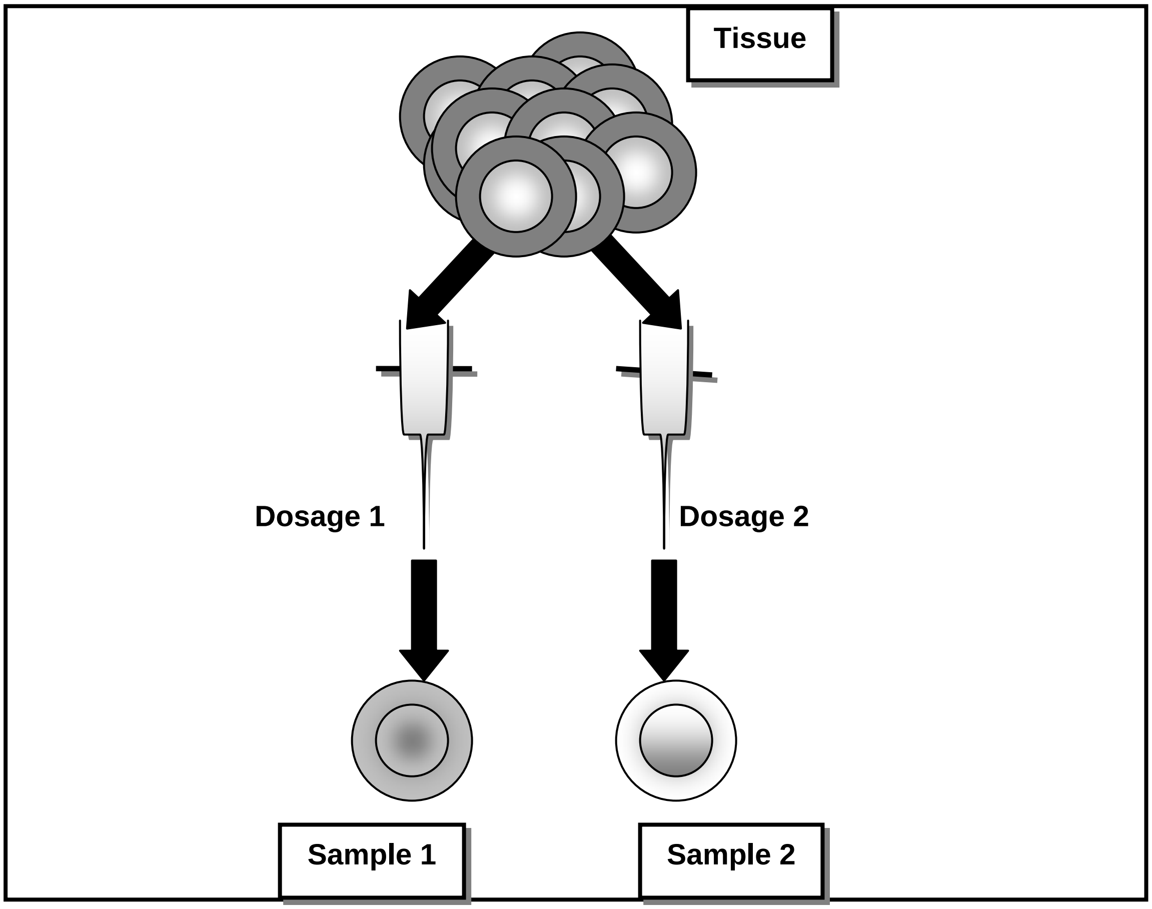


Figure 6: Examining the effects of experimental conditions
Sample 1 is taken from the tissue treated with drug 1, while Sample 2 is taken from the tissue treated with drug 2.

2. Sample 1 is a normal cell while sample 2 is a diseased cell. A simple example of this is sample 1 is a cell taken from normal tissue while sample 2 is a cell taken from the tissue of a tumor (Figure 7). Comparing these two samples, researchers may find genes/proteins that show a significantly different level of expression in the tumor cell. Discovering these significant genes or proteins can assist in providing a clue in determining disease susceptibility and will ultimately be useful in the diagnosis and treatment of various diseases.

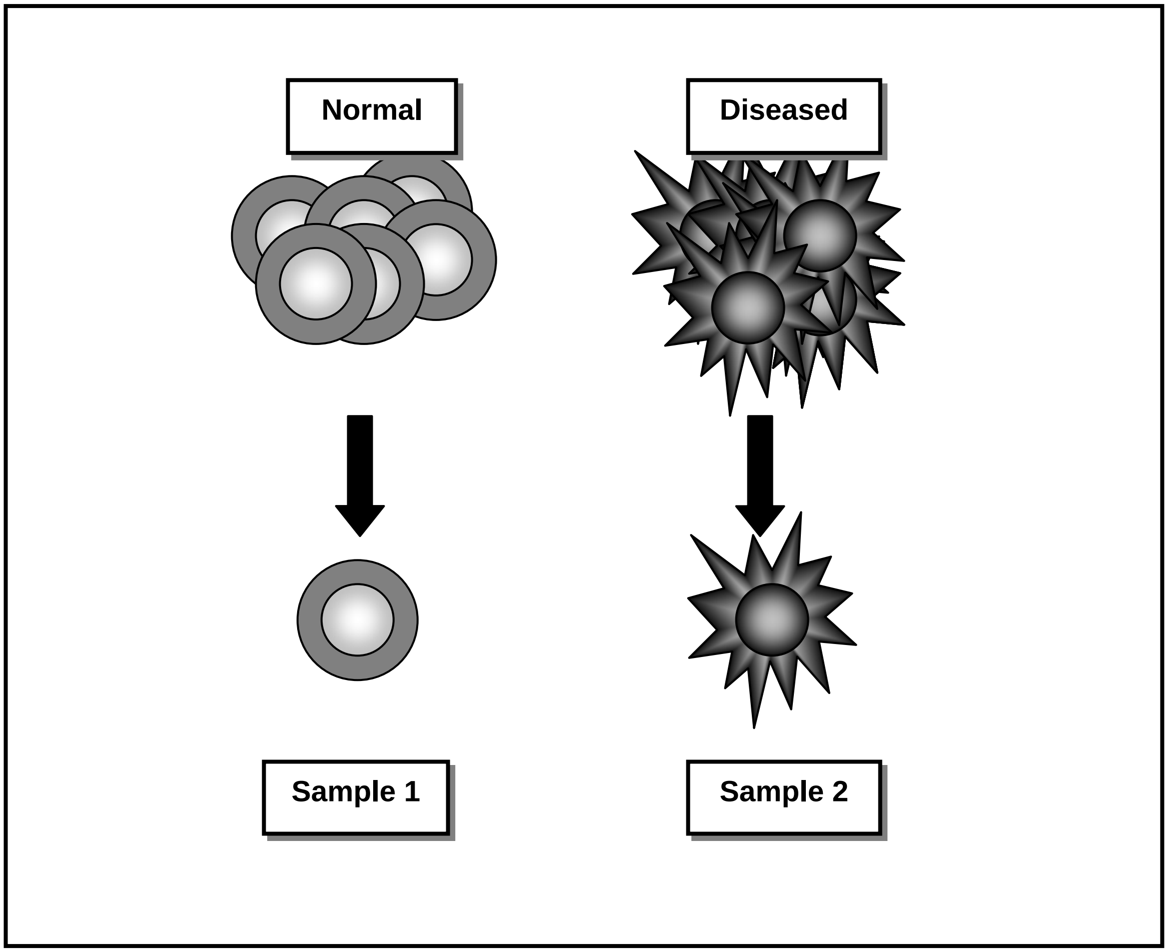


Figure 7: Comparing different cell populations
Sample 1 is taken from normal tissue while Sample 2 is taken from diseased tissue

3. Sample 1 and Sample 2 are taken from a cell or tissue at different times. An experiment of this type is known as a time-course experiment and is basically the monitoring of a cell/ tissue's gene expression over a time period.

2 RATIONALE

The introduction of microarray technology has been a significant asset to scientific research by saving researchers time and effort, but these researchers are faced with the challenge of the analysis of the microarray expression data. One challenge is finding and making sense of the gene expression patterns which result from differing experiments. Detecting considerable increases or decreases in mRNA expression between experiments may lead to the discovery of significant genes causing a condition. With such a plethora of data generated, it is difficult and time-consuming for researchers to compare and analyze microarray data by hand. To reduce the amount of data (and discover significant genes), restrictions are set on the data.

2D gels can be difficult to analyze because of the many possible sources of error that become involved (e.g., over staining, differences in migration in either or both dimensions between different experiments, etc.). Examining protein spots and detecting the changes in protein expression from the massive amount of protein expression data can be – as with the mRNA expression data analysis – lengthy and tedious when done manually. Without software, researchers have to depend on setting the gels to be compared next to each other and matching up protein spots to visually detect expression differences.[5]

In both mRNA and protein expression analysis, one of the main challenges is examining large amounts of data and identifying patterns (e.g. similarly expressed mRNA/proteins) to determine differentially expressed genes. This is where bioinformatics comes into play. Many computational tools have been developed to assist researchers in areas such as normalization, filtering, clustering and identification of differentially expressed genes among many other features.

This research focuses on the creation of a tool, DiffExpress, which takes as input a data matrix and provides as output, a list of variables (rows) that change by a specified threshold across two columns. More specifically, the tool filters microarray expression data or 2D gel data and lists mRNA/protein that change by a given user-defined threshold between samples. The tool also supplies a variety of graphs such as the box plot and scatter plot, enabling easier visualization of the results. Researchers can take the results, find patterns, co-regulation, etc. and use this information to find which genes are associated with certain responses (e.g. disease, drug effects, etc.). Identified genes may also be used in further statistical analysis (beyond the scope of this research).

3 METHODS

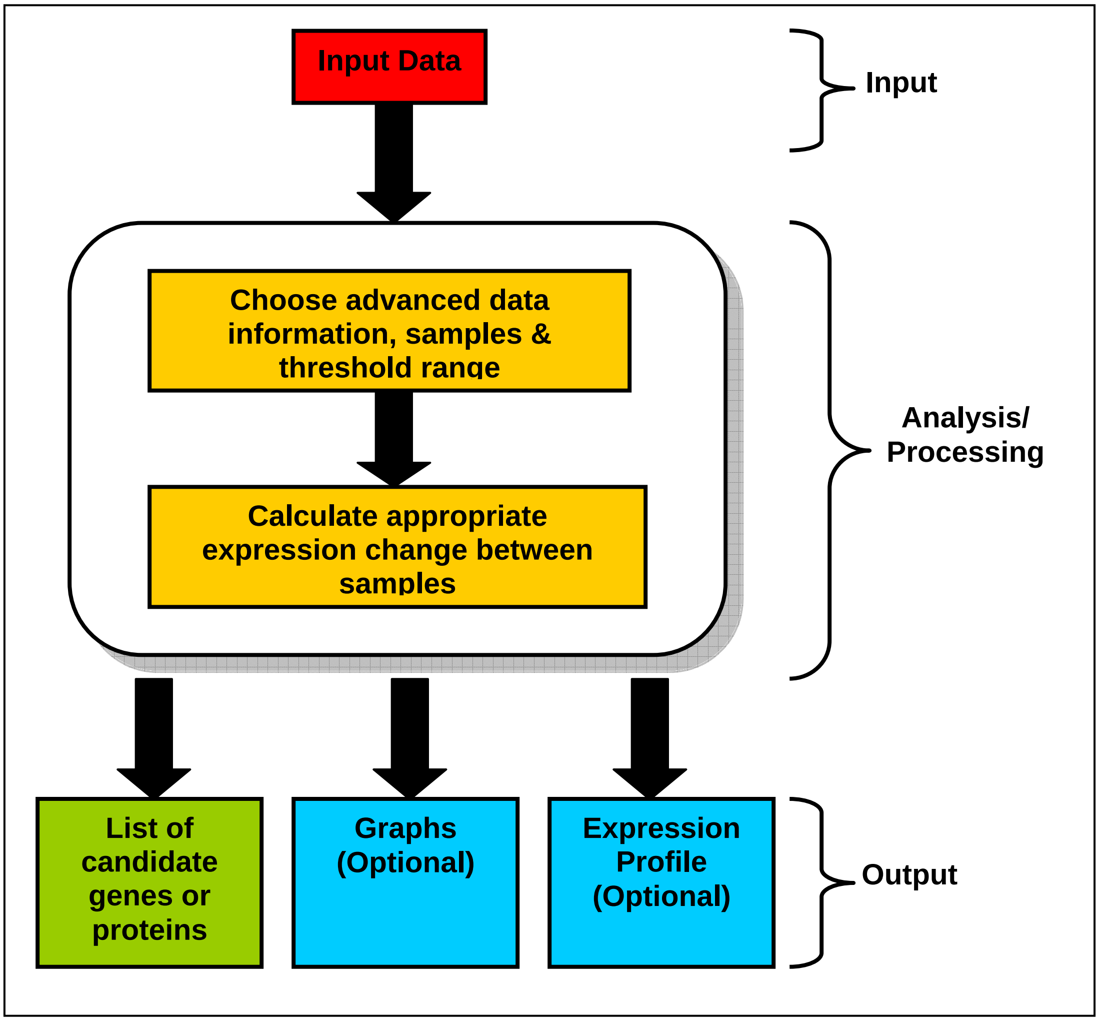

Input Data
Input
Choose advanced data information, samples & threshold range
Calculate appropriate expression change between samples
Analysis/ Processing
List of candidate genes or proteins
Graphs (Optional)
Expression Profile (Optional)
Output

Figure 8: Workflow of DiffExpress

3.1 Input

3.1.1 Input Data

Input for the tool is gene expression data in the form of an expression matrix (Table 1). Each column represents the expression levels from a single experiment, while each row represents the expression of a gene across all samples or experiments (gene expression vector).

3.1.2 Missing Values

Microarray data may often include missing values. There are a variety of reasons for missing values in the data, the majority of these reasons being errors during the experiment:

1. Hybridization may be poor or there may be spotting problems.
2. There may be printing problems (e.g. corruption of images or inadequate resolution).
3. Fabrication errors on slides, such as dust, fingerprints or scratches may also cause issues.
4. The spot may simply be empty resulting in an intensity equal to zero.
5. If the background intensity exceeds the spot intensity there will be low expression.
6. A researcher may have noticed suspicious values in the data and removed these values.

The problem with missing values is that complete data expression matrices are necessary for many types of data analysis (e.g. hierarchical clustering and classification algorithms). Since it is time consuming and expensive to repeat the whole experiment, researchers have come up with solutions to address the problem of these missing values. Imputation, which is the estimation of the missing values, is one of these solutions. Three of the most widely used imputation methods are:

1. Replacing the missing values with zeros.

2. Calculating the respective row or column averages and using these averages as replacement.

3. k-nearest neighbor: k genes are chosen that are most similar to the gene with the missing value. The missing value is then estimated as the weighted mean of the neighbors.

Another popular but less sophisticated solution for missing values involves removing any rows and columns of the matrix that contain a significant amount of missing values.

3.1.3 Input Data Limitations

As is the case with many tools, there is a specified format (Figure 9) for the input data which may be a limitation for some users:

1. The input file should be a text (.txt) file.

2. The rows in the expression data matrix should represent genes or spot ids.

3. The columns in the expression data matrix should represent samples or experiments.

4. Expression data matrix entries are real numbers.

5. Expression data matrix should be tab-delimited.

6. Newline or carriage return (\n) should be used between the rows. The data file should also end with a newline.

7. Any descriptions or comments should be at the beginning of the file with each line beginning with an ampersand and a space ("& ").

8. The first row after any comments should represent the number of variables. This tool only accepts a maximum of four variables and a minimum of two variables. These two variables are the genes or spotids and the samples. Dosage and time are examples of additional variables (i.e. third and fourth variables).

9. The row following the number of variables should display the sample ids.

10. If there is a third variable, the next row should display this third variable's ids.

11. If there is a fourth variable, the next row should display the fourth variable's ids.

12. All rows after the above initial rows correspond to the gene or spot ids (the first column of each row) and the expression levels (the remaining columns of each row) of the actual expression data matrix.

13. To conduct any statistical tests, the data should be normally distributed. If data that is not normally distributed is entered, they will still get results but these results may be inaccurate.

14. The tool does not support preprocessing (i.e. normalization, missing values imputation, etc) of data, so any preprocessing of the data should be done before it is entered into the tool.

15. If the data is normalized then it should be normalized using $\log_2$ transformation ($\log_{10}$ transformation is acceptable also, as long as it is used consistently).

16. There should be no missing values in the data. Any imputation or elimination of missing values should be executed before the data is entered into the tool.

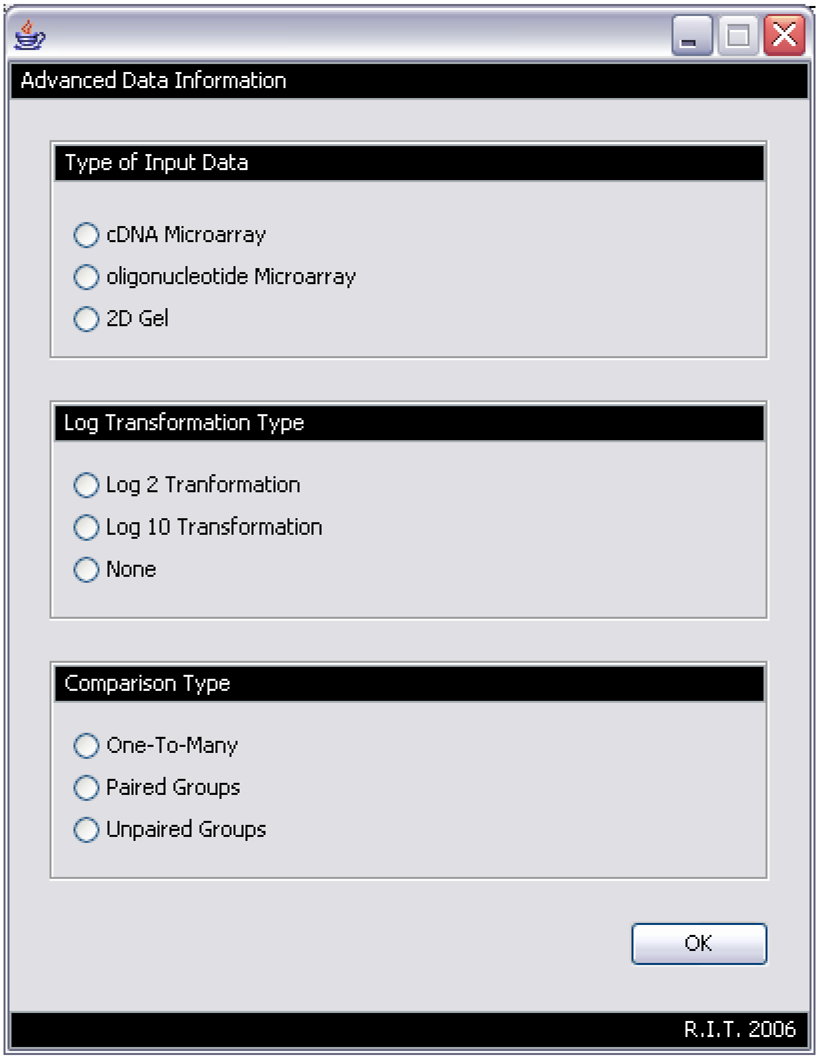
17. Regarding the naming convention for the gene/spot ids and the sample ids, there should be no spaces, i.e. each id should be a single string. For example a dosage id should be 10mg and not 10 mg.

```
& Example of Expression Data Format

& This is a comment line

3

spo_0X   spo_0.5X spo_2X   spo_5X   spo_7X   spo_9X   spo_11.5X spo_earlyX   spo_midX

10mg     17mg     13mg     5mg      15mg     16mg     7mg      18mg     19mg

YAL001C           -0.00    -0.40    -0.14    -0.26    -0.05    -0.16    0.03     0.31     0.07

YAL002W           0.08     0.37     0.15     -0.33    -0.99    -0.60    0.05     0.02     -0.58

YAL003W           0.27     -1.95    -1.28    -1.55    -2.03    -0.97    -1.00    -2.19    -3.09
```

Figure 9: Example of the format for Input Data

3.2 Analysis

3.2.1 Advanced Data Information



Advanced Data Information

Type of Input Data

○ cDNA Microarray
○ oligonucleotide Microarray
○ 2D Gel

Log Transformation Type

○ Log 2 Tranformation
○ Log 10 Transformation
○ None

Comparison Type

○ One-To-Many
○ Paired Groups
○ Unpaired Groups

Figure 10: DiffExpress - Advanced Data Information Options Window

Before certain tasks (for example graphing) can be performed in DiffExpress, the type of input data, log transformation and comparison must be selected (Figure 10). These selections allow the tool to know what type of data is being processed.

3.2.1.1 Input Data Type

DiffExpress can process cDNA microarray, oligonucleotide microarray or 2D gel data. Since oligonucleotide and 2D gel data use expression levels, while cDNA data uses a ratio of expression levels, processing between these input data types is different. Different graphs are created and the expression change is calculated differently.

3.2.1.2 Log Transformation Type

DiffExpress allows the user to enter log transformed data or raw data. There are two ways that the data can be log transformed: $\log_2$ transformation and $\log_{10}$ transformation. The calculations of the expression change will vary depending on which log transformation is used. To give the user less limitation on the type of data that can be entered, options for either $\log_2$ or $\log_{10}$ transformed data were given. This way, if the user's data is $\log_{10}$ transformed they will not have to convert it to data that is $\log_2$ transformed, and vice versa.

3.2.1.3 Data Comparison

Before any calculation of the expression change can occur, the user must select a type of data comparison i.e. which samples are to be compared. cDNA data is usually in the form of ratios representing expression changes, as a result there is no data comparison type to be selected before expression changes are calculated. Since oligonucleotide or 2D gel data consists of expression levels, DiffExpress provides the user with three choices for the selection of samples before the calculation of the expression change:

1. One-to-many comparison: The user selects a single sample as the baseline sample and one or more samples as the experimental samples (Figure 11 and Figure 12). The

baseline sample may be a gene knockout mouse sample, while the experimental samples may be wild type mice samples.

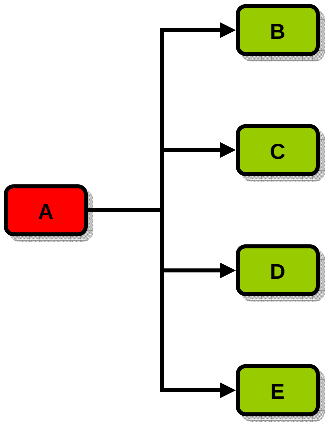
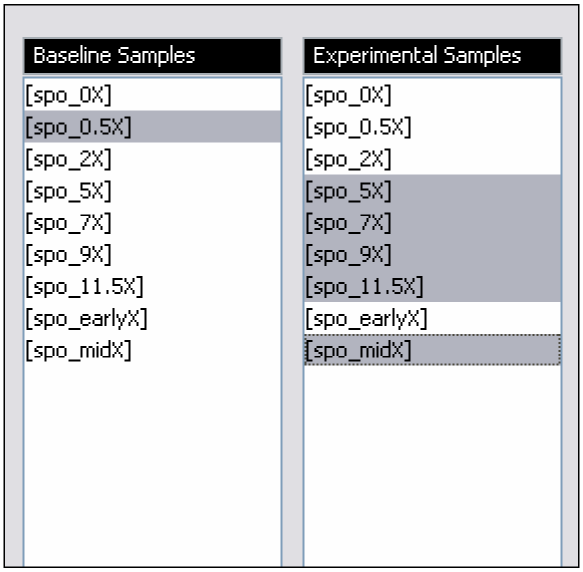


Figure 11: One-to-many comparison
Each of the samples B, C, D and E (experimental samples) will be compared to sample A (baseline sample).



Baseline Samples

[spo_0X]
[spo_0.5X]
[spo_2X]
[spo_5X]
[spo_7X]
[spo_9X]
[spo_11.5X]
[spo_earlyX]
[spo_midX]

Experimental Samples

[spo_0X]
[spo_0.5X]
[spo_2X]
[spo_5X]
[spo_7X]
[spo_9X]
[spo_11.5X]
[spo_earlyX]
[spo_midX]

Figure 12: DiffExpress - One-to-many comparison

2. Paired Groups comparison: The user selects one or more samples as the baseline group, and their corresponding sample pairs as the experimental group (Figure 13 and Figure 14). As an example, the baseline group may consist of samples from patients before a treatment while the experimental group may consist of samples from the same patients after a treatment has been administered. Another example is that where patients are paired based on some factor (e.g. age, or weight) and one member of the pair is given a drug treatment while the other member is given a placebo. Those patients who are given the drug treatment may be assigned to the baseline group while those patients who are given the placebo treatment may be assigned to the experimental group (or vice versa).

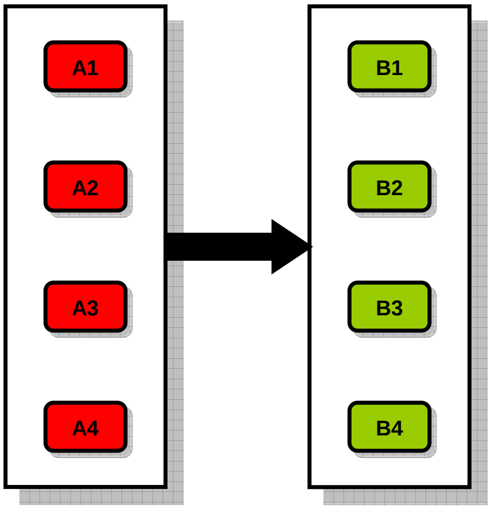
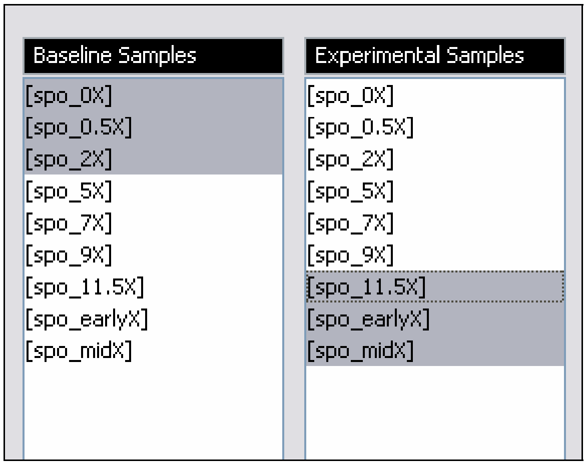


Figure 13: Paired Groups Comparison
The experimental group comprised of the B samples will be compared to the baseline group comprised of the A samples.



Baseline Samples	Experimental Samples
[spo_0X]	[spo_0X]
[spo_0.5X]	[spo_0.5X]
[spo_2X]	[spo_2X]
[spo_5X]	[spo_5X]
[spo_7X]	[spo_7X]
[spo_9X]	[spo_9X]
[spo_11.5X]	[spo_11.5X]
[spo_earlyX]	[spo_earlyX]
[spo_midX]	[spo_midX]

Figure 14: DiffExpress – Paired Groups Comparison

3. Unpaired Groups comparison: The user selects one or more samples as the baseline group and one or more samples as the experimental group (Figure 15 and Figure 16). These two groups should be unrelated, for example comparing the reaction in female patients (first group) and male patients (second group) who are given a drug.

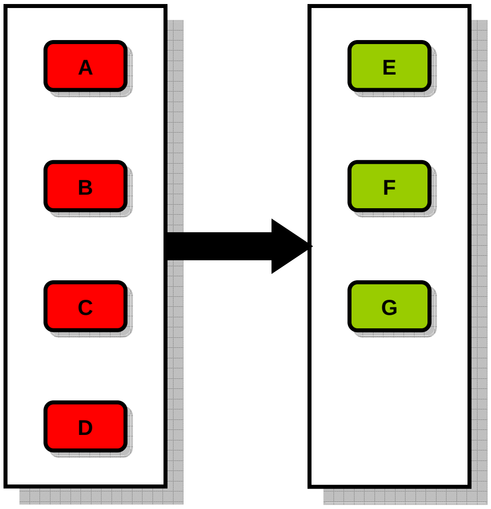
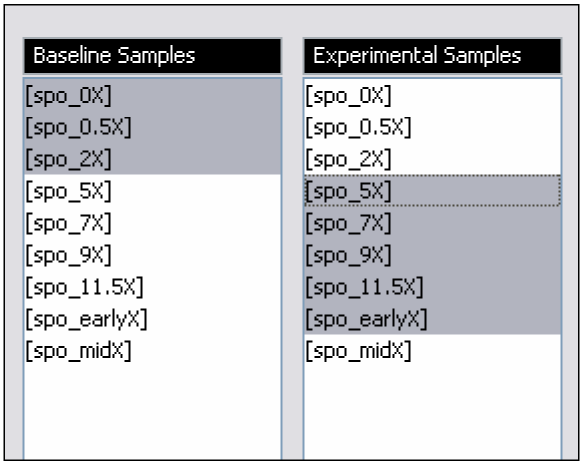


Figure 15: Unpaired Groups Comparison
The experimental group (Samples E, F and G) will be compared to the baseline group (Samples A, B, C and D).

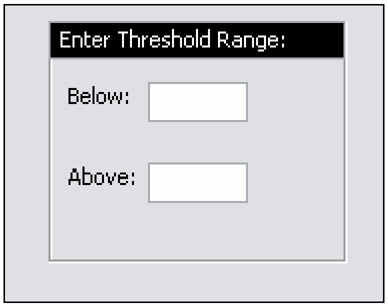


Baseline Samples	Experimental Samples
[spo_0X]	[spo_0X]
[spo_0.5X]	[spo_0.5X]
[spo_2X]	[spo_2X]
[spo_5X]	[spo_5X]
[spo_7X]	[spo_7X]
[spo_9X]	[spo_9X]
[spo_11.5X]	[spo_11.5X]
[spo_earlyX]	[spo_earlyX]
[spo_midX]	[spo_midX]

Figure 16: DiffExpress - Unpaired Groups Comparison

3.2.2 User Defined Threshold

DiffExpress gives the user a choice of entering an *above threshold*, a *below threshold*, or both (Figure 17). This choice provides the user with the option of focusing on the direction and magnitude of change in which they are interested, i.e. up-regulated genes or down-regulated genes, or both. The appropriate expression change is calculated and depending on the threshold range entered, the tool lists any gene whose calculated expression change is greater than or equal to the *above threshold* entered or is less than or equal to the *below threshold* entered.



Enter Threshold Range:

Below:

Above:

Figure 17: DiffExpress - Threshold Range

The user-defined threshold is advantageous because the user gets to set their personal restriction of what threshold they consider the genes to be differentially expressed. For example, if a threshold of two is entered, and there are too many resulting genes, the user may increase the threshold to a higher number to make the filtering process more sensitive.

3.2.3 Expression Change

Expression change reveals how much a gene's expression level varies across two different experimental conditions. If oligonucleotide microarrays are being used, the expression change has to be calculated. On the other hand, if cDNA microarrays are the microarray of choice the data is already represented as a ratio. The user may opt to convert this predefined ratio into another form of expression change (for example intensity ratio to log$_2$ ratio). In this case, one or more samples may be selected, and the appropriate expression change option is chosen.

DiffExpress provides three ways to calculate expression change: the intensity ratio, log ratio and fold change (Figure 18). The two latter measures are derived from the intensity ratio and are usually the preferred choices of measurement because they have more symmetrical qualities.[8]

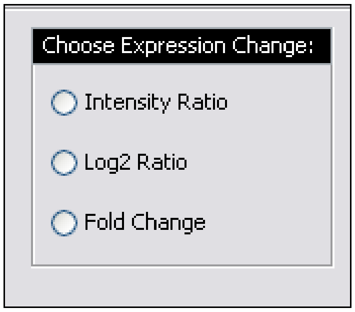


Figure 18: DiffExpress - Expression Change Option

The *intensity ratio* is the easiest way to calculate the expression change. For cDNA microarrays (two-color data) the calculation of the intensity ratio is as follows:

$$Intensity\ Ratio_{cDNA} = \left(\frac{Cy3'}{Cy5'} \right) \tag{1}$$

For oligonucleotide microarray data the intensity ratio is calculated by the formula (where expression level A is the expression level of a gene from the experimental (treatment) sample and expression level B is the expression level of the same gene from the baseline (control or reference) sample):

$$Intensity\ Ratio_{Oligonucleotide} = \left(\frac{Expression\ level\ A}{Expression\ level\ B} \right) \tag{2}$$

The values for up-regulated genes range from one to infinity while values for down-regulated genes range from zero to one. Because of this asymmetrical distribution, intensity ratios may be problematic in statistical data. In order to utilize many statistical methods, one of the assumptions is that the data is normally distributed (symmetric). Transformation – a technique using functions or formulae to derive a new variable from another variable – can be applied to make a distribution more normal. This technique is particularly useful when a ratio is involved because ratios tend to be skewed. A form of transformation, log transformation (which is usually taken in base 2), is common in DNA microarray experiments. A log transformation converts the original variable into a new variable called the *log ratio*. The log ratio (Equation 3) is the change in expression level between two samples (intensity ratio) expressed as a log ratio.

$$Log\ Ratio = \log_2(Intensity\ ratio) \tag{3}$$

The values for both up-regulated and down regulated genes range from negative infinity to positive infinity, while the value for an unchanged expression is zero.

The *fold change* is calculated in the same way as the intensity ratio (Equation 1 for cDNA data, and Equation 2 for oligonucleotide data) if expression level A is greater than or equal to expression level B. If expression level A is less than expression level B the fold change is calculated by negating the inverse of the intensity ratio (Equation 4).

$$Fold\ Change = \begin{cases} Intensity ratio, & if\ Intensity ratio \geq 1 \\ -\left(\dfrac{1}{Intensity\ ratio}\right), & if\ Intensity ratio < 1 \end{cases} \tag{4}$$

Much like the log ratio, the values for both up-regulated and down regulated genes range from negative infinity to positive infinity, but unlike the log ratio, the value for an unchanged expression is one.

	Expression Level A	Expression Level B	Intensity Ratio	Log Ratio	Fold Change
Gene A	100	50	2	1	2
Gene B	5	10	0.5	-1	-2
Gene C	150	150	1	0	1
Gene D	200	1	200	7.6439	200

Table 2: Expression Change Calculation - raw oligonucleotide microarray data

	cDNA Spot Value	Intensity Ratio	Log Ratio	Fold Change
Gene A	2	2	1	2
Gene B	0.5	0.5	-1	-2
Gene C	1	1	0	1
Gene D	200	200	7.6439	200

Table 3: Expression Change Calculation - raw cDNA microarray data

Note that the above calculations in Table 1Table 2 and Table 3 refer to raw microarray data that has not been transformed. If the expression levels from the oligonucleotide microarray data are normalized using a $\log_2$ transformation, this needs to be taken into account by un-logging the expression levels (i.e. $2^{(Expression\,level)}$) before any calculation (intensity ratio, log ratio or fold change) can be made. If the ratios from the cDNA have been $\log_2$ transformed, each spot will correspond to the log ratio, therefore in order to calculate the intensity ratio, the log ratio must be unlogged. The fold change will be calculated as usual, using the intensity ratio calculated by unlogging the log ratio (as shown in Table 4 and Table 5).

	Expression Level A	Expression Level B	Intensity Ratio	Log Ratio	Fold Change
Gene A	6.6439	5.6439	2	1	2
Gene B	2.3219	3.3219	0.5	-1	-2
Gene C	7.2288	7.2288	1	0	1
Gene D	7.6439	0	200	7.6439	200

Table 4: Expression Change Calculation - log transformed oligonucleotide microarray data

	cDNA Spot Value	Intensity Ratio	Log Ratio	Fold Change
Gene A	1	2	1	2
Gene B	-1	0.5	-1	-2
Gene C	0	1	0	1
Gene D	7.6439	200	7.6439	200

Table 5: Expression Change Calculation - log transformed cDNA microarray data

Table 6 displays a summary of the possible ranges for the three types of expression change and the type of regulation to be expected. If the intensity ratio is greater than 1, up-regulation has occurred. If the intensity ratio is equal to 1 then there is no change between the two samples. If the intensity ratio is less than 1, down-regulation has occurred.

If the log ratio is positive, up-regulation has occurred. If the log ratio is equal to 0, then there is no change between the two samples. If the log ratio is negative, down-regulation has occurred.

If the fold change is positive, up-regulation has occurred. If the fold change is equal to 1 then there is no change between the two samples. If the fold change is negative, down-regulation has occurred.

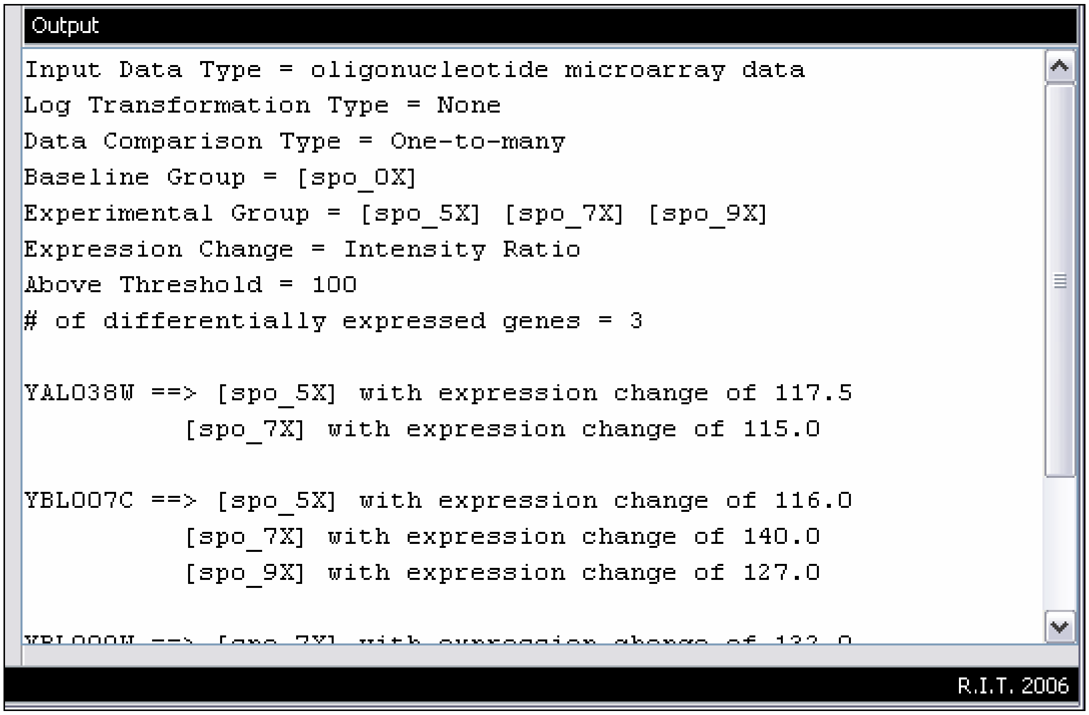
Intensity Ratio	Log Ratio	Fold Change	Regulation
>1	+	+	Up-regulation
1	0	1	No Change
<1	-	-	Down-regulation

Table 6: Summary of Expression Change and Types of Regulation

3.3 Output

3.3.1 Expression Change Detection

The task of identifying differentially expressed genes involves comparing samples and discovering differences in which genes are expressed and the level of expression of a given gene. Expression change detection is one of the simplest approaches used to find differentially expressed genes and is used when the researcher simply wants to know which genes have been over-expressed or under-expressed in the experiment. In this method the specified expression change (intensity ratio, $\log_2$ ratio or fold change) is calculated, and the user defined threshold is used as a cutoff. Any gene whose calculated expression change falls above the user-defined *above threshold* and/or below the user-defined *below threshold* is added to the list of candidate differentially expressed genes or proteins list (Figure 19).



```
Input Data Type = oligonucleotide microarray data
Log Transformation Type = None
Data Comparison Type = One-to-many
Baseline Group = [spo_0X]
Experimental Group = [spo_5X] [spo_7X] [spo_9X]
Expression Change = Intensity Ratio
Above Threshold = 100
# of differentially expressed genes = 3

YAL038W ==> [spo_5X] with expression change of 117.5
          [spo_7X] with expression change of 115.0

YBL007C ==> [spo_5X] with expression change of 116.0
          [spo_7X] with expression change of 140.0
          [spo_9X] with expression change of 127.0

YBL009W ==> [spo_7X] with expression change of 132.0
```

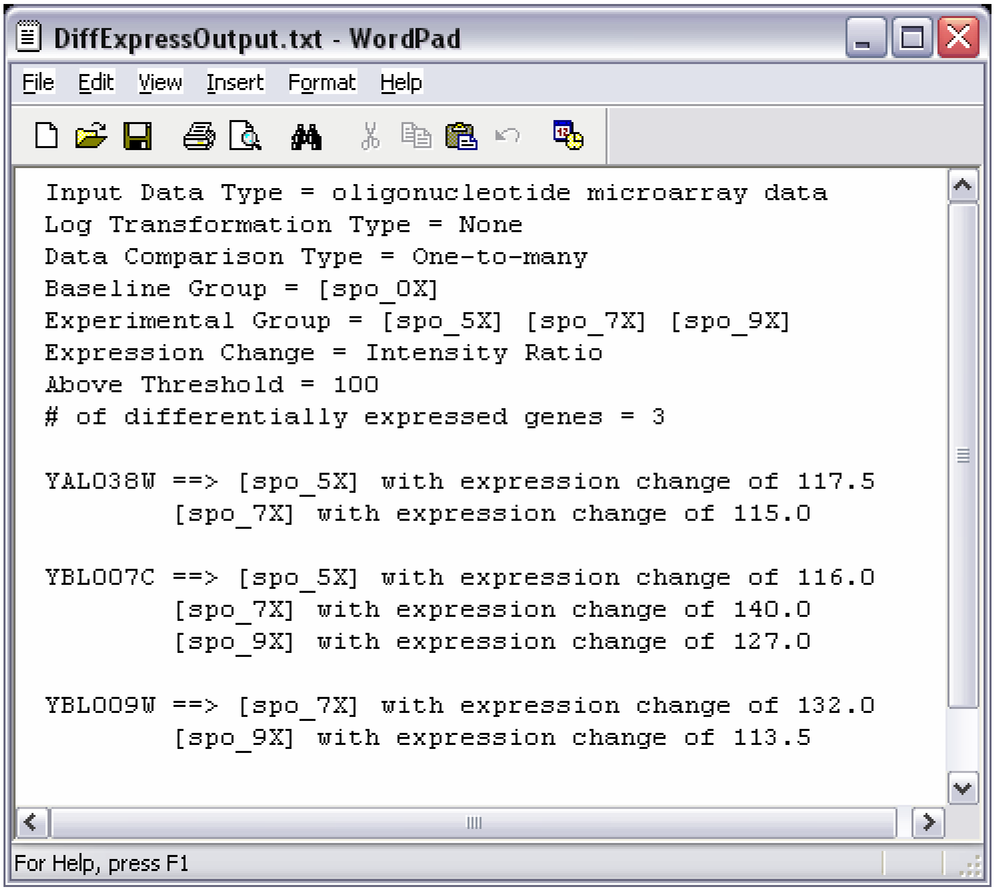
Figure 19: DiffExpress - Output Display

The results may be saved to a text file (Figure 20). This text file includes the following information:

1. The type of data
2. The type of transformation
3. The type of comparison (for oligonucleotide and 2D gel data)
4. The baseline sample and experimental samples (for a one-to-many comparison), or baseline groups and experimental samples (for paired and unpaired comparisons)
5. The expression change chosen
6. The threshold range entered

7. The number of candidate genes or proteins found to be differentially expressed

8. The candidate gene or protein and the corresponding samples, as well as the expression change


DiffExpressOutput.txt - WordPad
File Edit View Insert Format Help
For Help, press F1

```
Input Data Type = oligonucleotide microarray data
Log Transformation Type = None
Data Comparison Type = One-to-many
Baseline Group = [spo_0X]
Experimental Group = [spo_5X] [spo_7X] [spo_9X]
Expression Change = Intensity Ratio
Above Threshold = 100
# of differentially expressed genes = 3

YAL038W ==> [spo_5X] with expression change of 117.5
        [spo_7X] with expression change of 115.0

YBL007C ==> [spo_5X] with expression change of 116.0
        [spo_7X] with expression change of 140.0
        [spo_9X] with expression change of 127.0

YBL009W ==> [spo_7X] with expression change of 132.0
        [spo_9X] with expression change of 113.5
```

Figure 20: DiffExpress - Example of output text file

3.3.2 Graphs

DiffExpress offers various optional graphs which enable easier visualization of the relationships and patterns in data, thereby simplifying data analysis.

3.3.2.1 Outliers

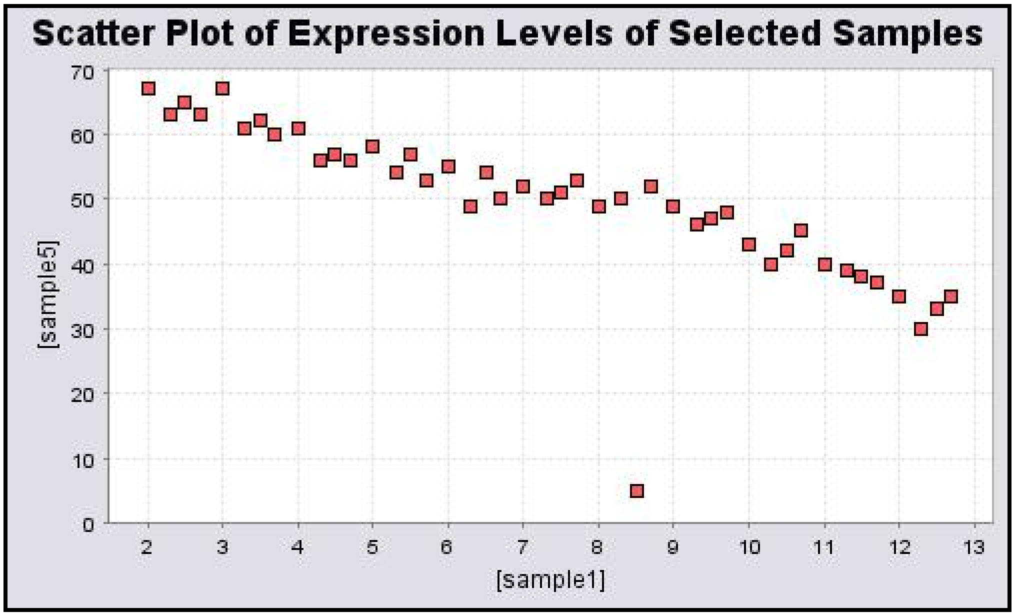


Figure 21: DiffExpress - An example of a Scatter Plot with an outlier.
Most of the data is more or less clustered around an imaginary line with a negative slope except for the value with an expression level of 8.5 for sample 1 and an expression level of 5.0 for sample 5

An outlier is a data value which appears to deviate from the distribution of the rest of the data. Outliers can be extremely problematic in data analysis if they are not properly dealt with. For example, they may cause an increase or decrease in the correlation coefficient or cause unreliable measures of spread. Before disposing of outliers, care must be taken to ensure that the outlier is in fact an error in the dataset and not valuable information that could possibly be a breakthrough in research.

In graphs, an outlier is usually represented as a data point that falls a significant distance from the remainder of the dataset. Two graphs which can assist researchers in visually identifying outliers are the Box Plot and the Scatter Plot.

3.3.2.2 Box Plots

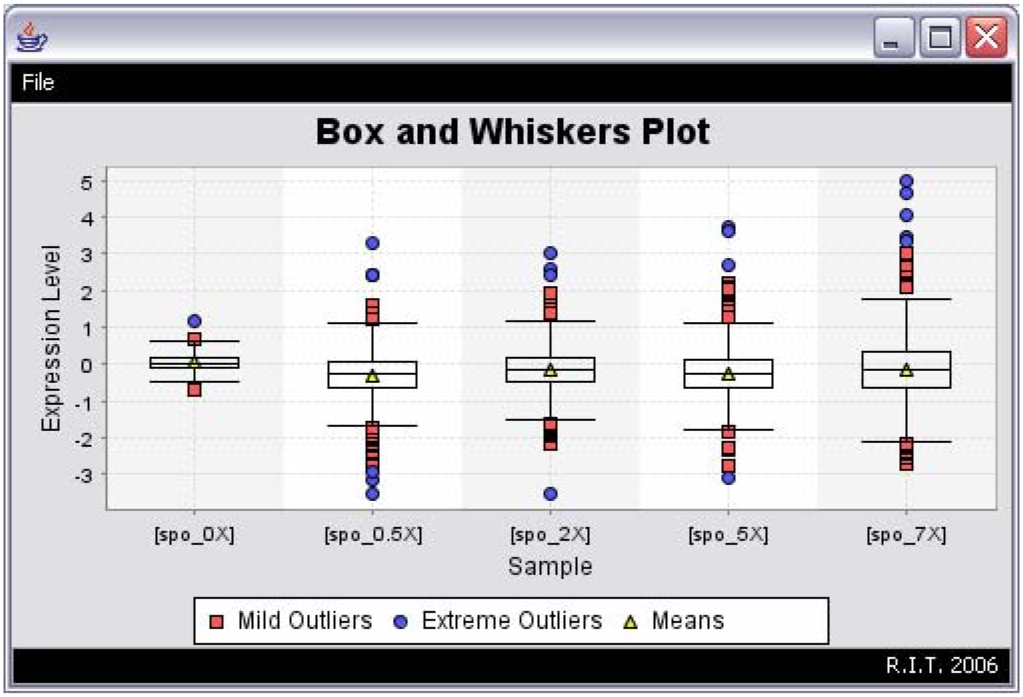


Figure 22: DiffExpress - Box and Whiskers Plot

A box plot can be used to graphically represent the minimum value, maximum value, lower quartile, upper quartile and median of a set of data.[9] This graph can also be used to calculate the mean of the data and for the identification of outliers (unusual observations). Placing two or more categorical box plots (one for each condition) side by side on the same graph (Figure 22) can assist in comparing the datasets' distributions and determining if there is any variation between the groups.

3.3.2.3 Scatter Plots

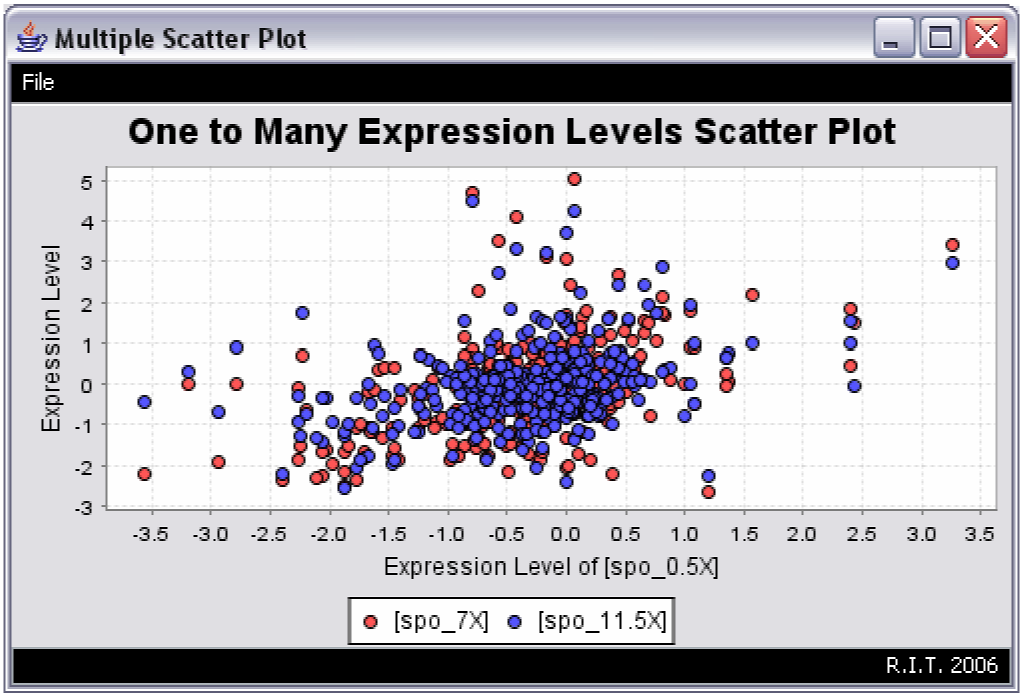


Figure 23: DiffExpress - Scatter Plot

Scatter plots (Figure 23) graphically display the spread of the data, illustrate the relationship between two variables, and are helpful when determining if it is appropriate to calculate the correlation coefficient or fit a regression curve. These graphs are also used for easier identification of outliers in datasets. In cDNA microarrays, a common scatter plot drawn is that of green versus red channel intensities.

The pattern in these types of graphs (scatter plots) is more apparent when there is a plethora of data. If the data points come close to forming a straight line, then the higher the correlation between the two variables. If the slope of the graph rises from left to right (a diagonal line from the origin to high x and y values) a positive correlation is represented, while a slope falling from left to right (diagonal line from high y values to high x values) represents a negative correlation.

A scatter plot may reveal some existing relationship between variables, but it does not mean that one variable is causing a change in another variable. Another variable may be the reason why the two variables seem related or their relationship may simply be coincidental.

3.3.2.4 Pearson Correlation Coefficient

When examining the relationship between variables the following questions can be asked:

1. Are two variables related in some way? (As one variable changes, does the other variable also change in a linearly consistent way?)

2. What is the strength of the relationship?

The Pearson Correlation Coefficient (r) (Equation 5) is a number that describes the strength and the direction of a relationship. The sign (+ or -) represents the direction (i.e. positive or negative) while the magnitude corresponds to the strength of the correlation (i.e. weak or strong correlation).

$$r = \frac{\Sigma XY - \frac{\Sigma X \Sigma Y}{N}}{\sqrt{(\Sigma X^2 - \frac{(\Sigma X)^2}{N})\ (\Sigma Y^2 - \frac{(\Sigma Y)^2}{N})}} \qquad (5)$$

Even if there is a correlation, it does not signify that there is a causal relationship. A significant correlation will only demonstrate that the two variables linearly vary together in a certain direction (positively or negatively).

3.3.2.5 RA Plot

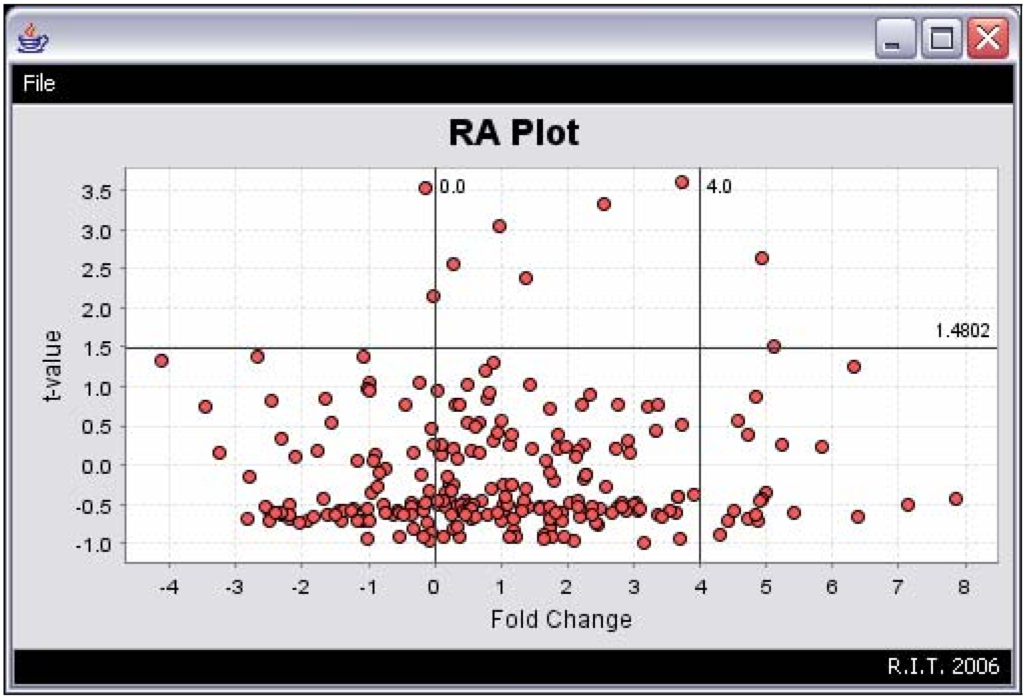


Figure 24: DiffExpress - RA Plot

The RA Plot of DiffExpress is a modified version of the volcano plot (Figure 24).

Volcano plots (Figure 25) graphically display the relationship between the expression change and statistical significance (using the t-test), thereby making it easier to detect significant differentially expressed genes. The volcano plot's horizontal axis(x) represents a measure of expression change (usually the log ratio) between the two groups while its vertical axis represents the negated log of the p-value (i.e. $-\log_{10}$ (p-value)). The p-value is calculated from the t-test, which is a parametric test used to assess whether there is a statistically significant difference between the means of two groups.[10]

The RA plot's horizontal axis also represents a measure of expression change (usually the log ratio) between the two groups, but unlike the volcano plot, its vertical axis represents the t-value calculated from the t-test, not a p-value.

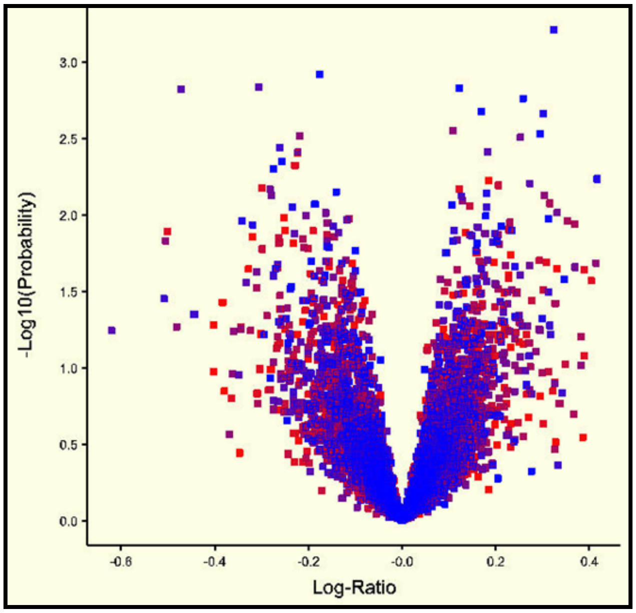


Figure 25: Volcano Plot (http://genstat.co.uk/doc/8doc/html/marray/VolcanoPlot.htm)

Region	Expression Change	Statistical Significance
Upper Left and Right	Greater than k-fold difference	Statistically Significant
Upper Middle	Less than k-fold difference	Statistically Significant
Lower Left and Right	Greater than k-fold difference	Not Statistically Significant
Lower Middle	Less than k-fold difference	Not Statistically Significant

Table 7: Summary of Regions of the RA Plot and the Statistical Significance

Genes/ Spot ids with statistically significant values based on the t-test and with a large log ratio will be identified as possibly being differentially expressed. The statistically significant values of interest are those that are in the upper left and right regions of the plot (Table 7).

Paired t-test

The paired t-test is used to compare the means between two of the same or related samples, and is commonly used when a subject is measured before and after some experiment.[11] For example, it may be interesting to test the significance of the differences of measurements of the pulse rate or blood pressure of a group of subjects before and after receiving a certain drug at different times during the day.

Unpaired t-test

The unpaired t-test, also known as the independent group t-test, is used to compare the means of two independent groups.[11] An example is the blood pressure between a group of patients who have received a certain medication and another group or patients who have received a placebo. Unlike a paired test which uses non-random samples, this test should be used when a replicate is randomly chosen from a population.

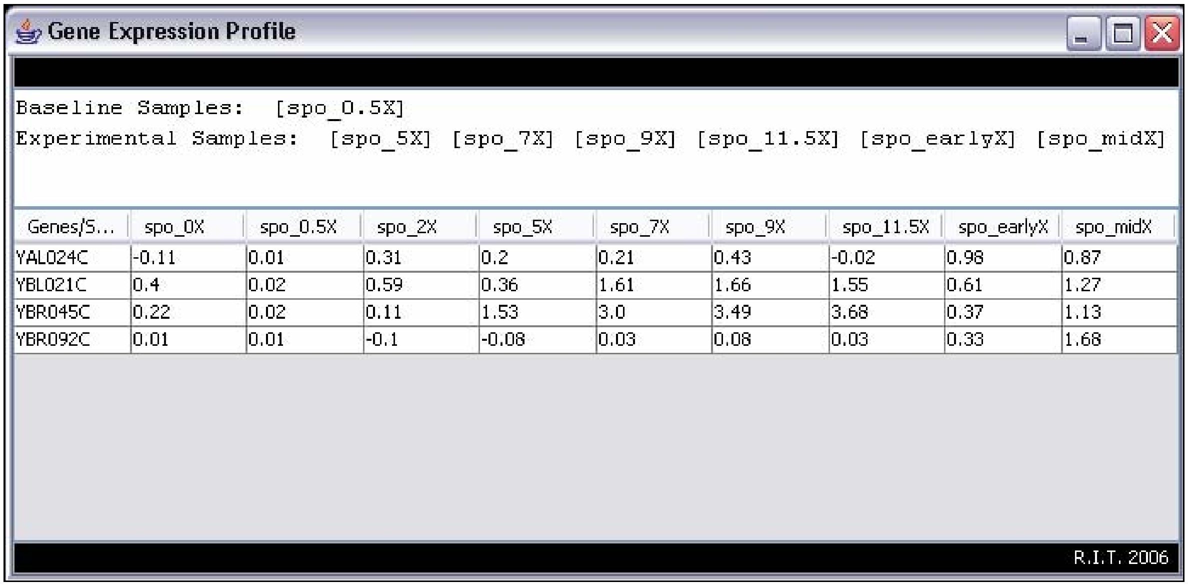
Expression Change

To calculate the expression change of the RA plot for both paired and unpaired groups, the log is taken of the mean of the y group divided by the mean of the x group (Equation 6).

$$Expression\,change = \log_2\left(\frac{\bar{y}}{\bar{x}}\right) \tag{6}$$

3.3.3 Expression Profile

When the candidate differentially expressed genes are identified, DiffExpress allows the user to generate an expression profile (an example is shown in Figure 26). Basically, this profile is a list of the candidate genes or proteins and the expression levels of the corresponding samples (expression changes when dealing with cDNA input data). After the profile has been created, the data can be analyzed and scanned for patterns (up regulated or down regulated genes) or unusual values.



```
Baseline Samples:  [spo_0.5X]
Experimental Samples:  [spo_5X] [spo_7X] [spo_9X] [spo_11.5X] [spo_earlyX] [spo_midX]
```

Genes/S...	spo_0X	spo_0.5X	spo_2X	spo_5X	spo_7X	spo_9X	spo_11.5X	spo_earlyX	spo_midX
YAL024C	-0.11	0.01	0.31	0.2	0.21	0.43	-0.02	0.98	0.87
YBL021C	0.4	0.02	0.59	0.36	1.61	1.66	1.55	0.61	1.27
YBR045C	0.22	0.02	0.11	1.53	3.0	3.49	3.68	0.37	1.13
YBR092C	0.01	0.01	-0.1	-0.08	0.03	0.08	0.03	0.33	1.68

Figure 26: DiffExpress - Gene Expression Profile

4 Implementation

4.1 Java

Java was used to implement DiffExpress because it is fast, robust and platform-independent (the same program can be executed on multiple operating systems). The complete Javadoc documentation for this tool can be referenced from the folder containing the DiffExpress program files.

4.2 JFreeChart

JFreeChart (used for the creation of all DiffExpress graphs) is a chart library founded by David Gilbert and written entirely in Java. JFreeChart supports the drawing of various graphs such as histograms, pie charts, bar charts and scatter plots, just to name a few. Details of this library can be found at: http://www.jfree.org/jfreechart/index.html.

4.3 Packages

A Java package is comprised of a group of related classes and interfaces. Five of the main packages in DiffExpress (Basics, Comparators, Datasets, Frames and Graphs), are described below.

4.3.1 Basics

The Basics package comprises of the majority of the classes designed to perform calculations in DiffExpress.

Class	Description
FoldChange	Calculates the intensity ratio, $\log_2$ ratio or fold change for the input data types (cDNA, oligonucleotide and 2D gels) and comparison types (one-to-many, paired groups, unpaired groups).

Outliers	Performs all calculations associated with identifying outliers using Tukey’s method. For each dataset, this class calculates the mean, median, interquartile range, maximum value, minimum value and cutoffs for mild and extreme outliers.
PCC	Calculates the pearson correlation coefficient between two samples.
ReadInData	Enters the input data into DiffExpress.
RAPlot	Calculates t-values and fold changes for the RA plot.

4.3.2 Comparators

This package consists of all the comparators.

Class	Description
BWDatasetComparator	Comparator to sort the box and whisker’s dataset.
FourthVarIDComparator	Comparator to sort the samples by the second condition.
SampleIDComparator	Comparator to sort the samples by the sample id.
ThirdVarIDComparator	Comparator to sort the samples by the first condition.

4.3.3 Datasets

This package comprises of all the dataset classes and basic classes.

Class	Description
BWDataset	Box and whiskers dataset object.
CustomXYDataset	Creates XY datasets for the RA Plot, Outliers Plot, Expression Levels Plot and Expression Change Plot.
Samples	Samples object.
Settings	Settings object.
xyDataset	XY dataset object.

4.3.4 Frames

This package contains the user interfaces for DiffExpress.

Class	Description
ExpressionFrame	Main application window. The user can select samples, choose the type of expression change, enter the threshold range, and obtain a list of the candidate differentially expressed genes or proteins. (Figure 27)
cDNAScatterPlotFrame	Options window for cDNA expression changes scatter plot. The user selects a sample from the "Choose Sample 1" list, and selects one or more samples from the "Choose Other Samples" list. (Figure 28)
ExpressionChangeScatterPlotFrame	Options window for oligonucleotide and 2D gels expression change scatter plots. The user can select a baseline sample and two experimental samples. They can draw intensity ratio, $\log_2$ ratio or fold change scatter plots, and calculate the pearson correlation coefficient for the samples selected. (Figure 29)
ExpressionLevelsBasicPlotFrame	Options window for expression levels basic plot. The user can select one or more samples to plot on the same graph. (Figure 30)
GeneProfileTableFrame	Window for the gene expression profile of candidate genes window.
GraphFrame	Window for all individual graphs (i.e. the user has not opted for "Multiple Plots in a Single Window").
OutliersFrame	Outliers and Box and Whiskers plot options window. The user can select one or more samples and select a plot to be drawn. From the "Display Outlier Information" tab they may also display the values for the non-outliers, outliers and statistical values. (Figure 31 and Figure 32)
PCCFrame	Pearson correlation coefficient options window. The user can select two samples and calculate their pearson correlation coefficient. (Figure 33)
RAErrorFrame	RA plot error dialog. If the two group sizes for a paired t-test are not equal, this window pops up. (Figure 34)
RAPlotFrame	RA plot options window. The user can select the samples for each of their two groups (paired or unpaired), enter a t-value cutoff percent rank and enter a threshold range for the RA Plot. (Figure 35)

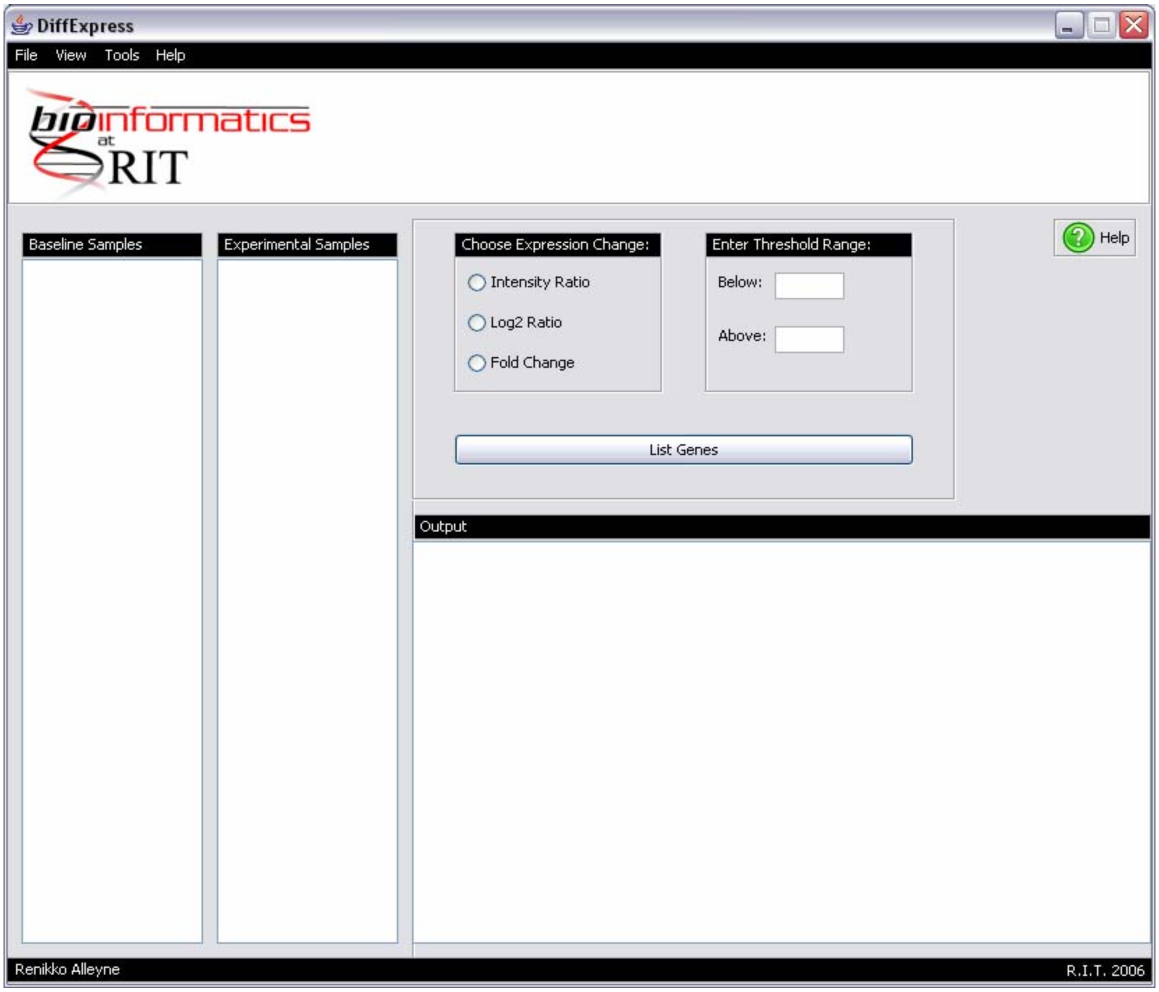


Figure 27: DiffExpress - Main Application Window

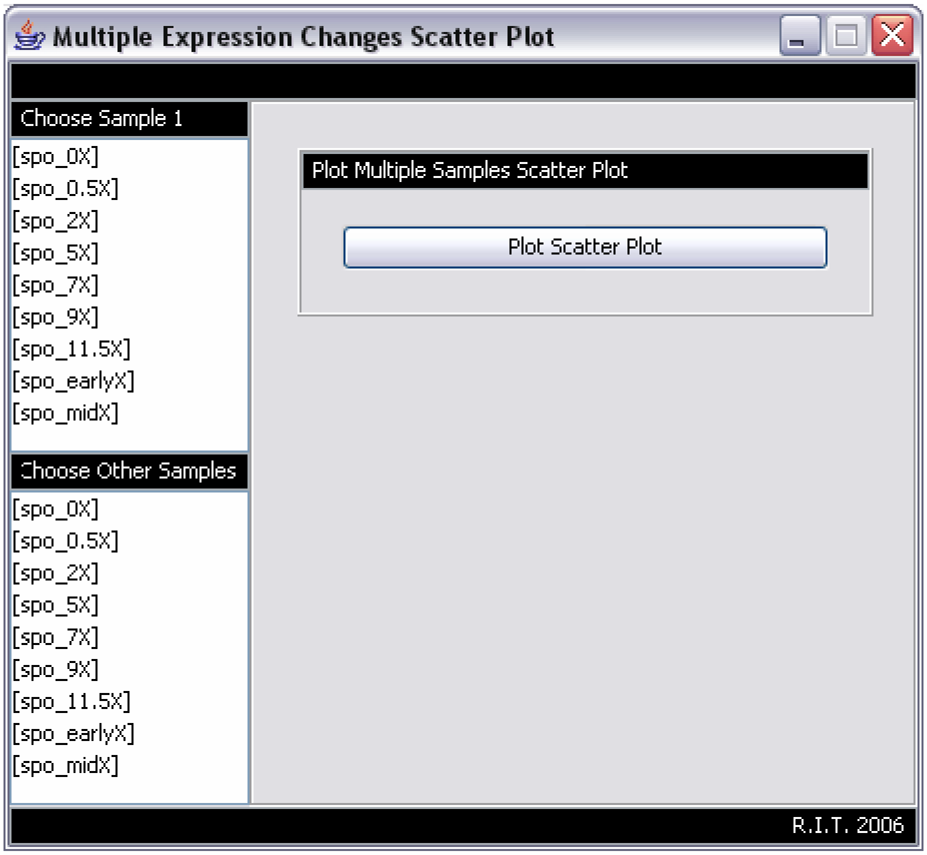
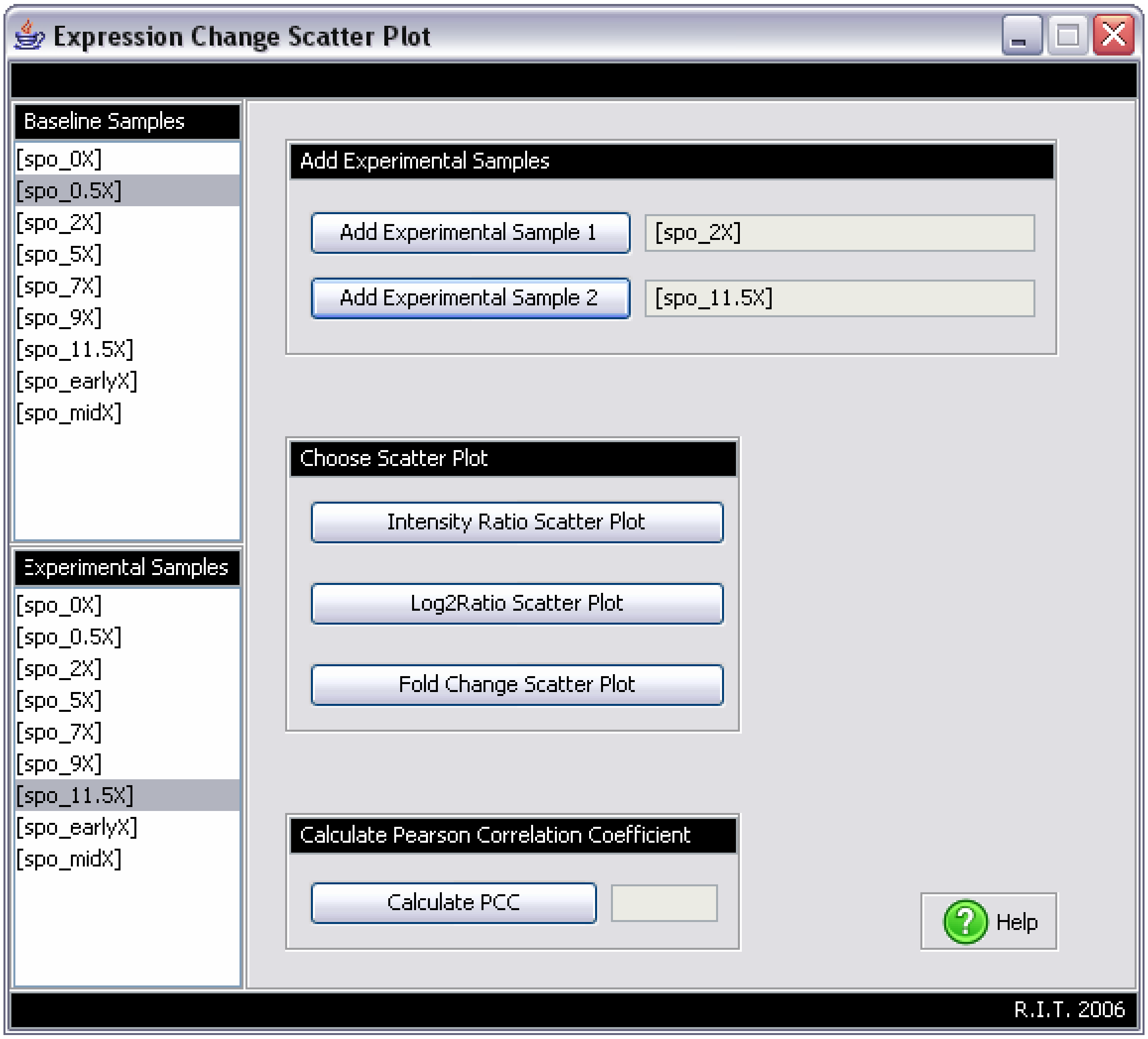


Figure 28: DiffExpress - cDNA Scatter Plot Options Window



Add Experimental Sample 1 [spo_2X]

Add Experimental Sample 2 [spo_11.5X]

Figure 29: DiffExpress – Expression Change Scatter Plot Options Window

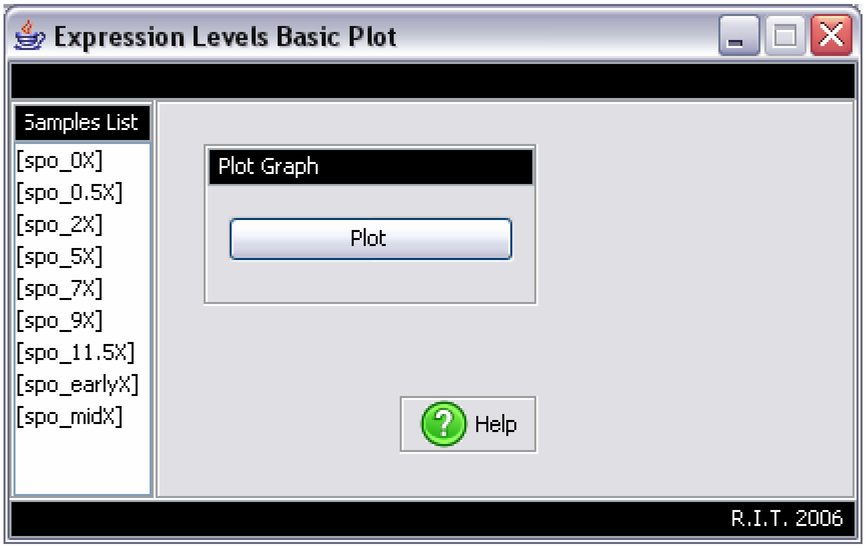


Figure 30: DiffExpress - Expression Levels Basic Plot Options Window

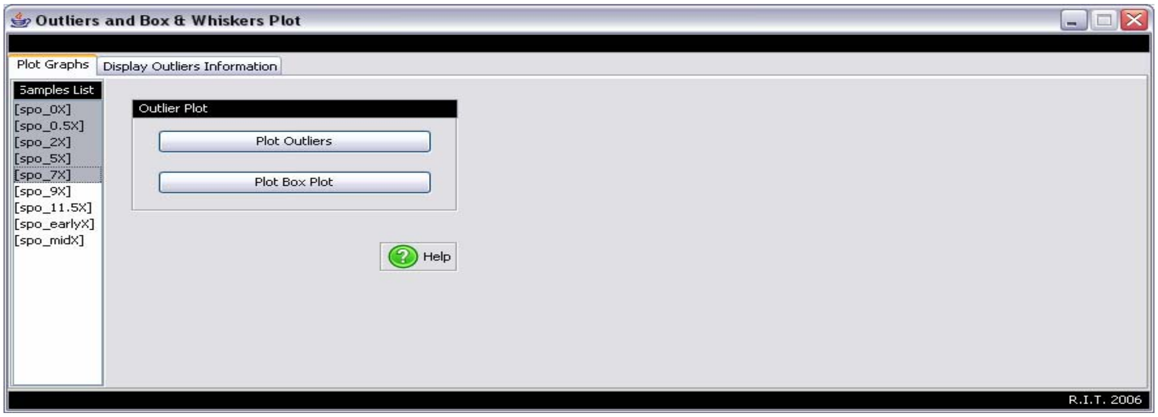
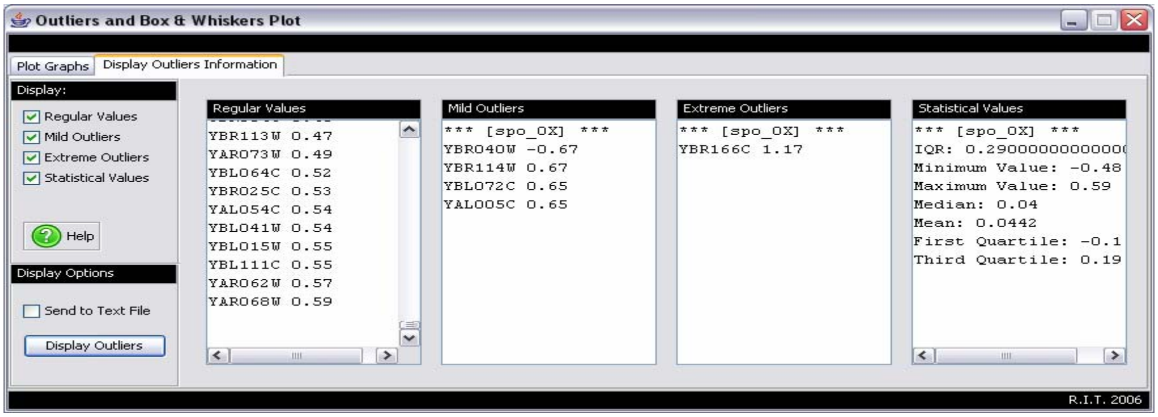


Figure 31: DiffExpress - Outliers and Box & Whiskers Plot: Plot Graphs


Outliers and Box & Whiskers Plot
Plot Graphs
Display Outliers Information
Help
Display Outliers
R.I.T. 2006

Display:

☑ Regular Values
☑ Mild Outliers
☑ Extreme Outliers
☑ Statistical Values

Display Options

☐ Send to Text File

Regular Values

```
YBR113W 0.47
YAR073W 0.49
YBL064C 0.52
YBR025C 0.53
YAL054C 0.54
YBL041W 0.54
YBL015W 0.55
YBL111C 0.55
YAR062W 0.57
YAR068W 0.59
```

Mild Outliers

```
*** [spo_0X] ***
YBR040W -0.67
YBR114W 0.67
YBL072C 0.65
YAL005C 0.65
```

Extreme Outliers

```
*** [spo_0X] ***
YBR166C 1.17
```

Statistical Values

```
*** [spo_0X] ***
IQR: 0.2900000000000(
Minimum Value: -0.48
Maximum Value: 0.59
Median: 0.04
Mean: 0.0442
First Quartile: -0.1
Third Quartile: 0.19
```

Figure 32: DiffExpress - Outliers and Box & Whiskers Plot: Display Outliers Information

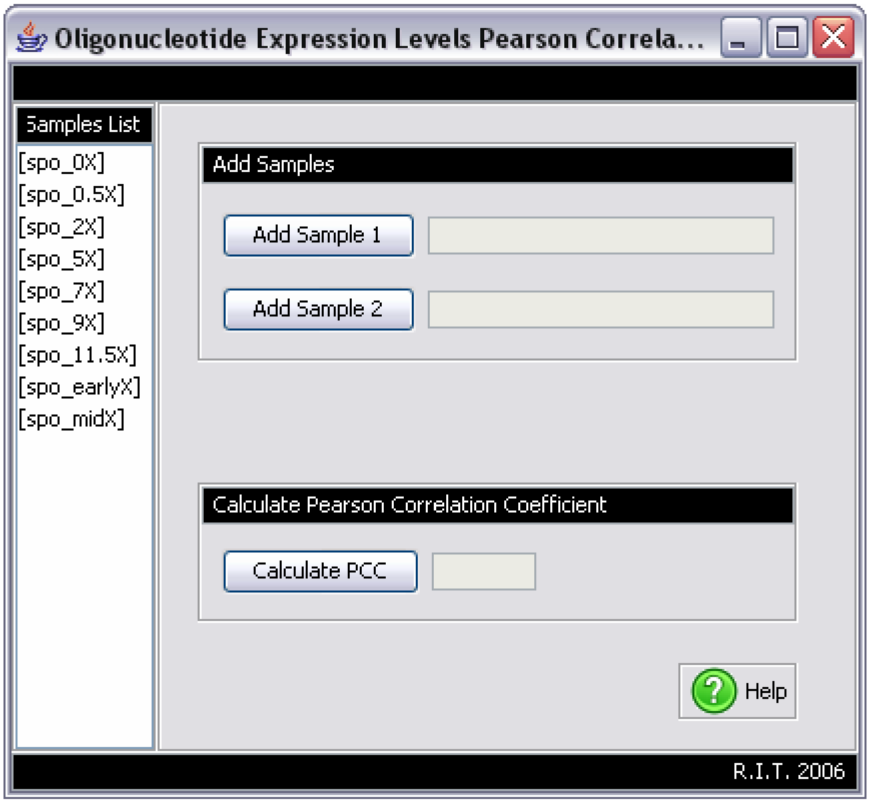
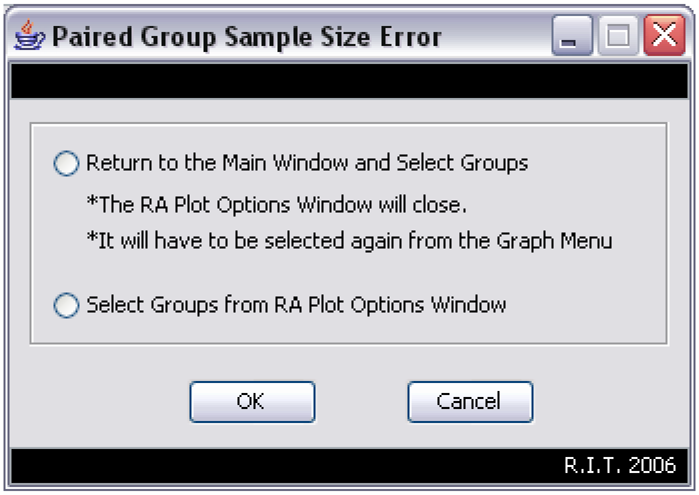


Figure 33: DiffExpress - Pearson Correlation Coefficient Options Window



○ Return to the Main Window and Select Groups

*The RA Plot Options Window will close.

*It will have to be selected again from the Graph Menu

○ Select Groups from RA Plot Options Window

Figure 34: RA Plot Error Frame

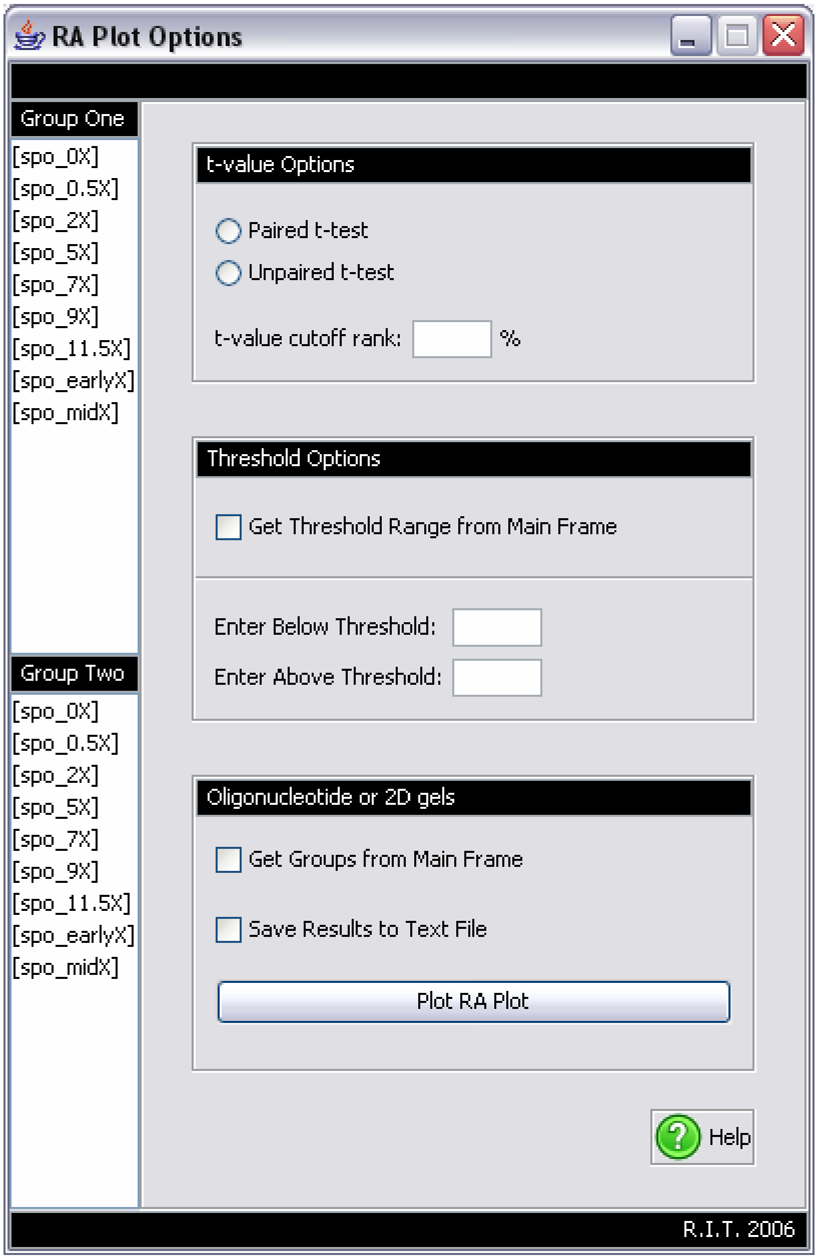


Figure 35: DiffExpress - RA Plot Options Window

4.3.5 Graphs

This package contains classes that plot the various graphs that DiffExpress supports.

Class	Description
MyBoxPlot	Draws the box and whiskers plot for any type of input data.
PlotBasicGraph	Draws the expression levels basic plot for oligonucleotide and 2D gel data, i.e. Expression level vs. gene.
PlotGraph	Draws the expression change basic plot for any type of input data, i.e. Expression change vs. gene.
PlotOutlier	Draws the outliers plot for any type of input data.
PlotRAPlot	Draws the RA plot for oligonucleotide or 2D gel data.
PlotScatterPlot	Draws the scatter plot for any type of input data.

5 PROBLEMS FACED

5.1 Missing Values

Missing values in the expression data matrix proved to be a problem initially. There are a variety of ways to account for missing values, but in the end it was decided that the user should do any preprocessing regarding missing values before the data file is loaded into the tool. This way allows the user some flexibility on how to deal with their missing values, rather than having the tool use a default that may not be suitable to their needs.

5.2 Infinity Values

Some infinity values appeared when the calculation of the expression change was performed. Infinity values are problematic when creating the graphs because they are out of range of the particular axis with which they belong. Initially the infinity values were converted to zeroes, but this led to inaccurate graphs. In the end it was easier to exclude the data points with infinity values from the graph completely.

6 FUTURE WORK

6.1 Preprocessing

Preprocessing is used to make data suitable for analysis purposes. DiffExpress performs no preprocessing; instead the user has to enter data that has already been preprocessed. Some suggestions for preprocessing methods that can be added to DiffExpress are as follows:

1. Imputation: for missing values.

2. Normalization: to ensure that differences in intensities are because of differential expression and not errors made when the experiment was carried out.

3. Averaging replicates.

4. Filtering bad data.

6.2 Volcano Plot Implementation

The current version of DiffExpress implements the RA Plot – a modified version of the Volcano Plot. An improvement to DiffExpress would be to implement an actual Volcano Plot which uses the p-value.

6.3 Clustering

Another addition to DiffExpress could be that of clustering. Clustering of data is advantageous because it helps to group genes according to patterns in their expression. Some commonly used clustering methods are: hierarchical clustering, self-organizing maps, k-nearest neighbor, k-means and principal component analysis.

6.4 Identification of Differentially Expressed Genes

Implementing more methods for finding differentially expressed genes is also an excellent addition. Chen's single slide method, Sapir and Churchill's single slide method, and Newton's single slide method are some of the methods used in this area.

REFERENCES

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APPENDIX A

DIFFEXPRESS USER GUIDE

1 GETTING STARTED

In order to begin using DiffExpress, double click the Threshold.jar file, or the following command: java –jar Threshold.jar may be run from the command line or terminal window.

2 DIFFEXPRESS INTERFACE

The interface (Figure A1) consists of a menu bar (Figure A2) and a work space (Figure A7).

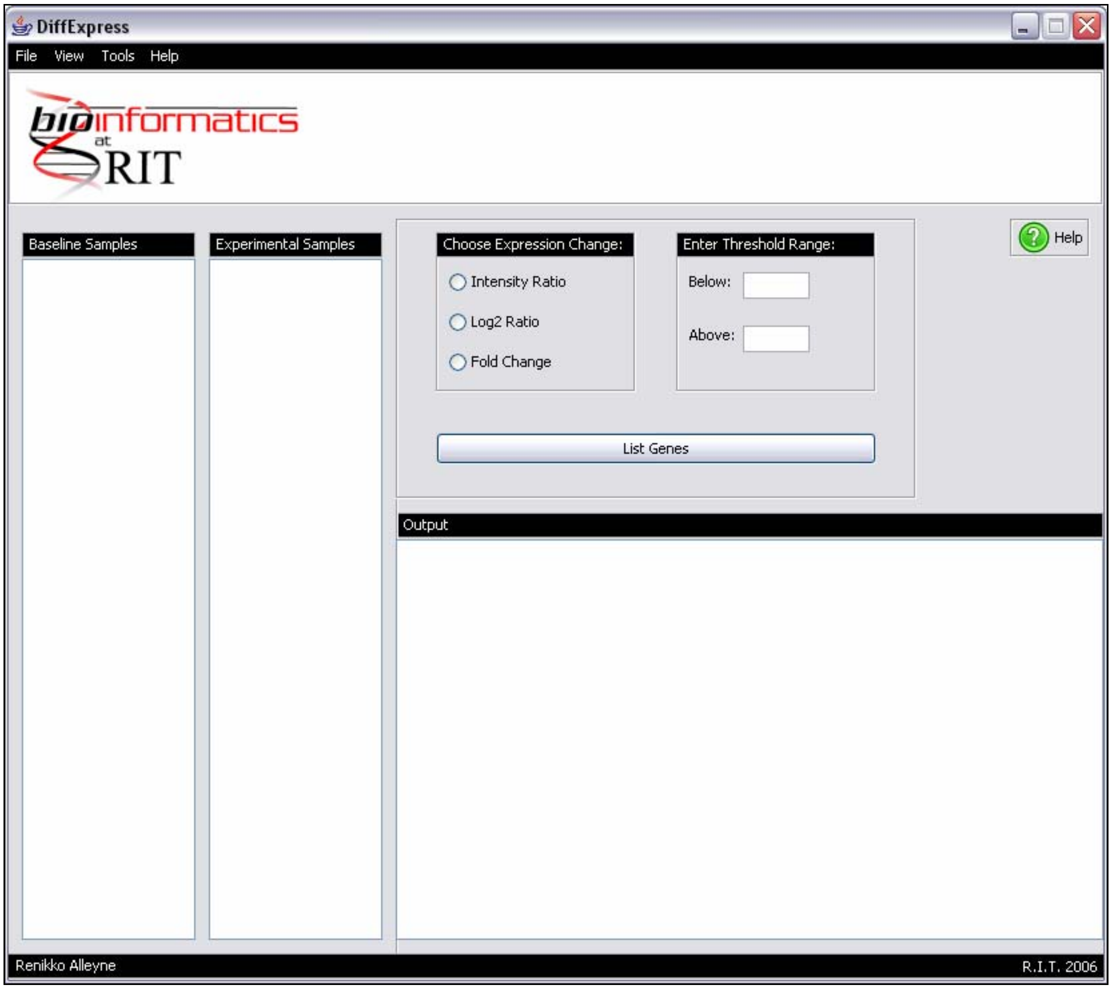


Figure A1: DiffExpress User Interface

2.1 Menu Bar

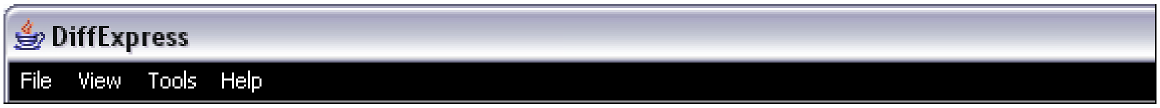


Figure A2: DiffExpress Menu Bar

2.1.1 File

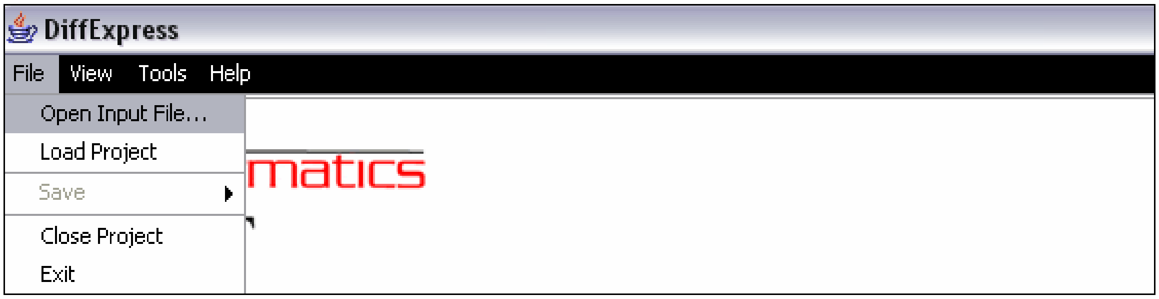


Figure A3: File Menu

Menu Item	Description
File/ Open Input File	Load a new expression matrix by opening a new file.
File/ Load Project	Load an existing project.
File/ Save/ Save Output	Save differentially expressed genes output.
File/ Save/ Save Project	Save current settings.
File/ Close Project	Close current project.
File/ Exit	Exit DiffExpress.

2.1.2 View

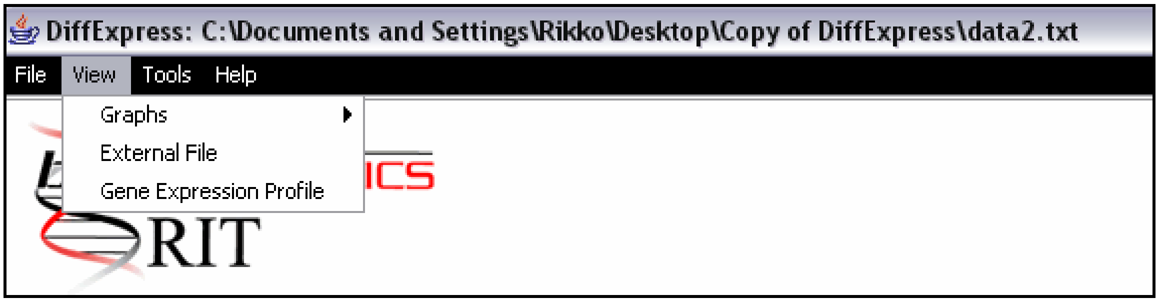

DiffExpress: C:\Documents and Settings\Rikko\Desktop\Copy of DiffExpress\data2.txt
File View Tools Help
Graphs
External File
Gene Expression Profile
ICS
RIT

Figure A4: View Menu

Menu Item	Description
View/ Graphs/ cDNA Graphs/ Expression Change Plot	Create cDNA expression change basic plot.
View/ Graphs/ cDNA Graphs/ Expression Change Scatter Plot	Create cDNA expression change scatter plot.
View/ Graphs/ Oligonucleotide or 2D Gel Graphs/ Basic Plots/ Expression Levels Plot	Create oligonucleotide or 2D gel expression levels basic plot.
View/ Graphs/ Oligonucleotide or 2D Gel Graphs/ Basic Plots/ Expression Change Scatter Plot	Create oligonucleotide or 2D gel expression change scatter plot.
View/ Graphs/ Oligonucleotide or 2D Gel Graphs/ Comparison Plots/ Expression Change Plot	Create oligonucleotide or 2D gel expression change basic plot.
View/ Graphs/ Oligonucleotide or 2D Gel Graphs/ Comparison Plots/ Expression Levels Scatter Plot	Create oligonucleotide or 2D gel expression levels scatter plot.
View/ Graphs/ RA Plot	Create RA plot.
View/ Graphs/ Box and Whiskers Plot	Create Outliers plot or Box and Whiskers plot.
View/ External File	View an external text file.
View/ Gene Expression Profile	View the gene expression profile of differentially expressed genes.

2.1.3 Tools

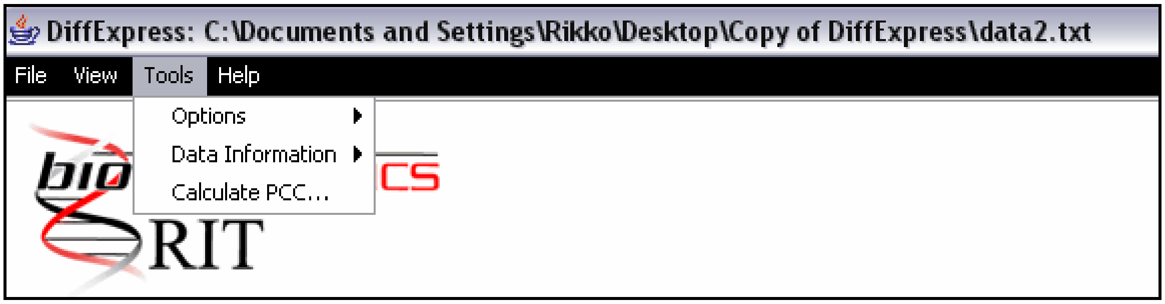

DiffExpress: C:\Documents and Settings\Rikko\Desktop\Copy of DiffExpress\data2.txt
File
View
Tools
Help
Options
Data Information
Calculate PCC...
bio
cs
RIT

Figure A5: Tools Menu

Menu Item	Description
Tools/ Options/ Sort Samples List	Sort sample list by sample id, first condition, or second condition.
Tools/ Options/ Multiple Plots in a Single Window	Group related graphs in a single window.
Tools/ Data Information/ Basic Data Information	View basic information about the input data, such as number of genes or spot ids, number of samples, gene ids or spot ids, and sample ids.
Tools/ Data Information/ Advanced Data Information	Enables user to specify the type of input data loaded.
Tools/ Calculate PCC	Calculate the Pearson correlation coefficient between two selected samples.

2.1.4 Help

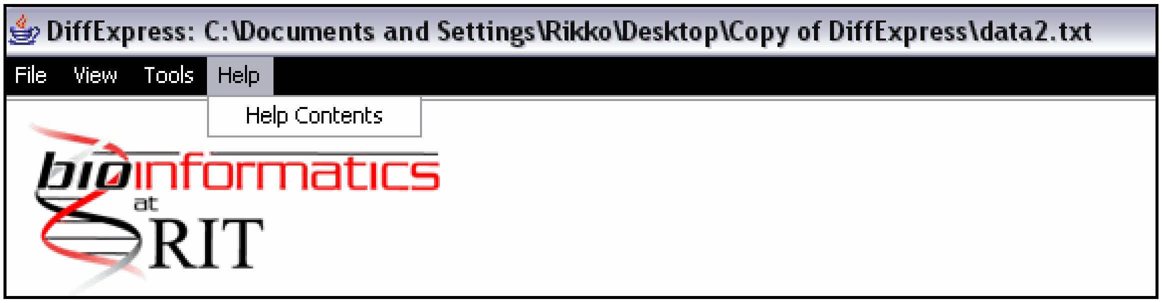

DiffExpress: C:\Documents and Settings\Rikko\Desktop\Copy of DiffExpress\data2.txt
File
View
Tools
Help
Help Contents
bioinformatics at RIT

Figure A6: Help Menu

Menu Item	Description
Help/ Help Contents	Displays a user guide for DiffExpress.

2.2 Work Space

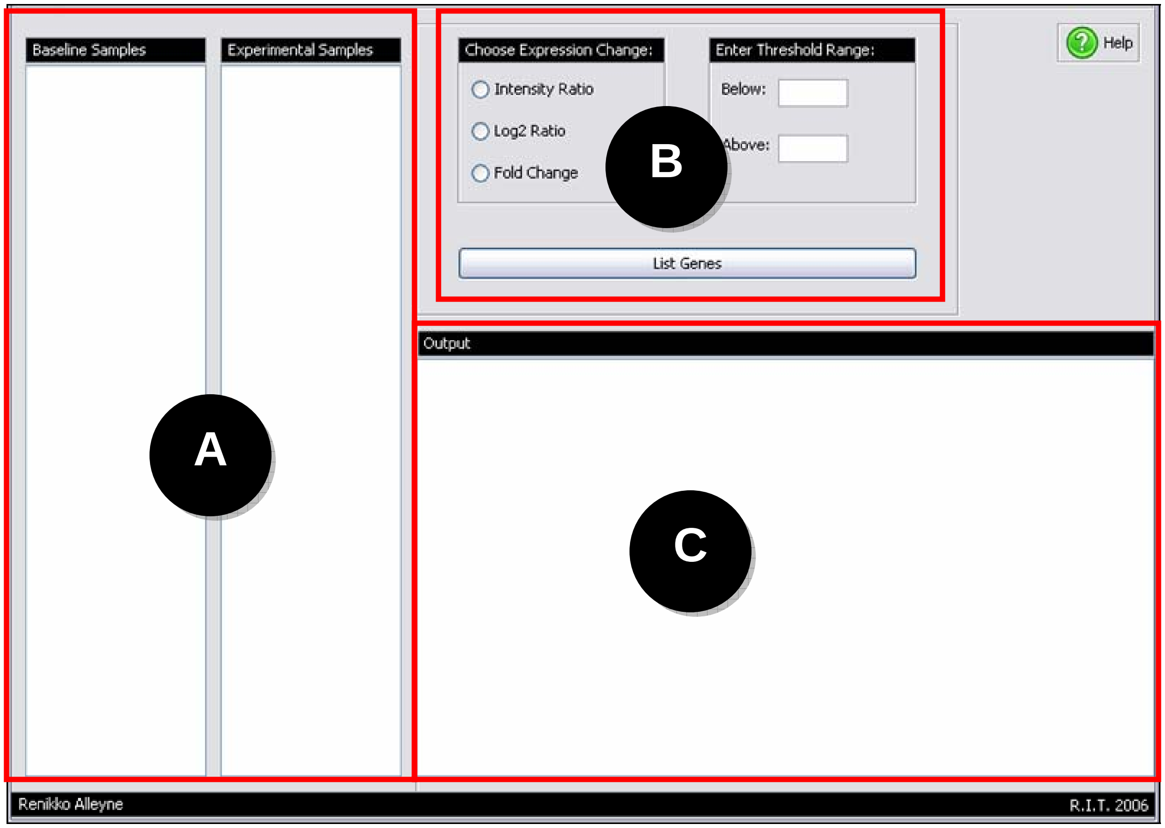
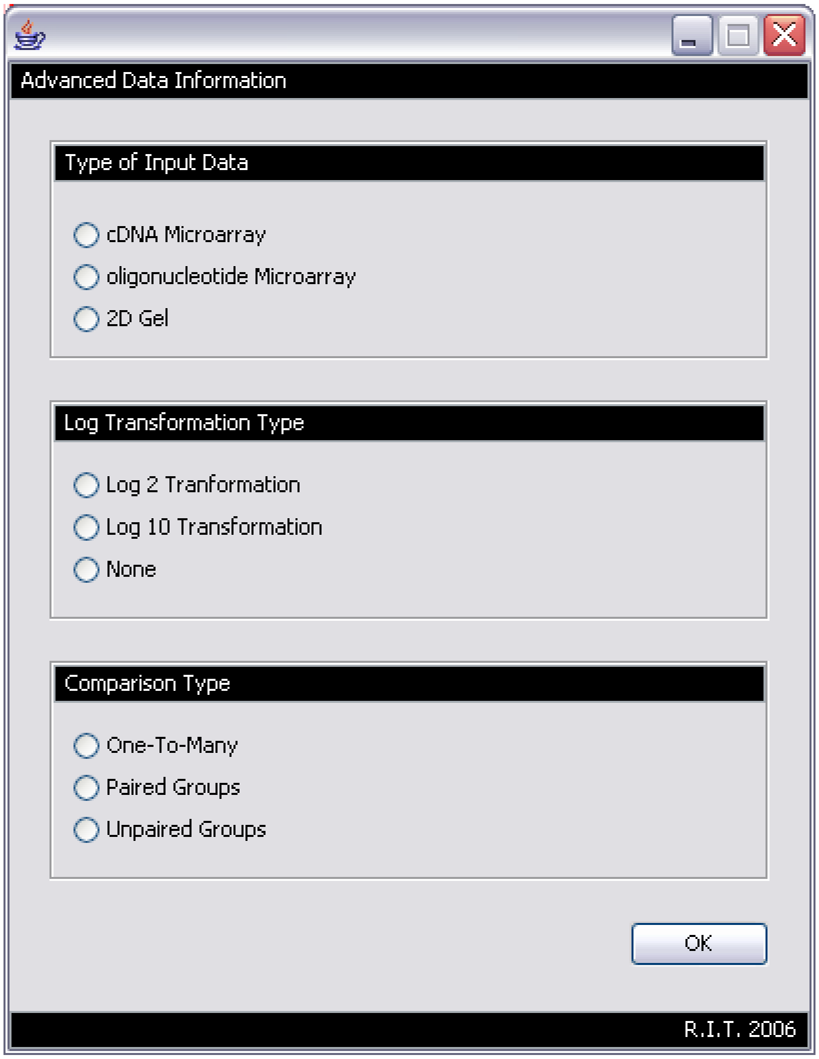


Figure A7: DiffExpress Workspace
(A) Samples Lists, (B) Expression change and Threshold Range options, (C) Output Window

The work space allows the user to select samples, choose an expression change, enter a threshold range, and view the differentially expressed genes.

3 LOADING DATA

Expression data can be loaded either by opening a new input file (File/ Open Input File) or loading an existing project (File/ Load Project). If a new input file is opened, the *Advanced Data Information* Option Window will open (Figure A8). This option window allows the user to specify the type of input data that has been loaded. This information may be entered now or at a later time, but it must be entered before any analysis and viewing of graphs can be performed.



Advanced Data Information

Type of Input Data

○ cDNA Microarray
○ oligonucleotide Microarray
○ 2D Gel

Log Transformation Type

○ Log 2 Tranformation
○ Log 10 Transformation
○ None

Comparison Type

○ One-To-Many
○ Paired Groups
○ Unpaired Groups

Figure A8: Advanced Data Information Option Window

If the user would like to change the advanced data information at anytime, they can do so by performing the following from the menu bar: Tools/ Data/ Information/ Advanced Data Information.

4 SAVING

4.1 Save Output

The information in the *Output Window* of the work space may be saved to a text file if desired (File/ Save Output).

4.2 Save Project

To save the current settings perform the following from the menu bar: File/ Save Project. These saved settings are the files used when loading existing projects.

4.3 Save Graphs

Graphs may be saved in jpeg format by using the File/ Save option on the respective graph's menu bar, or (on Windows systems) by right clicking on the graph and selecting the *Save as* option (Figure A9).

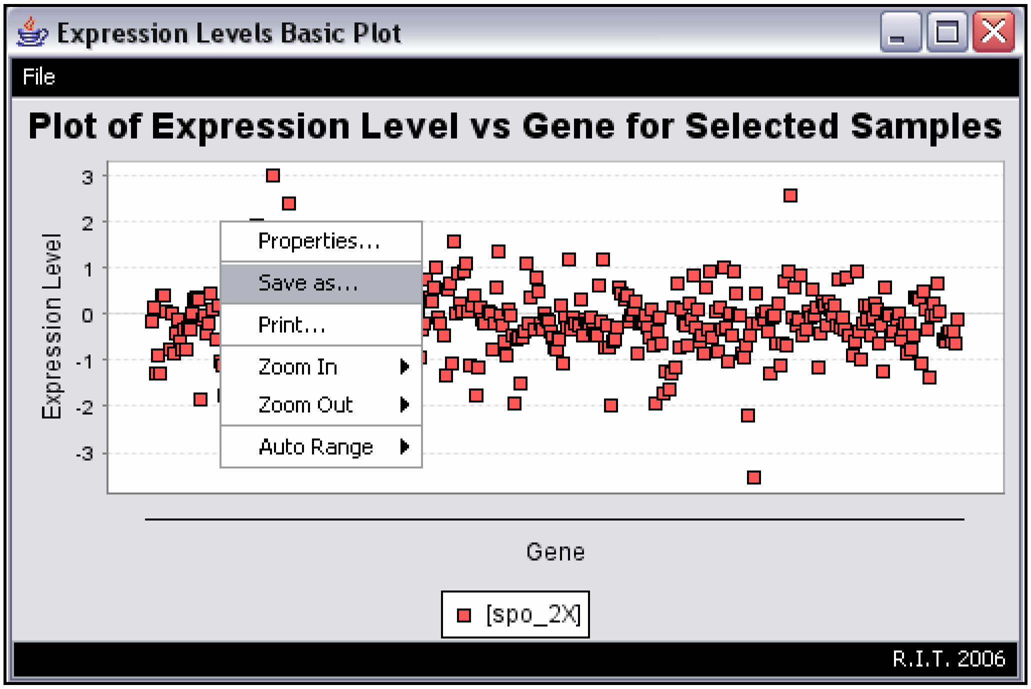


Figure A9: Saving a Graph

5 Viewing

5.1 View Graphs

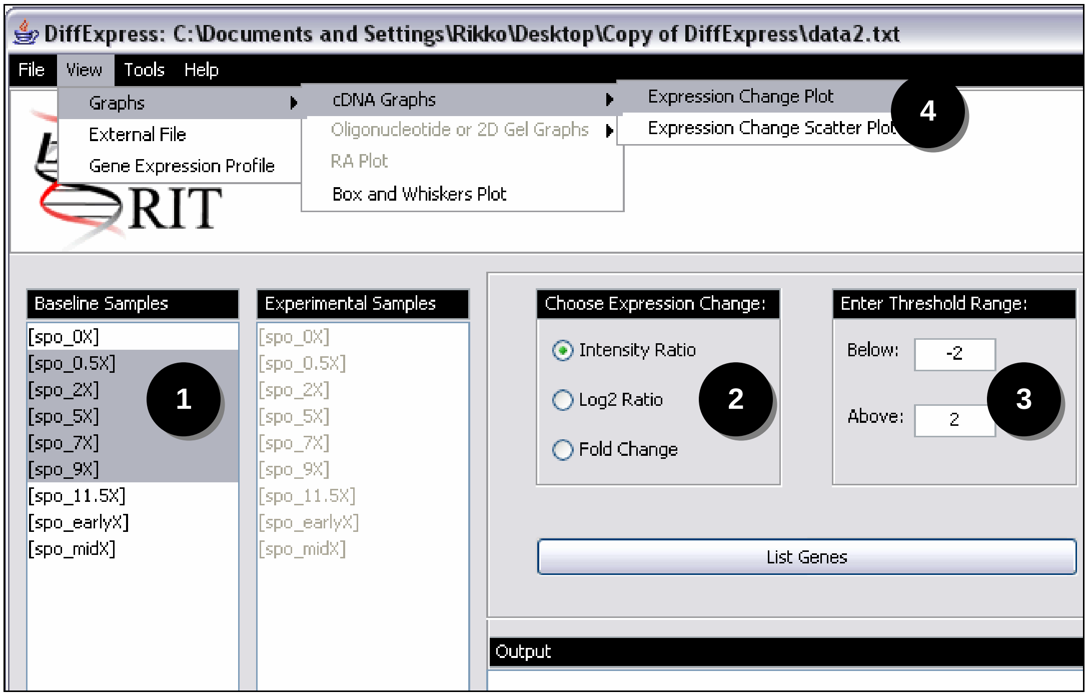
DiffExpress provides basic plots, scatter plots, outliers plots, box and whiskers plots and RA plots.

5.1.1 cDNA Data Graphs

Expression Change Plot

This is a plot of expression change versus genes for selected samples. Follow these steps to create an expression change plot (Figure A10):

1. Select one or more samples from the *Baseline Samples* list.
2. Choose a type of expression change.
3. Enter in a threshold range.
4. From the menu bar: View/ Graphs/ cDNA Graphs/ Expression Change Plot



DiffExpress: C:\Documents and Settings\Rikko\Desktop\Copy of DiffExpress\data2.txt

Baseline Samples: [spo_0X] [spo_0.5X] [spo_2X] [spo_5X] [spo_7X] [spo_9X] [spo_11.5X] [spo_earlyX] [spo_midX]

Experimental Samples: [spo_0X] [spo_0.5X] [spo_2X] [spo_5X] [spo_7X] [spo_9X] [spo_11.5X] [spo_earlyX] [spo_midX]

Choose Expression Change:
● Intensity Ratio
○ Log2 Ratio
○ Fold Change

Enter Threshold Range:
Below: -2
Above: 2

Figure A10: Drawing cDNA Expression Change Plot

Expression Change Scatter Plot

This is a scatter plot of the expression change of one or more selected samples versus the expression change of another sample. Follow these steps to create an expression change scatter plot (Figure A11):

1. From the menu bar: View/ Graphs/ cDNA Graphs/ Expression Change Scatter Plot.
2. Select one sample from the *Choose Sample 1* list.
3. Select one or more samples from the *Choose Other Samples* list.
4. Click the *Plot Scatter Plot* button.

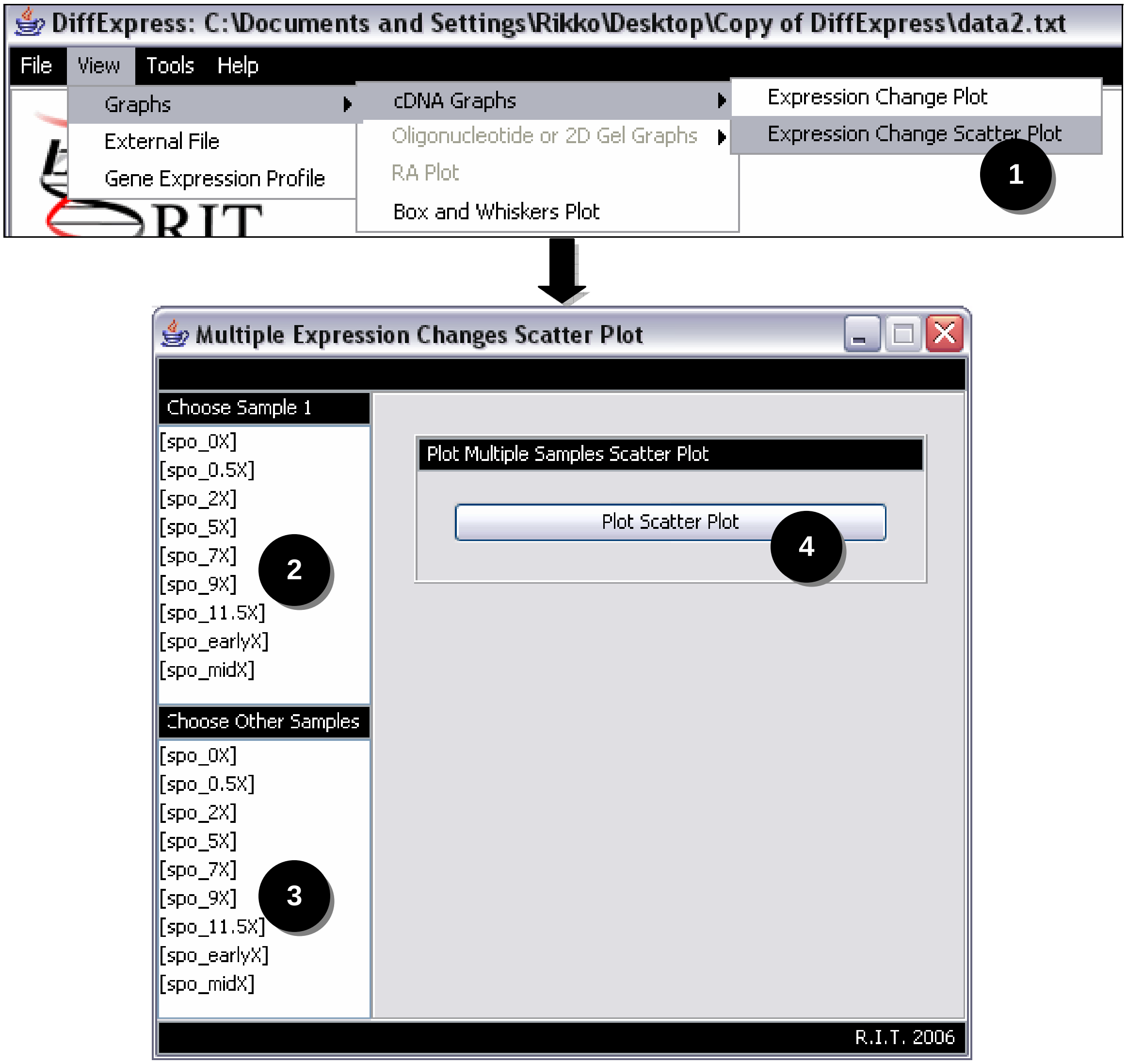


Figure A11: cDNA Scatter Plot Options Window

5.1.2 Oligonucleotide or 2D Gel Data Graphs

5.1.2.1 Basic Plots

Basic Plots do not require the user to make selections DiffExpress' work space. They have their own dialogs from which selections can be made.

Expression Levels Plot

This is a plot of expression levels versus genes for selected samples. Follow these steps to create an expression levels plot (Figure A12):

1. From the menu bar: View/ Graphs/ Oligonucleotide or 2D Gel Graphs/ Basic Plots/ Expression Levels Plot.
2. Select one or more samples from the *Samples List* list.
3. Click the *Plot* button.

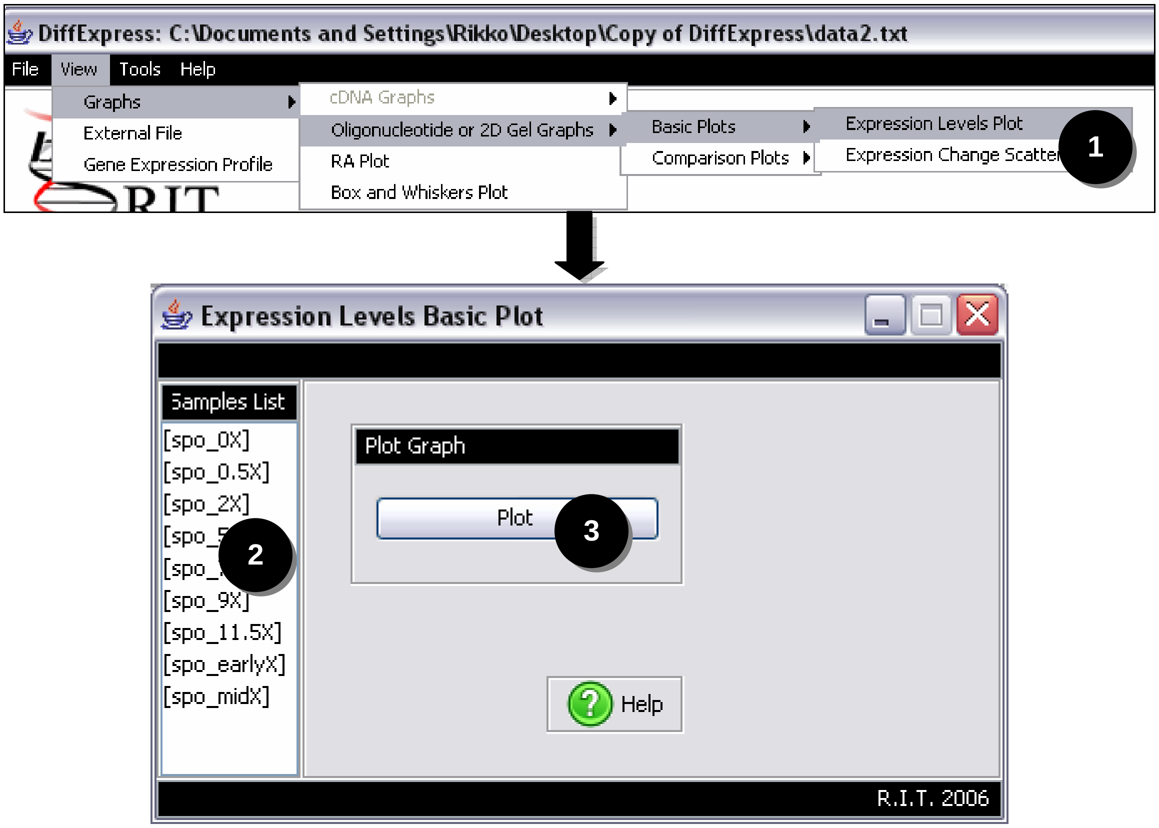
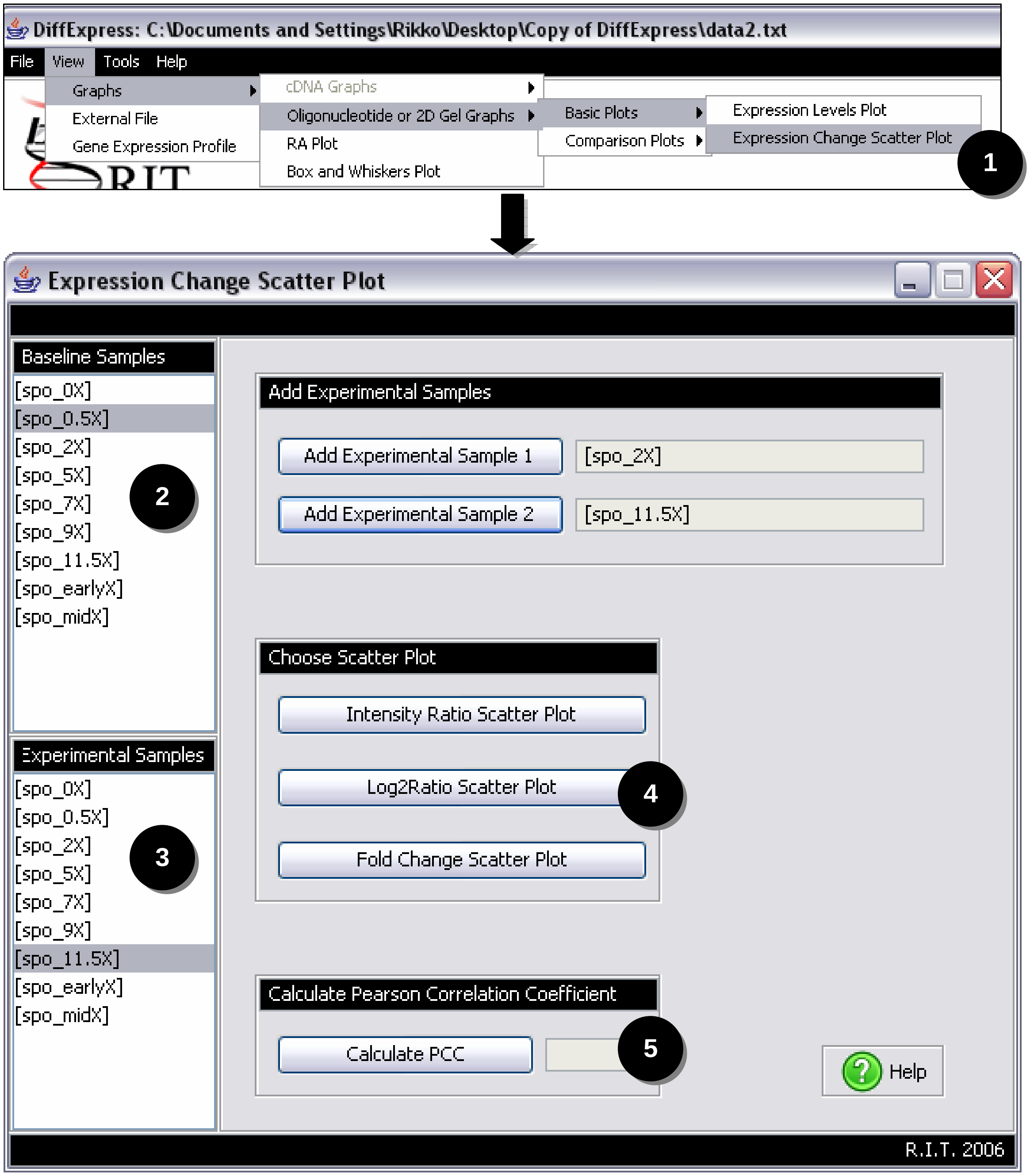


Figure A12: Expression Levels Basic Plot Options Window

Expression Change Scatter Plot

This is a plot of the expression change between the selected baseline sample and the first selected experimental sample versus the expression change between the baseline sample and the second selected experimental sample. Follow these steps to create an expression change scatter plot (Figure A13):

1. From the menu bar: View/ Graphs/ Oligonucleotide or 2D Gel Graphs/ Basic Plots/ Expression Change Scatter Plot.
2. Select one sample from the *Baseline Samples* list.
3. Select a sample from the *Experimental Samples* list, and click the *Add Experimental Sample 1* button. Select another sample from the *Experimental Samples* list, and click the *Add Experimental Sample 2* button.
4. Click one of the scatter plot buttons: *Intensity Ratio Scatter Plot*, *Log Ratio Scatter Plot*, or *Fold Change Scatter Plot*.
5. To calculate the Pearson correlation coefficient of the expression changes click the *Calculate PCC* button.



Add Experimental Sample 1: [spo_2X]

Add Experimental Sample 2: [spo_11.5X]

Figure A13: Expression Change Scatter Plot Options Window

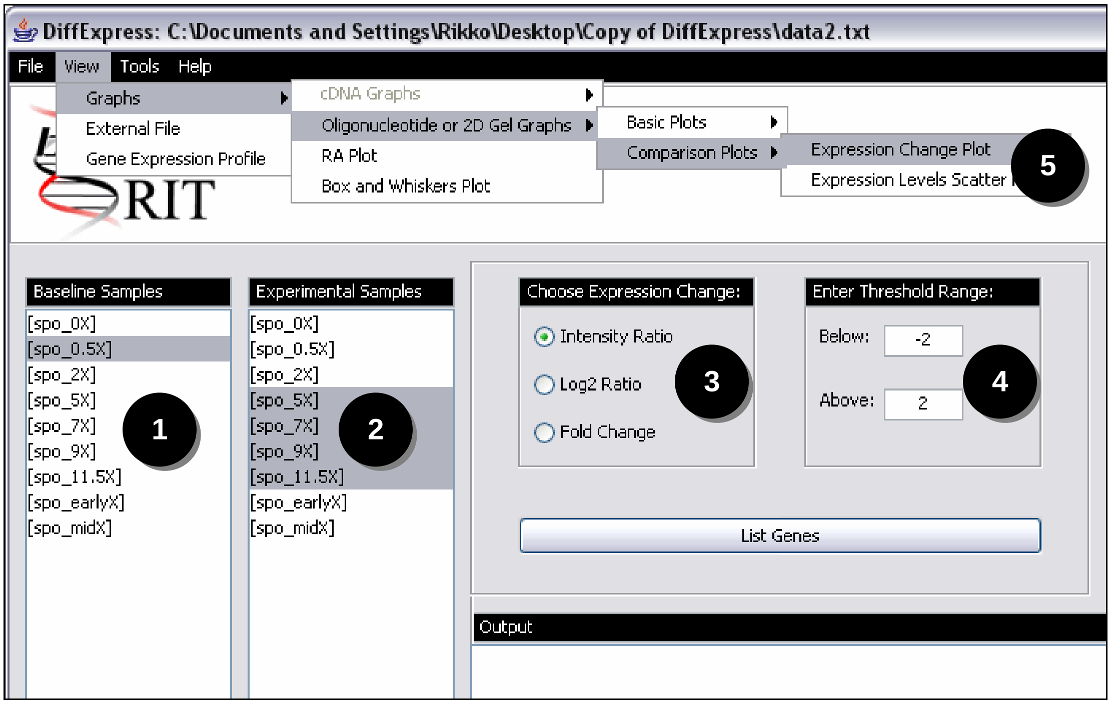
5.1.2.2 Comparison Plots

Comparison Plots require the user to make selections from the work space in order to draw the specified graph.

Expression Change Plot

This is a plot of expression changes between selected baseline samples and selected experimental samples versus genes. Follow these steps to create an expression change plot (Figure A14):

1. Select one or more baseline samples (dependent on the comparison type: one-to-many, paired, unpaired).
2. Select one or more experimental samples.
3. Choose expression change.
4. Enter a threshold range.
5. From the menu bar: View/ Graphs/ Oligonucleotide or 2D Gel Graphs/ Comparison Plots/ Expression Change Plot.


DiffExpress: C:\Documents and Settings\Rikko\Desktop\Copy of DiffExpress\data2.txt

Choose Expression Change: ● Intensity Ratio ○ Log2 Ratio ○ Fold Change

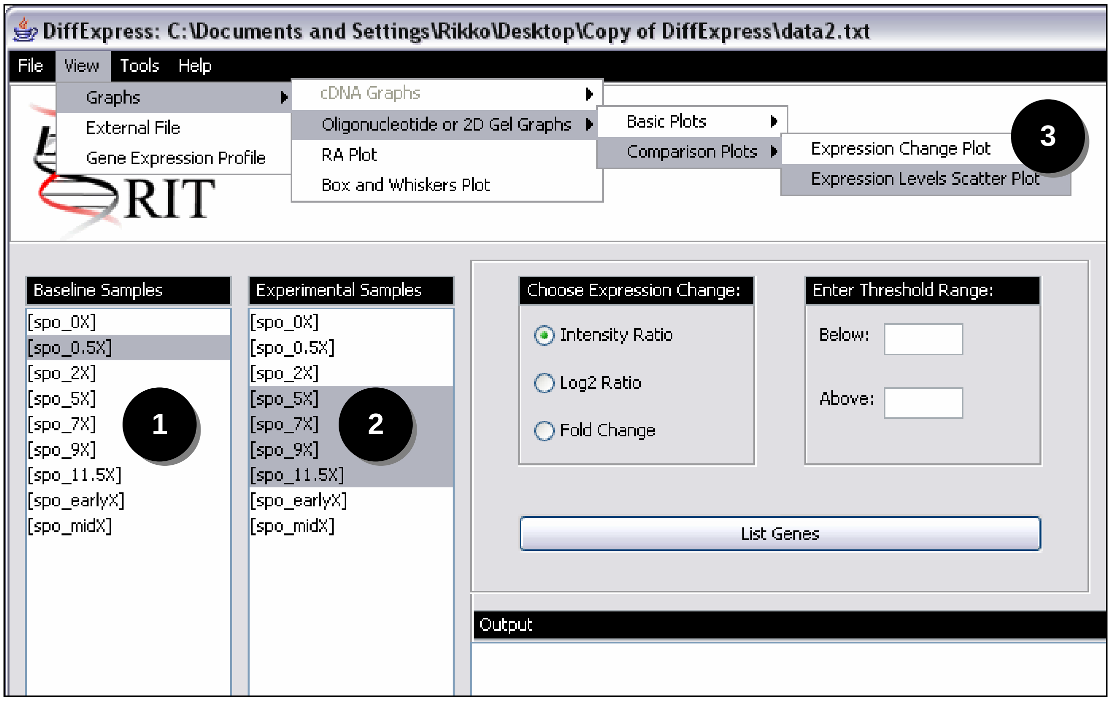
Enter Threshold Range: Below: -2 Above: 2

Figure A14: Create Expression Change Plot

Expression Levels Scatter Plot

This is a plot of selected experimental samples' expression levels versus selected baseline samples' expression levels. Follow these steps to create an expression levels scatter plot (Figure A15):

1. Select one or more baseline samples (dependent on the comparison type: one-to-many, paired, unpaired).
2. Select one or more experimental samples
3. From the menu bar: View/ Graphs/ Oligonucleotide or 2D Gel Graphs/ Comparison Plots/ Expression Levels Scatter Plot



Baseline Samples: [spo_0.5X]

Experimental Samples: [spo_5X] [spo_7X] [spo_9X] [spo_11.5X]

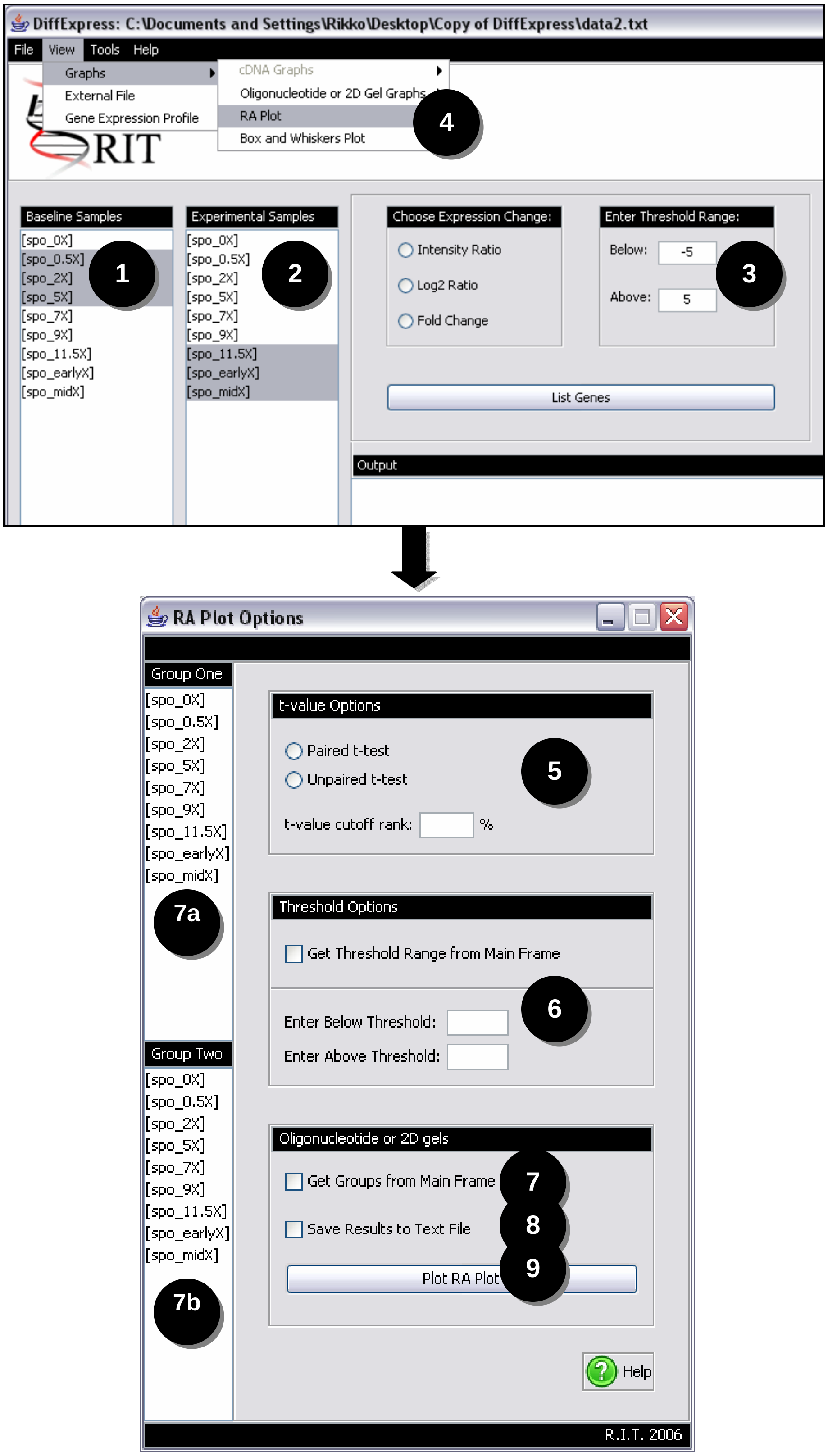
Choose Expression Change: ● Intensity Ratio ○ Log2 Ratio ○ Fold Change

Figure A15: Create Expression Levels Scatter Plot

5.1.3 RA Plot

This is a plot of t-values versus log2 ratio. Follow these steps to create an RA plot (Figure A16):

1. Select one or more baseline samples (dependent on the comparison type: paired or unpaired).
2. Select one or more experimental samples.
3. Enter a threshold range.
4. From the menu bar: View/ Graphs/ RA Plot.
5. Choose a t-test type (paired or unpaired), and enter a t-value cutoff rank.
6. To get threshold range from the work space, check the *Get Threshold from Main Frame* check box. The user may also enter a new threshold range by leaving the check box unchecked and entering new values in the allotted text fields of the RA Plot Options Window.
7. To get groups from the work space, check the *Get Groups from Main Frame* check box. The user may also reselect groups in the RA Plot Options Window:
    a. Select one or more samples for group one.
    b. Select one or more samples for group two.
8. (Optional) Save text results by checking the *Save Results to Text File* check box
9. Click the *Plot RA Plot* button.



Below: -5

Above: 5

Figure A16: RA Plot Options Window

5.1.4 Outliers Plot and Box and Whiskers Plot

The Outliers plot and Box and Whiskers plot both show the mild and extreme outliers. Follow these steps to create an Outlier plot or a Box and Whiskers plot (Figure A17 and Figure A18):

1. From the menu bar: View/ Graphs/ Box and Whiskers Plot.
2. Select one or more samples from the *Samples List* list.
3. Click the button for the type of plot to be drawn (*Plot Outliers* button or *Plot Box Plot* button).
4. To view the results in text format, go to the *Display Outliers Information* tab.
5. Check the check boxes of all the results to be viewed.
6. To save text results to a text file, check the *Send to Text File* button.
7. Click the *Display Outliers* button.

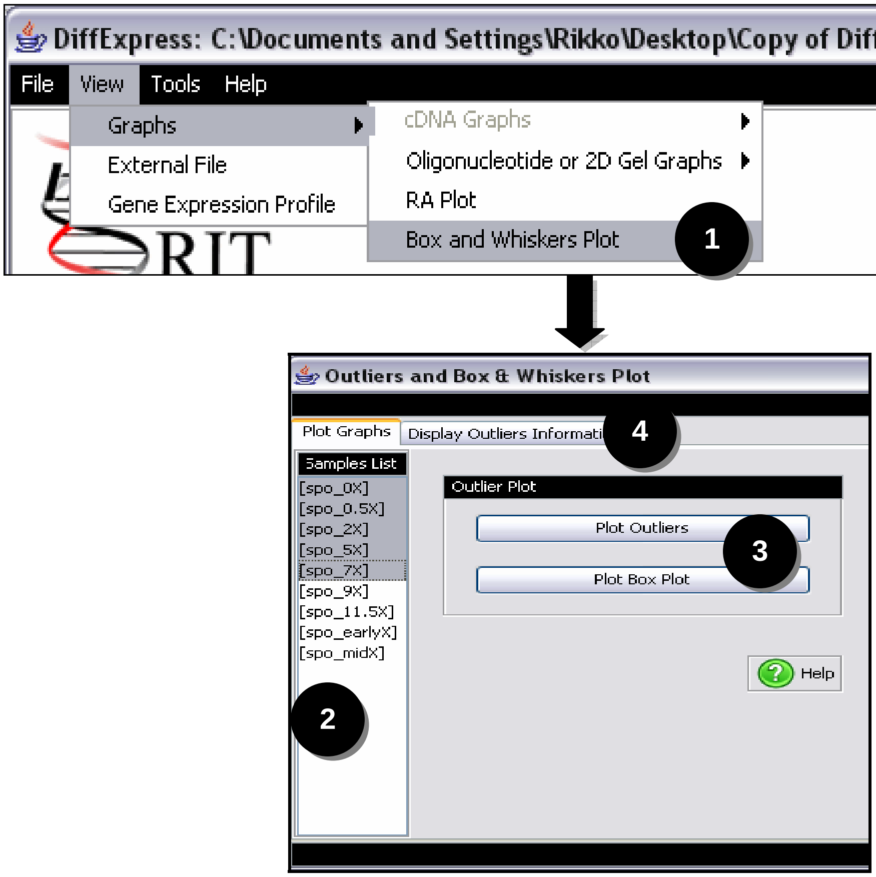
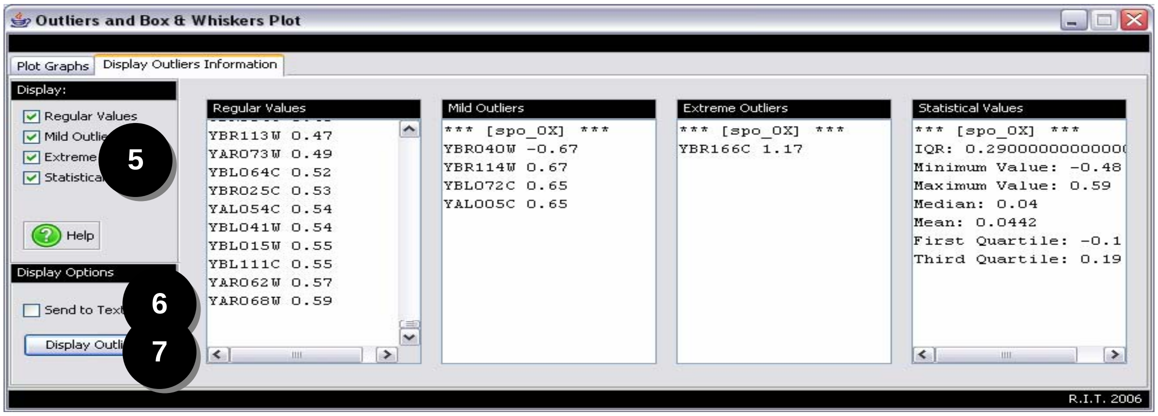


Figure A17: Outliers and Box & Whiskers Plot: Plot Graphs



Regular Values:

```
YBR113W 0.47
YAR073W 0.49
YBL064C 0.52
YBR025C 0.53
YAL054C 0.54
YBL041W 0.54
YBL015W 0.55
YBL111C 0.55
YAR062W 0.57
YAR068W 0.59
```

Mild Outliers:

```
*** [spo_OX] ***
YBR040W -0.67
YBR114W 0.67
YBL072C 0.65
YAL005C 0.65
```

Extreme Outliers:

```
*** [spo_OX] ***
YBR166C 1.17
```

Statistical Values:

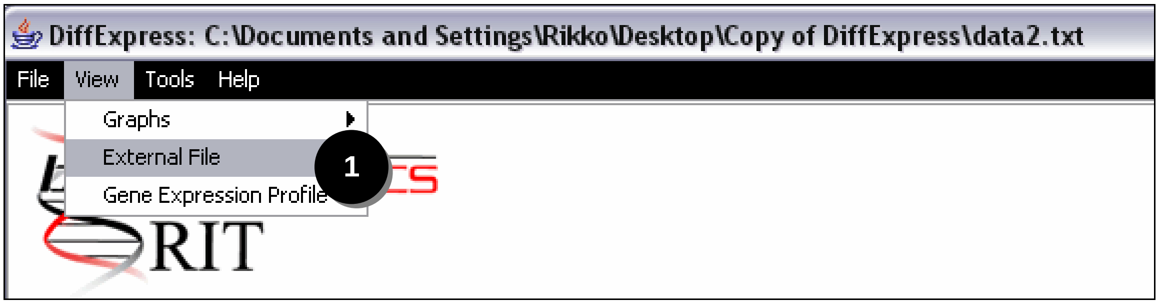
```
*** [spo_OX] ***
IQR: 0.2900000000000(
Minimum Value: -0.48
Maximum Value: 0.59
Median: 0.04
Mean: 0.0442
First Quartile: -0.1
Third Quartile: 0.19
```

Figure A18: Outliers and Box & Whiskers Plot: Display Outliers Information

5.2 View External Files

Follow these steps to view external text files:

1. From the menu bar: View/ External File.



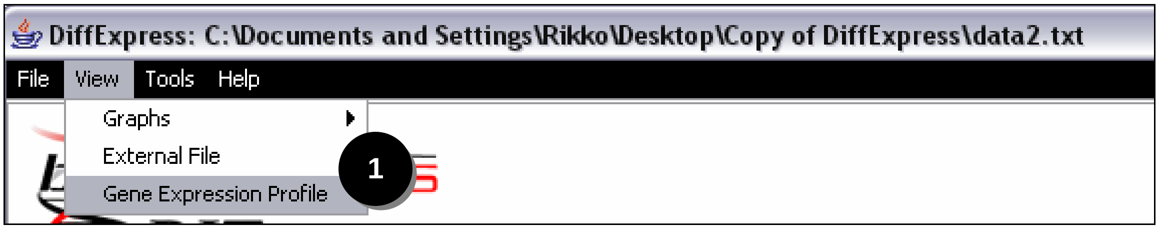
DiffExpress: C:\Documents and Settings\Rikko\Desktop\Copy of DiffExpress\data2.txt
File
View
Tools
Help
Graphs
External File
Gene Expression Profile
1
RIT

Figure A19: Viewing External Text Files

5.3 View Gene Expression Profile

Follow these steps to view the gene expression profile:

1. From the menu bar: View/ Gene Expression Profile.



DiffExpress: C:\Documents and Settings\Rikko\Desktop\Copy of DiffExpress\data2.txt
File
View
Tools
Help
Graphs
External File
Gene Expression Profile
1

Figure A20: Viewing Gene Expression Profile

6 Tools

6.1 Sorting

Follow these steps to sort the sample lists (Figure A21):

1. From the menu bar: Tools/ Options/ Sort Sample Lists/ (Sort By Sample ID, or Sort By 3rd Variable, or Sort By 4th Variable).

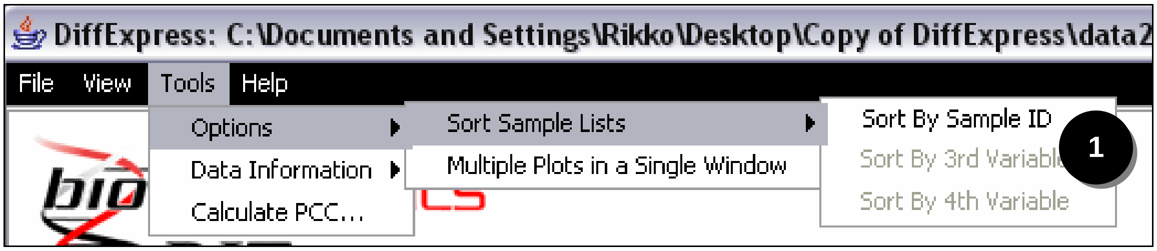


Figure A21: Sorting Sample Lists

6.2 Multiple Plots in a Single Window

Follow these steps to group plots of the same type in a single window (Figure A22):

1. From the menu bar: Tools/ Options/ Multiple Plots in a Single Window.

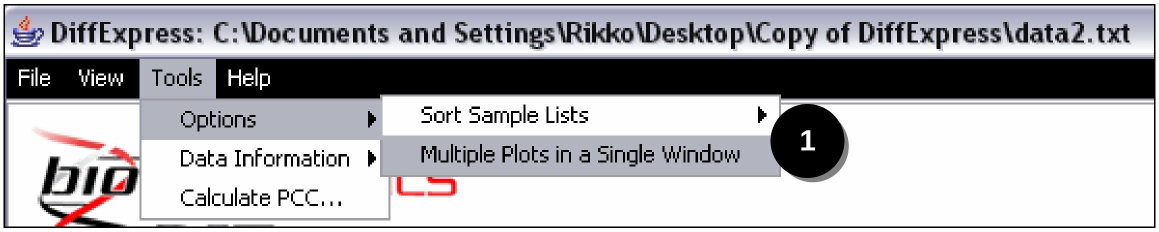

DiffExpress: C:\Documents and Settings\Rikko\Desktop\Copy of DiffExpress\data2.txt
File
View
Tools
Help
Options
Data Information
Calculate PCC...
Sort Sample Lists
Multiple Plots in a Single Window
1

Figure A22: Multiple Plots in a Single Window

6.3 Data Information

6.3.1 Basic Data Information

Follow these steps to get the number of genes or spot ids, number of samples, gene ids or spot ids, sample ids, and number of variables (Figure A23):

1. From the menu bar: Tools/ Data Information/ Basic Data Information.

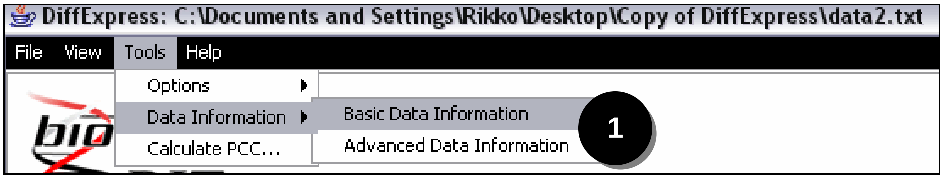

DiffExpress: C:\Documents and Settings\Rikko\Desktop\Copy of DiffExpress\data2.txt
File
View
Tools
Help
Options
Data Information
Calculate PCC...
Basic Data Information
Advanced Data Information
1
bio

Figure A23: Basic Data Information

6.3.2 Advanced Data Information

Follow these steps to specify the type of input data loaded (Figure A24):

1. From the menu bar: Tools/ Data Information/ Advanced Data Information.
2. Select type of input data.
3. Select log transformation type.
4. Select comparison type and Click the *OK* button.

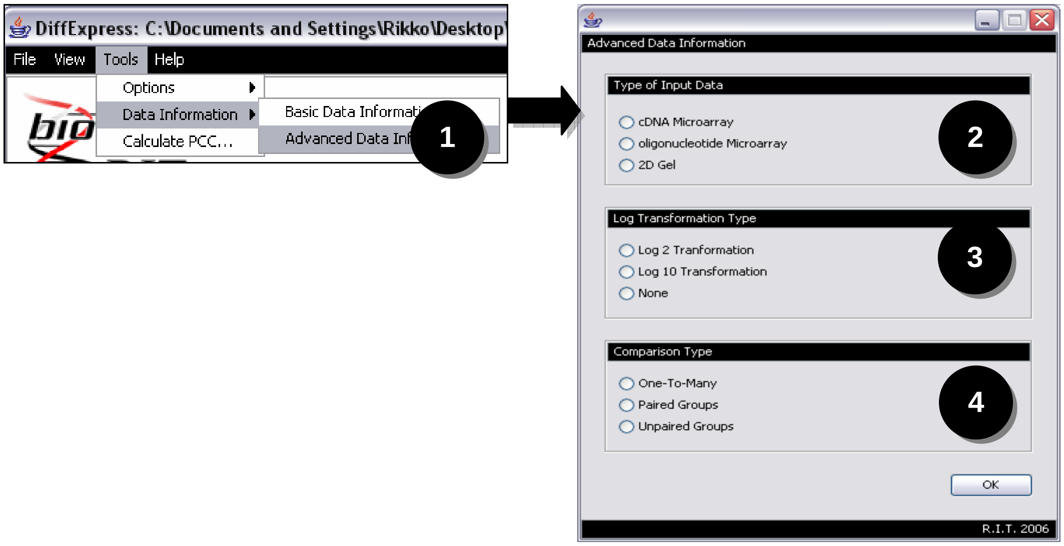


Figure A24: Advanced Data Information

6.4 Calculate PCC

Follow these steps to calculate the Pearson correlation coefficient between two samples (Figure A25):

1. From the menu bar: Tools/ Calculate PCC
2. From the *Samples List* list, select a sample and click the *Add Sample 1* button. Select another sample and click the *Add Sample 2* button.
3. Click the *Calculate PCC* button.

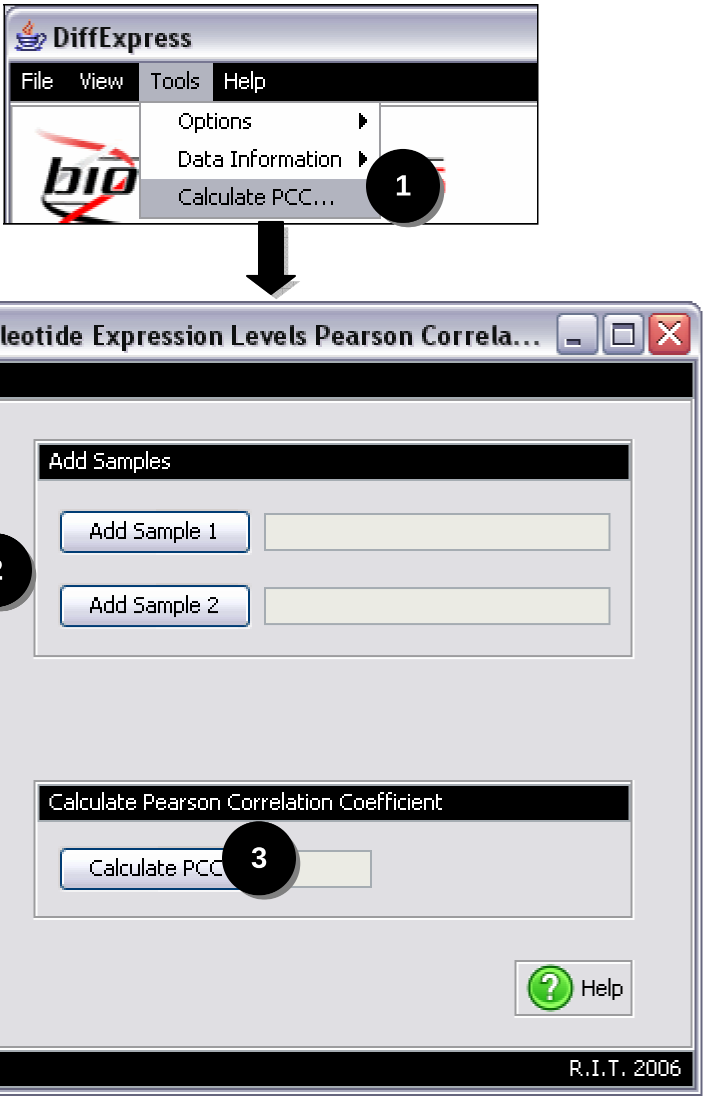


Figure A25: Pearson Correlation Coefficient Options Window

7 Listing Differentially Expressed Genes or Proteins

Follow the steps below to get a list of differentially expressed genes based on expression change (Figure A26):

1. Select one or more baseline samples.
2. If dealing with oligonucleotide or 2D gel data, select one or more experimental samples.
3. Choose an expression change.
4. Enter a threshold range.
5. Click the *List Genes* button.

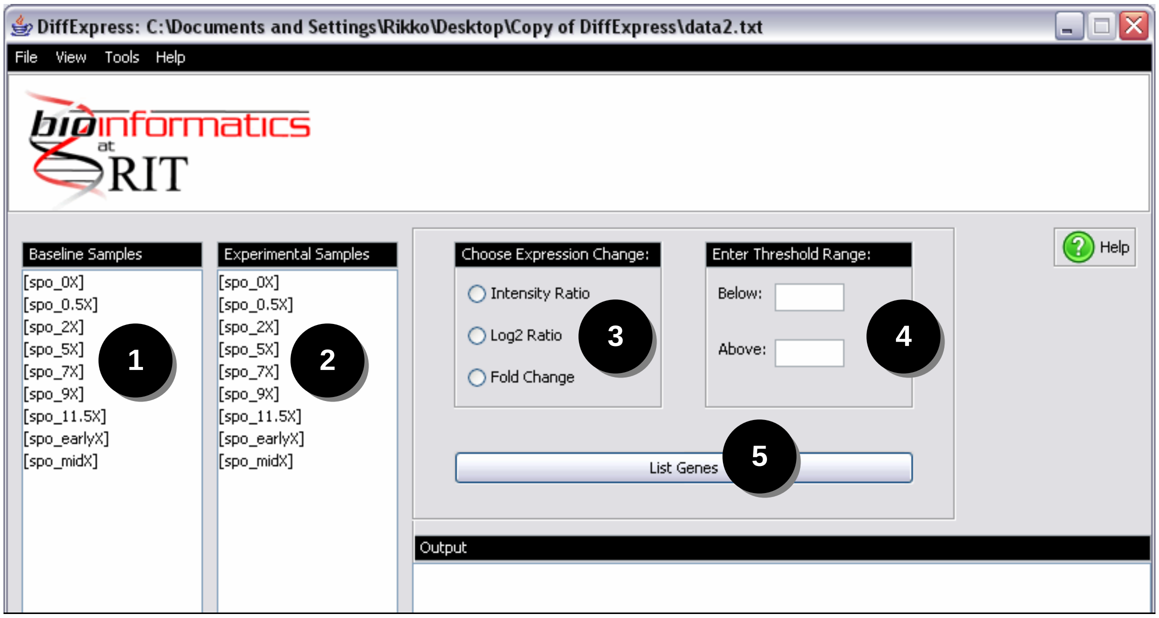


Figure A26: Identifying Differentially Expressed Genes

8 Help

When clicked, the *Help* button (Figure A27) featured throughout DiffExpress displays help for its respective window.

Figure A27: Help Button