

puma User Guide

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1 Abstract

Most analyses of Affymetrix GeneChip data (including traditional 3' arrays and exon arrays) are based on point estimates of expression levels and ignore the uncertainty of such estimates. By propagating uncertainty to downstream analyses we can improve results from microarray analyses. For the first time, the *puma* package makes a suite of uncertainty propagation methods available to a general audience. In addition to calculating gene expression from Affymetrix 3' arrays, *puma* also provides methods to process exon arrays and produces gene and isoform expression for alternative splicing study. *puma* also offers improvements in terms of scope and speed of execution over previously available uncertainty propagation methods. Included are summarisation, differential expression detection, clustering and PCA methods, together with useful plotting functions.

2 Citing *puma*

The *puma* package is based on a large body of methodological research. Citing *puma* in publications will usually involve citing one or more of the methodology papers (1),(2),(3),(4),(5),(6),(7),(8) that the software is based on as well as citing the software package itself. For the methodology papers, see <http://www.bioinf.manchester.ac.uk/resources/puma/>. *puma* makes use of the `donlp2()` function (9) by Peter Spellucci. The use of `donlp2()` must be acknowledged in any publication which contains results obtained with *puma* or parts of it. Citation of the author's name and `netlib-source` is suitable. The software itself as well as the extension of PPLR to the multi-factorial case (the `pumaDE` function) can be cited as:

puma: a Bioconductor package for Propagating Uncertainty in Microarray Analysis (2007) Pearson et al. BMC Bioinformatics, 2009, 10:211

The functionalities for processing of exon array data to calculate isoform and gene expression can be cited as:

puma 3.0: improved uncertainty propagation methods for gene and transcript expression analysis, Liu et al. BMC Bioinformatics, 2013, 14:39.

3 Introduction

Microarrays provide a practical method for measuring the expression level of thousands of genes simultaneously. This technology is associated with many significant sources of experimental uncertainty, which must be considered in order to make confident inferences from the data. Affymetrix GeneChip arrays have multiple probes associated with each target. The probe-set can be used to measure the target concentration and this measurement is then used in the downstream analysis to achieve the biological aims of the experiment, e.g. to detect significant differential expression between conditions, or for the visualisation, clustering or supervised classification of data.

Most currently popular methods for the probe-level analysis of Affymetrix arrays (e.g. RMA, MAS5.0) only provide a single point estimate that summarises the target concentration. Yet the probe-set also contains much useful information about the uncertainty associated with this measurement. By using probabilistic methods for probe-level analysis it is possible to associate gene expression levels with credibility intervals that quantify the measurement uncertainty associated with the estimate of target concentration within a sample. This within-sample variance is a very significant source of uncertainty in microarray experiments, especially for relatively weakly expressed genes, and we argue that this information should not be discarded after the probe-level analysis. Indeed, we provide a number of examples where the inclusion of this information gives improved results on benchmark data sets when compared with more traditional methods which do not make use of this information.

PUMA is an acronym for Propagating Uncertainty in Microarray Analysis. The *puma* package is a suite of analysis methods for Affymetrix GeneChip data. It includes functions to:

1. Calculate expression levels and confidence measures for those levels from raw CEL file data.
2. Combine uncertainty information from replicate arrays
3. Determine differential expression between conditions, or between more complex contrasts such as interaction terms
4. Cluster data taking the expression-level uncertainty into account
5. Perform a noise-propagation version of principal components analysis (PCA)

There are a number of other Bioconductor packages which can be used to perform the various stages of analysis highlighted above. The *affy* package gives access to a number of methods for calculating expression levels from raw CEL file data. The *limma* package provides well-proven methods for determination of differentially expressed genes. Other packages give access to clustering and PCA methods. In keeping with the Bioconductor philosophy, we aim to reuse as much code as possible. In many cases, however, we offer

techniques that can be seen as alternatives to techniques available in other packages. Where this is the case, we have attempted to provide tools to enable the user to easily compare the different methods.

We believe that the best method for learning new techniques is to use them. As such, the majority of this user manual (Section 4) is given over to case studies which highlight different aspects of the package. The case studies include the scripts required to recreate the results shown. At present there is just one case study (based on data from the *estrogen* package), but others will soon be included.

One of the most popular packages within Bioconductor is *limma*. Because many users of the *puma* package are already likely to be familiar with *limma*, we have written a special section (Section 5), highlighting the similarities and differences between the two packages. While this section might help experienced *limma* users get up to speed with *puma* more quickly, it is not required reading, particularly for those with little or no experience of *limma*.

The main benefit of using the propagation of uncertainty in microarray analysis is the potential of improved end results. However, this improvement does come at the cost of increased computational demand, particularly that of the time required to run the various algorithms. The key algorithms are, however, parallelisable, and we have built this parallel functionality into the package. Users that have access to a computer cluster, or even a number of machines on a network, can make use of this functionality. Details of how this should be set up are given in Section 6. This section can be skipped by those who will be running *puma* on a single machine only.

The *puma* package is intended as a full analysis suite which can be used for all stages of a typical microarray analysis project. Many users will want to compare different analysis methods within R, and the package has been designed with this in mind. Some users, however, may prefer to carry out some stages of the analysis using tools other than R. Section 7 gives details on writing out results from key stages of a typical analysis, which can then be read into other software tools.

We have chosen to leave details of individual functions out of this vignette, though comprehensive details can be found in the online help for each function.

This software package uses the optimization program donlp2 (9).

4 Introductory example analysis - estrogen

In this section we introduce the main functions of the *puma* package by applying them to the data from the *estrogen* package

4.1 Installing the *puma* package

The recommended way to install *puma* is to use the `install` function available via the BiocManager package. Installing in this way should ensure that all appropriate dependencies are met.

```
> if (!requireNamespace("BiocManager", quietly=TRUE))
+   install.packages("BiocManager")
> BiocManager::install("puma")
```

4.2 Loading the package and getting help

The first step in any *puma* analysis is to load the package. Start *R*, and then type the following command:

```
> library(puma)
```

To get help on any function, use the `help` command. For example, to get help on the `pumaDE` type one of the following (they are equivalent):

```
> help(pumaDE)
> ?pumaDE
```

To see all functions that are available within *puma* type:

```
> help(package="puma")
```

4.3 Loading in the data

To import data using the *oligo* package, the user must have data at the probe-level. This means that if Affymetrix data are to be imported, the user is expected to have CEL files.

Once sets of such files are available, the user can use two tools, depending on the array manufacturer, to import the data: `read.celfiles` function for CEL files. To assist the user on obtaining the names of the CEL files, the package provides the `list.celfiles` function, which accept the same arguments as the `list.files` function defined in the *R base* package. The basic usage of the package tools to import CEL files present in the current directory consists in combining the `read.files` functions with their `list.files` function counterparts, as shown below, in a hypothetical example:

```

> library(oligo)
> estrogenFileNames<-list.celfiles()
> oligo.estrogen<-read.celfiles(celFiles)
> pData(oligo.estrogen) <- data.frame(
+   "estrogen"=c("absent","absent","present","present"
+               ,"absent","absent","present","present")
+ ,   "time.h"=c("10","10","10","10","48","48","48","48")
+ ,   row.names=row.names(pData(oligo.estrogen))
+ )

```

When the user use the `read.celfiles` function to read the CEL files, the *oligo* package will attempt to identify the annotation package required to read the data in. If this annotation package is not available on BioConductor, the user should use the *pdInfoBuilder* package to create to create an appropriate annotation. In case *oligo* fails to identify the annotation package's name correctly, the user can use the `pkgname` argument available for `read.celfiles`.

```

> show(oligo.estrogen)

```

```

ExpressionFeatureSet (storageMode: lockedEnvironment)
assayData: 409600 features, 8 samples
  element names: exprs
protocolData
  rowNames: low10-1.cel low10-2.cel ... high48-2.cel
            (8 total)
  varLabels: exprs dates
  varMetadata: labelDescription channel
phenoData
  rowNames: low10-1.cel low10-2.cel ... high48-2.cel
            (8 total)
  varLabels: index
  varMetadata: labelDescription channel
featureData: none
experimentData: use 'experimentData(object)'
Annotation: pd.hg.u95av2
[1] TRUE

```

Here we can see some information about `oligo.estrogen`

```

> pData(oligo.estrogen)

```

```

           index
low10-1.cel      1

```

low10-2.cel	2
high10-1.cel	3
high10-2.cel	4
low48-1.cel	5
low48-2.cel	6
high48-1.cel	7
high48-2.cel	8

We can see from this phenotype data that this experiment has 2 factors (estrogen and time.h), each of which has two levels (absent vs present, and 10 vs 48), hence this is a 2x2 factorial experiment. For each combination of levels we have two replicates, making a total of $2 \times 2 \times 2 = 8$ arrays.

4.4 Determining expression levels for Affymetrix array

We will first use multi-mgMOS to create an expression set object from our raw data. This step is similar to using other summarisation methods such as MAS5.0 or RMA, and for comparison purposes we will also create an expression set object from our raw data using RMA. Note that the following lines of code are likely to take a significant amount of time to run, so if you are in a hurry and you have the *pumadata* library loaded simply type `data(eset_oligo_mmgmos)` and `data(eset_oligo_rma)` at the command prompt.

```
> eset_estrogen_mmgmos <- mmgmos(oligo.estrogen, gsnorm="none")
> eset_estrogen_rma <- rma(oligo.estrogen)
```

Note that we have `gsnorm="none"` in running `mmgmos`. The `gsnorm` option enables different global scaling (between array) normalizations to be applied to the data. We have chosen to use no global scaling normalization here so that we can highlight the need for such normalization (which we do below). The default option with `mmgmos` is to provide a median global scaling normalization, and this is generally recommended.

Unlike many other methods, multi-mgMOS provides information about the expected uncertainty in the expression level, as well as a point estimate of the expression level.

```
> exprs(eset_estrogen_mmgmos)[1,]
> assayDataElement(eset_estrogen_mmgmos, "se.exprs")[1,]
```

Here we can see the expression levels, and standard errors of those expression levels, for the first probe set of the `oligo.estrogen` data set.

If we want to write out the expression levels and standard errors, to be used elsewhere, this can be done using the `write.results` function.

```
> write.results(eset_estrogen_mmgmos, file="eset_estrogen_mmgmos.rda")
```

This code will create seven different comma-separated value (csv) files in the working directory. `eset_estrogen_exprs.csv` will contain expression levels. `eset_estrogen_se.csv` will contain standard errors. The other files contain different percentiles of the posterior distribution, which will only be of interest to expert users. For more details type `?write.results` at the R prompt.

We also provide a method, `PMmmgmos`, to calculate gene expression level use PM intensities only. `PMmmgmos` also return an expression set object.

```
> eset_estrogen_pmmgmos <- PMmmgmos(oligo.estrogen,gsnorm="none")
```

The extraction of expression mean and standard deviation is the same as above.

4.5 Determining expression levels for Affymetrix exon arrays

4.5.1 gmoExon function

We use `gmoExon` function to calculate gene and transcript expression levels. First you have to install *oligo* packages for read exon arrays. Second, `gmoExon` needs to use the relationships between genes, transcripts and probes which we put into *pumadata* R package. You need to download the latest *pumadata* package, and type `BiocManager::install("pumadata")`, `library(pumadata)` at the R prompt to have it installed.

The following scripts use example to show the use of `gmoExon` function.

```
> setwd(CEL.path) ## go to the directory where you put the CEL files.
> exonFileNames<-c("C0006.CEL","C021.CEL"
+                  ,"F023_NEG.CEL","F043_NEG.CEL") ## or you can use exonFileNames<-list.files(CEL.path,pattern="*.CEL",full.names=T)
> oligo.exon<-read.celfiles(exonFileNames)
```

And now we can use the `gmoExon` function to calculate the gene and transcript expression levels.

```
> eset_gmoExon<-gmoExon(oligo.exon,exontype="Human",gsnorm="none")
```

Note that we have `exontype="Human"`, `gsnorm="none"` in running `gmoExon`. The `exontype` option enables `gmoExon` to be applied to Human, Mouse and Rat exon arrays. The `gsnorm` option enables different global scaling (between array) normalizations to be applied to the data. We have chosen to use no global scaling normalization here so that we can highlight the need for such normalization (which we do below). The default option with `gmoExon` is to provide a median global scaling normalization, and this is generally recommended.

```
> exprs(eset_gmoExon$gene)[1,]
> assayDataElement(eset_gmoExon$gene,"se.exprs")[1,]
> exprs(eset_gmoExon$transcript)[1,]
> assayDataElement(eset_gmoExon$transcript,"se.exprs")[1,]
```


Here we can see the gene and transcript expression levels, and standard errors of those expression levels, for the first mapped gene and transcripts of the oligo.exon data set

If we want to write out the expression levels and standard errors, to be used elsewhere, this can be done using the `write.results` function.

```
> write.results(eset_gmoExon$gene, file="eset_gmoExon_gene")
```

This code will create seven different comma-separated value (csv) files in the working directory. File `eset_gmoExon_gene_exprs.csv` will contain gene expression levels. `eset_gmoExon_gene_se.csv` will contain gene standard errors. The other files contain different percentiles of the posterior distribution, which will only be of interest to expert users. For more details type `?write.results` at the R prompt.

```
> write.results(eset_gmoExon$transcript, file="eset_gmoExon_transcript")
```

This code will create seven different comma-separated value (csv) files in the working directory. File `eset_gmoExon_transcript_exprs.csv` will contain transcript expression levels. File `eset_gmoExon_transcript_se.csv` will contain transcript standard errors. The other files contain different percentiles of the posterior distribution, which will only be of interest to expert users. For more details type `?write.results` at the R prompt.

4.5.2 igmoExon function

Besides the `gmoExon`, we also can use `igmoExon` to calculate gene and transcript expression levels, which is effective than `gmoExon` function. The function requires following inputs:

cel.path The directory where you put the CEL files.

SampleNameTable It is a tab-separated table with two columns, ordered by "Celnames", "Condition". For our testing dataset, we can formulate the SampleNameTable like below: Celnames: C006.CEL C021.CEL F023_POS.CEL F043_NEG.CEL ; Condition: 1 1 2 2

exontype A string with value be one of "Human", "Mouse", "Rat", specifying the chip type of the data.

gnsorm The way you want to normalized, and the value is one of "median", "none", "mean", "meanlog".

condition Yes or No. Choose Yes means the `igmoExon` function separately calculate the gene expression values by the conditions and then combined the every condition's result, and normalized finally. Choose No means the `igmoExon` calculate the gene expression values as same as the `gmoExon` function.

Here are the two example as follows:

```
> cel.path<-"cel.path" ## for example cel.path<-"/home/gao/celData"
> SampleNameTable<-"SampleNameTable"
> eset_igmoExon<-igmoExon(cel.path="cel.path",SampleNameTable="SampleNameTable"
+                           , exontype="Human"
+                           , gsnorm="none", condition="Yes")
```

And now we can use the same methods to get the gene and transcript levels by `exprs` and `se.exprs`.

4.6 Determining expression levels for Affymetrix hta2.0 arrays

We use `gmhta` function to calculate gene and transcript expression levels. First you have to install *oligo* packages for read hta2.0 arrays. Second, `gmhta` needs to use the relationships between genes, transcripts and probes which we put into *pumadata* R package. You need to download the latest *pumadata* package, and type `BiocManager::install("pumadata")`, `library(pumadata)` at the R prompt to have it installed.

The following scripts use example to show the use of `gmhta` function.

```
> setwd(cel.path) ## go to the directory where you put the CEL files.
> library(puma)
> library(oligo)
> oligo.hta<-read.celfiles(celFiles)
> eset_gmhta<-gmhta(oligo.hta,gsnorm="none")
```

Note that we have `gsnorm="none"` in running `gmhta`. The `gsnorm` option enables different global scaling (between array) normalizations to be applied to the data. We have chosen to use no global scaling normalization here so that we can highlight the need for such normalization (which we do below). The default option with `gmhta` is to provide a median global scaling normalization, and this is generally recommended.

```
> exprs(eset_gmhta$gene)[2,]
> se.exprs(eset_gmhta$gene)[2,]
> exprs(eset_gmhta$transcript)[2,]
> se.exprs(eset_gmhta$transcript)[2,]
```

Here we can see the gene and transcript expression levels, and standard errors of those expression levels, for the first mapped gene and transcripts of the `oligo.hta` data set

If we want to write out the expression levels and standard errors, to be used elsewhere, this can be done using the `write.results` function.

```
> write.results(eset_gmhta$gene, file="eset_gmhta_gene")
```

This code will create seven different comma-separated value (csv) files in the working directory. File `eset_gmhta_gene_exprs.csv` will contain gene expression levels. File `eset_gmhta_gene_se.csv` will contain gene standard errors. The other files contain different percentiles of the posterior distribution, which will only be of interest to expert users. For more details type `?write.results` at the R prompt.

```
> write.results(eset_gmhta$transcript, file="eset_gmhta_transcript")
```

This code will create seven different comma-separated value (csv) files in the working directory. File `eset_gmhta_transcript_exprs.csv` will contain transcript expression levels. File `eset_gmhta_transcript_se.csv` will contain transcript standard errors. The other files contain different percentiles of the posterior distribution, which will only be of interest to expert users. For more details type `?write.results` at the R prompt.

4.7 Determining gross differences between arrays

A useful first step in any microarray analysis is to look for gross differences between arrays. This can give an early indication of whether arrays are grouping according to the different factors being tested. This can also help to identify outlying arrays, which might indicate problems, and might lead an analyst to remove some arrays from further analysis.

Principal components analysis (PCA) is often used for determining such gross differences. *puma* has a variant of PCA called Propagating Uncertainty in Microarray Analysis Principal Components Analysis (*pumaPCA*) which can make use of the uncertainty in the expression levels determined by multi-mgMOS. Again, note that the following example can take some time to run, so to speed things up, simply type `data(pumapca_estrogen)` at the R prompt.

```
> pumapca_estrogen <- pumaPCA(eset_estrogen_mmgmos)
```

For comparison purposes, we will run standard PCA on the expression set created using RMA.

```
> pca_estrogen <- prcomp(t(exprs(eset_estrogen_rma)))
```

```

> par(mfrow=c(1,2))
> plot(pumapca_estrogen, legend1pos="right", legend2pos="top", main="pumaPCA")
> plot(
+     pca_estrogen$x
+ ,     xlab="Component 1"
+ ,     ylab="Component 2"
+ ,     pch=unclass(as.factor(pData(eset_estrogen_rma)[,1]))
+ ,     col=unclass(as.factor(pData(eset_estrogen_rma)[,2]))
+ ,     main="Standard PCA"
+ )

```



Figure 1: First two components after applying `pumapca` and `prcomp` to the `estrogen` data set processed by multi-mgMOS and RMA respectively.

It can be seen from Figure 1 that the first component appears to be separating the arrays by time, whereas the second component appears to be separating the arrays by presence or absence of estrogen. Note that grouping of the replicates is much tighter with multi-mgMOS/pumaPCA. With RMA/PCA, one of the absent.48 arrays appears to be closer to one of the absent.10 arrays than the other absent.48 array. This is not the case with multi-mgMOS/pumaPCA.

The results from `pumaPCA` can be written out to a text (csv) file as follows:

```

> write.results(pumapca_estrogen, file="pumapca_estrogen")

```

Before carrying out any further analysis, it is generally advisable to check the distributions of expression values created by your summarisation method. Like PCA analysis,

this can help in identifying problem arrays. It can also inform whether further normalisation needs to be carried out. One way of determining distributions is by using box plots.

```
> par(mfrow=c(1,2))
> boxplot(data.frame(exprs(eset_estrogen_mmgmos)),main="mmgMOS - No norm")
> boxplot(data.frame(exprs(eset_estrogen_rma)),main="Standard RMA")
```



Figure 2: Box plots for **estrogen** data set processed by multi-mgMOS and RMA respectively.

From Figure 2 we can see that the expression levels of the time=10 arrays are generally lower than those of the time=48 arrays, when summarised using multi-mgMOS. Note that we do not see this with RMA because the quantile normalisation used in RMA will remove such differences. If we intend to look for genes which are differentially expressed between time 10 and 48, we will first need to normalise the mmgmos results.

```
> eset_estrogen_mmgmos_normd <- pumaNormalize(eset_estrogen_mmgmos)
> boxplot(data.frame(exprs(eset_estrogen_mmgmos_normd))
+         , main="mmgMOS - median scaling")
```



Figure 3: Box plot for `estrogen` data set processed by multi-mgMOS and normalisation using global median scaling.

Figure 3 shows the data after global median scaling normalisation. We can now see that the distributions of expression levels are similar across arrays. Note that the default option when running `mmgmos` is to apply a global median scaling normalization, so this separate normalization using `pumaNormalize` will generally not be needed.

4.8 Identifying differentially expressed (DE) genes with PPLR method

There are many different methods available for identifying differentially expressed genes. *puma* incorporates the Probability of Positive Log Ratio (PPLR) method (5). The PPLR method can make use of the information about uncertainty in expression levels provided by multi-mgMOS. This proceeds in two stages. Firstly, the expression level information from the different replicates of each condition is combined to give a single expression level (and standard error of this expression level) for each condition. Note that the following code can take a long time to run and a new faster version, IPPLR, is now available as described in section 4.9. The end result is available as part of the *pumadata* package, so the following line can be replaced with `data(eset_estrogen_comb)`.

```
> eset_estrogen_comb <- pumaComb(eset_estrogen_mmgmos_normd)
```

Note that because this is a 2 x 2 factorial experiment, there are a number of contrasts that could potentially be of interest. *puma* will automatically calculate contrasts which are likely to be of interest for the particular design of your data set. For example, the following command shows which contrasts *puma* will calculate for this data set

```
> colnames(createContrastMatrix(eset_estrogen_comb))

[1] "present.10_vs_absent.10"
[2] "absent.48_vs_absent.10"
[3] "present.48_vs_present.10"
[4] "present.48_vs_absent.48"
[5] "estrogen_absent_vs_present"
[6] "time.h_10_vs_48"
[7] "Int__estrogen_absent.present_vs_time.h_10.48"
```

Here we can see that there are seven contrasts of potential interest. The first four are simple comparisons of two conditions. The next two are comparisons between the two levels of one of the factors. These are often referred to as “main effects”. The final contrast is known as an “interaction effect”.

Don’t worry if you are not familiar with factorial experiments and the previous paragraph seems confusing. The techniques of the *puma* package were originally developed for simple experiments where two different conditions are compared, and this will probably be how most people will use *puma*. For such comparisons there will be just one contrast of interest, namely “condition A vs condition B”.

The results from *pumaComb* can be written out to a text (csv) file as follows:

```
> write.reslts(eset_estrogen_comb, file="eset_estrogen_comb")
```

To identify genes that are differentially expressed between the different conditions use the *pumaDE* function. For the sake of comparison, we will also determine genes that are differentially expressed using a more well-known method, namely using the *limma* package on results from the RMA algorithm.

```
> pumaDERes <- pumaDE(eset_estrogen_comb)
> limmaRes <- calculateLimma(eset_estrogen_rma)
```

The results of these commands are ranked gene lists. If we want to write out the statistics of differential expression (the PPLR values), and the fold change values, we can use the *write.reslts*.

```
> write.reslts(pumaDERes, file="pumaDERes")
```

This code will create two different comma-separated value (csv) files in the working directory. `pumaDERes_statistics.csv` will contain the statistic of differential expression (PPLR values if created using `pumaDE`). `pumaDERes_FC.csv` will contain log fold changes.

Suppose we are particularly interested in the interaction term. We saw above that this was the seventh contrast identified by `puma`. The following commands will identify the gene deemed to be most likely to be differentially expressed due to the interaction term by our two methods

```
> topLimmaIntGene <- topGenes(limmaRes, contrast=7)
> toppumaDEIntGene <- topGenes(pumaDERes, contrast=7)
```

Let's look first at the gene determined by RMA/limma to be most likely to be differentially expressed due to the interaction term

```
> plotErrorBars(eset_estrogen_rma, topLimmaIntGene)
```

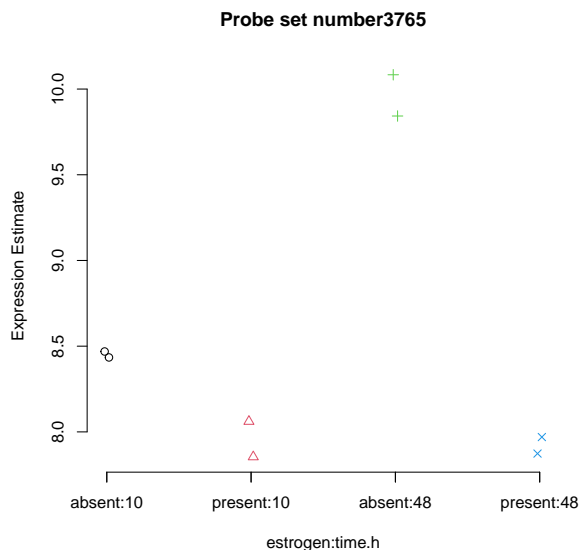


Figure 4: Expression levels (as calculated by RMA) for the gene most likely to be differentially expressed due to the interaction term in the `estrogen` data set by RMA/limma

The gene shown in Figure 4 would appear to be a good candidate for a DE gene. There seems to be an increase in the expression of this gene due to the combination of the `estrogen=absent` and `time=48` conditions. The within condition variance (i.e. between replicates) appears to be low, so it would seem that the effect we are seeing is real.

We will now look at this same gene, but showing both the expression level, and, crucially, the error bars of the expression levels, as determined by multi-mgMOS.

```
> plotErrorBars(eset_estrogen_mmgmos_normd, topLimmaIntGene)
```

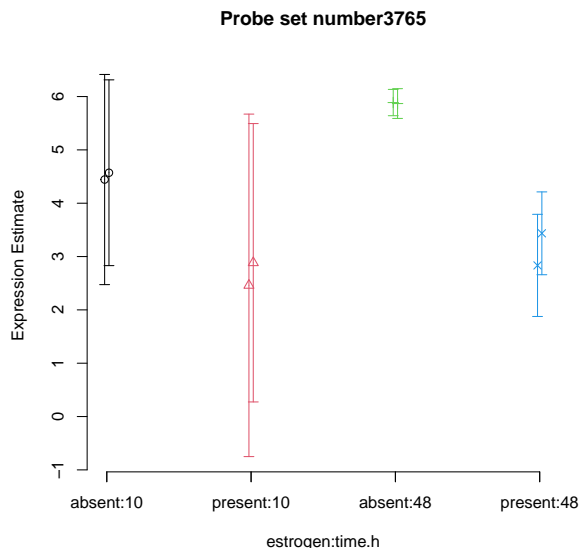


Figure 5: Expression levels and error bars (as calculated by multi-mgMOS) for the gene determined most likely to be differentially expressed due to the interaction term in the **estrogen** data set by RMA/limma

Figure 5 tells a somewhat different story from that shown in figure 4. Again, we see that the expected expression level for the absent:48 condition is higher than for other conditions. Also, we again see that the within condition variance of expected expression level is low (the two replicates within each condition have roughly the same value). However, from figure 5 we can now see that we actually have very little confidence in the expression level estimates (the error bars are large), particularly for the time=10 arrays. Indeed the error bars of absent:10 and present:10 both overlap with those of absent:48, indicating that the effect previously seen might actually be an artifact.

```
> plotErrorBars(eset_estrogen_mmgmos_normd, toppumaDEIntGene)
```



Figure 6: Expression levels and error bars (as calculated by multi-mgMOS) for the gene determined most likely to be differentially expressed due to the interaction term in the **estrogen** data set by mmgmos/pumaDE

Finally, figure 6 shows the gene determined by multi-mgMOS/PPLR to be most likely to be differentially expressed due to the interaction term. For this gene, there appears to be lower expression of this gene due to the combination of the estrogen=absent and time=48 conditions. Unlike with the gene shown in 5, however, there is no overlap in the error bars between the genes in this condition, and those in other conditions. Hence, this would appear to be a better candidate for a DE gene.

4.9 Identifying differentially expressed (DE) genes with IPPLR method

The PPLR method is useful as it effectively makes use of the information about uncertainty in expression level information. However, the original published version of PPLR uses importance sampling in the E-step of the variational EM algorithm which is quite computationally intensive, especially when the experiment involves a large number of chips.

The improved PPLR (IPPLR) method (7) also uses information about uncertainty in expression level information, but avoids the use of importance sampling in the E-step of the variational EM algorithm. This improves both computational efficiency and the IPPLR method greatly improves computational efficiency when there are many chips in

the dataset.

The IPPLR method identifies differentially expressed genes in two stages in the same way as the PPLR method described in Section 4.8. Firstly, the expression level information from the different replicates of each condition is combined to give a single expression level (and standard error of this expression level) for each condition. Note that the following dataset uses the `data(eset_mmgnos)`.

```
> data(eset_mmgnos)
> eset_mmgnos_100 <- eset_mmgnos[1:100,]
> pumaCombImproved <- pumaCombImproved(eset_mmgnos_100)
```

```
pumaComb expected completion time is 31 seconds
.....20%.....40%.....60%.....80%.....100%
.....
```

As described for the PPLR method, *puma* will automatically calculate contrasts which are likely to be of interest for the particular design of your data set. For example, the following command shows which contrasts *puma* will calculate for this data set

```
> colnames(createContrastMatrix(pumaCombImproved))

[1] "20.1_vs_10.1"
[2] "10.2_vs_10.1"
[3] "20.2_vs_20.1"
[4] "20.2_vs_10.2"
[5] "liver_10_vs_20"
[6] "scanner_1_vs_2"
[7] "Int__liver_10.20_vs_scanner_1.2"
```

From the results, we see that there are seven contrasts of potential interest. The first four are simple comparisons of two conditions. The next two are comparisons between the two levels of one of the factors. These are often referred to as “main effects”. The final contrast is known as an “interaction effect”.

The results from the `pumaCombImproved` can be written out to a text(csv) file as follows:

```
> write.results(pumaCombImproved,file="eset_mmgnos_combimproved")
```

The IPPLR method uses the PPLR values to identify the differentially expressed genes. This process is the same as the PPLR method, so the IPPLR method also uses the `pumaDE`.

```
> pumaDEResImproved <- pumaDE(pumaCombImproved)
```

The results of the command is ranked gene lists. If we want to write out the statistics of differential expression (the PPLR values), and the fold change values, we can use the `write.results`.

```
> write.results(pumaDEResImproved, file="pumaDEResImproved")
```

Section 4.9 gives further examples of how to use the results of this analysis.

4.10 Clustering with pumaClust

The following code will identify seven clusters from the output of `mmgmos`:

```
> pumaClust_estrogen <- pumaClust(eset_estrogen_mmgmos, clusters=7)
```

Clustering is performing
Done.

The result of this is a list with different components such as the cluster each probe-set is assigned to and cluster centers. The following code will identify the number of probesets in each cluster, the cluster centers, and will write out a csv file with probeset to cluster mappings:

```
> summary(as.factor(pumaClust_estrogen$cluster))
```

```
 1    2    3    4    5    6    7
2575 869 463 207 1438 147 6926
```

```
> pumaClust_estrogen$centers
```

```
  low10-1.cel low10-2.cel high10-1.cel high10-2.cel
1  -1.0683294 -0.9947219 -0.73765985 -0.54851597
2  -0.6151037 -0.5550043 -1.12338625 -0.86032118
3  -0.9760127 -0.9436758 -0.09253118  0.05522572
4  -0.9468928 -0.8241996 -0.93125464 -0.56268397
5  -0.9964496 -0.8539529 -0.93475236 -0.60904811
6  -0.8634344 -0.8159769 -1.01668664 -0.80343384
7  -0.9707527 -0.8794891 -0.94115054 -0.72965506
  low48-1.cel low48-2.cel high48-1.cel high48-2.cel
1   0.7057039  0.49703695   1.4006409   1.0255994
2   1.2420903  1.07428138   0.8376939   0.6127562
3   0.2800634  0.06561501   1.4233975   1.1885721
4   0.9812484  0.96317450   0.8045146   1.1875894
5   0.8591164  0.89476421   0.9977505   1.0973190
6   1.0843116  0.79769235   1.1733996   0.7120568
7   0.9075002  0.76717221   1.1784713   0.9236767
```

```
> write.csv(pumaClust_estrogen$cluster, file="pumaClust_clusters.csv")
```

4.11 Clustering with pumaClustii

The more recently developed `pumaClustii` method clusters probe-sets taking into account the uncertainties associated with gene expression measurements (from a probe-level analysis model like `mgMOS` and `multi-mmgMOS`) but also allowing for replicate information. The probabilistic model used is a Student's *t* mixture model (8) which provides greater robustness than the more standard Gaussian mixture model used by `pumaClust` (Section 4.10).

The following code will identify six clusters from the output of `mmgm`:

```
> data(Clustii.exampleE)
> data(Clustii.exampleStd)
> r<-vector(mode="integer",0)
> for (i in c(1:20))
+   for (j in c(1:4))
+     r<-c(r,i)
> cl<-pumaClustii(Clustii.exampleE,Clustii.exampleStd,
+               mincls=6,maxcls=6,conds=20,reprs=r,eps=1e-3)
```

Clustering is performing ...

Done.

In this example the vector `r` contains the labels identifying which experiments should be treated as replicate, and the `maxcls` and `mincls` are represented the maximum and minimum number of clusters respectively.

The result of this is a list with different components such as the cluster each probeset is assigned to and cluster centers. You can use the commands described in Section 4.10 to obtain information about the results.

4.12 Analysis using remapped CDFs

There is increasing awareness that the original probe-to-probeset mappings provided by `Affymetrix` are unreliable for various reasons. Various groups have developed alternative probe-to-probeset mappings, or “remapped CDFs”, and many of these are available either as `Bioconductor` annotation packages, or as easily downloadable `cdf` packages.

Importing the CEL files is achieved with the `read.celfiles` function. The function will, in general, correctly identify the annotation package to be used with the experimental data being imported, but the user can specify the `pkgname` argument to force the use of a particular one, if for some reason this is required. If the annotation package is not available from `BioConductor`, user can use the `pdInfoBuilder` package to build one.

If you have the (`pd.hg.u95a`) and `pd.hg.u95b` annotation packages, you can do it as follows:

```
> oligo.estrogen<-read.celfiles(celFiles,pkgname="pd.hg.u95a")
```

One of the issues with using remapped CDFs is that many probesets in the remapped data have very few probes. This makes reliable estimation of the expression level of such probesets even more problematic than with the original mappings. Because of this, we believe that even greater attention should be given to the uncertainty in expression level measurements when using remapped CDFs than when using the original mappings. In this section we show how to apply the uncertainty propagation methods of puma to the re-analysis the estrogen data using a remapped CDF. Note that most of the commands in this section are the same as the commands in the previous section, showing how straight-forward it is to do such analysis in puma.

To see the effect of the remapping, the following commands give the numbers of probes per probeset using the original, and the remapped CDFs:

```
> table(table(oligo::probeNames(oligo.estrogen)))
```

6	7	8	9	10	11	12	13	14	15
8	3	3	4	1	4	11	53	45	39
16	20	69							
12387	66	1							

Note that, while for the original mapping the vast majority of probesets have 16 probes, for the remapped CDF many probesets have less than 16 probes. With this particular CDF, probesets with less than 5 probes have been excluded, but this is not the same for all remapped CDFs.

Analysis can then proceed essentially as before. In the following we will compare the use of mmgmos/pumaPCA/pumaDE with that of RMA/PCA/limma.

5 *puma* for *limma* users

puma and *limma* both have the same primary goal: to identify differentially expressed genes. Given that many potential users of *puma* will already be familiar with *limma*, we have consciously attempted to incorporate many of the features of *limma*. Most importantly we have made the way models are specified in *puma*, through the creation of design and contrast matrices, very similar to way this is done in *limma*. Indeed, if you have already created design and contrast matrices in *limma*, these same matrices can be used as arguments to the `pumaComb` and `pumaDE` functions.

One of the big differences between the two packages is the ability to automatically create design and contrast matrices within *puma*, based on the phenotype data supplied with the raw data. We believe that these automatically created matrices will be sufficient for the large majority of analyses, including factorial designs with up to three different factors. It is even possible, through the use of the `createDesignMatrix` and `createContrastMatrix` functions, to automatically create these matrices using *puma*, but then use them in a *limma* analysis. More details on the automatic creation of design and contrast matrices is given in Appendix A.

One type of analysis that cannot currently be performed within *puma*, but that is available in *limma*, is the detection of genes which are differentially expressed in at least one out of three or more different conditions (see e.g. Section 8.6 of the *limma* user manual). Factors with more than two levels can be analysed within *puma*, but only at present by doing pairwise comparisons of the different levels. The authors are currently working on extending the functionality of *puma* to incorporate the detection of genes differentially expressed in at least one level of multi-level factors.

puma is currently only applicable to Affymetrix GeneChip arrays, unlike *limma*, which is applicable to a wide range of arrays. This is due to the calculation of expression level uncertainties within multi-mgMOS from the PM and MM probes which are specific to GeneChip arrays.

6 Parallel processing with *puma*

The most time-consuming step in a typical *puma* analysis is running the `pumaComb` function. This function, however, operates on a probe set by probe set basis, and therefore it is possible to divide the full set of probe sets into a number of different “chunks”, and process each chunk separately on separate machines, or even on separate cores of a single multi-core machine, hence significantly speeding up the function.

This parallel processing capability has been built in to the *puma* package, making use of functionality from the R package *snow*. The *snow* package itself has been designed to run on the following three underlying technologies: MPI, PVM and socket connections. The *puma* package has only been tested using MPI and socket connections. We have found little difference in processing time between these two methods, and currently recommend the use of socket connections as this is easier to set up. Parallel processing in *puma* has also only been tested to date on a Sun GridEngine cluster. The steps to set up *puma* on such an architecture using socket connections and MPI are discussed in the following two sections

6.1 Parallel processing using socket connections

If you do not already have the package *snow* installed, install this using the following commands:

```
> if (!requireNamespace("BiocManager", quietly=TRUE))
+   install.packages("BiocManager")
> BiocManager::install("snow")
```

6.1.1 Parallelisation of `pumaComb`

To use the parallel functionality of `pumaComb` you will first need to create a `snow` “cluster”. This can be done with the following commands. Note that you can have as many nodes in the `makeCluster` command as you like. You will need to use your own machine names in the place of “node01”, “node02”, etc. Note you can also use full IP addresses instead of machine names.

```
> library(snow)
> cl <- makeCluster(c("node01", "node02", "node03", "node04"), type = "SOCK")
```

You can then run `pumaComb` with the created cluster, ensuring the `cl` parameter is set, as in the following example, which compares the times running on a single node, and running on four nodes:

```
> library(puma)
> data(affybatch.estrogen)
```



```

> pData(affybatch.estrogen) <- data.frame(
+       "level"=c("twenty", "twenty", "ten")
+       ,       "batch"=c("A", "B", "A")
+       ,       row.names=row.names(pData(affybatch.example)))
> eset_mmgmos <- mmgmos(oligo.estrogen)
> system.time(eset_comb_1 <- pumaComb(eset_mmgmos))
> system.time(eset_comb_4 <- pumaComb(eset_mmgmos, cl=cl))

```

To run pumaComb on multi-cores of a multi-core machine, use a makeCluster command such as the following:

```

> library(snow)
> cl <- makeCluster(c("localhost", "localhost"), type = "SOCK")

```

We have found that on a dual-core notebook, the using both cores reduced execution time by about a third.

Finally, to run on multi-cores of a multi-node cluster, a command such as the following can be used:

```

> library(snow)
> cl <- makeCluster(c("node01", "node01", "node02", "node02"
+ , "node03", "node03", "node04", "node04"), type = "SOCK")

```

6.1.2 Parallelisation of gmoExon

To use the parallel functionality of gmoExon you also need to create a snow “cluster”. This can be done with the following commands:

```

> library(snow)
> cl<-makeCluster(c("node01", "node02", "node03", "node04"), type = "SOCK")

```

You can then run gmoExon with the created cluster, as in the following example:

```

> library(puma)
> library(oligo)
> object<-read.celfiles("filename.CEL")
> eset<-gmoExon(object, exontype="Human", GT="gene", gsnorm="none", cl=cl)

```

To run gmoExon on multi-core machine, use a makeCluster command such as the following:

```

> ibrary(snow)
> cl<-makeCluster(c("loaclhost", "localhost"), type = "SOCK")

```

Finally, to run on multi-cores of a multi-node cluster, a command such as the following can be used:

```

> library(snow)
> cl<-makeCluster(c("node01", "node01", "node02" , "node02"
+ , "node03" , "node03" , "node04" , "node04"), type = "SOCK")

```

6.1.3 Parallelisation of gmhta

To use the parallel functionality of gmhta you also need to create a snow “cluster”. This can be done with the following commands:

```
> library(snow)
> cl<-makeCluster(c("node01","node02","node03","node04"),type = "SOCK")
```

You can then run gmhta with the created cluster, as in the following example:

```
> library(puma)
> library(oligo)
> object<-read.celfiles("filename.CEL")
> eset<-gmhta(object,gsnorm="none",cl=cl)
```

To run gmhta on multi-core machine, use a makeCluster command such as the following:

```
> library(snow)
> cl<-makeCluster(c("localhost","localhost"),type = "SOCK")
```

Finally, to run on multi-cores of a multi-node cluster, a command such as the following can be used:

```
> library(snow)
> cl<-makeCluster(c("node01","node01","node02" ,"node02"
+ ,"node03" ,"node03" ,"node04" ,"node04"), type = "SOCK")
```

6.2 Parallel processing using MPI

First follow the steps listed here:

1. Download the latest version of lam-mpi from <http://www.lam-mpi.org/>
2. Install lam-mpi following the instructions available at <http://www.lam-mpi.org/>
3. Create a text file called **hostfile**, the first line of which has the IP address of the master node of your cluster, and subsequent line of which have the IP addresses of each node you wish to use for processing
4. From the command line type the command **lamboot hostfile**. If this is successful you should see a message saying

LAM 7.1.2/MPI 2 C++/ROMIO - Indiana University

(or similar)

5. Install R and the *puma* package on each node of the cluster (note this will often simply involve running R CMD INSTALL on the master node)
6. Install the R packages *snow* and *Rmpi* on each node of the cluster

The function `pumaComb` should automatically run in parallel if the `lamboot` command was successful, and *puma*, *snow* and *Rmpi* are all installed on each node of the cluster. By default the function will use all available nodes.

If you want to override the default parallel behaviour of `pumaComb`, you can set up your own cluster which will subsequently be used by the function. This cluster has to be named `cl`. To run a cluster with, e.g. four nodes, run the following code:

```
> library(Rmpi)
> library(snow)
> cl <- makeCluster(4)
> clusterEvalQ(cl, library(puma))
```

Note that it is important to use the variable name `cl` to hold the `makeCluster` object as *puma* checks for a variable of this name. The argument to `makeCluster` (here 4) should be the number of nodes on which you want the processing to run (usually the same as the number of nodes included in the `hostfile` file, though can also be less).

Running `pumaComb` in parallel should generally give a speed up almost linear in terms of the number of nodes (e.g. with four nodes you should expect the function to complete in about a quarter of the time as if using just one node).

7 Session info

This vignette was created using the following:

```
> sessionInfo()
```

```
R version 4.3.0 RC (2023-04-13 r84266)
Platform: aarch64-apple-darwin20 (64-bit)
Running under: macOS Monterey 12.6.1
```

```
Matrix products: default
```

```
BLAS: /Library/Frameworks/R.framework/Versions/4.3-arm64/Resources/lib/libRblas.0.dylib
```

```
LAPACK: /Library/Frameworks/R.framework/Versions/4.3-arm64/Resources/lib/libRlapack.dylib
```

```
locale:
```

```
[1] C/en_US.UTF-8/en_US.UTF-8/C/en_US.UTF-8/en_US.UTF-8
```

time zone: America/New_York

tzcode source: internal

attached base packages:

```
[1] stats4      stats      graphics  grDevices  utils
[6] datasets    methods    base
```

other attached packages:

```
[1] limma_3.56.1      pd.hg.u95av2_3.12.0
[3] DBI_1.1.3          RSQLite_2.3.1
[5] pumadata_2.35.0    puma_3.42.0
[7] mclust_6.0.0       oligo_1.64.1
[9] Biostrings_2.68.1  GenomeInfoDb_1.36.0
[11] XVector_0.40.0     IRanges_2.34.0
[13] S4Vectors_0.38.1   Biobase_2.60.0
[15] oligoClasses_1.62.0 BiocGenerics_0.46.0
```

loaded via a namespace (and not attached):

```
[1] Matrix_1.5-4      bit_4.0.5
[3] preprocessCore_1.62.1 compiler_4.3.0
[5] BiocManager_1.30.20 crayon_1.5.2
[7] SummarizedExperiment_1.30.1 blob_1.2.4
[9] affxparser_1.72.0  GenomicRanges_1.52.0
[11] bitops_1.0-7       splines_4.3.0
[13] fastmap_1.1.1      lattice_0.21-8
[15] S4Arrays_1.0.4     ff_4.0.9
[17] iterators_1.0.14   DelayedArray_0.26.2
[19] MatrixGenerics_1.12.0 affy_1.78.0
[21] GenomeInfoDbData_1.2.10 affyio_1.70.0
[23] rlang_1.1.0        cachem_1.0.7
[25] bit64_4.0.5        memoise_2.0.1
[27] cli_3.6.1          zlibbioc_1.46.0
[29] foreach_1.5.2      grid_4.3.0
[31] vctrs_0.6.1        codetools_0.2-19
[33] RCurl_1.98-1.12    pkgconfig_2.0.3
[35] matrixStats_0.63.0 tools_4.3.0
```

A Automatic creation of design and contrast matrices

The *puma* package has been designed to be as easy to use as possible, while not compromising on power and flexibility. One of the most difficult tasks for many users, particularly those new to microarray analysis, or statistical analysis in general, is setting up design and contrast matrices. The *puma* package will automatically create such matrices, and we believe the way this is done will suffice for most users' needs.

It is important to recognise that the automatic creation of design and contrast matrices will only happen if appropriate information about the levels of each factor is available for each array in the experimental design. This data should be held in an `AnnotatedDataFrame` class. The easiest way of doing this is to ensure that the `affybatch` object holding the raw CEL file data has an appropriate `phenoData` slot. This information will then be passed through to any `ExpressionSet` object created, for example through the use of `mmgmos`. The `phenoData` slot of an `ExpressionSet` object can also be manipulated directly if necessary.

Design and contrast matrices are dependent on the experimental design. The simplest experimental designs have just one factor, and hence the `phenoData` slot will have a matrix with just one column. In this case, each unique value in that column will be treated as a distinct level of the factor, and hence `pumaComb` will group arrays according to these levels. If there are just two levels of the factor, e.g. A and B, the contrast matrix will also be very simple, with the only contrast of interest being A vs B. For factors with more than two levels, a contrast matrix will be created which reflects all possible combinations of levels. For example, if we have three levels A, B and C, the contrasts of interest will be A vs B, A vs C and B vs C. In addition, from *puma* version 1.2.1, the following additional contrasts will be created: A vs other (i.e. A vs B & C), B vs other and C vs other.

If we now consider the case of two or more factors, things become more complicated. There are now two cases to be considered: factorial experiments, and non-factorial experiments. A factorial experiment is one where all the combinations of the levels of each factor are tested by at least one array (though ideally we would have a number of biological replicates for each combination of factor levels). The *estrogen* case study (Section 4) is an example of a factorial experiment. A non-factorial experiment is one where at least one combination of levels is not tested. If we treat the example used in the `puma-package` help page as a two-factor experiment (with factors “level” and “batch”), we can see that this is not a factorial experiment as we have no array to test the conditions “level=ten” and “batch=B”. We will treat the factorial and non-factorial cases separately in the following sections.

A.1 Factorial experiments

For factorial experiments, the design matrix will use all columns from the `phenoData` slot. This will mean that `combineRepliactes` will group arrays according to a combination

of the levels of all the factors.

A.2 Non-factorial designs

For non-factorial designed experiments, we will simply ignore columns (right to left) from the `phenoData` slot until we have a factorial design or a single factor. We can see this in the example used in the `puma-package` help page. Here we have ignored the “batch” factor, and modelled the experiment as a single-factor experiment (with that single factor being “level”).

A.3 Further help

There are examples of the automated creation of design and contrast matrices for increasingly complex experimental designs in the help pages for `createDesignMatrix` and `createContrastMatrix`.

References

- [1] Milo M., Niranjana M., Holley M.C., Rattray M., and Lawrence N.D. A probabilistic approach for summarising oligonucleotide gene expression data. *Technical report available upon request*, 2005.
- [2] Liu X., Milo M., Lawrence N.D., and Rattray M. A tractable probabilistic model for affymetrix probe-level analysis across multiple chips. *Bioinformatics*, 21:3637–3644, 2005.
- [3] Sanguinetti G., Milo M., Rattray M., and Lawrence N.D. Accounting for probe-level noise in principal component analysis of microarray data. *Bioinformatics*, 21:3748–3754, 2005.
- [4] Rattray M., Liu X., Sanguinetti G., Milo M., and Lawrence N.D. Propagating uncertainty in microarray data analysis. *Briefings in Bioinformatics*, 7:37–47, 2006.
- [5] Liu X., Milo M., Lawrence N.D., and Rattray M. Probe-level measurement error improves accuracy in detecting differential gene expression. *Bioinformatics*, 22:2107–2113, 2006.
- [6] Liu X., Lin K.K., Andersen B., , and Rattray M. Including probe-level uncertainty in model-based gene expression clustering. *BMC Bioinformatics*, 8(98), 2007.
- [7] Li Zhang and Xuejun Liu. An improved probabilistic model for finding differential gene expression. *the 2nd BMEI 17–19*, oct 2009.
- [8] Liu X. and Rattray M. Including probe-level measurement error in robust mixture clustering of replicated microarray gene expression. *Statistical Application in Genetics and Molecular Biology*, 9(1):Article 42, 2010.
- [9] Peter Spellucci. Donlp2 code and accompanying documentation. *Electronically available via <http://plato.la.asu.edu/donlp2.html>*, 2002.