Technical Training

Week 3

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What is Bioconductor?

- https://www.bioconductor.org
- **Open source** and **Open development**
- **Free**
- Software project for analysis of genomic data - and related tools, resources/datasets
- Current version 3.11, consists of 1903 packages

Example of packages can you find in Bioconductor?

- Annotation packages
- Experimental data packages

Bioconductor basics

- Install Bioconductor

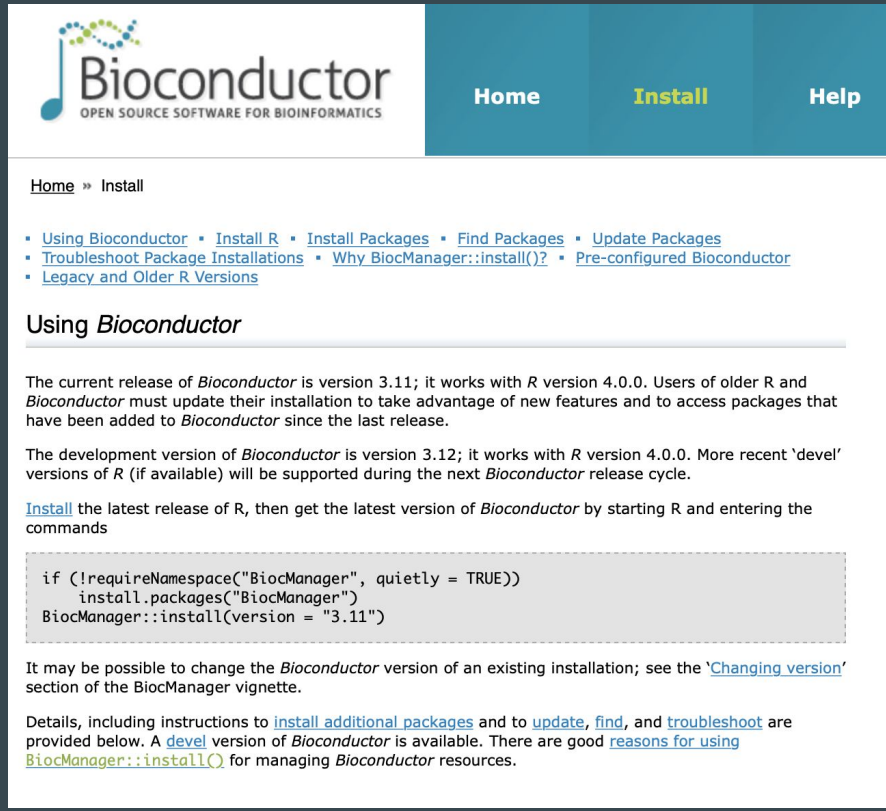
- Install the following libraries
  - affy
  - affyPLM
  - sva
  - AnnotationDbi
  - hgu133plus2.db
  - simpleaffy
  - arrayQualityMetrics
  - affyQCReport
  - gcrma

Using the code:

BiocManager::install('affy')

Load the library:

library(package_name)



Reading in the Data

1) Download the Raw.tar file from GEO website

2) Move the downloaded file to your working directory

3) Load affy package :

```
library(affy)
```

4) Read the .CEL.gz files directly from the folder containing them

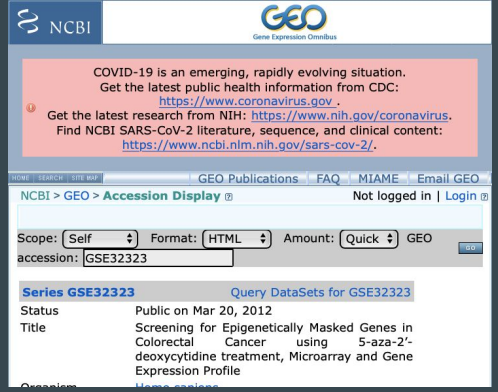
```
raw <- ReadAffy(celfile.path = "~/week_3/GSE32323_RAW")
```

5) Take a look at your data

```
df <- as.data.frame(exprs(raw))
```

NOT RECOMMEND TO VIEW THE WHOLE DATAFRAME

If you want to take a look at your data do: `View(head(df))`



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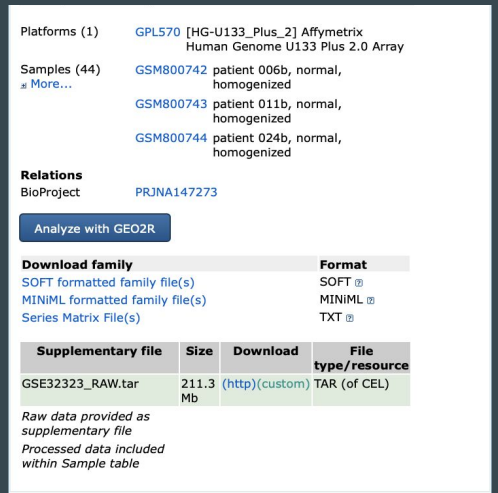


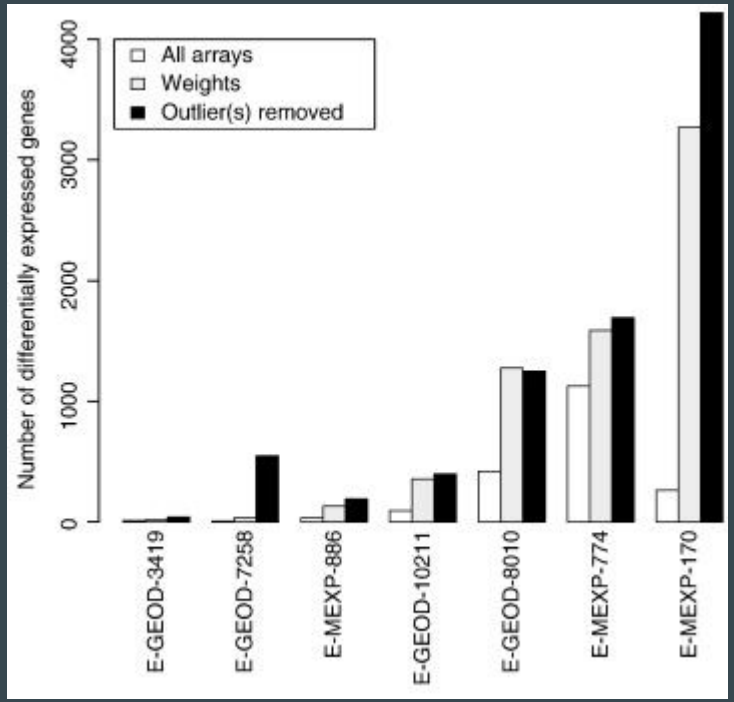
RNA Degradation, Hybridization, and Spike-in

- **Degradation of RNA** itself is a physiological process during the cell cycle to regulate RNA-dependent mechanisms.
  - Therefore, RNA degradation over time may affect the integrity of the samples and data
- **RNA Spike-in:** RNA transcript of known sequence and quantity used to calibrate measurements in RNA hybridization assays,
  - A **hybridization assay** comprises any form of quantifiable hybridization *i.e.* the quantitative annealing of two complementary strands of nucleic acids, known as nucleic acid hybridization.
  - **Spike-in controls** are needed in all types of genome-wide profiling analyses by microarray or sequencing where changes in absolute amounts of the total signal are suspected to occur between different experimental conditions.
- **Why is this relevant?** Such high-throughput methods can be error prone, and known controls are necessary to detect and correct for levels of error. RNA spike-in controls can provide a measure of sensitivity and specificity of an RNA-Seq experiment.

Why is this important?

- Being able to identify outlier data
- Help researchers identify the potential causes of outlier data
- Decide whether a sample needs to be repeated or removed from the set
- Improving the signal-to-noise ratio to improve power of test:
    - Signal-to-noise ratio (SNR) is a way to determine whether or not a peak should be considered when evaluating the data.

Note : systematic variation may be imparted by the experimental process itself and can be difficult to remove through standard normalization - may not be detected.

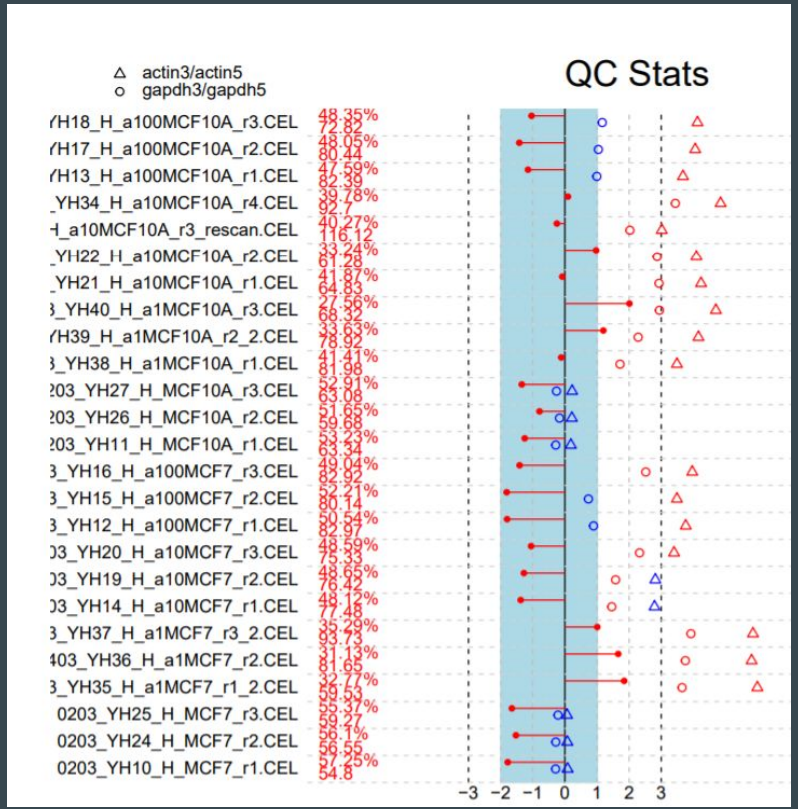


simpleAffy

“Provides high level functions for reading Affy .CEL files, phenotypic data, and then computing simple things with it, such as t-tests, fold changes and the like. Also has some basic scatter plot functions and mechanisms for generating high resolution journal figures”

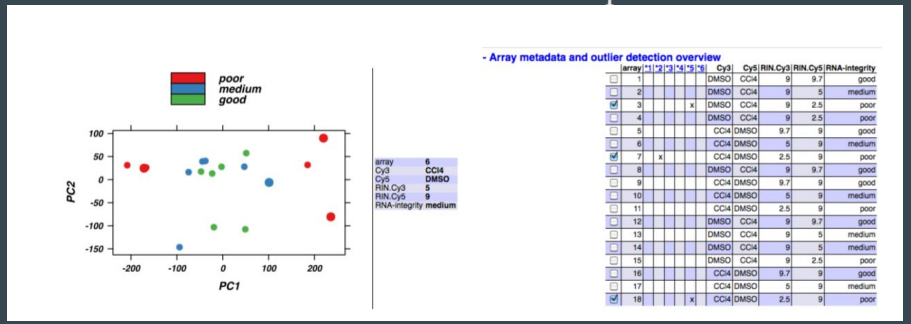
- A **CEL file** is a data **file** created by Affymetrix DNA microarray image analysis software. It contains the data extracted from "probes" on an Affymetrix GeneChip and can store thousands of data points, which may make it large in **file** size

QC Reports: The function qc produces an object of class ’QCStats’ containing QC metrics for each array in a project



Array Quality Metrics

- Provides a report with diagnostic plots for one or two colour microarray data.
  - assess reproducibility, identify apparent outlier arrays and compute measures of signal-to-noise ratio.
  - (i) assessing quality of a “raw” dataset, in order to get feedback on the experimental procedures that produced the data; (ii) assessing quality of a normalised dataset, in order to decide whether and how to use the dataset (or subsets of arrays in it) for subsequent data analysis.
- Note: You can use this function again after normalization of the dataset for assessing the difference in quality.
- **Goal**: find out correlation between samples and weed out bad data!



Affy QC Report

affyQCReport Package Link: affyQCReport

Example Report: affyQCExReport

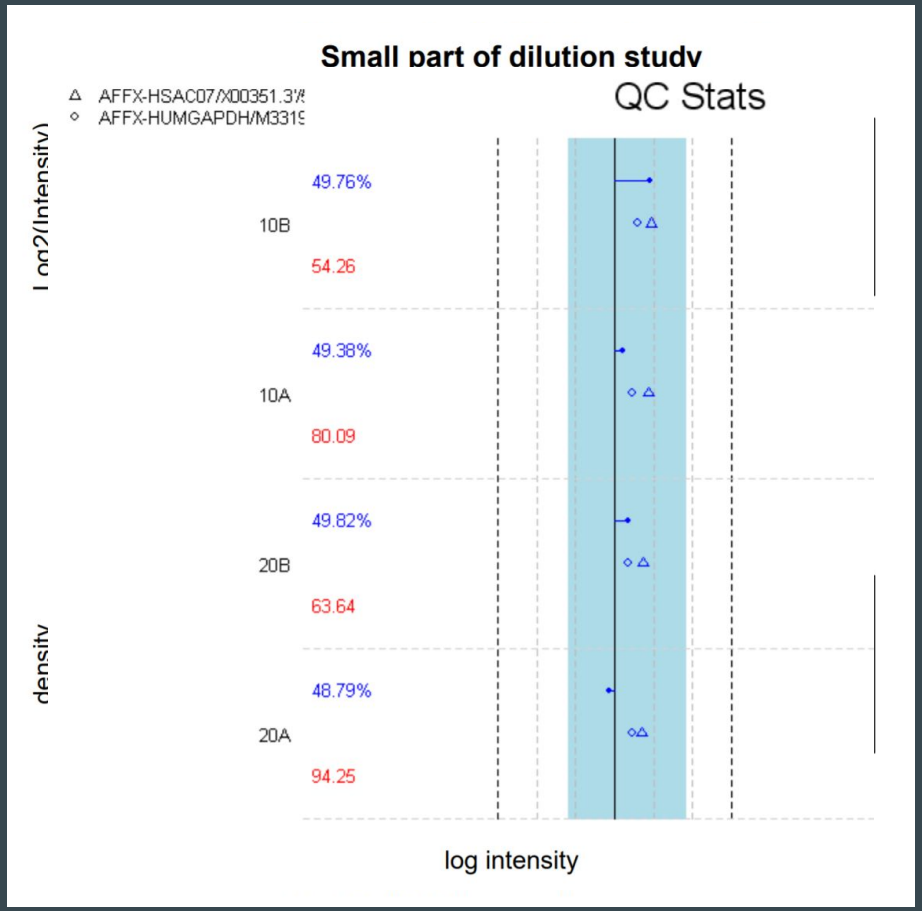
Installation

To install this package, start R (version "4.0") and enter:

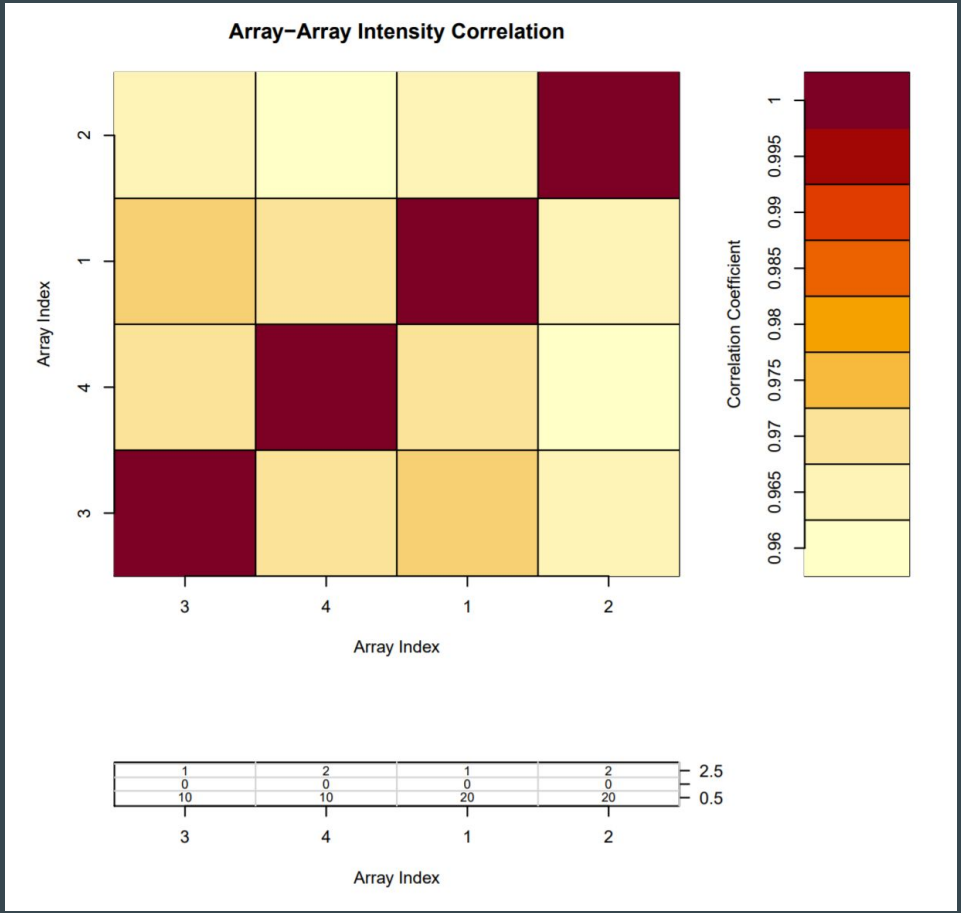
```
if (!requireNamespace("BiocManager", quietly = TRUE))
    install.packages("BiocManager")

BiocManager::install("affyQCReport")
```

- QC Report for an AffyBatch Object. The QC Report is intended to allow the user to quickly assess the quality of a set of arrays in an AffyBatch object
- The report generated consists of 6 pages:
  1) **First page:** list of the sample names and an index number that is used to identify each array in later plots.
  2) **Second page**: consists of two plots made using the affy package
  3) **Third page** is the QC plot generated with the simpleaffy package, which shows the 3' : 5' ratios for spiked-in and control genes specific to the array type.
  4) **Fourth page:** boxplots of the intensities of the positive and negative control elements on the outer edges of the Affymetrix arrays.
  5) **Fifth Page**: is a plot of the center of intensity" (COI) for the positive and negative border elements.
  6) **Sixth page:** is a heat map of the array-array Spearman rank correlation coefficients of the array intensities.

Affy QC Report [Cont.]



Affy QC Report [Cont.]



affyPLM + Relative Log Expression (RLE)

affyPLM Package Link: affyPLM

Installation

To install this package, start R (version "4.0") and enter:

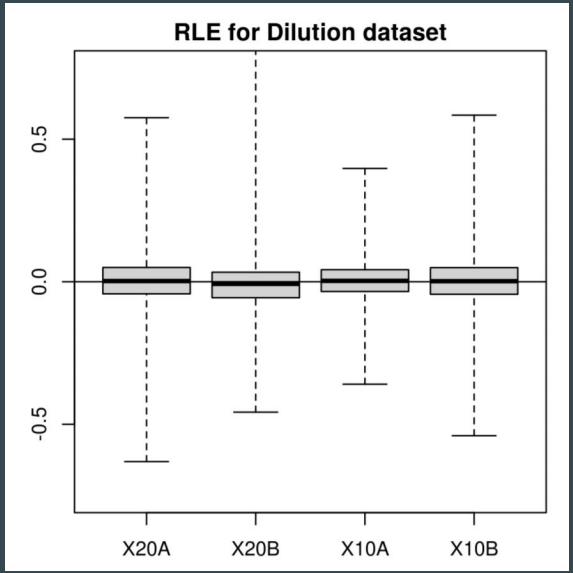
```
if (!requireNamespace("BiocManager", quietly = TRUE))
    install.packages("BiocManager")

BiocManager::install("affyPLM")
```

- This package creates a QC Report for an AffyBatch Object. The QC Report is intended to allow the user to quickly assess the quality of a set of arrays in an AffyBatch object
- The central focus of this package is on implementing methods for fitting probe-level models and tools (i.e. PLM-based Quality Assessment Tools) using these models.
- Relative Log Expression, also known as RLE, is a type of quality assessment tool.
    - RLE Values are computed for each probeset by comparing the expression value on each against the median expression value for that probeset across all arrays.
        - Assuming that most genes are not changing in expression across arrays means **ideally most of these RLE values will be near 0.**

affyPLM + Relative Log Expression (RLE) [Cont.]

- The user can create a boxplot of RLE Values (for each array) using:

```
> RLE(Pset,main="RLE for Dilution dataset")
```



affyPLM + Relative Log Expression (RLE) [Cont.]

- The user can also compute a summary of statistics using:

```
> RLE(Pset,type="stats")
```

	20A	20B
median	0.002359773	-0.007542337
IQR	0.092560253	0.089580148
	10A	10B
median	0.003051789	0.001902185
IQR	0.076335304	0.093406833

Normalized Unscaled Standard Error

- Useful for understanding how deparatures in quality of the data affect the expression estimates
- Want to combine residuals into estimated standard errors of expression estimates and summarize those a chip level

$$\text{unscaled SE } (c_{kj}) = 1/\sum_i w_{kij}$$

- For each chip, you get a vector of unscaled standard errors of estimated expression, one for each probe set
- To remove the source of heterogeneity, we can normalize the unscaled standard errors by diving the average value across the chips.

Different types of normalization methods

MAS5	RMA	GCRMA
Normalizes each array independently and sequentially.	RMA = robust multi-array, uses a multi-chip model. Generally, more commonly used today.	Gene chip RMA
Uses data from mismatch probes to calculate a "robust average", by subtracting mismatch probe values from match probe values. **However, subtracting mismatch data will result in loss of interesting signal in many probes and may cause noise at low intensity levels**	Doesn't use mismatch probes, because their intensities (positive values) are generally higher than match probes. Therefore they are unreliable as indicators of non-specific binding. With this method, normalizing at probe level avoids the loss of of information	Probe affinity calculated using position dependent base effects. Mismatch datta is based on probe affinity, and then subtracted from perfect match. In this way, no MM data is lost
Weights each probe intensity based on the distance from the mean (weighted mean uses one-step Tukey Biweight Estimate)	Data is a combination of background and signal. Assumes a strictly positive distribution for signal.	Combines intensity values from the probes in the probe set to get a single intensity value for each gene
Robust average means that it is insensitive to any small changes made from assumptions	Normalizes across all arrays to make all distributions the same. This protects against outliers	

Advantages & Disadvantages of RMA/GC-RMA vs. MAS5

Advantages	Disadvantages
Gives less false positives	May hide real changes, especially at low expression levels (i.e. false negatives)
Sees less variance at lower expression levels	Makes quality control after normalization more difficult
Provides more consistent fold change estimates	Normalization assumes equal distribution which may hide biological changes.
Inclusion of adjusted MM data in GC-RMA reduces noise, and retains MM data	Less precise than MAS5

Ideal solution - use standard MAS5.0 techniques for quality control. Then go back and perform probe level normalization on quality controlled genes

Principal Component Analysis (PCA)

- PCA is used for **exploratory data analysis**, allowing you to better visualize the variation within your dataset of many variables
- Particularly useful with **wide datasets**, where you have many variables in each sample

```
mtcars.pca <- prcomp(mtcars[,c(1:7,10,11)], center = TRUE,scale. = TRUE)

summary(mtcars.pca)

## Importance of components:
##                           PC1    PC2     PC3     PC4     PC5     PC6
## Standard deviation     2.3782 1.4429 0.71008 0.51481 0.42797 0.35184
## Proportion of Variance 0.6284 0.2313 0.05602 0.02945 0.02035 0.01375
## Cumulative Proportion  0.6284 0.8598 0.91581 0.94525 0.96560 0.97936
##                           PC7    PC8     PC9
## Standard deviation     0.32413 0.2419 0.14896
## Proportion of Variance 0.01167 0.0065 0.00247
## Cumulative Proportion  0.99103 0.9975 1.00000
```