Microarray

Lecture 2

Outline

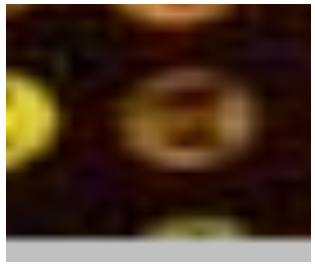
- Background
- Preprocessing of oligonucleotide microarray
- Quality Assessment for oligonucleotide Microarray
- Differential Expression Testing

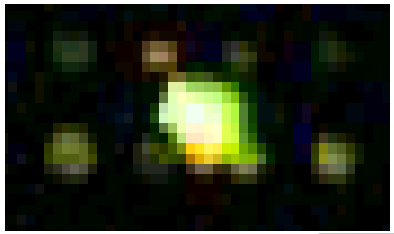
Some Causes of Technical Variation

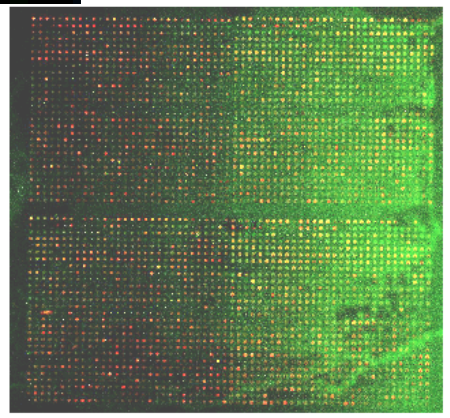
- Temperature of hybridization differs
- Amount of RNA differs
- RNA degraded in some samples
- Yield of conversion to cDNA differs
- Strength of ionic buffers differs
- Stringency of wash differs
- Scratches on some chips
- Ozone (affects Cy5) at some times

Spot QA for cDNA Spotted Arrays

- Spot Measures
  - Uniformity
  - Spot Area
- Inspect images for artifacts
- Global Measures
  - Qualitative assessments







Quality Assessment for oligonucleotide Microarray

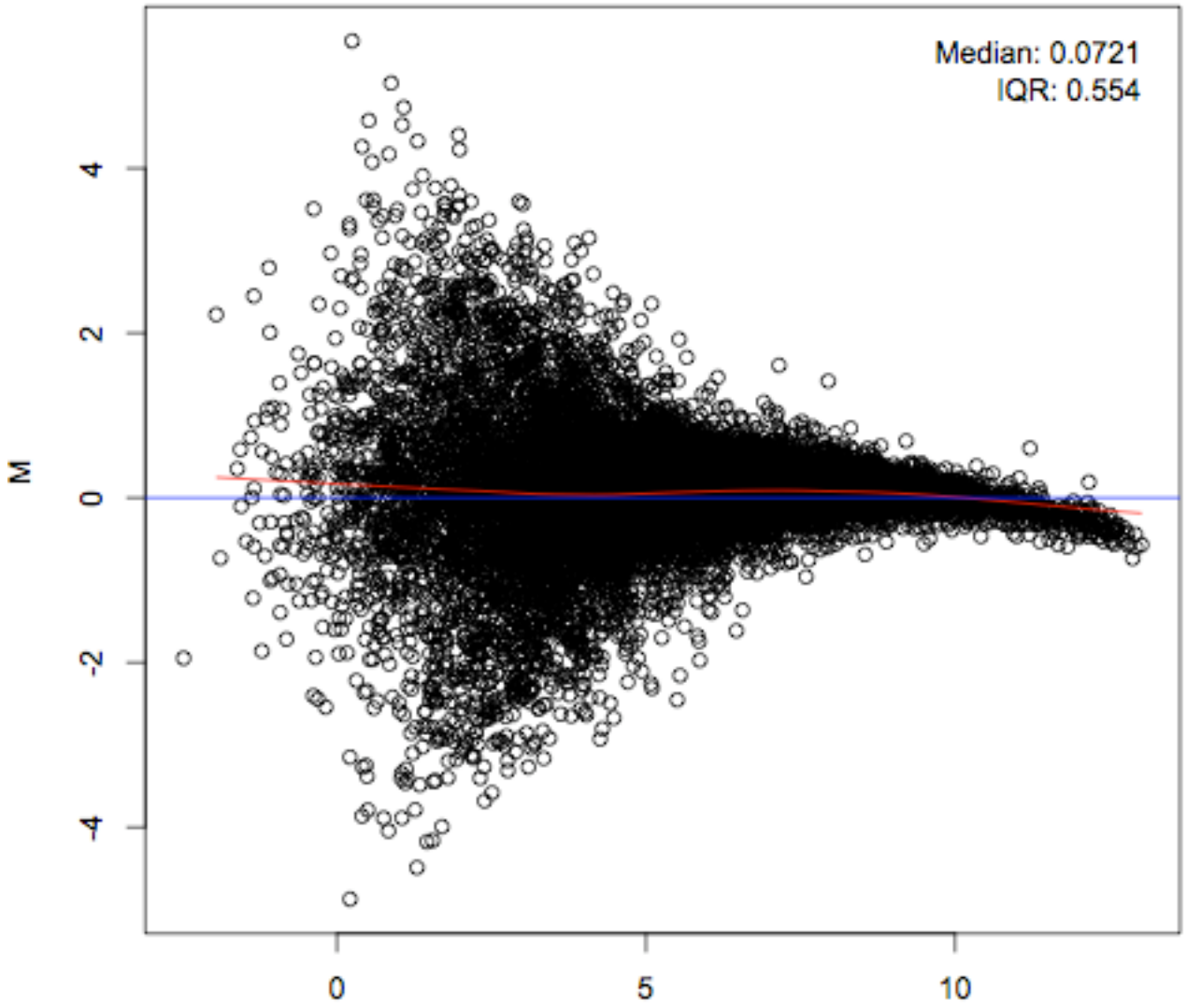
- Quality Assessment Plot
  - MA plot
  - Volcano plot
  - ….

- Quality Assessment Metric
  - 3’/5’ ratio
  - Covariation with Probe Position

MA plot - Ratio vs Intensity Plots

- "M" is the **log2 intensity ratio** for a probe in the two chips
- "A" is the **average log2 intensity** for a probe in the two chips
- The MA plot gives a quick overview of the distribution of the data.
- The general assumption is that most of the genes would not see any change in their expression.
- Therefore the majority of the points on the y axis (M) would be located at 0, since Log(1) is 0.
- How about for RNA-seq data?

MA plot

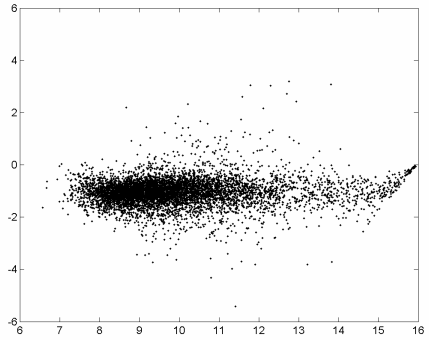


The general assumption is that most of the genes would not see any change in their expression.

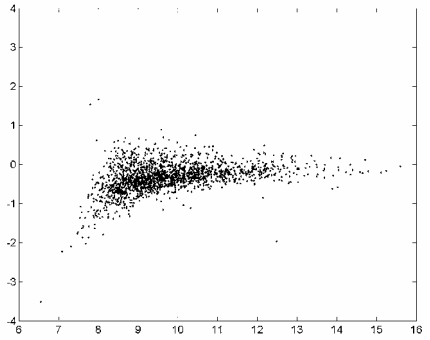
Common problems diagnosed using MA-plots

Cheung et al ***Nature*** (2005)

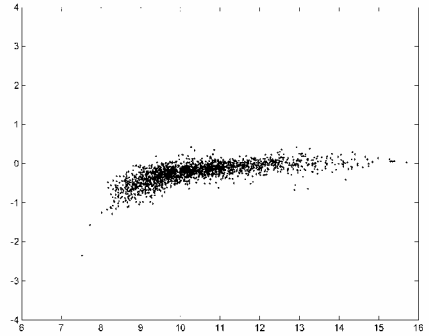
Saturation



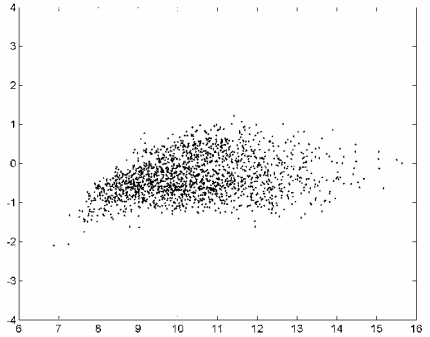
Low end variation



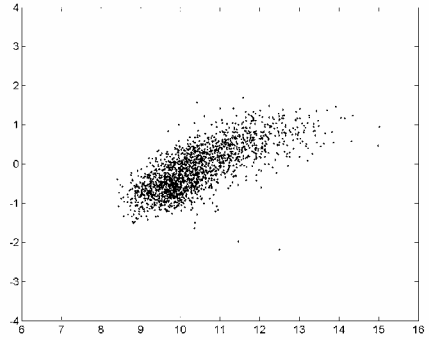
Curvature at Low intensity



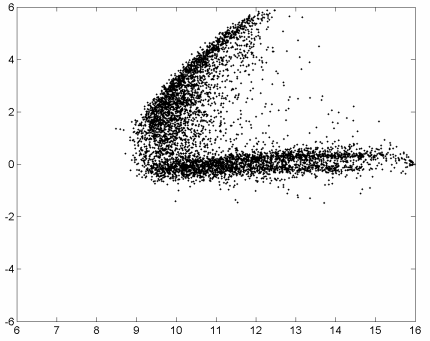
High end variation



Large curvature



Heterogeneity



MA Plots: Saturation & Quenching

- Saturation
  - Decreasing rate of binding of RNA at higher occupancies on probe
- Quenching:
  - Light emitted by one dye molecule may be re-absorbed by a nearby dye molecule
  - Then lost as heat
  - Effect proportional to square of density

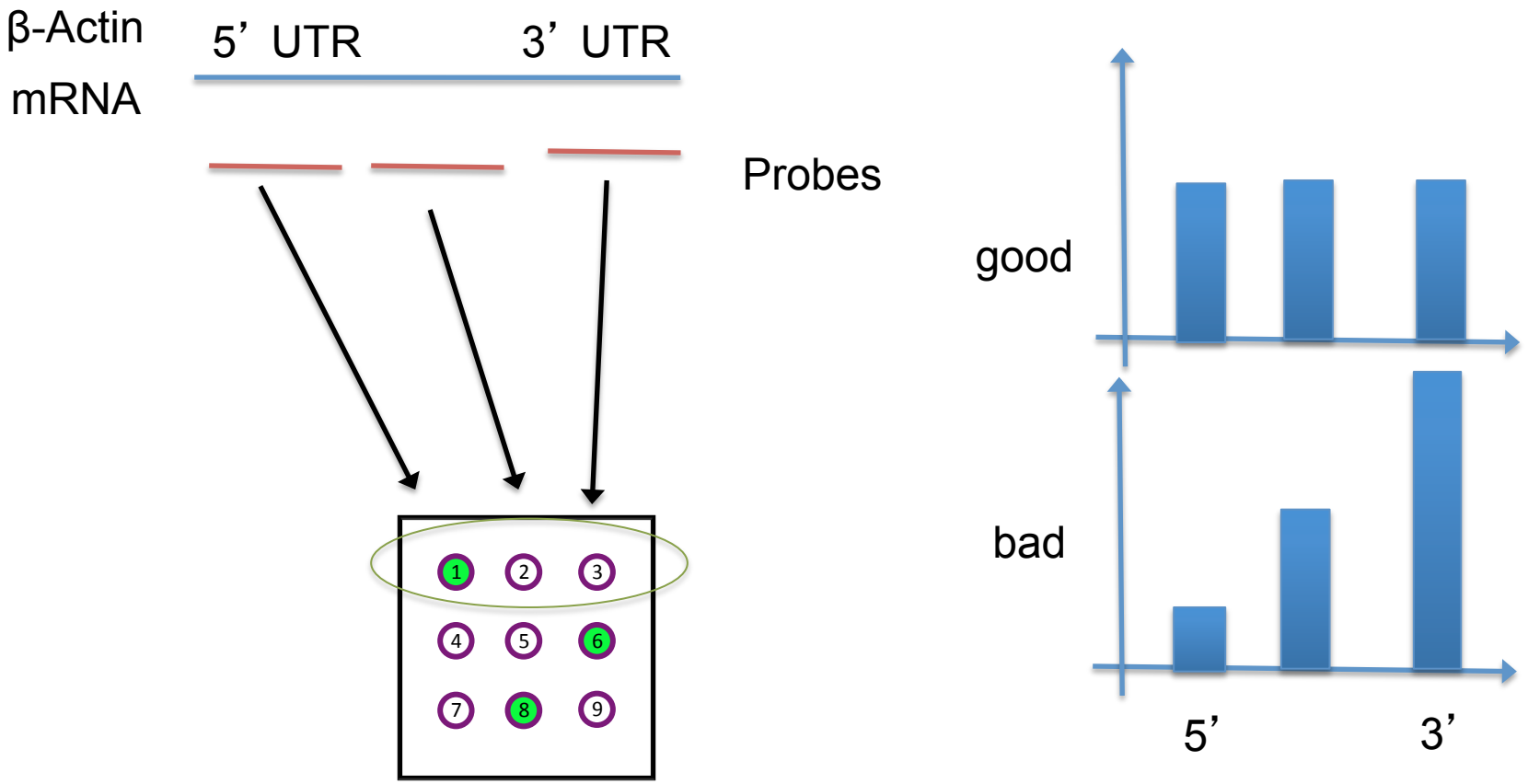
MA plot

```
> y <- exprs(Dilution)[, c("20B", "10A")]

> ma.plot( rowMeans(log2(y)), log2(y[, 1]/y[, 2]),
cex=1 )

> title("Pre-Norm Dilutions Dataset (array 20B v
10A)")
```

3’/5’ ratio: RNA quality

- The assumption is that RNA degradation, or problems during labeling, can lead to under intensity representation at the 5′ end of RNA, allowing the ratio between signals from 5′ and 3′ probesets to be used to assess RNA quality and labeling.
- Affymetrix genechip include a few RNA quality genes, each represented by 3 probe-sets, one at 5’ end of RNA, one at the middle, and one at the 3’ end of expressed RNA.
- The intensity ratio of 3’ probe-set to the 5’ probe-set for these genes can be used as a measure of RNA quality (i.e., the severity of RNA degradation).

3’/5’ ratio: RNA quality



The assumption is that RNA degradation, or problems during labeling, can lead to under intensity representation at the 5′ end of RNA

3’/5’ ratio: RNA quality

- Using Bioconductor, the “simpleaffy” package can compute these values.

  > library("simpleaffy")

  > d.qc=qc(Dilution)

  > ratios(d.qc)

	actin3/actin5	actin3/actinM	gapdh3/gapdh5	gapdh3/gapdhM
20A	0.6961423	0.1273385	0.4429746	-0.06024147
20B	0.7208418	0.1796231	0.3529890	-0.01366293
10A	0.8712069	0.2112914	0.4326566	0.42375270
10B	0.9313709	0.2725534	0.5726650	0.11258237

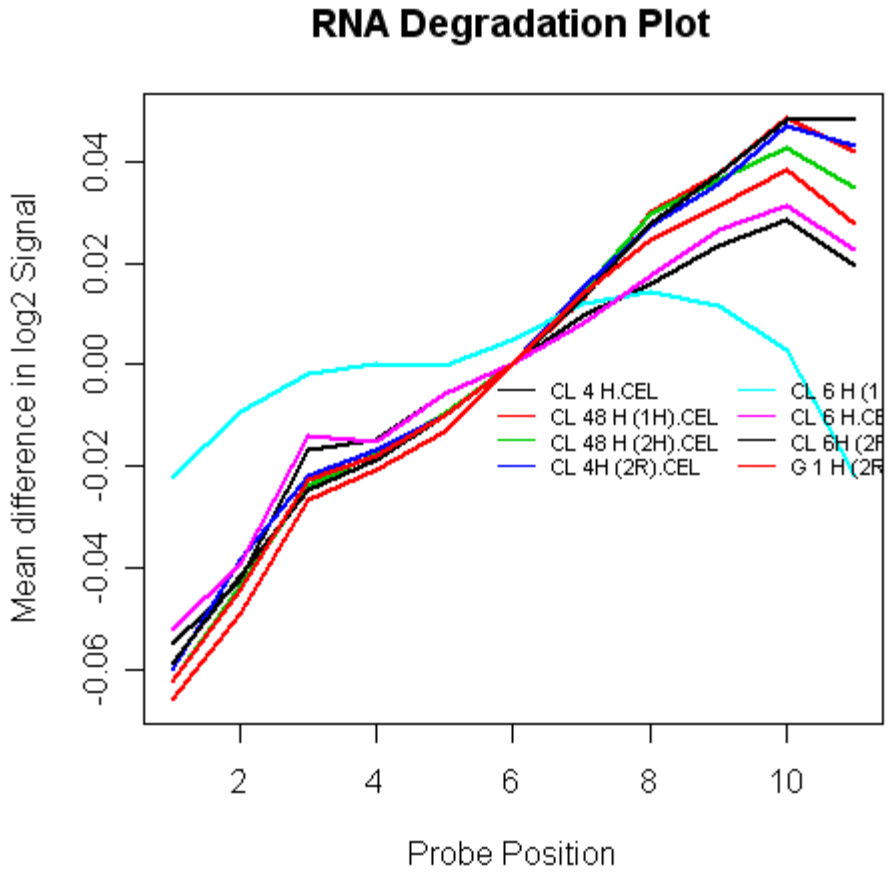
  - mostly β-Actin and GAPDH genes
  - 0.3 is the suggested safe threshold value for the 3’/5’ ratio.

Install “simpleaffy” first

- source("https://bioconductor.org/biocLite.R")
- biocLite("simpleaffy”)

Detect possible RNA degradation- Covariation with Probe Position

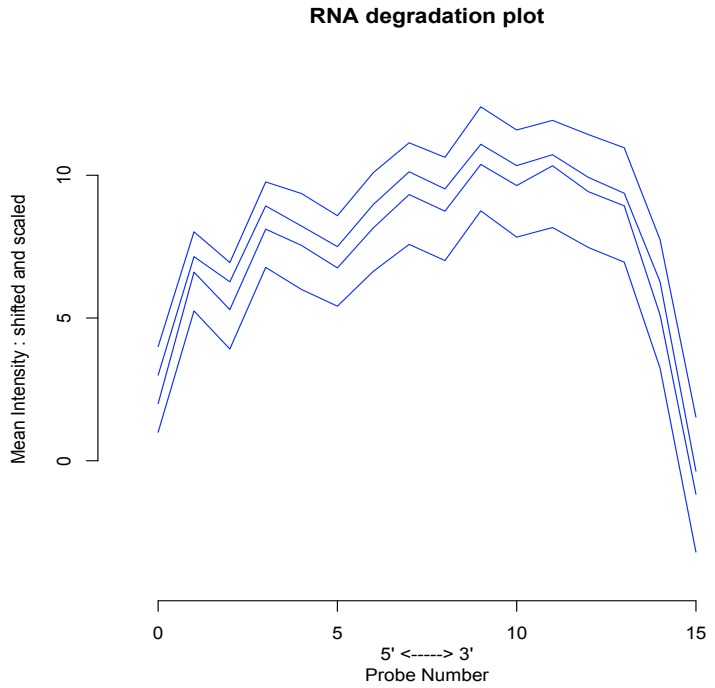
- RNA degrades from 5’ end
- Intensity should decrease from 3’ end uniformly across chips



Plot of average intensity for each probe position across all genes against probe position

Covariation with Probe Position

- `AffyRNAdeg` plots in ***affy*** package




```
> library(affy)
> RD<-AffyRNAdeg(Dilution)
> plotAffyRNAdeg(RD)
```

```
> summaryAffyRNAdeg(RD)
```

	20A	20B	10A	10B
slope	-0.0239	0.0363	0.0273	0.0849
pvalue	0.8920	0.8400	0.8750	0.6160

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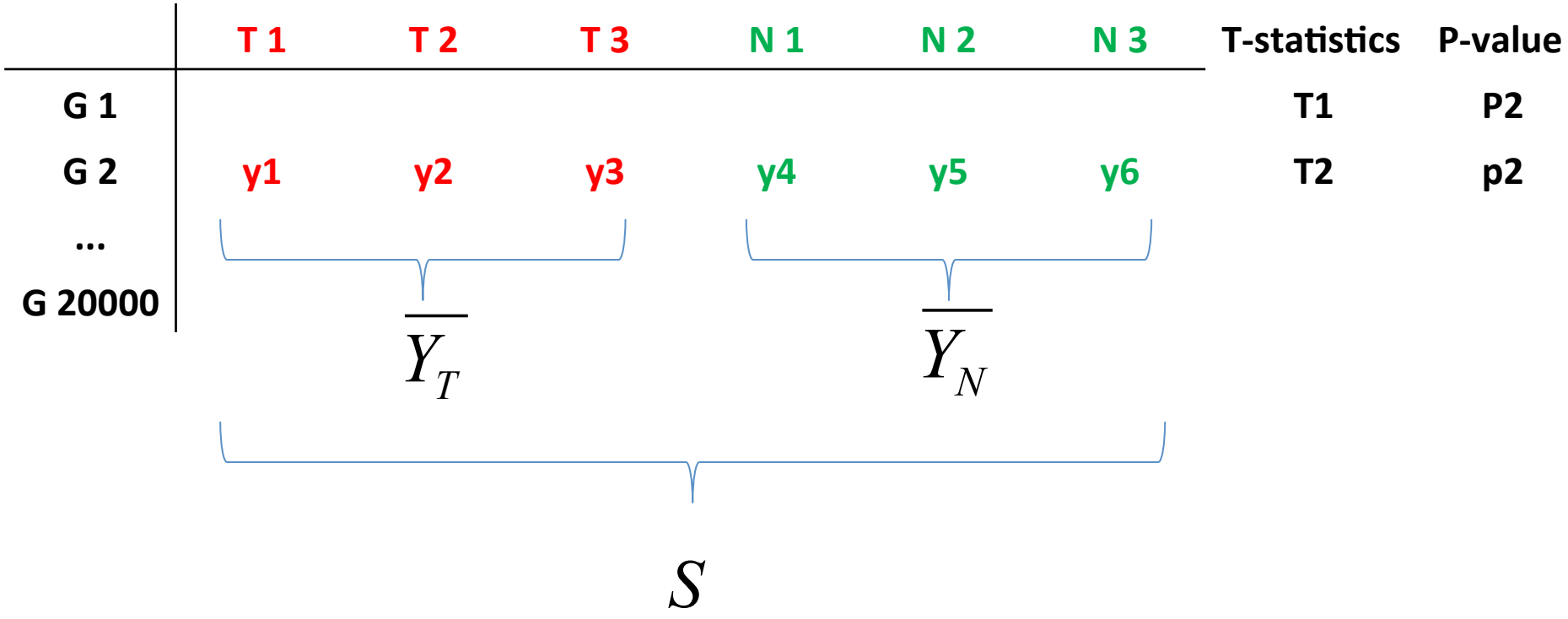
Types of problems

- To compare two groups
  - Treatment group vs. control group
- To compare multiple groups
  - Treatment A, Treatment B, Control group
- To consider multiple variables (factors) simultaneously
  - Treatment variable (Treatment vs. Control), age variables (>50 vs. <50), …

Two-group comparisons: Student's T-test

- Then, for gene2, calculated the difference between group means, divided by global standard error; obtain T2 and P2



Statistics methods for two-group comparisons

- T-test
  - Student's t-test: assumes normally distributed data in each group, equal variance within groups
  - Welch t-test: as above, but allows unequal vairance
- Univariate linear model
- Nonparametric test
  - Wilcoxon, or rank-sums test: non-parametric, rank-based
  - Permutation test: estimate the distribution of the test statistics under the null hypothesis by permutations of the sample labels

Univariate Linear Model

- The expression of gene x is modeled as a baseline expression level (from the normal group) plus the group effect (i.e., tumor vs. normal)

$$Y = Y_N + \beta Z + \varepsilon$$

Expression level of gene x

Baseline expression level of gene x

Group effect

Group vector
- ❑ 0 for normal group
- ❑ 1 for tumor group

- β represents the group effect. It is P-value (through F-test) can be used to test whether the expression in tumor is different from normal (i.e., showing group effect).

Univariate Linear Model

- Under R: > glm()
- Example output for a single gene:

Variable	Effect estimate	St.error	t-statistic	p-value
Intercept	0.4015	0.4334	0.926	0.381329
Group	-3.43	0.6129	-5.597	0.000512
Multiple R-square	0.7966		F-statistic	31.32
Adjusted R-square	0.7711		df	(1,8)
			p-value	0.000512

LIMMA Package

- The LIMMA package (one of the Bioconductor package) is for differential expression analysis of data arising from microarray experiments
  - The central idea is to fit a linear model to the expression data for each gene, with the experiment design information contained within the design matrix.
  - Also, empirical Bayes are used to borrow information across genes to estimate the standard deviation.

Limma for two-group case

	20A	20B	10A	10B
G 1				
G 2				
…				
G 12625				

```
> library("limma")
> d.exp=exprs(Dilution)
> design <- cbind(WT=c(1,1,0,0),MU=c(0,0,1,1))
> fit <- lmFit(d.exp, design)
> cont.matrix <- makeContrasts(MUvsWT=WT-MU, levels=design)
> fit2 <- contrasts.fit(fit, cont.matrix)
> fit2 <- eBayes(fit2)
> results=topTable(fit2, number=20, adjust="fdr", lfc=1)
```

Results

> results

	ID	logFC	AveExpr	t	P.Value	adj.P.Val	B
156253	156253	861.50	1687.750	14.351607	0.0001304622	0.885185	-4.595113
236914	236914	585.75	1252.375	12.166323	0.0002507076	0.885185	-4.595113
11614	11614	450.05	620.025	9.628034	0.0006270167	0.885185	-4.595113
21225	21225	358.50	587.250	8.843570	0.0008718059	0.885185	-4.595114
209366	209366	396.50	875.250	7.855832	0.0013746967	0.885185	-4.595114
212569	212569	431.10	718.950	7.086558	0.0020342135	0.885185	-4.595114
48215	48215	693.75	1243.525	6.988311	0.0021443453	0.885185	-4.595114
90347	90347	1571.35	2912.325	6.890651	0.0022611877	0.885185	-4.595114
28257	28257	545.75	1226.525	6.703351	0.0025080364	0.885185	-4.595114
47650	47650	543.10	890.450	6.609500	0.0026441801	0.885185	-4.595114
62342	62342	348.35	887.825	6.504775	0.0028069954	0.885185	-4.595114
52062	52062	320.75	641.525	6.494663	0.0028233597	0.885185	-4.595114
14171	14171	317.60	379.350	6.486309	0.0028369685	0.885185	-4.595114
53342	53342	262.75	383.125	6.293804	0.0031741131	0.885185	-4.595114

Limma Package and installation

- http://bioconductor.org/packages/release/bioc/html/limma.html

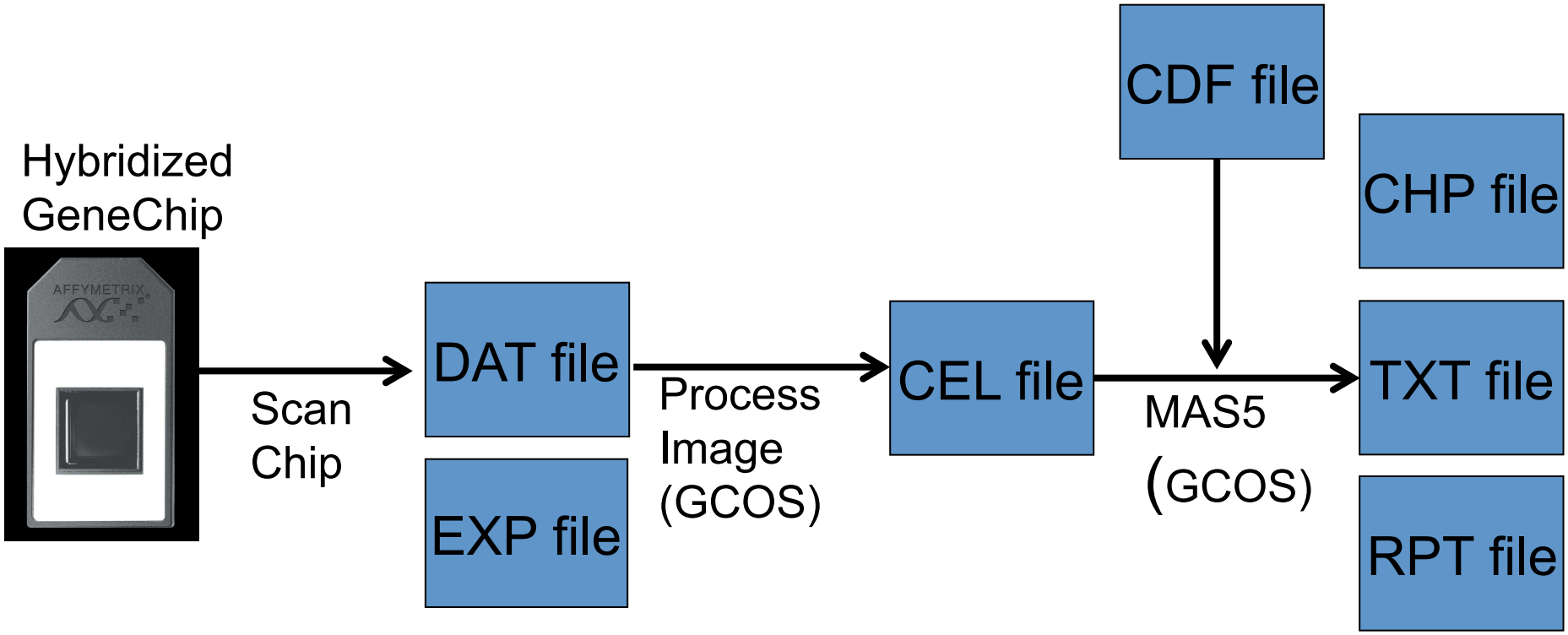
```
> source("https://bioconductor.org/biocLite.R")
> biocLite("limma")
```

How to deal with raw affy data

Affymetrix File Types

- DAT file:
  - Raw (TIFF) optical image of the hybridized chip
- CDF File (Chip Description File):
  - Provided by Affy, describes layout of chip. Each chip has a corresponding **CDF** which describes probe locations and probeset groupings on the chip.
- CEL File:
  - Processed DAT file (intensity/position values)
- CHP File:
  - Experiment results created from CEL and CDF files
- TXT File:
  - Probeset expression values with annotation (CHP file in text format)
- EXP File
  - Small text file of Experiment details (time, name, etc)
- RPT File
  - Generated by Affy software, report of QC info

Affymetrix Data Flow



Read CEL files

-Note we can read multiple CEL at the same time.

(1) need edit a plain text file saving all CEL names.

(2) read those affy files.

Read CEL files

-Edit a plain text file with the following format and save it as “targets.txt”.

```
FileName
Control_filename1.CEL
Control_filename2.CEL
Control_filename3.CEL
Treatment_filename1.CEL
Treatment_filename2.CEL
Treatment_filename3.CEL
```

Read CEL files

-(2) read those affymetrix CEL files.

```
> library(“limma”)
> # red target filetargets
> Targets <- readTargets("targets.txt")
> #read microarry
> dataab <- ReadAffy(filenames=targets$FileName)
> preprocessed_dataab=rma(dataab)
```

Limma for the two-group case

```
> design <- cbind(CT=c(1,1,1,0,0,0),TR=c(0,0,0,1,1,1))
> fit <- lmFit(preprocessed_dataab, design)
> cont.matrix <- makeContrasts(CT-TR, levels=design)
> fit2 <- contrasts.fit(fit, cont.matrix)
> fit2 <- eBayes(fit2)
> results=topTable(fit2, number=20, adjust="fdr", lfc=1)
```

Limma for two-group case

```
> design
    CT TR
[1,]  1  0
[2,]  1  0
[3,]  1  0
[4,]  0  1
[5,]  0  1
[6,]  0  1
```

```
> cont.matrix
       Contrasts
Levels CT - TR
    CT       1
    TR      -1
```

Log(fold change)=log(Control/Treatmetn)

Limma for two-group case

```
> results=topTable(fit2, number=20, adjust="fdr", lfc=1)

> results=topTable(fit2,  adjust="fdr", lfc=1)
```

Filter in R

> results

	ID	logFC	AveExpr	t	P.Value	adj.P.Val	B
13611	Zm.7576.1.A1_at	-7.294902	9.368417	-48.18529	2.227504e-10	3.950256e-06	13.440325
181	Zm.100.1.A1_at	-6.798488	9.147950	-35.79768	1.917041e-09	1.699841e-05	12.069663
3684	Zm.14489.1.S1_at	-5.545995	8.421467	-31.88446	4.427037e-09	2.520650e-05	11.443865
2002	Zm.12635.1.S1_s_at	-4.640604	10.588414	-29.71106	7.371049e-09	2.520650e-05	11.039271
9216	Zm.3633.1.A1_at	-4.731900	8.121015	-28.43470	1.011871e-08	2.520650e-05	10.779434
12870	Zm.6721.4.A1_s_at	-4.271442	8.528172	-27.17756	1.401924e-08	2.520650e-05	10.505617
12918	Zm.6757.1.S1_at	-5.111377	8.398503	-27.10415	1.429522e-08	2.520650e-05	10.489045
5250	Zm.16735.1.A1_at	-4.019595	8.592958	-27.02468	1.460095e-08	2.520650e-05	10.471031

> results[,6]<0.000001

```
 [1] TRUE FALSE FALSE FALSE FALSE FALSE FALSE FALSE
```

>results[results[,6]<0.000001,] ←what is the result?

Homework Assignment 7

- Learn to access data from GEO database
- Learn to read raw CEL files
- Learn to use limma package
- Due by Nov. 1, 2015