

Gene expression microarray: Differential expression, batch effect

Outline

- Scientific goal and potential problems.
- Review of basic statistical concepts:
 - Hypothesis testing.
 - Multiple comparison problem.
- Differential expression (DE) test methods:
 - Empirical Bayesian (EB) methods: limma.
 - SAM.
 - Complex designs.
 - Permutation.
- Batch effect.
- Introduction to tiling arrays (will be skipped).

Input data for DE test

- Assume data are correctly within- and between-array normalized.
- Input data for DE test is a matrix of positive number:
 - Rows for genes and columns for samples.
 - Usually work on logarithm of the data, which are normal-ish.

Goal of DE test

- Goal: find genes that are expressed differently between (among) conditions.
 - Assign a score for each gene to represent its statistical significance of being different.
 - Rank the genes according to the score.
 - Find a proper threshold for the score for calling DE.
- Easy solutions:
 - Hypothesis testing (t-test, ANOVA, linear model, etc.) to get p-values and use as scores.
 - Use canonical cutoff (0.05) to call DE.

Potential problems

- Hypothesis testing:
 - sample sizes are usually small, which lead to unstable test results.
- When data are not normal, p-values are not accurate.
- Use 0.05 as threshold of p-values to call DE - multiple comparison problem:
 - Tests are performed for 20,000 genes. Even if all are null (not DE), 1,000 will have p-value less than 0.05.

Review of statistical inference

- Two-group t-test:
 - data from two groups (cancer and normal): $X_1, \dots, X_M; Y_1, \dots, Y_N$.
 - Assume X 's and Y 's are normally distributed.
 - A “null hypothesis” is that the means of X 's and Y 's are identical.
 - Test statistics $t = (\bar{X} - \bar{Y}) / \sqrt{S_X^2 / M + S_Y^2 / N}$
where $S_X^2 = \frac{1}{M-1} \sum_{i=1}^M (X_i - \bar{X})^2$, $S_Y^2 = \frac{1}{N-1} \sum_{i=1}^N (Y_i - \bar{Y})^2$
 - t follows t-distribution.
 - P-value: under null hypothesis (that the means are the same), the probability to observe a t-statistics more extreme than the observed.

Why gene-by-gene t-test is a bad idea

- Small sample size (e.g., 3 vs. 3) leads to unstable estimates of variances.
 - By chance some genes have very small variance, which will result in large t-statistics and tiny p-values even when the difference is small.
 - Solution: SAM, EB methods.
- Sometimes data are not normally distributed, lead to incorrect p-values.
 - solution: non-parametric approach to obtain p-values.

SAM t-test

Tusher *et al.* (2001) PNAS

- Try to remove (or minimize) the dependence of test statistics on variances (because small variance tend to lead to bigger test statistics).
- Solutions: add a small constant to the denominator in calculating t statistics:

$$d_i = \frac{\bar{y}_i - \bar{x}_i}{s_i + s_0}$$

\bar{y}_i , \bar{x}_i : Means of two groups for gene i.

s_i : Standard deviation for gene i, assuming equal variace in both groups.

s_o : "Exchangeability factor" estimated using all genes.

The exchangeability factor

- Chosen to make signal-to-noise ratios independent of signal, e.g., the distribution of the statistics independent of the variance.
- Procedure:
 - Let S^α be the α percentile of the s_i values
 - For $\alpha \in (0, 0.01, 0.02, \dots, 1.0)$ compute $d_i^\alpha = (\bar{y}_i - \bar{x}_i) / (s_i + S^\alpha)$
 - Compute $v_j^\alpha = mad(d_i^\alpha | s_i \in [q_j, q_{j+1}])$, $j = 1, 2, \dots, 99$, here q_j are quantile.
 - Compute $cv(\alpha)$, the coefficient of variation of v_j^α
 - Choose $\hat{\alpha} = \operatorname{argmin}_a \{cv(a)\}$. $\hat{s}_o = \hat{s}^{\hat{\alpha}}$

SAM t-test

- Highly cited (>12,000 citations as of 2018),
<http://www-stat.stanford.edu/~tibs/SAM/>.
- Implemented as Bioconductor package
siggenes, and Excel plugin.
- Follow-up work: SAMSeq on RNA-seq DE test.
- Limitations: solutions for s_0 often sensitive to data.

Empirical Bayes method from limma

Smyth *et al.* (2004) *Statistical Applications in Genetics and Molecular Biology*

- Highly cited (~10,000 citations as of 2018).
- Use a Bayesian hierarchical model in multiple regression setting.
- Borrow information from all genes to estimate gene specific variances.
 - As a result, variance estimates will be “shrunk” toward the mean of all variances. So very small variance scenarios will be alleviated.
- Implemented in Bioconductor package “limma”.

The hierarchical model

Let β_{gj} be coefficient (difference in means in two group setting) for gene g, factor j, assume

$$\hat{\beta}_{gj} \mid \beta_{gj}, \sigma_g^2 \sim N(\beta_{gj}, v_{gj}\sigma_g^2) \quad s_g^2 \mid \sigma_g^2 \sim \frac{\sigma_g^2}{d_g} \chi_{d_g}^2 \quad \text{with priors:}$$

$$P(\beta_{gj} \neq 0) = p_j. \quad \beta_{gj} \mid \sigma_g^2, \beta_{gj} \neq 0 \sim N(0, v_{0j}\sigma_g^2). \quad \frac{1}{\sigma_g^2} \sim \frac{1}{d_0 s_0^2} \chi_{d_0}^2.$$

Posterior statistics

Posterior variance estimator: $\tilde{s}_g^2 = \frac{d_0 s_0^2 + d_g s_g^2}{d_0 + d_g}$.

Moderated t-statistics for
testing $\beta_{gj} = 0$:

$$\tilde{t}_{gj} = \frac{\hat{\beta}_{gj}}{\tilde{s}_g \sqrt{v_{gj}}}.$$

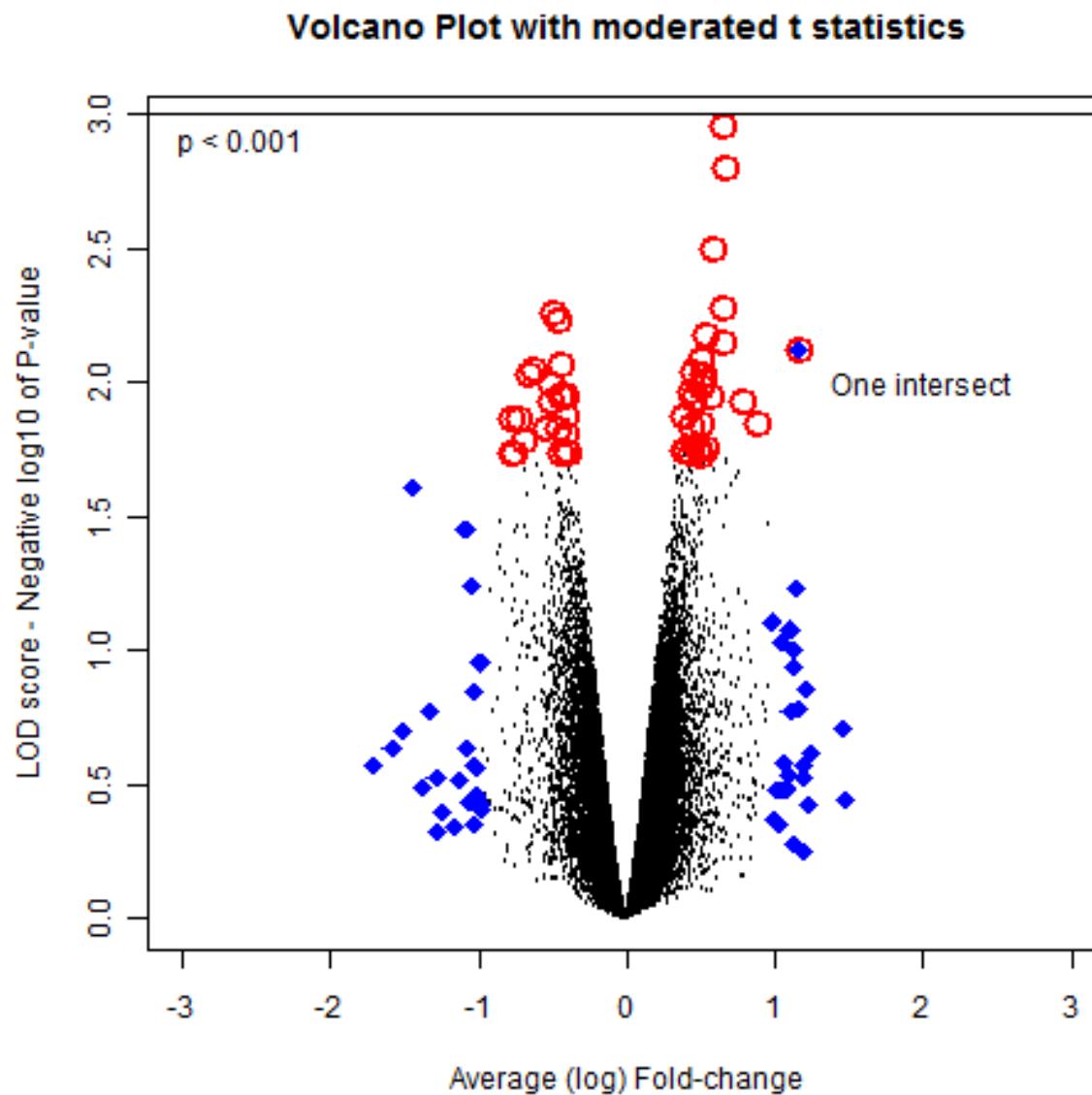
Summary on two-sample DE test

- Try to alleviate the “small sample variance” problem.
- Combine information from all genes.
- Many other variations of the model.
- In practice SAM and limma performs similarly.

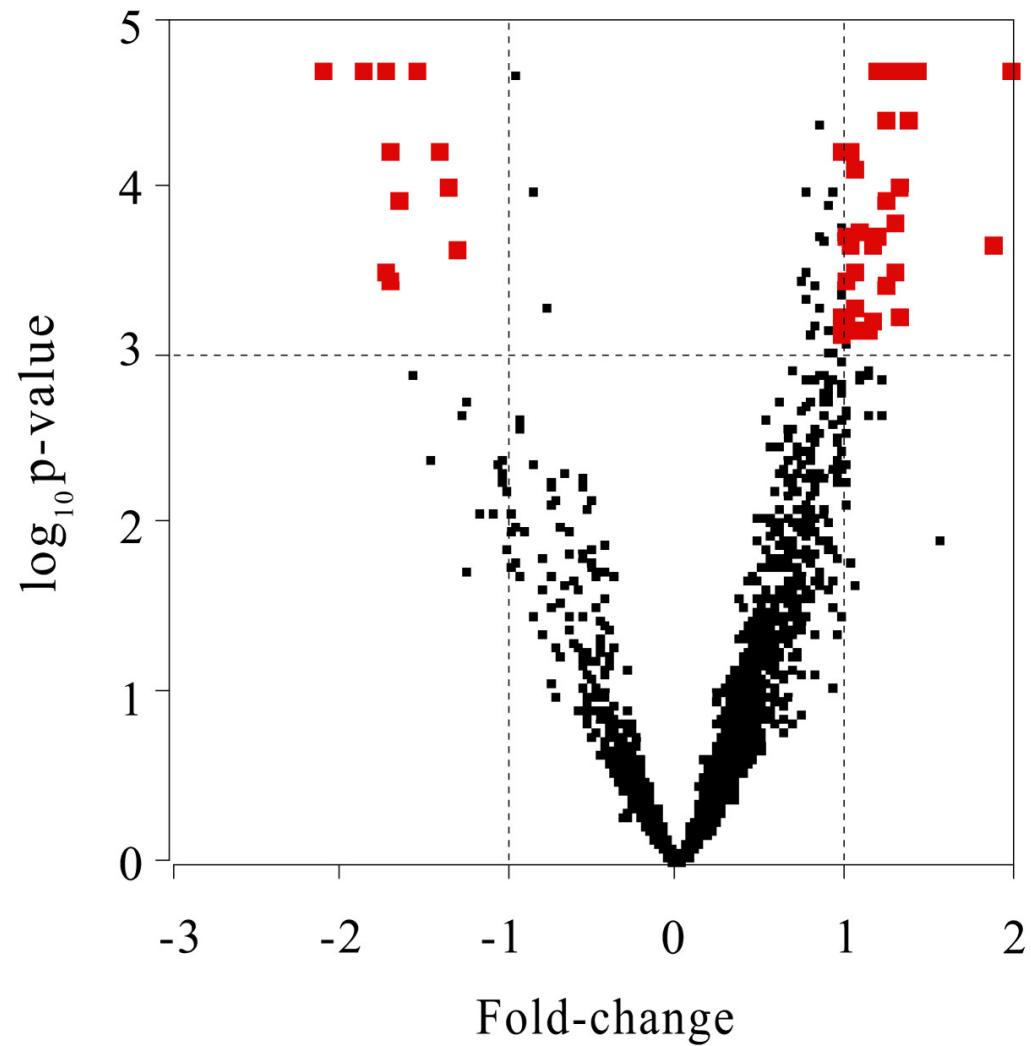
Volcano plot

- A diagnostic plot to visualize the test results.
- Scatter plot of the statistical significance (log p-values) vs. biological significance (log fold change).
- Ideally the two should agree with each other.

A bad volcano plot

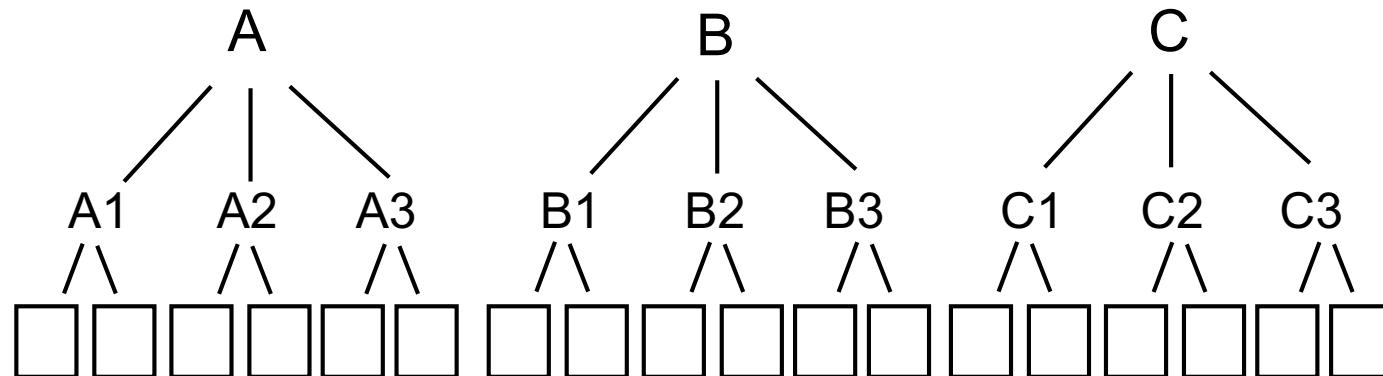


A good one

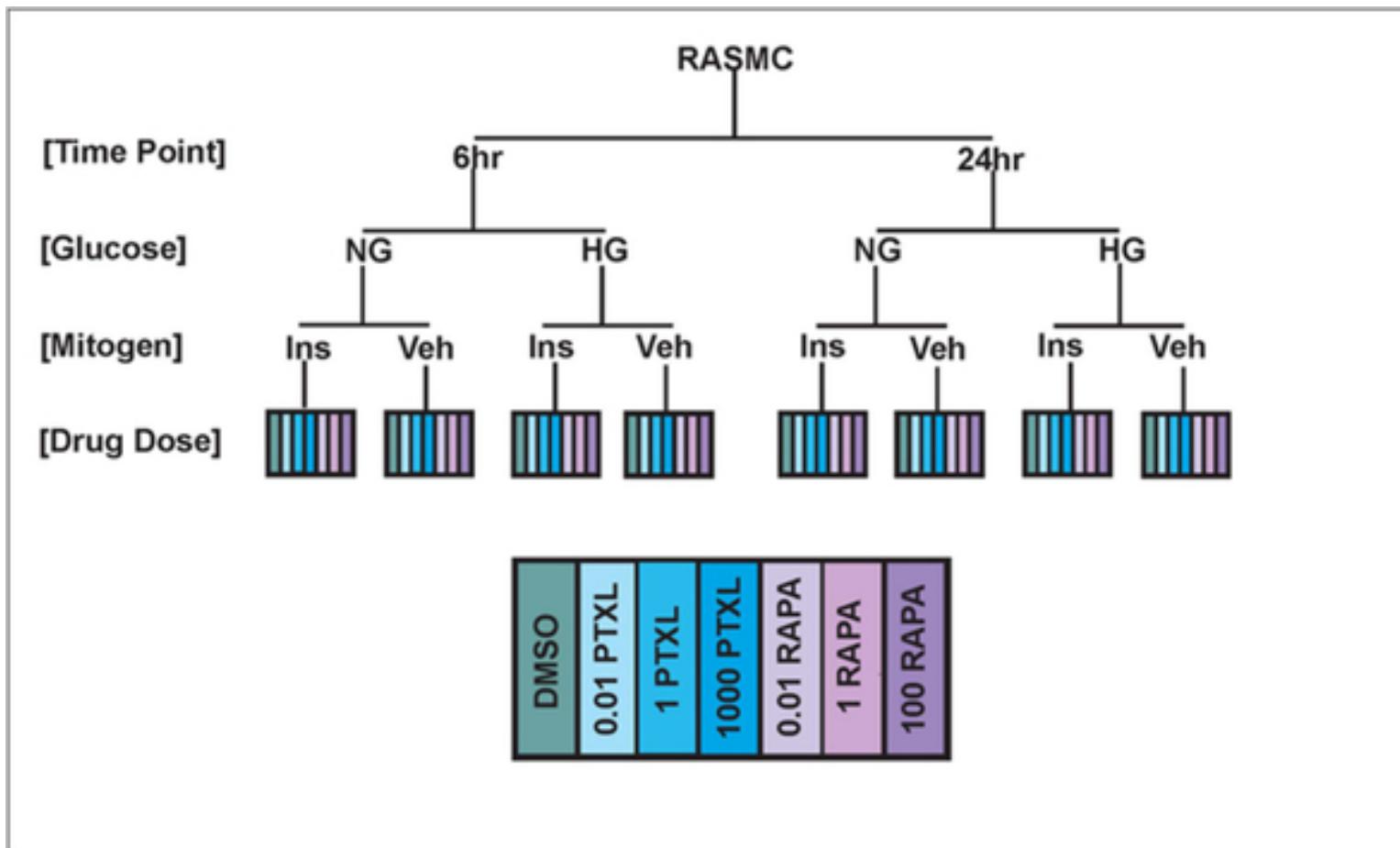


More complex experiments

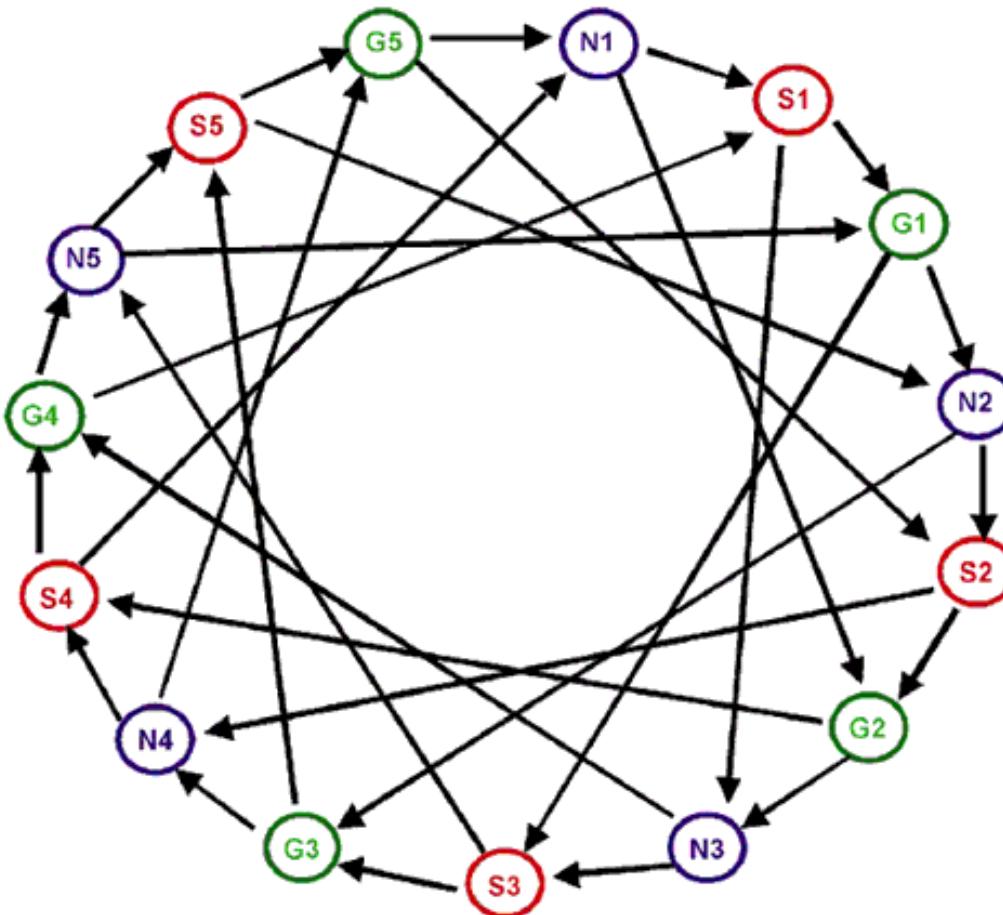
- Complex experimental designs:
 - multiple (>2) groups.
 - crossed/nested.
 - etc.
- Examples for multiple-group:



A crossed design



A complicated loop design on two-color array



DE test for complex design

- Two sample test -> multiple regression.
- The same problems still exist, and similar solutions can be applied.
- Mixed effect models can be used to capture heterogeneity among biological replicates.
- Both SAM and limma provide functions for complex designs.

P-values by randomization

- When the data don't satisfy normal assumption, permutation/bootstrap can be used to derive empirical p-values.
- Procedures for two sample comparison:
 - For each gene, randomly shuffle the data points.
 - Compute the t-statistics on the randomized data.
 - Repeat the procedure for N times, compute p-values as the percent of times that the permuted t-statistics more extreme than the observed.
- The procedure is a little complicated for multiple design.
Basically shuffle the data based on null model.

Multiple testing correction

- Multiple testing problem is severe in high throughput data analysis because a large number of tests were performed.
 - Under type I error $\alpha=0.05$, 1000 out of 20000 genes will be falsely declared DE (false positive) by chance.
 - If there are a total of 2000 genes declared DE, the false discovery rate (FDR) is 0.5!
- Multiple testing correction
 - Bonferroni correction: use $\alpha=0.05/20000$ (too conservative).
 - FDR control (Benjamini and Hochberg, 1995 JRSS-B)

Bioconductor packages for microarray analysis

Bioconductor for microarray data

- There're a rich collection of bioc packages for microarrays. In fact, Bioconductor started for microarray analysis.
- There are currently 228 packages for microarray.
- Important ones include:
 - affy: one of the earliest bioc packages. Designed for analyzing data from Affymetrix arrays.
 - limma and siggenes: DE detection using limma and SAM-t model.
 - oligo: preprocessing tools for many types of oligonucleotide arrays. This is designed to replace affy package.
 - Many annotation data package to link probe names to genes.

My suggestion

- Use `oligo` to reading in data, normalization and summarization.
- Use `siggenes` or `limma` for detecting DE genes.

An example of Analyzing a set of Affymetrix data

- Data generated by MAQC (MicroArray Quality Control) project.
- Five brain samples and five reference samples on human exon arrays.
- Raw data are CEL files (binary file generated by factory).
- Each CEL file is around 65Mb.
- The platform design package (`pd.huex.1.0.st.v2`) needs to be installed.

Read in data

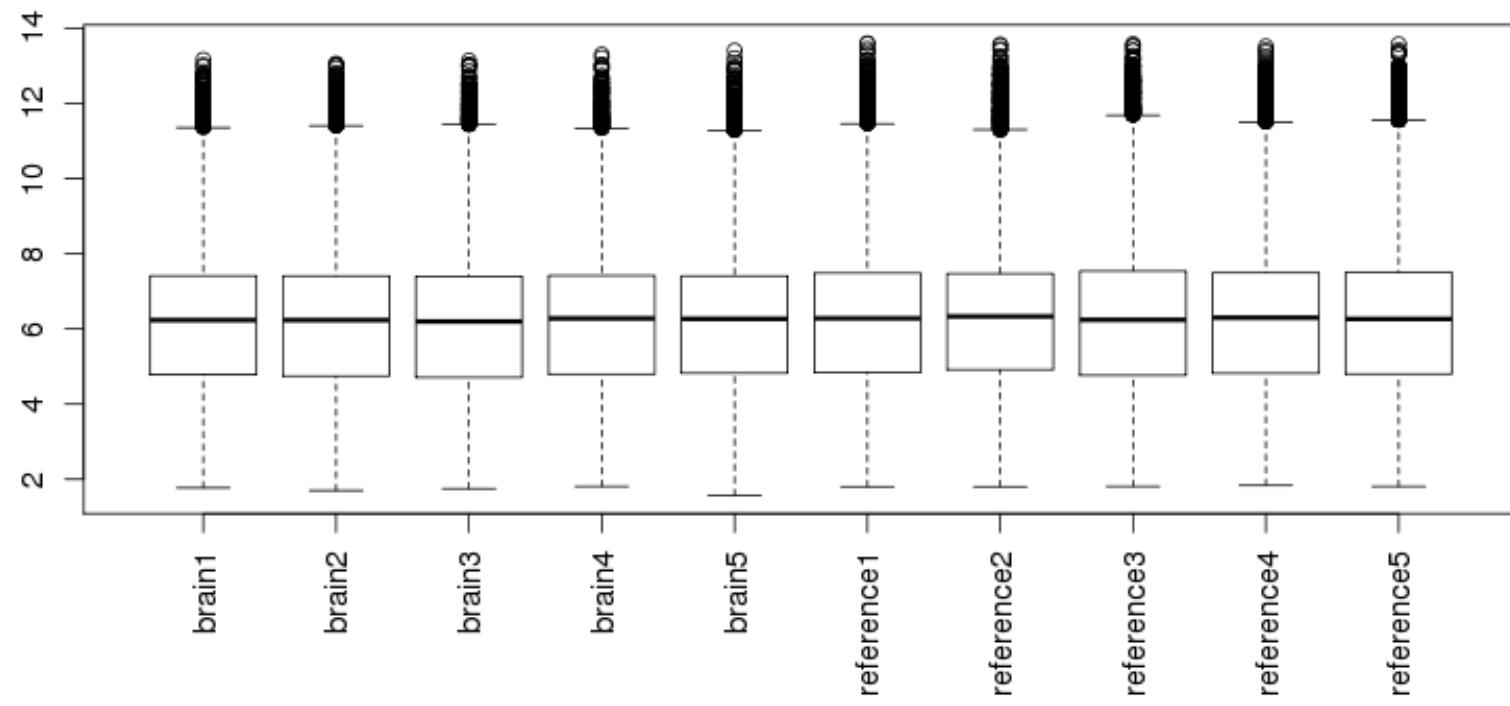
```
## load in necessary libraries
> library(oligo)
> library(limma)
## get a list of CEL files
> CELfiles=dir(pattern="CEL")
## read in all raw data
> rawdata=read.celfiles(CELfiles)
> rawdata
ExonFeatureSet (storageMode: lockedEnvironment)
assayData: 6553600 features, 10 samples
  element names: exprs
protocolData
  rowNames: ambion_A1.CEL, ambion_A2.CEL, ..., stratagene_K2.CEL
(10 total)
...
Annotation: pd.huex.1.0.st.v2
```

Normalization and summarization

```
## using RMA
> normdata=rma(rawdata, target = "core")
> normdata
ExpressionSet (storageMode: lockedEnvironment)
assayData: 22011 features, 10 samples
  element names: exprs
...
## extract expression values using expr function
> data=exprs(normdata)
> head(data)

      sample 1   sample 2   sample 3   sample 4
1007_s_at 10.160224 10.214496 10.090697 11.020649
1053_at    9.501826  9.500412  9.574311  7.361141
117_at     5.669447  5.478072  5.648788  6.048142
121_at     8.061479  8.154549  8.156215  7.902597
1255_g_at  4.307739  4.017903  3.992333  4.668972
1294_at    7.108730  7.185586  7.122404  6.597161
```

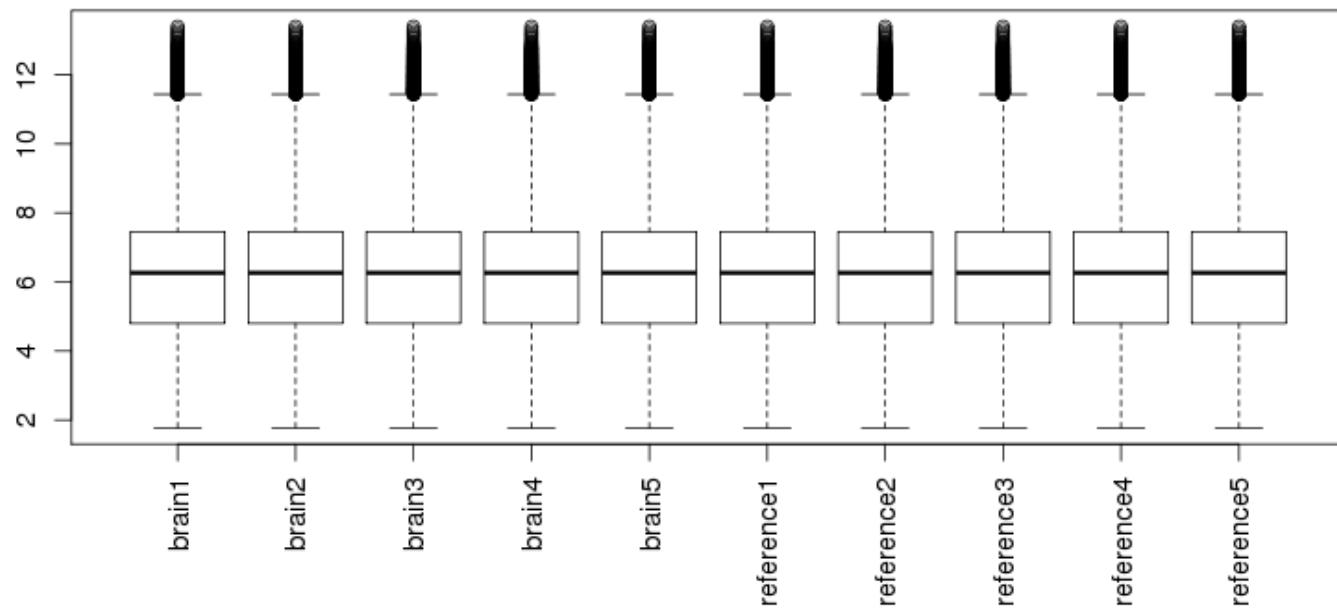
```
## check data distribution after RMA  
> boxplot(data)
```



The boxplot looks really good after RMA, so between array normalization is unnecessary. But in case you need it, use `normalizeQuantiles` function from `limma` for quantile normalization :

```
> data2=normalizeQuantiles(data)
```

Now the new boxplot after quantile normalization:



DE detection using SAM t-test

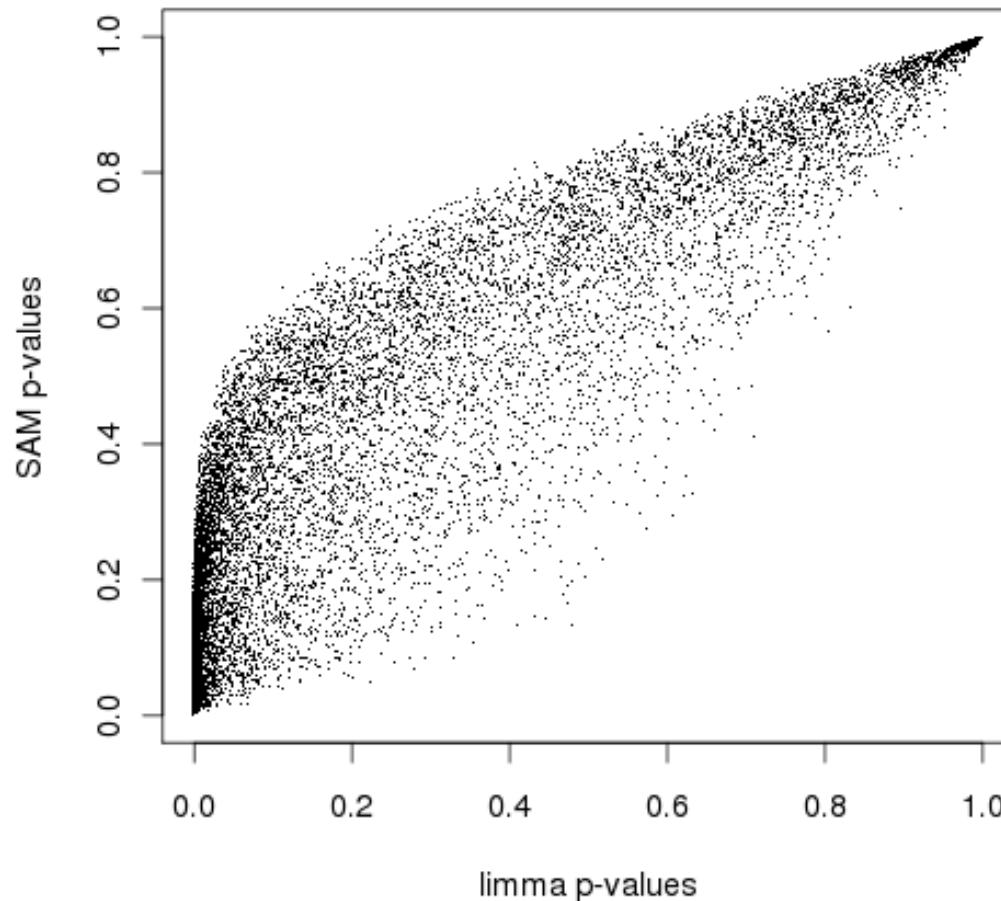
```
> library(siggenes)
## create a vector for design.
> design <- c(rep(0,5),rep(1,5))
> sam.result=sam(data2, cl=design)
> sam.result
SAM Analysis for the Two-Class Unpaired
Case Assuming Unequal Variances
```

DE detection using limma

```
## create design matrix. Intercept must be included  
> design=cbind(mu=1,beta=c(rep(0,5),rep(1,5)))  
## fit linear model and compute estimates  
> limma.result=lmFit(data2, design=design)  
## Empirical Bayes method to get p-values  
> limma.result=eBayes(limma.result)  
## get p-values for the comparison  
> pval=limma.result$p.value[, "beta"]
```

Compare results from limma and SAM

- Agreement is good, 0.95 Spearman rank correlation.
- Limma seems to be more liberal.



Obtain gene annotations

- Now you get p-values for all genes, but you also need gene names for generating report.
- There are many annotation packages available for different array platforms. For example, hgu133a.db is for HGU133A arrays.
- These packages contain comprehensive information for all probes, including their sequences, chromosome, position, corresponding gene IDs, GO terms, etc.

- A typical way to convert probeset names to accession number or gene alias is:

```
> library(hgu133a.db)
## convert to accession numbers:
> geneAcc=as.character(hgu133aACCTNUM[rownames(data)])
## convert to gene names
> geneNames=as.character(hgu133aSYMBOL[rownames(data)])
```

Finally generate a report table

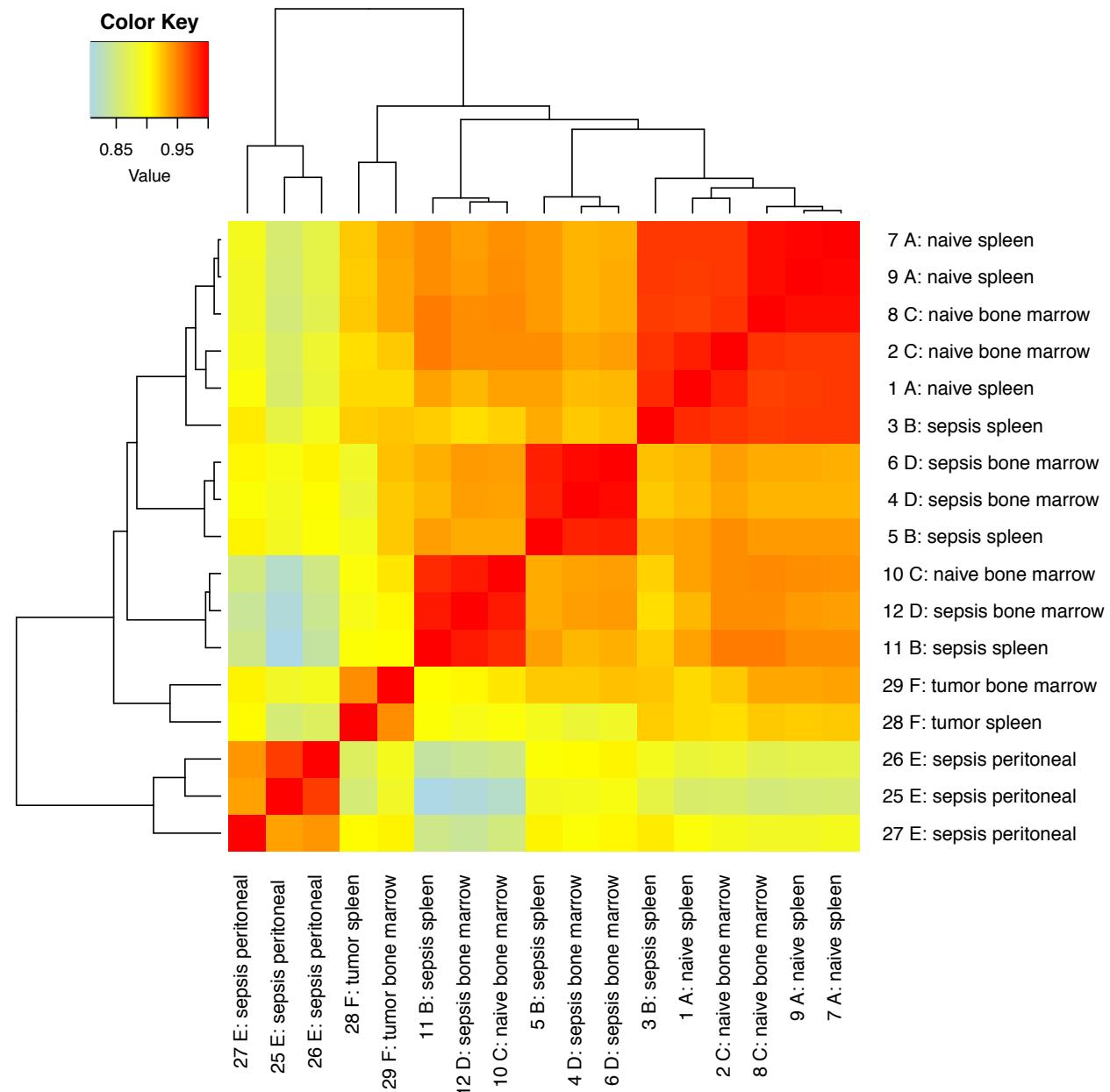
```
> ix=sam.result@q.value<0.1
> result=data.frame(gene=geneNames[ix],
  pvalue=sam.result@p.value[ix],
  fold=sam.result@fold[ix])
## sort by fold change
> ix2=sort(result$fold, decreasing=TRUE, index.return=TRUE)$ix
> result=result[ix2,]
> head(result)
      gene  pvalue     fold
2731192 NM_000477 0 185.5720
3457336 NM_006928 0 155.7143
2772566 NM_144646 0 152.8232
2731230 NM_001134 0 132.8515
> write.table(result, file="report.txt", sep="\t")
```

Data artifacts: batch effect and cell mixture

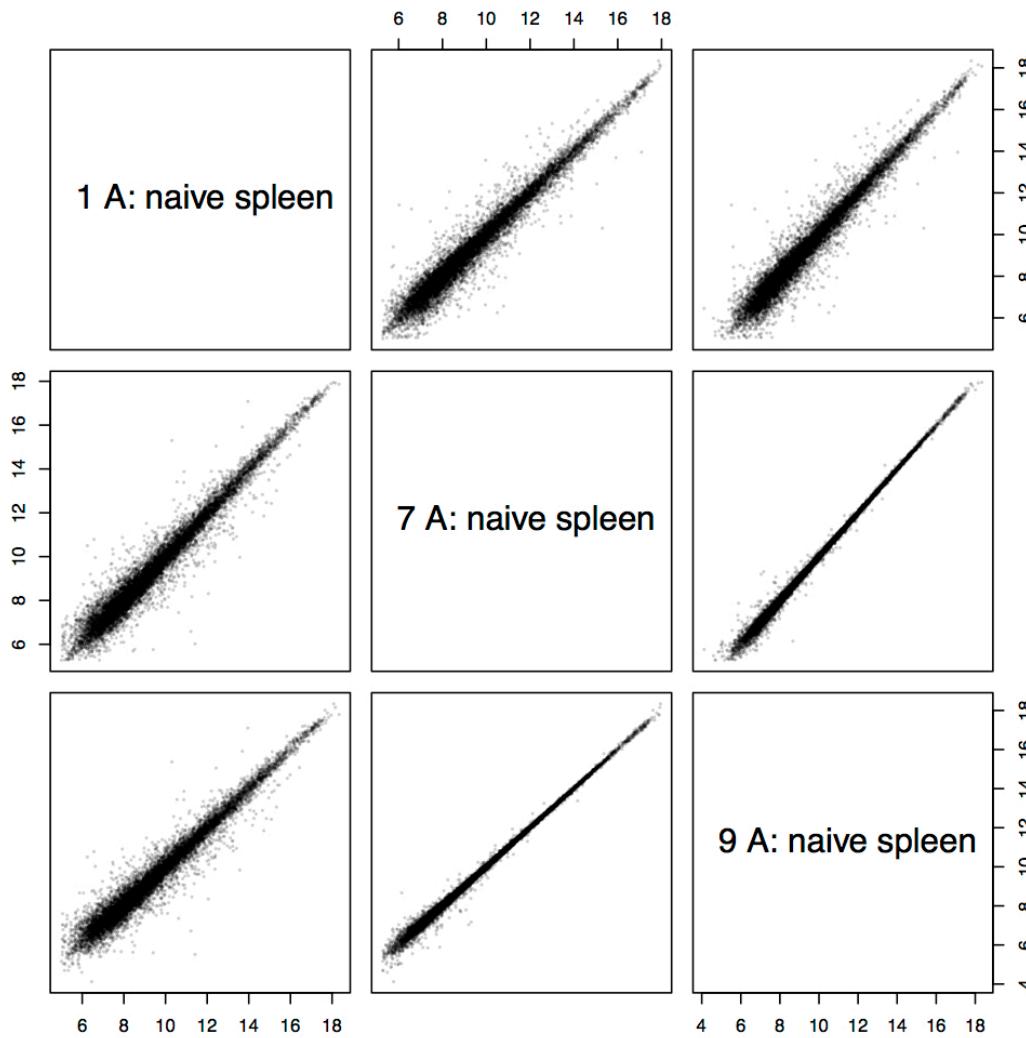
Technical artifact: batch effect

- Microarray experiments are very sensitive to experimental conditions:
 - Equipment, agents, technicians, etc.
- Data generated from different “batches” (lab, time, etc.) can be quite different, but data from the same batch tend to be more similar.
- So batch effects are structured noise/bias common to all replicates in the same batch, but markedly different from batch to batch.

Example



Variation within and between batches



Methods to remove batch effects

- Based on linear model: batches cause location/scale changes (e.g., combat).
- Based on dimension reduction technique: SVD, PCA, factor analysis, etc. (e.g., sva).
 - The singular vectors/PCs/factors that are correlated with batch are deemed from batch effects.
 - Remove batch effects from data, leftovers are biological signals.

sva package in Bioconductor

- Contains ComBat function for removing effects of known batches.
- Assume we have
 - edata : a matrix for raw expression values
 - batch : a vector named for batch numbers.

```
modcombat = model.matrix(~1, data=as.factor(batch))  
combat_edata = ComBat(dat=edata, batch=batch,  
                      mod=modcombat, par.prior=TRUE, prior.plot=FALSE)
```

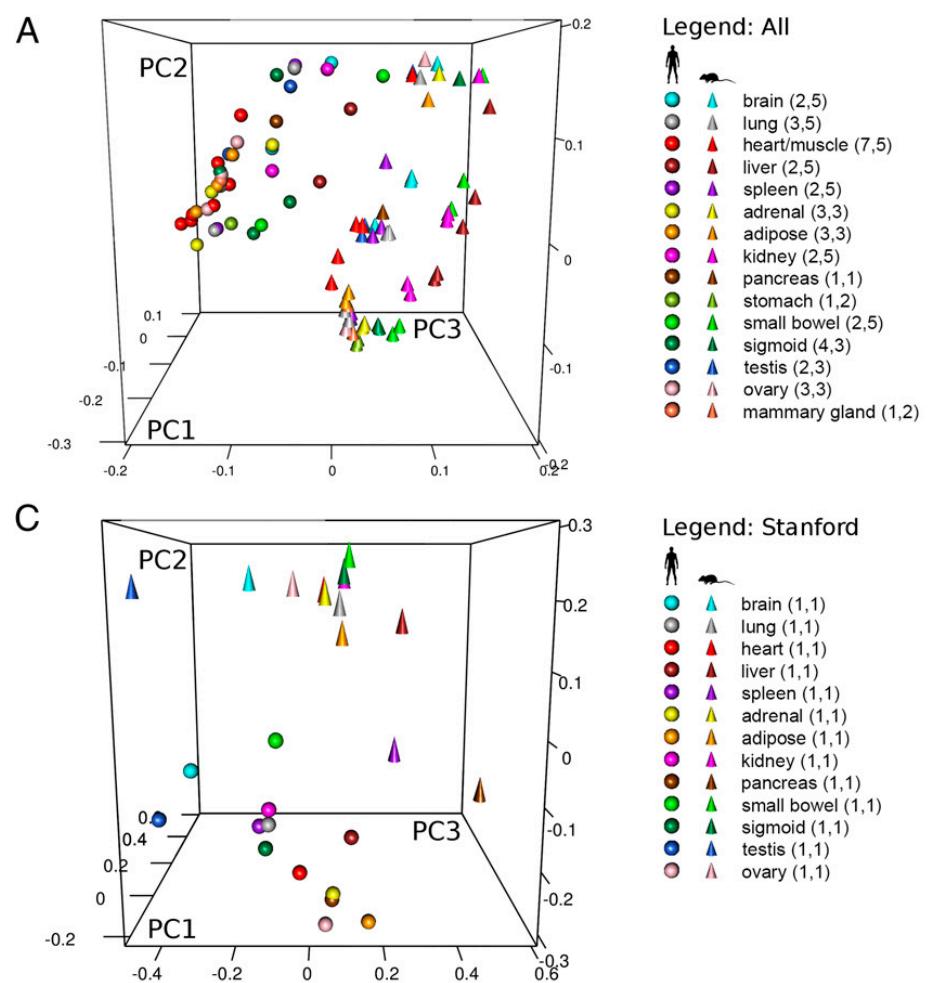
BatchQC - Batch Effects Quality Control

- A Bioconductor package with a GUI (shiny app).
- <http://bioconductor.org/packages/release/bioc/html/BatchQC.html>

Comparison of the transcriptional landscapes between human and mouse tissues

Shin Lin^{a,b,1}, Yiing Lin^{c,1}, Joseph R. Nery^d, Mark A. Urich^d, Alessandra Breschi^{e,f}, Carrie A. Davis^g, Alexander Dobin^g, Christopher Zaleski^g, Michael A. Beer^h, William C. Chapman^c, Thomas R. Gingeras^{g,i}, Joseph R. Ecker^{d,j,2}, and Michael P. Snyder^{a,2}

- One major conclusion is that tissues are more similar within a species, compared with the same tissue across species.





A reanalysis of mouse ENCODE comparative gene expression data [version 1; referees: 3 approved, 1 approved with reservations]

Yoav Gilad, Orna Mizrahi-Man

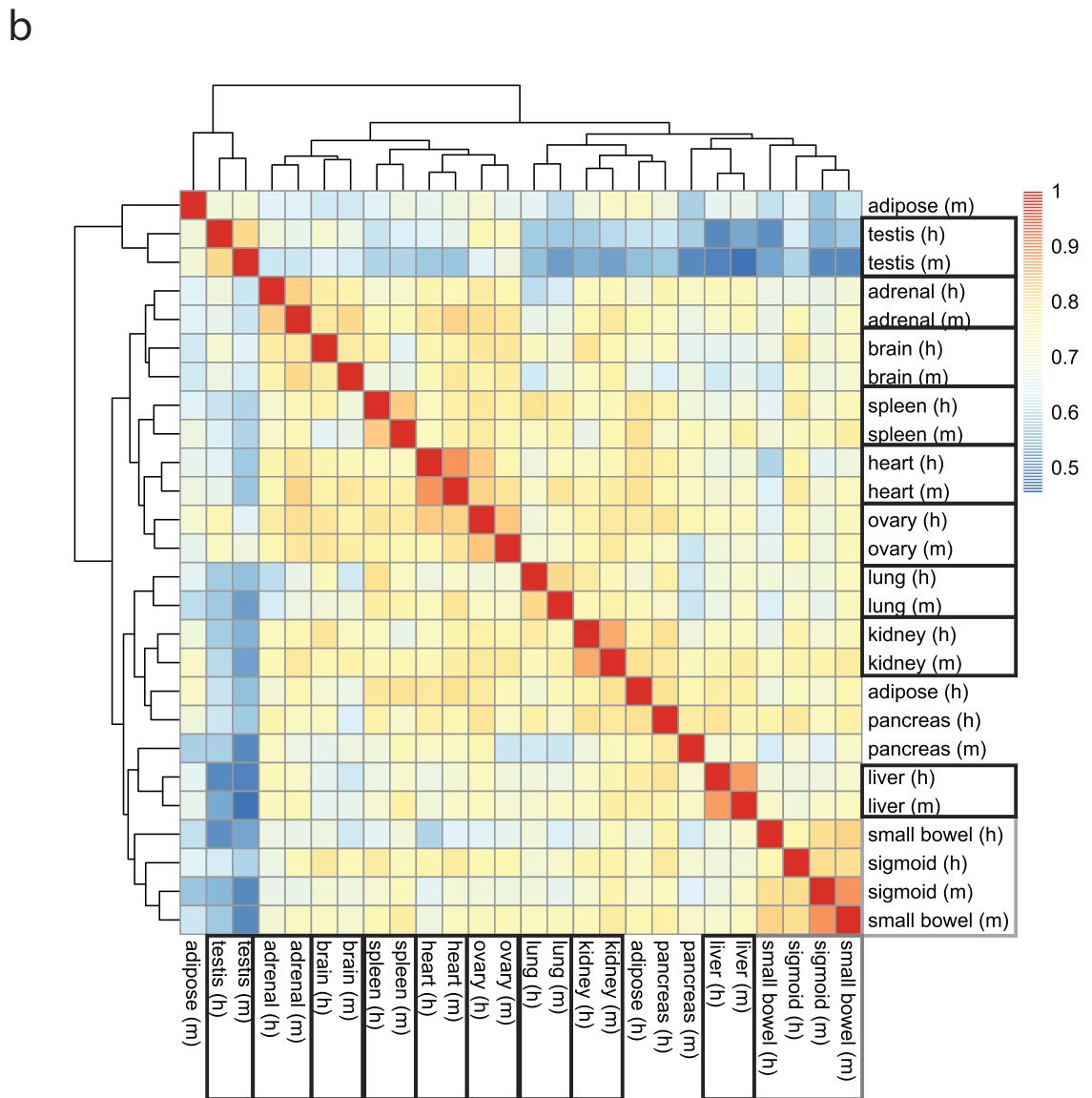
Department of Human Genetics, University of Chicago, Chicago, IL, 60637, USA

- Experimental design: data are from 5 batches.

D87PMJN1 (run 253, flow cell D2GUAACXX, lane 7)	D87PMJN1 (run 253, flow cell D2GUAACXX , lane 8)	D4LHBFN1 (run 276, flow cell C2HKJACXX , lane 4)	MONK (run 312, flow cell C2GR3ACXX , lane 6)	HWI-ST373 (run 375, flow cell C3172ACXX , lane 7)
heart	adipose	adipose	heart	brain
kidney	adrenal	adrenal	kidney	pancreas
liver	sigmoid colon	sigmoid colon	liver	brain
small bowel	lung	lung	small bowel	spleen
spleen	ovary	ovary	testis	● Human
testis		pancreas		● Mouse

After correcting for batch effects

- Tissues tend to cluster together more.

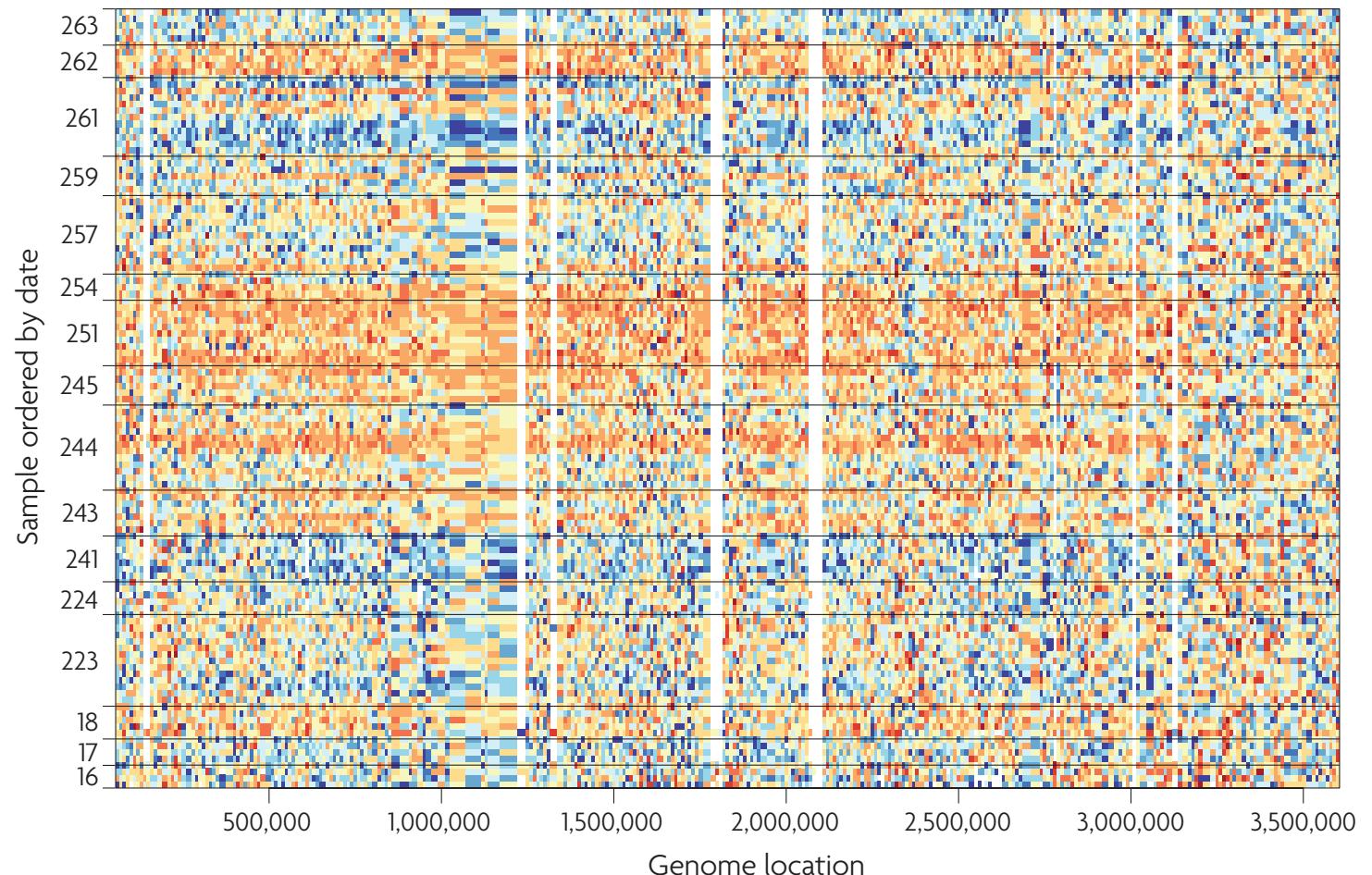


Batch effects are prevalent

- Observed in many high-throughput experiments: microarray, different types of sequencing, even brain imaging.
- Methods for identifying and removing batch effects is under continuous developments.

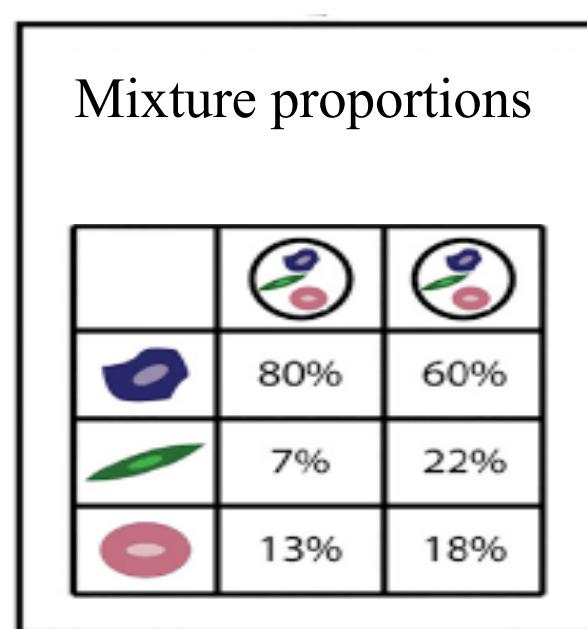
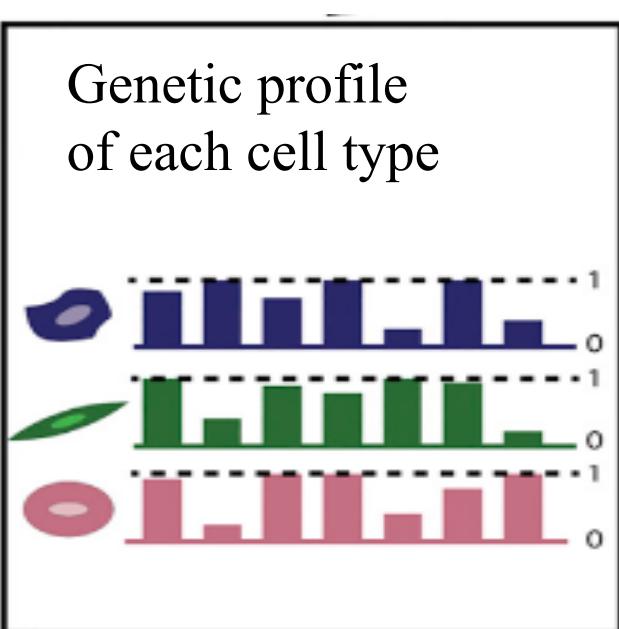
Tackling the widespread and critical impact of batch effects in high-throughput data

Jeffrey T. Leek, Robert B. Scharpf, Héctor Corrada Bravo, David Simcha,
Benjamin Langmead, W. Evan Johnson, Donald Geman, Keith Baggerly
and Rafael A. Irizarry



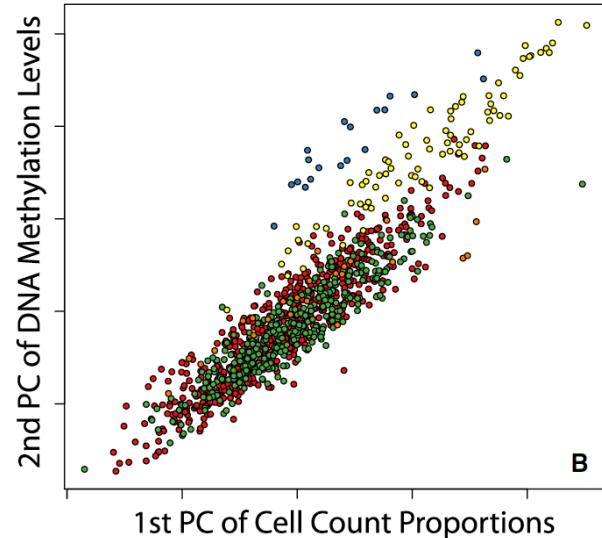
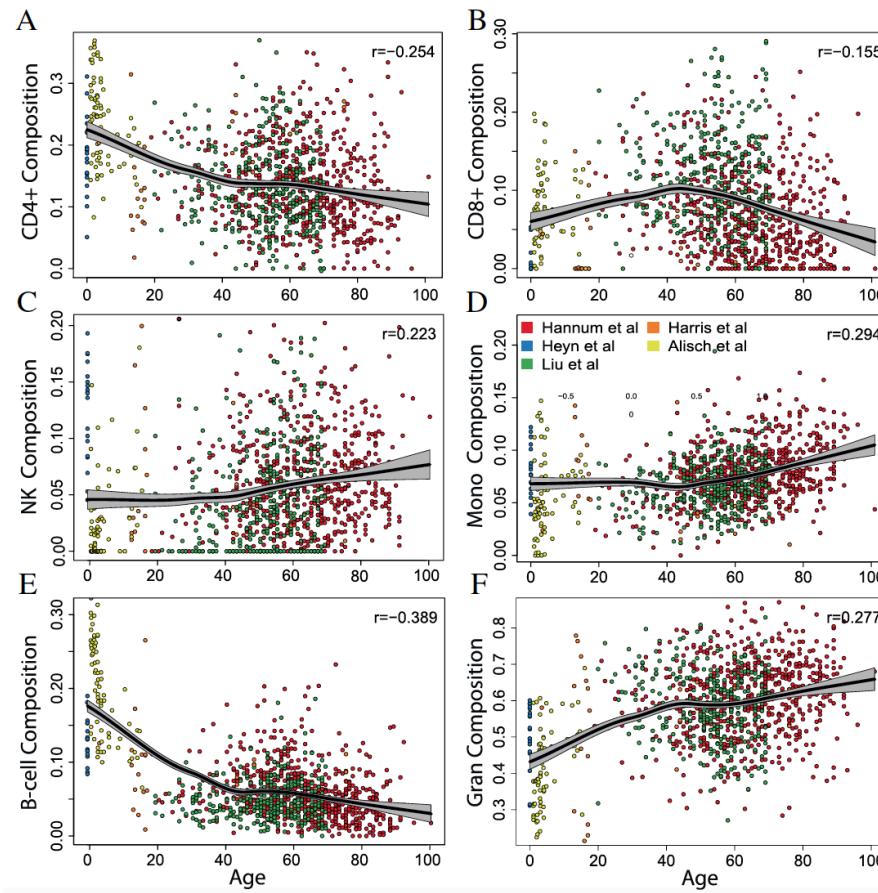
Biological artifact: cell mixture

- Tissue sample is a mixture of different cell types.
- Data collected are mixed signals.



An example: EWAS in aging study

- Cellular composition changes with age.
- Cellular composition is a major source of variability in DNA methylation datasets in whole blood.



Jaffe and Irizarry GB(2014)

Existing signal deconvolution methods

- **Reference-based** methods (some type of regression):
 - Require cell type specific signature: Abbas et al. 2009; Clarke et al. 2010; Gong et al. 2011; Lu et al. 2003; Wang et al. 2006; Vallania et al. 2018; Du et al. 2018;
 - Requires mixture proportions: Erkkila et al. 2010; Lahdesmaki et al. 2005; Shen-Orr et al. 2010; Stuart et al. 2004.
- **Reference free** methods (some type of factor analysis):
 - Gaujoux et al. 2011; Kuhn et al. 2011; Repsilber et al. 2010; Roy et al. 2006; Venet et al. 2001; Houseman et al. 2012, 2014, 2016; Rahmani et al. 2016, 2018; Lutsik et al. 2017; Xie et al. 2018;

Method to adjust for cell proportion

- In EWAS, add proportion as covariate in the model:
- More rigorous statistical modeling for DE/DM with sample mixture has been a popular topic recently, and a number of methods are developed:
 - csSAM: Shen-Orr et al. 2010 *Nature methods*
 - CellIDMC: Zheng et al. 2018 *Nature Methods*
 - TOAST: Li et al. 2019 *Bioinformatics*, 2019 *Genome Biology*

Review

- We have covered microarray analysis DE test, including:
 - SAM t-test.
 - EB method: Limma.
 - A little on complex design.
 - Permutation test.
 - Multiple testing.
 - R/Bioconductor packages for DE analysis.
- Batch effects.
- Cell type mixture in complex tissues