

Experimental design

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Brixen 2016

“To call in the statistician
after the experiment is done
may be no more than asking him
to perform a postmortem examination:
he may be able to say what the
experiment died of.”

Sir Ronald Fisher, Indian Statistical Congress, Sankhya, around 1938



Stephen John Senn

@stephensenn



Follow

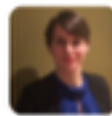
Statisticians are the bad fairies of research.
People forget to invite them until it's too late, at
which point they send everyone to sleep.

RETWEETS

92

LIKES

93



11:22 AM - 21 Feb 2016

What is experimental design?

- The organization of an experiment, to ensure that the **right type** of data, and **enough** of it, is available to answer the **questions of interest** as clearly and efficiently as possible.

What is **bad** experimental design?

Treatment I

M M M M M M M M

Treatment II

F F F F F F F F

What is **bad** experimental design?

Treatment 1

M M M M M M

Treatment 2

F F F F F F F

Confounding!

What is **bad** experimental design?

Analysis batch I / Study center I / Processing protocol I ...

Tr

Tr

Tr

Tr

Tr

Tr

Tr

Tr

Analysis batch II / Study center II / Processing protocol II ...

Ctl

Ctl

Ctl

Ctl

Ctl

Ctl

Ctl

Ctl

What is **bad** experimental design?

Analysis batch I / Study center I / Processing protocol I ...

Tr Tr Tr Tr Tr Tr

Analysis batch II / Study center II / Processing protocol II ...

Ctl Ctl Ctl Ctl Ctl Ctl Ctl

Confounding!

What can happen with bad experimental design?

- Example: gene expression study comparing 60 CEU and 82 ASN HapMap individuals
- 26% of the genes were found to be significantly differentially expressed (78% with less restrictive multiple testing correction)
- **But**: all CEU samples were processed (sometimes years) before all the ASN samples!

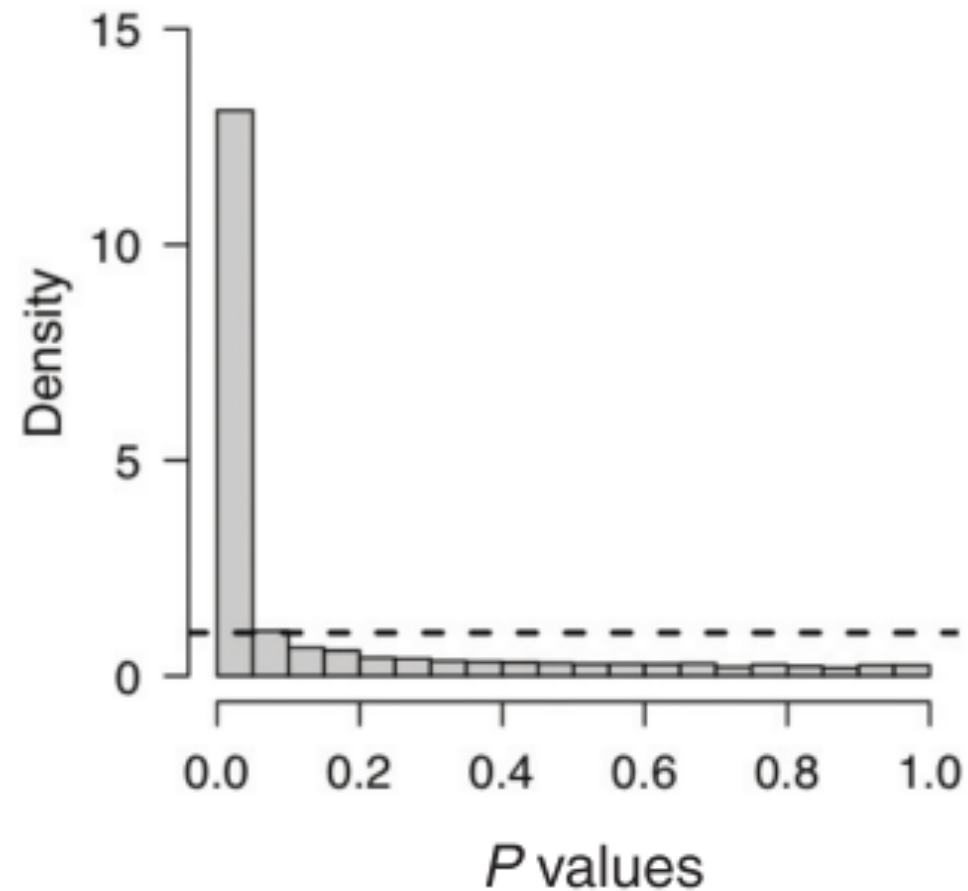
What can happen with bad experimental design?

- Example: gene expression study comparing 60 CEU and 82 ASN HapMap samples
- 26% of the genes could be significantly differentially expressed with less restrictive multiple testing (FDR = 0.05)
- **But**: all CEU samples were processed (sometimes years) before all the ASN samples!

Confounding!

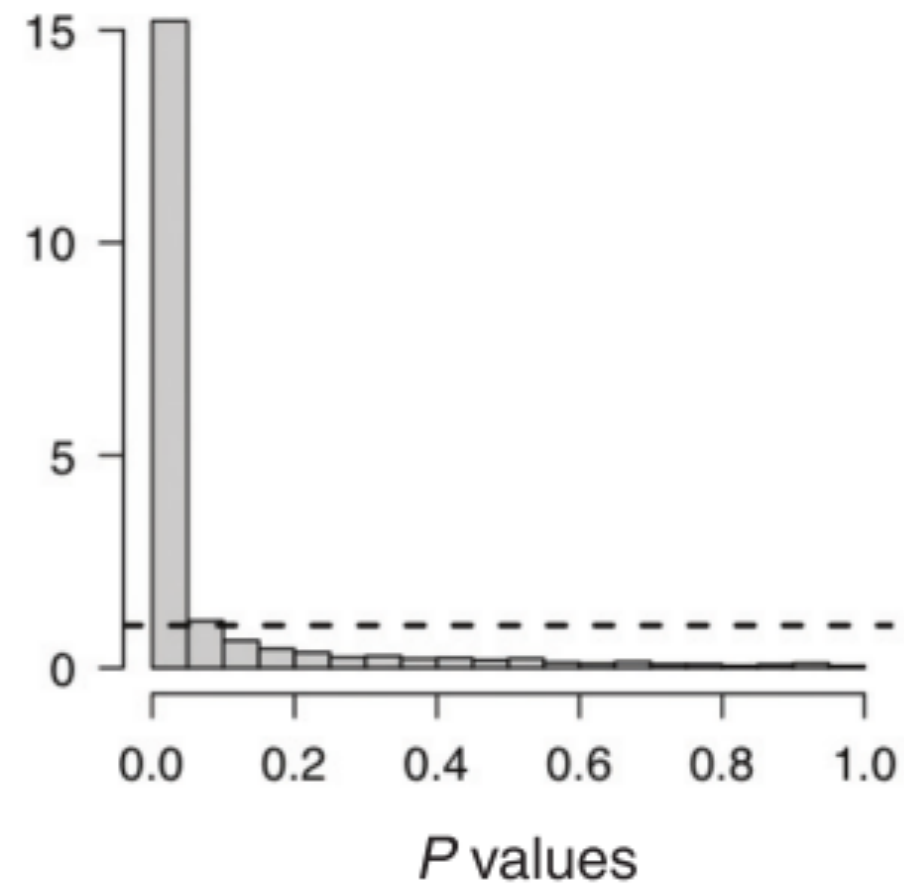
What can happen with bad experimental design?

a Comparing CEU and ASN



78% differentially expressed

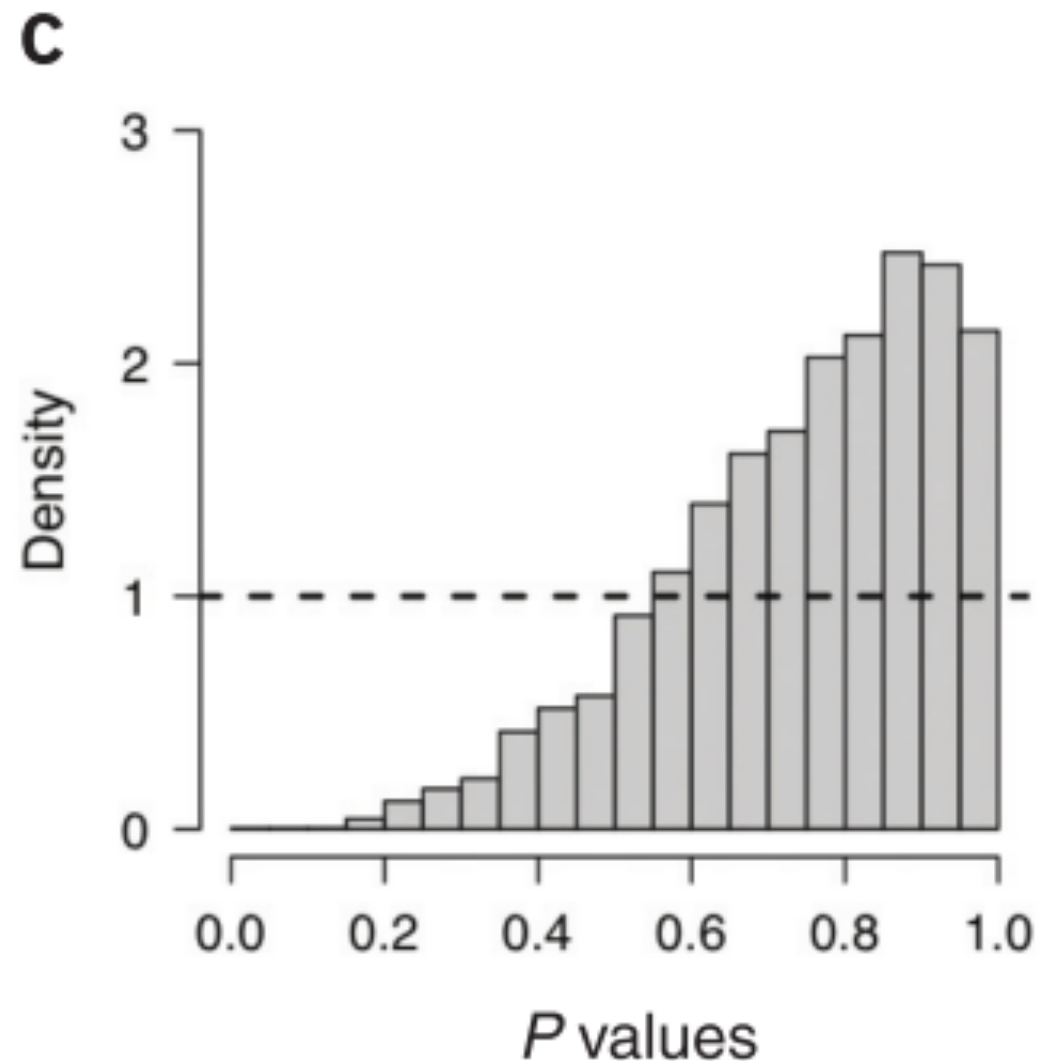
b Comparing processing times



96% differentially expressed

“Batch effect correction” won’t work here

p-values from test comparing CEU and ASN, after controlling for the processing year

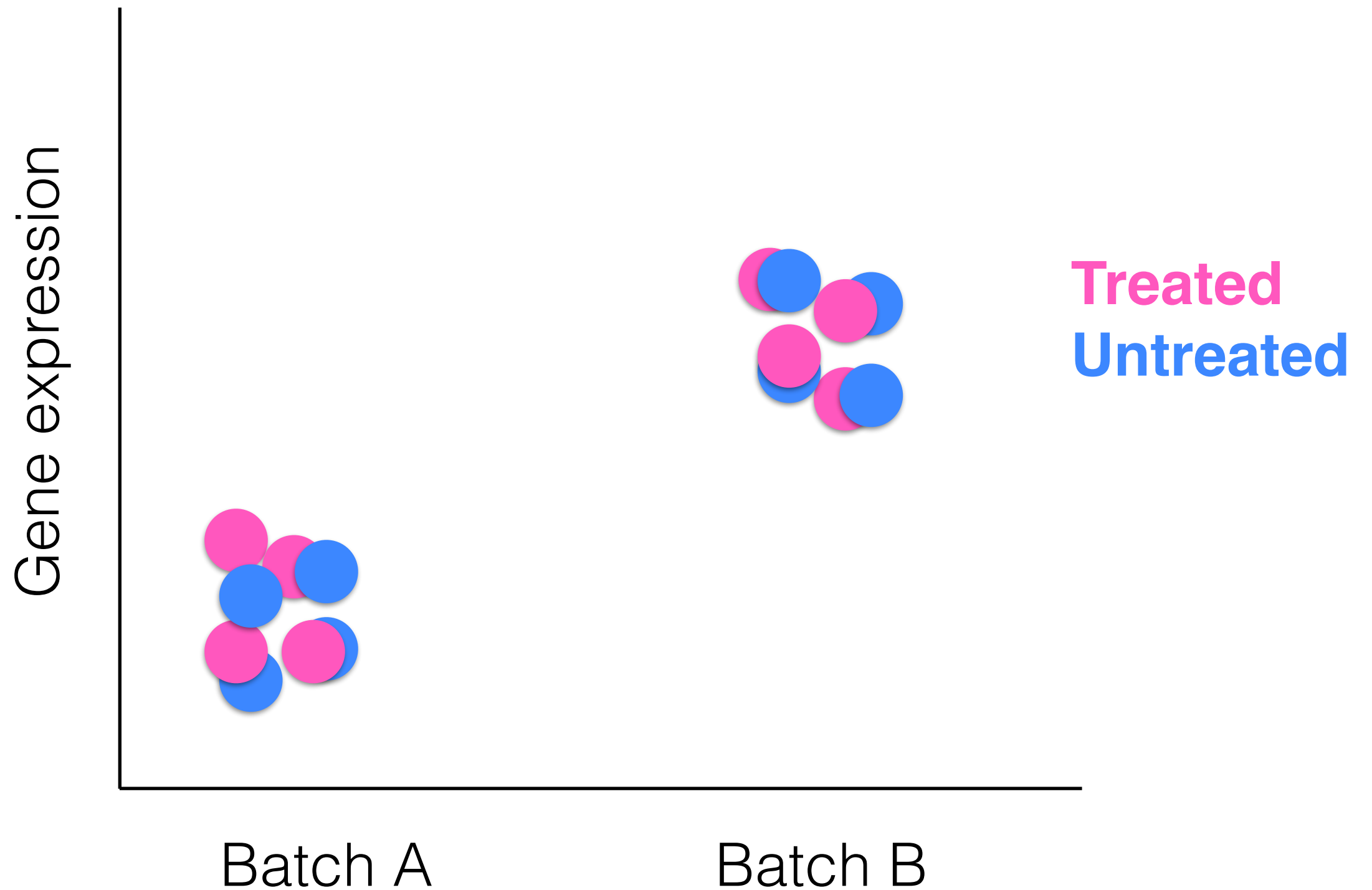


0% differentially expressed

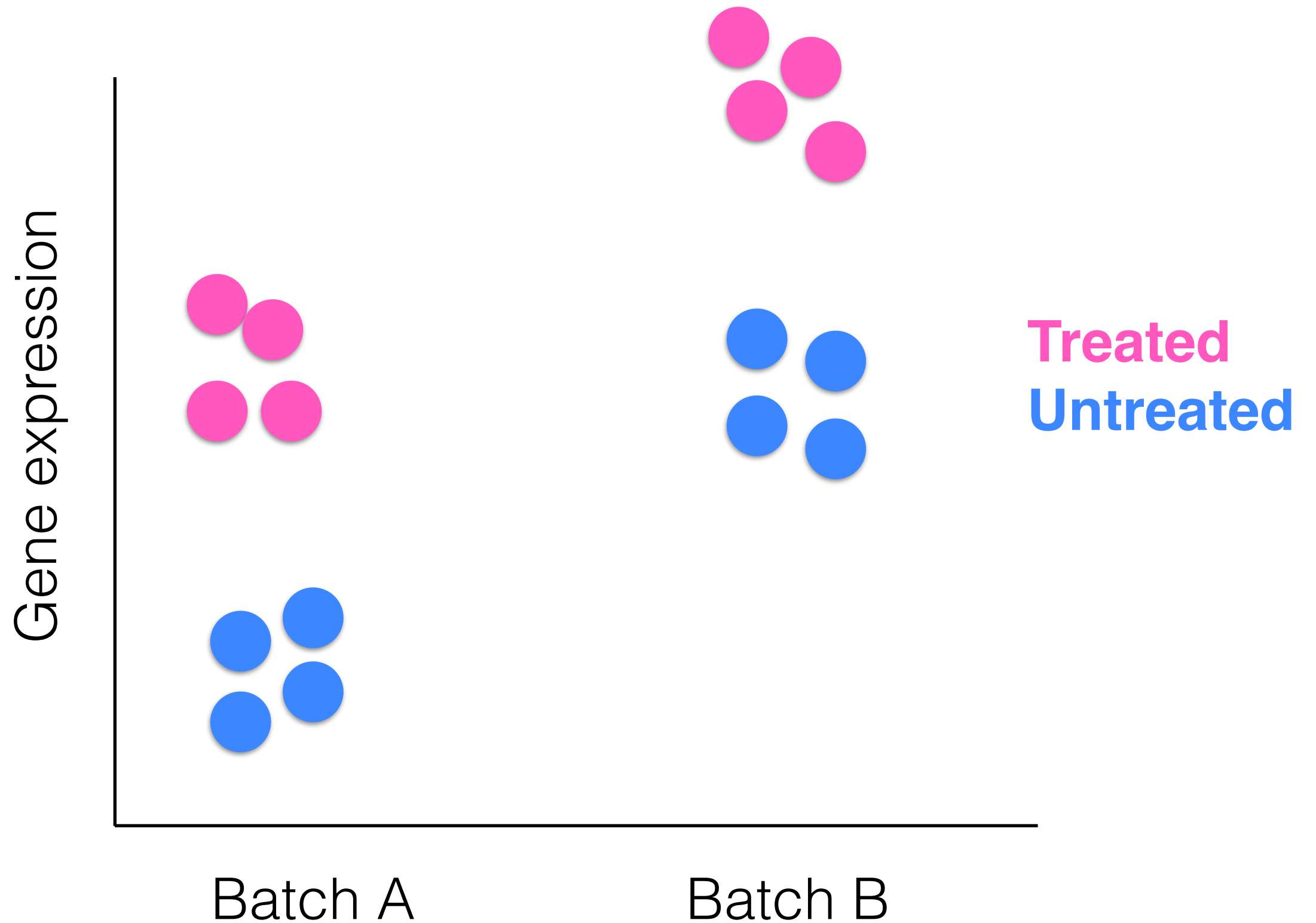
What could be a good experimental design?

- Process all samples at the same time (not always feasible)
- Minimize confounding as much as possible through
 - blocking
 - randomization
- The batch effect will still be there, but with an appropriate design we can account for it!

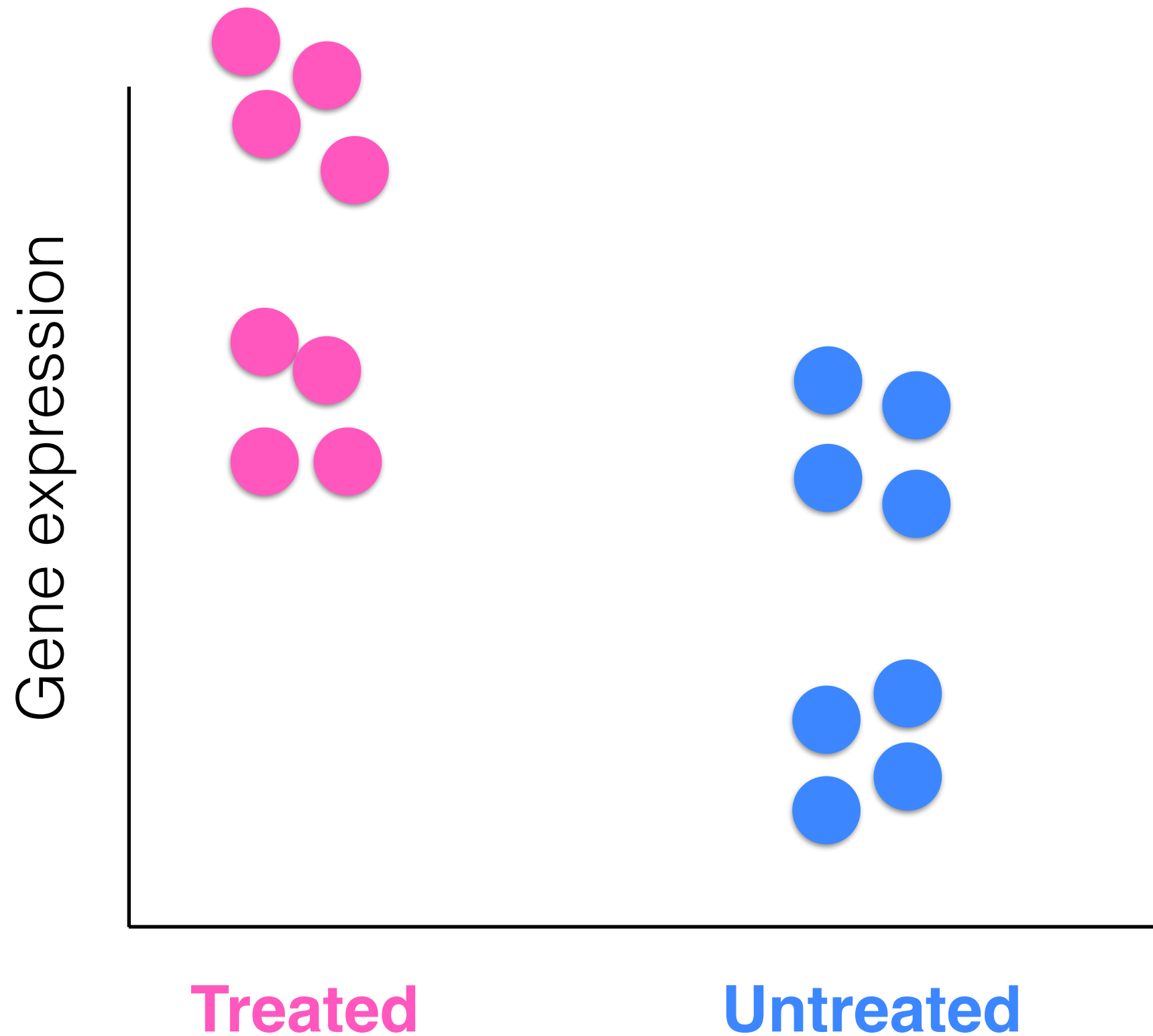
Non-confounded design



Non-confounded design



Non-confounded design

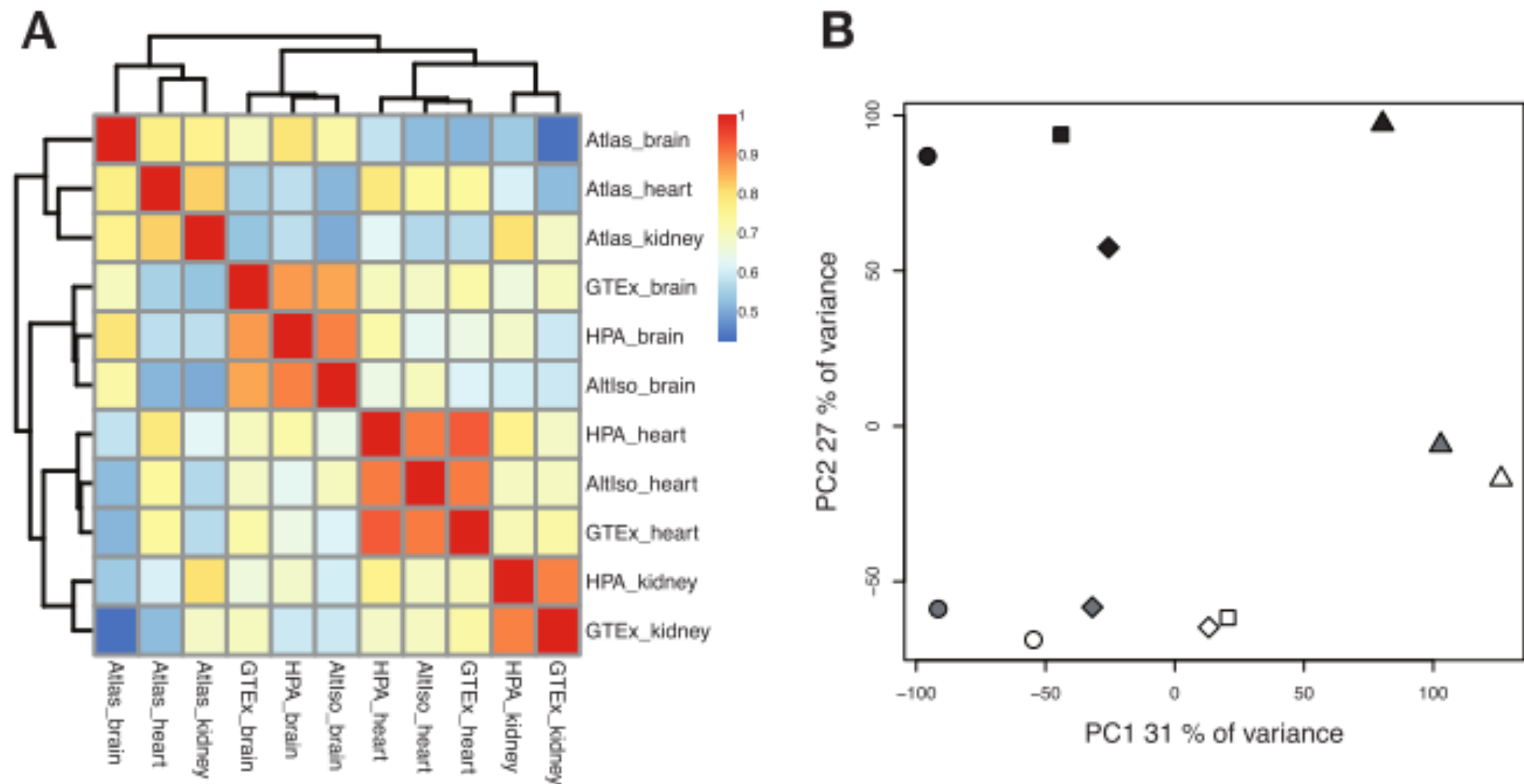


Accounting for batch effects

- In statistical modeling, batch effects are typically included as **covariates** (additional predictors) in the model.
- For exploratory analysis, we typically attempt to “eliminate” or “adjust for” such unwanted variation in advance, by subtracting the estimated effect from each variable.
- Even partial confounding between batch and signal of interest can lead to bias.

Accounting for batch effects in practice

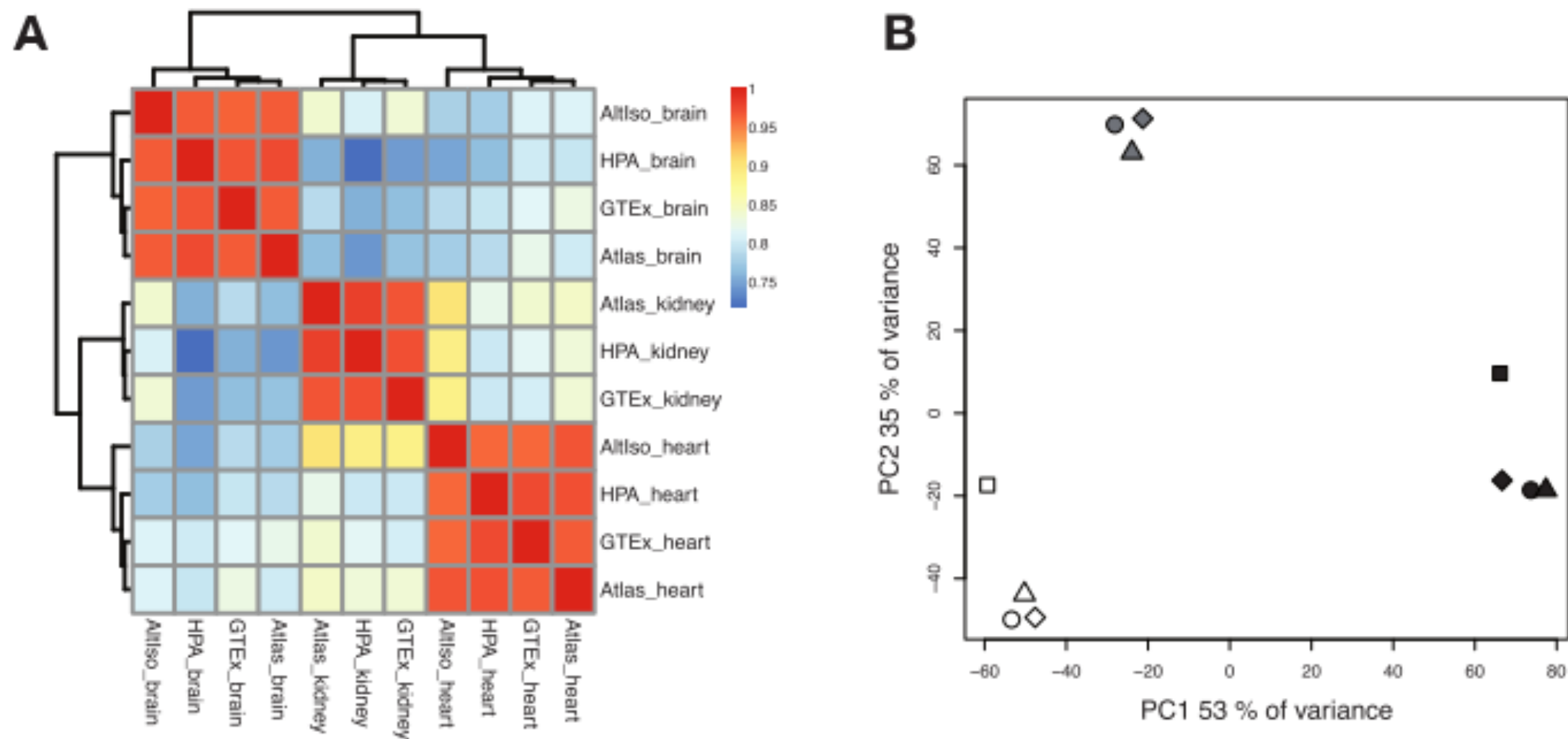
- Public, processed RNA-seq data from 3 tissues, 4 studies show strong association with study



color = tissue; symbol = study (batch)

Accounting for batch effects in practice

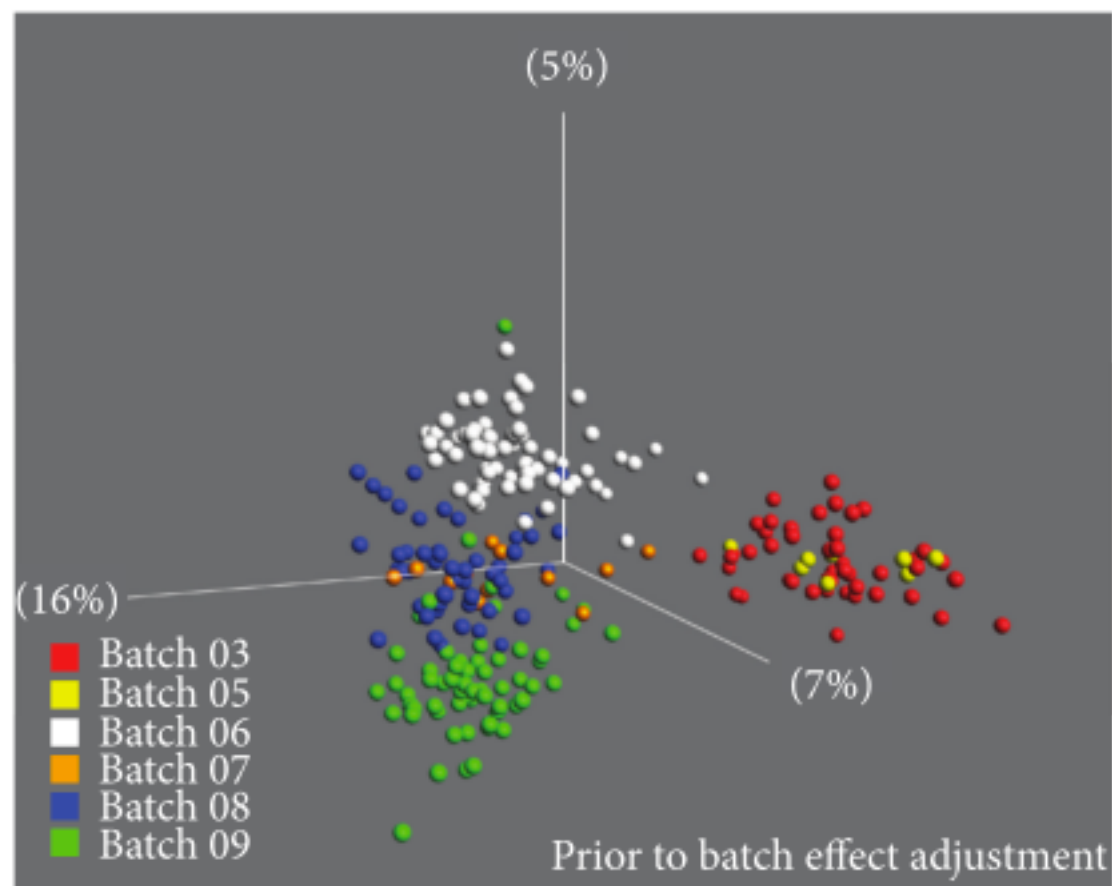
- Accounting for the batch effect brings out signal of interest.



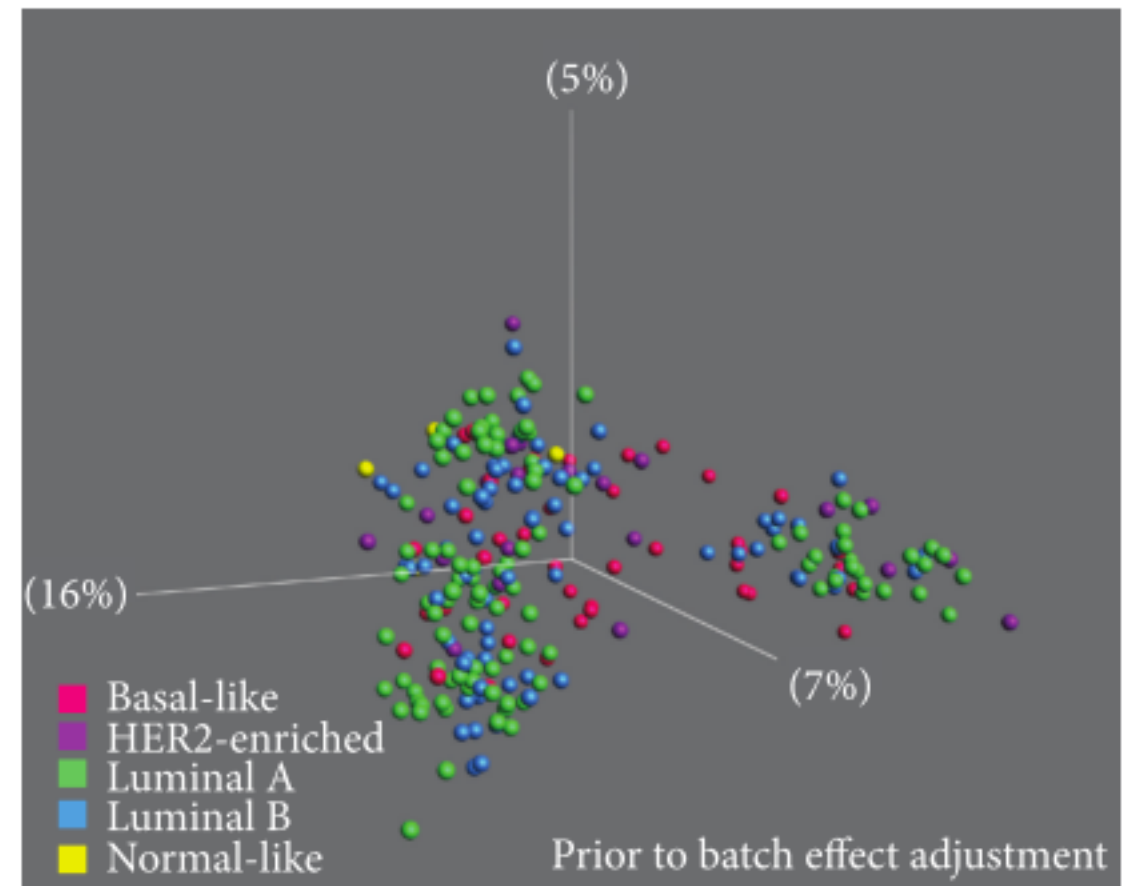
color = tissue; symbol = study (batch)

Accounting for batch effects in practice

- 5-subtype breast cancer microarray data processed in six batches.



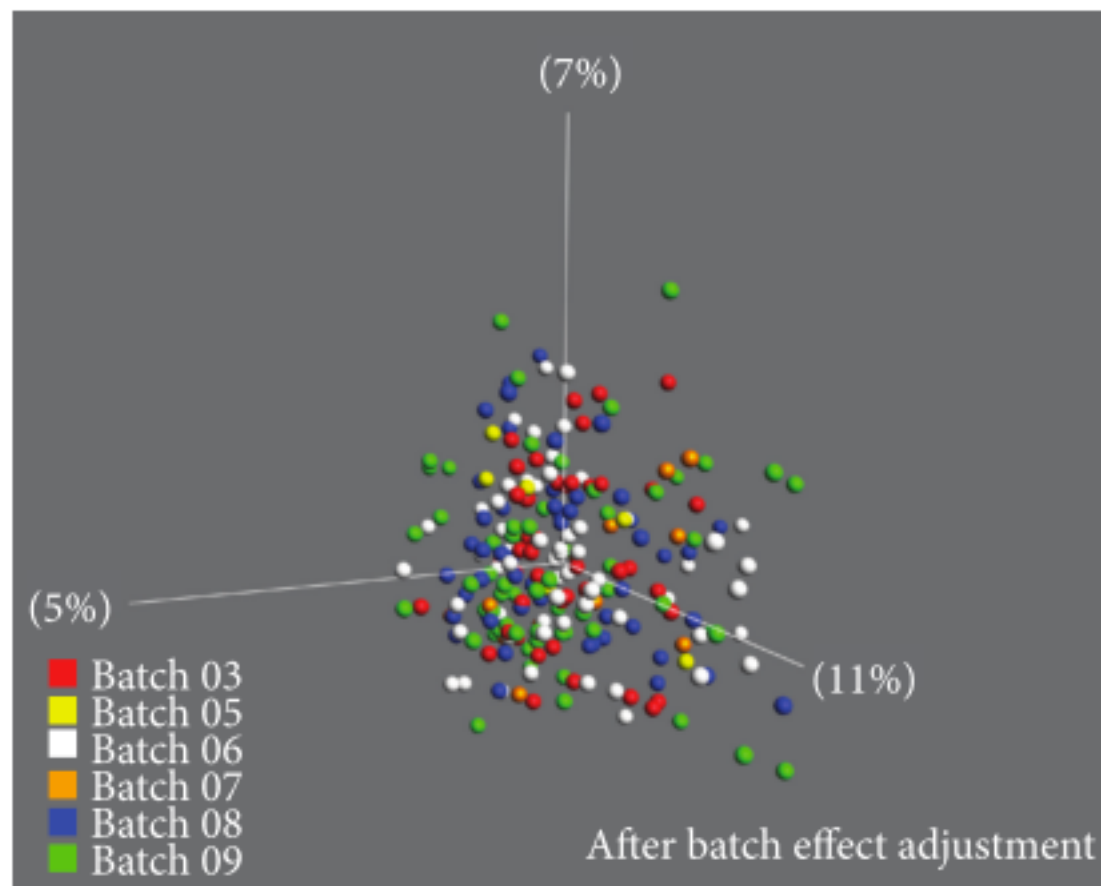
(a)



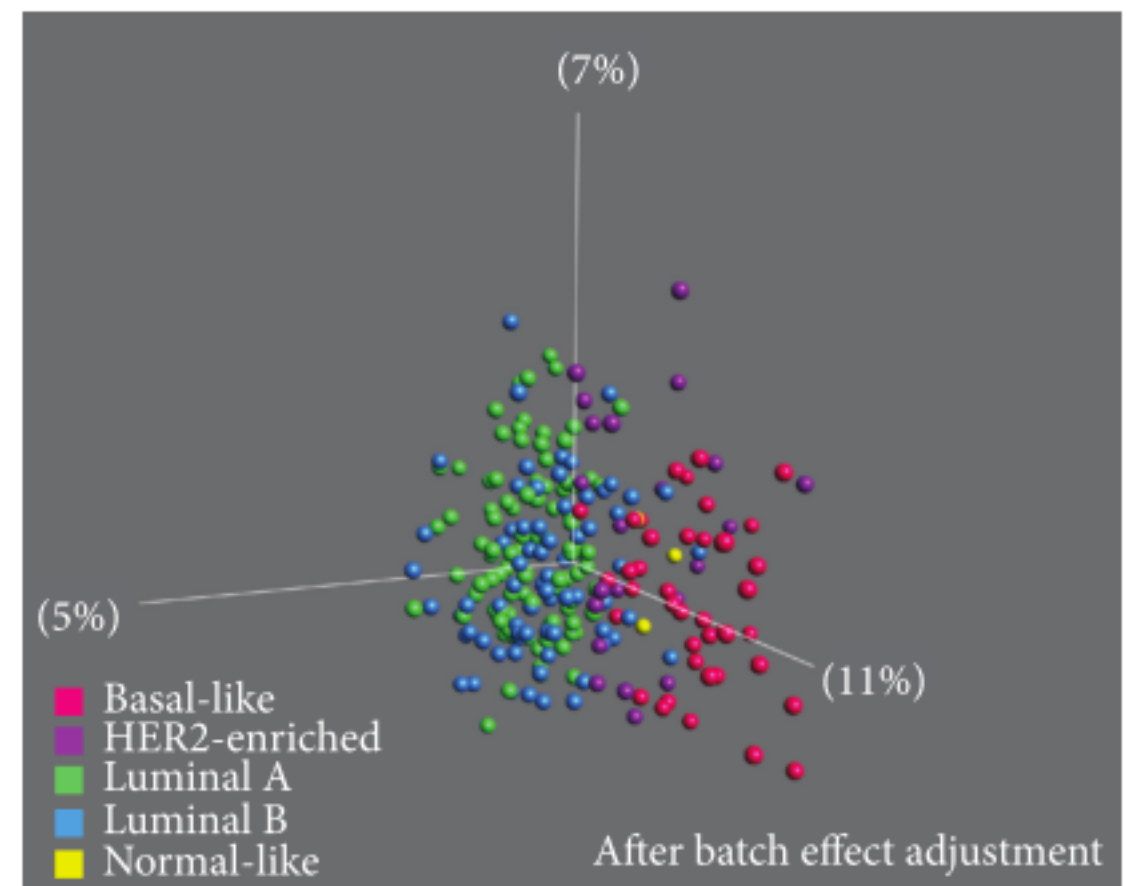
(c)

Accounting for batch effects in practice

- 5-subtype breast cancer microarray data processed in six batches.



(b)



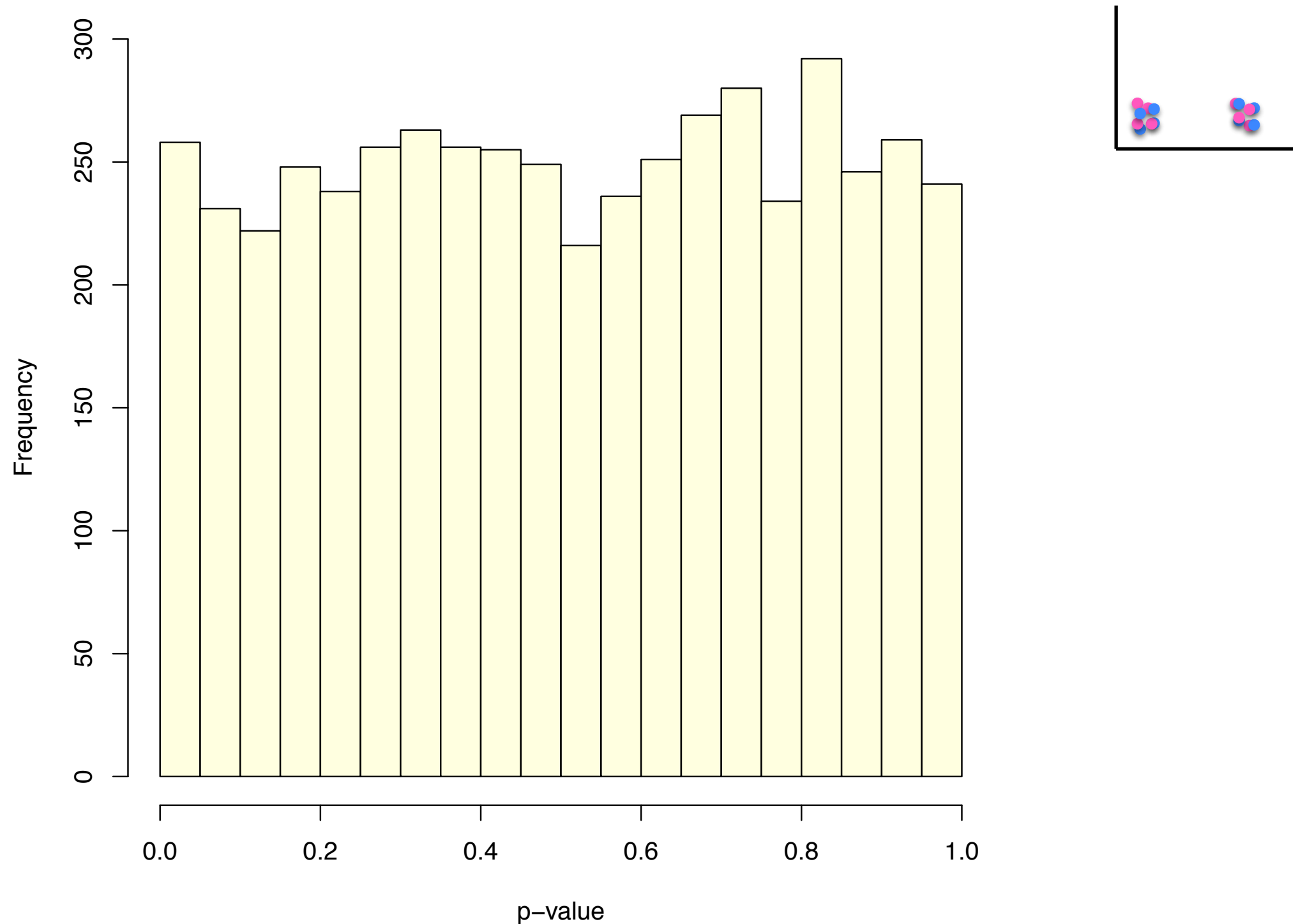
(d)

What if the batch variable is unknown?

- Manifests as systematic “unwanted variation” in data
- Identify using e.g.
 - control genes (“housekeeping” genes, spike-ins)
 - residuals after eliminating known signal
- Include estimated unwanted variation as covariate(s) in the statistical model
- **RUV**, **sva** packages commonly used in genomics

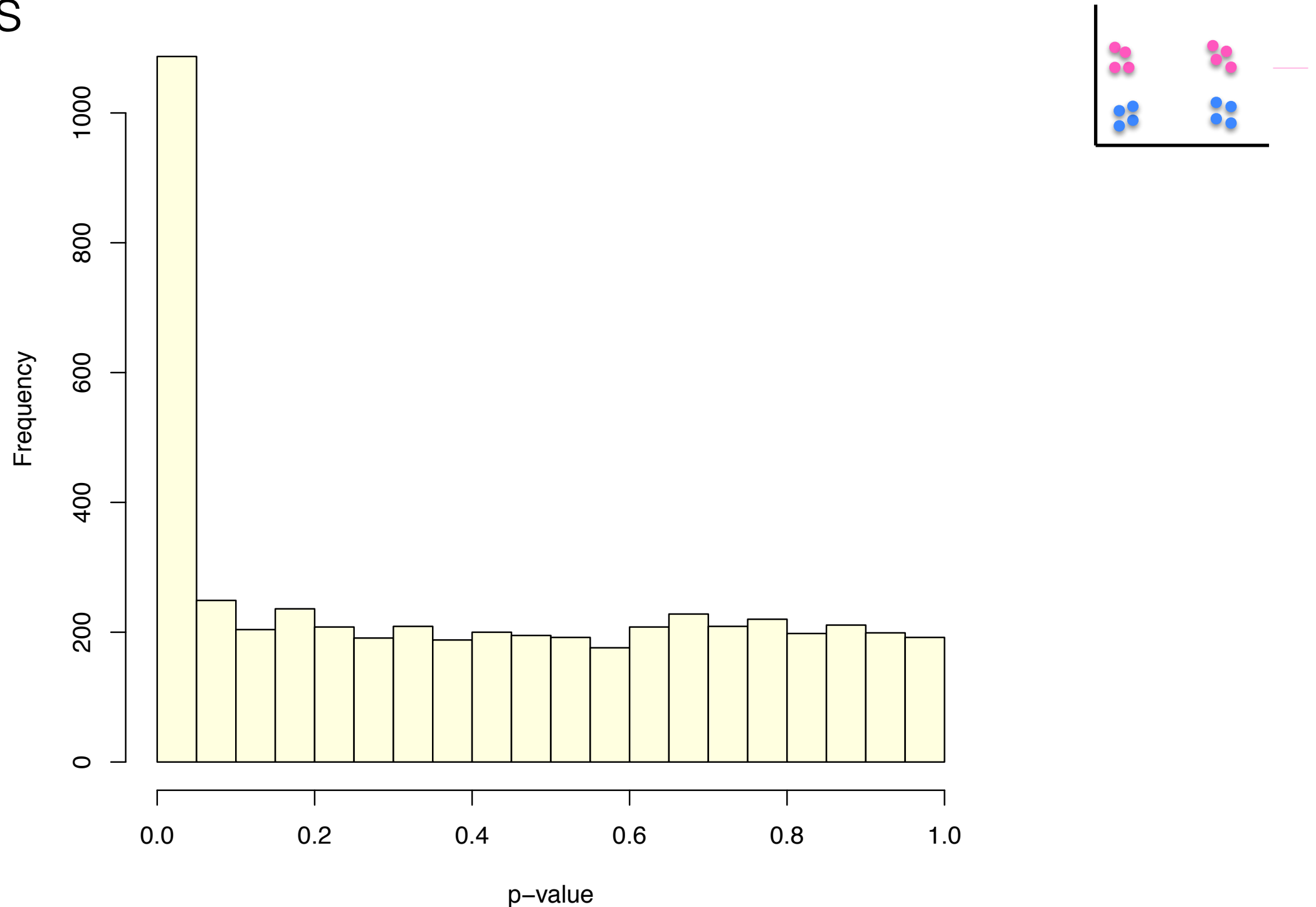
Impact of batch effect on p-value histogram

- No batch effect, no differentially expressed genes



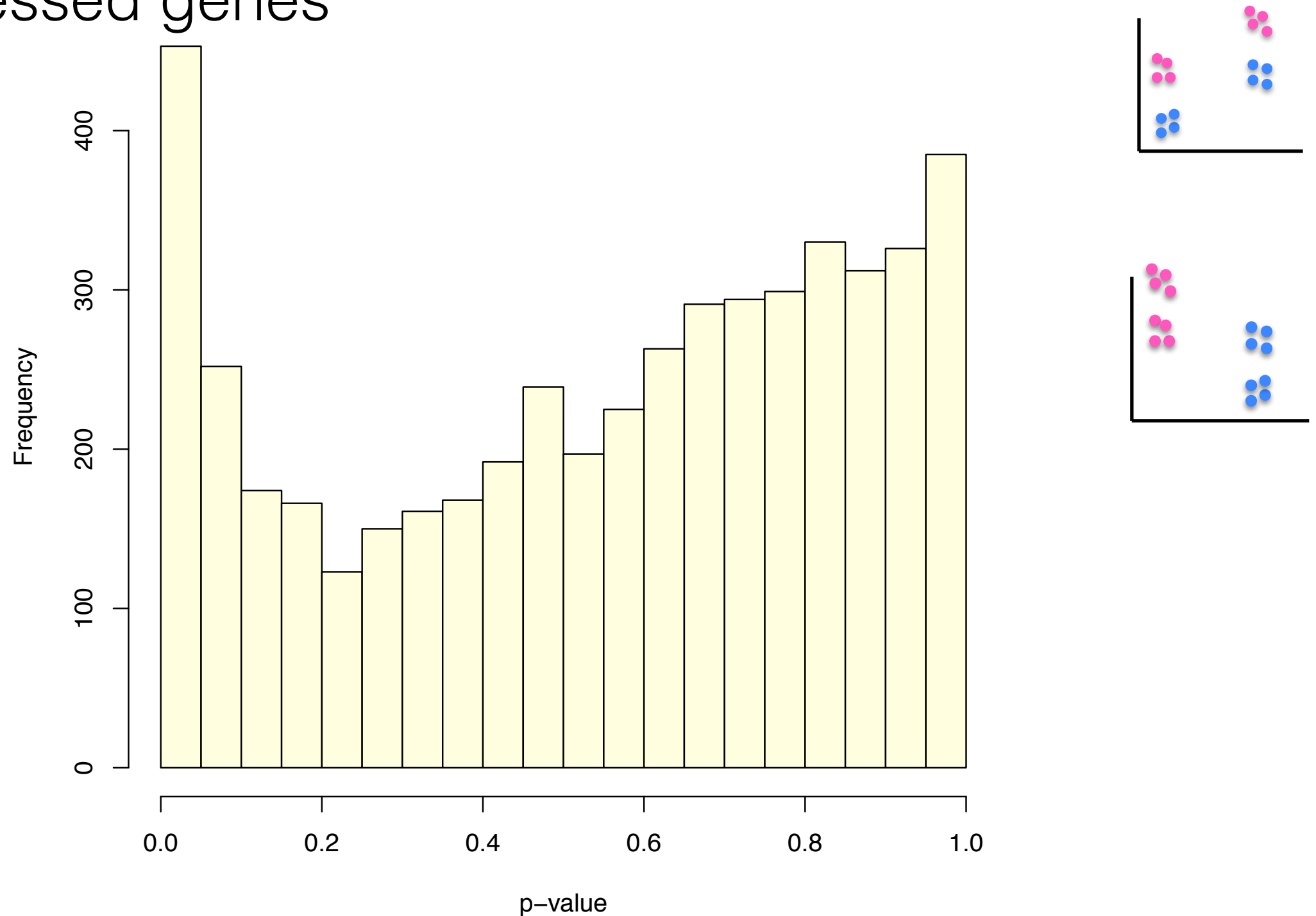
Impact of batch effect on p-value histogram

- No batch effect, some differentially expressed genes



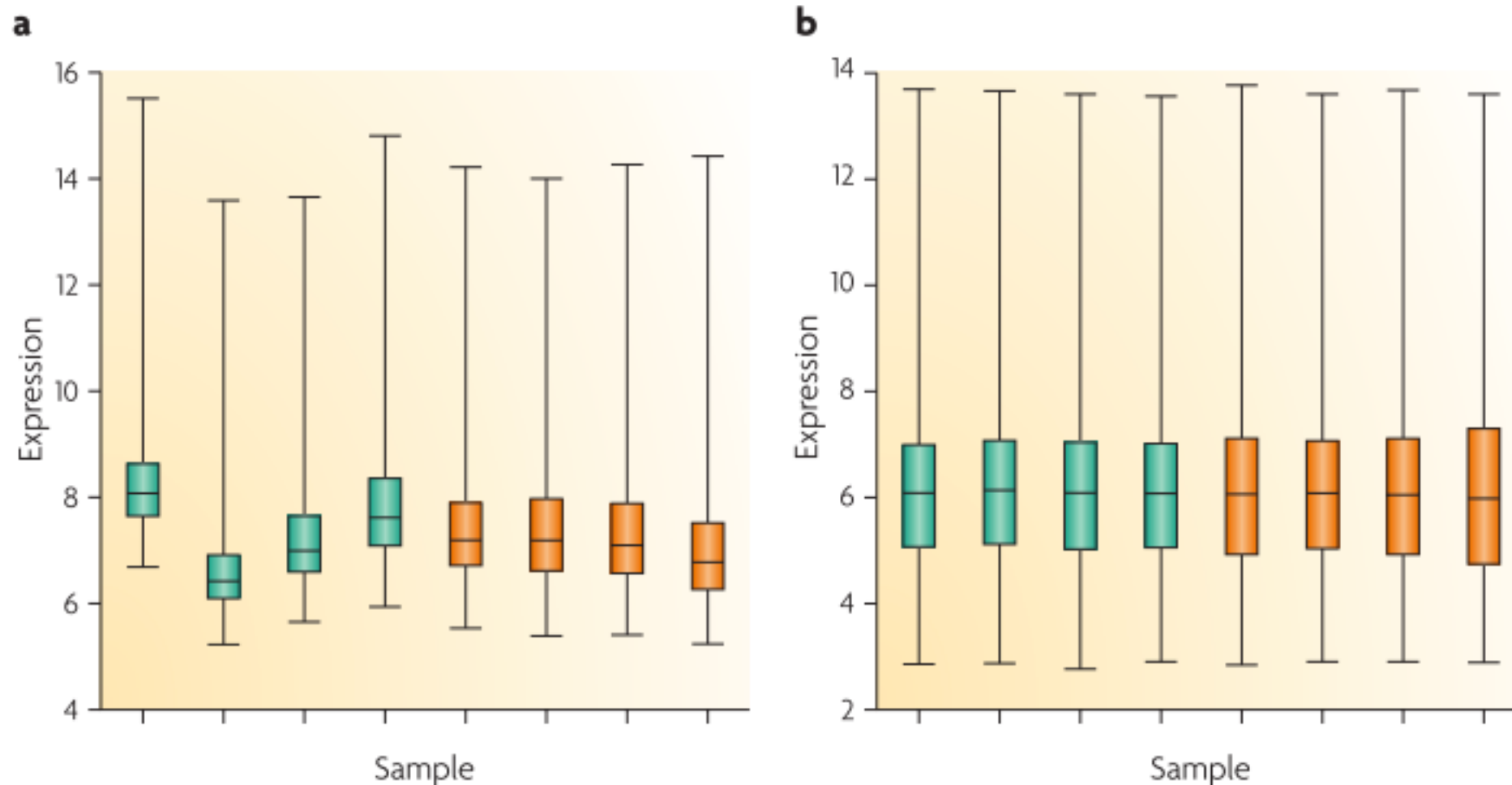
Impact of batch effect on p-value histogram

- Batch effect (no confounding), some differentially expressed genes



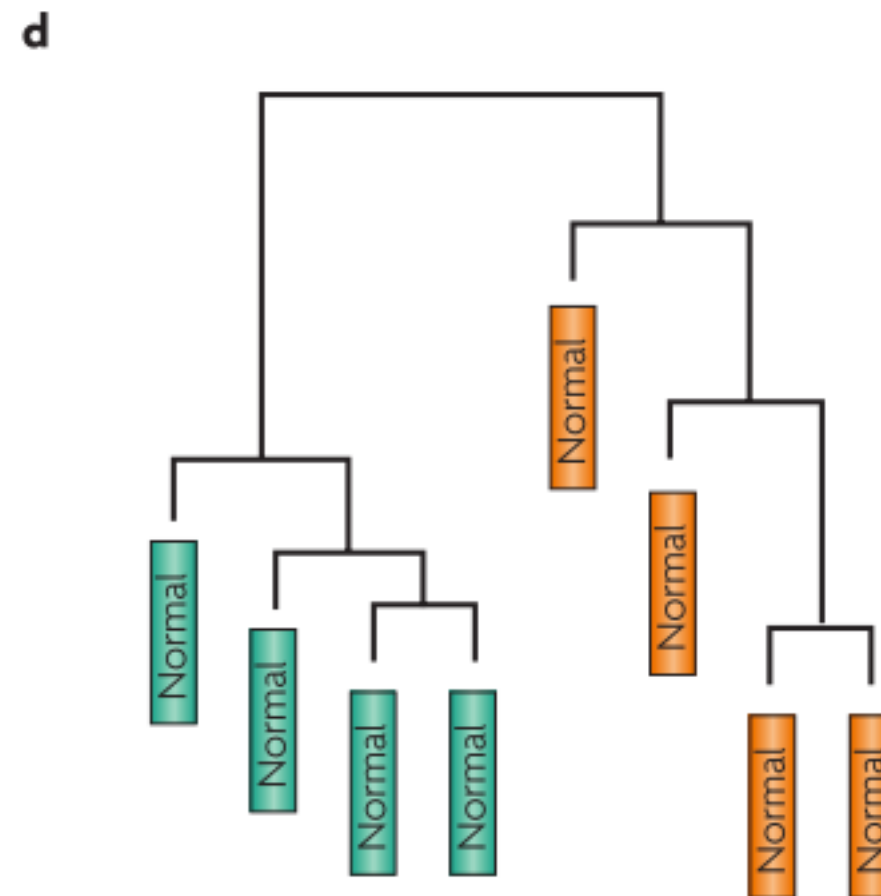
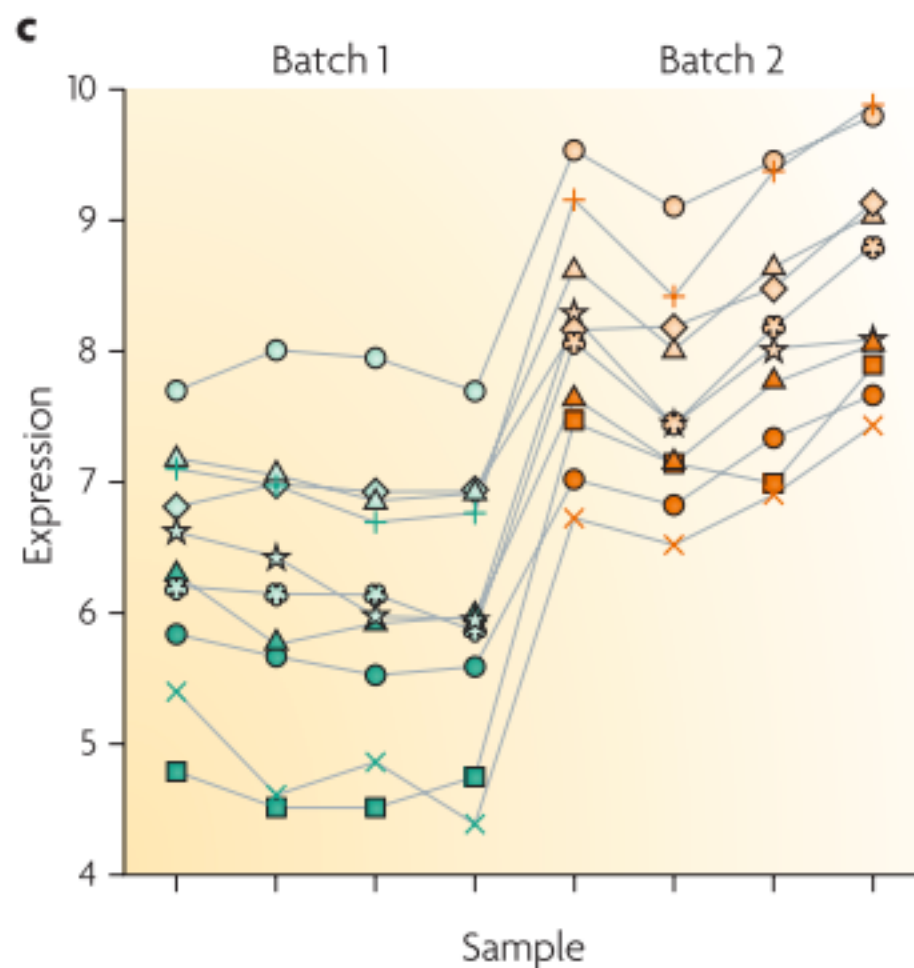
Batch effect adjustment vs normalization

- Batch effect adjustment goes **beyond** the “global” between-sample normalization methods.



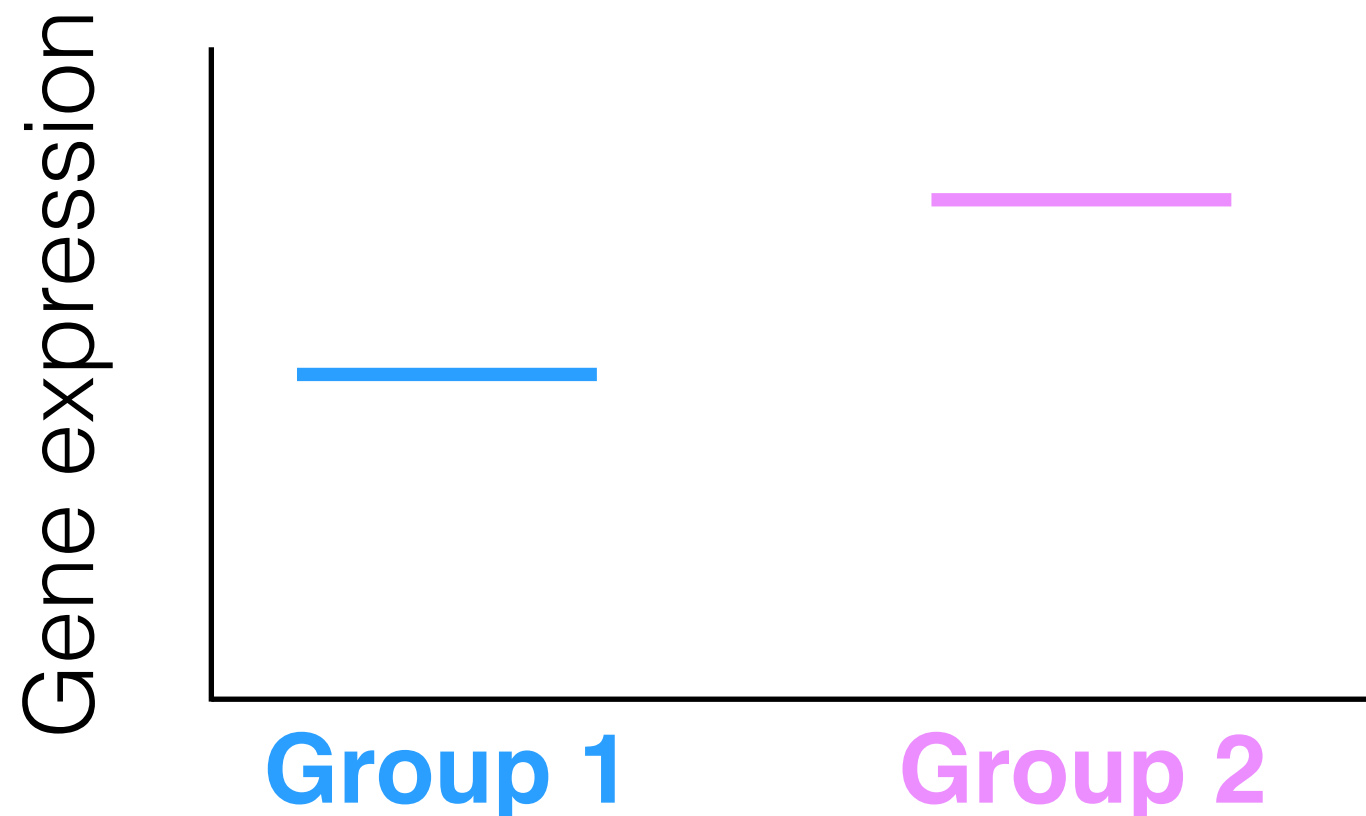
Batch effect adjustment vs normalization

- Batch effect adjustment goes **beyond** the “global” between-sample normalization methods.



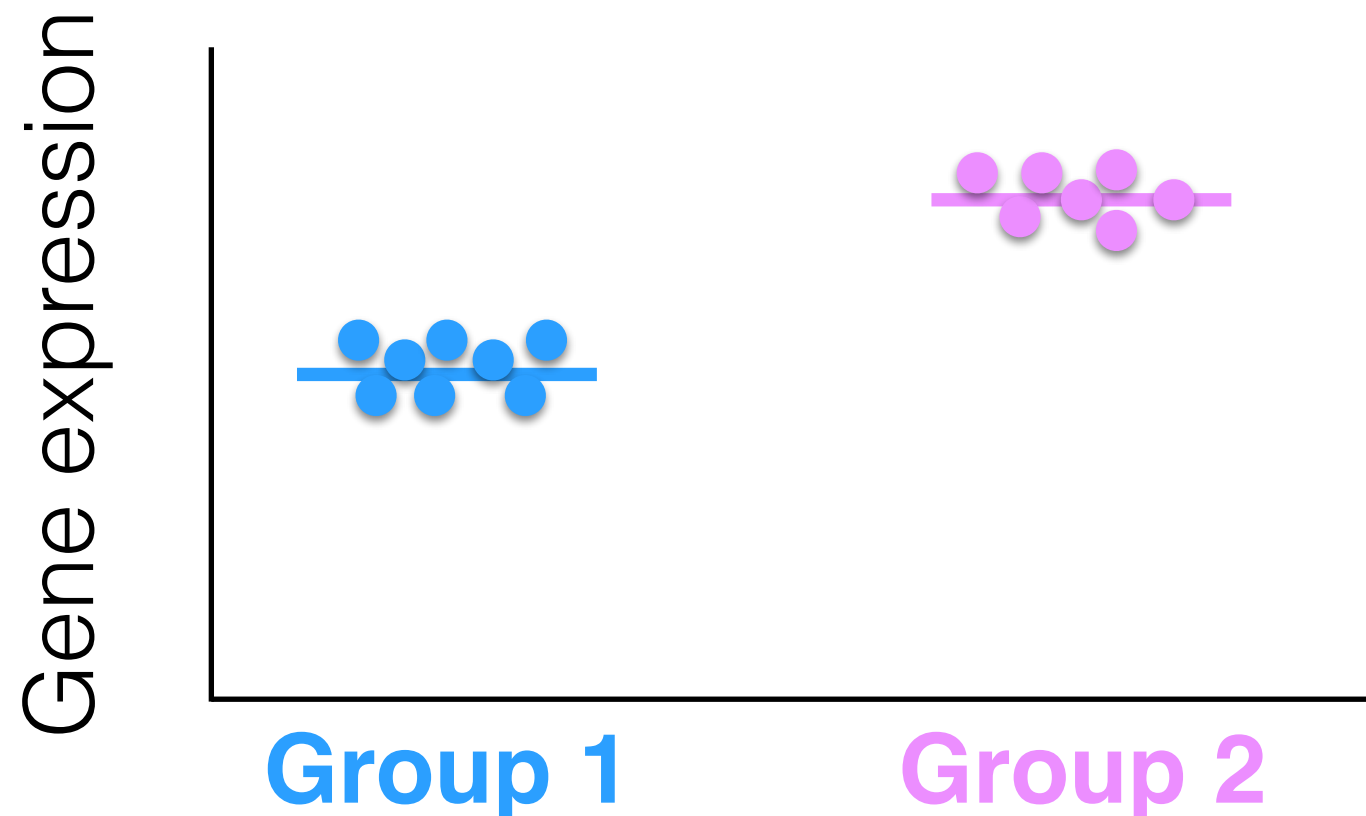
Other design issues: replication

- Replicates are **necessary** to estimate within-condition variability.
- Variability estimates are, in turn, **vital** for statistical testing.



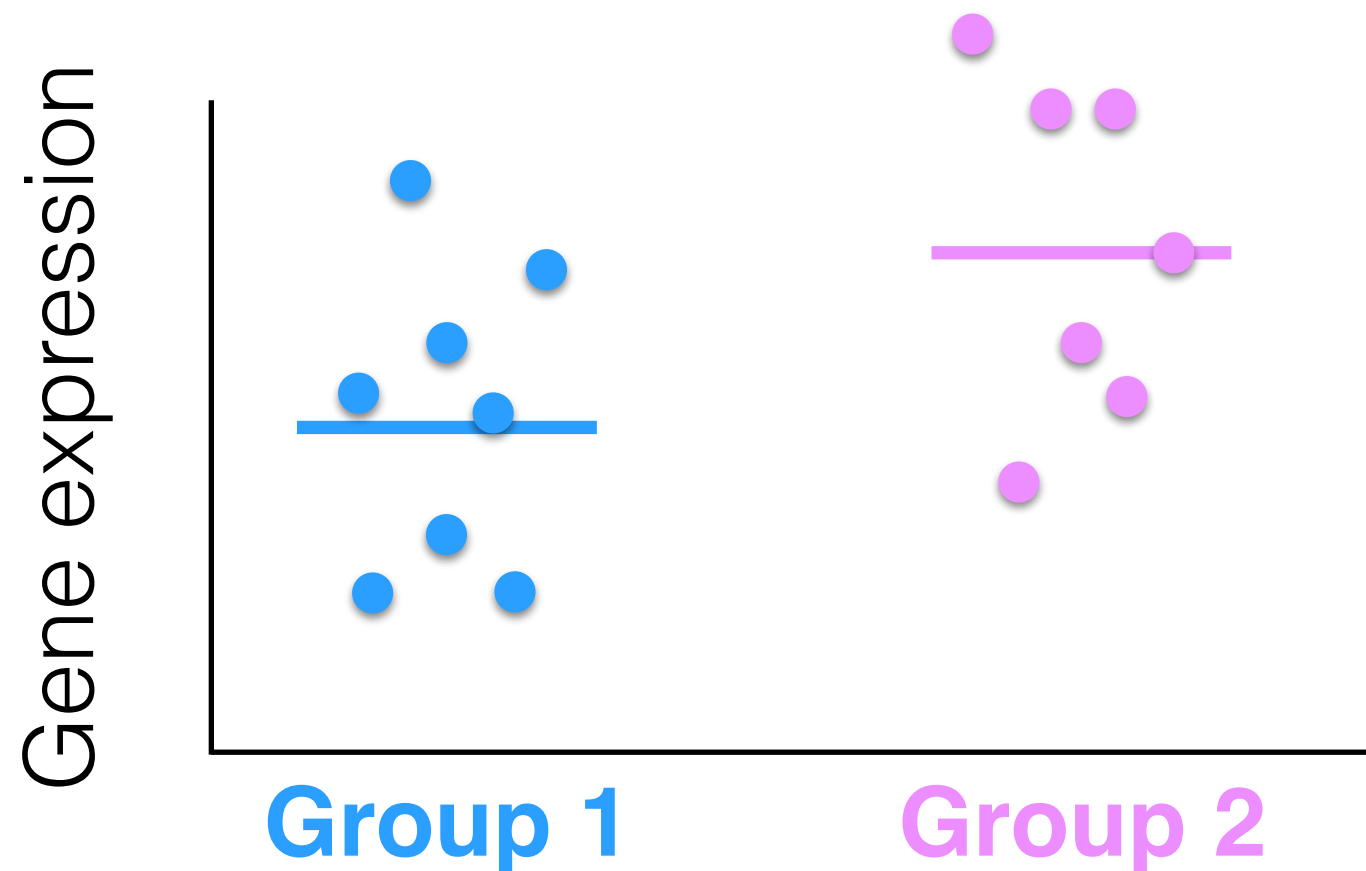
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Other design issues: replication

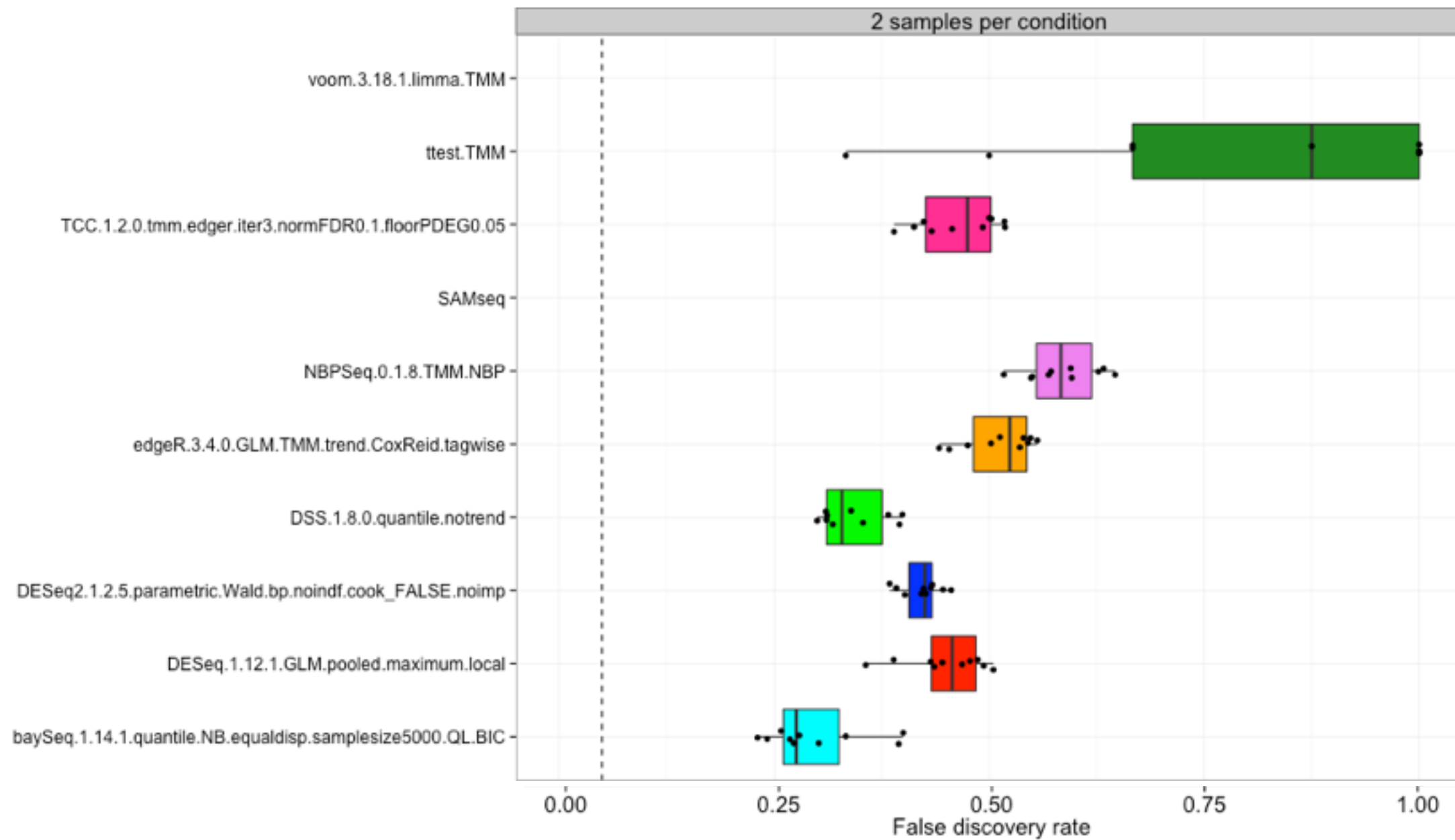
- Replicates are **necessary** to estimate within-condition variability.
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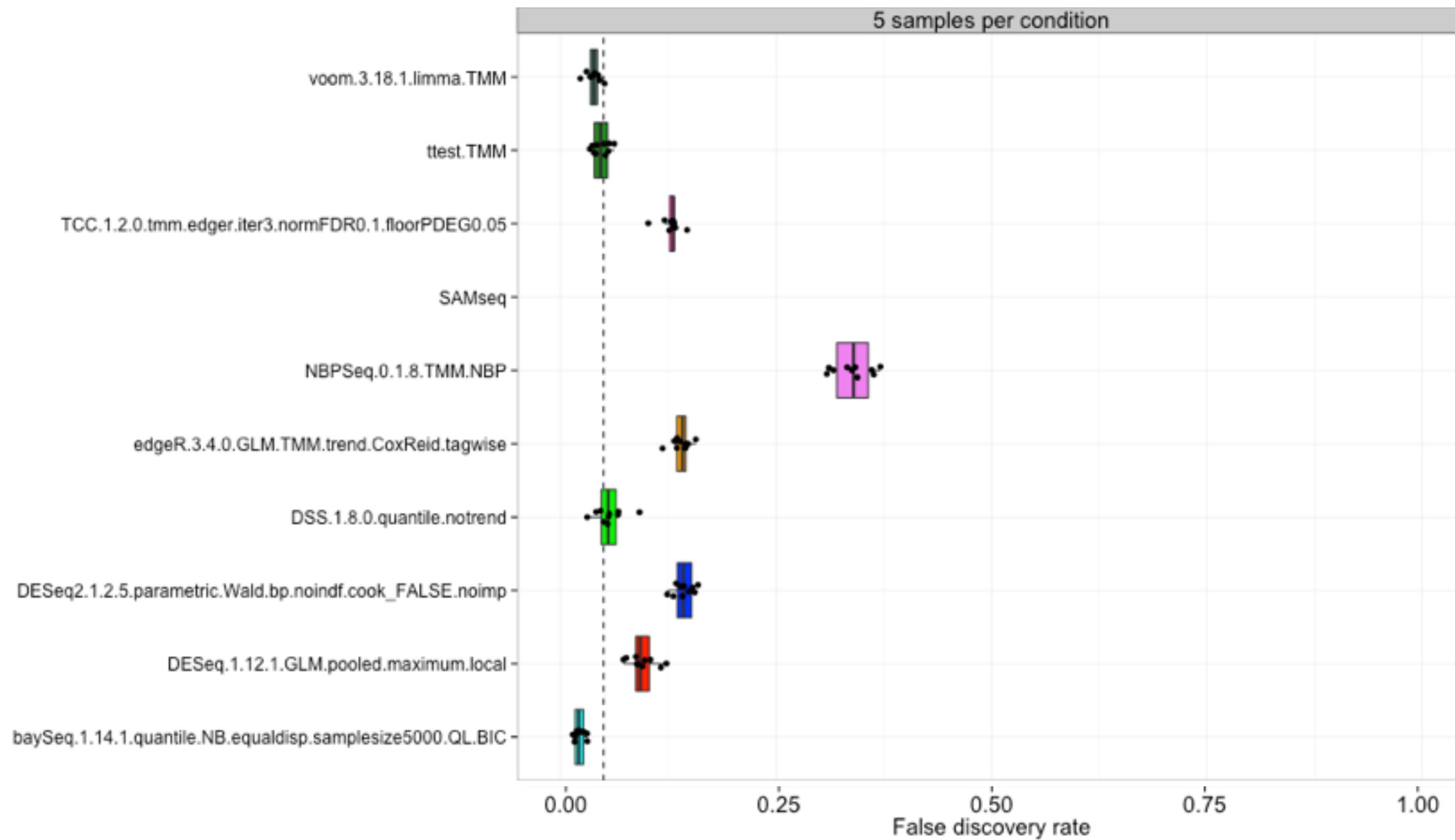
Other design issues: sample size

- As always, it depends...
 - on what we want to do (differential gene expression, variant detection, GWAS, ...)
 - on the variability between samples (cell lines, inbred animals, patients, ...)
 - on the magnitude of the expected effect

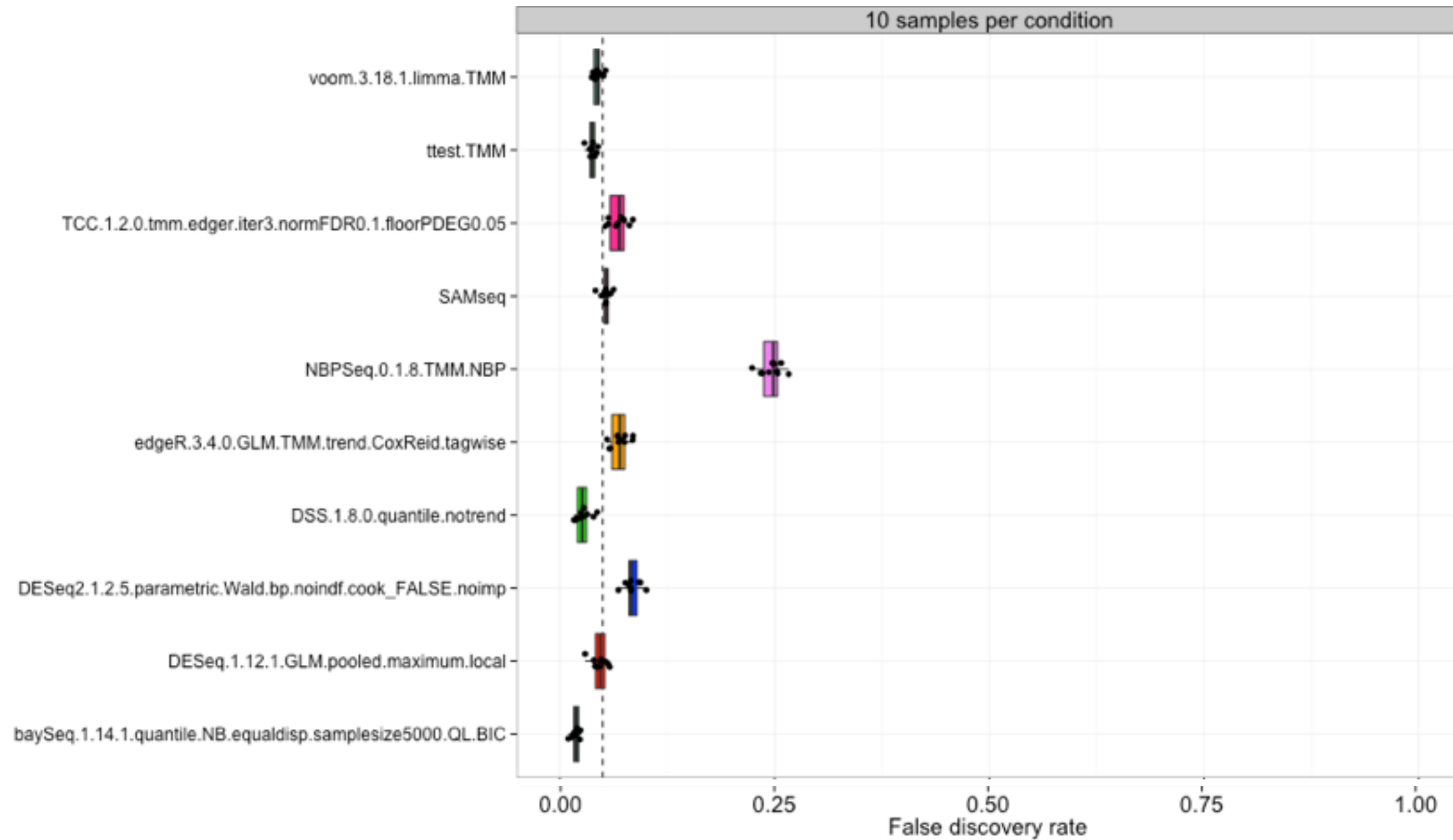
FDR, 2 replicates/condition



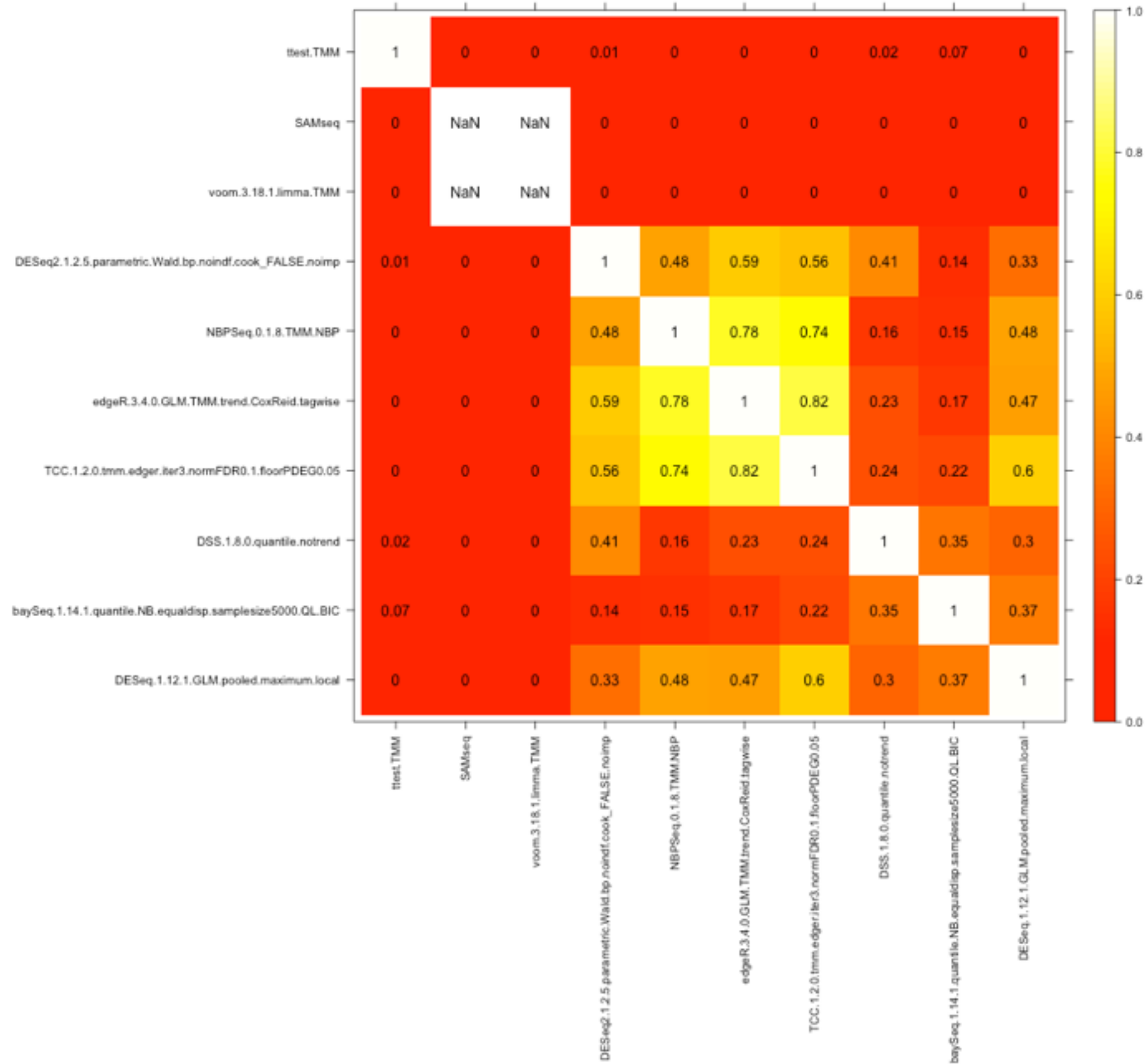
FDR, 5 replicates/condition



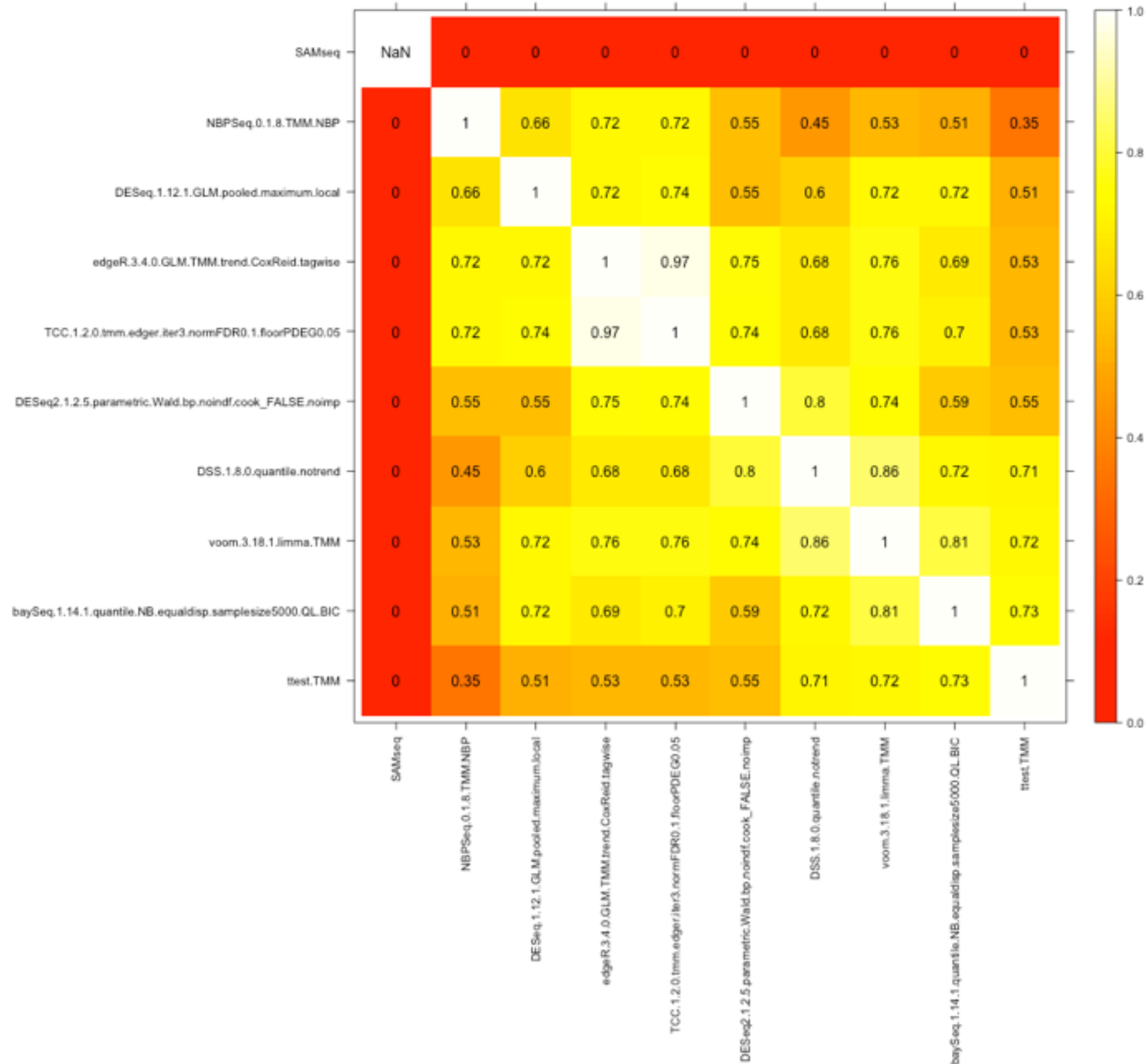
FDR, 10 replicates/condition



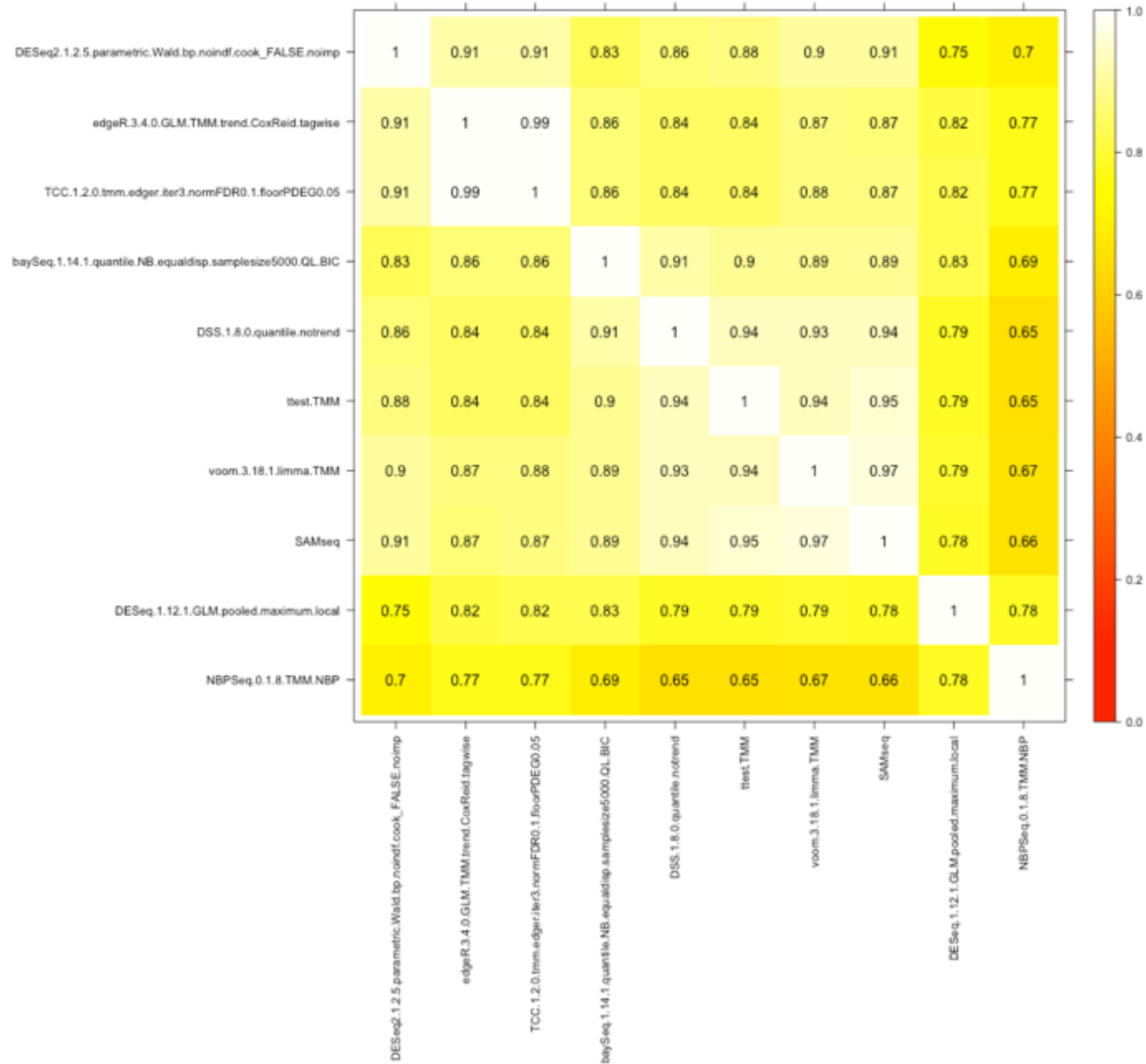
Similarity between sets of DEGs, 2 replicates/condition



Similarity between sets of DEGs, 5 replicates/condition



Similarity between sets of DEGs, 10 replicates/condition

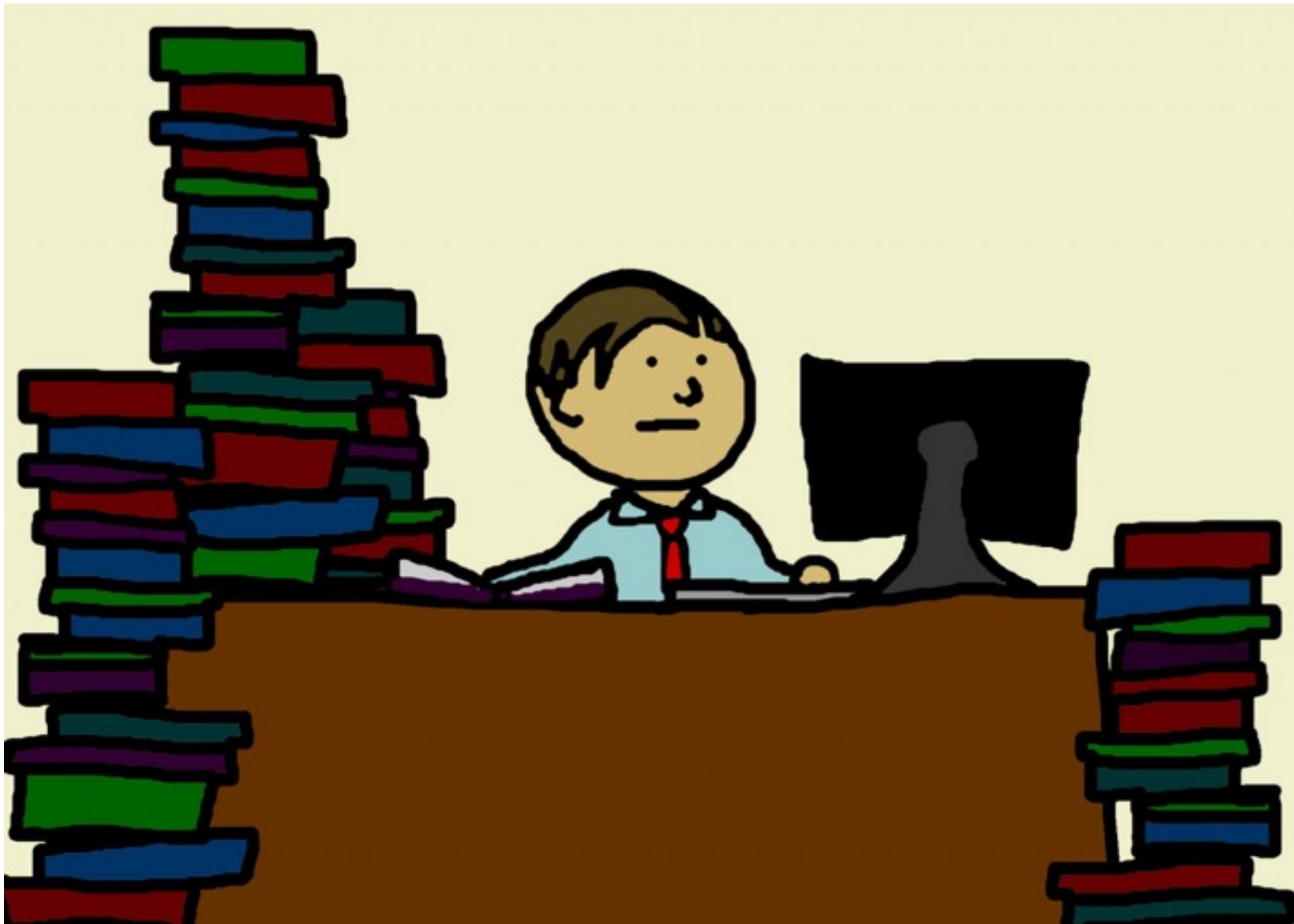


How many biological replicates are needed in an RNA-seq experiment and which differential expression tool should you use?

Nicholas J. Schurch^{1,6}, Pietá Schofield^{1,2,6}, Marek Gierliński^{1,2,6},
Christian Cole^{1,6}, Alexander Sherstnev^{1,6}, Vijender Singh², Nicola Wrobel³,
Karim Gharbi³, Gordon G. Simpson⁴, Tom Owen-Hughes², Mark Blaxter³ and
Geoffrey J. Barton^{1,2,5}

At least six replicates per condition for all experiments.
At least 12 replicates per condition for experiments where
identifying the majority of all DE genes is important.

**And now for something
completely different...**



No matter how carefully you design your experiment, data can still be compromised...

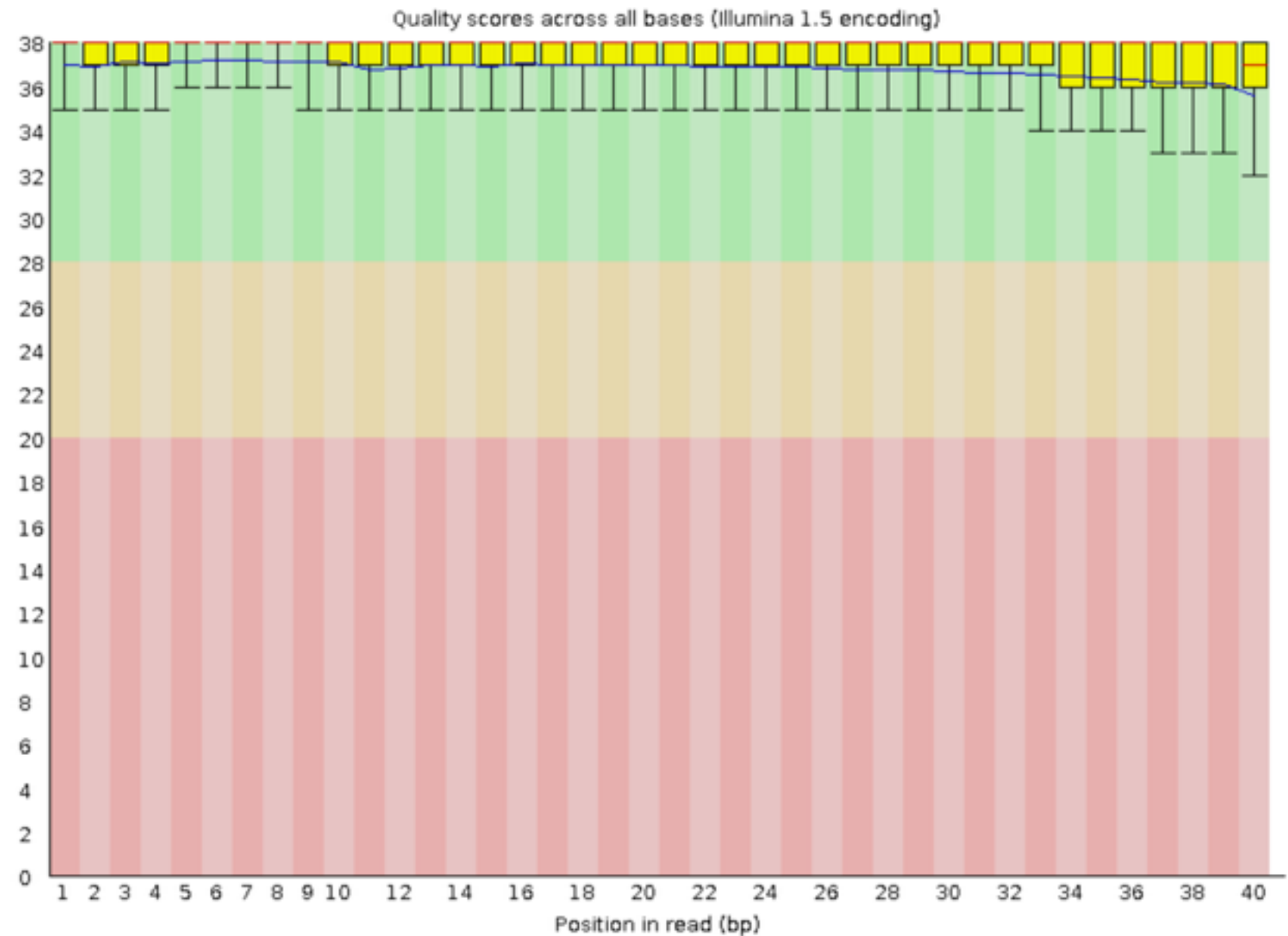
- Contamination
- Sequencing failures
- Remaining adapters
- PCR duplicates
- ...

QC software for NGS data - example

FastQC - raw read QC

<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>

✓ Per base sequence quality

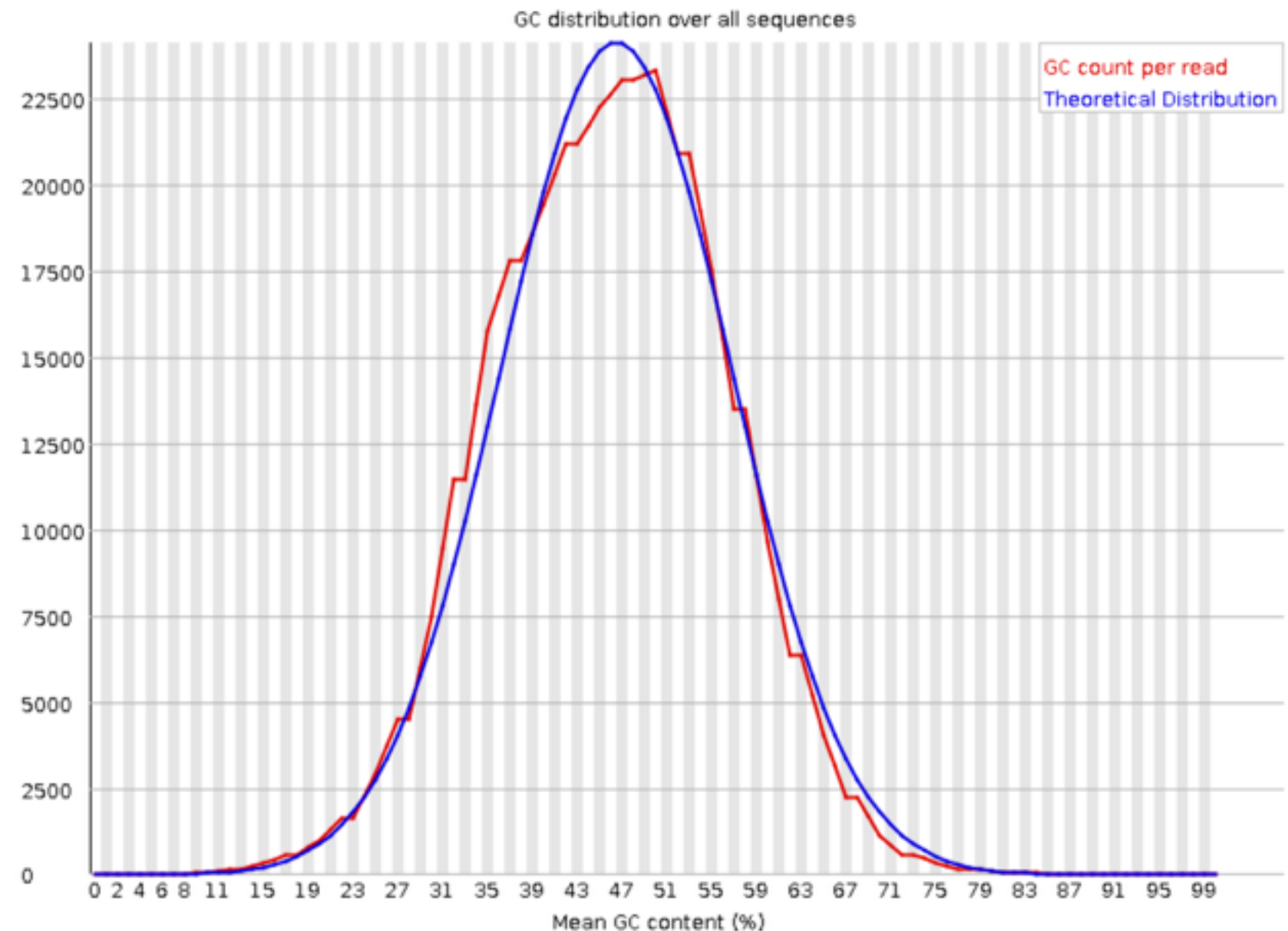


QC software for NGS data - example

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<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>

✅ Per sequence GC content

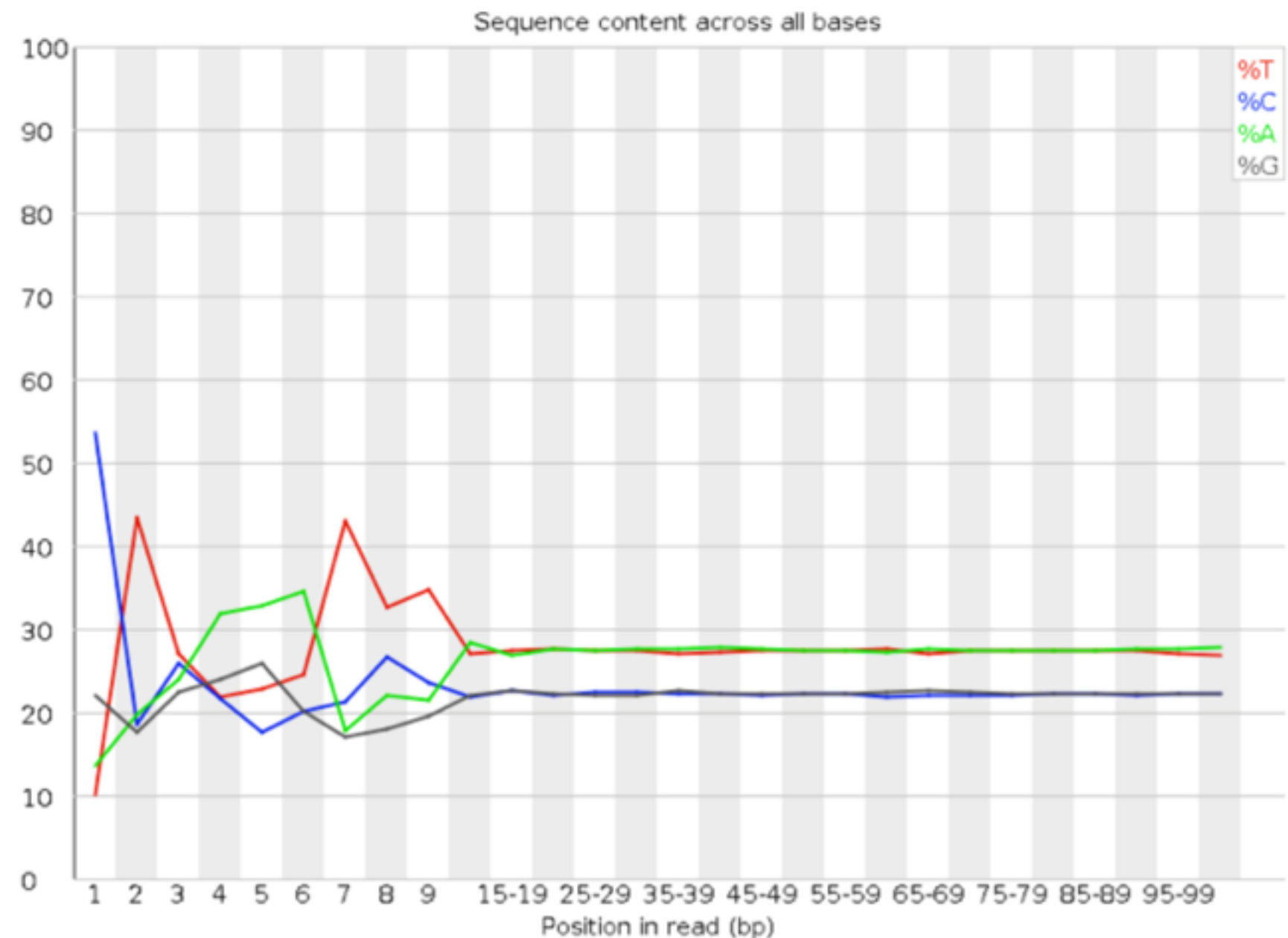


QC software for NGS data - example

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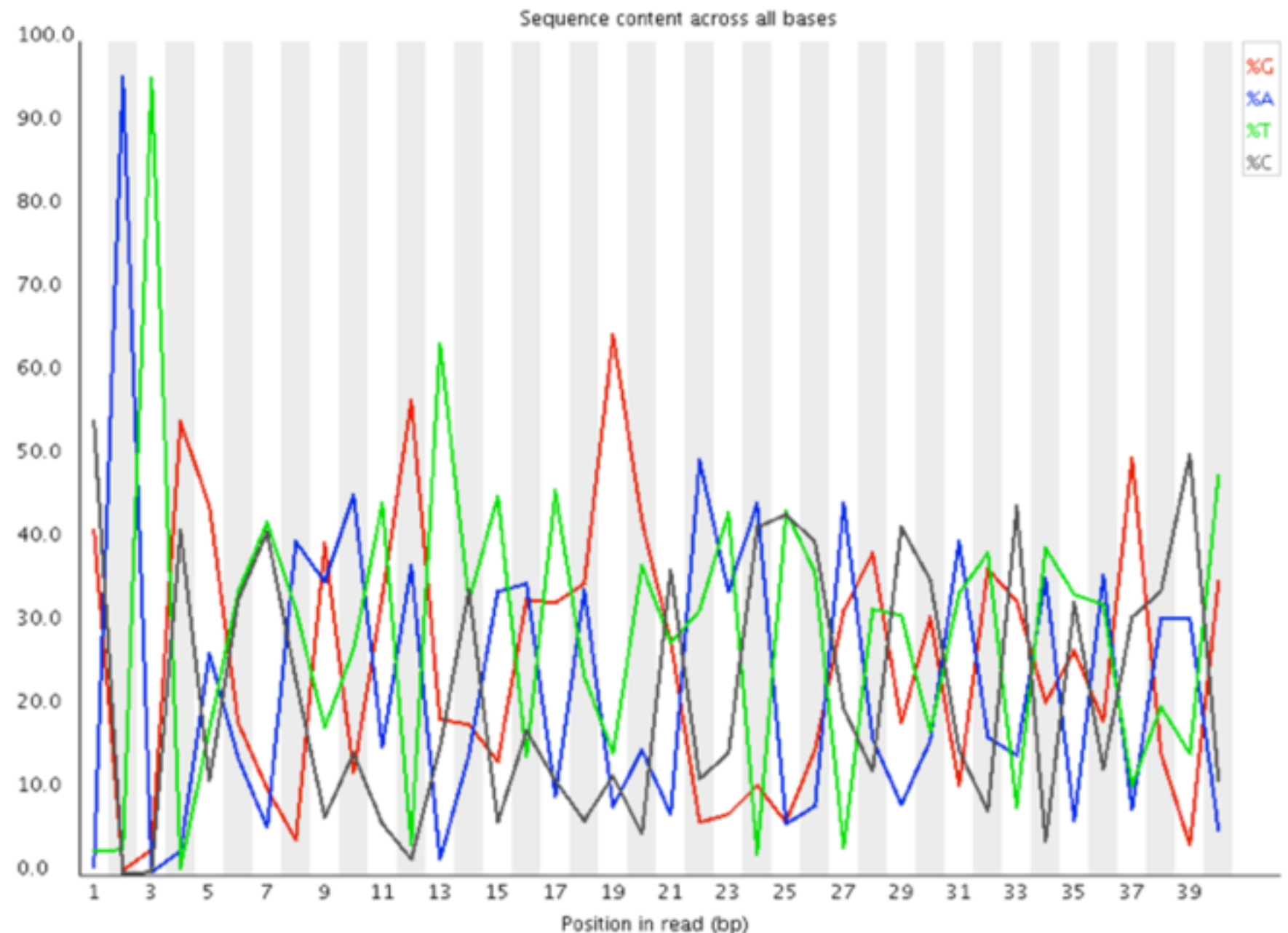
❌ Per base sequence content



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Overrepresented sequences

Sequence	Count	Percentage	Possible Source
GATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGATCT	8122	8.122	Illumina Paired End PCR Primer 2 (100% over 40bp)
GATCGGAAGAGCGGTTCAGCAGGAATGCCGAGATCGGAAG	5086	5.086	Illumina Paired End PCR Primer 2 (97% over 36bp)
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTAC	1085	1.085	Illumina Single End PCR Primer 1 (100% over 40bp)
GATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGGAAG	508	0.508	Illumina Paired End PCR Primer 2 (97% over 36bp)
AATTATACGGCGACCACCGAGATCTACACTCTTTCCCTAC	242	0.242	Illumina Single End PCR Primer 1 (97% over 40bp)
GATCGGAAGAGCGGTTCAGCAGGAATGCCGAAGATCGGAA	235	0.235000000000000001	Illumina Paired End Adapter 2 (96% over 31bp)
GATCGGAAGAGCGGTTCAGCAGGAATGCCGAGATCGGAAGA	228	0.227999999999999998	Illumina Paired End Adapter 2 (96% over 28bp)
GATCGGAAGAGCGGTTCAGCAGGAATGCCGAGACCGGACG	205	0.205000000000000002	Illumina Paired End PCR Primer 2 (97% over 36bp)
GATCGGAAGAGCGGTTCAGCAGGAATGCCGAGGATCGGAA	183	0.183	Illumina Paired End Adapter 2 (100% over 32bp)
GATCGGAAGAGCGGTTCAGCAGGAATGCCGAGGTCGGAAG	183	0.183	Illumina Paired End Adapter 2 (100% over 32bp)

QC software for NGS data - example

multiQC - summarize results from many analyses

<http://multiqc.info/docs/#>

MultiQC
v0.6

featureCounts

STAR

Cutadapt

FastQC

Sequence Quality Histograms

Per Sequence Quality Scores

Per Base Sequence Content

Per Sequence GC Content

Per Base N Content

Sequence Length Distribution

Sequence Duplication Levels

Adapter Content

MultiQC

A modular tool to aggregate results from bioinformatics analyses across many samples into a single report.

Report generated on 2016-05-03, 08:05 based on data in: `/Users/philewels/Work/MultiQC_website/public_html/examples/rna-seq/data`

General Statistics

Copy table

Configure Columns

Showing 8 rows.

Sample Name	% Assigned	M Assigned	% Aligned	M Aligned	% Trimmed	% Dups	% GC	Length	M Seqs
SRR3192396	67.5%	71.9	93.7%	97.8	4.0%	78.9%	51%	97	104.4
SRR3192397	66.6%	63.0	94.7%	87.1	3.5%	77.2%	49%	97	92.0
SRR3192398	50.9%	36.5	88.2%	58.7	5.0%	55.3%	47%	97	66.6
SRR3192399	52.3%	42.3	88.2%	65.6	5.0%	57.4%	47%	97	74.3
SRR3192400	70.3%	63.4	77.3%	73.4	7.2%	74.1%	45%	93	94.9
SRR3192401	71.2%	63.8	76.4%	72.8	6.3%	76.3%	45%	94	95.2
SRR3192657	73.1%	67.1	91.2%	85.0	3.1%	82.2%	51%	98	93.1
SRR3192658	71.2%	66.9	89.7%	87.1	3.4%	82.3%	52%	97	97.1

Toolbox

References

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- Nygaard et al.: Methods that remove batch effects while retaining group differences may lead to exaggerated confidence in downstream analyses. Biostatistics 17(1):29-39 (2016)
- Danielsson et al.: Assessing the consistency of public human tissue RNA-seq data sets. Briefings in Bioinformatics 16(6):941-949 (2015)
- Larsen et al.: Microarray-based RNA profiling of breast cancer: batch effect removal improves cross-platform consistency. BioMed Research International vol. 2014, article ID 651751 (2014)
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- Risso et al.: Normalization of RNA-seq data using factor analysis of control genes or samples. Nature Biotechnology 32(9):896-902 (2014)
- Gagnon-Bartsch & Speed: Using control genes to correct for unwanted variation in microarray data. Biostatistics 13(3):539-552 (2012)
- Schurch et al.: How many biological replicates are needed in an RNA-seq experiment and which differential expression tool should you use? RNA (2016)