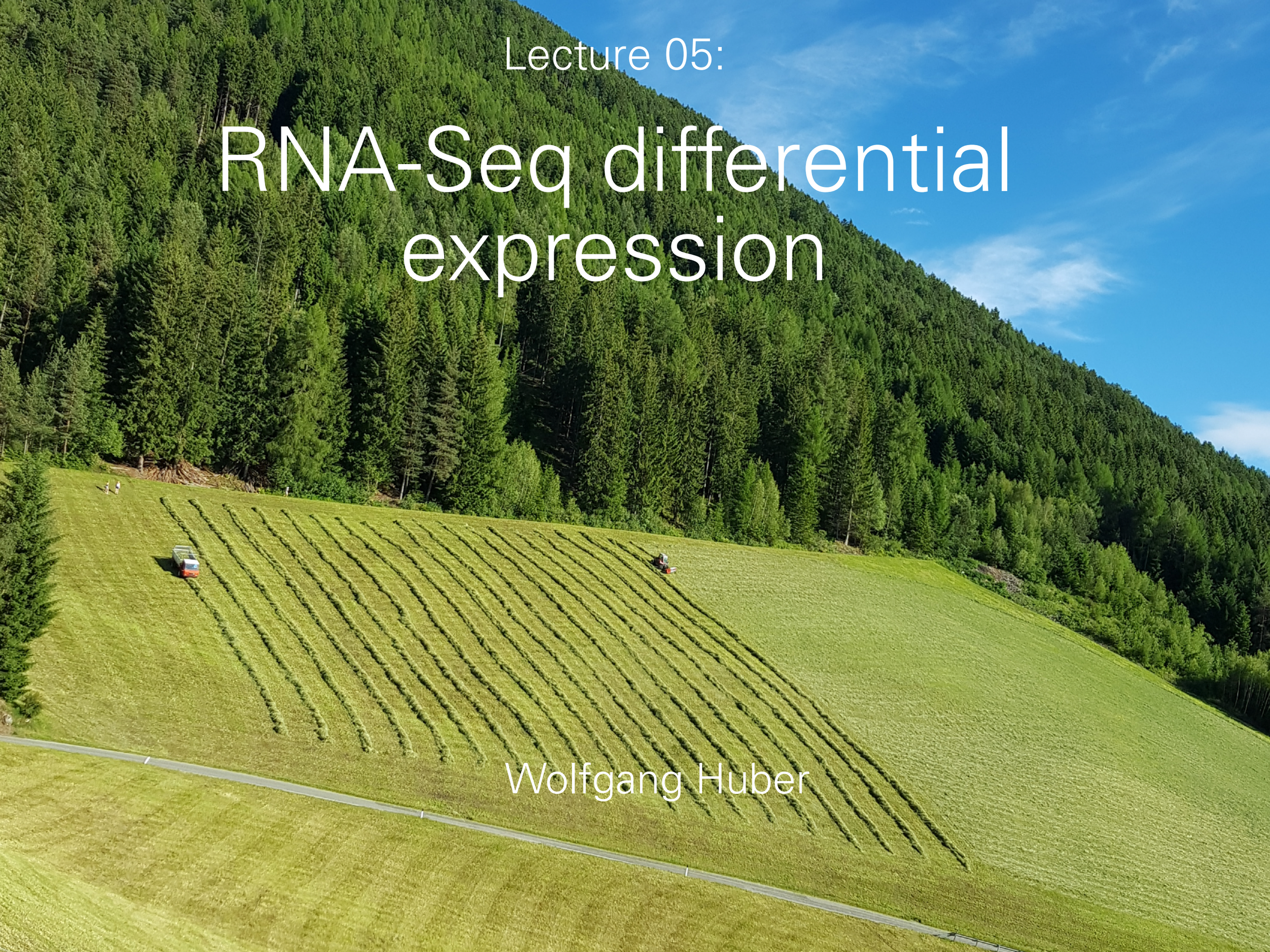
Lecture 05:

RNA-Seq differential expression



Wolfgang Huber

Aims for this lecture

Understand the peculiarities of count-data from high-throughput sequencing and the Gamma-Poisson model

Some basic concepts for generalized linear models (cf. Levi's lecture on Wed for more)

Understand shrinkage estimation and its application to modelling experiments using HT assays

Some further bells and whistles of DESeq2: transformation, outlier robustness, banded testing

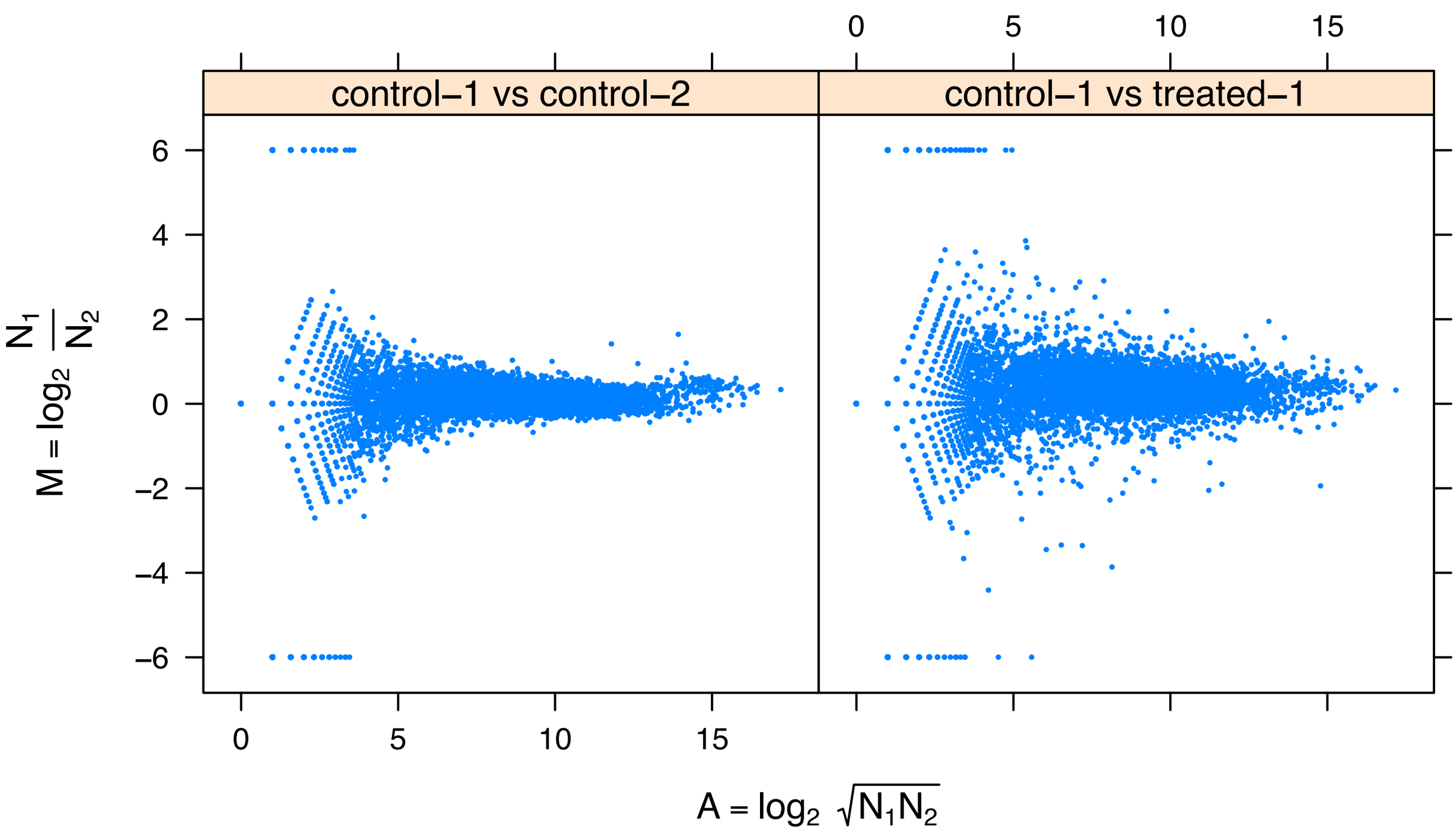
Testing differential exon abundance (→isoform usage)

Count data in HTS

Gene	GliNS1	G144	G166	G179	CB541	CB660
13CDNA73	4	0	6	1	0	5
A2BP1	19	18	20	7	1	8
A2M	2724	2209	13	49	193	548
A4GALT	0	0	48	0	0	0
AAAS	57	29	224	49	202	92
AACS	1904	1294	5073	5365	3737	3511
AADACL1	3	13	239	683	158	40
[...]						

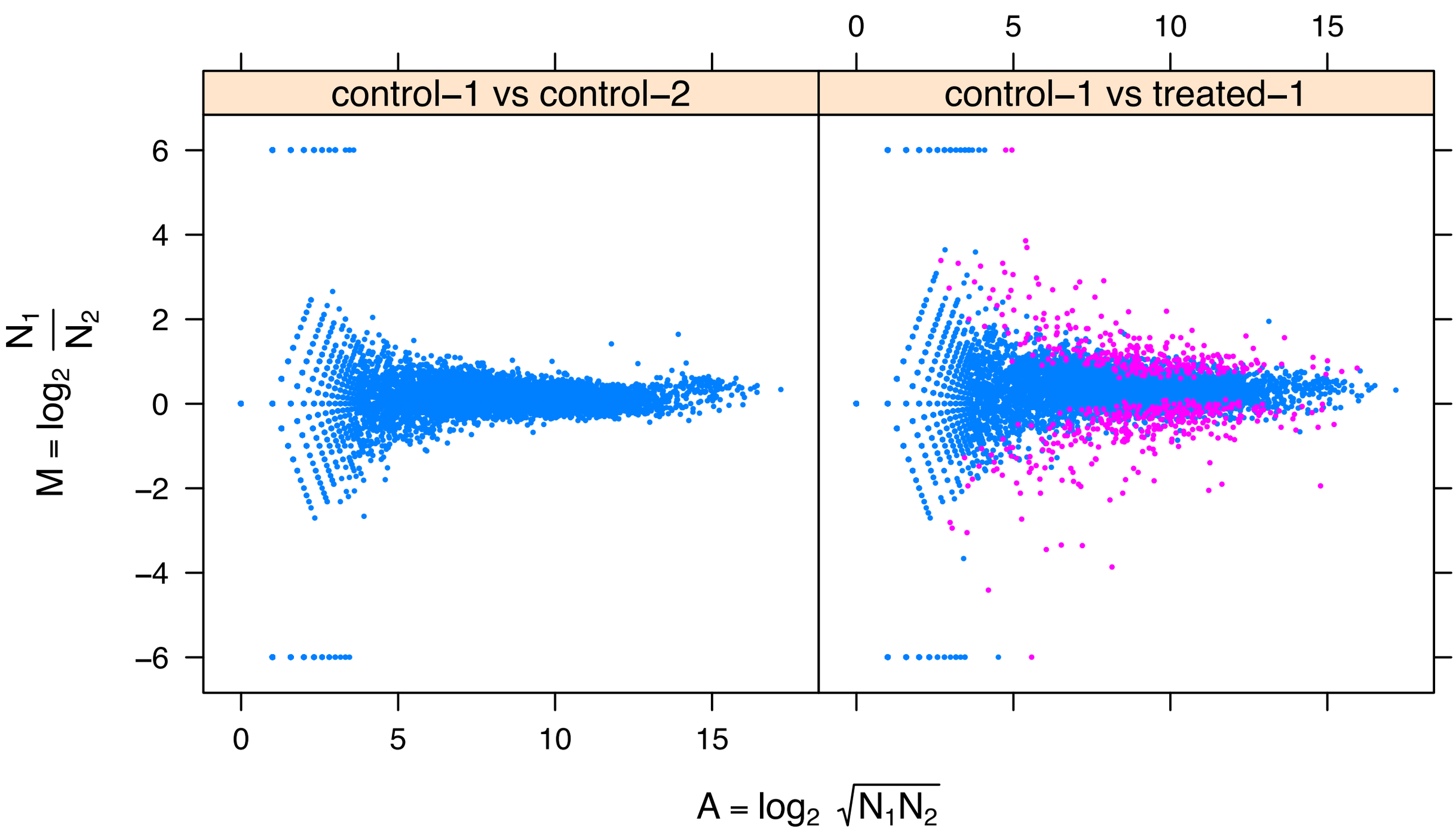
- RNA-Seq
- ChIP-Seq
- HiC
- Barcode-Seq
- Peptides in mass spec
- ...

Simon Anders



two biological replicates

treatment vs control



two biological replicates

treatment vs control

Challenges

Large dynamic range (0 ... 10^5)

➡ heteroskedasticity matters

Data are discrete, positive, skewed
Small numbers of replicates (…)

➡ no (log-)normal model

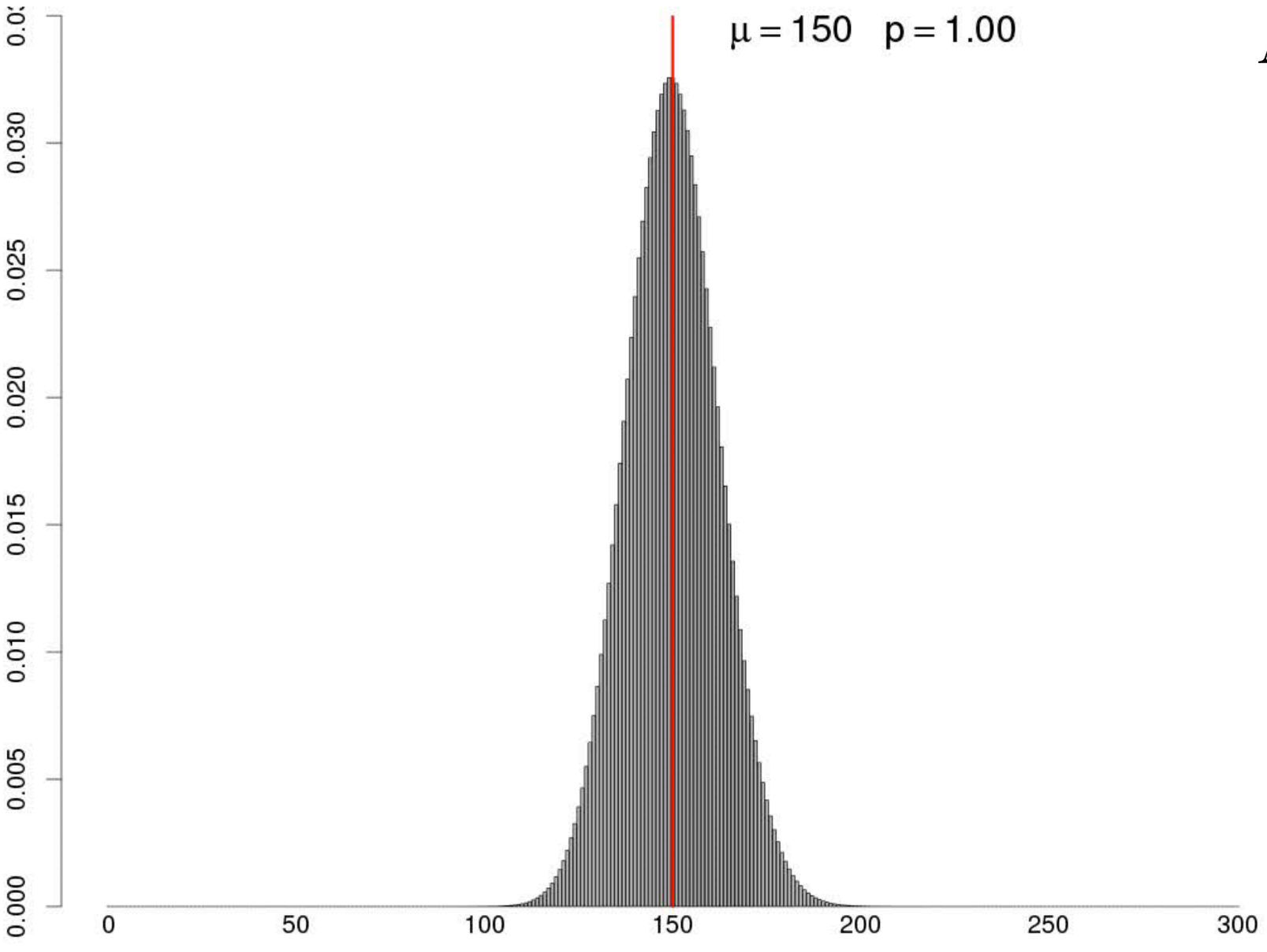
➡ no rank based or permutation methods

➡ use parametric stochastic model to infer tail behaviour (approximately) from low-order moments

➡ power sharing between genes (‘large-p small n’)

The Gamma-Poisson (a.k.a. Negative Binomial) distribution

$$\mathrm{P}(K = k) = \binom{k + r - 1}{r - 1} p^r (1 - p)^k, \qquad r \in \mathbb{R}^+,\ p \in [0, 1]$$



Alternative parameterisation

$$\begin{aligned} \alpha &= \frac{1}{r} \\ \mu &= \frac{pr}{1 - p} \end{aligned}$$

Moments

$$\begin{aligned} \text{mean} &= \mu \\ \text{variance} &= \mu + \alpha\mu^2 \end{aligned}$$

Bioconductor package
DESeq, since 2010

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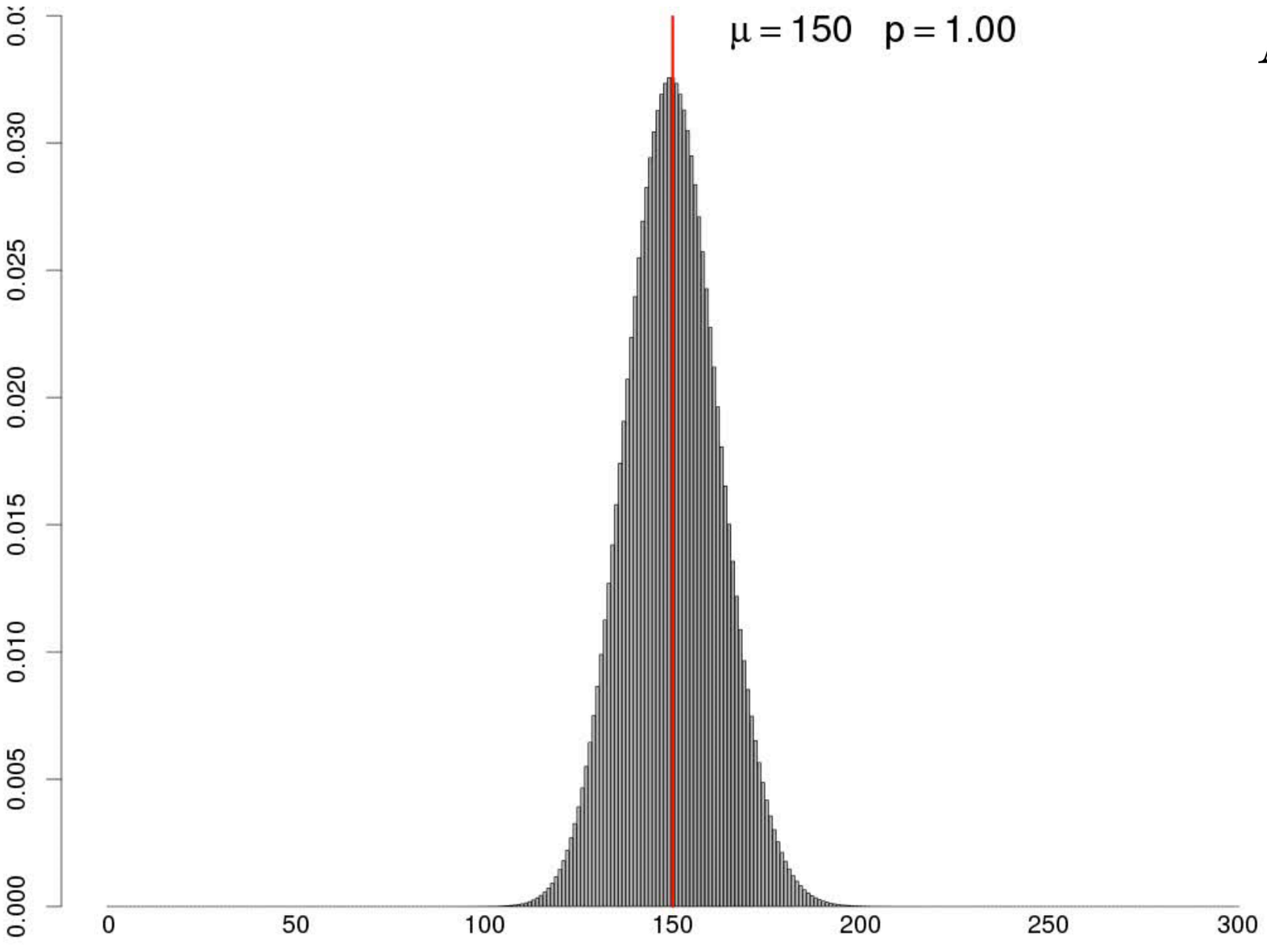

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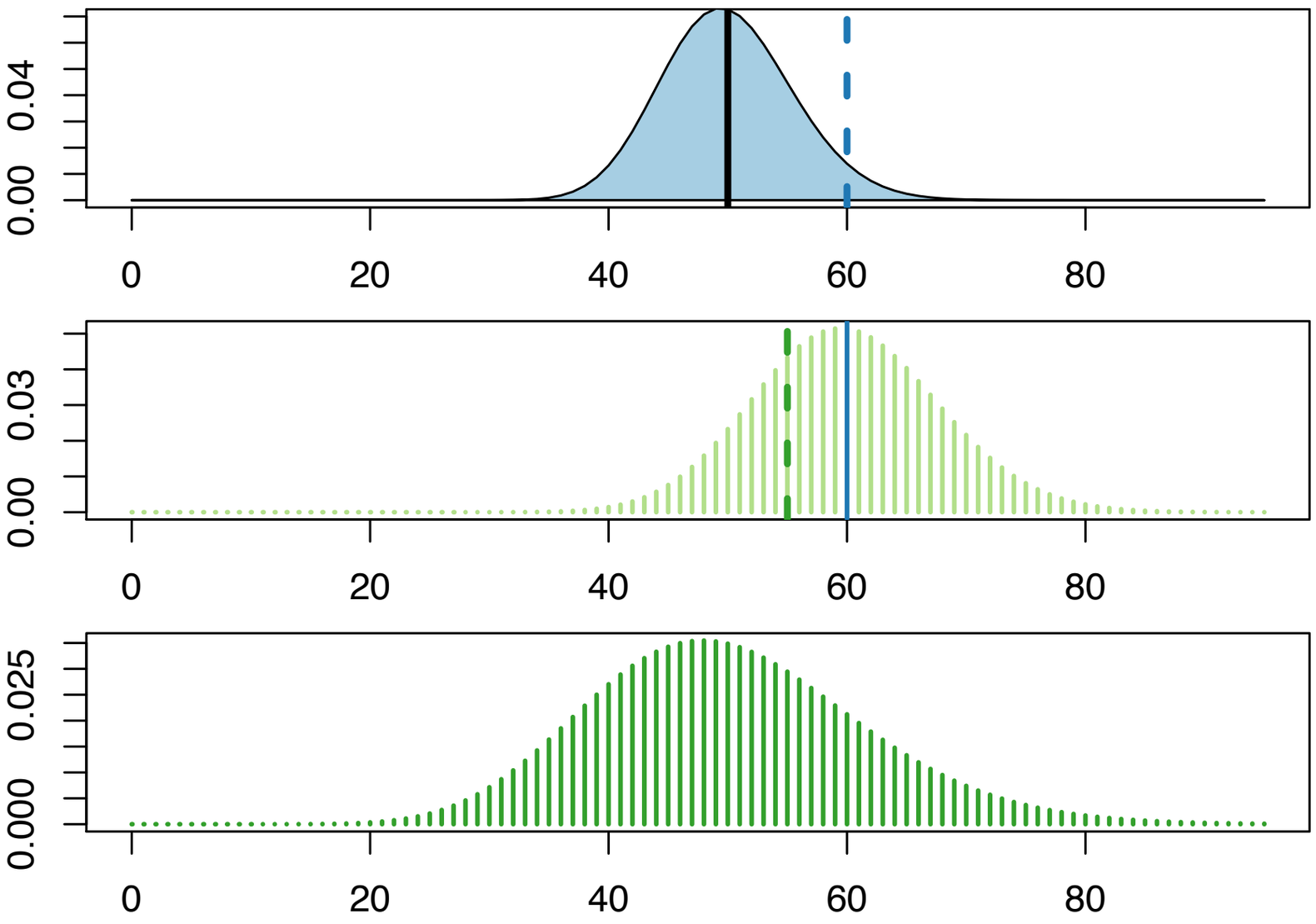
The Gamma-Poisson distribution models a Poisson process whose mean is itself randomly varying



Biological sample to sample variability Γ

Poisson counting statistics Λ

Overall distribution GP

$$NB(\mu, \sigma^2 + \mu) = \Lambda(\Gamma(\mu, \sigma^2))$$

Two component noise model

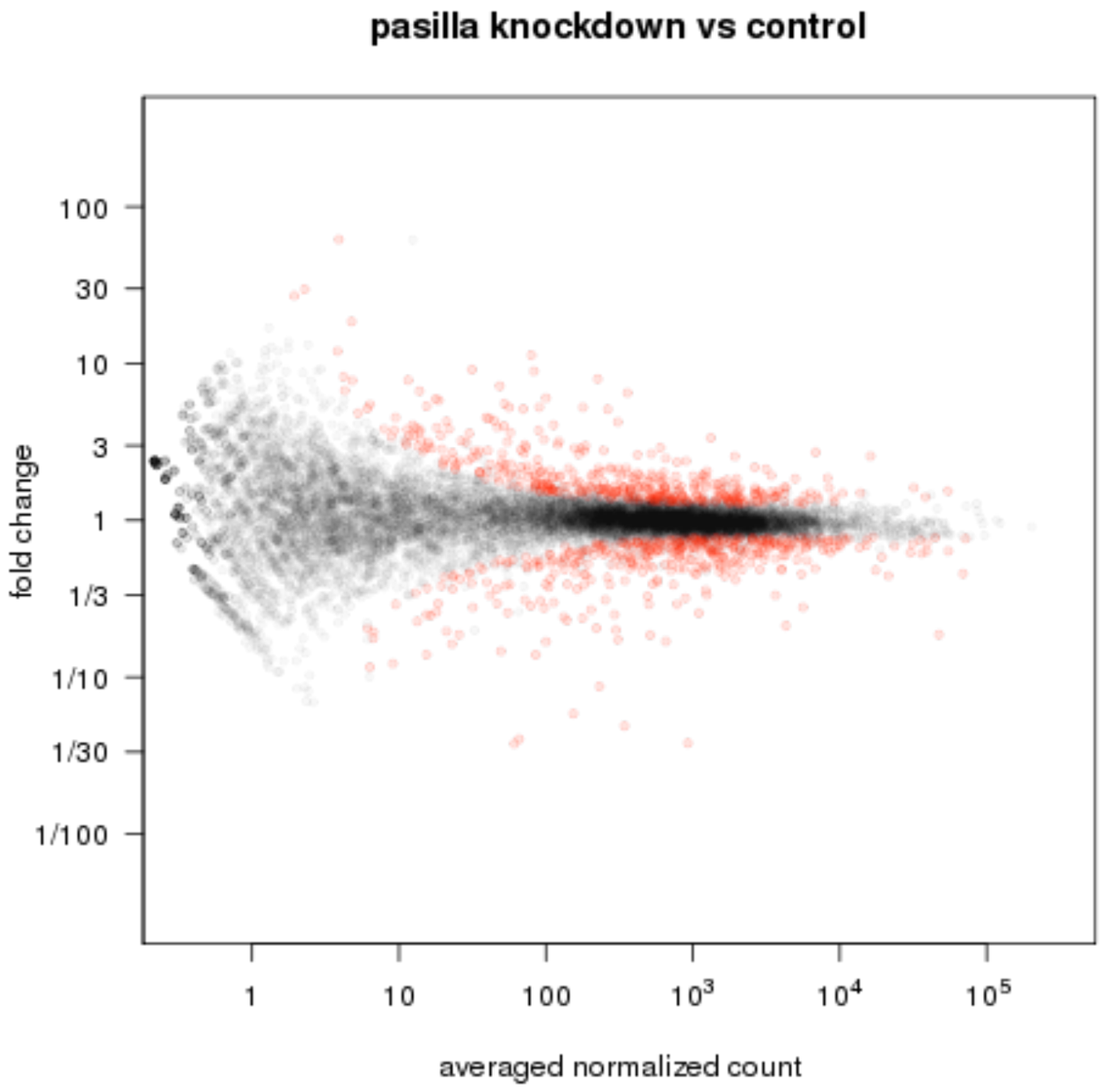
$$\text{var} = \mu + c\,\mu^2$$

shot noise (Poisson) — biological noise

Small counts

Sampling noise dominant

Improve power: deeper coverage



Large counts

Biological noise dominant

Improve power: more biol. replicates

Generalised linear model of the Gamma-Poisson (or NB) family

$$N_{ij} \sim \mathrm{NB}(\mu_{ij}, \alpha_{ij})$$ Noise part

$$\log \mu_{ij} = s_j + \sum_k \beta_{ik} x_{kj}$$ Systematic part

μ_{ij} expected count of gene *i* in sample *j*

s_j library size effect

x_{kj} design matrix

β_{ik} (differential) expression effects for gene *i*

Generalised linear model of the Gamma-Poisson (or NB) family

$$N_{ij} \sim \mathrm{NB}(\mu_{ij}, \alpha_{ij})$$ Noise part

$$\log \mu_{ij} = \sum_k x_{kj} \beta_{ik} + \log s_j$$ matic

Important special case: two groups

μ_{ij} expected count of gene *i* in sample *j*

s_j library size effect

x_{kj} design matrix

β_{ik} (differential) expression effects for gene *i*

What is a generalized linear model?

$$Y \sim D(\mu, \sigma)$$

A GLM consists of three elements:

1. A linear predictor $\eta = X\beta$
2. A non-linear transformation (link function) g such that $g(\mu) = \eta$
3. A probability distribution D (from the exponential family),
   with mean μ and scale σ

Ordinary linear model: g = identity, D = normal
DESeq(2), edgeR, ...: g = log, D = Gamma-Poisson

Design with a blocking factor

Sample	treated	sex
S1	no	male
S2	no	male
S3	no	male
S4	no	female
S5	no	female
S6	yes	male
S7	yes	male
S8	yes	female
S9	yes	female
S10	yes	female

GLM with blocking factor

$$K_{ij} \sim NB(s_j \mu_{ij}, \alpha_{ij})$$

i : genes
j : samples

full model for gene *i*:

$$\log \mu_{ij} = \beta_i^0 + \beta_i^{\mathrm{S}} x_j^{\mathrm{S}} + \beta_i^{\mathrm{T}} x_j^{\mathrm{T}}$$

reduced model for gene *i*:

$$\log \mu_{ij} = \beta_i^0 + \beta_i^{\mathrm{S}} x_j^{\mathrm{S}}$$

GLM: Interactions

$$K_{ij} \sim NB(s_j \mu_{ij}, \alpha_{ij})$$

full model for gene *i:*

$$\log \mu_{ij} = \beta_i^0 + \beta_i^{\mathrm{S}} x_j^{\mathrm{S}} + \beta_i^{\mathrm{T}} x_j^{\mathrm{T}} + \beta_i^{\mathrm{I}} x_j^{\mathrm{S}} x_j^{\mathrm{T}}$$

reduced model for gene *i:*

$$\log \mu_{ij} = \beta_i^0 + \beta_i^{\mathrm{S}} x_j^{\mathrm{S}} + \beta_i^{\mathrm{T}} x_j^{\mathrm{T}}$$

GLMs: paired designs

- Often, samples are paired (e.g., a tumour and a healthy-tissue sample from the same patient)

- Then, using pair identity as blocking factor improves power.

full model:

$$\log \mu_{ijl} = \beta_i^0 + \begin{cases} 0 & \text{for } l = 1(\text{healthy}) \\ \beta_i^{\mathrm{T}} & \text{for } l = 2(\text{tumour}) \end{cases}$$

reduced model:

$$\log \mu_{ij} = \beta_i^0$$

i gene
j subject
l tissue state

Recap: designs for generalized linear models

Simple design:

Two groups, e.g. *control* and *treatment*

Common complex designs:

- Designs with blocking factors
- Factorial designs
- Designs with interactions
- Paired designs

Dual-assay designs (e.g.: CLIP-Seq + RNA-Seq)

How does affinity of an RNA-binding protein to mRNA change under a (drug, RNAi) treatment?

For each sample, we are interested in the ratio of CLIP-Seq to RNA-Seq reads. How is it affected by treatment?

full model:

count ~ assayType + treatment + assayType : treatment

reduced model:

count ~ assayType + treatment

See https://support.bioconductor.org/p/61509/ ("DESeq2 testing ratio of ratios")
For an application example: e.g., Zarnack et al., Cell 2013

Benefitting from the many variables (‘big data’)

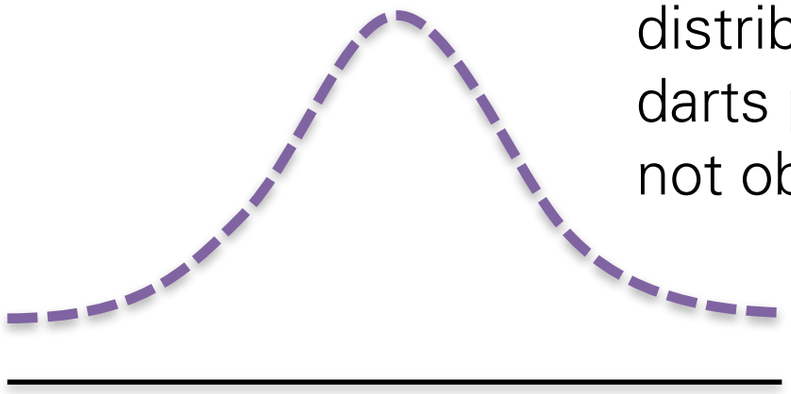
To assess signal and noise in the data from one gene, we have

- the data for that gene
- that of all the other genes
- user-defined parameters (e.g. cutoffs)

⇒ regularisation, (empirical) Bayes

Michael Love

Theoretical Interlude: Shrinkage estimation

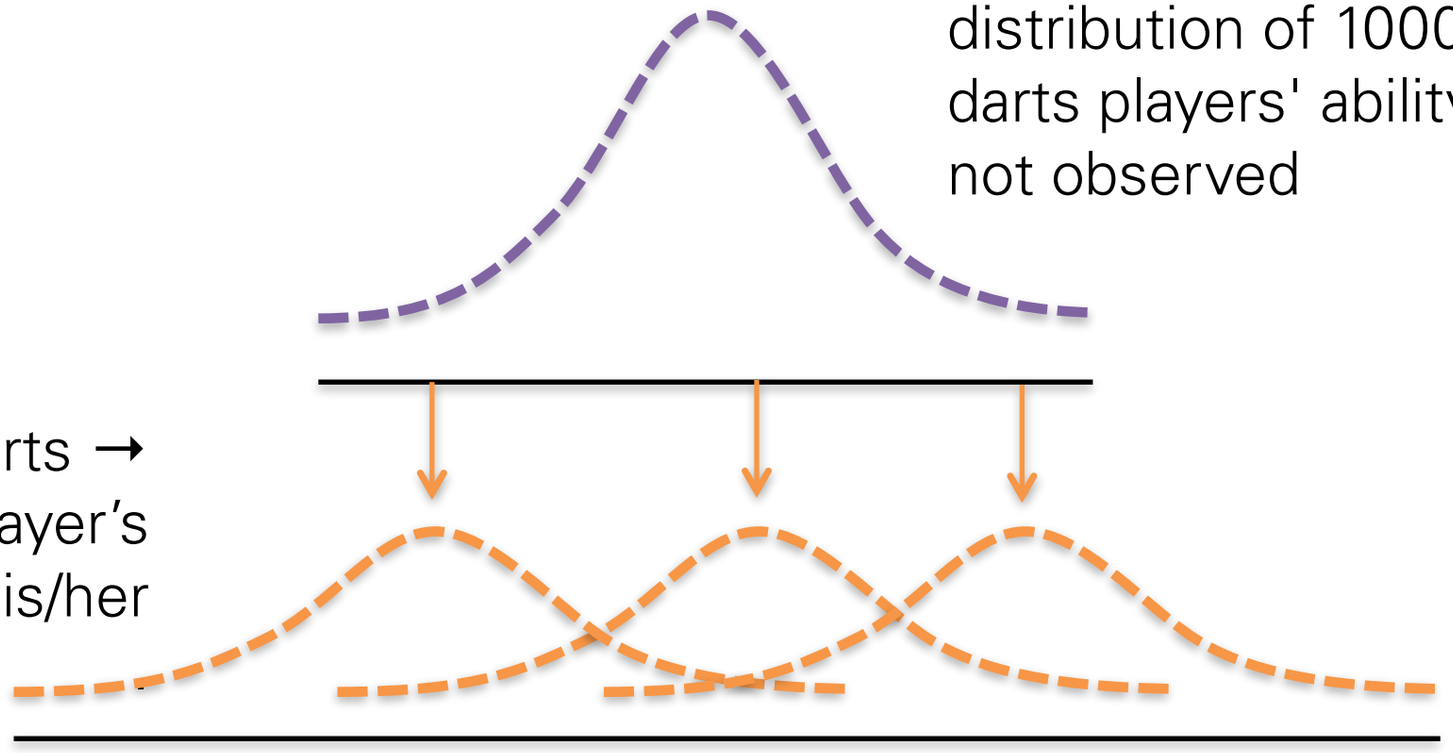


distribution of 1000
darts players' ability:
not observed

Theoretical Interlude: Shrinkage estimation

distribution of 1000 darts players' ability: not observed

each throws 3 darts → estimates of each player's ability & his/her distribution.



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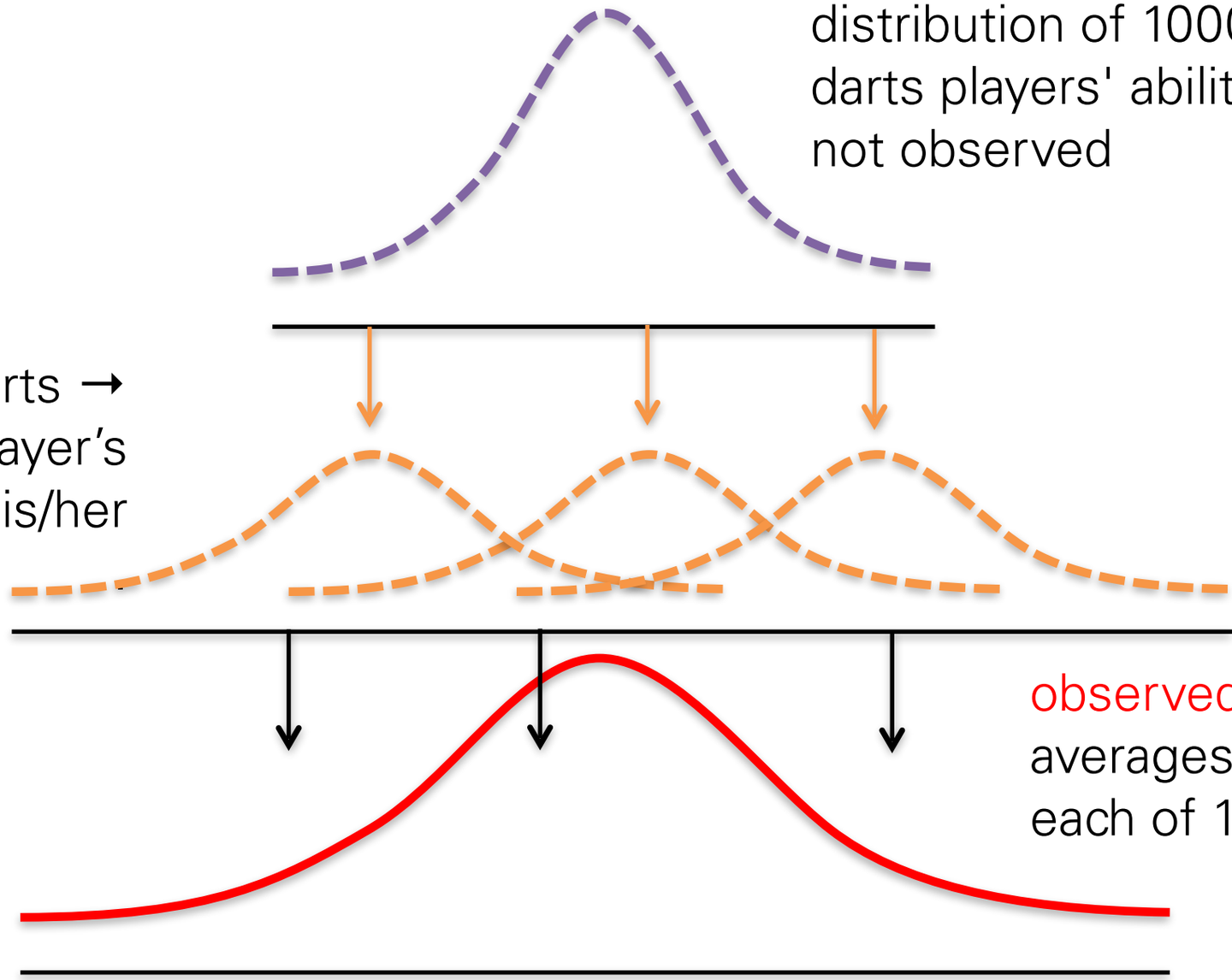

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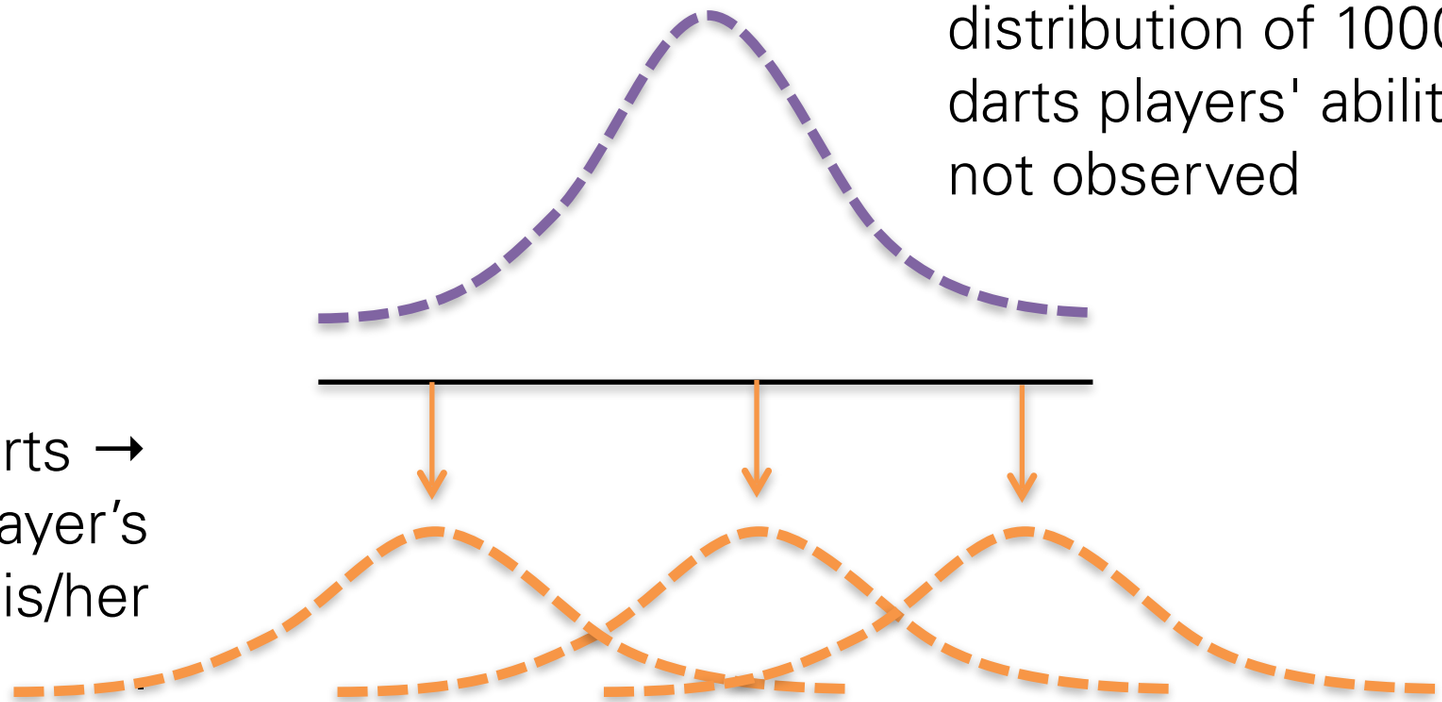

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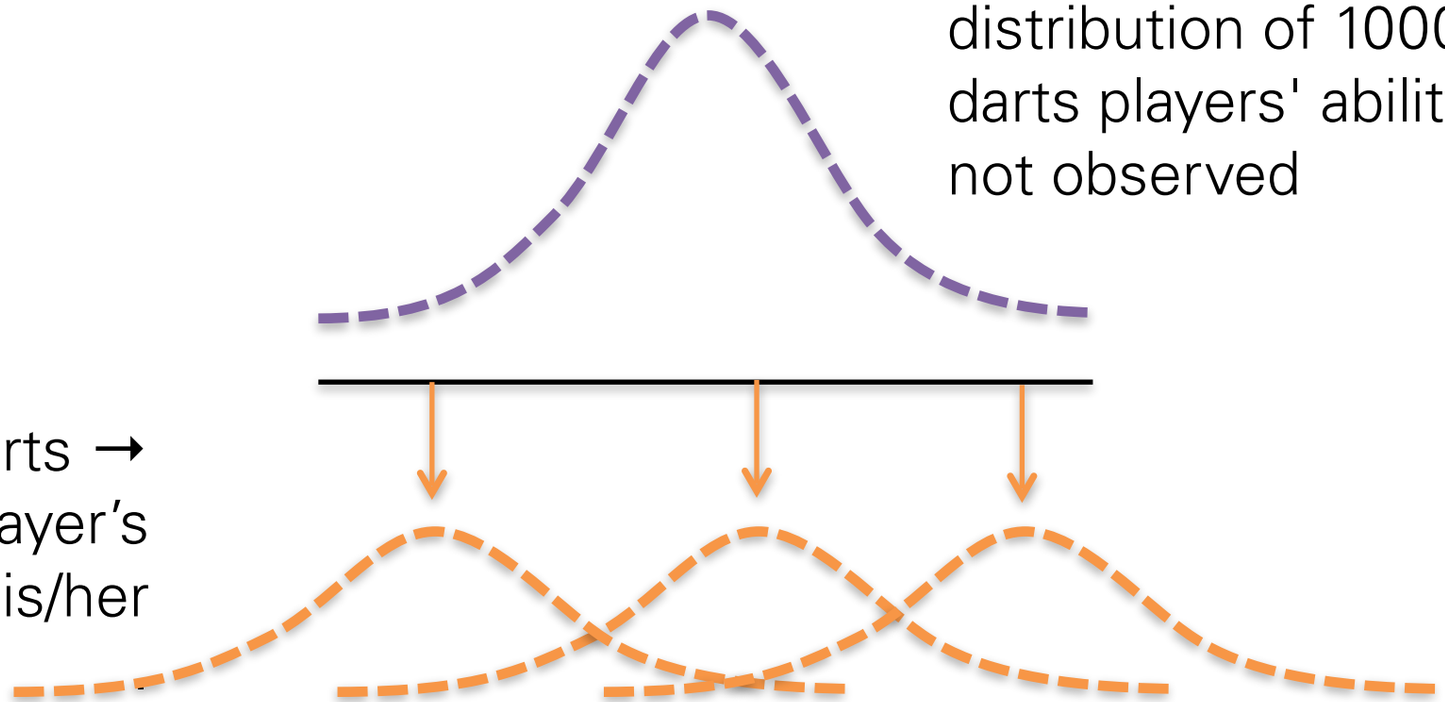
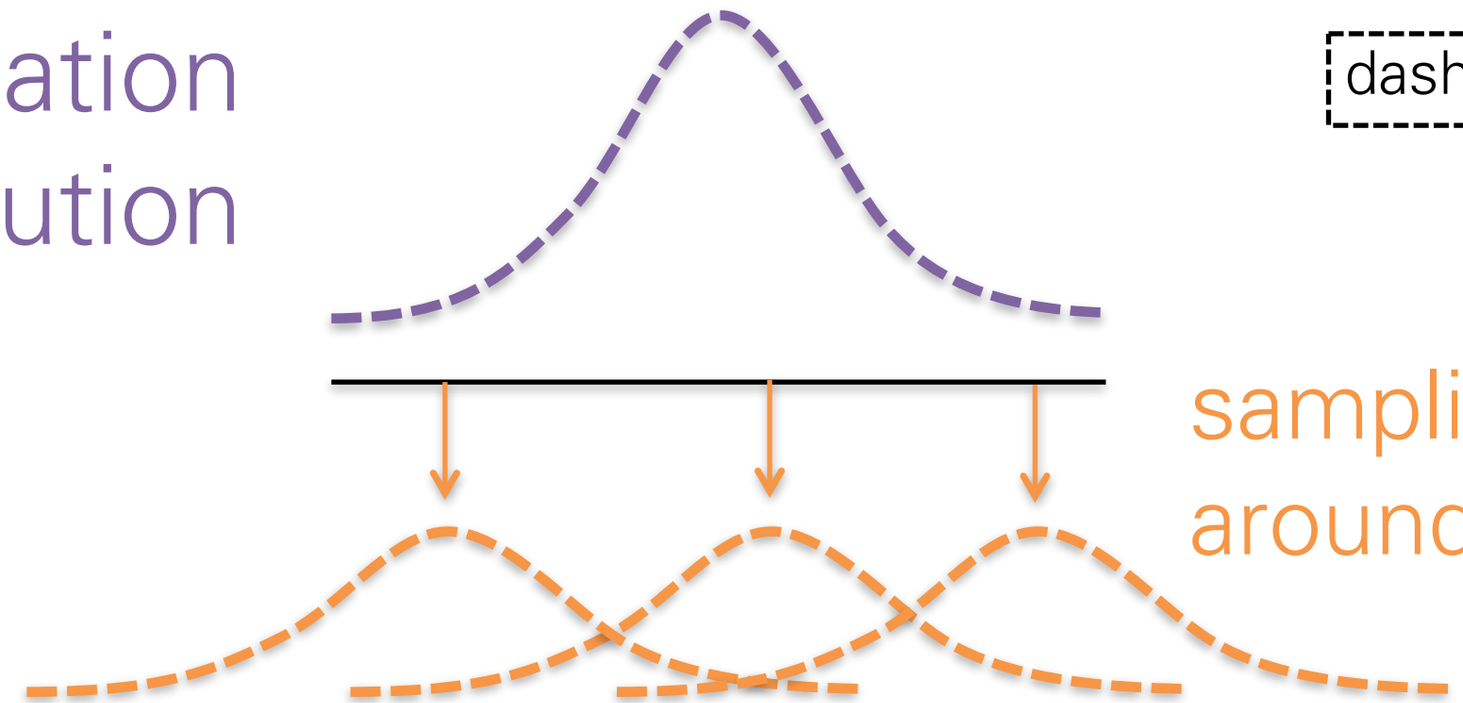


"shrunken" estimates
less error ***overall***
than **individual** estimates

Shrinkage estimation

population distribution

dashed = unobserved



sampling variance around true ability

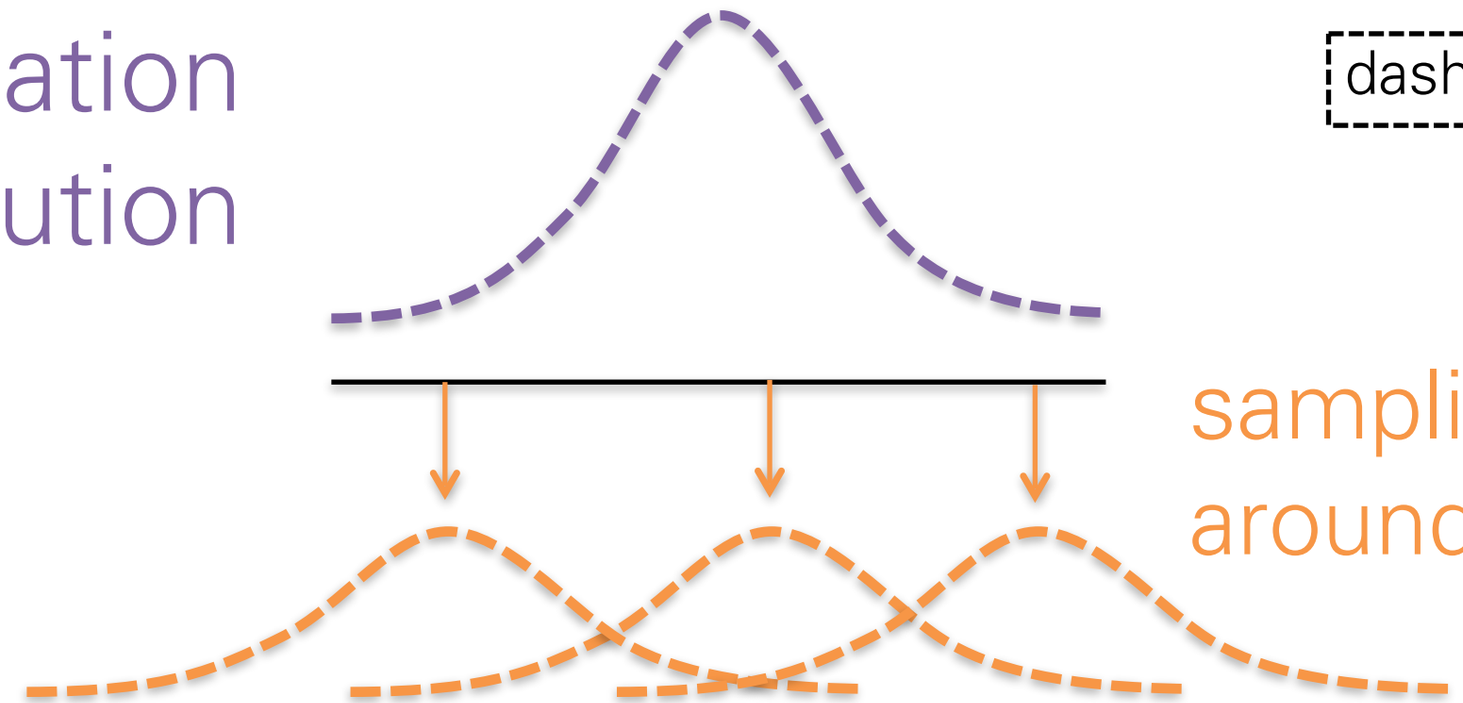
empirical distribution

the center defines the prior mean

Shrinkage estimation

population distribution

dashed = unobserved



sampling variance around true ability

empirical distribution

the center defines the prior mean

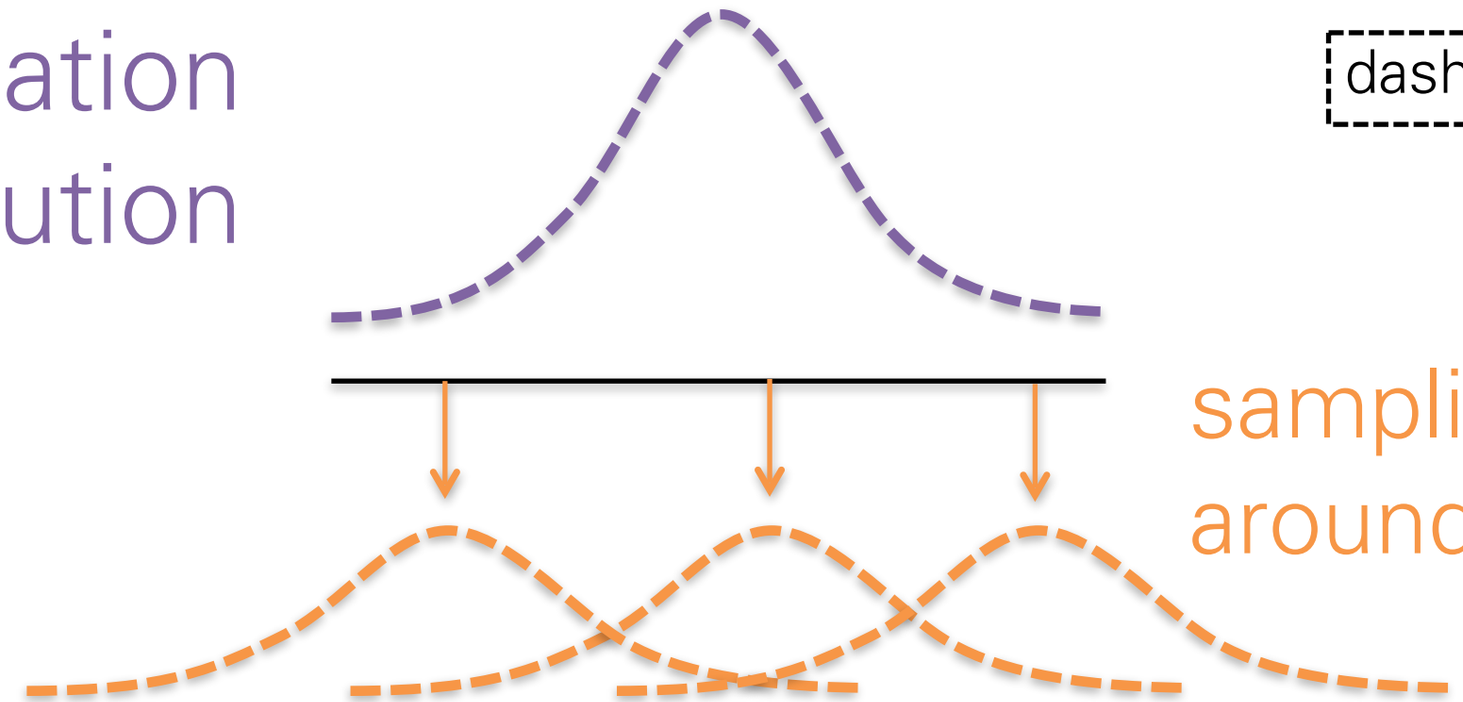
MLE

maximum likelihood estimates

Shrinkage estimation

population distribution

dashed = unobserved



sampling variance around true ability

empirical distribution

the center defines the prior mean

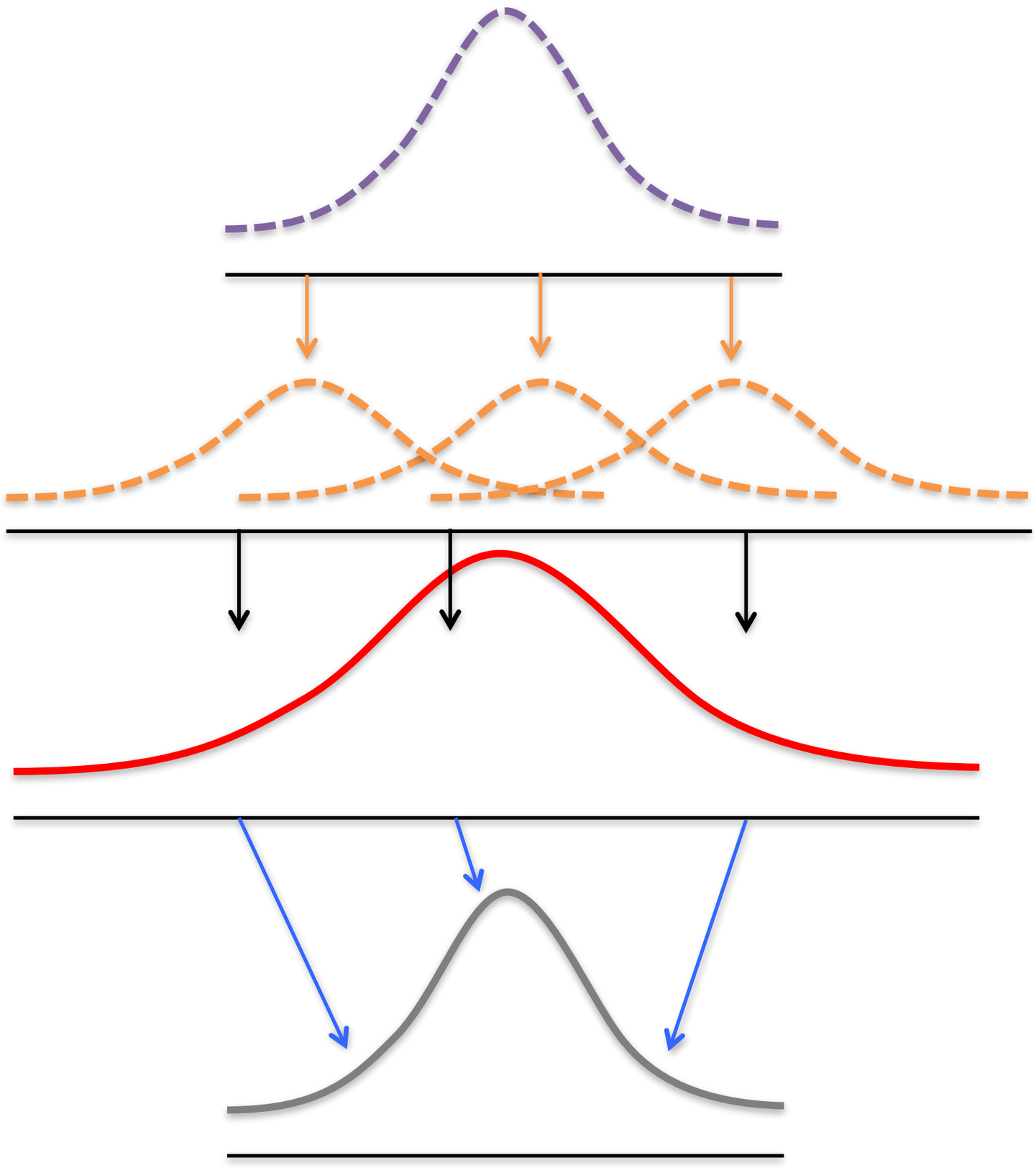
MLE

maximum likelihood estimates

shrunken estimates or MAP

maximum a posteriori

Shrinkage estimation



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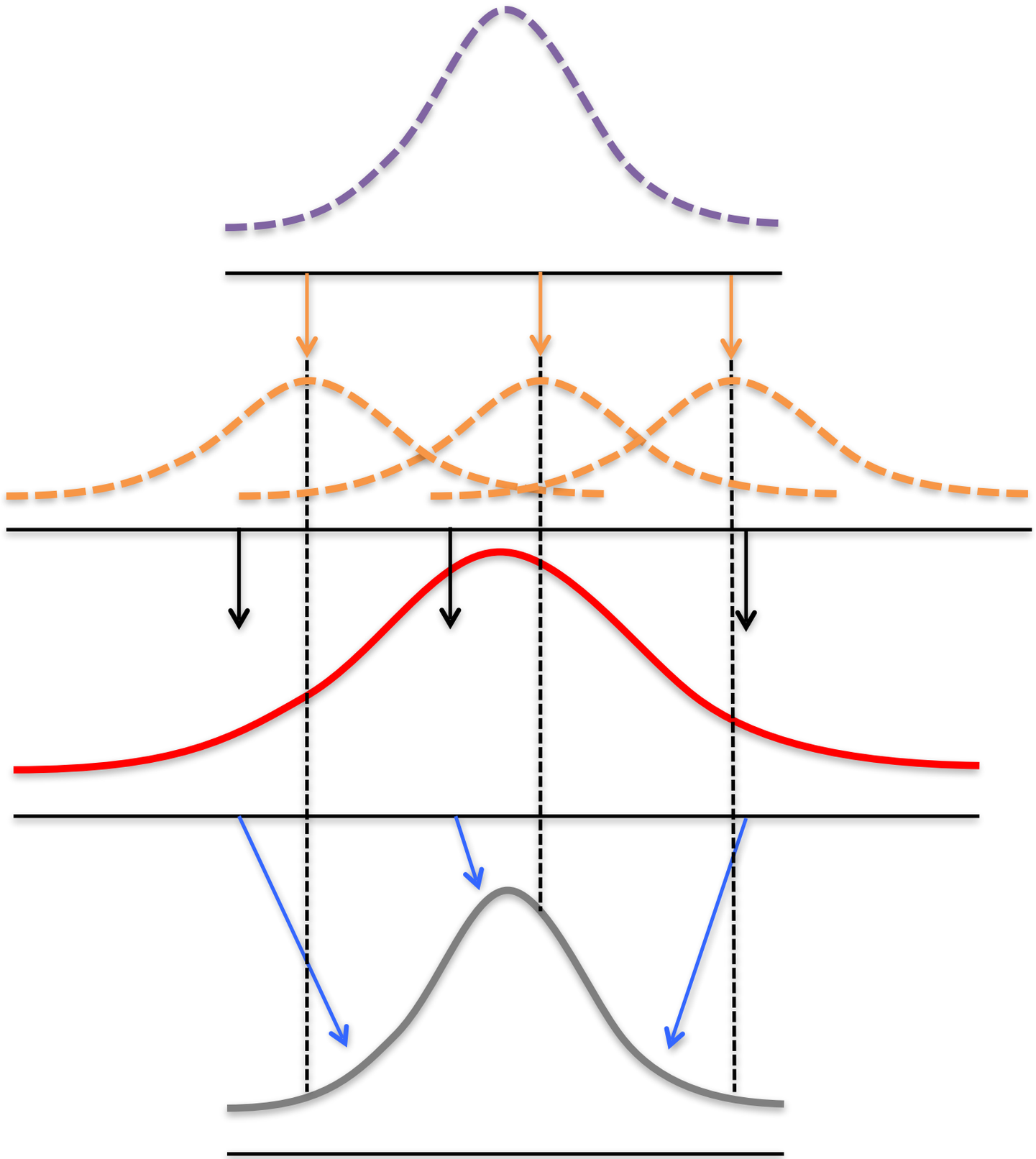

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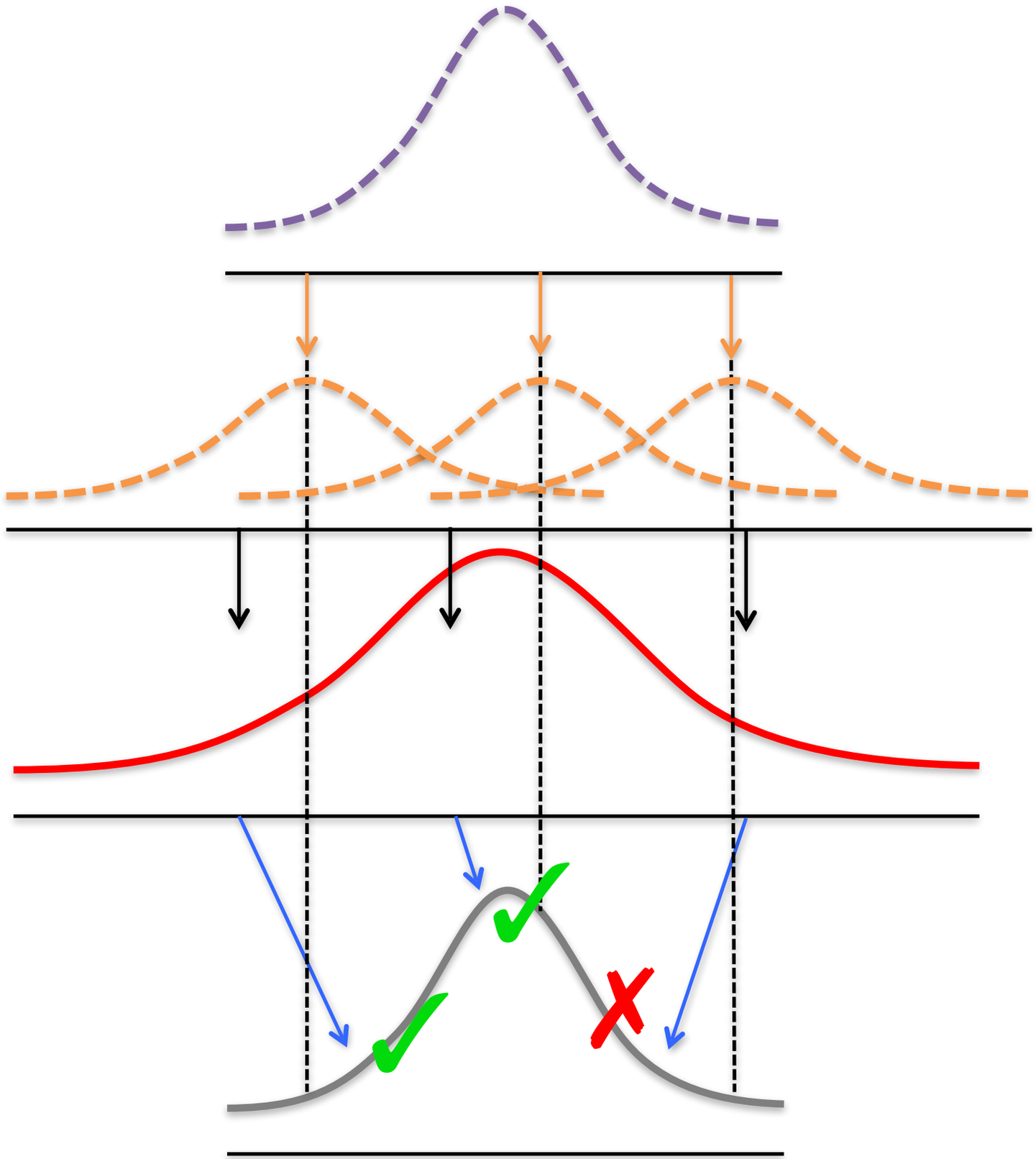

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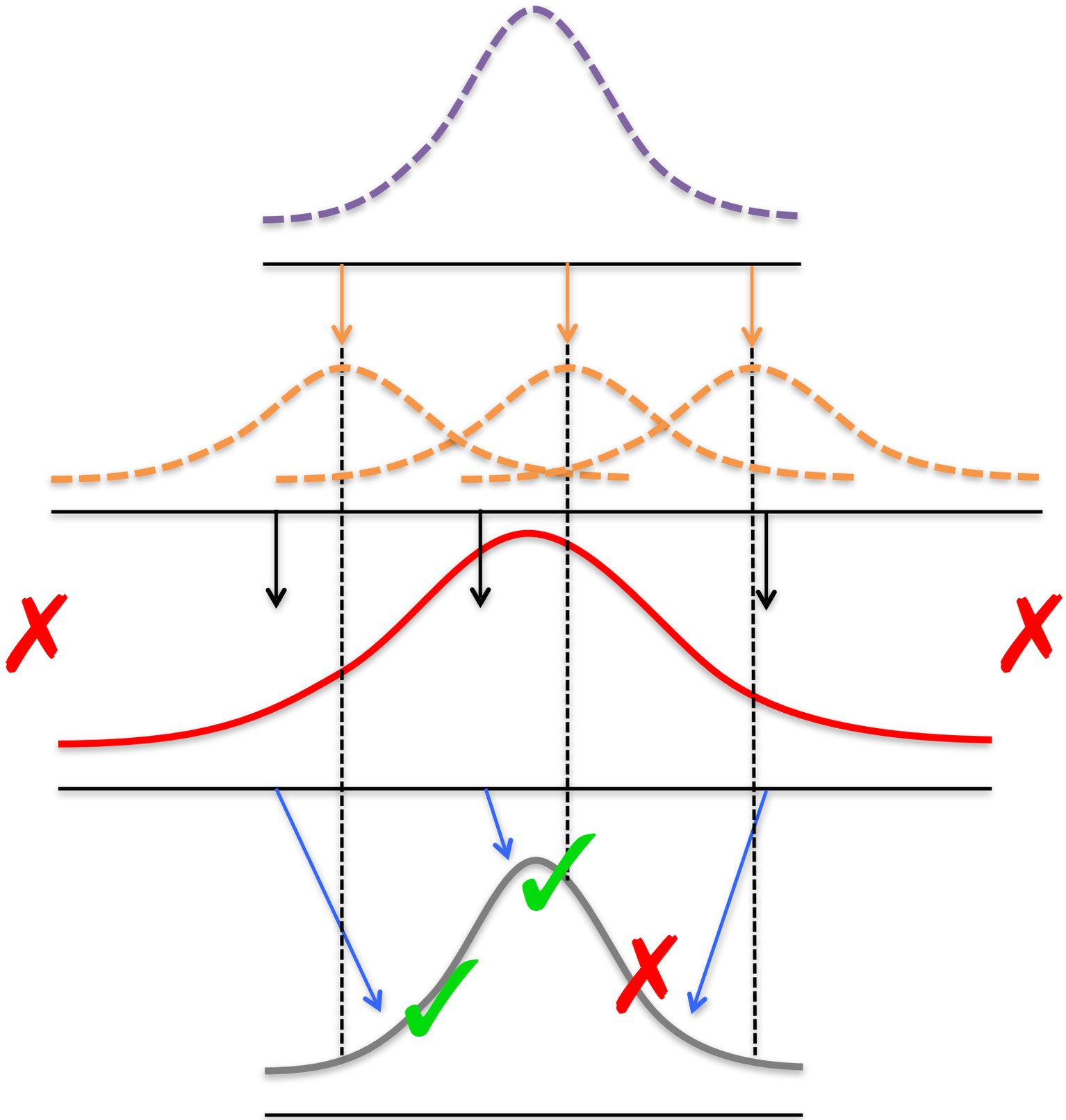
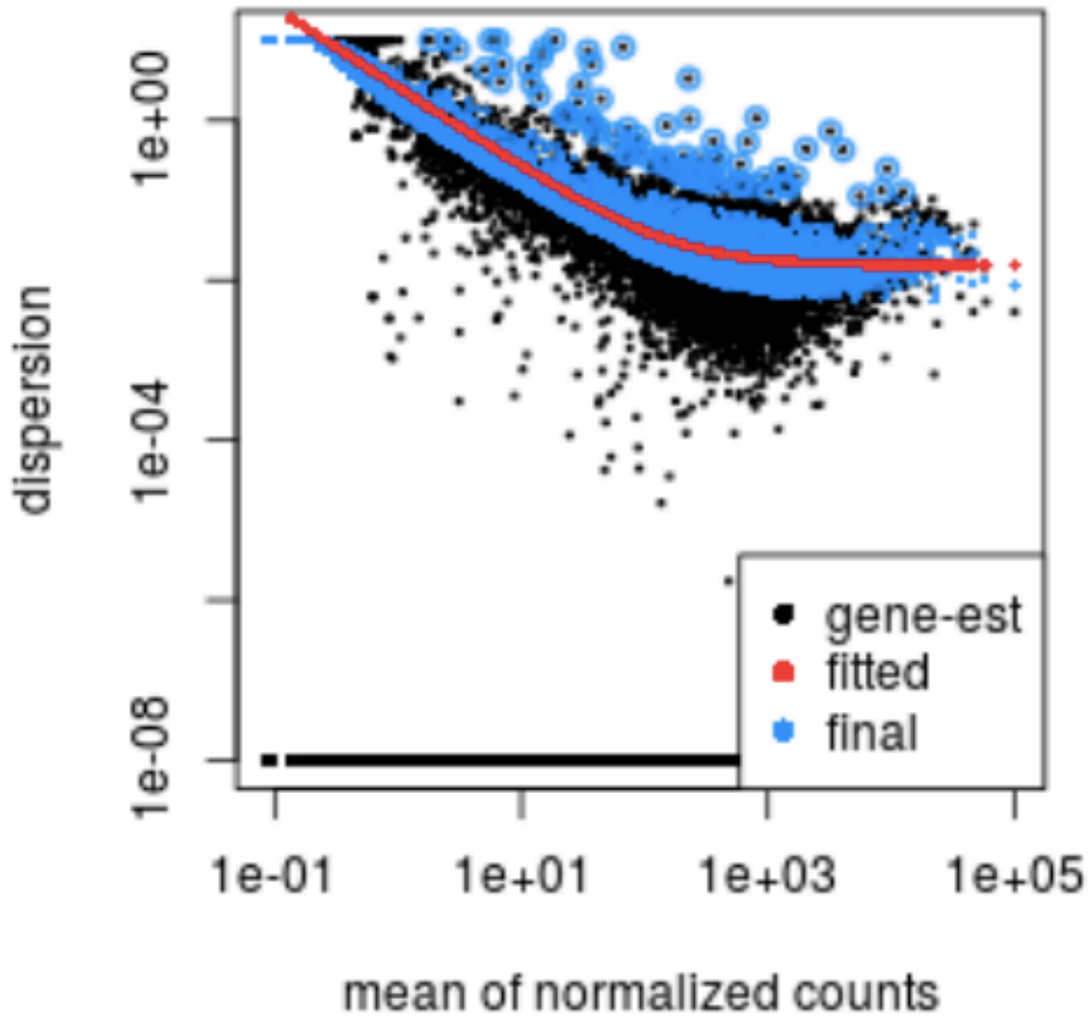


Shrinkage estimators in genomics

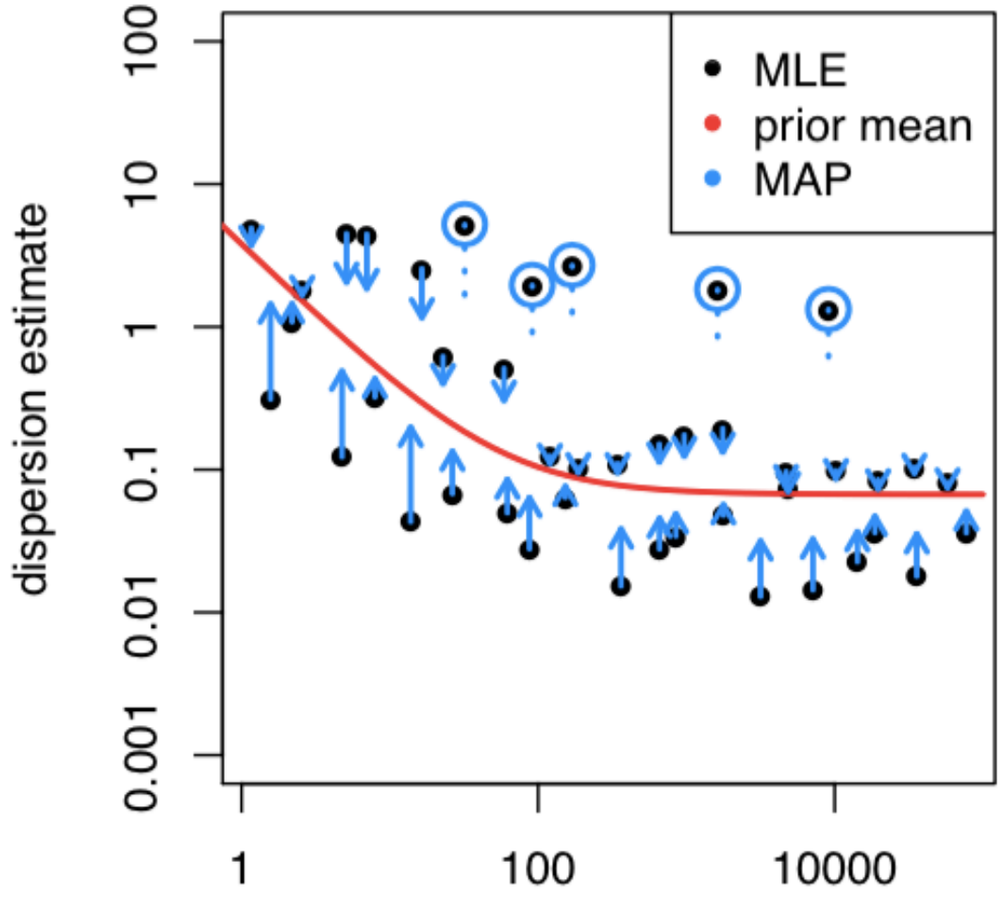
- Lönnstedt and Speed 2002: microarrays
- Smyth 2004: limma for microarrays
- Robinson and Smyth 2007: edgeR for SAGE and then applied to RNA-seq
- Many adaptations: DSS and DESeq2 use a similar approach, data-driven strength of shrinkage

Shrinkage of dispersion for RNA-seq

all genes (Pasilla)

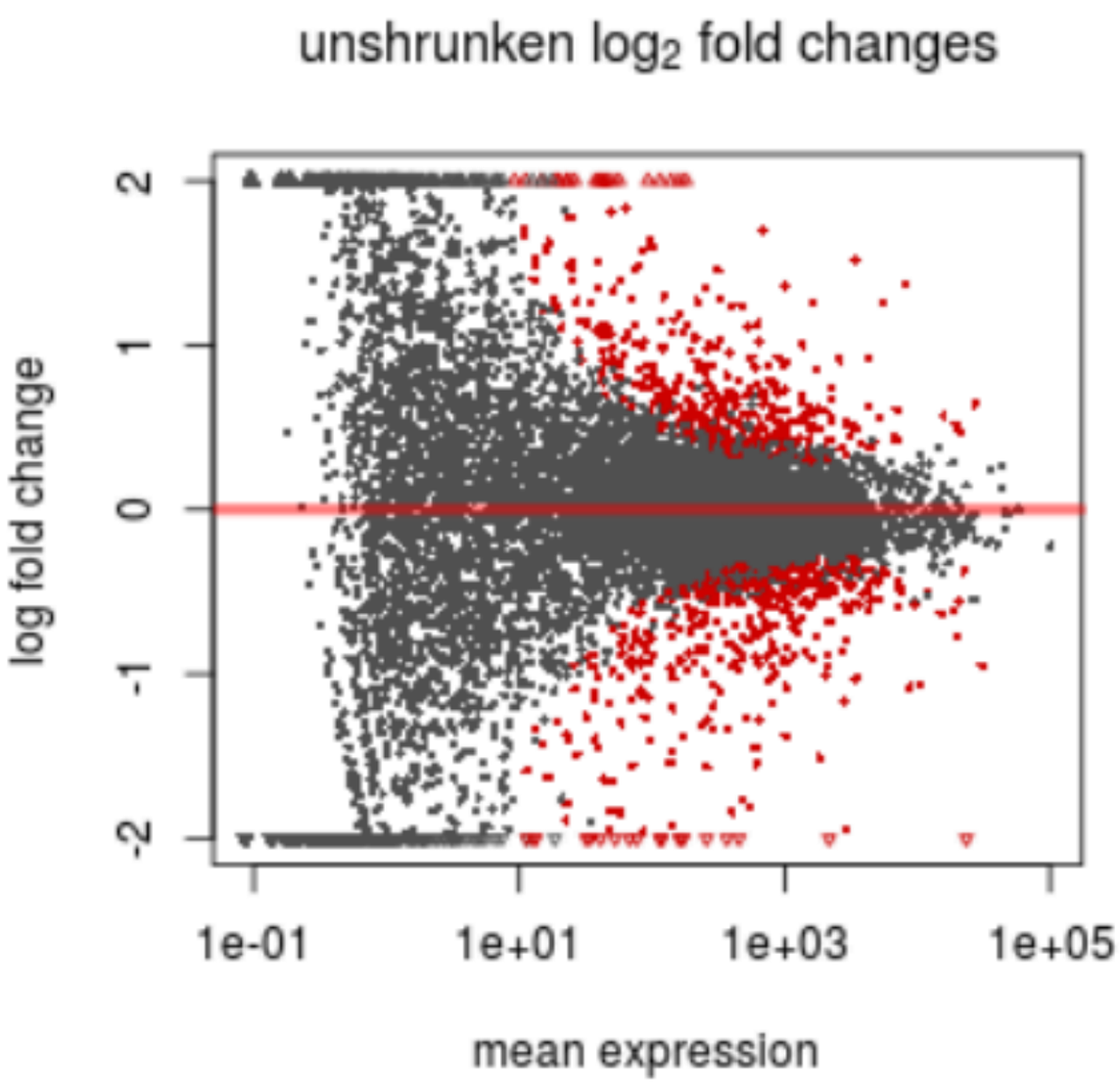


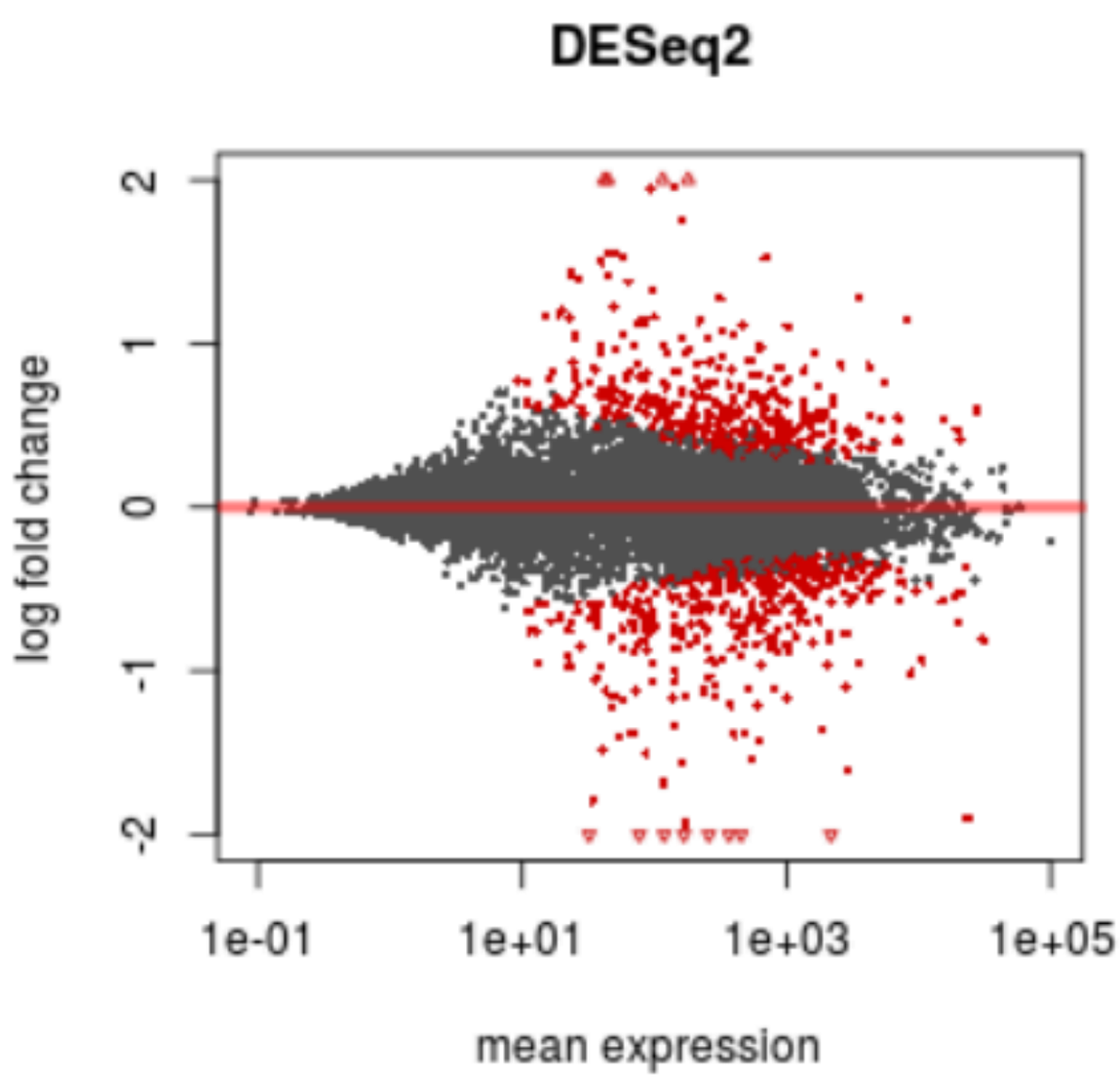
a subset of genes (Pickrell)



1. Gene estimate = maximum likelihood estimate (MLE)
2. Fitted dispersion trend = the mean of the prior
3. Final estimate = maximum a posteriori (MAP)

Shrinkage of fold changes for RNA-seq





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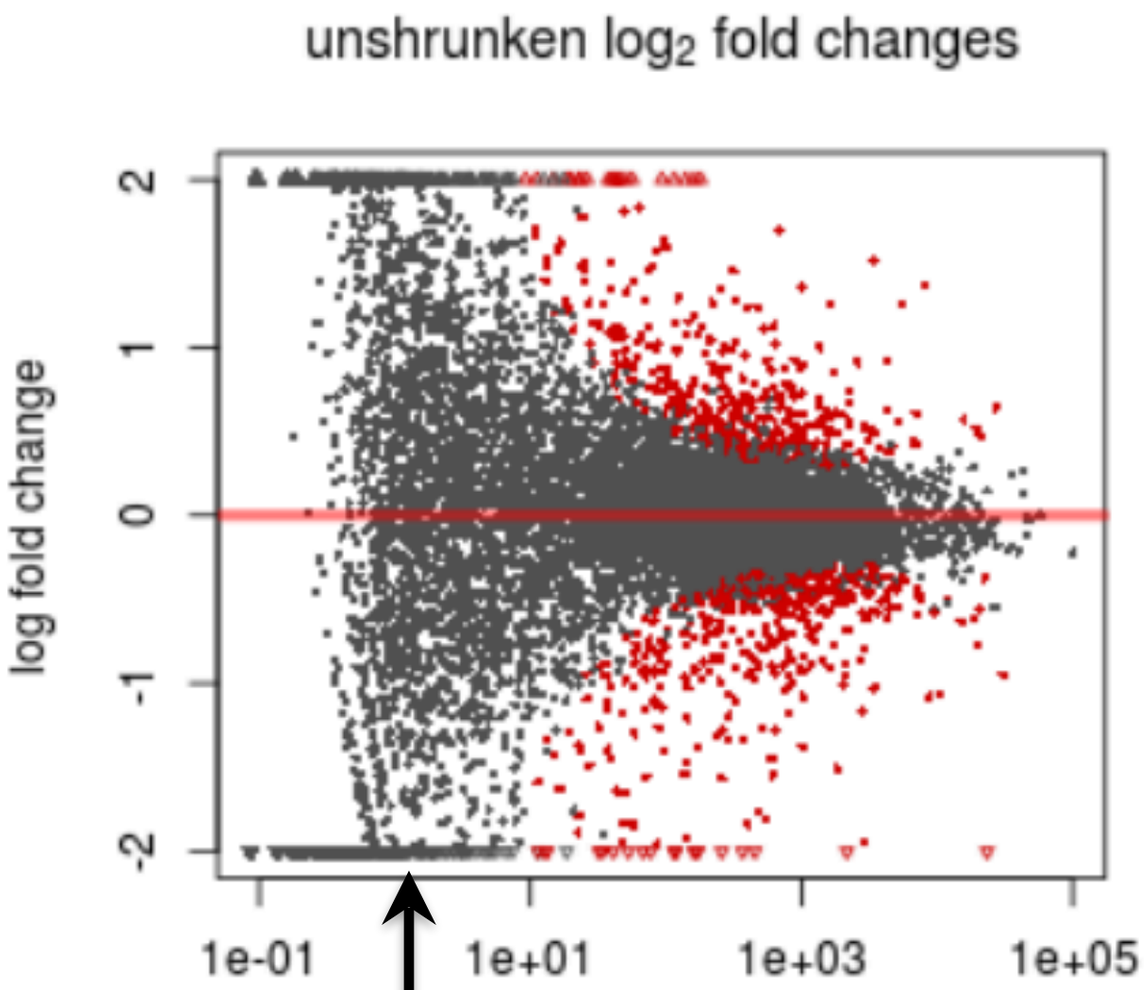
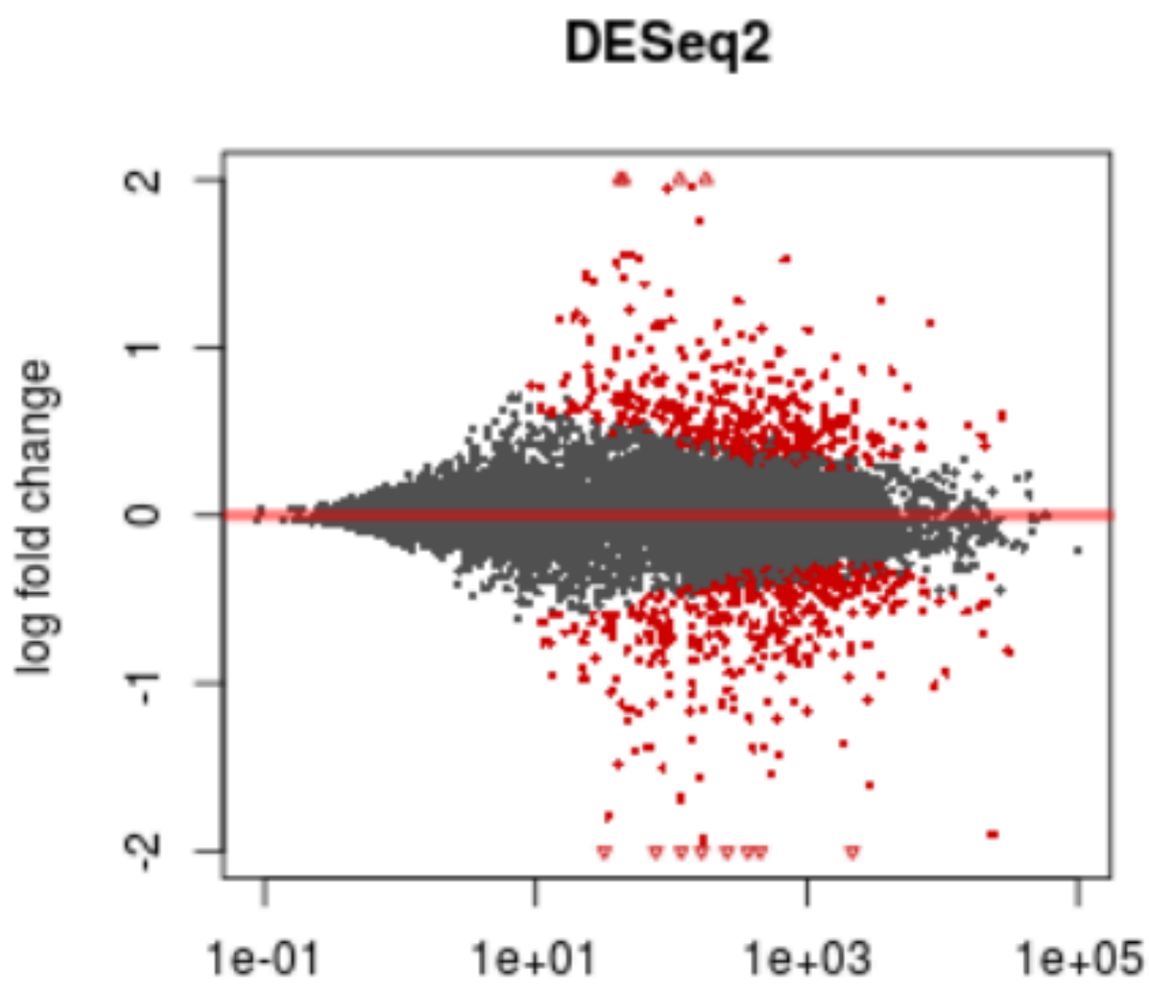
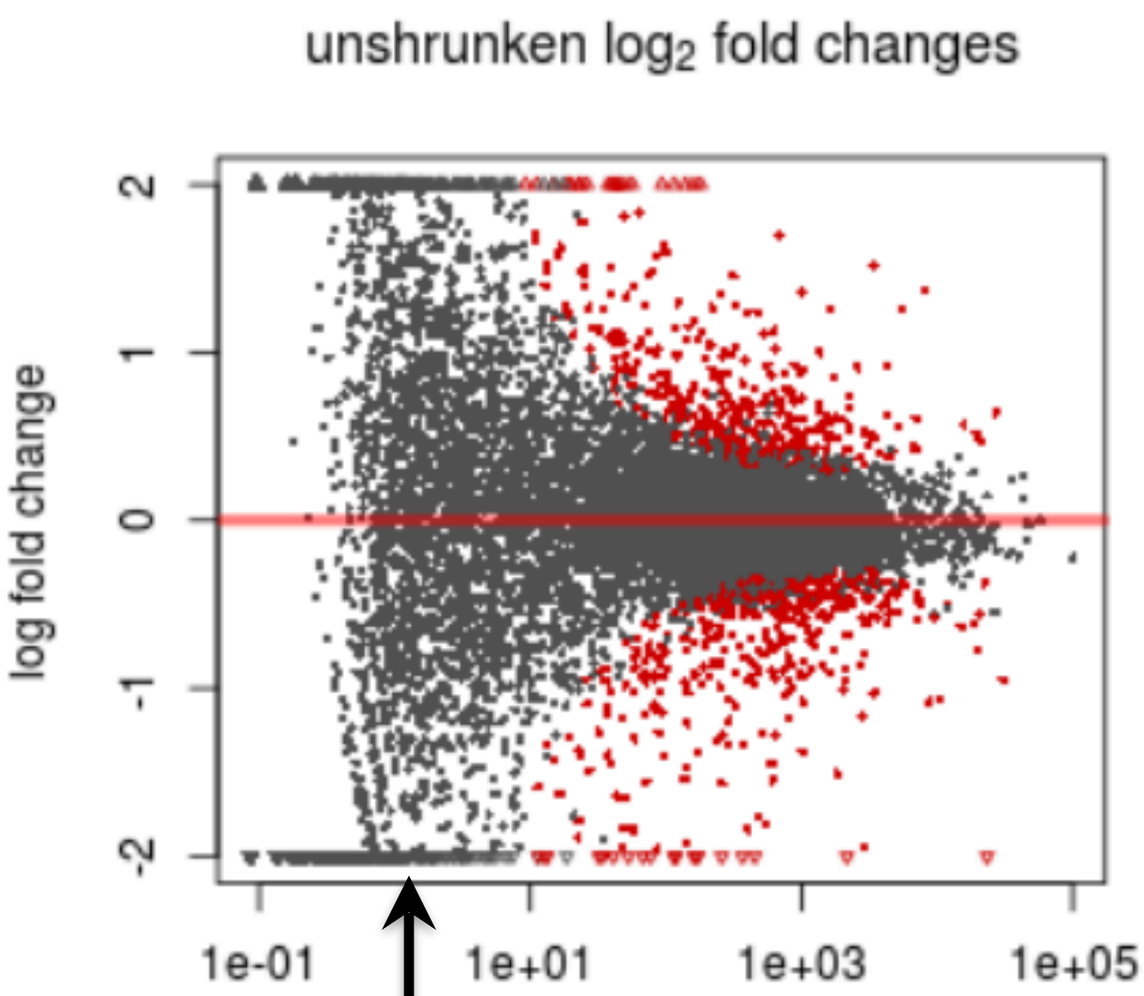
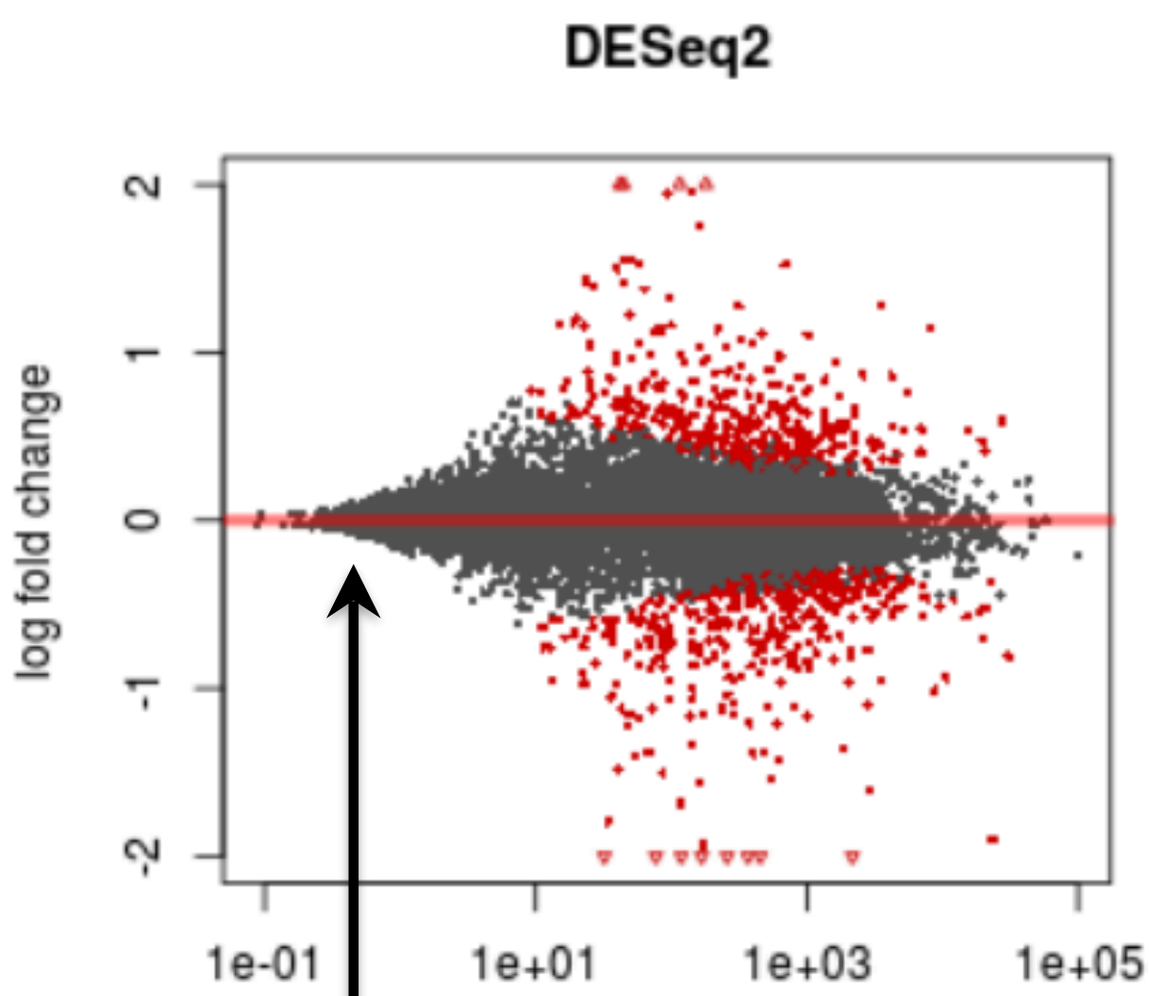




Noisy estimates due to low counts
statistical model will give large p-values, but also the FC estimates themselves are not trustworthy

Shrinkage of fold changes for RNA-seq

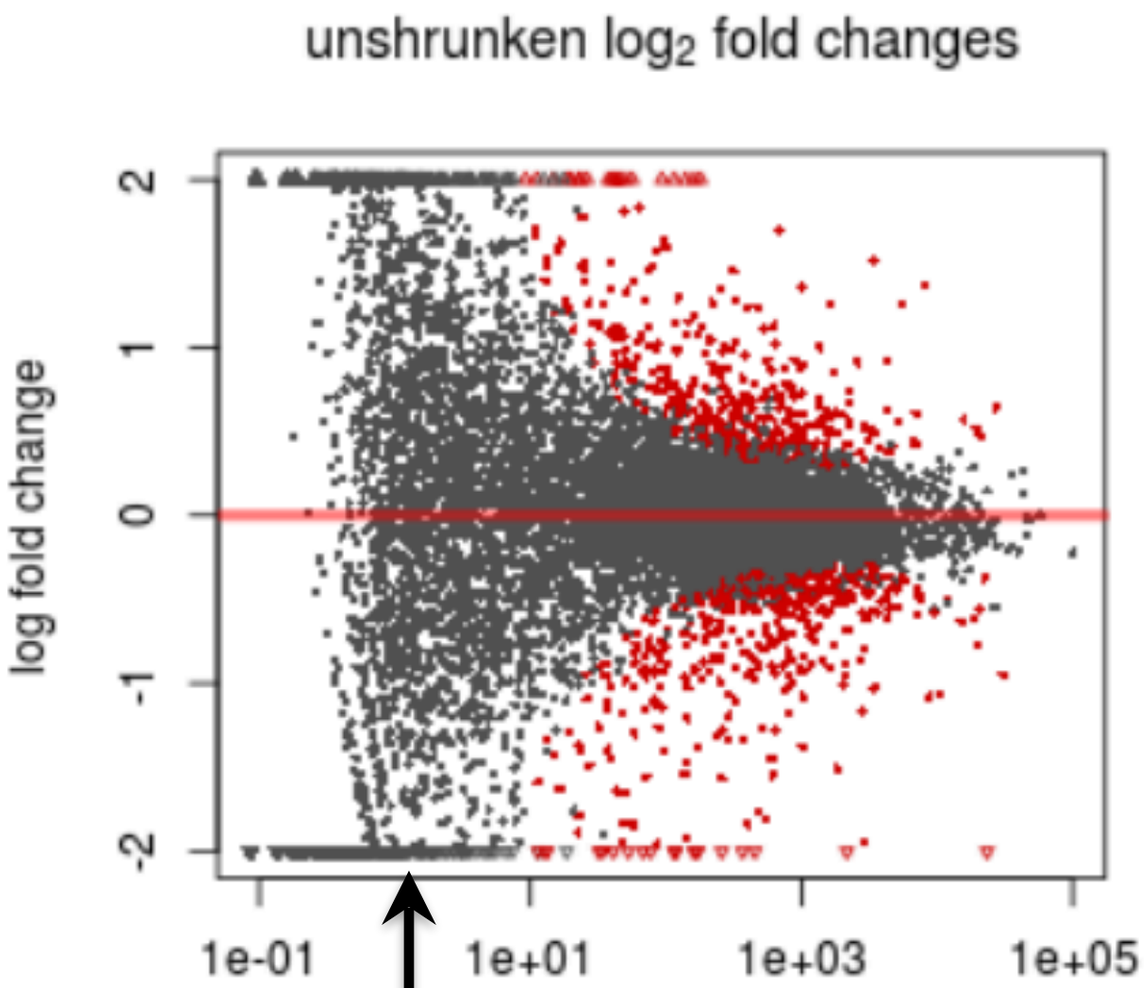


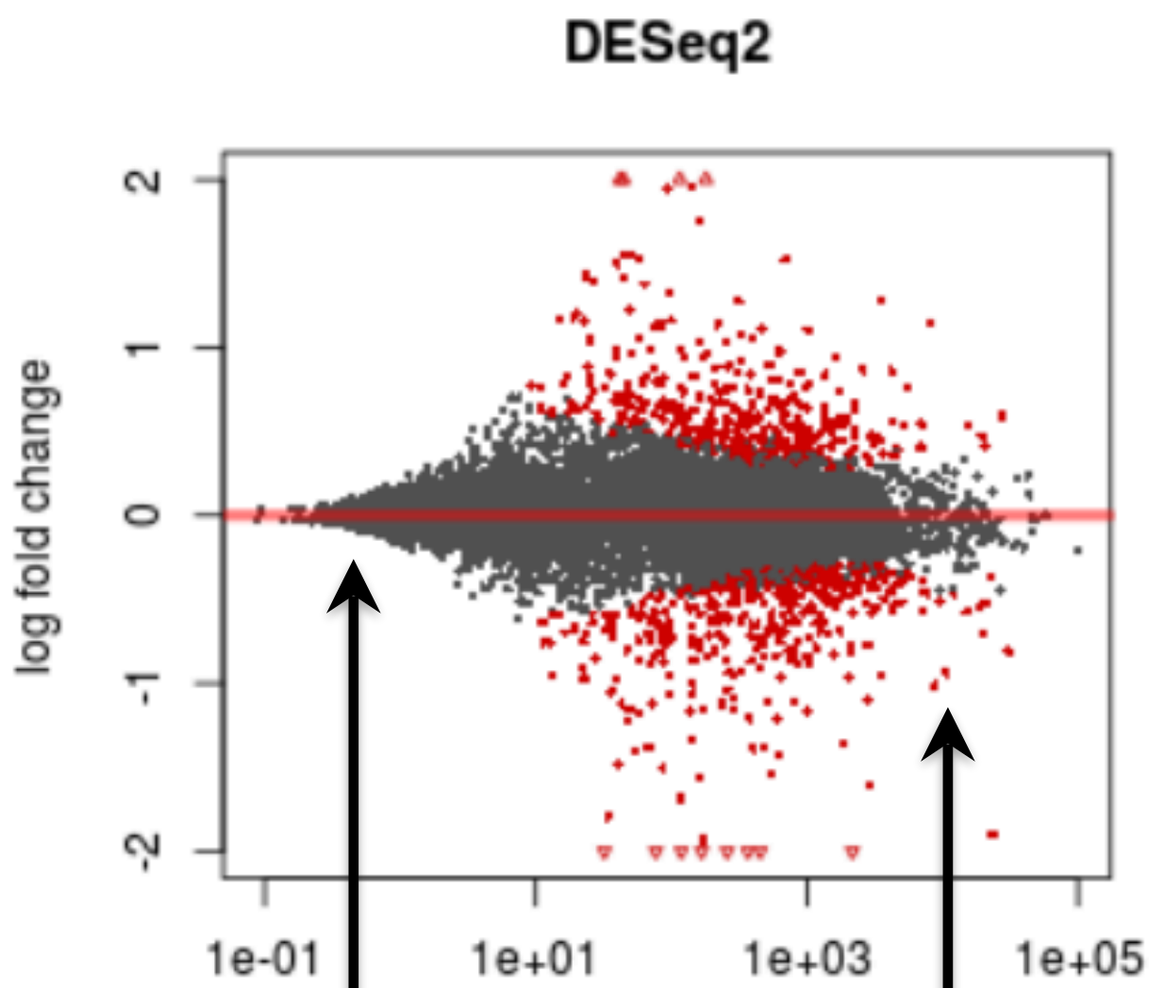


Noisy estimates due to low counts
statistical model will give large p-values, but also the FC estimates themselves are not trustworthy

shrinkage is not equal.
strong moderation for low information genes: low counts

Shrinkage of fold changes for RNA-seq



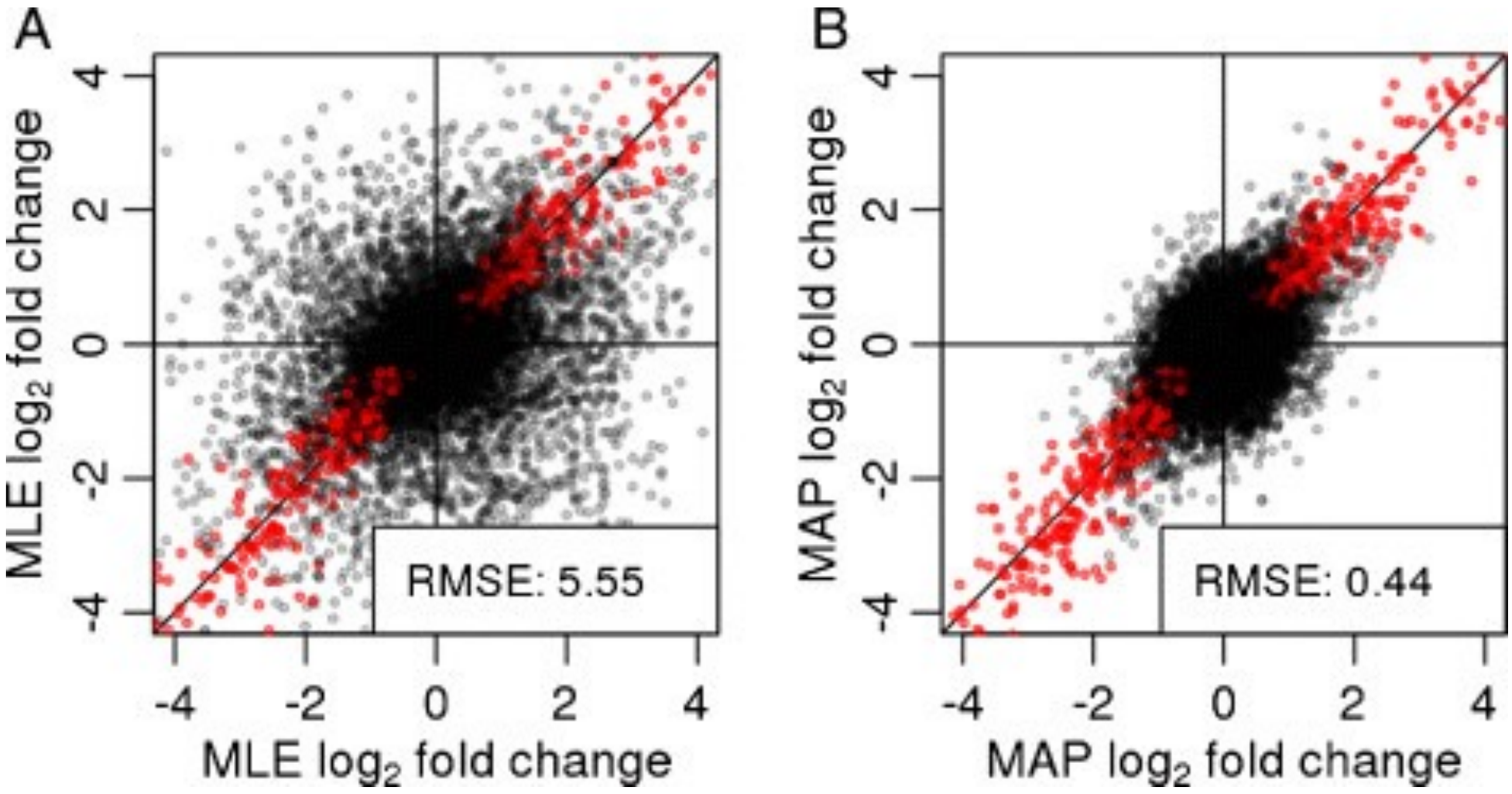


Noisy estimates due to low counts
statistical model will give large p-values, but also the FC estimates themselves are not trustworthy

shrinkage is not equal.
strong moderation for low information genes: low counts

almost no shrinkage

Why shrink fold changes?



Split a dataset into two equal parts, compare LFC

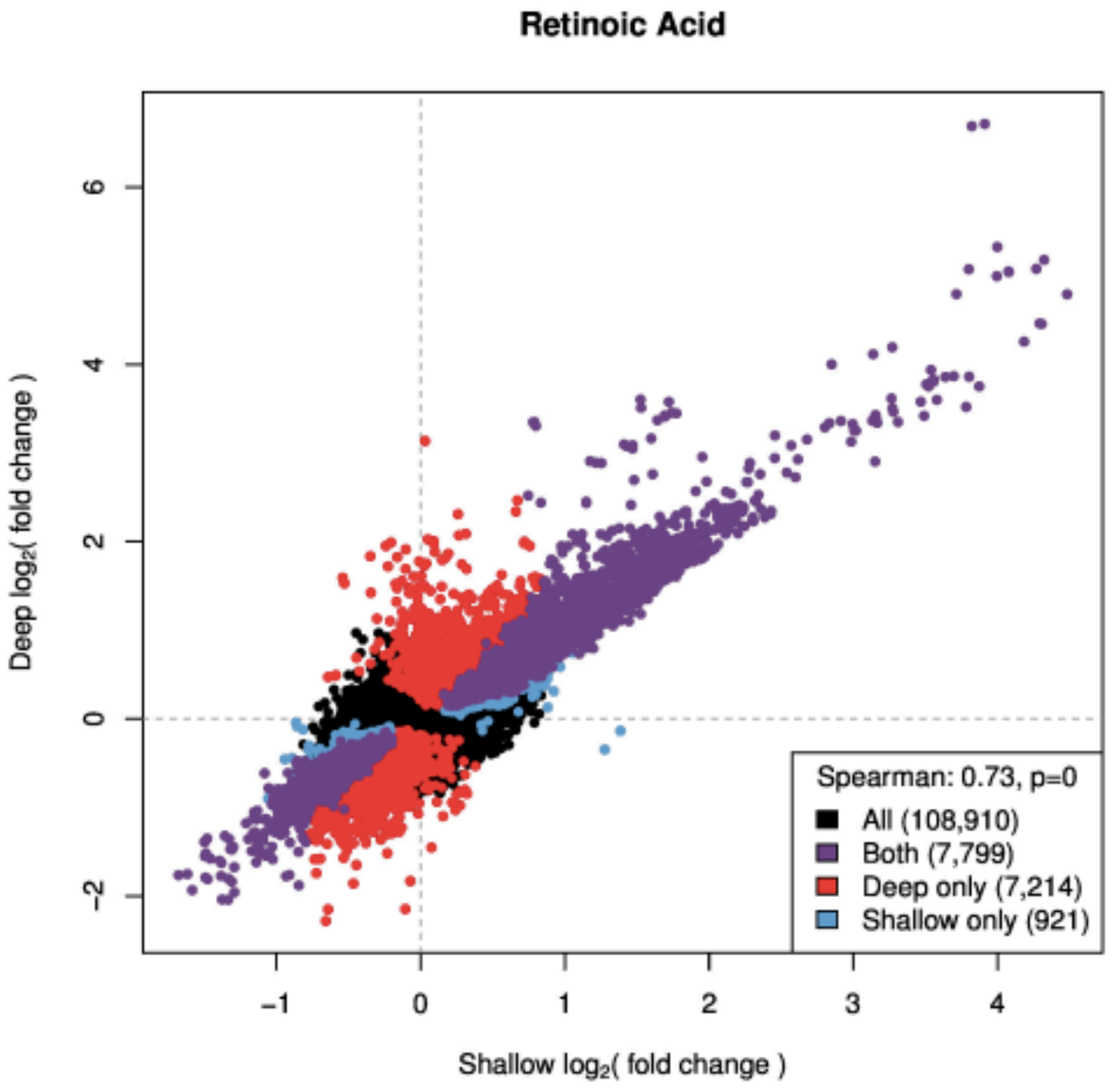
Why shrink fold changes?

Comparison of log fold changes across two experiments.

"A new two-step high-throughput approach:

1. gene expression screening of a large number of conditions

2. deep sequencing of the most relevant conditions"



G. A. Moyerbrailean et al. "A high-throughput RNA-seq approach to profile transcriptional responses" http://dx.doi.org/10.1101/018416

The maths: empirical Bayes shrinkage of gene-wise dispersion estimates

$$\hat{\alpha}_{\text{MLE}} = \underset{\alpha}{\text{argmax}}(\ell(\alpha|\vec{k}, \hat{\mu}))$$

$$\text{CR}(\alpha) = -\frac{1}{2}\log(\det(X^t W X))$$

$$\hat{\alpha}_{\text{CR}} = \underset{\alpha}{\text{argmax}}(\ell(\alpha|k, \hat{\mu}) + \text{CR}(\alpha))$$

$$\text{prior}(\alpha) = f_{\mathcal{N}}(\log(\alpha); \log(\alpha_{\text{fit}}), \sigma^2_{\alpha\text{-prior}})$$

$$\hat{\alpha}_{\text{CR-MAP}} = \underset{\alpha}{\text{argmax}}(\ell(\alpha|\vec{k}, \hat{\mu}) + \text{CR}(\alpha) + \log(\text{prior}(\alpha)))$$

Love, Anders, Huber, Genome Biology 2014

The maths: empirical Bayes shrinkage of gene-wise dispersion estimates

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"naive" GLM likelihood

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The maths: empirical Bayes shrinkage of gene-wise dispersion estimates

$$\hat{\alpha}_{\mathrm{MLE}} = \underset{\alpha}{\mathrm{argmax}}(\ell(\alpha|\vec{k},\hat{\mu}))$$ ← "naive" GLM likelihood

$$\mathrm{CR}(\alpha) = -\frac{1}{2}\log(\det(X^t W X))$$ ← Cox-Reid bias term

$$\hat{\alpha}_{\mathrm{CR}} = \underset{\alpha}{\mathrm{argmax}}(\ell(\alpha|k,\hat{\mu}) + \mathrm{CR}(\alpha))$$

$$\mathrm{prior}(\alpha) = f_{\mathcal{N}}(\log(\alpha); \log(\alpha_{\mathrm{fit}}), \sigma^2_{\alpha\text{-prior}})$$

$$\hat{\alpha}_{\text{CR-MAP}} = \underset{\alpha}{\mathrm{argmax}}(\ell(\alpha|\vec{k},\hat{\mu}) + \mathrm{CR}(\alpha) + \log(\mathrm{prior}(\alpha)))$$

Love, Anders, Huber, Genome Biology 2014

The maths: empirical Bayes shrinkage of gene-wise dispersion estimates

$$\hat{\alpha}_{\mathrm{MLE}} = \underset{\alpha}{\mathrm{argmax}}(\ell(\alpha|\vec{k},\hat{\mu}))$$ ← "naive" GLM likelihood

$$\mathrm{CR}(\alpha) = -\frac{1}{2}\log(\det(X^t W X))$$ ← Cox-Reid bias term

$$\hat{\alpha}_{\mathrm{CR}} = \underset{\alpha}{\mathrm{argmax}}(\ell(\alpha|\vec{k},\hat{\mu}) + \mathrm{CR}(\alpha))$$ ← bias-corrected likelihood

$$\mathrm{prior}(\alpha) = f_{\mathcal{N}}(\log(\alpha); \log(\alpha_{\mathrm{fit}}), \sigma^2_{\alpha\text{-prior}})$$

$$\hat{\alpha}_{\text{CR-MAP}} = \underset{\alpha}{\mathrm{argmax}}(\ell(\alpha|\vec{k},\hat{\mu}) + \mathrm{CR}(\alpha) + \log(\mathrm{prior}(\alpha)))$$

Love, Anders, Huber, Genome Biology 2014

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$$\mathrm{prior}(\alpha) = f_{\mathcal{N}}(\log(\alpha); \log(\alpha_{\mathrm{fit}}), \sigma^2_{\alpha\text{-prior}})$$ ← prior on **α** by 'information sharing' across genes

$$\hat{\alpha}_{\text{CR-MAP}} = \underset{\alpha}{\mathrm{argmax}}(\ell(\alpha|\vec{k},\hat{\mu}) + \mathrm{CR}(\alpha) + \log(\mathrm{prior}(\alpha)))$$

Love, Anders, Huber, Genome Biology 2014

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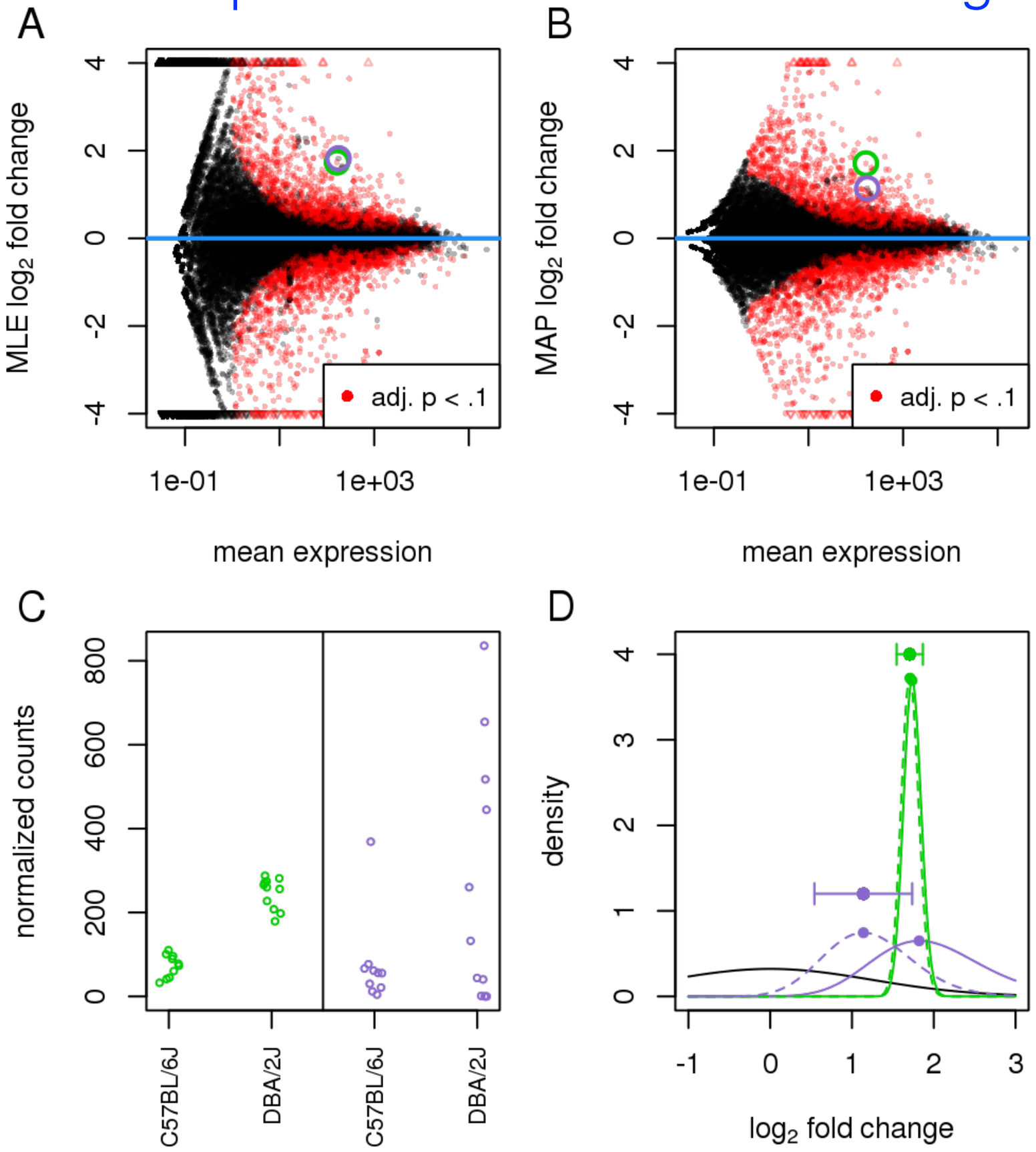
$$\hat{\alpha}_{\mathrm{CR}} = \underset{\alpha}{\mathrm{argmax}}(\ell(\alpha|\vec{k},\hat{\mu}) + \mathrm{CR}(\alpha))$$ ← bias-corrected likelihood

$$\mathrm{prior}(\alpha) = f_{\mathcal{N}}(\log(\alpha); \log(\alpha_{\mathrm{fit}}), \sigma^2_{\alpha\text{-prior}})$$ ← prior on α by 'information sharing' across genes

$$\hat{\alpha}_{\mathrm{CR\text{-}MAP}} = \underset{\alpha}{\mathrm{argmax}}(\ell(\alpha|\vec{k},\hat{\mu}) + \mathrm{CR}(\alpha) + \log(\mathrm{prior}(\alpha)))$$ ← penalized likelihood

Love, Anders, Huber, Genome Biology 2014

Example: difference between maximum likelihood and maximum a posteriori estimate for two genes



Banded hypothesis testing:
integrate testing with fold-change cutoff

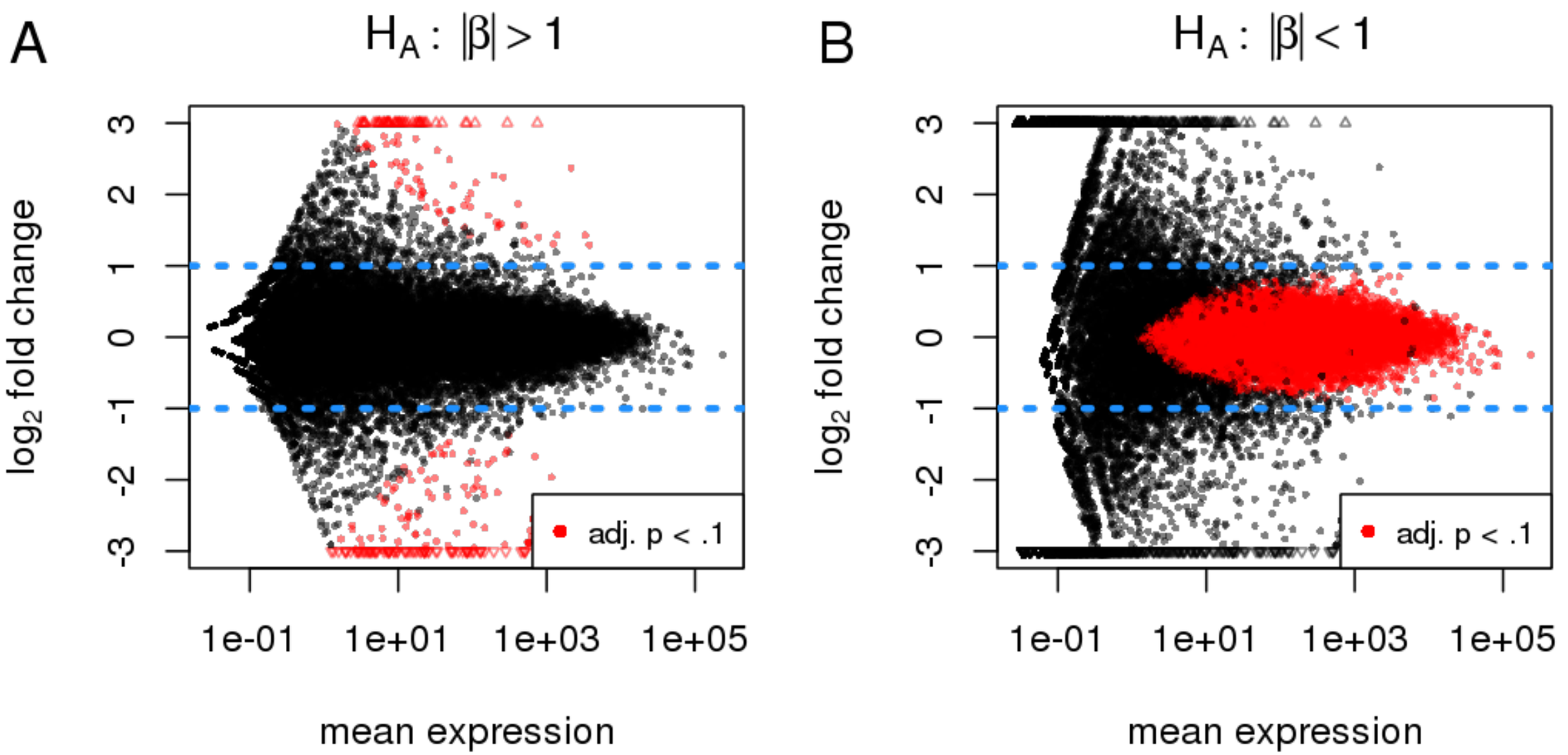
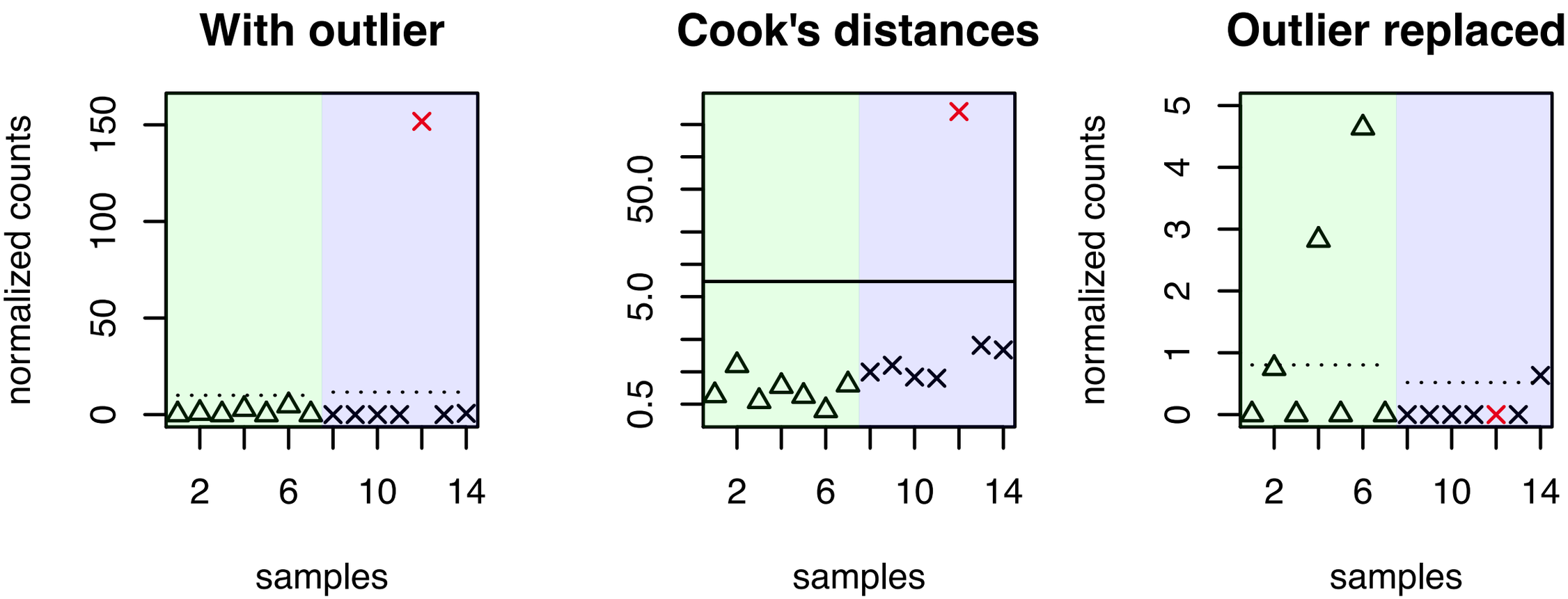


Figure 4 Hypothesis testing involving non-zero thresholds. Shown are MA-plots for a 10 vs 11 comparison using the Bottomly *et al.* [15] dataset, with highlighted points indicating low adjusted p-values. The alternate hypotheses are that logarithmic (base 2) fold changes are (A) greater than 1 in absolute value or (B) less than 1 in absolute value.

Outlier robustness

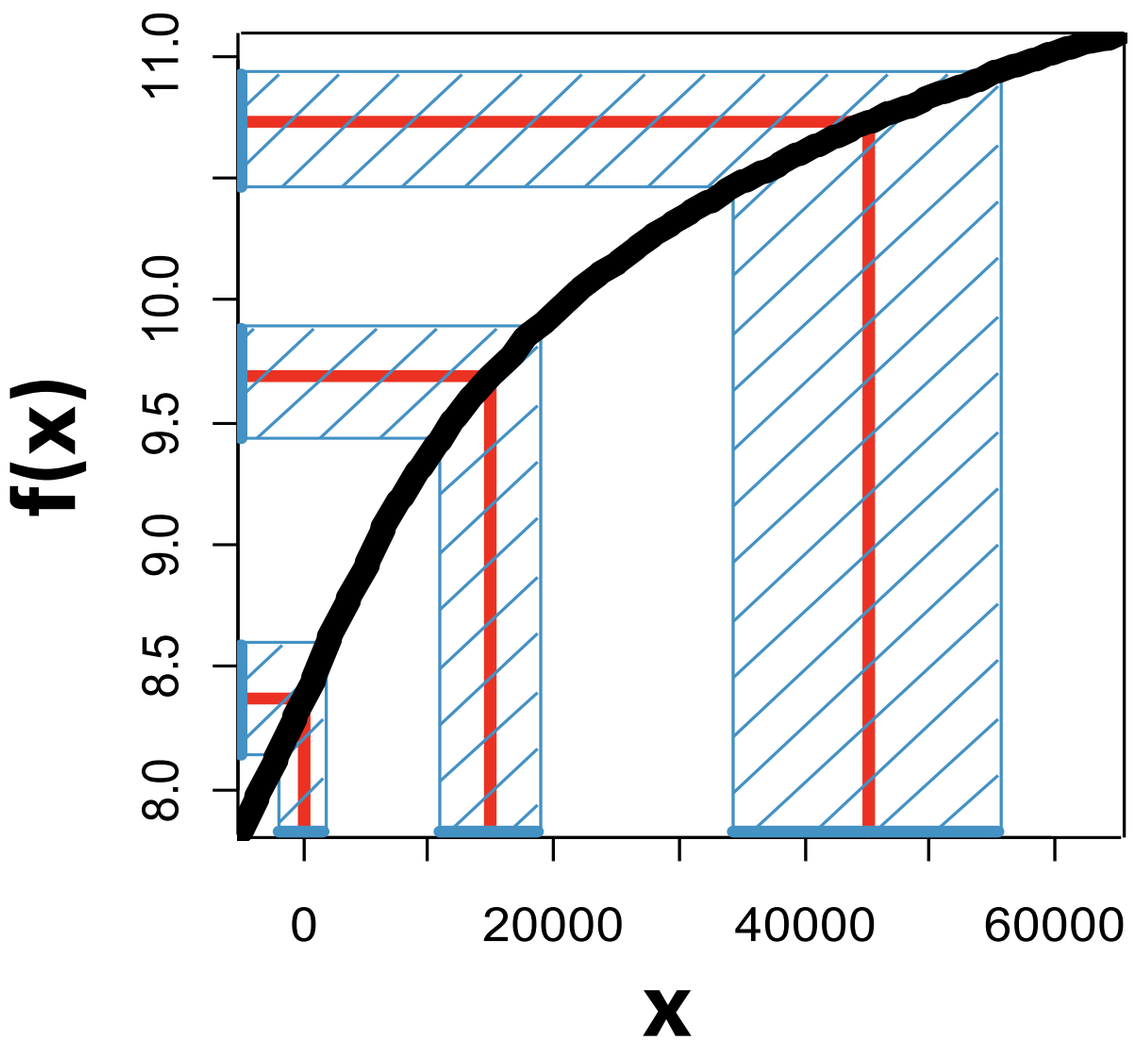


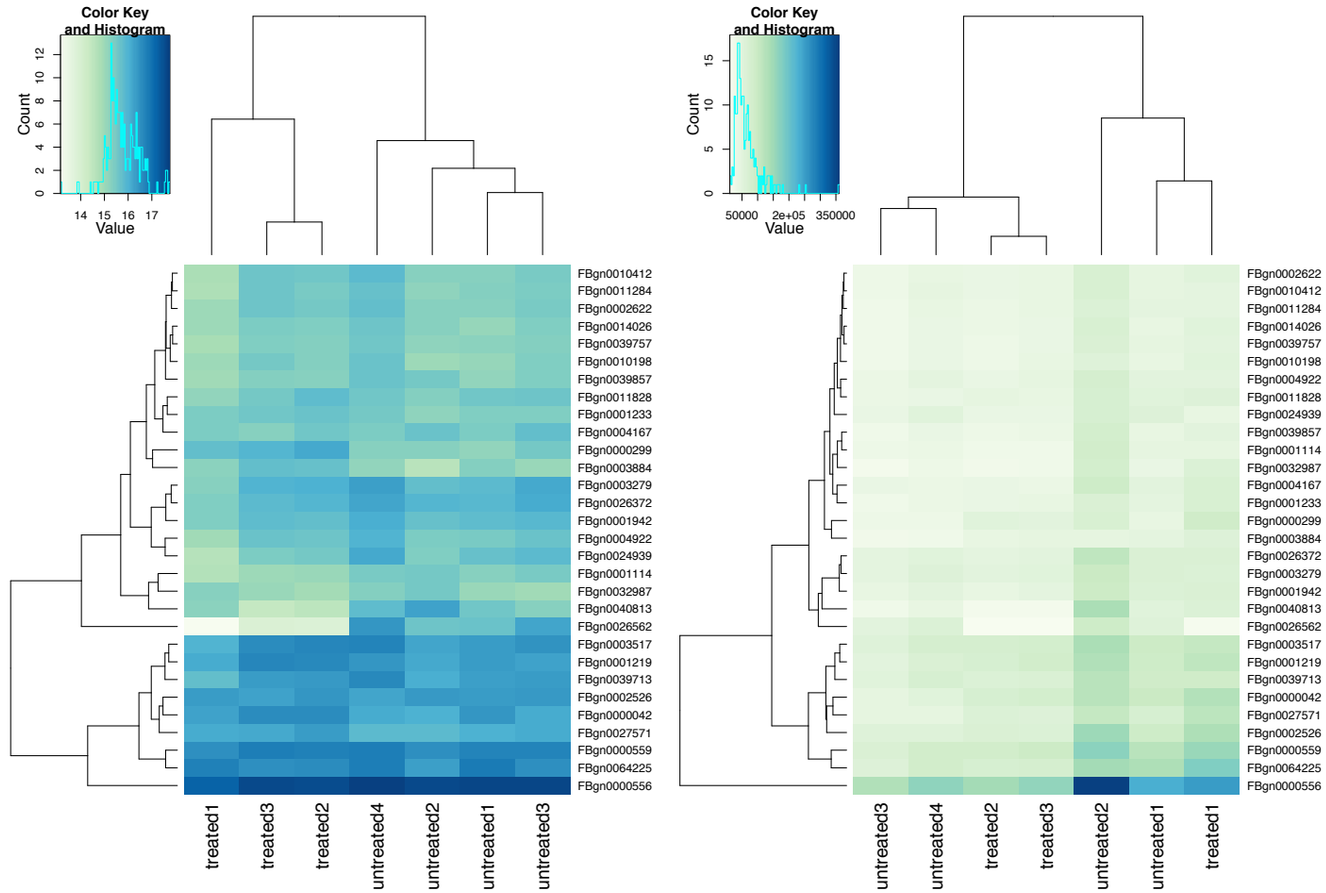
Cook’s distance:

Change in fitted coefficients if the sample were removed

Variance-stabilizing transformation

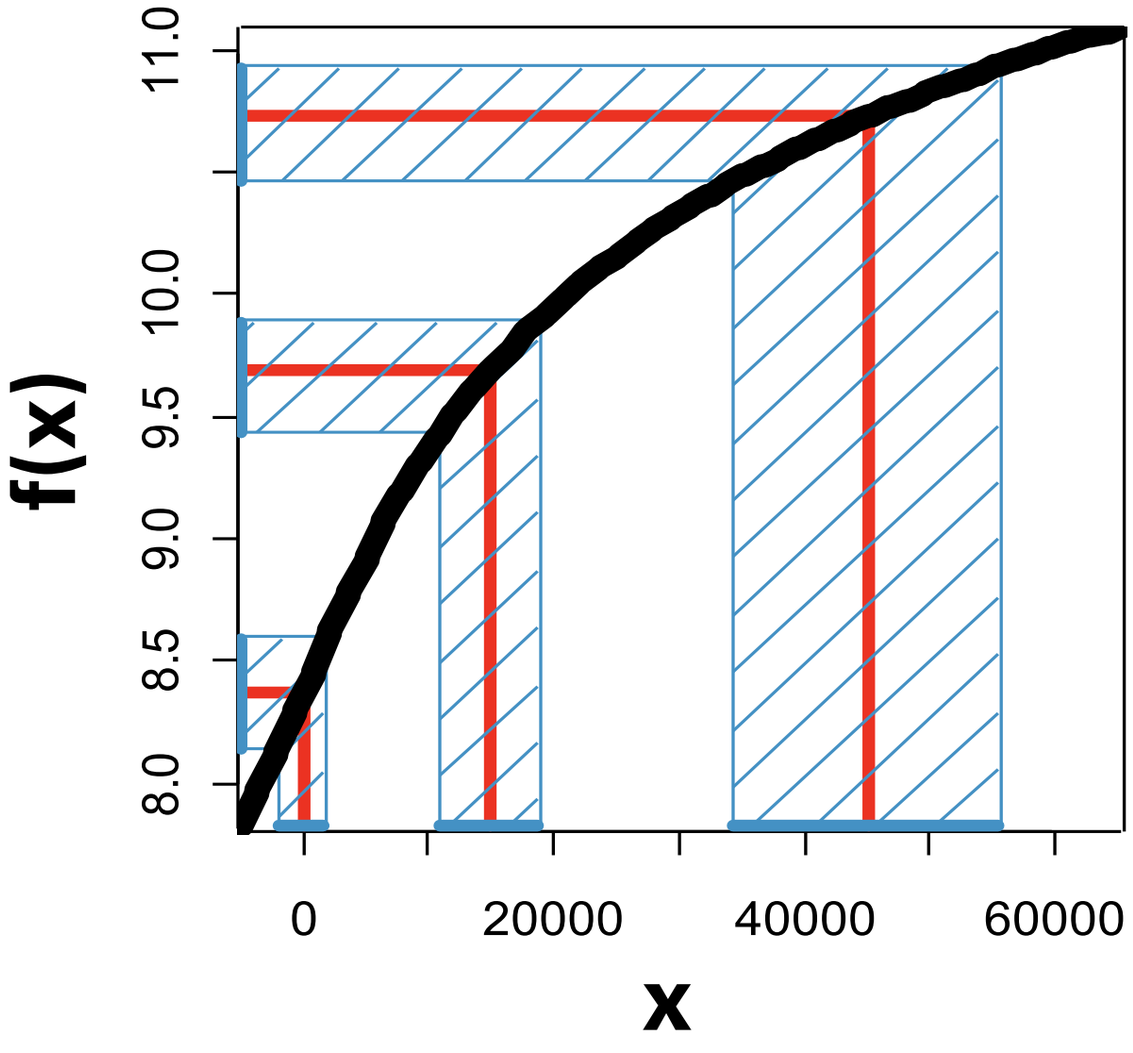
$$f(x) = \int^{x} \frac{du}{\sqrt{v(u)}}$$

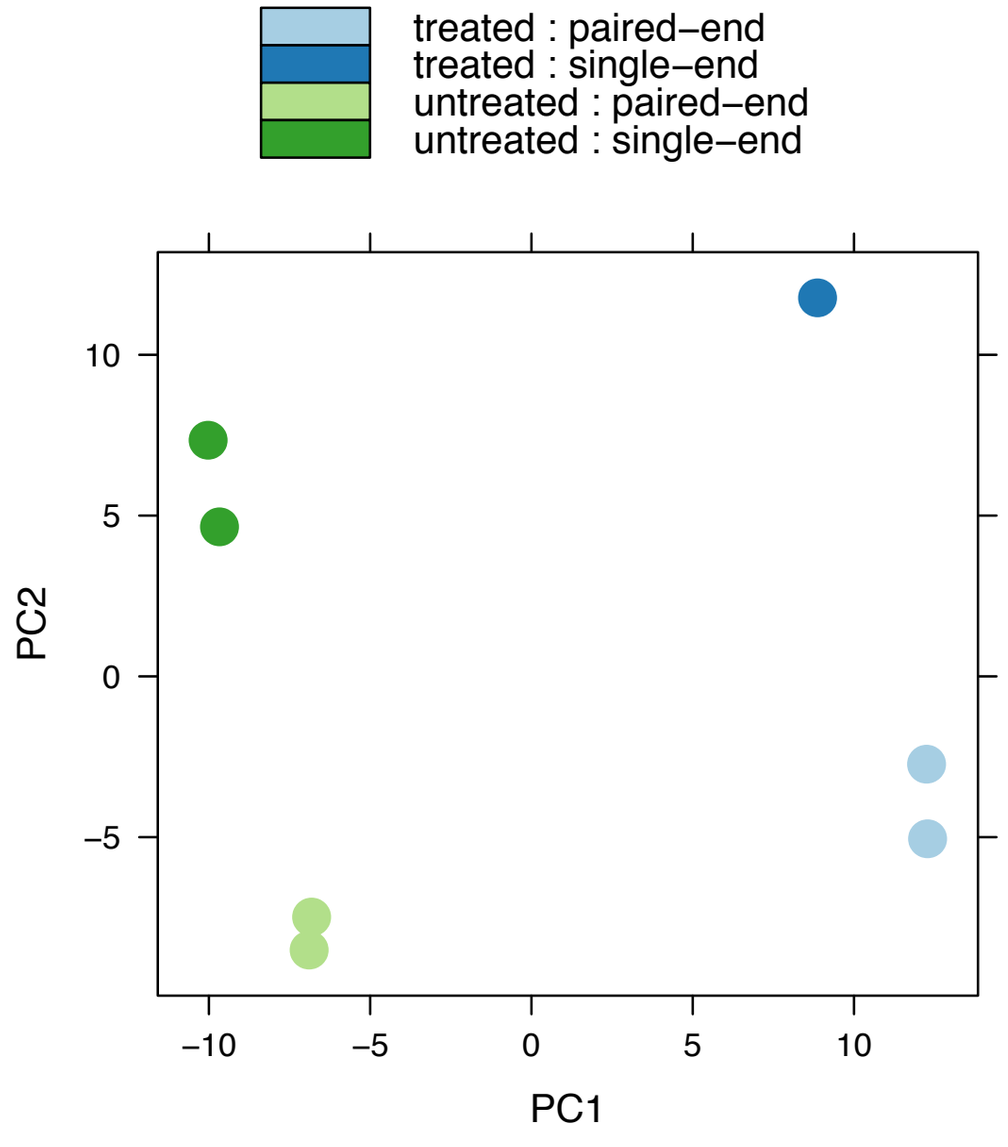




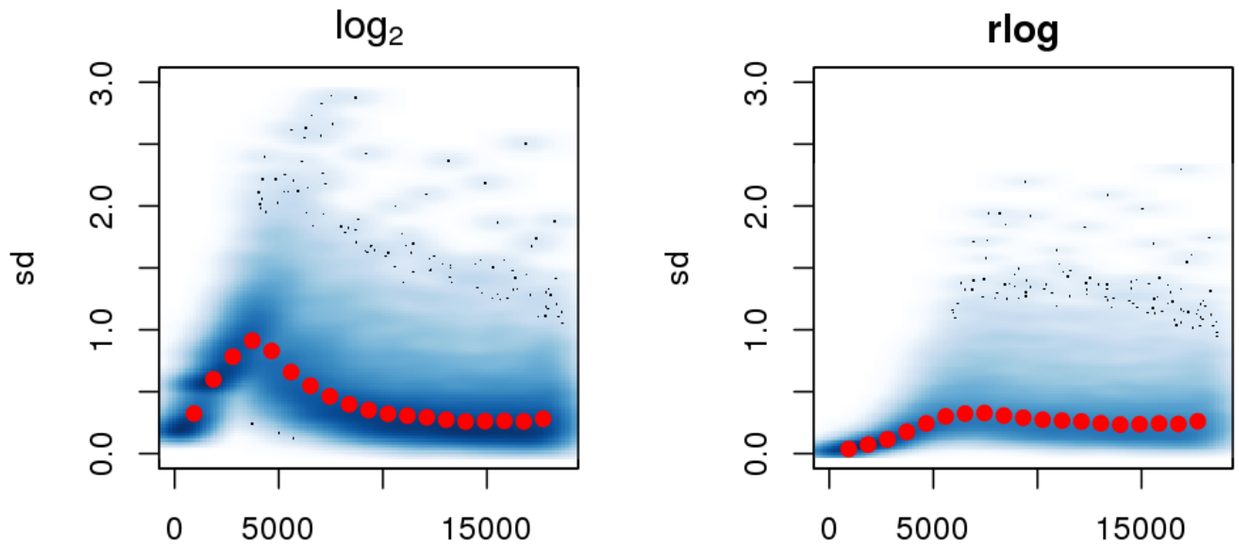
Variance-stabilizing transformation

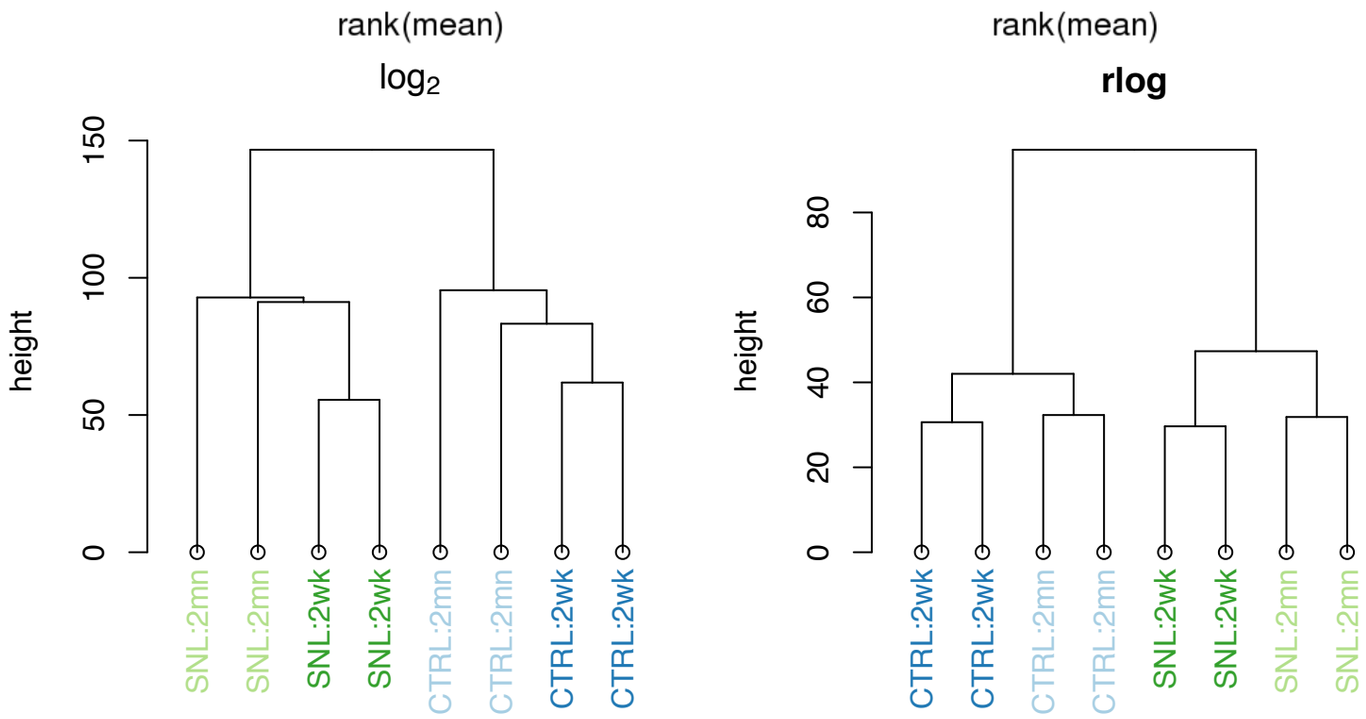
$$f(x) = \int^{x} \frac{du}{\sqrt{v(u)}}$$





Regularized log-transformation: Visualization, Clustering, PCA

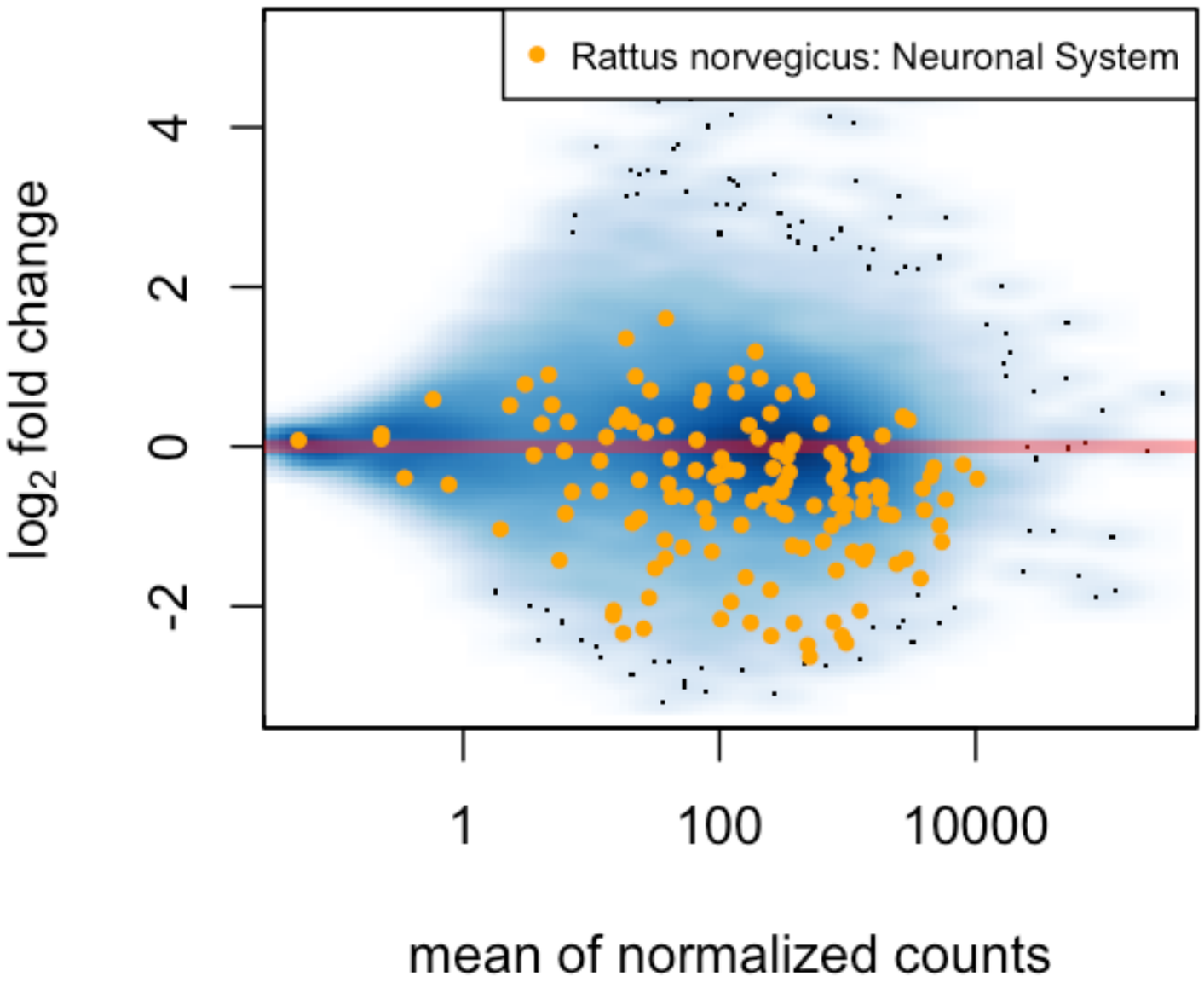




"rlog":
Shrunken log fold changes for every sample:
reduces effect of shot noise on inter-sample distances

RNA from the dorsal root ganglion of rats that underwent spinal nerve ligation and controls, 2 weeks & 2 months after the ligation. Hammer, …, Beutler AS, Genome Research 2010.

GSEA with shrunken log fold changes



Reactome gene set
one-sample t-statistic

Neuronal System
144 genes
avg LFC: -0.55
adjusted p-value: 10^{-8}

RNA from the dorsal root ganglion of rats that underwent spinal nerve ligation and controls, 2 weeks & 2 months after the ligation. Hammer, …, Beutler AS, Genome Research 2010.

Summary so far

- Text-book statistical concepts are (almost) sufficient for differential expression: ANOVA, hypothesis testing, generalized linear models
- In addition: small-n large-p - information sharing across genes, empirical Bayes, shrinkage
- Outliers
- Data transformation: visualisation, clustering, classification

- Next up: exon-level analysis

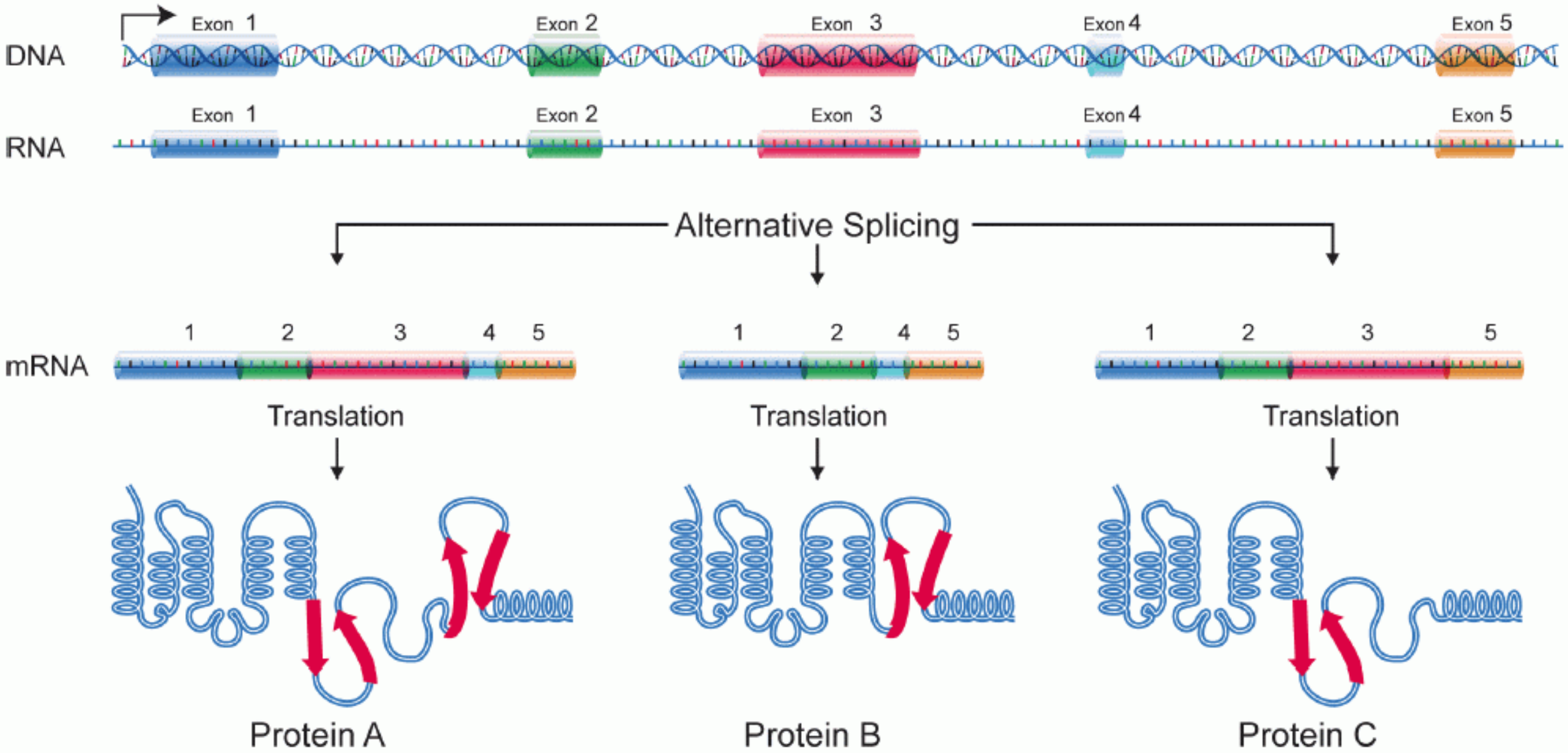
Genes and transcripts

So far, we looked at read counts per gene.

A gene's read count may increase

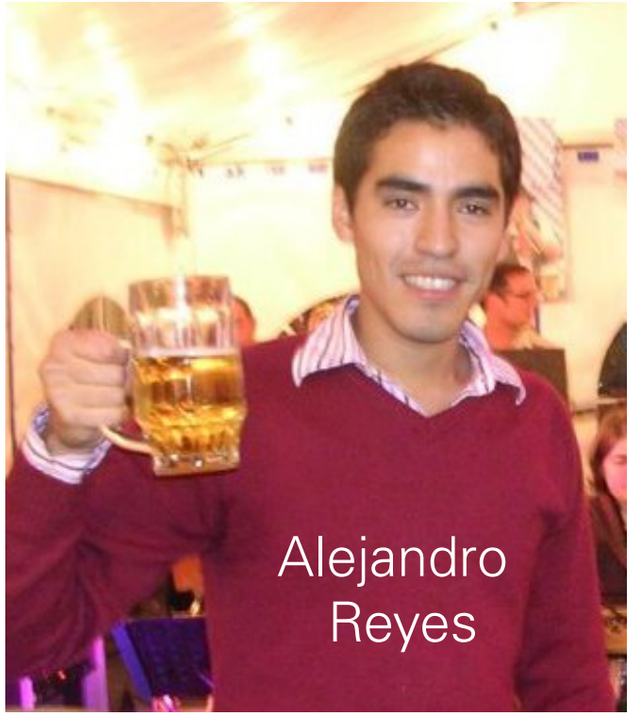
- because the gene produces more transcripts
- because the gene produces longer transcripts

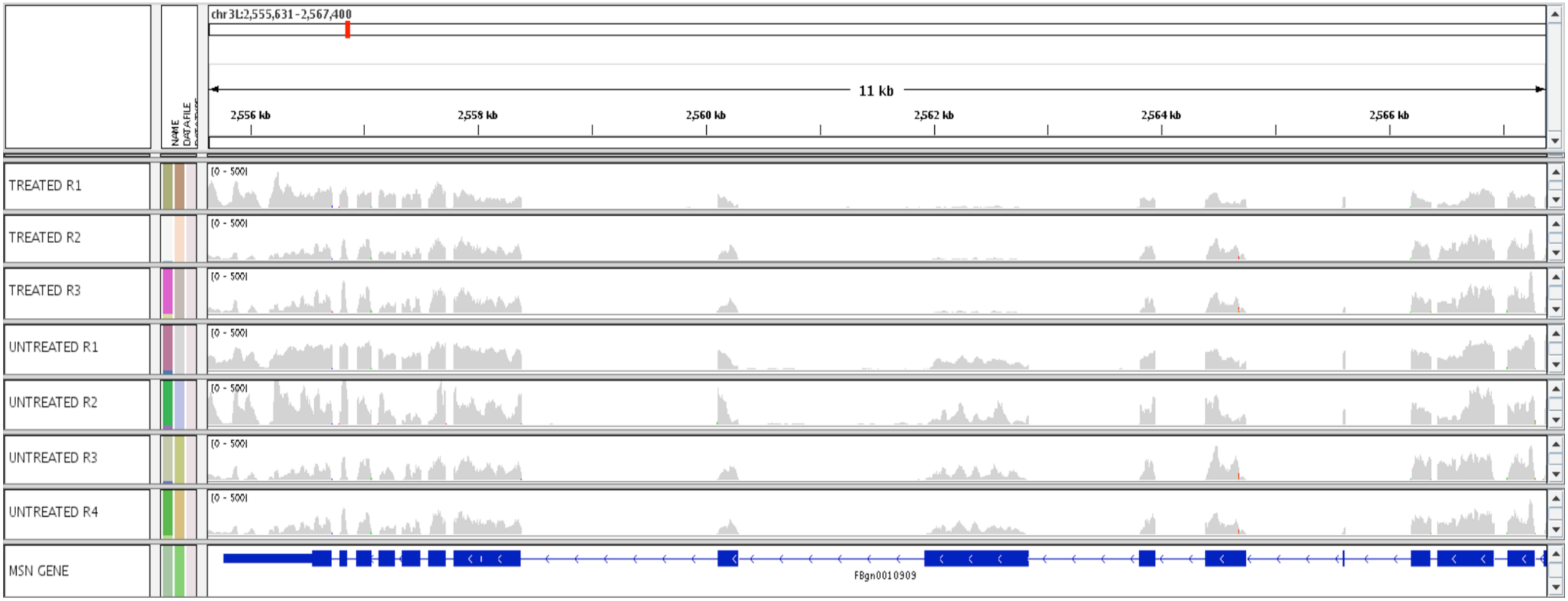
How to look at gene sub-structure?


DNA
Exon 1
Exon 2
Exon 3
Exon 4
Exon 5
RNA
Exon 1
Exon 2
Exon 3
Exon 4
Exon 5
Alternative Splicing
mRNA
1 2 3 4 5
1 2 4 5
1 2 3 5
Translation
Translation
Translation
Protein A
Protein B
Protein C

Wikipedia

Alternative isoform regulation





Data: Brooks, ..., Graveley, Genome Res., 2010

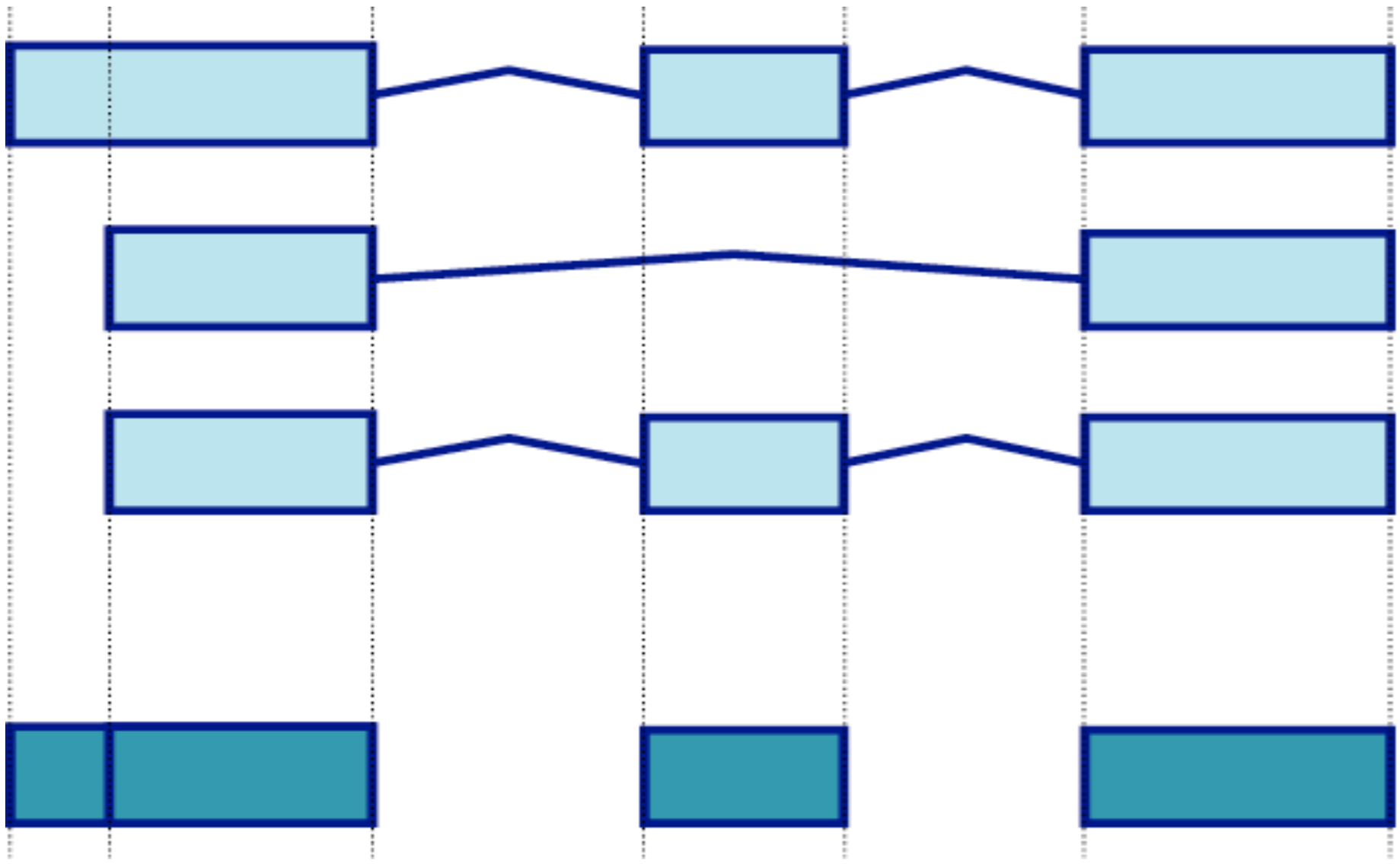
Count table for a gene

number of reads mapped to each exon in a gene

	treated_1	treated_2	control_1	control_2	
E01	398	556	561	456	
E02	112	180	153	137	
E03	238	306	298	226	
E04	162	171	183	146	
E05	192	272	234	199	
E06	314	464	419	331	
E07	373	525	481	404	
E08	323	427	475	373	
E09	194	213	273	176	
E10	90	90	530	398	<--- !
E11	172	207	283	227	
E12	290	397	606	368	<--- ?
E13	33	48	33	33	
E14	0	33	2	37	
E15	248	314	468	287	
E16	554	841	1024	680	

[...]

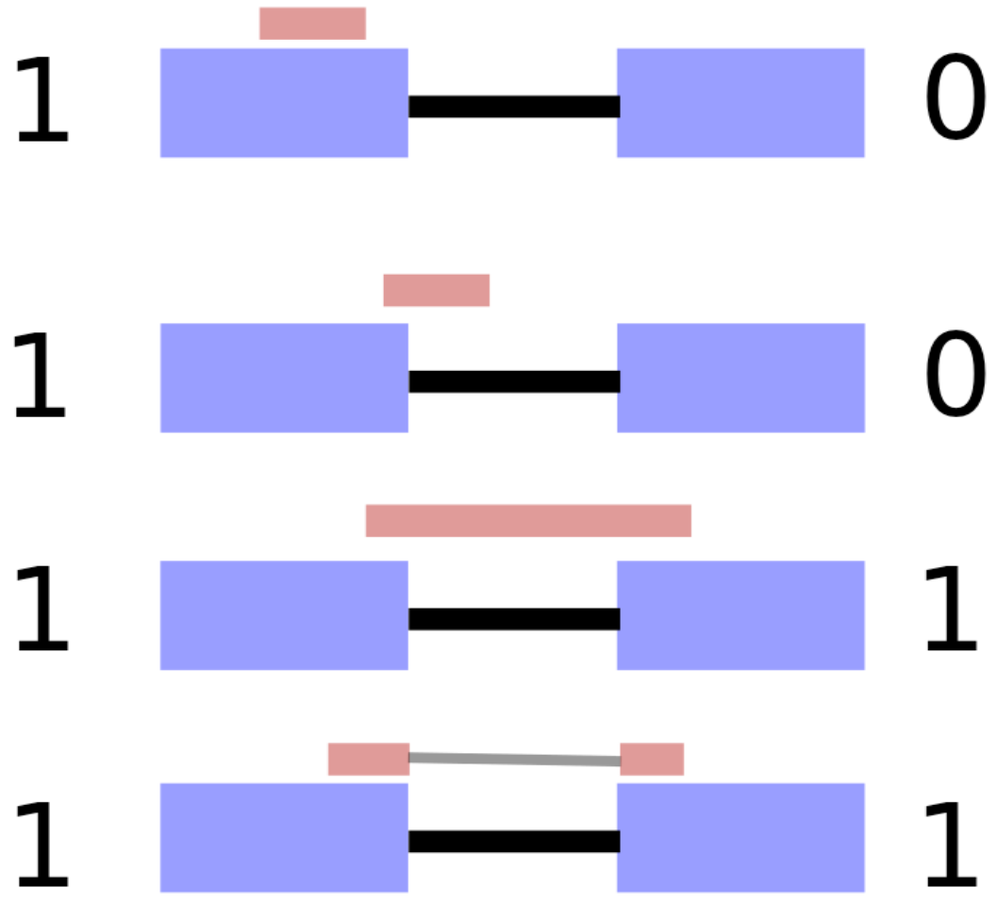
Exon counting bins



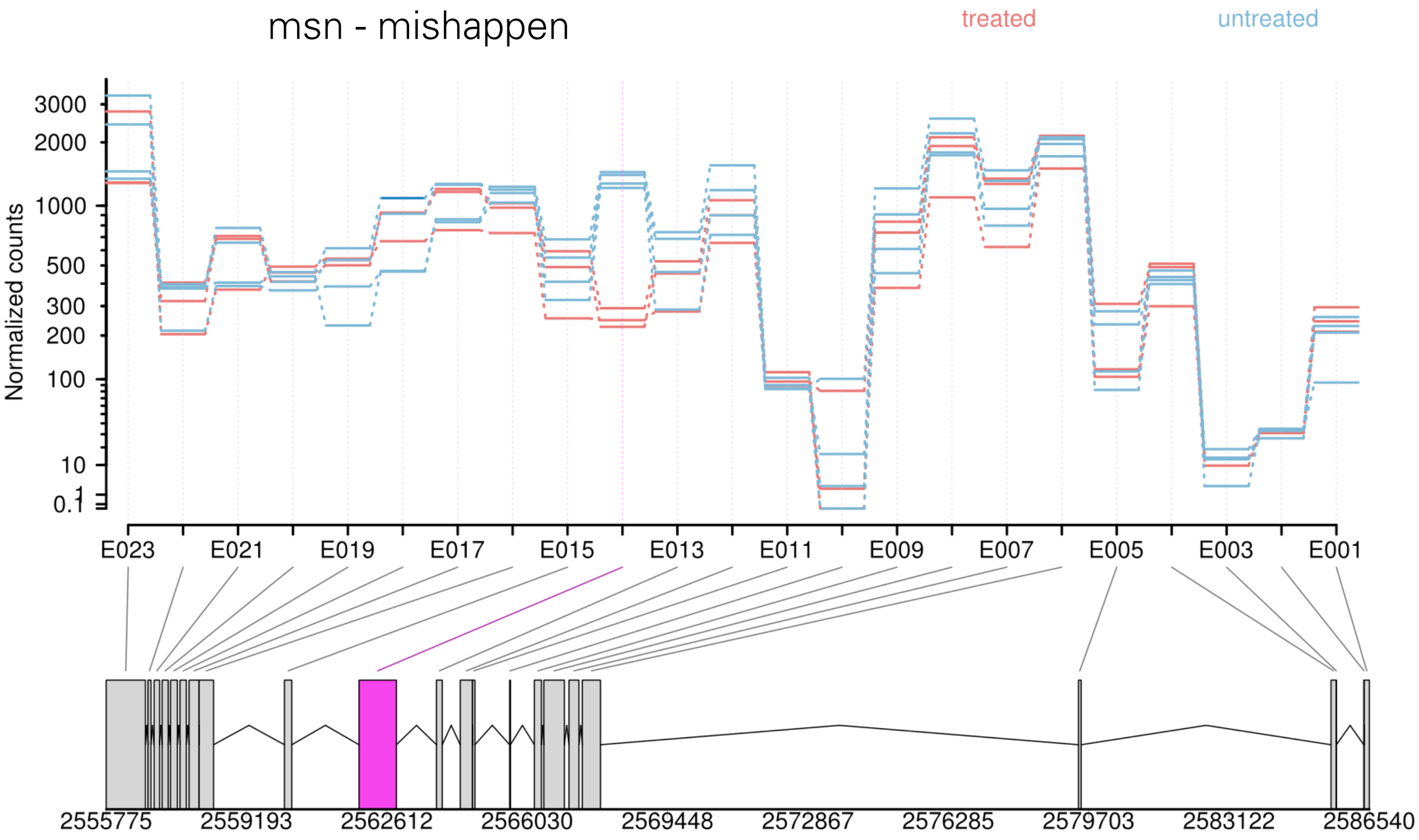
Anders, Reyes and Huber.
Genome Research 2012

Counting rules

- Alignment vs genome
- Uniquely aligned reads



Differential exon usage



DEXSeq

test for changes in the (relative) usage of exons:

$$\frac{\text{number of reads mapping to the exon}}{\text{number of reads mapping to the other exons of the same gene}}$$

DEXSeq

$$K_{ijl} \sim \mathrm{NB}(s_j \mu_{ijl}, \alpha_{il})$$

counts in gene *i*, sample *j*, exon *l*

size factor

dispersion

$$\log \mu_{ijl} = \beta_i^0 + \beta_{il}^{\mathrm{E}} x_l^{\mathrm{E}} + \beta_{ij}^{\mathrm{T}} x_j^{\mathrm{T}} + \beta_{ijl}^{\mathrm{ET}} x_l^{\mathrm{E}} x_j^{\mathrm{T}}$$

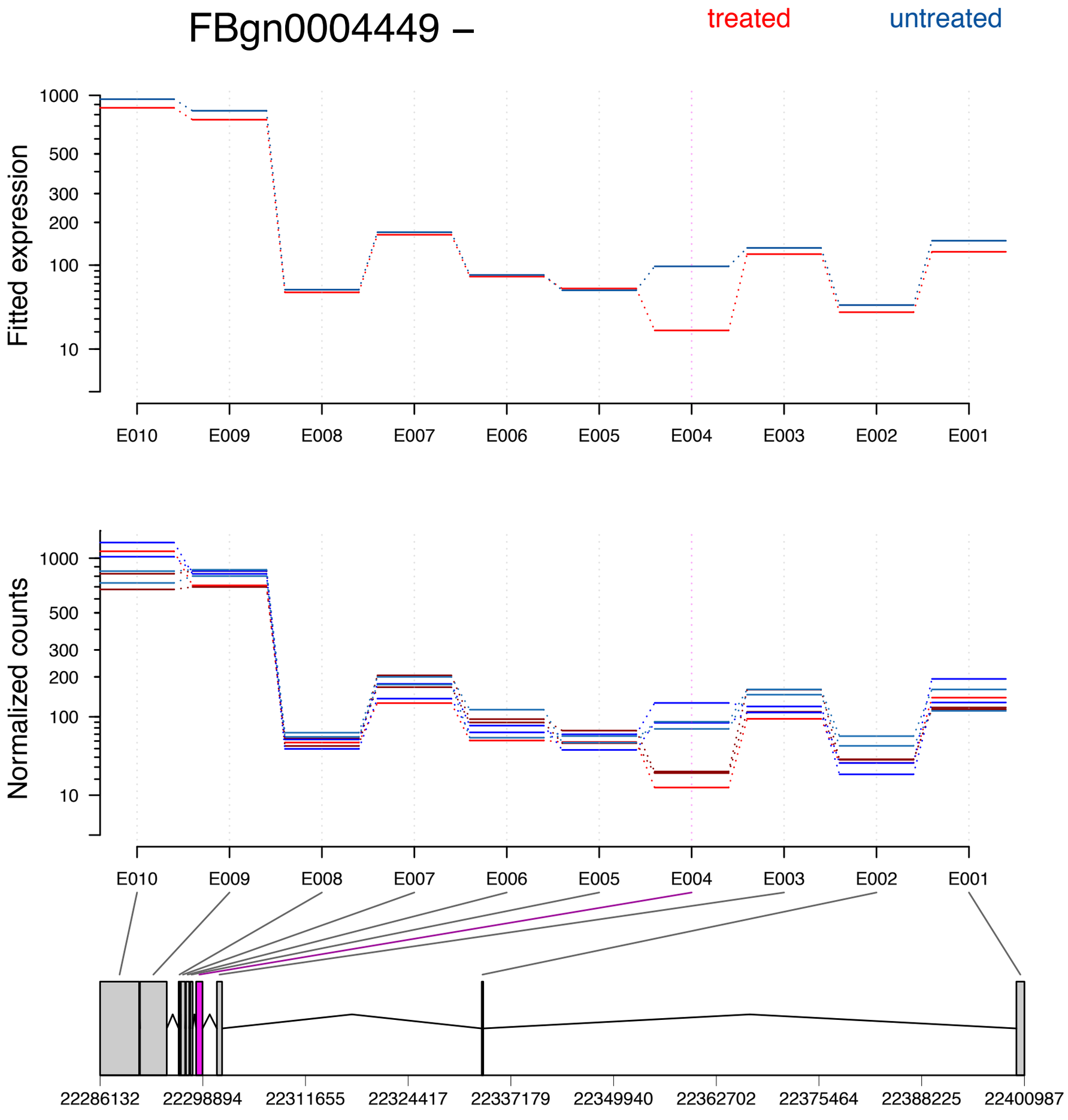
expression strength in control

fraction of reads falling onto exon *l* in control

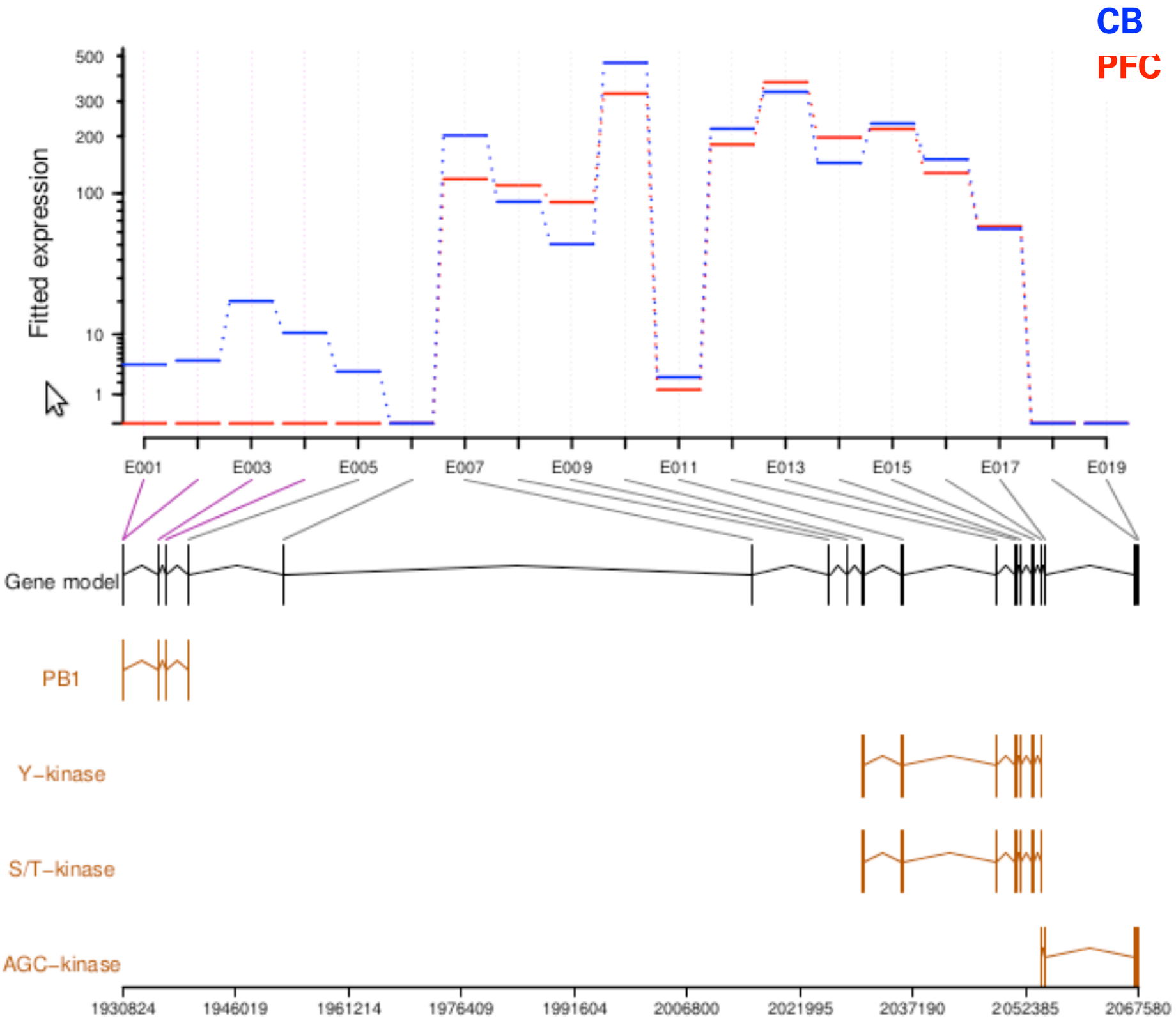
change in expression of gene *i* due to treatment

change to fraction of reads for exon *l* due to treatment

Ten-m


FBgn0004449 –
treated
untreated
Fitted expression
1000
500
300
200
100
10
E010
E009
E008
E007
E006
E005
E004
E003
E002
E001
Normalized counts
1000
500
300
200
100
10
E010
E009
E008
E007
E006
E005
E004
E003
E002
E001
22286132
22298894
22311655
22324417
22337179
22349940
22362702
22375464
22388225
22400987

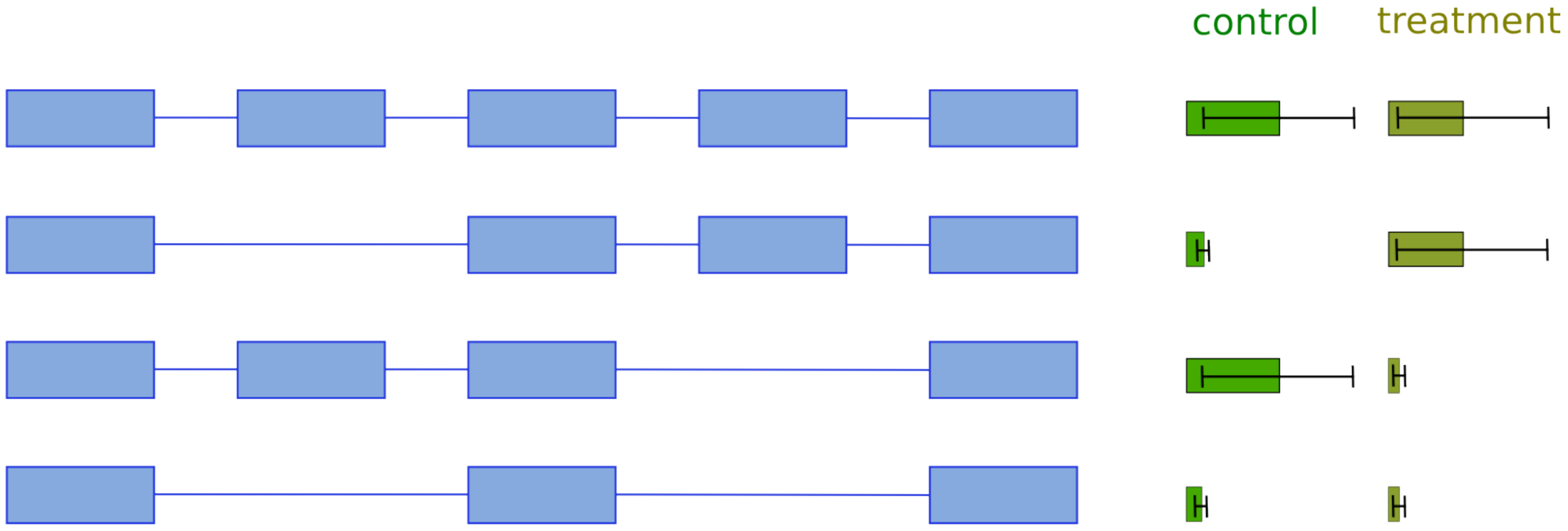
PKC ζ - PKM ζ



long form:
PKC-zeta

N-term.
truncated:
PKM-zeta

Why testing for differential exon usage rather than for isoform abundance changes?



control	90% in	50% in
treatment	10% in	50% in

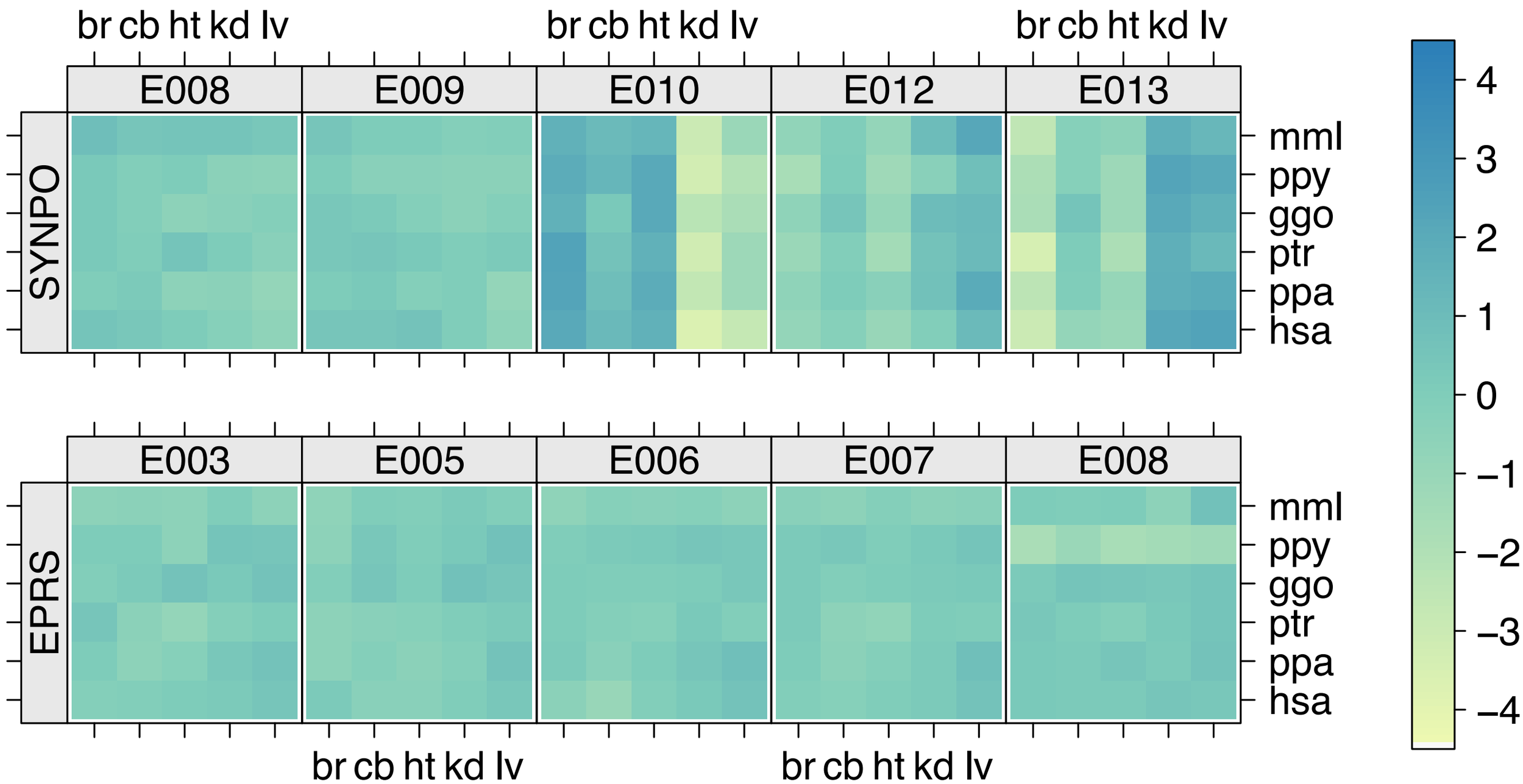
ARTICLE

doi:10.1038/nature10532

The evolution of gene expression levels in mammalian organs

David Brawand[1,2]*, Magali Soumillon[1,2]*, Anamaria Necsulea[1,2]*, Philippe Julien[1,2], Gábor Csárdi[2,3], Patrick Harrigan[4], Manuela Weier[1], Angélica Liechti[1], Ayinuer Aximu-Petri[5], Martin Kircher[5], Frank W. Albert[5]†, Ulrich Zeller[6], Philipp Khaitovich[7], Frank Grützner[8], Sven Bergmann[2,3], Rasmus Nielsen[4,9], Svante Pääbo[5] & Henrik Kaessmann[1,2]

- "Rate of gene expression evolution varies among organs, lineages and chromosomes, owing to differences in selective pressures"
- 9 species, 6 tissues, 2 individuals each: ~139 samples, ~414.145.196 high quality reads
- General goal: explore the functional and evolutionary aspect of the regulation of exon usage



Drift and conservation of differential exon usage across tissues in primate species

Alejandro Reyes[a,1], Simon Anders[a,1], Robert J. Weatheritt[b,2], Toby J. Gibson[b], Lars M. Steinmetz[a,c], and Wolfgang Huber[a,3]

PNAS 2013