More `limma` & multiple testing

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Announcements

- Midterm survey: Link posted in Discussion repo
  - Help us improve your learning experience!
  - Please fill out before Feb 14 at 11:59pm
- Projects: feedback from your project mentors is posted on your initial project proposals (see the issue in your team's repository)
  - finalized proposals due Friday

Learning objectives

- How to use `limma` to perform genome-wide differential expression testing on microarray data
- Understand the key differences between `limma` and `lm`
- Explain why multiple testing increases the number of errors we make by chance
- Be able to adjust for multiple comparisons by controlling the False Discovery Rate
    - e.g. using Benjamini-Hochberg or Storey's q-value

Recall: The hybrid estimator in `limma`

$$\tilde{s}_g^2 = \frac{d_0 s_0^2 + d s_g^2}{d_0 + d}$$

- recall that (s_0, d_0) are the *prior* parameters for σ_g^2 (random variable):

$$\frac{1}{\sigma_g^2} \sim \frac{1}{d_0 s_0^2} \chi_{d_0}^2$$

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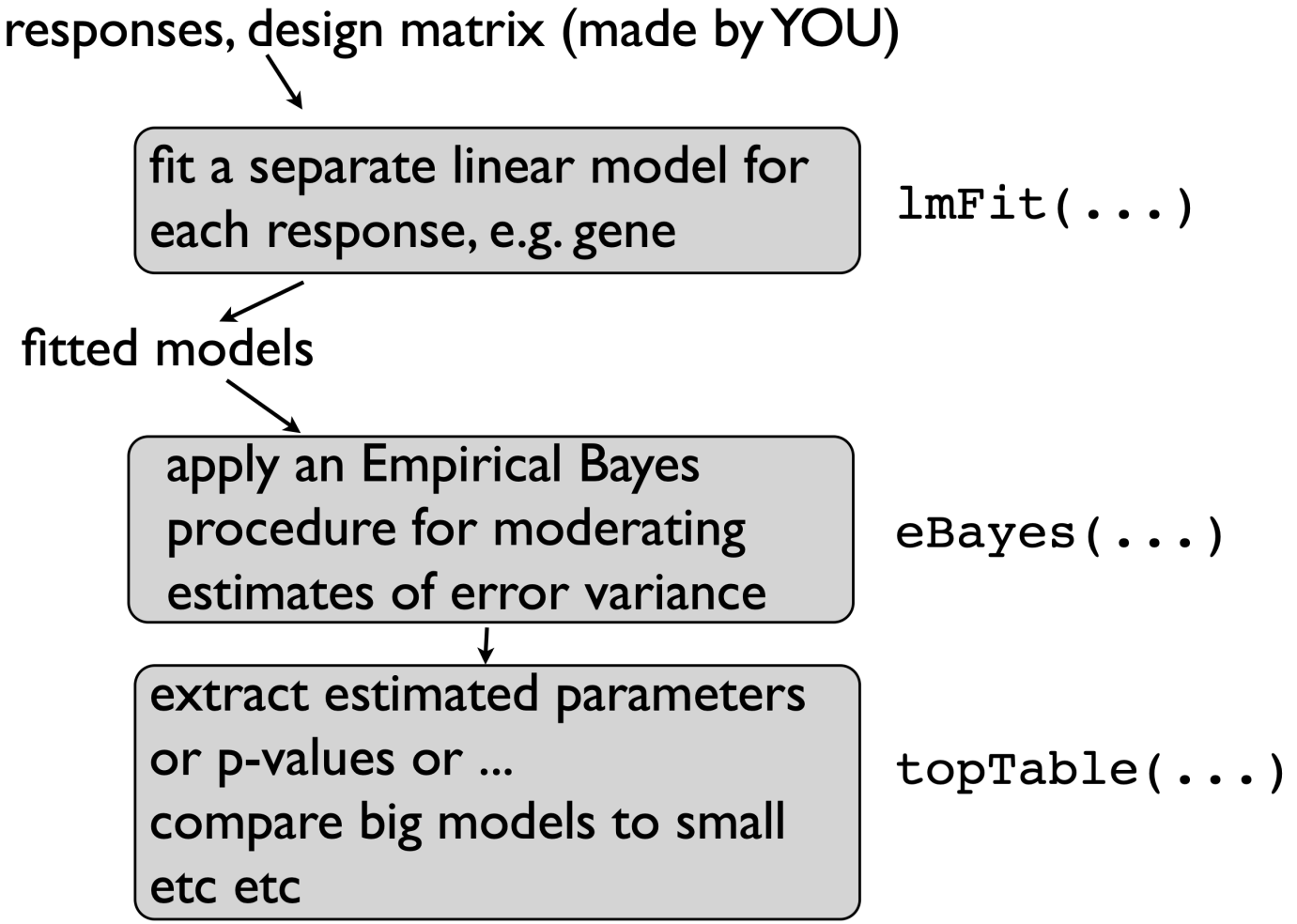
$$\frac{1}{\sigma_g^2} \sim \frac{1}{d_0 s_0^2} \chi^2_{d_0}$$

- the prior parameters incorporate information from all genes which allows us to shrink/nudge the gene-specific variances toward a common consensus
  - they are estimated from the data - the formulas for (s_0, d_0) and their derivations are beyond the scope of the course, but `limma` takes care of the details for us
  - note that (s_0, d_0) do not depend on g

Preview: `limma` workflow



Functions that make your life easier

Function	Description
`model.matrix`	Takes in your data frame and makes a design matrix
`limma::lmFit`	Fits the linear model to each gene separately - replace gene with "feature" depending on your data ('industrial scale' `lm`)
`limma::makeContrasts`	Create the contrast matrix C that you desire
`limma::contrast.fit`	Apply a contrast to your estimates
`limma::eBayes`	Use output of linear regression to compute moderated *t* statistics
`limma::topTable`	Query your results; sort your p-values; sort genes; Adjust for multiple comparisons
`limma::decideTests`	Identify which genes are significantly differentially expressed for each contrast

Getting help

Documentation

To view documentation for the version of this package installed in your system, start R and enter:

```
browseVignettes("limma")
```

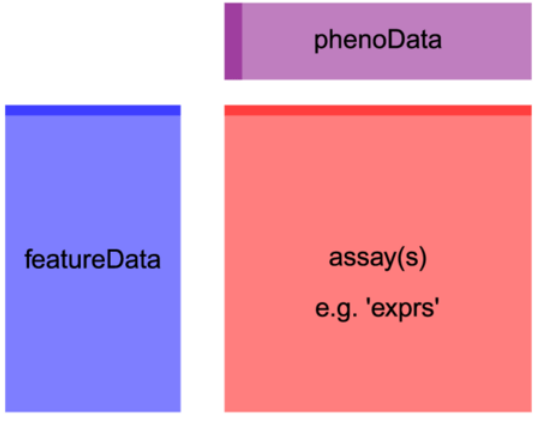
PDF		Limma One Page Introduction
PDF		usersguide.pdf
PDF		Reference Manual
Text		NEWS

- Bioconductor homepage for limma
- Bring up help pages for specific functions in RStudio, e.g. `?limma::topTable`

`limma` step one: `lmFit`

$$\mathbf{Y}_g = \mathbf{X}\boldsymbol{\alpha}_g + \boldsymbol{\varepsilon}_g,$$

- Within each gene observations are iid / constant variance
- `lmFit()` carries out multiple linear regression on each gene
- Usage: `lmFit(myDat, desMat)`
  - `myDat` is a data.frame or matrix with features in rows and samples in columns (*G* genes by *N* samples), or `ExpressionSet` object
  - `desMat` is a design matrix (output of `model.matrix(y ~ x)`; *N* samples by p parameters)



Bioconductor's ExpressionSet class

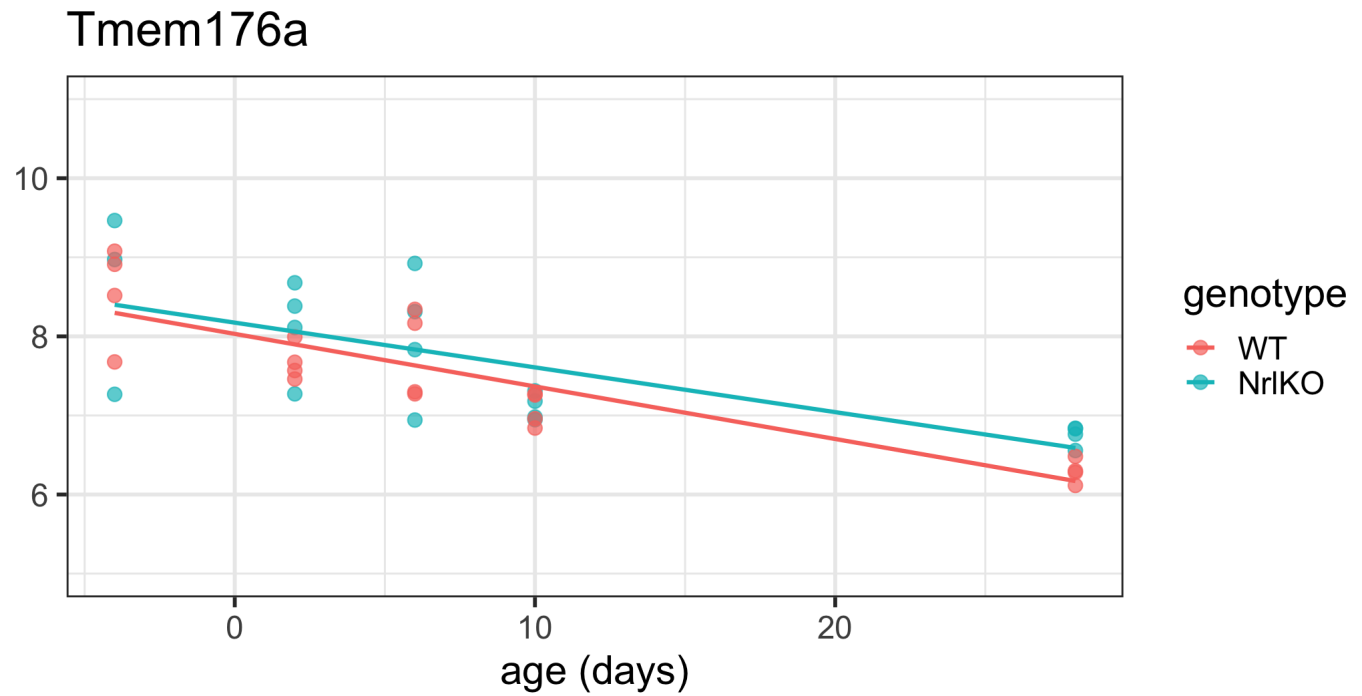
Let's run `limma` for the interactive model with age (continuous) and genotype (factor)

$$y_{ig} = \theta + \tau_{KO} x_{ig,KO} + \tau_{Age} x_{ig,Age} + \tau_{KO:Age} x_{ig,KO} x_{ig,Age}$$

- i indexes mouse, g indexes genes
- $x_{ig,KO}$ is the indicator variable for the NrlKO group
- $x_{ig,Age}$ is the continuous age variable

Interactive model with age and genotype

Example gene (but we want to fit this model on all genes):



Arranging input for `lmFit`: Bioconductor way

```
eset
```

```
## ExpressionSet (storageMode: lockedEnvironment)
## assayData: 45101 features, 39 samples
##   element names: exprs
## protocolData: none
## phenoData
##   sampleNames: GSM92610 GSM92611 ... GSM92648 (39 total)
##   varLabels: sample_id dev_stage genotype age
##   varMetadata: labelDescription
## featureData: none
## experimentData: use 'experimentData(object)'
##   pubMedIds: 16505381
## Annotation: GPL1261
```

Arranging input for `lmFit`: Separate expression & metadata

```
myDat <- assayData(eset)$exprs
myDat[1:5,1:5]
```

```
##             GSM92610 GSM92611  GSM92612 GSM92613  GSM92614
## 1415670_at   7.108863 7.322392  7.420947 7.351444  7.240428
## 1415671_at   9.714002 9.797742  9.831072 9.658442  9.708906
## 1415672_at   9.429030 9.846977 10.003092 9.914112 10.174548
## 1415673_at   8.426974 8.404206  8.594600 8.404206  8.835334
## 1415674_a_at 8.498338 8.458287  8.426651 8.372776  8.541722
```

```
myMeta <- pData(eset)
myMeta[1:5,]
```

```
##          sample_id dev_stage genotype age
## GSM92610  GSM92610        4W    NrlKO  28
## GSM92611  GSM92611        4W    NrlKO  28
## GSM92612  GSM92612        4W    NrlKO  28
## GSM92613  GSM92613        4W    NrlKO  28
## GSM92614  GSM92614       E16    NrlKO  -4
```

Formulating input for `lmFit`: Design Matrix

Bioconductor way:

```
desMat <- model.matrix(~ genotype*age,
                       data = pData(eset))
```

Equivalently, if using the separate way:

```
desMat <- model.matrix(~ genotype*age,
                       data = myMeta)
```

```
desMat
```

```
##           (Intercept) genotypeNrlKO age genotypeNrlKO:age
## GSM92610            1             1  28                28
## GSM92611            1             1  28                28
## GSM92612            1             1  28                28
## GSM92613            1             1  28                28
## GSM92614            1             1  -4                -4
## GSM92615            1             1  -4                -4
## GSM92616            1             1  -4                -4
## GSM92617            1             1  10                10
## GSM92618            1             1  10                10
## GSM92619            1             1  10                10
## GSM92620            1             1  10                10
## GSM92621            1             1   2                 2
## GSM92622            1             1   2                 2
## GSM92623            1             1   2                 2
## GSM92624            1             1   2                 2
## GSM92625            1             1   6                 6
## GSM92626            1             1   6                 6
## GSM92627            1             1   6                 6
## GSM92628            1             1   6                 6
## GSM92629            1             0  28                 0
## GSM92630            1             0  28                 0
## GSM92631            1             0  28                 0
## GSM92632            1             0  28                 0
## GSM92633            1             0  -4                 0
## GSM92634            1             0  -4                 0
## GSM92635            1             0  -4                 0
## GSM92636            1             0  -4                 0
## GSM92637            1             0  10                 0
## GSM92638            1             0  10                 0
## GSM92639            1             0  10                 0
```

Computation is fast

```
system.time(gFit <- lmFit(eset,
                          model.matrix(~ genotype*age, data = pData(eset))))
```

```
##    user  system elapsed
##   0.108   0.029   0.140
```

- Equivalently, using the 'separate' way:

```
gFit <- lmFit(myDat,
          model.matrix(~ genotype*age, data = myMeta))
```

- Using `lmFit` to fit an interactive model on 45K probesets takes a fraction of a second

Computation is fast

```
system.time(gFit <- lmFit(eset,
                          model.matrix(~ genotype*age, data = pData(eset))))
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##    user  system elapsed
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- Equivalently, using the 'separate' way:

```
gFit <- lmFit(myDat,
          model.matrix(~ genotype*age, data = myMeta))
```

- Using `lmFit` to fit an interactive model on 45K probesets takes a fraction of a second
- The time-intensive parts of an analysis lie in selecting the model and covariates, choosing how to parameterize it, and interpreting the output

Output of `lmFit`

```
summary(gFit)
```

```
##                  Length Class  Mode
## coefficients     180404 -none- numeric
## rank                  1 -none- numeric
## assign                4 -none- numeric
## qr                    5 qr     list
## df.residual       45101 -none- numeric
## sigma             45101 -none- numeric
## cov.coefficients     16 -none- numeric
## stdev.unscaled   180404 -none- numeric
## pivot                 4 -none- numeric
## Amean             45101 -none- numeric
## method                1 -none- character
## design              156 -none- numeric
```

```
nrow(eset)
```

```
## Features
##    45101
```

```
nrow(eset)*4
```

```
## Features
##   180404
```

Output of `lmFit`

```
summary(gFit)
```

```
##                 Length Class  Mode
## coefficients    180404 -none- numeric
## rank                 1 -none- numeric
## assign               4 -none- numeric
## qr                   5 qr     list
## df.residual      45101 -none- numeric
## sigma            45101 -none- numeric
## cov.coefficients    16 -none- numeric
## stdev.unscaled  180404 -none- numeric
## pivot                4 -none- numeric
## Amean            45101 -none- numeric
## method               1 -none- character
## design             156 -none- numeric
```

```
nrow(eset)
```

```
## Features
##    45101
```

```
nrow(eset)*4
```

```
## Features
##   180404
```

- OK... but where are the shrunken variable estimates?? How do I pull out p-values??
- Actually, we haven't carried out the empirical Bayesian computation yet -- need to run `eBayes()`!

Moderated *t*-tests using eBayes

```
summary(gFit)
```

```
##                  Length Class  Mode
## coefficients     180404 -none- numeric
## rank                  1 -none- numeric
## assign                4 -none- numeric
## qr                    5 qr     list
## df.residual       45101 -none- numeric
## sigma             45101 -none- numeric
## cov.coefficients     16 -none- numeric
## stdev.unscaled   180404 -none- numeric
## pivot                 4 -none- numeric
## Amean             45101 -none- numeric
## method                1 -none- character
## design              156 -none- numeric
```

```
summary(ebFit <- eBayes(gFit))
```

```
##                  Length Class  Mode
## coefficients     180404 -none- numeric
## rank                  1 -none- numeric
## assign                4 -none- numeric
## qr                    5 qr     list
## df.residual       45101 -none- numeric
## sigma             45101 -none- numeric
## cov.coefficients     16 -none- numeric
## stdev.unscaled   180404 -none- numeric
## pivot                 4 -none- numeric
## Amean             45101 -none- numeric
## method                1 -none- character
## design              156 -none- numeric
## df.prior              1 -none- numeric
## s2.prior              1 -none- numeric
## var.prior             4 -none- numeric
## proportion            1 -none- numeric
## s2.post           45101 -none- numeric
## t                180404 -none- numeric
## df.total          45101 -none- numeric
## p.value          180404 -none- numeric
## lods             180404 -none- numeric
## F                 45101 -none- numeric
## F.p.value         45101 -none- numeric
```

Components of the empirical Bayes estimators

math	plain english	`limma`	numerical result	also in `lm`?
s_g^2	gene-specific residual variance	`gFit$sigma^2`	45K numbers	✓
d	residual degrees of freedom $(n - p)$	`gFit$df.residual`	$39 - 4 = 35^*$	✓
s_0^2	mean of inverse χ^2 prior for s_g^2	`ebFit$s2.prior`	0.048	
d_0	degrees of freedom for the prior	`ebFit$df.prior`	2.61	
$\tilde{s}_g^2$	posterior mean of s_g^2 (i.e. moderated residual variance)	`ebFit$s2.post`	45K numbers	

* limma can handle more complicated models where this is not the same for each gene, so this is actually a vector of 45K copies of the number 35

topTable() will help us extract relevant output in a convenient format!

```
topTable(fit, coef=NULL, number=10, genelist=fit$genes, adjust.method="BH",
         sort.by="B", resort.by=NULL, p.value=1, lfc=0, confint=FALSE)
```

fit	list containing a linear model fit produced by lmFit, lm.series, gls.series or mrlm. For topTable, fit should be an object of class MArrayLM as produced by lmFit and eBayes.
coef	column number or column name specifying which coefficient or contrast of the linear model is of interest. For topTable, can also be a vector of column subscripts, in which case the gene ranking is by F-statistic for that set of contrasts.
number	maximum number of genes to list
adjust.method	method used to adjust the p-values for multiple testing. Options, in increasing conservatism, include "none", "BH", "BY" and "holm". See p.adjust for the complete list of options. A NULL value will result in the default adjustment method, which is "BH".
sort.by	character string specifying statistic to rank genes by. Possible values for topTable and toptable are "logFC", "AveExpr", "t", "P", "p", "B" or "none". (Permitted synonyms are "M" for "logFC", "A" or "Amean" for "AveExpr", "T" for "t" and "p" for "P".) Possibilities for topTableF are "F" or "none". Possibilities for topTreat are as for topTable except for "B".

... (truncated - see ?topTable for full listing)

Summary of `topTable` function

- `coef` is the argument where you specify the coefficient(s) you want to test for equality with zero (default is NULL; must be specified)
- `number` lets you control size of hit list (default is 10)
- `p.value` lets you specify a minimum adjusted p-value cutoff (default is 1)
- `lfc` lets you specify a minimum observed effect size - log2 fold change (default is 0)
- `sort.by` and `resort.by` give control over the ordering (default is by "B": log-odds that the gene is differentially expressed)
- `adjust.method` specifies how/if to adjust p-values for multiple testing (default is BH)

topTable in action: genotypeNrlKO

```
topTable(ebFit, coef = "genotypeNrlKO")
```

```
##                   logFC  AveExpr          t      P.Value    adj.P.Val        B
## 1450946_at   -4.7134891 8.733103 -15.583683 5.390931e-18 2.431364e-13 28.40938
## 1442070_at   -0.9669400 8.268122  -9.787407 6.896220e-12 1.555132e-07 16.46086
## 1443457_at   -1.1530057 6.049428  -9.091412 4.947029e-11 6.029428e-07 14.68843
## 1422679_s_at -2.1187394 9.495435  -9.002625 6.388954e-11 6.029428e-07 14.45703
## 1431708_a_at -2.1610625 8.591450  -8.967950 7.062007e-11 6.029428e-07 14.36634
## 1433050_at   -1.5083887 8.468891  -8.923932 8.021234e-11 6.029428e-07 14.25096
## 1426288_at   -4.2100657 9.323796  -8.772209 1.246491e-10 8.031144e-07 13.85106
## 1418108_at    0.8662091 8.260647   8.537984 2.475294e-10 1.395478e-06 13.22713
## 1450770_at    1.3103576 7.357033   8.128847 8.332369e-10 3.747976e-06 12.11870
## 1457802_at   -0.8362030 7.778189  -8.117112 8.629984e-10 3.747976e-06 12.08657
```

- `topTable(ebFit, coef = 2)` is equivalent here, but much less informative!!
- What is the null hypothesis here?

topTable in action: genotypeNrlKO

```
topTable(ebFit, coef = "genotypeNrlKO")
```

```
##                   logFC  AveExpr          t      P.Value    adj.P.Val        B
## 1450946_at   -4.7134891 8.733103 -15.583683 5.390931e-18 2.431364e-13 28.40938
## 1442070_at   -0.9669400 8.268122  -9.787407 6.896220e-12 1.555132e-07 16.46086
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## 1422679_s_at -2.1187394 9.495435  -9.002625 6.388954e-11 6.029428e-07 14.45703
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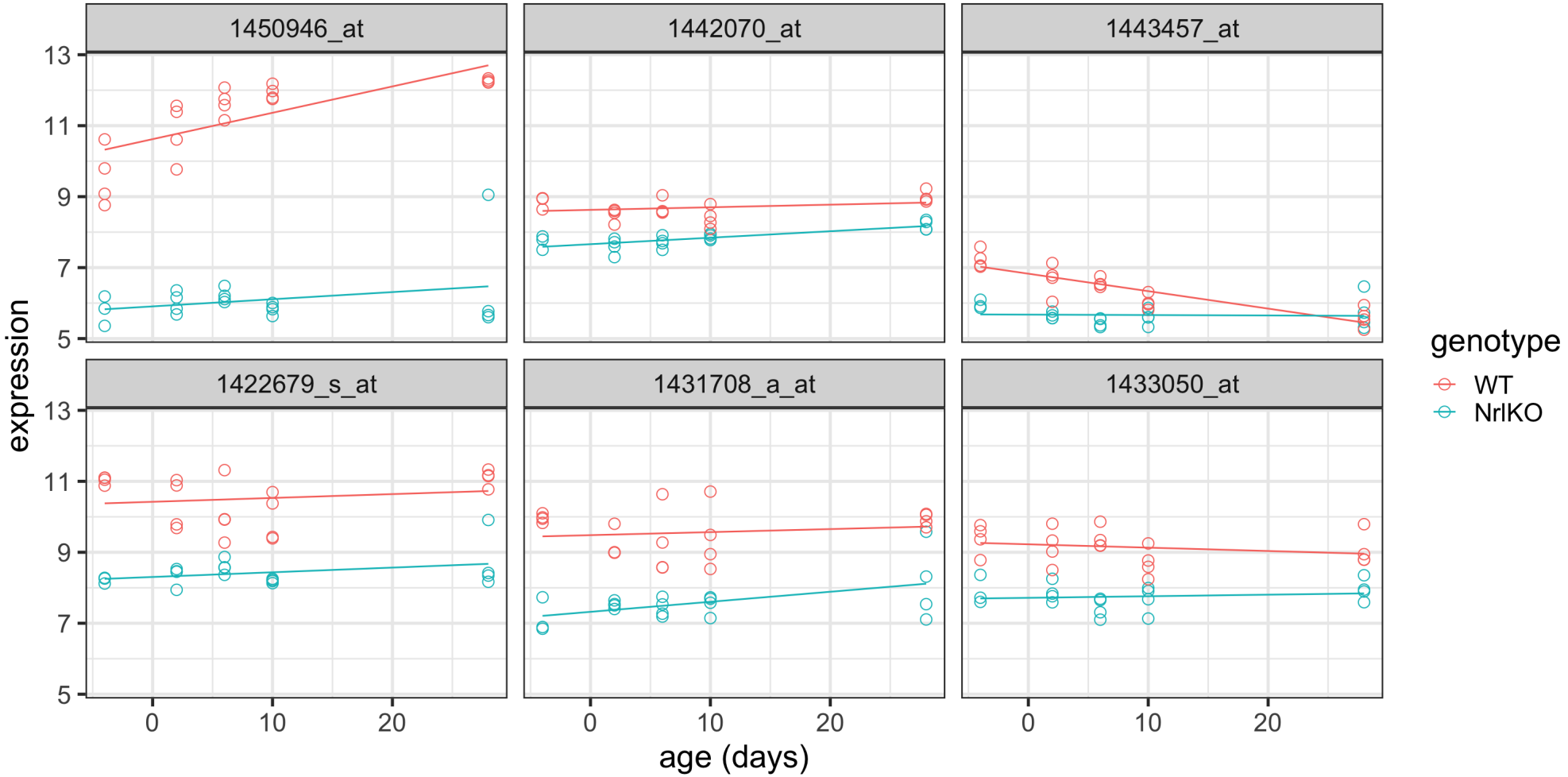
## 1433050_at   -1.5083887 8.468891  -8.923932 8.021234e-11 6.029428e-07 14.25096
## 1426288_at   -4.2100657 9.323796  -8.772209 1.246491e-10 8.031144e-07 13.85106
## 1418108_at    0.8662091 8.260647   8.537984 2.475294e-10 1.395478e-06 13.22713
## 1450770_at    1.3103576 7.357033   8.128847 8.332369e-10 3.747976e-06 12.11870
## 1457802_at   -0.8362030 7.778189  -8.117112 8.629984e-10 3.747976e-06 12.08657
```

- `topTable(ebFit, coef = 2)` is equivalent here, but much less informative!!
- What is the null hypothesis here?
  - $H_0 : \tau_{KO} = 0$; this finds genes where the KO differs from WT *when age is zero*

Plotting the top 6 probes for `genotypeNrlKO`



topTable in action: age

```
topTable(ebFit, coef = "age")
```

```
##                   logFC  AveExpr        t      P.Value    adj.P.Val        B
## 1451635_at   0.19294061 7.791237 20.78935 3.204717e-22 1.445359e-17 40.60768
## 1425222_x_at 0.17604111 6.514475 19.72633 1.962954e-21 4.426561e-17 38.79502
## 1433699_at   0.14875031 6.939973 17.80467 6.442530e-20 7.957605e-16 35.29412
## 1452243_at   0.15889179 6.840975 17.75658 7.057586e-20 7.957605e-16 35.20253
## 1435436_at   0.13067119 8.274676 17.61808 9.187120e-20 8.286966e-16 34.93763
## 1458418_at   0.21277027 6.270187 16.43686 9.326171e-19 7.006477e-15 32.60718
## 1424977_at   0.07814988 6.505464 16.36079 1.087456e-18 7.006477e-15 32.45260
## 1431174_at   0.16158579 7.418000 16.01901 2.183055e-18 1.230725e-14 31.75106
## 1421818_at   0.11250919 7.982278 15.66666 4.531133e-18 2.270651e-14 31.01562
## 1419069_at   0.09325234 8.222096 15.54775 5.813459e-18 2.621928e-14 30.76457
```

- `topTable(ebFit, coef = 3)` is equivalent here, but much less informative!!
- What is the null hypothesis here?

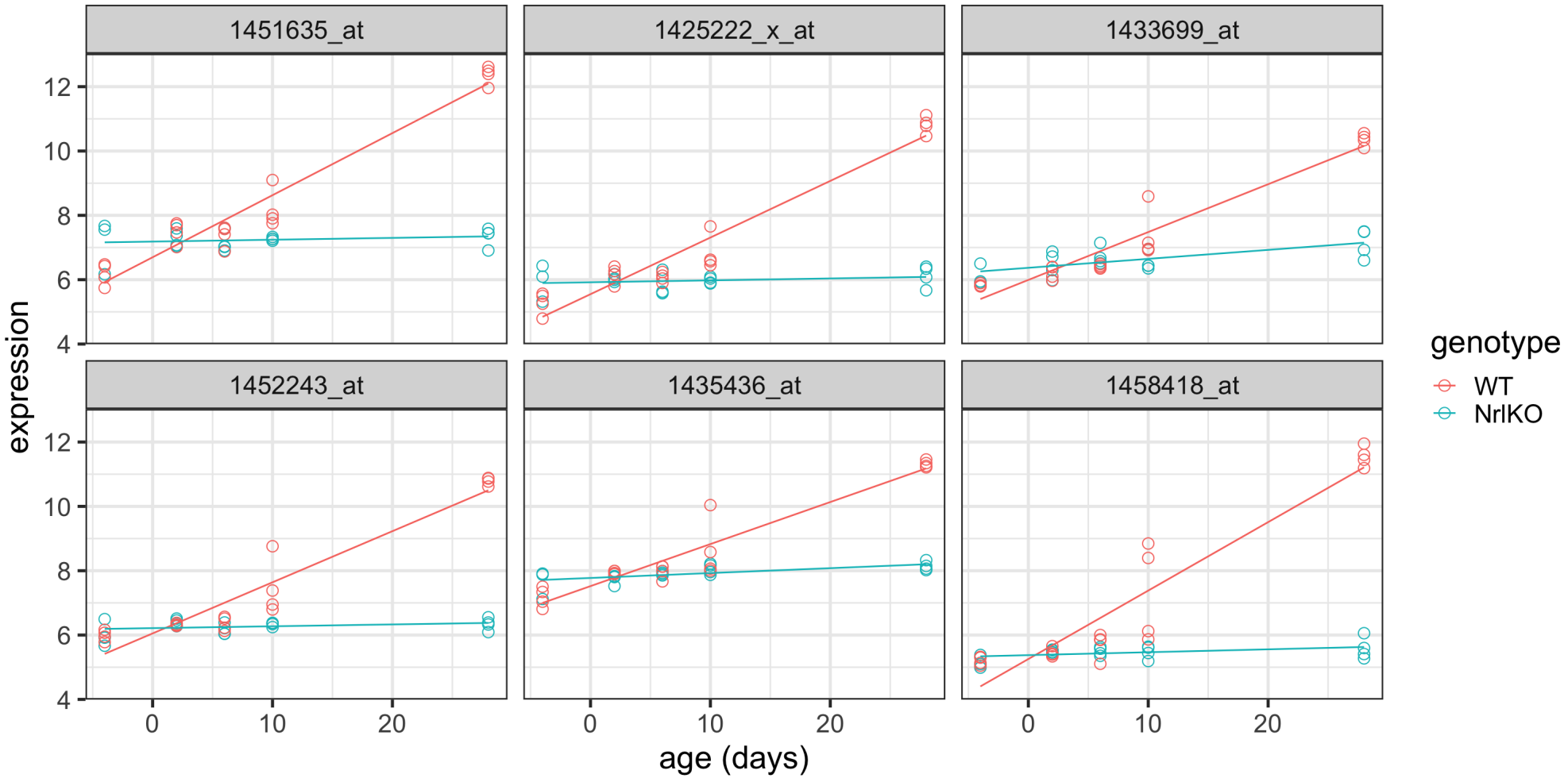
topTable in action: age

```
topTable(ebFit, coef = "age")
```

```
##                   logFC  AveExpr        t      P.Value    adj.P.Val        B
## 1451635_at   0.19294061 7.791237 20.78935 3.204717e-22 1.445359e-17 40.60768
## 1425222_x_at 0.17604111 6.514475 19.72633 1.962954e-21 4.426561e-17 38.79502
## 1433699_at   0.14875031 6.939973 17.80467 6.442530e-20 7.957605e-16 35.29412
## 1452243_at   0.15889179 6.840975 17.75658 7.057586e-20 7.957605e-16 35.20253
## 1435436_at   0.13067119 8.274676 17.61808 9.187120e-20 8.286966e-16 34.93763
## 1458418_at   0.21277027 6.270187 16.43686 9.326171e-19 7.006477e-15 32.60718
## 1424977_at   0.07814988 6.505464 16.36079 1.087456e-18 7.006477e-15 32.45260
## 1431174_at   0.16158579 7.418000 16.01901 2.183055e-18 1.230725e-14 31.75106
## 1421818_at   0.11250919 7.982278 15.66666 4.531133e-18 2.270651e-14 31.01562
## 1419069_at   0.09325234 8.222096 15.54775 5.813459e-18 2.621928e-14 30.76457
```

- `topTable(ebFit, coef = 3)` is equivalent here, but much less informative!!
- What is the null hypothesis here?
  - $H_0 : \tau_{Age} = 0$; this finds genes where age significantly affects gene expression *for WT*

Plotting the top 6 genes for age



topTable in action: genotypeNrlKO:age

```
topTable(ebFit, coef = "genotypeNrlKO:age")
```

```
##                   logFC  AveExpr         t      P.Value    adj.P.Val        B
## 1451635_at   -0.1871800 7.791237 -14.00463 1.684302e-16 7.596369e-12 27.52010
## 1425222_x_at -0.1700908 6.514475 -13.23451 9.961393e-16 2.246344e-11 25.74877
## 1441809_at    0.1336356 6.649440  12.55702 5.037221e-15 7.572790e-11 24.13110
## 1452243_at   -0.1531326 6.840975 -11.88284 2.670693e-14 3.011273e-10 22.46414
## 1431174_at   -0.1655894 7.418000 -11.39882 9.161231e-14 8.263614e-10 21.23123
## 1458418_at   -0.2037314 6.270187 -10.92850 3.122990e-13 2.347500e-09 20.00384
## 1435436_at   -0.1154500 8.274676 -10.80855 4.289363e-13 2.763637e-09 19.68613
## 1446715_at   -0.1409774 5.613122 -10.57909 7.912260e-13 4.325169e-09 19.07307
## 1442190_at   -0.1323038 5.896077 -10.54672 8.630965e-13 4.325169e-09 18.98601
## 1416306_at    0.1702059 6.560050  10.41285 1.238250e-12 5.584630e-09 18.62456
```

- `topTable(ebFit, coef = 4)` is equivalent here, but much less informative!!
- What is the null hypothesis here?

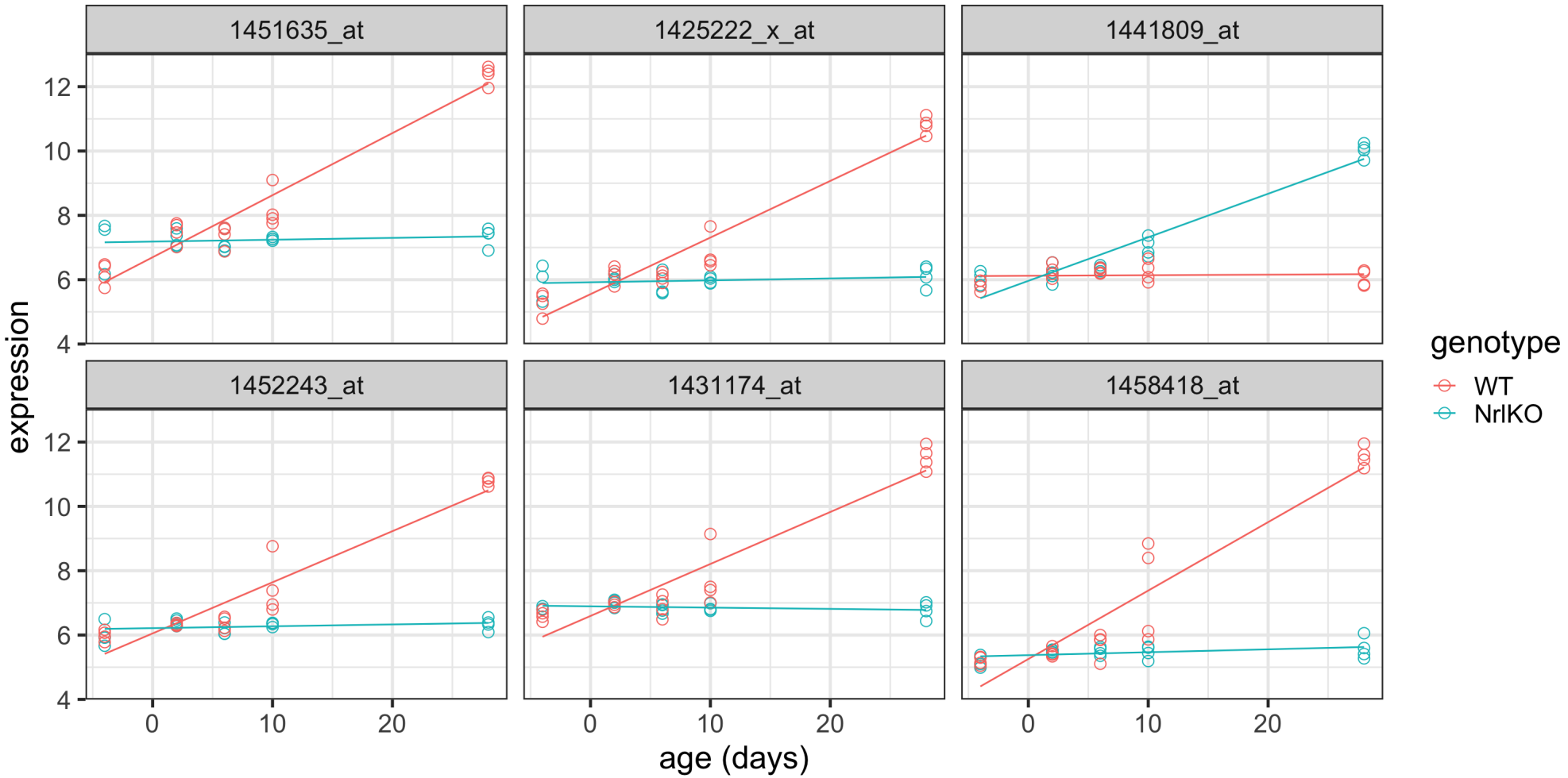
topTable in action: genotypeNrlKO:age

```
topTable(ebFit, coef = "genotypeNrlKO:age")
```

```
##                   logFC  AveExpr         t      P.Value    adj.P.Val        B
## 1451635_at   -0.1871800 7.791237 -14.00463 1.684302e-16 7.596369e-12 27.52010
## 1425222_x_at -0.1700908 6.514475 -13.23451 9.961393e-16 2.246344e-11 25.74877
## 1441809_at    0.1336356 6.649440  12.55702 5.037221e-15 7.572790e-11 24.13110
## 1452243_at   -0.1531326 6.840975 -11.88284 2.670693e-14 3.011273e-10 22.46414
## 1431174_at   -0.1655894 7.418000 -11.39882 9.161231e-14 8.263614e-10 21.23123
## 1458418_at   -0.2037314 6.270187 -10.92850 3.122990e-13 2.347500e-09 20.00384
## 1435436_at   -0.1154500 8.274676 -10.80855 4.289363e-13 2.763637e-09 19.68613
## 1446715_at   -0.1409774 5.613122 -10.57909 7.912260e-13 4.325169e-09 19.07307
## 1442190_at   -0.1323038 5.896077 -10.54672 8.630965e-13 4.325169e-09 18.98601
## 1416306_at    0.1702059 6.560050  10.41285 1.238250e-12 5.584630e-09 18.62456
```

- `topTable(ebFit, coef = 4)` is equivalent here, but much less informative!!
- What is the null hypothesis here?
  - $H_0 : \tau_{KO:Age} = 0$; this finds genes where the effect of age is significantly different in each genotype

Plotting the top 6 genes for `genotypeNrlKO:age`



topTable in action: any effect of genotype

```
topTable(ebFit, coef = c("genotypeNrlKO", "genotypeNrlKO:age")) %>% as_tibble()
```

```
## # A tibble: 10 × 6
##    genotypeNrlKO genotypeNrlKO.age AveExpr     F  P.Value adj.P.Val
##            <dbl>             <dbl>   <dbl> <dbl>    <dbl>     <dbl>
##  1        -4.71            -0.0541    8.73  247. 2.40e-22  1.08e-17
##  2         0.487           -0.187     7.79  130. 1.31e-17  2.18e-13
##  3        -0.989           -0.223     7.42  129. 1.45e-17  2.18e-13
##  4         0.370           -0.170     6.51  120. 4.72e-17  5.32e-13
##  5        -0.158            0.134     6.65  118. 6.59e-17  5.95e-13
##  6         0.428            0.170     6.56  113. 1.21e-16  9.11e-13
##  7         0.657            0.0617    5.86  111. 1.62e-16  1.00e-12
##  8         0.994            0.0423    7.37  111. 1.78e-16  1.00e-12
##  9         0.164           -0.153     6.84  106. 3.34e-16  1.68e-12
## 10        -0.682           -0.140     8.16  103. 5.77e-16  2.44e-12
```

- `topTable(ebFit, coef = c(2,4))` is equivalent here, but much less informative!!
- What is the null hypothesis here?

topTable in action: any effect of genotype

```
topTable(ebFit, coef = c("genotypeNrlKO", "genotypeNrlKO:age")) %>% as_tibble()
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```
## # A tibble: 10 × 6
##    genotypeNrlKO genotypeNrlKO.age AveExpr     F  P.Value adj.P.Val
##            <dbl>             <dbl>   <dbl> <dbl>    <dbl>     <dbl>
##  1        -4.71            -0.0541    8.73  247. 2.40e-22  1.08e-17
##  2         0.487           -0.187     7.79  130. 1.31e-17  2.18e-13
##  3        -0.989           -0.223     7.42  129. 1.45e-17  2.18e-13
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##  5        -0.158            0.134     6.65  118. 6.59e-17  5.95e-13
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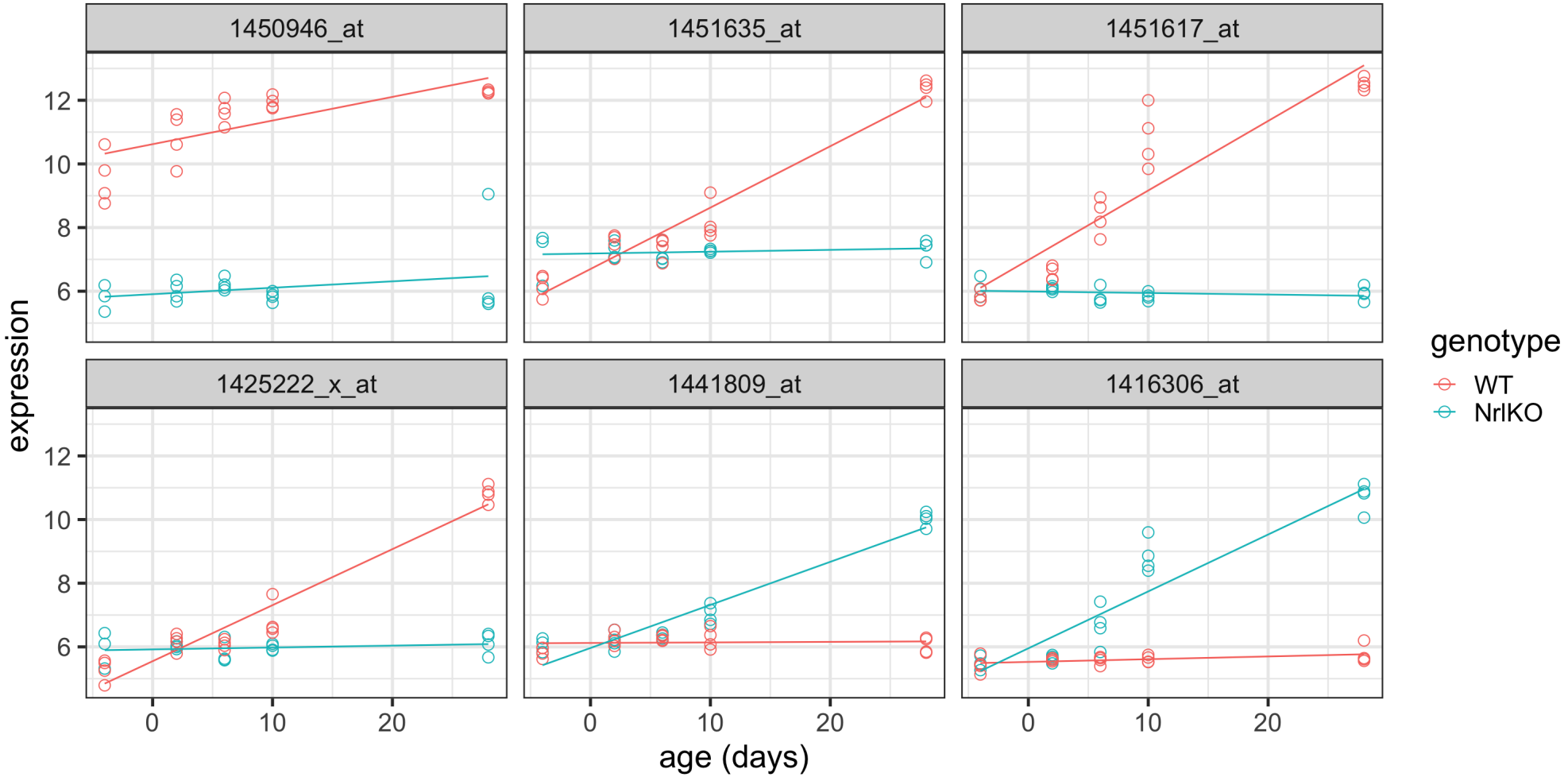
##  9         0.164           -0.153     6.84  106. 3.34e-16  1.68e-12
## 10        -0.682           -0.140     8.16  103. 5.77e-16  2.44e-12
```

- `topTable(ebFit, coef = c(2,4))` is equivalent here, but much less informative!!
- What is the null hypothesis here?
  - $H_0 : \tau_{KO} = \tau_{KO:Age} = 0$; this finds genes where any (additive and/or interaction) effect of genotype is significant

Plotting the top 6 genes for any effect of genotype



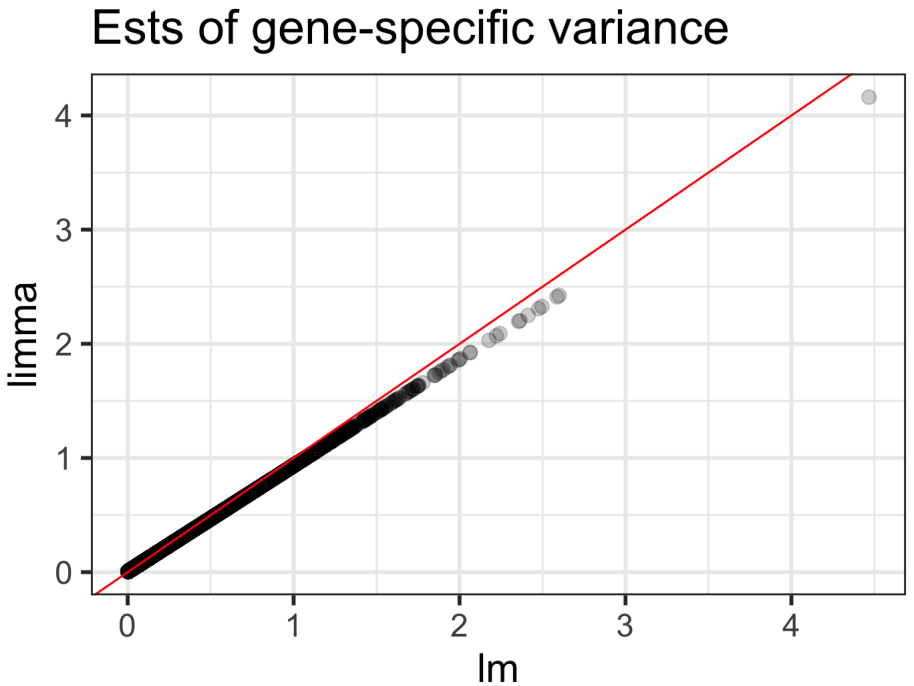
Comparison of s_g^2 and $\tilde{s}_g^2$ (shrinkage!)

Fill in the blank:

For large variances,
limma _____
the estimates.

(a) INCREASES

(b) DECREASES



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(a) INCREASES

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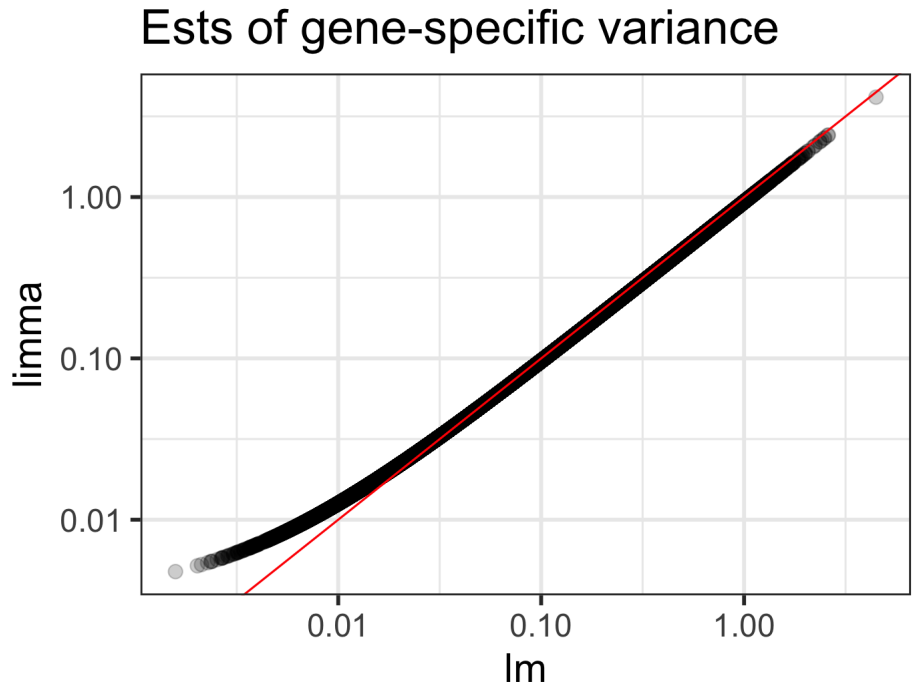
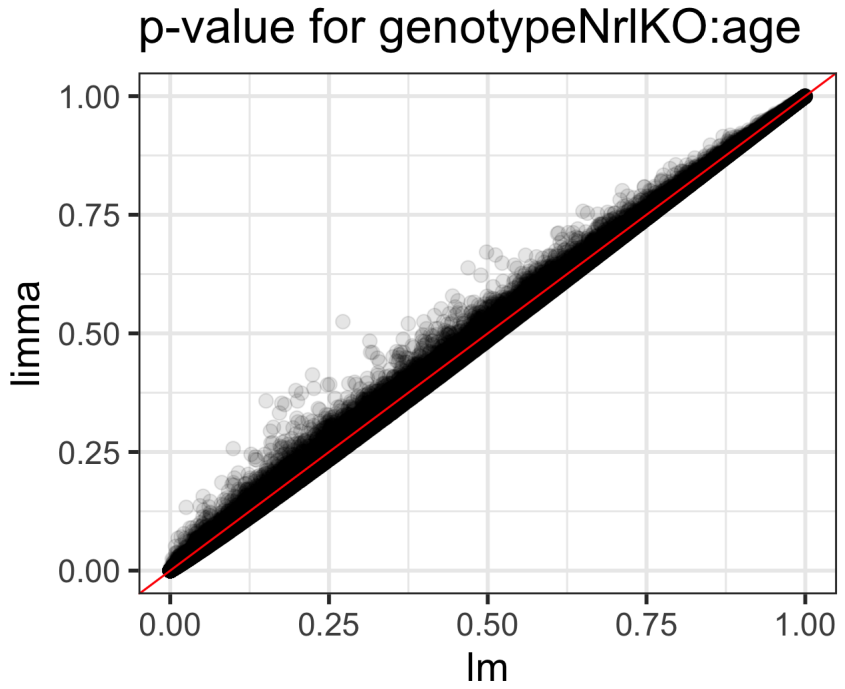


This plot is on the log-scale to 'zoom in' on the low range

Comparison of interaction coefficient p-values

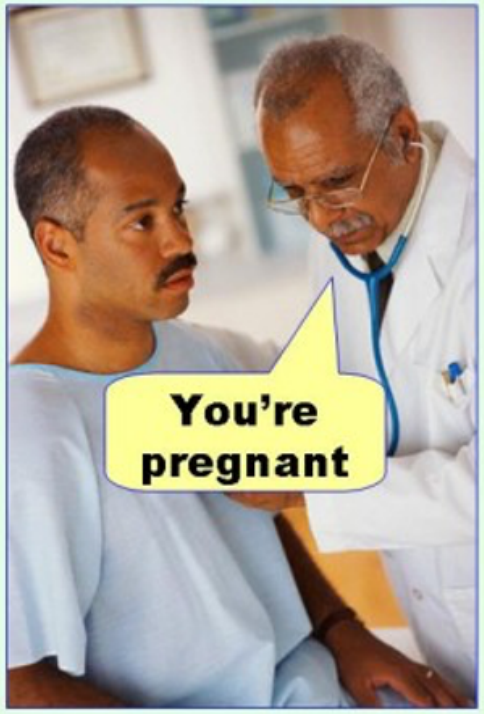


- 17479 genes where limma p-value is *larger* than lm
- 27622 genes where limma p-value is *smaller* than lm

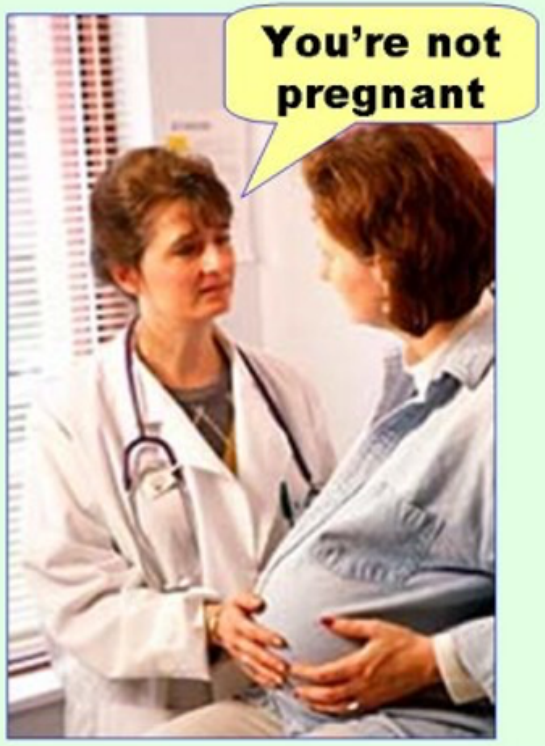
Multiple testing

Recall the two types of errors

Type I error
(false positive)



Type II error
(false negative)



Error rates

Decision	Actual Situation "Truth": H_0 True	H_0 False
Do Not Reject H_0	Correct Decision $1-\alpha$	Incorrect Decision Type II Error β
Reject H_0	Incorrect Decision Type I Error α	Correct Decision $1-\beta$

$$\alpha = P(\text{Type I Error}),\ \beta = P(\text{Type II Error}),\ \text{Power} = 1 - \beta$$

Type I Error rate for m tests

- $P(\text{incorrect decision}|H_0) = \alpha$
  - let $\alpha = 0.05$
- $P(\text{correct decision}|H_0) = 1 - \alpha = 0.95$

Type I Error rate for m tests

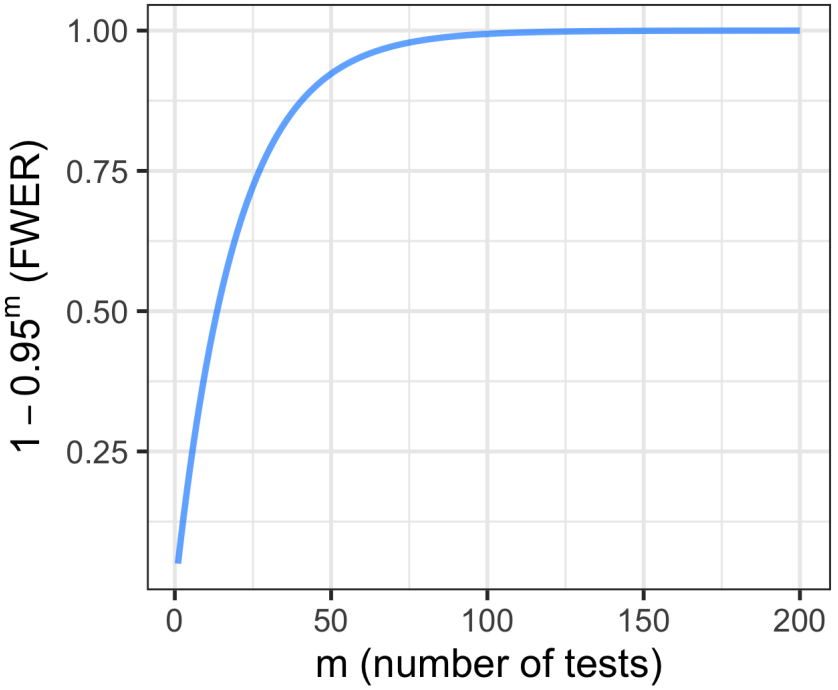
- $P(\text{incorrect decision}|H_0) = \alpha$
  - let $\alpha = 0.05$
- $P(\text{correct decision}|H_0) = 1 - \alpha = 0.95$
- $P(\text{correct decision on } m \text{ tests}|H_0) =$

$$(1 - \alpha)^m = 0.95^m$$

- $P(\text{at least one error on } m \text{ tests}|H_0) =$

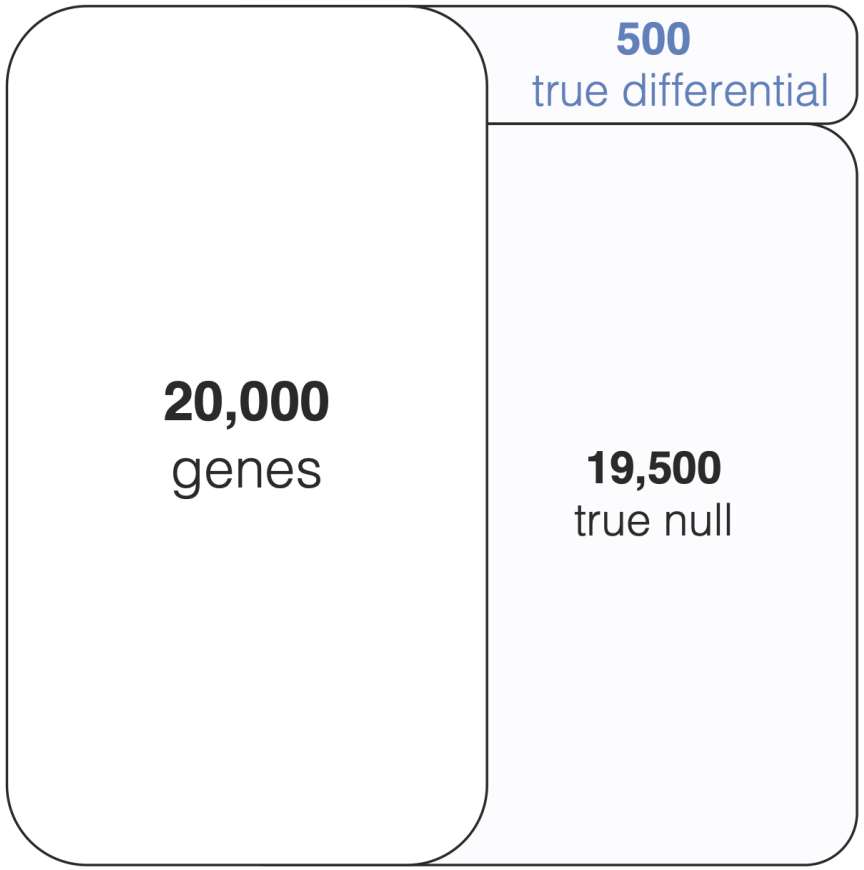
$$1 - (1 - \alpha)^m =$$

$$1 - 0.95^m = \alpha_{FWER}$$

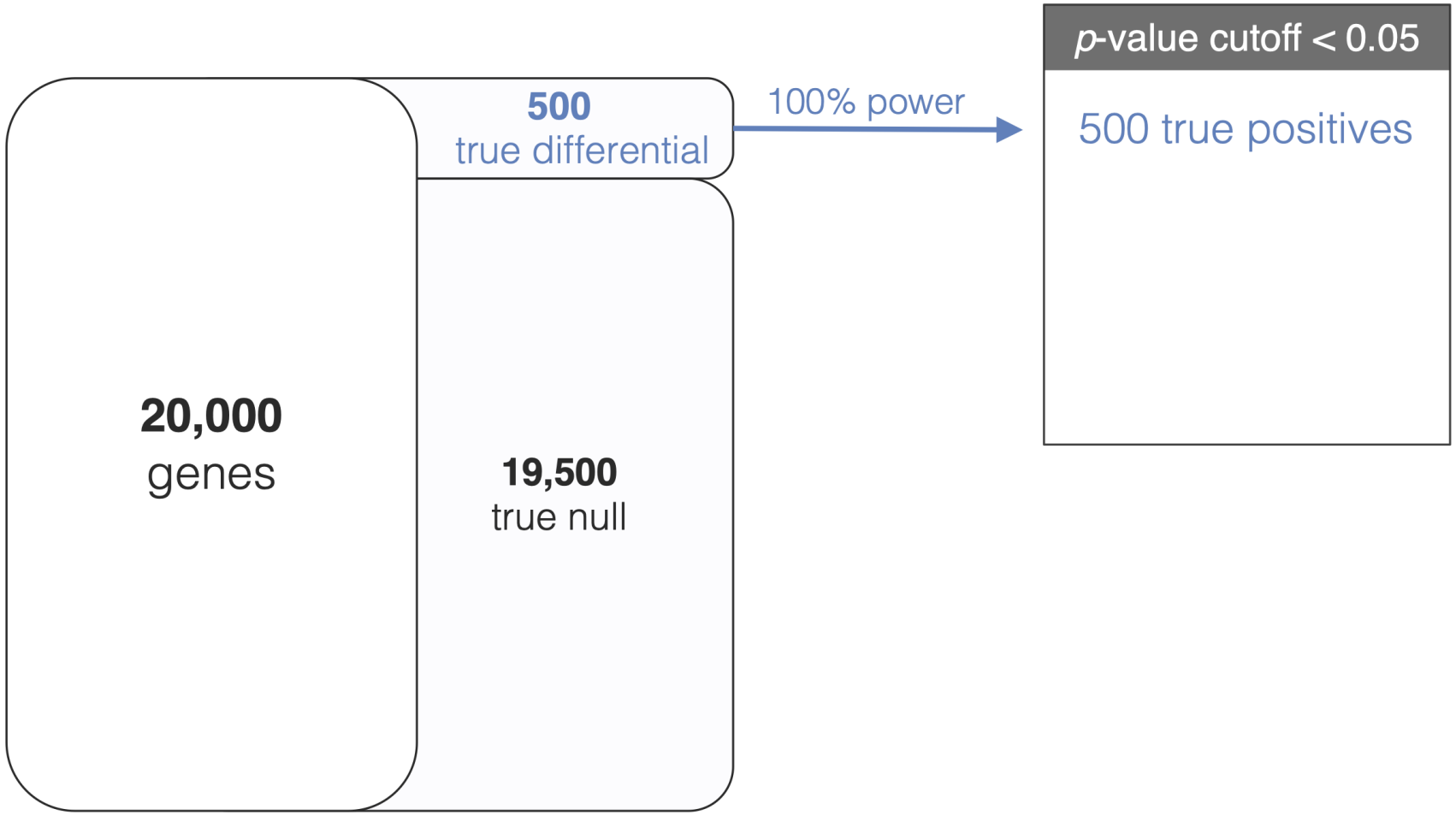


Multiple comparisons in genomics

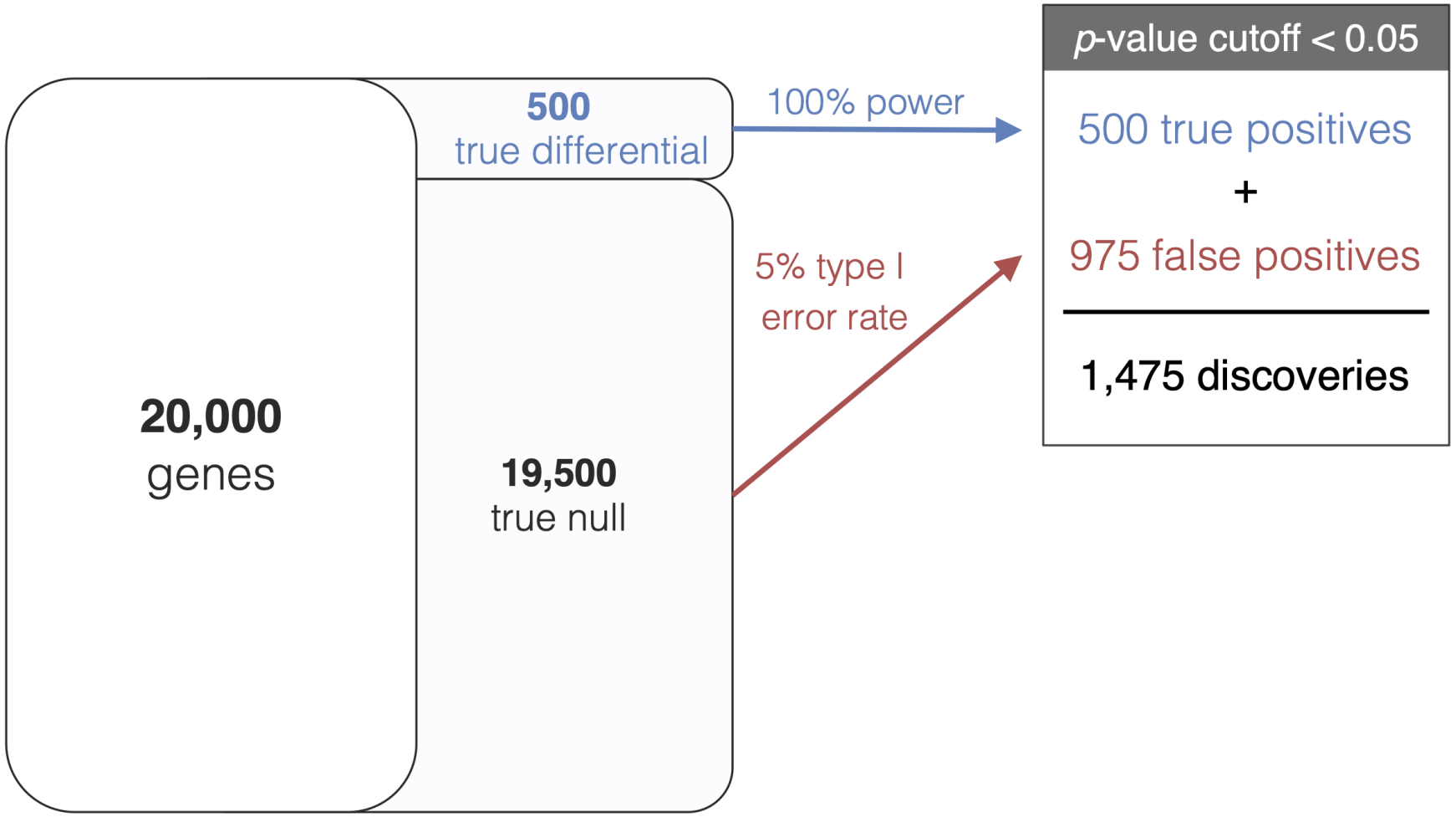
Multiple comparisons in genomics



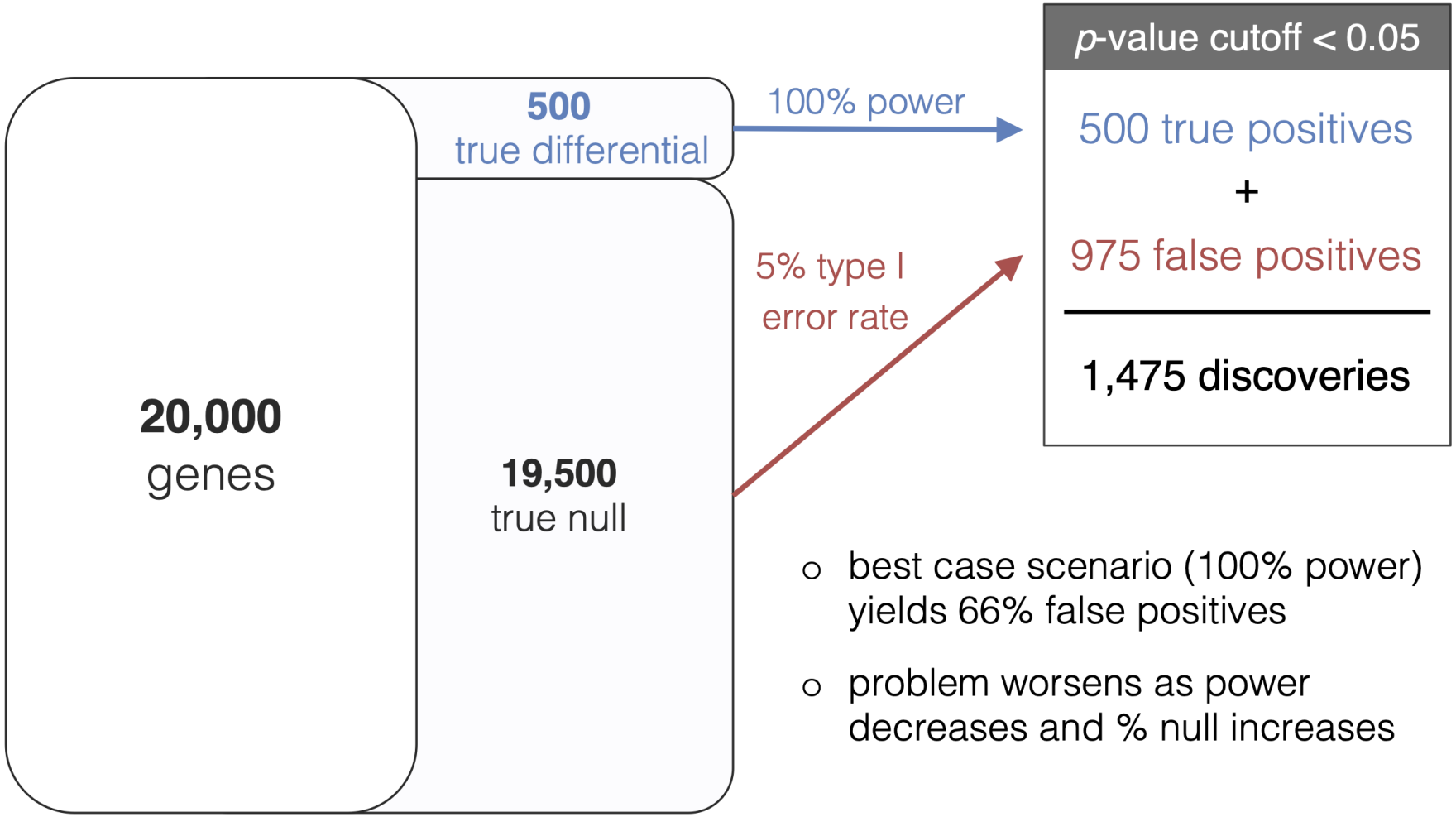
Multiple comparisons in genomics



Multiple comparisons in genomics



Multiple comparisons in genomics



- best case scenario (100% power) yields 66% false positives
- problem worsens as power decreases and % null increases

Family-Wise Error Rate (FWER)

- FWER is the probability of making at least one error when testing m tests

Family-Wise Error Rate (FWER)

- FWER is the probability of making at least one error when testing m tests
- Control the FWER: limit the probability of making at least one incorrect decision
- One example: the Bonferroni correction for $\alpha = 0.05$:

$$\text{If } P(\text{at least one error on } m \text{ tests}) < \alpha$$

$$\Rightarrow P(\text{at least one error on } m \text{ tests}) < \sum_{i=1}^{m} P(\text{error on test } i)$$

$$\sum_{i=1}^{m} P(\text{error on test } i) = m\alpha_{Bon}$$

$$\alpha_{Bon} = \frac{\alpha}{m} = \frac{0.05}{m}$$

Bonferroni correction: controlling the FWER

Can think of controlling the probability of at least one false positive in two ways:

1. Adjust the p-values; keep same α:

$$p_{Bon,i} = mp_i$$

$$(\text{to be more precise: } p_{Bon,i} = min(mp_i, 1))$$

$$\text{Then, threshold } p_{Bon,i} \text{ at } \alpha$$

2. Adjust the α threshold; keep same p-values:

$$\alpha_{Bon} = \frac{\alpha}{m}$$

$$\text{Then, threshold } p_i \text{ at } \alpha_{Bon}$$

Can we do better?

- Bonferroni correction is very conservative (i.e. controls the FWER even lower than α in many settings)
- Several other options are better
- For example, the Holm procedure: multiplier for p-value correction is not the same for all genes; more powerful

$$p_{Holm,1} = mp_1$$

$$p_{Holm,2} = (m-1)p_2$$

$$p_{Holm,3} = (m-2)p_3$$

$$\vdots$$

$$\Rightarrow FWER \leq \alpha$$

How practical is the FWER in high-throughput biology?

- Why do we care so much about making one single error??

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  - However, then our power is zero... 😭

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- Why do we care so much about making one single error??
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  - However, then our power is zero... 😭

Being overly strict about Type I error leads to greater Type II error (loss of power)

Radical idea: it's OK to make multiple errors, as long as you also have some true positives!

Enter: the False Discovery Rate (FDR)

J. R. Statist. Soc. B (1995)
57, *No.* 1, *pp.* 289–300

Controlling the False Discovery Rate: a Practical and Powerful Approach to Multiple Testing

By YOAV BENJAMINI† and YOSEF HOCHBERG

Tel Aviv University, Israel

[Received January 1993. Revised March 1994]

SUMMARY

The common approach to the multiplicity problem calls for controlling the familywise error rate (FWER). This approach, though, has faults, and we point out a few. A different approach to problems of multiple significance testing is presented. It calls for controlling the expected proportion of falsely rejected hypotheses—the false discovery rate. This error rate is equivalent to the FWER when all hypotheses are true but is smaller otherwise. Therefore, in problems where the control of the false discovery rate rather than that of the FWER is desired, there is potential for a gain in power. A simple sequential Bonferroni-type procedure is proved to control the false discovery rate for independent test statistics, and a simulation study shows that the gain in power is substantial. The use of the new procedure and the appropriateness of the criterion are illustrated with examples.

Benjamini Y, Hochberg Y. "Controlling the False Discovery Rate: a Practical and Powerful Approach to Multiple Testing." *Journal of the Royal Statistical Society: Series B (Methodological)*. 1995 Jan;57(1): 289-300.

Over 80K citations!!

False Discovery Rate

	Null True	Alternative True	Total
Not Called Significant	U	T	$m - R$
Called Significant	V	S	R
	m_0	$m-m_0$	m

V = # Type I errors [false positives]

FDR is designed to control the expected proportion of false positives (V) among all hypotheses where the null has been rejected (R)

False Discovery Rate

	Null True	Alternative True	Total
Not Called Significant	***U***	***T***	***m - R***
Called Significant	***V***	***S***	***R***
	m_0	$m-m_0$	***m***

V = # Type I errors [false positives]

FDR is designed to control the expected proportion of false positives (V) among all hypotheses where the null has been rejected (R)

$$FDR = E\left[\frac{V}{R}\right]$$

FDR vs FPR vs FWER

- False Discovery Rate (FDR) is the rate that significant features (R) are truly null

$$FDR = E\left[\frac{V}{R}\right]$$

- False Positive Rate (FPR) is the rate that truly null features (m_0) are called significant

$$FPR = E\left[\frac{V}{m_0}\right]$$

- Family-Wise Error Rate (FWER) is the probability that the number of truly null features rejected (V) is at least 1

$$\text{FWER} = P(V \geq 1)$$

Benjamini-Hochberg FDR (BH procedure)

- Proposed the idea of controlling FDR instead of FWER
- Proposed a procedure for doing so
  - note that we know R, but we don't know V
- Procedure: control FDR at level q
  1. order the raw p-values $p_1 \leq p_2 \leq \ldots \leq p_m$
  2. find test with highest rank j such that $p_j < \frac{jq}{m}$
  3. declare all smaller ranks up to j significant

Controlling FDR at level $q = 0.05$

Rank (j)	P-value
1	0.0008
2	0.009
3	0.127
4	0.205
5	0.396
6	0.450
7	0.641
8	0.781
9	0.900
10	0.993

Controlling FDR at level $q = 0.05$

Rank (j)	P-value	(j/m)*q
1	0.0008	0.005
2	0.009	0.010
3	0.127	0.015
4	0.205	0.020
5	0.396	0.025
6	0.450	0.030
7	0.641	0.035
8	0.781	0.040
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10	0.993	0.050

Controlling FDR at level $q = 0.05$

Rank (j)	P-value	(j/m)*q	Reject H_0?
1	0.0008	0.005	✓
2	0.009	0.010	✓
3	0.127	0.015	
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9	0.900	0.045	
10	0.993	0.050	

Controlling FDR at level $q = 0.05$

Rank (j)	P-value	(j/m)*q	Reject H_0?	$FWER_{Bon} < 0.05$?
1	0.0008	0.005	✓	✓
2	0.009	0.010	✓	
3	0.127	0.015		
4	0.205	0.020		
5	0.396	0.025		
6	0.450	0.030		
7	0.641	0.035		
8	0.781	0.040		
9	0.900	0.045		
10	0.993	0.050		

Where $\alpha_{Bon} = 0.05/10 = 0.005$

BH FDR values given in `limma` by default

```
topTable(ebFit, coef = "genotypeNrlKO")
```

```
##                   logFC  AveExpr          t      P.Value    adj.P.Val        B
## 1450946_at   -4.7134891 8.733103 -15.583683 5.390931e-18 2.431364e-13 28.40938
## 1442070_at   -0.9669400 8.268122  -9.787407 6.896220e-12 1.555132e-07 16.46086
## 1443457_at   -1.1530057 6.049428  -9.091412 4.947029e-11 6.029428e-07 14.68843
## 1422679_s_at -2.1187394 9.495435  -9.002625 6.388954e-11 6.029428e-07 14.45703
## 1431708_a_at -2.1610625 8.591450  -8.967950 7.062007e-11 6.029428e-07 14.36634
## 1433050_at   -1.5083887 8.468891  -8.923932 8.021234e-11 6.029428e-07 14.25096
## 1426288_at   -4.2100657 9.323796  -8.772209 1.246491e-10 8.031144e-07 13.85106
## 1418108_at    0.8662091 8.260647   8.537984 2.475294e-10 1.395478e-06 13.22713
## 1450770_at    1.3103576 7.357033   8.128847 8.332369e-10 3.747976e-06 12.11870
## 1457802_at   -0.8362030 7.778189  -8.117112 8.629984e-10 3.747976e-06 12.08657
```

BH FDR values given in `limma` by default

```
topTable(ebFit, coef = "genotypeNrlKO")
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```
##                   logFC  AveExpr          t      P.Value    adj.P.Val        B
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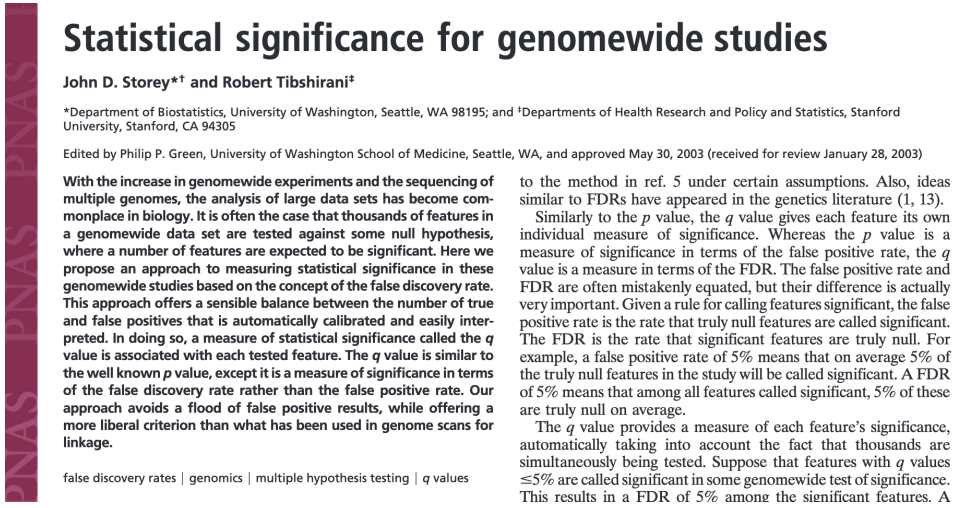
## 1457802_at   -0.8362030 7.778189  -8.117112 8.629984e-10 3.747976e-06 12.08657
```

Or, obtain them yourself for any vector of p-values `p` with `p.adjust(p, method="BH")`

Other ways to control FDR

- BH is just one (the first) method to control FDR
- Since the publication of the BH method, other methods have been proposed
- One of the most popular is Storey's q-value



Statistical significance for genomewide studies

John D. Storey*† and Robert Tibshirani‡

*Department of Biostatistics, University of Washington, Seattle, WA 98195; and ‡Departments of Health Research and Policy and Statistics, Stanford University, Stanford, CA 94305

Edited by Philip P. Green, University of Washington School of Medicine, Seattle, WA, and approved May 30, 2003 (received for review January 28, 2003)

With the increase in genomewide experiments and the sequencing of multiple genomes, the analysis of large data sets has become commonplace in biology. It is often the case that thousands of features in a genomewide data set are tested against some null hypothesis, where a number of features are expected to be significant. Here we propose an approach to measuring statistical significance in these genomewide studies based on the concept of the false discovery rate. This approach offers a sensible balance between the number of true and false positives that is automatically calibrated and easily interpreted. In doing so, a measure of statistical significance called the *q* value is associated with each tested feature. The *q* value is similar to the well known *p* value, except it is a measure of significance in terms of the false discovery rate rather than the false positive rate. Our approach avoids a flood of false positive results, while offering a more liberal criterion than what has been used in genome scans for linkage.

false discovery rates | genomics | multiple hypothesis testing | *q* values

to the method in ref. 5 under certain assumptions. Also, ideas similar to FDRs have appeared in the genetics literature (1, 13).

Similarly to the *p* value, the *q* value gives each feature its own individual measure of significance. Whereas the *p* value is a measure of significance in terms of the false positive rate, the *q* value is a measure in terms of the FDR. The false positive rate and FDR are often mistakenly equated, but their difference is actually very important. Given a rule for calling features significant, the false positive rate is the rate that truly null features are called significant. The FDR is the rate that significant features are truly null. For example, a false positive rate of 5% means that on average 5% of the truly null features in the study will be called significant. A FDR of 5% means that among all features called significant, 5% of these are truly null on average.

The *q* value provides a measure of each feature's significance, automatically taking into account the fact that thousands are simultaneously being tested. Suppose that features with *q* values ≤5% are called significant in some genomewide test of significance. This results in a FDR of 5% among the significant features. A

- `qvalue` package implementation: provides adjusted p-values

Storey's q-value vs BH (Conceptual)

- Just like BH, is focused on the proportion of discoveries that are false positives
- *Conceptual* difference between BH and Storey's q-value is:
  - BH controls the FDR
  - q-values give an unbiased estimate of the FDR (will control the FDR *on average*)

Storey's q-value vs BH (Mathematical)

- Mathematically, the difference between the two is in how m_0 is estimated
  - Or equivalently, how $\pi_0 = \frac{m_0}{m}$ is estimated (since m is known)
  - π_0 represents the overall proportion of tests that are truly null
- q-value:

$$\hat{q}(p_i) = \min_i \left(\frac{\hat{\pi}_0 m}{rank(p_i)} p_i, \, 1 \right)$$

- q-value and BH-adjusted p-values are equivalent when $\pi_0 = 1$

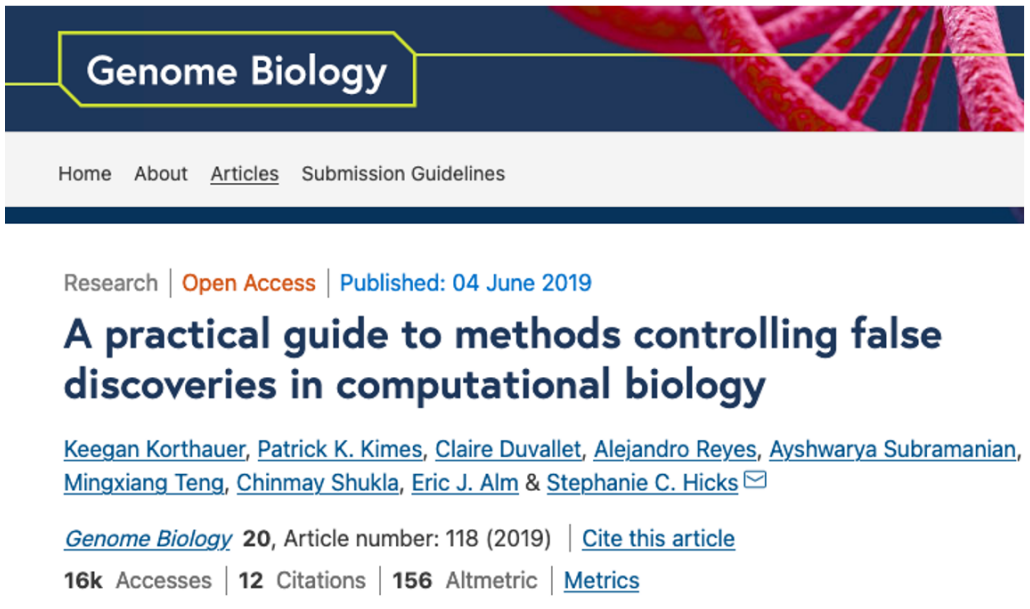
$$\hat{p}_{BH}(p_i) = \min_i \left(\frac{m}{rank(p_i)} p_i, \, 1 \right)$$

(BH conservatively assumes that $\pi_0 = 1$)

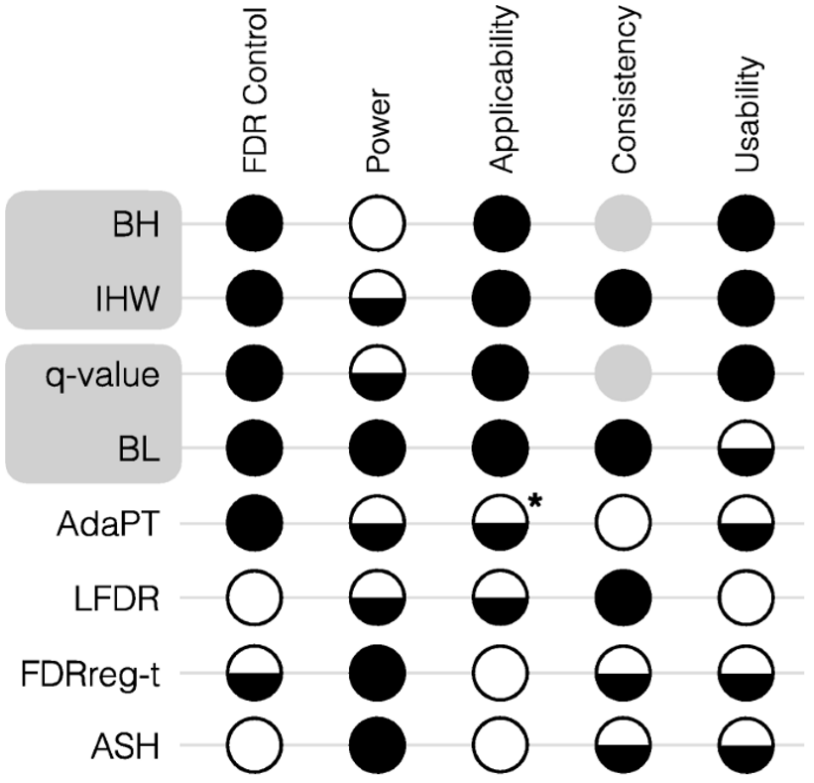
BH vs q-value in our example

Rank (j)	P-value	$\hat{p}_{BH}(p_i)$	$\hat{q}(p_i)$
1	0.0008	0.045	0.045
2	0.009	0.045	0.045
3	0.127	0.423	0.423
4	0.205	0.513	0.513
5	0.396	0.750	0.750
6	0.450	0.750	0.750
7	0.641	0.916	0.916
8	0.781	0.976	0.976
9	0.900	0.993	0.993
10	0.993	0.993	0.993

FDR control is an active area of research



Korthauer, *Kimes* et al. 2019



Compounding issues of multiple comparisons

- What if you're not only testing 20K genes, but also multiple tests per gene (e.g. multiple contrasts, such as several two-group comparisons)?
- Classical procedures for adjustment:
  - Tukey multiple comparison procedure
  - Scheffe multiple comparison procedure
  - Bonferroni or Holm FWER correction
- In our setting, we can also apply BH to all p-values globally
  - `limma::decideTests(pvals, method="global")` for a matrix of p-values or `eBayes` output (e.g. rows = genes, columns = contrasts)
  - p-values are combined, adjusted globally, then separated back out and sorted

Assumptions about p-values

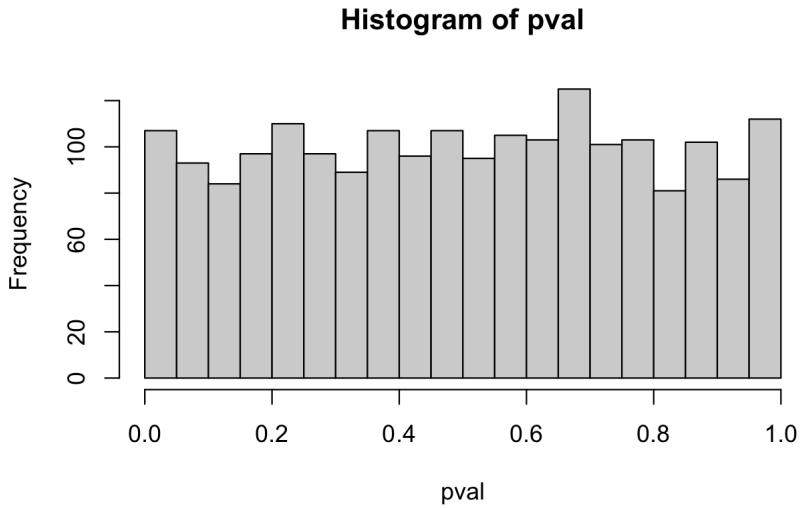
Implicit assumption for all multiple testing correction methods: p-value distribution is "well-behaved"

Assumptions about p-values

Implicit assumption for all multiple testing correction methods: p-value distribution is "well-behaved"

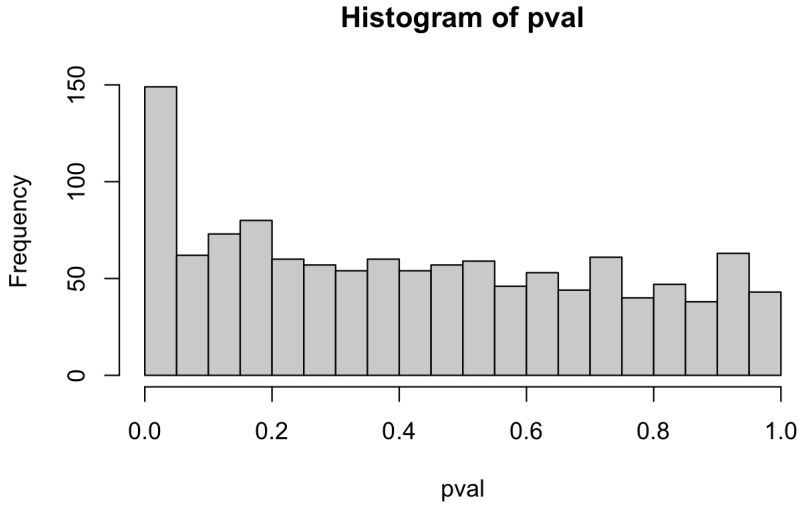
What does this mean?

Primarily, that the distribution of p-values *under the null* is uniform (i.e. flat)



p-value distributions

Spike of small p-values indicates non-null tests:



Great primer on how things can go wrong: http://varianceexplained.org/statistics/interpreting-pvalue-histogram/

What if p-values are *poorly behaved*?

- FDR estimates can be invalid (assumptions are violated)
- Possible solution: nonparametric test
- Alternative: estimate sampling distribution or p-values "empirically" using resampling techniques
    - Bootstrap: estimate sampling distribution of a statistic of interest (e.g. mean expression in each group) by taking repeated random samples *with replacement* from your dataset and recomputing. Use these to estimate confidence intervals without assuming data follows a particular distribution
    - Permutation: construct a simulated version of your dataset that satisfies the null hypothesis and compute statistic (e.g. shuffle group labels for a two-group comparison); repeat many times and use permutation statistics as your sampling distribution rather than a t, Normal, F, χ^2, etc
- Downsides: often computationally intensive for genomics; not optimal for small sample sizes