Statistical Methods for High Dimensional Biology

Continuous models and intro to limma

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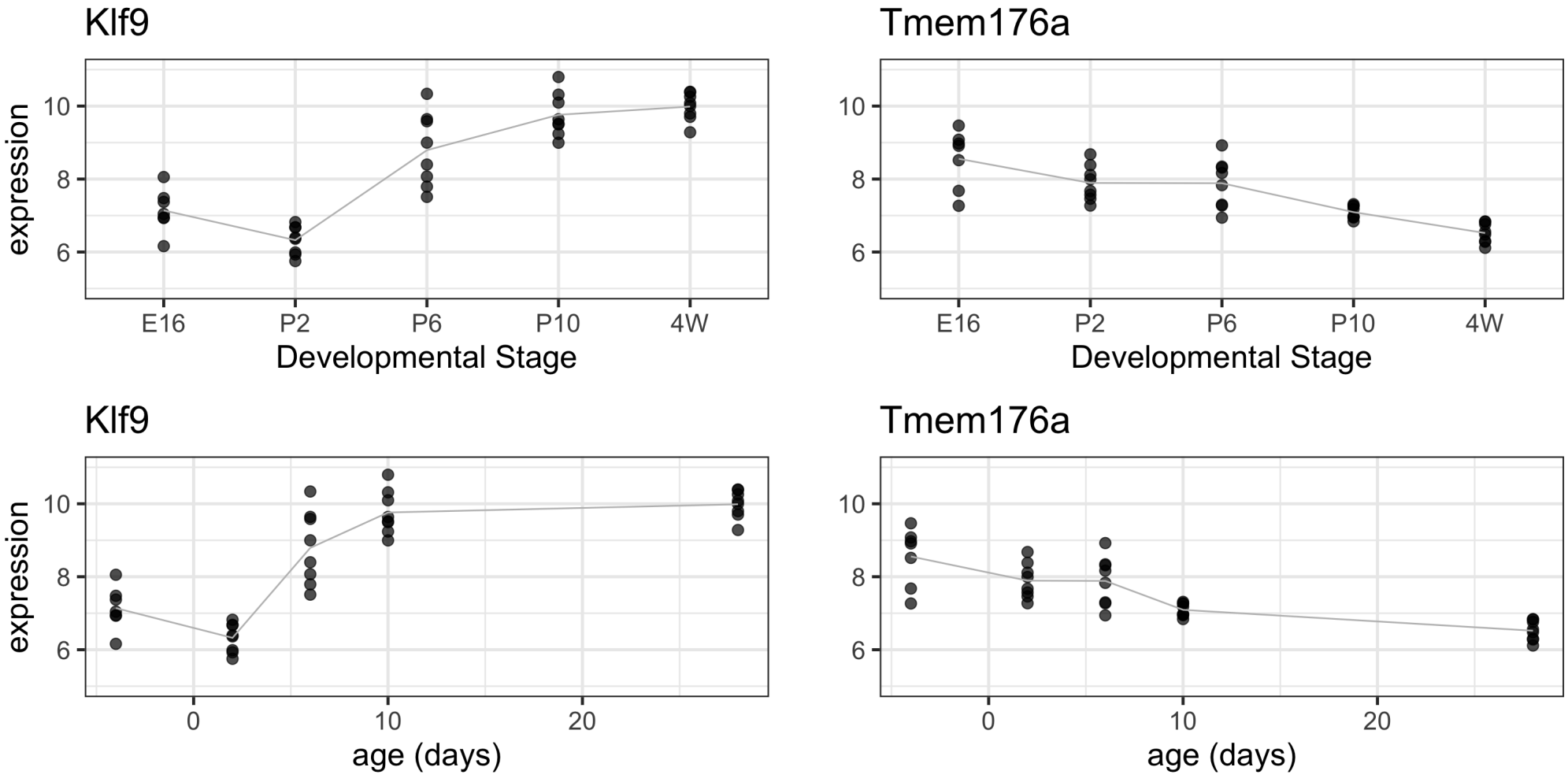
with slide contributions from Gabriela Cohen Freue and Jenny Bryan

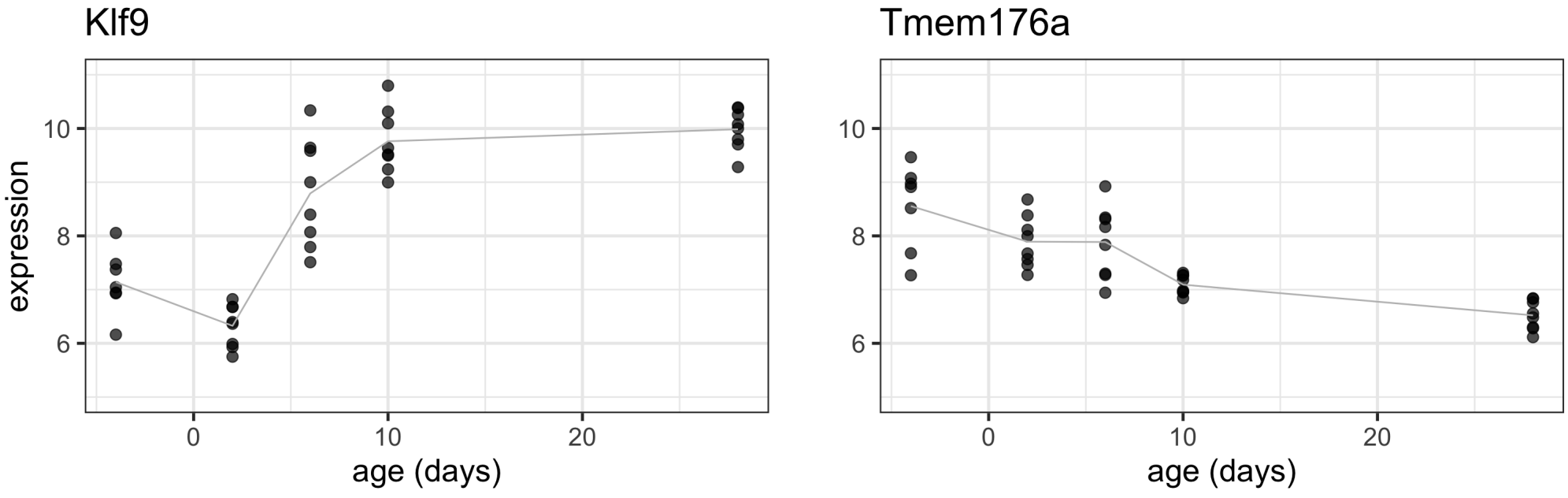
Summary so far

- ***t* tests** can be used to test the equality of 2 population means
- **ANOVA** can be used to test the equality of more than 2 population means
- **Linear regression** provides a general framework for modeling the relationship between a response variable and different types of explanatory variables
  - t tests can be used to test the significance of *individual* coefficients
  - F tests can be used to test the simultaneous significance of *multiple* coefficients (e.g. multiple levels of a single categorical factor, or multiple factors at once)

What if we represent age as a continuous variable?



Linear model with age as continuous covariate



- Linear looks reasonable for gene Tmem176a, but not so much for Klf9
- For now, assume linear is reasonable

Simple Linear Regression Model (Matrix formulation)

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

For 1 continuous/quantitative covariate:

$$\mathbf{Y} = \begin{bmatrix} y_1 \\ y_2 \\ \vdots \\ y_n \end{bmatrix}, \quad \mathbf{X} = \begin{bmatrix} 1 & x_1 \\ 1 & x_2 \\ \vdots & \vdots \\ 1 & x_n \end{bmatrix}, \quad \boldsymbol{\alpha} = \begin{bmatrix} \alpha_0 \\ \alpha_1 \end{bmatrix}, \quad \boldsymbol{\varepsilon} = \begin{bmatrix} \varepsilon_1 \\ \varepsilon_2 \\ \vdots \\ \varepsilon_n \end{bmatrix}$$

- $\alpha_0 =$ the **intercept** (expected value of y when x is equal to zero)
- $\alpha_1 =$ the **slope** (expected change in y for every one-unit increase in x)

Simple Linear Regression Model (Matrix formulation)

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

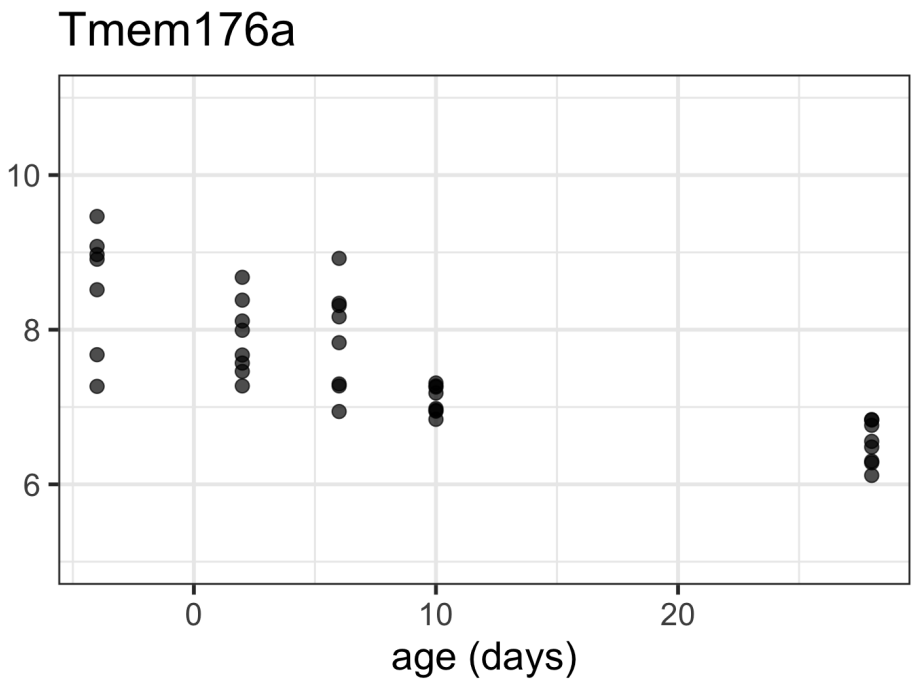
Remember / convince yourself that the matrix algebra does indeed reproduce simple linear regression:

$$\begin{bmatrix} y_1 \\ y_2 \\ \vdots \\ y_n \end{bmatrix} = \begin{bmatrix} 1 & x_1 \\ 1 & x_2 \\ \vdots & \vdots \\ 1 & x_n \end{bmatrix} \begin{bmatrix} \alpha_0 \\ \alpha_1 \end{bmatrix} + \begin{bmatrix} \varepsilon_1 \\ \varepsilon_2 \\ \vdots \\ \varepsilon_n \end{bmatrix} = \begin{bmatrix} 1 * \alpha_0 + x_1\alpha_1 \\ 1 * \alpha_0 + x_2\alpha_1 \\ \vdots \\ 1 * \alpha_0 + x_n\alpha_1 \end{bmatrix} + \begin{bmatrix} \varepsilon_1 \\ \varepsilon_2 \\ \vdots \\ \varepsilon_n \end{bmatrix}$$

$$= \begin{bmatrix} \alpha_0 + x_1\alpha_1 + \varepsilon_1 \\ \alpha_0 + x_2\alpha_1 + \varepsilon_2 \\ \vdots \\ \alpha_0 + x_n\alpha_1 + \varepsilon_n \end{bmatrix}$$

$$\Rightarrow y_i = \alpha_0 + x_i\alpha_1 + \varepsilon_i$$

SLR with age covariate

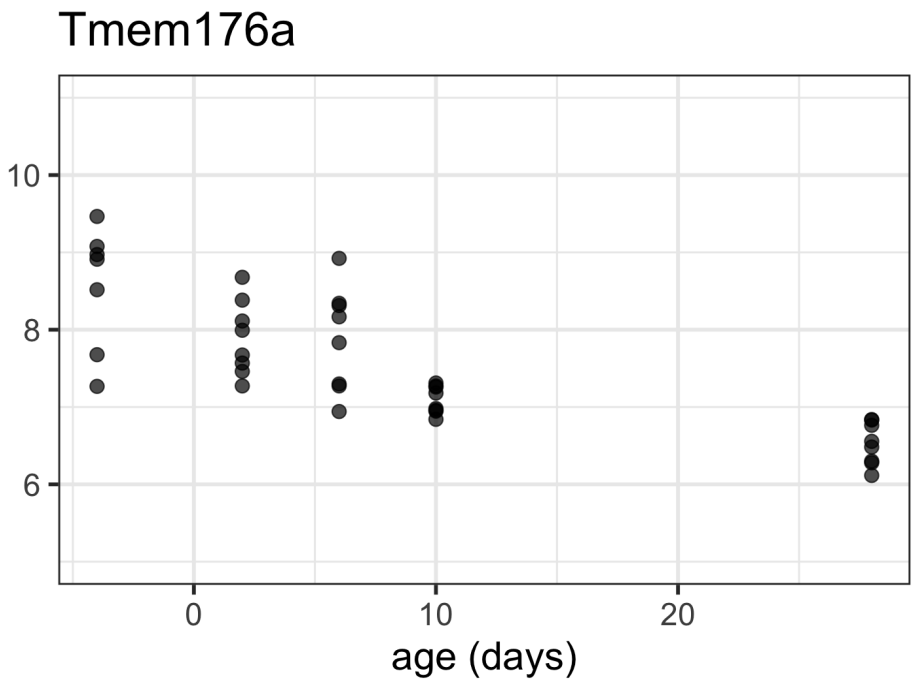



```
filter(twoGenes, gene == "Tmem176a") %>%
lm(expression ~ age, data = .) %>%
  summary() %>% .$coeff
```

```
##                Estimate  Std. Error  t value     Pr(>|t|)
## (Intercept)  8.10007931 0.113949630 71.08474 3.579302e-41
## age         -0.06137385 0.008214834 -7.47110 6.742526e-09
```

$H_0 : \alpha_0 = 0$ tests the null hypothesis that the intercept is zero - usually, not of interest

SLR with age covariate




```
filter(twoGenes, gene == "Tmem176a") %>%
lm(expression ~ age, data = .) %>%
  summary() %>% .$coeff
```

```
##                Estimate  Std. Error  t value     Pr(>|t|)
## (Intercept)  8.10007931 0.113949630 71.08474 3.579302e-41
## age         -0.06137385 0.008214834 -7.47110 6.742526e-09
```

$H_0 : \alpha_1 = 0$ tests the null hypothesis that there is no association between gene expression and age - usually of interest

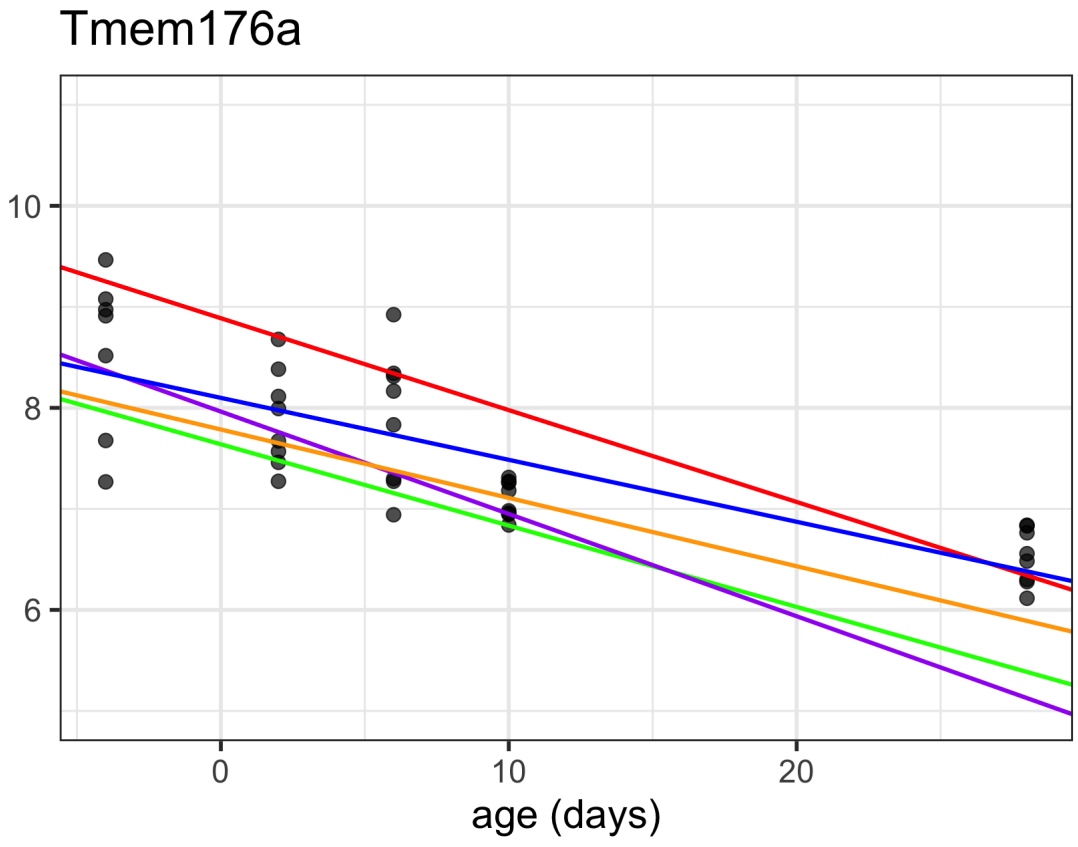
How do we estimate the intercept and slope?

Is there an *optimal* line?

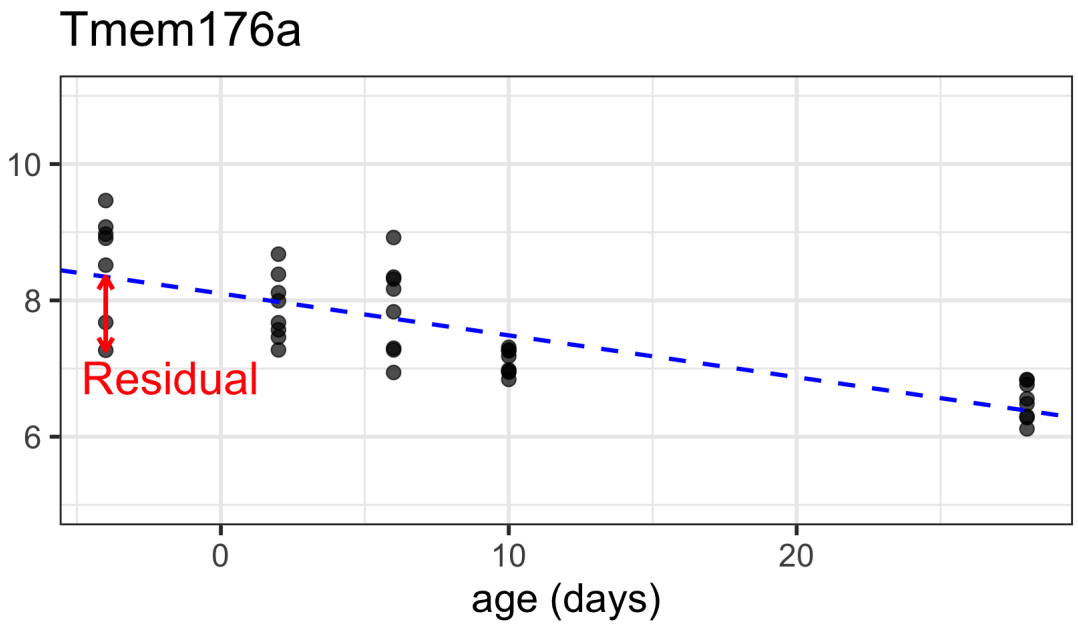
```
filter(twoGenes, gene == "Tmem176a") %>%
lm(expression ~ age, data = .) %>%
  summary()
```

```
## Coefficients:
##              Estimate Std. Error t value Pr(>|t|)
## (Intercept)  8.100079   0.113950  71.085  < 2e-16 ***
## age         -0.061374   0.008215  -7.471 6.74e-09 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 0.5535 on 37 degrees of freedom
## Multiple R-squared:  0.6014,    Adjusted R-squared:  0.5906
## F-statistic: 55.82 on 1 and 37 DF,  p-value: 6.743e-09
```

Which one is the **best** line?



Ordinary Least Squares



Ordinary Least Squares (OLS) regression: parameter estimates minimize the sum of squared errors

Error: vertical (y) distance between the true regression line (unobserved) and the real observation

Residual: vertical (y) distance between the fitted regression line and the real observation (estimated error)

OLS interactive demo

- Visual representation of the squared errors in OLS: http://setosa.io/ev/ordinary-least-squares-regression/
  - Visit the link and examine the first two plots
  - Drag points around in the first plot to see how the second plot changes
  - Adjust the slope and intercept for the regression line and observe how the second plot changes
- The squares of the errors are represented by the square areas in the second plot
  - which line minimizes the sum of these areas? OLS answers this question

OLS Estimator for Simple Linear Regression (1 covariate)

- Mathematically: ε_i represents the error

$$y_i = \alpha_0 + \alpha_1 x_i + \varepsilon_i, i = 1, \ldots, n$$

- We want to find the line (i.e. an intercept and slope) such that the sum of squared errors is minimized

$$S(\alpha_0, \alpha_1) = \sum_{i=1}^{n} (y_i - \alpha_0 - \alpha_1 x_i)^2$$

  - $\varepsilon_i = y_i - \alpha_0 - \alpha_1 x_i$ is the error
  - $S(\alpha_0, \alpha_1)$ is called an *objective function*

- How to obtain estimates $(\hat{\alpha}_0, \hat{\alpha}_1)$? Let's look at a more general case

OLS Estimator for Multiple Linear Regression (p covariates)

- Mathematically:

$$S(\alpha_0, \alpha_1, \alpha_2, \ldots, \alpha_p) = \sum_{i=1}^{n} (y_i - \alpha_0 - \alpha_1 x_{1i} - \alpha_2 x_{2i} - \ldots - \alpha_p x_{pi})^2$$

$$= (\mathbf{y} - \mathbf{X}\boldsymbol{\alpha})^T (\mathbf{y} - \mathbf{X}\boldsymbol{\alpha})$$

- We need to find values of $\boldsymbol{\alpha} = (\alpha_0, \alpha_1, \ldots, \alpha_p)$ that minimize the sum of squares S
- To do so, take partial derviatives with respect to each coefficient, set to zero, and solve the system of equations:

$$\frac{\partial S}{\partial \alpha_0} = \begin{bmatrix} \frac{\partial S}{\partial \alpha_0} \\ \frac{\partial S}{\partial \alpha_1} \\ \vdots \\ \frac{\partial S}{\partial \alpha_p} \end{bmatrix} = \begin{bmatrix} 0 \\ 0 \\ \vdots \\ 0 \end{bmatrix}$$

Properties of OLS regression

Regression model: $\mathbf{Y} = \mathbf{X}\boldsymbol{\alpha} + \boldsymbol{\varepsilon}$

OLS estimator: $\hat{\boldsymbol{\alpha}} = (\mathbf{X}^T\mathbf{X})^{-1}\mathbf{X}^T\mathbf{y}$

Fitted/predicted values: $\hat{\mathbf{y}} = \mathbf{X}\hat{\boldsymbol{\alpha}}$

$$= \mathbf{X}(\mathbf{X}^T\mathbf{X})^{-1}\mathbf{X}^T\mathbf{y} = \mathbf{H}\mathbf{y}$$

where $\mathbf{H} = \mathbf{X}(\mathbf{X}^T\mathbf{X})^{-1}\mathbf{X}^T$ is called the "hat matrix"

Additional assumptions (required for results on the next few slides):

1. $\boldsymbol{\varepsilon}$ have mean zero
2. $\boldsymbol{\varepsilon}$ are iid (implies constant variance)

If $\boldsymbol{\varepsilon}$ are iid **Normal**, then OLS estimator is also MLE (Maximum Likelihood Estimator)

Properties of OLS regression (cont'd)

Residuals: (note NOT the same as errors ε)

$$\hat{\boldsymbol{\varepsilon}} = \mathbf{y} - \hat{\mathbf{y}} = \mathbf{y} - \mathbf{X}\hat{\boldsymbol{\alpha}}$$

Estimated error variance:

$$\hat{\sigma}^2 = \frac{1}{n-p}\hat{\boldsymbol{\varepsilon}}^T\hat{\boldsymbol{\varepsilon}}$$

Estimated covariance matrix of $\hat{\boldsymbol{\alpha}}$:

$$\hat{Var}(\hat{\boldsymbol{\alpha}}) = \hat{\sigma}^2(\mathbf{X}^T\mathbf{X})^{-1}$$

Estimated standard errors for estimated regression coefficients: $\hat{se}(\hat{\alpha}_j)$, obtained by taking the square root of the diagonal elements of $\hat{Var}(\hat{\boldsymbol{\alpha}})$

Inference in Regression (normal iid errors)

How to test $H_0 : \alpha_j = 0$?

With a t statistic!

Under H_0,

$$\frac{\hat{\alpha}_j}{\hat{se}(\hat{\alpha}_j)} \sim t_{n-p}$$

So a p value is obtained by computing a tail probability for the observed value of $\hat{\alpha}_j$ from a t_{n-p} distribution

Inference - what if we don't assume Normal errors?

How to test $H_0 : \alpha_j = 0$?

With a t statistic!

Under H_0, asymptotically (by CLT)

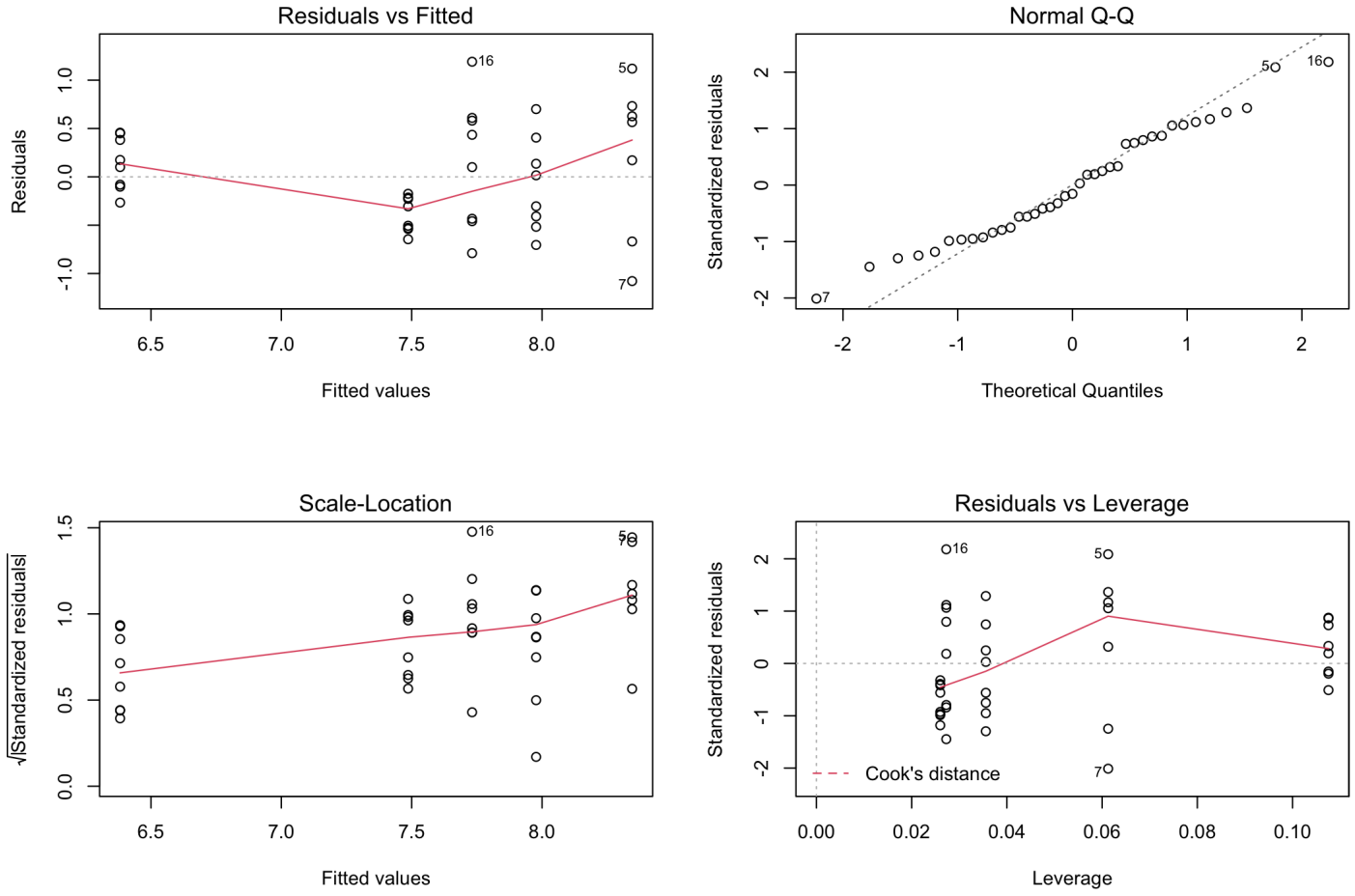
$$\frac{\hat{\alpha}_j}{\hat{se}(\hat{\alpha}_j)} \sim t_{n-p}$$

So with a large enough sample size a p value for this hypothesis test is obtained by computing a tail probability for the observed value of $\hat{\alpha}_j$ from a t_{n-p} distribution

Diagnostics: `plot(lm(y~x))`

Do our assumptions hold?

- Constant variance
- iid errors
- Normality of errors



Linear regression

- The nature of the regression function $f(x|\boldsymbol{\alpha})$ is one of the defining characteristics of a regression model
  - f is linear in $\boldsymbol{\alpha} \Rightarrow$ **linear model**
  - f is not linear in $\boldsymbol{\alpha} \Rightarrow$ **nonlinear model**
- For example, consider nonlinear parametric regression:

$$y_i = \frac{1}{1 + e^{(\phi - x_i)/\eta}} + \varepsilon$$

- We just did simple linear regression (a linear model): $y_i = \alpha_0 + \alpha_1 x_i + \varepsilon_i$
- What we could do instead: polynomial regression (also a linear model)

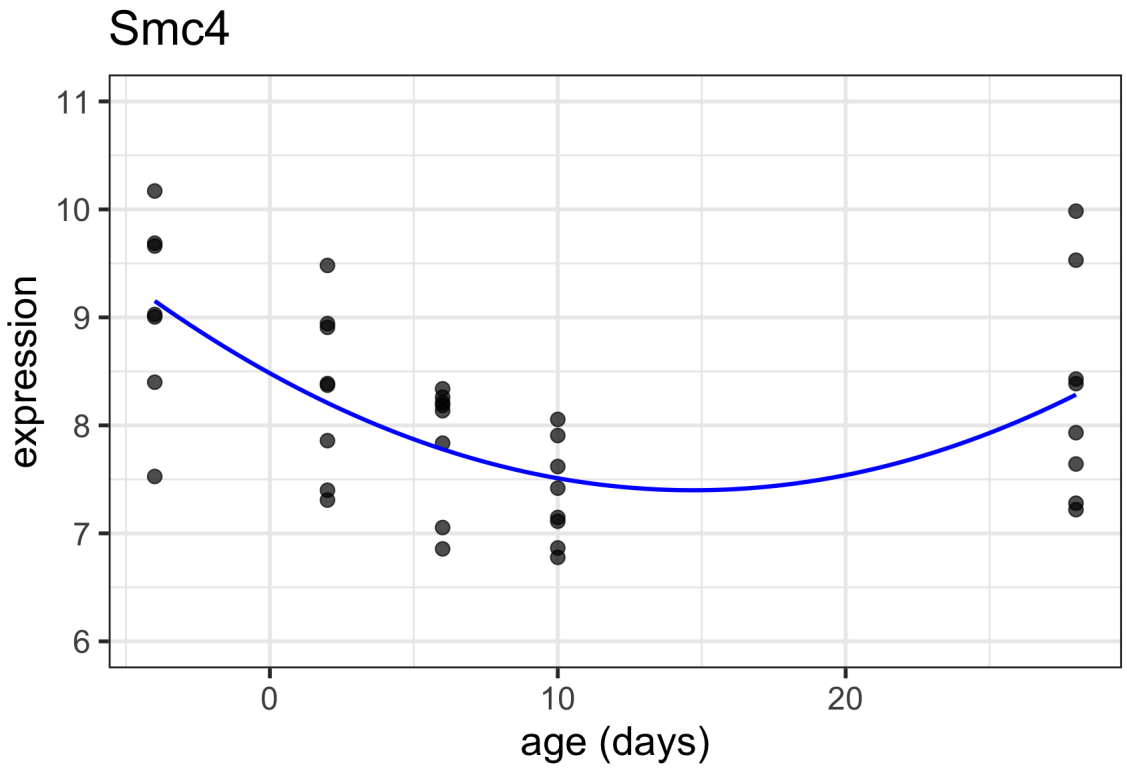
$$y_i = \alpha_0 + \alpha_1 x_i + \alpha_2 x_i^2 + \varepsilon_i$$

Polynomial regression

```r
quadfit <- lm(expression ~ age + I(age^2), data = oneGene)
summary(quadfit)
```

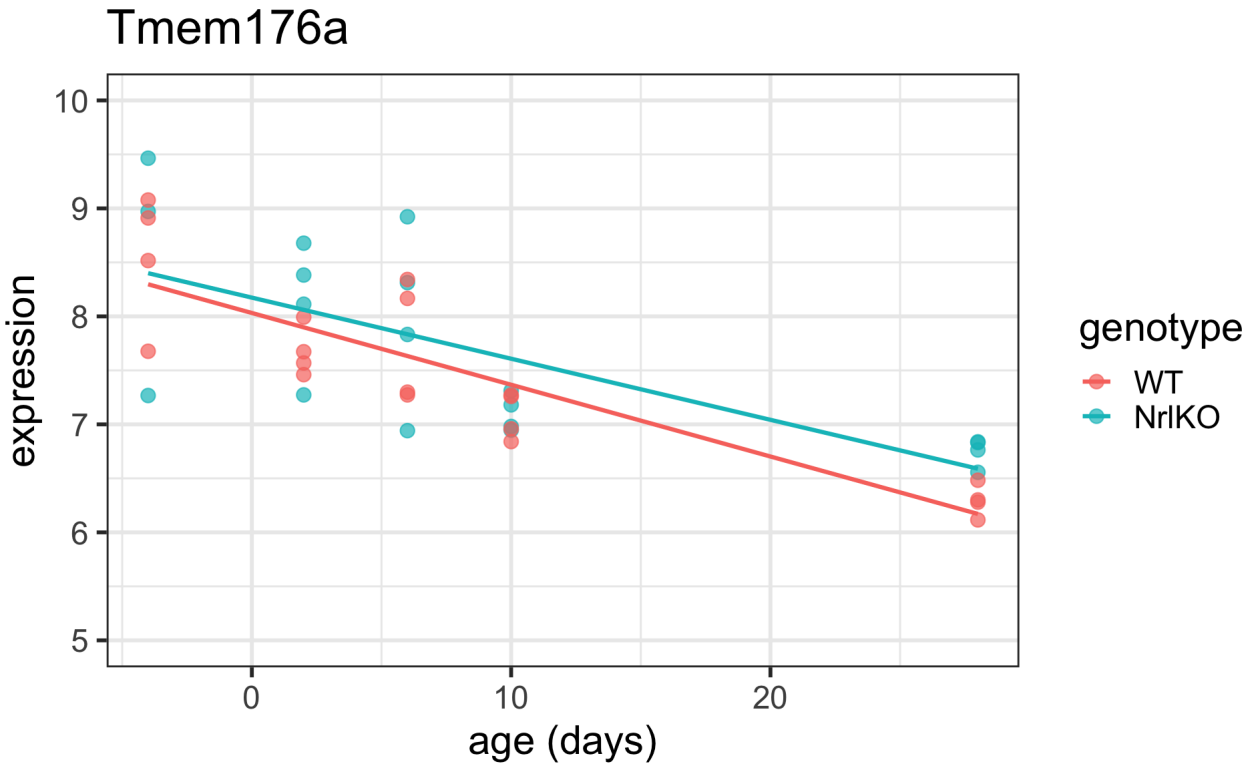
```
##
## Call:
## lm(formula = expression ~ age + I(age^2), data = oneGene)
##
## Residuals:
##     Min      1Q  Median      3Q     Max
## -1.6253 -0.6436  0.1023  0.4955  1.6996
##
## Coefficients:
##              Estimate Std. Error t value Pr(>|t|)
## (Intercept)  8.482542   0.160883  52.725  < 2e-16 ***
## age         -0.147339   0.032626  -4.516 6.52e-05 ***
## I(age^2)     0.005009   0.001164   4.303 0.000123 ***
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
## Residual standard error: 0.7527 on 36 degrees of freedom
## Multiple R-squared:  0.362,    Adjusted R-squared:  0.3265
## F-statistic: 10.21 on 2 and 36 DF,  p-value: 0.0003069
```

Polynomial regression



Note that **this is still a linear model**, because it is linear in the α_j

Putting it all together (continuous + categorical variables)



Interaction between continuous and categorical variables

```
lm(expression ~ age*genotype, data = filter(twoGenes, gene=="Tmem176a"))
  summary() %>% .$coeff
```

```
##                       Estimate Std. Error    t value     Pr(>|t|)
## (Intercept)         8.031510398 0.15654982 51.3032221 1.567640e-34
## age                -0.066454446 0.01141757 -5.8203672 1.331685e-06
## genotypeNrlKO       0.142283869 0.22824752  0.6233753 5.370794e-01
## age:genotypeNrlKO   0.009873243 0.01644292  0.6004556 5.520712e-01
```

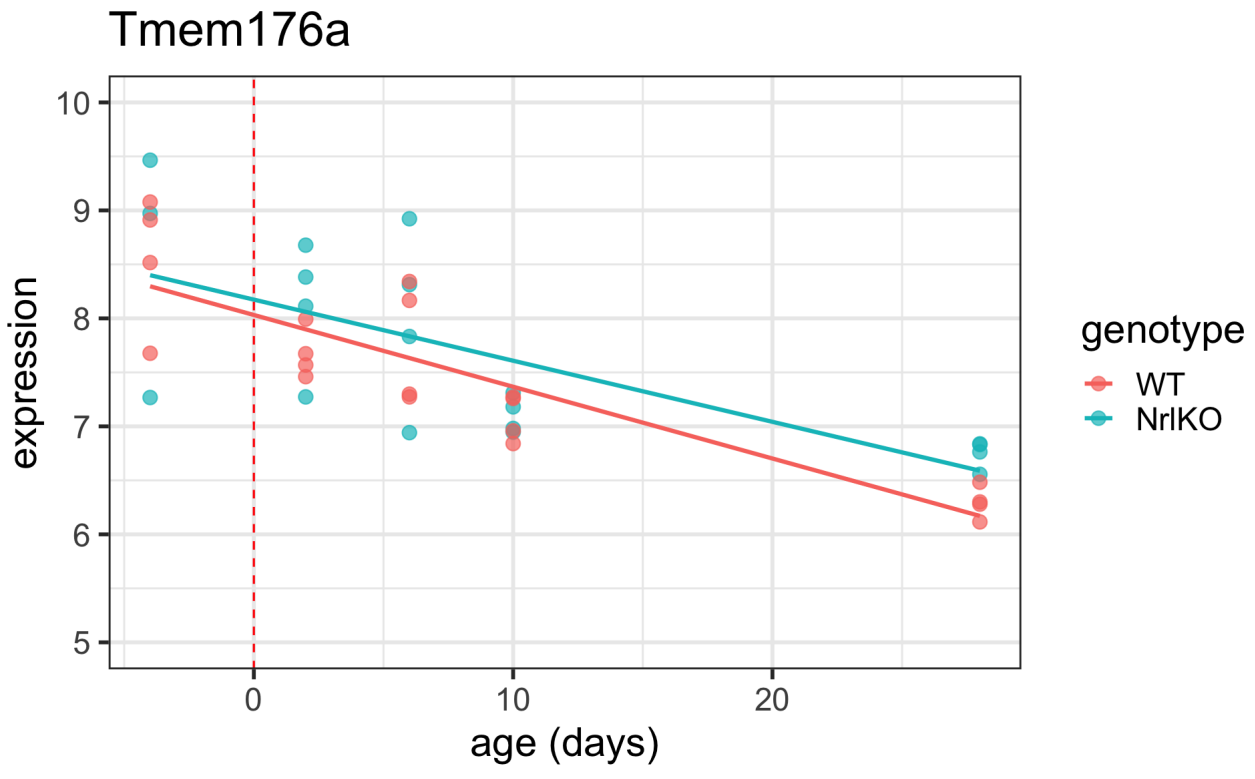
`(Intercept)`: Intercept of WT line

`age`: slope of WT line

`genotypeNrlKO`: difference in intercepts (KO vs WT)

`age:genotypeNrlKO`: difference in slopes (KO vs WT)

Reminder about the Intercept



Intercept terms refer to the estimates when the continuous covariate is equal to zero. Note that this is not usually very interesting on its own.

Interaction between continuous and categorical variables

$$y_{ij} = \alpha_0 + \tau_{KO} x_{ij,KO} + \tau_{Age} x_{ij,Age} + \tau_{KO:Age} x_{ij,KO} x_{ij,Age}$$

where

- $j \in \{WT, NrlKO\}, i = 1, 2, \ldots, n_j$
- $x_{ij,KO}$ is the dummy/indicator variable for WT vs KO ($x_{ij,KO} = 1$ for $j = NrlKO$ and 0 for $j = WT$)
- $x_{ij,Age}$ is the continuous age covariate

Interpretation of parameters:

- α_0 is the expected expression in WT for age = 0
- The "intercept" for the knockouts is: $\alpha_0 + \tau_{KO}$
- τ_{Age} is the expected increase in expression in WT for every 1 day increase in age
- The slope for the knockouts is: $\tau_{Age} + \tau_{KO:Age}$

Nested models

As always, you can assess the relevance of several terms at once - such as everything involving genotype - with an F test

```
anova(lm(expression ~ age*genotype, data = filter(twoGenes, gene=="Klf9"
      lm(expression ~ age, data = filter(twoGenes, gene=="Klf9")))
```

```
## Analysis of Variance Table
##
## Model 1: expression ~ age * genotype
## Model 2: expression ~ age
##   Res.Df    RSS Df Sum of Sq      F Pr(>F)
## 1     35 45.948
## 2     37 45.984 -2 -0.036415 0.0139 0.9862
```

We don't have evidence that genotype affects the intercept or the slope

F tests in regression

Model	Example	# params (df)	RSS
Reduced	expression ~ age	$p_{Red} = 2$	RSS_{Red}
Full	expression ~ age * genotype	$p_{Full} = 4$	RSS_{Full}

Full: $y_{ij} = \alpha_0 + \tau_{KO} x_{ij,KO} + \tau_{Age} x_{ij,Age} + \tau_{KO:Age} x_{ij,KO} x_{ij,Age}$

Reduced: $y_{ij} = \alpha_0 + \tau_{Age} x_{ij,Age}$

Under the null hypothesis (that the reduced model explains the the same amount variation in the outcome as the full model),

$$F = \frac{\frac{RSS_{Red} - RSS_{Full}}{p_{Full} - p_{Red}}}{\frac{RSS_{Full}}{n - p_{Full}}} \sim F_{p_{Fill} - p_{Red},\, n - p_{Full}}$$

A significant F test means we reject the null; we have evidence that the full model explains significantly more variation in the outcome than the reduced.

Linear regression summary

- linear model framework is extremely general
- one extreme (simple): two-sample common variance t-test
- another extreme (flexible): a polynomial, potentially different for each level of some factor
  - dichotomous variable? OK!
  - categorical variable? OK!
  - quantitative variable? OK!
  - various combinations of the above? OK!
- Don't be afraid to build models with more than 1 covariate

What about the other 45 thousand probesets??

```
eset
```

```
## ExpressionSet (storageMode: lockedEnvironment)
## assayData: 45101 features, 39 samples
##   element names: exprs
## protocolData: none
## phenoData
##   sampleNames: GSM92610 GSM92611 ... GSM92648 (39 total)
##   varLabels: title geo_accession ... age (40 total)
##   varMetadata: labelDescription
## featureData: none
## experimentData: use 'experimentData(object)'
##   pubMedIds: 16505381
## Annotation: GPL1261
```

Linear regression of many genes

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

- The g in the subscript reminds us that we'll be fitting a model like this *for each gene g* that we have measured for all samples
- Most of the time, the design matrices $\mathbf{X}_g$ are, in fact, the same for all g. This means we can just use $\mathbf{X}$
- Note this means that the residual degrees of freedom are also the same for all g

$$d_g = d = n - \text{dimension of } \boldsymbol{\alpha} = n - p$$

Linear regression of many genes (cont'd)

Data model:

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

Unknown error variance:

$$Var(\boldsymbol{\varepsilon}_g) = \sigma_g^2$$

Estimated error variance:

$$\hat{\sigma}_g^2 = s_g^2 = \frac{1}{n-p}\hat{\boldsymbol{\varepsilon}}_g^T\hat{\boldsymbol{\varepsilon}}_g$$

Estimated variance of parameter estimates:

$$\hat{Var}(\hat{\boldsymbol{\alpha}}_g) = s_g^2(\mathbf{X}^T\mathbf{X})^{-1} = s_g^2\mathbf{V}$$

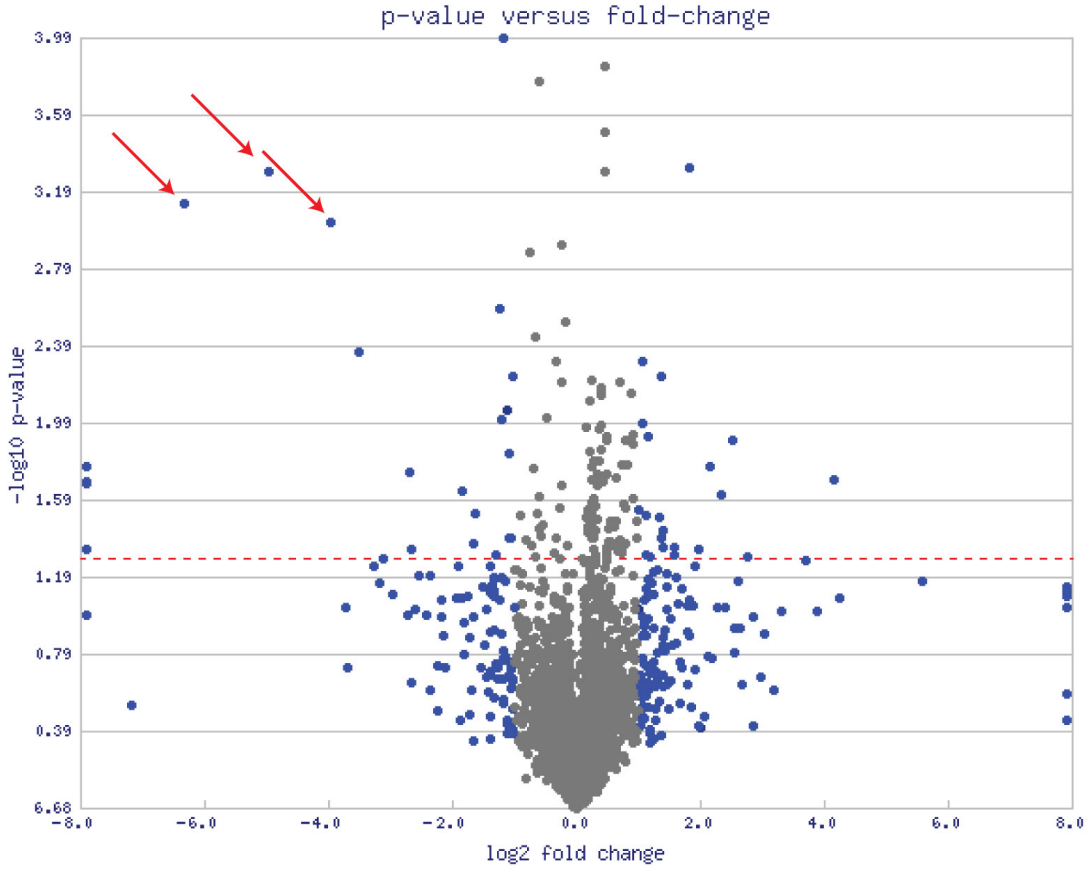
- $\mathbf{V}$ is the "unscaled covariance" matrix, and is the same for all genes!
- Estimated standard errors for estimated regression coefficients: $\hat{se}(\hat{\alpha}_{jg})$, obtained by taking the square root of the jth diagonal element of $\hat{Var}(\hat{\boldsymbol{\alpha}}_g)$, which is $s_g\sqrt{v_{jj}}$

So far, nothing is new - these are the "regular" *t* statistics for gene *g* and parameters *j*:

$$t_{gj} = \frac{\hat{\alpha}_{gj}}{s_g \sqrt{v_{jj}}} \sim t_d \text{ under } H_0$$

But there are so many of them!

Observed (i.e. empirical) issues with the "standard" *t*-test approach for assessing differential expression



Observed (i.e. empirical) issues with the "standard" *t*-test approach for assessing differential expression

Some genes with very **small p-values** (large -log10 p-values) are not **biologically meaningful** (small effect size)

How do we end up with small p-values but subtle effects?

$$t_{gj} = \frac{\hat{\alpha}_{gj}}{\hat{se}(\hat{\alpha}_{gj})} = \frac{\hat{\alpha}_{gj}}{s_g\sqrt{v_{jj}}} \sim t_d \text{ under } H_0$$

- Small variance estimate s_g leads to large t statistic $\rightarrow$ small p-value
- Estimates of variance from small sample sizes tend to under-estimate the true variance!
- This has led to the development of specialized methodology for assessing genome-wide differential expression

Empirical Bayesian techniques: `limma`

> Stat Appl Genet Mol Biol, 3, Article3 2004

Linear Models and Empirical Bayes Methods for Assessing Differential Expression in Microarray Experiments

Gordon K Smyth [1]

Affiliations + expand

PMID: 16646809 DOI: 10.2202/1544-6115.1027

Smyth 2004

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DOI: 10.18129/B9.bioc.limma

Linear Models for Microarray Data

Bioconductor version: Release (3.10)

Data analysis, linear models and differential expression for microarray data.

Author: Gordon Smyth [cre,aut], Yifang Hu [ctb], Matthew Ritchie [ctb], Jeremy Silver [ctb], James Wettenhall [ctb], Davis McCarthy [ctb], Di Wu [ctb], Wei Shi [ctb], Belinda Phipson [ctb], Aaron Lun [ctb], Natalie Thorne [ctb], Alicia Oshlack [ctb], Carolyn de Graaf [ctb], Yunshun Chen [ctb], Mette Langaas [ctb], Egil Ferkingstad [ctb], Marcus Davy [ctb], Francois Pepin [ctb], Dongseok Choi [ctb]

Maintainer: Gordon Smyth <smyth at wehi.edu.au>

Why use `limma` instead of regular *t*-tests?

- **Borrows information** from all genes to get a better estimate of the variance (especially in smaller sample size settings)
- Efficiently fits many regression models **without replicating unnecessary calculations**!
- Arranges output in a convenient way to ease further analysis, visualization, and interpretation

How does Empirical Bayes work?

- **Empirical**: observed
- **Bayesian**: incorporate 'prior' information
- Intuition: estimate prior information from data; *shrink* (nudge) all estimates toward the consensus

Shrinkage = borrowing information across all genes

Genome-wide OLS fits

- Gene by gene:
  - `lm(y ~ x, data = gene)` for each gene
  - For example, using `dplyr::group_modify` and `broom::tidy`
- All genes at once, using `limma`:
  - `lmFit(myDat, desMat)`
  - `myDat` contains all genes
  - `desMat` is a specially formatted design matrix (more on this later)
  - Or, even better, `lmFit(eset, desMat)` where `eset` is an `ExpressionSet` object

'Industrial scale' model fitting is good, because computations involving just the design matrix $\mathbf{X}$ are not repeated 30K unnecessarily

- **OLS estimator**:

$$\hat{\boldsymbol{\alpha}} = (\mathbf{X}^T\mathbf{X})^{-1}\mathbf{X}^T\mathbf{y}$$

- **Fitted/predicted values**:

$$\hat{\mathbf{y}} = \mathbf{X}(\mathbf{X}^T\mathbf{X})^{-1}\mathbf{X}^T\mathbf{y} = \mathbf{H}\mathbf{y}$$

OLS of first 2000 genes, using `lm` gene by gene

```
allGenes %>% head(10)
```

```
## # A tibble: 10 x 6
##    gene       sample_id expression dev_stage   age genotype
##    <chr>      <chr>          <dbl> <fct>     <dbl> <fct>
##  1 1415670_at GSM92610        7.11 4W           28 NrlKO
##  2 1415670_at GSM92611        7.32 4W           28 NrlKO
##  3 1415670_at GSM92612        7.42 4W           28 NrlKO
##  4 1415670_at GSM92613        7.35 4W           28 NrlKO
##  5 1415670_at GSM92614        7.24 E16          -4 NrlKO
##  6 1415670_at GSM92615        7.34 E16          -4 NrlKO
##  7 1415670_at GSM92616        7.38 E16          -4 NrlKO
##  8 1415670_at GSM92617        7.22 P10          10 NrlKO
##  9 1415670_at GSM92618        7.22 P10          10 NrlKO
## 10 1415670_at GSM92619        7.12 P10          10 NrlKO
```

```
system.time(lmfits <- allGenes %>%
    filter(gene %in% unique(allGenes$gene)[1:2000]) %>%
    group_by(gene) %>%
    group_modify(~ tidy(lm(expression ~ age + genotype,
                           data = .x))) %>%
    select(gene, term, estimate) %>%
    pivot_wider(names_from = term, values_from = estimate)
```

```
##    user  system elapsed
##  23.450   0.722  26.688
```

```
lmfits %>% head() %>% as.data.frame()
```

```
##           gene (Intercept)           age genotypeNrlKO
## 1   1415670_at    7.217851  0.0006225228 -0.0002861005
## 2   1415671_at    9.320083 -0.0018405479  0.1446053811
## 3   1415672_at    9.759959 -0.0039281143 -0.0421705559
## 4   1415673_at    8.404053  0.0039777804 -0.0436443351
## 5 1415674_a_at    8.517675 -0.0059840405  0.0192159017
## 6   1415675_at    9.665691 -0.0064185855  0.1330272055
```

OLS of **all** genes at once, using `limma`:

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

```
##    user  system elapsed
##   0.200   0.070   0.353
```

```
limmafits$coefficients %>% head()
```

```
##               (Intercept)           age genotypeNrlKO
## 1415670_at       7.217851  0.0006225228 -0.0002861005
## 1415671_at       9.320083 -0.0018405479  0.1446053811
## 1415672_at       9.759959 -0.0039281143 -0.0421705559
## 1415673_at       8.404053  0.0039777804 -0.0436443351
## 1415674_a_at     8.517675 -0.0059840405  0.0192159017
## 1415675_at       9.665691 -0.0064185855  0.1330272055
```

So far, no shrinkage.

How can we better estimate the SE?

$$t_{gj} = \frac{\hat{\alpha}_{gj}}{\hat{se}(\hat{\alpha}_{gj})} = \frac{\hat{\alpha}_{gj}}{s_g\sqrt{v_{jj}}} \sim t_d \text{ under } H_0$$

Small variance estimate leads to large t statistic, which leads to small p-value

Modeling in `limma`

limma assumes that for each gene g

$$\hat{\alpha}_{gj} \,|\, \alpha_{gj}, \sigma_g^2 \sim N(\alpha_{gj}, \sigma_g^2 v_{jj})$$

$$s_g^2 \,|\, \sigma_g^2 \sim \frac{\sigma_g^2}{d} \chi_d^2$$

which are the same as the usual assumptions about ordinary t-statistics:

$$t_{gj} = \frac{\hat{\alpha}_{gj}}{\hat{se}(\hat{\alpha}_{gj})} = \frac{\hat{\alpha}_{gj}}{s_g \sqrt{v_{jj}}} \sim t_d \text{ under } H_0$$

So far, nothing new...

Modeling in `limma` - shrinkage

- limma imposes a hierarchical model, which describes how the gene-wise α_{gj}'s and σ_g^2's vary **across the genes**
  - We are no longer considering genes in isolation
- this is done by assuming a **prior distribution** for those quantities
- Prior distribution for **gene-specific variances** σ_g^2: an inverse Chi-square with mean s_0^2 and d_0 degrees of freedom:

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

- this should feel funny compared to previous lectures - σ_g^2 is no longer a **fixed** quantity! (i.e. this is **Bayesian**)

How does this help us get a better estimate of the variance?

- The **posterior distribution** is an updated version of the observed likelihood based on incorporating the prior information
- The posterior mean for gene-specific variance:

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

- How to think about it: a weighted mean of the prior (indirect evidence) and the observed (direct evidence) gene-specific variances:

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

- More simply: "shrinking" the observed gene-specific variance towards the "typical" variance implied by the prior

Moderated *t*-statistic

- plug in this posterior mean estimate to obtain a 'moderated' *t*-statistic:

$$\tilde{t}_{gj} = \frac{\hat{\alpha}_{gj}}{\tilde{s}_g \sqrt{v_{jj}}}$$

- Under limma assumptions, we know the null distribution for the moderated *t*-statistic:

$$\tilde{t}_{gj} \sim t_{d_0+d} \text{ under } H_0$$

- parameters from the prior d_0 and s_0^2 are estimated from the data
- This is how limma is a **hybrid** of frequentist (*t*-statistic) and Bayesian (hierarchical model) approaches

Side-by-side comparison of key quantities and results

	OLS	**limma**
Estimated gene-wise residual variance:	$s_g^2 = \frac{1}{n-p}\hat{\boldsymbol{\varepsilon}}^T\hat{\boldsymbol{\varepsilon}}$	$\tilde{s}_g^2 = \frac{d_0 s_0^2 + d s_g^2}{d_0 + d}$
t-statistic for $H_0 : \alpha_{gj} = 0$:	$t_{gj} = \frac{\hat{\alpha}_{gj}}{s_g\sqrt{v_{jj}}}$	$\tilde{t}_{gj} = \frac{\hat{\alpha}_{gj}}{\tilde{s}_g\sqrt{v_{jj}}}$
distribution of the t-statistic under H_0:	$t_{gj} \sim t_d$	$\tilde{t}_{gj} \sim t_{d_0+d}$

*Not shown: estimation formulas for prior parameters d_0 and s_0^2

Moderated vs traditional tests

- moderated variances will be "shrunk" toward the typical gene-wise variance, relative to to raw sample residual variances

- degrees of freedom for null distribution **increase** relative to default (d vs $d_0 + d$)

  - $\rightarrow$ makes it closer to a standard normal
  - $\rightarrow$ makes tail probabilities (p-values) smaller
  - $\rightarrow$ easier to reject the null

- overall, when all is well *limma will deliver statistical results that are* more stable *and* more powerful*

Preview: `limma` workflow

responses, design matrix (made by YOU)

↘

fit a separate linear model for each response, e.g. gene — `lmFit(...)`

↙

fitted models

↘

apply an Empirical Bayes procedure for moderating estimates of error variance — `eBayes(...)`

↓

extract estimated parameters or p-values or ...
compare big models to small
etc etc — `topTable(...)`

Preview: 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 all genes (each gene separately) – replace gene with "feature" depending on your data
`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

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