# Statistical Methods for High Dimensional Biology STAT/BIOF/GSAT 540

Lecture 6 – Two Group Comparisons

Rob Balshaw January 20 2016

<sup>\*\*</sup>based on slides from Dr. Jenny Bryan, with edits by Sara Mostafavi\*\*

### Before we start ....

- Recap of last week
- Project groups

### Outline

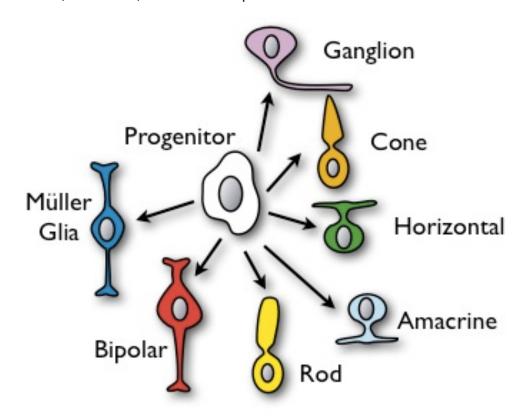
- Comparing Two Groups:
  - Deriving t-statistics/t-test
  - Reconstructing the null distribution based on simulation experiments
  - Brief intro: non parametric tests...
  - Statistical power

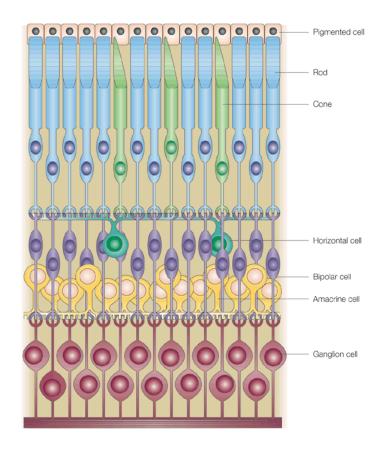
## **Comparing Two Groups**

#### We will analyze data from this study...

#### 🌄 Targeting of GFP to newborn rods by Nrl promoter and temporal expression profiling of flow-sorted photoreceptors

Masayuki Akimoto\*\*, Hong Cheng\*, Dongxiao Zhu<sup>§¶</sup>, Joseph A. Brzezinski<sup>|</sup>, Ritu Khanna\*, Elena Filippova\*, Edwin C. T. Oh<sup>‡</sup>, Yuezhou Jing<sup>¶</sup>, Jose-Luis Linares\*, Matthew Brooks\*, Sepideh Zareparsi\*, Alan J. Mears\*,\*\*, Alfred Hero<sup>§¶††‡‡</sup>, Tom Glaser<sup>||§§</sup>, and Anand Swaroop\*<sup>‡||¶¶</sup>





Nature Reviews | Neuroscience

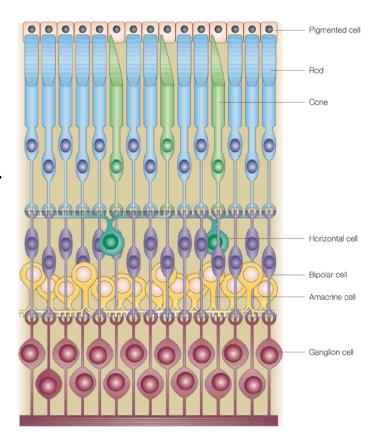
#### We will analyze data from this study...

# SANG

# Targeting of GFP to newborn rods by Nrl promoter and temporal expression profiling of flow-sorted photoreceptors

Masayuki Akimoto\*†, Hong Cheng\*, Dongxiao Zhu<sup>§1</sup>, Joseph A. Brzezinski<sup>||</sup>, Ritu Khanna\*, Elena Filippova\*, Edwin C. T. Oh\*, Yuezhou Jing<sup>1</sup>, Jose-Luis Linares\*, Matthew Brooks\*, Sepideh Zareparsi\*, Alan J. Mears\*,\*\*, Alfred Hero<sup>§11+12\*</sup>, Tom Glaser<sup>||§§</sup>, and Anand Swaroop\*\*<sup>||11|</sup>

- Retina presents a model system for investigating regulatory networks underlying neuronal differentiation.
- **Nrl** transcription factor (TF) known to be important for Rod development.
- What happens if you delete Nrl?
- Hypothesis: Gene expression levels pre/post deletion will inform us of regulatory network involved in rod/cone development.



Nature Reviews | Neuroscience

#### Developing mouse retina – time course for the experiment

5 distinct developmental stages:

Embryonic day 16 (E16)

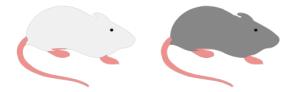
Postnatal days 2, 6 and 10 (P2, P6, P10)

4 week spostnatal (4\_weeks)

#### 2 genotypes

wild-type (wt) vs. Nrl knockout (KO)

NrIKO wt



#### **Experimental design**

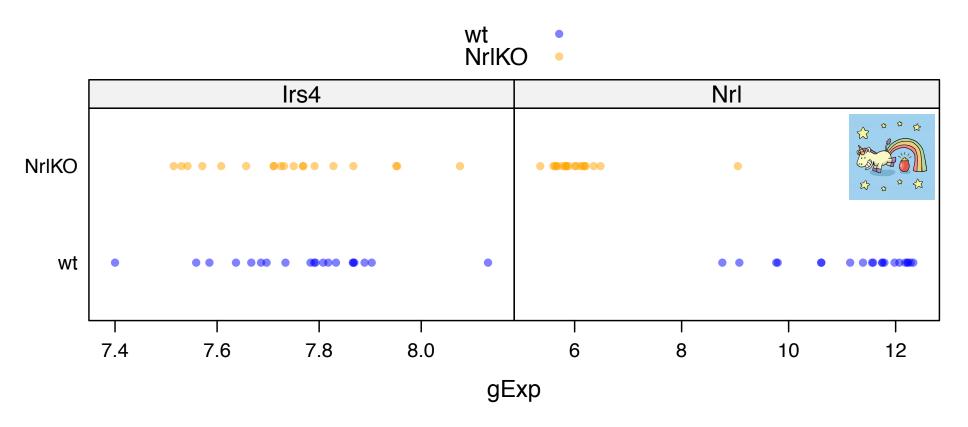
devStage	wt	NrlKO
E16	4	3
P2	4	4
P6	4	4
P10	4	4
4 weeks	4	4

```
> str(prDes)
'data.frame': 39 obs. of 3 variables:
 $ sample : num 20 21 22 23 16 17 6 24 25 26 ...
$ devStage: Factor w/ 5 levels "E16", "P2", "P6", ...: 1 1 1 1 1 1 2 2 2 ....
 $ qType : Factor w/ 2 levels "wt", "NrlKO": 1 1 1 1 2 2 2 1 1 1 ...
> str(prDat, max.level = 0)
'data.frame': 29949 obs. of 39 variables:
> peek(subset(prDat, select = 1:5))
           Sample 20 Sample 21 Sample 22 Sample 23 Sample 16
                      8.143
                              7.899
1416535 at
              8.133
                                       8.054
                                               7.867
            8.567 8.554 7.931 8.182
1437399 at
                                               6.257
1441587 at 6.134 5.745 6.137 5.953 6.575
1445975 at 6.022 5.960 5.994 6.069
                                               6.418
1446741_at 6.024 6.009 6.073 5.961 7.046
1450103 a at 8.376 8.902 8.570 8.755 7.991
1452844 at
          8.490 8.700 8.288 8.544 7.256
> with(prDes, table(devStage, gType))
       gType
devStage wt NrlKO
 E16
         4
                                  photoRec dataset
 P2
              4
 P6 4
                                  mouse photoreceptors
 P10 4
              4
 4 weeks 4
```

# Which genes are differentially expressed between WT and NrIKO?

Let's do it for 2 genes ... we can then apply the same procedure to all genes, one at a time

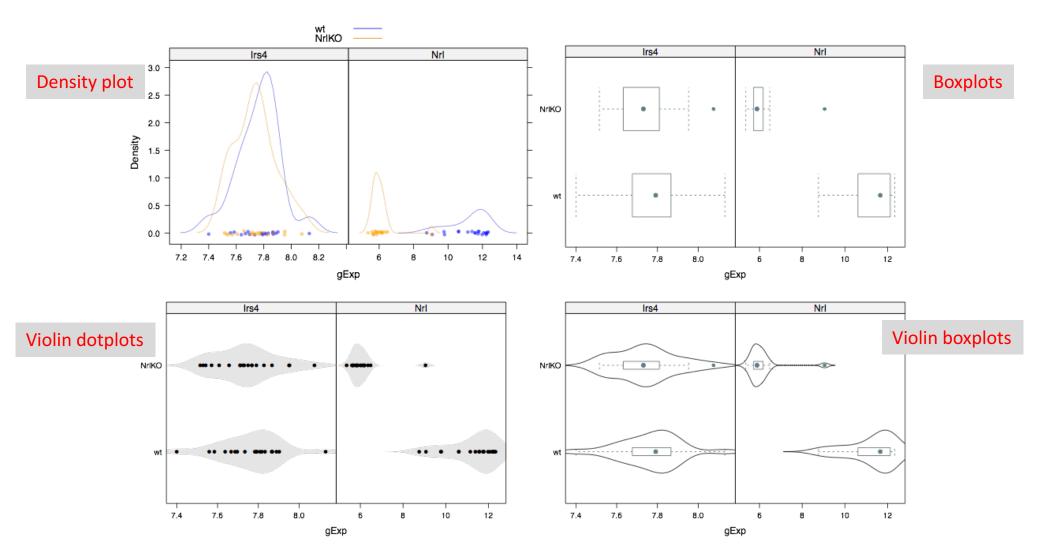
# Do we think the orange's and blue's are generated by different underlying distributions?

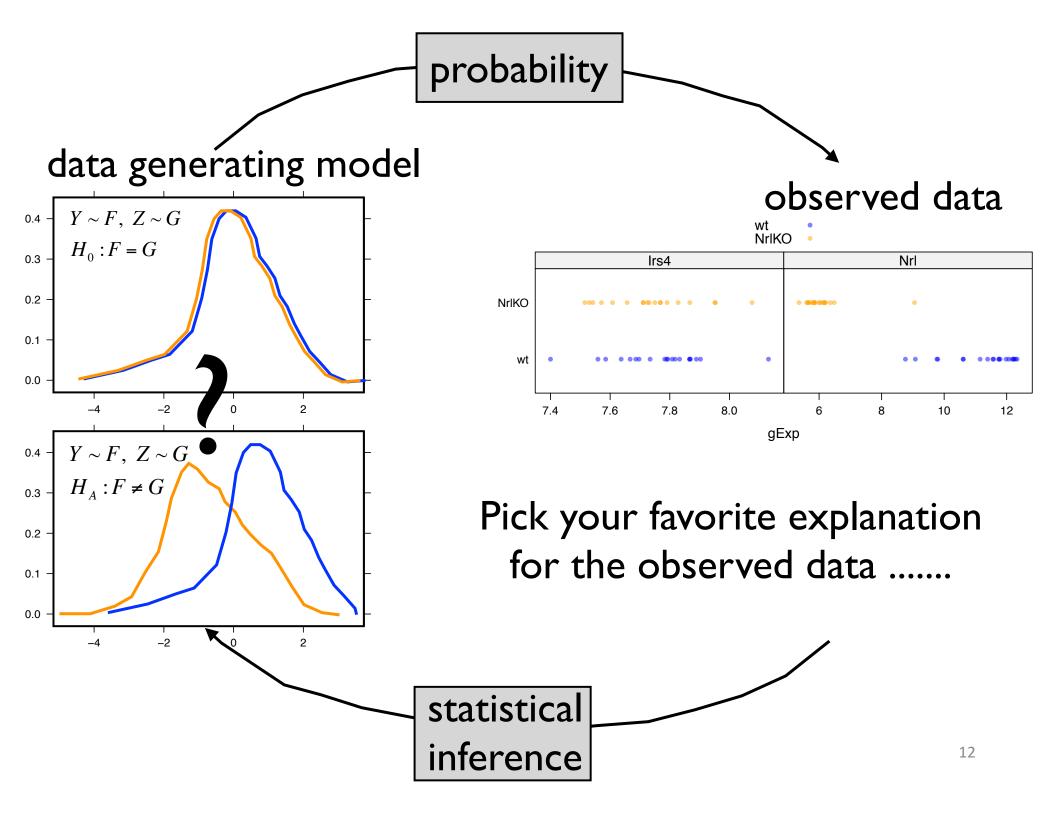


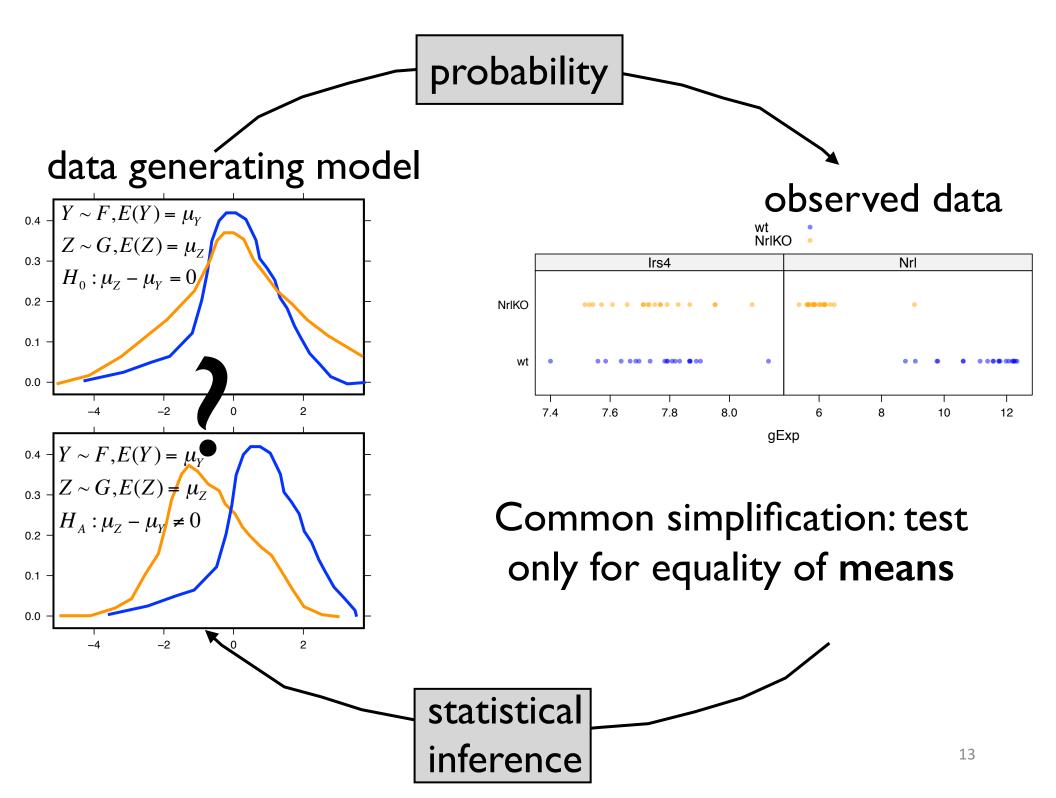
Irs4 (insulin receptor substrate 4) was selected at random as a boring non differentially expressed gene; NrIKO ~= wt Nrl (neural retina leucine zipper gene) is the gene that was knocked out in half the mice; obviously should be differentially expressed; NrlKO << wt

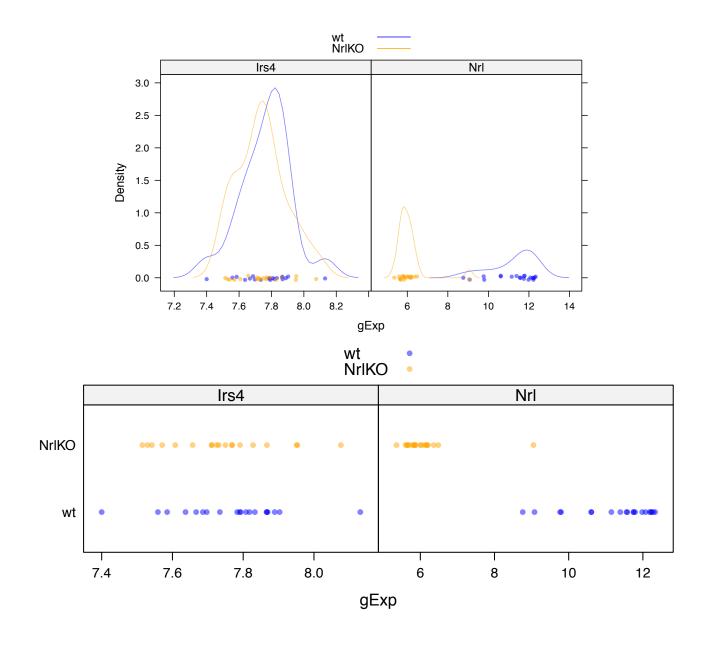
## Do we think expression of gene [Irs4|Nrl] in wild type vs. knockout mice comes from different underlying distributions?

First line of attack: let's look at the data in several ways!



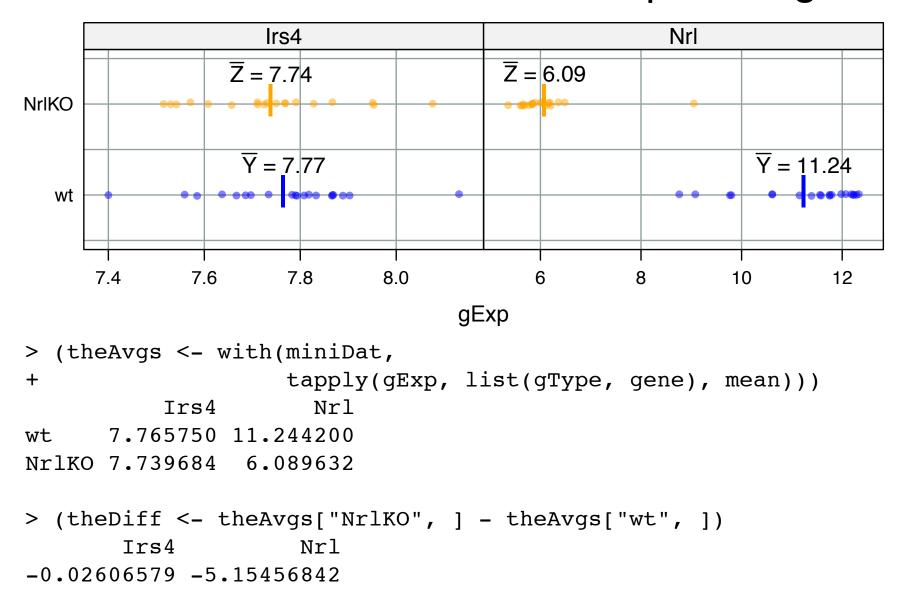






What's your quick-and-dirty best guess at  $\mu_Z - \mu_Y$ ?

#### ... the difference between the sample averages!



Are these observed differences convincing evidence that  $\mu_Z - \mu_Y \neq 0$ ?

### Are these differences convincing?

- How can we judge the size of the differences?
- The between groups difference for Irs4 looks small and for Nrl looks big...

 We judge size of differences between the group means relative to the differences within the groups (i.e., differences seen sample to sample)

### Are these differences convincing?

We need to know the background variability in the difference of sample averages under the null hypothesis that  $\mu_Z - \mu_Y = 0$ .

Then we can divide by the relevant standard deviation -- also called a standard error, in this setting -- and have a better idea.

$$\begin{split} V(\overline{Z}_n - \overline{Y}_n) &= V(\overline{Z}_n) + (-1)^2 V(\overline{Y}_n) + 2(-1) \operatorname{cov}(\overline{Y}_n, \overline{Z}_n) & \text{[I]} \\ &= V(\overline{Z}_n) + V(\overline{Y}_n) - 2 \operatorname{cov}(\overline{Y}_n, \overline{Z}_n) \\ &= V(\overline{Z}_n) + V(\overline{Y}_n) & \text{[2]} \\ &= \frac{\sigma_Z^2}{n_Z} + \frac{\sigma_Y^2}{n_Y} & \text{[3]} & \text{Note: variance of sample mean} \end{split}$$

- [1] basic probability result about variance of sums of scaled rvs
- [2] by assuming the Y's and Z's are independent from each other, we get that covariance is zero
- [3] basic result about variance of a mean of an iid sample
- \* See how independence assumptions are sprinkled everywhere?

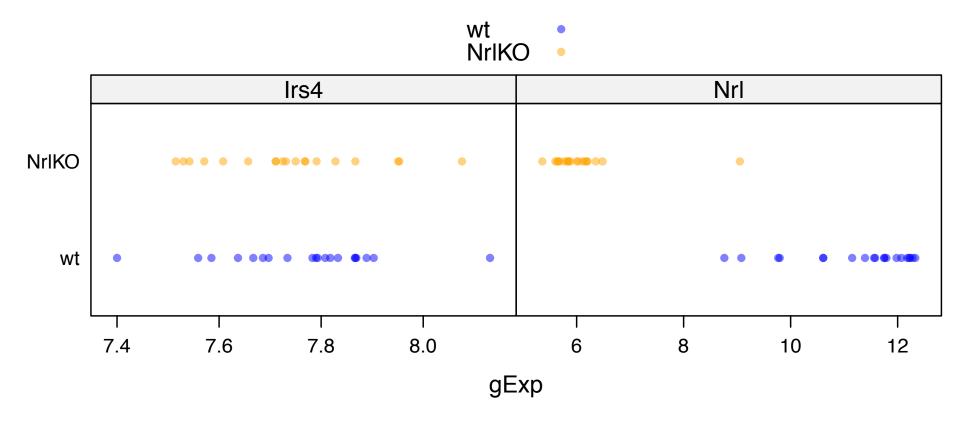
$$V(\overline{Z}_n - \overline{Y}_n) = \frac{\sigma_Z^2}{n_Z} + \frac{\sigma_Y^2}{n_Y}$$

if we assume that  $\sigma^2 Z = \sigma^2 Y = \sigma^2$ 

$$V(\overline{Z}_n - \overline{Y}_n) = \frac{\sigma^2}{n_Z} + \frac{\sigma^2}{n_Y}$$
$$= \sigma^2 \left[ \frac{1}{n_Z} + \frac{1}{n_Y} \right]$$

What's your quick-and-dirty best guess at  $\sigma^2$ ?

#### ... the sample variances (combined, somehow)!

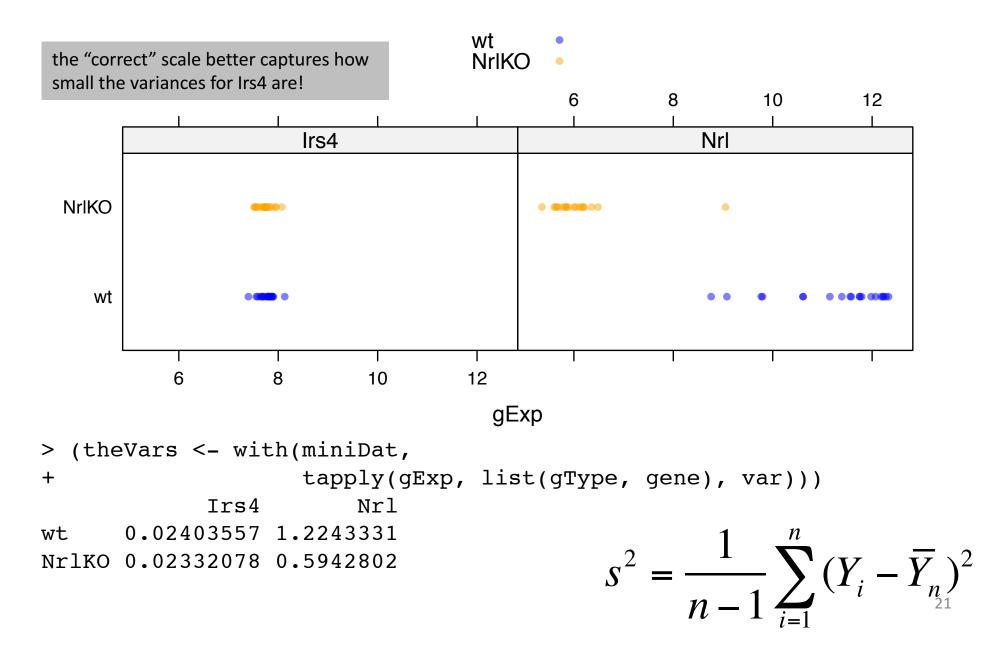


```
> (the Vars <- with (mini Dat, tapply (gExp, list (gType, gene), var)))

Irs4 Nrl

wt 0.02403557 1.2243331 0.5942802  s^2 = \frac{1}{n-1} \sum_{i=1}^{n} (Y_i - \overline{Y}_{n_2^0})^2
```

#### ... the sample variances (combined, somehow)!



Plug these sample variances into your chosen formula for the variance of the difference of sample means.

#### assuming equal variance of Y's and Z's

"pooled" 
$$\hat{\sigma}^2 = s_Y^2 \frac{n_Y - 1}{n_Y + n_Z - 2} + s_Z^2 \frac{n_Z - 1}{n_Y + n_Z - 2}$$
  
 $\hat{V}(\bar{Z}_n - \bar{Y}_n) = \text{"pooled" } \hat{\sigma}^2 \left[ \frac{1}{n_Y} + \frac{1}{n_Z} \right]$ 

assuming unequal variance of Y's and Z's

$$\hat{V}(\overline{Z}_n - \overline{Y}_n) = \hat{\sigma}_{\overline{Z}_n - \overline{Y}_n}^2 = \frac{S_Y^2}{n_Y} + \frac{S_Z^2}{n_Z}$$

```
> (nY <- with(miniDat, sum(qType == "wt" & gene == "Nrl")))</pre>
[1] 20
> (nZ <- with(miniDat, sum(qType == "NrlKO" & gene == "Nrl")))</pre>
[1] 19
                                            "pooled" \hat{\sigma}^2 = s_Y^2 \frac{n_Y - 1}{n_V + n_Z - 2} + s_Z^2 \frac{n_Z - 1}{n_V + n_Z - 2}
                                              \hat{V}(\overline{Z}_n - \overline{Y}_n) = \text{"pooled" } \hat{\sigma}^2 \left| \frac{1}{n_Y} + \frac{1}{n_Z} \right|
> (s2Pooled <- colSums(theVars * c((nY - 1) / (nY + nZ - 2),
                                                       (nZ - 1) / (nY + nZ - 2))))
 +
          Irs4
                            Nrl
 0.02368783 0.91782091
> (s2Diff <- s2Pooled * (1/nY + 1/nZ))
          Irs4
                            Nrl
 0.00243112 0.09419741
                                                                 \hat{V}(\overline{Z}_n - \overline{Y}_n) = \hat{\sigma}_{\overline{Z}_n - \overline{Y}_n}^2 = \frac{S_Y^2}{n} + \frac{S_Z^2}{n}
           > (s2DiffWelch <- colSums(theVars / c(nY, nZ)))</pre>
                      Irs4
                                          Nrl
           0.002429188 0.092494563
```

Let's calculate the relative difference by dividing the difference between the group means by our best guess at the standard deviation under the null hypothesis  $H_0$ : no difference between group means.

$$T = \frac{\overline{Z}_n - \overline{Y}_n}{\hat{\sigma}_{\overline{Z}_n - \overline{Y}_n}}$$

This permits us to describe the difference between the group means in terms of standard deviations (SD). This is a unitless quantity – and one we have encoutered before and whose properties are well studies.

We have just re-invented the t-test statistic!

$$T = \frac{\overline{Z}_n - \overline{Y}_n}{\hat{\sigma}_{\overline{Z}_n - \overline{Y}_n}}$$

R's default is to NOT assume equal variance, i.e. to perform "Welch's Two sample t-test"

```
> by(miniDat, miniDat$gene, function(theDat) {
     t.test(gExp ~ gType, theDat)
+ })
miniDat$gene: Irs4
    Welch Two Sample t-test
data: qExp by qType
t = -0.5289, df = 36.948, p-value = 0.6001
<snip, snip>
miniDat$gene: Nrl
    Welch Two Sample t-test
data: gExp by gType
t = -16.9486, df = 34.005, p-value < 2.2e-16
<snip, snip>
```

$$T = \frac{\overline{Z}_n - \overline{Y}_n}{\hat{\sigma}_{\overline{Z}_n - \overline{Y}_n}}$$

Now can we say the observed differences are "big"?

The difference is about half a standard deviation for Irs4 and 16 or 17 standard deviations for Nrl.

I predict we will conclude that true means are same for Irs4 and different for Nrl.

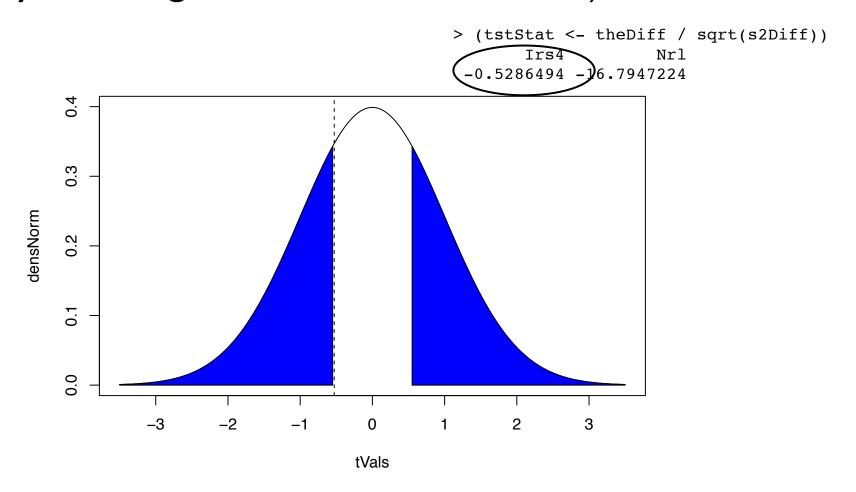
Theory now tells us specific null distributions for this test statistic, depending on your assumptions.

Willing to assume that F and G are normal distributions?

eq var 
$$T \sim t_{n_Y + n_Z - 2} \qquad \qquad T \sim t_{\text{sthg ugly}}$$
 "Welch's t test"

Unwilling to assume that F and G are normal distributions? But you feel  $n_Y$  and  $n_Z$  are "large enough"? Then go right ahead use the t dist'n above or even a normal distribution as a decent approximation.

Depicted here is the standard normal distribution (which is visually indistinguishable from t w/ 58 df).



We see that prob. of seeing a test stat as or more extreme than observed (T = -0.53) is pretty high.

```
> round(pt(-1 * abs(tstStat), df = nY + nZ - 2) * 2, 5)
   Irs4
             Nrl
0.60021 0.00000
                                                            densNorm
> round(pnorm(-1 * abs(tstStat)) * 2, 5)
   Irs4
             Nrl
0.59705 0.00000
                                                               0.1
                                                               0.0
                                                                            -2
                                                                                         0
                                                                                               1
                                                                                                     2
                                                                                                            3
                                                                      -3
                                                                                  -1
    miniDat$gene: Irs4
                                                                                        tVals
```

Two Sample t-test

Welch Two Sample t-test

# we knew we'd see extreme statistical significance for Nrl ... and we do

```
miniDat$gene: Nrl
    Two Sample t-test
data: gExp by gType
t = -16.7947, df = 37, p-value < 2.2e-16
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 4.532698 5.776439
sample estimates:
   mean in group wt mean in group NrlKO
          11,244200
                               6.089632
miniDat$gene: Nrl
    Welch Two Sample t-test
data: gExp by gType
t = -16.9486, df = 34.005, p-value < 2.2e-16
alternative hypothesis: true difference in means is not equal to 0
95 percent confidence interval:
 4.536507 5.772630
sample estimates:
   mean in group wt mean in group NrlKO
          11.244200
                               6.089632
```

In real life, working with just one (or two) genes, it's hard to believe in your gut that a difference of sample means or a two sample t statistic has a null *distribution*. It feels like it's just a particular number -- e.g. t stat = 0.53 for Irs4 in our current example.

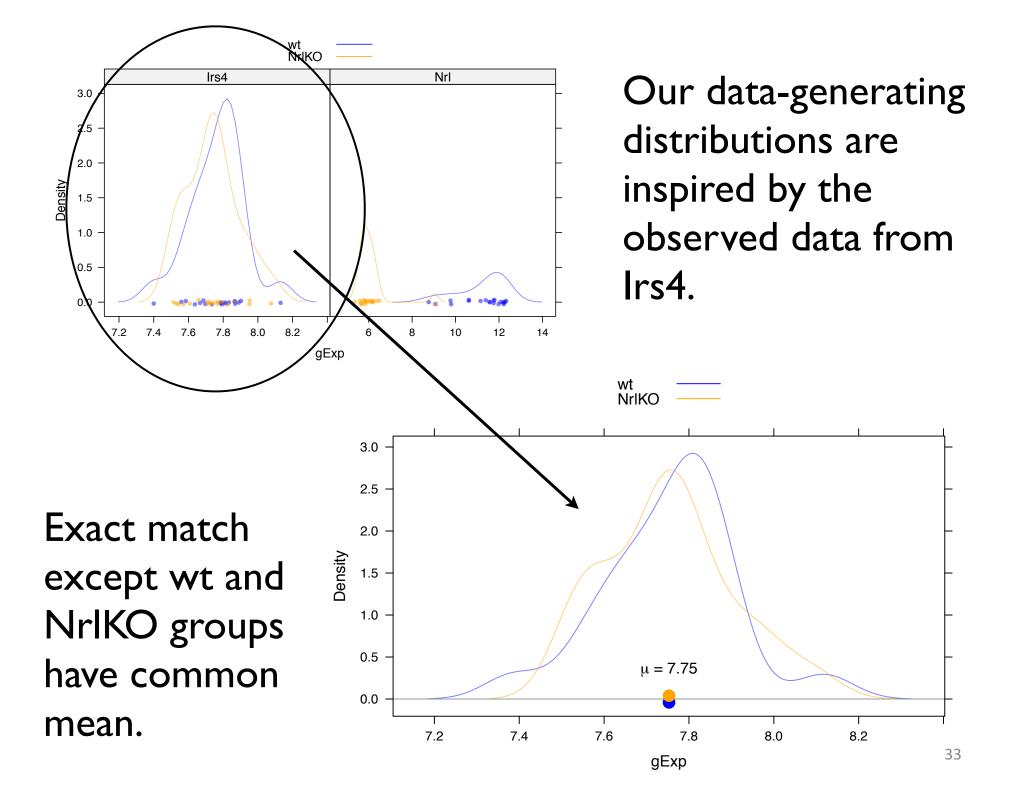
But you must think of it as a fleeting realization of a specific random variable.

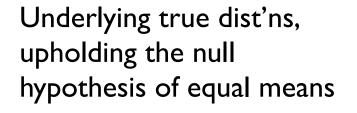
You've simply observed one of an infinity of possible values and it's the underlying null distribution that speaks to that and puts your specific observation into context.

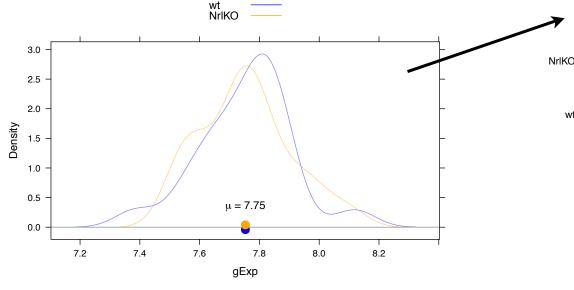
I will simulate data -- more blue Y's and more orange Z's -- and compute the observed difference of sample means and the t statistic.

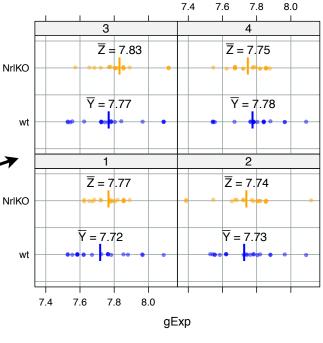
We'll compare the empirical distribution of this larger set of observations to the theoretical distributions just mentioned and used.

We'll feel really good about how this all works, at least when the assumptions truly hold.









... and many many more in silico repeats of this experiment ...

Here's the observed difference in sample means, the Welch's t statistic, and the associated p-value from the first 6 in silico datasets:

```
smDiff tStat pVal

1 -0.049219079 -1.1866161 0.2449818

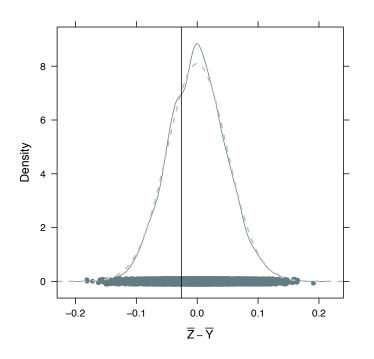
2 -0.012561184 -0.2422272 0.8099760

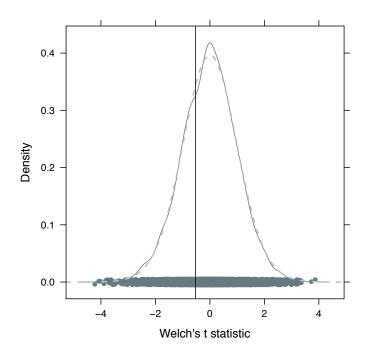
3 -0.063784868 -1.2212680 0.2298243

4 0.028180921 0.7100104 0.4827649

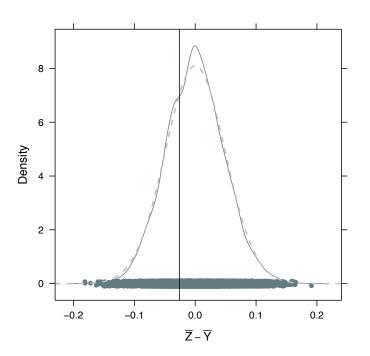
5 0.008151974 0.1881476 0.8525778

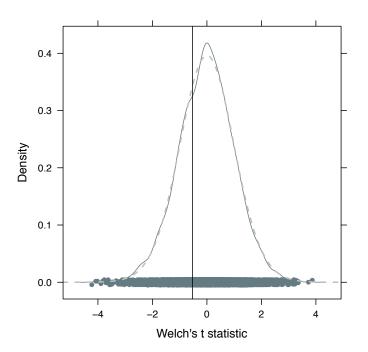
6 0.018928289 0.4349598 0.6661791
```





Empirical distribution of 10,000 observations, under the null of equal means, of the difference in sample means (left) and Welch's two sample t statistic (right). Overlaid w/ normal / t theoretical distributions (dashed line). Sample mean difference and t statistic from the real Irs4 data showed w/ vertical line.





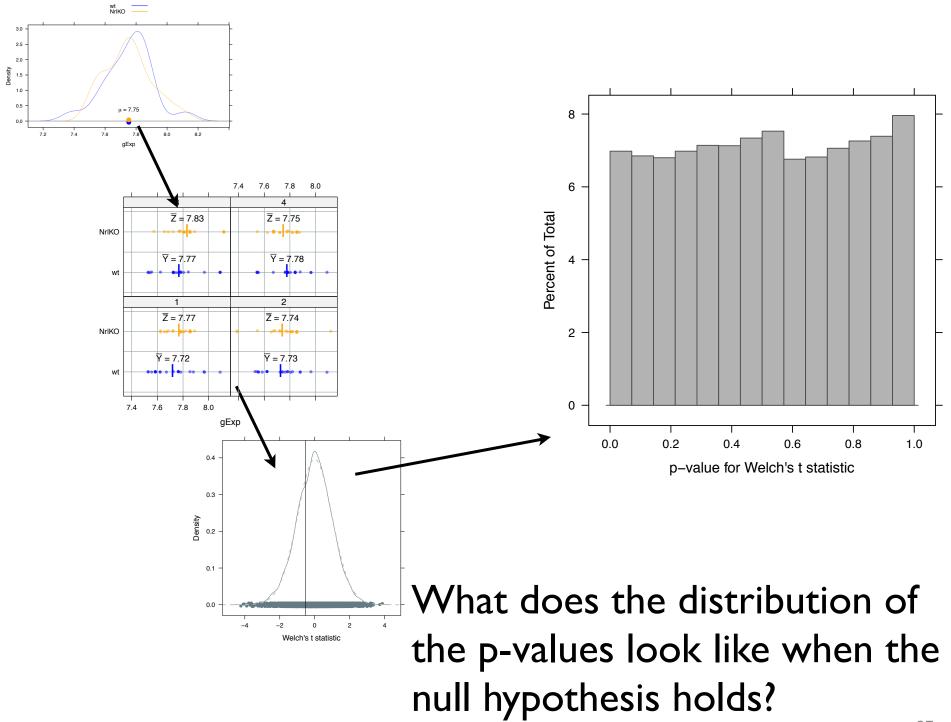
Let's sanity check the canned p-values. What proportion of these sample mean differences or Welch statistics are as or more extreme than what we observed?

```
miniDat$gene: Irs4
     Welch Two Sample t-test

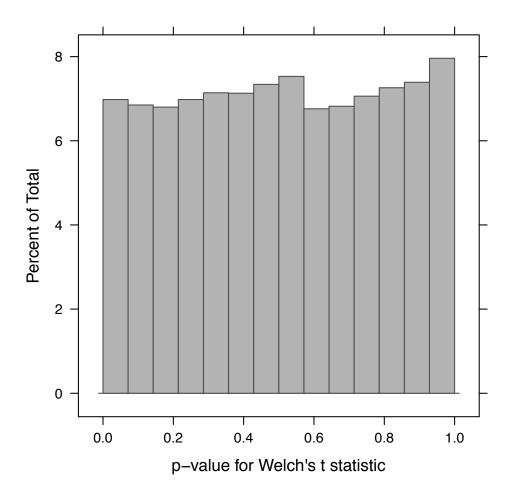
data: gExp by gType
t = 0.5289, df = 36.948, p-value = 0.6001
```

```
> mean(abs(bootTestStats$tStat) >= abs(welchStat))
[1] 0.5942
> mean(abs(bootTestStats$smDiff) >= abs(theDiff))
[1] 0.5818
```

Pretty bang on!



What does the distribution of the p-values look like when the null hypothesis holds?



$$V(\overline{X}_n - \overline{Y}_n) = \frac{\sigma_X^2}{n_X} + \frac{\sigma_Y^2}{n_Y}$$

True variance of difference of sample means depends on the underlying variance of the data and the sample sizes.

$$\hat{V}(\overline{X}_n - \overline{Y}_n) = \text{"pooled" } \hat{\sigma}^2 \left[ \frac{1}{n_X} + \frac{1}{n_Y} \right] \text{ assuming } \sigma_X^2 = \sigma_Y^2$$

$$\hat{V}(\overline{X}_n - \overline{Y}_n) = \hat{\sigma}_{\overline{X}_n - \overline{Y}_n}^2 = \frac{s_X^2}{n_X} + \frac{s_Y^2}{n_Y} \text{ assuming } \sigma_X^2 \neq \sigma_Y^2$$

Sample variance is used to estimate it.

$$T = \frac{\overline{X}_n - \overline{Y}_n}{\hat{\sigma}_{\overline{X}_n - \overline{Y}_n}} \quad \text{Under } H_0, T \sim t_{n_X + n_Y - 2} \text{ or } t_{\text{}}$$

What if the underlying variance could be reduced dramatically?

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# Less variance means same apparent effect is much more statistically significant.

```
> with(lDat,
      by(lDat, sigStat, function(yo) {
        t.test(obs ~ rv, yo)
                                                           \sigma^2 big
      }))
sigStat: big
                                                              \bigcirc
    Welch Two Sample t-test
data: obs by rv
t = 0.7314, df = 58, p-value = 0.4675
<snip, snip>
                                                                Χ
sample estimates:
mean in group x mean in group y
     0.1269433
                   -0.0618942
                                                         \sigma^2 small
sigStat: small
                                                                  Welch Two Sample t-test
data: obs by rv
t = 2.3128, df = 58, p-value = 0.02430
<snip, snip>
                                                                      Χ
sample estimates:
mean in group x mean in group y
     0.1269433
                   -0.0618942
                                               -3
                                                       -2
                                                                       0
                                                                obs
```

$$V(\overline{X}_n - \overline{Y}_n) = \frac{\sigma_X^2}{n_X} + \frac{\sigma_Y^2}{n_Y}$$

True variance of difference of sample means depends on the underlying variance of the data and the sample sizes.

$$\hat{V}(\overline{X}_n - \overline{Y}_n) = \text{"pooled" } \hat{\sigma}^2 \left[ \frac{1}{n_X} + \frac{1}{n_Y} \right] \text{ assuming } \sigma_X^2 = \sigma_Y^2$$

$$\hat{V}(\overline{X}_n - \overline{Y}_n) = \hat{\sigma}_{\overline{X}_n - \overline{Y}_n}^2 = \frac{s_X^2}{n_X} + \frac{s_Y^2}{n_Y} \text{ assuming } \sigma_X^2 \neq \sigma_Y^2$$

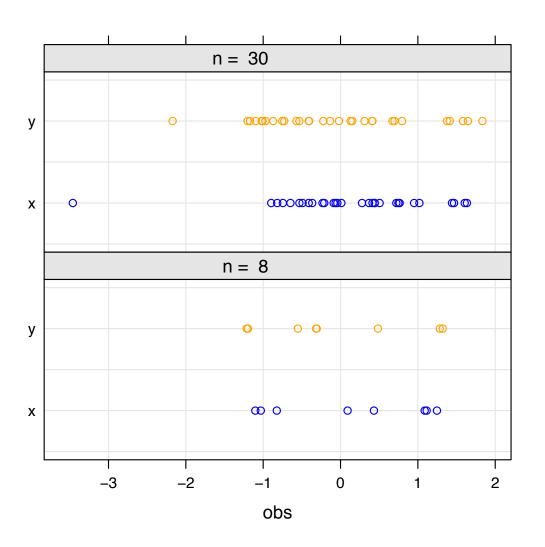
Note that sample sizes appear in denominators throughout.

$$T = \frac{\overline{X}_n - \overline{Y}_n}{\hat{\sigma}_{\overline{X}_n - \overline{Y}_n}} \quad \text{Under } H_0, T \sim t_{n_X + n_Y - 2} \text{ or } t_{\text{}}$$

What if the sample size gets cut way down?

# Smaller sample means same apparent effect is much less statistically significant.\*

```
> with(mDat,
       by(mDat, n, function(yo) {
       t.test(obs ~ rv, yo)
       }))
n: biq
    Welch Two Sample t-test
data: obs by rv
t = 0.7314, df = 58, p-value = 0.4675
<snip, snip>
sample estimates:
mean in group x mean in group y
      0.1269433
                     -0.0618942
n: small
    Welch Two Sample t-test
data: obs by rv
t = 0.3777, df = 14, p-value = 0.7113
<snip, snip>
sample estimates:
mean in group x mean in group y
      0.1269433
                     -0.0618942
```



<sup>\*</sup> I also held the sample variance constant here.

What if you don't wish to assume the underlying data is normally distributed AND you aren't sure your samples are large enough to invoke CLT?

What are alternatives to the t test?

First, one could use the t test statistic but use a bootstrap approach to obtain statistical significance. Later lecture on this. Plus, we basically demonstrated that today.

Alternatively, there are nonparametric tests that are available here:

Wilcoxon rank sum test, aka Mann Whitney, uses ranks

Kolmogorov-Smirnov uses the empirical CDF

### Wilcoxon test

Rank all the data, ignoring the grouping variable

Test stat = sum of the ranks for one group (optionally, subtract the minimum possible which is nY (nY + 1)/2)

(Alternative but equivalent formulation based on the number of yi, zi pairs for which yi >= zi)

Null distribution of such statistics can be worked out or approximated

#### miniDat\$gene: Irs4

Wilcoxon rank sum test with continuity correction

data: gExp by gType

W = 220.5, p-value = 0.3992

alternative hypothesis: true location shift is not equal to 0

\_\_\_\_\_

miniDat\$gene: Nrl

Wilcoxon rank sum test with continuity correction

data: gExp by gType

W = 379, p-value = 1.178e-07

alternative hypothesis: true location shift is not equal to 0

#### miniDat\$gene: Irs4

Welch Two Sample t-test

data: gExp by gType t = 0.5289, df = 36.948, p-value = 0.6001

<snip, snip>

-----

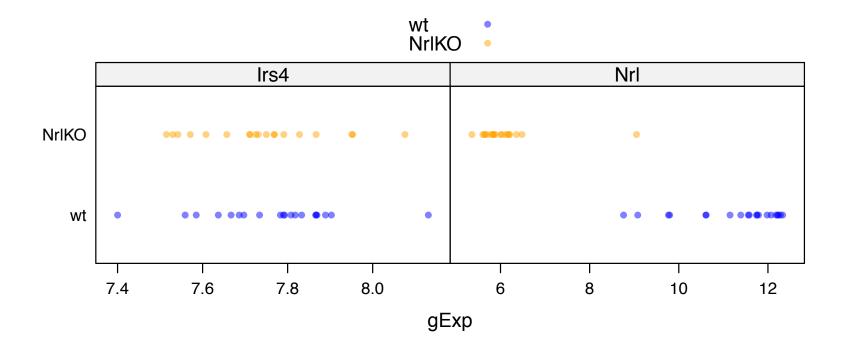
#### miniDat\$gene: Nrl

Welch Two Sample t-test

data: gExp by gType

t = 16.9486, df = 34.005, p-value < 2.2e-16

<snip, snip>



Kolmogorov-Smirnov test (two sample)

Null hypothesis: F = G, i.e. distributions are same

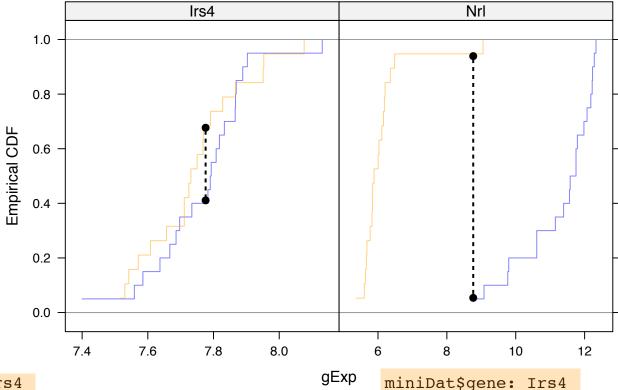
Estimate each CDF with the empirical CDF (ECDF)

$$\hat{F}(x) = \frac{1}{n} \sum_{i} I[x_i \le x]$$

Test statistic is the maximum of the absolute difference between the ECDFs

$$\max \left| \hat{F}(x) - \hat{G}(x) \right|$$

Null distribution does not depend on F, G (!) (I'm suppressing detail here.)



miniDat\$gene: Irs4

Two-sample Kolmogorov-Smirnov test

data: theDat\$gExp[theDat\$gType == "wt"] and theDat \$qExp[theDat\$qType == "NrlKO"] D = 0.2842, p-value = 0.4107 alternative hypothesis: two-sided

miniDat\$gene: Nrl

Two-sample Kolmogorov-Smirnov test

data: theDat\$qExp[theDat\$qType == "wt"] and theDat \$gExp[theDat\$gType == "NrlKO"] D = 0.95, p-value = 4.603e-08alternative hypothesis: two-sided

Welch Two Sample t-test

data: gExp by gType t = 0.5289, df = 36.948, p-value = 0.6001 <snip, snip>

miniDat\$gene: Nrl

Welch Two Sample t-test

data: qExp by qType t = 16.9486, df = 34.005, p-value < 2.2e-16 47

<snip, snip>

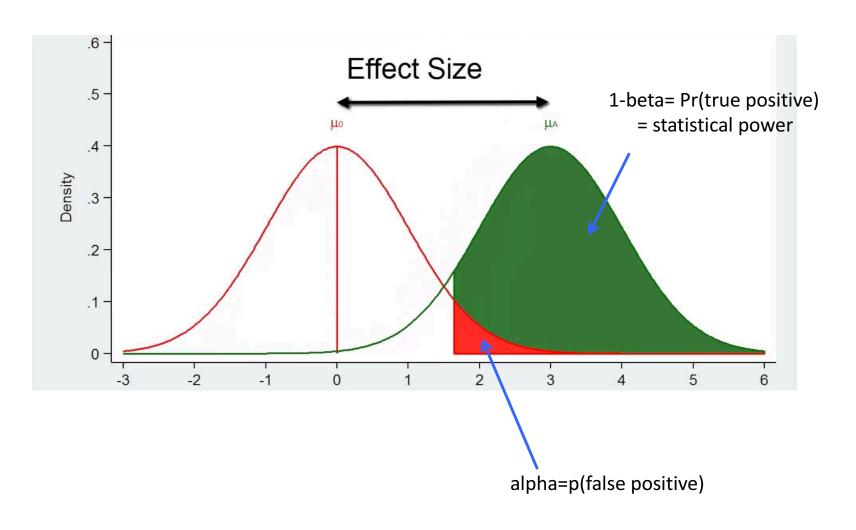
## Errors in hypothesis testing

	Actual Situation "Truth"	
Decision	H <sub>o</sub> True	H <sub>o</sub> False
Don Not Reject H <sub>0</sub>	Correct Decision 1-α	Incorrect Decision Type II Error B
Reject H <sub>0</sub>	Incorrect Decision Type I Error α	Correct Decision 1-β

$$\alpha$$
 = P(Type I Error)  $\beta$  = P(Type II Error)  
Power = 1 -  $\beta$ 

## Statistical power

The probability that your experiment will generate data (a result) that is convincing enough to lead you to reject the null hypothesis



### Statistical Power

- Power must be low when  $H_0$  is true
  - Think about it...
- Power will be higher when
  - The true difference is larger
  - The sample size is larger
  - The within-group variability is smaller