# Lecture 5 – Two Group Comparisons

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

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# Central dogma of statistics

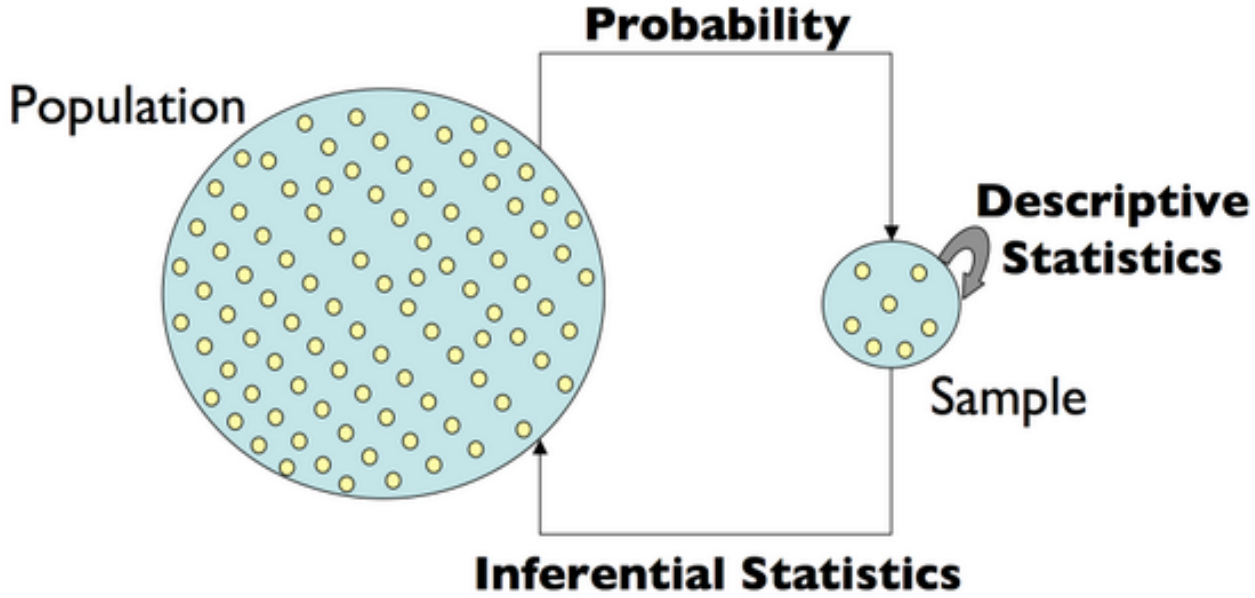



Image source: Josh Akey's Lecture notes

We want to understand a **population** (e.g., gene behaviour) but we can only study a **random sample** from it.

# Book and online resources

- Modern Statistics for Modern Biology by Susan Holmes and Wolfgang Huber, 2019 (free online book)
- Data Analysis for the Life Sciences by Rafael Irizarry and Michael Love, 2015 (free online book)
- Practical Regression and Anova using R by Julian Faraway, 2002 (free online book)
- Linear Models with R by Julian Faraway, Chapman & Hall/CRC Texts in Statistical Science, 2004

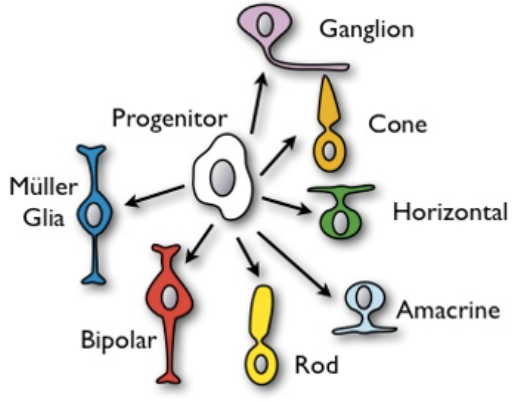
# Hypothesis Testing in Genomics

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

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

Akimoto et al. (2006)

- Retina presents a model system for investigating **regulatory networks** underlying neuronal differentiation.
- **Nrl** transcription factor is known to be important for Rod development




- **What happens if you delete Nrl?**

# Why a Hypothesis Test?

From the Akimoto et al. (2006) paper:

> "we hypothesized that Nrl is the ideal transcription factor to gain insights into gene expression changes ..."

**Biological question:** Is the expression level of gene A affected by knockout of the *Nrl* gene?

We can use statistical inference to answer this biological question!

# Statistical inference

**Statistical inference**:

We observe and study a random sample to make conclusions about a population (e.g., random sample of gene expressions from mice)

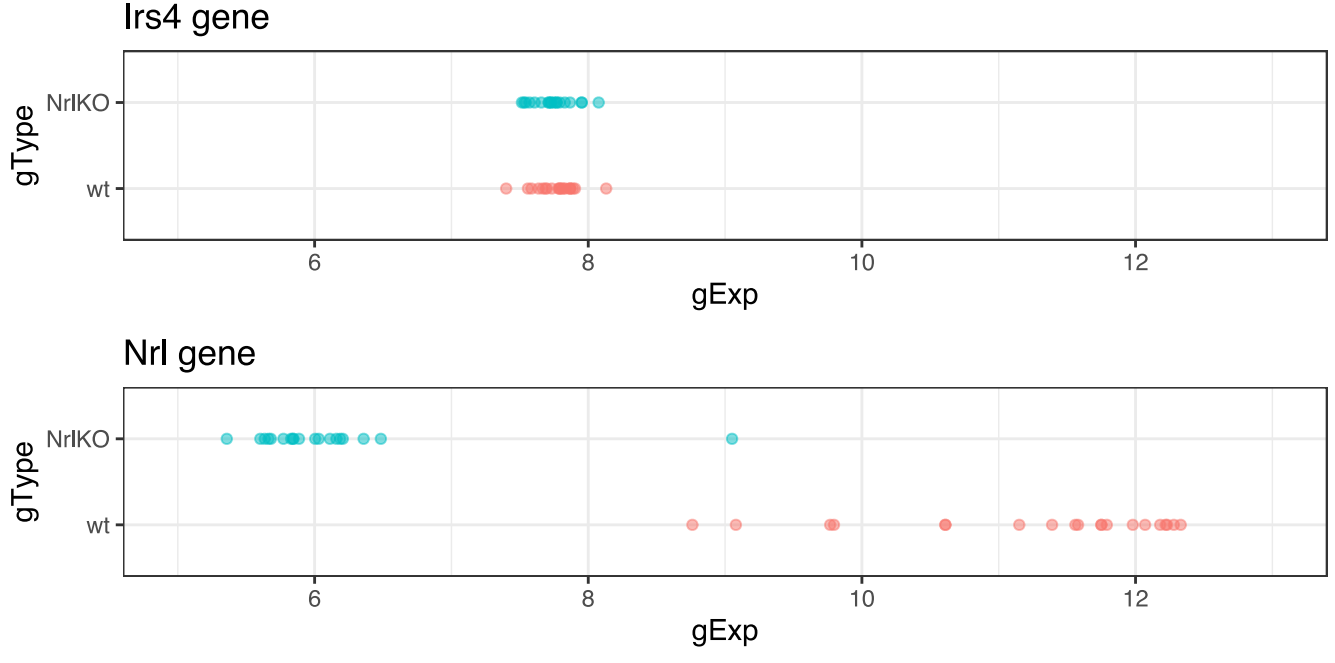
**Experimental design**:

- 4 developmental stages
- 2 genotypes: Wild type (WT), Nrl Knockout (NrlKO)
- 3-4 replicates for each combination

Let's take a look at 2 genes as an example: **Irs4 and Nrl**

**Biological question:** Are these genes truly different in NrlKO compared to WT?

We can't answer this question in general. We can *only* study these genes in collected data:



# Statistical Hypothesis

**Experimental design:**

- 2 conditions: WT *vs* NrlKO
- random sample: we observe the expression of many genes in all mice

**Biological hypothesis:** for *some* genes, the expression levels are different between conditions.

**Statistical hypotheses:** (for each gene $g = 1, \ldots, G$)

- $H_0$ (null hypothesis): the expression level of gene $g$ is the *same* in both conditions.
- $H_A$ (alternative hypothesis): the expression level of gene $g$ is *different* between conditions.

# Notation

## Random variables and estimates (we can observe):

$Y_i$ : expression of gene $g$ in the WT sample $i$

$Z_i$: expression of gene $g$ in NrlKO sample $i$

$Y_1, Y_2, \ldots, Y_{n_Y}$ : a random sample of size $n_Y$

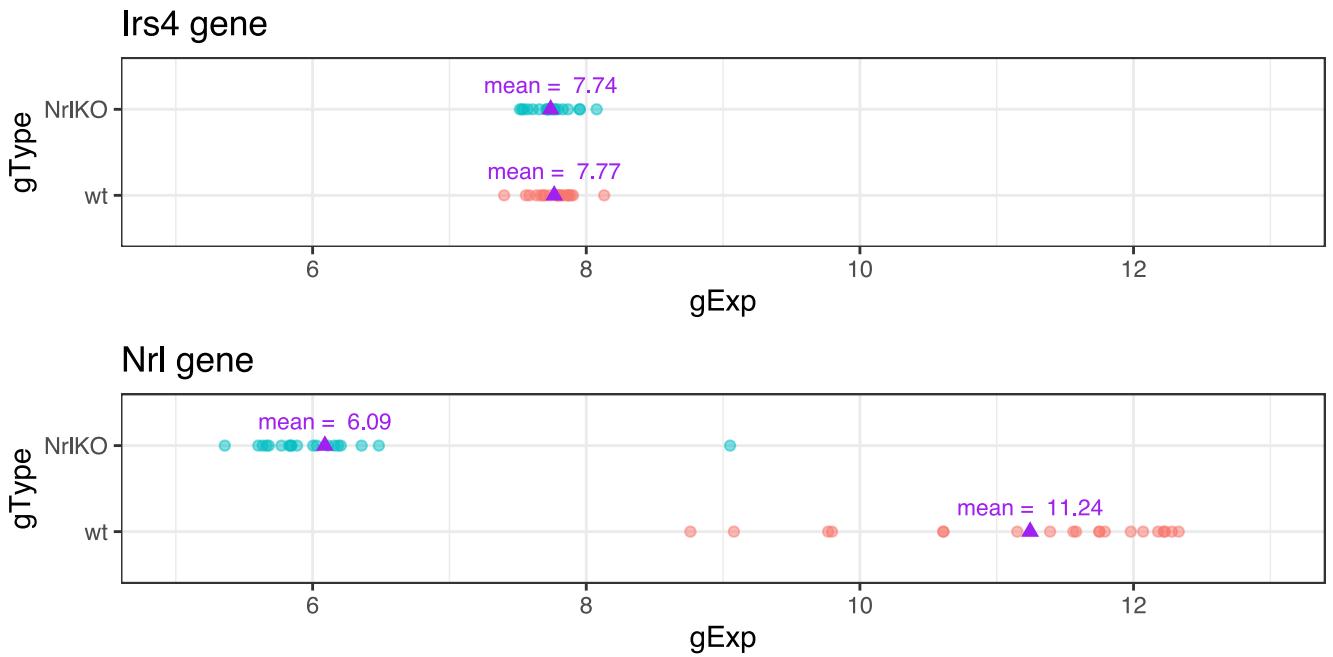
$\bar{Y} = \frac{\sum_{i=1}^{n_Y} Y_i}{n_Y}$: sample mean of gene $g$ expression from WT mice

## Population parameters (unknown/unobservable):

$\mu_Y = E[Y]$ : the (population) expected expression of gene $g$ in WT mice

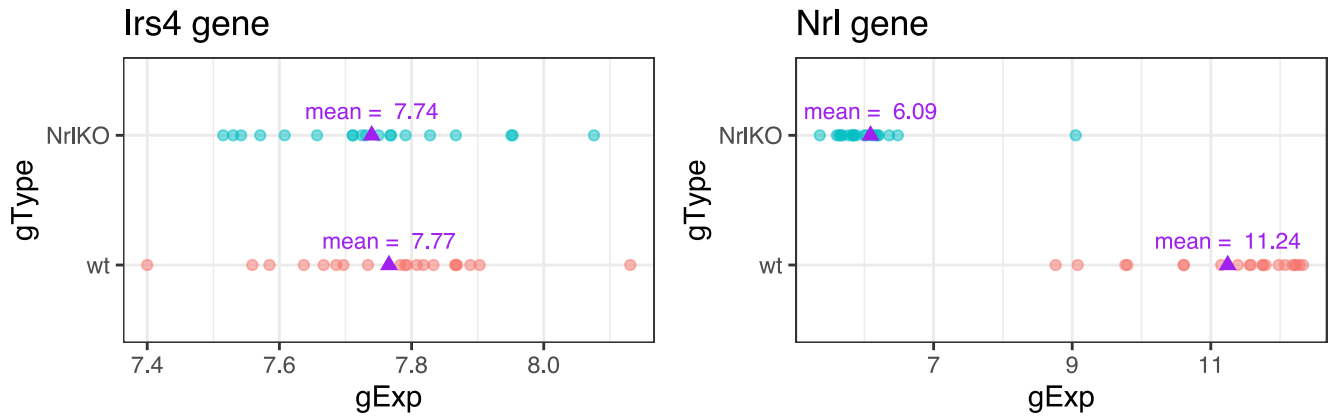
# Is there **enough** evidence in the data to reject $H_0$?

$$H_0 : \mu_Y = \mu_Z$$




**Statistical Inference**: random samples are used to learn about the population

# What we observe: the difference between the **sample averages**: $\bar{Y}$ vs $\bar{Z}$



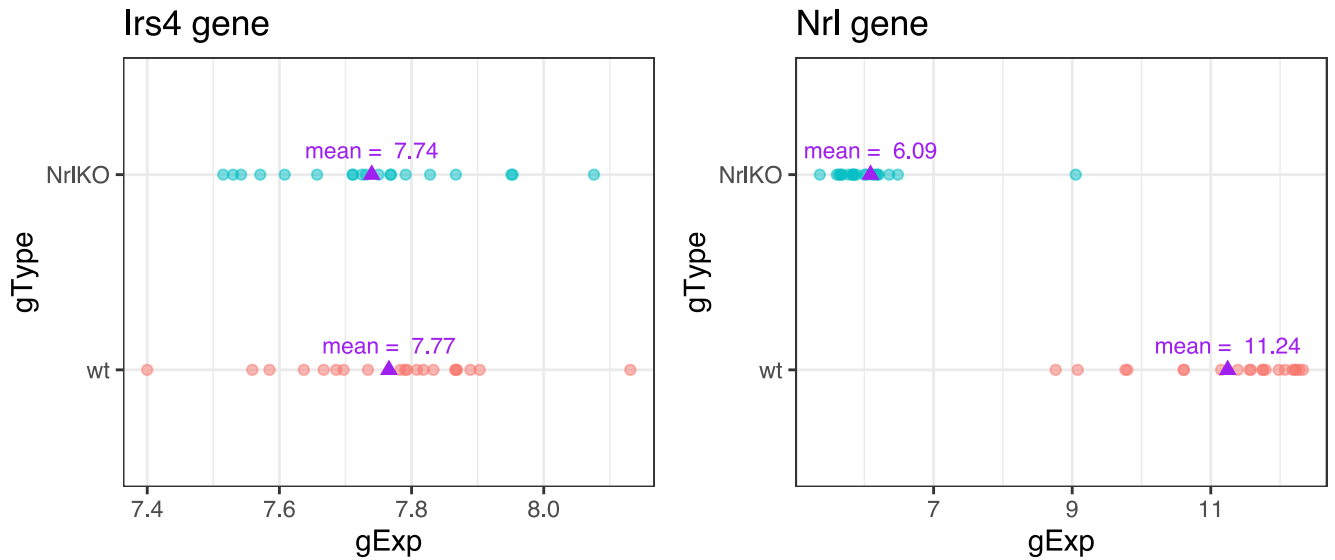

```
theAvgs <- with(miniDat,
                tapply(gExp, list(gType, gene), mean))
```

```
##         Irs4    Nrl
## wt    7.766 11.244
## NrlKO 7.740  6.090
```

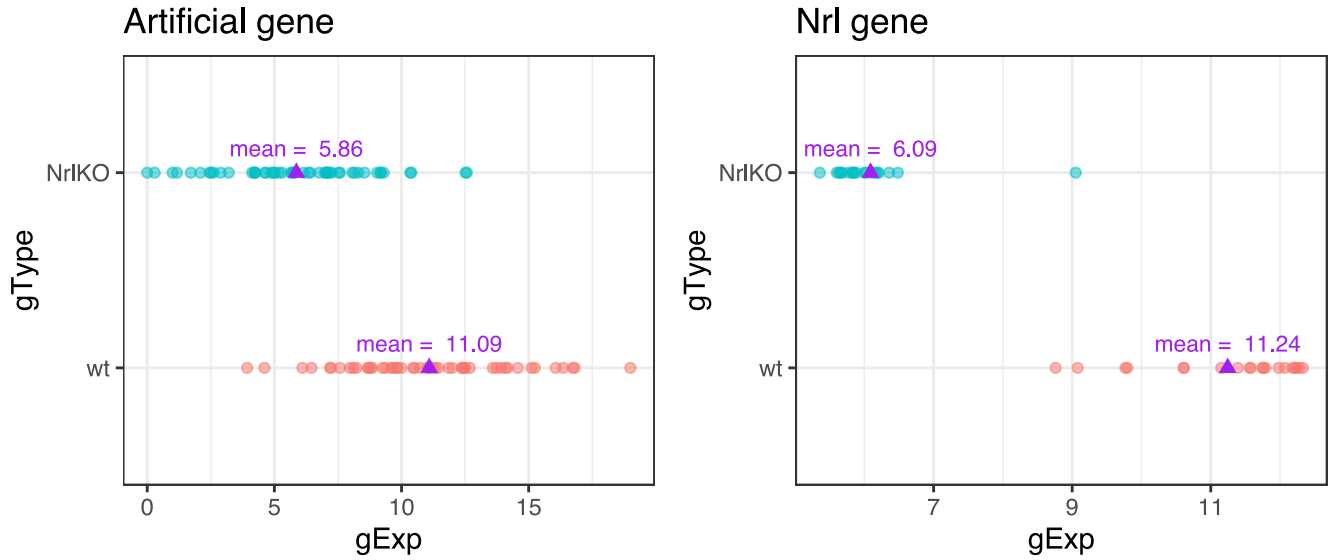
```
theDiff <- theAvgs["NrlKO", ] - theAvgs["wt", ]
```

```
##   Irs4    Nrl
## -0.026 -5.155
```

## Is the difference between $\bar{Y}$ and $\bar{Z}$ informative to reject $H_0$?




- The sample means, $\bar{Y}$ vs $\bar{Z}$, by themselves are not enough to make conclusions about the population
- What is a "large" difference? "large" relative to what?



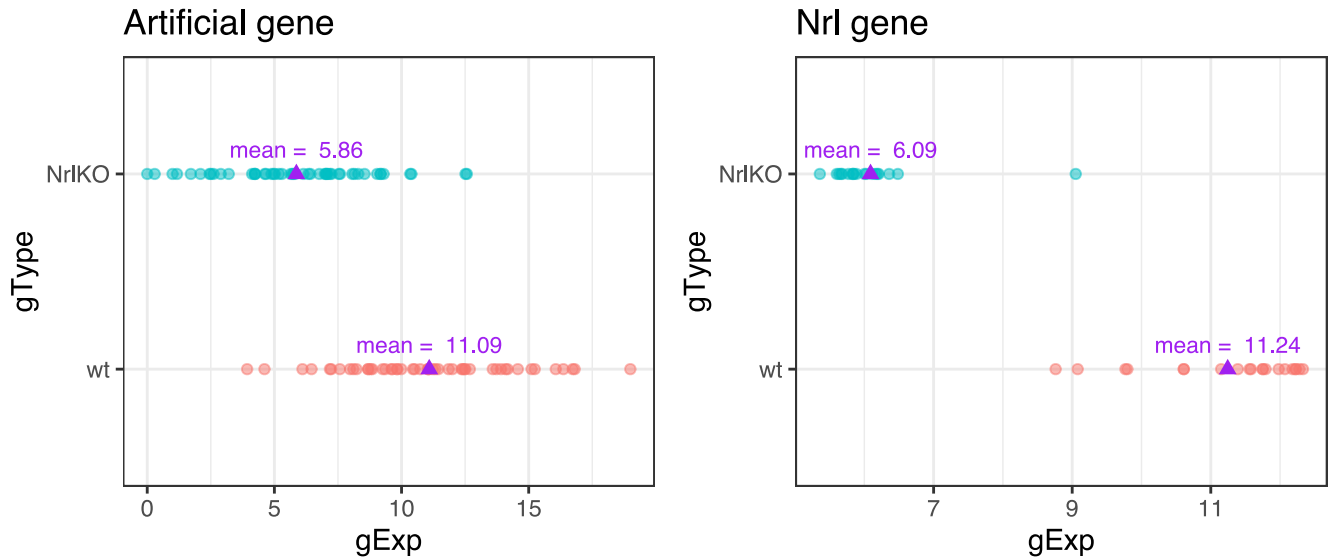

What can we use to interpret the size of the mean difference?

$$\frac{\bar{Y} - \bar{Z}}{??}$$

# What can we use to interpret the size of the mean difference?

"large" relative to the observed variation

$$\frac{\bar{Y}-\bar{Z}}{\sqrt{Var(\bar{Y}-\bar{Z})}}$$



# Quantifying observed variation

- Recall that if $Var(Y_i) = \sigma_Y^2$, then $Var(\bar{Y}) = \frac{\sigma_Y^2}{n_Y}$
- Assume that the random variables within each group are *independent and identically distributed* (iid), and that the groups are independent. More specifically, that

  1. $Y_1, Y_2, \ldots, Y_{n_Y}$ are iid,
  2. $Z_1, Z_2, \ldots, Z_{n_Z}$ are iid, and
  3. $Y_i, Z_j$ are independent. Then, it follows that

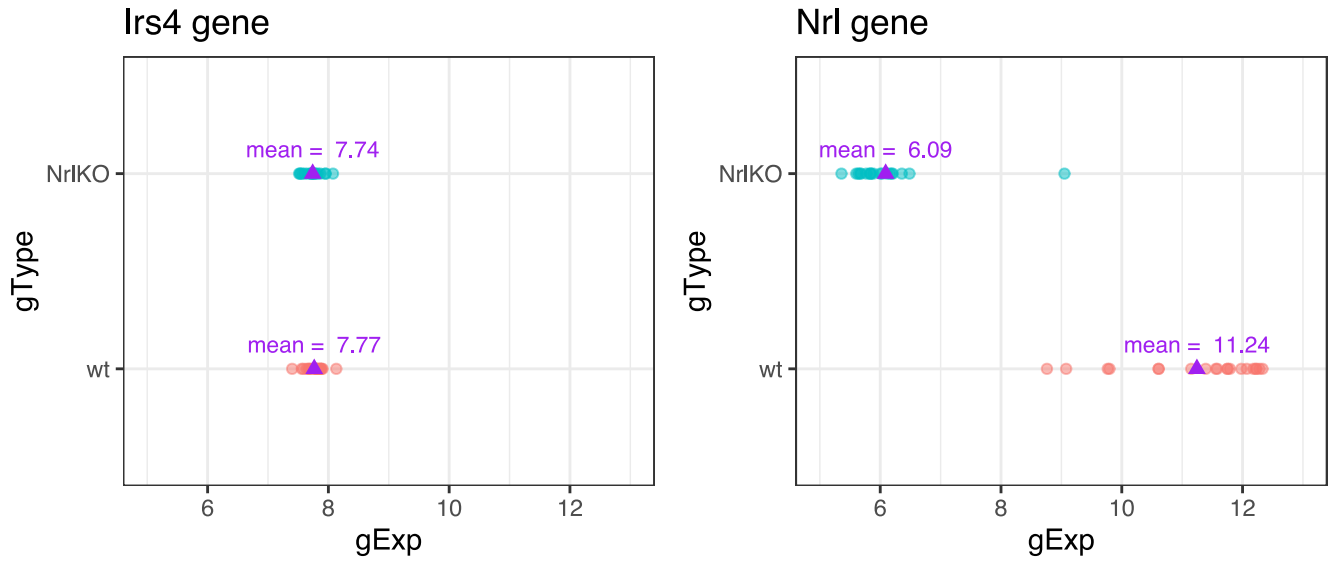
$$Var(\bar{Z} - \bar{Y}) = \frac{\sigma_Z^2}{n_Z} + \frac{\sigma_Y^2}{n_Y}$$

- If we also assume equal population variances: $\sigma_Z^2 = \sigma_Y^2 = \sigma^2$, then

$$Var(\bar{Z} - \bar{Y}) = \frac{\sigma_Z^2}{n_Z} + \frac{\sigma_Y^2}{n_Y} = \sigma^2 \left[\frac{1}{n_Z} + \frac{1}{n_Y}\right]$$

But how can we calculate population variance $\sigma$ if it is **unknown**?

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




```
theVars <- with(miniDat,
                tapply(gExp, list(gType, gene), var))
```

```
##          Irs4   Nrl
## wt     0.024 1.224
## NrlKO 0.023 0.594
```

e.g., for Nrl: $\hat{\sigma}^2_Y = S^2_Y = \frac{1}{n_Y}\sum_{i=1}^{n_Y}(Y_i - \bar{Y})^2 = 1.224$

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

$$\hat{\sigma}^2_{\text{pooled}} = S^2_Y \frac{n_Y - 1}{n_Y + n_Z - 2} + S^2_Z \frac{n_z - 1}{n_Y + n_Z - 2}$$

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

Assuming **unequal** variance of Y's and Z's

$$\hat{V}(\bar{Z}_n - \bar{Y}_n) = \hat{\sigma}^2_{\bar{Z}_n - \bar{Y}_n} = \frac{S^2_Y}{n_Y} + \frac{S^2_Z}{n_Z}$$

> Note: the 'hat' (^) is used to distinguish an 'estimate' from a 'parameter'.

# The Test Statistic: $T = \frac{\bar{Z}_n - \bar{Y}_n}{\hat{\sigma}_{\bar{Z}_n - \bar{Y}_n}}$

Assuming equal variances:

```
tstStat <- theDiff / sqrt(s2Diff)
```

```
##    Irs4     Nrl
##  -0.529 -16.795
```

Without assuming equal variances:

```
welchStat <- theDiff / sqrt(s2DiffWelch)
```

```
##    Irs4     Nrl
##  -0.529 -16.949
```

### Can we now say that the observed differences are 'big'?

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

The test statistic T is a random variable because it's based on our random sample.

We need a measure of its **uncertainty** to determine how big T is:

> If we were to repeat the experiment many times, what's the probability of observing a value of T **as extreme** as the one we observed?

We need to have a probability distribution!

However, this is unknown to us so we need to **make more assumptions**.

Theory now tells us specific **null distributions** for these test statistics, depending on your assumptions.

Let's call the unknown probability distributions F and G ($Y_i \sim F,$ and $Z_i \sim G$)

$\Rightarrow$ Willing to assume that F and G are normal distributions?

2-sample *t*-test:

(equal variances)

$$T \sim t_{n_Y+n_Z-2}$$

Welch test:

(unequal variances)

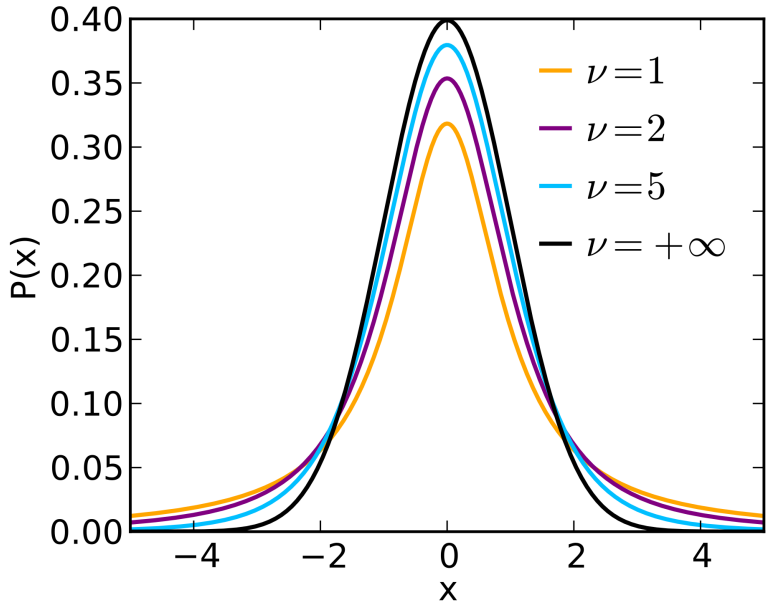
$$T \sim t_{<something\ ugly>}$$

$\Rightarrow$ Unwilling to assume that F and G are normal distributions? But you feel that $n_Y$ and $n_Z$ are large enough?

Then the t-distributions above or even a normal distribution are decent approximations.

# Student's *t*-distribution

Recall that T is a **random variable**. Under certain assumptions, we can prove that T follows a *t*-distribution.




where df = degrees of freedom.

# Hypothesis testing

1. Formulate your hypothesis as a statistical hypothesis:

$$H_0 : \mu_Y = \mu_Z \text{ vs } H_A : \mu_Y \neq \mu_Z$$

2a. Define a **test statistic**: 2-sample *t*-test

2b. Compute the observed value for the test statistic:

```
tstStat <- theDiff / sqrt(s2Diff)
```

```
##    Irs4     Nrl
##  -0.529 -16.795
```

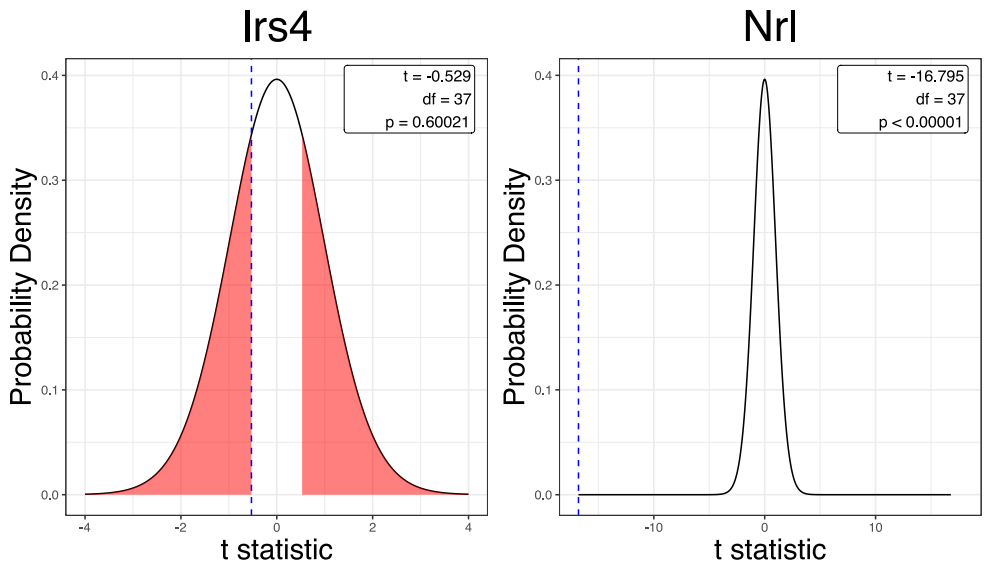
3. Compute the probability of seeing a test statistic at least as extreme as that observed, under the **null sampling distribution** (this is the definition of the p-value)

```
## miniDat$gene: Irs4
## [1] 0.6002058
## ------------------------------------------------------------
## miniDat$gene: Nrl
## [1] 6.764663e-19
```

In other words, assuming that $H_0$ is true:

For Irs4, the probability of seeing a test statistic as extreme as that observed $(t = -0.53)$ is pretty high $(p = 0.6)$.

But for Nrl, the probability of seeing a test statistic as extreme as that observed $(t = -16.8)$ is extremely low $(p = 6.76 \times 10^{-19})$



## 4. Make a decision about significance of results, based on a pre-specified value (alpha, significance level)

The significance level $\alpha$ is usually set at 0.05. However, this value is arbitrary and usually depends on the study.

Using $\alpha = 0.05$, since the p-value for the Irs4 test is greater than 0.05, we conclude that there is not *enough* evidence in the data to claim that Irs4 has a differential expression in WT compared to Nrl models.

We do not reject $H_0$!

## Assuming equal variances: *t*-test

```
> by(miniDat, miniDat$gene, function(theDat) {
+     t.test(gExp ~ gType, theDat, var.equal = TRUE)
+ })
miniDat$gene: Irs4

     Two Sample t-test

data:  gExp by gType
t = -0.5286, df = 37, p-value = 0.6002
<snip, snip>
------------------------------------------
miniDat$gene: Nrl

     Two Sample t-test

data:  gExp by gType
t = -16.7947, df = 37, p-value < 2.2e-16
```

## Without assuming equal variances: Welch *t*-test

```
> by(miniDat, miniDat$gene, function(theDat) {
+     t.test(gExp ~ gType, theDat)
+ })

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
```

## What is a p-value?

> Likelihood of obtaining a test statistic at least as extreme as the one observed, given that the null hypothesis is true (we are making a conditional probability statement)

## What is a p-value NOT?

- Not the probability that the null hypothesis is true
- Not the probability that the finding is a “fluke”
- Not the probability of falsely rejecting the null
- Does not indicate the size or importance of observed effects.

*[Credit to Dr. Fowler, UW]*

# "Genome-wide" testing of differential expression

- In genomics, we often perform thousands of statistical tests (e.g., a *t*-test per gene)
- The distribution of p-values across all tests provide good diagnostics/insights.
- Is it uniform (should be in most experiments) and if not, is the departure from uniform expected based on biological knowledge?

## Different kinds of *t*-tests:

- One sample *or* **two samples**
- One-sided *or* **two sided**
- Paired *or* **unpaired**
- **Equal variance** *or* unequal variance

# Types of Errors in Hypothesis Testing

| Decision | Actual Situation "Truth": $H_0$ True | $H_0$ False |
|---|---|---|
| Do Not Reject $H_0$ | Correct Decision<br>1-α | Incorrect Decision<br>Type II Error<br>β |
| Reject $H_0$ | Incorrect Decision<br>Type I Error<br>α | Correct Decision<br>1-β |

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

# $H_0$: "*Innocent until proven guilty*"

- The default state is $H_0 \rightarrow$ we only reject if we have enough evidence
- If $H_0$: Innocent and $H_A$: Guilty, then
  - Type I Error $(\alpha)$: Wrongfully convict innocent (*False Positive*)
  - Type II Error $(\beta)$: Fail to convict criminal (*False Negative*)

> 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 compute its p-value. We will cover this later on.

Alternatively, there are *non-parametric* tests that are available here:

- **Wilcoxon rank sum test**, aka Mann Whitney, uses ranks to test differences in population means.
- **Kolmogorov-Smirnov test** uses the empirical CDF to test differences in population cumulative distributions.

# Wilcoxon rank sum test

Rank all data, ignoring the **grouping** variable

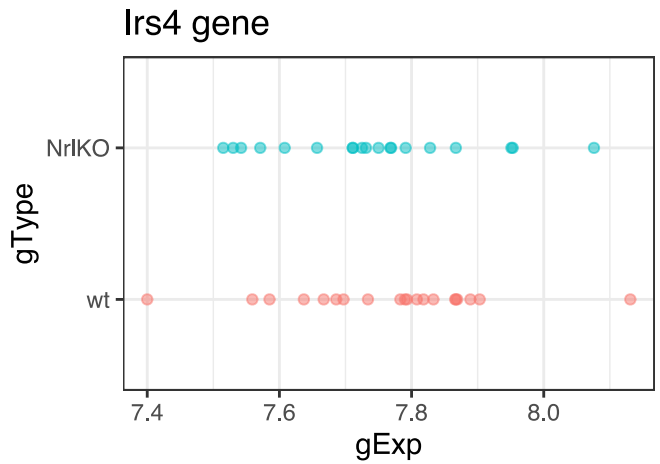
**Test statistic** = sum of the ranks for one group (optionally, subtract the minimum possible which is $\frac{n_Y(n_Y+1)}{2}$)

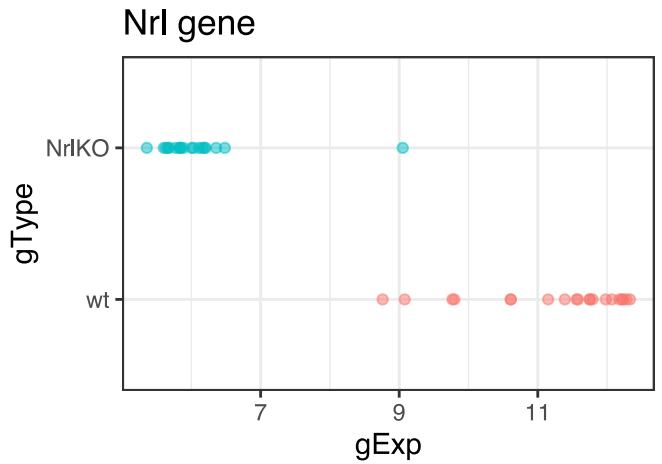
(Alternative but equivalent formulation based on the number of $y_i$, $z_i$ pairs for which $y_i \geq z_i$)

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. the distributions are the same

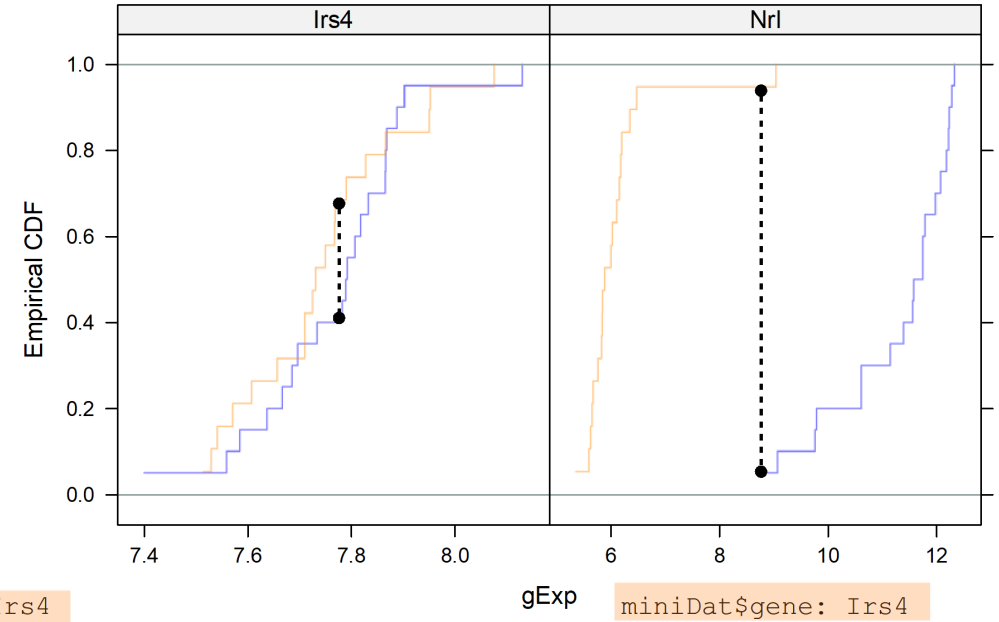
Estimate each CDF with the empirical CDF (ECDF)

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

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

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

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




```
miniDat$gene: Irs4

    Two-sample Kolmogorov-Smirnov test

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

```
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

    Two-sample Kolmogorov-Smirnov test

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

```
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>
```

# Discussion and questions ...

What if you are unsure whether your sample size is large enough? Outliers with small samples could be problematic

Which test result should one report ... the 2-sample *t*-test, the Wilcoxon, or the KS?

Treat p-values as one type of evidence that you should incorporate with others.

It is worrisome when methods that are equally appropriate and defensible give very different answers.