# Differential gene expression

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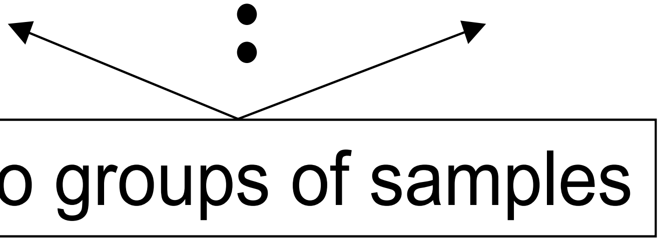
Slides partly adapted from S. Dudoit, Bioconductor short course

# Differential gene expression

Given: matrix of normalized expression data

| | samples | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| genes | 0.2 | 1.1 | -1.5 | 2.0 | 0.8 | 0.1 | -0.7 | 1.4 |
| | -0.7 | -0.1 | 0.4 | 1.1 | 1.3 | 1.7 | 0.8 | 1.5 |
| | 0.8 | 0.3 | -0.6 | -0.4 | -0.6 | 0.1 | -0.2 | -0.3 |
| | 0.2 | -0.5 | 0.3 | 0.9 | -1.2 | -0.7 | 0.5 | -0.2 |

⋮



two groups of samples

Which genes are differentially expressed between the groups?

# Identifying differentially expressed genes

- Aim: find genes that are differentially expressed between different conditions/phenotypes, e.g. two different tumor types.

- Estimate effects/differences between groups by (generalized) log–ratio, i.e., the difference between group means on the log scale.

- To assess the statistical significance of differences, conduct a statistical test for each gene.

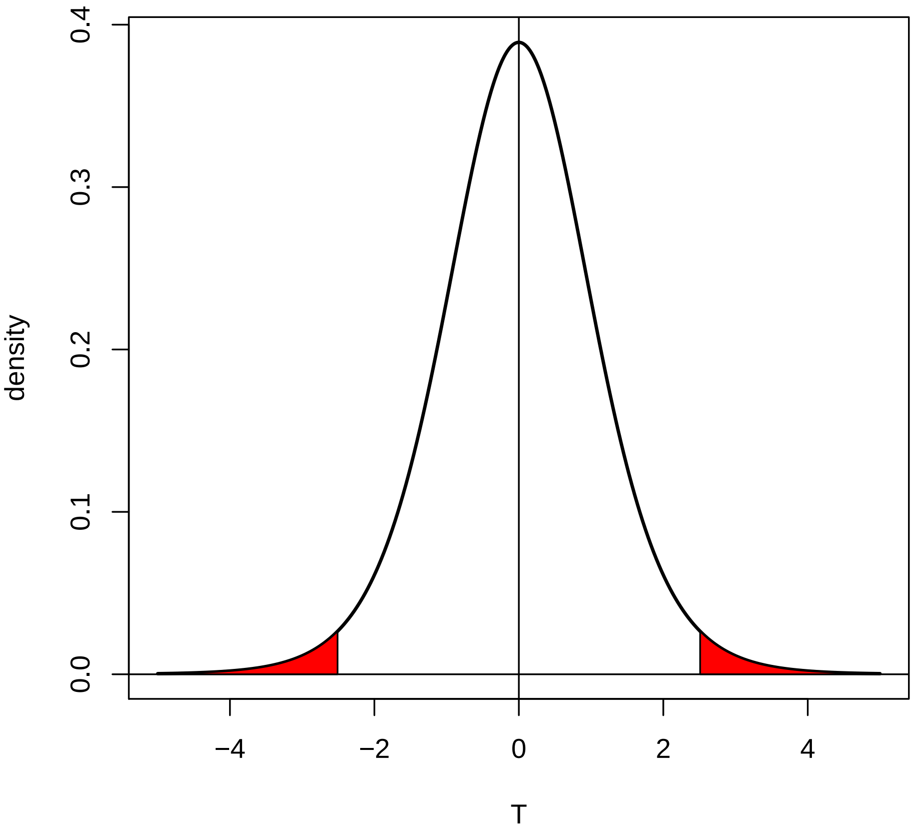
# Statistical tests

❍ Example: The two–sample $t$–statistic

$$T_g = \frac{\bar{X}_{g1} - \bar{X}_{g2}}{s_g \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

is used to test equality of the group means $\mu_1, \mu_2$.

❍ The $p$–value $p_g$ is the probability under the null hypothesis (here: $\mu_1 = \mu_2$) that the test statistic is at least as extreme as the observed value $T_g$. Under the null hypothesis, $Pr(p_g < \alpha) = \alpha$.



# Statistical tests: Examples

❍ standard $t$-test: assumes normally distributed data in each class (almost always questionable), equal variances within classes

❍ Welch $t$-test: as above, but allows for unequal variances

❍ Wilcoxon test: non–parametric, rank–based

❍ permutation test: estimate the distribution of the test statistic (e.g., the $t$-statistic) under the null hypothesis by permutations of the sample labels:
The $p$–value $p_g$ is given as the fraction of permutations yielding a test statistic that is at least as extreme as the observed one.

# Permutation tests

true class labels:

test statistic

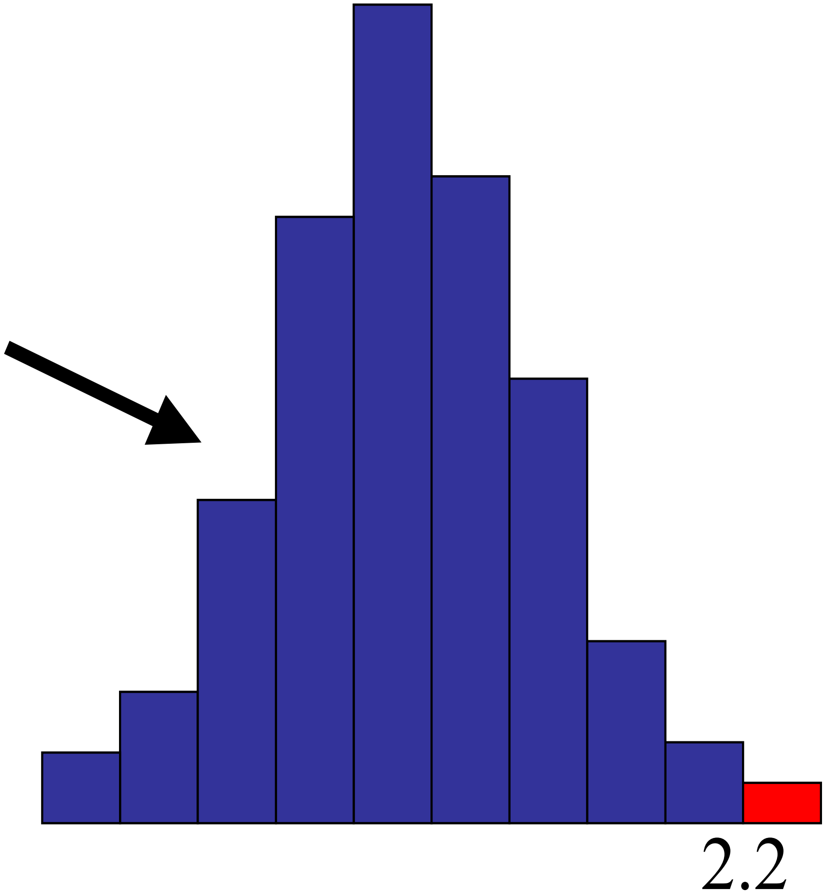
| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 🟨 | 🟨 | 🟨 | 🟨 | 🟨 | 🟩 | 🟩 | 🟩 | 🟩 | 🟩 | 2.2 |

(random) permutations of class labels:

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 🟨 | 🟨 | 🟨 | 🟩 | 🟩 | 🟩 | 🟨 | 🟩 | 🟩 | 🟨 | 1.5 |
| 🟩 | 🟩 | 🟨 | 🟨 | 🟨 | 🟨 | 🟩 | 🟩 | 🟩 | 🟨 | -0.4 |
| 🟨 | 🟩 | 🟨 | 🟨 | 🟨 | 🟨 | 🟩 | 🟩 | 🟩 | 🟩 | 2.3 |
| 🟨 | 🟨 | 🟨 | 🟩 | 🟩 | 🟨 | 🟩 | 🟨 | 🟩 | 🟩 | 0.7 |
| 🟨 | 🟨 | 🟩 | 🟨 | 🟩 | 🟨 | 🟩 | 🟩 | 🟩 | 🟨 | 0.2 |
| 🟩 | 🟨 | 🟩 | 🟩 | 🟩 | 🟨 | 🟨 | 🟨 | 🟨 | 🟩 | -1.2 |

⋮

null distribution of test statistic



# Statistical tests: Different settings

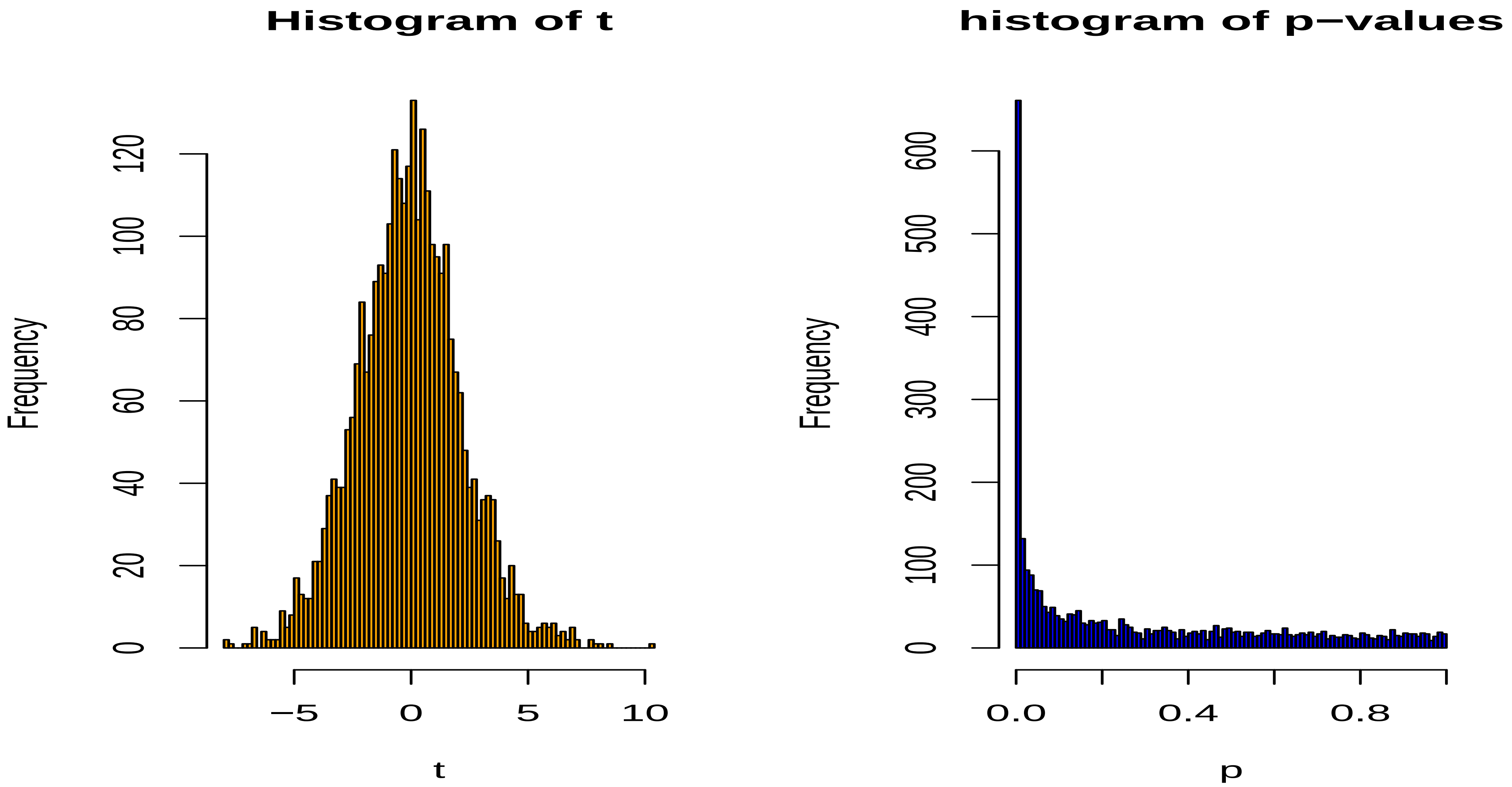
❍ comparison of two classes (e.g. tumor vs. normal), one class, paired observations from two classes: (permutation) t–test, Wilcoxon test

❍ more than two classes and/or more than one factor: tests may be based on ANOVA/linear models

❍ continuous response variable: linear models;
censored survival times: e.g. Cox proportional hazards models

# Example

Golub data, 27 ALL vs. 11 AML samples, 3,051 genes.



$t$-test: 1045 genes with $p < 0.05$.

# Multiple testing: the problem

Multiplicity problem: thousands of hypotheses are tested simultaneously.

- Increased chance of false positives.
- E.g. suppose you have 10,000 genes on a chip and not a single one is differentially expressed. You would expect $10000 * 0.01 = 100$ of them to have a $p$-value $< 0.01$.
- Individual $p$–values of e.g. $0.01$ no longer correspond to significant findings.

Need to **adjust for multiple testing** when assessing the statistical significance of findings.

# Multiple hypothesis testing

| | # non–rejected hypotheses | # rejected hypotheses | |
|---|---|---|---|
| # true null hypotheses<br>(non-diff. genes) | $U$ | $V$<br>Type I error | $m_0$ |
| # false null hypotheses<br>(diff. genes) | $T$<br>Type II error | $S$ | $m_1$ |
| | $m - R$ | $R$ | $m$ |

*From Benjamini & Hochberg (1995).*

# Type I error rates

1. **Family–wise error rate (FWER)**. The FWER is defined as the probability of at least one Type I error (false positive):

$$FWER = Pr(V > 0).$$

2. **False discovery rate (FDR)**. The FDR (Benjamini & Hochberg 1995) is the expected proportion of Type I errors (false positives) among the rejected hypotheses:

$$FDR = E(Q),$$

with

$$Q = \begin{cases} V/R, & \text{if } R > 0, \\ 0, & \text{if } R = 0. \end{cases}$$

# FWER: The Bonferroni correction

Suppose we conduct a hypothesis test for each gene $g = 1, \ldots, m$, producing

an observed test statistic: $T_g$

an unadjusted $p$–value: $p_g$.

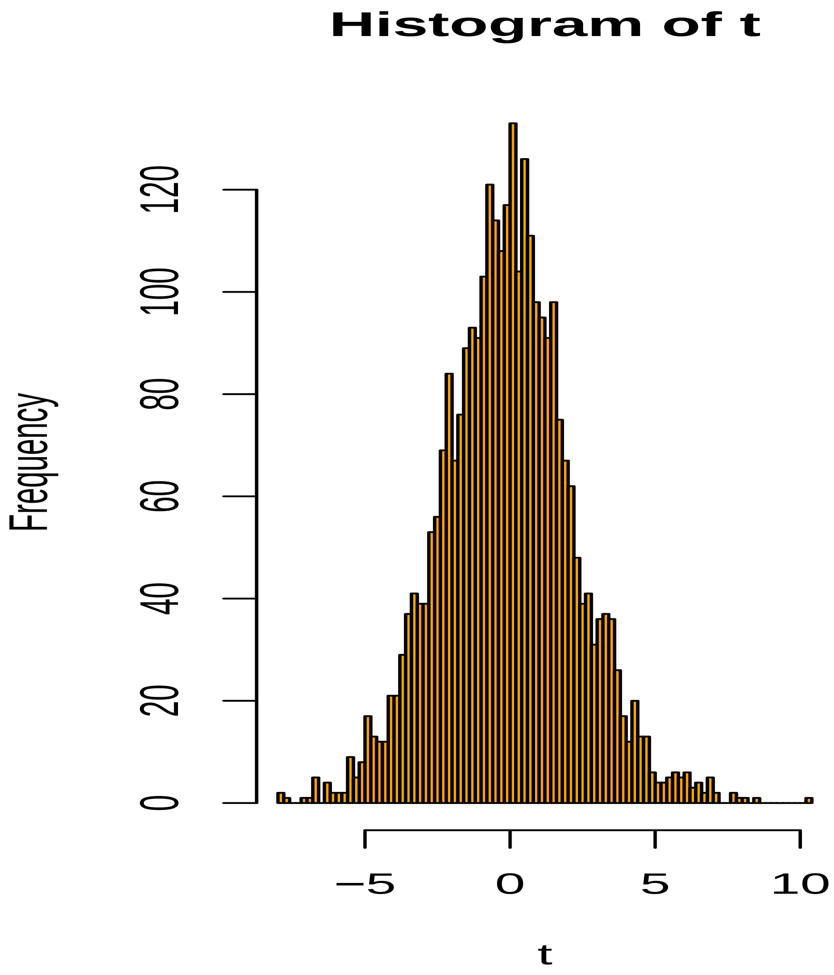
Bonferroni adjusted $p$–values:

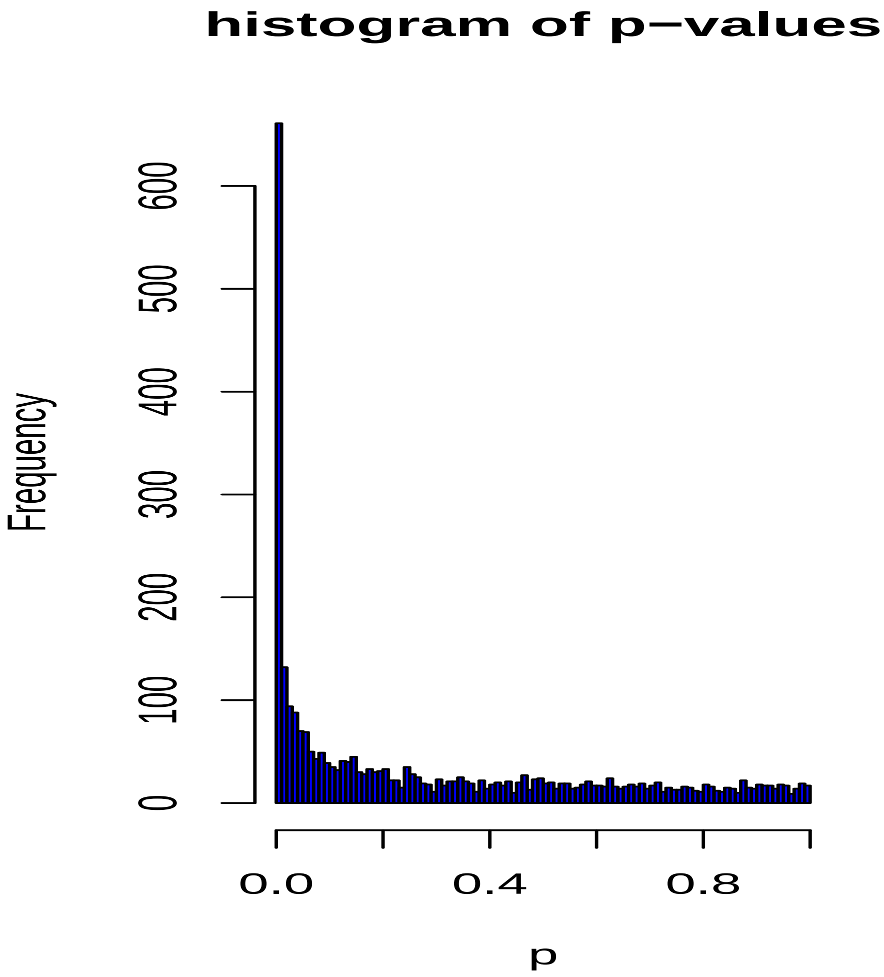
$$\tilde{p}_g = \min\big(mp_g, 1\big).$$

Selecting all genes with $\tilde{p}_g \leq \alpha$ controls the FWER at level $\alpha$, that is, $Pr(V > 0) \leq \alpha$.

# Example

Golub data, 27 ALL vs. 11 AML samples, 3,051 genes.





98 genes with Bonferroni-adjusted $\tilde{p}_g < 0.05 \Leftrightarrow p_g < 0.000016$ (t-test)

# FWER: Improvements to Bonferroni (Westfall/Young)

❍ The minP–adjusted p–values (Westfall and Young):

$$\tilde{p}_g = Pr(\min_{k=1,\ldots,m} P_k \leq p_g | H_0).$$

$H_0$ denotes the complete null hypothesis that no gene is differentially expressed.

❍ Selecting all genes with $\tilde{p}_g \leq \alpha$ controls the FWER at level $\alpha$.

❍ The probabilities $\tilde{p}_g$ are estimated through a permutation scheme.

# Westfall/Young FWER control

❍ Advantage of Westfall/Young: The method takes the dependence structure between genes into account, which gives in many cases (positive dependence between genes) higher power.

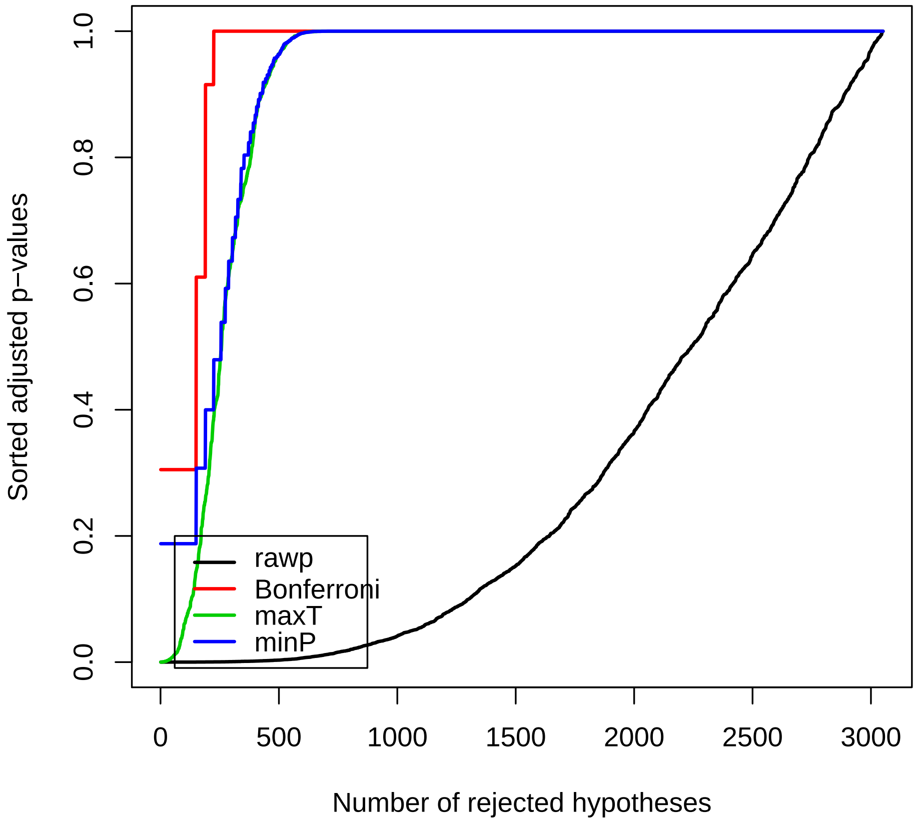
❍ Computationally intensive if the unadjusted $p$-values arise from permutation tests – two levels of permutations required.

❍ Similar method (maxT) under the assumption that the null distributions of the statistics $T_g$ are equal - replace $p_g$ by $|T_g|$ and $\min$ by $\max$. Computationally less intensive.

❍ All methods are implemented in the Bioconductor package multtest, with a fast algorithm for the minP method.

# FWER: Comparison of different methods

Golub data, 27 ALL vs. 11 AML samples, 3,051 genes.



Example taken from the multtest package in Bioconductor.

The FWER is a conservative criterion: many interesting genes may be missed.

# More is not always better

❍ Suppose you use a focused array with 500 genes you are particularly interested in.

❍ If a gene on this array has an unadjusted $p$-value of 0.0001, the Bonferroni-adjusted $p$-value is still 0.05.

❍ If instead you use a genome-wide array with, say, 50,000 genes, this gene would be much harder to detect, because roughly 5 genes can be expected to have such a low $p$-value by chance.

❍ Therefore, it may be worthwile focusing on genes of particular biological interest from the beginning.

# Controlling the FDR (Benjamini/Hochberg)

❍ Ordered unadjusted $p$–values: $p_{r_1} \leq p_{r_2} \leq \ldots \leq p_{r_m}$.

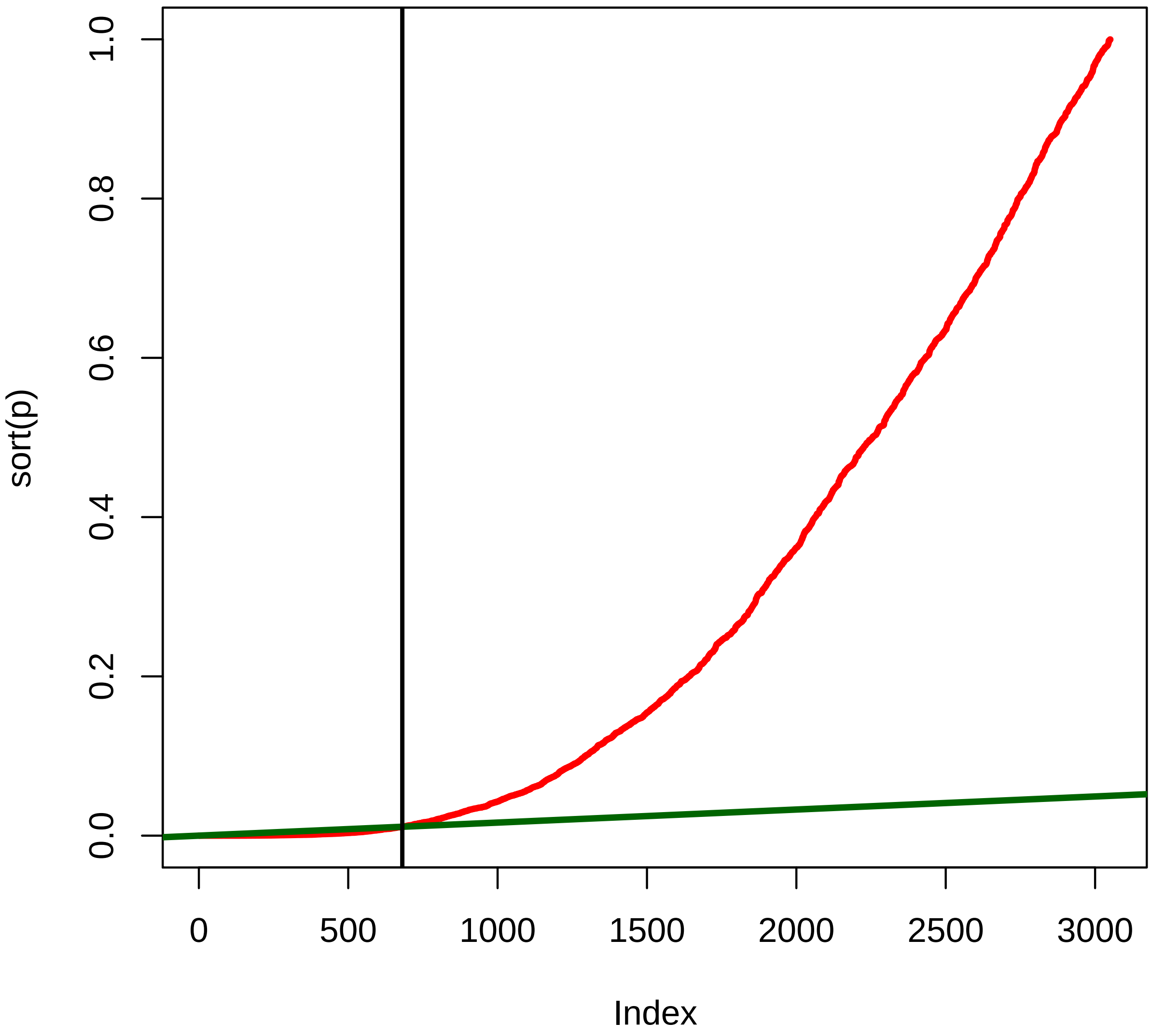
❍ To control $FDR = E(V/R)$ at level $\alpha$, let

$$j^\star = \max\{j : p_{r_j} \leq (j/m)\alpha\}.$$

Reject the hypotheses $H_{r_j}$ for $j = 1, \ldots, j^\star$.

❍ Is valid for independent test statistics and for some types of dependence. Tends to be conservative if many genes are differentially expressed. Implemented in multtest.

# Controlling the FDR (Benjamini/Hochberg)



Golub data: 681 genes with BH–adjusted $p < 0.05$.

# Estimation of the FDR (SAM, Storey/Tibshirani 2003)

Idea: Depending on the chosen cutoff-value for the test statistic $T_g$, estimate the expected proportion of false positives in the resulting gene list through a permutation scheme.

1. Estimate the number $m_0$ of non-diff. genes.

2. Estimate the expected number of false positives under the complete null hypothesis, $E(V_0)$, through resampling.
   Then, $\widehat{E(V)} = \frac{\hat{m}_0}{m}\widehat{E(V_0)}$ (because only the non-diff. genes may yield false positives).

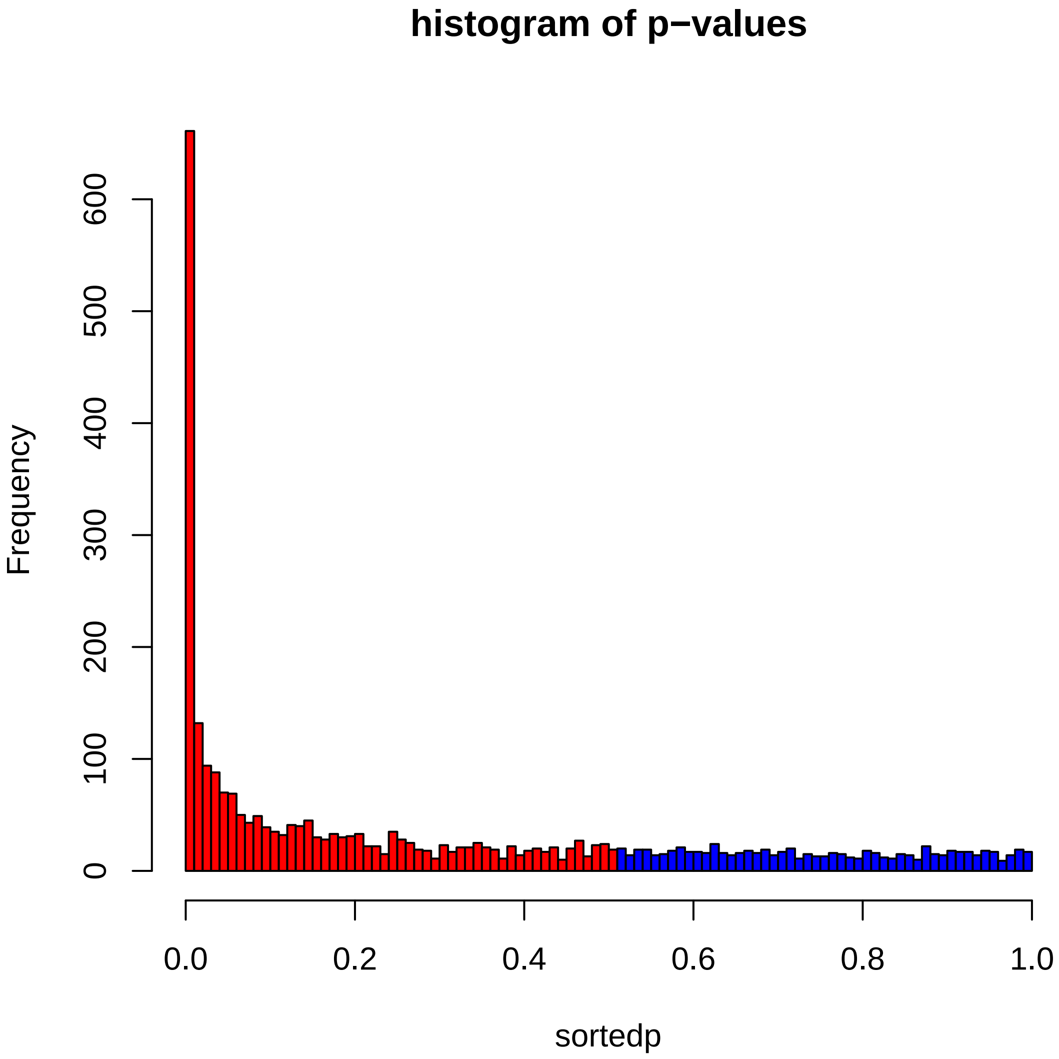
3. Estimate $FDR = E(V/R)$ by $\widehat{E(V)}/R$.

# FDR - 1. Estimating the number $m_0$ of not differentially expressed genes

❍ Consider the distribution of $p$-values: A gene with $p > 0.5$ is likely to be not differentially expressed.

❍ As $p$-values of non-diff. genes should be uniformly distributed in $[0, 1]$, the number $2 * \#\{g|p_g > 0.5\}$ can be taken as an estimate of $m_0$.

❍ In the Golub example with 3051 genes, $\hat{m}_0 = 1592$.



# 2. Estimation of the FDR

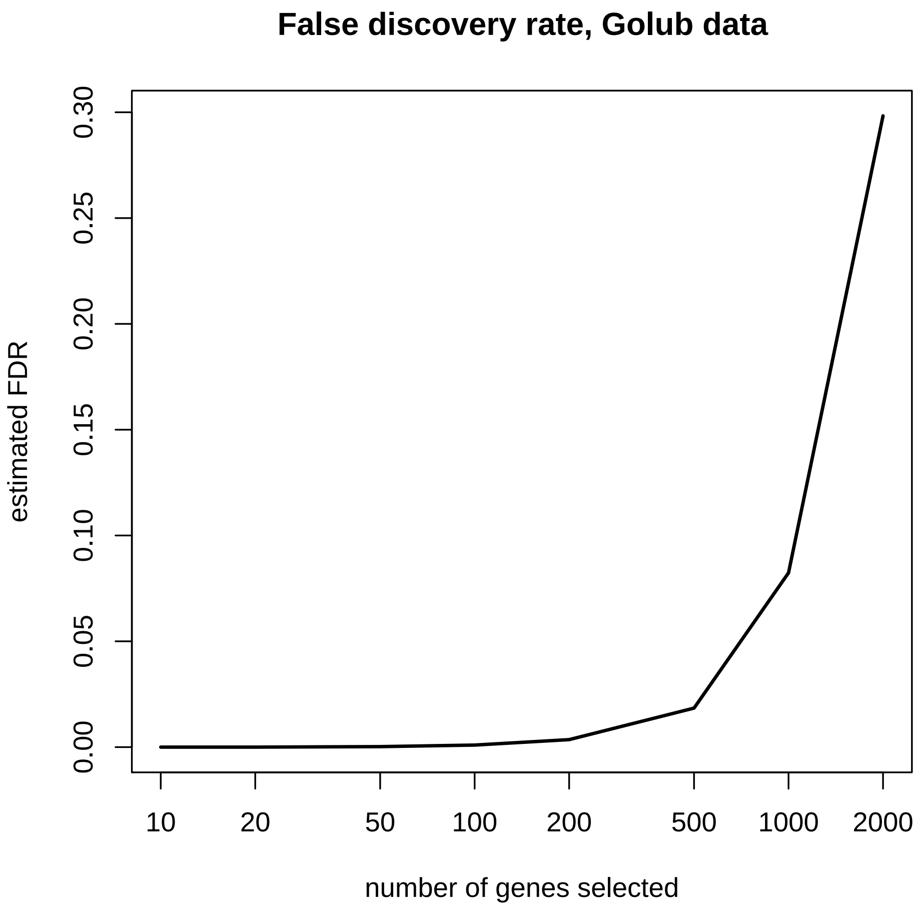
❍ For each iteration, $b = 1, \ldots, B$, (randomly) permute the sample labels, compute test statistics $T_{gb}$ – these correspond to the complete null hypothesis.

❍ For any threshold $t_0$ of the test statistic, compute the numbers $V_b$ of genes with $T_{gb} > t_0$ (false positives).

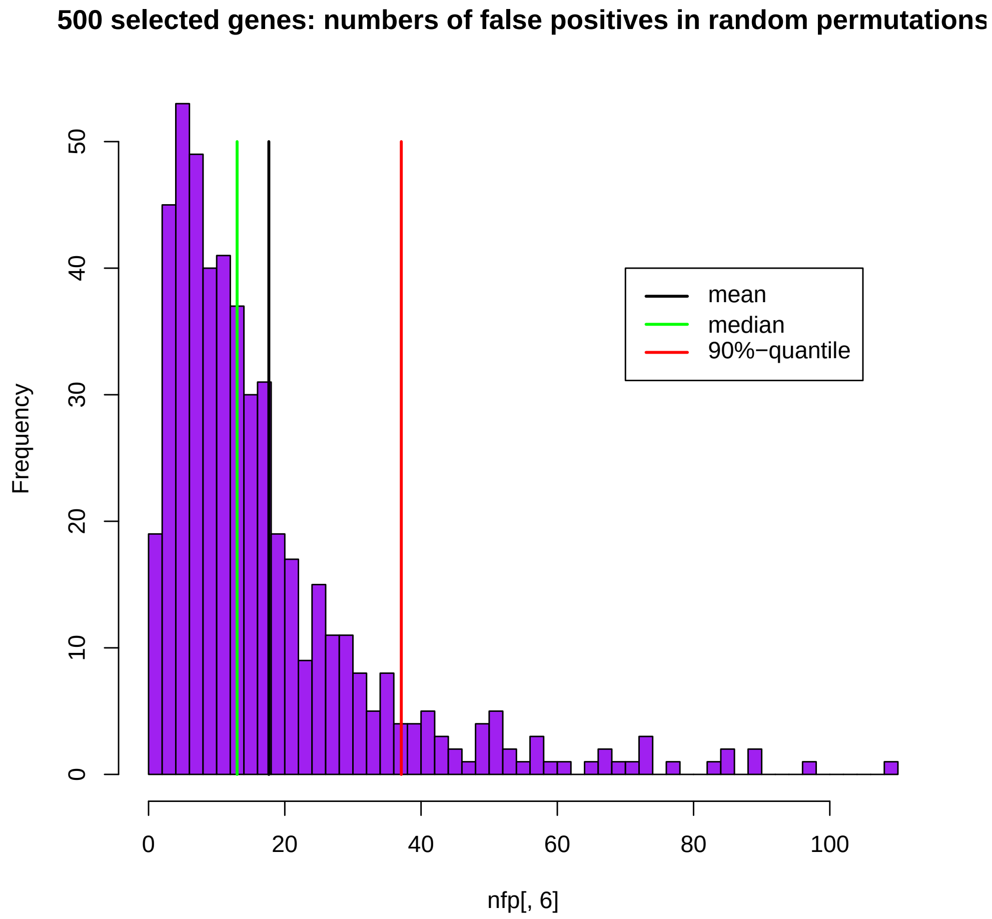
❍ The estimation of the FDR is based on the mean of the $V_b$. However, a quantile of the $V_b$ may also be interesting, as the actual proportion of false positives may be much larger than the mean.

# Estimation of the FDR: Example

## Golub data



False discovery rate, Golub data



500 selected genes: numbers of false positives in random permutations

# Estimation of the FDR

❍ The procedure takes the dependence structure between genes into account.

❍ In SAM, the $q$-value of a gene is defined as the minimal estimated FDR at which it appears significant.

# FWER or FDR?

❍ Choose control of the FWER if high confidence in all selected genes is desired. Loss of power due to large number of tests: many differentially expressed genes may not appear significant.

❍ If a certain proportion of false positives is tolerable: Procedures based on FDR are more flexible; the researcher can decide how many genes to select, based on practical considerations.

❍ For some applications, even the unadjusted $p$–values may be most appropriate (e.g. comparison of functional categories of affected vs. unaffected genes).

# Few replicates – moderated t–statistics

- With the t–test, we estimate the variance of each gene individually. This is fine if we have enough replicates, but with few replicates (say 2–5 per group), the variance estimates are highly variable.

- In a moderated $t$–statistic, the estimated gene–specific variance $s_g^2$ is augmented with $s_0^2$, a global variance estimator obtained from pooling all genes:

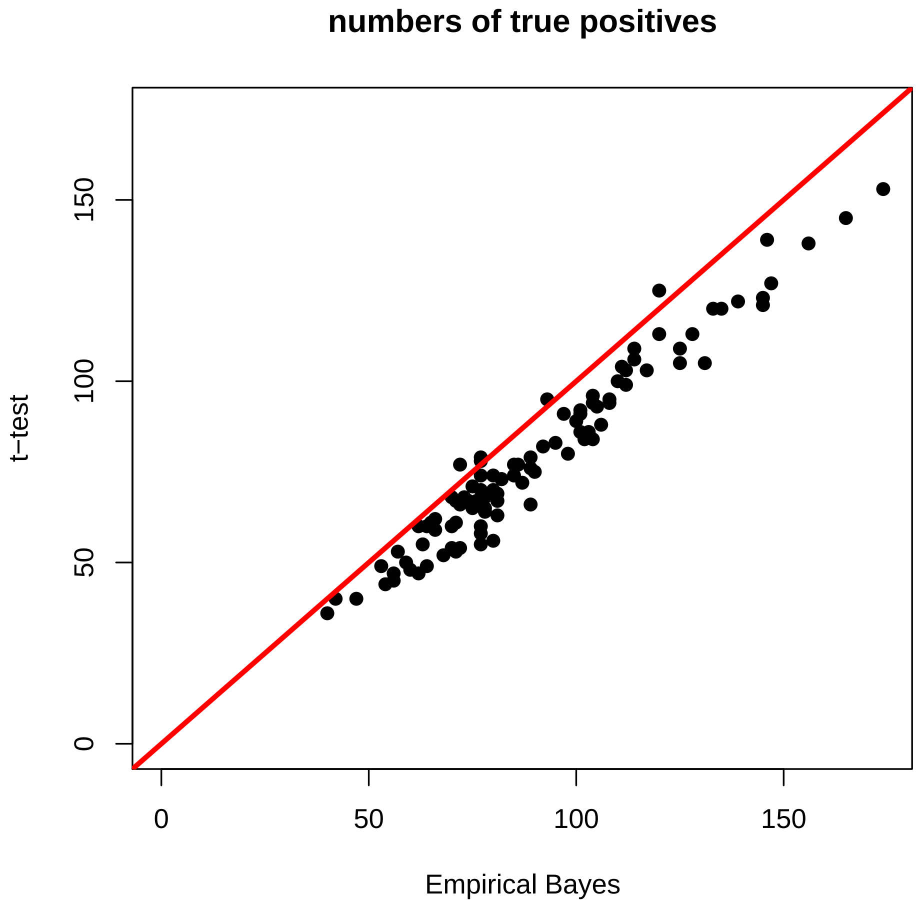
$$T_g \sim \frac{\bar{X}_{g1}-\bar{X}_{g2}}{\sqrt{\mu s_g^2+\lambda s_0^2}}.$$

- This gives an interpolation between the $t$–test and a fold–change criterion.

- Examples: packages limma, siggenes in Bioconductor, SAM.

# Moderated $t$–statistic

Repeatedly draw 4 ALL and 4 AML samples out of the total 38 samples and apply the usual and moderated $t$–test (Bioconductor package limma) to them. Using a cut–off of $p < 0.05$, “true positives” are defined on the basis of the analysis of the whole data set (681 genes with FDR $< 0.05$).



# Prefiltering

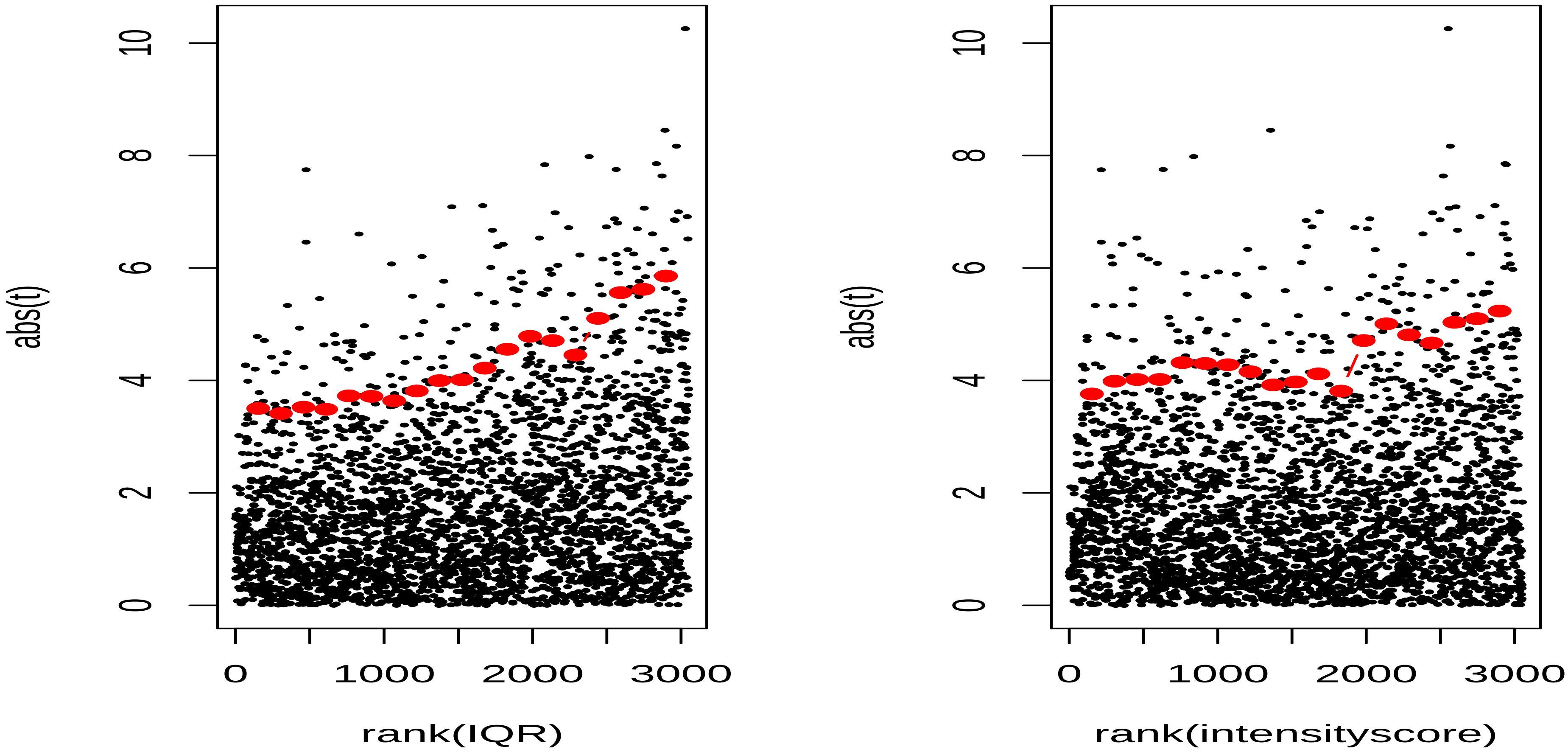
❍ What about prefiltering genes (according to intensity, variance etc.) to reduce the proportion of false positives?

❍ Can be useful: Genes with low intensities in most of the samples or low variance across the samples are less likely to be interesting.

❍ In order to maintain control of the type I error, the criteria have to be independent of the distribution of the test statistic under the null hypothesis.

# Prefiltering by intensity and variability

Golub data, 27 ALL vs. 11 AML samples, 3,051 genes.



Ranks of interquartile range and 75%–quantile of intensities versus absolute $t$–statistic.

# What else?

- ❍ Statistical tests rely on independent observations. For example, if you have 6 biological samples with 2 replicate hybridizations each, a $t$–test based on all 12 observations is not appropriate. Here, one may either i) average over the technical replicates or ii) use special methods (mixed effects models, see e.g. Bioconductor package limma for the case of duplicate spots).

- ❍ The Bioconductor package globaltest by J. Goeman provides a test whether a group of genes (e.g. a GO category) contains any differentially expressed genes.

# References

❍ Y. Benjamini and Y. Hochberg (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society B*, Vol. 57, 289–300.

❍ S. Dudoit, J.P. Shaffer, J.C. Boldrick (2003). Multiple hypothesis testing in microarray experiments. *Statistical Science*, Vol. 18, 71–103.

❍ J.D. Storey and R. Tibshirani (2003). SAM thresholding and false discovery rates for detecting differential gene expression in DNA microarrays. In: *The analysis of gene expression data: methods and software.* Edited by G. Parmigiani, E.S. Garrett, R.A. Irizarry, S.L. Zeger. Springer, New York.

❍ V.G. Tusher et al. (2001). Significance analysis of microarrays applied to the ionizing radiation response. *PNAS*, Vol. 98, 5116–5121.

❍ P.H. Westfall and S.S. Young (1993). Resampling–based multiple testing: examples and methods for p-value adjustment. Wiley.