

Multiple testing with gene expression array data

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Multiple hypothesis testing

- Suppose we want to find genes that are differentially expressed between different conditions/phenotypes, e.g. two different tumor types.
- We conduct a statistical test for each gene $g = 1, \dots, m$ (t -test, Wilcoxon test, permutation test, ...).
- This yields test statistics T_g , p -values p_g .
- p_g is the probability under the null hypothesis that the test statistic is at least as extreme as T_g . Under the null hypothesis, $Pr(p_g < \alpha) = \alpha$.

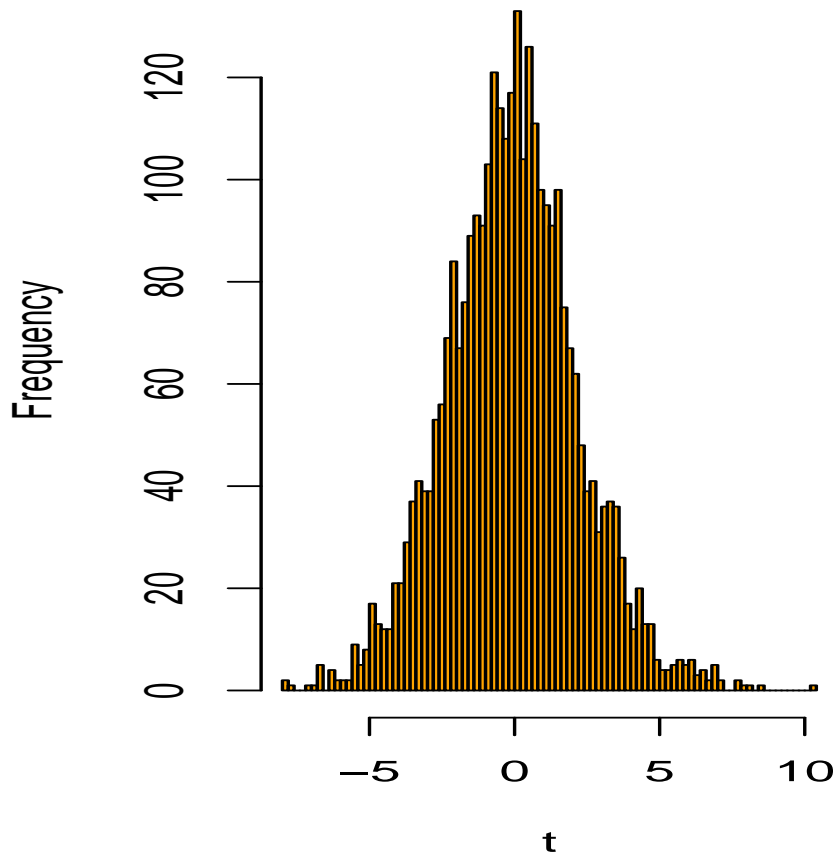
Statistical tests: Examples

- t -test: assumes normally distributed data in each class
- 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.

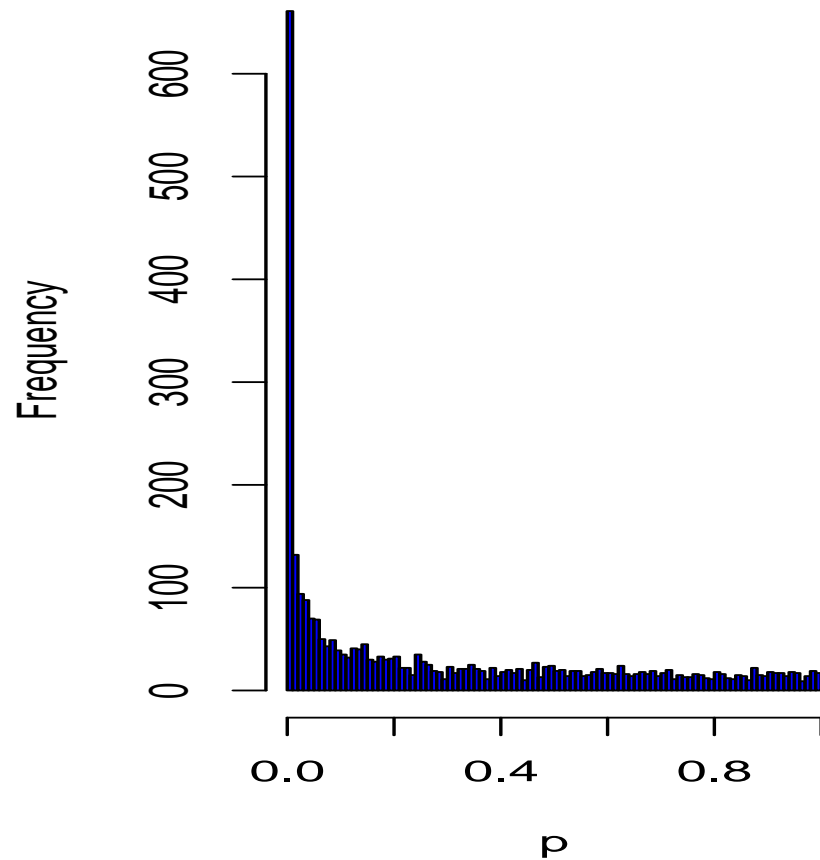
Example

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

Histogram of t



histogram of p -values



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

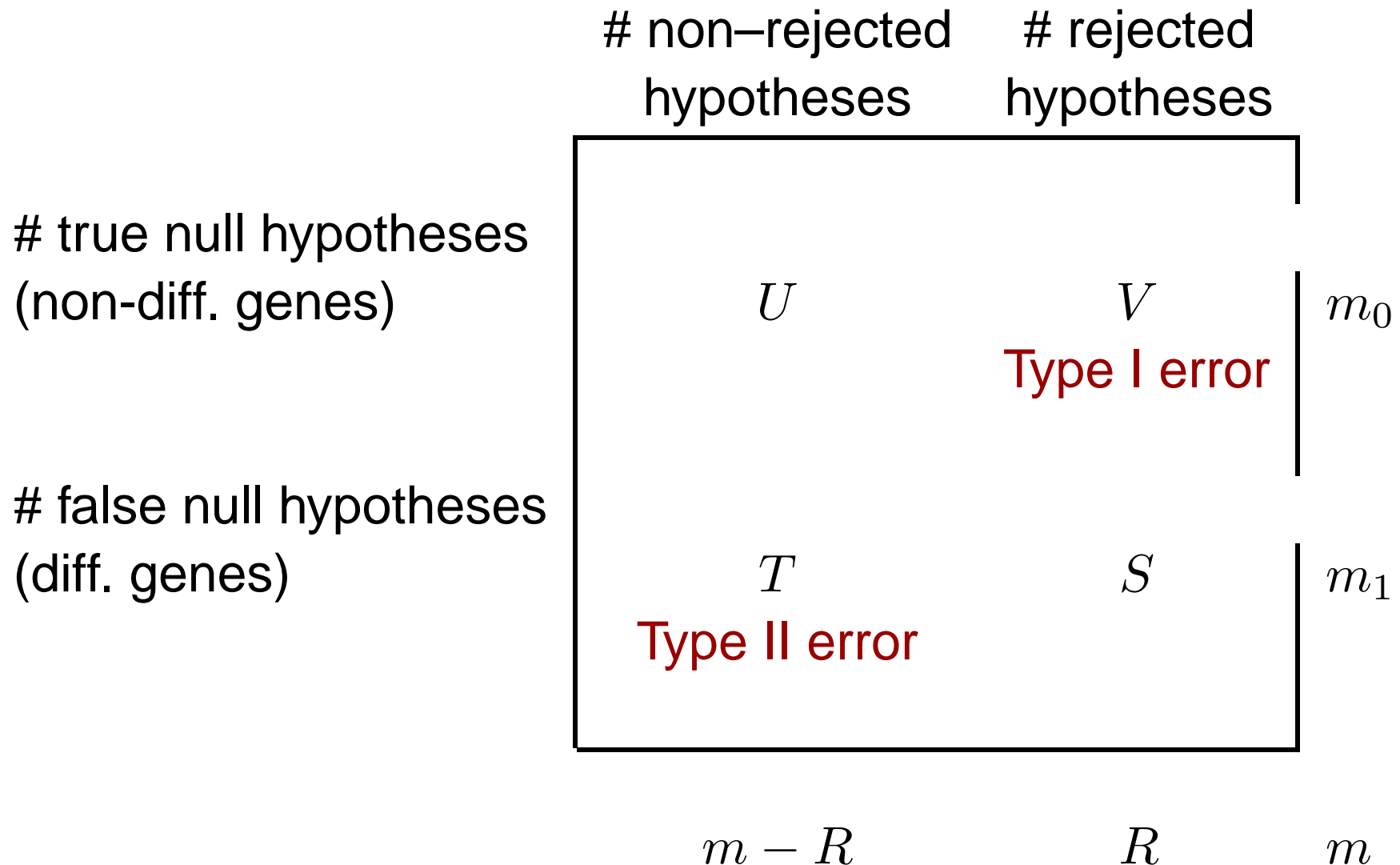
Multiple testing: the problem

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



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 among the rejected hypotheses:

$$FDR = E(Q),$$

with

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

Multiple testing: Controlling a type I error rate

- Aim: For a given type I error rate α , use a procedure to select a set of “significant” genes that guarantees a type I error rate $\leq \alpha$.
- The type I error is defined with respect to a given configuration of true and false null hypotheses.
- **Weak control** of type I error: only under the assumption that all null hypotheses are true (*complete null hypothesis*, H_0).
- **Strong control** of type I error: for all possible configurations of true and false null hypotheses.

FWER: The Bonferroni correction

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

an observed test statistic: T_g

an unadjusted p -value: p_g .

Bonferroni adjusted p -values:

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

FWER: The Bonferroni correction

Choosing all genes with $\tilde{p}_g \leq \alpha$ controls the FWER at level α :

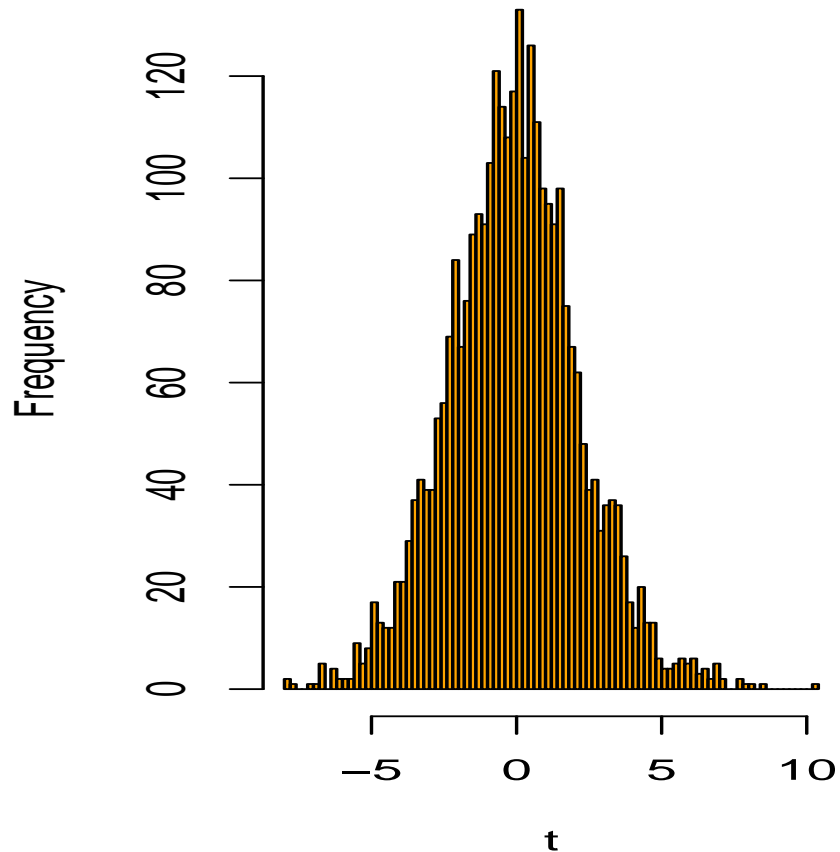
$$\begin{aligned} FWER = Pr(V > 0) &= Pr(\text{at least one } \tilde{p}_g \leq \alpha | H_0) \\ &= Pr(\text{at least one } p_g \leq \alpha/m | H_0) \\ &\leq \sum_{g=1}^m Pr(p_g \leq \alpha/m | H_0) \\ &= m * \alpha/m = \alpha. \end{aligned}$$

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

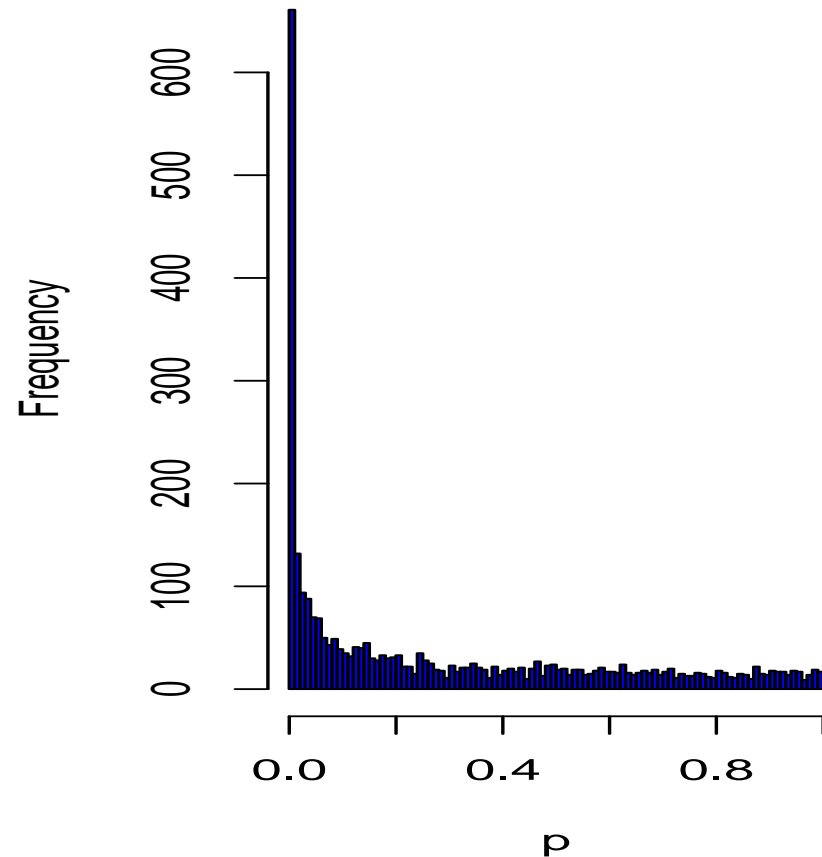
Example

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

Histogram of t



histogram of p-values



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

More is not always better

- Suppose you produce a small 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.

FWER: Improvements to Bonferroni (Westfall/Young)

- The minP adjusted p-values (Westfall and Young):
- $\tilde{p}_g = Pr(\min_{k=1, \dots, m} P_k \leq p_g | H_0)$.
- Choosing all genes with $\tilde{p}_g \leq \alpha \Leftrightarrow p_g \leq c_\alpha$ controls the FWER at level α :

$$\begin{aligned} FWER = Pr(V > 0) &= Pr(\text{at least one } \tilde{p}_g \leq \alpha | H_0) \\ &= Pr(\min \tilde{p}_g \leq \alpha | H_0) \\ &= Pr(\min p_g \leq c_\alpha | H_0) \\ &= \alpha. \end{aligned}$$

But how to obtain the probabilities \tilde{p}_g ?

Estimation of minP-adjusted p-values through resampling

- For $b = 1, \dots, B$, (randomly) permute the sample labels.
- For each gene, compute the unadjusted p -values p_{gb} based on the permuted sample labels.
- Estimate $\tilde{p}_g = Pr(\min_{k=1, \dots, m} P_k \leq p_g | H_0)$ by

$$\#\{b : \min_g p_{gb} \leq p_g\} / B.$$

Example

- Suppose $p_{\min} = 0.0003$ (the minimal unadjusted p -value).
- Among the randomized data sets (permuted sample labels), count how often the minimal p -value is smaller than 0.0003. If this appears e.g. in 4% of all cases, $\tilde{p}_{\min} = 0.04$.
- If there is a positive dependence between the non-diff. genes, this procedure can yield better results than the Bonferroni correction.

Westfall/Young FWER control

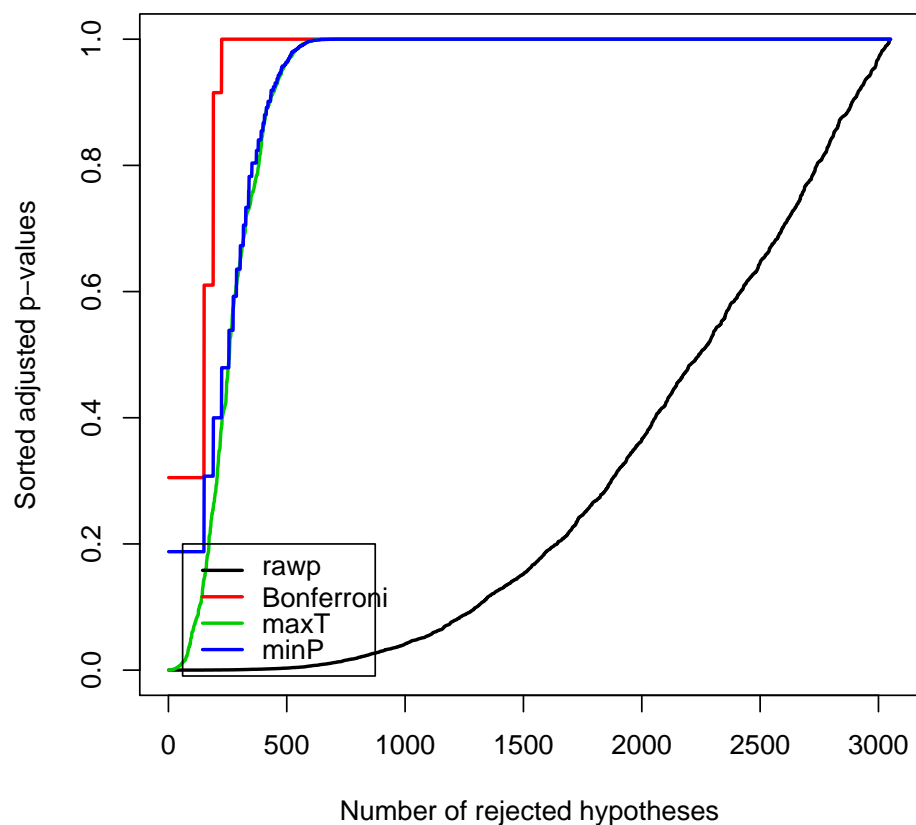
- **Step-down** procedure (Holm): Enhancement for Bonferroni and Westfall/Young: same adjustment for the smallest p -value, successively smaller adjustment for larger ones.
- Idea: Compare the k th-smallest p -value to the k th-smallest under the complete null hypothesis.

Westfall/Young FWER control

- Advantage: The method takes the dependence structure between genes into account, which gives in many cases higher power.
- Computationally intensive if the unadjusted p -values arise from permutation tests.
- Similar method (maxT) under the assumption that the statistics T_g are equally distributed under the null hypothesis - 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.

Estimation of the FDR (SAM, Storey 2001)

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.

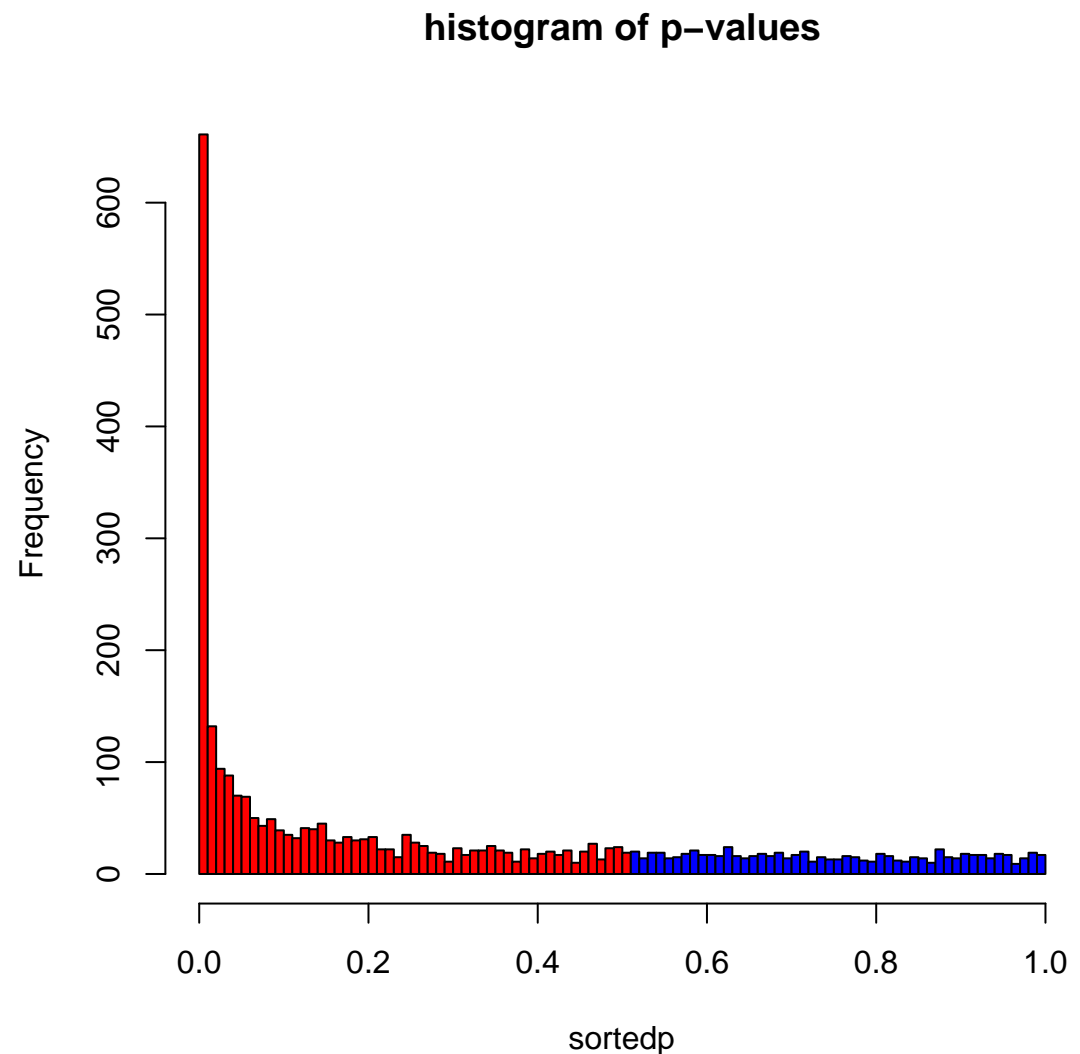
1. Estimate the number m_0 of non-diff. genes.
2. For each permutation b , compute the number of significant genes. The average of these numbers, multiplied with \hat{m}_0/m , gives an estimate of the number of false positives $E(V)$.
3. Estimate the FDR $E(V/R)$ by $\widehat{E(V)}/R$.

FDR - 1. Estimating the number m_0 of invariant 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, $\hat{m}_0 = 1592$.



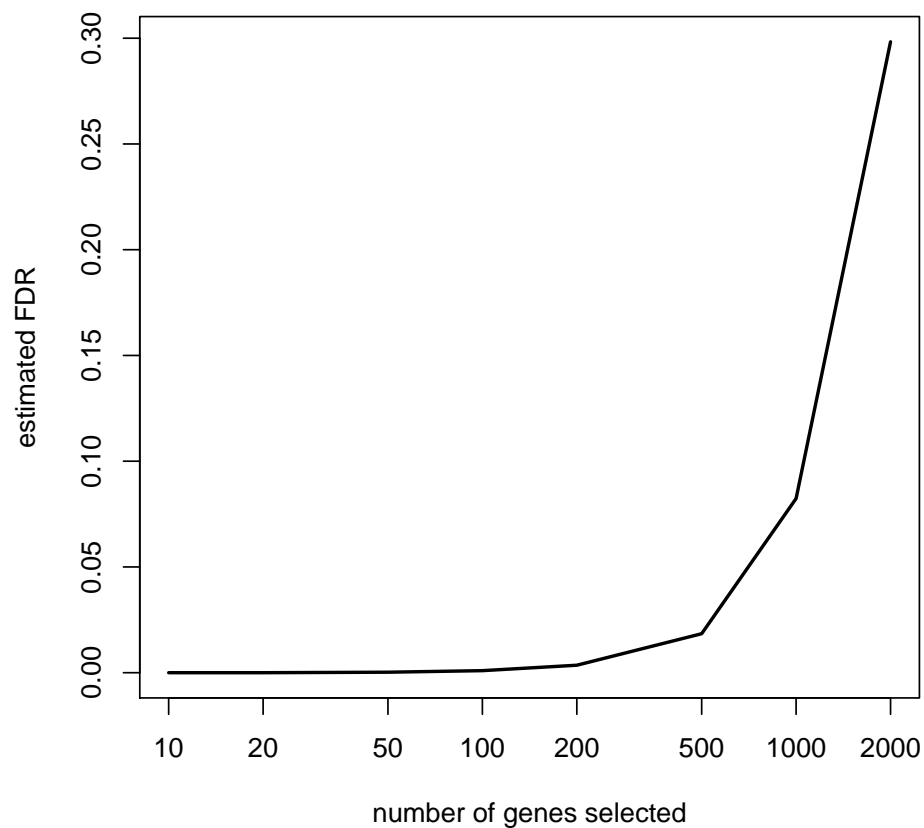
2. Estimation of the FDR

- For $b = 1, \dots, B$, (randomly) permute the sample labels, compute test statistics T_{gb} under the complete null hypothesis.
- For any threshold t_0 of the test statistic, compute the numbers n_b of genes with $T_{gb} > t_0$ (numbers of false positives).
- The estimation of the FDR is based on the mean of these numbers, but the median or 90%-quantile may also be interesting.

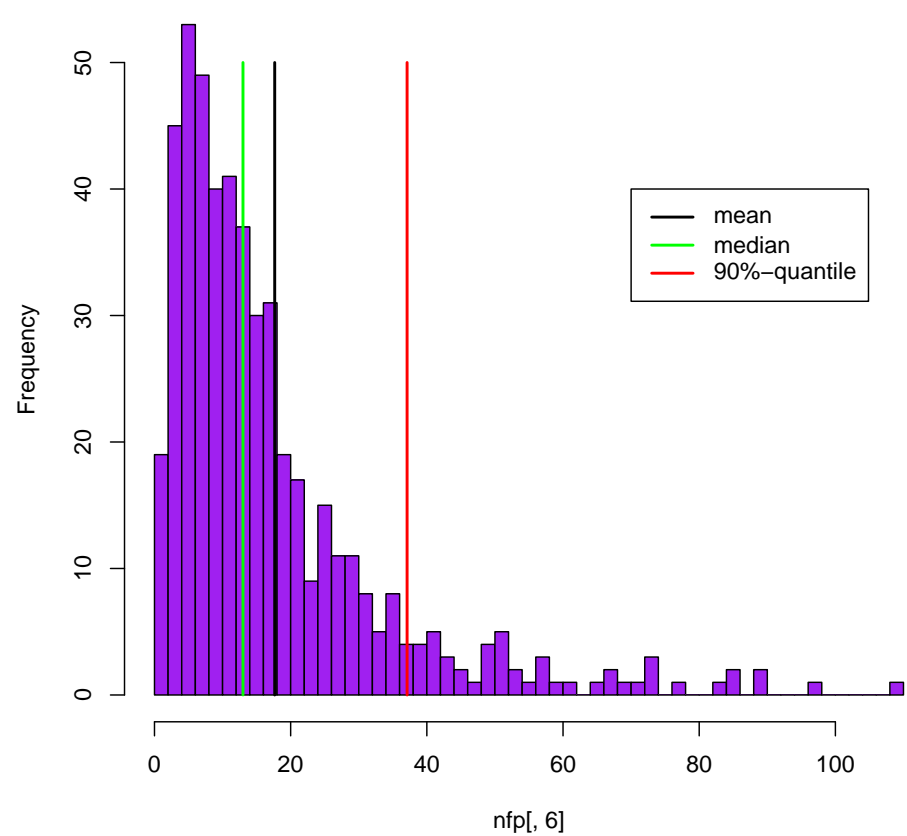
Estimation of the FDR: Example

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.
- The *q-value* of a gene is defined as the minimal FDR at which it appears significant.

FWER or FDR?

- Chose 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 as 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.

Prefiltering

- What about prefiltering genes (according to intensity, variance etc.) to reduce the proportion of false positives - e.g. genes with consistently low intensity may not be considered interesting?
- Can be useful, but:
- The criteria for filtering have to be chosen before the analysis - not dependent on the results of the analysis.
- The criteria have to be independent of the distribution of the test statistic under the null hypothesis - otherwise no control of the type I error.

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.
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- J.D. Storey and R. Tibshirani (2001). Estimating false discovery rates under dependence, with applications to DNA microarrays. Preprint, <http://www.stat.berkeley.edu/storey/>
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- P.H. Westfall and S.S. Young (1993). Resampling-based multiple testing: examples and methods for p-value adjustment. Wiley.