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

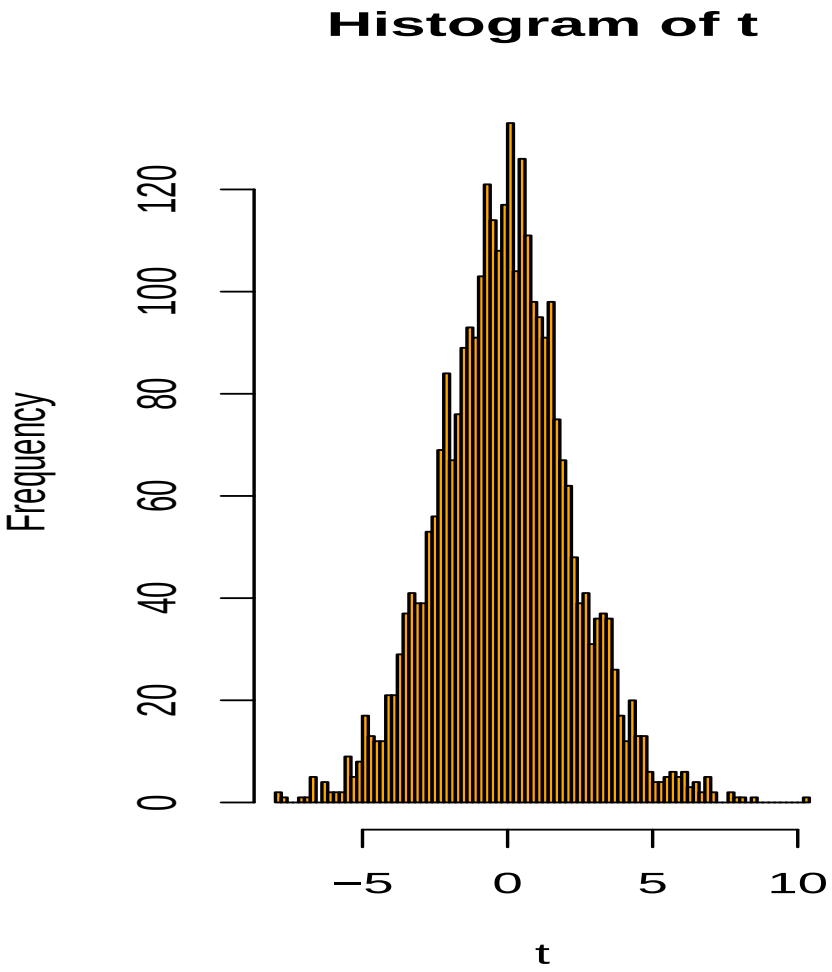
❍ We conduct a statistical test for each gene $g = 1, \ldots, m$ (t-test, Wilcoxon test, permutation test, ...).

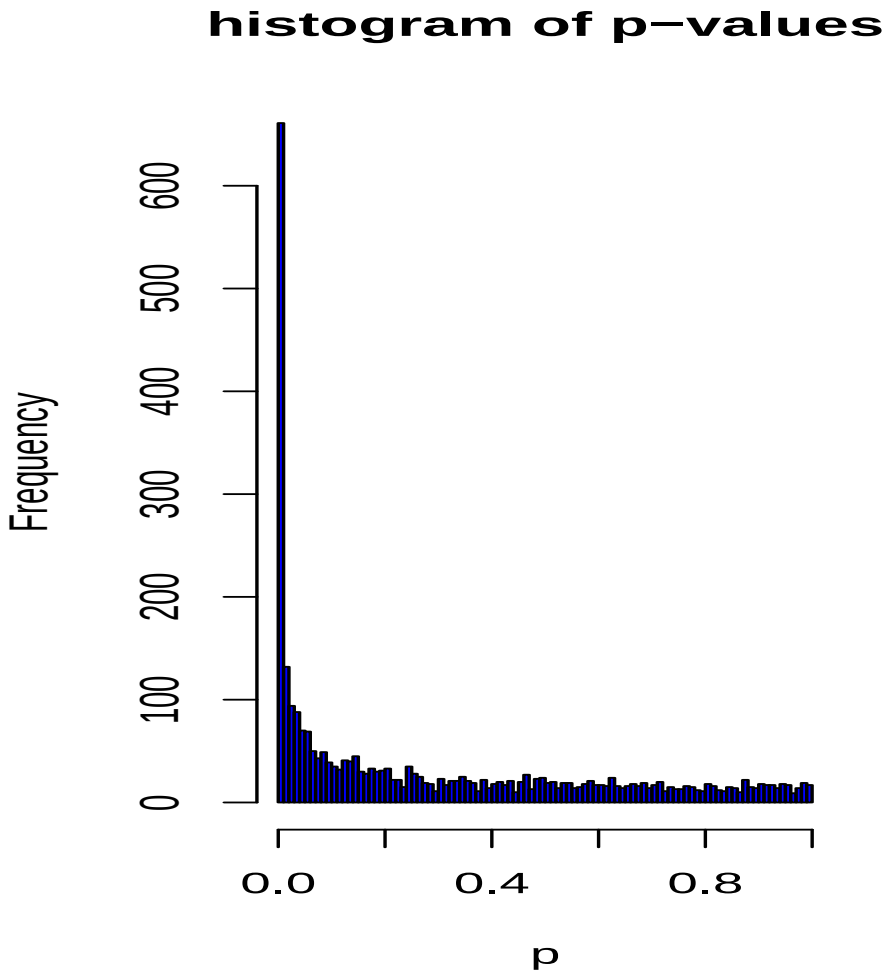
❍ This yields test statistics T_g, p-values p_g.

❍ 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, $Pr(p_g < \alpha) = \alpha$.

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 (non-diff. genes)	U	V Type I error	m_0
# false null hypotheses (diff. genes)	T 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 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 $g = 1, \ldots, m$, producing

- an observed test statistic: T_g
- an unadjusted p–value: p_g.

Bonferroni adjusted p–values:

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

FWER: The Bonferroni correction

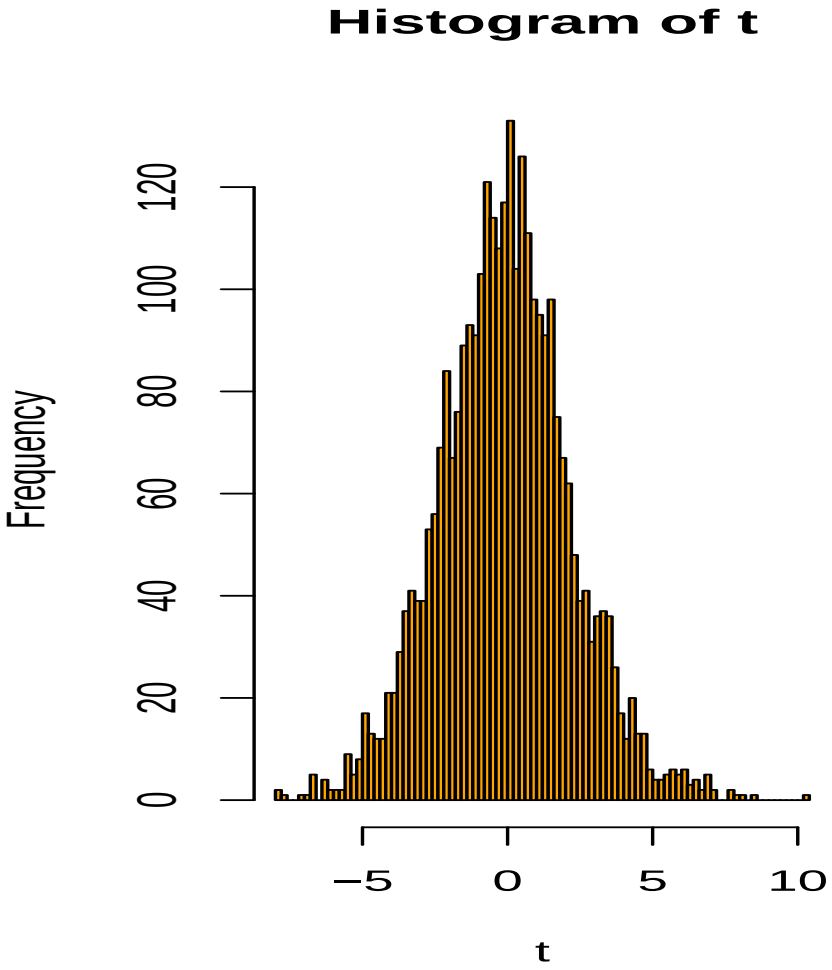
Chosing all genes with $\tilde{p}_g \leq \alpha$ controls the FWER at level α:

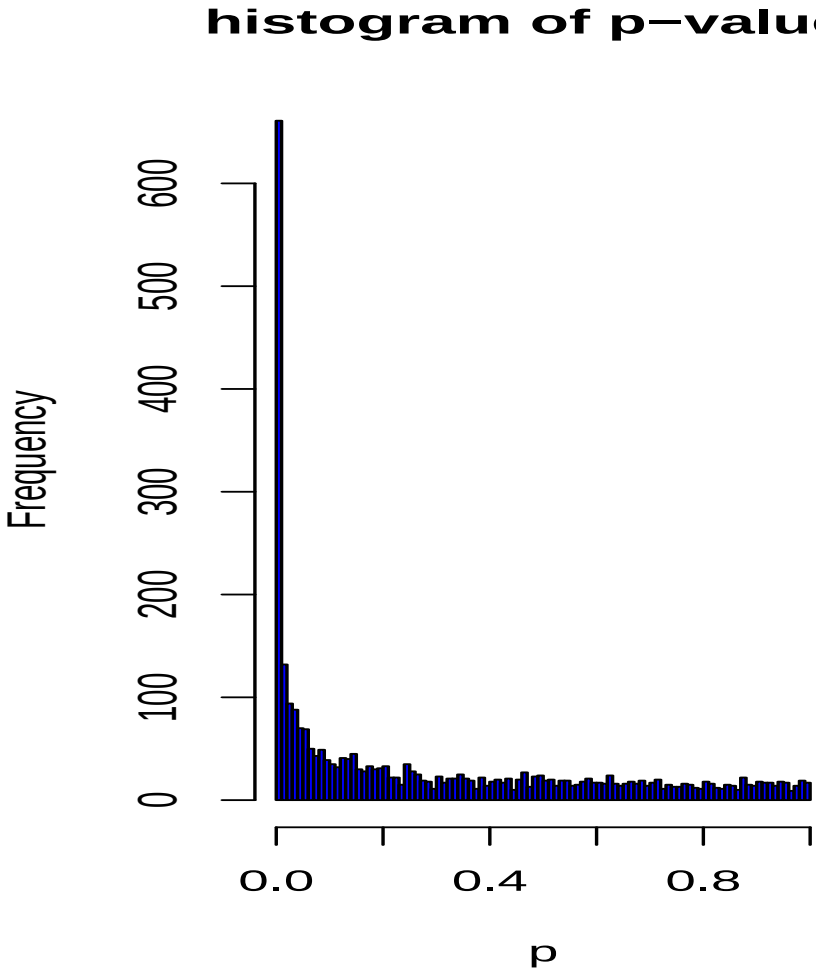
$$\begin{aligned} FWER = Pr(V > 0) = & \quad Pr(\text{at least one } \tilde{p}_g \leq \alpha | H_0) \\ = & \quad Pr(\text{at least one } p_g \leq \alpha/m | H_0) \\ \leq & \quad \sum_{g=1}^{m} Pr(p_g \leq \alpha/m | H_0) \\ = & \quad m * \alpha/m = \alpha. \end{aligned}$$

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.





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):
- $\tilde{p}_g = Pr(\min_{k=1,\ldots,m} P_k \leq p_g | H_0)$.
- Chosing all genes with $\tilde{p}_g \leq \alpha \Leftrightarrow p_g \leq c_\alpha$ controls the FWER at level α:

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

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

Estimation of minP-adjusted p-values through resampling

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

FWER control

❍ The Bonferroni and Westfall/Young methods give strong control of the FWER under mild assumptions.

❍ 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 kth-smallest p-value to the kth-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

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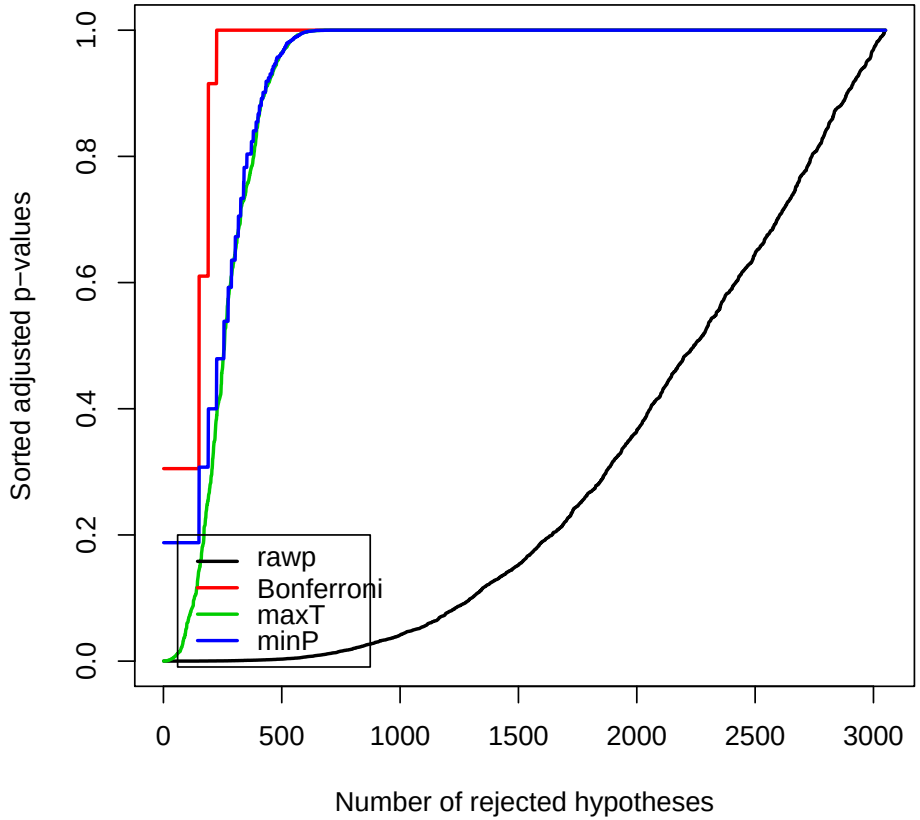


Example taken from the multtest package in Bioconductor.

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 $E(V)$.

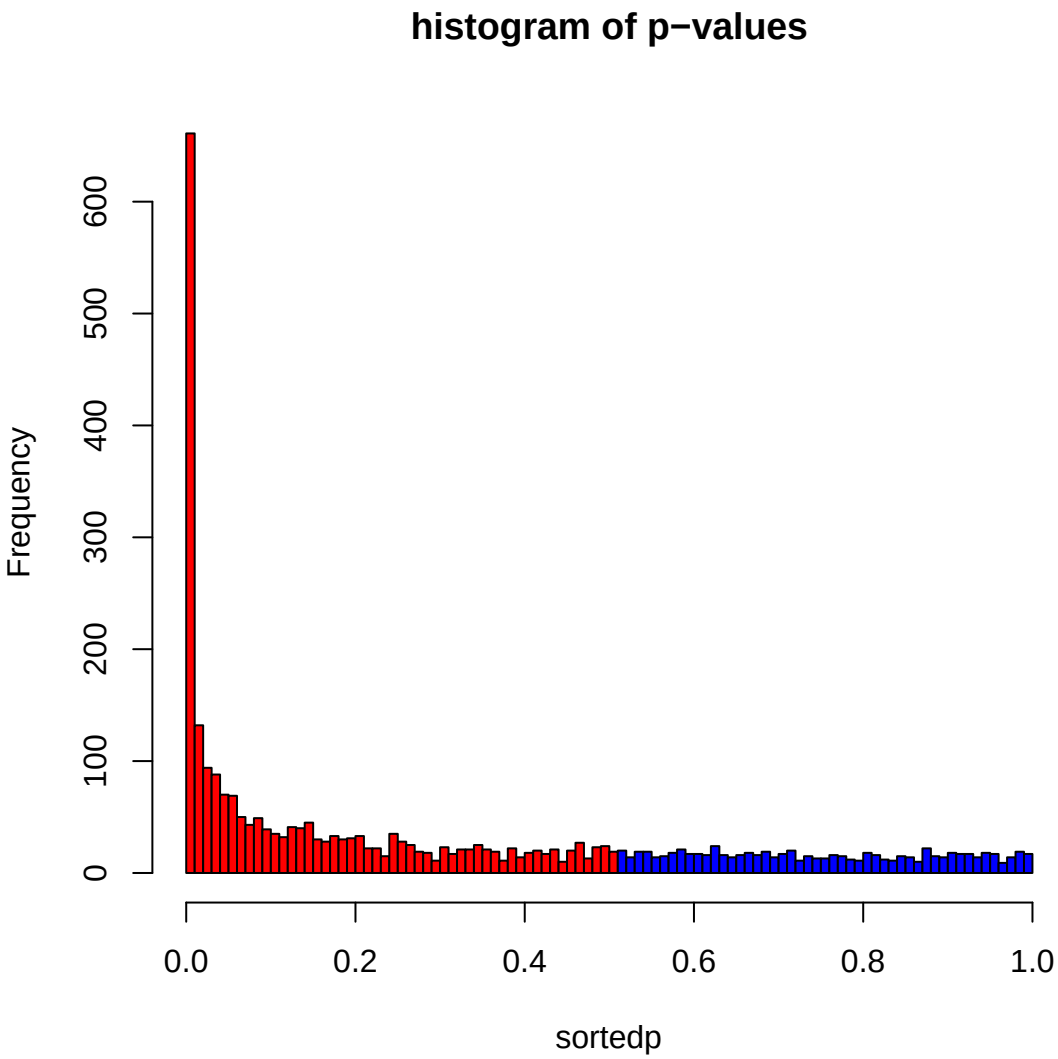
3. Estimate the FDR $E(V/R)$ by $\widehat{E(V)}/R$.

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

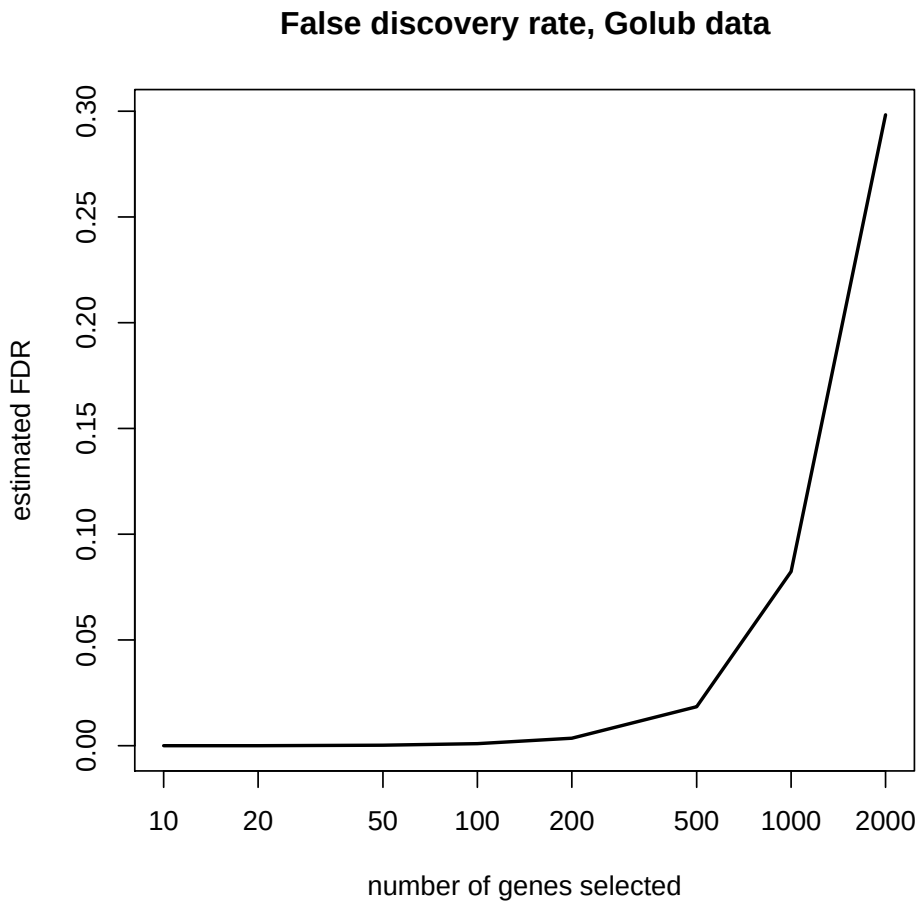
❍ For $b = 1, \ldots, B$, (randomly) permute the sample labels, compute test statistics T_{gb} under the complete null hypothesis.

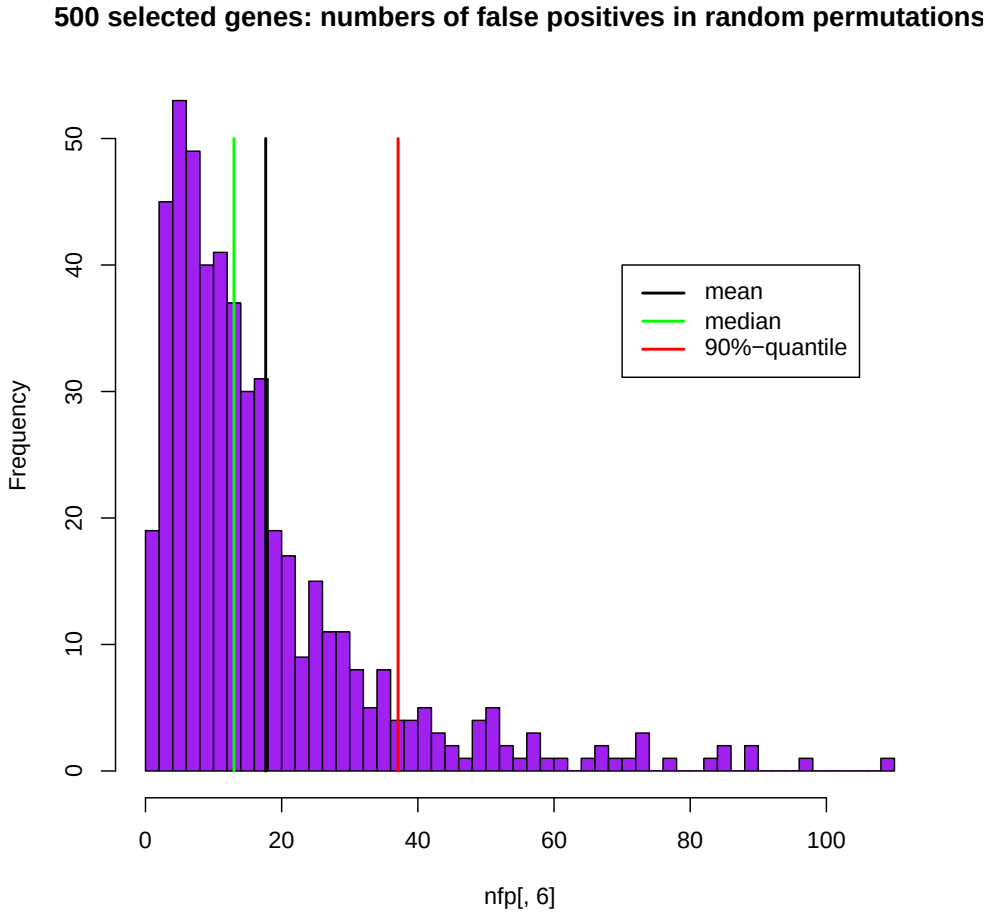
❍ For any threshold t_0 of the test statistic, compute the numbers n_b of genes with $T_{gb} > t_0$ (numbers of false positives).

❍ 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





Estimation of the FDR

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

❍ It is assumed that the distribution of the test statistic is the same for all genes that are not differentially expressed (may be a reasonable assumption for something like the t-statistic).

❍ 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

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❍ Dudoit et al. (2002). Statistical methods for identifying differentially expressed genes in replicated cDNA microarray experiments. *Statistica Sinica*, Vol. 12, 111–139.

❍ 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/

❍ 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.