Multiple Testing and Genomics

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Today's science: genomics

The microarray revolution

Microarray technology

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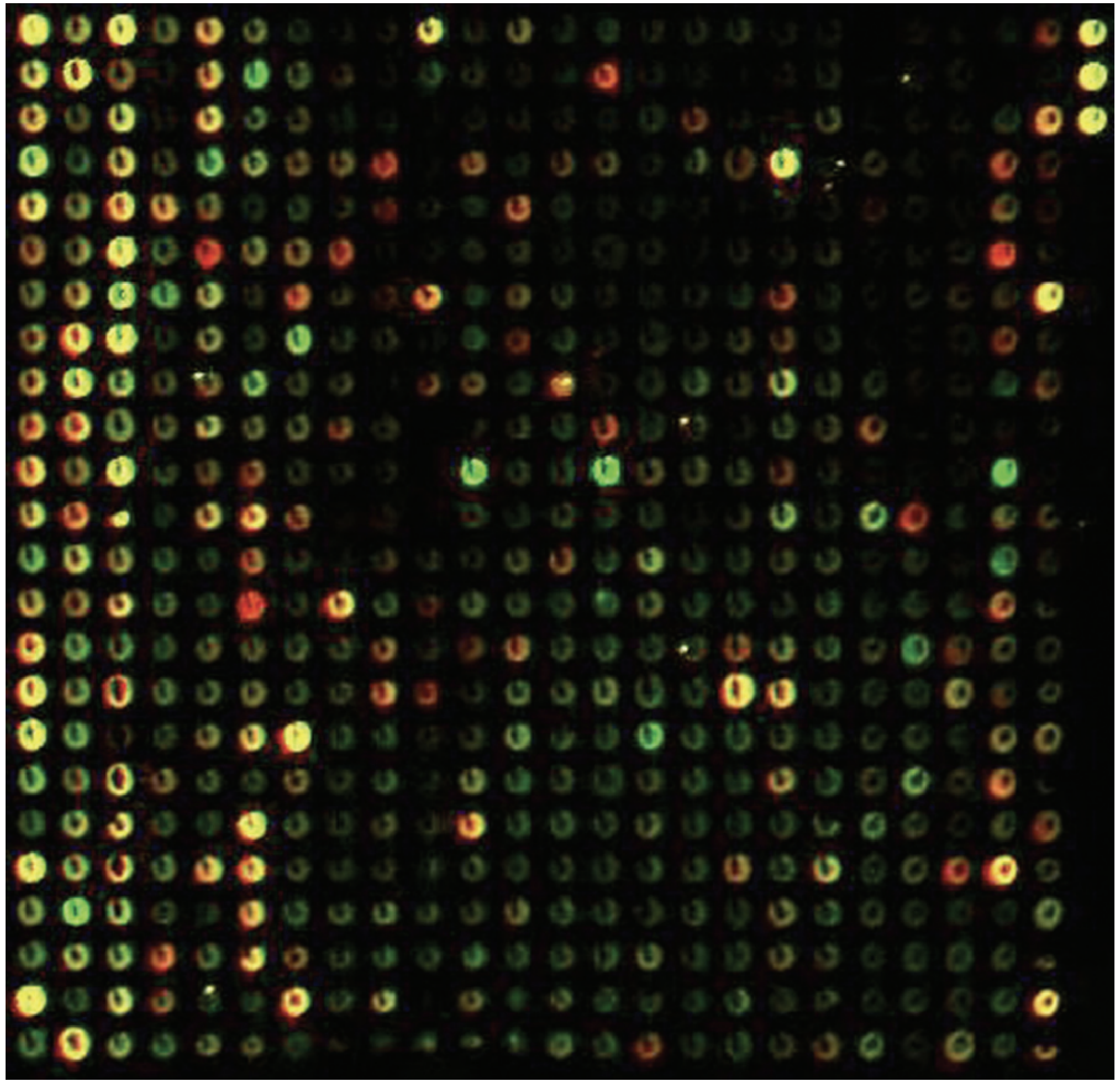
Imagine attempting to measure the expression level of every gene in the genome one at a time. Now imagine assaying thousands of genes all at once. It was the advent of microarray technology that made possible this leap from low to high throughput, allowing researchers to ask questions on a scale that was previously unattainable.



Differential gene expression

Suppose we have the expression level measurements for M genes on n observations, where observations belong to one of two (labelled) experimental conditions or populations.

Questions: Across the two (labelled) conditions/populations/etc., is gene j expressed at different levels, for all $j = 1, 2, \ldots, M$?

Statistically, a (large-scale) two-group testing problem:

- View observations in each group as sample from superpopulation
- Compare the marginal (per-gene) distributions for sampling from superpopulations (i.e. F_X and F_Y) with respect to a chosen functional, e.g. mean or (sort of) median

Scientific goals

What is the role of these hypothesis tests?

- In a nutshell, screening! Genes that “pass” through the filter are subjected to expensive and thorough validation experiments.

Overall philosophy:

- We want to get a moderately sized list of genes that seem like promising candidates for further investigation, and we want it fast and cheap.
- The “cost” of a false positive is that we waste resources on validating a red herring.
- **Key idea**: We can live with (say) 10% of our resources going to waste in the follow-up phase.
- After all, we have already saved a bunch of time and money by not investigating every gene in the full list!

Screen and clean in biology

This “screen and clean” philosophy is pervasive in high-throughput biological data contexts.

Ex. **Genome-wide association studies**: Measure tons of genetic markers and an observable characteristic. Test for association between all of the genetic variant and observable characteristics.

Ex. **Neuroimaging**: Simultaneously measure activity across the whole brain; divide up into 3d pixels (voxels), and test for differences between condition at all voxels.

What do we care about?

Naively, based on last week, might say we care about the FWER: probability of falsely rejecting at least one null hypothesis. That was (sometimes) sensible for last week.

This is not sensible this week.

- Why should we behave like we care so much about a single false positive? It's not a catastrophe, it's just inconvenient!
- With thousands or millions of tests, FWER controlling procedures can return very few rejections indeed. Is such an aggressive screen useful?

A new concept emerges: we care about the **proportion of resources wasted on red herrings in the validation experiments**, as well as the **number of validation experiments we get to do**.

False discovery proportion

On the **proportion of resources wasted on red herrings in the validation experiments**:

- Assume that the follow-up cost is the same for each gene
- Assume that cost is wasted for null genes

Let $X \sim F_X$ represent the data with which we test $H_{0j} : T_j(F_X) = 0$ for all $j = 1, 2, \ldots, p$. Let the follow-up cost for each gene be C.

Then, the "wasted" cost over the total cost is:

$$
\begin{aligned}
\mathrm{FDP}(X) &= \frac{C|\{j : F_X \in \mathcal{F}_{0j} \text{ and } p_j(X) \leq \alpha\}|}{C|\{j : p_j(X) \leq \alpha\}|}, \quad \text{for } \mathcal{F}_{0j} \equiv \{F : T_j(F) = 0\} \\
&= \frac{|\{j : F_X \in \mathcal{F}_{0j} \text{ and } p_j(X) \leq \alpha\}|}{|\{j : p_j(X) \leq \alpha\}|} \\
&= \frac{\text{Number of false discoveries}}{\text{Number of discoveries}} \quad \text{(nb. take convention of } 0/0 = 0.\text{)}
\end{aligned}
$$

FDP via contingency table

Suppose that we test $H_{0j} : T_j(F_X) \in \Theta_0$ for all $j = 1, 2, \dots, M$ using a random variable $X \sim F_X$. Define the following notation:

	H_{0j} **retained**	H_{0j} **rejected**	**Total**
H_{0j} true	$TN(X)$	$FD(X)$	M_0
H_{0j} false	$FN(X)$	$TD(X)$	M_1
Total	$N(X)$	$R(X)$	M

Then, can write the false discovery proportion as:

$$\mathrm{FDP}(X) = \frac{\mathrm{FD}(X)}{\max\{1, R(X)\}}.$$

This computes *vertically*. Contrast the *horizontal* false positive proportion ($\frac{\mathrm{FD}(X)}{M_0}$) or FWER.

FDP and FDR

We have a compelling reason to care about the FDP in this context:

$$\mathrm{FDP}(X) = \frac{\mathrm{FD}(X)}{\max\{1, R(X)\}}.$$

But we can't "control the FDP" in any single study: it's a random variable. Instead, think about controlling its expectation, the *false discovery rate*:

$$\mathrm{FDR} = \mathbb{E}_{F_X}[\mathrm{FDP}(X)].$$

More scientifically: if we were to **repeat** the process of a differential gene expression analysis study followed up by validation experiments on the discoveries, then the average proportion of resources wasted per study across these repeats is the FDR.

- Assuming again follow-up cost uniform across genes.

Your turn: interpreting FDR

Imagine you are working with a genome scientist to analyze their RNA sequencing data. You apply a procedure that controls FDR at level 10% to their data set, and reject 100 null hypotheses. Your collaborator says:

> "Oh, so no more than 10 genes out of that 100 really are differentially expressed?"

What is your answer?

What if your collaborator had said instead:

> "Oh, so about 10 genes out of that 100 really are differentially expressed?"

What is your answer?

Follow-up: interpreting FDR

$$\mathrm{FDR} = \mathbb{E}_{F_X}\left[\frac{\mathrm{FD}(X)}{\max\{1, R(X)\}}\right]$$

1. FDR = α clearly doesn't imply that $\mathrm{R(X)} \cdot \alpha$ upper bounds the number of false discoveries.

- In fact, since $R(X)$ is random, this is a nonsensical thing to say.
- More reasonable to want a probabilistic upper bound for $R(X)$.

2. We know averages across realizations. But this is asking about a single realization. We could only be confident in relating the two if we knew about *concentration*.

- How much of the distribution of FDP is concentrated around the mean?
- The variance of FDP also gives us some information about concentration.

Comparing FDR to FWER

$$\mathrm{FDR} = \mathbb{E}_{F_X}\left[\frac{\mathrm{FD}(X)}{\max\{1, R(X)\}}\right], \qquad \mathrm{FWER} = \mathbb{P}_{F_X}[\mathrm{FD}(X) \geq 1].$$

- Intuitively, the FDR is more adaptive to non-null:null ratio and number of tests
- For FDR, adding discoveries (rejecting true non-nulls) increases the denominator, allowing us to increase the numerator; logic holds no matter how many tests
- But for FWER, only ever allowed to reject one true null, no matter how many non-nulls and no matter how many tests
- Formally, FWER is always larger than the FDR, with equality when all tested hypotheses are null (Homework)

Your turn: interpretation redux

Imagine you are working with a genome scientist to analyze their microarray data. You apply a procedure that controls FDR at level 10% to their data set, and reject 100 null hypotheses. Your collaborator says:

> “I’ve decided to focus on just the 10 genes in that rejection list that have the smallest unadjusted p-values. What can we say about those 10 genes?”

What is your answer?

What if instead they said:

> “I’ve decided to focus on just the 10 genes in that rejection list that are in a particular biological pathway. What can we say about those 10 genes?”

What is your answer?

No subset property, FDR

We know that the following quantity is below 0.1:

$$\mathrm{FDR} = \mathbb{E}_{F_X}\left[\frac{\mathrm{FD}(X)}{\max\{1, R(X)\}}\right].$$

Your collaborator is tempted to think about the FDR among the 10 smallest:

$$\mathbb{E}_{F_X}\left[\frac{\#\{\text{genes that are among 10 smallest, are rejected in } X\text{, and are null}\}}{\max\{1, \#\{\text{genes that are among 10 smallest and were rejected in } X\}}\right]$$

But the numerator and the denominator have both changed, making it impossible to argue that one of them is always smaller than the other!

While the “biological pathway” version might feel intuitively more likely to be true, the logic above holds even when the subset of interest is fixed.

Subset property, FWER

By contrast, $\mathrm{FWER} = \mathbb{P}_{F_X}[\mathrm{FD}(X) \geq 1]$ **does** have the subset property.

Consider:

- the number of false discoveries based on X within all M genes
- the number of false discoveries based on X within a subset of genes

The former is ALWAYS larger!

That means that if there is ≥ 1 false discovery based on X within a subset of genes, then there is ≥ 1 false discovery based on X within all M genes. So

$$\text{FWER on subset} \leq \text{FWER on all.}$$

This logic holds even if the gene subset is selected using the data; this has implications for selective inference (next two weeks!)

Do we need to adjust?

Let $X \sim F_X$ represent the data with which we test $H_{0j} : T_j(F_X) = 0$ for all $j = 1, 2, \ldots, M$.

The naive, unadjusted idea for screening here is:

- Test H_{0j} for all $j = 1, 2, \ldots, M$.
- Reject all genes with p-value $\leq \alpha$.

This will not control the FDR. We know this for sure because:

- FWER = FDR when all tested hypotheses are null
- We established last week conditions where the naive approach does not control FWER.

(Not a very satisfying answer; we will get a more satisfying one on Thursday!)

How to control FDR?

We already know some ways, because FWER is always bigger than FDR. That means that any FWER controlling procedure controls FDR.

But doing so would be unnecessarily conservative.

We will focus on the landmark paper and method: Benjamini and Hochberg (1995).

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Controlling the False Discovery Rate

by Y Benjamini · 1995 · Cited by 101963 — 290 **BENJAMINI** AND **HOCHBERG** [No. 1,. (a)
Much of the methodology of FWER controlling MCPs concerns compari- sons of multiple…

- Showed that a procedure (independently developed by Eklund and Simes) controls the FDR.
- This procedure is now commonly called the “Benjamini-Hochberg”, or BH procedure.

BH procedure

The idea: Order p-values $p_{(1)} \leq \ldots \leq p_{(M)}$:

- If $p_{(M)} \leq \alpha$, then reject $H_{01}, \ldots, H_{0M}$ and stop; otherwise keep going
- If $p_{(M-1)} \leq \alpha\left(\frac{m-1}{m}\right)$, then reject $H_{01}, \ldots H_{0(M-1)}$ and stop; otherwise keep going
- Keep going until you run out of p-values

Corresponds to the following rule:

$$\text{Reject}(p_1, \ldots, p_M) = \left\{ j : p_j \leq \max\{p_{(j)} : p_{(j)} \leq \frac{\alpha j}{m}\right\}.$$

Comparing rejection boundaries

Here, Hochberg is a variant on Holm that controls FWER under independence; it uses the formula $\mathrm{Reject}(p_1, \ldots, p_M) = \left\{ j : p_j \leq \max\{p_{(j)} : p_{(j)} \leq \alpha/(M+1-j)\} \right\}$.

BH intuition

Why does BH work? To get some intuition, we'll set down a simple model and notation.

Define $H_{0j} : \mathcal{T}_j(F_X) = 0$ for $j = 1, 2, \ldots, M$, and let $X \sim F_X$ be the data that we use to test. Let $M_0 = |\{j : F_X \in \mathcal{F}_{0j}\}|$.

Let $p_j(X) \sim \text{Uniform}(0, 1)$ for all j with H_{0j} true, and $p_j(X) \sim F_p$ for all j with H_{0j} false.

We'll use the notation $\text{FD}(X; t)$ to denote the number of false discoveries if you reject all p-values $\leq t$.

FDR approximation

For any fixed threshold t and M big:

$$\begin{aligned}
\mathrm{FDR}_t &= \mathbb{E}_{F_X}\left[\frac{\mathrm{FD}(X;t)}{\max\{1, R(X;t)\}}\right] \\
&\approx \frac{\mathbb{E}_{F_X}[\mathrm{FD}(X;t)]/M}{\mathbb{E}_{F_X}[R(X;t)]/M} \\
&= \frac{\mathbb{E}_{F_X}[\sum_{j:H_{0j}\text{ true}} 1\{p_j(X) \le t\}/m]}{\mathbb{E}_{F_X}[\sum_{j=1}^{M} 1\{p_j(X) \le t\}/M]} \\
&= \frac{M_0\mathbb{P}(\mathrm{Uniform}(0,1) \le t)/M}{\mathbb{E}_{F_X}[\sum_{j=1}^{M} 1\{p_j(X) \le t\}/M]} = \frac{(M_0/M)t}{\mathbb{E}_{F_X}[\sum_{j=1}^{M} 1\{p_j(X) \le t\}/M]}
\end{aligned}$$

Estimating FDR

$$FDR_t \approx \frac{(M_0/M)t}{\mathbb{E}_{F_X}[\sum_{j=1}^{M} 1\{p_j(X) \leq t\}/M]}$$

We don't know what M_0/M is, but we can conservatively estimate it by 1.

We don't know what $\mathbb{E}_{F_X}[\sum_{j=1}^{M} 1\{p_j(X) \leq t\}/M]$ is, but given a realization x from $X \sim F_X$, we can estimate it with the empirical equivalent. Leads to:

$$F\hat{D}R_t(x) = \frac{t}{\sum_{j=1}^{M} 1\{p_j(x) \leq t\}/M}.$$

Assuming that there are no ties in p-values, and plugging in $p_{(j)}(x)$ to RHS yields $\frac{p_{(j)}}{j/m}$.

Reinterpretation of BH

Recall that BH says to reject every hypothesis with a p-value no larger than $p_{(\hat{j})}$, where

$$\hat{j} = \max\left\{j : p_{(j)} \leq \frac{\alpha j}{M}\right\}.$$

Rearranging terms, no larger than

$$\hat{j} = \max\left\{j : \frac{p_{(j)}}{j/m} \leq \alpha\right\}$$

Recall that $\frac{p_{(j)}}{j/m}$ is an estimate of the FDR if we reject at $p_{(j)}$!

BH procedure picks the biggest rejection threshold that yields an estimated FDR below targer α.

Empirical Bayes interpretation

Do you like Bayesian measures of uncertainty? Augment our simple model with a prior:

$$1\{H_{0j} \text{ false}\} \overset{iid}{\sim} \text{Bernoulli}(\pi_0)$$

Then for any threshold t, can derive the "Bayesian FDR":

$$\mathbb{P}(H_{0j} \text{ true} \mid p_j(x) \leq t) = \frac{t\pi_0}{\mathbb{P}(p_j(X) \leq t)}.$$

An empirical Bayesian then estimates with frequentist principles to get:

$$\mathbb{P}(H_{0j} \text{ true} \mid p_j(x) \leq t) \approx \frac{t}{\sum_{j'=1}^{M} 1\{p_{j'}(x) \leq t\}/M}.$$

Empirical Bayes interpretation

$$\mathbb{P}(H_{0j} \text{ true} \mid p_j(x) \le t) \approx \frac{t}{\sum_{j=1}^{M} 1\{p_j(x) \le t\}/M}.$$

This is our frequentist estimate of the approximation to FDR_t from earlier!

So another interpretation is that BH takes the largest threshold so that the estimated Bayesian FDR is $\le \alpha$.

If we buy into Bayesian notions of probability, can even say rejected cases "have estimated probability of being null lower than α".

BH under independence

Benjamini and Hochberg (1995) showed the following result.

Suppose that we test H_{0j} for $j = 1, 2, \ldots, M$ using $X \sim F_X$, and choose which hypotheses to reject by plugging p-values $p_1(X), \ldots, p_M(X)$ into the BH procedure.

Suppose that $p_1(X), \ldots, p_M(X)$ are valid .

Then,

$$\mathrm{FDR}_{BH} = \frac{M_0}{M}\alpha \leq \alpha.$$

BH under PRDS

Benjamini and Yekeutili (2001) showed that:

If the joint distribution of the p-values is independent, OR the subset of p-values corresponding to true nulls is ` `positive regression dependence on a subset" (PRDS), then

$$\mathrm{FDR}_{BH} = \frac{M_0}{M}\alpha \leq \alpha.$$

PRDS is technical and not so interpretable, but can show it holds for important subcases: e.g. p-values constructed with test statistics where the null distributions are jointly multivariate normal with all off-diagonal covariance matrix entries positive.

BH under general dependence

- Benjamini and Yekeutili (2001) also showed it's possible to make a small change to BH procedure to control the FDR under ANY dependence structure
- `p.adjust(..., method = "BY")` in R
- But this is typically very very conservative; not often used
- In practice, BH is hard to "break": FDR is typically controlled even if PRDS is not satisfied
- That being said, correlation can still throw a wrench by increasing variability of FDP. (Might see some of this on Thursday …)

Adjusted p-values

The idea: Convert $p_1, \ldots, p_M$ to $\tilde{p}_1, \ldots, \tilde{p}_M$ so that

$$\text{Reject}(p_1, \ldots, p_M) = \{j : \tilde{p}_j \leq \alpha\}$$

See e.g. `stats::p.adjust()`. General definition that applies to any rejection procedure.

Caution: interpretation issues

- Raw p-values have a natural interpretation: “If the null hypothesis were true, then how often would I see such an extreme value of the test statistic, were I to *repeat* the experiment?”.
- Justifies interpretation as “measure of evidence against the null”, and justifies thumb rules (< 0.001 = “overwhelming”, < 0.01 = “strong”, < 0.05 = “moderate”)
- Adjusted p-values have no such interpretation; so what can we read into (say) $\tilde{p}_j = 0.035$?
- In the case of FDR adjustment methods, q-values provide an interpretable alternative; see Storey and Tibshirani (2003) if you’re interested in learning more.

Food for thought

- We've really only scratched the surface; this was and is a very active research area!
- There are many other proposed error rates: positive FDR, directional FDR
- There are related research directions: e.g. exceedance probability control
- There are many methods for controlling FDR that try to improve on BH, e.g. by estimating the number of truly null hypotheses from the data
- There are many methods for controlling FDR that look quite different; a particularly popular one in recent years is knockoffs

Many options you can pick for your final presentation that can help you learn more!

Multiple testing wrapup

Multiple testing: Whenever we conduct more than one hypothesis test in a single analysis, and look at the outcome of all of them.

- Does this cause problems? If so, what are they?
- Do we need to adjust for multiple testing? If so, how should we adjust?

We saw very different answers to these questions within a single class last week, and then once again very different answers this week!

Does that make sense?

A review of the science

> **“I don’t know, it depends on the scientific problem at hand.”** - My annoying partner, whenever I ask him ANY question involving statistics.

	Clinical Trials	Genomics
Number of tests?	A few	Lots
Hypothesis selection	Carefully curated	Little to no screening
False positives?	Awful	Can live with a few

The science is different, so no wonder the answers are different.

> **“You better think (think) about what you’re trying to do …”** - Aretha Franklin, “Think”