Statistical methodology for the analysis of dye-switch microarray experiments

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UMR AgroParisTech/INRA 518
UMR INRA/ENVA/CNRS 1198, Jouy en Josas

Background

We consider a two-color microarray experiment where two treatments are compared, and where the number of slides is fixed.

We expect the experimental design to guarantee

- the balance between the two fluorochromes,
- a good compromise between technical and biological replicates.

We also need efficient methods for the statistical analysis of the proposed design.

Globally balanced design : dye-switch

array	1	2	3	4	5	6	7	8	9	10
Cy5	A1	B2	A3	B4	A5	B6	A7	B8	A9	B10
Cy3	B1	A2	B3	A4	B5	A6	B7	A8	B9	A10

- A_i : i^{th} biological sample in condition A
- The 10 arrays are independent
- 10 biological replicates per condition, no technical replicate.

The dye-switch design is often considered as optimal :

⋆ maximum number of biological replicates,

⋆ variance estimated with 9 *df*,

⋆ the statistical analysis is straightforward.

Individually-balanced design : dye-swap

array	1	2	3	4	5	6	7	8	9	10
Cy5	A1	B1	A2	B2	A3	B3	A4	B4	A5	B5
Cy3	B1	A1	B2	A2	B3	A3	B4	A4	B5	A5

- 5 pairs of biological samples
- Each pair is used two times (with reverse dyes)
- Each dye-swap is independent of the other ones

The dye-swap design is commonly used :

⋆ used when the technical error is high,

⋆ the statistical analysis (after averaging by swap) is straightforward,

⋆ variance estimated with only 4 *df*.

Individually-balanced : “hybrid” dye-switch

array	1	2	3	4	5	6	7	8	9	10
Cy5	A1	B1	A2	B2	A3	B3	A4	B4	A5	B5
Cy3	B1	A2	B2	A3	B3	A4	B4	A5	B5	A1

- 5 biological replicates per condition
- Each individual is used two times (one with Cy3, one with Cy5).
- Array k is correlated with $k-1$ and $k+1$

The hybrid design could be an alternative :

⋆ variance estimated with more than 4 *df*.

⋆ the statistical analysis is not straightforward,

$\Rightarrow$ Need of mixed models.

Statistical model for a gene

After log-transformation :

$$\begin{array}{lllllllll} X_i & = & \mu_A & + & \delta_{l(i)} & + & B_{j(i)} & + & M_i & + & T_i \\ Y_i & = & \mu_B & + & \delta_{l'(i)} & + & B_{j'(i)} & + & M_i & + & T'_i \end{array}$$

$B_{j(i)} \sim N(0, \sigma_B^2)$, $j(i)$: sample number corresponding to condition A and array i.

$M_i \sim N(0, \sigma_M^2)$ effect of the spot associated to the gene under concern in microarray i

$T_i \sim N(0, \sigma_T^2)$, includes the steps of labeling, hybridization and measure of intensity of fluorescence.

Model on the difference of expressions

For slide $i = 1, ..., 2n$:

$$\begin{aligned} D_i &= X_i - Y_i \\ &= \mu + (-1)^{i+1}\delta + BD_i + TD_i \end{aligned}$$

- $\mu = \mu_A - \mu_B$: true differential expression between A and B,
- $BD_i = B_{j(i)} - B_{j'(i)} \sim N(0, 2\sigma_B^2)$,
- $TD_i = T_i - T_i' \sim N(0, 2\sigma_T^2)$,
- $\delta = \delta_1 - \delta_2$: gene-specific dye bias.

$$\begin{aligned} E(D_i) &= \mu + (-1)^{i+1}\delta \\ V(D_i) &= 2\sigma_B^2 + 2\sigma_T^2 \\ cov(D_i, D_j) &= 0, \text{ except } cov(D_i, D_{i+1}) = \sigma_B^2. \end{aligned}$$

Unbiased estimate of $V(\overline{D})$

Unbiased estimator of μ : $\overline{D} = \frac{1}{2n}\sum D_i$

$$V_{\overline{D}} = \frac{1}{2n}(4\sigma_B^2 + 2\sigma_T^2)$$

Naive variance estimate : $S^2 = \frac{1}{2n-2}\sum_i (D_i - \overline{D}_{(i)})^2$

$$E(S^2) = 2\sigma_B^2 + 2\sigma_T^2 < 4\sigma_B^2 + 2\sigma_T^2$$

Unbiased variance estimate $C = \frac{1}{2n-4}\sum_i (D_i - \overline{D}_{(i)})(D_{i+1} - \overline{D}_{(i+1)})$

$$E(C) = \sigma_B^2$$

$$\begin{aligned} S_c^2 &= \frac{1}{2n}(S^2 + 2C) \\ E(S_c^2) &= \frac{1}{2n}(4\sigma_B^2 + 2\sigma_T^2) \end{aligned}$$

Test statistics (for each gene)

Mixed Model with ML estimation (ML)

Mixed Model with REML estimation (REML)

Naive Method (NM)

$$T_N = \sqrt{2n}\frac{\overline{D}}{\sqrt{S^2}}$$

Unbiased Paired Method (UP)

$$T_{UP} = \sqrt{2n}\frac{\overline{D}}{\sqrt{S_c^2}}$$

Level (in %) of the test procedures (simulation of 10000 genes, nominal level= 5%)

$$\sigma_B^2 = 0.5$$

Method	$n = 5$	$n = 10$	$n = 20$	$n = 30$
NM	7.0 (0.3)	7.3 (0.3)	7.3 (0.3)	7.6 (0.3)
UP	5.2 (0.2)	5.2 (0.2)	5.3 (0.2)	5.3 (0.2)
ML	8.5 (0.3)	8.6 (0.3)	8.3 (0.3)	8.3 (0.3)
REML	4.8 (0.2)	4.2 (0.2)	4.5 (0.2)	4.9 (0.2)

$$\sigma_B^2 = 2$$

Method	$n = 5$	$n = 10$	$n = 20$	$n = 30$
NM	13.2 (0.3)	14.0 (0.3)	14.0 (0.3)	14.2 (0.3)
UP	8.2 (0.3)	6.9 (0.2)	6.0 (0.2)	5.8 (0.2)
ML	12.5 (0.4)	11.1 (0.4)	9.9 (0.3)	9.9 (0.3)
REML	14.7 (0.4)	8.6 (0.3)	5.9 (0.2)	5.5 (0.2)

Power of UP and REML tests

$\mu = 1$

n	σ_B^2	UP	REML
5	0.5	13.6	10.6
5	2	5.0	12.9
10	0.5	39.3	34.0
10	2	7.8	9.1
20	0.5	80.1	78.1
20	2	14.5	13.9
30	0.5	95.5	95.0
30	2	22.5	21.7

$\mu = 3$

n	σ_B^2	UP	REML
5	0.5	92.1	86.7
5	2	29.4	34.7
10	0.5	100	99.6
10	2	63.5	63.1
20	0.5	100	100
20	2	94.8	94.5
30	0.5	100	100
30	2	99.6	99.5

Compared CPU time

n	UP CPU	REML CPU	no REML convergence
5	2.3	787	56.9
10	2.6	212	5
20	2.8	467	0
30	3.2	1046	0.16

User CPU time of procedures UP and REML ($\sigma^2 = 0.5$).
Last column : average number of genes for which REML did not converge.

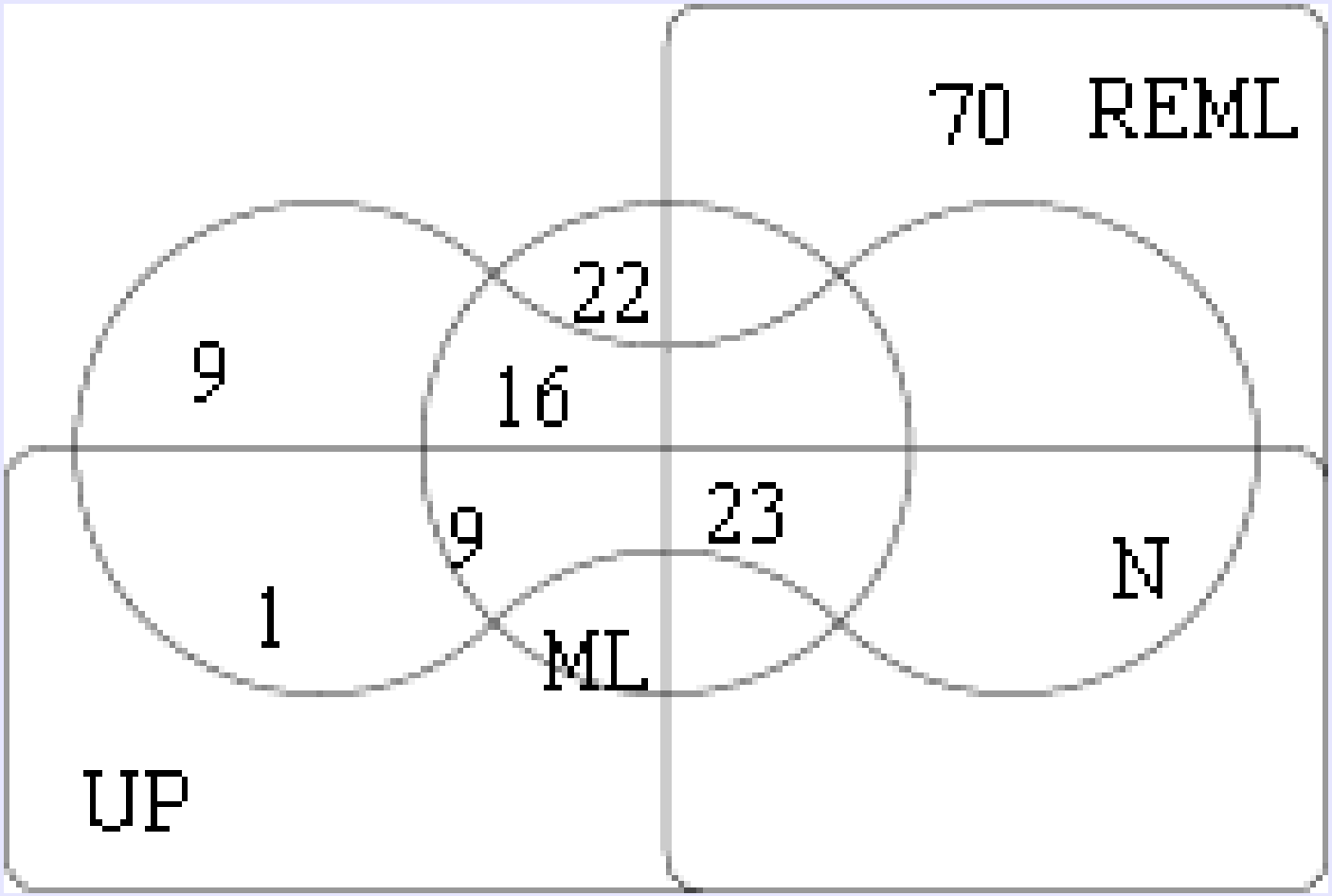
Embriogenomics experiment

In Mammals, the implantation of the embryo is a key event in the establishment of a pregnancy.

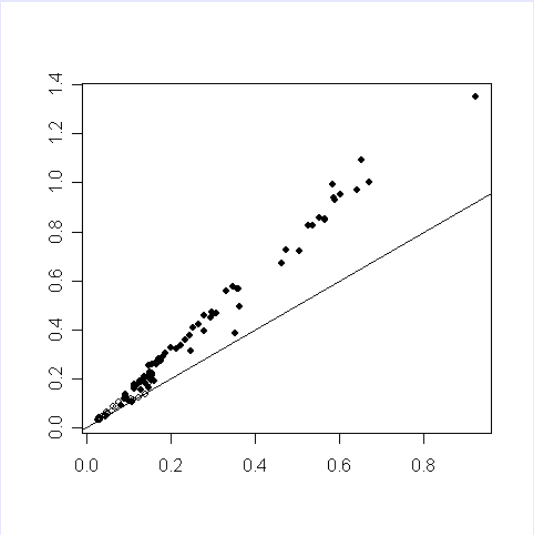
Microarray experiment : 13300 genes in the endometrium of cows. Only 5 animals were available for each condition $\Rightarrow$ hybrid dye-switch design.

Condition A : cyclic (day 20 of cycle)
Condition B : pregnant (day 20 of pregnancy).

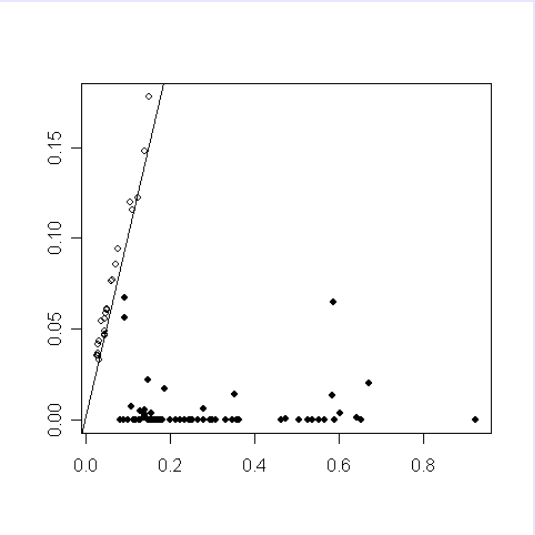
Number of genes DE found by 4 methods



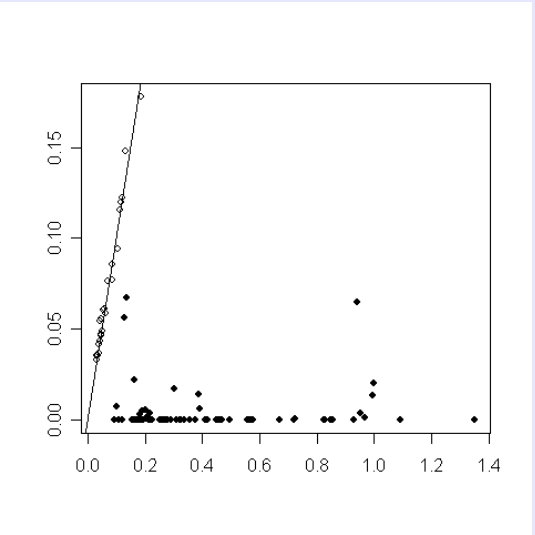
Plots of variance estimates for 93 genes



x-axis :ML
y-axis :UP



x-axis :ML
y-axis :REML



x-axis :UP
y-axis :REML

black points : classified DE only by REML

circle : classified DE by all methods.

Conclusions and perspectives

Practical guidelines :

- ⋆ Correlations must be taken into account !
- ⋆ UP should be preferred to REML (more robust, computationally efficient).
- ⋆ ANOVA estimators for variances should be considered.

Extension to more complex designs :

- ⋆ Loop designs
- ⋆ Interwoven loop designs

Often proposed for their theoretical properties (Wit et al 05, Altman & Hua 06) but with no practical considerations.

Bibliography

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