1. Experimental Layout:

- Define the goal of your experiment in a quantitative way: Make an appropriate structural compromise between the most informative and most robust set-up for your experiment.
- For cDNA microarrays comparisons made on the same slide are always more accurate than comparisons linked via several slides via common references. For experiments with only few comparisons of interest it is best to choose a design, where these comparisons are performed directly.
- For experiments with many comparisons of interest a design including all possible comparisons often becomes unfeasible. There is usually a choice between several different designs, which should be carefully considered.
- The most common design for two-color (competitively hybridized spotted) arrays is the 'reference design': each experimental sample is hybridized against a common reference sample. Although this effectively means that only one sample of interest is hybridized per chip, the reference design has several practical advantages over more efficient designs: extends easily to other experiments, if the common reference is preserved, facilitates inter-comparison of cross-array data, is robust to multiple chip failures, reduces incidence of laboratory mistakes, because each sample is handled the same way. But, be aware that the reference design may waste a lot of information which may be kept by an alternative design without using more resources.

2. Replication:

- Replication of biological samples is essential in order to draw conclusions that are valid beyond the scope of the particular samples that were assayed.
- There are several different levels of replication:
  1. First, replication of biological samples, in which the complete experiments is reproduced on independent biological samples. This is the most important level of replication and essential in order to understand the reproducibility of an experiment.
  2. During technical replicates only the hybridization and possibly RNA preparation steps are reproduced using the same biological sample. These may be used to get a better understanding of how big the variation introduced at the various steps is. It might also help to increase precision.
  3. Third, duplication of spotted clones on the microarray slides helps little to increase precision and to provide quality control and robustness to the experiment.
- Full disclosure of the details of sample preparation and handling is important to help identify the independent units in an experiment and to avoid inflated estimates of significance or artificial conclusions.
- Independent replication of the biological samples gives the most information and includes the other types of replication at lower levels. It is most important for the assessment of the quality of an experiment to include totally independent replicates.
- Statistical quantification of evidence is widely accepted as a standard requirement in scientific investigation and is preferred over the qualitative description of observations. A carefully designed experiment provides a sound basis for statistical analysis and lends itself to simple

and powerful interpretation. Putting experimental design principles into practice is not difficult, and there are often several design alternatives that will work well for any given situation.

- Give full account on the logical structure of your experiment by determining the special features of the process which influence the outcome of your experiment: sources of RNA, different production batches of microarrays, technical aspects of the microarray platforms (for example printheads for spotted cDNA arrays), etc. The structure of possible influences has to be considered into the structure of the experiment. It is highly recommended to use randomisation to exclude bias introduced by hidden variables not accounted for. The technical term is *randomized experimental design* or *randomized block design*. If possible, the groups in your experiments should be balanced – the same number of members of each group.
- Perform sample size calculations to see which set-up of your experiment can differentiate which diffenretial gene expression. A small experiment will only grasp a fold change of 10, while much larger sample sizes are needed to get differences which are of biological interest.
- Techniques like t-test or analysis of variance are often used and are appropriate for the statistical analysis of experiments. However, these methods are unstable using thousands of genes and only few (<20) repeats. Some modified versions of these methods exist, and are highly recommended, which use information of all the genes to make variance estimation more stable with only few repeats. Still a reasonable number (>3) is essential to be able to draw valid conclusions.

3. Quality control:

- Quality control on your experiment has to be performed.
- One approach is to use housekeeping genes whose expression is assumed to be constant in all conditions.
- Spiked in controls (positive dynamic range controls, negative controls, ratio controls) provide a good indication of the quality of an experiment.

The following are some important points to keep in mind:

- *Use adequate biological replication.* A common mistake is to generate an excess of technical replication with little or no independent replication of the biological samples. This is akin to studying the difference in heights of the two sexes by repeatedly measuring one man and one woman.
- *Make direct comparisons between samples* whose contrasts are of most interest and use short paths to connect any samples that might be contrasted.
- *Use dye swapping or looping to balance dyes and samples.*
- *Always keep the goals of the experiment in mind.* Experiments that are constructed to address a particular question are more likely to be simple and interpretable compared to experiments compiled from a haphazard set of conditions.

Conclusions:

Different studies have different objectives, and important aspects of design and analysis strategy differ for different types of studies. Experiments that are constructed to address a single particular question with very few factors (idealy 1 or 2) are more likely to be simple and interpretable compared to experiments compiled from a unsystematic set of conditions.

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