A look back: putting differences aside

Scientists have performed nucleic acid hybridization experiments since the early 1960s, but it was not until the late 1970s that an enterprising Stanford University graduate student named Fred Alt would demonstrate the potential of comparative hybridization for directly measuring changes in gene expression between two cell types. He and his colleagues were studying AT-3000, a murine sarcoma cell line known to greatly overexpress the enzyme dehydrofolate reductase (DHFR) relative to the parental line from which the cells had been derived. They generated radiolabeled cDNA from AT-3000 mRNA, which they hybridized to excessive quantities of parental cell line mRNA and purified on hydroxyapatite resin, separating out all double-stranded nucleic acid hybrids. This process readily yielded DHFR cDNA, left behind after 'subtraction' of transcripts common to both cell types owing to considerably reduced DHFR expression levels in parental lines. These findings would prove important as the first demonstration of both the phenomenon of gene multiplication and of the principle behind the method later known as 'subtractive hybridization'[1].

Shortly afterward, William Timberlake of Wayne State University presented a modified version of this procedure, which he dubbed 'cascade hybridization', for the isolation of genes differentially expressed at various developmental stages in the fungus *Aspergillus nidulans*[2,3]. His results would demonstrate the potential for performing broader comparative studies, yielding hundreds of previously unidentified life stage–specific transcripts, and the combined impact of his work with that of Alt and others in the field would make subtractive hybridization an immensely popular means for comparing gene expression states.

The method was still routinely being used in the 1990s, although some of the shine had by now worn off. At Harvard Medical School, Arthur Pardee was using two-dimensional electrophoresis to examine changes in protein expression, but bemoaned the absence of similar techniques for RNA-based expression analysis. Even though his wife and colleague, the late Ruth Sager, had found considerable success with subtractive hybridization in identifying cancer-related genes, he found the method needlessly laborious and sought a faster alternative. What he came up with was the concept for PCR amplification of expressed transcripts, using one relatively promiscuous primer of arbitrary sequence in conjunction with a second primer targeting the poly(A) tail. "If you [only] took primers at random, they'd be all over and you'd have too many pieces," explains Pardee. "Picking primers of the right size, and making them dependent on one place, led to the technique that we developed." Pardee gives much credit to former postdoc Peng Liang, whose biochemistry experience helped him to iron out the details of creating PCR-derived 'fingerprints' of the mRNA content of cells from various tissues or disease states. Liang found the ideal length for arbitrary primers that reasonably balance affinity and promiscuity, guided in part by recent DNA fingerprinting work[4], and he also came up with the idea of adding an 'anchor' sequence on the poly(dT) primer that ensures positioning near the start of the tail. Liang puts it modestly: "I made a lot of good educated guesses." Nevertheless, the method worked, and Liang and Pardee found that 'differential display' allowed them to rapidly compare RNA from several samples, with pattern variations revealing PCR bands that represent cell-specific transcripts[5].

Differential display was widely adopted—but also became the focus of controversy, specifically from some researchers who charged that it was plagued by false positives and limited detection of rare transcripts. Liang and Pardee acknowledge the challenges of their method, but also insist that success essentially comes down to mastering the great precision required by the technique. "Because you are comparing things," says Liang, "any errors introduced by pipetting or anything could introduce difference." Pardee agrees: "No method is perfect, but it is a matter of good technique as well as the method. You want to optimize every technique as best you can to avoid variations." And indeed, differential display was vindicated by at least one comparison study that downplayed the relative extent of these problems and favored the technique over other existing methods[6].

The potential for interexperimental variability has also been addressed in part by the development of automated differential display systems and improved understanding of proper experimental design—areas in which Liang has been actively involved[7]. And even in the age of the microarray, differential display still offers some unique advantages. "[It's] a fair amount of work," says Pardee, "but it's a way of discovering gene expression changes in all sorts of situations... [and] one can do it with very primitive equipment." Liang agrees, emphasizing the usefulness of differential display when microarrays may not be an option: "The method itself is basically a sequence-independent approach that does not require prior knowledge of the gene sequence, [and] a lot of people doing [differential display] these days are those that are working on systems where there is no genomic information... so basically, any eukaryotic systems can be compared."

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