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In situ hybridization to mRNA: from black art to guiding light

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ABSTRACT ***In situ* hybridization to mRNA in embryo sections or wholemount embryos is one of the most powerful analytical tools available to the molecular developmental biologist. For many workers, this procedure provides the first insights into the function of newly isolated genes, allowing the formulation of hypotheses and setting the course for further research. This paper presents a personal historical perspective of the development of *in situ* hybridization, looks at the theory and practice of the technique, summarizes the current state of the art, and speculates on possible directions for the future as a tool in functional genomics.**

KEY WORDS: *in situ hybridization, gene expression, mRNA detection, embryo.*

The year is 1986. A hopelessly underdressed Australian postdoc arrives in London with a backpack and a £6,000 stipend from an Australian charity with the unlikely name of Uncle Bob's Club. I am looking for the MRC Mammalian Development Unit, an institution of such lofty stature that my surprise at finding it occupying a run-down building of arresting ugliness in a cobbled back-alley near Euston station made me think this must surely be the wrong Wolfson House. But no, my A-Z had informed me correctly and my journey of discovery into London life, British/Australian terminological incompatibilities, and the secrets of mouse embryo development was about to begin.

Stories had been circulating about an amazing new technique that allowed researchers to visualise exactly when and where in an embryo individual genes are active. Publications had started to appear in the top journals showing dazzling, stripey patterns of gene expression in sections of *Drosophila* larvae (Akam, 1983; Ingham *et al.*, 1985), and rumour had it that the same technique could be applied to other species. At this point even the name for this technique was not decided—some called it hybridization histochemistry, but the name *in situ* hybridization was gaining hold.

At the time, molecular biology was in its heyday. The first volume of the classic molecular biology cookbook by Maniatis, Sambrook and Fritsch had been in use for four years (Maniatis *et al.*, 1982). DNA libraries were being constructed at a great rate, and homology screening was the Latest Thing. (Homeobox genes conserved in vertebrates? What next!) What developmental biologists needed was a way of deriving spatial information regarding gene activity in the developing embryo in order to get clues about the functions of all the newly cloned genes. Northern blotting was useful for adult tissues, and for studying the timing of gene expression in whole mouse embryos older than 9.5 days *post coitum* (dpc), but called for too much tissue to be useful for developmental studies. Really keen workers were able to use nuclease protection techniques to measure gene expression in painstakingly dissected embryonic tissues (Jackson *et al.*, 1985), but these techniques did not provide information at the single cell level.

In situ hybridization promised to provide all the answers. My mission was to establish this technique for use with mouse embryos at the MDU under the auspices of Anne McLaren, who had the curious impression that I knew what I was doing. I sought cover in the labyrinthine University College Library, and soon made a shocking discovery: here was a technique that took the best part of two weeks to perform, and called for expertise in histology, molecular biology, embryonic anatomy, even particle physics. The number of steps involved, and the number of variables at each step, was truly prodigious. What was worse, every paper I read on the technique seemed to recommend a completely different protocol.

How should the sample be fixed? Was it better to use paraffin sections or cryostat? What sort of probe should be used? How should it be labelled? What prehybridization treatments were necessary? What probe concentration should be used? How long should the hybridization step be? At what temperature? What sort of wash stringency was suitable? What photographic emulsion was the best use? How should the samples be counterstained? How would I make sense of the signal, even if I did get any? Where on earth should I start?

Abbreviations used in this paper: DIG, digoxygenin; dpc, days *post coitum*; mRNA, messenger ribonucleic acid; nt, nucleotides; RNA, ribonucleic acid.

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For a newcomer to molecular biology, it was hard to figure out the purpose of all those steps in the published protocols. Nevertheless, I was able to lay my hands on several papers in which the authors had taken the trouble to optimise the procedure, and describe the variables they had tested (Akam, 1983; Hafen *et al.*, 1983; Cox *et al.*, 1984; Gizang-Ginsberg and Wolgemuth, 1985; Berger, 1986). The greatest variability was in the prehybridization treatments, which involved sorcerous combinations of acid washes, hot buffer washes, alcohol washes, acetylation, protease treatment, neutralization and fixation; everything, it seemed, except eye of newt and something involving phases of the moon.

One of the nice things about working in London is that there is no shortage of expertise on almost any subject, a disarming willingness to share it, and many pubs in which to do so. Several other groups in London appeared to be making some progress with *in situ* hybridization to mouse embryos sections. These included Sigrid Lehnert and Rosemary Akhurst at St Mary's Hospital, Rebecca Haffner and Keith Willison at the Chester Beatty, Peter Holland and Brigid Hogan at MRC Mill Hill, and the future high priest of *in situ* hybridization, David Wilkinson, then working with Andy McMahon also at MRC Mill Hill. Talking to these groups provided great moral support but in practical terms made the situation even worse, with the added complication of anecdotal advice of the type extravagantly shared in beery conversation but never committed to print. American photographic emulsion, it transpired, gave higher background since it picks up cosmic rays in the aeroplane! Salt had to be added to the emulsion to prevent the probe falling off when the slides were dipped! This had to be a salt that could evaporate, otherwise crystals would ruin the result (and so on).

After what felt like only 13 months of this sort of caper (probably far longer), Anne gently suggested that I might like to try actually doing an experiment some time. I put together a protocol that was "averaged" from all available information. Some choices seemed quite straightforward. Cloning vectors that allowed *in vitro* RNA transcription (pGEM and, later, pBlueScript) had just hit the market, and it was clear that RNA probes represented the way forward in terms of sensitivity and low background, due to hybrid stability and the possibility of eliminating non-specific binding with RNase A. Paraffin sections seemed simplest, even though cryostat sections also seemed to work. Some hybridization cocktail involving salts, a buffer, a blocking agent (yeast RNA), an exclusion agent (dextran sulphate), a stringency agent (formamide), and so on seemed standard. The advantages of ^{35}S over ^{32}P (resolution, half-life) were abundantly clear.

Other variables needed to be tested empirically. I devised a test system using a probe for the abundantly expressed gene α-globin, and ^{32}P as the label to allow fast turn-around time. To describe the results as surprising would be a most British understatement. The only pre-treatment that made any perceptible difference was the protease digestion, which presumably improved access of the probe to target RNA molecules in the tissue section. The degree to which the probe was hydrolysed to reduce its size (or whether the probe was hydrolysed at all) did not seem to make any difference. Salt in the emulsion was bunk. In fact the discrepancies between our results and the conventional wisdom were so plentiful and so disturbing that we ended up doing most things the old way.

Meanwhile, papers started to appear in the literature describing the application of *in situ* hybridization to mouse embryo sections. These papers were typically rather tentative, confirming *in situ* data using Northern blots or other techniques, most likely to appease distrustful referees. A criticism of *in situ* hybridization was that it did not provide quantitative information, and many early papers went to great lengths to quantitate the signal by counting silver grains (Berger, 1986; Holland *et al.*, 1987). Further, black rectangles representing negative controls came to occupy lavish expanses of journal space.

Fortunately, those days of suspicion regarding *in situ* hybridization have now passed. *In situ* hybridization is now regarded as an accurate and trustworthy guide to the *in vivo* dynamics of gene expression. The passage of time, the availability of reagents and kits, and publication of several reliable guides to *in situ* hybridization (Wilkinson, 1998; Darby, 2000) have allowed workers to become more confident (and cut more corners) with this technique. New books have become available, and old books reprinted, that allow practitioners of the *in situ* art to make sense of the signals they observe (Rugh, 1968; Theiler, 1972; Kaufman, 1992; Kaufman and Bard, 1999). New labelling systems have become available, such as DIG, which circumvent the need for sitting in the darkroom for hours at a time (highly meditative, but the inability to read is a drawback to be sure), and waiting days or weeks to visualise the result.

So what are the essential ingredients of an *in situ* hybridization experiment? An answer to this question depends largely on the questions being asked and the characteristics of the gene being studied, but in practice it is most common to aim for an acceptable compromise of sensitivity, ease, speed, resolution, and specificity. The main options and considerations can be summarized as follows:

Probe type

RNA probes have remained the most common, since they are sensitive and specific. Curiously, single-stranded DNA probes made by asymmetric PCR have not gained popularity. In theory such probes are easier and cheaper to generate, circumvent the need for cloning into special transcription vectors, and offer flexibility in the positioning of primers. Oligonucleotide probes are used in some laboratories but are not considered sufficiently sensitive to detect less abundant transcripts.

Probe length

This is usually a compromise between strength of signal (long probes) and penetration into the tissue (short probes). Early protocols recommended generating a long primary labelled RNA transcript, and hydrolysing this probe to an average of 50-150nt in alkaline solution. However, alkaline hydrolysis is a random process that can generate a proportion of fragments too short to hybridize; it also results in significant loss of probe. We and many others have subsequently found hydrolysis to be unnecessary, and recommend using an intact, labelled RNA transcript of 600-1000nt. Depending on the gene and its level of expression, we have successfully used transcripts between 160 and 2000nt without hydrolysis.

Probe label

Many labs with a tradition of successful use of radioactive probes for section *in situ* hybridization continue to use this option. In this case, ^{35}S is by far the most common label, due to increased resolution and half-life over ^{32}P. However, DIG labeled probes have

become an extremely attractive option, particularly for workers with no previous experience with radiolabeled *in situ* probes, and are the only viable option for wholemount *in situ*s. Sensitivity is said to be less than ^{35}S-labelled probes, but the ease and speed of DIG visualization systems outweigh this disadvantage for most workers.

Tissue preparation

For sections, wax-embedded, 4% paraformaldehyde-fixed tissue gives good results for most investigators and is recommended. Cryostat sections also work well, but it is generally considered more difficult to find the desired region of the embryo or plane of sectioning using this method. Section thickness is often a compromise between signal and morphology, but 7-10μm is suitable in most cases. 4% paraformaldehyde is also recommended for wholemounts.

Slide coating

Of the many options used in early experiments, TESPA has emerged as the most popular slide coating to promote section adhesion. It is effective and easy to use, without generating high background signals.

Pre-hybridization treatments

Treatment of wholemount samples with alcohols serves the dual function of delipidization, which improves probe penetration, and bleaching, which improves the translucency and lightness of the sample for subsequent photography. Protease digestion is considered important for improving probe access to the mRNA in the sample, but needs to be carefully titrated to achieve a compromise between permeabilization and morphology. A prehybridization step that involves hybridization solution lacking probe is considered useful for reducing background.

Hybridization

It is possible to go to great lengths to calculate the T_m of the probe being used, in order to achieve the optimal hybridization temperature. However, since it is common to deal with a large number of probes, often in the same experiment, it is more practical to choose a temperature that suits "most" probes, such as 60°C (assuming 50% formamide), and adjust upwards or downwards (eg. to 55 or 65°C) in subsequent experiments if necessary, depending on levels of signal and background. Similarly, optimal probe concentration can be determined empirically. A concentration of 0·2-1μg/ml is often recommended, and we usually have best results at 1μg/ml or even higher. Dextran sulphate is no longer used, and detergents such as CHAPS and Triton-X100 have become popular for reducing background.

Post-hybridization treatments

Samples are usually washed extensively after hybridization to remove excess probe. Again, stringency of washing will affect signal and background, and we commonly wash to 0·2xSSC at a similar temperature to that used in the hybridization step.

Visualization of signal

Section *in situ*s hybridized with a radioactive probe need to be dipped in photographic emulsion (commonly Kodak NBT2 or Ilford K5) in a darkroom, dried, and exposed for several days (usually 5–10, but varies depending on gene expression levels), before developing in photographic solutions, counterstaining and mounting. The use of DIG-labelled probes simplifies the procedure, allowing a histochemical staining method that typically takes only a few hours. DIG is therefore highly recommended.

Undoubtedly the most significant advance in *in situ* technology has been the advent of wholemount *in situ* hybridization, which allows us to visualise the pattern of gene expression in a whole embryo in three dimensions. Wholemount *in situ* hybridization truly represents the Hubble telescope of developmental biology, providing spectacular insights into gene expression at a single view. For many workers, this technique is the first experiment performed when trying to characterize a novel gene. This approach can be followed by section *in situ* hybridization in cases where more detailed cellular localization of signal is required, or where the gene is expressed in a part of the embryo that may be inaccessible to the *in situ* reagents or difficult to visualize. In many situations, the required information can be obtained simply by cutting sections of a wholemount preparation after signal development and photography; overstaining of the sample is recommended in this case.

Where does one go for a good *in situ* protocol? A comprehensive set of protocols for DIG-labelled probes can be found in the chapter by Xu and Wilkinson in the latter's venerable *Practical Approach* volume (Wilkinson, 1998). An arguable drawback of this chapter is its comprehensiveness—too many options: the novice might prefer to use a protocol specifically tailored to the species of interest, and to the use of sections or wholemounts. The same volume contains and excellent chapter by Antonio Simeone dealing with the use of radioactively labelled probes on sections. Alternatively, the *Methods in Molecular Biology* volume edited by Ian Darby contains a number of chapters of interest to developmental biologists seeking protocols for *in situ* hybridization to mRNA (Darby, 2000).

In the early days of *in situ* hybridization, it was easy to get a gene expression paper published in *Cell*, *Nature* or *Science* (Ingham *et al.*, 1985; Jackson *et al.*, 1985; Wilkinson *et al.*, 1987; Wilkinson *et al.*, 1989). Sadly, those days have passed, and through the 1990s, descriptive *in situ* papers slid down the publication totem pole. It is fortunate that some journals, such as *Mechanisms of Development* and *Developmental Dynamics*, now have specials sections for gene expression patterns. This reflects a renewed interest in the type of information provided by *in situ* hybridization, perhaps fuelled by the various genome sequencing projects, and the need to attach functional (ie. expression) data to the vast numbers of novel gene sequences that are emerging.

What does the future hold? One of the current limitations of wholemount *in situ* hybridization is the size of the sample that can be analysed. It is likely that new techniques will be developed that will allow greater reagent penetration and the ability to visualise signal, either optically or digitally, to a greater depth within the sample. Advances in detergent technology are likely to lead to improved signal to noise ratios, which will further improve the sensitivity of the technique. Researchers continue to tinker with *in situ* hybridization protocols, to make them faster, more sensitive, and cleaner, and protocols are continuing to evolve. For example, Xu and Wilkinson list two different variations of the basic DIG *in situ* protocol that can be used in different situations and in different species (Wilkinson, 1998). Robotic technology will provide a quantum leap in the throughput of this labour-intensive technique. Automated *in situ* instruments are already available commercially, and may ultimately supersede DNA sequencers in prevalence and utility.

Several databases are being established to cope with the vast explosion in gene expression data. These include "GENEX", a joint initiative of the Medical Research Council (UK), the University of Edinburgh and the Jackson Laboratories. This multi-mode image bank is still under construction: http://genex.hgu.mrc.ac.uk/. Jackson Laboratories Informatics currently offers a mouse Gene Expression Database (GXD) at http://www.informatics.jax.org/menus/expression_menu.shtml, as part of its Mouse Genome Database (MGD); http://www.informatics.jax.org/. Other databases geared towards specific tissues are also available. These include databases for tooth (http://HONEYBEE.HELSINKI.FI/toothexp/), kidney (http://www.ana.ed.ac.uk/anatomy/database/kidbase/kidhome.html), and glandular organs (http://vonbaer.ana.ed.ac.uk/anatomy/database/orghome.html). A comprehensive list of expression databases in different species is provided in the chapter by Davidson *et al*. in Wilkinson's *Practical Approach* guidebook (Wilkinson, 1998). Information in these databases will be increasingly augmented by array-based expression profiling databases and EST databases.

This journal issue honours Anne McLaren, who has been an inspiration to countless developmental biologists worldwide. Among her many great qualities has been her foresight in embracing new technologies. Anne recognised at an early stage the potential of *in situ* hybridization, which now occupies an important place alongside PCR, microarrays, transgenesis and gene knockouts as essential tools for the developmental biologist. I owe her a personal debt of gratitude for coaxing me out of the library and into the darkroom (and teaching me that one does not have to answer the telephone). And we are all indebted to the pioneers of *in situ* hybridization for the eyes with which we are now able to view the molecular dynamics of embryonic development, surely the greatest show on earth.

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