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Developments in *in situ* hybridisation

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ABSTRACT

In situ hybridisation (ISH) is an established family of closely related methods for the detection and visualisation of specific nucleic acid sequences (DNA, RNA) in tissue sections, cytological preparations and whole organisms. The technique has a history of refinements and applications going back over several decades and is routinely employed in laboratories where visualisation of gene expression directly within the tissue of interest is necessary. This article will focus on ISH methods for the demonstration of messenger RNA (mRNA) and micro RNA (miRNA) in formalin-fixed paraffin-embedded (FFPE) tissues with emphasis on non-radioactive signal detection strategies currently available.

1. Introduction

ISH is an important tool for molecular and cellular pathology research and has been used in laboratories since the 1970s [1]. It can provide detailed spatial and contextual information about gene expression and chromosomes; for example identification of cell type, demonstration of chromosomal alterations (gains, losses or aberrations), presence of viral DNA/RNA genomes, and has been extended to the *in situ* detection of miRNA [2]. It is a fact that some characteristics of ISH methods include primarily qualitative results, multi-step protocols with varying levels of technical difficulty according to the particular method being used (e.g. ISH with fluorescent probes is much simpler to undertake than ISH using radioactive probes), and relatively low throughput. Thus alternative methods of analysing mRNA levels of large numbers of genes in parallel such as using microarrays, next generation sequencing (NGS) technologies or real time quantitative polymerase chain reaction (RT-qPCR) with laser micro-dissected material will give more quantitative data concerning mRNA expression levels in the tissue. However, obtaining expression data by these methods will necessarily involve destruction of the tissue specimen for mRNA extraction, and so information about cell type, for example, will be lost because the measured expression level is effectively an average derived from all the cells in the original sample e.g. consider the presence of stromal cells or infiltrating lymphocytes in a tumour biopsy. Improvements in reducing the input amount of mRNA needed to generate an expression profile using microarrays (e.g. <50 ng total RNA, of which 1–5% will be mRNA, for an Affymetrix GeneChip) means that profile data can be generated from very small amounts of tissue. Indeed a few or even single cells can be used to generate a profile (assuming 10–30 pg total per cell) if a PCR pre-amplification step is included but the resultant data is still not analysed in a histological context. Laser micro-dissection does allow for the identification and selection of cell populations *in situ* prior to capture and subsequent profiling but the process is relatively low throughput and is not readily amenable to automation. Note that when using ISH methods there are other considerations to take into account for example specificity and sensitivity of the probes used, and the signal detection system employed in the protocol (there has been a steady shift away from using radio labelled probes for ISH as alternative ways of detecting hybridised probe have become available, chiefly these use chromogenic or fluorescent endpoints). Hence there are advantages and disadvantages to whichever method is used for analysing gene expression. In a histopathology laboratory setting ISH comes into its own as a diagnostic tool when it is required to show the expression of a target gene or presence of a genetic marker over many samples. With the advent of tissue microarrays, hundreds of specimens can be analysed in parallel on a single microscope slide, and thus when combined with automated slide staining systems and improved image analysis software the ISH throughput can be increased significantly [3].

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2. Overview of selected ISH techniques

Before giving a description of the various ISH methods it is necessary to discuss some preliminary matters that are universally important.

2.1. Fixation

ISH techniques can, of course, be undertaken using unfixed material for example frozen tissue sections obtained using a cryostat but a fixation step will still be required before commencing the technique in order improve retention of nucleic acids. In these cases it should be recognised that when trying to detect mRNA, RNases are likely to be active within the tissue when it is brought up to room temperature and so steps should taken to inhibit RNase activity by prompt fixation and minimising the time until a mRNA duplex is formed with the hybridising probe (the double stranded duplex being much more resistant to RNase degradation). However this article is concerned with FFPE tissue sections (e.g. approximately 3–6 μm thickness on commercially available adhesive coated/electrostatically charged microscope slides) which brings its own set of challenges.

All nucleic acid probes, be they oligonucleotides, double stranded DNA (dsDNA) or single stranded anti-sense RNAs, will require access to the target sequence via diffusion within the cell. For FFPE tissues, formaldehyde solution (formalin) or a freshly prepared solution obtained from dissolving paraformaldehyde are the favoured fixatives, and typically 10% formaldehyde/PBS or 4% paraformaldehyde/PBS solutions are used. Fixation times vary according to tissue type, with only minutes being required for cell preparations compared to overnight for large tissue pieces (>1 cm^3). The fixing stabilises the tissue but means that the nucleic acid targets are shrouded by cross linked protein and are thus inaccessible to probe (note also that formaldehyde will cross link DNA/RNA to protein – a fact exploited by techniques such chromatin immunoprecipitation). Thus for most ISH protocols the tissue specimen is exposed to a solution of proteinase K (PK) to partially permeabilise the cross linked protein mesh e.g. incubation with a PK solution (5–15 μg/ml) for 5–30 min at 37 °C. This is sufficient to allow target access for hybridisation probes and conjugated linker antibodies but at the same time retain tissue morphology. It should be noted that over fixation will require extended proteinase K digestion before optimal hybridisation can be achieved and likewise under fixation will require less digestion. In order to obtain the best results proteinase K digestion times need to be determined empirically for each batch of enzyme on a given set of tissues. In addition, to help reduce variation in staining intensity, it is very helpful if the tissues are fixed in a consistent manner. Note also that large pieces of tissue will often demonstrate varying levels of fixation e.g. the interior of the specimen can be less fixed than regions near the outer surfaces. This may have consequences when performing ISH as less proteinase K digestion may be required to unmask target mRNA within the interior regions of a tissue section and over digestion can result in loss of tissue morphology.

2.2. Detection of hybridised probe and signal amplification

2.2.1. Radio labelled probes

The use of radiolabelled complementary RNA riboprobes is a sensitive ISH technique for detecting mRNA. Briefly, a riboprobe is constructed by cloning the target gene into a plasmid adjacent to a phage RNA polymerase promoter sequence (SPS, T3 or T7) [4]. By restriction digesting the recombinant plasmid at an appropriate place, purifying the linearised template DNA and then performing *in vitro* transcription (IVT) reaction in the presence of ^{35}S-UTP the anti-sense strand RNA (complementary to target mRNA sequence *in situ*) or sense strand RNA (not complementary to the target mRNA sequence, and useful as a control probe) can be synthesised. The probes are hybridised to the tissue section overnight, non-hybridised probe washed away in the presence of RNase A (which will not digest an mRNA/riboprobe duplex) and the slides coated in a photographic emulsion which forms visible particles of metallic silver where radioactive decay has occurred in the vicinity of riboprobe hybridised to its target (signal is best observed using dark field microscopy). Note that other isotopes can be used to radiolabel the nucleotide such as phosphorus (^{32}P) and tritium (^{3}H). The former emits highly energetic beta particles which increase sensitivity, reduce exposure times (days) but hinder precise signal localisation while tritium emits weakly energetic beta particles which increase resolution of signal as the particles do not travel so far but at the expense of greatly increased exposure times (weeks). Hence the use of ^{35}S as a radiolabel offers a good compromise between sensitivity, resolution and acceptable exposure time (exposure for 1–2 weeks is usually sufficient). The technique is very sensitive but suffers from a number of disadvantages (the most important of which is the difficulty in obtaining precise localisation of the signal) in addition to the requisite safe handling of radioisotopes. For example significant infrastructure is required for the use and disposal of radioactive materials, exposure times are generally long (days to weeks), and emulsion coating of the slides must be undertaken under safelight conditions necessitating the use of a dark room.

Consequently, because of the drawbacks with using radiolabelled probes, attention has focussed on non-radioactive methods that are able to offer similar levels of sensitivity as regards low abundance targets. Practically speaking this breaks down to two choices: chromogenic or fluorescent detection of bound probe. Chromogenic signal detection is suited to bright field microscopy and a light haematoxylin and eosin counterstain (H & E) will aid cell identification relative to location of signal. Fluorescent signals are observed using a suitably set up fluorescence microscope and an appropriate filter set, with DAPI used as a very effective counterstain in showing up the cell nuclei under UV illumination. Commercial preparations of mounting media are conveniently available with DAPI included.

2.2.2. Single molecule FISH

Detection of low abundance targets down to the single transcript level has successfully been achieved using mixtures of fluorescently labelled oligonucleotides whereby the signal is an aggregate measure detectable by fluorescence microscopy [5–7]. For this fluorescent ISH technique the oligonucleotide probe can be labelled with several dye molecules or with a single dye molecule so that there is a tradeoff between economy and efficiency of synthesis, with single labelled oligonucleotides being easier to produce on a larger scale [5,6]. However oligonucleotide probes labelled with a single fluorophore will generate less fluorescence than probes labelled with multiple fluorophores so by way of compensation more of them are required per target mRNA [7]. Mixtures of up to 48 individual oligonucleotide probes have been found to give good results but there are no constraints apart from cost and availability of suitable hybridisation sites within the target mRNA [7]. Since the oligonucleotides are generally <50 bp length care must be taken when designing the probes so that homology with other targets is avoided and, as far as is possible, to keep the G + C content and melting temperatures similar for all the probes in the mixture (note also that there are general formulae available that give good estimates of the optimal hybridisation temperature based on the G + C content and length of the probe). Self complementary regions should also be avoided within