Type of article : Review Article

In Situ Hybridisation-A Review

E.Rajesh[1], R.Hariharan[2], N.AravindhaBabu[3], L.Malathi[4],

[1]Reader, [2]Post graduate student, [3]Professor, [4]Reader, Department of Oral pathology and Microbiology, Sree Balaji Dental College and Hospital, Bharath Institute of Higher Education and Research

Abstract

In situ hybridisation (ISH) depends on the integral blending of labelled DNA or RNA probes with typical or irregular nucleic acid arrangements in intact chromosomes, cells or tissue segments. Compared with other molecular techniques appropriate to anatomical pathology, ISH appreciates better compatibility with histopathologists in view of its closeness to immunohistochemistry. It has the exceptional preferred position over other techniques to a great extent dependent on probe hybridization with nucleic acid removed from homogenized tissue probes - of permitting localization and representation of target nucleic acid sequences inside morphologically recognizable cells or cell structures. In spite of the fact that ISH is to a great extent an examination apparatus, it is as of now making solid advances into demonstrative histopathology. Other than recognition of infective specialists, it likewise permits localization of destinations of contamination, explanation of systems of infection transmission and spread also, examination of the connection between viral specialists and malignant growth. Sex composing, localization of qualities on chromosomes and recognition of basic and mathematical chromosomal changes in tumors are among different utilizations of ISH.

Key Words: *Probe, labelling, hybridization, nucleic acid, detection, denaturation.*

Introduction:

The utilization of molecular probes in anatomical pathology (including histopathology, cytopathology and electron microscopy) is based on the hybridization of named DNA and RNA tests with nucleic acid arrangements in tests of sick tissues. The primary methods are (1) insitu hybridization (ISH), where labelled probes are utilized to recognize integral DNA or RNA in tissue areas or smears, and (2) assessment by Southern or other blotting procedures of nucleic acids separated from homogenized biopsy material. Of these ISH appreciates better affinity with histopathologists in view of its similitude to immunohistochemistry. First portrayed in 1969 [1,2,3], its formative stage was to a great extent in the 1980s when recombinant DNA technology was applied to conquer the early issues of probe preparation.

From the useful view point, the most significant preferences of ISH to the histopathologist running a laboratory are

(1) simplicity

(2) specificity

(3) ease interpretation and

(4) its pertinence on tissue areas (frozen or formalin-fixed, paraffin-embedded) and spreads without the need for specimen collection or handling. ISH has the promising capability of turning out to be as "routine" as an immunohistochemical stain, particularly when nucleic acidprobes become financially accessible and methods for identification in formalin-fixed and paraffin-embedded tissue are consummated.

PRINCIPLE OF ISH:

ISH is the specific annealing of a labelled nucleic acid probe to complementary sequences in fixed tissues followed by visualization of the location of the probe. ISH – shows explicit nucleic acid arrangement in their cell environment[4].

STEPS IN ISH:

1. Probe preparation

2. Pre-treatment of specimens

3. Hybridization

4. Identification method[3].

Probe Preparation:

Probes:

A probe (a named complementary single strand) is joined with the DNA/RNA strands of intrigue. Strands will strengthen with integral nucleotides holding back along with their homologous components when cooled. Odds of a probe finding a homologous sequence other than the target sequence decline as the number of nucleotides in the test expands[5].

Double stranded DNA Probe:

Arranged by nicktranslation, arbitrary primer, PCR within the sight of a named nucleotide. Nick translation is a strategy for joining named nucleotides into DNA, for example, separated fragment. The strategy utilizes a blend of two enzymes, Deoxyribonuclease I which scratches the DNA making free 3 hydroxyls. DNA polymerase I, which processively includes nucleotides to the 3′ terminal hydroxyl[6]. Random preparing is a methods for marking DNA sections whereby, a blend of all potential mixes of hexamers, octamers, or nanomers are strengthened to denatured DNA. These little oligonucleotides then go about as primer that take into consideration the amalgamation of the reciprocal DNA strand by the Klenow protein and consolidation of both named and unlabeled nucleotides. More successful when the target is plentiful. Less touchy than single strand probe. Since two strands tend to hybridize to one another, accordingly diminishing the grouping of probe accessible for hybridization to the target[7].

Single stranded DNA Probe:

Single stranded DNA Probe spread an a lot bigger size range (200-500 bp) than oligonucleotide probes. They can be set up by primer expansion on singlestranded layouts by reverse transcription polymerase chain reaction (PCR) of RNA, or an enhanced primer expansion of a PCR-created fragment in the presence of a solitary antisense primer, or substance formation of oligonucleotides. PCR-based strategies are a lot simpler,probes can be combined from modest quantities of beginning material. In addition, PCR permits incredible adaptability in the decision of probe sequences by the utilization of proper primers[5].

RNA Probes:

RNA probes (cRNAprobes or riboprobes) are thermostable and are impervious to absorption by RNases. These probes are single-stranded and are the most broadly utilized in ISH. RNA tests are created by in vitro record from a linearized layout utilizing an advertiser for RNA polymerase. RNA polymerase is utilized to orchestrate RNA complementary to the DNA substrate. Single-stranded probes give favorable circumstances over doublestranded probes, the probesdoesn't self-toughen in solution, so the probe isn't depleted. Huge probe chains are not framed in solution; in this way, probe entrance isn't influenced. In the event that high sensitivity is required, singlestranded probes ought to be used[5].

Oligoprobes:

Normally, shorter 20-40 base pair length. They are created artificially by a mechanized substance combination. These probes are impervious to RNases and are little, along these lines permitting simple infiltration into the cells or tissue of interest. Little size has a burden in that it covers less targets. Label ought to be situated at the 3′ or the 5′ end. To increment sensitivity one can utilize a blend of oligonucleotides that are correlative to various districts of the target molecule. Another favorable position of oligonucleotide probes is that they are single stranded, accordingly barring the possibility of renaturation[8].

Properties of Probes:

• Probe construct: Oligonucleotide probes are better than conventional probes as a result of high specificity, single-stranded, and short probe length (10-50 nucleotides).

• The efficiency of labelling: Labeling by irregular preparing has been accounted for to be more proficient than nick translation.

• Percentage of G-C base pairs: Higher the substance of G-C matches, the higher the Tm (melting temperature).

• RNA versus DNA probes: Strength of the probetarget bond diminishes in the order of RNA-RNA, DNA-RNA, DNA-DNA.

• Probe length: Shorter the probe, the better its entrance into cells[3].

Purification of labelled probes:

There are a few techniques that can be utilized to test the purification. Here, a rundown of techniques that can be utilized yet it is fitting to follow the makers' suggestion on their utilization: Sephadex G-50 column, Sephadex G-50 chromatography, or Selective precipitation[5].

Probe concentration:

For DNA probes, the convergence of the probe will be 0.5-2 µg/ml. Oligonucleotide probes can be utilized with, or on the other hand without, acetylation. Probes without acetylation pretreatment of the sample will have a concentration of ~50-200 ng/ml and may give more extraordinary outcomes with an insignificant foundation. For probes with acetylation pre-treatment, a higher concentration of oligonucleotide probe might be utilized without bringing about vague background staining[5].

Length of Probe:

Ideal probe size for ISH is little pieces of about 200-300 nucleotides. Be that as it may, probes might be as little as 20-40 bp or as extensive as 1000 bp. As probes increment in length, they become more explicit. Longer probes may lead to more fragile sign. They infiltrates less productively the cross-linked tissues. The degree of more fragile signs and infiltration relies additionally upon the nature of the tissue, decision of fixative and whether a pre-treatment has been conveyed out[7].

Pretreatment of Specimens:

Tissue sections must hold fast well to extraordinarily treated glass slides to dodge loss of tissue during the hybridization measure. Different "adhesives" are accessible counting poly-l-lysine, gelatin chrome alum, and aminopropyltriethoxysilane[3].

Fixation:

Methanol/acetic acidfixation is suggested for metaphase chromosome spreads. Cryostat sections may be fixed with 4% formaldehyde (~30 min), Bouin's fixative, or paraformaldehyde vapor fixation. This fixation likewise assists with tying down the tissue to the slide. Most generally, tissue specimens are regularly fixed in 10% buffered formalin, processed overnight in a programmed tissue processor, and embedded in paraffin wax. Fixation season of 8-12 h is optimal[5].

Slide/sectionpreparation:

Sections are cut at 4-6 µm on aacohol cleaned microtome utilizing positively charged or hand-coated slides. Sections are depleted well and afterward air-dried at room temperature. After deparaffinization, slides are put in aalcohol cleaned staining compartment of diethyl pyrocarbonate water. The staining compartment is then positioned in the warmed water bath 23-37°C and held until the beginning of ISH. Gloves must be worn to prevent contamination, and all utensils, for example, brushes and forceps, ought to be cleaned with alcohol and kept inside the cleaned zone assigned for ISH[5].

Proteolytic digestion:

The utilization of formaldehyde-based fixatives before paraffin embedding of specimens will cover nucleic acid arrangements. Assimilation is a significant advance when performing ISH. Processing improves probe penetration by expanding cell penetrability with negligible tissue degradation[5].

Hybridization:

Molecular hybridization is the cycle whereby a singlestranded target sequence is tempered to a reciprocalsingle-stranded probe to frame a double stranded hybrid. Prior to hybridization, both the target and the probe, in the event that double stranded, must be denatured to deliver them single-stranded and this can be accomplished by warmth or alkali treatment. The accompanying denaturation, single-stranded target, and probe sequences are incubated in a hybridization blend, which gives an ideal situation to re-toughening of single-stranded sequences. Hybridization happens after denaturation, during cooling, in the presence of an integral probe, and allows hydrogen holding of the two

strands of nucleic acids[9].

Probe must shape stable hydrogen bonds with the target.All the while warming the probe and focus to high temperatures may build the consistency and sensitivity of discovery. This must be met if care is taken to accurately control this progression of the ISH procedure[9].

Post-hybridization washes:

Stringency washes after hybridization targets diminishing non specific binding. Notwithstanding, it is desirable over hybridize rigidly as opposed to wash stringently[10].

Detection Methods:

Different techniques are accessible for perception of the hybridization. Decision of detection framework will be basically controlled by the probe label utilized and second by the ISH methodology type. There are two techniques for probe labeling. Detectionmethods is of two types, direct and indirect method [11].

Oligonucleotide probe labeling:

• 5′-end labeling: The 5′ end of DNA or RNA goes through direct phosphorylation of the free 5′-terminal OH groups. The free 5′-OH substrates can be named utilizing T4 polynucleotide kinase. This strategy is normally utilized for radiolabeling. Non-radiolabels utilize a covalent linker [12].

• 3′-end labeling: Terminal dexoxynucleotidyl transferase (TdT) is utilized to include a labeled buildup to the 3′ end of an formed oligonucleotide that is around 14-100 nucleotides long. These tests give phenomenal explicitness yet just moderate sensitivity [5].

• 3′ tailing: A tail containing labeled nucleotides is added to the free 3′ end of double or single-stranded DNA utilizing TdT. These probes are more touchy than the 3′-end labeled adaptations, however can deliver more vague background [13].

Enzymatic detection:

Hybridized probes can be distinguished by enzymatic responses that produce a colored substrate at the site of hybridization. The most ordinarily utilized enzymes for this application are alkaline phosphatase (AP) Or horseradish peroxidase (HRP) Although these enzymes can be formed legitimately to nucleic acid probes, such enzyme coupled probes are regularly improper for ISH to tissue arrangements since probe infiltration is hampered by the presence of the conjugated enzyme. In this manner, indirect methods are preDNAprobe and a target grouping preferred[14].

Autoradiography detection:

Responses utilizing radioactive named probes are identified by autoradiography. This depends on the discharge of fastelectrons or beta-particles from the probe. Beta particles discharge a lot of energy when they hitwith atoms of an emulsion included to the section on the slide. The over energy delivered lessens ionic silver present in the emulsion to metallic silver. At the point when this occurs, a dedicated record of the area of the impact between an electron and the silver particles in the emulsion is created in the type of a latent image. This picture, when envisioned is the marker of the probe location in the tissue or cell. Autoradiography for radioactive labels is presumed to be more touchy than the immunoenzyme systems[3].

Fluorophores:

Fluorophores can be related with nucleic acid probes by compound formation to the nucleic acidor compound formation of the nucleic acid with a non-fluorescent particle that can bond fluorescent material after hybridization. The previous technique is called “direct labeling” and the last strategy is called “indirect labeling.” Chemical structures of four normal fluorophore classes (A-D) fluoresceins, rhodamines, cyanines, or coumarins[7].

Indirect method:

By implication through fuse of a nucleotide analog conveying a reactive group and ensuing biotinylation/ digoxigenylation. Coming about biotin-labeled probes are at that point distinguished utilizing streptavidin (KD = 10-15 M) formed with HRP or AP. Digoxigenylation is commonly pictured by HRP-or AP changed antibodies[15].

Multiple ISH:

More than one probe can be applied to a similar

tissue area to distinguish distinctive nucleic acid targets. By utilizing diverse identification systems with each probe, coming about in various colored end products, and perception of the diverse nucleic acid targets can be achieve[16]. The common advantages of ISH are,

1. Effortlessness of its procedure

2. Particularity of results got

3. Simplicity in understanding of discoveries

Its relevance on tissue sections (frozen or formalinfixed, paraffin-embedded) and spreads without the need for special specimen collection or processing[3].

Applications OF ISH:

1.Determination of Infective Agent:This depends on the discovery of the infective agents genome in the tissues or cells considered. Explicit composing of infective specialists likewise has significant ramifications for epidemiological reviews and episode investigations[17].

2 Location of Active Infection:The real cell or cell structures holding the infective genome can be explained by ISH, for example hepatitis B infection (HBV) in hepatocytes, parvovirus in cells of the lung[17].

3.Explanation of mechanism of Virus Dissemination and Transmission: common horizontal and vertical transmission courses of infections can be considered. For instance, the presence of Epstein-Barr infection (EBV) in epithelial cells of the oropharynx gives a way to transmission of the virus through saliva[18].

4. Localization of Persistent Virus Infection:Models are the constancy of JC virus in oligodendrocytes in progressive multifocal leukoencephalopathy and measles infection in neurons and glia cells in SSPE[19,20].

5.Link between Virus Agents and Carcinogenesis: Etiological function of different viruses in cancers and mechanism of malignant transformation of cells. The better realized affiliations are EBV and nasopharyngeal carcinoma and B-cell lymphomas[21], HBV and hepatocellular carcinomas[22],and human papillomavirus furthermore, cervical carcinoma[23].

6.Investigation of cell Development:ISH detection of cell-type explicit RNA in cells which do not display morphological separation can be applied to recognize the cell type[24].

7.Sex Determination:The Y chromosome can be distinguished through hybridization [25].

8.Human Gene Mapping:In situ Hybridisation (ISH) utilized in human quality planning[26].

9.Interphase Cytogenetics:ISH can be utilized to recognize numerical chromosomal variations in interphase cores. Probes perceiving profoundly monotonous sequencesin chromosomes 1, 7, 8, 9, 10, 15, 16, 17, 18, X and Y are currently available[3].

Conclusion

ISH has the extraordinary favorable position over other molecular biology procedures of permitting localization and representation of target nucleic acid sequences inside morphologically recognizable cells or cell structures in a heterogenous cell population. Such explicit localization has a stamped advantage over more seasoned molecular techniques where discovery of the nucleic acid objective from a tissue homogenate precludes identification of the real influenced cell, energy about cell detail and relations and subsequent reconstructive interpretation of findings.

Ethical Clearance – Not required since it is a review article

Source of Funding – Nil

Conflict of Interest – Nil

References

1. Buongiorno-Nardelli M, Amaldi F. Autoradiographic detection of molecular hybrids between rRNA and DNA in tissue sections. Nature 1969; 225: 946-7.
2. Gall G, Pardue ML. Formation and detection of RNA-DNA hybrid molecules in cytological preparations. Proc Natl Acad Sci 1969; 63: 378-81.
3. Looi LM, Cheah PL. In situ hybridisation: Principles and applications. Malays J Pathol1992;14:69-76.
4. Bishop R. Applications of fluorescence in situ hybridization (FISH) in detecting genetic aberrations of medical significance. Biosci Horizons 2010;3:85-95.

5. Bancroft JD, Gamble M. Theory and Practice of Histological Techniques. Amsterdam: Elsevier Health Sciences; 2008.

6. Rittié L, Perbal B. Enzymes used in molecular biology: A useful guide. J Cell Commun Signal 2008;2:25-45.

7. Morrison LE, Ramakrishnan R, Ruffalo TM, Wilber KA. Labeling fluorescence in situ hybridization probes for genomic targets. Methods Mol Biol2002;204:21-40.

8. Darby IA. In Situ Hybridization Protocols. New York: Springer Science & Business Media; 2004.

9. Tenover FC. Diagnostic deoxyribonucleic acid probes for infectious diseases. Clin Microbiol Rev 1988;1:82-101.

10. Poulsen L, Søe MJ, Snakenborg D, Møller LB, Dufva M. Multistringency wash of partially hybridized 60-mer probes reveals that the stringency along the probe decreases with distance from the microarray surface. Nucleic Acids Res 2008;36:e132.

11. de Muro MA. Probe design, production, and applications. Molecular Biomethods Handbook. Totowa, NJ: Humana Press; 2008. p. 41-53.

12. Alberts B, Johnson A, Lewis J, et al., Molecular Biology of the Cell. 4th edition. New York: Garland Science; 2002. Available from: http://www.ncbi.nlm.nih.gov/books/NBK21054/.

13. Hilario E. End labeling procedures: An overview. Mol Biotechnol2004;28:77-80.

14. Diamandis EP, Christopoulos TK. The biotin-(strept)avidin system: Principles and applications in biotechnology. Clin Chem 1991;37:625-36.

15. Nederlof PM, van der Flier S, Wiegant J, Raap AK, Tanke HJ, Ploem JS, et al. Multiple fluorescence in situ hybridization. Cytometry 1990;11:126-31.

16. Alonso MC, Cano I, Castro D, Perez-Prieto SI, Borrego JJ. Development of an in situ hybridisation procedure for the detection of sole aquabirnavirus in infected fish cell cultures. J Virol Methods 2004;116:133-8.

17. Morey AL, Porter HJ, Keeling JW, Fleming KA. Non-isotopic in situ hybridisation and immunophenotyping of infected cells in the investigation of human fetal parvovirus infection. J Clin Pathol1992;45:673-8.

18. Rostad SW, Olson K, McDougall J, Shaw CM, Alvord EC Jr. Transsynaptic spread of varicella zoster virus through the visual system: A mechanism of viral dissemination in the central nervous system. Hum Pathol1989;20:174-9.

19. Ironside JW, Lewis FA, Blythe D, Wakefield EA. The identification of cells containing JC papovavirus DNA in progressive multifocal leukoencephalopathy by combined in situ hybridization and immunocytochemistry. J Pathol1989;157:291-7.

20. Shapsak P, Tourtellotte WW, Wolman M, Verity N, Verity MA, Schmid P, et al. Search for virus nucleic acid sequences in postmortem human brain tissue using in situ hybridisation technology with cloned probes: Some solutions and results on progressive multifocal leukoencephalopathy and subacute sclerosing panencephalitis tissue. J Neurosci Res 1986;16:281-301.

21. Weiss LM, Movahed LA. In situ demonstration of Epstein-Barr viral genomes in viral-associated B cell lymphoproliferations. Am J Pathol1989;134:651-9.

22. Simon D, Searls DB, Cao Y, Sun K, Knowles BB. Chromosomal site of hepatitis B virus (HBV) integration in a human hepatocellular carcinoma-derived cell line. Cytogenet Cell Genet 1985;39:116-20.

23. Stoler MH, Rhodes CR, Whitbeck A, Wolinsky SM, Chow LT, Broker TR. Human papillomavirus type 16 and 18 gene expression in cervical neoplasias. Hum Pathol1992;23:117-28.

24. Martizez Arias A, Baker NE, Ingham PW. Role of segment polarity genes in the definition and maintenance of cell states in the Drosophila embryo. Development 1988;103:157-70.

25. Burns J, Chan VT, Jonasson JA, Fleming KA, Taylor S, McGee JO. Sensitive system for visualising biotinylated DNA probes hybridised in situ: Rapid sex determination of intact cells. J Clin Pathol1985;38:1085-92.

26. Rowley JD, Diaz MO, Espinosa R 3rd, Patel YD, van Melle E, Ziemin S, et al. Mapping chromosome band 11q23 in human acute leukemia with biotinylated probes: Identification of 11q23 translocation breakpoints with a yeast artificial chromosome. Proc Natl Acad Sci U S A 1990;87:9358-62.