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Fluorescence In Situ Hybridization with Concomitant Immunofluorescence in Human Pancreas

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Abstract

The ability to identify the presence of non-host cells in human pancreas with concomitant characterization of cell phenotype is particularly important to facilitate studies of transplantation and microchimerism resulted from pregnancy. The steps involved in processing tissue for fluorescence in situ hybridization (FISH) can however remove epitopes that are crucial for immunofluorescence and antigen retrieval strategies for immunofluorescence can negatively influence FISH. We describe a robust method to analyze X/Y chromosome constitution and cell phenotype simultaneously on the same pancreatic tissue section.

Keywords: Fluorescence in situ hybridization, Transplantation, Microchimerism

1 Introduction

In this chapter, the technique used to detect the X and Y chromosomes in sex-mismatched cells in pancreas (perhaps as a result of a pancreas or bone marrow transplant or maternal fetal cell transfer) and simultaneously determine the cell phenotype on the same cell is described. FISH employs fluorescently conjugated DNA probe to hybridize the centromeric regions of the X/Y chromosomes. Commercially available kits such as Vysis CEP X/Y Direct labeled fluorescent DNA probe kit are often used. Briefly, the alpha satellite sequence of the X chromosome and satellite III at the Yq12 of the Y chromosome contain highly repetitive tandem repeats. Binding to these repeats amplifies the FISH signal. In order to label immunomarkers concomitantly with FISH, DNA targets and protein epitopes must be retrieved under the same condition, for example the same heat-induced antigen retrieval (HIAR) buffer and condition, as illustrated in Fig. 1. This article describes a simple way of combining FISH and immunofluorescence on paraffin-embedded tissues.

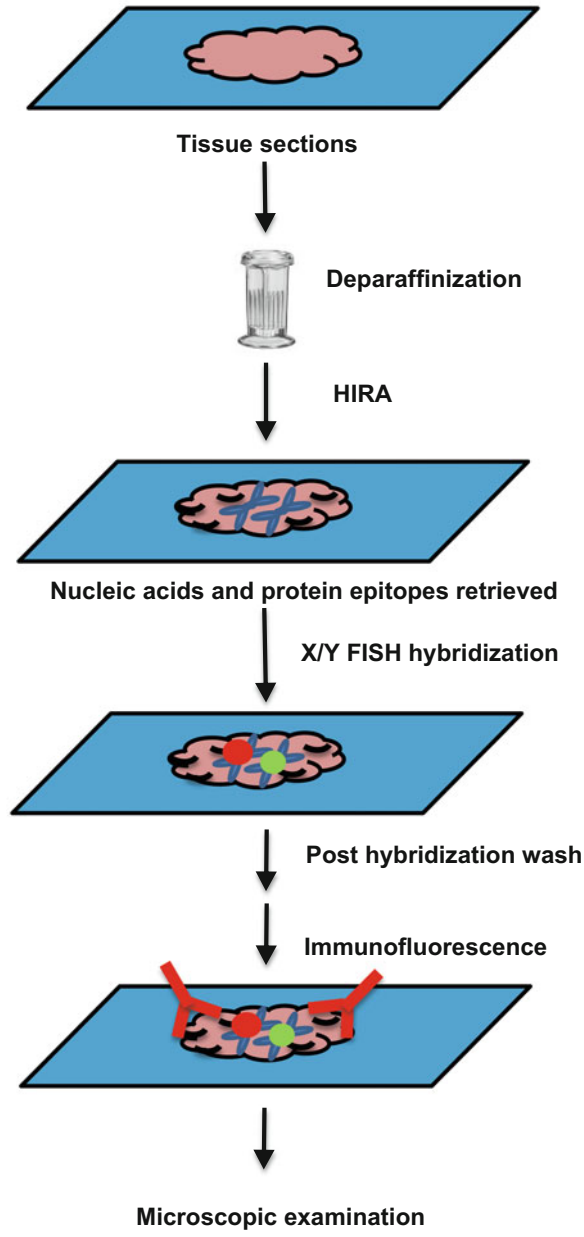

Fig. 1 Schematic illustration of concomitant X/Y chromosome fluorescence in situ hybridization and immunofluorescence on paraffin-embedded tissue sections. *HIAR* heat induced antigen retrieval; *black lines* indicate protein epitopes; *red dot* represents centromere of X chromosome and *green dot* represents centromere of Y chromosome; *dark blue* highlights chromosomes; *light blue* represent antibodies

2 Materials

2.1 Tissue Preparations

Cut formalin fixed paraffin-embedded human pancreas blocks at 4 μM per section and mount on positively charged slides.

2.2 Reagent and Materials for Fluorescence In Situ Hybridization

1. Xylene.
2. Vysis CEP X SpectrumOrange/Y SpectrumGreen Direct labeled fluorescent DNA probe kit (30-161050 and 32-161050, Abbott Molecular).
3. 1× sodium citrate buffer (10 mM, pH 6.3): dissolved 0.294 g tri-sodium citrate in 900 ml water, adjust pH to 6.0, bring total volume to 1 L and then add 0.5 ml Tween 20.
4. 20× saline sodium citrate (SSC) buffer (pH 5.3): dissolve 175.3 g sodium chloride and 88.2 g tri-sodium citrate in 800 ml deionized water (dH_2O), adjust pH to 5.3, and bring up the volume to 1 L. Solution can be filtered through a 0.45 μM filtration unit or autoclaved before storing at room temperature for up to 6 months.
5. Ethanol wash solutions: prepare v/v dilutions of 70 %, 85 %, and 100 % using 100 % ethanol in dH_2O.
6. 0.1 % Tween 20, 2× SSC wash buffer: dilute 20× SSC buffer (pH 5.3) in dH_2O at 1:10, adjust pH to 7.0–7.5, and add 0.1 % v/v pure Tween 20 solution to the buffer.
7. 0.4× SSC wash buffer: dilute 20× SSC buffer (pH 5.3) in dH_2O at 1:50 and adjust pH to 7.0–7.5.
8. Denaturing solution: 70 % formamide in 2× SSC solution (without Tween 20) (for separate denaturation and hybridization).
9. Slide warmer (for separate denaturation and hybridization).
10. Coverslips.
11. Rubber cement.
12. Digital slide hybridizer (for co-hybridization only).
13. Water bath (2 if performing separate denaturation and hybridization).
14. Microwave.
15. Coplin jars.
16. Digital thermometer.
17. Slide chamber.
18. Heat stable container for antigen retrieval.

2.3 Reagent for Immunofluorescence

1. Normal blocking serum.
2. 0.1 % Triton-X PBS antibody dilution reagent.
3. 1× PBS wash buffer.

4. Primary and fluorescent secondary antibodies.
5. VECTASHIELD DAPI mounting media.

3 Methods

The goal is to achieve optimum DNA target and protein epitope exposure under the same pretreatment condition.

3.1 Deparaffinization and Heat-Induced Antigen Retrieval

This procedure allows a maximum of five slides to be processed simultaneously (**Note 1**).

1. Immerse slides in xylene for 5 min.
2. Repeat in a second xylene wash.
3. Transfer slides to a 100 %, 85 %, and 70 % ethanol series; incubate for 1 min each.
4. Wash in dH_2O water.
5. Carefully preheat 10 mM sodium citrate buffer (pH 6.0) in microwave, bringing to the boil; quickly immerse slides in sodium citrate buffer and continue to heat up at full power ~850 W for at least 10 min (**Note 4**).
6. After antigen retrieval, slides are cooled down slides to room temperature in sodium citrate buffer.
7. Subsequently, slides are dehydrated in 70 %, 85 %, and 100 % ethanol series for 1 min each and air-dried on bench.
8. Slides can be stored overnight at room temperature but best to proceed with in situ hybridization straight after.

3.2 Fluorescence In Situ Hybridization (Co-hybridization)

1. Preheat digital slide hybridizer to 73 °C and water bath to 42 °C, this step requires accurate temperature settings and temperature cannot fluctuate more than 1 °C.
2. Depending on tissue size, pipette appropriate amount of FISH ready-to-use probe mixture onto a coverslip. As a general guidance, 10 μl FISH probe is used on a 2 cm^2 tissue section.
3. Reverse coverslip and place it on top of the tissue, make sure that the tissue is totally immersed in FISH probe, squeeze out any air bubbles.
4. Seal coverslip with rubber cement. From now on slides need to be protected from light, and incubated in slide hybridizer at exactly 73 °C for 10 min. This step allows double stranded DNA duplex to denature in the presence of single stranded FISH probe.
5. Immediately slides are transferred to a humidified slide chamber, allowing probes to re-hybridize with DNA in water bath at 42 °C overnight.

3.3 Fluorescence In Situ Hybridization (Separate Denaturation and Hybridization)

1. Pre-warm the water bath 1 to 73 °C, water bath 2 to 42 °C, and pre-warm the slide warmer to 45–50 °C.
2. Fill denaturation solution (70 % formamide in 2× SSC pH 7.0–8.0) into a Coplin jar and place it in the 73 °C water bath to warm up.
3. Denature specimen by immersing the prepared slides in the denaturation solution at 73 ± 1 °C for 5 min. Do not denature more than five slides.
4. Using forceps, remove slides from the denaturation solution and immediately place into 70 % ethanol, agitate gently, and allow slides to stand for 1 min.
5. Transfer slides to 85 % ethanol for 1 min and 100 % ethanol prior to FISH probe hybridization.
6. Place slides on a 45–50 °C slide warmer no more than 2 min.
7. Apply FISH probe solution on tissue, then place coverslip on top, seal with rubber cement.
8. Transfer slides to slide chamber, cover from light, and incubate in water bath 2 at 42 °C overnight.

3.4 Post Hybridization Wash

1. Preheat water bath to 73 ± 0.5 °C.
2. Preheat 0.4× SSC solution to exactly 73 ± 0.5 °C in a Coplin jar in water bath.
3. Prepare 2× SSC, 0.1 % Tween 20 solution in a Coplin jar.
4. Gently remove coverslip from tissue and immerse slides in 0.4× SSC solution for exactly 2 min. Gently agitate Coplin jar between each minute.
5. Transfer slides to 2× SSC, 0.1 % Tween 20 solution and incubate for 1 min, gently agitate for 30 s.

3.5 Immunofluorescence

1. At this point, immunofluorescence can be performed.
2. Block nonspecific binding with 3 % serum from appropriate species (the same species as secondary antibody was raised in) diluted in PBS buffer for 1 h at room temperature.
3. Incubate slides with desired primary antibodies at room temperature for 2 h or overnight at 4 °C.
4. Wash slides in PBS wash buffer twice for 3 min each with gentle agitation.
5. Incubate slides with desired fluorescent secondary antibodies at room temperature for 1 h.
6. PBS wash as described in step 4.
7. Counterstain and mount tissues in VECTASHIELD DAPI mounting media.
8. Seal coverslip with nail varnish.

3.6 Microscopic Examination

1. FISH and immunofluorescence signals can be visualized under regular and confocal microscope preferentially at 60× magnification lens or above.
2. Slides can be stored at −20 °C up to a year.

4 Notes

1. FISH can be performed either as co-hybridization or as separate denaturation and hybridization.
2. Deparaffinizing too many slides simultaneously can reduce the incubation temperature and inferior results.
3. At this stage slides can be stored in deionized water for less than 1 h.
4. Ten minutes are required to break up formalin-induced cross-link between peptides. Depending on target epitopes, the length of HIAR can vary between 10 and 20 min. For abundant antigens in islets such as insulin and glucagon, a 10-min antigen retrieval is sufficient to obtain strong signal. For proliferative markers such as Ki67, a 20-min antigen retrieval is recommended to achieve optimal staining.