Fluorescence in situ hybridization (FISH) for DNA replication origins

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Method Article

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Abstract

Introduction

Fluorescent in situ hybridization \(FISH) is a molecular cytogenetic technique used for the detection of specific chromosomal rearrangements and applicable to many different specimen types. FISH is widely used for several diagnostic applications: identification of numerical and structural abnormalities characterization of marker chromosomes, monitoring of effects of therapy, detection of minimal residual disease, origin of cells after bone marrow transplantation, identification of regions of deletion or amplification, chromosomes abnormalities in non-dividing or terminally differentiated cells, determination of lineage involvement of clonal cells. Moreover it has many applications in research: identification of non-random chromosome rearrangements, identification of translocation molecular breakpoint, identification of commonly deleted regions, gene mapping, characterization of somatic cells hybrids, identification of amplified genes, study of mechanism of rearrangements, RNA-FISH to study gene expression of 3D chromosome organization in interphase. DNA is a double stranded molecule, and when it is chemically denatured and separated into two strands, it quickly reanneals into a double stranded conformation. Thus, when a single stranded probe is incubated with a single-stranded \(denatured) metaphase chromosome \(or interphase), the probe will bind to complementary DNA sequences to reform the double stranded molecule. Overall, the most critical step when using FISH is the choice of adequate probes. A DNA probe is defined according to its target or complementary DNA in metaphase and interphase cells: \(1) repetitive sequence probe, \(2) whole chromosome \(painting), \(3) locus-specific probes. Technically the ideal probes especially for interphase FISH should give strong, specific signals with no backgrounds and should have a high hybridization efficiency \(> 90%).

Reagents

For slides: 0.005%Pepsin/0.001M HCl Post fixation wash \(5 ml PBS 10X, 5 ml MgCl2 0.5M, 40 ml H2O) Paraformaldehyde/PBS \(5ml PBS 10x, 5 ml MgCl2 0.5M, 15 ml H2O, 25 ml Paraf. 8%) For probes: Buffer 10X \(for 10ml: 5mL Tris-HCl 1M, 1mL MgCl2 0.5M, 0.005 gr BSA, 4 mL H_2O) Cot-1 DNA \(Invitrogen, 15279-011), Salmon sperm DNA Hybridization Mix:For 15 ml:7.5ml pure formamyde, 6.0 ml Dextran Solfate 25%,1.5 ml 20X SSC. Keep it refrigerated. LABELLING CY-3 \(Amersham, FLUOROLINK CY3-dUTP, code: PA53022-25005442) LABELLING FluorX \(Amersham FluorXdCTP code 11-0026-10) SSC solution DAPI\(ROCHE, 236 276) Antifade DABCO \(SIGMA, D2522)

Equipment

slides coplin jar Savant centrifuge HYBrite \(Vysis) LEICA DMRXA fuorescent mycroscope

Procedure

SLIDES PREPARATION 1)Clean slides with 70% ethanol \(to remove any grease or dust). 2)Place a few drops of pellet and let the slide air dry. 3) Place the slide in an incubator at 90°C for one hour and a half to age, or at 37°C overnight. In case they need to be used much later, place them at –20°C for indeterminate time. SLIDES TREATMENT 1)After 1.5 hours in the incubator, let the slide cool and then add 150-200 µl of 0.005%Pepsin/0.001M HCl and place them at 37°C for 15 min. 2)Place them in a coplin jar with PBS 1x for 5 min. 3) 5 min in post fixation wash 4) 5 min in Paraformaldehyde/PBS wash 5) 5 min in PBS 1x 6) 5 min each in ethanol series: 70%,90%, 100% \(slides can be left in 100% ethanol until ready to be hybridized). 7) Before hybridization with probe, slides are taken out and let air dry completely. LABELLING OF PROBE DIRECT LABELLING \(NICK TRANSLATION) FOR 1γ OF DNA 10ul DNA 3ul Buffer 10X 0.6ul dAGC \(for dUTP/CY-3, red) or 1.8ul dAGT \(for FluorX-dCTP, green) 0.3ul dUTP/CY-3 or 0.9ul for FluorX-dCTP 3ul B-mercaptoethanol 0.3ul DNApolymerase 6ul DNAse \(1:700 ul H2O) H_2O to reach 30ul final volume 1)After preparing the labelling mixtures with enzymes and DNA, place it in a water bath at 16°C for 2 hours. 2) After 2 hours, to PRECIPITATE THE PROBE take the appropriate quantity of labelled probe and place it in a new eppendorf \(for BAC probes 30 ul) 3)Add to the labelled probe: 3 µl Salmon Sperm DNA \(SSD) 10 µl Cot-1 DNA \(10µl of Cot per 30µl of labelled DNA, so for a cosmid add 15 µl) 1/10 Vol NaAC 3 Vol cold EtOH 100% PRECIPITATION 1)To precipitate, place eppendorf at –80°C for 15 min, or at –20°C for at least 30 min. 2) Centrifuge at +4°C for 20 min. 3)Take supernatant off and dry the pellet \(Savant centrifuge could be used to dry pellet in a better way) 4) Resuspend pellet in Hybridization Mix 5) Place in thermomixer at room temperature for 10 minutes to mix. 6) Place probes on dried slides 7)Place coverslip and seal it with rubber cement. 8)Place slides in Hybrite \(Vysis) and start cycle Hybrite temperatures: Melt: 69°C for 2 min \(human slide) Hyb: 37°C Overnight CO- HYBRIDIZATION Two probes are labelled with different fluorochromes and hybridized on the same slide: CY3-dUTP\(red) and FluorX-dCTP \(green). They can be easily used because they are both direct labelled probes. Each probe is labelled individually, and then they are mixed together and precipitated. Example: 30 ul labelled BAC A plus 30 ul labelled BAC B plus 3 ul SSD plus \(10ul plus 10ul) 20 ul Cot-1 DNA plus 8.3 ul NaAc plus 270 ul EtOH. The rest is the same as the single hybridization protocol. RE-HYBRIDIZATION A slide can be re-hybridized up to four times \(at least) with good results. 1) Remove coverslip from slide 2)Place slide for 2 hours at 42°C in 2x SSC 3) Rinse in PBS 1x at room temperature. 4)Place slide for 5 min in each Ethanol: 70%, 90%, 100% 5)Decrease denaturation time. Hybrite Temperatures: Melt: 69°C for 1 min \(human slide) Hyb: 37°C Overnight POST-HYBRIDIZATION WASHES • 3 washes at 57°C in 0.1x SSC for 5min each • 5 min in DAPI \(60 ml of 2x SSC, 120 ul DAPI) • Add a few drops of Antifade DABCO on the coverslip and make sure there are no air bubbles between slide and coverslip ANALYSIS Analyse with LEICA DMRXA Fluorescent microscope.Images can be acquired using applied spectral imaging \(ASI) camera and analyzed with FISH view 2.0 software

Timing

Critical Steps

Troubleshooting

1)Before probe resuspension in the Hybridization Mix it is important to pre-resuspend the precipitated probe in a few ul of H_2O. 2)Pepsin treatment is very important for chromosome quality: the amount of pepsin used and the length of pepsin treatment need to be very precise. 3) It is better to add cold paraformaldehyde in the paraformaldehyde/wash.

Anticipated Results

References

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