Fluorescent *in-situ* Hybridization
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• In situ hybridization is the method of localizing/detecting specific nucleotide sequences in morphologically preserved tissue sections or cell preparations by hybridizing the complementary strand of a nucleotide probe against the sequence of interest.

• If nucleic acids are preserved in a histological specimen, then it can be detected by using a complementary probe.
Principle

• Principle is same as that of ISH

• Use of a fluorescent labeled probe differentiates ISH & FISH

• FISH is a cytogenetic technique used to detect and localize the presence or absence of specific DNA sequences on chromosomes
FISH technique

Schematic representation of FISH technique. A DNA probe is tagged with a fluorescent marker. The probe and target DNA are denatured, and the probe is allowed to hybridize with the target. The fluorescent tag is then detected with a fluorescent microscope.
Types of Samples Used

- Fixed cell suspension
- Formalin fixed paraffin embedded tissues

FISH

A. FISH EGFR
B. FISH Break apart ALK
C. FISH HER2
Diagnostic Applications of FISH

- Prenatal diagnosis
- Cancer diagnosis
- Molecular cytogenetic of birth defects and mental retardation
- The identification of specific chromosome abnormalities
- The characterization of marker chromosomes
- Interphase FISH for specific abnormalities in cases of failed cytogenetic
- Monitoring the success of bone marrow transplantation
Protocol Outline

- Preparation of the fluorescent probes
- Denaturation of the probe and the target
- Hybridization of the probe and the target
- Detection
**FISH Procedure**

### Paraffin embedded tissue
1. Deparaffinized
   - Xylene 5 min×3
     - Room temperature
2. Dehydrate
   - 100% EtOH 5 min×2
     - Room temperature
3. Air dry
4. Pre-treatment
   - Paraffin
     - Pretreatment Solution
     - 65°C 30 min
   - Wash buffer (2×SSC)
     - 5 min×2
5. Protease treatment
   - Protease Solution
     - 37°C 10～20min
   - Wash buffer (2×SSC)
     - 5 min×2
   - *Protease Solution
     - Add 500μl protease in 50ml protease buffer
   - *Protease preservation
     - One month : 4°C
     - Over one month : -20°C
6. Dehydrate (Room temperature)
   - 70% EtOH
     - 1 min
   - 100% EtOH
     - 1 min
7. Air dry

### FISH protocol
1. Mark hybridizing area
   - Diamond pen
2. Apply 10μl FISH probe for 22mm x 22mm area
3. Cover with cover glass and seal with rubber cement
4. Denature
   - 2X SSC
     - Room temp.
     - 5 min
   - 2X SSC
     - 75°C 1-2min
5. Protease treatment
   - Wash buffer (2×SSC)
     - 5 min×2
6. Dehydrate (Room temperature)
   - 70% EtOH
     - 1 min
   - 100% EtOH
     - 1 min
7. Air dry

### Hybridization
1. Incubation
   - Humidified box
     - 37°C 16 – 72 hrs
2. Apply 10μl DAPI Solution to target area
3. Incubation
   - *DAPI Paraffin embedded tissue 1500ng/ml
4. Wash procedure
   - Remove rubber cement
     - Slide into 2X SSC and remove cover glass
5. Counter stain
   - DAPI 10μl
6. Put on cover glass
7. Seal with manicure
8. Examine
FISH Procedure

Frozen tissue
1. Frozen tumour tissue
2. Air dry
   Positive charged slides
3. Fix and Dehydrate
   95% EIOH 20min
4. Air dry
5. Protease treatment

   Protease Solution 37°C 10~20min
   Wash buffer (2xSSC) 5min x 2

   *Protease Solution
   Add 50μl protease in protease buffer

   *Protease preservation
   One month : 4°C
   Over one month : -20°C

6. Dehydrate (Room temperature)
   70% EIOH 1 min
   100% EIOH 1 min
7. Air dry

touch preparations of unfixed tumour tissue/cell smears/cytospins of cultured or blood cells are possible

FISH protocol
1. Mark hybridizing area
   Diamond pen
2. Apply 10μl FISH probe for 22mm x 22mm area

Hybridization
1. Incubation
   Humidified box 37°C 16~72 hrs

Wash procedure
3. Cover with cover glass and seal with rubber cement
4. Denature
   75°C 5 min
   2X SSC Room temp. 5 min
   2X SSC 0.3% NP-40 73~75°C 1.2min
   2X SSC Room temp. 1 min

Counter stain
1. Apply 10μl DAPI Solution to target area
   DAPI 10μl

   *DAPI
   Frozen tumour tissue
   150ng/ml

2. Put on cover glass
   Seal with manicure

Examine
FISH Procedure

Slide aging  Wax removal  Tissue rehydration  Heat pretreatment  Enzyme pretreatment  Tissue dehydration  Add probes  Co-denature  Overnight hybridization  Postwash  Counterstaining and visualisation

Additional steps for paraffin pretreatment

Enzyme pretreatment  Tissue dehydration  Add probes  Co-denature  Overnight hybridization  Postwash  Counterstaining and visualisation

Suspension pretreatment steps
Probes

• Complementary sequences of target nucleic acids

• Designed against the sequence of interest

• Probes are tagged with fluorescent dyes like biotin, fluorescein, Digoxigenin

• Size ranges from 20-40 bp to 1000bp

Fluorescein

Biotin
### Types of Probes

<table>
<thead>
<tr>
<th>Type</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td><strong>Centromere probes</strong></td>
<td>- Alpha and Satellite III probes</td>
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<tr>
<td></td>
<td>- Generated from repetitive sequences found in centromeres</td>
</tr>
<tr>
<td></td>
<td>- Centromere regions are stained brighter</td>
</tr>
<tr>
<td><strong>Telomere</strong></td>
<td>- Specific for telomeres</td>
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<tr>
<td></td>
<td>- Specific to the 300 kb locus at the end of specific chromosome</td>
</tr>
<tr>
<td><strong>Whole chromosome</strong></td>
<td>- Collection of probes that bind to the whole length of chromosome</td>
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<tr>
<td></td>
<td>- Multiple probe labels are used</td>
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<tr>
<td></td>
<td>- Hybridize along the length of the chromosome</td>
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<tr>
<td><strong>Locus</strong></td>
<td>- Deletion</td>
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<tr>
<td></td>
<td>- Translocation probes</td>
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<td></td>
<td>- Gene detection &amp; localization probes</td>
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<td>- Gene amplification probes</td>
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Denaturation & Hybridization

Denaturation

- Either by heat or alkaline method
- A prerequisite for the hybridization of probe and target

Hybridization

- Formation of duplex between two complementary nucleotide sequences
- Can be between
  - DNA-DNA
  - DNA-RNA
  - RNA-RNA
Hybridization

target DNA

probe

denaturation

hybridization
Detection & Visualization

Detection

- Direct labelling:
  - Label is bound to the probe
  - Less sensitive
- Indirect labelling:
  - Require an additional step before detection
  - Probe detected using antibodies conjugated to labels like Alkaline phosphatase
  - Results in amplification of signal

Hybridization

- Fluorescent probe attaches to the target sequence during hybridization
- This is visualized through a microscope with appropriate filters
Thank You

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