Organization of ISH procedure

- **Tissue preparation**
  - no fixation
  - PFA, Bouin’s Carnoy’s fixation
  - snap-freezing

- **Embedding of tissue and sectioning**
  - paraffin
  - cryomedia and frozen sections
  - whole mounts

- **Riboprobe synthesis**
  - $^{35}$S
  - digoxigenin
  - others: fluorescein, $^{33}$P

- **Hybridization and washes**
  - autoradiography
  - color reaction
  - fluorescence

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II. ISH procedure

A. Tissue preparation

B. Embedding and sectioning of tissue

C. Preparation and synthesis of probe

D. Hybridization and washes

E. Detection
A. Tissue Preparation

- Cells in tissue culture can be grown directly on slides or coverslips to which they are then fixed.

- Cell suspension can be spun down onto slides prior to fixation

- Tissues can be frozen and sectioned on a cryostat

- Tissues can be embedded in paraffin or plastic and sectioned on a mycrotome.

- Tissues or embryos can be fixed and hybridized in whole mounts
Fixation

Purpose of fixation is preservation of histological detail. The stronger the fixation the better is cellular morphology preserved, but accessibility of the target is lowered. Hence the conditions for fixation are a compromise.

Two types of fixation:

1) precipitation fixation (Methanol, Ethanol : problem is accessibility of the target Carnoy‘s fixative) precipitated proteins can cover the target RNA or DNA

2) cross-linking fixation (4% PFA, 4% FA, 1% glutaraldehyde): time of treatment alters degree of cross-linking and accessibility of the probe.

- carried out at 4°C under gentle agitation to inhibit endogenous ribonucleases
- proteases are used to increase target accessibility
- length of fixation depends on size of sample: isolated cells 20 min sufficient tissue < 1mm thick 2-3 hours tissue < 2cm thick over night large tissues need perfusion
- volume of fixative minimum 30 times volume of tissue
Bouin‘s fixative:

composed of picric acid, formalin and acetic acid, this fixative is suitable for larger embryos that contain calcified tissue. Bones become softened and can be sectioned on a microtome after embedding.

When Carnoy‘s and Bouin‘s fixative are used, amniotic fluid becomes very solid and is to remove after fixation. Therefore embryos are washed before in saline solution and embryonic membranes are removed.

Freezing of tissue:

frozen tissue is prone to dry out when stored for a longer time period. To avoid this problem tissue can be placed into a mold with cryomount medium and is then frozen on a slab of dry ice. In this form the tissue can be stored at -80°C without dehydration. **Advantage of freezing tissue:** Allows fixing the tissue after sectioning, so that different fixatives for immunohistochemistry and hybridization can be used on adjacent sections.

**Whole mount** embryos are fixed with MEMFA which contains 3.7% formaldehyde
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Embedding

Advantages of embedding tissue:

- better tissue morphology
- thin sections down to 1 µm, commonly 7 µm are used
- ease of orienting tissue for sectioning
- plastic embedding masks RNA/DNA but very thin sections for EM can be made
- for non EM purposes embedding is done in paraffin wax.
- tissue can be stored at room temperature for a long time since no air and water can access the tissue

Paraffin embedding:

Dehydration to remove water. Tissue is taken through a graded ethanol/salt series beginning with physiologic salt concentration and ending in 100% ethanol. Xylene is used for facilitating the penetration of wax into the tissue. For whole mounts methanol has to be used instead of ethanol for dehydration. Subsequently whole mounts can also be embedded and sectioned if necessary.
Paraffin embedding
Sectioning of tissue

ribbon of paraffin sections
Mounting of section onto glass slide

RNAse free water
45 - 50°C

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Drying of mounted sections

Slidewarmer set to 38°C
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Type of probe and method of synthesis

a) Double-stranded DNA: nick-translation, random priming or PCR
   disadvantage: less sensitive than single stranded probes

b) Single-stranded DNA: primer extension or PCR

c) Oligonucleotides: labelled during chemical synthesis
   disadvantage: only 20-30 nucleotides in length  few labelled
   nucleotides  low sensitivity. Oligomix can be
   used to circumvent this problem (expensive).

d) Single-stranded RNA: transcription reaction with RNA polymerases (T3, T7, Sp6)
   using a cloned fragment as a template.
   advantage: high specific activity, RNA-RNA hybrids stronger
   than DNA-RNA hybrids.
Probe length

The optimal probe length is determined by:

- penetration into tissue, which is dependent on fixation method of tissue
- intensity of signal, short probes give low signal and often high background

To optimize signal intensity and penetration:

- removal of cellular proteins (Proteinase K treatment)
- long probes can be cleaved by limited hydrolysis
Label of probe

a) **Radioactive labels** (quantitation possible)

- $^3$H probes subcellular resolution, but long exposure since probes have low specific activity. Labelled probe can be stored for months since $^3$H has long half-life (12 years).
- $^{35}$S probes give resolution of one cell diameter. Short exposure time. Reducing agent has to be used (e.g. DTT) to protect sulphur from oxidation. Probes can be stored for a week (half-life 85 days). **High sensitivity.**
- $^{32}$P probes not used since strong scattering on film and resolution very bad. Short half life (14 days). Alternatively $^{32}$P can be used. Quite expensive but high specific activity.

b) **Hapten labels**

- digoxigenin, biotin and others. Detected by specific antibody or other binding protein (avidin).
- probes are safe, **stable** and provide **single cell resolution.**
- suitable for **whole mount**
- suitable for chromosomes (fluorescence)
Single stranded RNA probes

Vector z.B. pCRII-TOPO

ColE1 ori

f1 Ori

Kan

Amp

P_{lac}
lacZ

SP6 Promoter

HindIII

KpnI

BamHI

EcoRI

EcoRI

NotI

XhoI

M13 forw primer

M13 rev primer

Gene (400-1000 bp)
1. Orientation of Insert

- M13 rev primer
- T7 Promoter
- SP6 Promoter
- Gene (400-1000 bp)
- EcoRI
- HindIII
- KpnI
- BamHI
- or
- M13 forw primer
- T7 Promoter

2. Linearization of Plasmid

- M13 rev primer
- T7 Promoter
- Gene (400-1000 bp)
- EcoRI
- HindIII
- KpnI
- BamHI
- or
- M13 forw primer
- T7 Promoter

linearization with XhoI
3. Transcription

**SP6 RNA Polymerase**

- SP6 Promoter
- HindIII
- KpnI
- BamHI
- EcoRI
- KpnI
- NotI
- XhoI
- EcoRI
- NotI
- XhoI

Gene (400-1000 bp)

- SP6 RNA Polymerase + rNTP + rUTP*

- labelled sense Riboprobe
  - *
  - *
  - *
  - *

- labelled antisense Riboprobe
  - **
  - *
  - *
  - **

Hybridization

- only antisense Riboprobe can hybridize
  - **
  - *
  - *
  - **

- or
  - *
  - *
  - *
  - **

Will be washed off
T7 Promoter Gene (400-1000 bp)

EcoRI

EcoRI

BamHI

NotI

XhoI

T7 polymer

** SP6 RNA Polymerase + rNTP + rUTP* **

labelled antisense Riboprobe

labelled sense Riboprobe

hybridization

only antisense Riboprobe can hybridize

will be washed off
Incorporation and amount of RNA synthetized

\[
\text{amount of } \mu\text{Ci added} \times \frac{\text{total counts}}{\text{incorporated counts}} \times 13.2 \times \frac{\text{number of rUTP in sequence}}{\text{specific activity of isotope}} = \text{amount of RNA synthesized}
\]

The approximate specific activity is expected to be around 10 \(9\text{ cpm/}\mu\text{g RNA}\)

The probe can be stored at -80°C for up to one week (not longer because of radiolysis)
RNA Polymerase
transcription buffer
sense antisense Templates
RNAase Inhibitor
dTT
rGTP rATP rCTP
α-35S rUTP