In situ hybridization and its application in clinical diagnosis: sarcomas

Ernst-Jan M. Speel

Department of Molecular Cell Biology
Department of Pathology
Maastricht UMC

ernstjan.speel@molcelb.unimaas.nl
ernstjan.speel@mumc.nl

In situ hybridization

- Interphase/metaphase cytogenetics
  - Molecular cytogenetics
  - Cancer research and diagnosis
  - Predictors of prognosis
  - Association with response to therapy
- Prenatal diagnosis, mola diagnostics
- Viral infections (EBV, HPV, etc)
- Mapping
- Cell biology: organization interphase nucleus
  - DNA localization
  - RNA transcription, processing and transport
- Gene/RNA expression, siRNAs

ISH applications in molecular diagnostics

- Numerical chromosomal alterations
- Gene amplifications and deletions
- Chromosomal translocations (lymphomas and sarcomas)
- Virus detection (DNA or RNA)

HER-2 FISH (n≥20 nuclei)

Ratio 1.0  Ratio > 2.0  Ratio >> 2.0

Green: 2Fc
Red: Her2

Her2 amplification detected by CISH in breast cancer (n≥20 nuclei)

No (<5 spots/nucleus)  Low (≥5-9)  High (≥10)
(Dis)advantages FISH vs CISH:

<table>
<thead>
<tr>
<th></th>
<th>FISH</th>
<th>CISH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection sensitivity</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Multiple probes</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Resolution</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Nuclear overlap</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Morphology</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Preparation storage</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>Evaluation time</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>Out of focus</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Autofluorescence</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Special microscope</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Virus detection

EBV RNA

“EBER”

Essential steps in ISH procedure

Specimen preparation
- Accessibility probe/target
- Preservation morphology

Probe selection and labeling

Denaturation
(probe and cellular DNA)

In situ hybridization

Probe detection

Microscopy

Speel et al. Histochemistry 1999

Preparation of biological specimen

- Solid support (glass, membrane) + coating

- Fixation:
  - Ethanol
  - Methanol/Acetic acid
  - (Para)formaldehyde

- Pretreatment:
  - Proteolytic digestion (pepsin, proteinase K, other) or microwaves
  - Detergents
  - Endogenous enzyme inactivation
  - RNase/DNase treatment

Optimal protocol for ISH on formalin-fixed, paraffin-embedded tissue sections (1 day)

- Déparaffination
- 85% formic acid / 0.3% H<sub>2</sub>O<sub>2</sub> treatment, 5-20 min RT or 0.2 M HCl, 20 min RT
- 1 M sodium thiocyanate (NaSCN) treatment, 10 min 80°C
- pepsin digestion, 4 mg/ml 0.02 M HCl 10-20 min 37°C
- (Acid) dehydration steps
- Post-fixation in 1% (para)formaldehyde, 10 min RT
- ISH (denaturation 75°C, hybridization 37°C)
- Stringent washings (2×SSC, 73°C) and probe detection

Hopman et al. Modern Pathol 4, 503-513, 1991; 1998; Vysis Inc
### Relation between nucleic acid target and probe sequence

<table>
<thead>
<tr>
<th>Target</th>
<th>Copy number</th>
<th>Probe</th>
<th>Probe label</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>High abundant</td>
<td>FISH</td>
<td>Fluorochrome&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Moderate low abundant</td>
<td>Repeat FISH</td>
<td>Fluorochrome&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>DNA</td>
<td>High unstable</td>
<td>Repeat FISH</td>
<td>Fluorochrome&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Unique</td>
<td>Repeat FISH</td>
<td>Fluorochrome&lt;sup&gt;*&lt;/sup&gt;</td>
<td>Tape&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Spreel, 1999*

### ISH probes
- Locus-specific (single-copy)
- Repeat (centromere, telomere)
- Paint (chromosome (arm))
- Translocation
  - Dual color, single fusion (2x flanking 1 side breakpoint)
  - Extra signal (1x spanning breakpoint, 1x flanking)
  - Dual color, dual fusion (2x spanning breakpoints)
  - Dual color, break apart (flanking 2 sides 1 breakpoint)

### Probe labeling and detection

**Label ((d)NTPs)**
- Fluorochrome
- Enzyme (HRP, APase)
- Hapten (bio, dig, FITC, DNP)

**Detection**
- Direct
- Indirect (antibody and avidine-biotin conjugates)

**Enzymatic probe labeling:** nick translation, random primed labeling, PCR, endlabeling, tailing, in vitro transcription

**Chemical probe labeling:** ULS etc.

### Parameters ISH
- Temperature (37-42°C)
- pH (7.0)
- Monovalent cations (Salt) (2xSSC)
- Formamide (50-60%)
- Probe
  - DNA or RNA
  - Length
  - CG content
  - Base mismatches
  - Concentration
  - Dextran sulphate
  - Build-in haptens

### Fluorochromes often used in ISH detection systems

<table>
<thead>
<tr>
<th>Fluorochrome</th>
<th>Excitation wavelength</th>
<th>Emission wavelength</th>
<th>Fluorochrome class</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy3</td>
<td>540</td>
<td>570</td>
<td>Red</td>
</tr>
<tr>
<td>Cy5</td>
<td>645</td>
<td>670</td>
<td>Red</td>
</tr>
<tr>
<td>Cy7</td>
<td>740</td>
<td>770</td>
<td>Red</td>
</tr>
<tr>
<td>Alexa</td>
<td>530</td>
<td>550</td>
<td>Yellow</td>
</tr>
</tbody>
</table>

*Now also Alexa, Spectrum, Platinum dyes, etc*

### FISH: 1c / 7c copy number evaluation

**Head and neck precursor lesions and tumor resection margins**

** Definition CIN:***
- copy number differences between 1c and 7c, or polysomy

*Bergsma et al., 2008*
Urine cytology: bladder cancer cell detection

Normal

Aneuploid (> 4 nuclei/preparation)

School for Oncology and Developmental Biology
Ernst-Jan M. Speel
Maastricht UMC

Spectral Karyotyping (SKY)

Lazar et al. 2006

Conventional karyotyping: G-banding

Advantages:
- Global genetic information in single assay
- Variants uncovered (undetectable by FISH and RT-PCR)
- Diagnostically useful: fine needle biopsy, sensitive, specific
- Provides direction for further molecular studies

Limitations:
- Requires fresh tissue
- Mostly cell culture 1-10 days
- Complex karyotypes, suboptimal morphology
- False negatives due to cryptic rearrangements
- Normal karyotypes (overgrowth normal fibroblasts, infiltrating cells)
- Low cell density

School for Oncology and Developmental Biology
Ernst-Jan M. Speel
Maastricht UMC

Fine needle biopsy from retroperitoneum: nonlipogenic area: desmoid tumor

Ring chromosomes:
Chrom 12: green
MDM2: red

Bridge, 2008

FISH on Lipo(sarco)ma

- Accounts for up to 40-45% of all liposarcomas
- Most prevalent in adults in the extremities and the retroperitoneum
- Tendency to recur when occurring in deep anatomic sites
- May dedifferentiate and metastasize in 2-20% of cases
- Cytogenetics: Ring and giant marker chromosomes: 12q13-15 amplification: MDM2 + CDK4 candidate genes

School for Oncology and Developmental Biology
Ernst-Jan M. Speel
Maastricht UMC

Alt / WDL

School for Oncology and Developmental Biology
Ernst-Jan M. Speel
Maastricht UMC

Conventional karyotyping: G-banding

Advantages:
- Global genetic information in single assay
- Variants uncovered (undetectable by FISH and RT-PCR)
- Diagnostically useful: fine needle biopsy, sensitive, specific
- Provides direction for further molecular studies

Limitations:
- Requires fresh tissue
- Mostly cell culture 1-10 days
- Complex karyotypes, suboptimal morphology
- False negatives due to cryptic rearrangements
- Normal karyotypes (overgrowth normal fibroblasts, infiltrating cells)
- Low cell density

School for Oncology and Developmental Biology
Ernst-Jan M. Speel
Maastricht UMC

Fine needle biopsy from retroperitoneum: nonlipogenic area: desmoid tumor

Ring chromosomes:
Chrom 12: green
MDM2: red

Bridge, 2008

FISH on Lipo(sarco)ma

- Accounts for up to 40-45% of all liposarcomas
- Most prevalent in adults in the extremities and the retroperitoneum
- Tendency to recur when occurring in deep anatomic sites
- May dedifferentiate and metastasize in 2-20% of cases
- Cytogenetics: Ring and giant marker chromosomes: 12q13-15 amplification: MDM2 + CDK4 candidate genes
Conclusions: study on 50 lipo(sarco)mas

- Strong (2+) immunostaining of both MDM2 and CDK4 proteins show a high amount of concordance with amplification of both gene loss in ALT / WDL.
- Lipomas show no amplification of MDM2 and CDK4 gene loci and predominantly no protein expression.

But:

- Weak (+) immunostaining of MDM2 correlates with the presence of fat necrosis and inflammation in lipoma and ALT / WDL, but not with amplification.
- A subset of ALT / WDL shows no MDM2 and CDK4 amplification.

Therefore:

- FISH might be preferred over IHC (and CDK4 over MDM2 IHC).
- What is genetic cause and implication of ALT without amplification?

Synovial sarcoma

- It accounts for up to 5-10% of all soft tissue sarcomas.
- 5 years survival 36-76%.
- Most prevalent in adolescent and young adults in deep soft tissue of the lower extremities.
- Specific t(X,18) translocation is found in 90% of synovial sarcoma.
- Genes affected by the t(X,18) are SYT from chromosome 18 and SSX1, SSX2 and SSX4 from the X chromosome.

T(X,18) FISH

- Vysis, Abbott Molecular
- n ≥ 50 nuclei

Evaluatie:

A+B: Geen translocatie
C: Translocatie
D-H: Kans op translocatie
Molecular cytogenetics: FISH

- Advantages:
  - Fresh, frozen, paraffin-embedded material (interphase and metaphase)
  - Localize alteration (translocation) in specific cells and tissue types
  - Useful if tumor is heterogeneous, or in case of MRD
  - Diagnostically useful: fine needle biopsy, sensitive, specific
  - Can provide results if karyotyping or RT-PCR is inconclusive
  - Rapid turn-around time
  - Validation and implementation easy
  - Normal tissue parts can serve as FISH control

- Limitations:
  - Targeted approach, except for CGH and SKY analysis
  - Relatively gross approach (no information on fusion genes and variants)
  - Number of commercially available probes is limited (Abbott Molecular, Kreatech)
  - Requires fluorescence microscope
  - Interpretation may be challenging, expertise required
  - Period of storage

Tyramide signal amplification

HPV

Speel et al., 1997; 1998; Hafkamp et al., 2003, 2008

Good luck with evaluation!

Acknowledgements

Maastricht
Molecular Cell Biology
Tom Heyman
Sandra Claessen
Annick Haeuseveldt
Frans Ramaekers

Pathology
Els Meulemans
Andrea Ruland
Guido Roemen
David Creytens

Clinical Genetics
Josefa Albrechts
Jannie Janssen
Merryn MacVille

Epidemiology
Adi Voogd

Maastricht Otorhinolaryngology & Head and Neck Surgery
Ewa Bergshoeff
Harriet Hafkamp
Hans Manni
Bernd Kremer

Nijmegen Pathology
Piet Slootweg

Rotterdam Pathology
Winand Driessen

Gent Pathology
Patrick Pauwels