American Society of Cytopathology
Core Curriculum in Molecular Biology
American Society of Cytopathology
Core Curriculum in Molecular Biology

Chapter 6

Fluorescence *in situ* Hybridization (FISH)
*Principles and Preparatory Techniques*

Amy Wendel Spiczka, M.S. SCT, MB, HTL
(ASCP)™
*Mayo Clinic*
*Scottsdale, Arizona*

Kara Hansing, SCT (ASCP)™
*Mayo Clinic*
*Rochester, Minnesota*
Objectives

- Describe use of FISH in the detection of neoplasia
- Characterize the stepwise progression through the FISH preparation process, detailing what is happening at the molecular level for each step
Outline of Presentation

• Background
  – FISH & Urothelial carcinoma
• Development & validation of UroVysion test
• How to perform the test
FISH – Current & Future Opportunities

• FISH, as a technique, continues to have an increasingly integral role in cytology laboratories

• Cytotechnologists, pathologists, and clinicians need to have an essential understanding the role FISH plays in detecting neoplasia and in guiding the management of patients who have cancer

• FISH frequently has higher sensitivity than conventional cytology
  – FISH may detect tumor cells in cytologic specimens that cannot be detected by conventional methods
What is FISH?

• **FISH** = Fluorescence *in situ* Hybridization

• Utilizes fluorescently labeled DNA “probes” that hybridize (stick to) specific chromosomal loci

• Technique that allows detection of chromosomal alterations in cells
FISH is a Type of Cytology

• FISH is a unique way to examine cells
• One examines cells for morphologic features typical of neoplasia (e.g., nuclear enlargement, nuclear irregularity, etc.), and chromosomal abnormalities indicative of neoplasia
FISH is an Excellent ‘Tool’ to Detect Cancer as Tumors have Chromosomal Abnormalities

**Normal Karyotype** – Normal cells have 46 chromosomes (visible only during mitosis)

**Karyotype of a Cancer Cell** – Tumor cells frequently have abnormal numbers of chromosomes (aneuploidy) & chromosomal structural abnormalities

Images Courtesy of FISH Education – Mayo Cytogenetics Laboratory
FISH Probes

• Centromeric probes
  – Hybridize to (i.e. attach to) specific chromosomal centromeres

• Locus-specific probes
  – Hybridize to other regions of chromosome
  – Usually designed to hybridize to specific gene regions
UroVysion Probe Set

Original Image Courtesy of FISH Education – Mayo Cytogenetics Laboratory
FISH Probes

• Centromeric probes
  – Allows one to count the number of copies of a chromosome in a cell
    • Why? Because if you lose the centromere of a chromosome, you lose the whole chromosome

• Locus-specific probes
  – Deletions or gains of specific gene regions
Cytologic Applications of FISH

- Urothelial carcinoma (urine)
- Biliary tract malignancy (biliary tract brushings)
- Barrett’s esophagus (endoscopic brushings)
- Cervical cancer (cervical cytology)
- Lung cancer (bronchoscopic brushings and secretions, sputum)
Urothelial Cell Carcinoma (UCC)

- ~55,000 cases/year
- ~12,500 deaths
- Very high prevalence
  - ~500,000 (U.S.)
- One of the most expensive cancers to diagnose and treat
Urothelial Carcinoma

- Papillary UC
  - ~80% of all UC
  - Tends to recur but not progress to invasion
  - Most are grade 1 and 2 tumors

- Flat UC (CIS)
  - ~20% of all UC
  - More aggressive, tends to progress to invasive tumor
  - High-grade tumors
Papillary UCC
In situ or flat UCC
Treatment of UCC

• Muscle-invasive
  – Cystectomy or nephroureterectomy

• Superficial
  – Excision, fulguration, BCG, other
  – Monitoring for tumor recurrence
    • Cystoscopy and cytology
Surveillance for Superficial UCC

• Cystoscopy
  – 0-2 years: Every 3 months
  – 2-4 years: Every 6 months
  – 5-on: Yearly

• Cytology
  – Obtained at time of cystoscopy
Pros & Cons of Cystoscopy vs. Cytology

• Cystoscopy
  – Detects papillary UC
  – Sometimes misses flat UC

• Cytology
  – Detects most flat UC (high grade)
  – Frequently misses papillary UC (low grade)
  – Overall low sensitivity
<table>
<thead>
<tr>
<th></th>
<th>Grade 1</th>
<th>Grade 2</th>
<th>Grade 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Studies before 1990 (n = 4)</td>
<td>37%</td>
<td>75%</td>
<td>94%</td>
</tr>
<tr>
<td>Studies from 1990 on (n = 16)</td>
<td>11%</td>
<td>31%</td>
<td>60%</td>
</tr>
<tr>
<td>All studies</td>
<td>21%</td>
<td>53%</td>
<td>78%</td>
</tr>
</tbody>
</table>

Halling et al., J Urol;164:1768, 2000
Laboratory Detection of UCC

- Cytology
- Antigen based methods
  - BTA-stat, NMP22, Immunocyt,...
- Molecular genetic methods
  - Telomerase, microsatellite analysis
- DNA ploidy analysis (flow cytometry/DIA)
- Molecular cytogenetic methods (FISH)
FISH for the Detection of Urothelial Carcinoma

- Mayo Clinic began working on the development of a FISH assay for the detection of UC in 1998 (in collaboration with Abbott Molecular)
- This led to a multi-target, multi-color FISH probe set for the detection of recurrent UC which is now referred to as UroVysion

Sokolova et al., J Mol Diagn;2:3, 2000
UroVysion Probes

CEP 3 (red)
CEP 7 (green)
CEP 17 (aqua)
LSI 9p21 (gold)
UroVysion

- These 4 probes chosen from 11 candidates
- The use of a multi-probe cocktail increases both the sensitivity and specificity of the assay over single or dual probes
UroVysion (Normal Cell)

- CEP 3
- CEP 7
- CEP 17
- LSI 9p21
UroVysion (Malignant Cell)

CEP 3  CEP 7
CEP 17  LSI 9p21
## Sensitivity of Cytology and FISH: Overall and by Stage*

<table>
<thead>
<tr>
<th></th>
<th>pTa</th>
<th>pTIS</th>
<th>pT1-pT4</th>
<th>All Tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cytology</strong></td>
<td>17/36 (47%)</td>
<td>14/18 (78%)</td>
<td>9/15 (60%)</td>
<td>40/69 (58%)</td>
</tr>
<tr>
<td><strong>FISH</strong></td>
<td>24/37 (65%)</td>
<td>17/17 (100%)</td>
<td>18/19 (95%)</td>
<td>59/73 (81%)</td>
</tr>
<tr>
<td><strong>P value</strong></td>
<td>0.058</td>
<td>0.046</td>
<td>0.025</td>
<td>0.001</td>
</tr>
</tbody>
</table>

*75 biopsy-proven UC patients

Halling et al., J Urol, 2000
<table>
<thead>
<tr>
<th></th>
<th>Grade 1</th>
<th>Grade 2</th>
<th>Grade 3</th>
<th>All Tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytology</td>
<td>3/11 (27%)</td>
<td>13/24 (54%)</td>
<td>24/34 (71%)</td>
<td>40/69 (58%)</td>
</tr>
<tr>
<td>FISH</td>
<td>4/11 (36%)</td>
<td>19/25 (76%)</td>
<td>36/37 (97%)</td>
<td>59/73 (81%)</td>
</tr>
<tr>
<td>P value</td>
<td>0.564</td>
<td>0.034</td>
<td>0.003</td>
<td>0.001</td>
</tr>
</tbody>
</table>

Halling et al., J Urol, 2000
FISH is more Sensitive than Conventional Urine Cytology

![Graph showing weighted mean comparison between Cytology and FISH for various categories such as Ta, T1, T2-4, TIS, Grade 1, Grade 2, Grade 3, Overall, Sensitivity, and Specificity.]
UroVysion

• FDA approved for:
  – Evaluation of patients with hematuria for bladder cancer (2005)
Performing the FISH Test:
Focus on Lab Preparation
FISH Urine Testing Overview

1. Obtain urine sample
2. Isolate cells from urine and place on slide (slide preparation)
3. Prepare cells for FISH hybridization (pre-hybridization)
4. Hybridize FISH probes to cells
5. Assess cells for chromosomal abnormalities using fluorescence microscope
Specimen Types

- Voided urine (FDA approved)
- Catherized urine
- Bladder washings
- Upper tract washings (ureters and renal pelvis)
- Stomal specimens
- Previously stained ThinPrep slides
Preservatives

- Ethanol/Methanol of varying concentrations
- PreservCyt and CytoLyt
- Fresh Specimen (no preservative)
UroVysion Slide Preparation

• Manual method (Abbott Molecular package insert)

• ThinPrep
  – 2.0 cm diameter (Non-gyn filters)
  – 1.0 cm diameter (UroCyte filter)

• Cytospin
Slide Preparation (Abbott Molecular Method)

• Cells sedimented in a centrifuge and washed with PBS buffer and fixative
• Cells resuspended in small volume (<0.5mL) of residual fixative
• Portion of this cell suspension (~10ul) dropped on a slide
Slide Preparation (Abbott Molecular Method)

• Cellularity of slide assessed with phase contrast microscope
• If necessary, additional cell suspension is added to get the desired result
• What is the desired result?
  – To get as many cells as possible on slide without having much cell overlap
Slide Preparation (Abbott Molecular Method)
Slide Preparation (Abbott Molecular Method)

• Some limitations with this method
  – Time consuming
  – Inconsistent cell dropping between different technologists
  – Hard to assess cellularity under phase contrast scope in specimens with a lot of debris (i.e. crystals, proteinaceous debris, etc.)
Slide Preparation (ThinPrep)

• Alternative preparation method – ThinPrep 2.0 cm slide
  – Problems
  • Cells spread over too large a surface area
  • Requires 10ul of UroVysion probe
  • Markedly increases time required to analyze slide
  • Increased cost
Slide Preparation (ThinPrep)

- Another alternative method – ThinPrep UroCyte filter/slide (1.0 cm)
  - Recently developed filter/slide to be used for FISH urine processing
  - Automates slide making for FISH
  - Reduces area over which cells are spread (1.0 cm)
    - Reduces amount of probe needed (3uL) and time required to analyze slide without the subjectivity of cell dropping
Slide Preparations

Manual Gold Seal® Slide

Cytyc ThinPrep®
2-cm NON-Gyn Slide/Filter

Cytyc 1-cm ThinPrep®
UroCyte™ Slide/Filter

Copyright 2010 American Society of Cytopathology
Prepare Cells for FISH Hybridization (Pre-hybridization)

- Pre-hybridization treatment is necessary to allow the FISH probes to enter the cell and hybridize to their target loci.
- Can use manual (Coplin jars) or automated (VP2000) method depending on number of specimens pre-hybridized by your laboratory.
VP2000
## Pre-hybridization Steps

<table>
<thead>
<tr>
<th>Step</th>
<th>Reagent</th>
<th>Time (min)</th>
<th>Temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2xSSC</td>
<td>2</td>
<td>73</td>
</tr>
<tr>
<td>2</td>
<td>Pepsin in HCL</td>
<td>10</td>
<td>37</td>
</tr>
<tr>
<td>3</td>
<td>PBS</td>
<td>5</td>
<td>RT</td>
</tr>
<tr>
<td>4</td>
<td>1% Form.</td>
<td>5</td>
<td>RT</td>
</tr>
<tr>
<td>5</td>
<td>PBS</td>
<td>5</td>
<td>RT</td>
</tr>
<tr>
<td>6</td>
<td>70% ETOH</td>
<td>1</td>
<td>RT</td>
</tr>
<tr>
<td>7</td>
<td>85% ETOH</td>
<td>1</td>
<td>RT</td>
</tr>
<tr>
<td>8</td>
<td>100% ETOH</td>
<td>1</td>
<td>RT</td>
</tr>
<tr>
<td>9</td>
<td>Air Dry</td>
<td>3</td>
<td>RT</td>
</tr>
</tbody>
</table>
Hybridization

• Following pre-hybridization, 3uL of FISH probe is carefully dropped on the target area
• A small coverslip (22x22 square or 12 mm circle) is then placed on the area
• Rubber cement is placed around the coverslip to prevent evaporation of the probe
Hybridization

- The hybridization of the FISH probes can be competed in a humidified chamber and 37°C oven or on a Denaturation System.
• DAPI I vs. II
  – There are unique formulas for DAPI
  – DAPI II is the manufacturer-recommended counterstain for most CEP and LSI probes. This concentration of DAPI provides weaker DAPI stain usually preferred with smaller probe signals such as CEP and LSI probes
Post-hybridization

• Following overnight hybridization
  – Slides are washed in 2xSSC to remove probe that is not specifically bound
  – Failure to do this long enough will result in background signals (starry sky pattern)

• DAPI counterstain added and slide cover slipped
  – 4’,6-diamidino-2-phenylindole
Please view the lecture “FISH Analysis”

Learn to assess cells for chromosomal abnormalities using fluorescence microscopy
Thank you!

Special recognition is given to Kevin Halling, M.D., Ph.D. and Ben Kipp, Ph.D. for the materials provided in this lecture.