Technology Transition Workshop| *Robert Driscoll, M.F.S.*

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Introduction to Fluorescent In Situ Hybridization (FISH)

Fluorescent In Situ Hybridization (FISH)

- **FISH is a cytogenetic technique used to detect the presence or absence of specific chromosomes and/or sequences**
- **Interphase FISH techniques incorporate probes which pass through cellular membranes and into the nucleus eliminating the need to lyse cells during processing**
- **Fluorescence compatible microscopes are typically employed to visualize the multicolor probes used in these hybridizations**

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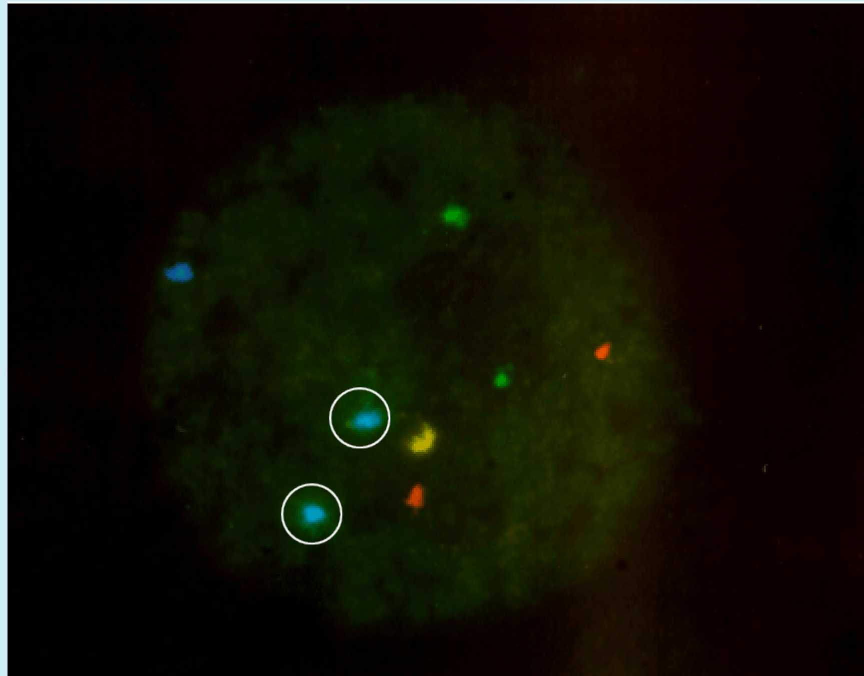
Traditional FISH Applications

- **Used to detect a variety of diseases caused by chromosomal abnormalities:**
  - **Prader-Willi Syndrome**
  - **Angelman Syndrome**
  - **Cri-du-chat**
  - **Down Syndrome**
  - **Chronic myelogenous leukemia**
  - **Acute lymphoblastic leukemia**
- **Used to compare genomes of two biological species to deduce evolutionary relationships**
- **Gene mapping**

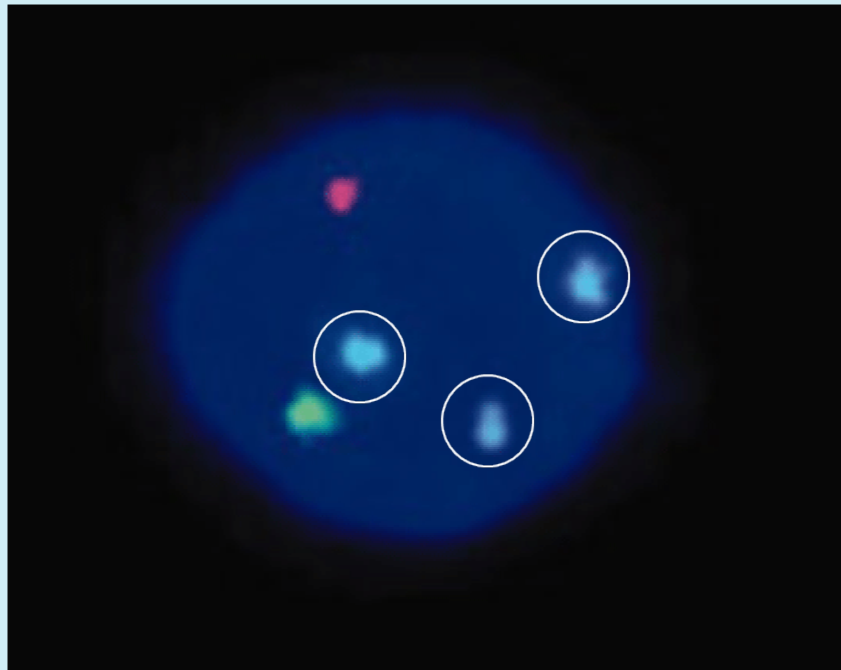
Prenatal Screening for Down Syndrome



Normal FISH assay displaying 2 copies of chromosome 21

Image courtesy of: http://www.parkavefertility.com/pre-implantation_genetic_diagnosis.html

Prenatal Screening for Down Syndrome



Trisomy 21 (Down Syndrome) - 3 copies of chromosome 21

Image courtesy of: http://www.parkavefertility.com/pre-implantation_genetic_diagnosis.html

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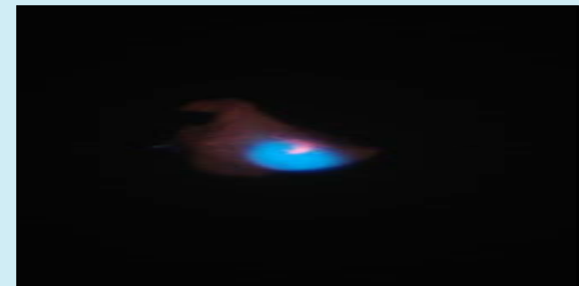
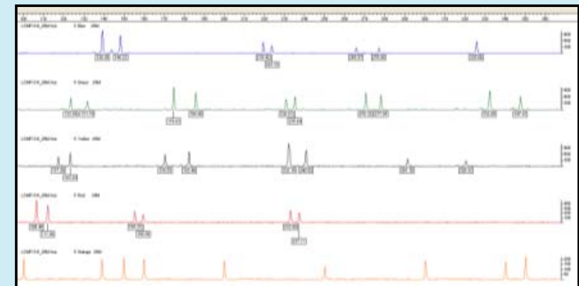
Methodology of LM-FISH Processing

- **Isolate cells of evidence mixtures**
- **Identify contributor by gender**
- **Capture, process, and generate clean profile**





Images courtesy of Rob Driscoll

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FISH Overview

- Preparation of slide
- Pre-treatment
  - Prepare sample for hybridization by reducing background fluorescence
- Hybridization
  - Apply probes and hybridization buffer to the sample
  - Denature and hybridize sample/probes
- Post-hybridization
  - Remove excess probe and salts
  - Apply DAPI counterstain to stain nucleus blue and increase fluorescence of probes

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Preparation of Slide

1. Elute cells from substrate.
2. Re-suspend cells in Carnoy's fixative.
   - 3:1 methanol/glacial acetic acid
   - Fixes cells to slides
   - Helps cells spread well
3. Steam slide for 5 seconds.
   - Allows for further membrane spreading
4. Dry on slide warmer.
5. Bake in biological oven.
6. Store in desiccator overnight.

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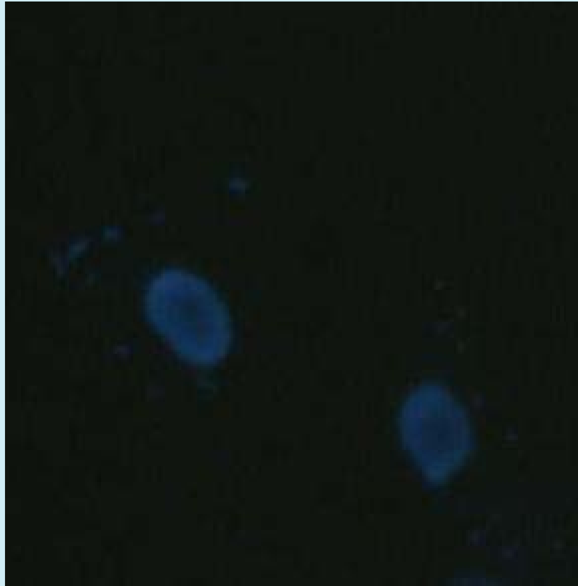
Pre-Treatment

- **SSC buffer wash**
  - **Isotonic buffer solution**
  - **Contains sodium chloride and sodium citrate**
- **Pepsin buffer wash**
  - **Protease digests the cell wall and cytoplasm, leaving the nucleus behind**
  - **Increases fluorescence of probes by reducing background fluorescence caused by cytoplasm**

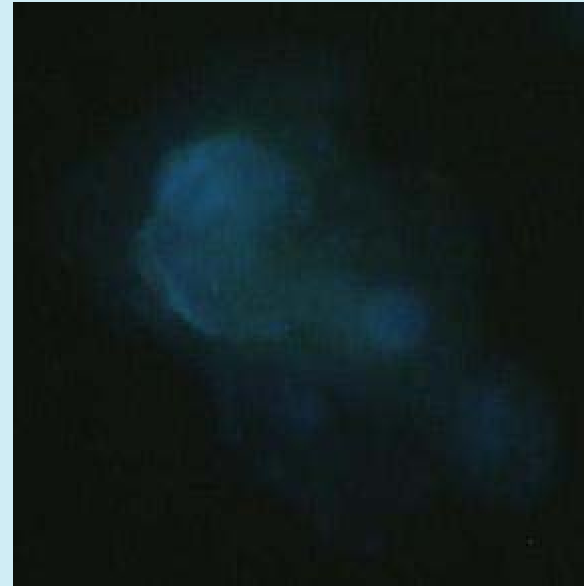
Pre-Treatment

- Effect of pepsin buffer on cells

10 minute pepsin buffer wash



No pepsin buffer wash



Images courtesy of Abby Bathrick

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Pre-Treatment Steps

- **PBS/MgCl2 wash**
  - Removes pepsin buffer from slide
- **Formalin (or formaldehyde) buffer wash**
  - Reduces background fluorescence
- **PBS wash**
- **Serial ethanol washes**
  - Dehydrates cells
- **Slides are stored in dessicator until hybridization**

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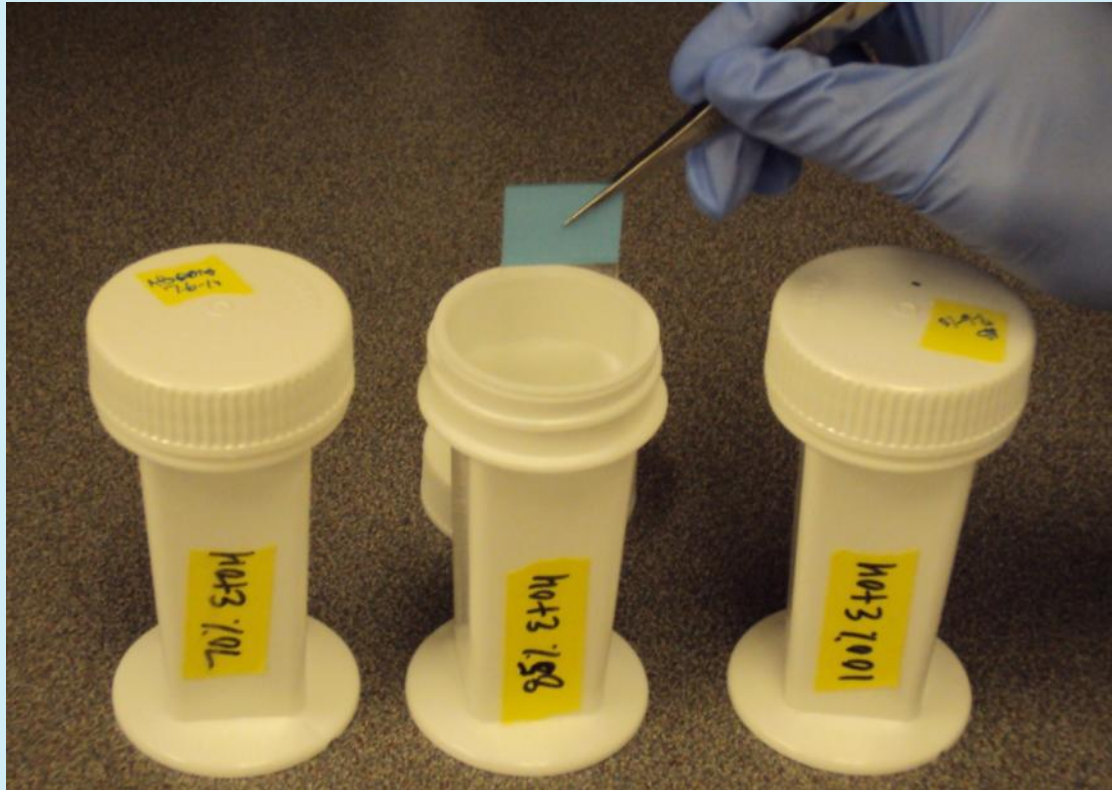
Pre-Treatment



Serial ethanol washes

Image courtesy of Rob Driscoll

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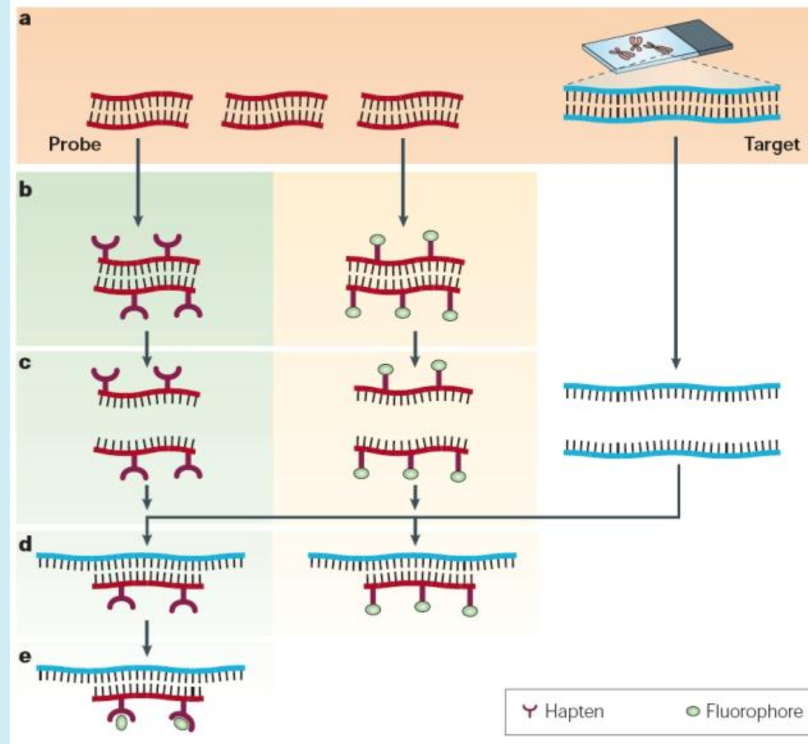
Hybridization



1. Identify the DNA probe and a target sequence.
2. Probe is labeled.
3. Labeled probe and target DNA are denatured.
4. Denatured probe and DNA are combined and complimentary DNA sequences anneal.
5. Indirectly labeled probe undergoes additional processing for visualization.

Image from reference #9 (See Handout, Relevant Scientific Literature)

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Hybridization Buffer

- Contains:
  - Formamide – denaturing agent
  - SSC – isotonic buffer, controls salt concentration
  - Dextran sulfate – high molecular weight polymer
- Buffer components and hybridization temperature affect the stringency
  - Stringency – adjustment of conditions to control hybridization of the probe binding to the target sequence
    - The higher the stringency, the lower the probability of hybridization
    - High stringency – high temp, lower salt concentration
    - Low stringency – low temp, higher salt concentration

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Hybridization Probes

- Chromosome enumeration probes (CEP®) bind to highly conserved sequences of individual chromosomes
  - Highly repetitive human satellite DNA sequences
  - Strong, crisp signals
- CEP Y® Satellite III SpectrumOrange™
  - Y chromosome hybridized with TRITC-labeled CEP Y® (DYZ1) probe (orange)
- CEP X® Alpha Satellite SpectrumGreen™
  - X chromosome hybridized with FITC-labeled CEP X® (DXZ1) probe (green)

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FISH – Male Epithelial Cells

CEP Y® Satellite III SpectrumOrange™

- CEP Y® (DYZ1) SpectrumOrange™ probe
  - Chromosome position Yq12
  - Aquired via Abbott Molecular®/Vysis™
  - TRITC fluorophore
- Blue DAPI counterstain

Image courtesy of Rob Driscoll

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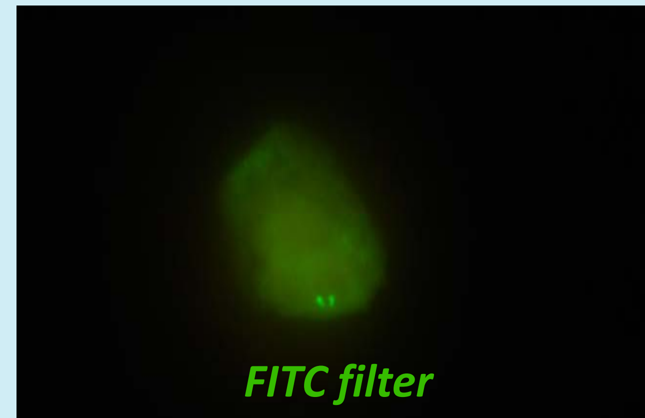
FISH – Female Epithelial Cells

CEP X® Alpha Satellite SpectrumGreen™

- **CEP X® (DXZ1) SpectrumGreen™ probe**
  - **Chromosome position Xp11.1**
  - **Aquired via Abbott Molecular®/Vysis™**
  - **FITC fluorophore**
- **Blue DAPI counterstain**



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Hybridization

- Denaturation: Breaks the hydrogen bonds between double-stranded segments of DNA, allowing new bonds to form between the probes and target DNA during hybridization
- Denaturant solution contains formamide, SSC, and water
- Process:
  1. Denature at 75 to 80°C in a water bath.
  2. Complete serial ethanol washes.
     - Dehydrates the cells
  3. Combine hybridization buffer, probes, and water.
     - Denature at 75 to 80°C in a water bath

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Hybridization

- **Hybridization: Probes specifically hybridize to their complementary sequences on the target DNA**
- **Process:**
  1. **Apply denatured probes to the denatured sample slide.**
  2. **Cover with coverslip.**
  3. **Seal with rubber cement.**
     - **Prevents evaporation of sample**
  4. **Hybridize at 37 to 42°C in a humidified chamber.**

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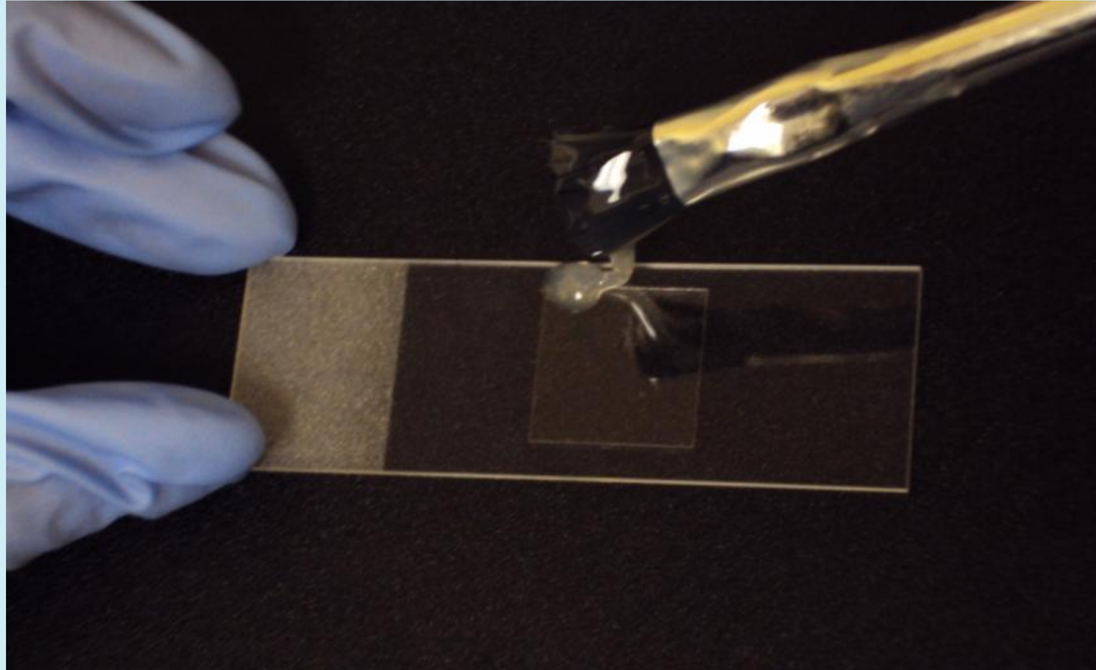
Hybridization



Sealing coverslip with rubber cement to prevent evaporation of sample

Images courtesy of Rob Driscoll

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Hybridization

- Co-denaturation and hybridization on StatSpin® ThermoBrite™ Slide Hybridization/Denaturation System
  1. Combine hybridization buffer, probes, and water.
  2. Apply to slide.
  3. Cover with coverslip.
  4. Seal with rubber cement.
  5. Denature at 73°C.
  6. Hybridize at 37 to 42°C.

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StatSpin® ThermoBrite™ Slide Hybridization/Denaturation System

- Microprocessor controlled bench top hot plate with lid
- Three operating modes: denaturation, hybridization, fixed temperature
- Stores 40 programs
- 12 slide capacity
- Humidity control to prevent evaporation of samples from slides
- Purchased to provide a more controlled environment for heating slides
  - Increased consistency and reproducibility of temperatures

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Hybridization

- **Use of re-useable perfusion chambers in combination with the ThermoBrite™ eliminates the need for water baths**
  1. **Place chamber on the slide.**
  2. **Fill chamber with buffer.**
  3. **Place slide on the ThermoBrite™ for incubation.**

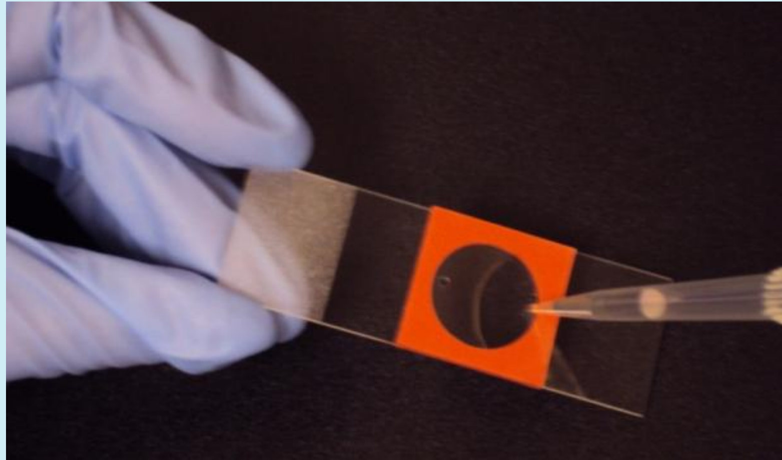

Image courtesy of Rob Driscoll

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Hybridization

Image courtesy of: http://international.abbottmolecular.com/ThermoBrite_7633.aspx#

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Post-Hybridization

- SSC/Tween® 20 wash buffer
  - High stringency wash
  - Removes excess unbound probe
- SSC wash
  - Low stringency wash
- Water rinse
  - Removes residual buffer salts to reduce nonspecific background fluorescence
- DAPI VECTASHIELD® counterstain
  - Fluorescent nuclear stain (blue)
  - Increases fluorescence of probes

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FISH Sample Visualization

1. **Utilize Vysis™ CEP X® Alpha Satellite and CEP Y® Satellite III probes to visually identify male and female cells.**
2. **Apply a DAPI counterstain to stain nuclei.**
3. **Detect fluorescently labeled nuclei using DAPI/FITC/TRITC bandpass filters.**
4. **Capture cells using a laser microdissection system.**

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FISH Sample Visualization

DAPI filter for detection of nuclei

Images courtesy of Abby Bathrick

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FISH Sample Visualization

Rhodamine filter for detection of Y chromosome

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FISH Sample Visualization

FITC filter for detection of X chromosome(s)

Images courtesy of Abby Bathrick

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Male White Blood Cells Stained with X and Y FISH Probes

Image courtesy of Rob Driscoll

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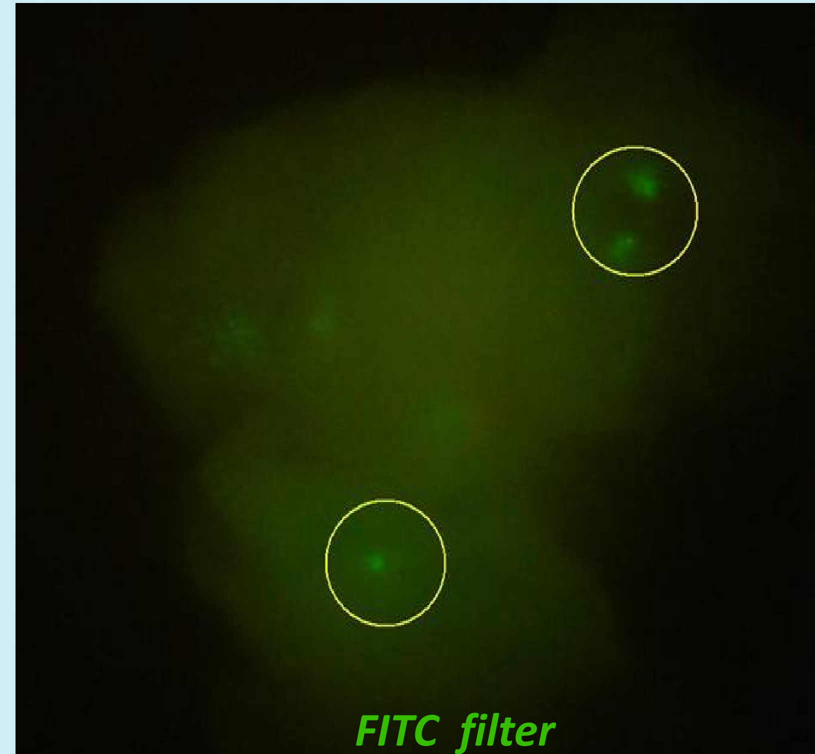
Male/Female Epithelial Cell Mixture



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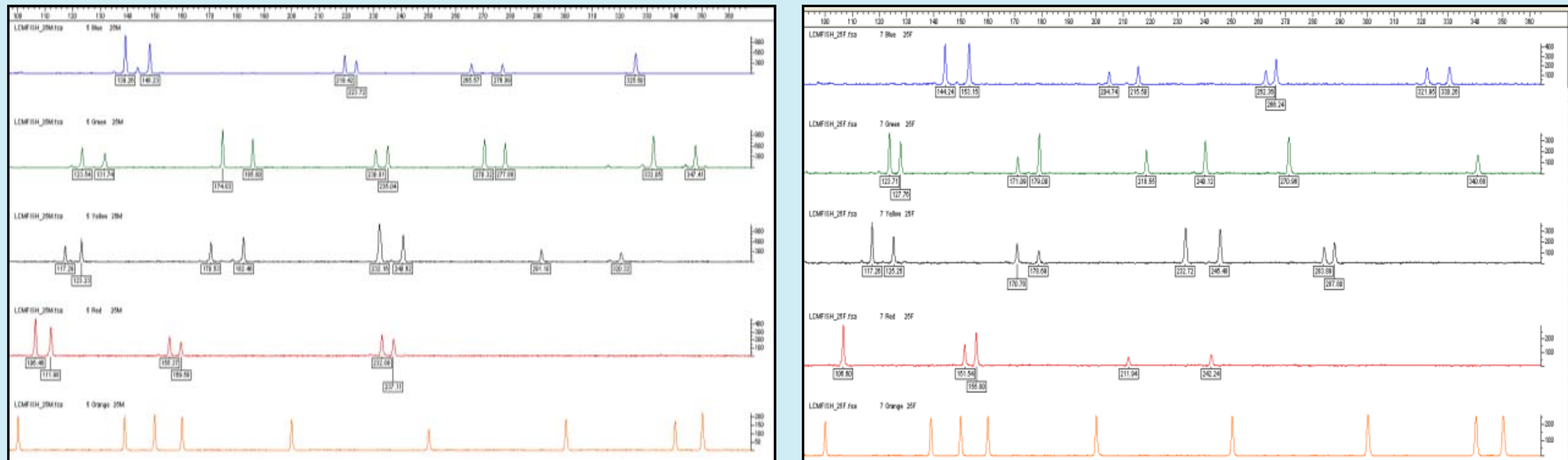
Initial STR Profiling of FISH Samples



- **Profiles generated from the separation of approximately 30 FISH-treated male and 30 FISH-treated female epithelial cells from a 1:1 mixture**
- **Extracts were amplified for 30 cycles in a 7 µl Identifiler® reaction**
- **Demonstrates a clean separation of male and female components**

Images courtesy of Rob Driscoll

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Initial STR Profiling of FISH Samples

15 FISH-Stained Male White Blood Cells Separated From a Male/Female White Blood Cell Mixture

Image courtesy of Rob Driscoll

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Initial STR Profiling of FISH Samples

Profile From 15 FISH-Stained Male Epithelial Cells Separated From a Male Epithelial/Female White Blood Cell Mixture

Image courtesy of Rob Driscoll

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Ongoing Research

- The current FISH X/Y probing technique is limited
  - Only allows for separation of cells of different gender
- Research is underway regarding the use of FISH and LM to separate same gender mixtures
- Key objective is to separate cellular mixtures of similar morphology and same gender by using sequence-specific FISH probes based upon the genetic polymorphisms associated with SNPs and the ABO blood groups

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Ongoing Research

- SNPs and the ABO blood groups were chosen as the basis for these assays because of the existence of their prevalent polymorphisms throughout multiple populations
- Both systems have multiple polymorphisms which can be targeted through multicolor probe assays
- Primer probes can be designed to target genetic loci associated with SNPs and the ABO blood groups to allow for visual identification of individuals in forensic mixtures

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Advantages of FISH

- **Allows for differentiation of male and female cells of similar morphological mixtures**
- **Allows for successful processing of previously unusable items of evidence**
- **Reduces need for complicated LCN mixture deconvolution**

Limitations of FISH

- 8 to 10 hours for FISH processing
- Probes are $30 per µl
- Difficult to troubleshoot because multiple factors can affect successful hybridization
- Formalin and hybridization buffers contain toxic chemicals

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Summary

- FISH processing utilizing X/Y probes is a novel method for differentiating male and female cells of similar morphological mixtures
- New systems are being explored that may allow for differentiation of cell mixtures of same morphology and gender

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Questions?

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