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

Laser Microdissection Methods

Carl Zeiss® PALM® MicroBeam System

- **Flexible applications from archival material to living cells for DNA isolation**
- **Patented laser catapult technology for contact- and contamination-free specimen capture**
- **Automated script search programs for items of interest**
- **Optimal workflow with simple component integration: from individual experiments to automation**

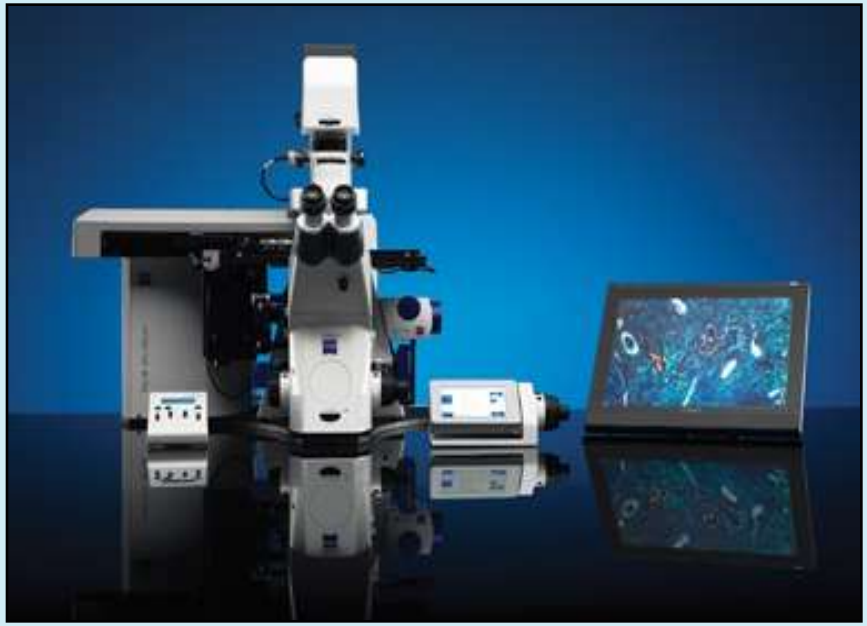

Image from: http://www.zeiss.com/c12567a10053133c/Contents-Frame/19ca656fd8df9adcc12575380027efea

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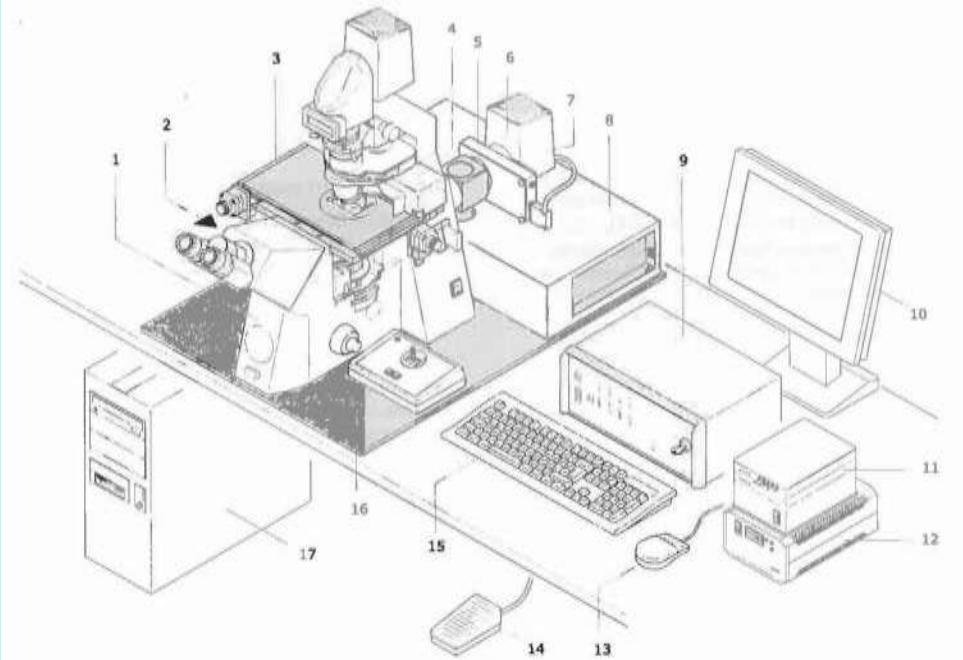
Carl Zeiss® PALM® MicroBeam System



- **PALM® MicroBeam System consists of:**
  - **Axiovert® 200 microscope**
  - **Fluorescence illumination unit**
  - **Laser interface and laser**
  - **Computer**

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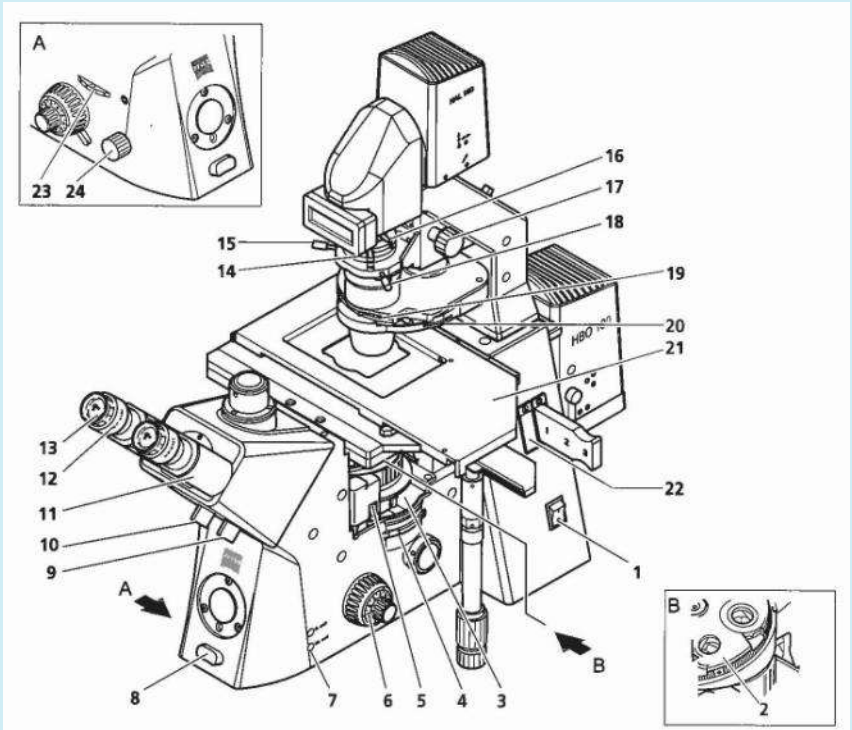
Image from: Carl Zeiss® PALM® MIcroBeam User Manual, Version 1105, page 5

Axiovert® 200 Microscope



1 On / Off switch
2 Objective nosepiece
3 Compartment for slider Analyzer
4 Setting wheel for Optovar turret
5 Reflector turret
6 Focusing drive coarse / fine
7 HAL on / off switch
8 Toggle switch for illumination intensity
9 Turning or sliding knob for vis / doc beam splitting
10 Turning or sliding knob for Bertrand lens and manual shutter
11 Binocular tube component
12 Setting ring of the eyepiece
13 Eyepiece
14 Polarizer D with 2-position filter changer
15 Centering screw for condenser
16 Setting lever for luminous-field diaphragm
17 Setting knob for vertical adjustment of the condenser
18 Centering screw for condenser
19 Turret disk of condenser
20 Setting wheel for aperture diaphragm on the condenser
21 Microscope stage
22 Compartment for aperture diaphragm slider
23 Setting wheel for Sideport
24 Setting knob for Frontport / Baseport

Image from: Operating Manual Axiovert® 200 / Axiovert® 200 M, page 0-13

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Carl Zeiss® PALM® MicroBeam System

- **Laser path**
  - **Guided through the microscope adapter into the epifluorescence channel of the microscope**
  - **Reflected by a special coated beam splitter**
  - **Focused by the objective**

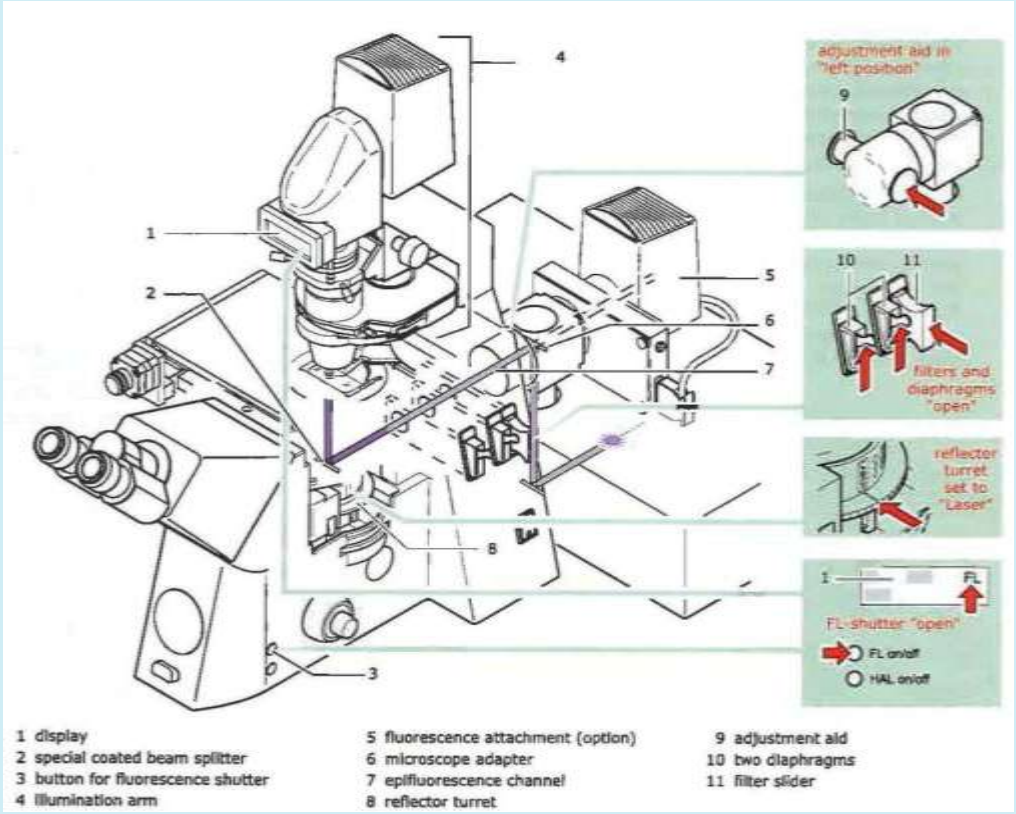

Image from: Carl Zeiss ® PALM® MicroBeam User Manual, Version 1105, Page 15

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Slides

- **Membrane-coated slides**
  - **Polyethylene naphthalate (PEN) membrane-coated slides**
    - **Requires less laser energy for cutting and catapulting**
    - **Pores in the membrane auto-fluoresce, making it difficult to detect true FISH signals**
  - **Polyethylene tetraphtalate (PET) membrane-coated slides**
    - **Requires more laser energy for cutting and catapulting**
    - **No membrane fluorescence**

Slides

- **Glass slides**
  - **No fluorescent background caused by the slide**
  - **Cells are catapulted without cutting**
  - **Once fixed to the slide, cells may difficult or impossible to remove from slide**
    - **High laser energy combined with multiple firings for successful catapulting**

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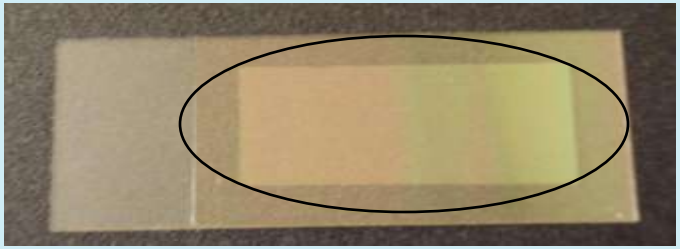
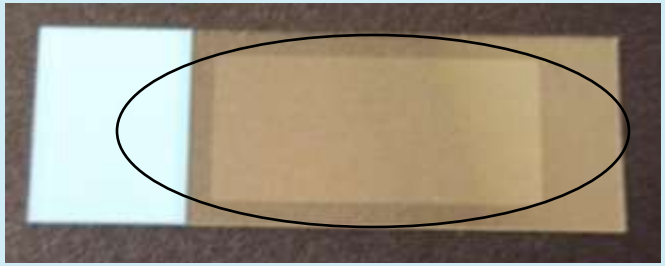
Slides



PEN membrane slide



PET membrane slide

Glass slide

Images courtesy of Abby Bathrick

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Collection Devices

- **AdhesiveCap 500**
  - **500 µl PCR tube**
  - **Cap filled with clear adhesive material for buffer-free sample capture**
- **0.5 ml microcentrifuge tubes**
  - **Flat top tube**
  - **Pipette 20 – 40 µl of H_2O or buffer in the cap**
  - **Surface tension keeps the liquid inside the cap**

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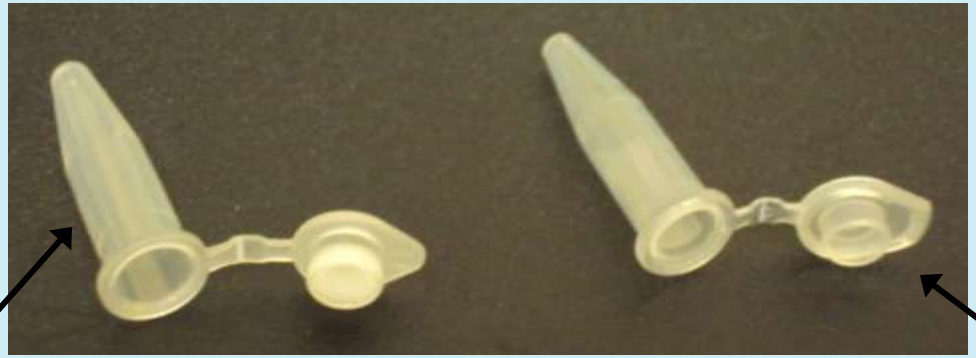
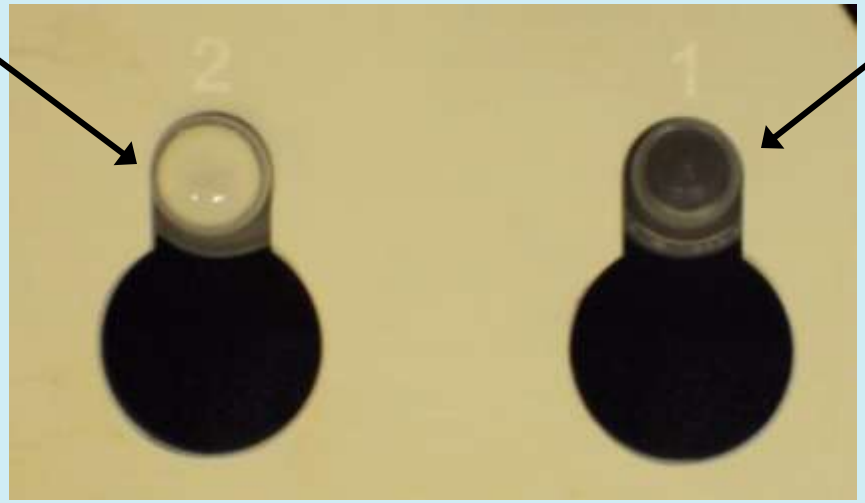
Collection Devices



AdhesiveCap 500



0.5 ml microcentrifuge tube

Images courtesy of Abby Bathrick

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Carl Zeiss® PALM® MicroBeam System

- **PALM® RoboMover**
  - **Automated positioning of caps over the sample to be collected**
  - **Interchangeable inserts allow for use of various collection vessels**
- **PALM® RoboStage**
  - **Motorized microscope stage**
  - **Various holders for Petri dishes, capillary tubes, and up to 3 slides**
- **Advanced Fluorescence Attachment**
  - **Module allows for simultaneous laser function under fluorescence illumination**

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Carl Zeiss® PALM® MicroBeam System

- **The PALM® EightTube Collector insert holds eight 0.5 ml microcentrifuge tubes**

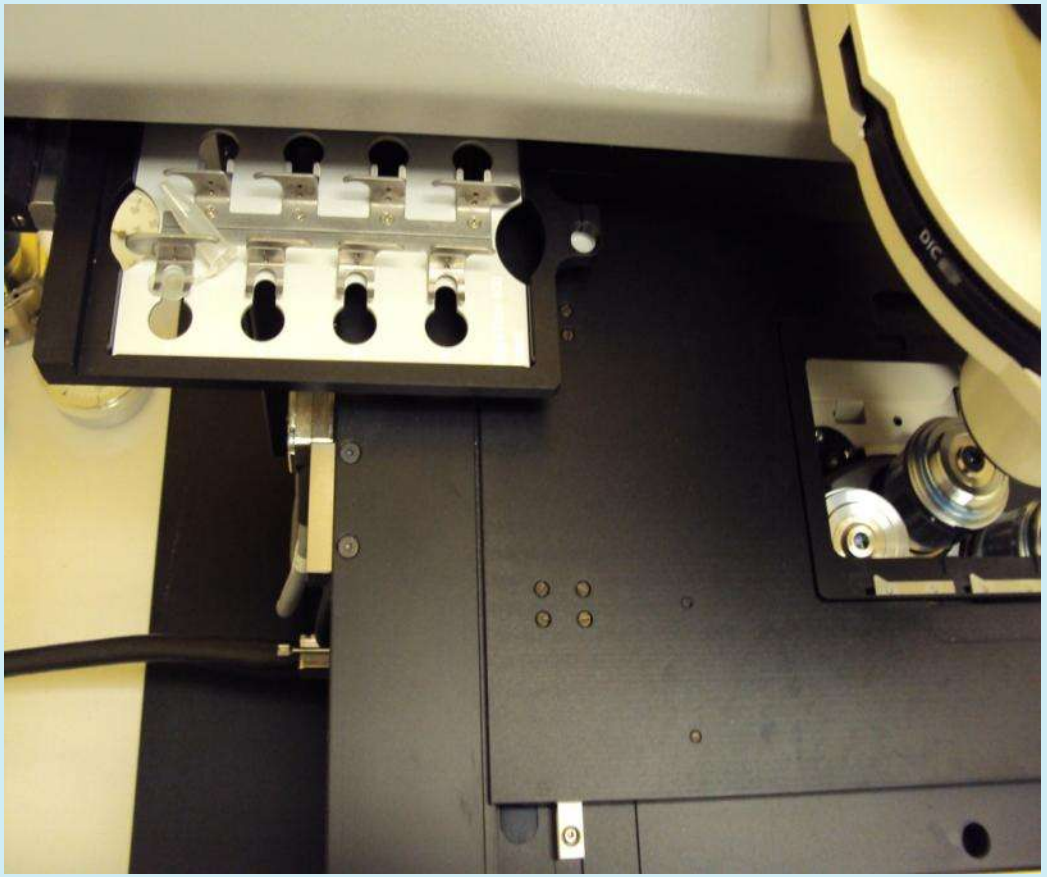

Image courtesy of Abby Bathrick

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PALM® RoboSoftware

- **Facilitates operation and control of the Carl Zeiss® PALM® MicroBeam System**
  - **Display of microscope image on the monitor**
  - **Storage of image viewed on the monitor**
  - **Software controlled movement of the stage**
  - **Definition of lines and areas (elements) for further processing with the laser**
  - **Automatic cutting along defined lines followed by catapulting**
  - **Creation of automated sperm search scripts**

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PALM® RoboSoftware

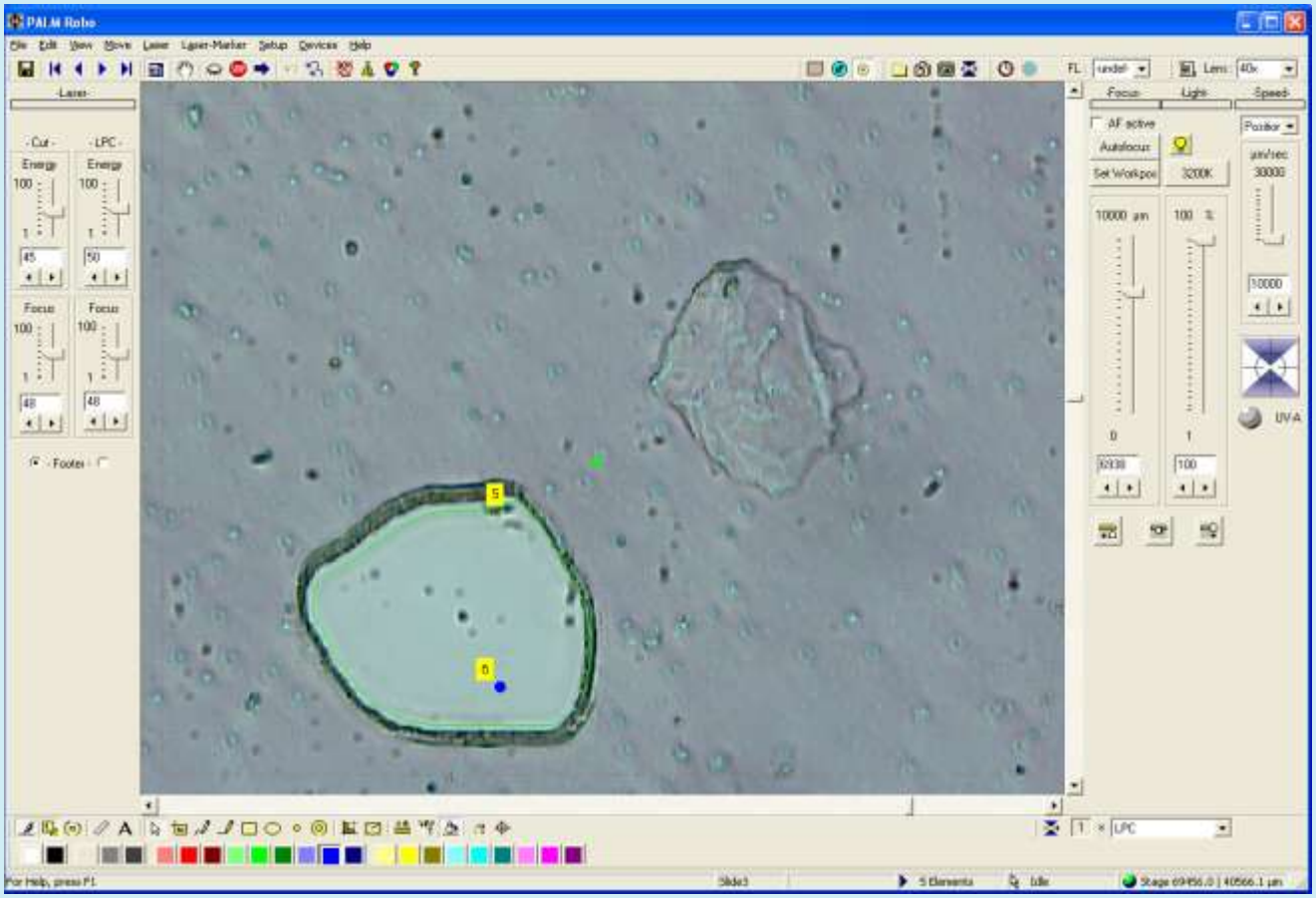

Image courtesy of Abby Bathrick

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LM Forensic Research
Sperm Search Script Development

- **The PALM® system includes automated scanning programs which allow one to differentiate and select targets based on size, shape and color**
- **Scripts were developed for the automated identification of sperm cells stained with:**
  - **Christmas tree stain**
  - **Haematoxylin**
- **Scripts scan an area of interest on a slide and automatically identify elements of interest**

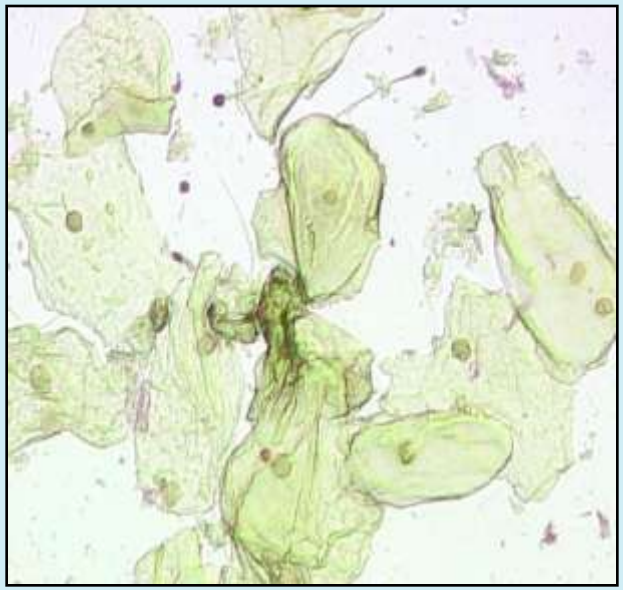

Image courtesy of Rob Driscoll

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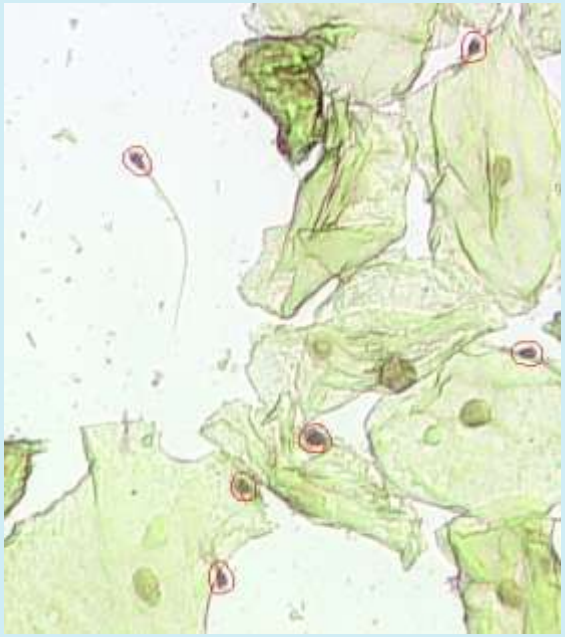
LM Forensic Research
Christmas Tree Sperm Search Script



- **The final version of the Christmas tree sperm identifier script exhibited:**
  - **76% true positive identifications**
  - **24% false negative identifications (i.e., missed sperm)**
  - **14.4% false positive identifications (i.e., incorrect labeling)**

Image courtesy of Rob Driscoll

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LM Forensic Research
Haematoxylin Sperm Search Script

- **The final version of the Haematoxylin stain sperm identifier script exhibited:**
  - **82% true positive identifications**
  - **18% false negative identifications (i.e., missed sperm)**
  - **12.8% false positive identifications (i.e., incorrect labeling)**

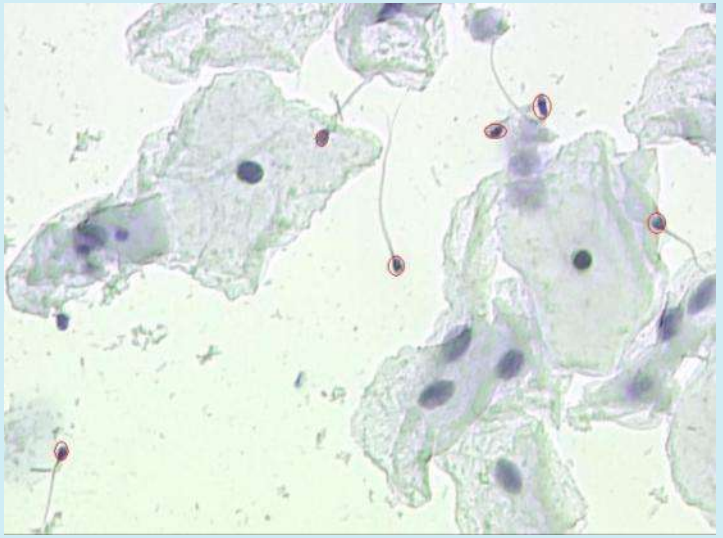

Image courtesy of Rob Driscoll

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LM Forensic Research

Final Conclusions for Sperm Search Scripts

- **Overall, the scripts serve as an effective sperm search method for automated purposes**
- **The use of these scripts will not eliminate the need of an analyst to review the findings of the software**
- **On average, the scripts will erroneously miss identification of 24.3% of all sperm in a scanned field**
- **The combination of software scanning and human manual review can ensure the efficient processing of slides containing sperm and epithelial cell mixtures**

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Optimization of LM Sample Processing Techniques

- **Evaluation of extraction and amplification methods**
- **Goals:**
  - **Improve DNA yield of low copy number (LCN) LM collected samples**
  - **Reduce time required to process LM collected samples**

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Optimization of LM Sample Processing Techniques: Elution of Cells from Swab

1. **Cut entire swab into 1.5 ml tube, and add 500 µl 1X PBS.**
2. **Incubate at room temperature with shaking at 900 rpm for approximately 2 hours.**
3. **Transfer sample to pre-assembled centrifuge filter basket in a 1.5 ml tube.**
4. **Spin for 10 minutes at 10,000 rpm.**
5. **Remove and discard supernatant.**
6. **Wash pellet with 500 µl 1X PBS.**
7. **Pipette up and down to mix, then spin for 10 minutes at 10,000 rpm.**
8. **Remove and discard supernatant.**
9. **Resuspend cells in Carnoy's fixative (3:1 methanol/acetic acid).**

Optimization of LM Sample Processing Techniques: Slide Preparation

1. **Spread 20 µl of sample onto a PEN membrane slide and fixate using a slide warmer set to 56°C for 2 minutes.**
2. **Incubate for 1 minute in 70% ethanol.**
3. **Using the Carl Zeiss, Inc.® PALM® MicroBeam System, cut and catapult cells into the caps of 0.5 ml tubes containing 20 to 25 µl ddH_2O.**
   - **AdhesiveCaps used for QIAamp® DNA Micro Kit extractions**

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Extraction Evaluation

- **QIAGEN® QIAamp® Micro Kit**
  - **Manual extraction**
  - **DNA binds to a silica membrane in the presence of chaotropic salt**
  - **DNA is washed and eluted from the membrane**
- **EZ1® DNA Investigator Kit**
  - **Robotic extraction**
  - **DNA binds to silica-coated magnetic beads in the presence of chaotropic salt**
  - **DNA is washed and eluted from the beads**
- **ZyGEM™ forensicGEM™**
  - **Single tube extraction method**
  - **Uses a thermophilic proteinase to lyse cells, degrade nucleases, and release DNA**
  - **Enzyme is heat inactivated**

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QIAGEN® QIAamp® Micro Kit Extraction Procedure

1. **Post LM, incubate caps in low volume Proteinase K/ Buffer ATL mixture at 56°C for 1 to 2 hours.**
2. **Add DTT to sperm extractions.**
   - **Laser-Microdissected Tissues Protocol via QIAGEN® Handbook**
3. **Spin caps down with attached tubes and additional QIAGEN® buffers (with carrier RNA), and add ethanol to increase sample volume for optimal column binding.**
4. **Concentrate sample via Microcon® down to approximately 5 to 10 µl.**

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LM Forensic Research Results

Identifiler® Profile Generated from 10 Captured Epithelial Cells

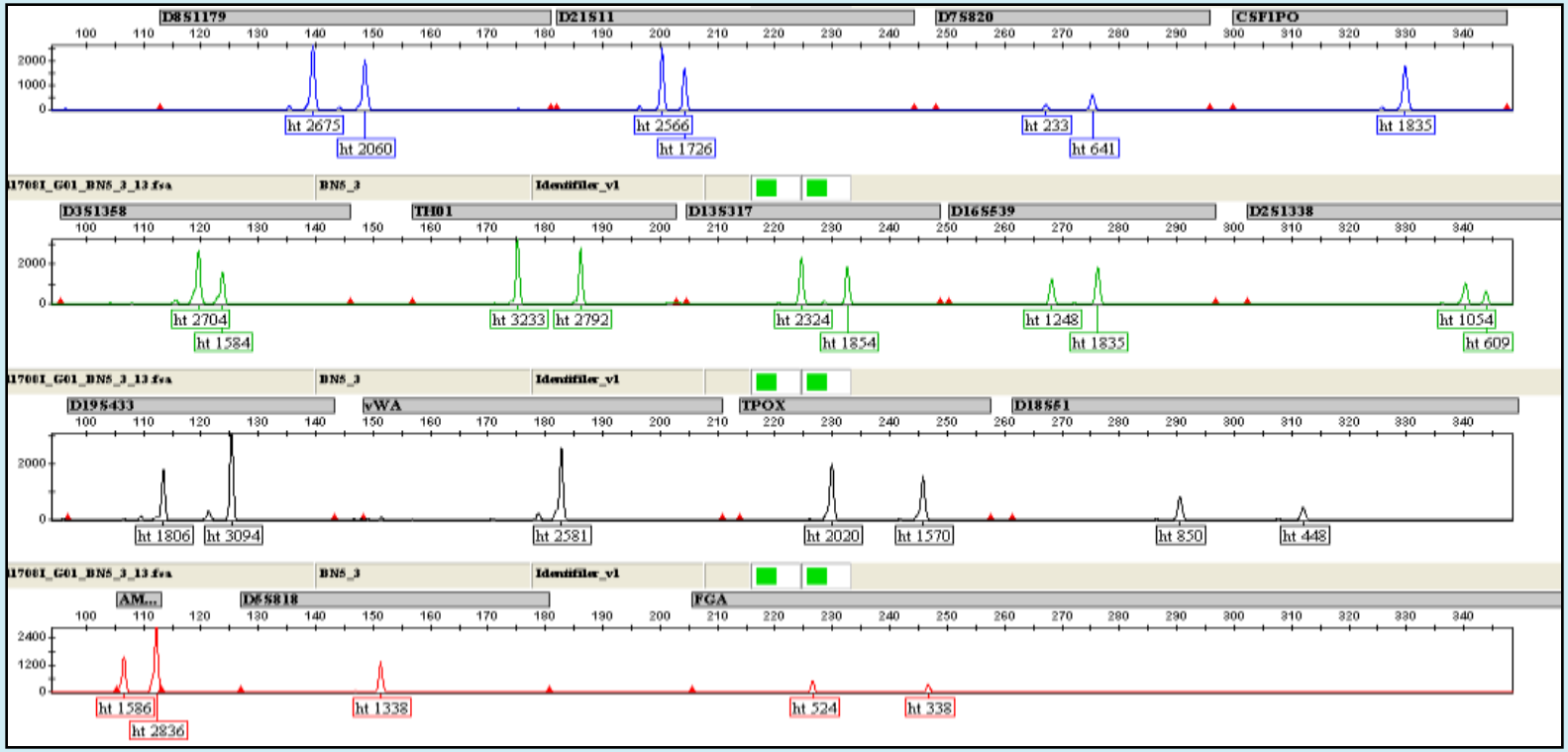

Image courtesy of Rob Driscoll

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QIAGEN® EZ1® DNA Investigator Kit Extraction Procedure

1. **Post LM, incubate sample in low volume Proteinase K/Buffer G2 mixture at 56°C for 1 to 2 hours.**
2. **Add carrier RNA to sample.**
3. **Transfer sample to EZ1® 2.0 ml skirted sample tubes and run on robot with EZ1® DNA Investigator cartridge.**
4. **Concentrate sample via Microcon® down to approximately 17.6 µl.**

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EZ1® – 25 cells

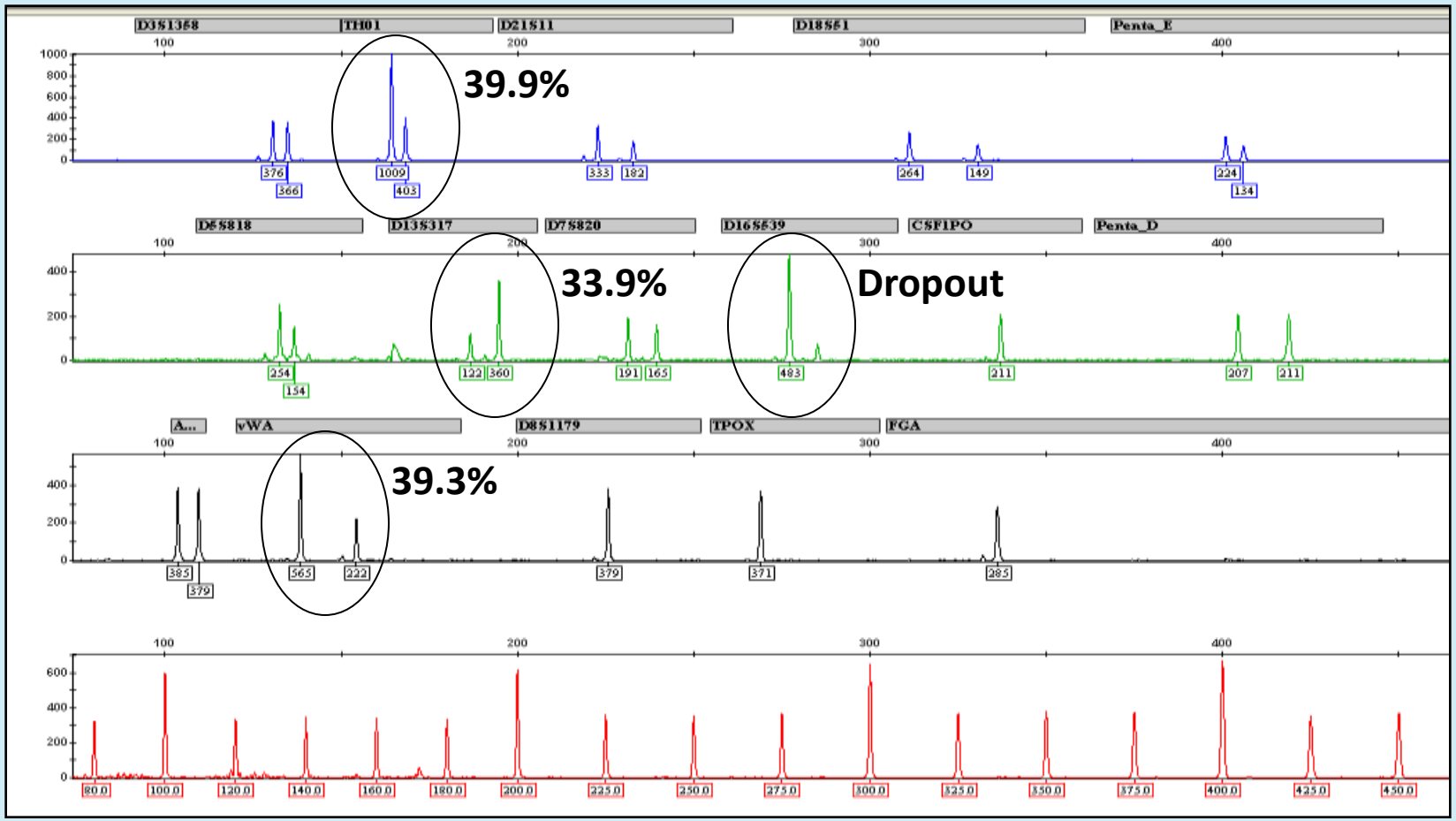

Image courtesy of Abby Bathrick

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ZyGEM™ forensicGEM™ Extraction Procedure

1. **Post LM, incubate samples with thermophilic enzyme, optimized buffer, and ddH_2O mixture at 75°C for 15 minutes and at 95°C for 5 minutes.**
2. **Concentrate sample via Microcon® down to approximately 17.6 µl.**

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ZyGEM™ – 25 cells

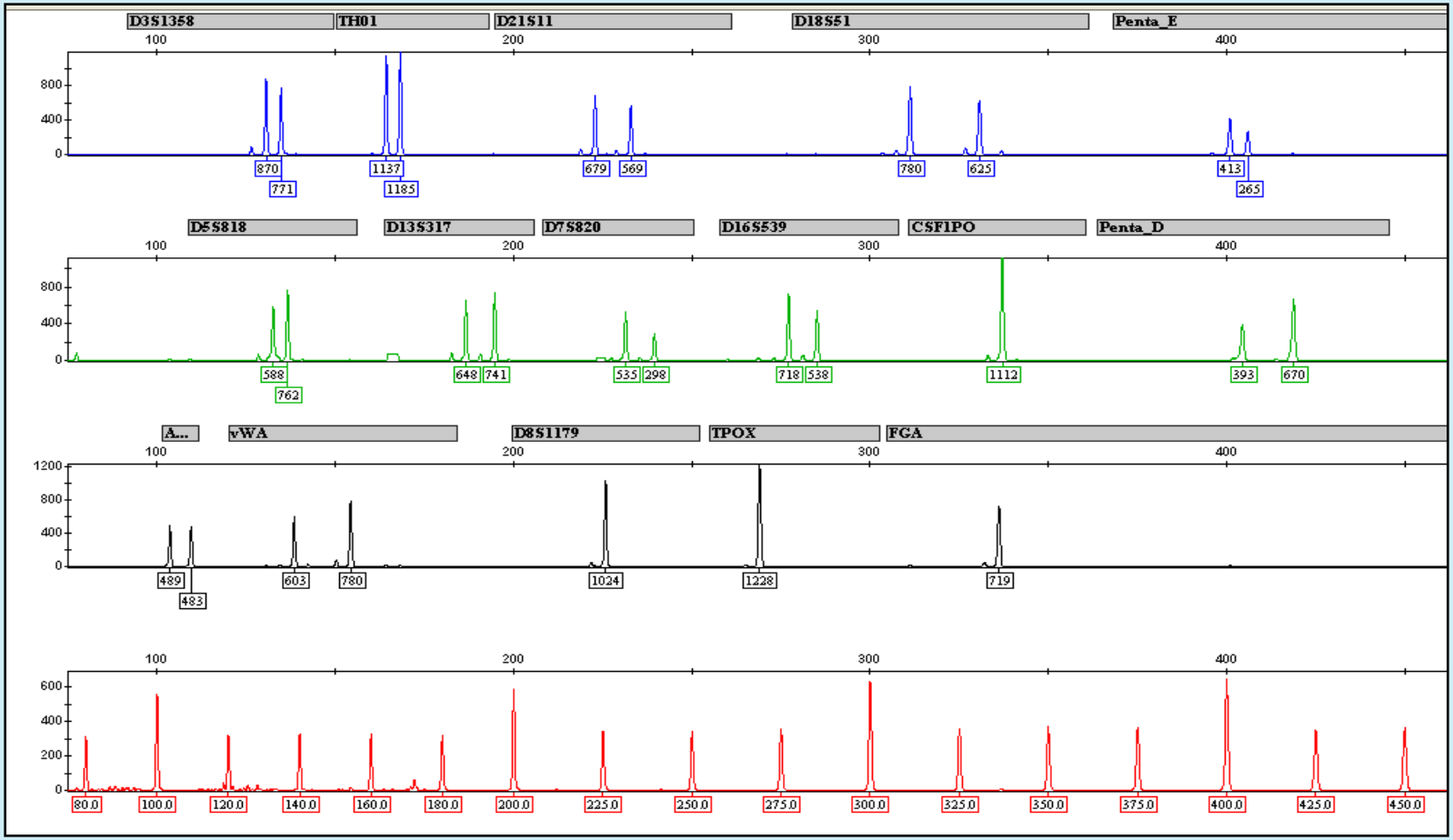

Image courtesy of Abby Bathrick

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EZ1® vs. ZyGEM™ Extraction Evaluation

- **25 to 50 cells collected in triplicate via LM**
- **ZyGEM™ and EZ1® extractions**
- **Concentrated using Microcon® YM-100 Columns**
- **Amplified using the PowerPlex® 16 HS amplification (25 µl/32 cycles)**

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EZ1® vs. ZyGEM™ Extraction Evaluation

	ZyGEM™	EZ1®
50 cells		
Average Height	338 RFU	435 RFU
Average Balance	80.0 %	72.5%
Dropout	5.2% of alleles*	0 alleles
Balance < 50%	4.2% of loci	6.3% of loci
25 cells		
Average Height	338 RFU	250 RFU
Average Balance	76.7%	63.7%
Dropout	4.2% of alleles	2.1% of alleles
Balance < 50%	2.1% of loci	16.7% of loci
Extraction Duration	20 minutes	1 hour 16 minutes
Extraction Type	Single tube	Robotic
Loci	Balanced	Imbalanced

***Dropout in ZyGEM™ samples seen at larger loci: D18, Penta E, Penta D**

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Amplification System Evaluation

- 50 cells were collected in triplicate using the PALM® MicroBeam System
- Cells were catapulted into the caps of 0.5 ml tubes containing 20 to 25 µl ddH_2O
- Extraction was performed using EZ1®
- Concentrated using Microcon® YM-100 Columns
- Amplified using the following amplification systems:
  - PowerPlex® 16 HS (25 µl/32 cycles)
  - PowerPlex® 16 (25 µl/30 cycles)
  - Identifier® (25 µl / 28 cycles)

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Amplification System Evaluation

Promega® PowerPlex® 16 HS

- **Amplifies 15 loci, plus Amelogenin**
- **32 cycles**
- **Kit components:**
  - **PowerPlex® 16 HS 10X Primer Pair Mix**
  - **PowerPlex® HS 5X Master Mix**
    - **Includes hot start Taq DNA polymerase**
  - **PowerPlex® 16 HS Allelic Ladder Mix**
  - **Internal Lane Standard 600**
  - **Water, amplification grade**
  - **9947A DNA**
- **3100 Genetic Analyzer parameters (manufacturer's recommendation):**
  - **3kv_10s**
  - **1 µl of amplification product**

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PowerPlex® 16 HS – 50 cells

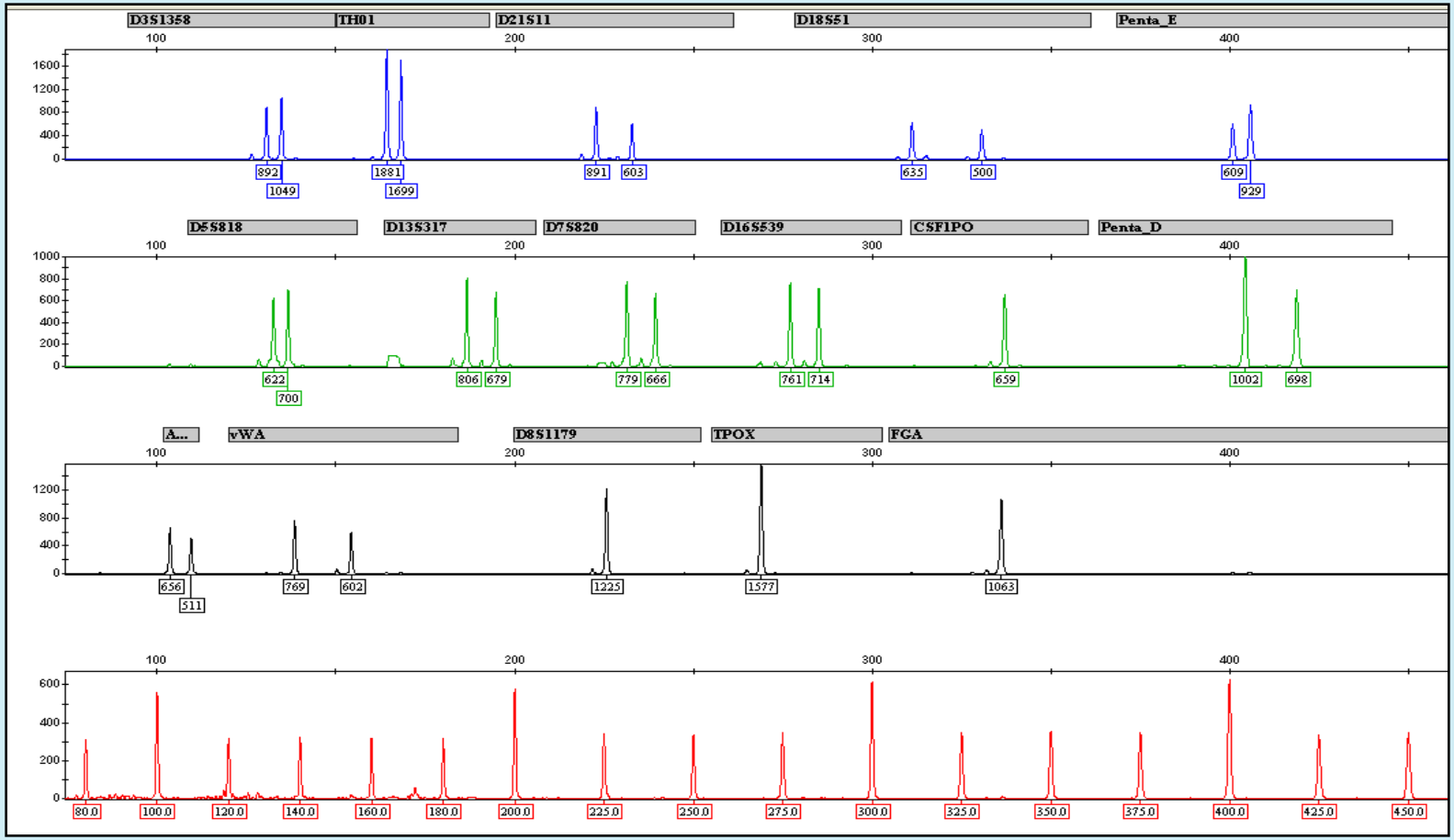

Image courtesy of Abby Bathrick

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Amplification System Evaluation

Promega® PowerPlex® 16

- **Amplifies 15 loci, plus Amelogenin**
- **30 cycles**
- **Kit components:**
  - **PowerPlex® 16 10X Primer Pair Mix**
  - **GoldST★R 10X Buffer**
  - **PowerPlex® 16 Allelic Ladder Mix**
  - **Internal Lane Standard 600**
  - **9947A DNA**
- **3100 Genetic Analyzer parameters:**
  - **3kv_10s**
  - **0.6 µl of amplification product**

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PowerPlex® 16 – 50 cells

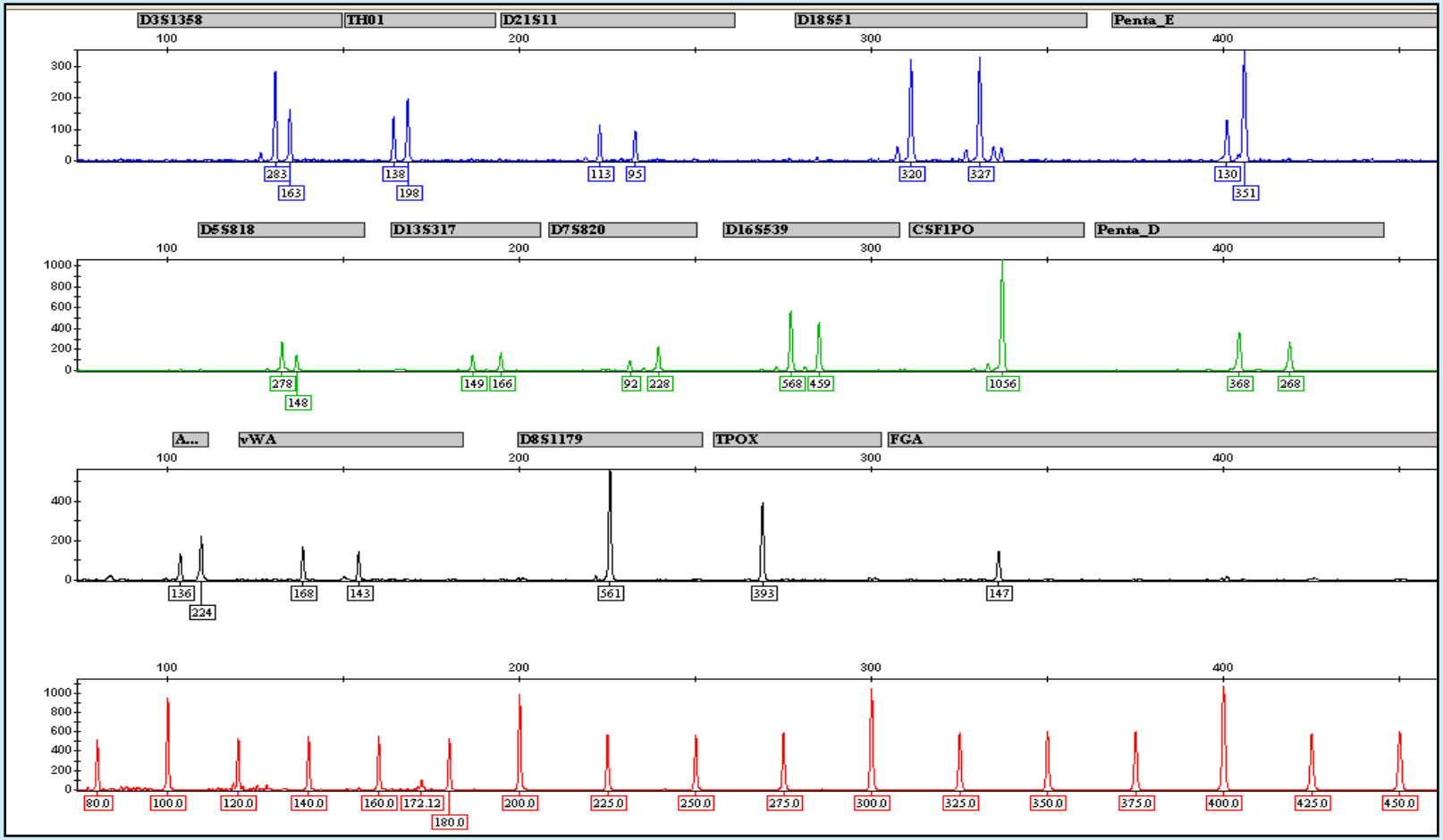

Image courtesy of Abby Bathrick

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Amplification System Evaluation

Applied Biosystems™ AmpFℓSTR® Identifiler®

- **Amplifies 15 loci, plus Amelogenin**
- **28 cycles**
- **Kit components:**
  - **Identifiler® Primer Set**
  - **PCR Reaction Mix**
  - **AmpliTaq Gold® DNA Polymerase**
  - **Identifiler® Allelic Ladder**
  - **Control 9947A DNA**
- **3100 Genetic Analyzer parameters:**
  - **3kv_10s**
  - **0.7 µl of amplification product**

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Identifiler® – 50 cells

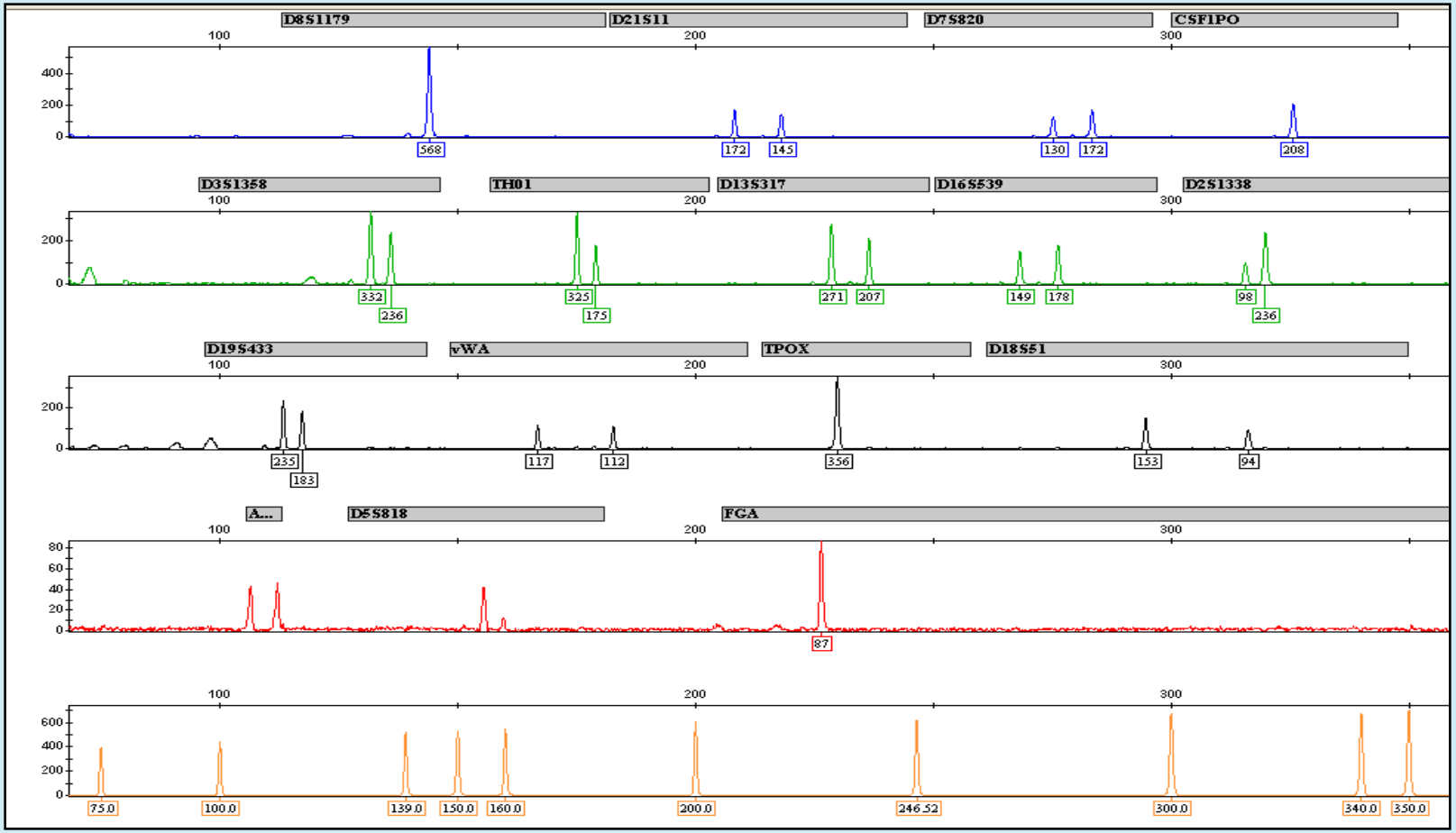

Image courtesy of Abby Bathrick

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Amplification System Evaluation

50 Cells	PowerPlex® 16 HS	PowerPlex® 16	Identifiler®
Average Height	554 RFU	221 RFU	142 RFU
Average Balance	80.6%	86.3%	74.2%
Dropout	8.3% of alleles	4.2% of alleles	16.7% of alleles
Balance < 50%	0 loci	2.1% of loci	4.2% of loci
Cycles	32 cycles	30 cycles	28 cycles
Cost	$17.61 per sample	$16.22 per sample	$17.33 per sample

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Summary of LM Sample Processing Optimization

- **Recommended procedure:**
  - **Cells → ZyGEM™ extraction → Microcon® concentration → PowerPlex® 16 HS amplification**
- **As few as 25 cells may be successfully amplified without any alterations to manufacturer's protocols**
- **Use of ZyGEM™ extraction may reduce some of the stochastic effects commonly seen in LCN samples**
- **ZyGEM™ extraction may result in allelic dropout at larger loci**

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Questions?

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