Laser Capture Microdissection and in-situ hybridisation.

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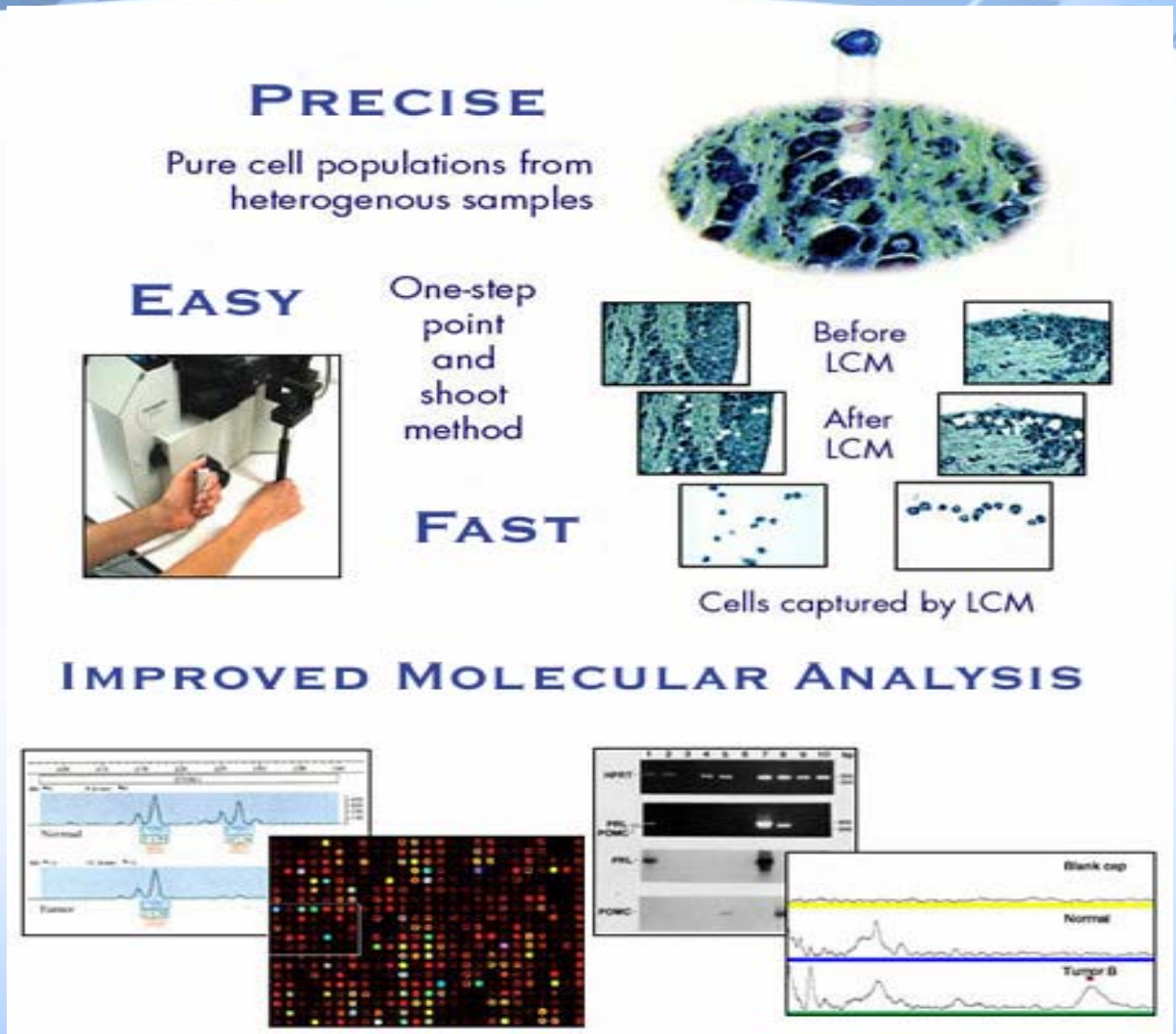
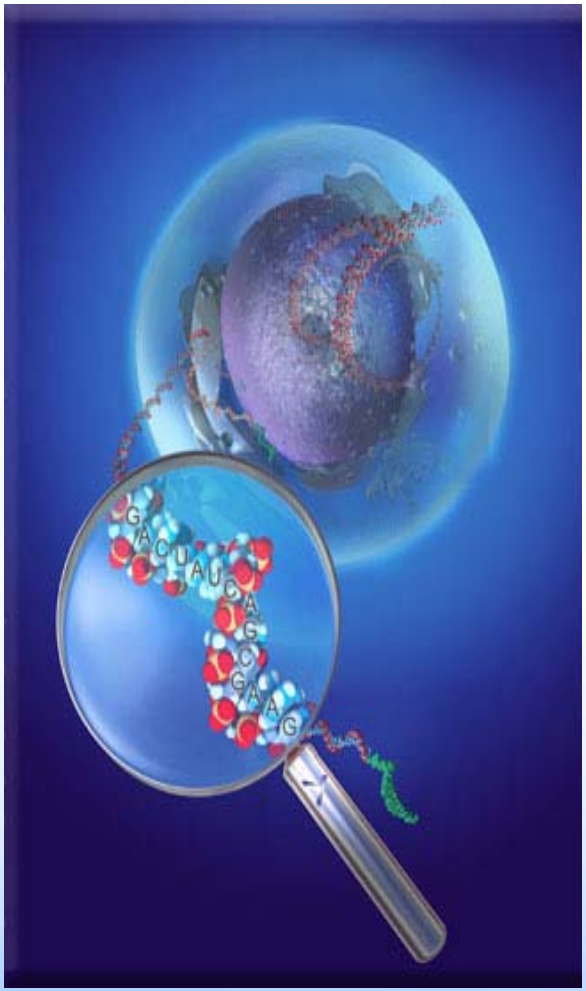
LASER CAPTURE MICRODISSECTION.



Types of LCM

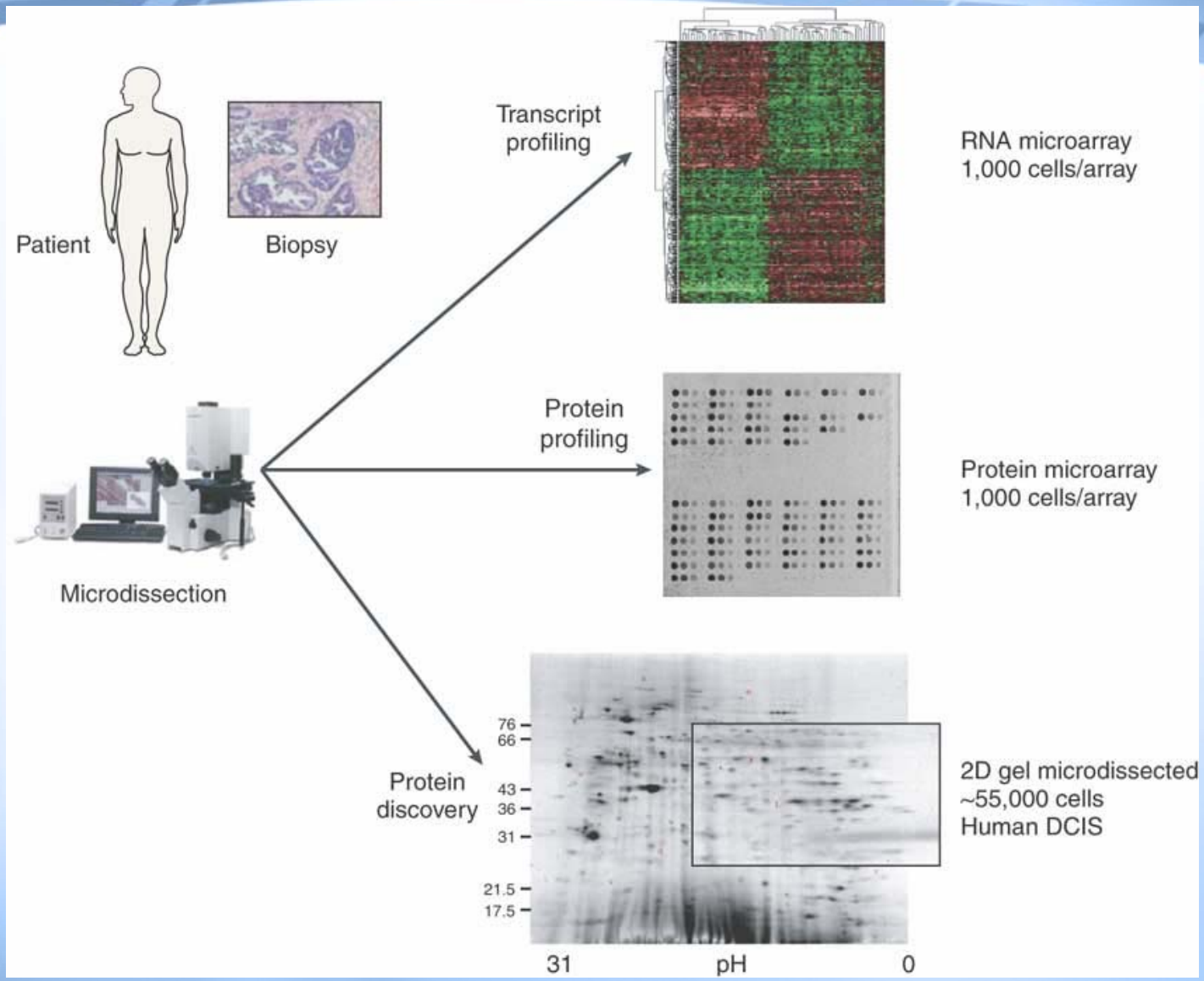
- Infrared (cold) Laser (PixCell –Arcturus)
- Ultraviolet (cutting) Laser (P.A.L.M. & *Leica*)

Why Laser Microdissect?



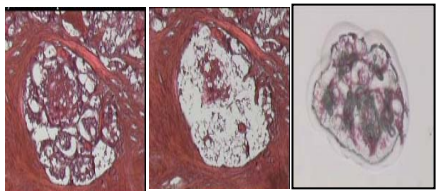
- With more sophisticated techniques generating data on expression and functional mutation analysis, there is an increasing need for absolutely pure cell populations to be entered into any testing protocol.

LCM allows downstream versatility

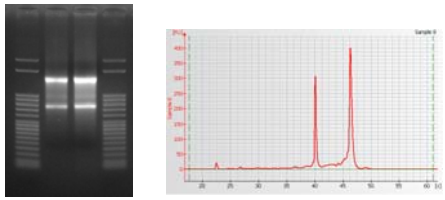


Sample Workflow

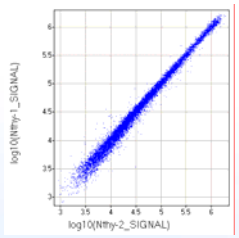
LCM of snap frozen tissue



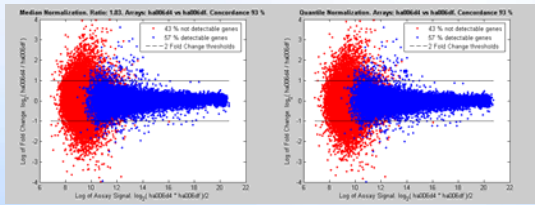
RNA purification



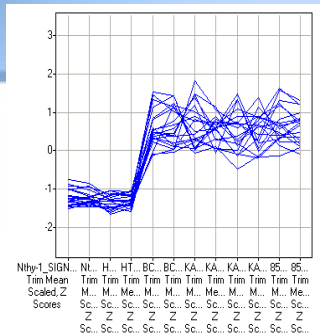
Array validation



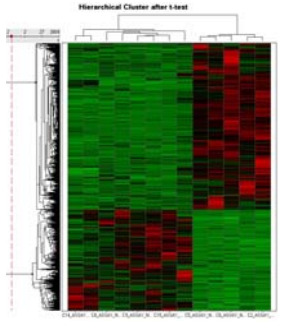
Normalisation of Data



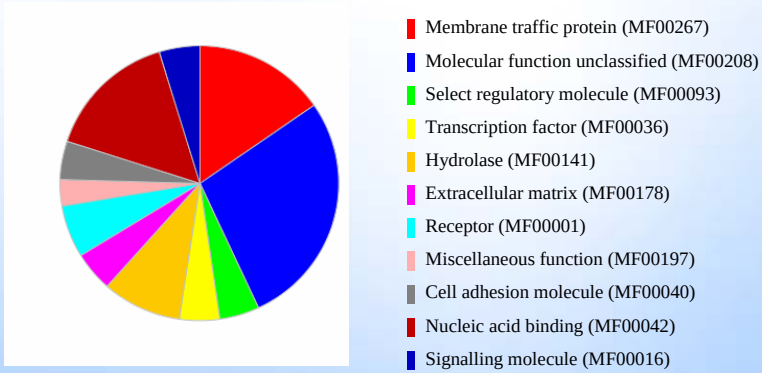
Identification of biomarker candidate genes For PTC



Use of hierarchical clustering to develop training sets



Correlation of detected genes with protein function



Laser Capture Microdissection

- Dramatically increases the sensitivity and accuracy of downstream molecular assays

- Starting Sample:
  - Homogeneous cell types
  - Multicellular structures isolated from whole tissue or cytology samples.

PixCell 11 Apparatus

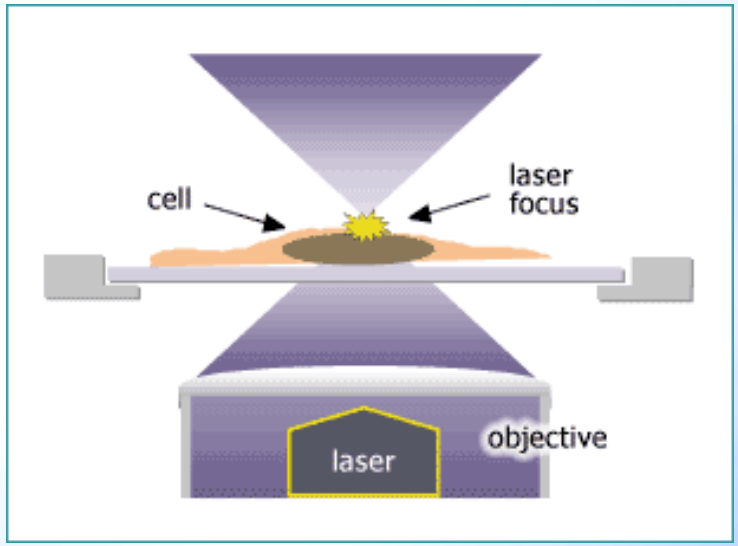
Laser Capture Microdissection

LCM is a means of isolating a pure population of cells from the tissue microenvironment.

Laser impulses directed at cells of interest activate a polymer film that expands and impregnates the cells which are then lifted off the slide. This technique enables isolation of pure cell populations from a heterogeneous tissue section.

PixCell II –Infrared laser

- LCM utilises a low-power infrared laser to melt a special thermoplastic film over the cell(s) of interest.
- CapSure™ HS or CapSure™ Macro Caps that are coated with this thermoplastic film are placed on the tissue section or cytology sample.
- The PixCell® II LCM instrument is then used to direct the laser through the cap to melt the film onto the cells of interest.
- When the cap is lifted, the selected cells remain attached and are captured for further analysis.



Laser Capture Microdissection

- The microdissected cells represent the *in vivo* state of the cells at the time of sample procurement.

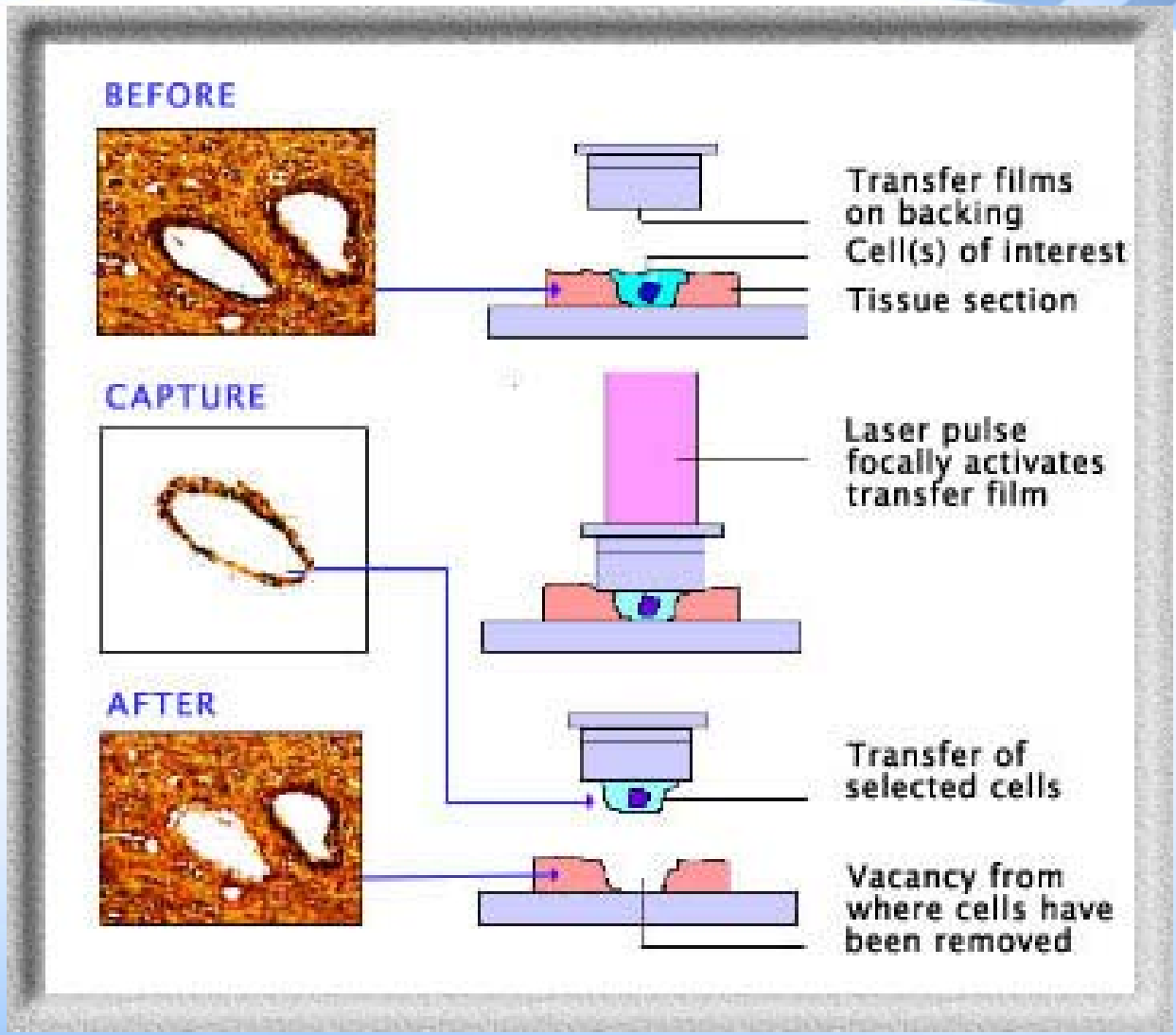
- Normal, pre-malignant and malignant cell populations may be analyzed in relation to their microenvironment.

Applications of LCM

The microdissected cells may be used for DNA, RNA or protein analysis.

- DNA sequencing
- DNA fingerprinting
- cDNA microarrays
- Quantitative RT-PCR
- Protein microarrays
- Western blots

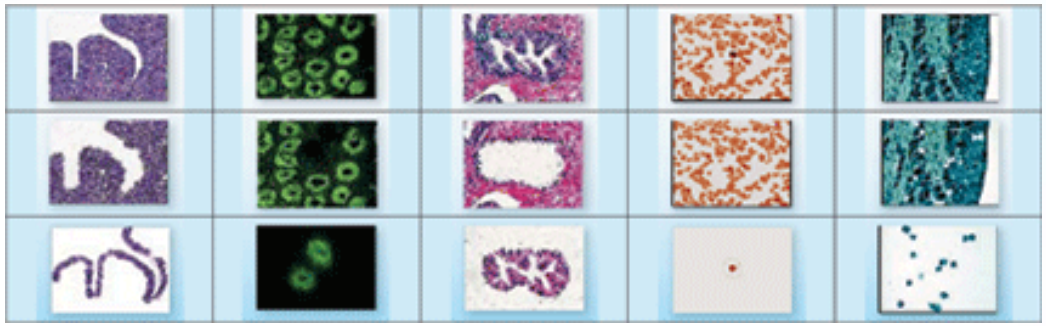
Arcturus LCM: Basic Principle



Before LCM

After LCM

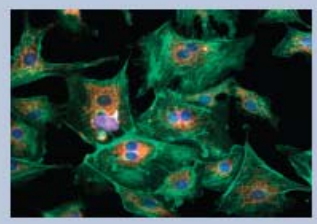
Captured Cells



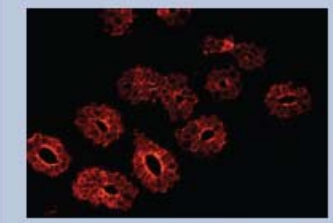
Frozen Rat Uterus H&E -stained 2-cell Surface Epithelium	Frozen Human Jejunum Cy2-Ab to Cytokeratin Epithelial Cells	Paraffin-embedded H&E-stained Prostate Gland	Human Blood Giemsa-stained White Blood Cell	Frozen Rat Nissl-stained Small Neuron

Specimen Requirements

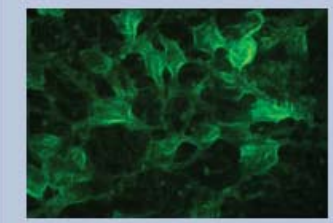
- Frozen or paraffin embedded tissue sections, cytospin preps; 2-15um thickness (5-8um optimal)
- Plain, uncharged uncoated glass microscope slides
- Compatible with most staining techniques:
  - Hematoxylin & Eosin (H&E)
  - Diff Quick
  - Toluene blue
  - Fluorescent dyes
  - In situ hybridization



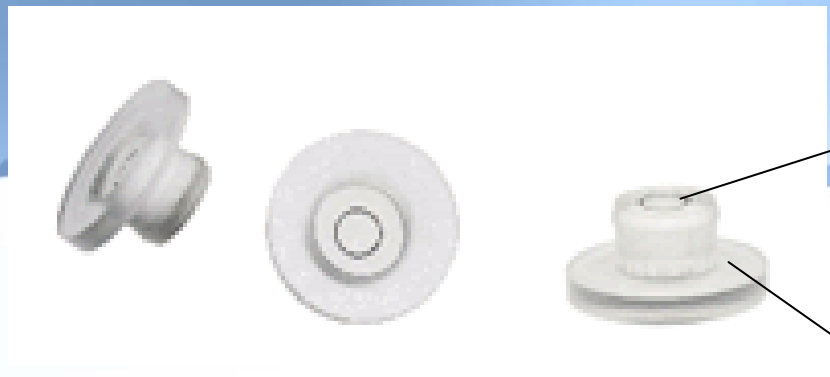
Triple labeled bovine pulmonary artery endothelial (BPAE) cells. Mitochondria = red, F-actin=green and Nuclei= blue. Visualized simultaneously using an Omega triple band dichroic filter.



Human Breast Carcinoma, anti-cytokeratin/Cy3



Cultured HELA cells exposed to BCECF, a cytoplasmic pH indicator.



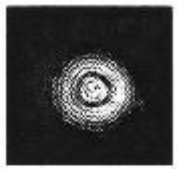
LCM Cap

- Plastic support with thermolabile polymer film
- Polymer = polyethylene vinyl acetate
- Dye impregnated in polymer

  Dye absorbs energy of laser

  Dye provides a means of visualizing each laser pulse

Laser spot sizes



30 um

30um spot = 5 cells

15um

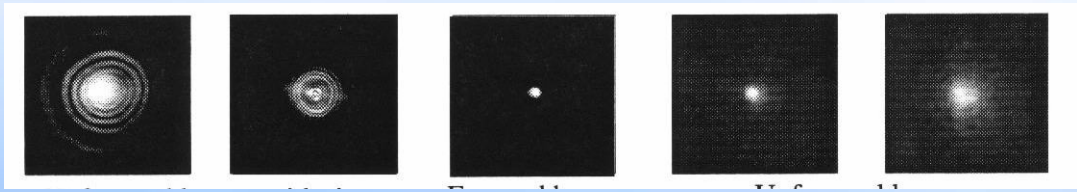
15um spot = 3 cells

7.5 um

7.5 um spot = 1 cell

Laser Focusing

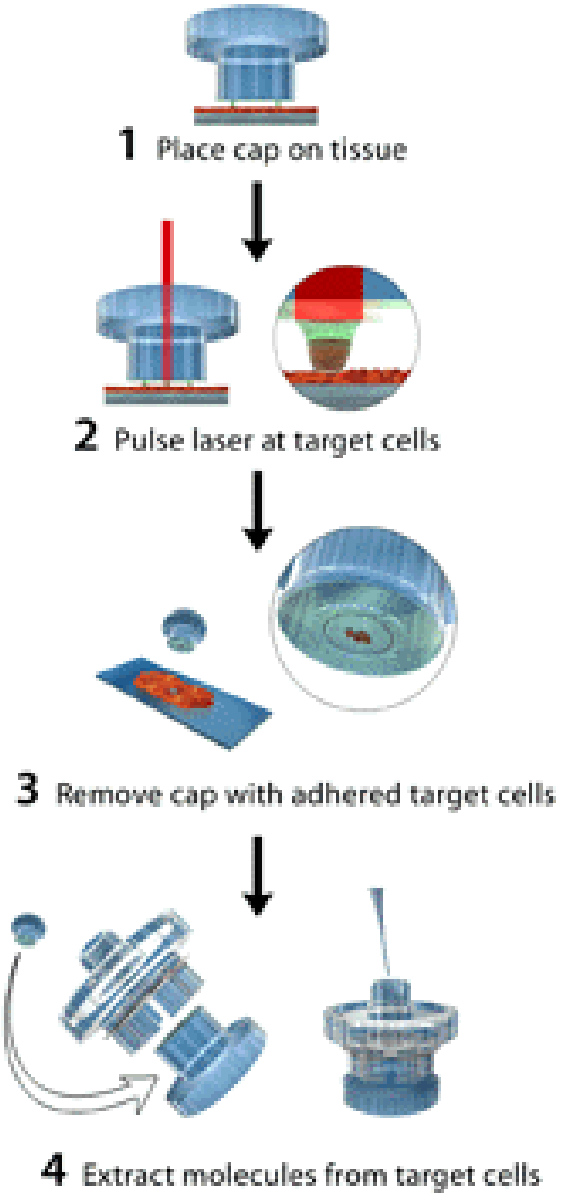
Unfocused Focused Unfocused



Steps involved in LCM

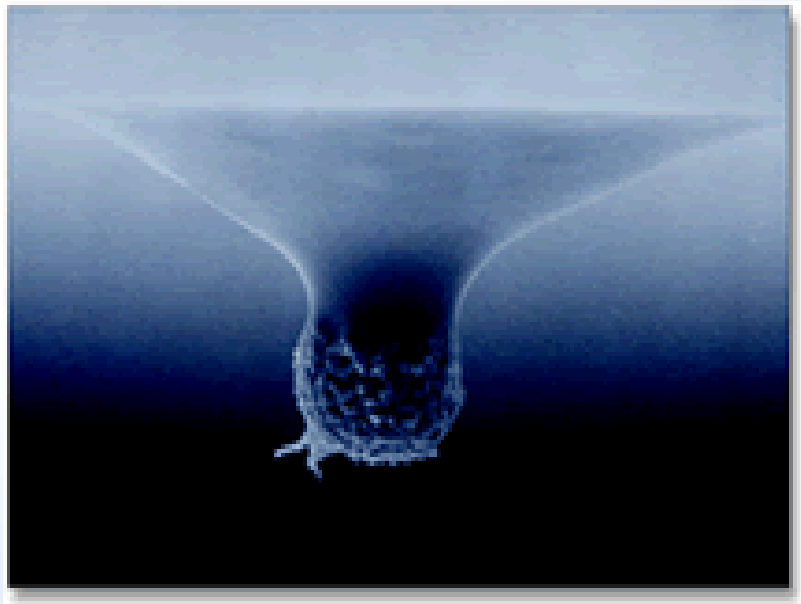
1. Load caps.
2. Joystick perpendicular to the table.
3. Position slide on microscope using fingers.
4. When tissue area is designated, apply vacuum.
5. Pick up a cap with swing arm and place on top of tissue.
6. Turn on the power to LCM and laser control box.
7. Enable laser. Set spot size to 7.5um and 10x objective.
8. Focus laser.
9. Test fire laser. Observe wetting of polymer for adequate contact with glass.
10. Locate cells to be dissected, fire laser.
11. Lift cap from tissue with swing arm.
12. Place cap in microcentrifuge tube. Proceed with downstream analysis.

The Laser Capture Microdissection Process

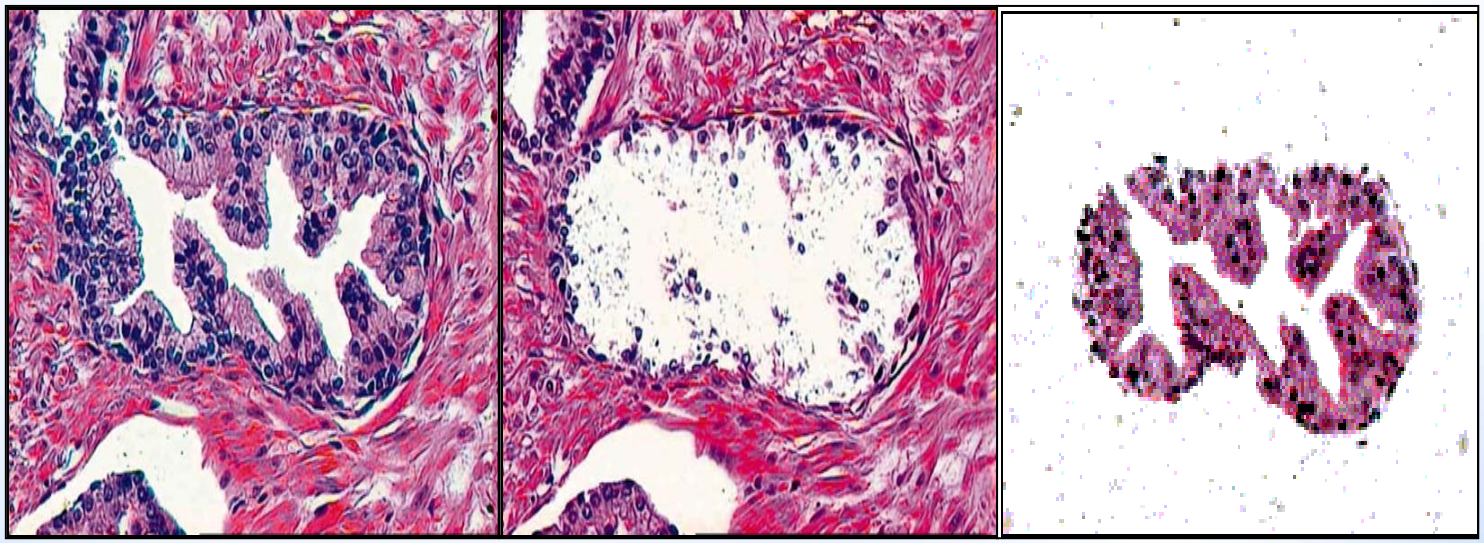


Single cell capture

- Single cell capture using LCM. Scanning electron micrograph of a CapSure LCM Cap with a single cell laser-captured onto the thermoplastic film.



LCM on prostate tissue



Before **After** **Dissected Material**

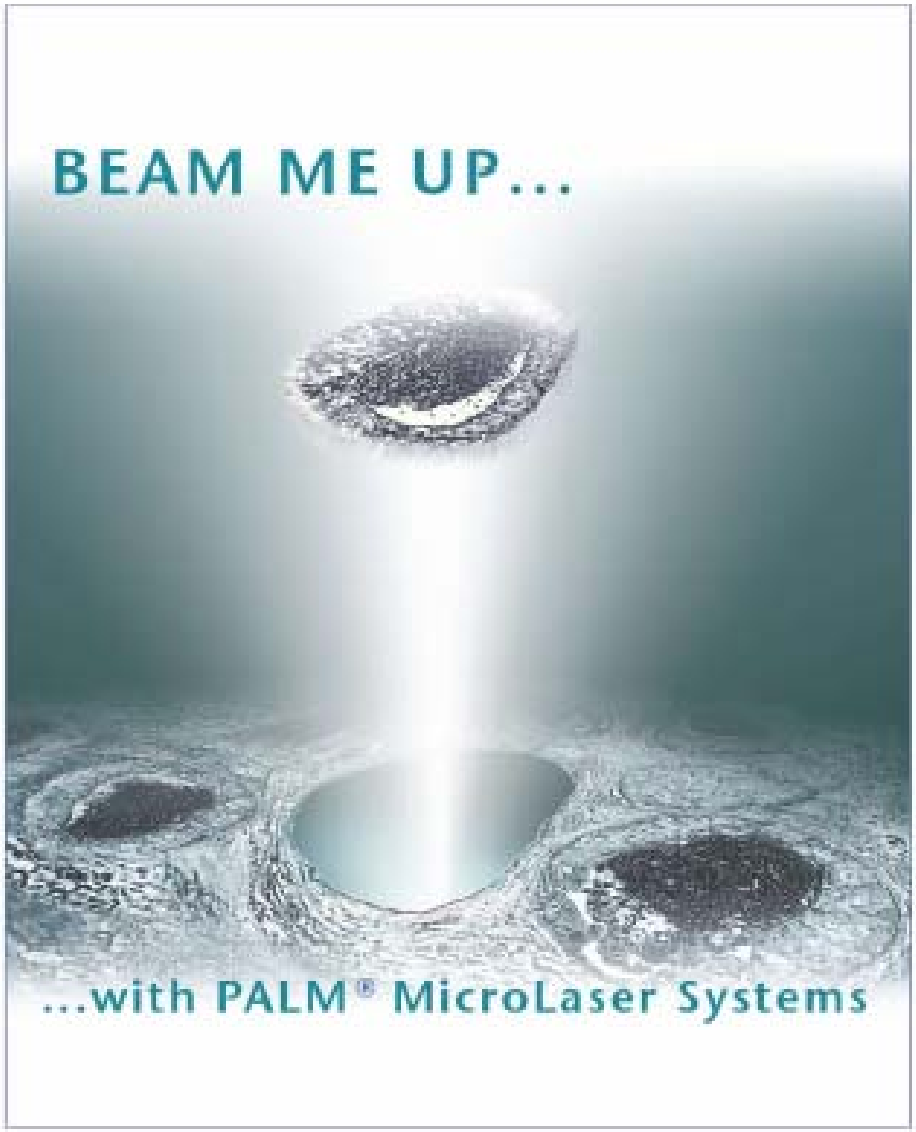
P.A.L.M. SYSTEM – UV laser

P.A.L.M. Microbeam

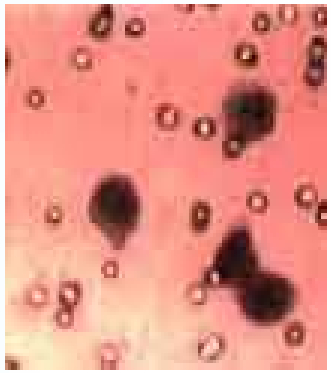
- Sole manufacturer of MicroBeam Micromanipulation Systems using LPC.
- Only the forces of light are used to dissect selected specimen from various sources and to eject them directly into a standard microfuge tube.
- These entirely 'non-contact' techniques eliminate the danger of contamination or infection.
- The PALM® MicroBeam is the "all in one" state of the art laser system for non-contact microsurgery, microdissection and microinjection.

What is LPC?

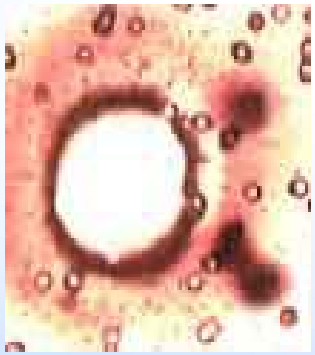
- LPC (Laser Pressure Catapulting) is PALM's patented technique for non-contact procurement of single cells, cell areas, chromosomes or parts of chromosomes ‘without any danger of contamination’.


BEAM ME UP...
...with PALM® MicroLaser Systems

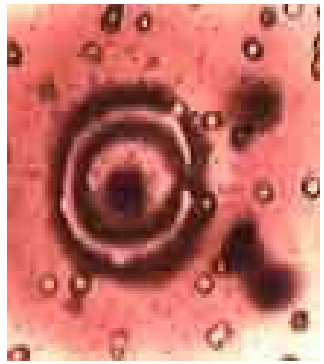
P.A.L.M. LPC



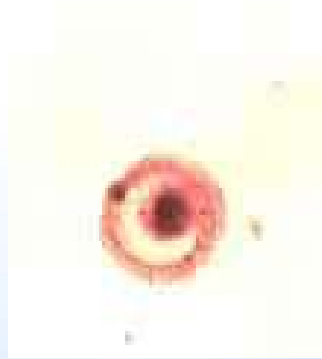
H&E Stained Cells on Filter



And catapulted by LPC



The membrane is precisely cut



Collected in the microfuge lid

LEICA System

Leica System

- The Leica AS LMD is based on the new fully automated microscope Leica DM LA
- Non-contact, stress-free preparation
- Transport by gravity
- No mechanical or physical forces are needed
- UV laser cutting. Laser movement by optics, not by mechanics for highest cutting precision
- Automated multiwell positioning
- Integrated sample checking function
- The smallest cutting area with the 100x objective has a diameter of 4-5µm with an accuracy of +/- 0,6 µm

Laser Capture Microdissection (LCM) and Laser Cutting Instrument

?best of both worlds:

UV Laser Cutting and IR Laser Capture

Microgenomics™

Arcturus platform

- **UV Laser Cutting** provides speed and precision.
  - Ideal for non-soft tissues and capturing large numbers of cells.
- **LCM** is a gentle technique, maximizing biomolecule integrity
  - Ideal for single cells or small number of cells.

Laser Capture Microdissection (LCM) and Laser Cutting Instrument

- Accurately Track Your Samples and Maintain Sample Integrity
- Use UV and IR Lasers for Precision and Performance
- Fast, Easy, and Efficient Three-Step Operation
- Ablate the Material You Don’t Need, Before and After Capture

In situ hybridisation

In Situ Hybridisation

- Method of localising, either
  - mRNA within the cytoplasm or
  - DNA within the chromosomes of the nucleus,
- by hybridising the sequence of interest to a complimentary strand of a nucleotide probe.
- Threshold levels of detection 10-20 copies of mRNA or DNA per cell.

In situ hybridisation

- unique set of problems as the sequence to be detected will be:
  - at a lower concentration,
  - be masked because of associated protein, or protected within a cell or cellular structure.
- in order to probe the tissue or cells of interest – need to increase
  - the permeability of the cell and
  - the visibility of the nucleotide sequence to the probe without destroying the structural integrity of the cell or tissue.

Other considerations

- the type of probe to use,
- how best to label it, to give the best level of resolution with the highest level of stringency.

Types of material

- Formalin fixed, paraffin embedded,
- Snap frozen, and embedded in a special support medium for cryosectioning.
- Cells in suspension
  - cytospun onto glass slides and fixed with methanol
- Preparation of metaphase chromosomal spreads,
  - normally fixed with a mixture of methanol and acetic acid.

Steps involved in ISH

- Preparation of slides and fixation of material
- Pretreatments of material on slides,
  e.g., permeabilisation of cells and tissues
- Denaturation of *in situ* target DNA
  (not necessary for mRNA target)
- Preparation of probe
- *In situ* hybridization
- Posthybridization washes
- Immunocytochemistry
- Microscopy

In-situ hybridisation

ds DNA

ss DNA

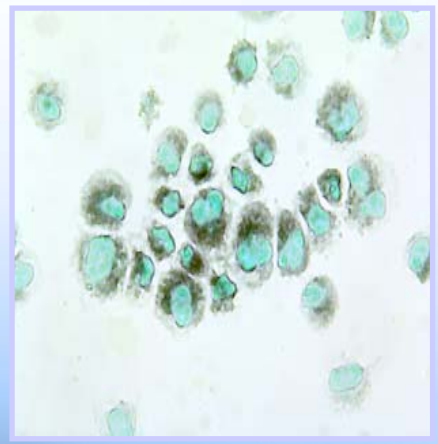
A T G C C T A

probe

T A C G G A T

= (Dig, Biotin etc.)

Detection using antibodies (1,3 or 5-step detection)

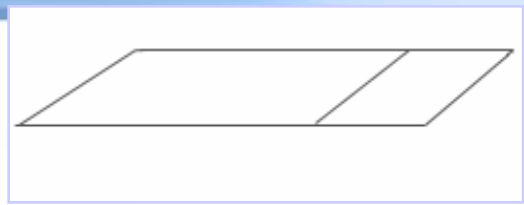


Variations of ISH

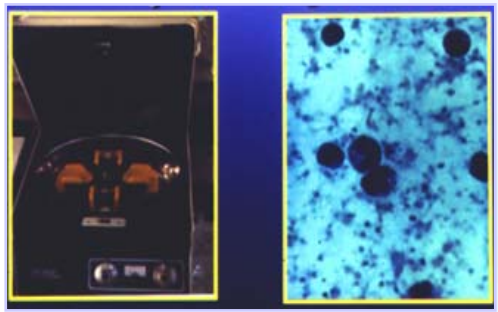
- In-cell PCR
- Multicolour FISH
- Comparative genome hybridisation genome analysis (CGH)
- M-FISH: 24 colour chromosome karotyping

In-cell PCR

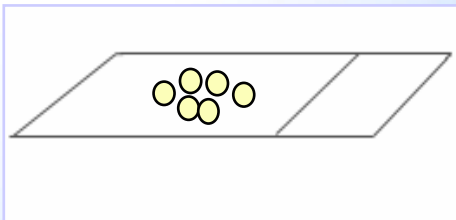
Slide preparation



Cytocentrifugation

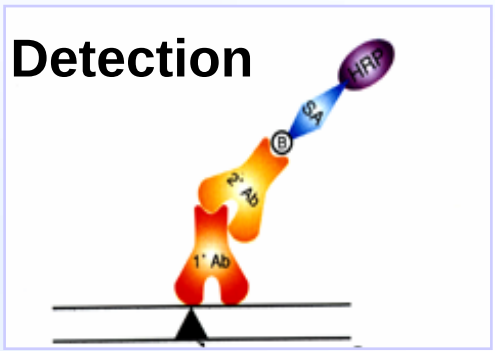


Fixation and Permeabilisation



RT PCR and Hybridisation

Detection



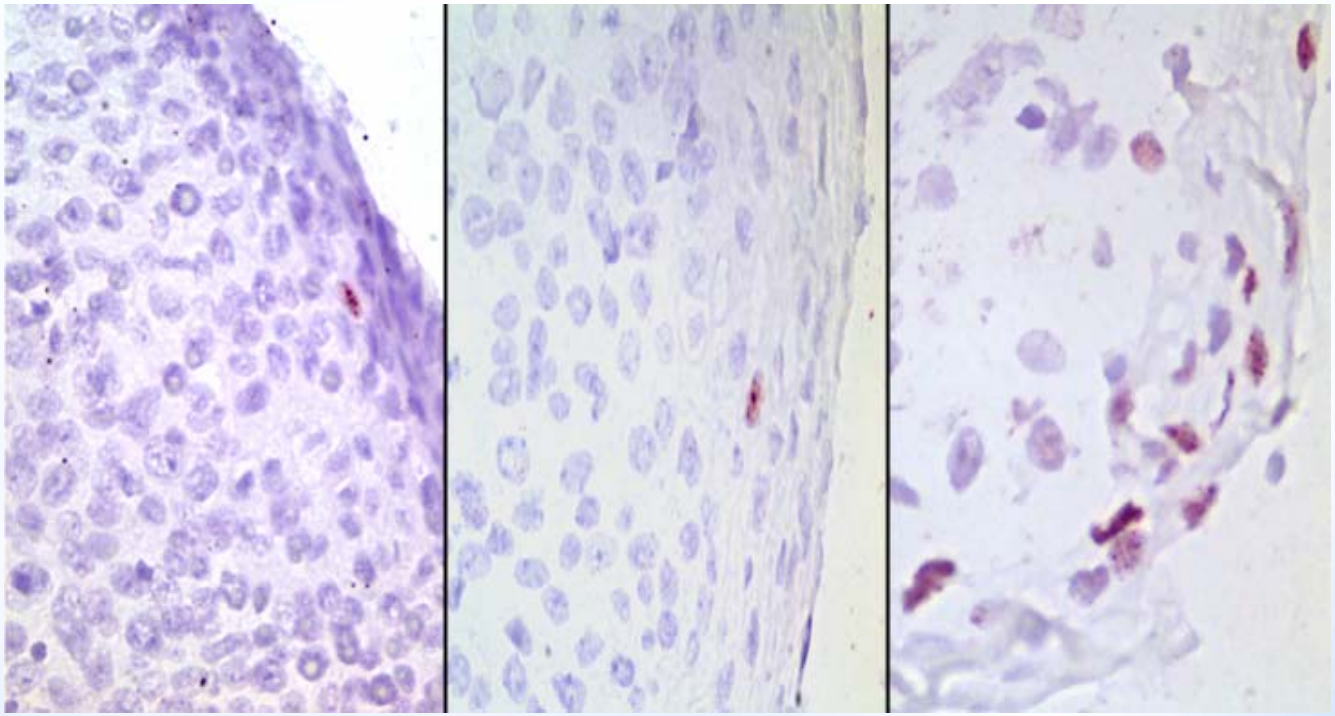
Definitions and Terminology

- DNA *in situ* PCR (direct PCR ISH).
  - PCR amplification of cellular DNA sequences in tissue specimens using either a labelled primer or labelled deoxy-nucleotide(dUTP)
  - The labelled product is then detected using standard detection techniques as for conventional ISH or immunocytochemistry.

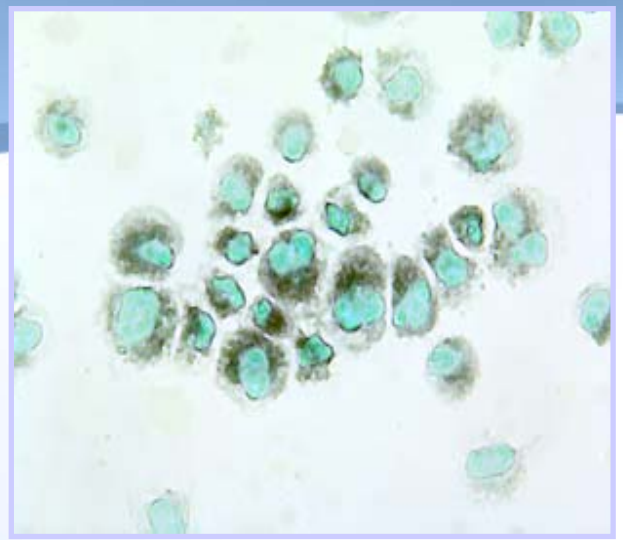
PCR *in situ* Hybridisation (indirect PCR ISH)

- PCR amplification of cellular DNA sequences in tissue specimens followed by ISH detection of the amplified product using a labelled internal or genomic probe.
- The labels can be isotopic (^{32}P, ^{35}S) or non-isotopic (biotin,digoxygenin, or fluorescein).
- Mostly non-isotopic labels are used.

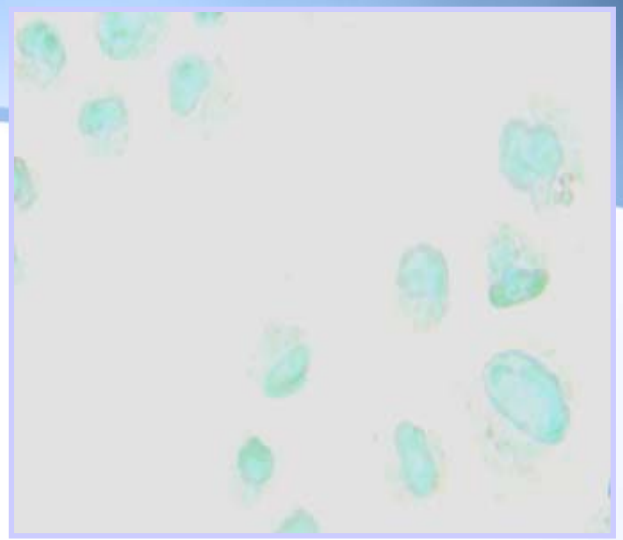
In-situ hybridisation and in-cell PCR



Detection of RNA by in-cell PCR

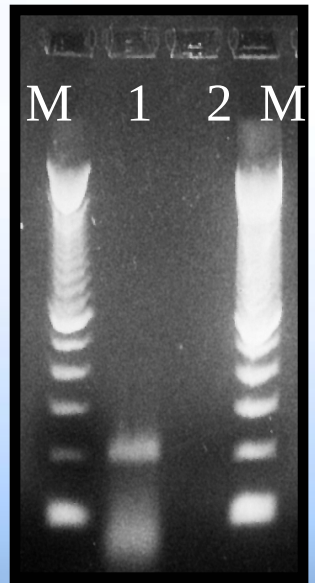


positive

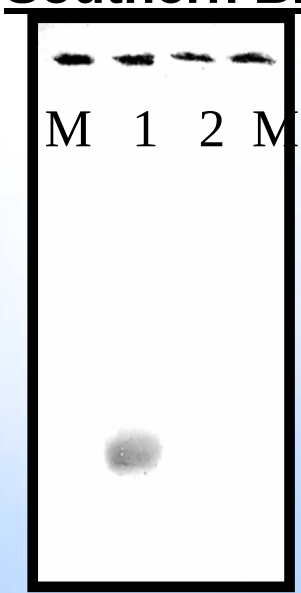


negative control

Supernatant

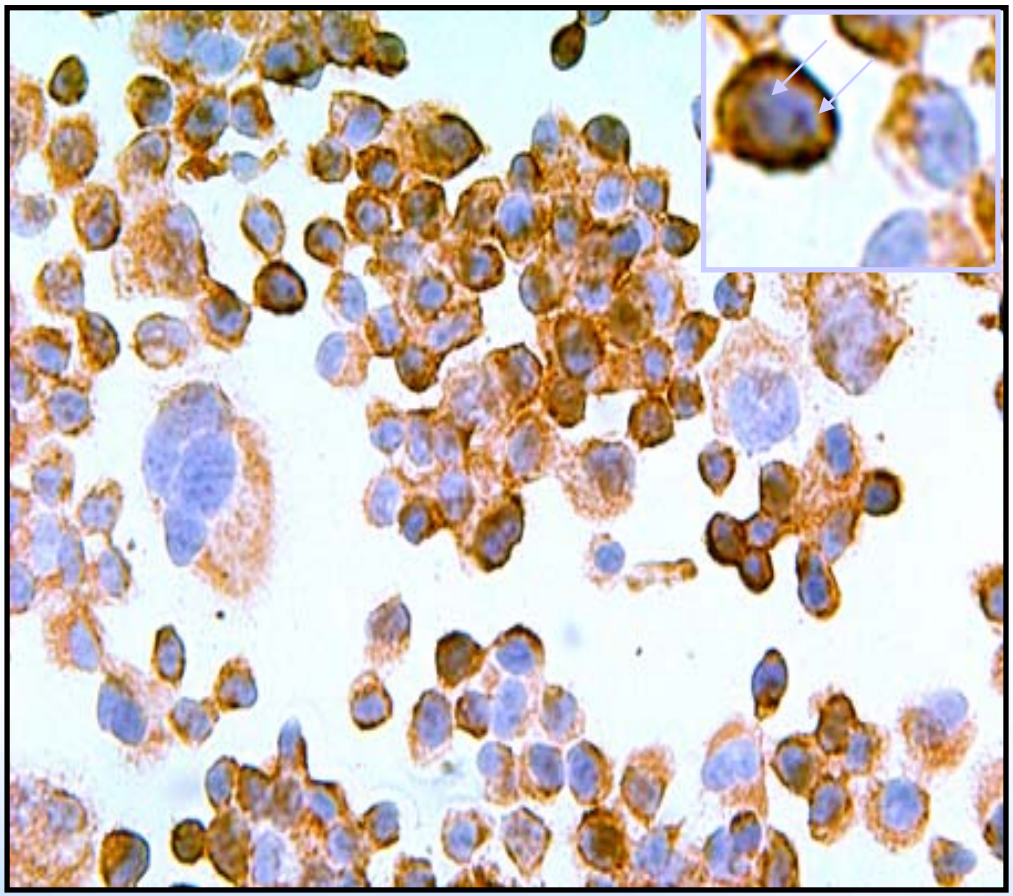


Southern Blot



30 cycles

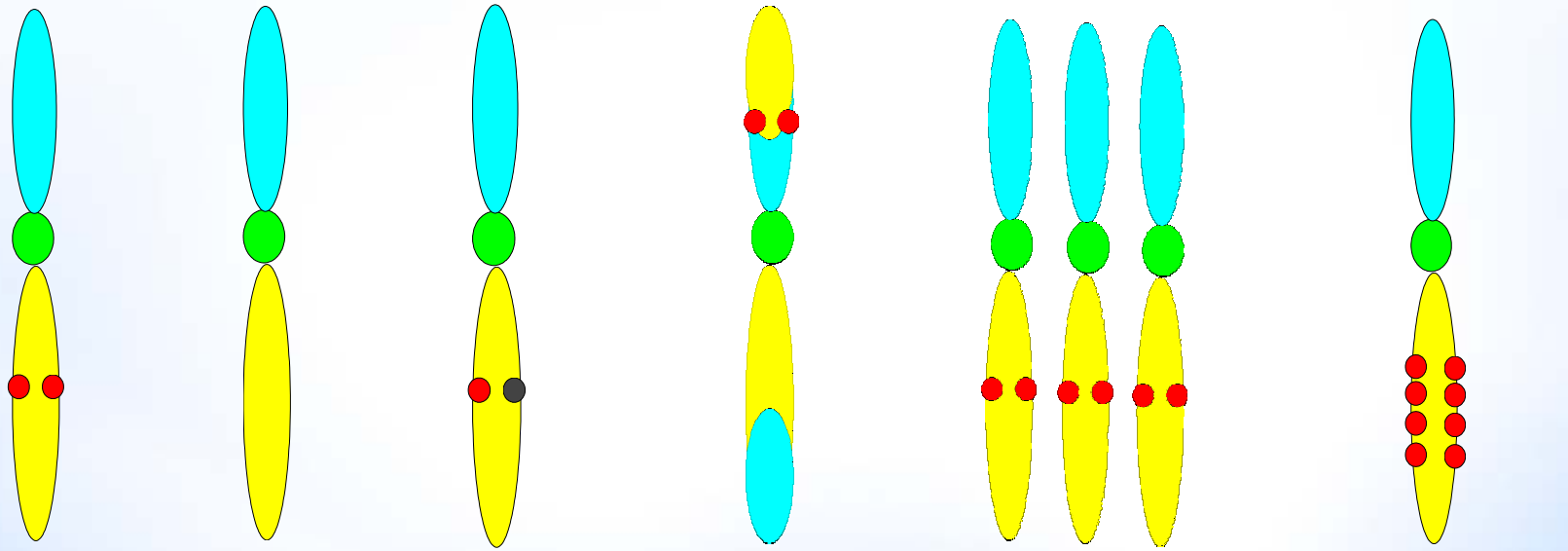
Simultaneous detection of DNA and RNA in-cells



Fluorescent In-situ hybridisation

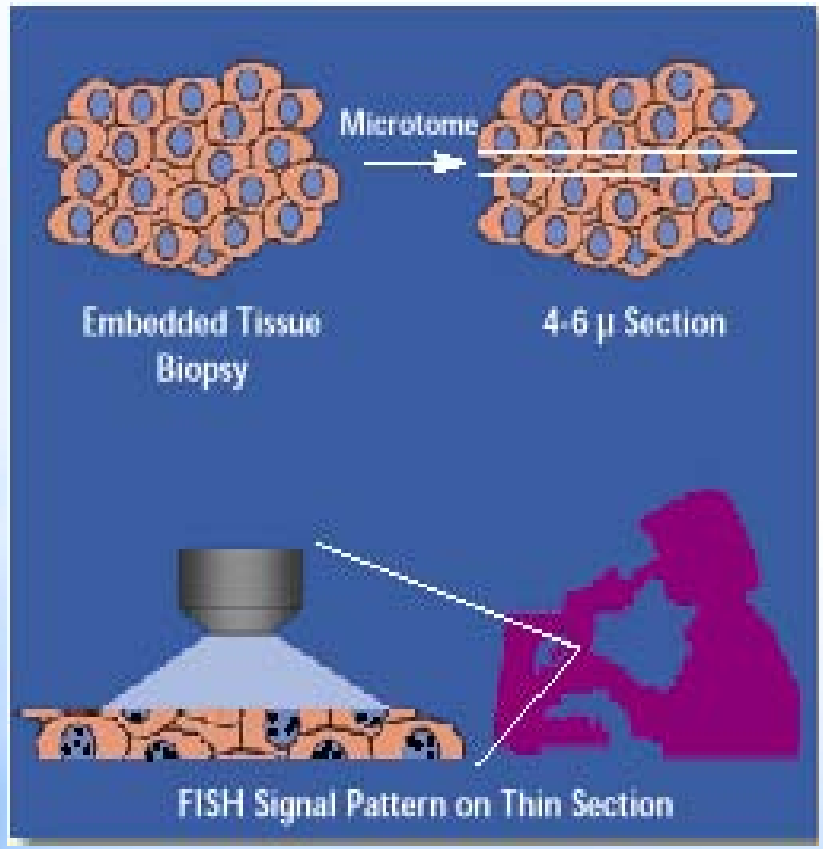
- Detect disease specific translocations.
- Chromosome numeration.
- Detect loss/gain in specific gene targets.
- Cytogenetics.
- Comparitive genome hybridisation.

Cancer – a genetic disease



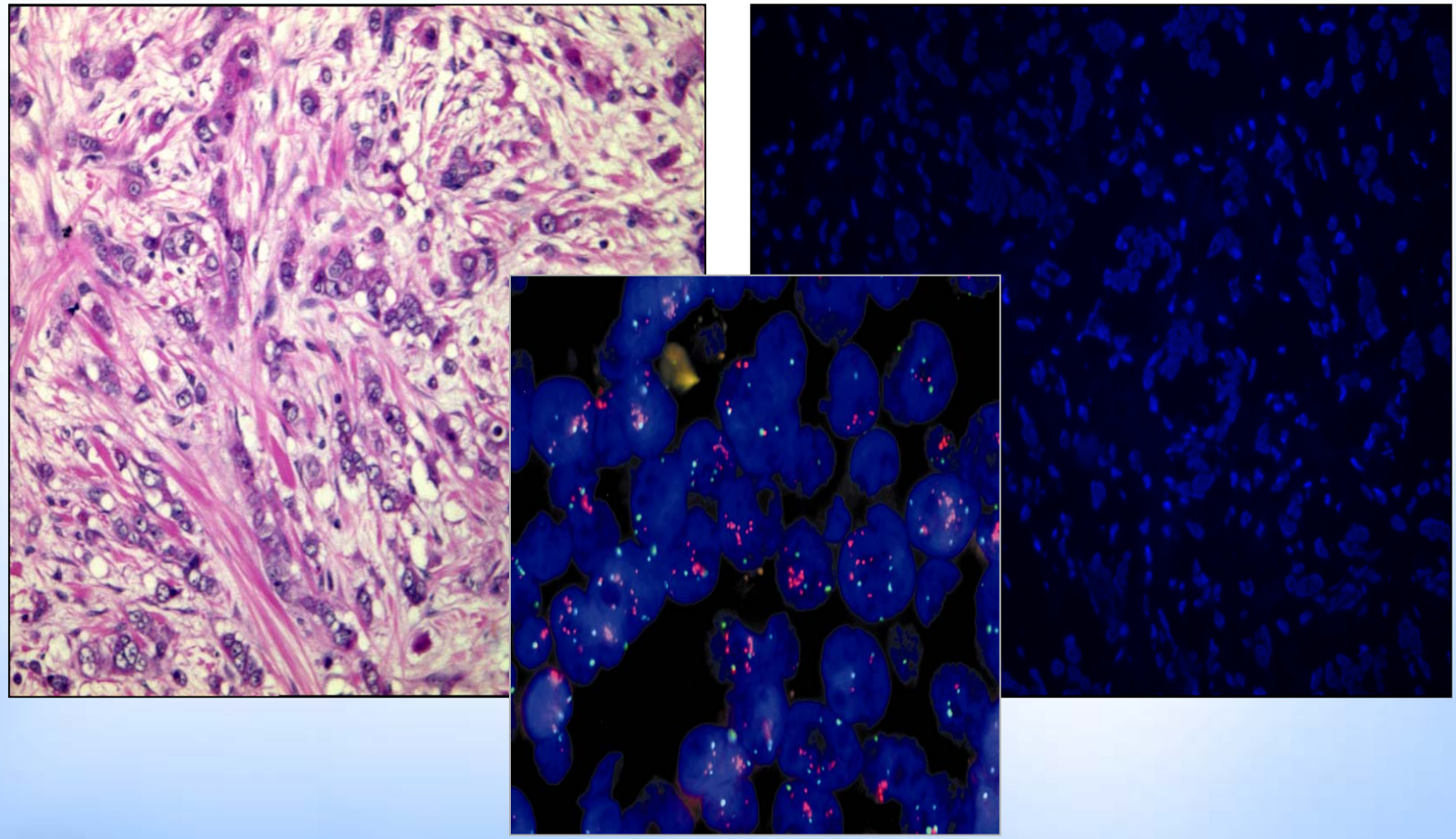
Normal **Deletion** **Mutation** **Rearrangement** **Replication** **Amplification**

FISH utilizing tissue sections:

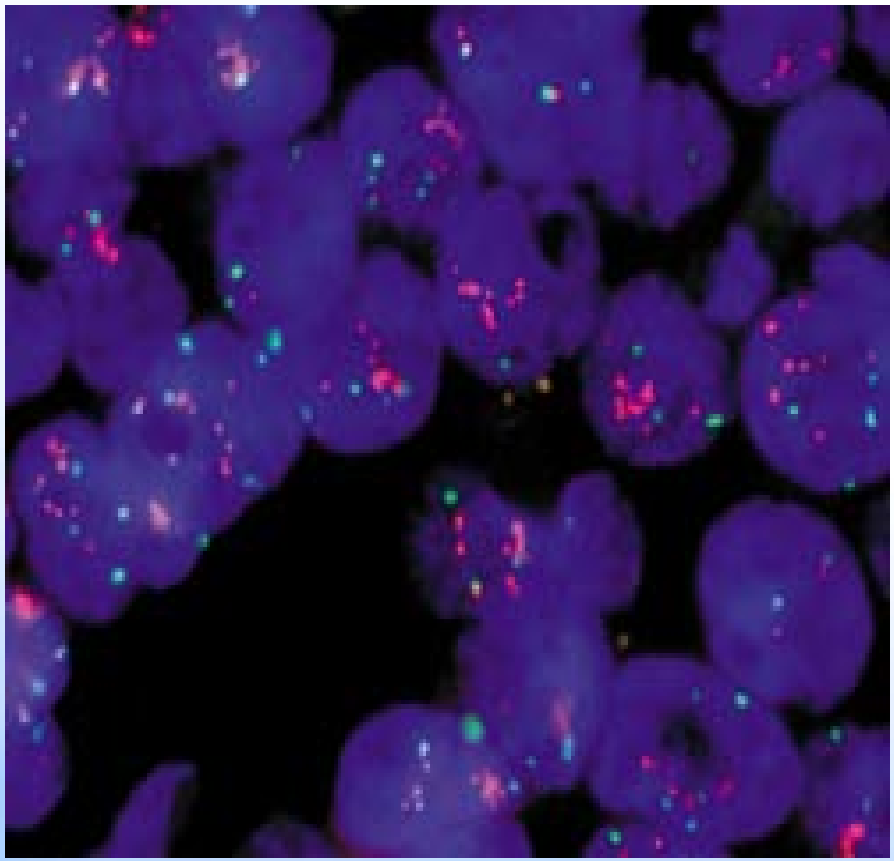


- the tissue is sectioned,
- then the probe is applied,
- then it is examined under the fluorescent microscope.

Benefits of FISH – comparable H&E



Examples: Breast Carcinoma – Her 2 Neu



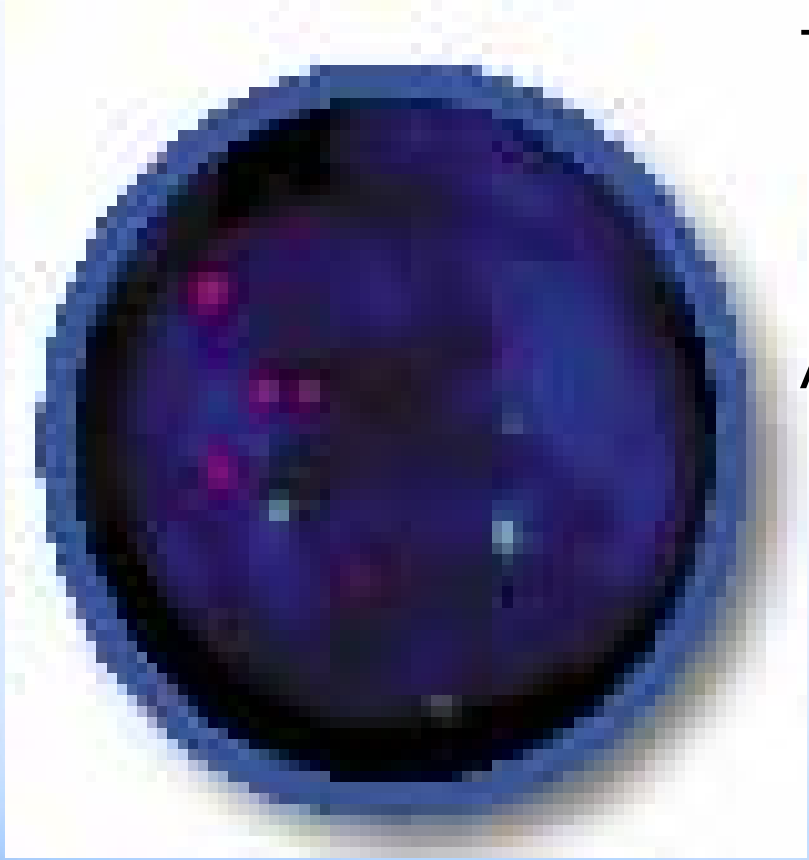
- Breast tissue hybridized with LSI HER-2 SpectrumOrange and CEP17 SpectrumGreen, demonstrating amplification of the HER-2 gene.

Non-amplified Her-2 neu

Two green signals indicate the presence of two copies of chromosome 17. Two orange signals indicate the presence of

two copies of HER-2 genes in the same nucleus. The ratio of HER-2 to CEP 17 is 1.0, which is non-amplified.

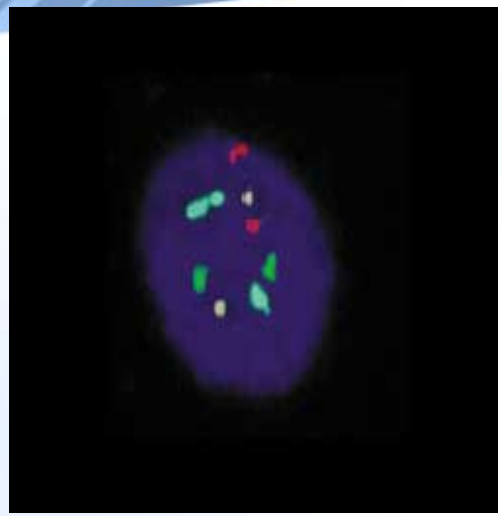
Amplified Signal

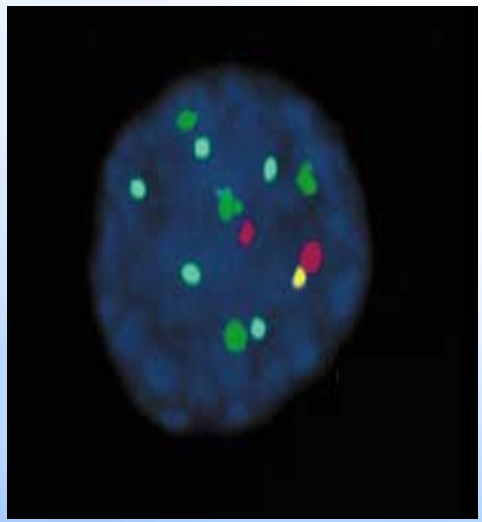


Three green signals indicate the presence of three copies of chromosome 17.

Approximately 13 orange signals indicate the presence of 13 copies of HER-2 genes in the same nucleus. The ratio of HER-2 to CEP 17 is approximately 4,which is amplified.

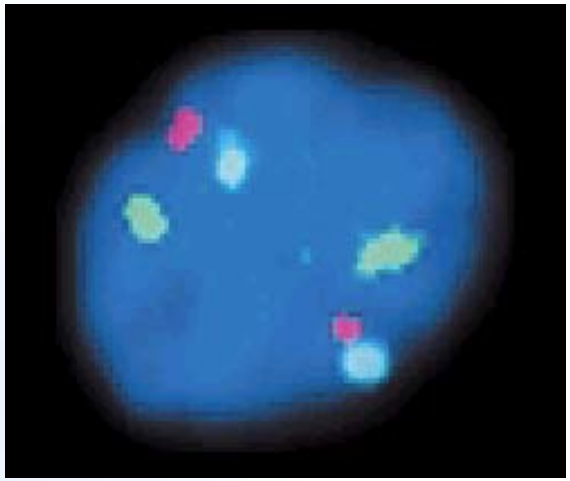
Other Examples

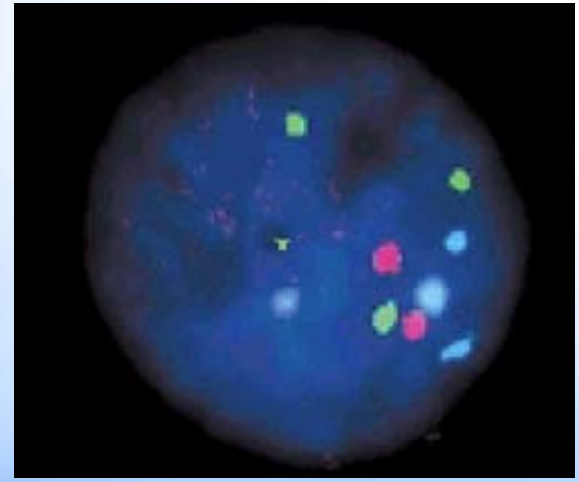




- Bladder Cancer
- UroVysion Multi-colour FISH probe
  - CEP 3 (Spectrum Red)
  - CEP 7 (Spectrum Green)
  - CEP 17 (Spectrum Aqua)
  - LSI 9p21 (Spectrum Gold)
- Interphase cell analysis
- Aneuploidy detection of Chromosomes 3,7,17 and deletion of 9p21
- Cells recovered from bladder washings – fixed on microscope slides.

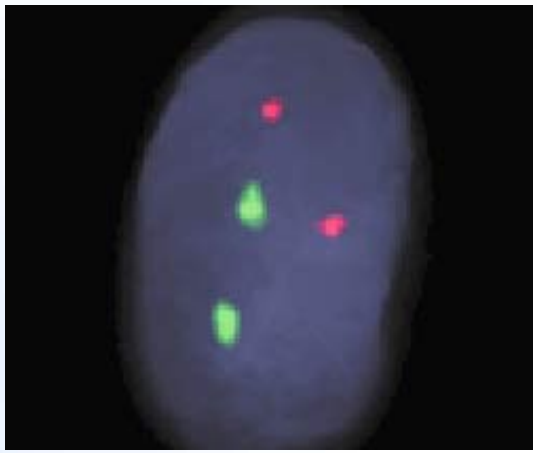
Other Examples

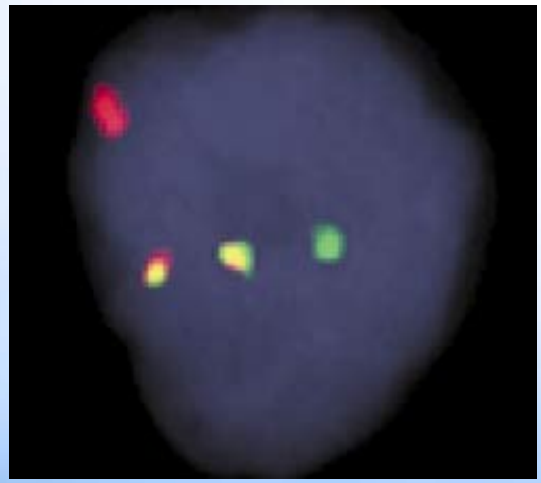




- Prostate Cancer
- Many genetic alterations documented
- Gain of band 8q24
- Loss of heterozygosity 8p21-22
- Genes mapped to 8p – early stage tumorigenesis.
- C-myc over-representation – tumour progression
- DNA FISH probes for:
  - 8p22 (LSI LPL)
  - c-myc (LSI c-myc)
  - Centromere Chromosome 8 (CEP 8)

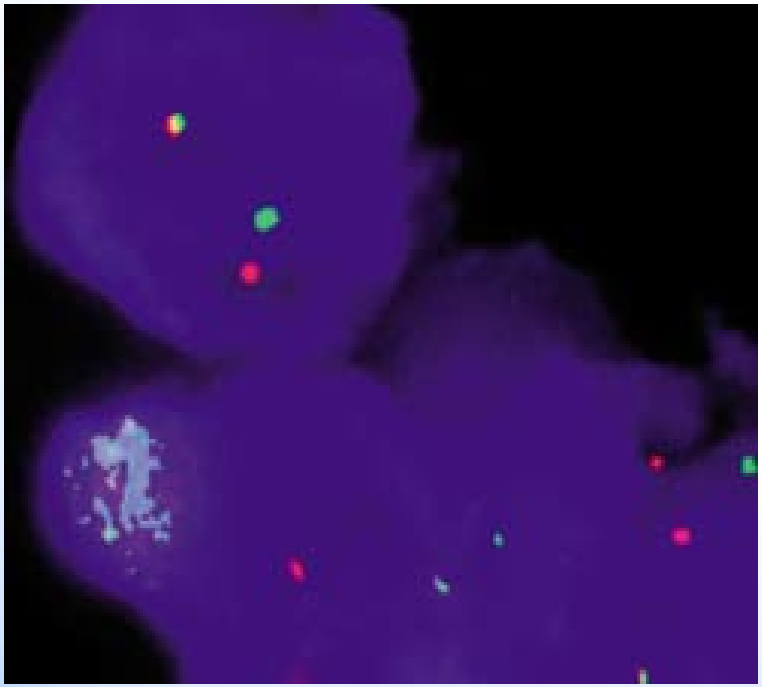
t(14/18) - follicular lymphoma





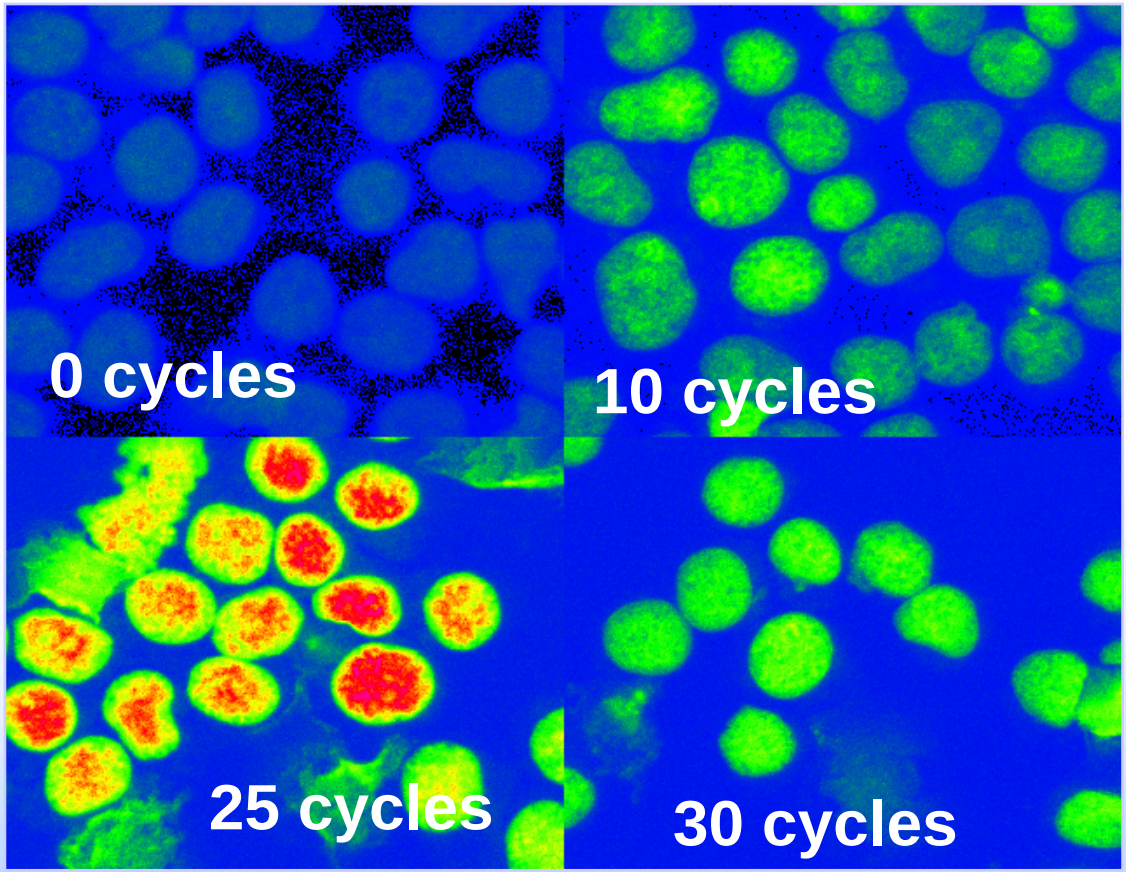
- LSI IGH/BCL2 Dual Color, Dual Fusion Translocation Probe hybridized to normal interphase nucleus.
- LSI IGH/BCL2 Dual Color, Dual Fusion Translocation Probe hybridized to a nucleus from a follicular lymphoma specimen.

t(2:5) - non-Hodgkins lymphoma



- 5-10% NHL
- also associated with anaplastic large cell lymphoma
- LSI ALK probe - 2 colours flanking breakpoint
- tissue without translocation > 2 adjacent/fused signals
- with translocation signals are separated.

In-cell Taq Man PCR: HHV 8: BC-3 cell line



IS-RT-TaqMan for *ret*/PTC-1 in TPC-1 cell line