Double Immunostaining Color Substrate Compatibility

PER OXIDASE \ ALK. PHOS.	Vector Red	Vector Blue	Vector Black	BCIP/NBT
Vector VIP	No	Yes	Yes	Yes
DAB	Yes	Yes	No	Yes
NovaRED	No	Yes	No	Yes
AEC	No	Yes	Yes	Yes

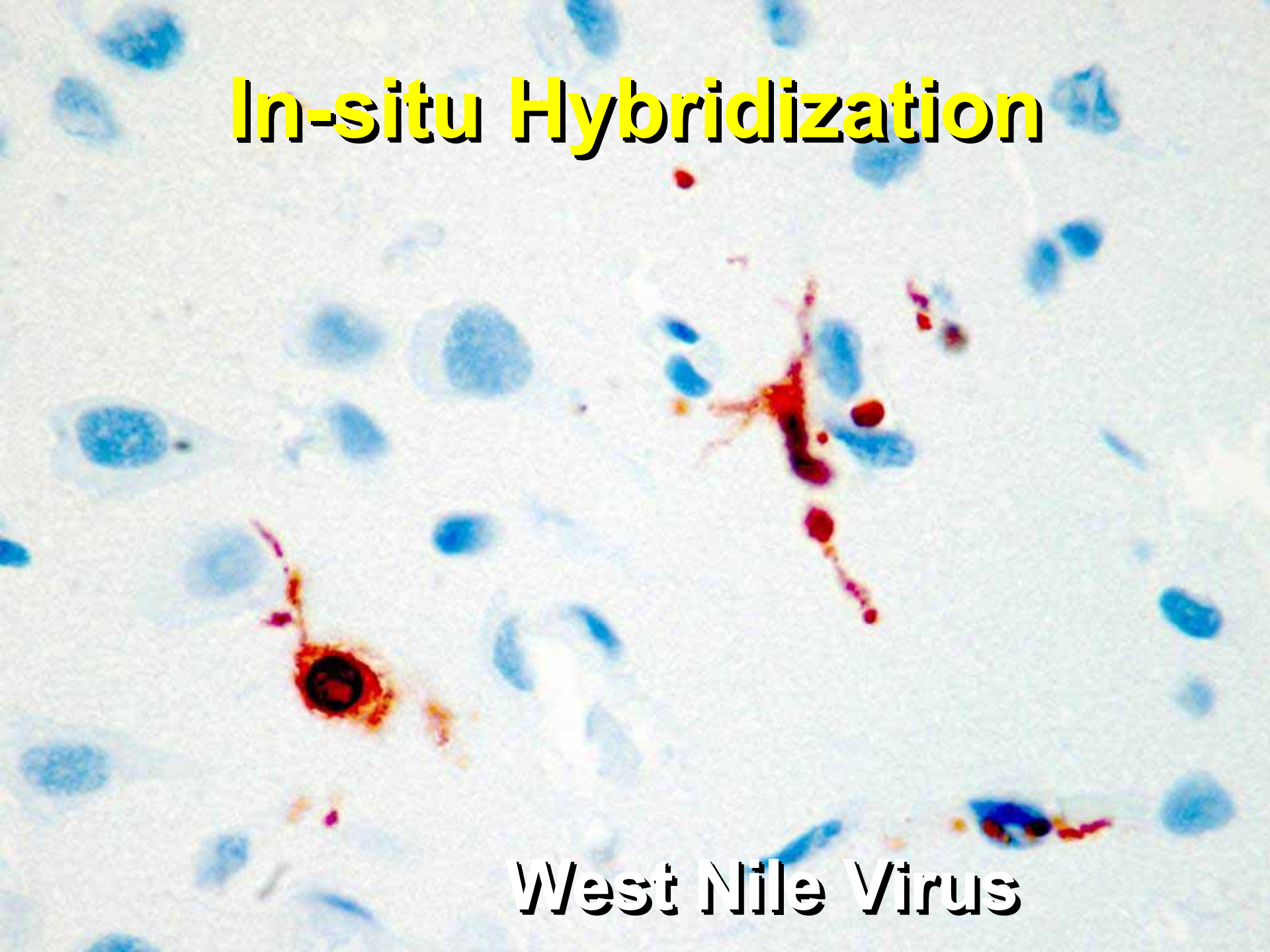
IHC Staining Pattern

- Knowledge of Ag location is essential
- Ag location compatible with biologic activity of Ag
- Some procedures (i.e. fixation, Ag retrieval) modify location (detection) of antigens
- Cytoplasmic (diffuse, paranuclear, perinuclear)
- Nuclear (diffuse, nucleolus)
- Plasma membrane (smooth, intermittent)
- Interstitial
- Mixed (nuclear/cytoplasmic, cytoplasmic/membrane)

Advantages and Disadvantages of IHC

- Routinely available, relatively inexpensive
- Rapid (48 hours), automated systems
- Can study antigens: protein levels and activities differ from those of RNA
- Connects visualized target with microscopic lesion
- Mainly used on fixed tissues:
  - Free of infectious agents so no human health risk
  - Good cell morphology preservation
  - Anchored antigens (no displacement like unfixed)
- Standardization is very difficult (antigen retrieval)
- Difficult to quantitate
- Must have well-trained pathologists and lab personnel (standardization & interpretation)
- Success depends on antibody (mono- vs. polyclonal)

In-situ Hybridization



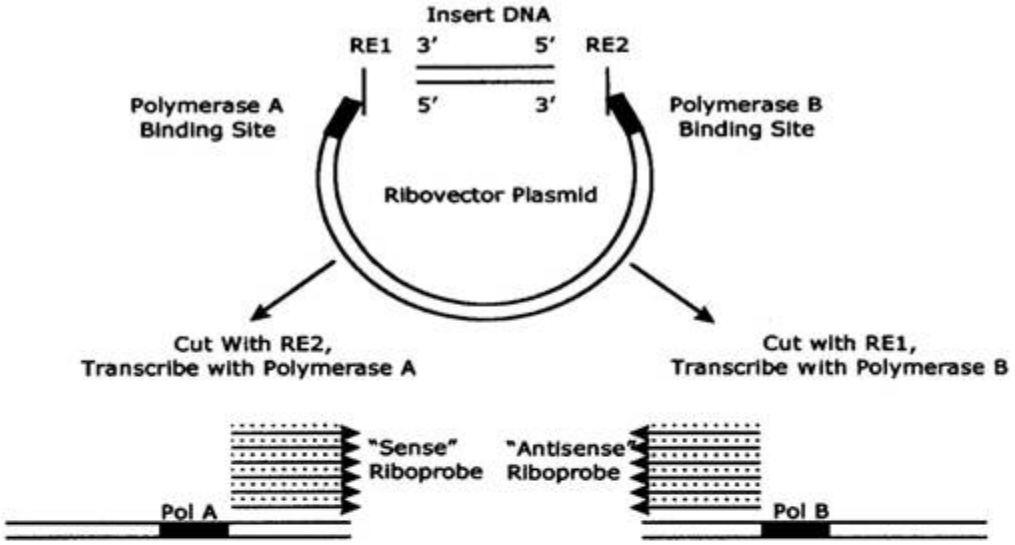
West Nile Virus

In-situ Hybridization

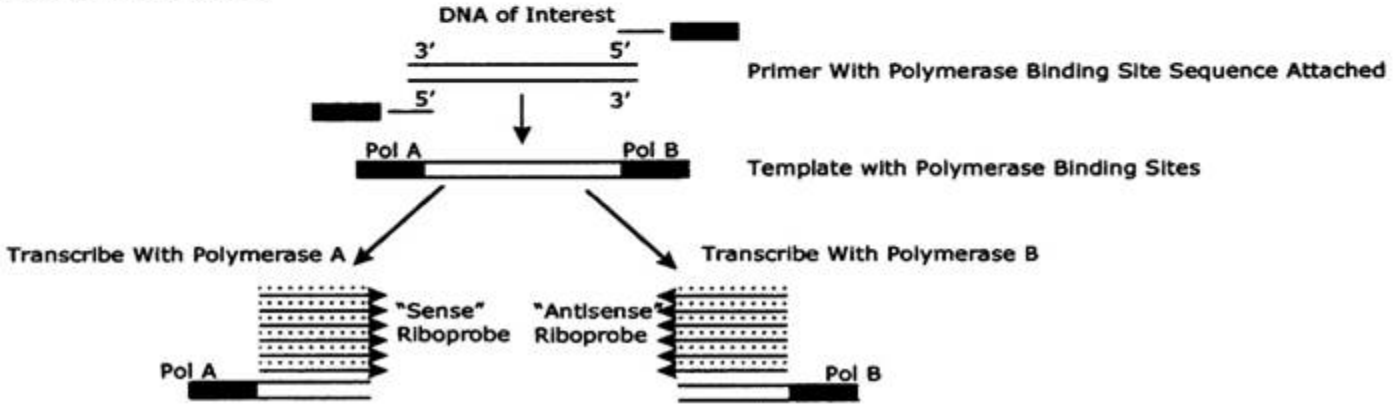
- Detects nucleic acids (DNA, RNA, mRNA)
  - Gene amplification, deletion, chromosome translocation, aneuploidy
- Visualize gene expression patterns
- Can provide spatial and temporal information on understanding gene function
- Sensitivity depends on detection system
  - Radioactive labeling (semiquantitative)
    - [^{35}S]-uridine triphosphatase
    - Less-accurate cellular localization
  - Biotin labeled probes – decreased sensitivity, background
  - Digoxigenin labeled probes
- Higher sensitivity with increased probe conc./time
- Reduce non-specific binding with 1000mM dithiothreitol
- Probes:
  - Purified DNA (labeled with nick translation or random priming)
    - Lower sensitivity, strands bind to each other
  - Oligoprobes through DNA synthesis, lower labeling efficiency
  - Riboprobes through genetic cloning, RNA probes
  - Selection of sense vs. antisense
  - Use HPLC purification of labeled probes

In-situ Hybridization

FROM PLASMID:



FROM PCR-PRODUCED TEMPLATE:

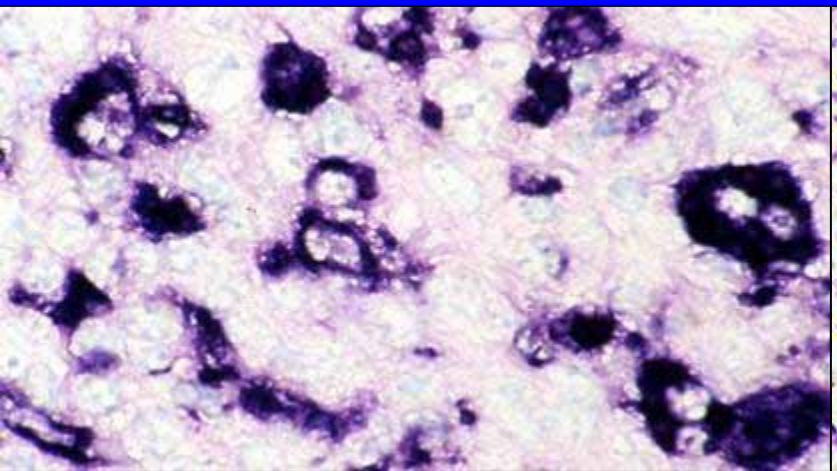


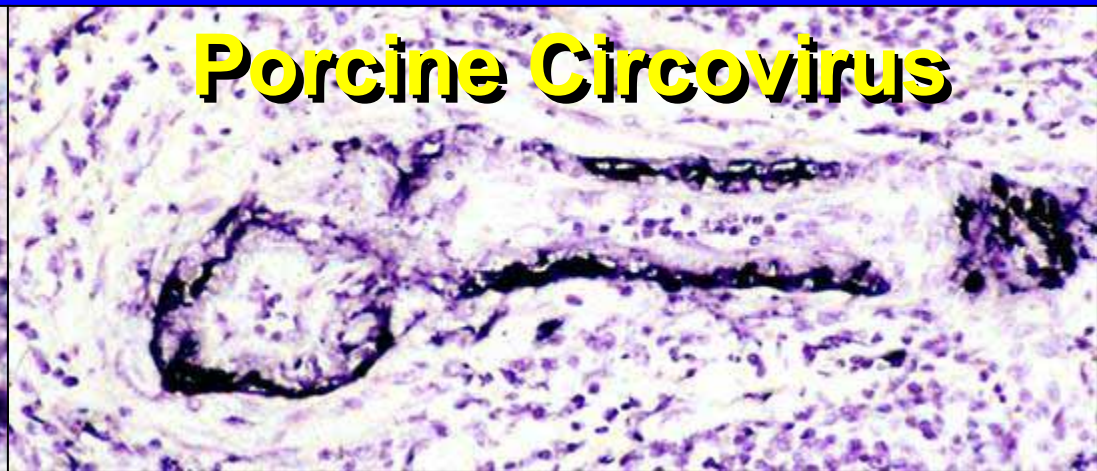
Criteria to Determining the Type of Probe

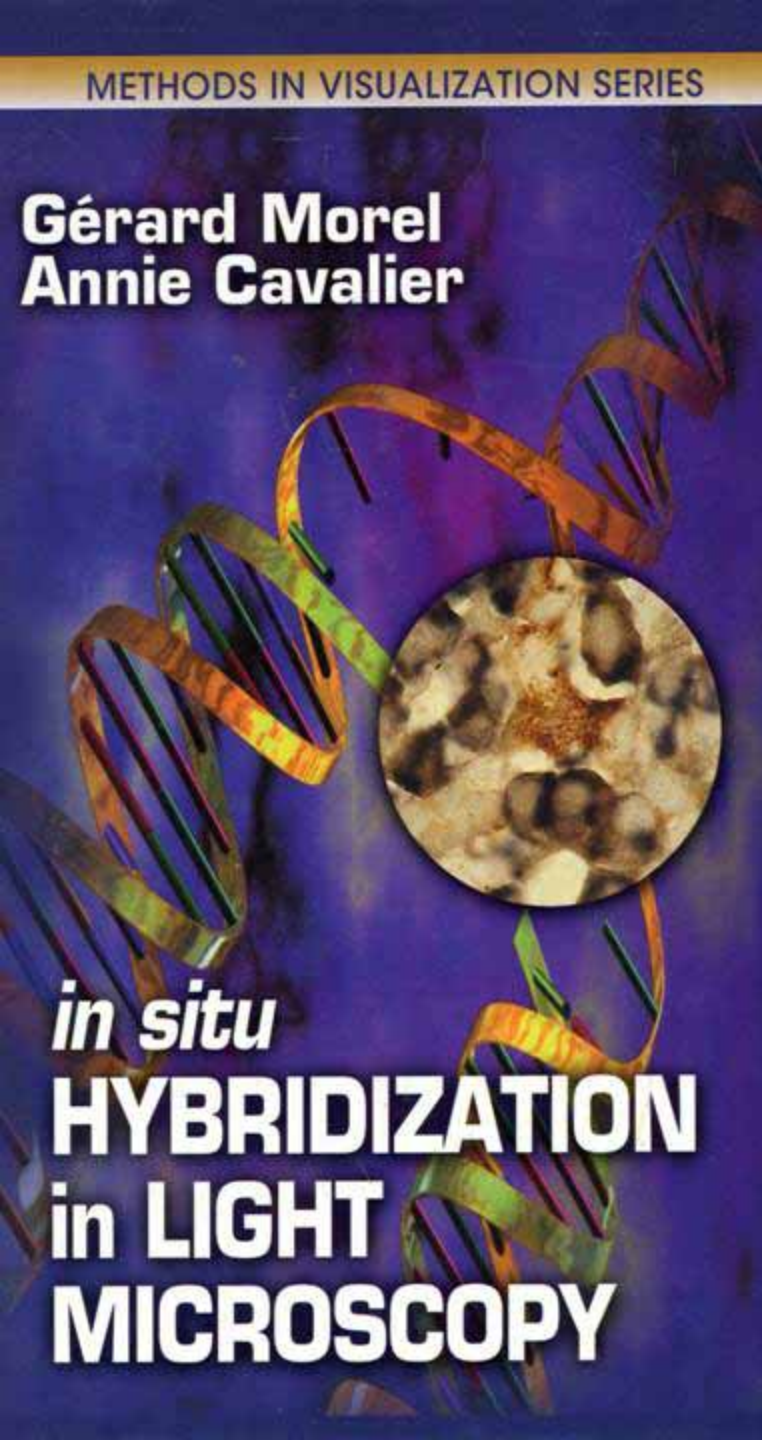
Criteria of Choice	Probes			
	Double Stranded DNA	Single Stranded DNA	Oligonucleotide	RNA
Availability	++	++	+++	+
Storage	+++	+++	+++	+
Stability	+++	+++	+++	+
Specific activity	++	++	++	+++
Manipulation	+++	+++	+++	+
Efficiency	+	++	++	+++
Controls	+	+	++	+++

In-situ Hybridization

- Fixation (preventing detachment)
- Pretreatment (Permeabilization, Deproteinization, Acetylation, Dehydration)
- Denaturation (breaking the double strand)
- Hybridization
- Post-hybridization stringency washes
- Detection system







Criteria		Signal	Back-ground	Sensi-tivity	Speci-ficity
Probe (vs. oligo)	cDNA	►	►	▼	►
	Ribo	▲	▼	▲	►
Homology	<100%	▼	▲	▼	▼
Purification		▲	▼	▲	▲
Fixation	+	▲	▼	▲	▼
	+++	▼	▲	▼	
Deproteinization		▲	▼	▲	
Acetylation		▼	▼	▼	
Prehybdridization			▼		▲
Probe conc.	<<	▼	▼	▼	▲
	>>		▲		▼
Hyb.-Temp.	+		▲	▲	▼
	++	▲		►	►
	+++	▼		▼	▲
Hyb.-Time	< 3h	▼	▼	▼	▲
	3h	▲		►	▲
	> 3h	►	▲	▲	▼
Washing	time	▼	▼	▼	▼
Detection		▲	▲	▲	▼

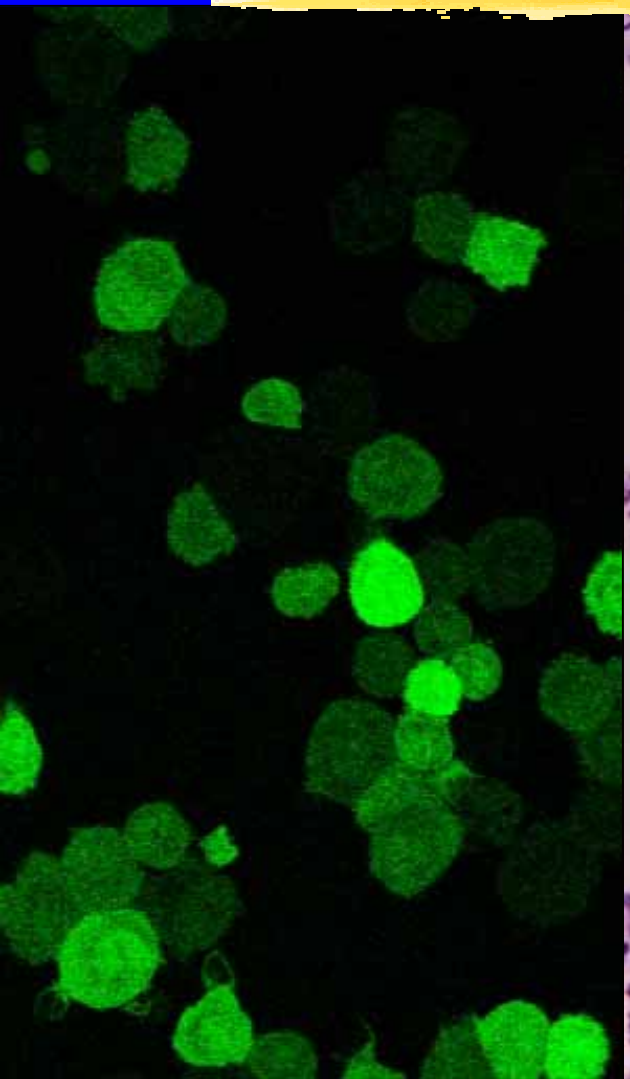
Advantages and Disadvantages of ISH

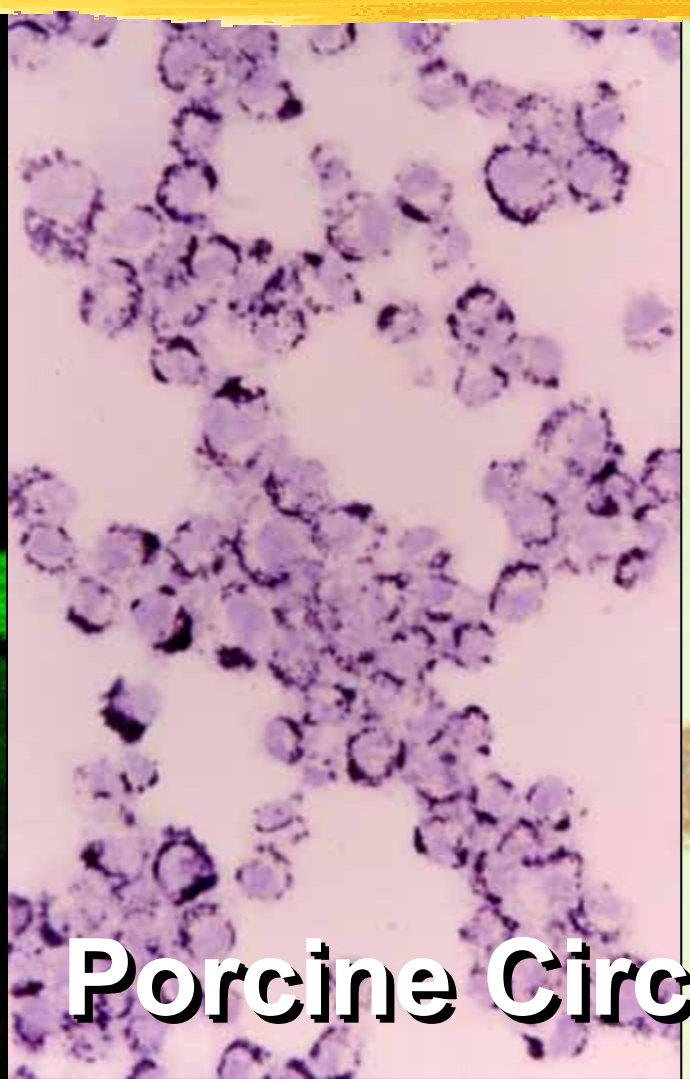
- Independent of antibody, unnecessary to have target/antigen available
- Highly sensitive and specific (target sequence!!!)
- Connects visualized target with microscopic lesion
- RNA easily degrades in tissues
- Fixation protects RNA, but cross-linking masks target
- Labor intensive, slow, difficult to automate
- Standardization is very difficult (antigen retrieval)
- Can be quantitated, except radioactive probes
- Does not detect post-translational changes
- Protein overexpression can be related to cell proliferation (different pathways with same result)
- Must have well-trained pathologists and lab personnel (standardization & interpretation)

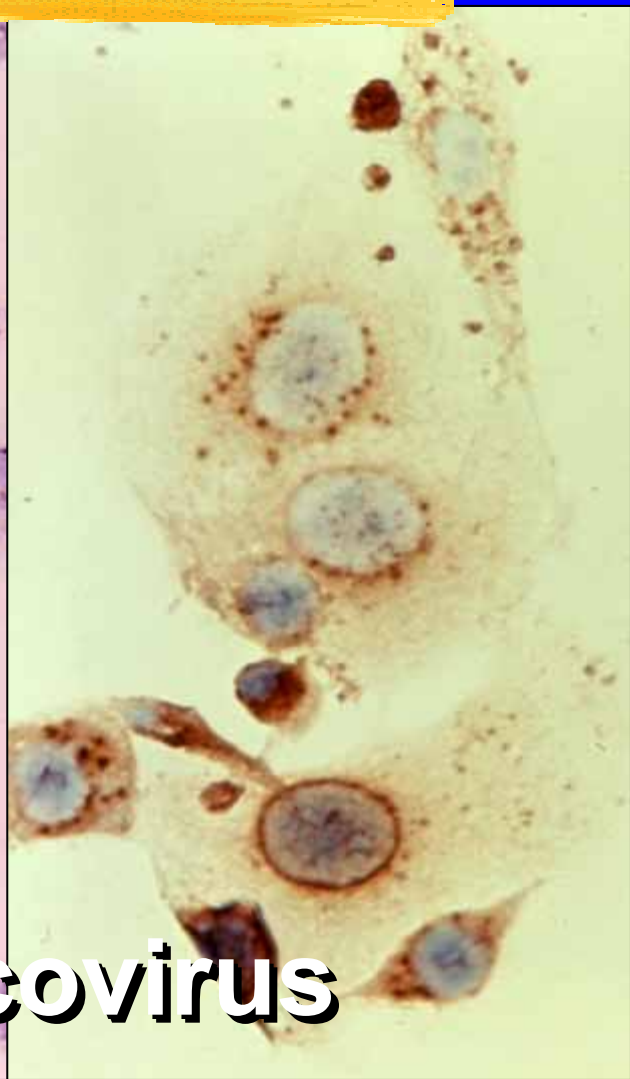
FISH versus CISH

- FISH:
  - Requires expensive specialized equipment
  - Fluorescent signals will commonly fade
  - Results are normally recorded with camera, time consuming and expensive
  - Direct detection, thereby faster
  - Easily used with many color systems
  - Autofluorescence
- CISH:
  - Can be interpreted on regular microscope
  - Lower ratio of signal to background staining
  - HPLC purified probes with sensitive detection system have overcome most of these problems
  - Restricted to 1-2 colors
  - Combines target detection with morphology
  - Can screen a section on low magnification
  - Permanent labeling and can be archived

IHC vs IFA vs ISH

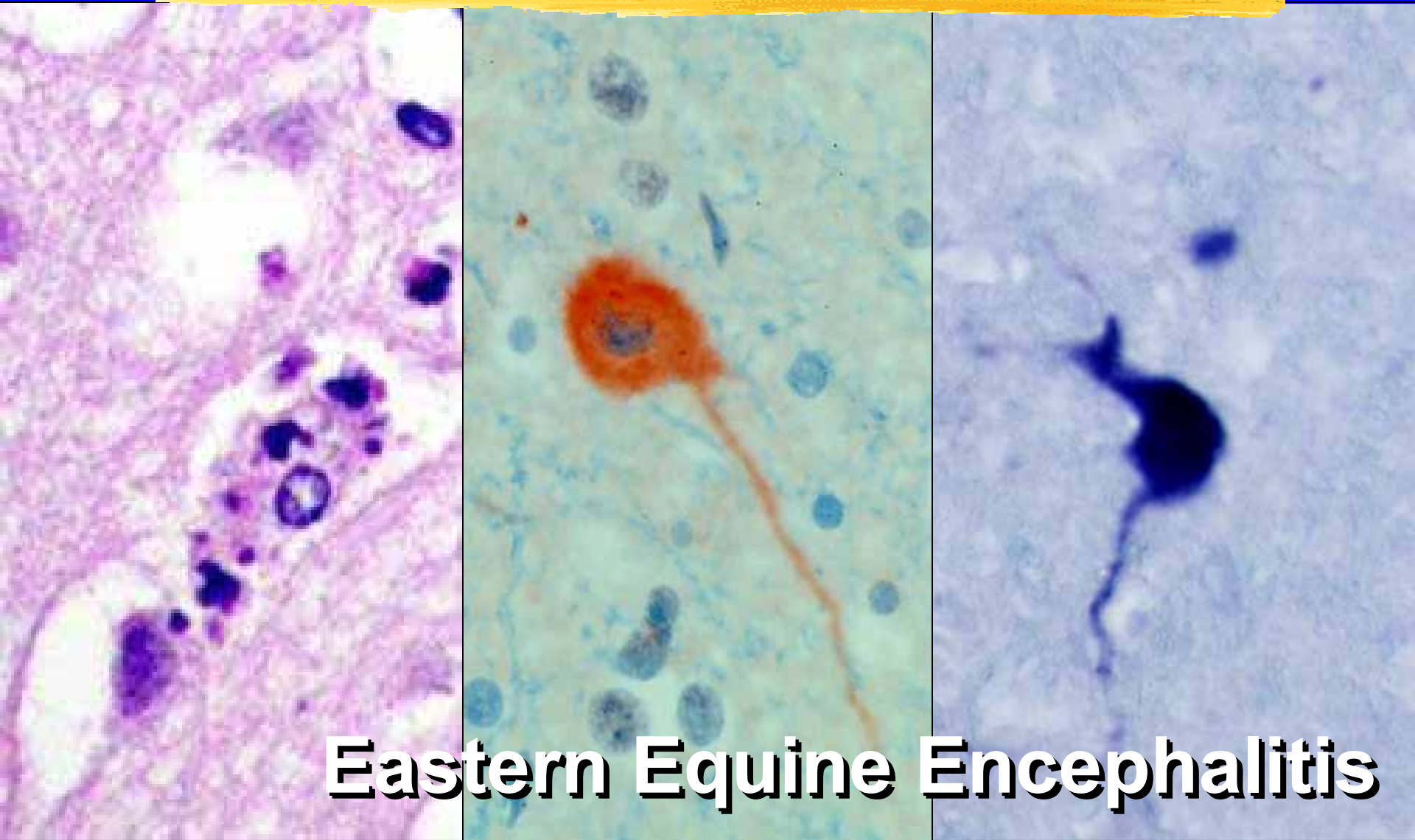






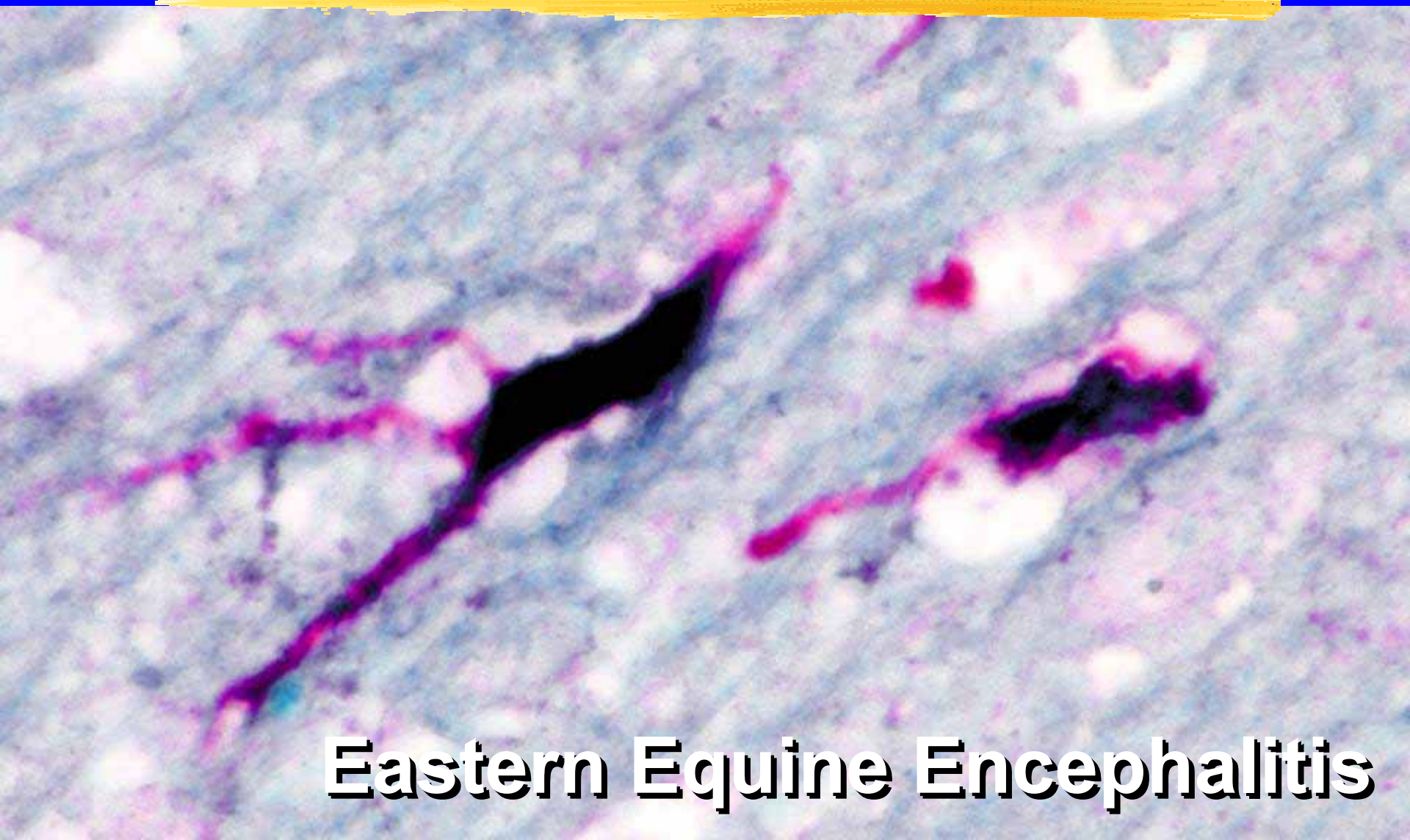
Porcine Circovirus

IHC vs ISH

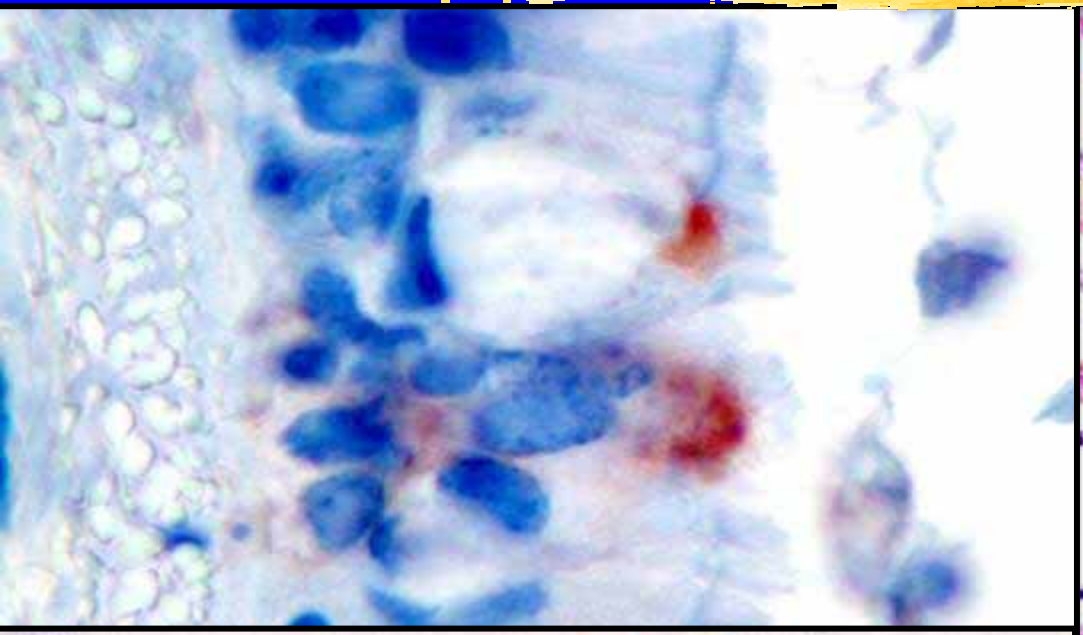


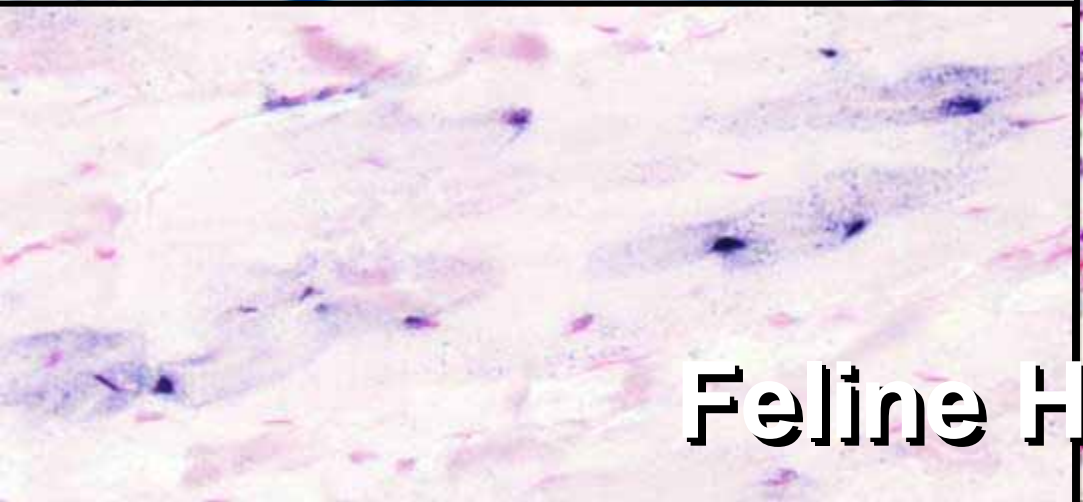
Eastern Equine Encephalitis

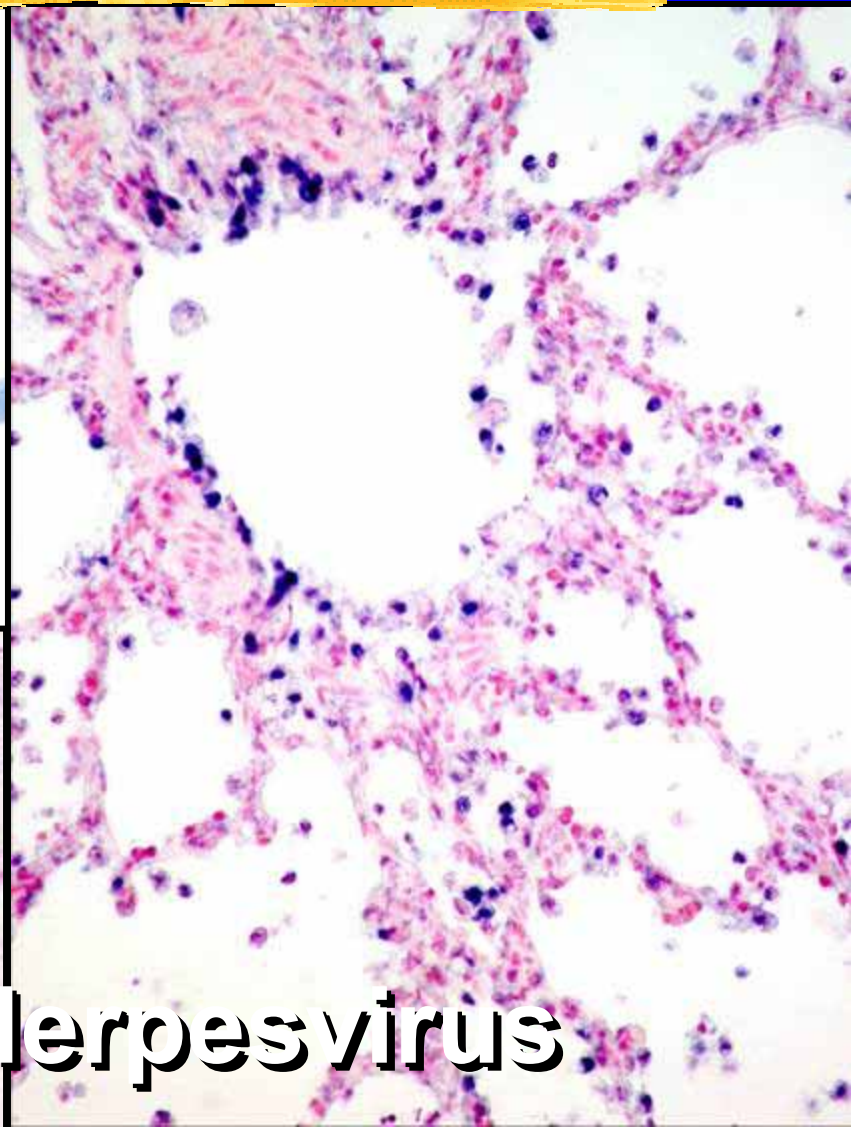
IHC combined with ISH



IHC vs ISH

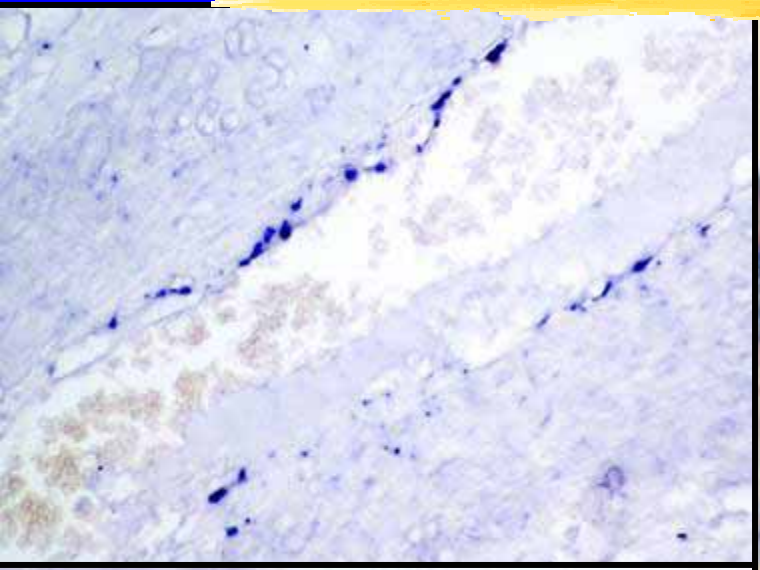


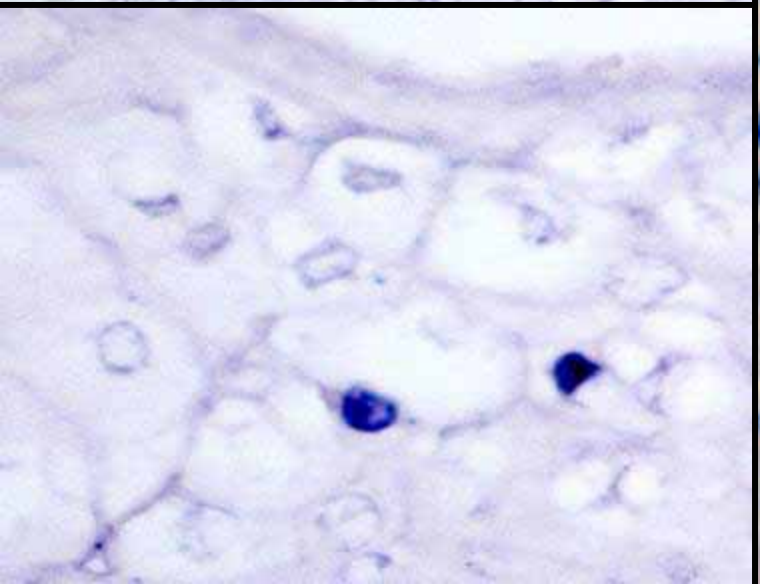


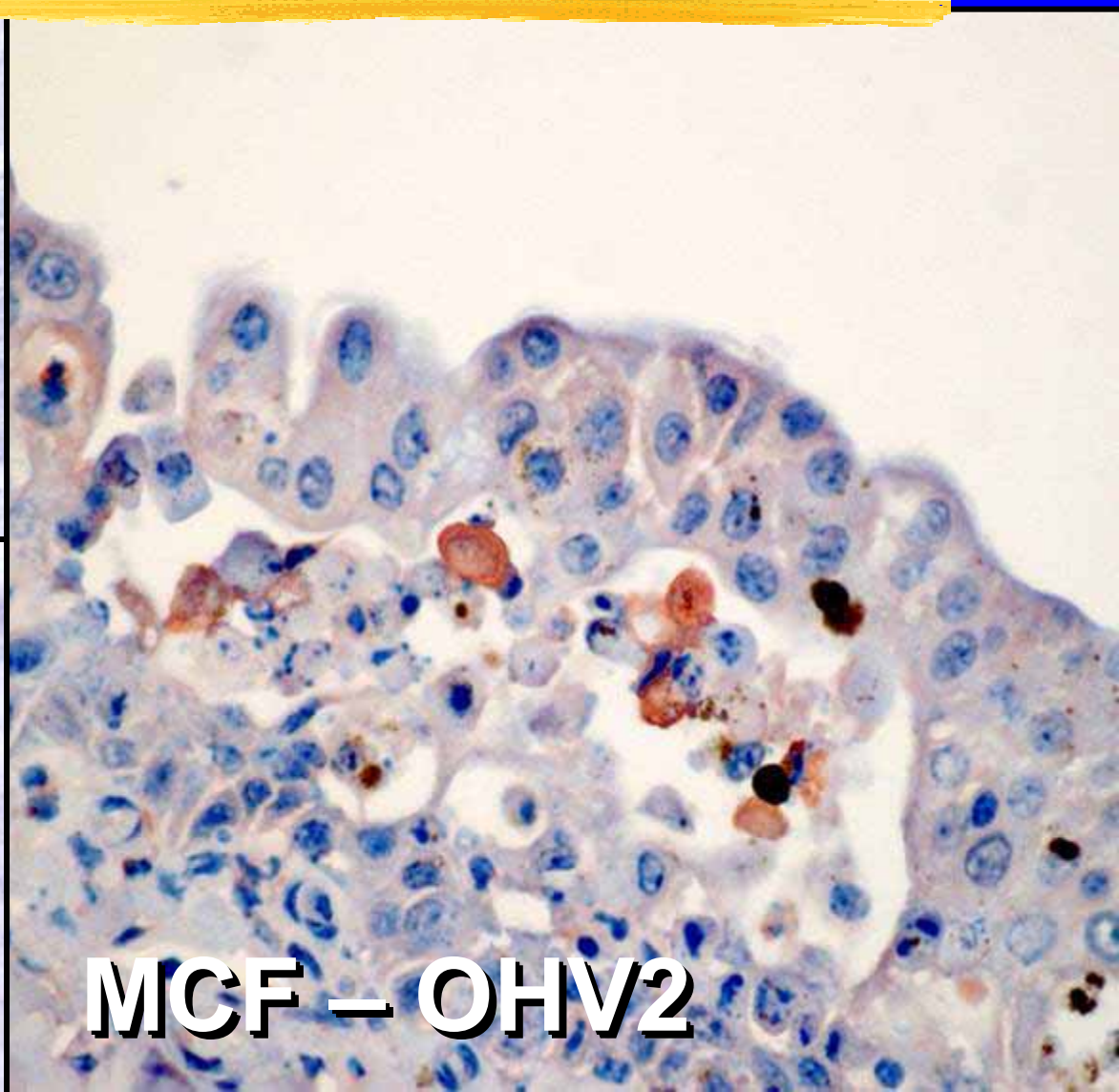


Feline Herpesvirus

IHC vs ISH







MCF – OHV2

IHC vs IFA vs ISH

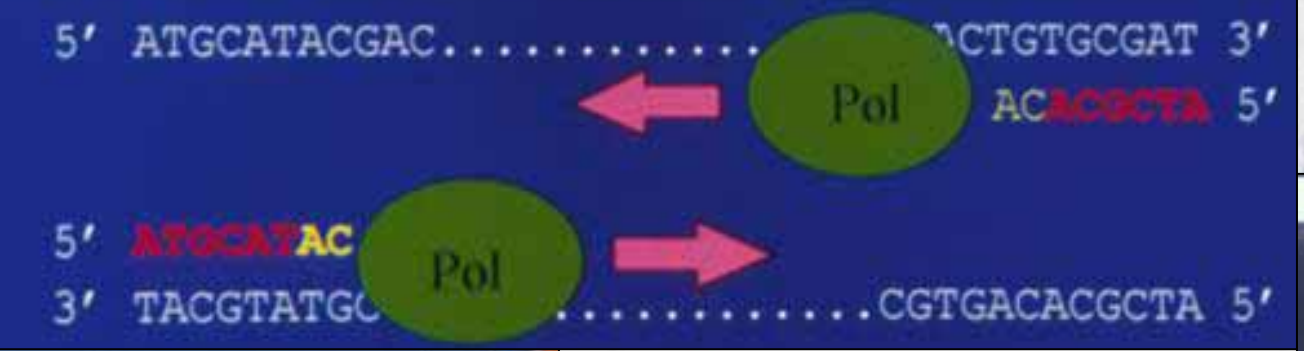
	Cost	Speed	Sensitivity	Specificity	Morphology	Target
IFA	low	fastest	variable	antibody dependent	no	protein
IHC	low	fast	variable	antibody dependent	yes	protein
FISH	high	slower	high	sequence dependent	no	nucleic acid
CISH	high	slowest	high	sequence dependent	yes	nucleic acid

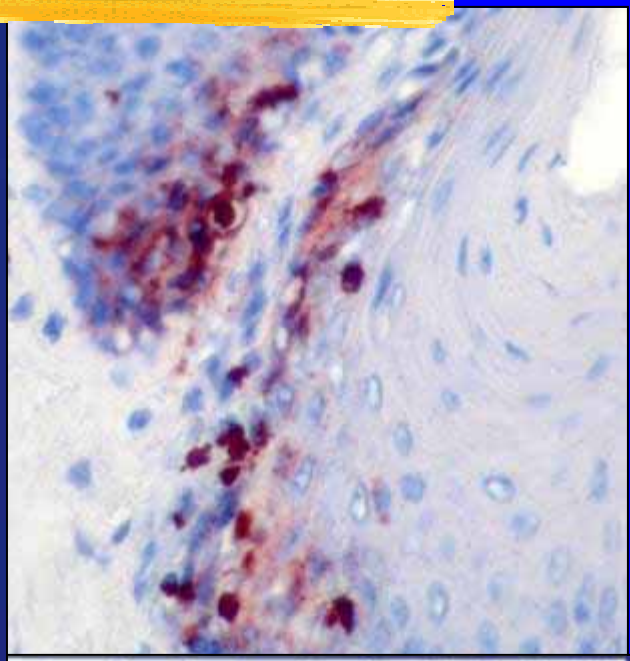
PCR and In-situ PCR

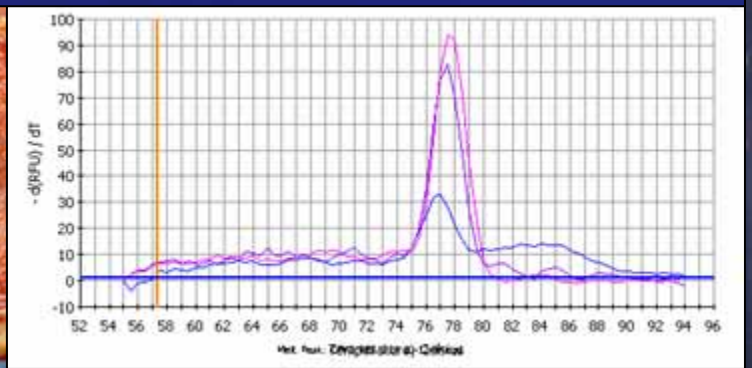
- **A. PCR cycle segments**

1. Denaturation	94-96C	1min
2. Annealing	50-60C	1min
3. Extension	72C	1min

- **B. Duplication of template DNA strands**







IN SITU PCR

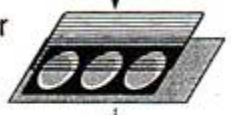
TECHNIQUES

OMAR BAGASRA
JOHN HANSEN

DNA Target Sequence		RNA Target Sequence
Thin tissue sections, cell suspensions, cells cultured on slide, or chromosome spreads		Same as for DNA reactions, but reagents must be RNAse-free (DEPC-treated)
Air dry, then heat @ 105°C for 90 - 120 sec.		Air dry
2% paraformaldehyde overnight, wash once in 3x PBS then twice in 1x PBS		Same as DNA protocol
Proteinase K treatment *(6 µg/ml for 10-60 min., must be optimized)*		Same as DNA protocol
Hydrogen peroxide treatment *(optional)*		Hydrogen peroxide treatment *(1 hour)*
		DNAse treatment to destroy endogenous DNA *(may not be necessary if target sequence is spliced)*
		Reverse transcription of RNA with appropriate primers

Add amplification cocktail & attach cover slip, seal with fingernail polish to keep solution concentrations consistent during thermal cycling

In situ amplification in a thermal cycler *(30 cycles, except for chromosome bands, which need 12-15 cycles)*



Dip in absolute ethanol for 5 minutes to loosen polish, pry off cover slip and place at 92° for one minute

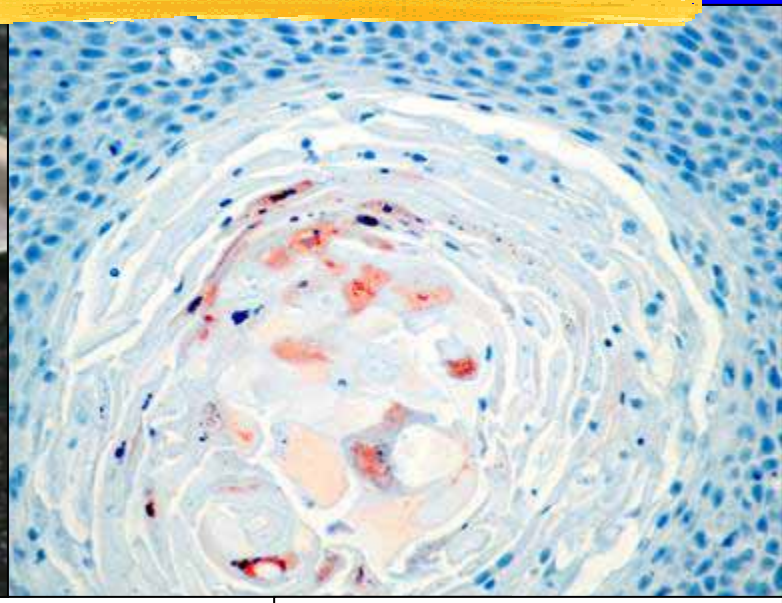
Thermal cycler that holds 16 slides & 24 tubes

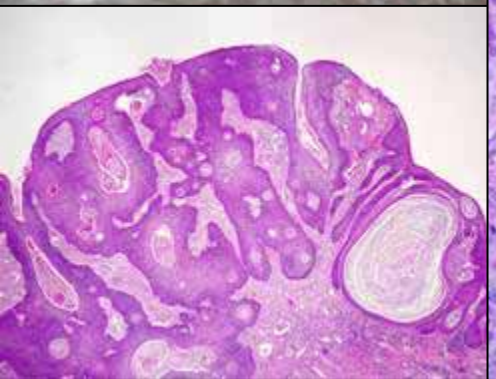
Perform in situ hybridization with a tagged probe that anneals to an internal region of amplified product

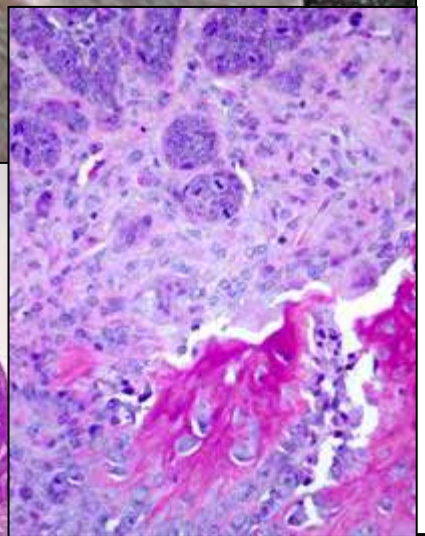
Use probe-detection system, look for color in cytoplasm, nuclei or bands, or count grains from radioactivity

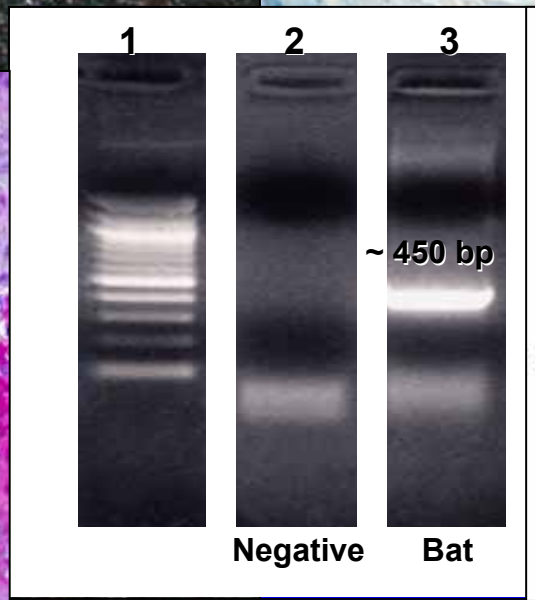
Good practice allows signal to be contained within the membrane-bound compartment of the original target

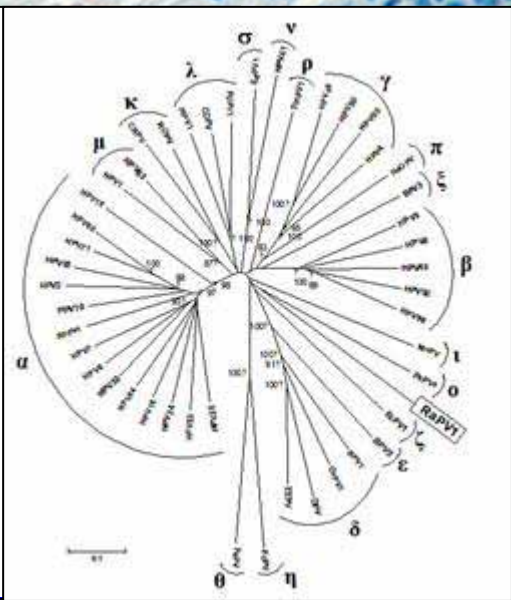
Combining Tools

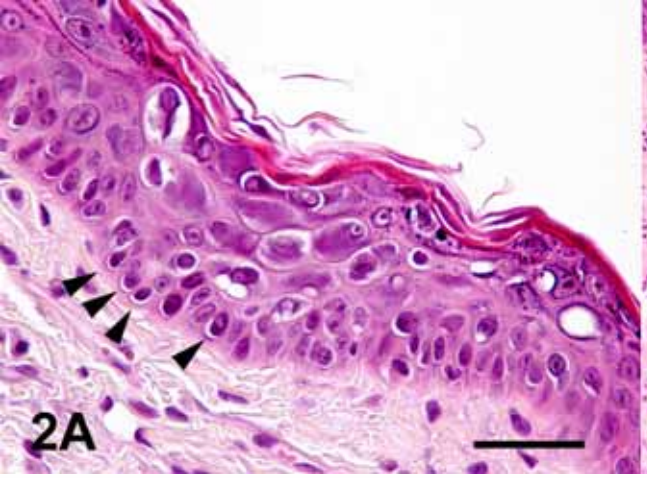








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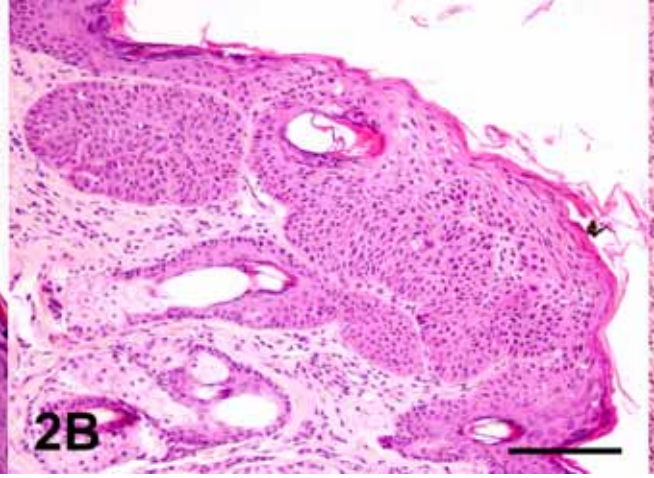
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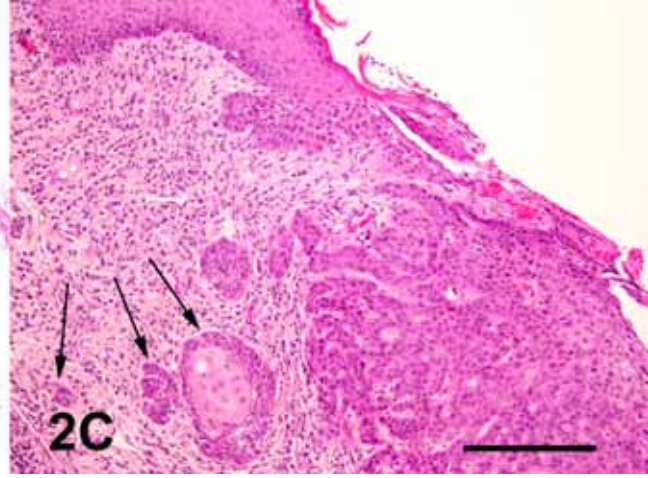
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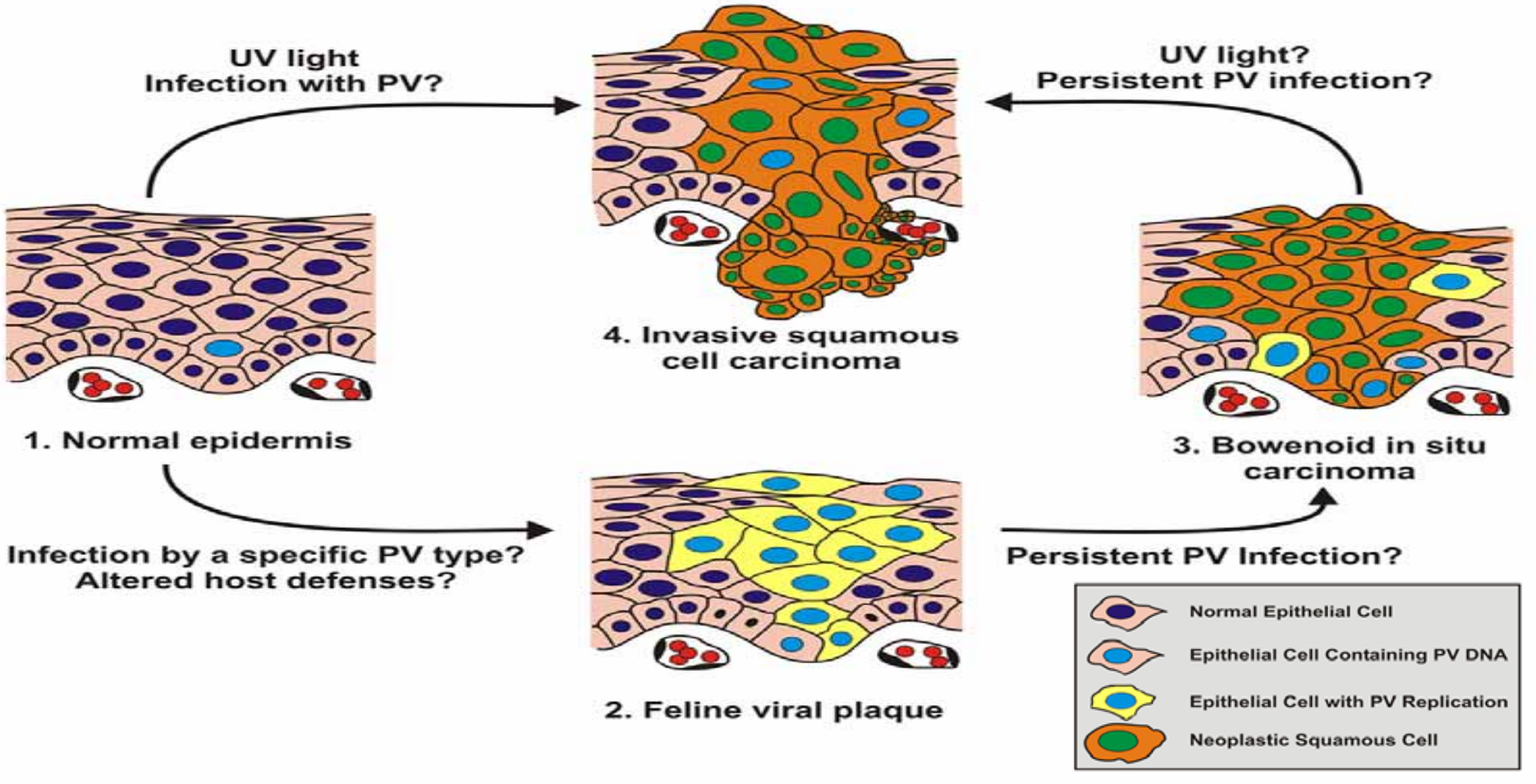
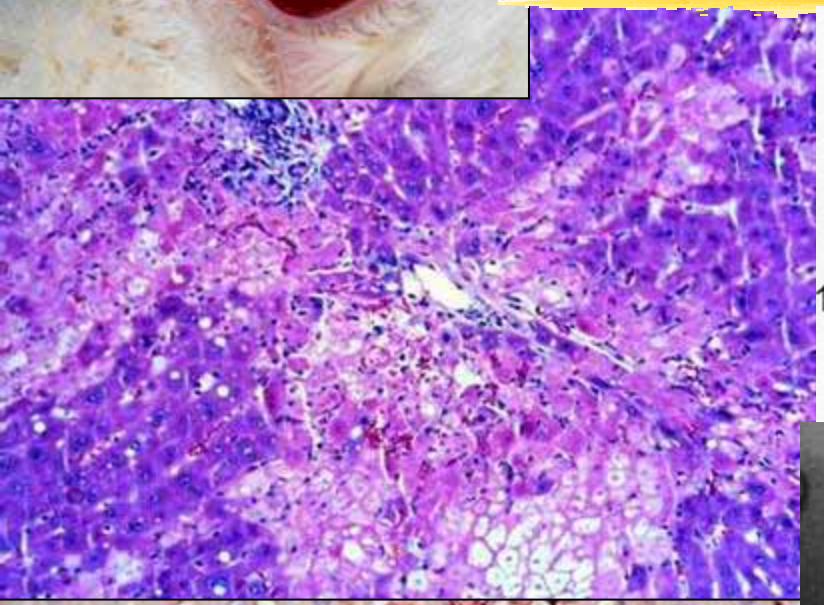
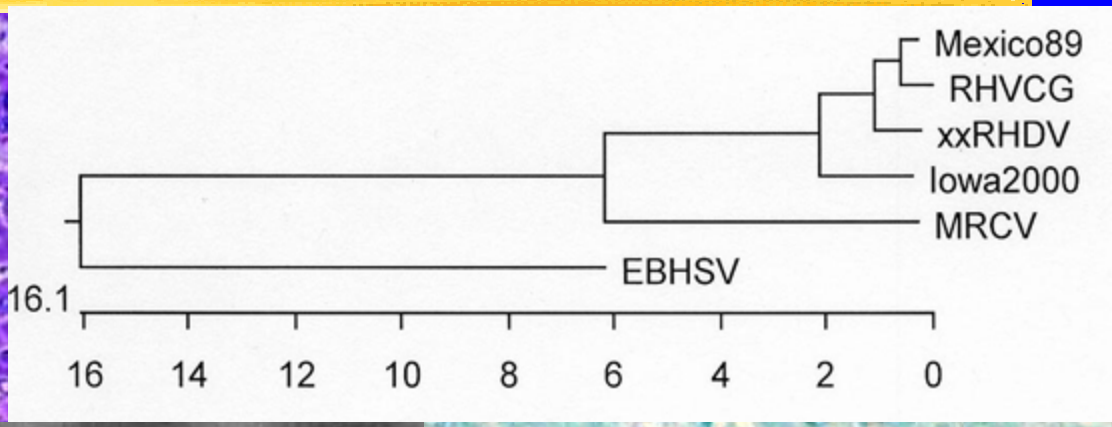
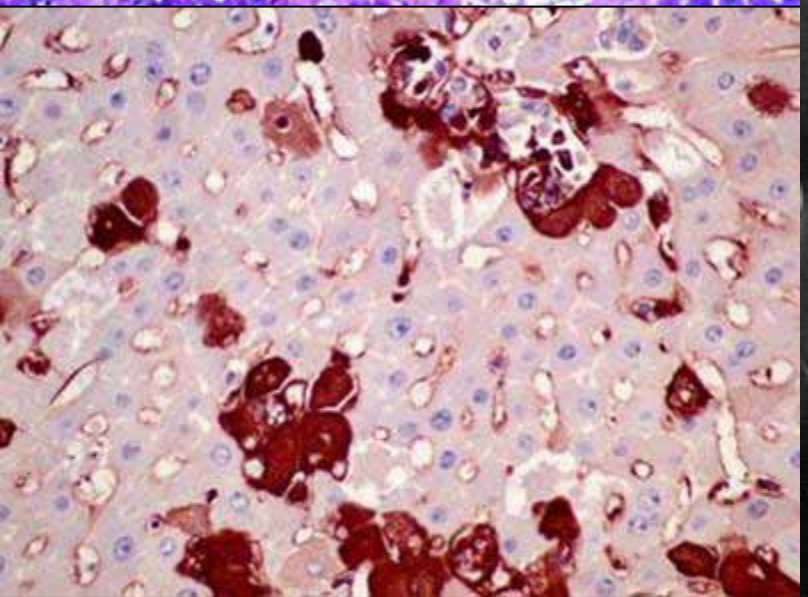
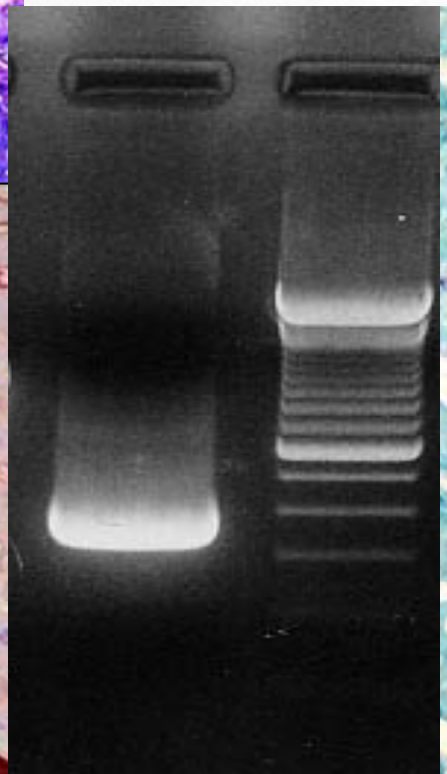

UV light
Infection with PV?
4. Invasive squamous cell carcinoma
UV light?
Persistent PV infection?
1. Normal epidermis
3. Bowenoid in situ carcinoma
Infection by a specific PV type?
Altered host defenses?
2. Feline viral plaque
Persistent PV Infection?
Normal Epithelial Cell
Epithelial Cell Containing PV DNA
Epithelial Cell with PV Replication
Neoplastic Squamous Cell

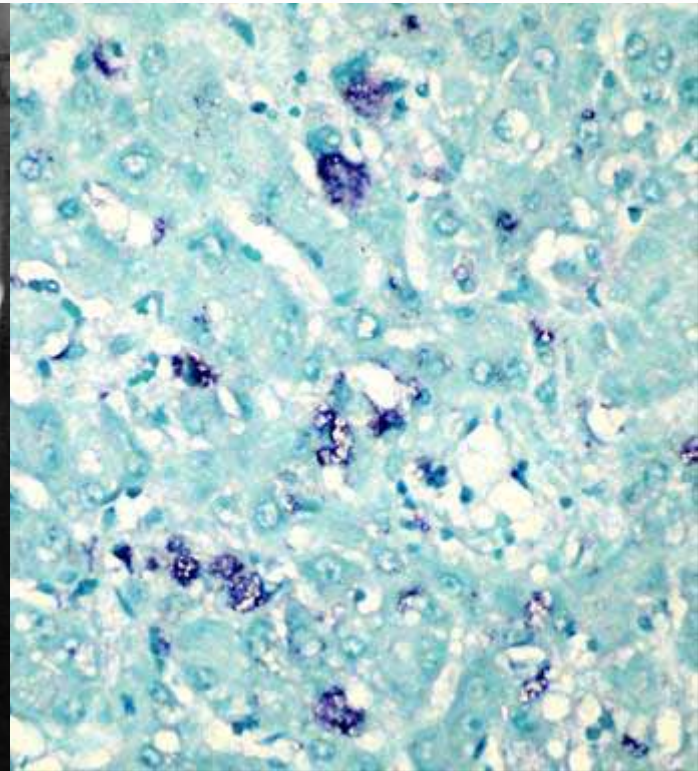
Combining Tools



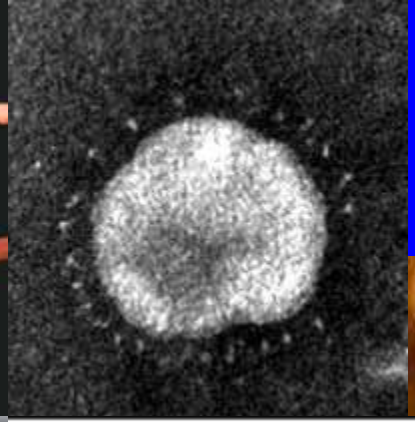


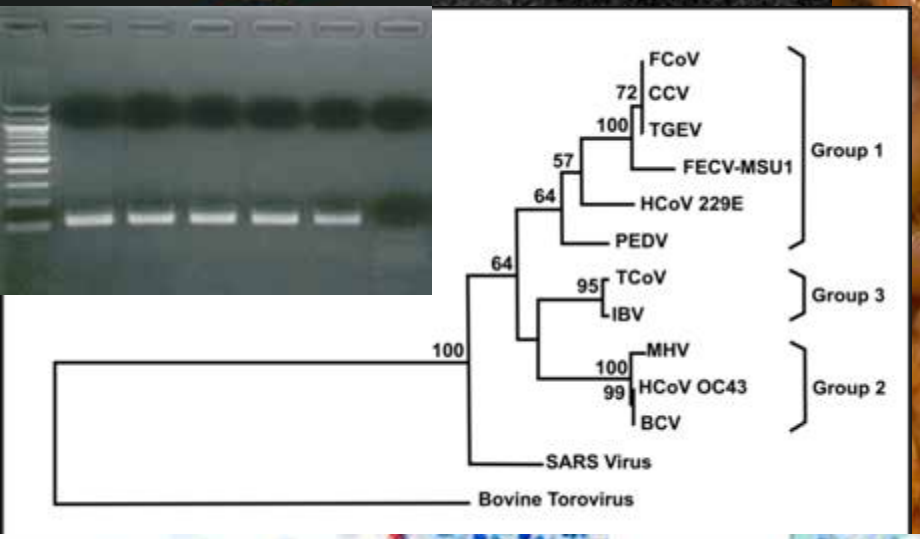


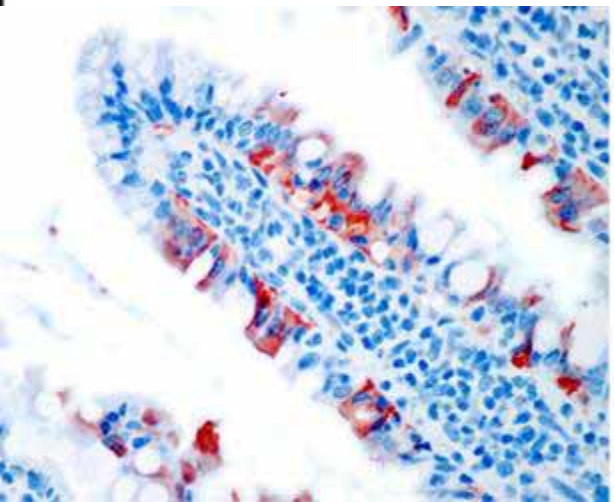


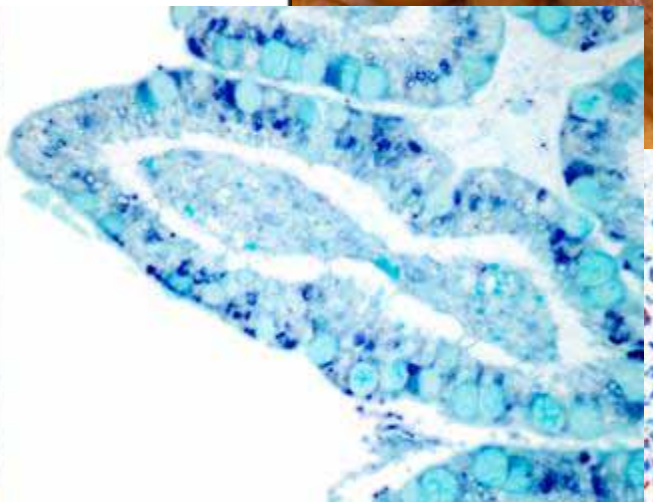


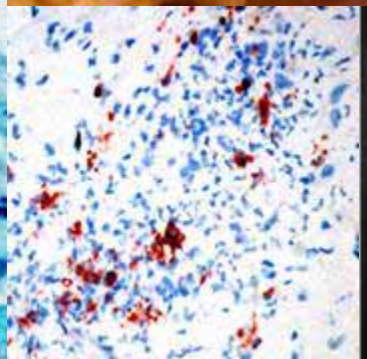
Combining Tools

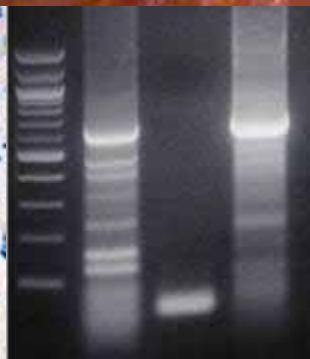













Are you stressed?

Do you feel stuck?

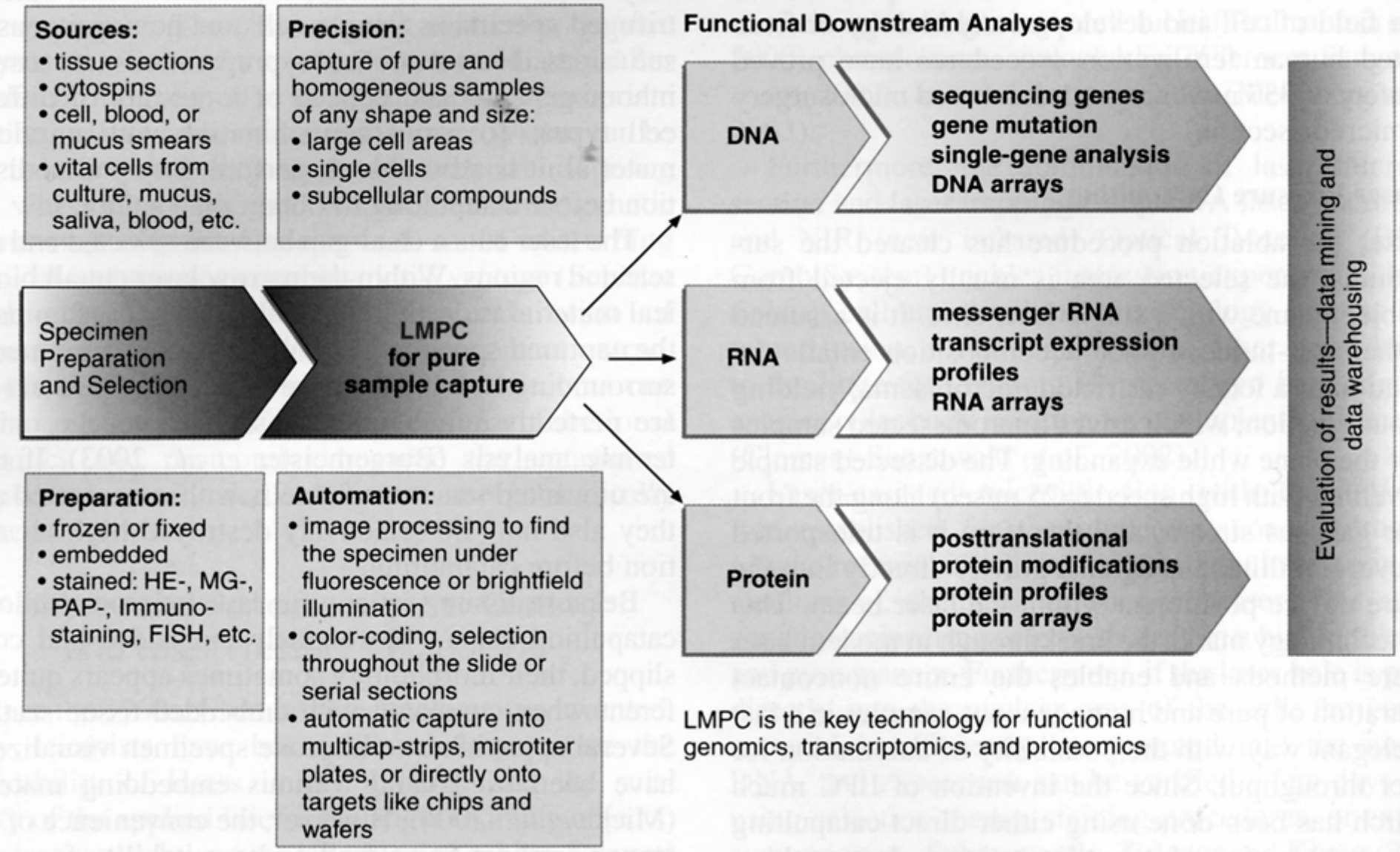
Relax!


Have confidence!

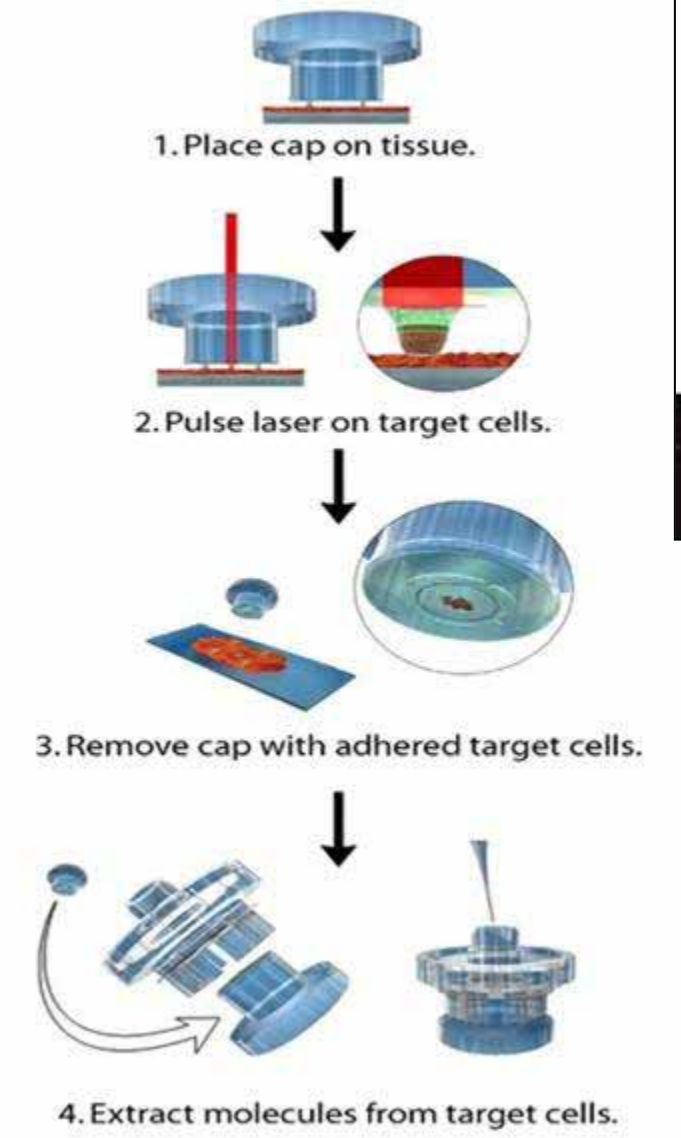

You can always call me!
517 432 2670

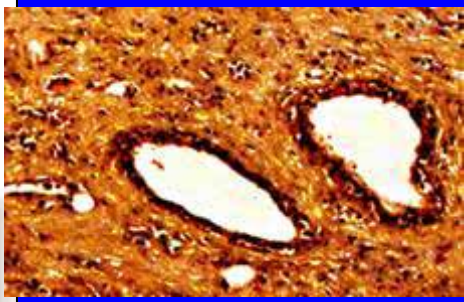
How else can we go after a Target?

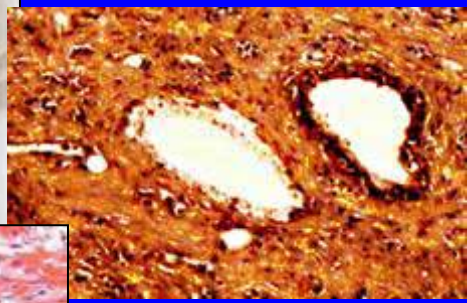
Laser Capture Microscopy

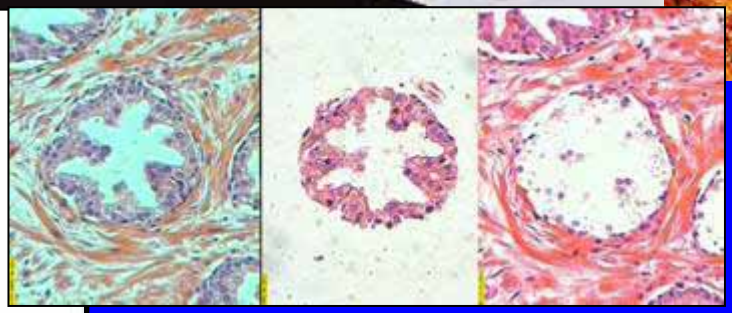


Laser Capture Microscopy









- **Powerful imaging tool that weds imaging to molecular methods and utilizes shared pathology informatics networks (SPIN)**

Laser Capture Microscopy

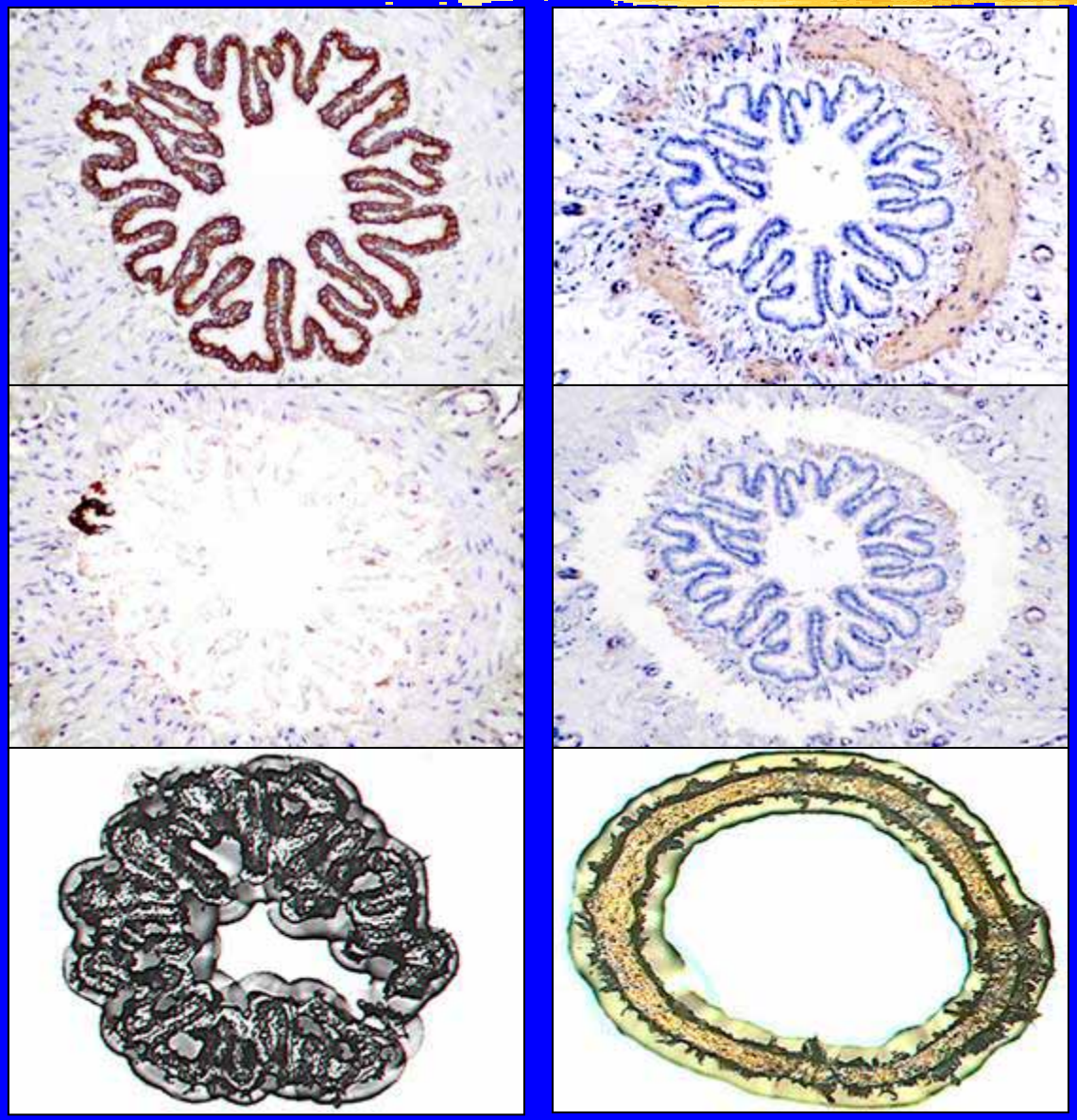
Fixation and slide preparation are the key

RNA – Isolation from Tissue

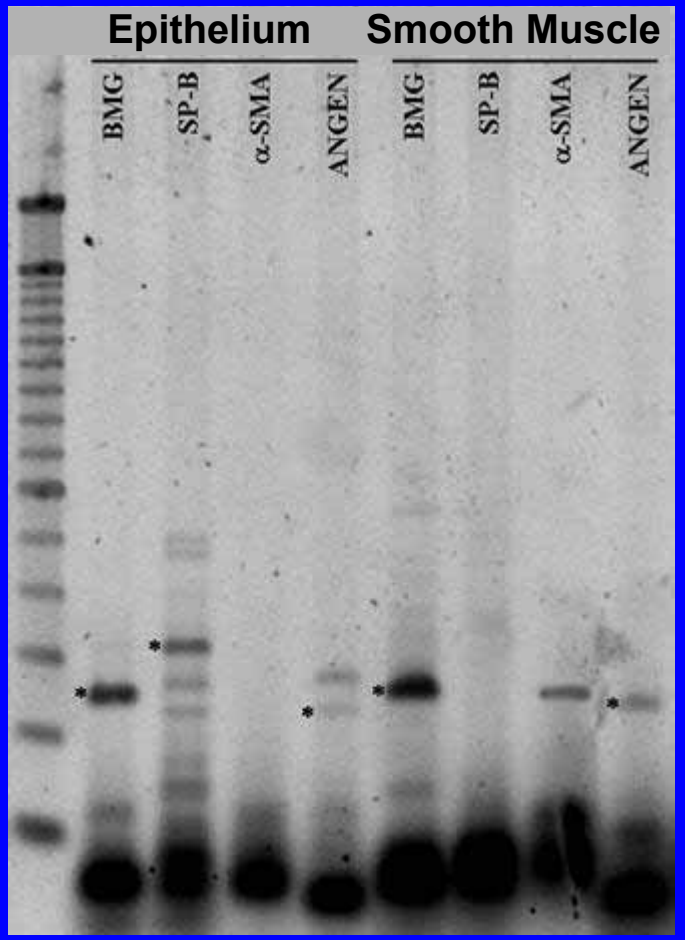
Vacuum systems with UV-sensitive tape transfer systems are an option

On approach is to change from a fixation model to a frozen section process …..

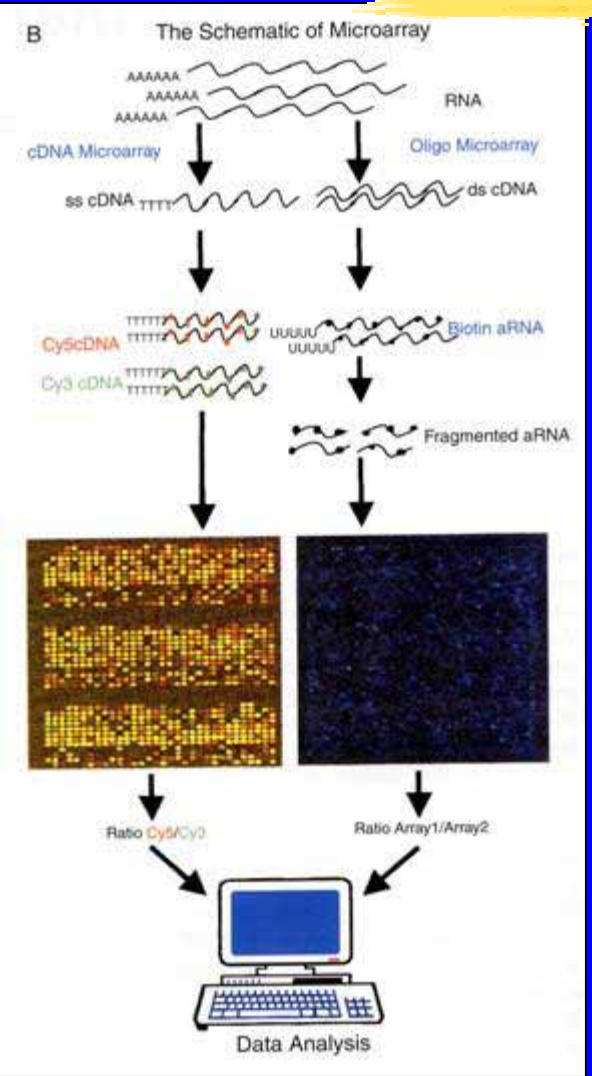
Laser Capture Microscopy



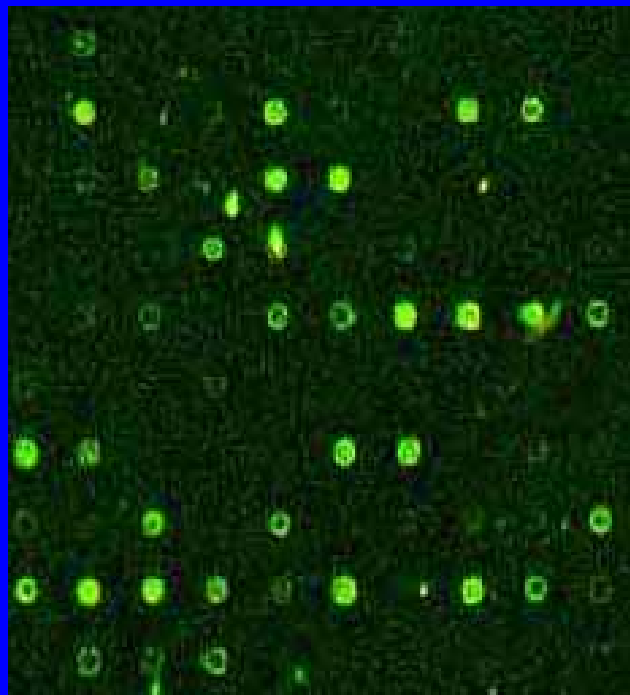
Pulmonary Fibrosis



Genes Associated with the Progression of Canine Cancer



- 60mer long cDNA microarray
- 851 genes associated with
  - Cancer, Inflammation, Stem cells, Osteogenesis
- Internal controls
  - Positive:
    - 5 housekeeping genes
  - Negative:
    - Empty
    - Nonsense, scrambled



Science Times

The New York Times

TUESDAY, APRIL 9, 2002

CONFRONTING CANCER

A New View of Malignancy

Custom Treatments

Turning Microscope Images That Look Alike ...

... Into a Digital Guide to a Better Outcome

How DNA Chips Work

Test Proves Fruitless, Fueling New Debate On Cancer Screening

Telling the Threatening Tumors From the Harmless Ones

Getting Used to Life, Long Life, With Cancer

'It's in our nature to defend against the fear of mortality.'

Inside

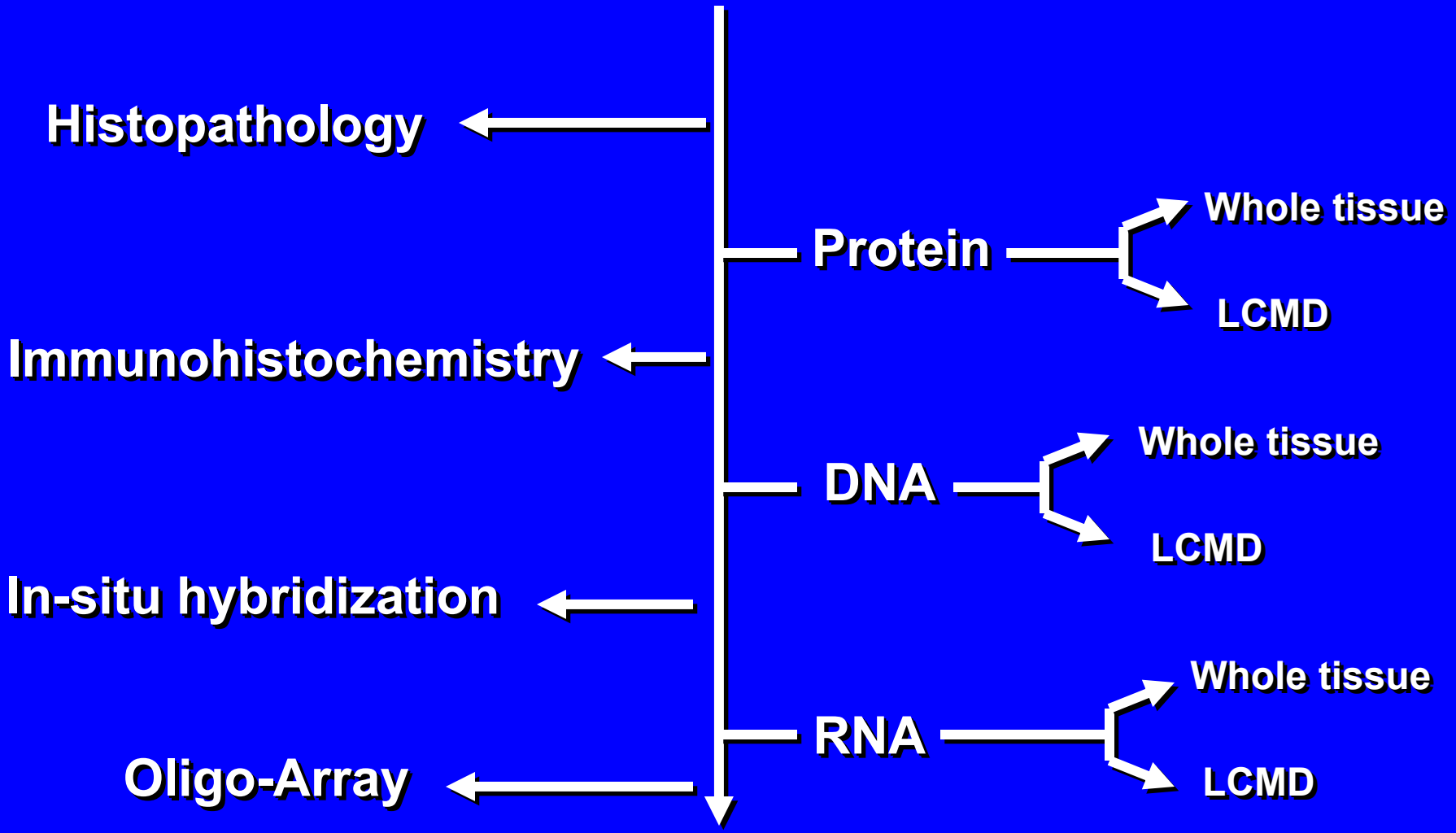
What have the following done to boost awareness and hope?

Glevec
Her2neu
P450 – Roche
cyp2d6
"poor metabolizer"
"extensive metabolizer"

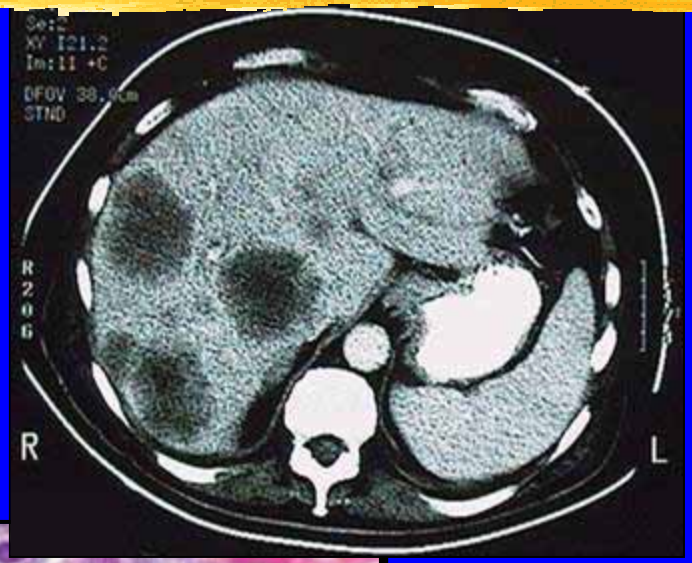
It is our hope … and not only ours … but of the science community and the public as well … that we can develop individualized diagnostic, prognostic and therapeutic procedures.

New Paradigm for Analysis

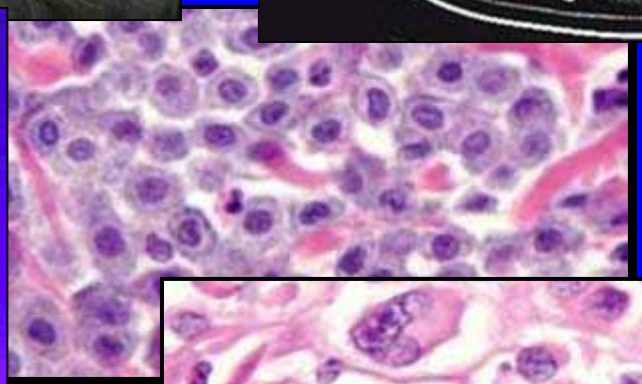
Fresh Frozen Tissues - OCT Embedded tissue

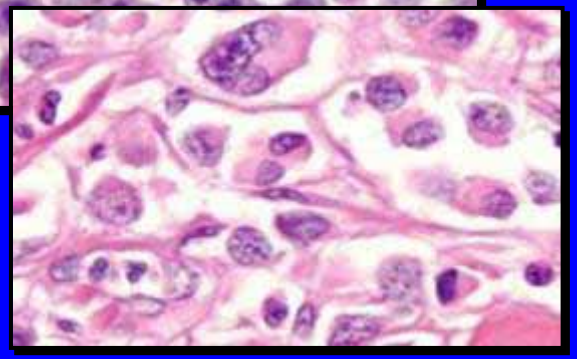


Anatomical Pathology: Past and Present
Mast Cell Tumor Prognosis



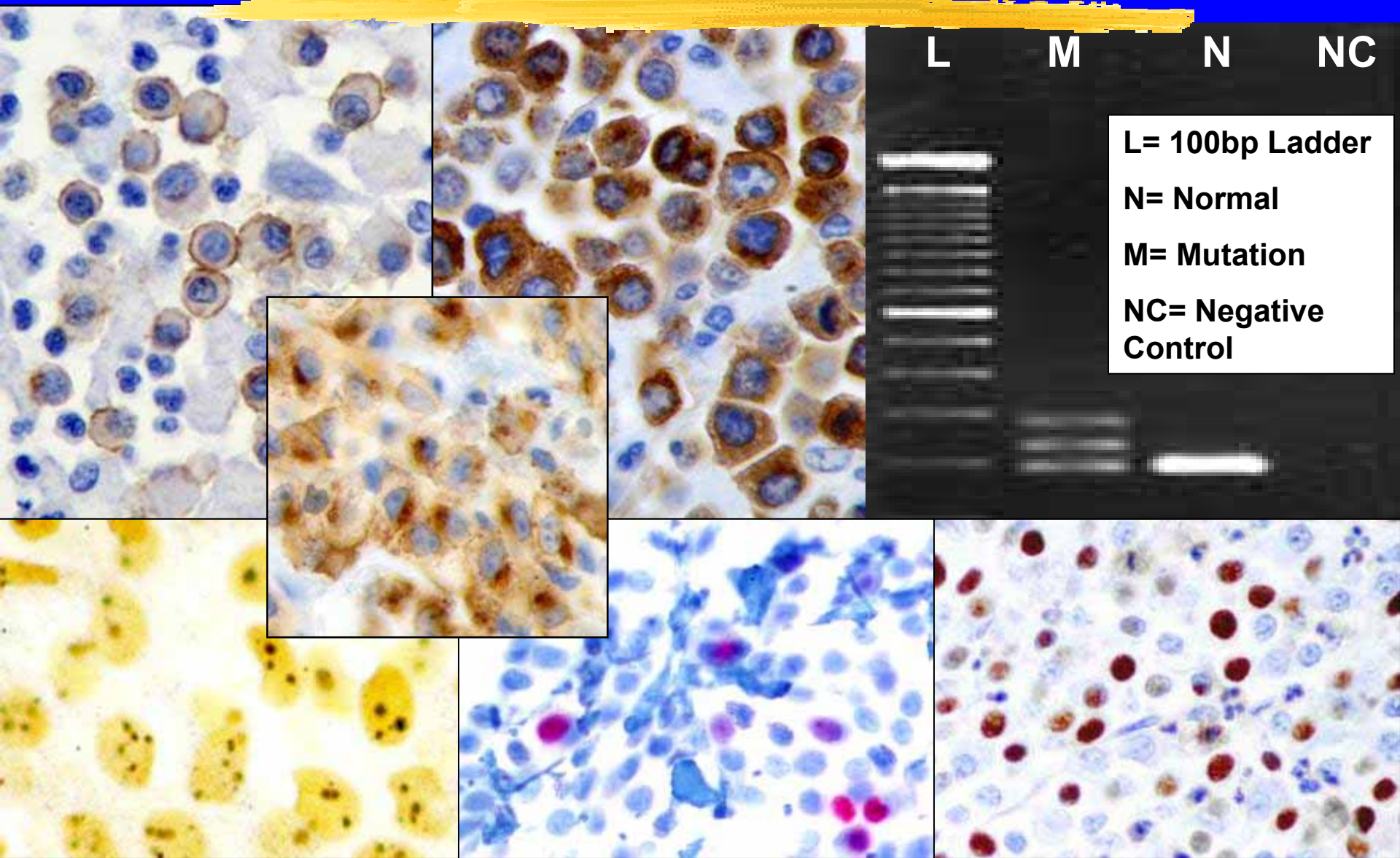
So what do we propose ?



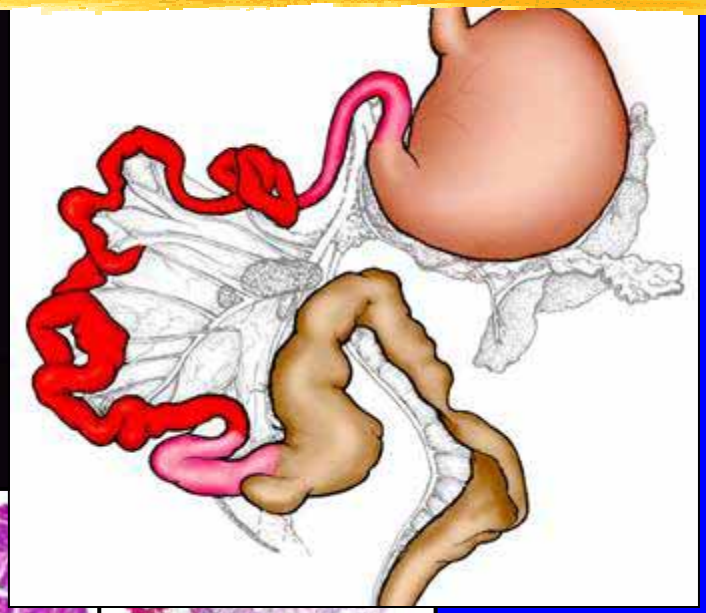


Histopathology is qualitative and excellent at assigning a case to a "class" . But it does not indicate the appropriate treatment with a great deal of assurance or indicate an "individual" prognosis.

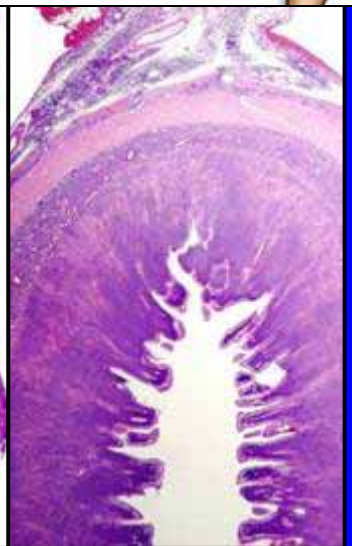
Anatomical Pathology: Past and Present
Mast Cell Tumor Prognosis



Anatomical Pathology: Past and Present
Feline Lymphoma Diagnosis

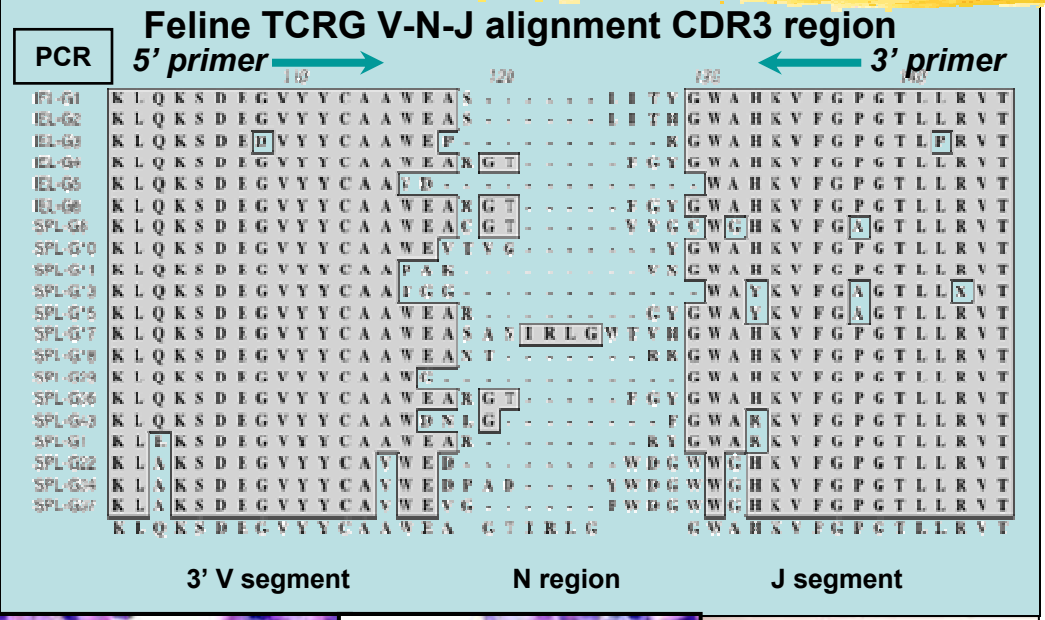


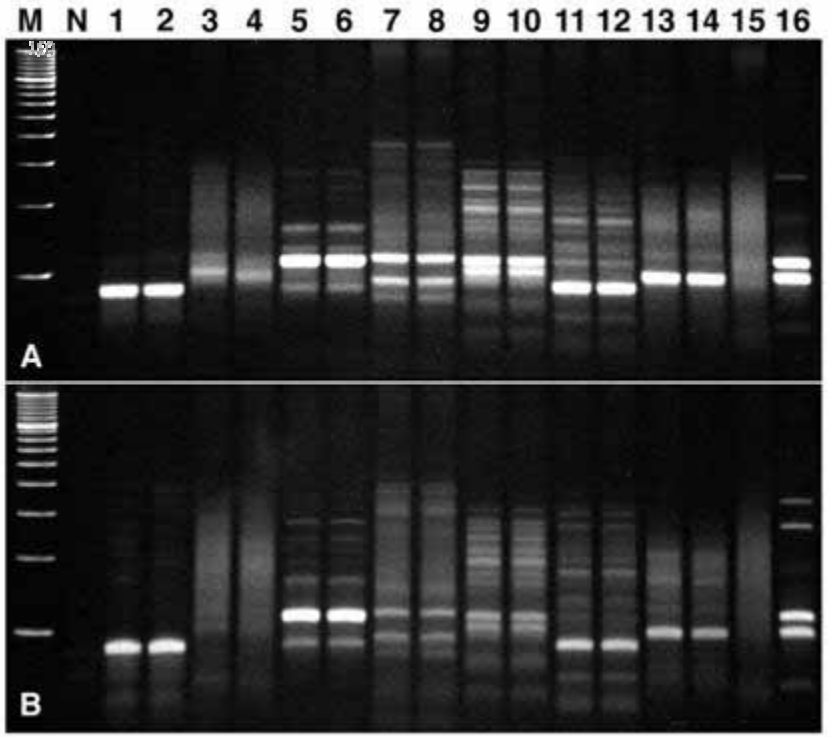
So what do we propose ?

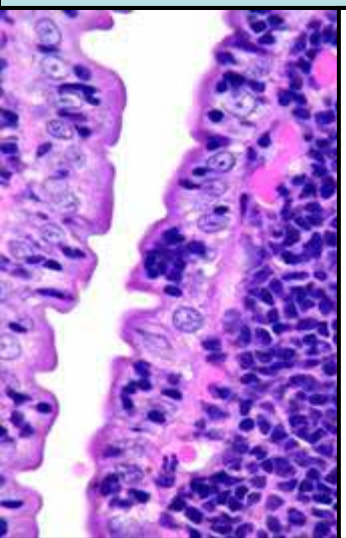


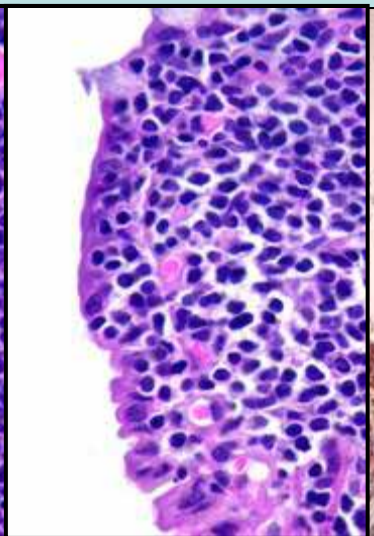
Inflammatory bowel disease and intestinal lymphoma (most are T-cell) commonly present morphological identical, especially in absence of muscularis involvement or endoscopic biopsies that don't allow for more detailed evaluation

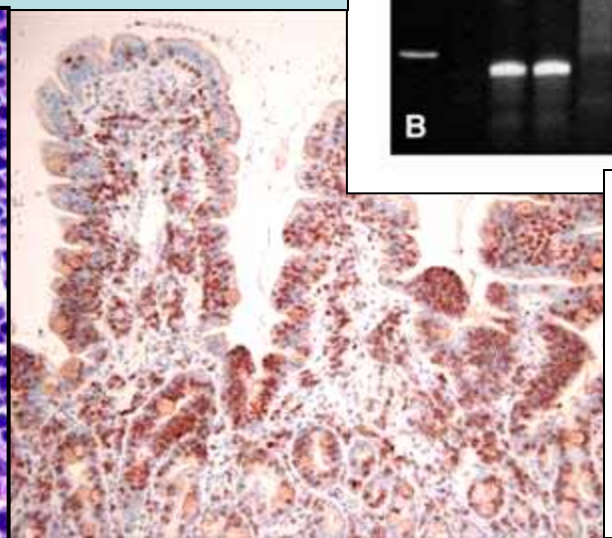
Anatomical Pathology: Past and Present
Feline Lymphoma Diagnosis

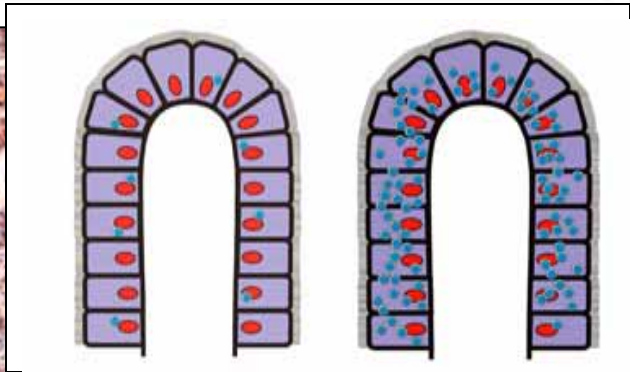








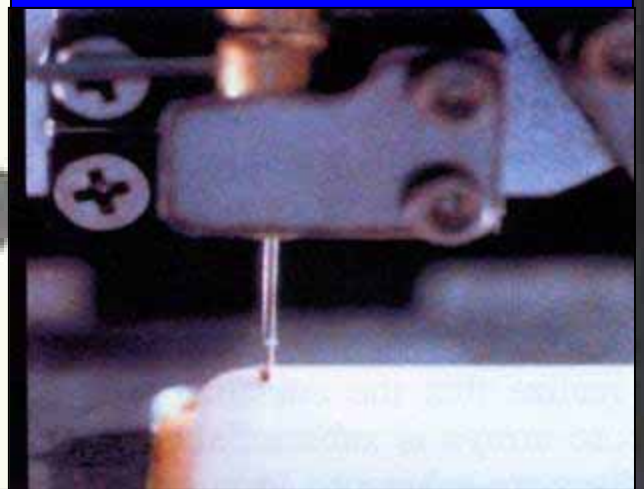




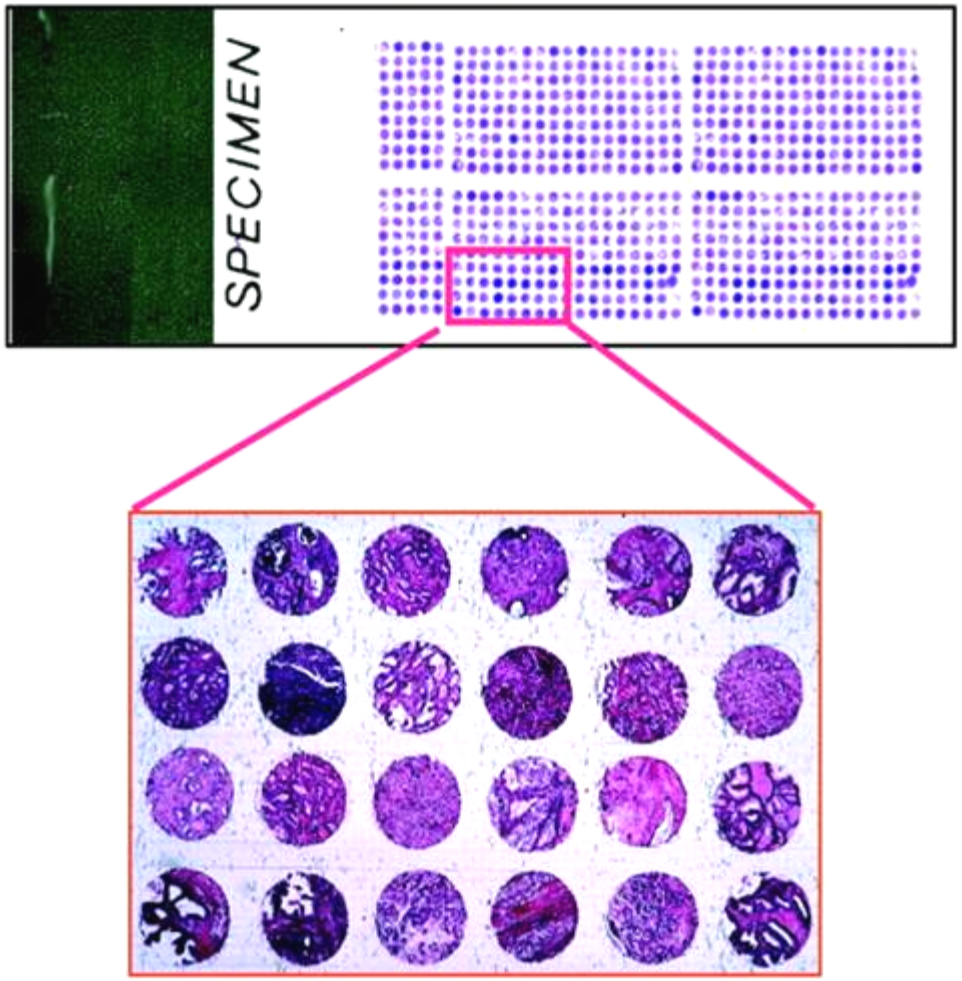
How do We Quantitate Reactions?

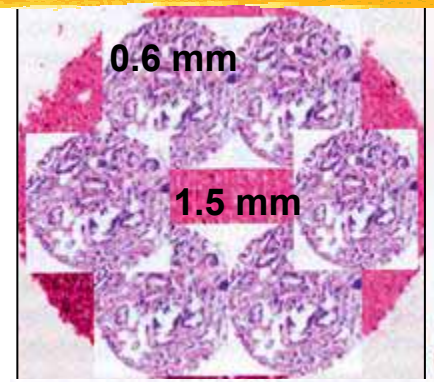
Tissue Mirco-Arrays

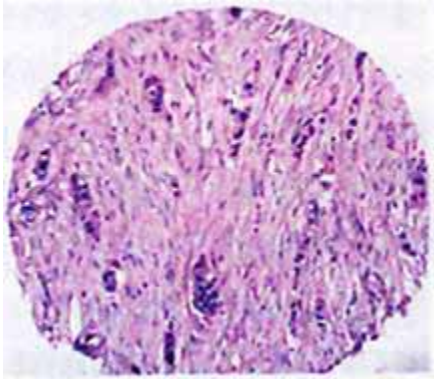
- Tissue microarray work station
- Stainless needles cores in mobile arm
- Digital precision devise
- 0.6 mm core samples

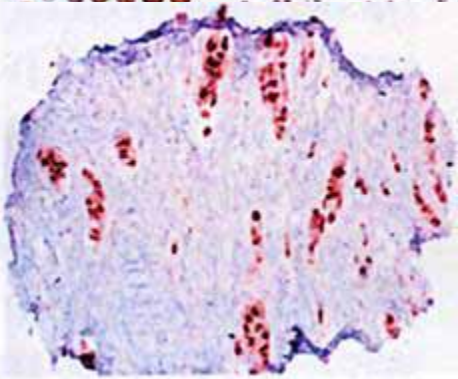


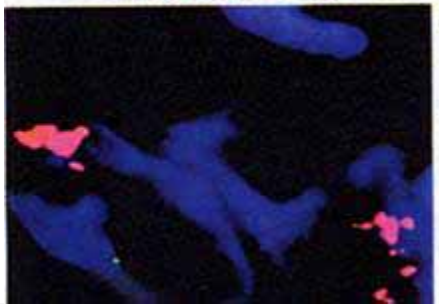
Tissue Mirco-Arrays



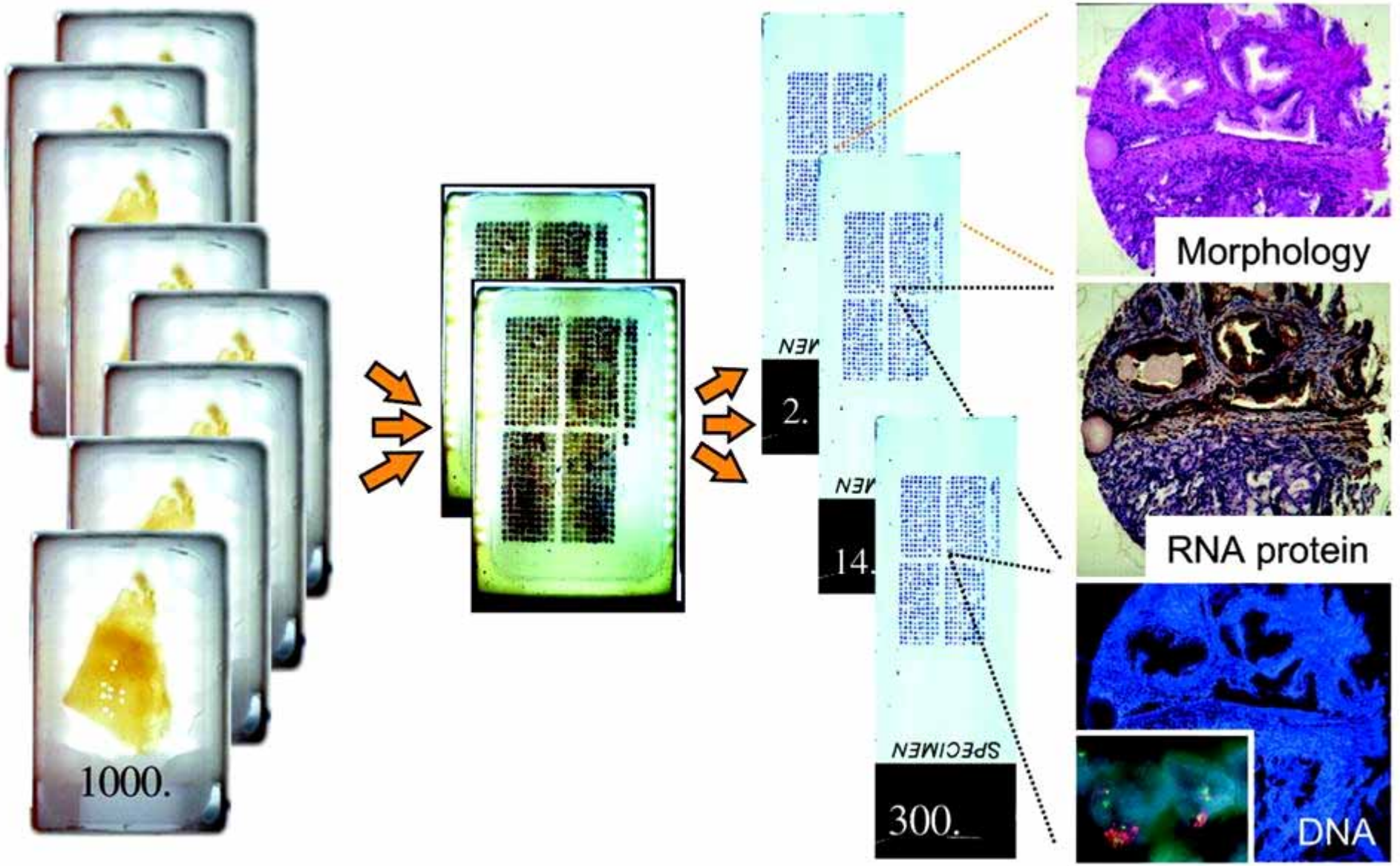




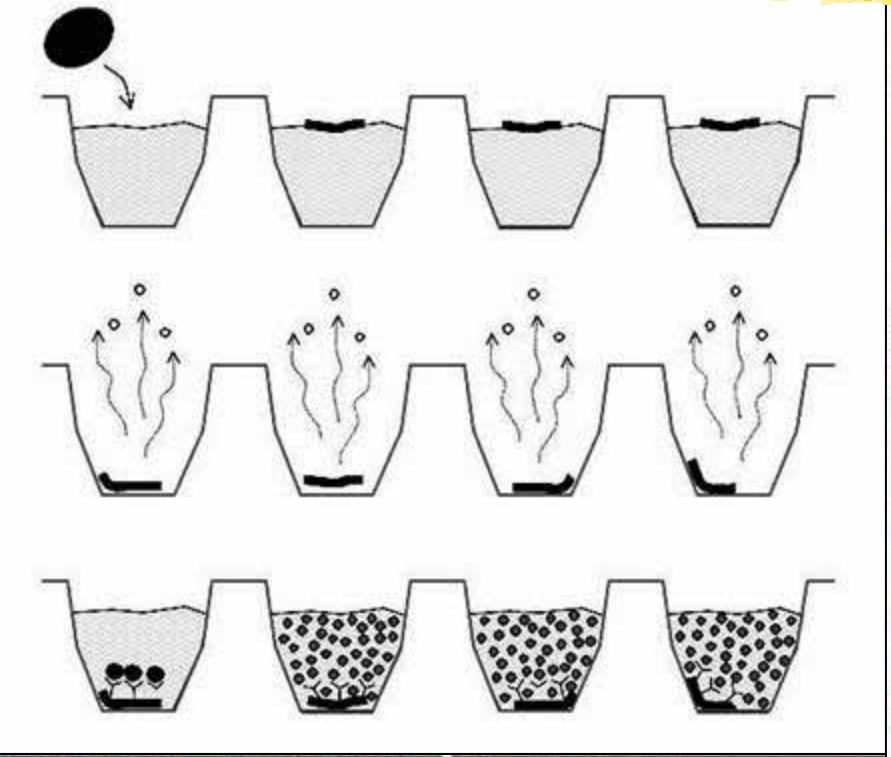


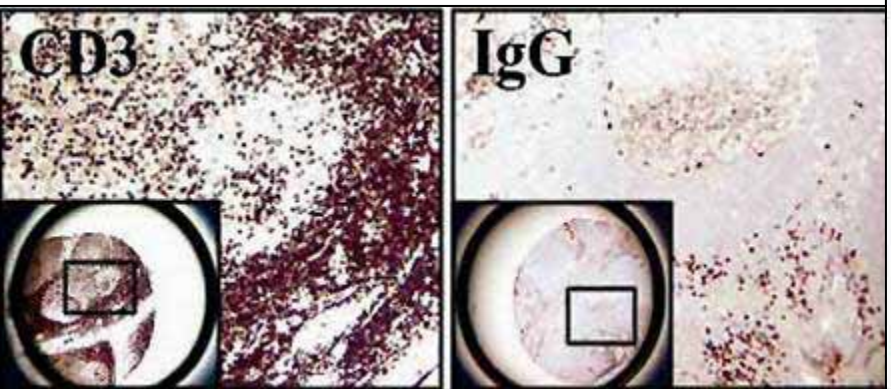


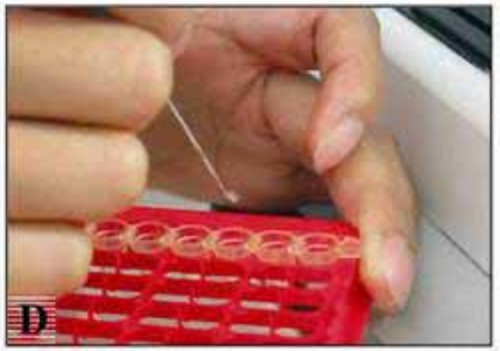
Tissue Array Technology: Paraffin or Frozen

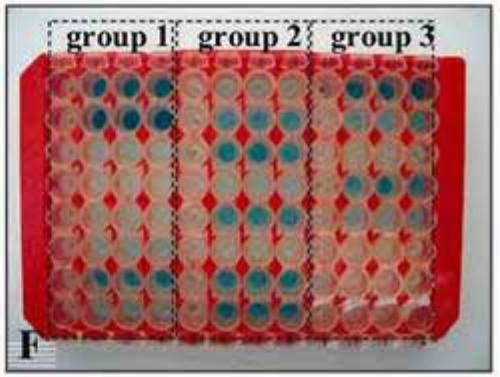


Quantitative ELISA-like IHC (QUELI)



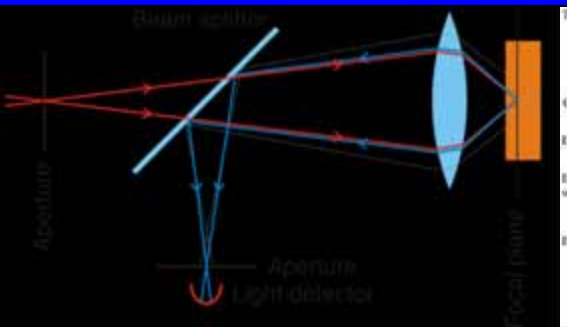


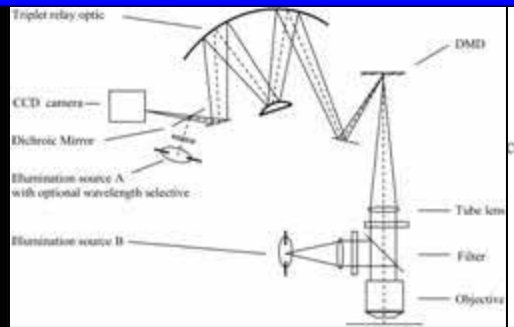


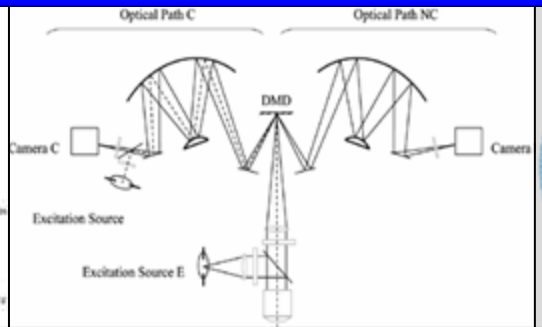


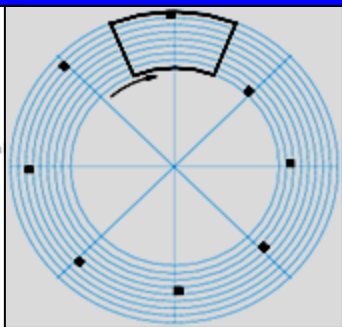
Confocal Microscopy

- Used to increase micrograph contrast and/or to reconstruct three-dimensional images
- Uses point illumination and a spatial pinhole to eliminate out-of-focus light or flares in specimens that are thicker than the focal plane
- Only the light within the focal plane can be detected
- 2D or 3D imaging requires scanning over a regular raster
- Three types of confocal microscopes:
  - Confocal laser scanning microscope
  - Spinning-disk (Nipkow disk) confocal microscope
  - Programmable Array Microscopes (PAM)

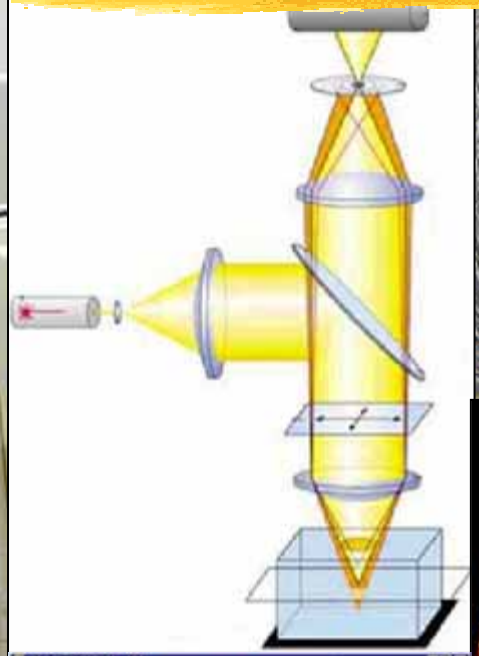


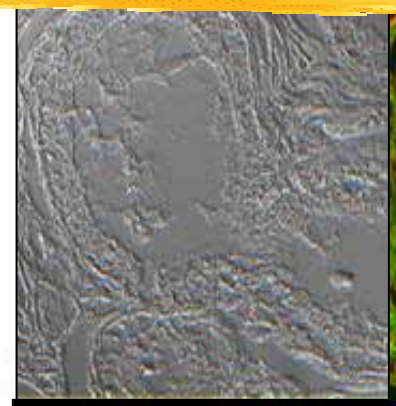


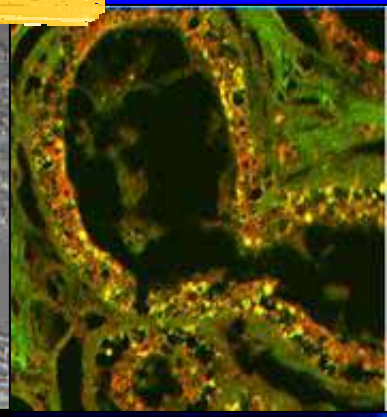


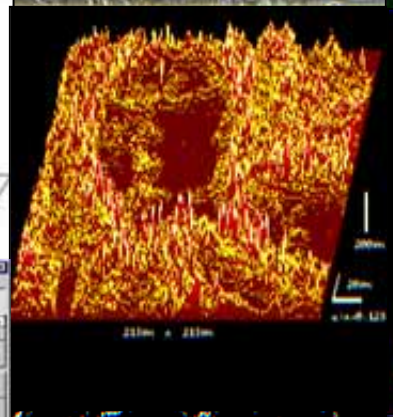


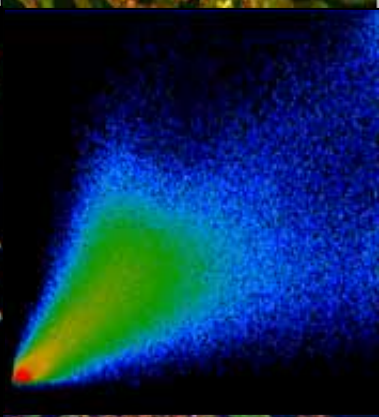
Confocal Laser Scanning Microscopy

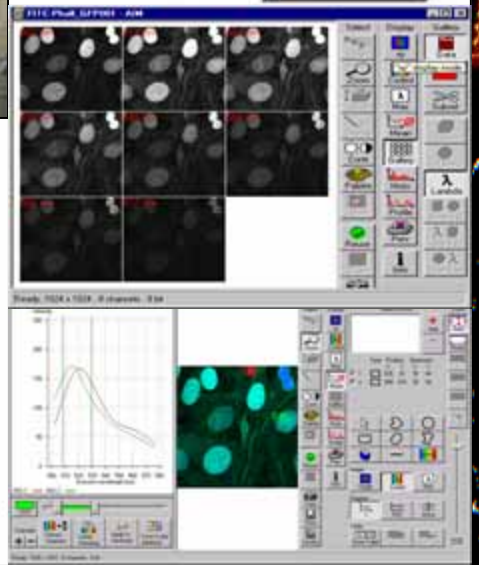


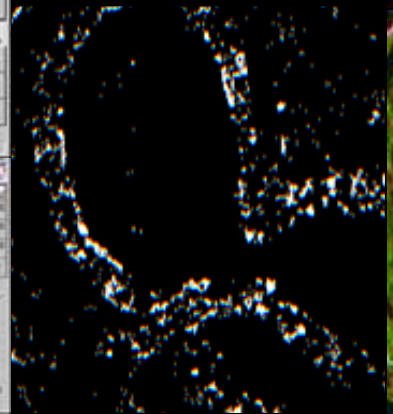


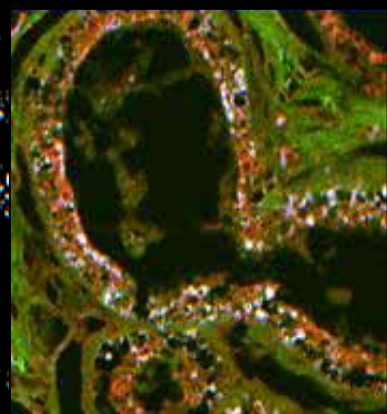


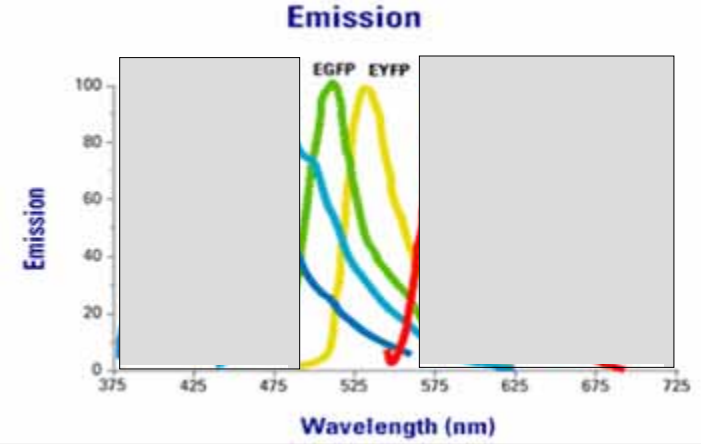




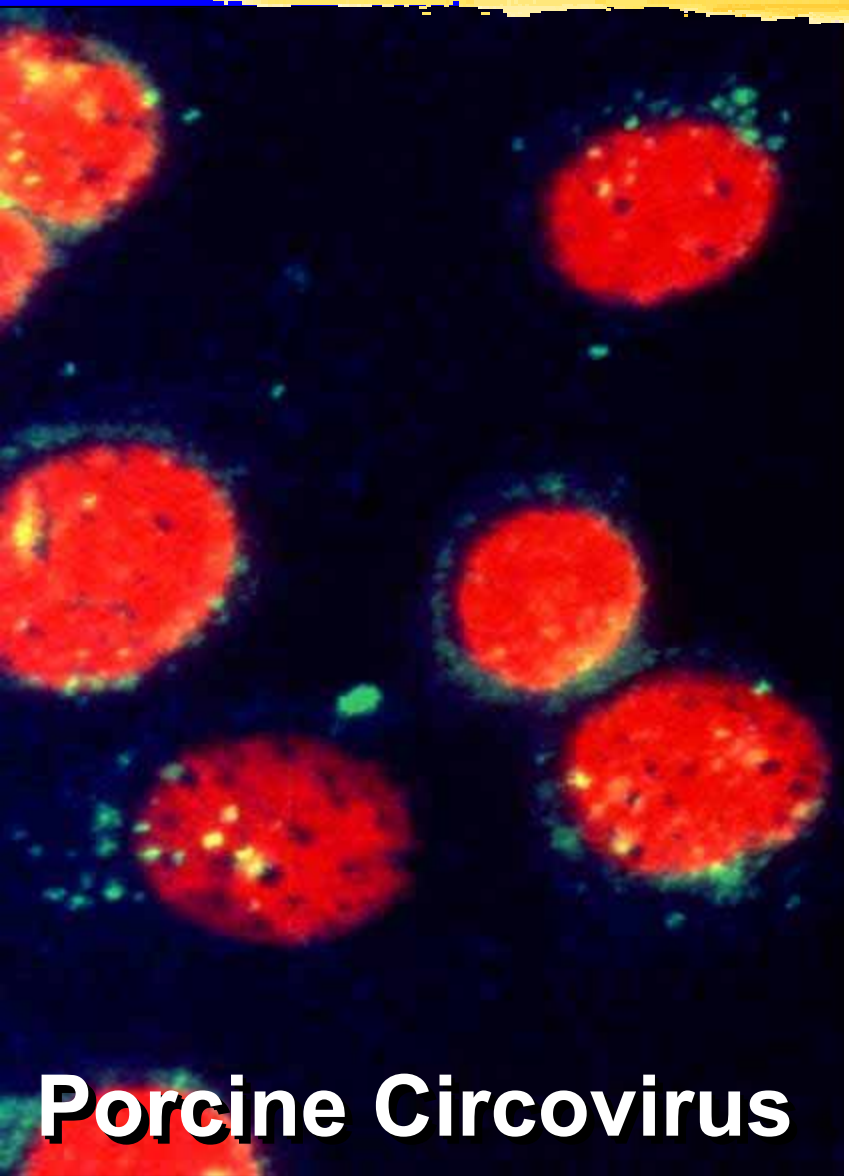




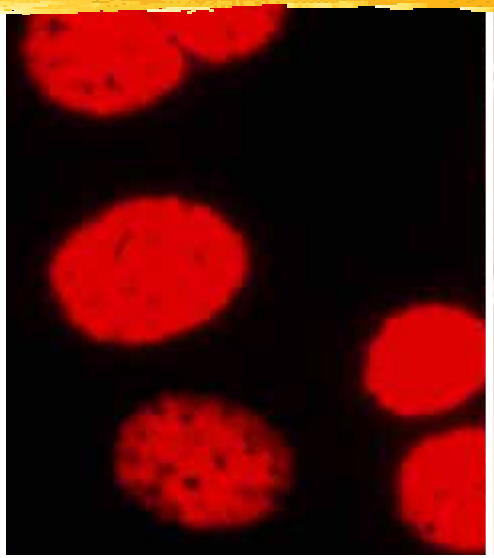


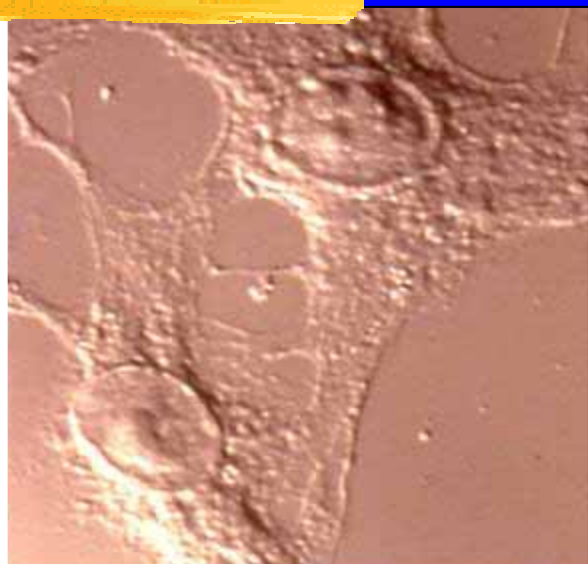


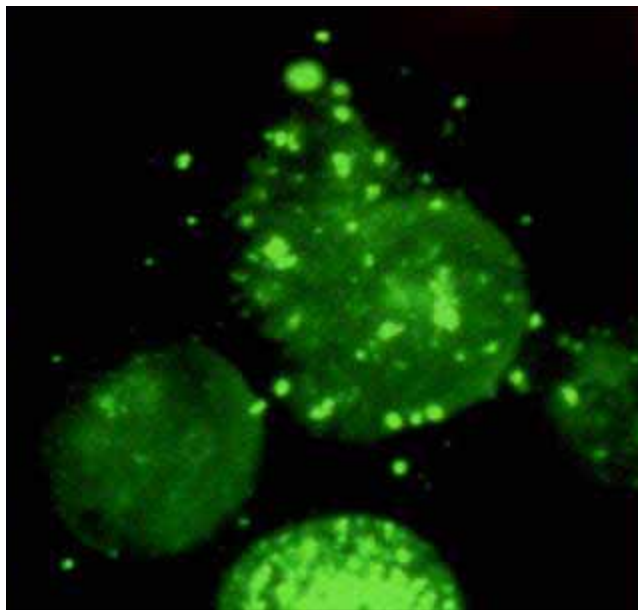
Confocal Laser Scanning Microscopy

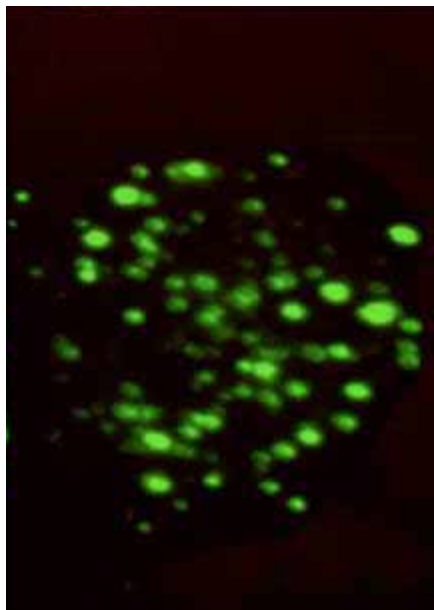


Porcine Circovirus









Aperio TECHNOLOGIES

Virtual Microscopy Solutions for Analysis & Discovery

Image Analysis

Typical ScanScope T3 System Configuration

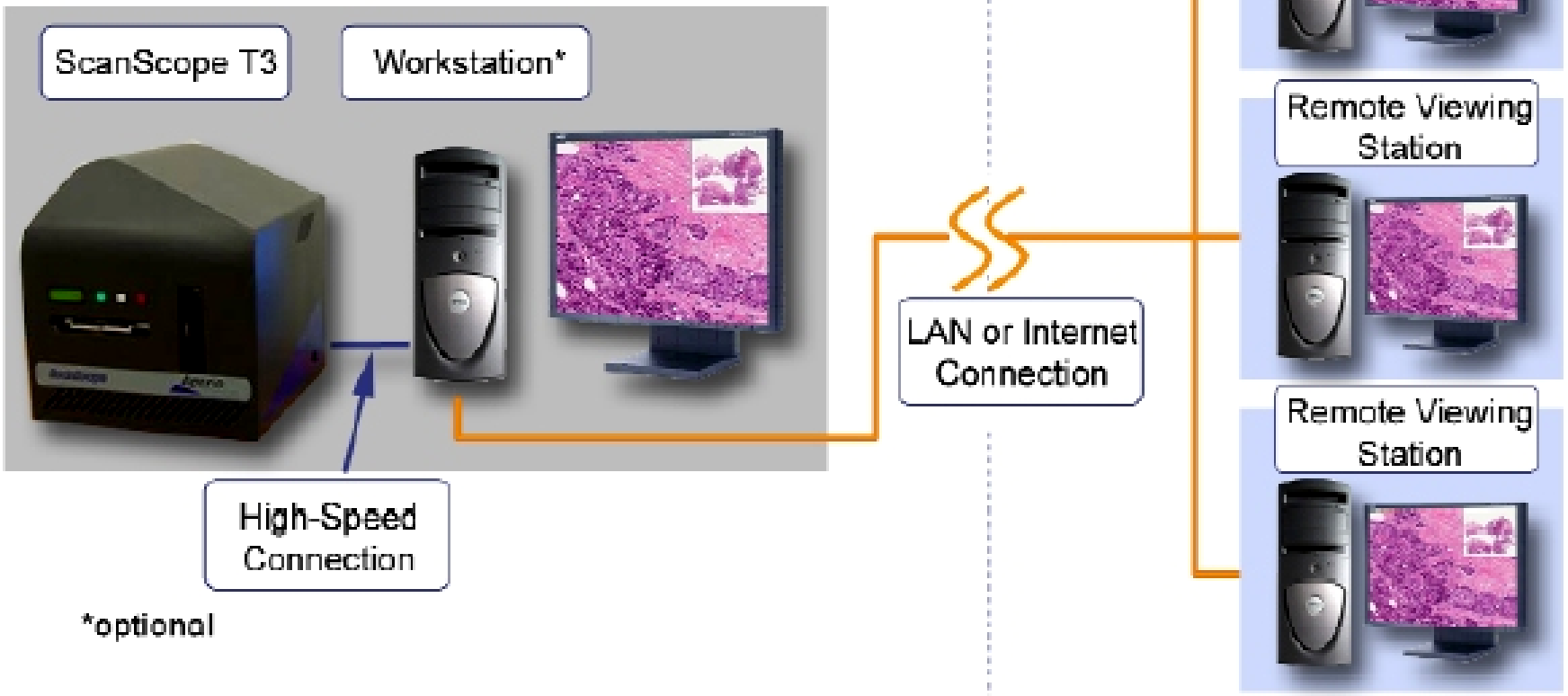


Image Analysis

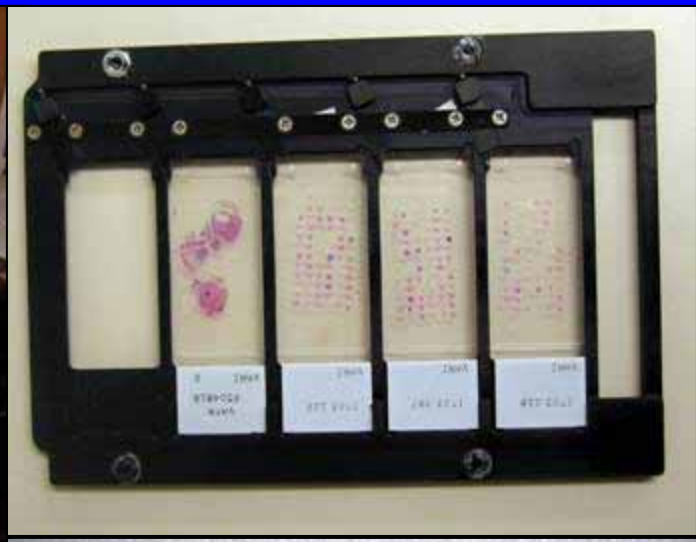


Image Analysis

The ScanScope® System

ScanScope Scanner

Digital Slide Information Management Software

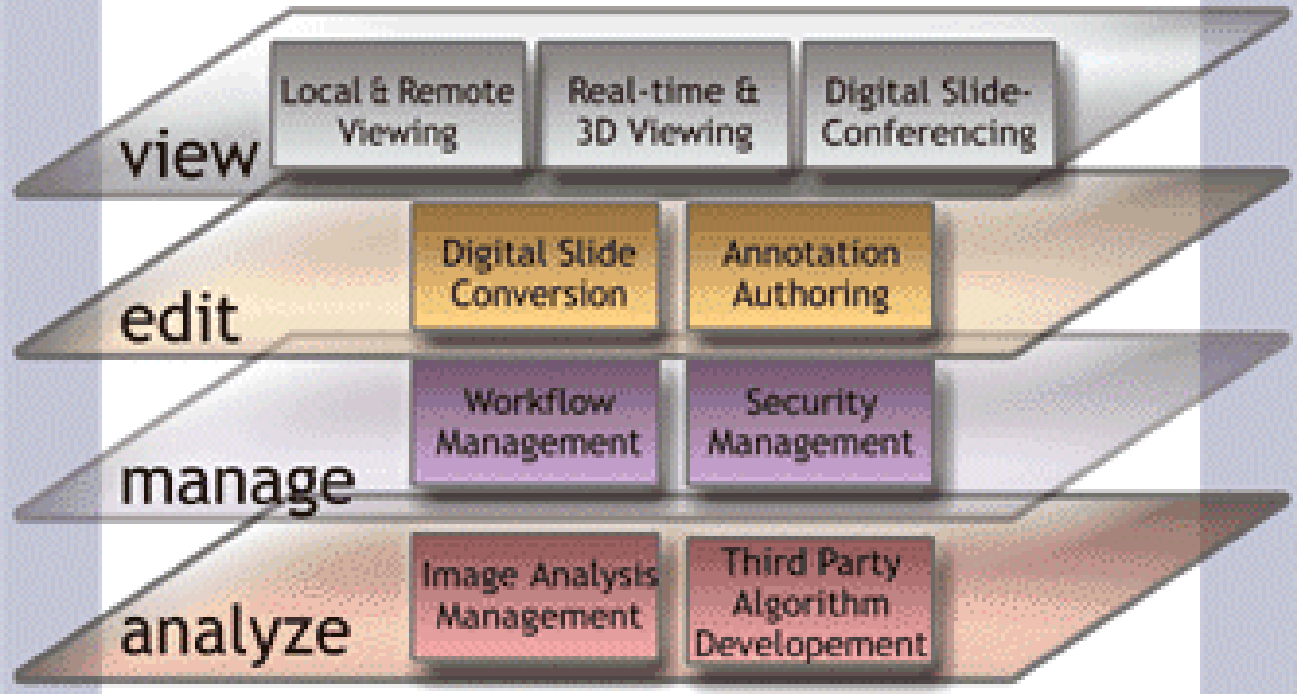
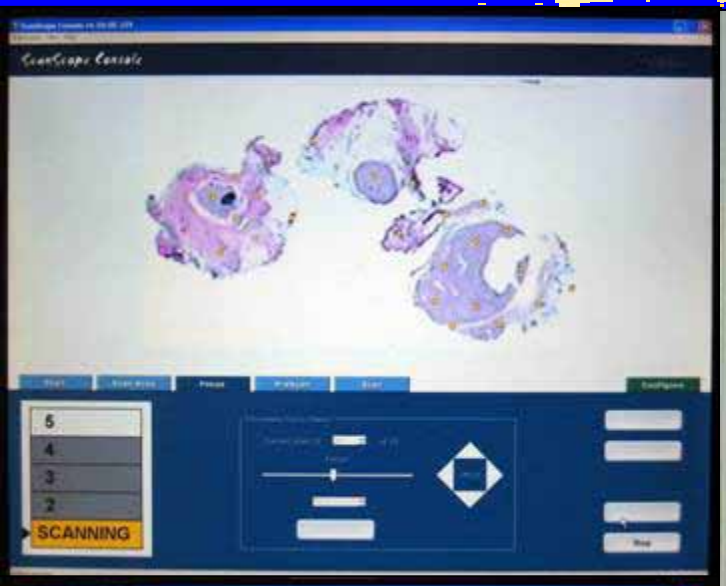
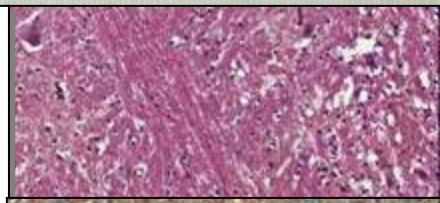
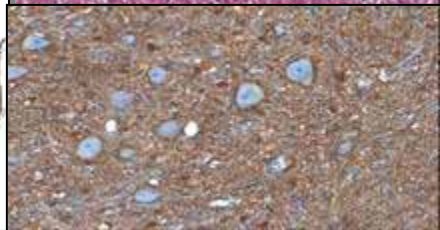


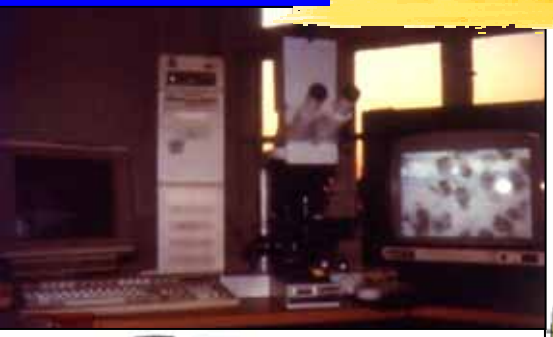
Image Analysis

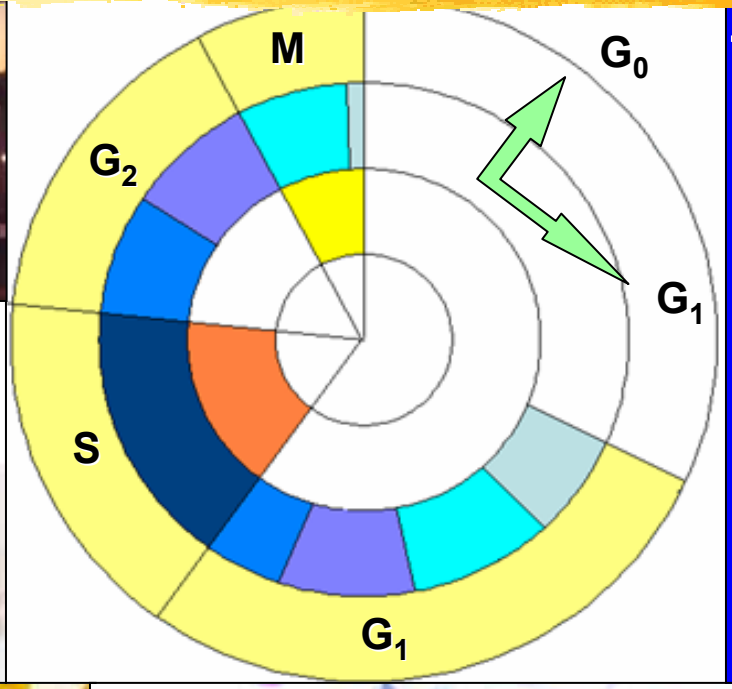






Analysis of Cell Proliferation





The intermediate step

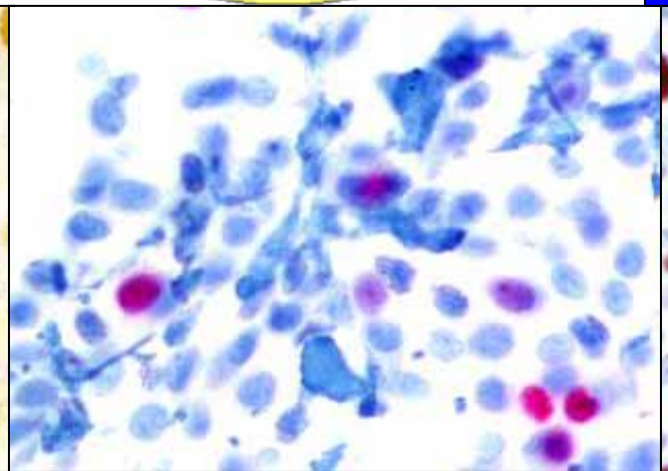
Ki-67

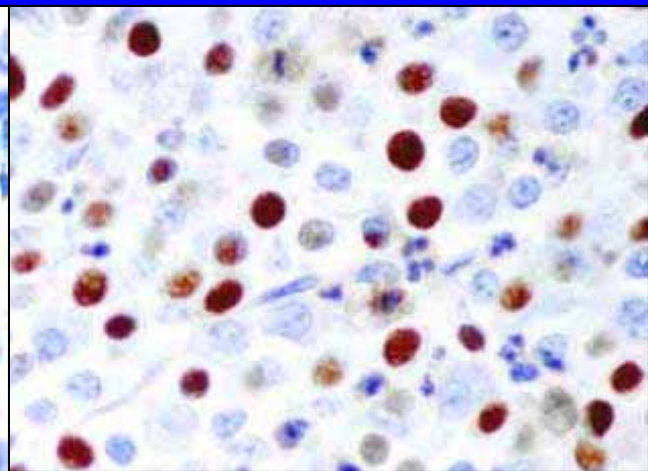
PCNA

increasing

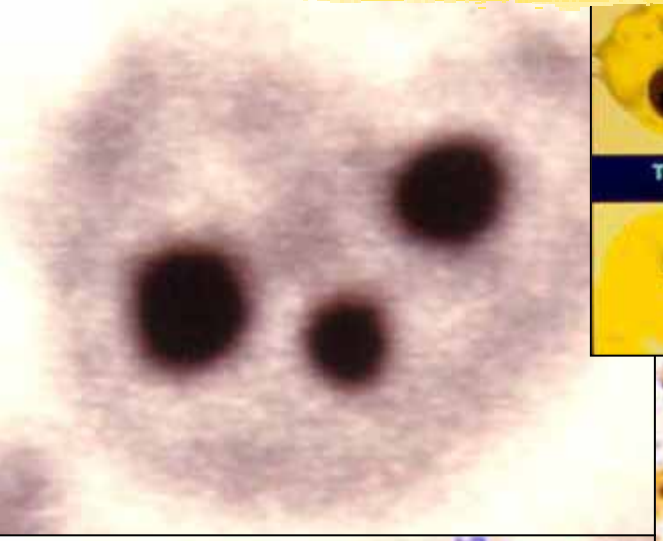
Mitotic Index

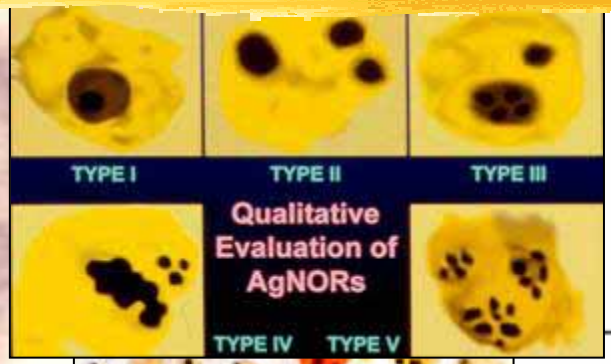
BrdU

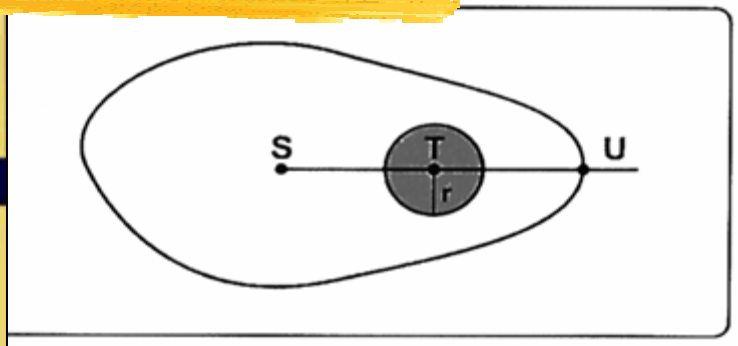


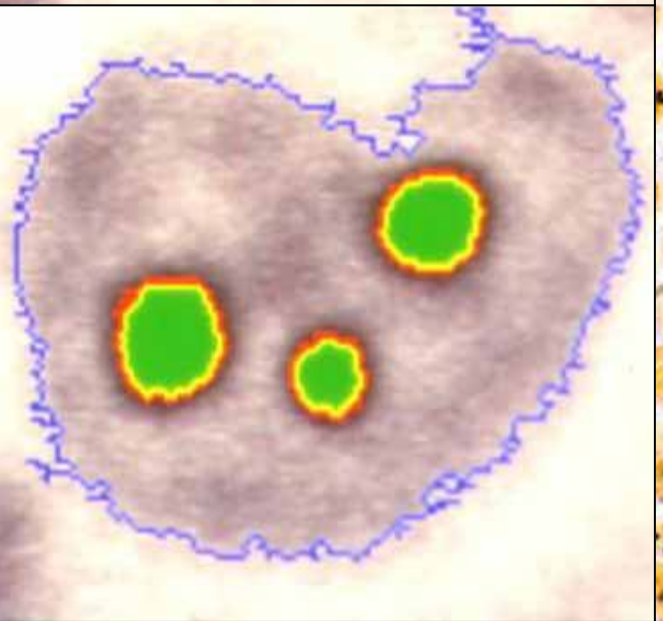


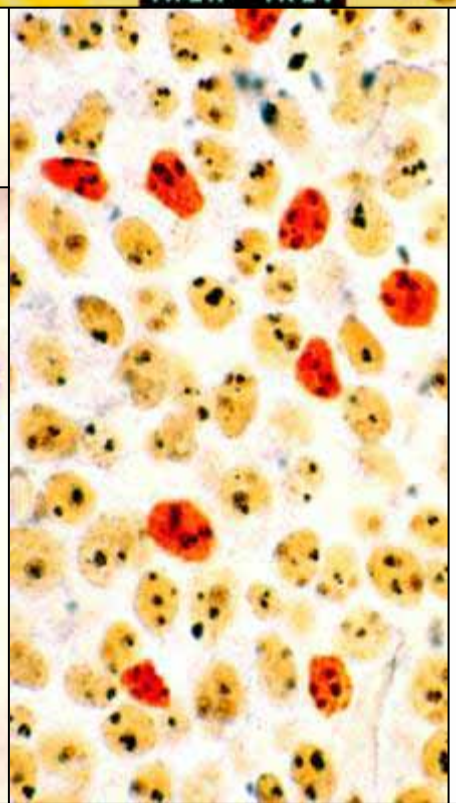
Analysis of Cell Proliferation

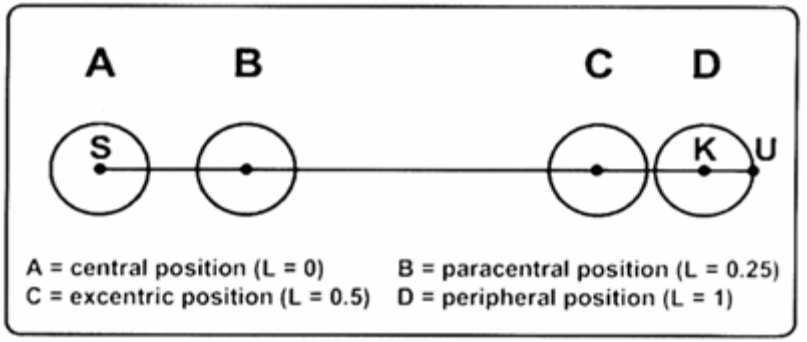


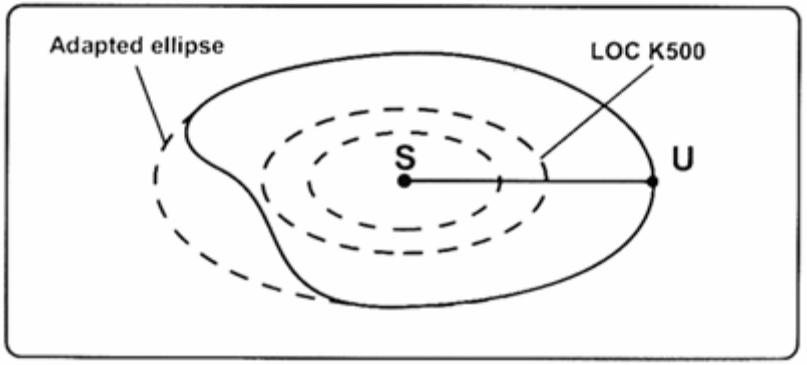




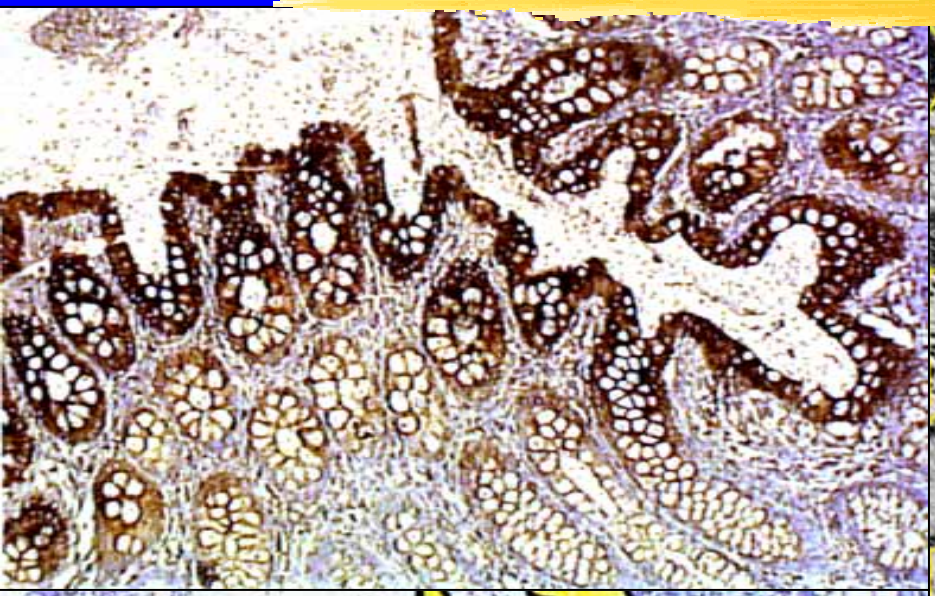


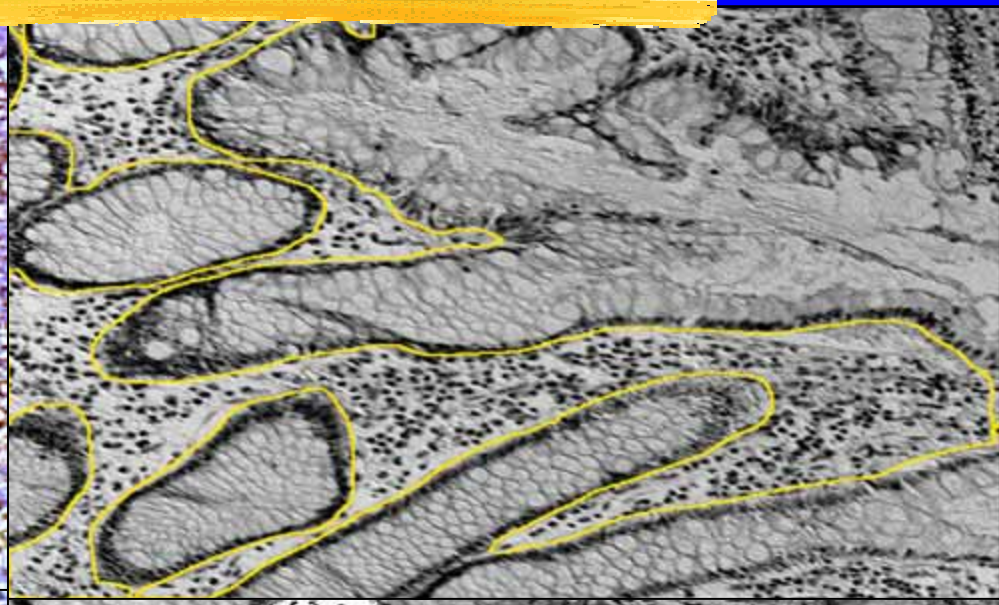


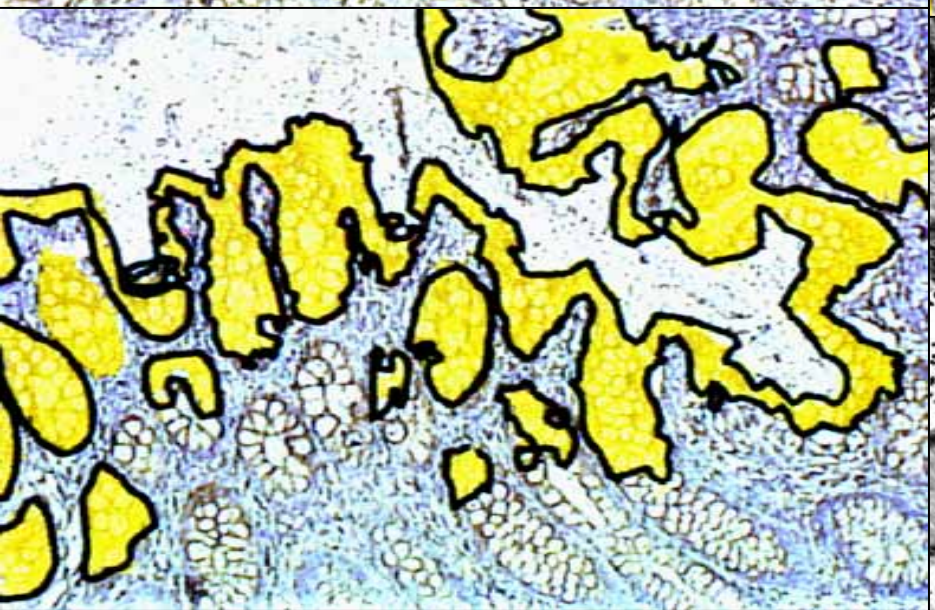


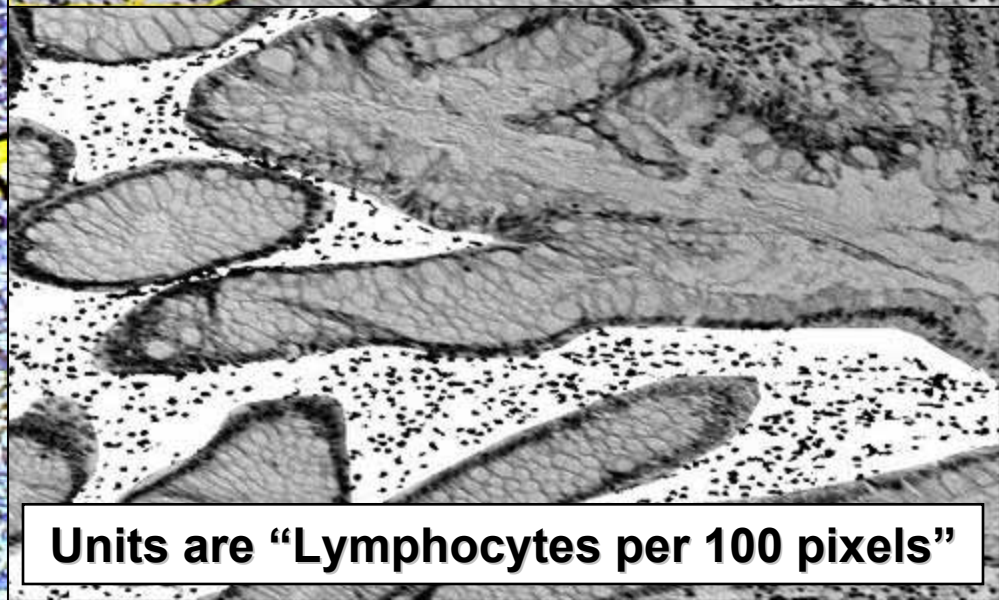


Quantification and Spatial Recognition



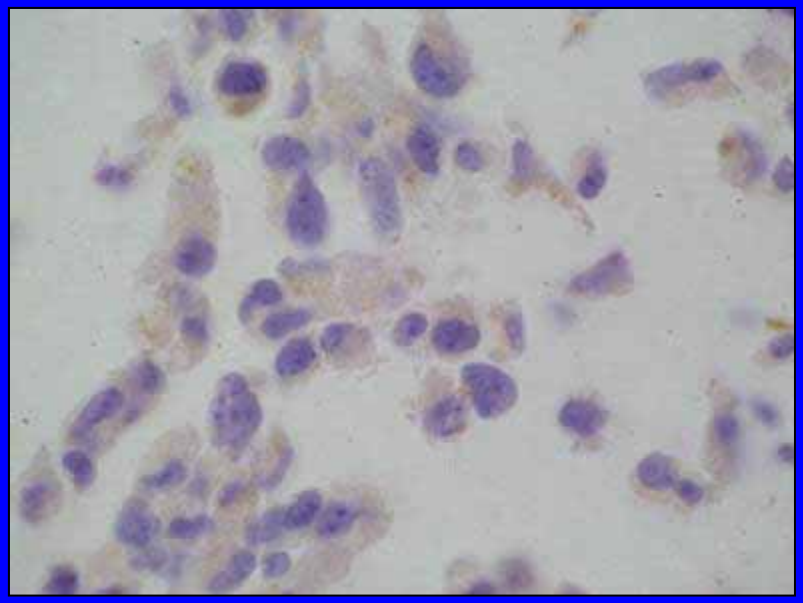


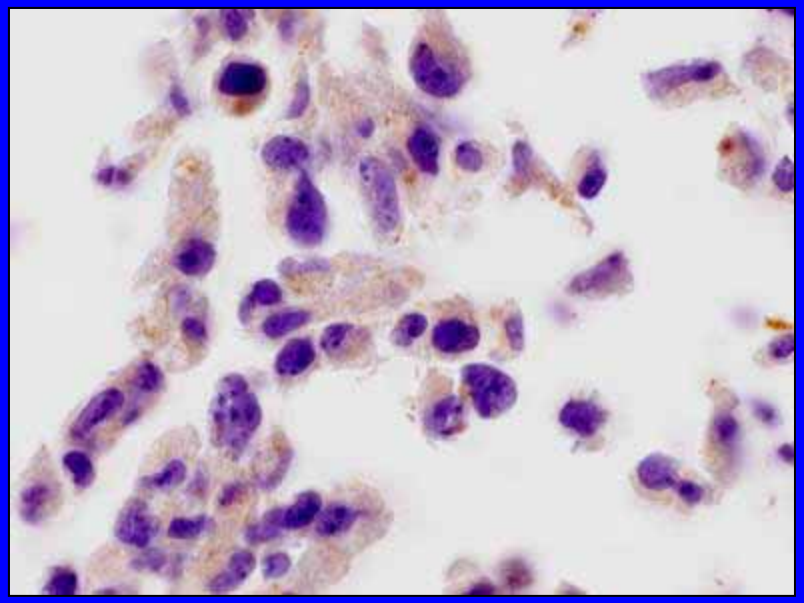




Units are “Lymphocytes per 100 pixels”

Nuance Multispectral Imaging

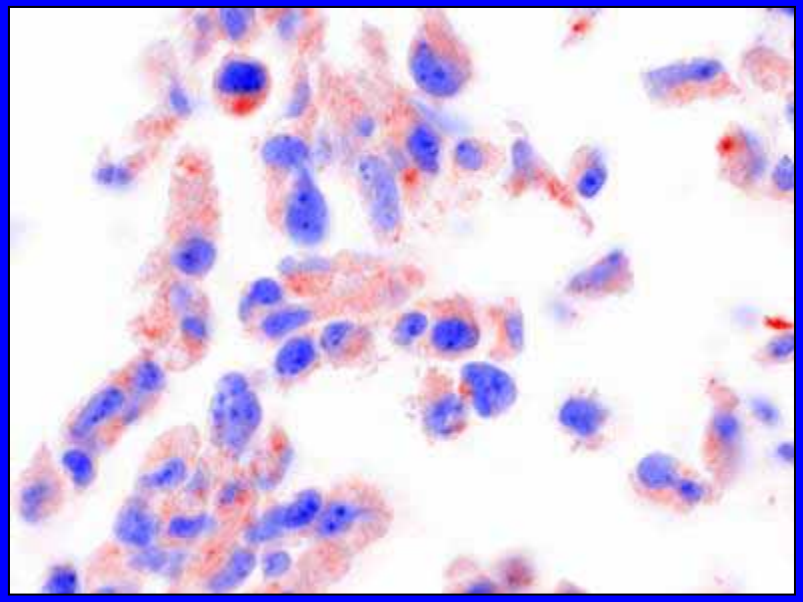




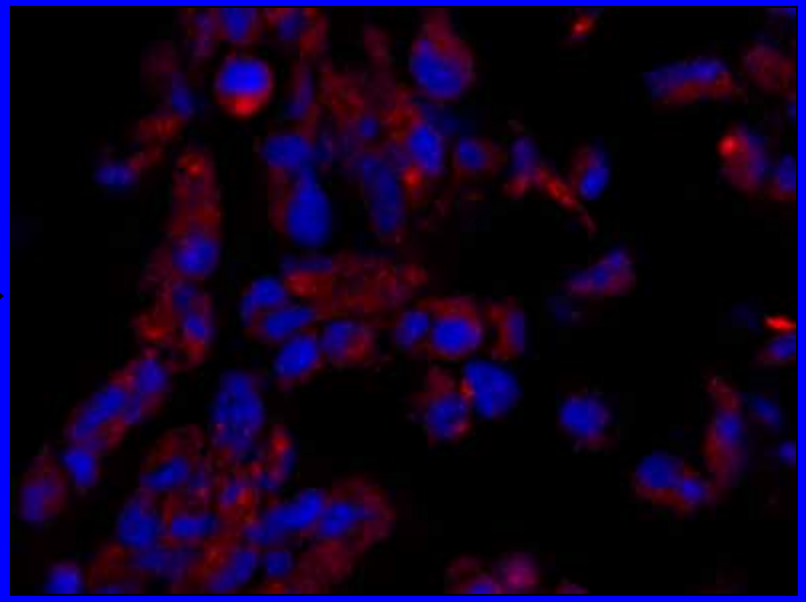
STEP 1: RGB image

STEP 2: Image Cube

Shown is the traditional image of IHC / DAB (step 1) and hematoxylin counter-stain with the spectral separation of the DAB from hematoxylin in step 2.



STEP 3: TIFF representation of the image cube



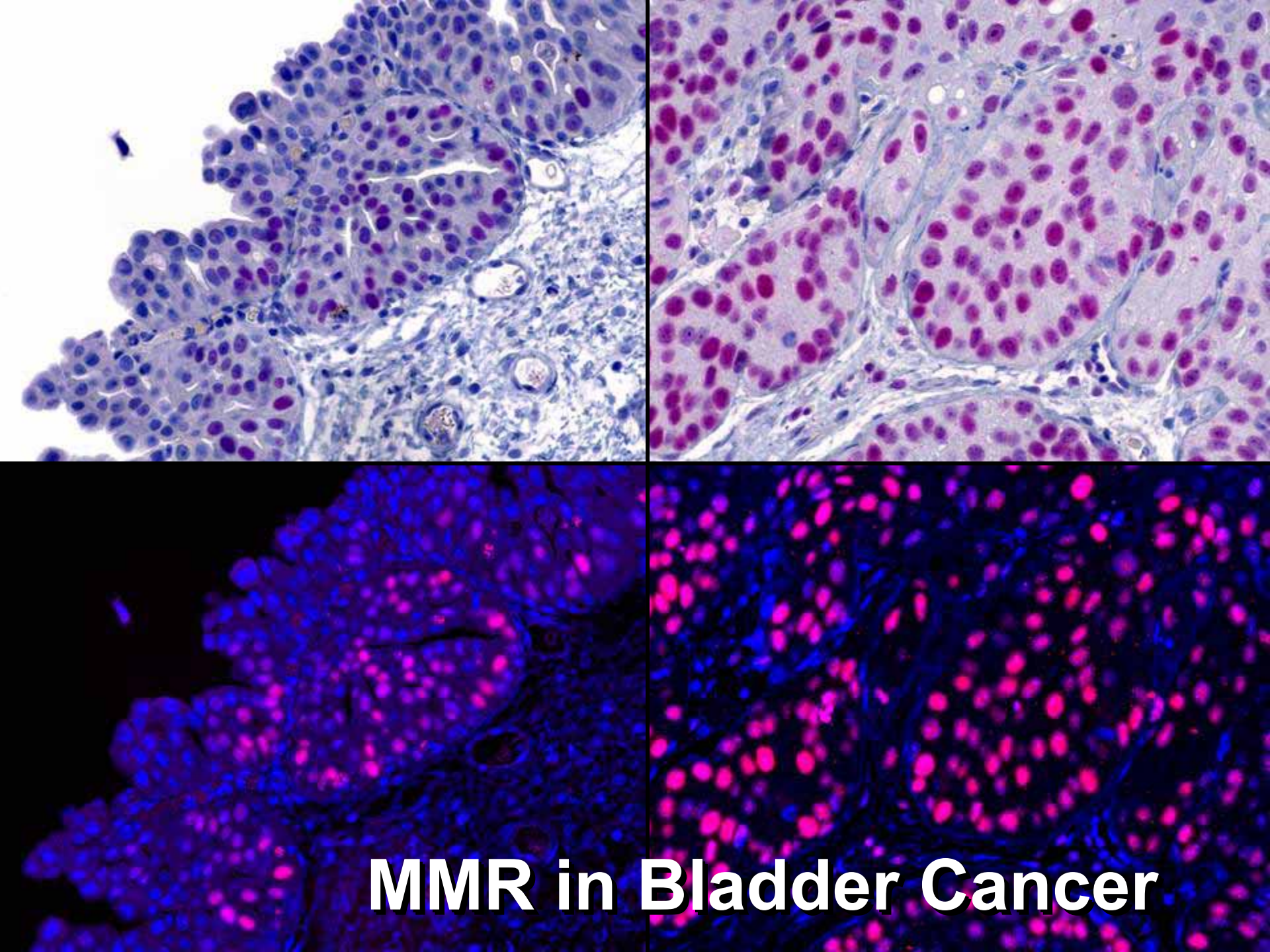
STEP 4: Pseudo-fluorescent image of the cube

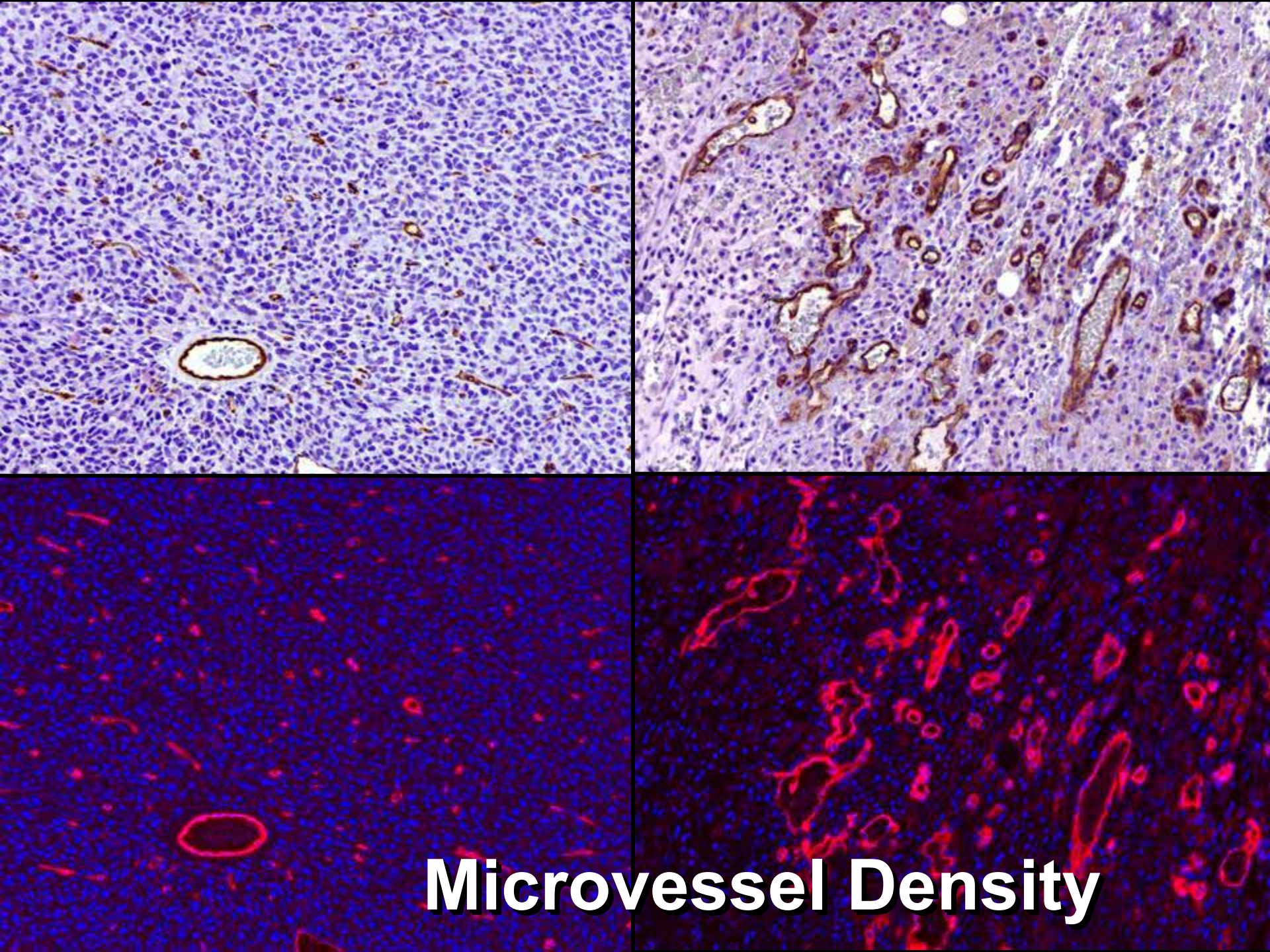
Brown has been changed to red by pseudo-color selection for ease of visualization and a pseudo fluorescent image created to facilitate quantification.

STEP 5: Quantification:

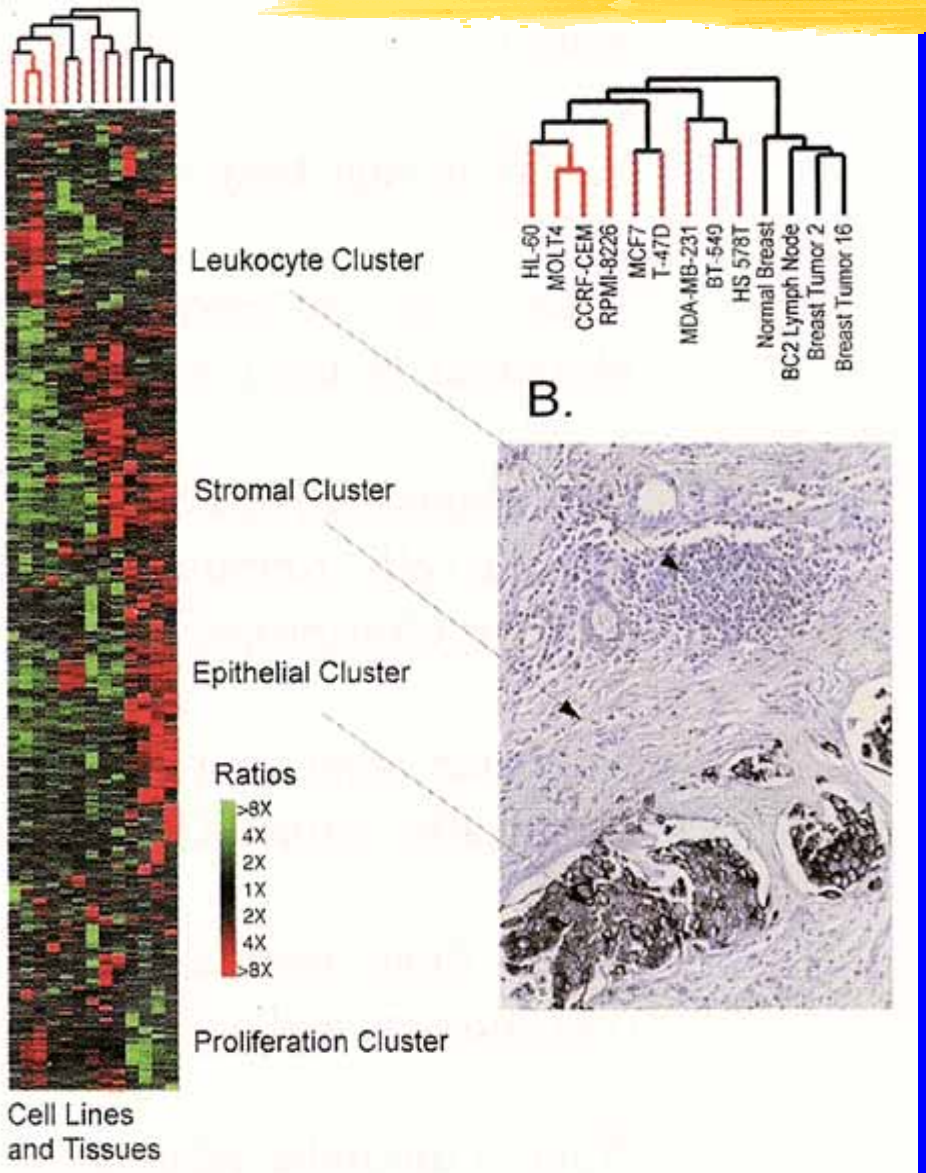
From the Pseudo-fluorescent image, the following may be quantified:

- **Number of cells**
- **Intensity per cell**
- **Intensity per number of cells**
- **Intensity per unit area**


MMR in Bladder Cancer


Microvessel Density

Pathology the Future: Needs



- Species specific monoclonal antibodies for diagnosis
- Oligo- and riboprobes directed against therapeutic and prognostic targets
- Tissue-array slides for controls
- Diagnostic oligo arrays specific for disease conditions as well as species and organ systems
- New instrumentation for molecular imaging
- Trained pathologists
- Trained technicians
- Collaborating scientists
- Informed clients and public