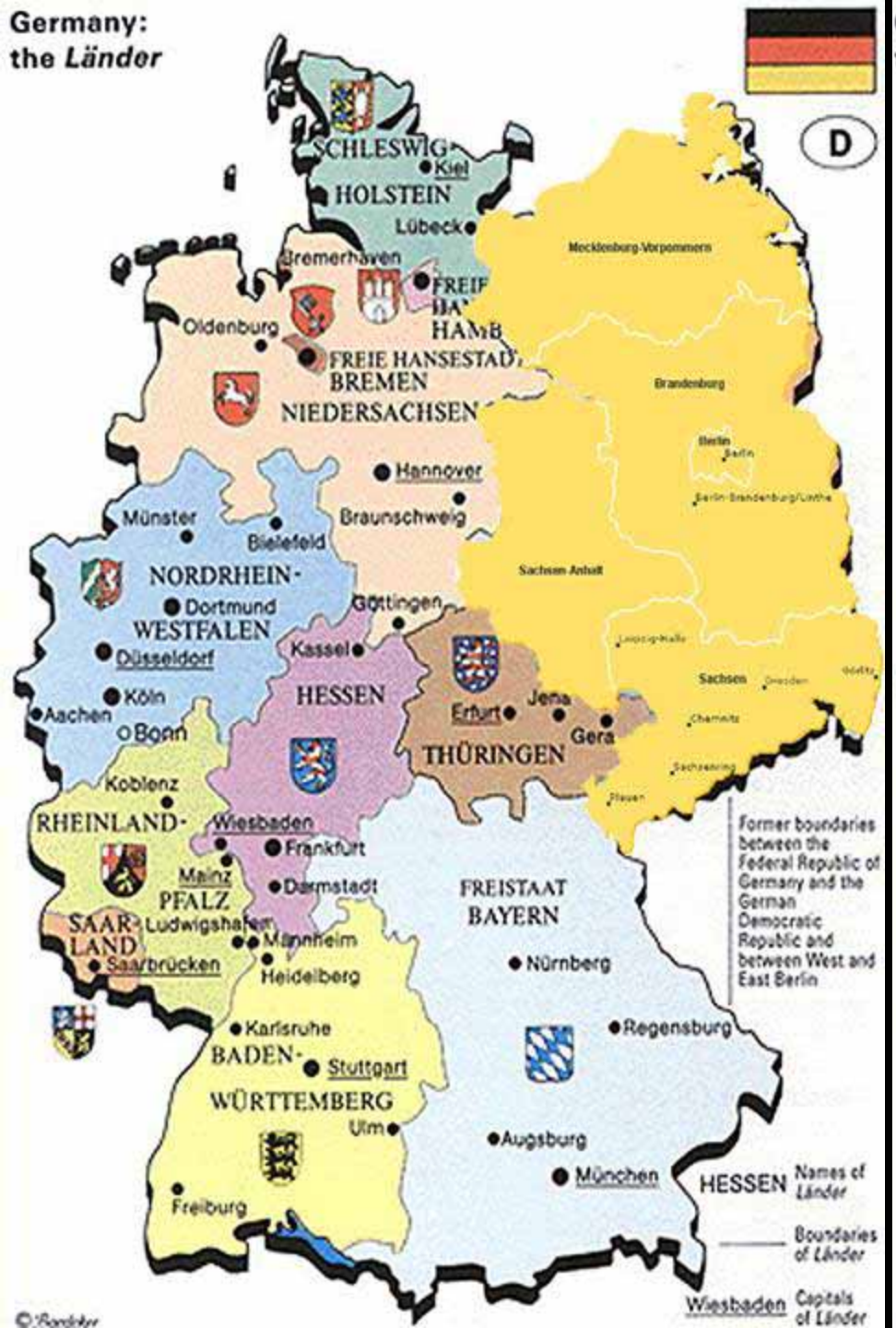
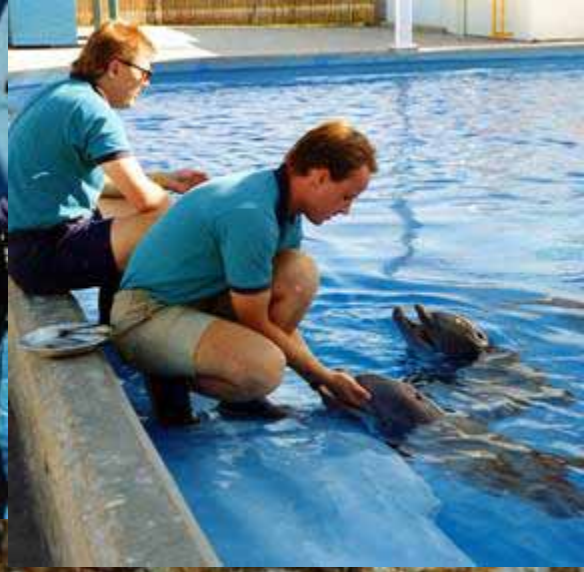

EUROPE
©GraphicMaps.com
15° E
30° E
45° E
60° E
Barents Sea
15° W
0°
ARCTIC CIRCLE
Norwegian Sea
White Sea
Iceland
Faroe Islands (Denmark)
Norway
Finland
Russian Federation
400 mi
400 km
60° N
Sweden
Estonia
Latvia
Lithuania
Atlantic Ocean
North Sea
Baltic Sea
Netherlands
Belgium
Ireland
UK
Belarus
Poland
Russian Federation (European Russia)
Ural Mountains
50° N
Luxembourg
Czech Rep
Slovakia
Ukraine
Kazakhstan
Liechtenstein
Switzerland
Austria
Hungary
Romania
Moldova
45° E
Bay of Biscay
France
Slovenia
Croatia
Bosnia and Herzegovina
Georgia
Caspian Sea
Monaco
Serbia and Montenegro
Black Sea
San Marino
Italy
Bulgaria
Andorra
Armenia
40° N
Corsica (France)
Vatican
Sardinia (Italy)
European Turkey
Turkey
Spain
Mediterranean Sea
Azerbaijan
Iran
Albania
Macedonia
Greece
Portugal
0°
Sicily (Italy)
Cyprus
Syria
N
Gibraltar (UK)
Malta
Crete (Greece)
ASIA
Lebanon
Algeria
15° E
30° E
Iraq
Israel
Jordan
Morocco
Tunisia
Libya
Egypt
30° N
AFRICA
Saudi Arabia
©GraphicMaps.com

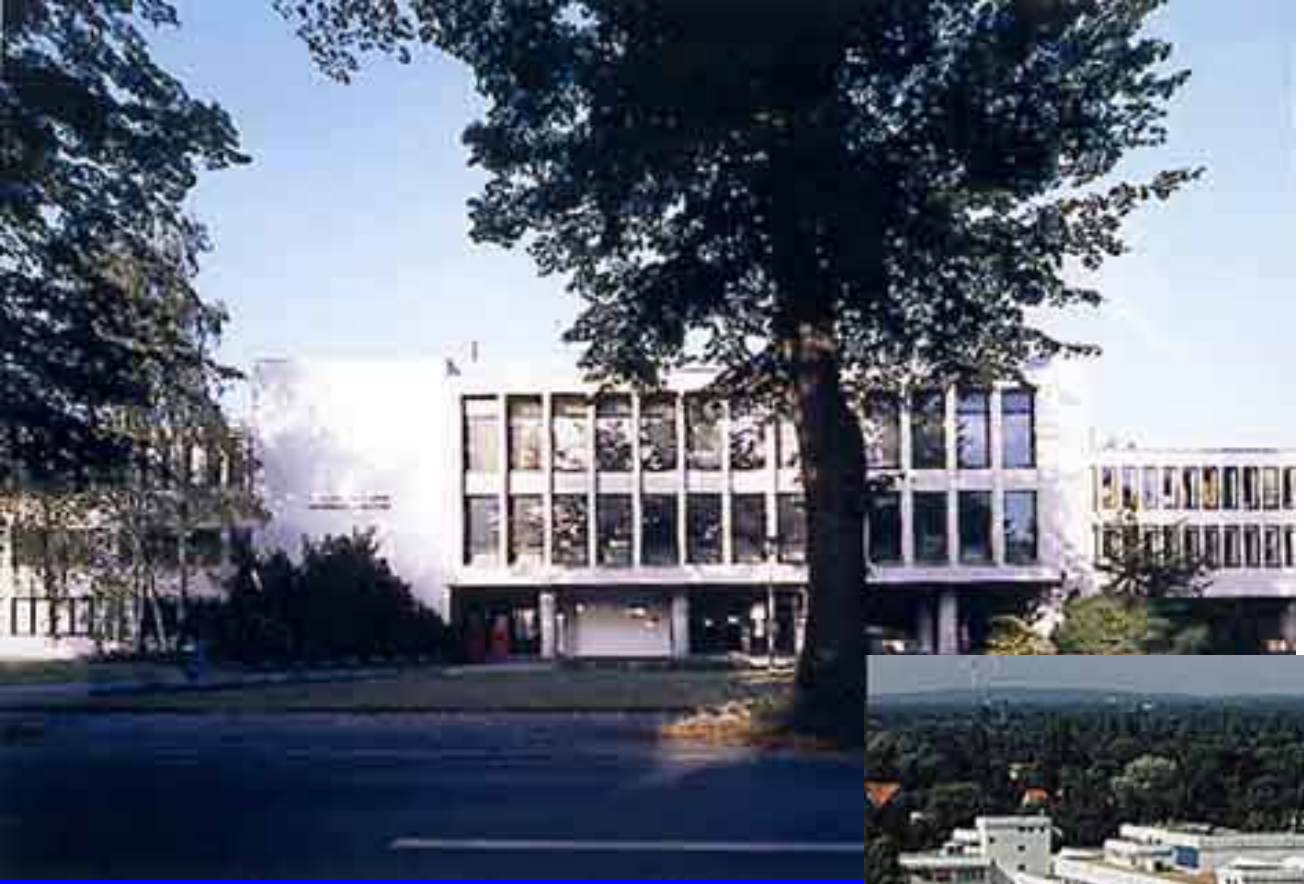

Germany:
the Länder
D
SCHLESWIG-HOLSTEIN
Kiel
Lübeck
Bremerhaven
Oldenburg
FREIE HANSESTADT BREMEN
NIEDERSACHSEN
Hannover
Braunschweig
Münster
Bielefeld
NORDRHEIN-WESTFALEN
Dortmund
Düsseldorf
Köln
Aachen
Bonn
Göttingen
Kassel
HESSEN
Koblenz
RHEINLAND-PFALZ
Wiesbaden
Frankfurt
Mainz
Darmstadt
Ludwigshafen
Mannheim
SAAR-LAND
Saarbrücken
Heidelberg
Karlsruhe
BADEN-WÜRTTEMBERG
Stuttgart
Ulm
Freiburg
Augsburg
München
Nürnberg
Regensburg
FREISTAAT BAYERN
Mecklenburg-Vorpommern
Brandenburg
Berlin
Sachsen-Anhalt
Erfurt
Jena
Gera
THÜRINGEN
Sachsen
Dresden
Chemnitz
Former boundaries between the Federal Republic of Germany and the German Democratic Republic and between West and East Berlin
HESSEN Names of Länder
Boundaries of Länder
Wiesbaden Capitals of Länder


Germany:
the Länder
D
SCHLESWIG-HOLSTEIN
Kiel
Lübeck
Rostock
MECKLENBURG-VORPOMMERN
Schwerin
Bremerhaven
Oldenburg
FREIE UND HANSESTADT HAMBURG
FREIE HANSESTADT BREMEN
NIEDERSACHSEN
BRANDENBURG
Potsdam
BERLIN
Hannover
Braunschweig
Magdeburg
SACHSEN-ANHALT
Dessau
Halle (Saale)
Cottbus
Münster
Bielefeld
NORDRHEIN-WESTFALEN
Dortmund
Düsseldorf
Köln
Aachen
Bonn
Göttingen
Kassel
HESSEN
Leipzig
FREISTAAT SACHSEN
Dresden
Chemnitz
Zwickau
Koblenz
RHEINLAND-PFALZ
Wiesbaden
Frankfurt
Mainz
Darmstadt
Ludwigshafen
Mannheim
SAAR-LAND
Saarbrücken
Heidelberg
Karlsruhe
BADEN-WÜRTTEMBERG
Stuttgart
Ulm
Freiburg
Augsburg
München
Nürnberg
Regensburg
FREISTAAT BAYERN
Erfurt
Jena
Gera
THÜRINGEN
Former boundaries between the Federal Republic of Germany and the German Democratic Republic and between West and East Berlin
HESSEN Names of Länder
Boundaries of Länder
Wiesbaden Capitals of Länder


RING TRUE


BODY GLO





FUBN

Matti Kiupel :

Prognostic factors for the evaluation of canine malignant lymphomas with special consideration of the importance of argyrophilic nucleolar organizer regions (AgNORs)

Prognostische Faktoren zur Beurteilung von caninen malignen Lymphomen unter besonderer Berücksichtigung der Bedeutung von argyrophilen Nukleolus organisierenden Regionen (AgNORs)

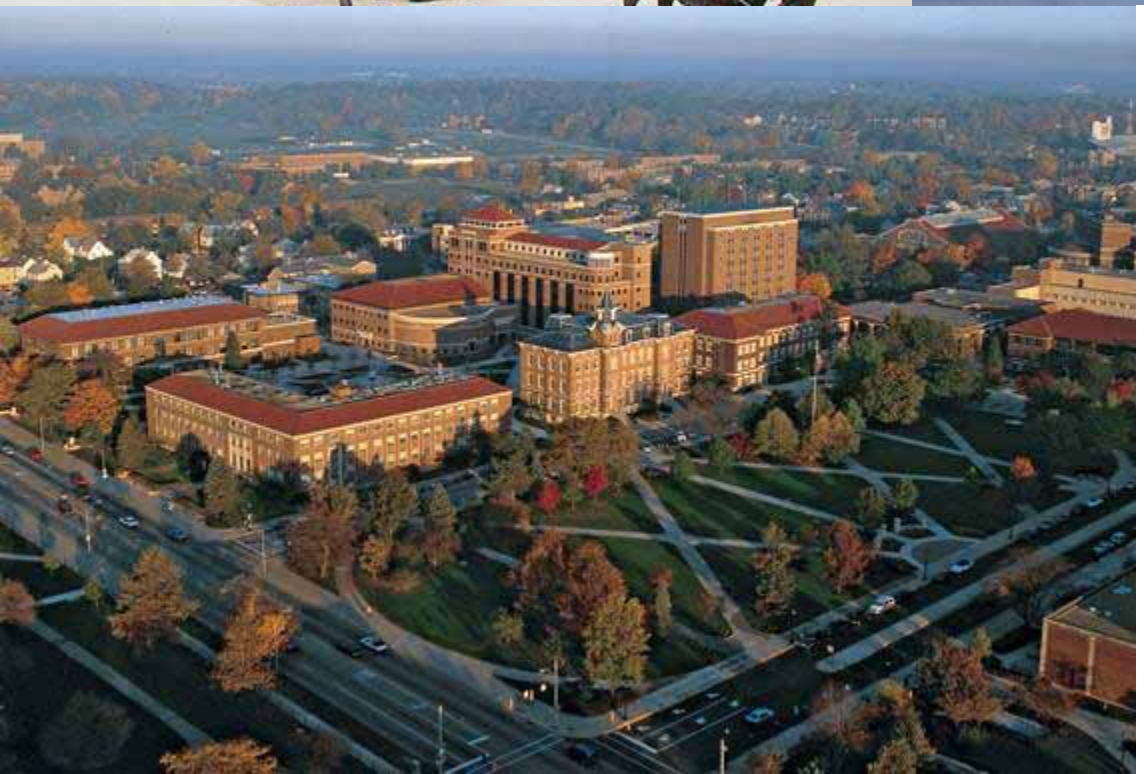
Vet Pathol **36**:292–300 (1999)

Prognostic Factors for Treated Canine Malignant Lymphoma

M. KIUPEL, E. TESKE, AND D. BOSTOCK

Institut für Veterinär-Pathologie der Freien Universität Berlin, Berlin, Germany (MK[1]);
Department of Clinical Sciences of Companion Animals, Utrecht University, Utrecht, The Netherlands (ET); and
Department of Clinical Veterinary Medicine, University of Cambridge, Cambridge, UK (DB)

Abstract. The aim of this study was to investigate the prognostic importance of different clinical, immunohistologic and tumorproliferation characteristics in dogs with malignant lymphoma treated with chemotherapy. From 74 dogs with malignant lymphoma at least one enlarged peripheral lymph node was taken for biopsy before chemotherapy following a standardized protocol (vincristine, cyclophosphamide, prednisolone, doxorubicin, and L-asparaginase). The variables evaluated as prognostic factors were age, sex, and tumor stage, as well as histomorphologic grade (Kiel classification, Working Formulation), immunophenotype (using markers for CD3 and CD79a), and cell proliferation (Ki-67, proliferation cell nuclear antigen, mitotic index, and argyrophil nucleolar organizer regions [AgNORs]) in extirpated lymph nodes. All markers were used on routinely formalin-fixed, paraffin-embedded tissues. The AgNORs were assessed qualitatively, based on the AgNOR



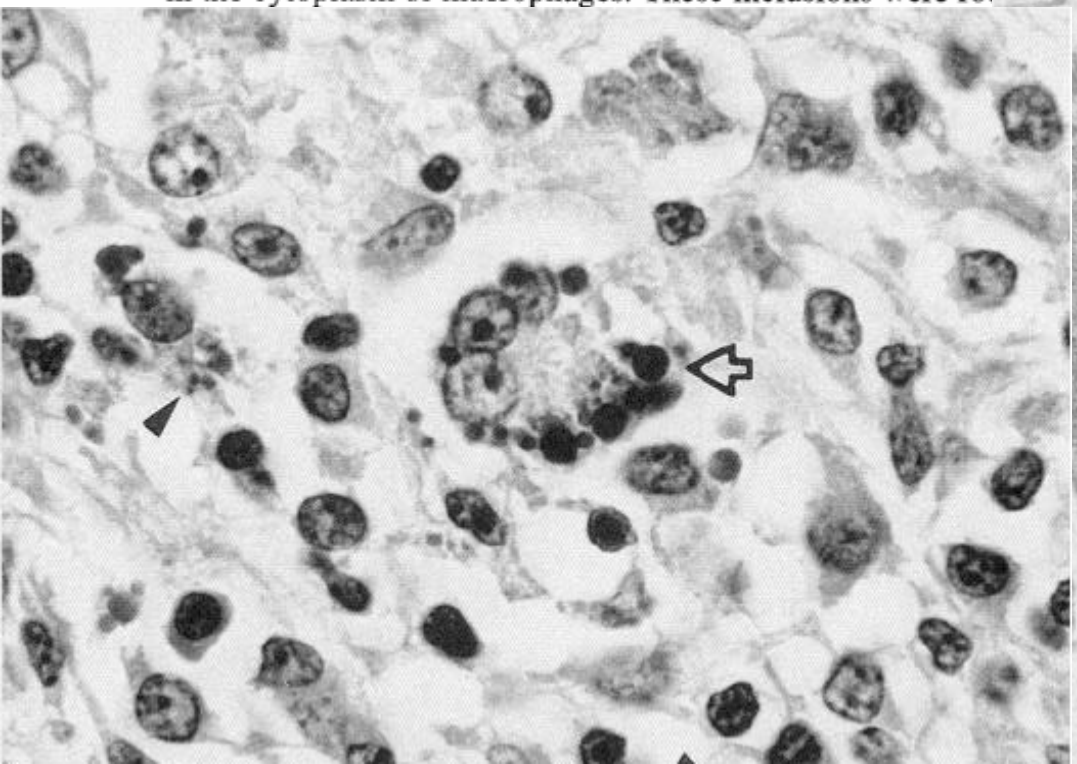
PURDUE™

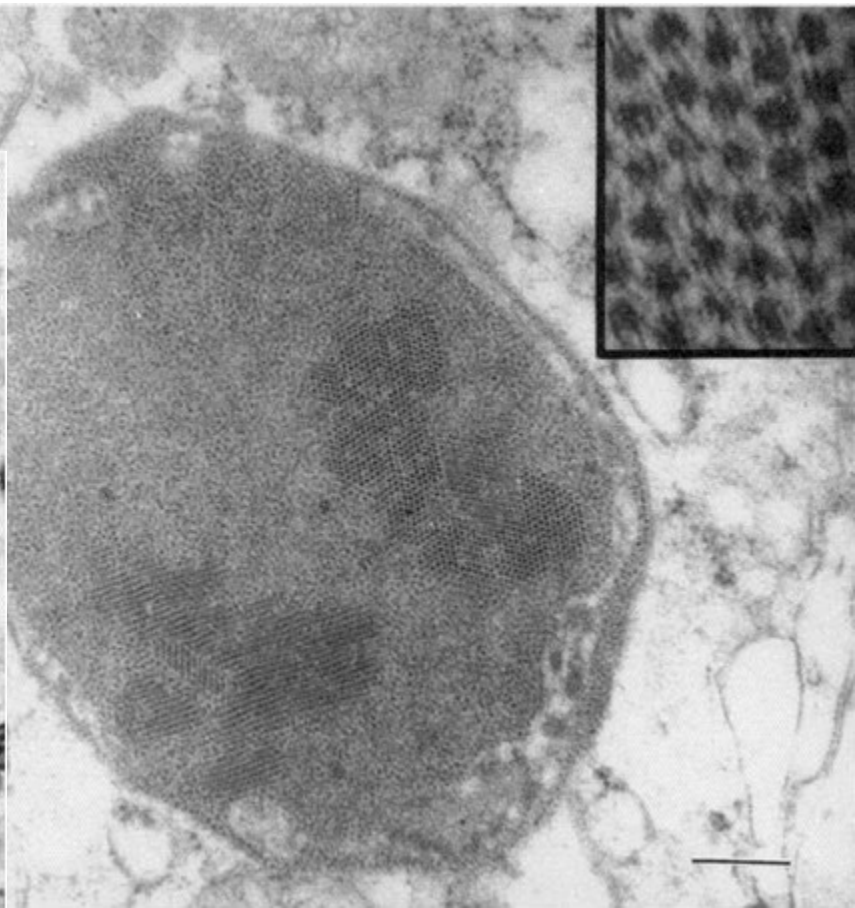
Vet Pathol **35**:303–307 (1998)

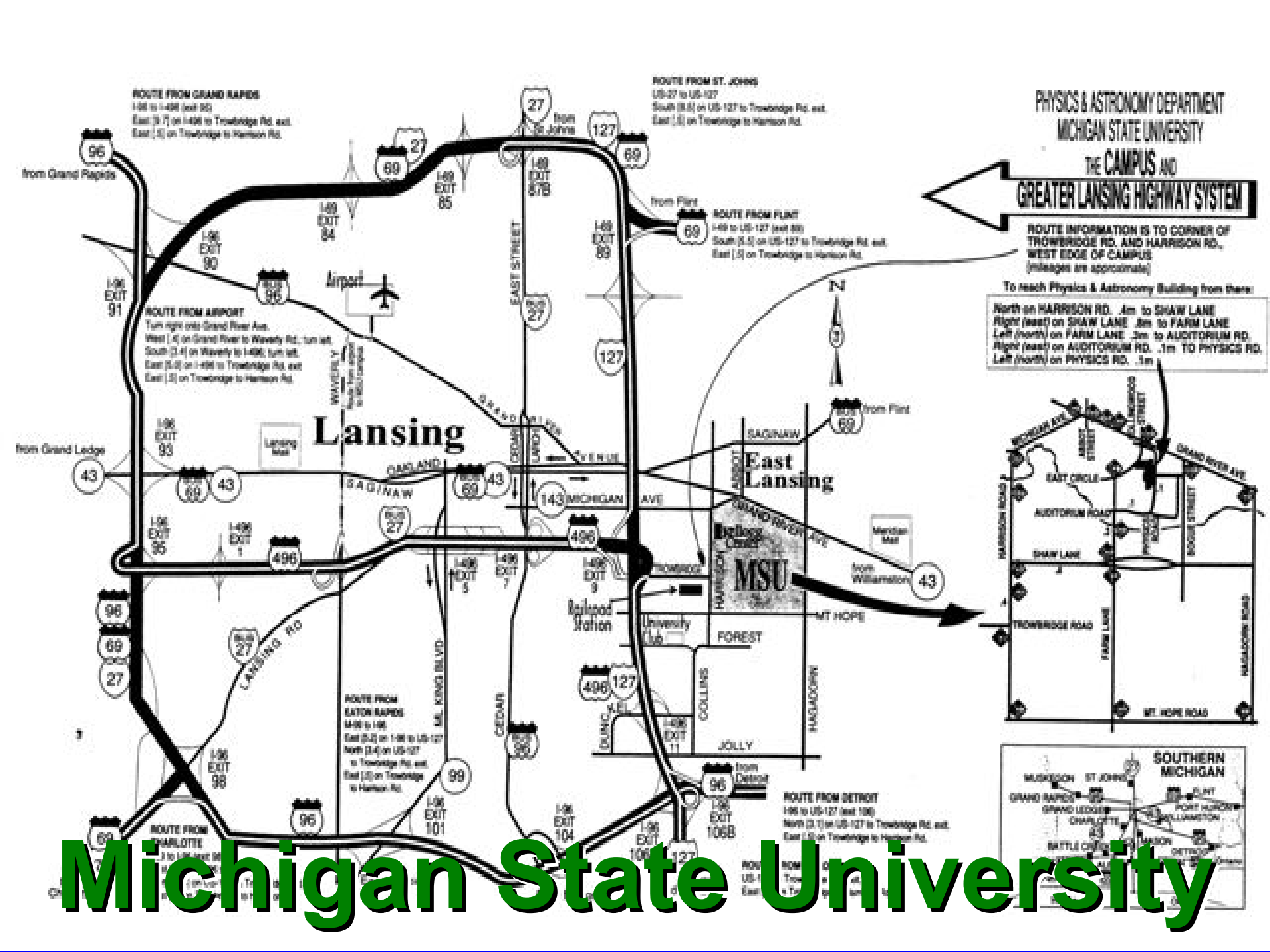
Circovirus-like Viral Associated Disease in Weaned Pigs in Indiana

M. KIUPEL, G. W. STEVENSON, S. K. MITTAL, E. G. CLARK, AND D. M. HAINES

Abstract. Inclusion bodies with staining affinity and ultrastructural characteristics typical of circoviruses that stained positive for porcine circovirus (PCV)-like virus were demonstrated in association with granulomatous lesions in multiple tissues of three clinically ill 10- to 12-week-old pigs. A syndrome of poor growth and wasting in 5–15% of weaned pigs was an intermittent problem on a 450-sow one-site farrow-to-finish swine farm in Indiana. Routine diagnostic testing did not demonstrate a cause. Gross examination of three representative weaned pigs from two farrowing groups over a 1-m
and interstitial pneumonia. A unique microscopic finding f
lymphoid tissues associated with large numbers of multin
in the cytoplasm of macrophages. These inclusions were ro







Michigan State University


Michigan State University


Michigan State University

Michigan State University


Michigan State University


Michigan State University


Diagnostic Center for
Population and Animal Health

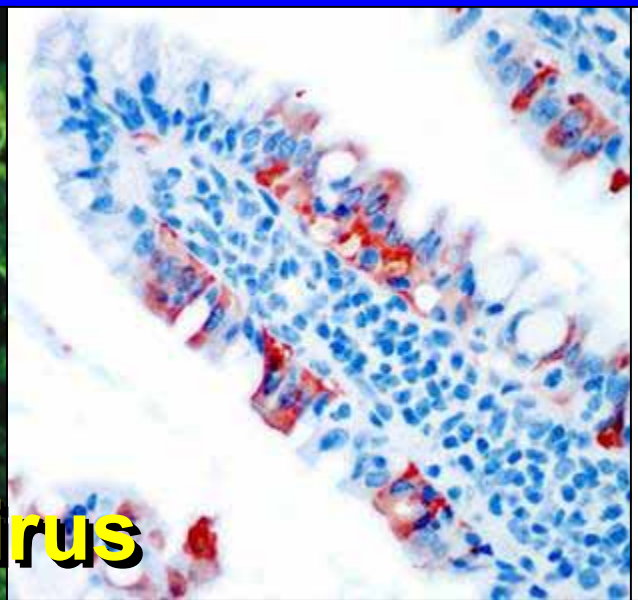
Diagnostic Molecular Pathology

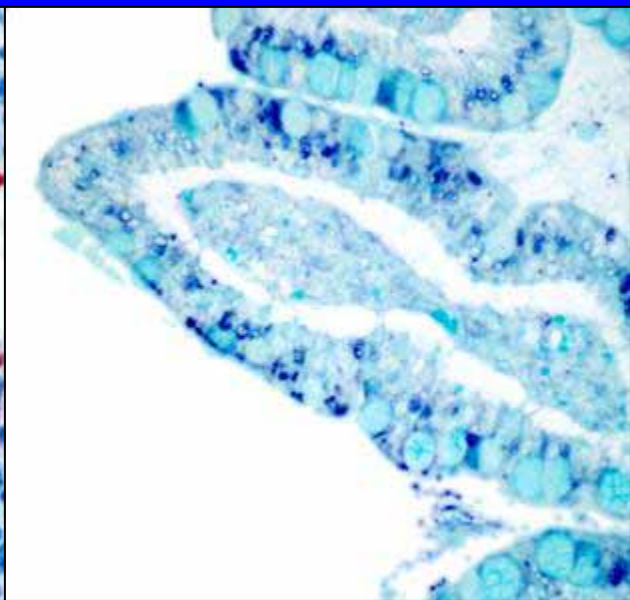
Dr. med. vet. Matti Kiupel, BS, MS, PhD, DACVP

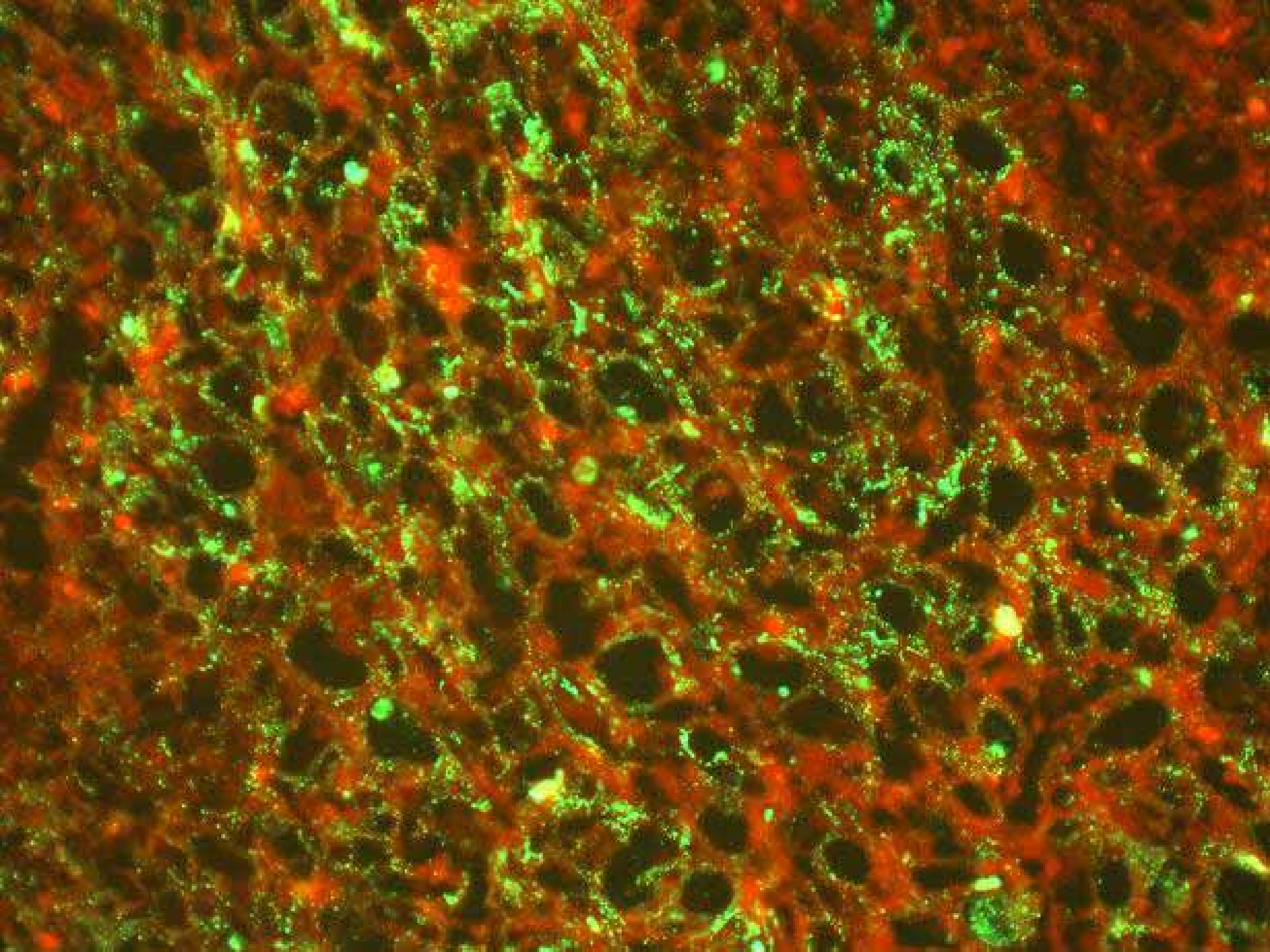
Diagnostic Center for Population and Animal Health
College of Veterinary Medicine, Michigan State University
4125 Beaumont Road Room 152A, Lansing, MI 48848, USA
Tel.: ** 517 432 2670; Fax: ** 517 432 6557;
E-mail: kiupel@dcpah.msu.edu

Visualizing a Target

- Fluorescent Antibody Staining
- Immunohistochemistry
- In-situ Hybdridization
  - Fluoresecent
  - Chromogenic







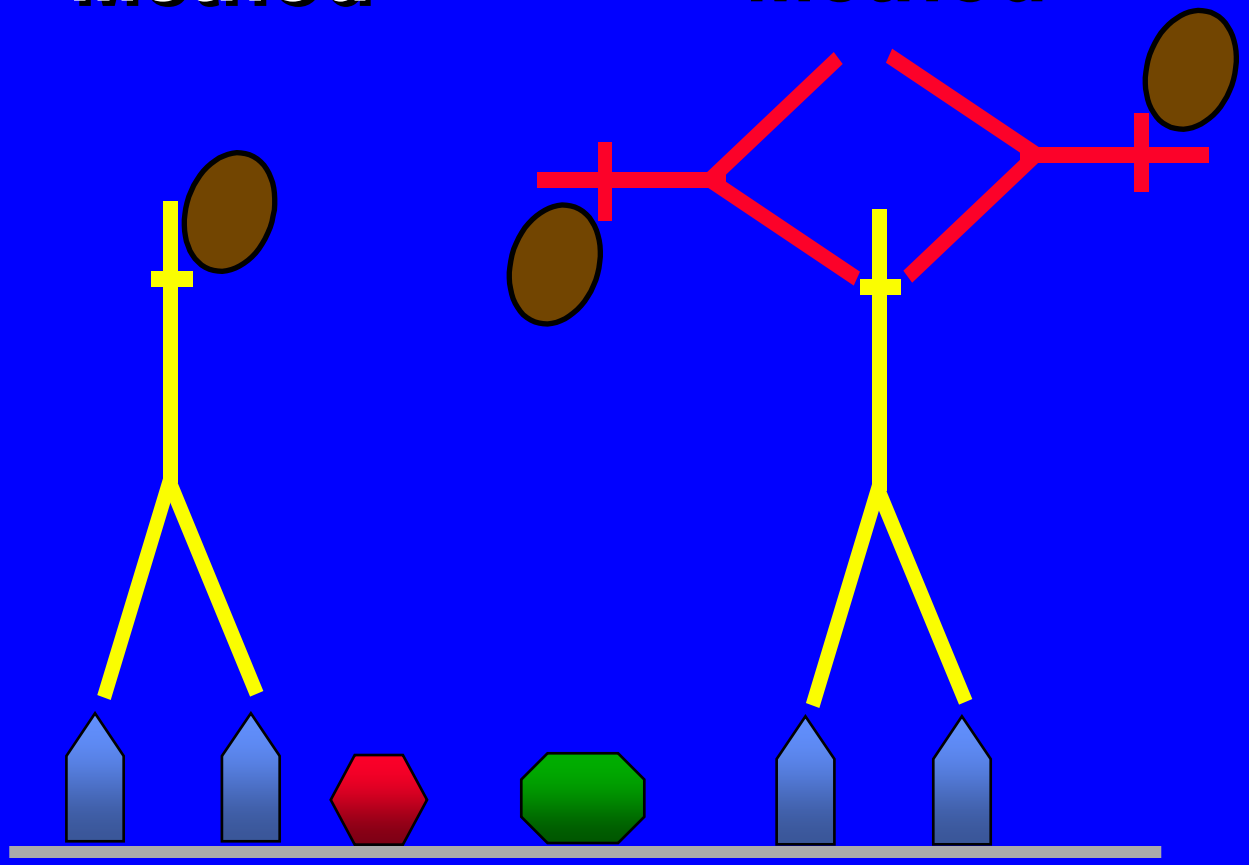
Fluorescent Antibody Staining

- Well established, widely used technique
  - Primary antibody to detect target
  - Either direct or indirect labeling
- Diagnostic tool for fresh/frozen material
- Identification of specific infectious organisms
- Commonly used on cell cultures
- Antigen: protein, glyco- or lipoprotein, carbohydrate

Fluorescent Antibody Testing

Direct Method

Indirect Method



Unlabelled Antibody

Labeled 1st Antibody

Labeled 2nd Antibody

Antigens

Advantages and Disadvantages of Fluorescent Antibody Staining

- Used on fresh/frozen samples
- Not useful for formalin fixed material
- Fast, relatively cheap
- Easy to detect, highly sensitive
- Signal can be quantitated
- Architecture difficult to evaluate
- Not a good tool for herd surveillance
- Requires expensive specialized equipment
- Fluorescent signals will commonly fade
- Autofluorescence
- Success depends on antibody (mono- vs. polyclonal)

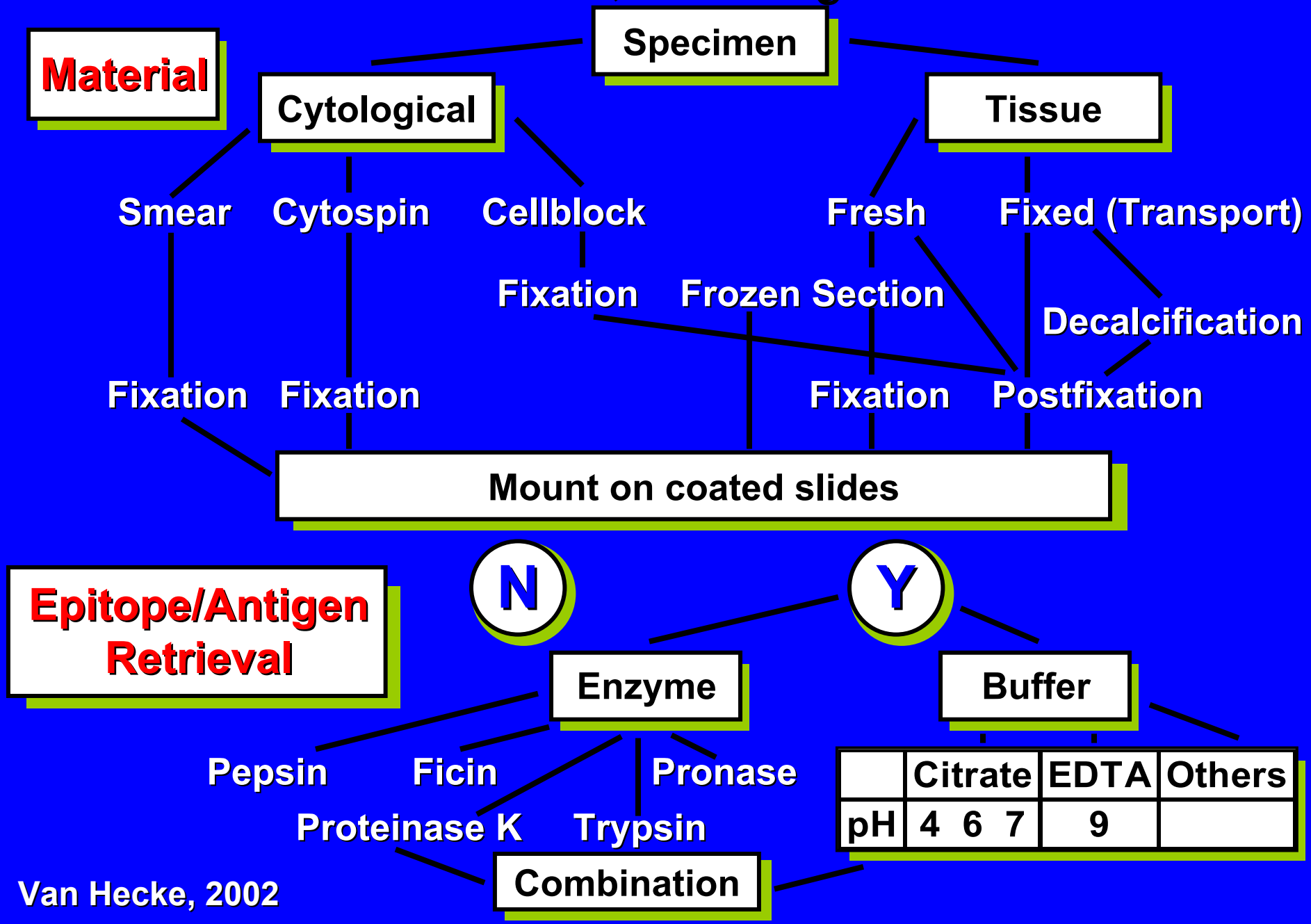
Immunohistochemistry

Lawsonia intracellularis

Immunohistochemistry

- Well established, widely used technique
  - Primary antibody to detect target
  - Detection system
  - Visualizing reagent
- Antigen: protein, glyco- or lipoprotein, carbohydrate
- Diagnostic tool for routine formalin fixed material
  - Identification of specific infectious organisms
  - Identification of cell types and tumor entities
  - Determination of primary site of tumor
  - Determination of tumor malignancy

Choices to Make, Challenges to Take



Van Hecke, 2002

4 Core Problems

- Fixation, Processing, Microtomy
- IHC Methodologies
- Quality of Primary Antibody
- Microscopic Interpretation

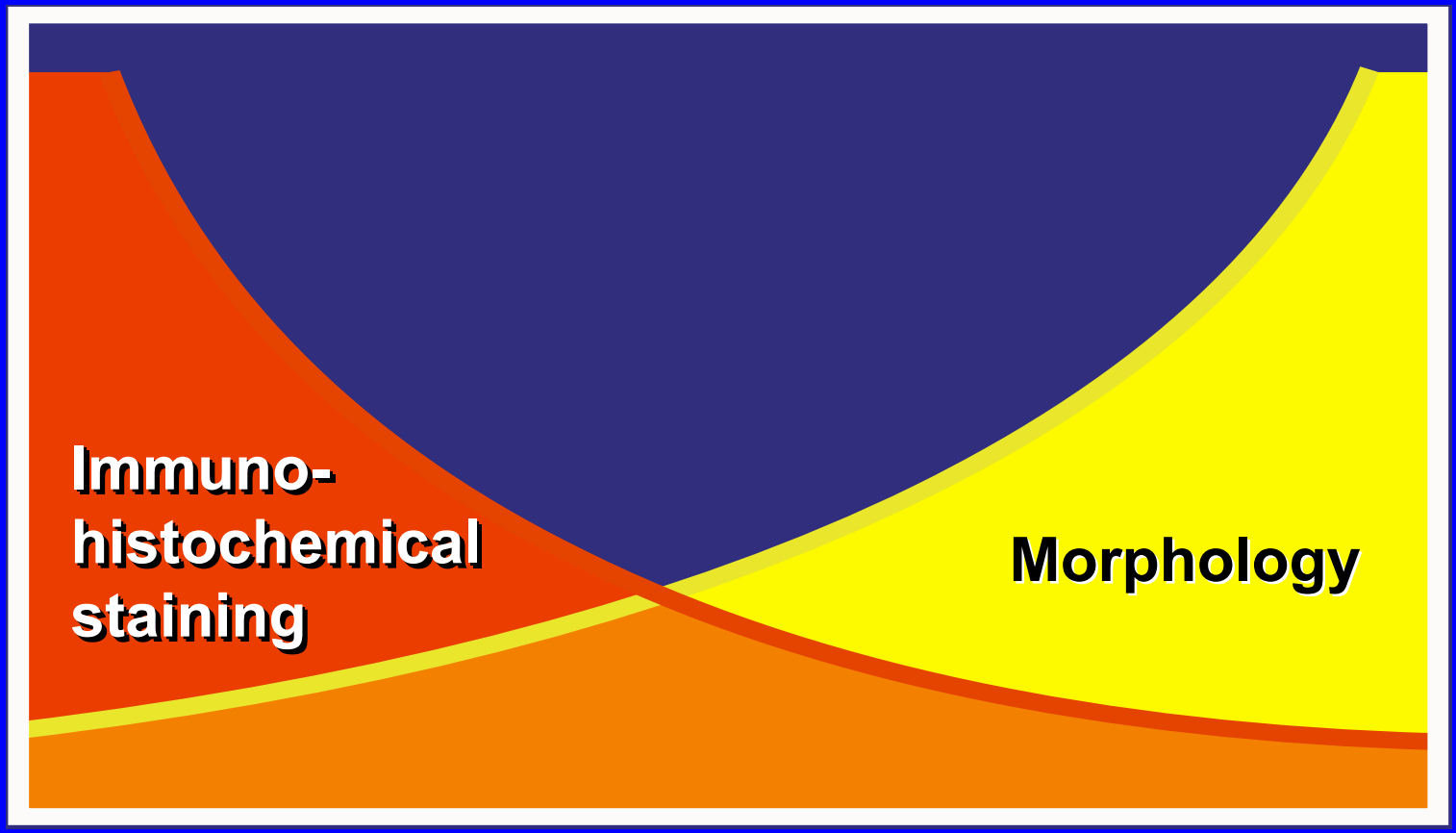
Sample Processing: Autolysis

- Effects
  - Diffusion of antigens
  - Degradation of antigens
  - Increased background
  - Loss of morphologic features
  - Not all antigens respond the same
- Solution
  - Take samples quickly
  - Fix samples adequately (ratio tissue/fixative; thickness)
  - No heat during fixation

Effects of Fixation

- Lack of detection (false negative)
- Reduced detection
- Unexpected detection (cross-reactivity)
- Increased background (overfixation adds more cross-links)
- Coagulation fixatives less deleterious than NBF
  - Suboptimal morphologic preservation
  - Always happens during routine processing!
- Options:
  - 3.5-18 hours in 10% NBF > 70% alcohol
  - Other fixative (Prefer™), Prolonged washing
  - Soaking in conc. ammonia + chloral hydrate
  - Use alcoholic formalin for 1 h on fatty tissues

Effects of Fixation and Processing on Morphology and Immunohistochemical Staining



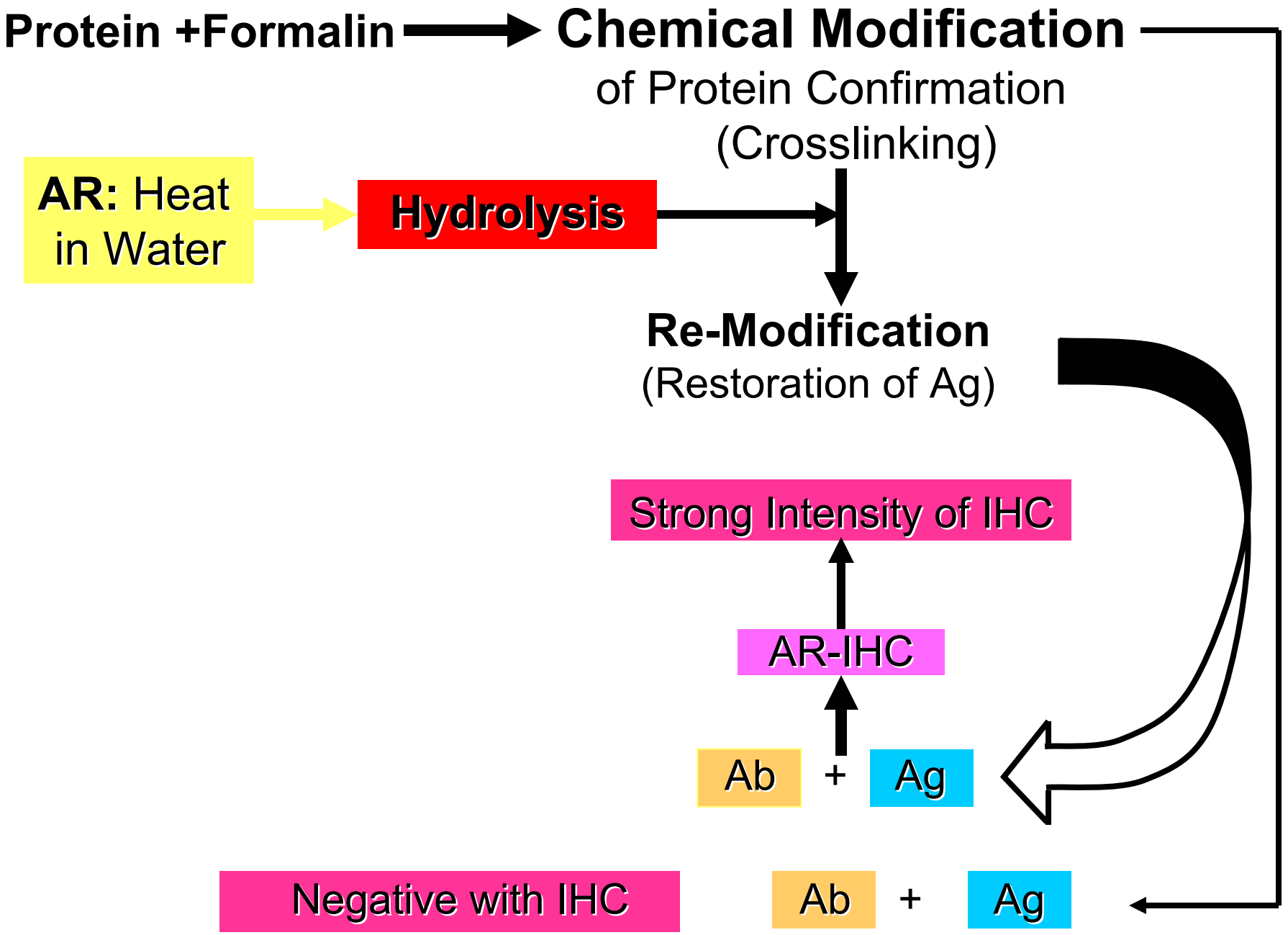
Black Magic or Haute Cuisine

-

all about boiling, steaming, microwaving, and of course, digestion

Mechanism of Antigen Retrieval

- Not clear at this time
- Loosening or breaking of cross-linkages
- Protein denaturation (some antigens are lost after AR)
- Multiple pathways: breaking of cross-linkage, extraction of diffusible blocking proteins, precipitation of proteins, rehydration of tissue (better penetration of antibody and increased accessibility to antigen)
- Mobilization of last traces of paraffin by microwave energy, allowing antibody to better penetrate tissue
- hydrolysis of Schiff bases
- Removal of cage-like calcium complexes bound during formalin fixation (calcium chelation).
- Heat induced reversal of various chemical modifications of protein structure caused by formalin fixation


Protein +Formalin
Chemical Modification
of Protein Confirmation
(Crosslinking)
AR: Heat
in Water
Hydrolysis
Re-Modification
(Restoration of Ag)
Strong Intensity of IHC
AR-IHC
Ab
+
Ag
Negative with IHC
Ab
+
Ag

High-Temperature Heating Method

The heating source is not significant to outcome, but a matter of convenience.

- vegetable steamer, microwave, pressure cooker
- microwave is faster, but more strenuous on tissue and requires more attention, calibration can be difficult

High-Temperature Heating Method

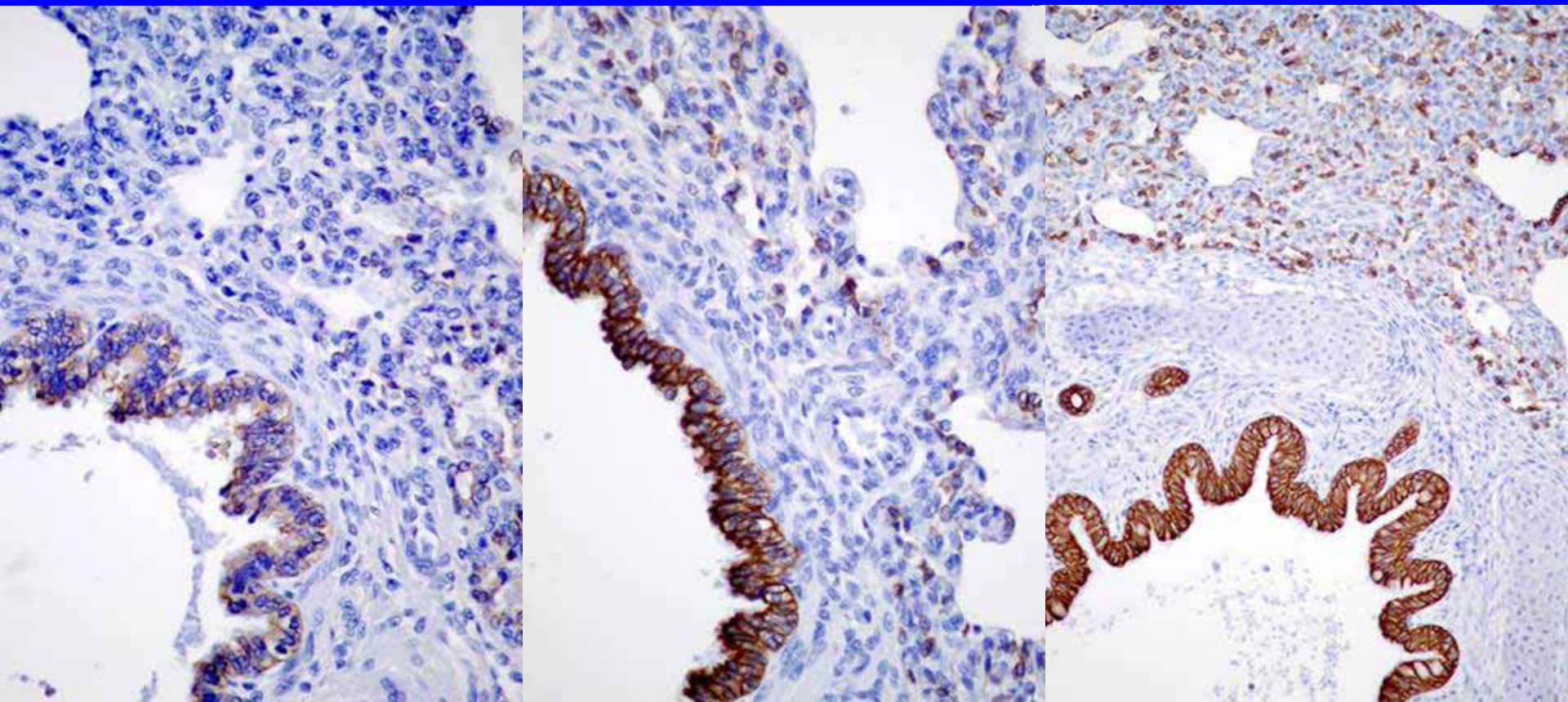
The relationship between temperature and time is inverse.

- the higher the temperature, the shorter the time need to achieve beneficial results
- temperature below 80C are not significantly beneficial
- a temperature of 100C is optimal
- example: 100C x 20 min
  90C x 30 min
  80C x 50 min
  70C x 10 hours
  achieved similar results
  (Roth et al. 2000)

Influence of Temperature on Antigen Retrieval

Cytokeratin AE1/AE3 staining in the lung

no heat | 20 min heat | 40 min heat

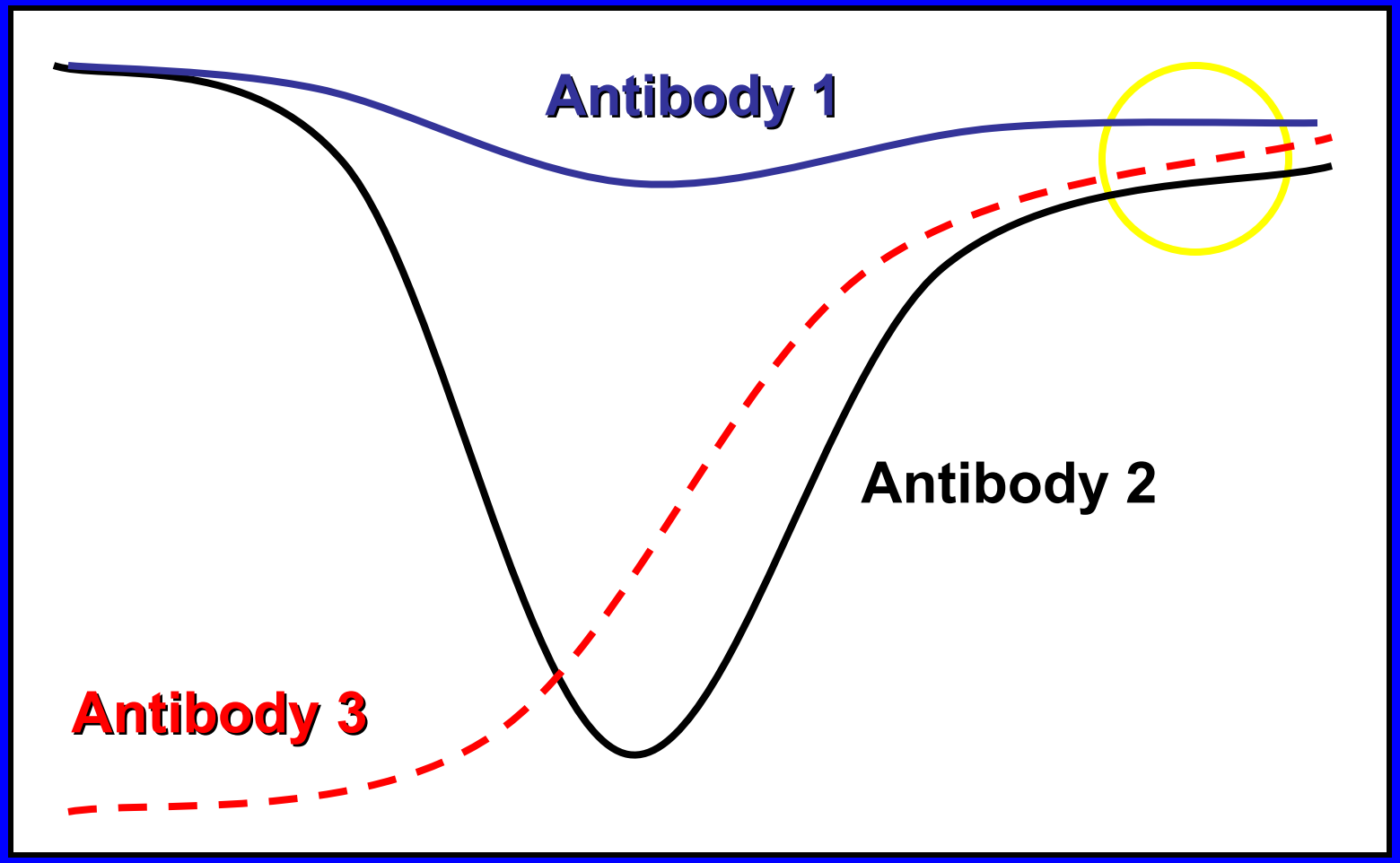


High-Temperature Heating Method

The retrieval solution pH is important.

- some antibodies stain well regardless of retrieval solution pH
- others exhibit diminished staining at neutral pH, but strong staining at very acidic or very alkaline pH
- high pH buffer appear especially useful for nuclear antigens
- example: acetate buffer pH 1.0-2.0
  glycin buffer pH 3.6
  citrate buffer pH 6.0-7.0
  Tris-HCl buffer pH 8.0-9.0
  (Speranza et al. 2001)

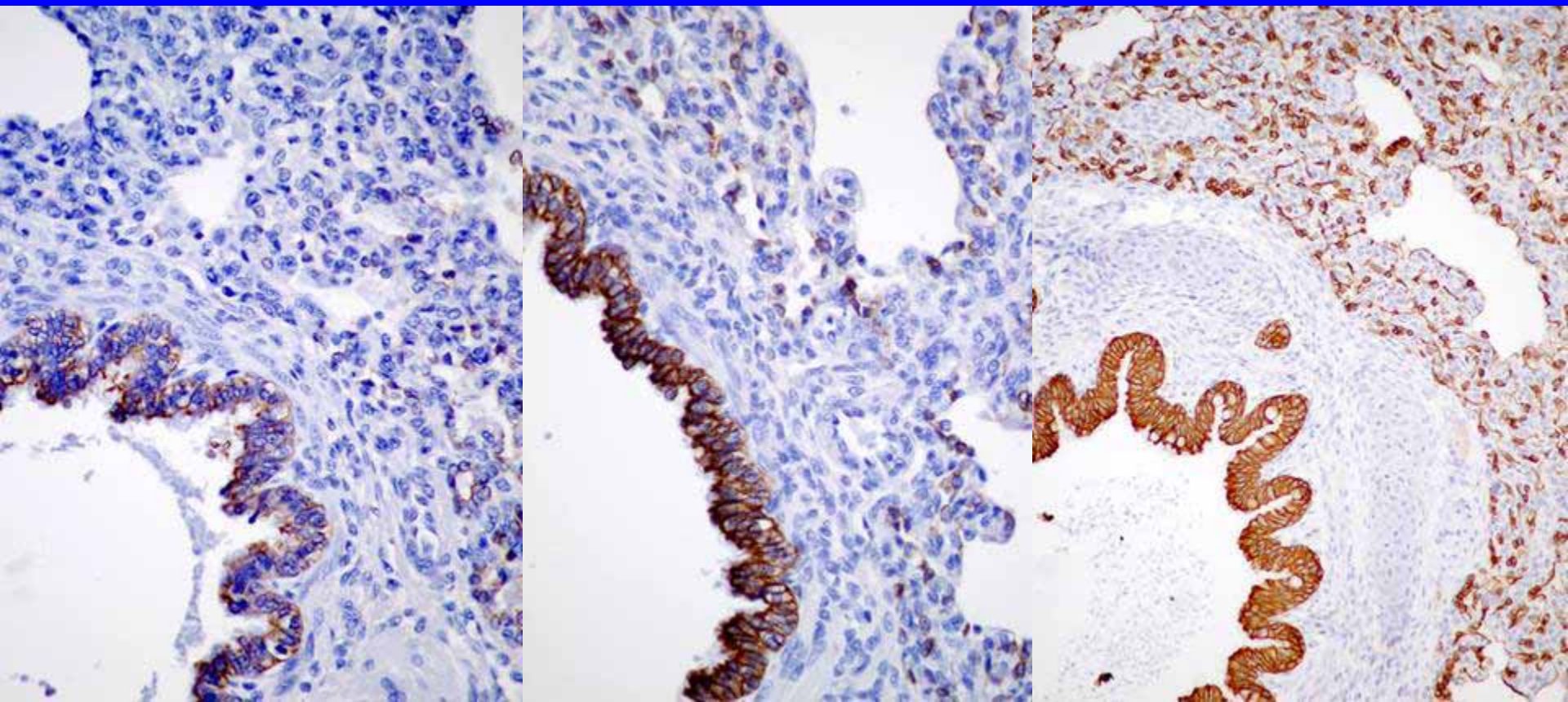
Influence of pH on Antigen Retrieval



Influence of pH on Antigen Retrieval

Cytokeratin AE1/AE3 staining in the lung

pH4 pH7 pH9



High-Temperature Heating Method

The significance of retrieval solution composition is unclear.

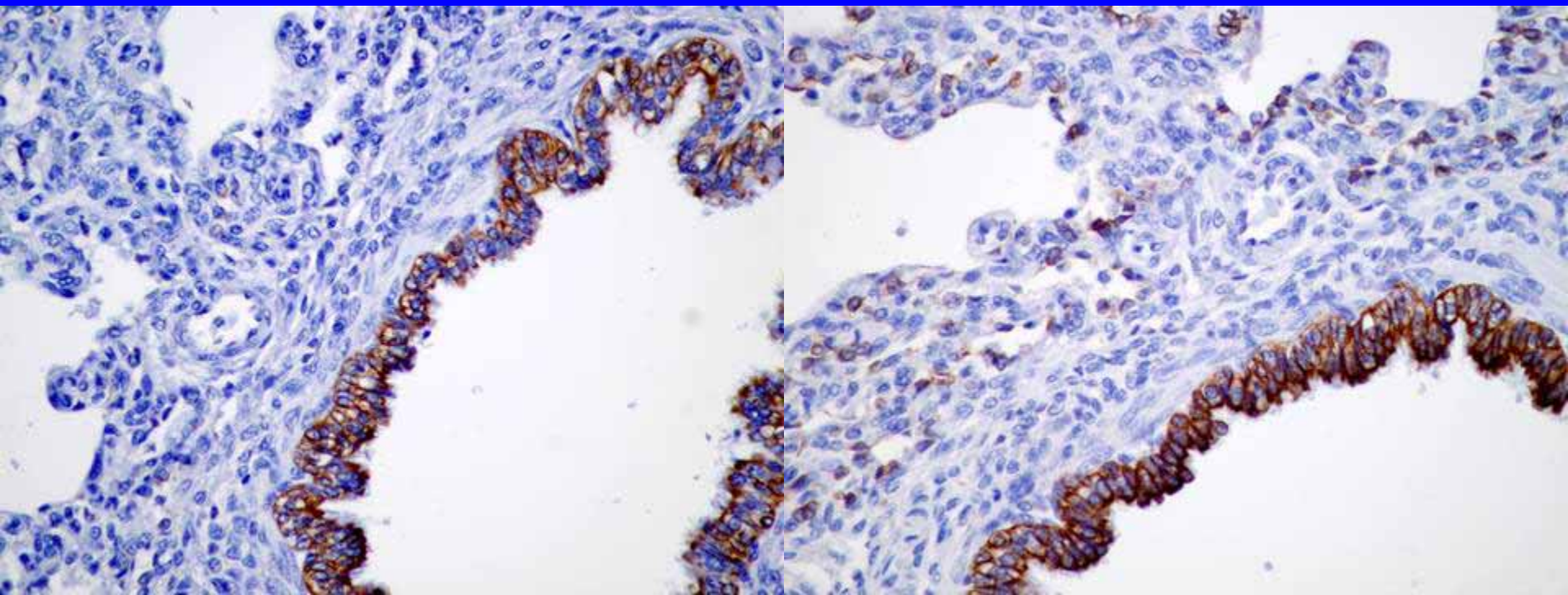
- high heat using distilled water alone will achieve improved IHC staining
- first solutions contained toxic salts such as zinc or lead, however there is no advantage over simple buffer solutions and such salts should therefore be avoided!
- many laboratories have developed their own buffer

Influence of Buffer on Antigen Retrieval

Cytokeratin AE1/AE3 staining in the lung

water

citrate buffer, pH7



Proteolytic Enzyme Antigen Retrieval

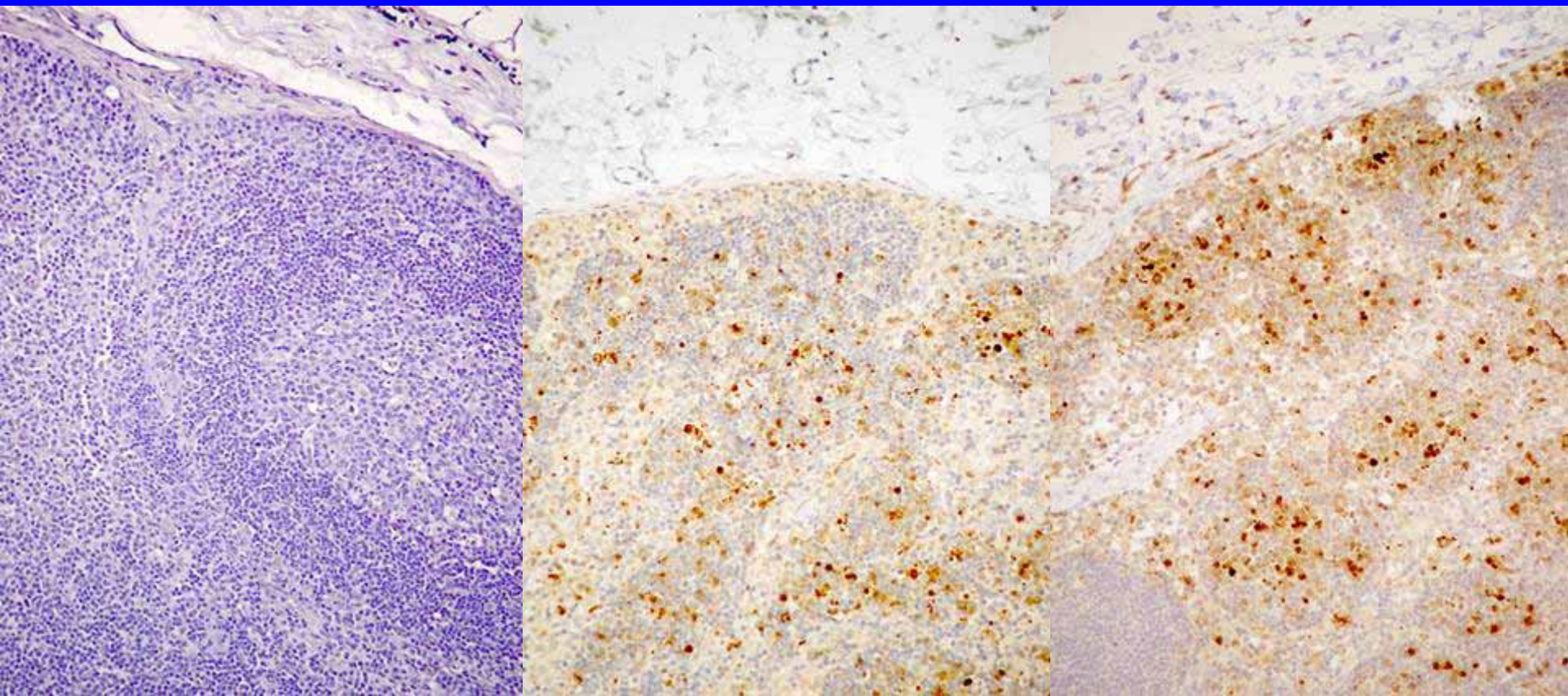
Their mechanism of action is not well known, but it is believed that they digest the tissue to some degree allowing antibodies to recognize antigenic sites.

- Types: trypsin, proteinase K, pepsin, pronase E, ficin
- Combinations of heat and protein digestion

Heat Retrieval and Protein Digestion

Lysozyme staining in the lymph node

no retrieval proteinase K trypsin



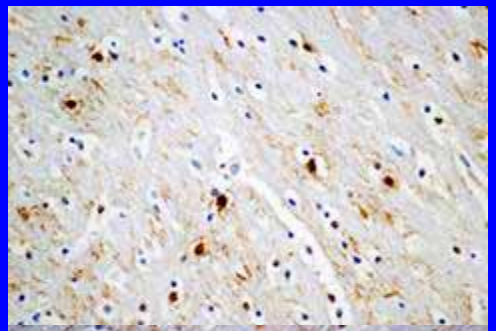
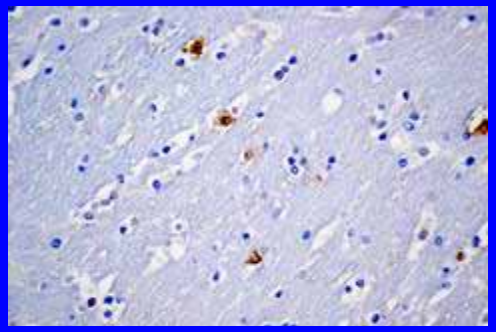
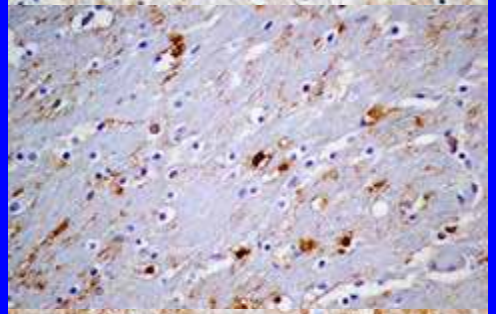
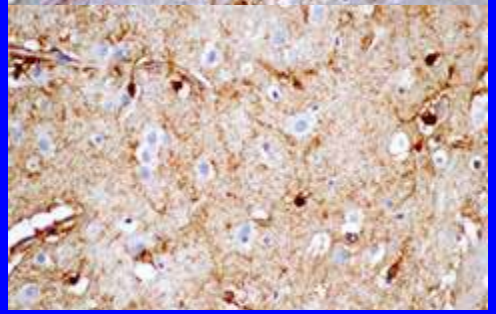
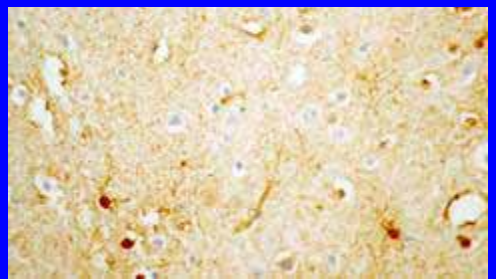
Test Battery Approach for Antigen Retrieval

TRIS-HCl Buffer	pH 1 to 2	pH 7 to 8	pH 10 to 11
Super high, 120C	Slide 1	Slide 2	Slide 3
High, 100C for 10 min	Slide 4	Slide 5	Slide 6
Low, 90C for 20 min	Slide 7	Slide 8	Slide 9

Test Battery Approach for Antigen Retrieval (our laboratory)

	100C for 20 min	no heat retrieval
Low pH retrieval buffer	Slide 1	Slide 5
Regular retrieval buffer	Slide 2	
EDTA, pH 10	Slide 3	
Proteinase K		Slide 4

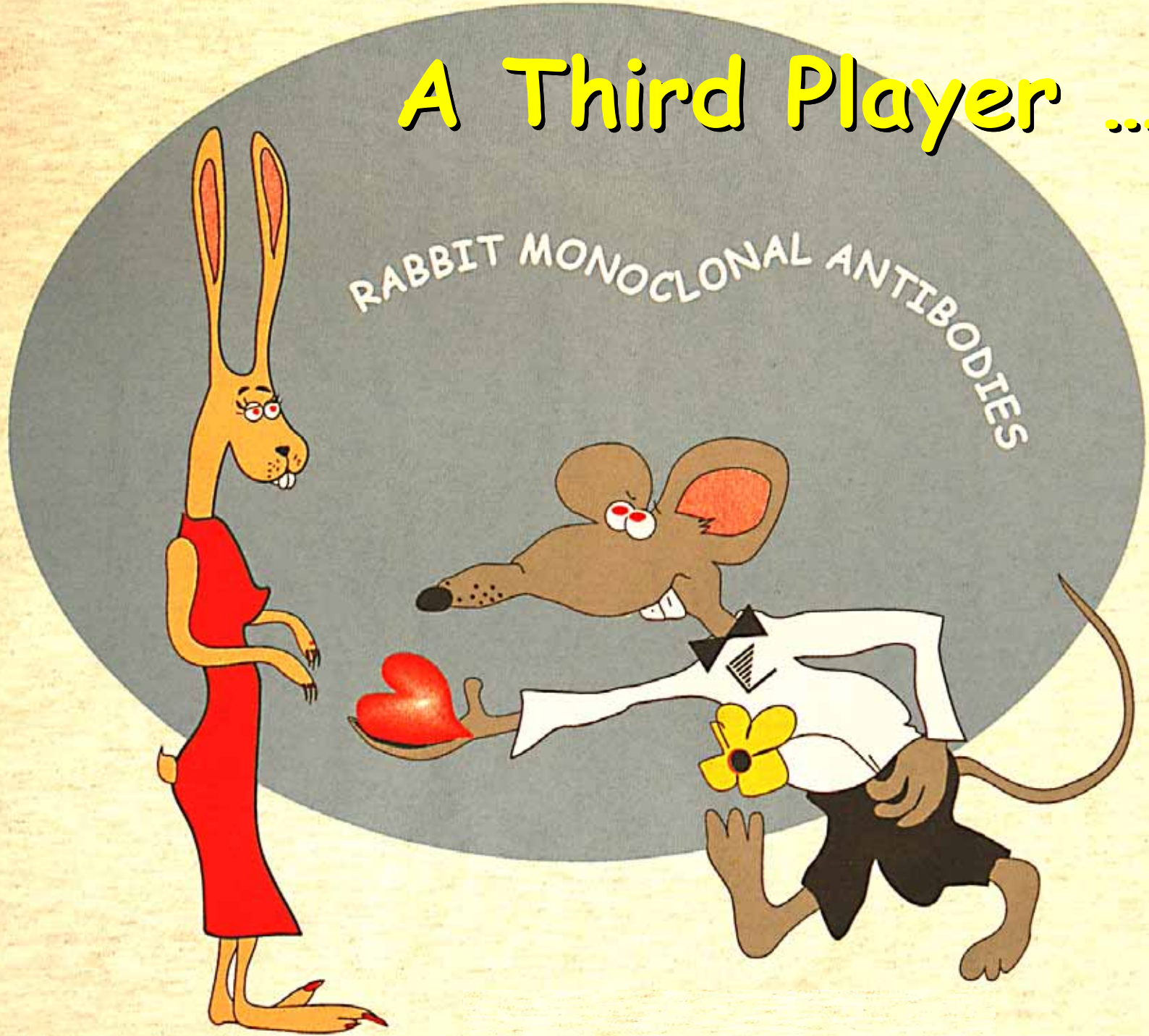
Test Battery Approach for Antigen Retrieval (S-100)

	100C for 20 min	no heat retrieval
Low pH retrieval buffer		
Regular retrieval buffer		
EDTA, pH 10		
Proteinase K		

Polyclonal vs Monoclonal

Characteristic	Polyclonal Ab	Monoclonal Ab
Origin	Multiple species	Mostly mouse (rabbit)
Affinity	Diverse (high and low)	Same affinity
Sensitivity	High	Low to moderate
Specificity	Low to high	High
Irrelevant Ig content	High (10 mg/ml)	Supernatant: none Ascites: 0.5-1.0 mg/ml
Fixation requirements	Broad	Strict
Fixation effects	Multiple	Single
Fixation tolerance	High	Low
Homogeneity	Batch variation	No batch variation
Cost	Moderate	High

A Third Player …



Rabbit Monoclonal Antibodies vs. Mouse Monoclonals

- Higher affinity
- Recognize a greater variety of epitopes that are not immunogenic in mice
- Many small compounds or peptides elicit good immune response only in rabbits
- Lack of background staining in mouse or rat tissues
- Less need for antigen retrieval
- Fewer monoclonal antibodies available
- Limited experience in canine and feline tissues

Sensitivity: Detection Methods

- Rule but not dogma: The more complex the IHC method, the more sensitive
- Establish the limit of detection (sensitivity) you need
- More sensitivity may produce more background
- Many good commercial kits available
- Special kits for mouse tissues or double immunostaining
- Brown, red, blue, etc. colors of the reaction: your choice

Which system should I choose?

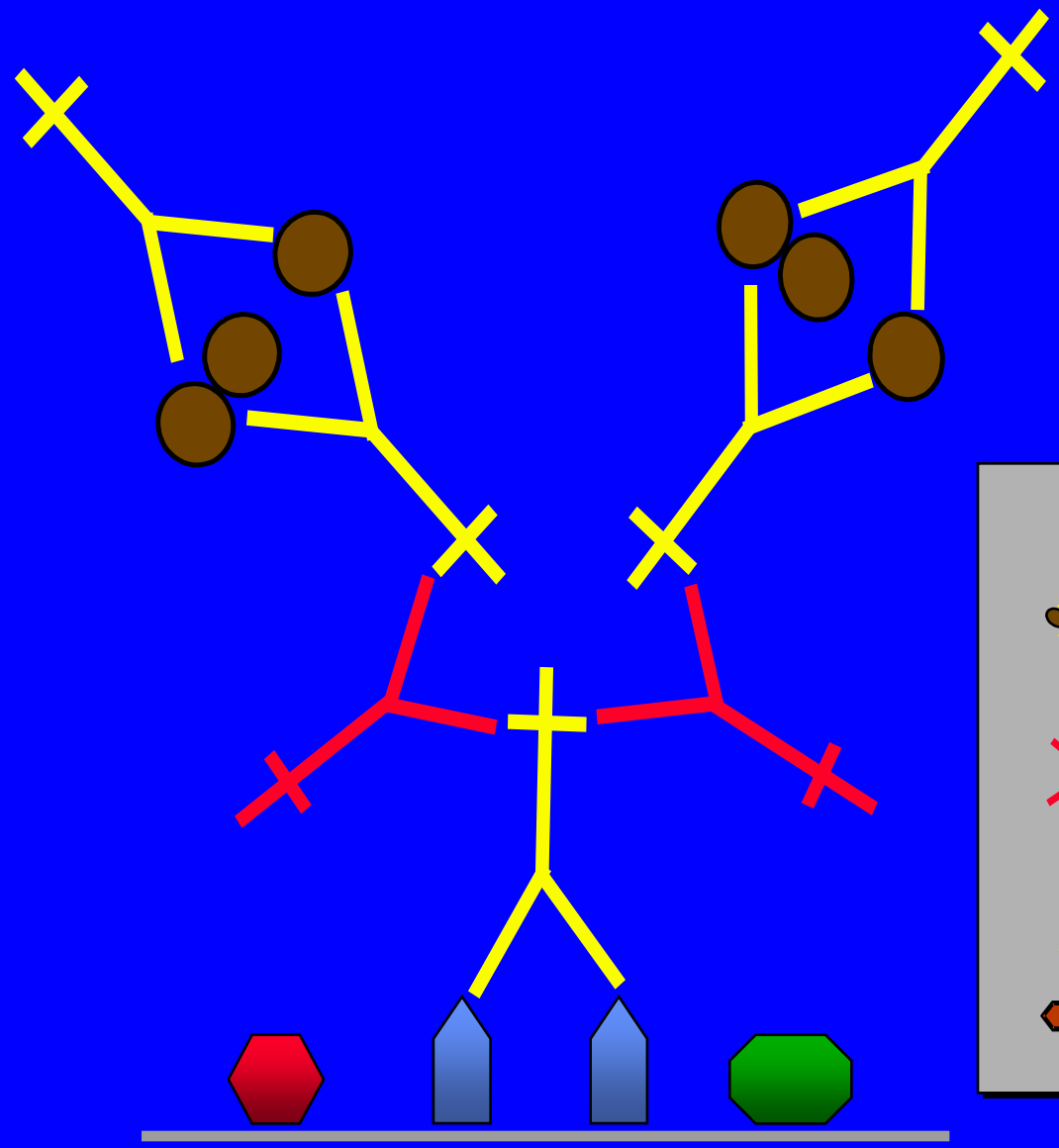
- Money available (including service agreement!)
- Case load
- Types of antigens to detect
  - Amount of antigen
  - Number of tests available
- Flexibility
- Technical support
- Technician's experience

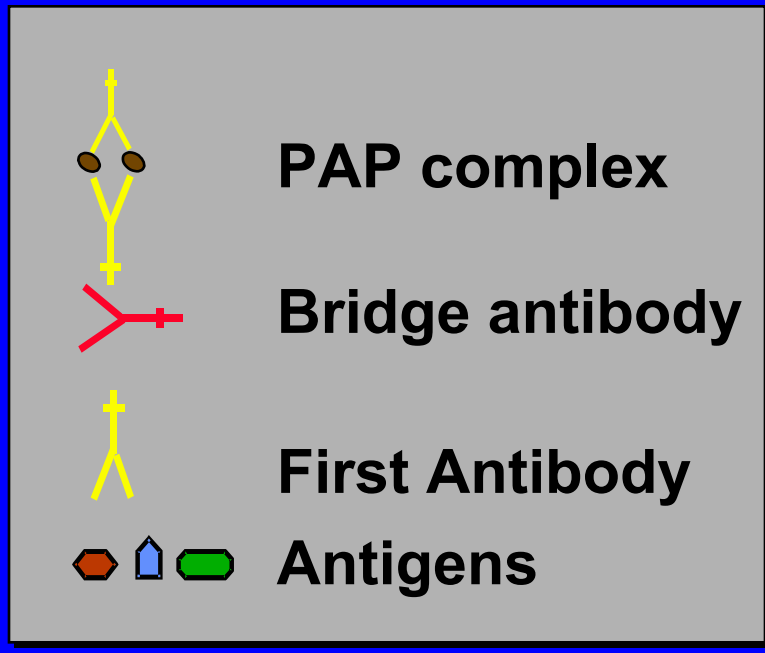

VENTANA

Detection Systems

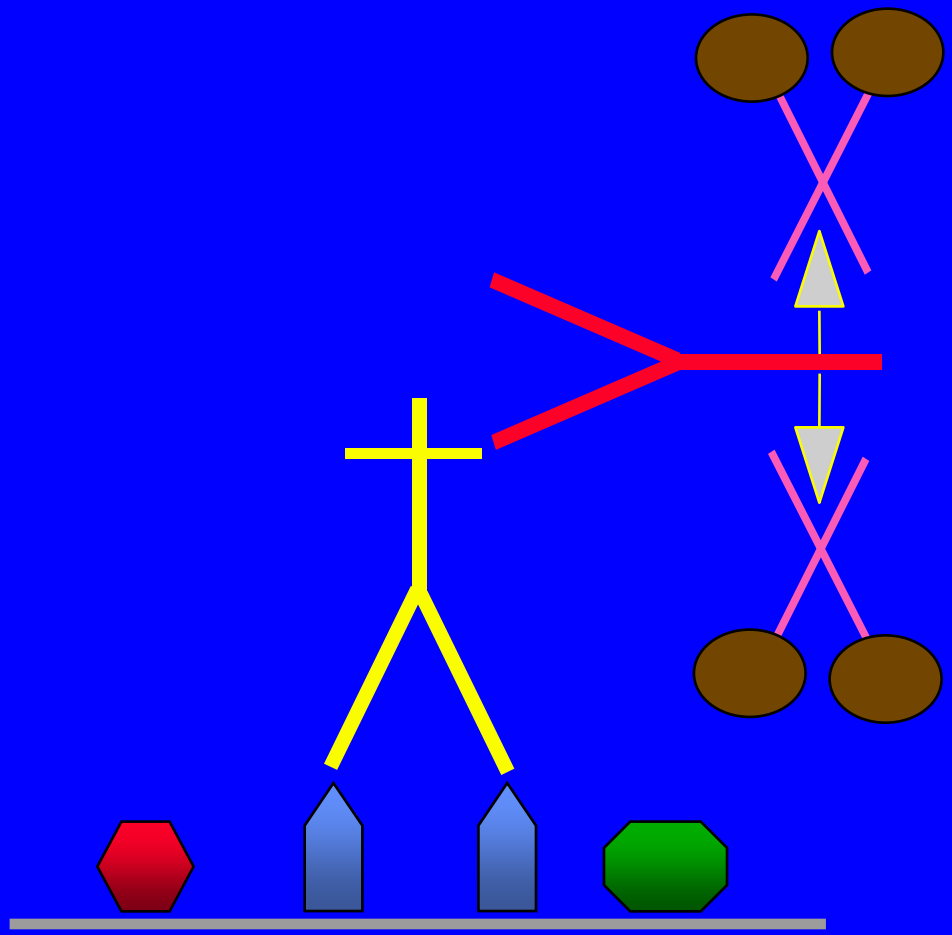
- Nonavidin-biotin immunoenzyme methods (1st generation)
  - One-, two-, or three-step methods
  - Moderately sensitive
  - Low background staining
- Avidin-biotin systems
  - High sensitivity
  - EAB background likely
- Nonavidin-biotin system (2nd generation)
  - Two-step procedures
  - Similar or more sensitive than avidin-biotin methods
  - Faster than avidin-biotin methods
  - Lower background

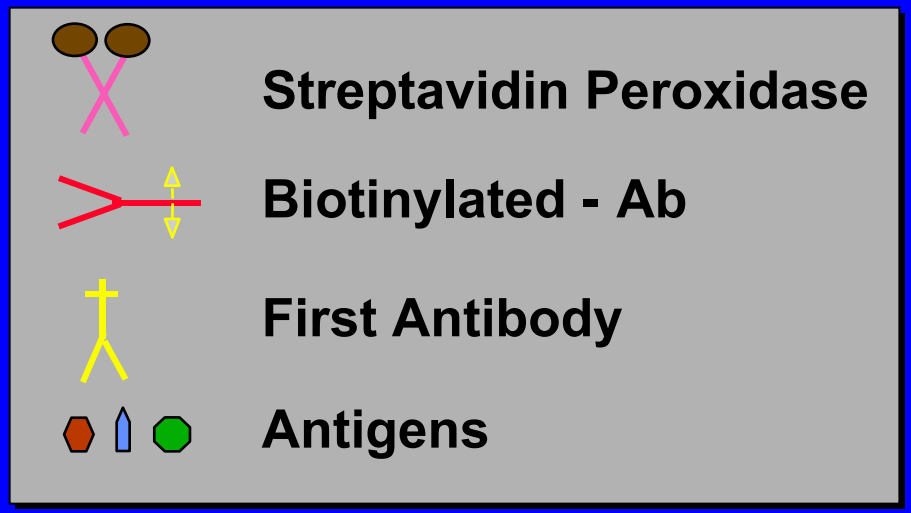
Peroxidase-anti-Peroxidase



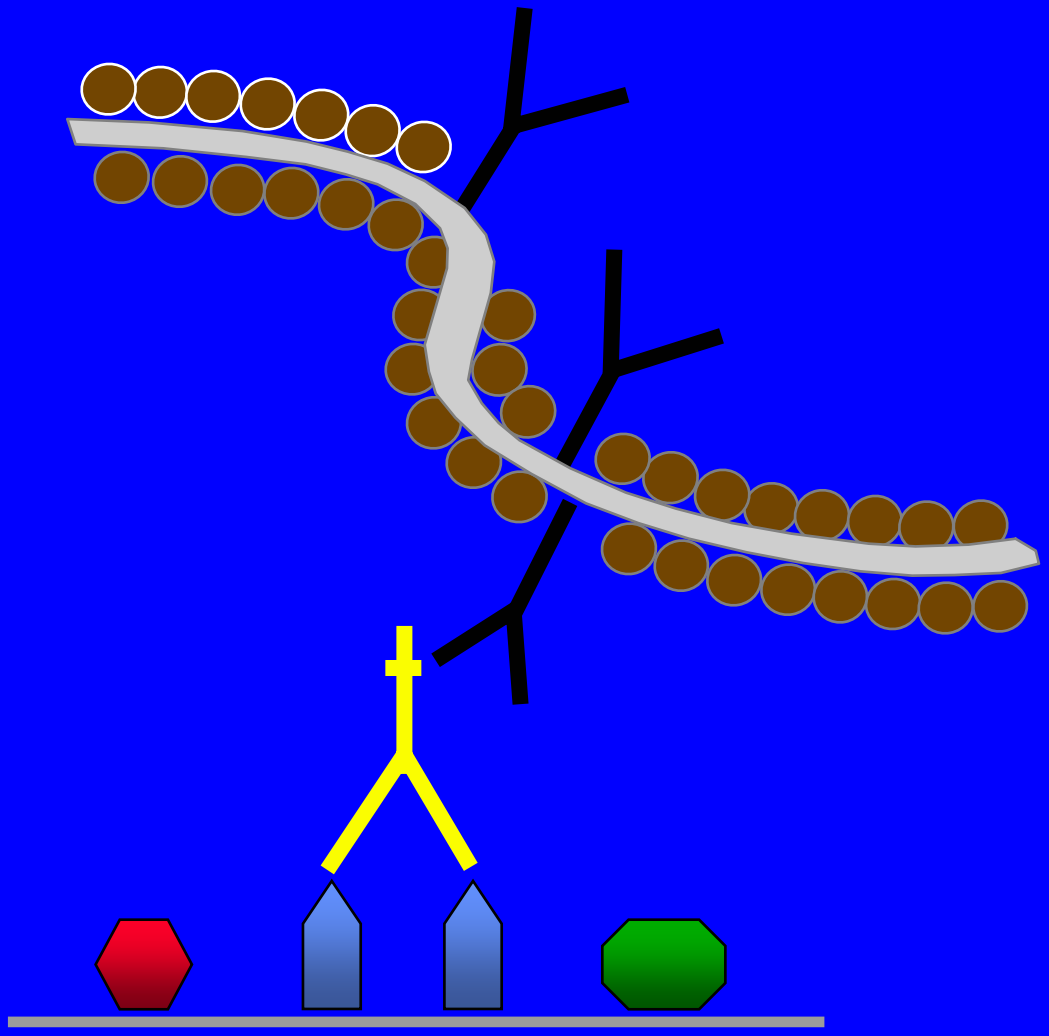


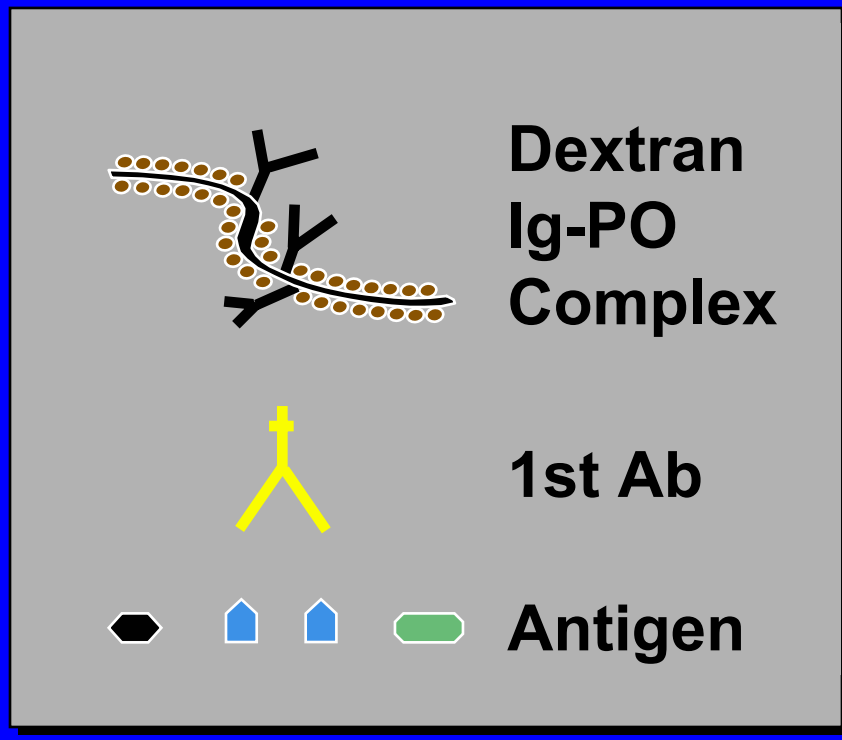
Labeled Streptavidin-Biotin Method





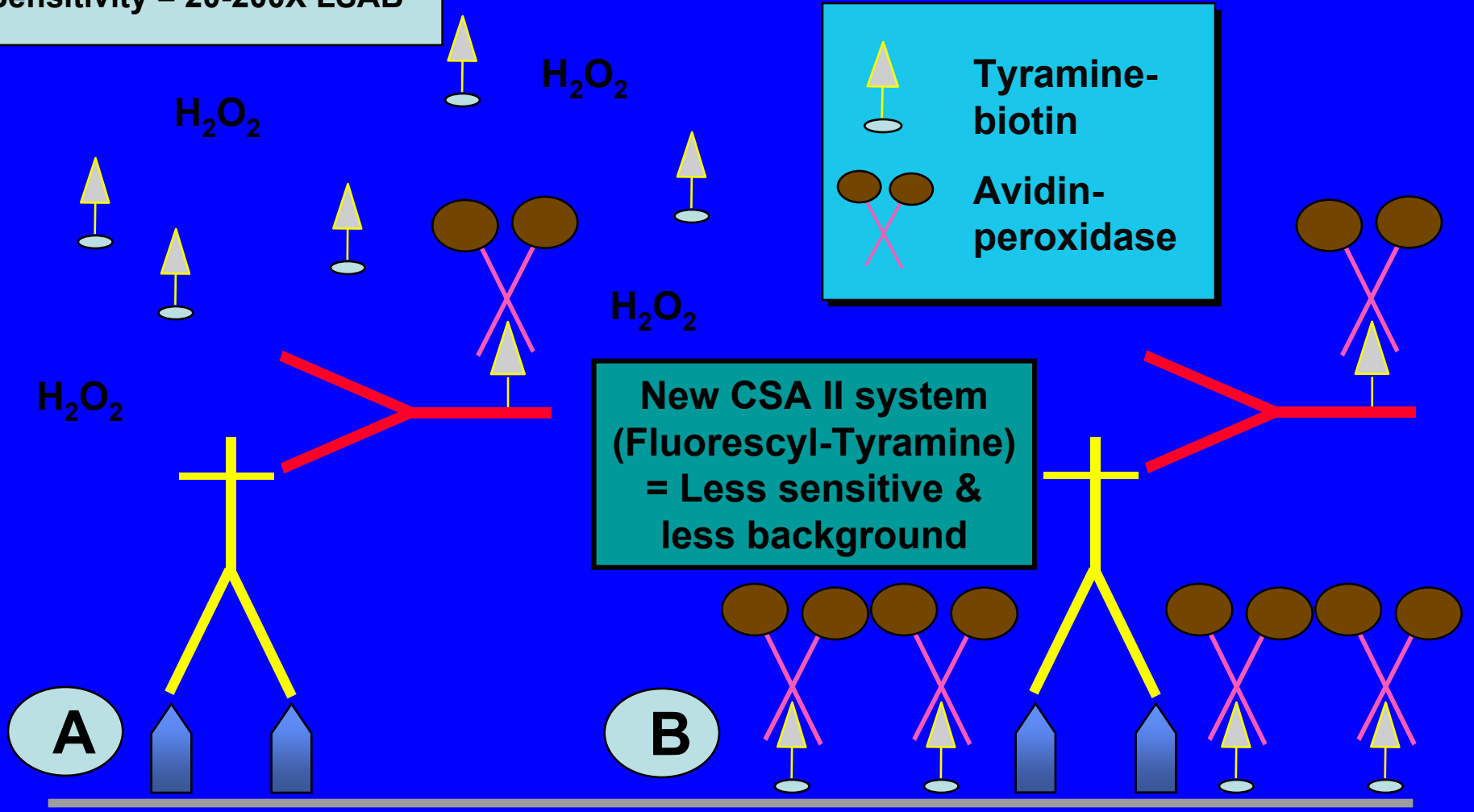
Chain Polymer-Conjugated Technology





Tyramine-Biotin Amplification

Sensitivity = 20-200X LSAB

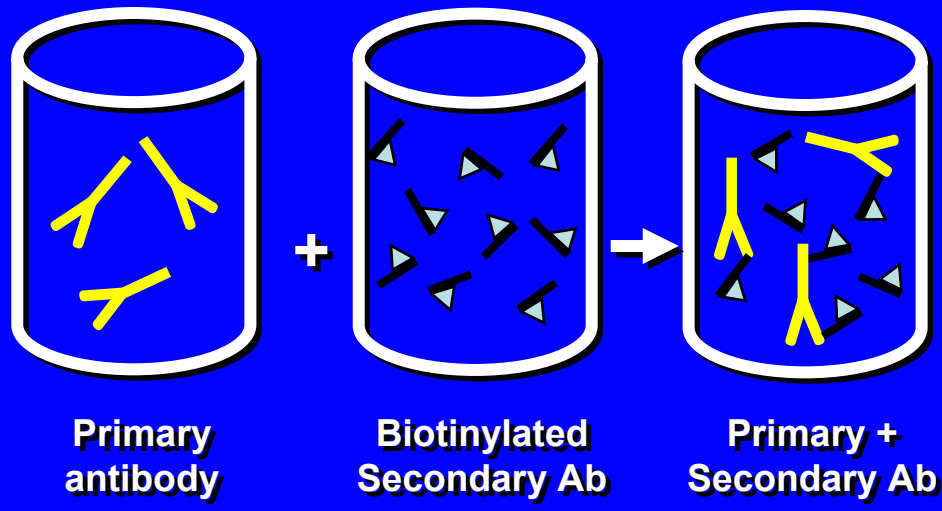


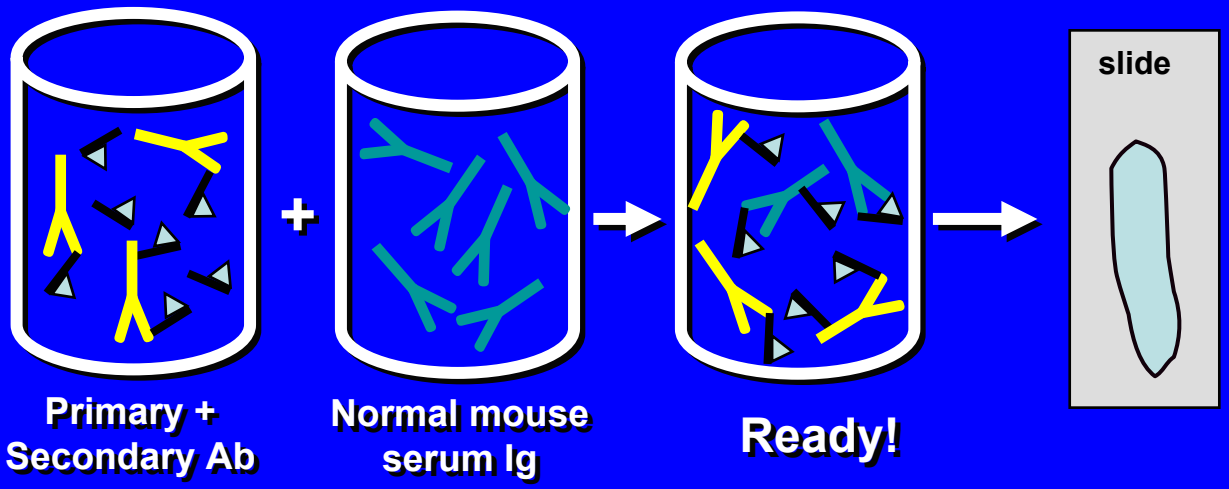
Chromogenes

Enzyme/Substrate	Chromogen	Color	Mountant	Counterstain
Peroxidase H_2O_2	DAB	Brown	Organic	Green, blue
	AEC	Red	Water	Blue, green
	4-Chloro-1-naphthol	Gray-blue	Water	Green, red
Alkaline P.				
Naphthol AS-MX	Fast Blue BB	Blue	Water	Red, green
	Fast Red TR	Red	Water	Blue, green
Indoxyl (BCIP)	TNBT	Blue-brown	Water	Green
Glucose oxid.	Phenazine MS	Dark blue	Organic	Red
β-d-Galactos.	K ferrocyanide	Blue	Organic	Red

Mouse-to-Mouse IHC

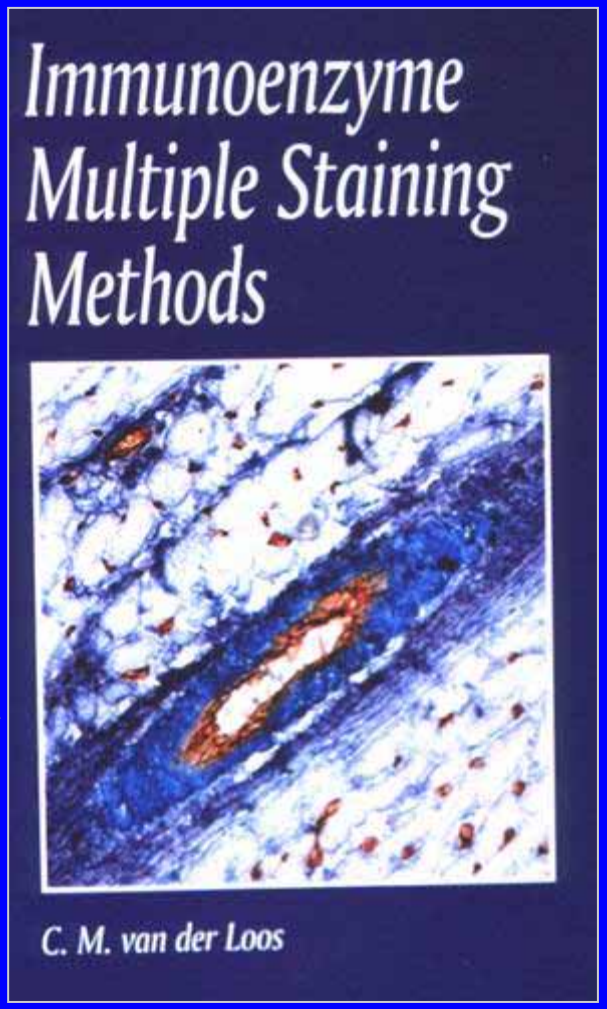
- Background due to endogenous mouse Igs
- Multiple commercial kits (avidin or non-avidin)
- Needs some fine tuning





Multiple Staining Methods

Read This Book!



Bios Scientific Publisher, Oxford, 1999

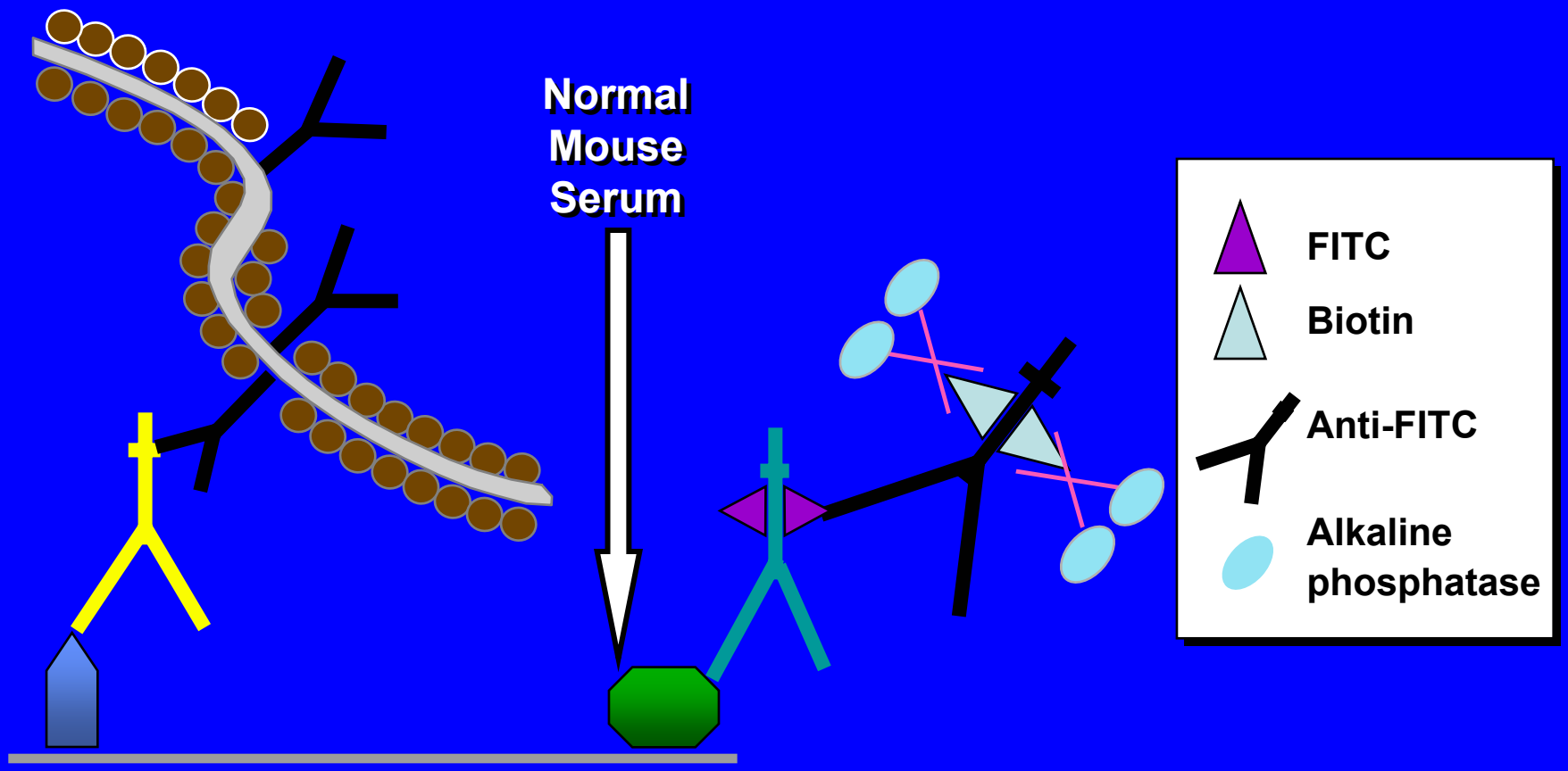
Multiple Immunostaining Troubleshooting

- More stringent controls needed
- Dissection of the immune reaction (step by step)
- Detection of 2 Ags in same cell compartment → unreliable
- Difficult when Ag retrieval differs for each antigen

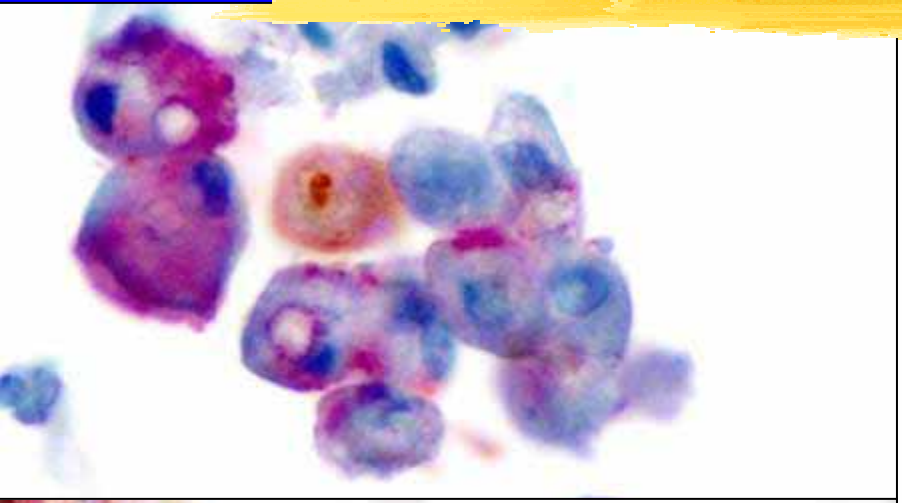
Double Immunostaining

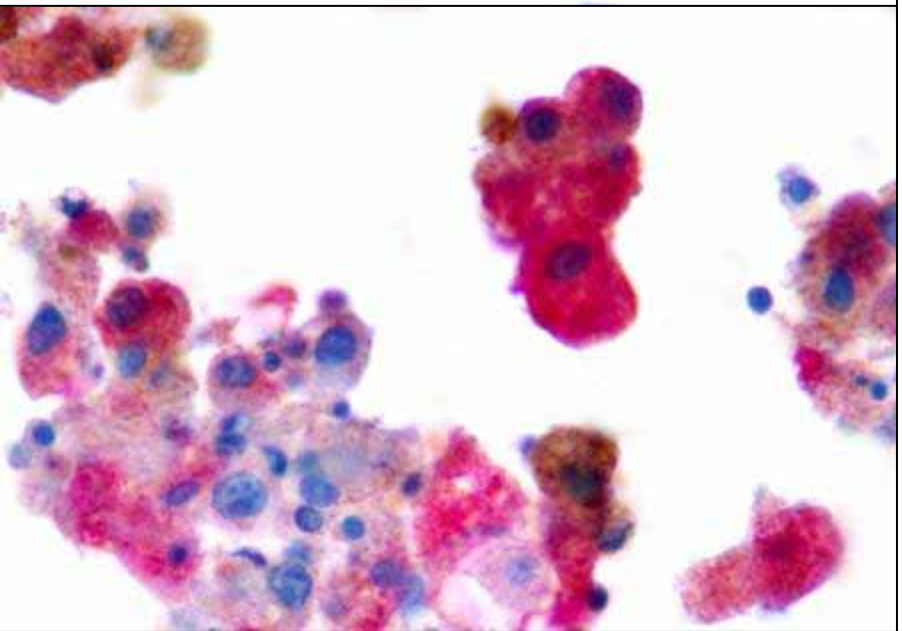
Two mouse monoclonal antibodies of the same Ig class/isotype

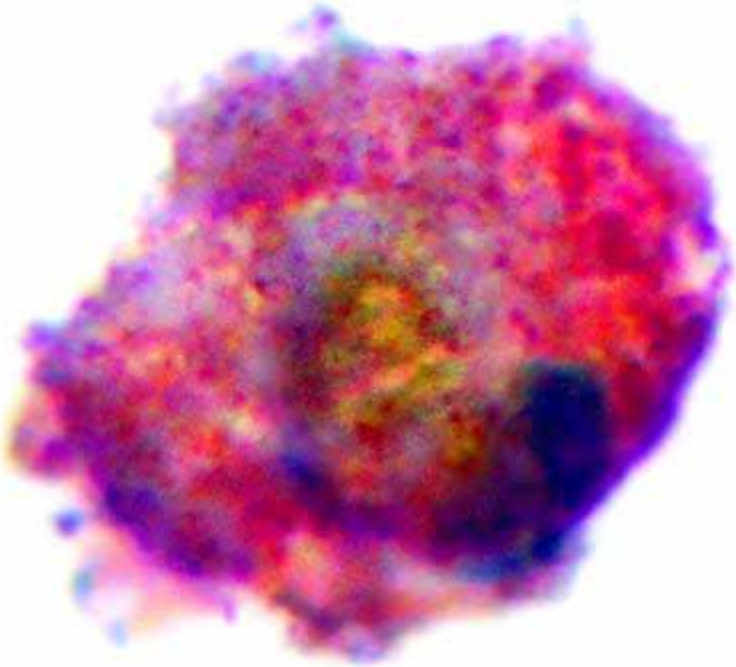
The Indirect/Direct Method



Double Staining







Avian Poxvirus and ILT

Double Staining