**An Introduction To IMMUNOHISTOCHEMISTRY**

C.L. Davis Foundation
2012 Descriptive Veterinary Pathology Course at Louisiana State University

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**Immunohistochemistry: What is it?**

“the identification of a tissue constituent in situ by means of a specific antigen-antibody interaction where the antibody has been tagged with a visible label”

*(Immunohistochemical Methods and Protocols, p.3)*

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**IHC Resources**

- The Journal of Histotechnology
- Applied Immunohistochemistry and Molecular Morphology
- DAKO, Serotec and other industry publications
- Lectures
- Internet
- Veterinary Pathology
**IHC Identification Targets**

- Cell Structural Components
- Enzymes and Hormones
- Extracellular Components
- Infectious Agents
- Cytokines, chemokines and receptors

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**Antibodies**

- Most are IgG
- Monoclonal or polyclonal
- High specificity
- High affinity

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**Antibodies**

- High specificity (specific staining)
  - The antibody reacts with a specific epitope on the antigen against which it was raised
  - Determined by Western blots, ELISA and then preadsorption with the desired antigen
Antibodies

- High affinity
  - The strength of the noncovalent binding of the immunoglobulin to a single site on the antigen molecule is strong
  - Rapid equilibrium with the tissue antigen is reached

Specimens

- Tissue sections, formalin-fixed or frozen
- Cell smears, suspensions, cytospins
- Cells or tissue sections for electron microscopy

Immunoelectron Microscopy-Ebola Virus
IHC TECHNIQUES

- **Direct Methods**
  - Little signal amplification
  - Uses large quantities of antibody

- **Indirect Methods**
  - Greater signal amplification
  - Uses smaller quantities of antibody
  - Multistep detections

**IHC Methods**

Direct Method

Two-step Indirect

- Enzyme
- Antigen
- Enzyme-labeled
- 2nd Antibody
- 1st Antibody
- Mouse Ab
- Rabbit Ab
- Polymer
Enzymology

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
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<tbody>
<tr>
<td>Horseradish Peroxidase</td>
<td>Diaminobenzidine</td>
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<tr>
<td></td>
<td>3-Amino-9-ethyl Carbozole(AEC)</td>
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<tr>
<td>Alkaline Phosphatase</td>
<td>Histomark Red</td>
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<tr>
<td></td>
<td>New Fuchsin</td>
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<td></td>
<td>BCIP/MBT</td>
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Endogenous Peroxidases

- Erythrocytes
- Neutrophils
- Eosinophils
- Hepatocytes
- Neutralize with hydrogen peroxide

Endogenous Alkaline Phosphatase

- Bone
- Liver
- Kidney
- Intestine
Example of an IHC Technique

1. Deparaffinize and rehydrate tissue
2. Antigen retrieval step
3. Block endogenous peroxidase
4. Serum/protein block
5. Add primary antibody
6. Add secondary antibody (labeled polymer)

Example of an IHC Technique

7. Add substrate-chromogen solution
8. Counterstain
9. Dehydrate tissue and coverslip

IHC Technique – Bench Top
Necessary to make a valid interpretation of staining
Helpful in troubleshooting
Positive control tissue contains the antigen of interest; negative tissue does not
Internal controls are structures in the tissue that can stain either positive or negative

IHC results are subject to more interpretation than other assays
Antibody reactivity differs among species
Fixation plays an important role:
  - Different susceptibility of antigens
  - Variable antigen retrieval
  - Time of fixation and fixation variable
  - Geographic differences, esp. altitude
Positive Internal Control

Which antibody? Factor VIII-Ag

Negative Control

Normal Rabbit Serum

Generalized Background Staining
Lymph node, nonhuman primate

Slide Interpretation

- Examine controls; determine whether staining run is valid
- Note any internal positive or negative controls
- Determine specific staining – state cell type, location in cell, intensity and distribution

Setting the Rules

- Always read the IHC together with the H&E
- Always interpret IHC with proper controls
- Use antibody panels rather than single tests
- Know your antibodies
- Don’t base your diagnosis on the absence of staining only
- IHC is a complimentary tool to H&E light microscopic diagnosis
The Key to Description

- Quantitation of staining (negative, light, medium, heavy)
- Distribution of positivity (cytoplasmic, nuclear, cell membrane, etc.) Is the location appropriate for the antibody?
- Presence or absence of an internal control

Tissue from a dog

- Neoplastic round cells arranged in ......
- Diffuse, strong cytoplasmic staining, positive internal control (vessel)
- Multifocal, moderate, cytoplasmic staining; no internal control

DIAGNOSIS AND CHARACTERIZATION OF NEOPLASMS
**Intermediate Filaments**

- Cytokeratin
- Vimentin
- Desmin
- Glial fibrillary acidic protein (GFAP)/Nestin
- Neural fibrillary protein (NFP)

**Intermediate Filaments – What are they?**

- Component of the cytoskeleton
- Have specific distributions in tissues and cells
- Possess a nucleic acid binding function
- May modulate calcium influx into the cell and nuclear transcription

**Limitations of Intermediate Filaments**

- They are sensitive to formalin fixation and standard tissue processing
- There is a possibility of cross-reaction
- There may be co-expression of two filaments in a single cell or tissue
Cytokeratin

- At least 20 different keratins
- Divided into 2 groups:
  - type I or acidic
  - type II or basic
- Many subtypes are expressed in normal and neoplastic epithelium
- Keratins can also be grouped by molecular weight

Cytokeratin

- Low Molecular Weight are in all epithelia, except squamous
- High Molecular Weight is located in squamous epithelia

Keratin Expression

- Normal epithelium
- Benign epithelial cutaneous tumors
- Carcinomas
- Mesothelium and mesothelioma
- Thymoma
Keratin Expression

- Chordoma
- Nonseminomatous germ cell tumors
- Synovial cell sarcoma
- Carcinosarcoma
- Carcinoids
- Meningioma, rarely

Respiratory epithelium, horse

Complex adenoma, cat
Colonic Adenocarcinoma, dog

Ocular neoplasm, cat

Ciliary body adenocarcinoma
Vimentin

- Most ubiquitous intermediate filament in the body
- First intermediate filament expressed embryologically
- Replaced by the intermediate filament specific for the cell type during development, but retained in mesenchymal cells

Vimentin Expression

- Myocytes
- Endothelial cells
- Fibroblasts
- Adipocytes
- Chondrocytes
- Melanocytes
- Macrophages
- Lymphocytes
- Sertoli cells
- Ovarian granulosa cells

Vimentin Expression

- Mesenchymal neoplasms
- Melanoma
- Mesothelioma
- Thymoma
- Synovial sarcoma
- Lymphoma (Lymphosarcoma)
- Transmissible venereal tumor
- Primitive neuroendocrine tumor
Splenic lymphoma, dog

Complex mammary gland adenoma, dog

Dual Expression of Intermediate Filaments
Hepatic capsule, cat

Vimentin

Keratin

DX: Mesothelioma
Desmin

- Primarily used to determine a possible myogenic origin for sarcomas
- Expressed in embryonic and adult skeletal, smooth and cardiac muscle
- Is NOT expressed in myoepithelium

Desmin Expression

- Most muscle tumors:
  - Rhabdomyoma
  - Rhabdomyosarcoma
  - Leiomyoma
  - Leiomyosarcoma
  - Juvenile rhabdomyosarcoma
Desmin

- May be expressed in the following neoplasms with divergent phenotypes:
  - Primitive neuroectodermal tumor
  - Epithelioid sarcomas
  - Rhabdoid-type tumors

Gingiva, dog

Gingiva, dog
Neural Fibrillary Protein (NFP)

- Major structural component of neurons and their processes
- Largely specific for cells of the neural crest and neuroectoderm
- Usually expressed in neural tumors with variable expression in neuroendocrine tumors

<table>
<thead>
<tr>
<th>NFP Expression</th>
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<tbody>
<tr>
<td>Neuromas</td>
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<tr>
<td>Ganglioneuromas</td>
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<tr>
<td>Neuroblastoma</td>
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<tr>
<td>Ganglioneuroblastoma</td>
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<tr>
<td>Carcinoid</td>
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<tr>
<td>Medulloblastoma</td>
</tr>
<tr>
<td>Esthesioneuroblastoma</td>
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<tr>
<td>Teratoma</td>
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<tr>
<td>Pheochromocytoma</td>
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Cerebral mass, dog

*Veterinary Pathology, 41:3, pp.282-84*
NFP Expression

Ganglioneuroblastoma

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Glia Fibrillar Acidic Protein (GFAP)

- The major protein of astrocytes
- Also present in developing and neoplastic ependymal cells and oligodendrocytes
- Poorly differentiated (high grade) astrocytomas usually do not express GFAP

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Mass, subcutis, cat
GFAP

Peripheral nerve sheath tumor

Nestin

- The main intermediate filament of immature astrocytes
- Used as a marker for astrocyte progenitors and neural stem cells
- Stains medium to high grade forms of astrocytoma vs. GFAP
Lysozyme

- Used as a macrophage marker

- In dogs it is expressed in:
  - Monocytes, neutrophils
  - Serous cells in mucosa-associated exocrine glands
  - Renal proximal tubular epithelium

Other Macrophage Markers

- CD 68: nonhuman primates, cat, dog
- Mac 387: nonhuman primate, cat
- CD18: dog

Kidney, dog
Multifocal vimentin + with a positive internal control

Lysozyme

Histiocytic sarcoma

Factor VIII Related Antigen

- 99% von Willebrand factor
- Expressed in endothelial cells, megakaryocytes and platelets
- Useful in staining poorly-differentiated hemangiosarcomas (sometimes)
Cutaneous neoplasm, dog

Factor VIII Antigen

Hemangiosarcoma

Platelet Endothelial Cell Adhesion Molecule (PECAM) (CD31)

• Also known as CD 31
• Expressed in endothelial cells, platelets and by some macrophages
• May be used in conjunction with Factor VIII to identify vascular origin
Thyroid Gland

- Thyroglobulin expressed in normal follicular epithelium and neoplasms arising from it
- Thyroid transcription factor-1 (TTF-1) expressed in follicular epithelium
- Calcitonin expressed in parafollicular C cells and neoplasms arising from them
- Chromogranin expressed in C cells

Neoplasm, neck, dog

Thyroglobulin

Thyroid follicular carcinoma
Thyroid mass, baboon

Calcitonin

Medullary carcinoma

TTF-1 Lung

Positive internal control
Primarily expressed by glial cells, but also expressed by:

- Adipocytes
- Chondrocytes
- Melanocytes
- Interdigitating reticular cells in LN

S-100 Protein

Neoplasms that express S-100

- Schwannomas
- Neurofibromas
- Astrocytomas
- Oligodendrogliomas
- Nerve sheath tumors
- Chordomas
- Chondrosarcoma
- Melanoma
- Liposarcoma
- Synovial sarcoma
Subcutaneous mass, cat

Peripheral nerve sheath tumor

S-100 Protein

- Not very specific
- Normally expressed in: neurons, nerve fibers, neuroendocrine cells
- Expressed in the following neoplasms:
  - Neuroendocrine tumors
  - Schwannomas
  - Melanomas
  - Astrocytomas
  - Gangliomas
  - Glioblastomas
  - Meningiomas
  - Gastrointestinal stromal tumors (GISTS)
- Must use in conjunction with other markers

Neuron Specific Enolase (NSE)
Synaptophysin and Chromogranin

- Used to demonstrate neuroendocrine origin
- Very sensitive to formalin fixation

Synaptophysin

Pancreatic islet cell, dog

Chromogranin

Adrenal medulla, dog
Pharyngeal neoplasm, dog

Synaptophysin

Neuroendocrine carcinoma

Muscle Markers

- Actin or muscle specific actin
- Smooth muscle actin
- Myoglobin
Actin

- Expressed by skeletal, smooth and cardiac muscle
- May also be expressed by actin-containing fibroblasts
- Used in conjunction with desmin to confirm myogenic origin

Smooth Muscle Actin

- Specifically expressed by smooth muscle cells
- May also stain actin-containing fibroblasts (myofibroblasts)
- Used to differentiate skeletal vs. smooth muscle origin

Myoglobin

- Expressed by normal skeletal and cardiac muscle
- Expressed late in cell differentiation
- Used to differentiate skeletal vs. smooth muscle origin
Neoplasm thoracic wall, rat

Actin

Desmin
Myoglobin

Rhabdomyosarcoma

Diagnosis?

Rhabdomyosarcoma

Gastric submucosa, dog
Diffuse vimentin immuno-reactivity

Diffuse desmin immuno-reactivity
Diffuse actin immunoreactivity

Diffuse smooth muscle actin immunoreactivity

Negative staining for CD117
Negative staining for GFAP

DIAGNOSIS?

Leiomyosarcoma

Spindle Cell Sarcoma Diagnosis

GFAP, S-100+  Actin, Desmin+  Factor VIII, CD31

Neurogenic  Myogenic  Vascular
Skeletal vs. Smooth Muscle

Actin or desmin +

Smooth muscle
- Actin +
- Myoglobin +

Skeletal muscle

Myoepithelial Markers

- Calponin
- Maspin
- p63

Mammary neoplasm, cat
Calponin

Complex adenoma

Leukocyte and Platelet Markers

- CD 1a: dendritic and Langerhans cells; some thymocytes
- CD 3: pan T cell marker
- CD 18: neutrophils, mast cells, macrophages
- CD 45RA: pan B lymphocyte
- CD 56: NK cells

Leukocyte and Platelet Markers

- CD 68: histiocytes and monocytes
- CD 79a: B lymphocytes, plasma cells
- BLA 36: B lymphocyte
- Lambda/kappa light chains: plasma cells
- CD 117 (c-kit): mast cells, Gastrointestinal stromal tumors (GIST)
Duodenal mass, dog
*Vet. Path.*, 39:5, pp.557-64

CD 117

Mast cell tryptase

Dx: Mast Cell Tumor
Cutaneous neoplasm, dog

CD 3

DX: Epitheliotropic lymphoma

Mass on the lip, dog
Interpretation?

CD 3

Interpretation?

CD 79a

DX: B-cell Lymphoma

Round Cell Neoplasm Diagnosis

CD 3+

T cell lymphoma

B cell lymphoma

CD79a, BLA 36, CD 45RA, CD20

Lysozyme, CD68, MAC 387

Histiocytic

Kappa, lambda Light chains

Plasma cell tumor
Diagnosis of Melanomas

- Have many different histologic appearances
- Many are vimentin and S-100 +
- Melan A has been used as a specific marker in cutaneous and oral melanomas

Oral neoplasm, dog

Melan A

DX: Malignant Melanoma
Infectious Disease Diagnosis

- IHC can be used retrospectively to identify bacteria, fungi, viruses, protozoa, algae.
- Some cross-reactivity with some of the antibodies for bacteria and protozoa
- Provides permanent preparation with the organism

Marburg Virus, liver

Coxiella burnetti, placenta
Yersinia pestis, lung

Pneumocystis carinii, lung

SOME ARTICLES in Veterinary Pathology
Feline Peripheral Nerve Sheath Tumors: Histologic, Immunohistochemical and Clinicopathologic Correlation (59 Tumors in 53 Cats)

- Vet Path: 46(6), 1166-1180 (Nov 2009); F.Y. Schulman, et al.
- All involved skin, subcutis, skeletal muscle and/or mucous membranes
- 75% on head, neck or limbs
- Three histologic patterns:
  - Benign with Antoni A areas S-100 and GFAP pos
  - Benign lacked Antoni A, but S-100 pos but GFAP neg
  - Malignant variant

Immunohistochemical Detection of CD34, E-cadherin, Claudin-1, Glucose Transporter 1, Laminin and Protein Gene Product 9.5 in 28 Canine and 8 Feline Meningiomas

- Vet Path: 47(4) 725-737 (Jul 2010); J.A. Ramos-Vara, et al.
- Staining rank:
  - Vimentin (100%)
  - CD34 (94%)
  - GLUT-1 (86%)
  - E-cadherin (81%)
  - S100 (75%)
  - Laminin (72%)
  - Claudin-1 (60%)
  - PGP 9.5 (55%)
  - Progesterone receptor (44%)
  - Pancytokeratins (39%)
Vimentin, CD34, GLUT-1, E-cadherin and S-100 were expressed in 75% of the meningiomas in this study.

Propose basic panel of vimentin, CD34 and E-cadherin for characterization of canine and feline meningiomas.

Examined detection of 21 different agents from different time points (1-12 weeks of fixation).

Found that generally fixation up to 7 weeks does not affect IHC detection of infectious agents.

Dependent on targeted antigen and selected antibody.
**Immunohistochemical Diagnosis of Canine Oral Amelanotic Melanocytic Neoplasms**

- Melan-A, PNL2, TRP-1 and TRP-2 highly sensitive and 100% specific for canine oral/lip amelanotic melanocytic neoplasms
- S-100 and Microphthalmia transcription factor (MiTF) highly sensitive, but less specific
- Recommend a cocktail of Melan-A, PNL2, TRP-1 and TRP-2

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**Canine Gastrointestinal Stromal Tumors: Immunohistochemical Expression of CD34 and Examination of Prognostic Indicators Including Proliferation Markers Ki67 and AgNOR**

- *Vet Path*: 48(1) 283-291 (Jan 2011); V. Gillespie, et al.
- GISTs are CD117 (KIT) positive and muscle markers are negative
- In this study, nearly 15% of the tumors identified as GISTs also staining positively for CD34

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**Myenteric plexus**

**GIST, Jejunum**

**Smooth muscle tumor**
**Immunohistochemical Characterization of 13 Canine Renal Cell Carcinomas; Vet Path, 48(2) 427-432, R.M. Gil da Costa, et al.**

- Papillary, tubulopapillary, papillary-cystic, solid or sarcomatoid
- 100% uromodulin +
- 12 of 13 c-KIT +
- 11 of 13 vimentin +
- 9 or 13 cytokeratin +
- Different histologic types stained differently

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**Immunohistochemical Characterization of 13 Canine Renal Cell Carcinomas**

- Papillary, tubulopapillary, papillary-cystic, solid or sarcomatoid
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- 11 of 13 vimentin +
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- Different histologic types stained differently

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**Immunohistochemical Identification of Canine Melanocytic Neoplasms with Antibodies to melanocytic Antigen PNL2 and Tyrosinase: Comparison with Melan A; Vet Path, 48(2) 443-450, J.A Ramos-Vara and M.A Miller.**

- Examined 113 samples; most were from the oral cavity or the skin
- PNL2 slightly more sensitive than Melan A and tyrosinase
- PNL2 has close to 100% sensitivity for melanocytic neoplasms
- PNL2 is resistant to prolonged fixation
Self-Evaluation

Haired skin, dog

Vimentin
GFAP

S-100

Diagnosis?

Peripheral Nerve Sheath Tumor
Self-evaluation #2 - Tissue from a dog

Morphologic Diagnosis:
- Mucous membrane:
  Rhabdomyosarcoma

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