Importance of Immunohistochemistry as a Tool for Research

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FRED HUTCHINSON CANCER RESEARCH CENTER

Importance of IHC

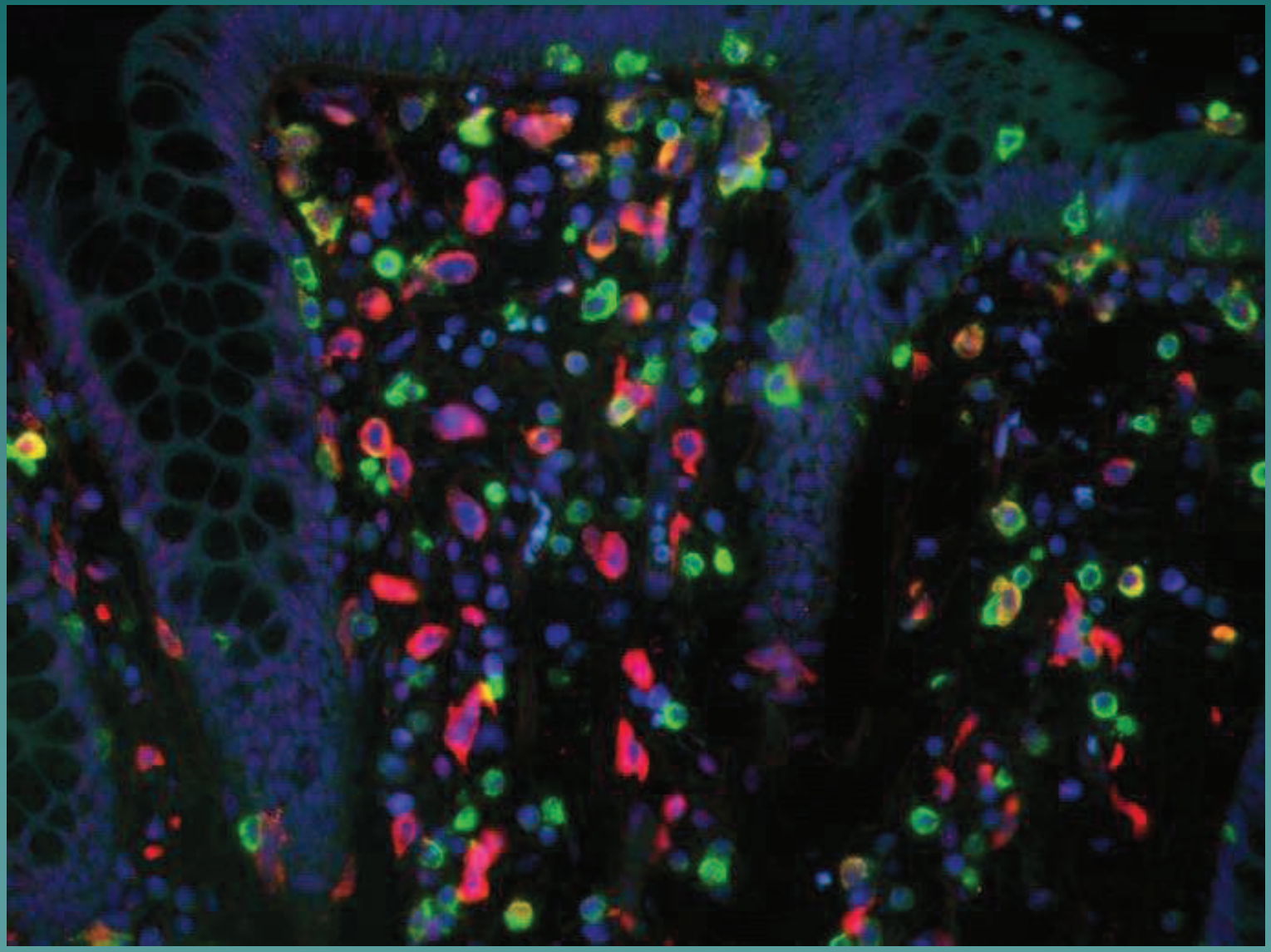
Part 2

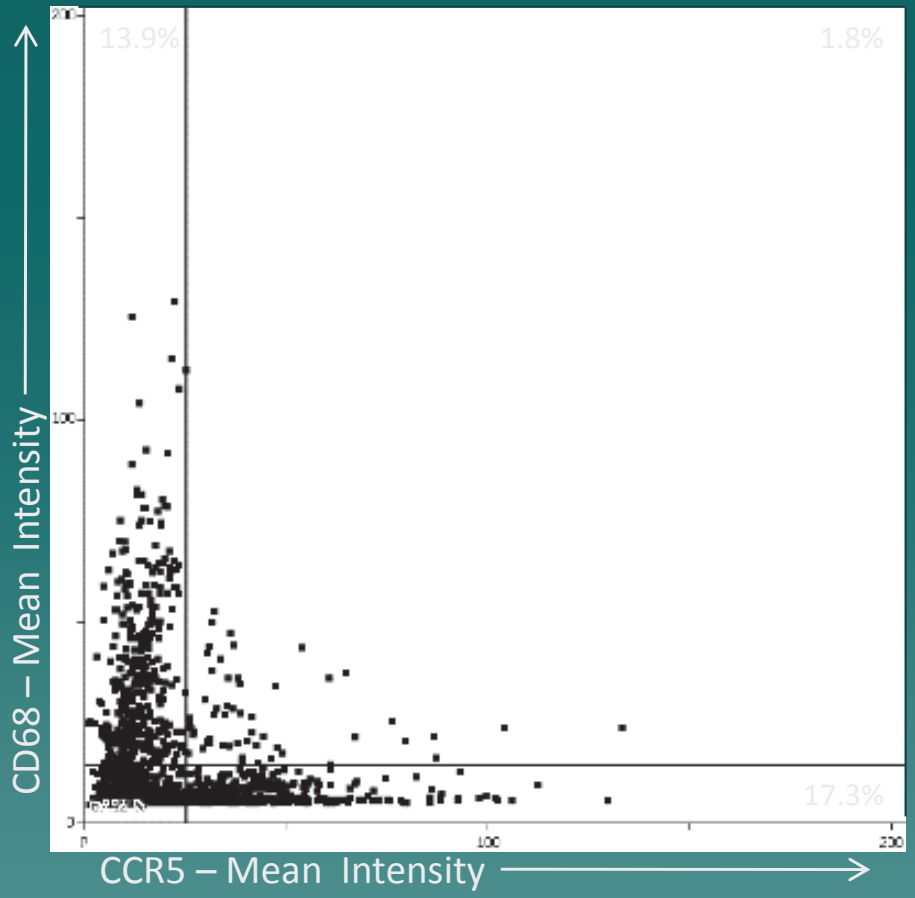
Multiplexing Antibodies

Immunofluorescence

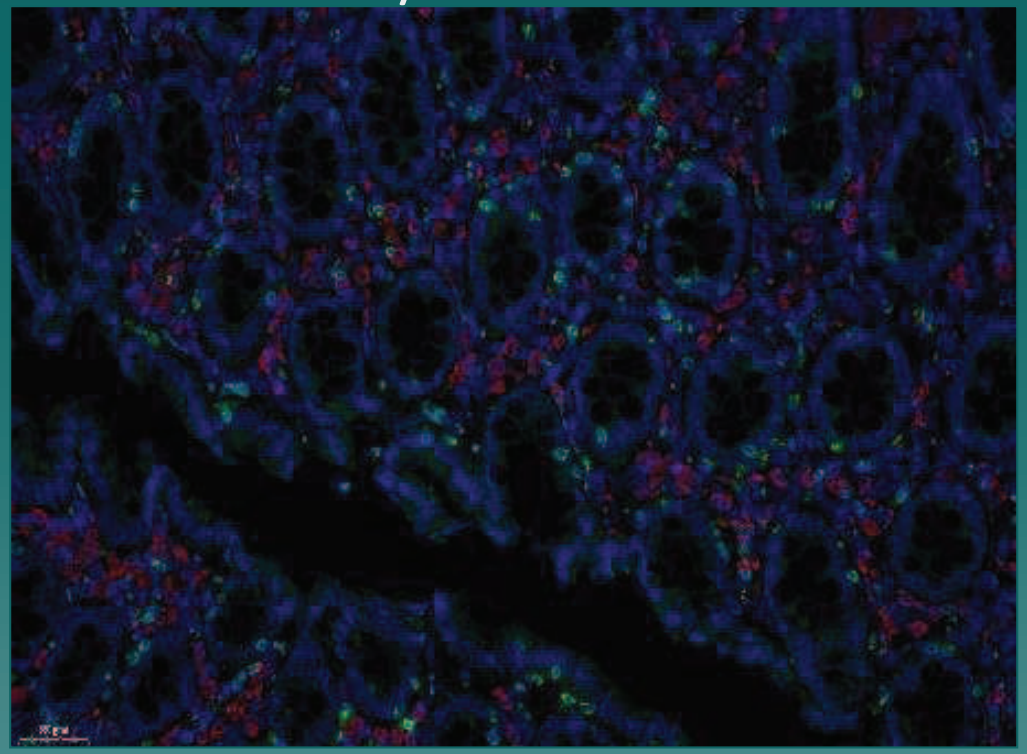
- Fluorochromes can be conjugated to proteins
  - Antibodies
    - Primary
    - Secondary
  - Avidin or Streptavidin

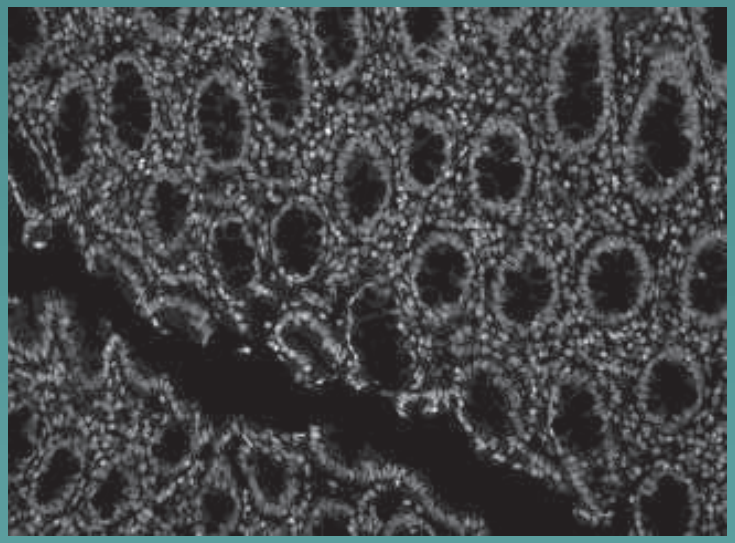
Co-localization





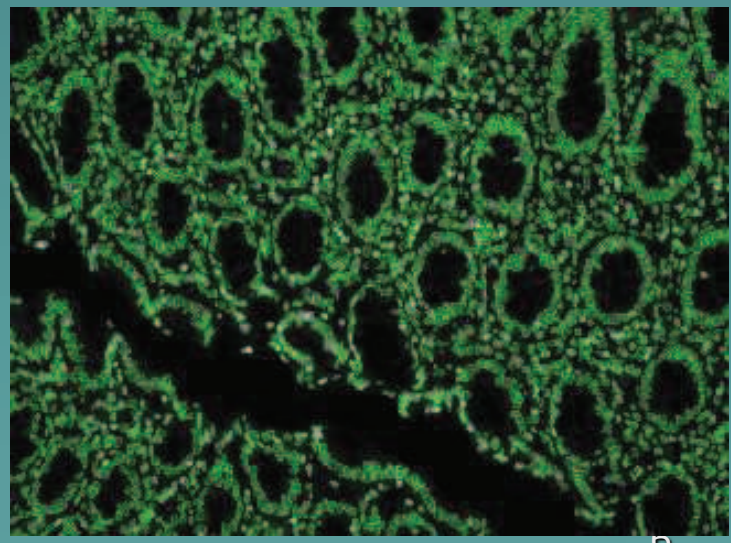
CCR5 / CD68 on Colon





Nuclei

“Recognized” Nuclei

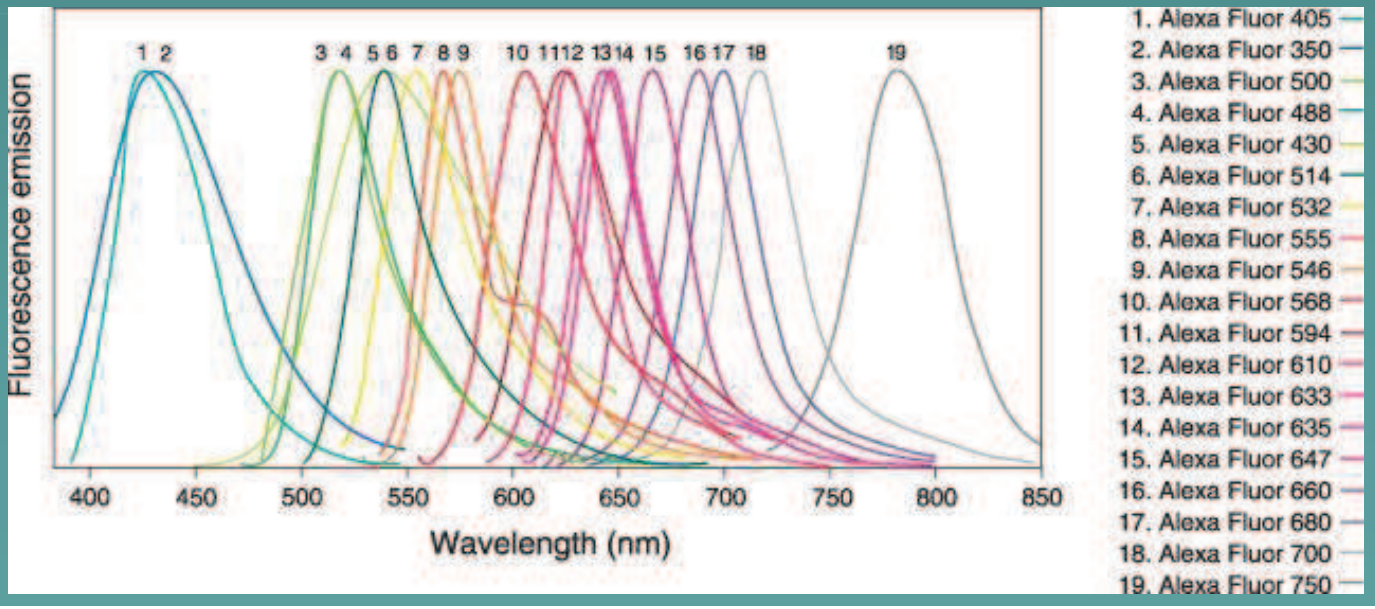


Challenges of IF

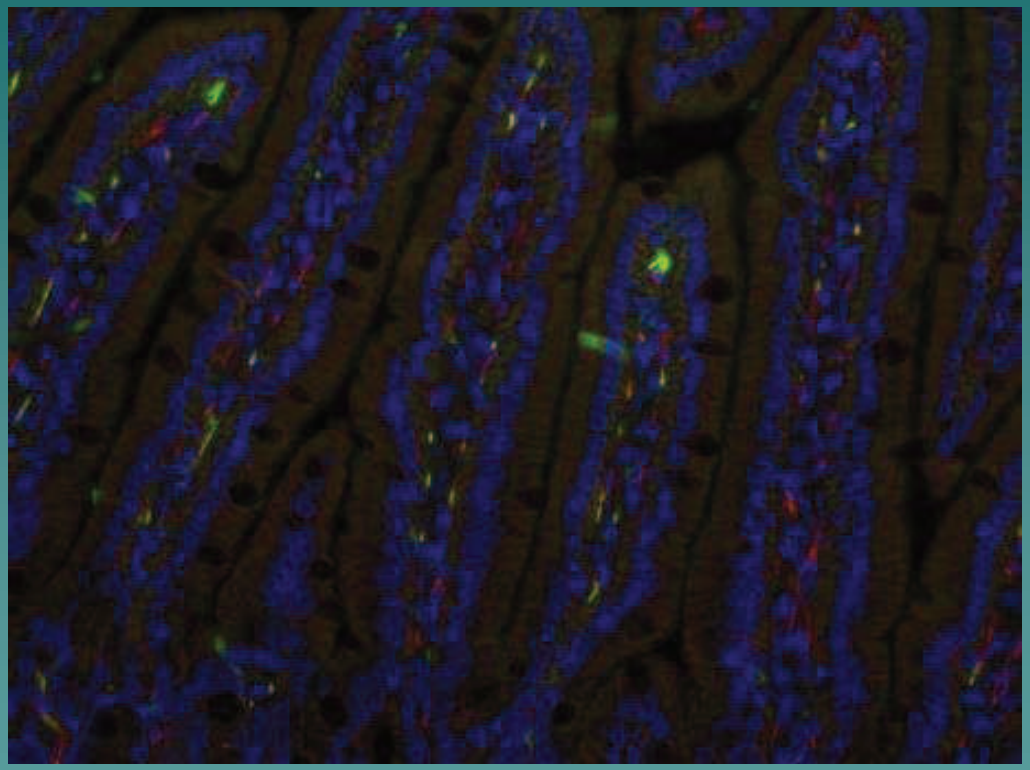
- Autofluorescence
- Photobleaching
- Quenching
- Special Equipment

Fading, Quenching, and Photobleaching

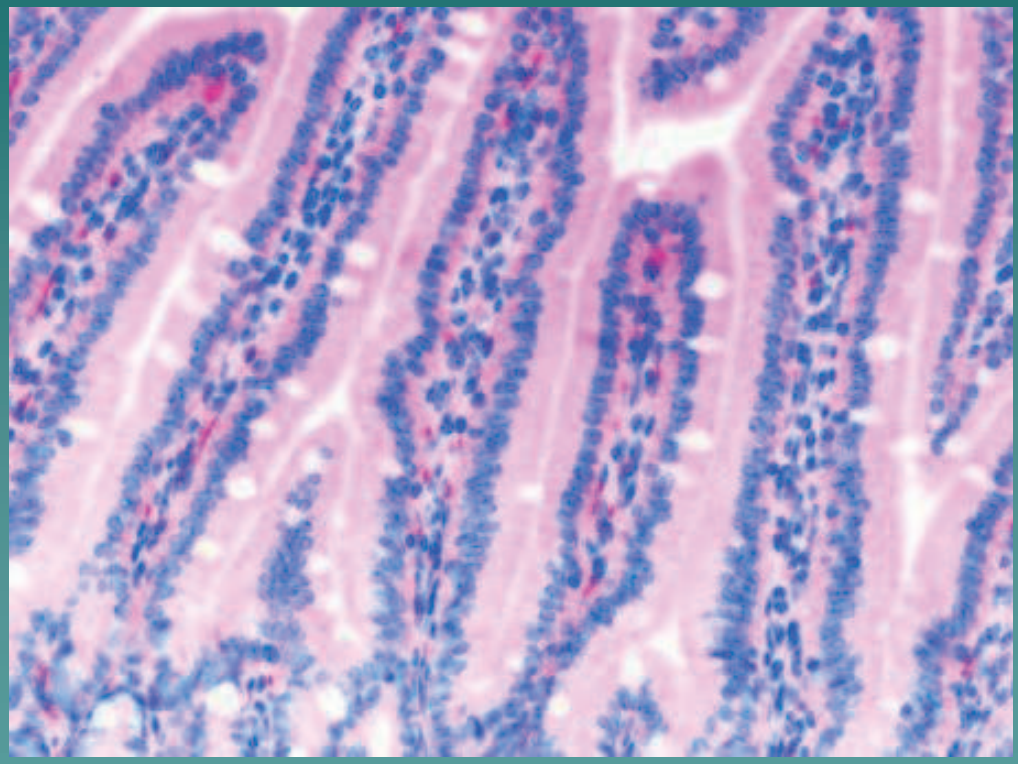
- Use anti-fading mounting media
- Use more stable fluorochromes
  - Alexa Dyes



Embrace your Autofluorescence!



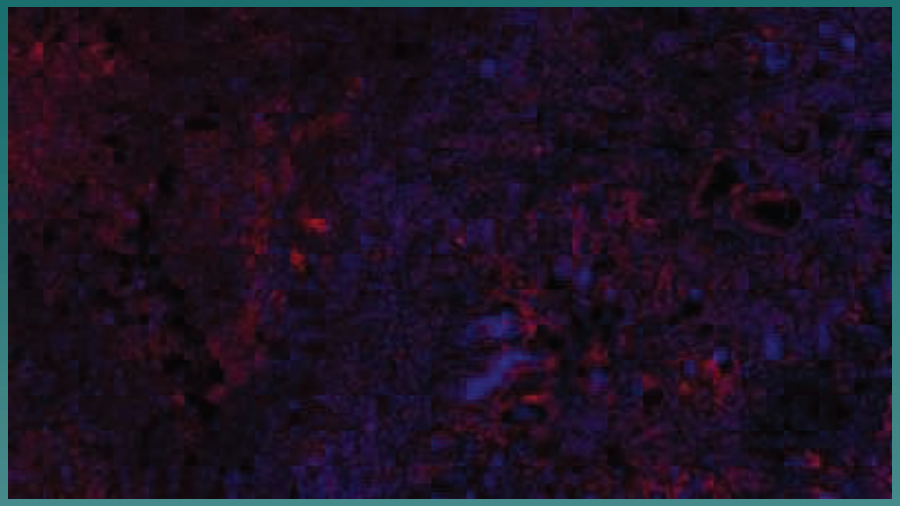
Caspase CD34



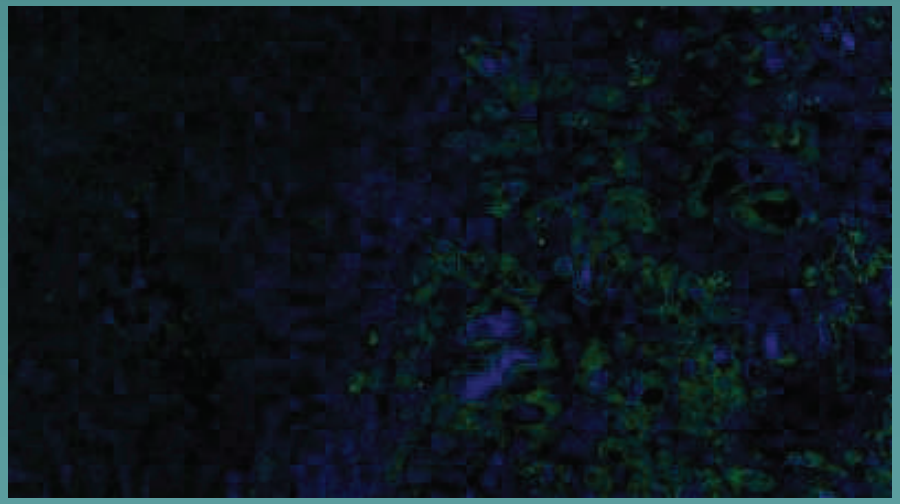
Pseudo H&E

TGF-β signaling alters the pattern of liver tumorigenesis induced by Pten inactivation

Hepatocyte

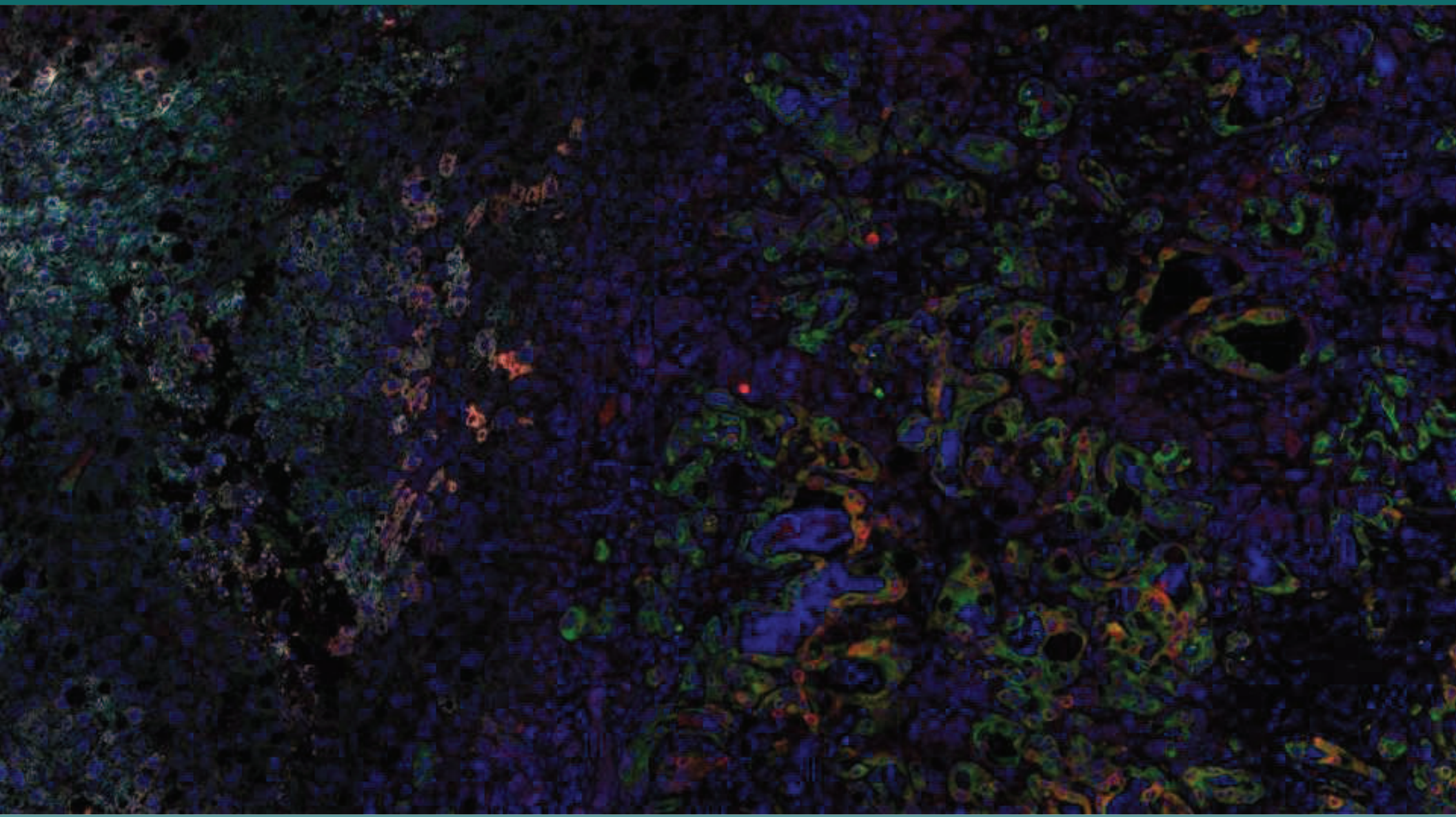


cKit



CK19

Concurrent HCC and CC



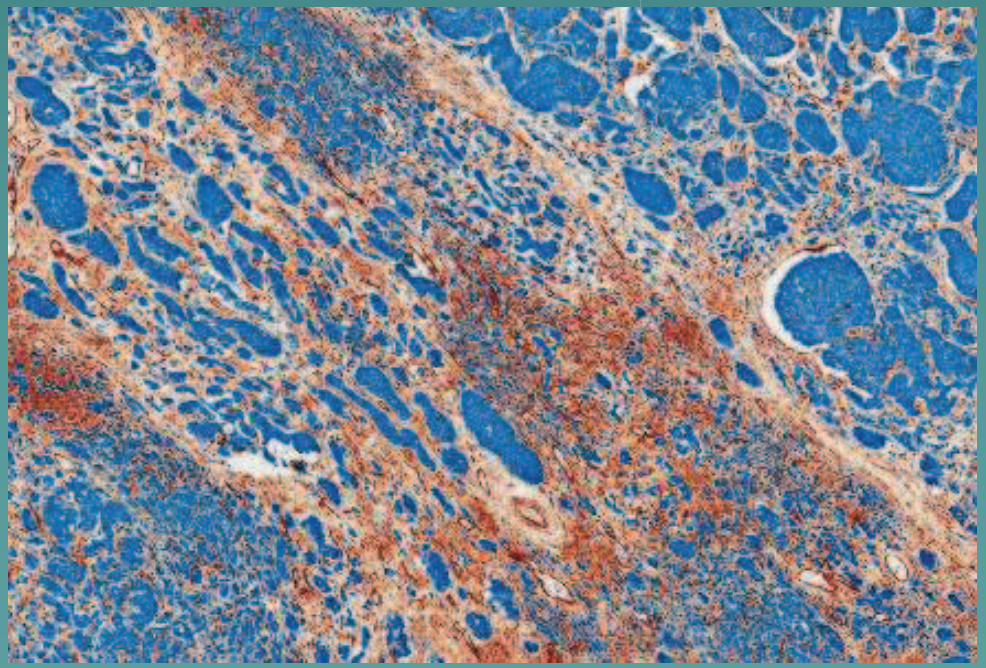
Oncogene (2014), 1–10; PMID: 2513227

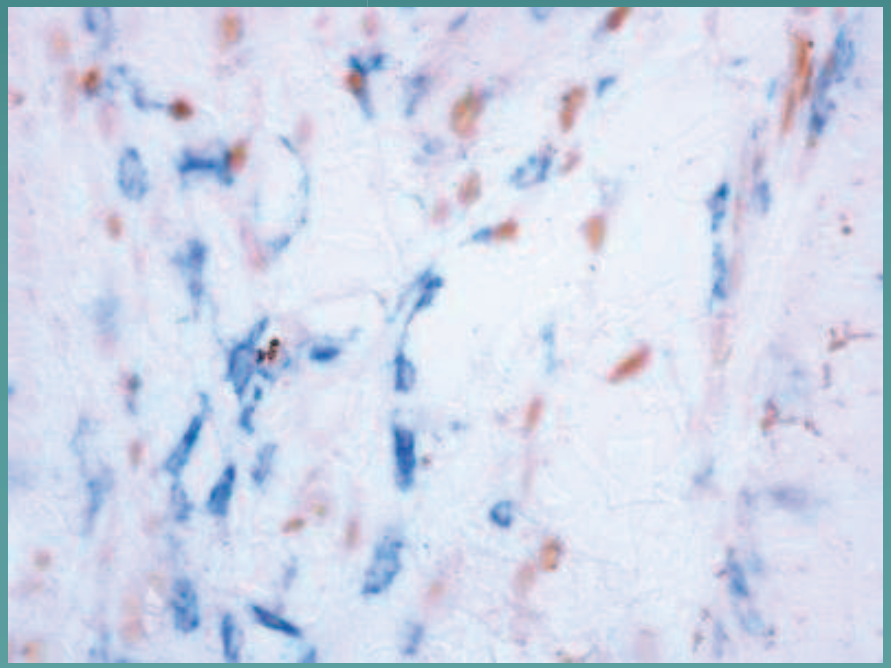
Less technically challenging then some multi-color IF

- Antibodies
  - Hepatocyte — Mouse
  - C-Kit (CD117) — Rabbit
  - Cytokeratin 19 — Rat
- Antigen Retrieval was compatible
  - All steam AR
  - No enzyme

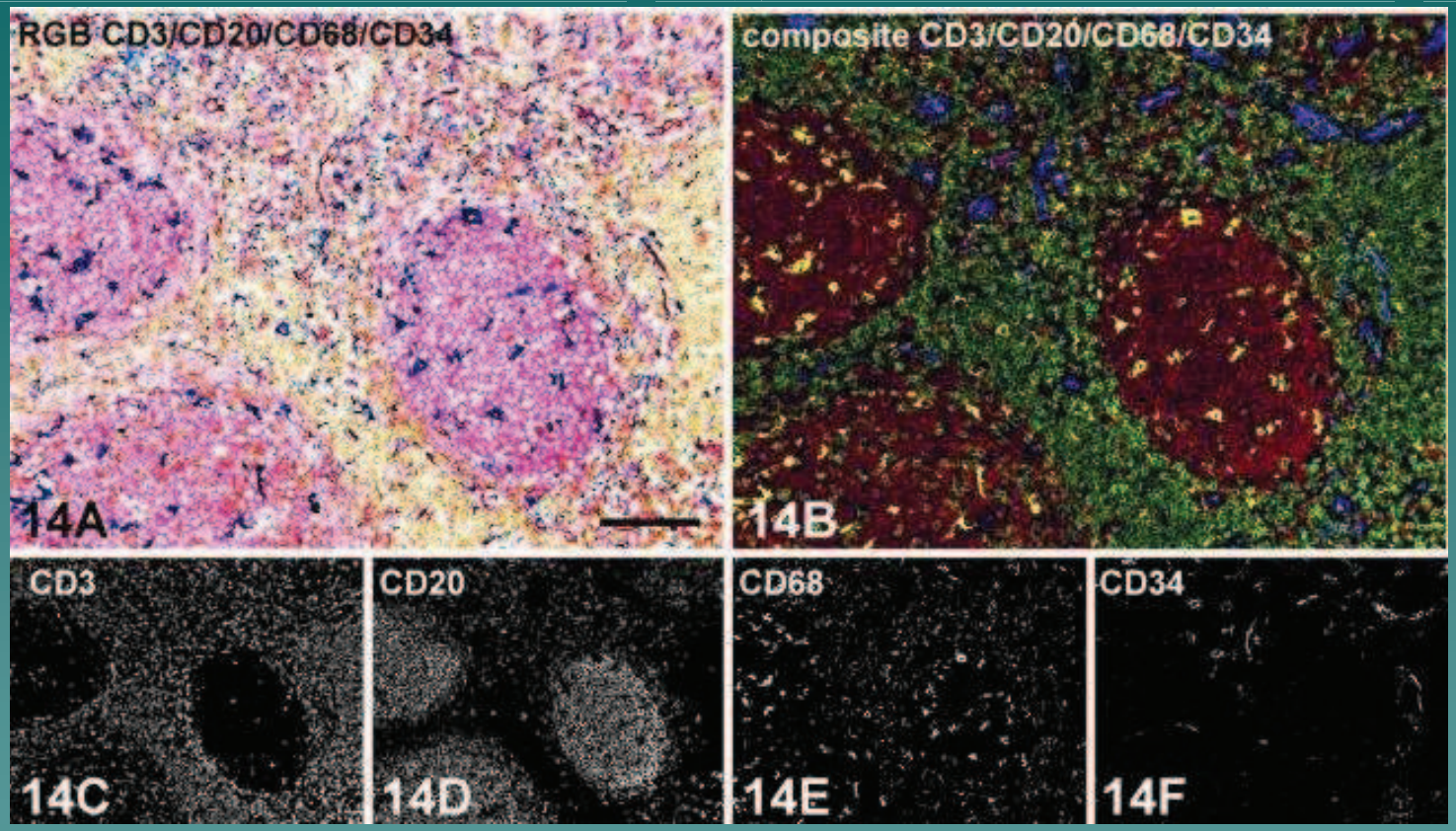
Brightfield Multiplexing

- Historically limited multi-chromagen IHC to distinct cells
  - Overlap looked muddy or one chromagen obscured other





Unmixing with spectral imaging creates a composite fluorescent-like image in pseudo-colors



RGB image of the original tissue section showing:

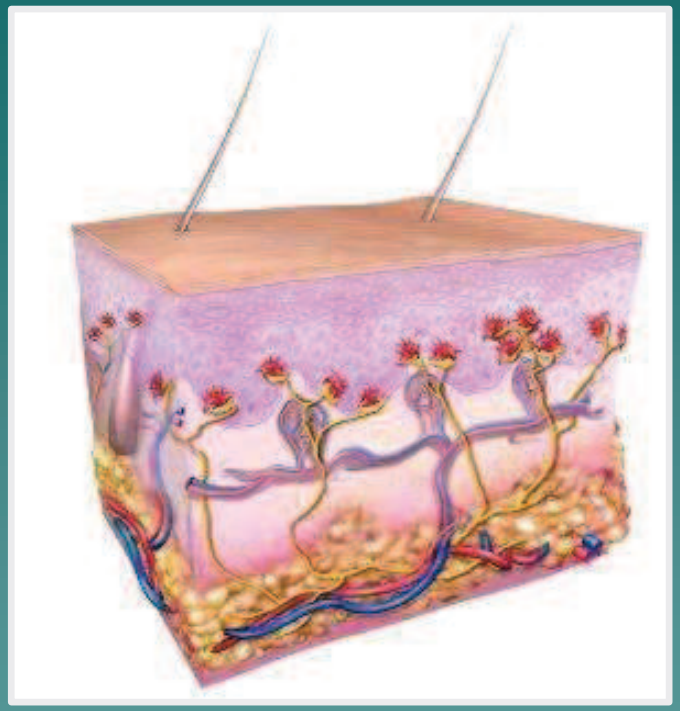
- T cells with CD3 in brown (DAB+)
- B cells with CD20 in red (LPR)
- Macrophages in blue (Vector Blue)
- Endothelium in purple (Vector VIP).

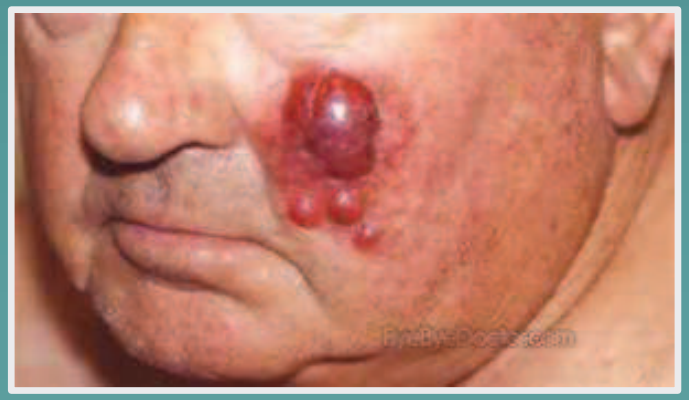
Chris van der Loos. J Histochem Cytochem 56:313–328, 2008

IHC and IF for a More Complete Picture

Why our Genomics and Proteomics folks need Histology!

Merkel Cell Carcinoma



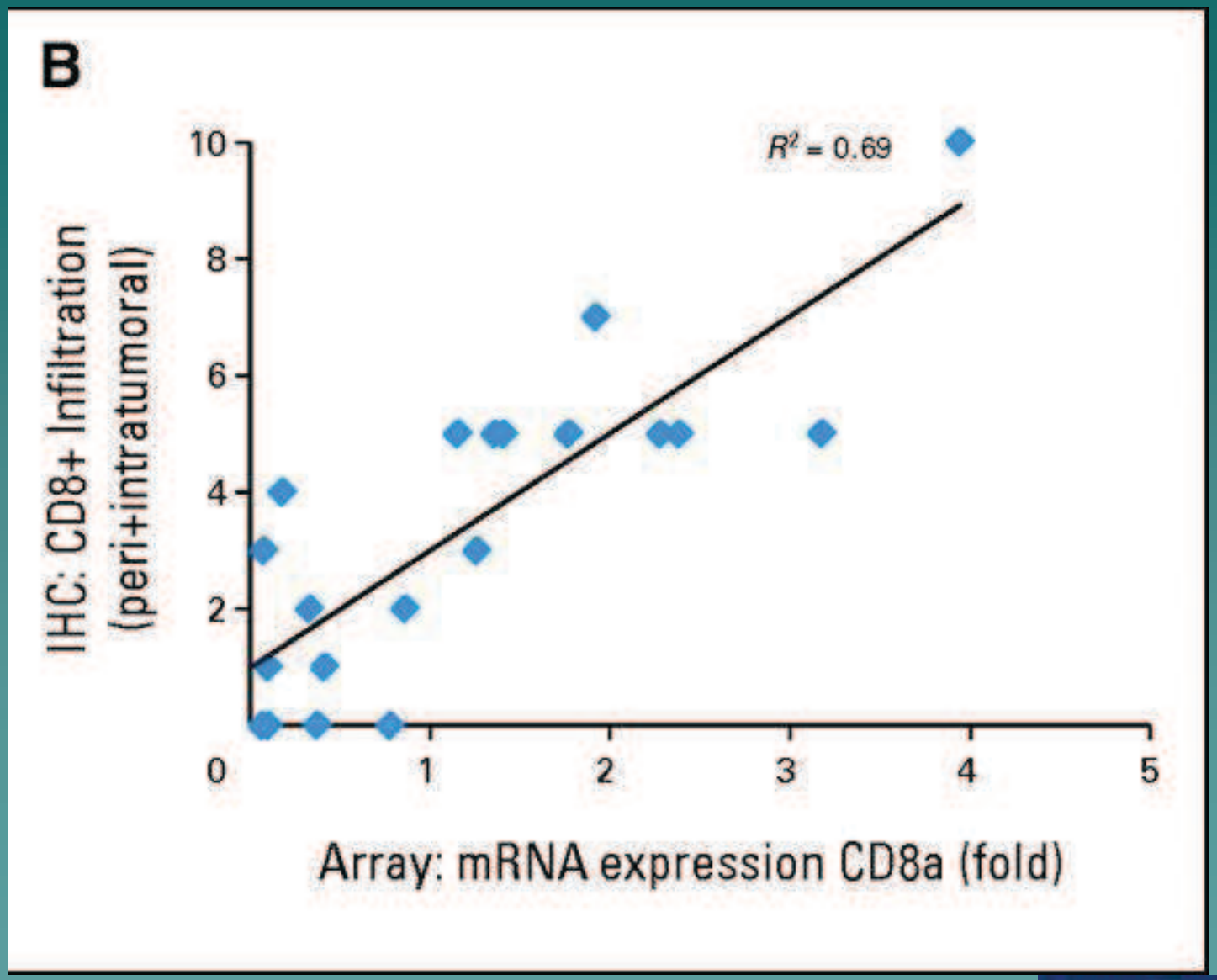


- Rare and highly aggressive skin cancer
- Most cases appear to be caused by the Merkel cell polyomavirus

Merkel Cell Carcinoma

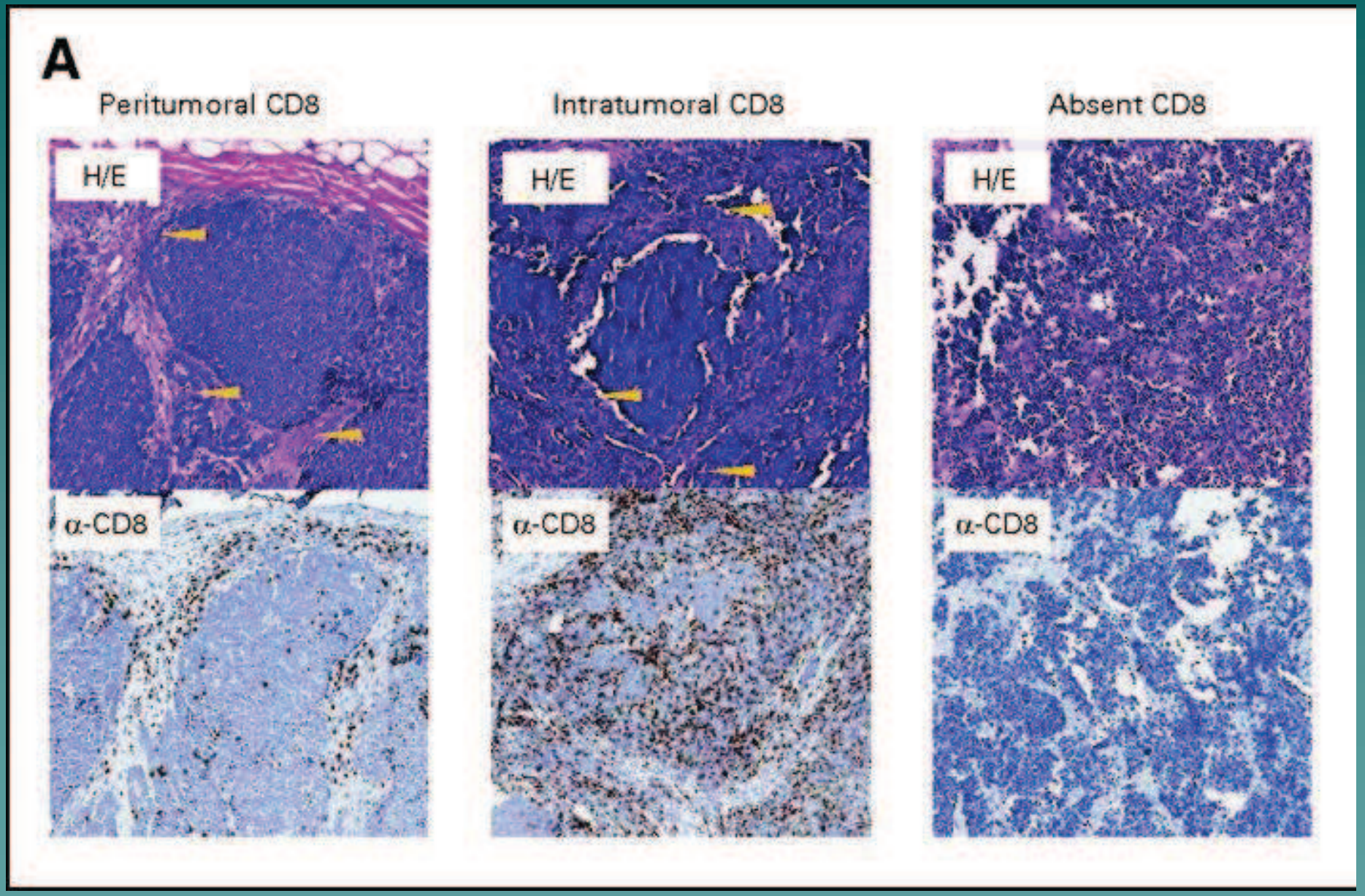
- RNA from total homogenized tissue
- Patients Negative for CD8a mRNA: Always poor outcome
- Patients Positive for CD8a mRNA: Mixed outcome – some good, some poor
- Why do some patients with lots of CD8 cells still do poorly?

CD8+ lymphocytic infiltration correlates with mRNA expression of CD8a.



Paulson K G et al. JCO 2011;29:1539-1546

It depends on where the CD8a cells are located!



Paulson K G et al. JCO 2011;29:1539-1546

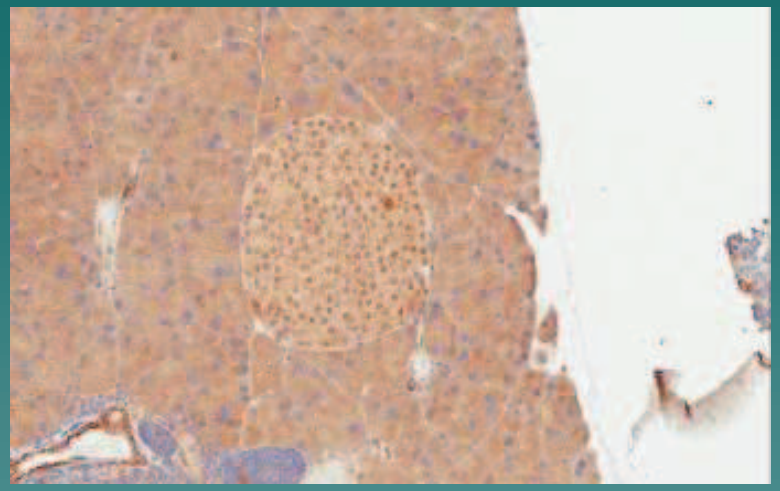
Islet Tumors

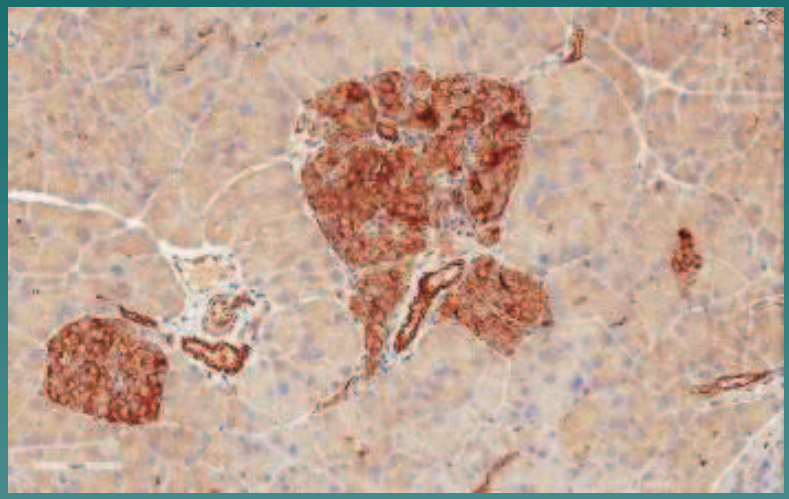
- RIP7-rt-TA mice have rat insulin 2 (*Ins2*) promoter
- Mated to a second transgenic strain carrying overexpression of Bcl-XL under the regulatory control of a tetracycline-responsive promoter element.
- Expression of the gene in pancreatic beta cells is induced with administration of the doxycycline.

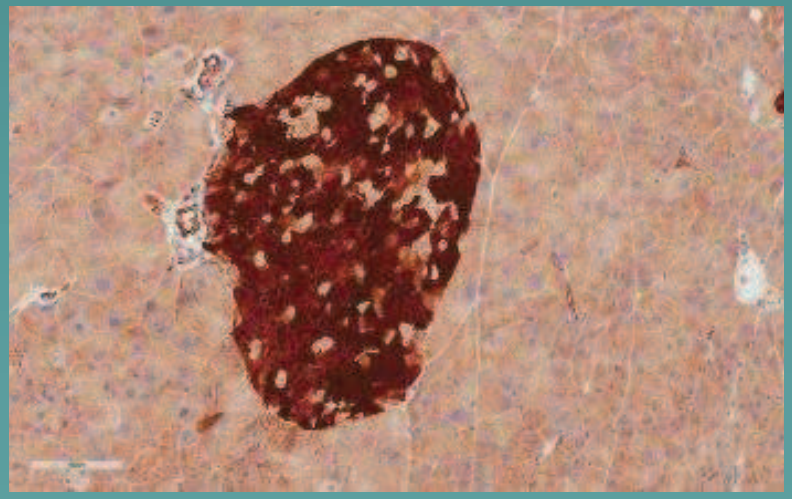
Problems

- Molecular analysis identified mice with proper genotype
- However, not all mice were developing tumors
- Turns out, variable level of Bcl-XL was being expressed by breeder mice

Bcl-XL Expression







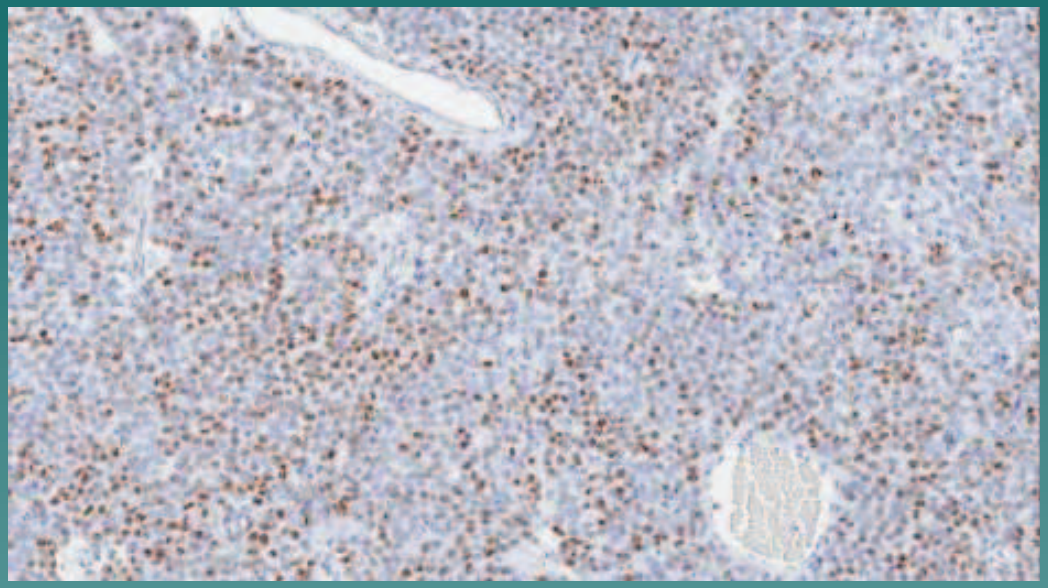
Choosing the Right Marker for your Question

Meduloblastoma Model

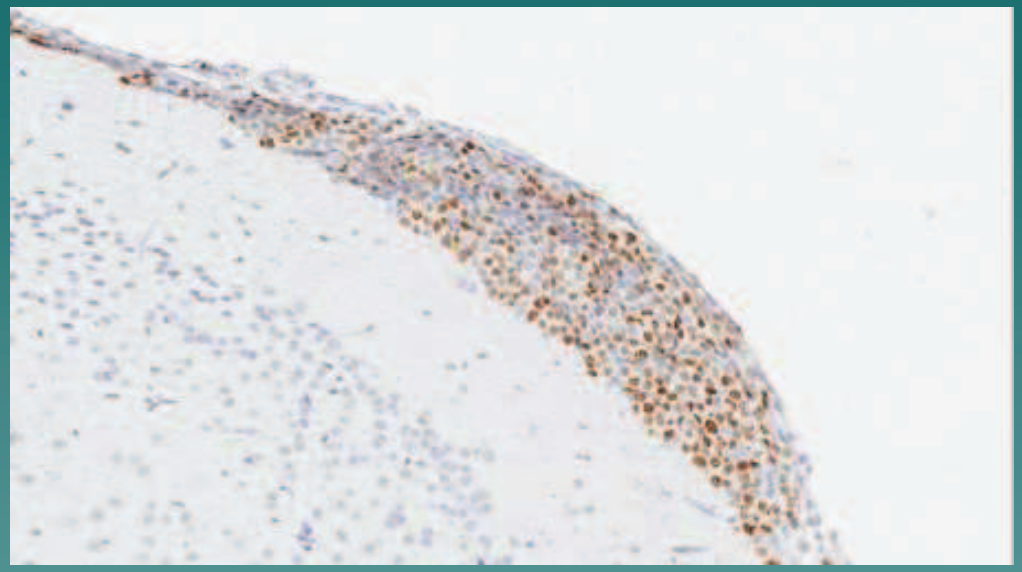
- Cyclin-Dependent Kinase (Cdk) 4/6 inhibitor:
  - Smaller tumors
  - Longer life
- Vehicle
  - No change from untreated
  - Large tumors
- Look at proliferation

Ki67 IHC to Measure Proliferation

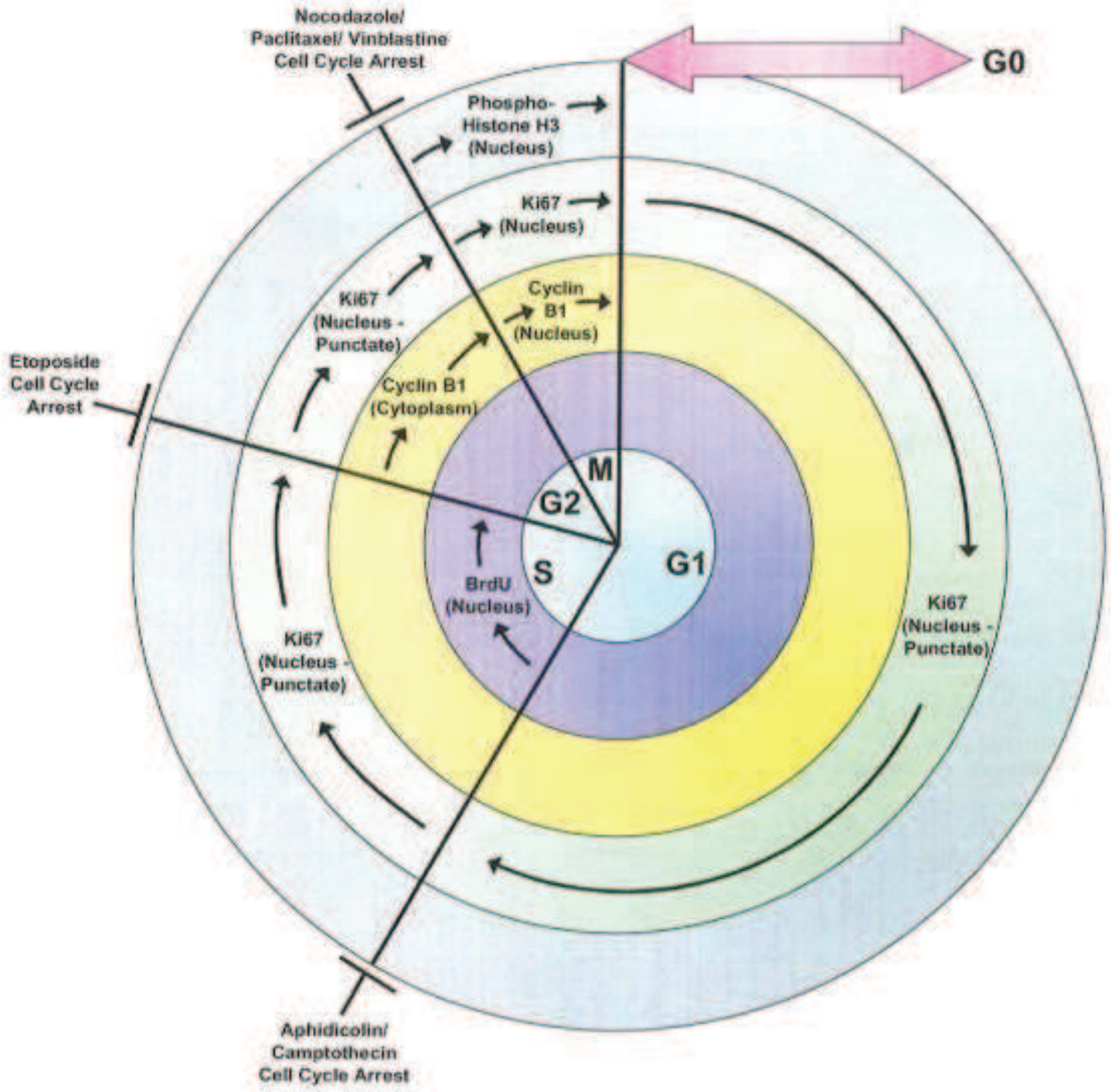
Vehicle



Drug



- Proliferation rate was the same. Why?
- Cdk4/6 inhibitor is expected to arrest the cells in the G1 phase of the cell cycle.

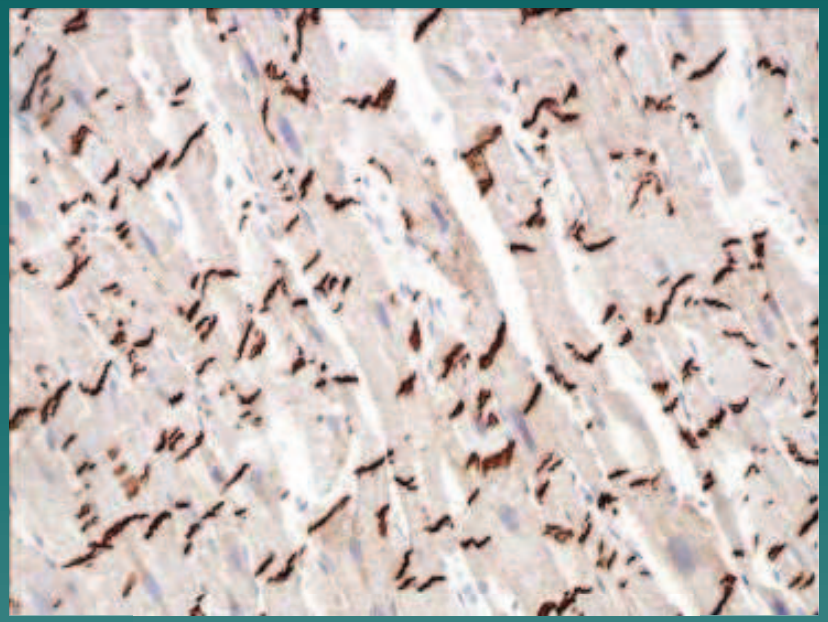

Nocodazole/
Paclitaxel/ Vinblastine
Cell Cycle Arrest
G0
Phospho-
Histone H3
(Nucleus)
Ki67
(Nucleus)
Ki67
(Nucleus -
Punctate)
Cyclin
B1
(Nucleus)
Etoposide
Cell Cycle
Arrest
Cyclin B1
(Cytoplasm)
M
G2
S
G1
BrdU
(Nucleus)
Ki67
(Nucleus -
Punctate)
Ki67
(Nucleus -
Punctate)
Aphidicolin/
Camptothecin
Cell Cycle Arrest

Validation of Antibody Specificity

Primary antibodies

How do you pick your antibody?

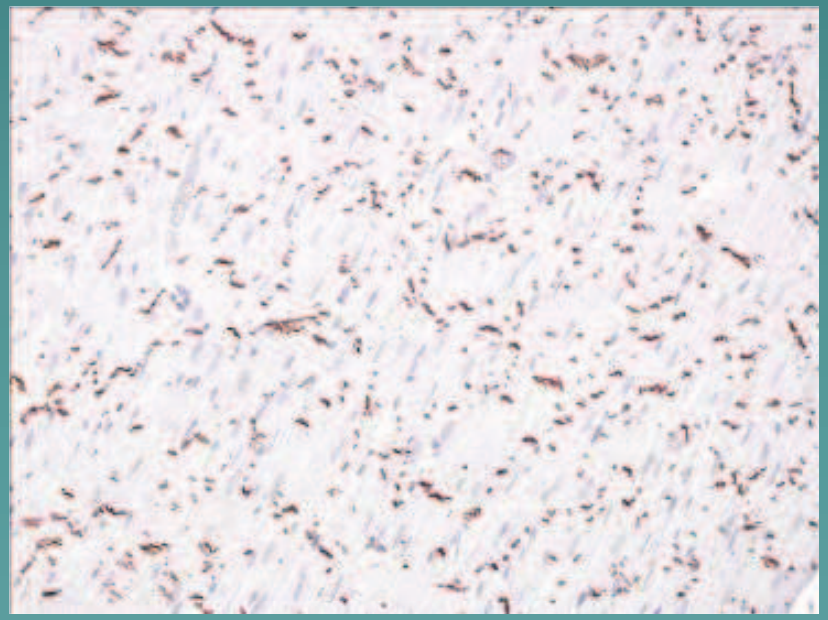
- It's been made against the species of interest or is known to cross-react
- Works in your system – nonfixed frozen, or formalin fixed, paraffin embedded
- Robust and consistent staining
- Cost effective and available



Human Heart

N-Cadherin: Made in mouse using Human Heart N-Cadherin as immunogen

Mouse Heart



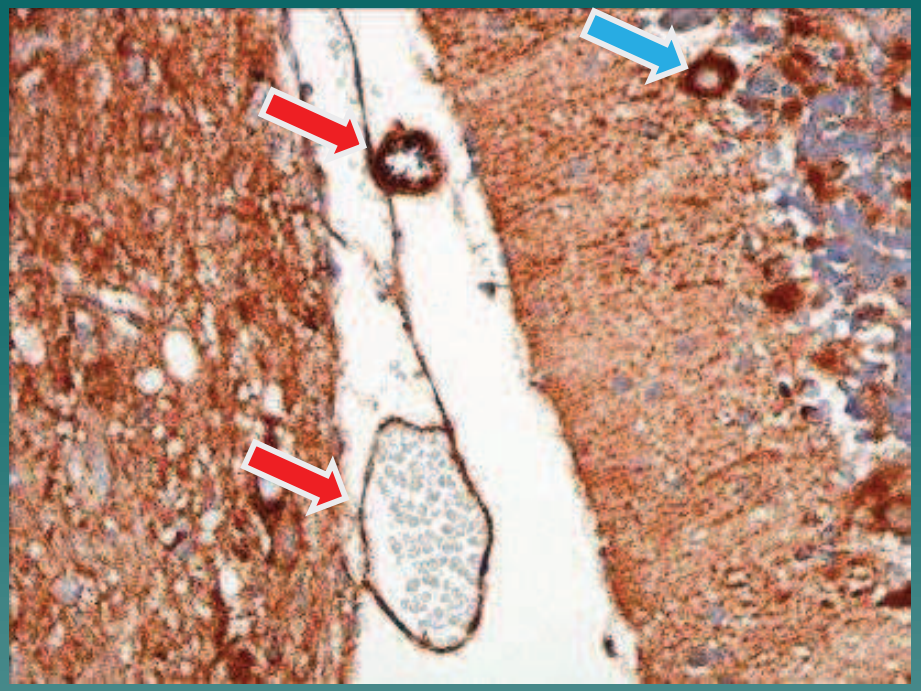
Canine Heart

BEWARE!

- "But **THEY** got it to work……
  - In this lab!"
    - On unfixed HUMAN cells! Not FFPE CANINE tissue!
  - In this paper!"
    - The methods failed to mention that they antigen retrieved in an autoclave for 20 minutes followed by proteinase K for 10 minutes and used the antibody at 50 ug/ml with a tyramide and all it is now is background!
    - With a photo shop expert
  - In flow!" or In a Western!"
    - Are you kidding me!

Interpreting the Staining

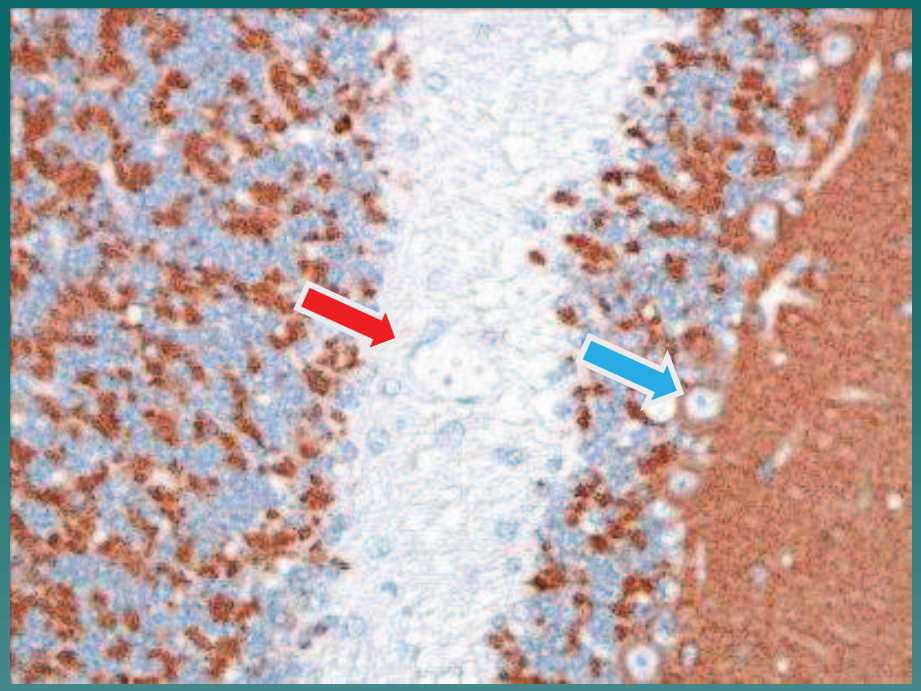
- Is it staining the right cells
  - With the right pattern
  - With the correct distribution
- Do not assume antibody has been correctly characterized
  - By another lab
  - By company
  - In the Literature



Synaptophysin
Novus NBP1-19361

Stains:
- Nerve tissue
- Purkinje
- Endothelial cells.

Inappropriate pattern.



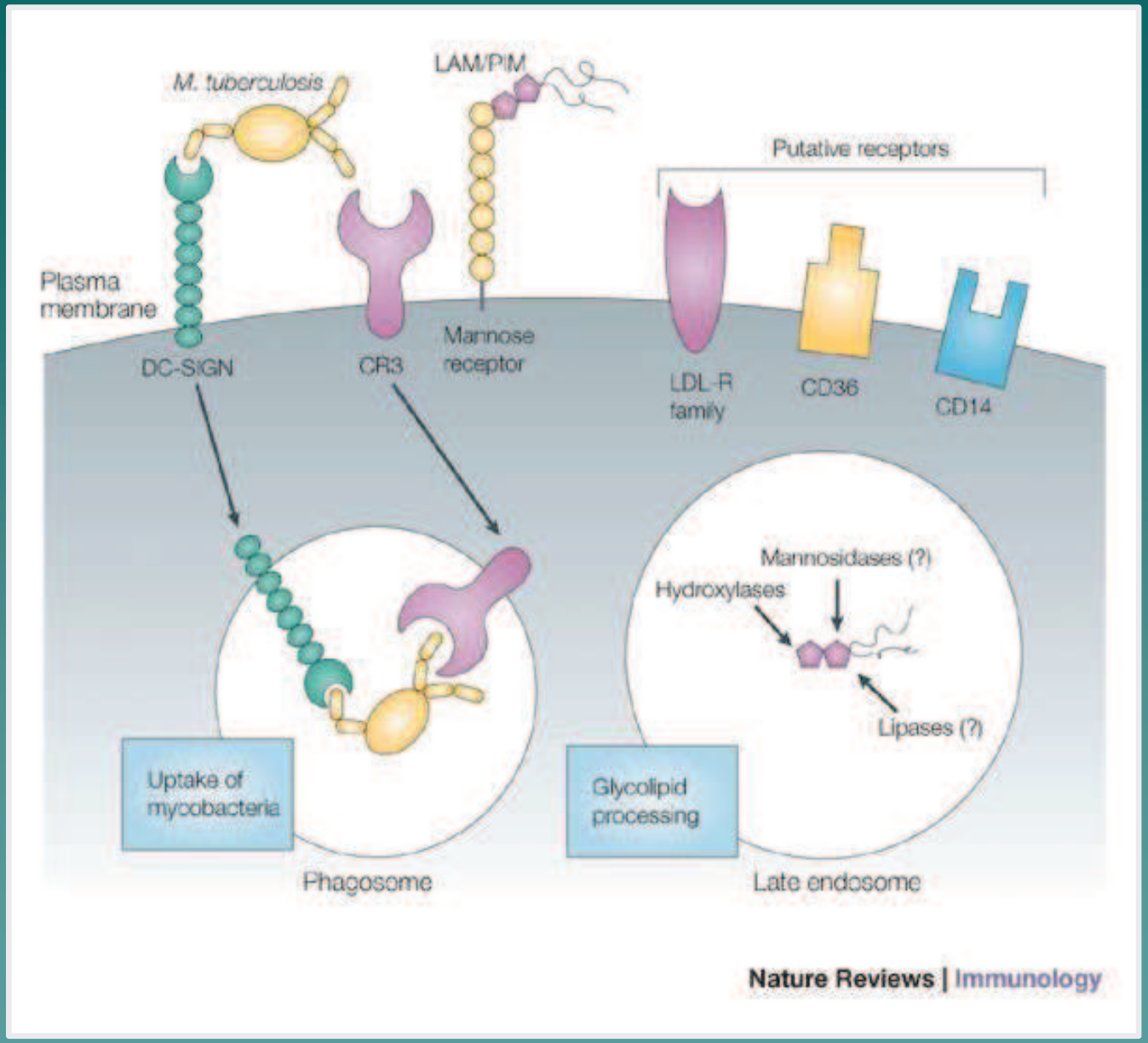
Synaptophysin
Invitrogen 18-0130

Stains:
- Nerve tissue

Appropriate pattern.

DC-SIGN

- **D**endritic **C**ell-**S**pecific **I**ntercellular adhesion molecule-3-**G**rabbing **N**on-integrin
  - also known as CD209
- On the surface of both macrophages and dendritic cells
- Activates recognition of pathogen and phagocytosis
- Mediates dendritic cell rolling interactions with blood endothelium and activation of CD4+ T cells

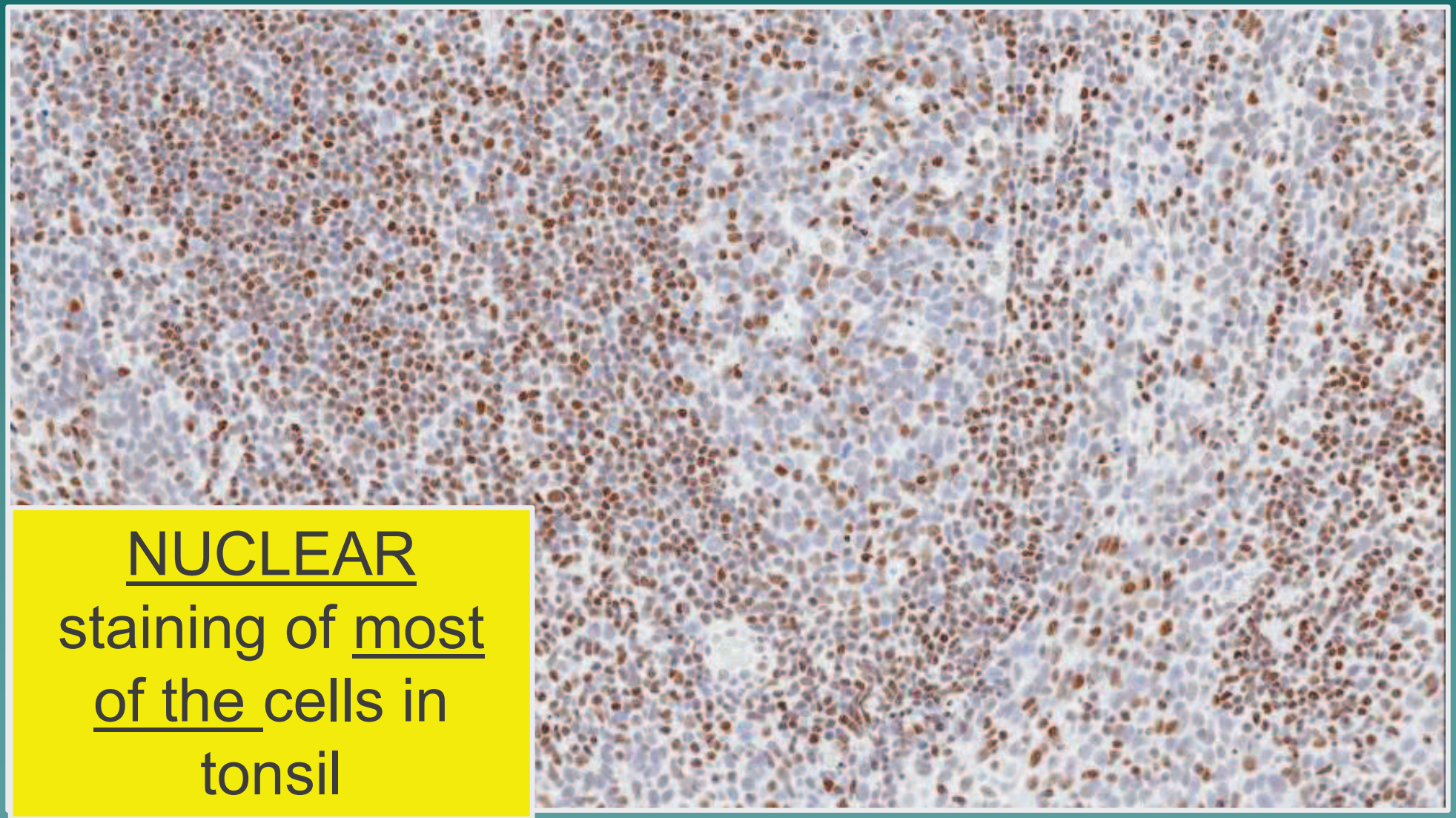


DC SIGN

- Macrophage
- Dendritic Cells

- Cell surface
- Cytoplasmic in phagosome

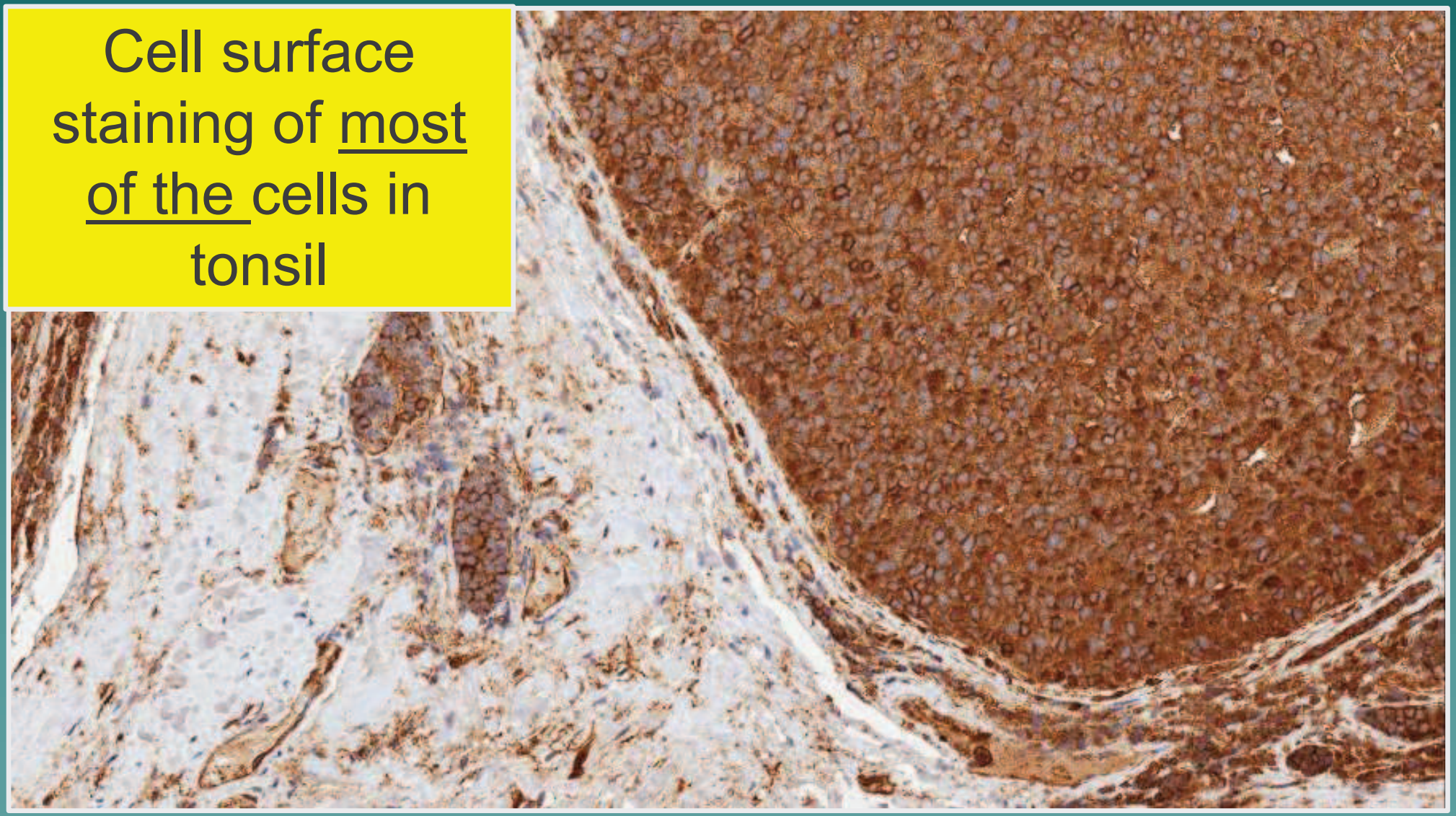
DC SIGN – Antibody #1



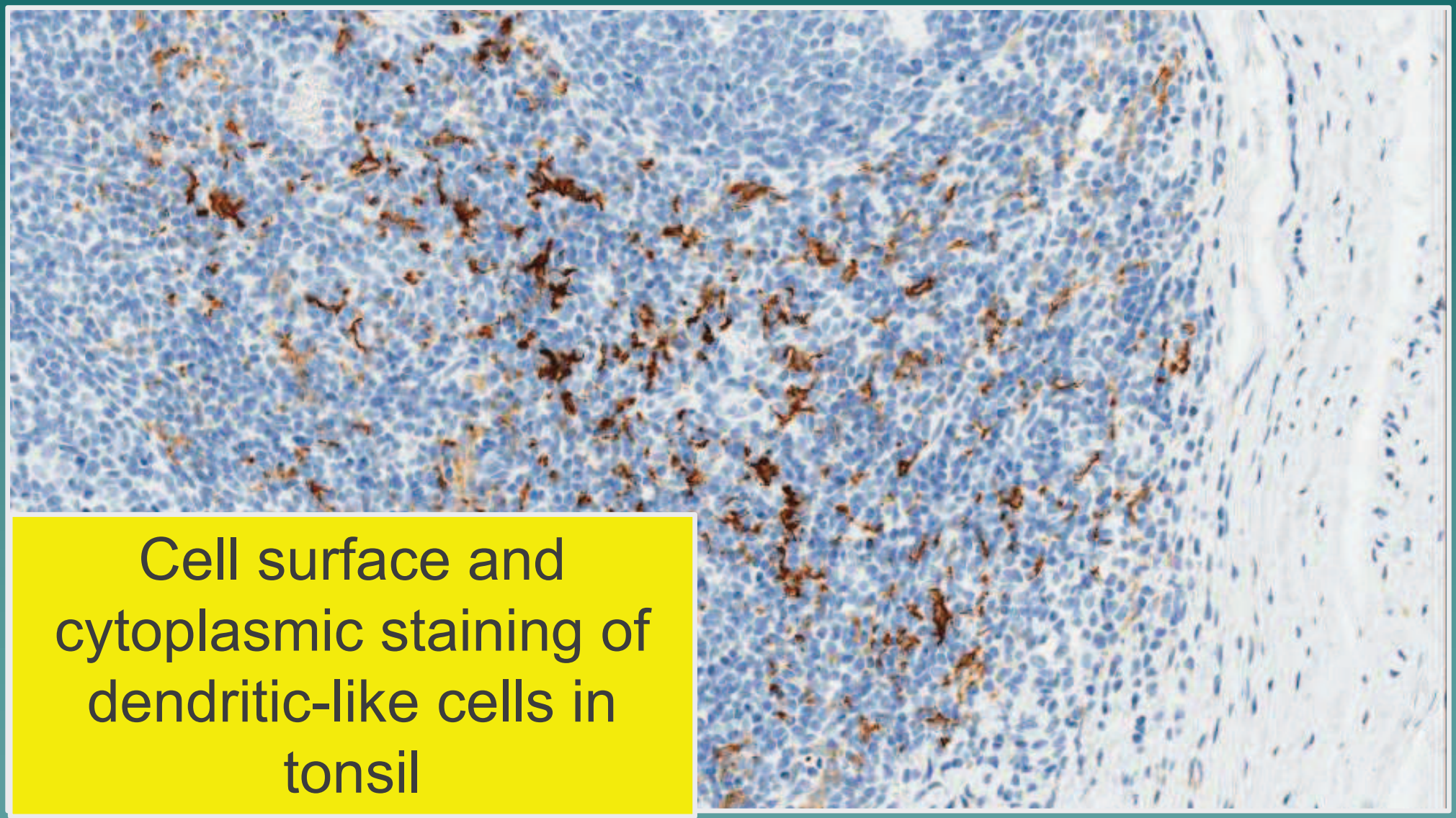
NUCLEAR staining of most of the cells in tonsil

DC SIGN – Antibody #2

Cell surface staining of most of the cells in tonsil

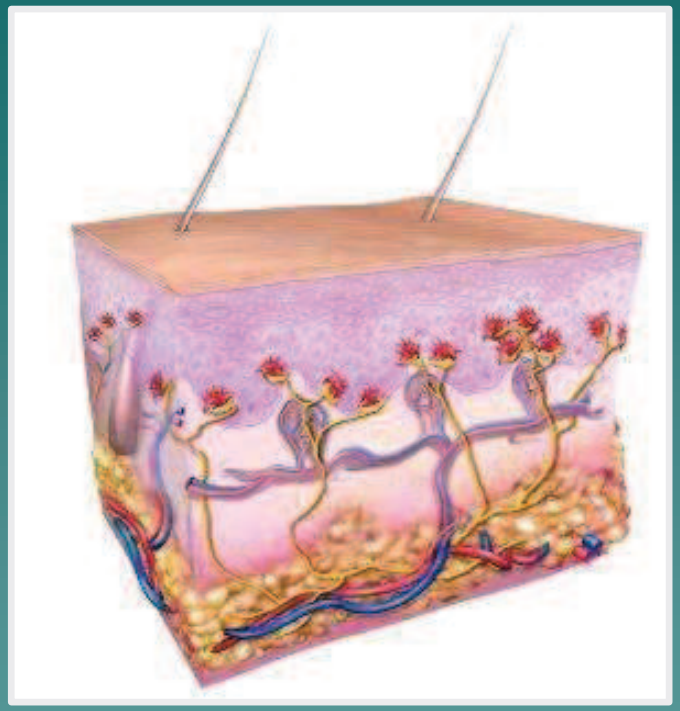


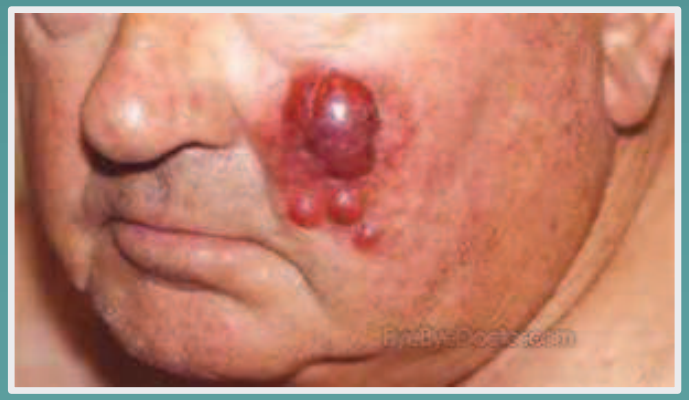
DC SIGN – Antibody #3



Cell surface and cytoplasmic staining of dendritic-like cells in tonsil

Merkel Cell Carcinoma





- Rare and highly aggressive skin cancer
- Most cases appear to be caused by the Merkel cell polyomavirus

CK20 and Merkel Cell Tumors

Cytokeratin 20: a marker for diagnosing Merkel cell carcinoma.

Scott MP[1], Helm KF.

Am J Dermatopathol. 1999 Feb;21(1):16-20.

Abstract

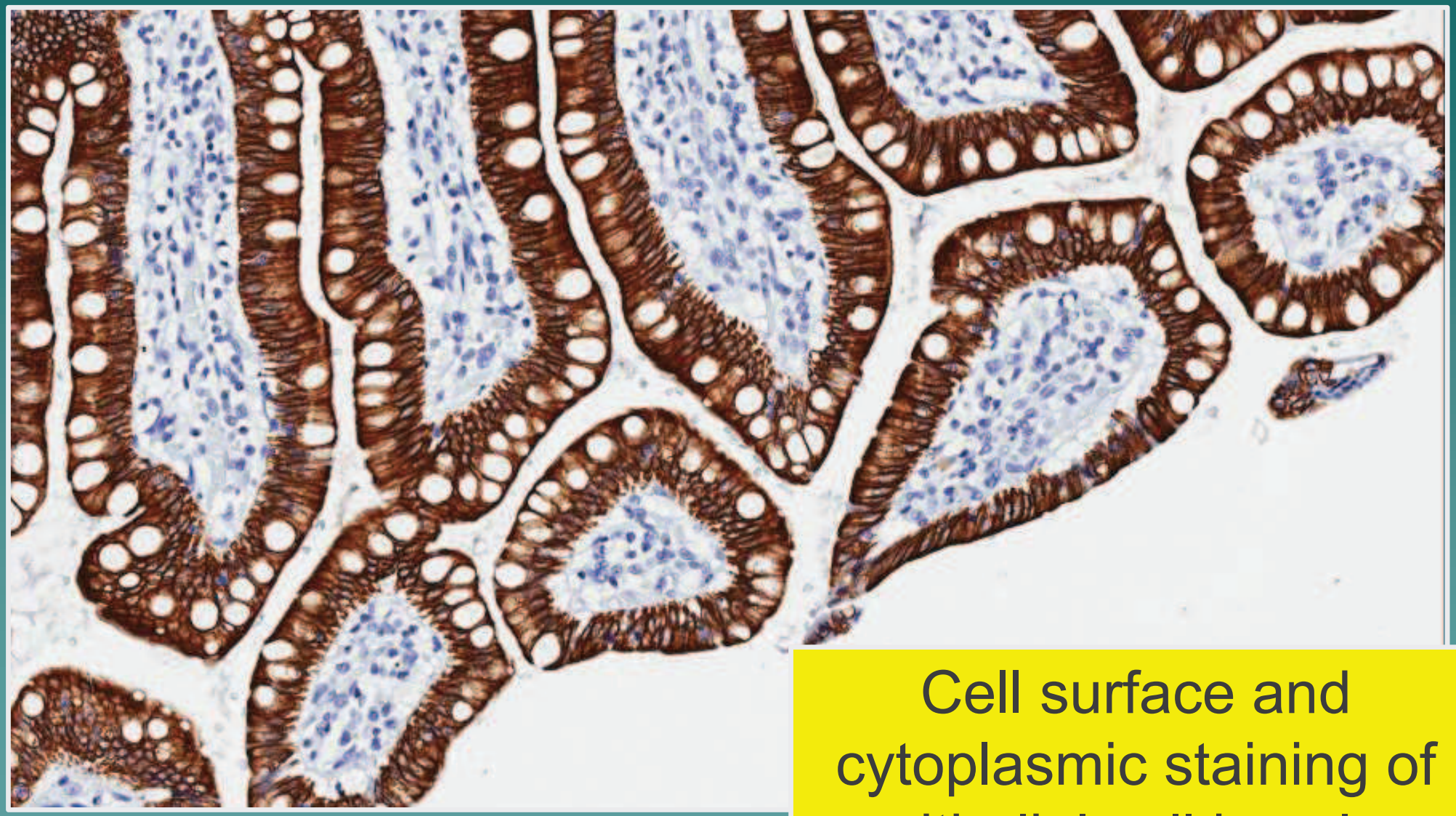
Merkel cell carcinoma is an aggressive cutaneous neoplasm that is often difficult to diagnose because of its histologic and immunohistochemical similarity to metastatic oat cell carcinomas and other cutaneous neoplasms. Our purpose was to determine the utility of immunoperoxidase staining of cytokeratin 20 (CK 20), a newly discovered intermediate filament protein, in Merkel cell carcinomas and other cutaneous tumors. Sixty-one tumors were sectioned and stained with antibodies directed at CK 20. The staining of Merkel cell carcinomas was compared with metastatic oat cell carcinomas, lymphomas, squamous cell carcinomas, basal cell carcinomas, melanomas, metastatic carcinoids, spiradenomas, eccrine carcinomas, adenoidcystic carcinoma, sebaceous carcinomas, hidradenomas, sebaceous epitheliomas, trichoblastomas, mixed tumors, and metastatic adenocarcinomas. Nine of 10 Merkel cell carcinomas stained with antibody to CK 20. Two metastatic carcinomas to the skin were also positive. One hidradenoma and one squamous carcinoma exhibited focal staining, but were otherwise negative. All other tumors were nonstaining.

Cytokeratin 20 is a sensitive and specific marker for Merkel cell carcinoma and is helpful in distinguishing between Merkel cell carcinoma and other malignant and benign neoplasms.

CK20

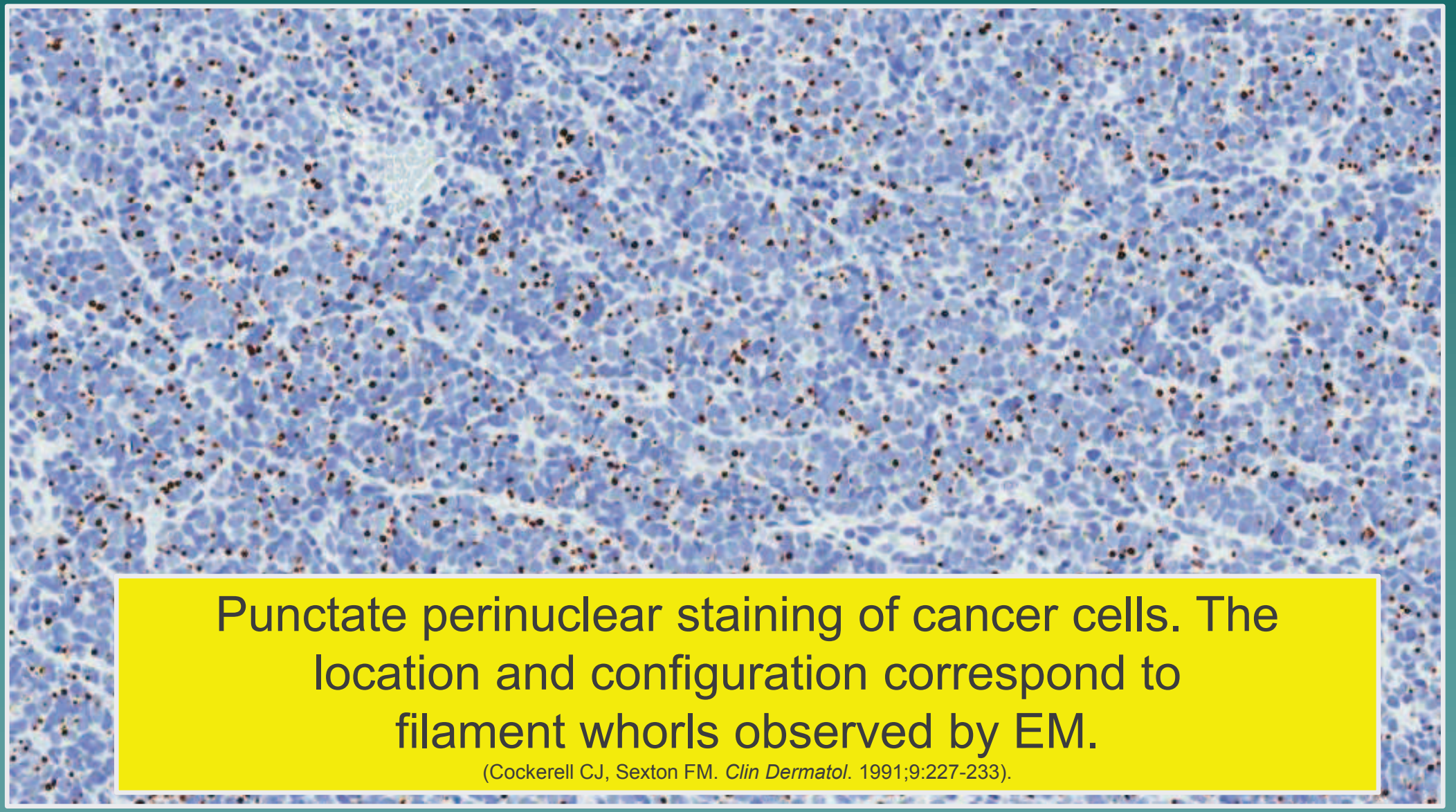
- **Keratin 20**, often abbreviated **CK20**
- Type I cytokeratin.
- Mature enterocytes and goblet cells and is specifically found in the gastric and intestinal mucosa.
- The protein is commonly found in colorectal cancer, transitional cell carcinomas and in Merkel cell carcinoma
- It is often used in combination with antibodies to CK7 to distinguish different types of glandular tumors

CK20 on Colon Control



Cell surface and cytoplasmic staining of epithelial cell in colon

CK20 On Merkel Cell CA



Punctate perinuclear staining of cancer cells. The location and configuration correspond to filament whorls observed by EM.

(Cockerell CJ, Sexton FM. *Clin Dermatol*. 1991;9:227-233).

Negative or Isotype Control

- Irrelevant immunoglobulin from the same species and subtype as primary
- Used to determine non-specific binding of primary or secondary antibody
- Must be concentration matched with the primary and use the same staining procedure
  - Primary antibodies in research are often used at very high concentrations
  - Caution when using Universal Isotype Controls

Inappropriate Negative Control

- "Secondary only" or "no primary"
  - Can be helpful during work-up and troubleshooting
    - Will show if problem with detection system
  - Secondaries and polymers very clean now

FHCRC Experimental Histopathology Shared Resources

Lab Contact Info

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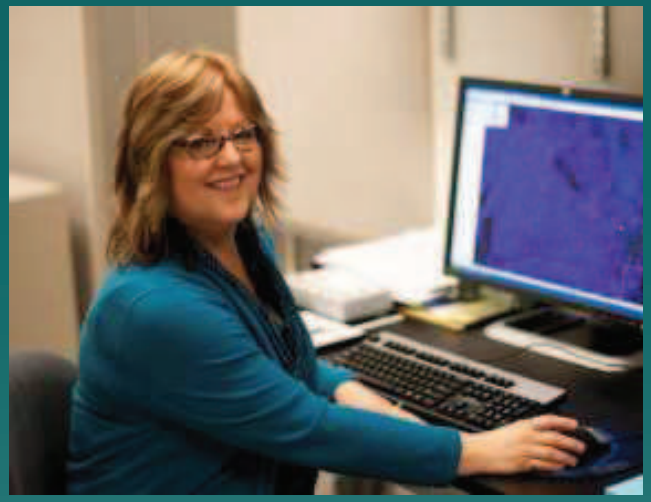
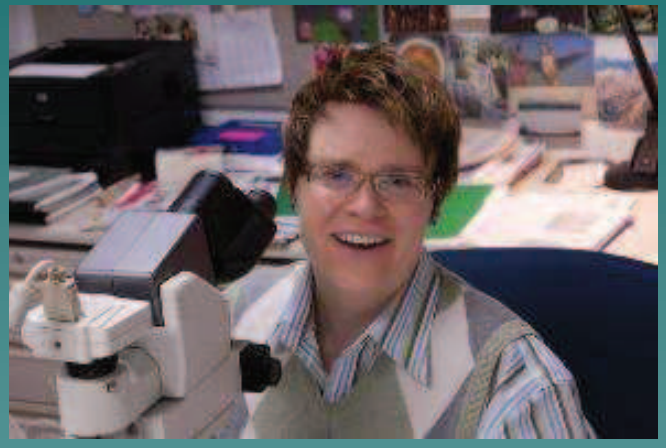
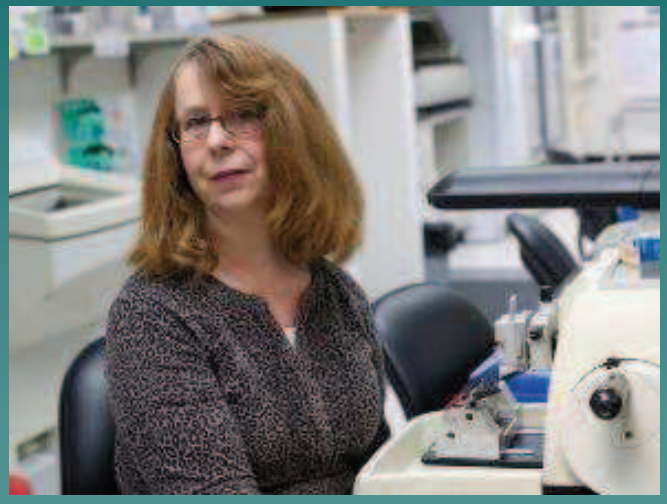
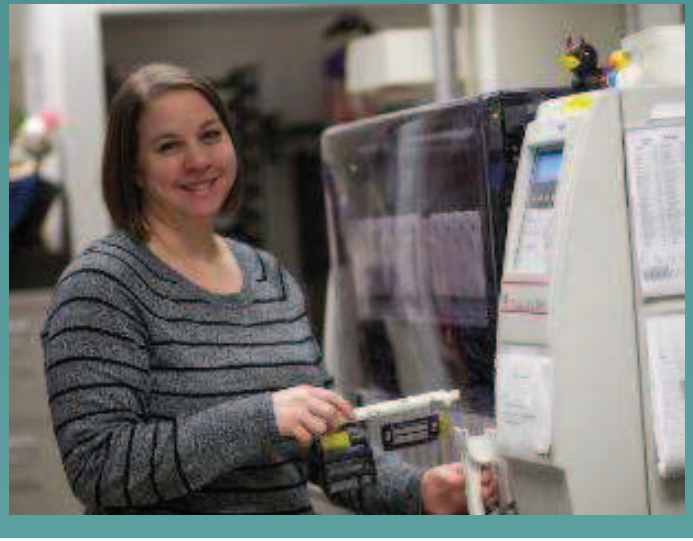
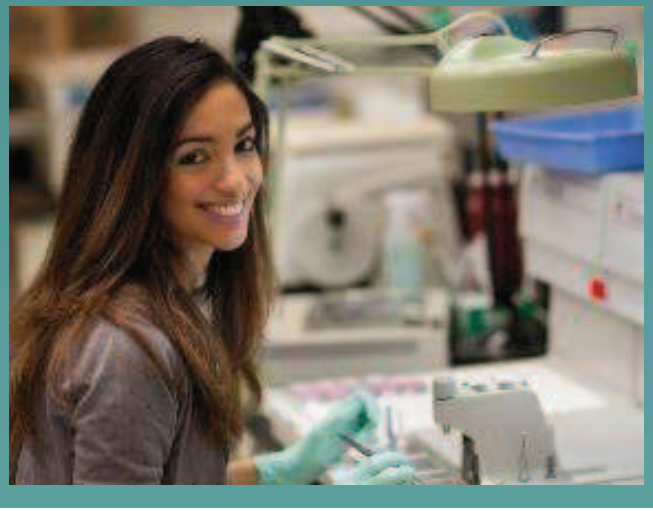
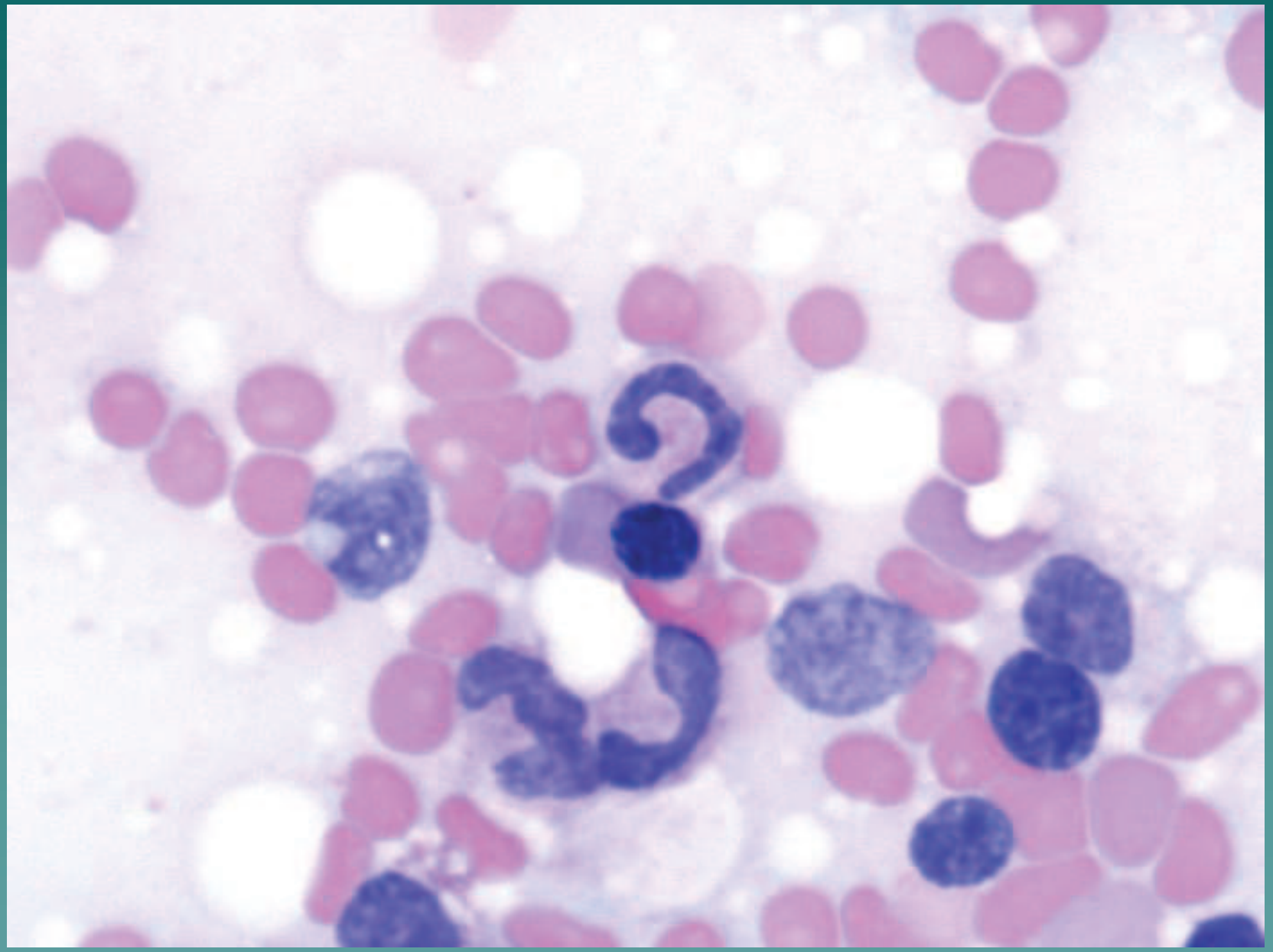





Questions?



Additional Notes

Tissue Preparation

Know the penetration and fixation rate of your fixative

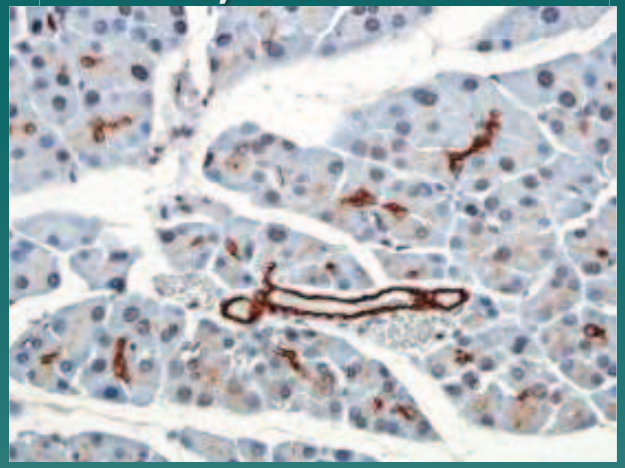
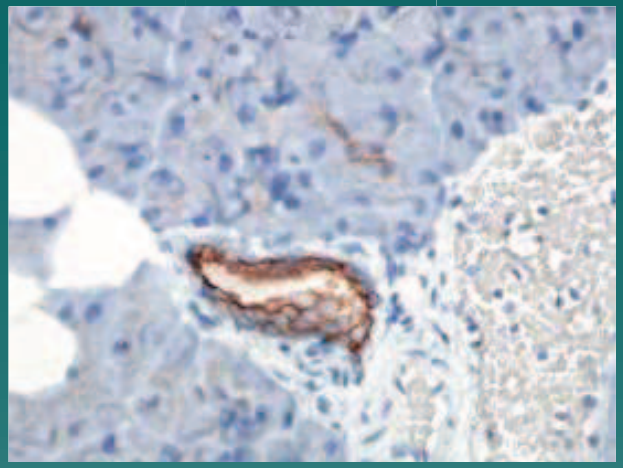
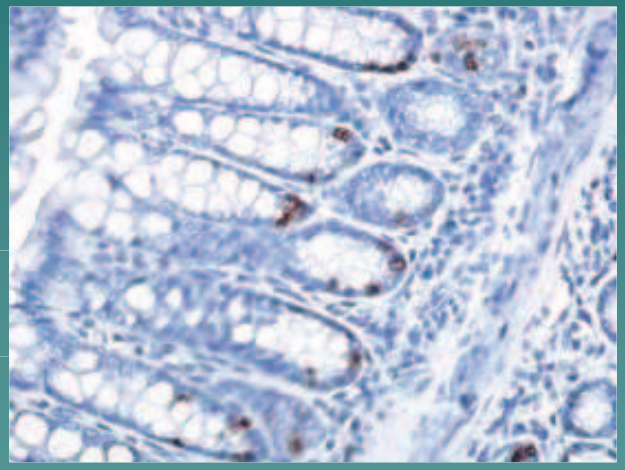
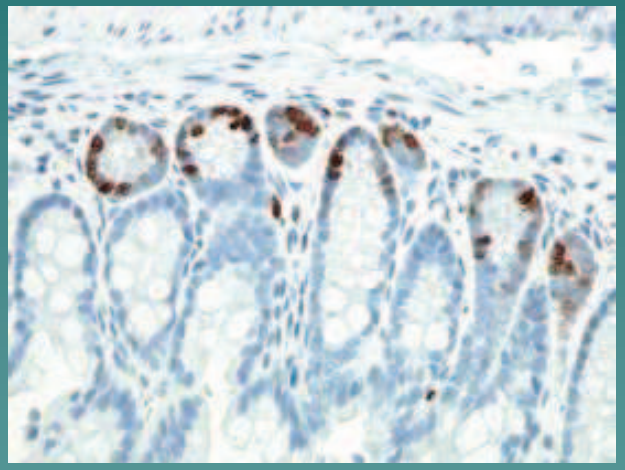
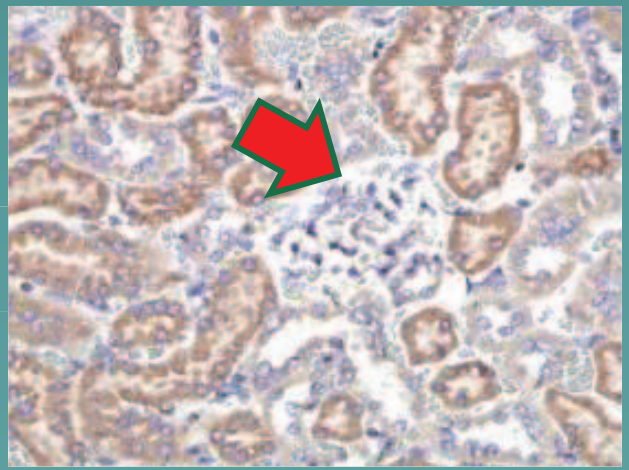
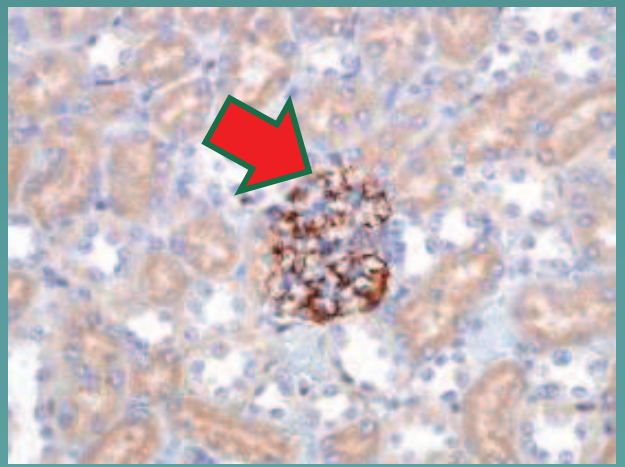
- Formalin is a paradox
  - Quick to penetrate
    - 0.5mm per hour at room temperature
  - Slow to fix
    - requires days to completely cross-link
- ^{14}C labeled formalin cross-linking times:
  - 16 um thick section — 24 hours
  - 1.5 mm thick tissue — 24.1 hours
  - 5 mm thick tissue — 24.5 hours
  - 4x4x4 mm cube — 25 hours
  - 8 mm thick tissue — 50 hours

Fox CH, Johnson FB, Whiting J, et al. Formaldehyde fixation. *J Histochem Cytochem.* 1985;33:845-853.

How long to fix

- Data from tissue fixed from 30 minutes to 1 year
  - 72 hours sufficient for reproducible results
    - Less time results in wide rage of staining variability
  - No difference in staining from fixing 3 days to 12 days
    - Most antigens can be detected after 1 year
    - May require amplification

Van der Loos. Biotech Histochem. 2007 Jun;82(3):141-54.

	Formalin less than 12 hours, then alcohol	Formalin 5 days, then alcohol
Cytokeratin WSS – not effected by underfixation		
Ki67 – Nuclei distorted and decreased staining in underfixed sample		
Vimentin – Lack of staining in glomeruli in underfixed sample		

Decalcification

- Must COMPLETELY FIX tissue before decal
- Decal reagents can damage antigens
  - Antigen dependant
- Choice of decal
  - HCl often damages antigens
  - Formic Acid is better
  - EDTA is best but takes forever

Decalcification Protocol

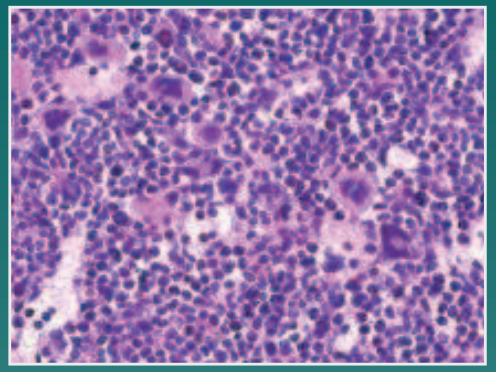
- Fix bone in formalin for 3-5 days
- Decal with Formic acid/EDTA solution
  - For example: Formical 2000
- Do not over decal, test your samples
    - Chemical end test can be too long
    - Can use mouse tail as guide for complete decal
- Rinse well with water to remove acid
  - IMPORTANT: at least 30 minutes

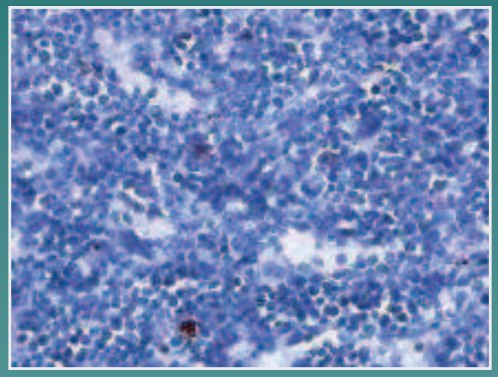
Combination fixative/decal NOT Recommended!

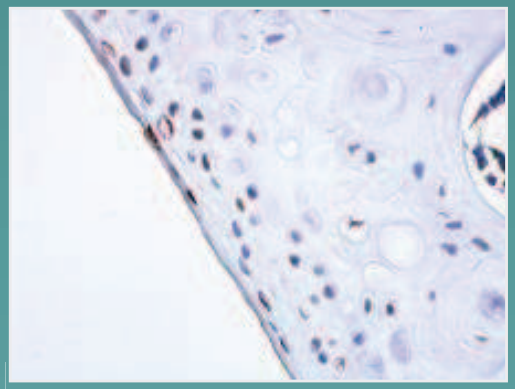
Poor Morphology

Decreased CD3 signal

Decreased Vimentin signal





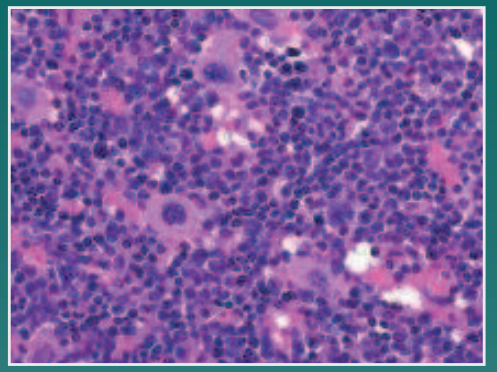


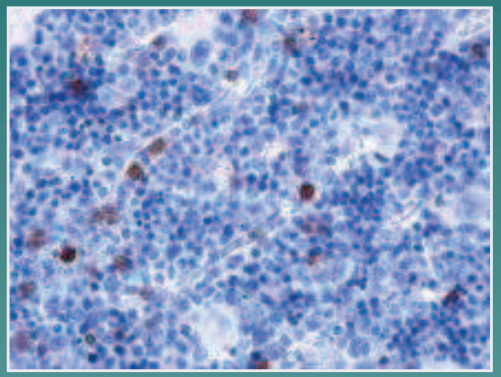
Combination Decal + Fixative

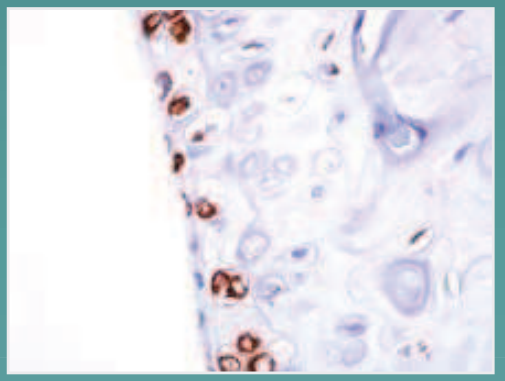
H&E

CD3

Vimentin







Fixed for 5 days then Decaled

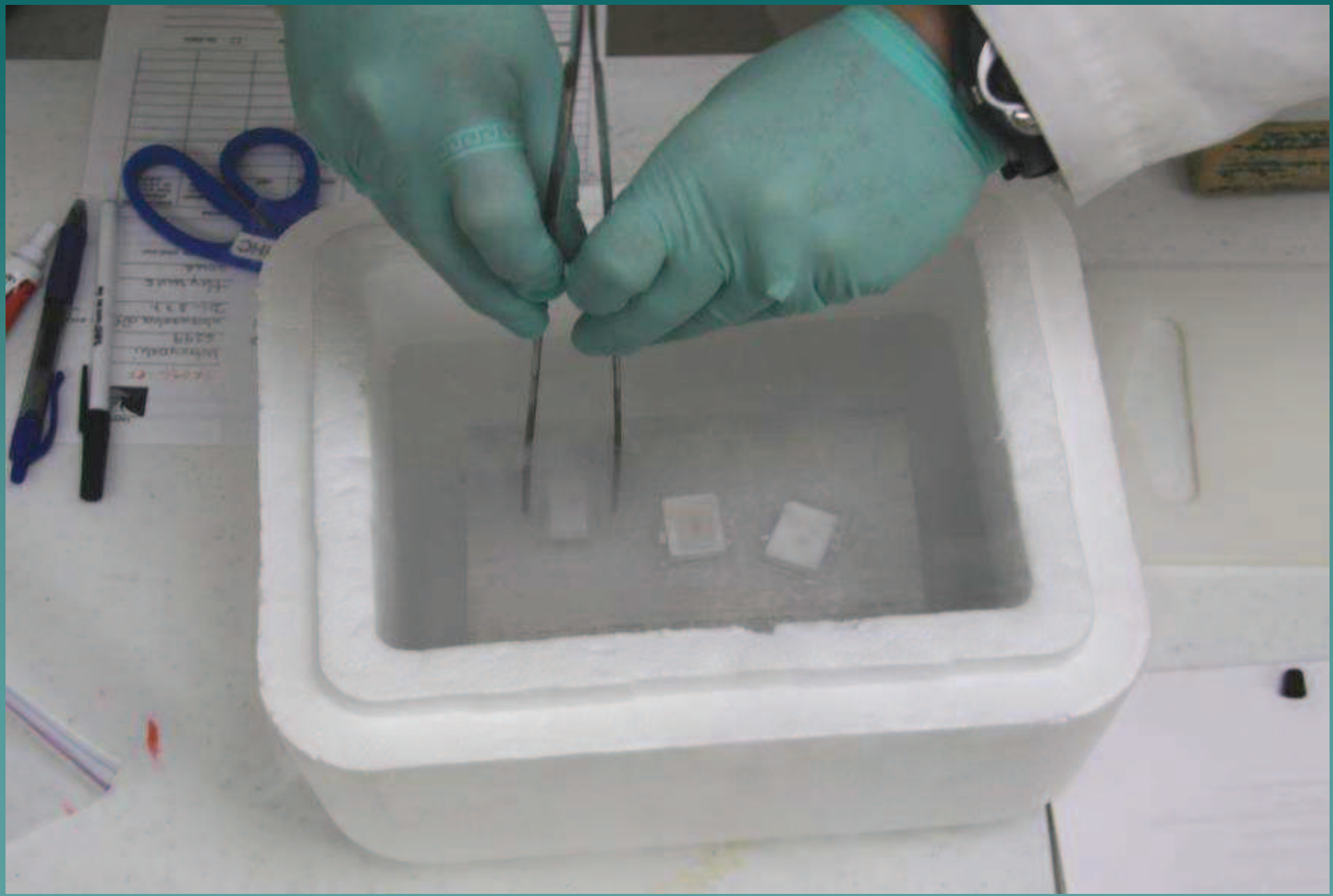
Decalcification - IHC

- Immunohistochemistry may need to be adjusted
- Control tissue must be Fixed, Decaled, and Processed in same manner as bone to test IHC protocols
  - Example: Spleen as a Ki67 control and thymus as a Cleaved Caspase 3 control

Frozen Tissue

- Do not use tissue frozen directly in liquid nitrogen!
  - Morphology is destroyed
  - Antigens washed away
- Get a good OCT procedure to preserve tissue architecture
  - Freezing must be done in a controlled manner

We use an Aluminum Block cooled in Liquid Nitrogen to prepare OCT samples



IHC Protocol Consideration

Antigen Retrieval

- Very critical step for consistent staining
- Standardized protocol is essential
- Must be done EXACTLY the same way every time

Protein or Serum Blocking Example

- Example:
  - Tissue: Mouse Lymph Node
  - Primary: Rat anti-Mouse CD3
  - Secondary: Biotinylated Goat anti-Rat
- Contains:
  - Tris Buffered Saline
  - 1 - 4% BSA
  - 5 - 15% Goat Serum
  - 3 - 5% Mouse Serum

Avidin/Biotin Background

- Tissue contains endogenous biotin, biotin receptors, and avidin binding sites
- Present in **ALL** tissue
  - Highest in Kidney, Liver, GI Tract, Spleen, Brain, Breast, Adipose Tissue, Lymphoid Tissue
- Reagents from ABC development can interact with these and cause background
- Higher background in antigen retrieved or unfixed samples
- Can be blocked with commercial kits
  - Strept Avidin
  - Monomeric biotin

Choice of secondary

- Choose secondary with limited cross-reactivity
  - No cross reactivity to **MOUSE** immunoglobulin

For example, Jackson ImmunoResearch makes:
- Goat anti-Rat IgG (112-065-003)

OR:

- Goat anti-Rat IgG (112-065-167) with minimal cross reactivity to Mouse, Human, Bovine, Horse, and Rabbit serum proteins

Other Mouse-on-Mouse Staining Strategies

Solution #1

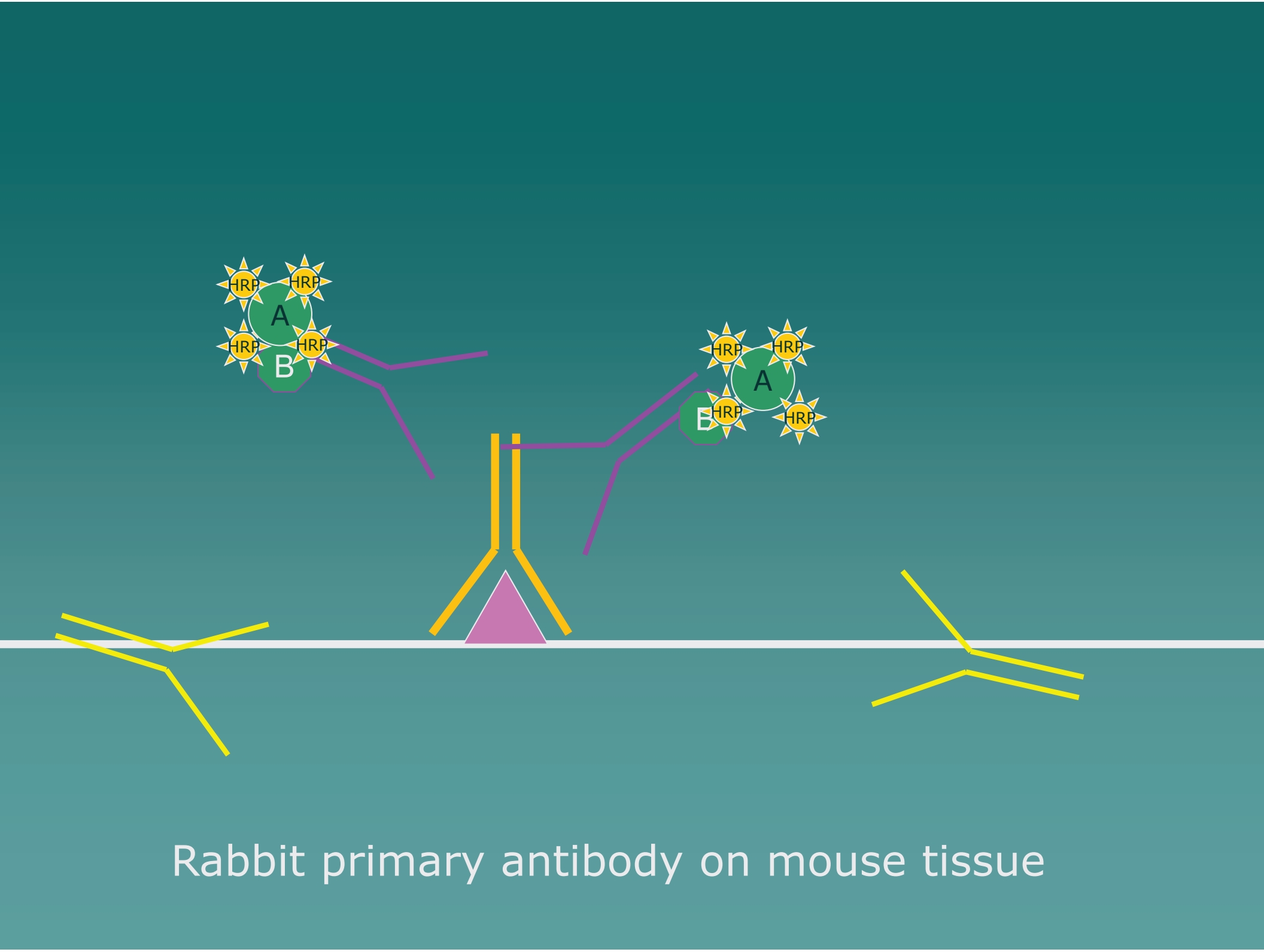
Don’t use a mouse monoclonal

- Can you use a different primary antibody made in a different species?

Example: CD3 on xenografts

Use Labvision (SP7) rabbit monoclonal

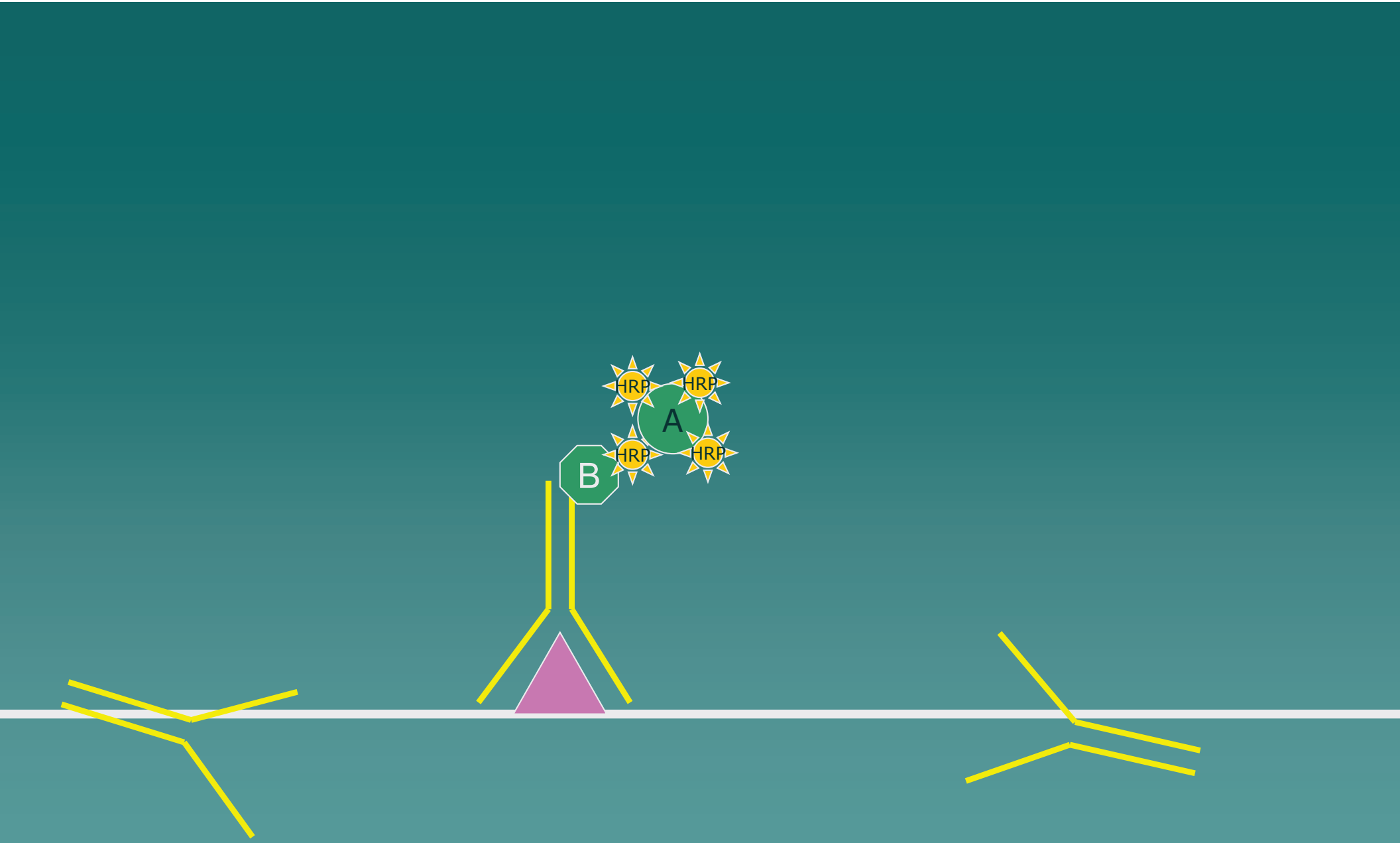
Instead of Dako mouse monoclonal



Rabbit primary antibody on mouse tissue

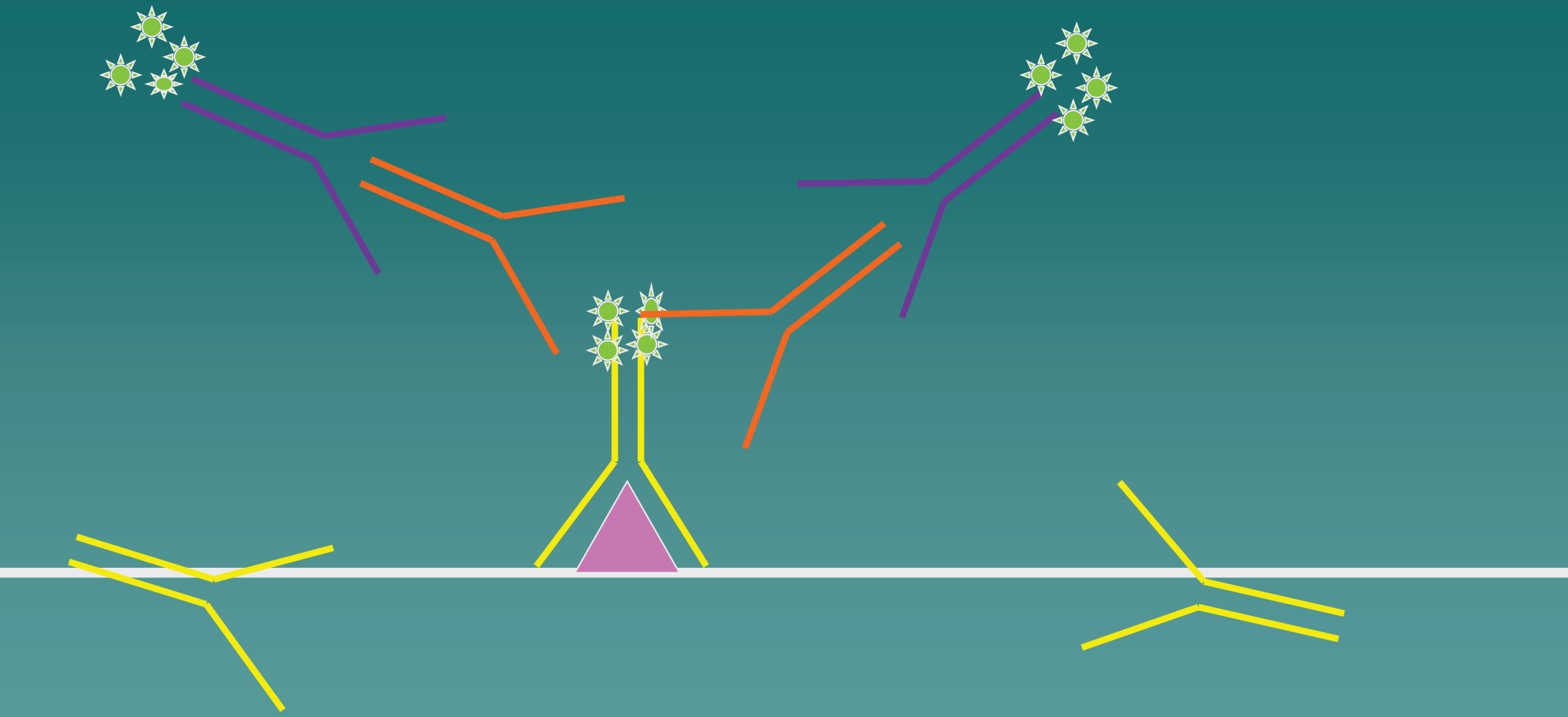
Solution #2

Use a directly labeled primary antibody!



Biotinylated Mouse primary on mouse tissue

Can amplify using antibodies directed against the label
For example: anti-FITC or anti-CY5 (647)



Fluorescently labeled mouse primary on mouse tissue

Solution #3

Block Endogenous Mouse antibodies

Anti-mouse Ig Block

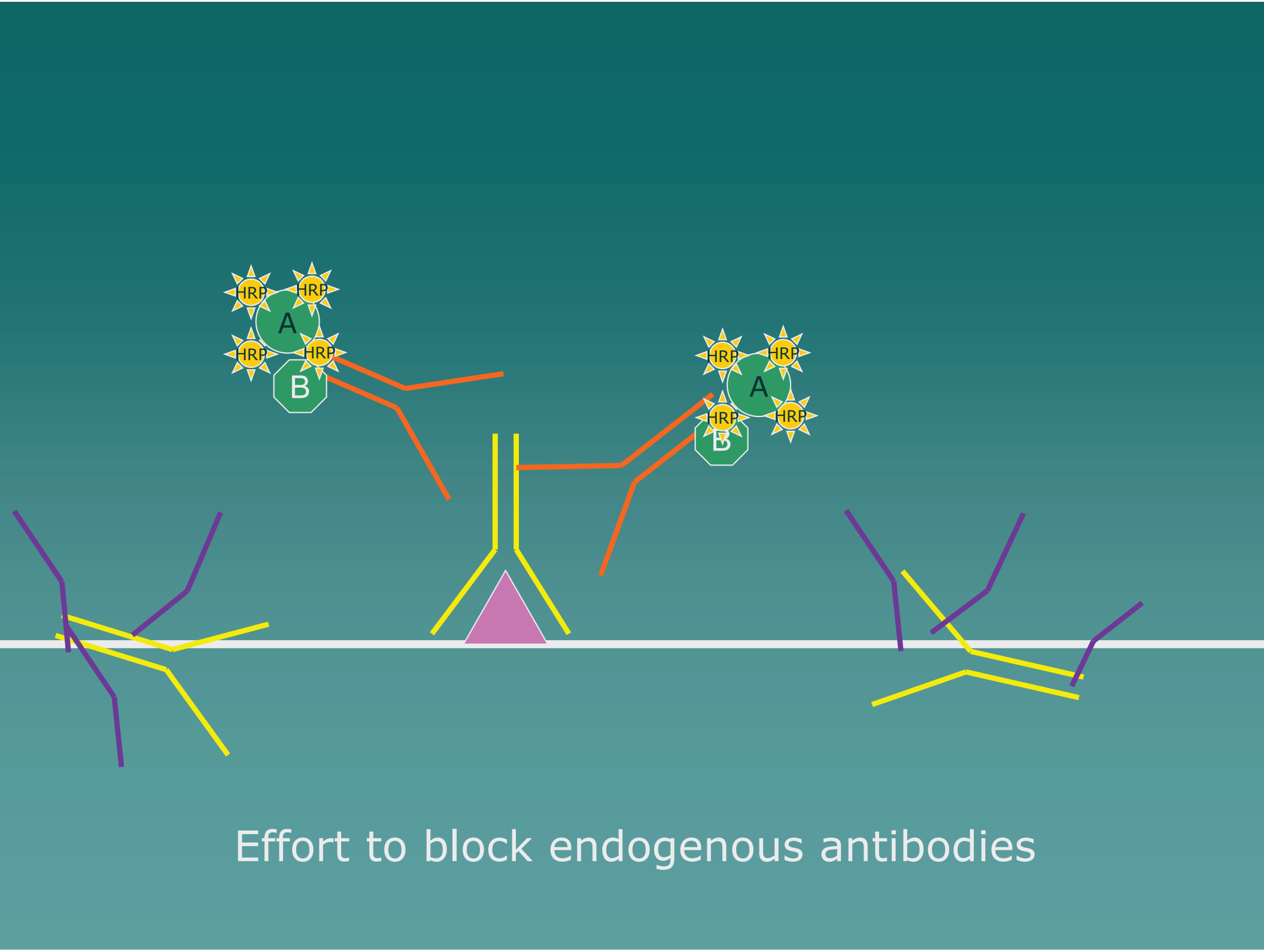
- Use an unlabeled anti-Mouse antibody to bind to all endogenous antibody

Examples of Commercial Kits that pre-block

- Chemicon Mouse-To-Mouse kit
- Vector Laboratories M.O.M. kit
- Invitrogen Histomouse-SP kit

Blocking Endogenous Antibodies

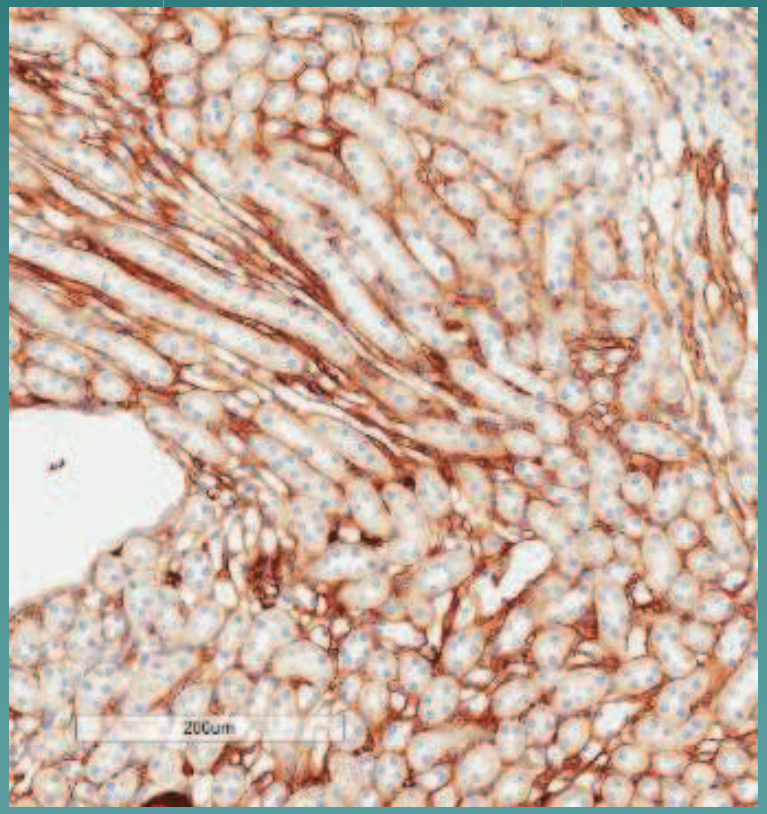
- Use an unlabeled anti-mouse antibody
- Use Fab fragments
- Test several different concentrations
  -Confirm reduction of background first with no primary antibody
  - Be careful of hindering primary binding
- Incubate Overnight in fridge
- Post-fix in NBF for 1 hour



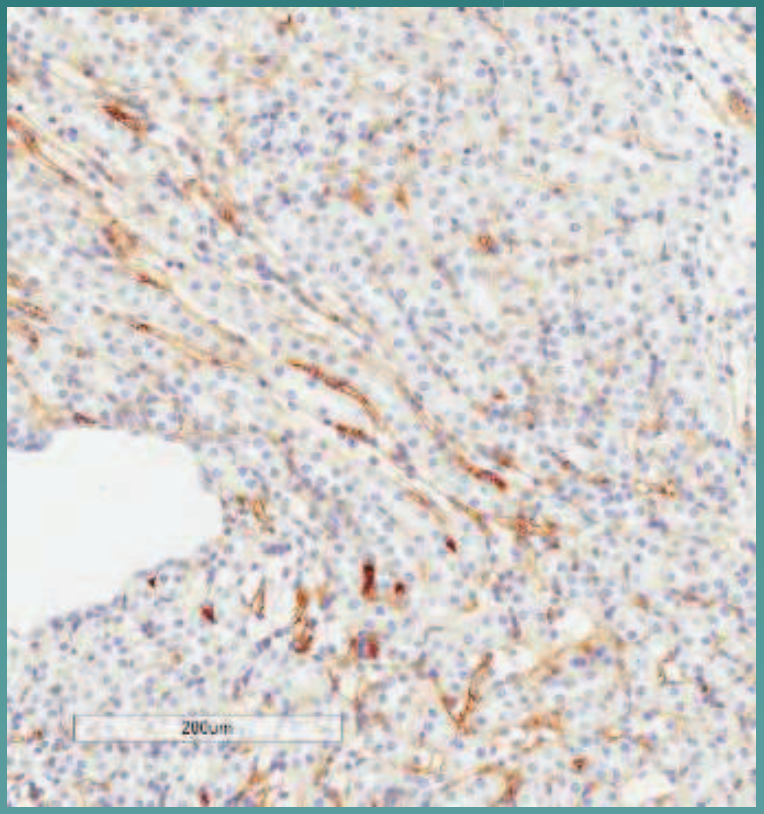
Effort to block endogenous antibodies

Blocking Endogenous Antibody on Mouse Kidney= reduced background

No block



Block



Solution #4

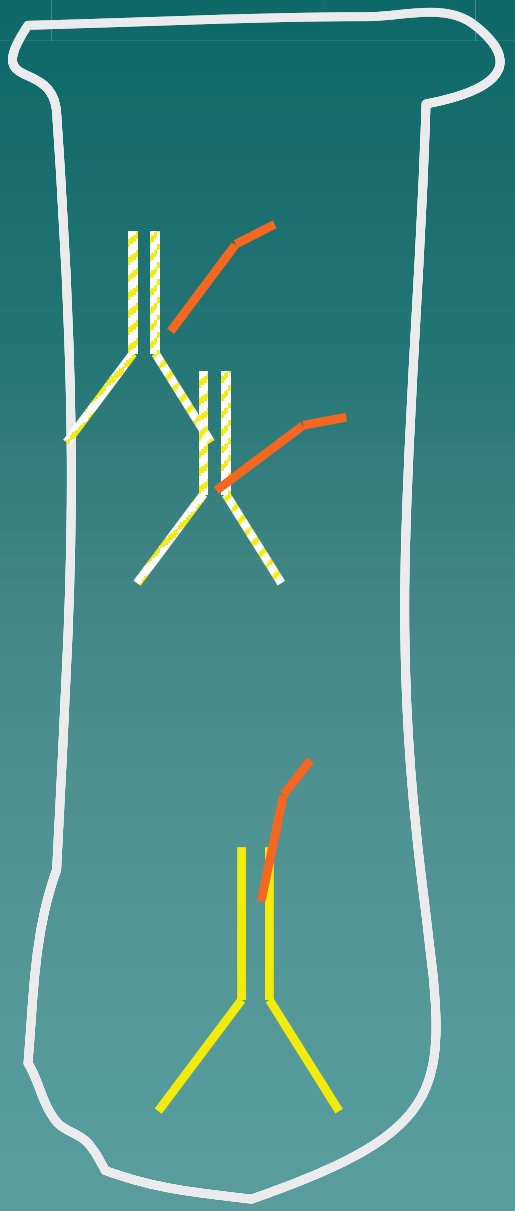
Mix the primary antibody with the secondary antibody in a tube first

- Allow the antibodies to form a complex
- Block free secondary with mouse serum
- Apply to tissue

Similar commercial Kits:

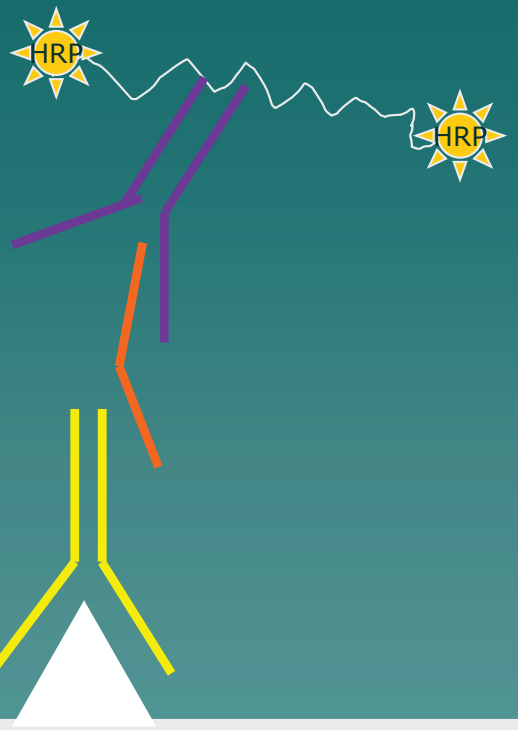
- Dako A.R.K.
- Ventana Discovery MoMap Kit
- Invitrogen Xenon Labeling Technology

Different Method Strategies:



Primary bound to Unconjugated secondary

Unconjugated Rabbit Secondary



Smooth Muscle Actin

1) rabbit anti-mouse bound to mouse anti-SMA
2) anti-rabbit polymer conjugated with HRP

Example: anti-SMA + Unconjugated Rabbit Secondary