Immunohistochemistry in investigative and toxicologic pathology

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ILS

"An expert is a man (/woman) *who tells you a simple thing in a confused way in such a fashion as to make you think the confusion is your own fault."*

"In a battle of the Titans, in which even the experts cannot agree, the democratic process tends to break down, and the pathologist with the loudest voice or "the highest CQ (credibility quotient) often carries the day." Under such charged circumstances it should not be forgotten that even experts offer only opinions, and the need to validate the diagnoses in difficult cases remains."

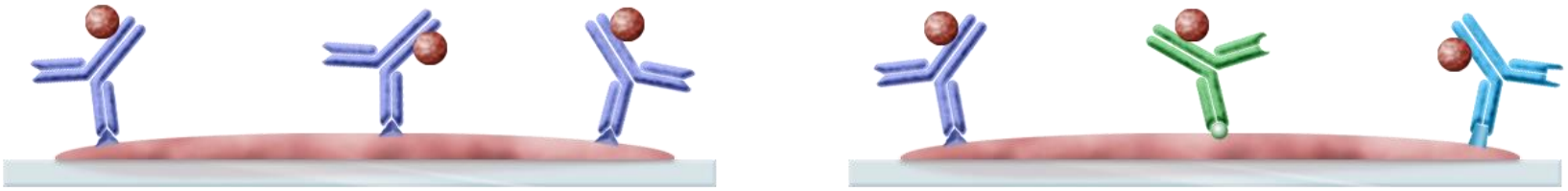
Hum Pathol. 1994 Jan;25(1):2-11
Castle WD: Harvard Med Alum Bull 29:18, 1955
Hum Pathol. 12:590-596 1981

Overview

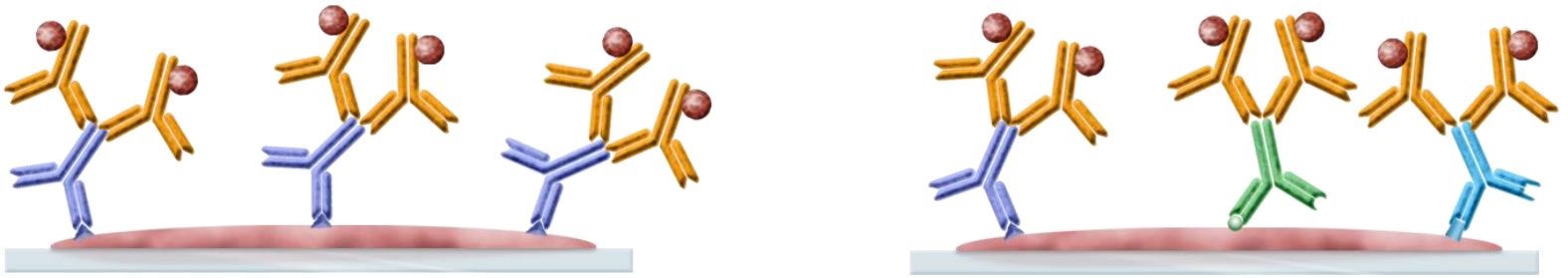
- Brief basics of IHC
- Variables
- Nonspecific staining
- Controls
- Optimization

Brief Basics of IHC

Detection methods



Direct method



Indirect method

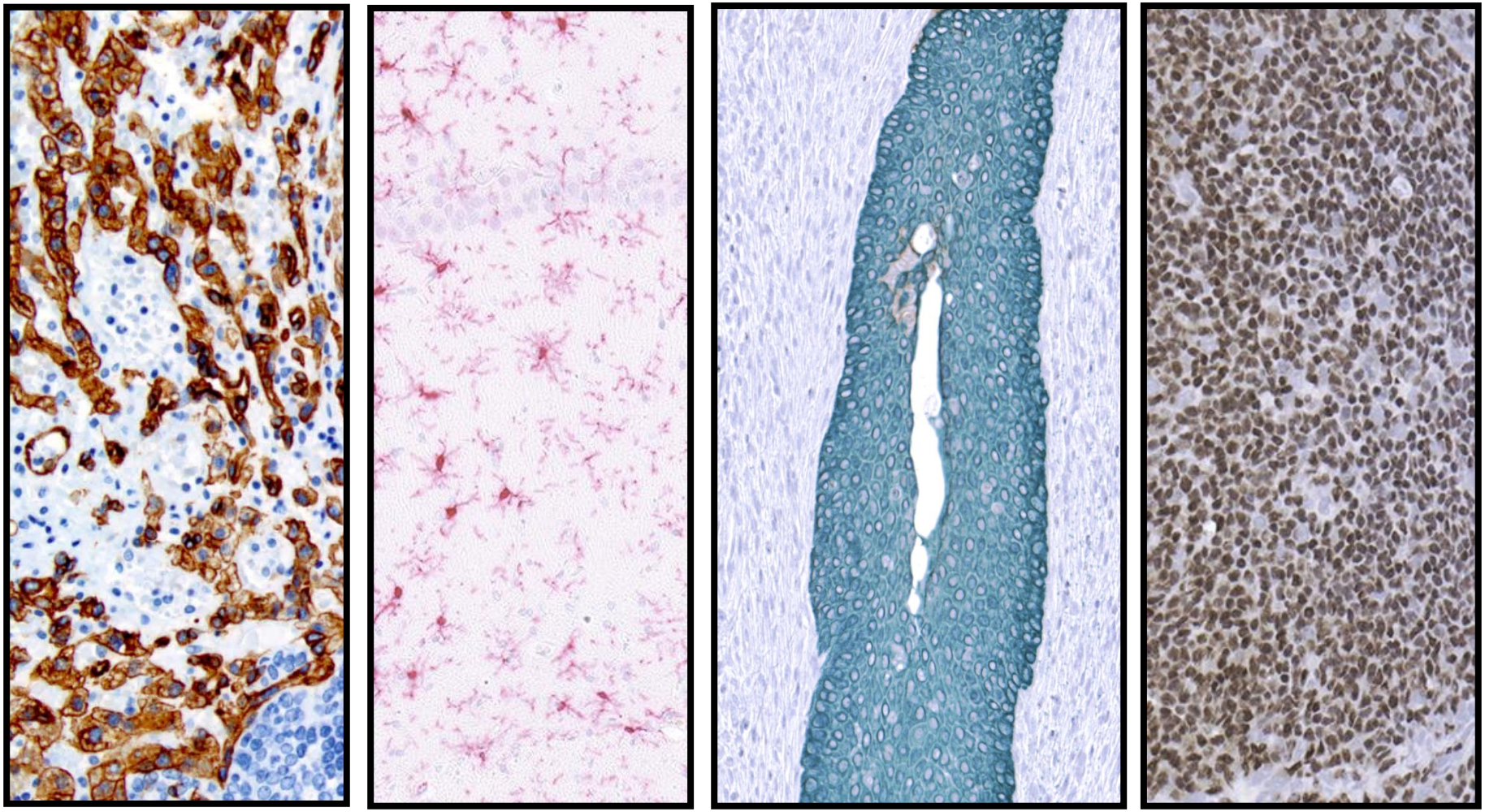
Indirect method

- Secondary antibody with an enzyme (HRP or AP) conjugate
- Peroxidase antiperoxidase method (PAP)
- **Avidin-Biotin Complex method (ABC)**
- Labeled Streptavidin Biton (LSAB) method
- **Polymer based technique**
- Catalyzed signal amplification/Tyramide signal amplification

Enzymes and Substrates

Horseradish Peroxidase	**Alkaline phosphatase**
3,3’ Diaminobenzidine (DAB;Brown)	**Fast red**
3-Amino-9-Ethylcarbazole (AEC; Red)	**Fast blue**
4-Chloro-1-Naphthol (Blue)	**New Fuchsin (red)**

Enzymes and Substrates



Variables that impact the results and reproducibility

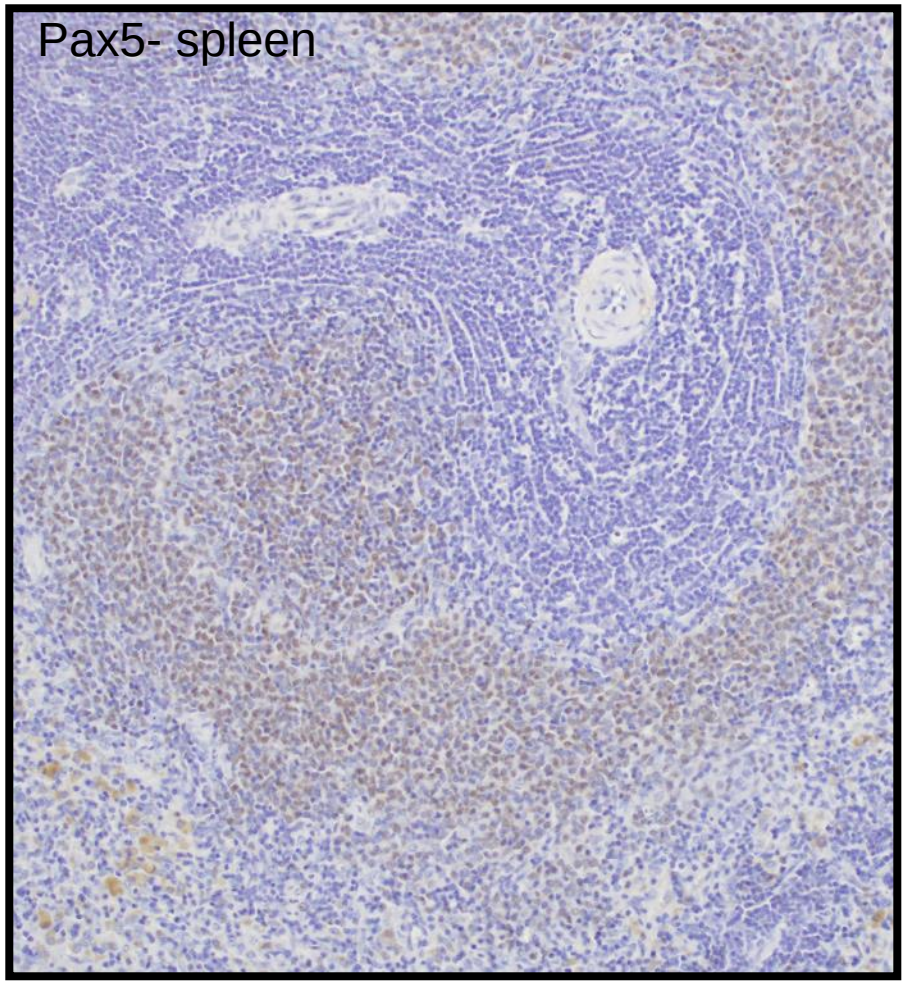
Fixatives

- 10% neutral buffered formalin
  - Optimal fixation time: 2-7 days
- Paraformaldehyde
  - Should be prepared fresh
- Ethanol
- Davidson’s fixative
- Bouin’s fixative

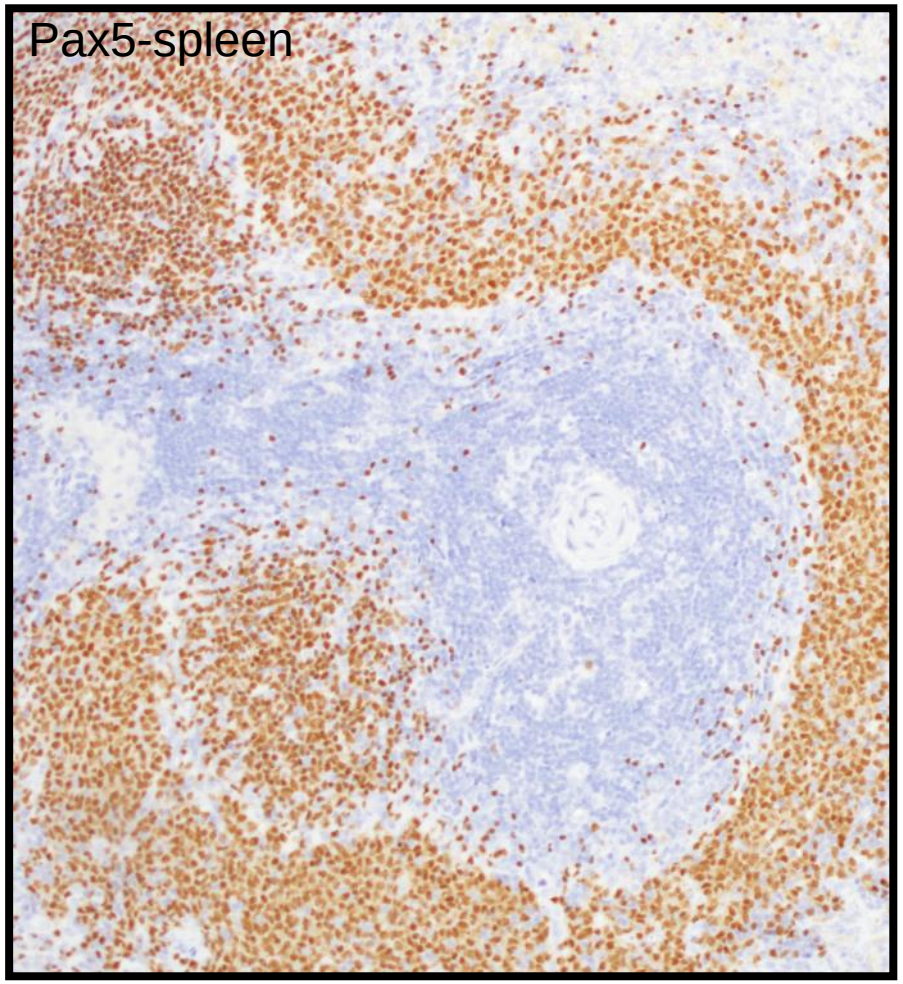
Antigen retrieval(AR)

- Enzymatic method
  - Protease, Pronase K, Pepsin, Trypsin
- Heat-induced method
  - Pressure cooker, steamer, microwave, water bath
- Some antigens do not require AR
- Not all antigens can be unmasked using AR

Antigen retrieval(AR)



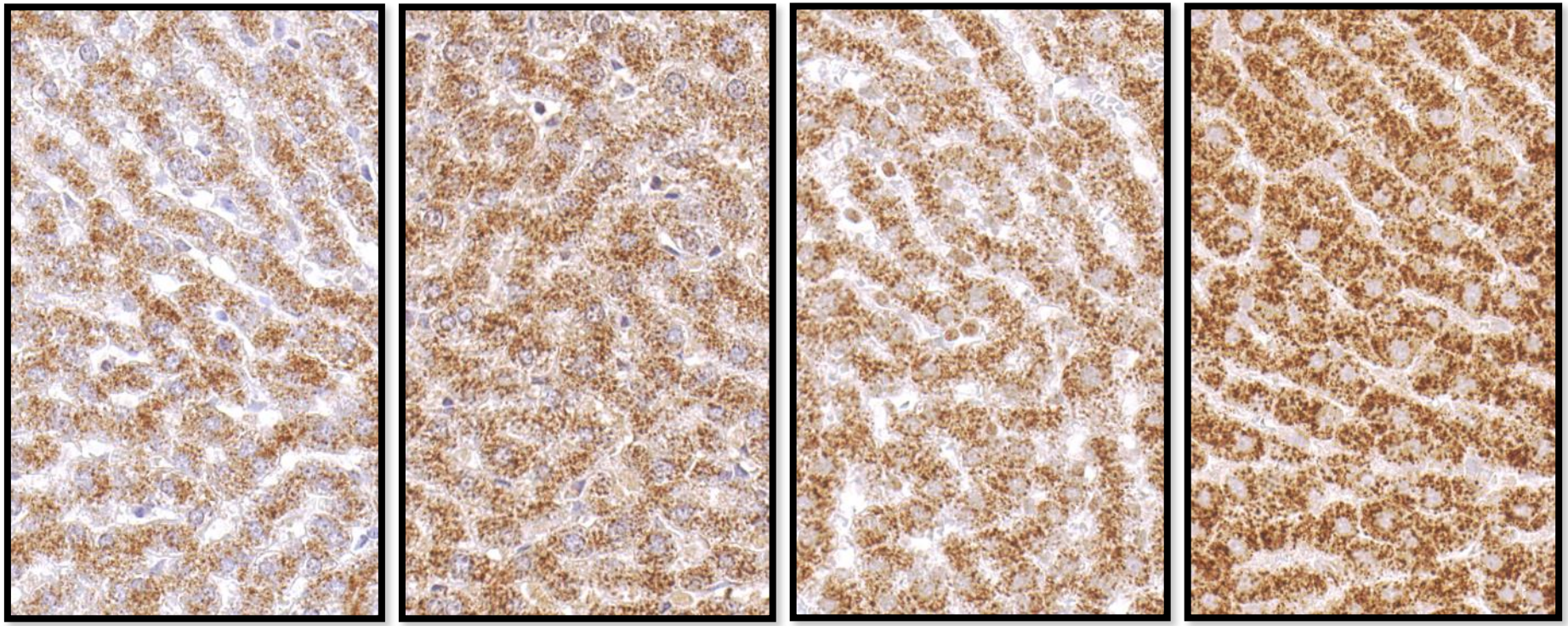
Without AR



With AR

Some antigens require AR

Antigen retrieval(AR)



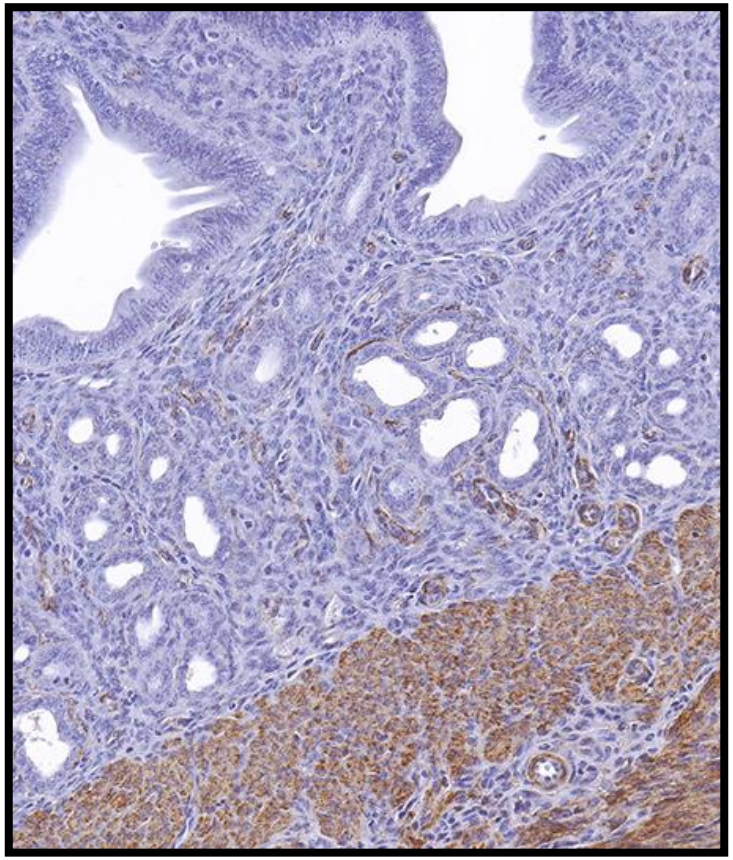
No retrieval | Pepsin | Decloaker/ Citrate buffer | Decloaker/ EDTA

Rat liver stained with PMP70 antibody

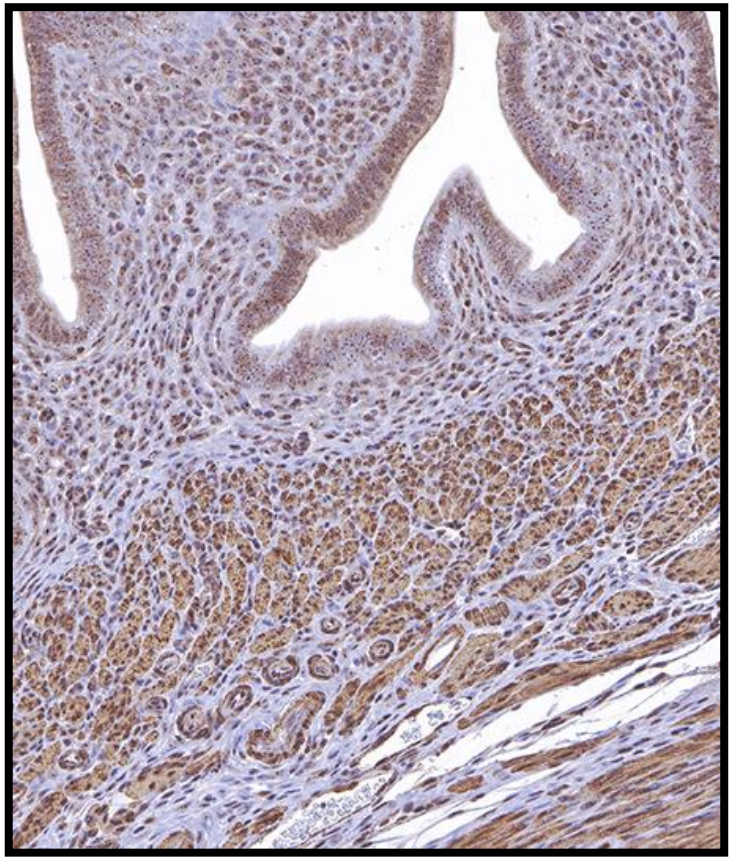
Some antigens do not require AR

Antigen retrieval(AR)

Smooth Muscle Actin



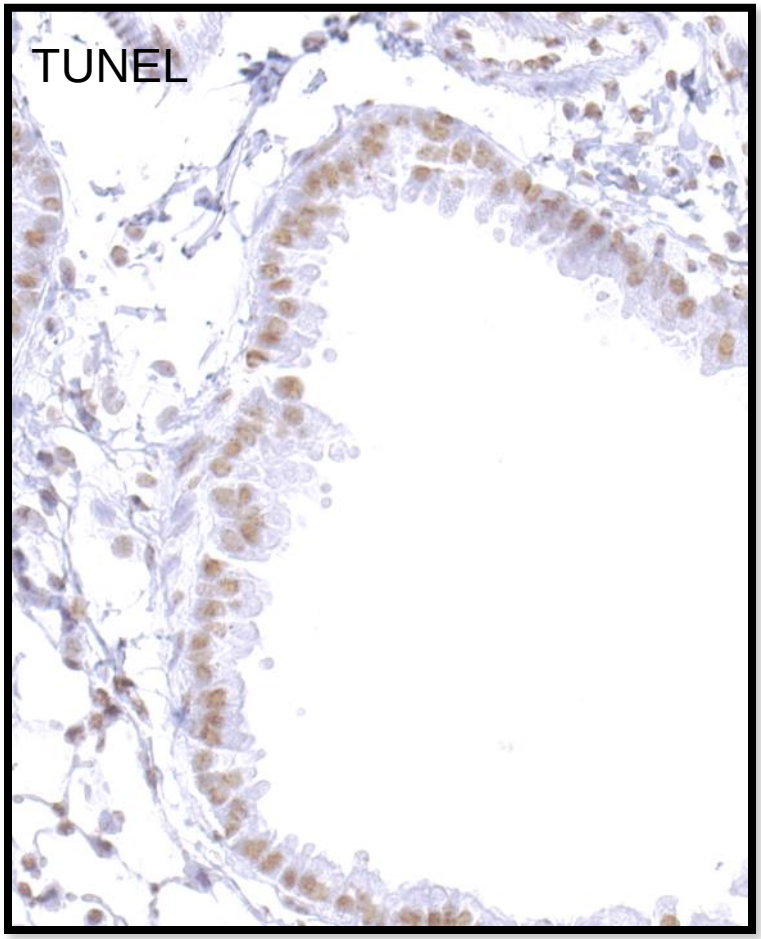
Without antigen retrieval



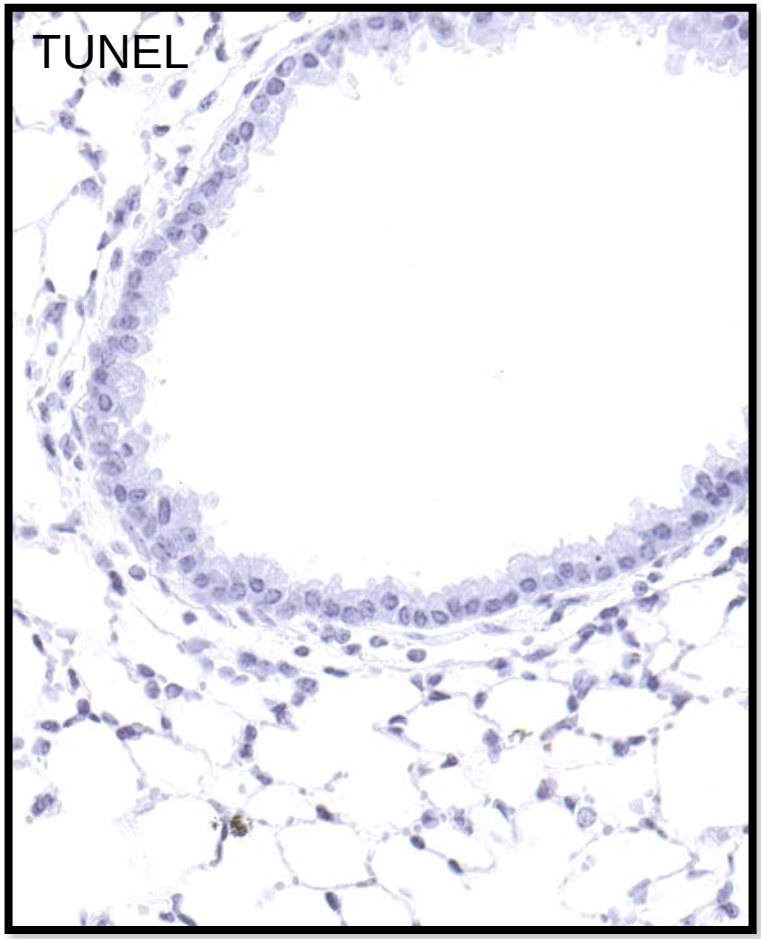
With antigen retrieval

Some AR methods induce nonspecific staining

Antigen retrieval(AR)



Decloaker/Citrate buffer



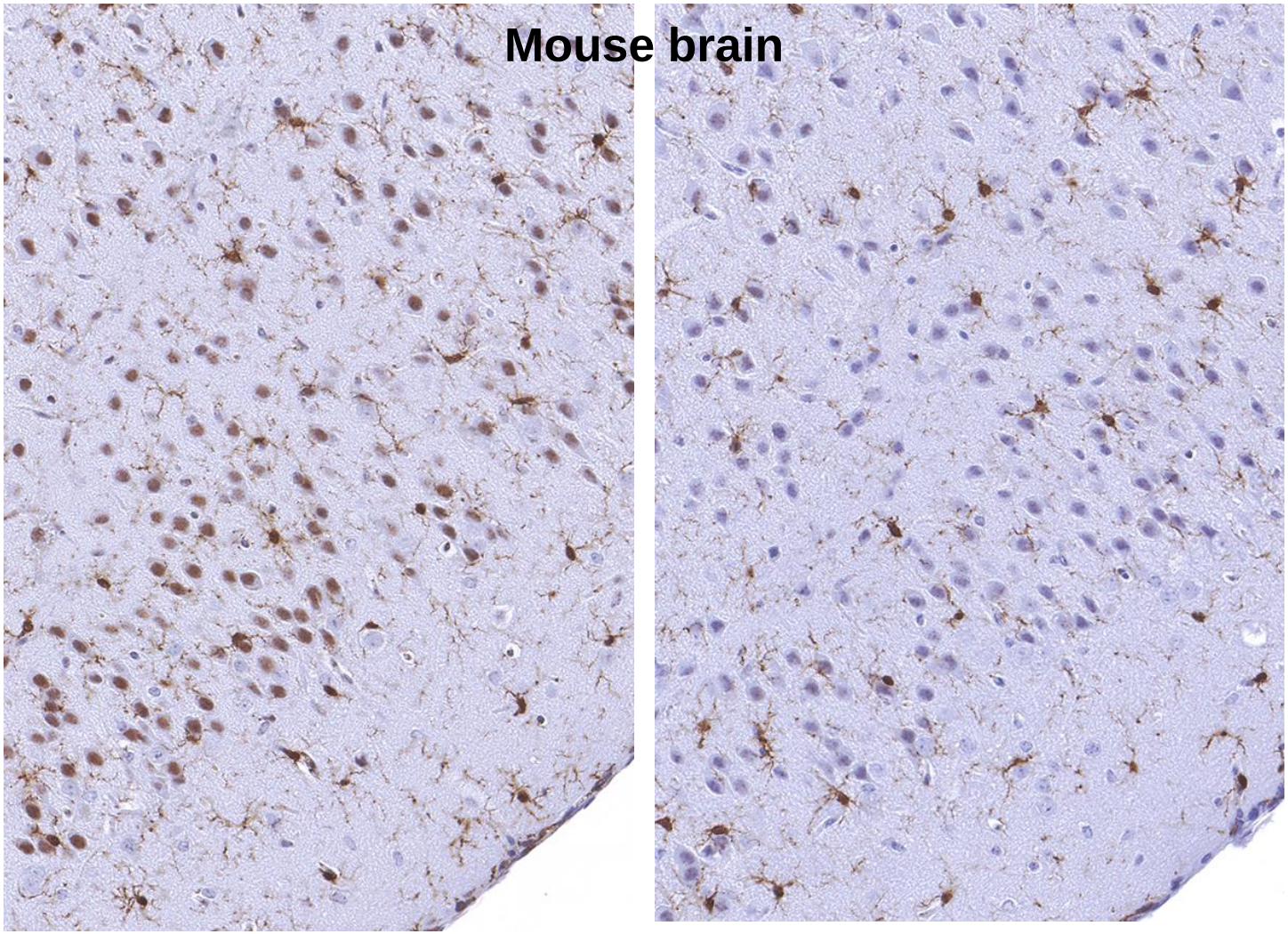
Proteinase K

Some AR methods induce nonspecific staining

Antigen retrieval(AR)

- Pressure cooker and enzymes are the most commonly used methods
- During optimization- try all possible AR methods
- Be aware of the protein biology
- If optimizing the method for a new protein, support the localization by other methods

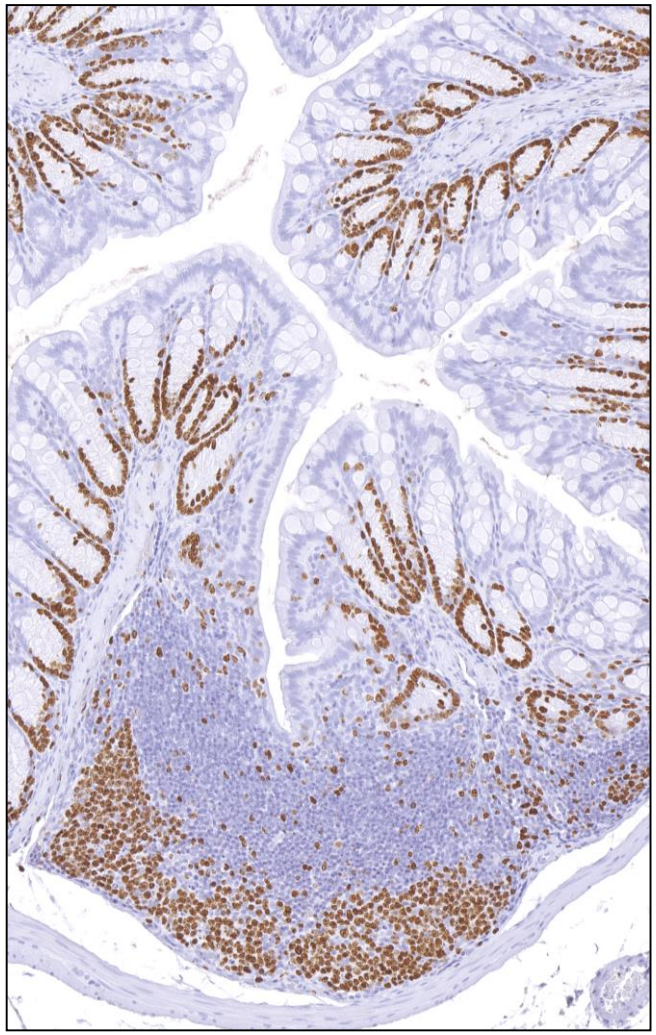
Detection method



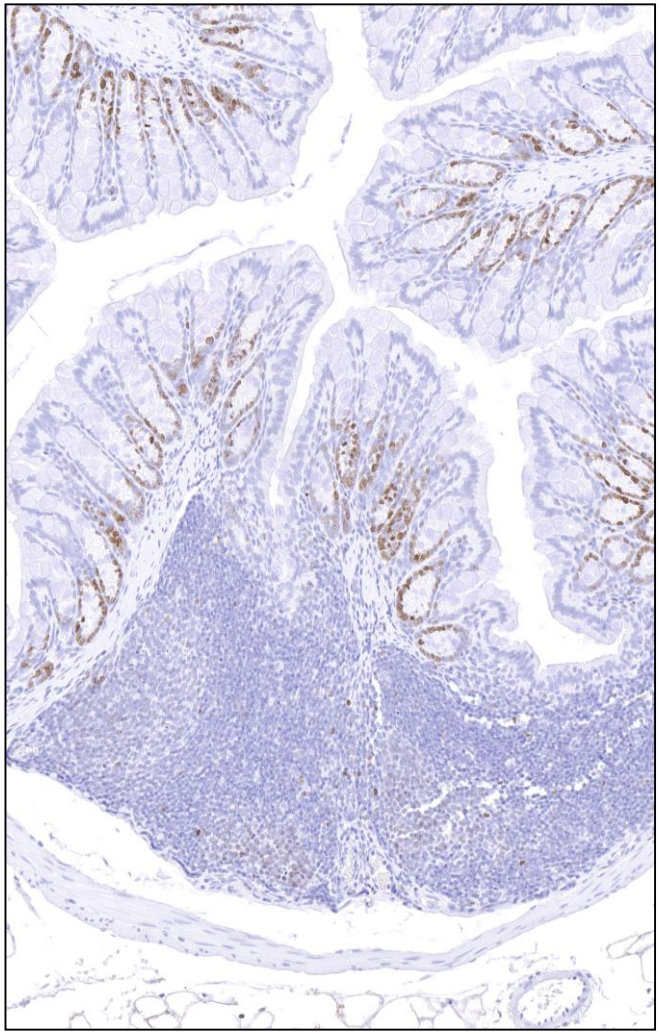
Iba1- using ABC

Iba1- using polymer

Detection method



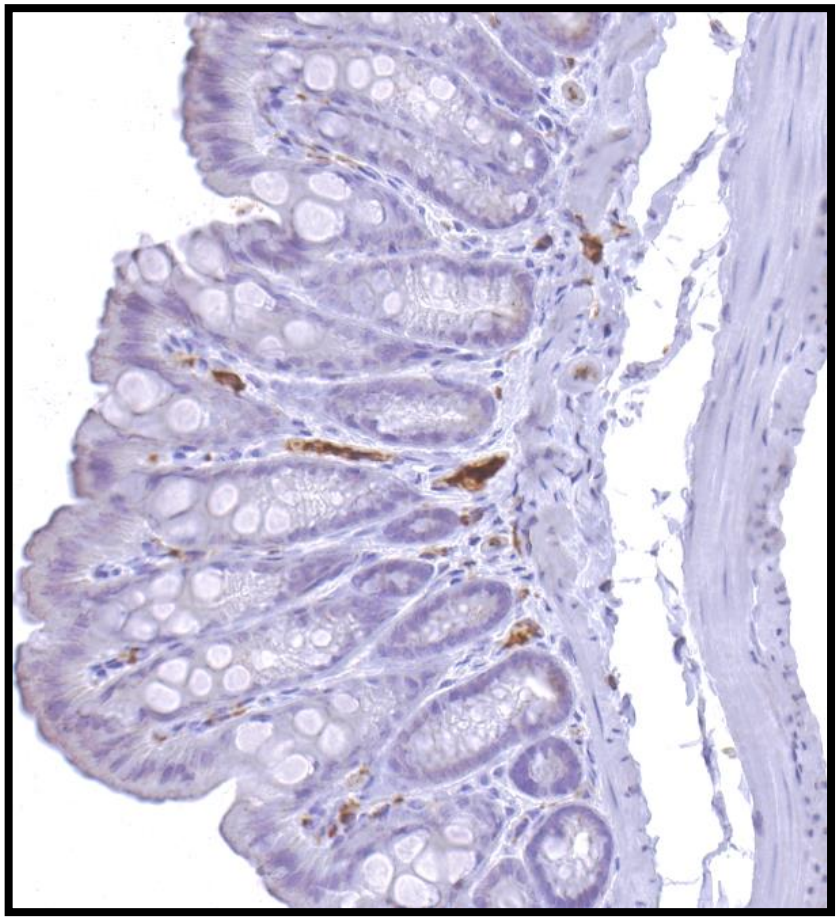
Brdu- using ABC



Brdu- using polymer

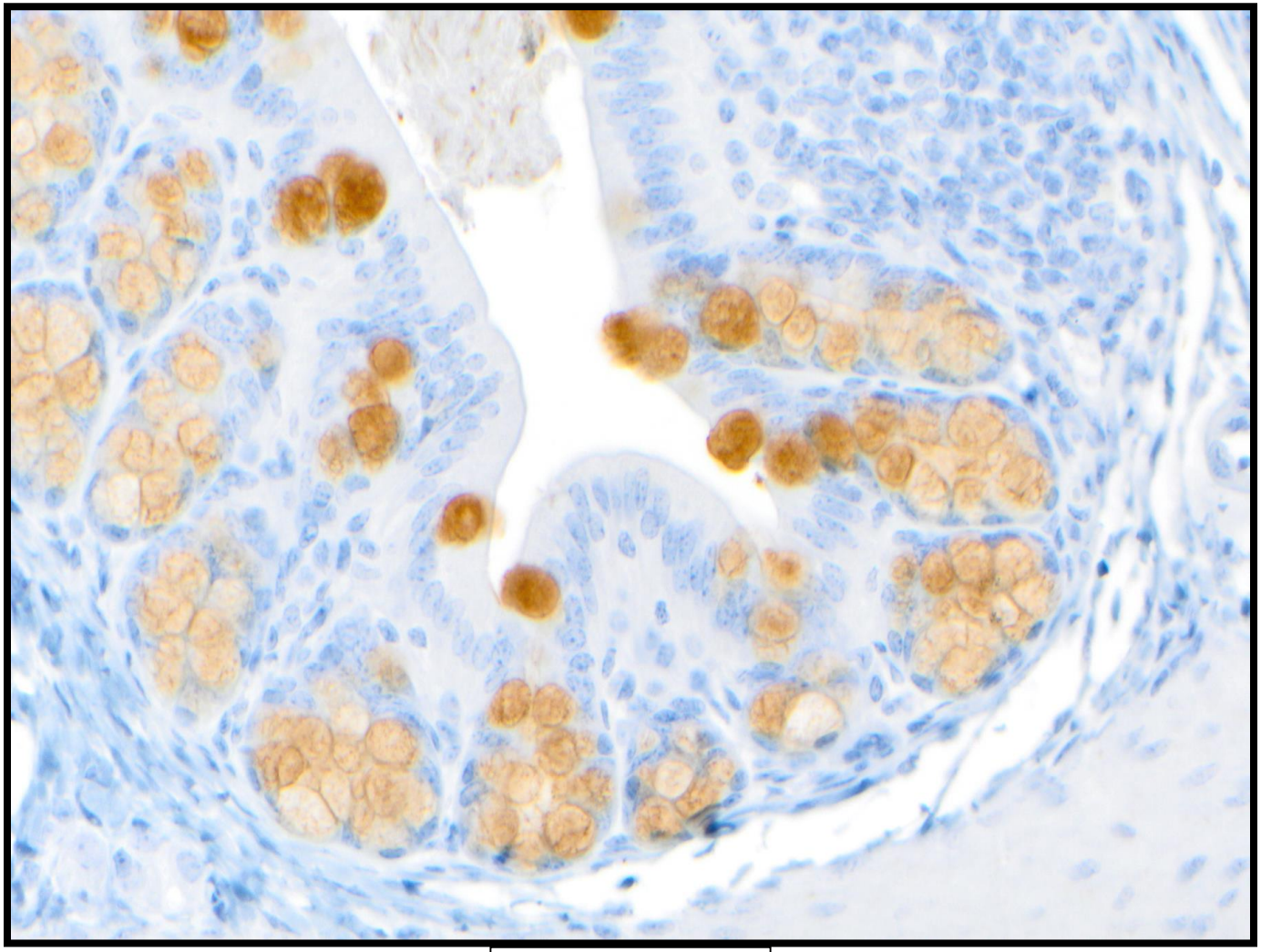
Choice of antibody

Muc2



Muc2 negative control

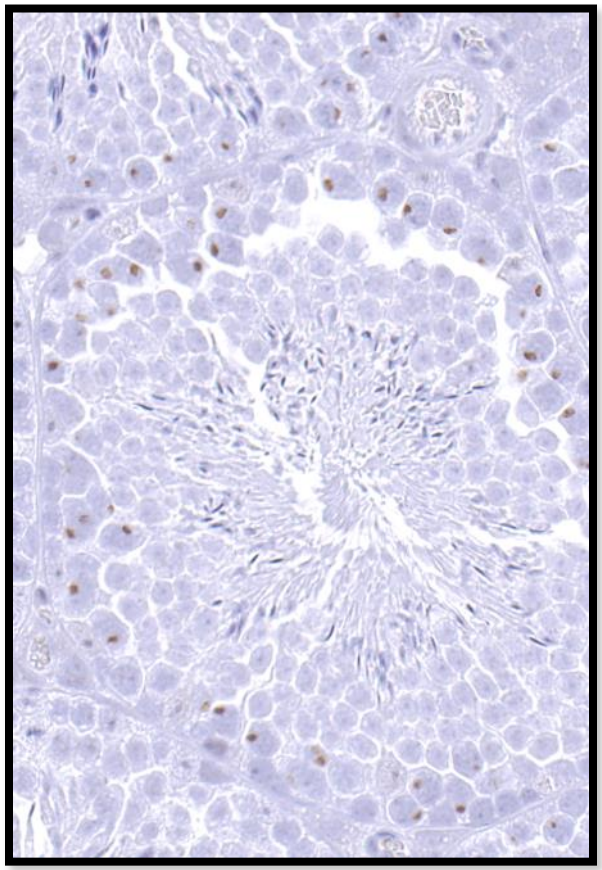
Choice of antibody



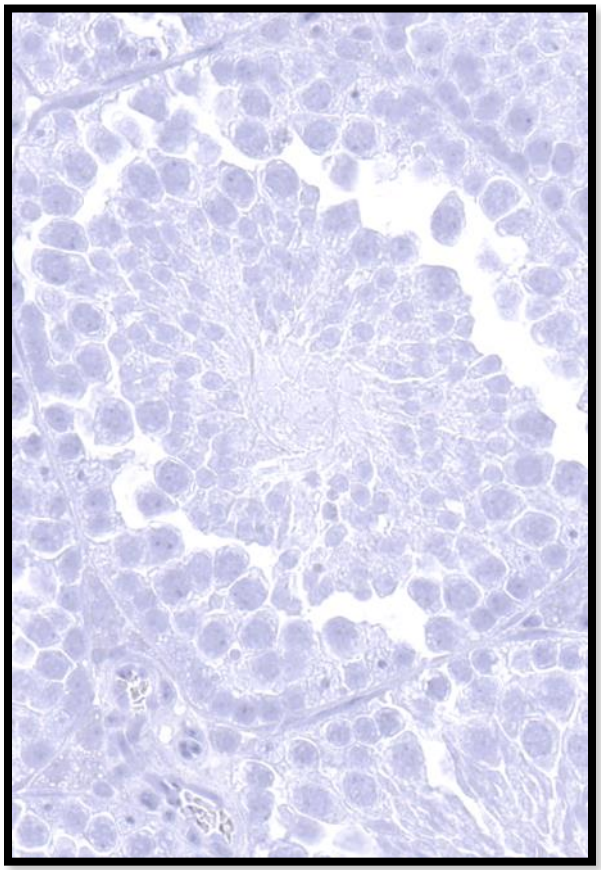
Muc2

Choice of antibody

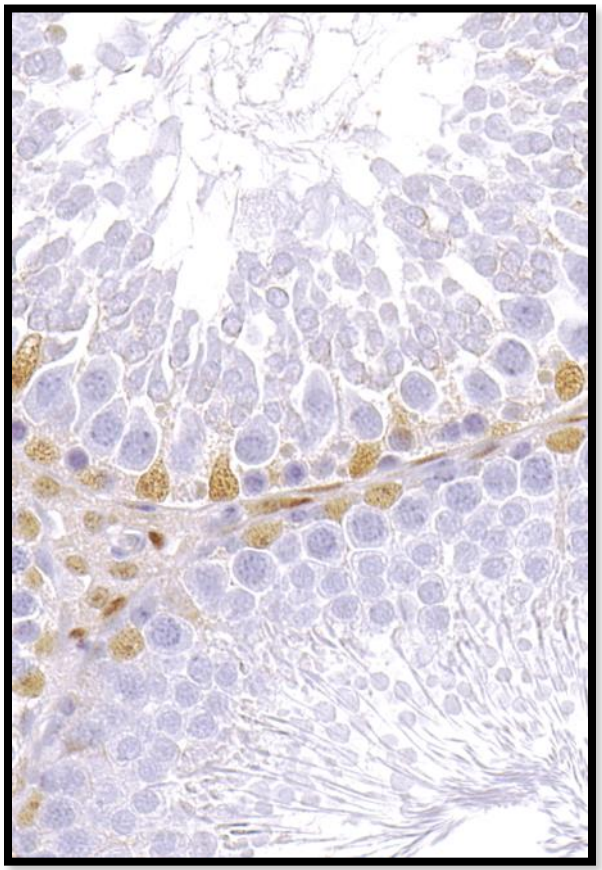
Mouse testis



Sox9 antibody



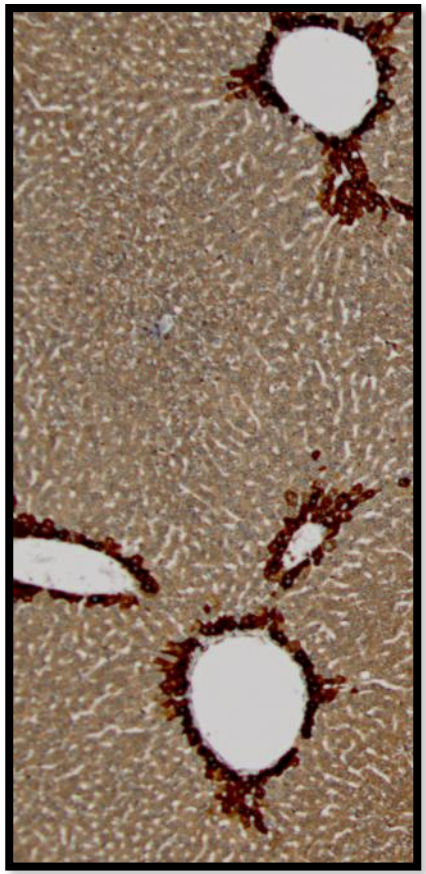
Sox9 negative control



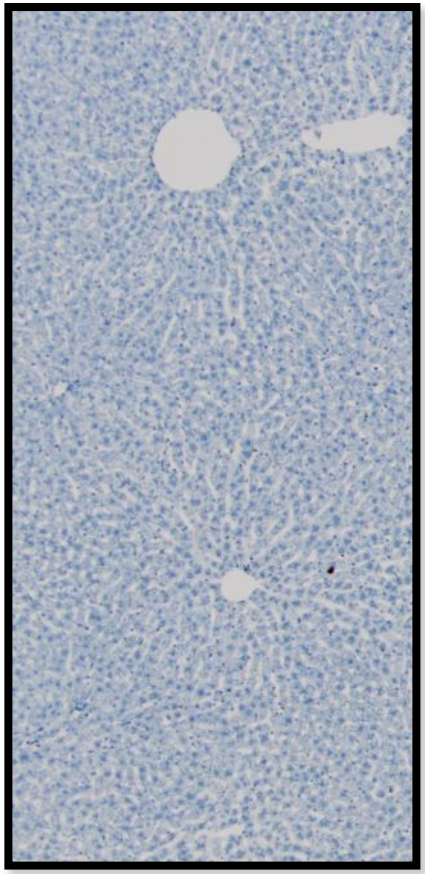
GATA4

Choice of antibody

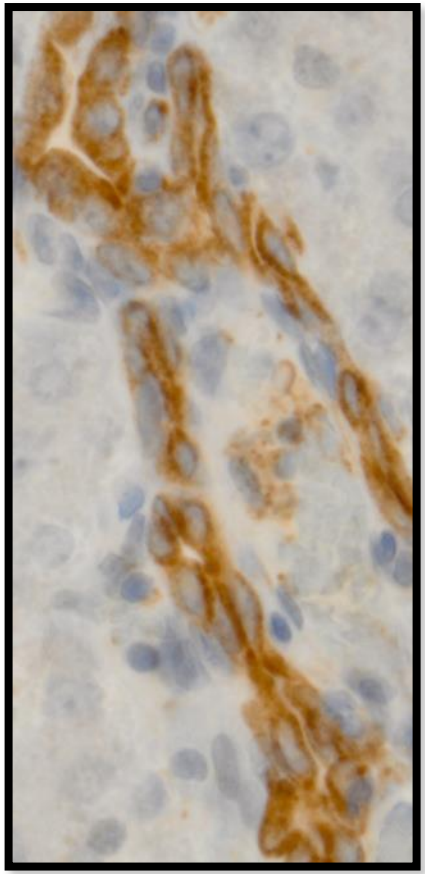
Rat liver: PPARα



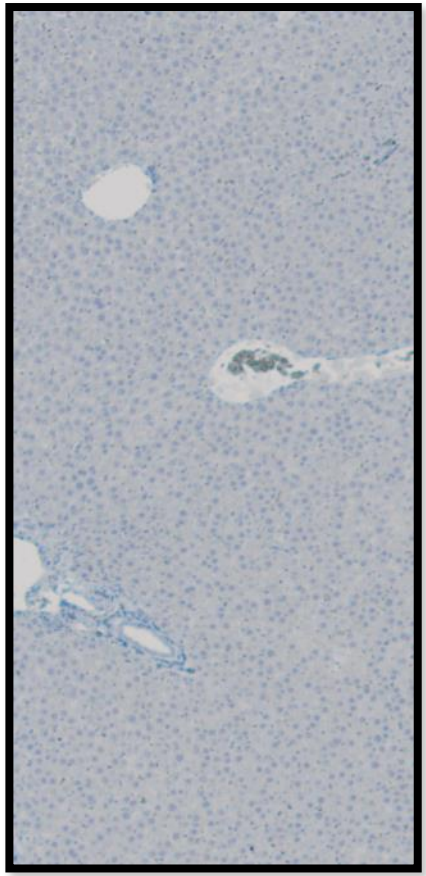
AR: Pepsin



Negative control

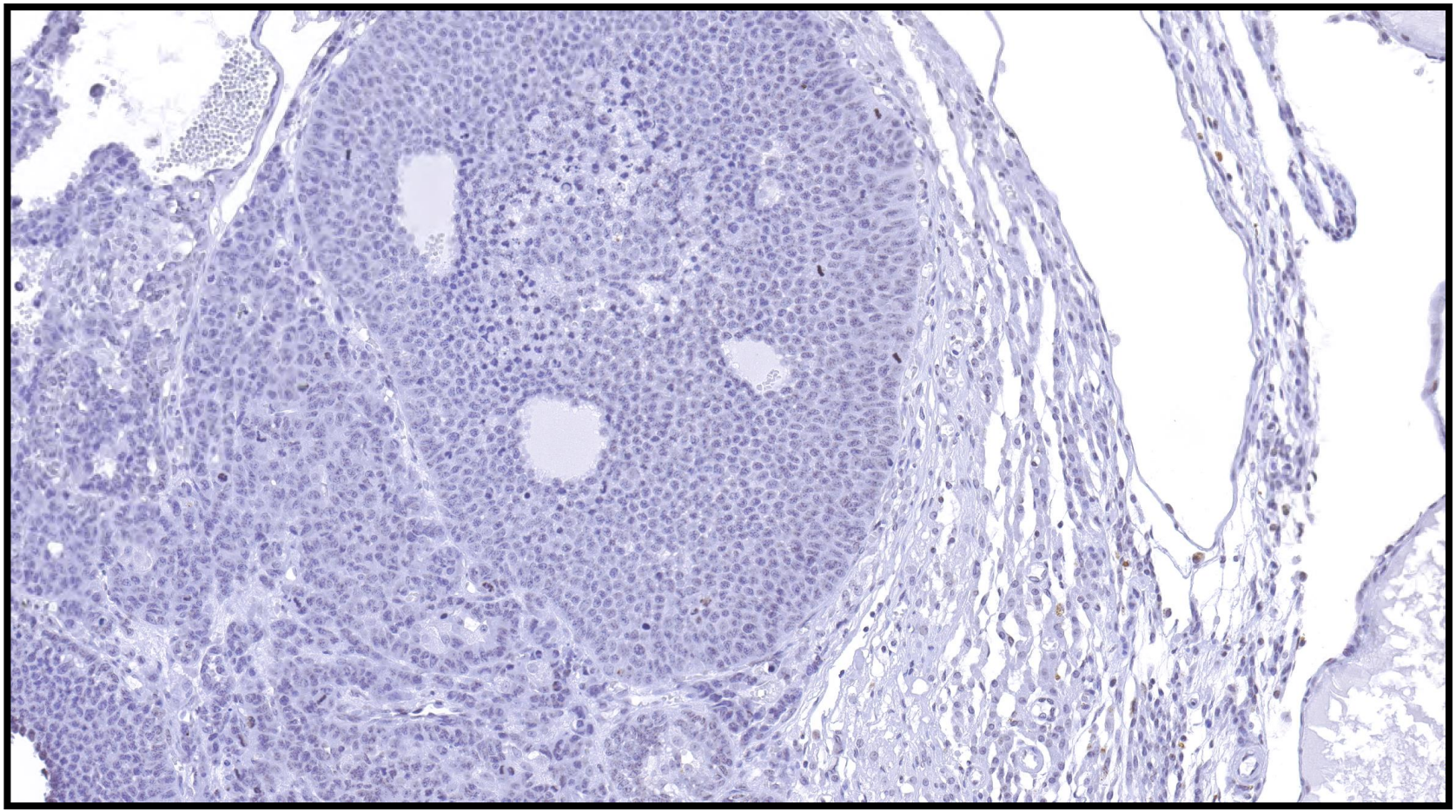


AR: Decloaker/ EDTA



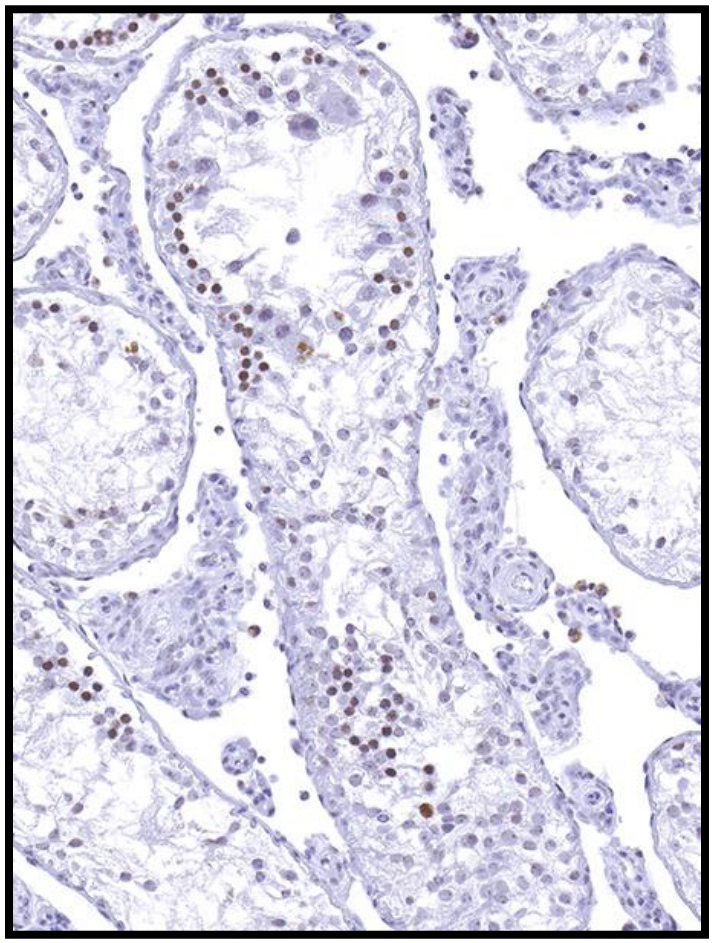
Negative control

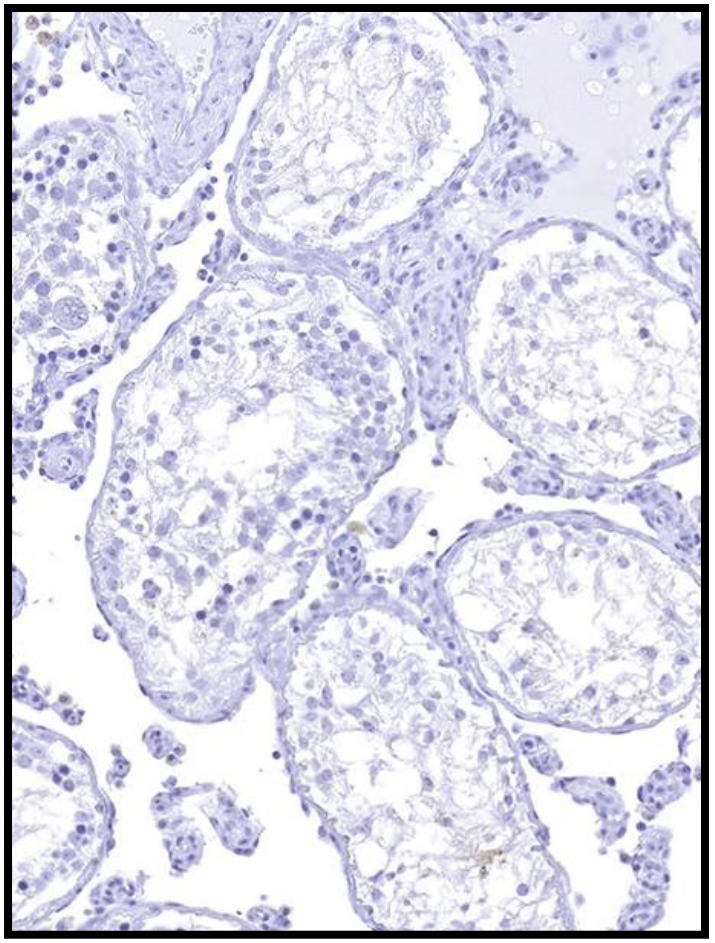
Choice of antibody



Rat seminoma: OCT 3/4

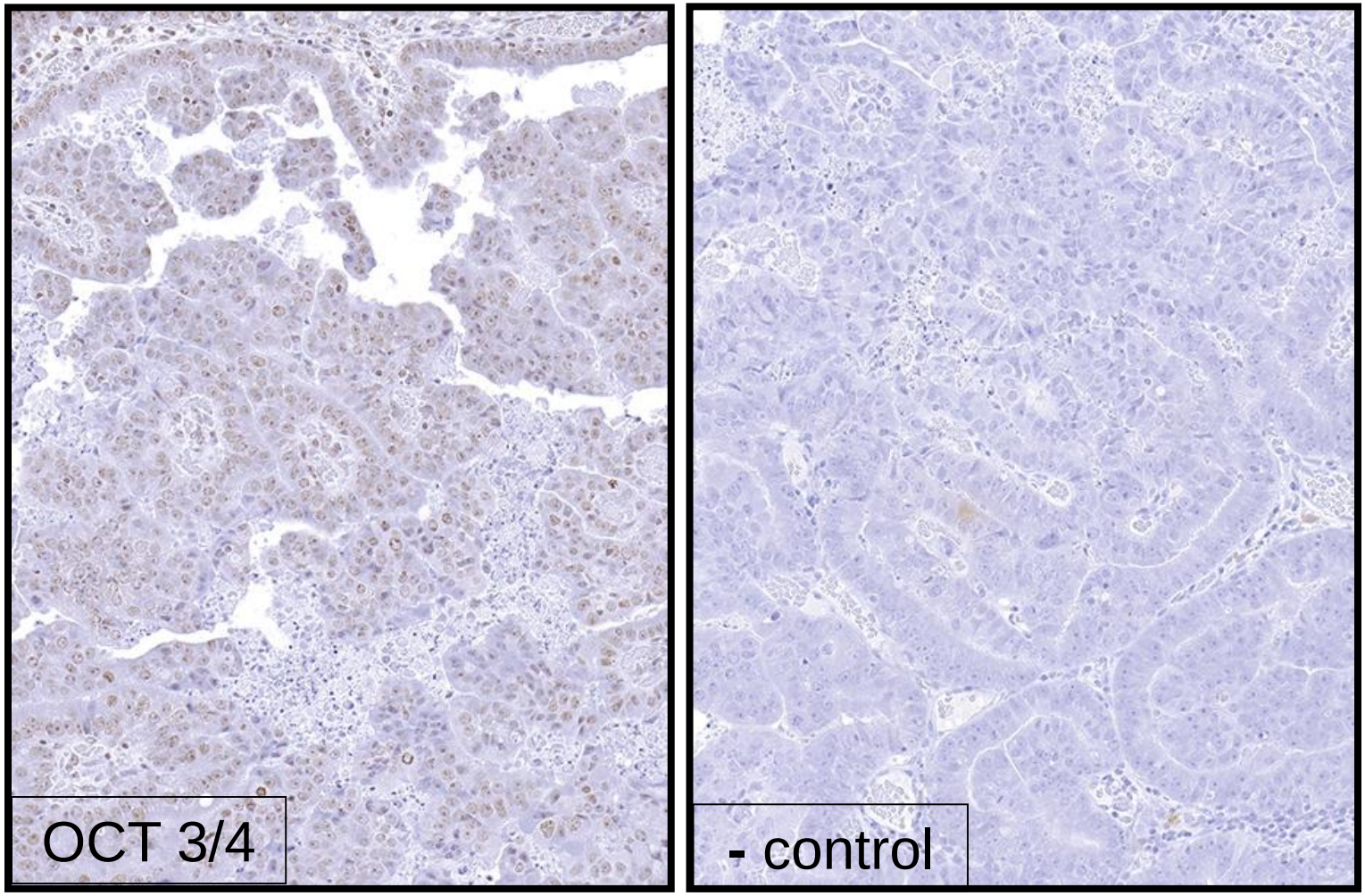
Choice of antibody





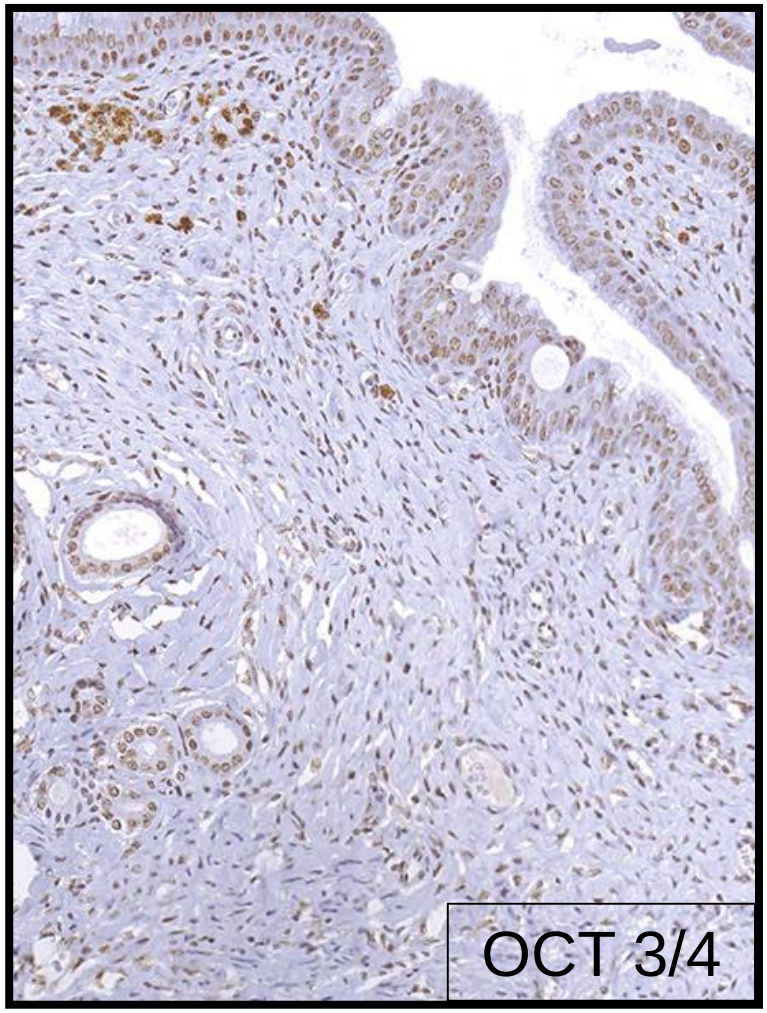
Rat testis: OCT 3/4

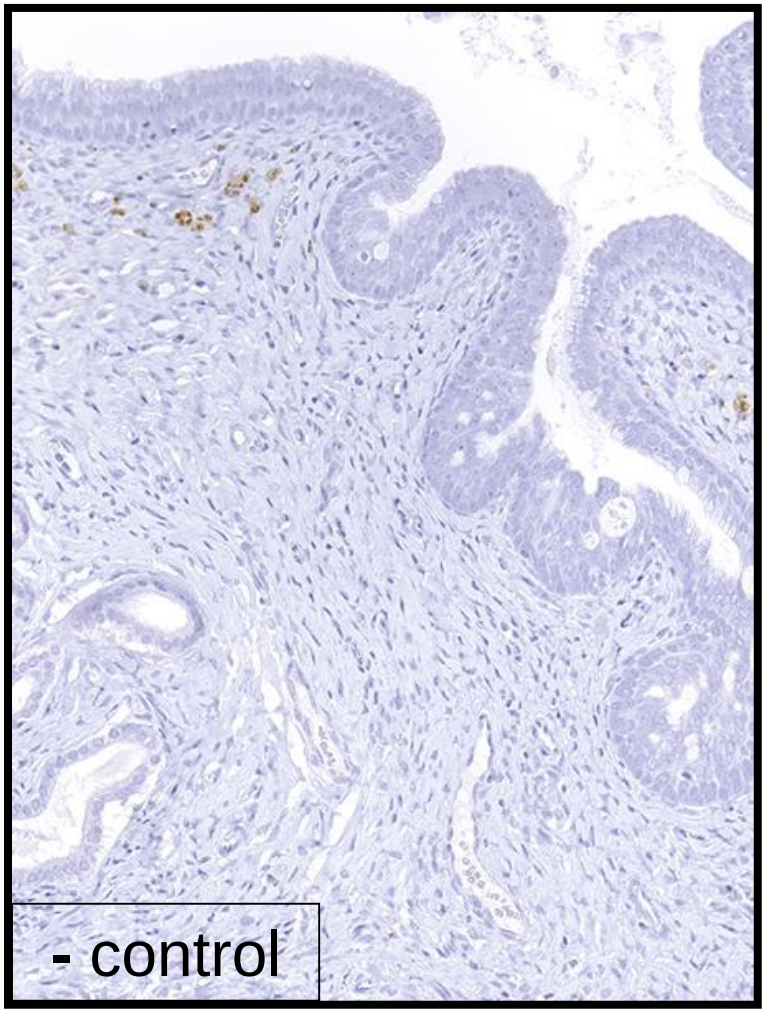
Choice of antibody



Rat: Uterine tumor

Choice of antibody

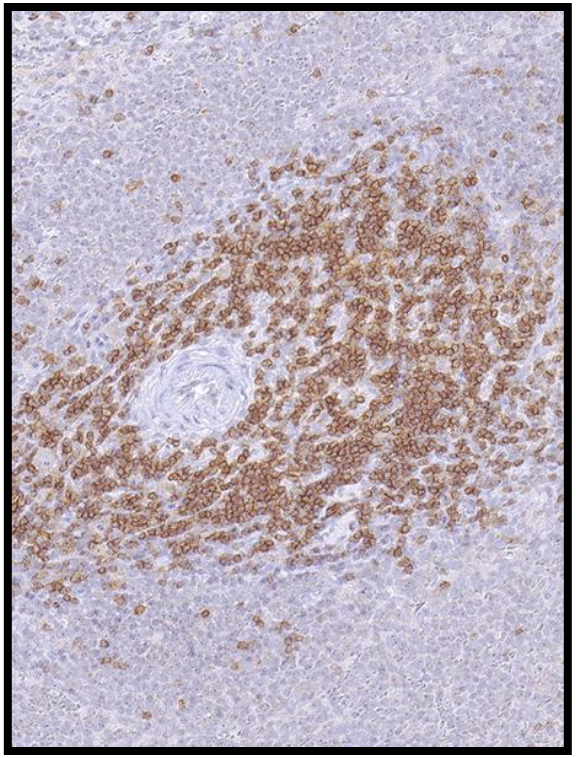




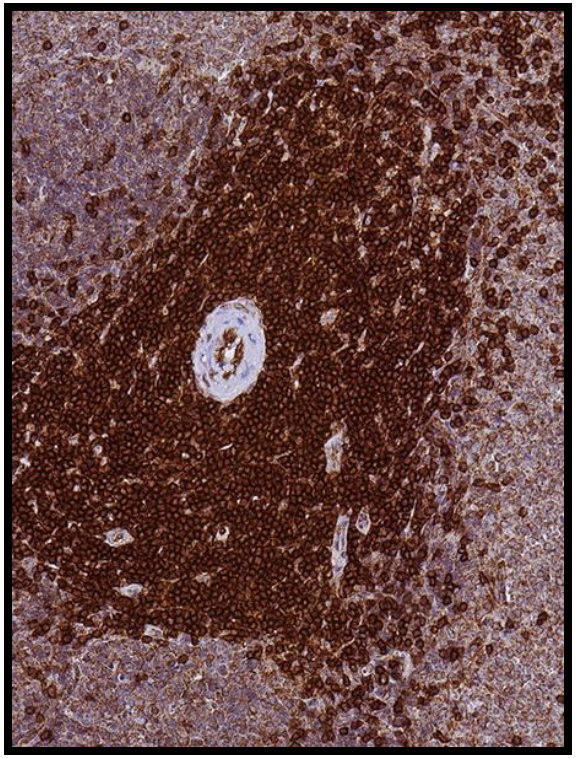
Rat: Uterus

Lot number

CD3 antibody



1:100



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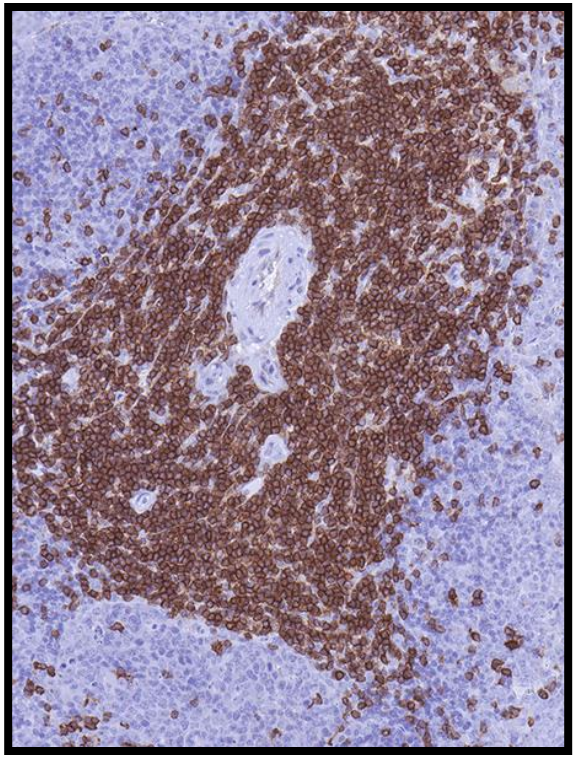
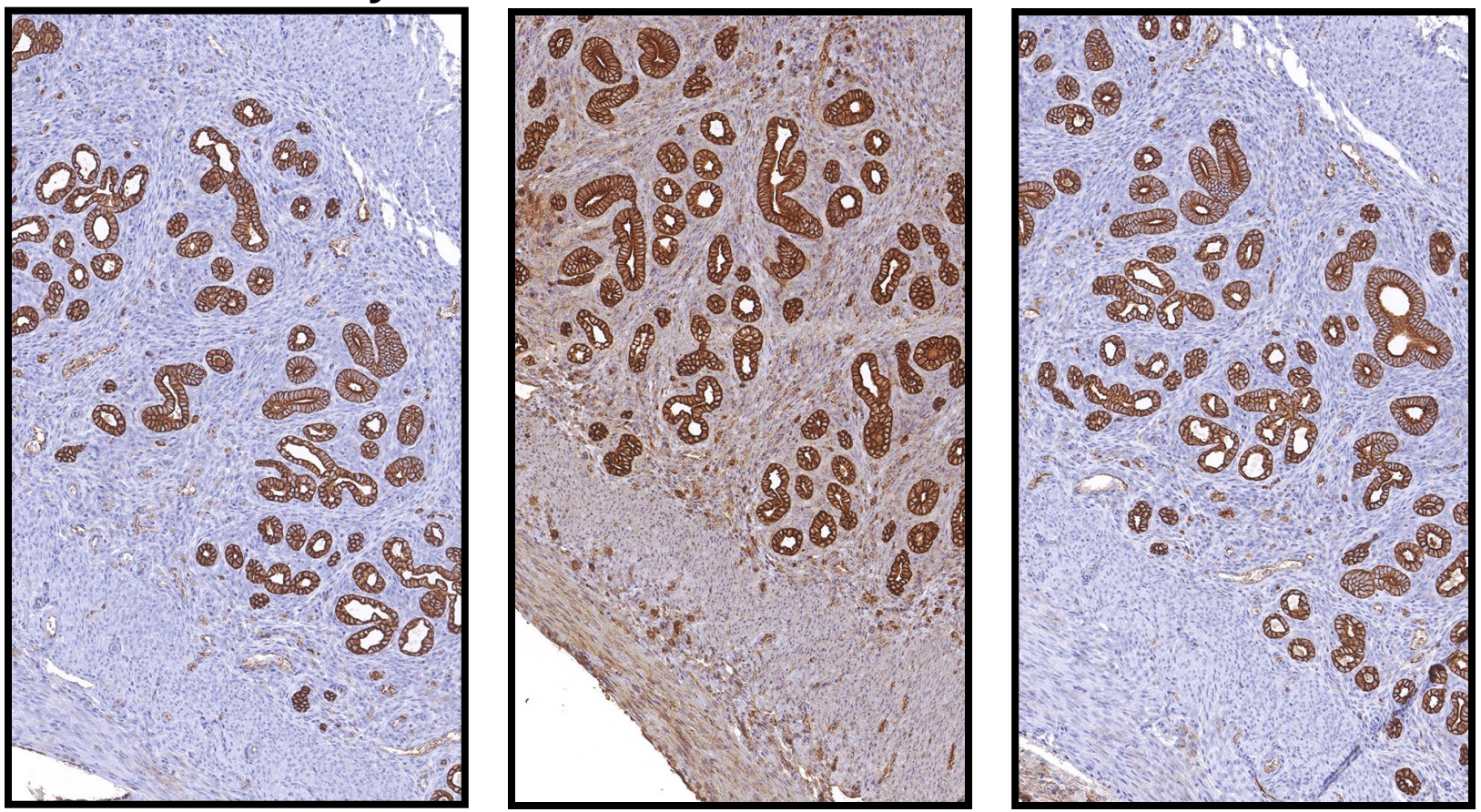


1:1000

Mouse Spleen

Lot number

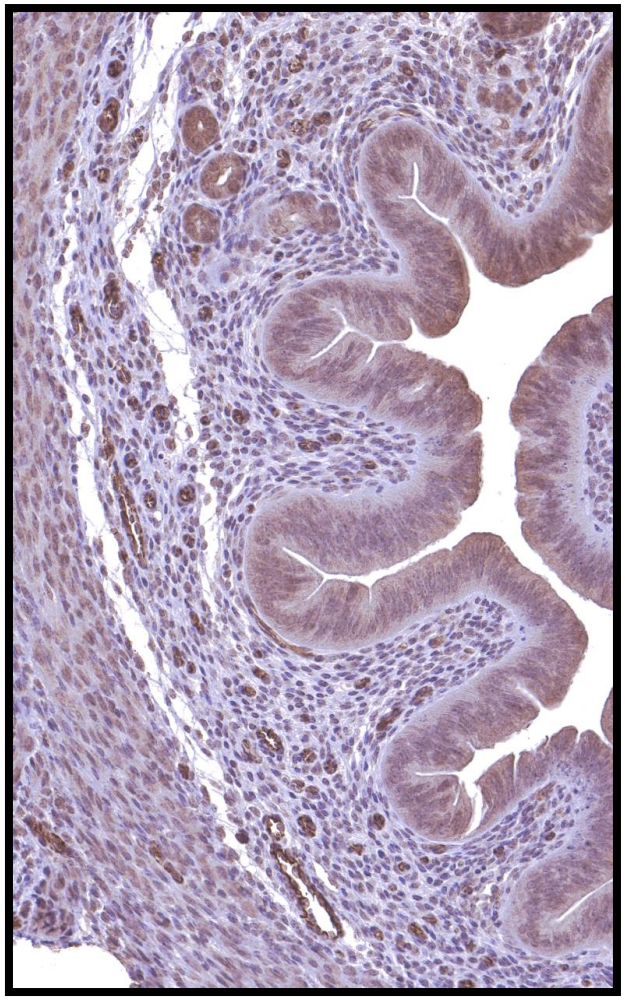
CK18 antibody



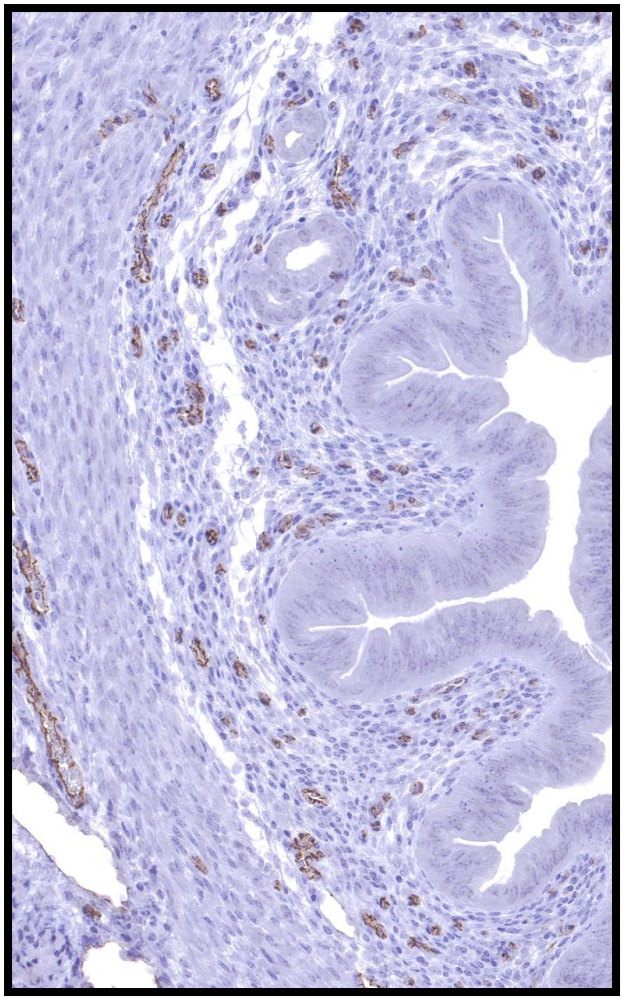
Mouse Uterus

Nonspecific staining: Sources and solutions

Technical error



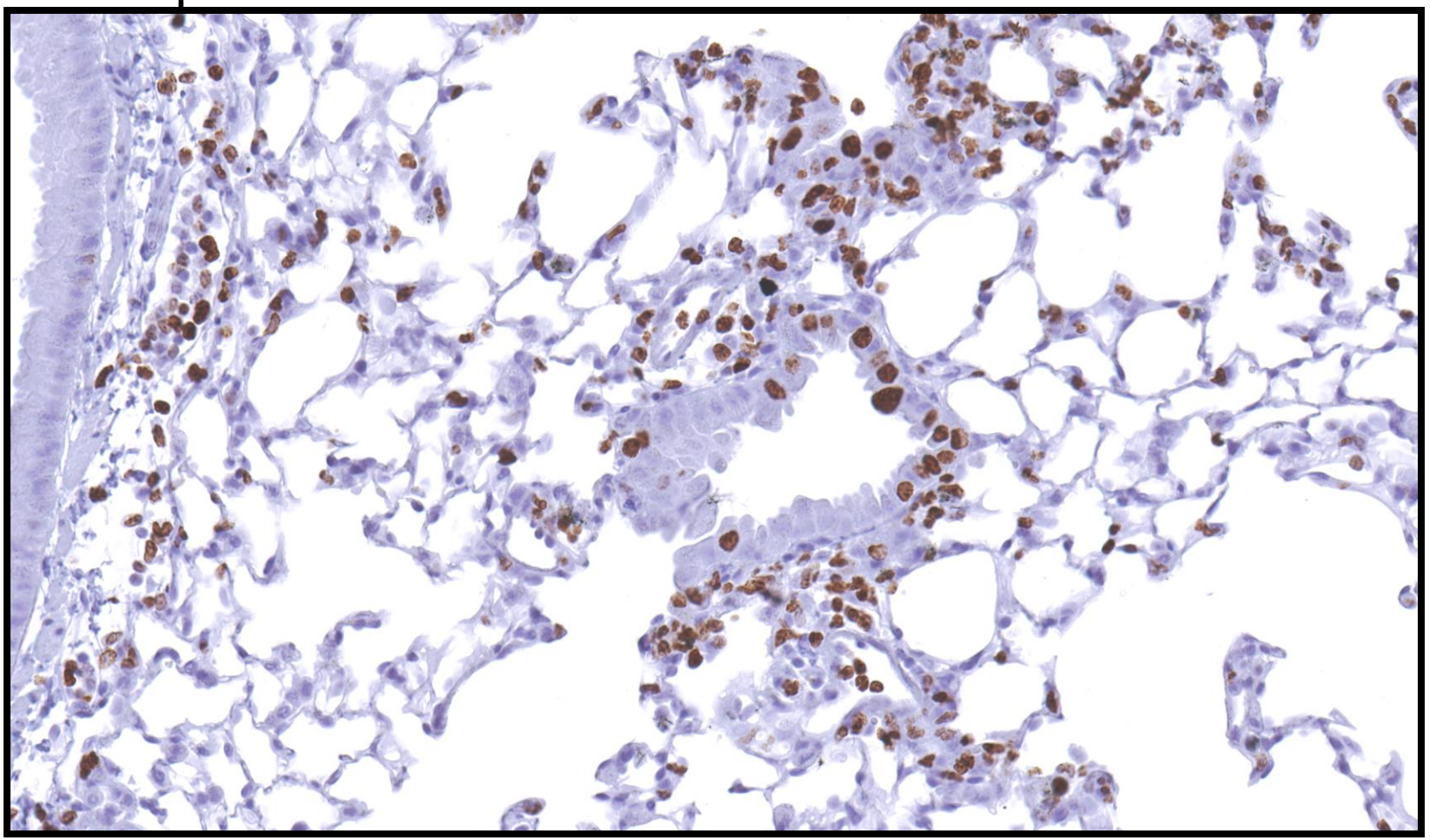
Nonspecific staining- CD31



Specific staining- CD31

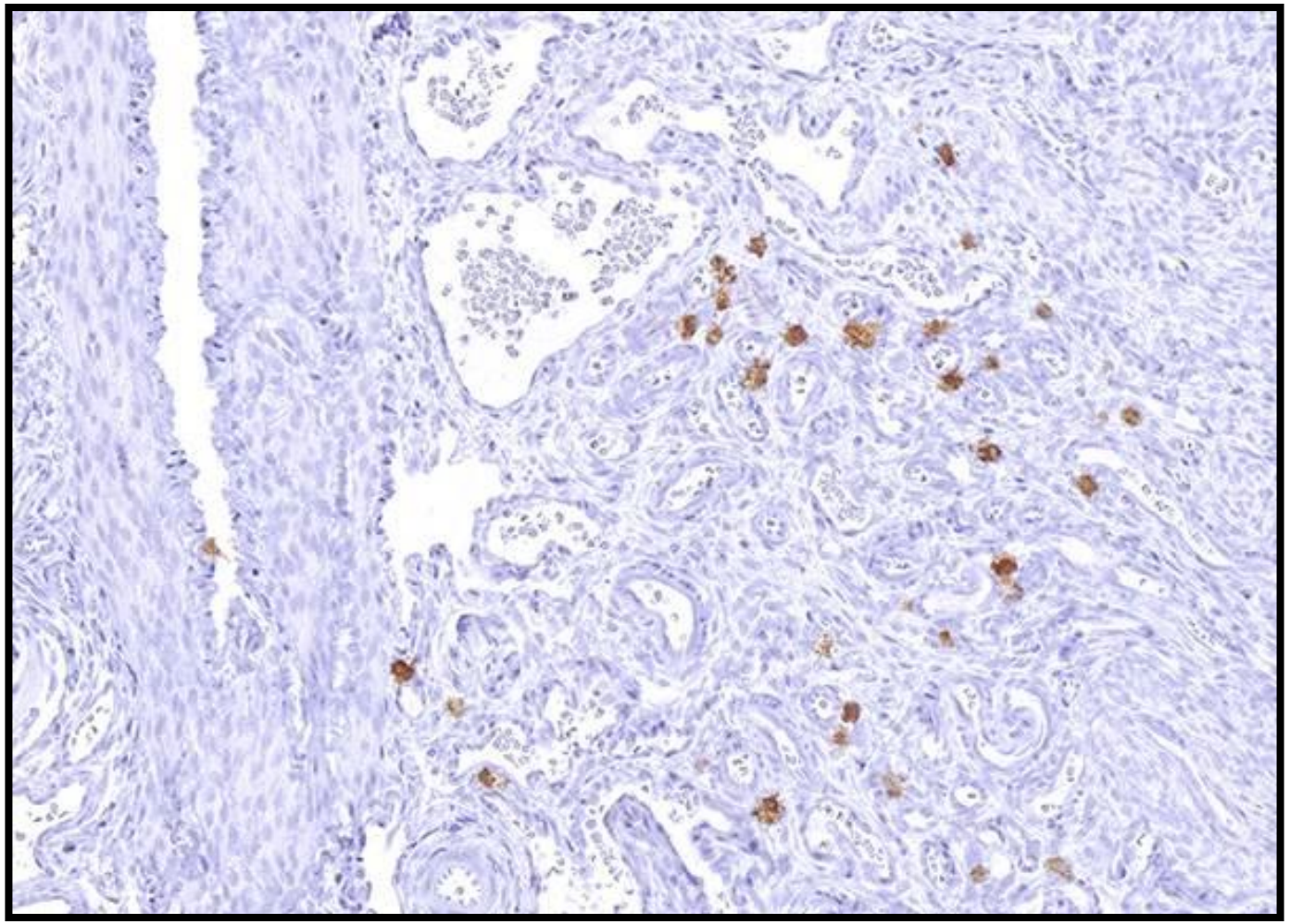
Certain cell types

Neutrophils & Ki67



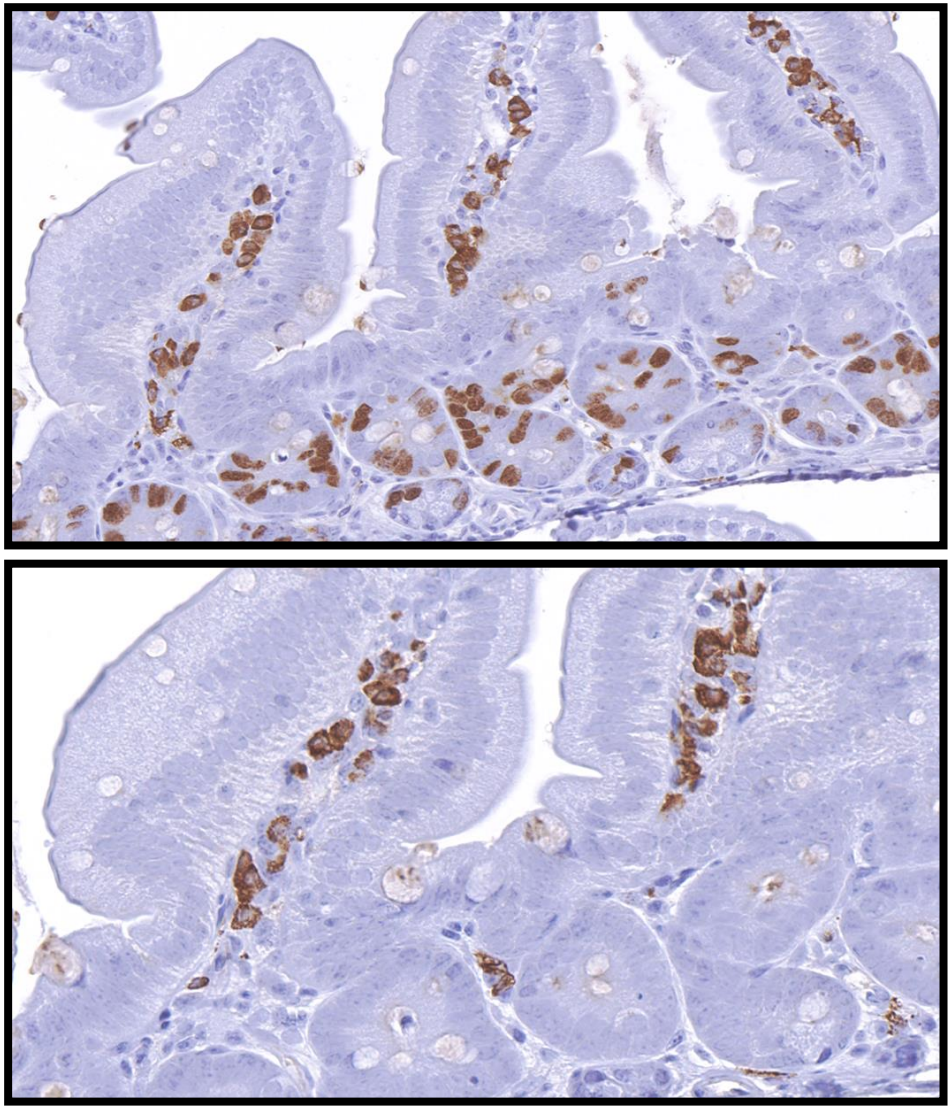
Certain cell types

Mast cells



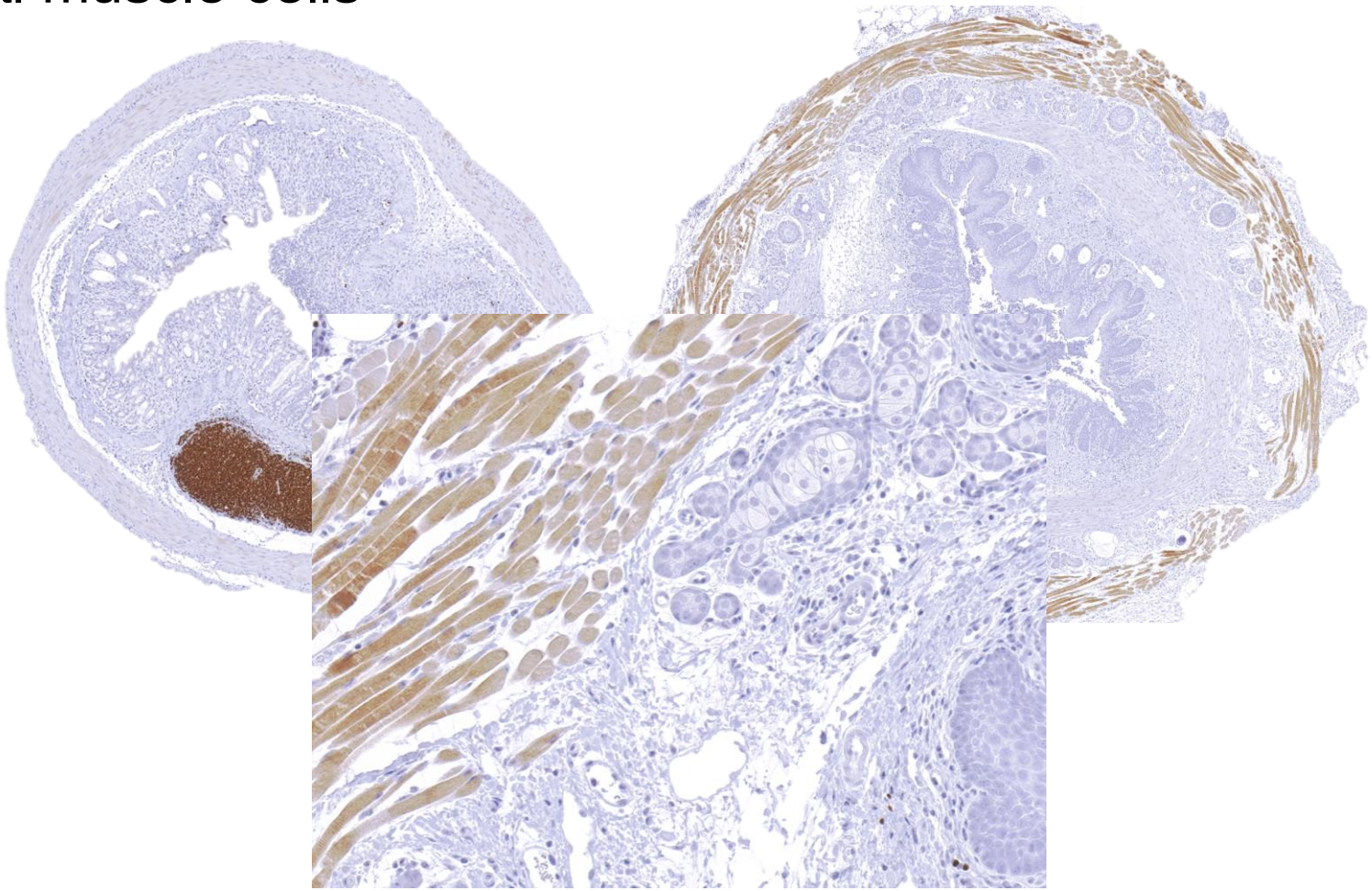
Certain cell types

Plasma cells

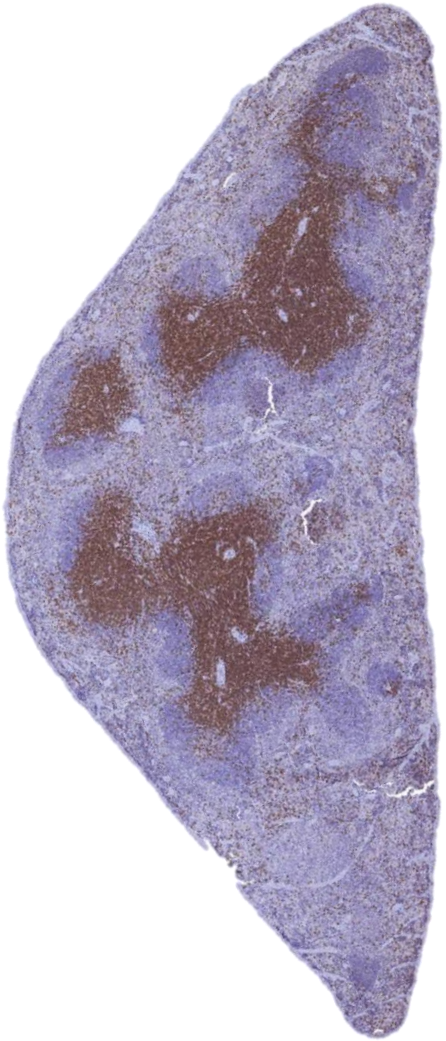


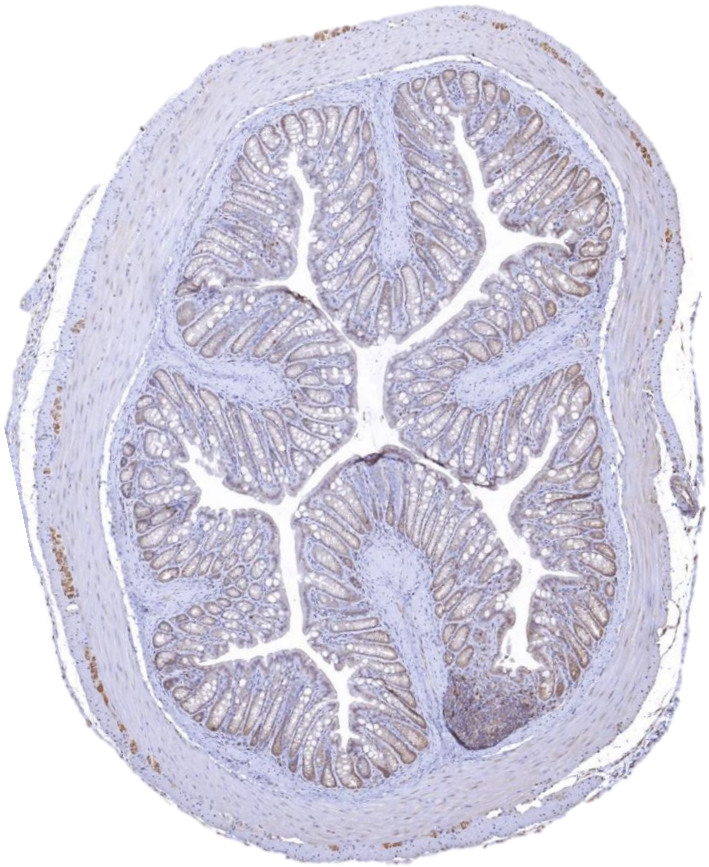
Certain cell types

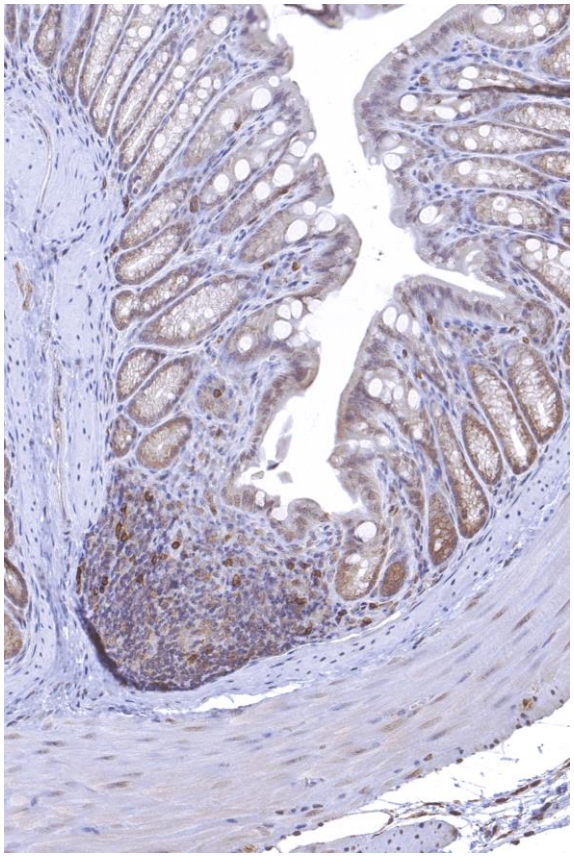
Skeletal muscle cells



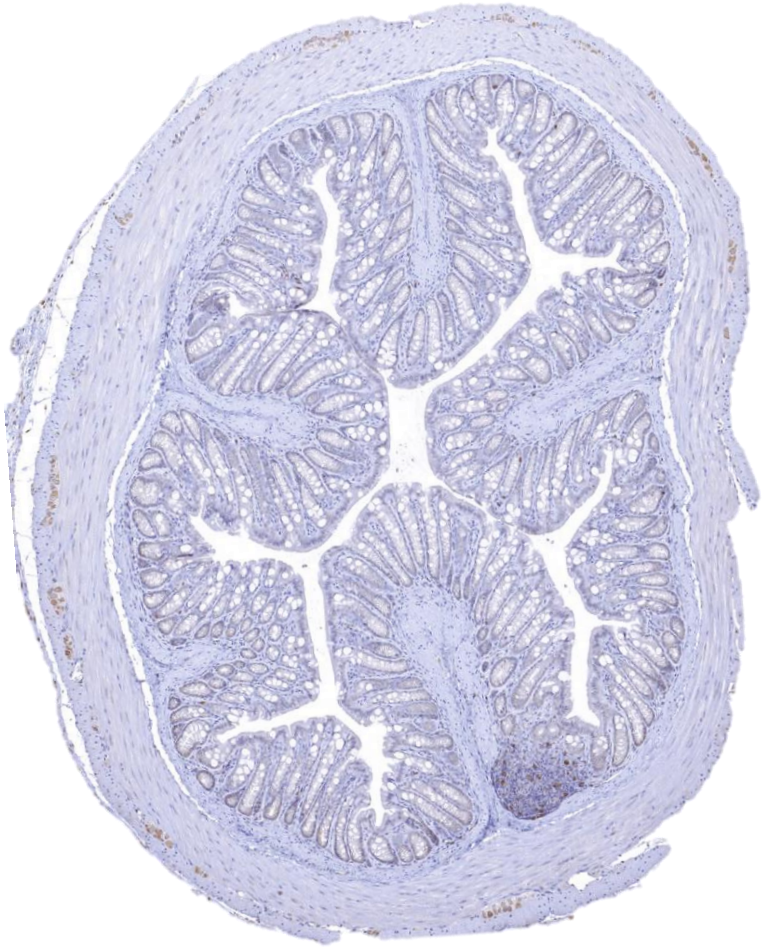
Tissue types

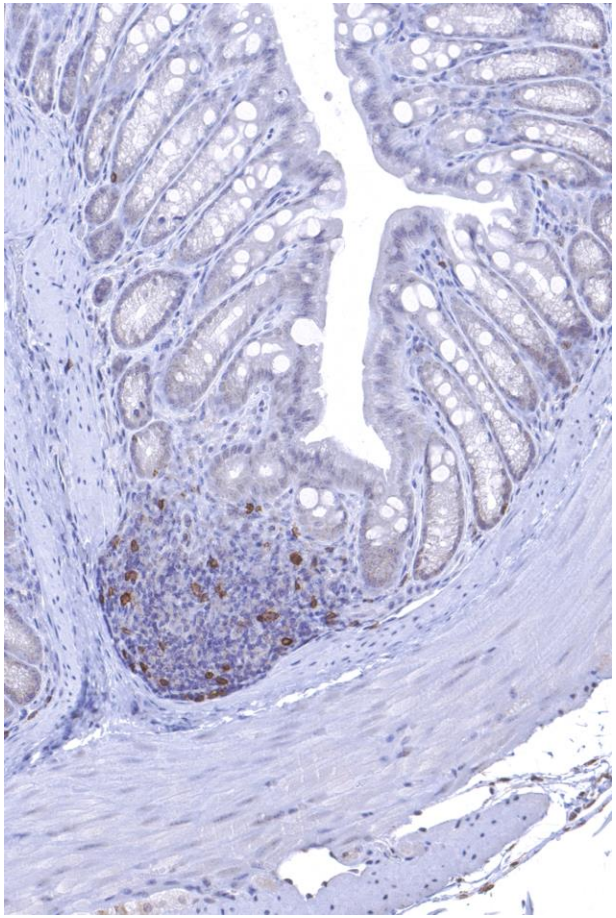






Tissue types





Hydrophobic interactions of proteins

- Blocking with proteins
- Is blocking really important?

Sci Rep. 2011; 1: 28.
Published online 2011 Jul 1. doi: 10.1038/srep00028
PMCID: PMC3216515

Non-specific binding of antibodies in immunohistochemistry: fallacies and facts

Igor Buchwalow,[a] Vera Samoilova, Werner Boecker, and Markus Tiemann

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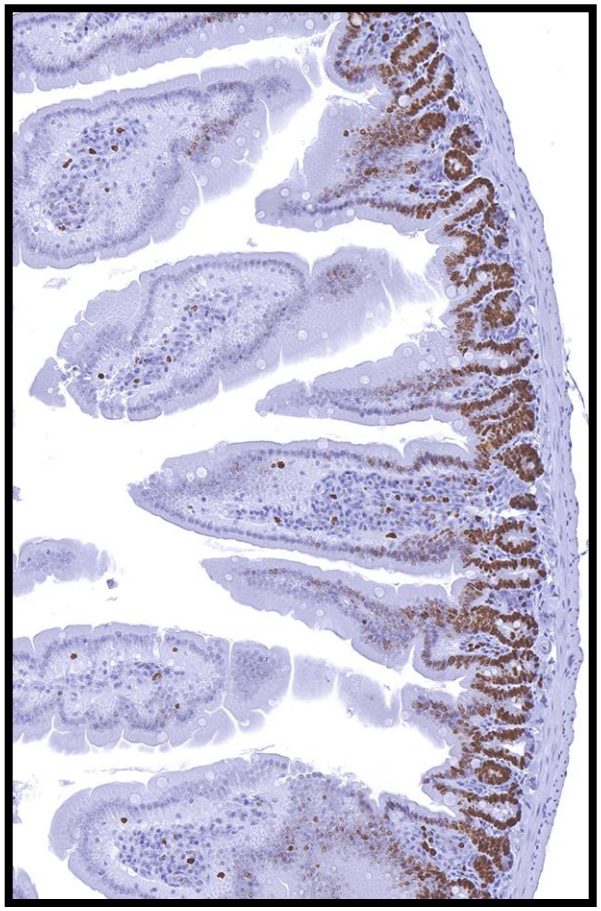
- 46 different antibodies
- 25 different tissues
- Conclusion: Blocking with serum or other reagents is not essential

Is blocking really important?

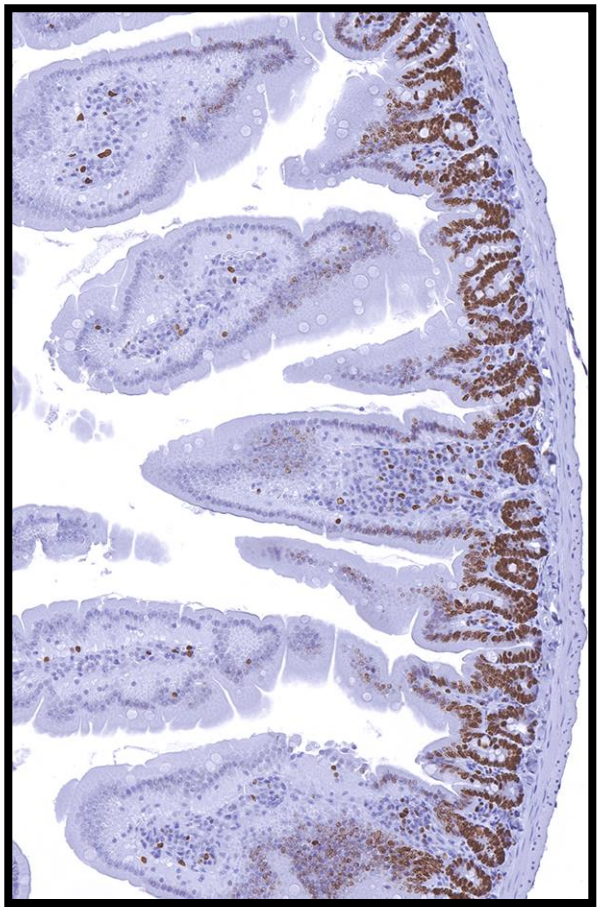
Blocking

No blocking

Is blocking really important?

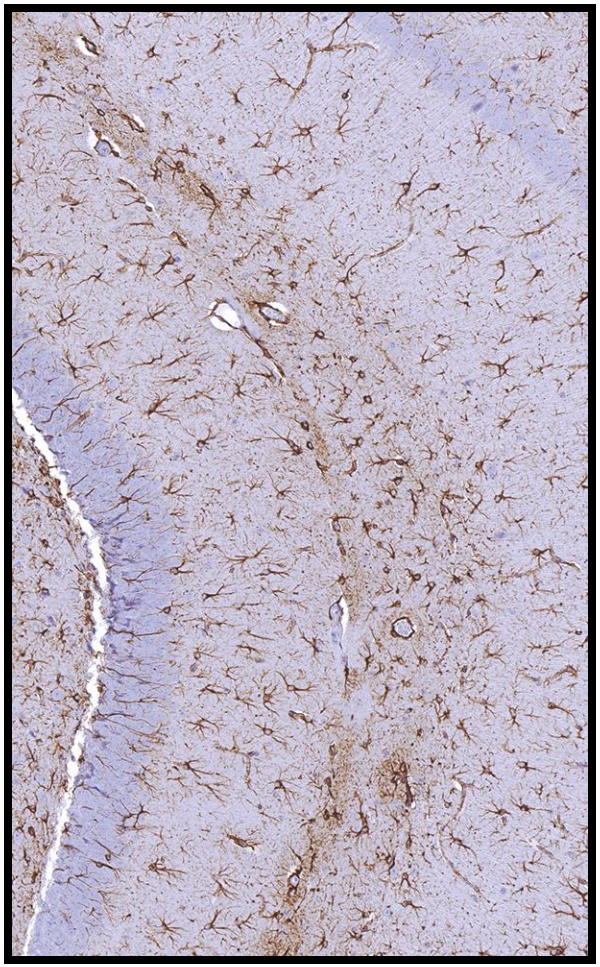


Blocking

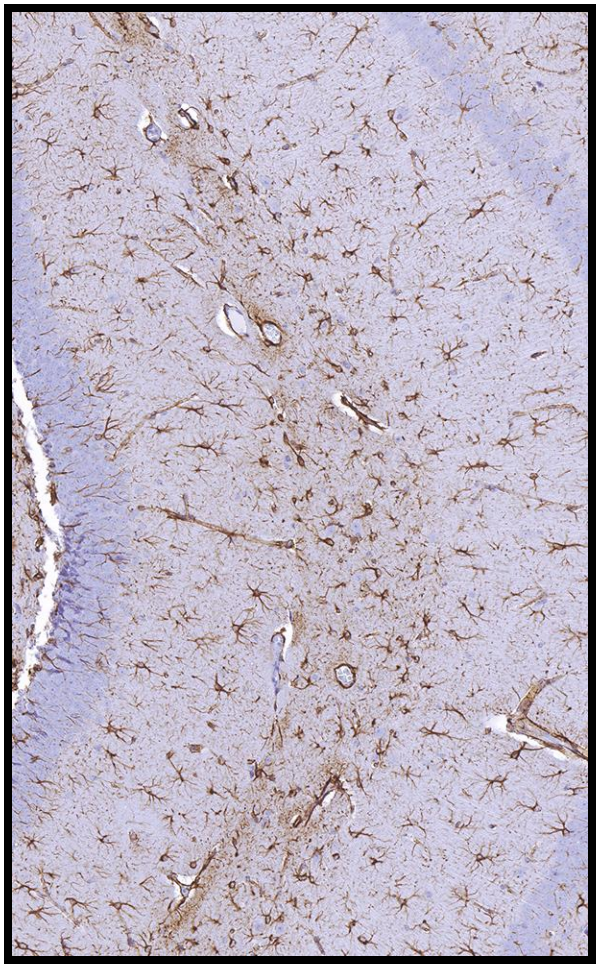


No blocking

Is blocking really important?



Blocking

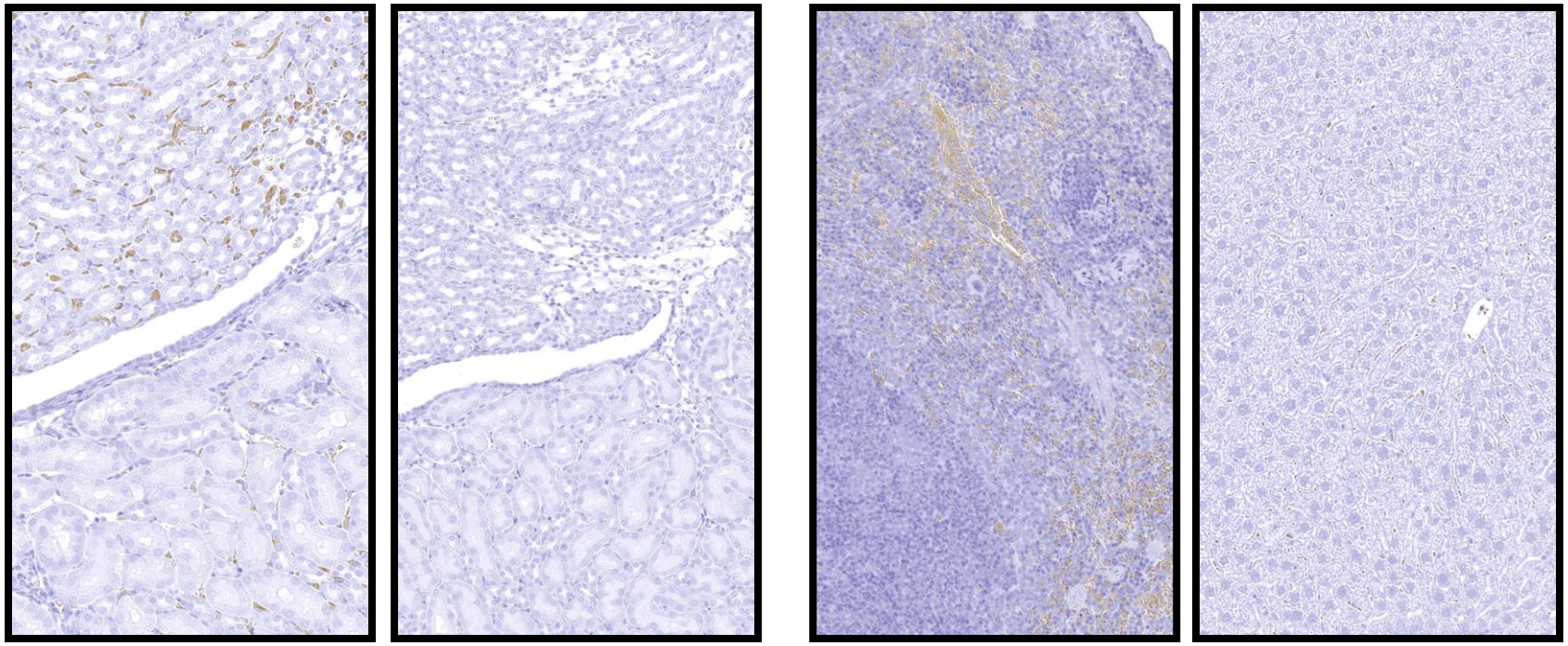


No blocking

Endogenous peroxidase and alkaline phosphatase activity

- Endogenous peroxidase activity
  - Red blood cells, granulocytes, monocytes, liver, kidney
  - Can be quenched using 3% Hydrogen peroxide in methanol
- Endogenous Alkaline phosphatase
  - Intestine, kidney, osteoblasts, endothelial cell surface, neutrophils.
  - Not a huge problem in formalin-fixed tissues
  - Can be quenched using 1mM levamisole
  - For intestinal alkaline phosphatase- 1% acetic acid

Is endogenous peroxidase really a concern?



Kidney

Spleen

Liver

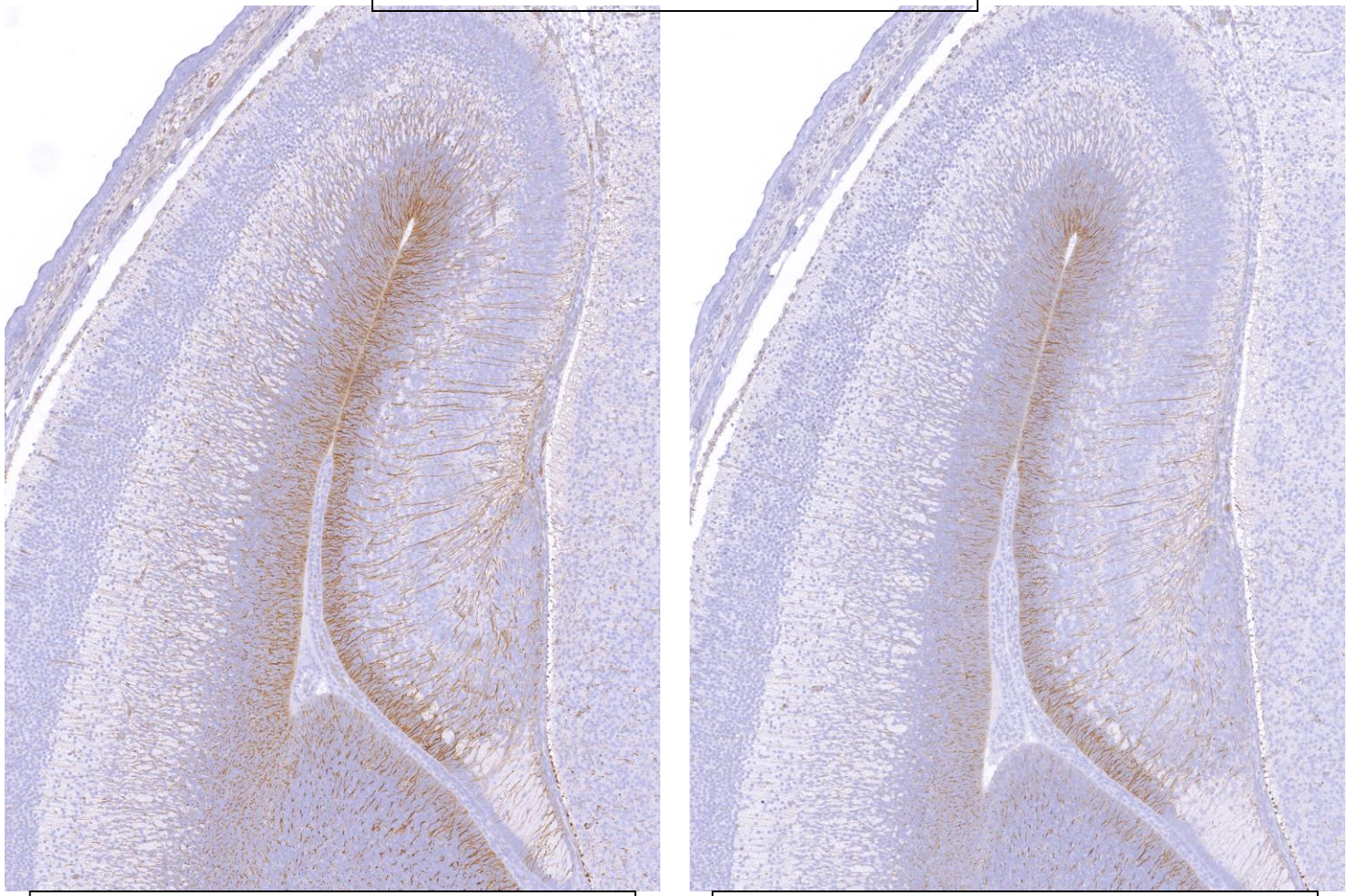
Endogenous biotin

- liver, lung, spleen, adipose tissue, mammary gland, kidney, brain
- Results in endogenous avidin-binding
- Formalin fixation diminishes endogenous biotin
- Antigen-retrieval exposes endogenous biotin

- Both avidin and biotin can be blocked by incubating sections with unlabeled avidin and biotin
- Use of polymer-based systems

Is endogenous biotin really a concern?

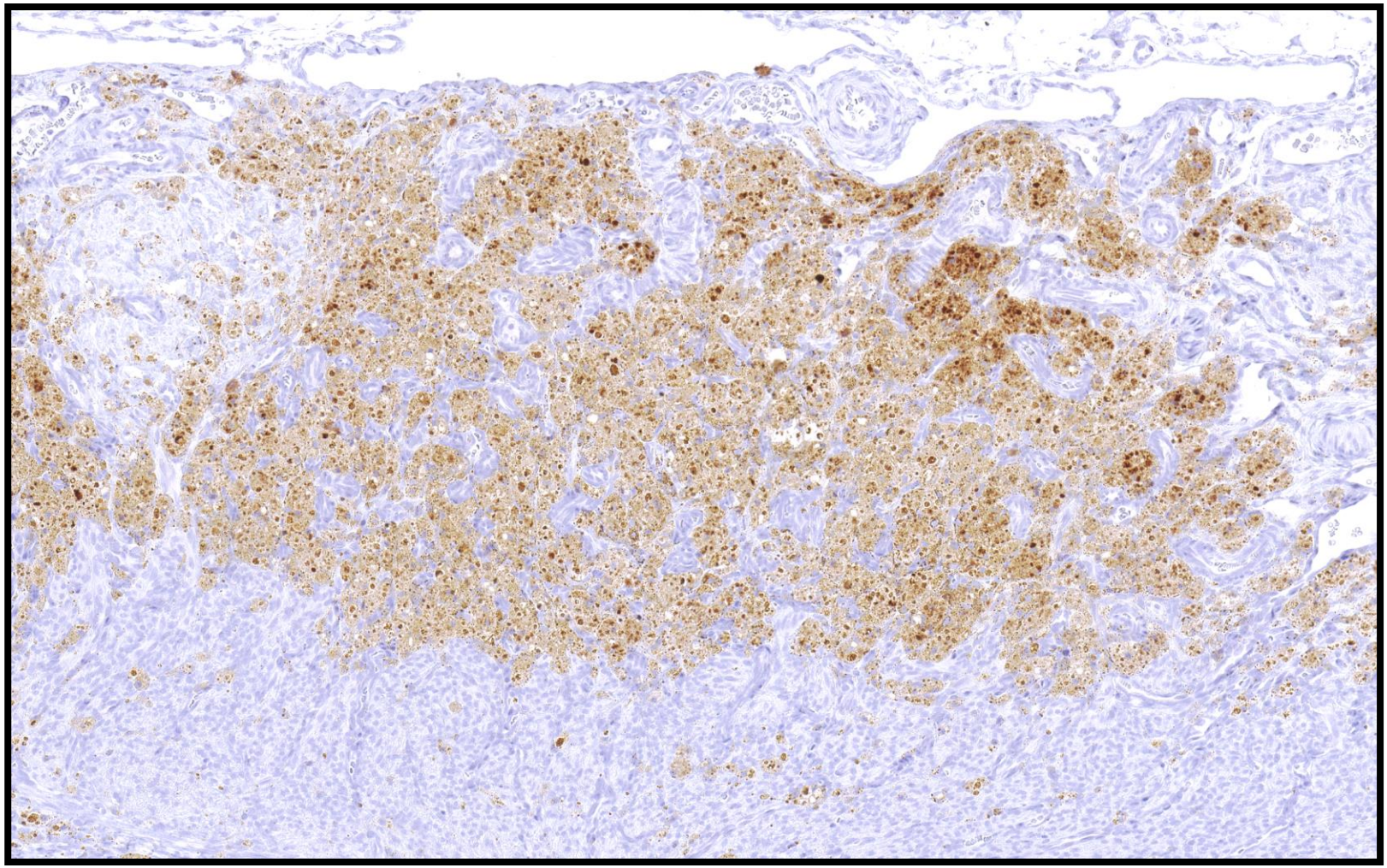
Mouse brain- Nestin



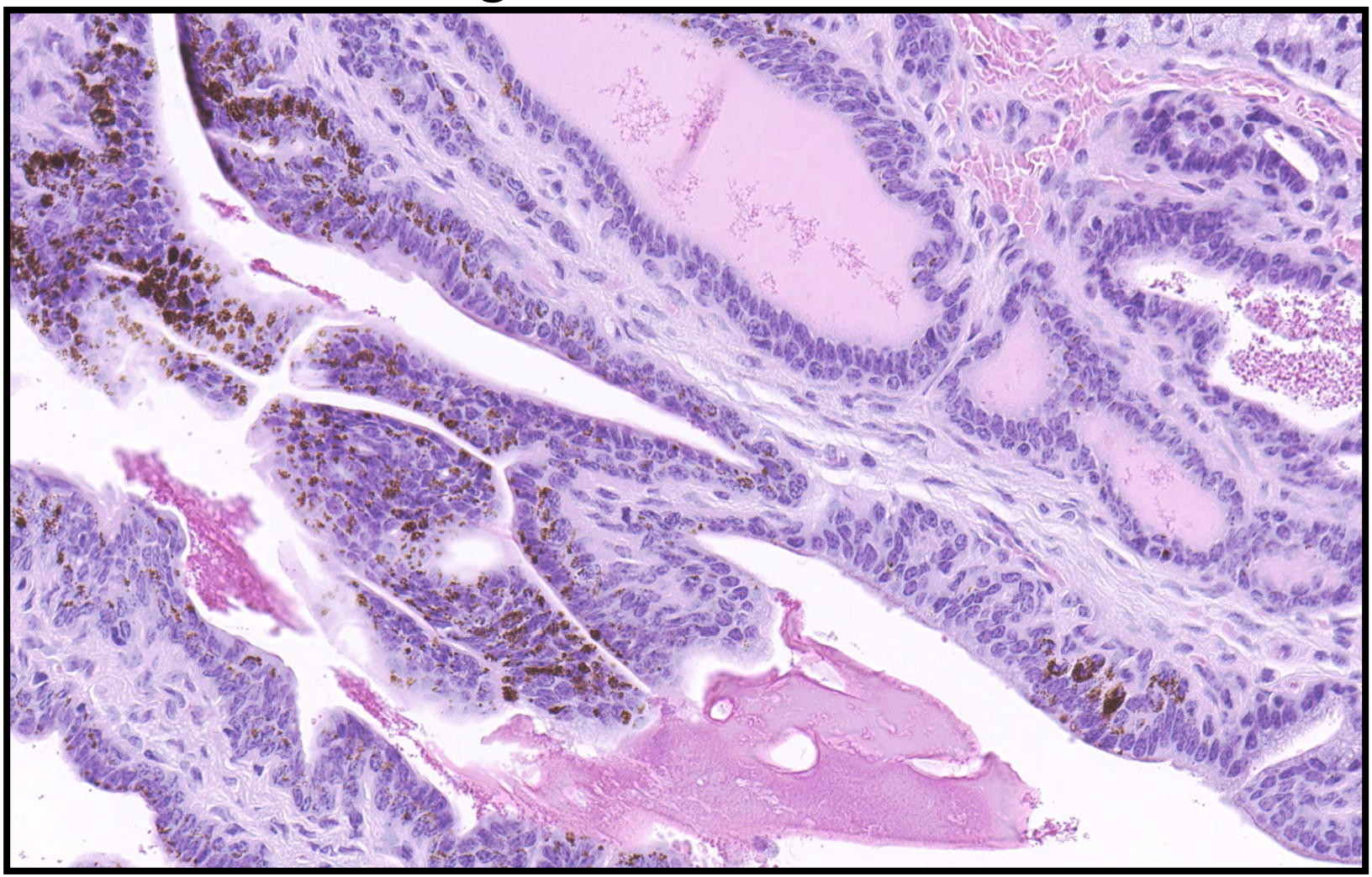
With blocking

Without blocking

Pigment: Hemosiderin



Pigment: Melanin



Controls

Controls

- Primary antibody controls
- Internal controls
- Secondary antibody controls
- Label controls

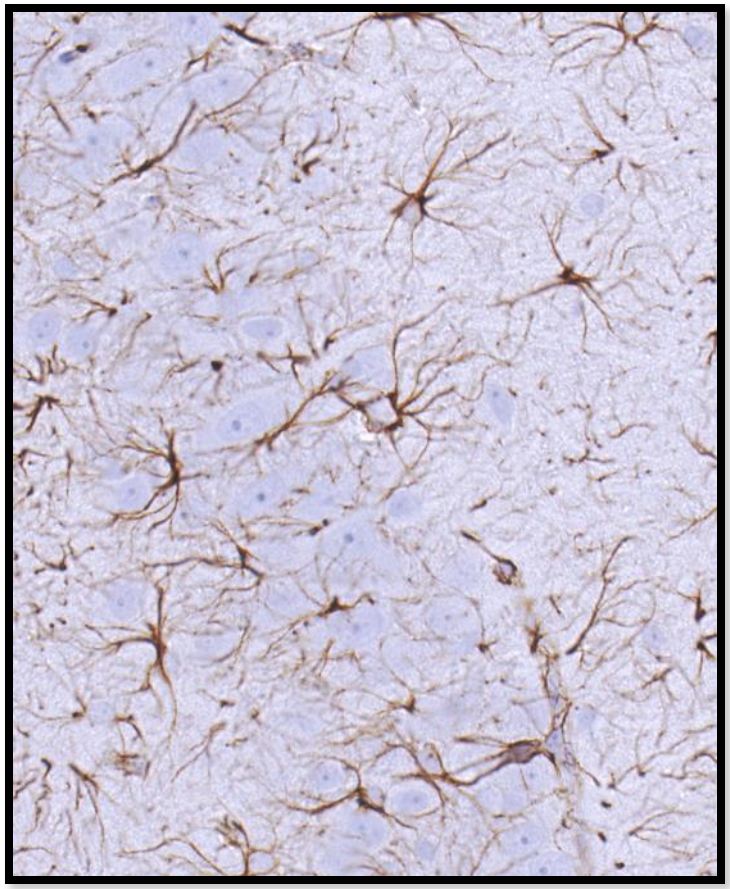
Richard Burry, J Histochem Cytochem 59:6–12, 2011

Primary antibody controls

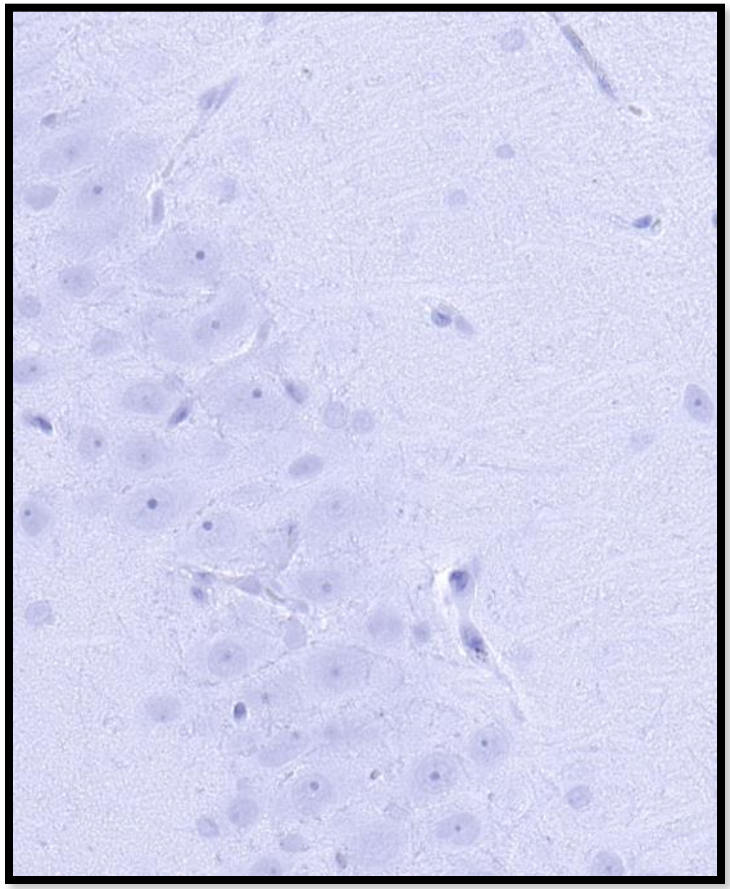
- Normal immunoglobulin/serum
- Genetic approach
  - Knockout animals
  - Transfected cell
- Absorption controls
- Western blot

Controls

Rat brain: GFAP

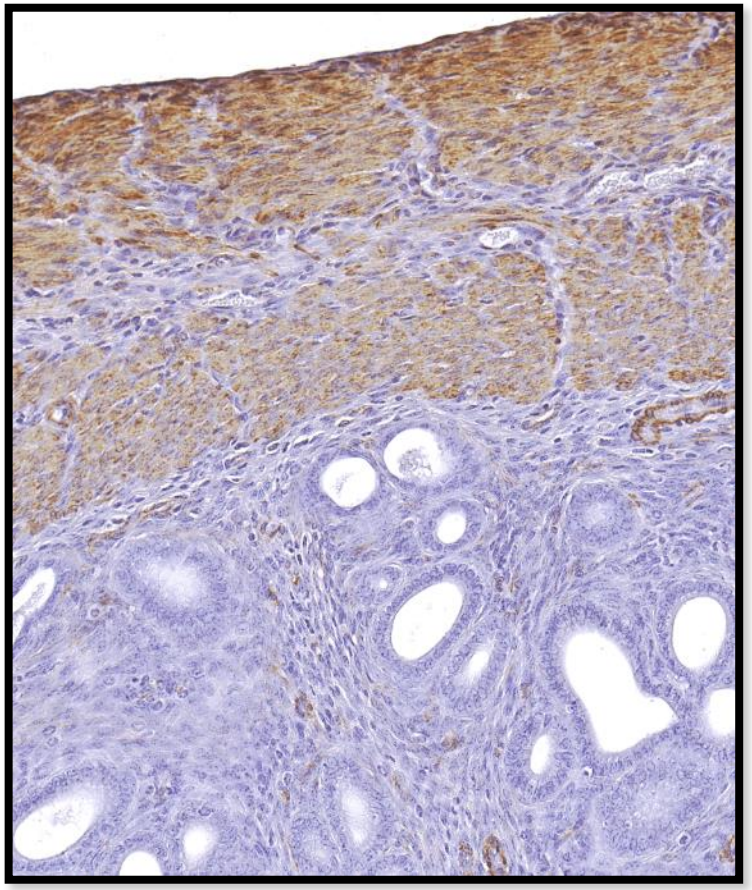


GFAP antibody

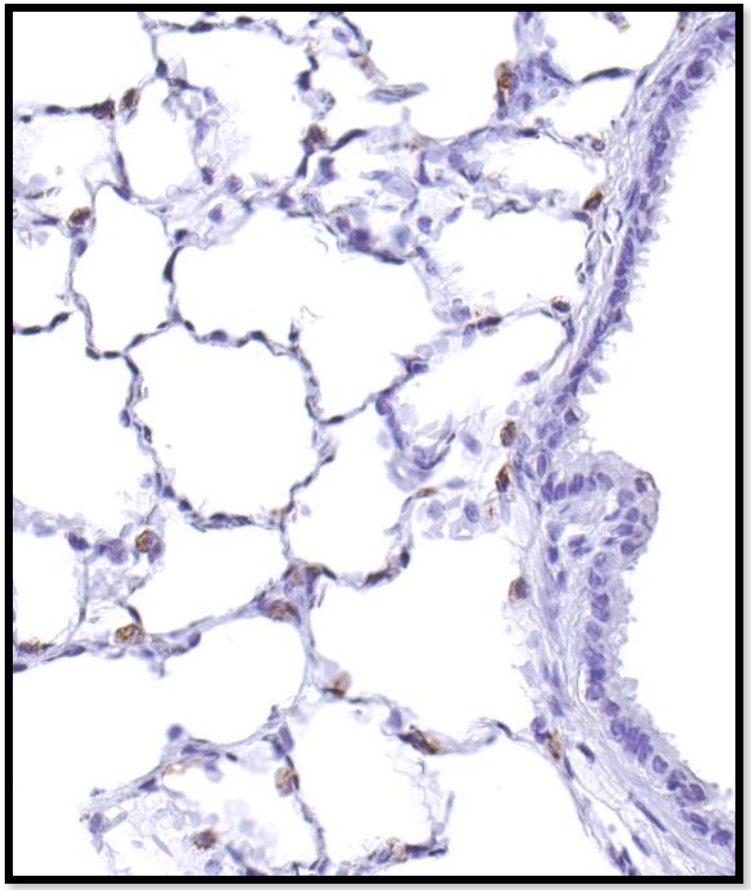


Normal immunoglobulin

Controls



Mouse uterus: SMA

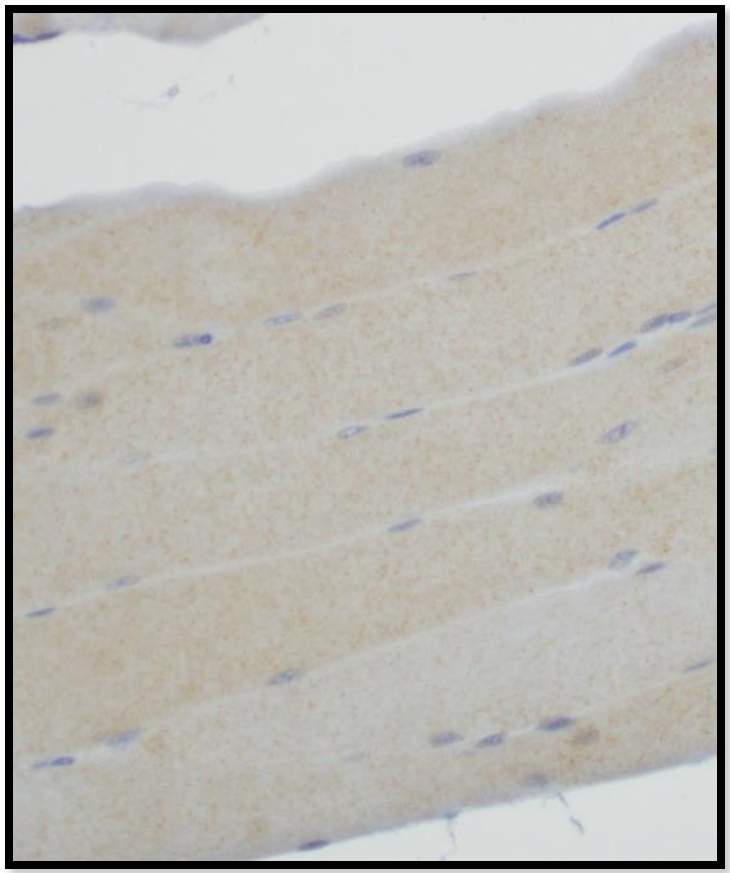


Rat lung: SPC

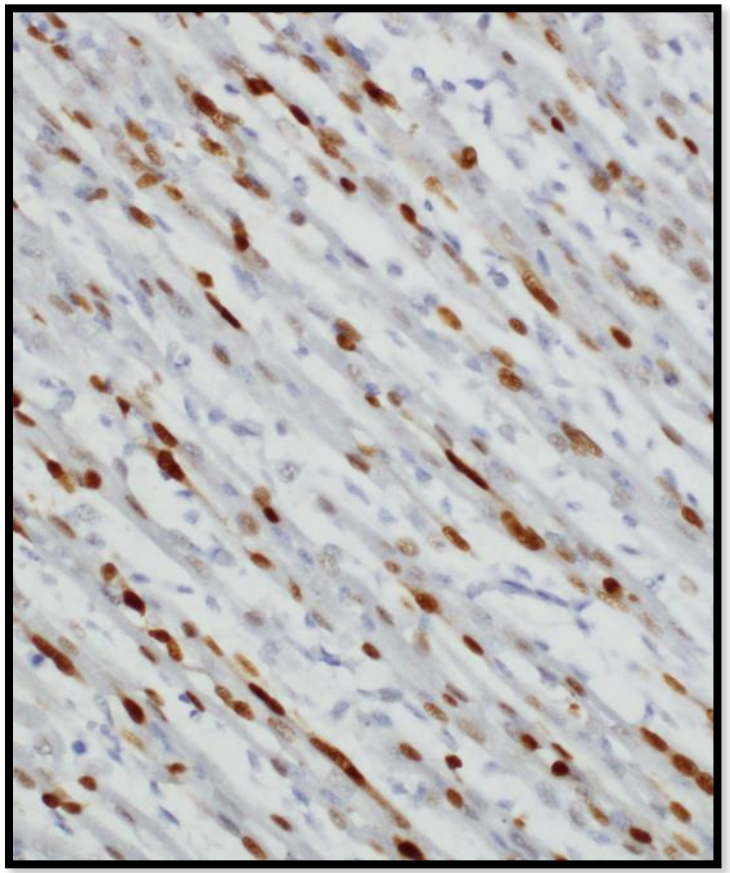
Internal controls

Controls

Myogenin



Adult

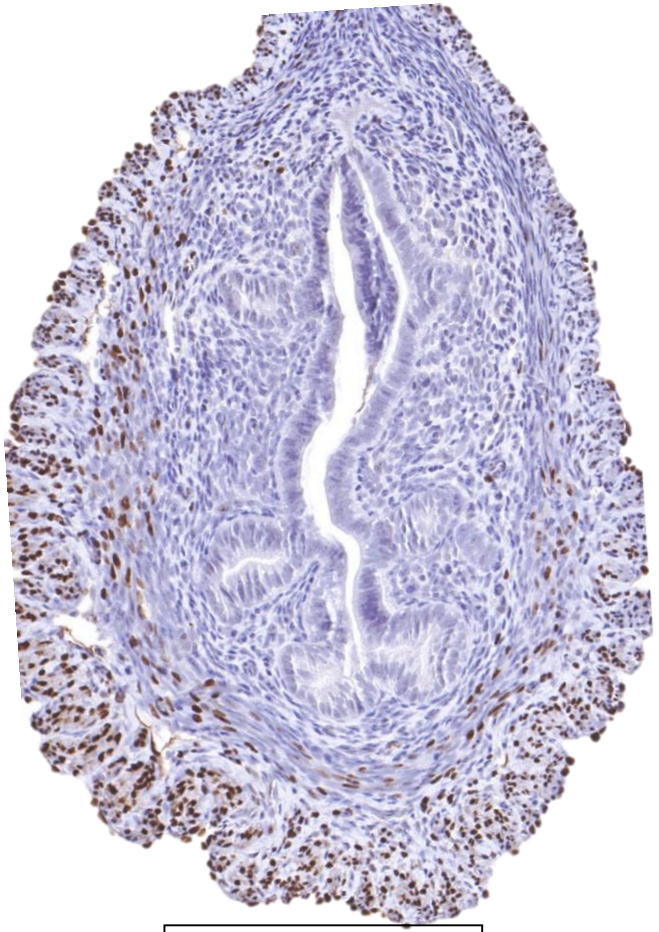


Embryo

Use appropriate control tissue for optimization

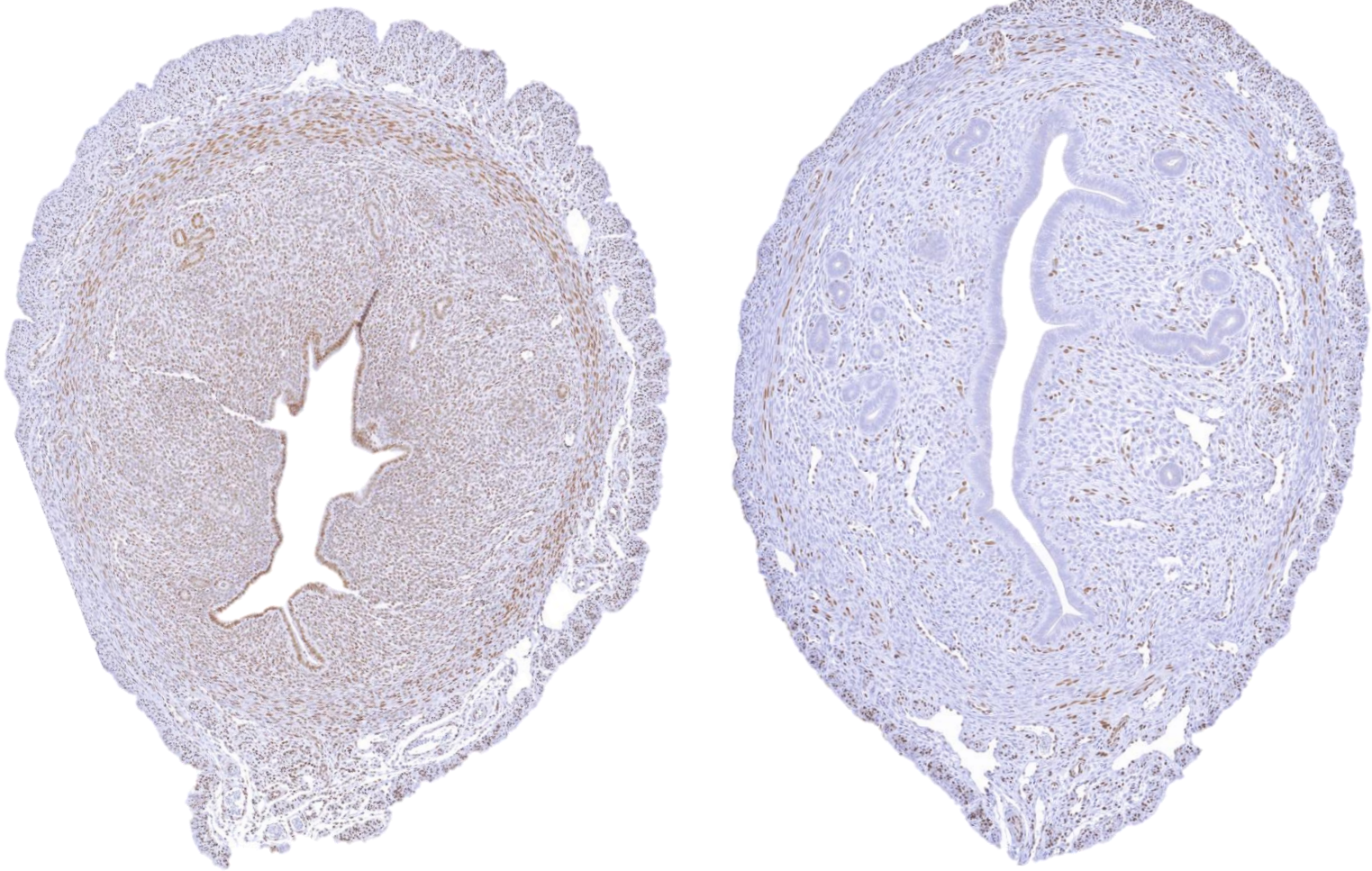
Primary antibody controls

ER- WT



ER- KO

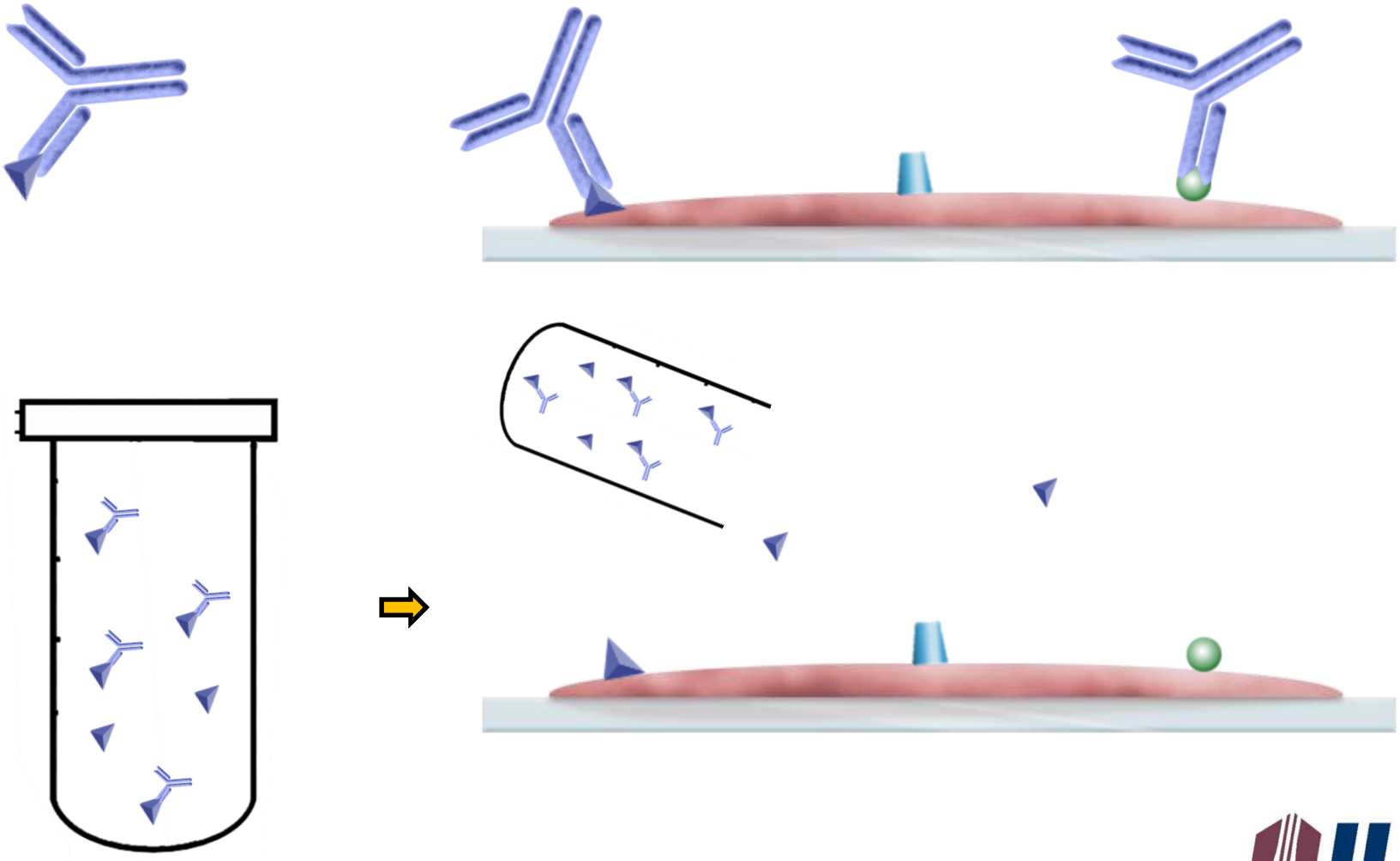
Primary antibody controls



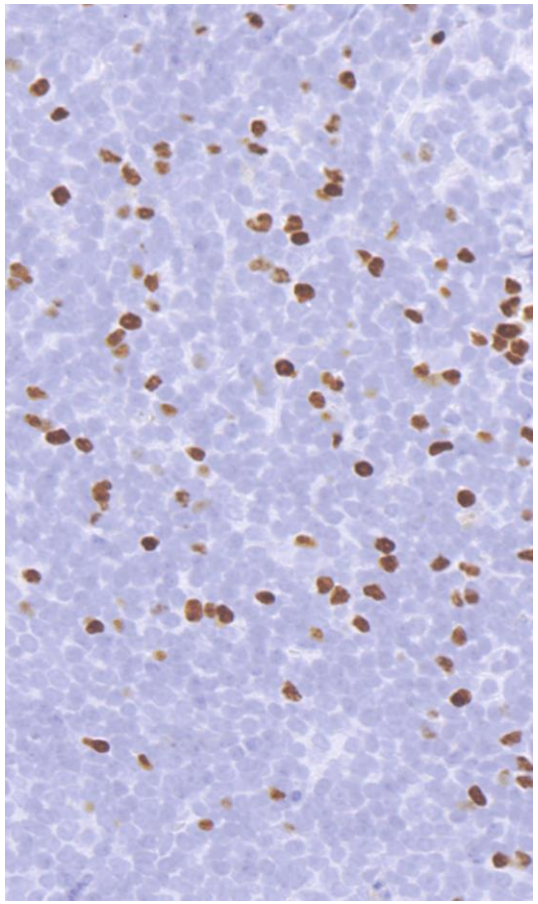
SRF-WT

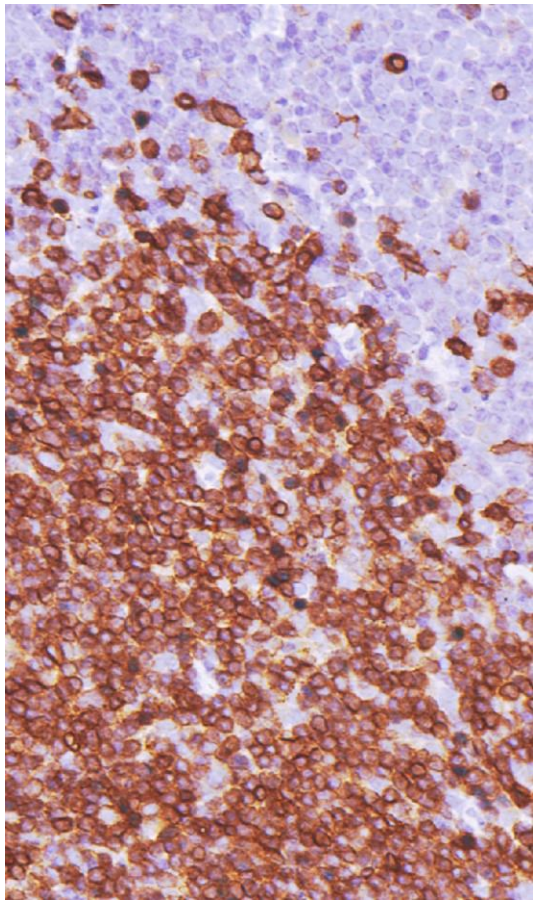
SRF-KO

Absorption controls



Other ways of confirming specificity

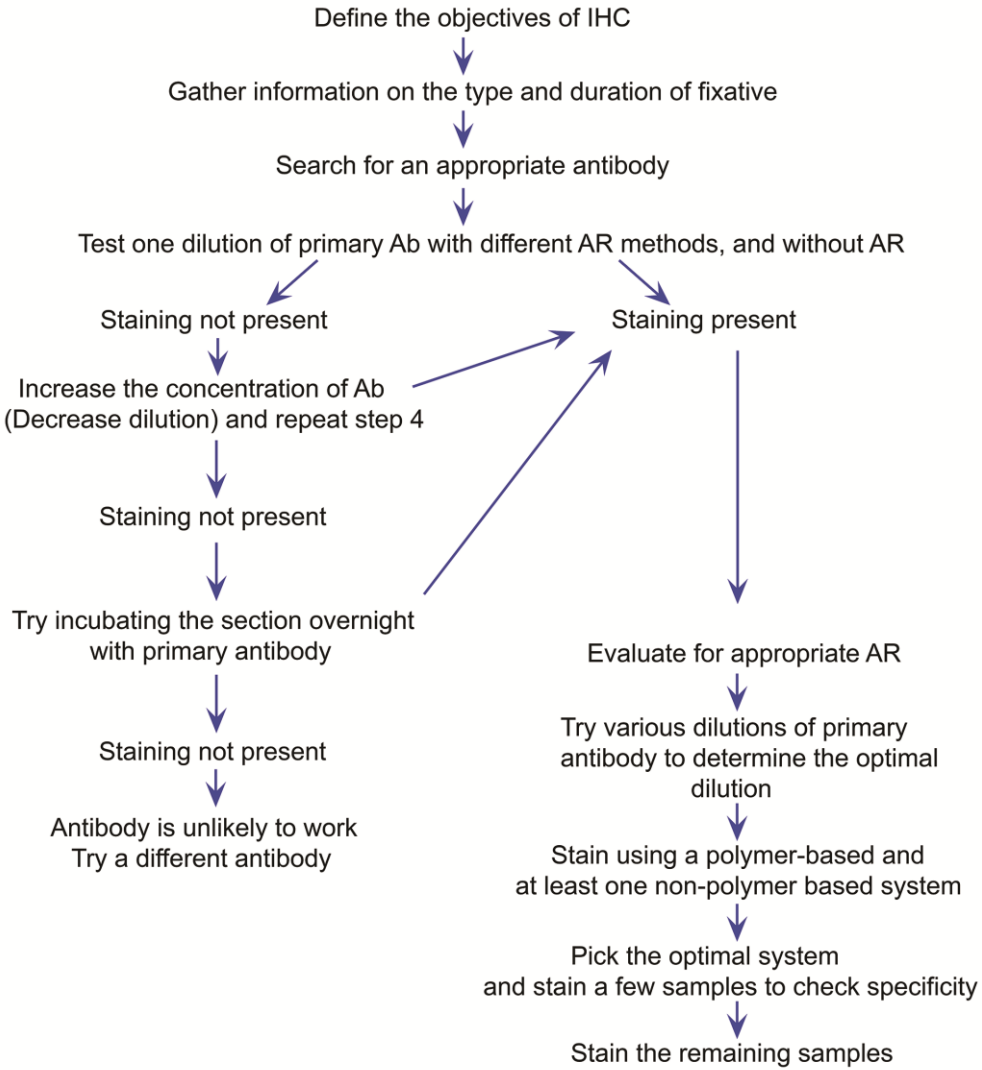




Controls

- Secondary antibody controls
  - Omission of primary antibody
- Labeling controls
  - Omission of enzyme-labeled antibody

Immunohistochemistry: method optimization



The truth is what you see on the slide and not what you were expecting to see
(When appropriate controls are in place)

Useful resources

- Janardhan, K.S., Jensen, H., Clayton, N.P. and Herbert, R.A. (2018). Immunohistochemistry in Investigative and Toxicologic Pathology. *Toxicol Pathol,* **46,** 488-510.
- Diagnostic Immunohistochemistry- David J. Dabbs.
- Antigen Retrieval Immunohistochemistry Based Research and Diagnostics- Shan-Rong Shi and Clive Taylor.
- Immunohistopathology: Apractical Approach to Diagnosis- Jules M. Elias.
- Diagnostic Histopathology of Tumors- Christopher D.M. Fletcher.
- Antigen Retrieval Immunohistochemistry: Review and Future Prospects in Research and Diagnosis over Two Decades. Shan-Rong Shi, Yan Shi, and Clive R. Taylor. Journal of Histochemistry & Cytochemistry. 2011, 59: 13 –32.
- Immunohistochemical Staining Methods- Dako.
- Technical Aspects of Immunohistochemistry- Ramos-Vara J.A. Veterinary Pathology. 2005, 42:405–426.
- Immunohistochemical Markers for The Rodent Immune System- Ward J.W. et al., Toxicologic Pathology. 2006,34:616-30.
- Rodent Immunohistochemistry: Pitfalls and Troubleshooting- Ward J.W. and Rehg J.E., Veterinary Pathology. 2014,51:88-101.
- Controls for Immunohistochemistry: An Update- Richard W. Burry, Journal of Histochemistry & Cytochemistry. 2011,59:6 –12.