Seminars in Histology

From basic principles to advanced histological techniques

“Immunofluorescence and immunohistochemistry”

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Histology Core Facility
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Common tissue processing steps

1. Sample collection

2. Fixation

Paraffin

Frozen

3. Embedding

4. Sectioning

5. Staining

Dyes, stains, and special probes in histology

Stain type	Reagent	Color	Cell structure
Acid dyes *synthetic*	Aniline blue Eosin Fast green	Blue Pink-red Green	Staining of acidophilic cell structures (anionic dye), f.e. cytoplasma
Basic dyes *synthetic*	Azures Methylene blue Toluidine blue	Blue	Staining ob basophilic cell structures (cationic dye), f.e. nuclei and RER (RNA); demonstration of metachromasia: color shift from orthochromatic to metachromatic color in the presence of polyanionic substances, f.e. granules in mast cells
Natural dyes *basic dyes*	Hematoxylin (must be oxidized and used together with a mordant)	Blue/black	Staining of basophilic cell structures such as nuclei and some cytoplasmic substances (cationic)
Chemical reaction *Feulgen stain*	Schiff reagent (basic fuchsin)	Magenta	Schiff reaction of aldehydes from previously hydrolyzed DNA with basic fuchsin for the specific demonstration of DNA
Chemical reaction *PAS stain*	Schiff reagent (basic fuchsin)	Magenta	Pretreatment with periodic acid for the conversion of 1-2 glycol linkages into aldehyde groups followed by the Schiff reaction for the demonstration of 1-2 glycol moieties
Lipid stains	Sudan Oil red (lipid soluble)	Black Red	Lipid droplets, unsaturated lipids, phospholipids: staining principle due to differences in solubility of the dye within two media, i.e. diffusion from low concentrated alcoholic solution into the specimen
Metal stains *impregnation* *oxidation*	Silver (Bodian, Gomori) Gold Osmium tetroxide	Brown Black Black	Silver impregnation of cell structures, f.e. for the demonstration of the Golgi apparatus, reticular fibres and neurofibrils Silver impregnation followed by gold chloride for stable and enhanced contrast Several distinct application, f.e. demonstration of lipids (unsaturated lipids, phospholipids) which reduce osmium tetroxide to give a black compound; fixation and contrasting of membranes; impregnation according to Golgi followed by $AgNO_3$ solution
Special stains: *acid and basic dyes* (Romanowsky type)	Giemsa Wright	Blue Purple Pink-red	Used for blood and bone marrow smears to demonstrate orthochromatic, polychromatic and metachromatic properties
Special stains: *acid and basic dyes* (defined pH)	Methylene blue and eosin	Blue Pink (light)	Buffered solution of acid and basic dye mixture to demonstrate cytoplasmic basophilia on tisue sections
Special stains: *acid and basic dyes* (polychrome stains)	Masson trichrome Mallory triple Movat pentachrome	Blue Green Blue-black	Selective staining of connective tissue compounds, muscle, fibrin
Special stains: *elastica stains*	Taenzer-Unna Weigert Orcein Resorcin fuchsin Verhoeff method	Brown (dark) Blue-black Purple Black	Elastic tissue, elastic fibrils
Special stains: *mucin stains*	Alcian blue at different pH values Combination with other cytochemical reactions (PAS etc.)	Blue Blue-green Magenta	Mucins (glycoconjugates), acid and neutral mucins in gastrointestial epithelium
Special stains: *neurohistology*	Weigert hematoxylin Methylene blue Cresyl fast violet Classical Nissl or fast Nissl methods Luxol fast blue (Klüver-Barrera)	Violet (nuclei, Nissl bodies) Dark blue (myelin sheaths) Blue-black (myelin sheaths) Deep-blue (nuclei, Nissl bodies) Bright blue (myelin sheaths)	Distinct applications for neuro-histology, staining of basophilic structures by basic dyes, f.e. perikaryon, nuclei, Nissl bodies, glial cells, fibers, myelin sheaths
Special stains: *colloidal susp.*	Trypan blue (vital staining)	Blue	Nontoxic colloidal particles which do not label living cells (vitality marker of cells in vivo and in vitro, cell suspensions, cultured cells); useful marker of phagocytic cells (cleared by phagocytic system)
Histochemistry *enzymes*	Specific enzyme substrates for endogenous enzymes	Chromogen dependent	Selective cell structure (site of endogenous enzyme)
Histochemistry *enzymes*	Specific probe and defined labels (immunohistology)	Chromogen dependent	Cell structure defined by the applied molecular probe (antigens, antibodies)
Fluorochromes: *vital staining or staining of sections*	Xanthenes Acridines Tetracycline (cationic, anionic and electroneutral fluorochromes)	Different colors (fluorescence)	Fluorescence: in vivo labeling of cells and tissue structures Fluorescence: tissue sections stained with fluorochromes

http://www.immunologie-labor.com/cellmarker_files/IET_introduct_05.pdf

Commercial reagents for in situ analysis

Invitrogen “The Molecular Probes Handbook”

Apoptosis Reagents
Autophagy Reagents
Calcium and Magnesium Indicators
Cell Cycle Reagents
Cell Proliferation Reagents
Cytoskeleton Reagents
Cytosol Reagents
Endocytosis, Phagocytosis and Internalization Reagents
Endosome, Lysosome and Peroxisome Reagents
Enzyme Substrates and Assays
Expression Vectors
Fluorescent Proteins
Golgi and ER Reagents
Labeling Reagents
Membrane and Lipid Reagents
Membrane Potential Indicators
Mitochondrial Reagents
Nucleic Acid Quantitation in Gels
Nucleic Acid Quantitation in Solution
Nucleus Reagents
Oxidative and Nitrative Stress Indicators
pH Indicators
Protein Detection and Quantitation
Receptor Probes
Reference Standards
Sodium, Potassium and Chloride Indicators
Tracking and Tracing Reagents
Viability, Vitality and Dead Cell Reagents

Introduction

The principle of IHC (IF) has existed since the 1930s, but it was not until 1941 that the first IHC study was reported.

Coons and his coworkers used Fluorescein isothiocyanate (FITC)-labeled antibodies to localize Pneumococcal antigens in infected tissues.

The immunohistochemistry is a methodology that uses antibodies to test for certain antigens (markers) in a sample of tissue.
It makes use of enzyme labeled (II) antibodies in combination with chromogenic substrates.

- Does not need ”special” microscopes for visualization
- Permanent staining
- More powerful signal amplification

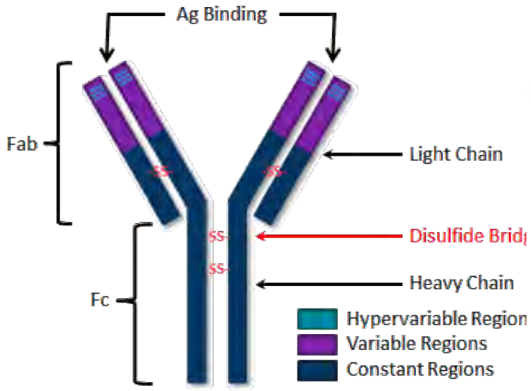
The immunofluorescence differs from the immunohistochemistry for the use of fluorophore-labeled (II) antibodies.

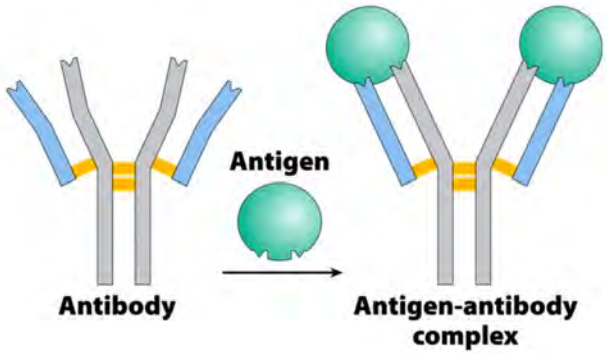
- Needs a fluorescence microscope
- Subject to decay
- More precise (Co-)localization

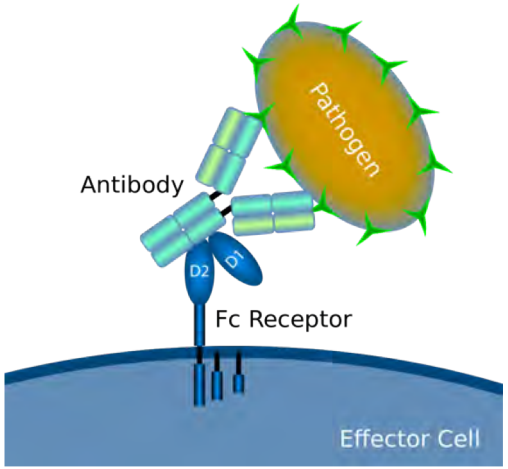
Coons AH, Creech HJ, Jones RN. Immunological properties of an antibody containing a fluorescent group. Proc Soc Exp Biol. 1941;47:200–2.
Coons AH, Kalpan MH. Localization antigens in tissue cells. Improvements in a method for the detection of antigen by means of fluorescent antibody. J Exp Med. 1950;91:1–13.

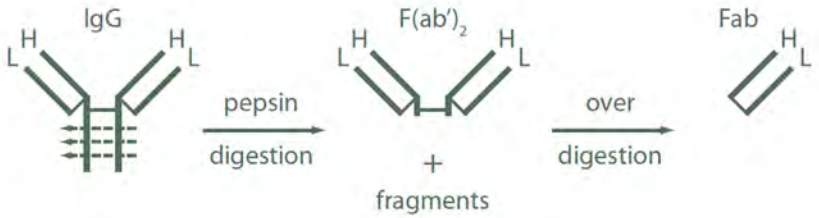
IgG

Immunoglobulin G (IgG) is a type of antibody. Representing approximately 75% of serum antibodies in humans, IgG is the most common type of antibody found in blood circulation. IgG molecules are created and released by plasma B cells. Each IgG has two antigen binding sites.

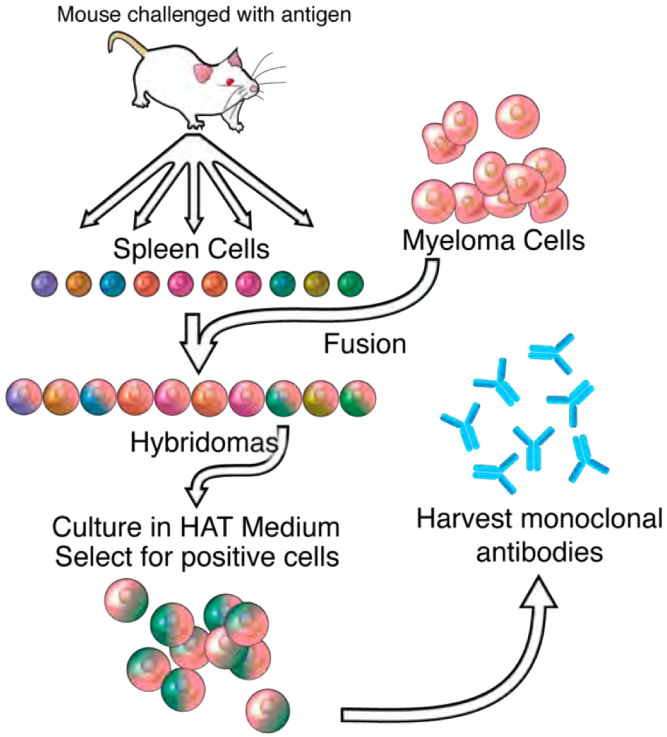


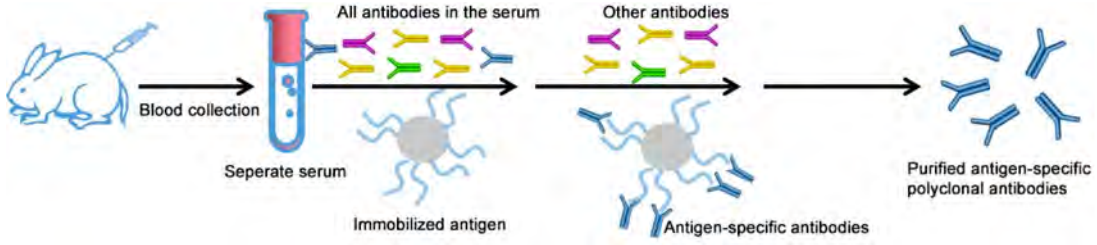


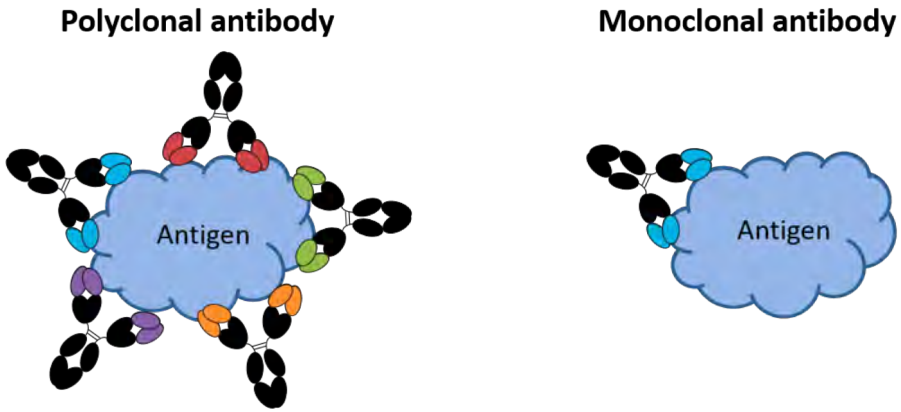




Polyclonal and monoclonal antibodies







Polyclonal and monoclonal antibodies

	Polyclonal Antibody	Monoclonal Antibody
What is it?	Antibodies generated from multiple B cell clones	Antibodies generated from a single B cell clone
What does it recognize?	Various epitopes of the same antigen	A single epitope
Advantages	•More resistant to changes in antigen conformation due to fixation or processing. •Recognition of multiple epitopes can enhance signal.	•Lower lot-to-lot variability •Less likely to cross react with other proteins. •Lower background
Disadvantages	•Higher background •Higher lot-to-lot variability	•Less tolerant to changes in antigen conformation due to fixation or processing.

Immunodetection strategies

Direct

Advantage	Limitation
Good for multicolor labeling	Lower sensitivity

Indirect

Advantage	Limitation
Higher sensitivity	Potential non-specific binding of secondary antibodies

KEY: Antigen, Label, Biotin, Secondary Antibody, Primary Antibody, Avidin/Streptavidin

Amplification - ABC Method

Advantage	Limitation
Increased signal intensity	Requires additional time & controls

Enzymes commonly used for detection:
Horseradish peroxidase (HRP)
Alkaline phosphatase (AP)

Common tissue processing steps

Frozen sample processing steps

1. Fixation
2. Washing
3. Protein blocking
4. I antibody incubation
5. Washing
6. II antibody incubation
7. Washing
8. Detection (IHC)
9. Washing
10. Counterstaining
11. Mounting

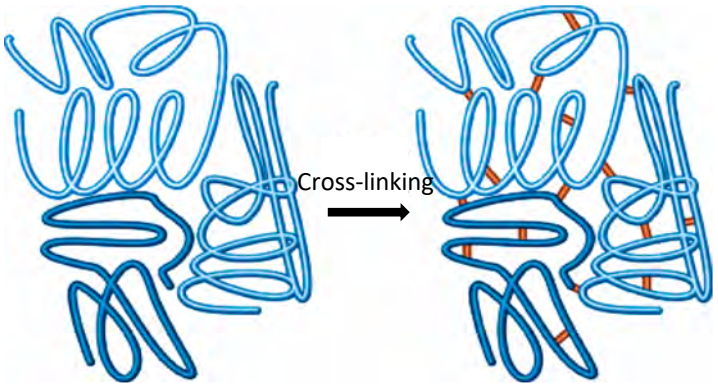
Common tissue processing steps

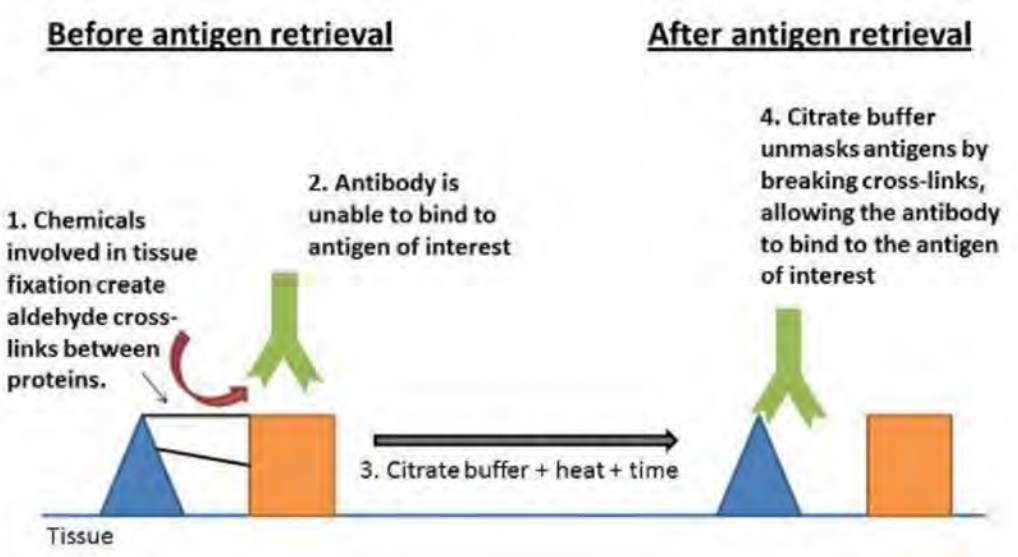
FFPE processing steps

1. Deparaffinization
2. Rehydration
3. Washing
4. Antigen retrieval
5. Washing
6. Endogenous enzyme activity blocking
7. Washing
8. Protein blocking
9. I antibody incubation
10. Washing
11. II antibody incubation
12. Washing
13. Detection
14. Washing
15. Counterstaining
16. Mounting

Fixation

In performing their protective role, fixatives denature proteins by coagulation, by forming additive compounds, or by a combination of coagulation and additive processes.





Antigen retrieval protocols

Protease-induced Epitope Retrieval (PIER)

Proteinase K

Trypsin

Pepsin

Pronase

Heat-induced Epitope Retrieval (HIER)

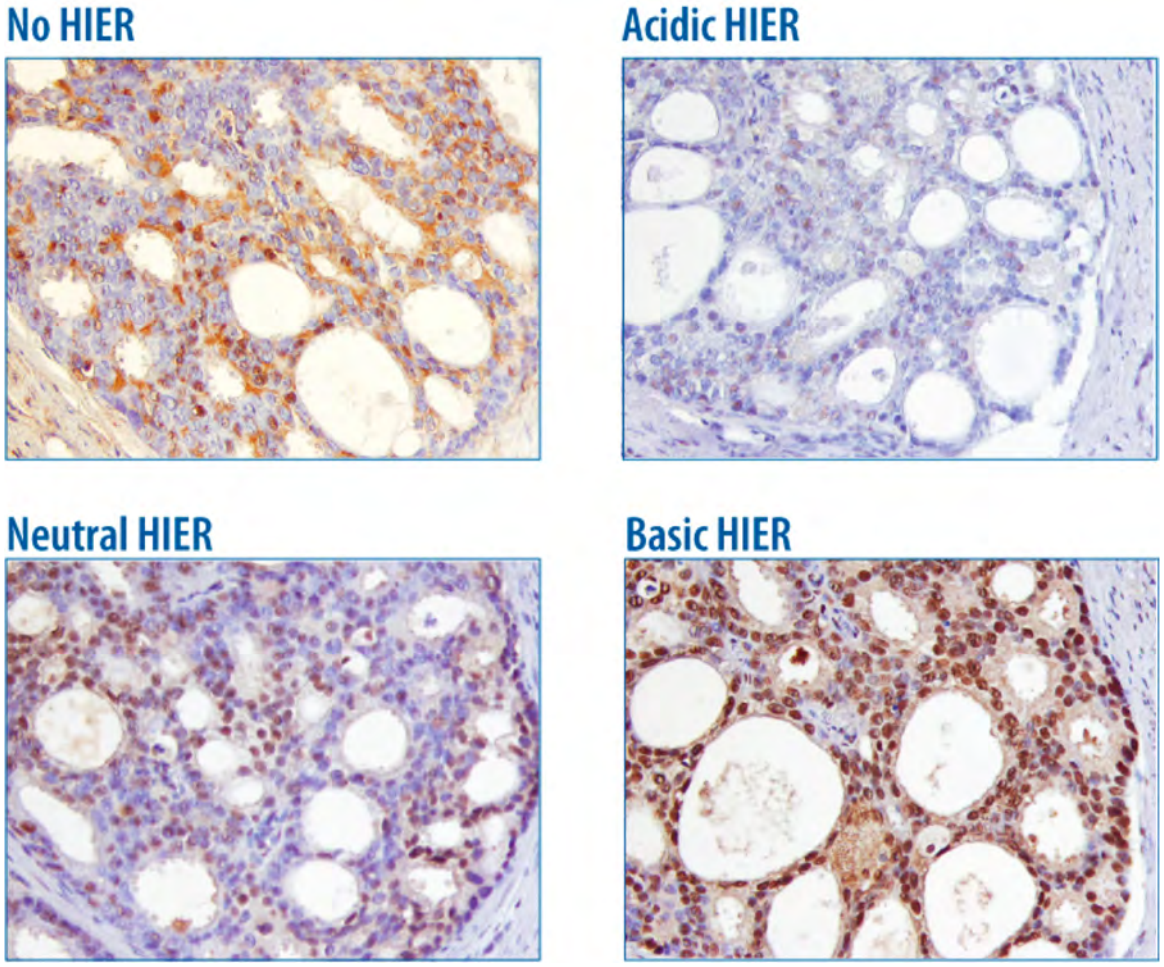
Sodium Citrate Buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0)

EDTA Buffer (1mM EDTA, 0.05% Tween 20, pH 8.0)

http://www.ihcworld.com/epitope_retrieval.htm

Time	Antigen Retrieval Solution pH		
	Acidic	Neutral	Basic
1 minute	Slide #1	Slide #2	Slide #3
5 minutes	Slide #4	Slide #5	Slide #6
10 minutes	Slide #7	Slide #8	Slide #9

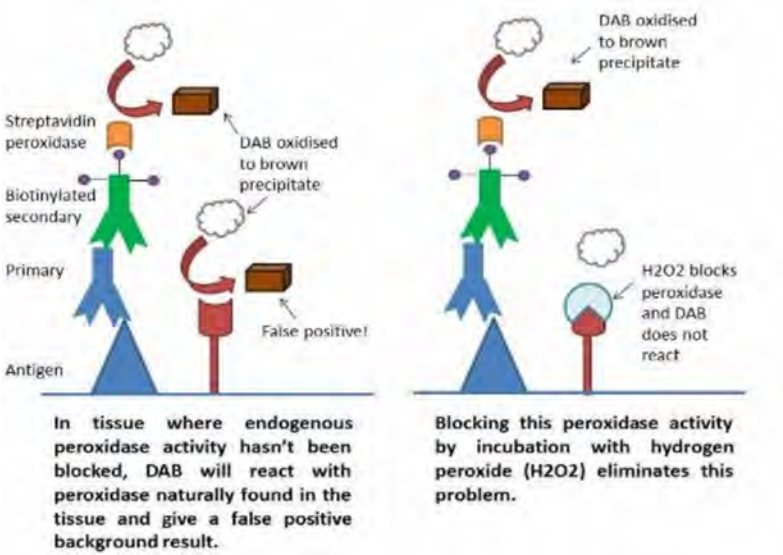
Antigen retrieval protocols



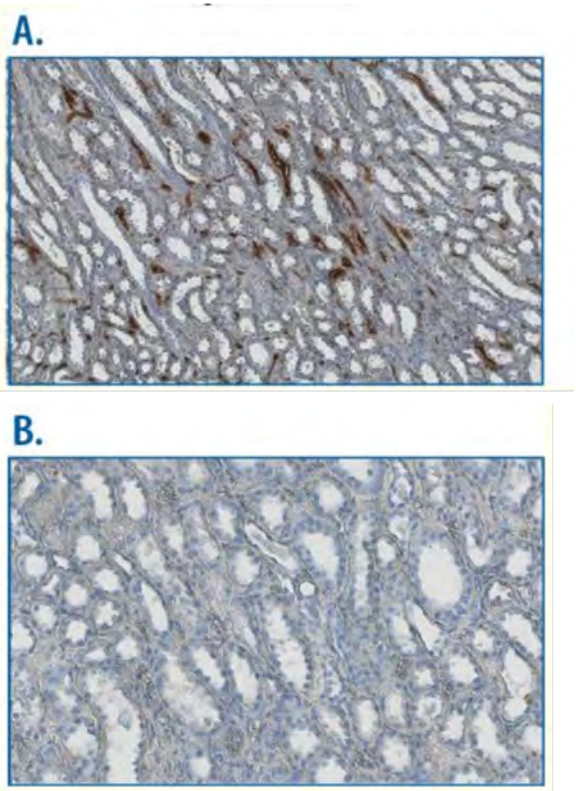
IHC images show the detection of p27 in paraffin-embedded human prostate cancer sections following incubation of tissue for 10 minutes at 95 °C in the specified antigen retrieval solution.

Blocking endogenous peroxidases and phosphatases

It is important to block endogenous peroxidases and phosphatases prior to using alkaline phosphatase (AP) / horseradish peroxidase (HRP) antibody conjugates.



www.sheffield.ac.uk/polopoly_fs/1.458351!/file/IHC_bitesize.pdf



Tissues such as kidney, liver and those containing red blood cells (such as vascular tissue) contain endogenous peroxidases.

Kidney, intestine, osteoblasts, lymphoid tissue and placenta contain AP. AP activity is higher in frozen tissue.

HRP blocking: 0.3%-3% hydrogen peroxide in methanol, PBS, distilled water or saline.

Levisamole is used for blocking AP. Intestinal AP is blocked with a weak acid (eg 1% acetic acid) before adding the primary antibody.

Protein blocking

Blocking with sera or a protein blocking reagent prevents non-specific binding of antibodies to tissue or to Fc receptors.

Theoretically, any protein that does not bind to the target antigen can be used for blocking.

In practice, some proteins bind more readily to non-specific sites:

- Serum is a common blocking agent as it contains antibodies that bind to non-specific sites. Using a serum matching the species of the secondary antibody is recommended
- Proteins such as BSA (1%-3%) or casein may also be used to block non-specific antibody binding.
- Specialized blocking buffers are also frequently used to block non-specific antibody binding.

Universal Blocking Buffer:

1%-3% BSA (blocking & stabilizer)
0.1% cold fish skin gelatin (blocking)
0.5% Triton X-100 (penetration enhancer)
0.05% sodium azide (preservative)
0.01M PBS, pH 7.2-7.4
Mix well and store at 4 ºC.

Primary antibody incubation

	Monoclonal Antibody	Polyclonal Antibody
Tissue	5-25 µg/mL, overnight at 4 °C	1.7-15 µg/mL, overnight at 4 °C
Cells	5-25 µg/mL, 1 hour at room temperature	1.7-15 µg/mL, 1 hour at room temperature

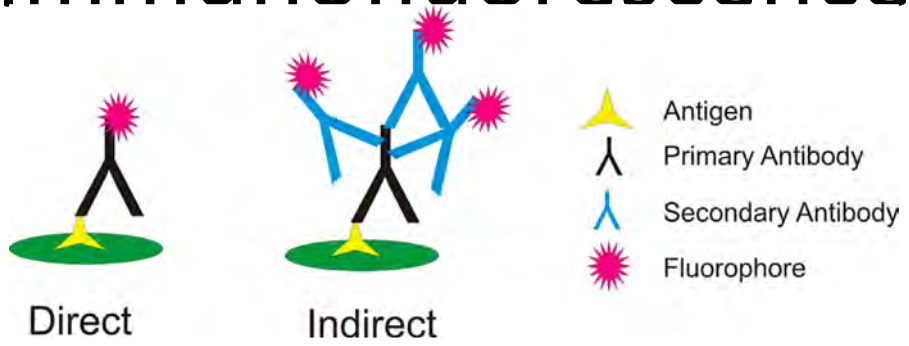
Secondary antibody

Secondary antibody dilution may vary according with the experimental setup and the detection method.
Generally secondary antibodies are incubated 1 hour at room temperature.

- Fluorophore labeled antibody (IF)
- Enzyme labeled antibody (IHC)
- Biotin labeled antibody (IHC, IF)
- Polymeric antibody (IHC, IF)

Secondary antibody must target IgGs from the species in which the primary antibody was produced

Immunofluorescence



Dye	Ex (nm)	Em (nm)	MW	Notes
Hydroxycoumarin	325	386	331	Succinimidyl ester
Aminocoumarin	350	445	330	Succinimidyl ester
Methoxycoumarin	360	410	317	Succinimidyl ester
Cascade Blue	(375);401	423	596	Hydrazide
Pacific Blue	403	455	406	Maleimide
Pacific Orange	403	551		
Lucifer yellow	425	528		
NBD	466	539	294	NBD-X
R-Phycoerythrin (PE)	480;565	578	240 k	
PE-Cy5 conjugates	480;565;650	670		aka Cychrome, R670, Tri-Color, Quantum Red
PE-Cy7 conjugates	480;565;743	767		
Red 613	480;565	613		PE-Texas Red
PerCP	490	675	35kDa	Peridinin chlorophyll protein
TruRed	490,675	695		PerCP-Cy5.5 conjugate
FluorX	494	520	587	(GE Healthcare)
Fluorescein	495	519	389	FITC; pH sensitive
BODIPY-FL	503	512		
G-Dye100	498	524		suitable for protein labeling and electrophoresis
G-Dye200	554	575		suitable for protein labeling and electrophoresis
G-Dye300	648	663		suitable for protein labeling and electrophoresis
G-Dye400	736	760		suitable for protein labeling and electrophoresis
Cy2	489	506	714	QY 0.12
Cy3	(512);550	570;(615)	767	QY 0.15
Cy3B	558	572;(620)	658	QY 0.67
Cy3.5	581	594;(640)	1102	QY 0.15
Cy5	(625);650	670	792	QY 0.28
Cy5.5	675	694	1272	QY 0.23
Cy7	743	767	818	QY 0.28
TRITC	547	572	444	TRITC
X-Rhodamine	570	576	548	XRITC
Lissamine Rhodamine B	570	590		
Texas Red	589	615	625	Sulfonyl chloride
Allophycocyanin (APC)	650	660	104 k	
APC-Cy7 conjugates	650;755	767		Far Red

Atto Dyes Overview

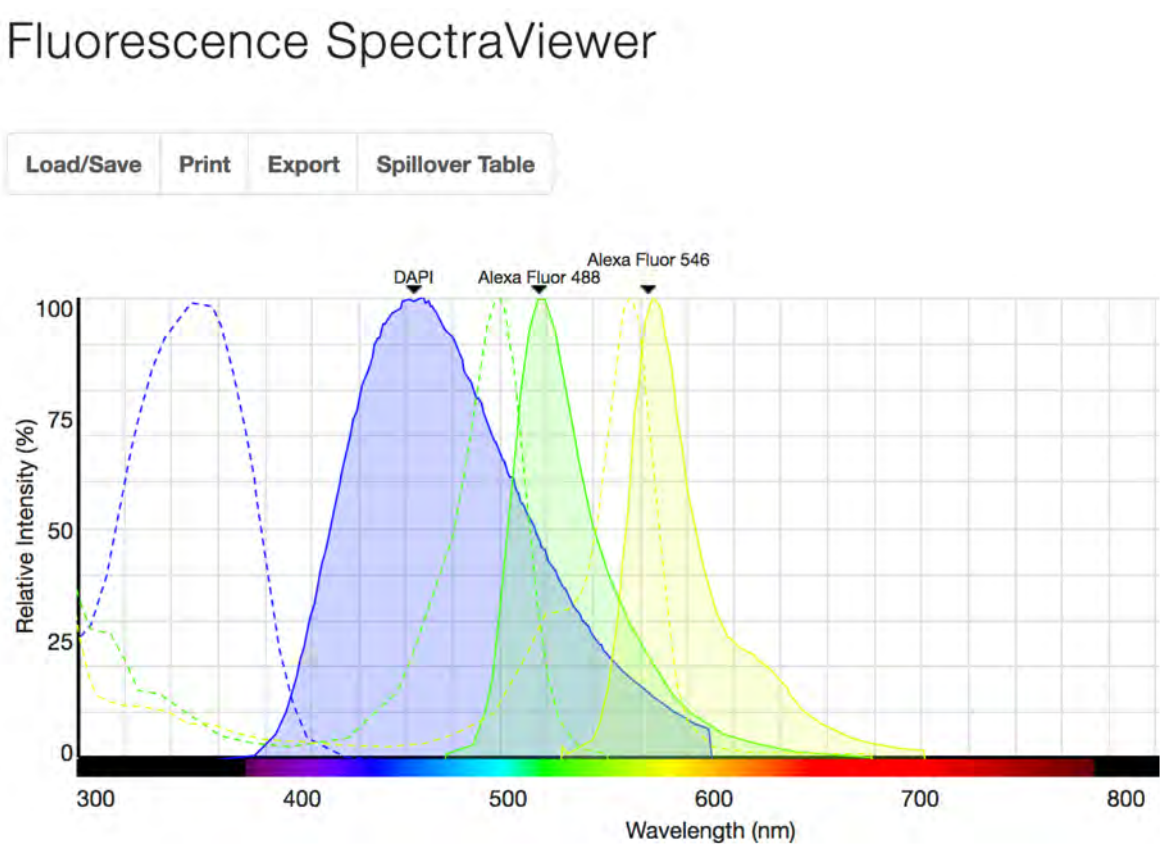
Atto Dye (and Quencher)	λabs [nm]	ε max [m-1 cm-1]	λem [nm]	ηem [%]	τem [ns]
Atto 390	390	24'000	479	90	3.8
Atto 425	436	45'000	484	90	3.5
Atto 430LS	433	32'000	547	65	4.0
Atto 465	453	75'000	508	55	2.2
Atto 488	501	90'000	523	80	3.2
Atto 490LS	496	40'000	661	30	2.6
Atto 495	495	80'000	527	45	2.4
Atto 514	511	115'000	533	85	3.0
Atto 520	516	110'000	538	90	3.8
Atto 532	532	115'000	553	90	3.8
Atto Rho6G	535	115'000	560	90	4.1
Atto 540Q	542	105'000			
Atto 550	554	120'000	576	80	3.2
Atto 565	563	120'000	592	90	3.4
Atto Rho3B	565	120'000	592	50	1.5
Atto Rho11	571	120'000	595	80	4.0
Atto Rho12	576	120'000	601	80	4.0
Atto Thio12	579	110'000	609	15	2.0
Atto Rho101	586	120'000	610	80	4.2
Atto 580Q	586	110'000			
Atto 590	594	120'000	624	80	3.7
Atto 594	601	120'000	627	85	3.5
Atto Rho13	600	120'000	625	80	3.9
Atto 610	615	150'000	634	70	3.3
Atto 612Q	615	115'000			
Atto 620	619	120'000	643	50	2.9
Atto Rho14	625	140'000	646	80	3.7
Atto 633	629	130'000	657	64	3.2
Atto 647	645	120'000	669	20	2.3
Atto 647N	644	150'000	669	65	3.4
Atto 655	663	125'000	684	30	1.9
Atto Oxa12	663	125'000	684	30	1.8
Atto 665	663	160'000	684	60	2.9
Atto 680	680	125'000	700	30	1.8
Atto 700	700	120'000	719	25	1.5
Atto 725	729	120'000	752	10	0.5
Atto 740	740	120'000	764	10	0.6
Atto MB2	658	100'000			

	Colour†	Absorb (nm)	Emit (nm)	MM (g/mol)	ε ($cm^{-1}M^{-1}$)	Quantum Yield
Alexa Fluor 350	blue	346	442	410	19,000	-
— 405	violet	401	421	1028	35,000	-
— 430	green	434	541	702	15,000	-
— 488	cyan-green	495	519	643	73,000	0.92
— 500	green	502	525	700	71,000	-
— 514	green	517	542	714	80,000	-
— 532	green	532	554	721	81,000	0.61
— 546	yellow	556	573	1079	112,000	0.79
— 555	yellow-green	555	565	~1250	155,000	0.1
— 568	orange	578	603	792	88,000	0.69
— 594	orange-red	590	617	820	92,000	0.66
— 610	red	612	628	1172	144,000	-
— 633	Far-red	632	647	~1200[7]	159,000	-
— 635	Far-red	633	647	-	140,000	-
— 647	Far-red	650	665	1155.06[8]	270,000	0.33
— 660	Near-IR	663	690	~1100	132,000	0.37
— 680	Near-IR	679	702	~1150	183,000	0.36
— 700	Near-IR	702	723	~1400	205,000	0.25
— 750	Near-IR	749	775	~1300	290,000	0.12
— 790	Near-IR	782	805	~1750	260,000	-

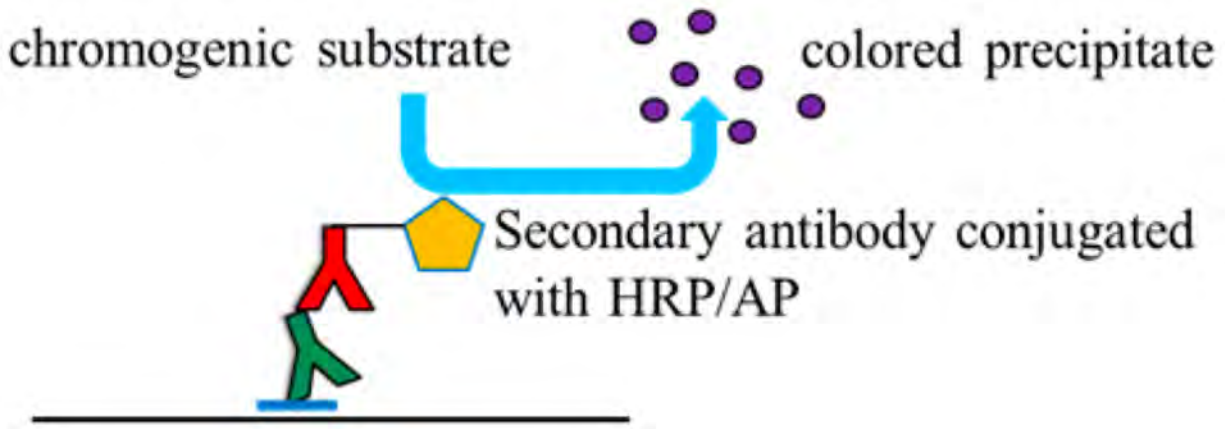
† = approximate color of the emission spectrum
ε = extinction coefficient

Multiple color IF

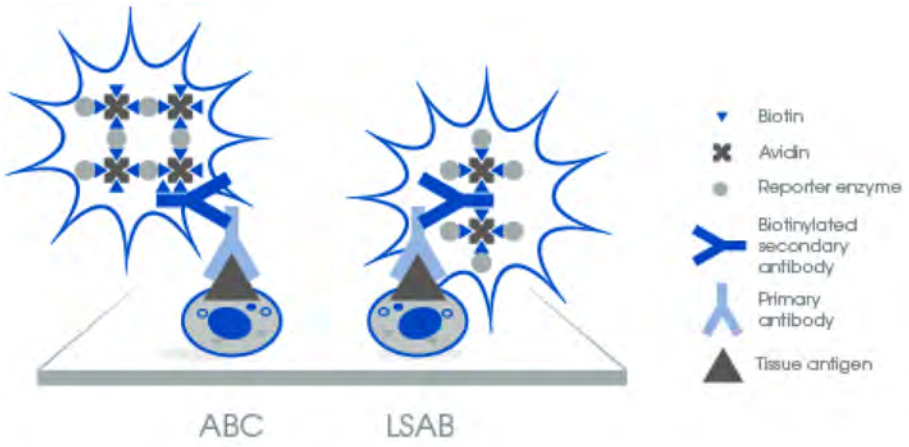
https://www.thermofisher.com/ch/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html



Enzyme labeled antibody



Biotinilated secondary antibody



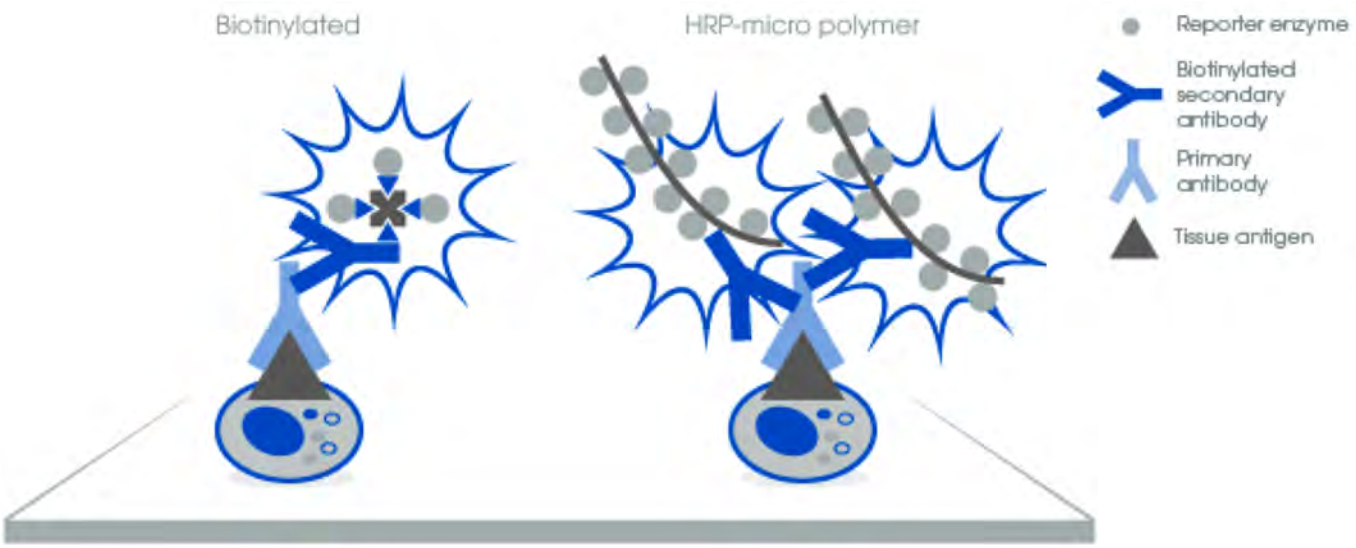
Avidin is a glycoprotein in egg white that combines stoichiometrically with Biotin

Streptavidin is purified from the bacterium *Streptomyces avidinii*, is not glycosylated, and exhibits lower non-specific binding than Avidin.

	Direct	Indirect	ABC	LSAB
First	Conjugated primary antibody	Unlabeled primary antibody	Unlabeled primary antibody	Unlabeled primary antibody
Second		Conjugated secondary antibody	Biotinylated secondary antibody	Biotinylated secondary antibody
Third			Avidin-biotin complex	Streptavidin complex

	ABC	LSAB	Comments
Specificity	Lower	Higher	Avidin may show non-specific binding due to its carbohydrate moieties and its high isoelectric point (pI). In contrast, streptavidin lacks carbohydrate moieties and has a more neutral pI.
Sensitivity	High	High	Both methods show greater sensitivity than direct or indirect detection.
Tissue penetration	Lower	Higher	The complex size in LSAB methods is smaller facilitating a greater tissue penetration.
Sample processing	More complex	Simpler	Both methods require three incubations steps, but ABC methods require an additional incubation of avidin with the reporter enzyme.

Polymeric II antibodies



Polymeric antibodies are available conjugated to:

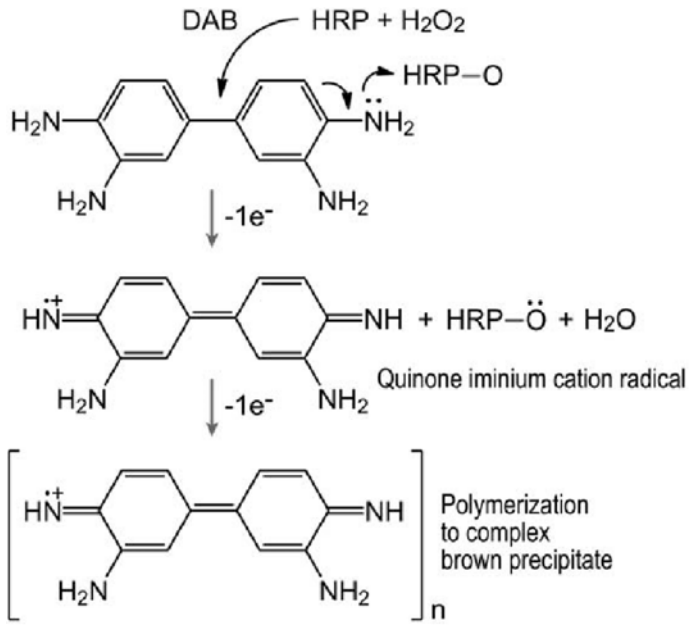
HRP

AP

Fluorophore

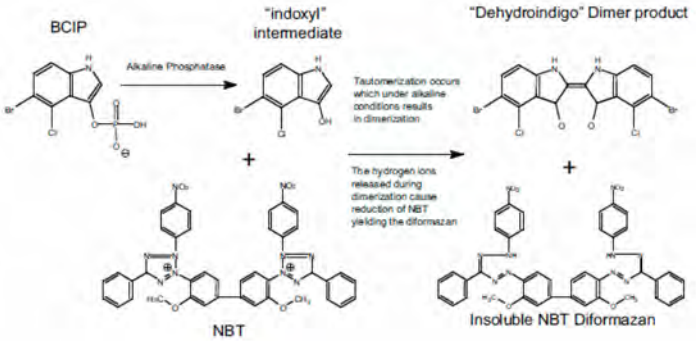
Detection

HRP-DAB

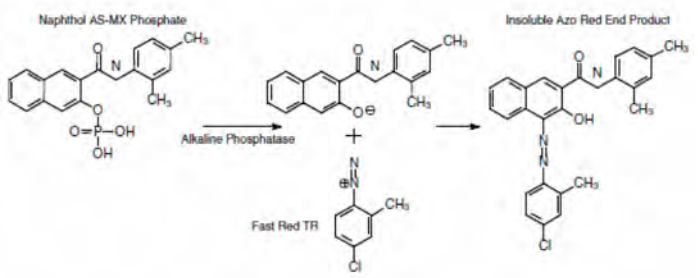


AP-substrate

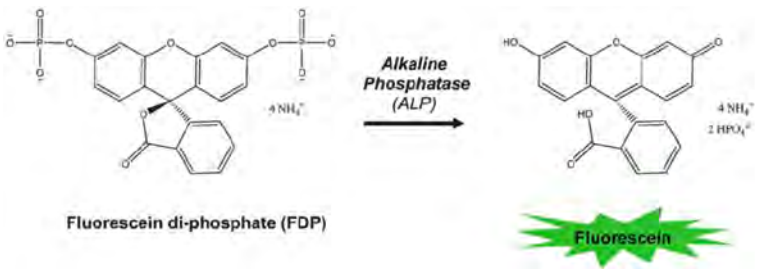
NBT-BCIP



Azo Red

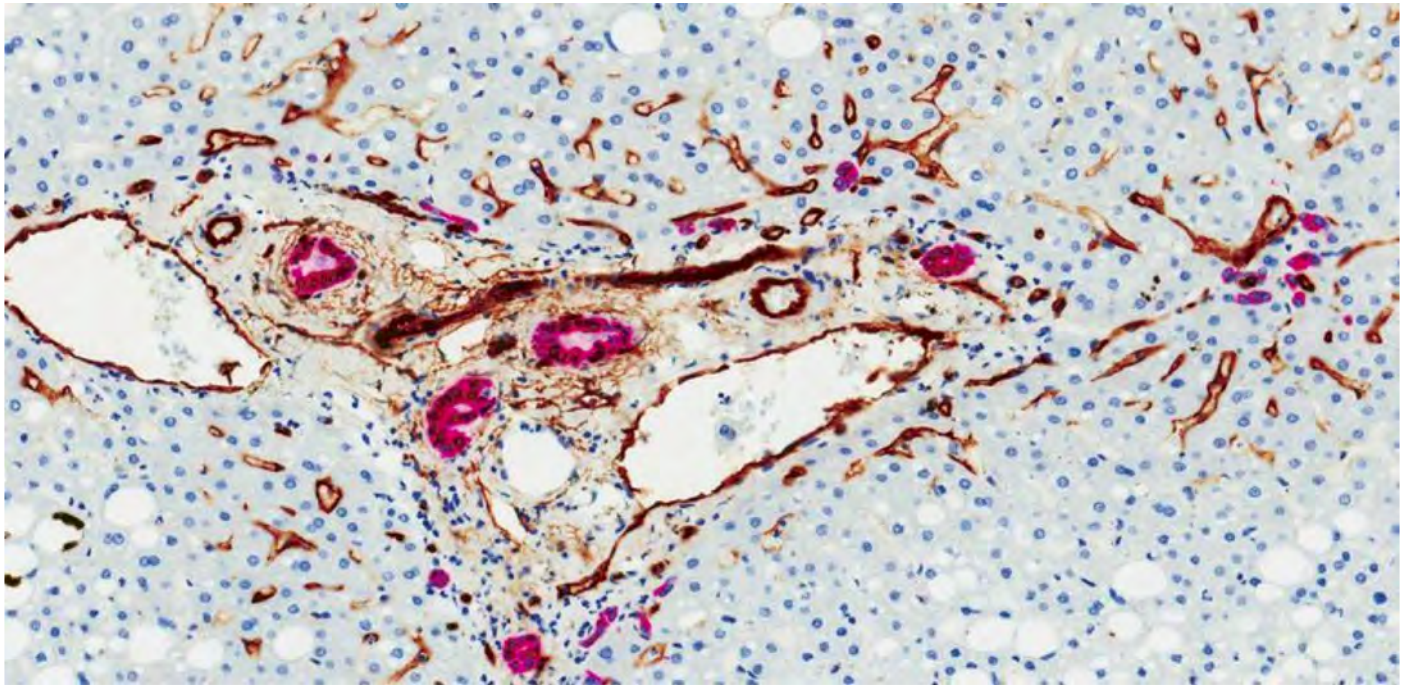


FDP



Warning: most of the AP substrates produce precipitates soluble in alcohol.
Slides should be them mounted with a water based mounting.

Multiplex IHC

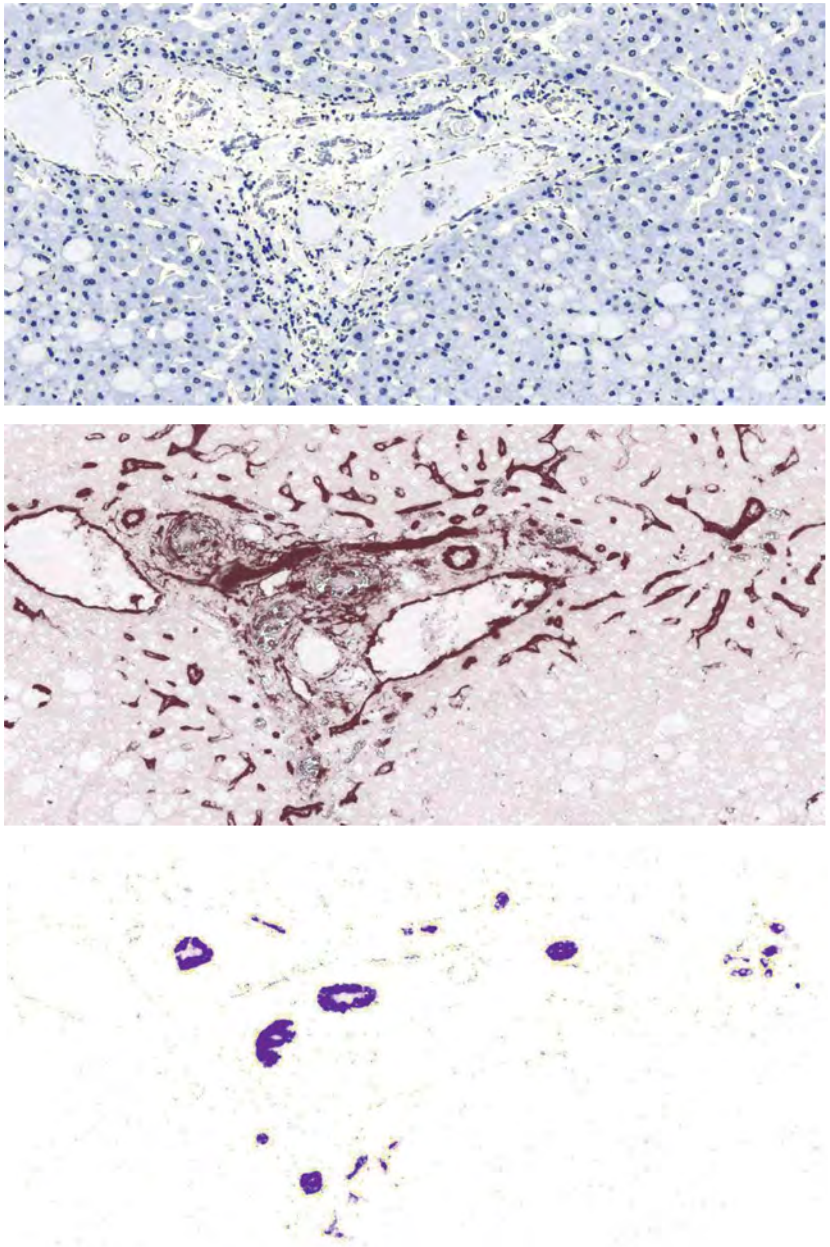


Dual Chromogen Staining of normal human liver. This figure shows dual chromogen staining for CD34 and CK19, for a normal human liver.

DAB CD34

Alkaline phosphatase CK19

Color deconvolution for image analysis



IF counterstaining

Dye	Ex (nm)	Em (nm)	MW	Notes
Hoechst 33342	343	483	616	AT-selective
DAPI	345	455		AT-selective
Hoechst 33258	345	478	624	AT-selective
SYTOX Blue	431	480	~400	DNA
Chromomycin A3	445	575		CG-selective
Mithramycin	445	575		
YOYO-1	491	509	1271	
Ethidium Bromide	210;285	605	394	in aqueous solution
Acridine Orange	503	530/640		DNA/RNA
SYTOX Green	504	523	~600	DNA
TOTO-1, TO-PRO-1	509	533		Vital stain, TOTO: Cyanine Dimer
TO-PRO: Cyanine Monomer				
Thiazole Orange	510	530		
CyTRAK Orange	520	615	-	(Biostatus) (red excitation dark)
Propidium Iodide (PI)	536	617	668.4	
LDS 751	543;590	712;607	472	DNA (543ex/712em), RNA (590ex/607em)
7-AAD	546	647		7-aminoactinomycin D, CG-selective
SYTOX Orange	547	570	~500	DNA
TOTO-3, TO-PRO-3	642	661		
DRAQ5	600/647	697	413	(Biostatus) (usable excitation down to 488)
DRAQ7	599/644	694	~700	(Biostatus) (usable excitation down to 488)

IHC counterstaining

Common counterstains and their targets

Type	Dye	Target	Color
Chemical stain	Mayer's Hematoxylin	Nuclei	Blue to violet
Chemical stain	Nuclear fast red (Kernechtrot)	Nucleic acids	Red
Chemical stain	Methyl green	Nucleic acids	Green
Chemical stain	Eosin	Cytoplasm	Pink to red

Mounting media

IF

Water based mounting media

Glycerol jelly.
Gelatin powder: 10 g
Water: 60 ml
Dissolve by warming and add:
Glycerol: 70 ml
Add *either* one drop of saturated aqueous solution of phenol ("liquid phenol") *or* 15 mg of sodium merthiolate as an antibacterial agent.

Buffered glycerol with anti-fade.
Buffer:
Either 0.1M Phosphate buffer (pH 7.4): 10 ml
or 0.1M TRIS buffer (pH 9.0): 10 ml
Anti-fading agent:
Either p-Phenylenediamine hydrochloride: 100 mg
or n-propyl gallate: 500 mg
Glycerol: 90 ml
Keeps for at least 3 months, probably much longer, in darkness (which protects the anti-fade agent) at -20C. The working bottle is kept at 4C, for a week or two.

IHC

Permanent mounting media

Pertex or similar
Require dehydration and clearing before their use
Not suitable for some AP substrates

For AP substrates:
Do not dehydrate tissues after staining with alcohol
Use a water based mounting
DIY
or
Sigma Crystal Mount™ Aqueous Mounting Medium

Controls

What controls to include and why?

•**Autofluorescence / endogenous tissue background staining control.** As certain cell and tissue types (especially those rich in elastin, collagen and lipofuscin) display natural fluorescence it is crucial to observe samples microscopically before every staining experiment.

•**Positive tissue control.** Include a tissue type that expresses your protein of interest. If you do not see a staining in the positive control something has gone wrong with the staining protocol.

•**Negative tissue control.** Include a tissue type in which your protein of interest is not expressed. Therefore if you see staining in this type of control you know that the staining is unspecific.

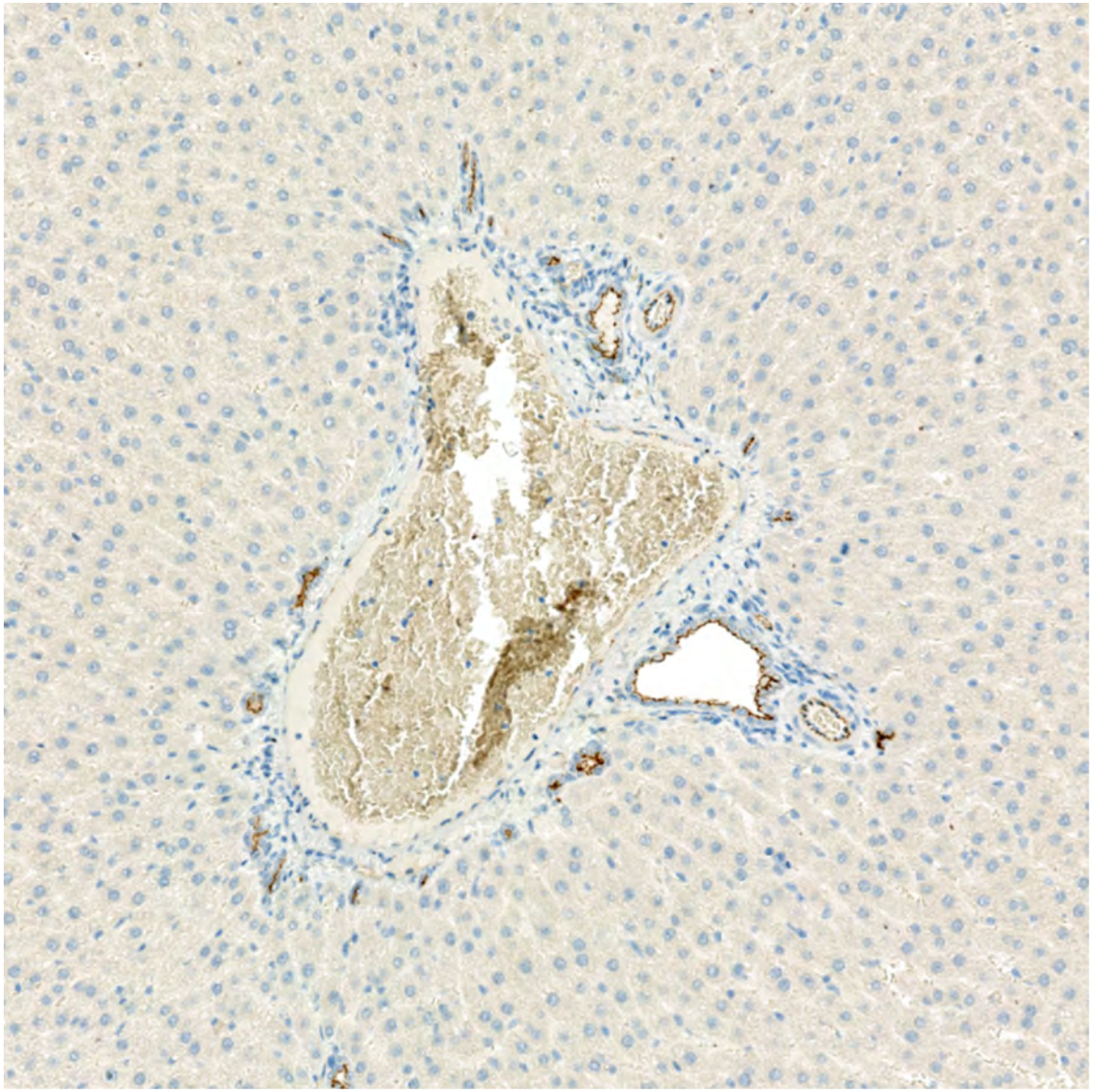
Controls

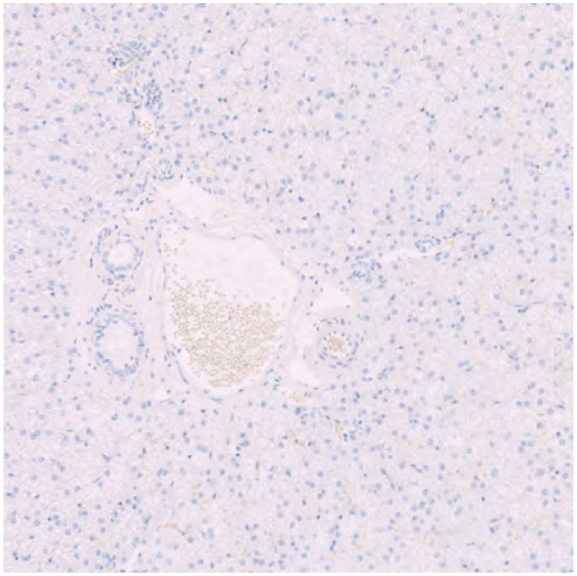
•Perform a **secondary antibody only control** (also called no primary antibody control; follow the same staining protocol without the addition of a primary antibody) in order to ensure that the secondary antibody does not unspecifically bind to certain cellular compartments.

•**Absorption controls** (inhibition of staining via adsorption of the primary antibody with the purified antigen/immunogen prior to use) indicate that the primary antibody used binds exclusively to the antigen it was raised against.

•**Isotype control.** This type of control is used to ensure that the observed staining is due to the antibody binding the desired antigen and not to some general unspecific binding of the immunoglobulin to the tissue.

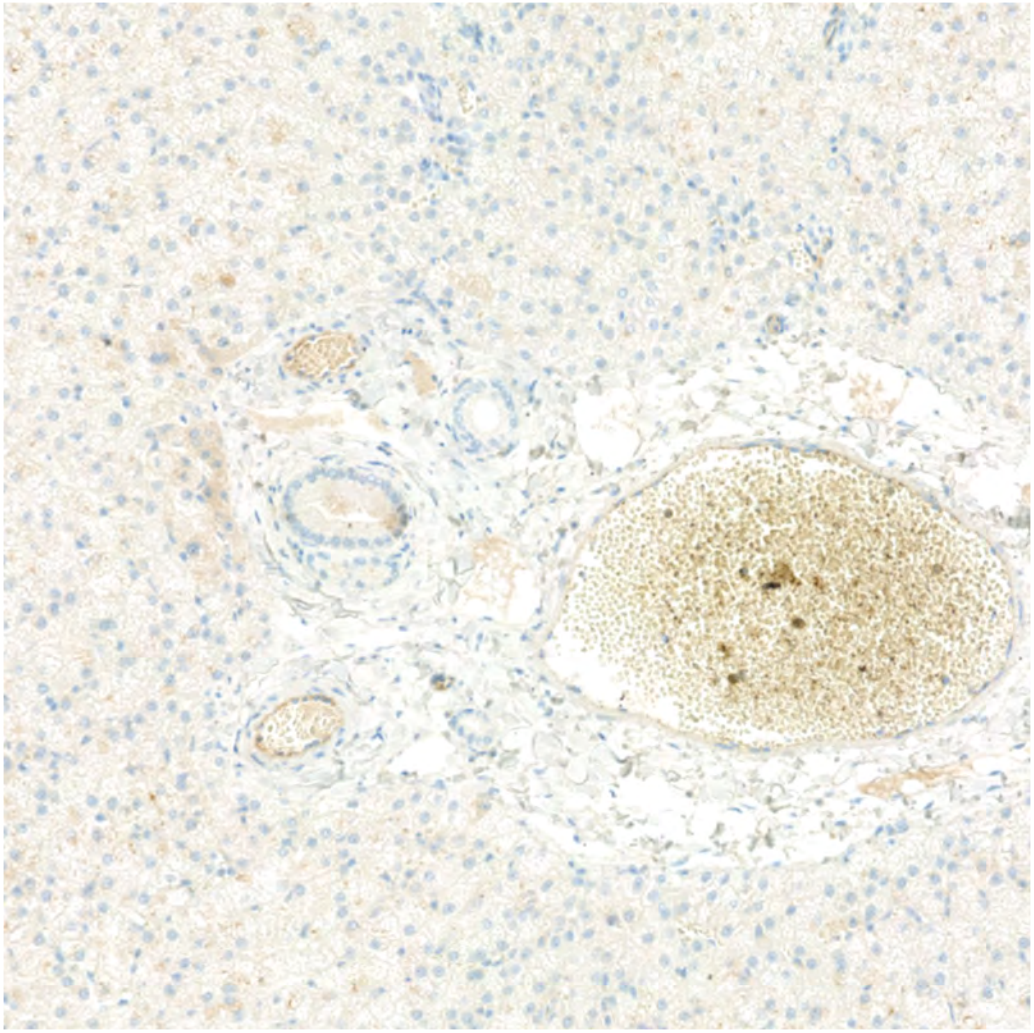
A case study: rat Cldn6



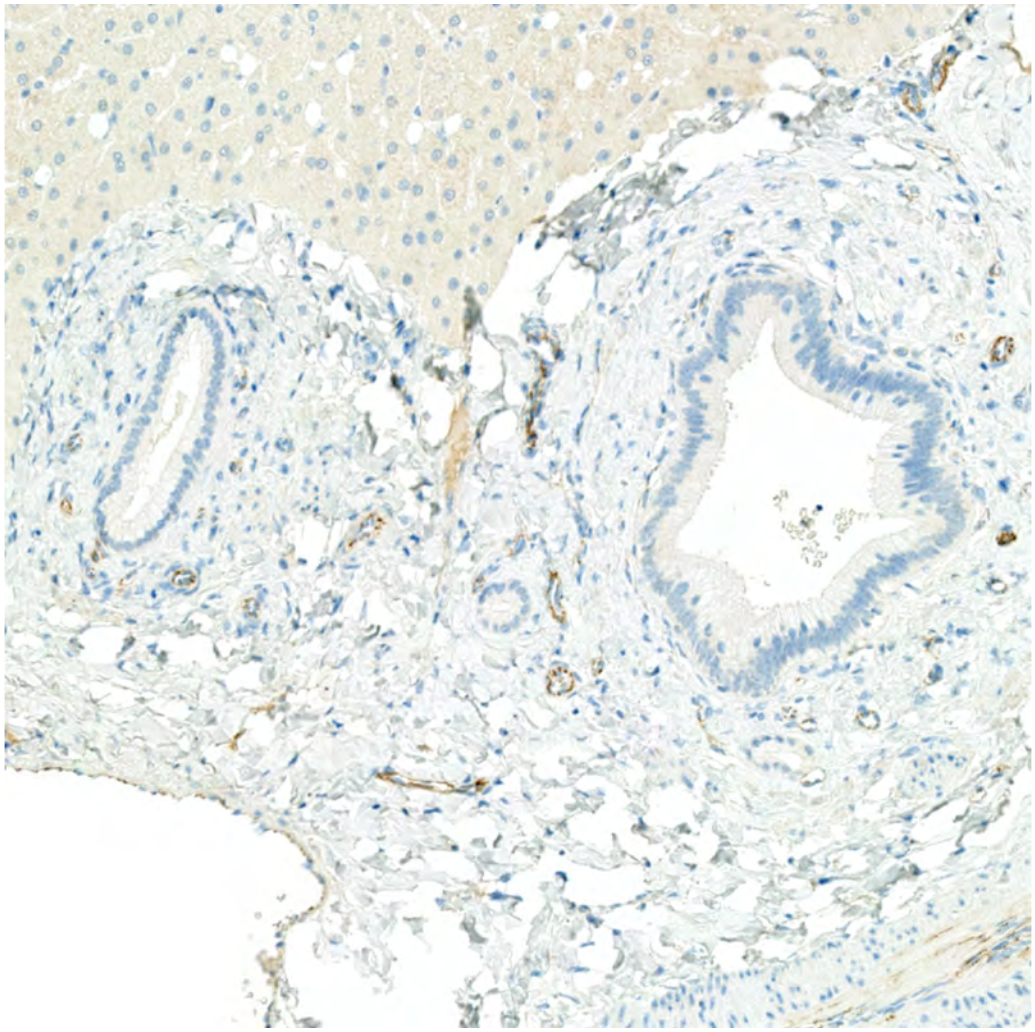


Isotype

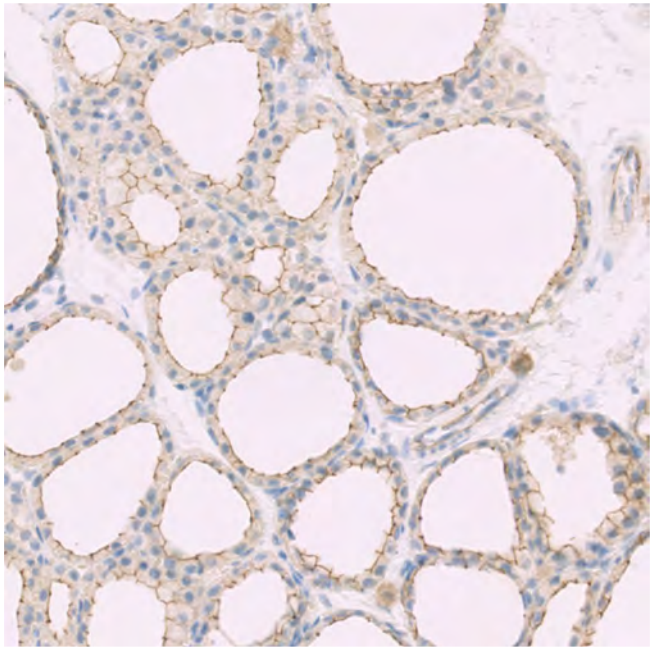
Dog Cldn6



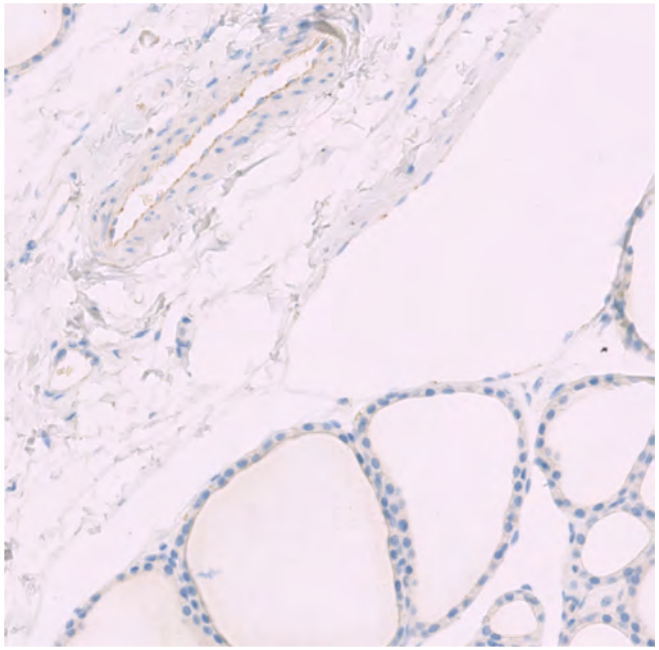
Monkey Cldn6



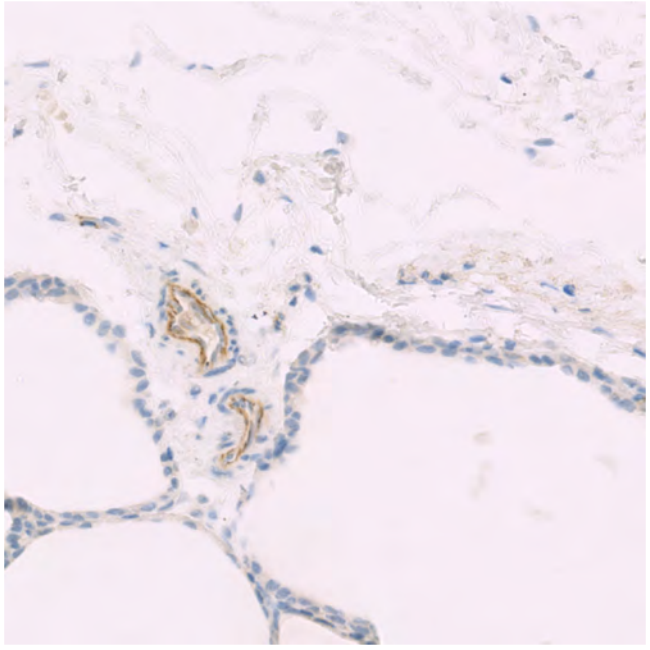
Positive controls



Rat thyroid

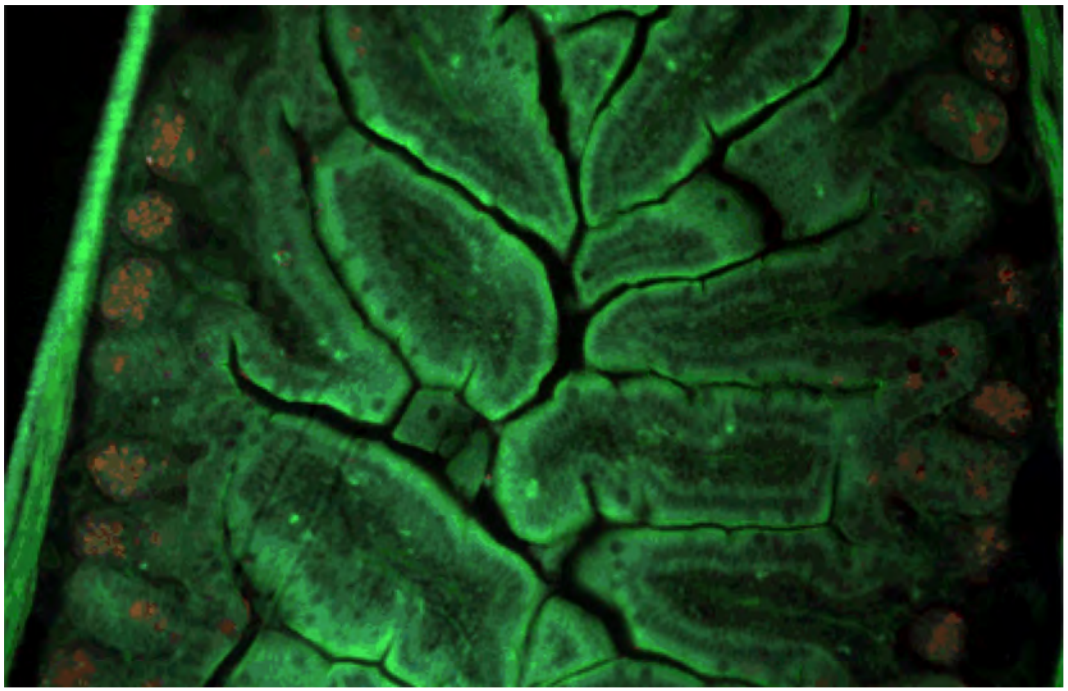


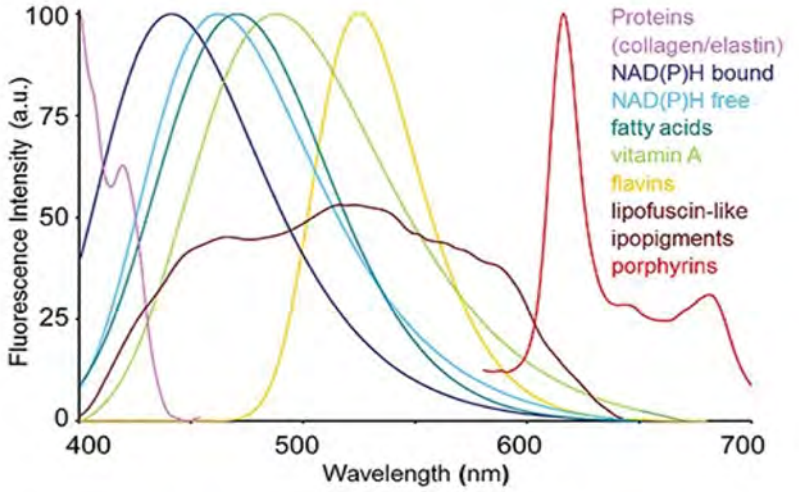
Dog thyroid



Monkey thyroid

Autofluorescence





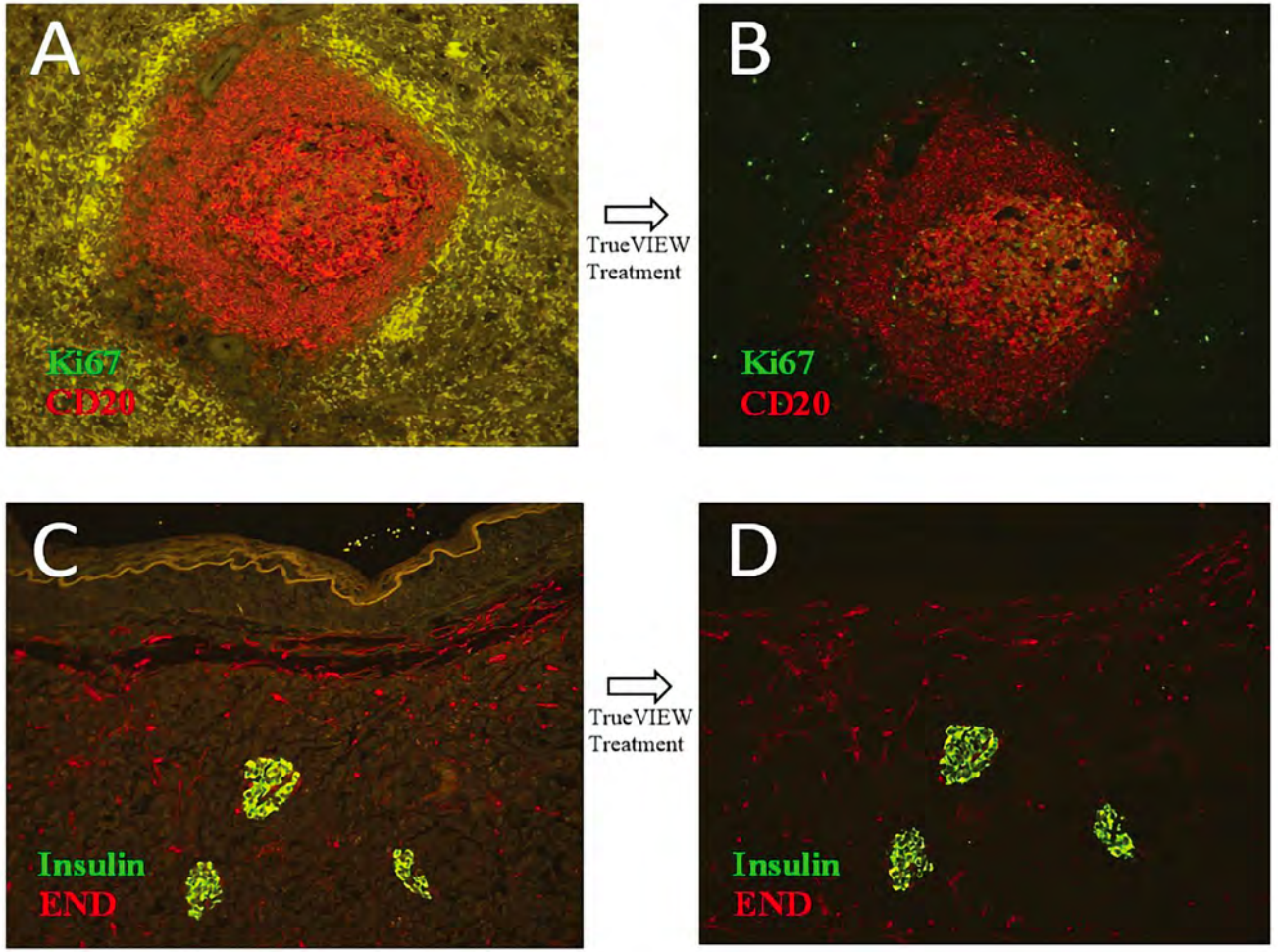
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4289852/

Autofluorescence

Endogenous fluorophores	Biological constituents	Autofluorescence (exc) / (em) ranges	Autofluorescence photophysical fingerprints and possible correlated alterations
Aromatic amino acids: Phe, Tyr, Trp	Functional proteins	(240-280 nm) / (280-350 nm)	Spectral shape and amplitude (near UV, blue region tail)
Cytokeratins	Intracellular fibrous proteins	(280-325 nm) / (495-525 nm)	Spectral shape and emission amplitude
Collagen/Elastin	Extracellular fibrous proteins	(330-340 nm) / (400-410 nm) (350-420 nm) / (420-510 nm)	Excitation light birifrangence effects spectral shape and emission amplitude, depending on maturation degree in eldering and fibrosis
NAD(P)H	Coenzymes of key enzymes in redox reactions	(330-380 nm) / (440, 462 nm, bound, free)	Spectral shape, emission amplitude
Flavins	Coenzymes of key enzymes in redox reactions	(350-370;440-450 nm) / (480/540 nm)	(NAD(P)Hbound/free, NAD(P)Htotal/oxidized $_{\text{flavins}}$ ratios, depending on aerobic/anaerobic energetic metabolism, antioxidant defense, inflammation, carcinogenesis
Fatty acids	Accumulated lipids	(330-350 nm) / (470-480 nm)	Spectral shape, emission amplitude and photosensitivity, depending on altered lipid metabolism
Vitamin A	Retinols and carotenoids	(370-380 nm) / (490-510 nm)	Spectral shape, emission amplitude and photosensitivity, depending on multiple functions including antioxidant and vision roles, and altered retinol metabolism
Protoporpyrin IX and porphyrin derivatives	Protein prostetic group	(405 nm) / (630-700 nm)	Spectral shape, emission amplitude and photosensitivity, depending on heme and iron altered metabolism
Lipofuscins/Lipofuscin like-lipopigments/ceroids	Miscellaneous (proteins, lipids, retinoids)	(UV, 400-500 nm) / (480-700 nm)	Spectral shape, emission amplitude depending on eldering, oxidation degree, cell stemness degree

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4289852/

Autofluorescence reduction



https://www.genengnews.com/gen-articles/reducing-tissue-autofluorescence/6265

Vector TrueVIEW Autofluorescence Quenching

Signal amplification and tertiary antibody

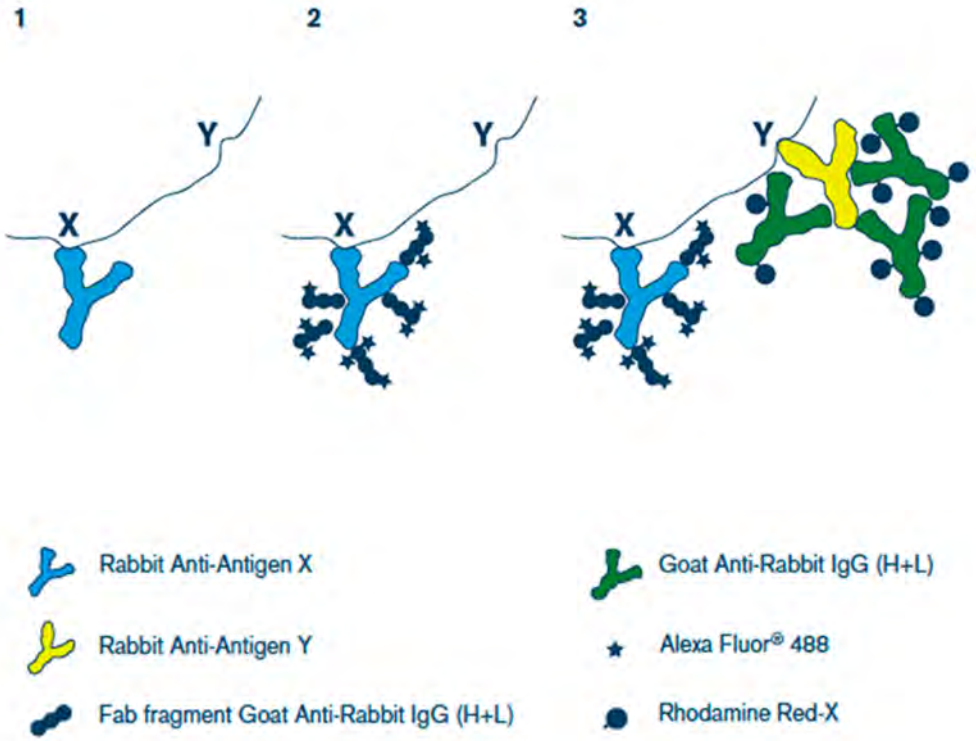
Antibody Description	Unconjugated	Horseradish Peroxidase	Alkaline Phosphatase	Biotin-SP (long spacer)	DyLight™ 405 A=400, E=421	Coumarin AMCA A=350, E=450	Alexa Fluor® 488 A=493, E=519	Fluorescein (FITC) A=492, E=520	Cyanine Cy™3 A=550, E=570	Rhodamine (TRITC) A=550, E=570	Rhodamine Red™-X (RRX) A=570, E=590	Alexa Fluor® 594 A=591, E=614	Alexa Fluor® 647 A=651, E=667	Alexa Fluor® 680 A=684, E=702	Alexa Fluor® 790 A=792, E=803
IgG Fraction ANTI-FLUORESCEIN															
Mouse Anti-Fluorescein (FITC)	1.0 mg 200-002-037 €169.00	0.5 ml 200-032-037 €215.00	0.5 ml 200-052-037 €216.00	0.5 ml 200-062-037 €206.00	0.5 mg 200-472-037 €264.00	0.5 mg 200-152-037 €208.00	0.5 mg 200-542-037 €271.00		0.5 mg 200-162-037 €247.00	0.5 mg 200-022-037 €189.00	0.5 mg 200-292-037 €189.00	0.5 mg 200-582-037 €271.00	0.5 mg 200-602-037 €271.00	0.3 mg 200-622-037 €246.00	0.3 mg 200-652-037 €246.00
IgG Fraction ANTI-DIGOXIN															
Mouse Anti-Digoxin	1.0 mg 200-002-156 €169.00	0.5 ml 200-032-156 €215.00	0.5 ml 200-052-156 €216.00	0.5 ml 200-062-156 €206.00	0.5 mg 200-472-156 €264.00	0.5 mg 200-152-156 €208.00	0.5 mg 200-542-156 €271.00	0.5 mg 200-092-156 €189.00	0.5 mg 200-162-156 €247.00	0.5 mg 200-022-156 €189.00	0.5 mg 200-292-156 €189.00	0.5 mg 200-582-156 €271.00	0.5 mg 200-602-156 €271.00	0.3 mg 200-622-156 €246.00	0.3 mg 200-652-156 €246.00
IgG Fraction ANTI-BIOTIN															
Mouse Anti-Biotin	1.0 mg 200-002-211 €169.00	0.5 ml 200-032-211 €215.00	0.5 ml 200-052-211 €216.00		0.5 mg 200-472-211 €264.00	0.5 mg 200-152-211 €208.00	0.5 mg 200-542-211 €271.00	0.5 mg 200-092-211 €189.00	0.5 mg 200-162-211 €247.00	0.5 mg 200-022-211 €189.00	0.5 mg 200-292-211 €189.00	0.5 mg 200-582-211 €271.00	0.5 mg 200-602-211 €271.00	0.3 mg 200-622-211 €246.00	0.3 mg 200-652-211 €246.00
AffiniPure ANTI-HORSERADISH PEROXIDASE															
Goat Anti-Horseradish Peroxidase	2.0 mg 123-005-021 €100.00		1.0 ml 123-055-021 €171.00	2.0 ml 123-065-021 €162.00	1.5 mg 123-475-021 €144.00	2.0 mg 123-155-021 €125.00	1.5 mg 123-545-021 €147.00	2.0 mg 123-095-021 €114.00	2.0 mg 123-165-021 €148.00	2.0 mg 123-025-021 €114.00	2.0 mg 123-295-021 €114.00	1.5 mg 123-585-021 €147.00	1.5 mg 123-605-021 €147.00	0.5 mg 123-625-021 €209.00	0.5 mg 123-655-021 €209.00
Rabbit Anti-Horseradish Peroxidase	2.0 mg 323-005-021 €125.00		1.0 ml 323-055-021 €178.00	1.5 ml 323-065-021 €162.00		1.5 mg 323-155-021 €125.00	1.0 mg 323-545-021 €147.00	1.5 mg 323-095-021 €114.00	1.5 mg 323-165-021 €148.00	1.5 mg 323-025-021 €114.00	1.5 mg 323-295-021 €114.00	1.0 mg 323-585-021 €147.00	1.0 mg 323-605-021 €147.00		

Uncommon setup

Case 1: we want to localize two targets, but the two available primary antibody were produced in the same animal (e.g. rabbit).

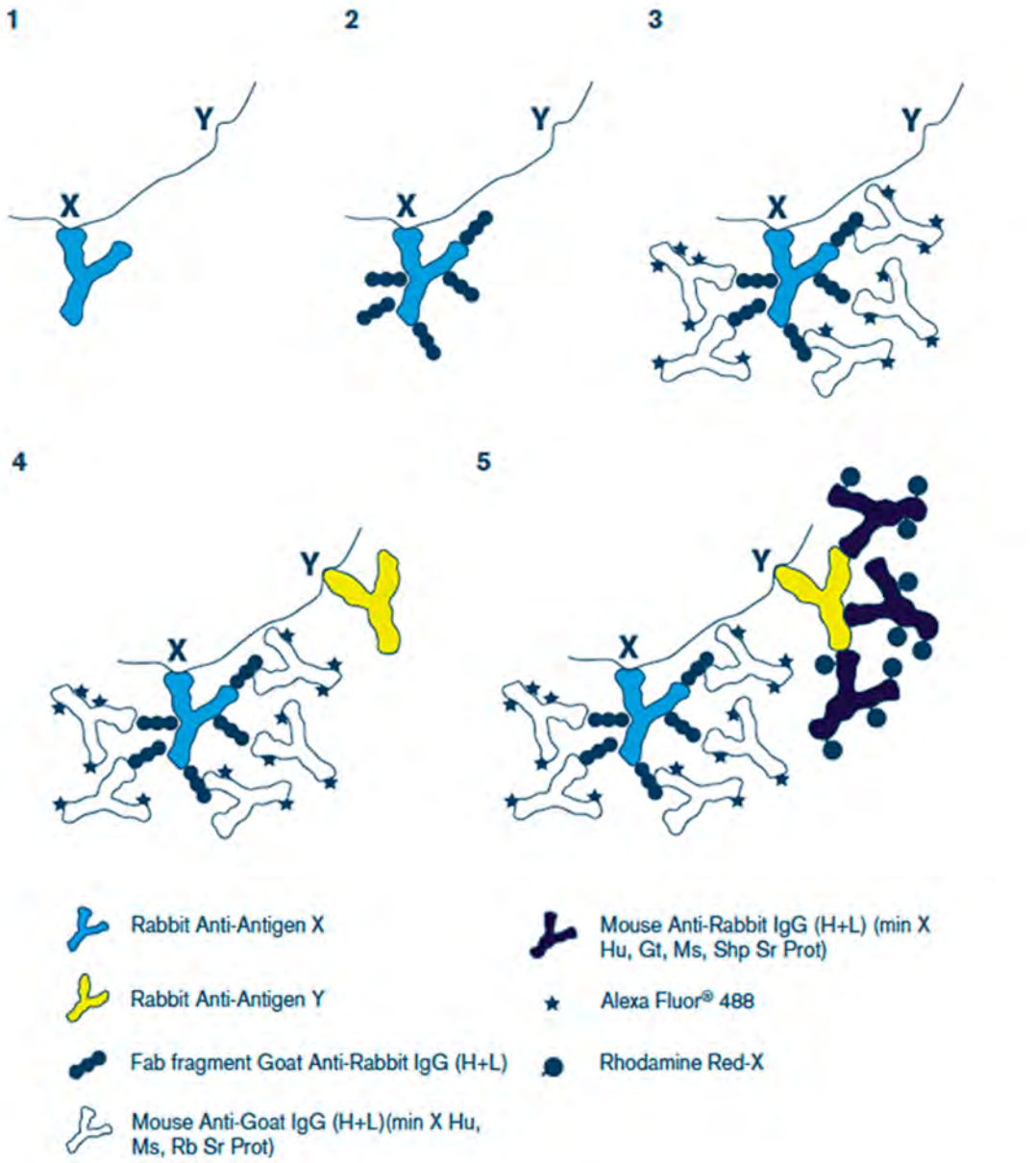
Case 2: use mouse monoclonal antibodies on mouse tissues

Fab Fragments for Blocking and Double Labeling of Primary Antibodies from the Same Host Species

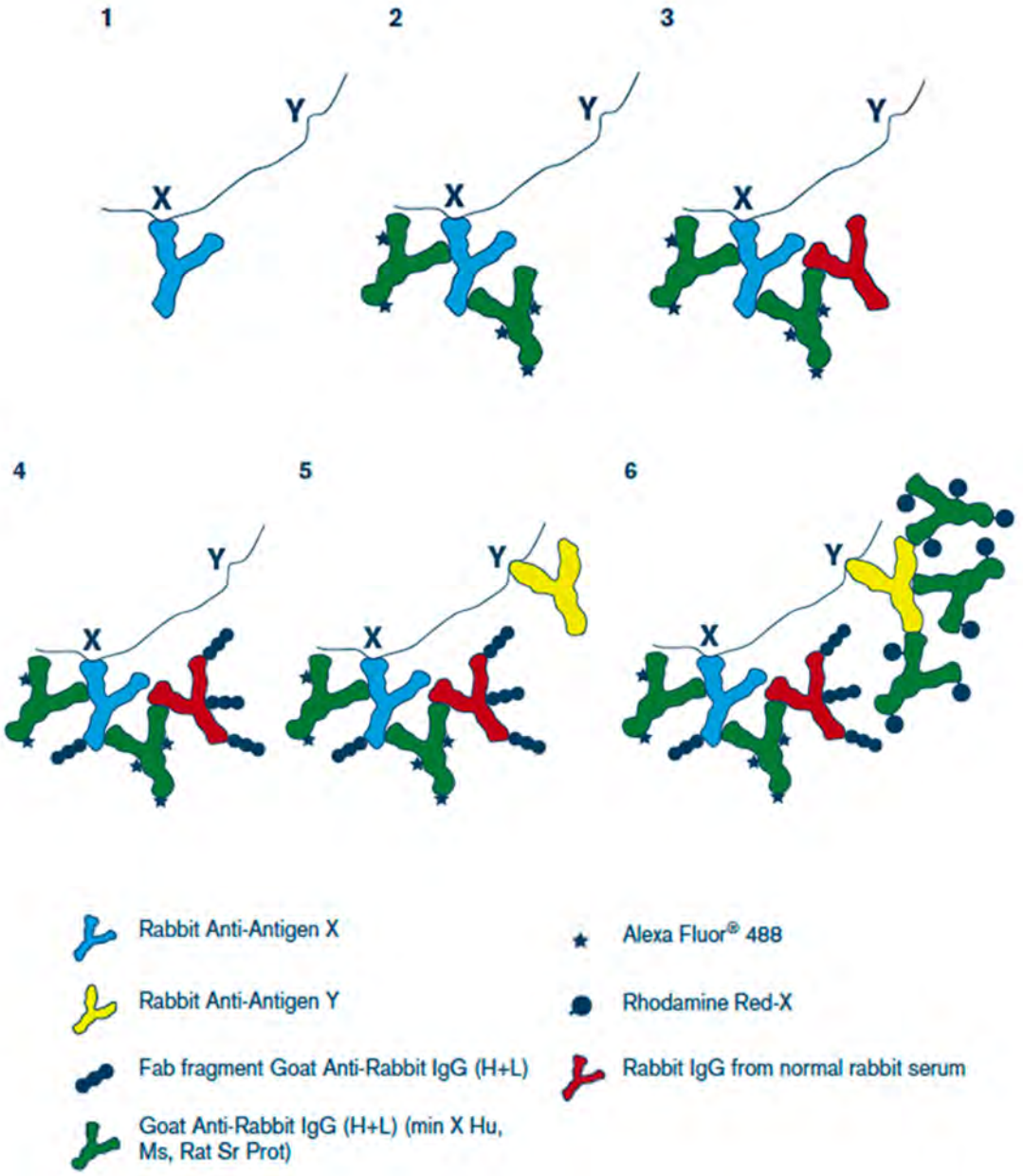


Use of conjugated Fab fragments for labeling and blocking.

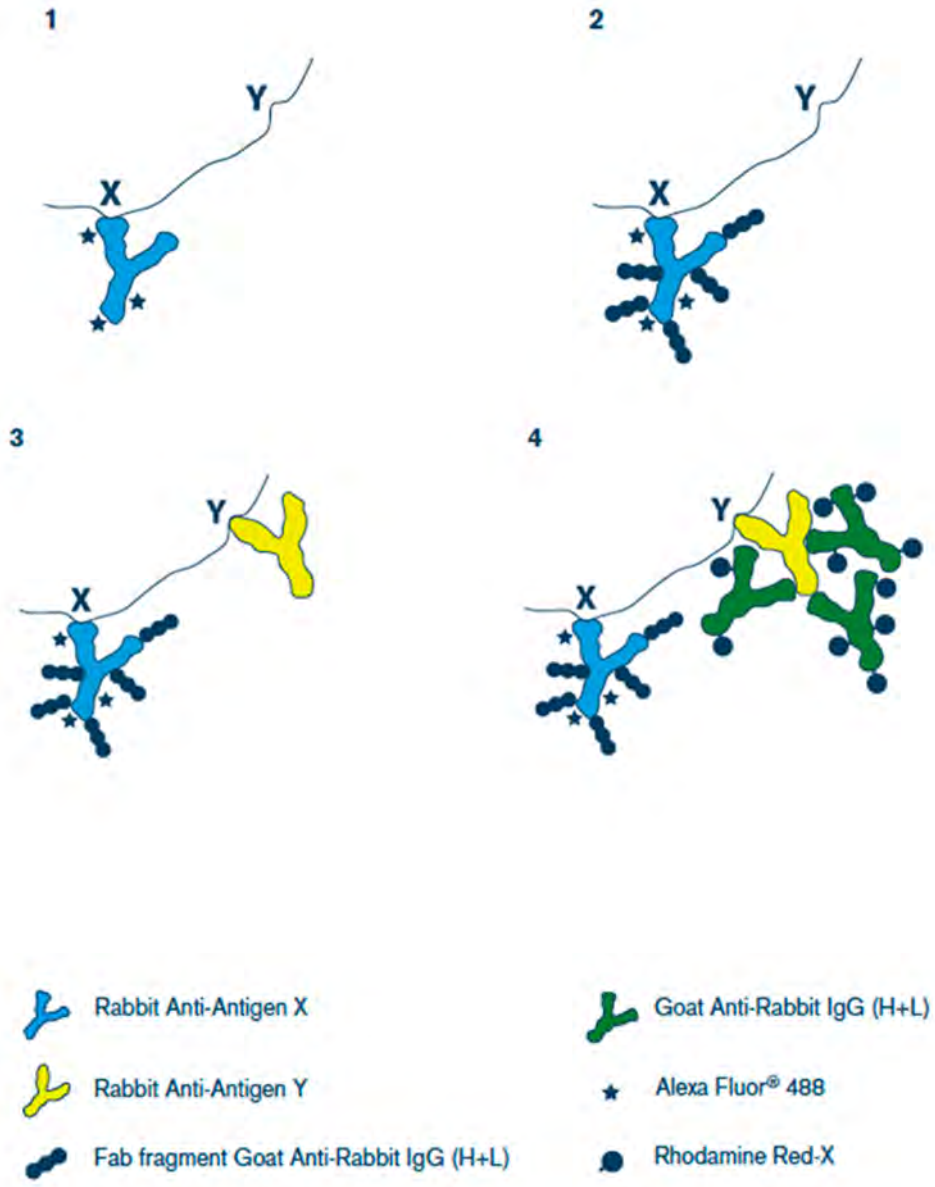
Use of unconjugated Fab fragments to cover the first primary antibody, presenting it as a different species.



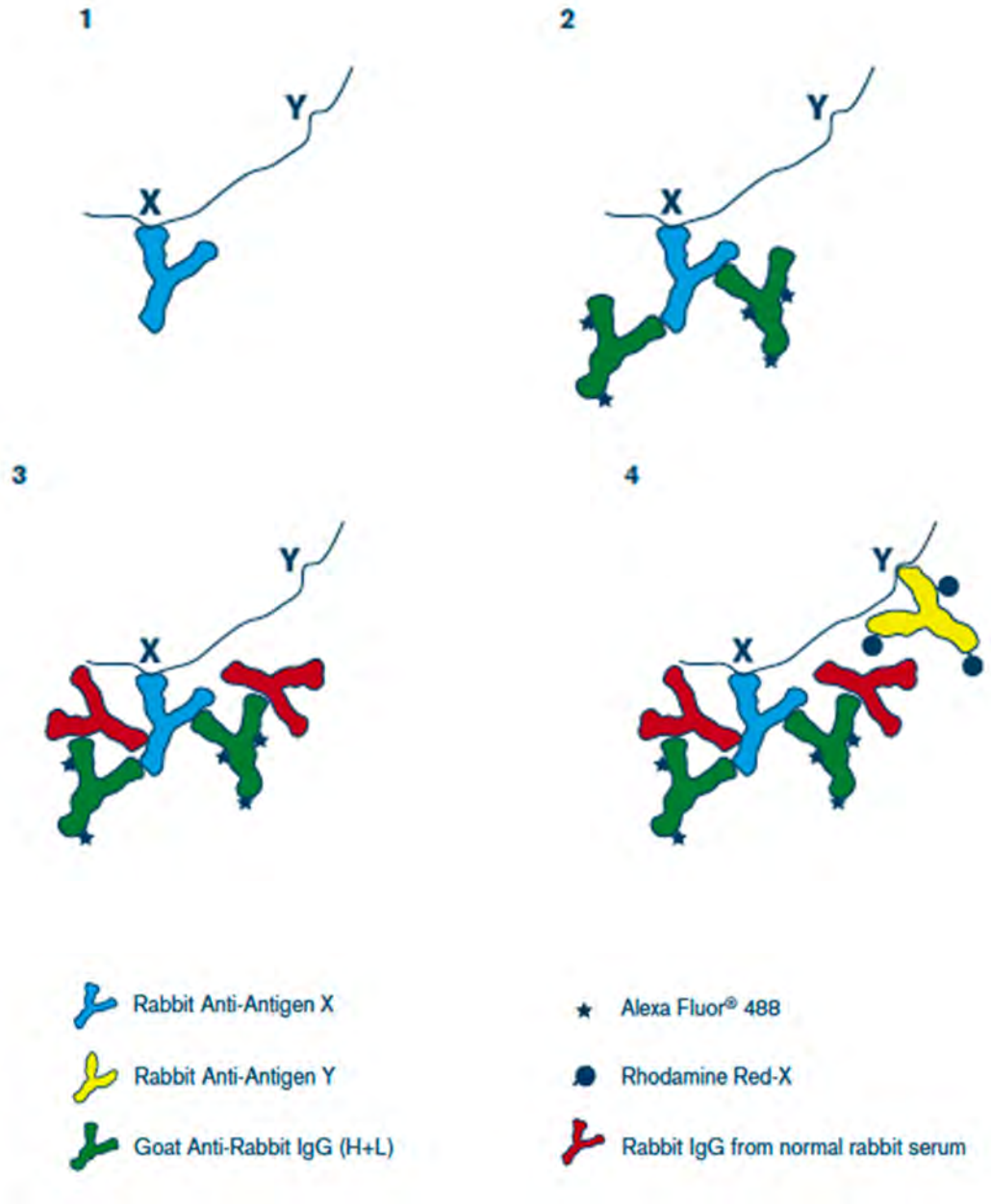
Use of unconjugated Fab fragments for blocking after the first secondary antibody



Use of *unconjugated* Fab fragments for detection of one unlabeled and one or more labeled antibodies



Detection of one unlabeled and one or more labeled primary antibodies without the use of Fab fragments



Mouse on mouse (MOM)

Much of the background is caused by secondary antibody binding to endogenous mouse IgG in the tissue being stained, and to Fc receptors on B cells, plasma cells and macrophages

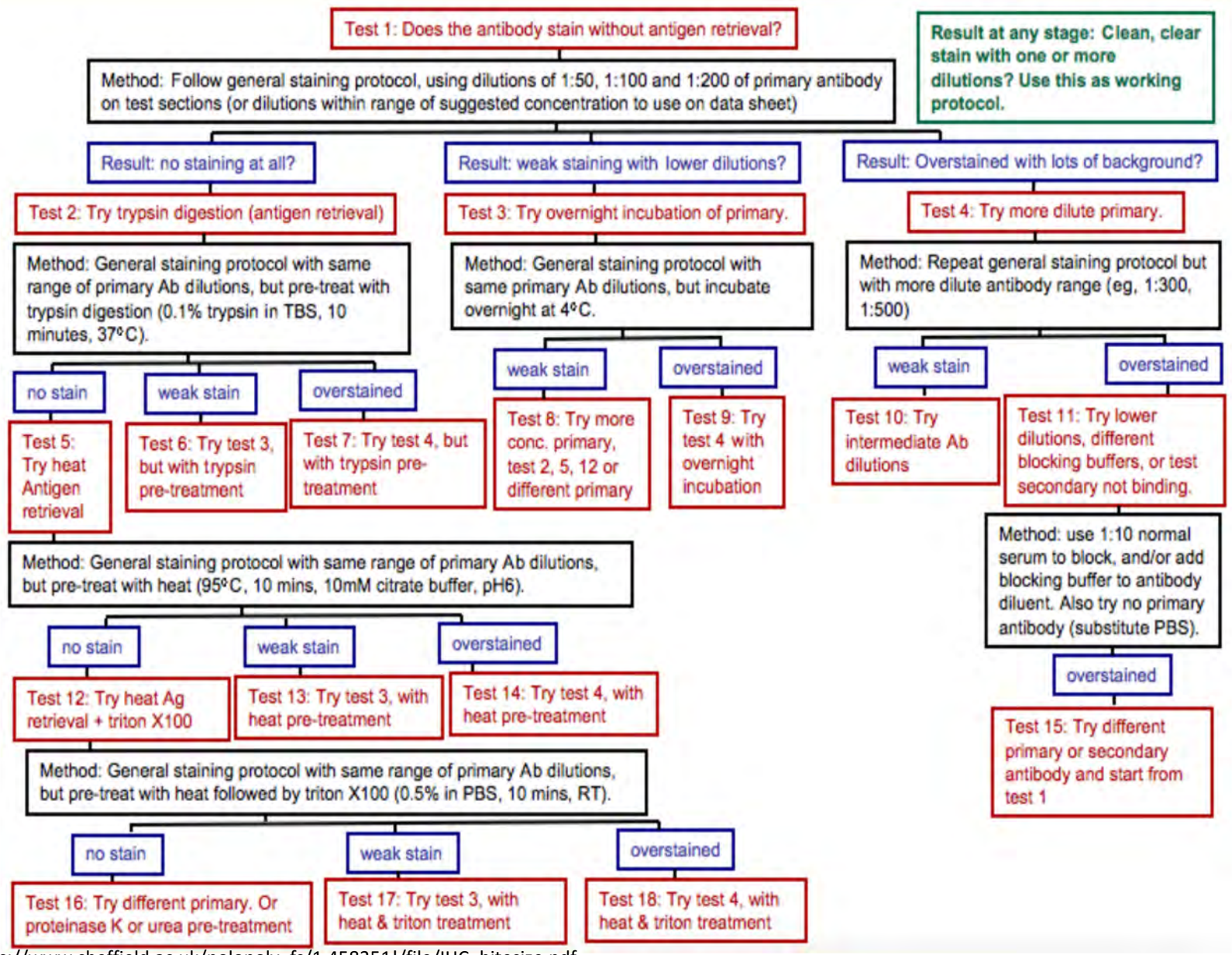
Blocking of endogenous IgG
Incubate sections with an unconjugated affinity purified F(ab) fragment anti-mouse IgG (H+L) for 1 hr at room temperature

- Standard blocking
- F(ab) endogenous IgG blocking
- I AB incubation
- II AB incubation

  FC fragment can still bind its own receptor

Blocking endogenous Fc receptors
Use F(ab) monomeric secondary antibodies to help reduce background.

New IF/IHC protocol development decision map



https://www.sheffield.ac.uk/polopoly_fs/1.458351!/file/IHC_bitesize.pdf

Ventana autostainer

Roche Ventana
Automated IHC/IF/ISH staining instrument

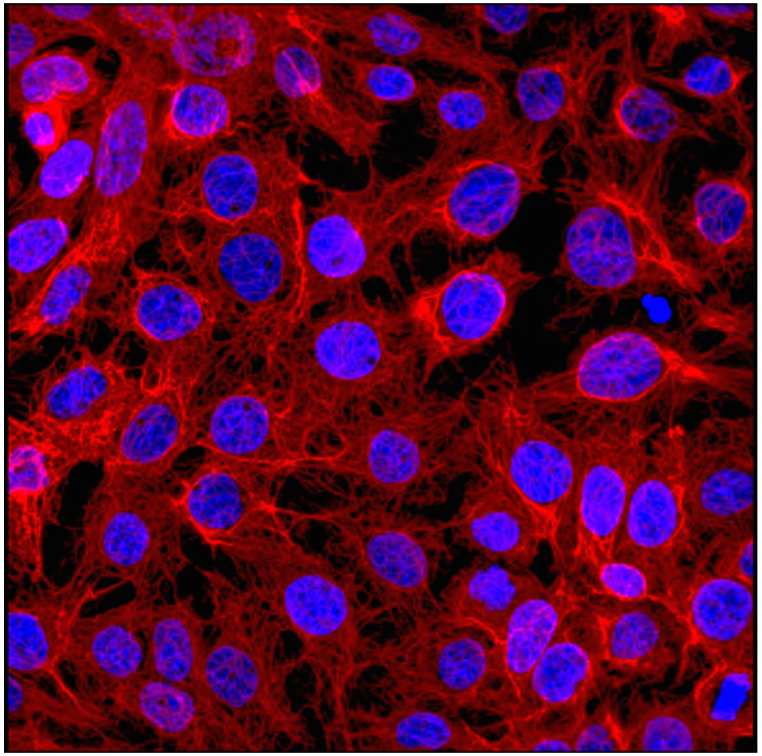
- Compatible with FFPE and frozen tissues, cytospin and cell smear.
- Simultaneous chemical dye, IHC (Dual Stain), IF, ISH stain.
- 24 special stains (H&E, Gram, Congo Red, Alcian Blue, Reticulin, etc.).
- Hundreds validated anti-human XX primary antibodies.
- ABs for other species can be used.
- 4 IHC detection systems (DAB, AP, normal secondary antibodies and polymeric AB).
- FITC, Rhodamine and Cy5 validated secondary AB.
- Compatible with DIG labeled probes for ISH
- Compatible with ADC RNAscope system for ISH

- **Consistent and reproducible results!**

Conclusions

IF and IHC are powerful techniques which allow for target in situ study.

The setup of a new methodology should take into account the biological characteristics of the tissue in analysis and should include always proper controls.



Q&A