

Overview of Immunohistochemistry and Histochemistry (Lecture in English)

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免疫組織染色と組織化学染色の原理と有用性について英語で概説する。免疫染色に使用する一次抗体や、二次抗体、発色による検出、蛍光による検出、抗原賦活化などを中心に説明する。

Histochemistry and immunohistochemistry are classic research techniques, yet they are still essential methods to identify expression of target molecules on tissue sections. By taking this class, you will learn basic principles of histochemistry and immunohistochemistry and will have ideas how to select primary antibodies, secondary antibodies, detection systems, and antigen retrieval.

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15:30-16:50

Overview of Immunohistochemistry and Histochemistry

免疫組織化学染色と組織化学染色の原理

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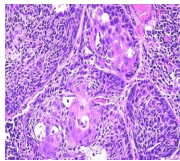
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Learning objectives

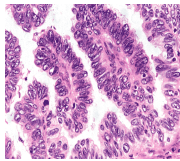
1. To learn how pathologists examine tissues and cells with different methods.
2. To understand the principle of histochemistry and immunohistochemistry (IHC).
3. To understand how to detect the antigen and antibody in IHC.
4. To learn how to improve the specificity and sensitivity of IHC.

Pathology

Pathology is a **morphology** based medical specialty that diagnoses diseases, mostly through analysis of tissue under the microscope.



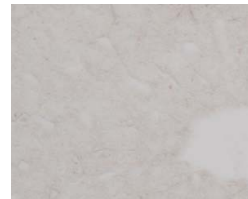
Squamous cell carcinoma



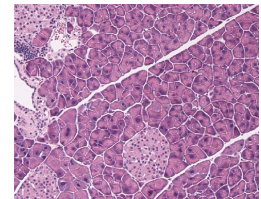
Adenocarcinoma: columnar shaped cells with glandular growth pattern

Histology

No Staining (translucent)



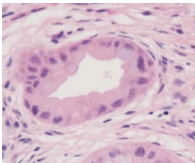
Hematoxylin and Eosin (H&E) staining



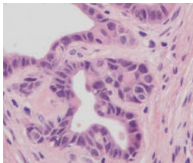
Staining gives a lot of information about tissue structure, cell types, cell morphology, and morphology and location of nucleus.

Grading pathological changes by histology Pancreatic ductal adenocarcinoma (PDAC)

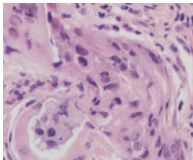
Pancreatic Intra-epithelial Neoplasia (PanIN-1A)



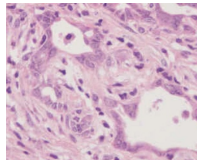
PanIN-2



PanIN-3

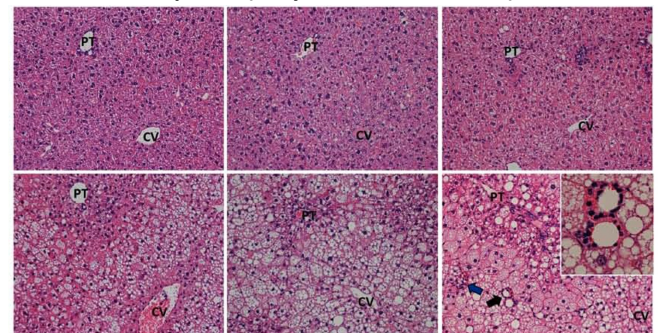


PDAC



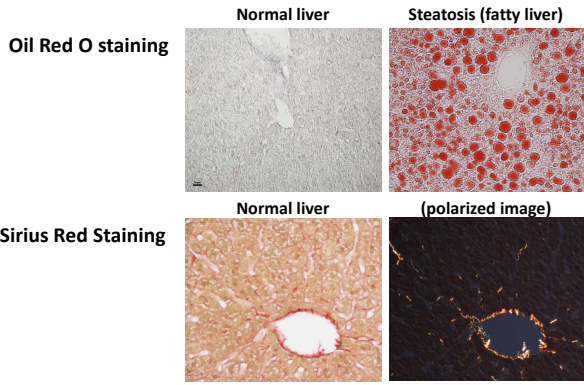
Asahina et al. Am J Physiol 2020

Grading pathological changes by histology Liver steatohepatitis (fatty liver, inflammation)



McGettigan et al. Hepatology 2019

Histochemistry

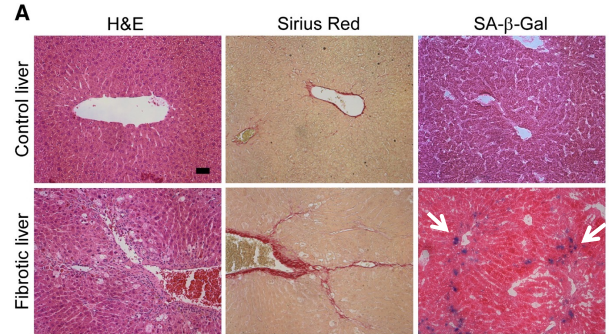


Detection of neutral lipids or collagen fiber in tissues

Balog *et al.* Hepatology 2020

Enzyme histochemistry

Detection of senescence associated β -galactosidase (SA- β -Gal) in liver fibrosis using X-gal substrate



Krizhanovsky *et al.* Cell 2008

Immunohistochemistry

Methods to visualize expression of antigens in tissue sections based on **antigen-antibody recognition**.

Antigens: proteins, sugars, lipids, nucleic acids, etc...

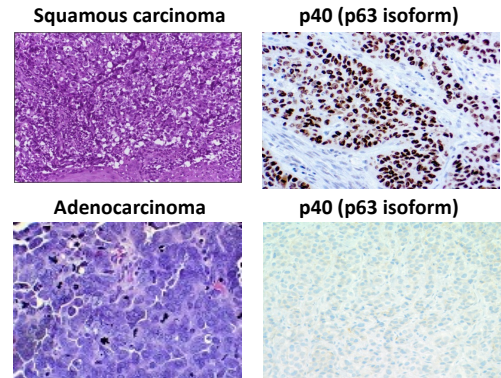
Immunohistochemistry will give you following information:

Which cells express the antigen you test.

Where the antigen localizes.

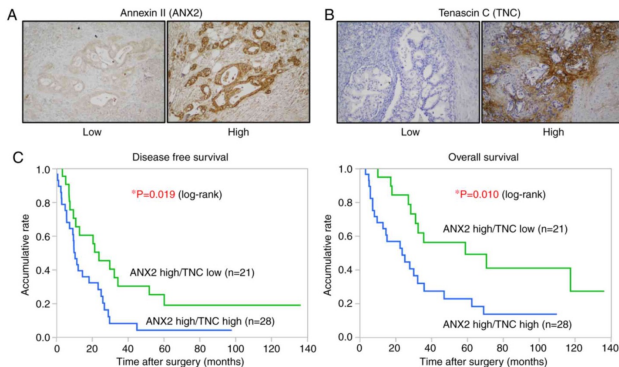
How the expression of the antigen changes.

IHC using p40 can differentiate squamous cell carcinoma versus adenocarcinoma despite similar morphology



Immunohistochemistry

Correlation of marker expression and survival of patients with PDAC



Yoneura *et al.* Int J Mol Med 2018

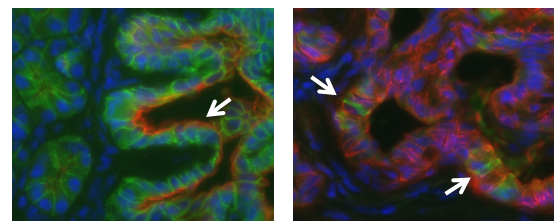
Immunohistochemistry

Stem cells may express unique antigens

Pancreatic cancer

E-cadherin (green)
Prominin-1 (red)

EPCAM (red)
DCLK1 (green)



Asahina *et al.* Am J Physiol 2020

Procedure of immunohistochemistry

Microscopic observation requirement:

Thin Section (5-7 μm)

How to make tissue section?

Frozen section

Formalin-fixed paraffin-embedded (FFPE) section

Frozen sections

Advantages

- Frozen samples can be prepared in short time with or without fixation of tissues.
- Proteins may keep their native structure in frozen sections.
- Many antibodies may work well in frozen sections.

Disadvantages

- Frozen sections give poor morphology.
- Cryostat (expensive) is necessary for making frozen sections.
- Frozen samples/sections are needed to be stored in freezers.

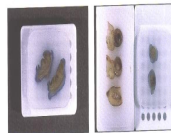
Paraffin blocks and sections

Advantages

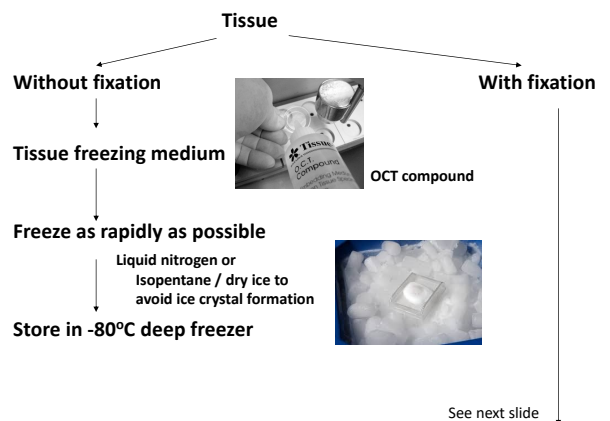
- Paraffin sections give excellent morphology.
- Thin paraffin sections can be prepared using microtome.
- Paraffin blocks can be stored in room temperature for decades.

Disadvantages

- Preparing paraffin block/sections consumes longer time.
- Paraffin sections may not work for immunohistochemistry.
- Antigen retrieval procedure may be necessary.



Immunohistochemistry using frozen sections



With fixation

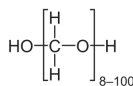
4% Paraformaldehyde (PFA)

Insoluble polymerization product of formaldehyde

10% Formalin (formaldehyde)

10% formalin solution, neutral buffered, containing 10-15% methanol (stabilizer)
Most common fixative solution

Paraformaldehyde



Decomposition

Formalin



- Formalin crosslinks primary amines of proteins and nucleic acids.
- Formalin inactivates endogenous enzymes (proteases, nucleases).
- Formalin prevents microbial contamination and inactivates virus.

With fixation

4% Paraformaldehyde (PFA) or 10% Formalin

Perfusion of organs/tissues with PFA (best)
or
Incubate tissues in PFA at 4°C, overnight

15% sucrose in PBS

A few hours-overnight

30% sucrose in PBS (to prevent ice crystal formation in frozen tissues)

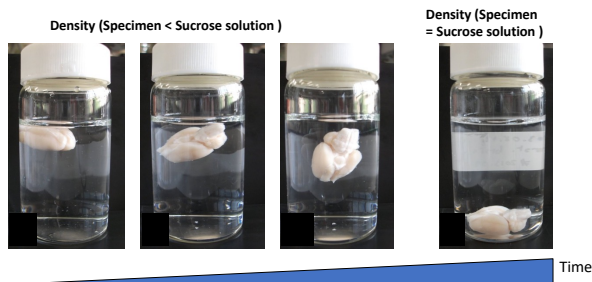
A few hours-overnight

Embed in freezing medium

Freeze as rapidly as possible

Store in -80°C deep freezer

Cryoprotection with 30% sucrose



Cryostat

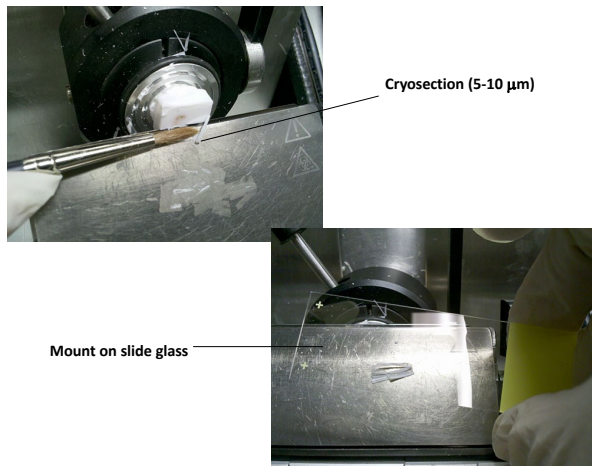
A microtome in a cold chamber which maintains very low temperature around -20°C .



A frozen sample embedded in freezing medium



Disposable blade



If frozen sections are prepared from unfixed samples, fixation is needed before histochemistry

Formalin

Formalin cross-links proteins in tissues.

Advantages: good morphology

Disadvantages: some antibodies may not work

Ice-cold acetone for 5-10 min

Acetone removes lipids and dehydrates tissues.

Advantages: conserves antigens for antibody binding

Disadvantages: poor morphology, loses soluble and lipid molecules

Ethanol (or methanol)

Ethanol dehydrates tissues without crosslinking.

Advantage: preserves the structure of proteins and tissues

Disadvantage: may lose reactivity to antibodies, poor morphology

Procedure of immunohistochemistry in frozen sections

1. Make cryosections ($7\ \mu\text{m}$)
2. Dry
3. Fix the sections if necessary
4. Remove freezing medium in PBS
5. Incubate the sections with blocking solution for 30 min (usually 5% goat or donkey serum and 0.2% bovine serum albumin in PBS)
6. Apply **primary antibody** for 1 hour room temperature or over night at 4°C
7. Wash with PBS
8. Apply **secondary antibody** for 30 min room temperature
9. Wash with PBS
10. Detect the secondary antibody
11. Counterstain nuclei if necessary

Polyclonal antibody vs. monoclonal antibody

Polyclonal antibody

Advantages

may recognize different epitopes of the antigen

Disadvantages

may vary its affinity to the antigen among a host animal

may be obtained limited amount from a host animal



Monoclonal antibody

Advantages

recognizes a single epitope of the antigen

can be produced stable quality from a hybridoma clone

Disadvantages

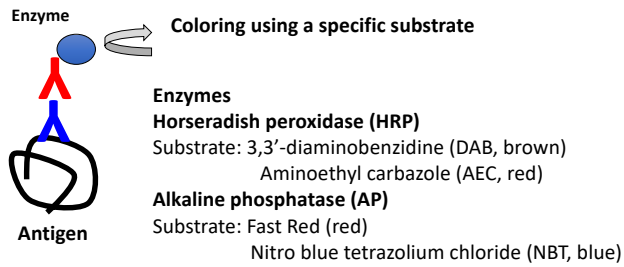
is often produced from mice

may give weak staining compared to polyclonal antibody



Detection of the primary antibody by secondary antibodies

Immunohistochemistry



Y Primary antibody
Y Secondary antibody
conjugated with enzymes

Inactivation of endogenous peroxidase or phosphatase before antibody incubation

Endogenous peroxidase

Incubate sections with 0.3-3% hydrogen peroxide (H_2O_2) in methanol (or water)

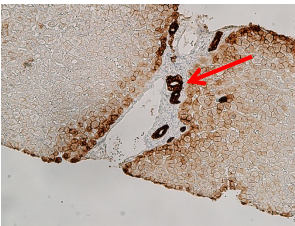
Endogenous phosphatase

Incubate with levamisole, an inhibitor for alkaline phosphatase

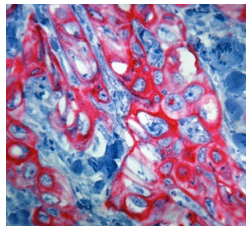


Immunohistochemistry

Human liver cirrhosis (biopsy)
Primary antibody: cytokeratin
Secondary antibody: anti-mouse-HRP
Color: DAB (brown)



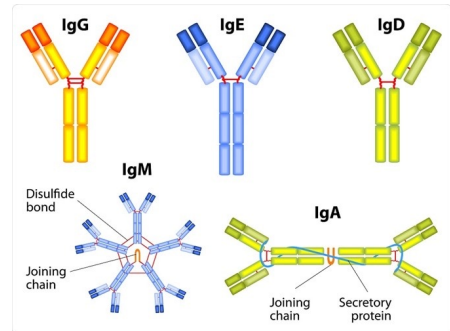
Human squamous carcinoma
Primary antibody: cytokeratin
Secondary antibody: anti-mouse-AP
Color: FastRed (Red)



Enzo

How to select primary antibodies?

IgG
or
IgM (rare)



Animal species for antibody generation

Mouse, rat, rabbit, goat, hamster, chicken, etc...

Check reactivity of the antibody you use

How to select secondary antibodies?

Find IgG subclasses if necessary

Mouse

IgG1, IgG2a, IgG2b, IgG2c, IgG3

Secondary antibodies for mouse IgG subclasses

Anti-mouse whole IgG antibody: recognize all IgG subclasses

Anti-mouse IgG1 antibody: only recognize IgG1

Anti-mouse IgG2a antibody: only recognize IgG2, etc...

Rat

IgG1, IgG2a, IgG2b, IgG2c

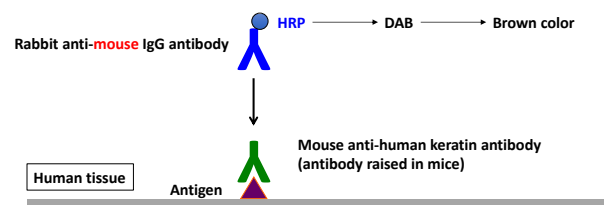
Rabbit

IgG (only 1)

Chicken

IgY

Immunohistochemistry



Blocking: 5% rabbit serum, 0.2% bovine serum albumin

Pretreatment: 3% H_2O_2 in methanol for inactivation of endogenous peroxidase

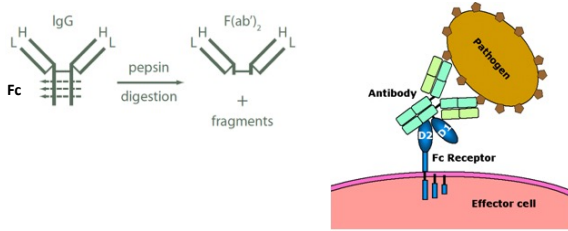
How to select **secondary** antibodies?

Whole Immunoglobulin or fragments?

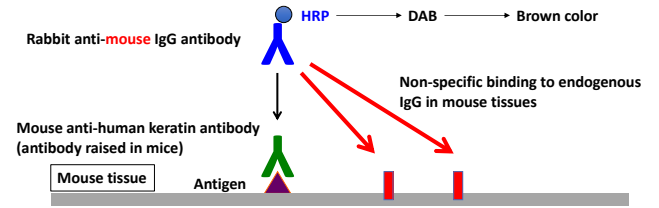
F(ab')₂ fragment of IgG

Remove the Fc fragment by pepsin digestion

Avoid binding to Fc receptors expressed on tissues



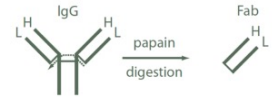
How to stain mouse tissues using mouse antibodies?



Available methods

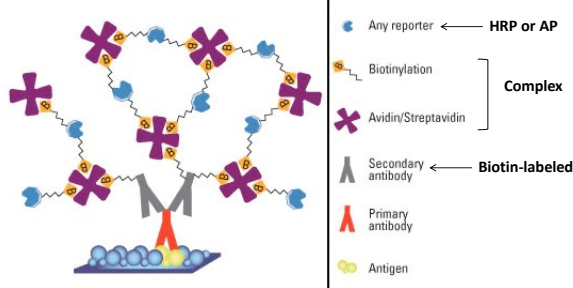
Use primary antibodies conjugated with fluorescence dye or enzyme without use of secondary antibody (limited availability of antibodies, relatively low signals)

Blocking endogenous mouse IgG with Fab



How can we enhance weak signals in IHC?

Avidin-biotin complex (ABC) method



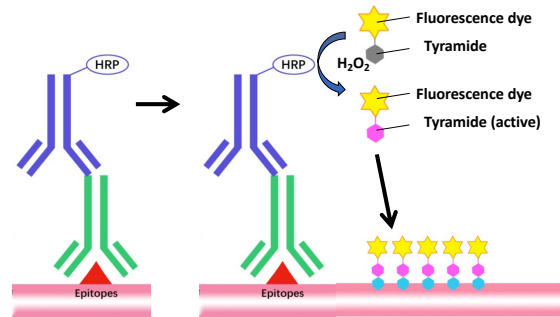
Protocol

Apply primary antibody
Apply secondary antibody conjugated with biotin
Apply Avidin-Biotin-reporter complex
Detect the complex through HRP or AP

ThermoFisher Scientific

How can we enhance weak signals in IHC?

Tyramide Signal Amplification (TSA) method

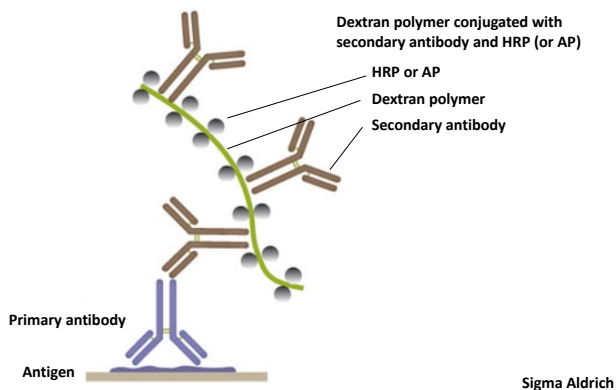


Radical tyramide (active) covalently binds to tyrosine residues near the antibody

APExBio

How can we enhance weak signals in IHC?

Polymer-based detection method



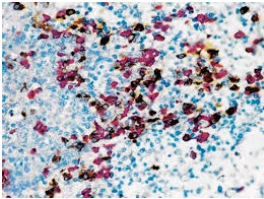
Sigma Aldrich

Comparison

Methods	Advantages	Disadvantages
Primary antibody conjugated HRP	Easy, short time	Low sensitivity Limited availability of antibodies
Use Secondary antibody	Easy, short time	Not so sensitive
ABC	Better sensitivity	Time consuming Need endogenous biotin blocking
Tyramide (TSA)	High sensitivity	Expensive Time consuming Non-specific signals
Polymer	High sensitivity Short time	Expensive May not be compatible to double staining

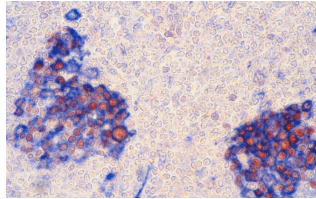
Immunohistochemistry using 2 different antibodies

Tonsil
anti- κ light chain (AP-FastRed, red)
anti- λ light chain (HRP-DAB, brown)



Dako

Spleen
BCL6 (HRP-AEC, brown)
Peanut agglutinin (AP-Fastblue, blue)

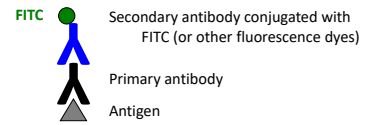


IHC world

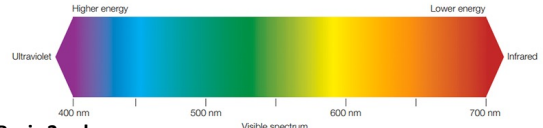
HRP and AP can be used for detection of 2 different antigens.
It is less clear to see co-localization of 2 antigens using these colors.

Immunofluorescence staining (Immunohistochemistry using fluorescence dyes)

Use of fluorescence dyes instead of enzymes (HRP or AP) for detection of antibodies



Spectrum of fluorescence



Basic 3 colors

Blue

Green

Red

Bio-Rad

How fluorophore gives fluorescence?

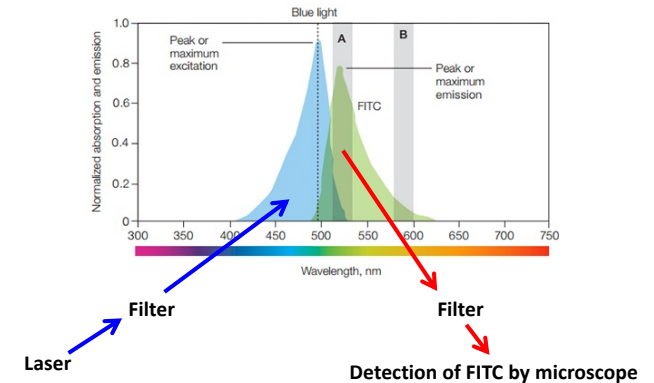
FITC (fluorescein, green)



- S_0 Resting state
Absorb 490 nm wavelength light from a laser
- S_2 Excited electronic singlet state (**Excitation**)
- S_1 Electronic singlet state
- Release the remaining energy as fluorescence (**Emission**)
Detect green fluorescence peaking at 525 nm using a filter

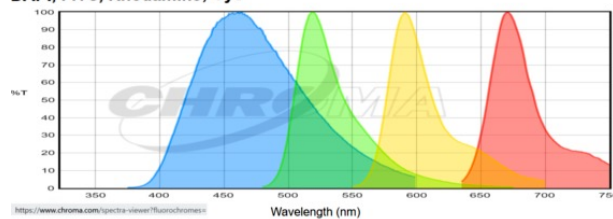
How fluorophore gives fluorescence?

FITC (fluorescein, green)



Use of different fluorescence colors

DAPI, FITC, Rhodamine, Cy5



	DAPI	FITC	Rhodamine	Cy5
Excitation (nm)	Blue	Green	Red	Near infrared red
Emission (nm)	365	490	560	640
	460	525	590	650

Choose appropriate fluorescence based on brightness and photostability

Blue

Alexa Fluore 350
DyLight 350
DAPI (DNA staining)

IHC (long laser exposure)
Better to use fluorescence having
longer photostability

Green

Fluorescein (FITC)
DyLight 488
Alexa Fluore 488

Flow cytometry (short laser exposure)
Better to use fluorescence with high
brightness

Red

Tetramethylrhodamine (TRITC)
Cy3
DyLight 550
Alexa Fluore 555
Propidium iodide (DNA staining)

Procedure of **fluorescence** immunohistochemistry in frozen sections

1. Make cryosections (7 μ m)
2. Dry
3. Fix the sections if necessary
4. Remove frozen medium with PBS (no need to inactivate endogenous peroxidase or alkaline phosphatase)
5. Block the sections with a blocking solution for 30 min (usually 5% goat or donkey serum and 0.2% bovine serum albumin in PBS)
6. Apply **primary antibody** for 1 hour room temperature or over night at 4°C
7. Wash with PBS
8. Apply **secondary antibody** conjugated with a fluorescent dye for 30 min room temperature
9. Wash with PBS
10. Counterstain nuclei with DAPI if necessary

Fluorescence immunohistochemistry

Primary antibody

rabbit anti-mouse type IV collagen (basement membrane)
goat anti-mouse Gata4 (nuclei)

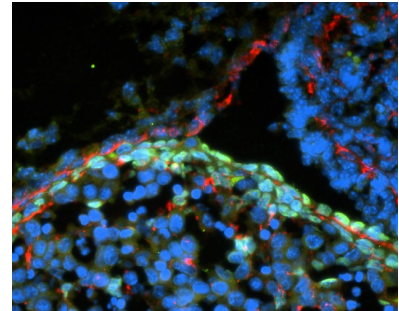
Secondary antibody

donkey anti-rabbit IgG-AlexaFluor 555
donkey anti-goat IgG-Alexafluor 488

Nuclei

DAPI

Mouse embryonic liver

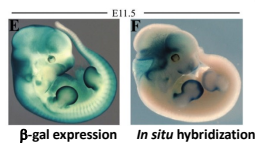
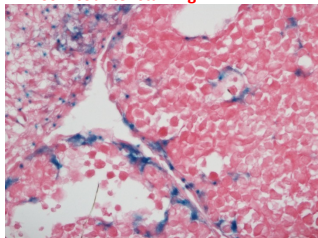


Detection of LacZ by fluorescence immunostaining

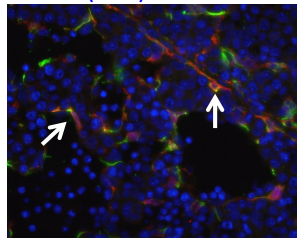
Transgenic mice carrying Msx2 promoter driven β -galactosidase (Asahina *et al.* 2009)

Question: Which cell types express β -galactosidase (Msx2) in the liver?

Enzymatic β -galactosidase activity
Eosin counter staining



rabbit anti- β -galactosidase
goat anti-mouse Desmin
DAPI (nuclei)



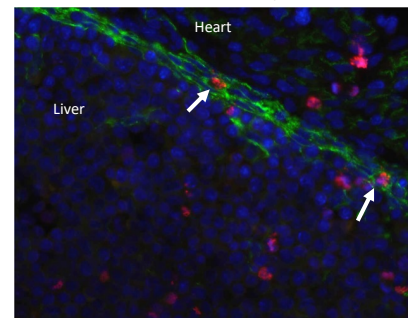
Detection of proliferating cells by fluorescence immunostaining

Protein markers for cell proliferation

Phosphorylated Histone H3
Proliferation cell nuclear antigen (PCNA)
Ki-67

M phase
G1 and S phases
G1, S, G2, M phases

Rabbit anti-phospho-H3
Rat anti-CD166
DAPI (nuclei)

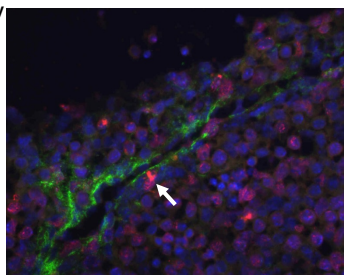


Detection of proliferating cells by fluorescence immunostaining

5-bromo-2-deoxyuridine:5-fluoro-2'-deoxyuridine (BrdU) incorporation assay

- Inject BrdU into the mouse peritoneal cavity
- Kill the mouse 2hrs later
- Fix tissues with 4% PFA and make cryosections
- Denature genome DNA with 2N HCl for 30 min
- Apply anti-BrdU antibody

Rat monoclonal anti-BrdU
Hamster anti-Podoplanin
DAPI (nuclei)



Microscopic observation requirement:

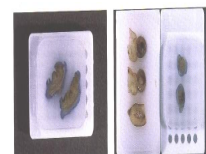
Thin Section (5-7 μ m)

How to cut thin tissue section

Frozen section

Formalin-fixed paraffin-embedded (FFPE) section

Archive pathology specimens

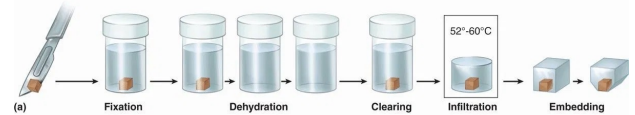


Advantages of FFPE

- FFPE sections provide excellent morphology for diagnosis.
- FFPE specimens can be kept in room temperature for a long term.
- Formalin sterilizes the specimen by killing all bacteria or virus.
- We may extract genomic DNA or RNA from FFPE sections for PCR or next generation sequencing.
- FFPE blocks have been used for more than 100 years in pathology.

Immunohistochemistry using **paraffin** sections

1. Collect the organ
(if possible, it is better to perfuse the organ with formalin or PFA)
2. Cut into small pieces
3. Fix with 4% PFA or 10% formalin at 4°C for over night
4. Wash with PBS
5. Replace to serial series of **ethanol** from 70% to 100%
6. Dehydrate the tissues with 100% ethanol 3 times
7. Replace to xylene, 3 times
8. Replace to **xylene/paraffin** in an oven at 52-60°C 3 times
9. Replace to paraffin in an oven at 52-60°C 3 times
10. Make paraffin blocks in room temperature



Automated tissue processor



Paraffin embedding workstation

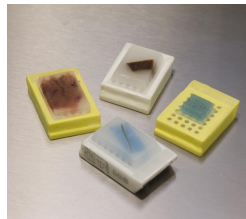


Cool down paraffin blocks

Pour liquid paraffin solution

Hot chamber

Paraffin blocks can be kept room temperature



Making paraffin sections using a microtome



Paraffin block

Disposal blade

Continuous paraffin sections (4-10 μ m)

Mount paraffin sections onto a slide glass

Tissue section
surrounding with
paraffin (white)



Use ID labels for
specimens



Immunohistochemistry using **paraffin** sections

1. Remove paraffin from the sections on the slide in **Xylene**
2. Replace to a serial series of **ethanol** from 100% to 70%
3. Wash with PBS
(Treat sections with **antigen retrieval solution** if necessary)
4. Inactivate endogenous peroxidase in **3% H₂O₂ in methanol**
5. Block with 5% serum, 0.2% bovine serum albumin
6. Incubate with primary antibody for 1 hour room temperature or 4°C overnight
7. Wash with PBS
8. Incubate with secondary antibodies
9. Detect the secondary antibody through HRP, AP, or fluorescence dye
10. Counterstain nuclei or tissues with appropriate dyes

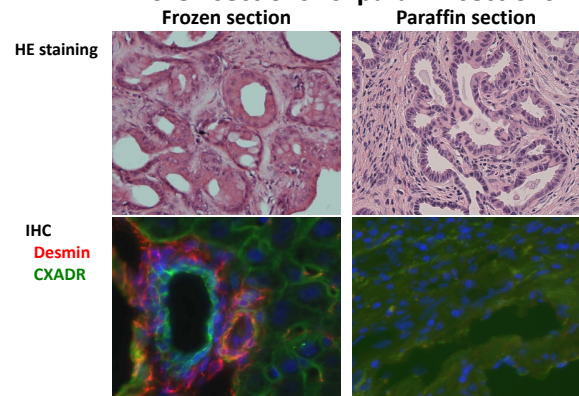
Manual slide process under a fume hood



Automated slide stainer



Frozen sections vs. paraffin sections



Paraffin sections give good morphology, but these antibodies do not work for IHC.

Why are there antibodies that do not work for formalin-fixed paraffin embedded (FFPE) sections?

Antigens in the section may lose antigenicity against antibodies

1. Proteins are denatured by heating during paraffin processing.
2. Proteins are denatured by organic solvents.
3. Cross-linking by formalin masks antigens.

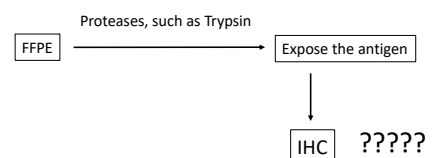
There are several methods to overcome these issues.

Use enzymes for partial digestion of sections.

Use an antigen retrieval solution for breaking crosslink in sections.

How to solve the problem- Approach 1

Pretreatment ----- Enzyme digestion

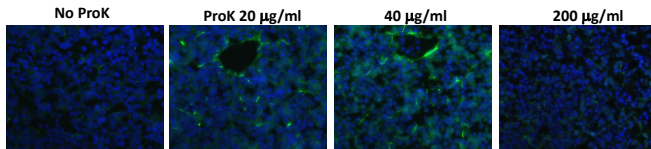


How to improve staining by enzyme treatment?

- ✓ **Proteinase K:** Digest proteins completely if incubate longer
Digestion should be stopped by washing with PBS
- ✓ **Trypsin:** Cleaves specific sites of proteins
Digestion should be stopped by washing with PBS
- ✓ **Pepsin:** Cleaves specific sites of proteins under a low pH
Digestion can be stopped by changing pH

Santa Cruz

Goat anti-desmin (Y-20) antibody



Pretreatment with Proteinase K increase the signals in IHCs in some antibodies (however, it not always works)

How to solve the problem – Approach 2

Remove formalin-induced modification and **Retrieve** the antigenicity in FFPE

Antigen retrieval solution

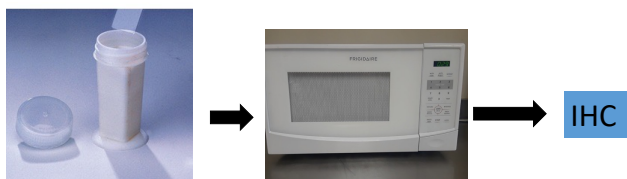
- ✓ **Citrate buffer** (10 mM citrate, 0.05% Tween 20, pH 6.0)
- ✓ **Tris-EDTA** (pH 9.0 or pH 10.0)

- Pathologists can continue to use formalin as fixative.
- All FFPE specimens accumulated in the past can be still useful for future research.

This is the solution everybody will love.

Antigen retrieval treatment

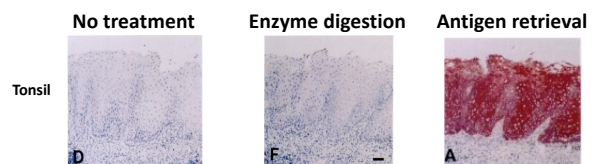
Seeing is Believing



Soaked FFPE sections in the antigen retrieval solution

Heated in microwave 5 min x 2 (high temperature)
or
Incubate at 95°C for 20 min

Monoclonal anti-cytokeratin antibody staining after antigen retrieval treatment in FFPE sections



Antigen Retrieval in Formalin-fixed, Paraffin-embedded Tissues: An Enhancement Method for Immunohistochemical Staining Based on Microwave Oven Heating of Tissue Sections

SHAN-RONG SHI, MARC E. KEY,¹ and KRISHAN L. KALRA
BioGenex Laboratories, San Ramon, California 94583.

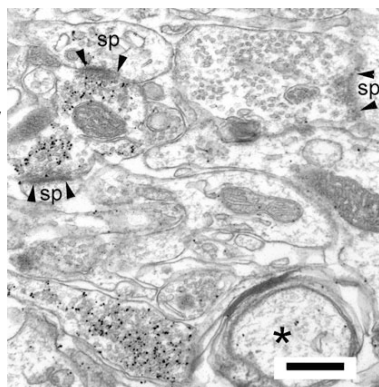
Received for publication January 15, 1991; accepted March 12, 1991 (IC2212).

Electron microscopy and immunohistochemistry

Immunogold labeling

Transmission electron microscopy

Synaptophysin in the synaptic vesicles

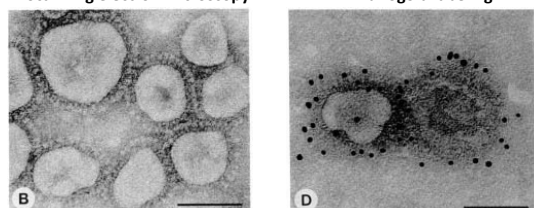


Immunoelectron microscopy 2010

Visualization of coronavirus by immunoelectron microscopy

Scanning electron microscopy

Immunogold labeling



Dea and Garzon. J Vet Diagn Invest 1991

Choice of an antibody for IHC

On September 9th, 2021, Antibodypedia.com is referencing 4,480,670 antibodies from 95 providers against 19,109 gene products...
It means about 234 antibodies are available by a gene product...

- Moser *et al.* (*J. Neurochem.* 2007;102:479-492)
13 working anti-nicotinic acetylcholine receptor (nAChR) antibodies were tested on nAChR-knockout mice.
Same immunoreactivity was observed in nAChR-knockout mice.
None of the antibodies was suitable for IHC!
- Egelhofer *et al.* (*Nat. Struct. Mol. Biol.* 2011;18:91-93.)
246 histone antibodies were tested:
25% were non-specific.
22% cannot precipitate chromatin by immunoprecipitation.
Some worked but recognized the wrong target histone.

Assessment of the antibody specificity

All immunohistochemical methods are prone to artefact and non-specific staining.

Researchers need to have appropriate controls and assess the specificity carefully.

Validation of the antibody is of critical importance in immunostaining.

About antibody validation

Magic peptides, magic antibodies: guidelines for appropriate controls for immunohistochemistry.
Saper CB, Sawchenko PE.
J Comp Neurol 2003;465:161-163. PMID: [12949777](#).

An open letter to our readers on the use of antibodies.
Saper CB.
J Comp Neurol 2005;493:477-478. PMID: [16304632](#).

Antibodies as valuable neuroscience research tools versus reagents of mass distraction.
Rhodes KJ, Trimmer JS.
J Neurosci 2006;26:8017-8020. PMID: [16885215](#).

Antibody validation.
Bordeaux J, Welsh A, Agarwal S, Killiam E, Baquero M, Hanna J, Anagnostou V, Rimm D.
Biotechniques 2010;48:197-209. PMID: [20359301](#)

Controls for immunohistochemistry: the Histochemical Society's standards of practice for validation of immunohistochemical assays.
Hewitt SM, Baskin DG, Frevert CW, Stahl WL, Rosa-Molinar E.
J Histochem Cytochem 2014;62:693-697. PMID: [25023613](#).

On the Necessity of Validating Antibodies in the Immunohistochemistry Literature.
Gautron L.
Front Neuroanat 2019;13:46. PMID: [31080409](#)