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 how to select primary antibodies, secondary antibodies, detection systems, and antigen retrieval.

Program of 2024 Intensive Course in Basic Science Fundamentals &
Multidisciplinary Seminars / Central Research Laboratory Special Seminars

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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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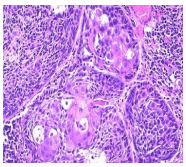
Learning objectives

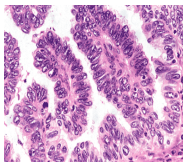
1. To learn how pathologists examine tissues and cells with different methods.
2. To understand the principle of histochemistry and immunohistochemistry (IHC).
3. To understand how to detect the antigen and antibody in IHC.
4. To learn how to improve the specificity and sensitivity of IHC.

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

Experimental Pathology

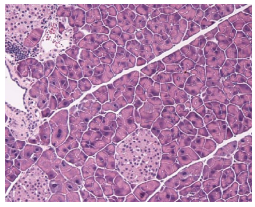
To reproduce pathogenesis of human disease using animals, such as mice, rats, or monkeys.

To understand cellular and molecular mechanisms underlying pathogenesis of the disease.

Histology

No Staining

Hematoxylin and Eosin (H&E) staining

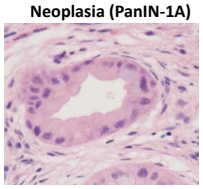


Staining gives a lot of information about tissue structure, cell types, cell morphology, and morphology and location of nucleus.

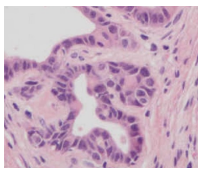
Grading pathological changes by histology

Pancreatic ductal adenocarcinoma (PDAC)

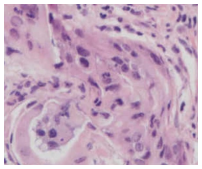
Pancreatic Intra-epithelial Neoplasia (PanIN-1A)



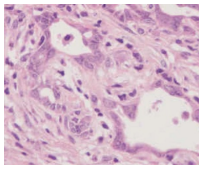
PanIN-2



PanIN-3



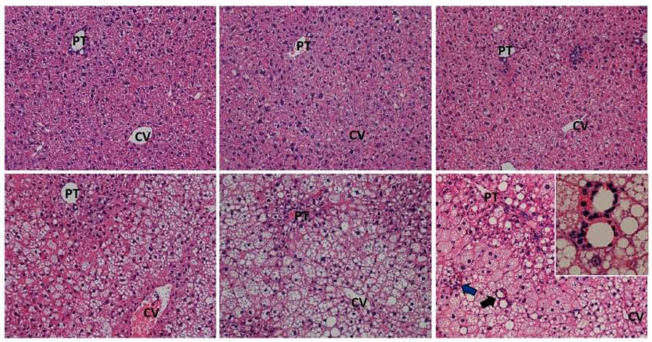
PDAC



Asahina *et al*. Am J Physiol 2020

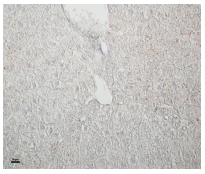
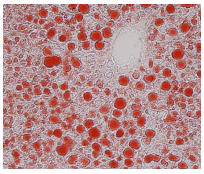
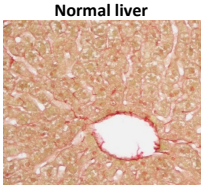
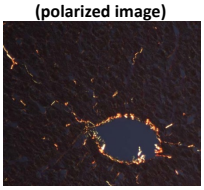
Grading pathological changes by histology

Liver steatohepatitis (fatty liver, inflammation)



McGettigan *et al*. Hepatology 2019

Histochemistry

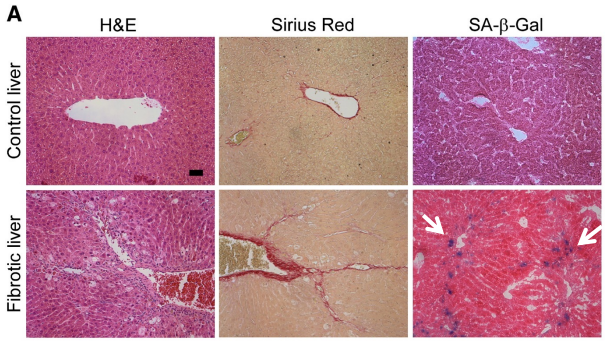
	Normal liver	Steatosis (fatty liver)
Oil Red O staining		
	Normal liver	**(polarized image)**
Sirius Red Staining		

Detection of neutral lipids or collagen fiber in tissues

Balog *et al*. Hepatology 2020

Enzyme histochemistry

Detection of senescent cells expressing β-galactosidase in liver fibrosis



Krizhanovsky *et al*. Cell 2008

Immunohistochemistry

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

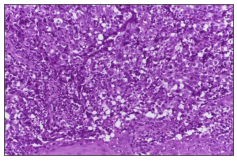
Antigens: proteins, sugars, lipids, nucleic acids, etc…

Immunohistochemistry will give you following information:

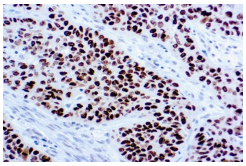
- **Which cells express the antigen you test.**
- **Where the antigen localizes.**
- **How the expression of the antigen changes.**

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

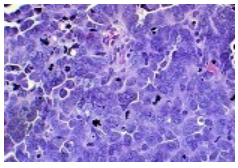
Squamous carcinoma



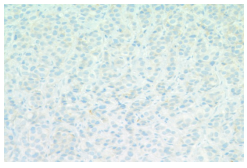
p40 (p63 isoform)



Adenocarcinoma

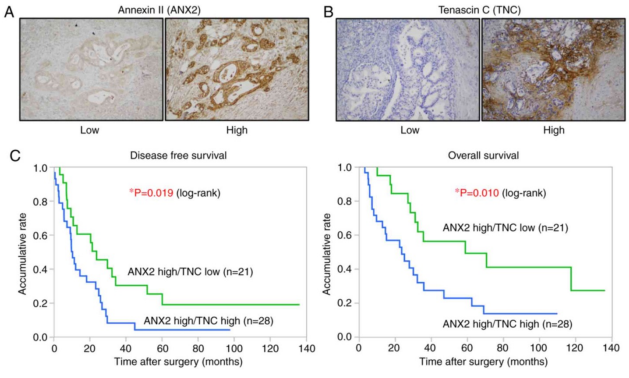


p40 (p63 isoform)



Immunohistochemistry

Correlation of marker expression and survival of patients with PDAC



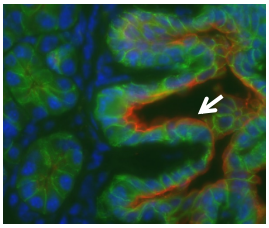
Yoneura *et al*. Int J Mol Med 2018

Immunohistochemistry

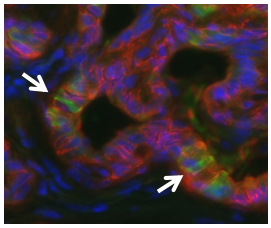
Stem cells may express unique antigens

Pancreatic cancer

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



EPCAM (red)
DCLK1 (green)



Asahina *et al*. Am J Physiol 2020

Procedure of immunohistochemistry

1. **Collect tissues**
2. **Make sections**
3. **Blocking sections**
4. **Apply primary antibodies**
5. **Detect primary antibodies**
6. **Observe the sections under microscope**

Microscopic observation requirement:

Thin Section (5-7 μm)

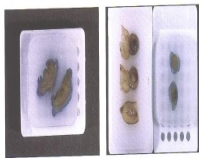
↓

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.

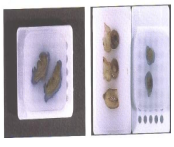
Paraffin blocks and sections

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

Tissue

Without fixation → **Tissue freezing medium** → **Freeze as rapidly as possible** → **Store in -80°C deep freezer**

OCT compound

Liquid nitrogen or Isopentane / dry ice to avoid ice crystal formation

With fixation → See next slide

With fixation

4% Paraformaldehyde (PFA)

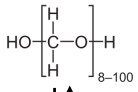
Insoluble polymerization product of formaldehyde

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

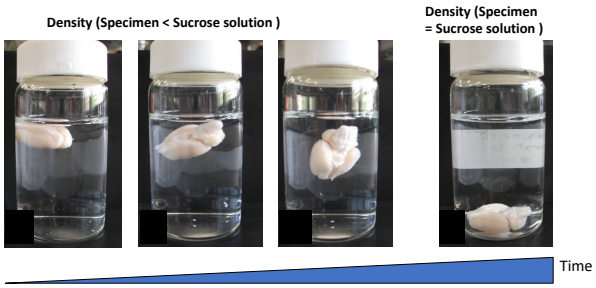
↓

Freeze as rapidly as possible

↓

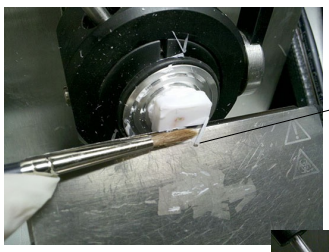
Store in -80°C deep freezer

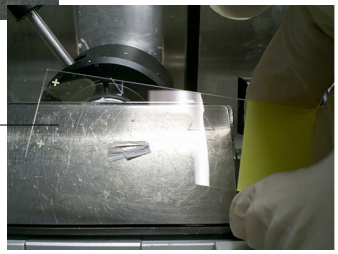
Cryoprotection with 30% sucrose



Cryostat

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





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 µm)
2. Dry
3. Fix the sections if necessary
4. Remove freezing medium in PBS
5. Blocking the sections 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. Detection of 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

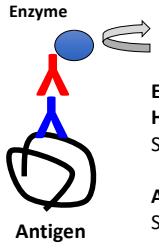
Monoclonal antibody

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



Coloring using a specific substrate

Enzymes

Horseradish peroxidase (HRP)
Substrate: 3,3'-diaminobenzidine (DAB, brown)
Aminoethyl carbazole (AEC, red)

Alkaline phosphatase (AP)
Substrate: Fast Red (red)
Nitro blue tetrazolium chloride (NBT, blue)

Y Primary antibody
Y Secondary antibody conjugated with enzymes

Inactivation of endogenous peroxidase or phosphatase before antibody incubation

Endogenous peroxidase

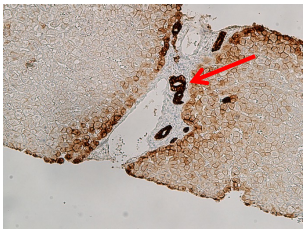
Incubate sections with 0.3-3% hydrogen peroxide (H_2O_2) in methanol (or water)

Endogenous phosphatase

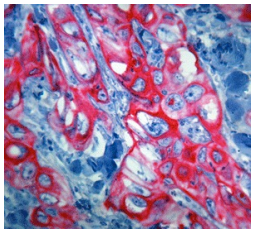
Incubate with levamisole, an inhibitor for alkaline phosphatase

Immunohistochemistry

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



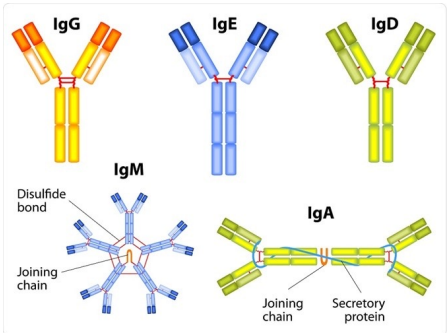
Human squamous carcinoma
Primary antibody: cytokeratin
Secondary antibody: anti-mouse-AP
Color: FastRed (Red)



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

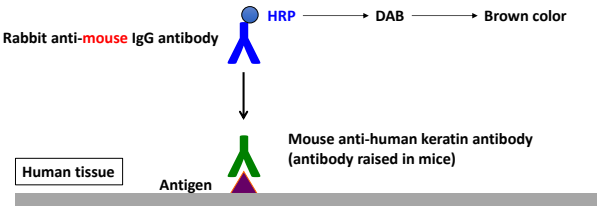
Secondary antibodies for mouse IgG subclasses
- Anti-mouse whole IgG antibody: recognize all IgG subclasses
- Anti-mouse IgG1 antibody: only recognize IgG1
- Anti-mouse IgG2a antibody: only recognize IgG2, ect…

Rat
- IgG1, IgG2a, IgG2b, IgG2c

Rabbit
- IgG (only 1)

Chicken
- IgY

Immunohistochemistry

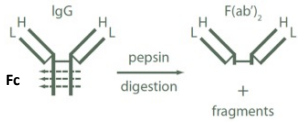


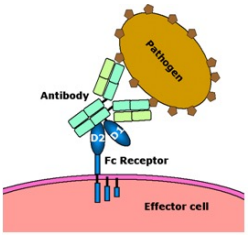
Blocking: 5% rabbit serum, 0.2% bovine serum albumin
Pretreatment: 3% H_2O_2 in methanol for inactivation of endogenous peroxidase

How to select secondary antibodies?

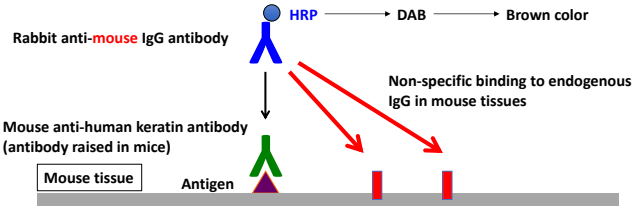
Whole Immunoglobulin or fragments?

F(ab')2 fragment of IgG
- Remove the Fc fragment by pepsin digestion
- Avoid binding to Fc receptors expressed on tissues





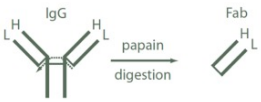
How to stain mouse tissues using mouse antibodies?



Available methods

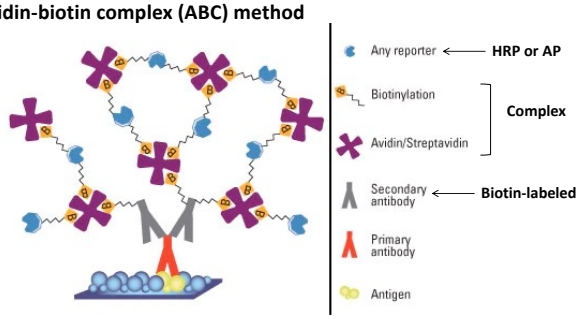
Use primary antibodies conjugated with fluorescence dye or enzyme
without use of secondary antibody (limited availability of antibodies, relatively low signals)

Blocking endogenous mouse IgG with Fab



How can we enhance weak signals in IHC?

Avidin-biotin complex (ABC) method

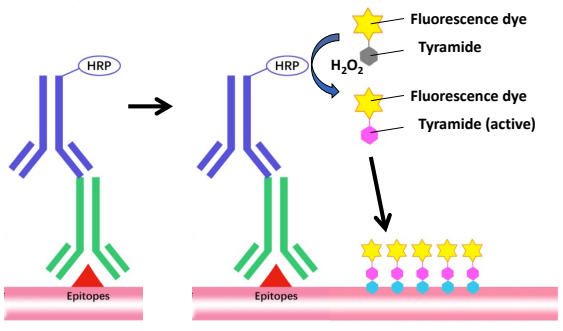


Protocol
Apply primary antibody
Apply secondary antibody conjugated with biotin
Apply Avidin-Biotin-reporter complex
Detect the complex through HRP or AP

ThermoFisher Scientific

How can we enhance weak signals in IHC?

Tyramide Signal Amplification (TSA) method

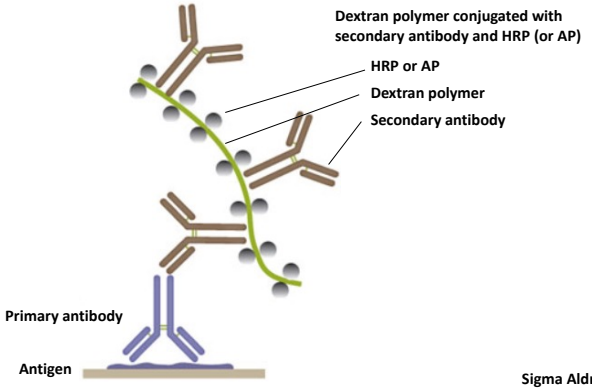


Radical tyramide (active) covalently binds to tyrosine residues near the antibody

APExBio

How can we enhance weak signals in IHC?

Polymer-based detection method



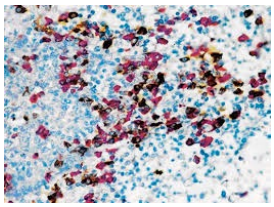
Sigma Aldrich

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

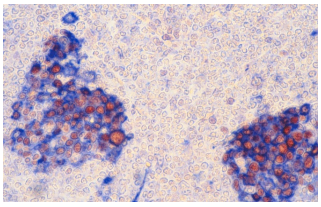
Immunohistochemistry using 2 different antibodies

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



Dako

Spleen
BCL-6 nuclear stain (HRP-AEC, brown)
PNA cytoplasmic stain (AP-Fastblue, blue)

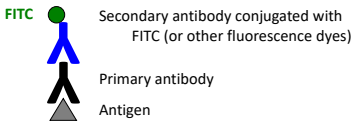


IHC world

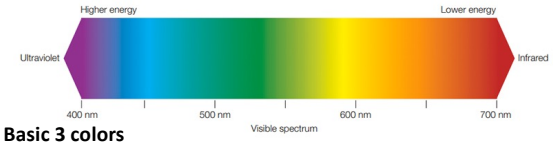
HRP and AP can be used for detection of 2 different antigens.
It is less clear to see co-localization of 2 antigens using these colors.

Immunofluorescence staining (Immunohistochemistry using fluorescence dyes)

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



Spectrum of fluorescence



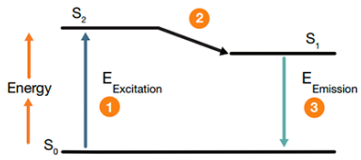
Basic 3 colors

Blue Green Red

Bio-Rad

How fluorophore gives fluorescence?

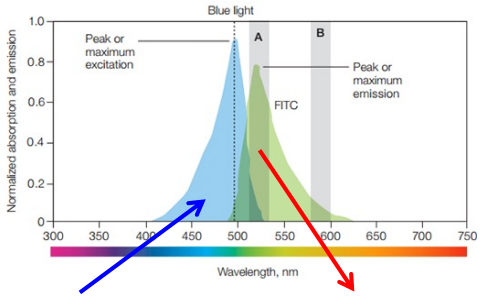
FITC (fluorescein, green)



- **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)



Filter

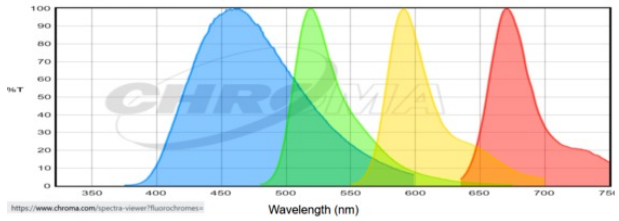
Filter

Laser

Detection of FITC by microscope

Use of different fluorescence colors

DAPI, FITC, Rhodamine, Cy5



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

Choose appropriate fluorescence based on brightness and photostability

Blue
- Alexa Fluore 350
- DyLight 350
- DAPI (DNA staining)

Green
- Fluorescein (FITC)
- DyLight 488
- Alexa Fluore 488

Red
- Tetramethylrhodamine (TRITC)
- Cy3
- DyLight 550
- Alexa Fluore555
- Propidium iodide (DNA staining)

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

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

Procedure of fluorescence immunohistochemistry in frozen sections

1. Make cryosections (7 µm)
2. Dry
3. Fix the sections if necessary
4. Remove frozen medium with PBS (no need to inactivate endogenous peroxidase or alkaline phosphatase)
5. Blocking the sections 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

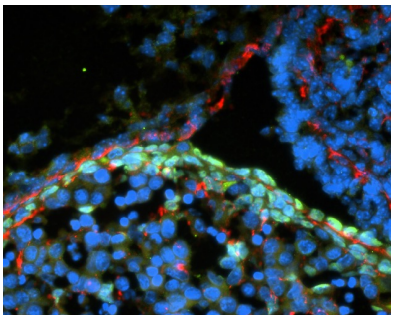
Fluorescence immunohistochemistry

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

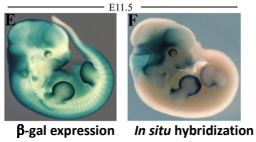
Mouse embryonic liver



Detection of LacZ by fluorescence immunostaining

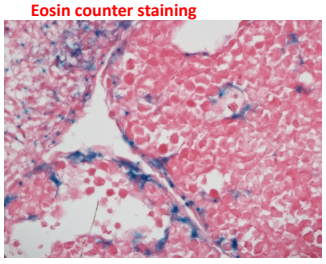
Transgenic mice carrying Msx2 promoter driven β-galactosidase (Asahina *et al*. 2009)

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

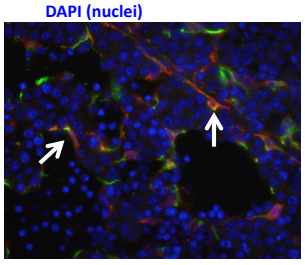


β-gal expression *In situ* hybridization

Enzymatic β-galactosidase activity
Eosin counter staining



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

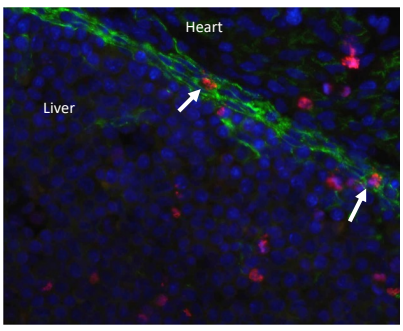


Detection of proliferating cells by fluorescence immunostaining

Protein markers for cell proliferation

Phosphorylated Histone H3	M phase
Proliferation cell nuclear antigen (PCNA)	G1 and S phases
Ki-67	G1, S, G2, M phases

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

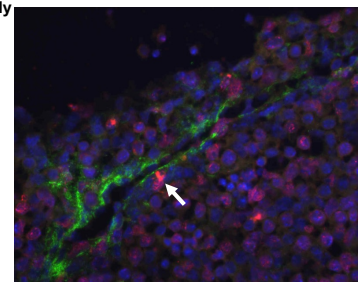


Detection of proliferating cells by fluorescence immunostaining

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 μm)

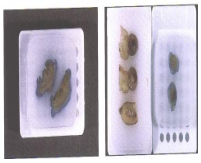
↓

How to cut thin tissue section

↓ **Frozen section**

↓ **Formalin-fixed paraffin-embedded (FFPE) section**

Archive pathology specimens

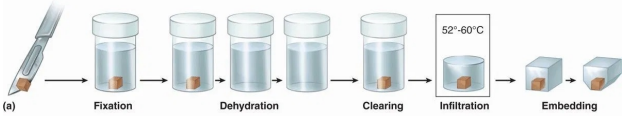


Advantages of FFPE

- **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.**

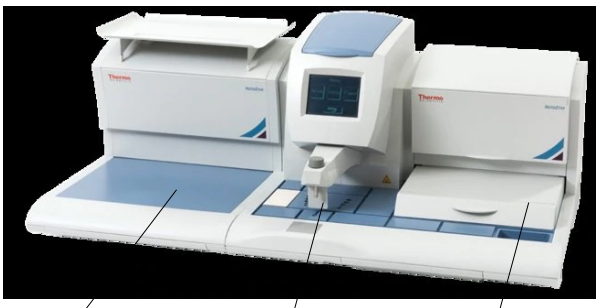
Immunohistochemistry using paraffin sections

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



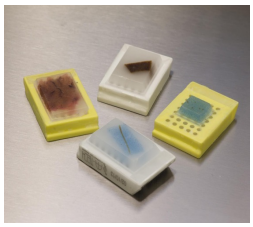
Automated tissue processor

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

Mount paraffin sections onto a slide glass

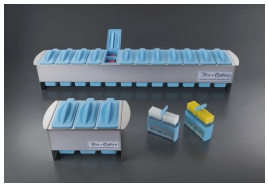
Tissue section surrounding with paraffin (white)

Use ID labels for specimens

Immunohistochemistry using paraffin sections

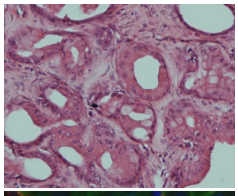
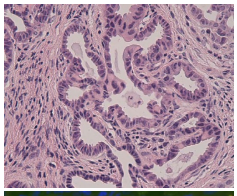
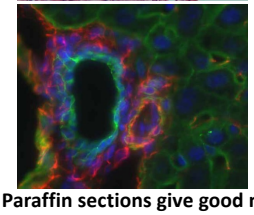
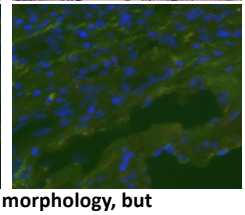
1. Remove paraffin from the sections on the slide in Xylene
2. Replace to a serial series of ethanol from 100% to 70%
3. Wash with PBS
   (Treat sections with antigen retrieval solution if necessary)
4. Inactivate endogenous peroxidase in 3% H_2O_2 in methanol
5. Block with 5% serum, 0.2% bovine serum 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

	Frozen section	Paraffin section
HE staining		
IHC Desmin CXADR		

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.

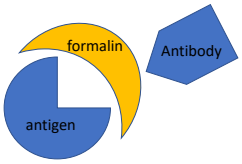
There are several methods to overcome these issues.

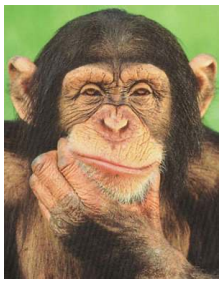
Use enzymes for partial digestion of sections.

Use an antigen retrieval solution for breaking crosslink in sections.

Drawback of FFPE

Formalin induces chemical modification to "mask" antigens, so most antigens on the tissue sections become inaccessible for immunohistochemistry (IHC).





How to solve the problem?

How to solve the problem- Approach 1

Replace formalin with non-formalin fixatives like ethanol, methanol, or acetone.

Pathologists hate to use a new fixative.
It changes the morphology and they cannot make the diagnosis.

Researchers hate to use a new fixative.
FFPE specimens in pathology accumulated in the past 100 years become useless.

How to solve the problem – Approach 2

Develop formalin-resistant antibody

↓

Not always possible

How to solve the problem – Approach 3

Pretreatment ------- Enzyme digestion

FFPE → (Trypsin) → Destroy the crosslink

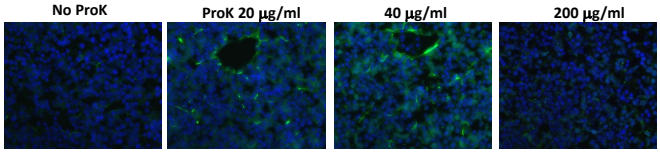
↓

IHC ?????

How to improve staining by enzyme treatment?

Proteinase K: Digest proteins completely if incubate longer
Digestion should be stopped by washing 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 (not always works)

How to solve the problem – Approach 3

Pretreatment ------ Enzyme digestion

FFPE —Trypsin→ ????? Destroy the crosslink

Limited Application

- Only worked for a limited number of antigens
- Digestion condition is difficult to control:
  - Too little digestion: cannot recover the antigen
  - Too much digestion: tissue section is destroyed by proteinase.

How to solve the problem – Approach 4

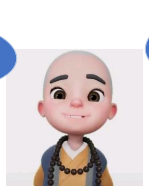
Remove formalin-induced modification and
Retrieve the antigenicity in FFPE

- 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.

Does it work????
How to make it work???

- Formalin induced chemical modification of protein (crosslinkage) can be reversed by:
  - high-temperature heating
  - strong alkaline treatment.



It is reversible!!!

Can antigens be cooked without damage???

Antigen retrieval treatment

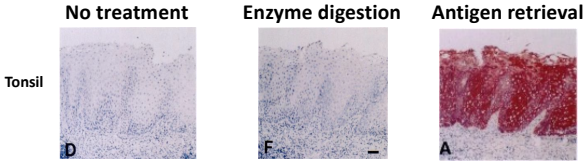
Seeing is Believing

Soaked FFPE sections in the solution

Heated in microwave 5 min x 2 (high temperature)

→ IHC

Monoclonal anti-cytokeratin antibody staining by 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 (1C2212).

Enzyme digestion vs. Antigen retrieval

- Both are pretreatment of IHC
- Different approach
- Different antigen recovering effect

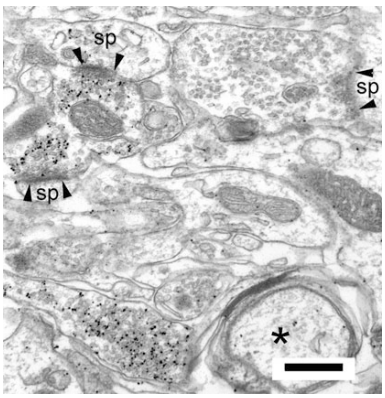
Pretreatment	Enzyme Digestion	Antigen Retrieval
Approach	Proteinase-induced destruction	High temperature-induced recovery
Effect	Destruction is difficult to control Effective in some antigens	Simple, effective and widely applied in almost all antigens recovery in FFPE

Electron microscopy and immunohistochemistry

Immunogold labeling

Transmission electron microscopy

Synaptophisin in the synaptic vesicles

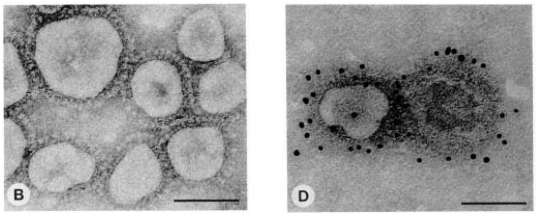


Immunoelectron microscopy 2010

Visulalization 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-nAChR antibodies were tested on nAChR-KO mice.
  Same immunoreactivity was observed in wildtype and KO 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.
  Some worked but recognized the wrong target histone.

Assessment of the antibody specificity

All histochemical and immunohistochemical methods are prone to artefact and unspecific staining.

Researchers need to have apropriate 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

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