化學及免疫組織化學染色於組織病理的應用

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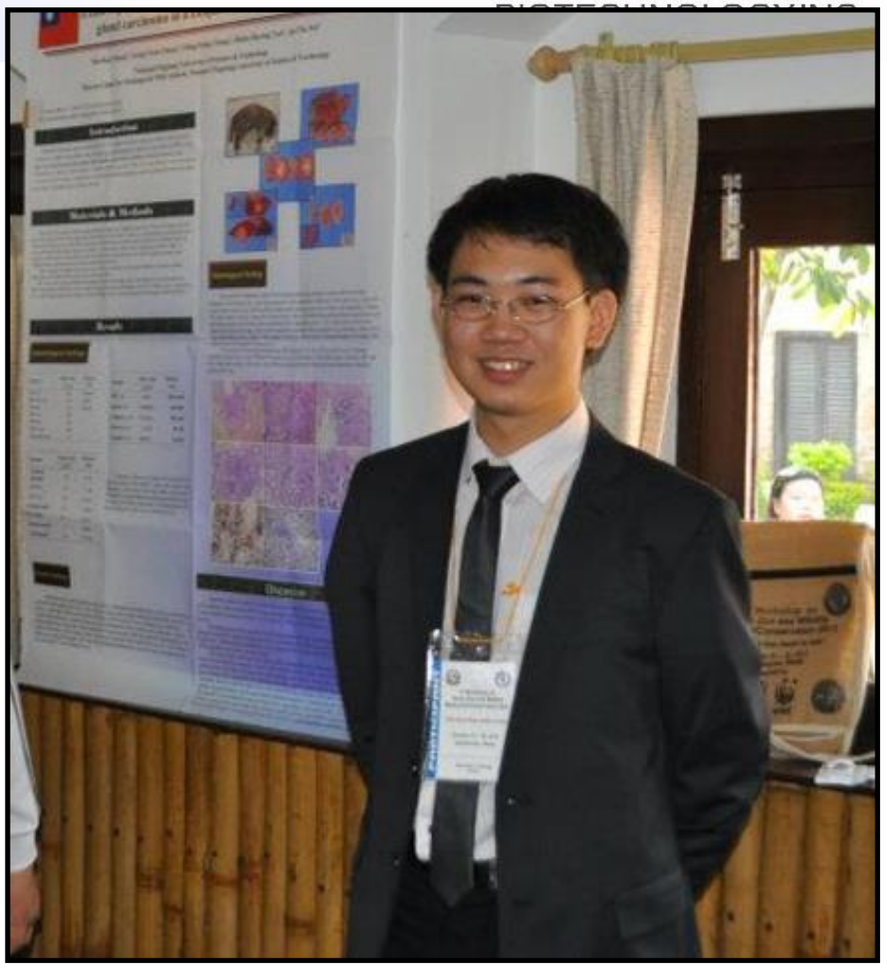
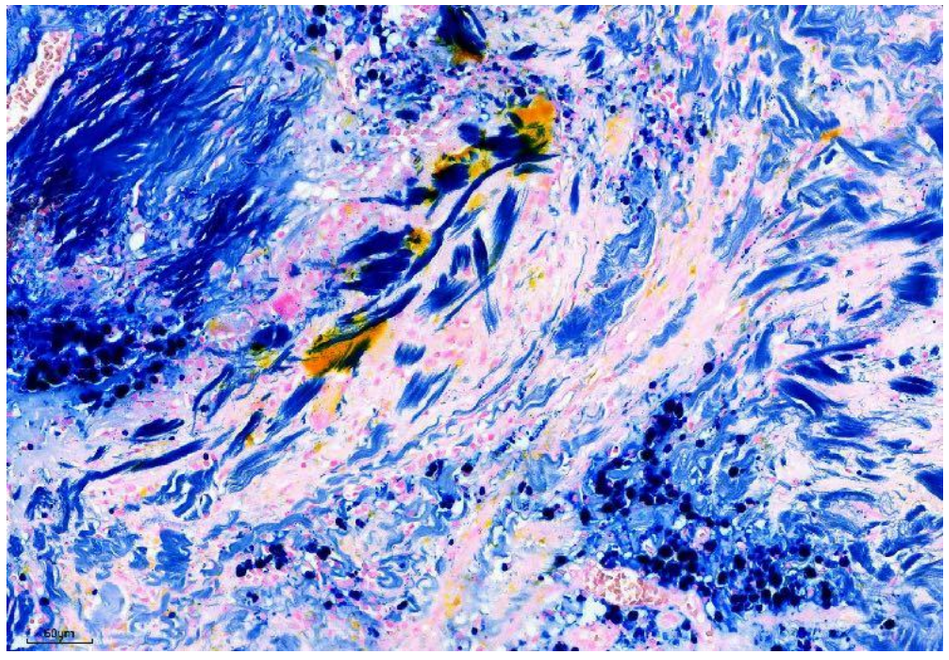


Outline

- Introduction: 組織切片製作
- 常見化學染色(histochemical stain)
  - 染色原理、結果、常見問題
- 免疫組織化學染色(immunohistochemical stain, IHC)
  - IHC操作技術拆解
  - IHC染色結果？
  - 我就要一次N個marker !
- 原位雜合試驗(in situ hybridization, ISH)
- ISH × IHC × histochemical stain 各種混搭



Introduction

組織切片製作流程

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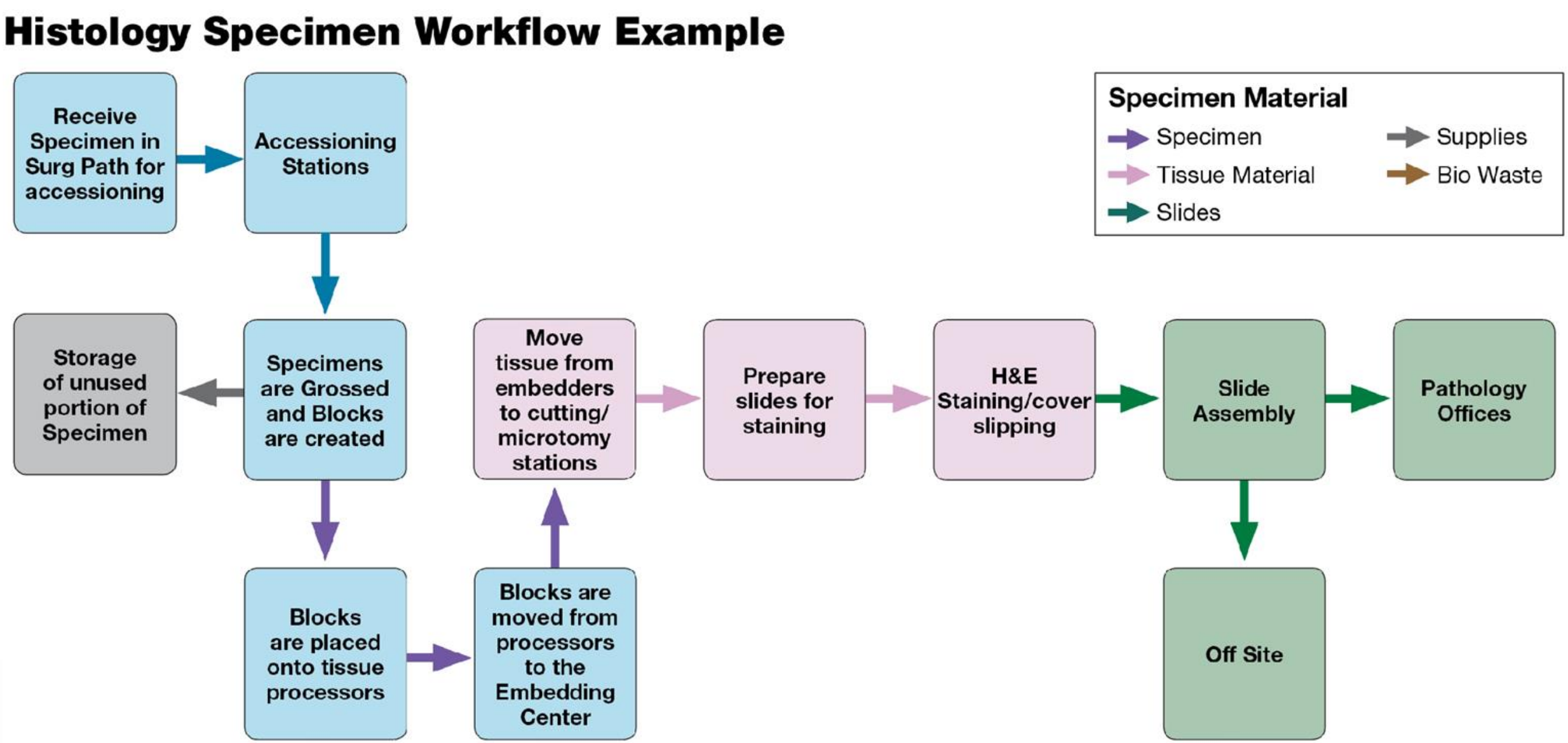

https://www.medlabmag.com/article/1436

組織固定

- 常見固定液
  - 福馬林(Formaldehyde, CH_2O)
  - Paraformaldehyde (PFA, = higher polymers of formaldehyde)
  - Helly’s solution
  - Bouin’s solution
  - Davidson’s solution
  - Zenker’s solution
  - Glutaraldehyde
  - Osmium tetroxide

Armamentarium

TOXICOLOGIC PATHOLOGY, vol 30, no 4, pp 524–533, 2002

DOI: 10.1080/01926230290105721

Fixation of Testes and Eyes Using a Modified Davidson's Fluid: Comparison with Bouin's Fluid and Conventional Davidson's Fluid

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Bouin's solution

modified Davidson's solution

不同固定液的效果

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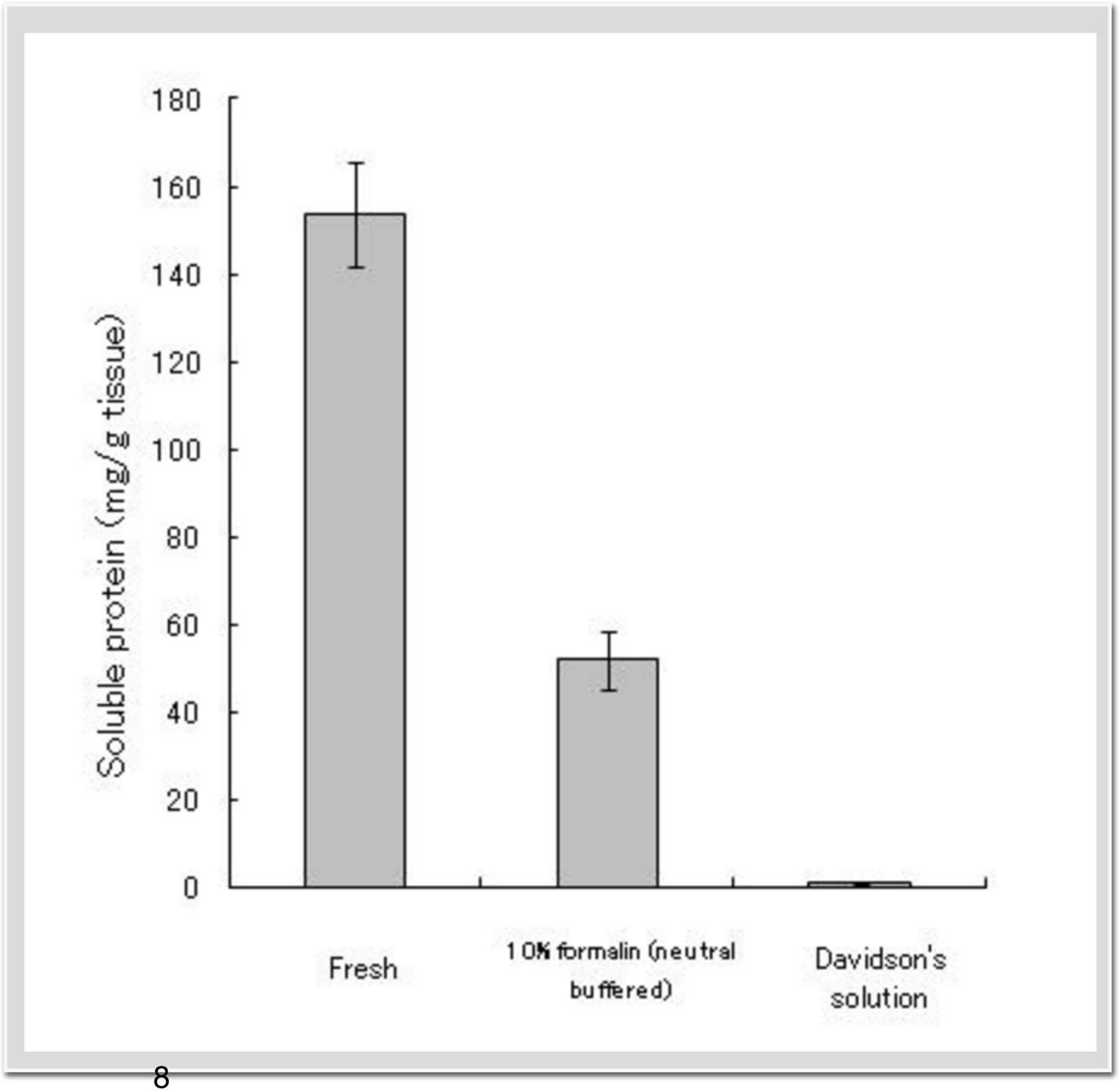
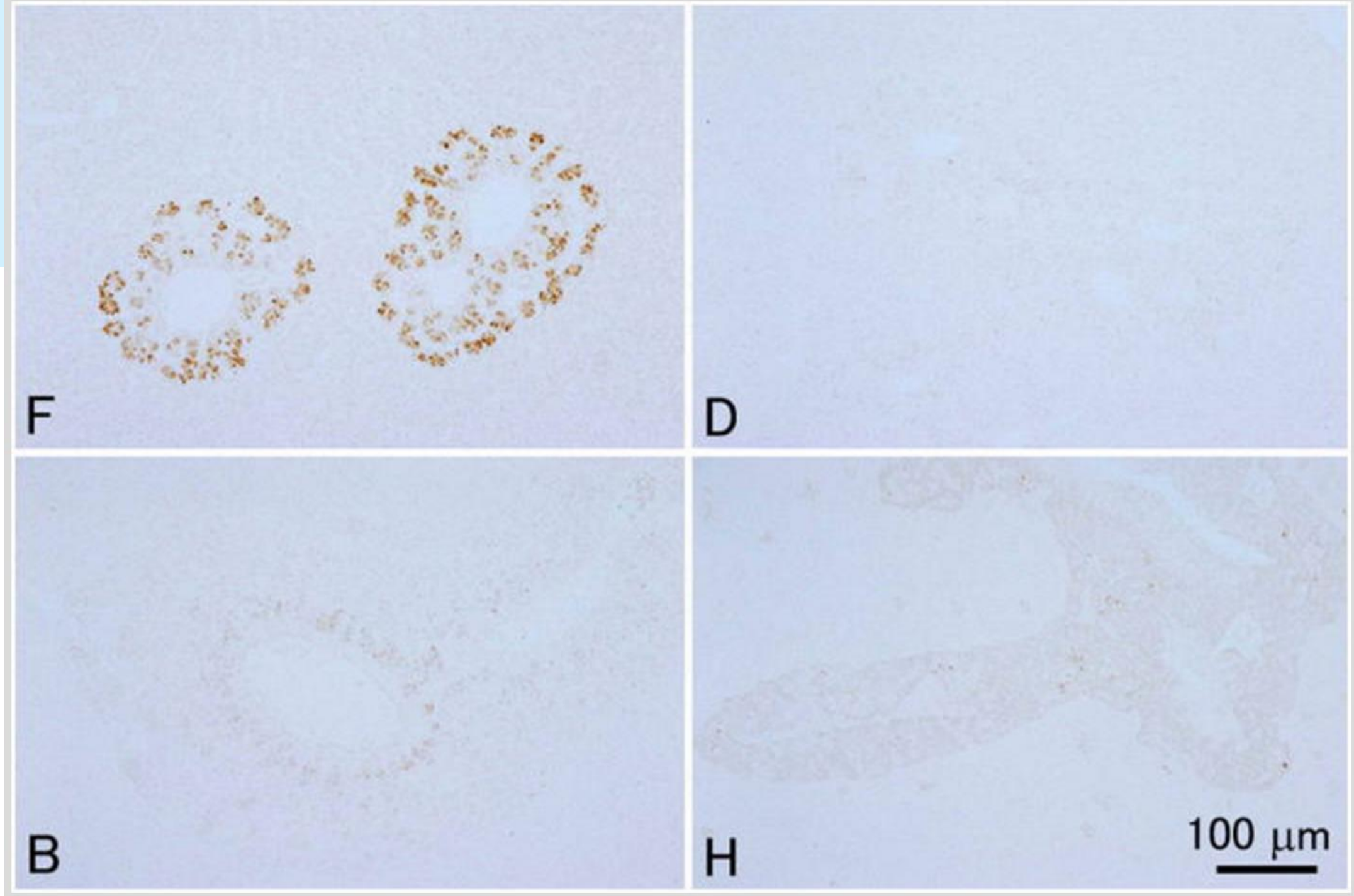
FRA Fisheries Research Agency National Research Institute of Aquaculture

When the liver of trout is fixed in either 10% neutral buffered formalin or Davidson's solution for 35 minutes, a substantial amount of soluble protein still remains in the tissue fixed in 10% formalin, whereas virtually no soluble protein remains in the tissue fixed in Davidson's fixative





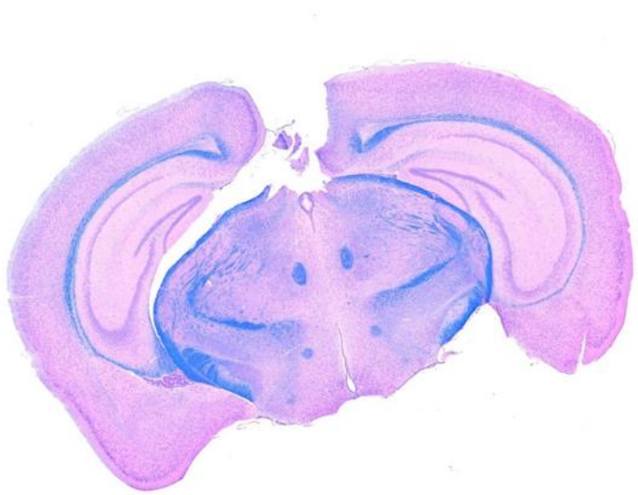
IHC for tripsinogen in the pancreatic tissue in the liver. Rabbit polyclonal antibody was used as the primary antibody.

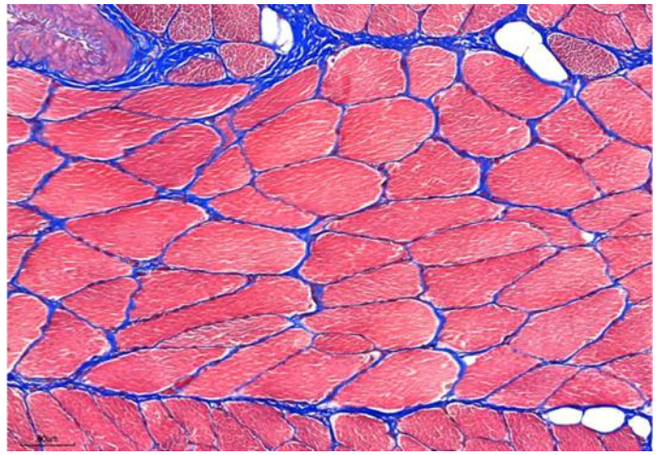
Antigenicity remains only in the tissue fixed in NBF

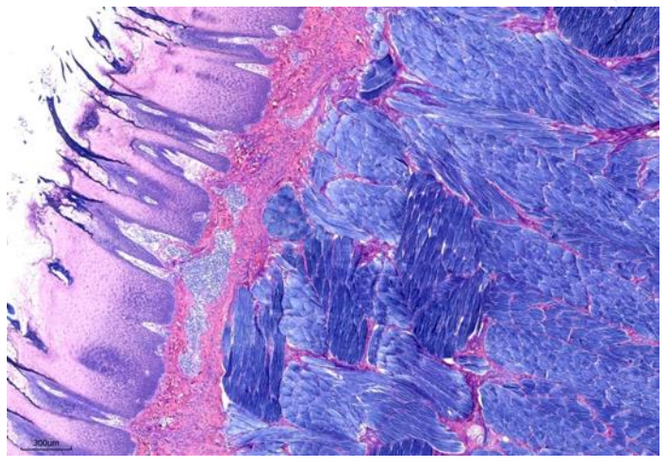
化學染色
(histochemical stain)

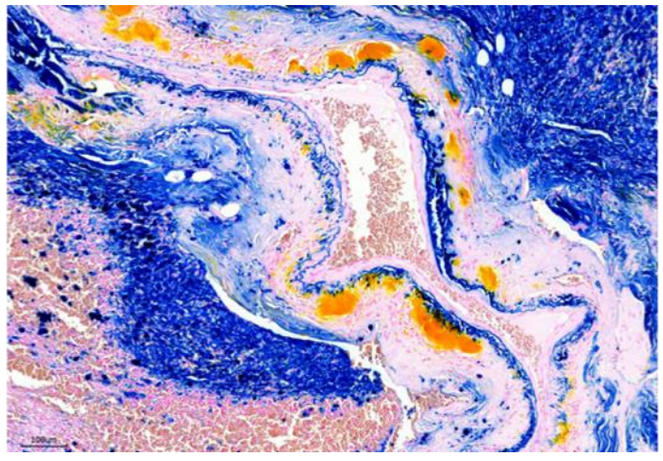
常見化學染色

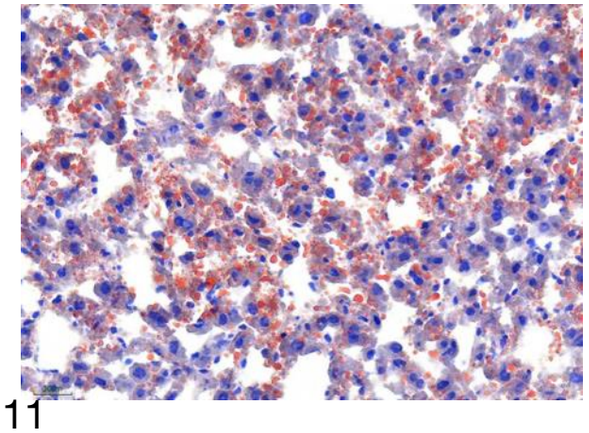
- Masson Trichrome - 膠原纖維 & 肌肉
- Picro-sirius red - 膠原纖維
- Periodic acid Schiff (PAS) - 醣類、黏液
- Alcian blue - 黏液、軟骨
- Toluidine blue - mast cell, 生長板, etc.
- Luxol fast blue (LFB) - 神經髓鞘
- Nissl stain - 神經元、nissl body
- Congo red - 類澱粉 (需偏光鏡)
- Alizarin Red S - 鈣鹽
- Von Kossa stain - 鈣鹽
- PTAH - 橫紋肌、纖維素、骨
- Oil Red O - 油滴 (frozen section only)
- Prussian blue stain - Fe^{3+}

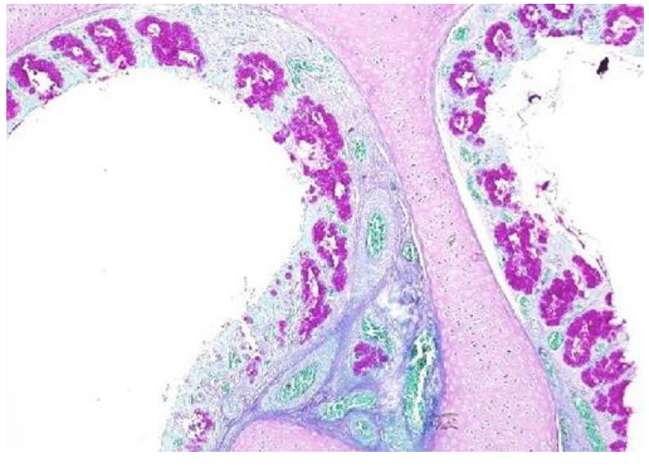












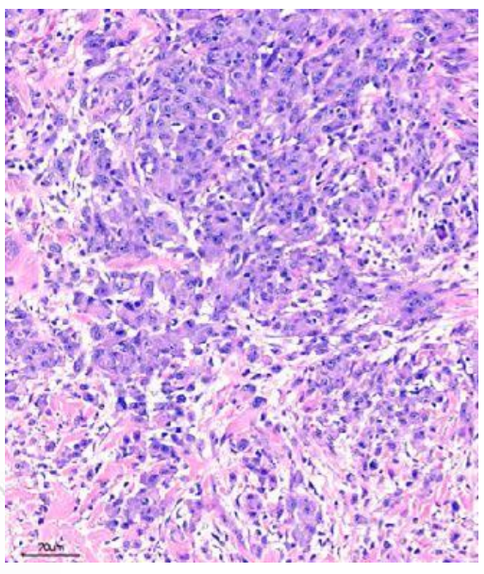
影響化學染色的因子

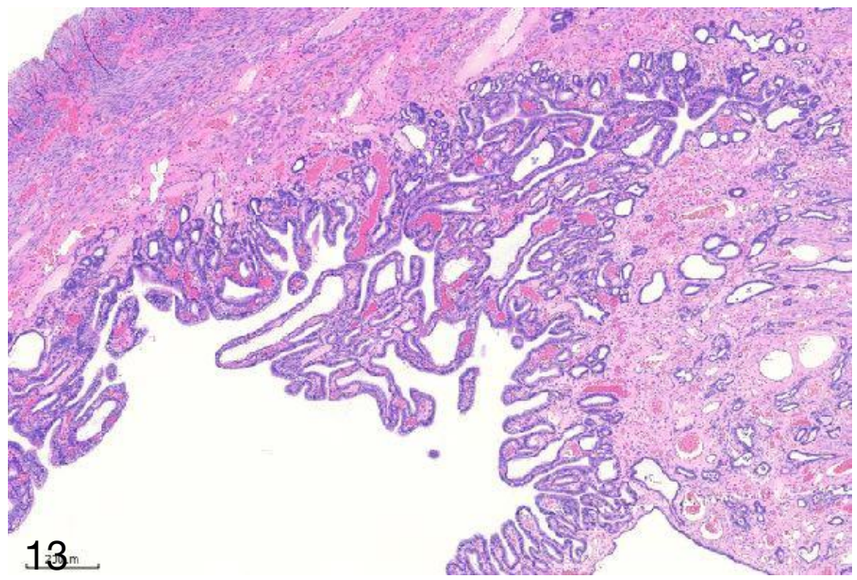
- 物理性質
  - 溶解度(solubility)
  - 吸附性(adsorption)
  - 組織滲透度 (tissue permeability)

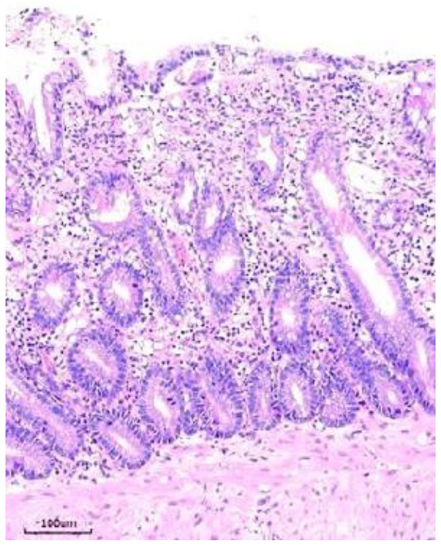
- 化學性質
  - 染劑酸鹼性質
  - 化學鍵結 ⟶ 帶電性
    - Covalent bond, van der waal forces, hydrogen bond

H&E 染色

- Hematoxylin and Eosin stain (蘇木紫-伊紅染色)
  - 組織學最常規的染色方法
  - 蘇木紫：鹼性染劑，帶正電，染核酸(核醣體、細胞核等)，呈藍色
  - 伊紅：酸性染劑，於pH = 6.7 - 6.8時可與蛋白質胺基的正電荷結合，使細胞質呈紅色







Masson trichrome

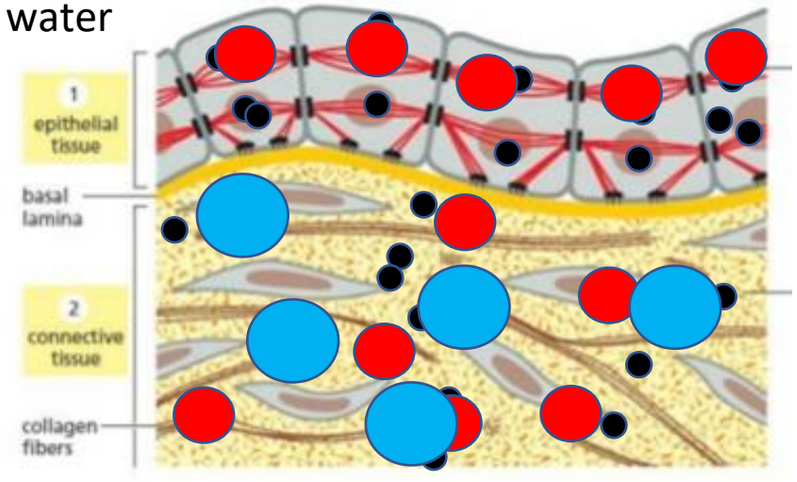
- 最廣泛使用的化學染色之一，主要用以鑑別組織中的肌肉及膠原纖維，但亦會對所有組織產生不同程度的染色
- 主要原理是先使用Bouin’s solution酸化組織，並分別使用不同染色劑進行染色。染劑會因各組織的pH值、分子孔隙大小等因素而有不同的親合性。

Phosphomolybdic/Phosphotungstic Acid Solution (1%)

Acetic Acid Solution (1%)

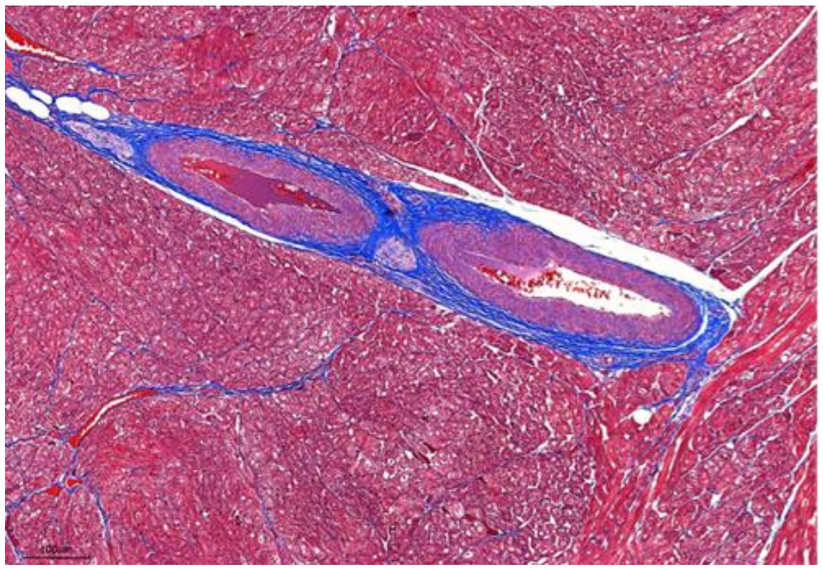
Tap water

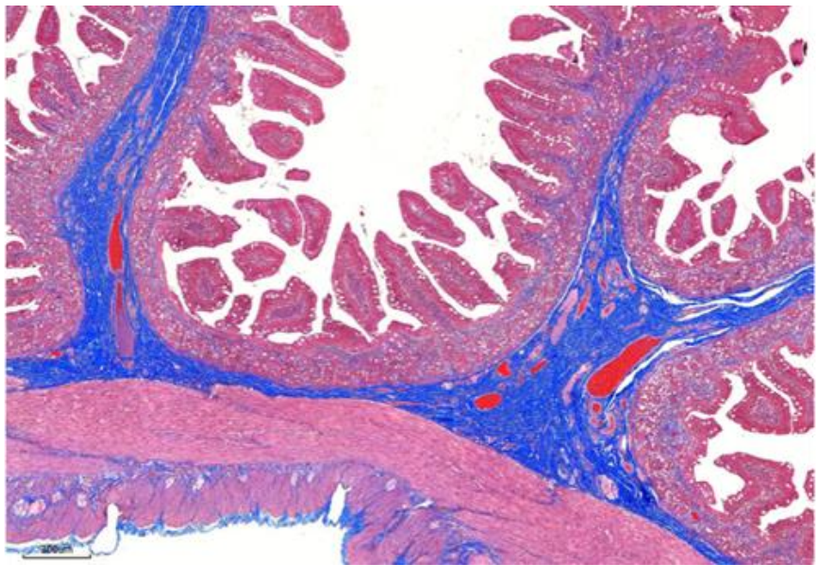
- Weigert's Iron, Hematoxylin
- Biebrich Scarlet/Acid Fuchsin Solution
- Aniline Blue Solution

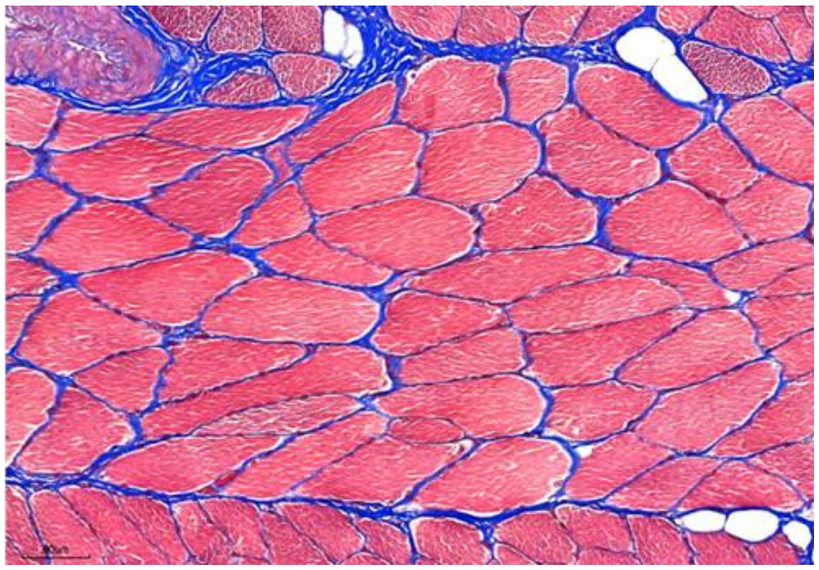


Laslowski, A., *A modified silver methenamine Masson trichrome stain using methyl green for staining of renal biopsies.* Journal of Histotechnology, 2016. **39**(2): p. 66-71.

Trichrome Masson染色結果





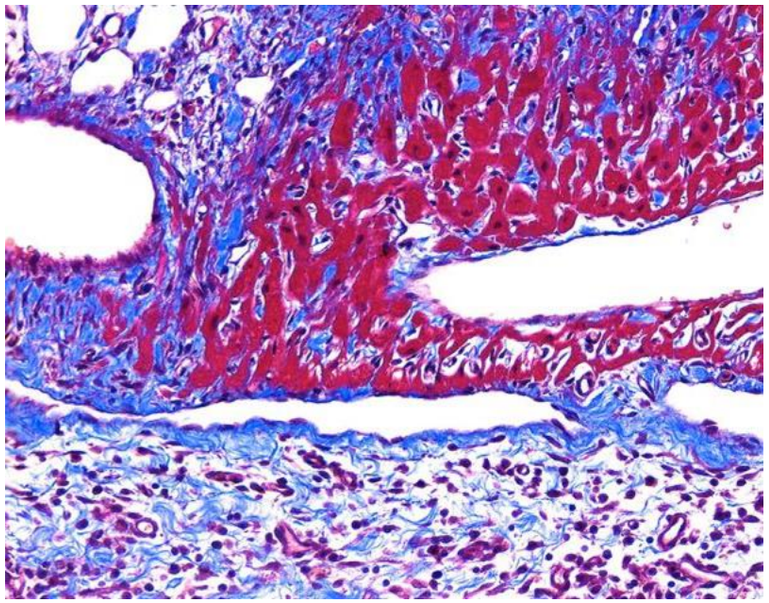


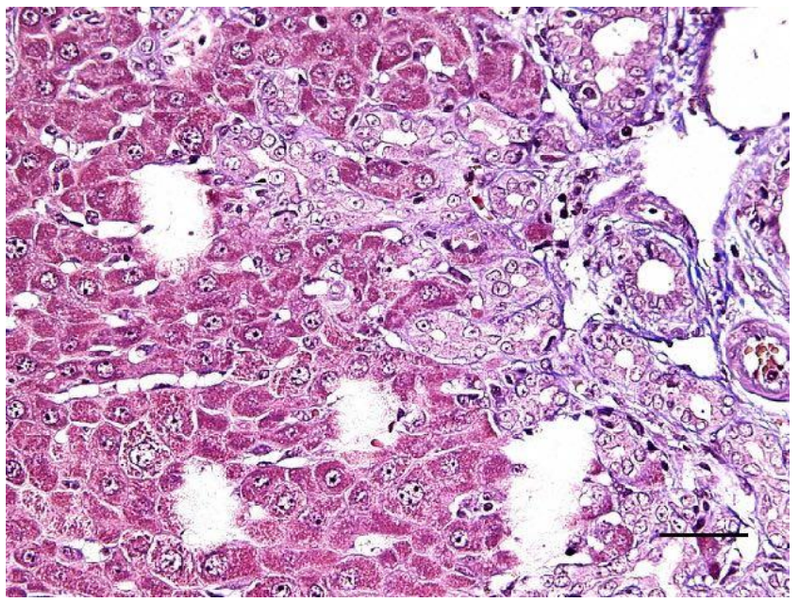
Staining Interpretation

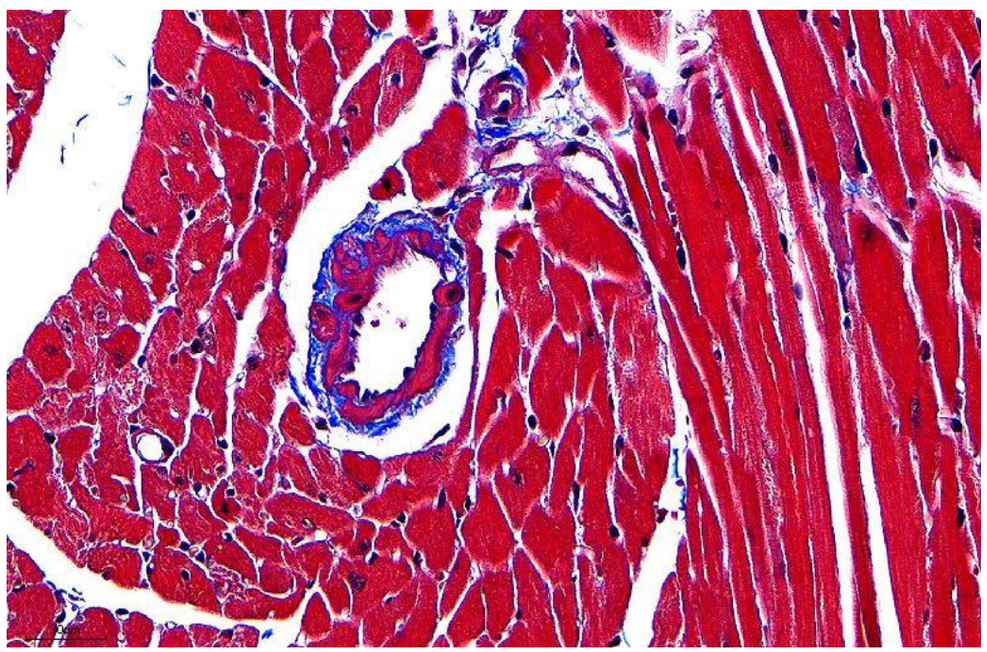
Collagen：Blue
Muscle Fibers：Brick red (橫紋肌會較深)
Nuclei：dark blue

Trichrome Masson染色常見問題

- 藍色無法表現
  - 組織酸化不足
  - 最後wash的水pH值錯誤
- 肌肉不紅 or 變成紫色
  - 組織酸化不足
  - 染劑pH值錯誤
  - 磷鎢酸-磷鉬酸浸泡時間不足
  - 過度沖水
- 細胞核沒有顏色
  - 染劑pH值錯誤
  - 水轉化時間不足
  - 猩紅-酸性復紅浸泡時間過久

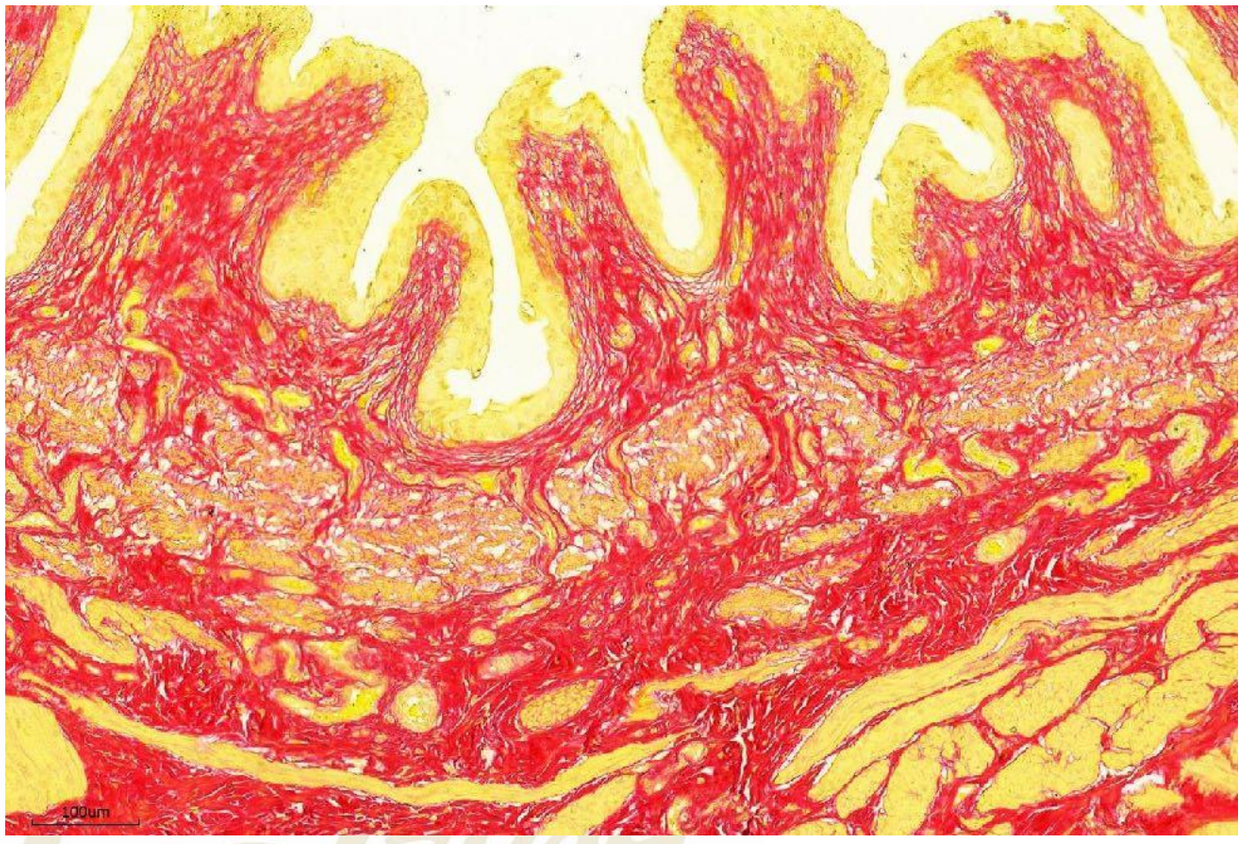


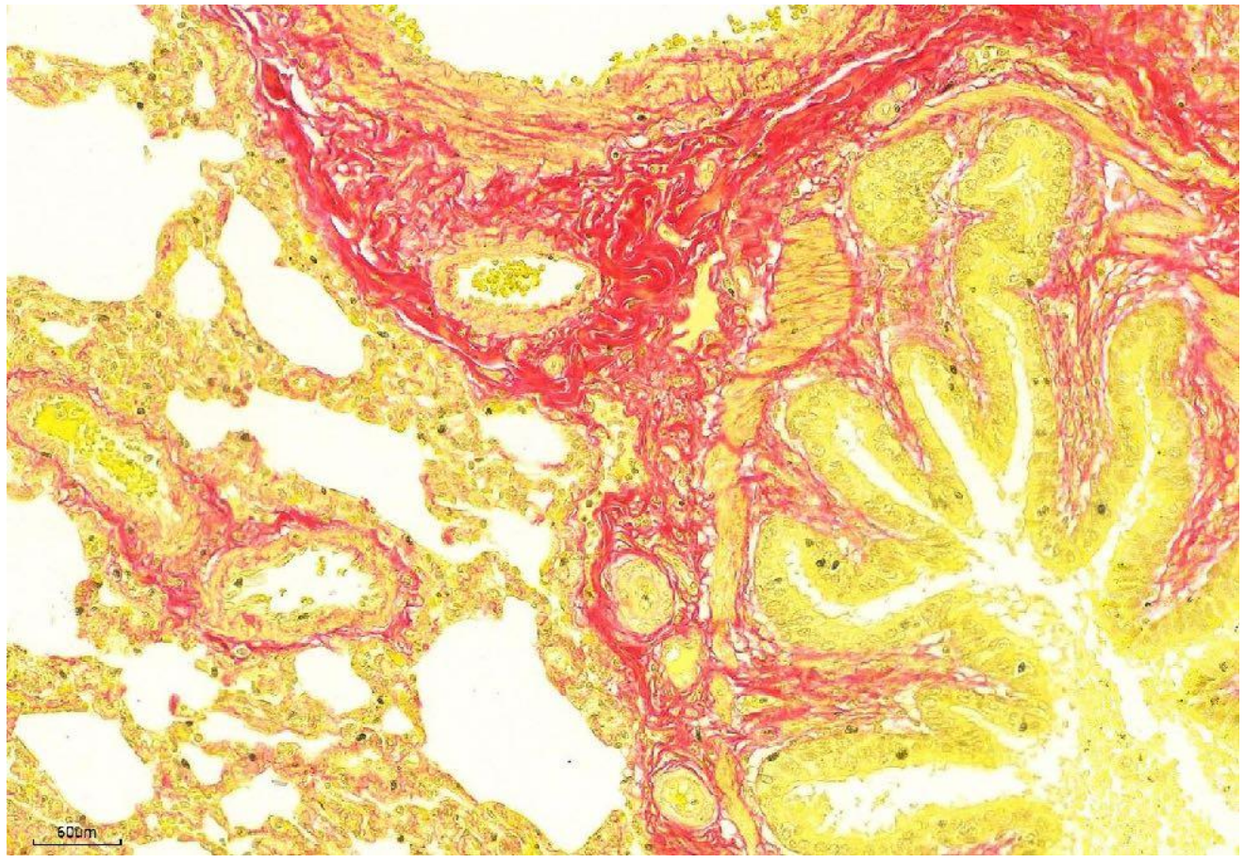




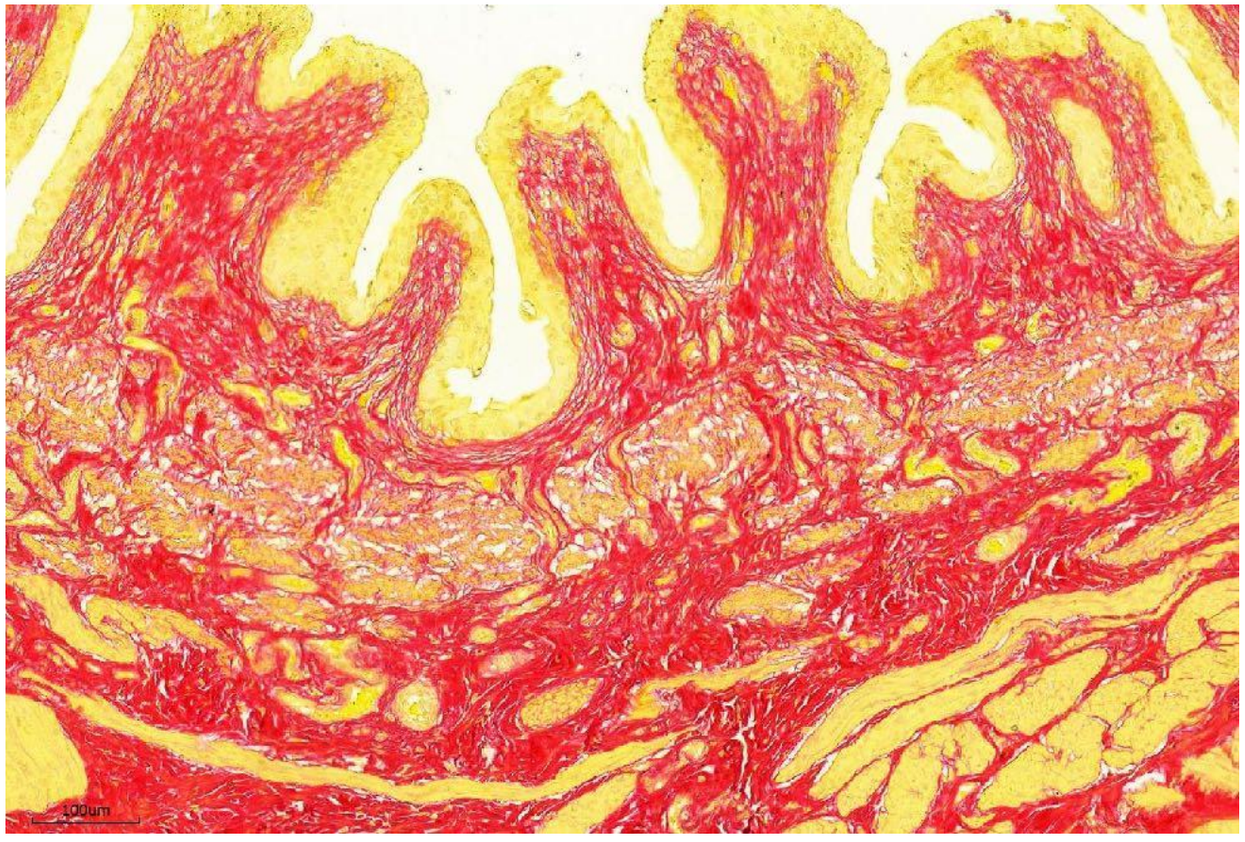
Picro-sirius red

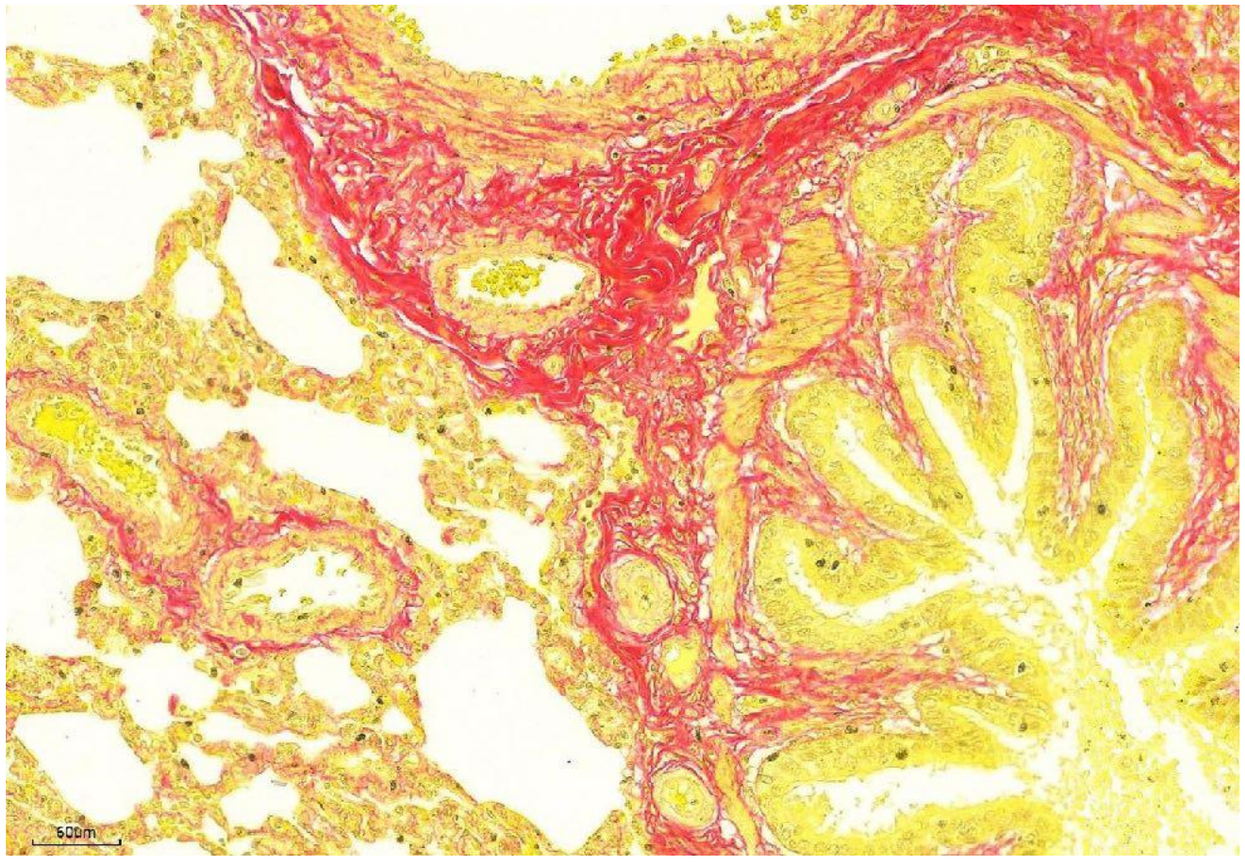
- 天狼星紅，主要用於觀察膠原纖維
- 原理：染劑呈强酸性，易與膠原纖維分子中鹼性基團结合





Picro-sirius red





Staining Interpretation

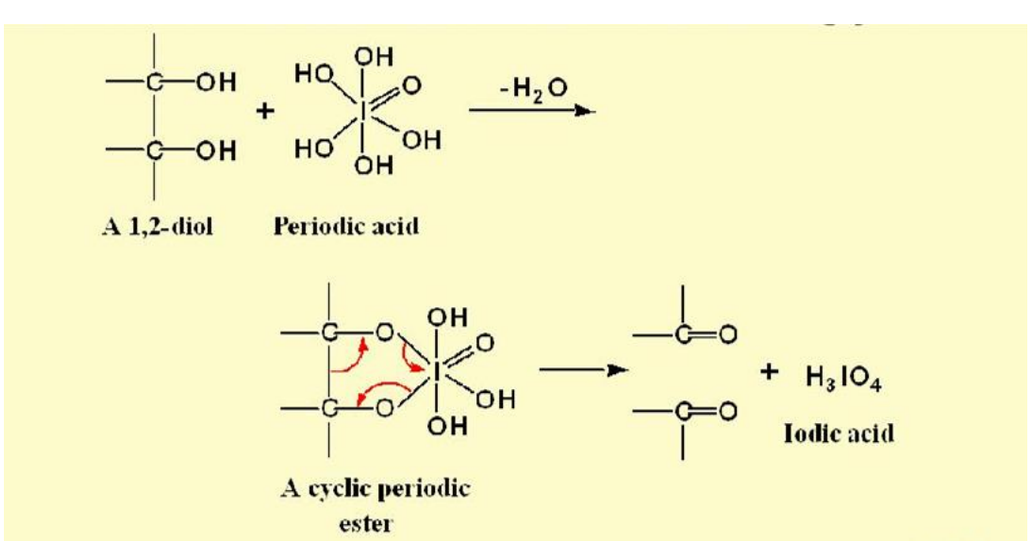
Collagen：Red

Nuclei：dark blue

Other tissue: yellow (苦味酸)

Periodic-acid Schiff (PAS)

- 最廣泛使用的化學染色之一，主要用以鑑別組織中的醛類分子，於組織病理學上，常用於檢驗中性黏液素(neutral mucin)、黏多醣、肝醣、基底膜及黴菌細胞壁等。



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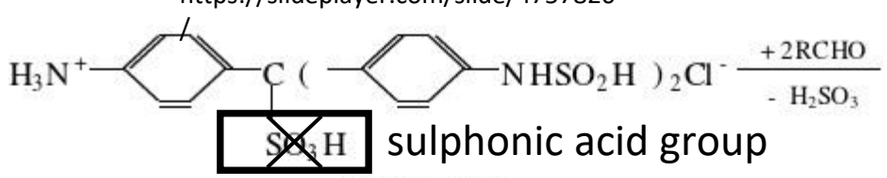
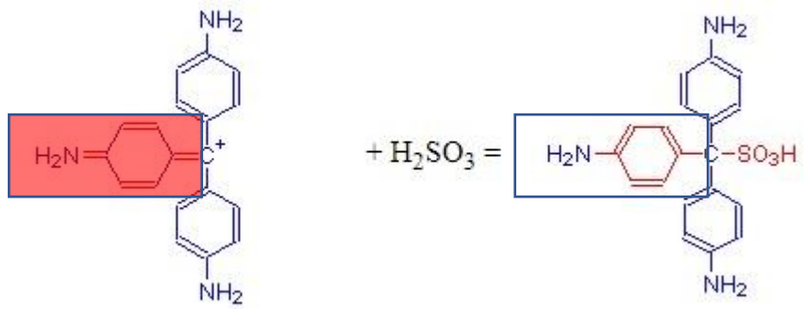
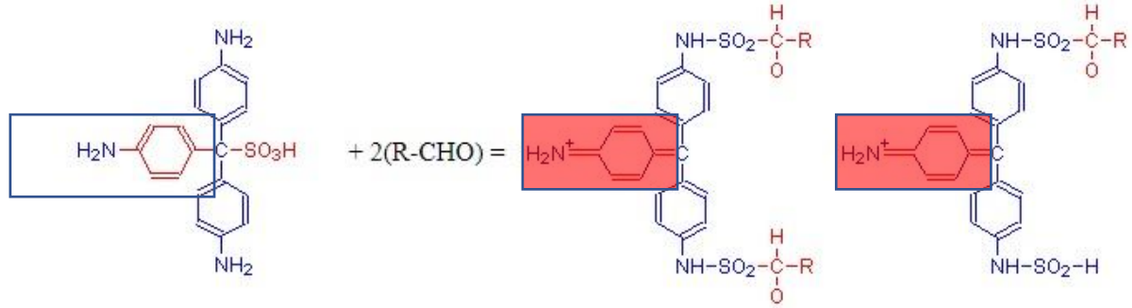


Schiff 试剂(无色)





http://stainsfile.info/stain/schiff/schiffwhatis.htm

https://wenku.baidu.com/view/19806d24bd64783e09122b2a?pcf=2

Periodic-acid Schiff (PAS)

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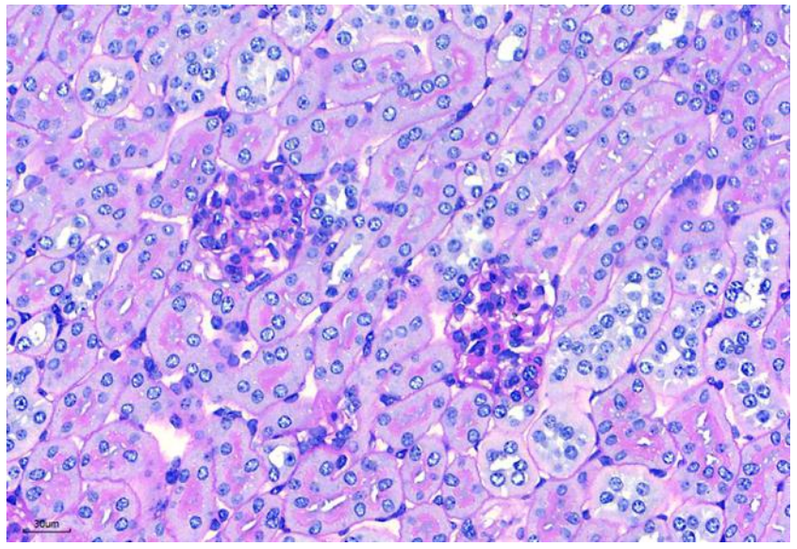
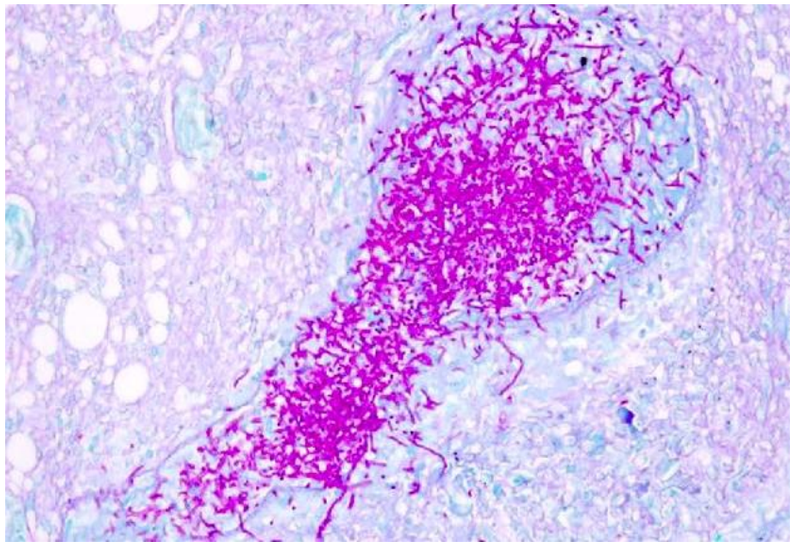
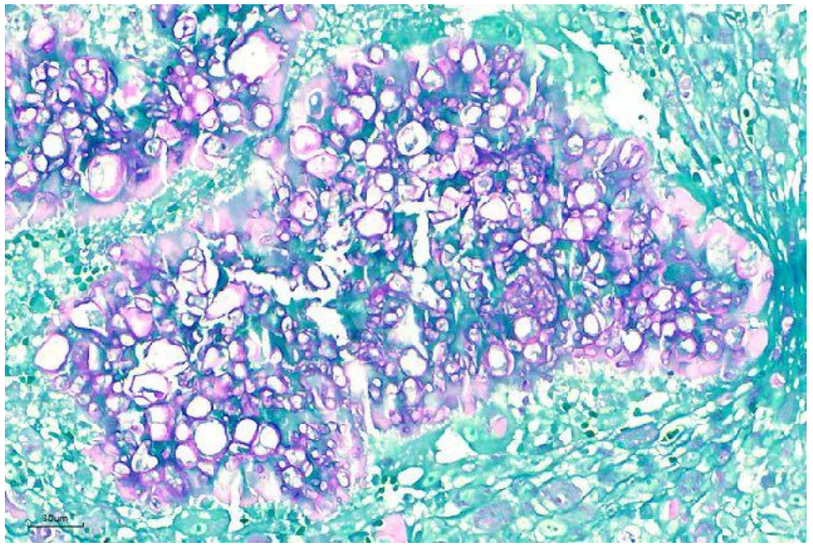
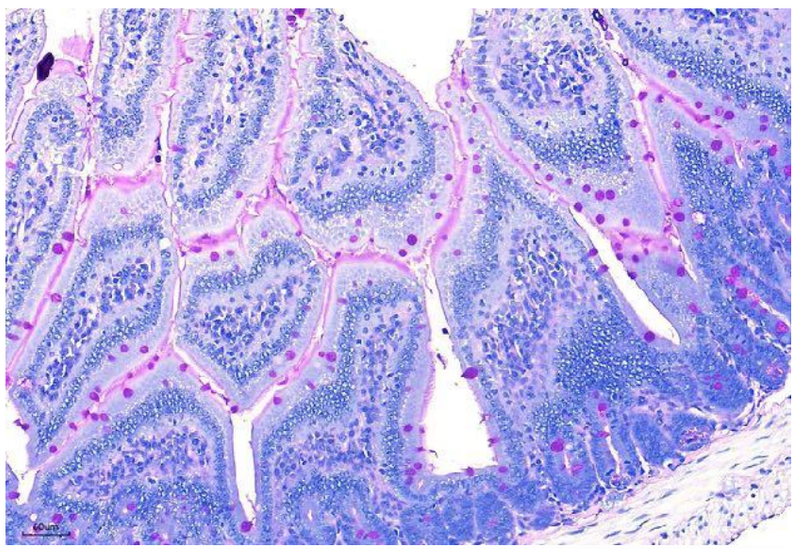
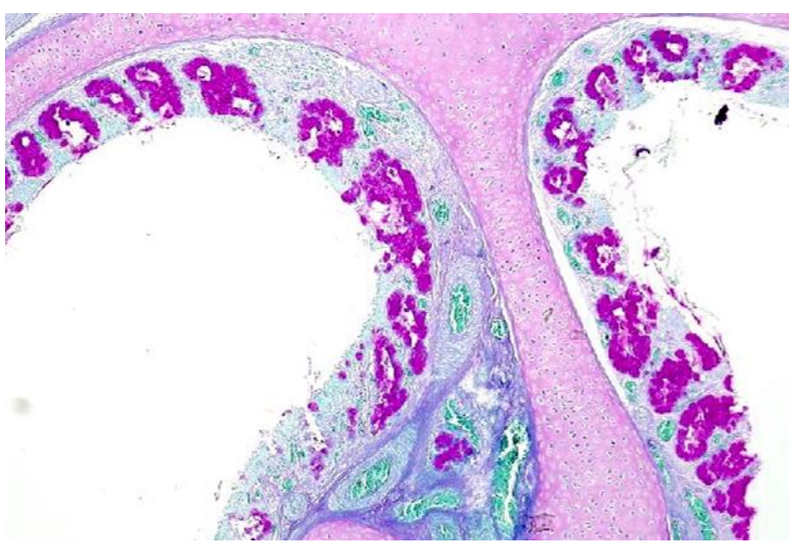










Staining Interpretation
Positive tissue：Pink
Background：blue (hematoxylin) or green (light-green)

PAS常見問題

- 陽性物質不表現
  - 過碘酸(periodic acid)失效或濃度不足
  - Schiff solution失效

- 背景過高
  - Schiff solution的pH值改變
  - 組織pH值不正確 (多見於非福馬林固定組織)

PAS常見問題

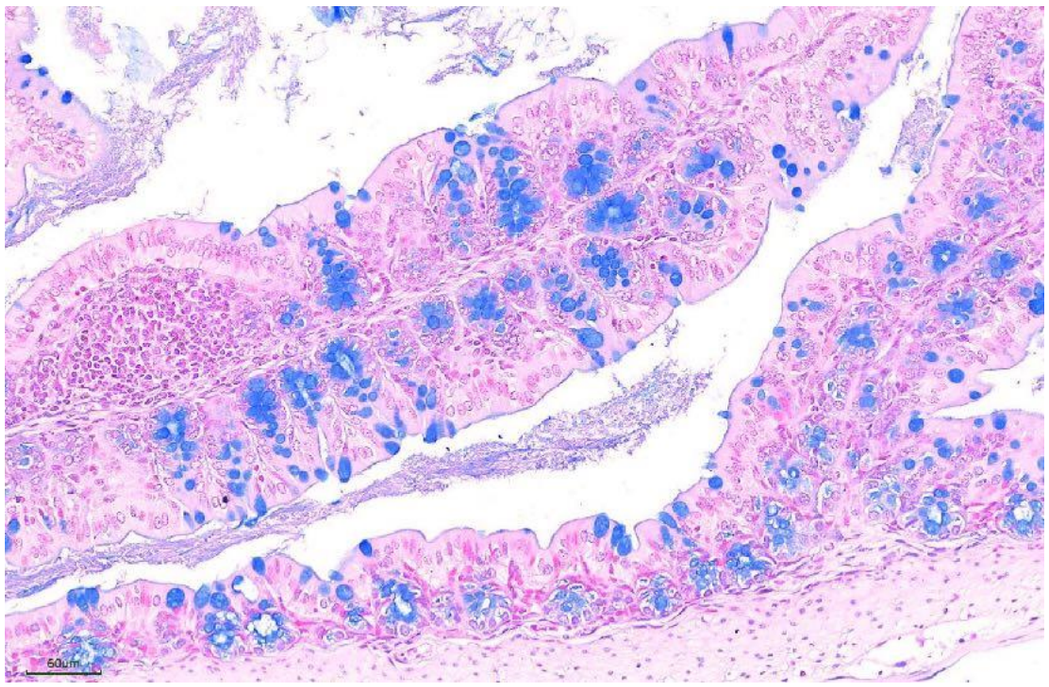
- 石蠟組織切片下能不能染肝醣??

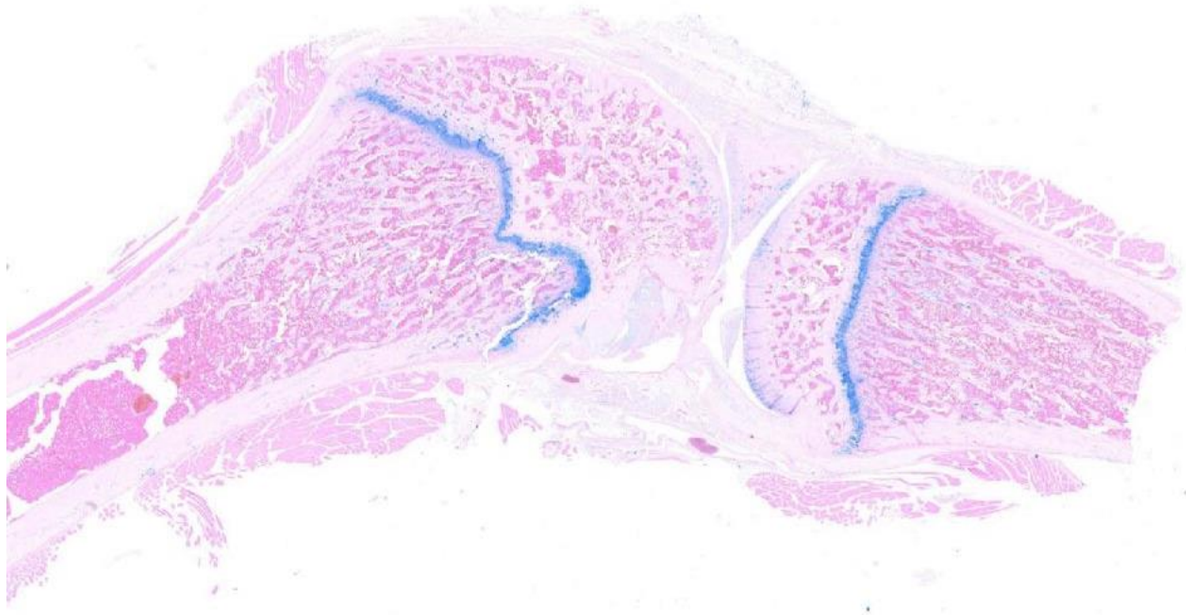
Alcian blue

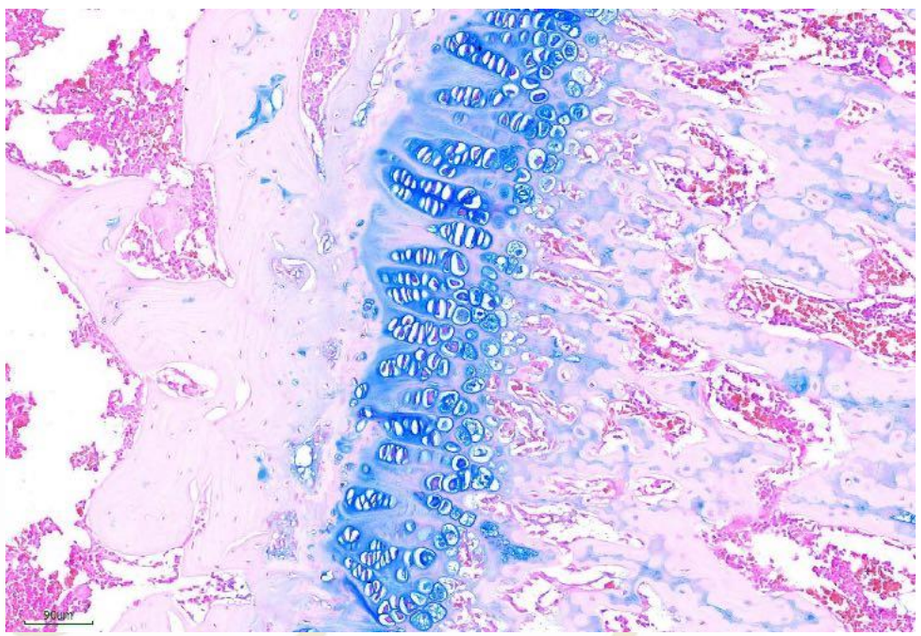
- 1940年首次被合成，1975年其化學結構才被解析出來。主要染色標的為酸性黏液素，最常用於觀察軟骨組織
  - pH 2.5: 硫化醣胺素(sulfated GAGs)，醣蛋白(glycoprotein)，玻尿酸(hyaluronic acid)
  - pH 1.0: 硫化醣胺素(sulfated GAGs)，醣蛋白(glycoprotein)

- 染色標的與PAS相似，但有些許差異，於腸道組織的研究可與PAS染色併用，變成Alcian - PAS染色

Alcian blue



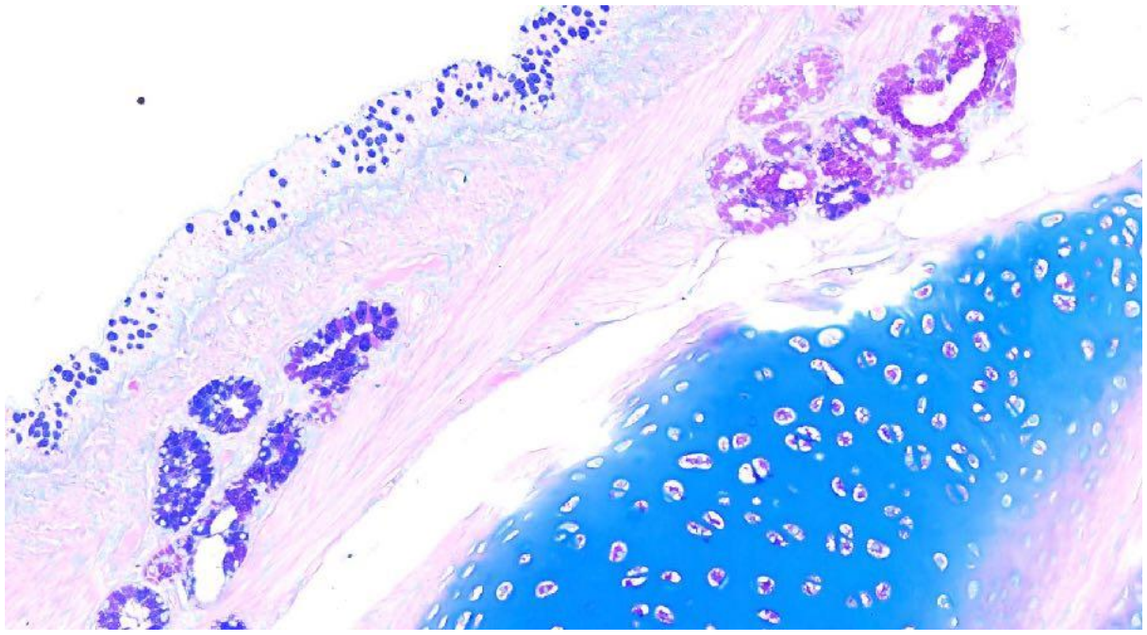


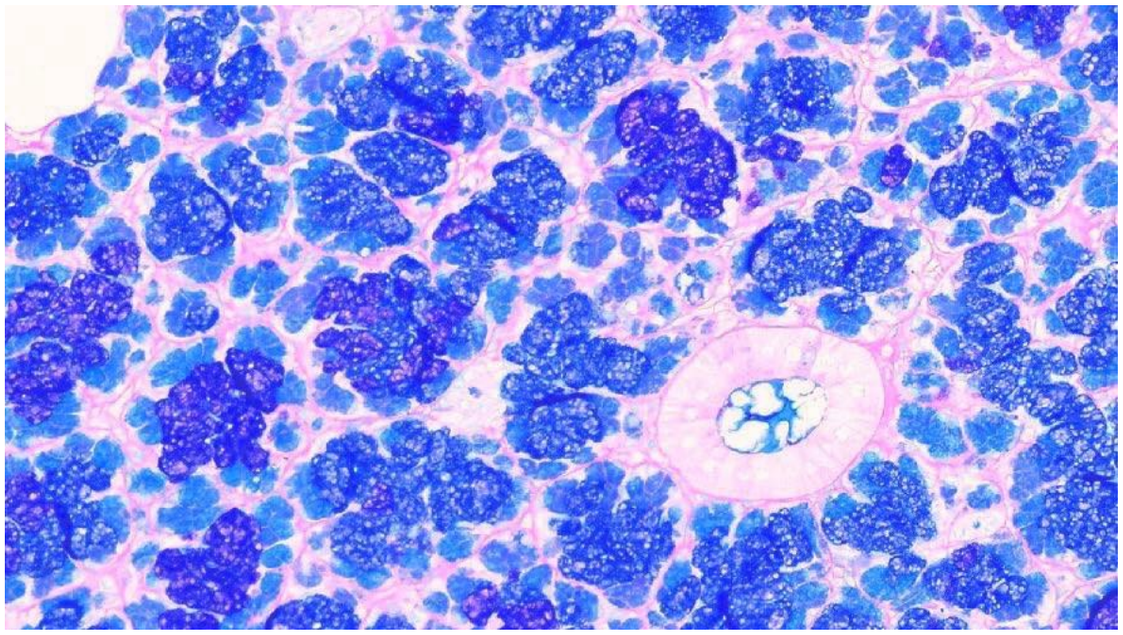


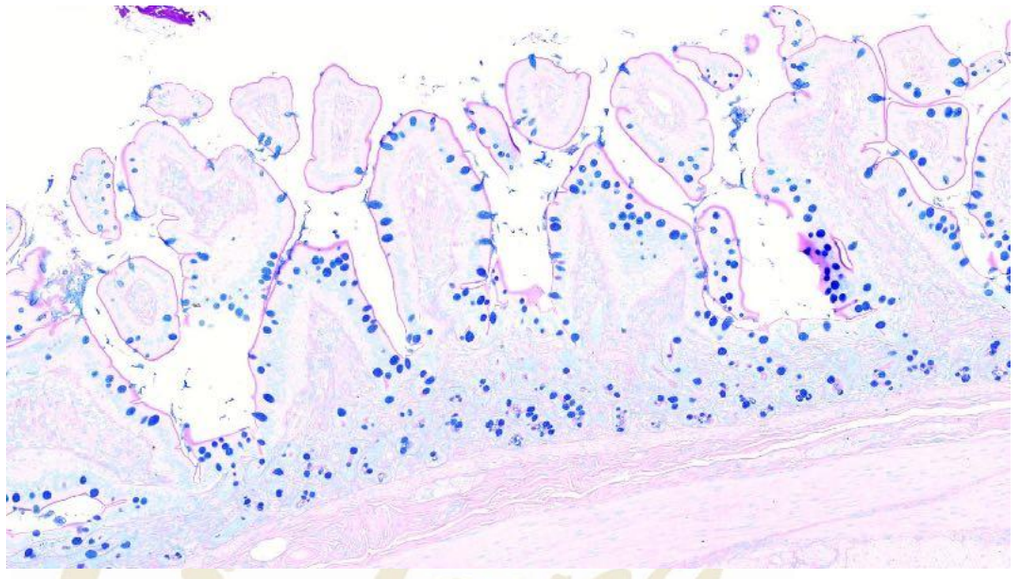
Staining Interpretation

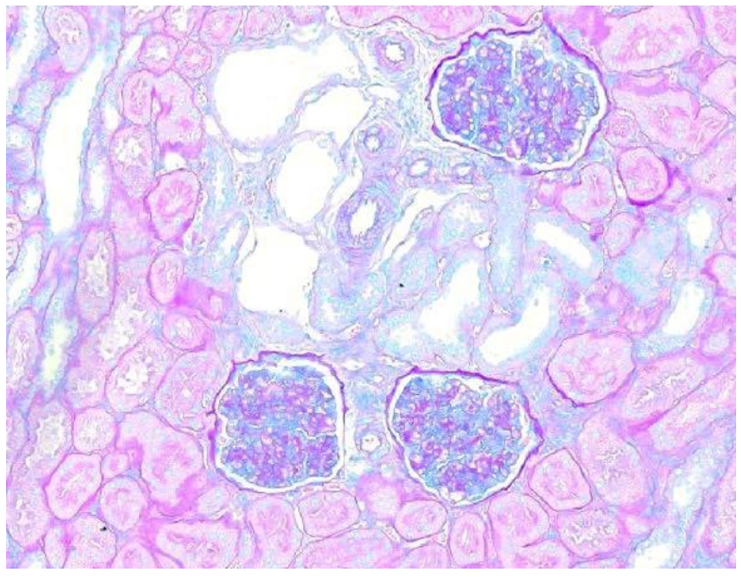
Acidic mucin: Blue
Counter stain: Nuclear fast red

Alcian blue -PAS









Staining Interpretation
Acidic mucin: Blue
Neutral mucin: Pink

Toluidine blue

- metachromatic dye，對酸性組織成份具有較高的親合性，在不同的組織中呈深淺不一的藍色
  - 肥大細胞內含的histamin等物質結合呈現桃紅色
  - 常用於檢驗肥大細胞瘤(mast cell tumor)
  - 因對不同的酸性物質(sulfates, carboxylates, phosphate radicals等)會呈不同程度的深淺，常用於骨組織研究時

Toluidine blue

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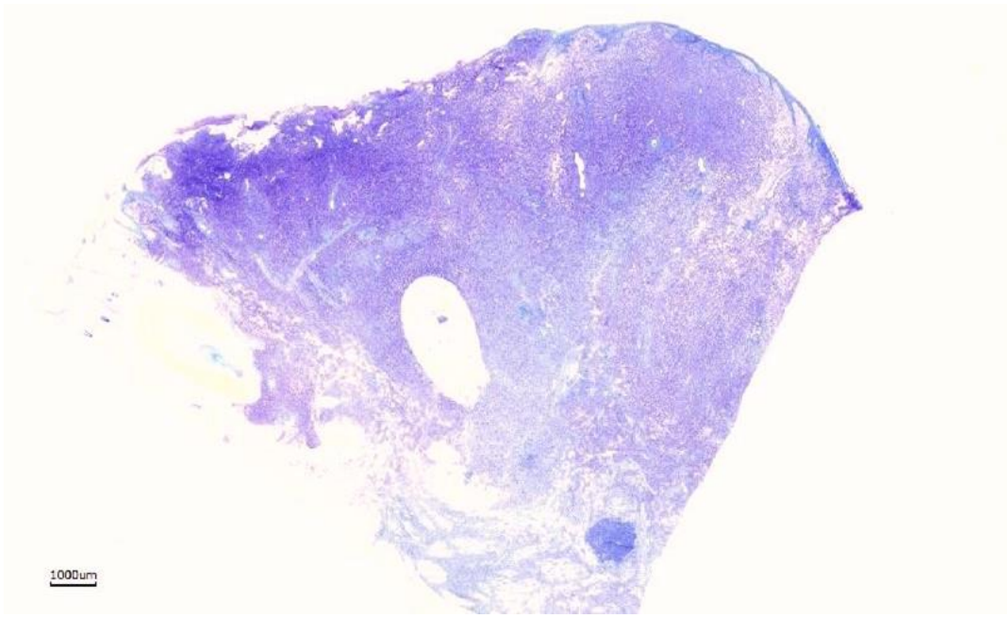
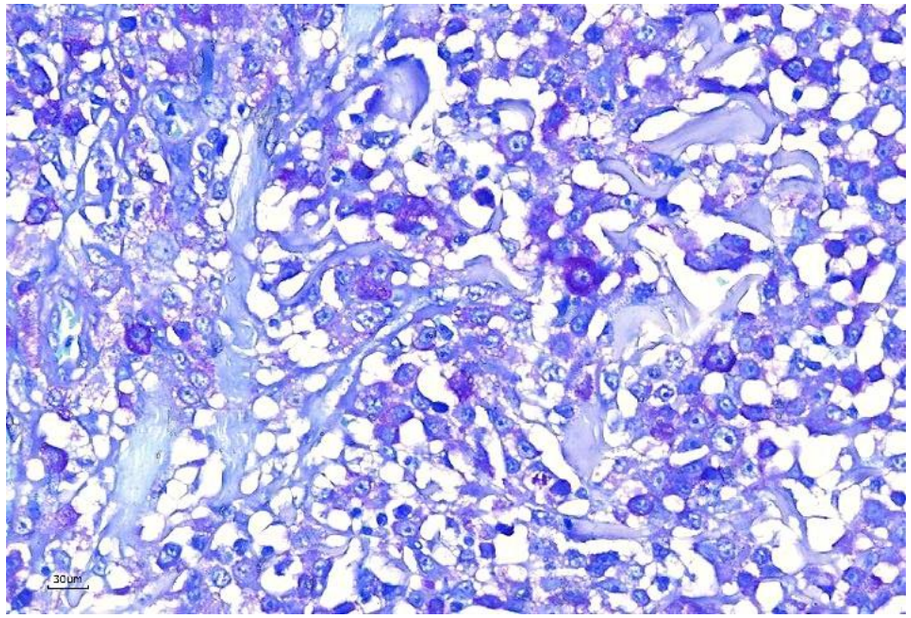
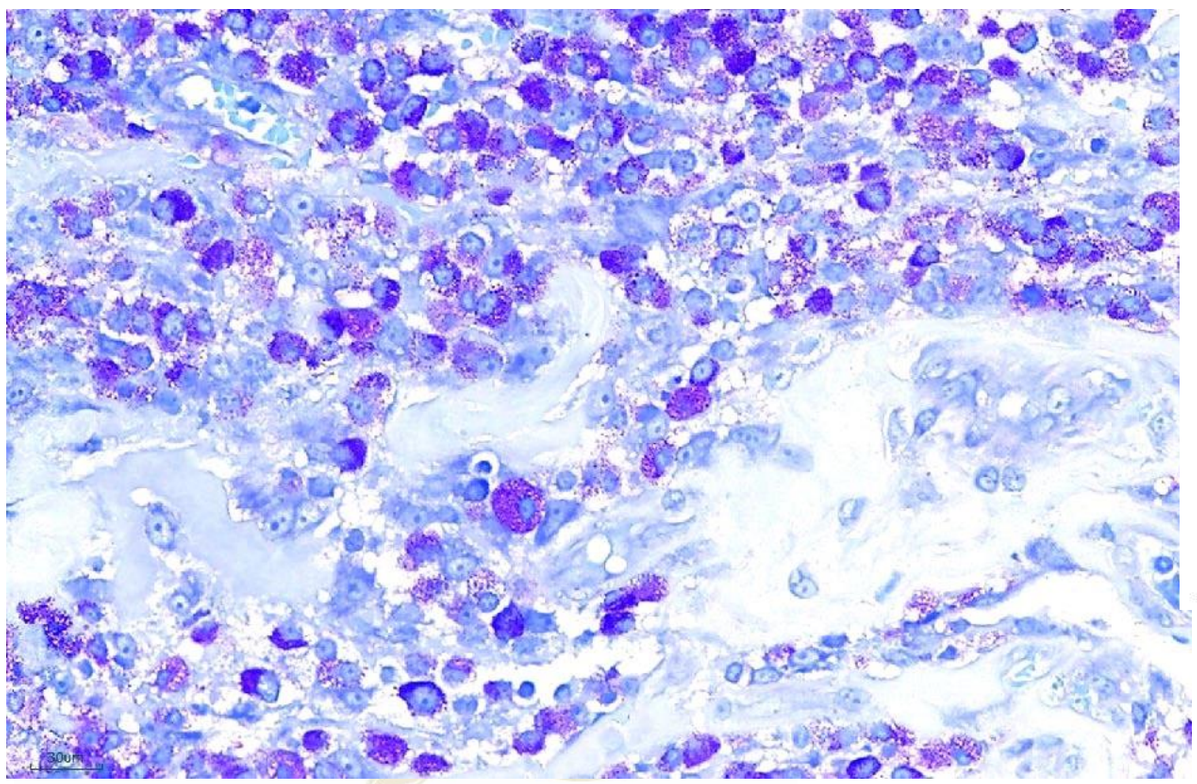
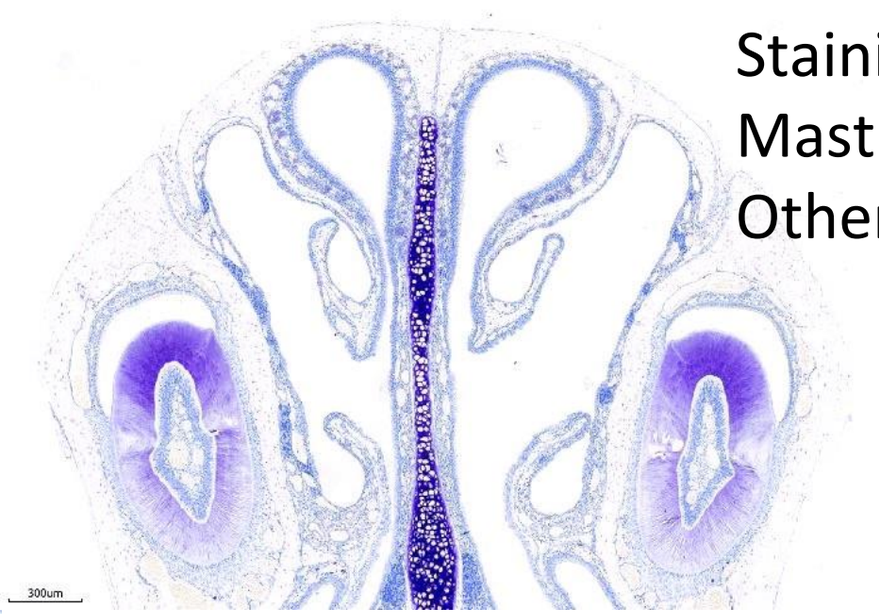






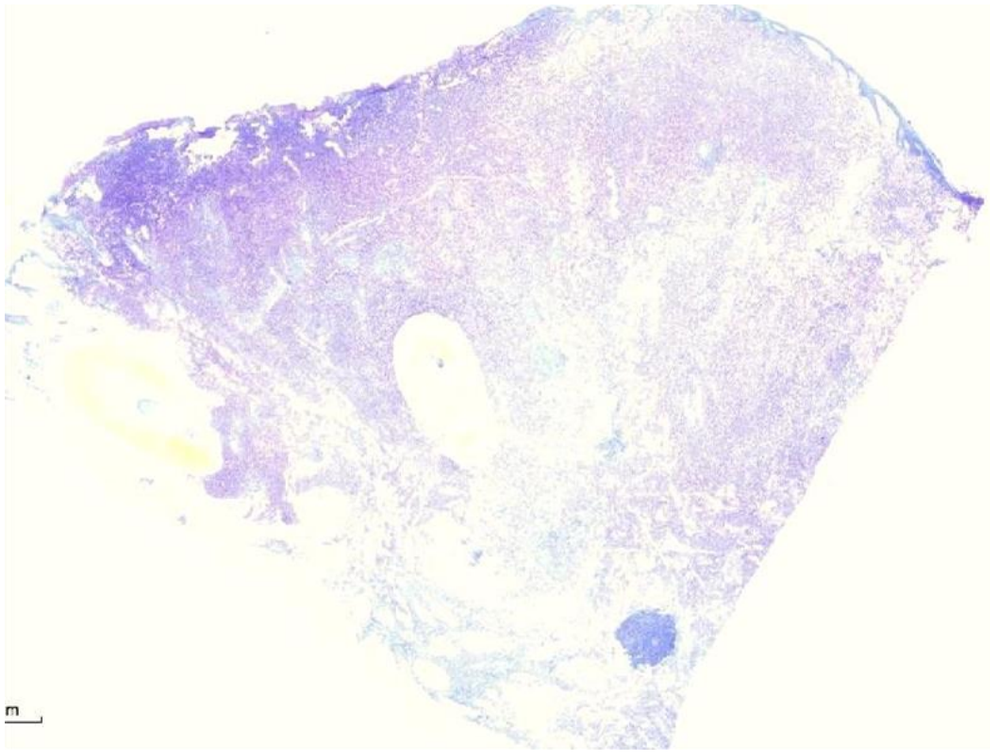


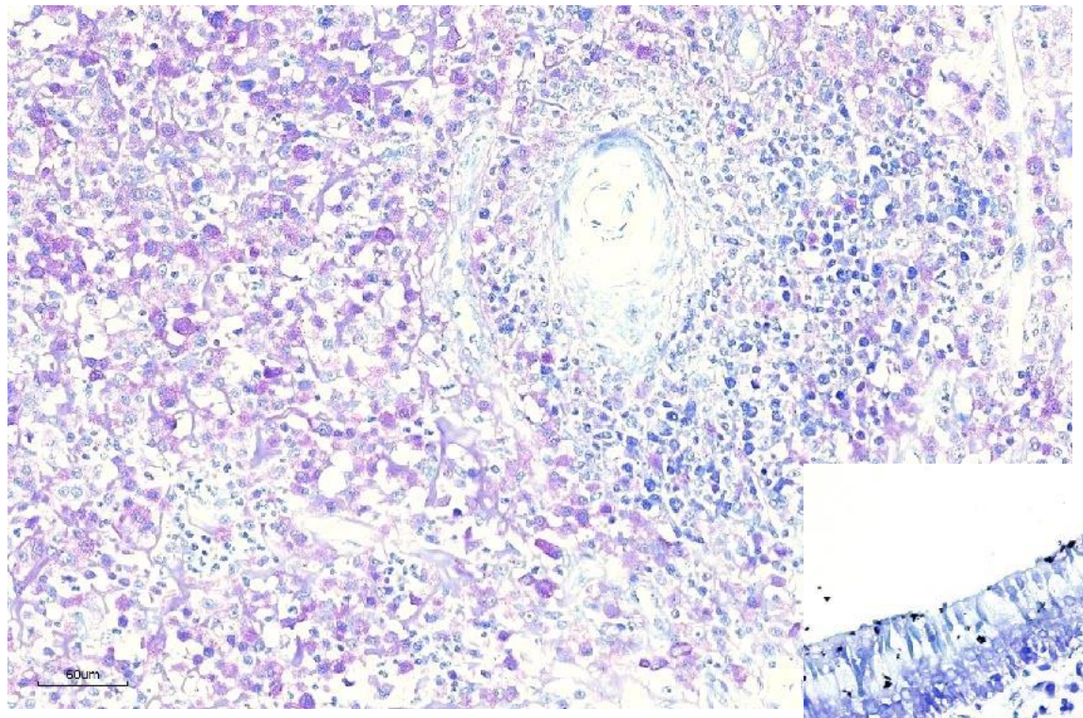
Staining Interpretation

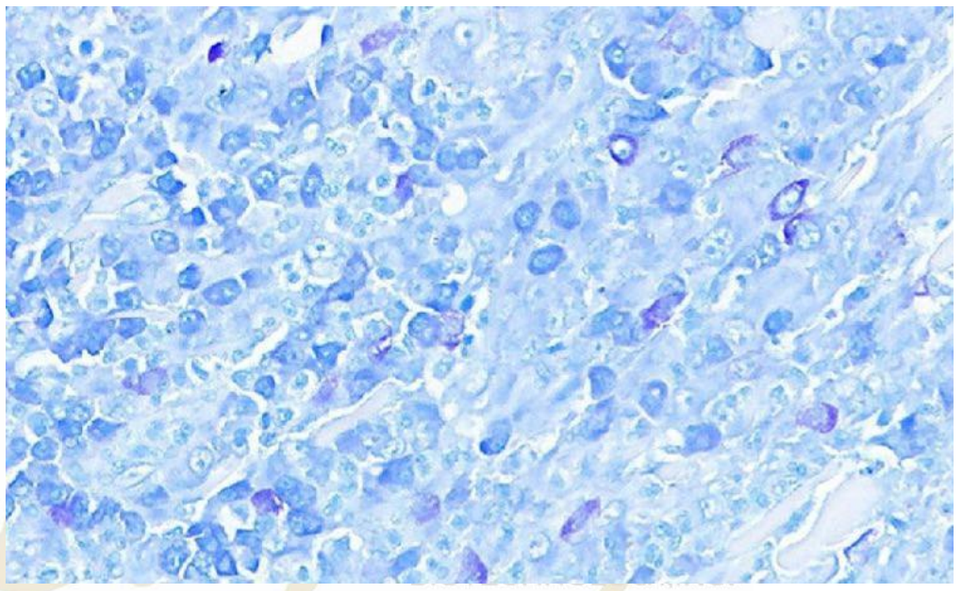
Mast cell: Purlple

Other tissues: blue

Toluidine blue 常見問題







A. 染色性過淡
B. 陽性組織與背景無法區分

Luxol-fast blue (LFB)

- 使用銅酞青(銅苯二甲藍)進行的染色，對神經髓鞘(myeline)的脂蛋白有很高的親合性，多用以觀察中樞神經的髓鞘
  - 使用碳酸鋰及70%進行脫色，需注意脫色時間
  - 可搭配各種染色做為對比(counter stain)，較常見為使用H&E、PAS或Nissl stain

Luxol-fast blue (LFB)

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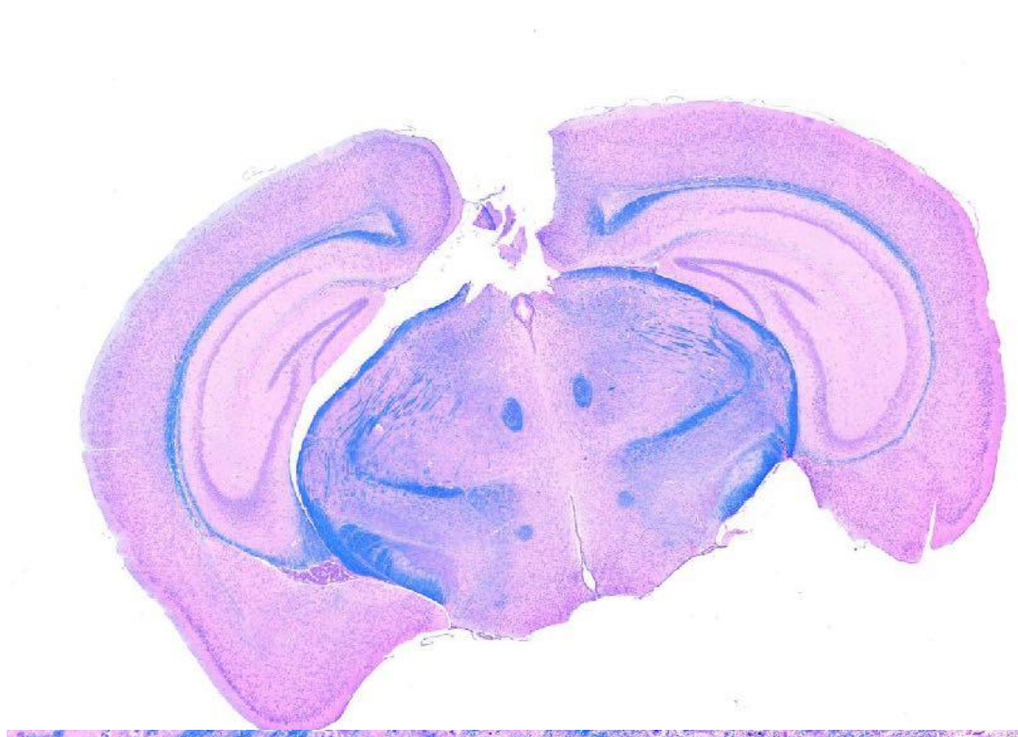
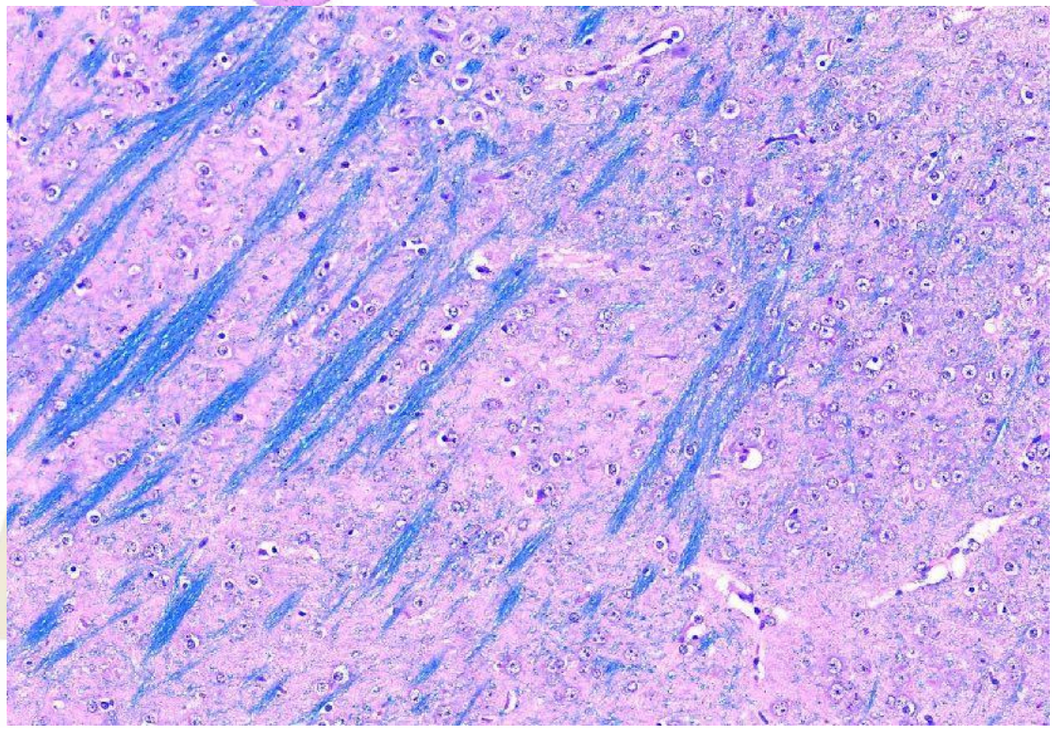
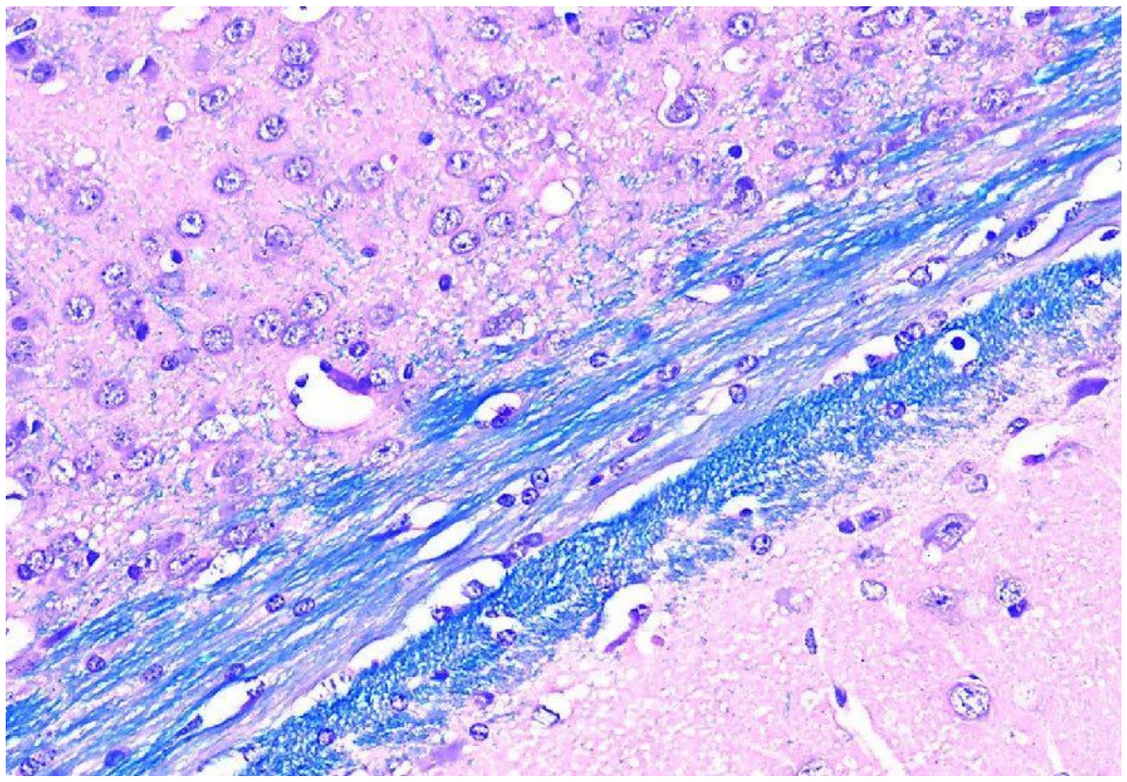


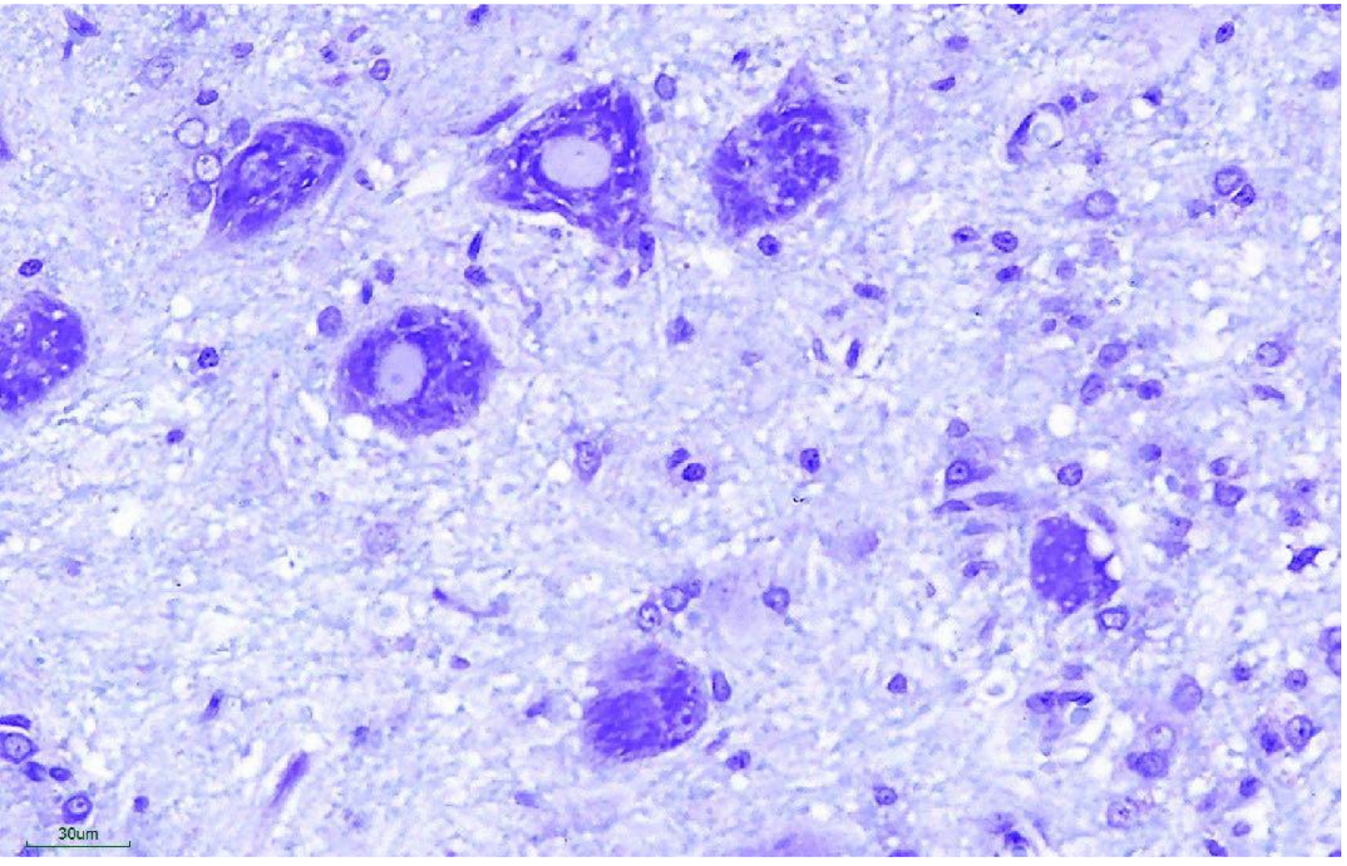


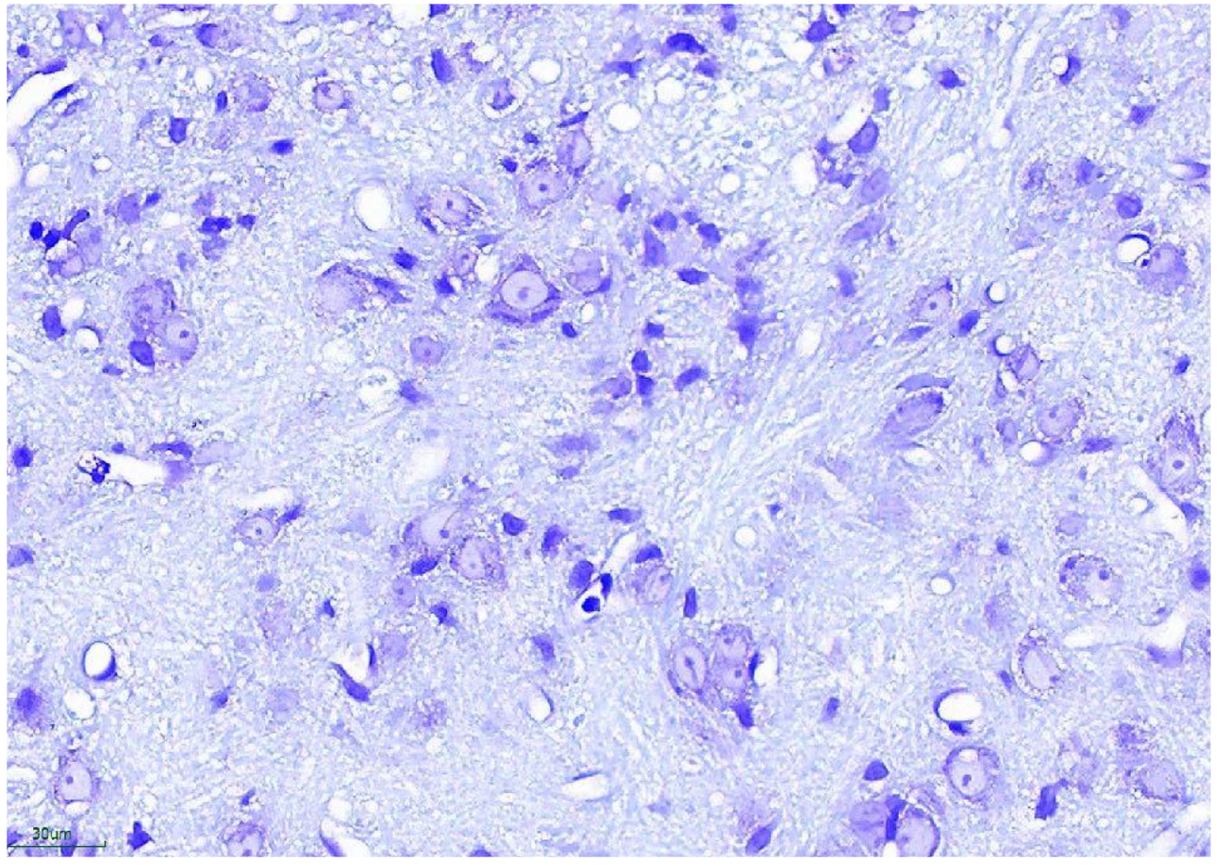


Staining Interpretation
Myeline: sky blue
Other tissues: H&E (counter stain)

Nissl stain

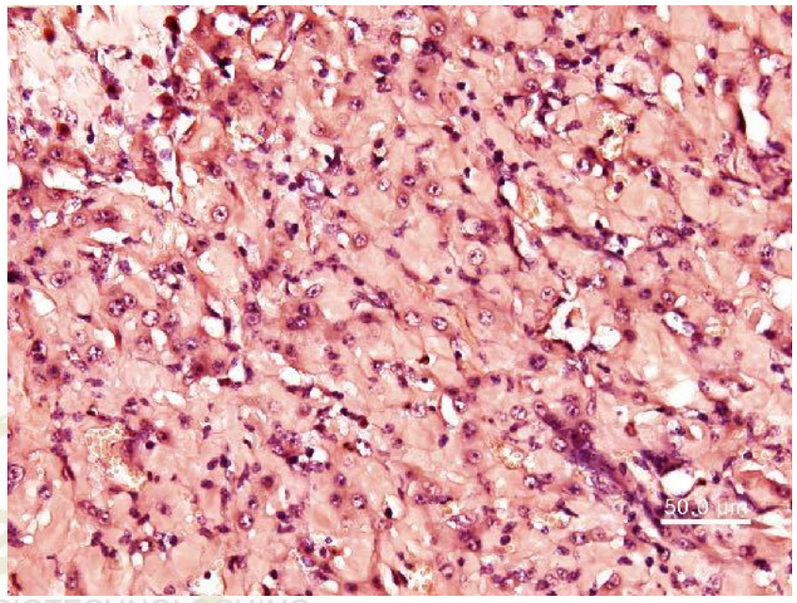
- 使用cresyl violet對神經元的內質網(endoplasmic reticulum)進行染色，其結果為神經元細胞核及尼氏體(Nissl bodies)會被染成藍紫色
  - pH值會顯著影響染色結果

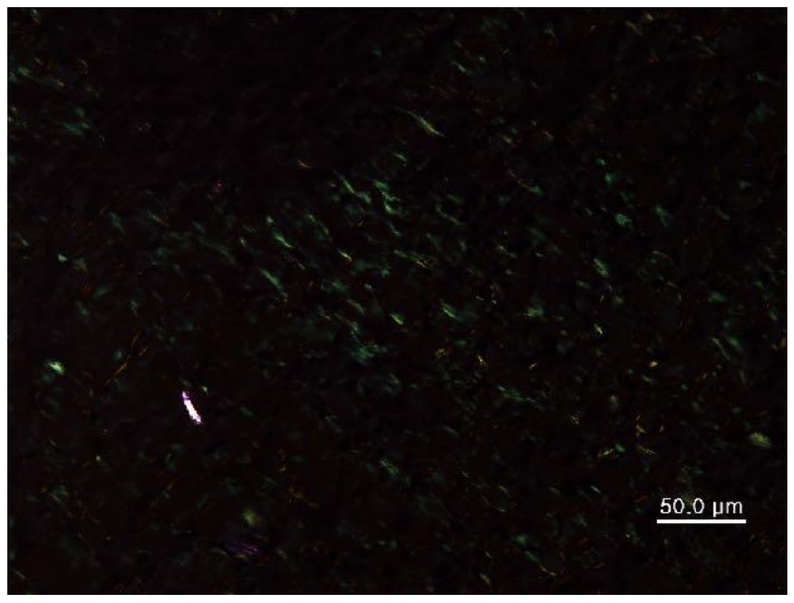




Congo red

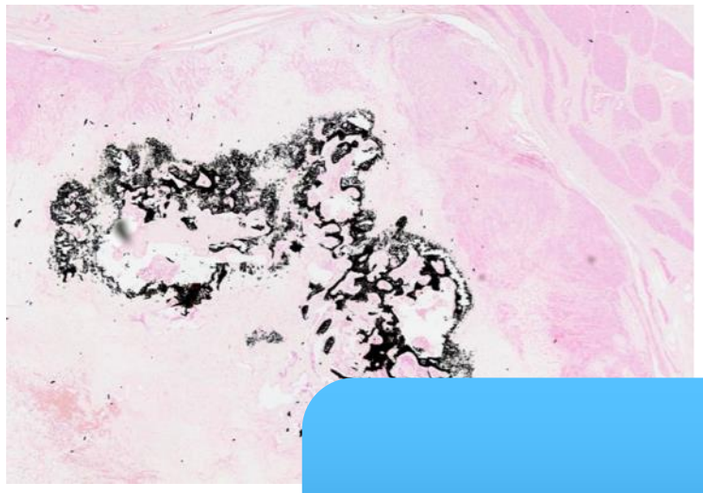
- 剛果紅染色，本身為一種酸鹼指示劑，pH<3.0時呈藍色，pH>5.2時呈紅色。與類澱粉(amyloid)反應後，能在偏光鏡下呈蘋果綠螢光
  - 需厚切片 (8 - 10 μm)
  - 敏感性相當低

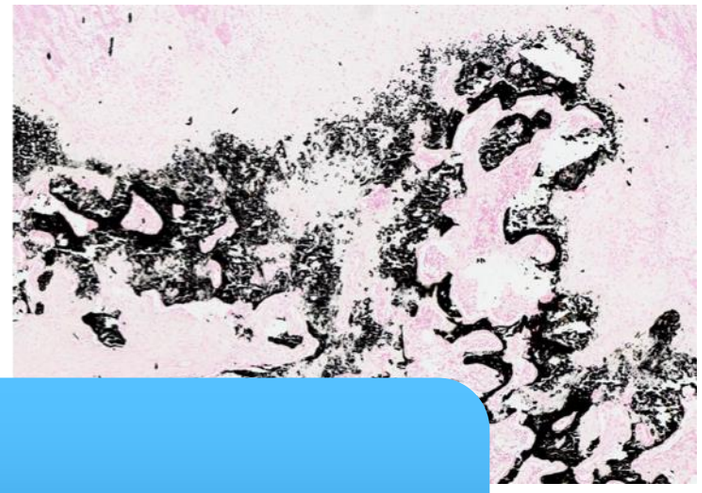




組織中的鈣鹽染色

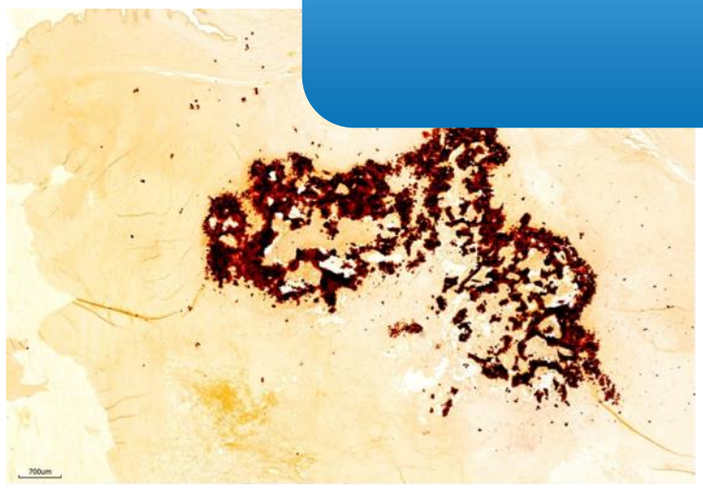
- Von Kossa
  - 使用硝酸銀
  - 需紫外光轉色

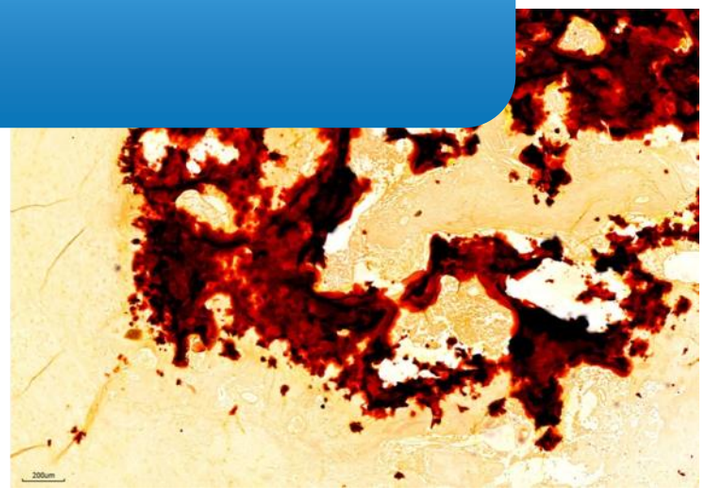


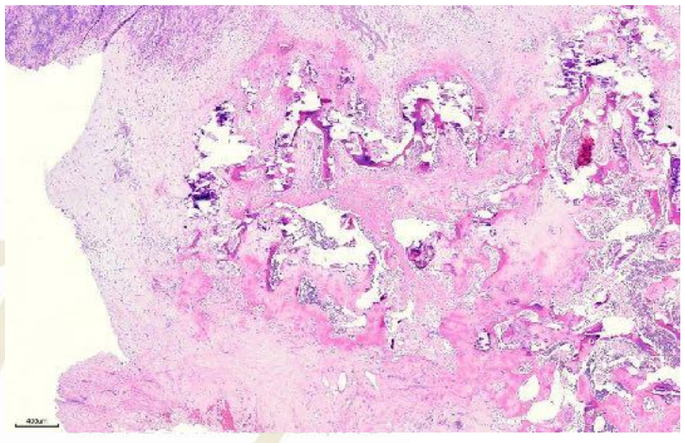


- Alizarin Red S (茜素紅)
  - 染色受pH值影像極大

脫鈣後的組織不能染!!

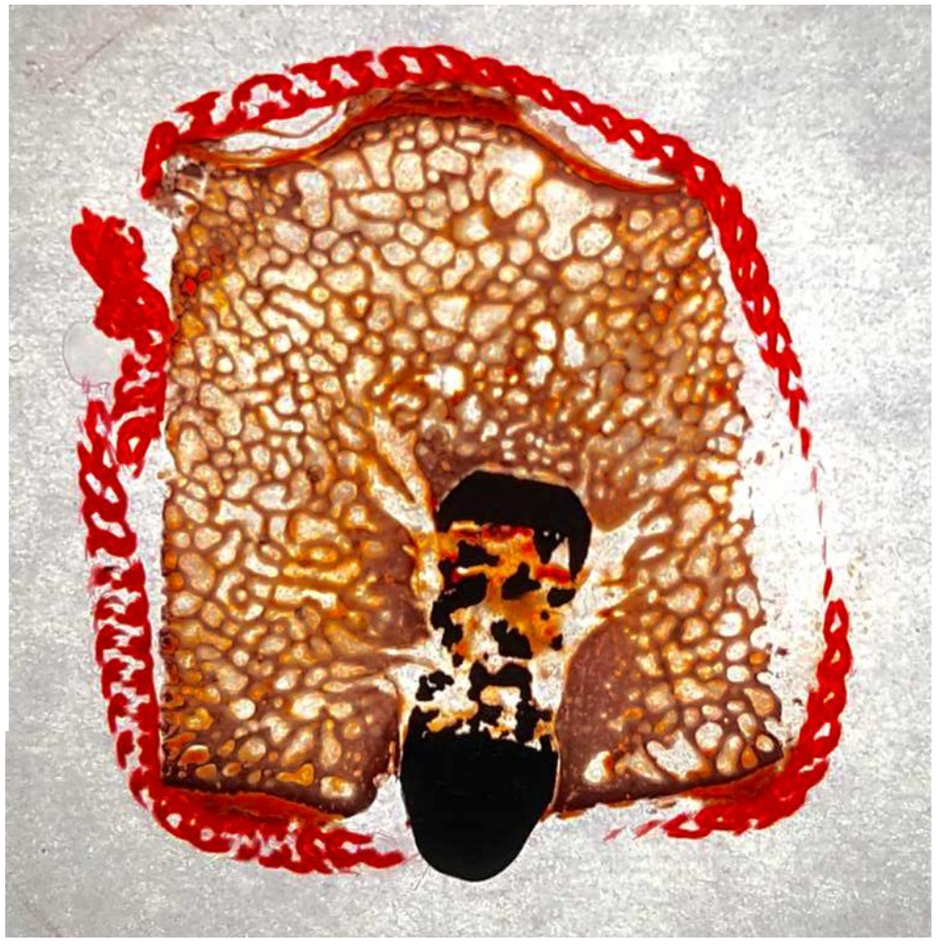






不脫鈣下看組織中的鈣!?

- 塑酯包埋組織
  - 研磨片
  - 切片



PTAH染色

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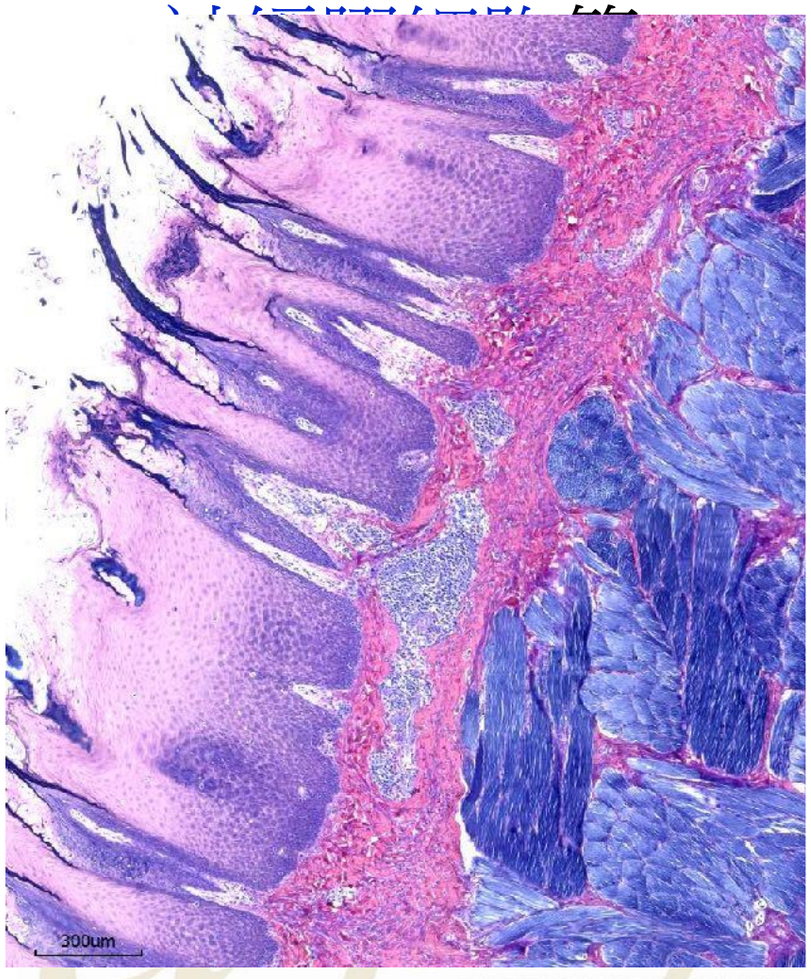
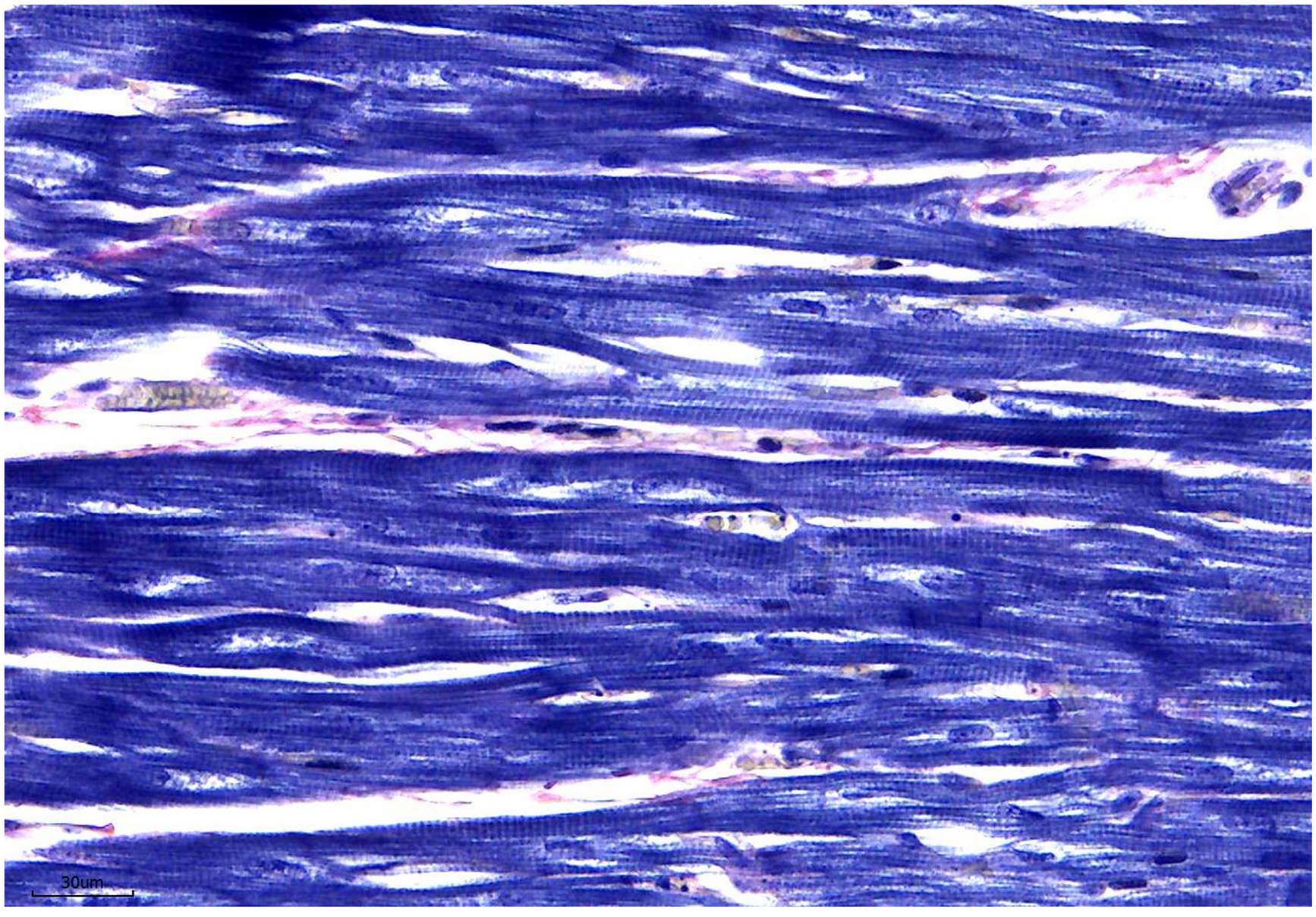
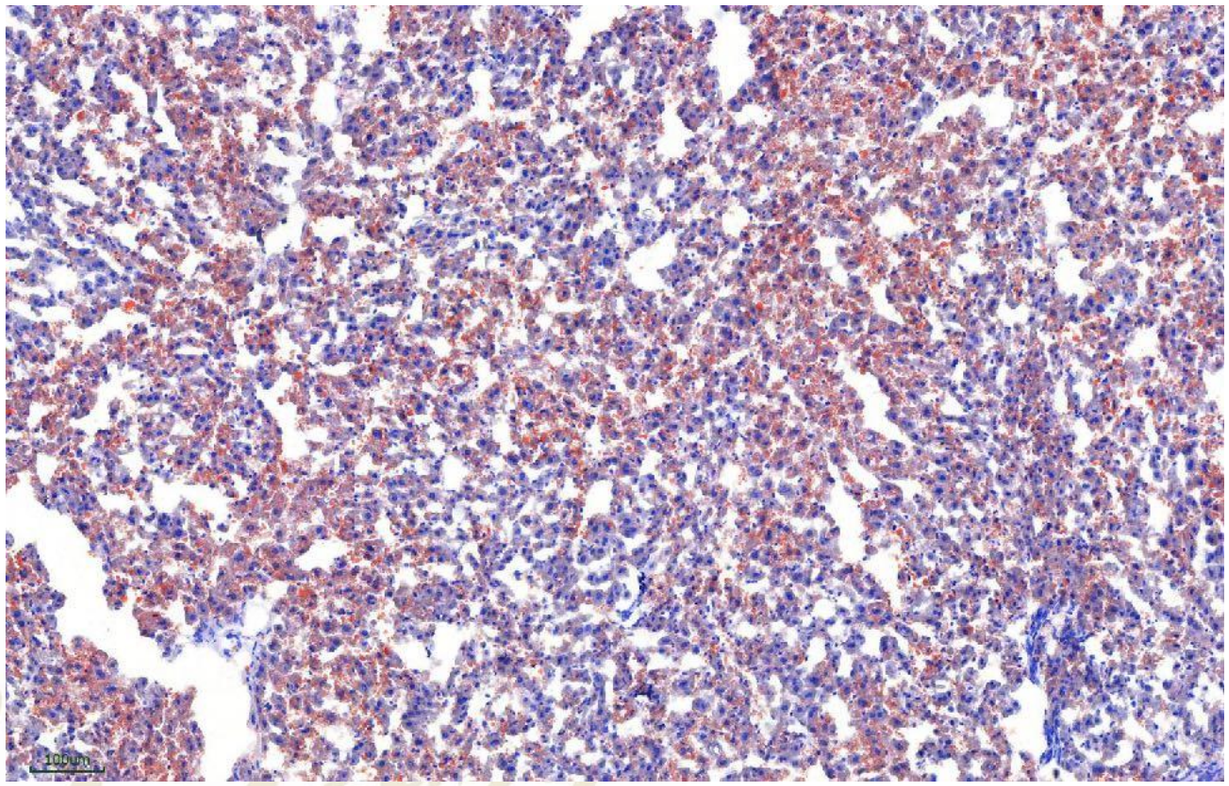
- Phosphotungstic acid-haematoxylin staining，磷鎢酸
(phosphotungstic a
色料(lake pigment

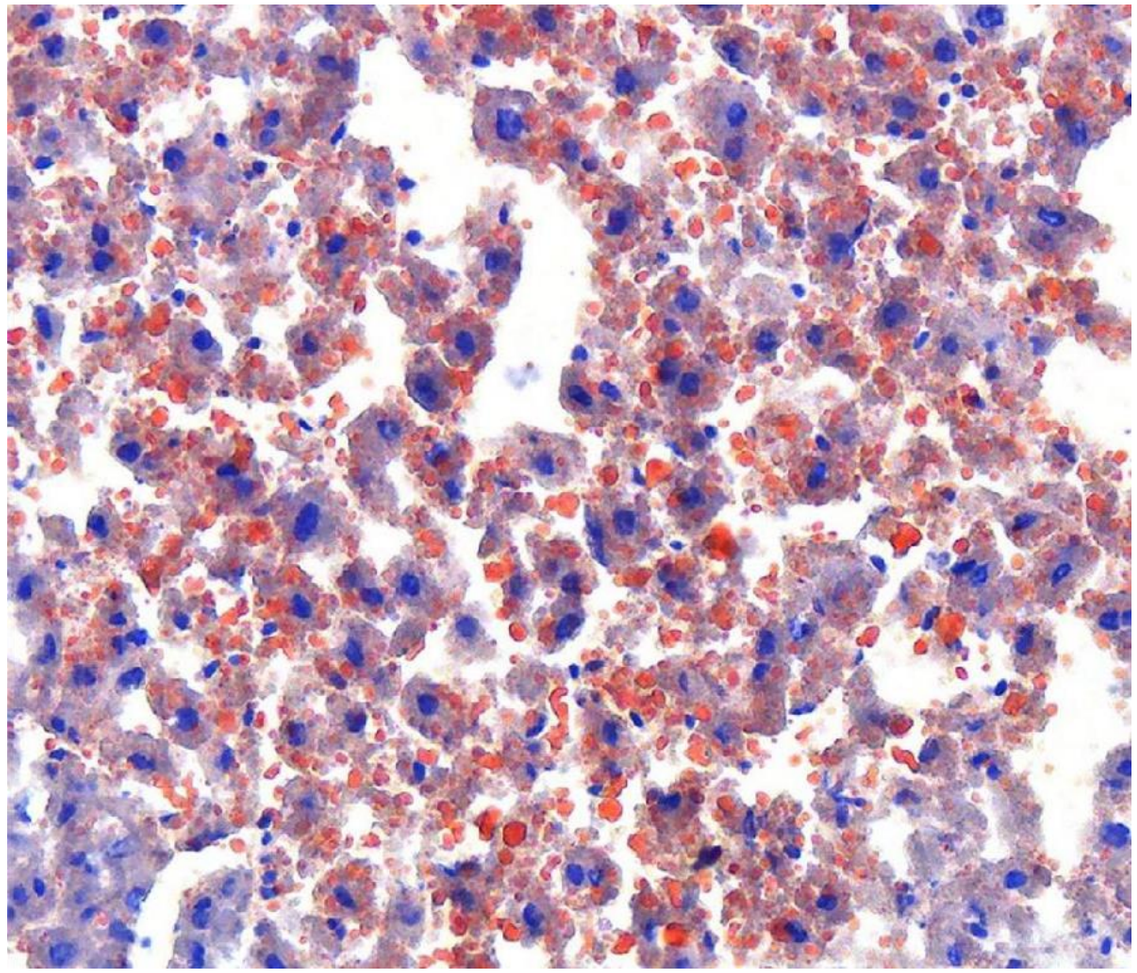




Oil-Red O

- 染組織中的油滴
  - frozen section only !
  - 福馬林固定組織亦可染色，但效果會略差

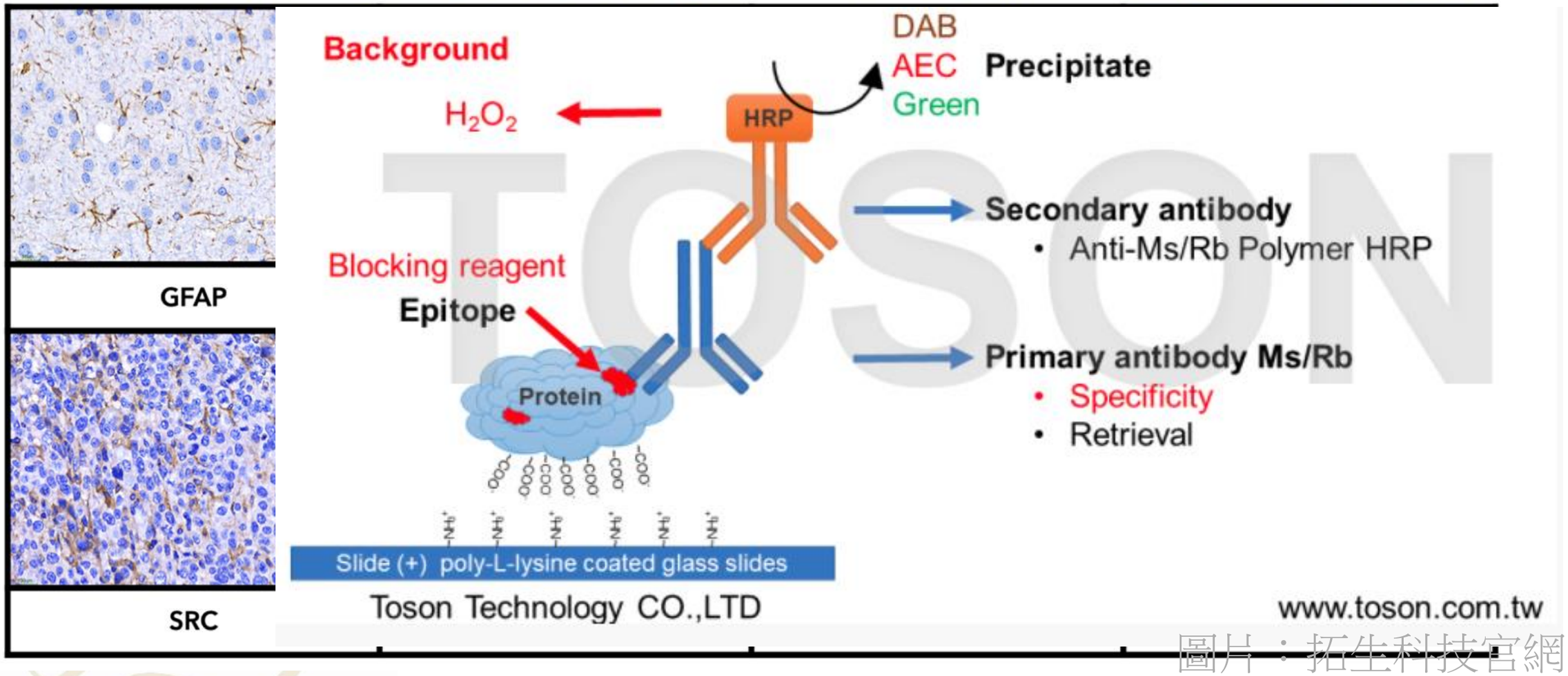




免疫組織化學染色 (immunohistochemical stain, IHC)

IHC基本原理

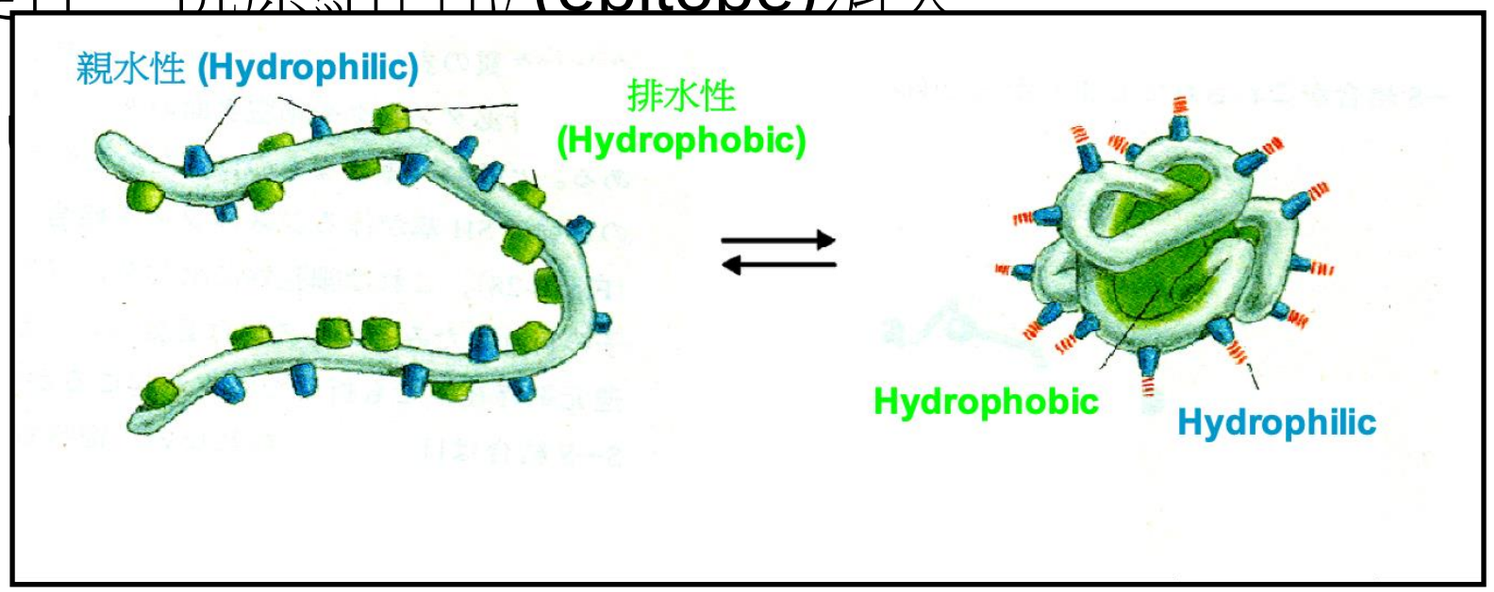
利用抗體, 與細胞上的抗原發生專一性的結合作用，並於抗體上連結有酵素，與呈色劑引發多步驟的化學反應, 而形成有色沉澱得以顯現出來



圖片：拓生科技官網

IHC操作技術拆解

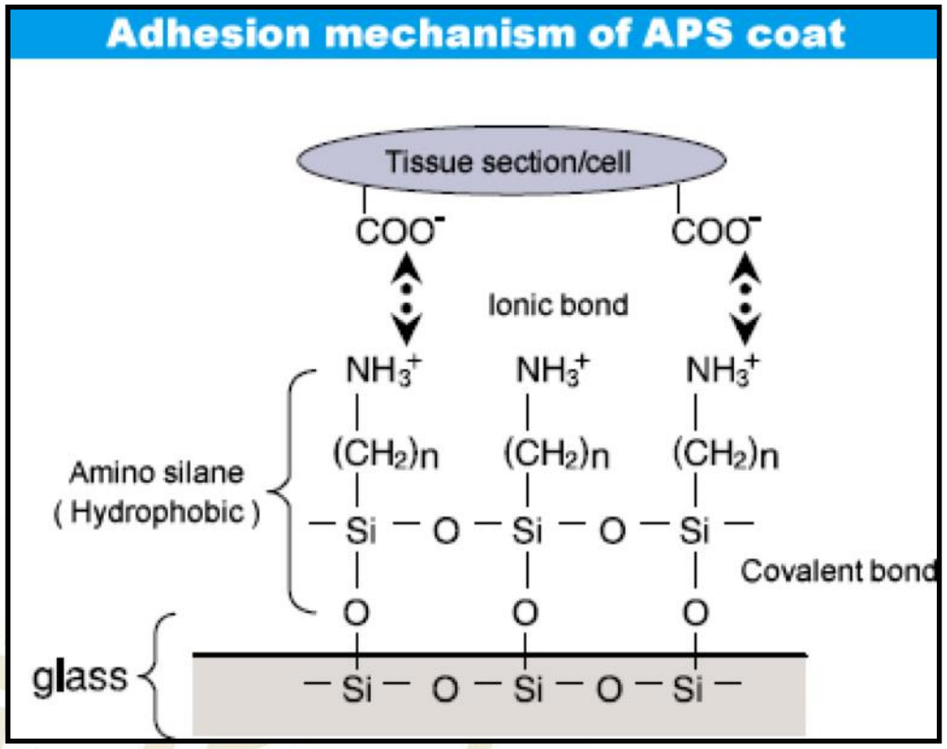
- 中性福馬林組織固定影響
  - 造成蛋白質變性
    - 福馬林(甲醛)中的醛基與抗原胺基交聯，使抗原決定位的三維結構改變
    - 抗原(antigen, Ag)產生變性，抗原結合位(epitope)消失
    - Modified Davidson's sol
      性
    - 增加組織疏水性

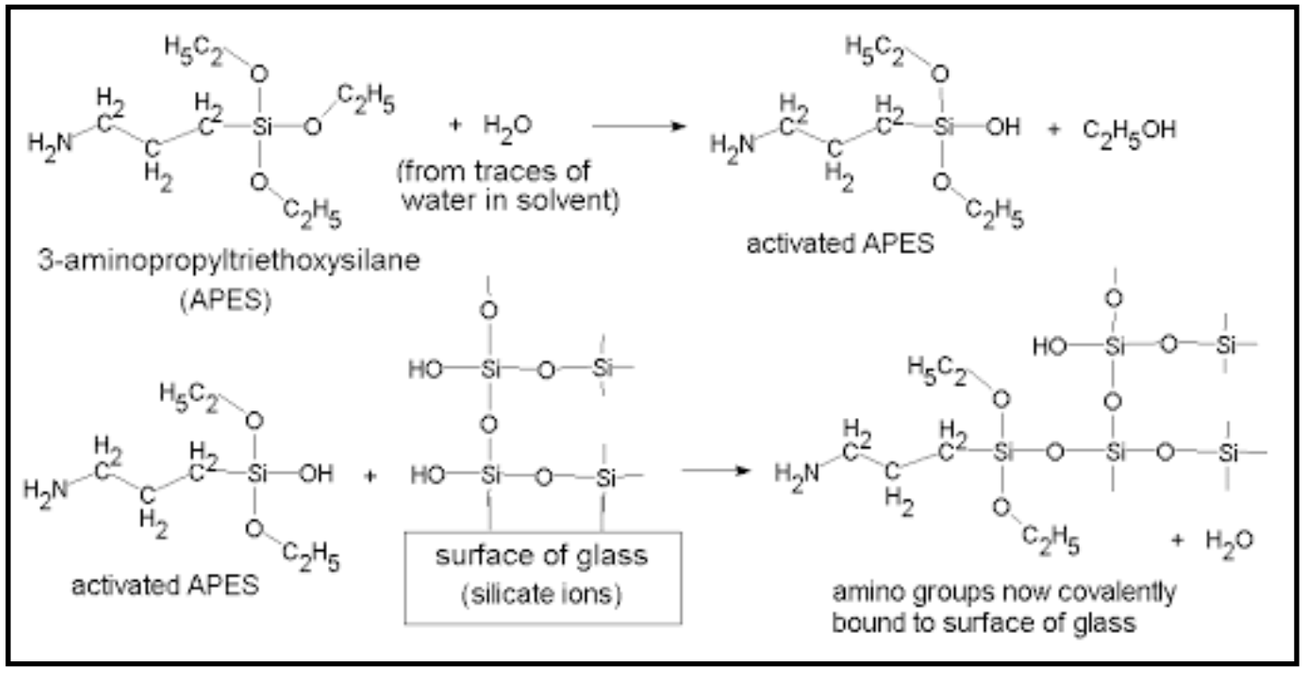


- 冷凍切片 可較好的保留抗原

IHC操作技術拆解

- Coated slides:
  - 使玻片表面帶電荷，與組織的蛋白質形成共價鍵
  - 讓組織不易脫落

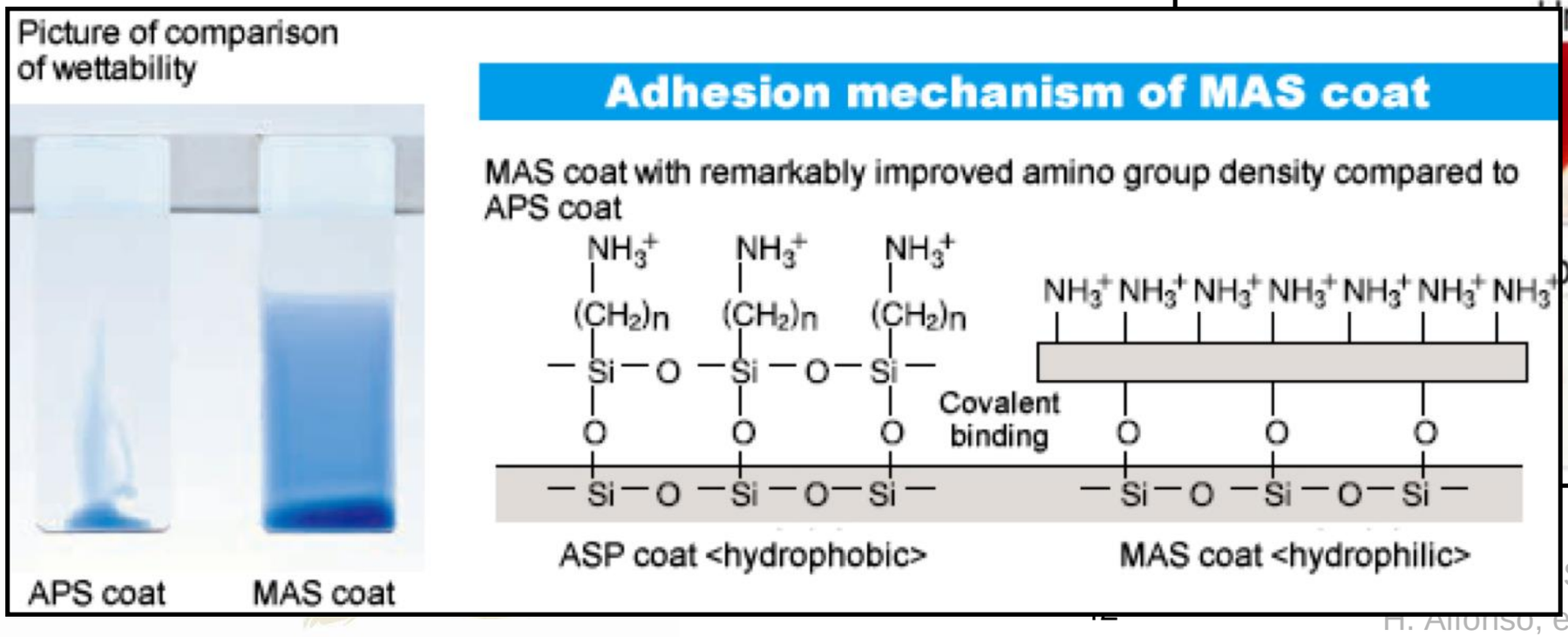


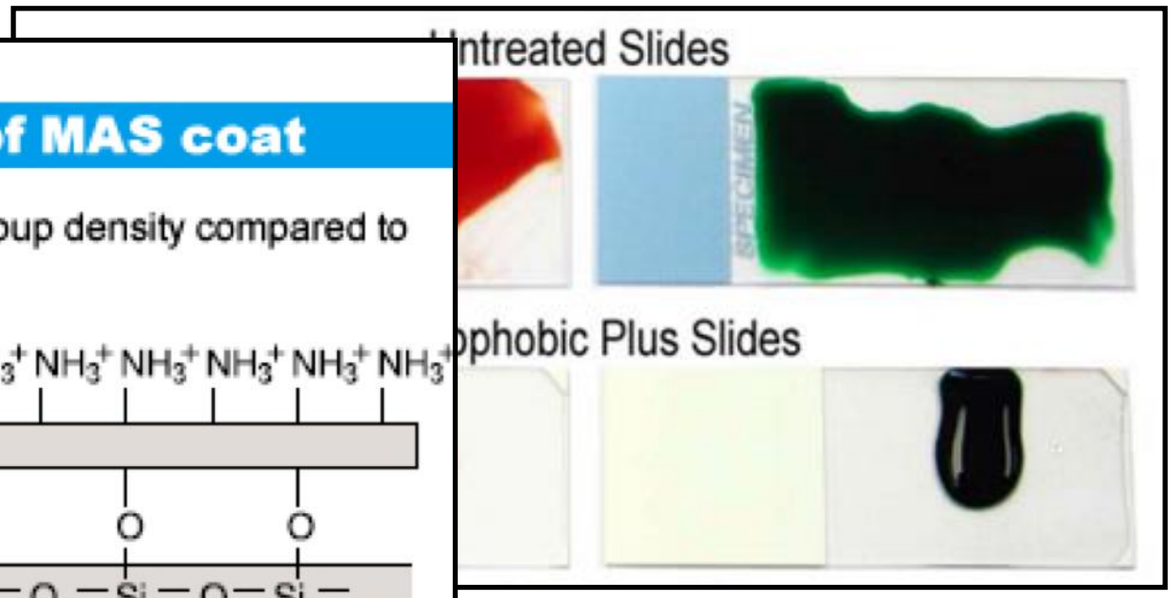


IHC world.
http://www.ihcworld.com/_technical_tips/prevent_section_fall.htm

IHC操作技術拆解

- Coated slides:
  - 使玻片表面帶電荷，與組織的蛋白質形成共價鍵
  - 讓組織不易脫落
  - 多具有疏水性





Plus Slides: A Surface-Modified Slide for Improved Tissue Adhesion. H. Alfonso, et al.

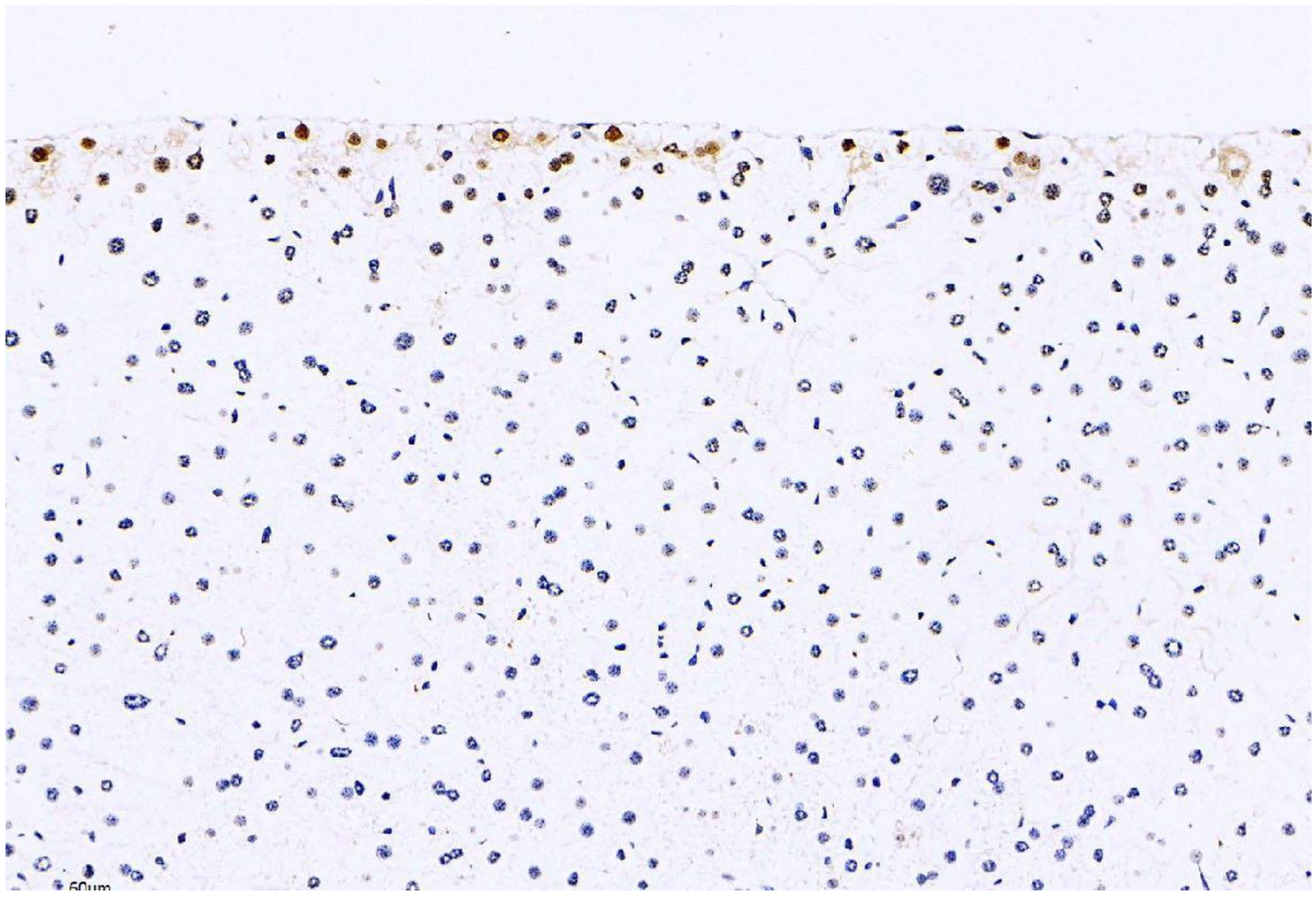
IHC操作技術拆解

- Coated slides:
  - 使玻片表面帶電荷，與組織的蛋白質形成共價鍵
  - 讓組織不易脫落
  - 多具有疏水性

- APS(Amino Silane)coating slide
- MAS-GP coating
- Gelatin coating
- Silane coating
- Poly-L-lisin coating
- Non-coating

IHC操作技術拆解

- Coated slides:
  - 使玻片表面帶電荷，與組織的蛋白質形成共價鍵
  - 讓組織不易脫落
  - 多具有疏水性



抗原修復(antigen retrieval)

- Heat-induced epitope retrieval (HIER)
  - Citrate buffer (pH 6.0)
  - EDTA (pH 8.0, pH 9.0, pH 10.0)

Boiling（＞95℃）for 10~40 minutes
Cool down > 15 minutes

- Enzyme-induced epitope retrieval (EIER)
  - Trypsin
  - Ficain
  - Pepsin
  - Proteinase K

RT：15~40min
or 37℃：10min

抗原修復(antigen retrieval)

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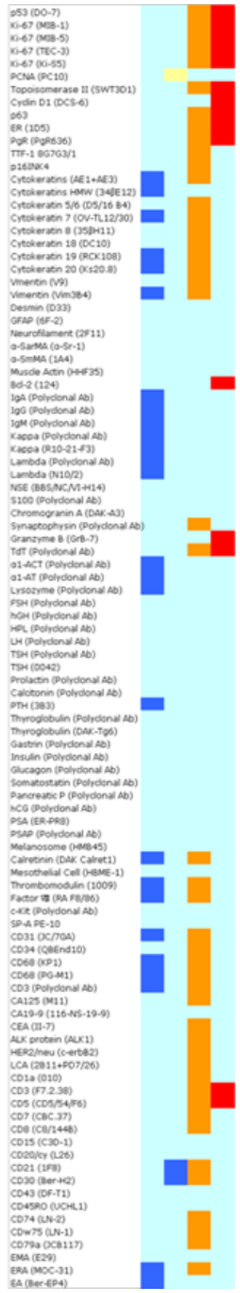
HIER by EDTA

HIER by CB

HIER by dH_2O

Pro.K treatment



細胞核蛋白
(Nuclear proteins)

細胞質蛋白(Cytoplasmic proteins)

細胞膜蛋白 (Cell membrane proteins)

去過氧化酶 (Deperoxidase)

- 消除組織內源性過氧化氫酶(endogenous peroxidase)
  - 減少背景雜訊(background signals)

過度的H_2O_2處理會造成抗原被破壞 !!!

去過氧化酶 (Deperoxidase)

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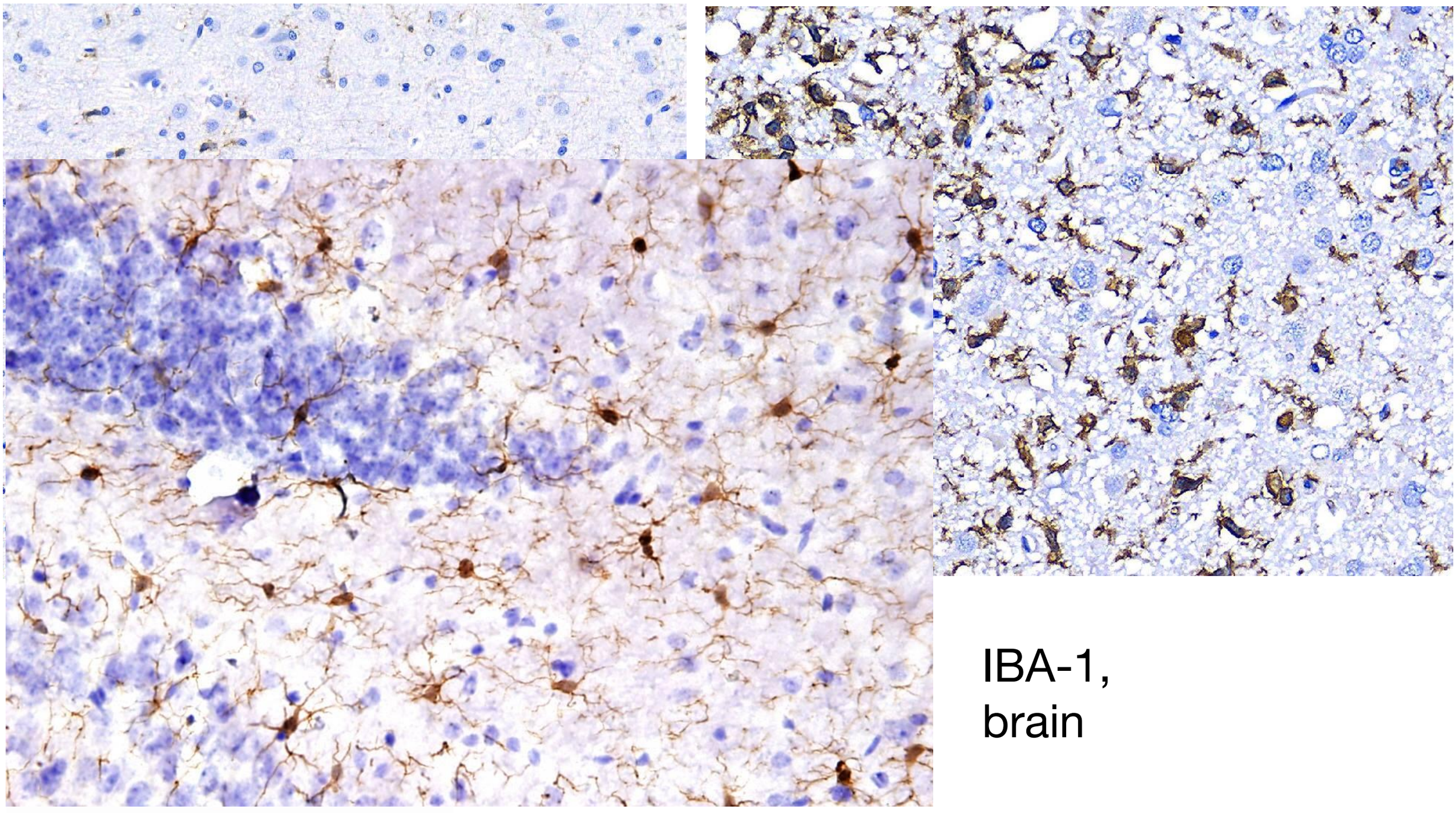
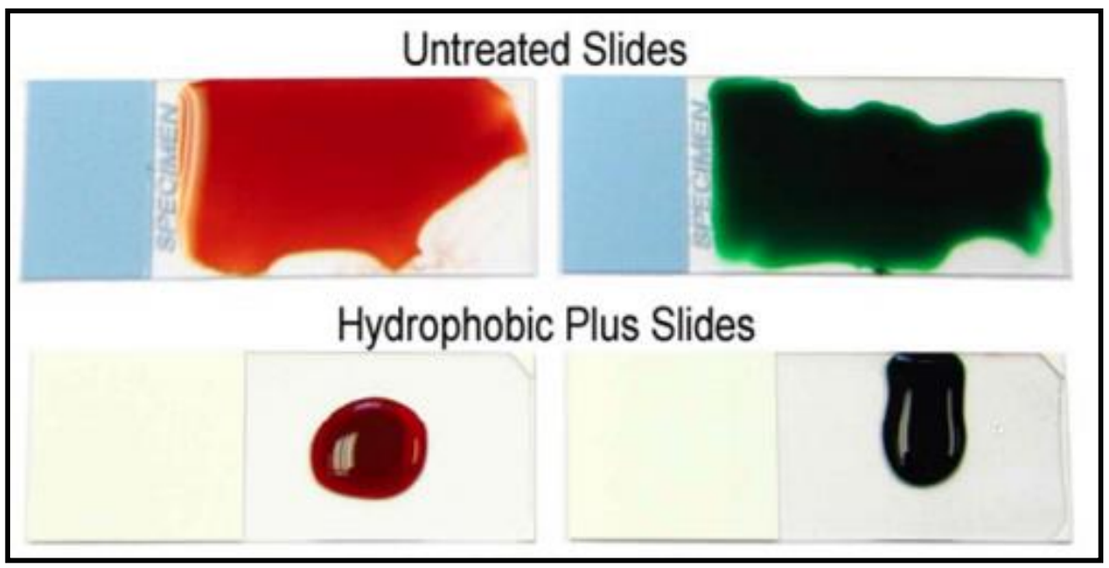


IBA-1,
brain

Blocking

- 消除組織內源性酵素
  - peroxidases, phosphatases等
- 消除玻片上的電荷，減少玻片的疏水性
  - 因IHC多使用coated slides，而coated slides多具有較強的疏水性
- 阻斷內源性的IgG及IgM



初級抗體 (primary antibody)

- 多株(polyclone)及單株(monoclone)
- 來源物種(host)不同: rabbit, mice, goat, etc.
  - 抗體host 跟染色目標的物種不能相同!!
- 稀釋液(diluent): PBS, citrate buffer, BSA dilution, etc.
- 需測試反應溫度、時間、濃度，同一支抗體於不同批的切片組織，反應條件可能不盡相同

初級抗體 (primary antibody)

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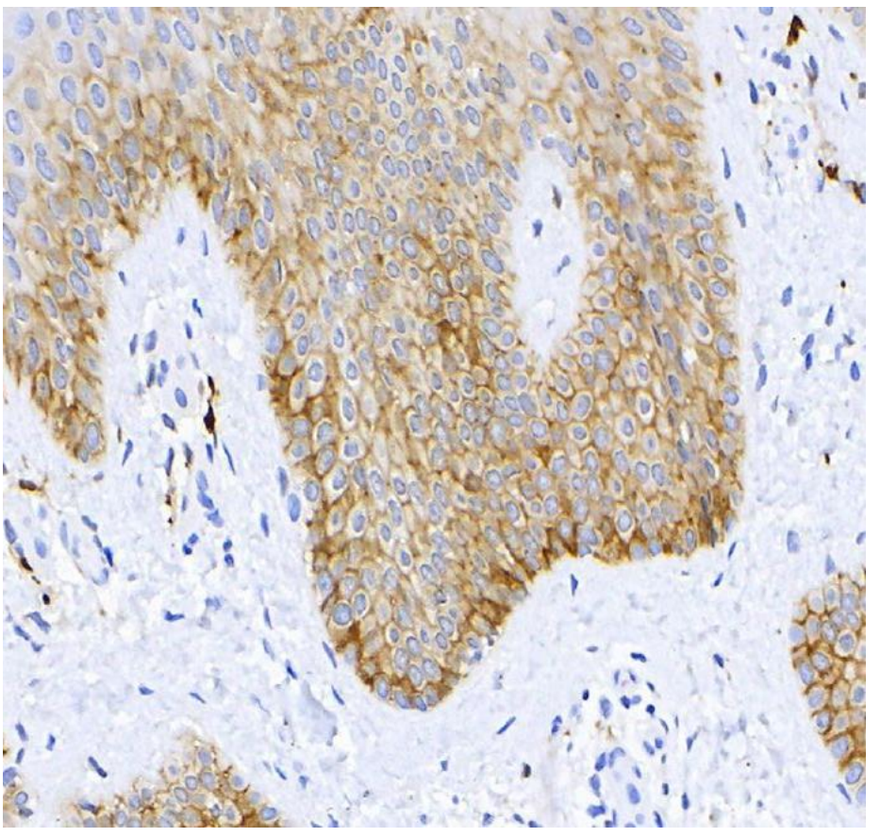
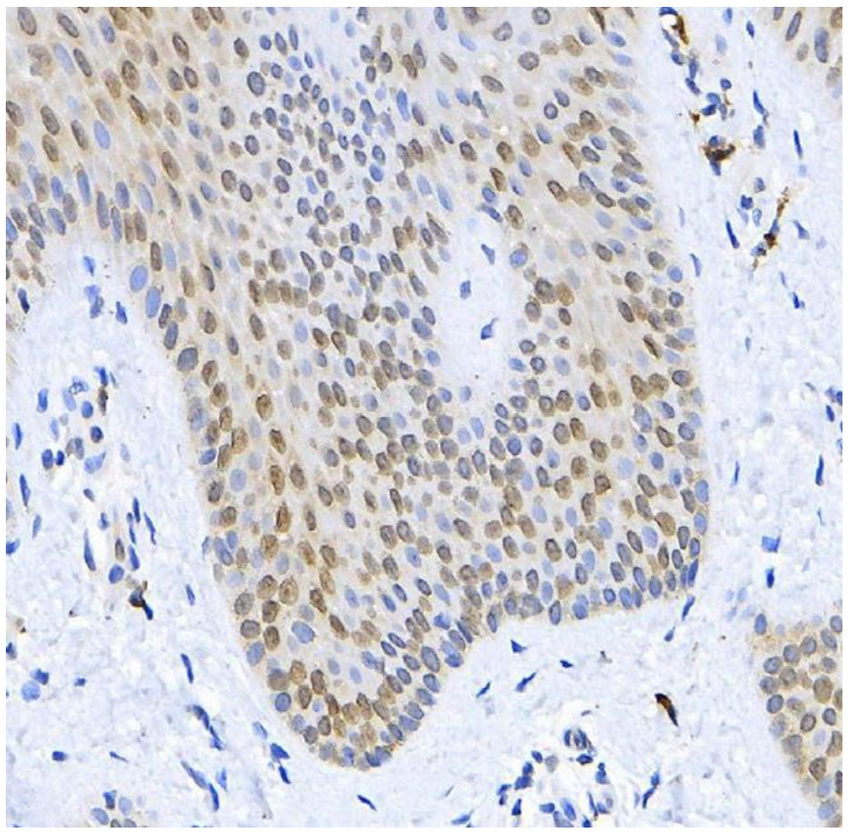
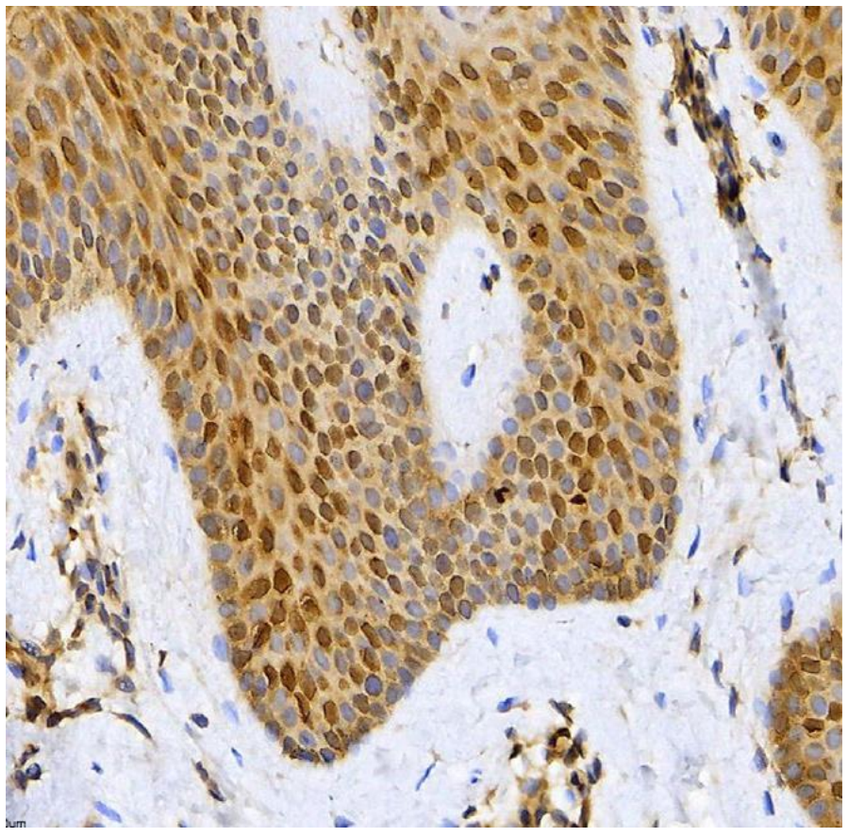


RT



37℃



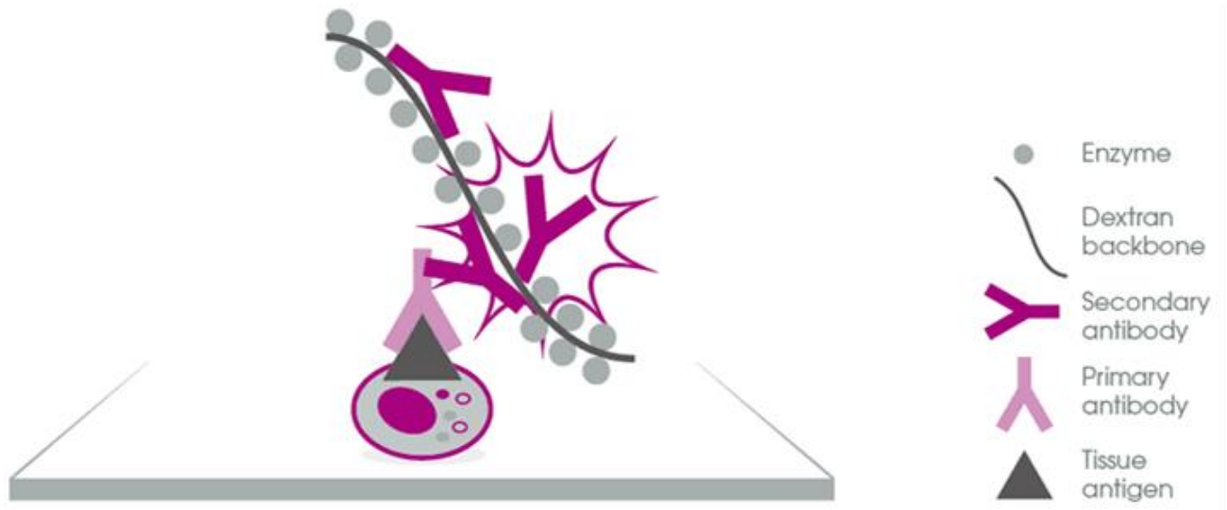
4℃

不同抗體有其適合的條件，沒有固定不變的染色條件!!

anti-EGFR antibody

次級抗體 (secondary antibody)

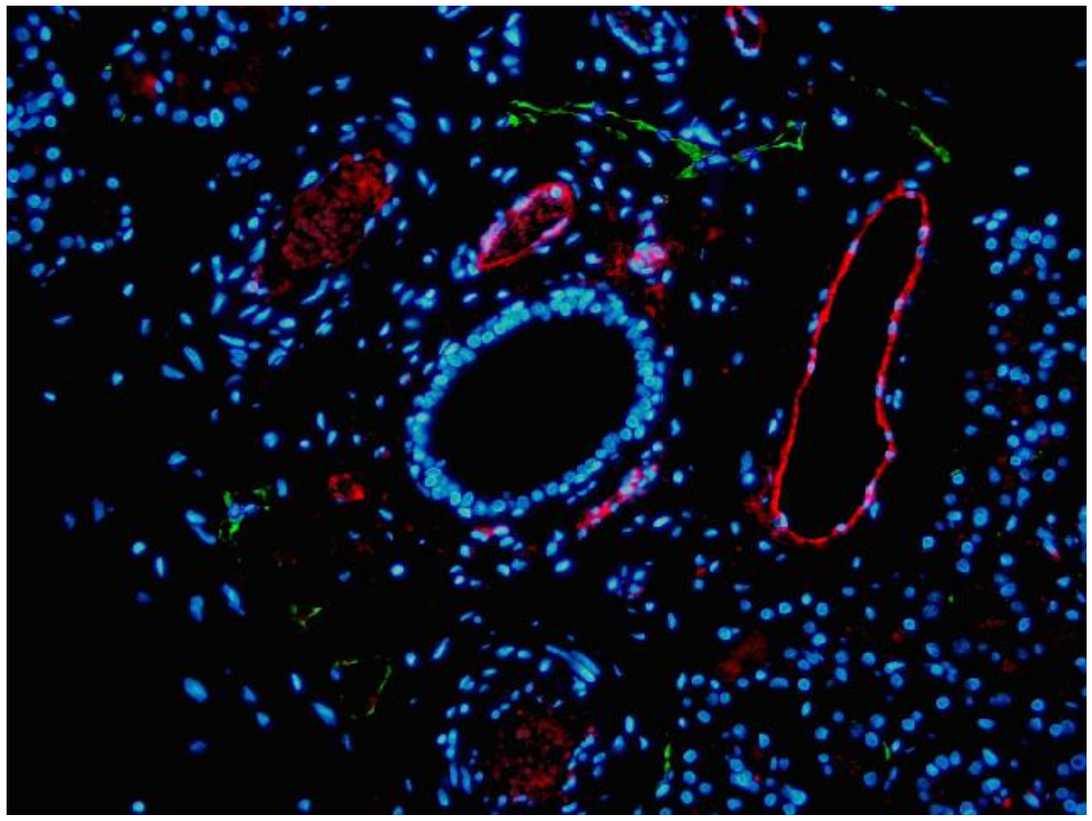
- 依初級抗體host，選擇針對該host的抗體
  - Goat anti-mouse
  - Goat anti-rabbit
  - rabbit anti-mouse, etc.
- 呈色系統(chromogen)
  - ABC (avidin-biotin complex)
  - LSAB (labeled streptavidin-biotin)
  - Polymer - 可避免內源性biotin干擾，特異性及敏感性皆更高
  - HRP/ AP conjugated (HRP-DAB, AP-AEC)



http://www.blossombio.com/eNews/20190708/index.html

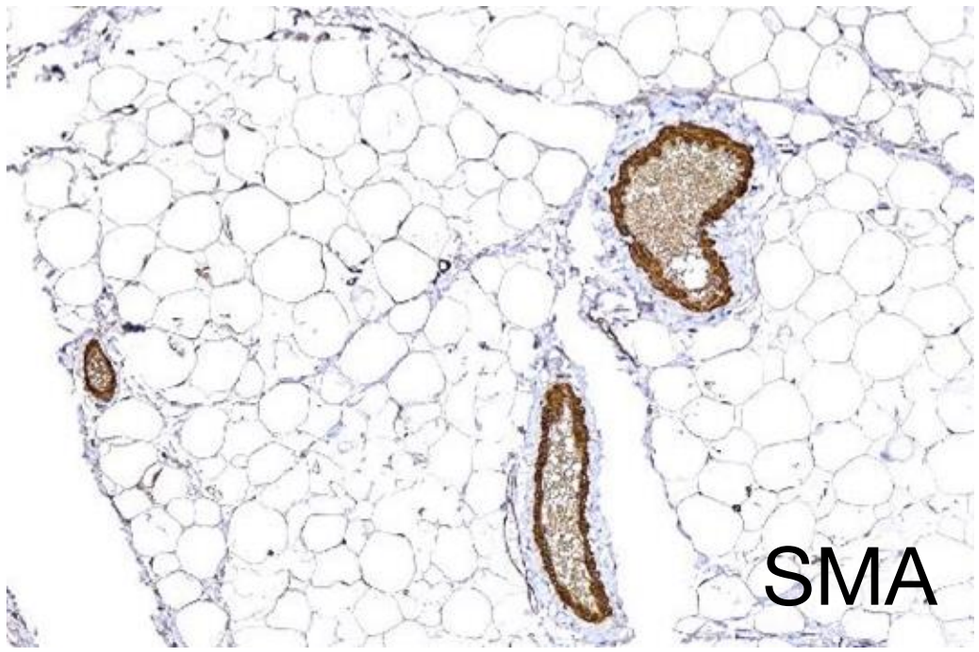
次級抗體 (secondary antibody)

- 依初級抗體host，選擇針對該host的抗體
  - Goat anti-mouse
  - Goat anti-rabbit
  - rabbit anti-mouse, etc.

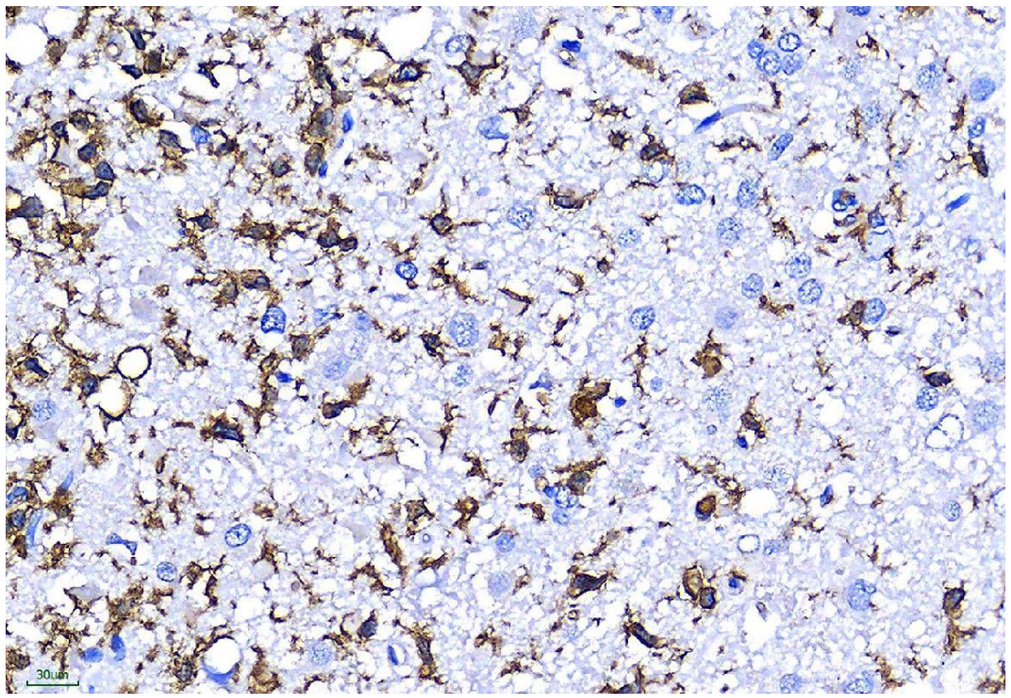


- 呈色系統(chromogen)
  - ABC (avidin-biotin complex)
  - LSAB (labeled streptavidin-biotin)
  - Polymer - 可避免內源性biotin干擾，特異性及敏感性皆更高
  - HRP/ AP conjugated (HRP-DAB, AP-AEC)

染色結果?

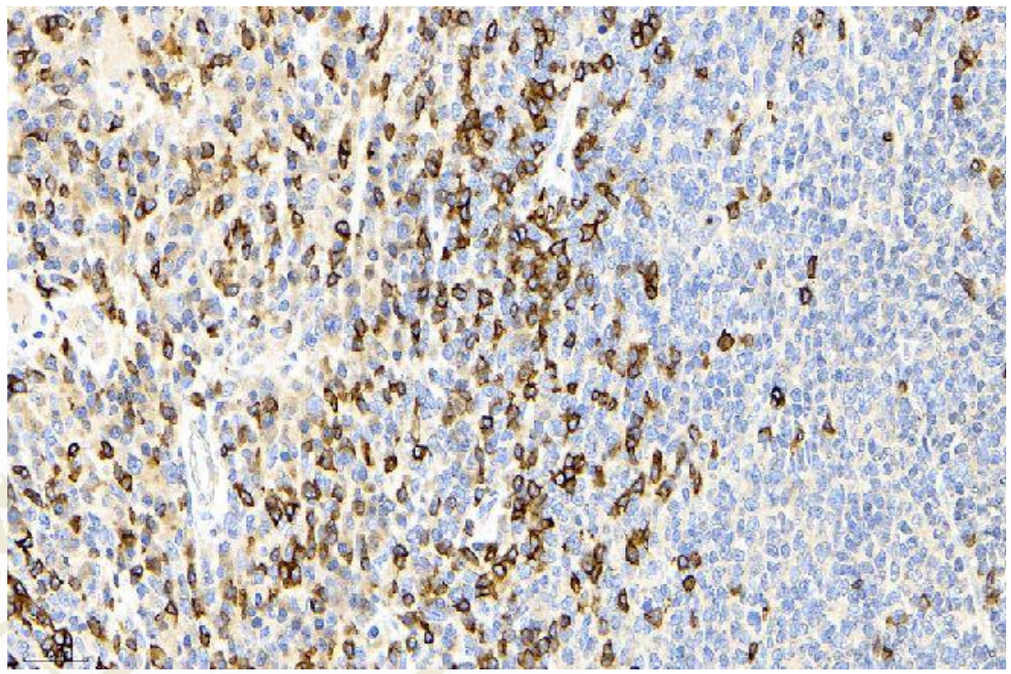


SMA

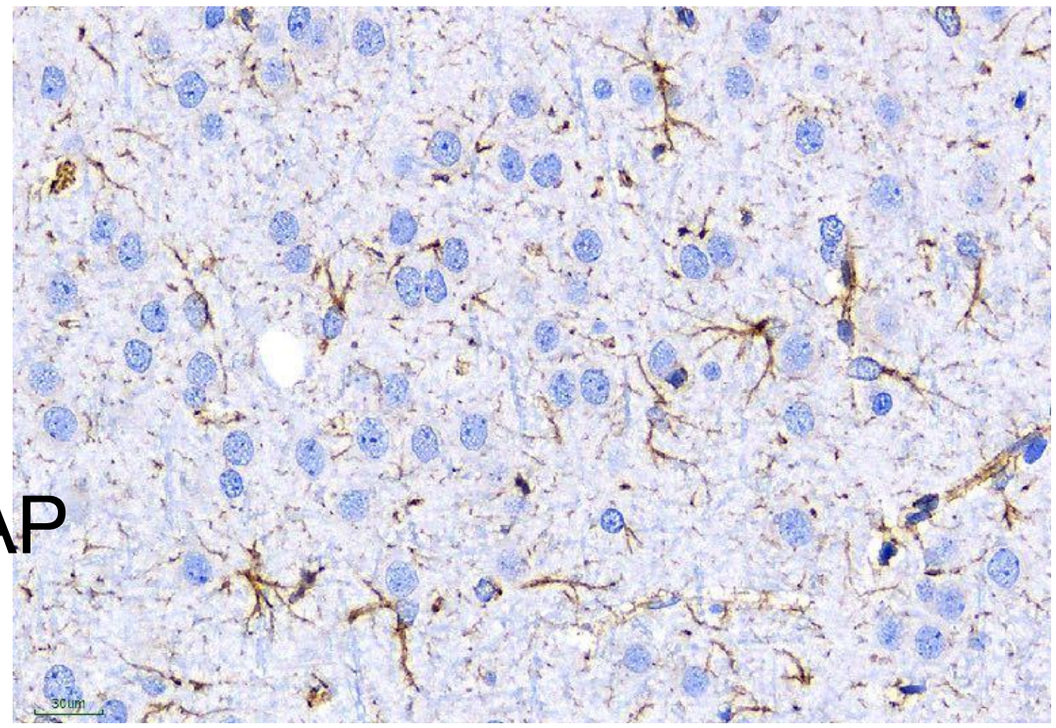


IBA-1

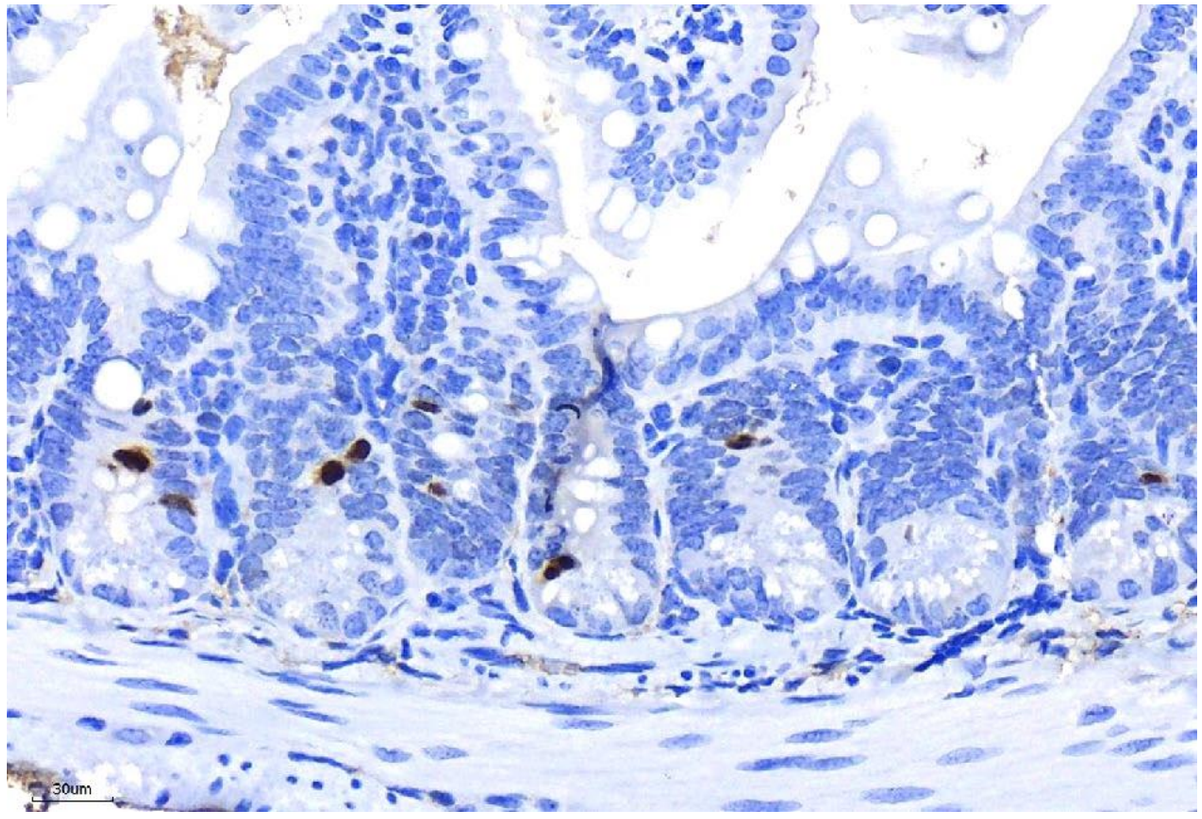
CD3

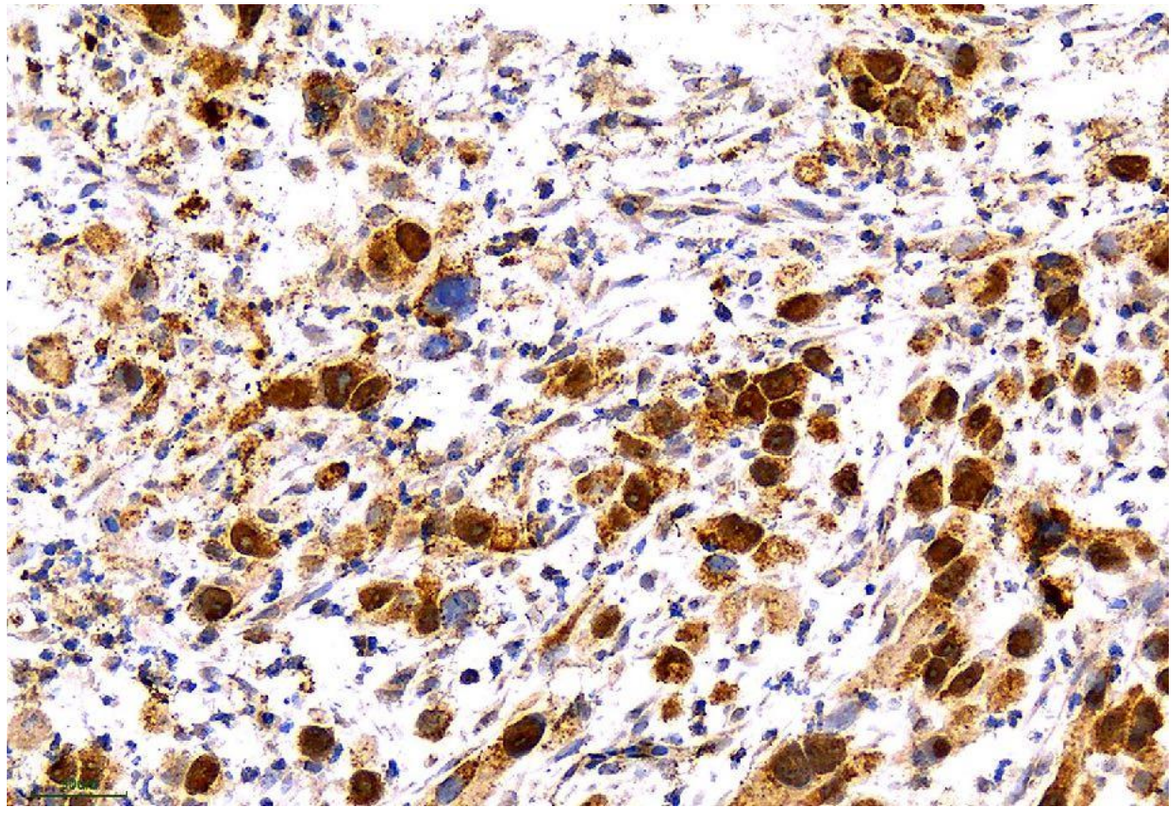


GFAP



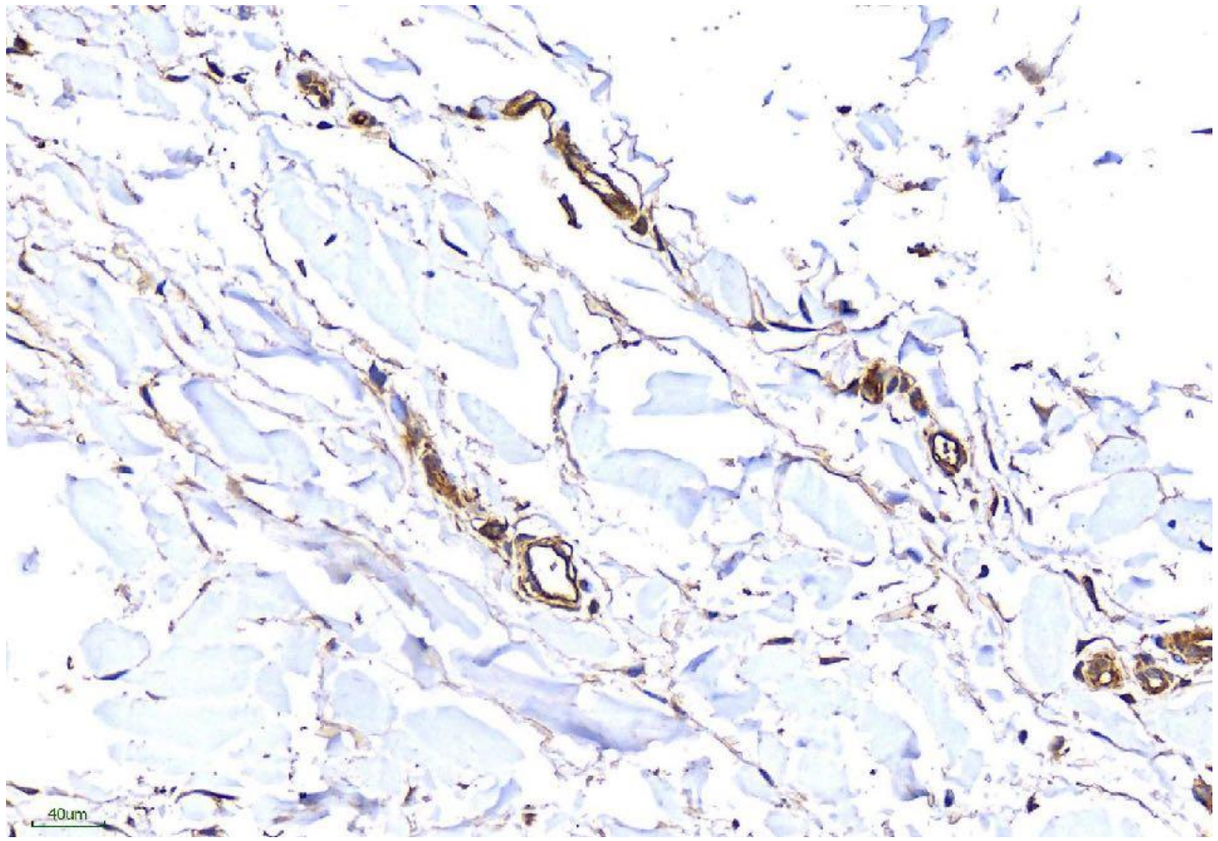
染色結果?

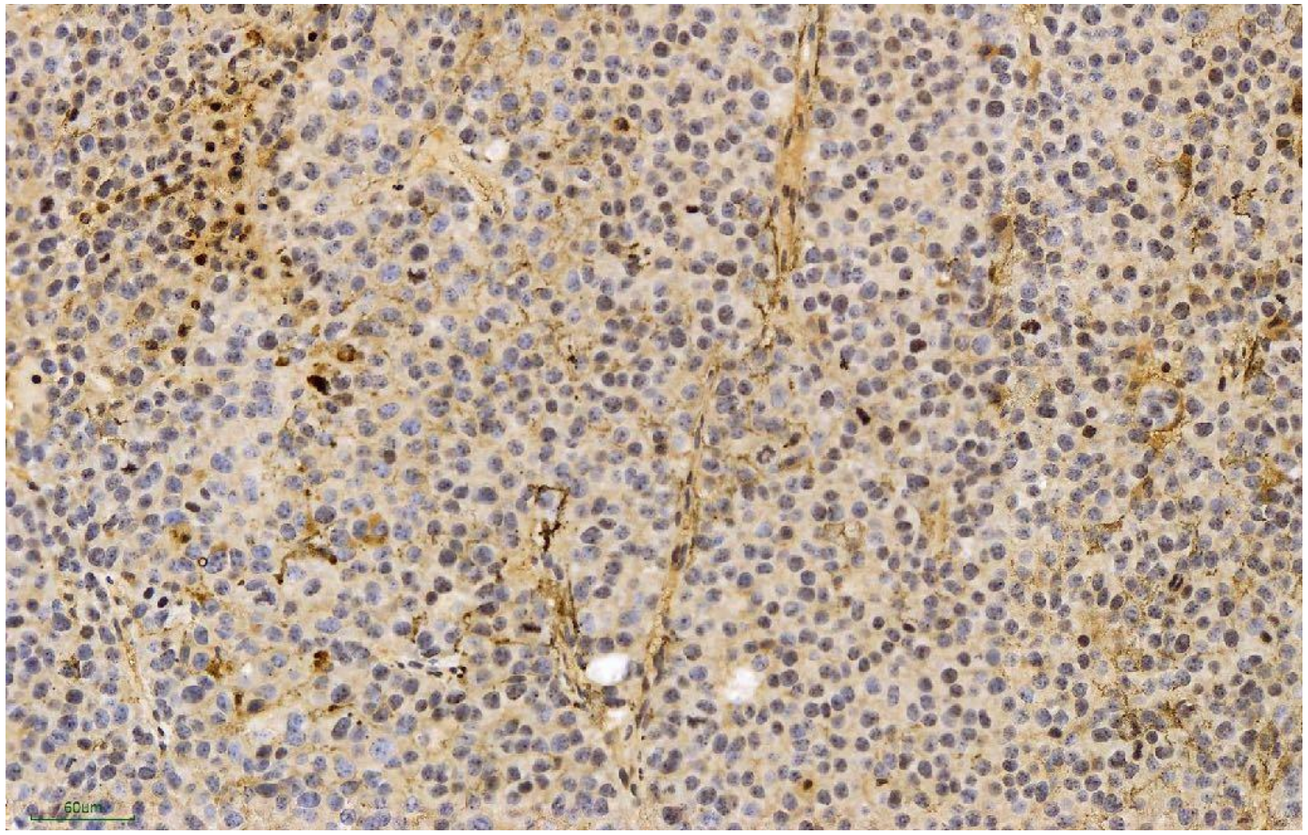




Ki-67

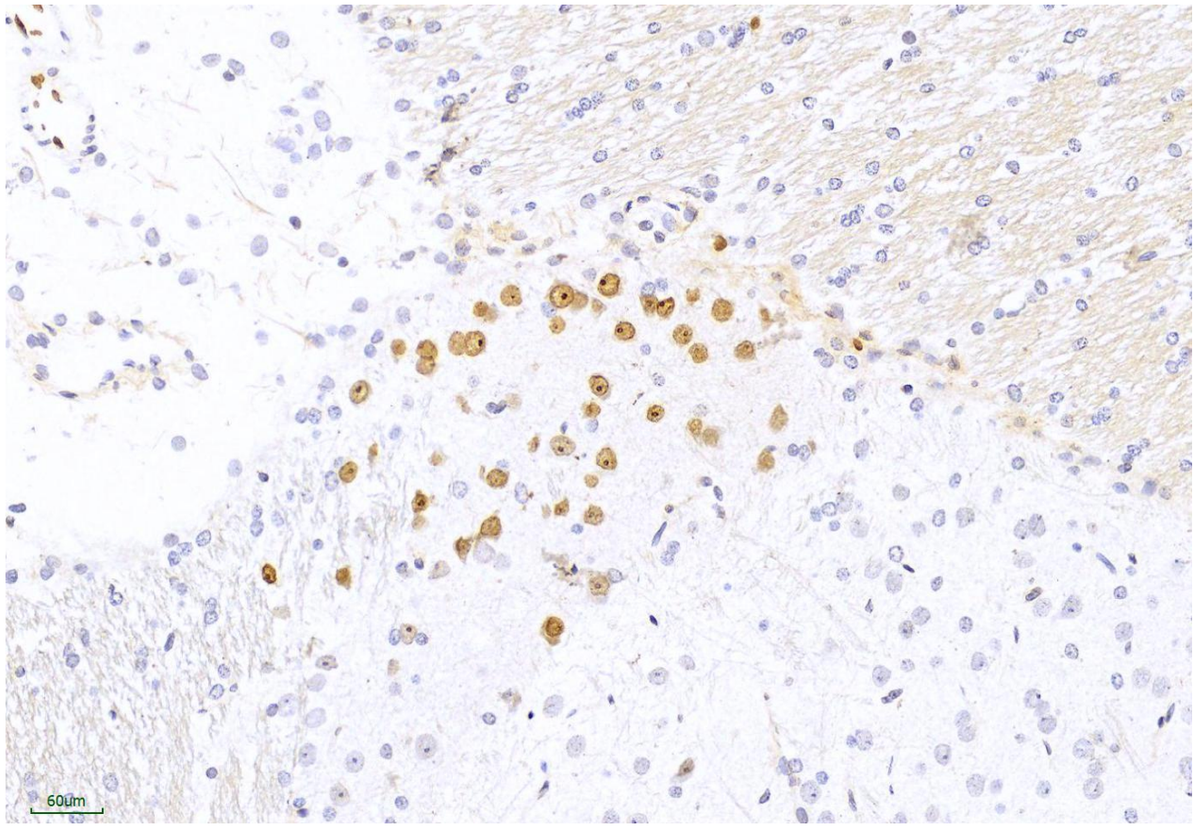
染色結果?

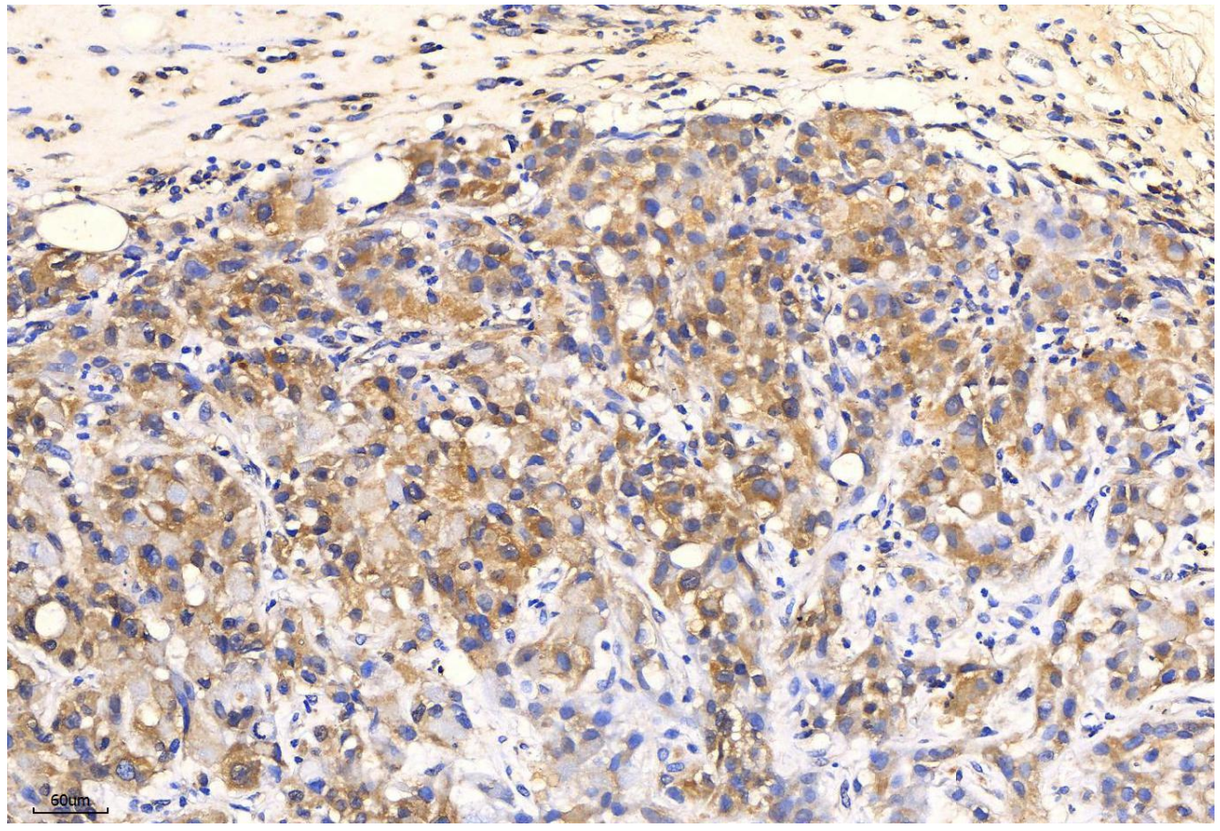




CD31

染色結果?





TUNEL assay

染色結果?

- 背景太深- 抗原修復/Blocking/一抗/二抗/呈色
- 顏色太淡-抗原修復/特殊blocking/一抗/二抗/呈色
- 沒有呈色-過度固定/抗原修復/一抗/二抗/呈色
- 位置不對-抗原修復/一抗/
- 物種不同產生的背景-blocking/二抗
- 不均勻的背景-切片/blocking/玻片/稀釋液不足/染色過程乾掉

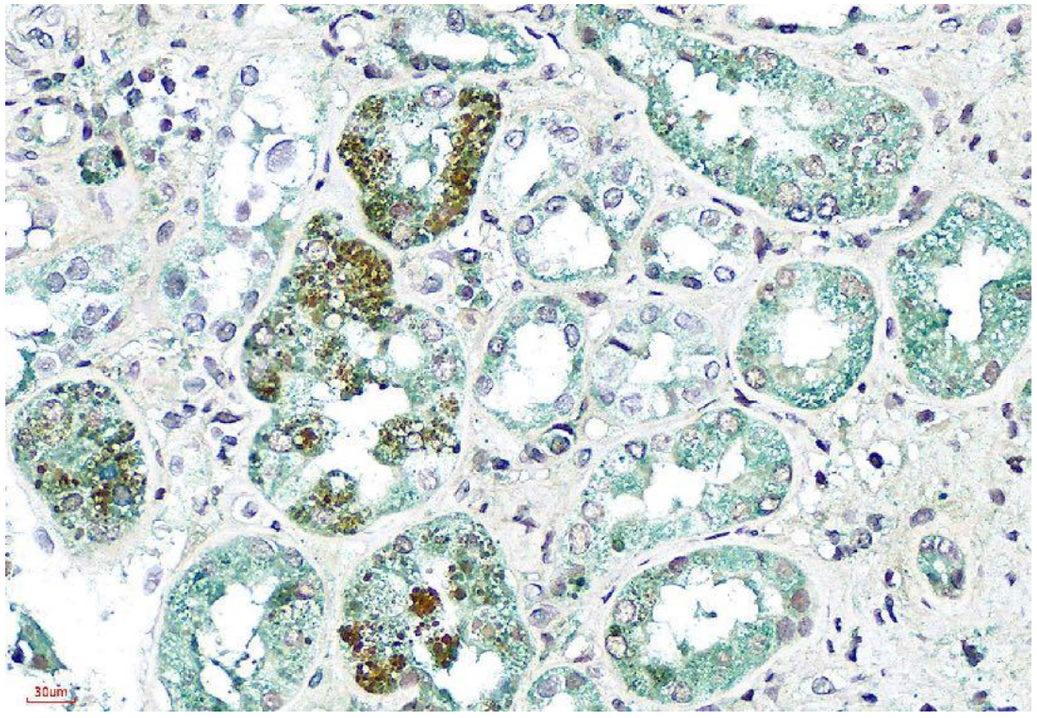
固定液對抗原的影響

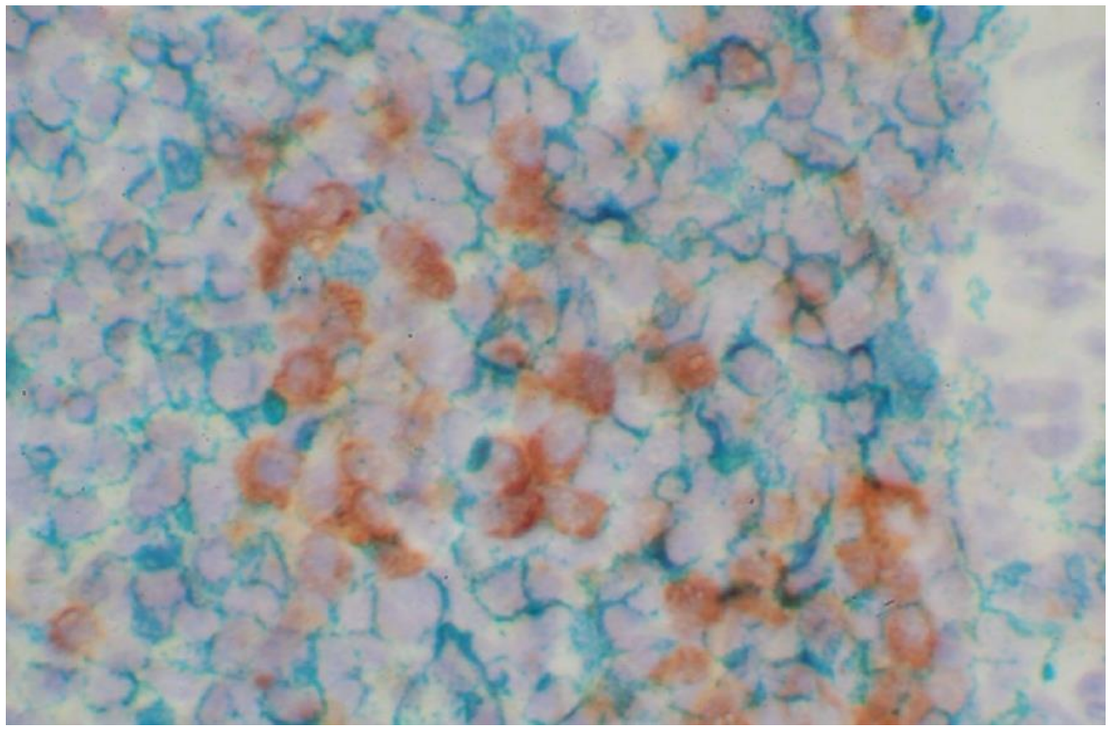
Target	Labeling Intensity		Target	Labeling Intensity	
	Formalin	Davidson		Formalin	Davidson
Brn-3	++	+	Interleukin-1β	++	++
Brn3a	++	++	Interleukin-6	++	++
Calbindin	++	++	Inducible nitric oxide synthase	++	+
Calretinin	++	++	Laminin	++	++
CD3	++	++	Monocarboxylate transporter-3	++	++
Choline acetyl transferase	++	++	Major histocompatibility complex II	++	++
Chx10	++	++	Myeloperoxidase	++	++
Cyclooxygenase-2	++	++	Nestin	++	+
Ciliary neurotrophic factor	++	++	Parvalbumin	++	++
αB-crystallin	++	+	Proliferating cell nuclear antigen	++	++
ED1	++	++	Protein gene product 9.5	++	++
Collagen VI	++	++	Protein kinase C-α	++	++
Fibroblast growth factor-2	++	++	RPE-65	++	++
γ-Aminobutyric acid	++	—	Rhodopsin (RET-P1)	++	++
Glial fibrillary acidic protein	++	++	Synaptophysin	++	++
Glutamine synthetase	++	++	Tumor necrosis factor -α	++	++
Heat shock protein-27	++	+	β_3-tubulin	++	++
Heat shock protein-32	++	++	Tyrosine hydroxylase	++	++
Heat shock protein-70	+	+	Vimentin	++	++
Iba1	++	+			

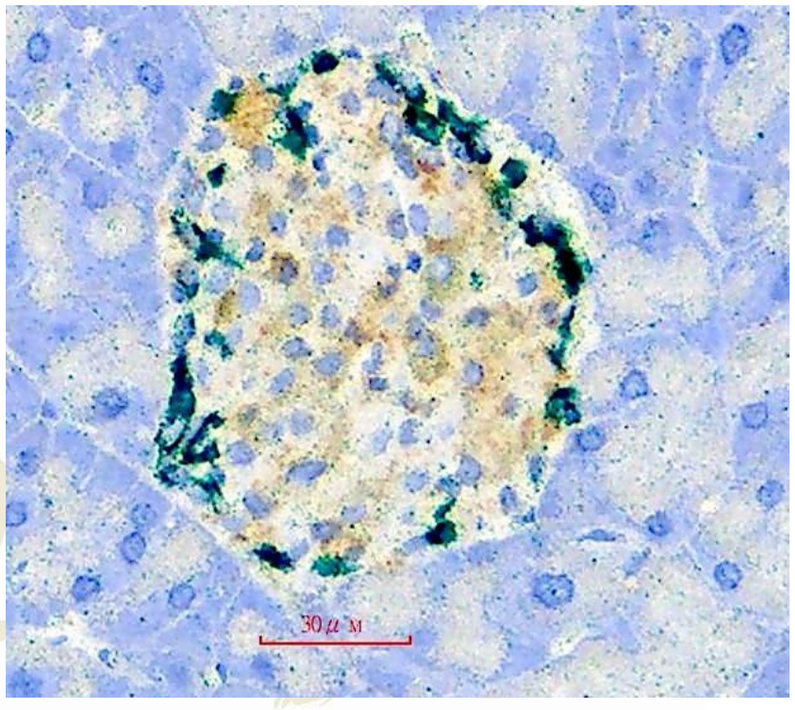
Grading scheme: – = minimal specific labeling, + = weak, specific labeling, ++ = intense, specific labeling.

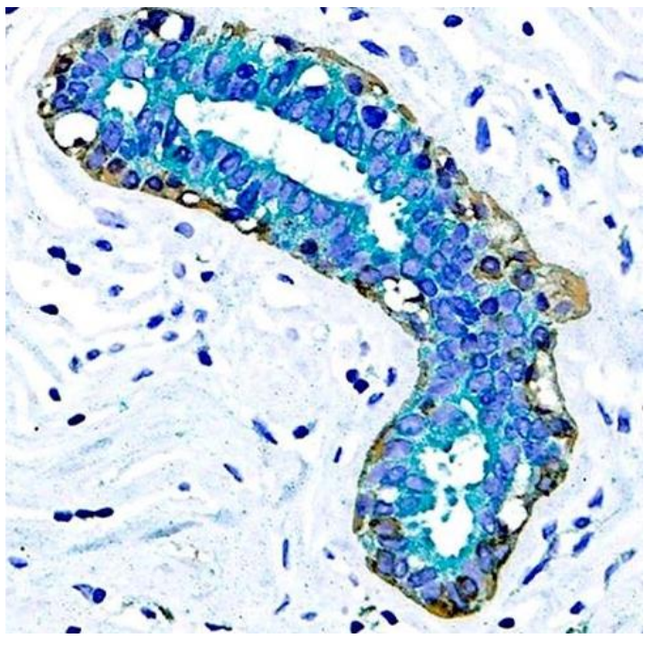
Chidlow. G., et al., 2011. Localization of a wide-ranging panel of antigens in the rat retina by immunohistochemistry: comparison of Davidson's solution and formalin as fixatives.

就是要一次N個marker ?









- Double stain 條件
  - 1抗要不同host
  - 抗原復性方法要相同
  - 視情況甚至可以3染

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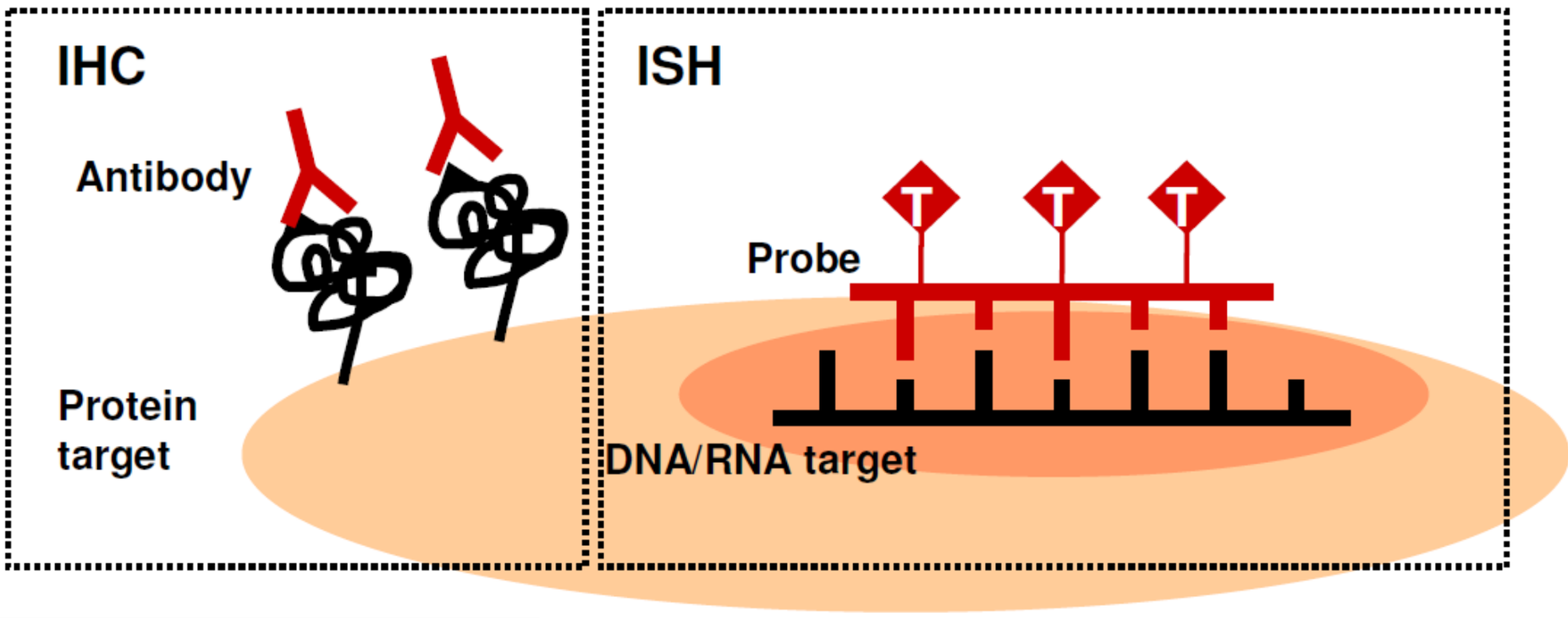
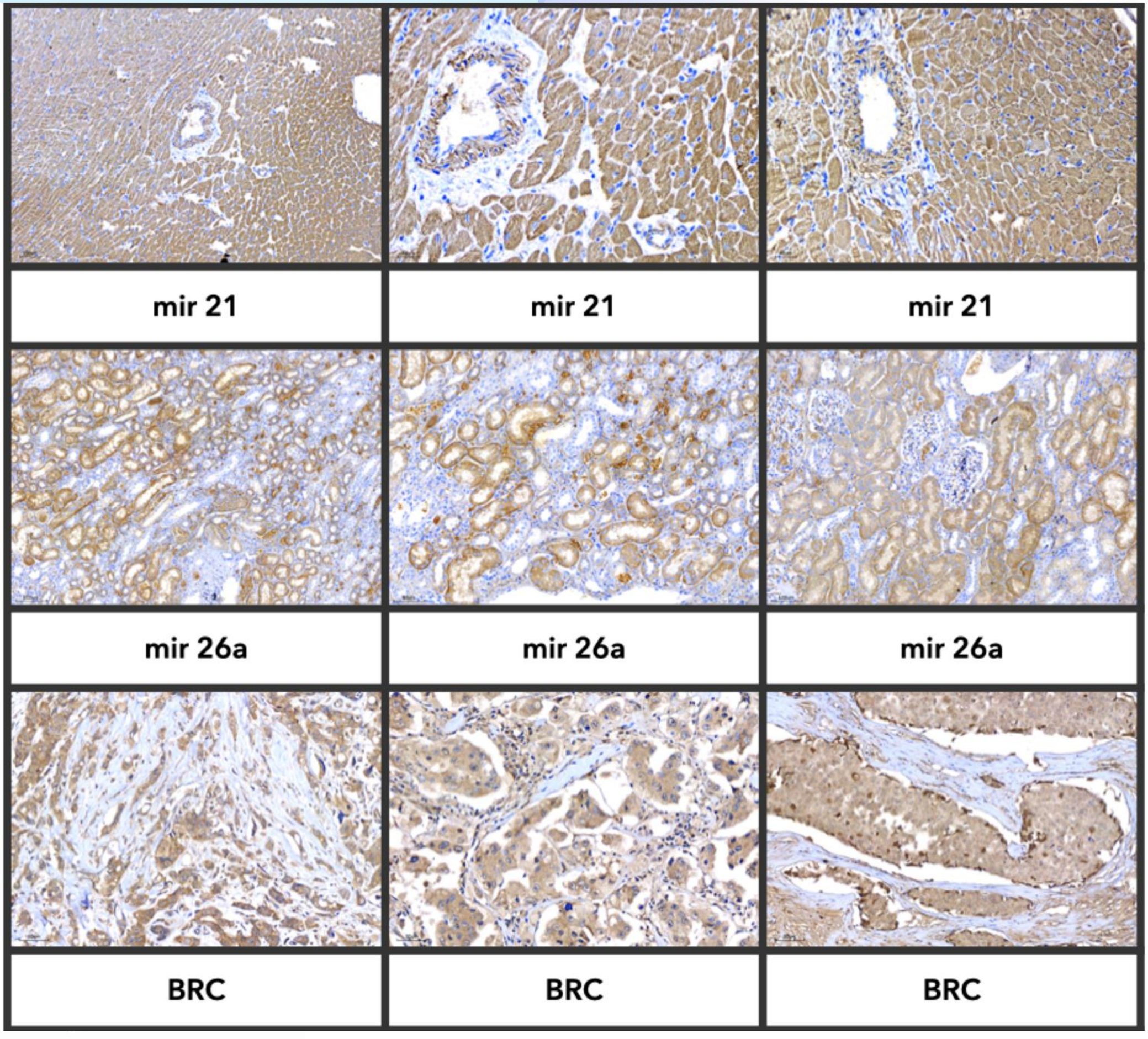
原位雜合反應
(in situ hybridization)

In situ hybridization

- 檢測細胞內DNA or RNA 標靶內的基因物質(genetic material)
  - 使用標靶的探針(probe)來檢測所需要的基因序列
  - 藉由fluorescence (FISH)或 chromogenically (CISH)方法來顯現檢測

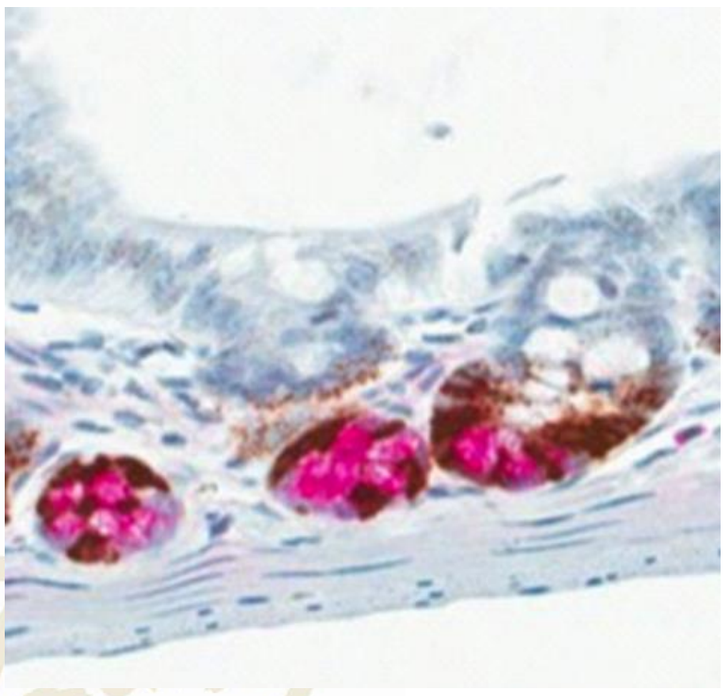


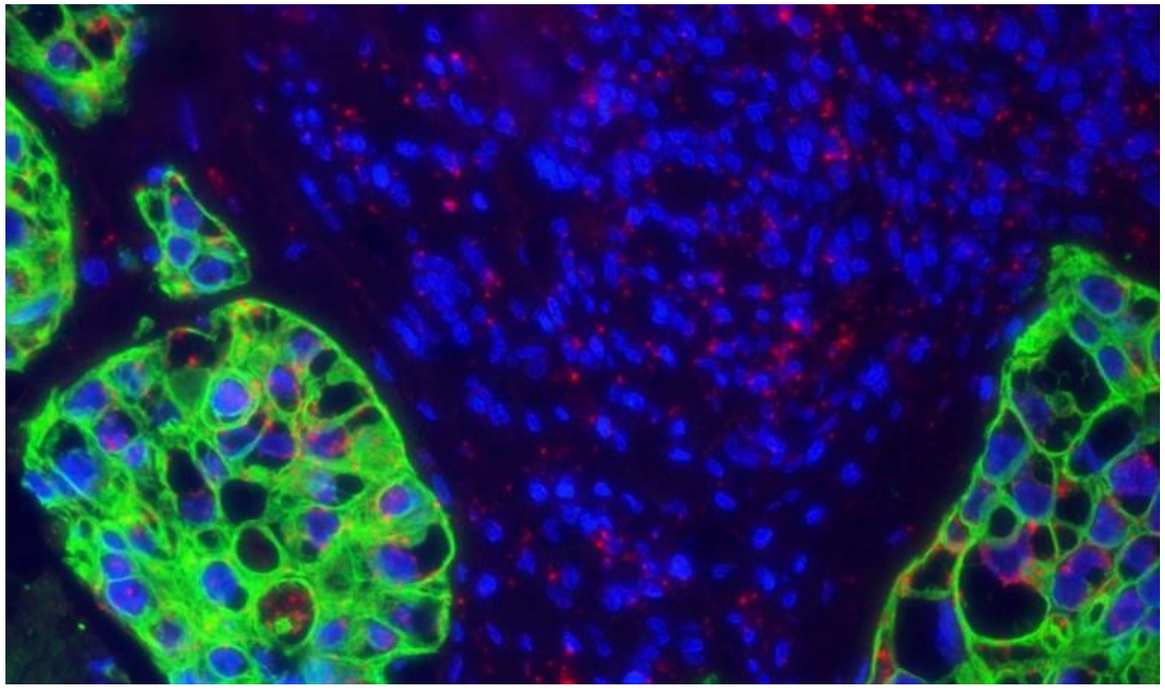
- ISH – Advantages over IHC
- 檢測細胞內蛋白合成前的基因階期
- 檢測潛伏期的病毒感染(無法藉由IHC檢測)
- 鑑定基因的異常


mir 21
mir 21
mir 21
mir 26a
mir 26a
mir 26a
BRC
BRC
BRC

ISH X IHC X Histochemistry

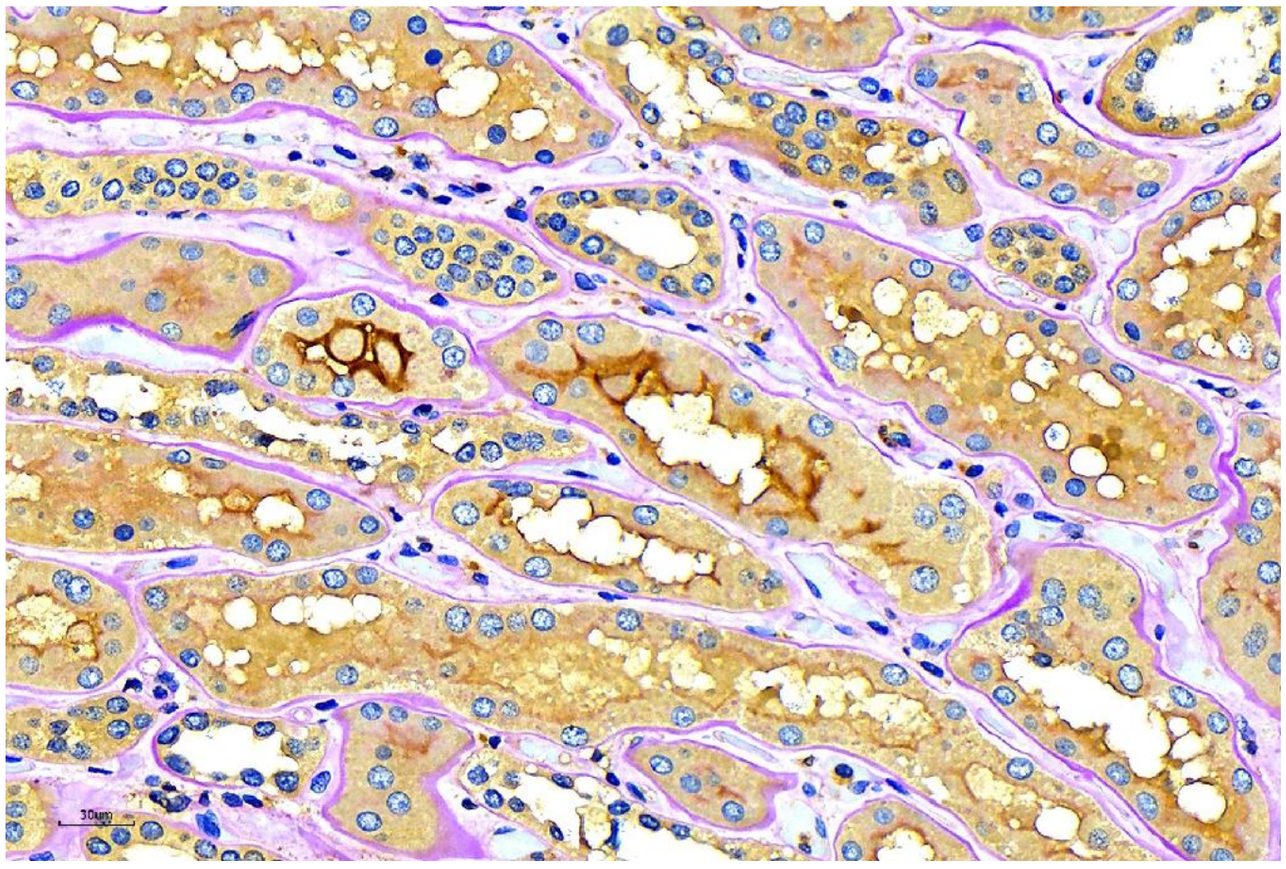
- ISH + IHC: 較常見使用螢光染色
- 因ISH會經過較常時間加熱，可能影響IHC的抗原呈現，一般流程需IHC先做

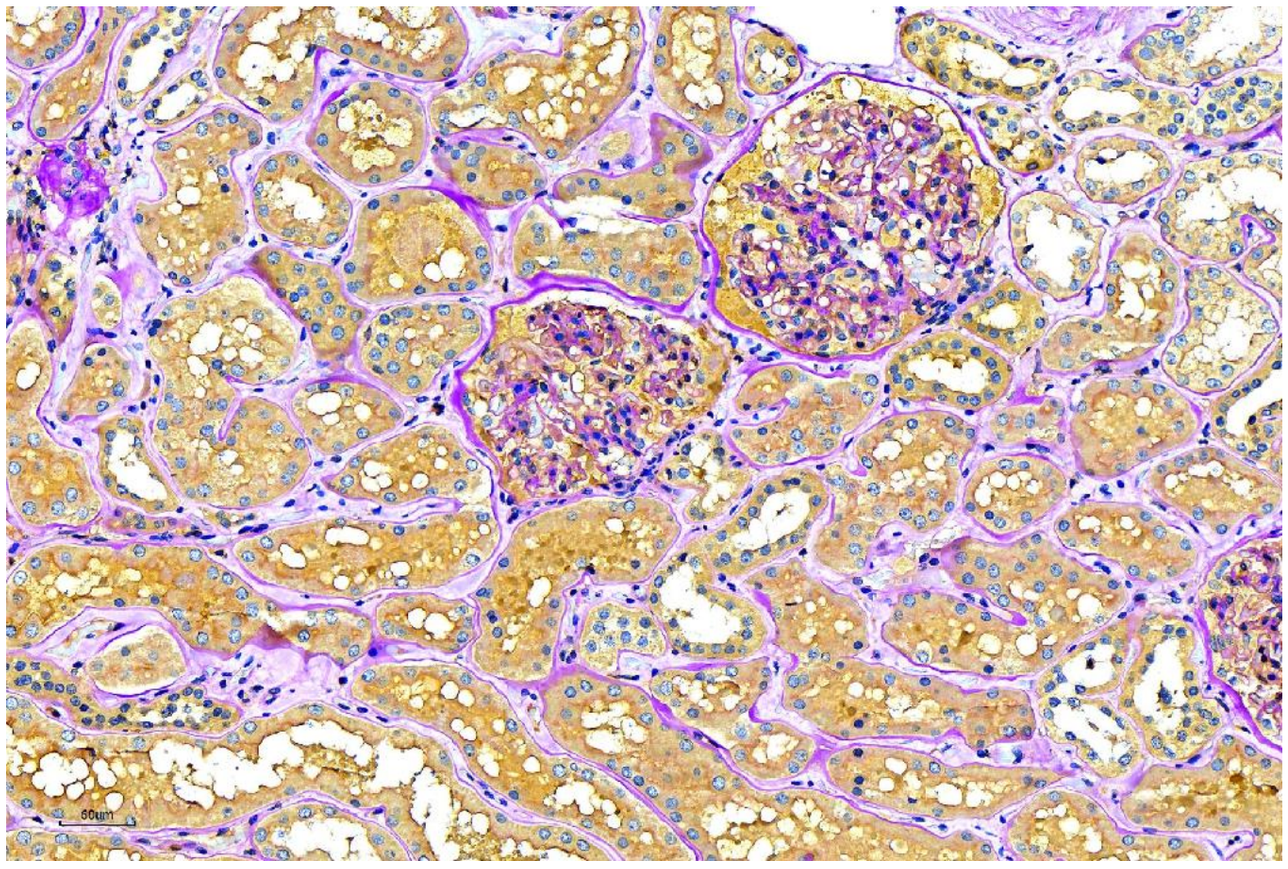




https://acdbio.com/science/applications/research-solutions/dual-ish-and-ihc

IHC + Histochemistry





ACE2 + PAS

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Q & A