

Pitfalls and trouble shooting in immunostaining

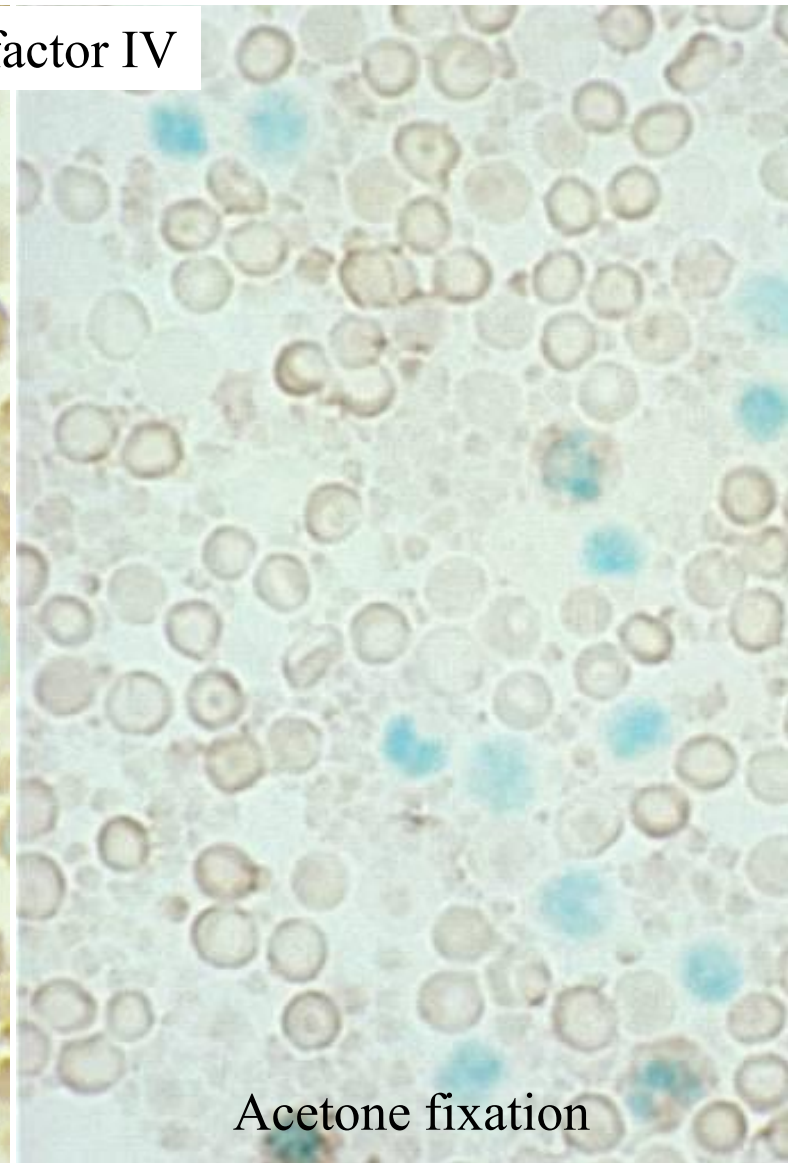
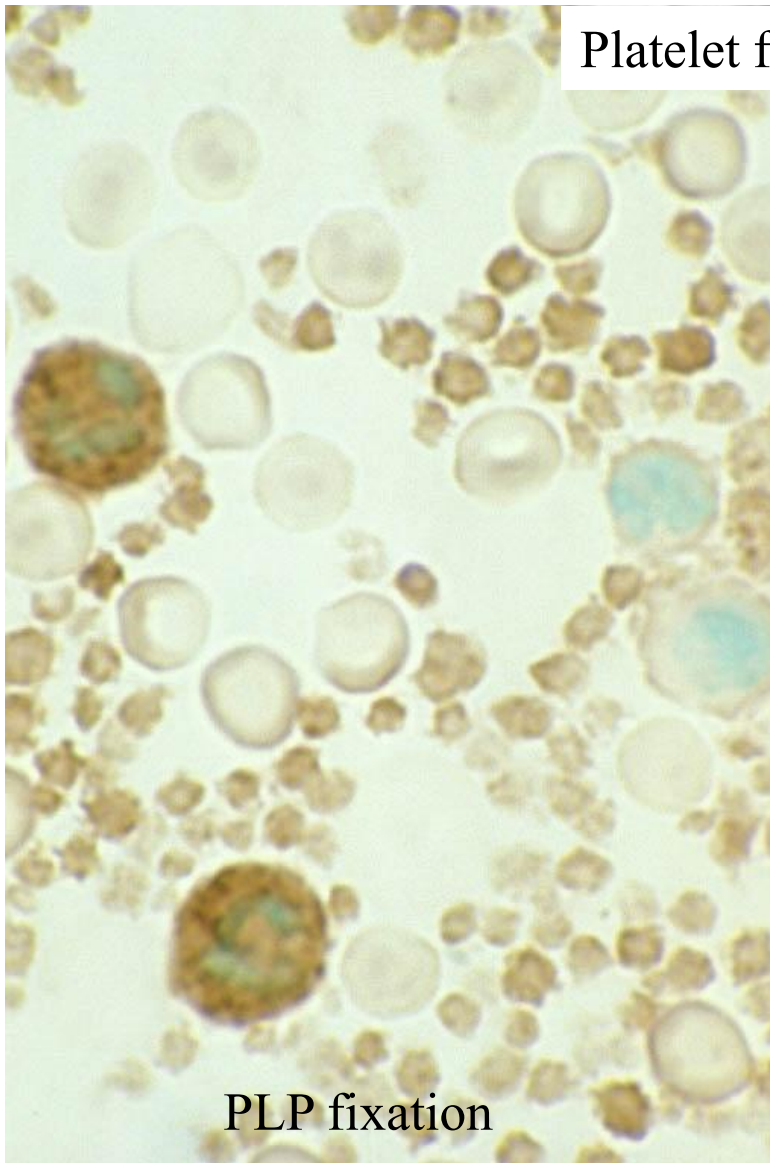
Chromogenic immunohistochemistry is an essential histochemical technique for analyzing pathogenesis and making a histopathological diagnosis in routine pathology services. In neoplastic lesions, immunohistochemistry allows the study of specific clinical and biological features such as histogenesis, behavioral characteristics, therapeutic targets, and prognostic biomarkers. The needs for appropriate and reproducible methods of immunostaining are prompted by technical development and refinement, commercial availability of a variety of antibodies, advanced applicability of immunohistochemical markers, accelerated analysis of clinicopathological correlations, progress in molecular targeted therapy, and the expectation of advanced histopathological diagnosis. However, immunostaining does have various pitfalls and caveats. Pathologists should learn from previous mistakes and failures and from results indicating false positivity and false negativity. Here, the author describe various devices, technical hints, and trouble-shooting guides to keep in mind when performing immunostaining.

Ref.: Tsutsumi Y. Pitfalls and caveats in applying chromogenic immunostaining to histopathological diagnosis. *Cells* 2021; 10: 1501. doi: 10.3390/cells10061501

Fixation

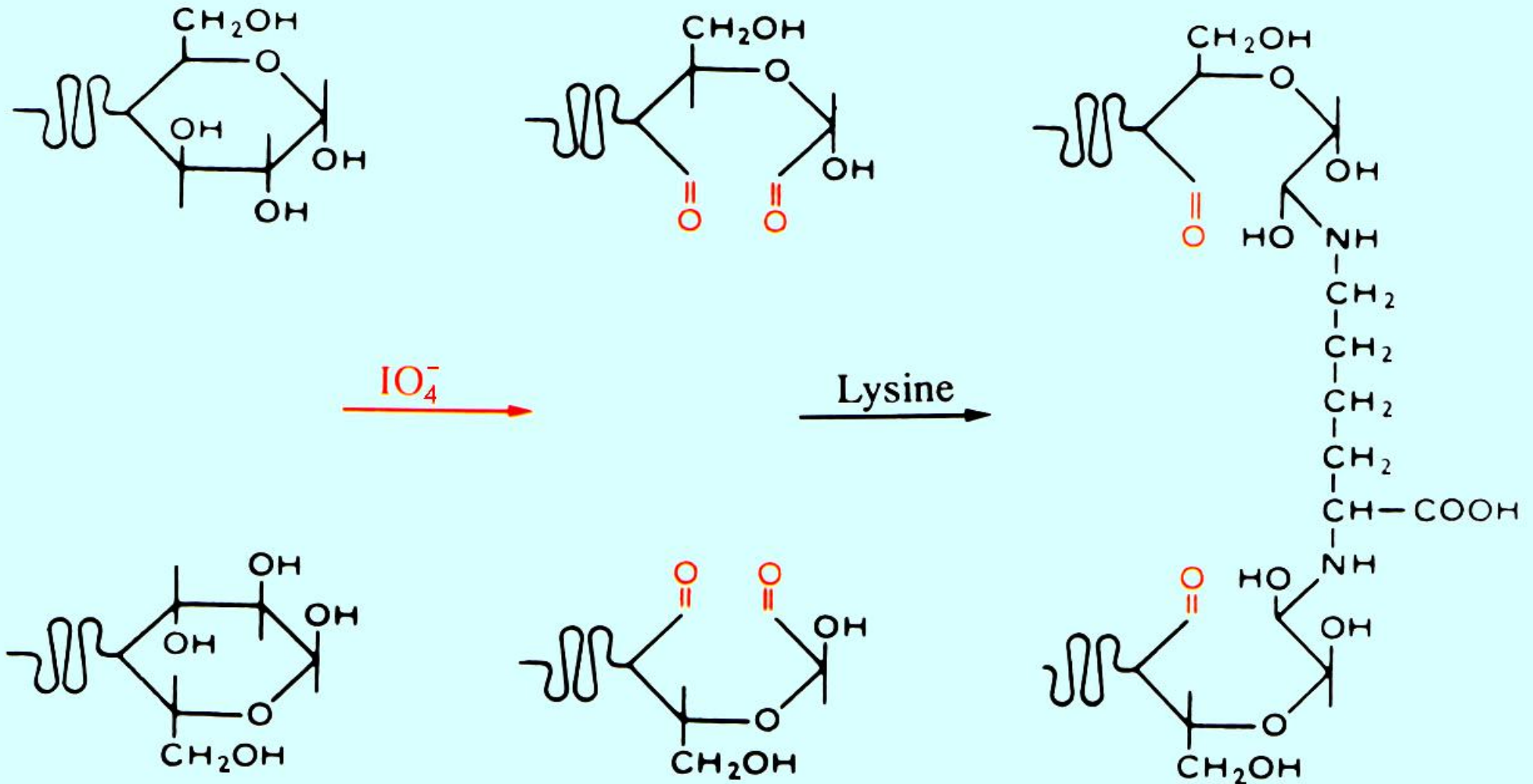
1. Fresh frozen sections (post-fixed in organic solvent such as alcohol or acetone)
2. Organic solvent-fixed paraffin-embedded sections
3. Formalin-fixed paraffin-embedded sections

Platelet factor IV

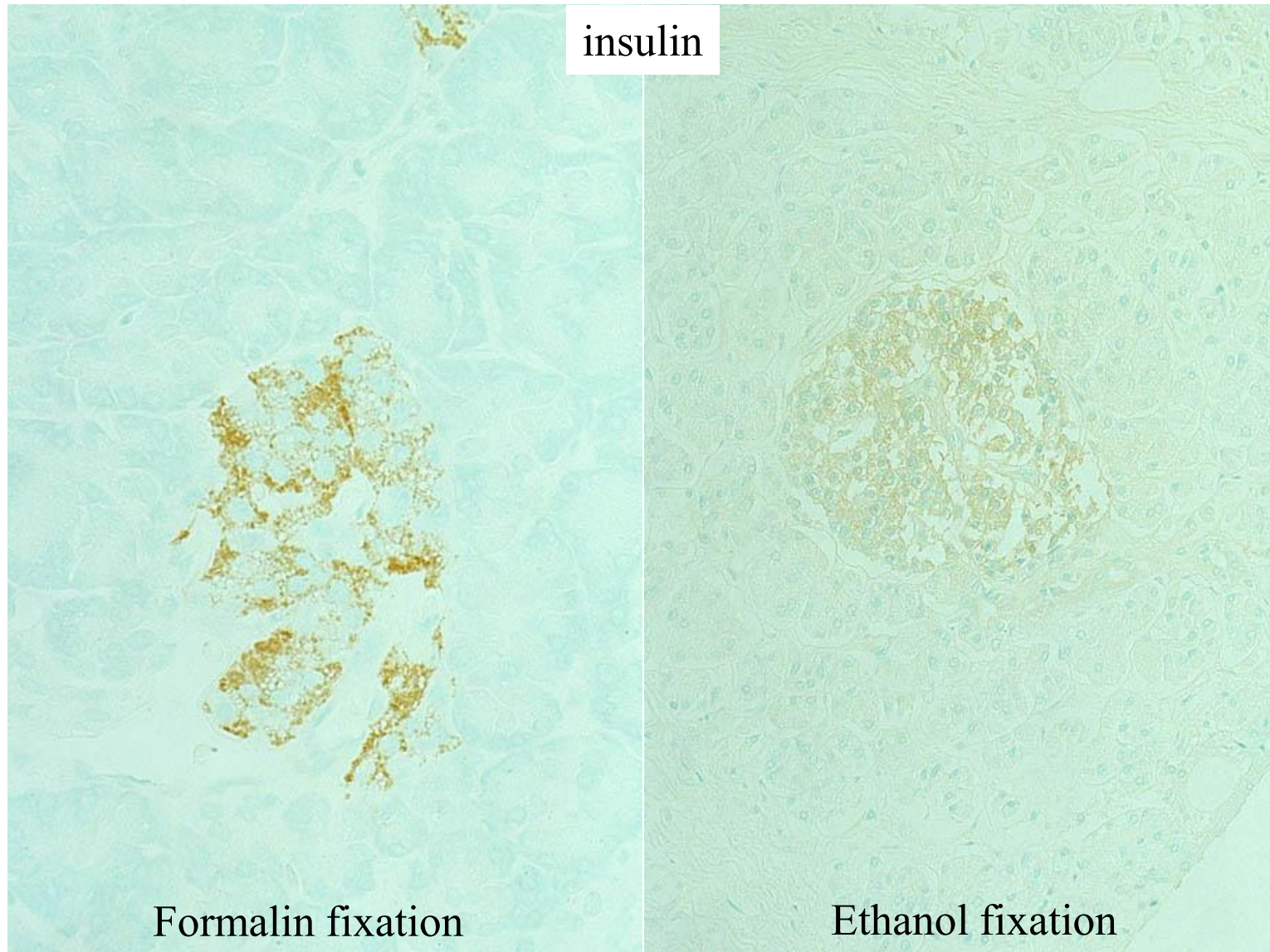


Antigenicity of platelet factor IV in the buffy coat of the peripheral blood is retained with PLP fixation but lost in acetone fixation. Platelet factor IV in the platelet granules are extracted during fixation in the organic solvent.

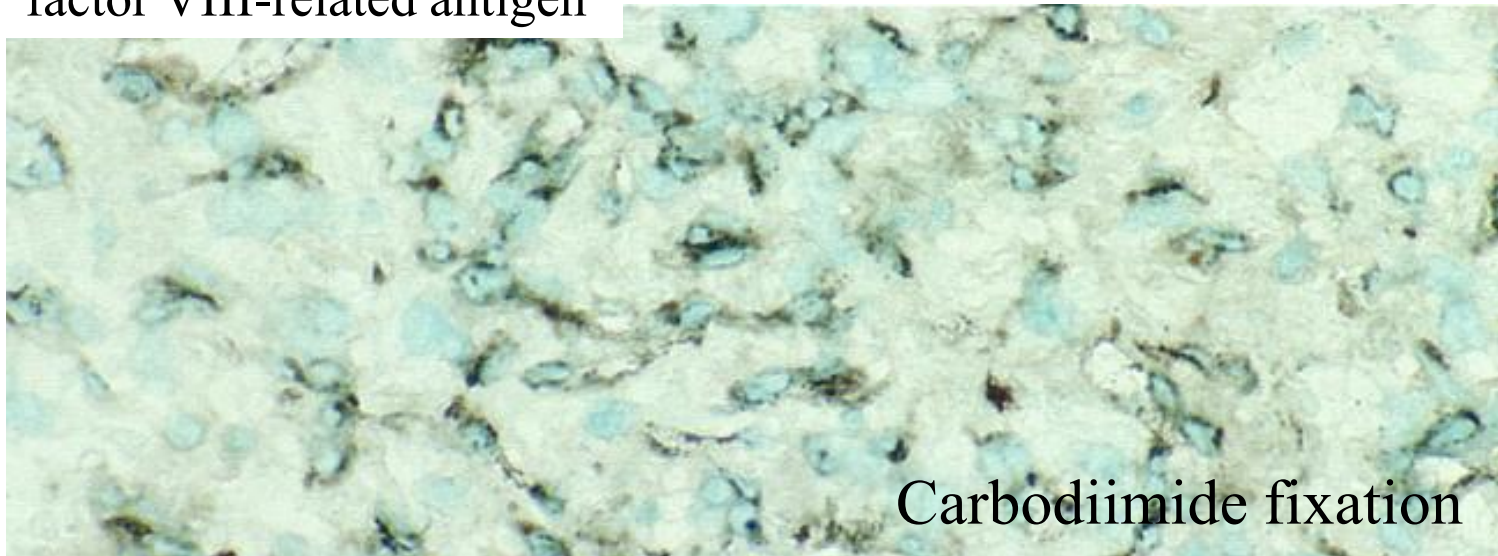
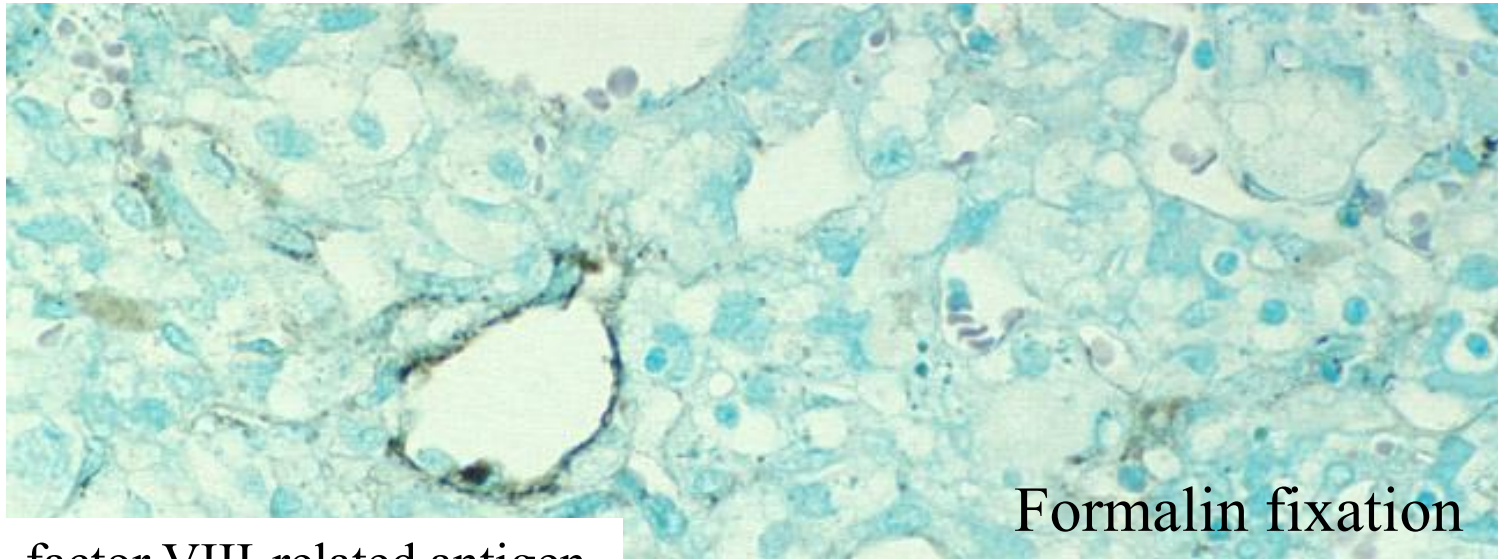
PLP fixation



Periodic acid oxidizes the vicinal diol groups on sugar to aldehydes, and the aldehydes then react with lysine molecules containing two amino residues. As a result, the sugar molecules are cross-linked and fixed.

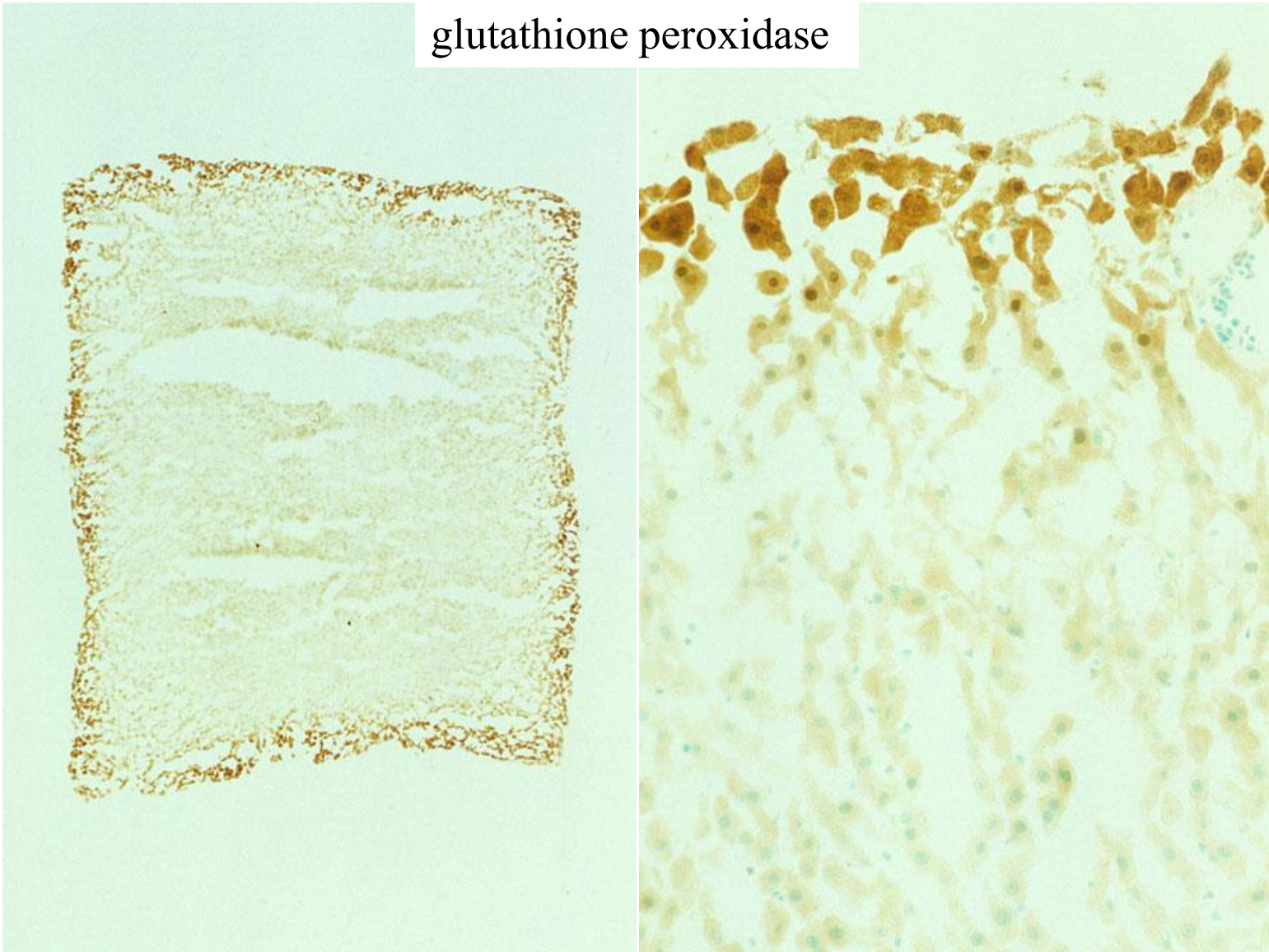


Antigenicity of insulin in the normal pancreatic islets. Insulin immunoreactivity is well-detained by formalin fixation but markedly reduced after ethanol fixation. Insulin in the neuroendocrine granules are extracted during fixation in the organic solvent.



Factor VIII-related antigen in hemangioblastoma of the cerebellum. Carbodiimide fixation well retains the antigenicity, while the antigenicity is mostly lost after formalin fixation.

glutathione peroxidase

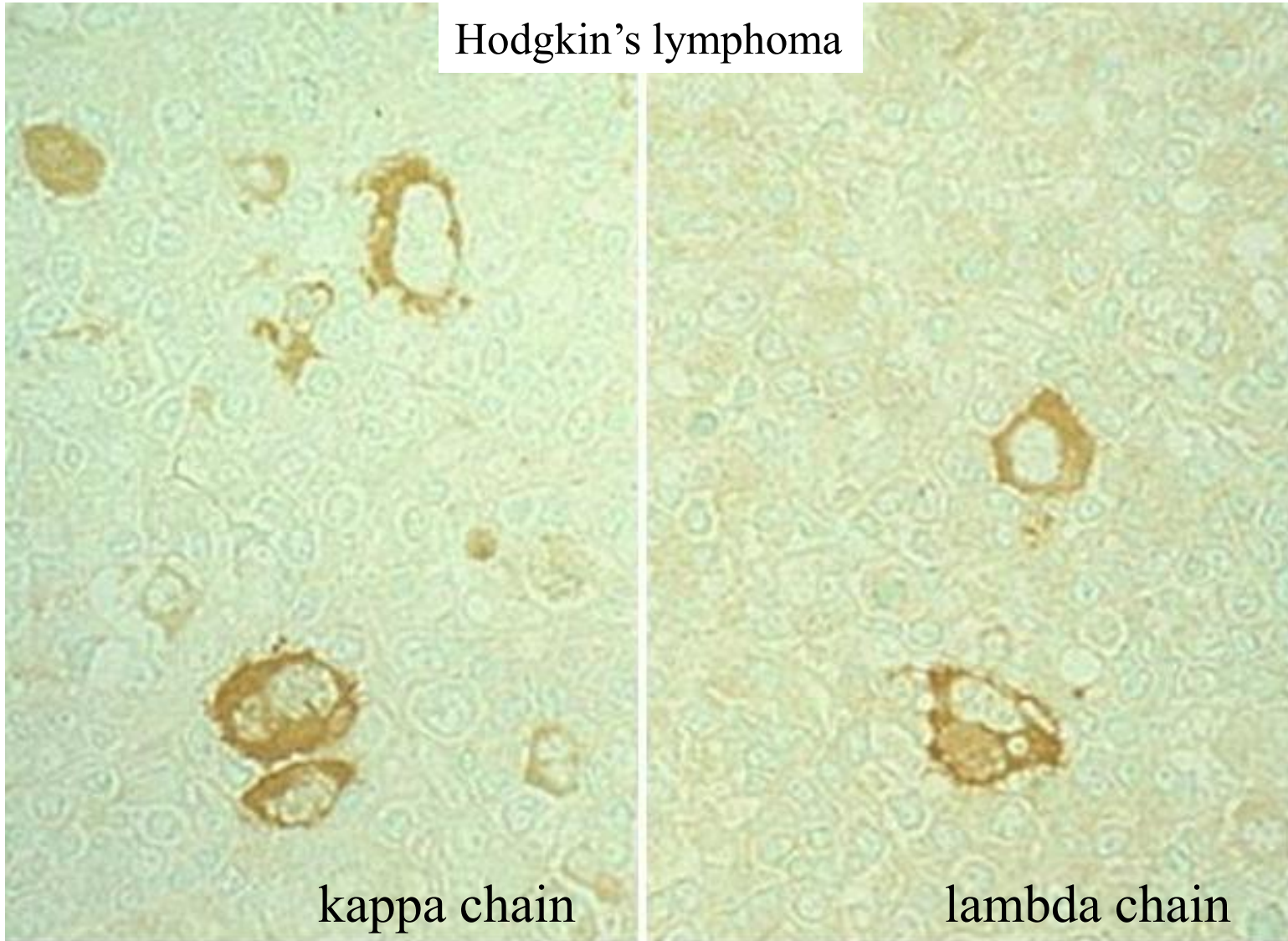


Microwave fixation: glutathione peroxidase localization in PLP-fixed frozen sections of the rat liver. Only the peripheral part of the liver tissue block shows positive signals.

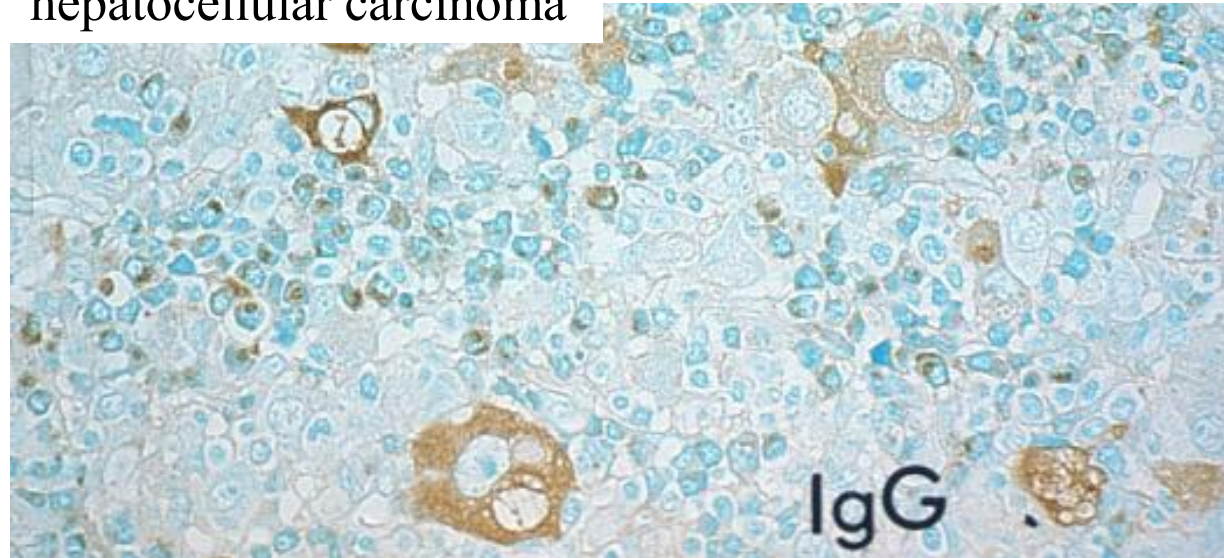
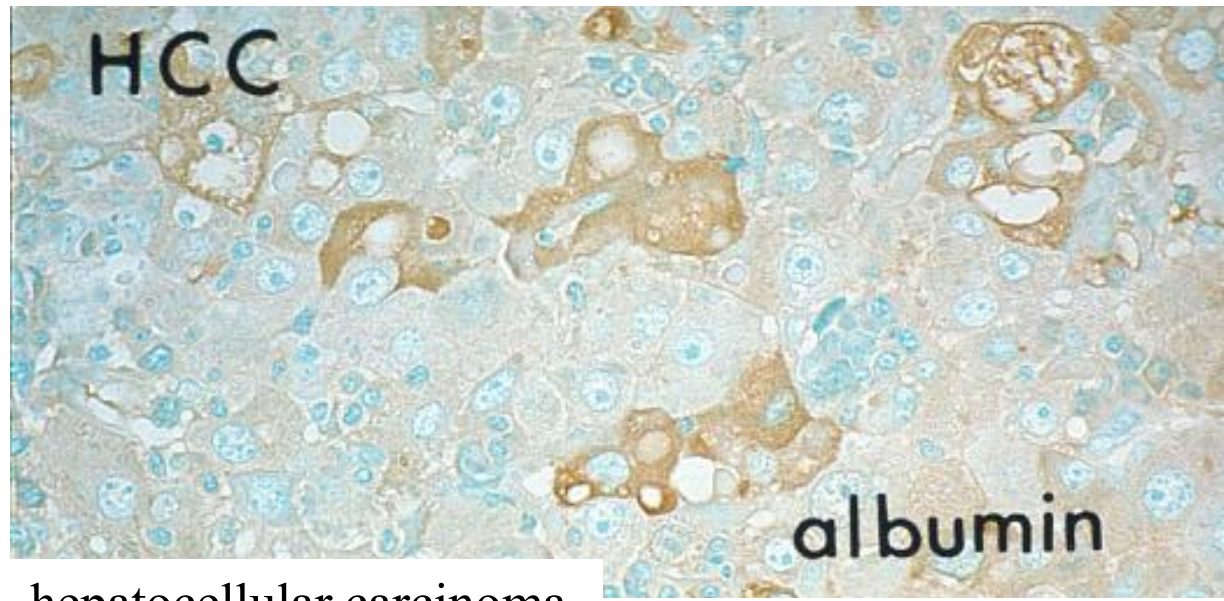
Artifacts and effects of formalin fixation

- 1) Diffusion artifact of plasma proteins into the cytoplasm of cells
- 2) False negativity due to improper fixation
- 3) False positivity due to improper fixation
- 4) Results in decalcified or necrotic tissue
- 5) Formalin fixation can retain the immunoreactivity for years.

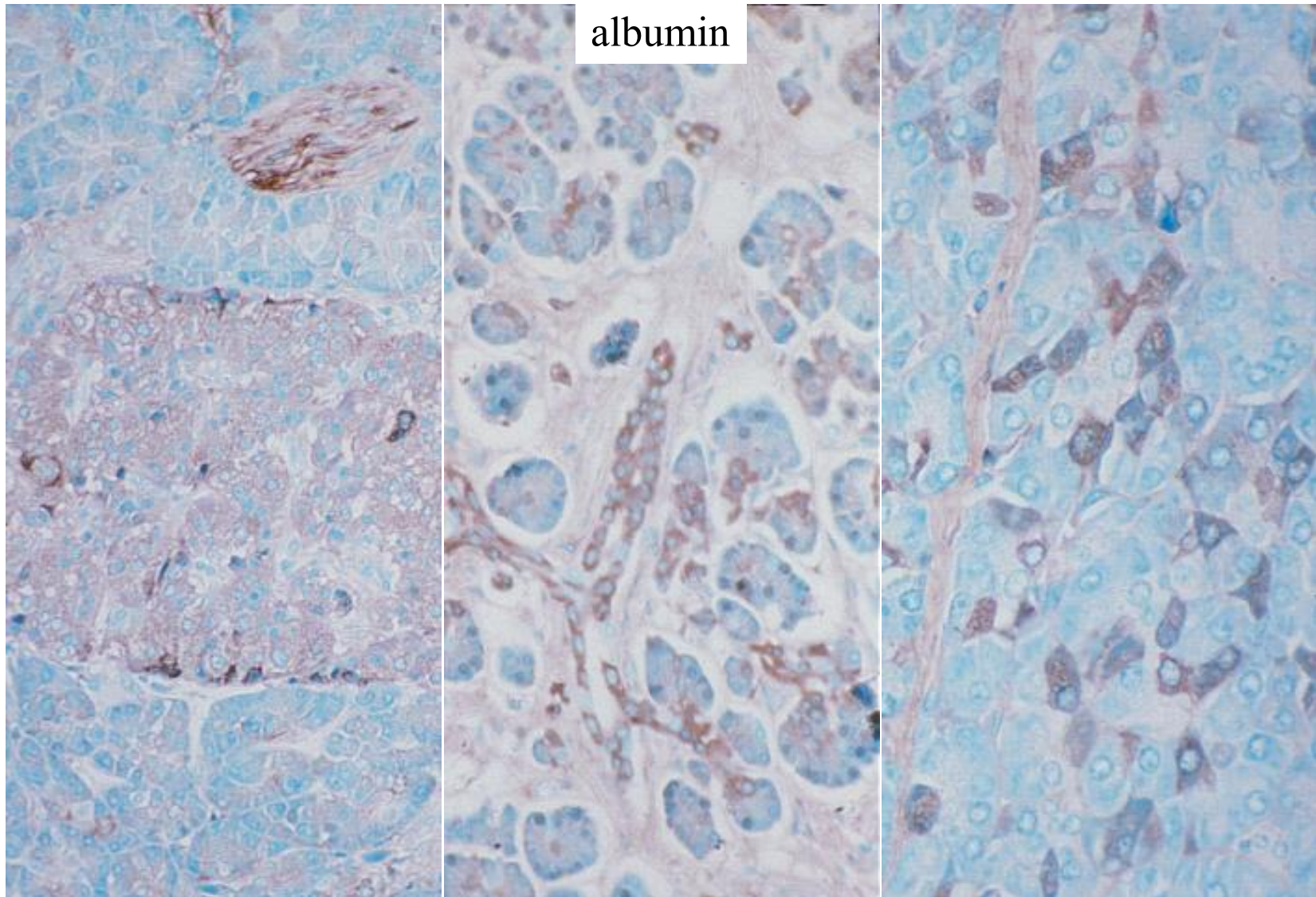
Hodgkin's lymphoma



Diffusion artifact of plasma proteins in Hodgkin's lymphoma. Both kappa and lambda chains are positive in the cytoplasm of Hodgkin's cells and Reed-Sternberg cells.

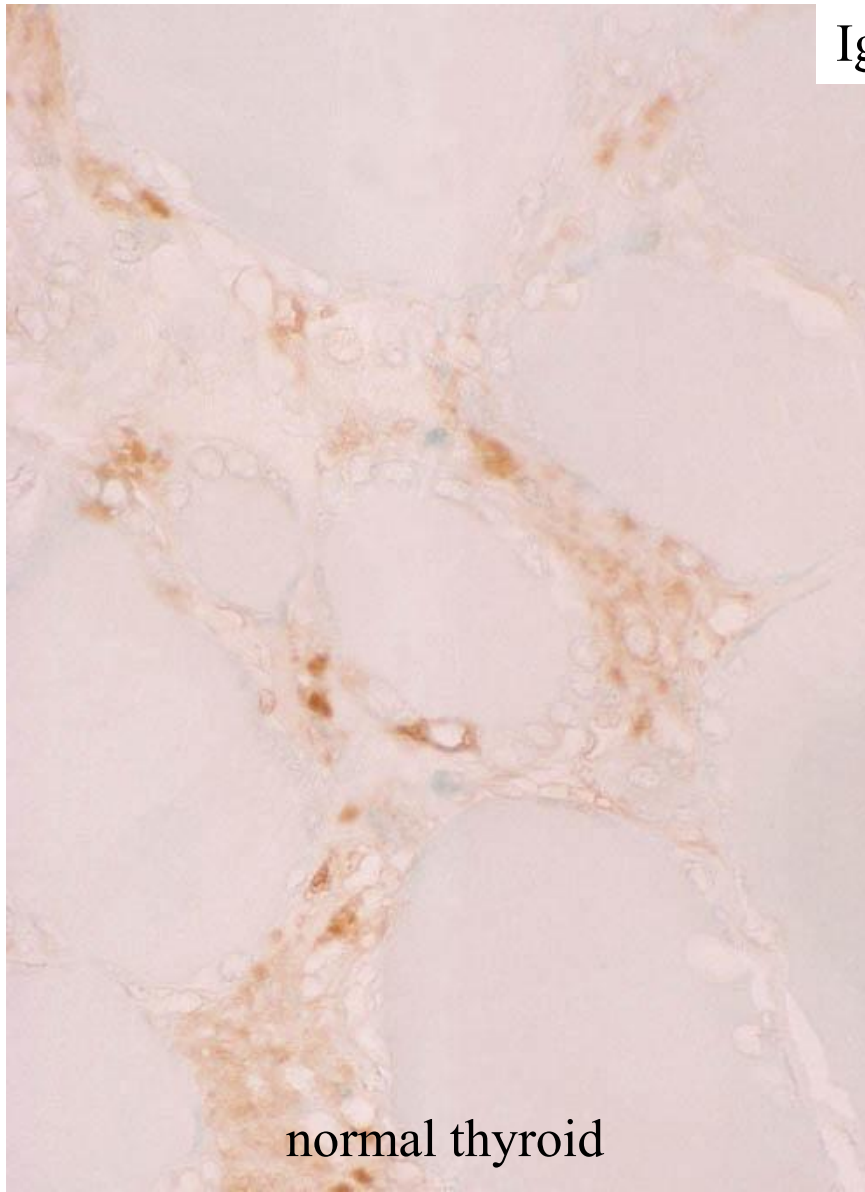


Diffusion artifact of plasma proteins in hepatocellular carcinoma metastatic to the node. Both albumin and IgG immunoreactivities are detected in the cytoplasm of tumor giant cells.

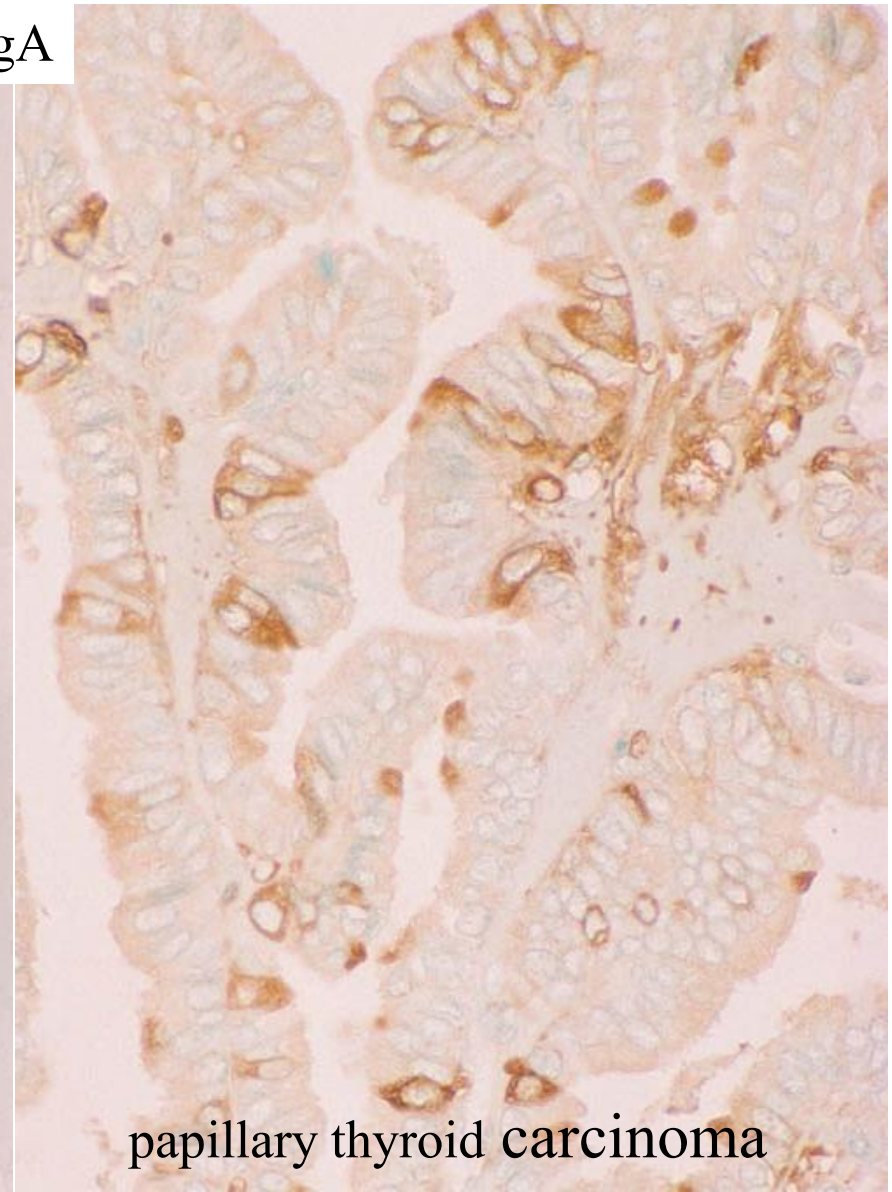


Diffusion artifact of plasma proteins in the autopsied normal pancreas. Albumin immunoreactivity in the pancreas is varied from place to place. Right: islets and nerve fibers, center: ductular cells, right: acinar cells.

IgA

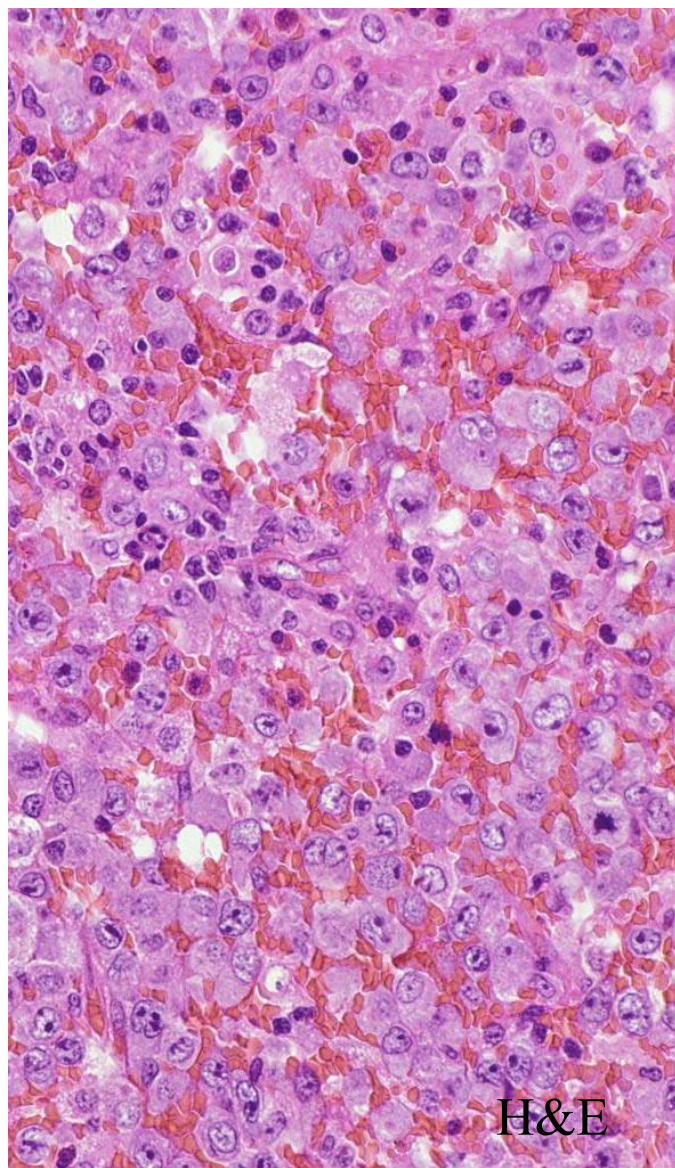


normal thyroid

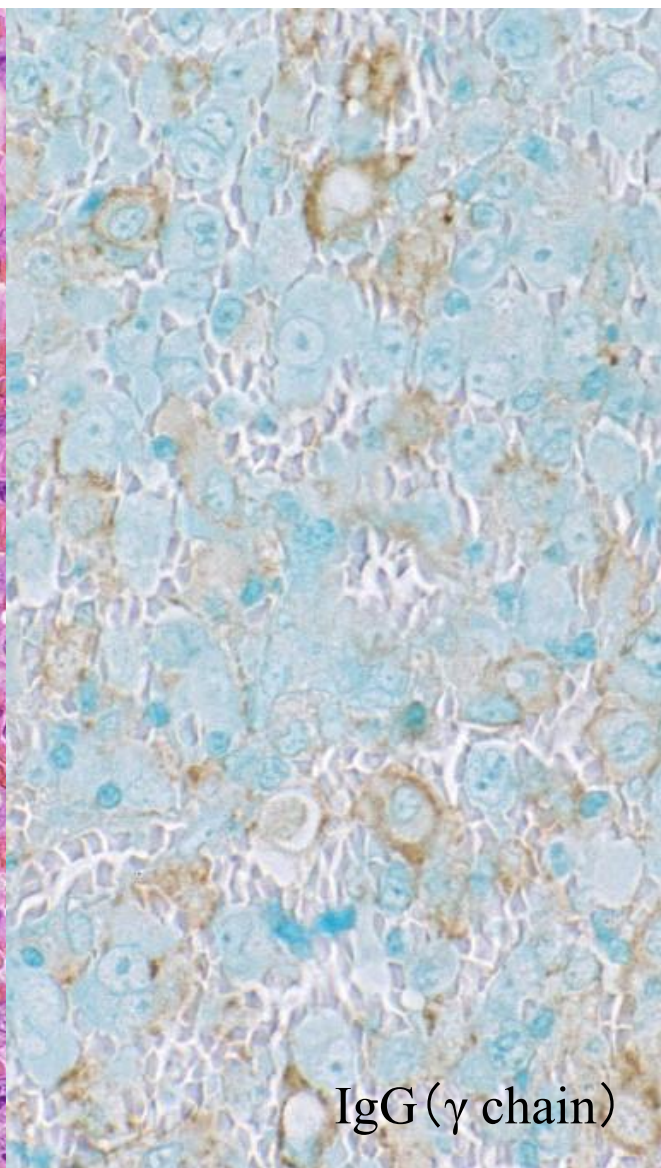


papillary thyroid carcinoma

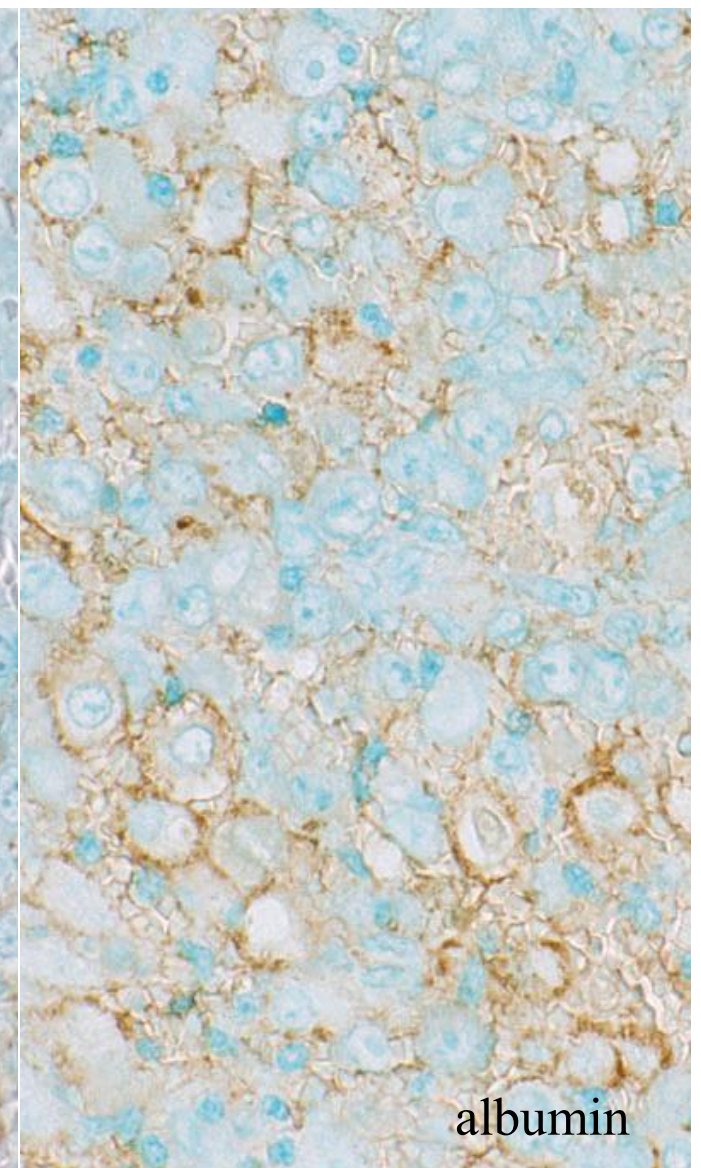
Diffusion artifact of plasma proteins in the thyroid. IgA immunoreactivity is seen in the cytoplasm of some of the normal and cancerous thyroid cells. Non-specific diffusion of IgA before effective fixation is demonstrated.



H&E

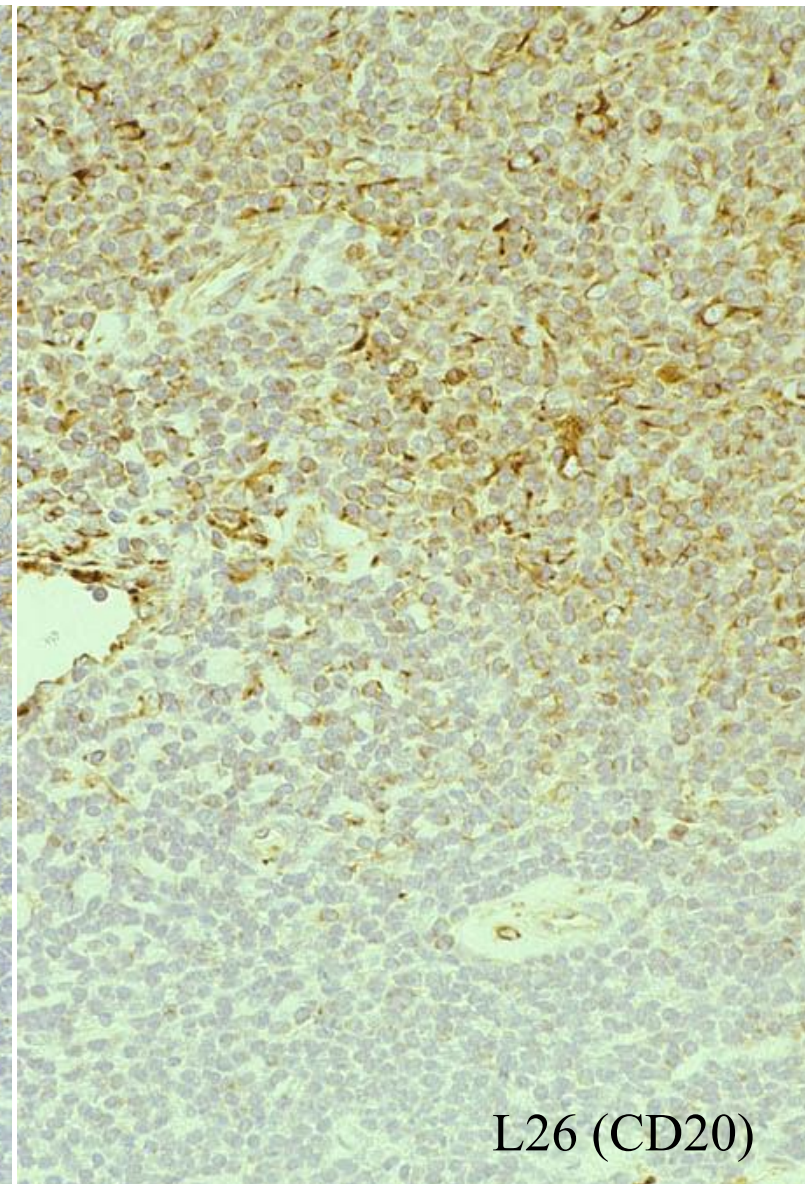
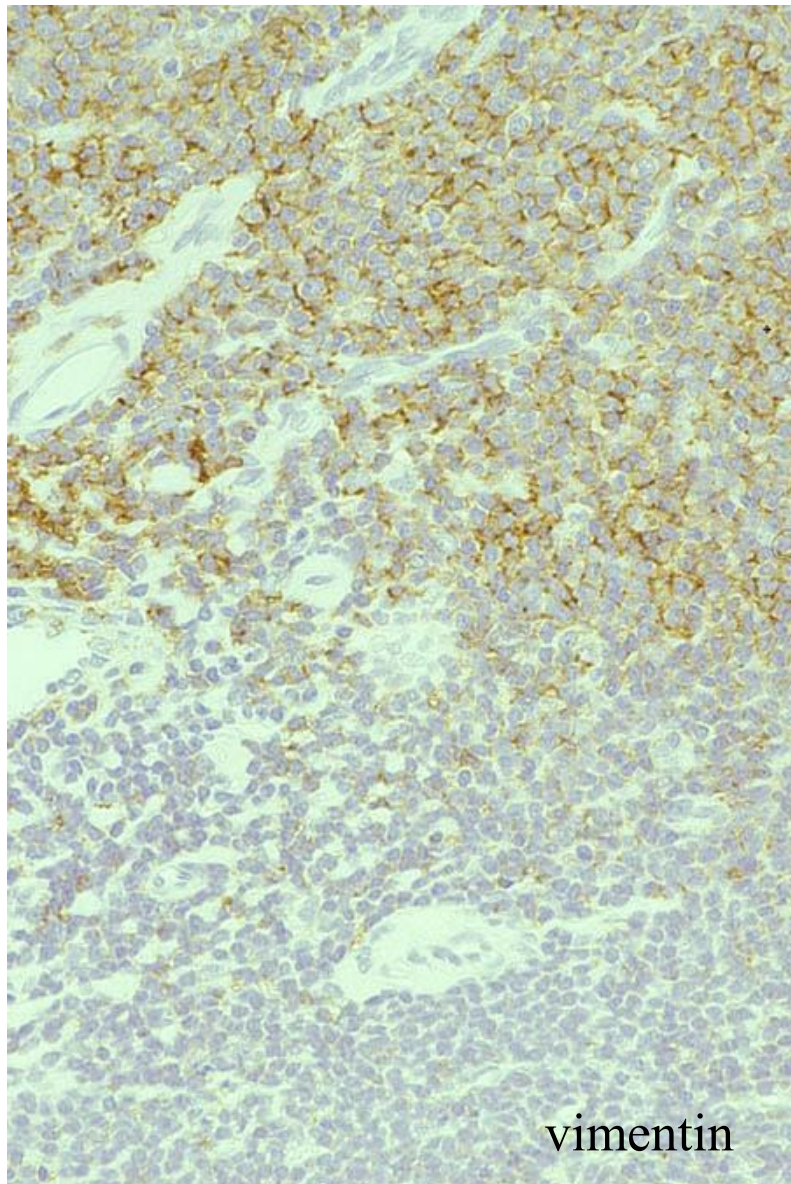


IgG (γ chain)

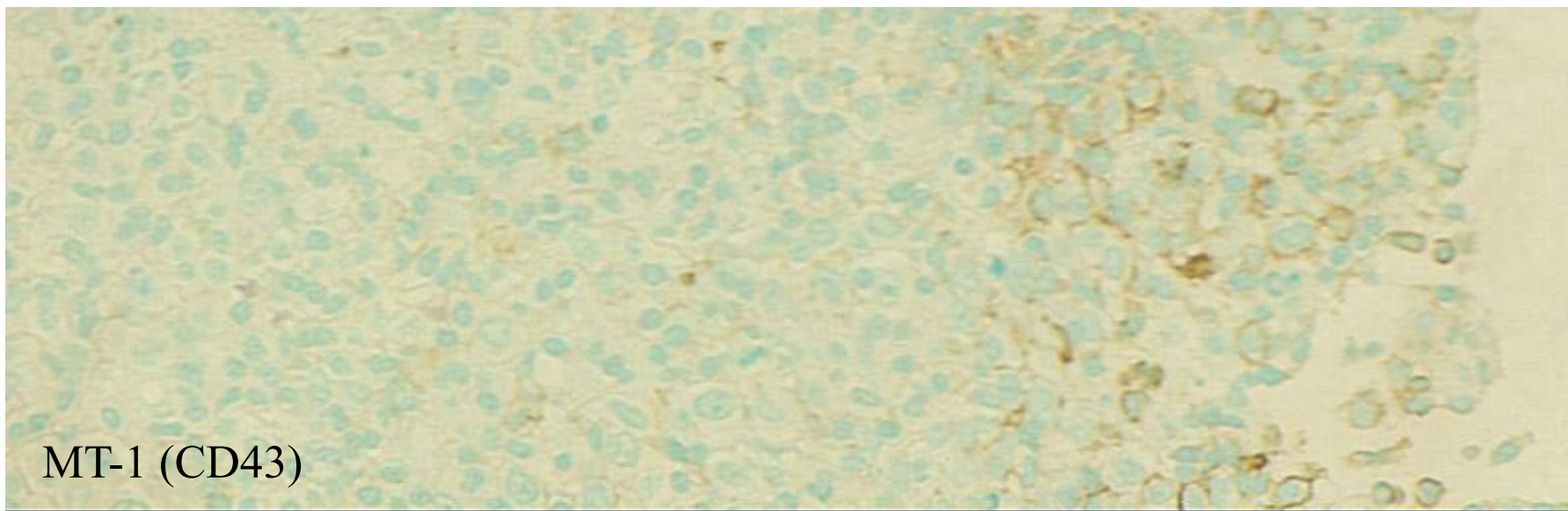


albumin

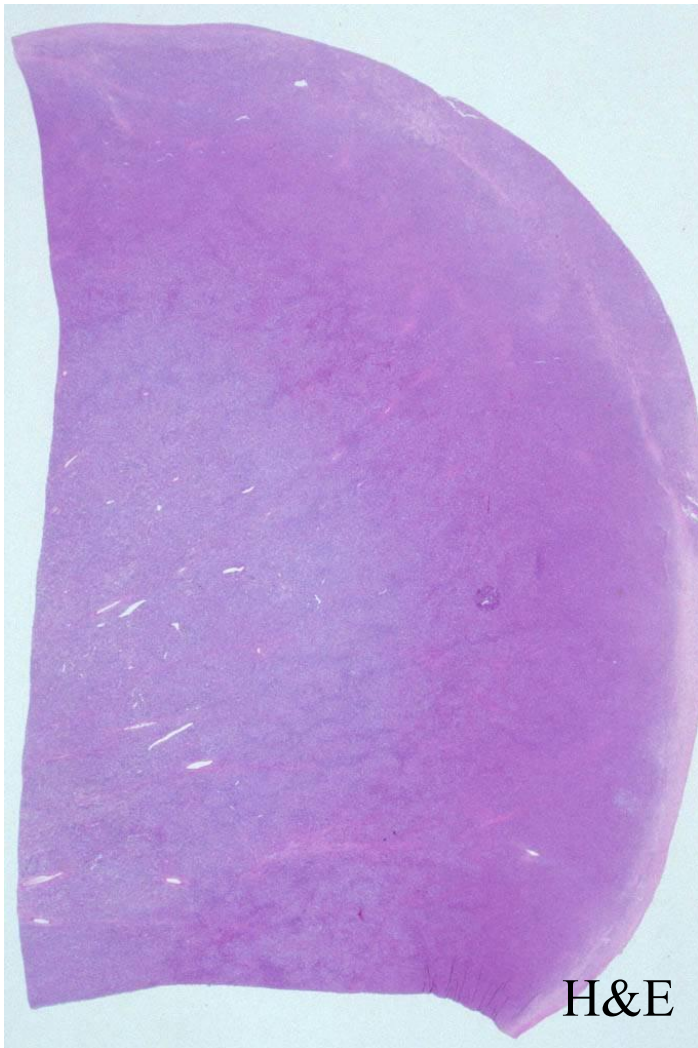
Diffusion artifact of plasma proteins in diffuse large B-cell lymphoma (left: H&E). IgG immunoreactivity in the cytoplasm of the lymphoma cells (center) should be a diffusion artifact, because albumin immunoreactivity is also detectable (right).



Vimentin control in B-cell lymphoma: Vimentin immunostaining can be a marker of appropriate fixation. The overfixed areas (bottom parts) are negative for vimentin, and the same area is also negative for CD20.



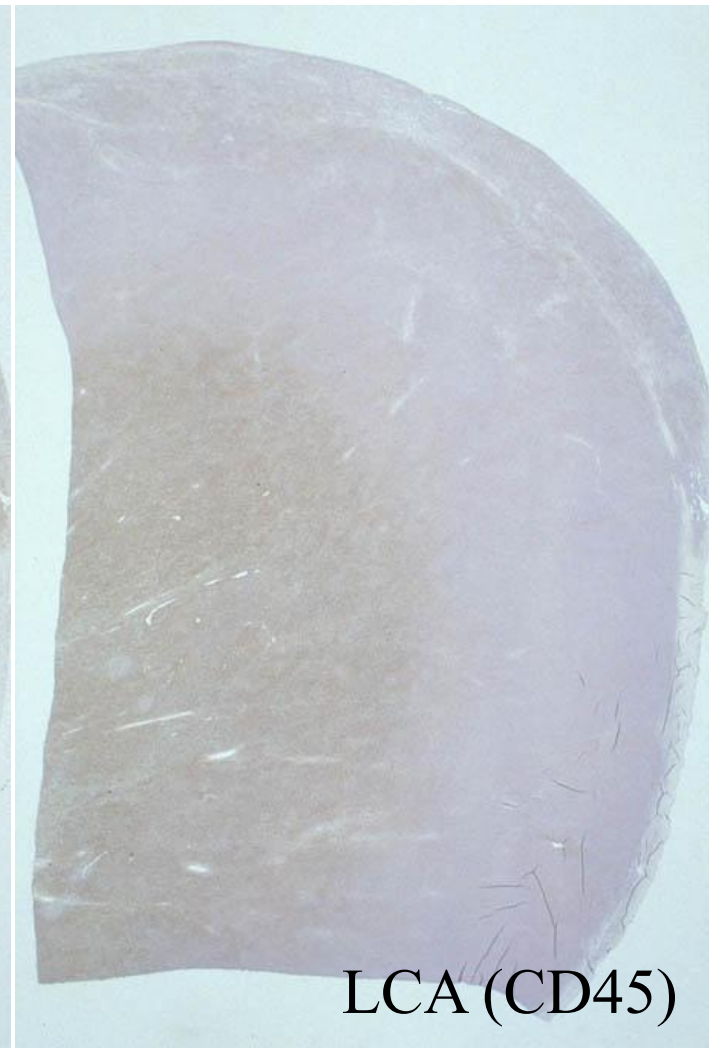
Effect of formalin fixation on the malignant lymphoma. The outermost part of the T-cell lymphoma is positive for CD43 (top), while the outermost part of the follicular lymphoma is negative for CD45 (bottom). The preservation of the antigenicity is dependent upon the degree of overfixation or poor fixation.



H&E



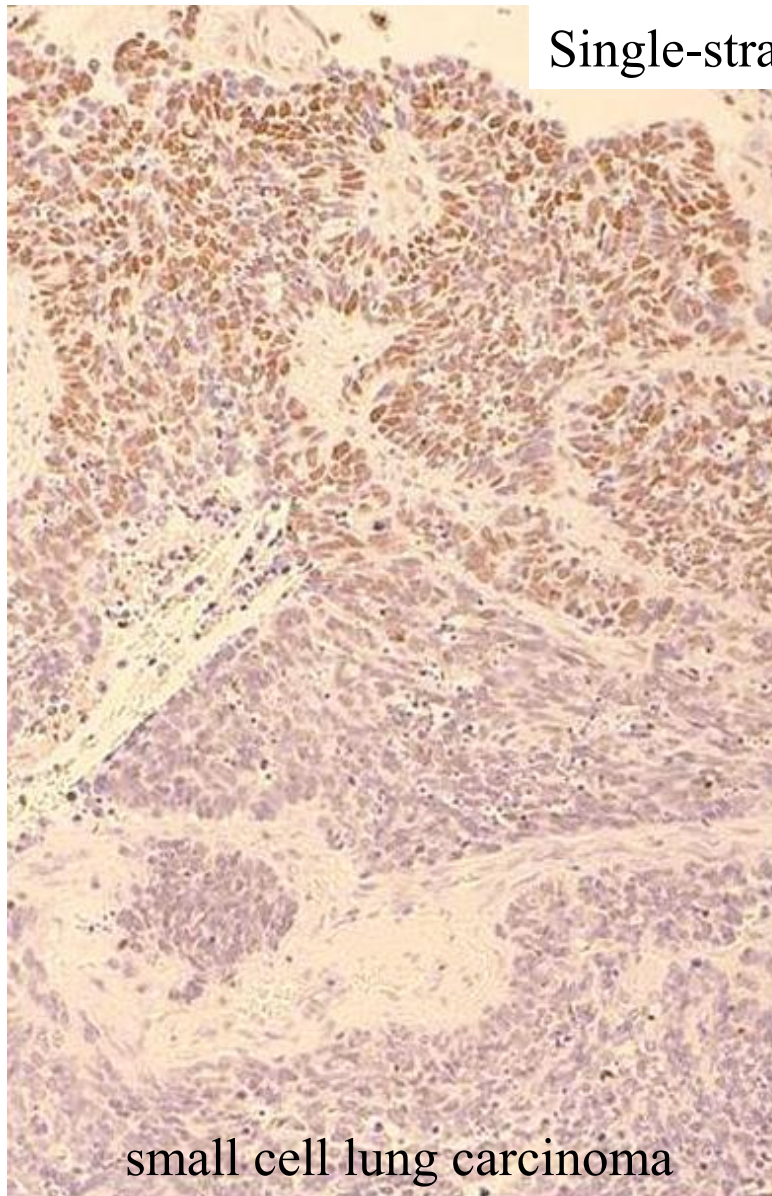
vimentin



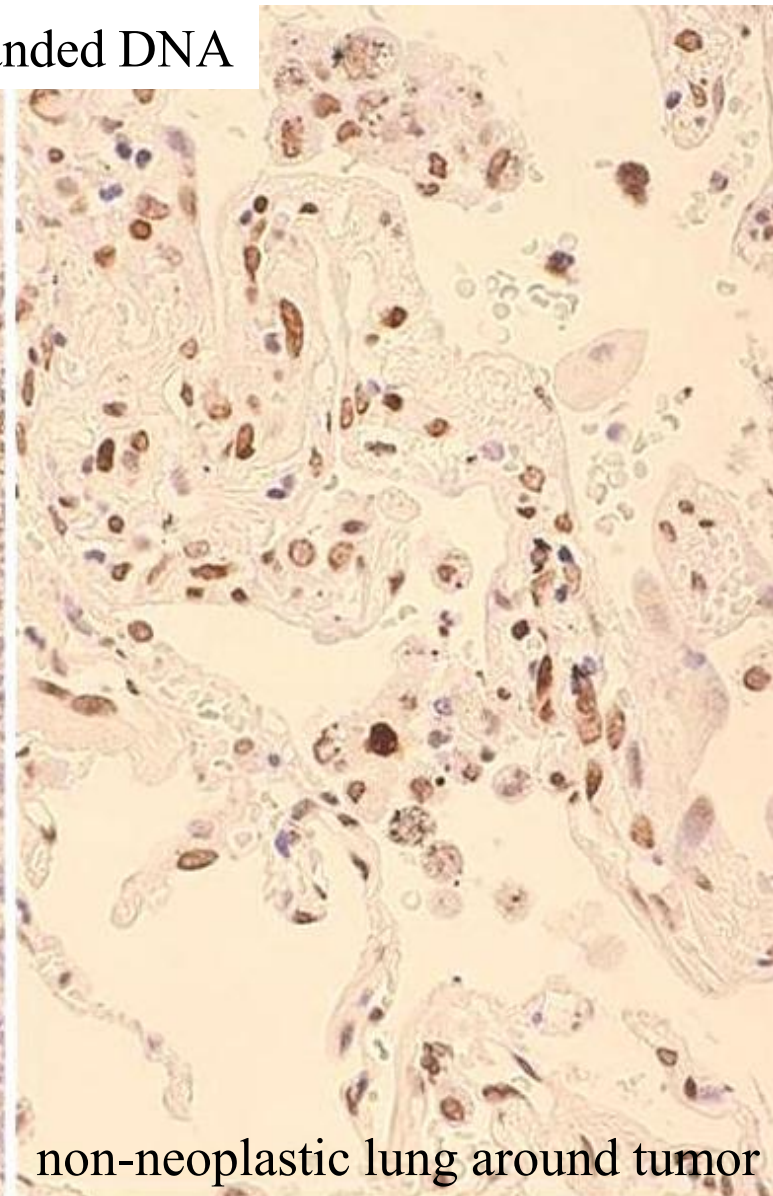
LCA (CD45)

Immunostaining for B-cell lymphoma. The central part appears to be poorly fixed (left: H&E). Vimentin is expressed in the peripheral area, and the poorly fixed central area reveals false negativity (center). In contrast, CD45 is expressed in the central area (right). The overfixed peripheral area reveals false negativity.

Single-stranded DNA

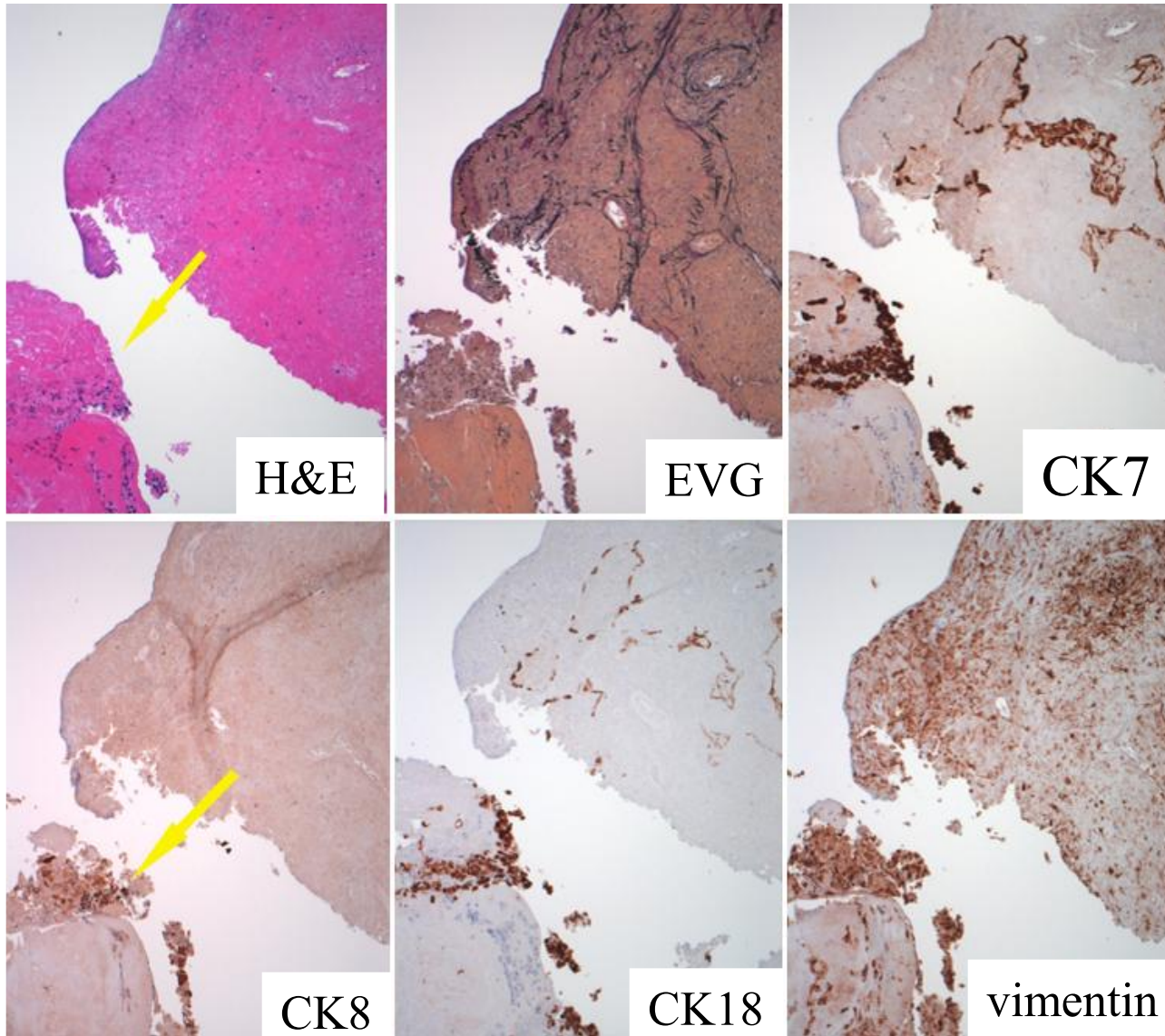


small cell lung carcinoma

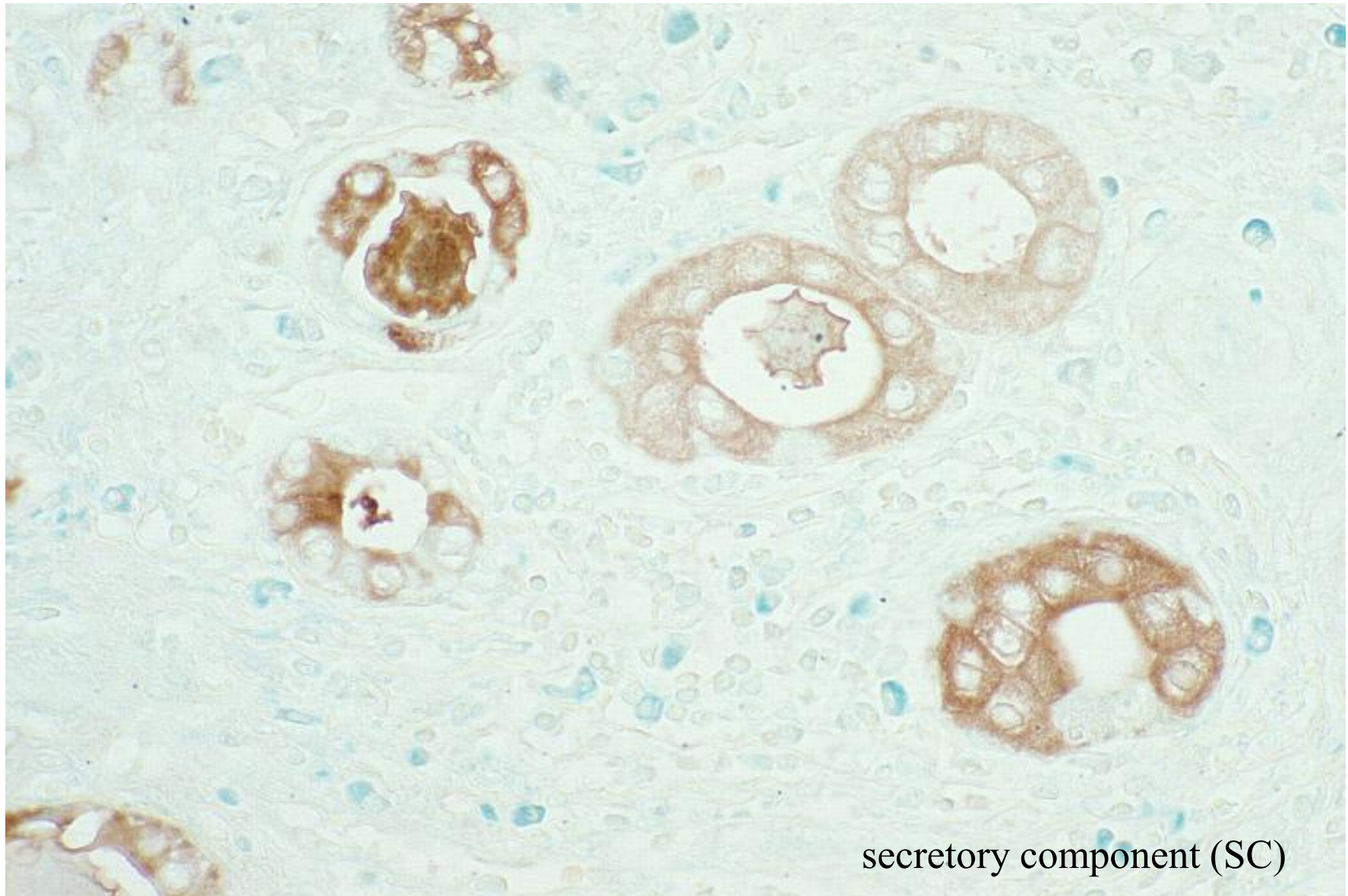


non-neoplastic lung around tumor

Nonspecific single-stranded DNA immunoreactivity in normal and neoplastic lung tissues, caused by DNA fragmentation (artificial ssDNA formation) by formalin fixation. Under an ideal fixation condition, ssDNA should be a marker of apoptotic cells.

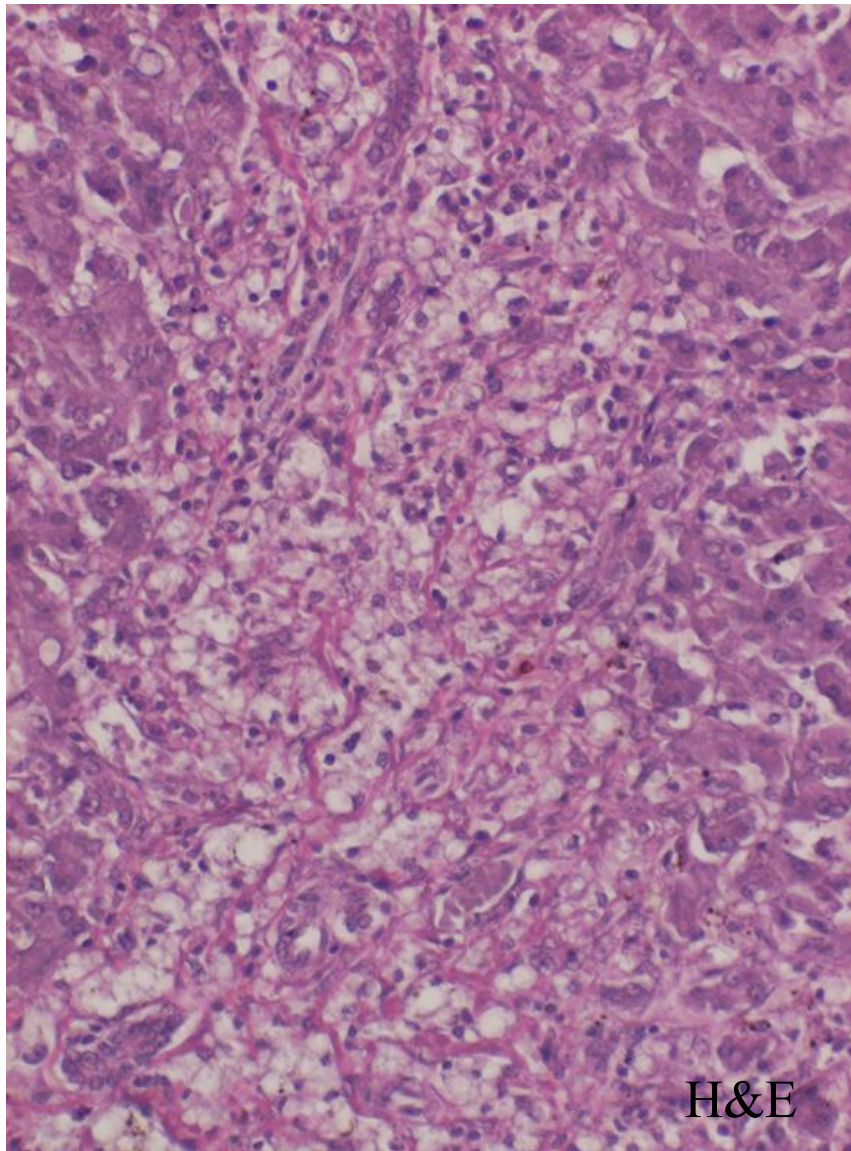


Transbronchial lung biopsy containing adenocarcinoma cells with coagulation necrosis (arrows). The necrotic tumor cells express low-molecular weight cytokeratins and vimentin.

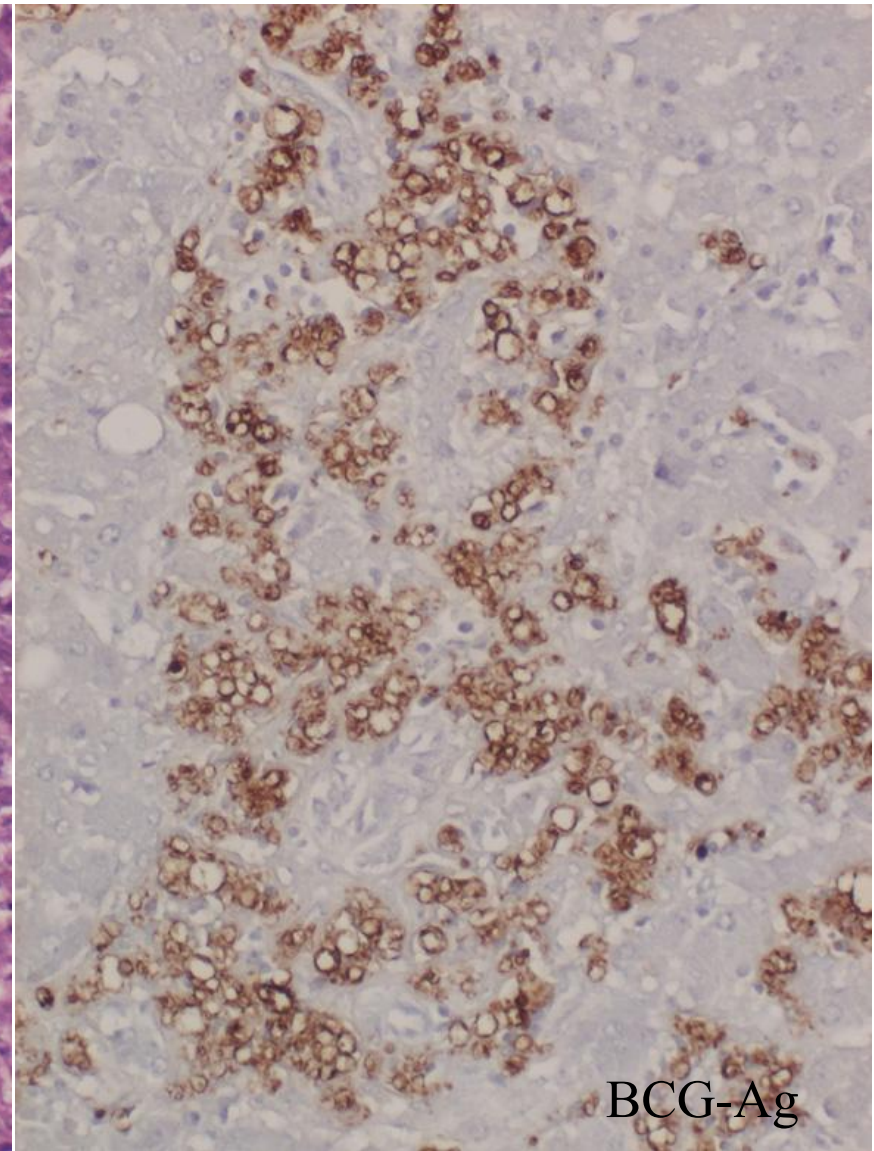


secretory component (SC)

Immunoreactivity resistant to long-period fixation. Secretory component immunoreactivity in the renal medulla after formalin fixation for 5 years

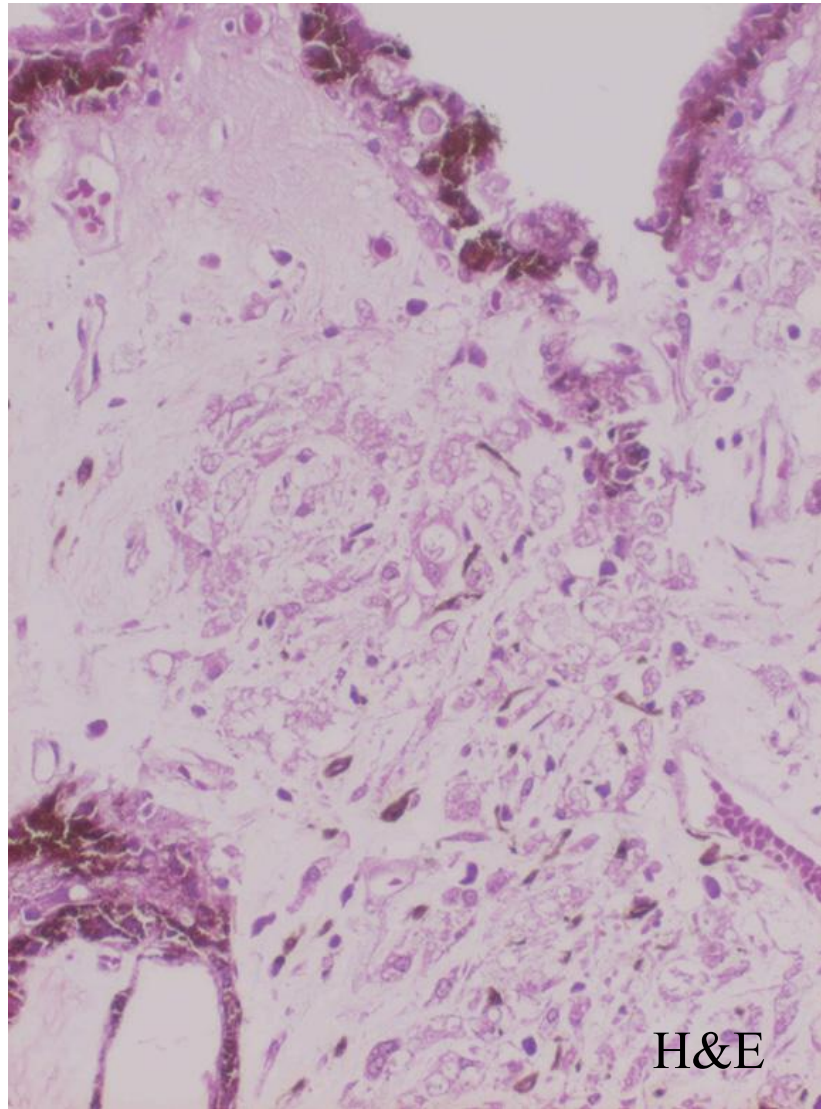


H&E

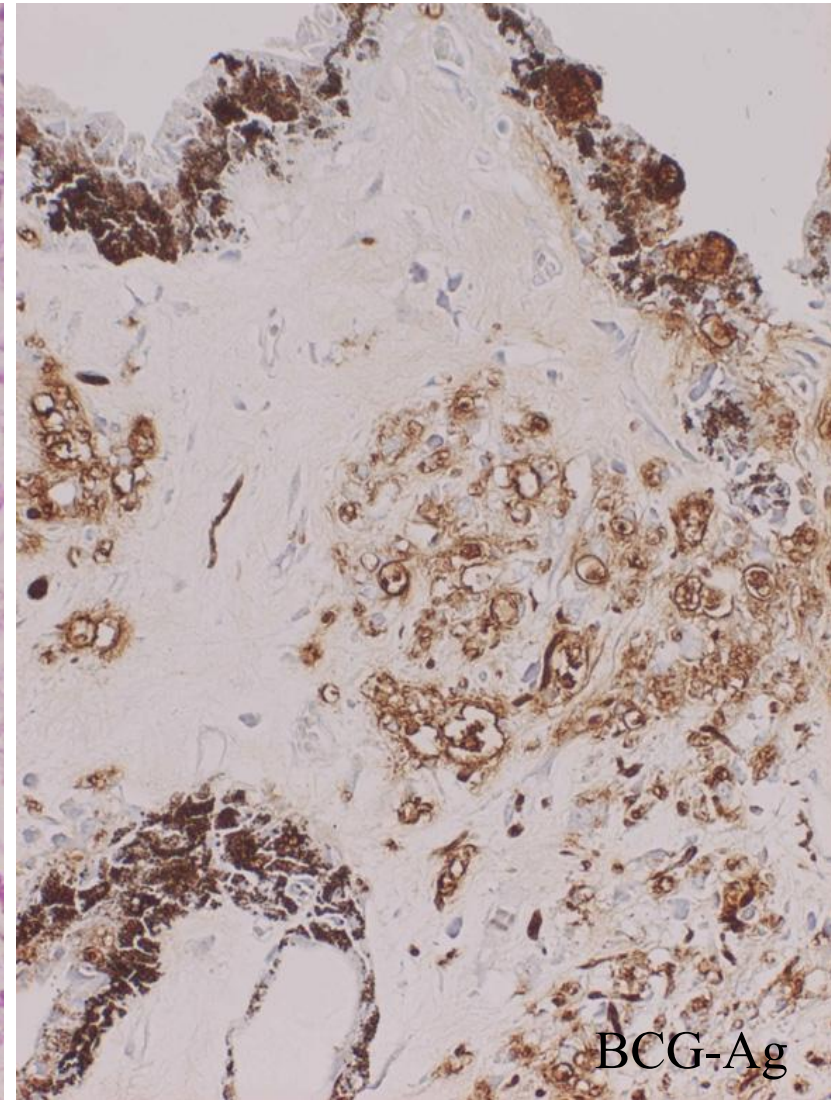


BCG-Ag

Immunoreactivity resistant to long-period fixation. In an autopsy case, BCG immunoreactivity in lepromatous hepatitis after formalin fixation for 40 years. The leproma cells in the portal triad are clearly labeled for the BCG antigens.

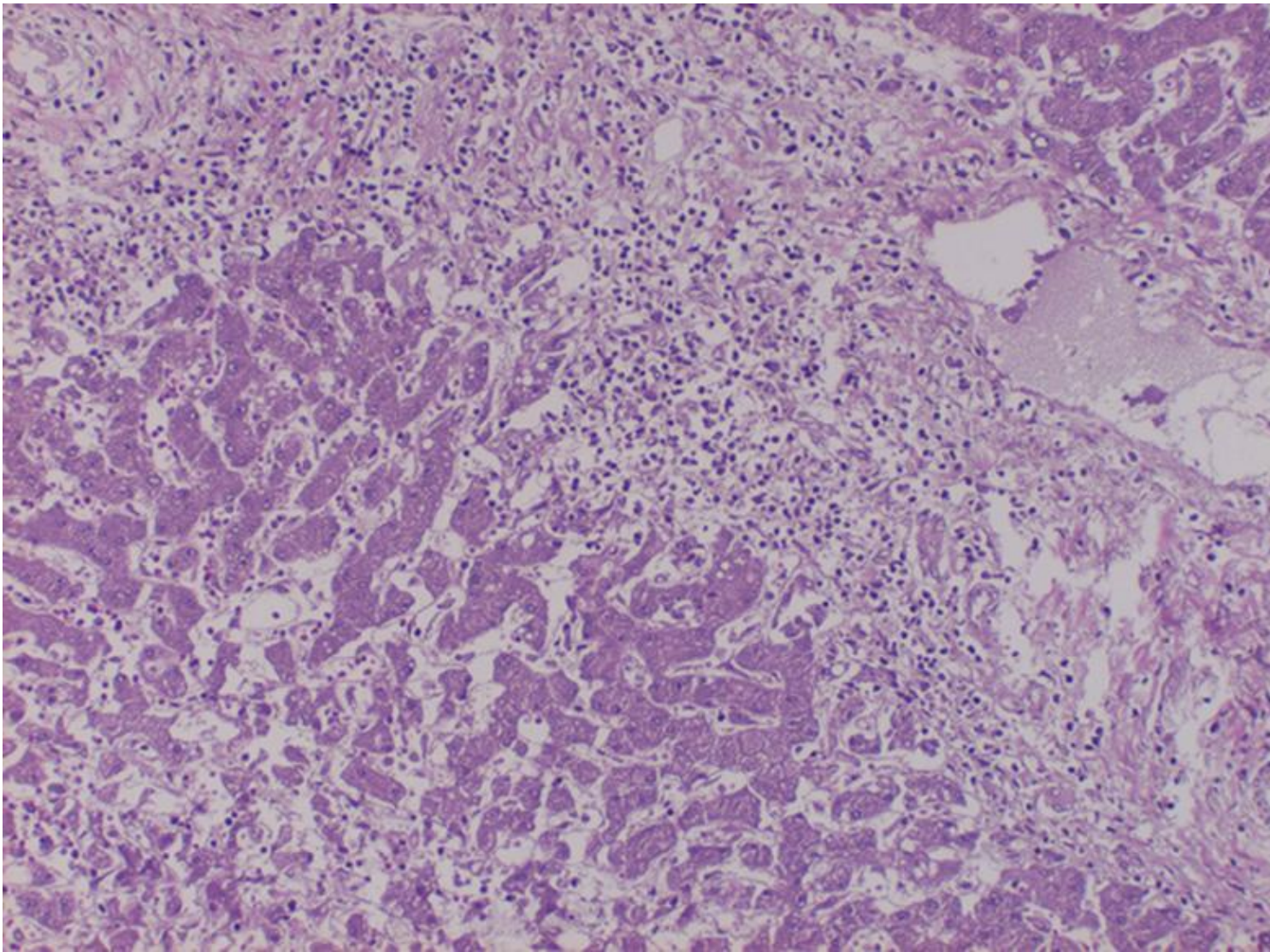


H&E

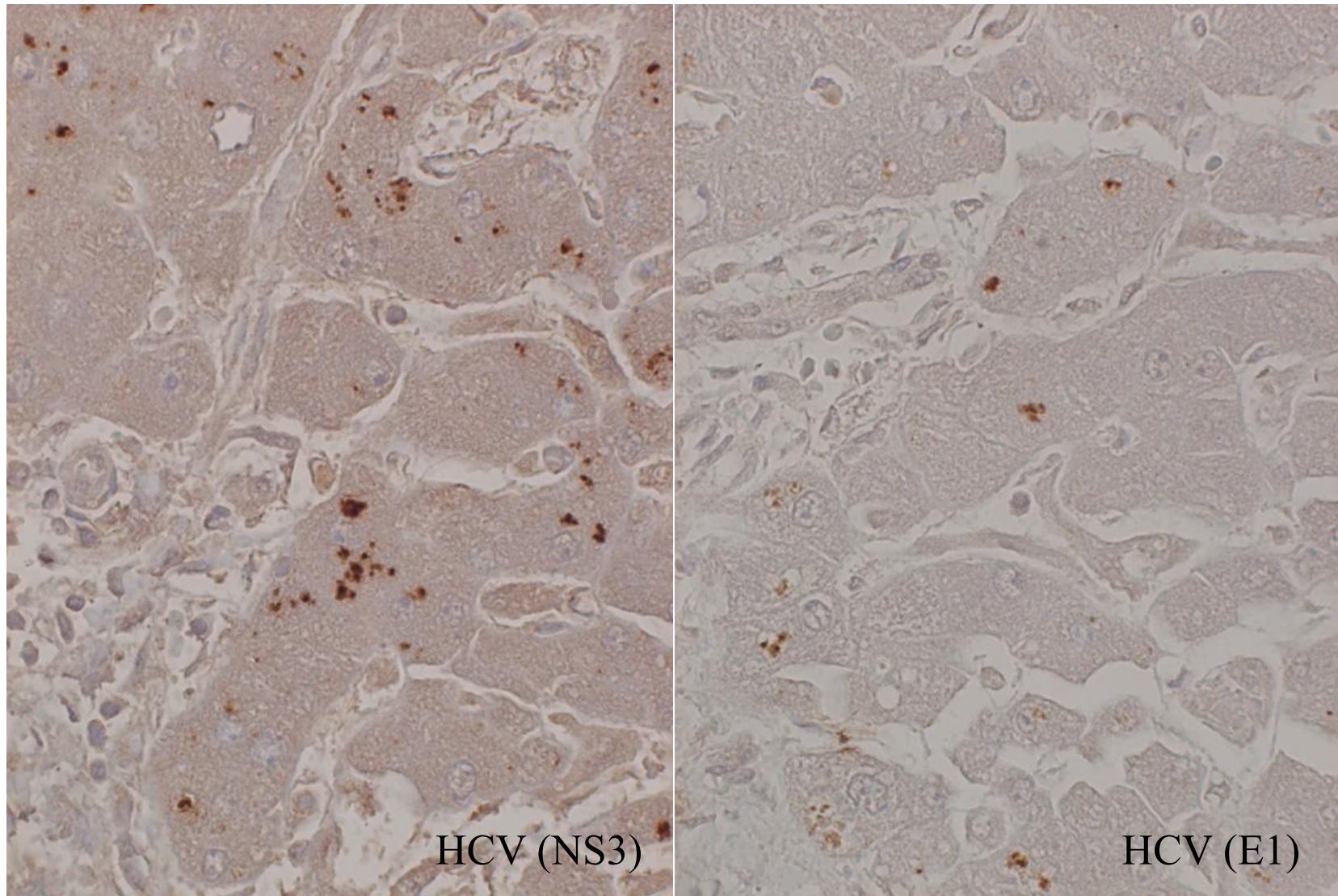


BCG-Ag

Immunoreactivity resistant to long-period fixation. In an autopsy case, BCG immunoreactivity in lepromatous iridocyclitis after formalin fixation for 70 years. The leproma cells in the iris are clearly labeled for the BCG antigens.



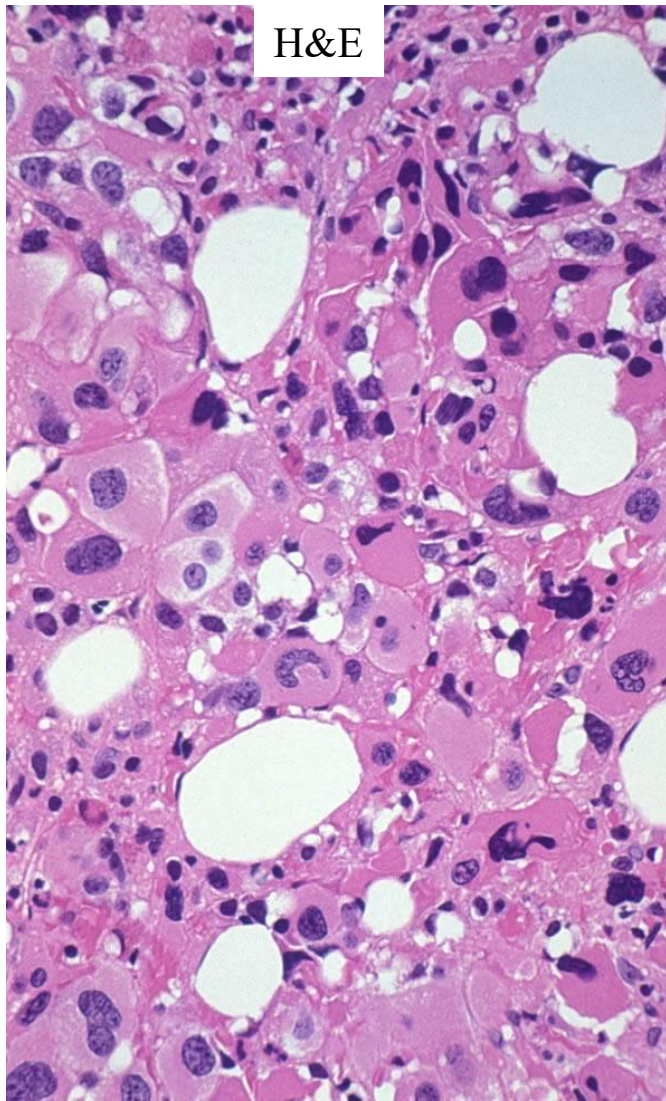
Immunoreactivity resistant to long-period fixation. In an autopsy case of lepromatous leprosy, features of chronic hepatitis are commonly observed (H&E). The specimen was fixed in formalin for 50 years. HCV was transmitted by therapeutic injection of Promin without changing the needles.



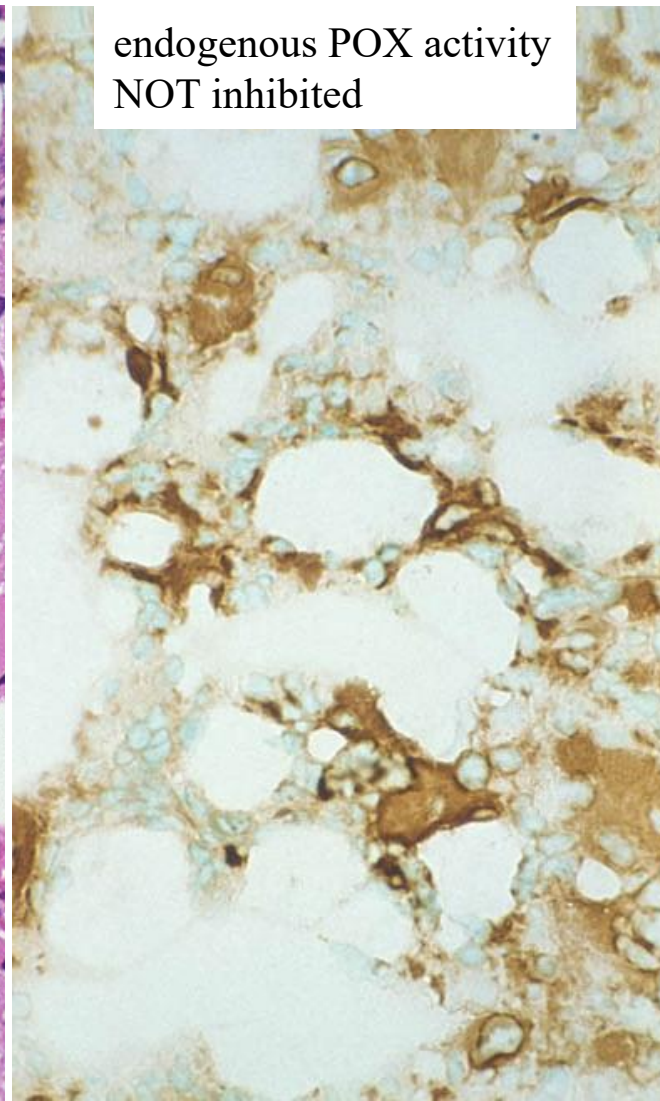
Immunoreactivity resistant to long-period fixation. Chronic hepatitis C was common among the patients with lepromatous leprosy. In an autopsied liver fixed in formalin for 50 years, immunostaining using monoclonal antibodies against HCV antigen discloses viral infection as dotted signals after heat-induced Ag retrieval (left: NS3, right: E1 of HCV).

Staining notes

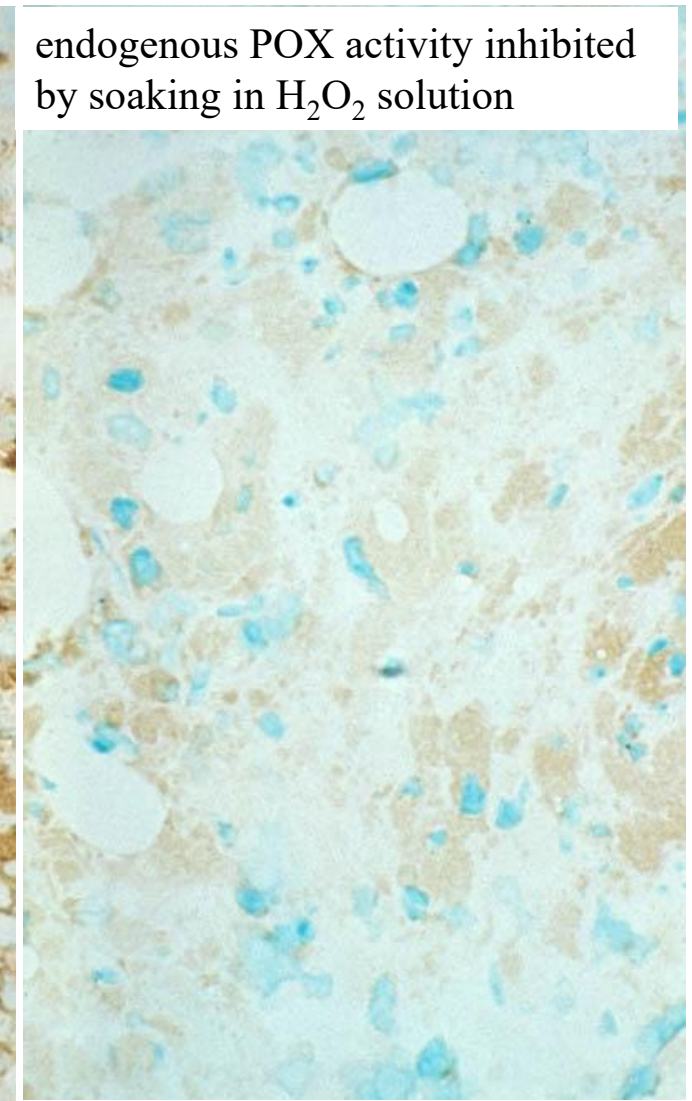
1. Endogenous peroxidase activity
2. Endogenous biotin activity
3. Endogenous pigments



H&E



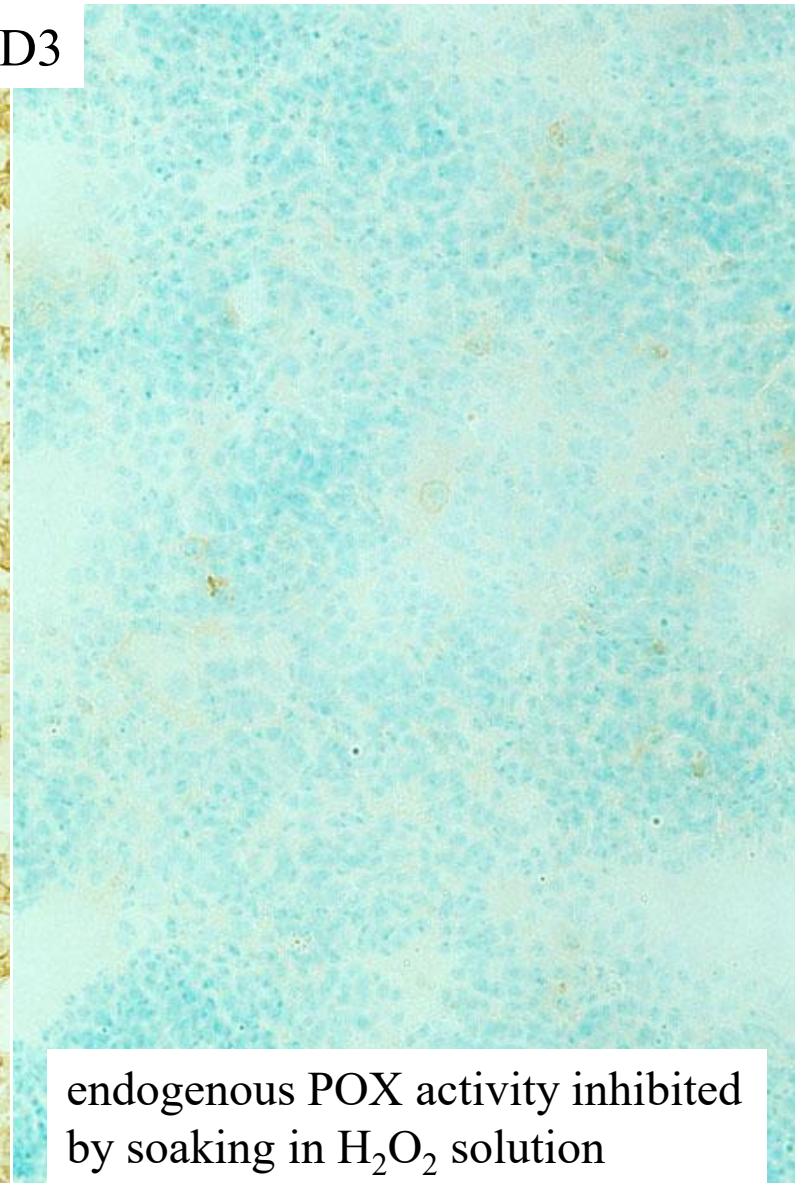
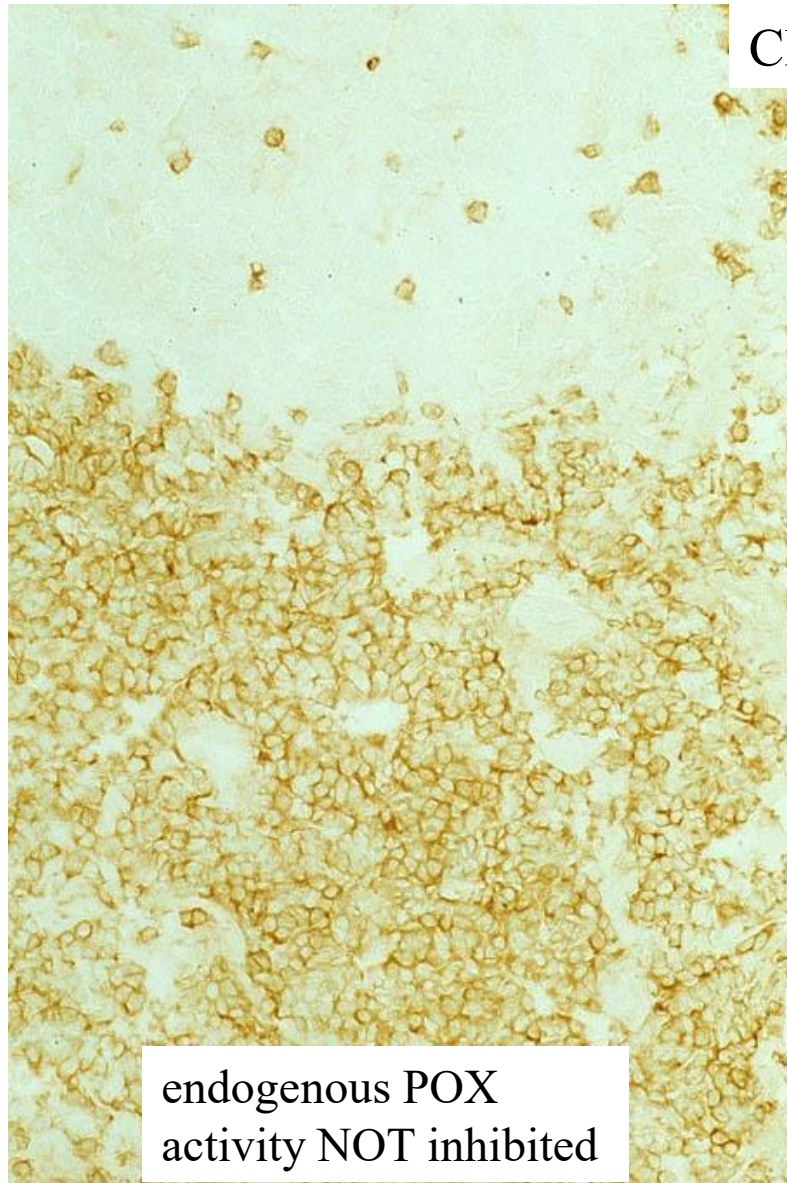
endogenous POX activity
NOT inhibited



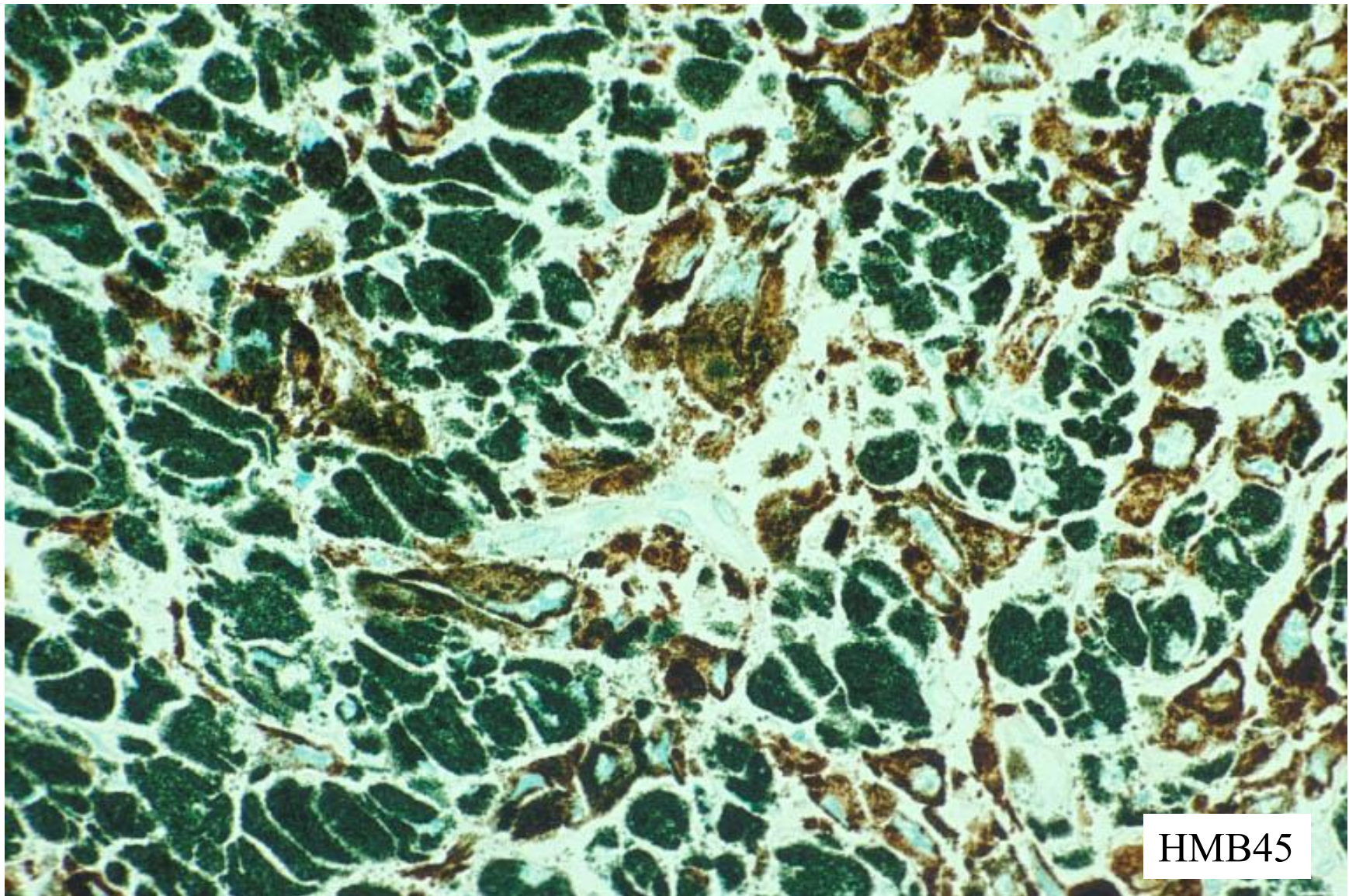
endogenous POX activity inhibited
by soaking in H_2O_2 solution

Fresh frozen sections of the bone marrow aspirate fixed in ethanol. Essential thrombocythemia (left: paraffin section, H&E). Monoclonal antibody against GpIIb/IIIa (integrin α IIb/ β 3) detects megakaryocytes (center), but after inhibition of peroxidase activity, the immunoreactivity is significantly weakened (right). The carbohydrate antigen detected by the monoclonal antibody is susceptible to H_2O_2 oxidation.

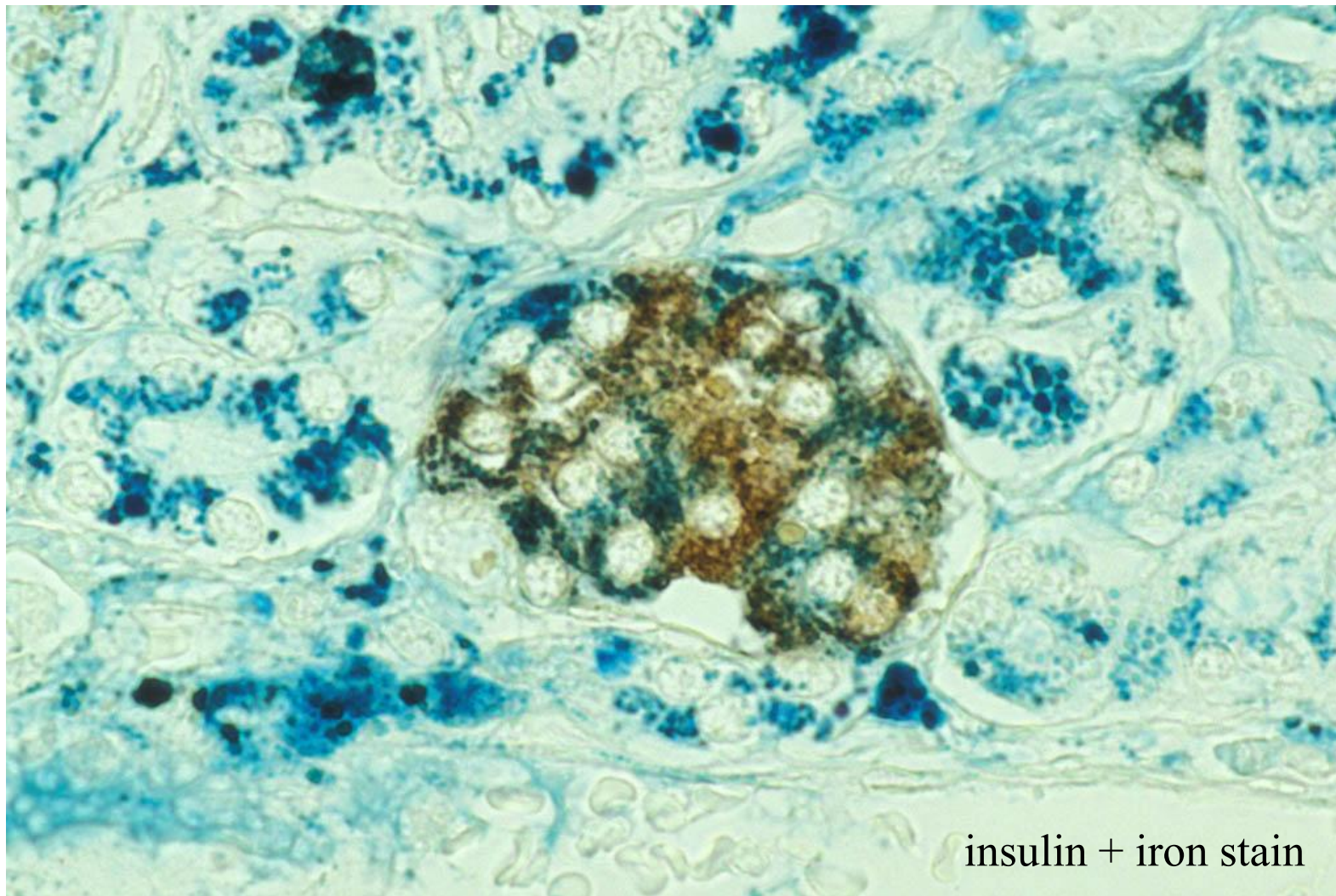
CD3



Another example of inactivation of carbohydrate antigen by hydrogen peroxide pretreatment. In fresh frozen sections of the palatine tonsil fixed in ethanol, CD3 detected with Leu 4 monoclonal is markedly inactivated by H₂O₂ pretreatment.



Immunostaining for HMB45 in nasal malignant melanoma. Nuclear staining with methylgreen is effective to distinguish the HMB45 immunoreactivity from the melanin pigment produced by the tumor cells. The melanin pigments show greenish metachromasia.



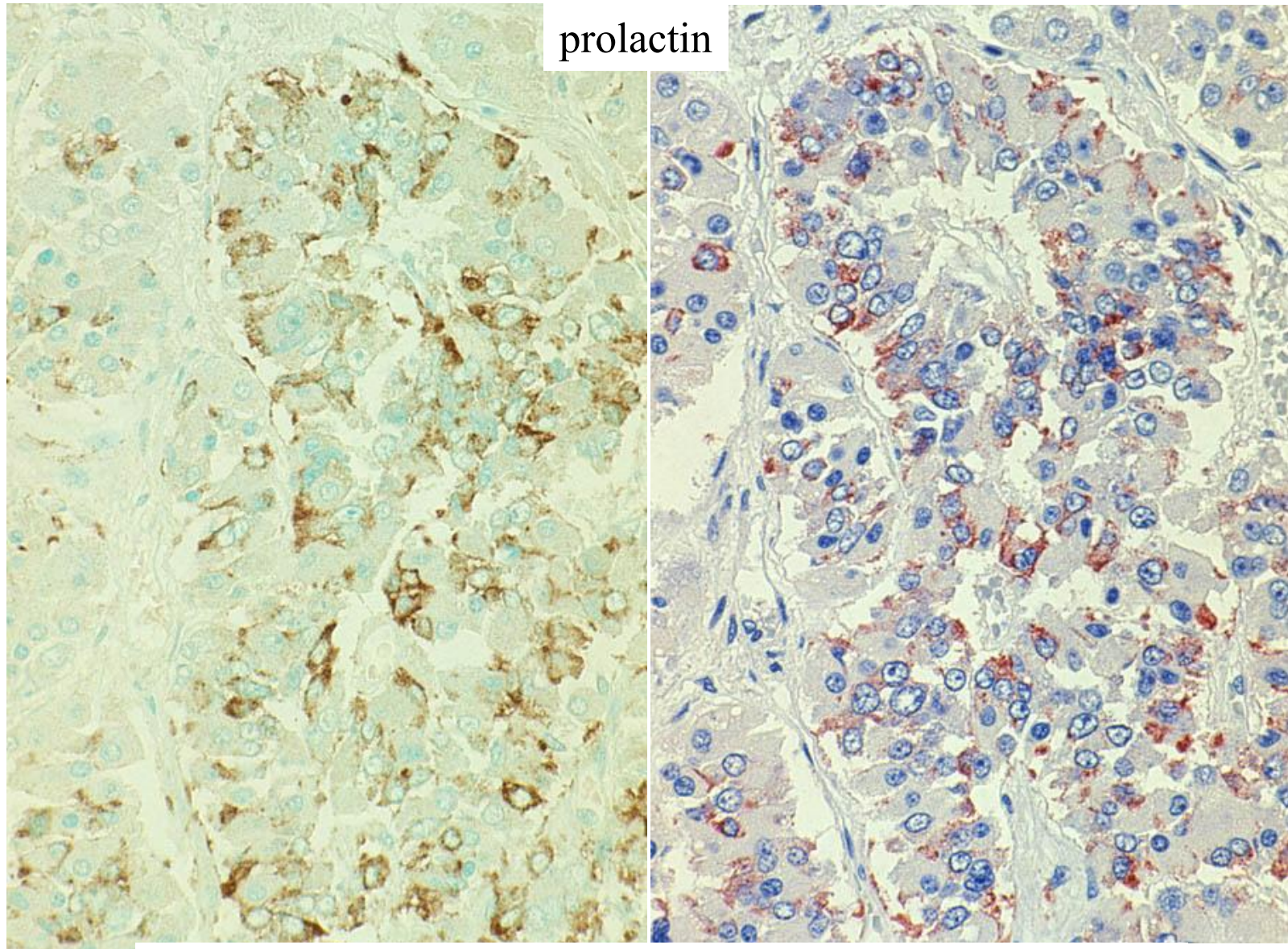
insulin + iron stain

Insulin immunoreactivity and Berlin blue reactivity is demonstrated simultaneously. In a case of hemochromatosis manifesting diabetes mellitus, hemosiderin reposition is seen in insular beta cells, in addition to pancreatic acinar cells. Distinction between brown-colored DAB products (the insulin signals) and blue-stained hemosiderin pigments can be obtained with this double staining procedure.

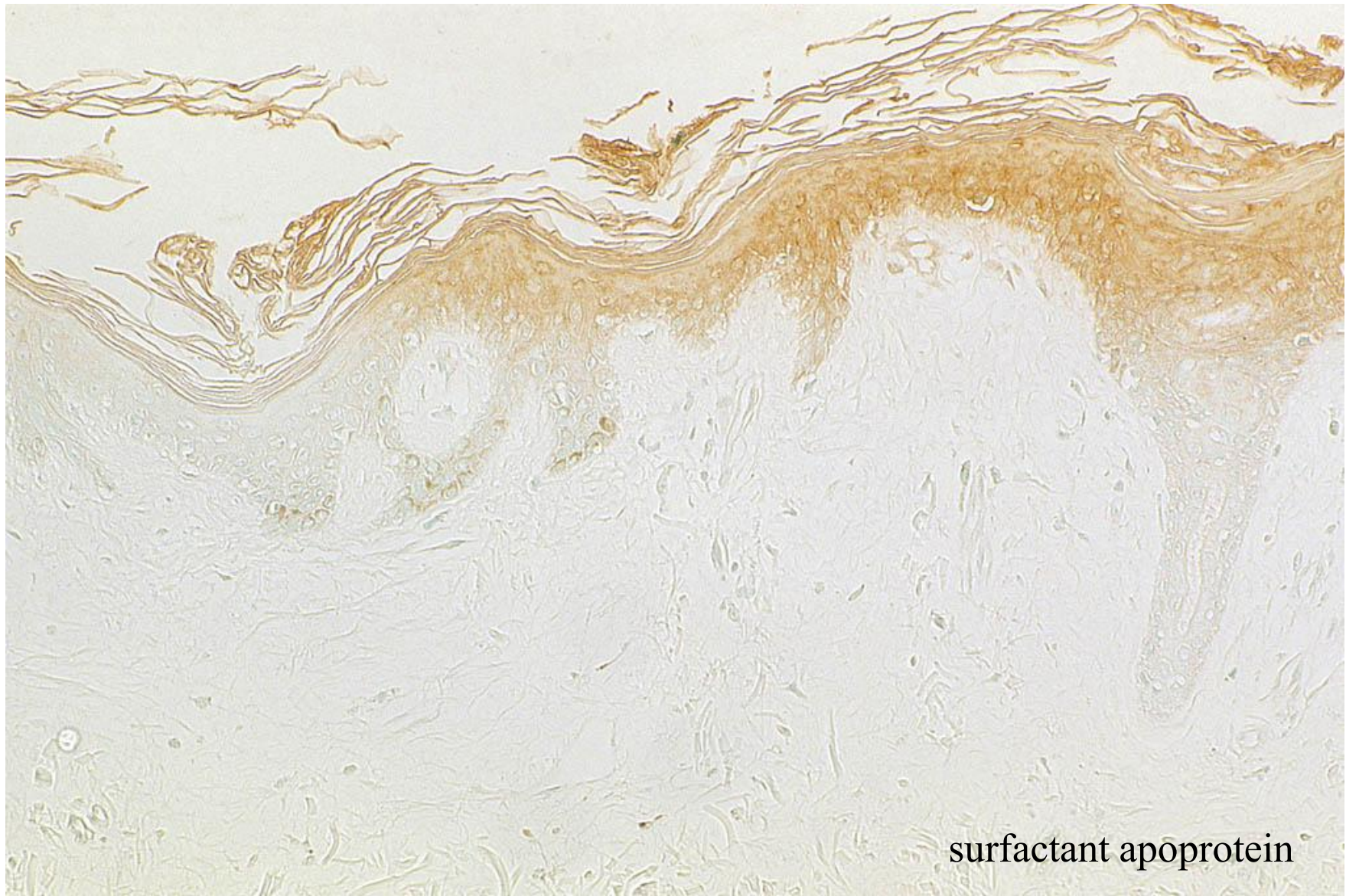
Techniques in immunohistochemistry

1. Drying during antibody incubation and effect of air bubbles and improper dewaxing
2. High-sensitivity techniques
3. Antigen (epitope) retrieval
 - ① Protease treatment
 - ② Hydrated heating
 - ③ Others

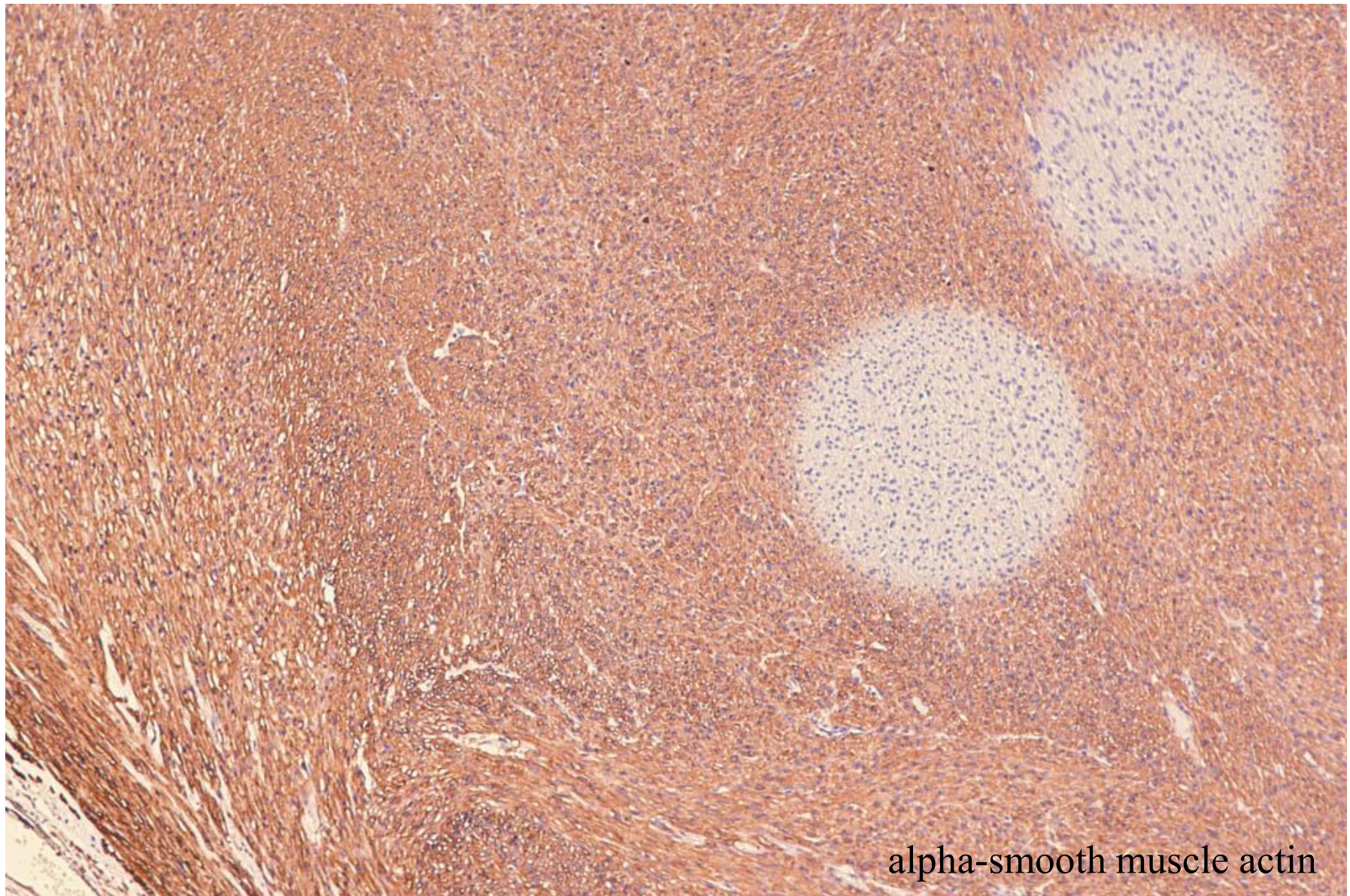
DAB coloration, methylgreen counterstaining AEC coloration, hematoxylin counterstaining



Combinations of coloration and nuclear counterstaining:
prolactin immunoreactivity in the normal pituitary gland

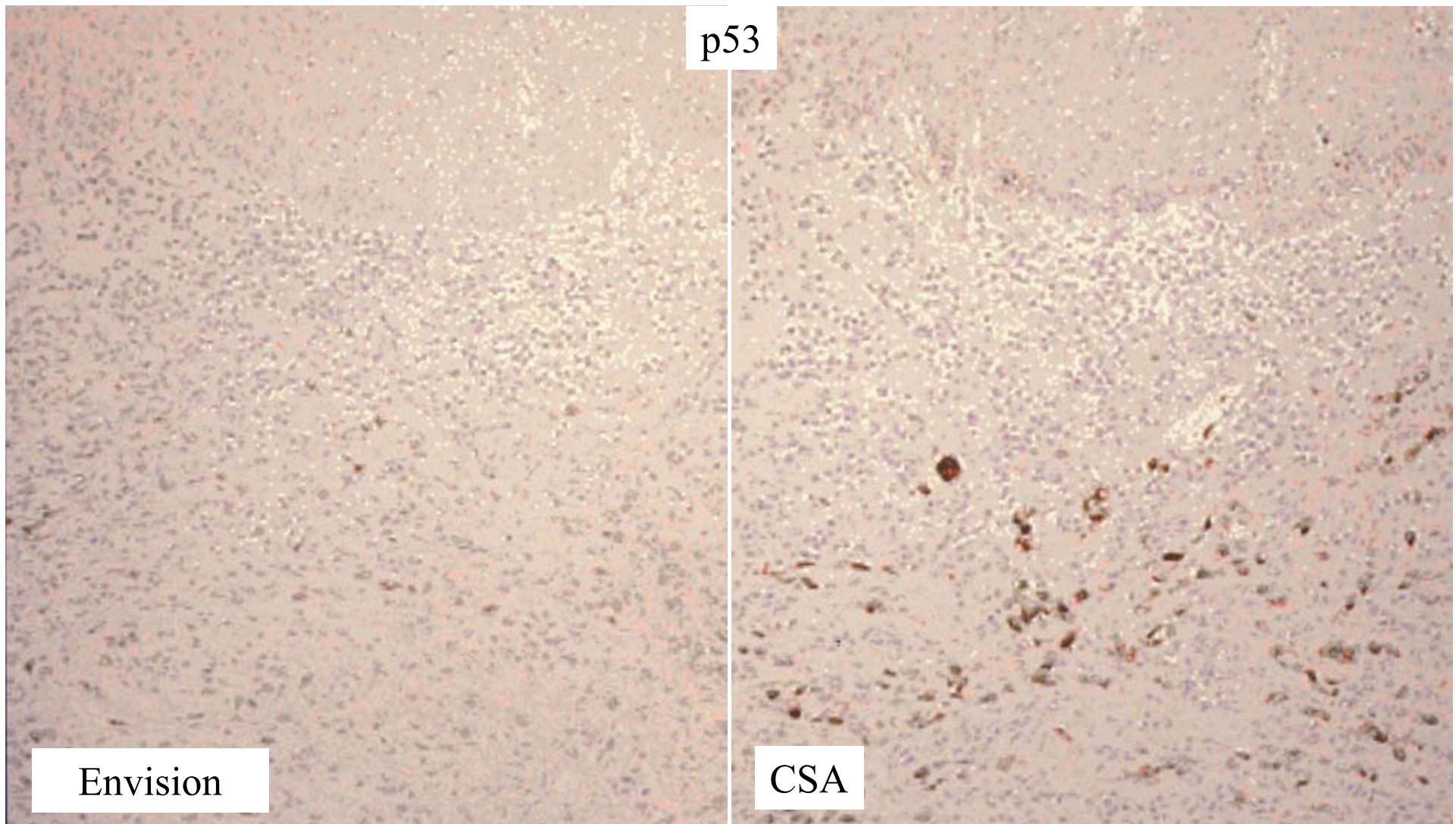


Nonspecific staining in a dried area: immunostaining for surfactant apoprotein in the normal skin.

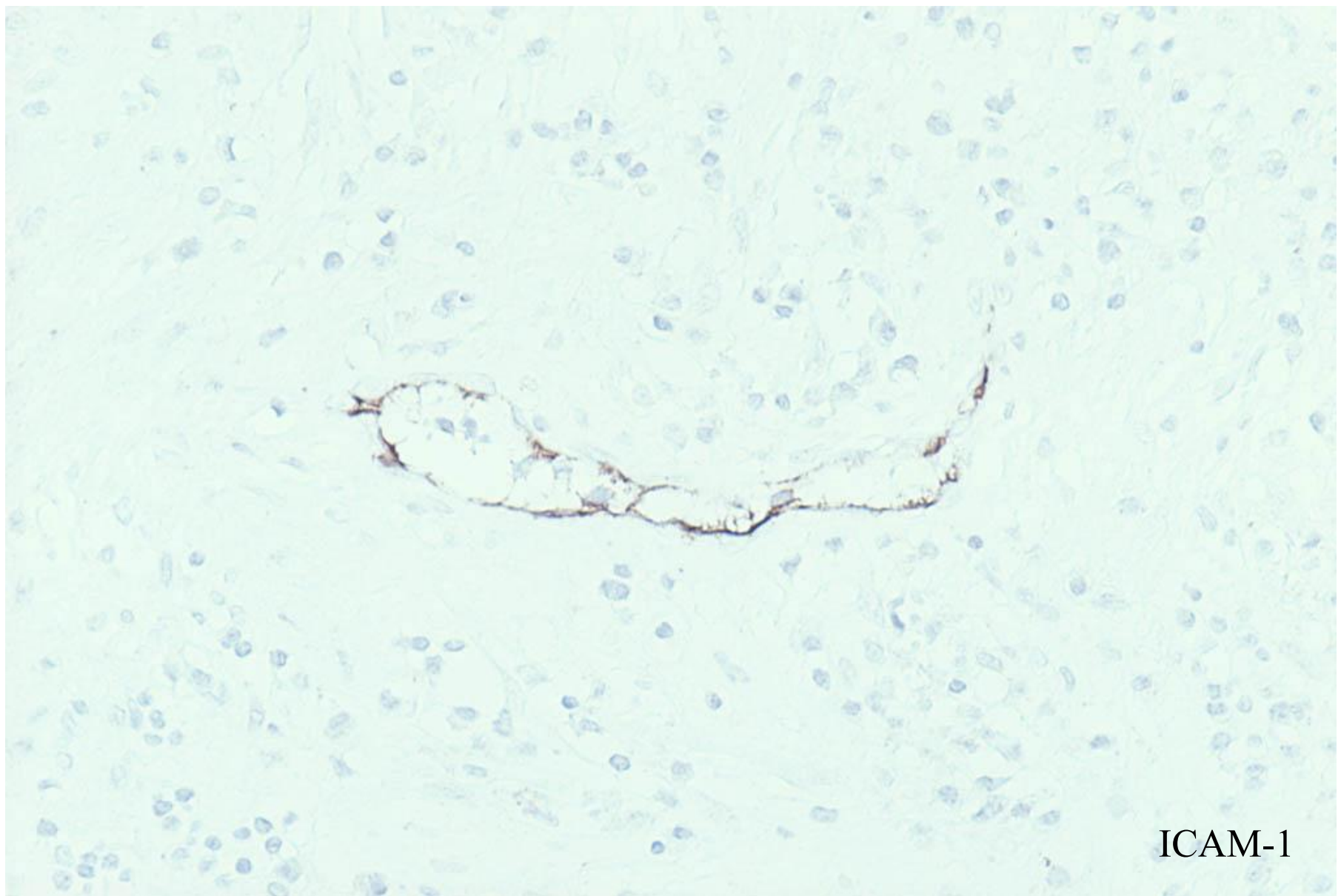


alpha-smooth muscle actin

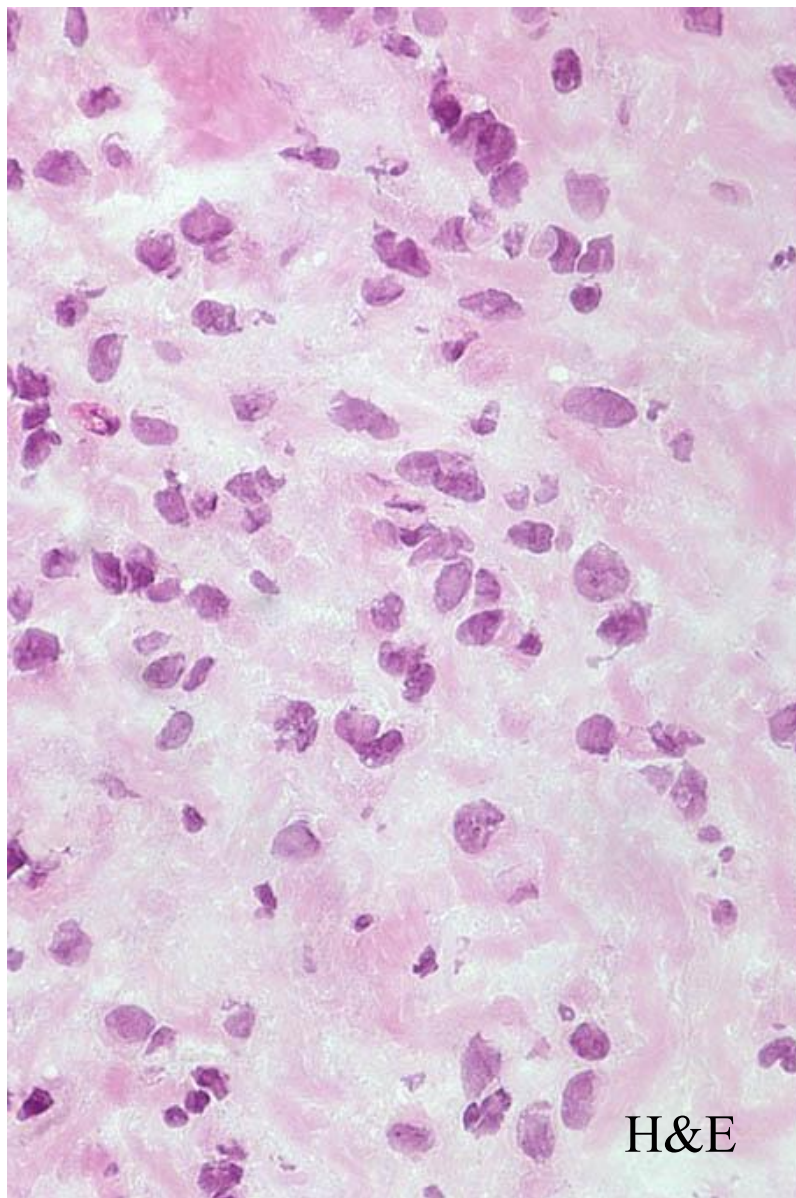
Improper dewaxing: alpha-smooth muscle actin immunostaining in leiomyosarcoma.
Improperly dewaxed areas show artifactual negativity.



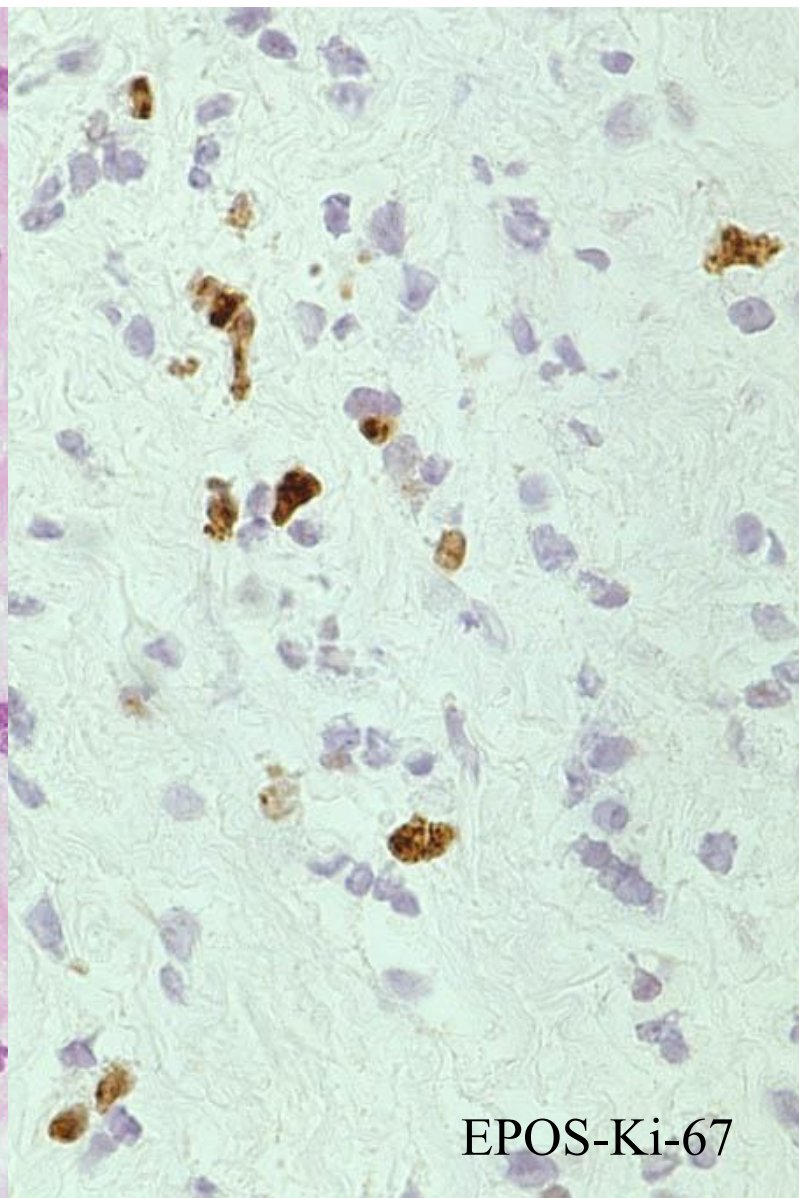
Application of ultrasensitive technique (the CSA method) for demonstrating p53 immunoreactivity in small cell carcinoma of the esophagus. In sections without heat-induced antigen retrieval, ultrasensitive CSA method can detect p53 protein in the cancer cell nuclei, while Envision (the polymer method) scarcely detects the signals.



ICAM-1 immunoreactivity in endothelial cells can be demonstrated in formalin-fixed, paraffin-embedded sections by using the CSA method.



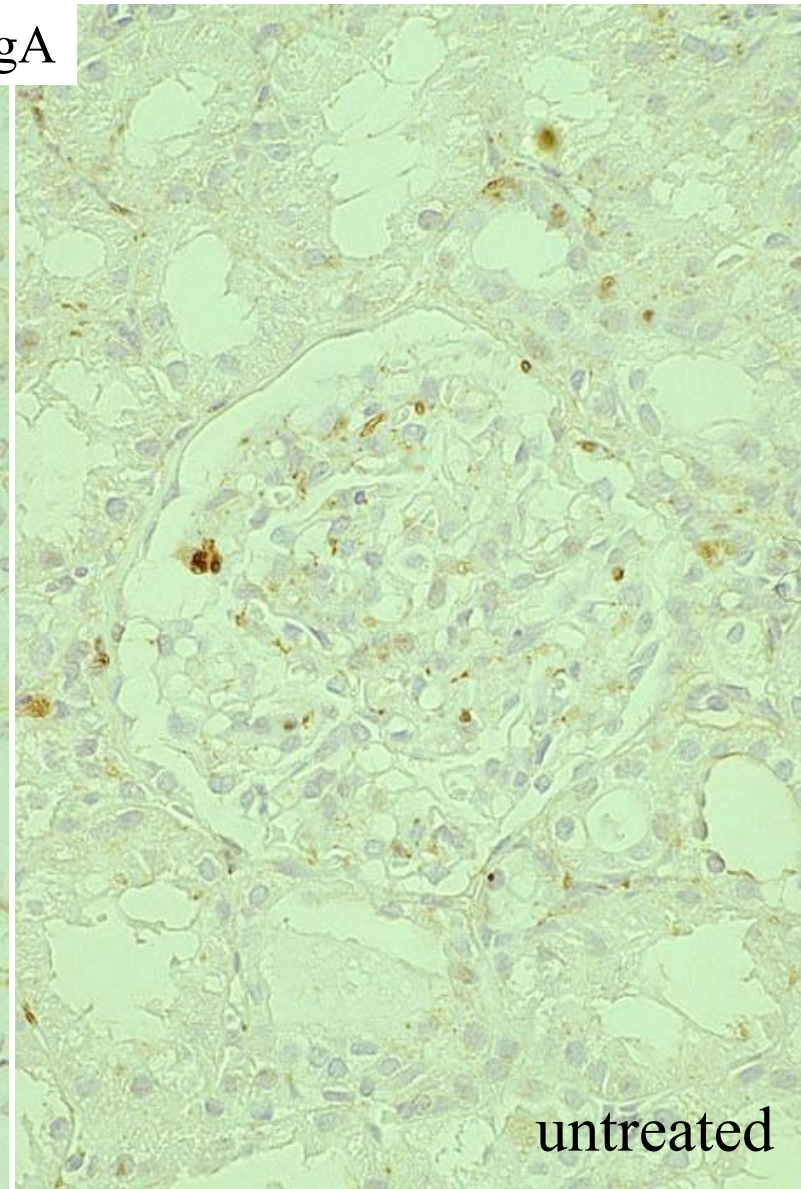
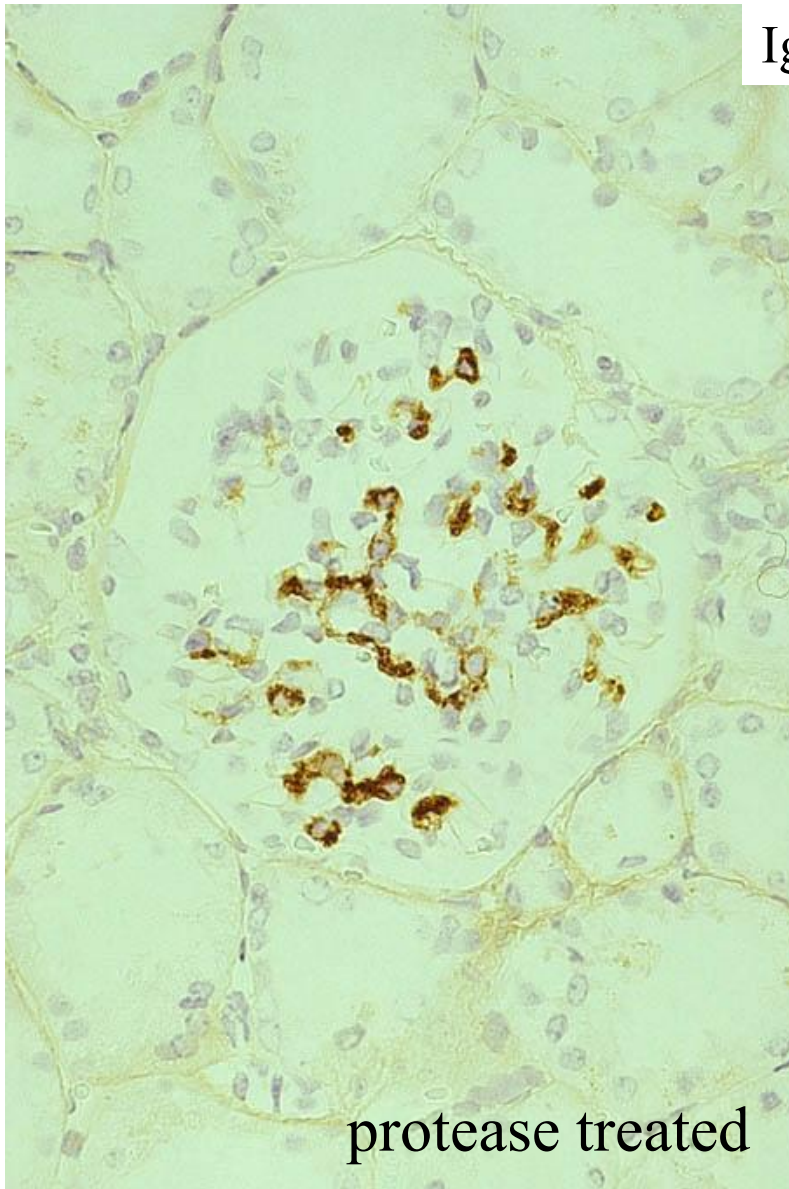
H&E



EPOS-Ki-67

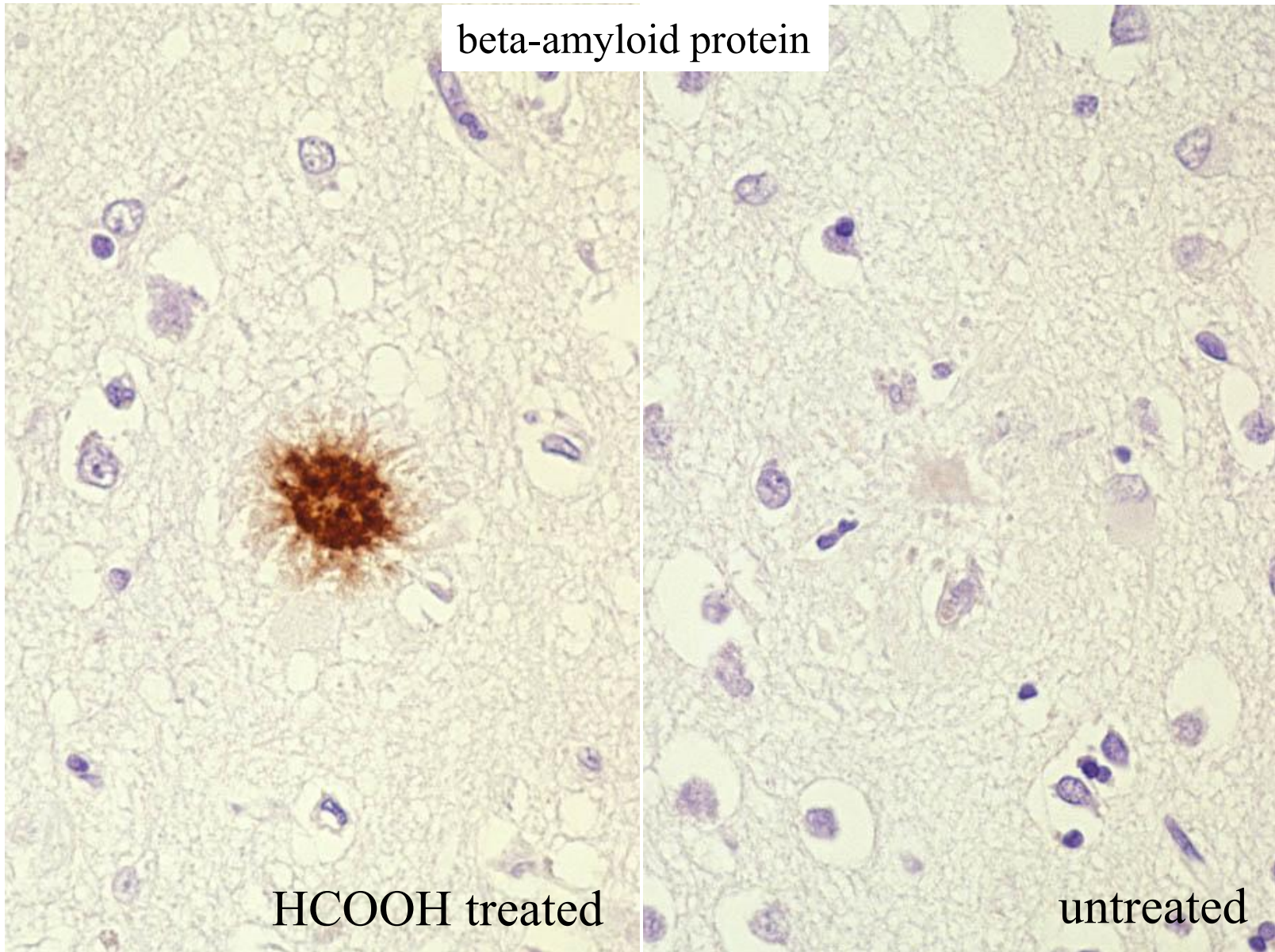
Application of EPOS (enhanced polymer one-step staining) method to intraoperative frozen section diagnosis: Ki-67 in peritoneal metastasis of breast cancer. Left: H&E, right: EPOS for Ki-67

IgA



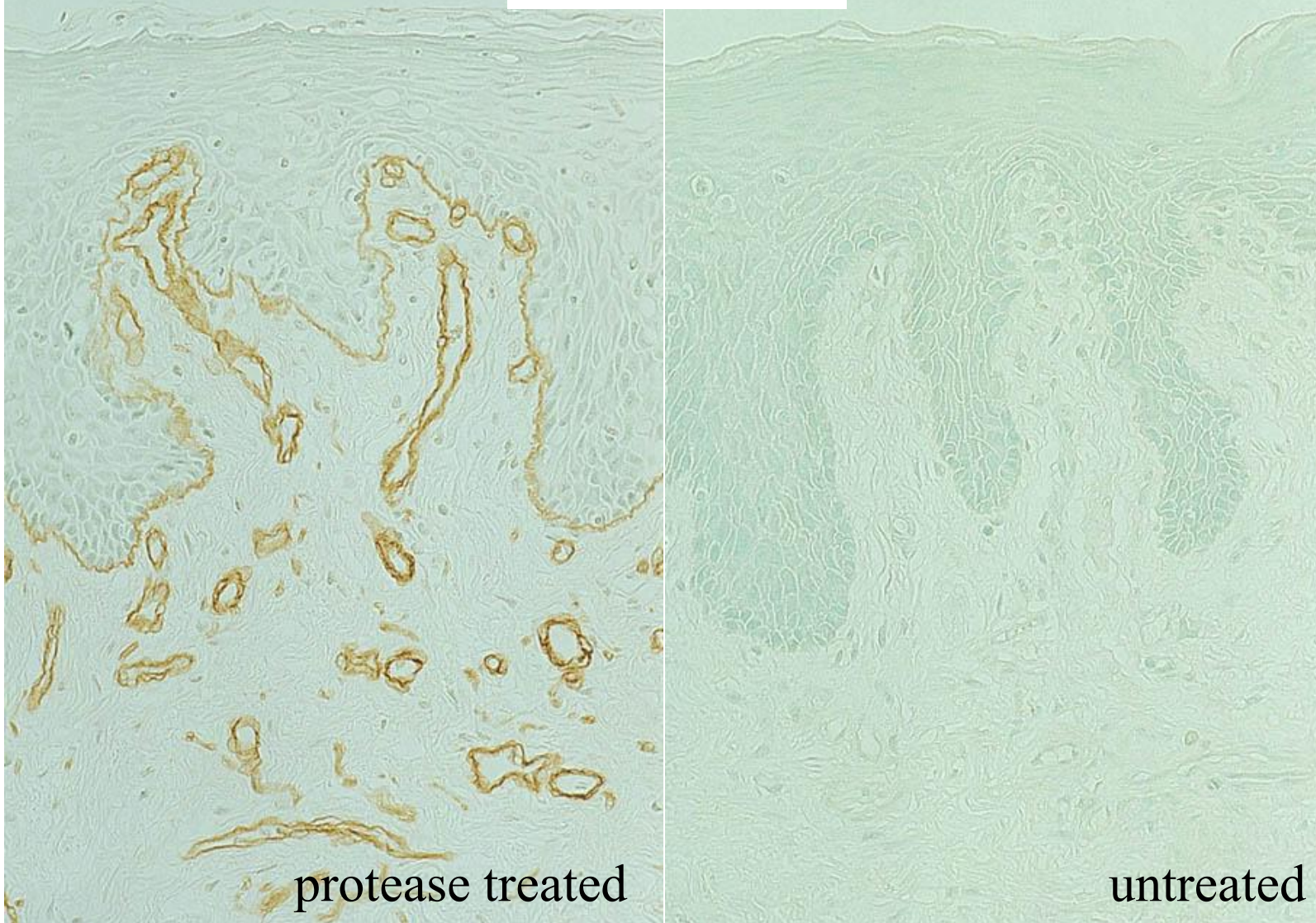
Effectiveness of proteinase treatment for demonstrating immunoglobulins and complements in formalin-fixed, paraffin-embedded renal biopsy sections with glomerular lesions. Mesangial IgA deposition in IgA nephropathy. Untreated section is negative for the deposits.

beta-amyloid protein

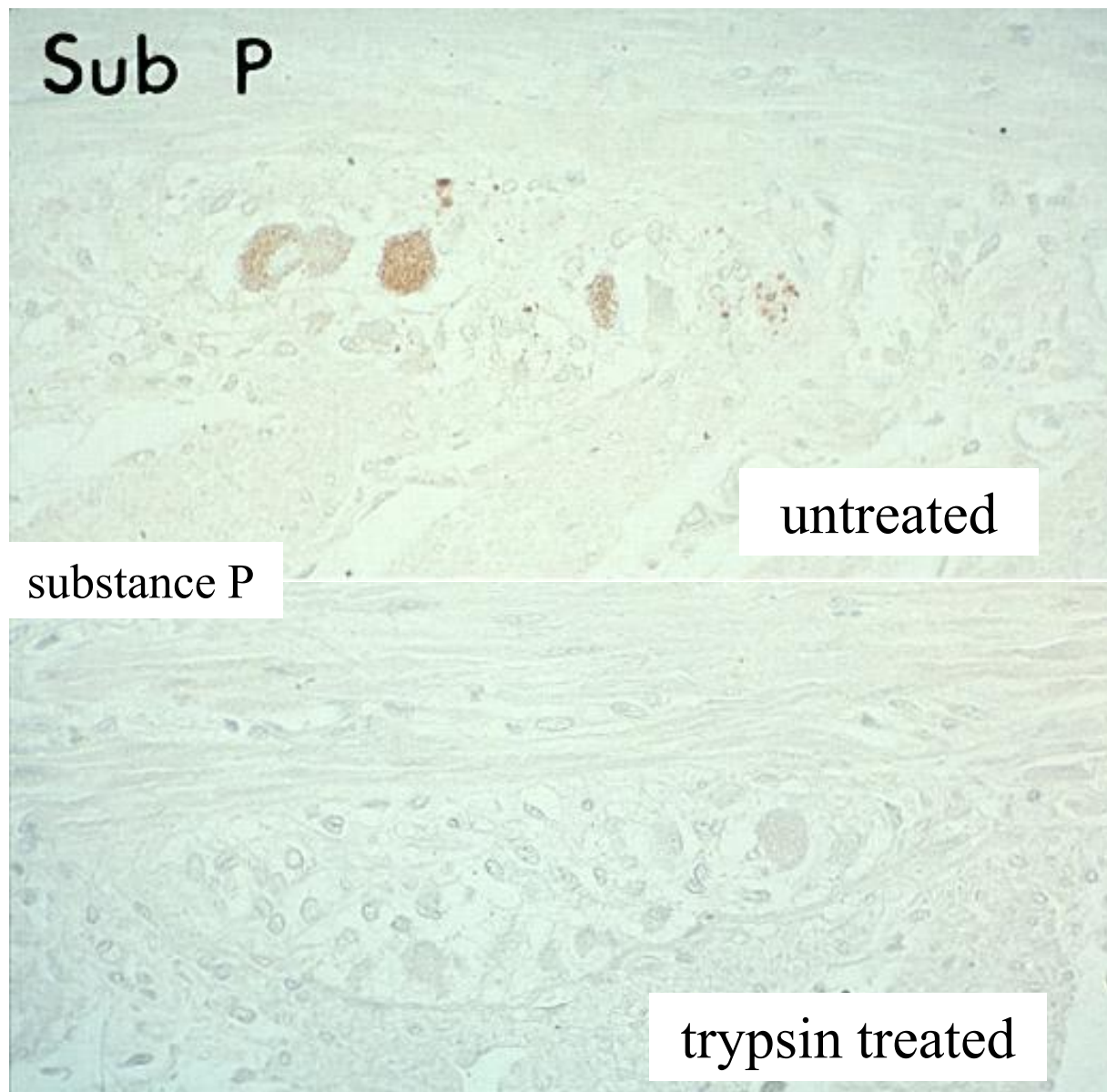


Formic acid treatment to visualize beta-amyloid protein in senile plaques in Alzheimer's disease. In this case, brief treatment with 100% formic acid is quite effective, while proteinase and heating pretreatments are ineffective.

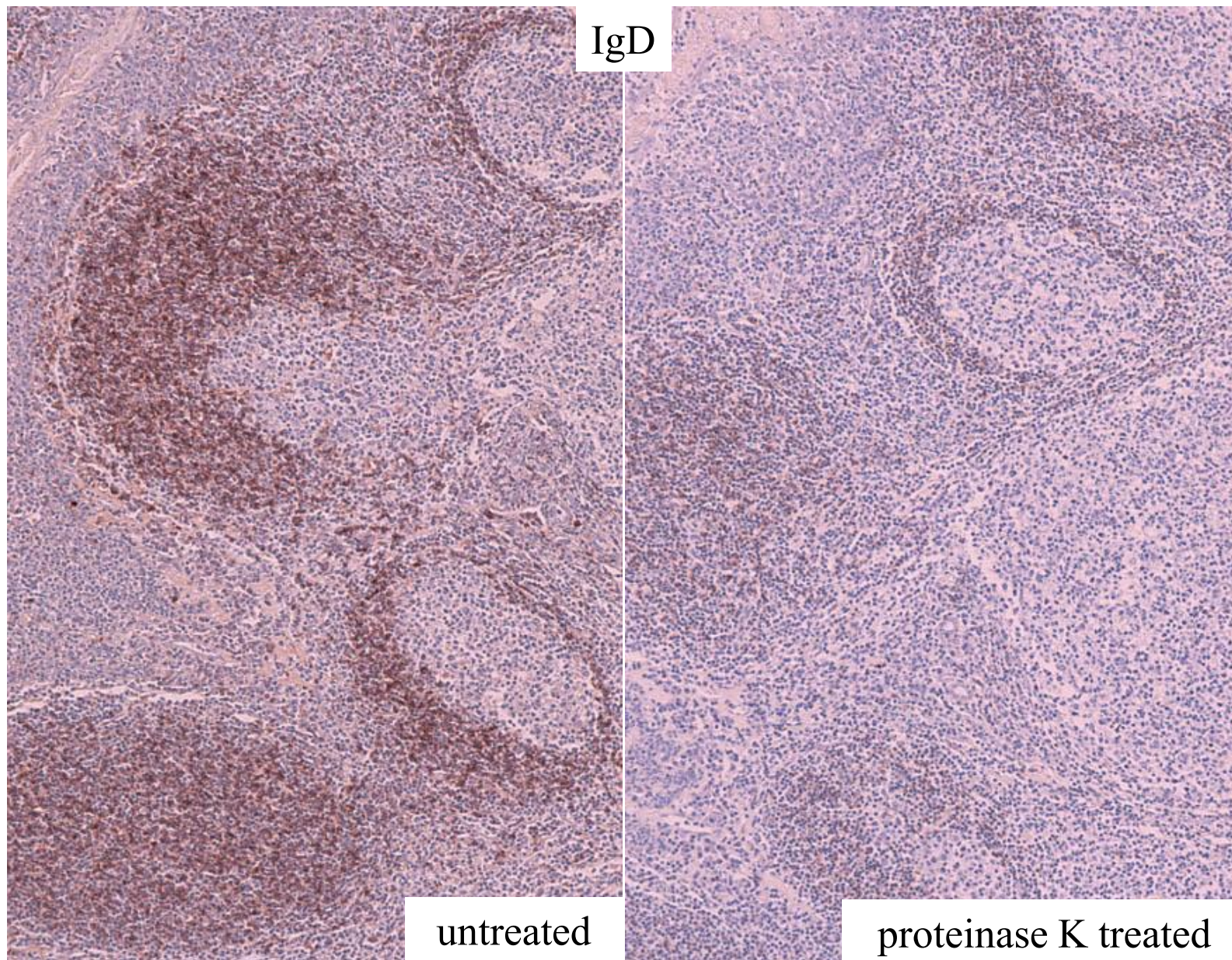
type IV collagen



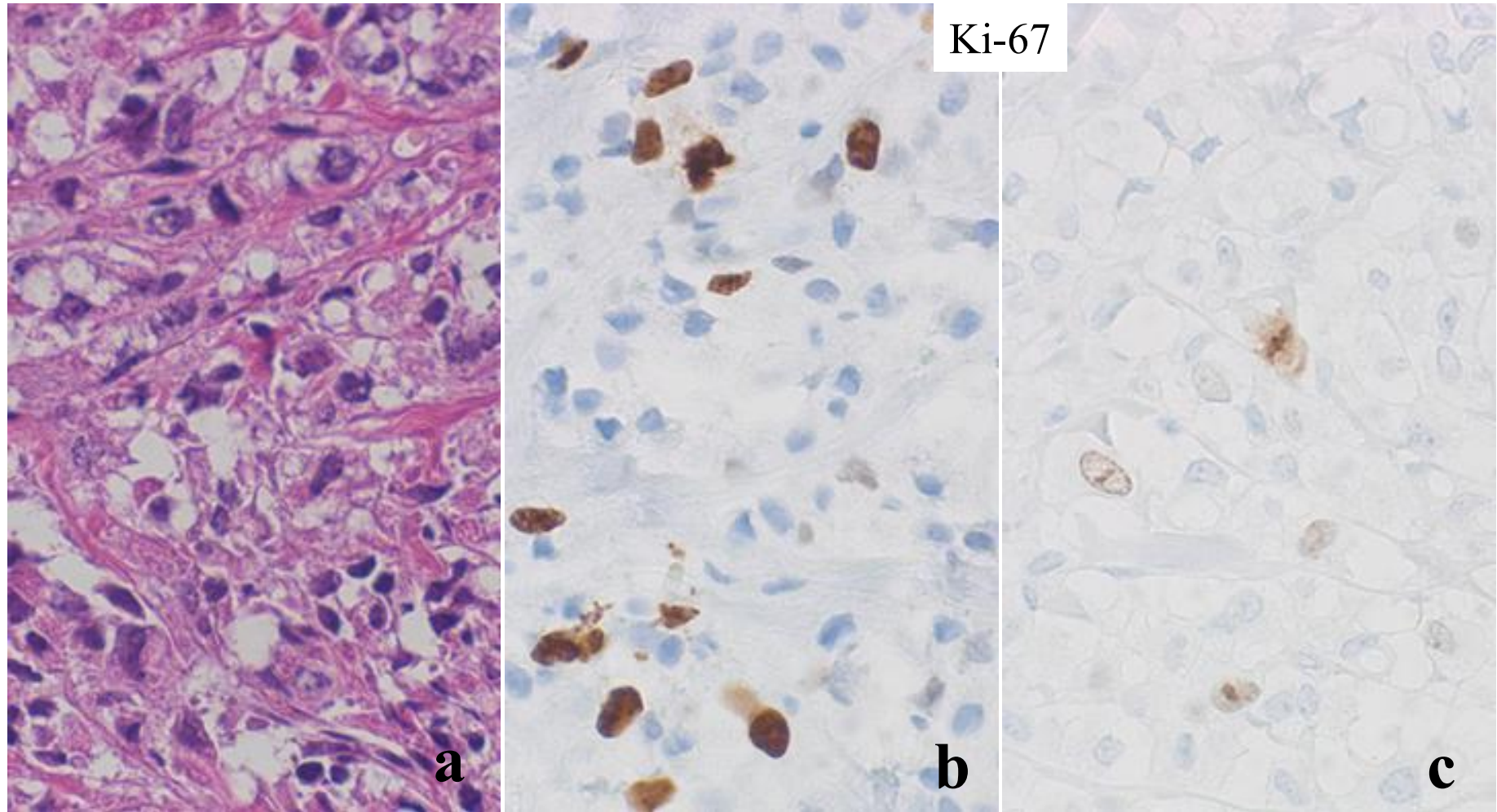
Effectiveness of protease treatment for demonstrating Type 4 collagen in the normal skin. The basement membranes of the epithelial cells and endothelial cells are clearly stained only after the enzymatic digestion.



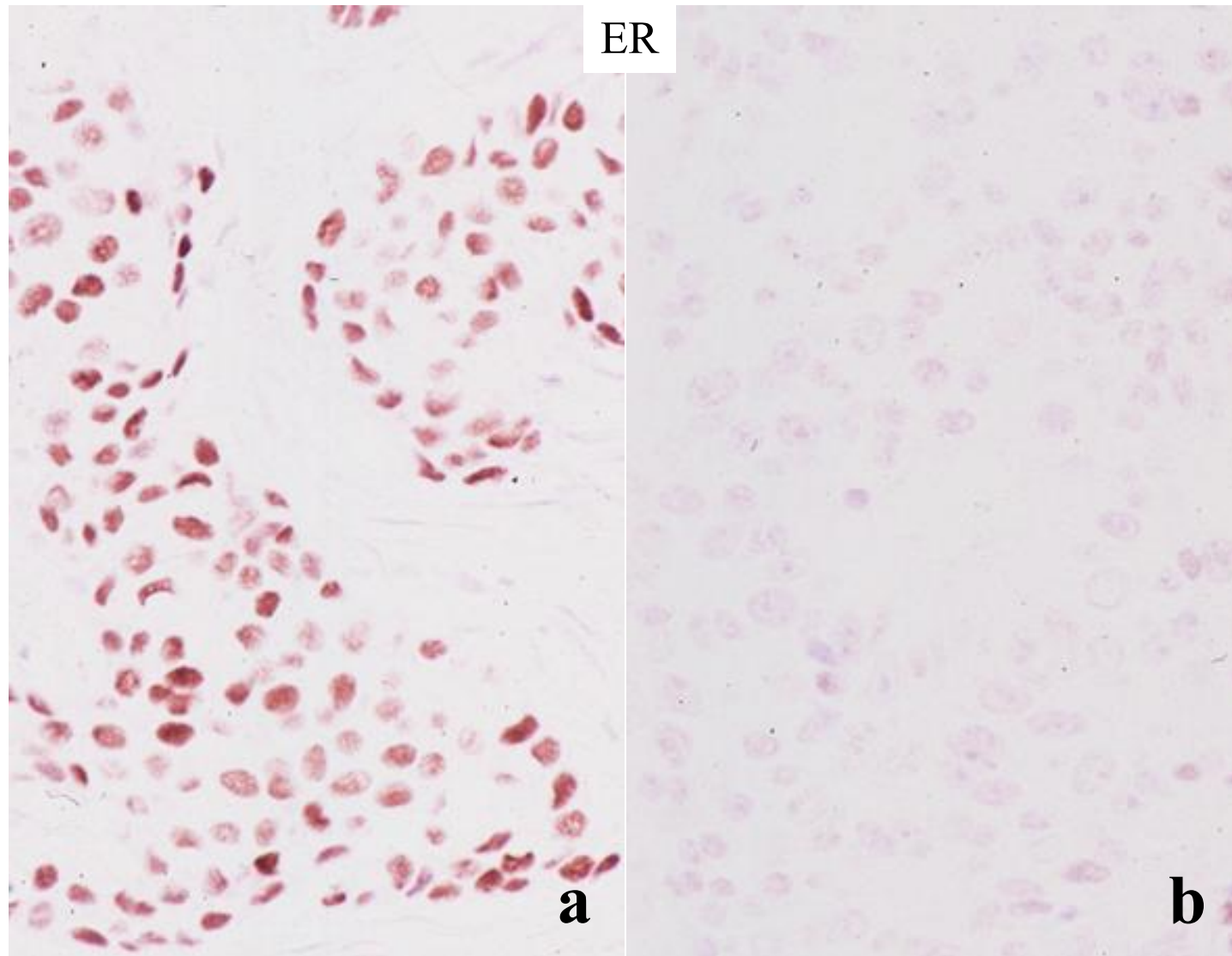
Trypsin digestion destroys the immunoreactivity of substance P in the myenteric plexus of the small intestine.



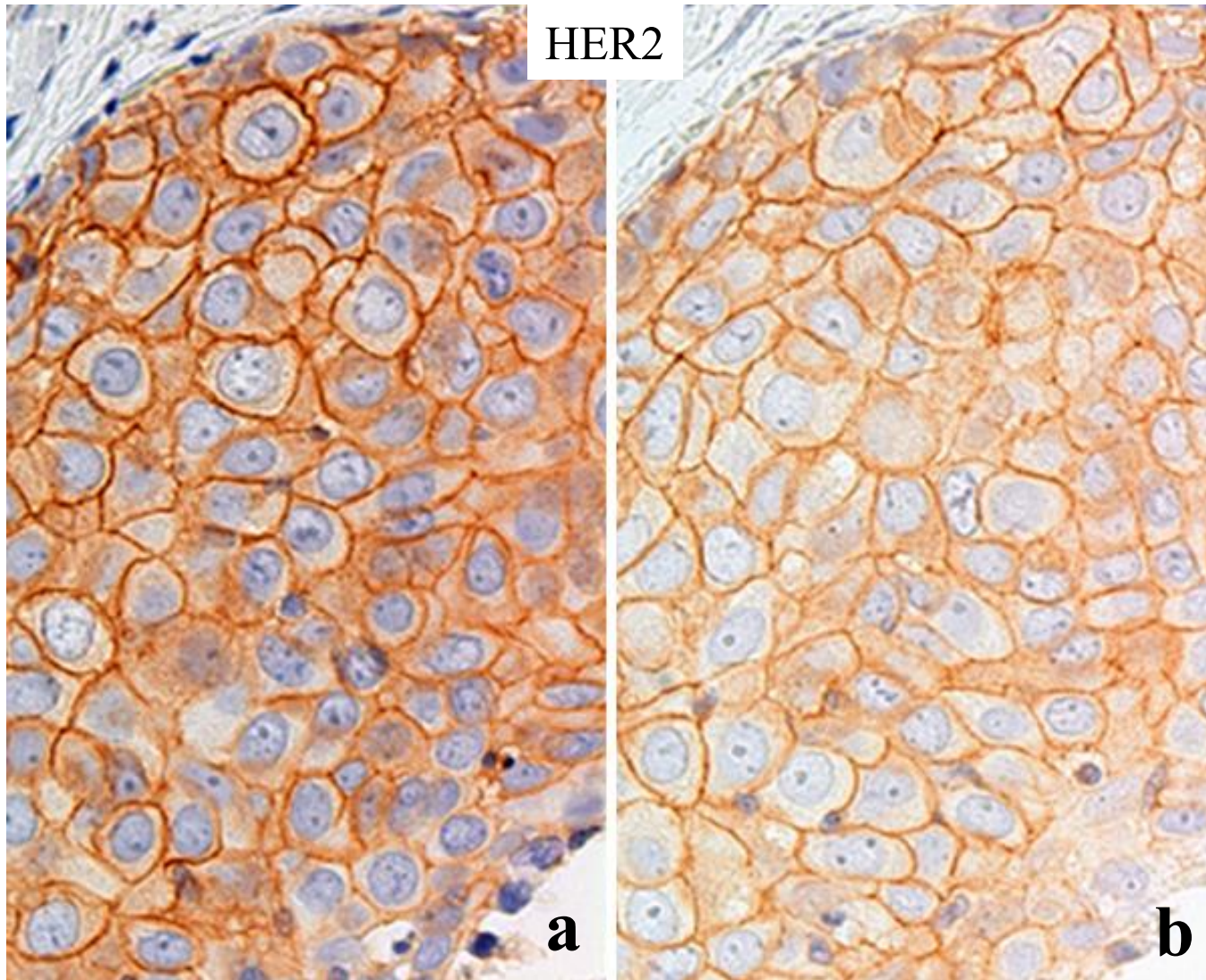
IgD immunoreactivity on the mantle zone lymphocytes of the palatine tonsil is significantly weakened by proteinase K digestion.



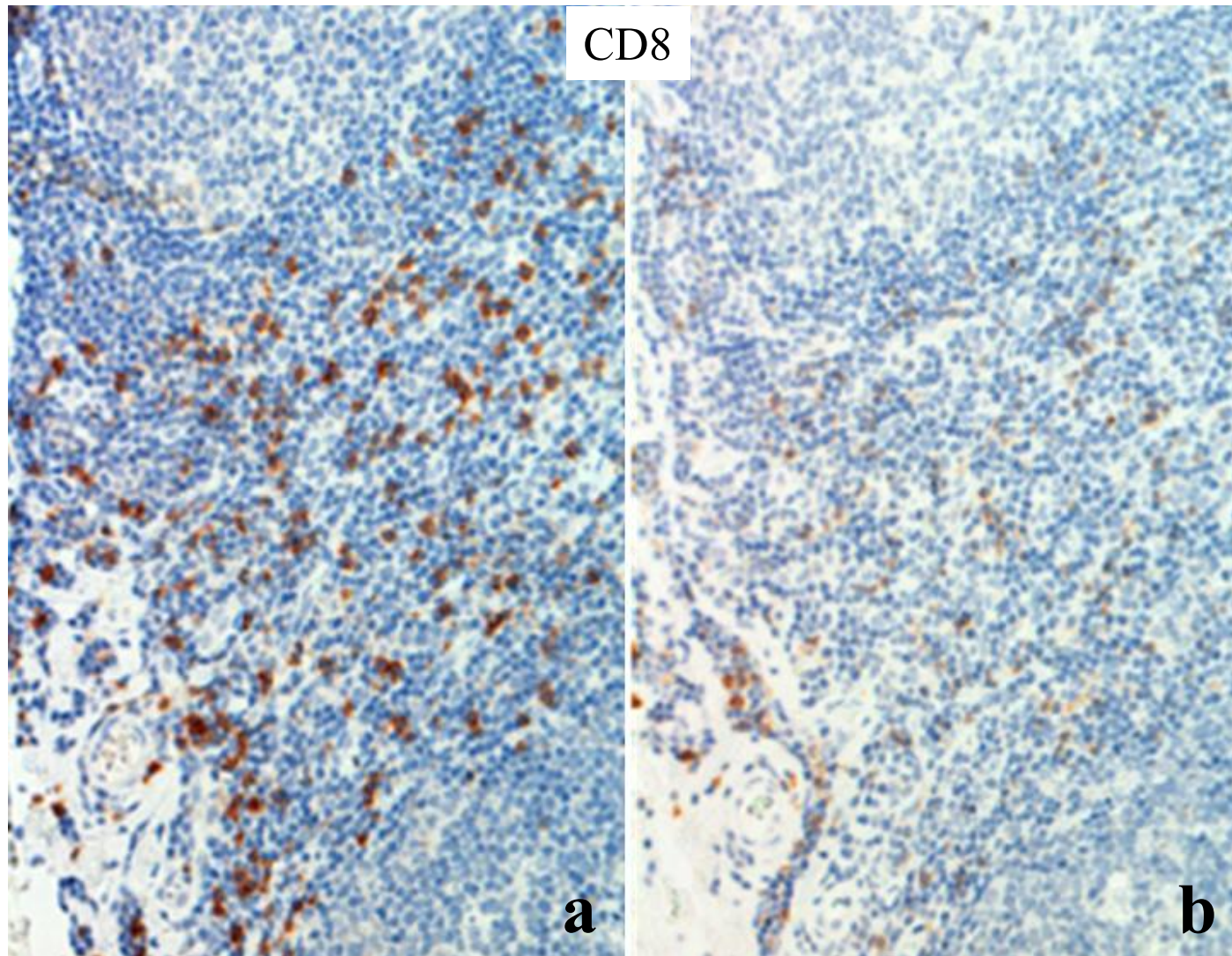
Negative effect of the surface decalcification procedure on demonstrating Ki-67 labeling in breast cancer. Surface decalcification of the paraffin block using trichloroacetic acid significantly deteriorates Ki-67 immunoreactivity. Mild decalcification using hydrochloric acid or EDTA is recommended. a: H&E, b: untreated, c: surface-decalcified with trichloroacetic acid.



Loss of estrogen receptor (ER) immunoreactivity by keeping unstained sections at room temperature for a long period of time. ER signals in the nuclei of breast cancer (a) is almost lost 1 year after cutting (b). The immunoreactivity is no longer retrieved by heating pretreatment.

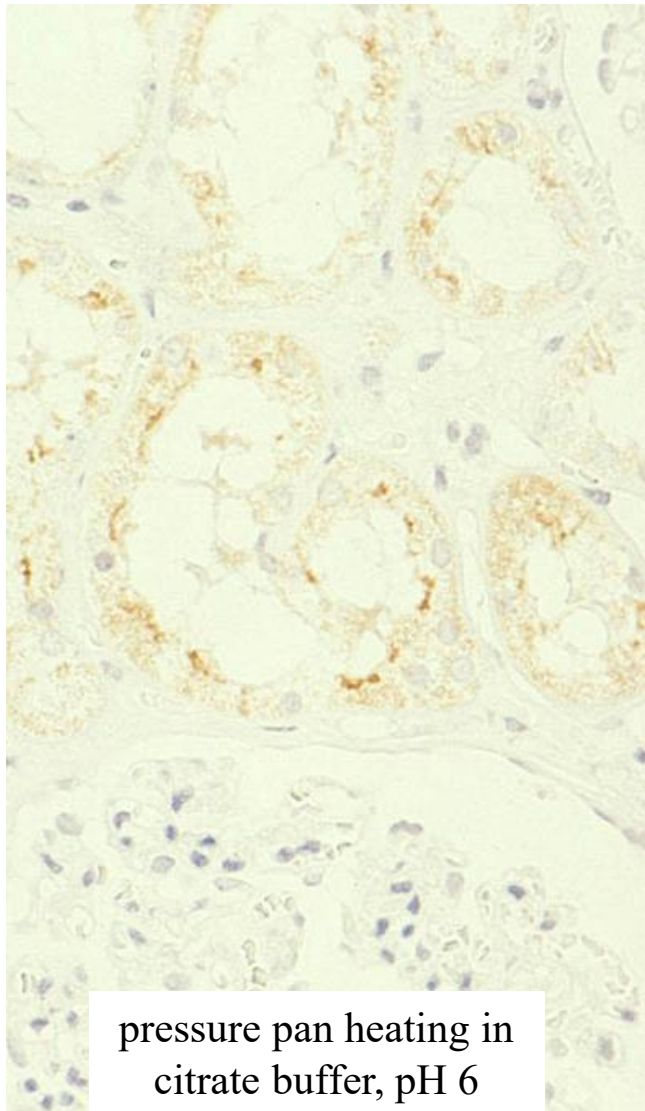


Decreased immunoreactivity of HER2 after prolonged drying in an incubator oven. Incubation of the unstained section at 40C for 3 days significantly weakens the overexpressed HER2 immunoreactivity on the breast cancer cells. a: drying at 40C for 1 hour, b: drying at 40C for 3 days.

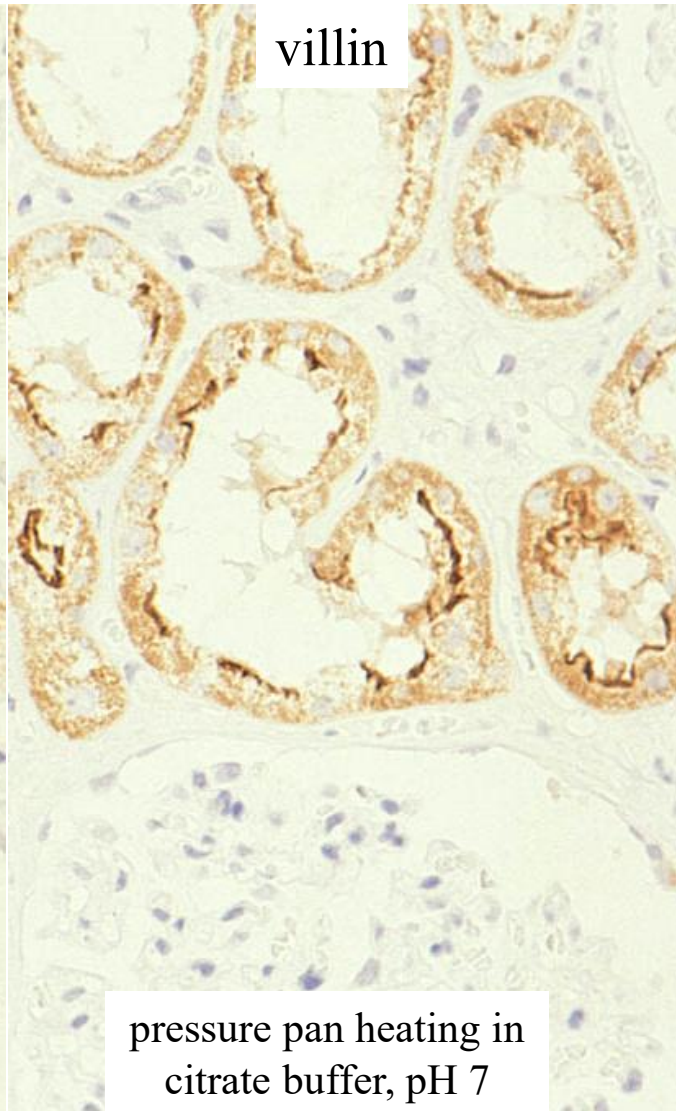


Influence of the temperature of the hotplate for stretching the sections (to minimize wrinkles). CD8 immunoreactivity of the palatine tonsil is negatively influenced by stretching at 70C for 3 seconds. a: stretching at 40C for 20 seconds, b: stretching at 70C for 3 seconds

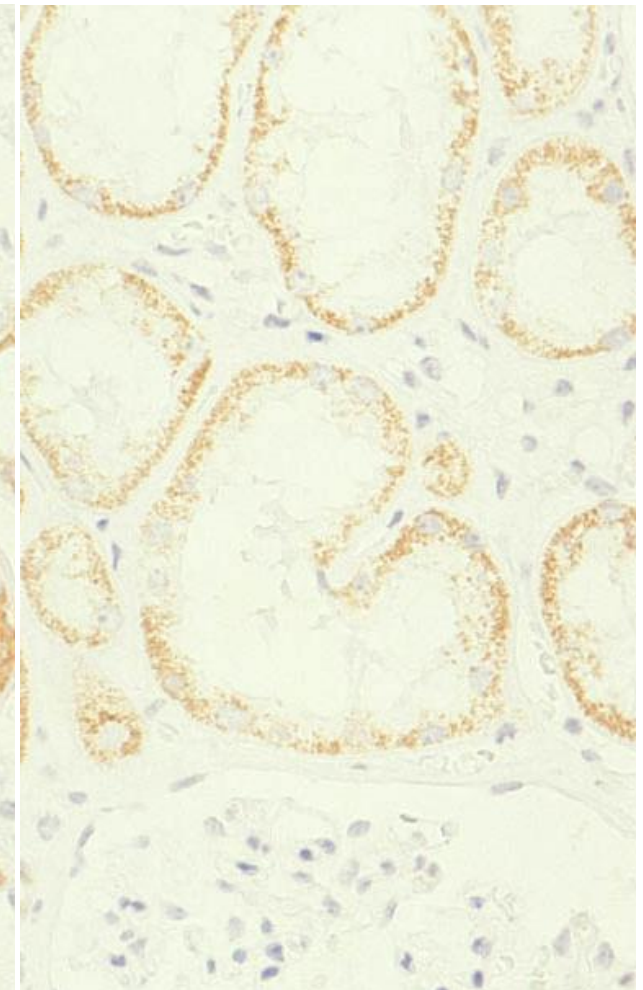
villin



pressure pan heating in
citrate buffer, pH 6



pressure pan heating in
citrate buffer, pH 7



pressure pan heating in citrate buffer,
pH 7, without primary antibody

Retrieval of endogenous biotin activity in the kidney detected with the LSAB method. Villin immunoreactivity is demonstrated in the apex of the proximal renal tubules (left). Heating treatment in citrate buffer, pH7, retrieves the endogenous biotin activity in the proximal renal tubules rich in mitochondria (center). Mitochondria are positive even without primary antibody (right).

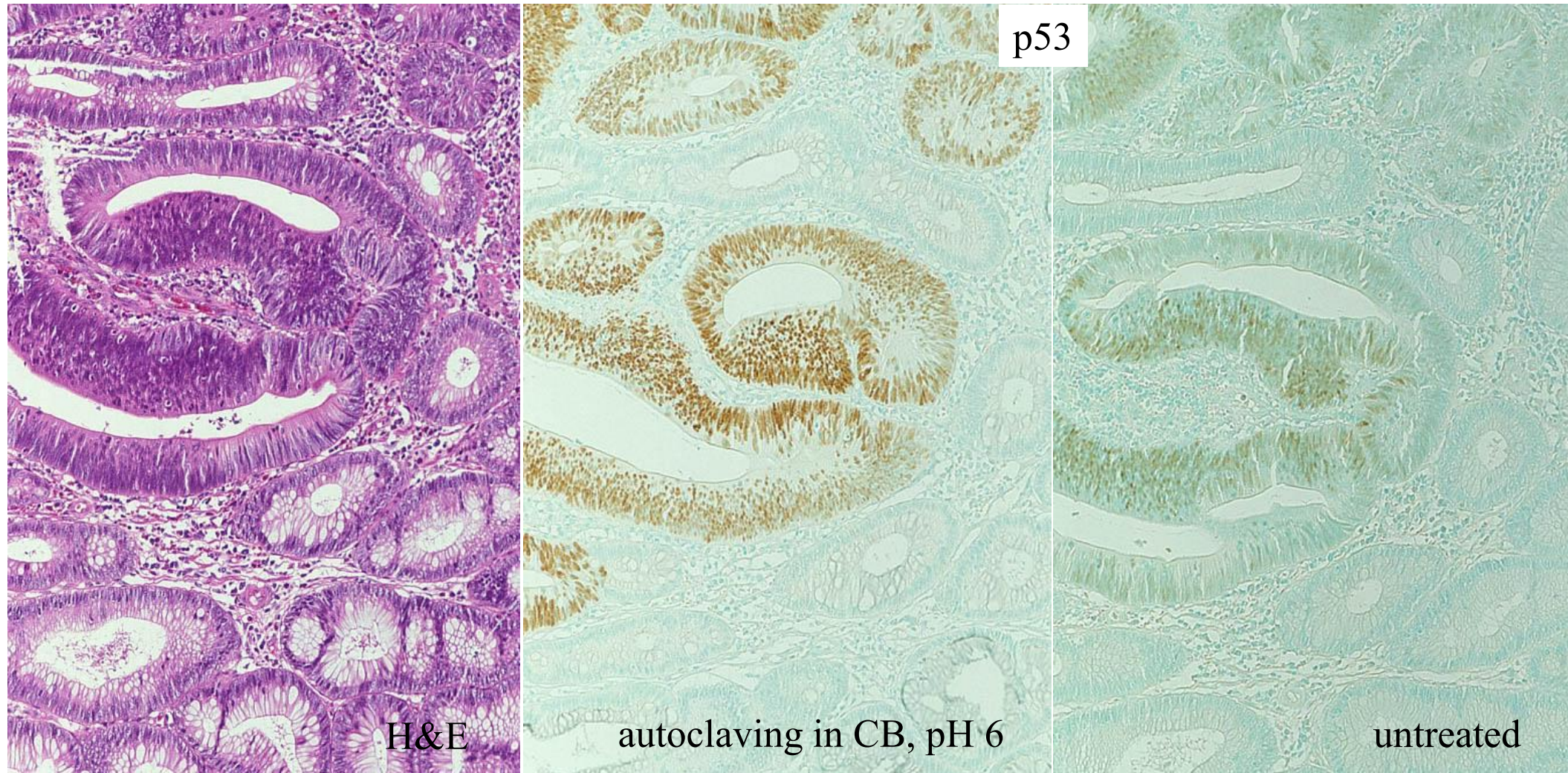
Methods of antigen retrieval

How to heat

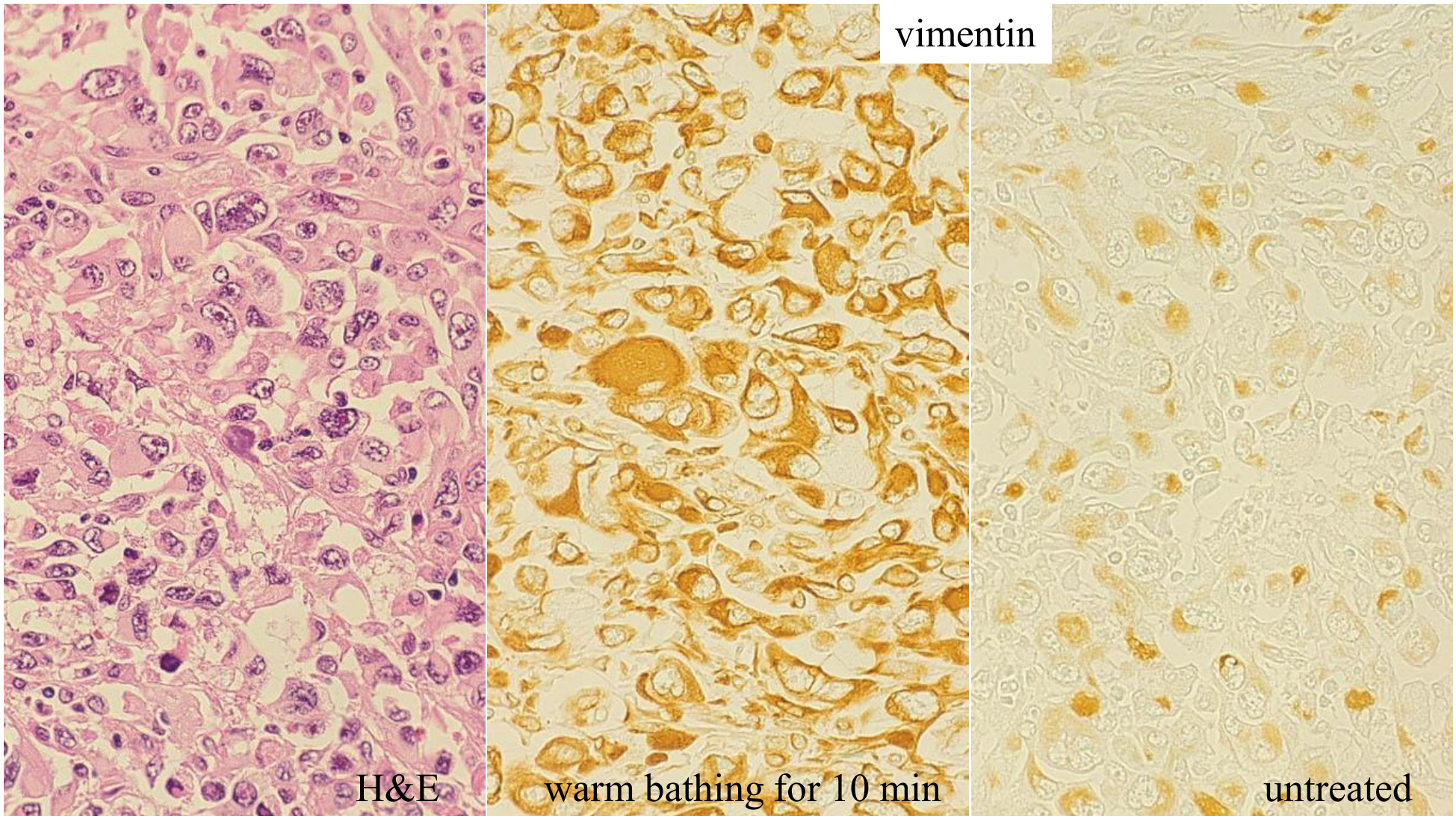
- 1) water bath (60°C, 95°C, 100°C)
- 2) Microwave (100°C)
- 3) Autoclave (121°C)
- 4) Pressure pan (110°C, 125°C)

Soaking solutions

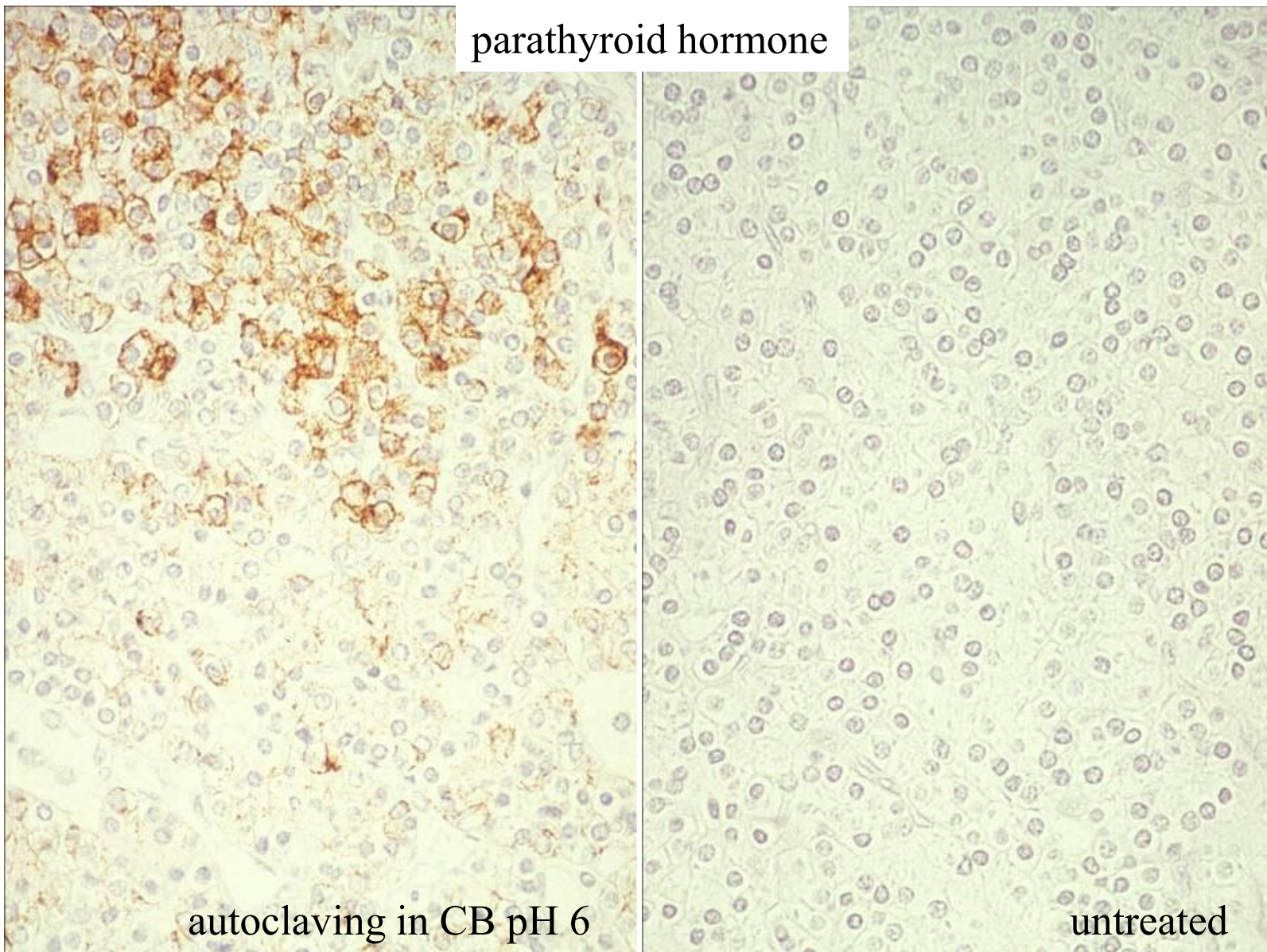
- 1) Water
- 2) 5-20% zinc sulfate solution
- 3) 10 mM citrate buffer, pH 6 or pH 7
- 4) 1 mM EDTA solution, pH 8



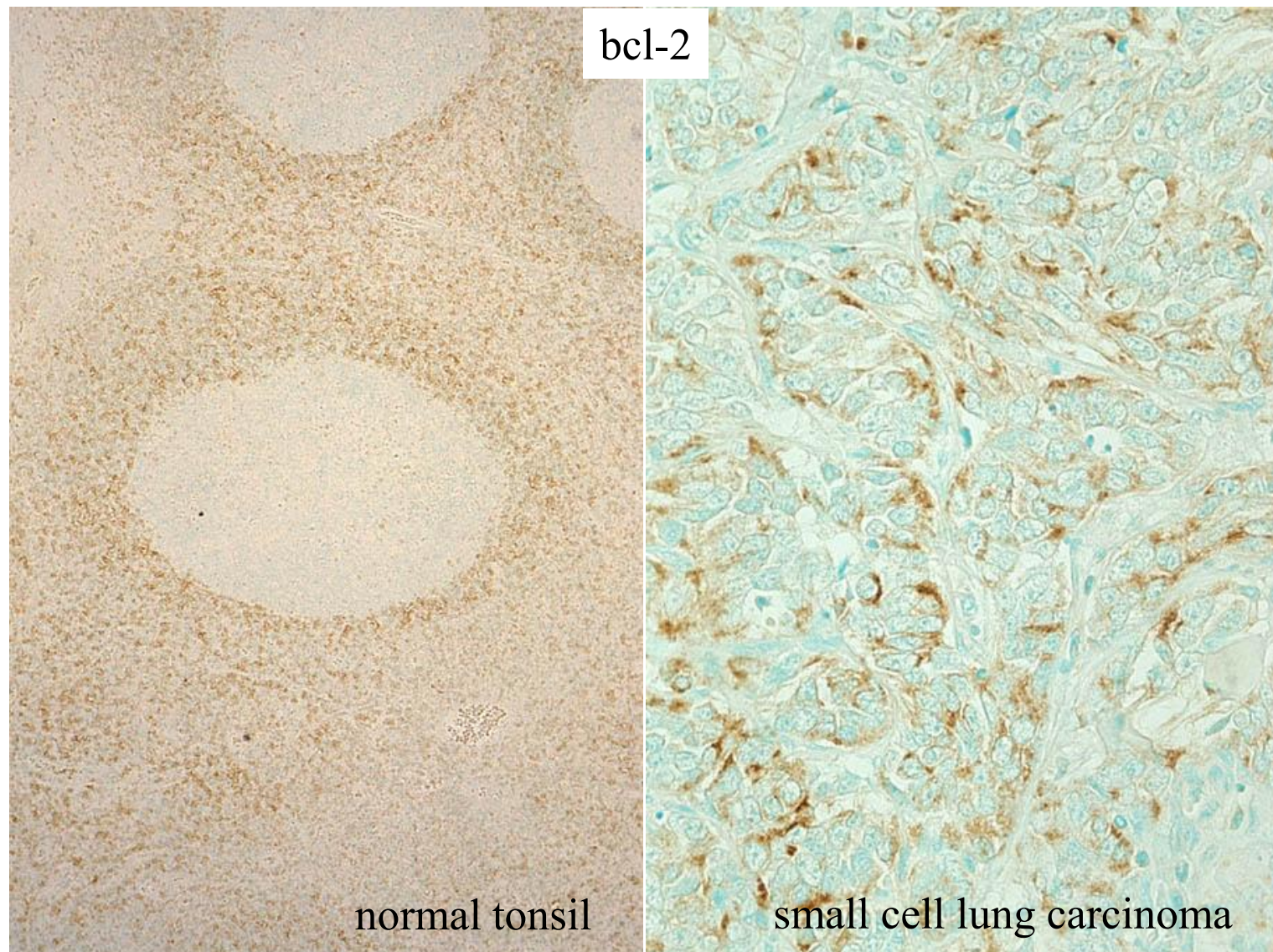
p53 overexpression in cancer in adenoma of the colon (H&E). Polypectomized specimen reveals focal intramucosal malignancy in tubular adenoma. After autoclaving pretreatment in 10 mM citrate buffer, pH 6, for 10 minutes, intranuclear overexpression of p53 turned to be clearly visible in the cancerous glands but with negative expression in the adenoma cells.



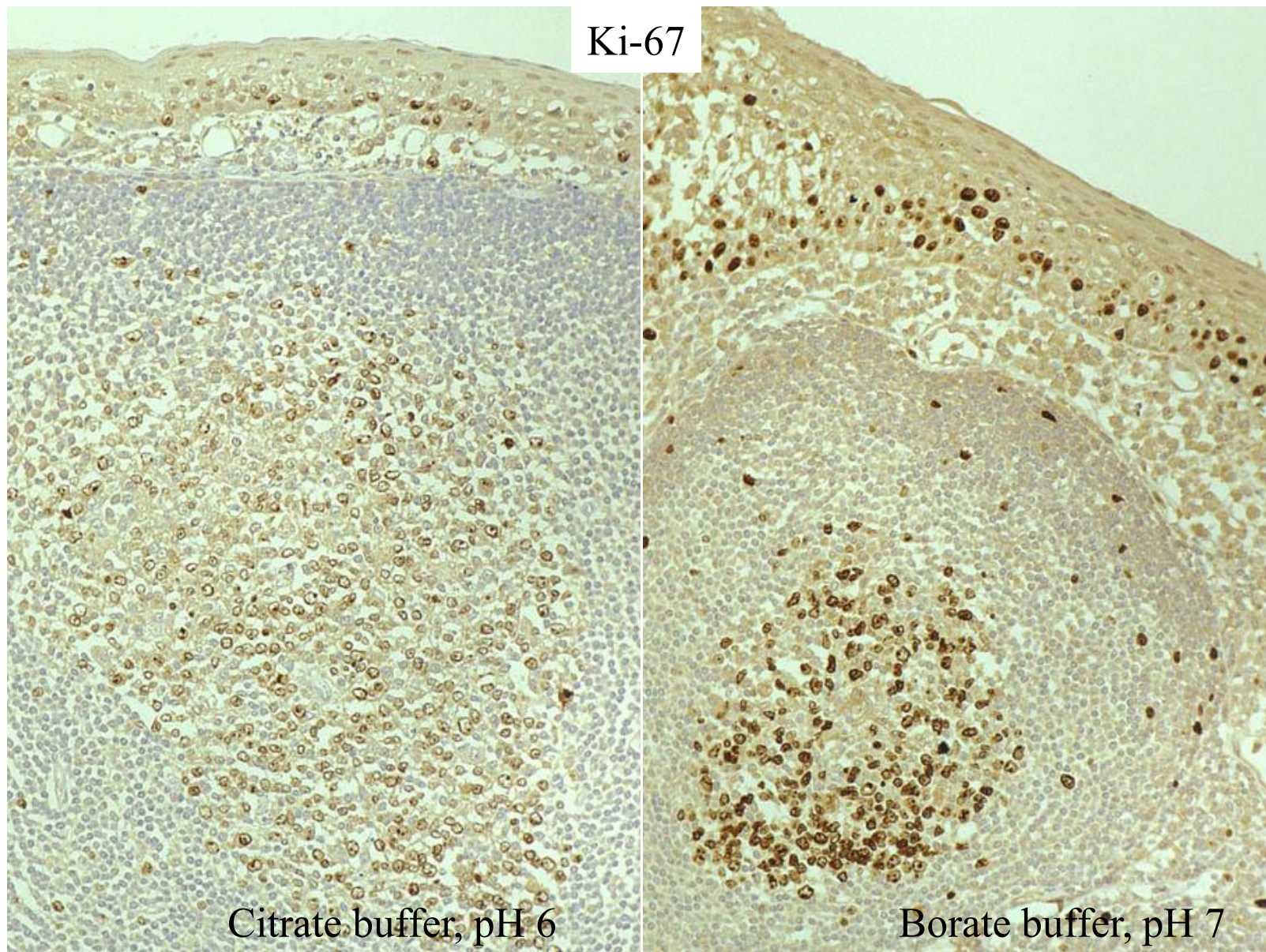
Vimentin immunoreactivity in anaplastic carcinoma of thyroid (lung metastasis). Heating procedure is necessary for consistent localization of vimentin. In this case, warm bathing at 100C for 10 minutes is effective.



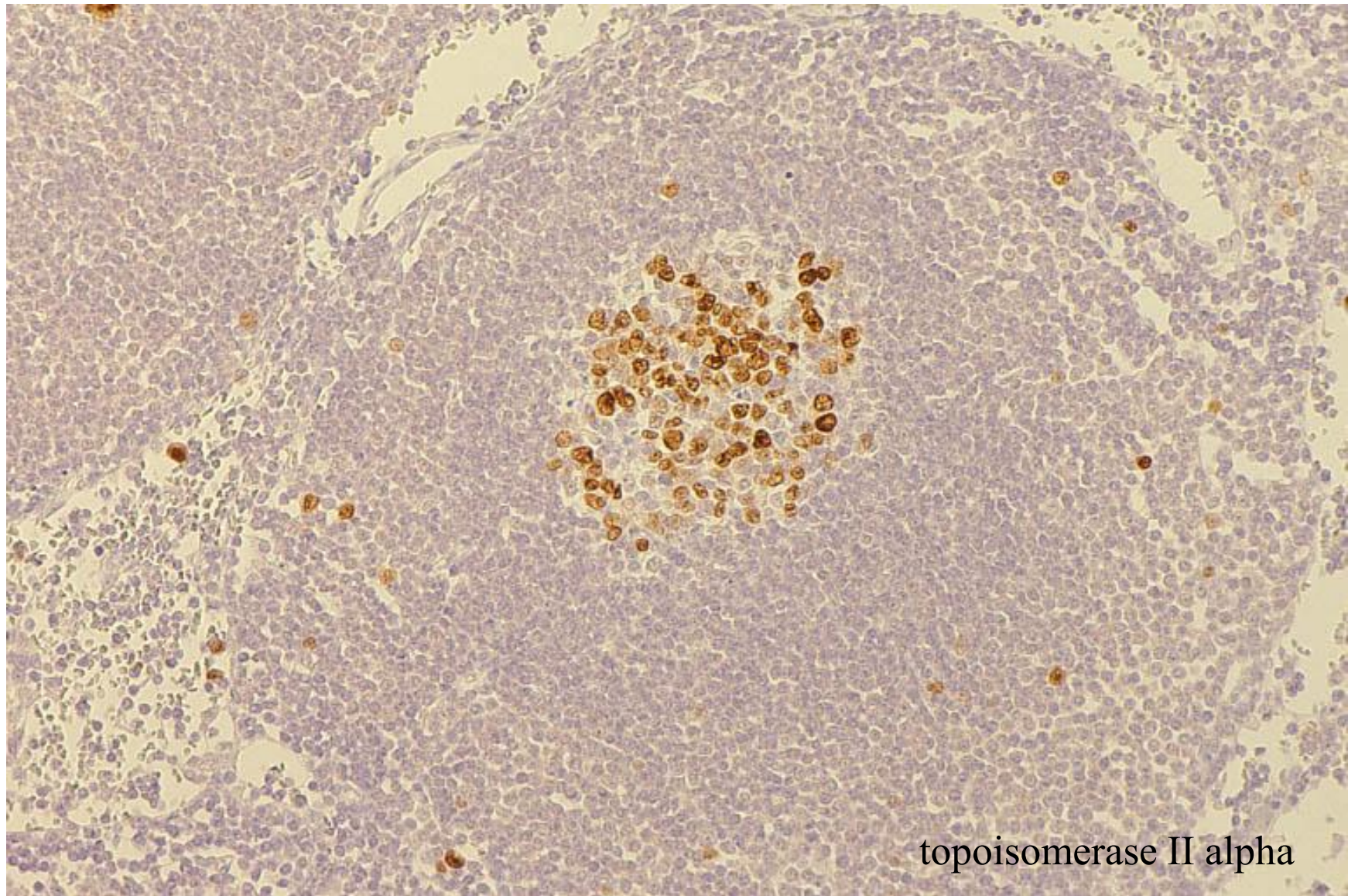
Parathyroid hormone (PTH) localization in the normal parathyroid. Heating pretreatment is needed for consistent localization of PTH. Autoclaving in 10 mM citrate buffer, pH 6, is effective in this case.



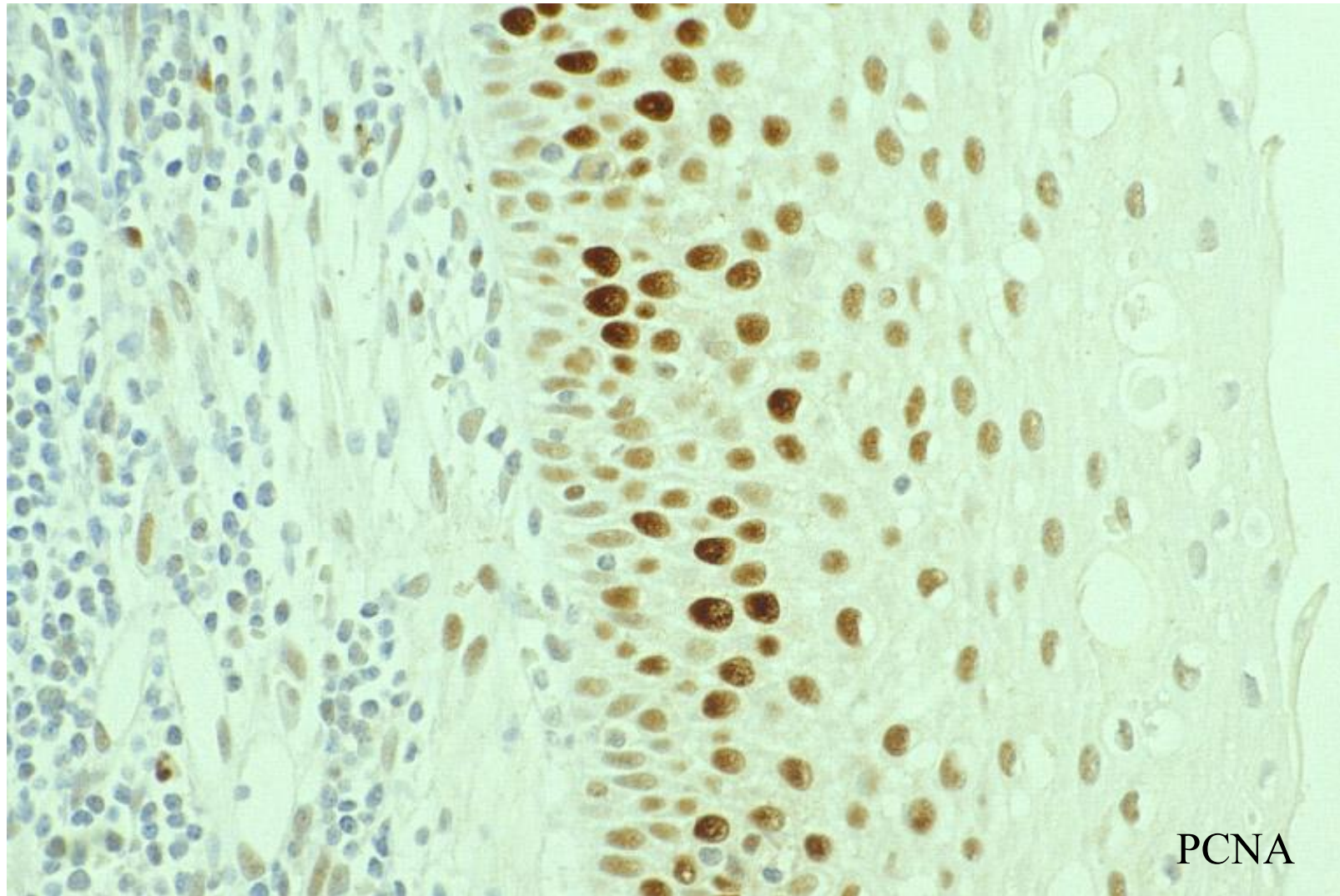
Localization of **bcl-2 protein** after autoclaving in distilled water. Expression of bcl-2 protein, a mitochondrial protein, is consistently visualized after heating pretreatment. In this case, distilled water is used as a soaking solution. The normal germinal center lymphocytes are negative for bcl-2, while bcl-2 is expressed in small cell lung carcinoma.



Ki-67 localization in normal palatine tonsil after autoclaving. As a soaking solution for heating, 10 mM borate buffer, pH7, is more effective than 10 mM citrate buffer, pH 6.

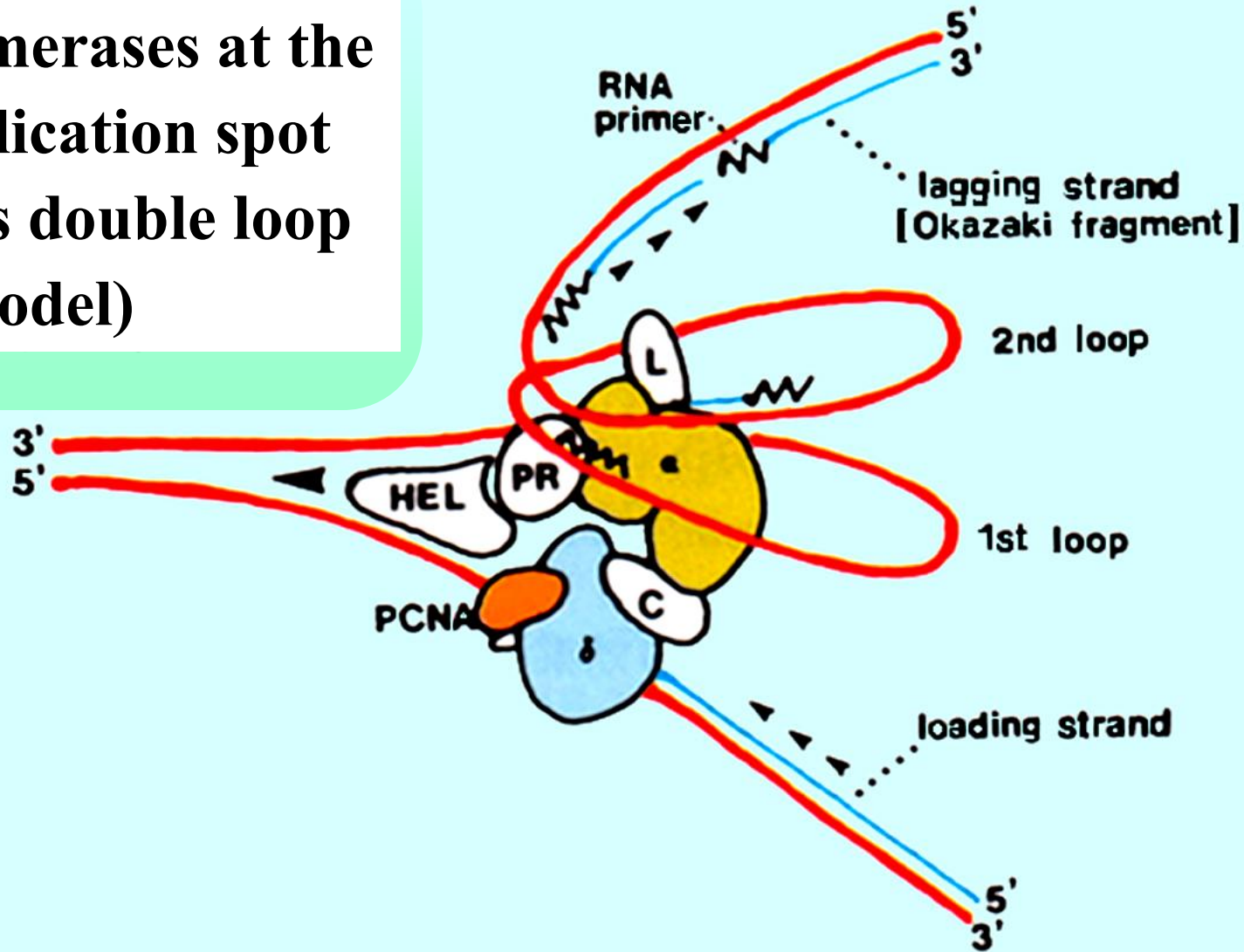


Topoisomerase II alpha localization in the palatine tonsil after autoclaving in 10 mM citrate buffer, pH 6. The germinal center lymphocytes are labeled, a comparable result with Ki-67 labeling.

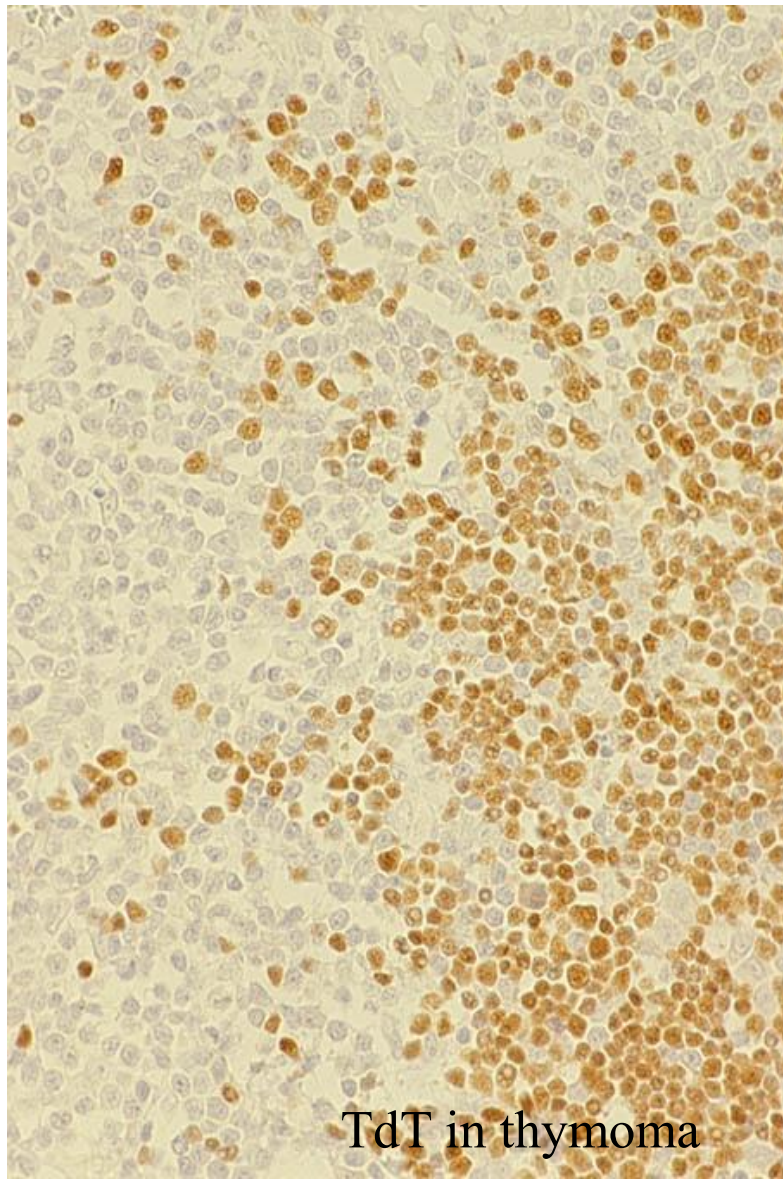


Proliferating cell nuclear antigen (PCNA) localization in the tonsillar squamous mucosa after autoclaving in 10 mM citrate buffer, pH 6. PCNA antigenicity remains in post-mitotic cells, and, therefore, PCNA is not a suitable marker for demonstrating proliferating cells.

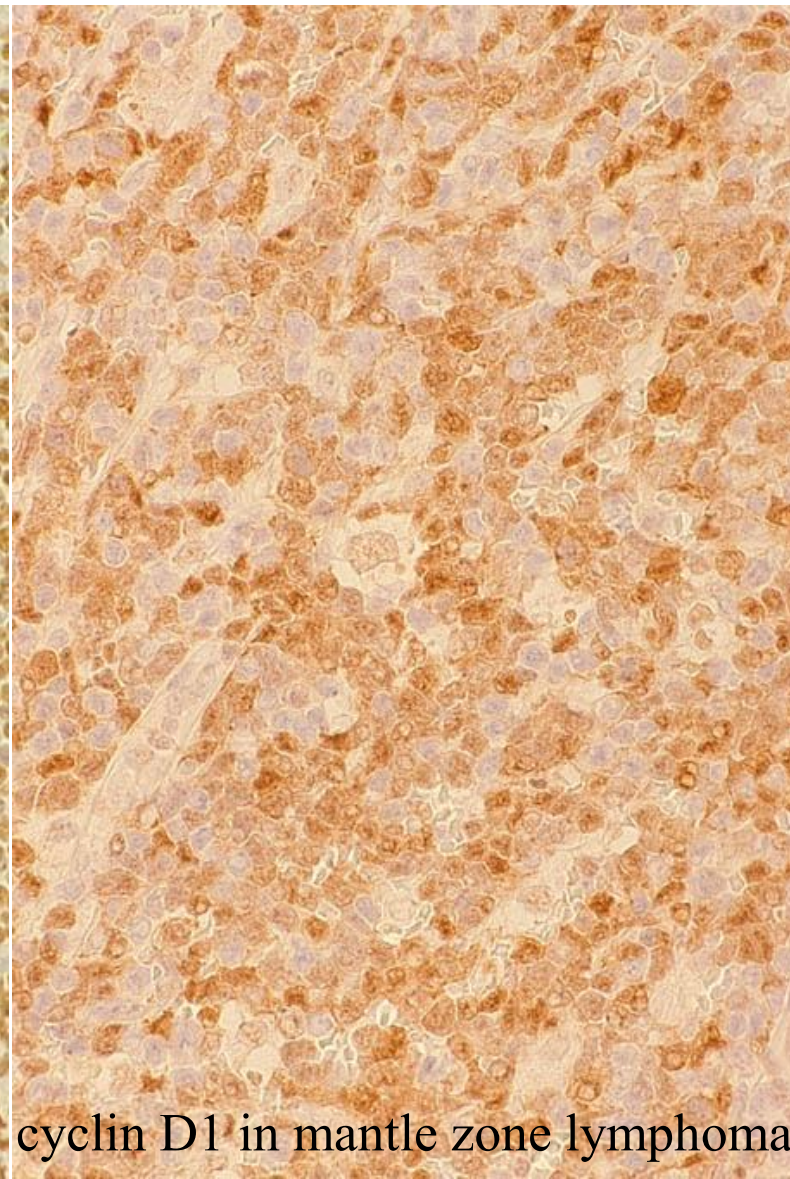
DNA polymerases at the DNA replication spot (Spadari's double loop model)



Proliferating cell nuclear antigen (PCNA), a kind of cyclin, is an auxiliary protein of DNA polymerase delta to form DNA replication complex with DNA polymerase alpha at the DNA replication spot.



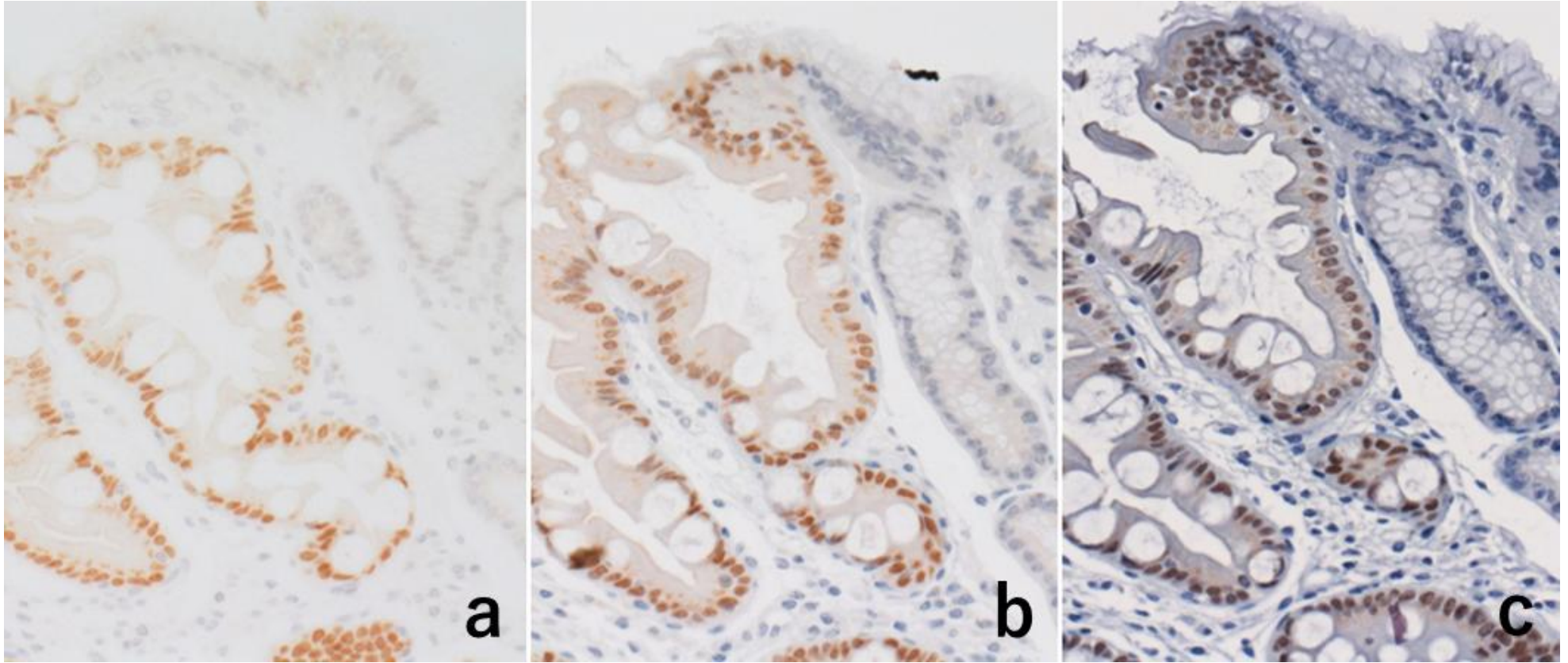
TdT in thymoma



cyclin D1 in mantle zone lymphoma

Localization of nuclear antigens in lymphoid tumors after pressure pan treatment in 10 mM citrate buffer, pH 7. TdT in thymoma and cyclin D1 in mantle cell lymphoma.

CDX2 expression in intestinal metaplasia



Nuclear staining with Mayer's hematoxylin after pressure pan pretreatment with 1 mM EDTA, pH 8. After heating in EDTA, pH 8, the nuclei turned to be resistant to hematoxylin staining. Prolonged staining period is required. Immunostaining for CDX2 in intestinal metaplasia of the stomach. a: 10 seconds, b: 1 minute, c: 10 minutes.

The author recommends French cuisine using a pressure pan.

Use of a pressure pan (T-FAL, France)
for heat-induced antigen retrieval

It is handy and easy to reduce pressure after treatment to get a consistent result.

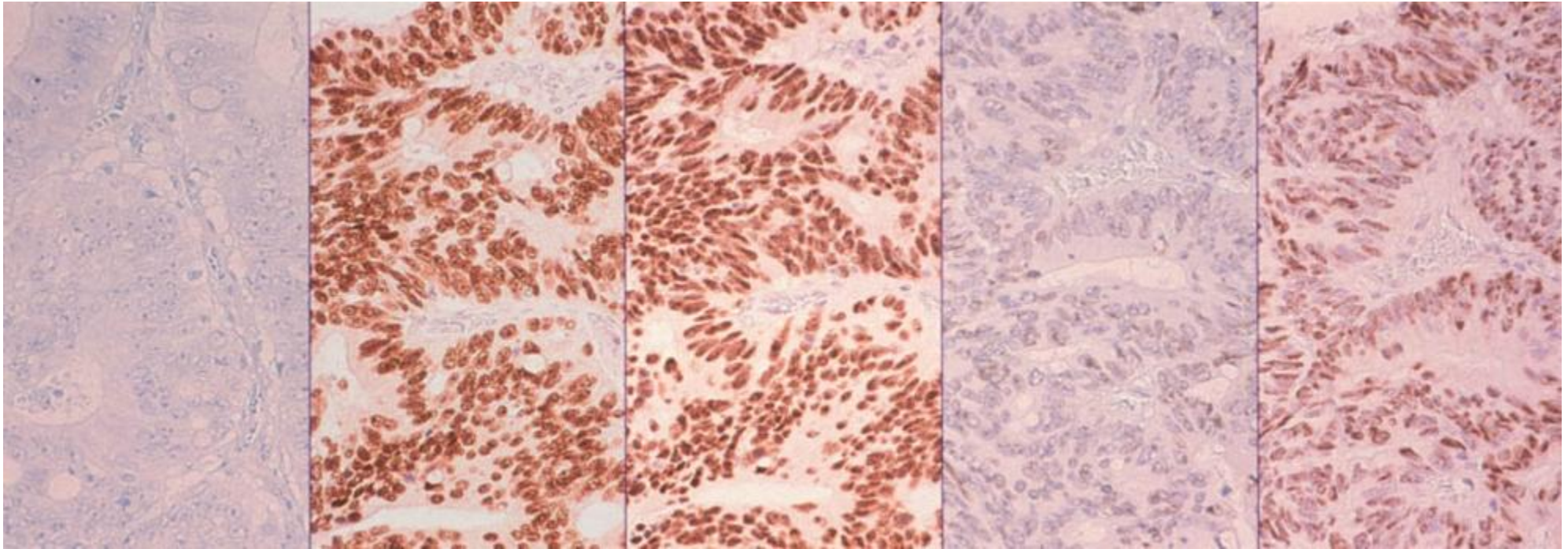


Pressure pan (T-FAL, France)



How to use the pressure pan (T-FAL, France). Sections can be soaked in an appropriate solution in a small container.

p53 in colon cancer



pH 5

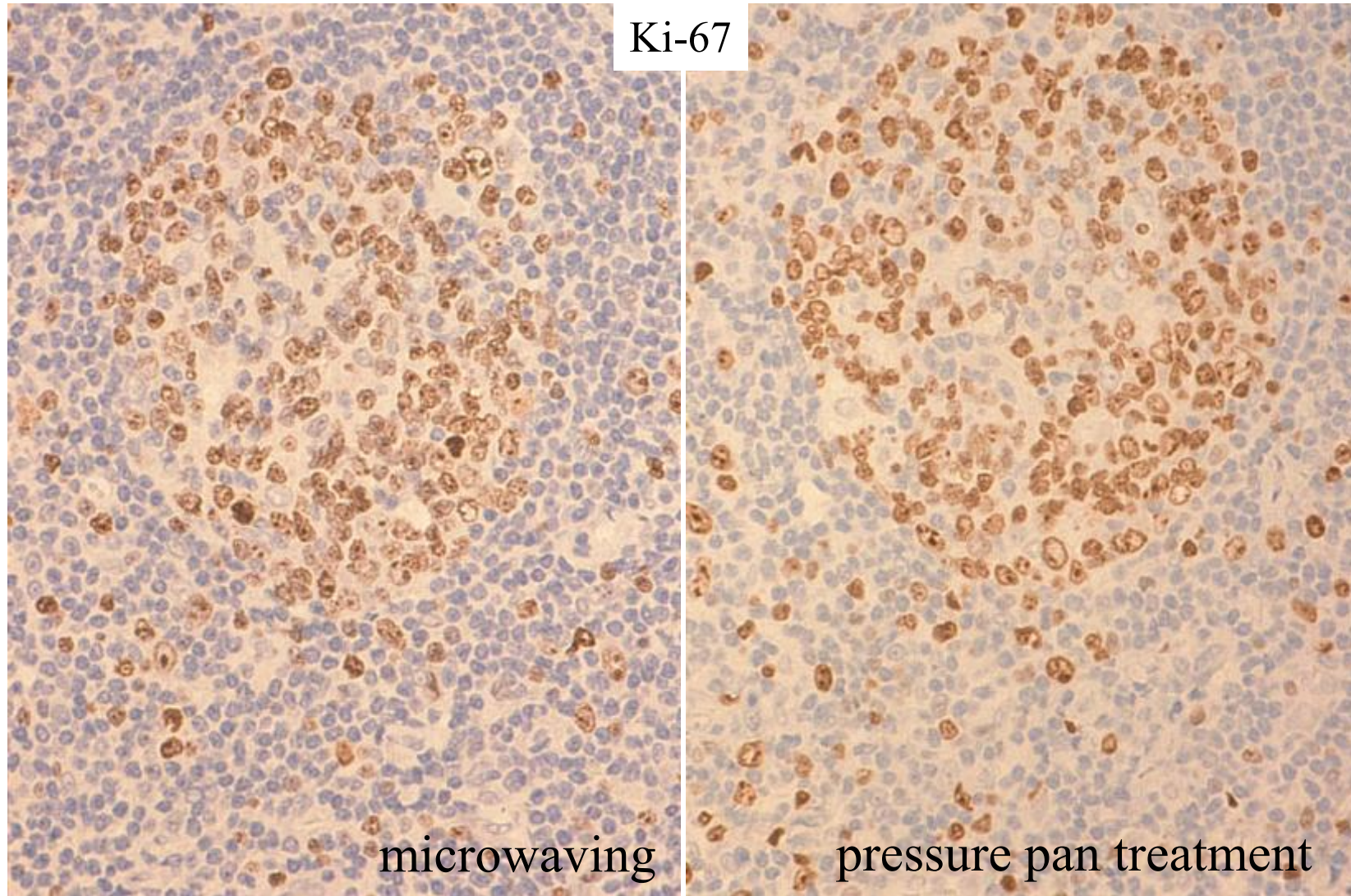
pH 6

pH 7

pH 8

pH 9

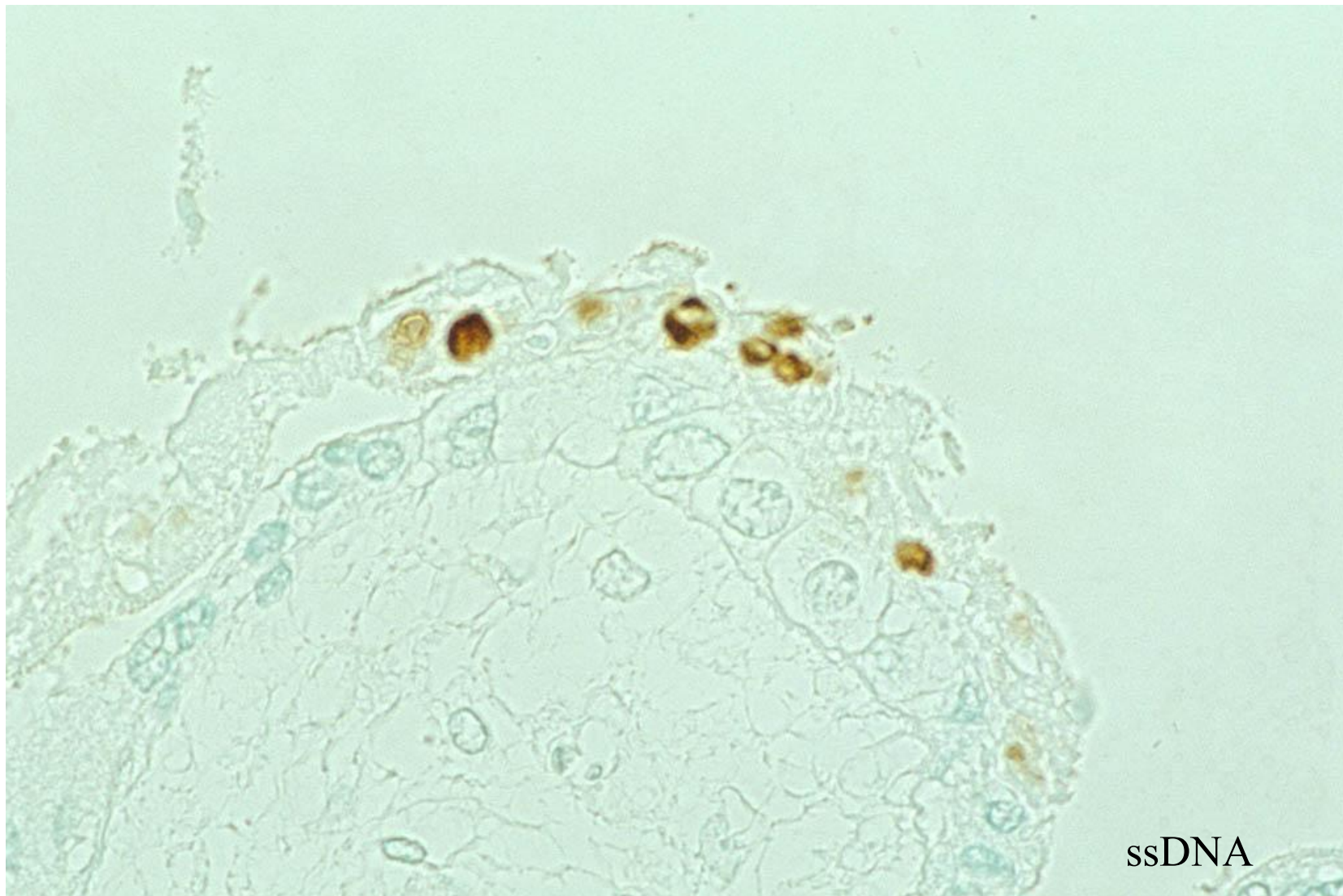
Intranuclear **p53 localization** in colon adenocarcinoma after pressure pan cooking in 10 mM citrate buffer, pH 5~9. pH 6 or 7 is suitable for reproducible visualization of overexpressed p53 protein in the nuclei.



Ki-67 localization in palatine tonsil after heating in 10 mM citrate buffer, pH 6, by using the amino acid polymer method. Both microwaving and pressure pan heating are effective.

Immunohistochemical visualization of apoptosis in formalin-fixed, paraffin-embedded sections

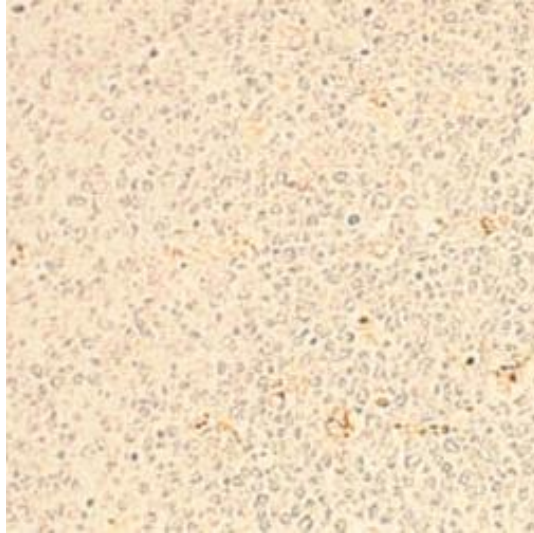
Apoptotic cell death can consistently be visualized in routinely prepared paraffin sections. Recognize the appropriate pretreatments and pitfalls in the immunostaining for apoptosis, as described below.



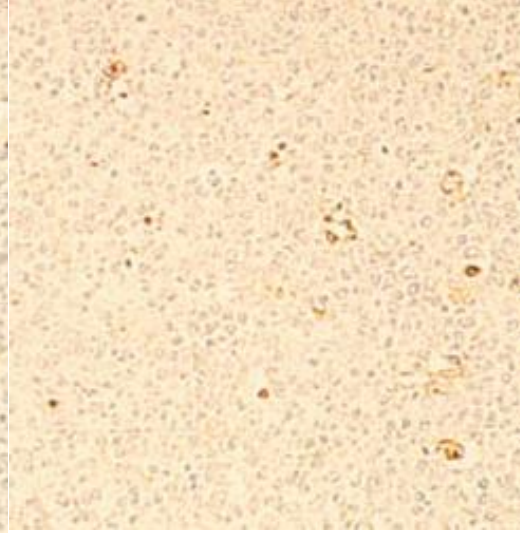
Apoptosis in chorionic villi seen in abortion: immunostaining for single-stranded DNA (ssDNA). Syncytiotrophoblastic cells at the tip of the chorionic villi show apoptosis.

Different effects of pretreatments for ssDNA localization

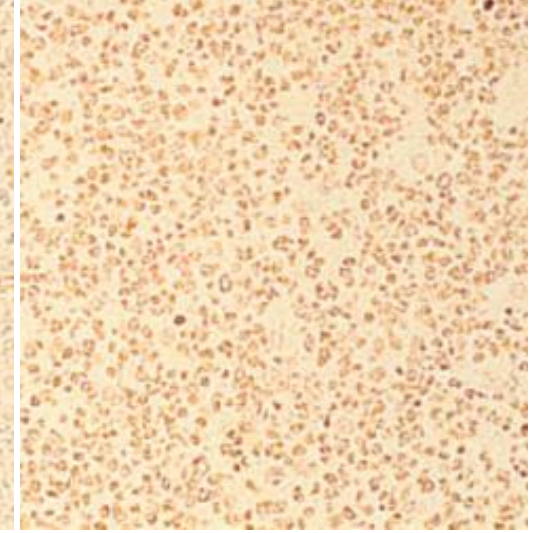
Palatine
tonsil



untreated

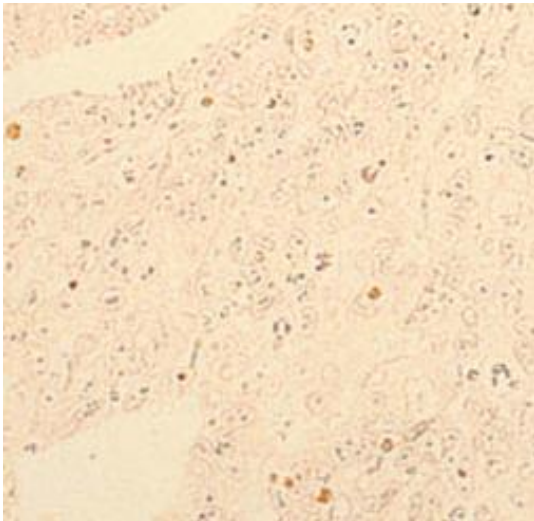


60C 10 min in CB7

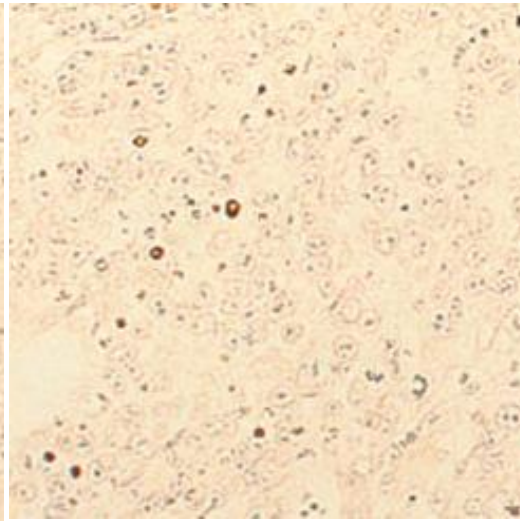


121C 10 min in CB7

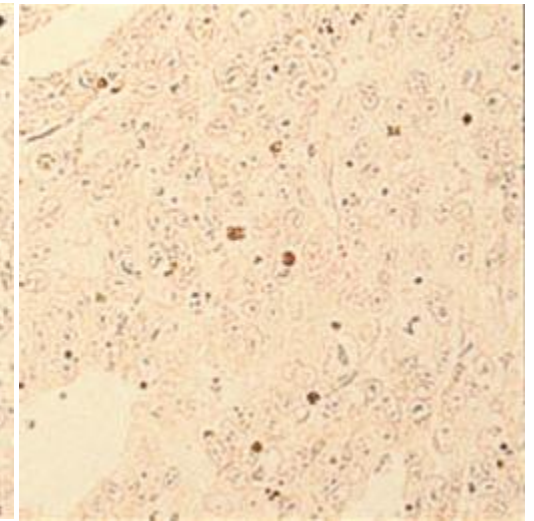
Embryonal
carcinoma



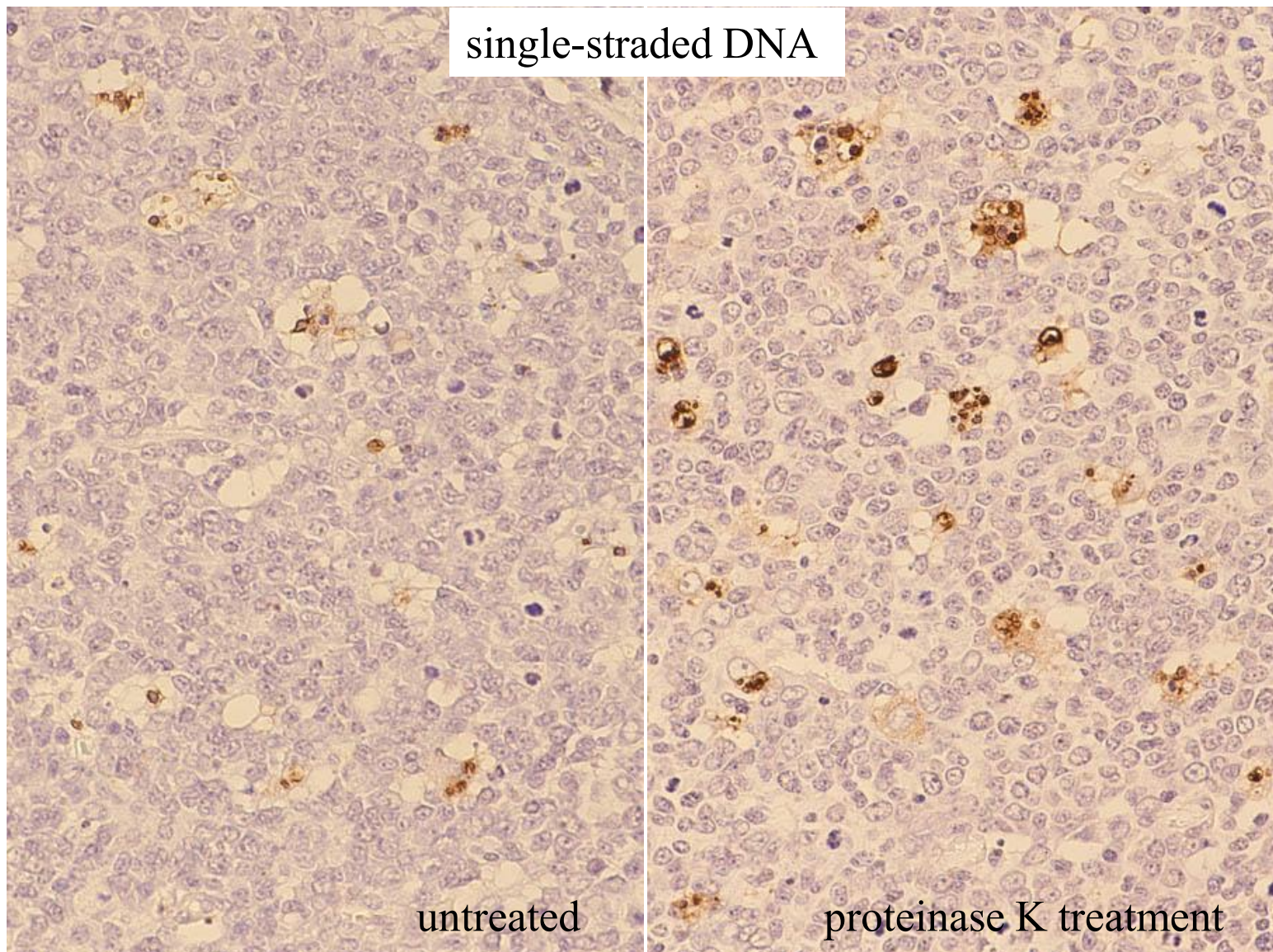
untreated



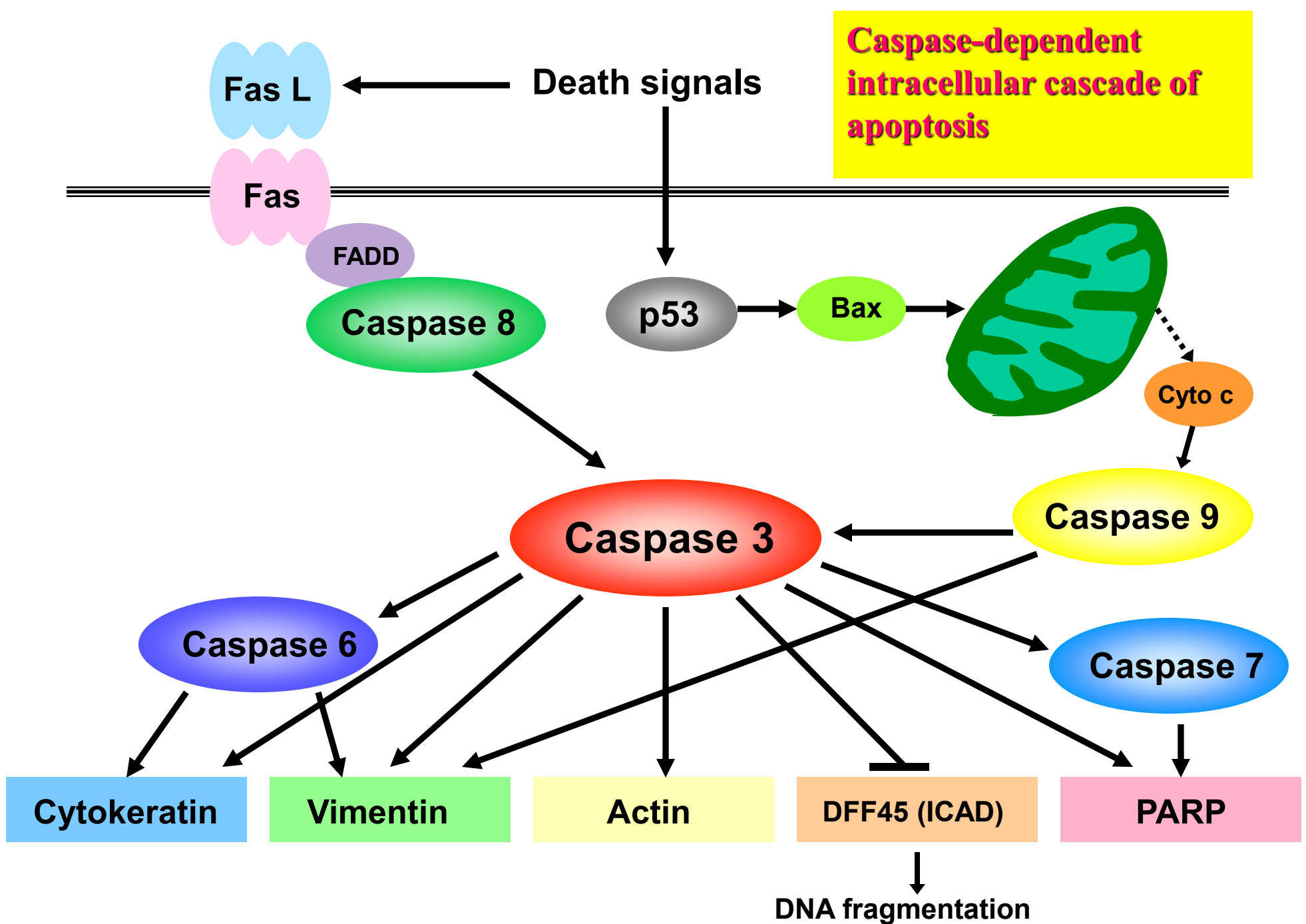
proteinase K



DNase

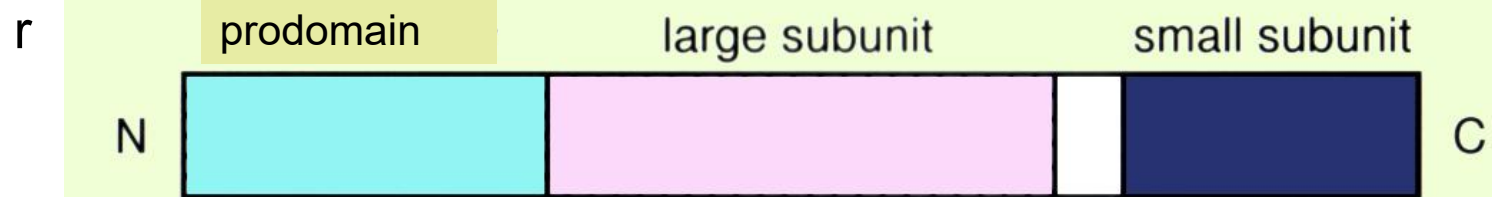


Apoptotic cells in the germinal center of the palatine tonsil, visualized by immunostaining for **single-stranded DNA (ssDNA)**. Proteinase K treatment enhances both the sensitivity and specificity of the immunostaining.

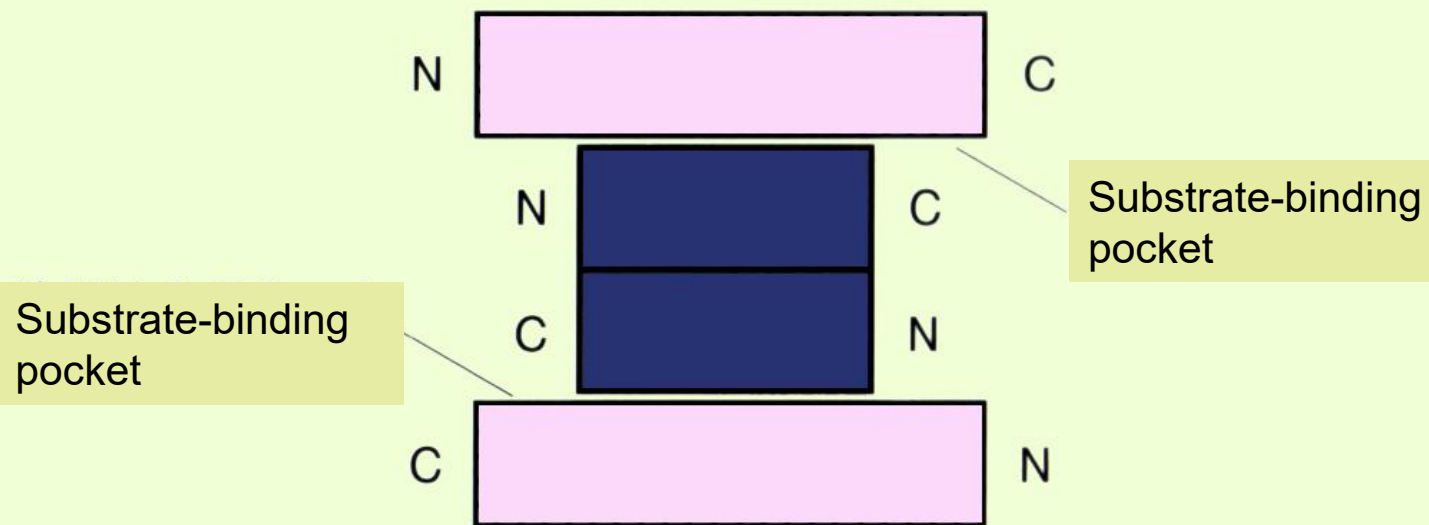


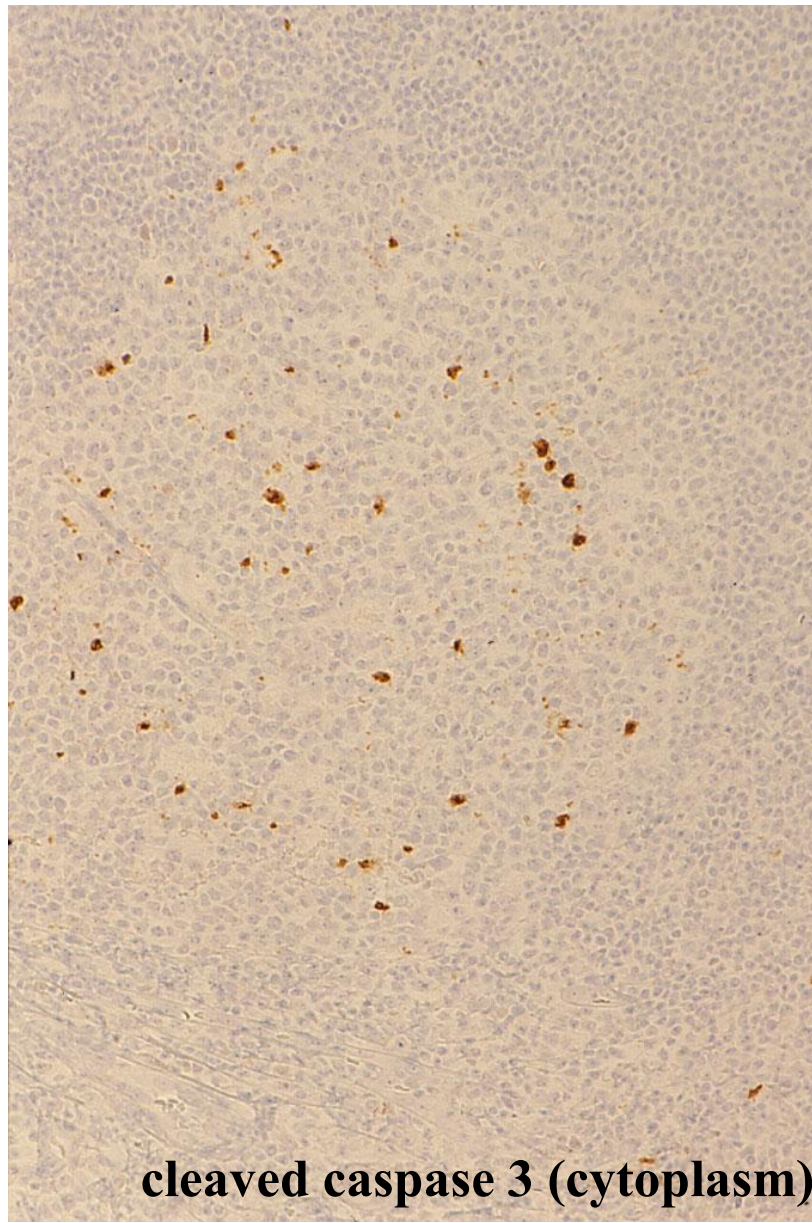
Structure of caspases

Precursor (pro-caspase)

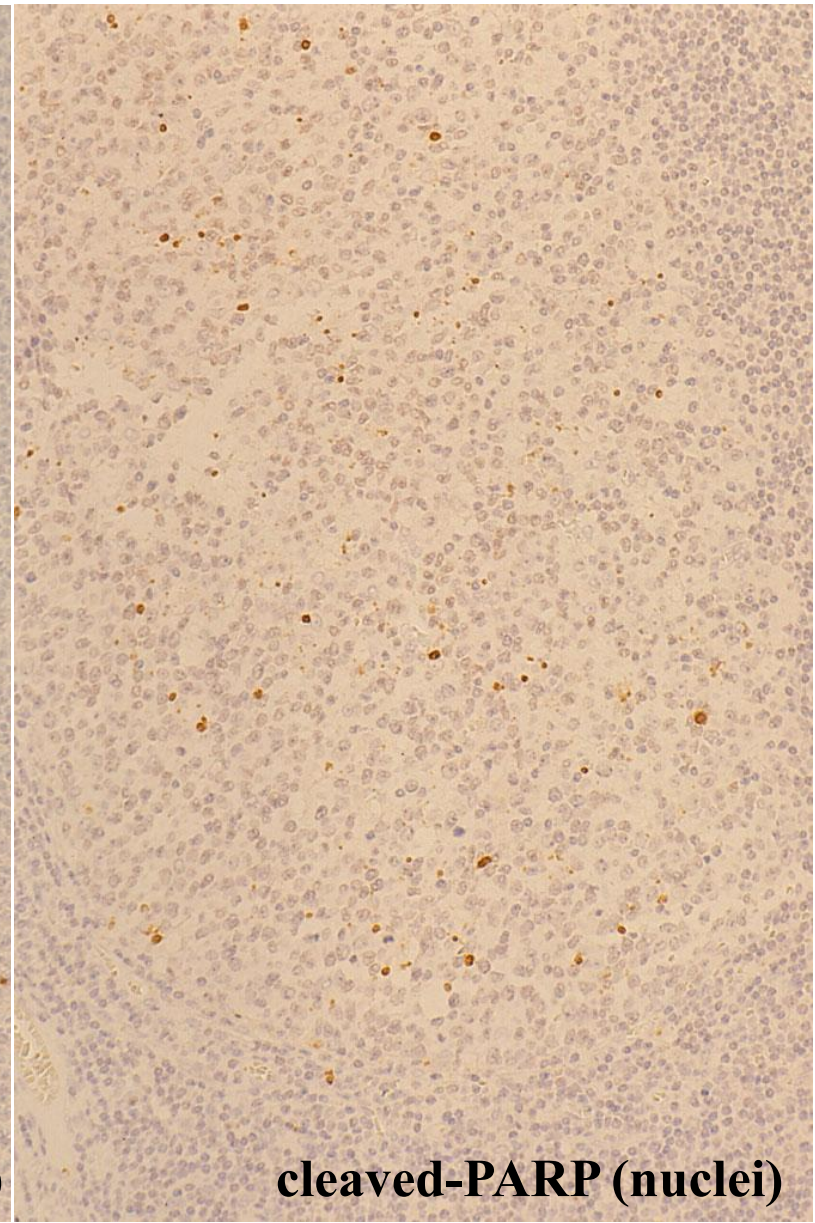


Activated form (active caspase)



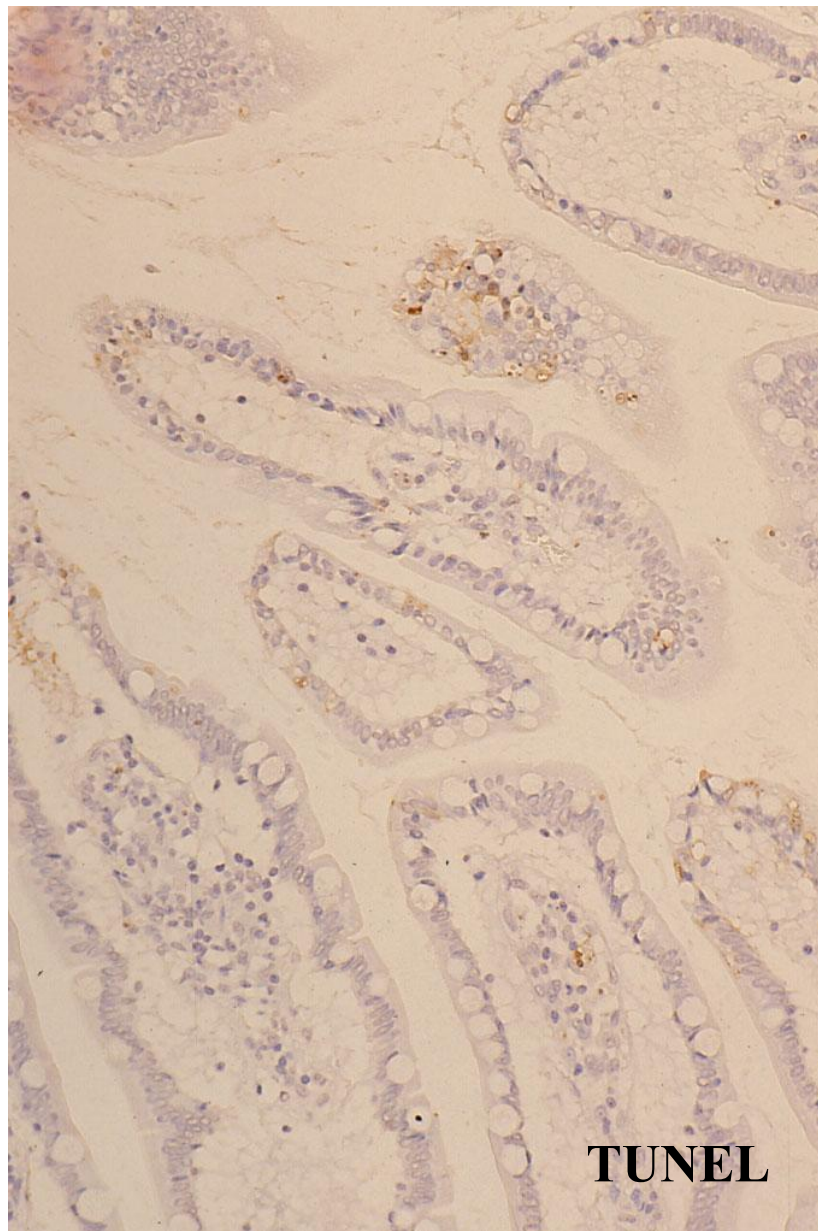


cleaved caspase 3 (cytoplasm)

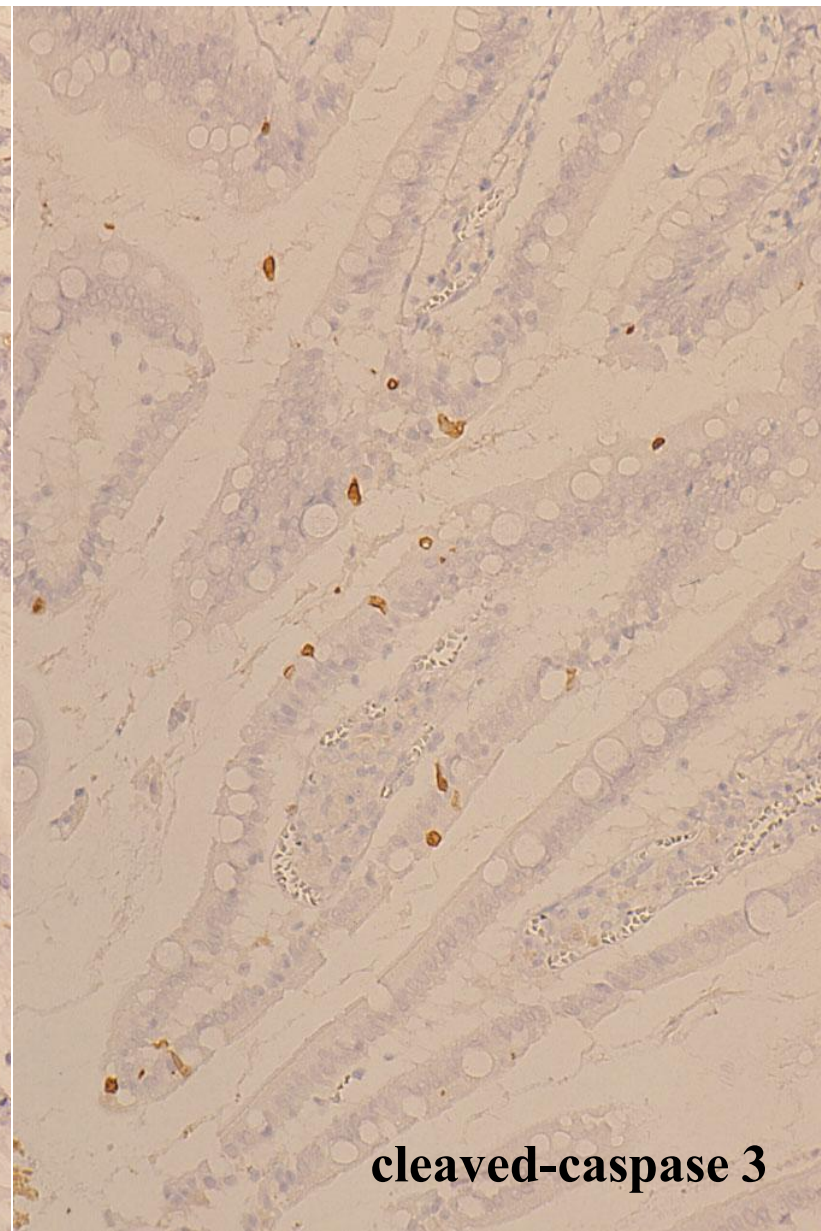


cleaved-PARP (nuclei)

Visualization of apoptosis with immunostaining for cleaved caspase 3 and cleaved PARP in the germinal center of the palatine tonsil. Heating treatment is effective.

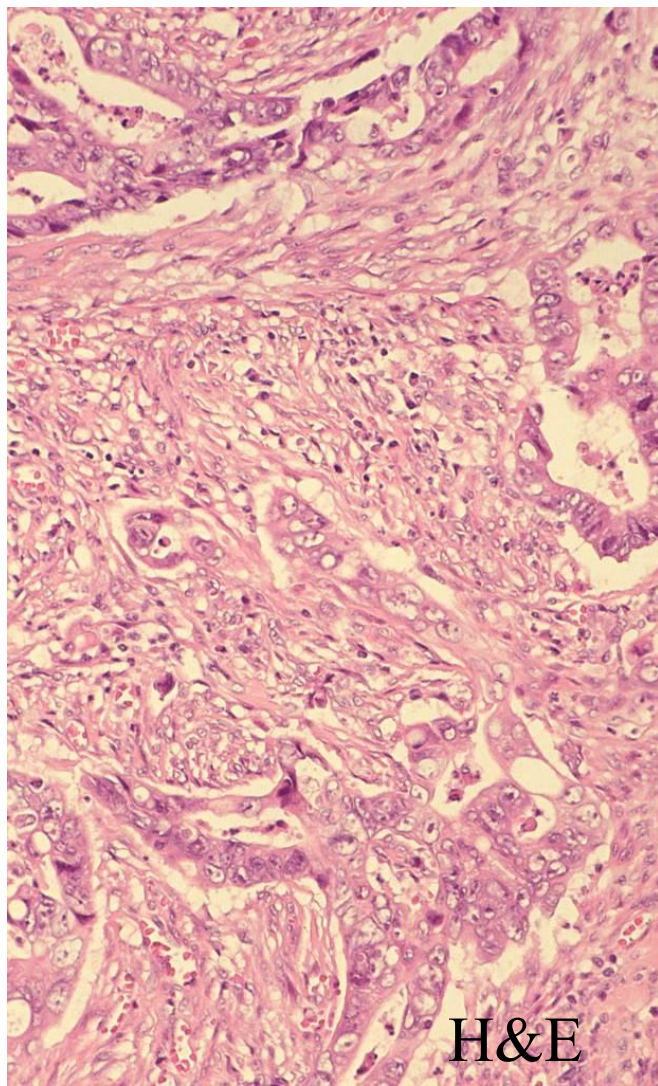


TUNEL

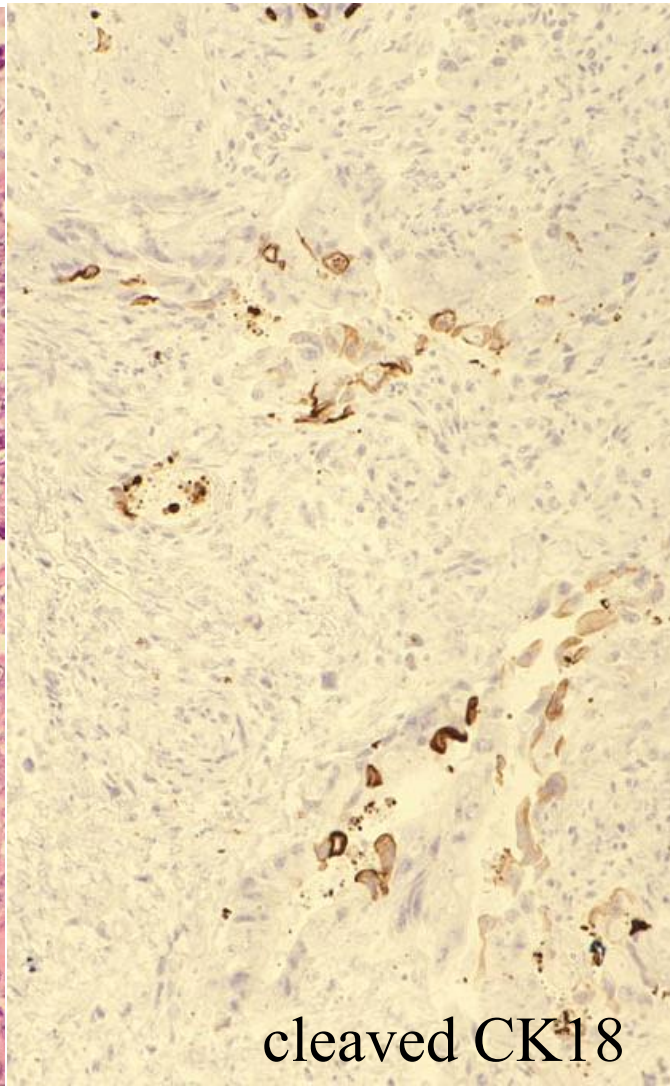


cleaved-caspase 3

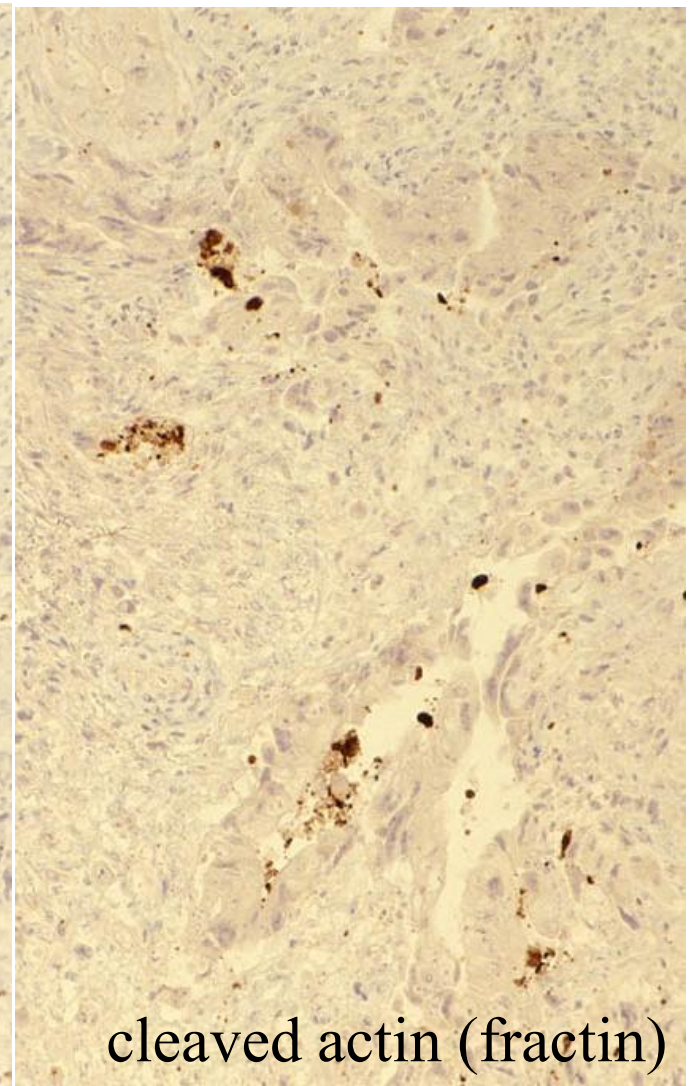
Visualization of apoptosis at the tip of villi of the normal ileal mucosa with TUNEL and immunostaining for cleaved caspase 3.



H&E

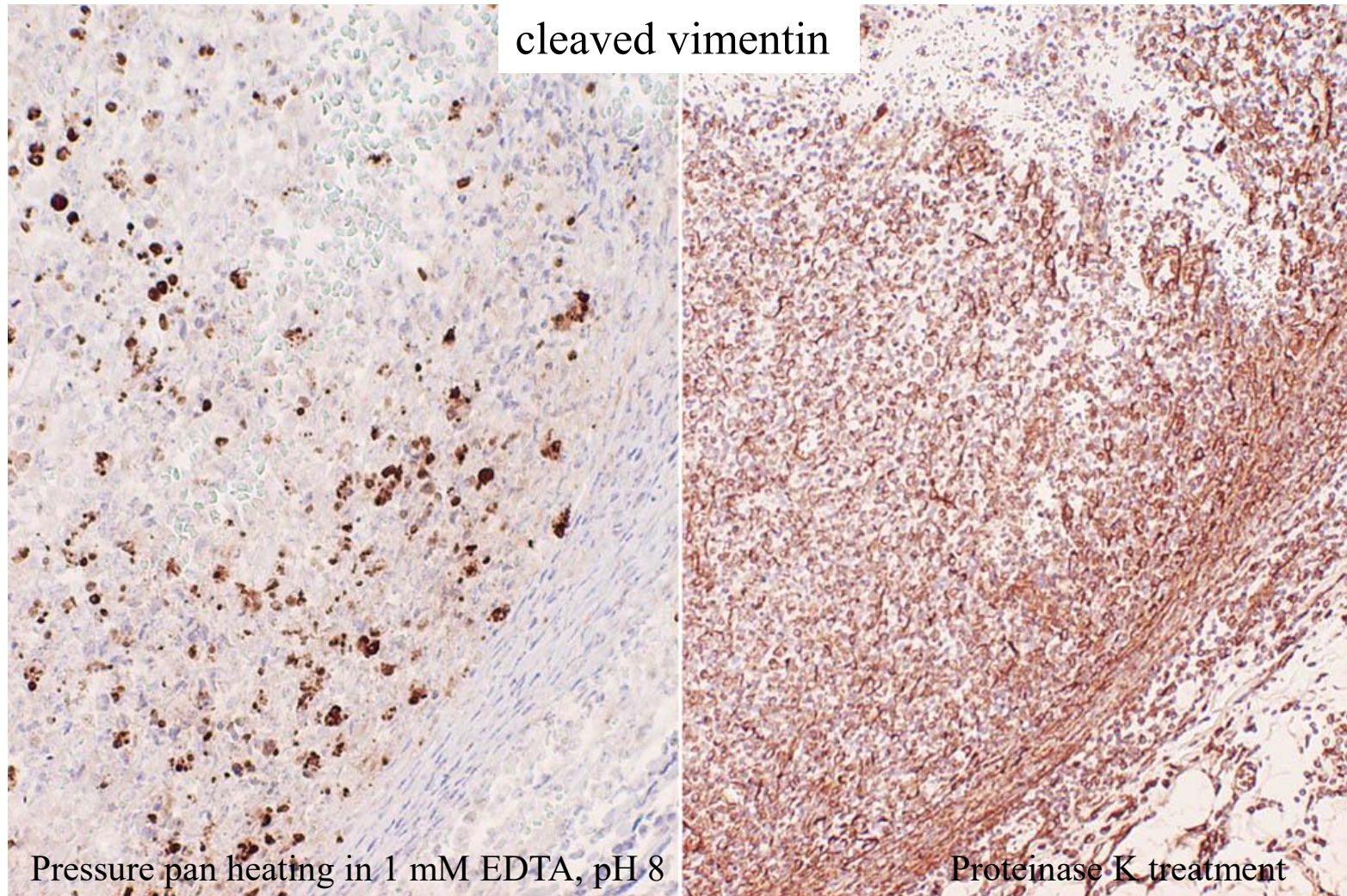


cleaved CK18



cleaved actin (fractin)

Visualization of apoptosis in colon cancer after preoperative 5-FU treatment (pressure pan treatment in EDTA, pH 8). Apoptotic cancer cells are positive for cleaved CK18 (center), while apoptotic inflammatory cells are immunoreactive for cleaved actin (fractin) (right).



Immunostaining for cleaved vimentin for visualizing apoptotic cells in necrotizing lymphadenitis. Pressure pan heating in 1 mM EDTA, pH 8, is effective in visualizing apoptotic cells in necrotizing lymphadenitis, while proteinase K treatment induces a loss of specificity.

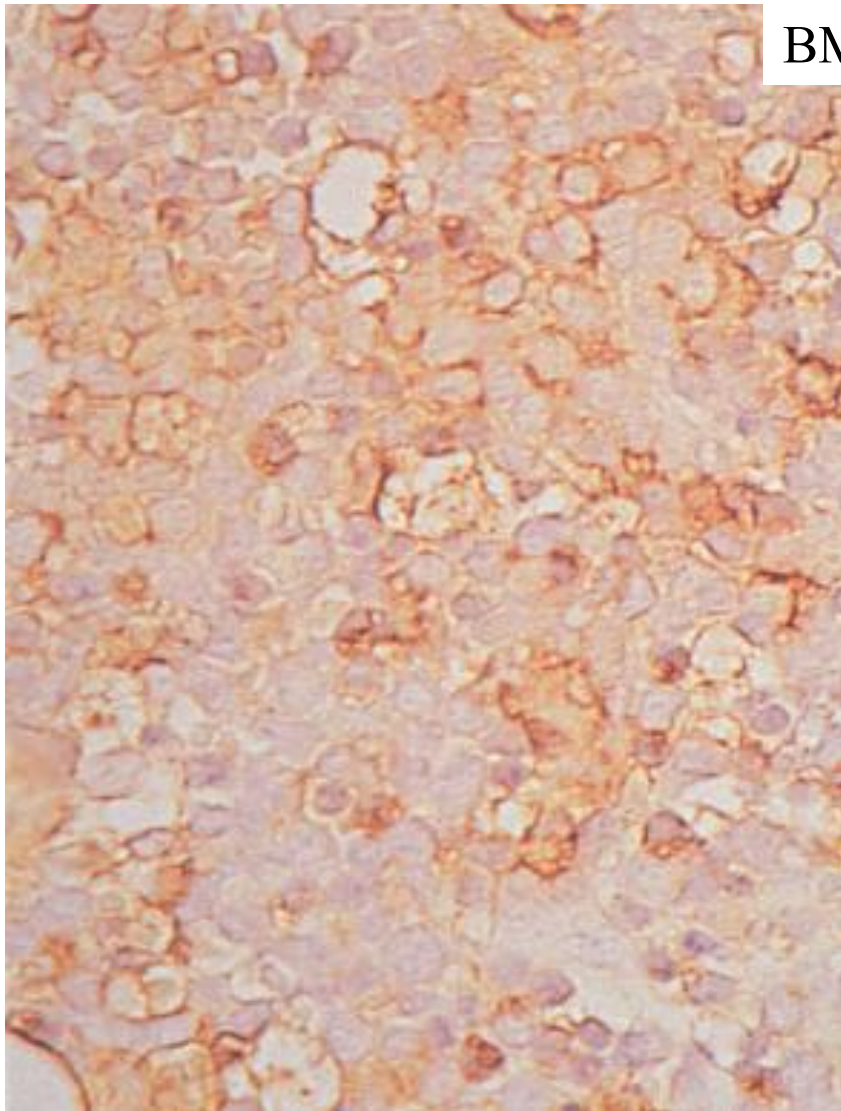
Treatments for increase of sensitivity in apoptosis histochemistry

Method of detection		Treatment for increased sensitivity
TUNEL method		1) Treatment of mung bean nuclease (single-stranded DNA-digesting enzyme) 2) Heating at 120°C for 2–10 min in 10 mM citrate buffer (pH 6.0) or heating at 120°C for 2 min in 1 mM EDTA (pH 8.0)
ISNT method		No report
Single-stranded DNA		Heating at 60°C for 10 min in 10 mM citrate buffer (pH 7.0) Ficin treatment DNase treatment
Cleaved caspases 3&6		Heating at 120°C for 2 min in 1 mM EDTA (pH 8.0)
Cleaved CK18, vimentin		
Cleaved actin (fractin)		
Cleaved PARP		

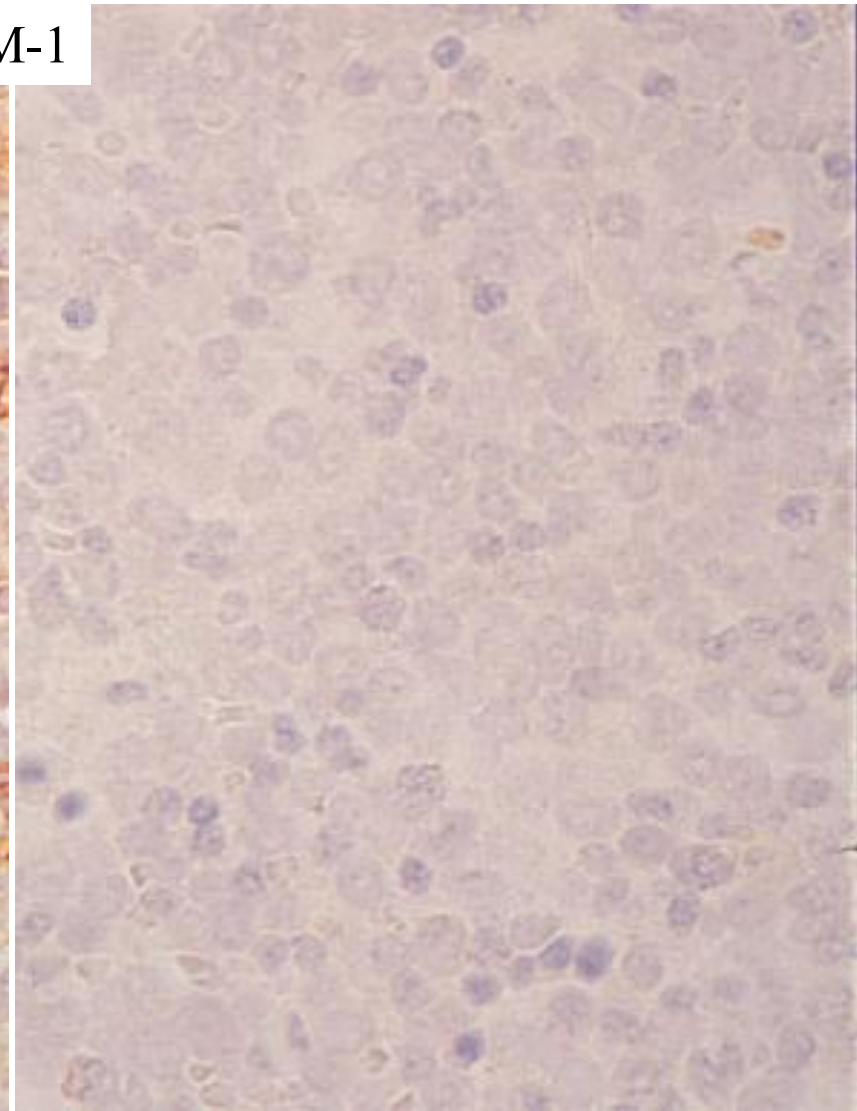
Pitfalls in heating treatment

1. Some antigens lose the antigenicity after heating treatment.
2. Antigenicity weakens when unstained sections are kept in room temperature, particularly true for nuclear antigens.
3. Staining intensity may weaken when antiserum is incubated overnight.
4. Antigenicity may weaken when heated sections are cooled quickly.
5. False negative results may occur when high-molecular weight secondary reagent is used for immunostaining
6. Nuclear antigens such as Ki-67 and progesterone receptor may show unusual localization in the cytoplasm.
7. Heating effect may be seen also in alcohol-fixed cytology specimens.

Negative effect of heating treatment



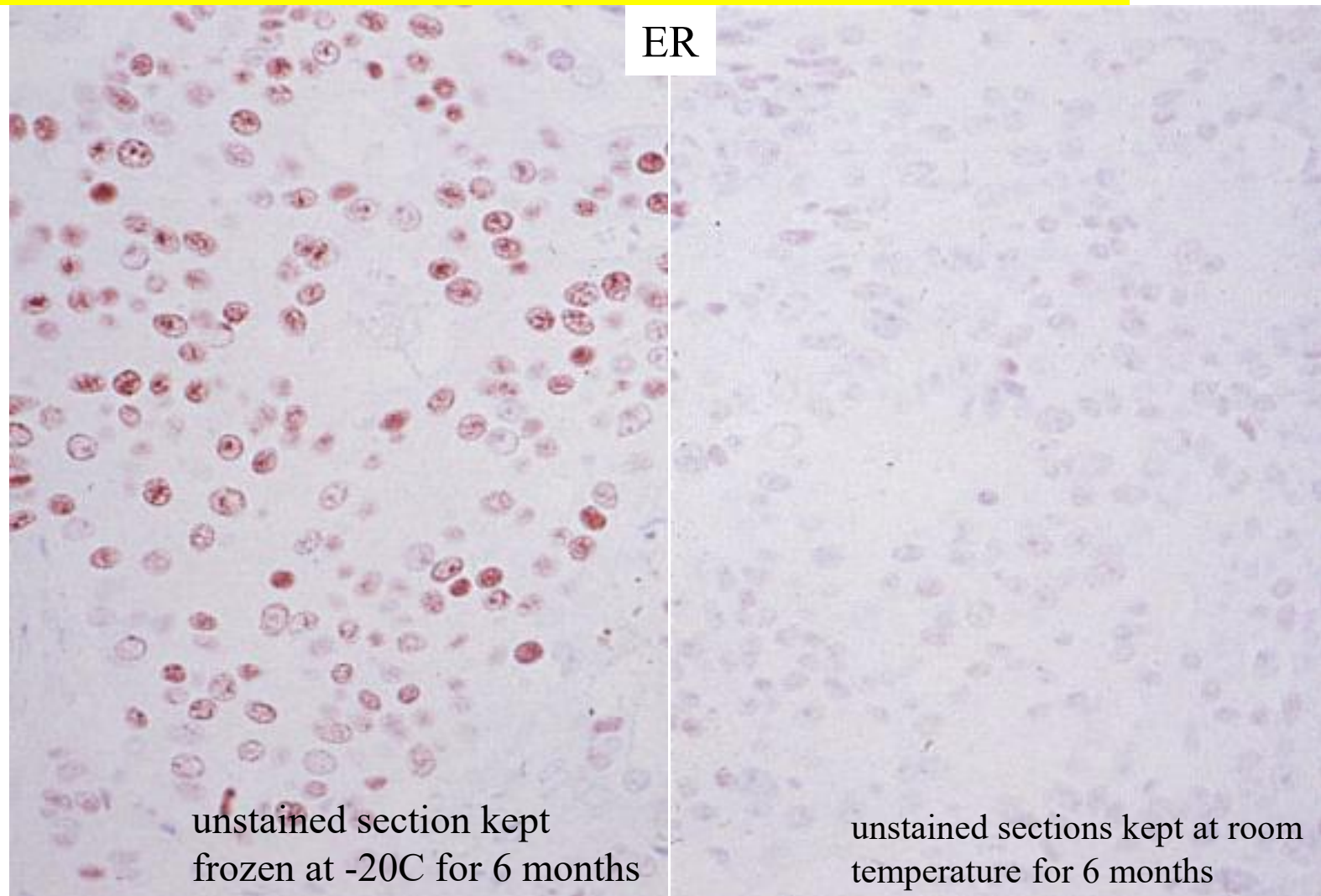
BM-1



Immunostaining for BM-1 (a myeloid marker) . Untreated specimen of bone marrow aspiration

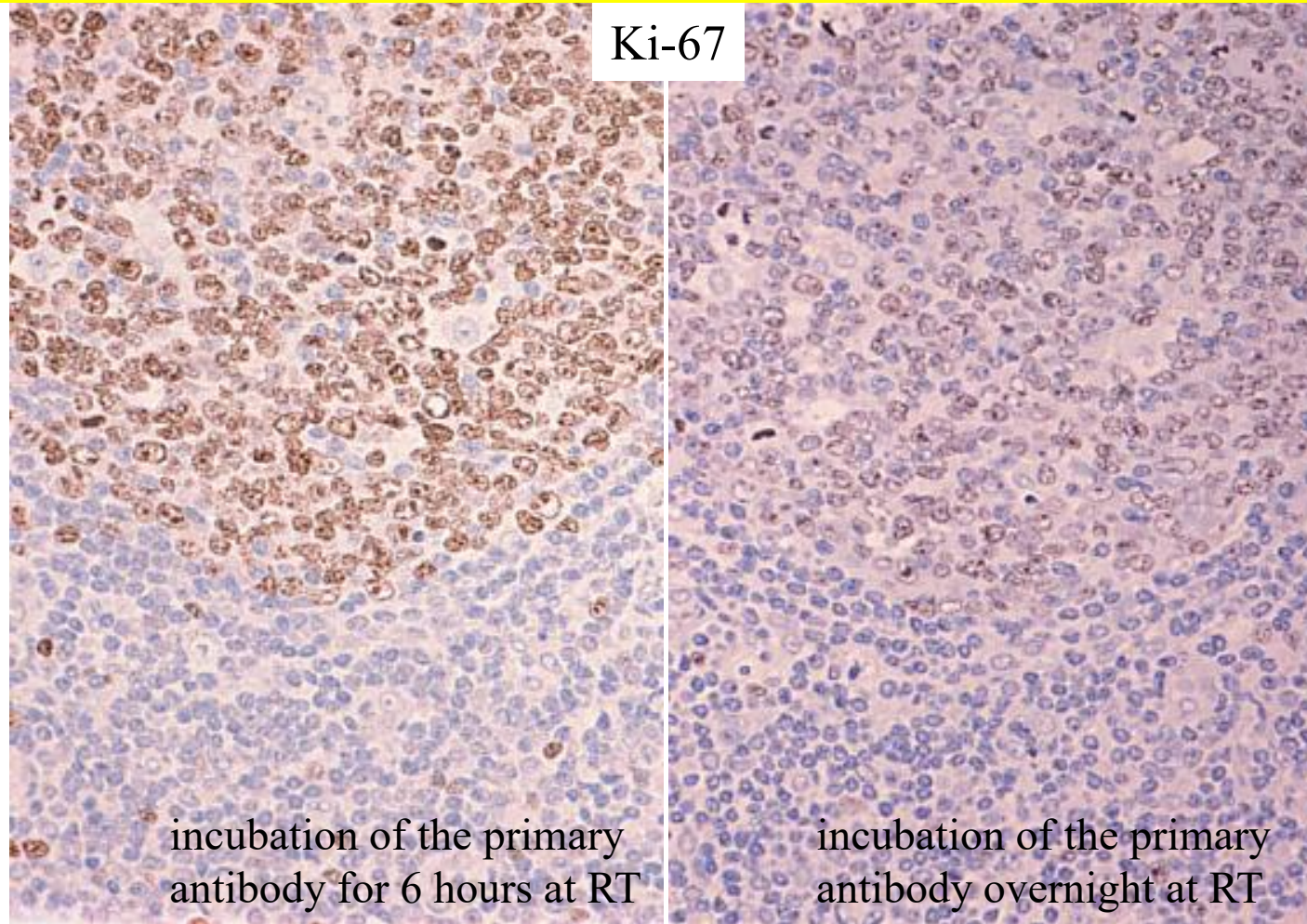
Immunostaining for BM-1 in the bone marrow aspiration after pressure pan heating in 10 mM citrate buffer, pH 6

Immunostaining after long-period storage of paraffin sections

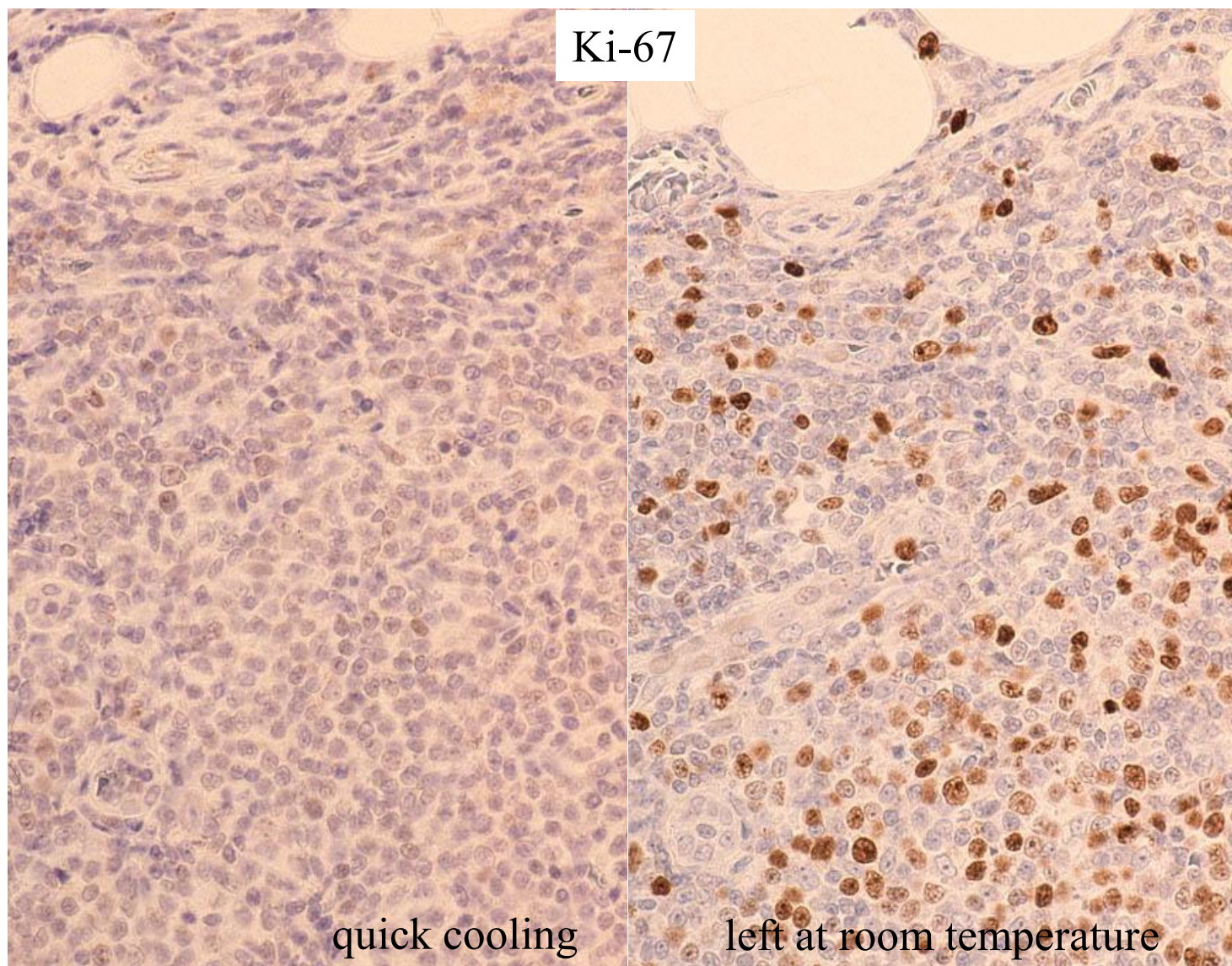


Prolonged storage of unstained paraffin sections. Estrogen receptor (ER) in breast cancer after pressure pan-treatment in 10 mM citrate buffer, pH 7. The storage sections at room temperature for a long time deteriorates immunoreactivity of ER. The paraffin section kept at -20C retains the ER immunoreactivity.

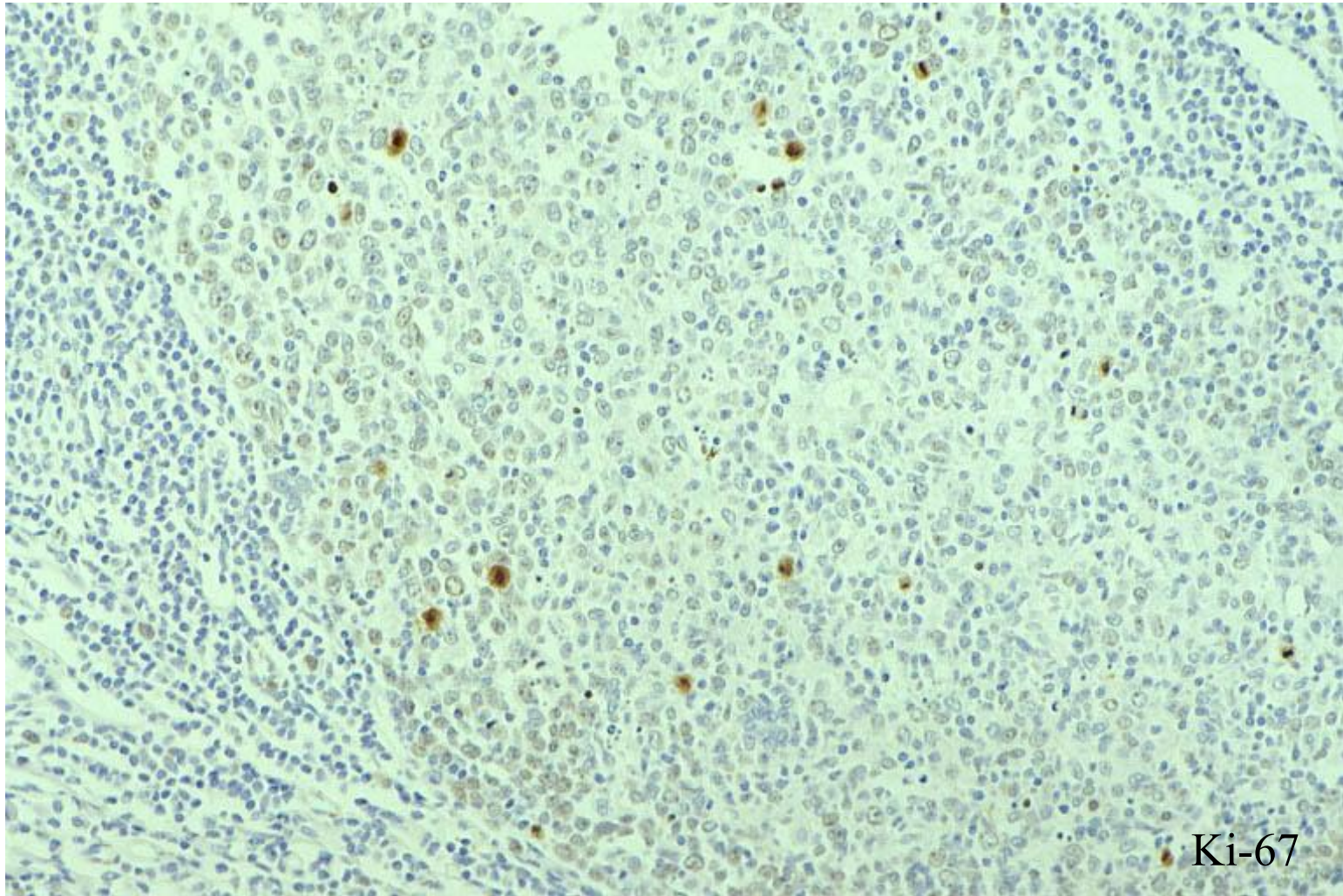
Negative effect of prolonged antibody incubation for Ki-67 immunolocalization



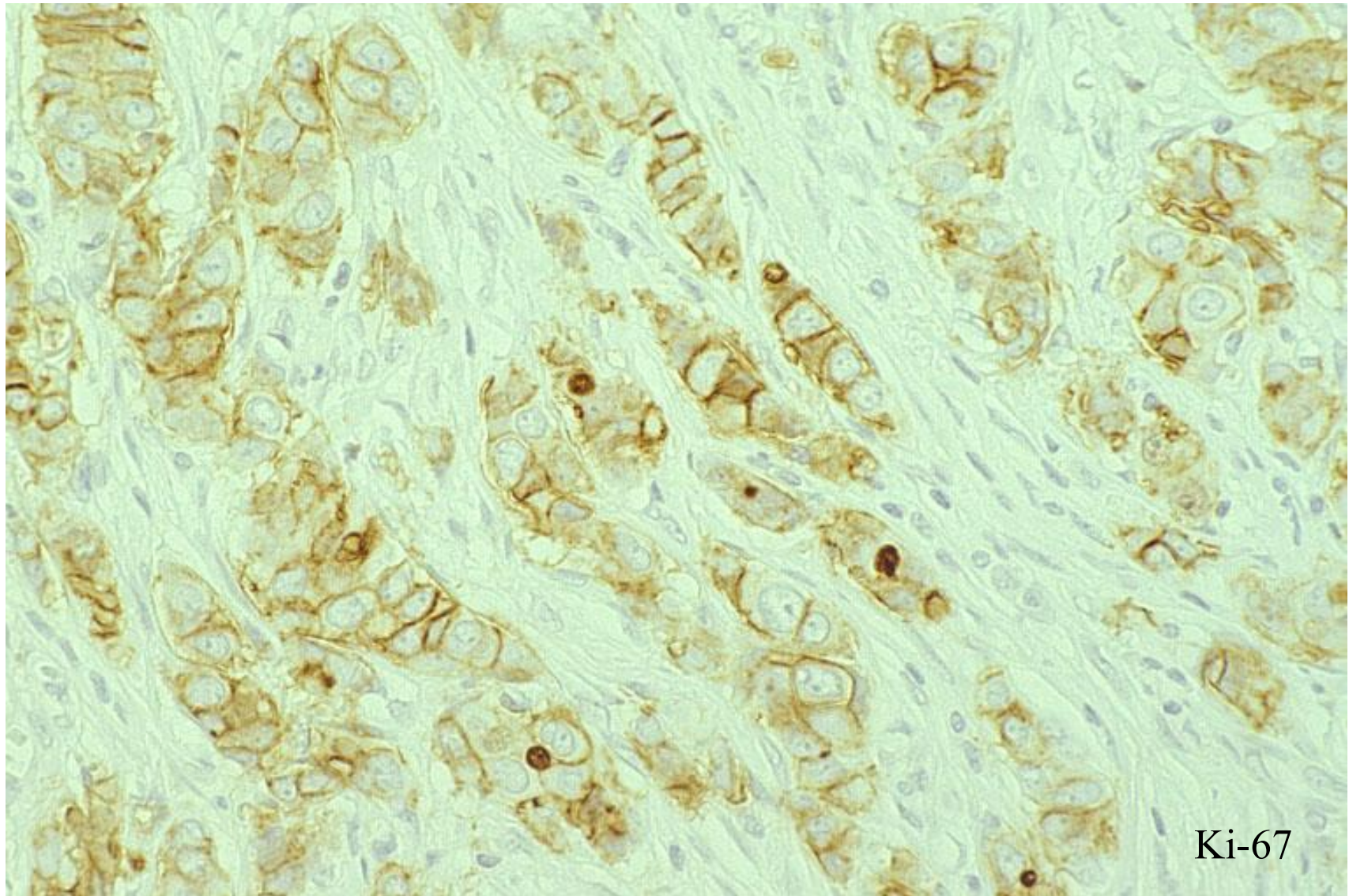
Localization of Ki-67 in the palatine tonsil after water bath heating in 10 mM citrate buffer, pH 6, using the amino acid polymer method. Overnight antibody incubation at room temperature results in marked weakening of Ki-67 immunoreactivity, when compared with 6-hour incubation. During prolonged incubation, single-stranded DNA returns to double-stranded DNA, hampering the access of antibody to the Ki-67 antigen embedded in the DNA structures.



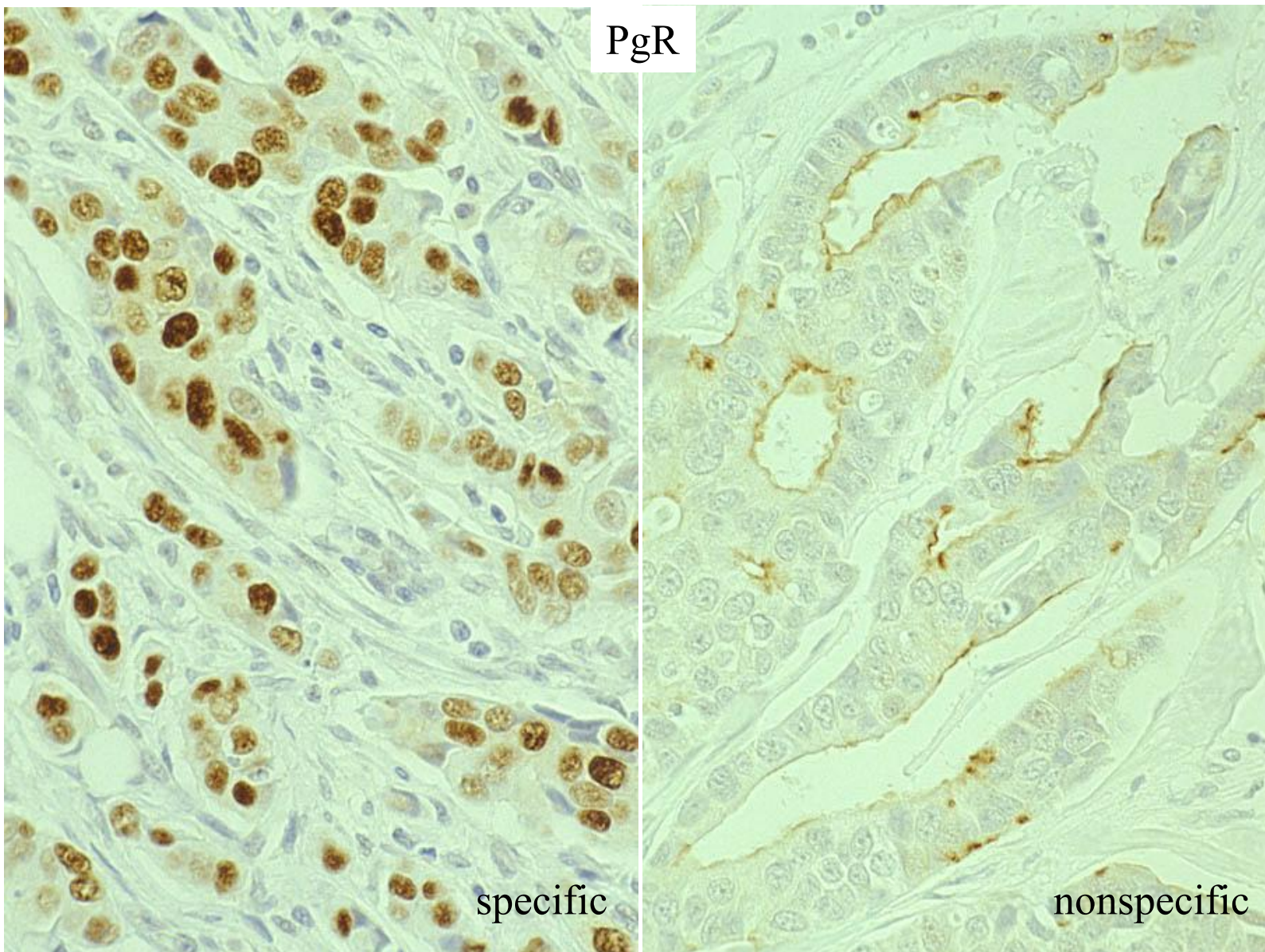
Negative effect of quick cooling after heating: Ki-67 immunostaining in the lymph node). When the heated sections are quickly cooled down in 10 mM phosphate buffer at room temperature, false negativity of Ki-67 is encountered. Quick cooling accelerates reformation of double-stranded DNA in the nuclei.



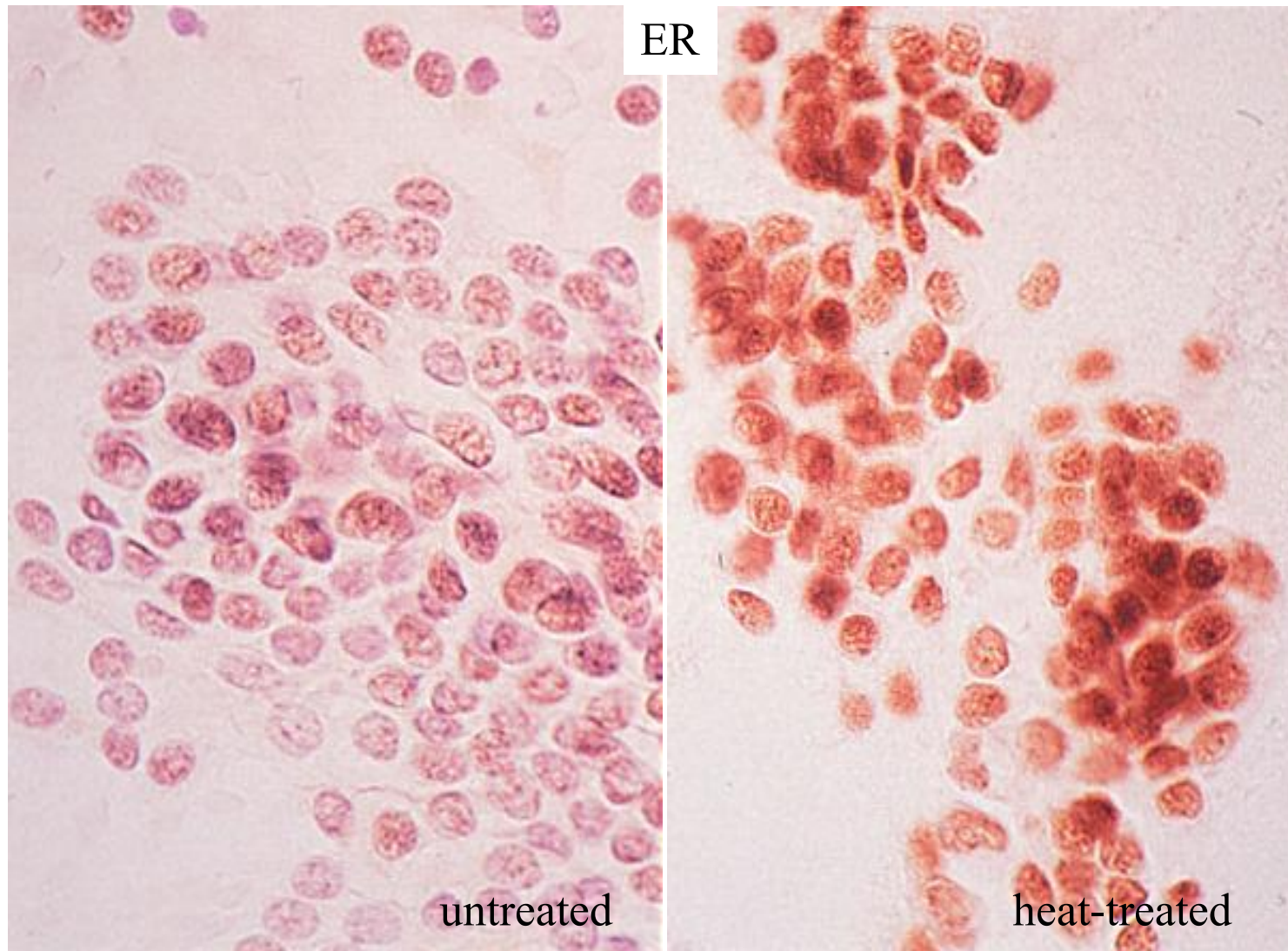
Ki-67 localization in mitotic cells in the germinal center of the palatine tonsil. When the Envision (Dako) is used as the secondary labeled reagent, only mitotic cells may be labeled. Probably because of the large molecular size of the Envision reagent, the this secondary reagent can not penetrate into the intact nuclei. The mitotic cells lack the nuclear membrane to hamper the penetration.



Nonspecific Ki-67 labeling of the plasma membrane. In breast cancer, nonspecific staining of Ki-67 may be seen on the plasma membrane, in addition to low-percentage nuclear labeling.



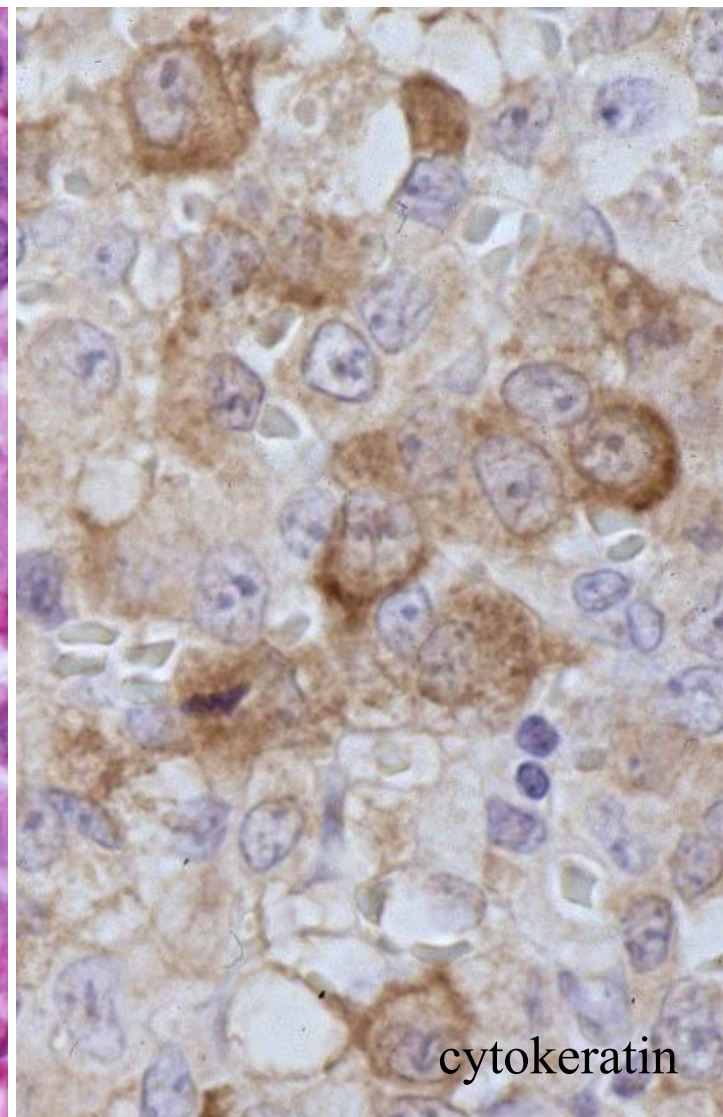
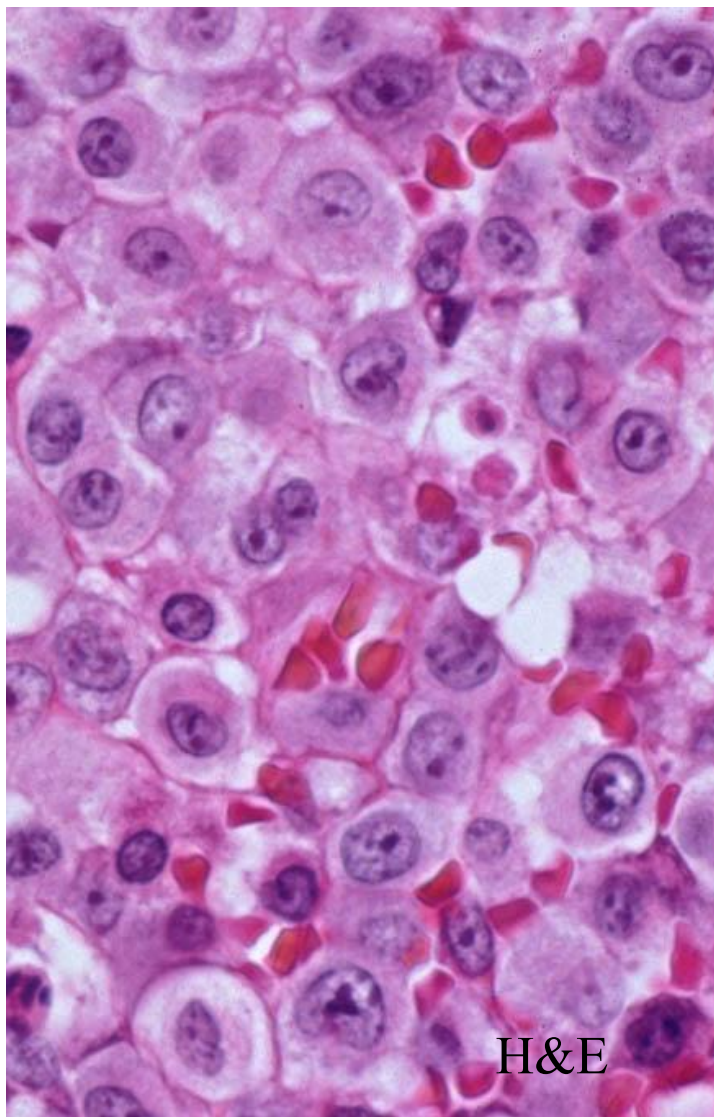
PgR immunoreactivity in breast cancer after pressure pan heating. In addition to specific nuclear labeling (left), nonspecific membrane positivity of PgR is focally observed (right).



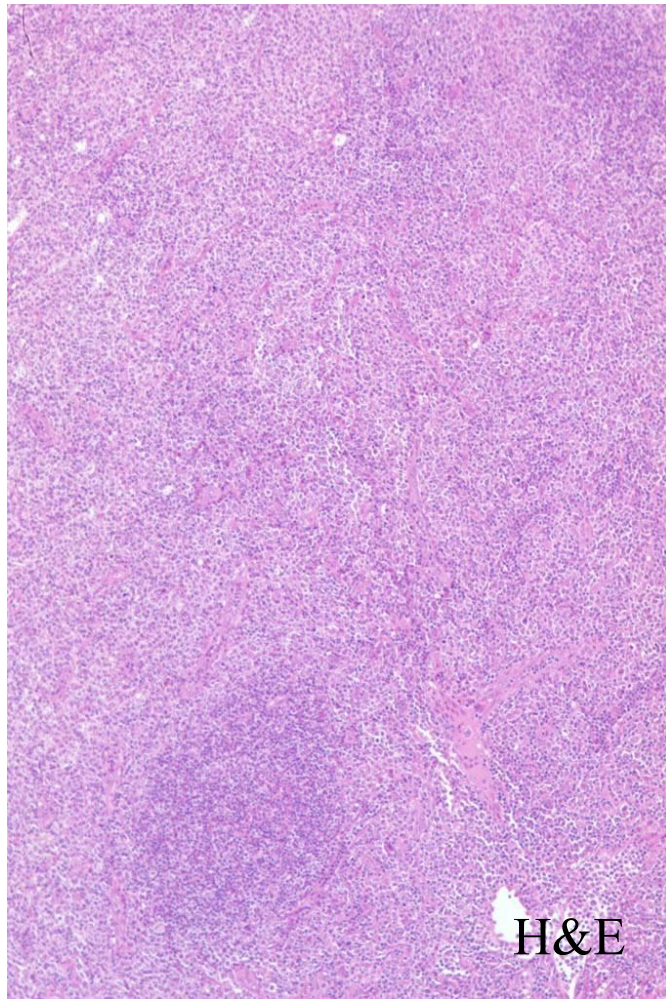
Heating effect in cytology specimens: estrogen receptor (ER) in breast cancer. Heating in 10 mM citrate buffer, pH 7, enhances the immunoreactivity of ER in the cytology specimen demonstrated by monoclonal antibody 6F11.

Judgment of results of immunostaining

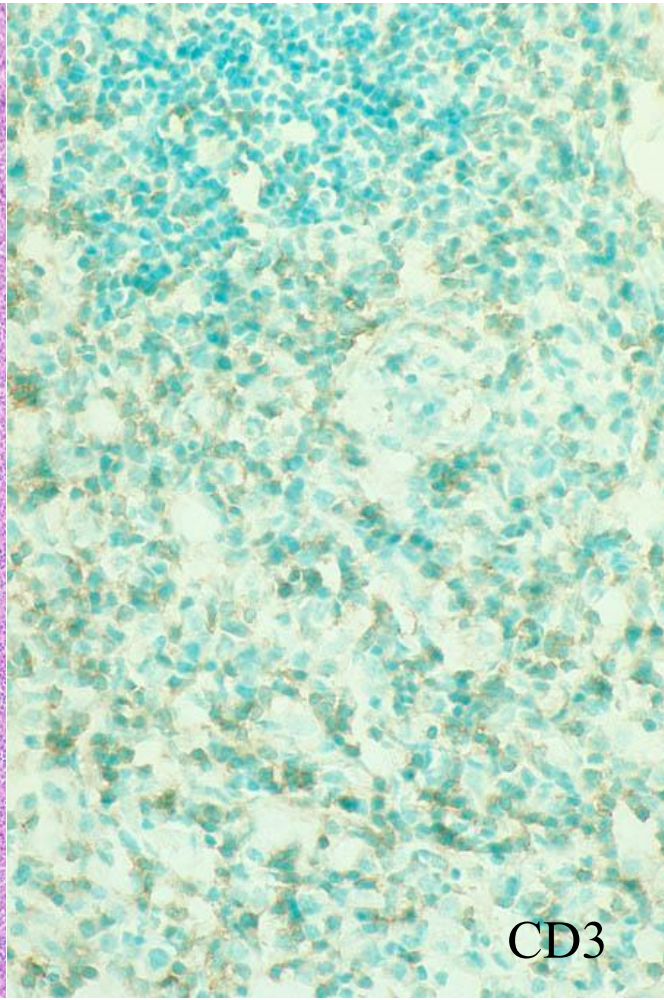
1. False negativity and equivocal staining.
2. Judgment of the specificity of immunostaining
 - a) Proper intracellular localization of the antigen
 - b) Specificity of the antibody
 - c) Nonspecific adsorption of IgG by certain cells, such as mast cells, parietal cells, endocrine cells, Purkinje cells and HBV-infected hepatocytes
3. When unexpected results are obtained.



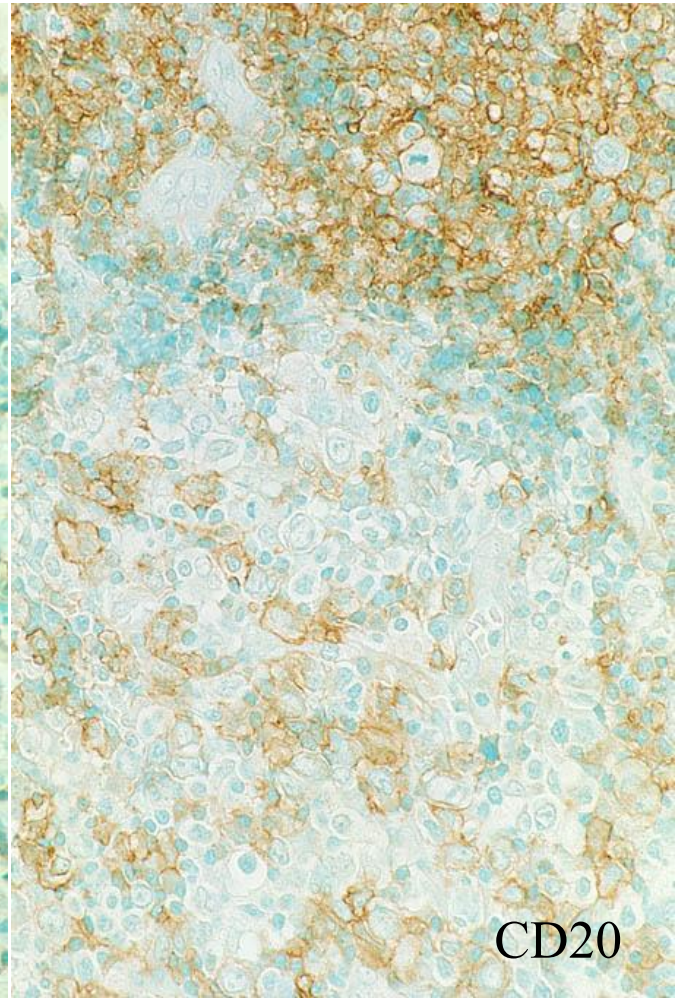
Cytokeratin expression in Ki-1 lymphoma (anaplastic large cell lymphoma) detected with a cytokeratin antiserum. Cytokeratin expression and epithelioid appearance of this CD30-positive lymphoma necessitate the differential diagnosis from metastatic carcinoma.



H&E

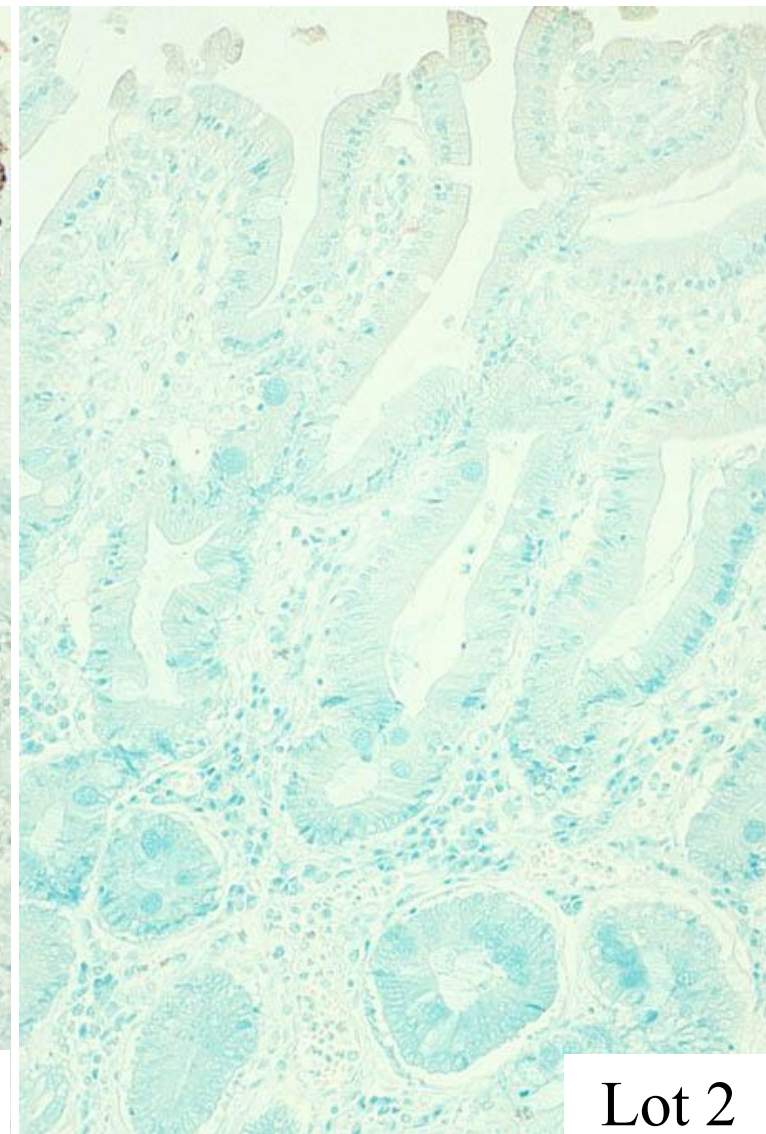
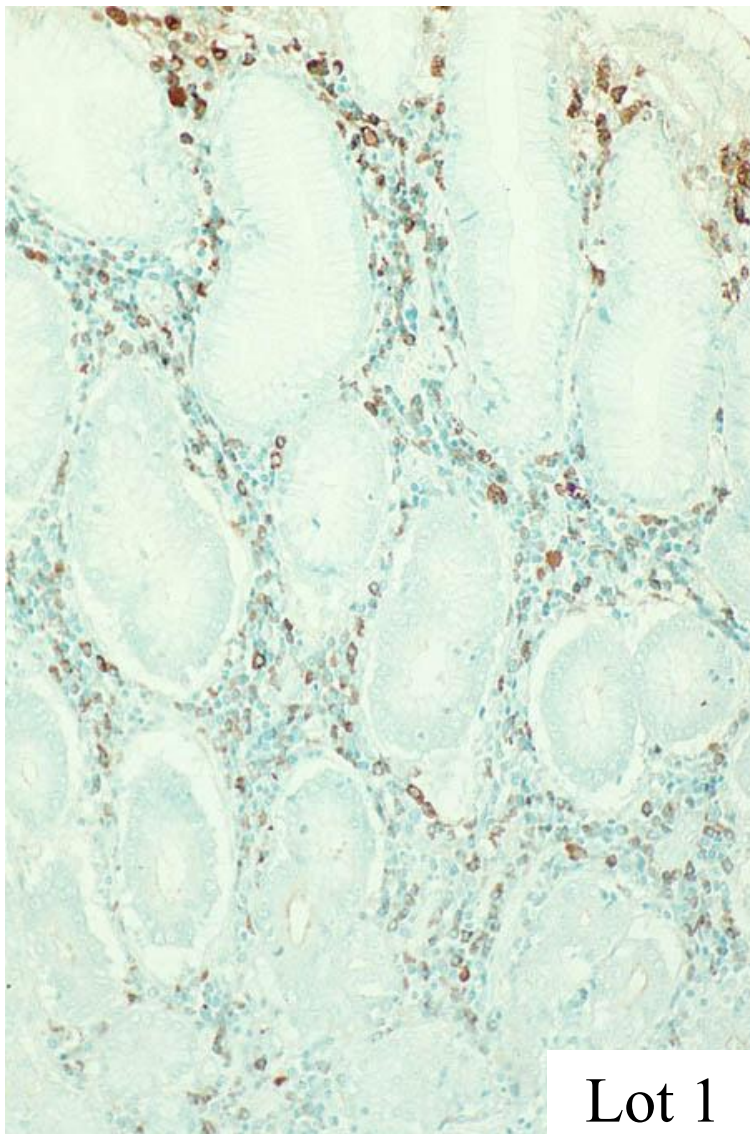


CD3

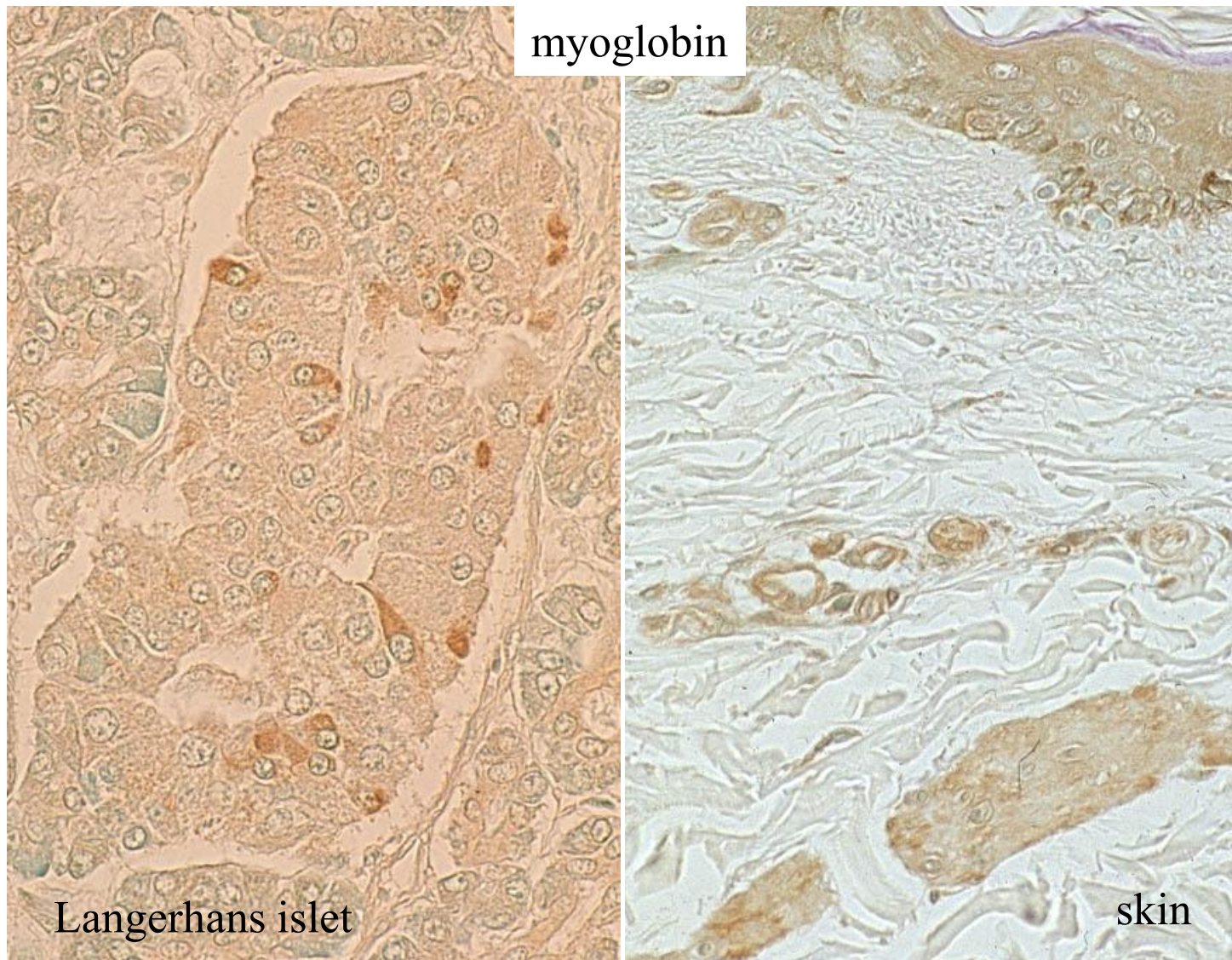


CD20

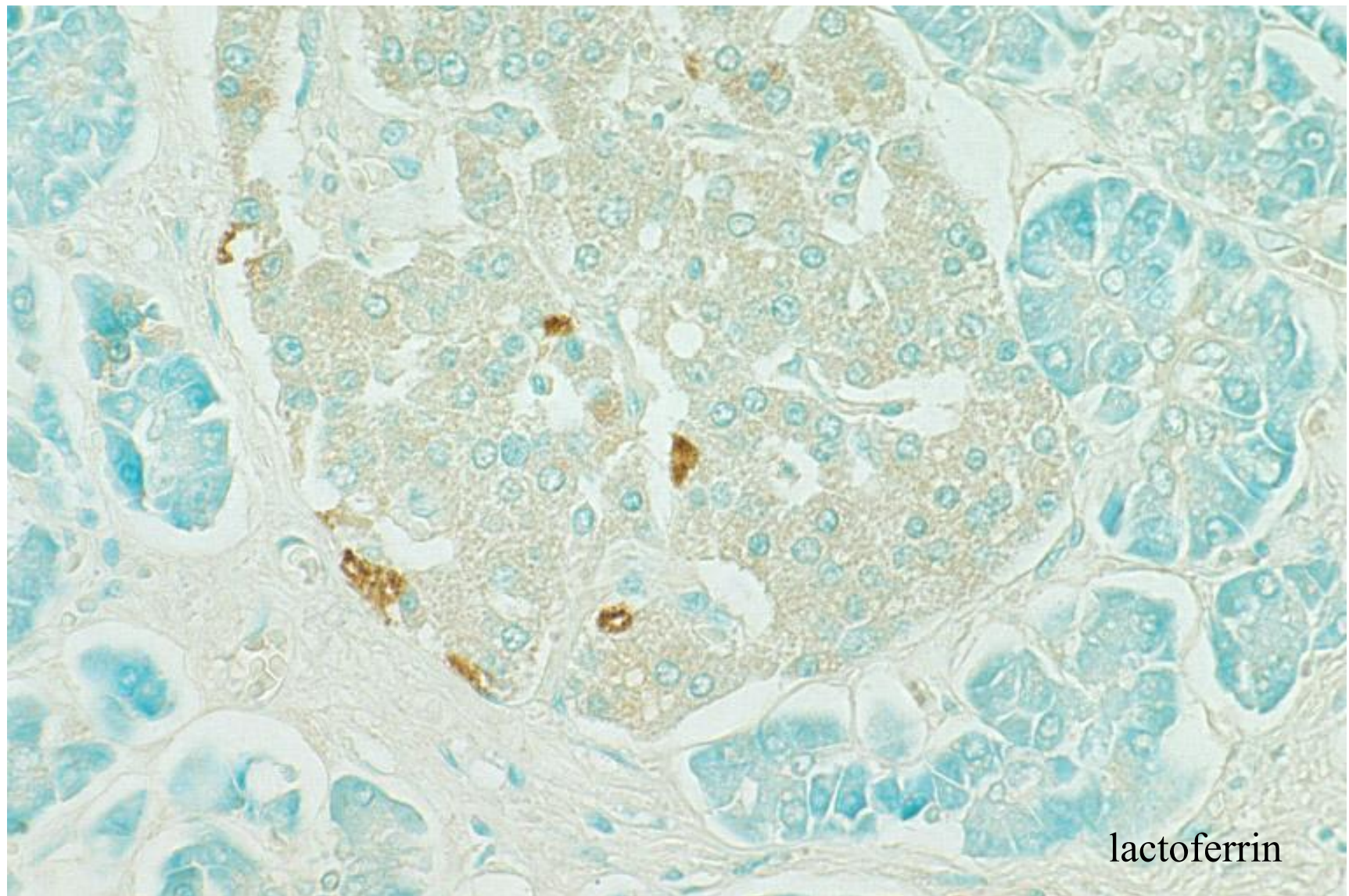
B-cell lymphoma involving the parafollicular zone of the lymph node. Numbers of CD3-positive small T-lymphocytes are observed, while a smaller number of large-sized CD20-positive B-lymphoma cells are distributed in the parafollicular zone. The judgment of the lymphoma subtype should not be confused.



IgD localization in the gastric mucosa. IgD antiserum Lot 1 demonstrates numbers of plasma cells in the lamina propria, while the other IgD antiserum Lot 2 fails to demonstrate IgD plasma cells in the gastric mucosa. Contamination of light chain reactivity should be considered in Lot 1.

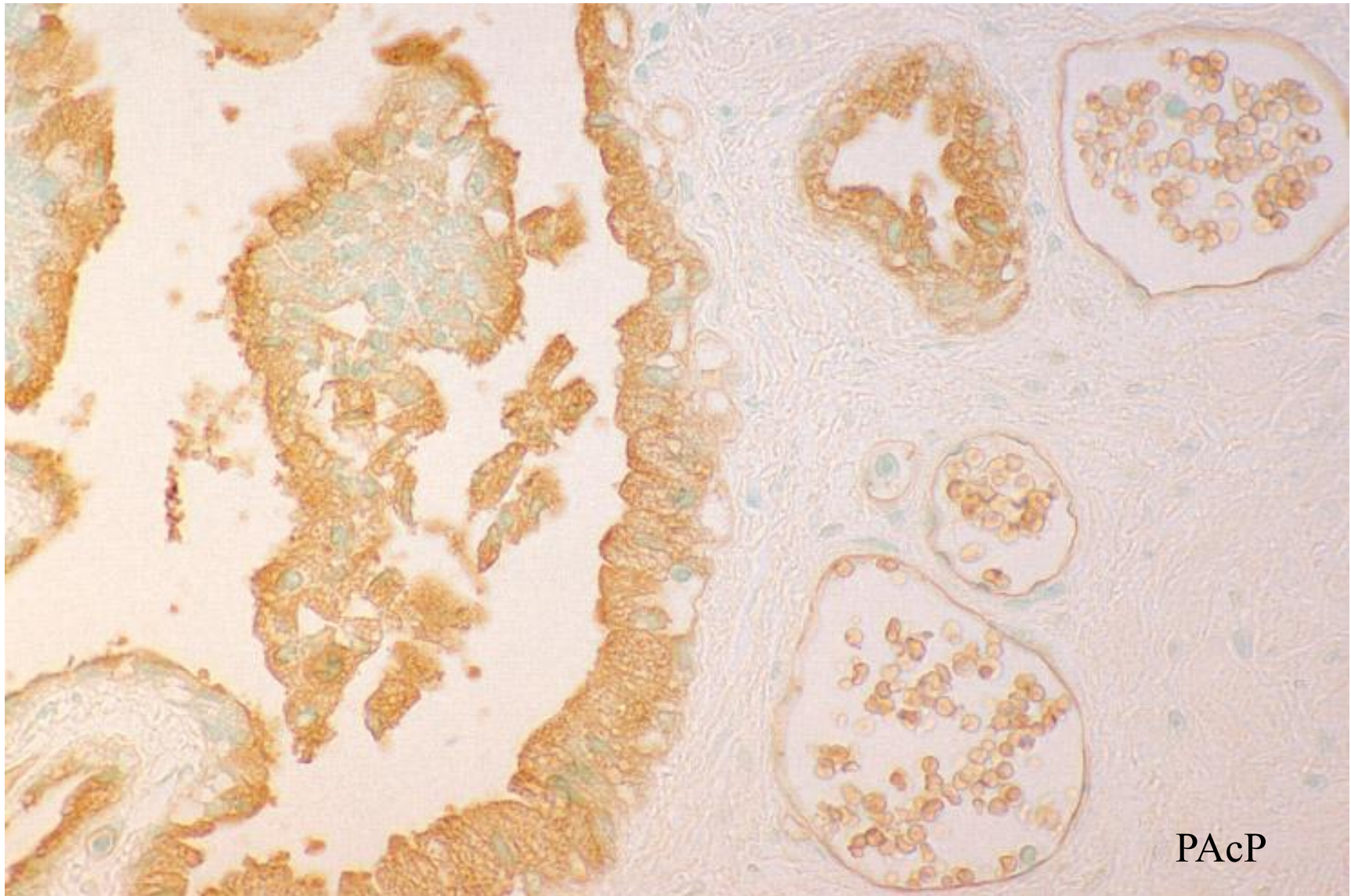


Nonspecific staining using myoglobin antiserum. Some of the cells in Langerhans islets of the pancreas and endothelial cells and erector pili in the skin show nonspecific reactivity. This must be caused by contaminating anti-intermediate filament activity (natural antibody) in the rabbit serum.



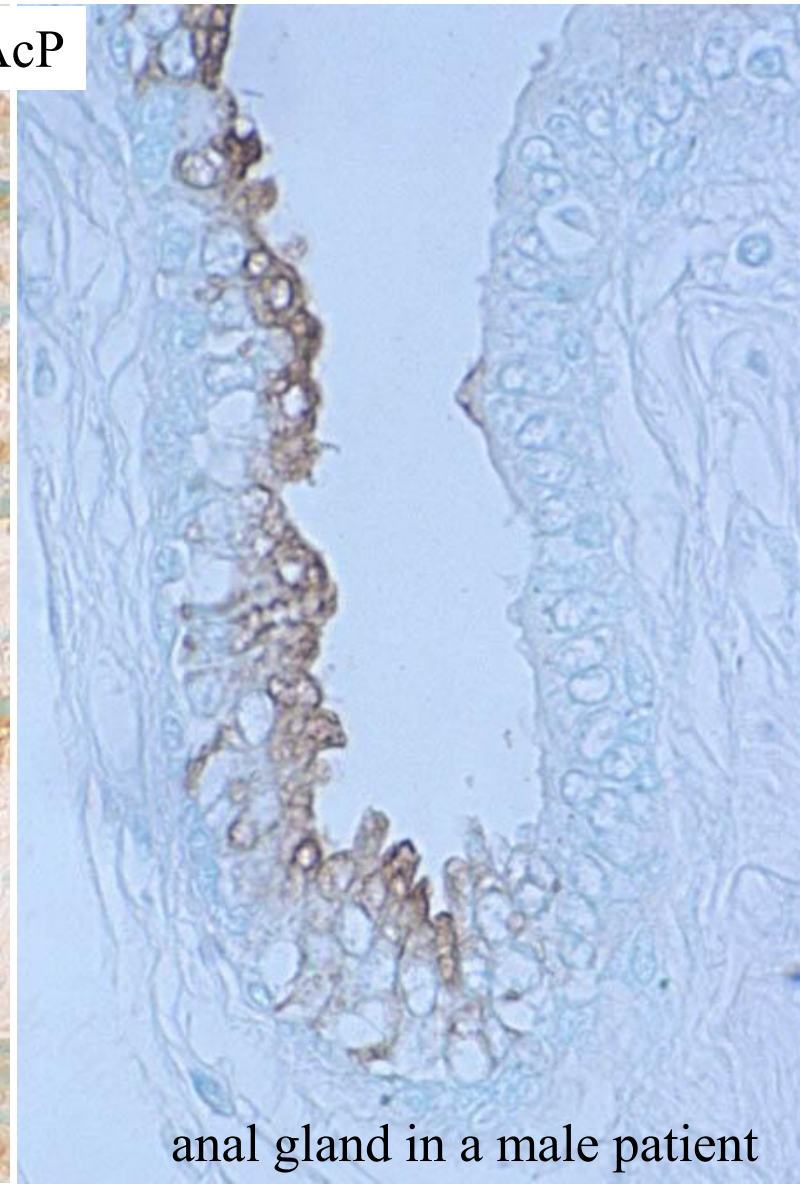
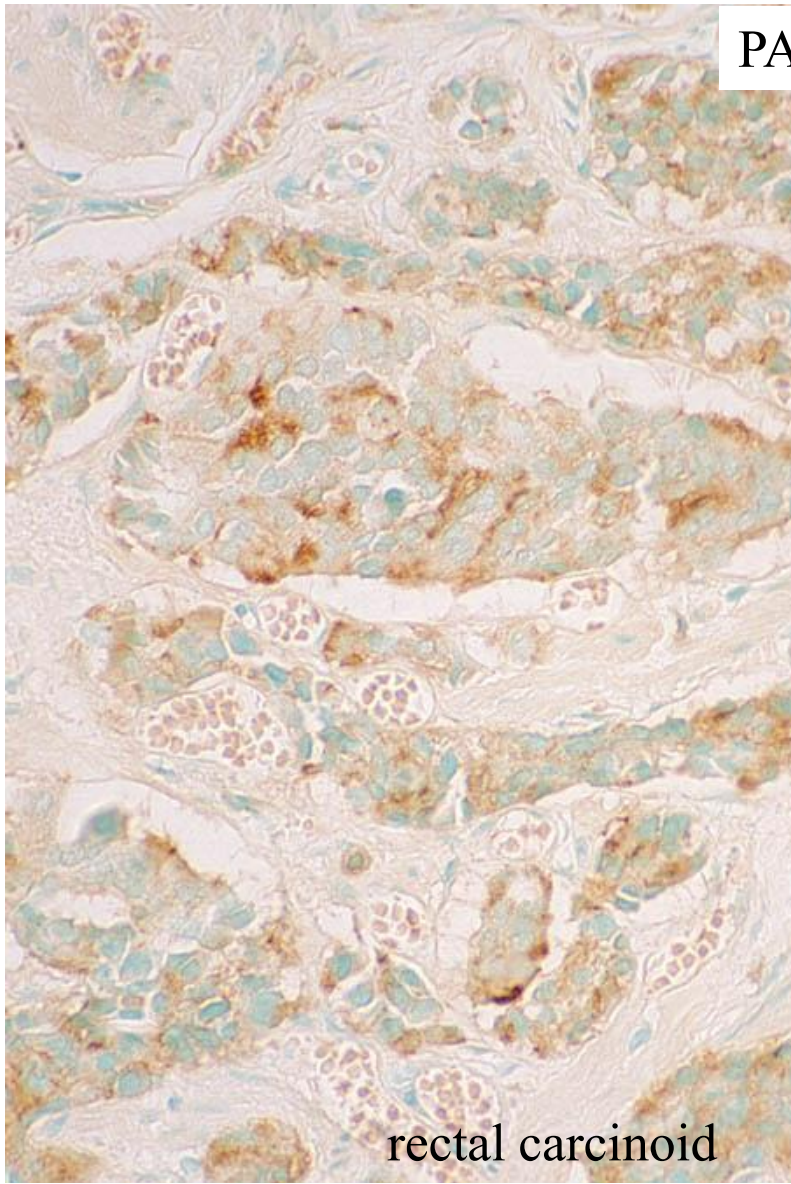
lactoferrin

False lactoferrin immunoreactivity in the pancreatic islet, detected with lactoferrin antiserum. This artifact is also caused by contaminating anti-intermediate filament activity (natural antibody) in the rabbit serum.

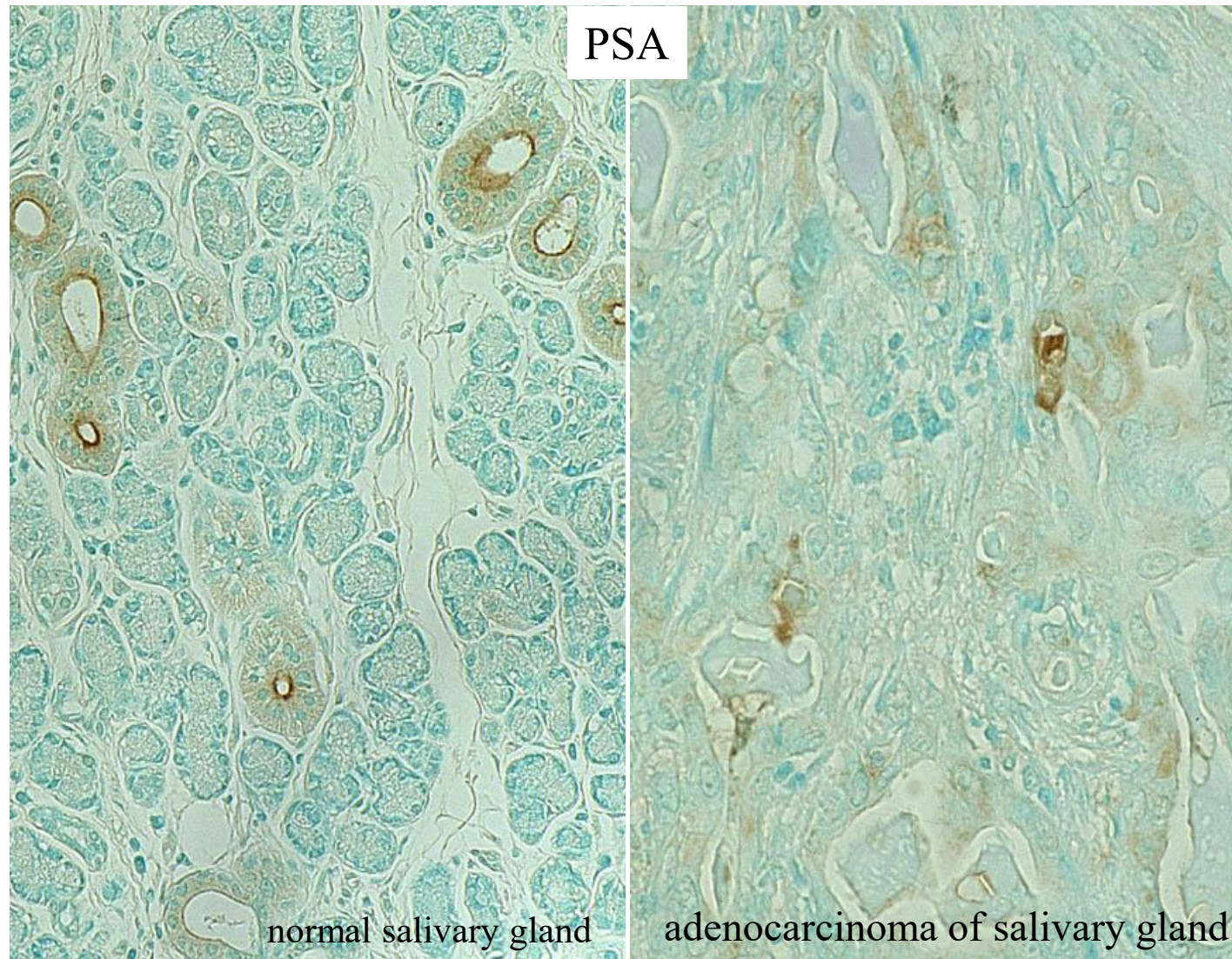


Contamination of anti-blood group substance activity in prostatic acid phosphatase (PAP) antiserum. In addition to normal prostatic epithelial cells, endothelial cells and red blood cells are positively labeled.

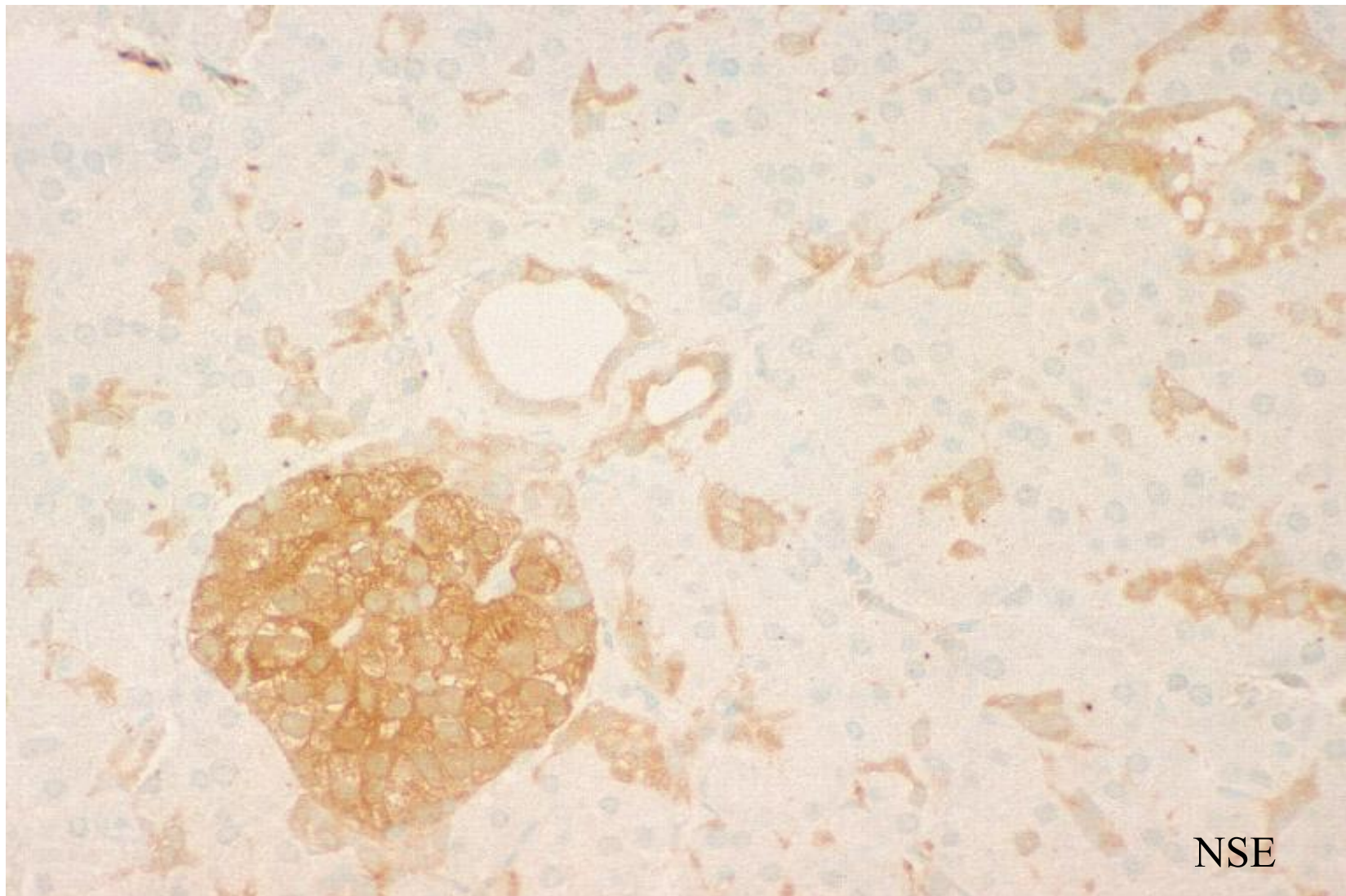
PAcP



Specific immunostaining for prostatic acid phosphatase (PAcP). It should be noted that PAcP immunoreactivity can be demonstrated in rectal carcinoid tumor (left) and in normal anal gland acinar cells in male individuals (right).

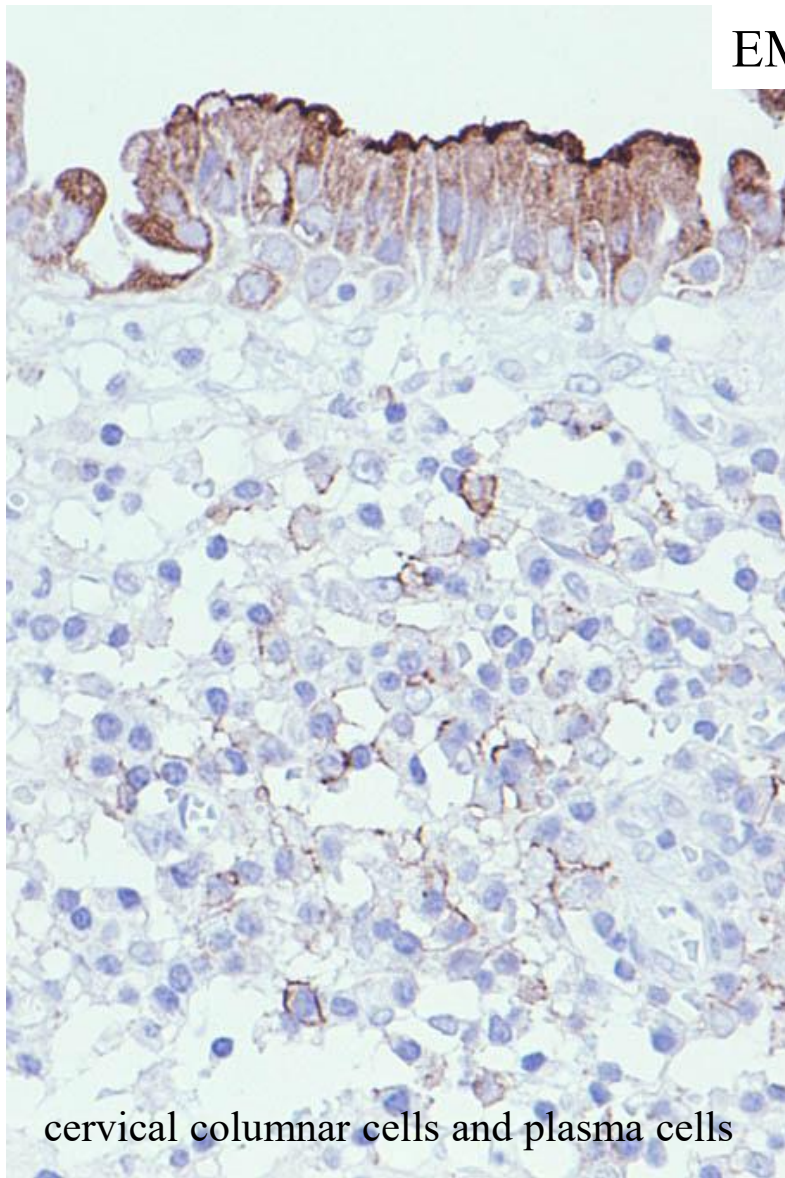


Prostate-specific antigen (PSA) in the salivary gland. PSA antiserum cross-reactively detect normal ductal cells in the salivary gland (left) and some salivary gland tumors such as adenocarcinoma (right). This is caused by the presence of common epitopes between PSA and kallikreins. **Ref.:** Tazawa K, et al. Localization of prostate-specific antigen-like immunoreactivity in human salivary gland and salivary gland tumors. *Pathol Int* 1999; 49(6): 500-505. doi: 10.1046/j.1440-1827.1999.00900.x

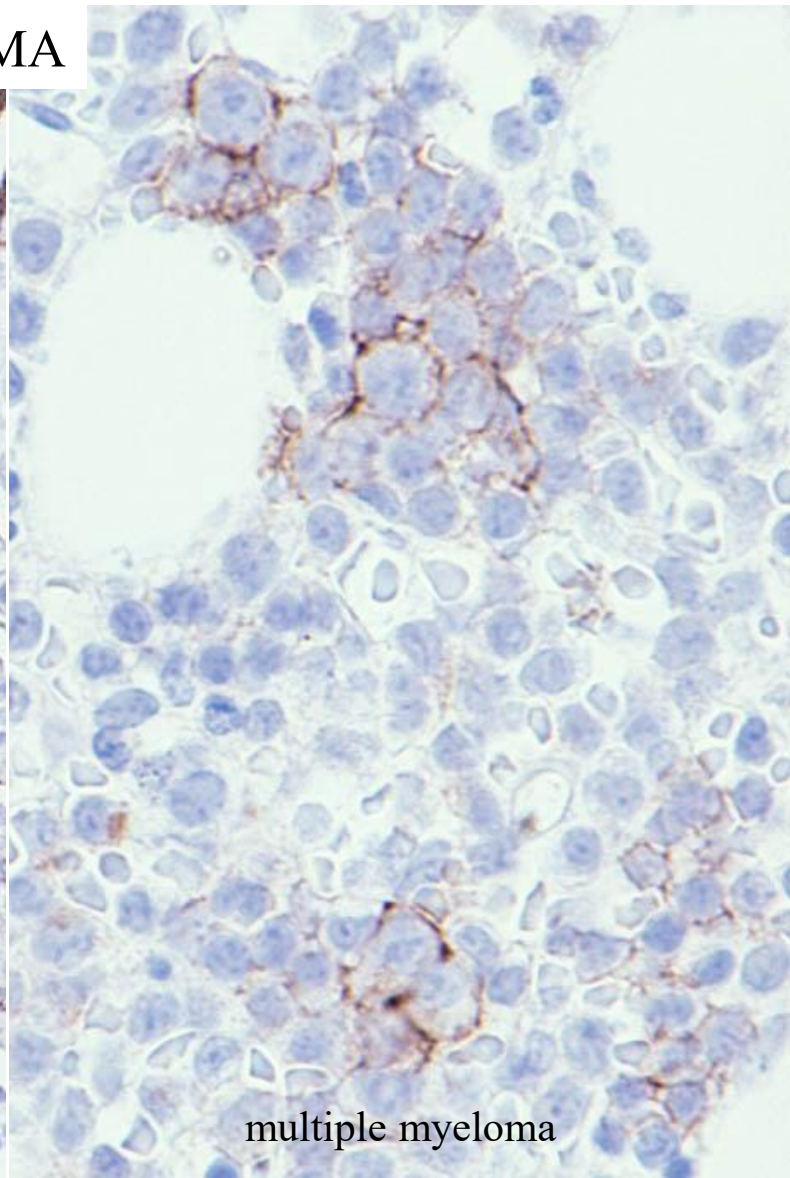


Neuron-specific enolase (NSE) expression in the normal pancreas. Not only islet (endocrine) cells but also exocrine cells such as ductal and acinar cells show positivity in the cytoplasm. NSE should be abbreviated as “nonspecific enolase” from the immunohistochemical point of review.

EMA

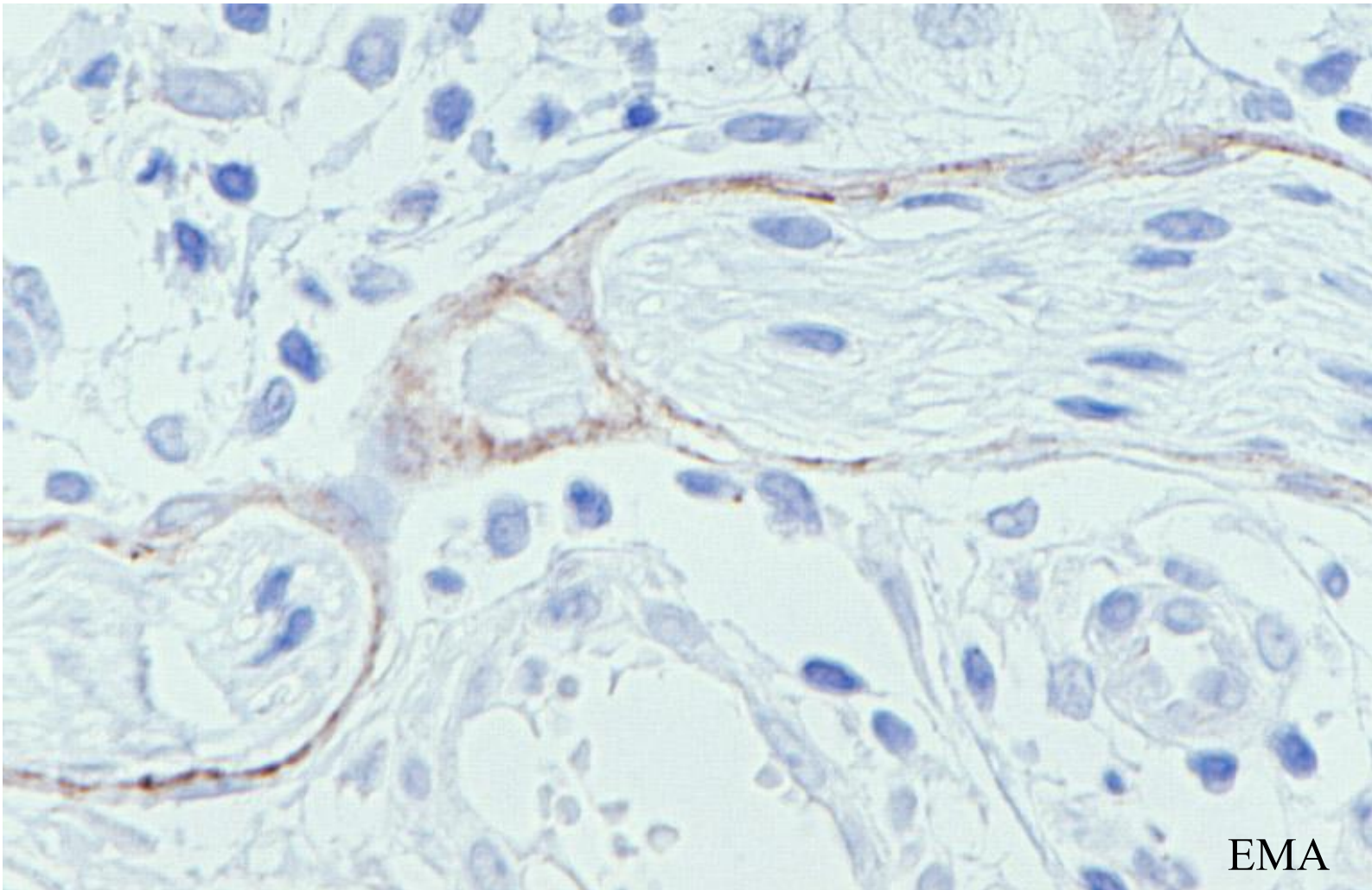


cervical columnar cells and plasma cells

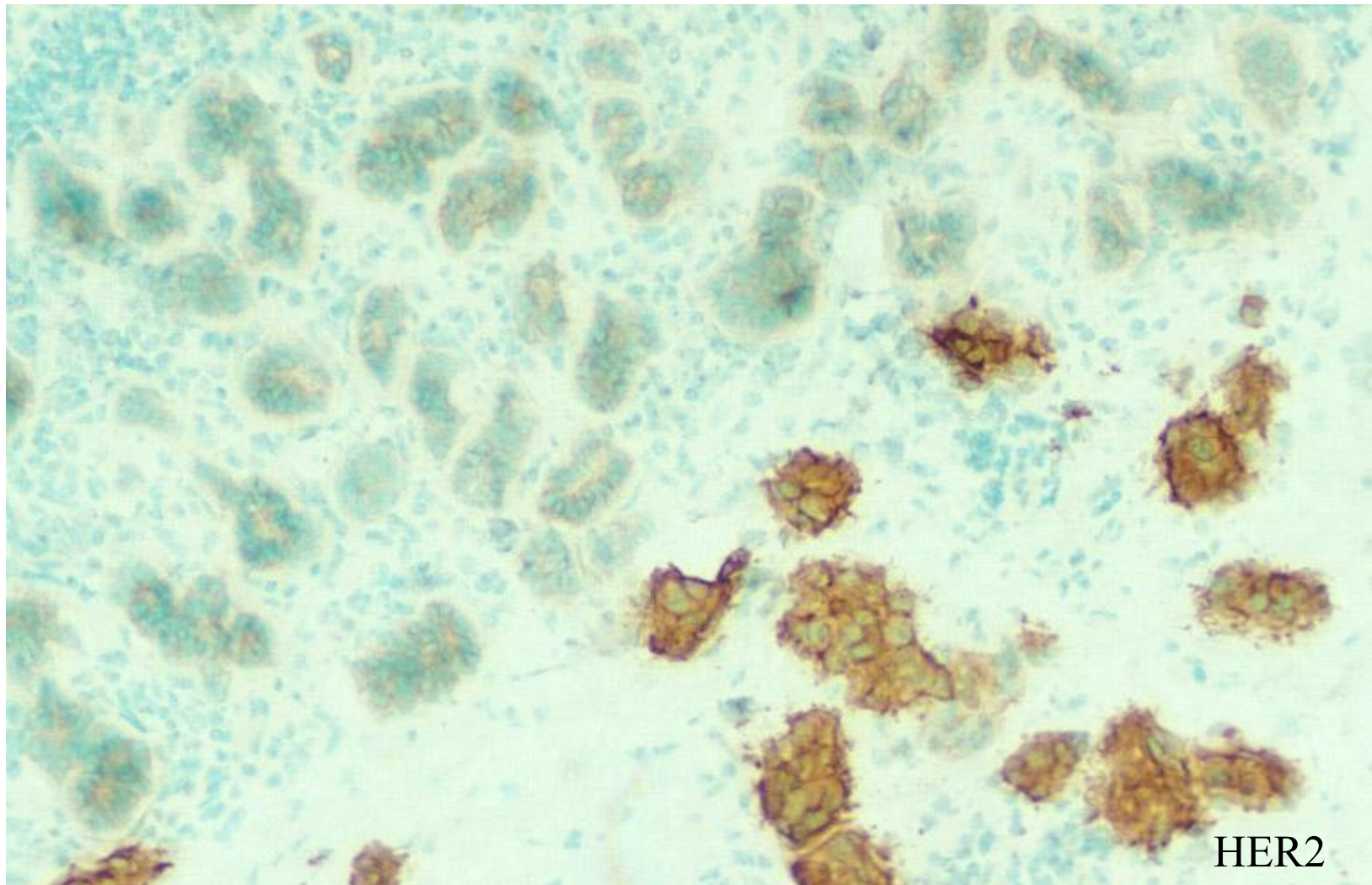


multiple myeloma

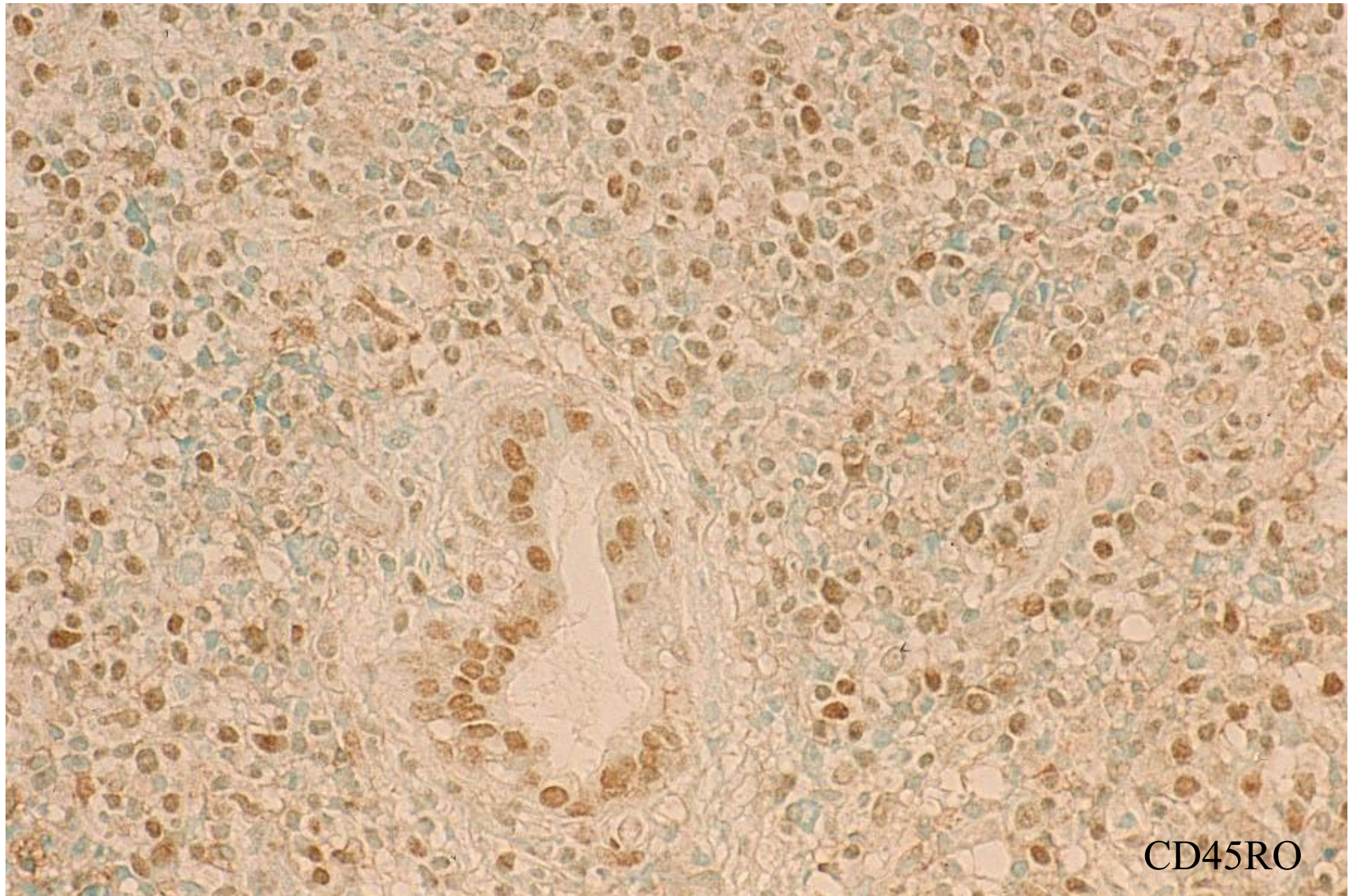
Expression of epithelial membrane antigen (EMA), a group of MUC-1 protein. EMA is expressed not only in normal columnar cells (left), but also normal (left) and neoplastic (right) plasma cells.



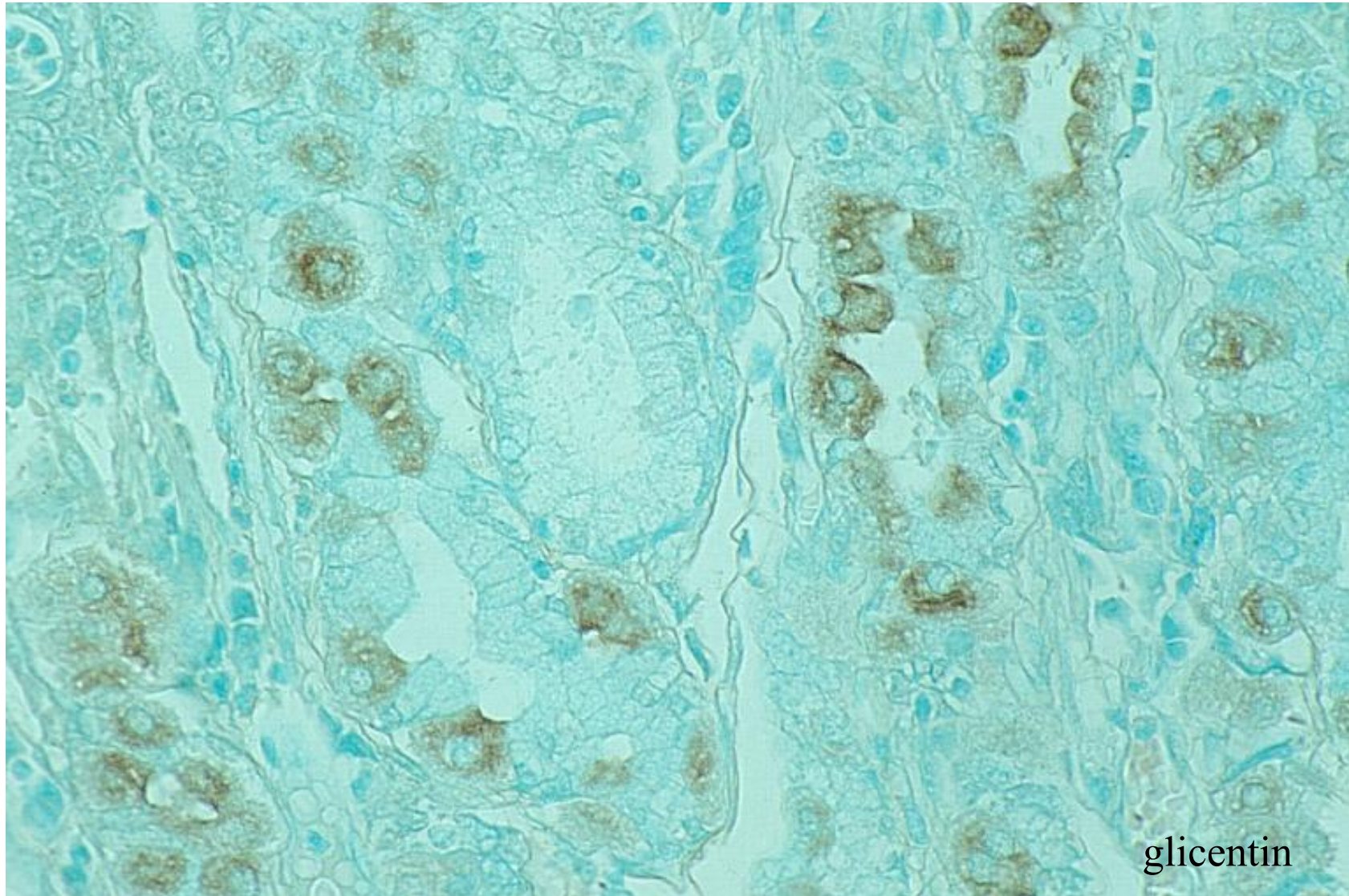
EMA expression in perineurial cells in the skin. After heating treatment, the normal perineurial cells are immunoreactive for EMA. The perineurial cells represent the meningeal cells in the peripheral nerves. **Ref.:** Kamoshida S, Tsutsumi Y. Expression of MUC-1 glycoprotein in plasma cells, follicular dendritic cells, myofibroblasts and perineurial cells: immunohistochemical analysis using three monoclonal antibodies. *Pathol Int* 1998; 48(10): 776-785. doi: 10.1111/j.1440-1827.1998.tb03837.x



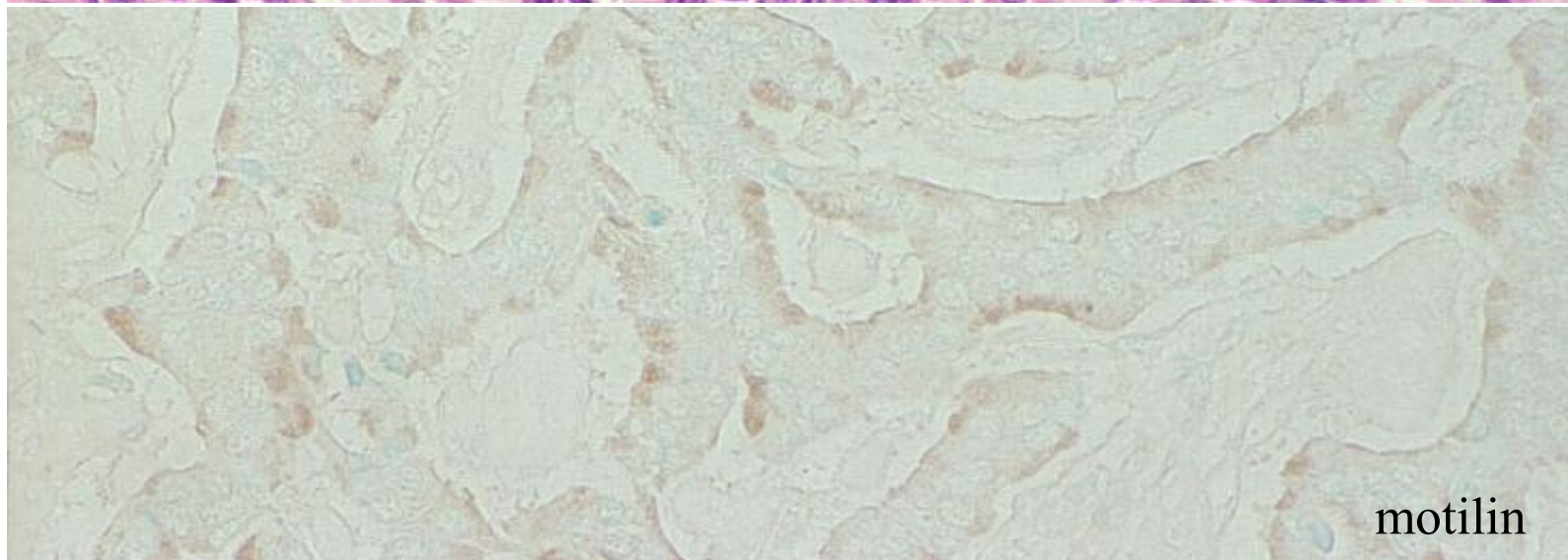
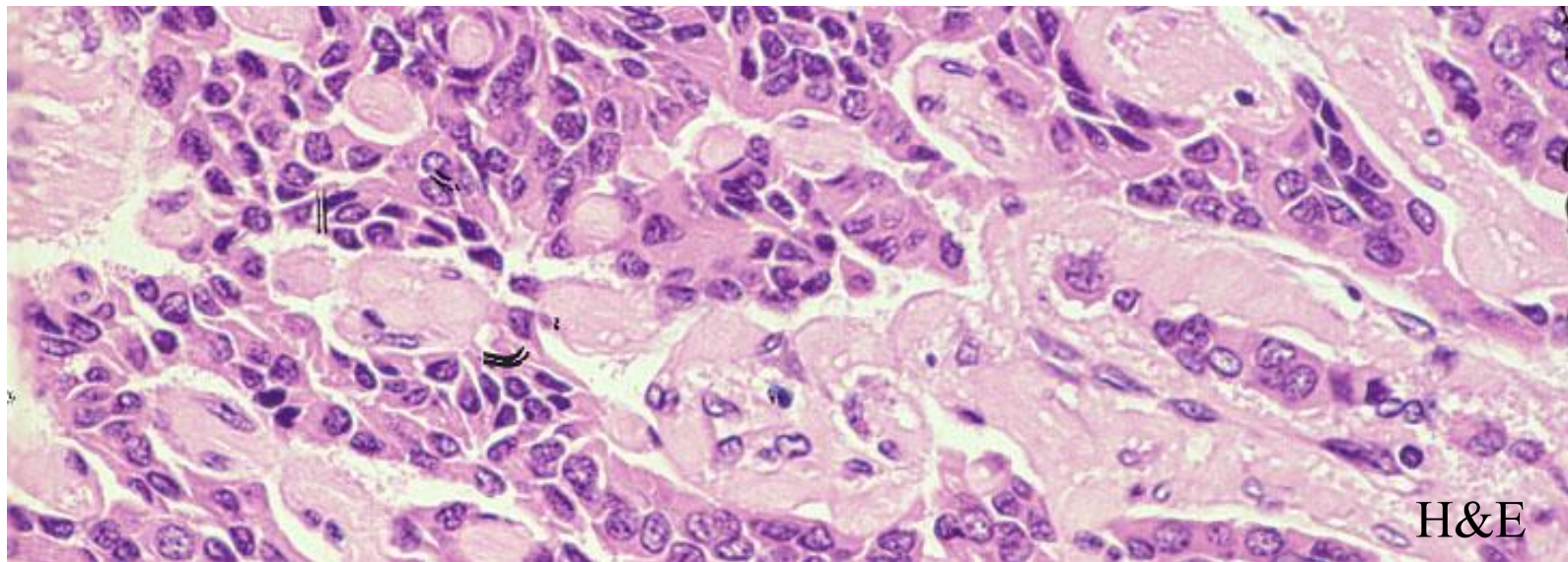
HER-2 expression in normal and cancerous mammary ductal cells in paraformaldehyde-fixed frozen section. In addition to overexpressed breast cancer cells, the non-neoplastic mammary ductal cells weakly but consistently express HER2. Ref.: Naber SP, et al. Strategies for the analysis of oncogene overexpression. Studies of the neu oncogene in breast carcinoma. Am J Clin Pathol 1990; 94(2): 125-136. doi: 10.1093/ajcp/94.2.125



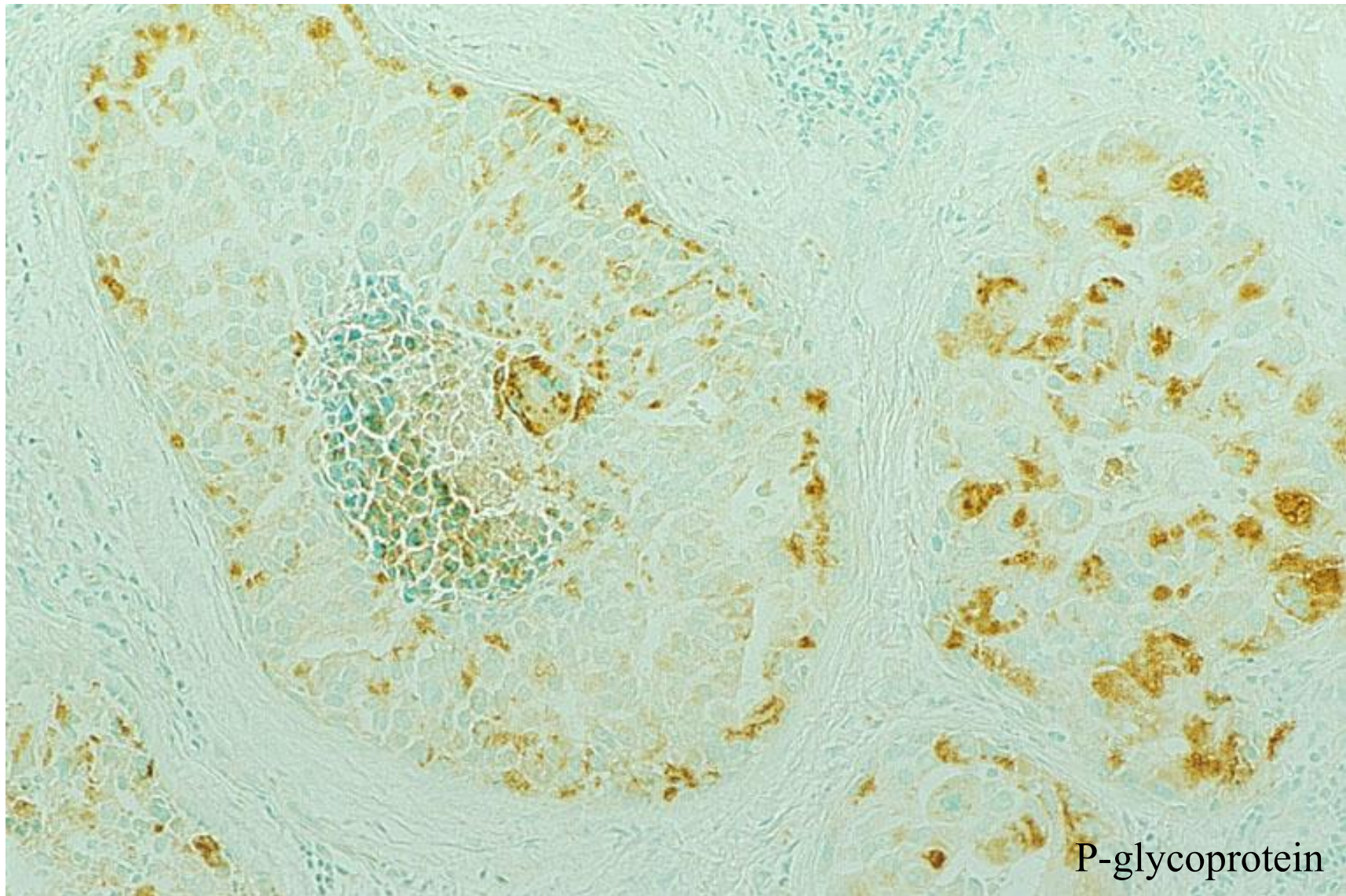
Nonspecific (false) localization of CD45RO in the nuclei of nasal NK-cell lymphoma. Non-neoplastic nasal gland cells also show nuclear reactivity. CD45RO, a T-cell marker, should not be expressed in the nucleus.



Glicentin immunoreactivity in gastric parietal cells: nonspecific adsorption of IgG in the antiserum in the cytoplasm of the parietal cells.



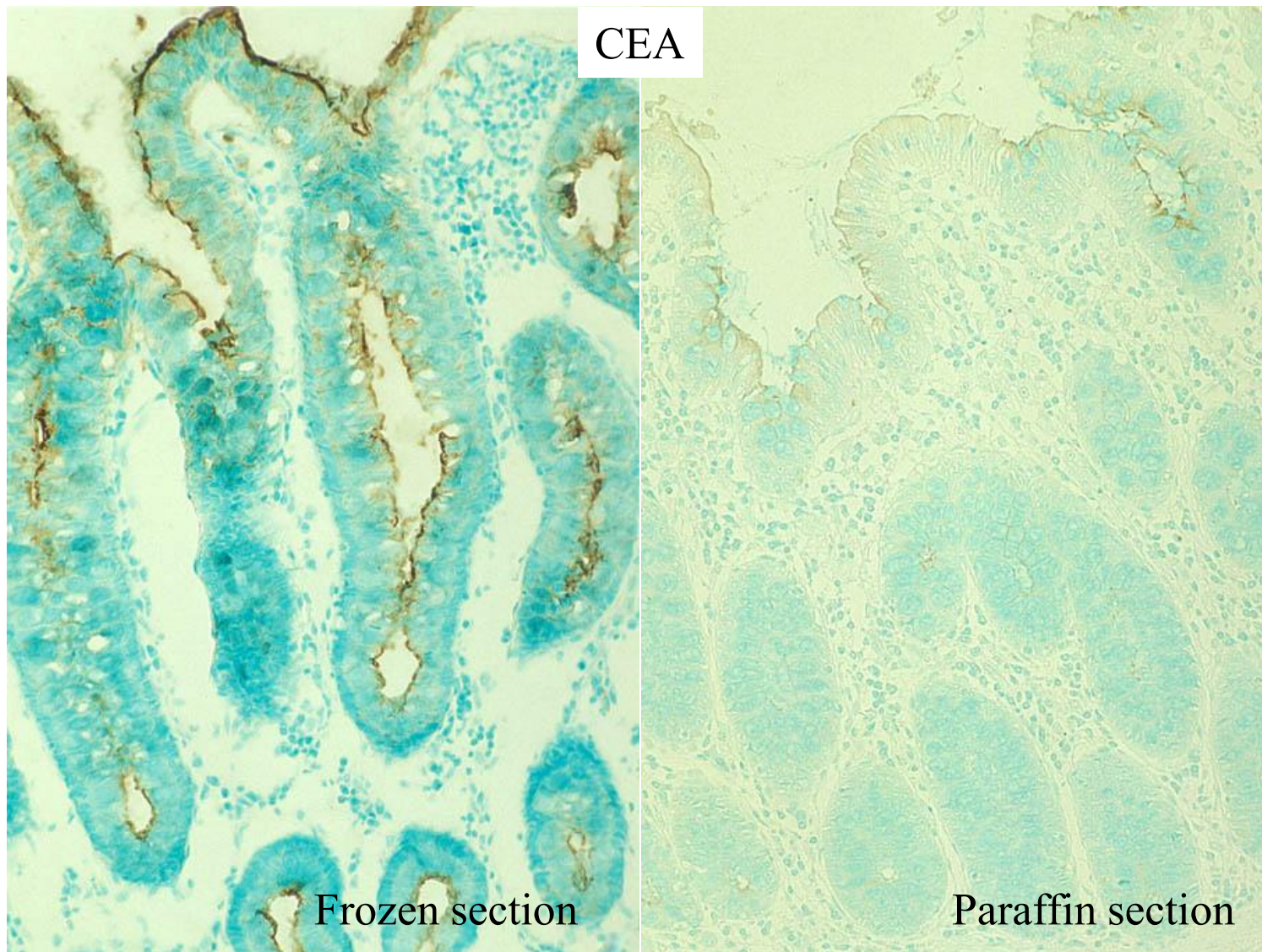
Nonspecific motilin immunoreactivity in rectal carcinoid. The immunoreactivity is not absorbed by synthesized motilin molecules, and should be regarded as nonspecific adsorption of the antiserum to the carcinoid tumor cells. There has been no report of motilin-producing neuroendocrine tumors.



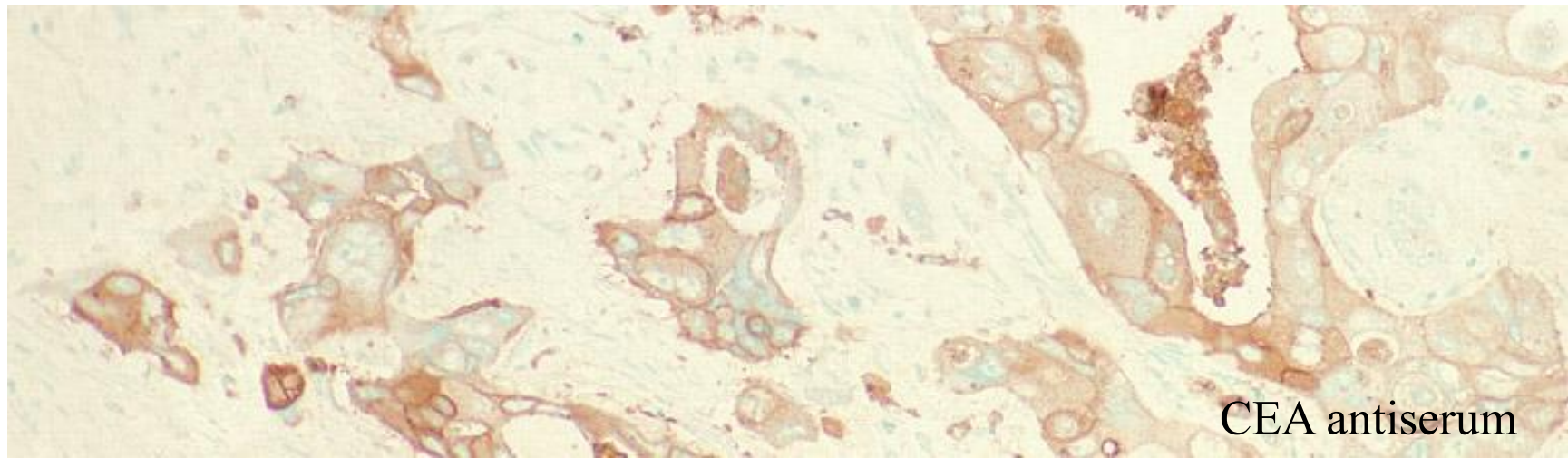
P-glycoprotein (C219) immunostaining in breast cancer: nonspecific staining is seen in the cytoplasm. Note that P-glycoprotein should be expressed on the plasma membrane.

Immunostaining for CEA and cytokeratins: Demonstration of the sensitivity and specificity

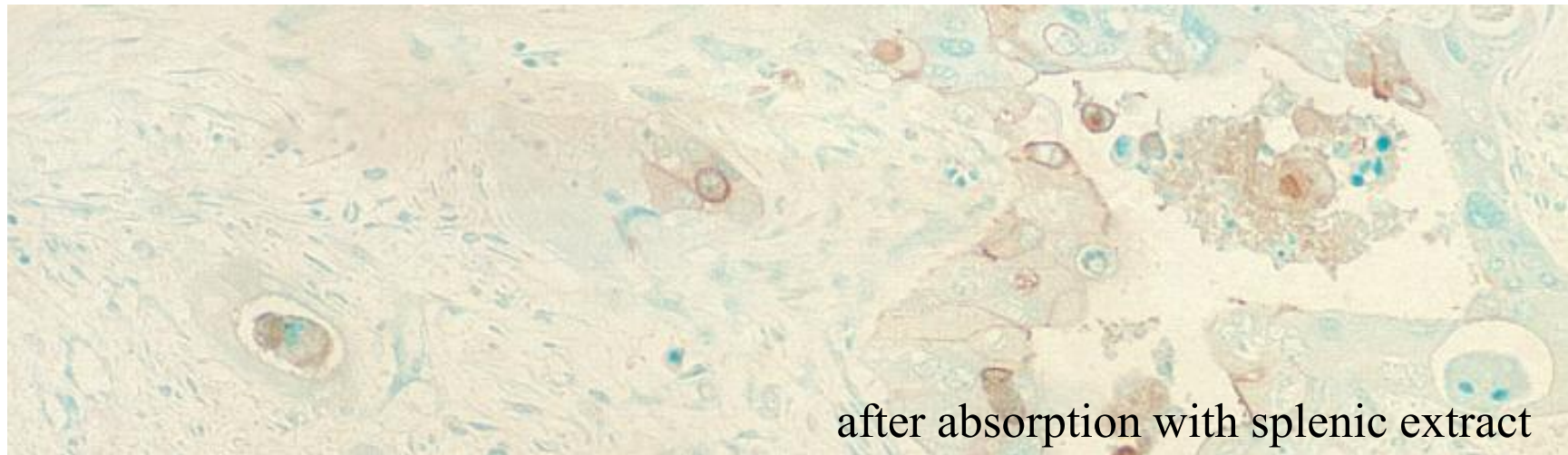
- 1) The effect of formalin fixation
- 2) The use of absorbed antiserum
- 3) The use of monoclonal antibodies



CEA expression in the normal gastric mucosa. In PLP-fixed frozen sections, CEA is consistently expressed on the apical membrane of the foveolar cells. After formalin fixation and paraffin embedding, the reactivity is significantly weakened.

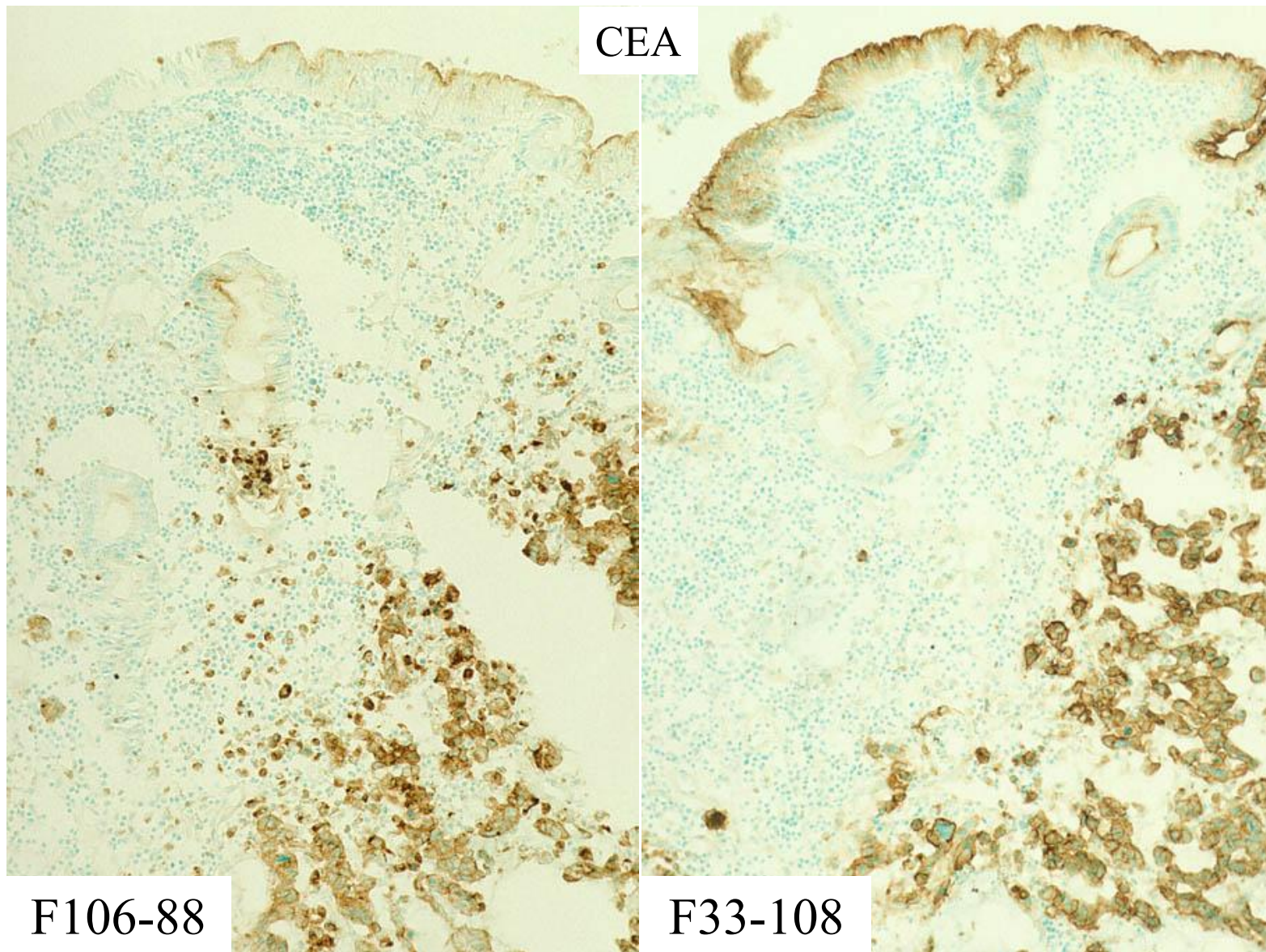


CEA antiserum

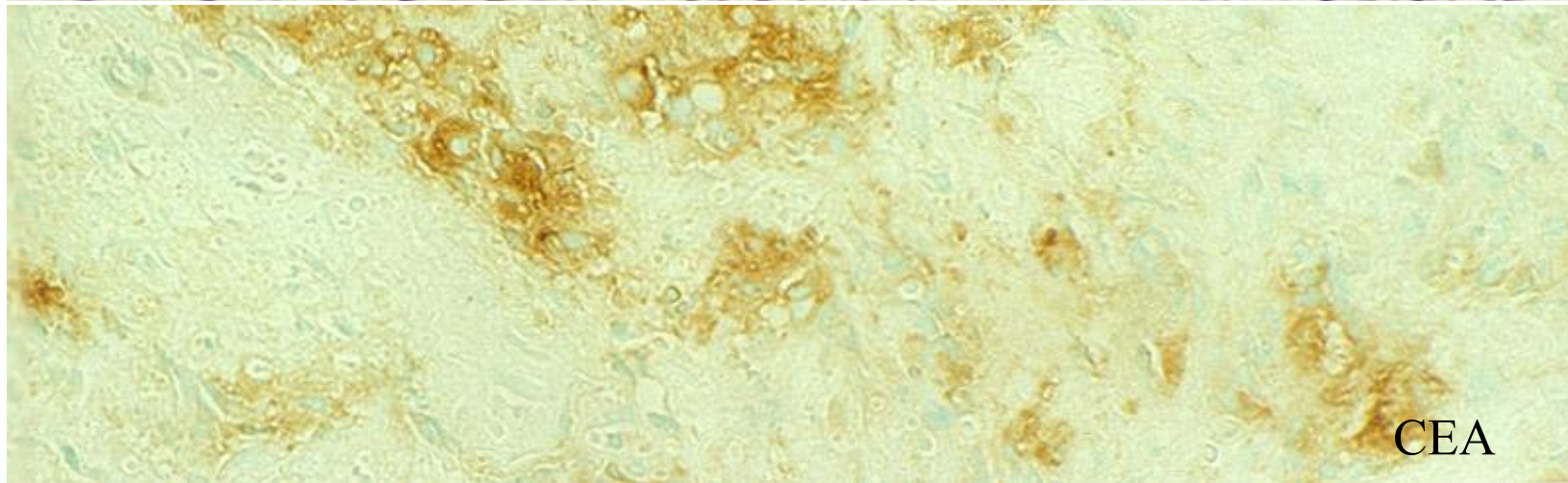
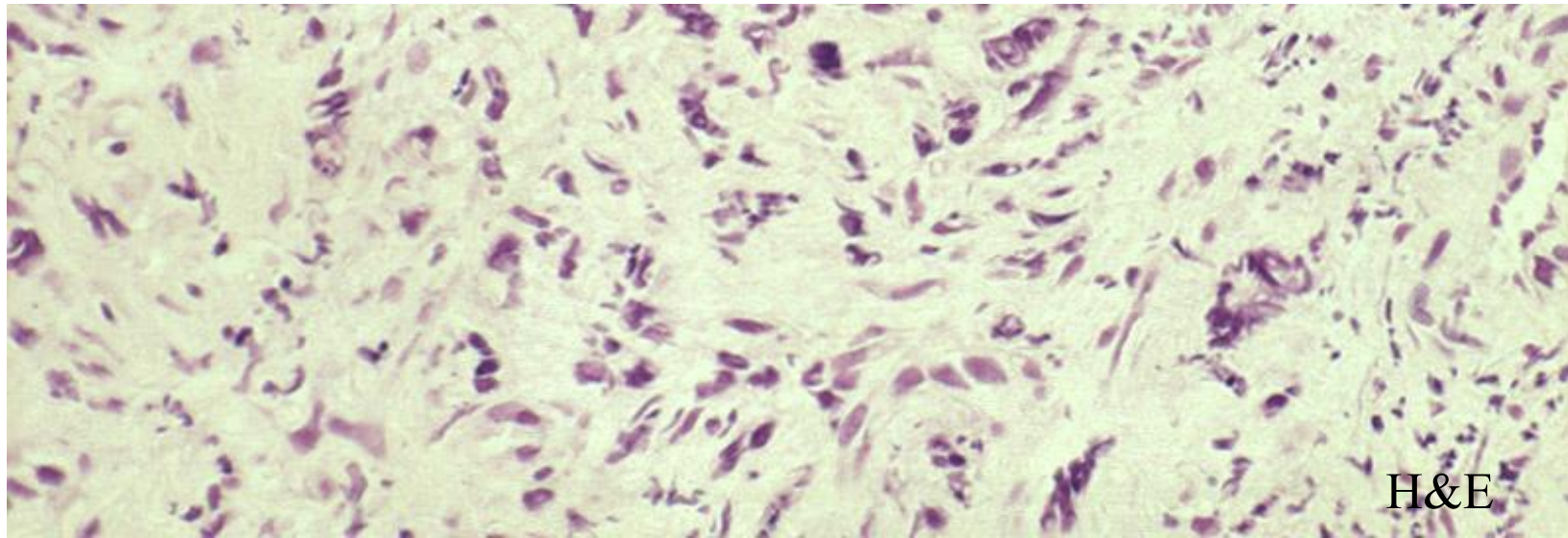


after absorption with splenic extract

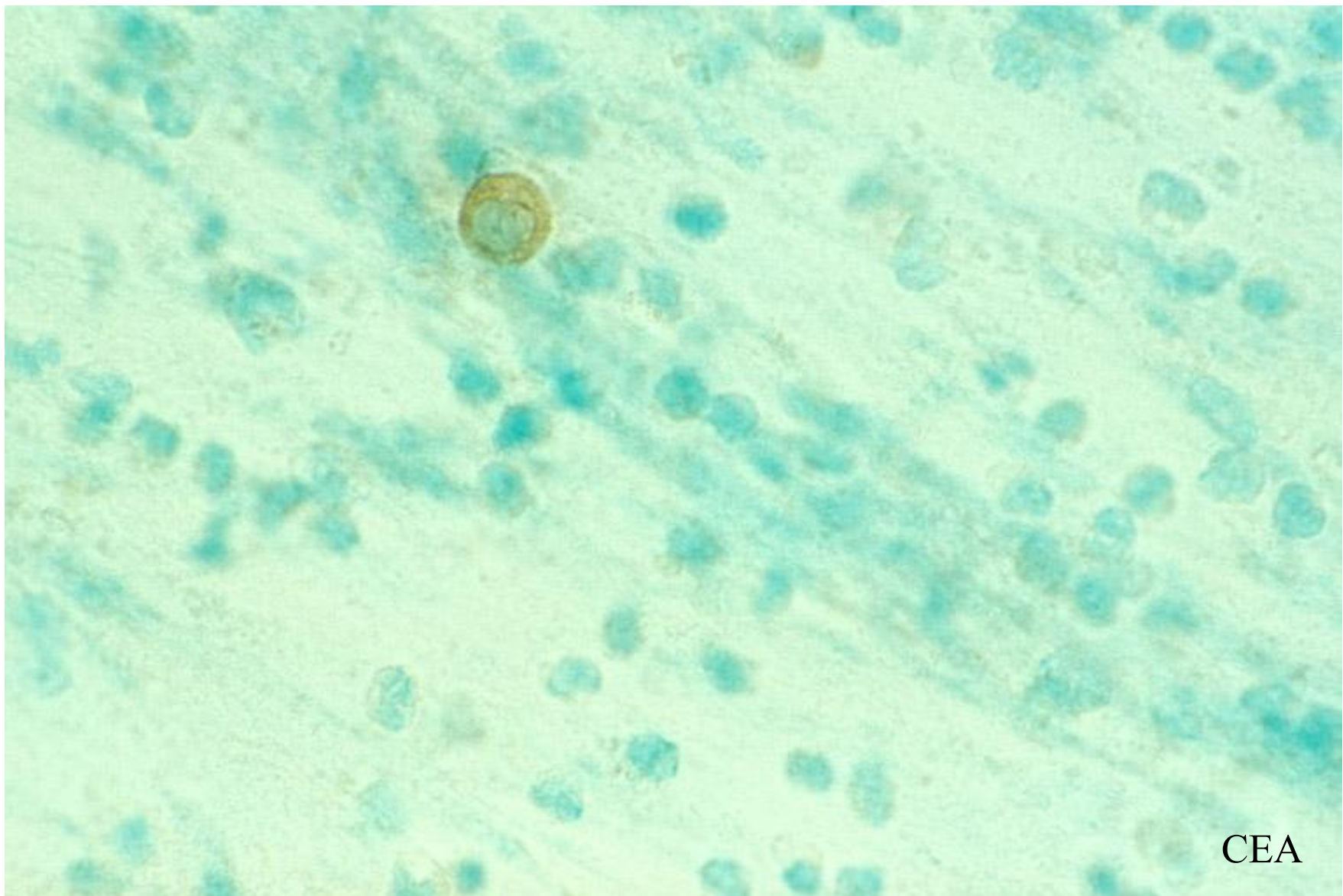
CEA-related antigens in pancreatic adenocarcinoma. Immunoreactivity with CEA antiserum in pancreatic adenocarcinoma is significantly weakened with CEA antiserum absorbed with splenic extract. The immunoreactivity is mostly related to the expression of “nonspecific cross-reacting antigen (NCA)”, rich in neutrophils.



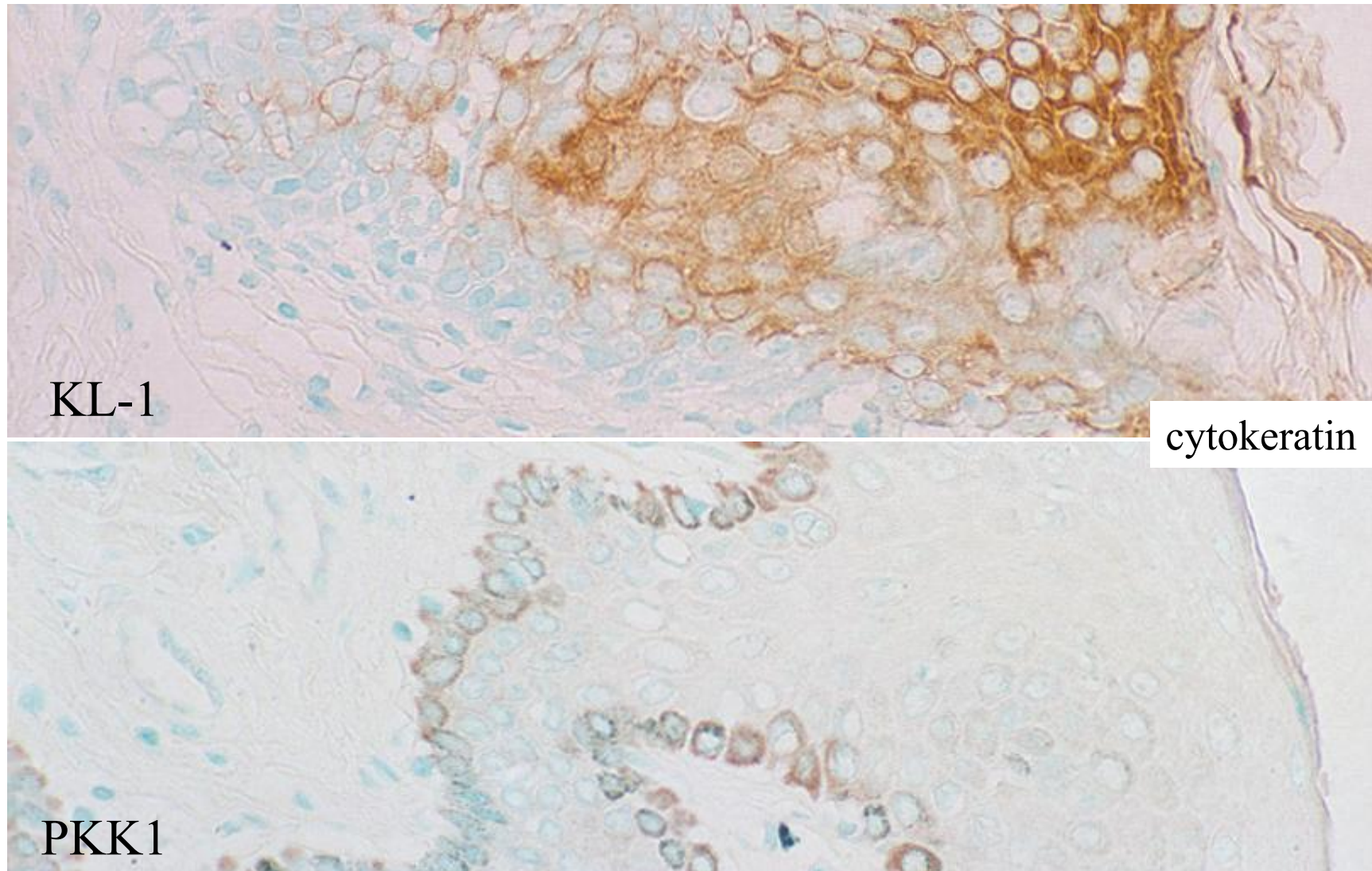
Immunostaining with two monoclonal antibodies against CEA in PFA-fixed frozen sections of signet ring cell carcinoma of the stomach. The monoclonal F106-88 is cross-reactive with nonspecific cross-reacting antigen (NCA), while the monoclonal F33-108 is specific to CEA.



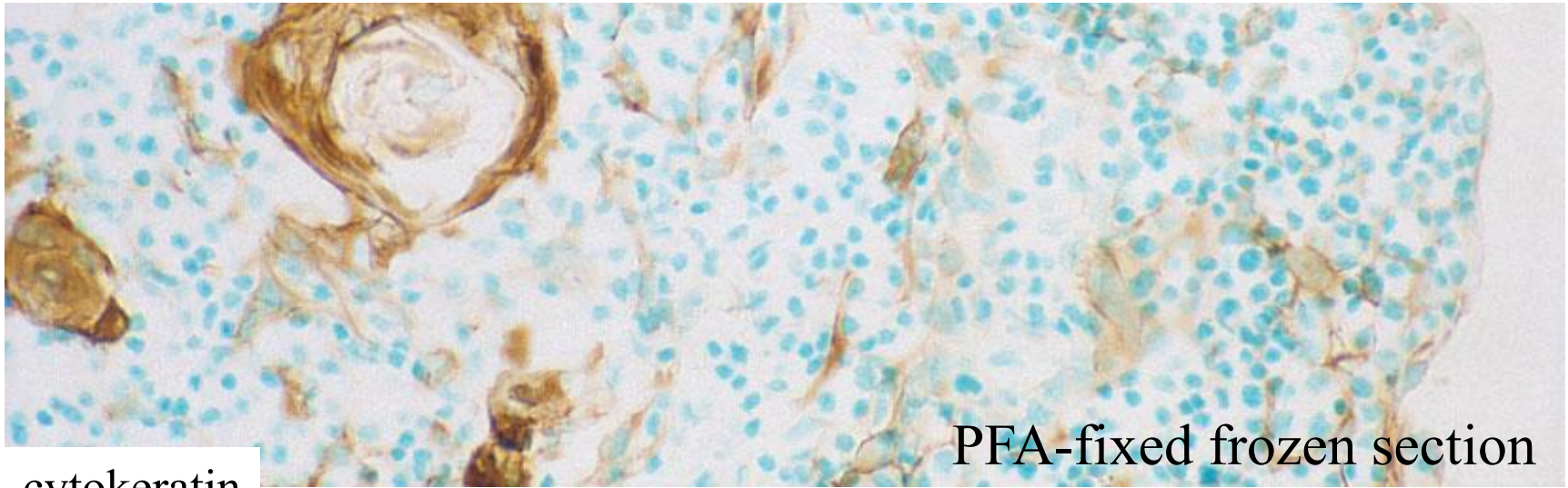
Immunostaining with CEA antiserum preabsorbed with the splenic extract in crushed ulcer base tissue in a gastric biopsy specimen. The crushed atypical cells express genuine CEA, confirming the diagnosis of gastric adenocarcinoma.



Application of immunostaining with CEA antiserum preabsorbed with the splenic extract to cytology diagnosis. An isolated adenocarcinoma cell in pleural effusion shows CEA immunoreactivity, confirming the diagnosis of malignancy.

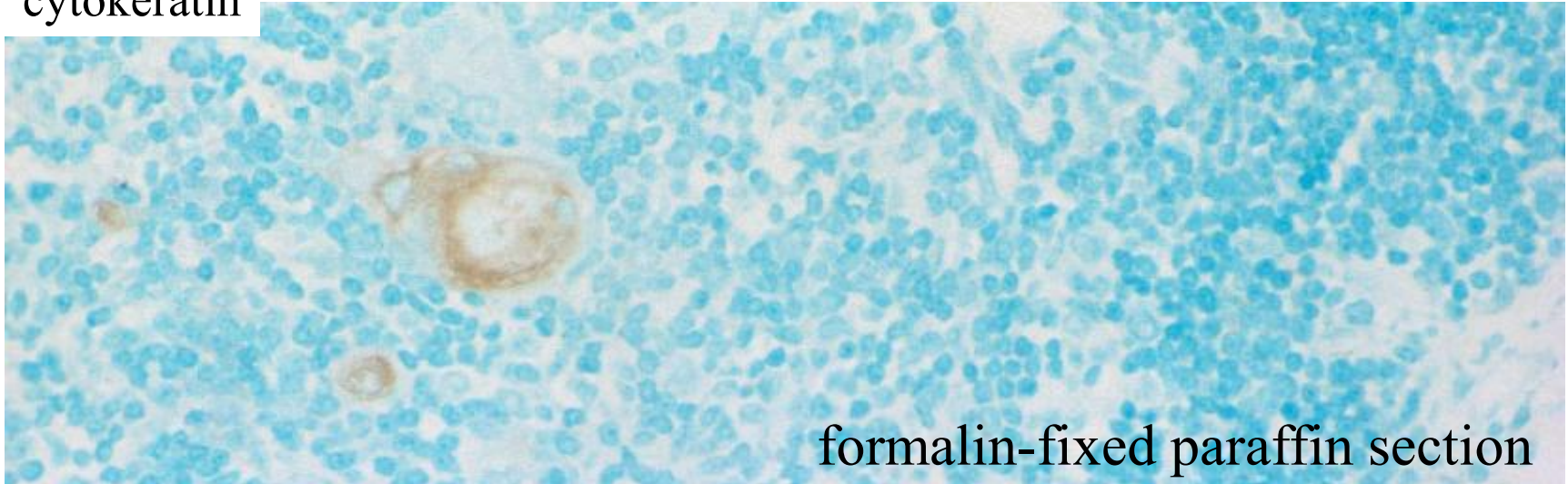


Immunostaining with cytokeratin monoclonals in the skin. The monoclonal KL-1 detects suprabasal keratinocytes (top), while the monoclonal PKK1 stains basal keratinocytes (bottom). You should know the subtype specificity of the monoclonal antibodies used.



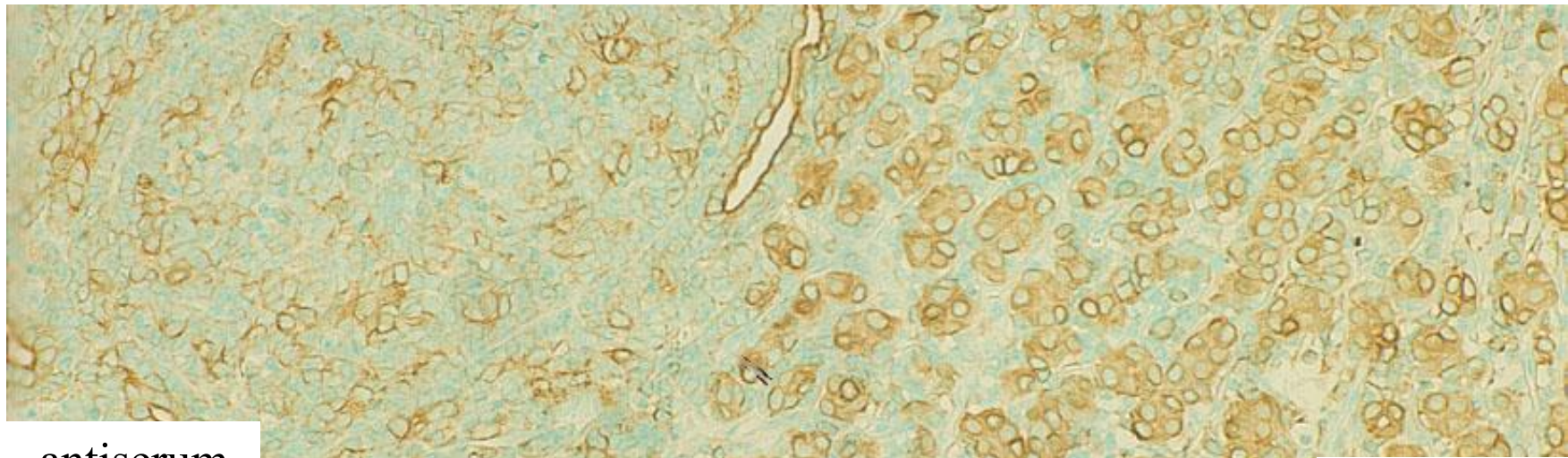
cytokeratin

PFA-fixed frozen section



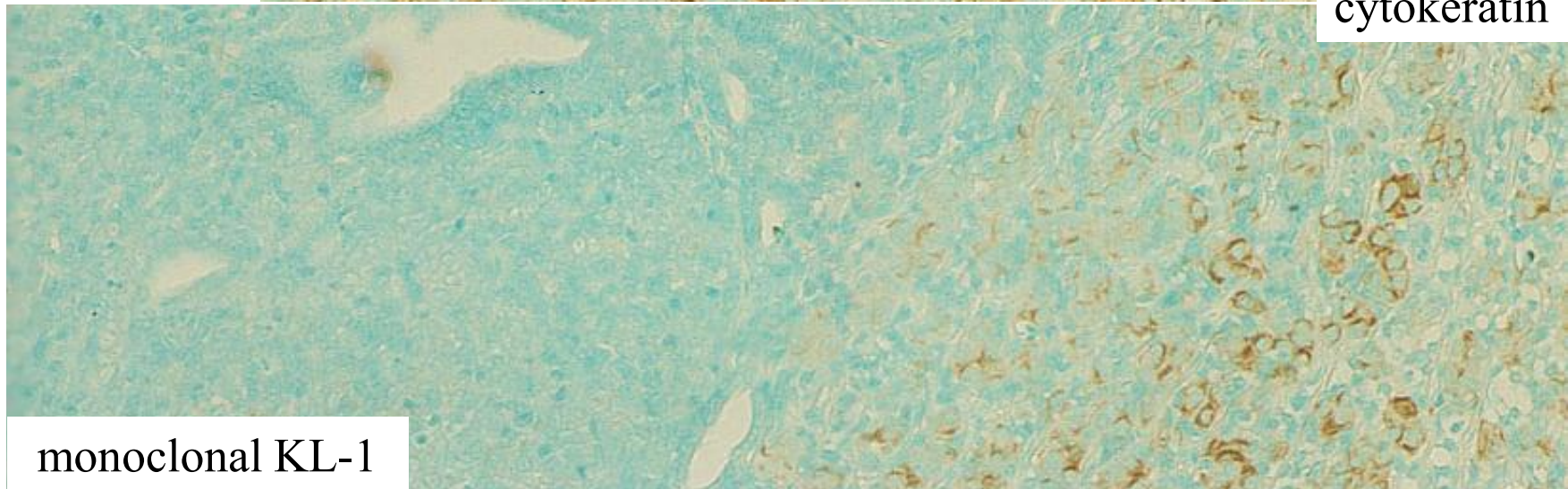
formalin-fixed paraffin section

Cytokeratin immunoreactivity in the thymus detected with monoclonal KL-1. In frozen sections, all the thymic epithelial cells including Hassall bodies are immunoreactive, while after formalin fixation and paraffin embedding, only Hassall bodies are stained.



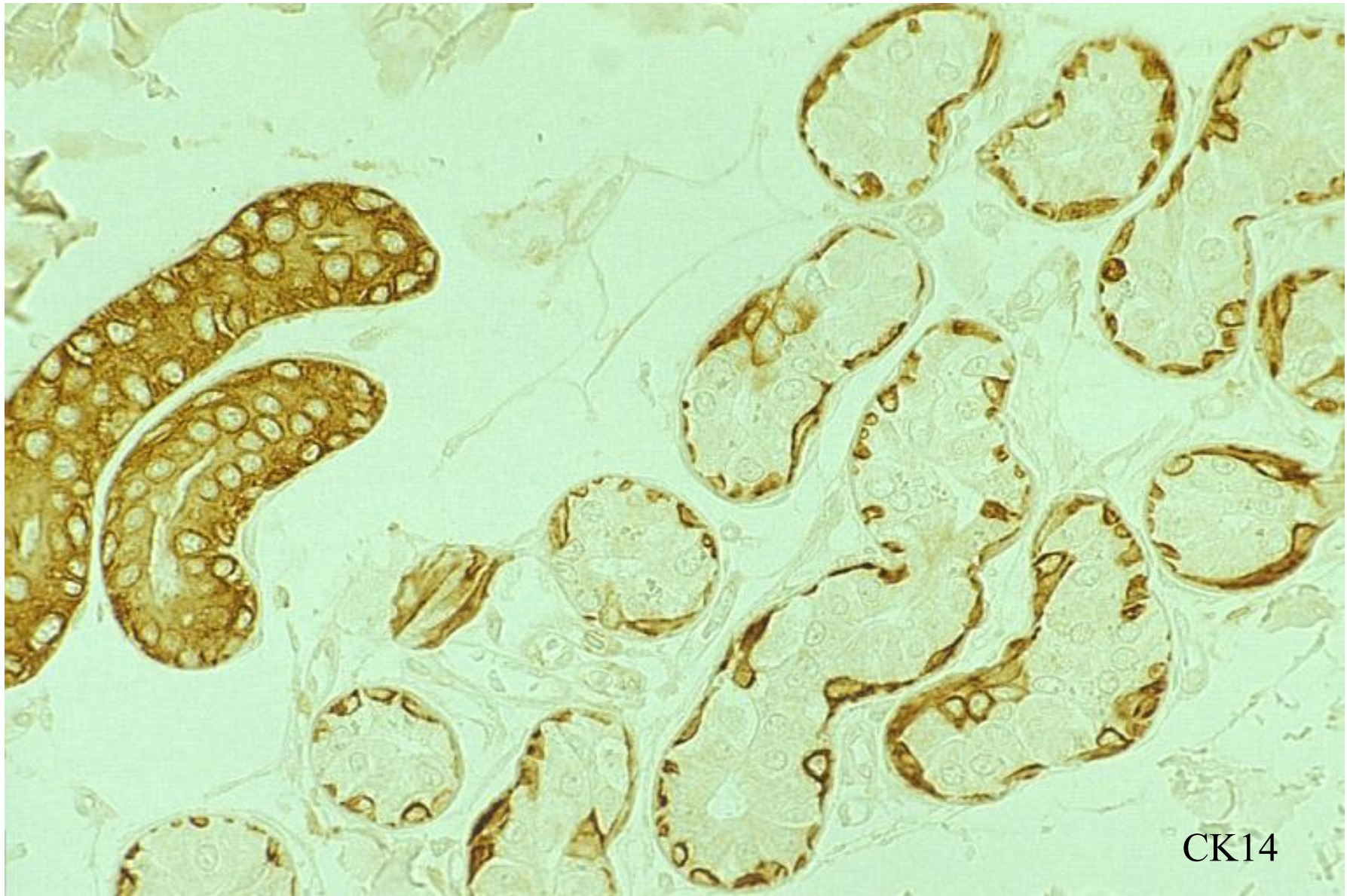
antiserum

cytokeratin

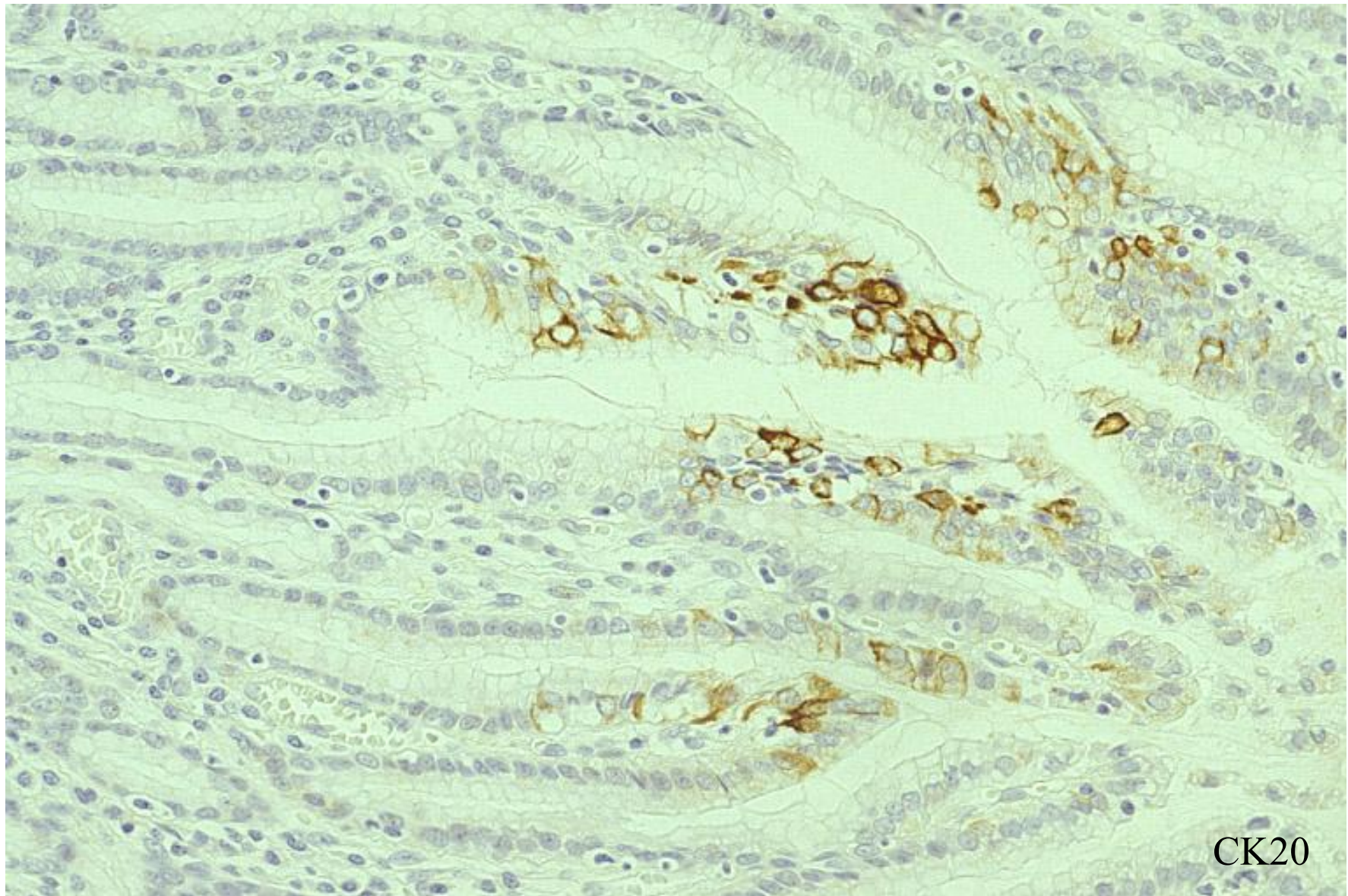


monoclonal KL-1

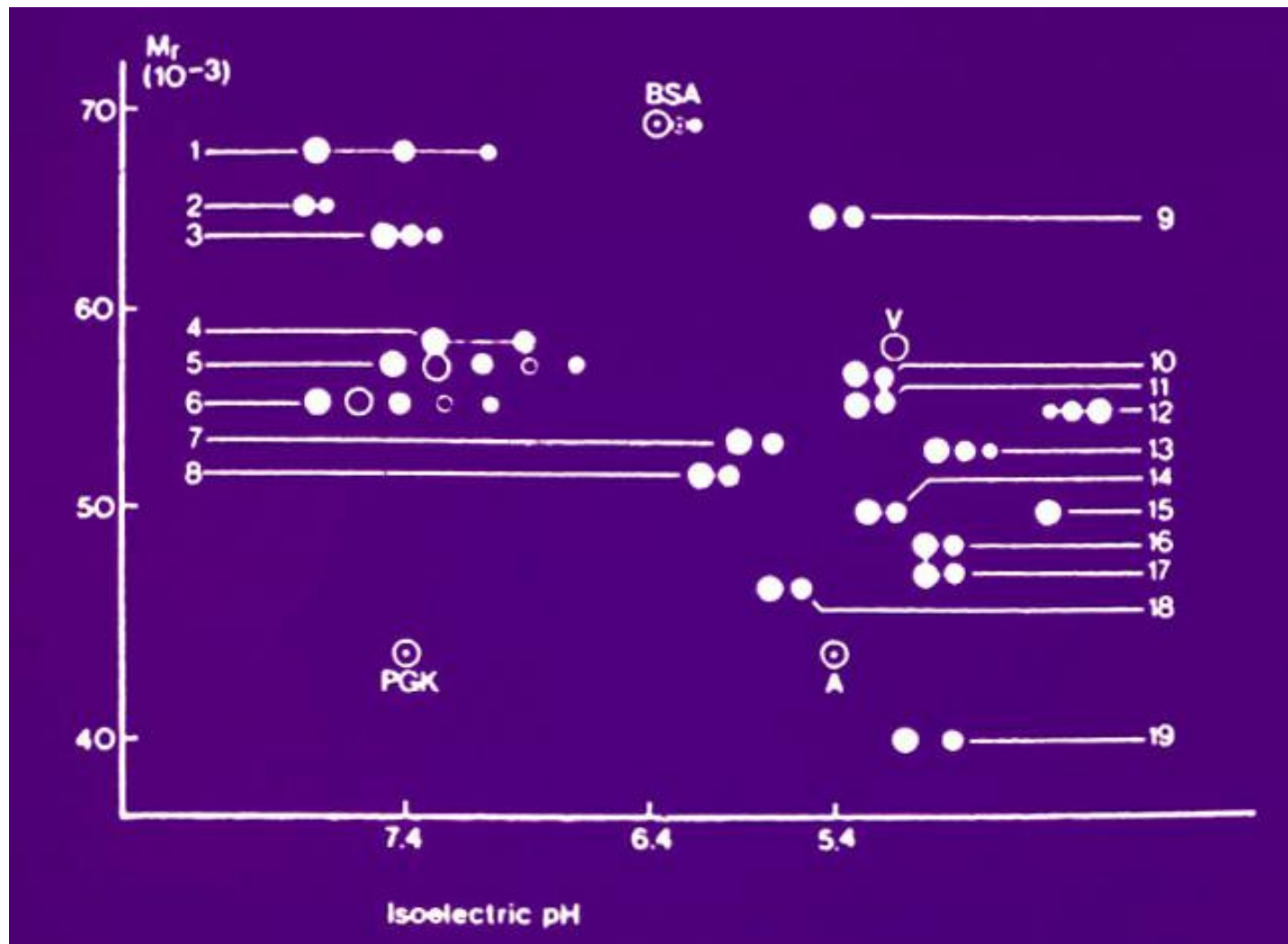
Cytokeratin immunostaining in eccrine spiradenoma of the skin. All the tumor cells are immunoreactive with cytokeratin antiserum (top), while the monoclonal KL-1 detects part of the tumor cells (bottom).



CK14 expression in the normal eccrine sweat gland of the skin. CK14 is expressed mainly in the myoepithelial cells, while some ductal cells also express CK14.



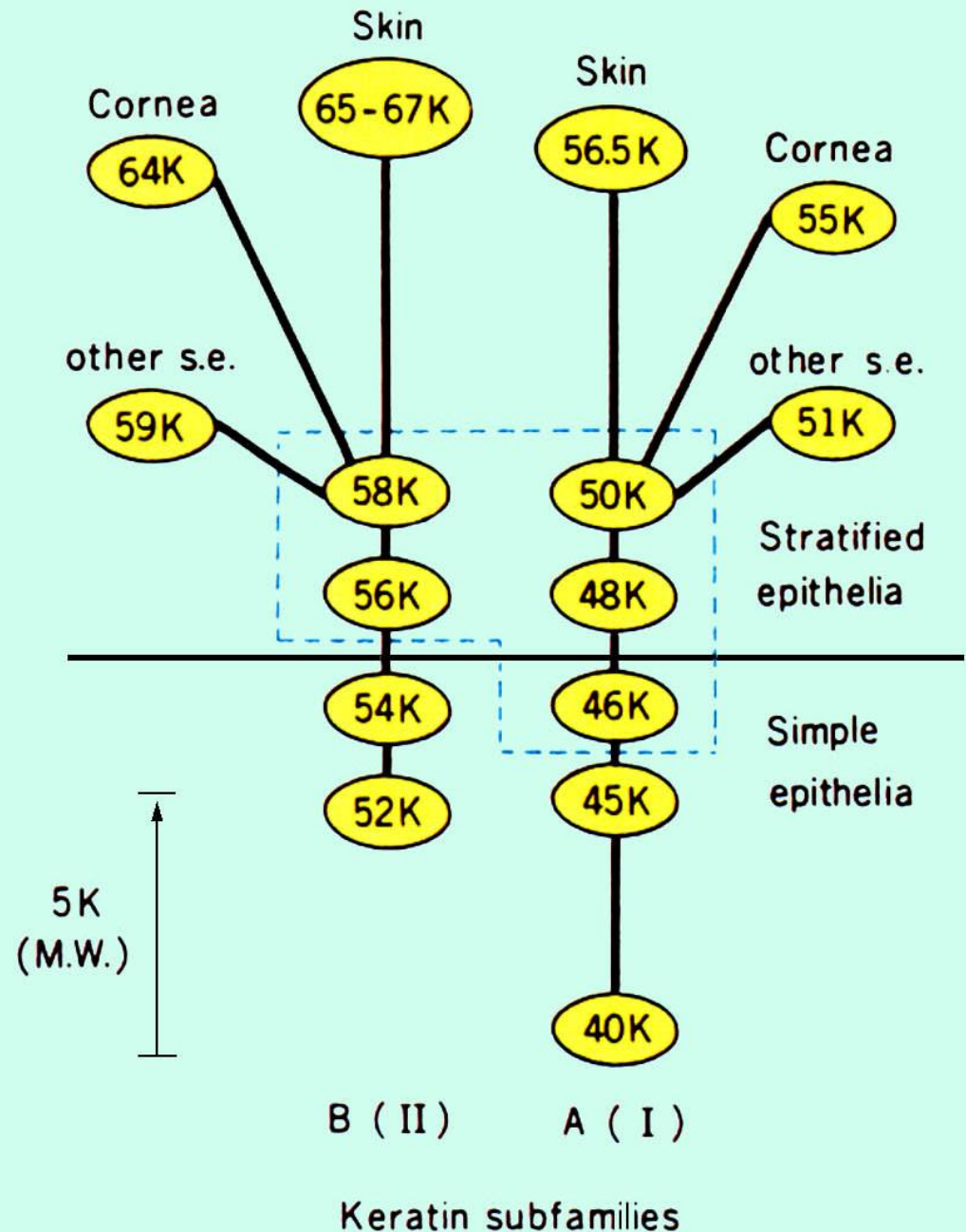
CK20 expression in the normal gastric mucosa. CK20 is expressed in certain types of epithelial cells, including gastrointestinal, urothelial and some neuroendocrine-type cells.



Schematic chart of human cytokeratin polypeptides. Human cytokeratins can be divided into acidic and basic groups. The basic cytokeratin polypeptides tend to possess higher molecular weight. Ref.: Moll R, et al. The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. Cell 1982; 31: 11-24. doi:10.1016/0092-8674(82)90400-7

Model of cytokeratin expression in epithelial cells

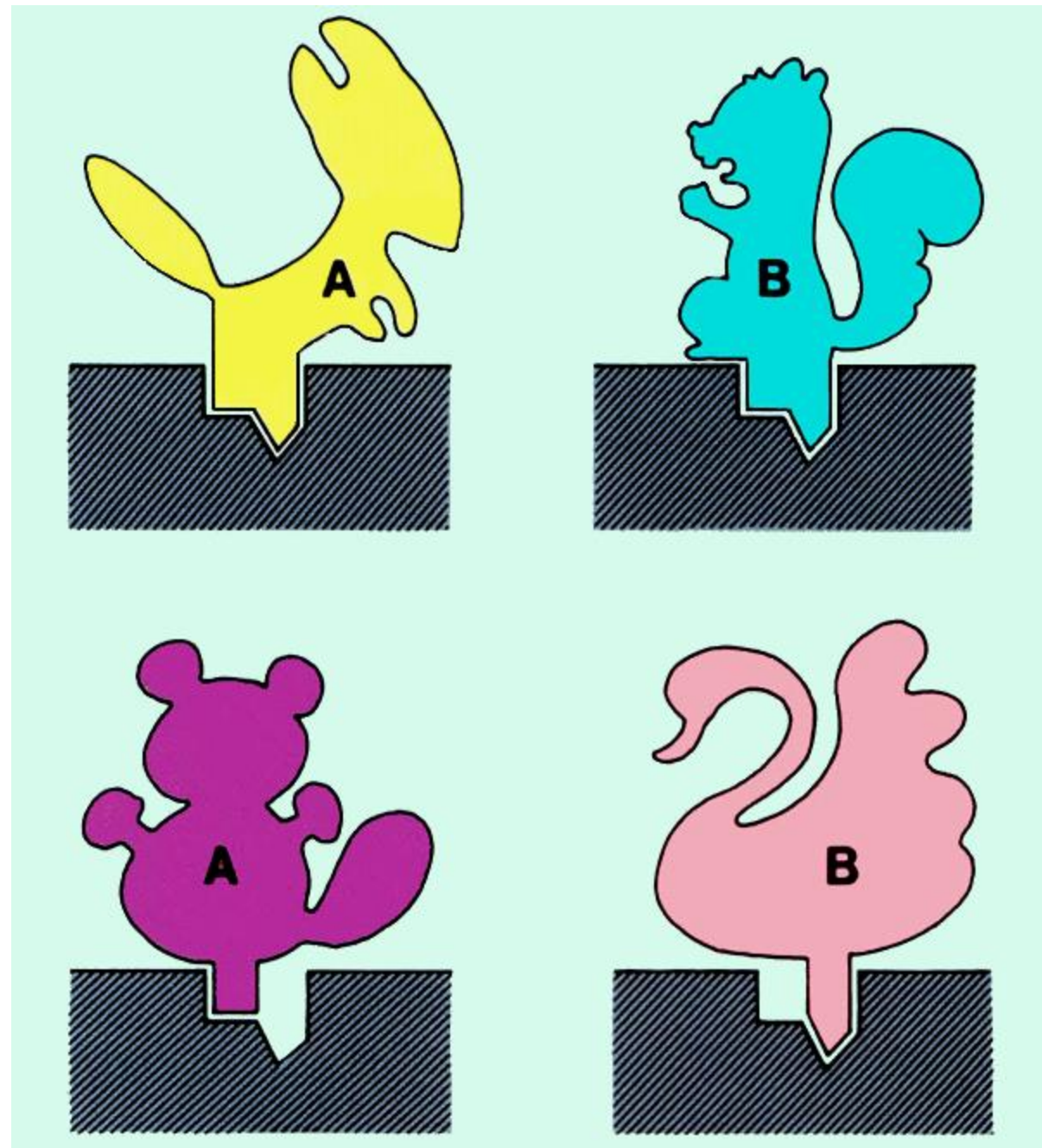
The epithelial cells express a pair of acidic (A) and basic (B) cytokeratins in the cytoplasm. Basic cytokeratins possess higher molecular weight than the paired acidic cytokeratins.



Cross-reactivities of monoclonal antibodies

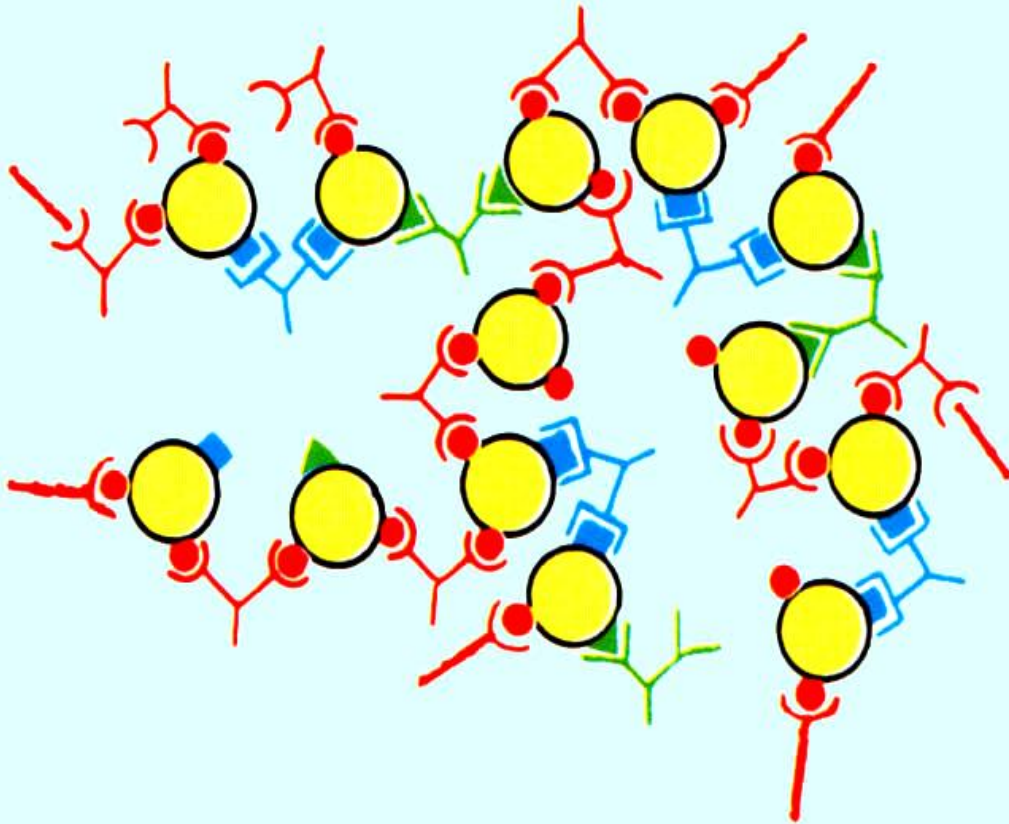
Schematic representation of the epitope recognized by a monoclonal antibody. When the different molecules have the same epitope, the monoclonal strongly reacts (top). Even if the monoclonals do show the totally different reactivity, partial identity to the epitope will give a weak reactivity to the monoclonal (bottom).

Modified from: Lane D, Koprowski H. Molecular recognition and the future of monoclonal antibodies. Nature 1982; 296(5854): 200-202.
doi: 10.1038/296200a0

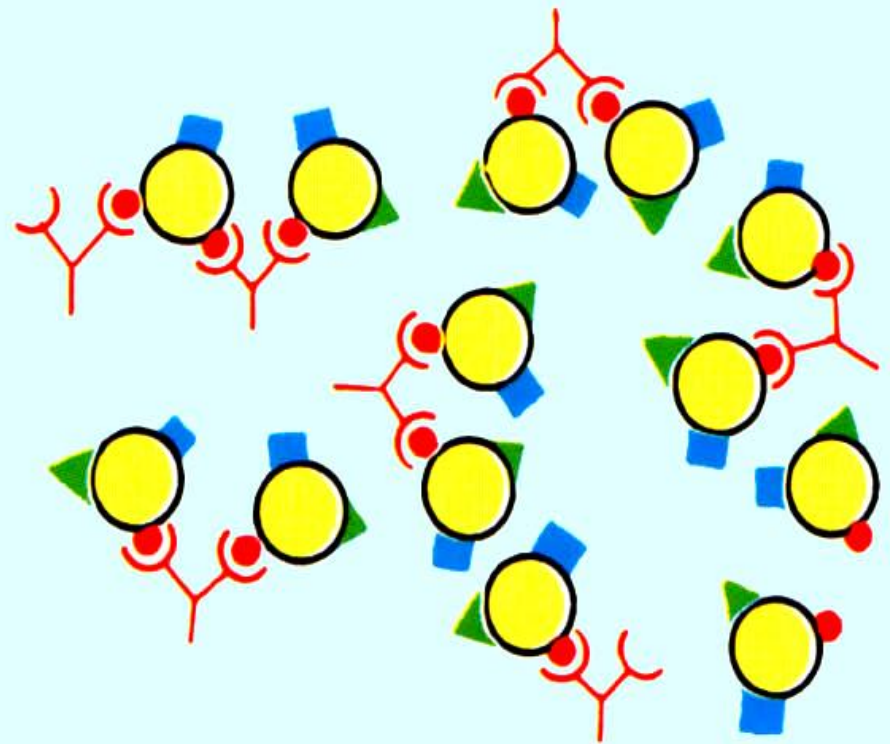


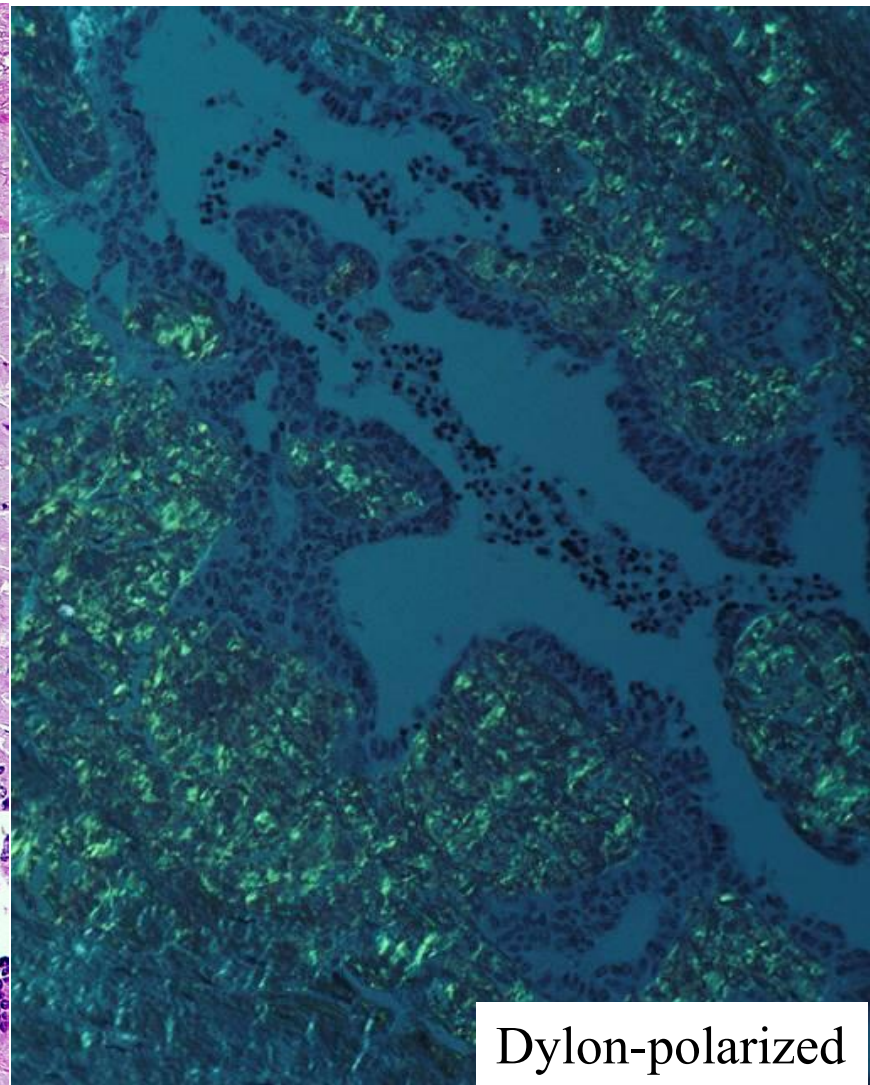
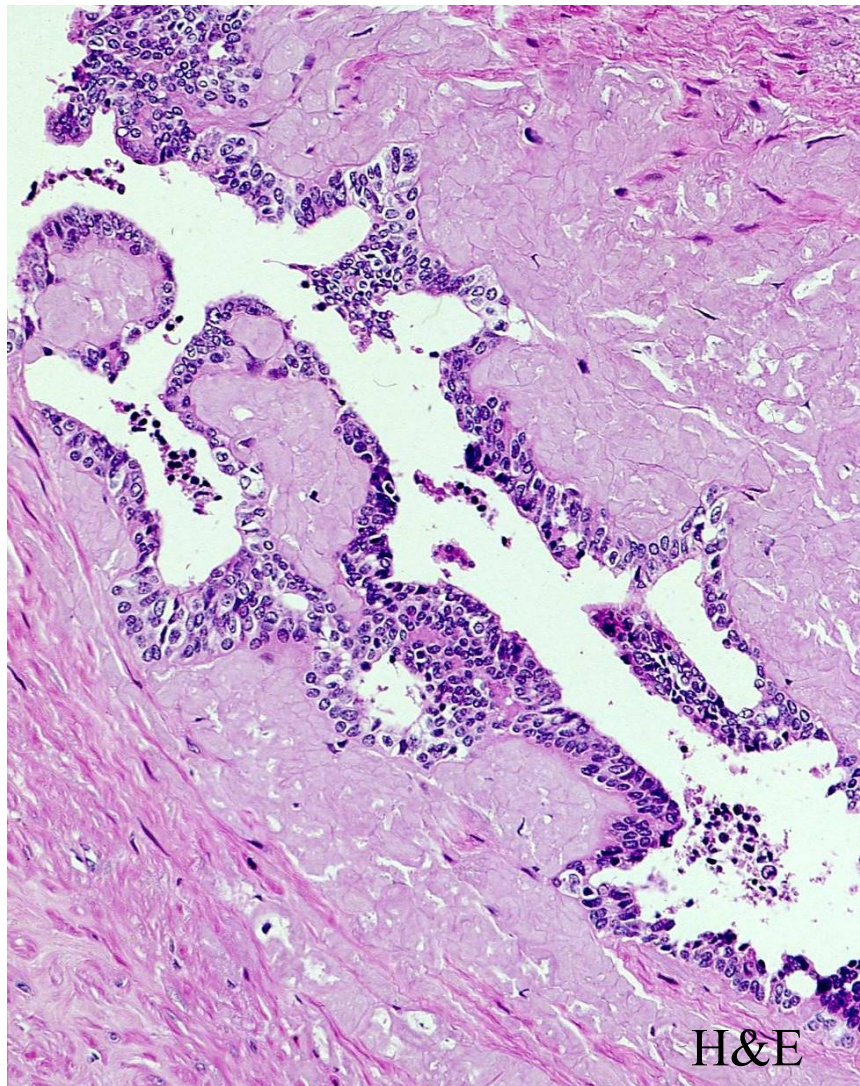
Antigen antibody reactions

polyclonal antibody

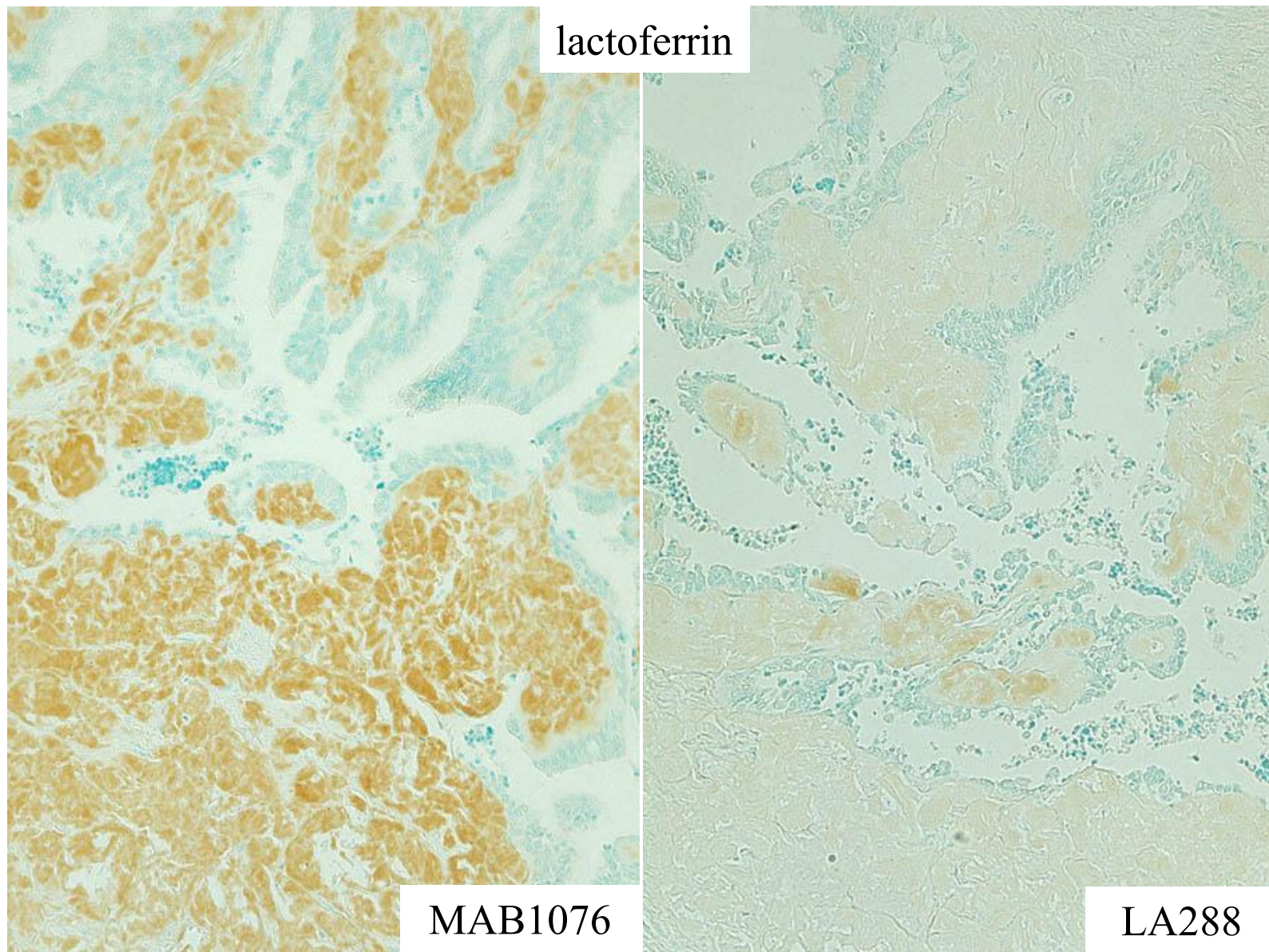


monoclonal antibody



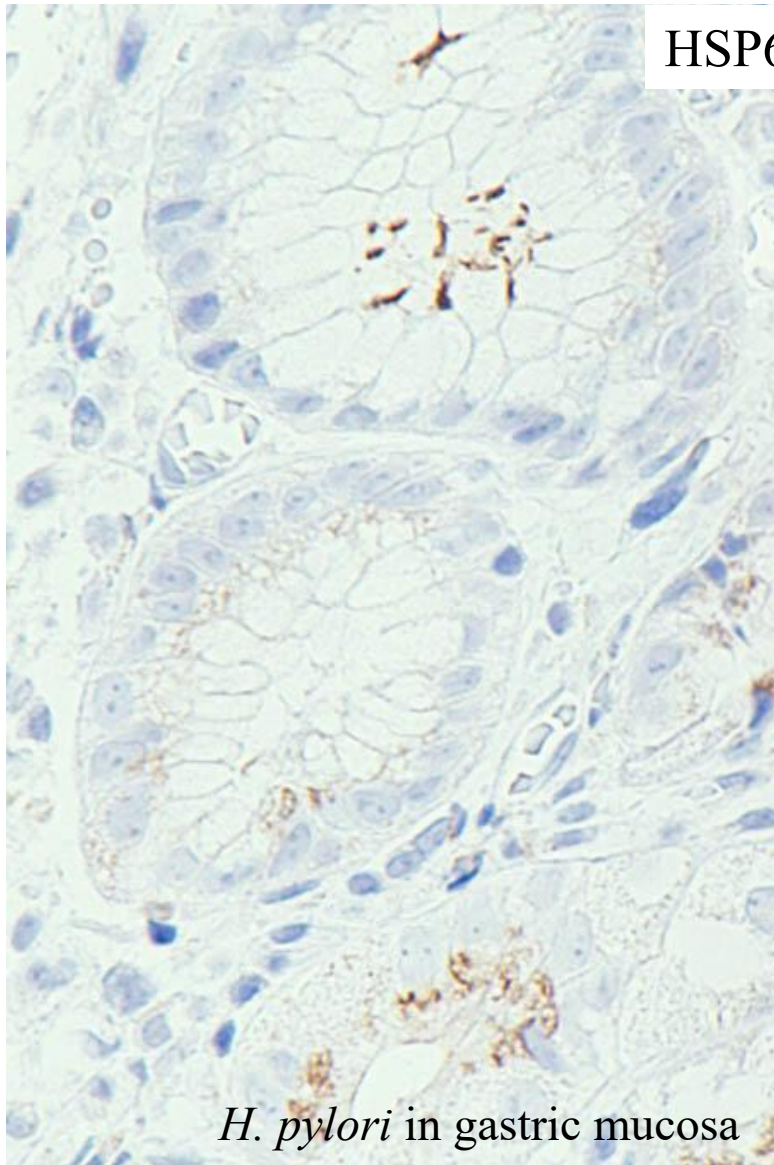


Localized amyloidosis of seminal vesicle. Left: H&E, right: polarized microscope after Dylan staining. Deposition of amorphous amyloid material is seen in the subepithelial stroma of the seminal vesicle.

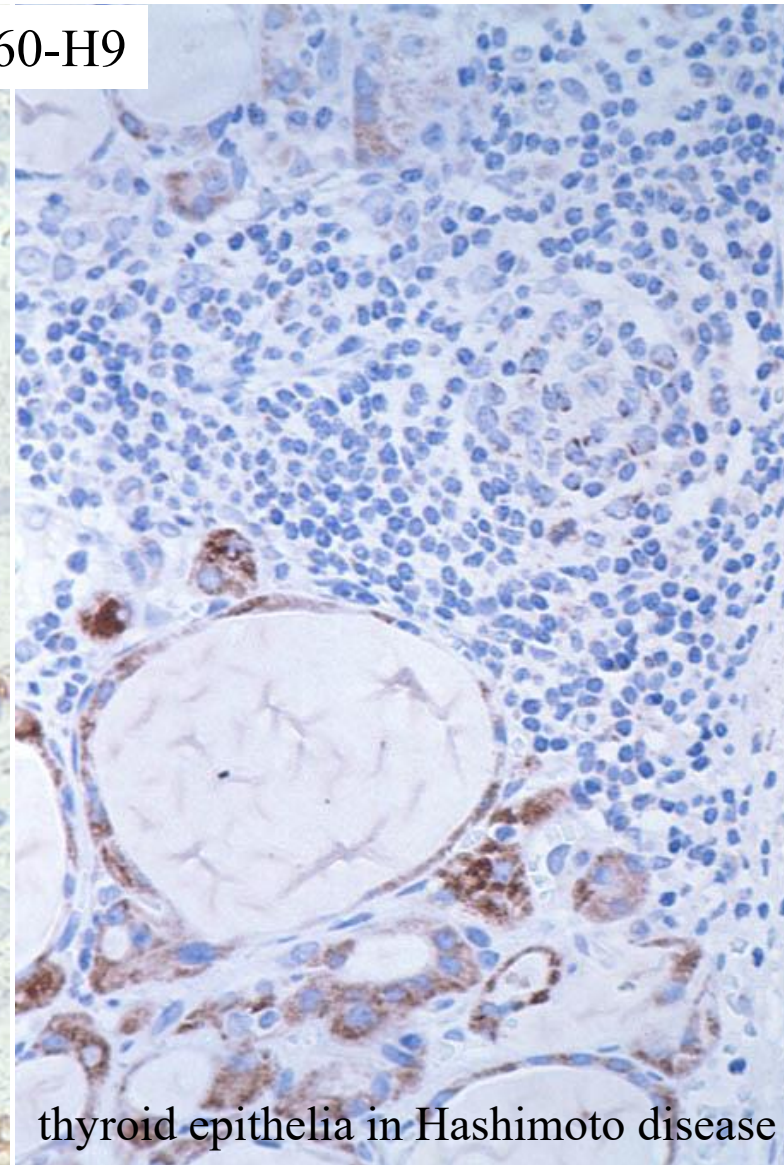


Immunostaining for lactoferrin in localized seminal vesicle amyloidosis using different monoclonal antibodies. The amyloid is diffusely positive with the monoclonal MAB1076, while the other monoclonal LA288 is only partially reactive to the amyloid material.

HSP60-H9



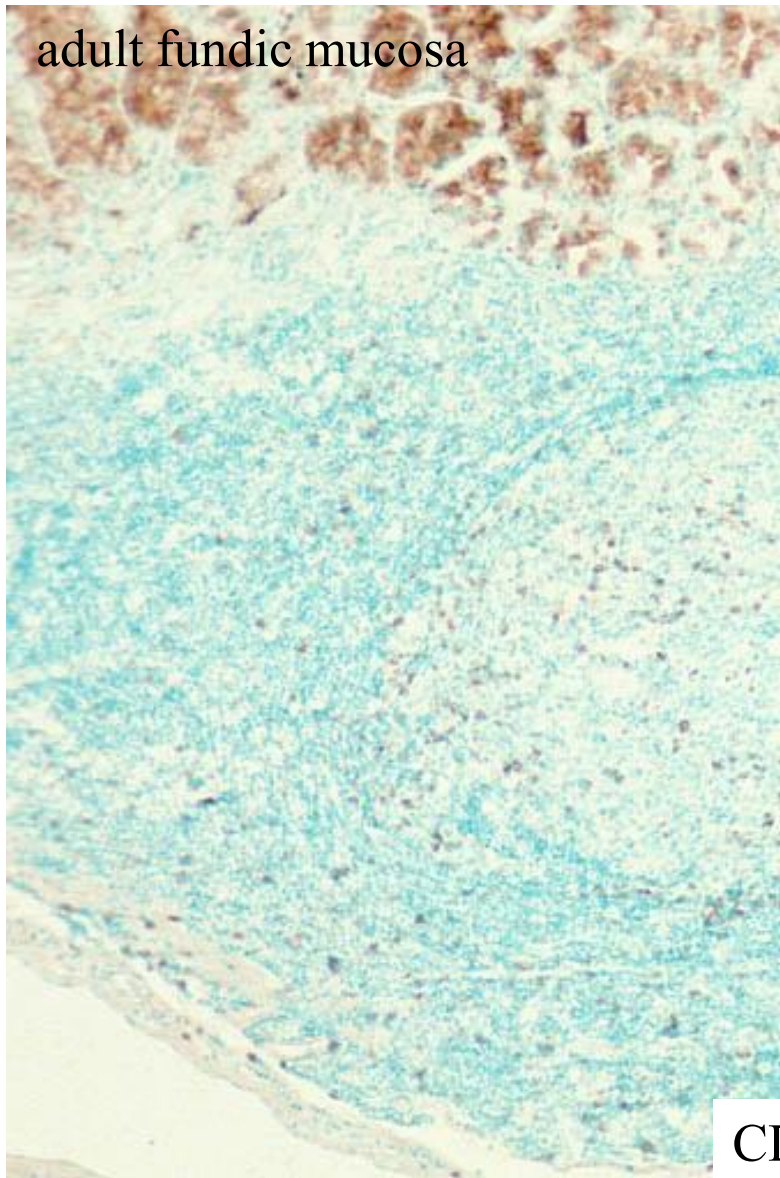
H. pylori in gastric mucosa



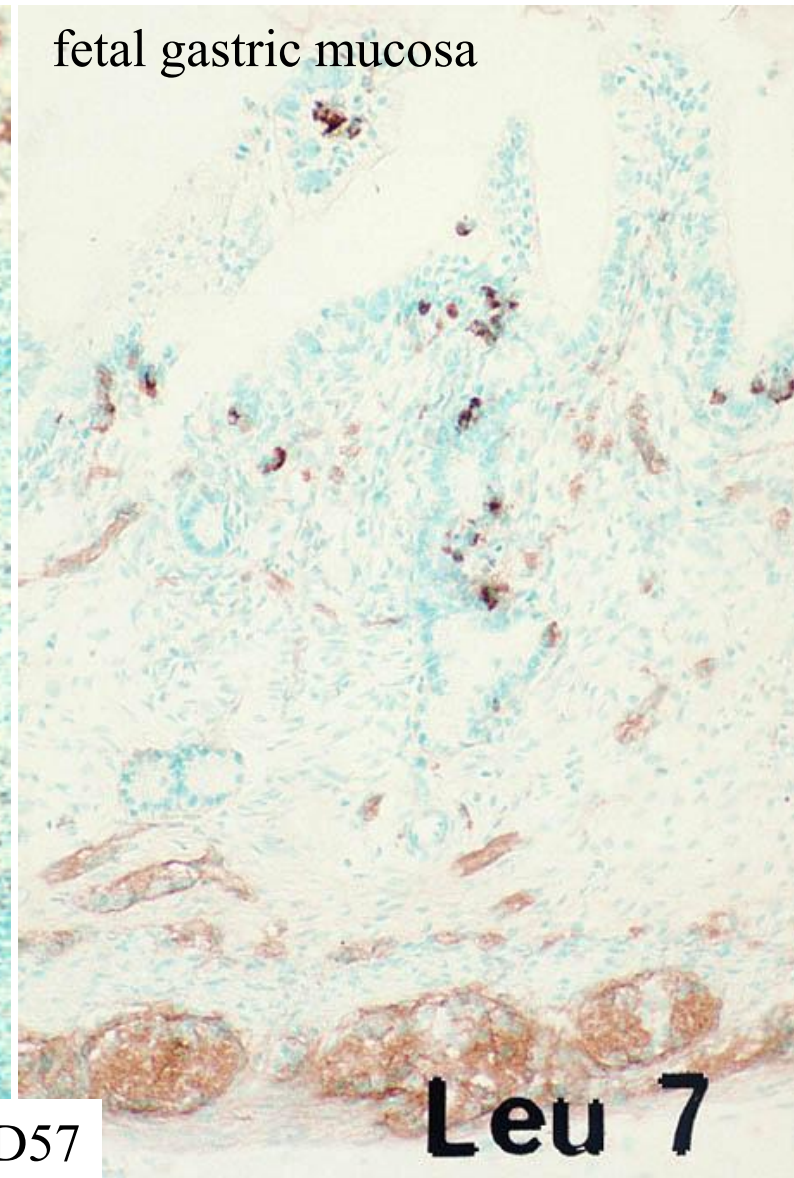
thyroid epithelia in Hashimoto disease

Cross-reactivity of bacterial heat shock protein 60 (HSP60). Monoclonal antibody H9 stains *Helicobacter pylori* infected on the gastric mucosa (left), while some human epithelial cells such as thyroid follicular cells are cross-reactively positive with the same antibody (right).

adult fundic mucosa



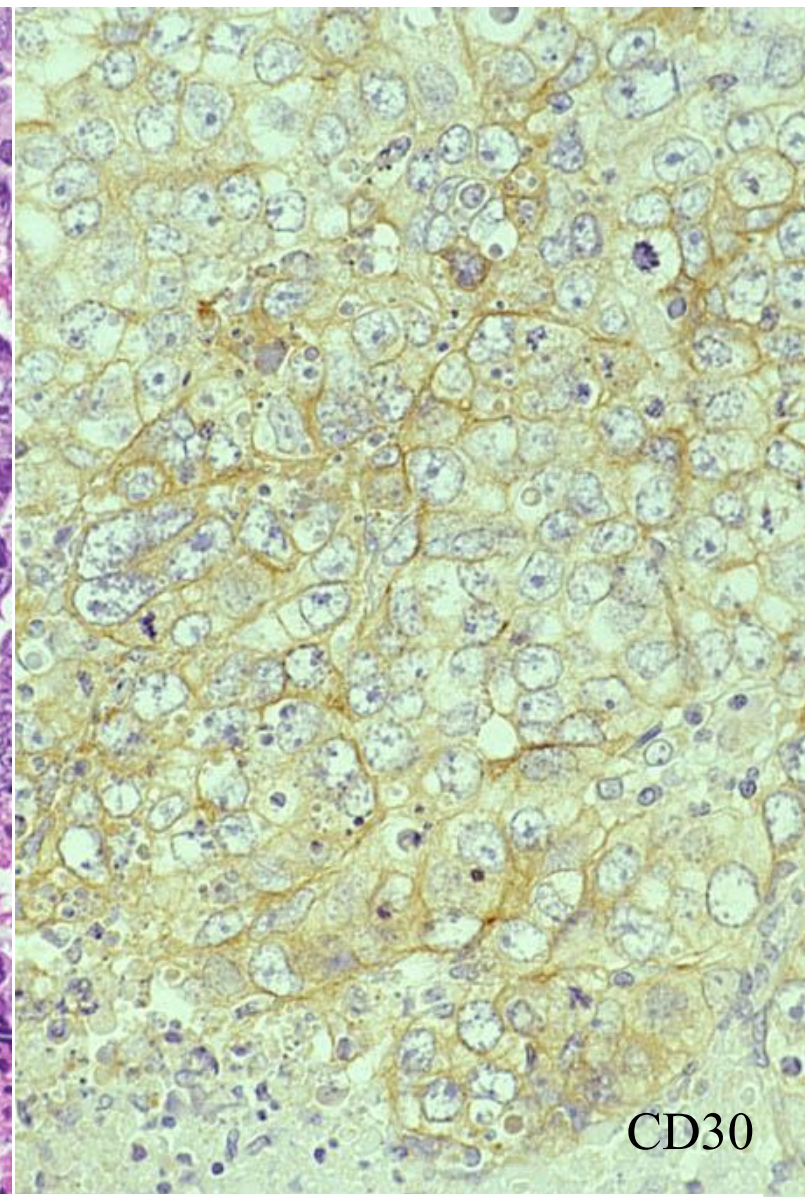
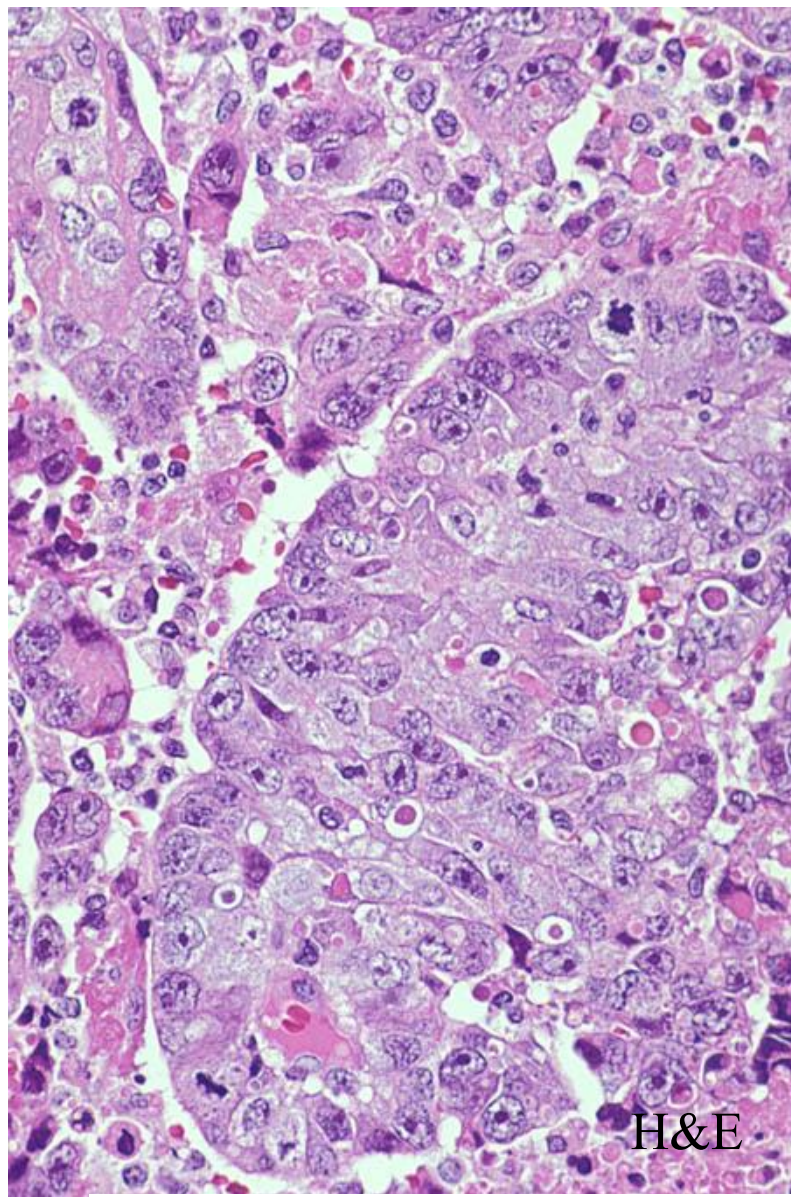
fetal gastric mucosa



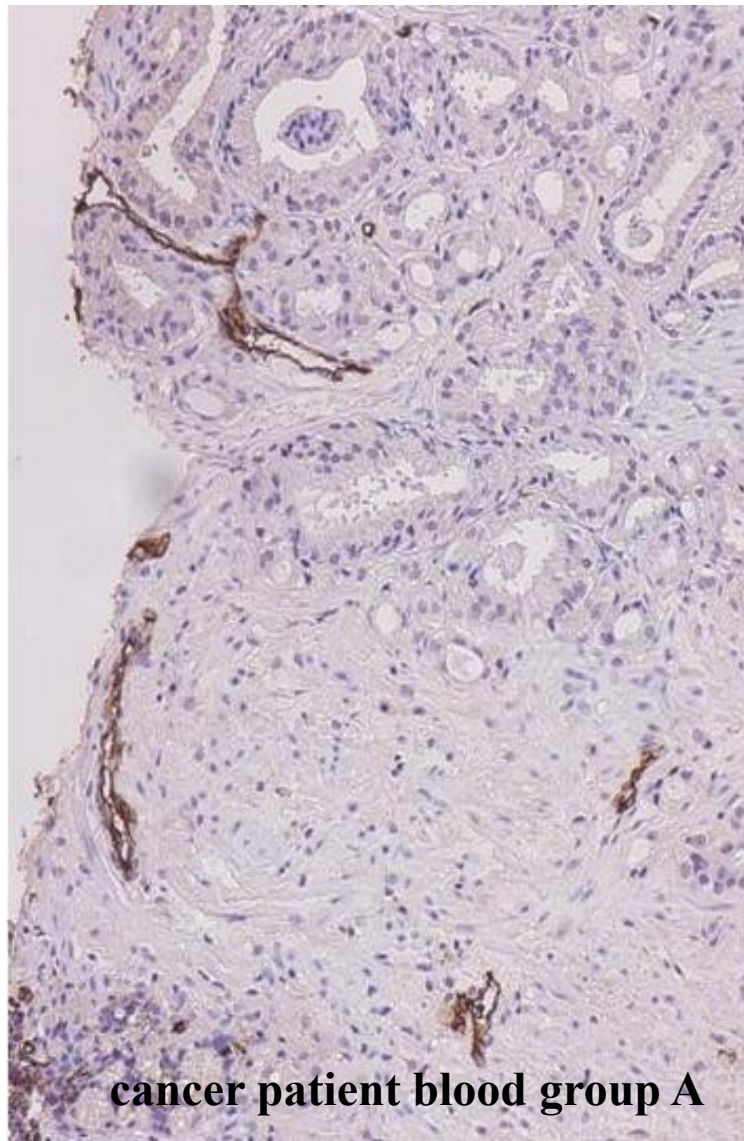
CD57

Leu 7

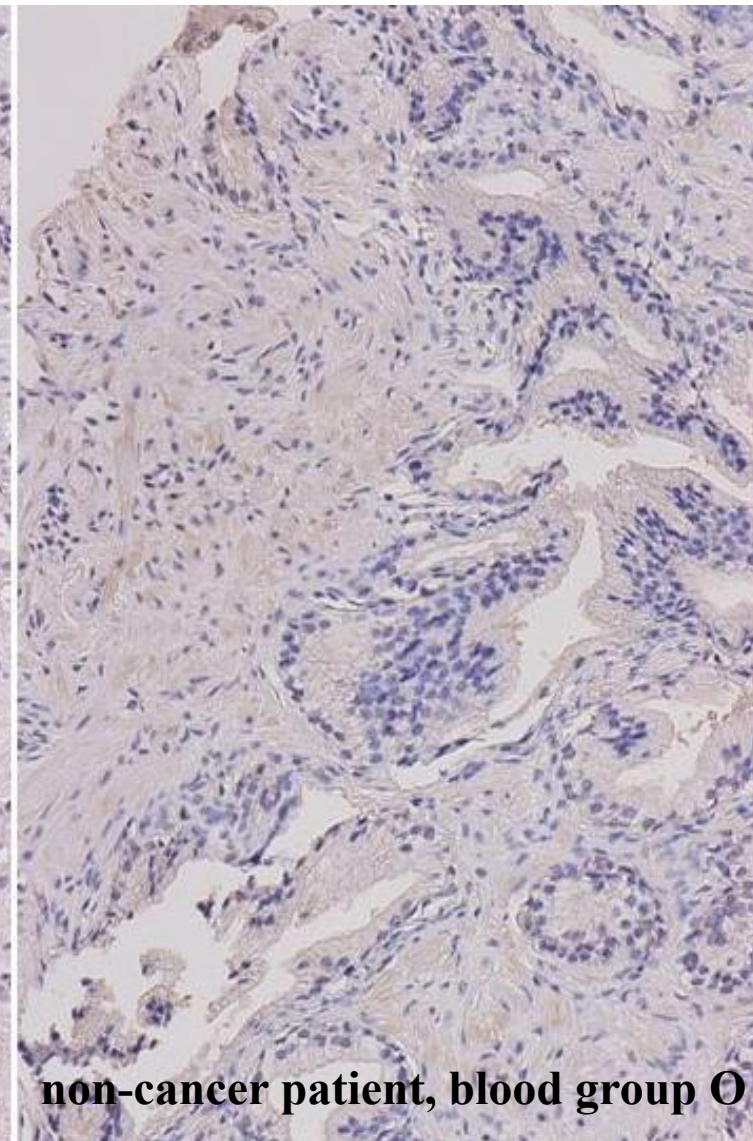
CD57 (Leu 7) immunoreactivity in the gastric mucosa. CD57 is a marker of NK cells (left) and neuroendocrine cells (right). However, the monoclonal is cross-reactive with the normal fundic glands (left) and peripheral nerve fibers (right).



CD30 expression in embryonal carcinoma. CD30, a marker of Ki-1 lymphoma and Hodgkin's lymphoma, is diffusely expressed on the tumor cells of embryonal carcinoma.



cancer patient blood group A

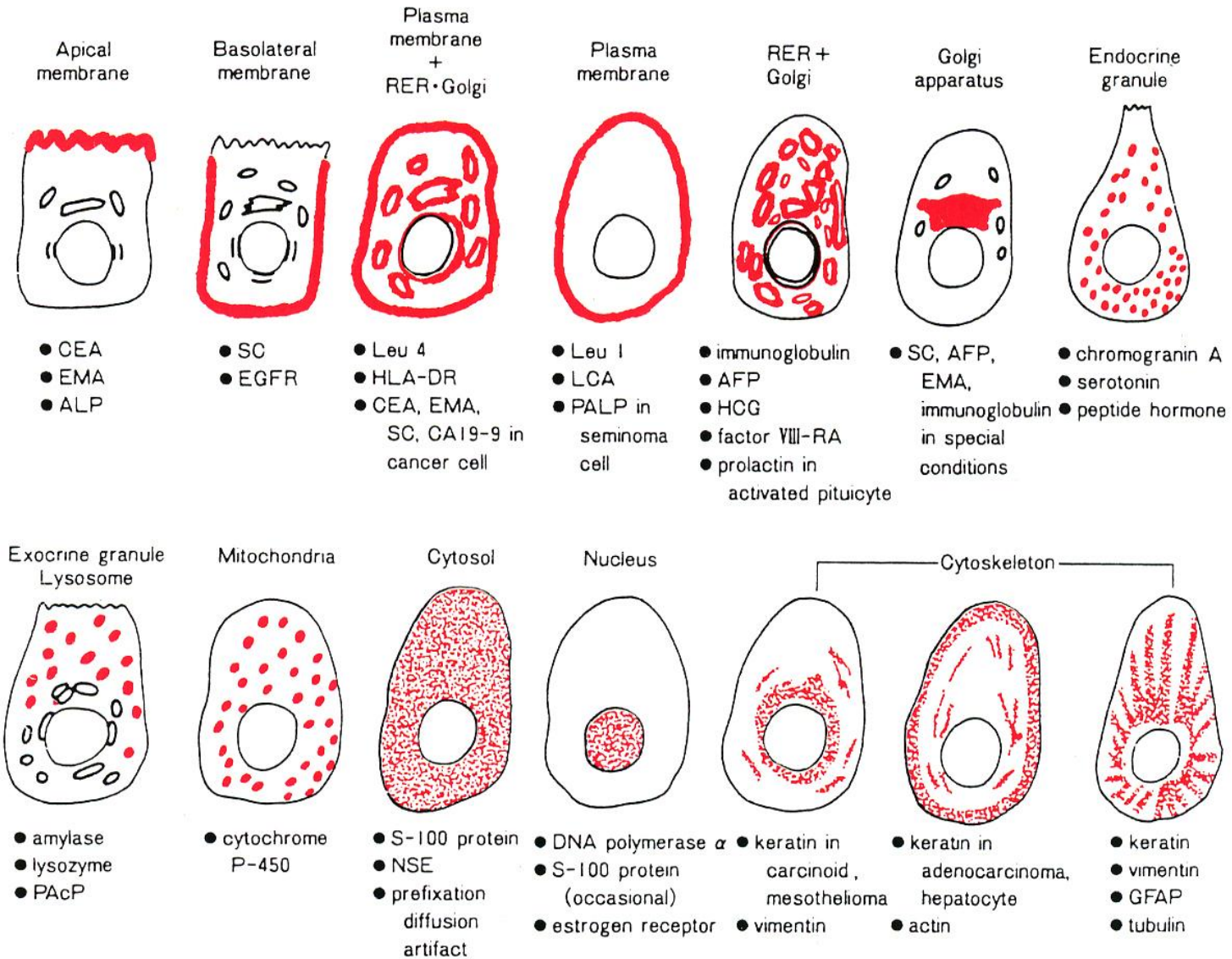


non-cancer patient, blood group O

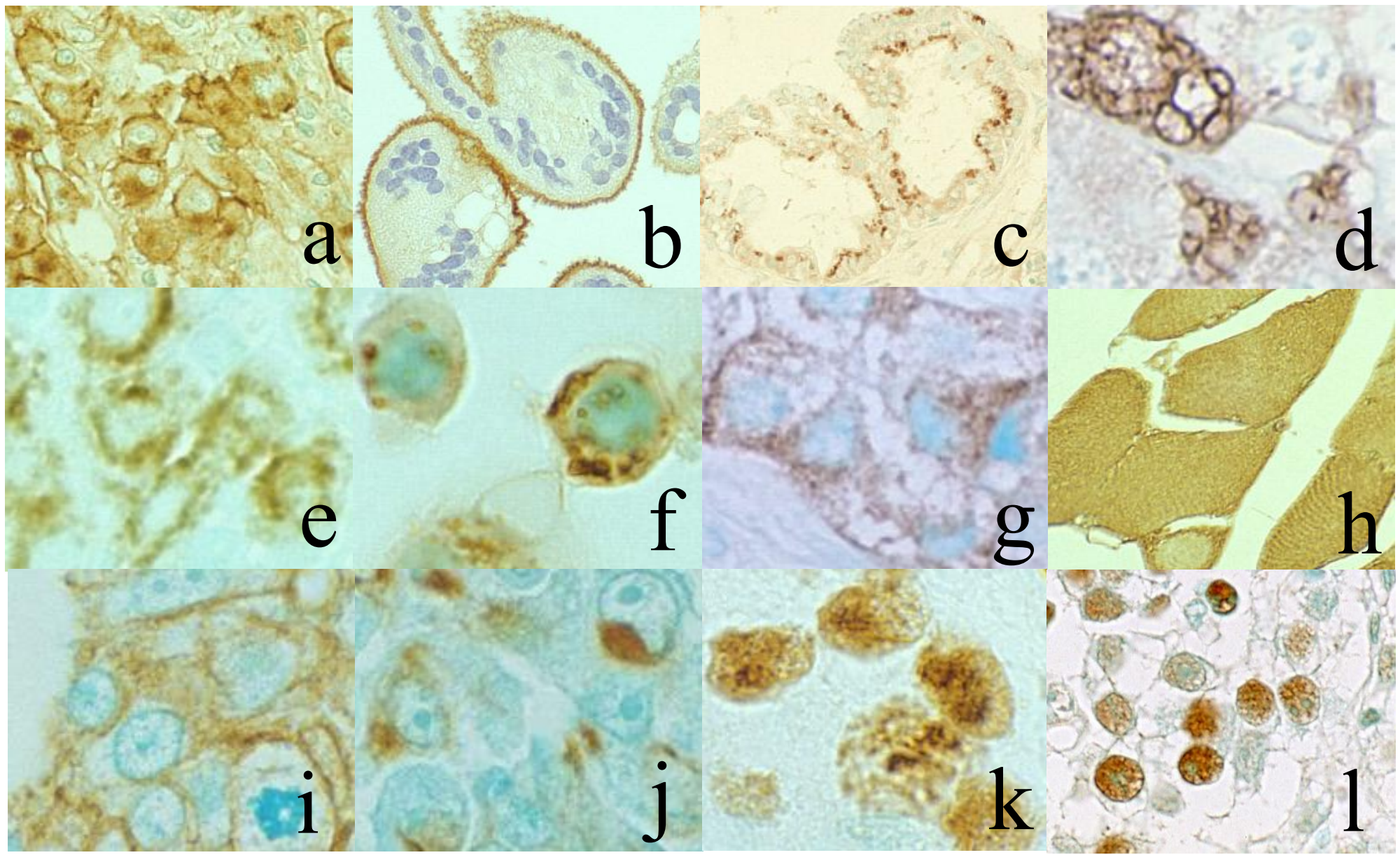
Contamination of the prostate biopsy specimens sampled from two patients with either blood group A and blood group O. Blood group determination of the biopsy specimens is applicable to identification of the patient with cancer. Immunostaining for blood group A discloses that the specimen with adenocarcinoma belongs to the individual with blood group A.

Intracellular localization pattern of antigens

The specificity of immunostaining can be judged by the intracellular localization pattern of the antigenic substances. Peptide hormones and chromogranin A are localized as intracytoplasmic fine granules. Lysosomal and mitochondrial proteins are visualized as intracytoplasmic coarse granules. Secretory proteins such as alpha-fetoprotein, human chorionic gonadotropin and immunoglobulins are seen as fine or coarse vesicles in the cytoplasm, representing the ultrastructural localization in the rough endoplasmic reticulum and Golgi apparatus. Particularly in neoplastic cells, immunoreactivity of secretory proteins may be accentuated and clustered in the Golgi area. Diffuse cytoplasmic reactivity is seen for cytosolic proteins such as myoglobin, NSE, creatine kinase-mm isozyme and GST. S-100 protein, heat-shock proteins, ubiquitin and β -catenin are distributed diffusely in both the cytoplasm and nucleus. The nuclei are diffusely positive for Ki-67 (MIB-1), p53, CDX2, ER and PgR, but nucleolar staining is frequently accentuated for Ki-67. Plasma membrane proteins reveal distinct membrane staining, and the Golgi area is also frequently positive. Carcinoembryonic antigen (CEA) is expressed along the apical plasma membrane in normal gastric and colonic mucosa, while in adenocarcinomas of the stomach and colon, the plasma membranes are circumferentially positive. Such intracellular antigen distribution patterns are clearly recognized in formalin-fixed, paraffin-embedded sections.



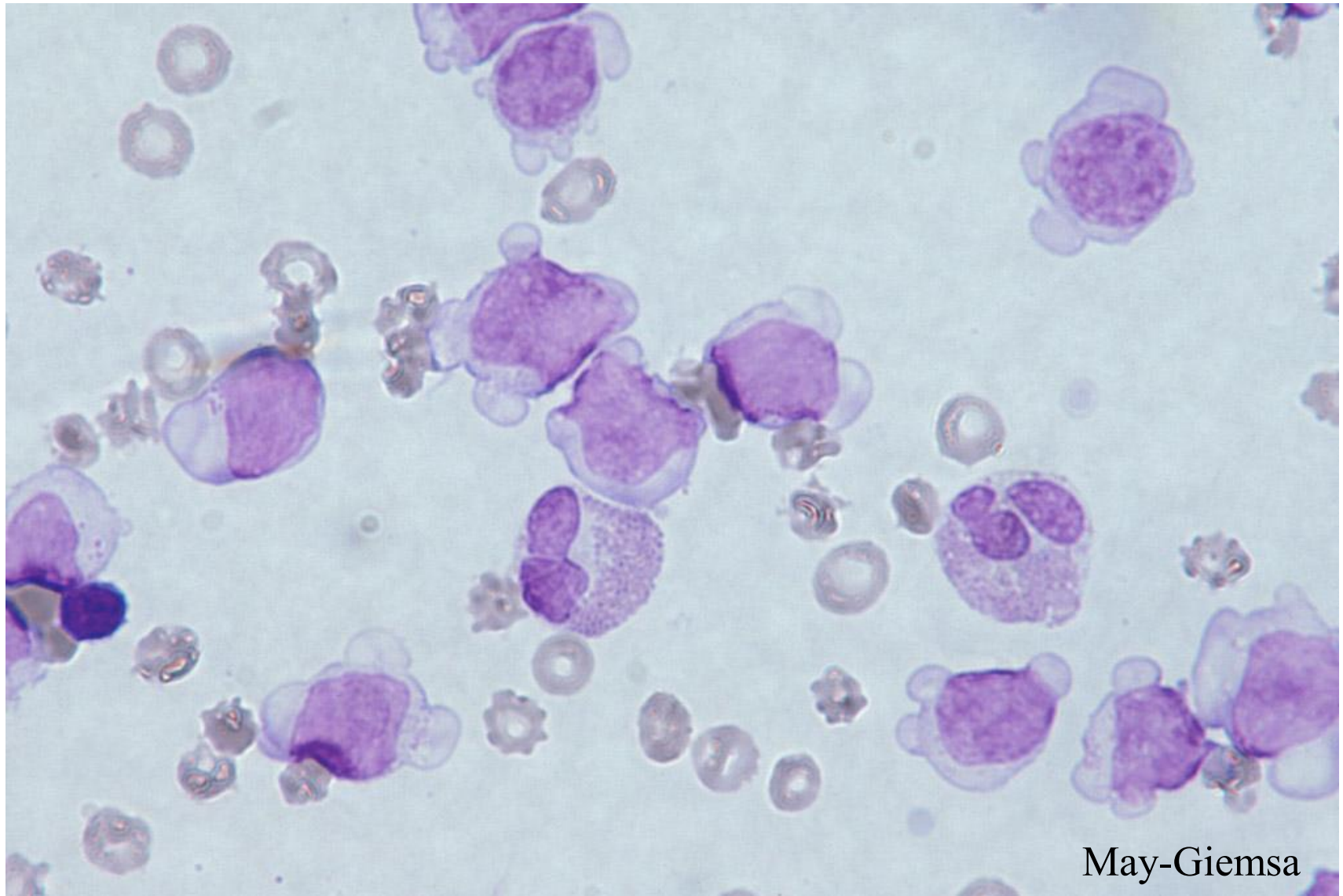
Antigen expression pattern in the cells



Representative intracellular localization of antigens. a: CD30 (Ki-1 lymphoma), b: PALP (placenta), c: CA125 (serous ovarian adenocarcinoma), d: IgG (plasmacytoma), e: intrinsic factor (parietal cells), f: NCA (lung adenocarcinoma cells PC-9), g: insulin (insulinoma), h: CPK-mm (striated muscle), i: cytokeratin (well-differentiated gastric adenocarcinoma), j: cytokeratin (poorly differentiated gastric adenocarcinoma), k: Ki-67 (breast cancer), l: PCNA (seminoma)

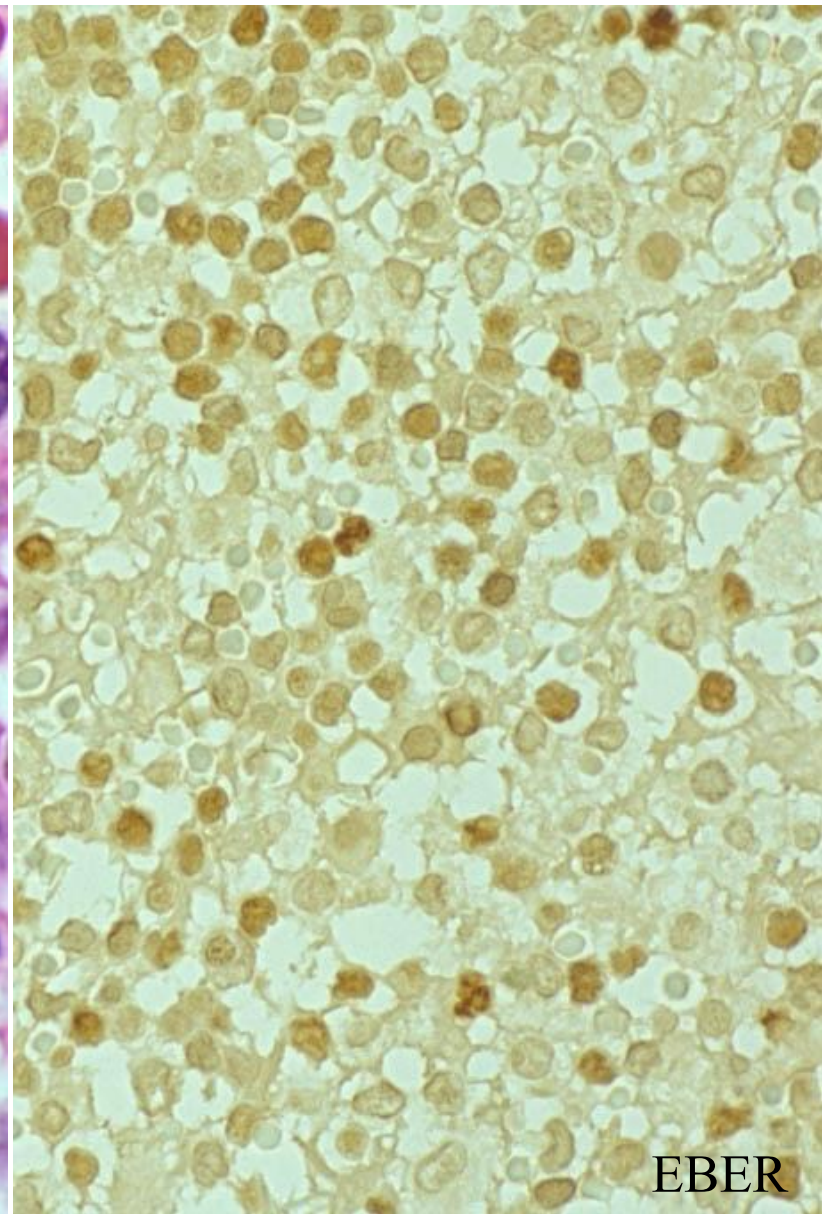
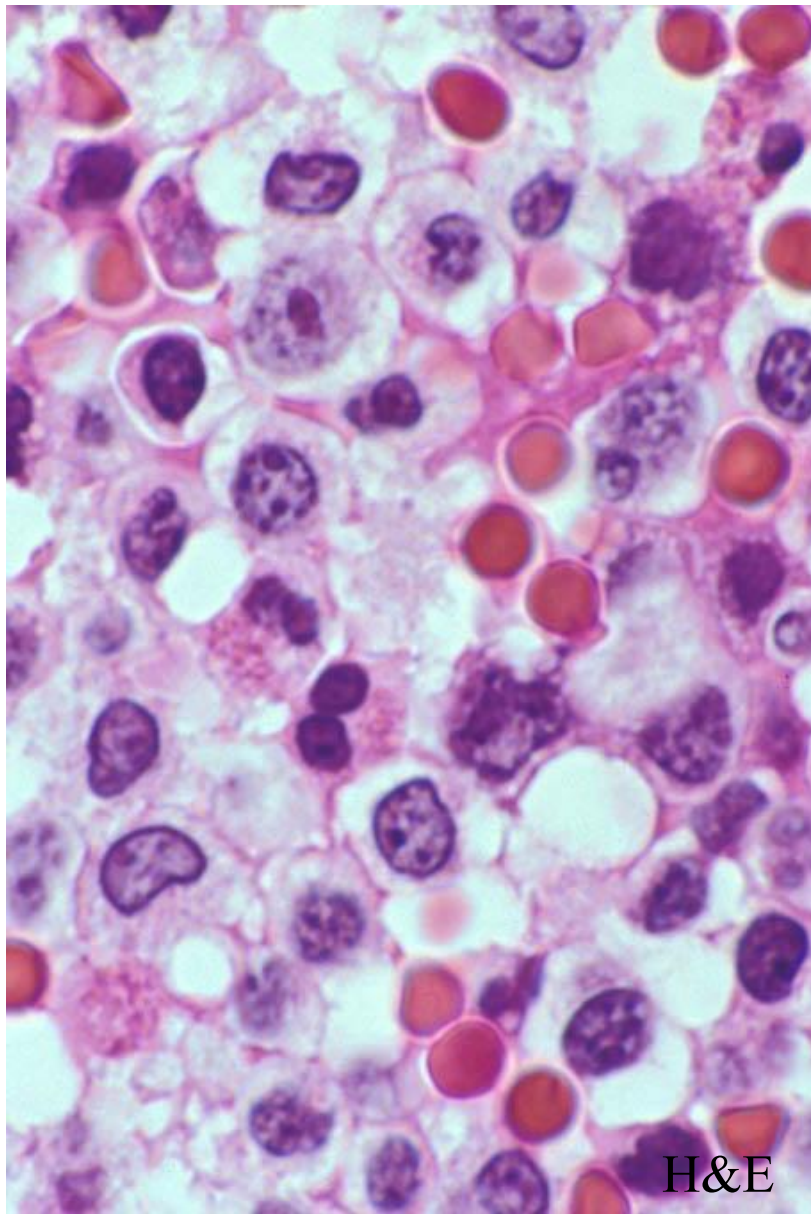
Immunocytochemistry using cytology specimens

- 1) Use of cell block
- 2) Re-staining method using a single slide
- 3) Cell transfer method
(Mounting material, such as Malinol and MountQuick)
- 4) Application of the cell transfer method
(Transfer of cultured cells, repair of broken glass slide,
DNA sampling for PCR)



May-Giemsa

Large granular lymphocytes and eosinophils in hemorrhagic ascitic fluid in a young male case of chronic active EBV infection (May-Giemsa)

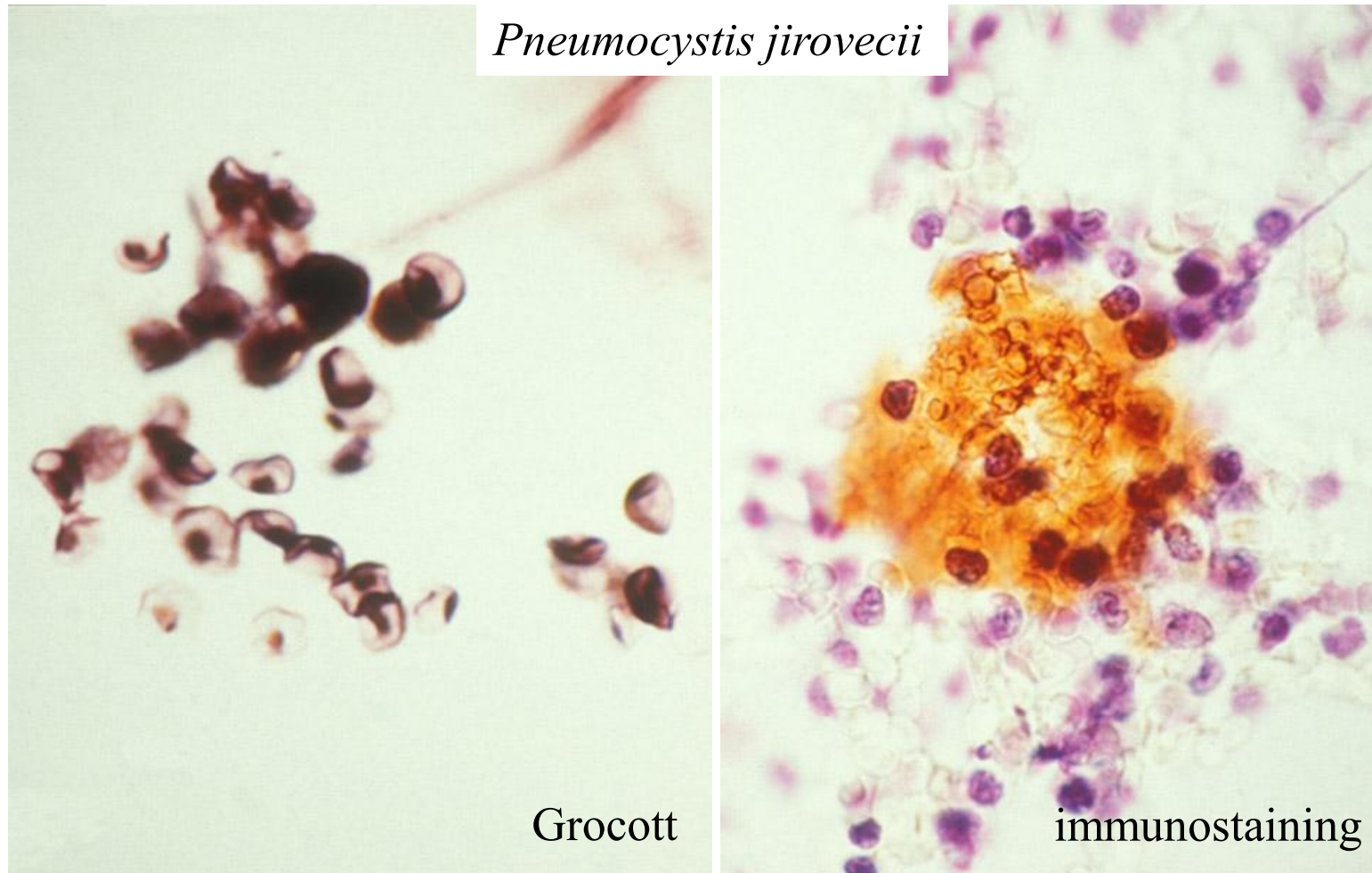


Cell block prepared from the ascites, demonstrating EBER reactivity in the nuclei. The diagnosis of chronic active EBV infection was made.

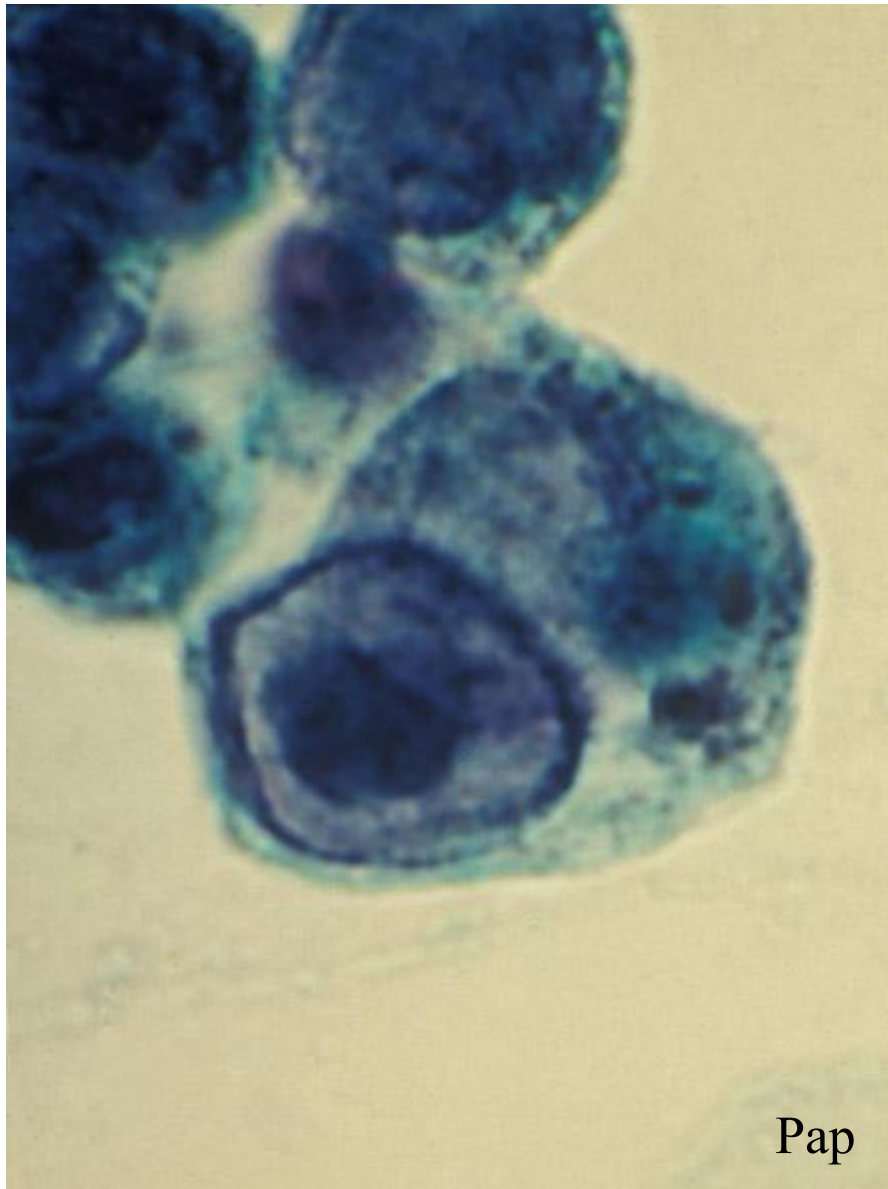
How to use a single cytology slide for immunocytochemistry

- a) Re-staining procedure
- b) Transfer of smears to another glass slide

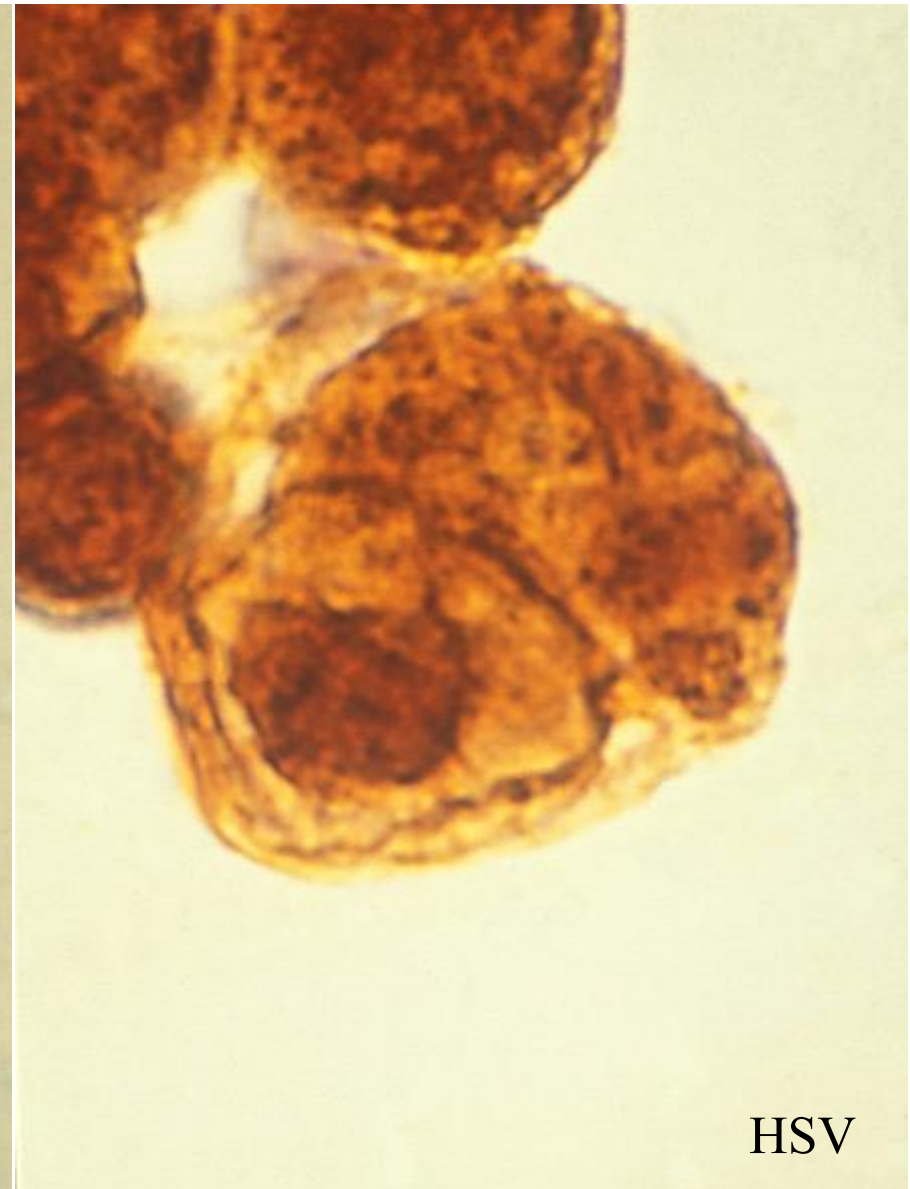
When plural specimens can be prepared



Pneumocystis jirovecii pneumonia. Grocott staining in broncho-alveolar lavage (BAL) material (left). Dot-like structure is observed in the cysts. In an additional cytology material, immunostaining for *P. jirovecii* using a monoclonal antibody can be performed (right).

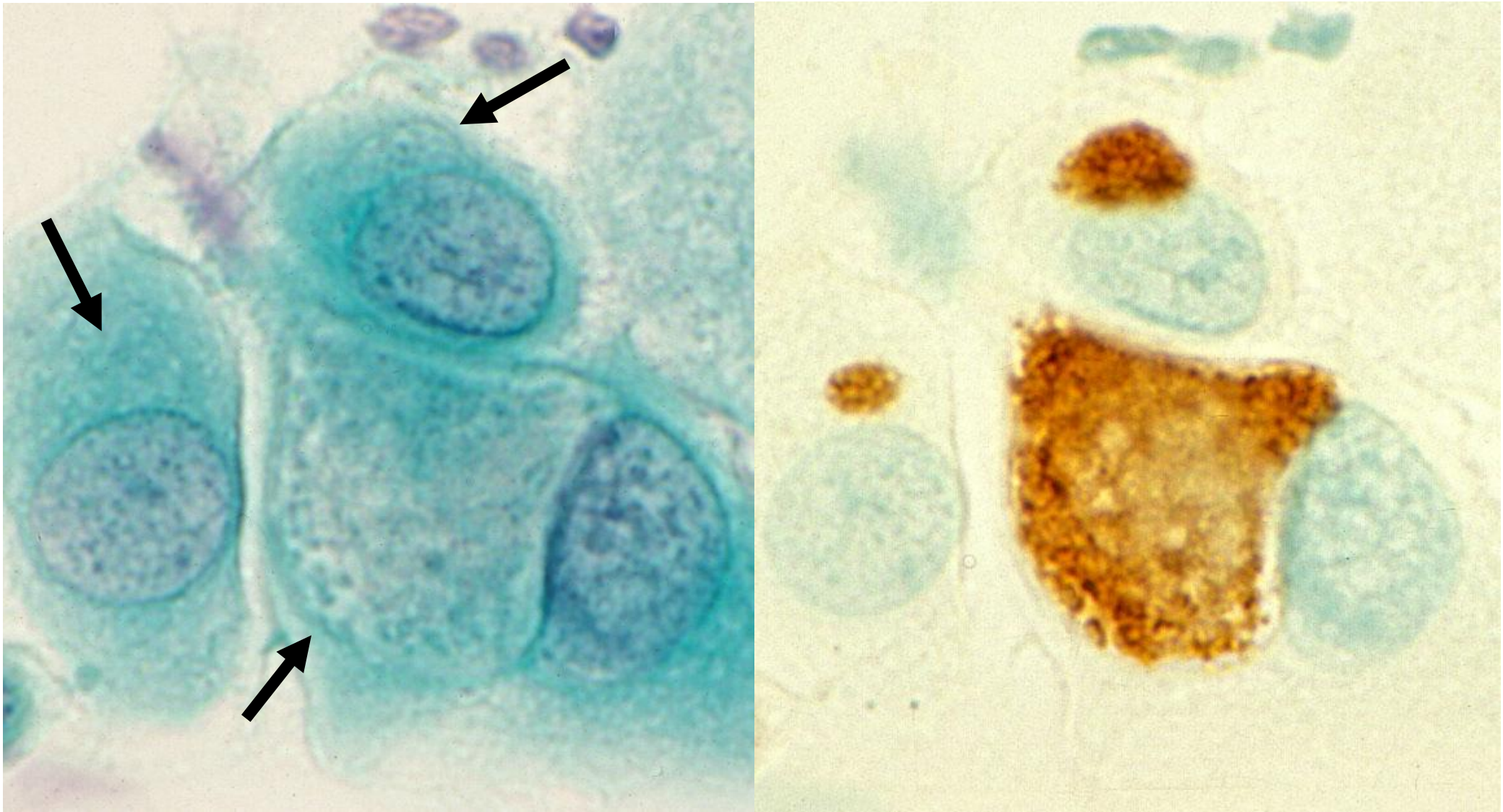


Pap

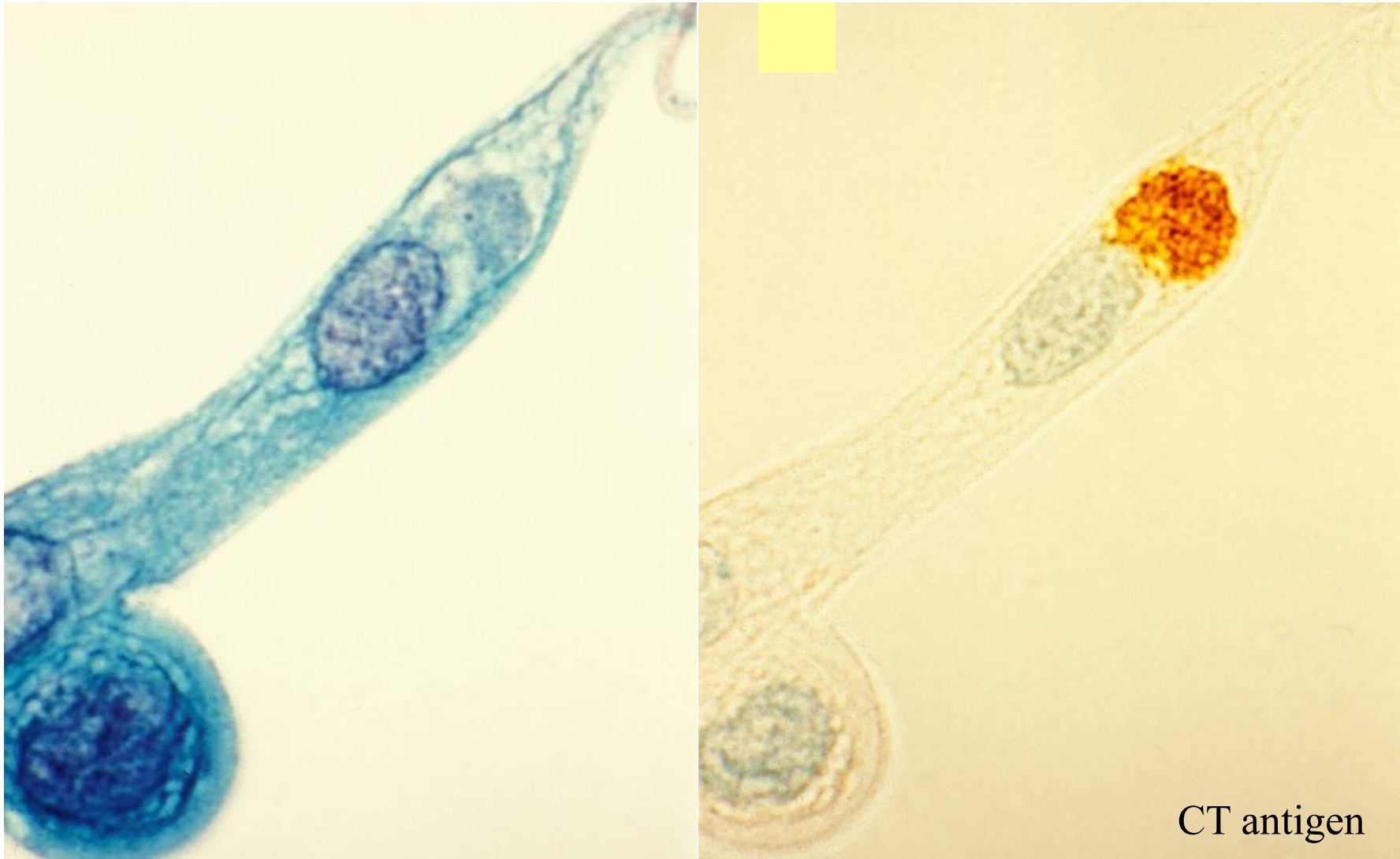


HSV

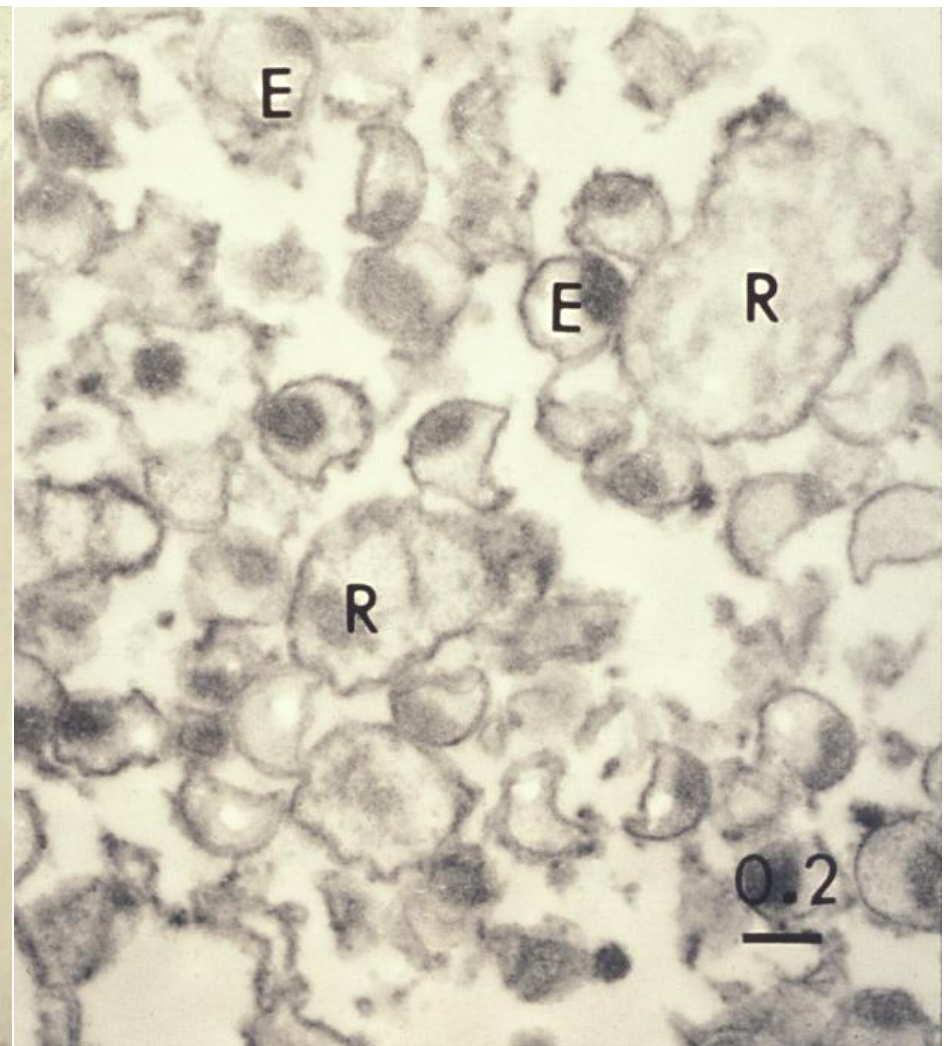
Re-staining method: herpes simplex viral antigen was restained using a single slide of the cervical smear. The HSV antigen is localized both in the nuclei and cytoplasm.



Demonstration of *Chlamydia trachomatis* antigen in the nebular inclusion bodies in the cervical smear preparation. A 20 y-o female patient complained of increased fluor. Pap smear reveals nebular inclusion bodies (arrows, left). The restaining method demonstrates chlamydial antigen in the inclusions (right).

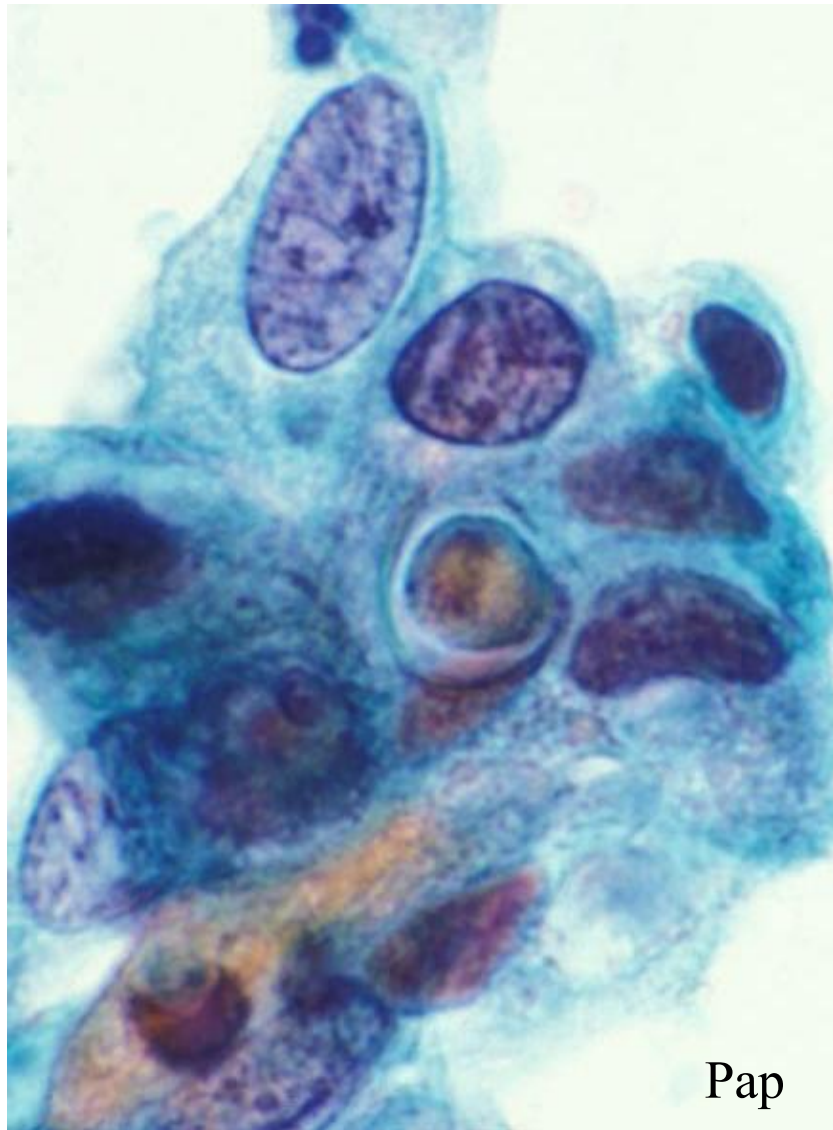


Restaining method: *Chlamydia trachomatis* antigen is visualized in the neovular inclusion body in the cytoplasm of a metaplastic cell seen in the cervical smear.

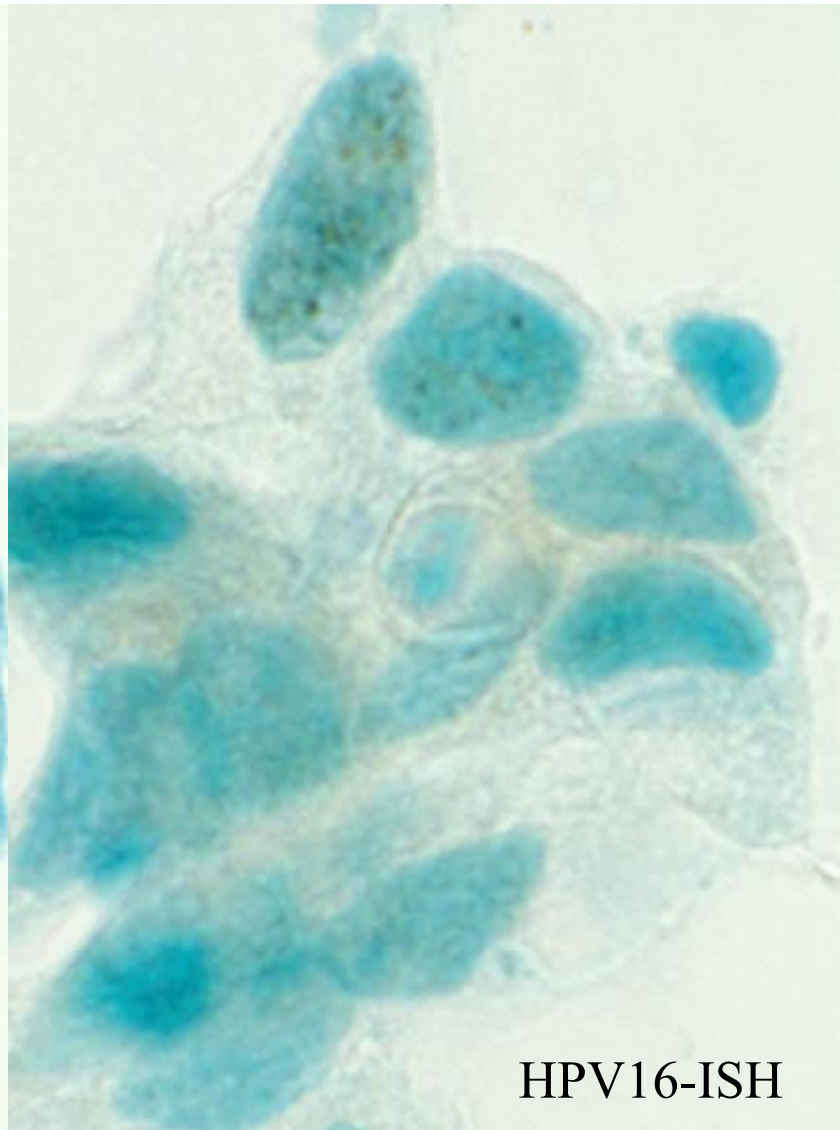


Immunoelectron microscopy for CT antigen

Immunoelectron microscopy, pre-embedding method, using an ethanol-fixed cervical smear of chlamydial cervicitis (see also the previous slide). The cell membrane of *Chlamydia trachomatis* is clearly labeled even after routine alcohol fixation. E: elementary bodies (infectious form), R: reticulate bodies (proliferative form). **Ref.:** Hori S, et al. Immunoelectron microscopic detection of chlamydial antigens in papanicolaou-stained routine vaginal smears. Acta Cytol 1995; 39(4): 835-837. PMID: 7631568

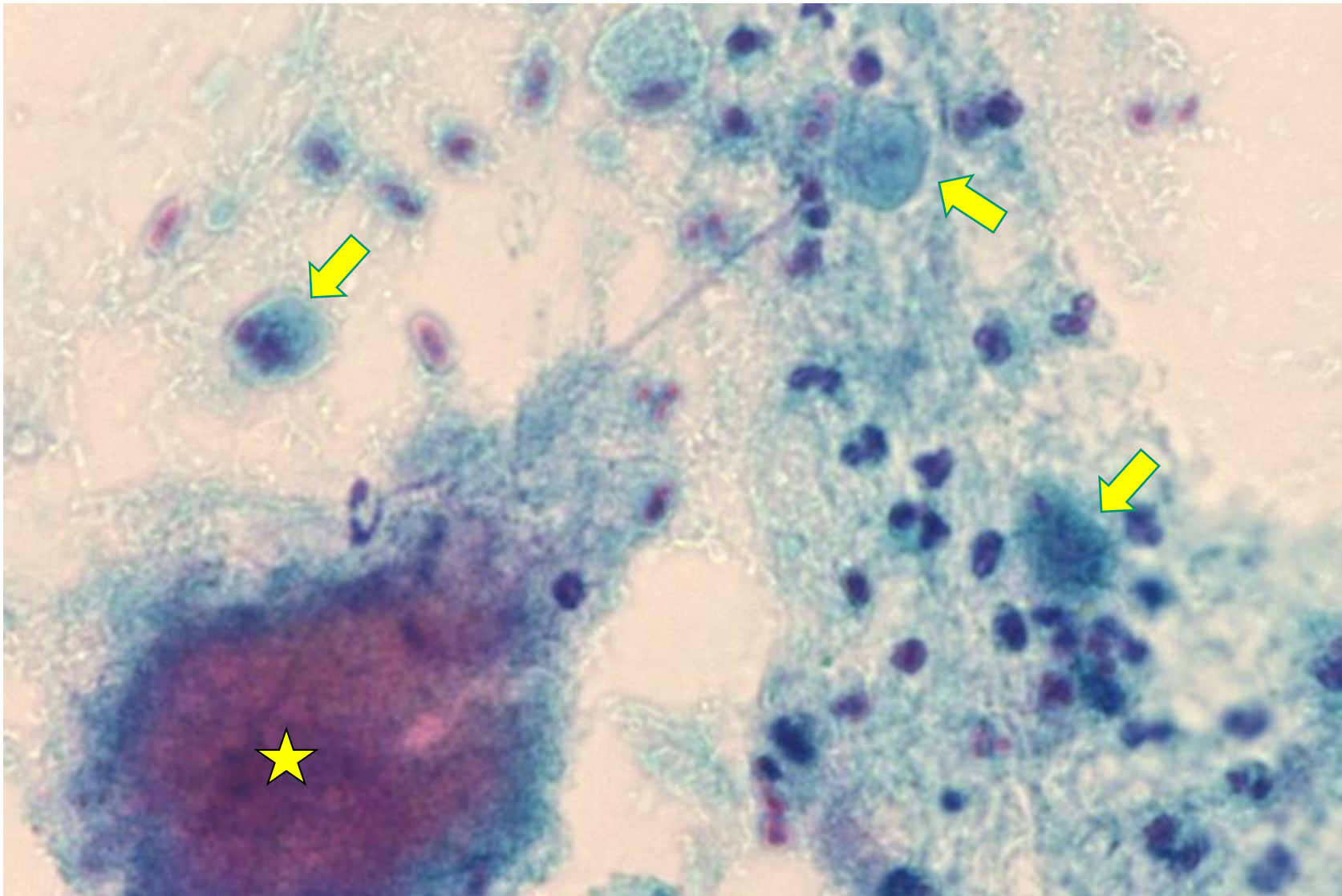


Pap



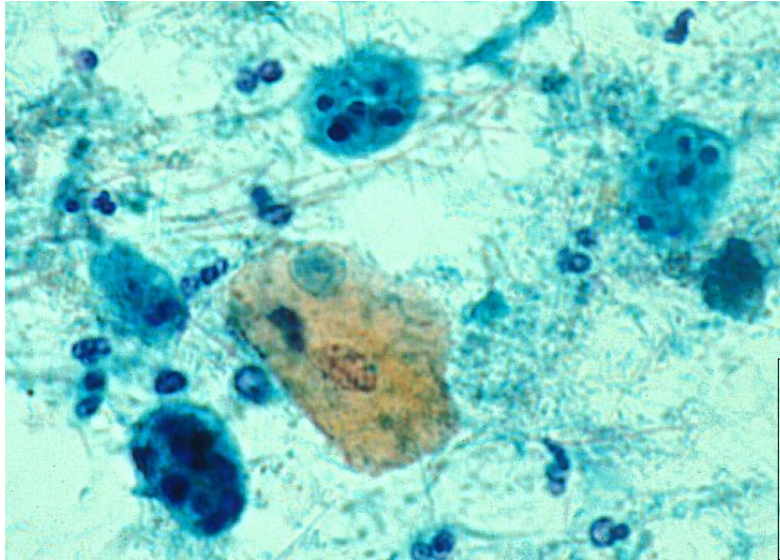
HPV16-ISH

Re-staining method after cell transfer to silane-coated glass slide. Dot-like intranuclear signals of HPV16 genome are observed in the same cells of severe dysplasia of the cervix. The cell transfer technique is indispensable for performing ISH, because a heating step for making double-stranded DNA into single-stranded is included in ISH.



A 50 y-o female patient with prolonged insertion of contraceptive device into the uterine cavity complained of abnormal fluor. The device was removed, and the cytology specimen of exudates around the device shows actinomycotic grains (asterisk) and infection of amebic trophozoites phagocytizing neutrophils (arrows) (pap)

PCR for *Entamoeba* species



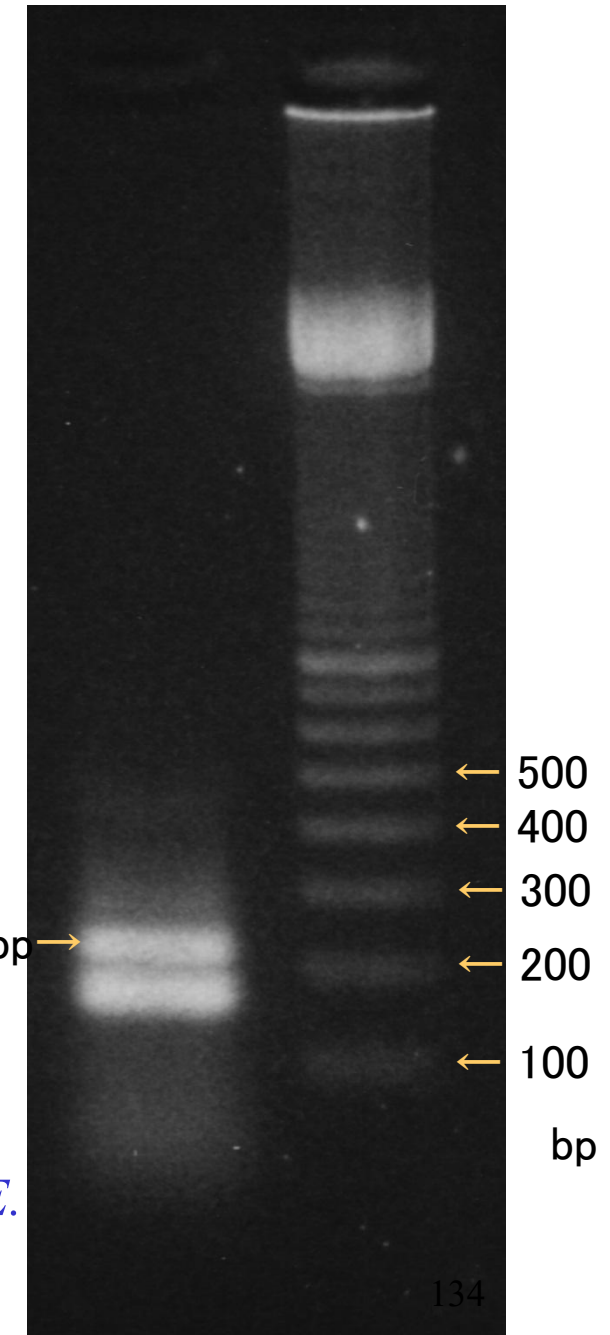
Entamoeba gingivalis
seen in the dental
plaque, phagocytizing
neutrophils

Primers sense 5'– tcagataccgtcgtagtcct – 3'
 antisense 5'– cctggtgtgcccttcct – 3'

PCR consitions: 94°C15 sec、55°C30 sec、72°C30 sec : 35 cycles

221bp →

The 221bp fragment: High homology with *E. gingivalis* genome

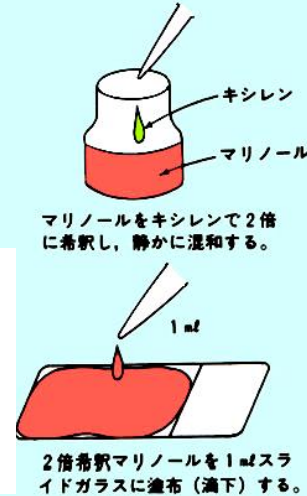


Colonization of *E. gingivalis* in the uterine cavity was confirmed. The colonization was mediated by oral sex. Both *Actinomyces* and *E. gingivalis* are normal flora of the oral cavity. The presence of the foreign material (IUD) accelerated the sexually transmitted infection.

Techniques for cell transfer

The multistep procedures are time-consuming, but valuable for extended cytological evaluations.

Cover the slide with Malinol mounting solution



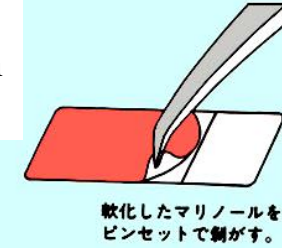
Solidification of the Malinol membrane on a hot plate



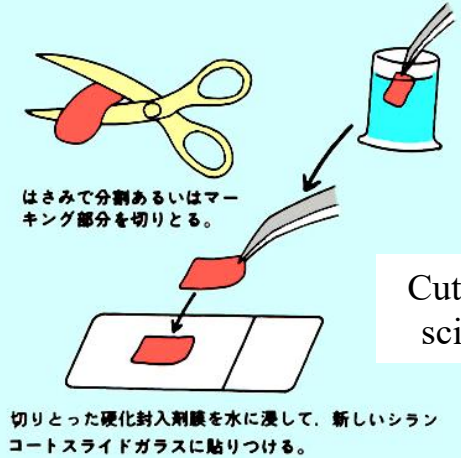
Soak the section in warm water



Detach the cover slip in xylene

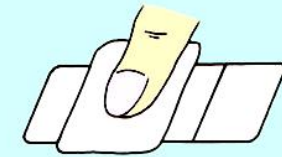


Peel off the membrane

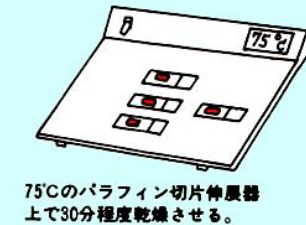


Cut with scissor

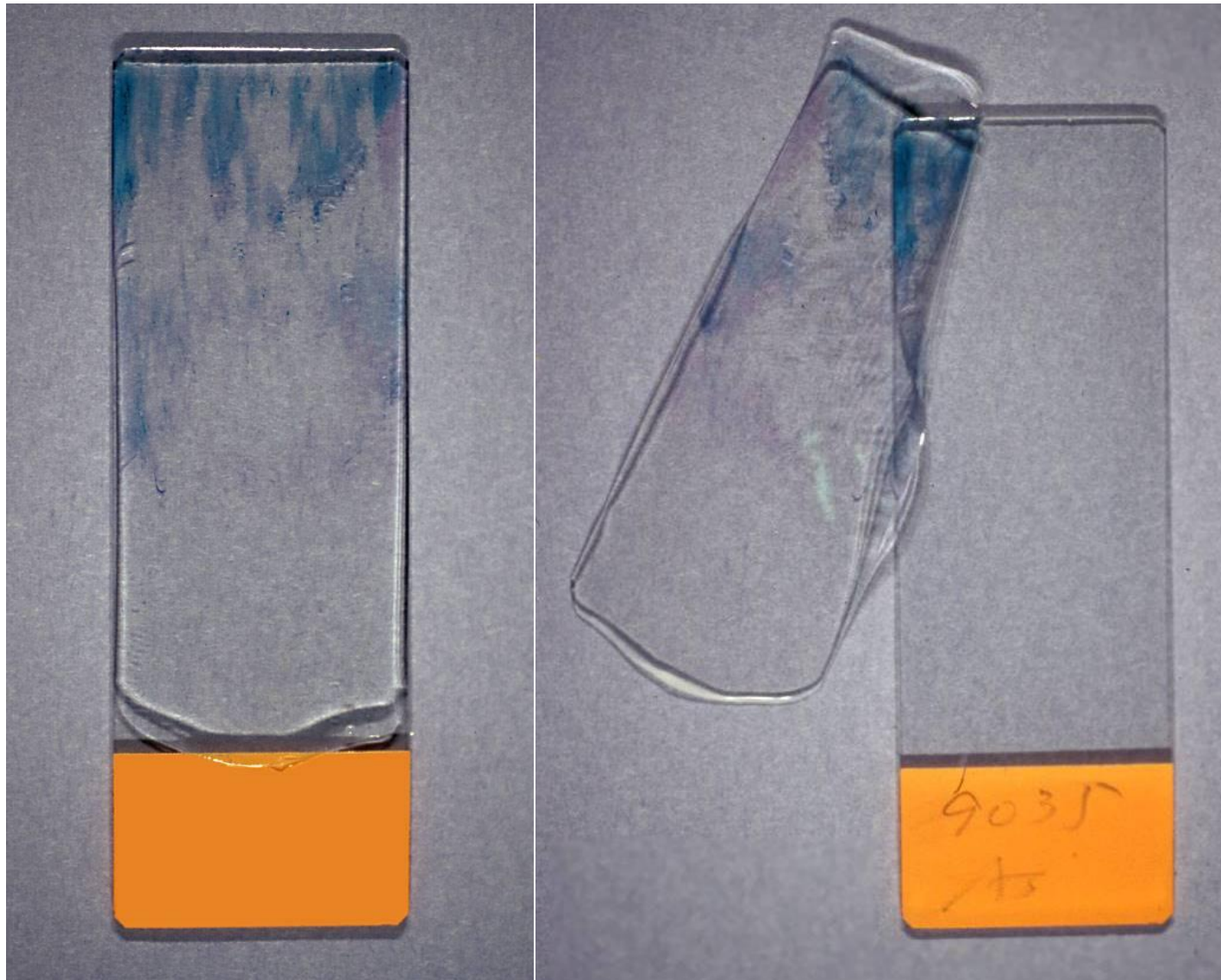
Wipe off excessive water with paper



Place onto silane-coated glass slide and dry

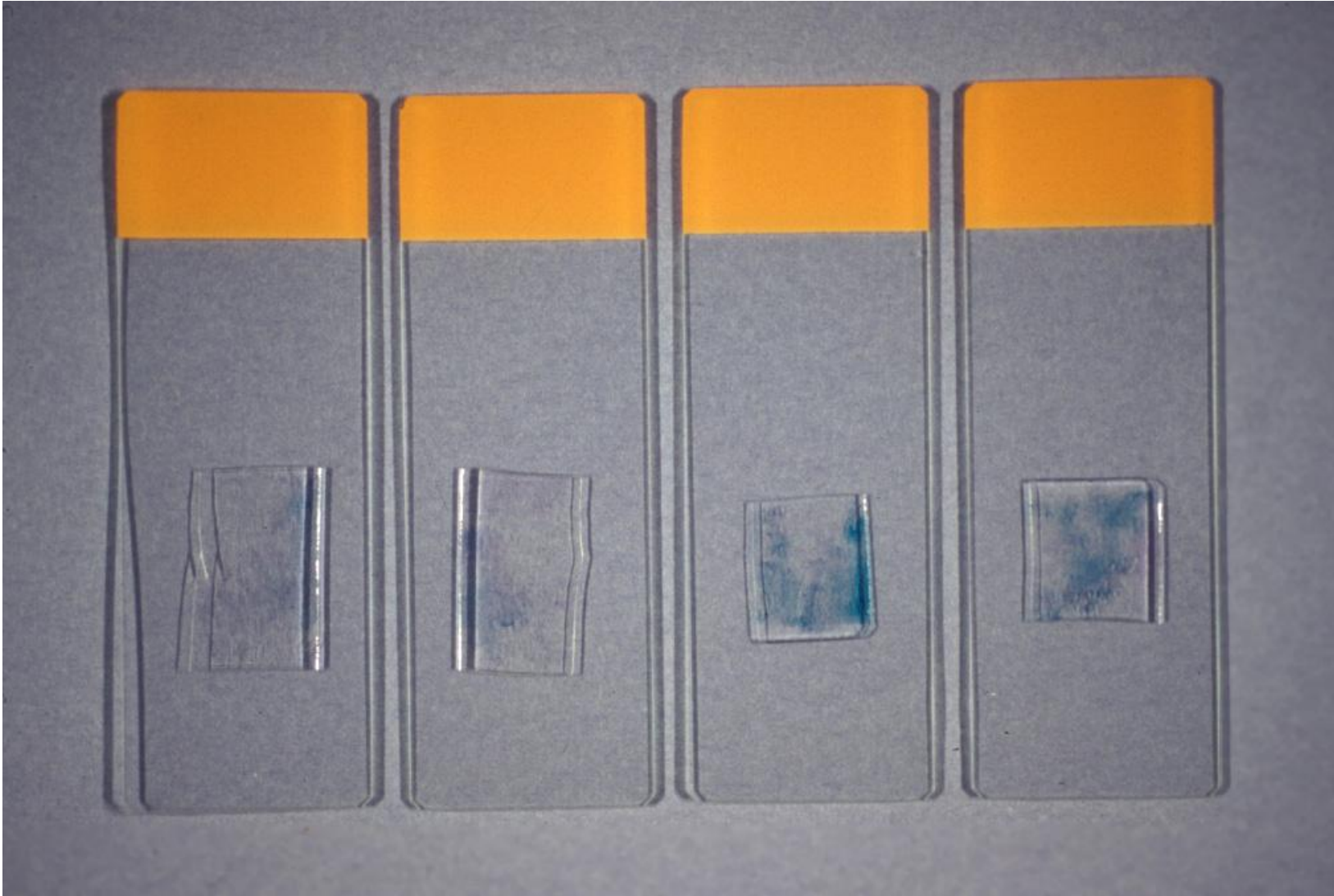


Soak in xylene, then in ethanol and finally in water to be ready for the next step



After detaching the cover slip, Malinol mounting solution is covered and solidified to form a Malinol membrane (left). The specimen is dipped in warm water, and then the solidified membrane can be peeled off. All the cells are transferred on the Malinol membrane (right).

Cell transfer, followed by cutting into pieces



Several slides are ready for histochemical analysis by cutting the solidified Malinol membrane. The pieces should be placed onto Silane-coated glass slides.

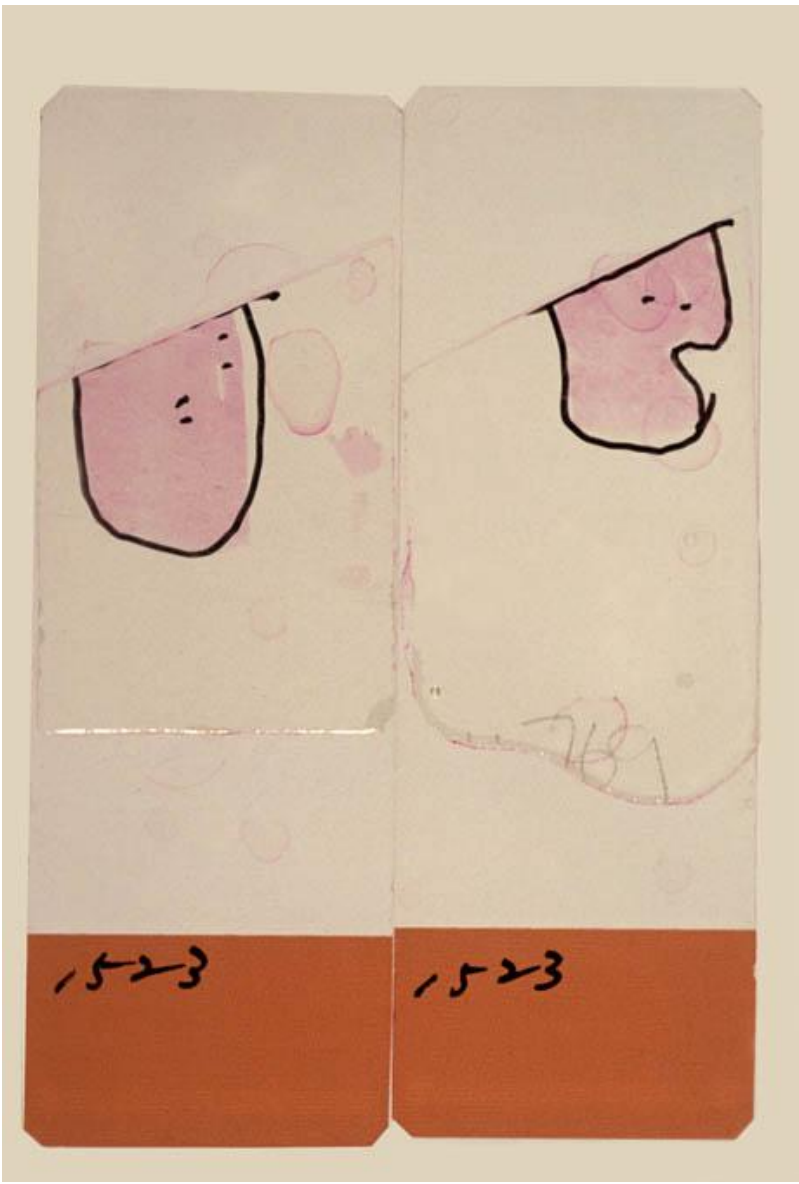
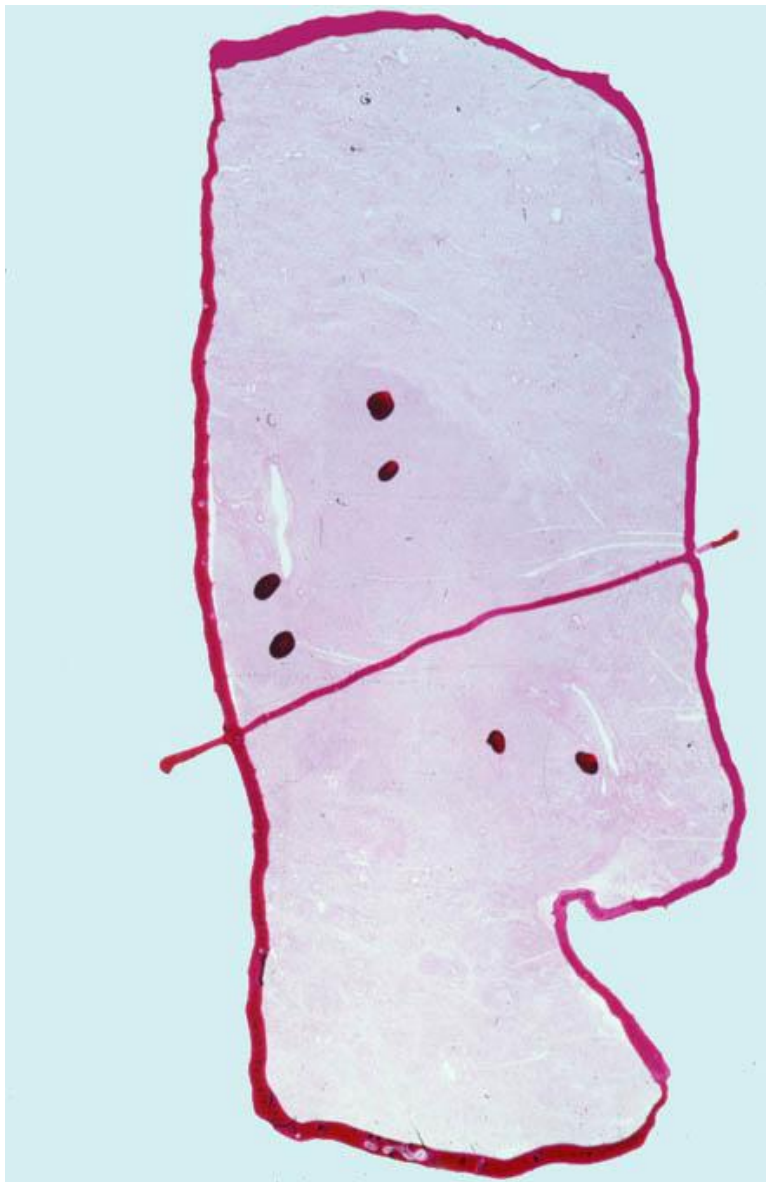
**Demonstration of CD15
and EBER in the archival
pathology specimens of
Hodgkin's lymphoma
autopsied by Thomas
Hodgkin himself in 1820's.
Use of the cell transfer
technique.**

*Dr. Thomas Hodgkin
(1797-1866)
Guys Hospital, London*

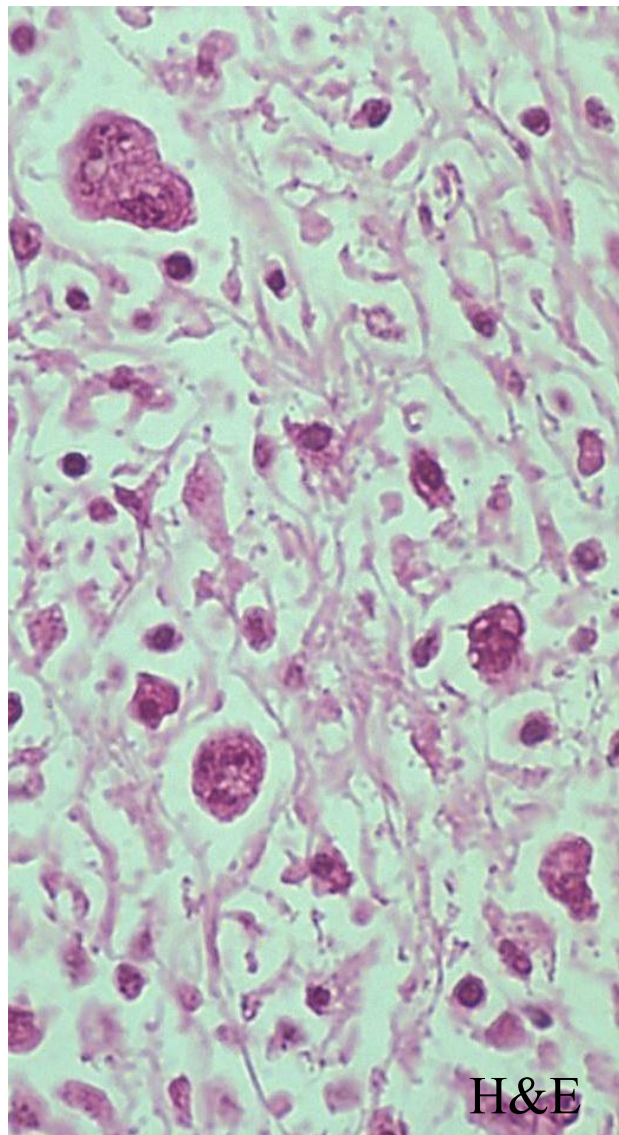




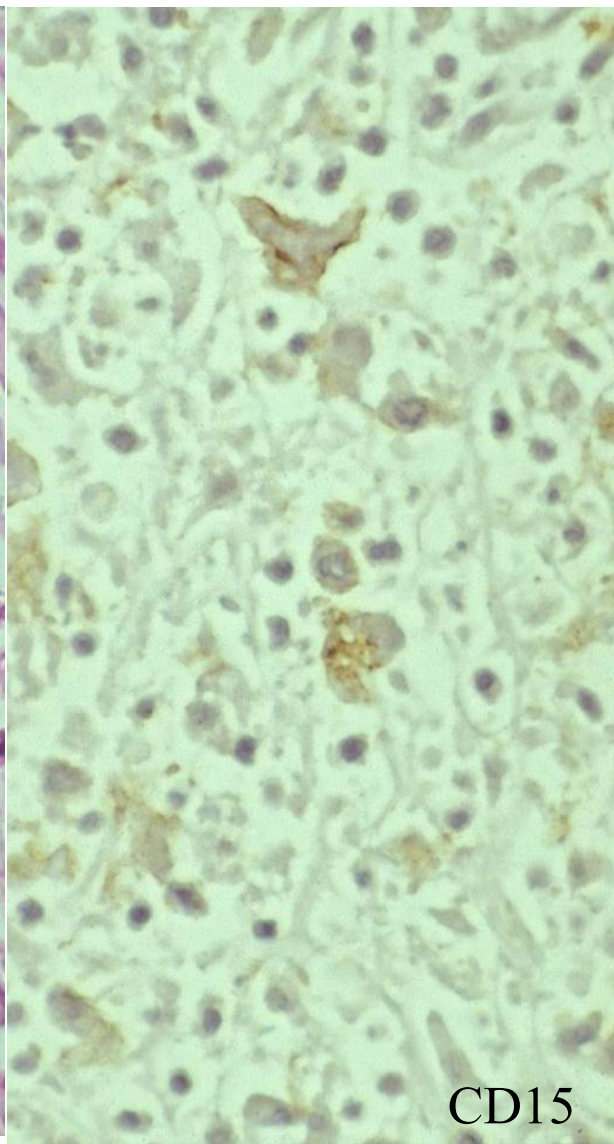
Presentation of samples of 3 cases autopsied by Thomas Hodgkin. Gordon museum, Guys Hospital, London. Single unstained glass slides (a total of 6 slides) were the precious gifts to the author from the President of the Museum. The specimens were fixed in ethanol for 80 years and thereafter in formalin for 90 years.



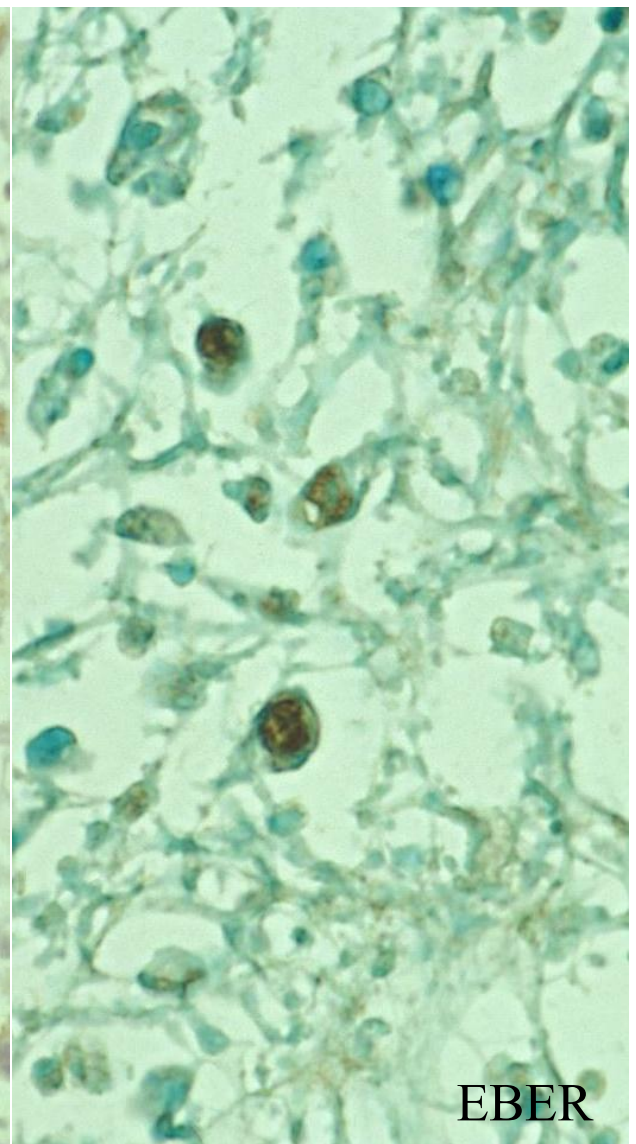
The precious single slide of the liver was stained with H&E, and then cell transferred onto the Silane-coated glass slides to get two slides for staining. Hodgkin' cells and Reed-Sternberg cells were identified in the dotted areas.



H&E



CD15



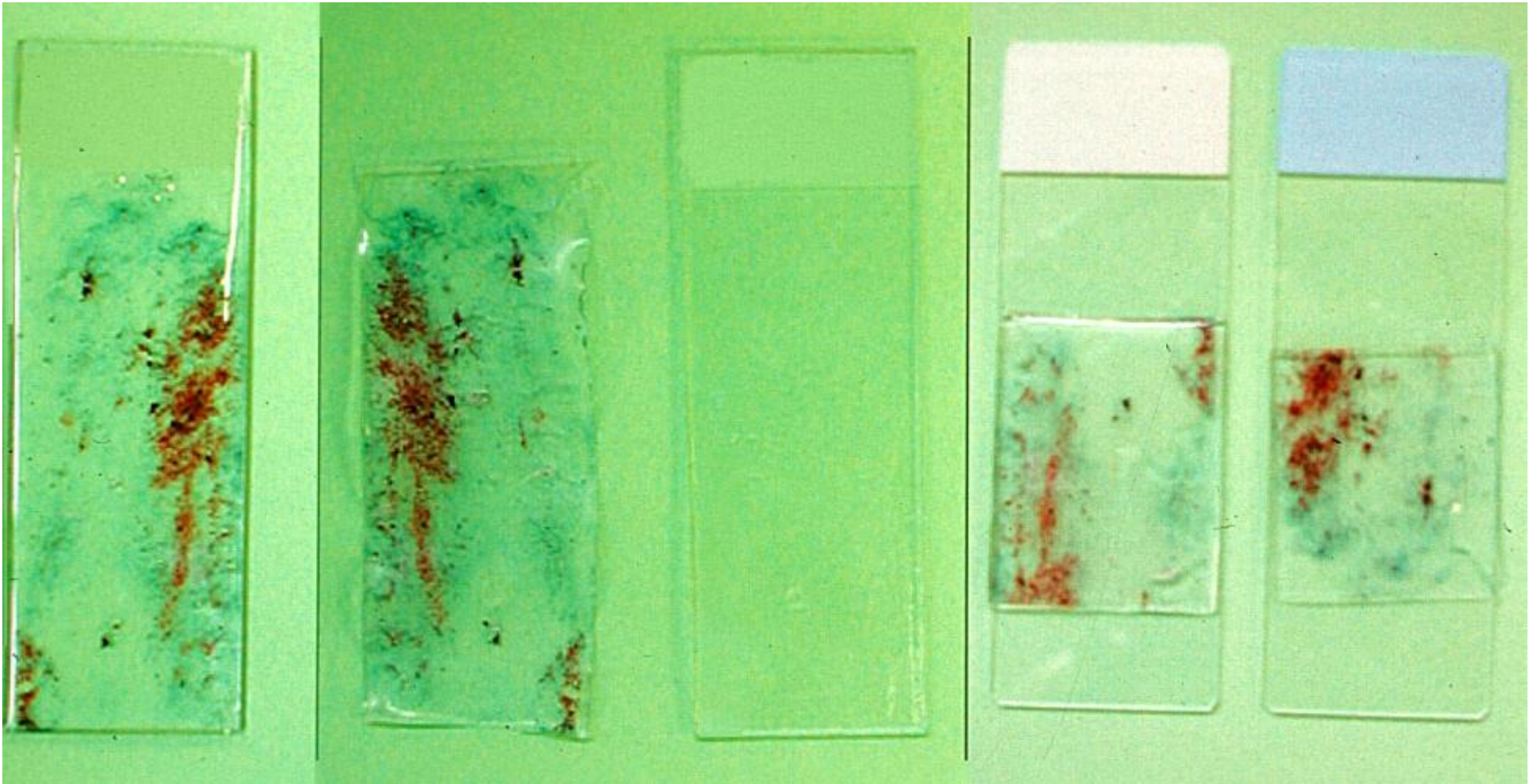
EBER

Hodgkin's cells and Reed-Sternberg cells in the lymph node autopsied by Dr. Thomas Hodgkin. The plasma membrane expression of CD15 (Leu M1) and nuclear expression of EBER can be confirmed after long fixation in ethanol and formalin. **Ref.:** Tsutsumi Y. Demonstration of Epstein-Barr virus genome in archival paraffin sections of Hodgkin's lymphoma autopsied by Dr. Thomas Hodgkin nearly 170 years ago. *Acta Histochem Cytochem* 2003; 36: 511-514. doi: 10.1267/AHC.36.511

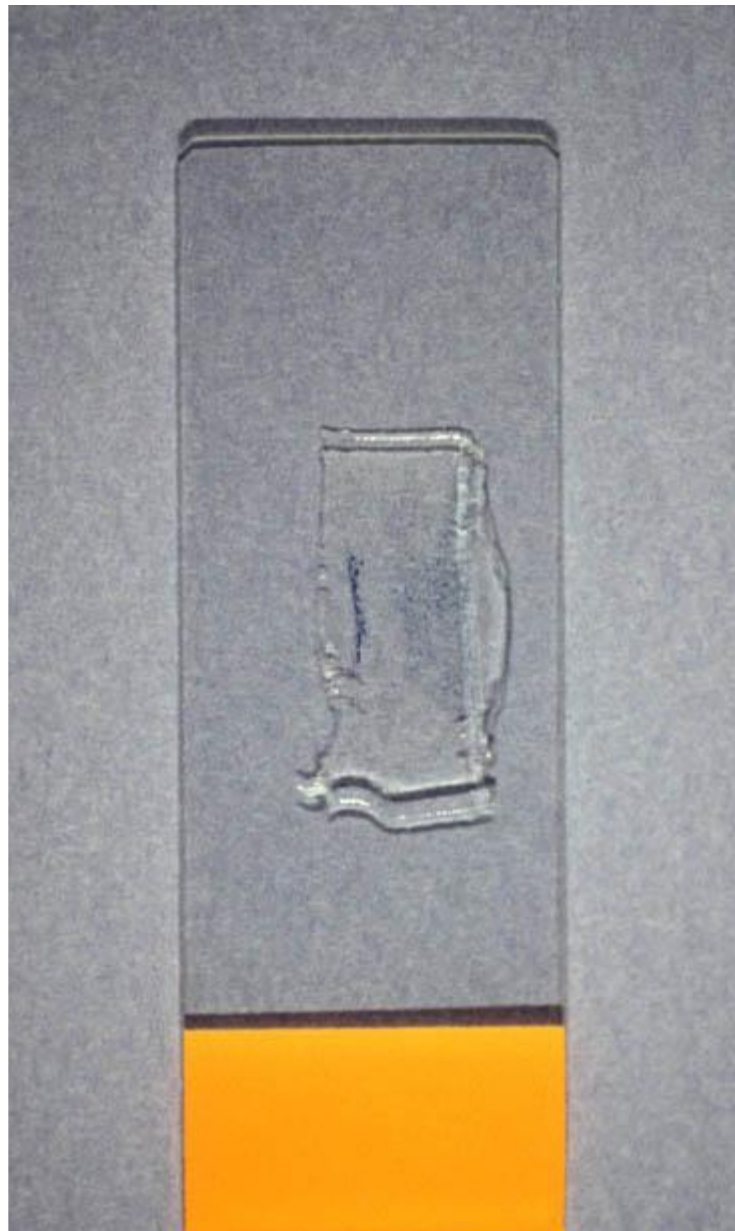
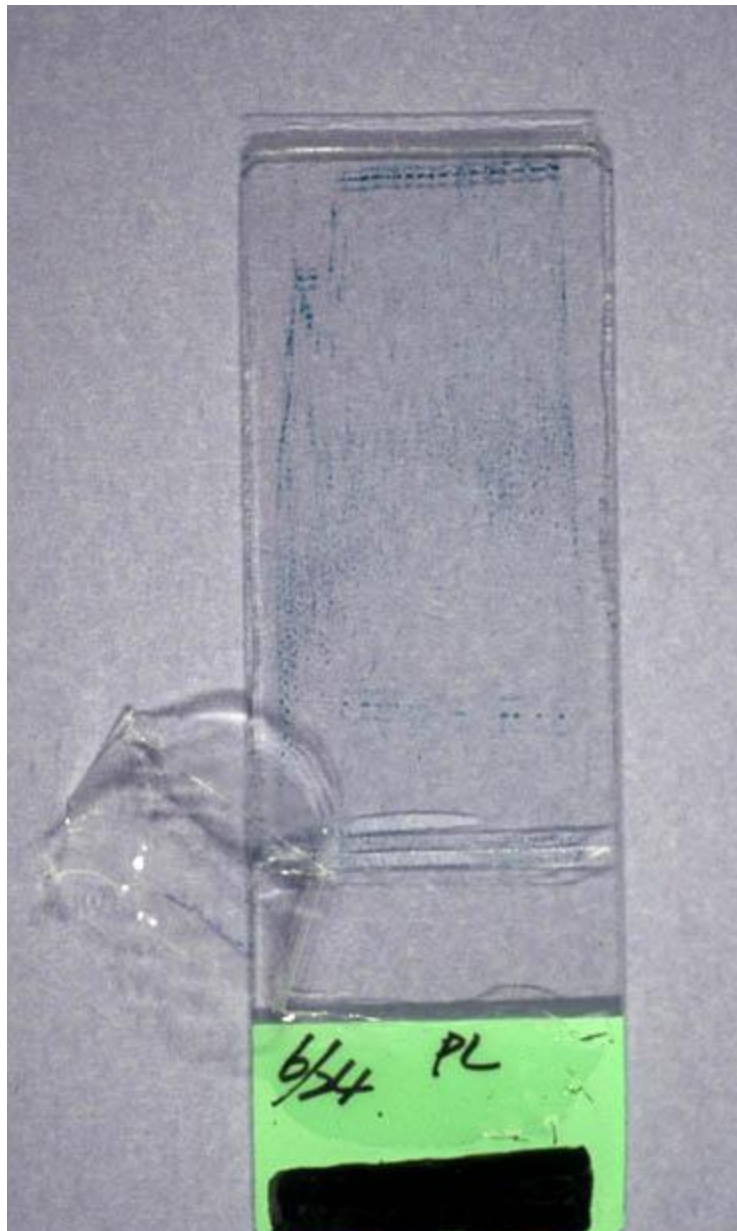
Quick cell transfer technique (Ito's method)

1. Detach cover slip by dipping in warm xylene
(for rapid detachment, injure cover slip by a diamond pen)
2. Drop 1 ml diluted Malinol (mounting solvent) onto specimen
(Dilution: Mix Malinol with the same volume of xylene)
3. Dry and solidify Malinol on a 70°C hot plate for 30 min
4. Dip slide in warm water for 15 min for softening
5. Detach sheet of Malinol by forceps
(If necessary, cut specimen into several pieces by scissors)
6. Place sheet of Malinol on Silane-coated glass slides
(Check carefully the side of the sheet)
7. Dry slides on a 70°C hot plate
8. Clear in xylene to be ready for immunostaining

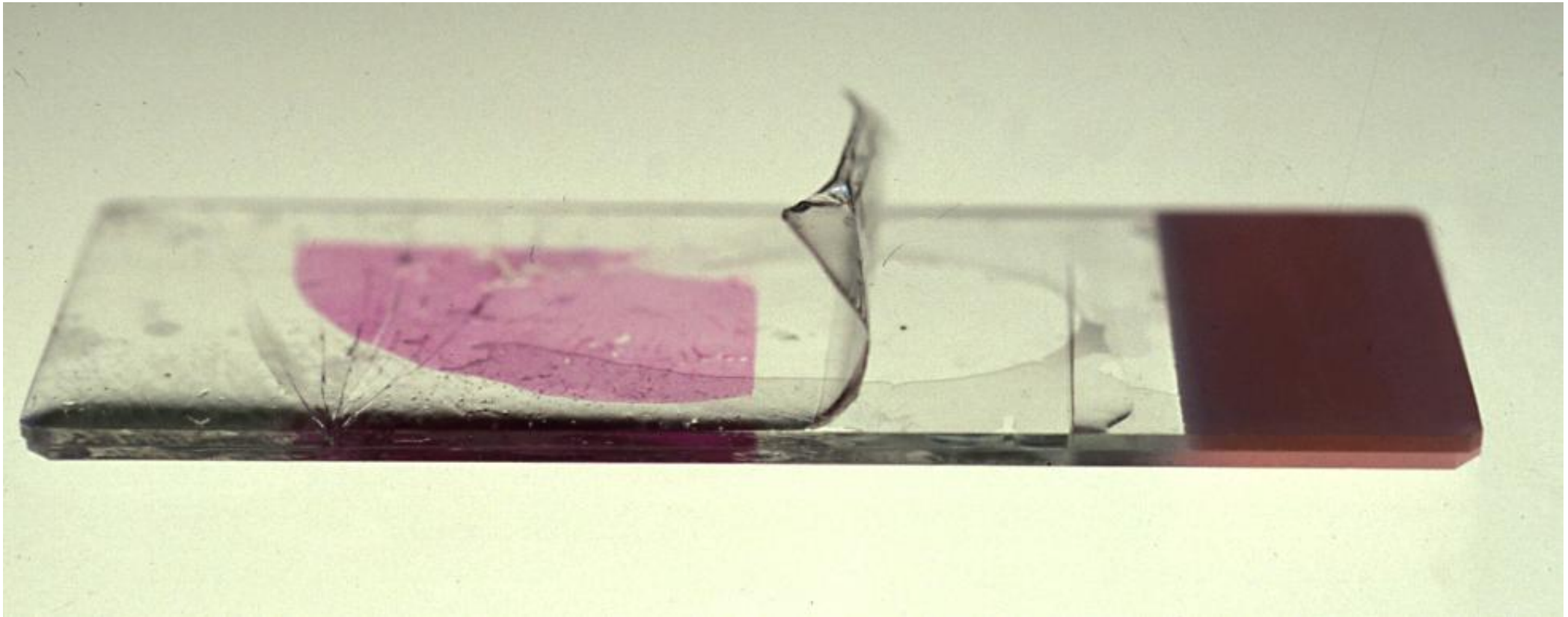
Ref.: Itoh H, et al. Development of the rapid cell transfer technique. Nihon Rinsho Saibo Gakkai Zasshi 2002; 41: 302-303 (in Japanese with English abstract).



Cell transfer technique. Detached Malinol membrane is cut into plural pieces by knife and attached onto the silane-coated glass slides. The Pap-stained cytology specimens on uncoated glass slides can be transferred. It should be noted that Giemsa-stained glass slides or Pap-stained preparations on coated glass slides can not be transferred.



The technique without detaching the cover slip: the shoulder portion of the cervical smear preparation can be used for the cell transfer.



The cell transfer technique can be applied to repairing a broken glass slide. Here, a broken H&E preparation of the kidney was repaired by supporting the broken glass slide with another glass slide attached.

Rapid detaching of cover slip (Harada's method)

1. Dip glass slide in 65°C warmed xylene for 30 min
2. Wipe off excessive xylene by Kim Wipe
3. Stick tightly size-adjusted Gum Tape on cover slip
4. Detach cover slip in a breath by peeling Gum Tape
5. Dip glass slide in xylene to be ready for transfer

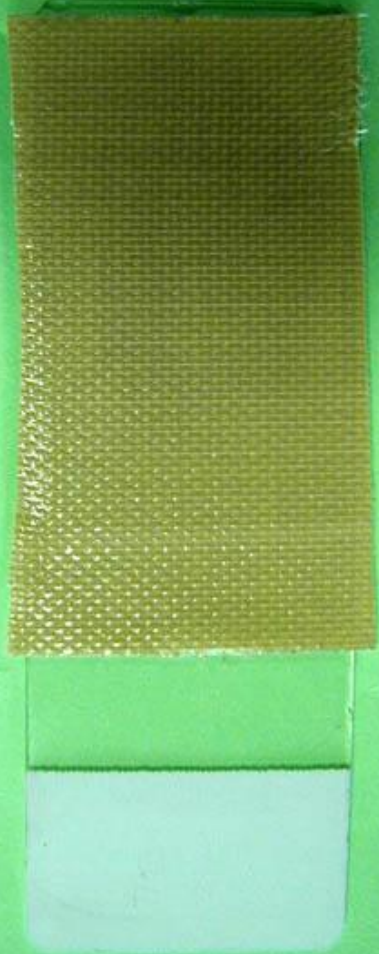
Ref.: Harada E, et al. Rapid detachment of cover slips. Byori-to-Rinsho 2003; 21(11): 1306-1307 (in Japanese).

Clean up on the surface of the cover slip
of the pre-warmed glass slide



Rapid detaching of cover slip 1

Gum tape is evenly stuck on cover slip



When seen from the reversed side



Rapid detaching of cover slip 2

Detach cover slip in a breath
by peeling Gum Tape



Rapid detaching of cover slip 3