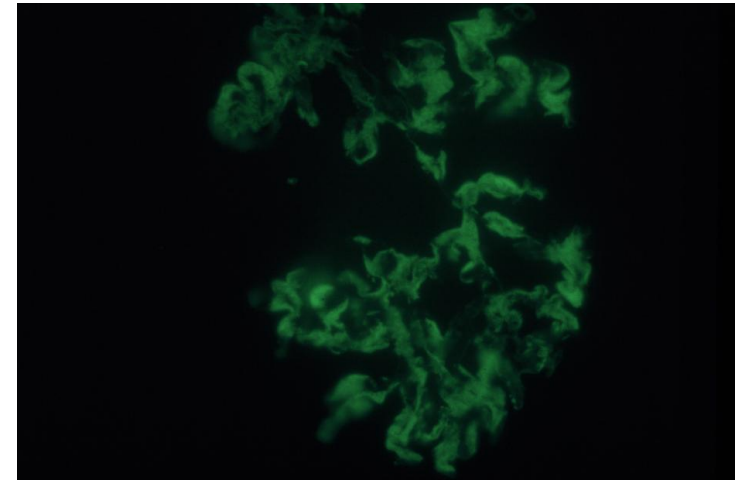
Past, present and future of immunohistochemistry

Immunohistochemistry, particularly immunoperoxidase method, is indispensable for both the morphological research works and histopathological diagnosis. Formalin-fixed, paraffin-embedded sections can be analyzed not only for immunostaining but also for molecular study using *in situ* hybridization and polymerase chain reaction (PCR). High sensitivity detection techniques and a variety of technical inventions have contributed to the practical use of this histochemical method. Here, the past, present and future of immunohistochemistry is briefly reviewed.

History of immunohistochemistry



IgA nephropathy

1955: Coons: immunofluorescence

1966: Nakane: immunoperoxidase

Method of antigen localization at EM level:

Frozen sections were used for research

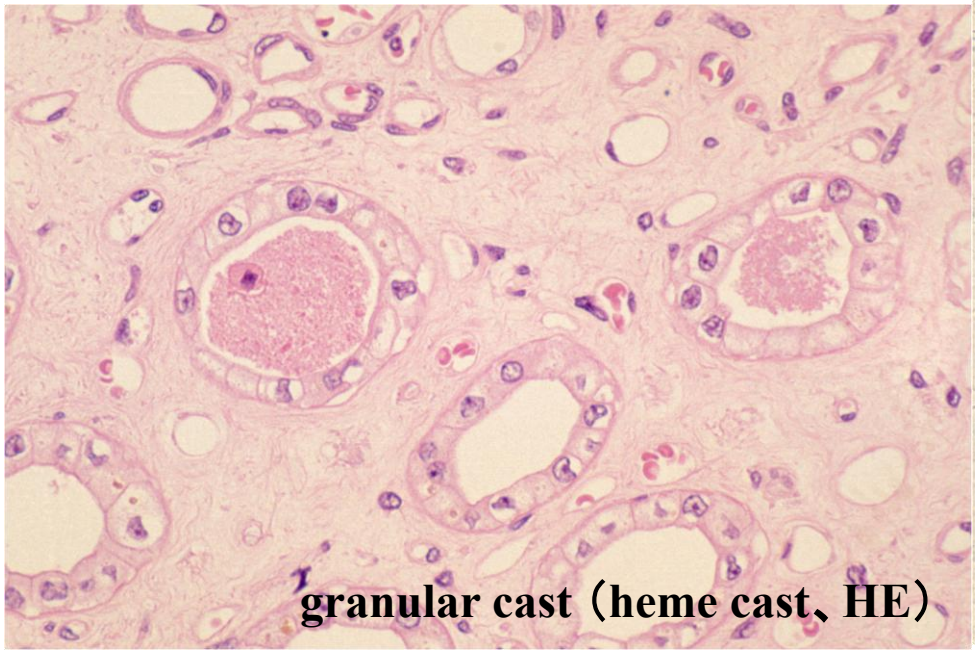
1984: Tsutsumi: application of the immunoperoxidase staining to histopathological diagnosis

Byori-to-Rinsho 2(5): 703-725 (in Japanese)

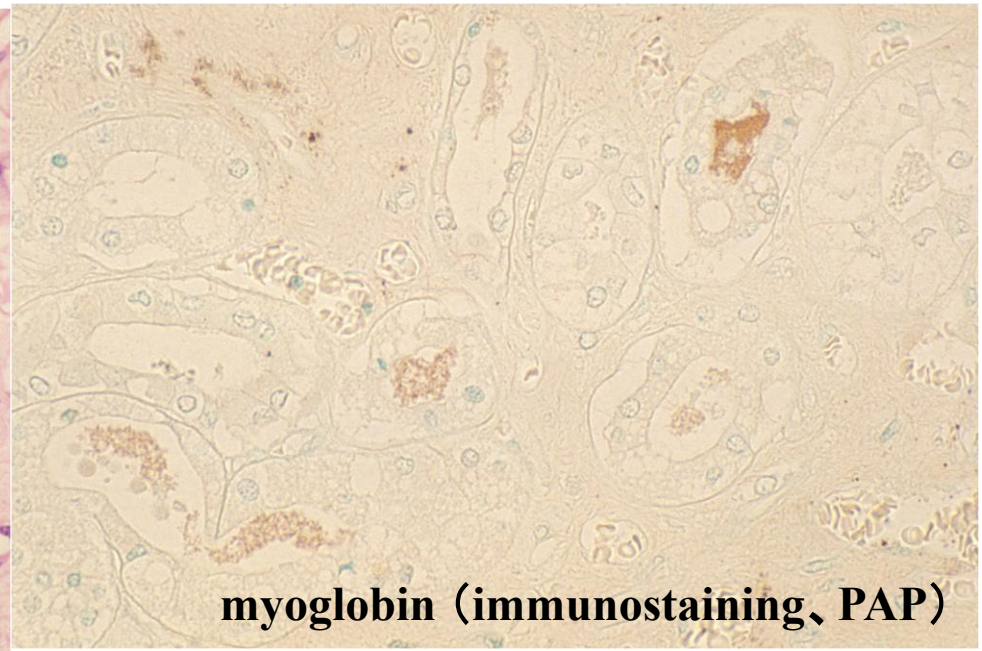
(Use of routinely prepared paraffin sections)

An autopsy case of idiopathic myoglobinuria (1979, 4 years after graduation of medical school)

A specialist of renal pathology said to me that for detecting myoglobin in renal tissue, either frozen sections or alcohol-fixed sections should be indispensable. However, I tried and succeeded to detect the antigen!



granular cast（heme cast、HE）



myoglobin（immunostaining、PAP）

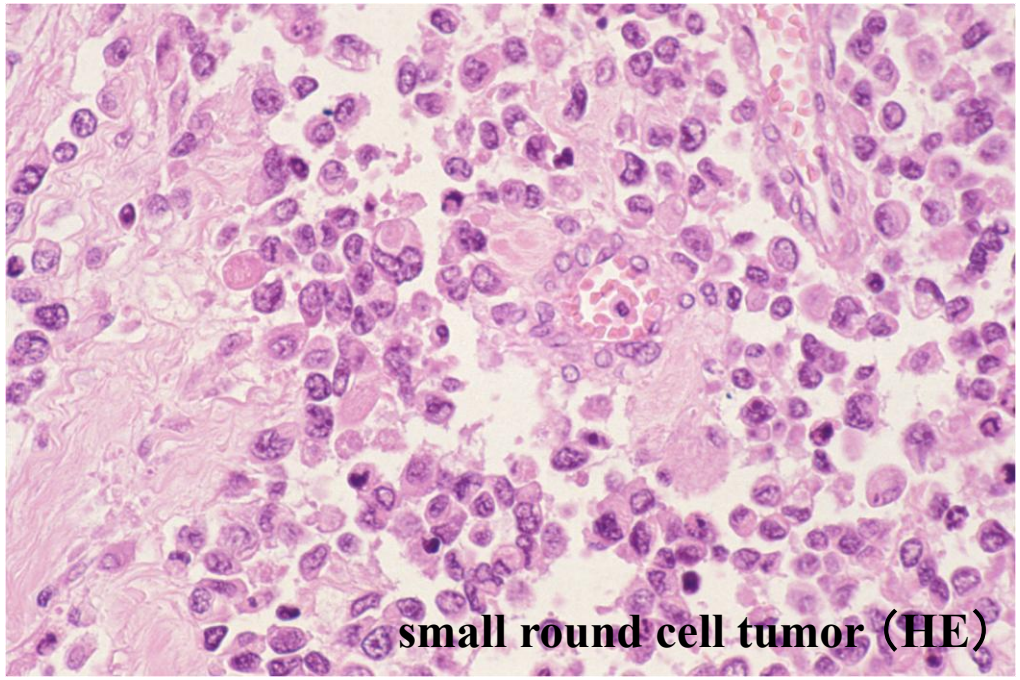
Danish male: Fatigue manifested in the air plane（rhabdomyolysis of unknown origin causing acute renal failure）

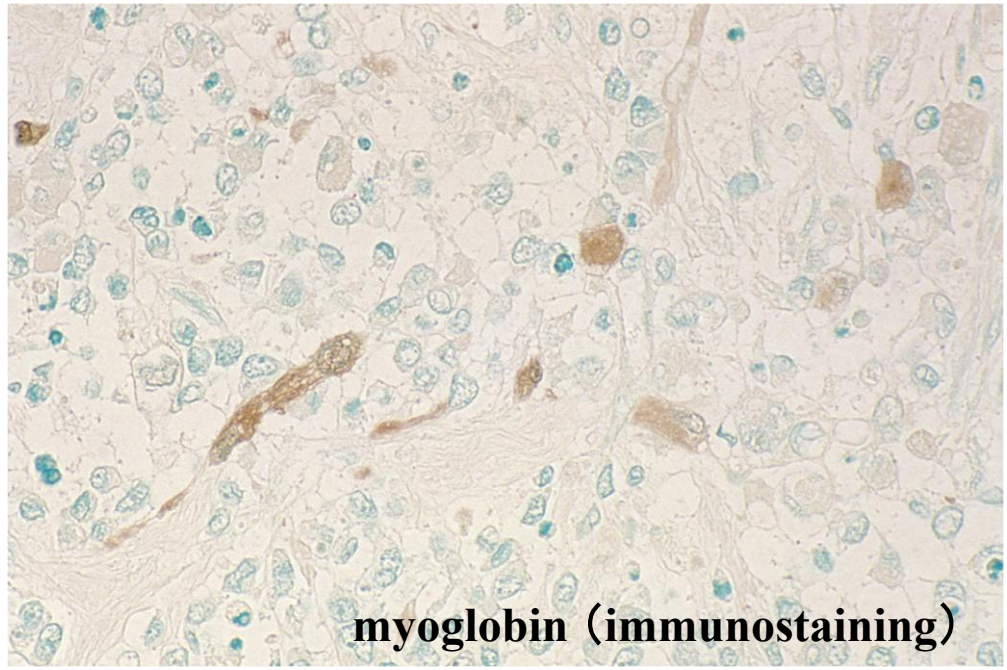
Ref.: Tsutsumi Y. Tsutsumi Y. An autopsy case of idiopathic rhabdomyolysis in 1979: immunoperoxidase detection of myoglobin casts in formalin-fixed, paraffin-embedded sections of the kidney. Cureus 2021; 13(10): e18922. doi: 10.7759/cureus.18922

I applied the method to rhabdomyosarcoma.

At that time, PAS、Azan and PTAH stains were used.
(We must detect glycogens and cross-striation!)

Immunostaining is simple and quick! We can keep the stained sections for ever. *Myoglobin is specific to striated muscle cells!*

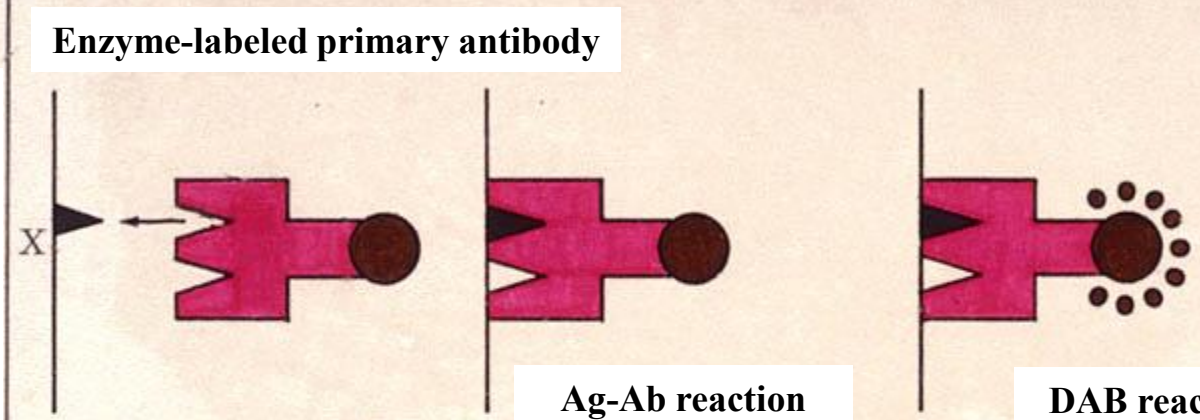

small round cell tumor（HE）


myoglobin（immunostaining）

Rhabdomyosarcoma was rapidly diagnosed immunohistochemically, since the cytoplasm of the spindled tumor cells was positive for myoglobin!

Immunoperoxidase method (direct and indirect)

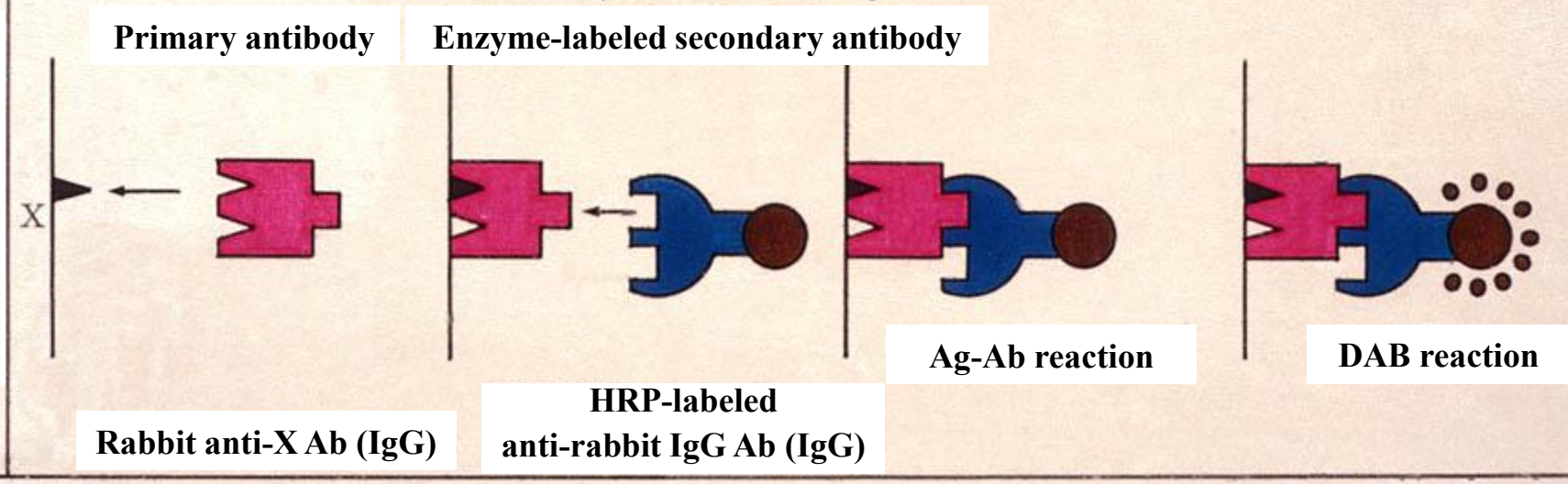
Direct method



HRP catalizes: $H_2O_2 \rightarrow H_2O + (O)$

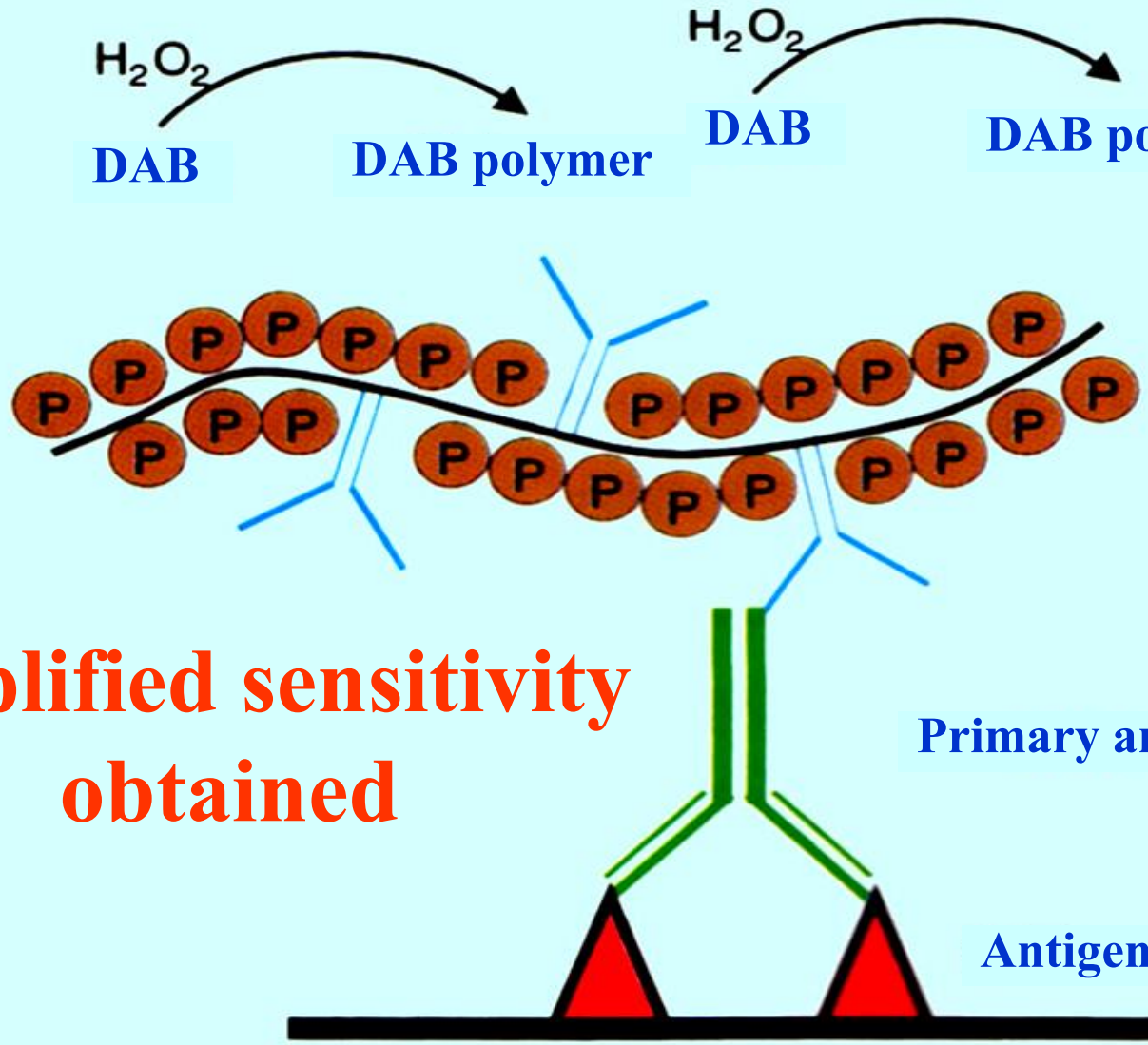
Oxydized DAB gives brown color at the site of reaction

Indirect method



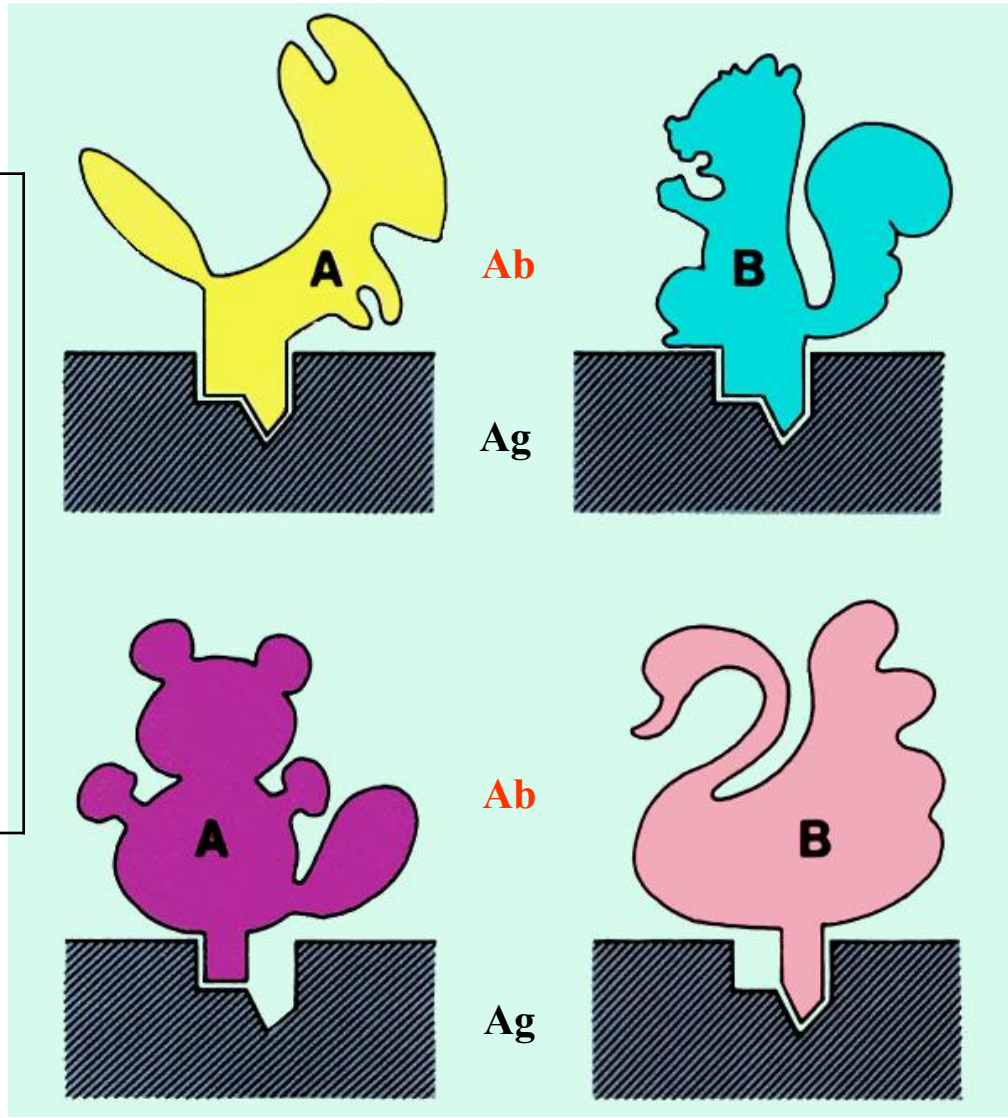
HRP＝horseradish peroxidase、DAB ＝diaminobenzidine

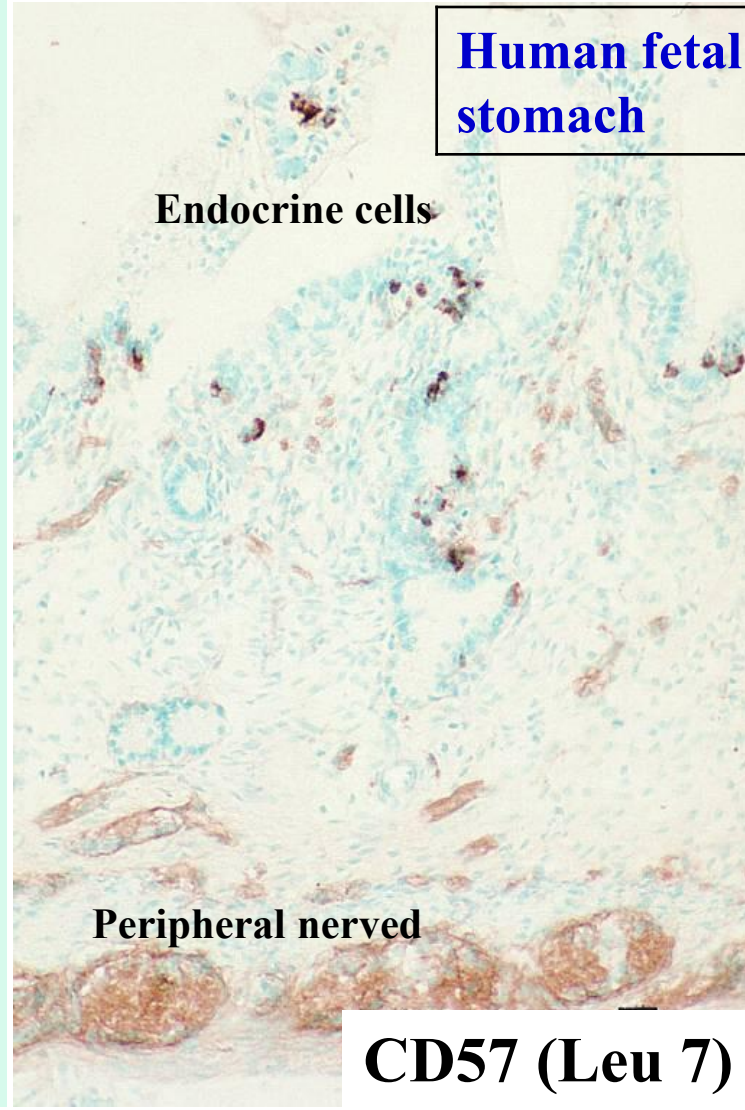
Enzyme-labeled polymer method



Cross-reactivity by monoclonal antibodies (Köhler &Milstein, 1975, Nature)

Monoclonal antibodies detect a single epitope on the molecule rather than the antigen molecule itself.





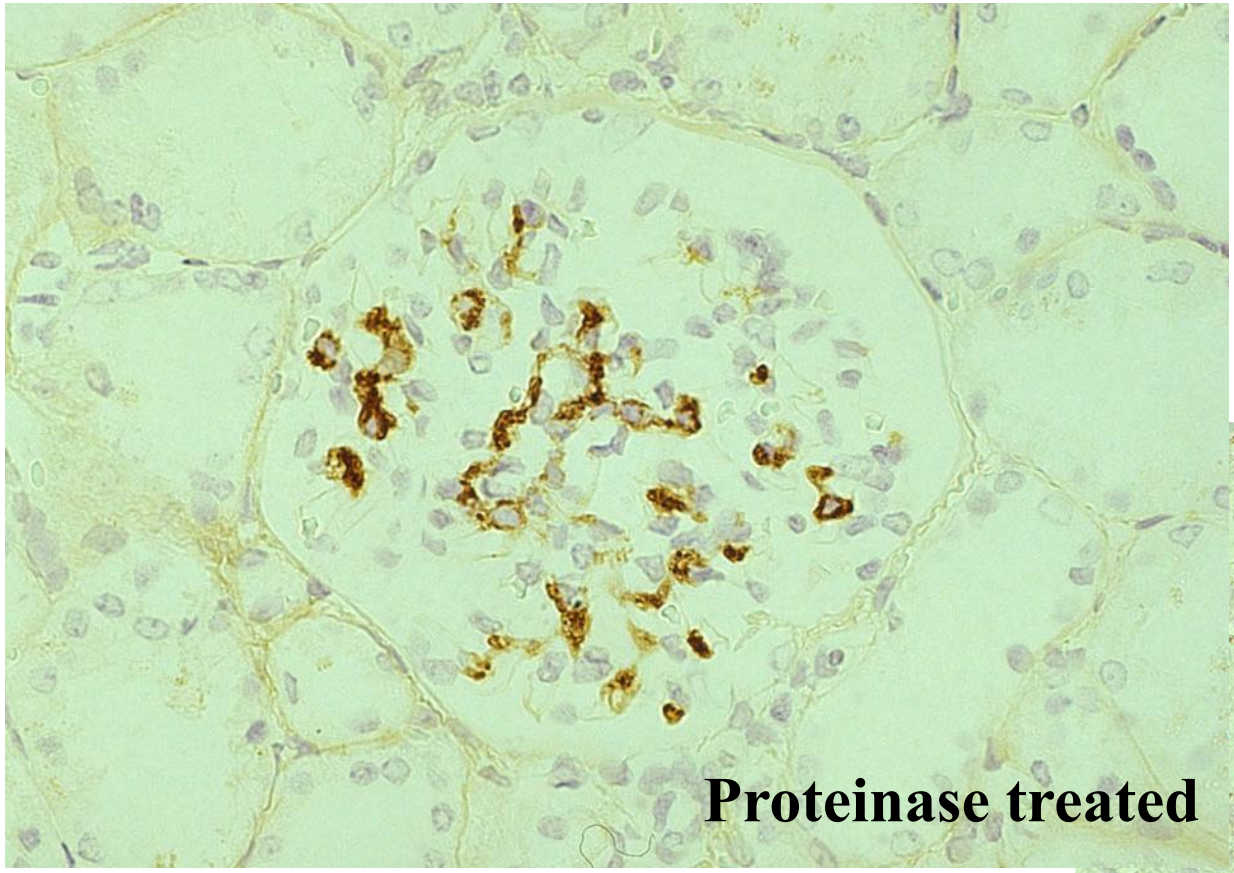
Methods of antigen retrieval

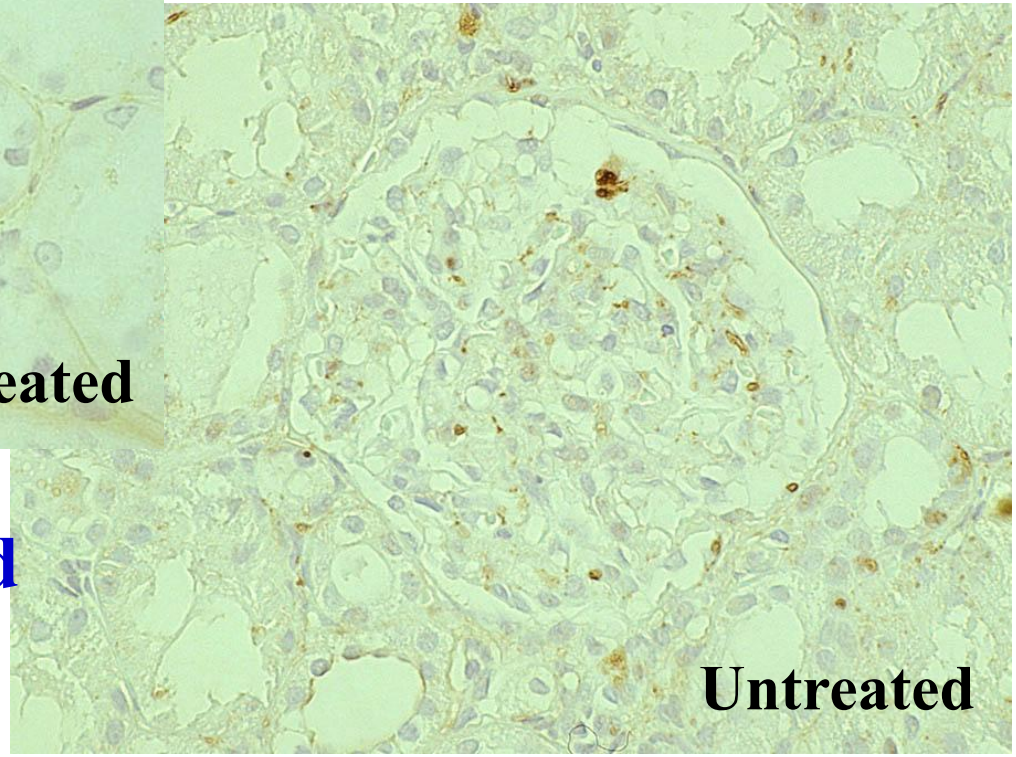
1. Proteinase treatment
2. Hydrated heating（Shi、1991）

In the past, histochemists believed that heating of the specimens was contradictory in any situation!

Heating in a pressure pan

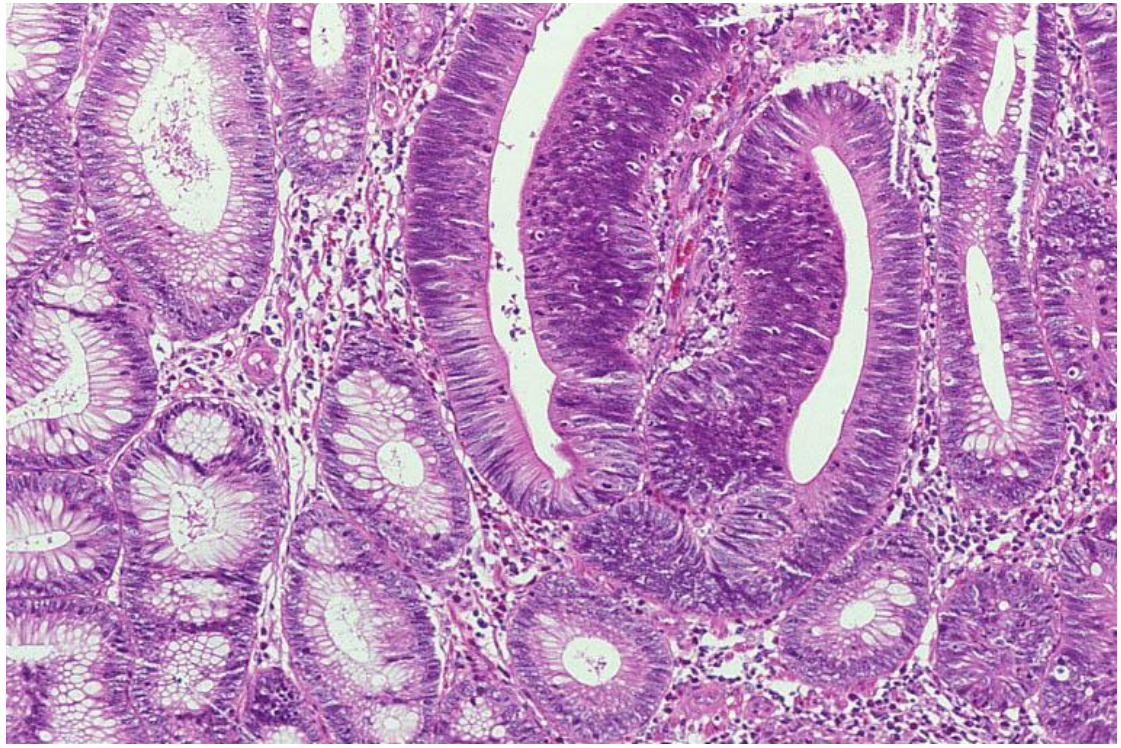
Proteinase treatment（IgA nephropathy）

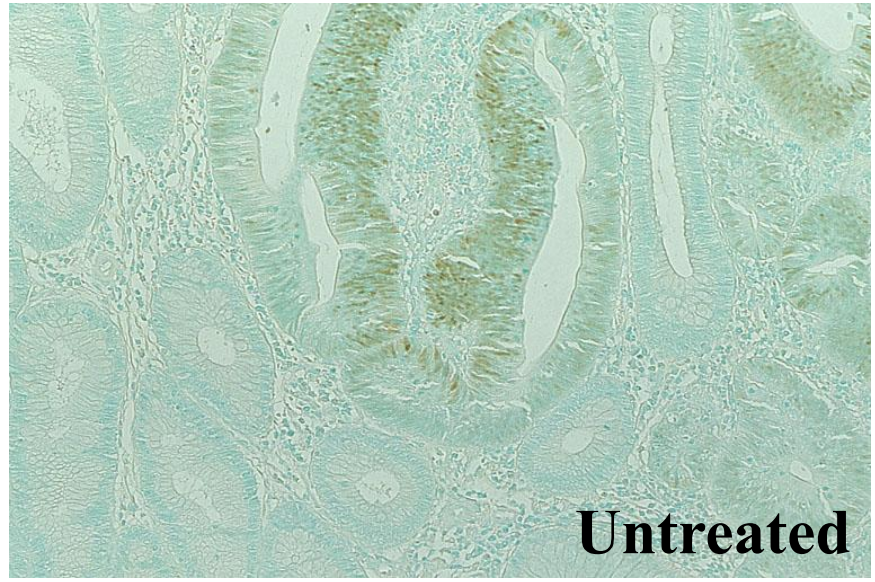


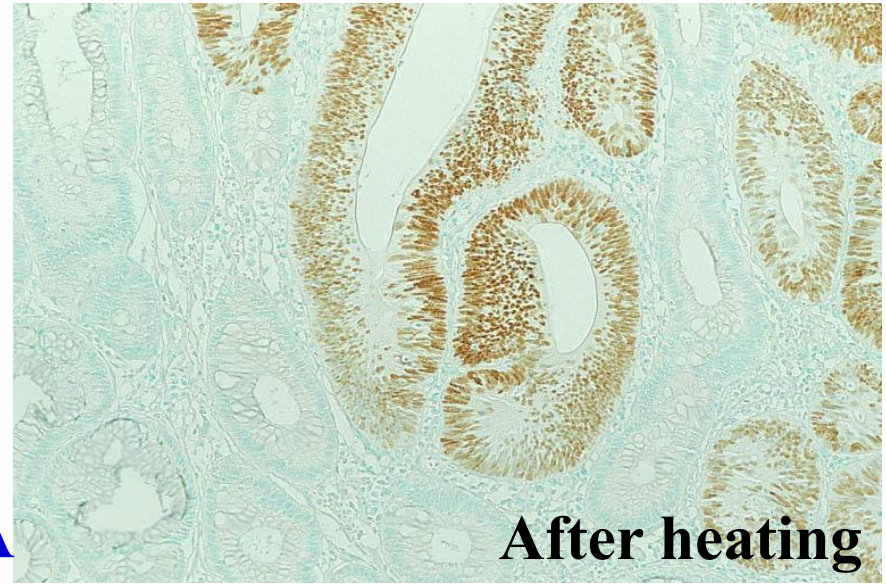


Formalin-fixed paraffin-embedded section（renal biopsy）

Heating treatment（cancer in adenoma, colon, p53 protein）







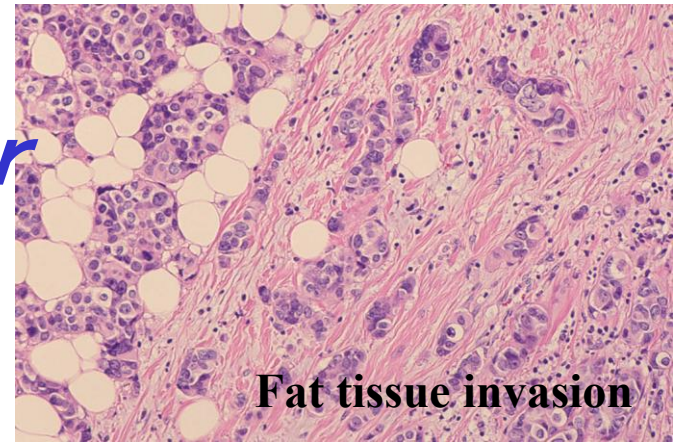
Method of heating：warm bath, microwave oven, autoclave and pressure pan

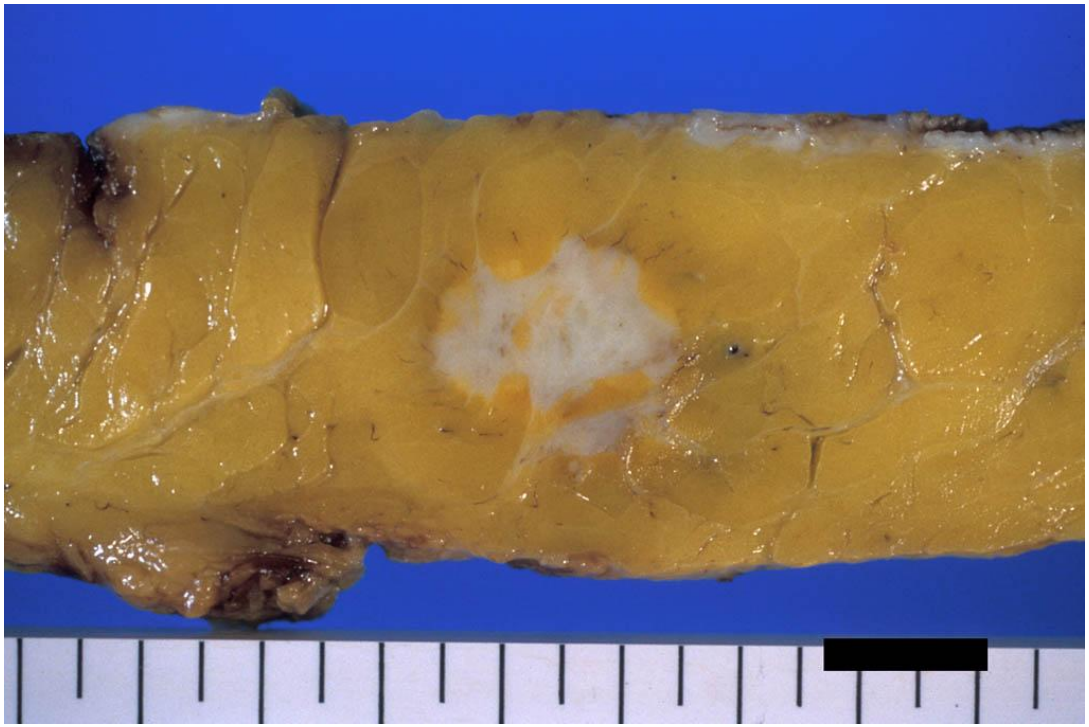
Solution: water, citrate buffer, EDTA

Usefulness of immunostaining in modern medicine : Contribution to tailor-made medicine

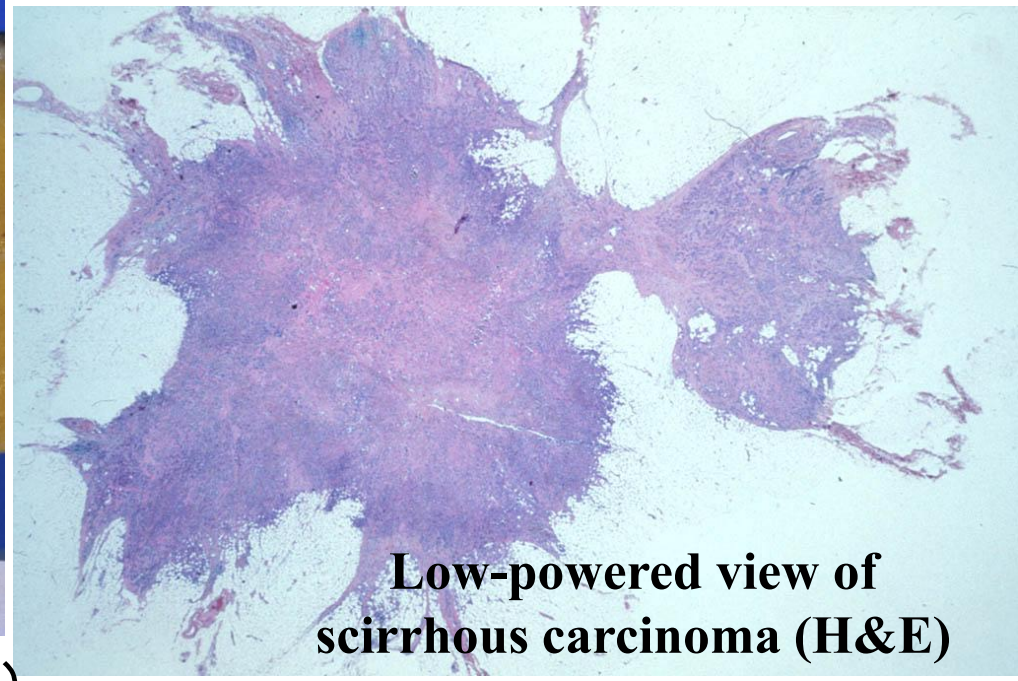
ER、PgR and HER2 in breast cancer

Treatment is dependent on the histopathological diagnosis !



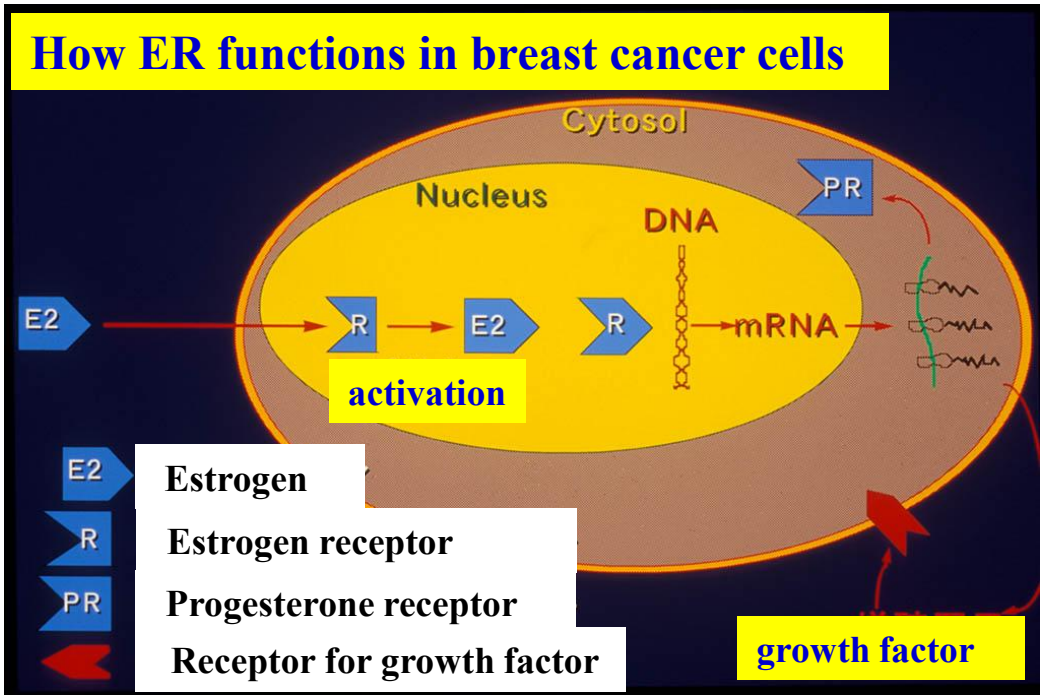


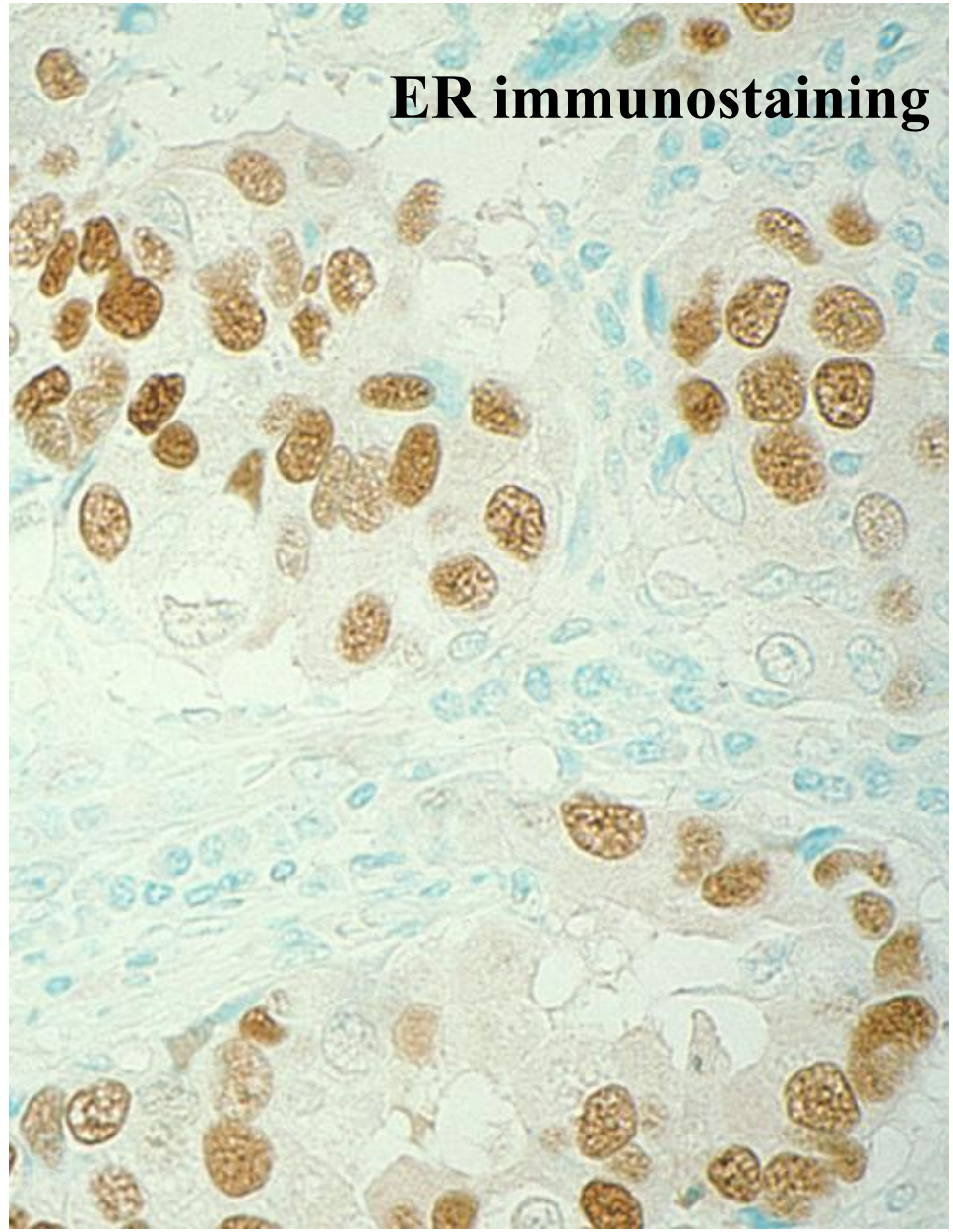
Cut surface of scirrhous cancer（formalin-fixed）



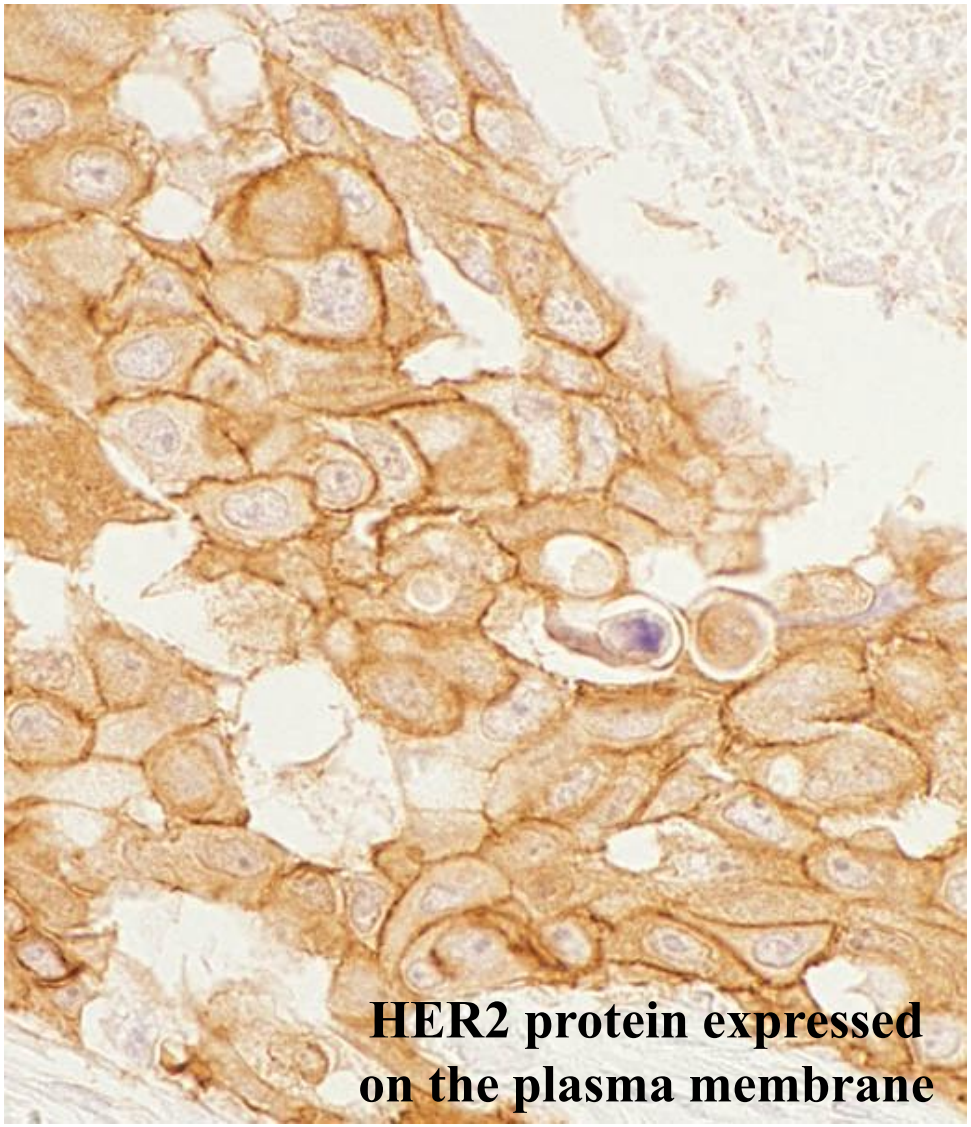
Estrogen receptor in the nucleus of breast cancer cells（immunostaining after heating treatment）

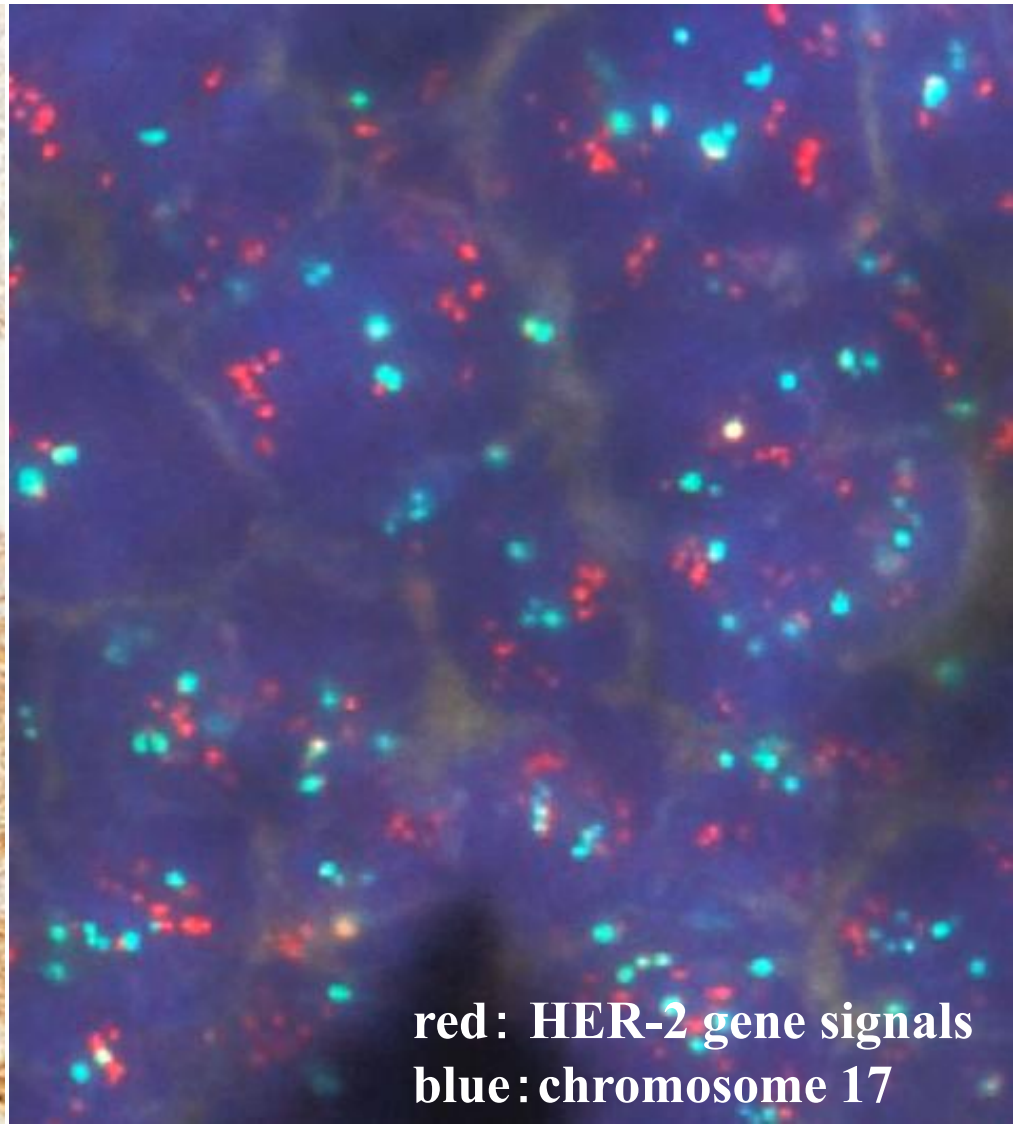
When ER is immunoreactive, hormone therapy is given to the patient.





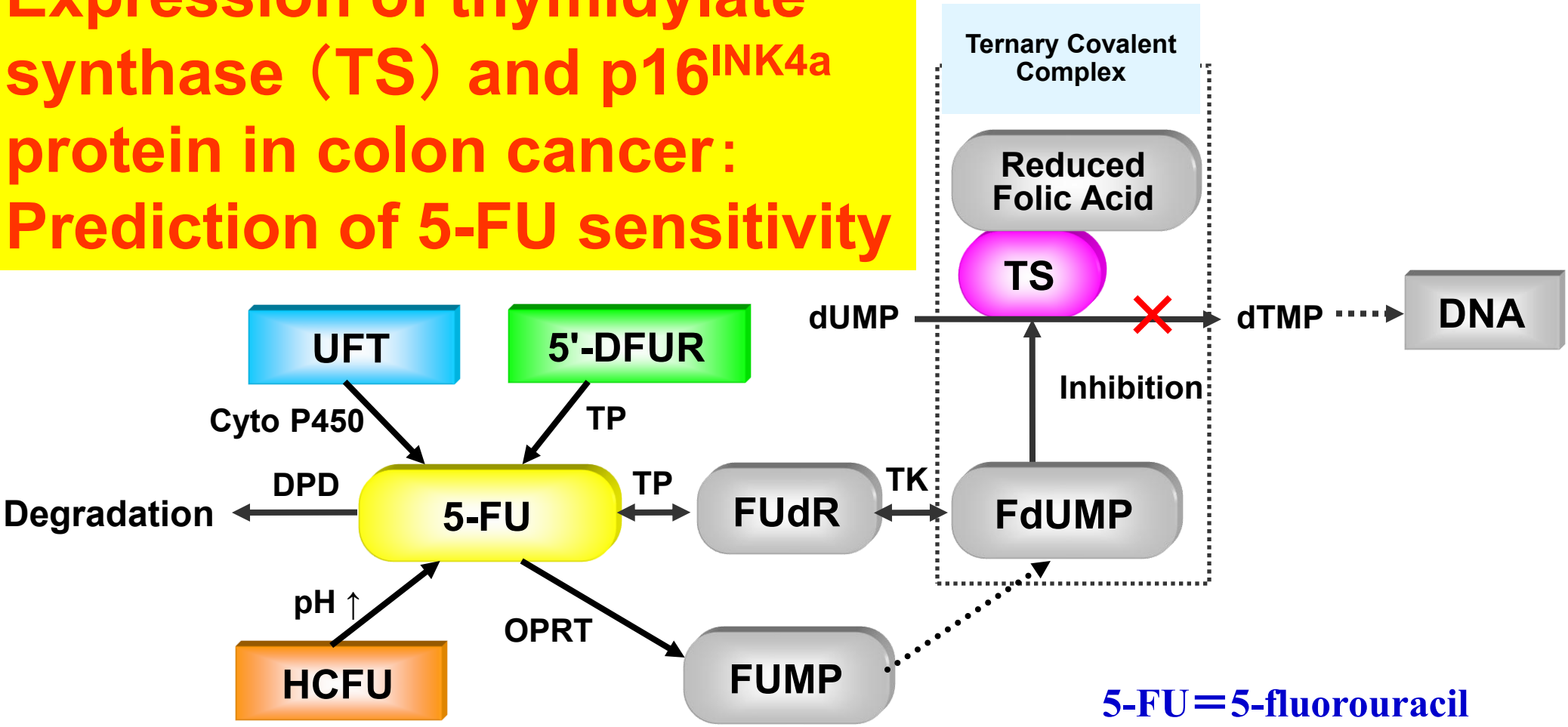
HER-2 expression in breast cancer (left：immunostain, right：FISH)





When HER2 is positive, Herceptin treatment is given to the patient.

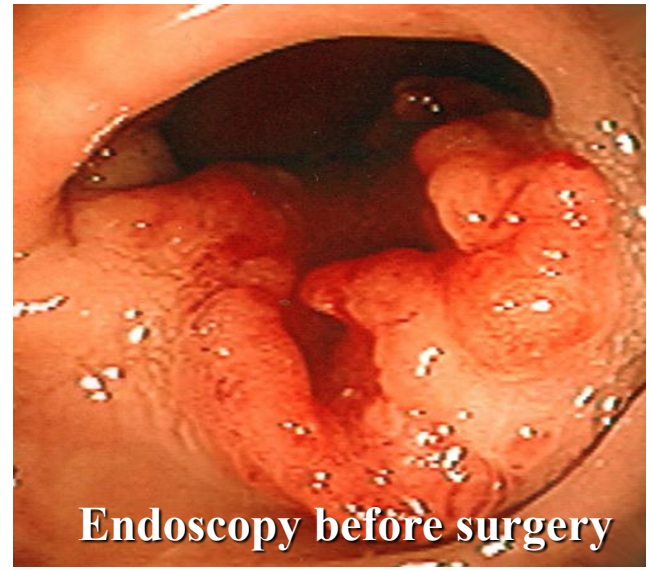
Expression of thymidylate synthase（TS）and p16^{INK4a} protein in colon cancer：Prediction of 5-FU sensitivity

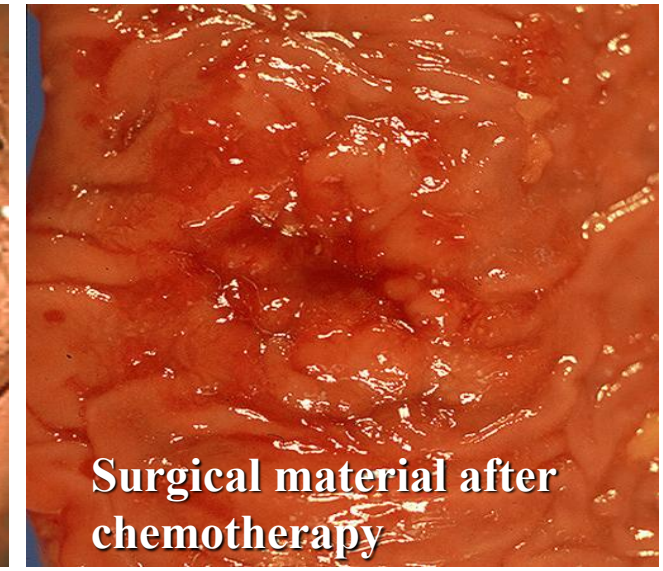


DNA injury by 5-FU and 5-FU prodrugs

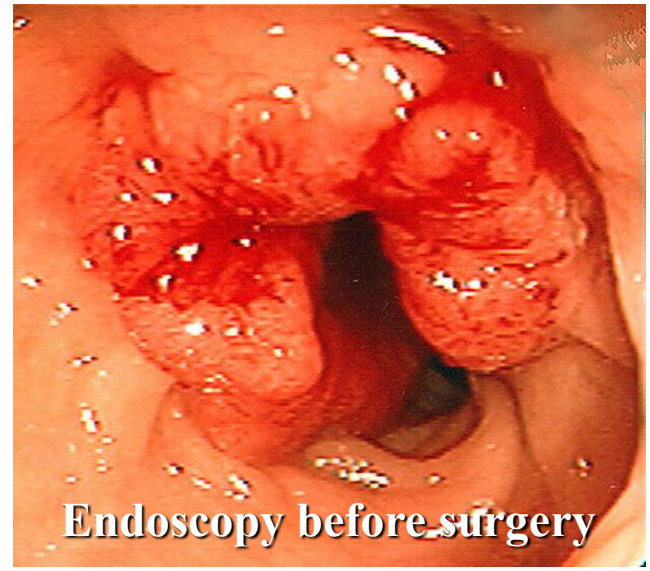
Part of colon cancers well reacts to 5-FU chemotherapy before surgery

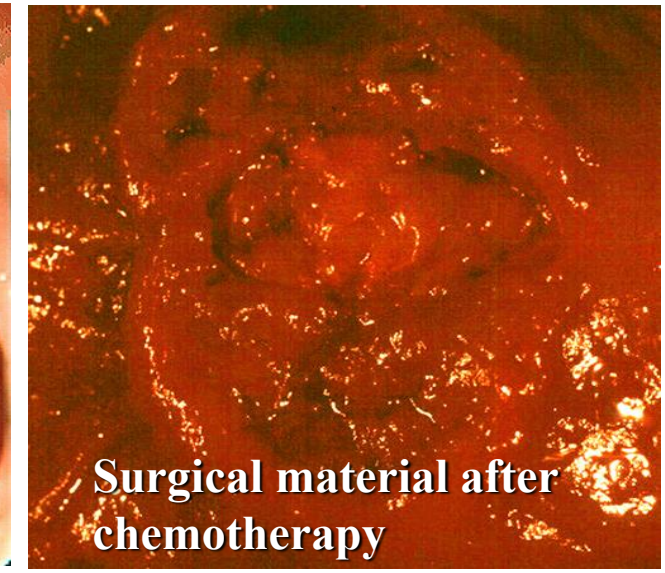
Case 1





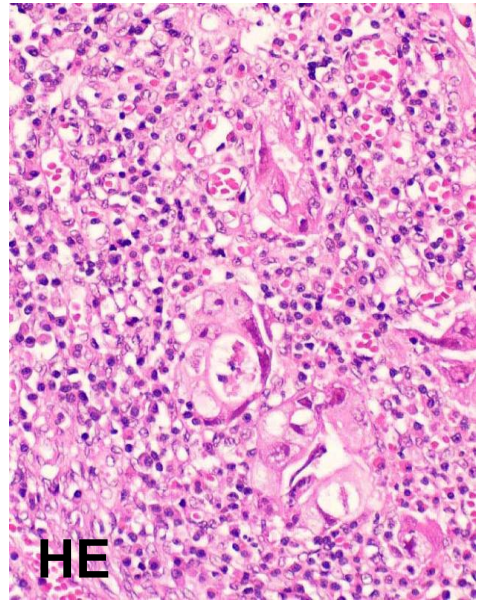
Case 2

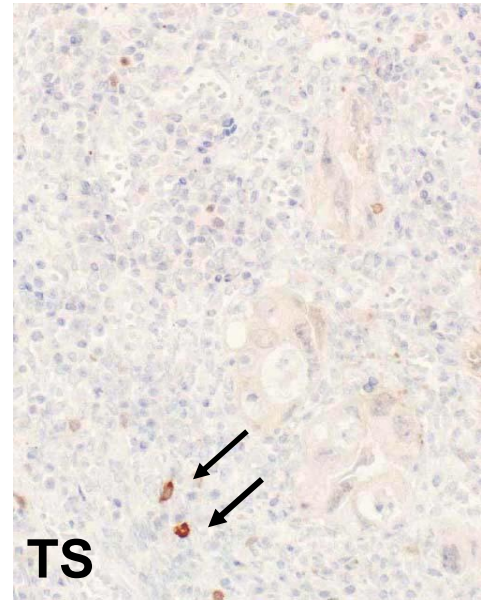


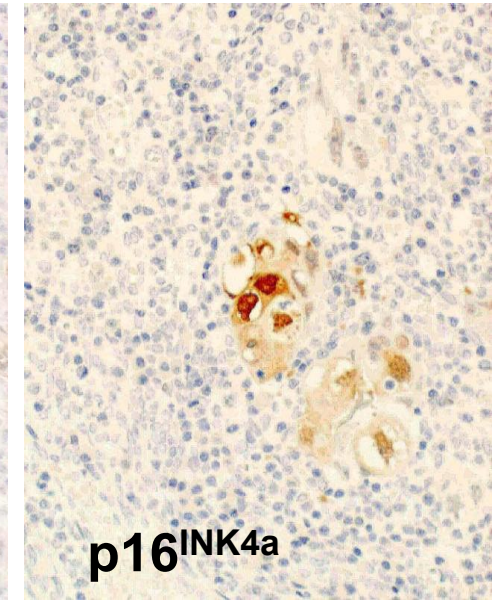


Preoperative chemotherapy:
Grade 2 reactivity seen in two cases of colon cancer

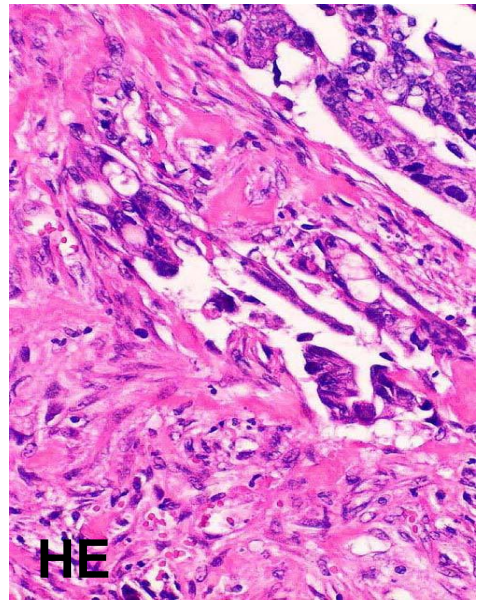
Case 1

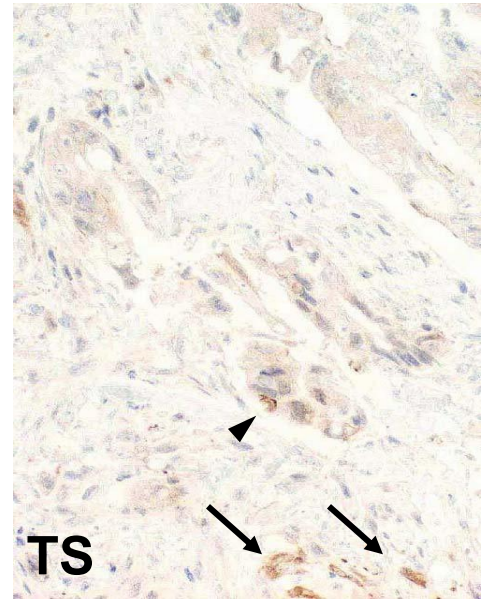


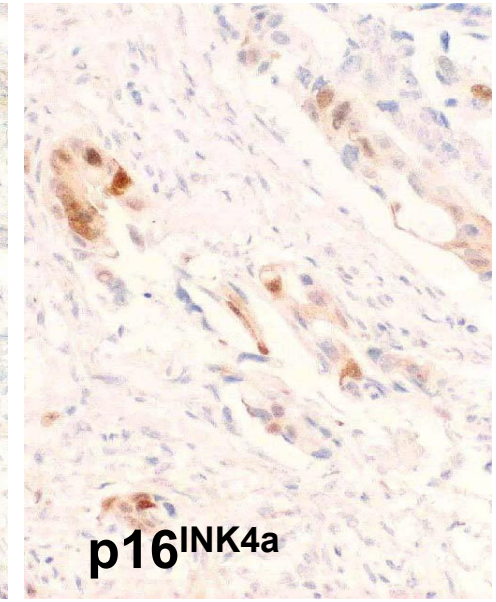




Case 2

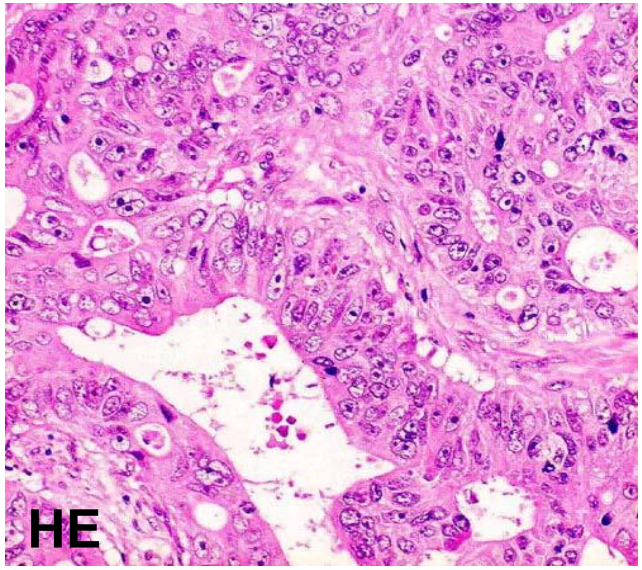


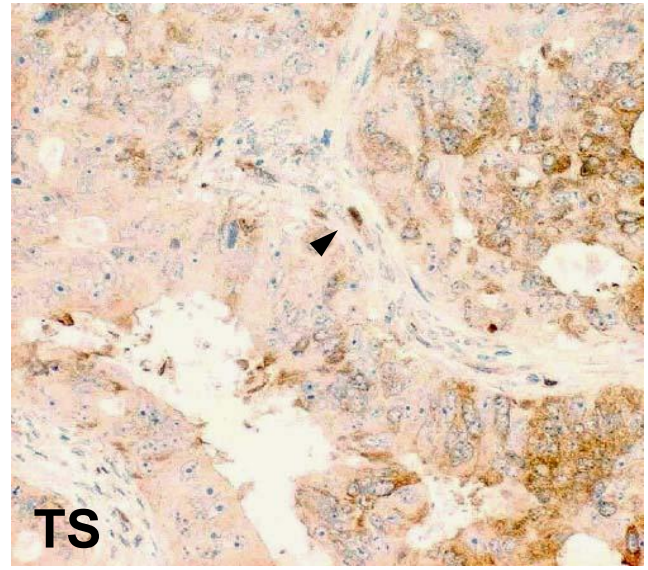


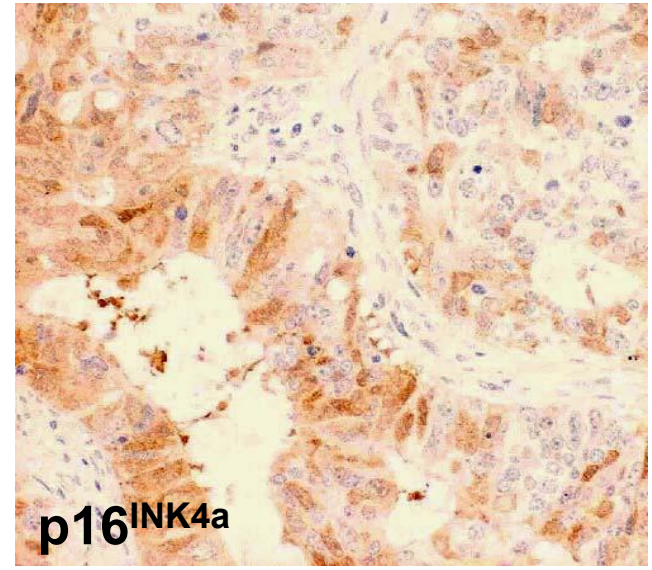


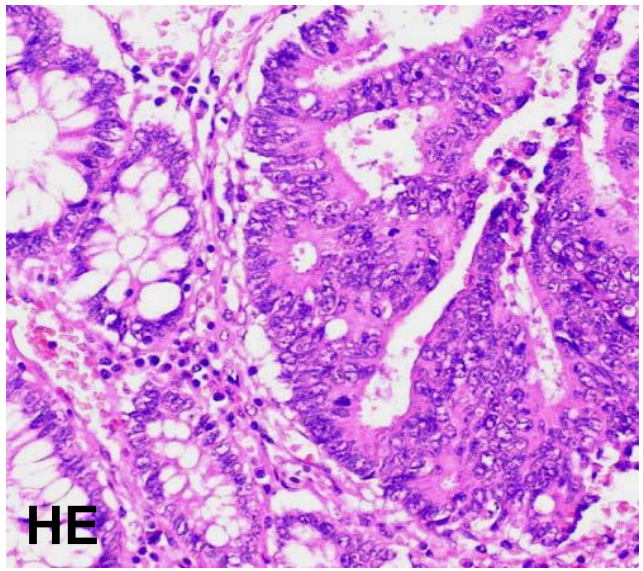
TS＝
thymidylate
synthase

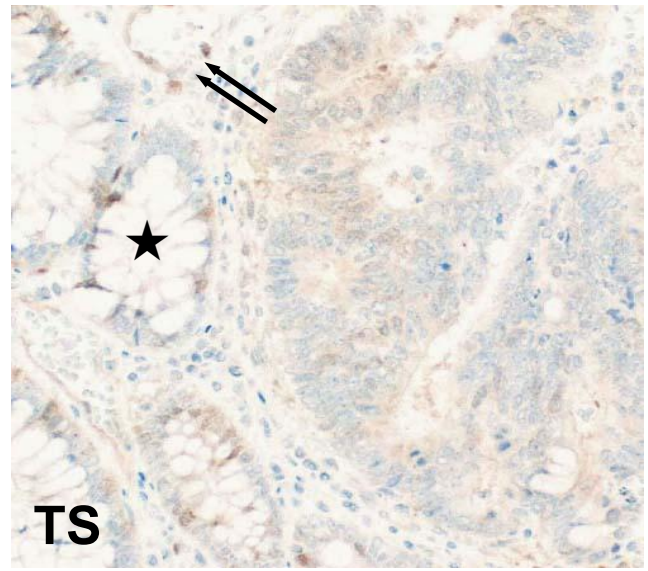
Preoperative chemotherapy-reactive colon cancer:
Expression of TS and $p16^{INK4a}$ (TS-negative and p16-positive)

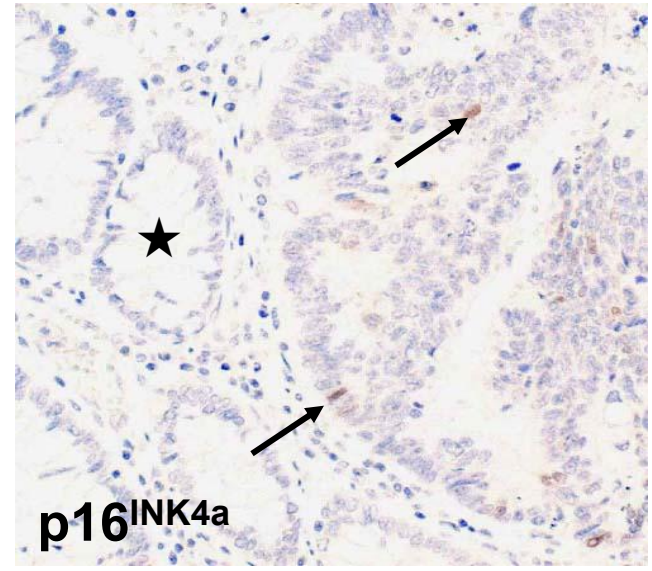












Preoperative chemotherapy-unresponsive colon cancer:
Expression of TS and $p16^{INK4a}$ (TS-positive or p16-negative)

Choice of anti-cancer drugs in colon cancer cases

1. Overexpression of TS represents 5-FU tolerance.
2. When low expresson of TS and high expression of $p16^{INK4a}$, causing inhibition of cell cycling, are seen, the colon cancer is sensitive to 5-FU.
3. In the 5-FU responsive cancer, $p16^{INK4a}$ was often induced after chemotherapy.
4. TS immunostaining in colon biopsy specimen will predicts 5-FU tolerance of the cancer cells.

Special techniques in immunohistochemistry

a) Detection of cell kinetics in sections

～Immunostaining for cell proliferation and apoptosis～

b) Immunostaining using a single section

～Re-staining method and cell transfer technique～

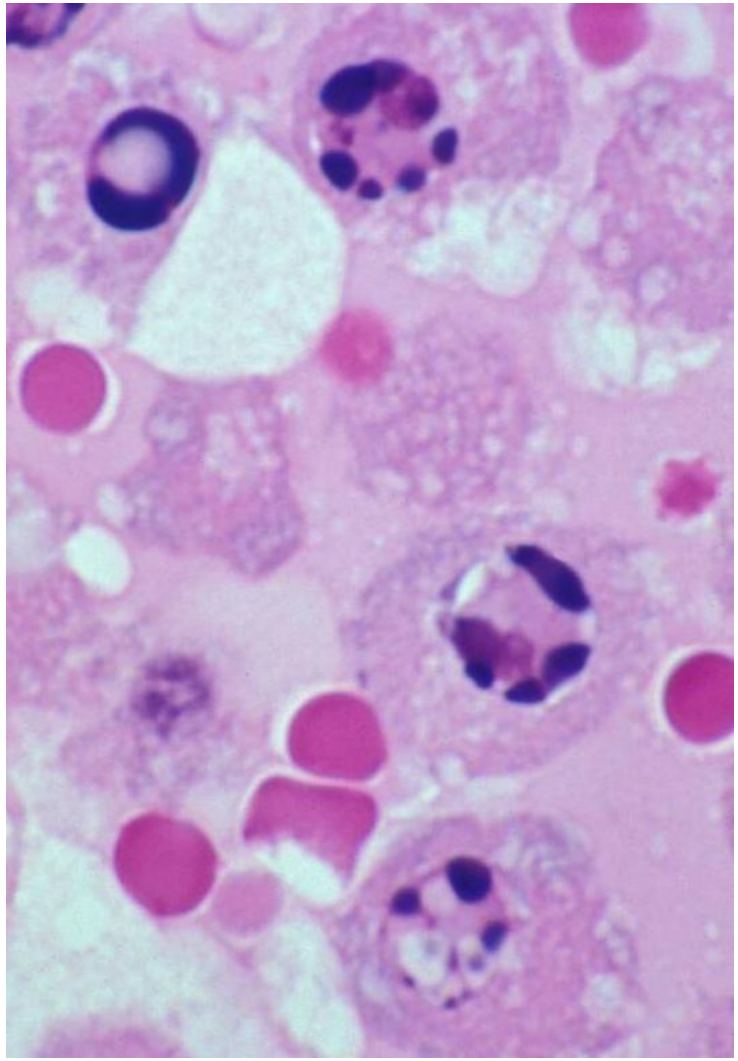
c) Use of patients' sera for immunostaining

～Histopathological diagnosis of infectious diseases～

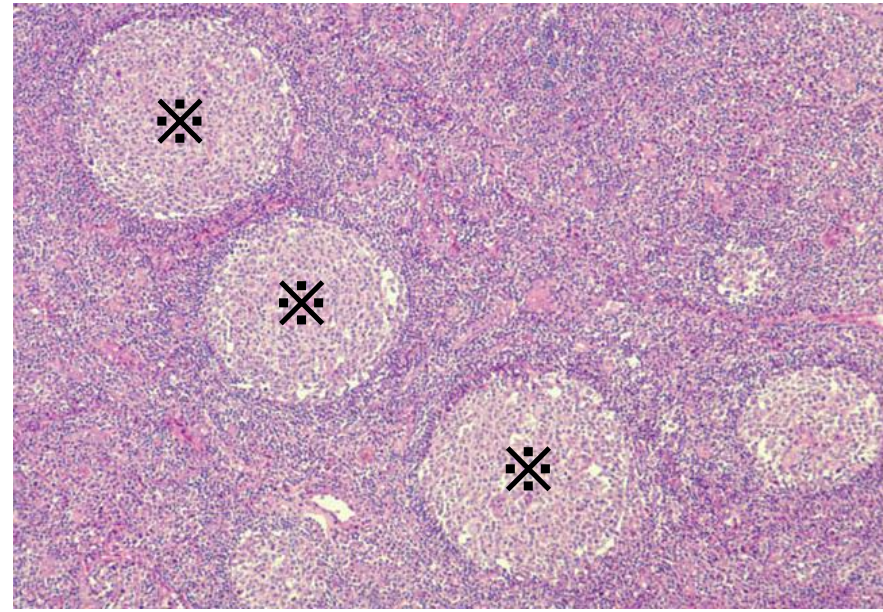
d) Staining using tissue fixed for a long period of time

～Hansen's disease and Hodgkin's lymphoma～

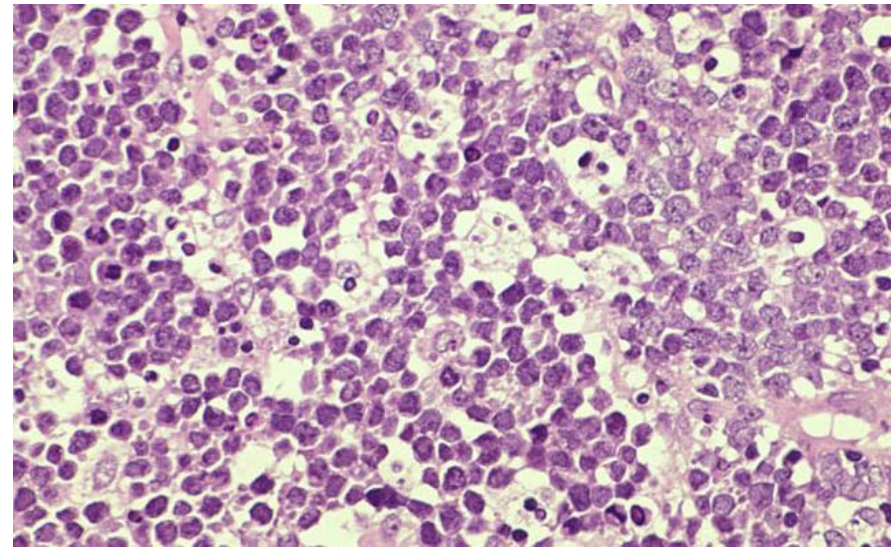
Analysis of cell kinetics：cell growth and apoptosis



Apoptosis of plasmacytoma cells in pleural effusion

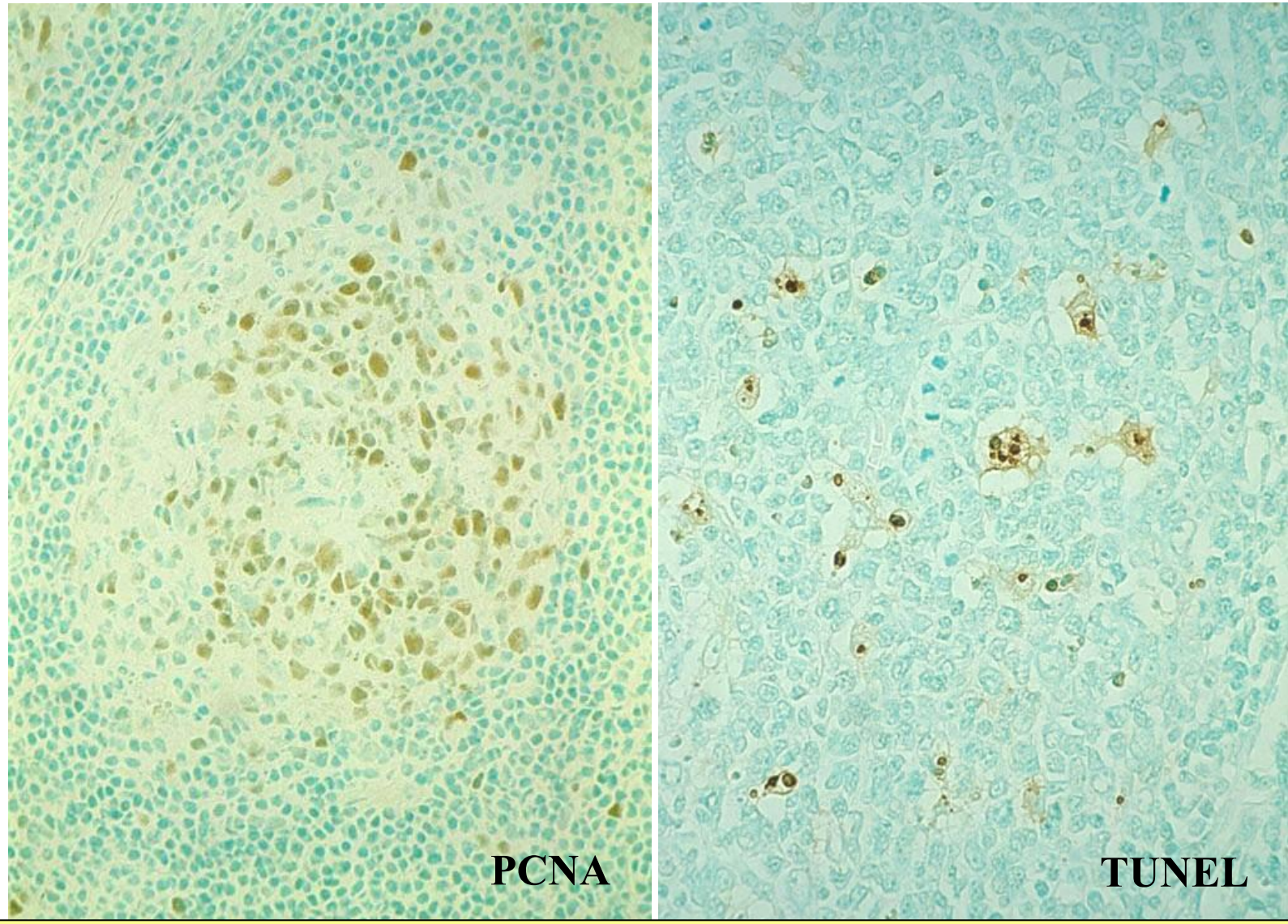


Gesrminal centers of the LN: the site of cell growth and apoptosis



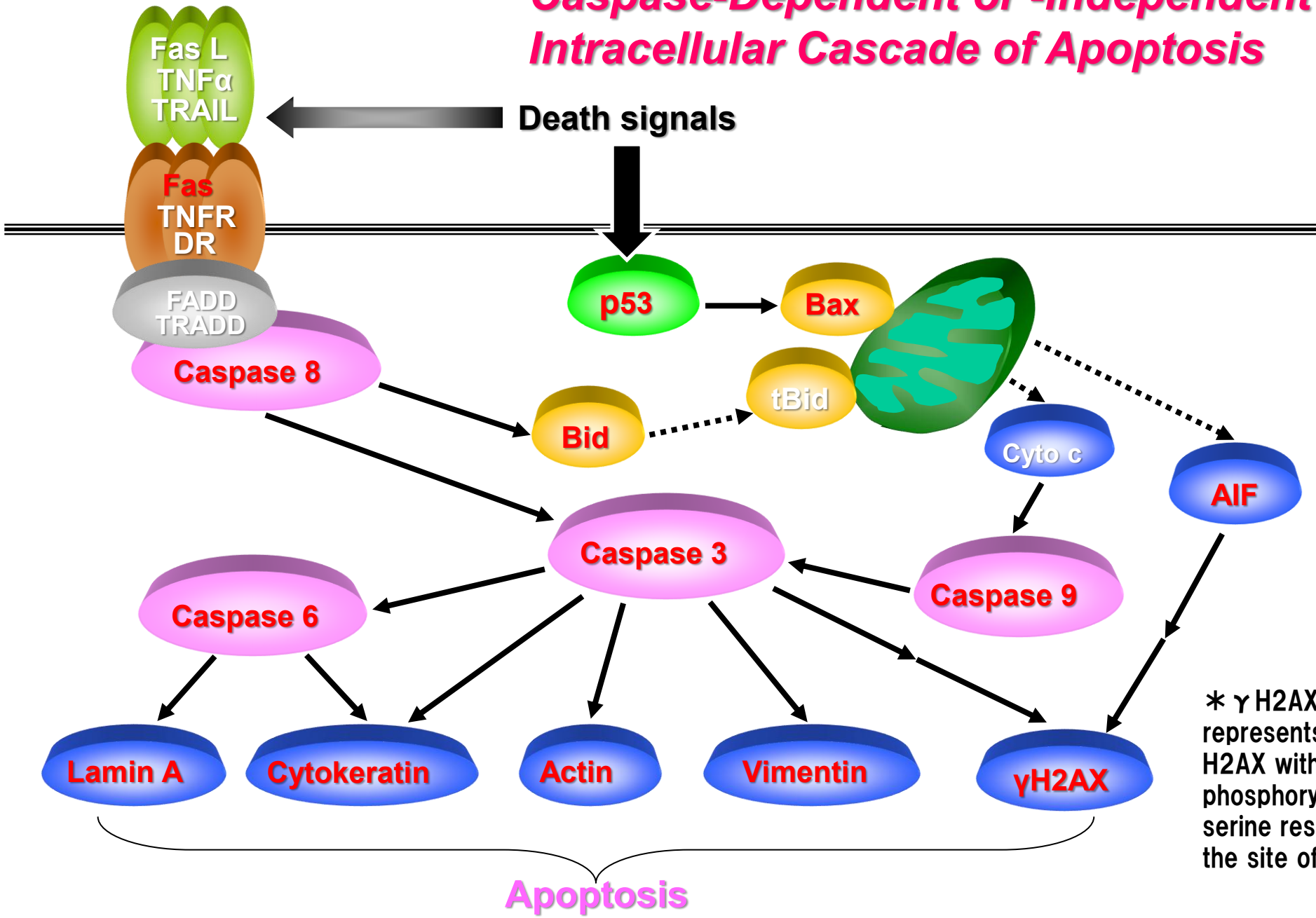
Apoptotic bodies in the germinal center

Both cell growth and apoptosis are activated in the germinal center



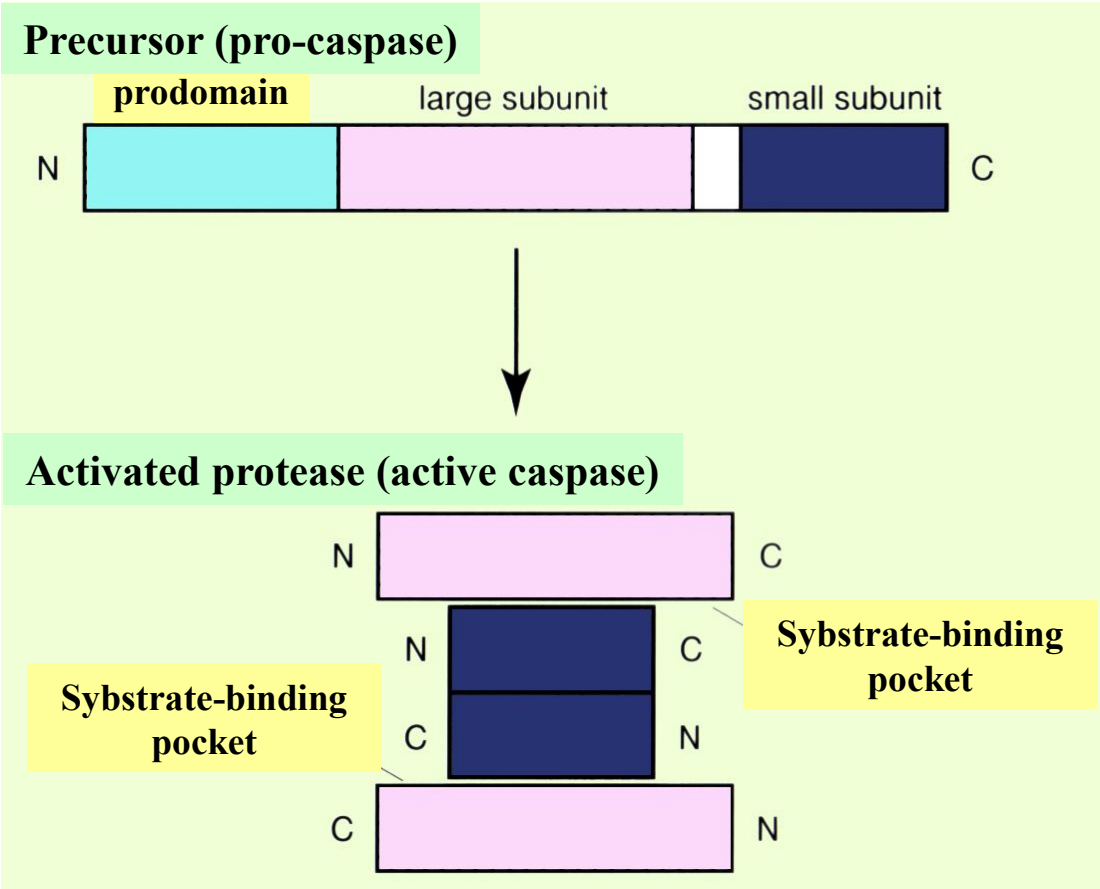
Germinal center of the lymph node：
left：cell growth (PCNA immunostaining)、right：apoptosis（TUNEL method）

Caspase-Dependent or -Independent Intracellular Cascade of Apoptosis

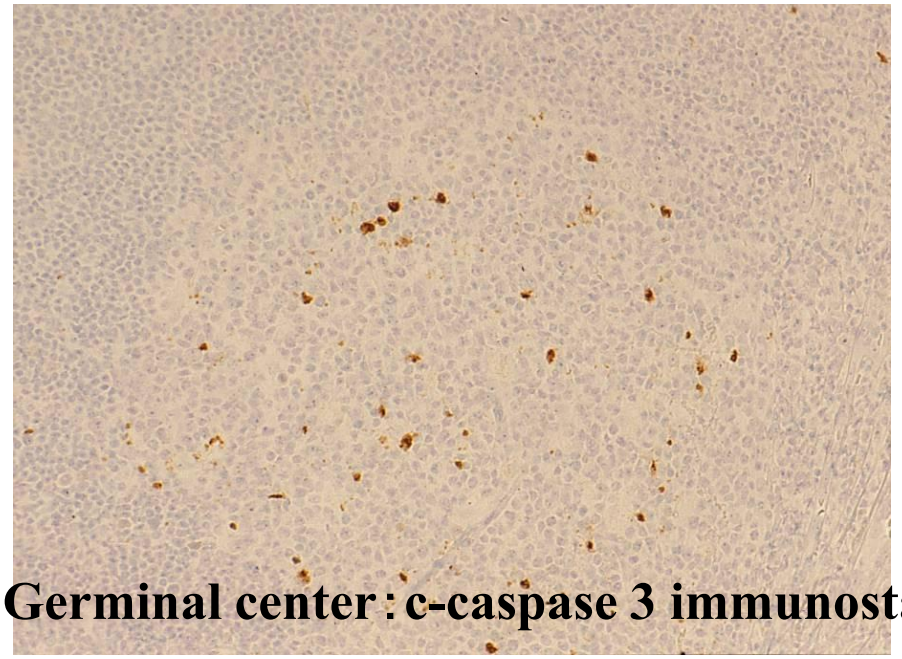


＊γH2AX represents histone H2AX with phosphorylated serine residues at the site of 139

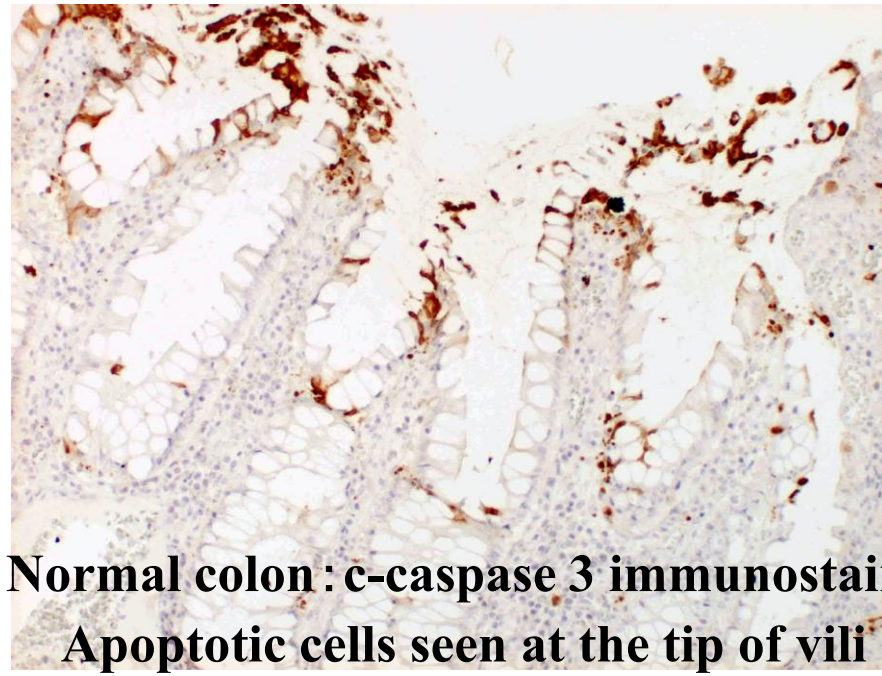
Activation of caspases



Antibody against cleaved (activated) caspase 3 (c-caspase 3) reacts with the cytoplasm of apoptotic cells.

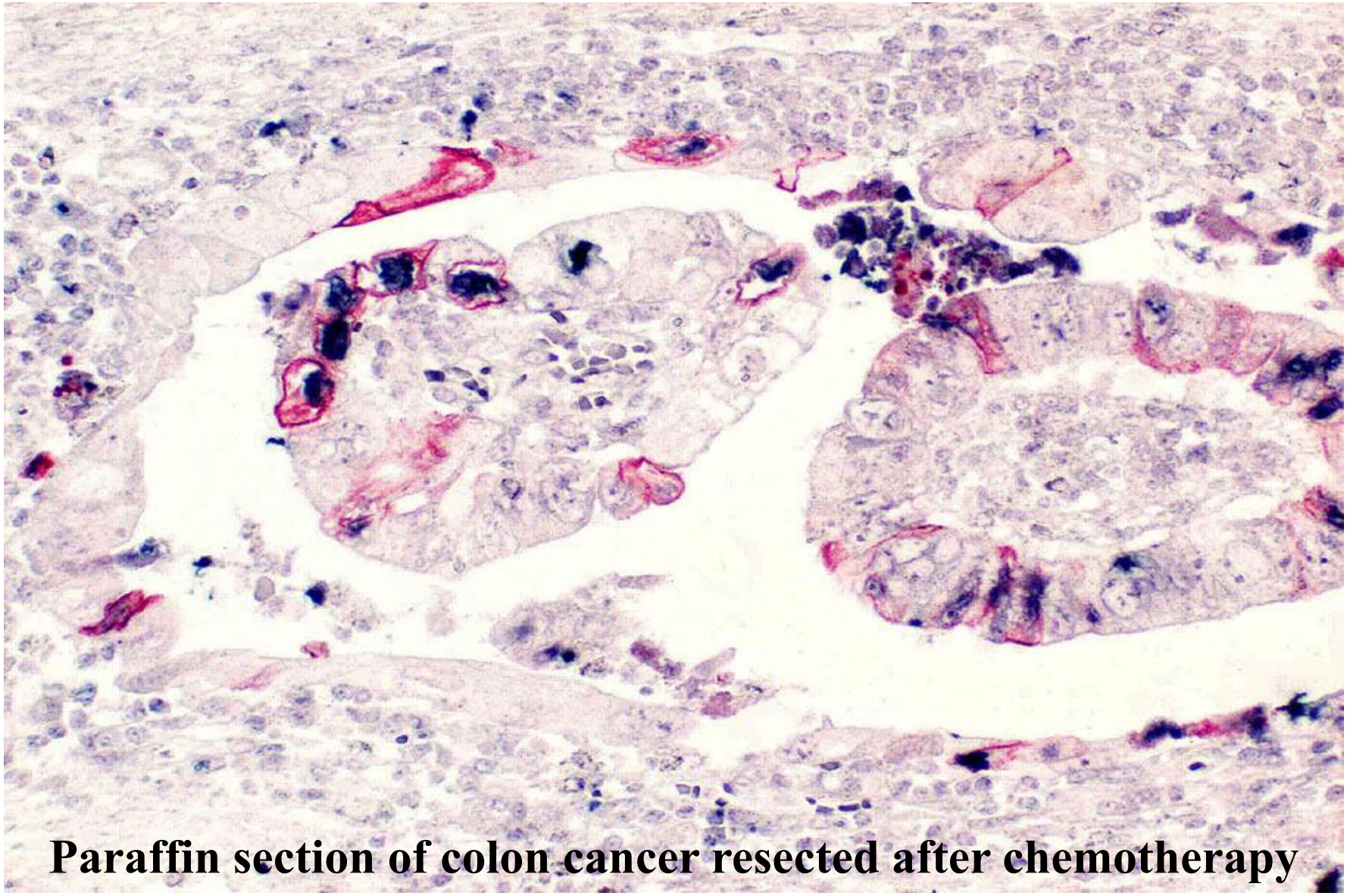


Germinal center : c-caspase 3 immunostain



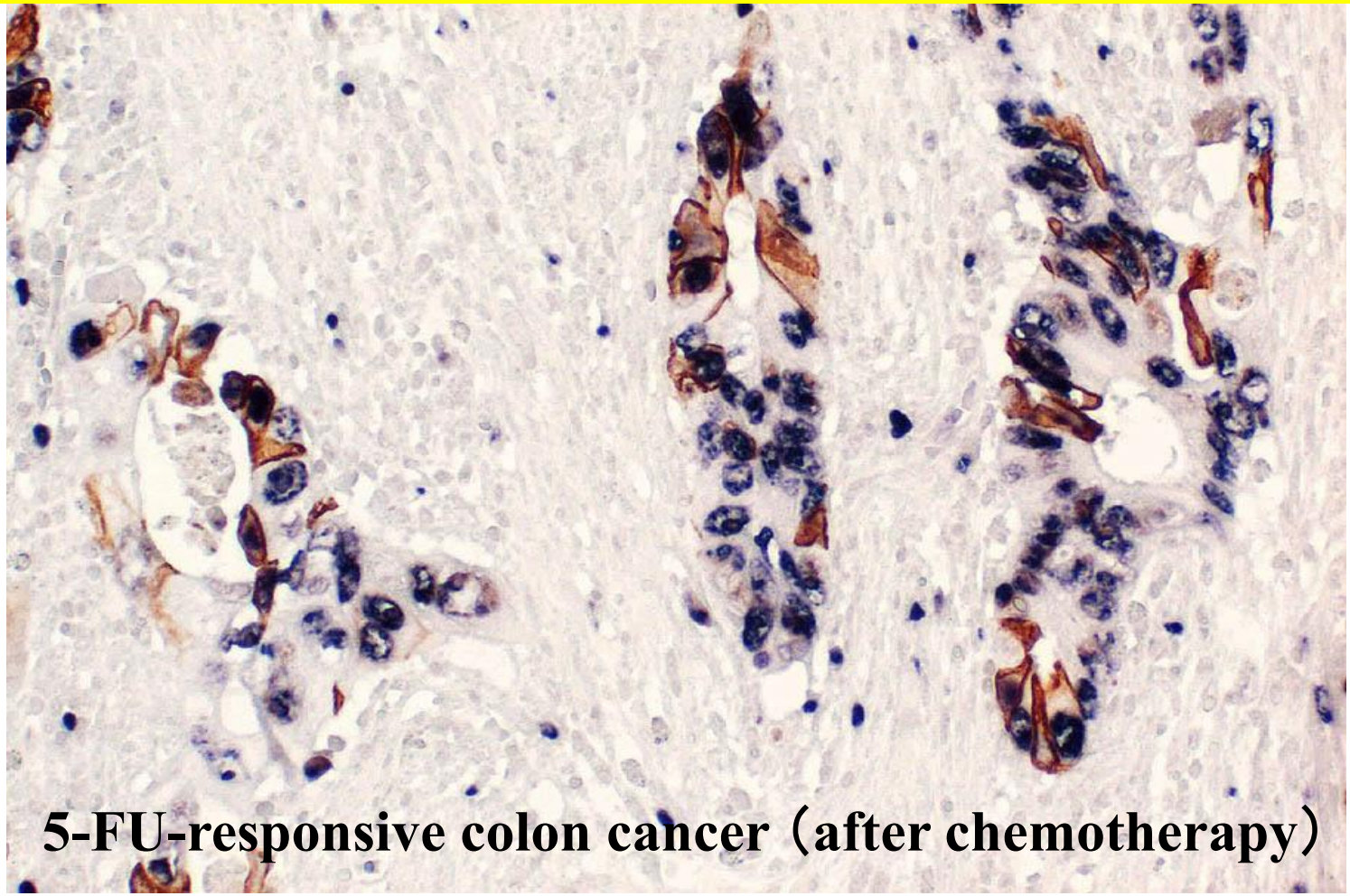
Normal colon : c-caspase 3 immunostain. Apoptotic cells seen at the tip of vili

Double stain for TUNEL（blue） and cleaved cytokeratin 18 (c-CK18： red)



Paraffin section of colon cancer resected after chemotherapy

Double stain for proliferating cells（Ki-67：blue）and c-CK18（brown）



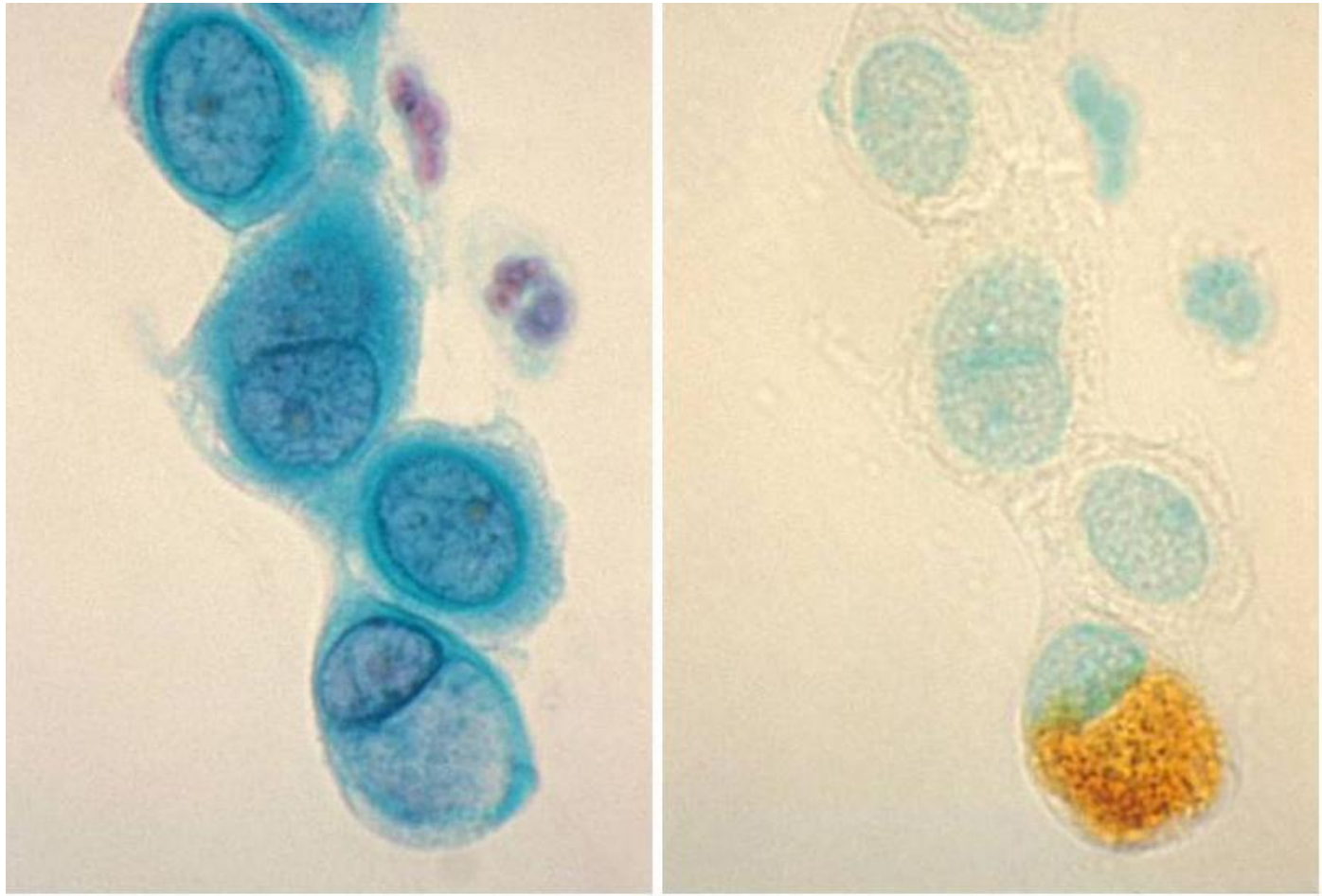
5-FU-responsive colon cancer（after chemotherapy）

Part of Ki-67-positive proliferating cancer cells show apoptosis represented by cleaved CK18 positivity

Techniques in immunostaining

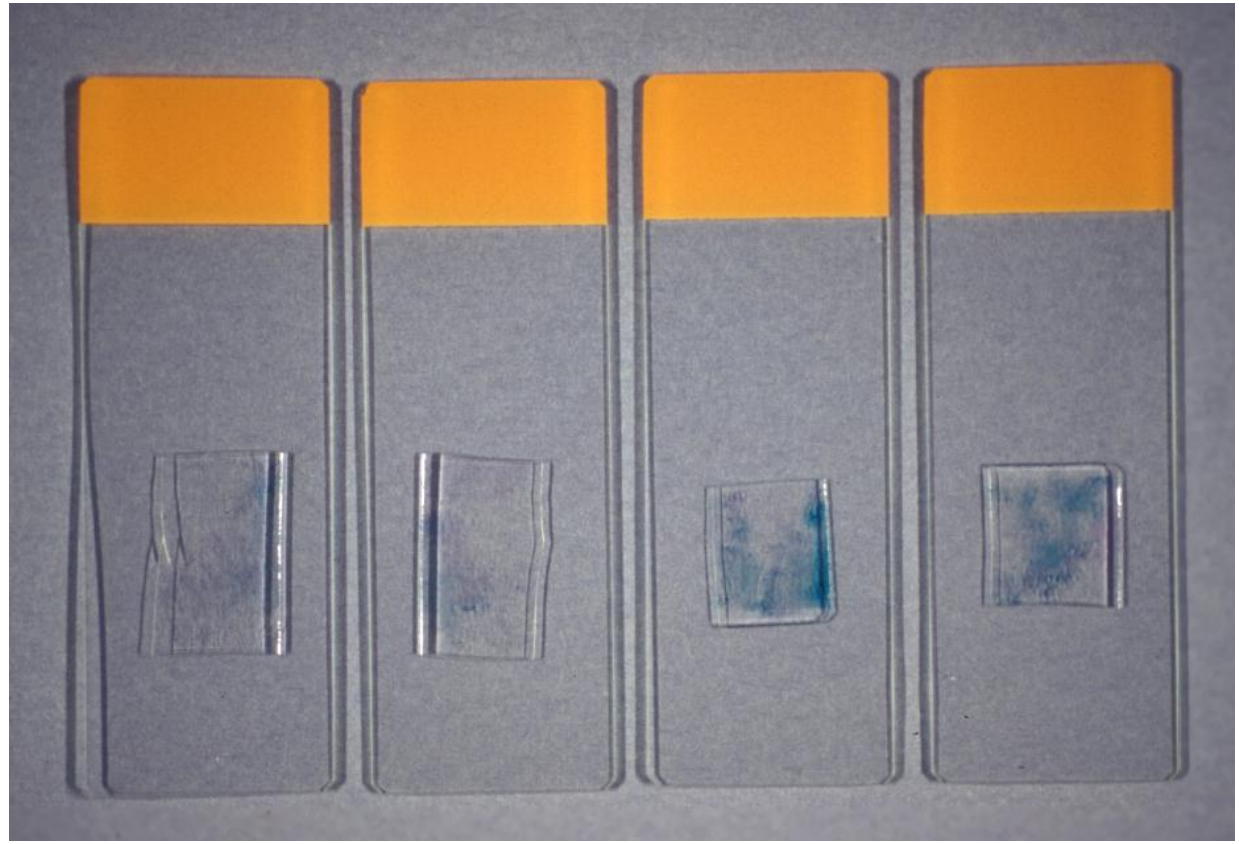
1) Re-staining method
2) Cell transfer technique
3) Use of patients' sera for detecting pathogens in paraffin sections
4) Use of widely cross-reactive antisera against pathogens to detect unknown pathogens in paraffin sections
5) Detections of pathogens in archival pathology specimens
6) The enzyme-labeled antigen method

Chlamydia trachomatis infection demonstrated in a single cytology slide（re-staining method）



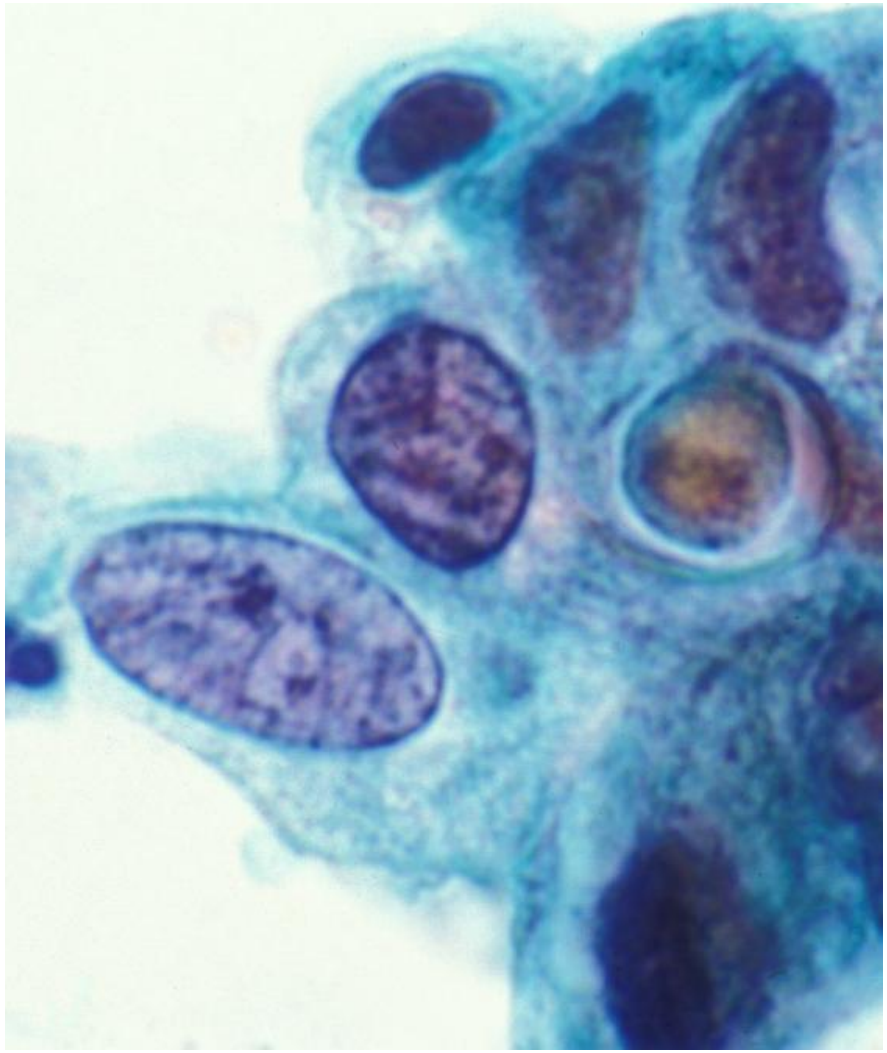
Nebular inclusion in chlamydial cervicitis（pap）and *C. trachomatis* antigen localization in the same cell

Immunostaining using cells on a single glass slide

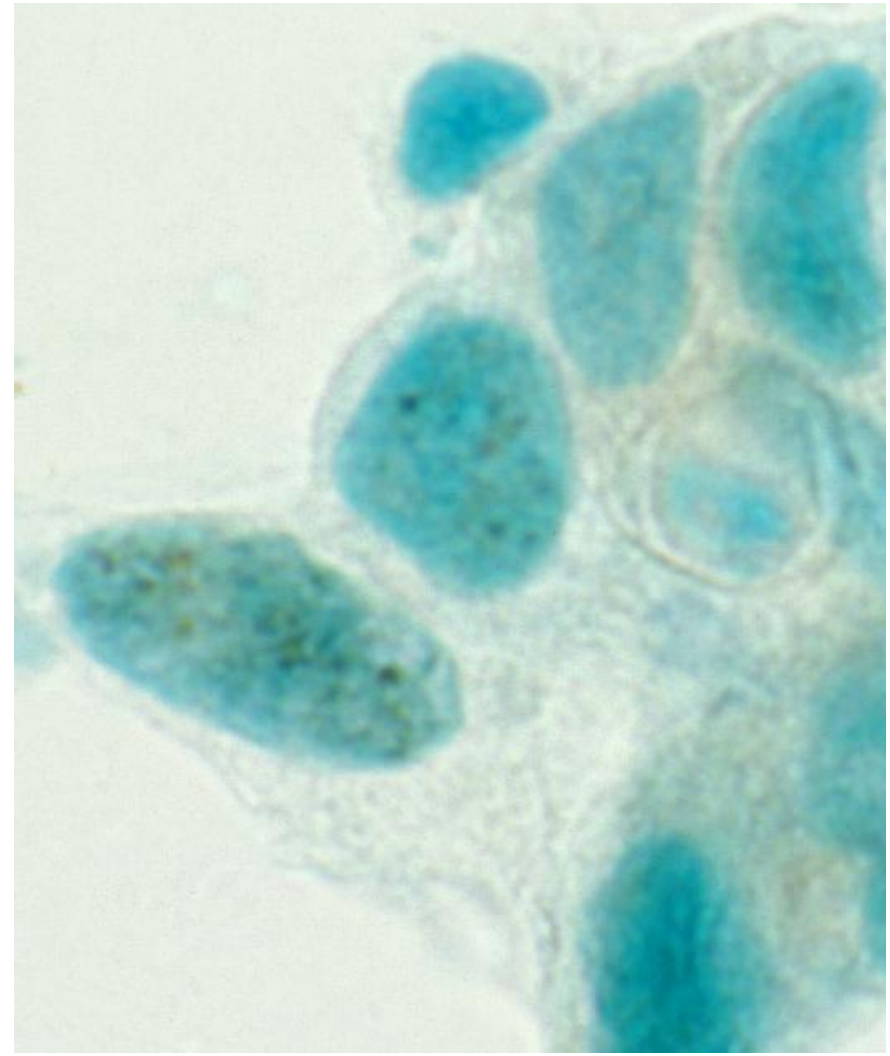


Cell transfer technique: Solidified Malinol membrane is cut and placed on the silane-coated glass sllides

HPV genome in severe dysplasia of the cervix



Severe dysplasia in cervical smear（pap）



The same cells transferred to silane-coated glass slide:
ISH method for HPV type 16, requiring a heating step

Cell transfer onto the silane-coated glass slide must be done to avoid detaching off the cells during the ISH method requiring heating step. The silane-coated glass slide can retain the cells on the slide.

Use of patients' sera for immunostaining

1) High antibody titer is expected when host response to pathogens (abscess or granuloma) is confirmed histologically.

2) Indirect immunoperoxidase method should be applied to formalin-fixed, paraffin-embedded sections.

3) Commercial antibodies against pathogens are scarcely available and not cost-effective.

4) High specificity of the patients' serum is expected particularly in case of protozoan and helminthic infections.

5) Biohazardous sera such as carriers of hepatitis virus and HIV must not be used.

Case: visceral leishmaniasis (Kala Azar)

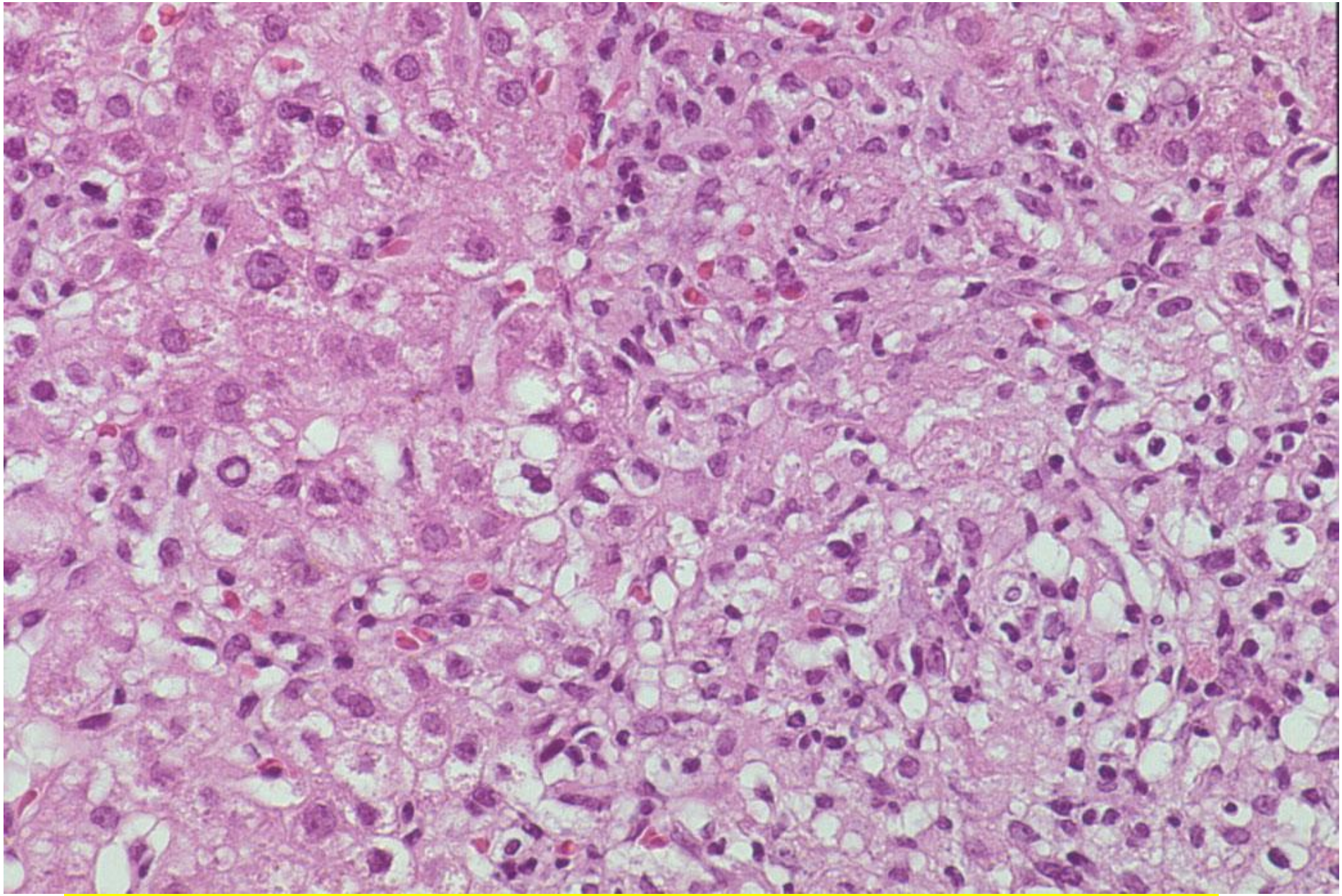
50 y-o Japanese male

He stayed a long period of time for business in Australia, Thailand and India.

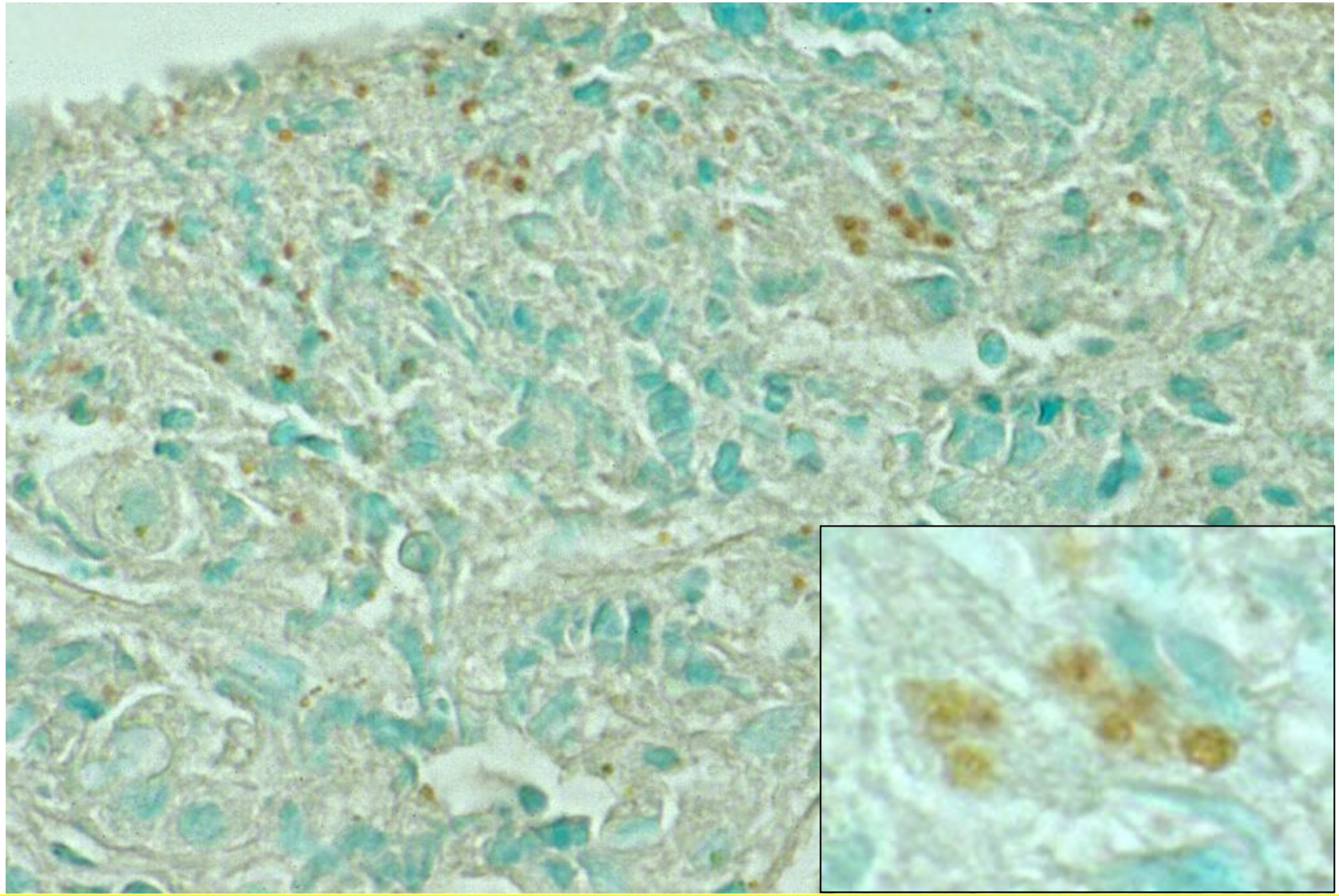
He suffered from high fever, liver dysfunction, thrombocytopenia and anemia.

Liver biopsy was performed.

Immunostaining using patient own serum was applied.



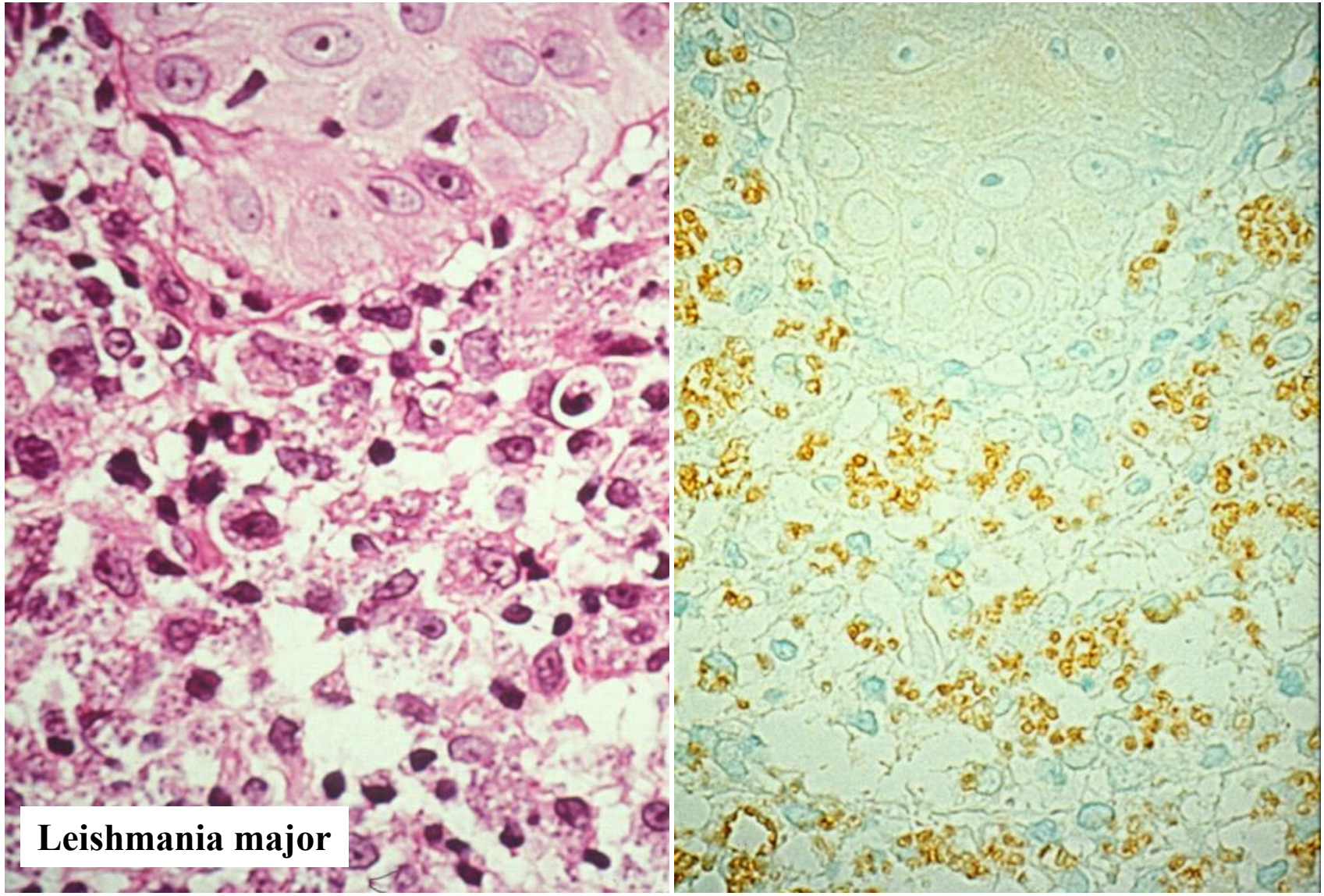
Liver biopsy: non-caseous small granuloma seen (H&E）



Protozoan bodies reactive with the diluted patient own serum: The cytoplasm of Kupffer cells or epithelioid cells is positive, and the size, shape and localization of the pathogens are compatible with protozoan infection.

The patient general condition was not good with thrombocytopenia and DIC. The histopathological diagnosis using the patient's own serum saved the patient's life. Treatment with Miltefosine and Paromomycin was effective.

African type cutaneous leishmaniasis（infected in Mali）



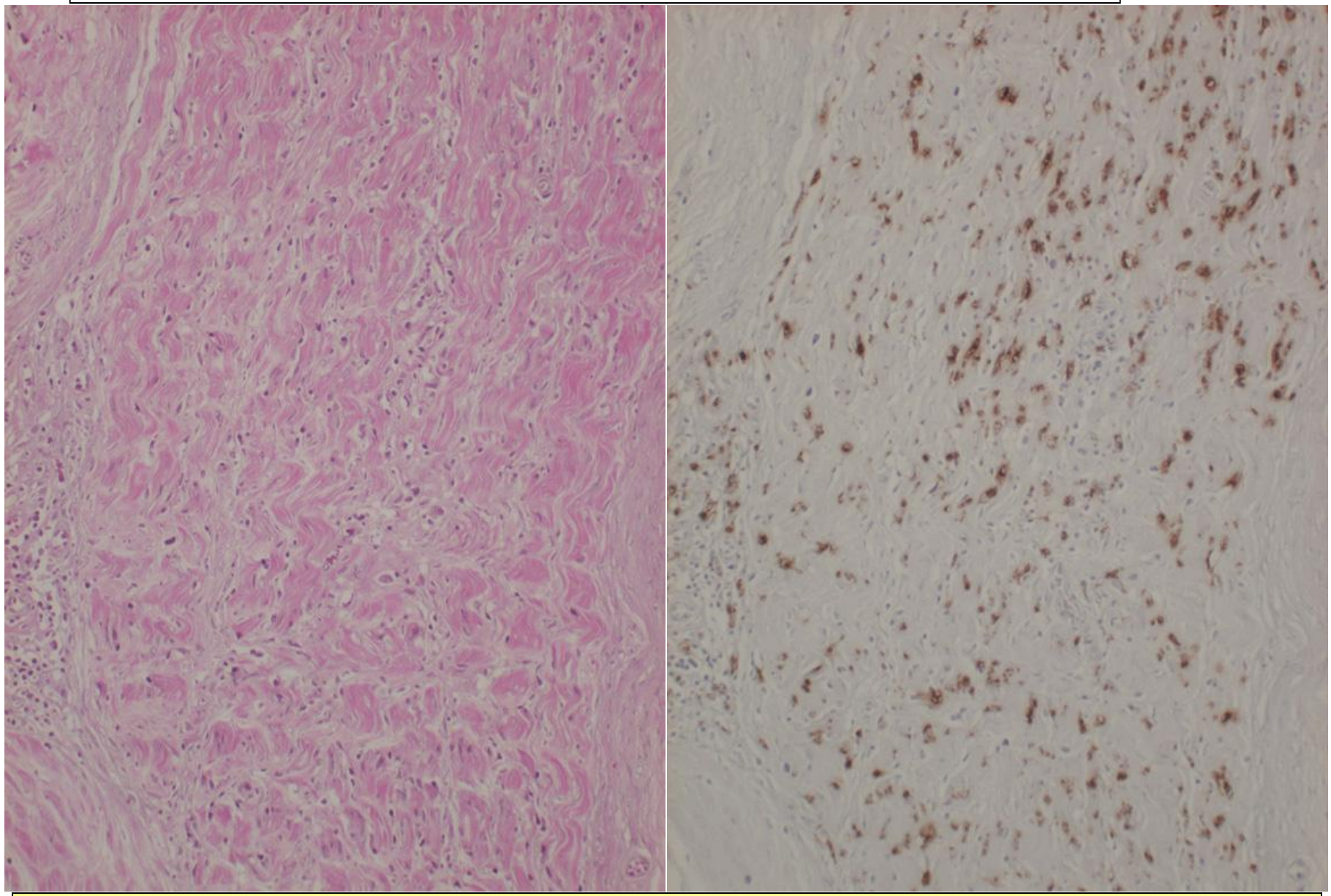
Leishmanian bodies in the cytoplasm of macrophages are clearly demonstrated by 1:500 diluated patient’s own serum.

Detection of pathogens in tissue samples of Hansen's disease autopsied during 1940's and 1990's

Materials: Formalin-fixed organs
(Fixation periods: 10～70 years)
Paraffin-embedded sections

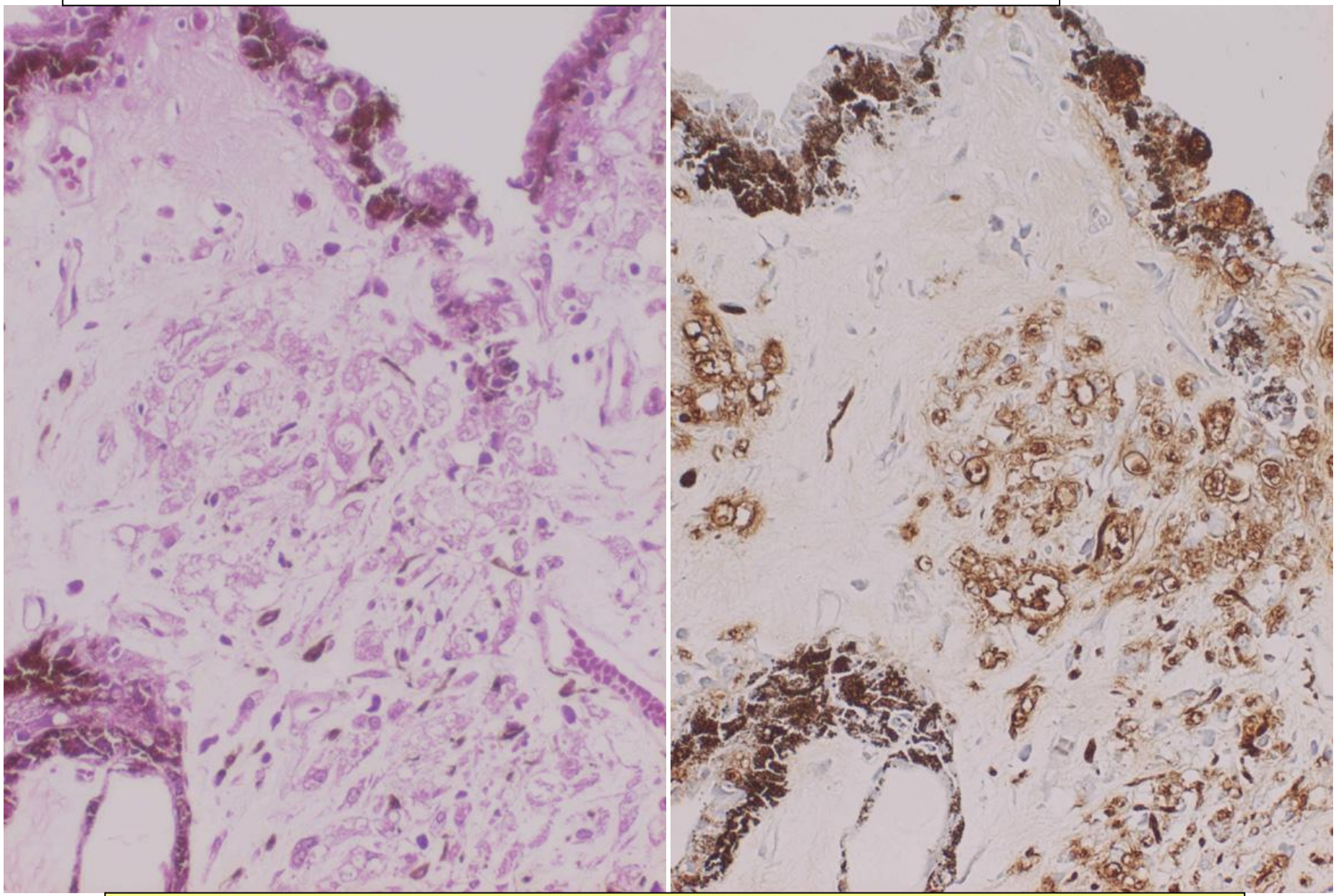
1) Detection of *M. leprae* with BCG immunostain
2) Detection of HCV: HCV-Ag and HCV-RNA

Sampling was done after formalin fixation for 40 years



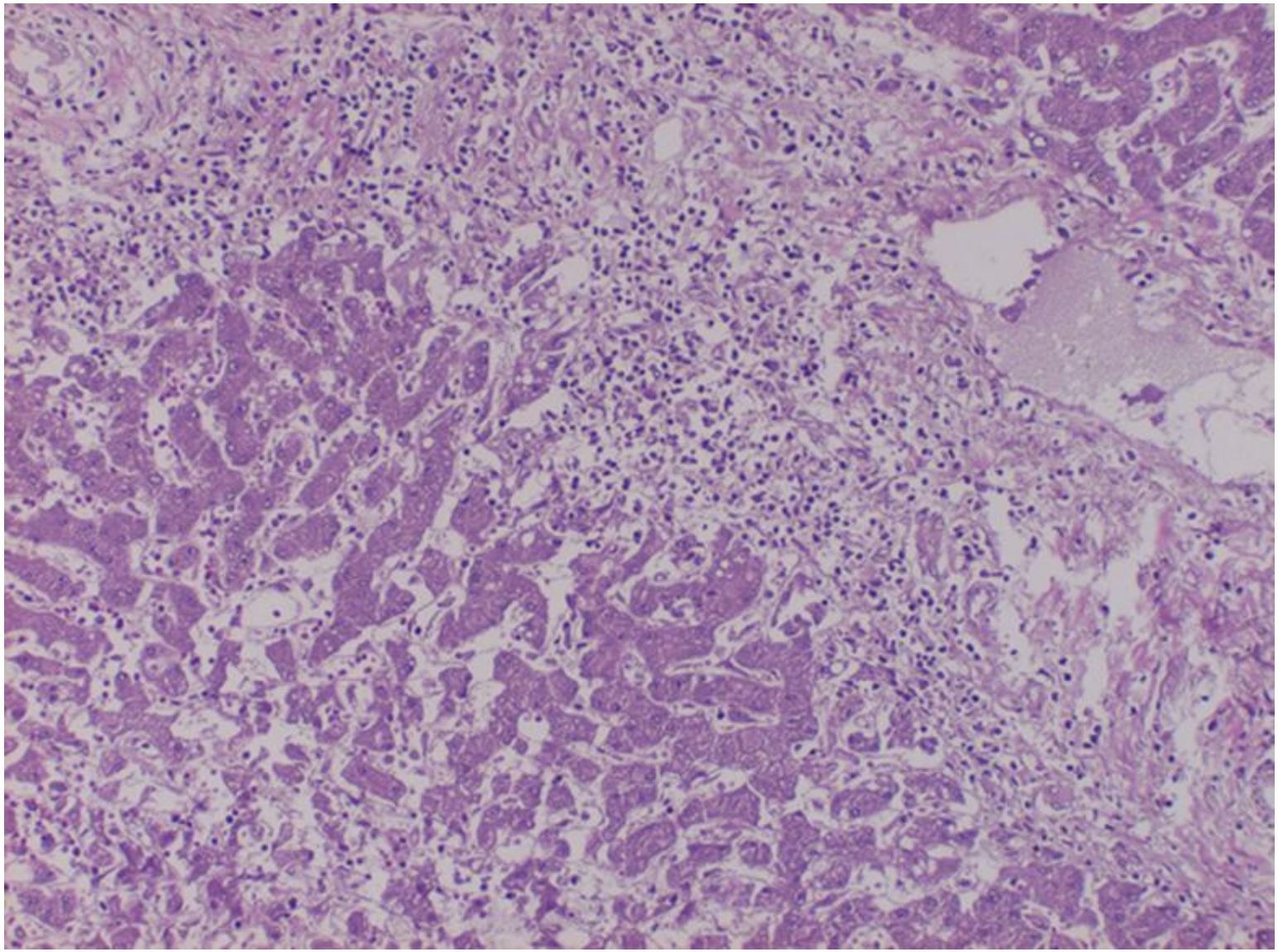
Peripheral neuritis in leprosy（left: H&E、right: BCG immunostain）

Sampling was done after formalin fixation for 70 years



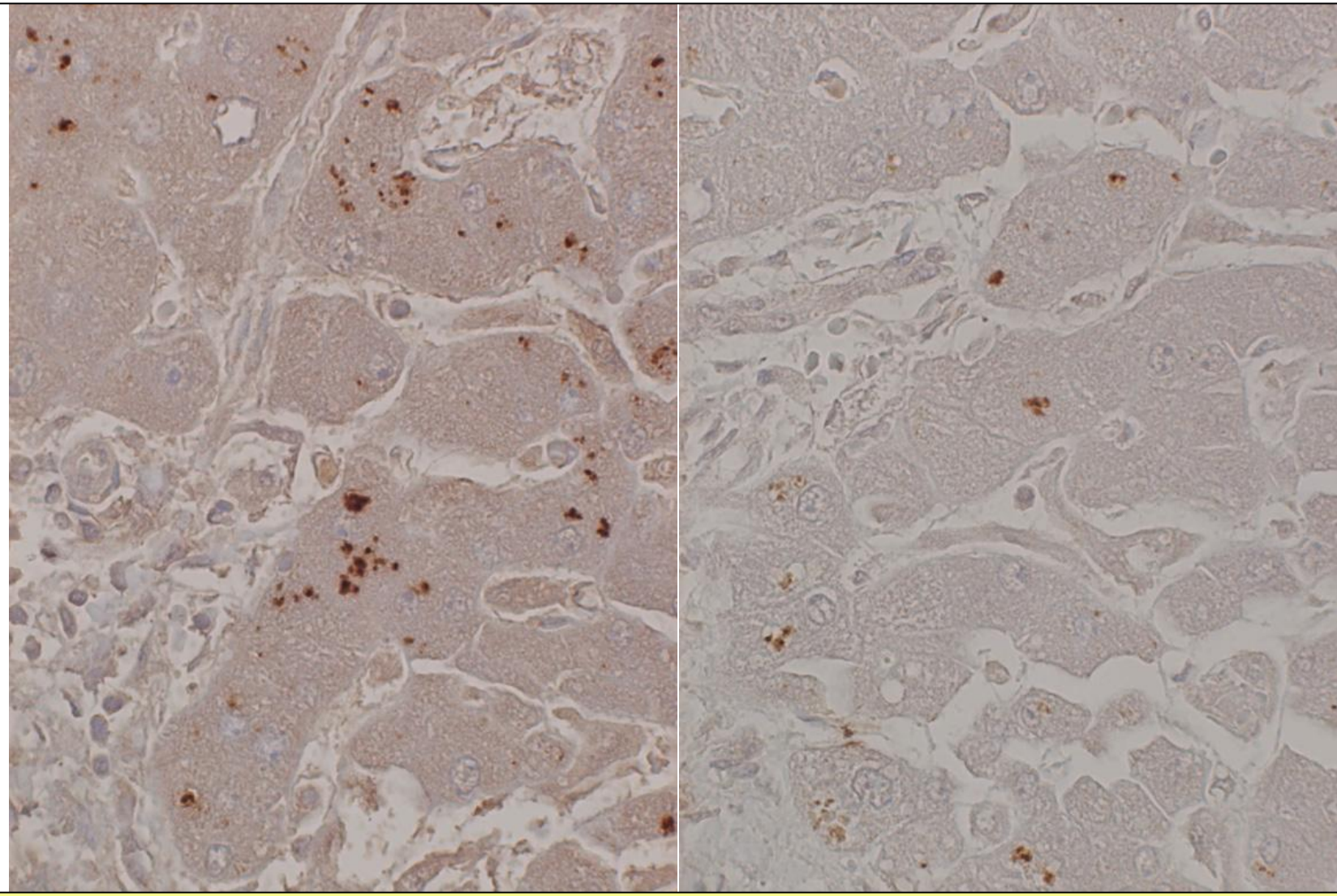
Iridocyclitis in leprosy（left: H&E、right: BCG immunostain）

Sampling was done after formalin fixation for 40 years

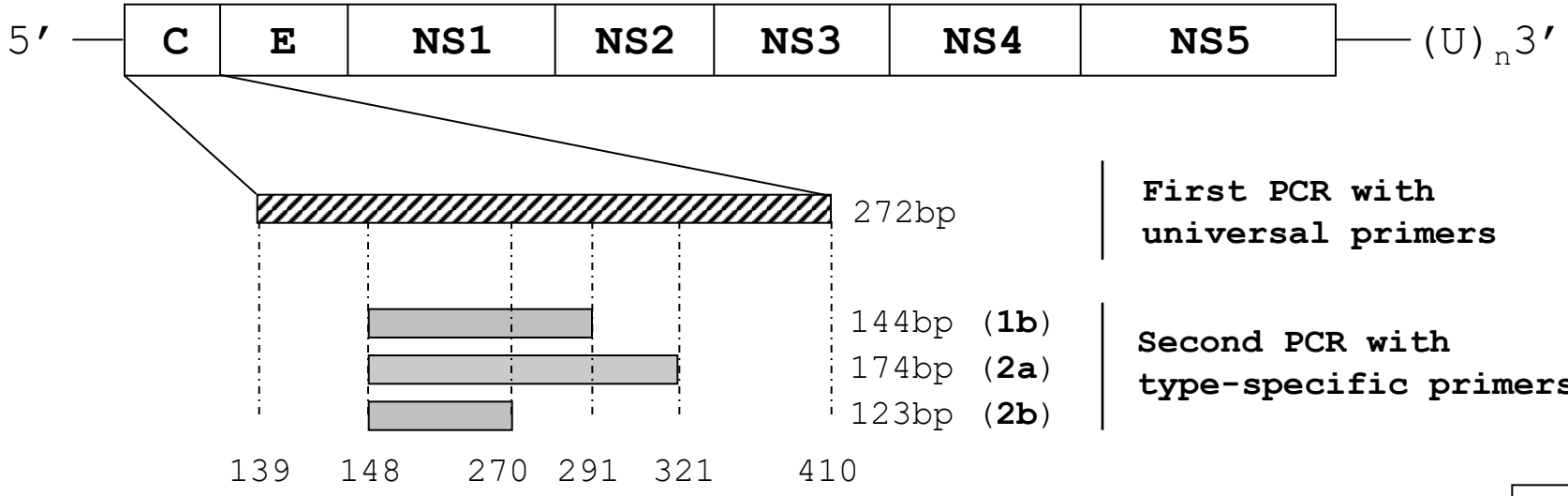


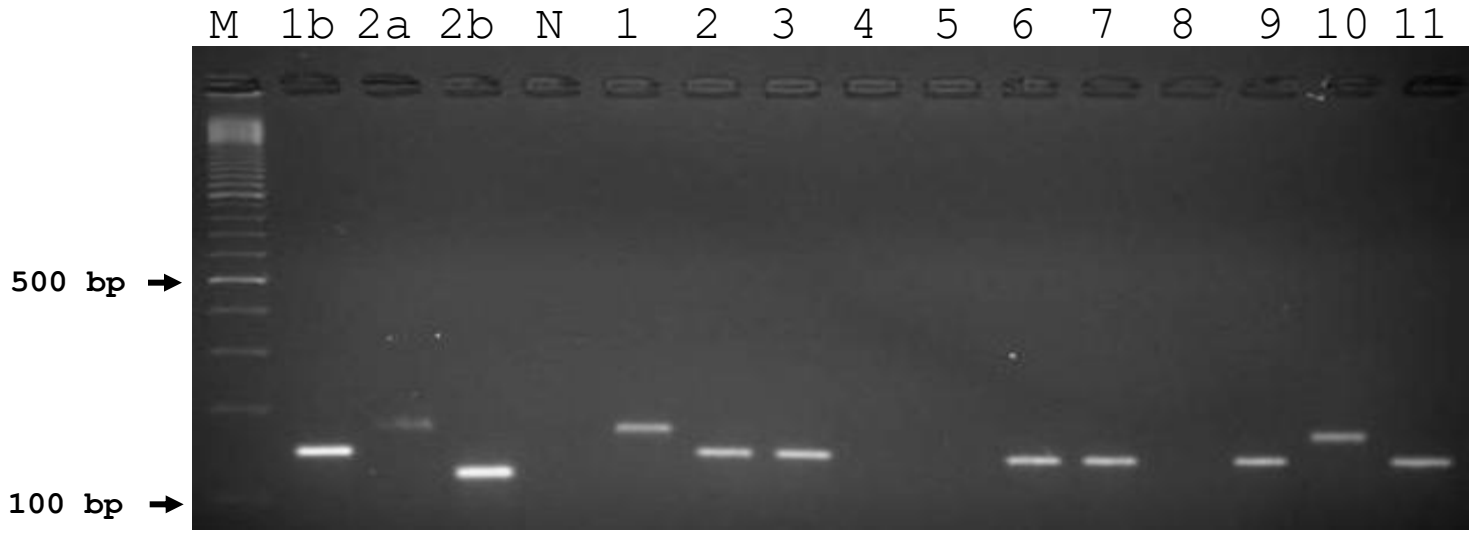
Type C hepatitis in a leprosy patient（H&E）: hospital-acquired infection due to intravenous injection of Promin without changing needles is indicated.

HCV antigen localization in the liver sampled after prolonged formalin fixation



Immunostaining after hydrated heating, amino acid polymer method.
HCV antigen is visualized in the cytoplasm of hepatocytes as granular positivities. left: NS3 of HCV, right: E1 of HCV





Lane 1: 1940 (LC)
Lane 2: 1942 (LC)
Lane 3: 1942 (LC)
Lane 4: 1964 (LC)
Lane 5: 1964 (LC)
Lane 6: 1970 (LC+HCC)
Lane 7: 1970 (LC)
Lane 8: 1987 (LC)
Lane 9: 1988 (LC)
Lane 10: 1989 (LC+HCC)
Lane 11: 1996 (LC)

Nested RT-PCR using HCV type-specific primers

(Total RNA was extracted from formalin-fixed, paraffin-embedded sections)

M: size marker、1b, 2a and 2b: positive control (patient serum)、N: normal liver

HCV-RNA was preserved after prolonged formalin fixation

HCV subtype demonstrated by nested RT-PCR

genotyping	1b	2a	2b	Total
1940-1949	6	1	0	7
1950-1959	0	0	0	0
1960-1969	9	1	0	10
1970-1979	5	0	0	5
1980-1989	8	3	0	11
1990-1999	5	0	0	5
Total	33	5	0	38

HCV typing was succeeded by the nested RT-PCR.

Paraffin sections of the liver prepared after formalin fixation for 10 to 70 years were the target of analysis.

Detection of EBER (Epstein-Barr virus-encoded small RNA) in Hodgkin's lymphoma tissue autopsied by Thomas Hodgkin himself in 1820's

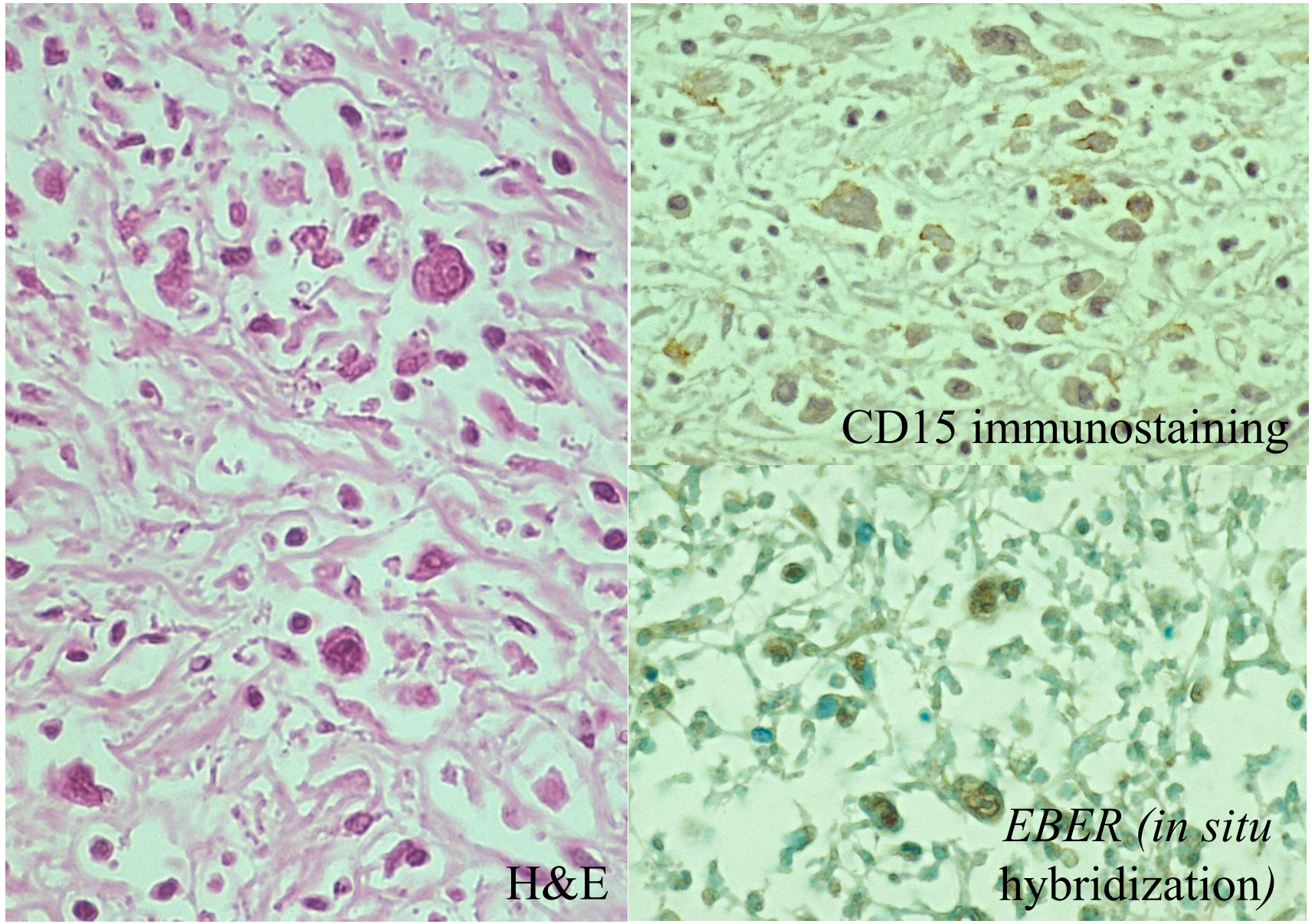
（Alcohol fixation 80 years and formalin fixation 80 years）

Autopsy sketch drawn by Carlswell, Dr. Hodgkin's friend (1828)

Autopsy tissues sampled by Thomas Hodgkin himself exhibited in the Gordon Museum (Guy's Hospital, London)

Tissue was fixed in alcohol for 80 years and then soaked in formalin for additional 80 years.

Unstained slides, one slide for each tissue, were given to me. I tried to detect EBER in the very original tissue of Hodgkin's lymphoma.

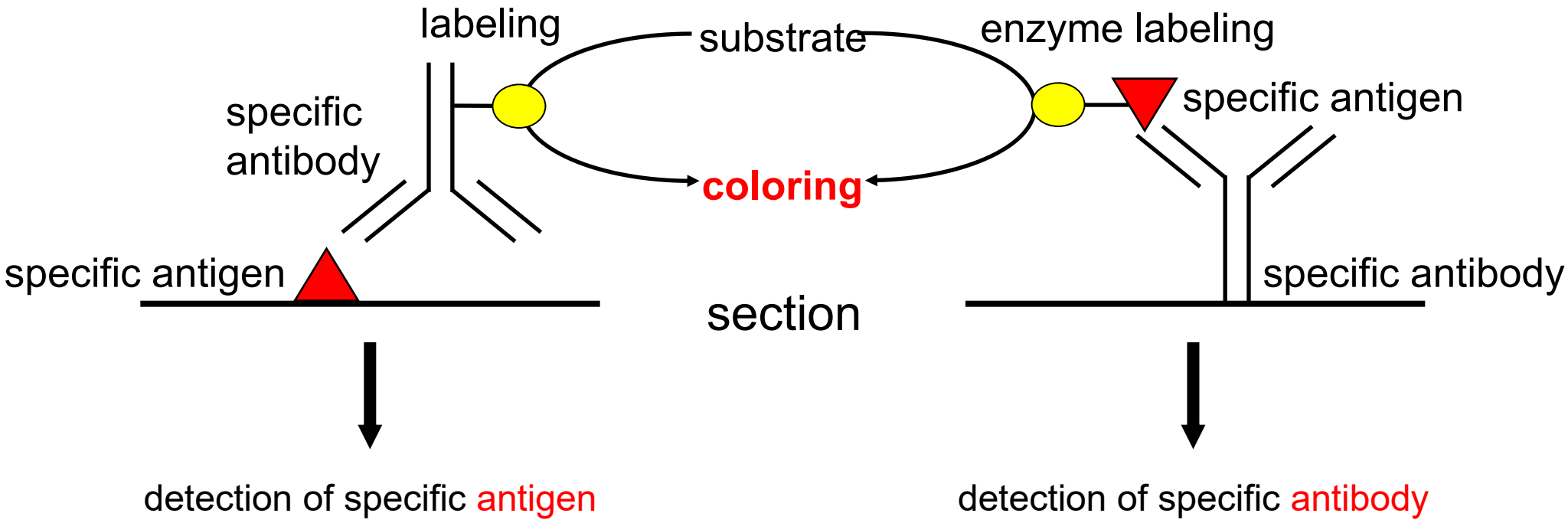


Liver involved by Hodgkin's lymphoma (50 y-o male case) autopsied by Thomas Hodgkin himself. CD15 is one of the markers of Hodgkin's and Reed-Sternberg cells, and many Hodgkin's lymphomas are positive for EBER.

Enzyme-labeled antibody method

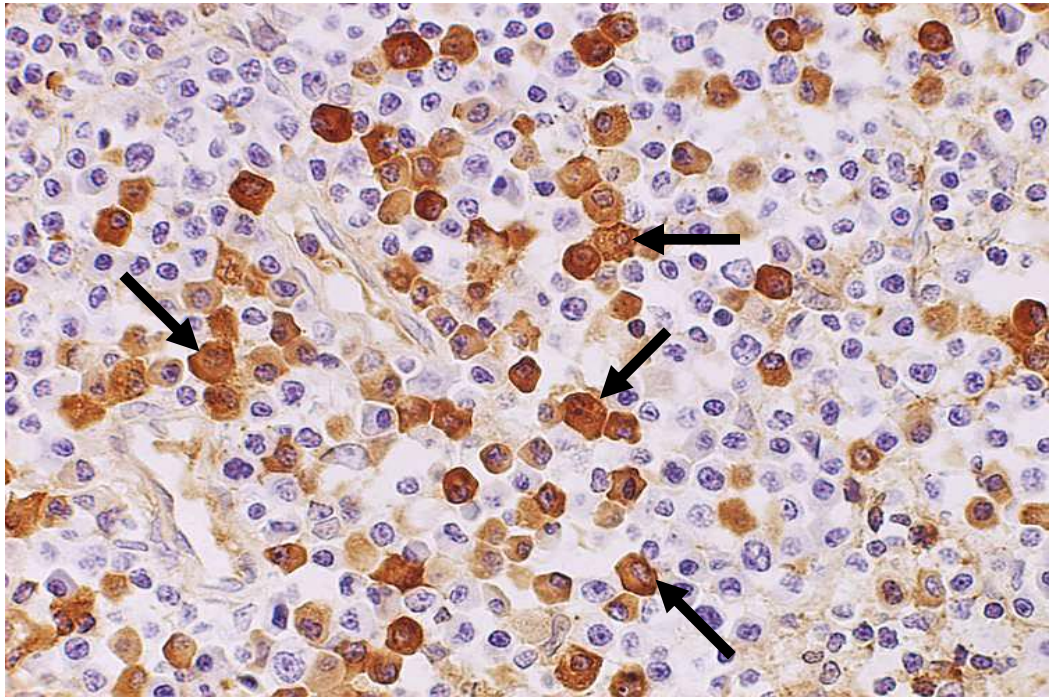
Enzyme-labeled antibody method（immunostaining）

Enzyme-labeled antigen method

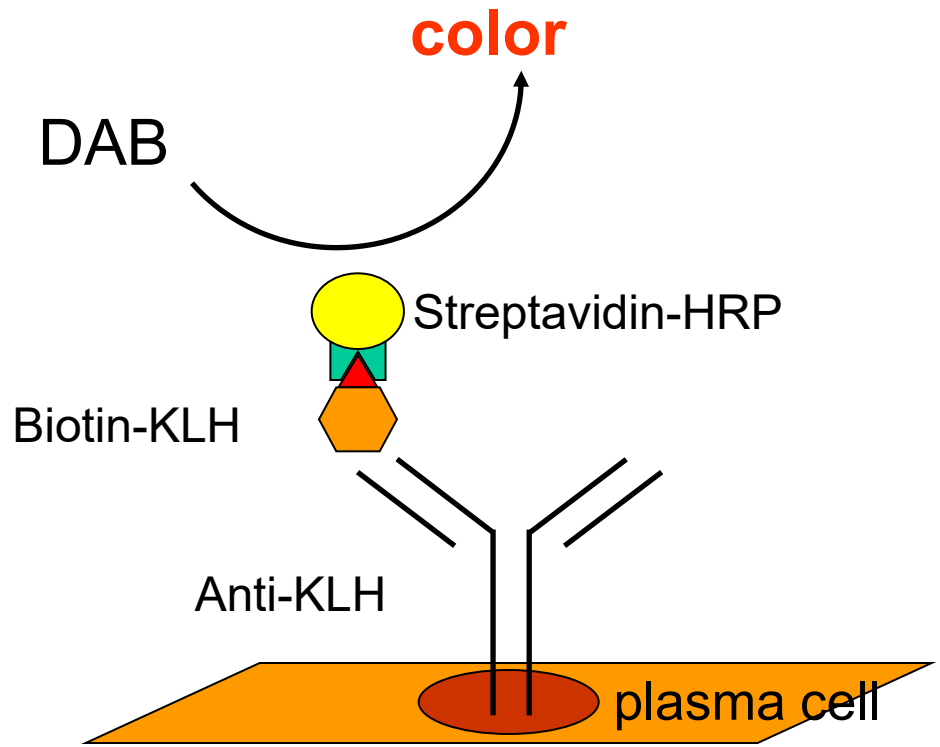


The enzyme-labeled antigen method is a reversed form technique of the enzyme-labeled antibody method.

The enzyme labeled antigen method visualizes the site of antibody production.

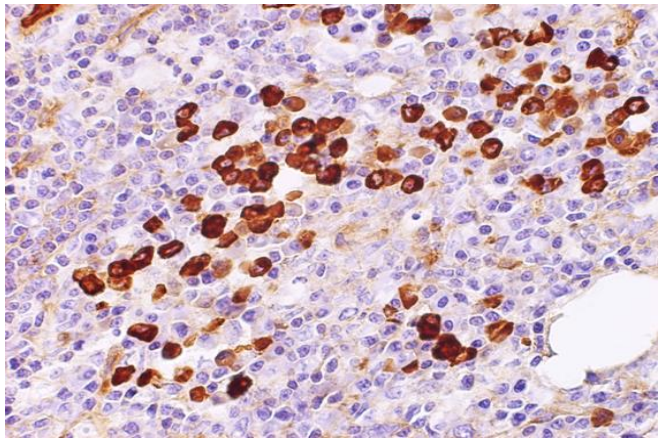


Plasma cells producing antibodies against keyhole limpet hemocyanin（KLH）in the rat lymph node

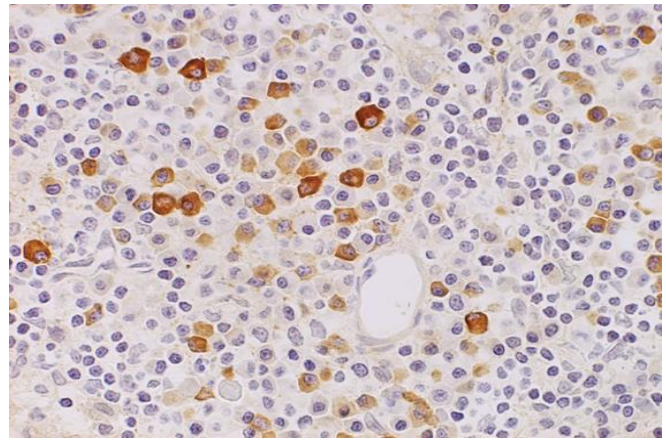


section of lymph node

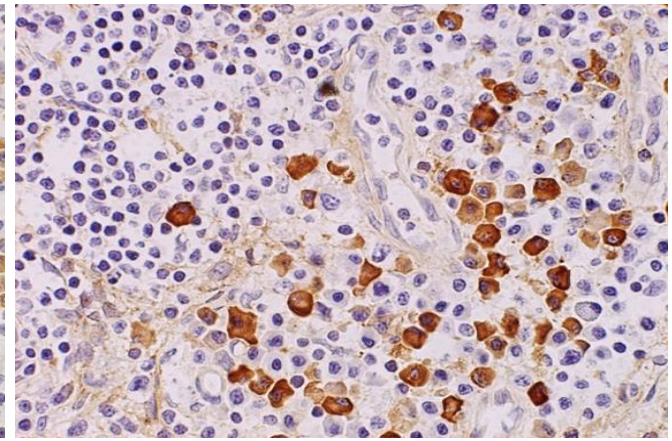
Variety of specific antibodies can be detected.



Horseradish peroxidase



Ovalbumin



Keyhole limpet hemocyanin

Lymph nodes of the rat experimentally immunized with the antigens

We need the target antigen!

↓

We should detect and purify the target antigen.

How should we do it?

Techniques of Sawaski's lab, Ehime University, Matsuyama, Japan

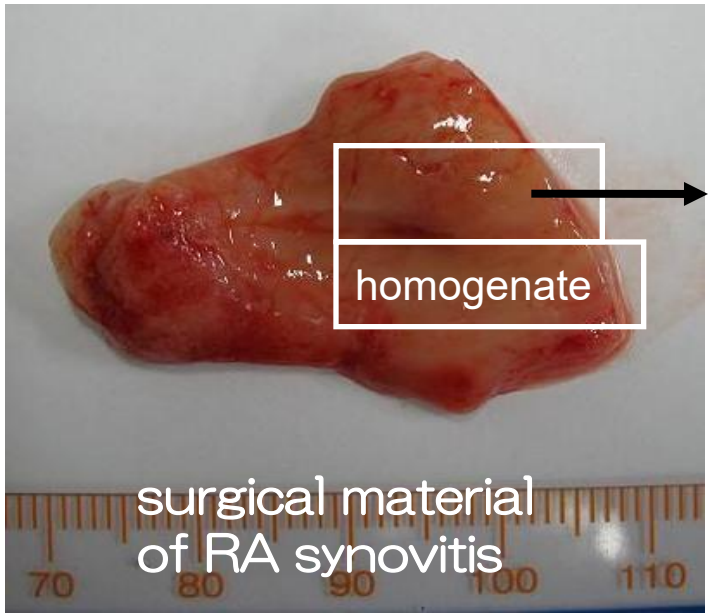
By using the acellular protein synthesis system, Sawasaki's group has established a human/mouse libraries containing more than 2,000 biotinylated proteins.

The target antigens can be screened from the library by using the patient's serum : This is called as the AlphaScreen method.

Acellular protein synthesis system:
Wheat germ extract effectively synthesizes proteins encoded by cDNA amplified by RT-PCR. *E. coli* or cultured cells are NOT used in this system.

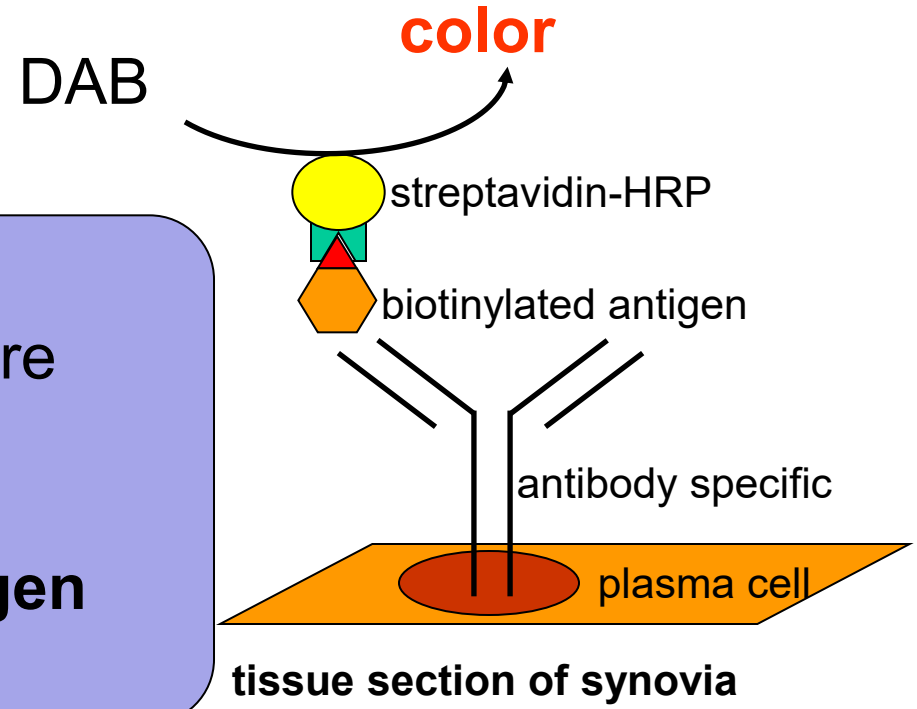
We can thus detect the target antigens of the antibodies produced within the lesion.

Use of the screened antigens for the enzyme-labeled antigen method



4% paraformaldehyde-fixed frozen sections

The screened antigens were used as probes for the enzyme-labeled antigen method

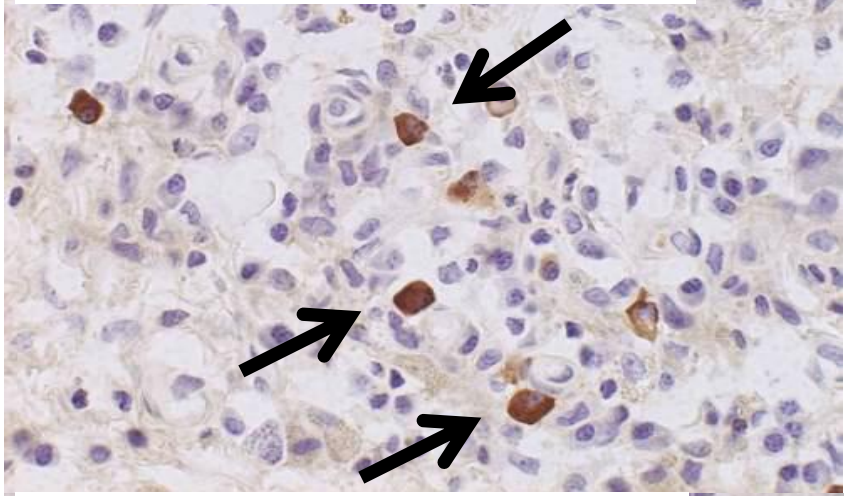


Five biotinylated antigens showing high signals with the AlphaScreen method were expressed by the wheat germ mediated "acellular protein synthesis system".

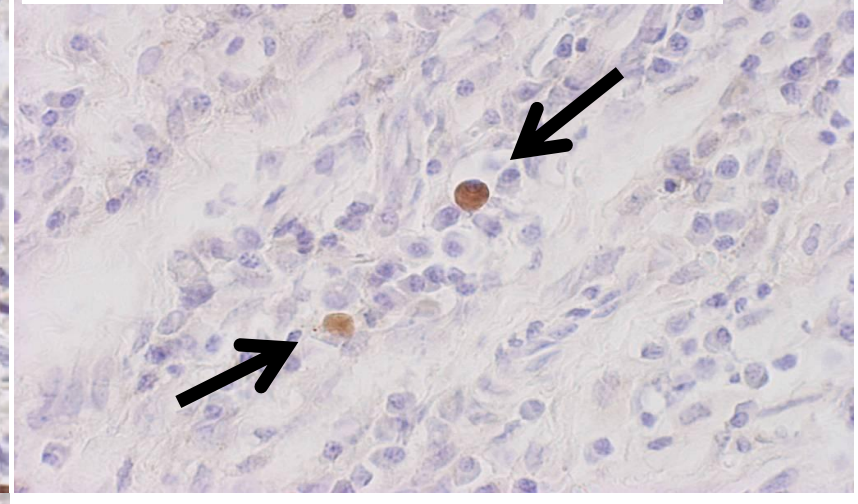
→**Probes for the enzyme-labeled antigen method used without purification**

Samples of the enzyme-labeled antigen method in rheumatoid arthritis (RA) synovitis

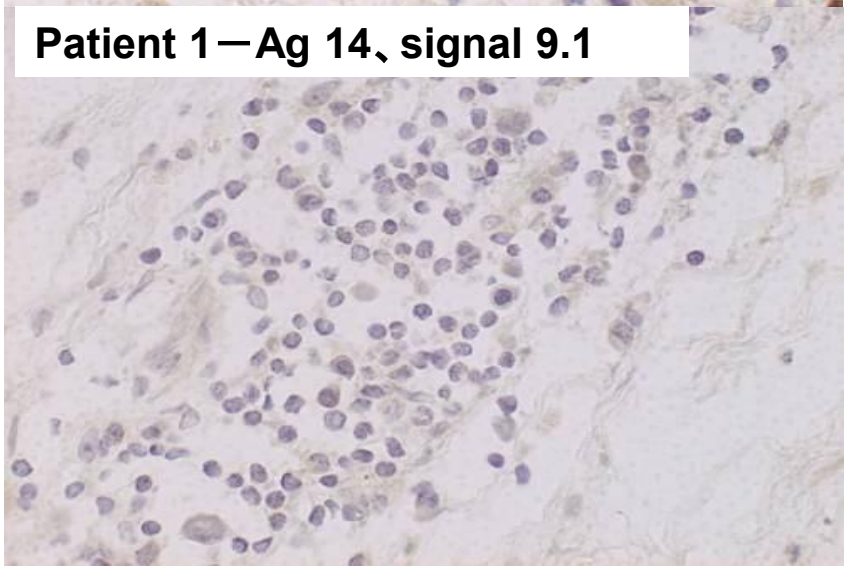
Patient 1－Ag 2、signal 77.6



Patient 3－Ag 10、signal 96.4



Patient 1－Ag 14、signal 9.1



・Plasma cells infiltrating in RA synovitis showed positivity against antigens with the highest signal intensity in patients 1 and 3.

・All the other combinations gave negative results. Enhancement of the detectability of the technique is needed.

The possible target lesions of the enzyme-labeled antigen method

1. **Autoimmune diseases**, such as rheumatoid arthritis, Hashimoto's thyroiditis, Sjoegren's syndrome and autoimmune gastritis (type A gastritis)
2. **Infectious diseases**, such as *Helicobacter pylori*-induced gastritis and syphilis
3. **Malignant tumors** showing heavy infiltration of plasma cells in the stroma, including **EBV-related tumors** (nasopharyngeal carcinoma, gastric carcinoma with lymphoid stroma, Hodgkin's lymphoma and nasal NK/T lymphoma), HPV-related uterine cervical carcinoma, MALT lymphoma, and even multiple myeloma (plasmacytoma).