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Takao Suzuki[*] Genshu Take[†]

Katsuhide Ikeda[‡] Toshiyuki Mitsuya[**]

[*]Showa University,

[†]Showa University,

[‡]Showa University Fujigaoka Hospital,

[**]Showa University,

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Takao Suzuki, Genshu Take, Katsuhide Ikeda, and Toshiyuki Mitsuya

Abstract

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KEYWORDS: multicolor immunofluorescence, heat inactivation, confocal laser scanning microscope

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Original Article

A Novel Multicolor Immunofluorescence Method Using Heat Treatment

Takao Suzuki*, Genshu Tate, Katsuhide Ikeda, and Toshiyuki Mitsuya

Department of Surgical Pathology, Showa University Fujigaoka Hospital, Yokohama 227-8501, Japan

We describe a novel method for immunofluorescent detection of multiple antigens in a single paraffin-embedded tissue section. We hypothesized that if fluorescent dyes are resistant to heat treatment, then thermal inactivation of immunoglobulins during antigen detection procedures might make it possible to use multicolor immunofluorescence detection even if the primary antibodies are from the same species. We found that several fluorescent dyes, including fluorescein isothiocyanate (FITC), Cy3 and Cy5, were resistant to heating at 90°C for 15 min, whereas the antigenicities of the primary antibodies were lost completely. This novel method, which uses heat treatment between staining steps, has great advantages for multicolor immunofluorescence because unlabeled primary antibodies from the same species can be used. Therefore, by using this method not only 3 unlabeled mouse monoclonal antibodies but also 3 unlabeled rabbit antisera can be used as primary antibodies for multicolor immunofluorescence.

Key words: multicolor immunofluorescence, heat inactivation, confocal laser scanning microscope

Primary antibodies coupled to fluorescent dyes or obtained from different species have been used for conventional multicolor immunofluorescence analyses. However, application of the conventional method is limited because the protocol is quite complex and many primary antibodies originate from the same species [1-4]. In addition, during the sequential steps of the conventional method, specimens must be kept in a cool and dark environment to prevent decay of the fluorescence. We hypothesized that the decay of fluorescence is due to light but not heat. On the basis of this notion, we developed a new multicolor immunofluorescence method. This method involves heat-inactivation of the antibodies without inactivation of the fluorescent dyes, and we used this method to perform a multicolor immunofluorescence analysis of the same tissue section with 3 different primary mouse monoclonal antibodies.

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*Corresponding author. Phone: +81-45-974-6383; Fax: +81-45-972-6242
E-mail: takabo@ya2.so-net.ne.jp (T. Suzuki)

Materials and Methods

Specimens. Specimens were formalin-fixed paraffin-embedded sections of human pheochromocytoma, thymoma and normal pancreas or ethanol-fixed touch smears of human pheochromocytoma and bronchioalveolar lung cancer.

Reagents. The mouse monoclonal antibodies, fluorescent dyes and other reagents used in the experiments are listed in Tables 1 and 2. An electric warmer (PDH-F200, Tiger, Osaka, Japan) was used for heat treatment, and a fluorescence microscope (BX-60, Olympus, Tokyo, Japan) and a confocal laser scanning microscope (CLSM, FluoView, FV500, Olympus) were used to examine the immunofluorescent staining.

Assessment of heat treatment on

antigenicity. Formalin-fixed paraffin-embedded sections of human pheochromocytoma were soaked in 10 mM citrate buffer (pH 6.0) and heated at 98 ˚C for 45 min for antigen retrieval. After incubation with primary antibody against CD56, sections were heated in the same buffer at 90 ˚C for 15 min to eliminate primary antibody antigenicity [5]. Antigenicities of other primary antibodies used in the present study were also lost under this condition (data not shown). Sections were then incubated with biotin-labeled anti-mouse immunoglobulin and detected by the fluorescein isothiocyanate (FITC)-labeled streptavidin (FITC-LSAB) method or by the Cy3-labeled anti-mouse immunoglobulin indirect method. The results obtained from the sections treated at 90 ˚C for 15 min were compared with those obtained without heat treatment.

Fluorescence intensity after heat treatment. Formalin-fixed paraffin-embedded sections of human pheochromocytoma were used for immunofluorescence studies to analyze the decay of

Table 1 Primary antibodies used in this study

Antibodies	Clone	Source	dilution	pretreatment
CD56 (mouse monoclonal antibody)	1B6	Novocastra, Newcastle,UK	1:50	Heating (pH6)
Insulin (guinea pig polyclonal antibody)		DakoCytomation, Glostrup, DK	1:200	no
Glucagon (rabbit polyclonal antibody)		DakoCytomation, Glostrup, DK	1:200	no
Somatostatin (rabbit polyclonal antibody)		DakoCytomation, Glostrup, DK	1:200	no
CD4 (mouse monoclonal antibody)	1F6	Novocastra, Newcastle,UK	1:20	Heating (pH8)
CD8 (mouse monoclonal antibody)	4B11	Novocastra, Newcastle,UK	1:20	Heating (pH8)
Cytokeratin (mouse monoclonal antibody)	AE1/AE3	DakoCytomation, Glostrup, DK	1:50	Heating (pH8)*[a]
EMA (mouse monoclonal antibody)	E29	DakoCytomation, Glostrup, DK	1:50	Heating (pH6)
TTF-1 (mouse monoclonal antibody)	8G7G3/1	DakoCytomation, Glostrup, DK	1:50	Heating (pH6)
Synaptophysin (mouse monoclonal antibody)	SY38	DakoCytomation, Glostrup, DK	1:10	Heating (pH6)
Chromogranin A (mouse monoclonal antibody)	LK2H10	Diagnostic Biosystems, Pleasanton, USA	1:100	Heating (pH6)

EMA, Epithelial Membrane Antigen; TTF-1, Thyroid Transcription Factor-1; no, no treatment.
Heating (pH6), High temperature antigen unmasking technique (98 ˚C 45 min) using 10 mM citrate buffer (pH 6.0).
Heating (pH8), High temperature antigen unmasking technique (98 ˚C 45 min) using 1 mM EDTA (pH 8.0).
*[a], Using 10 mM citrate buffer (pH 6.0) in case of ethanol-fixed touch smears.

Table 2 Secondary antibodies and other reagents

Secondary antibodies and reagents	Source	Dilution
Anti-rabbit immunoglobulin antibodies*		
Biotin-labeled	DakoCytomation, Glostrup, DK	1:100
Cy3-labeled	Chemicon, Temecula, CA, USA	1:100
Cy5-labeled	Chemicon, Temecula, CA, USA	1:100
Anti-mouse immunoglobulin antibodies		
Cy2-labeled	Chemicon, Temecula, CA, USA	1:50
Alexa Fluor 488-labeled	Molecular Probes, Eugene, OR, USA	1:200
Rhodamine-labeled	Chemicon, Temecula, CA, USA	1:100
Cy3-labeled	Chemicon, Temecula, CA, USA	1:100
Alexa Fluor 555-labeled	Molecular Probes, Eugene, OR, USA	1:200
Cy5-labeled	Chemicon, Temecula, CA, USA	1:100
Alexa Fluor 647-labeled	Molecular Probes, Eugene, OR, USA	1:200
Biotin-labeled	DakoCytomation, Glostrup, DK	1:100
FITC-labeled streptavidin	DakoCytomation, Glostrup, DK	1:50
Fluoro Guard (mounting reagent)	Bio-Rad, Hercules, CA, USA	

*Cross-react with guinea pig immunoglobulin, Biotin labeled anti-rabbit immunoglobulin goat serum used here showed cross reactivity against guinea pig immunoglobulin.

fluorescence after heat treatment. FITC was labeled by the LSAB method, and rhodamine, Cy2, Cy3, Cy5 and Alexa Fluors 488, 555 and 647 were labeled by the indirect method. Sections were incubated with anti-CD56 antibody, washed and then incubated with fluorescent dye-labeled secondary antibodies. Sections were then heated at 90 ˚C for 15 min or 98 ˚C for 45 min in 10 mM citrate buffer (pH 6.0). The fluorescence intensities were compared between sections with and without heat treatment. CLSM was used to observe the intensity of Cy5 and Alexa Fluor 647, and the fluorescence microscope was used for evaluation of the other fluorescent dyes.

Multicolor immunofluorescence method with heat treatment. Sections were heated at 90 ˚C for 15 min after each step to inactivate the primary antibody used in the previous step (Fig. 2). Insulin,

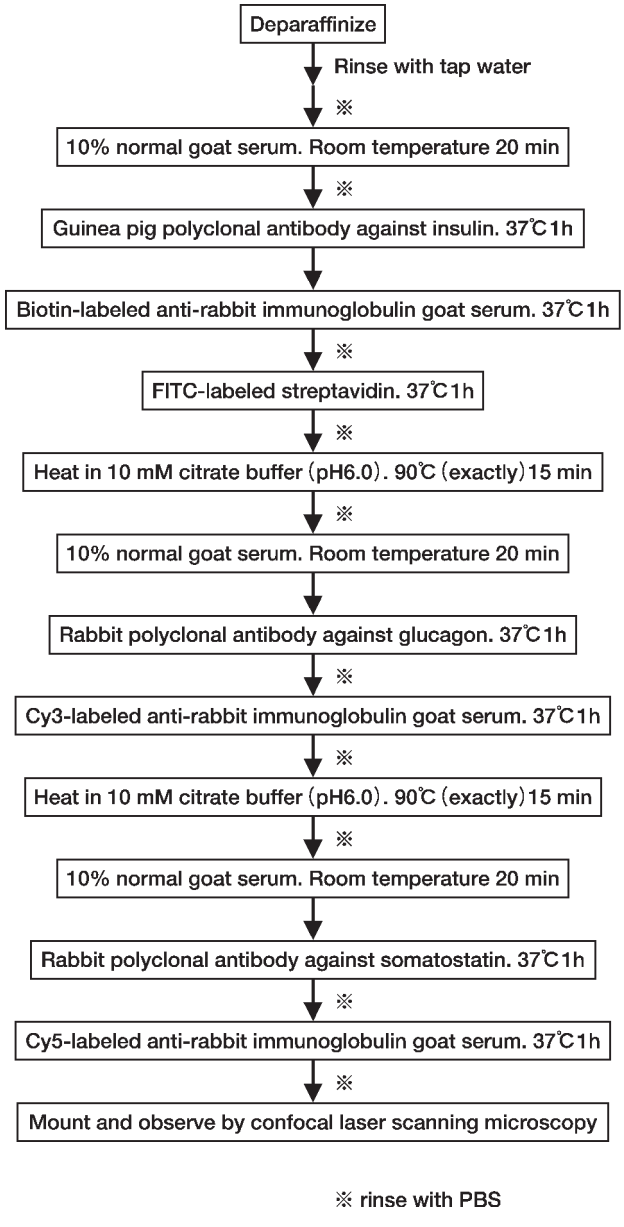

Fig. 2 Protocol for triple immunofluorescent staining of insulin, glucagon and somatostatin.

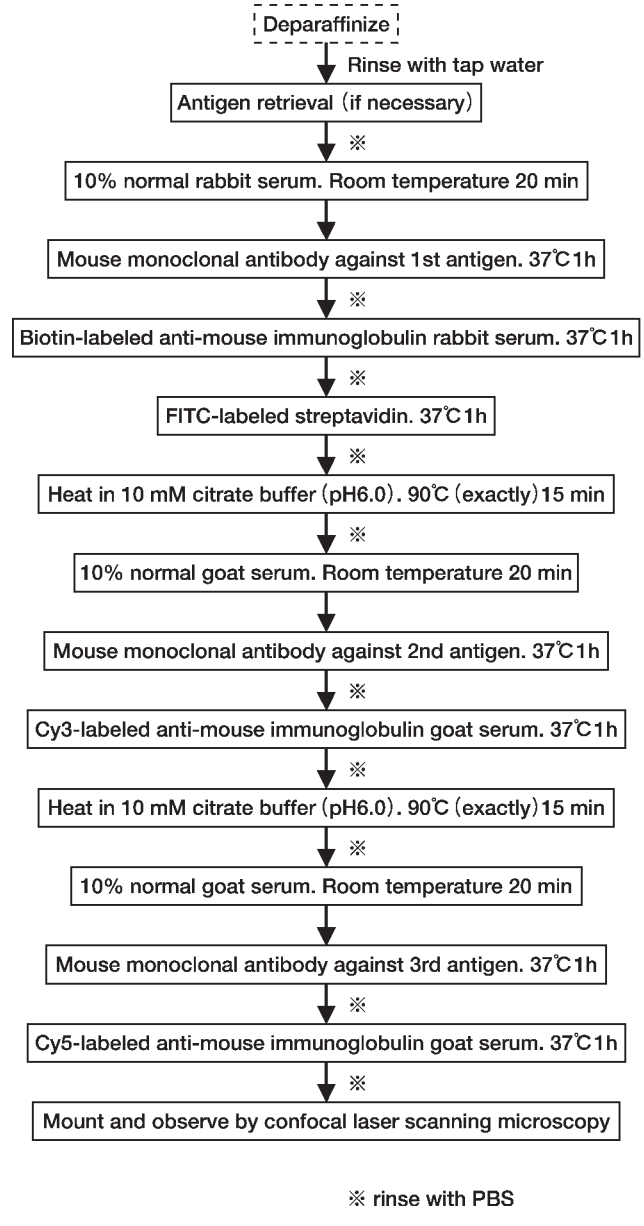

Fig. 3 Protocol for triple immunofluorescent staining when all primary sntibodies are mouse monoclonal antibodies.

glucagon and somatostatin were stained in formalin-fixed paraffin sections of normal human pancreas. Primary antibodies were unlabeled guinea pig polyclonal anti-insulin, unlabeled rabbit polyclonal anti-glucagon and anti-somatostatin antibodies. We also performed a tricolor immunofluorescence analysis using the procedure outlined in Fig. 2, but we used a combination of 3 different unlabeled mouse monoclonal antibodies (Fig. 3). CD4, CD8 and cytokeratin were stained in formalin-fixed paraffin sections of a human thymoma. Cytokeratin, epithelial membrane antigen (EMA) and thyroid transcription factor-1 (TTF-1) were stained in ethanol-fixed touch smears of bronchioalveolar lung cancer. Synaptophysin and chromogranin A were visualized in ethanol-fixed touch smears, and propidium iodine (PI) was used for nuclear staining.

Results

Inactivation of the primary antibody antigenicity after heat treatment. Antigenicity of the primary antibody was lost after the sections of human pheochromocytoma were heated at 90 ℃ for 15 min (Fig. 1). We obtained similar results with both the FITC detection method (Fig. 1B) and the Cy3 detection method (Fig. 1D). Results of routine immunofluorescence analysis with the FITC and Cy3 detection systems are shown in Figs. 1A and C, respectively.

Fluorescence intensity after heat treatment. Fluorescent dyes including FITC, Cy3 and Cy5 showed no or only a slight decay of fluorescence intensity after treatment at 90 ℃ for 15 min (Table 3). Under these conditions, primary antibodies completely

Table 3 Fluorescence decay after heat treatment

	90 ℃ 15 min	98 ℃ 45 min
FITC	→～↓	↓～↓↓
Cy2	↓～↓↓	lost
Alexa Fluor 488	↓↓～↓↓↓	lost
rhodamine	↓～↓↓	↓↓～↓↓↓
Cy3	→～↓	↓～↓↓
Alexa Fluor 555	↓～↓↓	↓↓～↓↓↓
Cy5	→～↓	↓～↓↓
Alexa Fluor 647	↓～↓↓	lost

→, no decay; ↓, mild decay; ↓↓, moderate decay; ↓↓↓, marked decay.

Fig. 1 Inactivation and elimination of primary antibody antigenicity after heat treatment as observed by a confocal laser scanning microscope (CLSM) (**A-D**). A formalin-fixed paraffin section of pheochromocytoma was stained with anti-CD56 mouse monoclonal antibody. Routine examination of the fluorescence without heat treatment after reaction with anti-CD56 and followed by biotin-labeled anti-mouse immunoglobulin rabbit serum and FITC-labeled streptavidin-biotin (FITC-LSAB) staining is shown in **A** (× 400). Heat treatment at 90 ℃ for 15 min after incubation with CD56 and followed by FITC-LSAB staining eliminates the primary antibody antigenicity, and no fluorescence is visible (**B**). Examinations of immunofluorescence with the Cy3 labeling method without and with heat treatment are shown in **C** and **D**, respectively. Loss of antigenicity of the primary mouse monoclonal antibody against CD56 was observed after heat treatment (**D**). Decay of fluorescence intensity (**E-L**). Formalin-fixed paraffin-embedded sections of pheochromocytoma were stained with anti-CD56 mouse monoclonal antibody and detected by FITC-LSAB (**E**, **F**), Alexa Fluor 488 labeling (**G**, **H**), Cy3 labeling (**I**, **J**) and Cy5 labeling (**K**, **L**). Fluorescence staining findings of sections heated at 90 ℃ for 15 min after incubation with FITC-labeled streptavidin (× 400, **F**) or fluorescent dye-labeled secondary antibodies (× 400, **H**, **J** and **L**). Features of conventional immunofluorescent staining without heat treatment are shown in **E**, **G**, **I** and **K**. Immunofluorescent tricolor staining of insulin, glucagon and somatostatin in the pancreas (**M-P**). According to the method shown in Fig. 2, formalin-fixed paraffin-embedded sections of normal human pancreas were incubated with anti-insulin antibody and detected by the FITC-LSAB method (**M**), the anti-glucagon antibody and Cy3-labeled indirect method (**N**) and the anti-somatostatin antibody and Cy5-labeled indirect method (**O**). A merged image of panels **M**, **N** and **O** is shown in **P**. Insulin, glucagon and somatostatin appear yellow-green, orange-red and blue, respectively, by confocal laser scanning microscopy (CLSM) (× 400). A tricolor immunofluorescence analysis of human thymoma observed by the CLSM (**Q**). Formalin-fixed paraffin-embedded sections were stained with anti-CD8 mouse monoclonal antibody and the FITC-LSAB method, the anti-CD4 mouse monoclonal antibody and Cy3-labeled indirect method and the anti-cytokeratin mouse monoclonal antibody and Cy5-labeled indirect method. Cytokeratin-positive epithelial cells in the thymoma appear blue. CD4-positive cells appear orange-red, and CD8-positive cells appear yellow green. CD4/CD8 double-positive cells are visible (**Q**. × 1000). Tricolor immunofluorescence of human bronchioalveolar lung cancer (**R**). According to the method shown in Fig. 3, ethanol-fixed touch smears of human bronchioalveolar lung cancer were stained with anti-cytokeratin mouse monoclonal antibody and the FITC LSAB method (yellow-green), the anti-EMA mouse monoclonal antibody and Cy3-labeled indirect method (orange-red) and the anti-TTF mouse monoclonal antibody and Cy5-labeled indirect method (blue). CLSM was used for the observation (**R**. × 1000). Ethanol-fixed touch smears of human pheochromocytoma were stained with anti-synaptophysin mouse monoclonal antibody and the FITC-LSAB method (yellow-green), PI (orange-red) and anti-chromogranin A mouse monoclonal antibody and Cy5-labeled secondary antibody (blue) (**S**. × 1000).

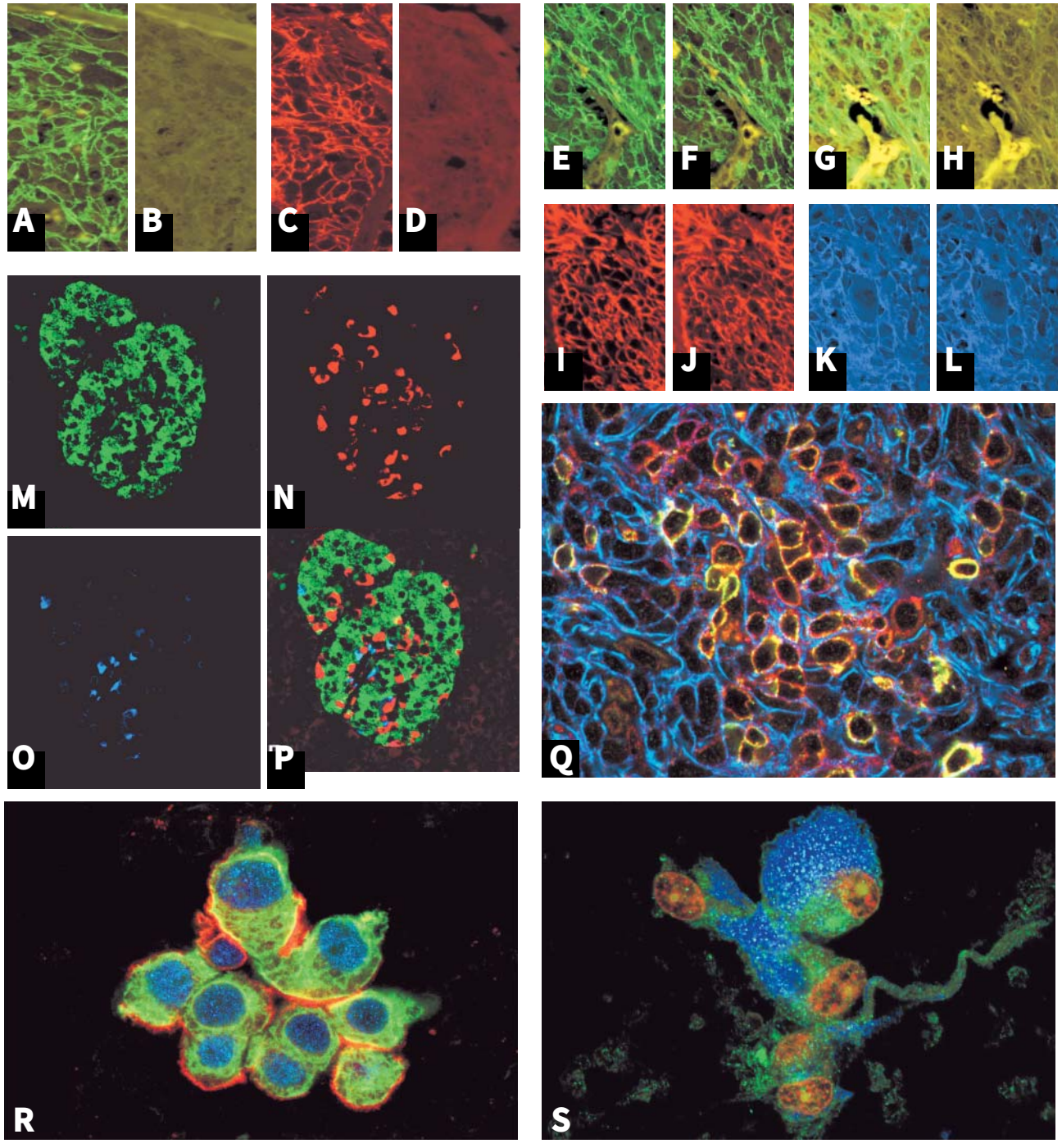

Fig. 1 Legend on the opposite page.

lost their antigenicities. We then compared the fluorescence intensities with (Fig. 1F, H, J and L) and without (Fig. 1E, G, I and K) heat treatment. Alexa Fluor 488 showed reduced fluorescence intensity after heat treatment (Fig. 1H); however, FITC (Fig. 1F), Cy3 (Fig. 1J) and Cy5 (Fig. 1L) maintained their intensities after treatment at 90 °C for 15 min. All dyes showed dramatic reductions or total loss of fluorescence intensity after antigen retrieval at 98 °C for 45 min. Therefore, we used heat treatment at 90 °C for 15 min between the antigen detection steps to successfully perform tri-color immunofluorescence analysis.

A multicolor immunofluorescence method with heat treatment. We used the procedure outlined in Fig. 2 to detect 3 peptide hormones, insulin (yellowish green), glucagon (red) and somatostatin (blue), in pancreatic islets (Fig. 1 M, N and O, respectively). The merged image (Fig. 1P) revealed independent distribution of these peptides. As shown in Fig. 1Q, CD8 (yellow-green), CD4 (orange-red) and cytokeratin (blue) were stained in the formalin-fixed paraffin-embedded sections of a human thymoma, which contained epithelial cells and lymphocytes. In Fig. 1Q, epithelial cells positive for cytokeratin (blue) are visible around CD4-positive (orange-red) and CD8-positive (yellow-green) T lymphocytes. CD4/CD8 double-positive cells, which are characteristic of thymomas, were also visible. Triple staining of ethanol-fixed touch smears of bronchioalveolar lung cancer (Fig. 1R) revealed a granular distribution of TTF-1 in nuclei (blue) and fibrillary distribution of cytokeratin in cytoplasm (yellow-green). EMA was expressed on the cell surface (orange-red). Double staining of synaptophysin and chromogranin A in ethanol-fixed touch smears of pheochromocytoma (Fig. 1S) revealed different distributions of synaptophysin (yellow-green) and chromogranin A (blue). Under our experimental conditions, the multicolor immunofluorescence method described here showed no loss of antigenicity and no decay of the fluorescent intensity in any steps, including the final step.

Discussion

At present, many commercially available primary antibodies are either mouse monoclonal antibodies or rabbit antisera; therefore, it is not unusual that the primary antibodies we would want to use for multicolor immunofluorescence analyses come from the same species. If 2 primary antibodies are derived from the same species, the secondary antibody can react with both primary antibodies because the primary antibodies have the same antigenicity.

For the conventional multicolor immunofluorescence analysis reported by Nakane, sections were soaked in glycine buffer (pH 2.5) to dissociate the primary antibodies and avoid cross reactivity [1]; however, it is difficult to dissociate primary antibodies completely. Recently data resulting from use of the multiple immunostaining method using primary antibodies from the same species were reported [2–4]. These methods, however, have disadvantages such as requiring formaldehyde vapour treatment [2], treatment at 130 °C [3] or blocking of an antigen by an unlabeled antibody [4].

Compared with these conventional methods, our new method does not need any harmful reagents, and the experimental conditions are quite simple. We previously applied the heat treatment for antigen retrieval [5–8] to inactivate primary antibodies and for labeling enzymes. We used combinations of the heat treatment and a highly sensitive polymer to develop an immunoenzyme-based triple-staining method [9]. This method is advantageous because primary antibodies derived from the same species can be used to detect antigens localized in different areas, such as the cytoplasm and the cell surface. However, when the antigens are colocalized, it is difficult to identify the signals because they are merged. The novel immunofluorescence method described herein made it possible to resolve this problem with immunoenzyme-based triple staining [7]. In addition, we were able to identify antigens present in the same location if the appropriate microscope filter was used. Our new method is also advantageous because we can use a combination of three different unlabeled mouse monoclonal antibodies as primary antibodies for the tri-color fluorescence immunohistochemistry and because we can use this method with formalin-fixed paraffin-embedded sections, alcohol-fixed touch smears and cultured cells. Unlabeled primary antibodies used for immunoenzyme staining in the pathological diagnosis are available to perform our methods described here, which makes this method quite useful.

Even though primary antibodies are derived from the same species, it is possible to carry out a multicolor immunofluorescence analysis by using the direct method. However, there are not many commercially available fluorescence-labeled primary antibodies, and those that are available are more expensive compared to the unlabeled antibodies. In our novel method it is unnecessary

to label the fluorescence dye with primary antibodies, and our method is more sensitive than the direct method. Detailed analysis of multiple antigen localizations in a cell is possible when sections stained by this novel method are scanned by confocal laser scanning microscopy. We believe that this method will prove useful in histochemistry, cell biology and pathology.

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