Simultaneous immunohistochemical detection of gangliosides and neuronal markers in paraformaldehyde-fixed nervous tissues by acetone etching*

Yasuhiko Sakumoto¹, Hisashi Ueta¹, Nobuhiro Yuki², and Kenjiro Matsuno¹

¹Department of Anatomy (Macro), Dokkyo Medical University, Mibu, Tochigi; and ²Department of Neurology and Clinical Research, Niigata National Hospital, Kashiwazaki, Niigata, Japan

Summary. A need for identifying ganglioside-positive cells with neuronal markers prompted us to establish a reliable method for double or triple immunostaining nervous tissues. Perfusion fixation with paraformaldehyde is typically performed for the routine immunostaining of various neuronal markers but is not suitable for immunostaining gangliosides. Acetone fixation of fresh cryosections is frequently used for ganglioside immunodetection; thus, we tested the effect of acetone treatment for unmasking the antigen epitope of gangliosides (acetone etching) on sections of paraformaldehyde-fixed nervous tissue from rats. Acetone etching significantly retrieved ganglioside immunoreactivity while preserving the immunoreactivity of neuronal markers. Various combinations of gangliosides and neuronal markers could be double-stained by the immunoenzyme method or triple-stained by the immunofluorescence method. This new method may provide additional information regarding the relationship between gangliosides and various neuronal markers from routinely paraformaldehyde-fixed nervous tissues, both freshly prepared specimens and those stocked in the laboratory.

Introduction

Gangliosides, sialic acid-containing glycosphingolipids, are normal membrane constituents in nervous tissue and have been implicated as functional molecules in neuronal activities (Yu and Saito, 1989). Autoantibodies to gangliosides play a central role in the development of immune-mediated neuropathies (Willison and Yuki, 2002). The IgG antibodies against GM1 and GD1a are associated with Guillain-Barré syndrome, and IgG antibodies against GQ1b are associated with Fisher syndrome. The IgM antibodies against GD1b, GD3, and GQ1b are associated with chronic sensory ataxic neuropathy. For these reasons, the mapping of gangliosides in nervous tissues contributes to advancing basic and clinical neuroscience.

Immunohistochemical staining has been used to reveal the localization of gangliosides (Kotani et al., 1993; Kusunoki et al., 1994; Gong et al., 2002). However, detailed analysis of the localization of gangliosides has been limited because neuronal markers rarely co-stain. One of the obstacles in co-staining is a difference between the fixatives used in ganglioside and neuronal marker immunostaining; though fresh cryosections are used for ganglioside immunostaining (Kotani et al., 1993; Kusunoki et al., 1994), sections fixed with aldehyde are used for the neuronal marker immunostaining (Lawson et al., 1984; Silverman and Kruger, 1990; Averill et al., 1995). The intensity of ganglioside immunostaining using aldehyde-fixed cryosections decreases considerably...
compared with those without aldehyde fixation (Gong et al., 2002). Because antigen epitopes of gangliosides are considered to be sugar components (Willison and Yuki, 2002), weak staining after aldehyde fixation may be due to epitope masking by bridging membrane proteins with aldehyde. In contrast, acetone treatment, which is the most suitable fixation for ganglioside immunostaining in fresh cryosections (Kotani and Tai, 1997), might partially remove the lipid components of the plasma membrane, resulting in exposure of the ganglioside epitopes (acetone etching) (Kusunoki et al., 1994). Accordingly, we speculated that, if target tissues are fixed with aldehyde first and then etched with acetone, ganglioside epitopes might be exposed.

In the present study, we examined the effects of acetone etching on ganglioside immunostaining in paraformaldehyde-fixed nervous tissue. We then compared the ganglioside and neuronal marker co-staining patterns among these fixatives. The rat cerebellum, lumbar dorsal root ganglion (DRG), sciatic nerve, and ventral root were selected as targets because the localizations in these tissues are well studied (Gong et al., 1996) of neurons, ganglioside was co-stained with type IV collagen.

In the present study, we demonstrate that acetone etching is applicable for ganglioside immunostaining in paraformaldehyde-fixed nervous tissue. We then compared the ganglioside and neuronal marker co-staining patterns among these fixatives. The rat cerebellum, lumbar dorsal root ganglion (DRG), sciatic nerve, and ventral root were selected as targets because the localizations in these tissues are well studied (Gong et al., 2002). The immunoreactivity was evaluated using the enzyme-labeled antibody method first because it shows the tissue architecture more readily than the immunofluorescence method. Then the availability of the immunofluorescence method was examined. To outline the basal lamina (Matsuno et al., 1996) of neurons, ganglioside was co-stained with type IV collagen.

Materials and Methods

Animals

Adult female Wistar rats (80 to 160 days old) were purchased from Japan SLC, Inc. (Shizuoka). Handling and care were in accordance with Dokkyo Medical University’s Regulation for Animal Experiments and Japanese Governmental Law (No. 105) and approved by the Dokkyo Medical University Animal Experiment Committee.

Tissue preparation

1) Simple acetone fixation (A-fixation)

After sacrifice by exsanguination under ether anesthesia, target organs were removed, dissected on ice, and put into Tissue-Tek cryomolds (Miles Inc., Elkhart, IN) filled with cold OCT compounds (Miles Inc.). Samples were snap-frozen in cold hexane (−80°C). The time from sacrifice to freezing was approximately 10 min. Six micrometer-thick cryosections were prepared, mounted on silane-coated glass slides (Dako Corp., Santa Barbara, CA), and air-dried for more than 2 h at room temperature. Cryosections were dipped in acetone at different temperatures (−20 to 0°C) and for different periods of time (5 to 10 min). The conditions were determined for each antibody and probe so as to yield optimal ganglioside immunostaining using fresh cryosections. Sections were air-dried for 1 h.

2) Simple paraformaldehyde fixation (P-fixation)

Under anesthesia, rats were perfused with physiological saline solution via the left ventricle, followed by 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.3) for 10 min at a perfusion pressure of 80 to 120 mmHg. Target organs were dissected in the same manner as A-fixation. Samples were washed in 0.01 M phosphate-buffered saline (PBS; pH 7.2) and cryoprotected with a series of sucrose in PBS up to 30% at 4°C. Mounting, freezing, and cryosection preparation was performed in the same manner as A-fixation.

3) Simple paraformaldehyde fixation with acetone etching (P-A-fixation)

Paraformaldehyde-fixed cryosections were prepared in the same manner as P-fixation. Acetone dipping was performed in the same manner as A-fixation.

4) Prolonged paraformaldehyde fixation (P-P-fixation)

Paraformaldehyde fixation and dissection were performed in the same manner as P-fixation. After dissection, post-fixation with 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.3) was performed for 1 h to 3 h at 4°C. Washing and cryoprotection were performed in the same manner as P-fixation. Mounting, freezing, and cryosection preparation was performed in the same manner as A-fixation.

5) Prolonged paraformaldehyde fixation with acetone etching (P-P-A-fixation)

Cryosections were prepared in the same manner as P-P-fixation. Acetone dipping was performed in the same manner as A-fixation.
**Immunohistochemistry**

1) **Enzyme-labeled antibody method**

Sections for each fixative group were simultaneously immunostained at room temperature. Sections were rehydrated in PBS, rinsed in 0.2% Tween 20 in PBS, and incubated with 5% bovine serum albumin (BSA) (Sigma, St. Louis, MO) in PBS for 15 min to reduce background staining. Sections were incubated with either primary antibodies or biotinylated probes diluted with 0.2% BSA in PBS for 1 h or overnight (Table 1). After washing and rinsing, the sections were incubated with secondary conjugates diluted with 1% normal rat serum in 0.2% BSA in PBS for 1 h (Table 1) and then washed. Alkaline phosphatase activity was developed with Vector Blue Alkaline Phosphatase Substrate Kit

| Table 1. Antibodies, biotinylated probes, and streptavidins. |
|---------------------------------------------------------------|
| **Primary**                                                   |
| **Antigen** | Antibody/probe | Dilution | Source/reference |
| GM1         | biotinylated CTB | 1:1000 | Invitrogen (Eugene, OR) |
|             | mAb GB2         | 1:50   | Yuki et al. (2004) |
| GD1b        | mAb GGR12       | 1:100  | Seikagaku Corporation (Tokyo) |
| GD3         | mAb GMR19       | 1:100  | Seikagaku Corporation |
| GQ1b        | mAb GMR13       | 1:100  | Seikagaku Corporation |
| Neuronal markers |                |        |                 |
| a-D-galactosyl residues | biotinylated IB4  | 1:300 | Invitrogen |
| Neurofilament 200kD | pAb               | 1:200 | Chemicon International Inc. (Temecula, CA) |
| Parvalbumin | pAb             | 1:500  | Affinity BioReagents (Golden, CO) |
| Tyrosine kinase receptor A | pAb       | 1:100  | Chemicon International Inc. (Temecula, CA) |
| Type IV collagen | pAb            | 1:1500 | Cosmo Bio (Tokyo) |
| **Secondary**                                               |
| **Product** | Conjugate | Dilution | Source |
| Goat Ig to mouse IgG | ALP       | 1:125 | A9316 Sigma (Saint Louis, MO) |
|               | Alexa Fluor 488 | 1:100 | A11029 Molecular Probes (Eugene, OR) |
| Goat F(ab')2 to mouse IgM | ALP        | 1:50 | 59353 Cappel (West Chester, PA) |
| Goat F(ab')2 to rabbit IgG | HRP       | 1:125 | 55693 Cappel |
|               | AMCA         | 1:150  | 711-155-152 Jackson ImmunoResearch Laboratories Inc. (West Grove, PA) |
| Streptavidin  | ALP          | 1:200  | 43-8322 Zymed Laboratories Inc. (San Francisco, CA) |
|               | HRP          | 1:500  | 016-030-084 Jackson ImmunoResearch Laboratories Inc. |
|               | Alexa Fluor 594 | 1:500 | S11223 Molecular Probes |

ALP, alkaline phosphatase; AMCA, 7-amino-4-methylcoumarin-3-acetic acid; CTB, cholera toxin subunit B; HRP, horseradish peroxidase; IB4, biotinylated *Griffonia simplicifolia* isoelectin B4; mAb, mouse monoclonal antibody; pAb, rabbit polyclonal antibody.
Dojin Chemicals, Kumamoto) in 40 ml PBS with 1 ml 0.3 M imidazole (Nacalai Tesque Inc., Kyoto) and 10 µl 30% H₂O₂. Double immunostaining was carried out by repeating two cycles of indirect immunostaining as described above for gangliosides with alkaline III (Vector Laboratories Inc., Burlingame, CA) in 2.5 ml 0.1 M Tris-HCl buffer (pH 8.2) with 200 µl 40 mM levamizole (Sigma) in the dark for 5 to 10 min. Horseradish peroxidase activity was developed for 5 to 10 min with 5 mg 3’-diaminobenzidine hydrochloride (Dojin Chemicals, Kumamoto) in 40 ml PBS with 1 ml 0.3 M imidazole (Nacalai Tesque Inc., Kyoto) and 10 µl 30% H₂O₂. Double immunostaining was carried out by repeating two cycles of indirect immunostaining as described above for gangliosides with alkaline
Multiple immunostaining for ganglioside 81

**Table 2.** Ganglioside immunoreactivities in each fixation.

| Ganglioside | Structure                  | Fixation | A- | P- | P-A- | P-P- | P-P-A- | Reference       |
|-------------|----------------------------|----------|----|----|------|------|--------|----------------|
| GM1         | Cerebellum                 |          |    |    |      |      |        | Kotani *et al.* (1993) |
|             | Granular layer             | +++      | ++ | +++| +++  | ++   | +++    | Kotani *et al.* (1993) |
|             | White matter               | ++       | -  | ++ | -    | ++   | -      | Sheikh *et al.* (1999) |
|             | Sciatic nerve              | +++      | -  | +++| -    | ++   | -      | Kotani *et al.* (1993) |
| GD1b        | Cerebellum                 |          |    |    |      |      |        | Kotani *et al.* (1993) |
|             | Molecular layer            | +        | +  | +  | +    | +    | +      | Kotani *et al.* (1993) |
|             | Cerebellar glomeruli       | +        | +  | +  | -    | -    | -      | Kotani *et al.* (1993) |
|             | Small granular cell        | ++       | -  | -  | -    | -    | -      | Kotani *et al.* (1993) |
| GD3         | Cerebellum                 |          |    |    |      |      |        | Kawai *et al.* (1994) |
|             | Small granular cell        | ++       | -  | -  | -    | -    | -      | Kotani *et al.* (1993) |
|             | White matter               | +++      | +  | +++| +    | +++  | +      | Kotani *et al.* (1994) |
|             | Ventral root               | +        | +  | +++| +    | +    | +      | Ogawa-Goto *et al.* (1992) |
| GQ1b        | Cerebellum                 |          |    |    |      |      |        | Kotani *et al.* (1993) |
|             | Granular layer             | +++      | +  | +++| ND   | ND   | ND     | Chiba *et al.* (1993) |
|             | Cerebellar nuclei          | ++       | +  | ++ | ND   | ND   | ND     | Chiba *et al.* (1993) |

Staining intensity: +++ strong; ++ bright; + weak; − negative; ND not determined.
GM1 immunoreactivities were determined using mouse monoclonal antibody GB2, but similar activities were found using biotinylated cholera toxin subunit B in each fixative.
All the cited references show positive immunoreactivities in corresponding structures.

Phosphatase substrate (blue) first and neuronal markers or type IV collagen with horseradish peroxidase substrate (brown) second. For type IV collagen immunostaining in paraformaldehyde-fixed cryosections, the sections were pre-treated with 0.001% pepsin in 0.01 N HCl (Ezaki *et al.*, 1995) after fixation with 1% glutaraldehyde in PBS for 30 sec to avoid section detachment. Sections were mounted in Aquatex (Merck, Darmstadt, Germany). Negative controls were incubated in 0.2% BSA in PBS instead of the primary probes.

2) **Immunofluorescence method**
Immunofluorescence stainings were carried out in the same manner as the enzyme-labeled antibody method but performed in the dark. Triple immunostainings were carried out by repeating three cycles of indirect immunofluorescence staining. Sections were mounted in Fluorescent Mounting Media (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Negative controls were prepared in the same manner as the enzyme-labeled antibody method.

**Measurement of immunoreactive DRG neurons**
Sections co-stained for gangliosides and type IV collagen were used to outline the shape of DRG cell bodies. The DRG neurons were classified according to the length of the longer diameter of the cell bodies as in a previous
Ganglioside immunostaining after each fixation

Ganglioside immunostaining was carried out after each fixation in triplicate using three rats. An exception was for the sciatic nerve where only one rat was used. Results are summarized in Figure 1 and Table 2. All the cited references in Table 2 show positive immunoreactivities in corresponding structures. The frequencies of each cell body-size range in ganglioside-positive DRG neurons after A-, P-A-, and P-P-A-fixation are shown in Figure 2. Either faint staining or none at all was seen in negative controls.

Fig. 2. Frequency of ganglioside-positive dorsal root ganglion in each cell body-size range stained after A-, P-A-, P-P-A-fixations. The number written at the top of each bar graph is the frequency of ganglioside-positive dorsal root ganglions. A: GM1 detected by cholera toxin subunit B. B: GM1 detected by mouse monoclonal antibody GB2. C: GD1b. D: GQ1b.
Table 3. Neuronal marker and type IV collagen immunoreactivities in A-, P-A-, and P-P-A-fixation.

| Antigen Structure                  | Antigen                     | Fixation | Reference                        |
|------------------------------------|-----------------------------|----------|----------------------------------|
| Dorsal root ganglion               | a-D-galactosyl residues     | A-        | Silverman and Kruger (1990)      |
| Small sized neuron                 |                             | P-A-      |                                  |
| ++                                 |                             | P-P-A-    |                                  |
| Neurofilament 200 kD               | Cerebellum                  | A-        | Gotow and Tanaka (1994)          |
| Axonal component                   |                             | P-A-      |                                  |
| +++                                |                             | P-P-A-    |                                  |
| Dorsal root ganglion               | Large sized neuron          | ND       | Lawson et al. (1984)             |
| Medium sized neuron                |                             | +++       | Lawson et al. (1984)             |
| Ventral root                       | Myelinated axon             | +++       | Križ et al. (2000)               |
| Parvalbumin                        | Cerebellum                  | A-        | Celio (1990)                     |
| Basket cell                        |                             | P-A-      |                                  |
| ++                                 |                             | P-P-A-    |                                  |
| Stellate cell                      |                             |           |                                  |
| Purkinje cell                      |                             |           |                                  |
| Medium sized neuron                |                             |           |                                  |
| Dorsal root ganglion               | Large sized neuron          | ND       | Antal et al. (1990)              |
| Medium sized neuron                |                             | ND       | Antal et al. (1990)              |
| Tyrosine kinase receptor A         | Dorsal root ganglion        | A-        | Averill et al. (1995)            |
| Small sized neuron                 |                             | P-A-      |                                  |
| ++                                 |                             | P-P-A-    |                                  |
| Type IV collagen                   | Cerebellum                  | A-        | Vosko et al. (2003)              |
| Basal lamina                       |                             | P-A-      |                                  |
| ++                                 |                             | P-P-A-    |                                  |
| Basal lamina                       | Dorsal root ganglion        | A-        | Dubový et al. (2006)             |
| ++                                 |                             | P-P-A-    |                                  |
| Sciatric nerve                     | Basal lamina                | A-        | La Fleur et al. (1996)           |
| ++                                 |                             | P-P-A-    |                                  |
| Ventral root                       | Basal lamina                | A-        | La Fleur et al. (1996)           |
| ++                                 |                             | P-P-A-    |                                  |

Staining intensity: +++ strong; ++ bright; + weak; − negative; ND not determined.
All the cited references show positive immunoreactivities in corresponding structures.
Differences in ganglioside immunostaining of paraformaldehyde-fixed cryosections with or without acetone etching

Ganglioside immunostaining after P-A-fixation (Fig. 1C, F, I) and P-P-A-fixation was better than that after P-fixation (Fig. 1B, E, H) and P-P-fixation (Table 2). This staining was clear in the white matter of the cerebellum (Fig. 1C) and myelin sheath of Schwann cells in the ventral root (Fig. 1I). Ganglioside immunoreactivity in DRG neurons was stably detected after P-A- and P-P-A-fixation compared with that after P- and P-P-fixation. The frequency of ganglioside-positive DRG neurons after P-A- and P-P-A-fixation were similar to that after A-fixation (Fig. 2).

Differences in ganglioside immunostaining with or without paraformaldehyde fixation

Because the morphological preservation was better, immunostaining for gangliosides after P-A- and P-P-A-fixation was clearer than that after A-fixation (compare Fig. 1A with Fig. 1C). However, the staining pattern after these fixations sometimes differed from the pattern in A-fixation, being either less (e.g., small granular cells; Table 2) or more (e.g., scattered dotty staining in DRG cell bodies; Fig. 1F versus Fig. 1D).

Neuronal marker and type IV collagen immunostaining in A-, P-A-, and P-P-A-fixation

To test whether the fixing conditions for ganglioside immunostaining are also applicable to neuronal markers, staining was performed after A-, P-A-, and P-P-A-fixation. The results are summarized in Table 3, where all the cited references show positive immunoreactivities in corresponding structures. Faint staining or none at all was seen in negative controls. Staining for neuronal markers after P-A-fixation was the best among the three fixations.

Immunostaining for type IV collagen clearly stained the basal lamina, outlining the nodes of Ranvier (Fig. 3A, arrow head) and neuron-satellite cell units of the DRG (Fig. 3B) after all fixations (Table 3). Interestingly, clustered DRG neurons (Pannese et al., 1993) were not separated from each other by type IV collagen (Fig. 3C). This may not be a false negative because clustered DRG neurons were not separated from each other by type IV collagen (Fig. 3C). This may not be a false negative because clustered DRG neurons were not separated from each other by type IV collagen.
Multiple immunostaining for ganglioside 85

Fig. 4. Legend on the opposite page.
neurons do not seem to possess a basal lamina between neighboring cells (Pannese et al., 1993).

**Double and triple immunostaining of gangliosides for neuronal markers**

Double and triple immunostaining succeeded in P-A-fixation, which provided the best results for single staining gangliosides and neuronal markers. Double immunostaining was performed to test whether both gangliosides and neuronal markers could be specifically stained using the enzyme-labeled antibody method. Triple immunostaining was carried out to confirm the applicability of the immunofluorescence method.

The localization of GM1 in the cerebellum was examined by co-staining for the neurofilament 200kD that is present in the axons (Gotow and Tanaka, 1994). The GM1 immunostaining appeared to correspond with the cerebellar glomeruli and some nerve fibers (Fig. 4A). To identify the localization of GM1 in the sciatic nerve, co-staining was performed for GM1 and type IV collagen. Type IV collagen immunostaining clearly identified the nodes of Ranvier, where GM1 was localized (Fig. 4B, arrow head) as previously reported (Sheikh et al., 1999).

Neurofilament 200kD, tyrosine kinase receptor A, and α-D-galactosyl residues divided DRG neurons into three populations: large light, peptidergic small dark, and nonpeptidergic small dark neurons, respectively (Averill et al., 1995). To classify the ganglioside-positive neurons, we co-stained for neurofilament 200kD (Fig. 4C, F), tyrosine kinase receptor A (Fig. 4D), and α-D-galactosyl residues (Fig. 4E). The co-staining pattern of both GM1 and GD1b with neurofilament 200kD (Fig. 4C and F, asterisk) were in agreement with the frequency of ganglioside-positive neurons classified based on the length of the longer diameter of the DRG cell bodies (Fig. 2). To compare the localization of GQ1b with parvalbumin-positive proprioceptors (Ichikawa et al., 2005), co-staining was performed for GQ1b and parvalbumin. GQ1b was found in some proprioceptors (Fig. 4G, asterisk). GM1, neurofilament 200kD, and α-D-galactosyl residues were also specifically detected in each DRG subpopulation using the immunofluorescence method (Fig. 4H).

**Discussion**

The aims of this study were to examine the applicability of acetone etching for ganglioside immunostaining in paraformaldehyde-fixed cryosections and to establish a co-staining method for gangliosides and neuronal markers. Co-staining was successfully performed for gangliosides in nervous tissue perfused with 4% paraformaldehyde and etched with acetone (P-A-fixation).

The quality of ganglioside immunostaining in each fixative was judged from previous reports describing the localization and function of gangliosides (Table 4). We also used human data for this evaluation because the ganglioside immunoreactivity in human peripheral nerves is similar to that of rats (Gong et al., 2002). We have summarized our findings and others in Tables 2 and 3. Most of our results after P-A- and P-P-A-fixation were in agreement with previous studies. To our knowledge, GM1 immunostaining in the cerebellar glomeruli and some nerve fibers of the granular layer (Fig. 4A) has not been previously reported. The former staining does not contradict the report of GM1 regulatory function in the synapse membrane (Tanaka et al., 1997). The latter staining suggests GM1 localization in the myelin sheath of oligodendrocytes because GM1 may localize in oligodendrocytes (Kotani et al., 1993). Accordingly, we consider these observations as specific stainings. Although scattered dotty staining in DRG cell bodies was seen after P-A- (Fig. 1F) and P-P-A-fixation, we consider the specificity for DRG neurons to be preserved because the tendencies of each ganglioside-positive DRG subpopulation did not contradict the results after A-fixation (Fig. 2) and previous reports for GM1 (Tong et al., 1999), GD1b (Gong et al., 2002), and GQ1b localization (Kusunoki et al., 1999).

Paraformaldehyde fixation may combine hydrated formaldehyde with the free amino group (Berod et al., 1981). Because the molecular structures of the gangliosides investigated in this study have no free amino group (Tettamanti and Riboni, 1994; Popa et al., 2007), the direct effect of paraformaldehyde on the ganglioside epitope may be limited. The possible cause of the negative staining in paraformaldehyde-fixed nervous tissue may be due to the masking of the ganglioside epitope by the methylene bridging between lipoproteins in proximity to the epitope.

Molander et al. (2000) did not perform acetone etching in ganglioside immunostaining because they expected antigen loss with the acetone. However, our results indicate that acetone etching is useful in paraformaldehyde-fixed cryosections. This usefulness was clear in the white matter and myelin sheath of Schwann cells where lipid components are rich (Fig. 1 and Table 2). Acetone may expose the ganglioside epitopes of paraformaldehyde-fixed tissues by removing the lipoprotein masking. The immunoreactivity on small granular cells, however, did not recover after
**Table 4.** Evaluation of ganglioside and neuronal marker immunoreactivities in each fixation.

| Tissue/antigen | Fixation   | A- | P- | P-A- | P-P- | P-P-A- |
|---------------|------------|----|----|------|------|--------|
| Cerebellum    |            |    |    |      |      |        |
| Ganglioside   |            |    |    |      |      |        |
| GM1           | excellent  | poor | excellent | poor | excellent |
| GD1b          | excellent  | poor | poor | poor | poor |
| GD3           | excellent  | poor | poor | poor | poor |
| GQ1b          | excellent  | poor | excellent | poor | poor |
| Neuronal marker | Neurofilament 200 kD | excellent | excellent | poor |
| Parvalbumin   | poor       | excellent | excellent |          |
| Type IV collagen | excellent | excellent | excellent |          |
| Dorsal root ganglion |            |    |    |      |      |        |
| Ganglioside   |            |    |    |      |      |        |
| GM1           | excellent  | poor | excellent | poor | excellent |
| GD1b          | excellent  | poor | excellent | poor | excellent |
| GQ1b          | (excellent) | (poor) | (excellent) | (poor) | (excellent) |
| Neuronal marker | a-D-galactosyl residues | excellent | good | poor |
| Neurofilament 200 kD | poor | excellent | excellent |
| Parvalbumin   | poor       | excellent | excellent |
| Tyrosine kinase receptor A | excellent | good | good |
| Type IV collagen | excellent | excellent | excellent |
| Sciatic nerve |            |    |    |      |      |        |
| Ganglioside   |            |    |    |      |      |        |
| GM1           | excellent  | poor | excellent | poor | good |
| Type IV collagen | excellent | excellent | excellent |          |
| Ventral root  |            |    |    |      |      |        |
| Ganglioside   |            |    |    |      |      |        |
| GD3           | (good) | (poor) | (excellent) | (poor) | (good) |
| Neuronal marker | Neurofilament 200 kD | excellent | excellent | excellent |
| Type IV collagen | excellent | excellent | excellent |          |

( ) based on human data.
acetone etching (Table 2). This may be due to structural differences in which acetone exposure in this area is incomplete. A variety of techniques have been devised to retrieve the immunoreactivity (Ezaki et al., 1995). The exposure of the ganglioside epitope by removing lipid components from nervous tissue with acetone after paraformaldehyde fixation is a novel method of antigen retrieval.

Although we performed acetone etching after paraformaldehyde fixation, some structures did not show bright staining (e.g., small granular cell). Exposing the ganglioside epitope by the acetone etching condition for fresh cryosections may be not enough because the fixation of lipoproteins with aldehyde may strongly mask the epitope. Finding an appropriate acetone etching condition for paraformaldehyde fixation may improve the immunostaining. Another approach would be to use permeabilization (Molander et al., 2000) with acetone etching, which may be helpful because extensive acetone etching may extract many gangliosides (Schwarz and Futerman, 1997).

The co-staining method established in this study may enable the accurate locating of gangliosides and clarify their correlation with important markers. For instance, the simultaneous visualization of GM1 and tyrosine kinase receptor A in vivo may be informative because GM1 modulates nerve growth factor-tyrosine kinase receptor A signaling (Tanaka et al., 2007). A comparison of ganglioside location between normal mice and ganglioside synthase knockout mice by co-staining for neuronal markers may be also useful for analyzing the function of affected gangliosides. Furthermore, this approach can detect the localization of gangliosides suspected as target antigens that might be expressed by a certain subpopulation of DRG neurons (Gong et al., 2002) and a subregion at the nodes of Ranvier (Susuki et al., 2007) in autoimmune neuropathies. Co-staining for type IV collagen readily outlines the tissue framework (Matsuno et al., 1996), including the nerve cell body, myelin sheath, and blood vessels, enabling us to locate gangliosides easily.

This method is also applicable for the immunofluorescent staining of paraformaldehyde-fixed tissues routinely performed in neuroscience. In contrast, immunoenzyme staining of thin serial sections is feasible and may enable us to comprehend various markers in one target cell identified by type IV collagen co-staining. Another benefit is that, because perfusion fixation with paraformaldehyde quickly preserves the whole body of animals, many tissues can be excised at once without postmortem changes and be put into one cryomold together. This approach facilitates the examination of many tissues simultaneously under the same immunostaining conditions. A practical benefit is that frozen tissue stocks after routine P-P-fixation in neuroscience laboratories may be re-examined for ganglioside localization, or co-stained for ganglioside and neuron markers, adding information to the previous results.

In conclusion, acetone etching for paraformaldehyde-fixed nervous tissue readily retrieved ganglioside immunoreactivity by removing lipoprotein masking. Furthermore, we simultaneously detected ganglioside-positive cells and neuronal markers in nervous tissue. This new method may enable us to examine the relationship between gangliosides and various neuronal markers as well as identify the phenotype of ganglioside-positive cells by using routinely paraformaldehyde-fixed nervous tissues.

Acknowledgements
We are grateful to Keiichiro Susuki for critically reading this manuscript. We thank Tatsuo Ushiki, Kazunori Toida, Kei Funakoshi, Yoshihiro Morikawa, Shin-ichi Sakakibara, Changde Shi, Toshio Terashima, and Masahiko Watanabe for their valuable discussions and suggestions.

References
Antal M, Freund TF, Polgár E: Calcium-binding proteins, parvalbumin- and calbindin-D 28k-immunoreactive neurons in the rat spinal cord and dorsal root ganglia: A light and electron microscopic study. J Comp Neurol 295: 467-484 (1990).
Averill S, McMahon SB, Clary DO, Reichardt LF, Priestley JV: Immunocytochemical localization of trkA receptors in chemically identified subgroups of adult rat sensory neurons. Eur J Neurosci 7: 1484-1494 (1995).
Berod A, Hartman BK, Pujol JF: Importance of fixation in immunohistochemistry: Use of formaldehyde solutions at variable pH for the localization of tyrosine hydroxylase. J Histochem Cytochem 29: 844-850 (1981).
Celio MR: Calbindin D-28k and parvalbumin in the rat nervous system. Neuroscience 35: 375-475 (1990).
Chiba A, Kusunoki S, Obata H, Machinami R, Kanazawa I: Serum anti-GQ1b IgG antibody is associated with ophthalmoplegia in Miller Fisher syndrome and Guillain-Barré syndrome: Clinical and immunohistochemical studies. Neurology 43: 1911-
Dubovy P, Jancalek R, Klusakova I: A heterogeneous immunofluorescence staining for laminin-1 and related basal lamina molecules in the dorsal root ganglia following constriction nerve injury. *Histochem Cell Biol* 125: 671-680 (2006).

Ezaki T, Yao L, Matsuno K: The identification of proliferating cell nuclear antigen (PCNA) on rat tissue cryosections and its application to double immunostaining with other markers. *Arch Histol Cytol* 58: 103-115 (1995).

Gong Y, Tagawa Y, Lunn MPT, Laroy W, Hefferman M, Kotani M, Kawashima I, Ozawa H, Ogura K, Ishizuka I, Kusunoki S, Chiba A, Shimizu T, Kanazawa I: Unique glycoconjugate expression in sensory and autonomic ganglia: Relation of lectin reactivity to peptide and enzyme markers. *Arch Histol Cytol* 187: 331-334 (1993).

Ichikawa H, Jin HW, Terayama R, Yamaai T, Jacobowitz DM, Sugimoto T: Calretinin-containing neurons which co-express parvalbumin and calbindin D-28k in the rat spinal and cranial sensory ganglia; triple immunofluorescence study. *Brain Res* 1061: 118-123 (2005).

Kawai K, Mori M, Watarai S, Yasuda T: Immunohistochemical demonstration of ganglioside GD3 in the central nervous system. *Neurosci Res* 19: 119-124 (1994).

Kotani M, Tai T: An immunohistochemical technique with a series of monoclonal antibodies to gangliosides: Their differential distribution in the rat cerebellum. *Brain Res Protoc* 1: 152-156 (1997).

Kotani M, Kawashima I, Ozawa H, Terasima T, Tai T: Differential distribution of major gangliosides in rat central nervous system detected by specific monoclonal antibodies. *Glycobiology* 3: 137-146 (1993).

Kotani M, Kawashima I, Ozawa H, Ogura K, Ishizuka I, Terasima T, Tai T: Immunohistochemical localization of minor gangliosides in the rat central nervous system. *Glycobiology* 4: 855-865 (1994).

Križ J, Zhu Q, Julien J-P, Padjen AL: Electrophysiological properties of axons in mice lacking neurofilament subunit genes: Disparity between conduction velocity and axon diameter in absence of NF-H. *Brain Res* 885: 32-44 (2000).

Kusunoki S, Chiba A, Shimizu T, Kanazawa I: Unique localization of fucosyl GM1 in rabbit spinal cord and peripheral nerve: Immunohistochemical study using monoclonal anti-fucosyl GM1 antibody CRD73-6. *Biochim Biophys Acta* 1214: 27-31 (1994).

Kusunoki S, Chiba A, Kanazawa I: Anti-GQ1b IgG antibody is associated with ataxia as well as ophthalmoplegia. *Muscle Nerve* 22: 1071-1074 (1999).

La Fleur M, Underwood JL, Rappolee DA, Werb Z: Basement membrane and repair of injury to peripheral nerve: Defining a potential role for macrophages, matrix metalloproteinases, and tissue inhibitor of metalloproteinase-1. *J Exp Med* 184: 2311-2326 (1996).

Lawson SN, Harper AA, Harper EI, Garson JA, Anderton BH: A monoclonal antibody against neurofilament protein specifically labels a subpopulation of rat sensory neurones. *J Comp Neurol* 228: 263-272 (1984).

Matsuno K, Ezaki T, Kudo S, Uehara Y: A life stage of particle-laden rat dendritic cells in vivo: Their terminal division, active phagocytosis and translocation from the liver to the draining lymph. *J Exp Med* 183: 1865-1878 (1996).

Molander M, Berthold C-H, Persson H, Fredman P: Immunostaining of ganglioside GD1b, GD3 and GM1 in rat cerebellum: Cellular layer and cell type specific associations. *J Neurosci Res* 60: 531-542 (2000).

Ogawa-Goto K, Funamoto N, Ohta Y, Abe T, Nagashima K: Myelin gangliosides of human peripheral nervous system: An enrichment of GM1 in the motor nerve myelin isolated from cauda equina. *J Neurochem* 59: 1844-1849 (1992).

Pannese E, Procacci P, Ledda M, Conte V: The percentage of nerve cell bodies arranged in clusters decreases with age in the spinal ganglia of adult rabbits. *Anat Embryol (Berl)* 187: 331-334 (1993).

Popa I, Pons A, Mariller C, Tai T, Zanetta J-P, Thomas L, Portoukalian J: Purification and structural characterization of de-N-acetylated form of GD3 ganglioside present in human melanoma tumors. *Glycobiology* 17: 367-373 (2007).

Schwarz A, Futerman AH: Determination of the localization of gangliosides using anti-ganglioside antibodies: Comparison of fixation methods. *J Histochem Cytochem* 45: 611-618 (1997).

Sheikh KA, Deerinck TJ, Ellisman MH, Griffin JW: The distribution of ganglioside-like moieties in peripheral nerves. *Brain* 122: 449-460 (1999).

Silverman JD, Kruger L: Selective neuronal glycoconjugate expression in sensory and autonomic ganglia: Relation of lectin reactivity to peptide and enzyme markers. *J Neurocytol* 19: 789-801 (1990).

Susuki K, Rasband MN, Tohyama K, Koibuchi K, Okamoto S, Funakoshi K, Hirata K, Baba H, Yuki N: Anti-GM1 antibodies cause complement-mediated disruption of sodium channel clusters in peripheral motor nerve fibers. *J Neurosci* 27: 3956-3967 (2007).
Tanaka T, Furutama D, Sakai R, Fujita A, Kimura F, Tagami M, Ohsawa N, Hanafusa T: Biological roles of anti-GM1 antibodies in patients with Guillain-Barré syndrome for nerve growth factor signaling. *Biochim Biophys Acta* 1772: 543-548 (2007).

Tanaka Y, Waki H, Kon K, Ando S: Gangliosides enhance KCl-induced Ca\(^{2+}\) influx and acetylcholine release in brain synaptosomes. *Neuroreport* 8: 2203-2207 (1997).

Tettamanti G, Riboni L: Gangliosides turnover and neural cells function: A new perspective. *Prog Brain Res* 101: 77-100 (1994).

Tong Y-G, Wang HF, Ju G, Grant G, Hökfelt T, Zhang X: Increased uptake and transport of cholera toxin B-subunit in dorsal root ganglion neurons after peripheral axotomy: Possible implications for sensory sprouting. *J Comp Neurol* 404: 143-158 (1999).

Vosko MR, Busch E, Burggraf D, Bültemeier G, Hamann GF: Microvascular basal lamina damage in thromboembolic stroke in a rat model. *Neurosci Lett* 353: 217-220 (2003).

Willison HJ, Yuki N: Peripheral neuropathies and anti-glycolipid antibodies. *Brain* 125: 2591-2625 (2002).

Yu RK, Saito M: Structure and localization of gangliosides. In: *Neurology of glicoconjugates* (Margolis RU, Margolis RK, ed), Plenum Press, New York, 1989 (p. 1-42).

Yuki N, Susuki K, Koga M, Nishimoto Y, Odaka M, Hirata K, Taguchi K, Miyatake T, Furukawa K, Kobata T, Yamada M: Carbohydrate mimicry between human ganglioside GM1 and *Campylobacter jejuni* lipooligosaccharide causes Guillain-Barré syndrome. *Proc Natl Acad Sci USA* 101: 11404-11409 (2004).