Microwave-assisted immunostaining: a new approach yields fast and consistent results

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Abstract

Advances in microwave technology permitted the development of new antigen labeling techniques. The recent microwave development of a true variable wattage unit designed for laboratory use and an apparatus for dampening standing wave radiation patterns have allowed investigators to better control the conditions within a microwave cavity. Thus, operating limits thought to be endemic to microwave-assisted protocols could be effectively mitigated. Standard protocols for histochemistry call for prolonged incubations and numerous rinses that add considerable time to the procedure. Here, we present microwave-assisted staining protocols for floating rat brain sections and cultured rat hippocampal cells. Acetylcholinesterase (ACHE) histochemistry and immunocytochemistry were conducted inside a specially designed and configured laboratory microwave oven. As a control additional tissue sections were stained on the bench and treated in the same manner as those in the microwave. Labeling was minimal in the control tissue, but specific, high contrast staining was present in the microwave group. Tissues were evenly stained with minimal background, and anatomical structures were easily detected. Also, the differences between lesioned and intact sides of the brain were obvious and agreed with previous observations. Microwave-assisted methods resulted in significantly shorter protocol times (∼10-fold) resulting in staining patterns of equal or superior quality to those obtained using conventional methods.

Keywords: Microwave technology; Histochemistry; Acetylcholinesterase; Immunocytochemistry; Fluorescence; Floating sections; Cultured neurons; Cultured astrocytes

1. Introduction

Histochemical and immunolabeling techniques are widely employed in anatomy, physiology and cell biology. They are valuable tools in the identification of proteins, visualization of cellular components and the study of signaling pathways. The application of the microwave for immunological stains was first introduced by Leong and Maliao (1986) and was expanded on by others (Chiu, 1987; Chiu and Chan, 1987). Work by Chicoine and Webster (1998) described microwave-assisted labeling of ultrathin cryosections using protein A gold in an 800 W laboratory microwave oven. These authors reported increased specific labeling, yet each antibody required different microwave exposure times. However, it seemed that regardless of the type of protocol, the basic formula for success depended on the degree of microwave-induced sample heating (Boon and Kok, 1994; Choi et al., 1995; Giberson and Demaree, 1995; Giberson et al., 1997; Gu et al., 1995; Login and Dvorak, 1985, 1994; Kok and Boon, 1992). One impediment was that all microwave ovens produced an uneven distribution of radiation within the cavity (Giberson and Demaree, 1995; Giberson et al., 1997; Login and Dvorak, 1994; Kok and Boon, 1992). Further, microwave power output was not uniform between manufacturers or even identical models from the same manufacturer. However, advances in temperature control (Choi et al., 1995), microwave exposure and sample configuration (Gu et al., 1995) have been reported. These studies suggested that a significant microwave radiation effect existed independent of sample heating (Choi et al., 1995). Recent work by Sanders and Gartner (2001) described in vivo labeling of Allium sp. root tip and Drosophila embryos with vital stains, and demonstrated tissue viability after microwave-assisted processing using an oven with the present modifications. The unique results of Sanders and Gartner (2001) were obtained using a continuous low power microwave...
(variable wattage) oven in conjunction with an accessory called the ColdSpot™ (Patent #US6329645, Ted Pella, Inc., Redding, CA). This equipment has demonstrated that uniform microwave irradiation can be maintained during microwave-assisted protocols and that specimen heating can be all but eliminated (Giberson, 2001; Giberson et al., 2003; Sanders, 2002). This report details the use of low power microwave energy in combination with the ColdSpot™ for acetylcholinesterase (ACHE) histochemistry and immunohistochemical labeling. A widely used ACHE protocol and a basic antibody dependent labeling method were successfully adapted for microwave-assisted staining using this equipment.

Floating tissue sections and adherent primary neural cell cultures were labeled using similar protocols, to examine applicability of the microwave effect on various preparations. We report here that a variable wattage microwave oven can be used to obtain staining patterns as good or better than those seen using the conventional non-microwave protocols. Microwave-assisted staining has dramatically reduced the amount of time to carry out chromatogen and fluorophor based labeling. We concluded that continuous low power microwave energy combined with uniform control of the microwave environment produced consistent results and that this method can be used to accelerate a variety of staining techniques.

2. Materials and methods

2.1. Tissue preparation

All procedures were carried out in accordance with IACUC guidelines. Adult rats from the breeding facilities at California State University, Chico were anesthetized with Nembutal (50 mg/kg of body weight) and a stereotaxic lesion of the hippocampus was performed using a Scounten Knife (Beck et al., 1993). Animals were decapitated 14 days after lesion. The brains were removed and fixed overnight in 4% paraformaldehyde at 4°C. Brains were equilibrated with 30% sucrose containing 0.1% sodium azide at 4°C. They were sectioned at 30 μm using a sliding microtome. Sections were stored at 4°C in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, and 1.4 mM KH₂PO₄ at pH 7.4) containing 0.1% sodium azide.

Adherent primary neuronal cell cultures from postnatal day 1 rat pups were prepared according to Banker and Goslin (1998). This procedure yields a confluent layer of astrocytes that support an overhanging population of hippocampal neurons.

2.2. Histochemistry

All incubations and rinses were carried out in a Pelco Model 3451 Microwave Processor with variable power control and ColdSpot™ (Ted Pella, Inc., Redding, CA) unless otherwise stated (Fig. 1). The temperature of the recirculating water was set to 35°C. To determine the effectiveness of the microwave process, control tissue was stained on the bench in identical containers using the same times, steps, and solutions as those used in the microwave-assisted protocols. Brain sections and coverslips with adherent cells were placed in a polypropylene cup (10 ml) or a 24-well polystyrene plate. Unless otherwise stated all chemicals were obtained from Fisher Scientific, Houston, TX. A comparison of the microwave-assisted and the conventional protocols used in these experiments are shown in Tables 1–3.

2.3. Acetylcholinesterase staining

Microwave power was set at 150 W (adjustable range 80–750 W). Floating brain sections were incubated in Tris-malate buffer (0.1 M tris-base and 0.1 M maleic acid, pH 5.7) containing tetraisopropyl pyrophosphoramide

<table>
<thead>
<tr>
<th>Step</th>
<th>Standard protocol (time)</th>
<th>Microwave protocol (time)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Tris-malate buffer incubation</td>
<td>30 min</td>
<td>1 min</td>
</tr>
<tr>
<td>(2) K–R incubation</td>
<td>30 min</td>
<td>1 min</td>
</tr>
<tr>
<td>(3) PBS rinse²</td>
<td>5 min</td>
<td>40 s</td>
</tr>
<tr>
<td>(4) Imidazole rinse²</td>
<td>5 min</td>
<td>40 s</td>
</tr>
<tr>
<td>(5) DAB incubation</td>
<td>5 min</td>
<td>~6 min</td>
</tr>
<tr>
<td>(6) Color-reaction</td>
<td>3–5 min</td>
<td>Until desired color is reached</td>
</tr>
<tr>
<td>(7) Imidazole rinse²</td>
<td>5 min</td>
<td>40 s</td>
</tr>
<tr>
<td>(8) PBS rinse²</td>
<td>5 min</td>
<td>40 s</td>
</tr>
<tr>
<td>Total time</td>
<td>86–90 min</td>
<td>~12 min</td>
</tr>
</tbody>
</table>

K–R medium (13.5 in PBS of a solution containing the following and added in the order listed: 8.5 ml Tris-malate buffer, 0.5 ml 0.1 M sodium citrate, 1 ml 0.03 M copper sulfate, 1 ml distilled water, 1 ml 5 mM potassium ferricyanide, 5 mg acetylcholine iodide. ² Repeat once. ³ 2 min on, 2 min off, 2 min on.
Table 2

Immunolabeling with DAB on floating sections

<table>
<thead>
<tr>
<th>Step</th>
<th>Standard protocol</th>
<th>Microwave-assisted protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Antigen retrieval</td>
<td>3 min</td>
<td>1 min</td>
</tr>
<tr>
<td>(2) PBS rinse</td>
<td>5 min</td>
<td>On bench</td>
</tr>
<tr>
<td>(3) H2O2 incubation</td>
<td>20 min</td>
<td>On bench</td>
</tr>
<tr>
<td>(4) PBS rinse</td>
<td>5 min</td>
<td>On bench</td>
</tr>
<tr>
<td>(5) 10% Horse block incubation</td>
<td>30 min</td>
<td>On bench</td>
</tr>
<tr>
<td>(6) Primary antibody incubation</td>
<td>Over night in cold room</td>
<td>On bench</td>
</tr>
<tr>
<td>(7) 1% Horse block rinse</td>
<td>10–15 min</td>
<td>On bench</td>
</tr>
<tr>
<td>(8) Secondary antibody incubation</td>
<td>60 min</td>
<td>On bench</td>
</tr>
<tr>
<td>(9) PBS rinse</td>
<td>10–15 min</td>
<td>On bench</td>
</tr>
<tr>
<td>(10) ABC incubation</td>
<td>60 min</td>
<td>On bench</td>
</tr>
<tr>
<td>(11) PBS rinse</td>
<td>10–15 min</td>
<td>On bench</td>
</tr>
<tr>
<td>(12) Imidazole rinse</td>
<td>5 min</td>
<td>On bench</td>
</tr>
<tr>
<td>(13) Color reaction</td>
<td>3–7 min</td>
<td>On bench</td>
</tr>
<tr>
<td>(14) Imidazole rinse</td>
<td>5 min each</td>
<td>On bench</td>
</tr>
<tr>
<td>(15) PBS rinse</td>
<td>5 min each</td>
<td>On bench</td>
</tr>
<tr>
<td><strong>Total time</strong></td>
<td>&gt;24 h</td>
<td>52 min</td>
</tr>
</tbody>
</table>

* Repeat step two times.
  b 2 min on, 2 min off, 2 min on.
  c 2 min on, 2 min off, 2 min on.
  d Repeat step once.

(iso-ompa, Sigma, St. Louis, MO, one drop of 3 mM iso-ompa in 1 ml Tris-malate buffer) for 1 min. The sections were incubated in K-R medium (see Table 1) for 1 min, rinsed twice in PBS and twice in imidazole buffer (0.175 M sodium acetate, pH 7.2 and 0.01 M imidazole, pH 9.2) for 40 s each. The sections were incubated for 6 min in a solution containing 3,3′-diaminobenzidine tetrachloride (DAB, Sigma, St. Louis, MO, 20 mg/45 ml PBS), and 3% ammonium–nickel sulfate. For the first 2 min, the microwave power was on, for the next 2 min off, and on for the final 2 min (2 min on, 2 min off, 2 min on). One microliter of 30% hydrogen peroxide was added to the floating sections for each 5 ml of DAB solution. Sections were incubated in this solution and checked every 40 s until the desired intensity was reached. They were rinsed twice in imidazole buffer and twice in PBS (40 s each). The sections were mounted using aqueous mounting media (Dako, Carpinteria, CA).

2.4. Immunocytochemistry

2.4.1. Floating sections

All incubations and rinses were carried out in the microwave unless otherwise stated. Microwave power was set at 450 W and sections were incubated in 10 mM sodium citrate (pH 6.0) for 3 min for antigen retrieval (adapted from DeHart et al., 1996). Microwave power was changed to 150 W, and sections were rinsed three times in PBS (1 min each). Sections were incubated in 0.3% hydrogen peroxide for 1 min to remove endogenous peroxidase activity. Sections were rinsed again in PBS three times (1 min each). Tissue was incubated for 1 min in 10% horse serum with 1% bovine serum albumin (BSA) and 0.3% Triton X-100 (Sigma, St. Louis, MO) in PBS to remove non-specific binding. Blocking serum was removed and the sections were incubated in the primary antibody for a total of 8 min (3 min...
The primary antibody was removed and the tissue was rinsed three times (1 min each) in dilute blocking solution (1% horse serum, 1% BSA, and 0.3% Tween 20, Sigma, St. Louis, MO, in PBS). Sections were then incubated in biotinylated secondary antibody diluted at 1:50 (anti-mouse IgG affinity purified rat absorbed or universal secondary antibody, Vector laboratories, Burlingame, CA) for a total of 6 min (2 min on, 2 min off, 2 min on). They were rinsed three times in PBS (1 min each), and incubated in avidin–biotin peroxidase complex (Vectastain Elite Kit, Vector Laboratories, Burlingame, CA) for 6 min (2 min on, 2 min off, 2 min on). Following three 1 min rinses with PBS and two 1 min rinses with imidazole buffer, sections were incubated for 6 min (2 min on, 2 min off, 2 min on) in a solution containing 0.5 mg/ml DAB, 2.5% ammonium–nickel sulfate, and 7.0 μl of hydrogen peroxide (30% stock) per 40 ml imidazole buffer (note: DAB was added less than 10 min before use and hydrogen peroxide was added immediately before use). Sections were rinsed two times in imidazole buffer and two times in PBS (1 min each) and then mounted on slides using Dako’s Ultramount, aqueous permanent mounting media.

2.4.2. Cell cultures

Primary neurons and astrocytes were harvested from hippocampus of postnatal day 1 Fisher 344 rat pups according to Banker and Goslin (1998) and plated at 2500 cells/cm² on 12 mm round poly-d-lysine/laminin coated coverslips (Becton Dickinson). After 5 days in culture, cells were rinsed with cold phosphate buffered saline (PBS), fixed in 4% paraformaldehyde, and processed using the procedure shown in Table 3. Cell were incubated with monoclonal antibodies (anti-beta-tubulin, 1:200, or anti-glial fibrillary acidic protein (GFAP), 1:400, Chemicon, Temecula, CA), followed by rinses with PBS. FITC-conjugated rat anti-mouse IgG antibody (1:200, Jackson Immuno Research, West Grove, PA) was used to complete the labeling. The cells were also counter-stained with bisbenzimide (Sigma, 1:1000, see Fig. 5). Cells were then rinsed with PBS, mounted on slides with Fluorescent Mounting Medium (Dako) and imaged using an Olympus IX-70 inverted epifluorescent microscope equipped with a Pixera 600CL CCD camera.

3. Results

All protocols presented here produced stains of equal or superior quality to those produced by protracted conventional protocols. The microwave-assisted acetylcholinesterase histochemistry accurately stained neuronal fibers in the hippocampus. Hippocampal structures such as the dentate gyrus were stained better in the microwave oven compared to those stained using the standard protocol. In the control tissue, regardless of the protocol, staining was negligible. The microwave-assisted procedure was sensitive enough to detect responses after injury. The differences between the lesioned and unlesioned sides of the rat brain were evident. In 26-day post-lesion animal a loss of cholinergic fibers was observed in the inner molecular region of the dentate gyrus. This observation agreed with previous studies (Lynch et al., 1972; Schauwecker and McNeill, 1995) (see Fig. 2).
Microwave-assisted immunocytochemistry was also successful with a variety of antibodies. Fig. 3 shows a comparison of the results from microwave-assisted immunohistochemistry of mouse GFAP and those obtained from the conventional and the control protocols. Both protocols produced specific staining with little background.

This method worked well with both monoclonal and polyclonal antibodies. Fig. 4 shows examples of six different primary antibodies, three monoclonal specific for: growth associated protein-43 (Zymed GAP-43, 1 μg/ml), synaptophysin (Dako, 1:10), NMDA receptor subunit-1 (Chemicon, 1:10), and three polyclonal specific for glutamate receptor subunits 1, 2 and 3, and 4 (Chemicon, 1 μg/ml), which were processed with the aid of the multiwattage microwave.

This technique was adapted for fluorophore conjugated secondary antibodies (see Table 3). Antibodies specific for mouse anti-tubulin III isoform and GFAP were used to label primary neurons and astrocytes cultured from postnatal day.
Fig. 5. Microwave protocol for immunohistochemistry was adapted for use with fluorescent secondary antibodies. Parts (A) and (B) show neurons stained with anti-β-tubulin III isoform (1:200, Chemicon). Parts (C) and (D) show astrocytes counterstained with bisbenzimide and labeled with anti GFAP (1:400, Chemicon), respectively. FITC-conjugated goat anti-mouse IgG antibody (1:200, Jackson Immuno Research) was used to visualize the epitope. Bar: 200 μm (A), 50 μm (B–D).

1 rat brains. The cells shown in Fig. 5 were also counterstained with bisbenzimide. The method was highly efficient and required less than 20 min to complete.

4. Discussion

Our results show that low-wattage microwave irradiation in conjunction with effective temperature control can be adapted to a number of staining procedures and dramatically reduce processing times (Tables 1–3). These procedures yield staining of equal or better quality as those produced by conventional protocols. From the comparison of the experimental tissue and the control tissue, it is evident that low-power wattage microwave radiation is an integral part of these shorter protocols.

The microwave has already proven to be an important tool for science. Its uses have been reported in a number of procedures. However, early techniques were unsuccessful because the methodology depended primarily on sample heating. Antigen retrieval, a method to restore immunolabeling efficiency to formalin-fixed tissues, is a process that relies on boiling a solution, usually a citrate buffer at a pH of 6, for a specified period of time (DeHart et al., 1996; Shi et al., 1991). Special staining methods for histology, as described by Brinn (1983), relied on a relatively high final solution temperature to be attained. Similar results were obtained using other non-microwave heating methods suggesting that microwave irradiation was not necessarily a factor in the process.

Microwave-assisted processing of tissues for electron microscopy has also been very successful. Beginning with the first report by Mayer (1970), which described microwave stabilization of tissue, it took a number of years before Login and Dvorak (1985) reported that microwave-assisted chemical fixation was a viable method of tissue preservation for electron microscopy. It took another 12 years for the processing of tissue for electron microscopy to gain renewed attention. In 1997, microwave-assisted processing reduced processing times for electron microscopy by 90% (Giberson et al., 1997). The efficacy of the technique is evidenced by its wide use in recently published reports (e.g. Almari et al., 2000; Fiala et al., 1998; Giberson, 2001; Giberson and Demaree, 2001; Micheva et al., 2001; Tyler and Pozzo-Miller,
2001; Von Dobeln et al., 2001). Microwave-assisted methods for light level immunocytochemistry are showing similar results. The development of the ColdSpot™ allows for uniform radiation and temperature control. This suggests that sample heating is not a significant factor in the success of these abbreviated protocols.

The present authors believe that the microwave-assisted protocols introduced here will become the preferred tool of many investigators. The quality of the microwave-assisted stains is at least as good as that of the standard protocols, yet considerably shorter. The variable power microwave oven used here may also be used to improve current methods used in vital stains of living cells and tissues. These methods are currently under development by the present authors. Investigations on the effects of microwaves at the molecular level are also under development to determine how the microwave effect works in the absence of heating. The determination of specific mechanisms by which microwave irradiation facilitates these interactions will lead to the development of other useful protocols.

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References

Giberson RT, V on Dohlen CD, Kohler S, Alsop ST, McManus WR. Mealybug P. politella endosymbionts contain γ-proteobacterial symbionts.