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Microwave processing of gustatory tissues for immunohistochemistry

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Abstract

We use immunohistochemistry to study taste cell structure and function as a means to elucidate how taste receptor cells communicate with nerve fibers and adjacent taste cells. This conventional method, however, is time consuming. In the present study we used taste buds from rat circumvallate papillae to compare conventional immunohistochemical tissue processing with microwave processing for the colocalization of several biochemical pathway markers (PLC 2, syntaxin-1, IP₃R3, -gustducin) and the nuclear stain, Sytox. The results of our study indicate that in microwave versus conventional immunocytochemistry: (1) fixation quality is improved; (2) the amount of time necessary for processing tissue is decreased; (3) antigen retrieval is no longer needed; (4) image quality is superior. In sum, microwave tissue processing of gustatory tissues is faster and superior to conventional immunohistochemical tissue processing for many applications.

Keywords

Microwave fixation; Gustation; Immunohistochemistry; Taste bud; Taste; Microwave tissue processor

1. Introduction

Combining a fluorescent dye with an antibody was first performed in 1941 (Coons et al., 1941). Since then, the technique of immunohistochemistry has been carried out using various fixatives and protocols (Giberson and Demaree, 2001; Galvez et al., 2006; Ferris et al., 2009). For years we have been using conventional immunohistochemistry to elucidate biochemical pathways and identify neurotransmitters in mammalian taste buds. The disadvantages of this technique are that the results are capricious and the length of the experiment after fixation and labeling takes large amounts of time, typically three days. For many antibodies, an additional antigen retrieval step is required. This, however, can be problematic, because the tissue is easily damaged during antigen retrieval and tissue preservation can be compromised. Recently, however, microwave tissue processing has begun to emerge as an efficient and, in some cases, superior technique when compared with conventional immunohistochemical preparation techniques.

Microwave irradiation was first utilized as a fixation method in the laboratory in 1970 (Mayers, 1970). Since then, microwave processing has been incorporated into many techniques, resulting in a significant decrease in the amount of time necessary to complete a

procedure, better fixation, improved preservation of tissue, and enhanced immunostaining (Leong et al., 1985, 1988; Miura et al., 1988; Boon et al., 1990; Takes et al., 1989; Leong and Milios, 1990; Krug and Takes, 1991; Kok and Boon, 1990, 1994, 2003; Login and Dvorak, 1988, 1994; Leong and Sormunen, 1998; Giberson and Demaree, 2001; Giberson et al., 2003; Munoz et al., 2004; Leong and Price, 2004; Galvez et al., 2006; Ferris et al., 2009). When microwaves are applied to a solution, they cause any electrically charged molecule to rapidly rotate throughout the sample, resulting in an increase in diffusion rates. In a typical microwave processor, this would result in an increase in temperature, thereby heating the sample. Specialized microwave tissue processors allow one to control variables such as temperature, wattage, and time. In these processors, microwaves are applied to tissue samples that are suspended in solution. Electrically charged molecules begin to move rapidly throughout the sample; however, the temperature is controlled to prevent overheating or “cooking” the sample. The use of a vacuum in microwave tissue processing enhances tissue preservation and facilitates diffusion resulting in deeper penetration of antibodies.

There were initially many limitations to the use of microwaves in laboratories. Temperature and wattage had to be closely monitored throughout the procedure and it was difficult to provide a uniform distribution of microwave irradiation throughout the sample. With the current generation of microwave processors, these problems are greatly reduced. Temperature, wattage, and time can be controlled automatically. Although all the antibodies in this study reacted similarly to microwave irradiation, there is evidence that some antibodies react differently (Hjerpe et al., 1988; Takes et al., 1989; Munoz et al., 2004; Temel et al., 2006). The temperature, wattage, and time must be adjusted based on the nature of the antibody. Other studies have shown that controlling temperature and power output can affect results (Sanders and Gartner, 2001; Sanders, 2002; Munoz et al., 2004; Ferris et al., 2009). Using a modern laboratory microwave tissue processor, these settings can be automated and easily changed before and after an experiment. Protocols are entered and stored on the microwave’s touch screen system so that the procedure can be carried out automatically with the press of a button. Variables for each protocol can be changed at any time.

In the present study we have studied the efficacy of microwave processing for immunohistochemistry on circumvallate taste buds of rodents. We have compared results from tissues using the conventional method of immunohistochemistry with those from tissues using a microwave processor to evaluate the effects of microwave-assisted immunohistochemistry on taste cells.

2. Materials and methods

2.1. Animal care

Adult Sprague-Dawley male rats (250–350 g) were used for these studies. Animals were cared for and housed in facilities approved by the Institutional Animal Care and Use Committee of the University of Denver. All animals were anesthetized with a mixture of sodium ketamine (200 mg/kg) and xylazine (70 mg/kg) (I.P.). Primary antibodies, secondary antibodies, and nuclear stain are listed in Tables 1 and 2.

2.2. Conventional immunohistochemistry for confocal microscopy

Four rats were perfused for 10 s through the left ventricle with 0.1% sodium nitrate, 0.9% sodium chloride and 100 units of sodium heparin in 100 ml of 0.1 M phosphate buffer (pH 7.3). This was followed by perfusion fixation with PLP (75 mM Lysine, 1.6% PFA, and 10 mM Sodium Periodate) in 0.1% phosphate buffer for 10 min (Weedman et al., 1996). All perfusates were warmed to 42 °C before use. After perfusion, the excised circumvallate

papillae were fixed in fresh fixative for 3 h at 4 °C. The tissues were then cryoprotected with 30% sucrose in 0.1 M phosphate buffer overnight at 4 °C.

2.3. Double labeling and nuclear staining

Tissue was frozen in OCT and then sliced on a cryostat into sections 20 µm thick. Cryostat sections containing circumvallate taste buds were washed in 0.1 M PBS (pH 7.3) for 30 min, then blocked in 5% normal goat serum and 0.3% Triton X-100 in 0.1 M PBS (pH 7.3) for 2 h on ice. An additional step was added to the procedure when the primary antibody to IP₃R3 was applied: sections were incubated in 10 mM sodium citrate (pH 9) for 20 min at 80 °C for antigen retrieval, prior to blocking. The sections were incubated in a combination of two primary antibodies: either rabbit polyclonal antibody α -gustducin with mouse monoclonal antibody IP₃R3 or rabbit polyclonal antibody PLC α 2 with mouse monoclonal syntaxin-1. Both combinations were mixed with 0.1 M PBS (pH 7.3) and refrigerated overnight at 4 °C. Tissues were then rinsed in 0.1 M PBS (pH 7.3) for 30 min. The sections were treated in a combination of two secondary antibodies in 0.1 M PBS (pH 7.3): Alexa-Fluor 546 goat anti-mouse IgG and Dylight 649 goat anti-rabbit IgG. The tissues were left in this treatment for 1 h. Following this treatment, the tissues were washed in 0.1 M PBS (pH 7.3) for 30 min. For nuclear staining, the tissues were washed in 0.1 M PBS (pH 7.3) for 10 min, followed by 1 min of incubation in a mixture of Sytox nuclear stain and deionized distilled H₂O. Following this incubation, tissues were washed for 30 min in deionized distilled H₂O. The sections were then mounted onto glass slides using Fluorogel with Tris Buffer (Electron Microscopy Sciences).

All sections were processed in polystyrene 6 wells plates, with approximately 2.5 ml of the appropriate solution in each well for rinsing. During antibody incubation steps, tissue was immersed in 500 µl of solution in a 1 ml centrifuge tube.

2.4. Microwave processed immunohistochemistry for confocal microscopy

Four rats were perfused for 10 s through the left ventricle with 0.1% sodium nitrate, 0.9% sodium chloride and 100 units sodium heparin in 100 ml 0.1 M phosphate buffer (pH 7.3). This was followed by perfusion fixation with PLP (75 mM Lysine, 1.6% PFA, and 10 mM Sodium Periodate) or 4% PFA in 0.1% phosphate buffer for 10 min (Weedman et al., 1996). All perfusates were warmed to 42 °C before use. After perfusion, the excised circumvallate papillae were fixed in fresh fixative in a Ted Pella Biowave Microwave Processor inside the Pelco EM Pro Vacuum Chamber, connected to the microwave processor by the Pelco ¼ Silicone Vacuum Hose, for 6 min. The vacuum power remained on for the entire 6 min. During this time, the microwave power was turned on at 150 W for 2 min, turned off for 2 min, and then turned back on at 150 W for 2 min. Alternating the on–off state of the microwave power helps to maintain temperature at 23 °C or below and increase the speed of antibody interactions, resulting in increased antibody penetration over a reduced time span. The tissue was then cryoprotected in 30% sucrose in phosphate buffer (pH 7.3) in the microwave processor with vacuum for 6 min. During this time, the microwave power was turned on at 150 W for 2 min, turned off for 2 min, and then turned back on at 150 W for 2 min. These steps were carried out in polystyrene 6 well plates, with approximately 0.5 ml of the appropriate solution in each well.

2.5. Double labeling and nuclear staining

Cryostat sections (20 µm thick) containing circumvallate taste buds were washed in 0.1 M PBS (pH 7.3) in the microwave processor for 1 min with the microwave power on at 150 W. The tissue was then blocked in 5% normal goat serum and 0.3% Triton X-100 in 0.1 M PBS (pH 7.3) in the microwave processor for a total of 3 min. During this time, the microwave power was on at 150 W for 1 min, off for 1 min, then on again at 150 W for 1 min. The

sections were incubated in a combination of two primary antibodies: either rabbit polyclonal antibody -gustducin with mouse monoclonal antibody IP₃R3, or rabbit polyclonal antibody PLC 2 with mouse monoclonal syntaxin-1. Both combinations were mixed with 0.1 M PBS (pH 7.3) and placed in the vacuum chamber inside the microwave processor for 8 min. During this time, the microwave power was on at 150 W for 3 min, off for 2 min, then on at 150 W for 3 min. The tissues were then washed in 0.1 M PBS (pH 7.3) in the microwave three times for 1 min each, with the microwave power on at 150 W. Following these washes, the tissues were incubated in a combination of two secondary antibodies in 0.1 M PBS (pH 7.3): Alexa-Fluor 546 goat anti-mouse IgG and Dylight 649 goat anti-rabbit IgG. This treatment was done in the microwave processor with vacuum for 8 min. During this time, the microwave power was on at 150 W for 3 min, off for 2 min, then on at 150 W for 3 min. The tissues were then washed in 0.1 M PBS (pH 7.3) in the microwave two times for 1 min each, with the microwave power on at 150 W. Following this treatment, two additional washes were performed in 0.1 M PBS (pH 7.3) in the microwave, 30 s per wash. Nuclear staining was performed conventionally, outside of the microwave; sections were incubated in Sytox Green in deionized, distilled H₂O for 1 min, followed by three washes in deionized, distilled H₂O 1 min each, inside the microwave, 10 min per wash. The tissues were then mounted onto glass slides using Fluorogel with Tris Buffer (Electron Microscopy Sciences). The temperature in the microwave processor was programmed to a set point of 23 °C. All sections were processed simultaneously using polystyrene 48-well plates (BD Falcon, NJ) in 500 µl of solution.

2.6. Microscopy and image processing

Slides were examined using a Zeiss Axioplan II with an Apotome confocal attachment. Images were acquired using Zeiss Axiovision software and later adjusted using Adobe Photoshop. Adjustments were made in brightness only.

3. Results

3.1. Significant time saving

The amount of time necessary to complete fixation and staining of tissue is significantly less in the microwave versus conventionally prepared tissues (Table 3). Microwave processing takes only 42 min, while conventional processing takes three days. Post-fixation time decreases from 3 to 4 h conventionally versus 6 min in the microwave. Conventional cryoprotection in 30% sucrose typically occurs overnight at 4 °C. In the microwave processor, this step only takes 6 min. These steps alone dramatically reduce processing times. In the microwave, incubation times for primary and secondary antibodies are only 8 min. For conventional processing, primary antibodies incubate overnight and secondary antibodies incubate for 1 h. All washes in the microwave are only 1 min and the technique does not require as many washes as conventional processing. Antigen retrieval was not necessary for any tissue processed in the microwave. This is significant for IP₃R3 because this antibody requires 20 min of high heat (80 °C) antigen retrieval in sodium citrate solution (pH 9) when processed conventionally. Clapp et al. (2004) showed the morphologic characterization of IP₃R3 using antigen retrieval in sodium citrate (pH 9). During this study, they tested IP₃R3 expression with antigen retrieval in sodium citrate (pH 6) versus sodium citrate (pH 9). They found sodium citrate (pH 9) to be more effective for antibody expression. Since this study was published in 2004, our lab has adopted this method for the best expression of IP₃R3 immunoreactivity. For this reason, we do not treat IP₃R3 with antigen retrieval in sodium citrate (pH 6).

3.2. Double label studies of PLC 2 with Syntaxin-1 and Sytox

PLC 2 is present in Type II cells and syntaxin-1 is present in the nerve fibers and some Type III cells. Fig. 1 shows a confocal image of conventionally processed PLC 2 with syntaxin-1 and Sytox. The nuclei are visible with the Sytox nuclear stain (Fig. 1A). PLC 2 clearly stains Type II cells (Fig. 1B). Nerve fibers are stained with syntaxin-1, along with some Type III cells (Fig. 1C). Fig. 2 shows the same conditions as Fig. 1, with tissue that was processed in the microwave rather than conventionally. Once again, the nuclei are visible with Sytox nuclear stain (Fig. 2A). PLC 2 stains Type II cells (Fig. 2B) and syntaxin-1 stains the nerve fibers and some Type III cells (Fig. 2C). The tissue processed in the microwave is of the same quality or better than tissue processed conventionally. The results of both techniques show staining of the same cell types. When comparing the merged images, the tissue processed in the microwave (Fig. 2D) appears to be sharper than the tissue processed conventionally (Fig. 1D). It is possible that tissue processed in the microwave appears to be sharper because it has less exposure time to high heat and spends less processing time incubating in solutions than tissue processed conventionally, resulting in less shrinkage.

3.3. Double label studies of α -gustducin with IP₃R3 and Sytox

Both α -gustducin and IP₃R3 are present in Type II cells. Fig. 3 shows a confocal image of conventionally processed α -gustducin with IP₃R3 and Sytox in rat circumvallate papillae. Nuclei are visible with Sytox nuclear stain (Fig. 3A). α -Gustducin stains a small subset of Type II cells (Fig. 3B). IP₃R3 stains a larger subset of Type II cells (Fig. 3C). Fig. 4 shows a confocal image under the same conditions as Fig. 3, with tissue processed in the microwave instead of conventionally. The sections that were fixed and labeled in the microwave processor did not undergo any antigen retrieval step like the sections that were fixed and labeled conventionally. IP₃R3 is a Type II cell marker that normally requires antigen retrieval. Even with elimination of the antigen retrieval step in the microwave processor, IP₃R3 immunoreactivity is still excellent in Type II cells (Fig. 4C). During conventional fixation, tissue is exposed to the formaldehyde fixative for prolonged periods of time. This results in cross-linkage of proteins that mask antigen binding sites. In the microwave processor, fixation times are dramatically reduced due to increased diffusion rates and temperature controls. This leads us to conclude that microwave-assisted immunohistochemistry preserves the antigenicity of the tissue. α -Gustducin stains a subset of Type II cells (Fig. 4B) and Sytox clearly stains the nuclei (Fig. 4A). Once again, tissue processed in the microwave is of the same quality or better than tissue processed conventionally. Merged images show the microwave processed tissue (Fig. 4D) to be crisper than the conventionally processed tissue (Fig. 3D).

4. Discussion

4.1. Mechanism of microwave processing

The exact mechanism of how microwave energy positively affects the tissue is not yet fully elucidated, however, it is known that microwave irradiation processes tissue through dielectric heating, also known as the “thermal effect,” causing a rise in temperature inside the sample. This is contrary to conventional heating, which begins outside the sample (Leonard and Shepardson, 1994; Giberson and Demaree, 1995; Kok and Boon, 2003; De La Hoz et al., 2005; Schroeder et al., 2006; Zechmann and Zellnig, 2009). Due to this phenomenon, tissue processed in the microwave promotes deeper penetration of reagents. Due to the properties of the microwave processor, the temperature is controlled and heating is uniform throughout the sample. Tissue is not “cooked” as it would be in a classic microwave. In conventional methods, penetration occurs from the outside and might never reach the middle of the sample, resulting in variable fixation and staining. Due to the nature

of gustatory tissue, proper fixation can be very difficult. The properties of the microwave have helped to overcome this difficulty. The results of this study show a successful post-fixation after only 6 min under vacuum in the microwave processor.

4.2. Important variables for the microwave method

Tissue thickness and the amount of solution utilized for each step are important variables in the microwave processing technique. Microwave energy excites dipolar molecules at a frequency of 2.45 GHz, thereby causing a uniform distribution of heat (Kok and Boon, 1990; Sun et al., 2009). If the tissue exceeds the penetration depth, heat will no longer be distributed uniformly, resulting in inconsistent immunostaining. In this study, we achieved optimum results using 500 μ l of solution for each step and tissue sliced from 20 to 40 μ m. We did not experiment with larger sample sizes, however, we did run many experiments in which the amount of solution used in each step exceeded 500 μ l. These results yielded little to no signal within the taste cells (results not shown). Thus, not only do tissue thickness and amount of solution affect microwave processing, but also it is important to have a uniform level of solution in each well of the well plate. Finally, it is critical that the materials used in this technique to hold the samples allow the passage of microwaves (e.g., Teflon). Otherwise, the microwaves will bounce off the materials and never reach the sample.

4.3. Significant time savings

The fixation and labeling of tissue processed in the microwave is much faster than tissue processed conventionally. Conventional immunohistochemistry lasts approximately three days, using a protocol that has been successful in our lab for more than 15 years, while microwave processing techniques last only 42 min following the perfusion. Microwaves cause an increase in the amount of collisions of molecules, speeding up the reactions that occur in the microwave processor (Leong and Sormunen, 1998). This then accelerates the rate of diffusion, increasing the speed of tissue processing. Table 3 shows the comparison of time for each step between the microwave and conventional methods. Cryoprotection in sucrose lasts only 6 min in the microwave processor, versus overnight when processed conventionally. This step alone reduces processing time by approximately 12 h.

4.4. Preservation of antigenicity

Fixation of tissue for immunohistochemistry can greatly reduce detection of some antigens. This happens because fixatives cause modifications of antigens due to a change in their structure, thereby masking antigen binding sites (Boon and Kok, 1994). For this reason, certain antibodies require an antigen retrieval step to overcome the masking of the antigen binding sites. The problem with this step is that it requires high heat, which can break down the tissue and cause damage. Previous studies have shown that microwave irradiation preserves antigenicity (Patterson and Bulard, 1980; Leong et al., 1988; Moran et al., 1988; Jamur et al., 1995; Rohr et al., 2001; Morales et al., 2004; Emerson et al., 2006). In the present study, IP₃R3 is an antibody that usually must undergo antigen retrieval in order to see any immunostaining. With the tissue processed in the microwave, antigen retrieval was not needed. The results for conventional processing of IP₃R3 without the antigen retrieval yield almost no signal in the taste cells. For tissue processed in the microwave, however, results were successful without the use of antigen retrieval. These results suggest that antigenicity is better preserved using the microwave processing method. This is most likely a result of microwave fixation. Tissue that is conventionally fixed is exposed to formaldehyde for prolonged periods of time. During this long fixation proteins cross-link, masking antigen binding sites. Fixation times in the microwave are dramatically reduced, thereby increasing antigenicity.

4.5. Microwave versus conventional results

The results of this study indicate that tissue processed in the microwave is of equal or better quality than tissue processed conventionally. These results support the finding of previous studies on other cell types (Rohr et al., 2001; Morales et al., 2002, 2004; Hafajee and Leong, 2004; Leong and Price, 2004; Munkholm et al., 2008; Zechmann and Zellnig, 2009). Based on the confocal images taken in the present study, the tissues processed in the microwave appear to be superior to the conventionally processed tissues. The microwave tissues are crisper than the tissues processed conventionally. This is likely a result of decreased tissue shrinkage. Microwave processed tissues are immersed in solutions and exposed to high heat for much less time than conventionally processed tissues. Lengthy exposure to solutions and heat can result in tissue shrinkage and damage. These results, along with the reports from previous studies suggest that microwave processing results in improved tissue preservation (Leong et al., 1985; Login et al., 1987; Login and Dvorak, 1988; Rassner et al., 1997; Zechmann and Zellnig, 2009).

As microwave processing becomes more widely accepted for immunohistochemical techniques, it will greatly improve the final results while significantly reducing processing times. Our laboratory consistently employs the microwave processor for immunofluorescence. This technique has allowed for optimization of tissue and staining quality, while maximizing the amount of experiments that can be completed. Part of the convenience of the microwave processor is that it can be used in conjunction with conventional methods. It is possible to stop at any step and switch methods.

5. Conclusion

Although microwave processing has been applied to various techniques and tissue types, it has never before been studied in gustatory tissue for immunohistochemistry. In this study, we have developed a protocol for processing gustatory tissue in a microwave processor. We have compared the images of microwave-processed tissues with images of conventionally processed tissues and found the images of the microwave-processed tissues to be equal or superior to the conventionally prepared tissues. Microwave processing significantly reduces turn-around times and increases antigenicity. The properties of microwaves ensure better preservation of tissue. Based on time, image quality, tissue preservation and antigenicity, we believe that microwave processing is a superior method for immunohistochemistry.

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Abbreviations

PLC 2	phospholipase C 2
IP₃ R3	inositol 1,4,5-trisphosphate receptor 3
I.P	intraperitoneal
PLP	periodate–lysine–paraformaldehyde
PFA	paraformaldehyde

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HIGHLIGHTS

- Microwave fixation of gustatory tissue improves fixation quality.
- Microwave processing decreases the time necessary to complete experimental procedures.
- Antigen retrieval is not needed when using a microwave processor.
- Image quality from the microwave processor is superior to conventional methods.

Fig. 1. Confocal images of conventionally processed rat circumvallate taste buds. (A) Nuclei stained with Sytox (green). (B) PLC 2 (red). (C) Syntaxin-1 (purple). (D) Merge. Scale bar = 20 μ m.

Fig. 2. Confocal images of microwave processed rat circumvallate taste buds. (A) Nuclei stained with Sytox (green). (B) PLC 2 (red). (C) Syntaxin-1 (purple). (D) Merge. Scale bar = 20 μm .

Fig. 3. Confocal images of conventionally processed rat circumvallate taste buds. (A) Nuclei stained with Sytox (green). (B) Gustducin (red). (C) IP₃ R3 (purple). (D) Merge. Scale bar = 20 μ m.

Fig. 4. Confocal images of microwave processed rat circumvallate taste buds. (A) Nuclei stained with Sytox (green). (B) Gustducin (red). (C) IP₃ R3 (purple). (D) Merge. Scale bar = 20 μ m.

Table 1

Primary antibodies.

Antibodies	Species	Dilution	Source	Code no.
-Gustducin	Rabbit	1:100	Santa Cruz Biotech.	SC-395
IP ₃ R3	Mouse	1:100	Transduction Lab.	610313
PLC 2	Rabbit	1:100	Santa Cruz Biotech.	SC-206
Syntaxin-1	Mouse	1:100	Sigma	S0664

Table 2

Secondary antibodies and nuclear stain.

Antibodies/stains	Species	Dilution	Source	Code no.
Dylight 649 IgG	Rabbit	1:100	Jackson	111495144
AlexaFluor 546 IgG	Mouse	1:100	Molecular Probes	A11030
Sytox	N/A	1:100	Molecular Probes	S7020

Table 3

Processing times for conventional and microwave immunohistochemistry.

Conventional	Microwave
3–4 h post-fixation in PFA or PLP	6 min post-fixation in PFA or PLP
30% Sucrose Overnight	6 min in 30% sucrose
3 × 10 min PBS washes	1 min PBS wash
2 h in blocking solution	3 min in blocking solution
20 min antigen retrieval	No antigen retrieval necessary
Primary antibody overnight	8 min primary antibody
3 × 10 min PBS washes	3 × 1 min PBS washes
2 h in secondary antibody	8 min in secondary antibody
3 × 10 min PBS washes	2 × 1 min PBS washes
41 min for Sytox staining	5 min for Sytox staining
Total: 3 days	Total: 42 min