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ORIGINAL PAPER

Microwave oven-based technique for immunofluorescent staining of paraffin-embedded tissues

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Abstract Immunohistochemical analysis of formalin-fixed paraffin-embedded tissues can be challenging due to potential modifications of protein structure by exposure to formalin. Heat-induced antigen retrieval techniques can reverse reactions between formalin and proteins that block antibody recognition. Interactions between antibodies and antigens are further enhanced by microwave irradiation, which has simplified immunohistochemical staining protocols. In this report, we modify a technique for antigen retrieval and immunofluorescent staining of formalin-fixed paraffin-embedded tissues by showing that it works well with several antibodies and buffers. This microwave-assisted method for antigen retrieval and immunofluorescent staining eliminates the need for blocking reagents and extended washes, which greatly simplifies the protocol allowing one to complete the analysis in less than 3 h.

Keywords Microwave oven · Immunofluorescence · Antigen retrieval · Pituitary gland · Uterus · Insulin receptor · Insulin-like growth factor receptor · Adipose

Introduction

Preparation of thin sections of tissue for immunohistochemistry often requires formalin fixation followed by paraffin embedding (http://www.dakousa.com/ishbantigenretr.pdf). Immunohistochemical staining of specific cellular proteins in tissue sections capitalizes on interactions between antibodies and antigens (Cook 2000); however, formalin fixation can modify the tertiary structure of proteins, which may interfere with these interactions (Ramos-Vara 2005). Therefore, immunohistochemistry was not commonly performed on formalin-fixed sections until after the introduction of antigen retrieval. Initially, a protease-induced antigen retrieval approach was used to unmask antigens in formalin-fixed tissues, but it had limited success due to non-specific digestion of antigens (Huang 1975). Subsequently, a heat-based antigen retrieval technique was introduced (Shi et al. 1991), and this method utilized the concept that chemical reactions between proteins and formalin can be reversed by heat or alkaline hydrolysis (Fraenkel-Conrat et al. 1947; Fraenkel-Conrat and Olcott 1948a; b). Still, it was important to determine the optimal buffer and heating condition for successful antigen retrieval (Shi et al. 1991; Kumada et al. 2004; Namimatsu et al. 2005, U.S. Patent 5,244,787 and http://www.herpesvirus.tripod.com/research/protoc.htm).

Traditional immunohistochemical techniques involve multiple reagents and a lot of time (Shi et al. 1991). A typical immunohistochemical protocol has the following steps: inactivating endogenous peroxidase activity, blocking tissue with serum, incubating tissue with a primary antibody, and applying a secondary antibody which contains an enzyme whose activity is detected after the addition of a substrate. More reagents may be required to enhance detection via a reporter enzyme. Immunofluorescent antibodies have greatly simplified analysis of antibody-antigen interactions, in addition to allowing detection of multiple proteins in one tissue section. Microwave irradiation has been employed to decrease incubation times by enhancing antibody-antigen interactions (Leong and Milos 1986) and advances in temperature control have significantly improved speed and reliability (Kumada et al. 2004; Munoz et al. 2004; Temel et al. 2006). Microwave

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irradiation has been employed in experiments involving immunofluorescence (Chiu and Chan 1986; Sheriffs et al. 2001), however, its success has primarily been with those probing frozen sections. Standard immunofluorescent protocols, which involve blocking agents, washes and lengthy antibody incubations, are still commonplace.

In this report, we modify a technique for antigen retrieval and immunofluorescent staining of formalin-fixed paraffin-embedded tissues that works well with several antibodies and buffers. The incubation of tissue sections with antibodies takes only minutes with microwave-assisted irradiation and the need for blocking reagents and extended washes are both eliminated.

Materials and methods

Tissue preparation

Pituitary, adipose and uterine tissues were isolated from adult CD-1 mice and fixed in 10% formalin prior to paraffin embedding. Five micron sections were prepared and mounted on slides.

Deparaffinization

Paraffin-embedded sections were placed at 60°C for 15 min, incubated in xylene at RT for 15 min, and then transferred sequentially into 100% EtOH, 95% EtOH, 70% EtOH, and 50% EtOH for 4 min at RT. Sections were rinsed in deionized water and stored in PBS.

Antigen retrieval

To determine the optimal condition for antigen retrieval, three different buffers were used. Buffer 1 (100 mM Tris, pH 10) was recommended for immunofluorescence using a microwave oven (http://www.herpesvirus.tripod.com/research/protoc.htm); Buffer 2 (0.05% citraconic anhydride) was recently reported as a superior buffer for antigen retrieval (Namimatsu et al. 2005), and Buffer 3 (10 mM citrate, pH 6.2, 2 mM EDTA, and .05% Tween 20) was previously shown to give optimal results with immunofluorescence (Sheriffs et al. 2001). Slides were immersed in antigen retrieval buffer using an uncovered 470 ml Rubbermaid tray and heated in an 1100 W GE microwave oven (http://www.herpesvirus.tripod.com/research/protoc.htm) for three sequential 5-min cycles at power levels 5, 5 and then 4. The tray was removed from the microwave oven and allowed to cool from 90°C to RT. Slides were rinsed in PBS.

Immunofluorescent staining

Tissues were outlined with a liquid Blocker Super Pap Pen to minimize the volume (20–30 µl) of antibody solution needed for staining. Several antibodies were used: 1:25 dilution of anti-insulin-like growth factor 1 receptor beta subunit (IGF-1Rβ, Santa Cruz #sc-713); 1:5 dilution of anti-insulin receptor alpha subunit (IRα, Santa Cruz #sc-710); 1:50 dilution of anti-rLH beta-Ic-3 antibody (LHβ, NIDDK) and 1:20 dilution of human IgG Fc-fragment (hIgG, Upstate #AG714). Antibodies were diluted in PBS containing 1% BSA and .01% Triton X-100 and added to each tissue section. Slides were placed in a tray (Research Products International #248270) and water added to a level within 0.5 inch of the bottom of the slides. The tray was covered and placed into the microwave oven for a three-minute heating cycle at power level 4. Two minutes after the heating cycle was completed, slides were rinsed in PBS and then 1:100 dilution of a secondary antibody was applied (Santa Cruz Biotechnology, #SC-2012). Slides were placed back into the tray, containing fresh deionized water, and incubated in the microwave oven for a three-minute heating cycle at power level 4. Two minutes after incubation, the slides were rinsed with PBS, stained with DAPI (Sigma-Aldrich #D-9542) and examined using an Olympus BX51 microscope.

Results

Paraffin-embedded sections of mouse tissues were deparaffinized and subjected to antigen retrieval using three different buffers. Immunofluorescent analysis using an IRα antibody shows staining of the plasma membrane of adipocytes, consistent with the expected location of the insulin receptor (Fig. 1). DAPI highlights nuclei, located along the periphery of the adipocytes (Fig. 1). There was comparable staining of the IRα subunit with all three buffers but no detectable staining with a hIgG antibody (Fig. 1A–C compared to Fig. 1D).

The same protocol and buffer 3 was used to stain formalin-fixed, paraffin-embedded mouse pituitary sections with IGF-1Rβ (Fig. 2A) and LHβ antibodies (Fig. 2B). The LHβ antibody shows a clear cytoplasmic-staining pattern (Fig. 2B), whereas, the IGF-1Rβ antibody shows a distinct cytosolic membrane-staining pattern (Fig. 2A). No staining was observed with a hIgG antibody (Fig. 2C).

To broaden the scope of investigated tissues, uteri from CD-1 mice were prepared using buffer 1 and stained with IGF-1Rβ antibody. The IGF-1 receptor shows a strong membranous staining pattern of uterine epithelial cells (Fig. 3A). In addition, a strong membranous staining pattern is observed on the epithelial cells of the uterine glands (Fig. 3C). No staining was observed with a hIgG antibody (Fig. 3B, D).

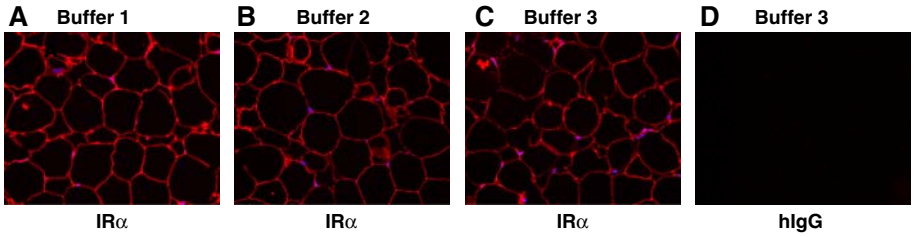

Fig. 1 Immunofluorescent analysis of formalin-fixed paraffin-embedded sections of mouse adipose tissue. Antigen retrieval was performed with three different buffers (**A**, Buffer 1; **B**, Buffer 2; **C**, Buffer 3; **D**, Buffer 3) and subsequently analyzed by immunofluorescence with IRα (**A–C**) and hIgG (**D**) antibodies and DAPI. Representative photomicrographs are shown at 200×

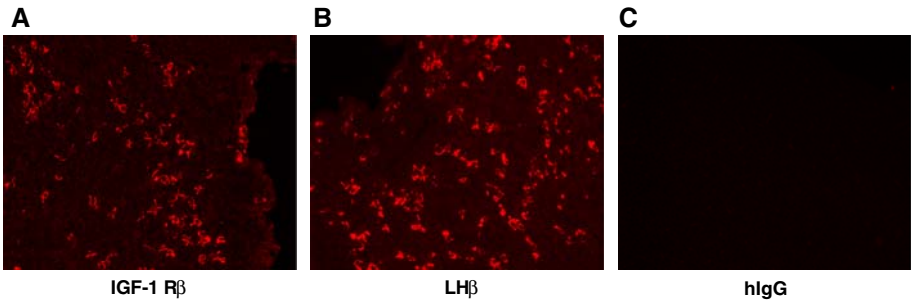

Fig. 2 Immunofluorescent analysis of formalin-fixed paraffin-embedded sections of mouse pituitary. Antigen retrieval was performed with Buffer 3 and subsequently analyzed by immunofluorescence with (**A**) IGF-1Rβ, (**B**) LHβ, and (**C**) hIgG antibodies. Representative photomicrographs are shown at 200×

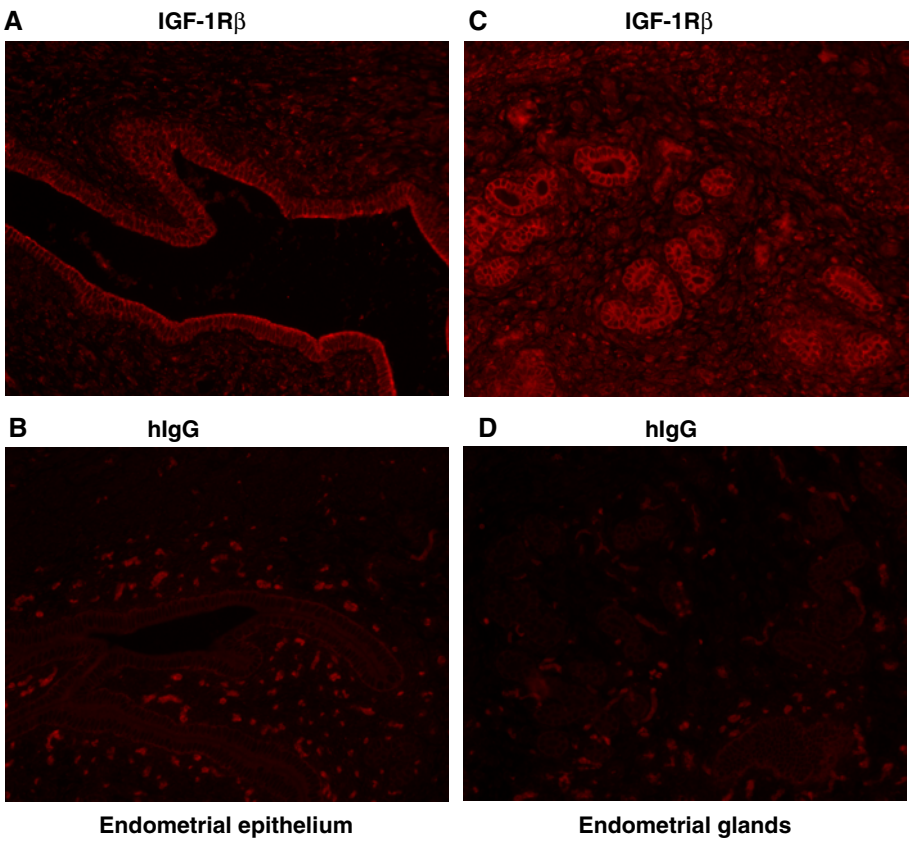

Fig. 3 Immunofluorescent analysis of formalin-fixed paraffin-embedded sections of mouse uteri. Antigen retrieval was performed with Buffer 1 and subsequently analyzed by immunofluorescence with IGF-1Rβ antibody (**A** & **C**) and hIgG (**B** & **D**). Representative photomicrographs are shown at 200×

Discussion

We report a technique that allows immunofluorescent staining of formalin-fixed tissues in less than 3 h. Deparaffinized slides are immersed in a buffered solution and heated via microwave irradiation. We used different buffers listed in the literature for antigen retrieval and found that tissue staining was comparable. Although the method can be applied to different tissues, we found that pituitary tissue is more easily damaged than skeletal muscle or liver during the antigen retrieval step. Therefore, it is important to consider, with some tissues, that solutions should not be allowed to reach a vigorous boil during the heating stage of antigen retrieval. In this study, microwave irradiation was used after deparaffinization; however, there is one report that shows it can be used during deparaffinization (Temel et al. 2005).

This modified method for antigen retrieval and immunofluorescent staining using microwave-assisted irradiation has several advantages compared to standard immunofluorescent protocols. First, it reduces incubation times with primary and secondary antibodies. Second, it eliminates the need for repeated washings. Third, it requires no blocking reagents. Finally, it is an inexpensive and sensitive technique that can be applied to various tissues that require formalin-fixation and paraffin embedding. Therefore, this simple and rapid method combining antigen retrieval and immunofluorescent analysis may be a very useful technique for both basic science and clinical research.

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References

Chiu KY, Chan KW (1986) Rapid immunofluorescence staining of human renal biopsy specimens using microwave irradiation. J Clin Pathol 40:689–692

Cook H (2000) Evolution of histology. Biomed Scientist 44:825–827

Fraenkel-Conrat H, Brandon B, Olcott H (1947) The reaction of formaldehyde with proteins. IV. Participation of indole groups. Gramicidin J Biol Chem 168:99–118

Fraenkel-Conrat H, Olcott H (1948a) The reaction of formaldehyde with proteins. V. Cross-linking between amino and primary amide or guanidyl groups. J Am Chem Soc 70:2673–2684

Fraenkel-Conrat H, Olcott H (1948b) Reaction of formaldehyde with proteins. VI. Cross-linking of amino groups with phenol, imidazole, or indole groups. J Biol Chem 174:827–843

Huang S (1975) Immunohistochemical demonstration of hepatitis B core and surface antigens in paraffin sections. Lab Invest 33: 88–95

Kumada T, Tsuneyama K, Hatta H, Ishizawa S, Takano Y (2004) Improved 1-h rapid immunostaining method using intermittent microwave irradiation: practicability based on 5 years application in Toyama Medical and Pharmaceutical University Hospital. Mod Path 17:1141–1149

Leong AS-Y, Milos J (1986). Accelerated immunohistochemical staining by microwaves. J Pathol 148:183–187

Munoz T, Giberson R, Demaree R, Day J (2004) Microwave-assisted immunostaining: a new approach yields fast and consistent results. J Neuro Meth 137:133–139

Namimatsu S, Ghazizadeh M, Sugisaki Y (2005). Reversing the effects of formalin fixation with citraconic anhydride and heat: a universal antigen retrieval method. J Histochem Cytochem 53(1):3–11

Ramos-Vara J (2005) Technical aspects of immunohistochemistry. Vet Pathol 42:405–426

Sheriffs I, Rampling D, Smith V (2001). Paraffin wax embedded muscle is suitable for the diagnosis of muscular dystrophy. J Clin Pathol 54:517–520

Shi S, Key M, Kaira K (1991) Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. J Histochem Cytochem 39(6):741–748

Temel S, Minbay F, Kahveci Z, Jennes L (2006) Microwave-assisted antigen retrieval and incubation with cox-2 antibody of archival paraffin-embedded human oligodendroglioma and astrocytomas. J Neuro Meth 156:154–160

Temel SG, Noyan S, Cavusoqlu I, Kahveci Z (2005) A simple and rapid microwave-assisted hematoxylin and eosin staining method using 1,1,1 trichloroethane as a dewaxing and clearing agent. Biotech Histochem May-Aug 80(3–4):123–132