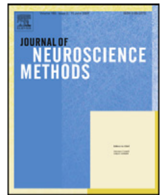
Journal of Neuroscience Methods 182 (2009) 157–164

Contents lists available at ScienceDirect

Journal of Neuroscience Methods

journal homepage: www.elsevier.com/locate/jneumeth



# Advanced laboratory techniques for sample processing and immunolabeling using microwave radiation

Adam M. Ferris[a], Richard T. Giberson[b], Mark A. Sanders[c], Jonathan R. Day[a,*]

[a] Department of Biological Sciences, California State University, Chico, CA 95929-0515, USA
[b] Ted Pella, Inc., 4595 Mountain Lakes Blvd., Redding, CA 96003, USA
[c] Twin Cities Campus, University of Minnesota, St. Paul, MN 55108, USA

A R T I C L E   I N F O

*Article history:*
Received 9 March 2009
Received in revised form 30 May 2009
Accepted 1 June 2009

*Keywords:*
Immunohistochemistry
GFP expression
*HeLa* cells
Formaldehyde fixation
Antibodies
Double labeling
Hippocampus
Cell culture

A B S T R A C T

A better understanding of improved microwave technology has increased the benefits and versatility of the technique as it applies to all aspects of immunohistochemistry. The role of continuous magnetron power output (wattage) combined with precise control of sample heating demonstrated their significance to complex labeling protocols. Here, we present results for microwave-assisted formaldehyde fixation and its effect on GFP expression in transfected *HeLa* cells. Rat brain sections and cultured hippocampal cells were labeled with 11 different primary antibodies using a unified microwave protocol. Microwave-assisted immunohistochemistry made it possible to sequentially label tissues and cells with several primary antibodies in a very short period of time with excellent labeling characteristics.
© 2009 Elsevier B.V. All rights reserved.

## 1. Introduction

Immunolabeling success relies on at least two separate and distinct processes. The first is fixation which is typically either chemical (e.g. formaldehyde, acetone, alcohol, etc.) or freezing. The importance of an adequate fixation in clinical immunohistochemistry is well established (Battifora, 1999; Leong and Wright, 1987; vonWasielewski et al., 1998; Werner et al., 2000). The second is the labeling protocol that follows the fixation step. This process is comprised of numerous steps that include antigen retrieval, blocking to reduce nonspecific antigen–antibody interactions, antibody incubations and signal detection.

Of the chemical fixatives, formaldehyde is considered to be the best choice for immunohistochemistry (Battifora, 1999; vonWasielewski et al., 1994, 1998; Werner et al., 2000; Tubbs et al., 2004). Formaldehyde penetrates tissues rapidly but is slow to stabilize the intracellular matrix (Medawar, 1941; Fox et al., 1985). Historically, high quality fixation required 24 h at room temperature (RT) or 16 h at 37 °C (Lillie, 1948; Fox et al., 1985; Helander, 1994, 1999).

Immunohistochemistry, independent of the fixation step, can require over 24 h for the entire labeling process (Munoz et al., 2004). Microwave-assisted methods have reduced turnaround times for both fixation and immunohistochemistry (Munoz et al., 2004; Galvez et al., 2006). Originally, microwave methods were introduced as a means to shorten the fixation step (Mayers, 1970) and reduce antigen–antibody interaction times by heating the sample (Leong and Milios, 1986). However, over the years the value of increased temperature during fixation and labeling has been questioned. Independent control of the sample temperature and the ability to change the power output of the magnetron during fixation, reduced fixation times to as little as 20 min with no decrease in antigenicity, morphology, or ultrastructure (Galvez et al., 2006). Effective temperature control and variable power output significantly improve immunohistochemical results independent of fixation (Sanders and Gartner, 2001; Sanders, 2002; Munoz et al., 2004).

The present study had two goals: the first was to evaluate the effects of microwave-assisted formaldehyde fixation on cellular morphology and immunohistochemistry. The second was to demonstrate that a unified microwave protocol could be applied to a variety of immunolabeling methods. The first goal examined the effect of microwave-assisted formaldehyde fixation on expression of a tubulin-green fluorescent protein construct in transfected *HeLa* cells. The use of this construct allowed for comparison of cellular morphology between living, immersion fixed, and microwave-assisted fixation treatments. The second aim tested single and multiple immunolabeling protocols using 11 different primary anti-

* Corresponding author. Tel.: +1 530 898 6303; fax: +1 530 898 4363.
*E-mail address:* jday@csuchico.edu (J.R. Day).

0165-0270/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jneumeth.2009.06.002

bodies on free-floating brain sections and adherent neural cell cultures.

Microwave methods for fixation and labeling yielded significant time savings compared to conventional procedures. The benefits of microwave-assisted formaldehyde fixation were improved cellular morphology, enhanced GFP fluorescence, and superior anti-GFP immunohistochemistry. Regardless of the primary antibody or chromophore, the unified microwave protocol developed in this study provided a standardized method with results typical of more protracted non-microwave methods.

## 2. Materials and methods

### 2.1. Transfection of HeLa cells

*HeLa* cells (Invitrogen, Carlesbad, CA) were grown (plated on day 1, transfected on day 2, imaged on day 3) on MatTek® glass bottom dishes (http://www.glass-bottom-dishes.com/) and transfected with Cellular Lights Tubulin-GFP (Invitrogen, Carlesbad, CA) following the manufacturers recommendations. Cellular Lights Tubulin-GFP (tub-GFP) is a fusion of Emerald GFP to the N terminus of beta-tubulin. Prior to observation, living *HeLa* cells were rinsed in their culture dishes with 80 mM PIPES buffer, containing 5 mM EGTA, 2 mM $MgCl_2$, pH 7.2 (PIPES buffer) at 37 °C.

### 2.2. Fixation and immunolabeling of HeLa cells

Coverslips with live adherent *HeLa* cells expressing tub-GFP were rinsed, as noted above, and then fixed with 3% (w/v) paraformaldehyde in PIPES buffer at 37 °C for 30 min. Cells were blocked in buffer, consisting of PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4{\cdot}7H_2O$, 1.4 mM $KH_2PO_4$), pH 7.2, with 5% normal goat serum, 1% glycerol, 0.1% bovine serum albumin (Fraction V; Sigma), 1% fish skin gelatin, and 0.04% sodium azide, for 30 min at RT. Cells were incubated with anti-mouse GFP (mAb 3E6; Invitrogen, Carlesbad, CA) diluted 1:2000 in blocking buffer for 2 h at RT. Coverslips were washed three times for 5 min each in PBS and incubated with Alexa-488 goat anti-mouse IgG (Invitrogen, Carlesbad, CA) diluted 1:800 in blocking buffer for 2 h at RT. Coverslips were rinsed in PBS and slide mounted with Prolong Gold mounting medium (Invitrogen, Carlesbad, CA) containing 0.2 μg/ml 4′,6-diamidino-2-phenylindole (DAPI). Total processing time was approximately 5.5 h.

### 2.3. Microwave fixation and immunolabeling of Hela cells

All steps were preformed in a PELCO Biowave® Pro with SteadyTemp™ (Ted Pella, Inc., Redding, CA). The SteadyTemp™ was used to control the incubation temperature in conjunction with the PELCO ColdSpot® providing a uniform pattern of microwave radiation (Munoz et al., 2004). Microwave power settings are expressed as continuous power in watts (W). Interrupted microwave irradiation cycles were used for primary and secondary antibody incubations. For example 2-1-2 at 150 W denotes that the magnetron was on for 2 min at 150 W, off for 1 min at 0.0 W, and on for 2 min at 150 W.

Coverslips were washed in PIPES buffer at 37 °C. The cells were fixed with 3% (w/v) paraformaldehyde in PIPES buffer for 1 min at 150 W at 37 °C. Cells were treated in blocking buffer for 2-1-2 at 150 W at RT. All incubations and rinses were done at 150 W and RT in the microwave. Coverslips were incubated with mouse anti-GFP diluted 1:2000 in blocking buffer for 5-2-5. Coverslips were washed three times for 30 s each in PBS, and incubated with Alexa-488 goat anti-mouse IgG diluted 1:800 in blocking buffer for 5-2-5. All coverslips were rinsed in PBS three times for 30 s each. Coverslips were slide mounted in Prolong Gold mounting medium containing 0.2 μg/ml DAPI. Total processing time was approximately 0.5 h.

### 2.4. Confocal microscopy

Cells were viewed with a Nikon C1si spectral confocal microscope attached to a Nikon TE3000 motorized inverted microscope (Nikon USA, Melville, NY) using the 488 nm line of the 40 mW Argon laser. Fluorescent images were collected through an emission filter (505–535 nm). DIC images were collected using a 100×, 1.40 na PlanApo oil-immersion objective. Optical sections were collected at 0.15 μm increments and 2D or 3D projections were made using Nikon C1 viewer software (version 3.2). Image stacks were processed with Huygens2 deconvolution software and viewed with Imaris software (version 4.0; Bitplane AG; Zurich, Switzerland). Images were collected from at least two sets of individual experiments.

### 2.5. Free-floating brain sections

Adult Sprague-Dawley derived rats from the California State University, Chico breeding colony were deeply anesthetized with Nembutal (50 mg/kg of body weight). Brains were removed and immersed in 4% (w/v) paraformaldehyde in PBS overnight (18–24 h) at RT. Free-floating brain sections were cut at a thickness of 50 μm in ice cold PBS (pH 7.4) using a Vibratome® 1000 Plus (Ted Pella, Inc., Redding, CA). Prior to staining all tissue sections were stored at 4 C in PBS with 0.1% sodium azide. All procedures involving live animals were approved by the California State University, Chico Institutional Animal Care and Use committee in accordance with federal guidelines.

Preparation of Neuronal Cultures: primary neuron and astrocyte cell dissociations were prepared from the hippocampus of postnatal day 1 rat pups as described by Banker and Goslin (1998). Hippocampal neurons were cultured for 7–9 days and plated at a density of 10,000–12,000 cells/cm$^2$ on 12 mm round poly-D-lysine/laminin coated glass coverslips. This procedure yields a confluent layer of astrocytes on the bottom of the plate that supported the growth of hippocampal neurons on the coverslip.

### 2.6. Immunolabeling of rat neurons

Labeling for bright field microscopy was done with the chromagen 3,3′-diaminobenzidine tetrachloride (DAB; Sigma, St. Louis, MO). All primary antibodies were from Bioscience Research Agents (Temecula, CA). The Vectastain ABC Elite Kit and biotinylated secondary antibodies were from Vector Laboratories (Burlingame, CA; see Table 1). Depletion of endogenous peroxidase activity was done by incubating the sections with 0.3% hydrogen peroxide in water. Blocking prior to primary antibody incubation was done in 1% horse serum, 1% BSA and 0.3% Tween 20 (Sigma, St. Louis, MO) in PBS. All DAB color reactions were done at RT on the bench in a solution containing 0.5 mg/ml DAB, 2.5% ammonium-nickel sulfate, and 3.0 μl of 30% hydrogen peroxide per 20 ml imidazole buffer (175 mM sodium acetate, pH 7.2; 10 mM imidazole, pH 9.2). DAB was added less than 10 min before use and hydrogen peroxide was added immediately prior to use. All sections were subjected to an antigen retrieval step in 10 mM sodium citrate (pH 6.0) for 5 min in the microwave at 550 W (adapted from DeHart et al., 1996). During antigen retrieval the temperature of the solution never exceeded 50 C. Conventional immunolabeling followed the methods of Munoz et al. (2004). Briefly, primary antibody incubations were overnight at 4 C followed by 10–15 min rinses. Secondary antibody and subsequent ABC reagent incubations done were at RT for 60 min with gentle agitation. As outlined in the previous work the process required in excess of 24 h to complete. Antibody dilutions for all protocols are

Download English Version:

https://daneshyari.com/en/article/4335775

Download Persian Version:

https://daneshyari.com/article/4335775

Daneshyari.com