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A simple fluorescent double staining method for distinguishing neuronal from non-neuronal cells in the insect central nervous system

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Abstract

Being able to discriminate between neurons and non-neuronal cells such as glia and tracheal cells has been a major problem in insect neuroscience, because glia-specific antisera are available for only a small number of species such as *Drosophila melanogaster* and *Manduca sexta*. Especially developmental or comparative studies often require an estimate of neuron numbers. Since neuronal and glial cell bodies are in many cases indiscernible in situ, a method to distinguish neurons from non-neuronal cells that works in any given species is wanting. Another application is cell culturing. Cultured cells usually change their outward shape dramatically after being isolated so that it is frequently impossible to tell neurons and glia apart.

Here, we present a simple method that uses a commercially available antiserum directed against horseradish peroxidase, which specifically stains neurons but no other cell type in every insect species investigated. Counterstaining with DAPI, a fluorescent chromophore that binds to double-stranded DNA in the nuclei of all cells, yields the total number of cells in a given sample. Thus, double labeled cells can be identified as neurons, cells that carry only DAPI staining are non-neuronal.

Keywords: Horseradish peroxidase; DAPI; Confocal laser-scanning microscopy; Immunocytochemistry; Double fluorescent staining; Cell culture; Insect brain; Glia

1. Introduction

While morphological, physiological, and other functional properties of neurons in the insect nervous system have been studied extensively during the last decades, the role of insect glia has drawn less attention. This is reflected by the fact that a PubMed-search for "insect neuron" yields more than 10 times more hits than does a search for "insect glia".

Besides such obvious tasks like being part of the blood–brain barrier, providing nourishment for neurons, and helping to regulate the extracellular milieu of the brain (Treherne and Schofield, 1981) glia has been found to play a critical role in neural development. For example, glia cells act as struts for migrating neurons and growing axons, and they form barriers that restrict neuritic growth, thereby defining the boundaries of neuropils (Oland and Tolbert, 2003). Glia has been investigated only in a very limited number of insect species, namely in the fly *Drosophila melanogaster* (Chotard and Salecker, 2004; Parker and Auld, 2004; Edenfeld et al., 2005; Jones, 2005), the moth *Manduca sexta* (Tolbert et al., 2004), and to a lesser extend in the locusts *Schistocerca gregaria* (Bicker et al., 2004), and *Locusta migratoria* (Vanhems, 1985).

The reason why there is not more information on glia structure and abundance in a wider variety of species is in part due to the fact that glia cells in insects are hard to distinguish from neurons especially when they do not form glia-specific structures such as the neurolemma or sheaths surrounding the neuropils (Hahnlein and Bicker, 1996). While there are some markers that specifically recognize insect glia in some species, like repo in *D. melanogaster* and *S. gregaria* (Halter et al., 1995), they usually do not work in another species or only at certain developmental stages, rendering comparative studies of glia distribution almost impossible. Especially in tiny insects or early developmental stages it is furthermore impossible to use the sometimes smaller amount of perinuclear cytoplasm as an indication that one is looking at a glial cell, since here both neurons and glial cells have small cell bodies and do not contain much cytoplasm. In fact, there are no reliable estimates of the ratio between glial

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and neuronal cells to be found in the literature at all. Edenfeld et al. (2005) conclude that "insects are characterized by having only a small number of these [glia] cells". In contrast, Oland and Tolbert have estimated that the antennal lobe of *M. sexta* contains approximately 7000 glial cells but only little more than 1000 neurons (personal communication). Similar problems are encountered in cell cultures of insect neuronal tissue because cultured cells usually change their outward shape dramatically after being isolated (Oland and Oberlander, 1994). Cultured neurons as well as glial cells frequently grow similar protrusions which makes the discrimination of these two cell types problematic.

In this account, we present an affordable and easily applicable method to distinguish neurons from non-neuronal cells such as glia and treacheal cells in situ as well as in culture. By using an antiserum against horseradish peroxidase (HRP), a neuronal cell surface marker, neurons but no other cell type can be recognized specifically (Jan and Jan, 1982). Counterstaining with DAPI, that binds to DNA (Morikawa et al., 1981) in the nuclei of all cells, yields the total number of cells in a given sample. Thus, double labeled cells can be identified as neurons, cells that carry only DAPI staining are non-neuronal.

2. Materials and methods

2.1. Staining chemicals

The anti-HRP serum, raised in rabbits using purified peroxidase from horseradish as immunogen was purchased from Sigma (St. Louis, MO, Product No. P-7899) and used at a dilution of 1:10000. DAPI (4′,6-diamidino-2-phenylindole, dilactate) was also purchased from Sigma (Product No. D-9564) and used at a dilution of 1:1000. DAPI is the classic nuclear counterstain. Its fluorescence emission does not overwhelm signals from green- or red-fluorescent secondary antibodies. When using a confocal laser-scanning microscope the use of DAPI requires a UV laser. Users who do not have access to a UV laser can select from a variety of commercially available fluorescent nuclear markers that can be excited by lasers of a longer wavelength. In such a case, the fluorophore of the secondary antiserum that recognizes the anti-HRP antibodies has to be selected accordingly in order to prevent cross-talk. Access to a confocal laser-scanning microscope, however, is no prerequisite. A conventional microscope equipped with epifluorescence illumination is sufficient for the application of this method.

2.2. Staining procedure

This protocol is applicable to all ganglionic tissue, nerve tissue, and primary cell cultures derived from insects. Thus, details of the dissection procedure or dissociation of neuronal tissue for cell culturing will not be elaborated here but have to be adjusted to the requirements of the individual experiment.

2.2.1. In situ stainings

Supraoesophageal ganglia (brains) of the cockroach *Leucophaea maderae* ($n = 20$) and thoracic ganglia of the locust *L. migratoria* ($n = 4$) were dissected out of the head capsule or thorax, respectively, under fixative. The fixative used was 4% paraformaldehyde (freshly prepared from powder) in phosphate buffered saline (PBS). After dissecting, brains were further fixed over night at room temperature. After rinsing in PBS the tissue was embedded in gelatin/albumin. The gelatin/albumin blocks were fixed in 10% formalin at 8 °C over night. Sections of 50 μm thickness were obtained with a vibratome (VT1000S from Leica Microsystems, Wetzlar, Germany) and collected in 24-well multidishes and rinsed in PBS containing 0.1% Triton X-100 to remove excessive fixative. For reducing nonspecific background staining, sections were incubated in PBS containing 0.5% Triton X-100 and 5% normal swine serum at room temperature over night. The next day, a volume of primary anti-HRP serum that resulted in a final dilution of 1:10000 was directly added to this blocking solution. Sections were incubated for 48 h at room temperature. After rinsing again in PBS containing 0.1% Triton X-100, sections were incubated with secondary antiserum (Cy3-conjugated goat anti-rabbit, Jackson, West Grove, PA) used at a dilution of 1:1000 in PBS containing 0.5% Triton X-100 and 1% normal swine serum over night at room temperature. Then, the secondary antibody solution was removed and the sections were incubated with DAPI at a dilution of 1:1000 in PBS for 12 min. Subsequently, sections were rinsed again in PBS containing 0.1% Triton X-100, and mounted on chrome alum/gelatin-coated glass slides under glass coverslips using Elvanol (mounting medium for fluorescent stainings after Rodriguez and Deinhard, 1960).

2.2.2. Cell culture stainings

The same protocol applied for tissue stainings can be used for paraformaldehyde fixed cell cultures. However, rinsing must be carried out extremely carefully in order to not detach cells from the surface of the culture dish. Since antibody solutions penetrate individual cells or thin cellular layers much faster than tissue sections, incubation times can be reduced. We suggest over night-incubation in primary antiserum, 2–4 h incubation in secondary antiserum, and incubation in DAPI solution for 10 min. The cells for the primary cell cultures shown in this account ($n = 8$) were dissociated from meso- and metathoracic ganglia of adult *L. migratoria* and cultured in modified Leibovitz L-15 medium. Cultures were fixed at an age of 4 days after dissociation. For details of the protocol see Weigel (2005).

2.3. Imaging

Double-labeled preparations were analyzed with a confocal laser-scanning microscope (TCS SP2, Leica Microsystems, Wetzlar, Germany). For imaging of Cy3 fluorescence the helium/neon laser (excitation wavelength 543 nm, detection range 555–700 nm, filter DD 488/543), for imaging of DAPI fluorescence the diode-laser (excitation wavelength 405 nm, detection range 410–550 nm, substrate filtered) was used. To visualize cell ramifications in the cultures, the transmitted light scan mode of the confocal microscope was utilized. Images were further processed with Adobe Photoshop using global imaging enhancement procedures (contrast and brightness).