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Janus Green B as a rapid, vital stain for peripheral nerves and chordotonal organs in insects

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Effective staining of peripheral nerves in live insects is achieved with the vital stain Janus Green B. A working solution of 0.02% Janus Green B in saline is briefly applied to the exposed peripheral nervous system. The stain is then decanted and the dissection flooded with fresh saline, resulting in whole nerves being stained dark blue in contrast to surrounding tissues. This simple and reliable technique is useful in describing the distribution of nerves to their peripheral innervation sites, and in locating small nerve branches for extracellular physiological recordings. The stain is also shown to be useful as a means of enhancing the contrast between scolopale caps and surrounding tissues in chordotonal organs, staining chordotonal organ attachment strands, and the crista acustica (tympanal organ) of crickets and katydids. The advantages of Janus Green B over traditional peripheral nerve stains, in addition to its shortcomings, are discussed.

Introduction

It is frequently desirable for insect neurobiologists to stain peripheral nerves in situ in order to differentiate them from surrounding tissues, such as muscles or tracheae. Peripheral nerve stains may be employed in drawing topographical maps of the nervous system (e.g., Nüesch, 1957; Pipa and Cook, 1959; Eaton, 1974); in describing the innervation of peripheral structures such as muscles, organs, or receptors (e.g., Eggers, 1928; Hustert, 1974; Yack and Fullard, 1990); for preparing demonstration materials used in teaching; or locating small branches for physiological recordings.

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Two methods commonly used for staining peripheral nerves in situ (by collectively staining the nerve's constituent neurones) are the methylene blue and cobalt chloride techniques. Although methylene blue offers beautiful results when successful, it is notorious for being difficult and unpredictable, with success rates of about 20% (Pantin, 1964; Plotnikova and Nevmyvaka, 1980). Axonal filling with cobalt chloride is also highly successful in tracing individual neurones to their peripheral targets but, again, is known to be time consuming and fickle (Altman and Tyrer, 1980).

Here I describe how the vital stain Janus Green B (JG-B), is effectively used as a simple and rapid technique for staining whole peripheral nerves in insects. Observations concerning its usefulness for a number of other applications, including the staining of chordotonal organ strands and scolopale caps, cricket and katydid tympanal organs, and identifying small nerve branches for

extracellular physiological recordings are also discussed.

Materials and methods

Animals used in this study for representative photographs include two species of Lepidoptera (*Manduca sexta* L. (Sphingidae); *Actias luna* L. (Saturniidae)) and one species of Orthoptera (*Cyphoderris monstrosa* Uhler (Haglidae)). Peripheral nerves and/or chordotonal organs of several other orthopteran, lepidopteran, dictyopteran, and neuropteran species were also examined.

All preparations were performed using a 0.02% solution of JG-B (Sigma Chemical Co.) prepared in insect saline (150 mM NaCl; 3 mM KCl; 4.9 mM MgCl; 1.5 mM NaH_2PO_4; 0.6 mM $NaHCO_3$, pH 7.4). This solution could be used immediately following its preparation, or stored in the refrigerator for several months. During a dissection both the stain and saline solutions were kept cool.

To stain peripheral nerves and/or chordotonal organ strands, a few drops of JG-B solution were applied (with a 1 ml syringe) to the exposed tissue for a short duration (5–60 s), and then rinsed with saline. When employed in identifying nerves for extracellular recordings (see Yack and Fullard, 1990; Yack, 1992 for details of recording procedure), the stain was applied only once. When used for dissection purposes, the tissue was pinned to Sylgard (Dow Corning, Midland, MI) in a petri dish, and the stain reapplied during the course of the dissection as unstained nerves were exposed, or adequate contrast between the stained branches and surrounding tissues was achieved.

Differentiation of chordotonal organ scolopale caps was carried out by first placing the unstained organ in a few drops of saline on a microscope

Fig. 1. In vivo dissection (viewed with incident light) of a peripheral nerve branch (white arrow) and chordotonal organ strand (black arrow) at the wing-hinge of a moth (*Manduca sexta*) before (a), and after (b) applying JG-B. Sc, scutum of the metathorax; M, muscle; Tr, tracheal tissue. Scale bars = 600 μm.

slide. Several drops of JG-B were then applied to one end of the cover glass, and the stain drawn through the saline by placing a rolled piece of tissue at the other end. The stain remained in place until a desirable amount of differentiation was achieved between the scolopale caps and surrounding tissues (20–60 s). The JG-B was then replaced with saline in a similar manner, and the scolopale caps, were examined and photographed.

Dissection of the katydid tympanal organ (crista acustica) was carried out by first removing the foreleg from the animal and pinning it to Sylgard in a petri dish. The tympanal membrane and surrounding cuticle was then carefully peeled away and 1 or 2 drops of the stain were applied for 20–30 s, then rinsed away with saline. The stained nerve and cap cells of the crista acustica could then be observed and photographed.

Results

Within 60 s of applying the JG-B to fresh insect tissue, peripheral nerves were sufficiently differentiated from surrounding tissue to easily locate small branches for extracellular recordings. Recordings from lightly stained branches showed no obvious differences from those of unstained nerves, and were often held for periods of 6 h or longer.

When used for dissection purposes, the JG-B would generally fade after about 30 min, and then the stain was simply reapplied in order to renew contrast. The effectiveness of the stain was reduced with the age of the preparation: after several hours the nerves stained blue initially, but quickly turned a pink colour and faded. However, if the dissecting bath was changed periodically (every 5–10 min) with saline, the life of the dis-

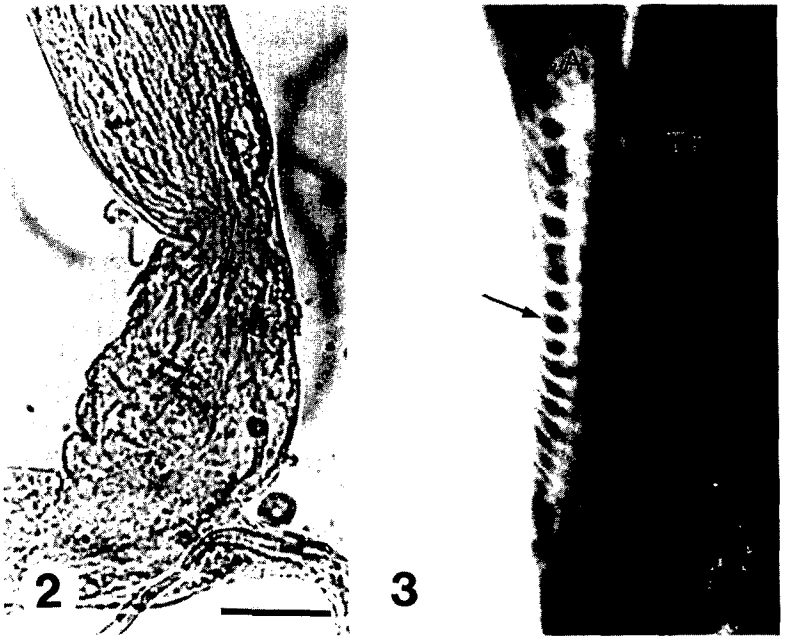

Fig. 2. Wholemount preparation of the JG-B-stained wing-hinge chordotonal organ in a moth (*Actias luna*) viewed with a compound microscope. An arrow points to 1 of the 3 differentiated scolopale caps. Scale bar = 30 μm. Fig. 3. The foreleg tympanal organ of an adult katydid (*Cyphoderris monstrosa*) stained with JG-B, and viewed with a dissection microscope (transmitted light). The crista acustica (CA), comprised of numerous scolopidia, extends along the anterior tracheal air sac (Tr). The attachment cells (an arrow points to one) of the scolopidia are differentiated from surrounding structures. Scale bar = 200 μm.

section could be extended. The chordotonal organ strand appeared to take up the stain more readily than did the peripheral nerves, and would remain a dark blue colour for long periods (1 h or more). Fig. 1 illustrates a JG-B stained nerve branch and chordotonal strand in a moth.

Care was taken not to expose the preparation to JG-B for long periods, since this led to non-specific staining of other tissues, particularly the cut ends of muscles. Therefore, when the stain was used for lengthy dissections, such as for the purpose of preparing anatomical drawings, it was important to make sketches during the course of the dissection as nerve branches covered by overlying tissues were exposed. JG-B proved to be equally reliable as a peripheral nerve stain in all species examined.

Fig. 2 demonstrates the differentiation of scolopale caps in the wing-hinge chordotonal organ of *A. luna*. When lightly stained, scolopale caps appear dark blue in contrast to surrounding structures. It was necessary to begin with a small amount of stain and watch the organ under the microscope until adequate differentiation occurred, since too much stain would darken the sheath surrounding the organ and obscure the scolopale caps within.

When the stain was applied to the opened foreleg of the cricket or katydid, the crista acustica could be seen clearly under the dissection microscope as a neat row of darkly stained units (Fig. 3). Upon inspection of this structure with higher magnification, it was apparent that the nuclei of the attachment cells were the structures taking up the stain.

Discussion

When used for the purpose of describing or identifying peripheral nerves in insects, JG-B has the advantage of being extremely easy to prepare and employ, resulting in effective differentiation of whole nerves from surrounding tissues. The method is especially useful in situations where small branches are obscured by surrounding tissues such as muscles or tracheae, as the stain may be reapplied repeatedly during the course of the dissection as branches are exposed. Because JG-B is used on fresh tissue, nerves retain their elasticity, and do not become brittle as is often the case with fixed tissue. The stain is also very predictable, as it has worked successfully as a peripheral nerve stain in all species examined to date. Perhaps its principle disadvantage as a method for anatomical description, is that it stains the entire nerve rather than individual neurones. Therefore details concerning the number of axons, or types of sensory cell bodies in a nerve, cannot be determined accurately with the JG-B method.

As a vital stain, JG-B is useful in isolating small branches for extracellular physiological recordings (Miller, 1979). Although I have found brief exposures of the stain to have no harmful effects on recordings, others (P. Faure, T.A. Miller (personal communication)) have suspected the dye to be somewhat hard on the tissue and, although classified as a vital stain (one of the conditions being harmlessness), it has been suggested to be one of the more toxic of the vital dyes (Baker, 1958). Therefore, for live nerve preparations, it is recommended that the stain be used sparingly to assist the researcher in identifying the proper nerve branches, and that eventually the investigator 'weans' him or herself of the dye after the preparation becomes familiar.

The stain's effectiveness in differentiating chordotonal organ strands is useful for both locating and photographing these structures. In addition, the technique is potentially useful in observing chordotonal organs in live, active preparations. For example, movements of the stained moth wing-hinge chordotonal organ (Fig. 1) have been observed during stationary flight. Since the mechanical processes leading to sensory transduction in chordotonal organs are not clearly understood (e.g., Wolfrum, 1990), such observations could lead to important insights into these processes.

The material component of the nerve being stained with JG-B is not completely understood. JG-B has been traditionally used as a vital stain for mitochondria (Lazarow and Cooperstein, 1953a). However, these authors suggest that the stain is not just taken up by mitochondria, but by

diverse proteins, and that the mitochondrial activities are able to maintain the oxidized state (blue form) the longest. In the present study, it appears that the stain is taken up by the sheath surrounding the peripheral nerve or chordotonal organ. The sheath in both is known to consist of a non-cellular neural lamella within which is embedded collagen fibrils (Smith, 1968; Moulins, 1976; Yack and Roots, 1992). It is possible that the collagen fibrils are taking up the JG-B stain (Lazarow and Cooperstein, 1953b) and the oxidized form of the stain is maintained due to its proximity to the periphery.

In this study JG-B has also been shown to be effective in differentiating scolopale caps from surrounding tissues. This is helpful when photographing or counting the caps in a chordotonal organ, circumventing more involved histological procedures that may be used for this purpose (e.g., Young, 1970; Yager and Hoy, 1987). The technique is most useful for examining smaller chordotonal organs, with fewer than 50 scolopidia, since the density of the surrounding tissues in larger organs tends to obscure the visibility of the caps. Again, it is not known what material is being stained, since the composition of the scolopale cap is unknown (see Yack and Roots, 1992). Since the cap material is of high density, the stain may be simply enhancing the contrast between the cap and surrounding structures of lower density.

Finally, JG-B has been shown useful in differentiating the attachment cell nuclei of the crista acustica in crickets and katydids. This is useful as a simple means of counting the number of scolopidia in a tympanal organ (there being one attachment cell per scolopidium in these structures), and for photographing the crista acustica. Also, when used in low concentrations, the stained attachment cells could provide orientation for intracellular recordings from the attachment cells themselves, or adjacent neural elements (e.g., Oldfield and Hill, 1986). JG-B has been reported to stain nuclei in other insect cells (Chambers, 1915), but again, the reason for the stain's specificity is not clear.

In conclusion, JG-B offers a simple and effective means of staining whole peripheral nerves in living insect tissue. When used carefully, it can be useful in following nerves to their peripheral innervation sites, in isolating branches for extracellular physiological recordings, and in the description of chordotonal organs.

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