STAINING OF THE CRAB LEG JOINT RECEPTORS

By

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1. Purpose

To learn histological techniques for staining nerve cells to aid in describing the general organization of a chordotonal organ.

2. Preparation

The chordotonal organs in the joints of walking legs of the blue crab *Callinectes sapidus* or the Dungeness crab *Cancer magister* will be employed. Other large *Cancer* species are also suitable.

3. Introduction

The walking leg of a crab has six joints, each having a means of detecting joint proprioception (**Figure 1**). The proprioceptive organs that monitor joint position and movement in crustaceans are called chordotonal organs. Using Alexandrowiz's (1967) designation, these receptors are named according to which joint they are monitoring (i.e., "PD-organ" is the organ between the propodite (P) and the dactylopodite (D)). Joint receptors consist of an elastic strand into which are inserted the dendrites of neurons. These neurons signal joint movement, direction of movement, and static position (Wiersma, 1959). Analysis of individual cells in the chordotonal organ spanning the propodite-dactylopodite joint (PD) revealed an orderly arrangement of these neurons along the elastic strands according to function

(Hartman and Boettiger, 1967) as well as in other chordotonal organs (Cooper and Hartman, 1993; Cooper, 2008).

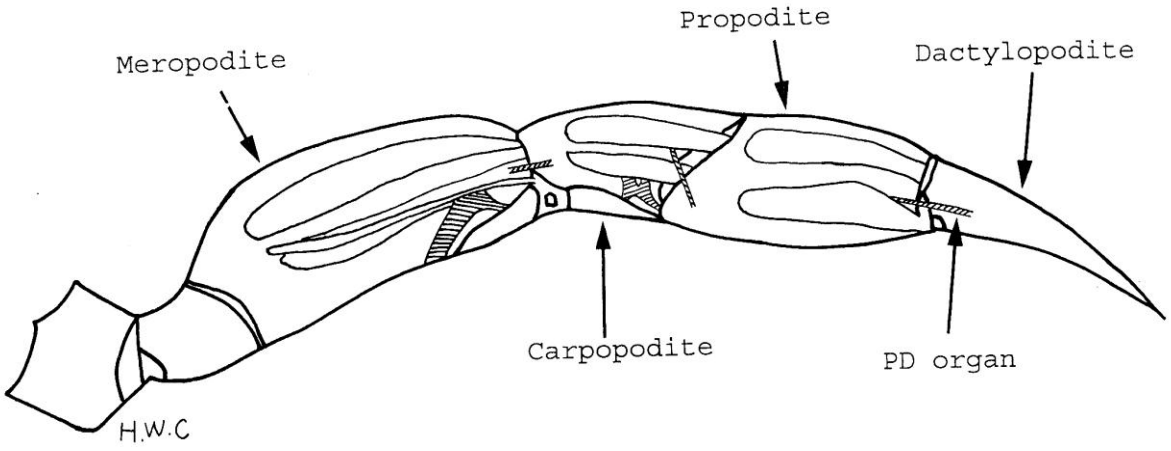

Figure 1: First walking leg of a crab with the tendon shown as an X-ray. The chordotonal organs are the hatched regions.

The anatomical arrangement of chordotonal organs in crabs allows one to analyze each individual neuron according to function (**Figure 2**). In addition, developmental questions can be addressed as the animal grows or when the animal regenerates a limb (Cooper, 2008; Cooper and Govind, 1991; Hartman and Cooper, 1994).

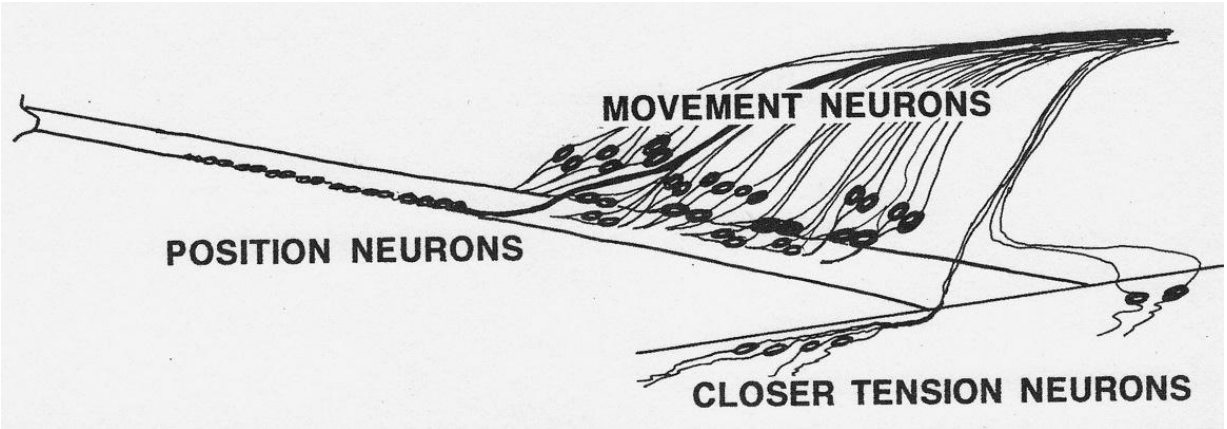

Figure 2: The functional arrangement of the sensory neurons on the PD organ and the tension receptors associated with the closer muscle.

4. Methods

4.1 Materials

1. Crab Saline (see Tables 1 & 2)
2. Methylene blue: This is made of crayfish saline at a concentration of 0.25%
3. Cobalt chloride (300 mM)
4. 4-di-2-ASP or Lucifer yellow stain (10 µM).
5. Sylgard coated dishes (Dow Corning, SYLGARD® 184 silicone elastomer kit; Dow Corning Corporation, Midland, MI. USA)
6. Dissecting tools
7. Insect pins
8. Glass rod/tools for dissecting and manipulating nerves
9. Pipettes and beakers
10. Petroleum jelly (clear)

4.2 Setup

1. The microscope, high intensity illuminator, and the saline bath is needed

2. Setup the microscope in a position where it is overlooking the microscope stage.

3. Position the high intensity illuminator in a convenient position.

4. Place the previously prepared crab saline in the Sylgard dish and position the dish on the stage of the microscope. Staple the dissected leg in the dish.

4.3 Dissection

Walking legs of the swimming crab *Callinectes sapidus* caught in the Gulf of Mexico (http://www.crabplace.com/crabs.asp) or from *Cancer magister*, found off the northwest coast of the USA, will be used. If the crab is dropped, its carapace will crack and the animal will bleed to death. Therefore, handle them carefully.

Using a net obtain a crab from the aquarium and place into an empty bucket. While holding the crab with the net or large tongs across the carapace from behind, and avoiding the claws, cut across the merus of the second-right walking leg with a stout pair of scissors. The animal will/should autotomize the remaining basal portion of that limb thus sealing the wound and preventing blood loss. If the crab does not break it off, help it by inserting the scissor tips into the wound and twisting the stump. Return

the animal to its aquarium. Put the leg in the Sylgard-lined dissecting dish and cover it with the species correct cooled (12-19^{o}C) crab saline.

With scissors, make a cut between the propus and carpus. Discard the carpus and the attached merus. Use **Figure 3** to guide you through the rest of this dissection. Cut a large window in the cuticle on the pigmented (anterior) side of the propus with a scalpel with a #11 blade. (Note: Do not cut deeply). Remove the cuticle layer by sliding the scalpel blade beneath and parallel to the cuticle. This severs the opener and closer muscle fibers attached to the cuticle. Using the same technique cut a smaller window on the pigmentless (posterior) side of the propus, but leaving the condyle attachment intact.

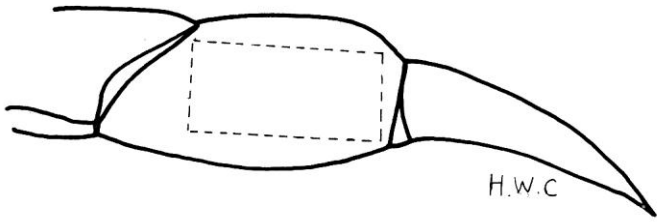

Figure 3: Cut along the dotted line on the propodite

You must exchange the saline in the bath with fresh cold saline throughout the dissection so that the neurons stay alive. Pin the preparation to the recording dish in the locations illustrated using stainless-steel staples with the pigmented side facing upwards. For further dissection, place the preparation dish under a dissecting microscope and use fiber-optic illumination and transmitted light through the base of the dissection platform.

Using sharp-pointed intermediate-size scissors carefully cut the opener tendon from its attachment to the dactyl. Be very careful not to disturb the main leg nerve which should be clearly visible, remove and discard the opener muscle and tendon. This will require minimal cutting and trimming.

Locate the PD organ by careful probing with the glass needles. The elastic strand spanning the joint has a silver appearance. Ask for help if you cannot find it. Now pin the main leg nerve so that the PD organ nerve is exposed (see **Figure 4**). The fine dissection that follows requires iris scissors. They are only used to cut small soft tissue. You will receive guidance with this part of the dissection because it is not

initially easy. Remove muscle fibers that obscure your view of the organ and its nerve from both sides of the tendon being very careful not to injure the PD organ or its nerve. Once this has been accomplished, firmly re-attach the preparation to the dish with the pigment side facing you.

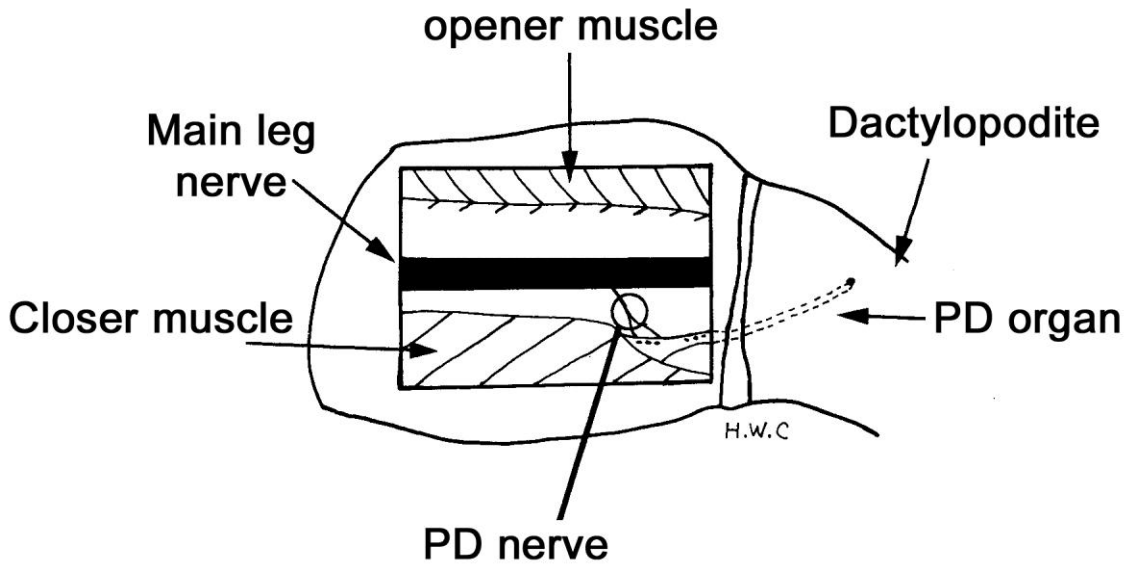

Figure 4: Exposed PD organ and nerve.

Using a fragment of a plastic millimeter ruler, measure and record in your notebook the length of the elastic strand when the dactyl is in the opened and closed positions. Use the attachment points on the protuberance and the tendon as reference markers.

Trace the PD organ nerve in the propus as far proximally as possible in order to free-up a long length of nerve (1.5 cm) for recording purposes. This is best done while the PD nerve is still attached to the main leg nerve for a cm or so since leaving the nerve attached to the main leg nerve adds support. After separating the PD nerve from the main leg nerve with the aid of glass needles, sever the PD nerve proximally with the iris scissors. (Note: Do not stretch or pull on the nerve during the dissection).

After finding the PD nerve the rest of the preparation not required can be cut way. The opener muscle and the carpopodite can be removed. The distal half of the dactylopodite can also be removed. Carefully remove the proximal half of the closer muscle and apodeme as well as the ventral part of the propodite cuticle. One should

have gone through a series of steps as shown in **Figure 5**.

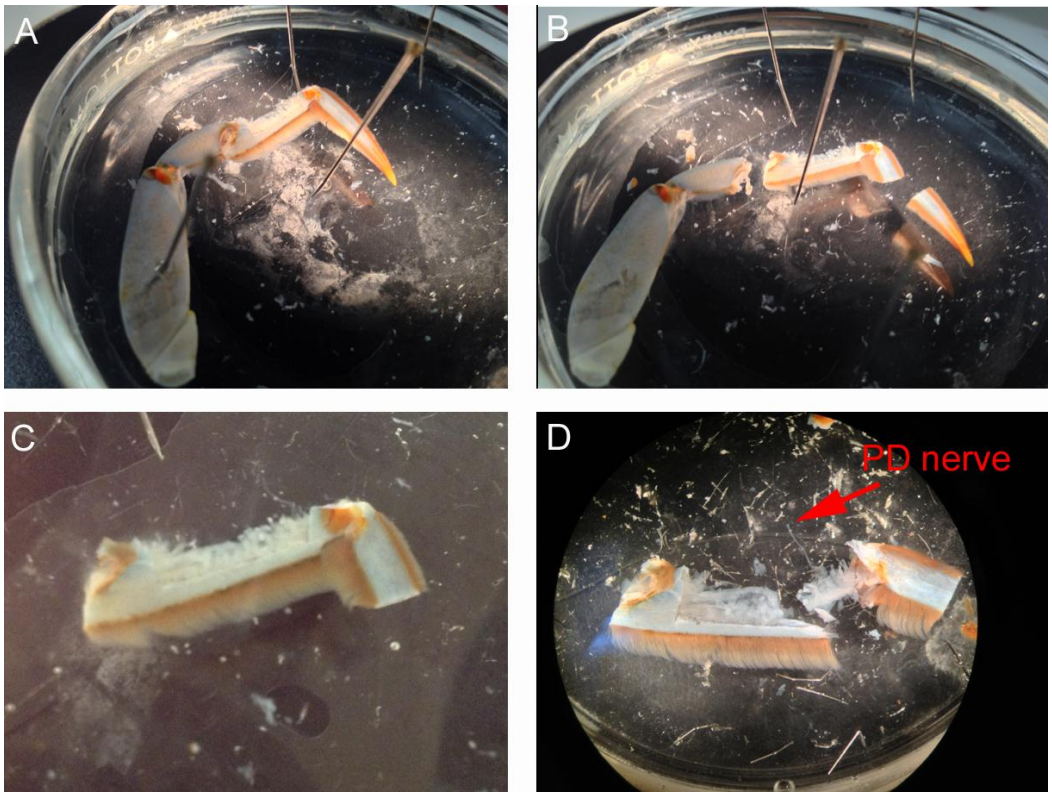

Figure 5: Trimming the closer apodeme to the expose the PD organ and nerve.

4.4 Staining

4.5.1 Methylene Blue:

This technique was explained in the physiology protocol for the PD organ. Refer to this earlier text for details.

4.5.2 Cobalt back-fill of the PD nerve:

Using the *in situ* preparation, back-fill the entire PD organ nerve according to the following procedure. A one-day back-fill in the incubator at 13 (^{o}C) is suggested.

Expose the nerve. Keep it immersed in cold saline that is refreshed every 10-15 minutes during the operation and in all the steps to follow except where noted. A **petroleum jelly** well is required to be made to hold the $CoCl_2$. If any $CoCl_2$ spills into the saline bath the entire preparation will stain black, and the preparation should be discarded. One needs to slip a small cut of a polystyrene sheet, to make a plastic platform, and pin it in such a manner that it will not float away or become immersed in the saline bath (**Figure 6**).

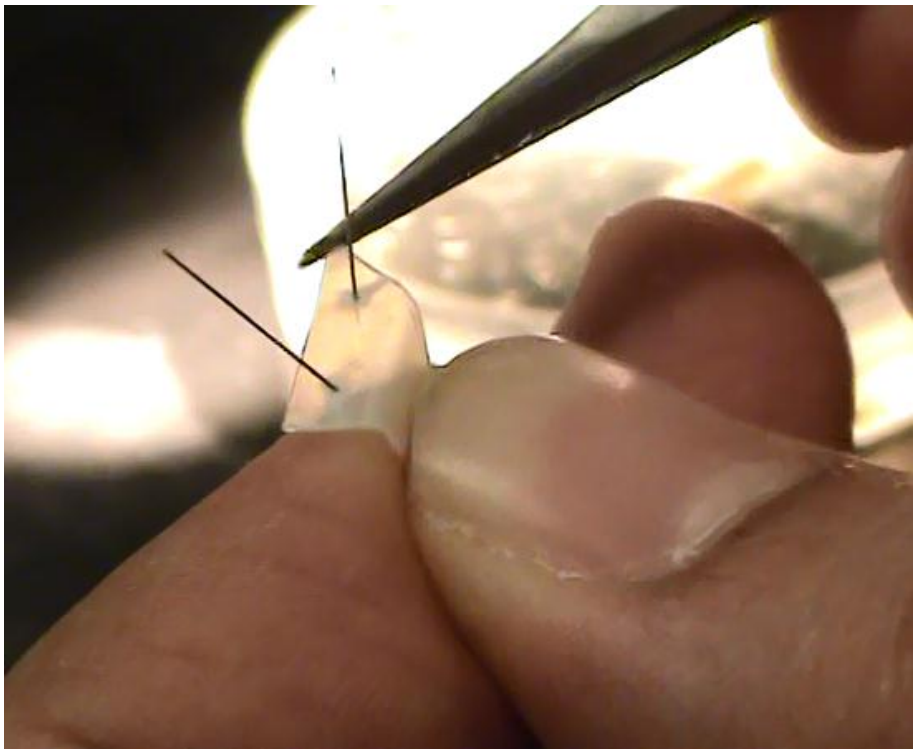

Figure 6: Polystyrene sheet with pins to hold in place in dish.

Ejecting petroleum jelly or silicone stopcock grease from a fine hypodermic needle fastened to a disposable syringe, make a barrier (a circle might work well) on top of the slip of polystyrene. The barrier should be about 1-1.5 mm high except for a shallow "V" at midpoint where the nerve will be draped across. Make a small puddle of saline on either side of the barrier near the "V". Taking care not to stretch or pinch the nerve. Lift the nerve carefully from the dish and place it in the saline puddle in the

petroleum jelly well. Working quickly so that the section of nerve does not dry out, carefully eject petroleum jelly or stopcock grease to cover the exposed nerve. Now test the barrier with saline and make sure it is not leaking. To assure yourself that the two sides are isolated from one another, blot away saline on the inside of the barrier and see if it fills up when the bath saline is high around the wall of petroleum jelly. If saline does not leak across in a few seconds, the barrier is probably sealed (**Figure 7**).

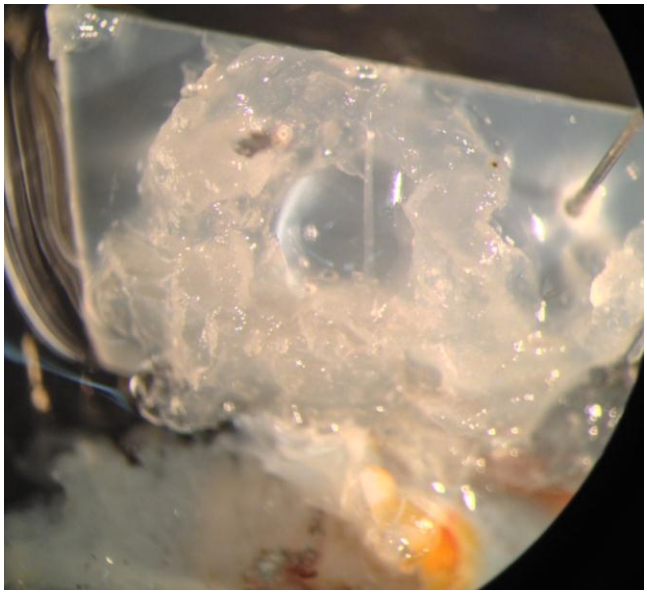

Figure 7: Petroleum jelly well with saline and PD nerve spanning the well

Using the rolled up point of a piece of tissue paper, blot up the saline in the well without the paper wrapping up the nerve. Make a new puddle using a few small drops of distilled water, and then cut the nerve end. Be very careful not to pull the nerve cord through the barrier when making the cut. The osmotic shock of the distilled water will "balloon" the axons of the connectives. Within 30 seconds add a small drop of $CoCl_2$ to the water, blot up this solution, and then add enough $CoCl_2$ to form a puddle over this shortened section of nerve (**Figure 8**). Preparations are best kept refrigerated at 4^oC for 12-24 hrs

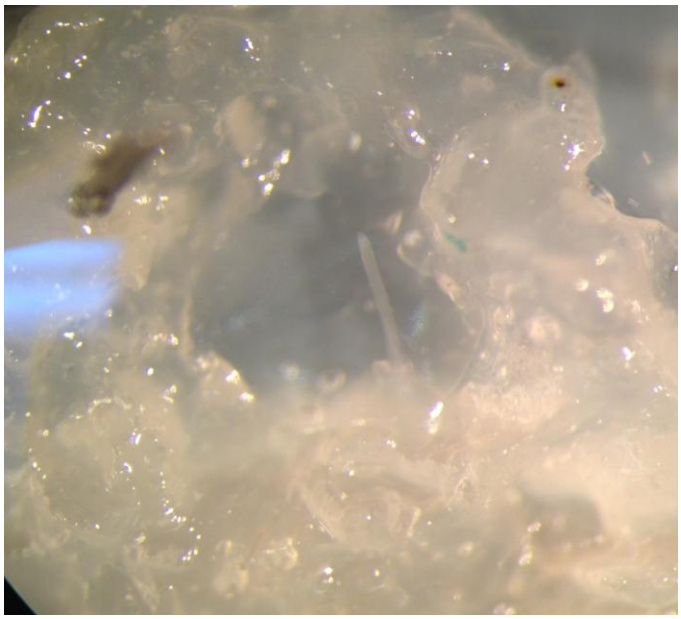

Figure 8: The cut PD nerve being exposed to $CoCl_2$.

The rest of the procedure involves precipitation of $CoCl_2$ which has been taken up by the axons and transported to neural processes and cell bodies. Remove the humidifying puddles. Blot away the cobalt solution using a tissue and wash away the remnants of cobalt with several changes of saline. Transfer the isolated nerve cord to a small glass Petri dish containing about 10 ml of saline. The neurons are washed and the following steps are done in situ. Good metal tools are not to be used to handle the preparation after this step (you should use specific tools for this). Add 1-2 drops of ammonium sulfide $(NH_4)_2S$ to the saline. Cap the bottle tightly and place back into the hood. Observe the reaction in the preparation under a dissecting microscope (**Figure 9**).

Within a minute or two, cobalt filled neurons and their processes should begin to appear. After 5-10 min, replace the development solution with fresh saline. Make certain that the development solution you have poured into the sink drain is followed by running tap water for a few minutes.

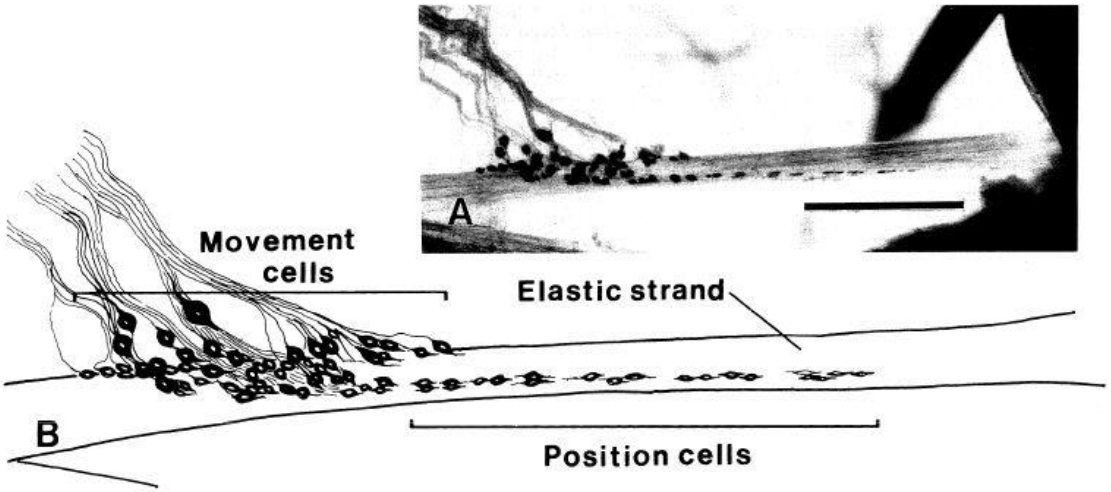

Figure 9: Neurons that were filled with $CoCl_2$ and processed (A). Traced outline of the stained preparation shown (B).

Pour out the saline and fix the nerve preparation for about 15 minutes with two changes of Bouin's solution fixative (Sigma). For larger tissues, increase the duration of fixation. Dehydrate in an ascending order of ethanol concentration beginning at 70% (i.e. 70%, 80%, 90%, 100%). About 10 minutes at each concentration is sufficient for small tissues. After about 10-15 minutes in two changes in 100 % alcohol clear the tissue by replacing alcohol with methyl salicylate. The preparation will stay in this solution permanently for repeated viewing. With time the filled cells will be come more apparent because the surrounding tissue will become clearer.

4.5.3. 4-di-2-ASP or Lucifer yellow stain:

Fluorescent dyes can also be used to back-fill the PD neuron; however a microscope with abilities to view the fluorescent stain is required.

Use the same approach as described above for cobalt filling in making a *petroleum jelly well* to contain the dye and the nerve of interest. In the well place a 0.01 to 0.04 mM 4-Di-2-ASP solution or a 0.02 mM Lucifer yellow solution and leave the preparation in the refrigerator for 12 to 24 hours. These fluorescent dyes have the

advantage over the cobalt dye, in that the amount of filling can be assessed at various stages of filling, although, these fluorescent dyes do fade relatively quickly. So photograph the preparations quickly and avoid over exposure to the mercury light (**Figure 10**). If one has a high enough magnification the sensory endings can be seen inside of the supportive scolopales (Whitear, 1962,1965; Hartman and Cooper, 1994; **Figure 11-13**).

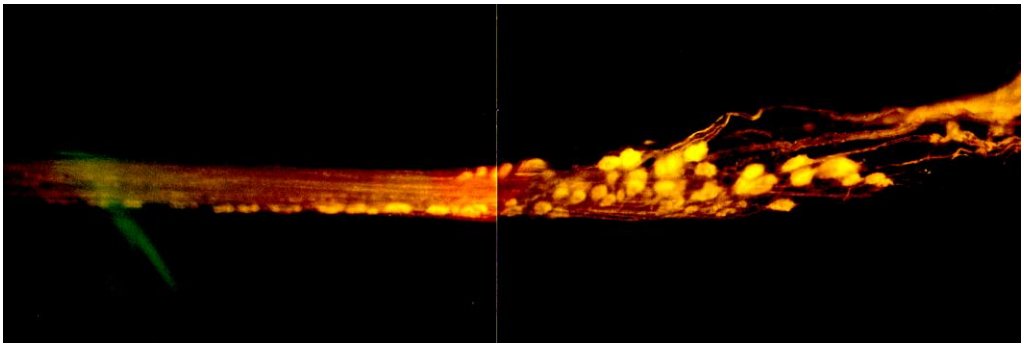

Figure 10: A back-fill of the PD nerve in Cancer magister with 4-Di-2-ASP.

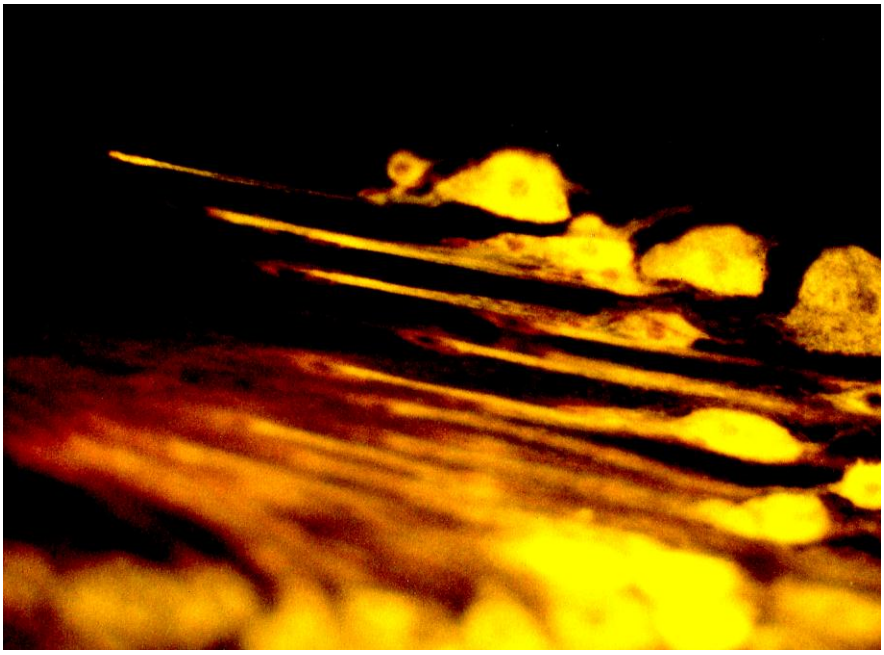

Figure 11: Higher magnification of neurons shown in Figure 14. The PD nerve in Cancer magister was back filled with 4-Di-2-ASP.

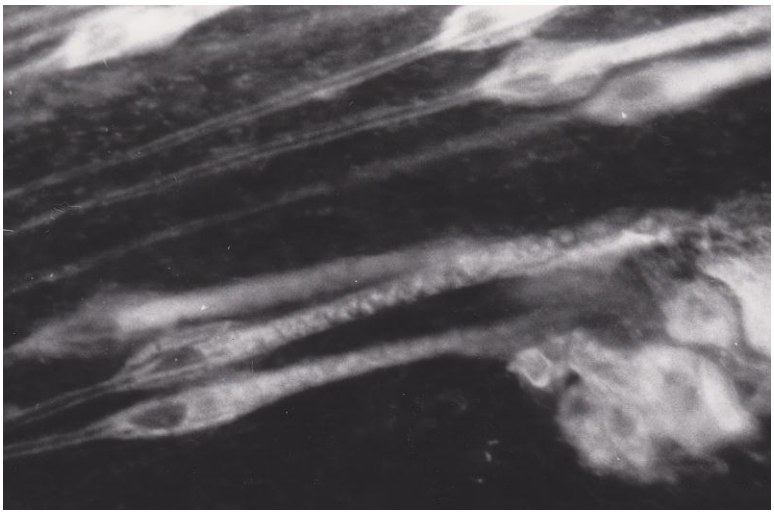

Figure 12: Movement sensitive sensory endings in the PD organ of Cancer magister.

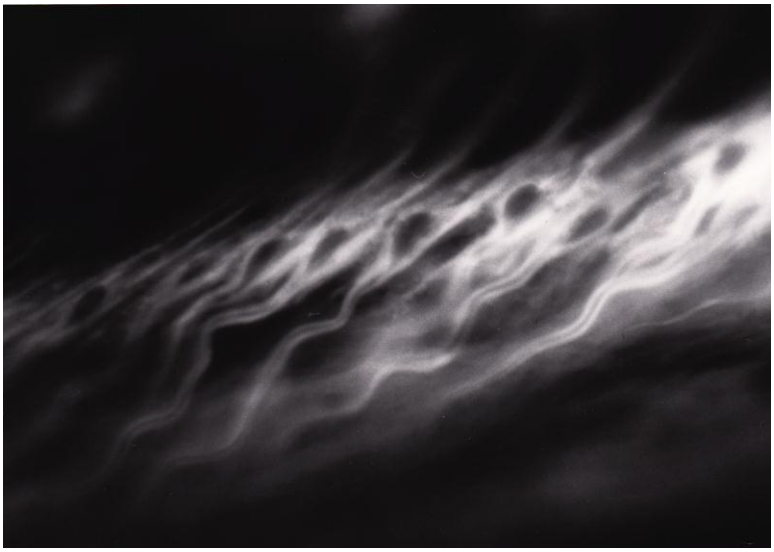

Figure 13: Sensory endings of position cells in the distal end of the PD organ in Cancer magister.

5. Discussion

The anatomical arrangement of the neurons based on function and the size of the soma are similar in the various chordotonal organs within the crab legs. One might ask themselves is it also similar in other crustacean species and in insects? Combining physiological recordings from single cells and mapping the location allows

direct structure function relationships. The large somata located proximally on the strand tend to belong to the dynamic movement sensitive neurons; the neurons signaling static positions by firing tonically have small somata and are located distally (**Figure 9**).

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Table 1: *Solution for C. sapidus saline (Blundon 1989, J. Comp. Physiol B 158: 689-696)*

SALT	**g/l**	**g/2l**	**g/3l**
NaCl	27.47	54.94	82.41
KCl	0.59	1.18	1.77
$MgCl_2$ $6H_20$	1.421	2.842	4.269
$CaCl_2$ $2H_20$	2.205	4.41	6.615
Dextrose	1.982	3.964	5.945
HEPES acid (5mM)	1.19	2.38	3.57
HEPES salt (5mM)	1.30	2.60	3.90

Adjust to pH 7.5 with NaOH or HCl.
With an osmotic pressure of 1000 mm/l.

Table 2*: Solution for C. magister saline (Macmillan & Dando, 1972; Hartman and Cooper, 1994)*

SALT	**g/l**
NaCl	27.29
KCl	0.81
$MgSO_4$ $7H_20$	4.81
$CaCl_2$ $2H_20$	1.85
Na_2SO_4 $10H_20$	0.97
Dextrose	1.982
HEPES acid	0.476
HEPES salt	2.08

Adjust to pH 8.1 with NaOH or HCl.