Optical monitoring of activity of many neurons in invertebrate ganglia during behaviors

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Summary. Optical methods for monitoring neuron activity were developed because these methods lend themselves to simultaneous multiple-site measurements. With the use of new voltage-sensitive dyes, the dye-related pharmacology and photodynamic damage appear to be relatively unimportant. Using multiple-site measurements made with a 124-element photodiode array, we estimated that approximately 30 of the 200 neurons present in the *Navanax* buccal ganglion make action potentials during feeding and that approximately 300 of the 1100 neurons present in the *Aplysia* abdominal ganglion are active during the gill-withdrawal reflex. The fact that a light mechanical touch to the siphon skin activated such a large number of neurons in the abdominal ganglion suggests that understanding the neuronal basis of the gill-withdrawal reflex and its behavioral plasticity may be forbiddingly difficult.

Key words. Voltage-sensitive dye; abdominal ganglion; buccal ganglion; neuronal activity; gill-withdrawal reflex; *Aplysia; Navanax.*

Introduction

Neurobiologists study the activity of individual neurons with the long-range goal of describing the neuronal activity which underlies behavior and understanding how changes in the activity and interactions of neurons can account for simple forms of behavioral plasticity. The use of microelectrodes to monitor activity is limited in that one can observe single cell activity in only as many cells as one can impale with electrodes. In the first attempt to use optical methods [14], we were fortunate to be able to monitor activity in a single leech neuron. Now, however, optical methods for monitoring membrane potential are available in which the activity of hundreds of individual neurons may be recorded simultaneously [9, 10, 18]. Preparations of opisthobranch molluscs were used because these animals have central nervous systems with relatively few, relatively large neurons [1].

In this article we will describe optical monitoring techniques for recording activity of neurons in invertebrate ganglia. We also discuss the extent of the pharmacological and photodynamic damage caused by the dyes, and analyze some of the problems associated with light scattering by the preparation. Finally, we will show some results of multi-unit monitoring during behavior. Several reviews describing optical recording have appeared [3–5, 13].

In general, the optical monitoring technique is based on a system for monitoring changes in the absorption or fluorescence of a dye that has stained a piece of nervous tissue. In recordings from intact, stained invertebrate ganglia, the signal-to-noise ratio in absorption measurements is larger than in fluorescence measurements [19]; therefore, all of the signals that we describe are from measurements of absorption. The ganglion is placed in a recording chamber which is positioned on the stage of a microscope (fig. 1). A long working distance objective forms a magnified real image of the ganglion at the objective image plane where we have positioned one or more photodiodes. In the presently used 12×12 array, each of the 1.4×1.4 mm^2 active elements of the array is separated by 0.1 mm insulating regions. The output of each photodiode is amplified independently; the signals are high-frequency filtered and the amplifiers are AC coupled, so that only changes in intensity are recorded. The amplifier outputs are multiplexed and passed to an analog-to-digital converter and subsequently stored and analyzed in a computer.

Figure 2 compares the absorption signals and microelectrode recordings from a large neuron in a barnacle supraesophageal ganglion. The changes in absorption mirrored sub- and suprathreshold events recorded with the microelectrode. On the left, a small sub-threshold membrane potential change as a result of injection of depolarizing current is recorded electrically (bottom trace). A small change in the light absorption (top traces) of the cell is recorded simultaneously. When the cell is stimulated (shown on the right) to give an action potential the light trace also reflects this activity. The electrode artifacts that appear at the beginning and end of the current step do not occur on the optical recording.

Pharmacological and photodynamic effects of the dye

Pharmacological effects, photodynamic damage and bleaching have been examined in three invertebrate preparations. In the isolated ocellus-supraesophageal ganglion preparation from the barnacle a decrease in the illumination on the ocellus elicits an increase in spike activity monitored by suction electrode recordings from peripheral nerves. This response is referred to as the off-response. By screening several dyes at several concentrations it was possible to find staining conditions (e.g. 1 mg/ml NK2367 for 20 min) that did not affect the off-response, but did give relatively large optical signals, suggesting that the pharmacologic effects of the dyes could be controlled [6]. It was noted that the off-response of the preparation did decline after a long recording session

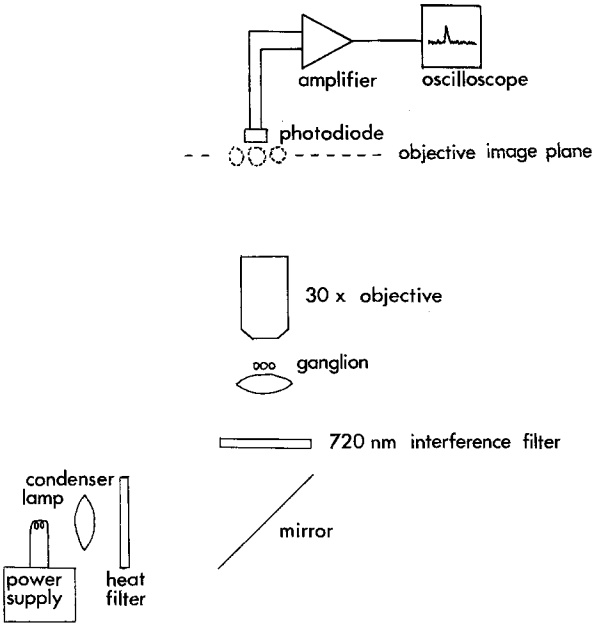


Figure 1. Schematic diagram of apparatus used for monitoring absorption changes in ganglia. The image of the ganglion appears at the objective image plane, the plane at which photodiodes were also situated. A DC power supply is used for the 12 V, 100-W tungsten-halogen filament lamp. The light is heat-filtered and made quasi-monochromatic with an interference filter. The peak transmission wavelength and width-at-half-height of the interference filter was chosen to optimize the signal-to-noise ratio. The output of the photodiode was fed into a current-to-voltage converter, amplified, and displayed.

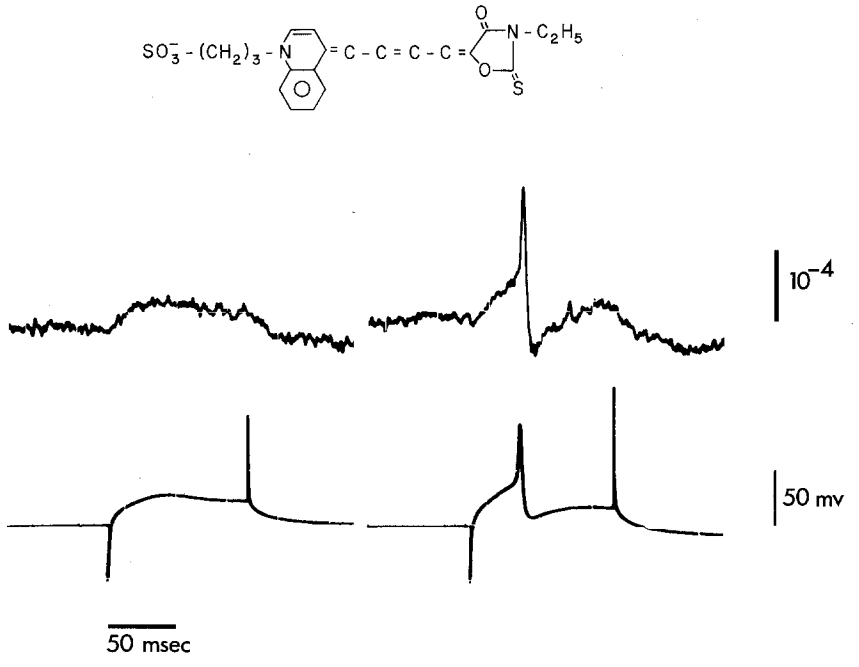


Figure 2. Comparison of simultaneous changes in light absorption at 720 nm with membrane potential changes in a stained barnacle neuron. These signals were recorded in single trials. For both sub-threshold and action potential activity, the optical signals (top traces) have a shape that is essentially identical to the electrode signals (bottom traces) ignoring the stimulation artifacts on the electrode signals. The structure of the merocyanine dye used in this experiment is shown at the top. (Modified from Salzberg et al. [15]).

indicating that there was some photodynamic damage. In addition, bleaching of the dye would occur after several minutes of constant illumination. However, both of these effects could be minimized by using restricted illumination periods. In later experiments, using a semi-intact *Navanax* preparation (see below) and a different dye (0.5 mg/ml RH155 [NK3041] for 30 min), photodynamic damage and bleaching appeared to be insignificant [10]. Constant illumination of a preparation for 10 min resulted in little if any change in the pattern of neuronal activity or in signal size. The pharmacological effects of dyes were examined in behavioral experiments. Response to food and subsequent feeding were compared in animals whose buccal ganglia either had or had not been stained. Few, if any, changes in behavior were observed as a result of staining.

Preliminary experiments on the *Aplysia* gill-withdrawal reflex suggest that pharmacological effects may be present in some preparations using the *Navanax* staining procedure [18]. In other preparations, gill withdrawal and its habituation and sensitization (see Marcus et al., this review) appeared normal after staining. Photodynamic damage also appears to be more noticeable in the Aplysia experiments.

Spatial resolution

Using individual photodiodes placed at the objective image plane, a large signal was recorded only in the detector positioned directly over the stimulated cell body, indicating that the optical signal was confined to the expected area of the image. However, when the image of a ganglion is formed on an array of very regularly arranged diodes, then the correspondence of detectors and cells will be lost. A schematic drawing of the projection of several neurons onto an array is shown in figure 3. Each square represents the area of the preparation to which a single photodiode is sensitive. The light of larger cells will fall on several detectors. These cells' activity will be recorded as simultaneous events on neighboring detectors. Some cells cover the entire area of a diode and therefore the signal recorded from these cells on that detector will be relatively large. Several detectors have only a small part of the cells' image projected onto them, and the signal on these detectors from these cells will be small. Finally, it can be seen that one detector may record the activity of several cells, for example, when sections of 3 different cells are projected onto 1 detector.

The concepts suggested by the schematic drawing are illustrated in figure 4 which is a small portion of an array measurement from an *Aplysia* abdominal ganglion. In the top section original recordings from seven of the photodiodes from the array are shown. At the number 1 there are syn-

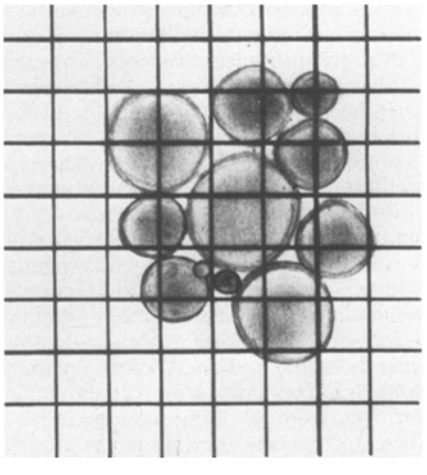


Figure 3. Drawing of the projected image of an idealized cluster of cells onto the array. Note how various sized areas of one cell may fall on different detectors, giving rise during a recording to duplicate spike activity of different size on neighboring detectors. Note also how areas of more than one cell project their images onto a single detector, thus one detector may record the activity of several cells. We thank Dr A. Grinvald for allowing us to use this drawing.

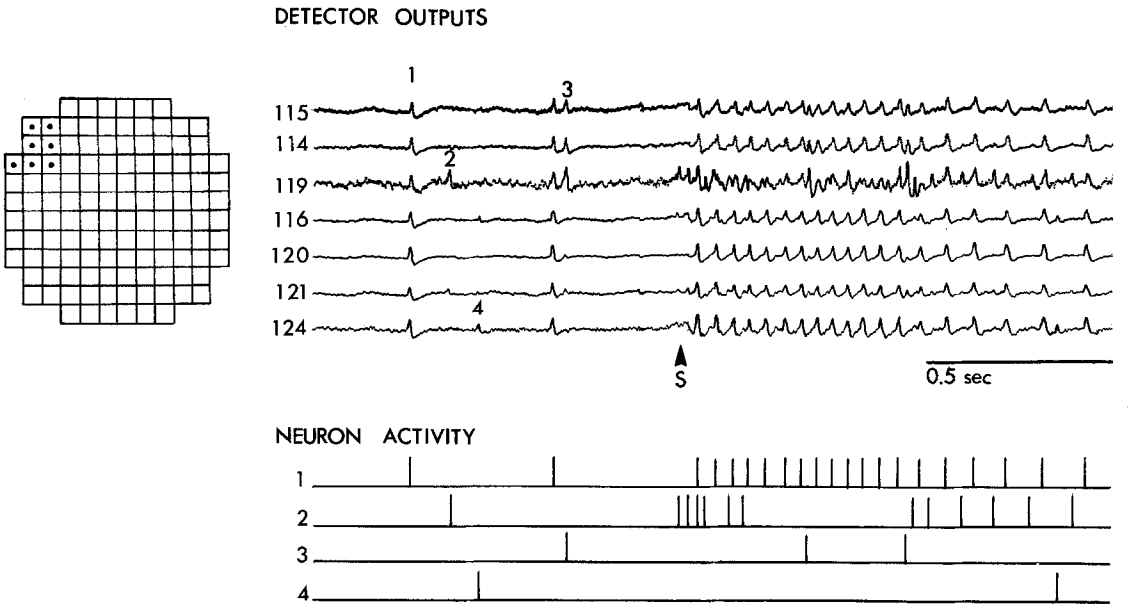


Figure 4. Optical recordings from a portion of a 12 × 12 array from an *Aplysia* abdominal ganglion. The number to the right of each trace identifies the detector from which the trace was taken. The drawing to the left represents the relative position of the detectors whose activity is displayed. In the top section, the original data from 7 detectors is illustrated. In the bottom section the results of our analysis of this data into the spike activity of four neurons is indicated. (Modified from London et al.[9] and Wu et al.[18])

chronously occurring action potentials on all seven detectors. This synchronous event occurs many times (> 20) and we presume that each synchronous event represents the activity of one relatively large neuron. The activity of this cell is represented by the vertical lines on trace 1 of the bottom section. The activity of a second cell is indicated by the spike on 119 and a very small signal on its neighbor, 121. The activity is represented on the second trace in the bottom section. The activity of two additional neurons was similarly identified. Two of the problems associated with optical recording are also illustrated by this figure. One problem arises from the signal-to-noise ratio. There may be an additional spike on detector 116 just before the stimulus (at the arrow), but the signal-to-noise ratio was not large enough to be certain. In addition, following the stimulus, there is a great deal of activity and it would be easy for this activity to obscure small signals. The kind of analysis illustrated in figure 4 has been used to generate the raster data shown in figures 8–10. The complete analysis for trials like that shown in figure 10 takes from 1 to 3 days.

Resolution in the Z axis. We also tried to determine how well a single detector would discriminate between cells that were in the same *x-y* position but were not in the same focal plane. To determine the resolution in the *z* axis, the size of the optical signal was measured when a neuron was moved both above and below the plane of focus. The signal size was reduced by 50% when the neuron was moved +/− 300 µm out of focus (fig. 5) using a nominal numerical aperture[15] of 0.4. Clearly, activity of neurons not in the plane of focus can also be monitored. This change in signal size as a function of focus will depend on the numerical aperture of the objective, with a larger numerical aperture there will be a more rapid decrease in signal size for the same change in focus. In a ganglion of 600-µm thickness, with 0.4 N.A. optics, one could focus in the middle of the ganglion and record signals from the activity of neurons at the top and at the bottom of the ganglion with a reduction in signal size of about 50%.

Light scattering. We were concerned about two effects of light scattering. Our estimate of the size of the neurons was made from the number of detectors on which the activity of

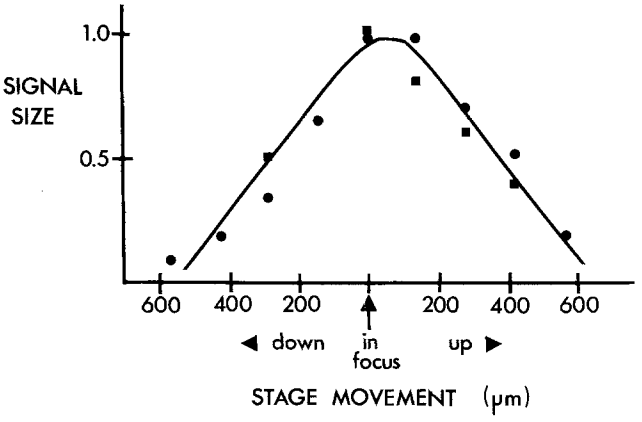


Figure 5. Size of the optical signal depends on the position of the cell body relative to the plane of focus. A 50% decrease in signal size is seen when the soma is moved either 300 µ up or down from the plane of focus. (Modified from Salzberg et al.[15])

the cell was seen. Light scattering will reduce the amount of light that reaches a cell and reduce the amount that is collected by the objective to form its image. The blurring effect of scattering will be largest for cells on the bottom of the ganglion because the light that will form their image has a relatively long path in the ganglion. It is possible that the light from a cell at the bottom of the ganglion whose image would normally fall on only one detector, would become dispersed enough to result in an image that would fall on several detectors. This would lead to an erroneous estimate of cell size. Substantial scattering would also decrease the signal-to-noise ratio by spreading the light that would otherwise reach a single detector over many detectors. In order to measure the scattering in *Navanax*, a small spot of light was focused on the object plane. The image of the spot fell on a single detector of the array (fig. 6, top section). A ganglion was

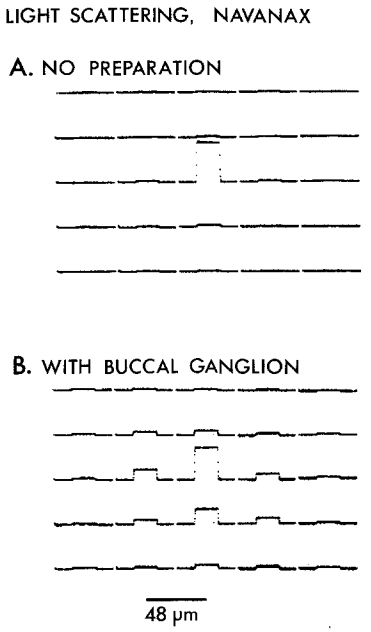


Figure 6. Measurement of light scattering by a *Navanax* buccal ganglia. The top section is a measurement of the response of 25 adjacent detectors when a small spot of light was focused in the object plane on a single detector. The bottom section is the response of the same 25 detectors, but now a buccal ganglion has been placed between the object plane and the objective. (Modified from London et al.[10])

then placed in the path of the light and the number of detectors receiving light was again recorded (fig. 6, bottom section). It can be seen that while the largest intensity was recorded on the detector which originally had the light focussed on it, some of the surrounding detectors now received small levels of light. However, because the intensity on the surrounding detectors is less than ⅓ the intensity on the central detector, the signals from the scattered light might be lost in the noise. Thus, scattering in *Navanax* ganglia apparently would not seriously distort estimates of cell sizes. However, in *Navanax,* there is a substantial intensity loss due to scattering. In the experiment of figure 6, the intensity reaching the central detector was reduced about 10-fold by the presence of the ganglion. While some of the light that is lost from the central detector can be seen on the adjacent detectors, there must be additional light loss due to scattering at relatively large angles. A 10-fold light loss causes a reduction in the signal-to-noise ratio by a factor of three.

In *Aplysia,* the scattering by abdominal ganglia from large animals (> 100 g) is markedly worse. For this reason, the experiment described below and shown in Figure 11 was carried out on a 20-g animal. We are now trying to do similar experiments on 2-g animals. The scattering of abdominal ganglia from 2–5-g animals is similar to the scattering we obtained in *Navanax* buccal ganglia[18].

Recording from Navanax buccal ganglia during feeding

We have used the diode array to monitor activity from the nervous system of a minimally dissected preparation in

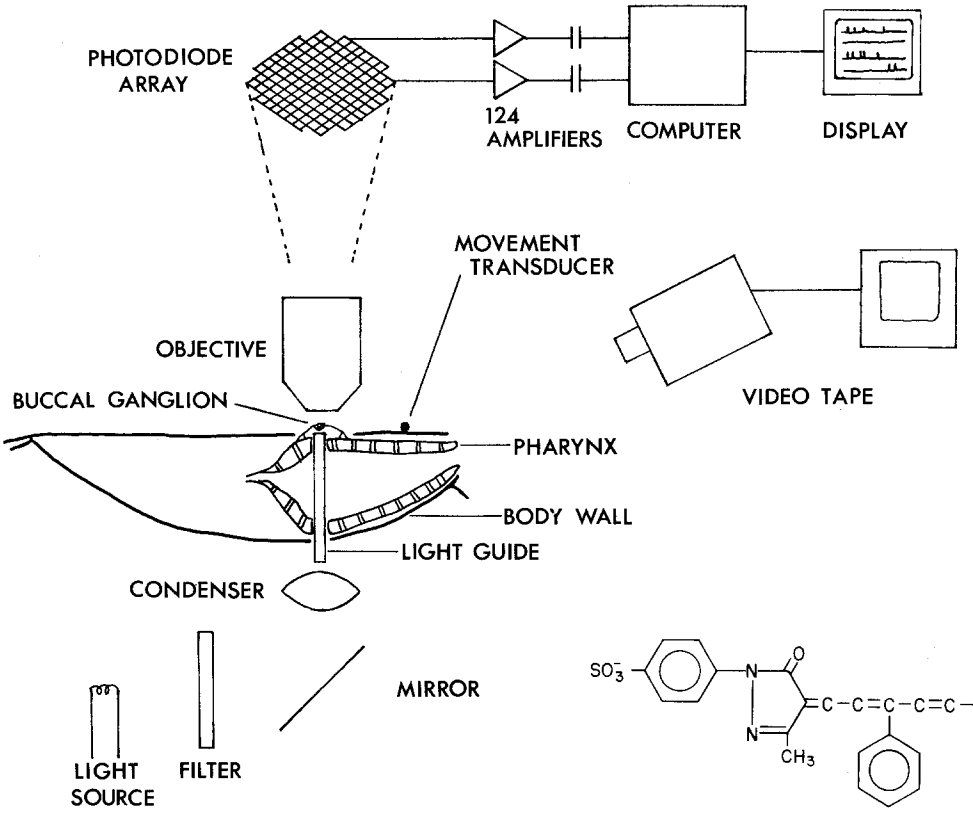


Figure 7. Schematic drawing of whole animal recording apparatus for *Navanax*. The light is focused by a condenser onto the light guide which passes through the body wall and two slits in the pharynx. The light guide was constructed from a 1 mm clad quartz cylinder of glass encased in a metal tube. The portion of the light quick next to the ganglion was surrounded by a Sylgard (Dow) platform. After staining, the ganglion was then pinned to the platform and covered with a 3% solution of agar dissolved in sea water to decrease movement artifacts. The image of the ganglion was projected onto the 12 × 12 photodiode array. The pharyngeal expansion was recorded with a movement transducer and with a video camera. (Modified from London et al.[10])

which the animal behaves. Activity of the buccal ganglion of *Navanax inermis,* was monitored while the animal exhibited spontaneous feeding movements and during actual feeding activity. *Navanax* is a carnivore which feeds on several species of opisthobranchs including other *Navanax,* and some species of fish [12]. Briefly, the feeding movements consist of the protraction of the mouth, prey contact and grasping with the lips, followed by an explosive expansion of the pharynx. This expansion creates a negative pressure so that the prey is sucked into the pharynx. Neurons controlling the expansion have been described and reside in the buccal ganglion [16, 17].

A minimally dissected whole animal preparation was used to monitor feeding behavior and neuronal activity simultaneously (fig. 7). An incision was made in the skin on the ventral surface above the buccal ganglion and the skin retracted to expose the ganglion. A 1 mm clad glass light guide was used to carry the light from the microscope condenser to the ganglion. A movement transducer was placed on the pharynx to record movements. A video camera and recorder were also used to monitor the behaviors.

The action potential data from these experiments were displayed in raster diagrams (see figs 8 and 9). The bottom trace is a recording from the movement transducer which had been positioned on the pharynx. An upward deflection indicates expansion. The column of small numbers on the left represents the number of detectors on which this particular neuron's activity was recorded. The larger numbers represent the identification number of the detector used to enter this neuron's activity into the computer and to generate the raster trace.

Animals in this recording situation sometimes would exhibit spontaneous pharyngeal expansions (feeding-like) and in many preparations food-induced feeding could also be observed. During spontaneous expansions, we detected activity in 6–19 cells. In the example shown in figure 8, there is a large unit which is active during the beginning of the expansion; this cell's activity is recorded on 13 detectors. There is also a large cell whose activity was observed on 8 detectors which is active after the initial expansion occurs. Several cells are active during the expansion and become quiet during the maintained expansion and the contraction phase. Other cells, like those displayed on detectors 57 and 15 become active at the end of the expansion. There are 13 small cells that are active during this spontaneous expansion; the activity of each of these cells was seen on only 1 detector.

During feeding (fig. 9) the pattern of muscular activity is similar in form to that of the spontaneous expansions; however, there were usually more neurons active during feeding than during spontaneous expansions. In the experiment shown, 22 cells are active. As in the spontaneous expansion, during this feeding cycle there is a burst of activity reflected as increased spike activity prior to the expansion. Several large-sized cells, on detectors 121 and 18, are active early during the expansion; one of these, on detector 121 may be the same large cell that was active during the spontaneous expansion. We think that this cell is the G-motorneuron previously identified by Levitan et al. [8] and Spira et al. [16].

We needed to determine the completeness of this optical recording. Was activity detected in 22 neurons because only 22 were active, or, because the apparatus was only sensitive enough to detect activity in 22 neurons? To determine the completeness we compared the number of cells we could identify by their optical activity when we attempted to activate all of the cells in the ganglion with the number of cell bodies present in the ganglion. To obtain the number of cells whose activity could be detected optically, the ganglion was activated by stimulating each of the peripheral nerves and connectives with suction electrodes. To determine the number of cell bodies, ganglia were stained with methylene blue and the number of neurons counted using a computer-interfaced microscope [1, 11]. The results of this comparison for three preparations are shown in the table. On average we

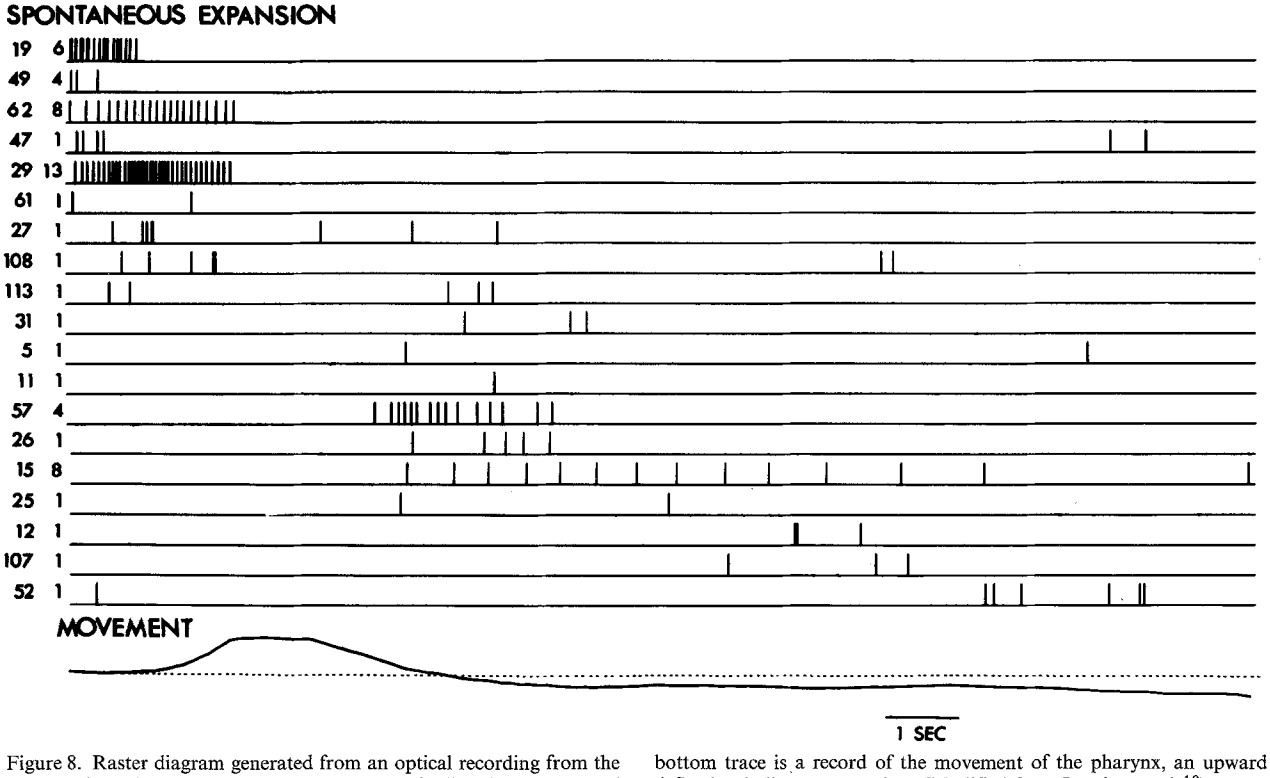


Figure 8. Raster diagram generated from an optical recording from the *Navanax* buccal ganglion during a spontaneous (feeding-like) pharyngeal expansion. All the neurons whose activity was detected are shown. The bottom trace is a record of the movement of the pharynx, an upward deflection indicates expansion. (Modified from London et al. [10])

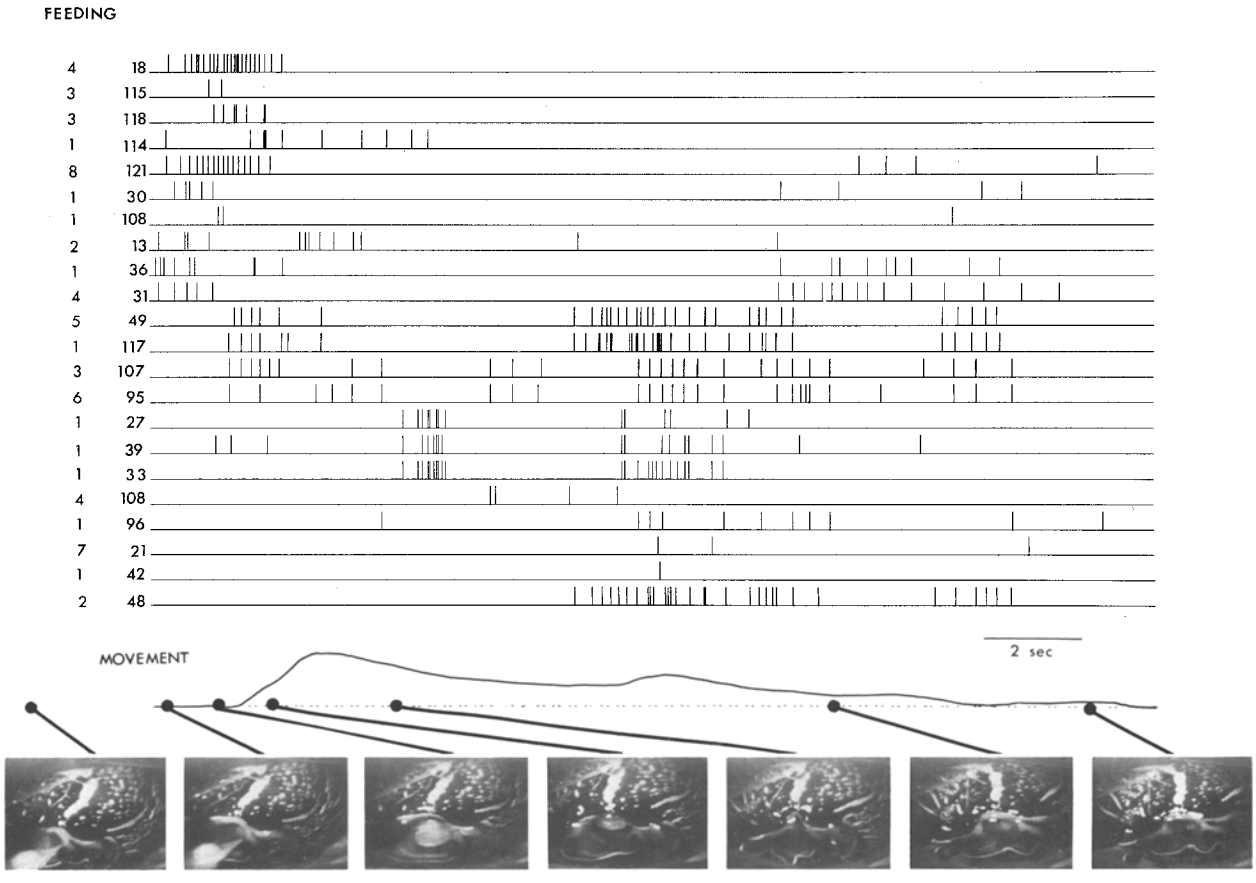


Figure 9. Raster diagram generated from an optical recording of the buccal ganglion during an actual feeding episode. Below the movement recording are shown individual frames from the video-tape recording of the movement. The timing of the frames are indicated by the dots. There is a small piece of food that can be seen in the lower left corner of the first three frames which is completely engulfed by the fifth frame. (Modified from London et al.[10])

detected activity in approximately 70% of the total number of cells present in the buccal ganglia. We think that this number may underestimate the actual completeness of the optical recording. One possible cause of an underestimation is that we may not correctly separate the activity of small cells. Several small cells' activities may occur on one detector; their small optical action potentials might appear too similar to allow us to differentiate between them. Another possible cause of an underestimation is that the electrical stimulation may not activate 100% the neurons. For example, the incoming volley could have inhibitory effects on some neurons, or local interneurons may not be activated. In any case, if we use 70% for the completeness of the recording, then the actual number of active neurons during feeding would be about 30.

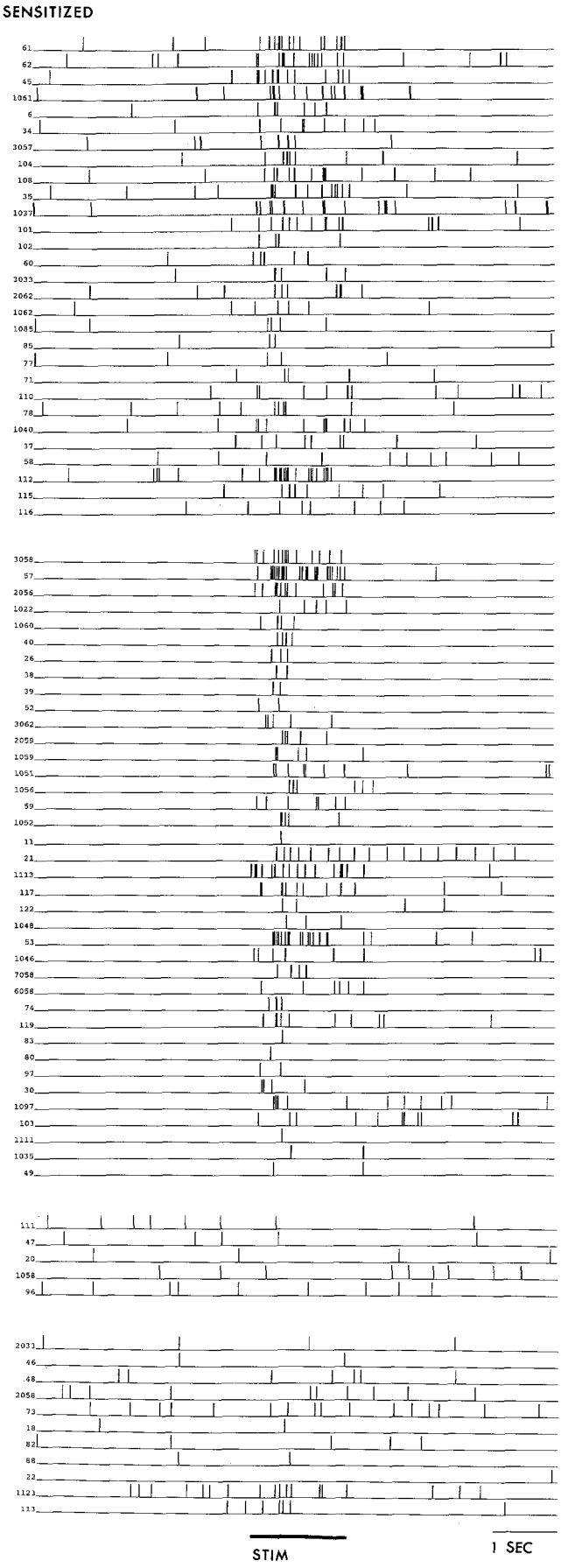
Completeness of the optical recordings from *Navanax*.

Preparation	Number of neurons whose activity was detected optically	Cell bodies in the ganglion	%
89	140	183	77
93	128	162	79
95	112	203	55

Recording from the Aplysia abdominal ganglion during the gill-withdrawal reflex

We have used the same dye and same apparatus to make similar recordings from the *Aplysia* abdominal ganglion during the gill-withdrawal reflex. In this case we used a more reduced preparation consisting of siphon, siphon nerve, ganglion, genital and brachial nerves and gill[7]. The siphon skin was given a light mechanical touch and the gill movements were recorded on video-tape. An eight second raster diagram of the spike activity measured during a withdrawal in a preparation that had been acutely sensitized is illustrated in figure 10. The mechanical stimulus occurred at the time indicated by the bar at the bottom. The activity of more than 80 neurons was detected optically. The active neurons are arranged in four groups. The first are spontaneously active and have increased activity following the mechanical stimulus. The second group was silent before the stimulus but activated by it. The third group appears to be inhibited by the stimulus. And the fourth group has activity that apparently ignores the stimulus.

One important difference between the *Aplysia* experiment and the *Navanax* experiment is the number of neurons present in the ganglion. Coggeshall[2] counted about 1100 neurons in *Aplysia* abdominal ganglia from animals weighing 5–40 g. With a photodiode array of only 124 elements it is clear that the number of neurons per detector in the *Aplysia* experiment is relatively large and thus it will often be difficult to distinguish activity in neighboring small neurons.



We have made preliminary attempts to determine the completeness of this *Aplysia* recording. Again we used electrical stimuli to try to activate as many neurons as possible in the ganglion. Preliminary analysis of optical recording made during electrical stimulation in two preparations indicated that we were able to detect activity in 250–300 neurons. This result would indicate that the recordings made during the gill-withdrawal reflex were only about 25% complete. While the number is, of course, subject to considerable uncertainty, we estimate that there were actually more than 300 neurons in the abdominal ganglion which were activated by the light touch to the siphon.

We were surprised at the large number of neurons that are involved in the response to the mild stimulation of the siphon skin. With this number of involved neurons it would seem to be difficult to determine the role of any given neuron or synaptic connection in generating the behavior. Certainly it is possible, or even likely, that not all of the more than 300 neurons are actually involved in gill withdrawal, but providing the evidence that they are not would seem to be impractical with presently available neurobiological tools. In fact, it is not easy to think of a practical experiment that will allow one to determine the role of any particular synaptic connection or group of synaptic connections in generating the gill withdrawal or in accounting for its plasticity. It will be a challenge to neurobiologists to develop methods for understanding circuits of this complexity.

Conclusions

The optical monitoring system has reached the level of development where activity from a network of cells can be recorded in a minimally dissected behaving animal. We monitored activity in the buccal ganglion of *Navanax* during feeding and the *Aplysia* abdominal ganglion during the gill-withdrawal reflex. Deleterious pharmacological effects and photodynamic damage appear to be negligible in these preparations. The signal-to-noise ratio is large enough to detect the activity of many individual neurons without signal averaging, providing the cell soma is of sufficient size (> 30 μm). The effect of light scattering on cell size estimates would appear to be small in *Navanax* buccal ganglia and in *Aplysia* abdominal ganglia from small animals. However, scattering does substantially reduce the signal-to-noise ratio obtained in these recordings. Our measurements suggest that the recordings from *Navanax* buccal ganglia are more than 70% complete. The *Aplysia* recordings apparently are less complete.

Future directions

One objective is to continue to improve the optical technique. We hope that better dyes will become available, ones that will produce larger signals in response to voltage changes in the membrane. With increased signal-to-noise ratios we would be able to record synaptic potentials consistently and therefore map synaptic interactions. We also plan to increase the spatial resolution of the apparatus by incorporating an array with more elements. This should be espe-

Figure 10. Raster diagram of the action potential activity recorded optically from an *Aplysia* abdominal ganglion during a gill-withdrawal reflex. In this recording, from an acutely sensitized preparation, we detected activity in about 80 neurons. But since we estimated that the recording was only about 25% complet the number of activated neurons in the ganglion was probably close to 300. (Modified from London et al.[9]; and Wu et al.[18])

cially helpful in experiments on ganglia like that from *Aplysia* with relatively large numbers of neurons.
We plan to further characterize the cells participating in the behavior. One way in which we hope to do this is by monitoring the direction of spike propagation in the peripheral nerves and matching their timing with soma recordings. Activity of cells with peripheral nerve spikes proceeding outward (centrifugal) are of possible motoneuron origin; activity of axons spikes proceeding inward (centripetal) may be from sensory neurons. With improvements in cell characterization and identification, and with the recording of synaptic potentials we hope that optical recording will become a useful tool in working out the neuron interactions that underlie simple behaviors.

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Switching among functional states by means of neuromodulators in the lobster stomatogastric ganglion

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Summary. The lobster stomatogastric ganglion contains the central pattern generators (CPGs) for the pyloric and gastric mill rhythms. All of the neurons and their synaptic connections have been identified for each rhythm and serve as the basis for understanding the mechanisms by which chemical neuromodulators are able to alter the functional state of each CPG. Using examples of different amines and peptides, I show how these substances can be found within specific neurons and how their application to the CPG can alter the motor patterns in specific ways. I also discuss what changes in cellular and synaptic properties occur as a result of bath application and particularly in the case of proctolin, how these changes may have behavioral correlates. The various outputs appear to be the result of a functional 'rewiring' of anatomically defined neural circuits and this may be a widespread mechanism for the production of closely-linked but behaviorally distinct movement patterns.
Key words. Lobster stomatogastric system; neuromodulation.

Many classes of rhythmic behaviors do not exist as discrete entities but as a continuum of various more or less stable states. An example is vertebrate locomotion. Separate functional modalities such as walking, trotting, galloping etc. can exist as recognizable behaviors but are not completely regular due to cycle by cycle changes imposed on each state by sensory feedback and descending and coordinating fibers extrinsic to the particular pattern generator involved. Given this complexity, an important consideration in the neuronal analysis of networks producing rhythmic movements is whether there is a separate central pattern generator (CPG) for each variation of a fundamental movement, or whether different functional states are carved out of one large network which has the potential for producing all possible rhythmic movements a particular effector system is capable of. The two possibilities are not meant to be mutually exclu-