Experiment NB-6: Morphological Identification of Neurons

Allow two lab periods to complete this experiment.

Background

In this experiments, dyes will be injected into specific neurons and then viewed under a fluorescence compound microscope to see the structural details of the neurons. This anatomical study will confirm:

- The identity of the neurons.
- The location of their axons
- The extreme complexity of the branching pattern of these neurons, especially in the central part of the ganglion where synaptic contacts are made among neurons.

The dyes used to fill the cells in the ganglion are delivered through the same type of microelectrode that used in the previous experiment to record and stimulate the neurons. Students will learn about neuronal dyes, their delivery into cells, and the observation of these dyes with a microscope.

Neuronal Dyes

There are a variety of morphological techniques used to trace the connections among neurons. Most of these involve the selective staining of one or a class of neurons and observing the projection of their axons. In general, the dyes used to stain tissue are complex organic chemicals which often show some variability in performance. They may be classified in numerous ways, but the simplest approach is to base the classification upon use, with regard to tissue and cell components. Dyes may be of general use, staining either the nucleus or the cytoplasm, or they may be more specific with regard to particular components. The purpose of the following summary is to classify and describe some of the tissue tagging procedures now available to the neuroscientist. All of the methods known for labeling particular tissue types rely on features of the tissue and of the tag for specificity. In addition to understanding the properties of the dye itself, one needs to understand how the dye is rendered visible, either in the light or electron microscope. There are a variety of methods used to add tracers to biological probes. These are classified and reviewed below.

Structural Identifiers

These types of markers include:

- General Markers: Dyes that adhere to membranes or intracellular structures; e.g., cresyl violet acetate, Toluidine blue, haematoxylin.
- Specific Markers: Dyes that adhere to specific structures; e.g., Weil stain for myelin.
- Variable Markers: Dyes that adhere to a variety of cell types; e.g., Nauta silver stain for nerve cells.
- Specific for Cell Types: Monoclonal and polyclonal antibodies, or plant lectins that label cell surface glycoconjugates.
- Intracellular or Cytoplasmic Markers: Injected via electrodes or cell processes, diffusion, or through membrane disruption or sectioning.

Functional Identifiers

These markers include:

- Neuroactive substance tags: Antigens have been immunocytochemically localized.
- Metabolic activity tags: a) Labelled metabolic substrates, e.g., 2-deoxy-glucose; b) Mitochondrial activity tags; e.g., cytochrome oxidase; c) Uptake by active cells; e.g., HRP, sulforhodamine.
- Molecular processing tags: Hybridization of tagged probes like RNA.
- Proliferating cell tags: Nucleotide substitution during cell division to mark new DNA to be followed during future divisions; e.g., ^{3}H-thymidine, 5-bromo-2'-deoxyuridine (BrUdR), retroviruses

Tracers

These are substances that attach to the probes to make the probes more visible. They include:

- Enzymatic tracers: Horseradish peroxidase (HRP), cobaltus lysine.
- Particulate tracers: Particles attached to biological tracers, e.g., ferritin, iron-dextran complexes, colloidal gold, polystyrene latex, polymethacrylate latex spheres.
- Emissive tracers: Fluorescent, e.g., Lucifer Yellow or DiI; or, radioactive tracer tags.
- Lipid soluble tracers: DiA (red), DiI (yellow), DiN (blue).

Lucifer Yellow (LY)

This dye was created specifically as a bright and convenient marker to be injected into neurons (Stewart, 1978, 1981). Lucifer Yellow is:

- Negatively charged, so that LY can be injected into cells by passing a negative current.
- A small, highly mobile molecule that diffuses readily to all parts of the cells.
- Highly fluorescent, so that a small amount of LY will produce a very bright signal
- Designed with the appropriate chemical groups to link it to intracellular proteins with aldehyde fixatives
- Non-toxic.

For these reasons, LY has become a workhorse in identifying neurons. Routinely, LY-filled microelectrodes are used to record from neurons, then hyperpolarizing current is passed to mark the cell for later identification.

However, Lucifer Yellow is not soluble in solutions with high concentrations of Sodium or Potassium, like the concentrations in the solutions used to fill electrodes. Fortunately, LY is soluble in concentrated solutions containing Lithium. Typically, the Lucifer Yellow solution used to dye cells is made as a highly concentrated solution (3-10% by weight in distilled water). Only a small amount of Lucifer Yellow is used in the tip of the electrode because it is an expensive dye. The rest of the shaft of the electrode is filled with a concentrated solution of LiCl that facilitates recording from the cell and pushing the LY into the cell. The electrode filled in this manner has an impedance that is 3-5 times greater than a matching electrode filled with KCl or potassium acetate. A good LY-filled electrode will have an impedance in the range of 60-120 MΩ.

Also, Lucifer Yellow fades with viewing under a fluorescent microscope. This is a problem that is inherent in this type of microscopy. The electrons of each fluorescent molecule absorb light at one wavelength and emit it at another wavelength; but, the molecule goes into a state where it cannot absorb any more light energy. This means that every light-absorbing molecule can only give off one photon, at best. Therefore, it is critical to minimize the length of time that the fluorescent-labeled cell is exposed to bright UV light. Slides containing cells filled with LY should be stored in the dark because there is enough UV in daylight or even room light to cause the dye to fade. When using the fluorescent microscope, close the shutter between the UV light source and the preparation whenever the preparation is not being viewed.

Rhodamine dextran

When examining the processes of two different neurons that lie close to each other, it is advantageous to use another type of dye that emits a second color. A particularly good dye for this purpose is rhodamine, which emits a bright red fluorescence. However, rhodamine needs to be bonded covalently to another molecule that is large enough to remain within the neuron and can be fixed to protein. Without the other molecule, rhodamine would leak quickly out of the cell.

In this experiment, a compound containing rhodamine bound to lysinated dextran is used. Dextran is a polymer of sugars, available in a variety of molecular weights. The dextran in the compound used in this experiment has a molecular weight of 3000. The amino acid, lysine, that is conjugated dextran permits the dye molecule to be fixed to the intracellular proteins by exposing the filled neurons to paraformaldehyde.

Equipment Setup

This experiment uses some of the same equipment and procedures used in Experiment NB-5 to record and stimulate neurons. Refer to the directions in Experiment NB-5 to remind yourself of the techniques that will also be used in this experiment.

Exercise 1: Filling Neurons with Lucifer Yellow

Aim: To identify and fill a specific neuron with the fluorescent dye, Lucifer Yellow, in order to examine the structure of the neuron and its connections to other cells.

Setup - Neuron Identification

1 Pull down the **Settings menu**. Select the **LeechCNS-2A-NBK** settings file. LabScribe will appear on the computer screen as configured by the **LeechCNS-2A-NBK** settings. This settings file programs LabScribe2 to stimulate and record in **Repetitive Scope mode** (Table NB-6-1 on page NB-6-2).

Table NB-6-1: Settings Programmed on the Channels Preference Dialogue window for the LeechCNS-1C-NBK Settings File.

Parameter	Units/Title	Setting	Mode/ Function
Acquisition Mode		Scope-Repetitive	
Start		User	
Stop		Timed, 0.150 sec	
Display Time	Sec	0.150	
Speed	Samples/Sec	20000	
Channel A1	Cell Potentials	√	BNC
Channel S1	Stimulus	√	Record

2 Click the **Stimulator Preferences icon** on the LabScribe **toolbar** (Figure NB-6-1 on page NB-6-2) to open the **stimulator control panel** (Figure NB-6-2 on page NB-6-2) on the **Main window**.

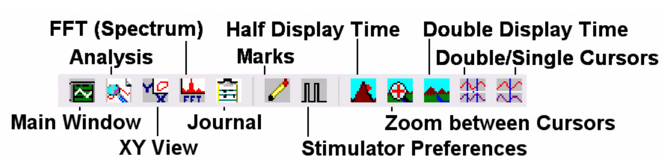

Figure NB-6-1: The LabScribe toolbar.

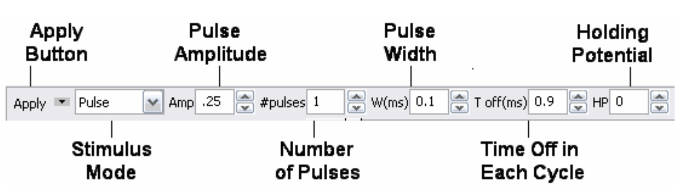

Figure NB-6-2: The stimulator control panel

3 Check the settings on the **stimulator control panel** against the settings for this portion of the exercise that are listed in Table NB-6-2 on page NB-6-3.

Table NB-6-2: Settings Programmed on the Stimulator Dialogue Window for Determining the Characteristics of Action Potentials.

Parameter	Units/Title	Setting
Stimulus Mode		Pulse
Stimulator Start		With Recording
Time Resolution	msec	0.01
Toolbar Step Frequency	Hz	1
Toolbar Step Amplitude	Volts	0.01
Toolbar Step Time	Variable	0.01
Delay	Sec	0.005
Amplitude (Amp)	Volt	0.000
Pulses (#pulses)	Number	1
Pulse Width (W)	msec	30
Time Off (T Off)	msec	900
Time Off Amplitude	Volts	0
Holding Potential (HP)	Volts	000

4 Make sure the stimulus amplitude on the **stimulator control panel** is set to **0.000V**.

5 Click the **Apply button** to finalize any changes that were made to the stimulator control panel.

6 Set the µ**A knob** on the amplifier to zero and the **CURRENT INJECTION switch** is **OFF**.

Procedure - Neuron Identification

1 Obtain a microelectrode with a small amount of concentrated solution of LY in the tip. Fill the rest of the shank of the microelectrode and the electrode holder with the 1M LiCl solution.

2 Test the resistance of the microelectrode while its tip is in the bath solution.

3 Set the baseline of the recording using the **DC OFFSET** of the Model 3100 amplifier while the microelectrode is outside the neuron.

4 Impale the cell and record. If any action potentials are occurring in the absence of current injection, hyperpolarize the cell by flipping the **CURRENT POLARITY switch** to **-** (negative**)**. Turn the µ**A knob** on the Model 3100 clockwise until the action potentials stop firing.

5 Change the stimulus amplitude on the **stimulator control panel** to **0.020V**. An amplitude of **0.020V** sets the current injected into the neuron at **2nA**.

6 Click the **Apply button** to finalize any changes that were made to the stimulator control panel.

***Note:** The conversion factor for the EXTERNAL input of the Model 3100 is 100nA of current for each volt sent to the input from a voltage source like a stimulator. A positive voltage generates a current with a positive polarity.*

7 Before measuring the response of the neuron, use the **DC BAL knob** to eliminate the voltage drop across the electrode:

- Flip the **CURRENT INJECTION switch** to **CONT** position (continuous). When a sweep occurs a stimulus pulse is sent from the IWX/214 stimulator to the electrode through the **EXTERNAL** BNC input of the Model 3100.

- Adjust the position of the **DC BAL knob** between each of the repetitive sweeps until the artifact of the current pulse is eliminated from the recording.

- The only indications of the current pulse on the recording should be the two transient artifacts at the onset and offset of the stimulus pulse.

8 Once **DC BAL** is set for a current pulse, record an artifact-free sweep.

9 Click the **Stop** buton to halt the recording.

10 Select **Save** in the **File menu**.

11 Confirm the identity of the neuron by its electrical properties. Be sure the neuron generates a good action potentials.

12 Keep the microelectrode in the neuron and setup quickly for the next section of the exercise

Setup - LY Injection

1 Pull down the **Settings menu**. Select the **LeechCNS-2B-NBK** settings file. LabScribe will appear on the computer screen as configured by the **LeechCNS-2B-NBK** settings. This settings file programs LabScribe2 to stimulate and record continuously in **Chart mode** (Table NB-6-3 on page NB-6-3).

Table NB-6-3: Settings Programmed on the Channels Preference Dialogue window for the LeechCNS-2B-NBK Settings File.

Parameter	Units/Title	Setting	Mode/ Function
Acquisition Mode		Chart	
Start		User	
Stop		User	
Display Time	Sec	10.000	
Speed	Samples/Sec	20000	
Channel A1	Cell Potentials	√	BNC
Channel S1	Stimulus	√	Record

2 Check the settings on the **stimulator control panel** against the settings for this portion of the exercise that are listed in

Table NB-6-4 on page NB-6-4.

Table NB-6-4: Settings Programmed on the Stimulator Dialogue Window for Injecting Lucifer Yellow.

Parameter	Units/Title	Setting
Stimulus Mode		Pulse
Stimulator Start		With Recording
Time Resolution	msec	0.01
Toolbar Step Frequency	Hz	1
Toolbar Step Amplitude	Volts	0.01
Toolbar Step Time	Variable	0.01
Delay	Sec	0.000
Amplitude (Amp)	Volt	-0.050
Pulses (#pulses)	Number	0 (Continuous)
Pulse Width (W)	msec	600
Time Off (T Off)	msec	400
Time Off Amplitude	Volts	0
Holding Potential (HP)	Volts	0

3 Make sure the stimulus amplitude on the **stimulator control panel** is set to **-0.050V**, so that 5**nA negative** (hyperpolarizing) pulses will be generated. These pulses should have a **duration** of **600 msec**, a frequency of 1Hz (**Time Off** is **400 mesec**), and be delivered throughout the complete recording period (**#Pulses** is **0 for continuous**).

4 Click the **Apply button** to finalize any changes that were made to the stimulator control panel.

Procedure - LY Injection

1 Click on the **Record button** to pass the current that pushes the LY in to the cell. Continue to record for 5 minutes.

2 Turn off the illuminator during the dye injection, except to briefly check on the injection of LY. If the neuron is no more yellow than any other neuron in the ganglion:

- **Ring** the electrode briefly.
- Continue to inject the dye for 5 more minutes at a higher stimulus current.
- Continue ringing and increasing the stimulus current until the neuron is distinctly more yellow than any other cell, or until the cell is distinctly dead. Cell death should occur after 15-30 minutes of dye injection

3 When the cell looks yellow:

- Click on the **Stop button** to halt the recording and the injection.
- Pull the electrode out of the cell.
- Let the ganglion sit for another 15 minutes to allow the dye to diffuse into all the processes.

Warning: (If the preparation sits for more than 60 minutes, the dye will start to leak out of the cell.)

Exercise 2: Filling Neurons with Rhodamine Dextran

Aim: To identify and fill a neuron with possible connectives to the neuron just injected with LY. This neighboring neuron will be injected with another fluorescent dye, Rhodamine Dextran.

Setup - Neuron Identification

Use the settings file, **Leech CNS-2A-NBK**, to configure the LabScribe2 software to record from and identify a neuron that is believed to be associated with the neuron injected with Lucifer Yellow.

Procedure - Neuron Identification

Use the same techniques described in Exercise 1 to record and identify the cell to be injected with Rhodamine Dextran.

Setup - Rhodamine Dextran Injection

1 Use the settings file, **Leech CNS-2A-NBK**, to configure the LabScribe2 software to inject the neuron with Rhodamine Dextran.

2 Change the stimulus amplitude on the **stimulator control panel** is set to **0.050V**, so that 5nA positive (depolarizing) pulses will be generated.

3 Click the **Apply button** to finalize any changes that were made to the stimulator control panel.

Procedure - Rhodamine Dextran Injection

Use the same techniques described in Exercise 1 to inject the cell with Rhodamine Dextran.

Exercise 3: Fixing, Clearing, and Viewing the Ganglion

Warning: 1. Wear gloves whenever fixing and clearing ganglia. 2. Perform these steps in the hood. 3. Pour the paraformaldehyde into a waste container, not the drain.

1 Fix the ganglion in 4% paraformaldehyde for 30 minutes.

- Pour the saline from the recording dish into a waste container.
- Fill the recording dish with paraformaldehyde.
- Replace the paraformaldehyde solution twice during the 30 minutes.

2 To view the ganglion immediately, place the ganglion on a slide in a drop of buffered glycerol and cover it with a coverslip.

Warning: Once the ganglion is placed in glycerol, it cannot be put into methyl salicylate for permanent storage.

3 For permanent mounting of the ganglion on a slide:

• Dehydrate the ganglion with EtOH while it is still pinned in the prep dish. Change the alcohol through the following series: 50%, 70%, 95%, 100%. Each step in the series is 3 minutes long, and the change at 100% EtOH is performed twice.

• Clear the ganglion in methyl salicylate for 5 minutes. Change the methyl salicylate and clear again for 5 more minutes.

• Place the ganglion on a slide in a drop of methyl salicylate and cover with a coverslip.

4 View and photograph the dye-filled neurons. Examine their morphological features.

• Before viewing the neuron in the microscope, look at drawings in papers listed in the bibliography of Experiments NB-5 and NB-6 to appreciate the complexity of the structures filled neurons. The paper by Muller & McMahan paper is particularly useful.

• Observe the ganglion in the fluorescence microscope. The Instructor will assist you in setting the microscope for optimal viewing.

• Even though photographs of the ganglion will be taken, it is also useful to make drawings of complex structures, because not all the processes of a neuron are in the same focal plane.

Note: *Remember to minimize the exposure of the filled cells to the UV light, to avoid fading of the fluorescence.*

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