

Chapter 2

Methods

This chapter describes in detail the methods employed to gather and analyze data for this thesis. Described are the specific protocol for Golgi staining, which is a method of random neuronal impregnation with silver. Also described are the electrophysiological and dissection procedures involved in filling individual neurons with fluorescent dyes. Analysis and computer routines are delineated in the final section.

Some data analysis included in this thesis was performed on Bodian stains prepared by Dr. Gilles Laurent (Caltech, Pasadena, CA) and Jochen Pflüger (Free University, Berlin).

All experiments described here were performed on the locust, *Schistocerca americana*. Adult animals of both sexes were chosen from a crowded colony and processed at room temperature, unless otherwise noted.

2.1 Golgi Staining

Locust heads were severed into a solution of 2.5% potassium dichromate containing sucrose (see Table 2.1, solution 2). A small window was dissected in the head capsule between the compound eyes and the eye sheaths were removed. The head was then immersed in a mixture containing glutaraldehyde (solution 3) and allowed to pre-fix for 30 minutes. The brain was then dissected out in solution 2 and all extraneous tissue was cleaned off. In some cases, all dissections were carried out on ice to slow penetration of potassium dichromate into the tissue. Also, in some cases, the brains

were desheathed at this dissection stage. The isolated brain was then immersed once again in solution 2 and left in the dark for one to two days.

Table 2.1: Golgi Solutions

Solution 1	2.5% Potassium Dichromate	
Solution 2	Solution 1	1.3% Sucrose
Solution 3	4 parts Solution 2	1 part 25% gluteraldehyde
Solution 4	99 parts Solution 1	1 part 1% osmium tetroxide
Solution 5	.75% AgNO ₃	water
Solution 6	.75% AgNO ₃	95% Boric Acid + 5% Na Tetraborate

Next, a second round of fixation was carried out. Brains were washed three times in 2.5% potassium dichromate (solution 1) and immersed into a mixture containing osmium tetroxide (solution 4). Incubations were carried out for a period of one to nine days in the dark.

For silver impregnation, AgNO₃ was mixed into either water or a borate buffer (solutions 5 and 6 respectively, Table 2.1). Brains were transferred into solution 3 and then into several rinses of the silver containing solution. Again, incubation was carried out in the dark, this time for one to four days.

Finally, brains were washed two times in water for five minutes and dehydrated in increasing concentrations of ethanol, from 10% to 100% (by 10% increments), for 10 minutes each. To prepare brains for plasticization, they were then incubated two times for five minutes in propylene oxide. Brains were then left in a 1:1 mixture of propylene oxide to Durcupan (FLUKA, prepared according to package directions) for one hour. The mixture was then left open to air overnight to allow all propylene oxide to evaporate. After one more four hour incubation in 100% Durcupan (left open to air), brains were embedded in Durcupan in small cylindrical molds and baked at 60 degrees Celsius for 12-16 hours. After cooling, brains were sectioned on a microtome into 10-20 μm thick slices, baked for five minutes onto glass slides, mounted and sealed with Permount (Fisher Scientific), and covered with a glass coverslip.

Varying the lengths of time for different incubation stages in general produced

staining of different clusters of cells. In this study, the most useful results were obtained from slides where subsets of cell populations were stained simultaneously. For staining of KC groups, the best protocols involved a 2-day incubation in the gluteraldehyde solution (solution 3), a 7-day incubation in the osmium tetroxide solution (solution 4), and a 3-day incubation in either silver solution (solution 5 or 6).

Slides were viewed through a Zeiss axioscope equipped with a Zeiss Axiocam upright microscope for acquisition of digital images. Digital images were analyzed and sections aligned using Photoshop software (Adobe Corp.).

2.2 Intracellular Recording and Dye Fills

For electrophysiology, locusts were immobilized and fixed to a Plexiglas holder, positioned with their dorsal side facing up. A cup was built around the head using dental wax to hold saline (table 2.2). Following perfusion of saline, a small window was cut into the head capsule between the eyes, and the tissue surrounding the brain was dissected away. In those experiments where neuronal responses to odors were to be recorded, the antennae were left intact, and the bases were inserted into a small section of polyethylene tubing to restrict antennal movement; most of the antennae were left uncovered for odor stimulation. In preparations where only staining was to occur, both antennae were cut at the base. To further reduce movement in all preparations, the esophagus was cut beneath the brain and the entire gut was removed through the abdomen, which was sealed to prevent leakage of saline from the head. Finally, a wax-coated platform was placed under the brain and secured to the cup at the posterior end of the brain. The brain was then desheathed following a 10 second treatment with protease type XIV (Sigma).

Electrophysiology was carried out using sharp glass microelectrodes pulled on a Sutter horizontal puller, using borosilicate capillary glass with an outer diameter of 1 mm and an inner filament diameter of 0.058 mm (Warner Instrument Corp.)

Extrinsic neuron experiments all included the recording of responses to odors, and these cells were filled with the dye Lucifer Yellow (LY). Stock LY was obtained from

Table 2.2: Physiological Saline for Locust

140 mM	NaCl
5 mM	KCl
5 mM	CaCl ₂
4 mM	NaHCO ₃
1 mM	MgCl ₂
6.3 mM	HEPES
pH	7.4

Molecular Probes as a premixed 10% solution. Electrodes were tip-filled with LY and then backfilled with a solution of .5 molar potassium acetate. Typical electrode resistances for extrinsic cells with this solution ranged between 100 and 200 M Ω .

KCs and PNs were filled using the fluorescent dyes, LY (as above) and Alexa Fluor (Molecular Probes). Alexa Fluor (AF) of two types — AF-488 and AF-546 — was obtained in pre-mixed solutions (10 mM in 200 mM potassium chloride). For these experiments electrodes were tip-filled with one of the above dyes and backfilled with 1 molar lithium chloride. Typical resistances for electrodes used to fill KCs ranged between 100 and 150 M Ω , while PN electrode resistances were between 60 and 100 M Ω .

To record odor responses from extrinsic cells, an odor delivery system was constructed such that a 1/8" diameter polyethylene tube was placed .5 cm from the antenna ipsilateral to the recording side. The other end of the tube was connected to a 20 ml odor vial containing 10% odor mixed into mineral oil (10 ml total). The odors used were hexanol, octanol, cherry and mint (Aldrich Chemicals). Vials were then connected to an olfactometer which could bubble air through the odor vial, which in turn, pushed odorized air onto the antenna. The air pressure was adjusted so that no visible movement of antenna occurred and mechanosensory responses were minimized (flowrate of .9 l/min).

Neuronal responses were recorded via an Axoclamp 2B (Axon Instruments) in bridge mode, with 3 KHz sampling rate. Data was acquired using a National Instruments data acquisition board (BNC-2090), and Labview software on a standard Dell

workstation running Windows 2000. This system was also used to trigger delivery of odor pulses through the olfactometer and current pulses for cell iontophoresis through the Axoclamp.

For each type of neuron (extrinsic cells, PNs, and KCs), a slightly modified iontophoresis protocol was used. The pulse protocol for all three was a .5 s hyperpolarizing current pulse at 1 Hz. Extrinsic cells were filled using a 1-2 nA current for 20-30 minutes. PNs were filled for 30 minutes with a 6-7 nA current, and KCs were filled 2-7 minutes with a 1-2 nA current. Because extrinsic and projection neurons are large cells, the dye was allowed to diffuse 30 minutes before fixation. KCs dye-diffusion time was 10 minutes.

Following diffusion, the brain was fixed *in situ* using a 5% solution of paraformaldehyde in water (EM-grade 10% stock solution from Electron Microscopy Sciences) for 30 minutes at room temperature. Brains were then washed overnight at 4 degrees Celsius in distilled, de-ionized water and then dissected out of the head capsule. Finally, the tissue was dehydrated through a series of ethanol solutions of increasing concentration (from 50% by 10% increments to 100%) and cleared in methyl salicylate (Electron Microscopy Sciences). Brains were mounted whole on glass slides in methyl salicylate, coverslipped and sealed with clear nail polish (Revlon) for imaging. Slides were kept at -20 degrees Celsius for up to 24 hours before imaging.

2.3 Imaging

Brains were imaged using a confocal system (Caltech Biological Imaging Center) with a 12-bit scan module on a Zeiss Axiovert 2000. Data was acquired using the Zeiss LSM 3.0 software on a Dell workstation running Windows NT. A laser line at 488 nm was used to image LY and AF-488, and light was gathered through a 505 nm highpass filter. AF-546 was imaged with a 543 nm laser line, and light was gathered through a 560 nm highpass filter.

Typically, using a Zeiss 20x objective with a numerical aperture of .75, optical slices of .9 μm were used to scan through the brain, creating between 200 and 300

digital images for an average brain size. For some stacks, a digital zoom of 2 was applied through the LSM software.

Stacks were viewed and converted to TIFF files using the open source software, ImageJ (<http://rsb.info.nih.gov>). Ray tracing was used to create 3D-reconstructions in ImageJ using the 3D-project function.

To obtain a clearer view of cellular morphology, cells were isolated from background in images using MATLAB (The Mathworks, Inc.) in an automated process as described below (section 2.4). Series of stacks following the extent of an individual neuron (between 4 and 10 stacks, each with 50-100 images) were stitched together in three axes using MATLAB (code is reproduced in Appendix B). Brain structures were traced from background by hand using the paintbrush function in the Scion Image software (Scion Corp., trace-stack macro written by Charles P. Daghljan) and exported to ImageJ.

For volume renderings, traced cells and brain structures from each brain were exported to the software Imaris (Bitplane AG Scientific Solutions) as separate channels of the same image. These could then be reconstructed as 3D images using the Surpass function which allows the generation of a volume image. Brain structure reconstructions were filtered with the Gaussian filter within the Surpass function to create smoother surfaces. Pictures of these images were saved as TIFF files and prepared for publication using Photoshop.

2.4 Isolating Filled Neurons

The insect brain, especially that of the locust, has a high degree of autofluorescence even without any stain, and even when the tissue has been cleared. This background fluorescence occurs when the tissue is excited by a 488 nm laser light, and to a lesser degree, by a 543 nm laser light. For this reason, in order to adequately view and reconstruct cells filled with the aforementioned dyes, it was necessary to extract the cell from the background in the digital images. Though this is done easily by the human eye, for the number and complexity of the cells in this study, this activity

is too time consuming to be done manually. Therefore, a program was designed to automate the tracing process using MATLAB equipped with the imaging toolbox.

The basics of this algorithm (named *nimage*) are illustrated in figure 2.1. The code itself is reproduced in Appendix A. The stack of confocal images is loaded into MATLAB as a single 3D matrix, and all operations are performed in 3D on the entire stack. The user defines several variables and the algorithm's starting pixel(s). These values are used to determine if a certain pixel, p in the set of pixels to search P , is part of the neuron. At the outset, P contains only the user-defined starting pixel(s). Three user-defined values determine the distance of a shell of pixels from each p (N , M and R in figure 2.1). The mean of the values of the pixels that define this shell is then calculated. If the value of each p is 2.5 to 3 standard deviations above the mean calculated for its shell, then p is ruled to be part of the cell and becomes a 'hot' pixel, h , and part of the set of all hot pixels, H . The standard deviation multiplier, X , is determined by the user at the outset of the algorithm. The set of neighboring pixels to each h is then defined as the new set of search pixels P . This neighborhood, B , is a cube whose side length is defined by the user. The algorithm progresses in this way, searching pixels it defines as 'hot', until no more new hot pixels are found. The user then has the choice of terminating the program or adding new search pixels. This is useful when there is a break in the data, but the cell can clearly be seen by the eye to continue at a point not within the neighborhood of any currently hot pixels.

The combination of a user defined search neighborhood and ability to add new pixels to the search covers all combinations of breaks in data. Larger values of B increase the search space to compensate for missed pixels between points in the cell; however, if there are only a few such 'missed' points, it is more time efficient for the user to set a lower value for B and enter these missed points manually as new search pixels. If the value of B is too high, search time increases, and more noise will be picked up by the algorithm as it searches too far outside the cell.

The determination of the search shell defined by N , M and R allows each pixel to be judged based on its immediate surroundings, and avoids other neighboring pixels within the neuron. Certain parts of the brain tissue (like the calyx) are especially

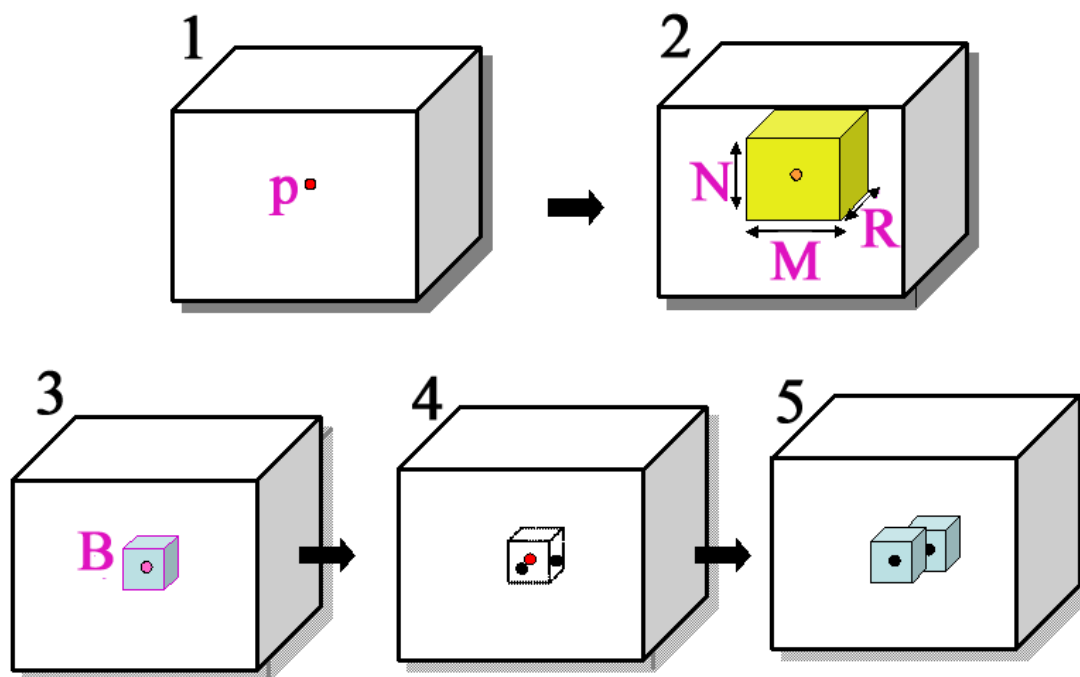


Figure 2.1: A flowchart of the *nimage* algorithm. The cube outlined in black represents the image stack. p in step 1 is the starting pixel. In step 2, the yellow shell around p determines pixels to consider as background for comparison to p . In step 3, the blue cube B defines the neighbors of p to be searched. In step 4, two pixels in B were searched and are found ‘hot’ using 2. Step 5 shows the neighbors of those two pixels that will be searched next. The algorithm continues to search neighbors of hot pixels until no more hot pixels are found and no more p are entered by user.

dense and have high autofluorescence, and this method allows for the simultaneous searching of pixels within such areas and areas with less intrinsic fluorescence. The user-defined standard deviation multiplier allows for adjusting to each particular stack and its level of noise.

The idea of this algorithm is to combine human expertise with the computational power available today. Visual judgment can be made by the user to the extent that s/he wishes, and the repetitive computation can be left up to the processing unit.

Once all hot pixels have been defined, the binary hot pixels matrix, H , can be used as a mask to extract original pixel values. This preserves the relative staining intensities throughout the cell which can be important in morphological analysis (see following chapters).

This algorithm has allowed the extraction of a neuron with very little labor on the part of the experimenter; one KC can be extracted with as little as 10 minutes of user interaction and 5 hours of computation. A comparable tracing of the cell by hand takes roughly 8 hours of labor. Figure 2.2 shows a comparison for a pair of filled KCs; part (a) shows the ray-traced 3D reconstructions on the raw data and part (b) shows the same reconstruction on the extracted data for the same pair of cells. Notice that the background structures are completely eliminated in (b), and the cell dendrites are clearly visible.

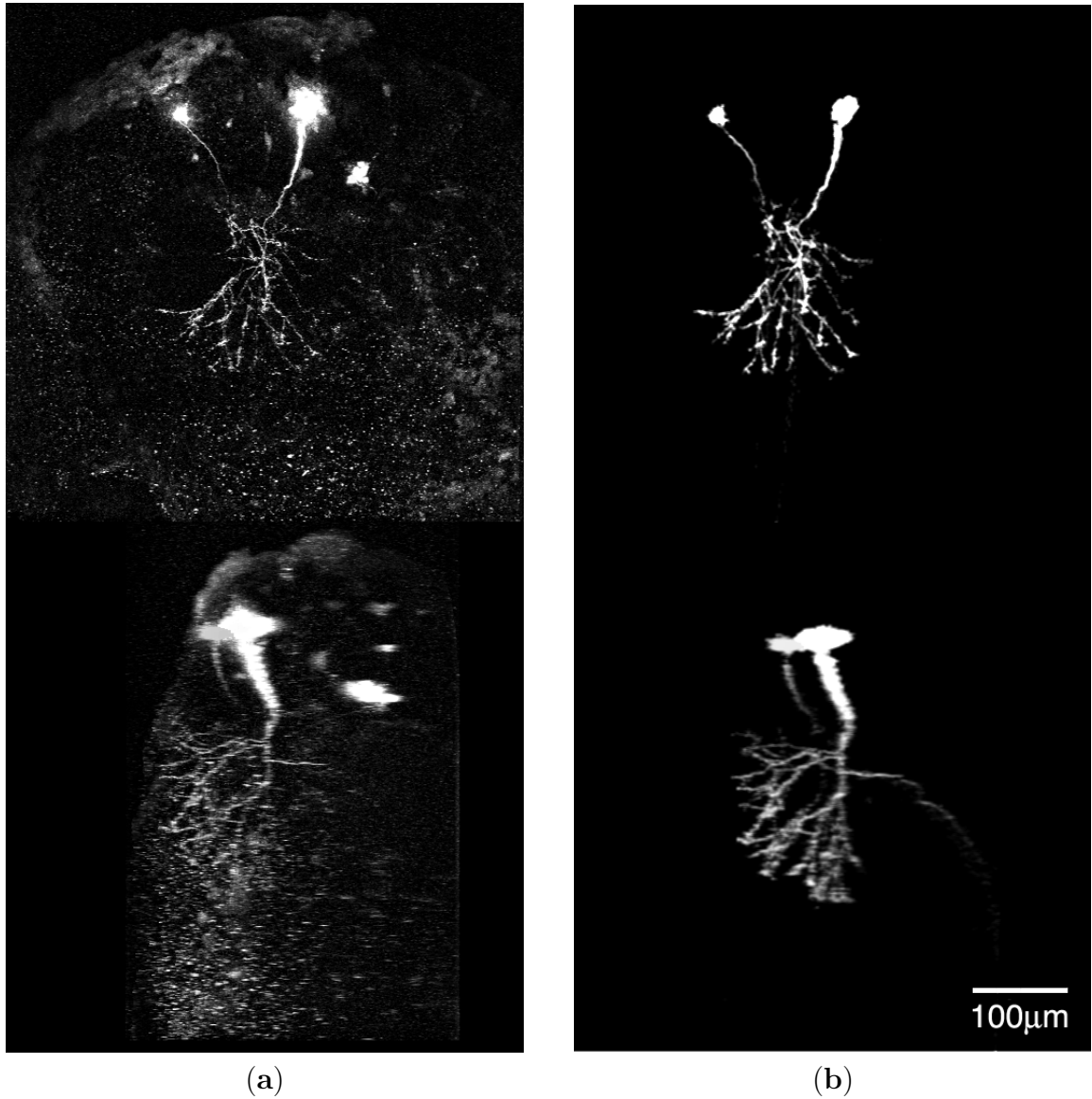


Figure 2.2: Ray-traced reconstructions of a pair of KCs filled with LY. (a) Ventral and lateral views of the reconstructed raw data. (b) Ventral and lateral views of the reconstructed *nimage* extracted data.