Analyzing Cultured Purkinje Cells from GFP Mice Using Sholl Analysis and ImageJ Software

Presented to the faculty of Lycoming College in partial fulfillment of the requirements for Departmental Honors in Biology

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Lusha Xu

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I. Introduction

The Purkinje cell is one of the most wellstudied nerve cells; however the morphological study of cultured Purkinje cells from Green Fluorescent Protein (GFP) animals with real-time imaging is rarely reported in the literature. The Purkinje cells from the GFP mice are used in this experiment because they can glow green under ultraviolet light, making them easy to identify in co-culture with non-GFP cerebellar cells from wild-type mice. This should allow us to capture the images and study the growth of living Purkinje cells *in vitro* over time without having to kill the cells to allow antibody staining.

Purkinje cells are neurons located only in the cerebellum, and they produce the main output from the cerebellum to coordinate movement. The cerebellum is located in the brain under the cerebral hemispheres. It controls locomotion, balance, and eye movement. Improper development of the cerebellum could lead to symptoms such as ataxia (Sugawara et al., 2008). Ataxia can be caused by gene, neural degeneration and many other factors. Symptoms include poor coordination of limbs, difficulty pronouncing words, and abnormal eye movements. The age of affected patients can vary (Schulz et al., 2010). Ataxia patients have difficulty in controlling their movements and performing many other everyday life functions (Manto, 2009).

The Purkinje cell in the GFP mice is used in this study because it contains Green Fluorescent Protein which will glow green under ultraviolet light (Okabe et al., 1997). Green fluorescent protein was first discovered in the jellyfish *Aequorea victoria*. Many GFP transgenic animals have been produced including the GFP transgenic mice used in this experiment. The GFP mice for this study were first produced by Okabe et al. in 1997 in Japan (Fig. 1). Okabe et al. modified the original GFP gene into an Enhanced Green Fluorescent Protein (EGFP) gene by changing the codons in jellyfish into more preferable codons for the same amino acid in mammals. The GFP expression in EGFP transgenic mice increased four-fold compared to the unenhanced GFP transgenic mice. In this honors thesis, the EGFP will be referred to as GFP.

To make the GFP mice, Okabe et al. started with a cDNA of the GFP gene which only includes the axon sequences that are used for making the protein. Primers 5′-ttgaattcgccaccatggtgagc-3′ and 5′-ttgaattcttacttgtacagctcgtcc-3′ were used to amplify the cDNA in a Polymerase Chain Reaction (Okabe et al., 1997). Then the sequence was cloned into a pCAGGS expression vector containing the chicken beta-actin promoter and cytomegalovirus enhancer using restriction enzymes BamHI and SalI. The recombinant plasmid was then injected into fertilized mouse eggs (Okabe et al., 1997). Then fertilized eggs were transferred into the uterus of female mice. After the transgenic mice were born, all tissues glowed green under ultraviolet light except for the red blood cells and hair, because such cells lack nuclei, thus cannot express the GFP protein (Okabe et al., 1997). In our mouse facilities, at Lycoming College heterozygous GFP mice and homozygous wild-type mice were used to produce the FI generation, making

approximately one half of the generation heterozygous for the GFP gene. No homozygous GFP mice were bred because of their poor survival.

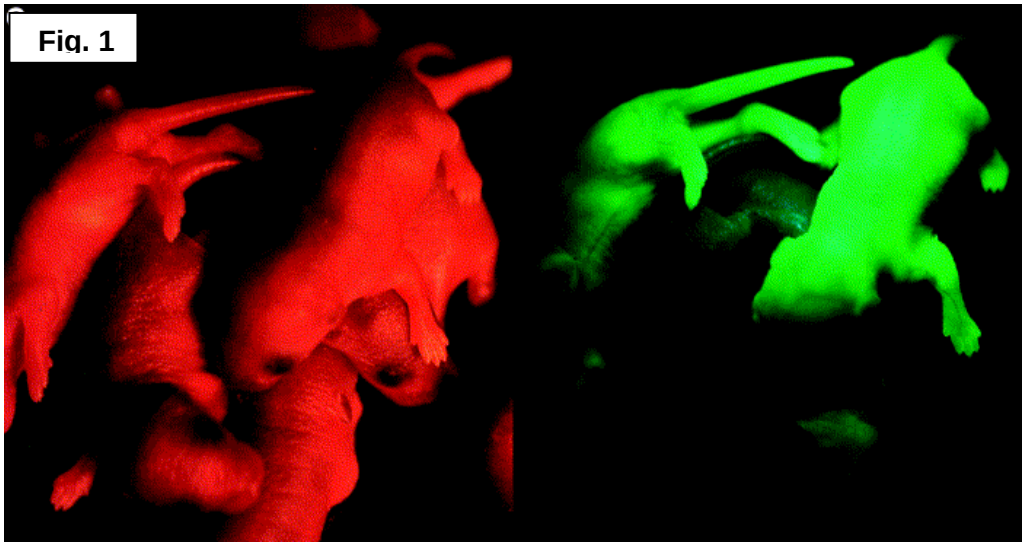


Figure 1. Mice pups not under UV light (left) and mice pups under UV light. (Right) GFP mice pups appear to be green under UV light (Okabe et.al., 1997).

The Purkinje cell communicates with other cells and its environment frequently using its functional components. The three main components of the Purkinje cell that could help it to communicate with its environment are the axon, the dendrites, and the dendritic spines. In a well-developed Purkinje cell (Fig. 2), the axon is a thin afferent process which includes a growth cone and can transmit signals to the next cell in the circuit. Dendrites are branch-like structures that mostly receive signals from the environment and can also send signals to other associated cells. The dendritic spines are needle-like, mushroom-like, or thorn-like structures where dendrites connect with other neurons and axons and where excitatory synapses occur (Baptista et al., 1994; Mason et al.,1997; Sorra and Harris., 2000).

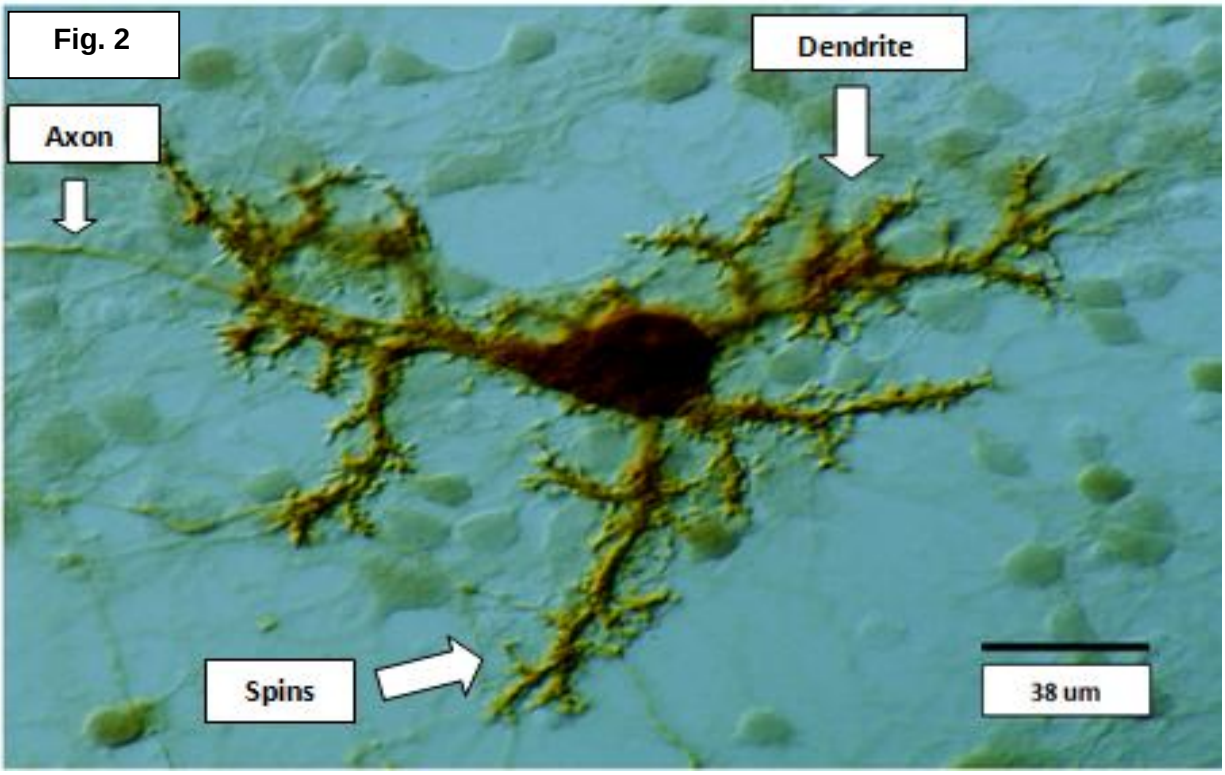


Figure 2. Mature Purkinje cell stained with anti-calbindin in cell culture. (Morrison Unpublished data)

In order to understand more about the Purkinje cell dendrite, the developmental stages of the Purkinje cell must first be understood (Fig. 3). Purkinje cell division stops by embryonic day 14 (E 14). The development of the Purkinje cell can be divided into 4 stages. First, the Purkinje cells migrate under the external granular layer, the olivocerebellar climbing fibers attach to the Purkinje cell body, and the axons start to develop. This stage has already taken place by the 17th embryonic day (E17 day) in the mice. At 3 postnatal days (P3), neurites start to develop around the Purkinje cell soma and granule cells develop axons, which later become parallel fibers. In the third stage (7 postnatal days, P7), apical dendrites develop and extend and dendritic spines start to develop. Also, climbing fibers begin to make contact with the dendrites. In the final stage, well-developed Purkinje cell dendrites develop spines where synapses with the parallel fibers occur by postnatal 14 days (P14 days) and these continue to develop though postnatal 21 days (P 21 days) (Mason et al., 1997).

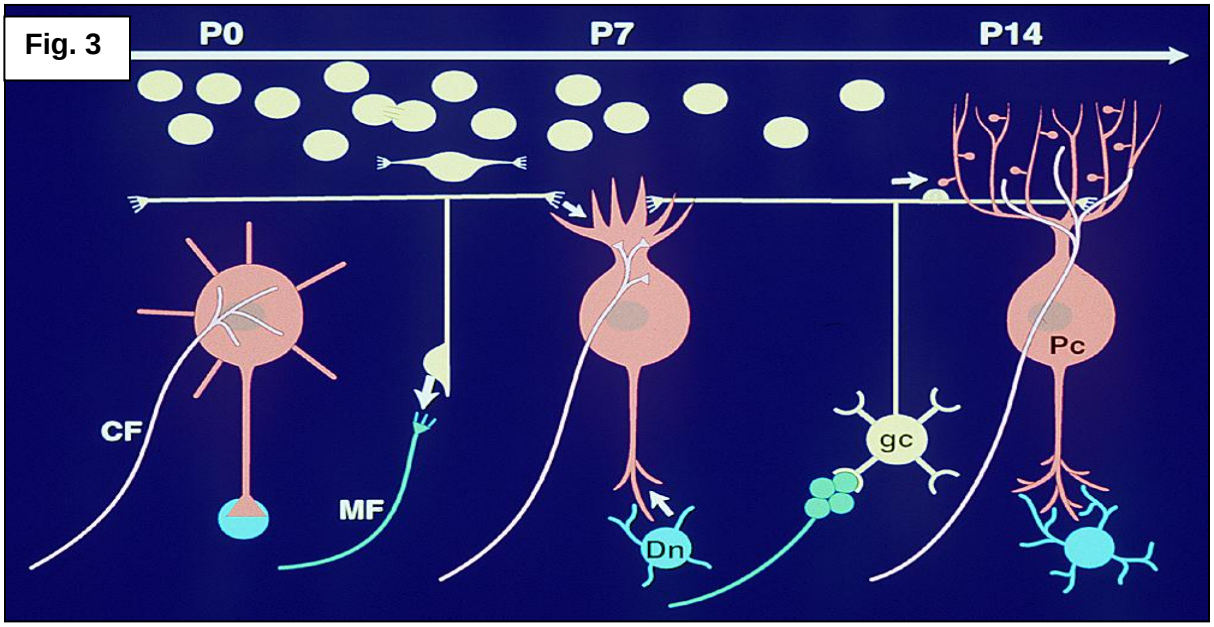


Figure 3. Purkinje cell development in the cerebellum. (Morrison unpublished data)

A Purkinje cell needs to communicate with its environment at all stages of development. Purified and isolated mice Purkinje cells do not survive well *in vitro* and only develop axons. However, when purified Purkinje cells are cultured with granule cells, the survival of the Purkinje cells increases and normal axons, dendrites, and spines form (Baptista et al., 1994). Even though experimental results show that granule cells contribute to Purkinje cell survival, the signals and mechanisms are still unknown, BDNF/TrkB might be part of this mechanism. In another experiment, when the Purkinje cells were cultured along with different growth factors, NT4 (Neurotrophin 4) and BDNF (Brain-derived neurotrophic factor) were two types of growth factors which increased the survival of Purkinje cells compared to the control and other growth factors. However, when BDNF was added to the Purkinje and granule cell coculture, Purkinje cells survived poorly. Different molecules such as trkB-IgG were used to block BDNF signaling, thereby increasing Purkinje cell survival. The conclusion was that although low level of BDNF boosted development Purkinje cell, excess BDNF could be toxic to Purkinje cells and cause cell death (Morrison and Mason, 1998).

DCC (Deleted in Colorectal Cancer) is one receptor for netrin, and they could also have an effect in mouse Purkinje cells during development (Morrison unpublished data). The netrin family includes: netrin-1, netrin-3, netrin-4, and netrin-G. Those signaling factors are mostly found in the midline of all bilateral symmetrical animals, and they can act as attractants or repellents depending on the signal receptor of the cell and the developmental stage. Netrin-1 is the most studied member of the netrin family. It is highly expressed in the floor plate of the spinal cord and also in parts of the cerebellum (Alcantara et al., 2000). Netrin-1 can attract many different types of axons and simulate their outgrowth. Netrin-1 can also repel axons, such as those that are not allowed to

cross the midline, like parallel fibers (Alcantara et al., 2000; Barallobre et al., 2005; Guijarro et al., 2006). DCC is highly expressed in CNS and epithelial cells of some tissues, and it can interpret the netrin signal as an attractant or repellant. The expression of netrin-1 and DCC were both absent in adult mice, which indicates that netrin-1 and DCC only have an effect on the axon and neuron migration during the early developmental stages (Livesey and Hunt, 1997). Recent studies in mice show that when netrin1 signaling is blocked via knockout, the Purkinje cells have less well-developed dendrites (Morrison unpublished data, Fig. 4).

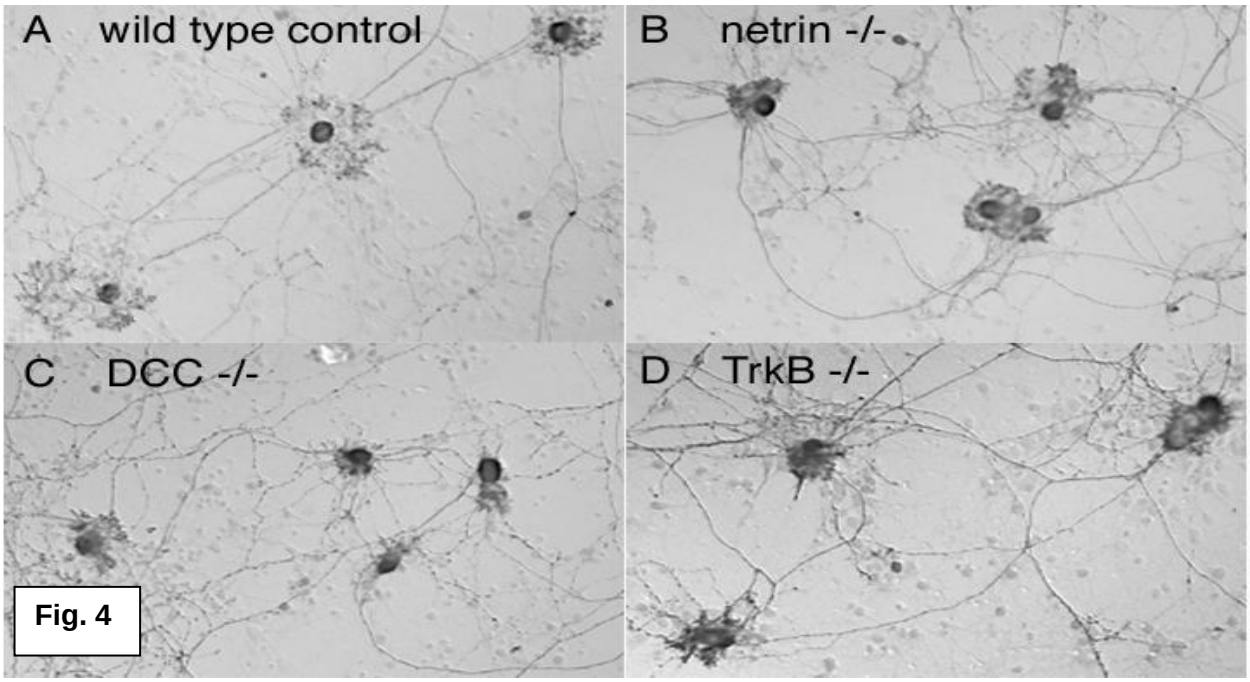


Figure 4. Mice Purkinje cells in cerebellum culture. (A, wild type Purkinje cell control; B, homozygous netrin Purkinje cell cutlure; C, homozygous DCC Purkinje cell culture; and D, homozygous TrkB Purkinje cell culture (Morrison unpublished data. magnification: 10X eye piece and 63X objective).

The homozygous mutant strains of mice deficient in netrin, DCC, and TrkB will have underdeveloped Purkinje cells, which leads to death in early development of the mice (Moore et al., 2007; Morrison and Mason, 1998; Morrison unpublished data). Ataxia patients have abnormal Purkinje cells in their cerebellum like the Purkinje cells in mutant mice, which may cause them to have uncoordinated movements (Manto, 2009). In order to learn more about cerebellum disease associated with Purkinje cells, the dendrite morphology needs to be studied and analyzed using DCC and netrin mutant mice.

In order to analyze Purkinje cell morphology, it is important to identify the Purkinje cell first. Staining is an important technique to identify the Purkinje cell. The Purkinje cell contains a cytoplasmic protein calbindin which cannot be found in other cell types

(Mason et al., 1997). After mixed cerebellum cultures are made, an antibody against calbindin D-28K derived from rabbit is used as the primary antibody to bind calbindin in the Purkinje cells (Morrison and Mason, 1998; Schrenk et al., 2002). Then a secondary antibody such as anti-rabbit antibody with a colored or fluorescent substrate is applied to the culture which will bind to the primary antibody (Morrison and Mason, 1998; Schrenk et al., 2002). After a series of staining steps, the Purkinje cell will have a distinct color among other cells in the culture, which makes identification and analysis convenient.

Even though specific staining can help with identifying the Purkinje cell, it will also kill the cell and prevent the study of the living cell in culture. Long-term observation of the living Purkinje cell from GAD67-GFP mice cultured with non-GFP mouse cerebellum culture *in vitro* has been done to study the extension and retraction of the Purkinje cell dendrites between P10 to P25 days (Tamamaki et al., 2003; Tanaka et al., 2006). Fluorescent images of the cell culture were acquired at 10, 15, 20, and 25 days *in vitro* using the Zeiss confocal laser scanning microscope LSM510 (Tanaka et al., 2006). The Zeiss LSM Ver. 3.0 software was used for analyzing the image, cell morphology, and measuring the length of dendrites, the number of dendrites was manually counted, and a Student‟s t-test was performed for the statistical analysis (Tanaka et al., 2006). At P10 days, Purkinje cells had several primary dendrites, and the level of GFP expressed in the Purkinje cells, as well as the morphology was distinguishable among other cells. This study indicated that both extension and retraction of the Purkinje cell dendrites occurred during P10 to P15; usually one primary dendrite elongated while the others shortened, which was consistent with the fact that each mature Purkinje cell only had one primary dendrite *in vivo*. This study also concluded that the location of the dendrites on the cell showed no relation to its growth, but dendrites with shorter length and fewer branches had a tendency to retract. Therefore, the final morphology of the Purkinje cell was attributed to both extension and retraction of the primary dendrites (Tanaka et al., 2006).

After the images are collected, an efficient and affordable method is needed to analyze the Purkinje cell morphology, such as Sholl analysis. Sholl analysis was invented by D. A. Sholl in 1953. It is a method which can study neuron morphology quantitatively and efficiently (Sholl, 1953). Image J software could be used to process the cell image or drawing for Sholl analyses (Image J). Concentric circles centered on the cell soma are drawn on the cell (Fig. 5). The intersections of the circles with the dendrites are counted, and the relationship between the numbers of intersection with the radii of the circles is graphed. Statistical tests such as ANOVA, F test, and Student‟s t-test can be performed to demonstrate the correlation between the numbers of intersections and the radii of the circles (Fig. 6 & 7) (Cook and Wellman, 2003; Martinez-Tellez et al., 2005; Ristanović et al., 2006; Vega et al., 2004).

Fig. 5

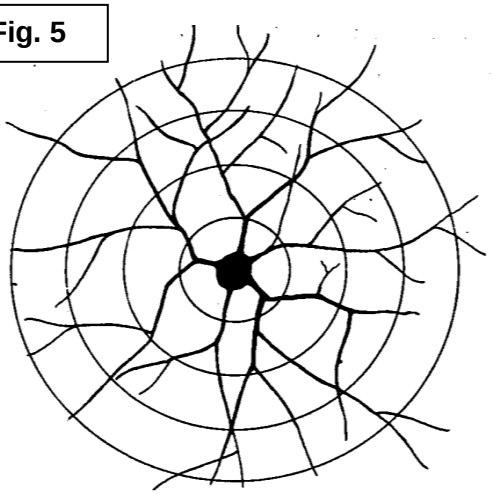


Fig. 6

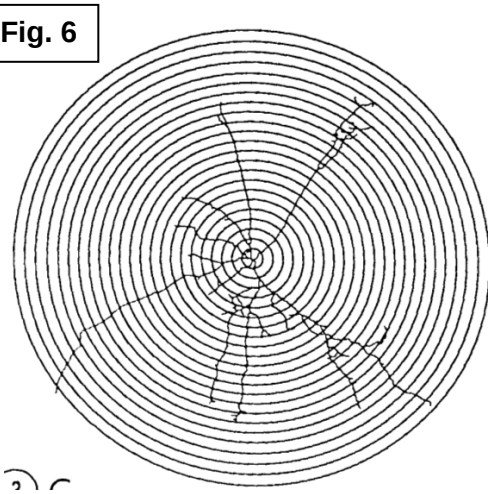


Figure 5 & 6. Fig. 5 (left): Drawing of a neuron cell study by the Sholl analysis. Concentric circles intersect with the dendrites. (Sholl, 1953, text Figure 2) Fig 6 (right): Skeletonized neuron image study by the Sholl analysis. (Neale et al., 1993, Figure 3, C)

Fig. 7

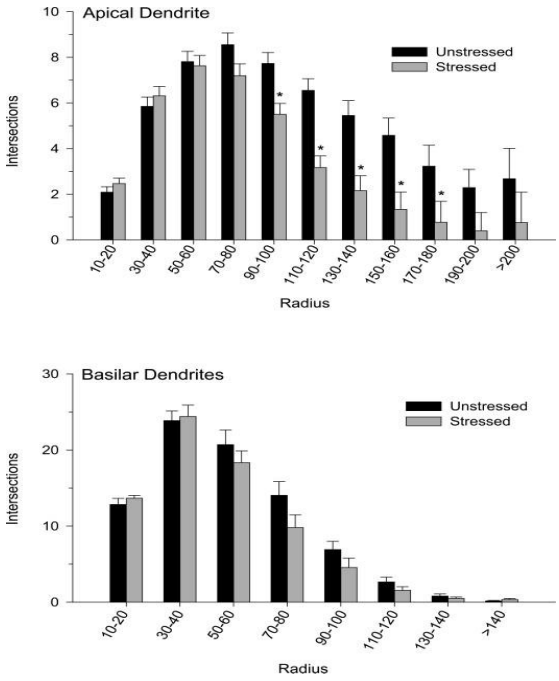


Fig. 8

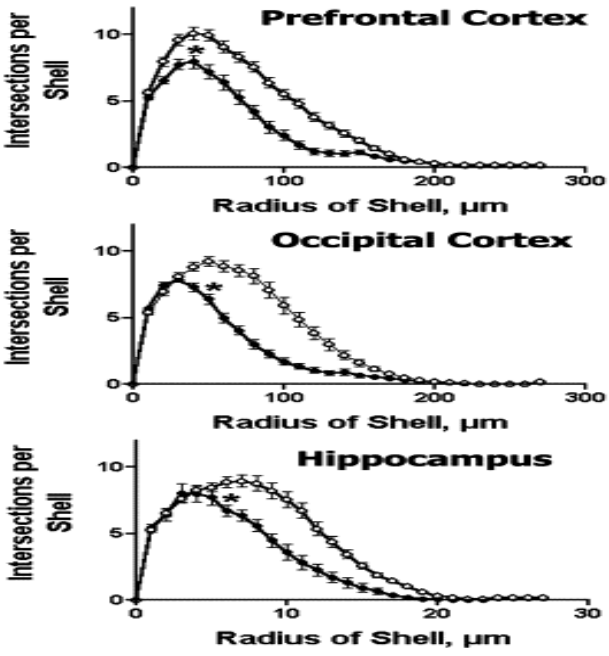


Figure 7. Sample of graphs that will be used after analyzing the relationship between the number of intersections and the radius of the circles by statistic tests. Left: histogram analyzed by two-way repeated-measure ANOVAs test. Right: Scattered plot analyzed by 2-tailed Kruskal-Wallis and Mann-Whitney tests. (Cook and Wellman, 2003; Martinez-Tellez et al., 2005)

The initial goal for this experiment is to make mouse mixed cerebellar culture, fix and stain the culture, and then capture images of the culture. The final goal for this

experiment is to culture Purkinje cells from the GFP mice with mixed cerebellar cells from wild-type mice, as well as to study the growth of the GFP Purkinje cells in culture.The images of the Purkinje cells from GFP mice will be taken using a Nikon ET 2000 microscope, and the morphology of the neurons will be analyzed by Sholl analysis using Image J software. It is important to study and analyze the Purkinje cell in order to gain greater knowledge about the cerebellum. This experiment will also allow our lab to conduct further studies on living Purkinje cells, which will extend the lab‟s previous work with fixed, stained Purkinje cells.

II. Methods and Materials

Media Recipes

CMF-PBS: Per liter: NaCl, 8.00 g; KCl, 0.30 g; glucose, 2.00 g; $NaH_2PO_4.H_2O$, 0.50 g; KH_2PO_4, 0.25 g; $NaHCO_3$, 2 ml of 2% stock; phenol red, 0.5 ml of 0.5% solution; pH to 7.4; filter-sterilize.

BME stock solution: powdered complete BME mix for 1L (Gibco #41100); 980 ml dH2O; 20 ml 1.0 M NaHCO3; pH to 7.4; filter-sterilize. Or: Mediatech (800-Cellgro) cat 50-005-PB (Basal Medium Eagle with Earle‟s salts and L-glutamine without sodium bicarbonate) 9.19 g in 980 ml water, add 1.68 g sodium bicarbonate, pH to 7.4 bring volume to 1L total; filter-sterilize.

Trypsin solution: Per 15 ml: 150 mg trypsin; 15 mg DNAse; 15 ml CMF+ Mg (0.15 g $MgSO_4.7H_2O$ in 100 ml of CMF-PBS); 90 ul 1 N NaOH; filter-sterilize. (Box in -20 refrigerator)

DNAse solution: Per 15 ml: 7.5 mg DNAse; 14.5 ml BME stock solution; 0.15 ml 10% glucose; filter-sterilize. (Box in -20 refrigerator)

Medium with horse serum: Per 100 ml: 84.0 ml BME stock solution; 4.8 ml 10% glucose; 200 ul penicillin-streptomycin; 160 ul L-glutamine; 10.0 horse serum (Gibco # 16050); 71 ul 10% NaCl; 769 ul dH2O; pH to 7.4; filter-sterilize.

Serum-free medium: Per 100 ml: 1 g bovine serum albumin (Sigma A-9418); add 93 ml BME stock solution, 4.8 ml 10% glucose, 1.0 ml serum-free supplement (Sigma 1-1884), 200 ul penicillin-streptomycin, and 70 ul L-glutamine; 79 ul 10% NaCl; 851 ul dH2O; pH to 7.4; filter sterilize.

Buffers and Fixatives Recipes

0.2 M Sorensen‟s phosphate buffer: Make 0.2 M monobasic phosphate stock solution (27.59 g/L $NaH_2PO_4.H_2O$). Make 0.2 M dibasic phosphate stock solution (28.39 g/L NaHPO4). Add 1 part monobasic to 4 parts dibasic for 0.2 M Sorensen‟s; pH 7.2-7.3.

4% parafomaldehyde in 0.1 M phosphate buffer: Make 8% paraformaldehyde in distilled water by heating to 60 ℃ while stirring; do not exceed 60 ℃ . Add drops of 10 N NaOH until clear (usually 1-4 drops per 100 ml). Cool to room temperature. Add 1:1 0.2 M Sorensen‟s phosphate buffer to 8% paraformaledhyde. (We usually make larger amounts of 8% paraformaldehyde to aliquot and store at 20 ℃ . Thaw in heated beaker of water until clear, and dilute with 0.2 M buffer.

Tok PBS: Per liter: NaCl, 8.0 g; KCl,0.2 g; Na_2HPO_4, 1.15 g; $NaH_2PO_4.H_2O$, 0.23 g; pH 7.3.

Immunocytochemical staining recipes

Preblock: 0.05% triton and 10% normal goat serum dilute in 1x Tok-PBS.

Mock Solution: 0.05% triton and 1% normal goat serum dilute in 1x Tok-PBS.

Primary antibody: 0.05% triton, 1% normal goat serum and the needed concentration for primary antibody dilute in 1x Tok-PBS.

Secondary antibody: 0.05% triton, 1% normal goat serum and 1:3000 anti-rabbit (G): goat- anti- rabbit IGG Alexa 488 dilute in 1x Tok-PBS

Mating and Plug Checking

1. Select appropriate animal for mating. Minimum eight months old, maximum 18 months old.
2. Put one male mouse in his own cage the night before the breeding. Add with two female mice in the same cage, write down the birth date of the mice, and the date the mating is set up.
3. Use autoclaved (sterile) probe to check for plug early in the next morning. (During mating, the male mice place a protein substance in female mice‟s vagina. The plug will prevent the female from mating with other males, and increase the chance of fertilization. But the plug will dissolve within 10 to 16 hours.)
4. Check plug by inserting the probe into the female‟s vagina, if you feel resistance before the bend on the probe reaches the labia, then the female is plugged.

5. Separate plugged female from the male. Label the number of females that are plugged and the date of the plug found on the cage card and the calendar. The gestation period for mice is approximately 19 days. Write down when the pups will be born on the calendar.
6. Not all plugged female mice will be pregnant (about 75% of them will be pregnant), check the plugged female a few days in advance to confirm whether they are pregnant, in order to prepare for practice dissection and making cell culture.

Prepare Lab-Tek chamber slide

Coat LabTek chamber slides with sterile 500 ug/ml poly-D-lysine in water overnight at 4 ℃ . Wash with sterile distilled water three times, and then air-dry in the hood before making cerebellar cultures.

Dissecting procedure

1. Turn on the hood and use 75% ethanol to wipe down the hood. Let the hood run at least 15 minutes before working in there.
2. Make a pup warmer by putting down paper towel, then Kimwipe in a small paper box. Put the box on a heating block set for 37 ℃ . (We try to decrease their stress level by mimicking the environment when the pups are under the protection of their mom. If the pups are under stress, their cortisol level will increase which can alter the result of Purkinje cell development in the cell culture.)
3. Put an ice bucket, dissecting microscope, dissecting light, glass pipet, Petri dish, and autoclaved dissecting tools in the hood. Put the CMF-PBS 50 ml tube in the ice bucket. Also prepare a small sterile tube and fill it up with CMF-PBS to use later.
4. Take the mice pups from the mouse house and put them into the pup warmer. Do not take them away from the female mouse if you do not plan to dissect right away. Taking them away from the female will cause the pups become stressed which will decrease the survival of the Purkinje cells.
5. Capture the pup by its lower back, position its head towards the curved-out side of the curved scissors. Position the pup"s head deep into the curved scissors; make one quick cut to decapitate the pup.
6. Use the curved forceps to hold the pup"s nose by the left hand. Use one side of the straight scissors insert into the spinal channel and to the front of the head. Make a cut under the ear, over the eye on each side of the head.
7. Use the straight forceps to peel off the top of the skull which contains the brain.

8. Put one big drop of CMF-PBS in the Petri dish. Use the straight forceps to gently tease out the brain into the CMF-PBS. Always work above the fluid.
9. Put the Petri dish under the dissecting microscope. Use straight forceps to orient the brain to make it ventral side up.
10. Use the forceps in the left hand to hold the cortex, use the forceps in the right hand to peel off the pia on the upper and lower edge of the cerebellum on ventral side.
11. Reposition the brain dorsal side up. Use the forceps in the left hand to hold the cortex, use the forceps in the right hand to gently clean off the pia on the cerebellum without stabbing the brain.
12. Position the forceps in the right hand vertically and make a cut at the hind brain. Use forceps in the left hand to strip off the hind brain.
13. Repeat the same process in step 12 to separate the cortex. Now only a part of the hind brain (peduncle) is attached to the cerebellum.
14. Clean off any pia that is left on the cerebellum. Orient the left brain matter anterior side up. Use the forceps in the left hand to hold the peduncles, use the forceps in the right hand to cut the peduncle by placing one end of the forceps in the hole between the cerebellum and the peduncles, and the other end between the cerebellum and middle position one of the peduncles. Repeat this procedure for cutting the other peduncle.
15. Flip the left brain matter posterior side up. Repeat the same procedure in step 14 and cut the parts of peduncle that are still connected to the cerebellum.
16. Clean off any pia that is left on the cerebellum. Then transfer it into the small tube on ice with CMF-PBS.

Make mixed cerebellar cultures

1. Thaw trypsin, DNAse and horse serum medium (HSM) in a 37 ℃ hot water bath before beginning the dissection.
2. After dissecting out cerebella from animals, collect in ice cold CMF-PBS until all cerebella are ready.
3. Remove CMF-PBS; add 1 ml trypsin solution. Incubate three minutes at room temperature. Prepare small, medium, and large fire-polished pipettes ready, make sure HSM is being equilibrated at 37 ℃ , check centrifuge rotor and balance buckets.
4. Remove CMF-PBS; add 1 ml DNAse. Triturate with large, medium, and small fire-polished Pasteur pipettes until a single cell suspension is obtained, avoiding

creating bubbles. (Small- 300 micro meter, medium-600 micro mete, large-polished enough to smooth edges)

5. Set timer and spin mixture in IEC tabletop centrifuge for three minutes on setting three. Prepare 30 um nylon mesh filter and syringe.
6. Remove the supernatant, add DNAse to fill the narrow length of a 9 inch Pasteur pipette. Add CMF-PBS and triturate again with large, medium and small fire-polished pipettes. Pass mixture though a nylon mesh filter to eliminate chunks of debris. Wash out the original tube with 1-2 ml of CMF-PBS, and pass this wash through the filter.
7. Spin the mixture in IEC tabletop centrifuge for three minutes on setting three. Prepare hemacytometer by cleaning it with water and 70% ethanol. Allow ethanol to evaporate while you prepare a uniform suspension of cells.
8. Remove a sample of cells using a sterile unpolished pipette. Place the tip of the pipette on the loading groove and allow the sample to enter the hemacytometer by capillary action. Only load as much as will enter by capillary action.
9. Count the cells in the hemacytometer through the microscope. View the cell at 10X objective magnification x 10X eyepiece magnification, focus to see cells and hemacytometer etched lines.
10. Count all cells in four squares of the 16-square areas that are in diagonal. Count from the top left side of the outer etched lines only, do not count cells laying on the bottom or right outer etched lines.
11. By this procedure, the counted volume is 1 mm x 1 mm x 0.1 mm. To calculate cells per ml: 0.1 cm x 0.1 cm x 0.01 cm = 0.0001 cm^3 = 0.0001 ml. Raw count x dilution factor x 10^4 = cells/ml in your original cell suspension.
12. Add appropriate volume of horse serum medium into the original cell suspension so for every 100ul of solution, there are approximately 330,000 cells present.
13. Plate 100ul of cell suspension in each Lab-Tek well coated with poly-D-lysine. Grow cell culture in a humidified 37 ℃ incubator with 35% carbon dioxide concentration (in air).
14. Replace the horse serum medium with serum free medium the next day. Change medium every 3-4 days until cell culture are ready for fixing.

Fixation of Lab-Tek chamber slide cultures

1. Prepare fixative: 4% paraformaldehyde in 0.1 M Sorensen‟s phosphate buffer.

2. Set timer for 30 minutes and start.
3. Add two drops of fixative to each culture well.
4. Repeat step 3.
5. Remove solution from each well, leave a little at the bottom of the well to prevent drying of cells.
6. Fill each well with fixative.
7. When timer sounds, remove fixative from all wells, and add 1X Tok-PBS at room temperature. Wait 10 minutes.
8. Repeat step 7 twice more.
9. Fixed culture can be refrigerated in Tok-PBS if immediate staining is inconvenient. Otherwise, remove the well dividers and proceed with staining.

Immunocytochemical Staining for Lab-Tek Cultures

1. Break off the top part of the Lab-Tek chamber slides, tap slide on paper towel to remove extra fluid. Add 50 ul of preblock onto each cell culture well, incubate at room temperature for at least 30 minutes.
2. Tap slide on paper towel to remove the preblock, and add 50ul of primary antibody solution. To control sample add 50ul of mock solution. Incubate overnight at 4 ℃ .
3. Wash slides in a tray with 250 ml Tok-PBS, 10-15 minutes each time and repeat it for two to three times at room temperature.
4. Drain most fluid off the wells, and then overlay 50ul of secondary antibody solution. Incubate at room temperature for 30 minutes.
5. Wash slides same as step 3.
6. Use laser blade and forceps to remove silicon on the slide
7. Mount slides by adding six drop of Gelmount on the slide, then place a thin cover slip on the top. Try to avoid making air bubbles.
8. Store slides properly. If the secondary antibody contains a fluorescent substance, store slide in fridge and away from light source.

Imaging slide

1. Clean slide with Kimwipe and lens cleaning fluid. Mark back of the slide with marker for easy orientation.
2. Turn on the electric equipments in the order of mercury lamp (then push ignite button), microscope, camera power supply, camera, monitor, computer, and back up hard drive. Turning on the mercury lamp will create a electric shock, which can destroy other operating electric supplies.
3. Open Metavue software.
4. Go to “Journal – Taskbar- Load taskbar”
5. Open “Main Control JTB” , then click “Acquire”
6. Place culture slide on the microscope, cover slip side down, use 20X objective first. Send light to eye pieces by adjusting the knob on the right of the microscope to setting “1”. Adjust eyepiece distance by using both eyepieces. Adjust light with knob on the left of the microscope.
7. Send light to the camera by twisting the knob on the right side of microscope to “5”. When capturing an image using 60x objective, clean the slide and objective then put a drop of lens oil on the cover slip, quickly turn it upside down, place the oil drop right on the objective.
8. Turn off room lights (they cause reflection that interfere with the image).
9. For taking fluorescent image: select “fluorescent” setting on the window. Spin the filter wheel to appropriate color. For taking a green fluorescent image, adjust filter to “blue” in order to see green fluorescent cells. Open the shutter when ready to capture the image. For taking a phase image: Filter at top left: put D in and ND out; filter at top right: put GIF out and NCB in. Set condenser on A. Spin the filter wheel so white light can pass though. For DIC image: Filter top left: put D out and ND out; filter at top right: put GIF out a NCB in. Set condenser on N2. Push polarizer in to the left. Adjust polarizer to optical depth perception. Insert DIC disk into the microscope objective.
10. Click “show live” on window, then adjust focus using the fine adjusting knob, click “auto expose” on the window for the computer to suggest the best exposure time for taking the picture. For the fluorescent image, two filters in the fluorescent light source can be used to adjust the light intensity. For phase and DIC image, a knob on the left side of microscope can be used to adjust the light intensity. When all adjustments are made, click “Acquire” to capture the image.
11. Wait for the image to appear, and then shut the shutter to prevent fading of the fluorescent substance.

12. Covert the 12 bit image to 8 bit by clicking “copy to 8-bit image.” Because other computer on campus can only read 8-bit image. Save the image by clicking “file”- “save as,” name the file then save under appropriate folder.
13. After finished acquiring all images, clean the 60x objective using lens tissue with lens cleaning fluid, clean slide with Kimwipe with lens cleaning fluid.
14. Save all images in the back up hard drive. Turn off the electric equipment in the opposite order of turning on. Put the cover over the microscope and camera.
15. Store slides properly. If the secondary antibody contains a fluorescent substance, store the slide in refrigerator and away from light source. (It will fade in the light over time.)

III. Results

In order to decrease the duration of the dissection, practice dissecting is crucial. Two months of practice dissecting was used before making mixed cerebellar cell culture. Dissection was performed at least once every week, for a total of 12 dissection practice sessions, to improve dissecting techniques and decrease the duration of the dissection. Different adjustments were made during practice sessions, such as adjusting instrument position while making the under-the-ear, over-the-eye cut; gently insert forceps into the pia then peeling it off; relax arms and shoulders while dissecting; and etc. Dissection duration was significantly reduced after two months of practice, from more than 10 minutes every pup initially to 4 minutes every pup while making the mixed cerebellar culture.

During the experiment, three sets of cultures including seven Lab-Tek chamber slides and total 34 wells of cerebellar mixed culture were made. All cultures have been fixed, but only one set of culture was stained with fluorescent antibody and images have been captured so far because fluorescent antibody will fade overtime. For the first set of cultures, the negative control wells were only stained with the secondary antibody: goat-anti- rabbit IGG Alexa 488 at a concentration of 1: 3000. Other wells were stained with both primary antibody and secondary antibody, but at different concentrations of the primary antibody of 1:1000 or 1:4000.

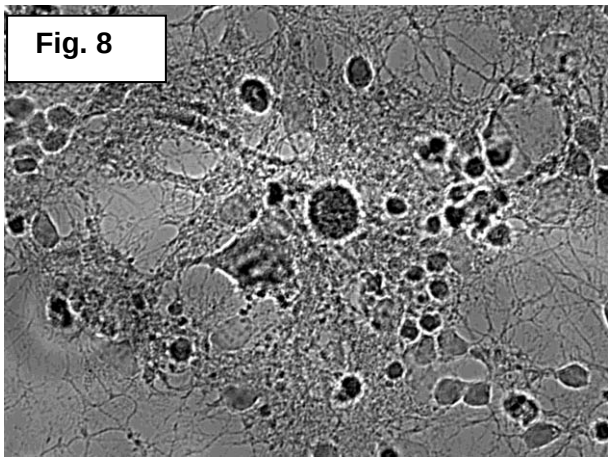


Fig. 8 LX1Cb21dno1abAlexa488-600X193msbf

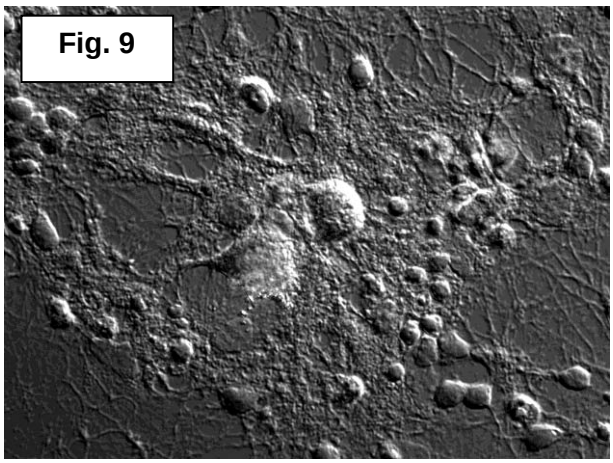


Fig.9 LX1Cb21dno1abAlexa488-600X4msdic

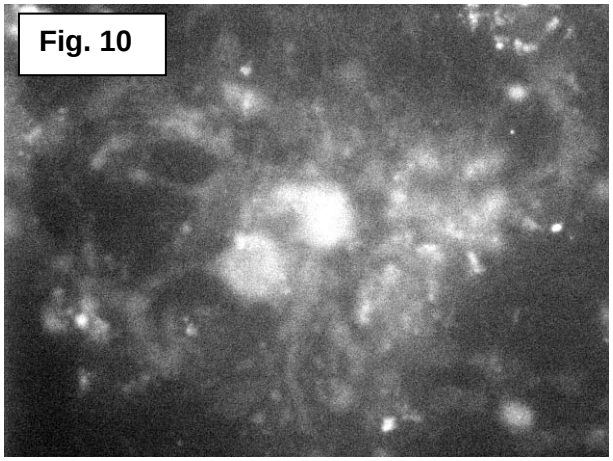
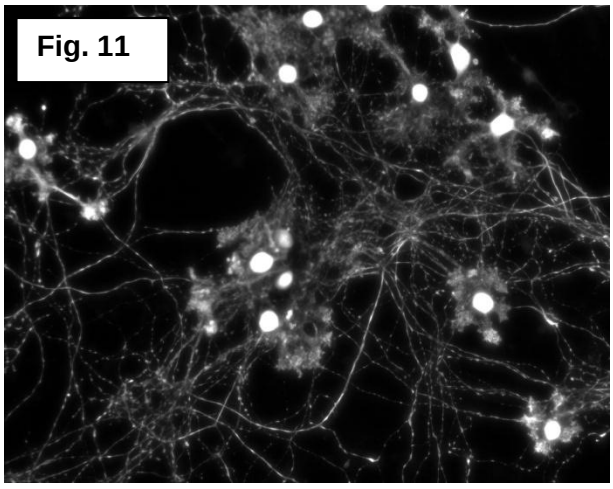


Fig.10 LX1Cb21dno1abAlexa488-600X193ms

Figure 8-10. All Figures are showing the same visual field with different methods. Figures are showing culture 1 stained with only the secondary antibody (Alexa488) taking at 21 div (days *in vitro*), 600 x magnifications. Fig. 8 is the bright field image, Fig. 9 is the DIC image and Fig. 10 is the fluorescent image, showing the level of background staining due to secondary antibody alone.

Figure 8, 9 and 10 are showing the same visual field of the control well, which was only stained with the secondary antibody, using bright field, DIC and fluorescent imaging. Distinct cells and processes can be identified in Figures 8 and 9. But in Figure 10, the fluorescent image of the cells shows fluorescent substances are present in the cell culture, but no clear cell morphology can be identified.



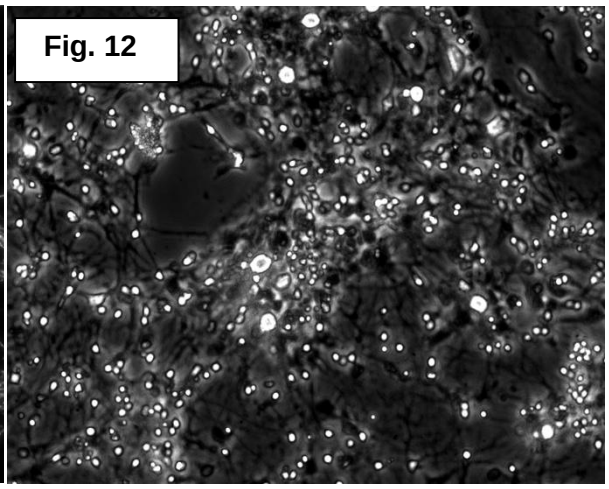


Fig.11 LX1Cb21dCaBPAlexa488-200X193ms Fig.12 LX1Cb21dCaBPAlexa488-200X193msph

Figure 11 & 12. Both Figures are showing the same visual field with different methods Figures are showing culture 1 stained with primary antibody (anti-calbindin) and secondary antibody (Alexa488) taking at 21 div, 200 x magnifications and 193 ms exposure time. Fig. 11 is the fluorescent image and Fig. 12 is the phase image.

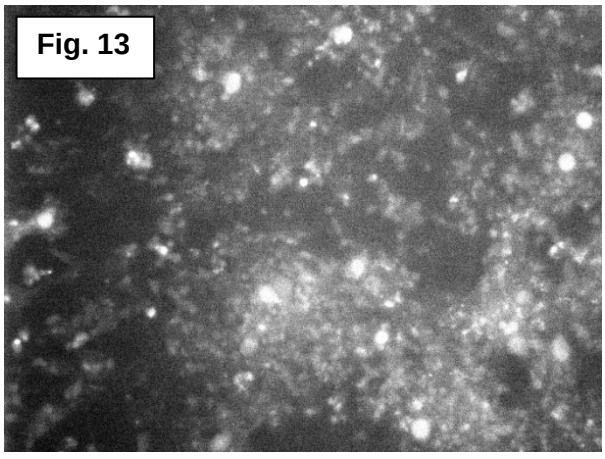
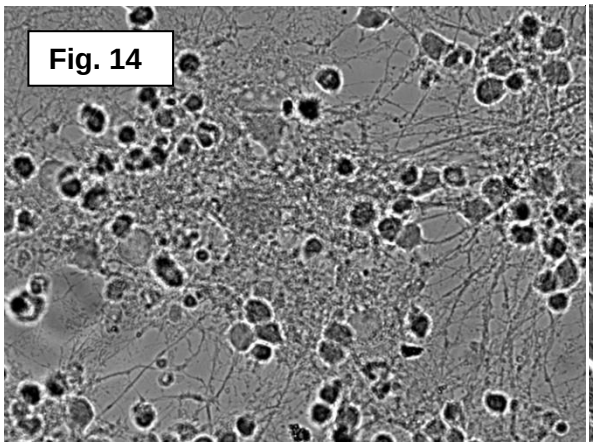


Fig.13 LX1Cb21dno1abAlexa488-200X193ms

Figure13. The fluorescent image of culture 1 stained with only the secondary antibody (Alexa488) taking at 21 div, 200 x magnifications and 193 ms exposure time.

Figures 11 and 12 are the fluorescent image and phase image of the same field in the same culture well. In Figure 12, there are many cells including Purkinje cells and granule cells and processes visible in the field. In Figure 11, there are only a few Purkinje cells and fewer processes visible in the field. Many cells and processes in Figure 12 were absent in the fluorescent image because only Purkinje cell contains calbindin, which will allow the binding of the primary antibody. In Figure 13, the fluorescent image of the cell culture stained only with the secondary antibody shows the fluorescent substances are present in the cell culture, but no specific staining for cells is reviewed compared to Figure 11 while under the same exposure time.



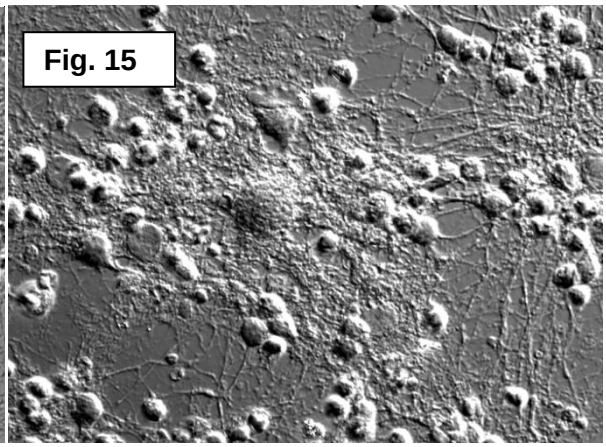
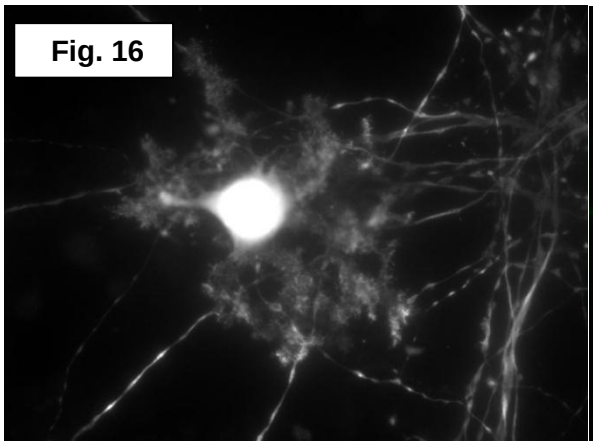


Figure 14. LX1Cb21dCaBPAlexa488-600X4msbf Figure 15. LX1Cb21dCaBPAlexa488-600X4msdic

Figure 14 & 15. Both Figures are showing the same visual field with different methods. Figures are showing culture 1 stained with primary antibody (anti-calbindin) and secondary antibody (Alexa488) taking at 21 div using 600 x magnifications. Fig. 14 is the bright field image taking with 4 ms exposure time. Fig. 15 is the Differential Interference Contrast (DIC) image taking with the same exposure time.



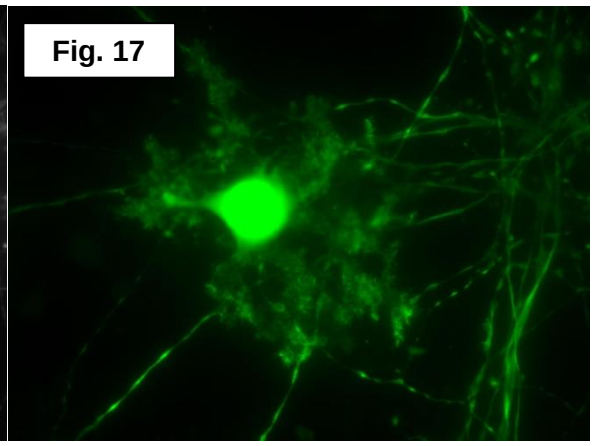
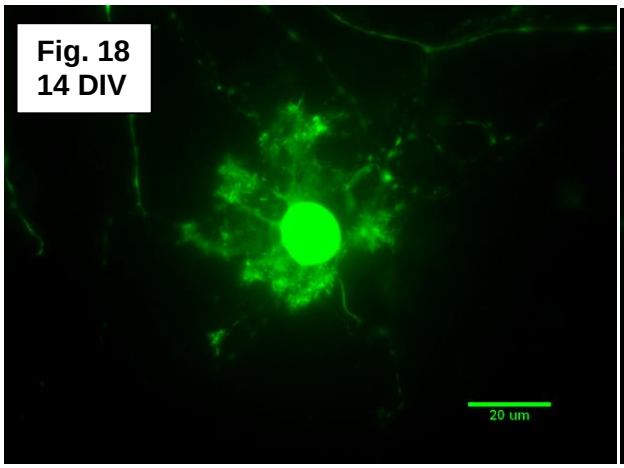


Figure 16. LX1Cb21dCaBPAlexa488-600X193ms Figure 17.LX1Cb21dCaBPAlexa488-600X193mscolor

Figure 16 & 17. Both Figures are showing the same visual field with different methods. Figures are showing culture 1 stained with primary antibody (anti-calbindin) and secondary antibody (Alexa488) taking at 21 div using 600 x magnifications. Fig. 16 is the black and white fluorescent image taking with 193 ms exposure time. Fig. 17 is the colored fluorescent image of Fig.16.

Figures 14 to 17 are the bright field, DIC, black and white, and the colored fluorescent image in the same field in the same culture well. In Figure 14, there are many cells including Purkinje cells and granule cells, and processes visible in the field. In Figure 15, similar situation is observed except the cells in the culture are three dimensional due to the DIC optics. In Figures 16 and 17, there is only one Purkinje cell and fewer processes visible in the field, except Figure 16 is a black and white image captured by the black and white Nikon camera. This black and white Nikon camera has very high resolution, and a colar camera of this high resolution would have been too expensive to be bought by the college at this time. Figure 17 is the same image as

Figure 16, but the green color is added by using the Matavue software to simulate the cell culture is appearance through the microscope using the naked eye. Many cells and processes in Figures 14 and 15 were absent in the fluorescent Figures 16 and 17.



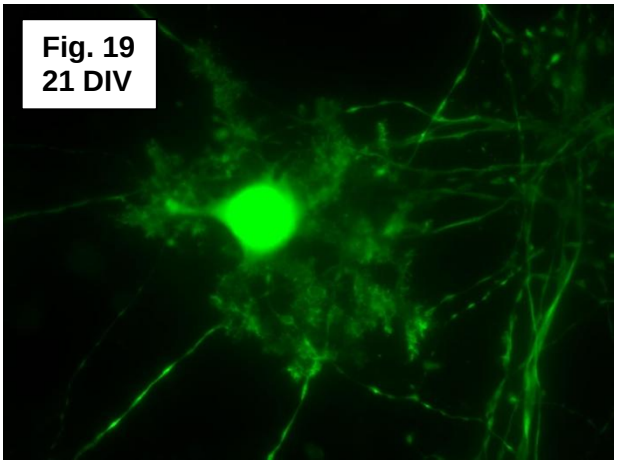
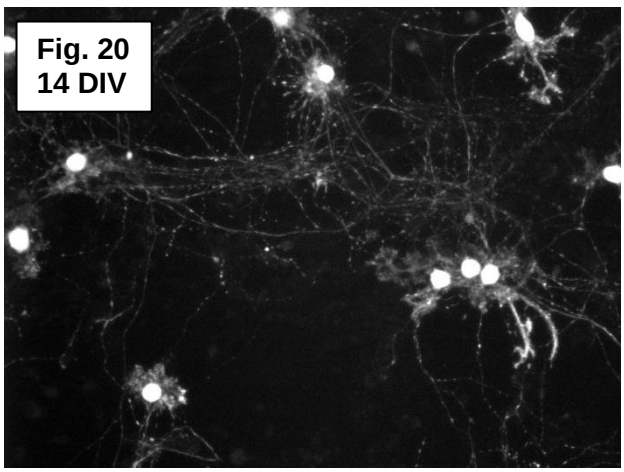


Fig 18 LX1Cb14d1CaBPAlexa488-600X100msxcolor Fig.19 LX1Cb21dCaBPAlexa488-600X193mscolor



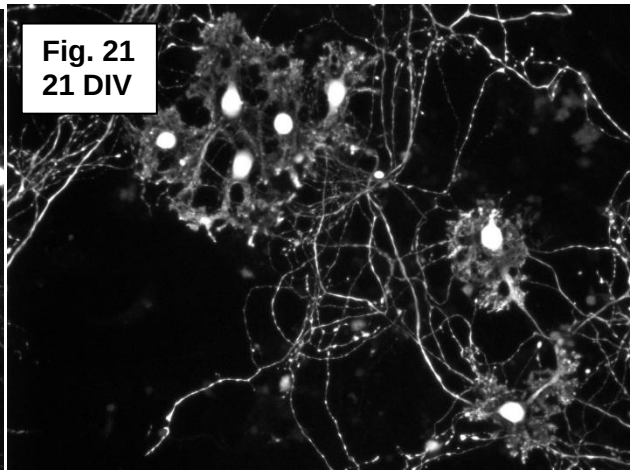


Fig.20 LX1Cb14d3CaBPAlexa488-200X88ms Fig.21 LX1Cb21d9CaBPAlexa488-200X37ms

Figure 18- 21. Fig. 18 is the fluorescent image of a Purkinje cell 14 DIV under 60X objective, and Fig. 20 is showing Purkinje cells 14 DIV under 20X objective. Fig.19 is the fluorescent image of a Purkinje cell 21 DIV under 60X objective, and Fig. 21 is showing Purkinje cells 21 DIV under 20X objective.

Figures 18 and 19 are both fluorescent images of two different Purkinje cells grown in culture for 14 days and 21 days under 60 X objective. All growth conditions are identical except for the time the cells were allowed to grow. The Purkinje cell in Figure 19 grew in culture for 21 days had more extensive and complete dendrites compared to the Purkinje cell in Figure 18, which only grew in cell culture for 14 days. A similar situation is obtained for comparing fluorescent images of Purkinje cells growing in culture for 14 days and 21 days under 20 X objective (Fig. 20 & 21). Purkinje cells grew in culture for 21 days, the dendrites occupied larger area in the culture and were more branched compared to cells that only grew in culture for 14 days.

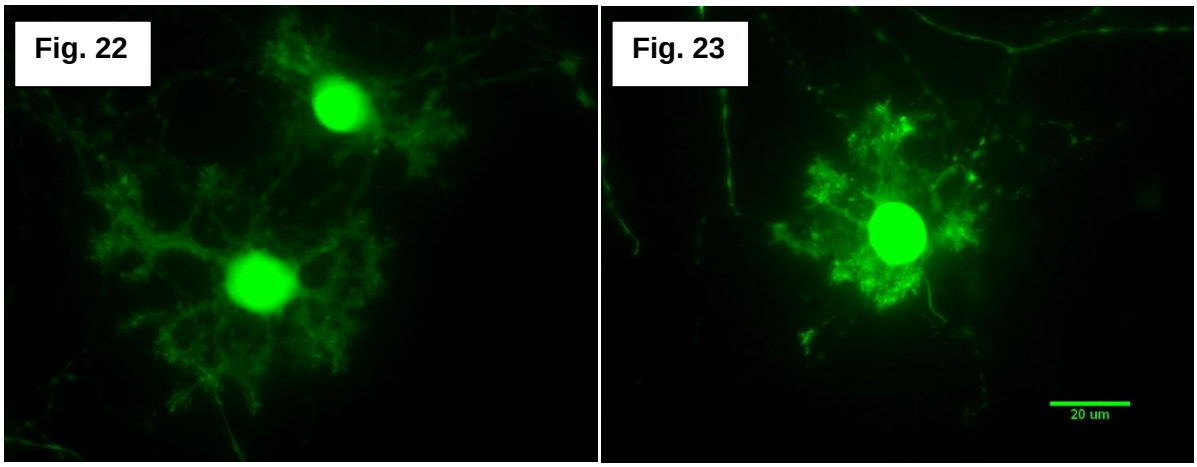


Fig 22 LX1Cb14d2CaBPAlexa488-1-600X100ms Fig 23 LX1Cb14d1CaBPAlexa488-600X100msx

Figure 22 & 23. Fig. 22 is the fluorescent image of Purkinje cell culture 14 DIV stained with primary antibody at a 1/4000 dilution and secondary antibody at 1/3000 dilution. Fig. 23 is the fluorescent image of Purkinje cell culture 14 DIV stained with primary antibody at a 1/1000 dilution and secondary antibody at 1/3000 dilution.

Figures 22 and 23 show the fluorescent image of Purkinje cell culture stained at 14 days in vitro with the same concentration for secondary antibody (1/3000 dilution) but a different concentration for primary antibody (Fig. 22 primary antibody 1/4000 dilution, and Fig. 23 primary antibody 1/1000 dilution). All growth conditions of cells are identical and the exposure time for capturing the image is the same. Purkinje cell in Figure 23 shows slightly stronger fluorescent light intensity in both dendrites and axons compared to Purkinje cells in Figure 21. The cell bodies had similar light intensity in both images.

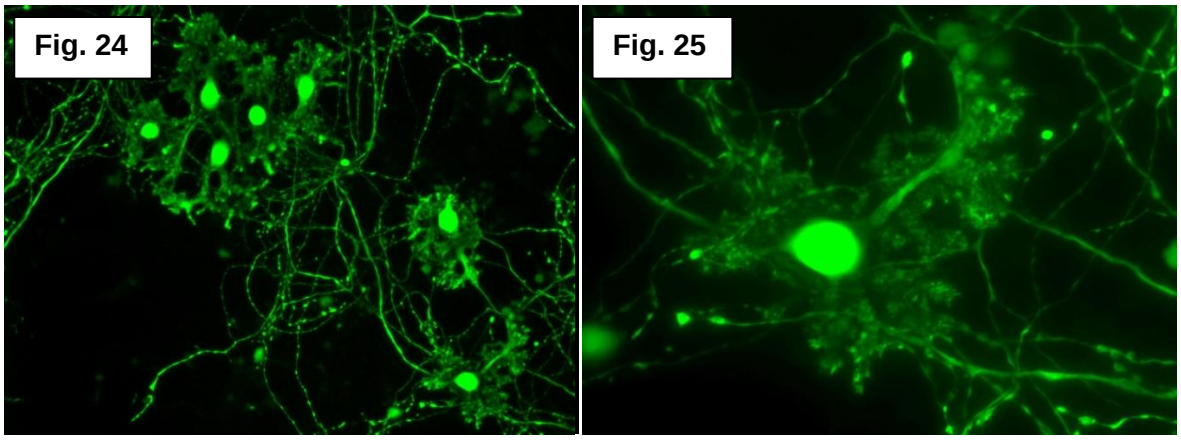


Fig.24 LX1Cb21d9CaBPAlexa488-200X37ms Fig 25.LX1Cb21d9CaBPAlexa488-600X193mscolor

Figure 24& 25. Fluorescent image of the same Purkinje cell culture field using different magnification. Fig. 24 is the image captured using 20 x objectives. Fig. 25 is the image captured using 60 x objectives.

Figures 24 and 25 are the fluorescent images of the same Purkinje cell culture field. Figure 24 shows the cell culture under 20 X objective. Figure 25 shows the cell culture under 60 x objective and focuses on the cell in lower right in Figure 24. More cells and processes are present in the visual field in Figure 24 using the 20 X objective. More detail of the Purkinje cell can be observed in Figure 25, captured using the higher magnification. Spins on the dendrites can be seen in Figure 25 which is hard to observe in Figure 24.

IV. Discussion

During this honors project, Purkinje cells from the mouse cerebellum were cultured, fixed and stained, while images of the cell cultures were captured. A few questions have to be raised during the experiment: Is the primary antibody against calbindin specific for Purkinje cell staining? What is the morphology of the Purkinje cell at different stages of development? What is the best concentration of the primary antibody for staining the Purkinje cell with a fluorescent secondary antibody? And finally, what is the best magnification for observing the Purkinje cell?

The negative control of this experiment was only using secondary antibody to stain the culture. The initial hypothesis was there will be no specific staining on the negative control because the secondary antibody binds specifically to the primary antibody. The bright field and DIC image were taken to show there were Purkinje cells and granule cells present in the visual field (Fig. 8 & 9), but no specific staining was obtained in the fluorescent image (Fig. 10). Therefore, in the absence of the primary antibody, the secondary antibodies will not have specific bindings. In culture wells that recerved both primary and secondary antibody, fluorescent images have been captured under 20 X objective, and there were cells that glowed green, which were Purkinje cells (Fig. 11). Other cells (mostly granule cells) present in the same visual field were captured by phase image, but they were not present in the fluorescent image (Fig. 12). This indicated that the binding of the primary antibody was specifically against calbindin in the Purkinje cell. The fluorescent image of the negative control has been taken using the same exposure time as Figure 11; a low background and no specific staining was obtained in the negative control (Fig. 13). Bright field, DIC, and fluorescent images were also captured under 60 X objective (Fig. 14-17), and the same conclusion was drawn that the antibody staining was specifically for the Purkinje cell. The conclusion was consistent with the results in the literature (Baptista et al., 1994).

In order to obtain the morphology of the Purkinje cells at different developmental stages, images of the fixed and stained cell culture need to be captured at different times. Purkinje cell cultures were fixed and stained at 14 DIV (days in vitro) and 21 DIV. Fluorescent images of the cell at two different times were captured under 20 X objective and 60 X objective (Fig. 18-21). Purkinje cells that were 21 DIV had more extensive and branch dendrites compared to Purkinje cells that were 14 DIV. Longer growth time in

culture allow Purkinje cells to develop more mature and make more connections among associated cells (Mason et al., 1997).

A high quality fluorescent image is crucial for analyzing the cell morphology, therefore, knowing the best concentration for the antibodies is important. During immunocytochemical staining, the primary antibody against calbindin was applied to the culture, and then the fluorescent secondary antibody was added to bind to the primary antibody (Morrison and Mason, 1998; Schrenk et al., 2002). Because the secondary antibody specifically binds to the primary antibody, the concentration of the primary antibody will determine the brightness of the fluorescent cells under ultraviolet light while using the same concentration of the secondary fluorescent antibody. The Purkinje cell culture stained with the primary antibody at a 1/1000 dilution had a higher fluorescent light intensity compared to cultures stained with the primary antibody at a 1/4000 dilution while using the same exposure time for capturing the images (Fig. 22 & 23). Minute structures on the dendrite (such as spins) were more visible on the Purkinje cell that was stained with a higher concentration of the primary antibody (Fig. 23). According to the results, the 1/1000 dilution of the primary antibody is better for staining the Purkinje cell than using 1/4000 dilution in green fluorescent staining. More experiment may be required to discover the best primary antibody dilution.

To obtain a good cell image for analyzing, the magnification for imaging the cell is also important. More cells are present in the image while using the 20 X objective, but more minute structures and the morphology of the Purkinje cell are obtained by using the 60 X objective (Fig. 24 & 25). For studying individual morphology or fine structures on the Purkinje cell, 60 X objective is more appropriate because more detail can be obtained under higher magnification. For studying the Purkinje cell development with its associated cells, 20 X objective could be used since larger visual field can be captured under lower magnification and more cells and processes can be found in the visual field.

During this honors project, I became skilled at dissecting a mouse brain in order to isolate the cerebellum, making mixed mouse cerebellum culture, fixing and staining cerebellum cell culture, and capturing images of the cell culture. Becoming proficient at dissection is difficult, and it takes a long period of time. The first set of dissection took me more than 10 minutes for every mouse pup. After two months of practice, I can complete the dissection in approximately four minutes for every pup. Finding a mouse pup to practice dissection on was problematic. The female mice usually did not give birth on the predicted days, or they gave birth but the pups were eaten. There were three research students who needed mice for their research project, but the mouse house has a limited capacity and not many mouse pups were available for all three students. Fortunately, a schedule for practice dissection and research had been set up, and all three research students were able to complete their experiments. Three sets of mixed mouse cerebellum culture were made. One set of culture was fixed and stained, and images of the culture were captured. The Purkinje cell in the mixed cerebellar culture had a good growth tested by the fluorescent imaging.

In conclusion, making, fixing and staining mixed cerebellar cell culture, as well as capturing the images of the cell culture during this honors project were successful. The

project confirmed that the stain of the primary antibody against calbindin and the secondary antibody anti-rabbit (G): goat- anti- rabbit IGG Alexa 488 for the Purkinje cell was successful. The appropriate concentration of the primary antibody was preliminarily identified. More research may be required to discover the best concentration of the primary antibody for fluorescent staining. The morphology of the Purkinje cell at different developmental stages was compared visually. More work is required to compare the cell morphology at different developmental stages using quantitative methods, such as the Sholl analysis (Sholl, 1953). The best magnification for studying and analyzing individual Purkinje cell is using 60 X objective, and 20 X objective is better for studying a Purkinje cell with its associate cell. The GFP and wild-type cerebellar mixed culture was prepared; because of a limitation on time, cell culture has not been analyzed. Future work will be needed to analyze the GFP and wild-type cerebellar mixed culture. In order to discover the best ratio of the cell mixture for Purkinje cell development.

V. Future Work

1. Culture Purkinje cells from the GFP mice with cerebellar cells from wild-type mice, and discover the best ration for the mixed cell culture.
2. Capture images of the GFP Purkinje cell alive in culture.
3. Finish developing the method for Sholl analysis using ImageJ software
4. Analyze the images of the developing Purkinje cell morphology by Sholl analysis using ImageJ software

VI. Appendix

Analyzing Mouse Purkinje Cell Image by Sholl Analysis Using ImageJ Software

Background

The Purkinje cells communicate with other cells and their environment frequently. The three main components of the Purkinje cell that could help it to communicate with its environment are the axon, the dendrites, and the dendritic spines. In a well developed Purkinje cell (Fig. 1), the axon is a thin afferent process which includes a growth cone and can transmit signals to the cell body. Dendrites are branch-like structures that mostly receive signals from the environment and can also send signals to other associated cells. The dendritic spines are needle-like, mushroom-like or thorn-like structures where dendrites connect with other neurons, axons and where excitatory synapses occur. (Baptista et al., 1994; Mason et al., 1997; Sorra and Harris, 2000)

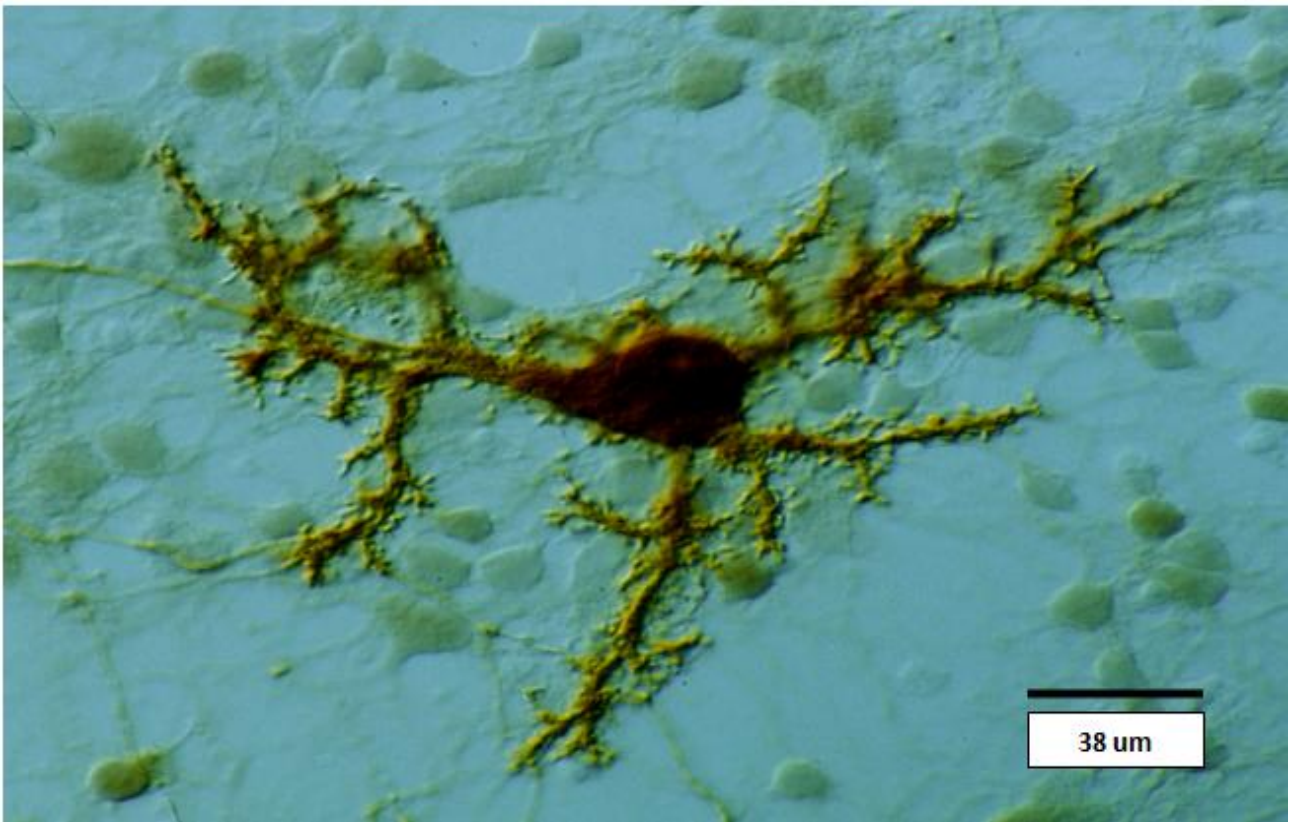


Figure 1. Mature Purkinje cell stained with calbindin in cell culture. (Morrison Unpublished data)

Dendrites are critical structures for communication in the Purkinje cell. Mutations of signals and signal receptors in mice such as BDNF (Brain-derived neurotrophic factor), TrkB (Neurotrophic tyrosine kinase B), netrin, and DCC (Deleted in Colorectal Cancer) could lead to irregular Purkinje cell dendrite growth (Fig. 2) and could cause the Purkinje cell to survive poorly. (Moore et al., 2007; Morrison and Mason, 1998; Morrison Unpublished data) Ataxia patients have abnormal Purkinje cells in their cerebellum, which cause them to have uncoordinated movements (Manto, 2009). In order to help patients with abnormal Purkinje cells, such as ataxia patients, Purkinje cell dendrite morphology was needs to be studied and analyzed.

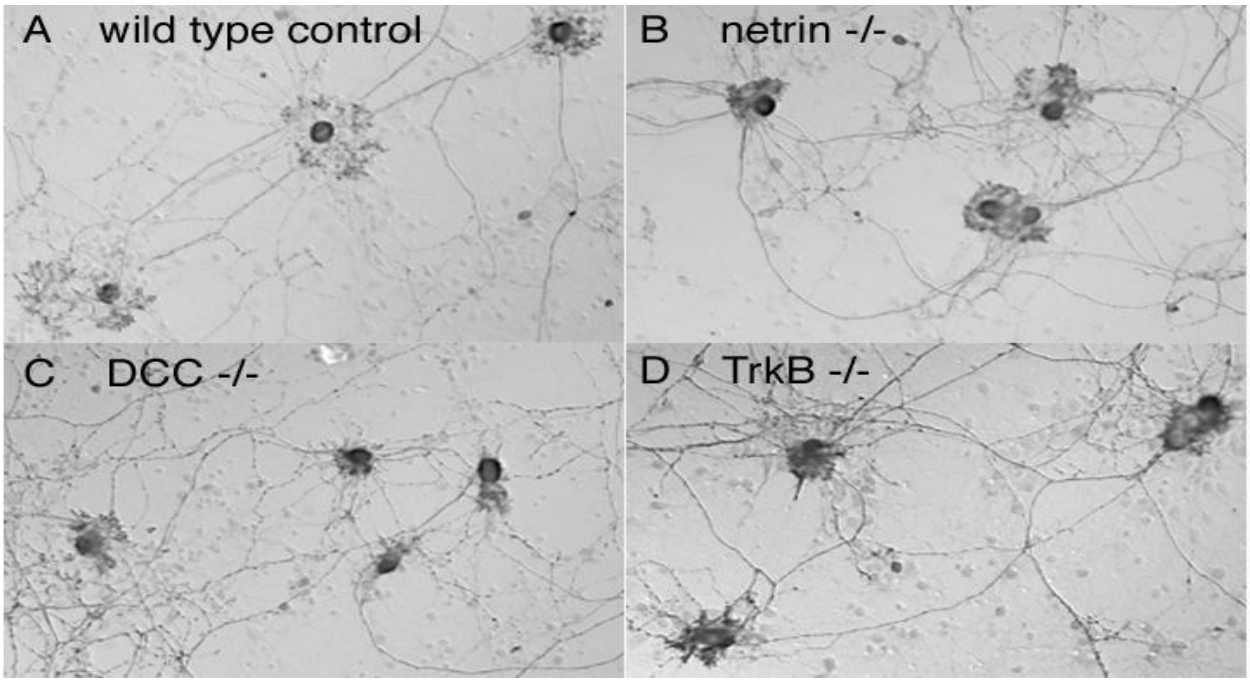


Figure 2. Mice Purkinje cells in cerebellum culture. (A, wild type Purkinje cell control; B, homozygous netrin Purkinje cell cutlure; C, homozygous DCC Purkinje cell culture; and D, homozygous TrkB Purkinje cell culture (Morrison unpublished data. magnification: 10X eye piece and 63X objective).

In order to understand more about the Purkinje cell dendrite, the developmental stages of the Purkinje cell need to be understood first. (Fig. 3) The development of the Purkinje cell was divided into 4 stages. First, the Purkinje cells migrate under the external granular layer, the olivocerebellar climbing fibers attach to the Purkinje cell body, and the axon started to develop. This stage happens on approximately the 17th embryonic day (E17 day) in the mice. At 3 post-natal days (P3), neurites start to develop around the Purkinje cell soma and granule cells developed axons, which later become parallel fibers. In the third stage (7 postnatal days, P7), apical dendrites develop and extend and dendritic spines start to develop. Also, climbing fibers begin to

make contact with the dendrites. In the final stage, well developed Purkinje cell dendrites and parallel fibers develop synapses on the spines at P14 days (Mason et al., 1997).

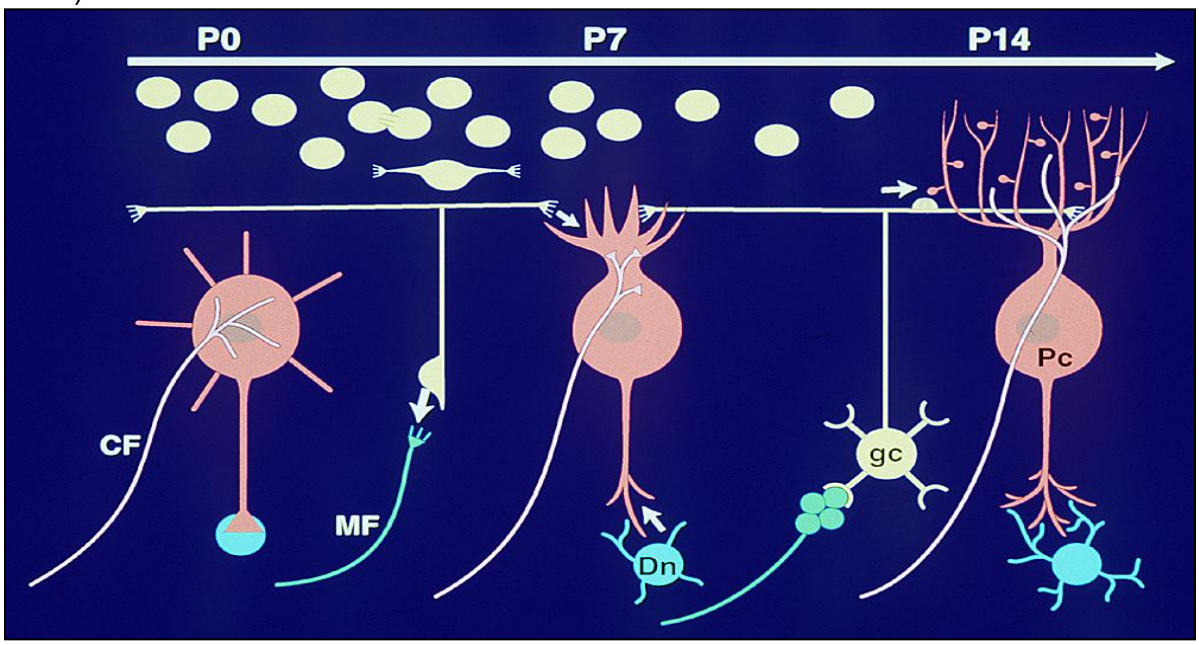


Figure 3. Purkinje cell development in the cerebellum. (Morrison Unpublished data)

A well developed Purkinje cell needs to communicate with its environment at all stages of development. Purified and isolated mice Purkinje cells do not survive well *in vitro* and only develop axons. However, when purified Purkinje cells are cultured with granule cells, the survival of the Purkinje cells increases and normal axons, dendrites and spines develop (Baptista et al., 1994). Even though the experiment results show that granule cells are significant for Purkinje cell survival, the signals and mechanisms are still unknown. In another experiment, the Purkinje cells were cultured along with different growth factors. NT4 (neurotrophin 4) and BDNF were two types of growth factors which increased the survival of Purkinje cells compared to the control and other growth factors. However, when BDNF was added to the Purkinje and granule cell coculture, Purkinje cells survived poorly. Different molecules such as trkB-IgG were used to block BDNF signaling, thereby increasing Purkinje cell survival. The conclusion was that excess BDNF could be toxic to Purkinje cells and cause cell death (Morrison and Mason, 1998).

Netrin and DCC could also have an effect in mouse Purkinje cells during development (Morrison Unpublished data). The netrin family includs: netrin-1, netrin-3, netrin-4 and netrin-G. They are mostly found in the midline of all bilateral symmetrical animals and they can act as attractants or repellents depending on the signal receptor of the cell and the developmental stage. Netrin-1 is the most studied member of the netrin family. It is highly expressed in the floor plate of the spinal cord and also in other

parts of the cerebellum (Alcantara et al., 2000). Netrin-1 can attract many different types of axons and simulate their outgrowth. Netrin-1 can also repel axons, such as those that are not allowed to cross the midline such as parallel fibers (Alcantara et al., 2000; Barallobre et al., 2005; Guijarro et al., 2006). DCC is one receptor for netrin. DCC is highly expressed in CNS and epithelial cells of some tissues, and it can interpret the netrin signal as an attraction or repulsion. The expression of netrin-1 and DCC were both absent in adult mice, which indicates that netrin1 and DCC only have an effect on the axon and neuron migration during the early developmental stages (Livesey and Hunt, 1997). Recent studies in mice show that when netrin1 signaling is blocked via knockout, the Purkinje cells have less well developed dendrites (Morrison Unpublished data).

To analyze dendrite morphology differences between mutant Purkinje cell cultures and wild type cultures, specific methods are needed such as Sholl analysis. Sholl analysis was invented by D. A. Sholl in 1953. It is a method which can study neuron morphology quantitatively and efficiently (Sholl, 1953). Image J software could be used to process the cell image or drawing for analyses (Image J). Concentric circles are drawn on the cell and the center of the cell soma is used as the center of the circles (Fig. 4). The intersections of the circles with the dendrites or skeletonized dendrites (Fig. 5) are counted, and the relationship between the numbers of intersection with the radii of the circles is graphed. Statistical tests such as ANOVA, F test and student‟s t test can be performed to demonstrate the correlation between the numbers of intersections and the radii of the circles (Fig. 6) (Cook and Wellman, 2003; Martinez-Tellez et al., 2005; Ristanović et al., 2006; Vega et al., 2004).

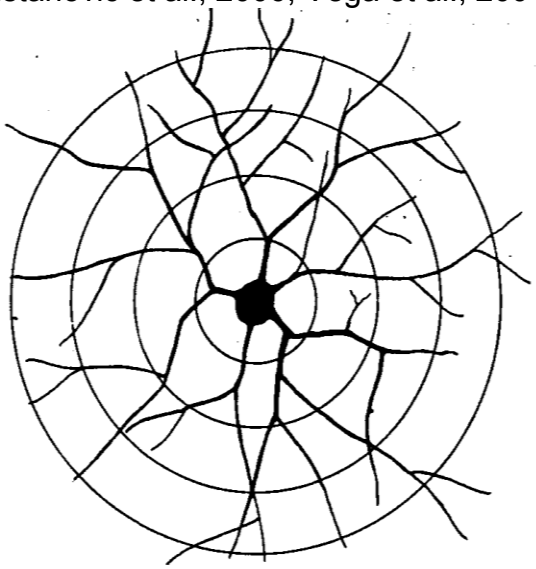


Figure 4. Drawing of a neuron cell study by the Sholl analysis. Concentric circles intersect with the dendrites. (Sholl, 1953, text Figure 2)

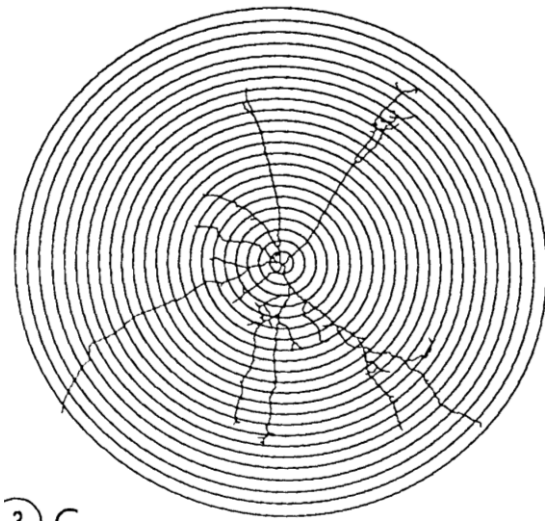


Figure 5: Skeletonized neuron image study by the Sholl analysis. (Neale et al., 1993, Figure 3, C)

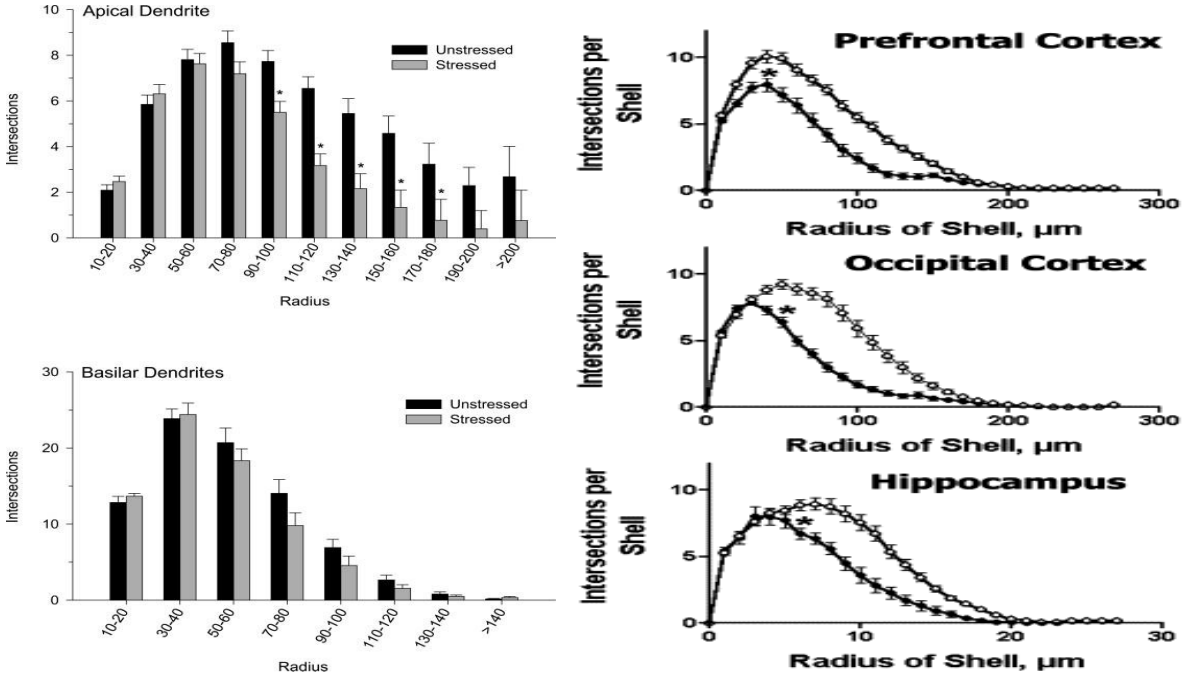


Figure 6. Sample of graphs that will be used after analyzing the relationship between the number of intersections and the radius of the circles by statistic tests. Left: histogram analyzed by two-way repeated-measure ANOVAs test. Right: Scattered plot analyzed by 2-tailed Kruskal-Wallis and Mann-Whitney tests. (Cook and Wellman, 2003; Martinez-Tellez et al., 2005)

It is important to study the morphology of Purkinje cell dendrite because the dendrites are crucial for the development and survival of the Purkinje cells. During Purkinje cell development, it is important for the Purkinje cells to stay healthy and alive in order to help the cerebellum to control one"s eye movement, balance and locomotion. The Purkinje cells in ataxia patients are abnormal and these patients have difficulty in controlling their movements and performing many other everyday life functions (Manto. 2009). Because the interactions of the Purkinje cell with many molecules and growth factors are still unclear, it is very important to carry out these experiments to develop a method to analyze the data more efficiently. Performing this experiment can also help students gain more understanding about the Purkinje cells and develop more research skills when working with the Purkinje cell or Purkinje cell images.

Method and Result

1. Edit the LUT (lookup table) of an Image

Because the image was taken by the Nikon TE2000 Microscope while shining a polarized light on the surface of the cell culture, some areas of the cell will have a higher pixel value (lighter color) compared to the rest of the cell. In order to analyze the cell as

a whole with ImageJ, the areas with lighter pixel values on the cell need to have a lower pixel value (darker color). To use the ImageJ software process the cell image, open the ImageJ program by double clicking its icon. The ImageJ menu will open up (Figure 7).To open an image file, go to “File” and “Open”, a new window will open up. Select the wanted image by double clicking the image‟s icon (Figure 8). First, make sure the cell image is “8-bit”, to do this, go to “Image”, “Type” and select “8-bit”. To change the pixel value of the image, go to “Image”, “Color” and then “Edit LUT”. A new window, “LUT Editor”, will open up with different pixel values in each square on the lookup table (Figure 10). Use the mouse to select the squares that have higher pixel values, they will be the squares that look white. For most images, select the bottom three rows on the lookup table but this can vary between different images. Then a small window will pop up with an addressed value for “Red”, “Green” and “Blue” (Figure 11). Change all the values to “0” either by dragging the bar tool to the left or replacing the addressed value by “0”. Pop up windows might come up more than one time; just repeat the process to change all pixel value to “0”. After finishing changing the pixel value, click “OK” on the “LUT Editor” window. Now the area of the cell with a higher pixel value (white) will become black (Figure 9). Save the edited LUT image by “File”, “Save as” and chose one image type. For this experiment, all edit images were saved as “Jpeg…”, a pop up window “Save as Jpeg” will open up, name the file and save the file by clicking “Save”.

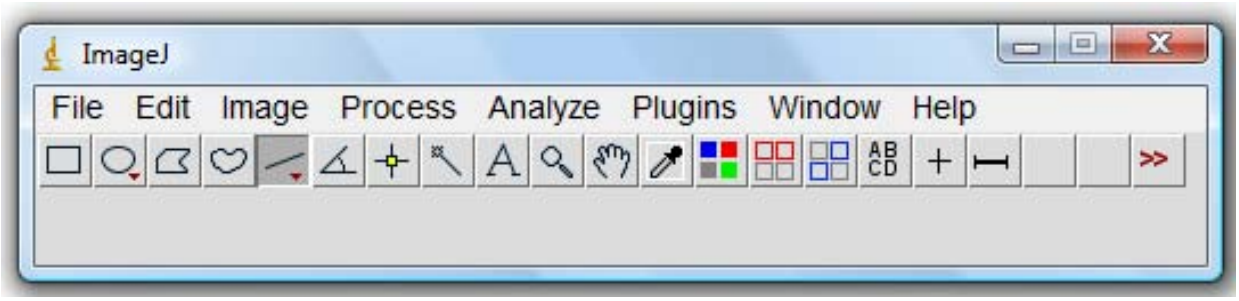


Figure 7. The ImageJ Manu.

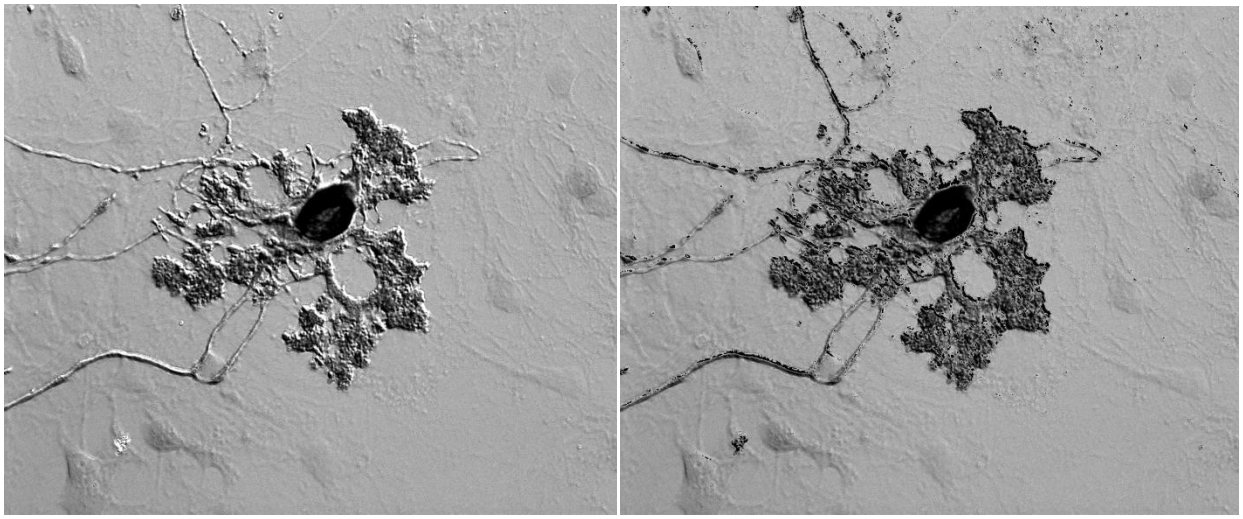


Figure 8 & 9: The original image (left); image after edit LUT (right) (Morrison unpublished data)

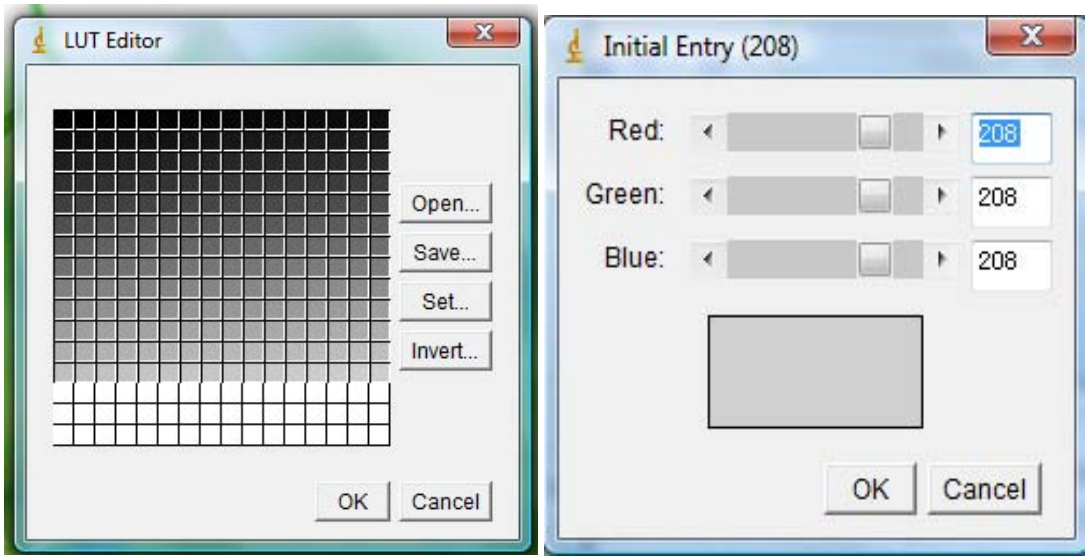


Figure 10 & 11. The lookup table editor (left) and pixel value editor (right)

2. Lightening the background and threshold a cell image

Lightening the background of an image is necessary since it will make a better threshold of the cell image. Open the previous edited LUT image file and also the original image by clicking “File”, “Open” and select the wanted image. To lighten the background of the edited LUT image, go to “Image”, “Adjust” and “Brightness/Contrast”, a new window named “B&C” will pop up (Figure 12). Adjust the “Minimum” and “Maximum” tool bar by dragging it left and right. Compare with the original image while adjusting and try not to lose any detail on the cell. Do not adjust “Brightness” and “Contrast”, this will lead to pixel loss. Then click “Apply” and close the window. The

change will be made on the edited LUT image and the background will be lightened (Figure 14). To threshold the image, go to “Image”, “Adjust” and select “Threshold”. A new window named “Threshold” will pop up (Figure 13). Select “Default” and then “B&W” for the two selection bars. Do not adjust the adjustable tool bar; it will lead to pixel loss. Then click “Apply” to confirm the image (Figure 15).

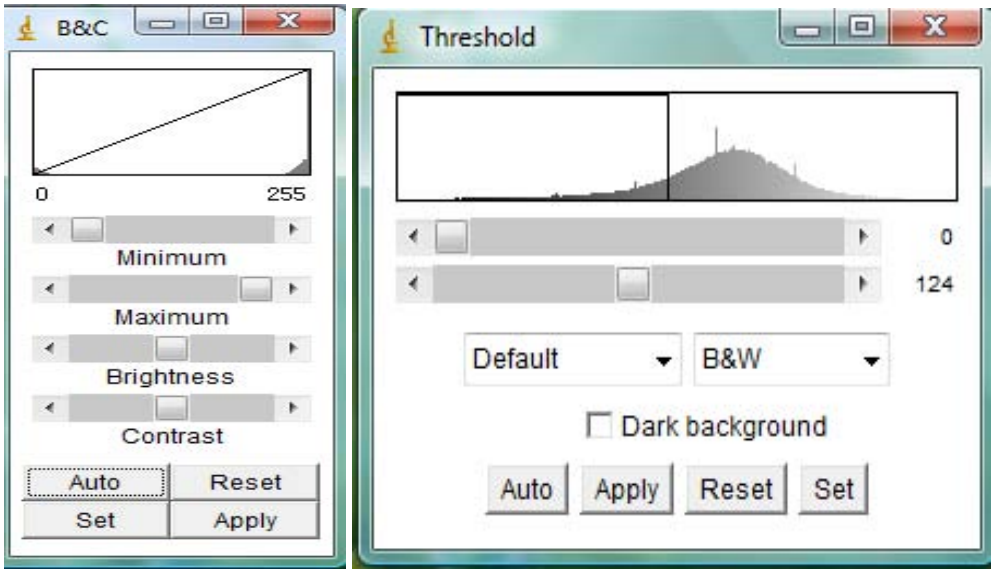


Figure 12 & 13. The Brightness/Contrast window (left) and Threshold window (right).

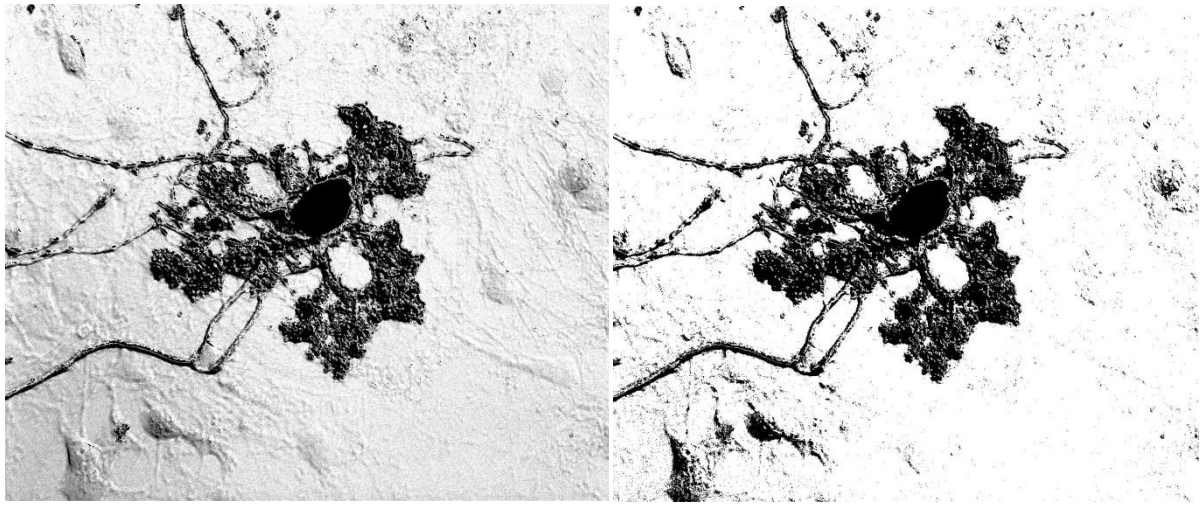


Figure 14 & 15. Edit LUT Image with lightened background (left); Threshold image after edit LUT and lighten background (right).

3. Set Scale and Draw a Scale Bar for the Image

To set a scale for the image, click “Straight line selection”, draw a straight line on the image while holding down the “Shift” key on the keyboard. Then go to “Analyze” and “Set Scale”,

a “Set Scale” window will open up (Figure 17). Before entering numbers in the blanks, find out the calibration distance for the image. For a Nikon TE2000 Microscope, the calibration distance for the image is in table 1. A 60x lens was used to take all the Purkinje cell images, and the calibration distance for 60x lens for a Nikon TE2000 Microscope is x=y= 0.106464 um/pixel. In the “Set Scale” window, the value in “Distance in Pixel” indicates the length of the straight line selection. Enter “um” in “Unit of Length”. To find out the “Known Distance” in micrometers (um), use the value in “Distance in Pixel” multiply by 0.106464 um/pixel, and enter the result into “Known Distance”. Make sure the value for “Pixel Aspect Ratio” is “1.0”. Check “Global” to apply the scale to all images that will be processed by ImageJ and click “OK” to apply the calibration scale to the image. To confirm whether calibration is correct, a scale bar could be drawn on the image. Click “Macro Toolset Switcher” to the far right on the “Toolbar” (Figure 16). Then select “Magic Montage”, the icons on the right side of the “Toolbar” will change. Click “Bar” icon and the “ScaleBar Plus” window will pop up (Figure 18). Enter the length of the scale bar in “Width in UM”, and a value in “Height in pixels”, which determines the thickness to the scare bar. For example, enter “20” in “Width in UM”, “8” in “Height in pixels”, do not change the value for “Font Size”. Select “Black” in “Color”, “None” in “Background”, “Lower Right” in “Location”, check “Bold Text” box and click “OK”. A scale bar will be drawn on the image (Figure 19).

Microscope Lens Setting	Calibration Distance of Pixel (X=Y, unit: um/pixel)
10X lens	X=0.653594
20X lens	X=0.326085
40X lens	X=0.159362
60X lens	X=0.106464
10X lens with 1.5 zoom	X=0.434782
20X lens with 1.5 zoom	X=0.219298
40X lens with 1.5 zoom	X=0.108991
60X lens with 1.5 zoom	X=0.0715559

Table 1. Calibration distance for different settings of Nikon TE2000 Microscpe.

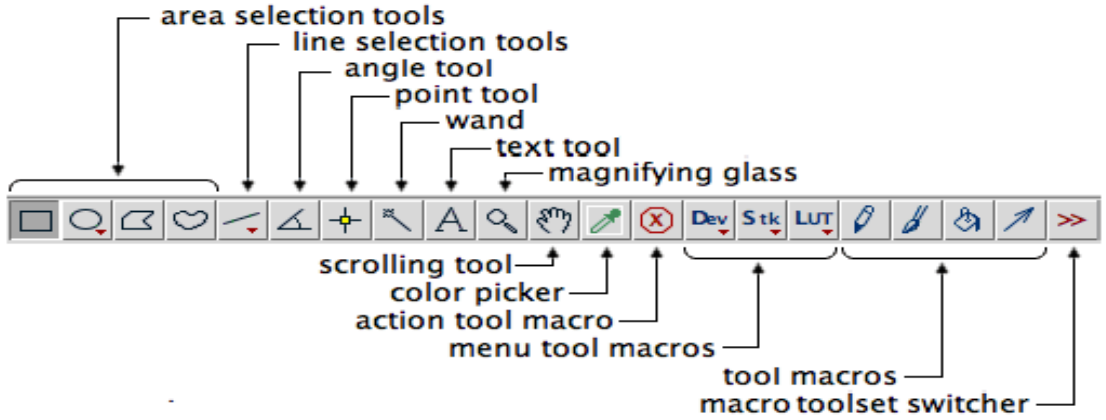


Figure 16. the toolbar.

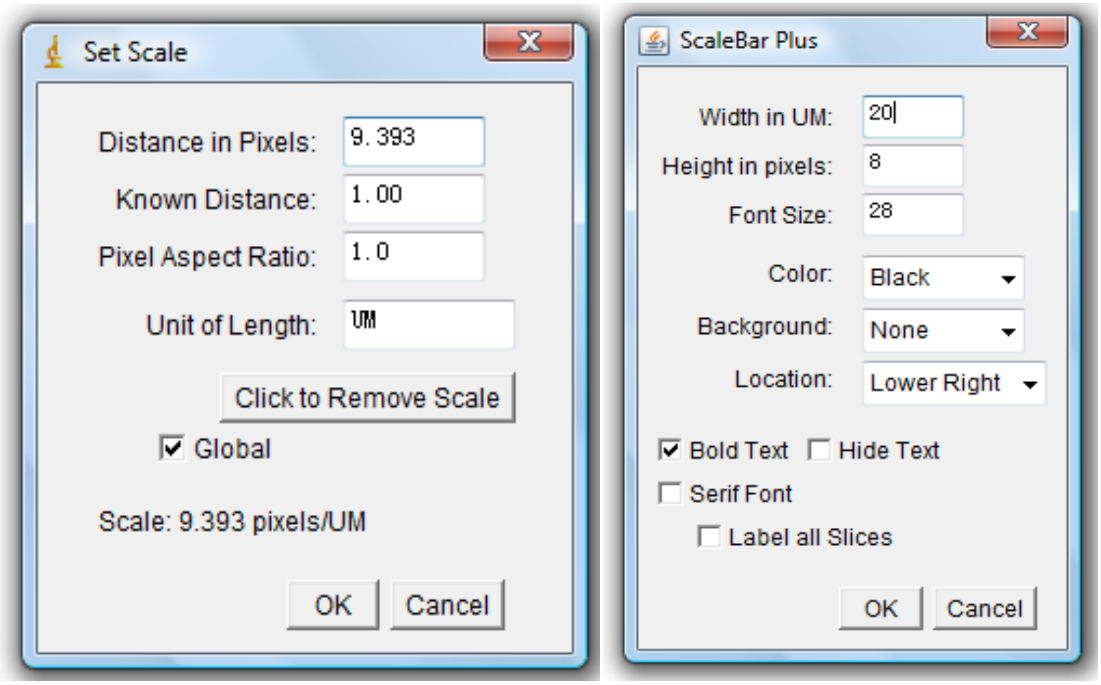


Figure 17 & 18. The Set Scale window (left) and the ScaleBar Plus window (right)

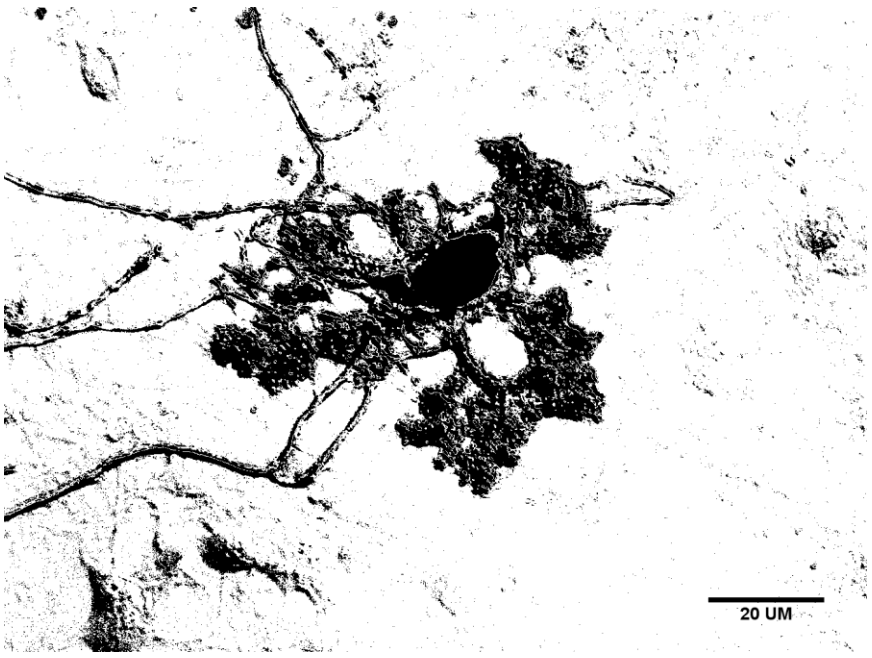


Figure 19. Calibrated image with 20 um scale bar.

4. Manual Tracing and Analyze Particle

After thresholding the image, click “Freehand selections” and hold down the left key of the mouse to trace around the cell. Eliminate any particle that is not a part of that cell body, dendrite or dendritic spin. If it is hard to decide whether the particle is a part that needs to be included in the tracing, use “Magnifying glass” to zoom in and zoom out and compare the shape and color of that particle to the rest of the image. Compare the size of the structure with the scale bar as well. Practice manual tracing on many cell images will help to become efficient with this procedure. Enclose the tracing area, and then go to “Analyze” and “Analyze Particle”. A new window, “Analyze Particles”, will open up (Figure 20). Check “Pixel Unit”, enter “50” in the blank after “Size”, do not change the value for “Circularity”, select “Masks” in “Show”, check “Clear Result” and click “OK”. A new image window will open up with the mask of the traced cell (Figure 21). Save the image by “File”, “Save as” and “Jpeg…”, a pop up window “Save as Jpeg” will open up, name the file and save the file by clicking “Save”.

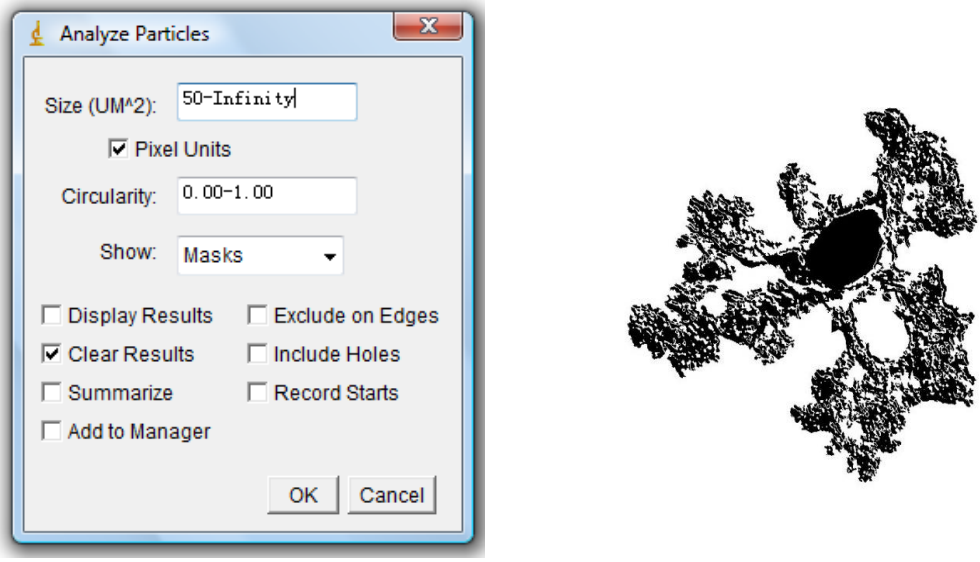


Figure 20 & 21.The Analyze Particles window (left) and the mask of the traced cell (right)

5. Connect the Particles of the Cell

In order to perform Sholl analysis on a neuron image, a continuous cell is required. To connect all the particles of a cell image, first go to “Process”, “Binary” and “Make Binary”. Now all the pixels of the image are converted to black and white pixels. Go to “Process”, “Binary” and “Options…”, a new window, “Binary Options”, will pop up (Figure 22). Enter “1” in blank “Iteration (1-25)” and “Count (1-8)”, and select “Overwrite” in “EDM” output. Settings can be altered according to the user. Then go to “Process”,

"Binary" and "Dilate", this command allows adding 1 pixel to all the pixels on the edge of the cell and connect the particles. Repeat this command by hold "Ctrl" + "Shift" + "R". Repeat this command, trying best to connect all the particles; also do not connect the particles that are not supposed to connect. Record the number of times that this command has been used, and go to "Process", "Binary" and "Erode". This command allows erasing 1 pixel to all the pixels on the edge of the cell, but it will not erase the pixels that are already connected. Use this command the same number of times as "Dilate". The typical number for repeating "Dilate" or "Erode" should be no less than 2 times and no more than 6 times. Figure 23 shows the result after using the "Dilate" and "Erode" command.

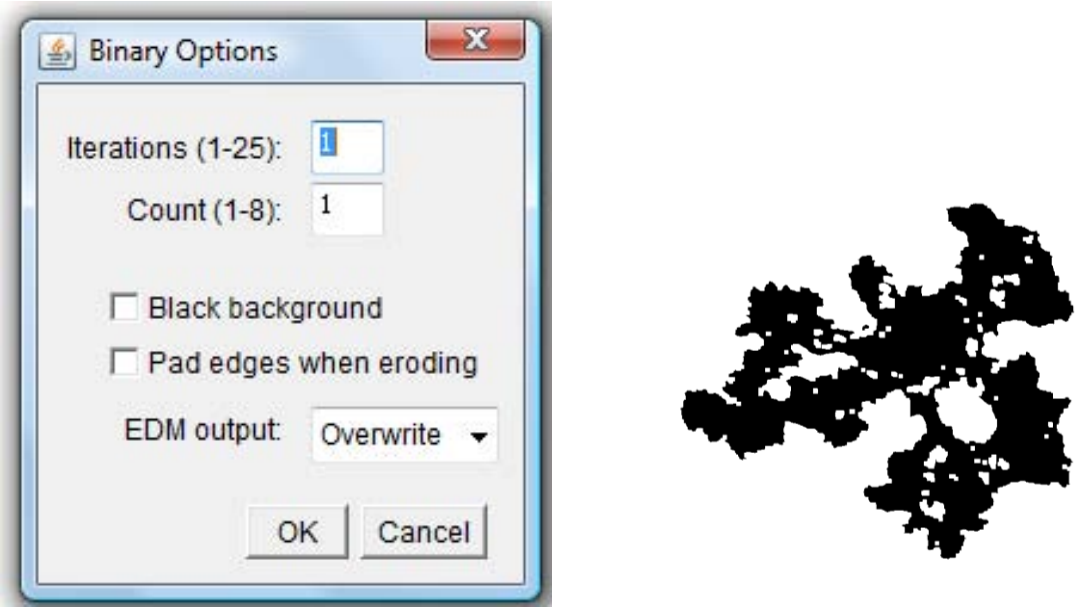


Figure 22 & 23.The Binary Options window (left) and Purkinje cell image after dilate and erode (right).

6. Compare Edit Cell Image with the Original Cell Image

After processing the Purkinje cell image with ImageJ, it is necessary to compare the result to the original image. Go to "File" and "Open", a new window will open up, double click the icon of the original cell image to open. Then go to "Image", "Stack" and "Concatenate", a new window, "Concatenator", will pop up (Figure 24). Select the original in "Stack 1", select the computer processed image in "Stack 2", check "Keep Source Stacks" and click "OK". Now the original image and the computer processed image will be present on the same stack with a sliding bar on the bottom of the image. To see the original image, slide the bar to the left; to see the computer processed image, slide the bar to the right (Figure 25 and 26). To combine and compare two images, go to

"Image", "Stack" and "Z Project…", a new window "Z Projection" will pop up (Figure 27). Enter "1" in "Start slice", "2" in "Stop slice", select "Sum Slices" in "Projection Type" and click "OK". The combination of the original image and the computer processed image will be shown on a new image (Figure 28). The computer processed image is the area with higher pixel value (white) and the original image remains unchanged, except with a slightly lower pixel value (darker). Use "Magnifying glass" on the tool bar to zoom in or zoom out on the image to compare the overlap of the Purkinje cell on the original image and computer processed image. Decide whether the computer processed image is accurate, which the Purkinje cell in computer processed image should have less than 5% difference with the Purkinje cell in the original image. If the computer processed image is accurate, go on to the next step. If the computer processed image is missing too much area of the cell, repeat step 4 and 5 and adjust the number of times for dilating and eroding, then make a new computer processed image, then compare with the original image with step 6.

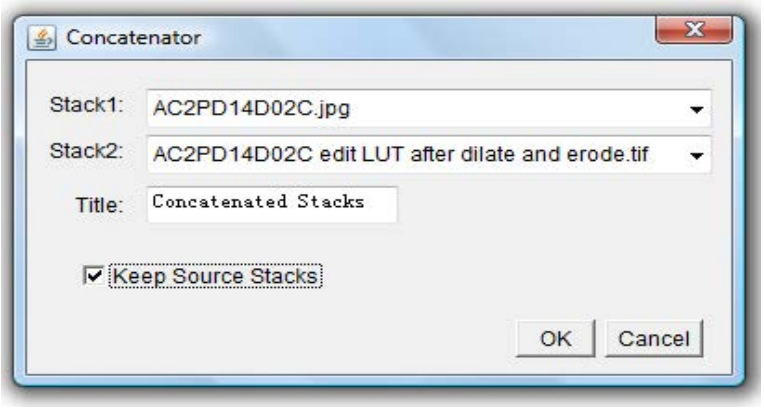


Figure 24. The Concatenator window.

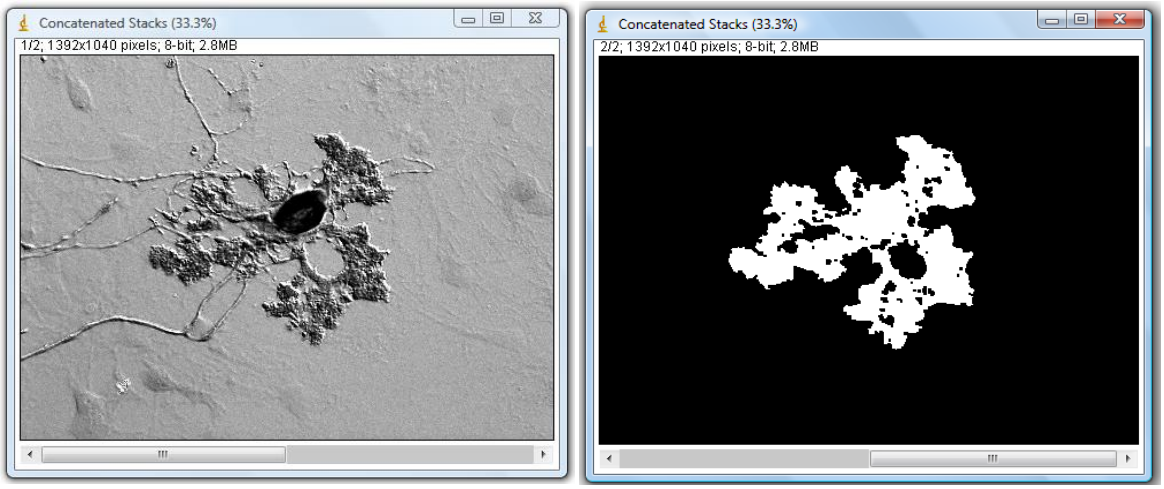
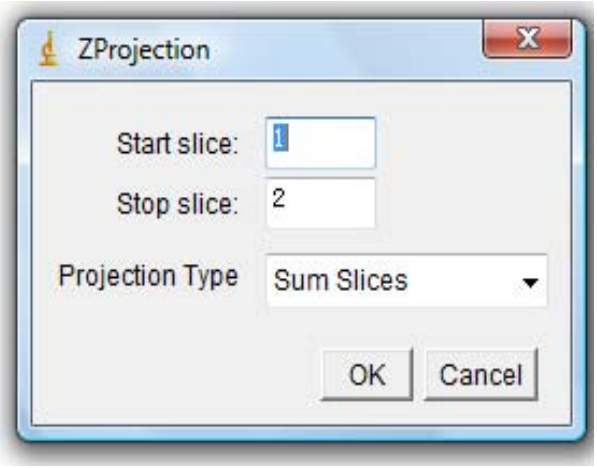


Figure 25 & 26. Stack of the original image (left) and stack of the computer processed image (right)



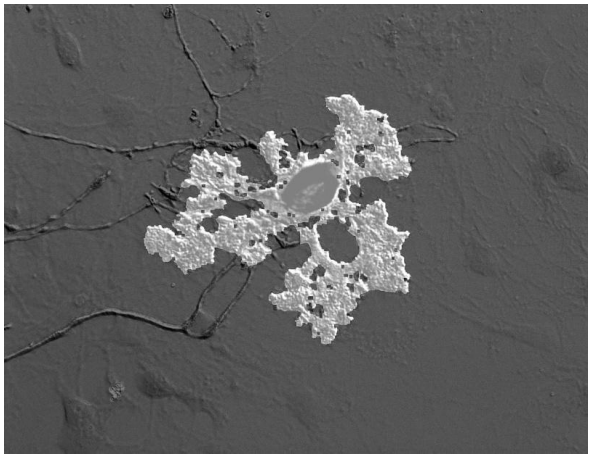
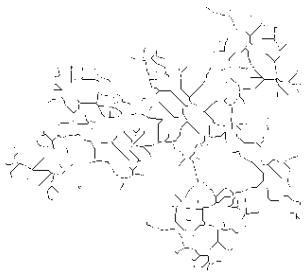


Figure 27& 28. The Z Projection window (left) and the combination of original and computer processed image (right).

7. Skeletonize the Cell after Dilate and Erode (Image processed in procedure 5)

In order to perform Sholl analysis on a Purkinje cell image, a skeletonized Purkinje cell is needed. To skeletonize the Purkinje cell, go to “Process”, “Binary” and “Skeletonize”. The Purkinje cell will be skeletonized on the same image. Figure 29 shows the result after skeleonization. However, not all branches of the cell are connected. To make a fully connected cell, go to “Process”, “Binary” and “Dilate”. Now most of the unconnected branches will be connected (Figure 30).



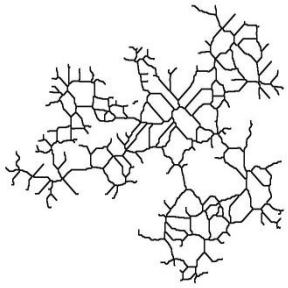


Figure 29 & 30. The skeletonized Purkinje cell image (left) and the dilated skeletonized Purkinje cell image (right).

8. Clear the Area of the Soma

Because only dendrite morphology is of interest, it is necessary to eliminate the cell soma area in the dilated skeletonized cell (Figure 30). Use the previous procedure from #6 to create a stack of the dilated skeletonized image with the original cell image. Go to “Image”, “Stack” and “Concatenate”, a new window, “Concatenator”, will pop up. Select the dilated skeletonized image in “Stack 1”, select the original image in “Stack 2”, check “Keep Source Stacks” and click “OK” (Figure 31). Now the dilated skeletonized image and the original image will be present on the same stack with a sliding bar on the bottom of the image. To see the dilated skeletonized image, slide the bar to the left; to see the original image, slide the bar to the right. Click “Freehand selection” and slide the bar to the right. Trace around the cell soma on the original image by holding down the left key on the mouse. After enclosing the trace of the cell soma on the original image, a yellow enclosed outline will be shown on the cell soma (Figure 32). Slide the bar to left, and the enclosed outline will also appear on the dilated skeletonized cell image at the same region (Figure 33). Go to “Edit” and “Clear”, a window will open up, click “No” to process only the selected image. Now the dilated skeletonized cell image will not have the cell soma area (Figure 34).

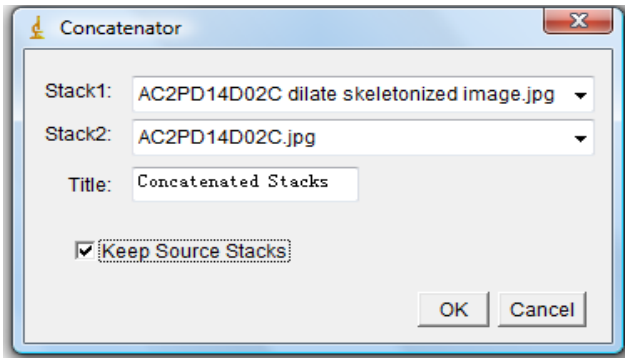


Figure 31. The Concatenator window.

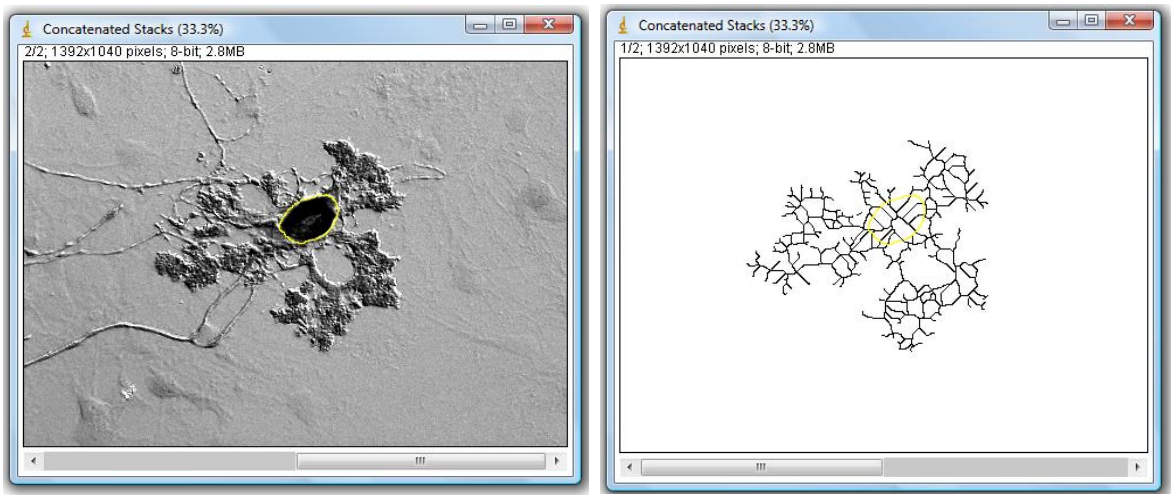


Figure 32 & 33. The original image with freehand tracing around soma (left) and the dilated skeletonized image with freehand tracing around soma (right).

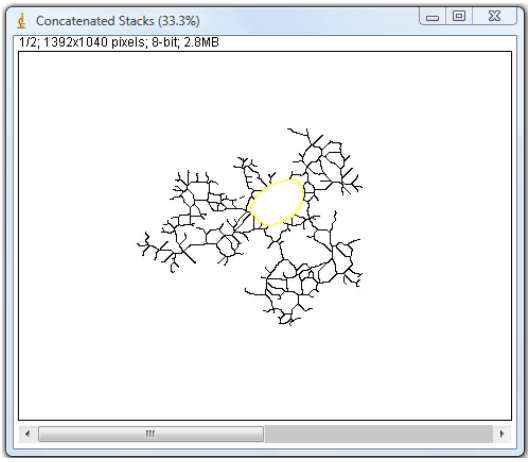


Figure 34. Dilated skeletonized cell image without cell soma area.

9. Find the Centroid of the Soma

In ImageJ language, centroid is the center of the selected area. Follow procedure #8, go to “Analyze” and “Set Measurements…”. A window named “Set Measurements” will open up, check “Area” ,“Centroid” and click “OK” (Figure 35). To find the centroid of the dilated skeletonized cell soma, make the stack of the dilated skeletonized cell image the active window by clicking on the frame of the stack, and go to “Analyze” and “Measure”. A result window will open up with the area and the X and Y value of the centroid of the cell soma area (Figure 36). Save the dilated skeletonized cell image without soma area by clicking “File”, “Save as…”, “Jpeg…” and name the image.

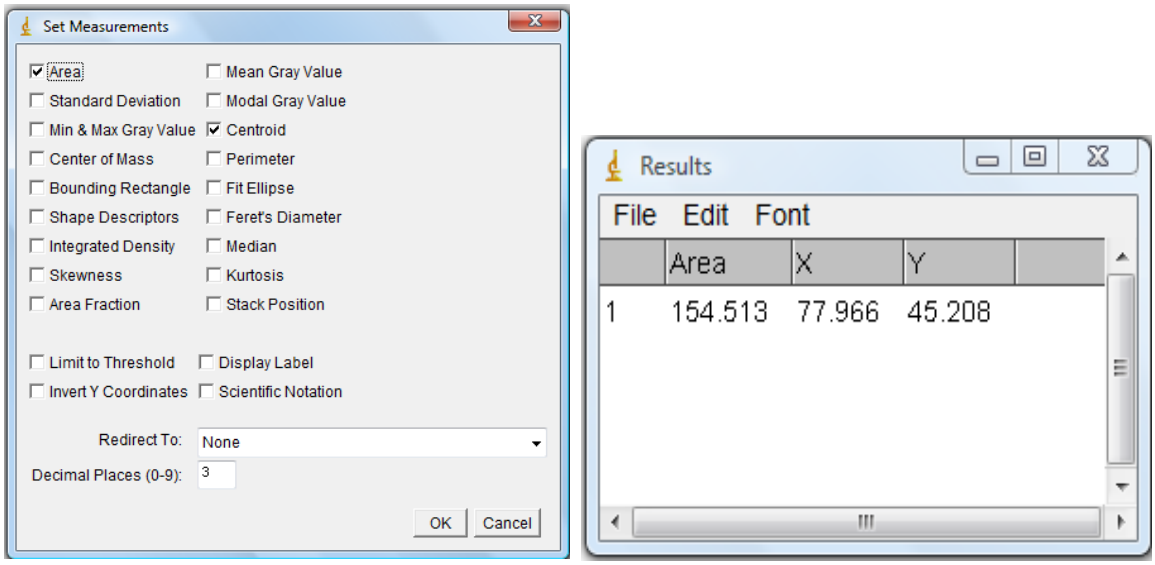


Figure 35 & 36. The Set Measurement table (left) and the result window.

10. Sholl Analysis

Go to “File” and “Open” to open the dilated skeletonized cell image without cell soma area that just saved. To perform Sholl analysis on this image, first use “Point selection” and try best to select the centroid of the cell soma as shown on the result window by looking at the position of the mouse on the bottom left of the ImageJ Menu bar. Then go to “Plugins”, “Analysis” and “Sholl Analysis”. A new window, “Sholl Analysis Options”, will pop up (Figure 37). Enter “10” for “Starting Radius” and set “10” for “Radius Step Size”, this command set the interval between the concentric circles. Enter “100” for “Ending Radius”. Select “Median” in “Span Type”. Values for commands may vary for different images, depending on the size of the Purkinje cell. Click “OK” when setting is finished. The result of the Sholl analysis will be present on a graph in a new window (Figure 38). The x-axis represents the distance (in um) from the center of the soma (point selection) and the y-axis represent the number of the intersections of the concentric circles with the skeletonized dendrites.

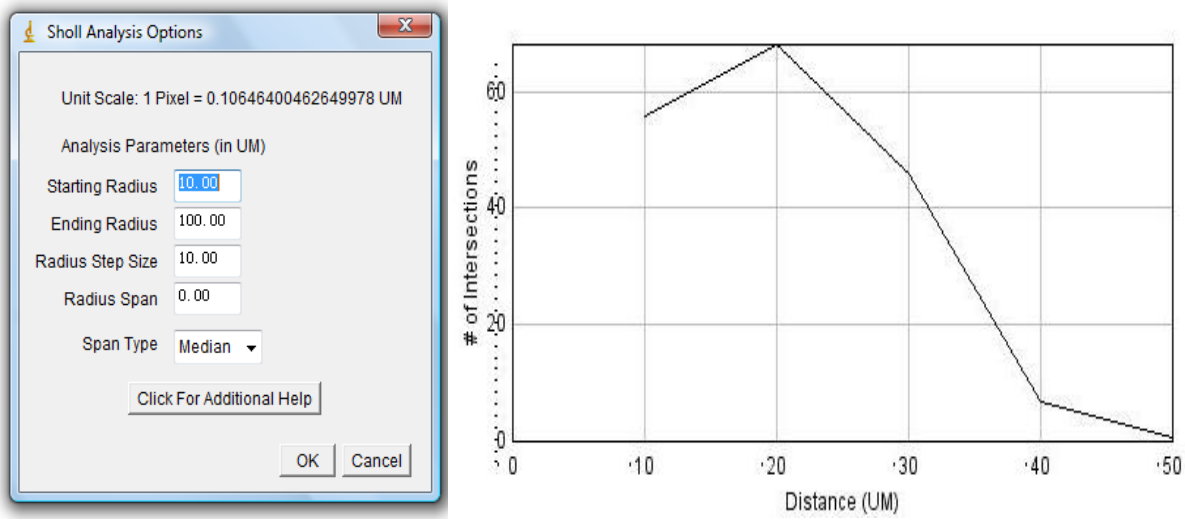


Figure 37 & 38.. The Sholl analysis Option window (left) and the Sholl analysis result for the dilated skeletonized without cell soma area Purkinje cell image (right).

A flow chart of the whole procedure for analyzing Purkinje cell images by Sholl analysis using ImageJ software is also showing in Figure 39.

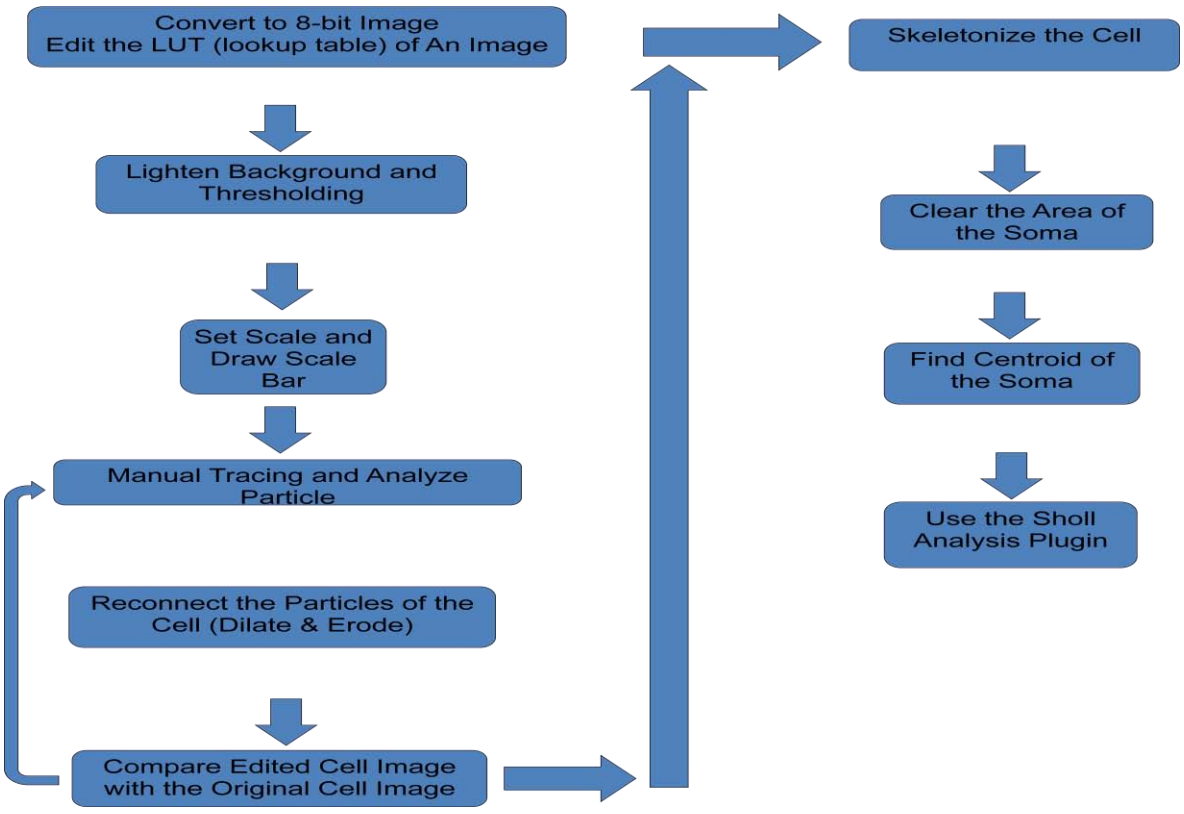


Figure 39. Flow chart of the procedure for analyzing Purkinje cell image by Sholl analysis using ImageJ software.

Discussion

This experiment developed a procedure for analyzing the dendrite morphology of the Purkinje cell. At the beginning of the experiment, there were a few major questions: Does ImageJ process the drawing of the cell or the image of a cell? If the drawing of the cell is needed, how to make the drawing; if the image of the cell is needed, how to erase the background or isolate the Purkinje cell from the background and skeletonize the Purkinje cell. Finally, how to find the center of the cell soma and draw the concentric circles.

In order to make the procedure efficient, the image was processed by the ImageJ program instead of making a drawing. The experiment started with skeletonizing a fluorescent Purkinje cell. This process was not too difficult since the cell in the image was continuous and the background was black. However, most Purkinje cell images that needed to be analyzed were not fluorescent cell images. Those cell images were taken by the Nikon TE2000 microscope while shining a polarized light on the Purkinje cell culture stained with calbindin to make small structures visible, such as spines (Baptista et al,, 1994). On the other hand, using this technique increased the difficulty for analyzing the Purkinje cells because the polarized light created light areas and dark areas on the Purkinje cell, and it was difficult for the computer to recognize the light areas as parts of the Purkinje cell in which the Purkinje cell was considered as many discontinuous particles. This problem was solved by editing the pixel values in the LUT, which change the areas in the Purkinje cell that had high pixel values to a low pixel value, and then dilate and erode the cell to make it as a continuous object.

In addition to editing the pixel value and connecting all parts of the Purkinje cell, clearing the background of the image was also complicated. There were granule cells, axons from different cells and other noises in the background. Adjusting the brightness and thresholding helped lighten the background and convert the image to black and white. Then “Freehand selection” and “Analyze particles” commands were used to isolate and present just the Purkinje cell on another slice. Special care was taken while manually tracing to make sure all parts of the Purkinje cell were included, and areas that did not belong to the cell were eliminated. Many quality controls were done to insure the computer processed image was reliable, such as compare the computer processed cell with the original cell image. The skeletonized cell was made from the analyzed Purkinje cell after “Dilate” and “Erode” commands. Most of the skeletonized cells needed one additional dilation since many lines of the skeletonized cells was unconnected. Other questions also arose during the experiment, such as how to set a scale for an image and how to draw a scale bar for an image, but those problems were all solved during the process.

Besides problems from processing the cell image, the concentric circles were not able to be drawn by using ImageJ to perform Sholl analysis. However, the Sholl analysis plugin was found instead (Sholl analysis plugin). Installing the plugin was a little complicated because the plugin “.class” file could not be downloaded into the ImageJ file directly. The file had to be saved on the desktop then copy and paste into the

ImageJ file. After installing the Sholl analysis plugin, the plugin was tested on 15 different images. According to the literatures (Cook et al., 2004; Martínez-Tellez et al., 2005; Vega et al., 2004), the interval of the concentric circles for the Sholl analysis was 10um. Because no known literature presents the study of Purkinje cell using Sholl analysis, intervals of 10um and 5um were both tested on the skeletonized cells. After comparing the result, an interval of 10um was used as the standard concentric circle interval for the Sholl analysis.

There are still questions remaining and future researches needs to be done. While using the Sholl analysis plugin on the skeletonized cell image and the stack of the same skeletonized cell image with the original cell image, the results of the same image under two different conditions had a significant difference. The number of intersections of the concentric circles with the skeletonized dendrites was significantly larger in the skeletonized cell image than the stack of the same skeletonized cell image with the original cell image. The center of the cell soma was identified by using centroid measurement and then using the mouse to select the closest point to the centroid. This method is more advanced than physically picking with human eyes, but improvement still could be made on selecting the point.

In conclusion, this experiment of developing the procedure for studying the Purkinje cell by Sholl analysis using ImageJ software is incomplete. The procedure provided a good method for processing the Purkinje cell image by ImageJ. However, more experiments need to be done to test the Sholl analysis plugin, improvement could be made for selecting the center of the cell soma, and more images need to be analyzed to confirm the reliability of this method. Future work needs to be done to improve and complete this procedure.

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Image J: http://rsbweb.nih.gov/ij/

Sholl Analysis Plugin: www-biology.ucsd.edu/labs/ghosh/software