ABSTRACT

Neurodevelopmental diseases drastically impact the lives of millions. Not only are the lives of these people altered, but it also poses an increasing public health challenge. Finding new measures to prevent and treat such diseases is the main priority of this research. Immunohistochemistry (IHC) is a technique that enables the visualization (using confocal microscopy) of the tissue distribution of specific antigens. The process localizes protein targets of interest by applying specific monoclonal or polyclonal antibodies to tissue surfaces in a process called antibody incubation (Histochem, 2006). A major obstacle in IHC is keeping the vital brain slices alive long enough to observe them as experimental models. By assessing the mouse brain slices by using IHC, slices can be examined under which conditions they maintain optimal functions. The goals of this research project is to assess the health of mouse brain slices and figure out under which conditions do these slices survive and thrive the best for their use in further experiments. This later translates in the use of these slices as experimental disease models for the purpose of neurological studies. Results were found with the acute and the cultured slices. The cultured experimental forms of microtubule associated protein 2 (MAP2) and Tyrosine hydroxylase (TH) had significant fluorescence indicating maintained slice health and the presence of pyramidal cells as characterized by the MAP2 staining and dopaminergic cells as characterized by the TH staining. Preliminary results show some promise of using these methods to culture brain slices to serve as disease models.

INTRODUCTION

The human brain is still one of the most misunderstood organs in the human body to this date. Vast amounts of research ranging from the sensory and motor aspects of the brain to the chemical reactions and functions responsible during certain activities of the brain have been ventured by many. The brain, all together, has four distinct sections called lobes, which in their own aspects have distinct functions and responsibilities. They are the: Frontal lobe – involved in motor function, problem solving, spontaneity, memory, language, initiation, judgment, impulse control, and social and sexual behavior (Levin et al., 1987); Parietal lobe – The first function integrates sensory information to form a single perception (cognition). The second function constructs a spatial coordinate system to represent the world around us (Kandel et al., 1991); Temporal lobe – involved in the primary organization of sensory input and hearing (Read, 1981); and the Occipital lobe – center of our visual perception system (Westmoreland et al., 1994).

Another section of the brain not considered a lobe, but never the less important in the proper function of the human body is the limbic lobe which is responsible for judgment, insight, motivation and mood. It is also important for conditioned emotional reactions (Swenson, 2006). All of these sections are vital in the proper and normal function of the human body.

Damage to any parts of the brain can result in numerous health problems ranging from speech and sight impediments, to memory/cognition problems. In the case of this study, certain detrimental neurological disease that impact the brain is caused by the damage of the certain neurons. Parkinson's disease (PD), second in incidence to Alzheimer's disease, is a progressive neurodegenerative disorder characterized by the loss of substantia nigra pars compacta (SNpc) dopaminergic neurons (Dauer et al., 2003). It is still unclear why these neurons are damaged.

PD is a disorder that affects nerve cells, or neurons, in a part of the brain that controls muscle movement. In Parkinson's, neurons that make a chemical called dopamine die or do not work properly. Dopamine normally sends signals that help coordinate movements (NIH, 2009). Another disorder associated with loss of dopamine is schizophrenia. Schizophrenia is a chronic, severe, and disabling brain disorder that affects about 1.1 percent of the U.S. population age 18 and older in a given year. People with schizophrenia sometimes hear voices others do not hear, believe that others are broadcasting their thoughts to the world, or become convinced that others are plotting to harm them (NIMH, 2009).

It is noted that such disorders manifest in the area of the substantia nigra. The substantia nigra (literally meaning "black substance") is a small region in the brain stem, located just above the spinal cord. It is one of the centers that help control body movements. Cells within the substantia nigra (SN) produce and release a chemical called dopamine. Dopamine is a neurotransmitter that helps control movement and is essential to the proper functioning of the central nervous system (CNS). Dopamine assists in the effective communication (transmission) of electrochemical signals from one nerve cell (neuron) to another. Dopamine released by SN neurons lands on the surface of neurons in other brain centers, controlling their activities or inciting the area and thus regulating movement. In SZ, cells of the SN degenerate, and therefore can no longer produce adequate amounts of dopamine for proper motor functions. When this occurs, neurons elsewhere in the brain are no longer regulated and do not behave in a normal manner. This results in a loss of controlled movements, leading to slowed movements, tremor, and rigidity (WE MOVE, 2008).

The study plan is to assess brain slice health of the SN of c57/bl6 mice by conducting immunohistochemistry. IHC involves the localization of antigens or proteins in tissue sections by the use of labeled antibodies as specific reagents through antigen-antibody interactions that are visualized by a marker (IHC WORLD, 2007). To study the amount of dopamine receptors present within brain slices, IHC will be used and visualized by florescence. The goal is to maintain optimal amounts of dopamine within brain slices for several days. These slices will be evaluated for healthiness and conditions under which they are kept. The antibodies used for this research include MAP2, TH, and Alexa Fluor-488-conjugate goat anti-mouse IgG. These antibodies are necessary for this type of research because it specifically binds to the proteins that are necessary in helping to determine the health of such brain slices. Binding and fluorescing these antibodies serves as indicators to aid in the assessment of brain slice health. Acute brain slices will be used to compare with cultured slices. If fluorescence remains constant during the time period, then that means the methods had successfully kept the brain slices alive and healthy enough for further experimentation intent. However, if the fluorescence fades then that indicates the slices are not doing so well and are dying rendering them useless for the cause. The goal of this research is to maintain these brain slices for several days by successfully keeping them healthy for the use of further research and studies.

The possible medical breakthrough this research may hold for people harboring mental disorders associated with dopamine loss can help pave the way into understanding conditions in which dopamine survives and in instances when they die. This can help doctors, scientists, and people gain the one step advantage of preventing and treating such disorders and create better quality of life throughout the world.

HYPOTHESIS

1. It is expected that the cultured slices will be nearly as healthy as the acute slices after one day in culture.
2. It is hypothesized that pyramidal cells of the prefrontal cortex and dopaminergic neurons in the midbrain can survive together in 14-day old cultures.

MATERIALS

NeuroBasal Medium with 25% Horse Serum (100 mls):

Materials needed for making the NeuroBasal Medium with 25% Horse Serum include NeuroBasal medium, horse serum, B-27 supplement, pen/strep stock (1%), GlutaMax stock (200 mM (100X concentrate); 2 mM final) nitrile exam gloves, and 70% ethonol.

Paraformaldehyde (PFA) Solution (4% in PBS):

Materials needed for making this solution include PFA powder, balance, fume hood, hot plate/stirrer, spoon, pH strips, H_2O (Millipore), beakers, 10X phosphate-buffered saline (PBS), 1.0N NaOH, pH paper, large beaker, containers, stir bar, Whatman filter papers, 5.0 NaOH, ring-stand, permanent markers, and a thermometer.

PBS / 0.2% Triton / 5% Serum Blocking Solution (P/T/S):

Materials needed for making this solution include pipettes, pipette tips, 0.2% triton, 5% goat serum, 1X PBS, scissors, permanent markers, and test tubes.

Explant Preparation:

Materials needed for performing such a method include PFA solution, Neurobasal media,

c57/bl6 mice, ice, cut buffer, mouse box, adhesive, petri dishes, well plates, pipettes, pipette tips, PBS, scissors, foreceps, fine paint brush, microscissors, and an EMS 5000 vibrating-blade tissue slicer.

Antibody (AB) Solutions:

Materials needed for making this solution include the P/T/S solution, pipette, pipette tips, test tubes, permanent markers, and necessary ABs.

MAP2 / TH Primary AB with Alexa-488 Fluorescent Secondary AB:

Materials needed for the performing this method include pipettes, pipette tips, 12 well plates, timer, PBS, PFA, P/T/S, primary antibodies (AB^1), secondary antibodies (AB^2), microscope with fluorescent capabilities, tape, parafilm, aluminum foil, and permanent markers.

Brain Slice Mounting:

Materials needed for performing this method include PBS, pipettes, pipette tips, transparent slides, mounting solution, and fine-tip paintbrush.

Confocal Microscopy:

Materials needed for performing this method include mounted slides of slice specimens, confocal microscope, digital camera, Kimwipes, computer, confocal microscopy computer software, and slide transpotation box.

METHODS

NeuroBasal Medium with 25% Horse Serum (100 mls):

Obtain the necessary materials in their proper concentrations from their proper storage location. Let the materials thaw out in a warm water bath for about 10 – 15 minutes. Since the concentrations have been measured out prior to the creation of the solution, just add all the

components into an appropriate sized beaker and mix the solution on a stir plate to ensure proper dilution of all materials. Once satisfied results become apparent place made up solution into a bottle and label appropriately.

PFA Solution (4% in PBS):

One must prepare the solution in a fume hood, for the PFA is a level three hazard and can cause severe health problems if it comes in contact with. Safety is very important and thus the reason for using a fume hood. This method is for a final volume of 100 ml. While under the hood, weigh four grams of PFA into a 250 ml beaker. Add 50 ml distilled water and thermometer and start heating it on a hot plate/stirrer. Heat the solution up to 60 – 65^{o}C. DO NOT OVERHEAT the solution (temperature will increase rapidly after reaching 40 – 50^{o}C). Once the temperature reaches 60^{o}C, add 1.0N NaOH drop-wise until solution clears. Add slowly, since the solution does not clear immediately. Keep the pH around 7 (test with strips). Add 50 ml 2X PBS and stir well. Chill the solution in ice bath to room temperature. Filter the solution with Whatman filter paper into another 100 ml bottle. The newly made solution should be good for about two weeks.

PBS / 0.2% Triton / 5% Serum Blocking Solution (P/T/S):

Calculations must be made to make the correct amounts and concentrations of solutions, to make sure no materials go to waste. The calculations are based on the number of brain slices present in each well. Since each well should receive 500 µl, multiply that to the number of brain slices you have for each well. This calculation should give the exact amount of PBS solution to use to figure out the rest of the dilution. Now that there is a known value of the amount of PBS needed, the triton and serum concentrations can be added. Multiply the 0.002 of triton by the total amount value of PBS and multiply that to 1000µl to get the total amount of triton needed

for the solution. For this step make sure to cut the tip off of the regular pipette tip to allow easy passage of liquid substance. To figure out the total amount of serum needed, multiply the 0.05 value by the total amount of PBS and multiply that amount by 1000µl to receive the total amount of 5% serum needed for the entire solution. Pull all the amounts together in a test tube, label, and store in refrigerator until use.

Ex: Amount of PBS

→24 well plates × 0.5ml = 12ml of PBS

PBS 1X 0.2% triton

→ 0.002 × 12ml = 0.024ml × 1000µl = 24µl amount needed of triton for solution

5% serum

→ 0.05 × 12ml = 0.6ml × 1000µl = 600µl amount needed of serum for solution

***Expant Preparation*:**

Tissue slices were prepared from 1-6 day old C57 BL/6 mouse pups from the breeding colony at the UNC animal facility. All procedures were performed according to The University of Northern Colorado Institutional Animal Care and Use Committee (IACUC) approved protocol, and was in accordance with NIH guidelines. Animals were euthanized via immersion in an ice water bath were they would be anesthetized due to the pups lack of thermo-regulators and then immediately decapitated. The brain was rapidly removed and placed in 4°C oxygenated physiological saline (PS) solution for 3-4 minutes before slicing. The brain was then glued to a platform and immersed in cold PS via a chamber surrounding the slicing platform. Slices containing prefrontal cortex and ventral striatum, and slices from the midbrain ventral tegmental area were then prepared using an EMS 5000 vibrating-blade tissue slicer at 350 µm. The tissue slices were transferred onto membrane inserts that rest in 6-well plates containing 1 mL of

culture medium (Neurobasal Medium supplemented with 20% Horse serum) per well. The cultures were maintained in an incubator with a 5% CO_2-enriched atmosphere. Culture medium was replaced twice weekly to provide a continuous supplement of necessary nutrients. The slice explants were cultured for 1-2 weeks before immunohistochemical experiments are preformed.

AB Solutions:

The method of calculations for making the AB solution is similar to that of making the P/T/S solution. The amount of AB needed for the solution is based on the concentration of the solution and the total amount of brain slices there are present for experimentation. Based on these amounts one can calculate the necessary amounts of AB needed for the correct concentration of the solution. For this experiment the concentration needed is 1:500, take this ratio and multiply it to the total amount of PBS needed for the solution (this number is based on the total number of brain slices) and multiply that number by 1000µl. This calculation should give the exact quantity of AB to use in the solution.

Ex: Amount of PBS

→ 12 well plate × 0.5ml = 6ml of PBS

PBS 1 0.2% triton

→ 0.002 × 6ml = 0.012ml × 1000µl = 12µl amount needed of triton for solution

5% serum

→ 0.05 × 6ml = 0.3ml × 1000µl = 300µl amount needed of serum for Solution

AB amount

→ 0.002 × 6ml = 0.012ml × 1000µl = 12µl amount of AB needed for solution

MAP2 / TH Primary AB with Alexa-488 Fluorescent Secondary AB:

Rinse the cultured wells twice with 1X PBS to remove serum proteins. Fix the cells in a

4% PFA solution in PBS for one hour, with about 1ml per well, at room temperature. This step is necessary to permanently "fix" the cells, in which they no longer grow or shrink. After the fixing step, rinse the slices three times, 15 minutes each, with about 1ml of PBS solution. Extract the PBS wash and add 500µl of the P/T/S solution for one hour to each well at room temperature with gentle rocking. Do not rinse immediately following the blocking step. Incubate the wells in primary AB^1 solution of 1:4000 dilution in P/T/S for 1 hour at room temperature with gentle rocking. Remember for the control to incubate the wells in P/T/S with no primary AB^1. After the designated time period, remove all solution from the wells. Save the primary AB^1 solution for it is re-usable. Label the test tube with the appropriate labels. Rinse all the wells with the PBS wash, three times for 15 minutes each. Incubate all the wells now with the secondary AB^2 of 1:4000 dilution in P/T/S for one hour at room temperature with gentle rocking. From this point on protect the cells from light by shielding the dishes with aluminum foil to avoid quenching the fluorophore. After the hour mark, remove the Secondary AB^2 and save the solution into a new test tube, appropriately labeling it as such. Repeat the rinsing step three more times for 15 minutes each again. After the final wash add more PBS, just enough to completely cover the brain slices. Now observe the cells with fluorescent microscope (green filter set). Take notice of fluorescence within the wells. There should be noticeable fluorescence in all the wells except for the control.

Brain Slice Mounting:

Figure out the best slices out of the acute and cultured well plates for the purpose of confocal microscopy. Once designated slices are picked out, remove them one by one from their wells and place them on individual transparency slides. Remove all the remaining solution on the slide by a pipette and wipe the remaining solution left off by using kimwipes. Once the brain

slice specimen is cleaned, add the mounting solution to the slice. Place the slide mount coverslip over the mounting solution and wait overnight for the transparency slips to be completely fixed and mounted for observation purposes.

Confocal Microscopy:

Once brain slices are mounted to transparency slips, they are ready for the process of confocal microscopy. Turn on the microscope and place the transparency slip onto the microscope slide mount. Focus on the portion of the brain slice that suits best for the experimental design. Use the special software to take digital pictures of the brain slices to help in determining the results of the experiment.

RESULTS

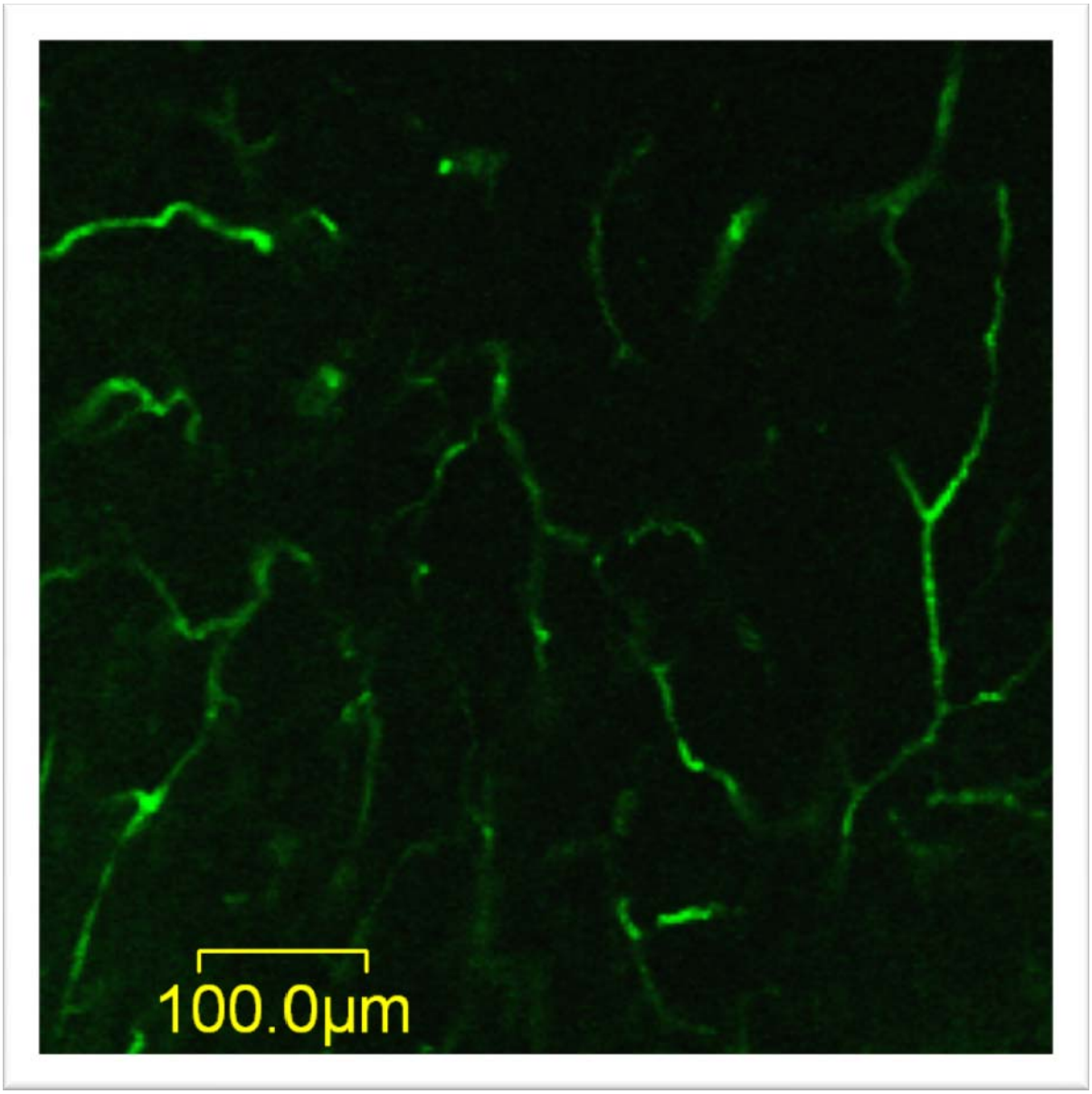

Figure 1: Acute Pyramidal Cell in the Prefrontal Cortex Experimental

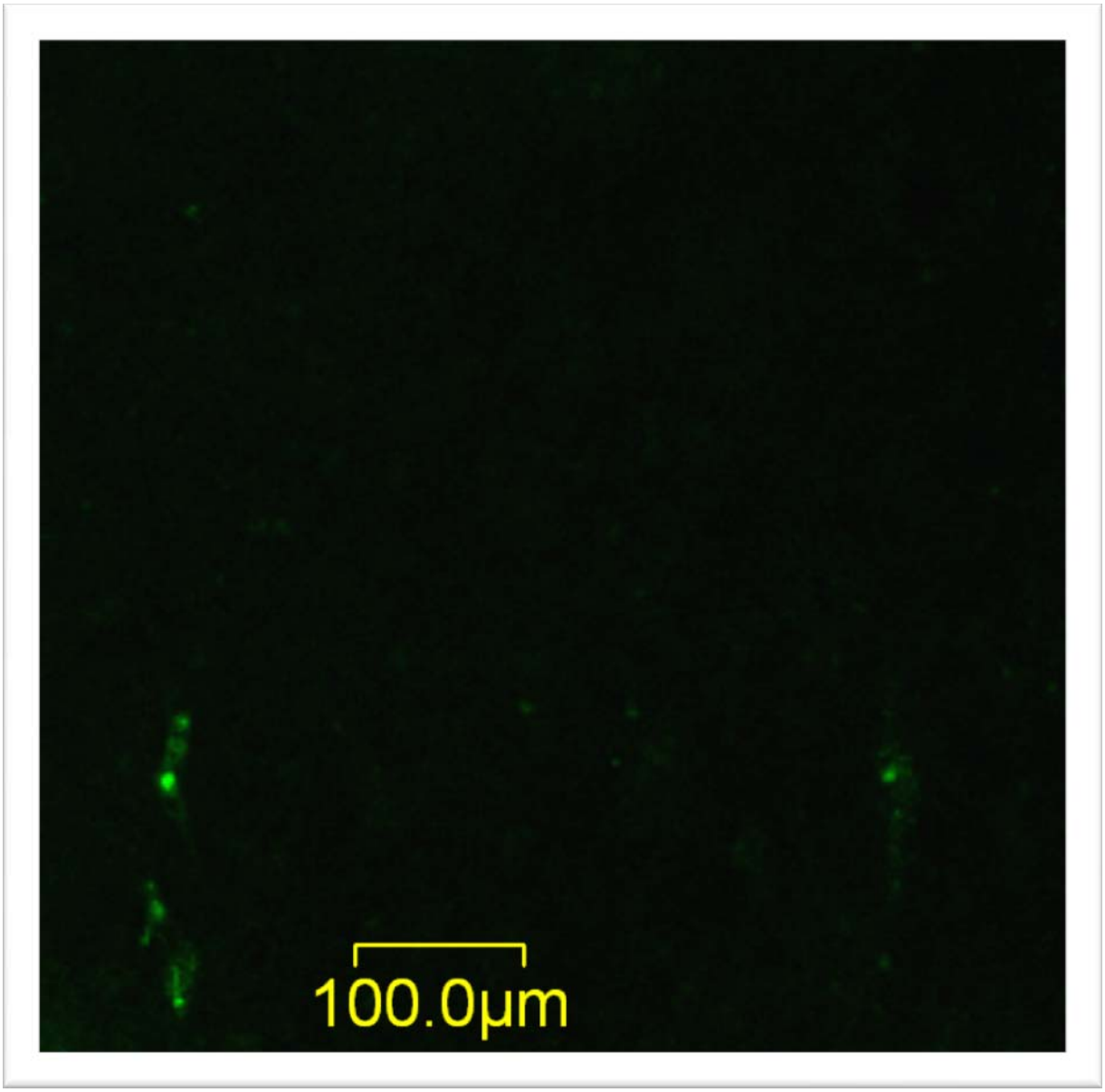

Figure 2: Acute Pyramidal Cell in the Prefrontal Cortex Control

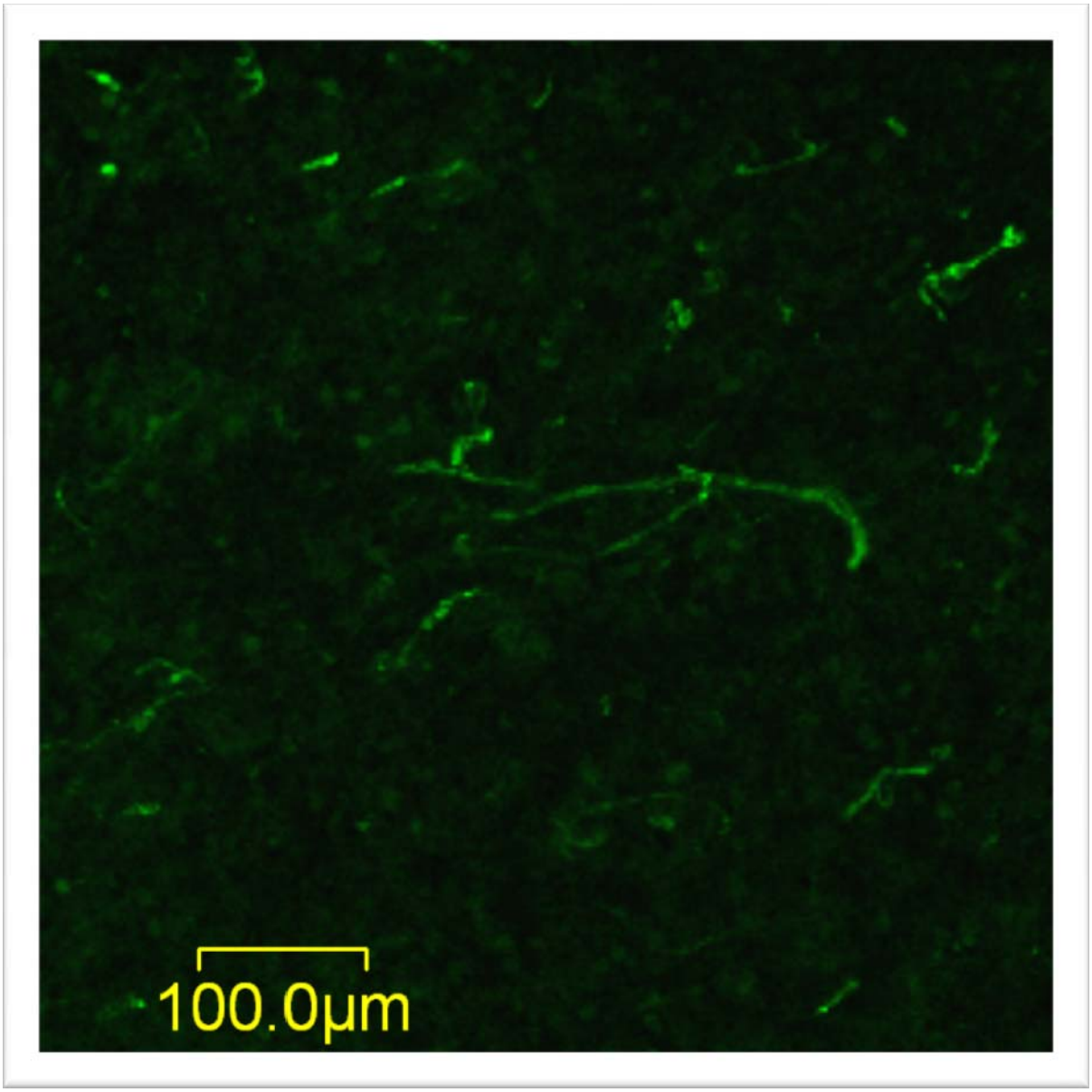

Figure 3: Cultured Pyramidal Cell in the Prefrontal Cortex Experimental

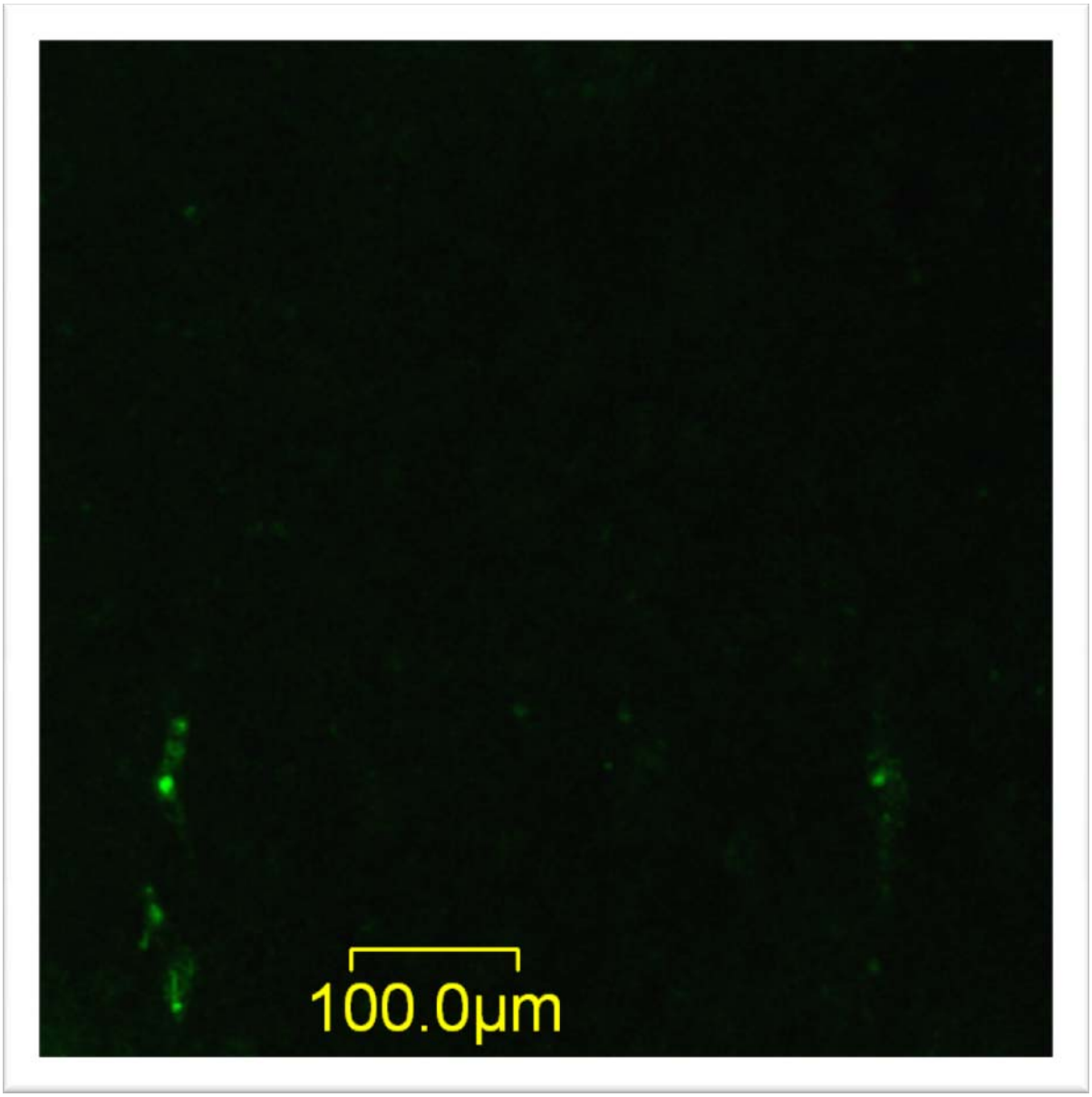

Figure 4: Cultured Pyramidal Cell in the Prefrontal Cortex Control

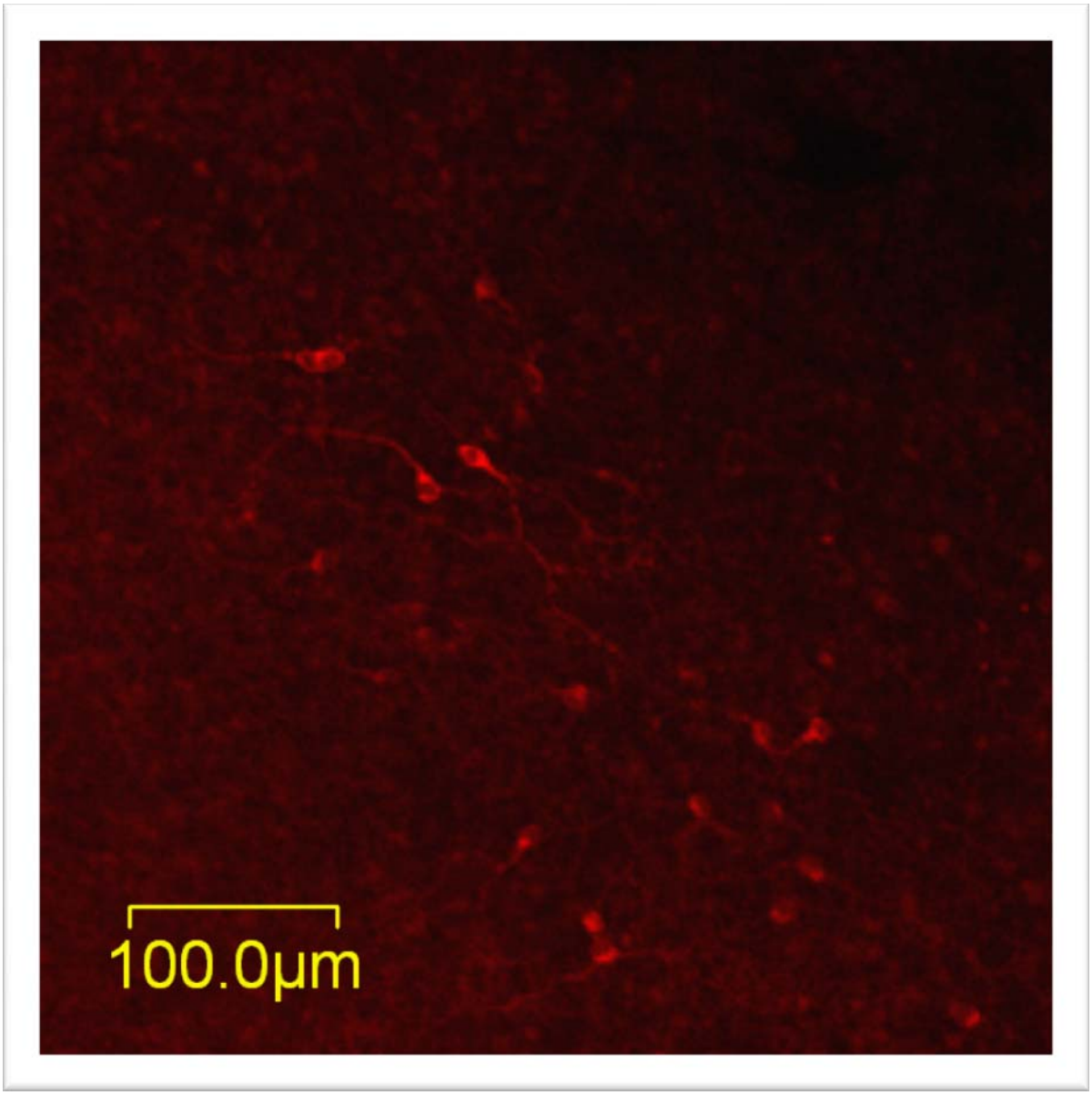

Figure 5: Cultured TH 1:500 Dopaminergic Cells Experimental Located in the Midbrain Region

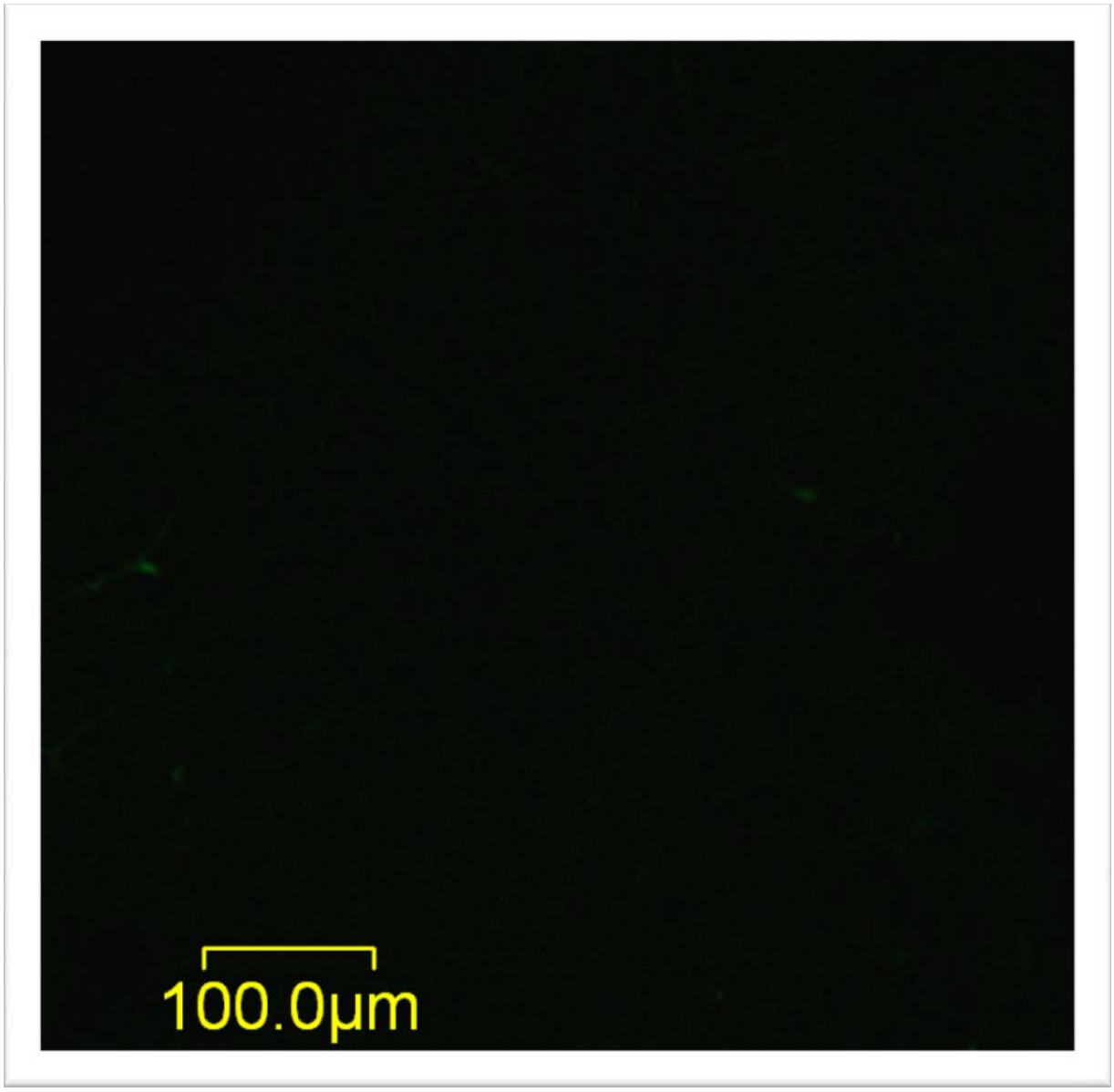

Figure 6: Cultured TH 1:500 Dopaminergic Cells Control Located in the Midbrain Region

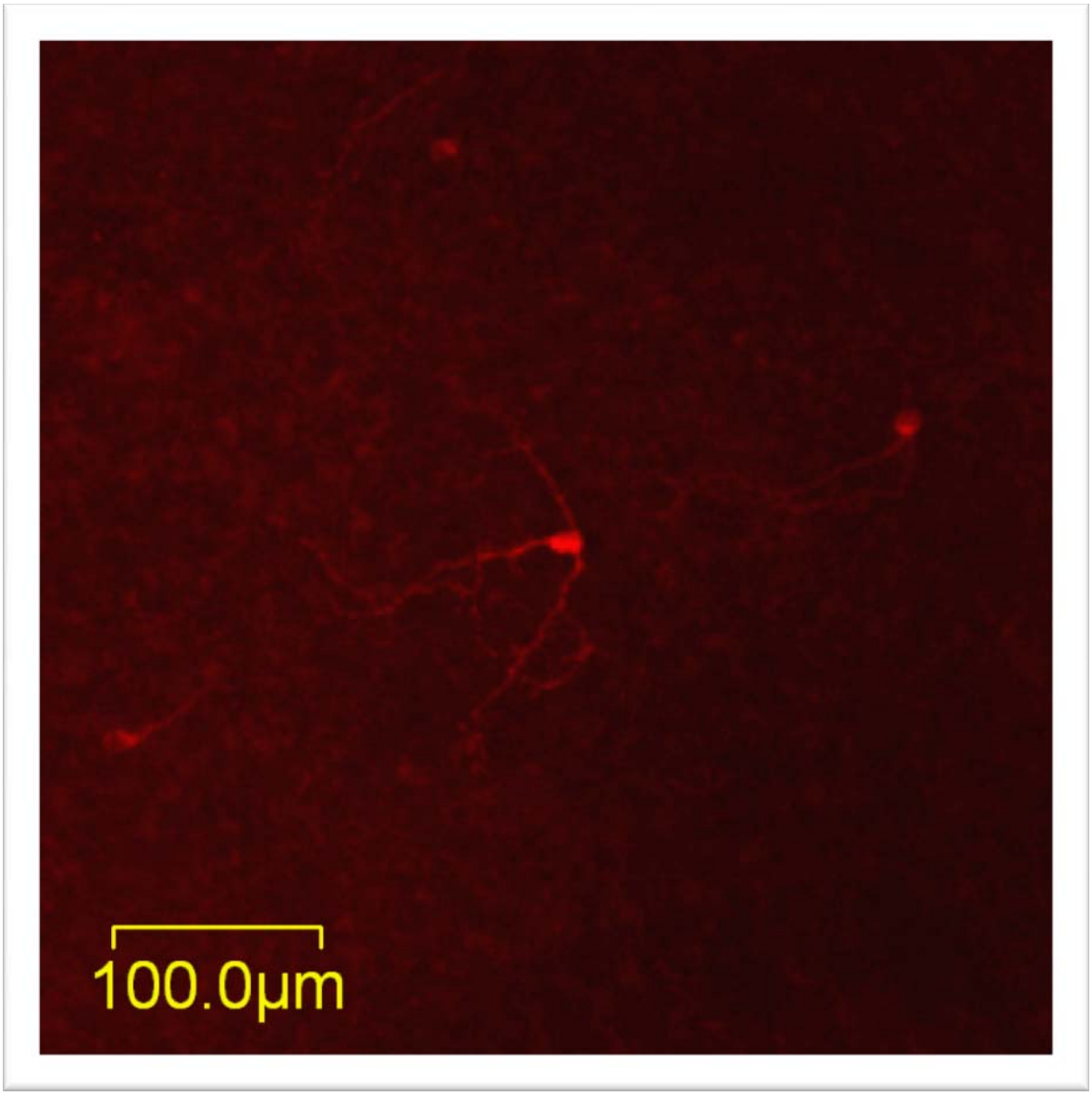

Figure 7: Cultured TH 1:500 Dopaminergic Cells Experimental Located in the Midbrain Region

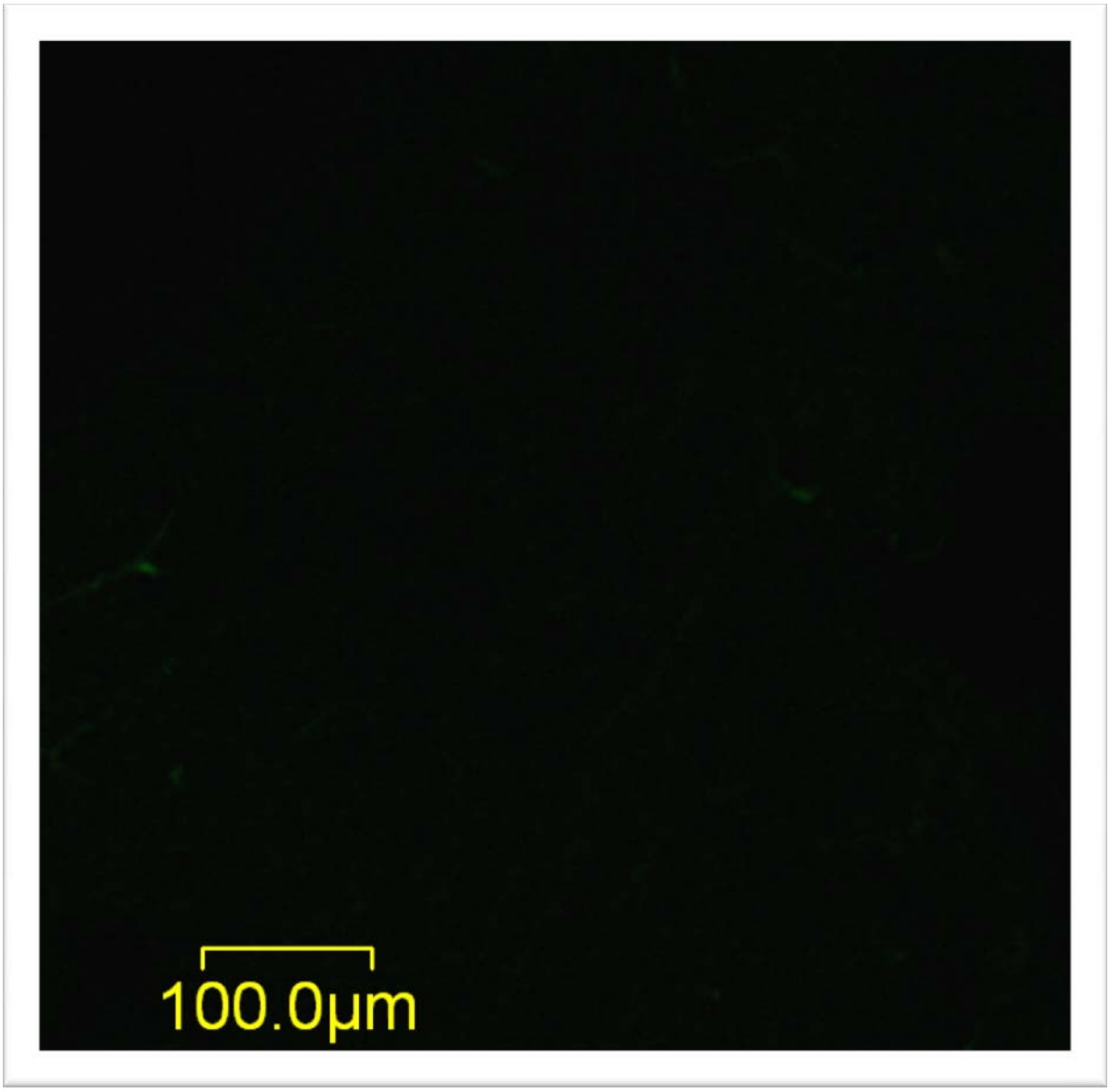

Figure 8: Cultured TH 1:500 Dopaminergic Cells Control Located in the Midbrain Region

DISCUSSION

The results of this experiment showcased fluorescence, which indicated tagged antibodies. These results showed that since there is fluorescence present, we can compare and assess brain slice health by qualitatively looking at the amount of fluorescence. The results of these experiments showcased fluorescence, which indicated tagged antibodies in the Pyramidal cells indicated by the MAP2 (Figures 1-4) and in the Dopaminergic Cells (Figures 5-8). The comparative analysis of the cultured slices show that these cells can survive, at least for a short periods of time, in the media solution utilized in our experiments.

CONCLUSION

The research goal of this project was assessing brain slice health by using methods of IHC. It was hypothesized that the cultured slices will be nearly as healthy as our acute slices after one day in culture, and that the fourteen day old slices will be nearly as healthy as the one day old slices. Our hypotheses were supported by the results of fluorescence present within the brain slice specimens. These results showed that brain slices can live in culture for at least fourteen days, however, due to the limited number of trials, more research is recommended. The data demonstrates the probable viability of using these slices for experimental disease models. This is an important contribution to this field of research because it is one of the major obstacles associated in keeping a brain slice tissue alive long enough for the duration of an experiment. With brain slices dying within a day, it is not logically viable to carry out research involving these specimens. That is why these results are promising, and gives hope of actually finding the

proper methods and conditions under which these slices survive. Also by locating these different neurons, the neuronal pathways can be examined to study whether theses pathways stimulate or connect to one another. This opens much more in the field of neurology and helps pave the way in research involving neurological diseases such as Schizophrenia.

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