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Rapid Golgi Stain for Dendritic Spine Visualization in Hippocampus and Prefrontal Cortex

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TITLE:

Rapid Golgi Stain for Dendritic Spine Visualization in Hippocampus and Prefrontal Cortex

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KEYWORDS:
dendritic spines, synaptic plasticity, golgi impregnation, pyramidal cell, hippocampus, prefrontal cortex

SUMMARY:
The protocol describes a modification of the rapid Golgi method, which can be adapted to any part of the nervous system, for staining neurons in the hippocampus and medial prefrontal cortex of the rat.

ABSTRACT:
Golgi impregnation, using the Golgi staining kit with minor adaptations, is used to impregnate dendritic spines in the rat hippocampus and medial prefrontal cortex. This technique is a marked improvement over previous methods of Golgi impregnation because the premixed chemicals are safer to use, neurons are consistently well impregnated, there is far less background debris, and for a given region, there are extremely small deviations in spine density between experiments. Moreover, brains can be accumulated after a certain point and kept frozen until further processing. Using this method any brain region of interest can be studied. Once stained and cover slipped, dendritic spine density is determined by counting the number of spines for a length of dendrite and expressed as spine density per 10 µm dendrite.

INTRODUCTION:
The method of using potassium dichromate and silver nitrate to label neurons was first described by Camillo Golgi [1,2] and subsequently used by Santiago Ramon y Cajal to produce an immense body of work differentiating neuronal and glial subtypes. A recently published book with his illustrations is now available[3]. Following Ramon y Cajal's studies, which were published more than 100 years ago, very little Golgi impregnation was used. Golgi impregnation is a laborious process that allows three-dimensional visualization of neurons with a light microscope. There have been numerous modifications of the Golgi method over the years to make the method

easier and the staining more consistent[4]. In 1984, Gabbott and Somogyi[5] described the single section Golgi impregnation procedure which allowed for more rapid processing. This Golgi impregnation method requires perfusion with 4% paraformaldehyde and 1.5% picric acid, post-fixation followed by vibratome sectioning into a bath of 3% potassium dichromate. Sections are mounted onto glass slides, the four corners of coverslips glued so that when immersed in silver nitrate, diffusion is gradual. Coverslips are then popped off, sections are dehydrated, and eventually cover slipped permanently with mounting medium. This technique was successfully used to label neurons and glia[6-8] in the hippocampus. The rapid Golgi method described here is an improvement because there is far less exposure to both potassium dichromate and silver nitrate and no paraformaldehyde and picric acid are used. In addition, although cells that were impregnated using modifications of the Gabbott and Somogyi[5] method could be analyzed, often the sections were over-or under-exposed or fell off the slides during the dehydration step and generally, several experiments had to be pooled to have enough cells for analysis.

The present protocol describes the use of the Golgi staining kit (see **Table of materials**) to label dendrites and dendritic spines in the hippocampus and medial prefrontal cortex (mPFC) of the rat. The advantages of this method over previous ones are that it is rapid, there is less exposure to noxious chemicals for the researcher and there is consistent staining of neurons. The protocol described below has been used with minor modifications to assess dendritic spine density in the hippocampus and mPFC of the rat in many studies[9-15].

PROTOCOL:

All experimental procedures are approved by the Sacred Heart University Institutional Animal Care and Use Committee and are in accordance with the NIH Guide for the Care and Use of Animals.

1. Isolation and infiltration of brain tissue

1.1. Premix solutions A and B of the Golgi staining kit 24 h prior to use and keep in dark bottles and/or in dark. Make approximately 80 mL of solution A and B mix which is sufficient to change the solution after 24 h. Store in airtight bottles.

NOTE: Perfusion with either saline or paraformaldehyde is not necessary.

1.2. Sacrifice rats by guillotine following carbon dioxide euthanasia and remove brains within seconds. Rinse brains in saline if required but is not necessary.

1.2.1. Place the brain, cortex down so that the hypothalamus is visible because the cuts are made anterior and posterior to it, on a non-porous surface. Cut into an anterior (contains the prefrontal cortex) and posterior (contains the hippocampus) block as shown in **Figure 1**.

1.3. Place blocks into the premixed solutions of A and B, making sure that they are well immersed in the solution. Ensure that the volume of solutions A and B is sufficient to immerse the blocks. Store blocks in either brown bottles or clear bottles covered with foil (to keep light

out) in the dark at room temperature.

1.4. Replace solution after 24 h and keep in the dark for another 13 days at room temperature.

NOTE: If used with mouse brains, there is no need to block, and the entire brain can be placed in the solution. If staining brain areas, which are different in size, the time in solutions A and B may have to be determined by trial and error.

1.5. After two weeks the tissue is infiltrated well with solutions A and B, transfer the blocks to the cryoprotectant solution (solution C in the Golgi staining kit), and leave them for 48–72 h at 4 °C.

NOTE: After cryoprotection, blocks may be frozen until further processing. Also, note that all solutions from the kit are collected and disposed of as hazardous waste.

1.6. Freeze brain, cortex down, on a glass slide on dry ice. Once frozen either cut on the cryostat or store at -80 °C until sectioning using a cryostat.

NOTE: Do not freeze in liquid nitrogen as this produces cracks in the tissue.

2. Sectioning of brain tissues

2.1. Place a small amount of tissue medium on a pre-cooled cryostat chuck. Mount blocks on cryostat chucks by thawing one side of the block slightly in hand (gloved) and placing it on the tissue medium.

NOTE: It is not necessary to embed the tissue. The block containing the hippocampus is somewhat easier to cut than the block with the prefrontal cortex because the latter is anterior to the corpus callosum and the two halves are separate.

2.2. Cut 100 µm sections on a cryostat at -22 °C and mount onto subbed slides. Sections up to 150 µm of thickness can be used. Try to mount 3–4 coronal sections per slide as this decreases the number of slides that require processing. Use one of the following techniques to keep the sections frozen till they get on the slide.

NOTE: These sections are not fixed in the usual way and therefore they tend to melt quickly. allow the sections to melt on the slide. If they begin to melt before being placed on the slide, it is impossible to get them to be flat on the slide. There are several techniques to do this.

2.2.1. Use a freezing spray on the knife and the block. Depending on the temperature and humidity in the room this may be necessary for every section. Use either the antiroll plate or a brush to keep the section flat while cutting.

NOTE: Make sure to have enough freezing spray. Several cans can be used when cutting 20

blocks.

2.2.2. Thaw mount, if possible, by using a room temperature slide and quickly appose it to the section. With practice, one can mount several sections at once. If this doesn't work, keep slides in the cryostat so they are very cold and transfer the section with cold forceps or a cold paintbrush and then thaw mount.

2.3. Clean the knife with paper wipes between slices. If the knife requires more cleaning, use 100% ethanol (EtOH), and let it dry before cutting the next slice.

2.4. Once mounted onto the slide, place the slide flat on cardboard slide trays and allow sections to dry at room temperature (for several hours to a maximum of 48 h) in the dark. Do not cover the slide tray. Ensure that the sections are completely dry before Golgi impregnation. Store flat, in the dark, on slide trays until Golgi impregnation.

NOTE: Drying the sections does not cause any damage.

3. Staining and dehydration of brain tissue

3.1. Perform staining in glass staining dishes. Mix solutions D and E immediately prior to staining. As per the protocol, add one part of solution D, one part of solution E, and two parts of distilled water. For glass staining dishes, make a 200 mL solution to completely submerge the sections. For Koplin jars, this requires less volume.

3.2. Place slides in racks spaced far enough apart to allow solution access to sections.

3.3. Place sections in distilled water for 4 min (2x) before placing them into the Golgi impregnation staining solution for 10 min. Change the staining solution every 70 sections. Change the distilled water when it turns yellow.

NOTE: The timing for the staining step is critical. Too short does not allow enough staining and too long causes over staining, making the dendrites hard to separate when doing analysis. Once out of the staining solution the timing is less critical.

3.4. Dehydrate the sections as follows: 70% EtOH (5 min), 95% EtOH (5 min; 2x), 100% EtOH (5 min; 2x), clearing agent (5 min; 3x). Change all solutions often, especially the 100% EtOH and clearing agent to ensure the sections remain anhydrous.

NOTE: It is not necessary to go through a 50% EtOH step. Counterstaining is not needed for cell visualization because the Golgi impregnation provides sufficient contrast. The use of other clearing agents has not worked as well as the one used here (see **Table of materials**).

3.5. Coverslip with glass coverslips that are 60 mm long with a generous amount of mounting medium. Make sure that there are, as few air bubbles as possible. If needed, carefully remove,

and redo the coverslip (can be done even weeks later). Do this very slowly in order not to damage the section (can be done because of the large amount of mounting medium).

NOTE: A large amount of mounting medium is somewhat messy but still necessary because enough mounting medium must be used to cover the thick 100 µm sections.

3.6. To ensure that only one coverslip is placed on the sections, separate the coverslips in advance of the process.

3.7. Once cover slipped, dry slides flat on any non-porous paper for 3–5 days, moving them slightly, especially after the first day, to avoid sticking. After 3–5 days transfer the slides to slide holders and, ideally, dry slides for at least 3 weeks before examining them. Keep slides flat to decrease the possibility of air bubbles forming.

4. Determination of dendritic spine density

4.1. For analysis of dendritic spine density in pyramidal neurons of both the mPFC and the CA1 region of the hippocampus, examine the most lateral secondary basal dendrites and the most lateral tertiary apical dendrites as described in step 4.1.1 (**Figure 2**).

4.1.1. Choose a dendrite, measure the length of the dendrite using an image analysis program, count the spines on the dendrites using a hand counter, and record both length and number of spines.

4.2. Study and analyze six cells per region (mPFC, CA1) per brain. Quantify a minimum of six brains per group as previously described[7,8]. Choose neurons that meet the following criteria for analysis: cell bodies and dendrites are well impregnated; dendrites are distinguishable from adjacent cells and are continuous.

4.3. Count the spines at 1000x (oil-immersion) by hand counting with a light microscope and measure dendritic length using an image analysis program. Calculate spine density by dividing the spine number by the length of the dendrite and express data as the number of spines/ 10 µm dendrite.

NOTE: There are far more sophisticated methods for differentiating spine subtypes and dendritic architecture that can be used, but hand counting with a light microscope at 1000x can give a rapid result that can then determine if further investigation is necessary. Although every effort is made to consistently sample similar dendrites, there are variations in thickness that may affect the counting.

REPRESENTATIVE RESULTS:

Using the rapid Golgi method, cells are consistently well impregnated so that there are plenty of cells to analyze. This is a marked improvement over prior methods where experiments had to be pooled to have enough data for analysis. Therefore, more samples can be processed at once and

brains can be stored frozen until processing. Examples of Golgi impregnated cells in the CA1 region of the hippocampus are shown at low and high power in **Figure 3**. Counting of spines in a given region yields consistent results with small standard errors. This is also important because one can make comparisons between experiments. **Figure 4** illustrates an experiment in which basal dendritic spine density was increased on pyramidal cells in adolescent male and female rats after environmental enrichment (EE) in both CA1 and the mPFC. Briefly, male and female rats were weaned at postnatal day (PND) 21 and assigned to control or enriched groups. The EE group spent 2 h/day in enriched housing from PND 24–42 while the control group was housed in ordinary cages during this time. EE induced, in adolescents of both sexes, an increase in basal dendritic spine density in both CA1 and the mPFC. Note the small standard error of the mean (SEM) in all the dendritic spine values.

FIGURE AND TABLE LEGENDS:

Figure 1: Ventral surface of rat brain. Photograph of the ventral surface of a fresh rat brain indicating where to cut into anterior and posterior blocks before submerging blocks into initial solutions.

Figure 2: Typical Pyramidal Cell. Schematic of a pyramidal cell, illustrating apical and basal dendrites which are analyzed for dendritic spine density.

Figure 3: Golgi Impregnated Neuron. Examples of Golgi impregnated neurons in the CA1 region of the rat hippocampus**.** Left: Several impregnated pyramidal cells. Scale bar = 25 µm. Upper right: Basal dendrites. Scale bar = 12.5 µm. Lower right: Example of a secondary basal dendrite. Arrows denote spines. Scale bar = 5 µm.

Figure 4: Spine Density. A data set illustrating basal and apical dendritic spine density in the mPFC and CA1 region of the hippocampus (CA1) following environmental enrichment (EE) compared to control (CON) in male (M) and female rats (F). Histograms show the average number of spines/10 µm dendrite $\pm$ SEM. Data were analyzed using statistical analysis software (see **Table of materials**). Two-way (sex X EE) ANOVAs were used to test for group differences and Fisher's LSD tests were used for post-hoc analysis. Significant effects are $p<0.05$. t denotes a significant difference.

DISCUSSION:

The present protocol describes a method of Golgi impregnation that allows for rapid simultaneous processing of many sections. It is an improvement over previously described[5] more labor-intensive methods and consistently yields impregnated neurons for analysis. In addition, there is less exposure to toxic chemicals used in Golgi impregnation. The most challenging part of the process is getting the sections to be flat on the slides, which takes considerable practice. Keeping everything as cold as possible with the use of freezing spray is essential.

Once slides are dry and analysis can be performed, it is very important to be consistent in selecting the cells that are counted. Hippocampal pyramidal cells are chosen from CA1. For the mPFC, which has several subparts, pyramidal cells from the infralimbic cortex are used. For

reasons that are not clear, fewer cells in the mPFC are stained than in the CA1 region of the hippocampus. In addition, it is possible to combine experiments when all the sections cannot be processed together for logistical reasons. For consistency, the same person should count a given set of cells.

The limitations of this method are similar to all Golgi impregnation methods. The fact that only a small number of cells are stained is an advantage. The small number of cells impregnated allows for visualization of the entire cell in three dimensions. The disadvantage of the Golgi method is that it is not clear which subset of cells is labeled. Therefore, in experiments, one must assume that the cells impregnated for both control and experimental groups are the same. Even though this method results in far better staining than previous methods, there are always cells that cannot be analyzed because they are covered by debris, an air bubble, or have broken dendrites.

In conclusion, the rapid Golgi method described here is a fast, safe method for consistent and reproducible labeling of neurons that can be used for any brain region. In addition to labeling cells in the prefrontal cortex[17,18] and hippocampus [10,12,19], it has also been used in the amygdala[20], cerebellum[21], and cortex[22]. Assuming one is familiar with the anatomy and can quickly identify cells, hand counting of spine density can provide a quick result, but it does not provide information on dendritic subtypes which requires more sophisticated methods of analysis.

ACKNOWLEDGMENTS:
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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1. Photograph of the ventral surface of a fresh rat brain indicating where to cut into anterior and posterior blocks before submerging blocks into initial solutions.

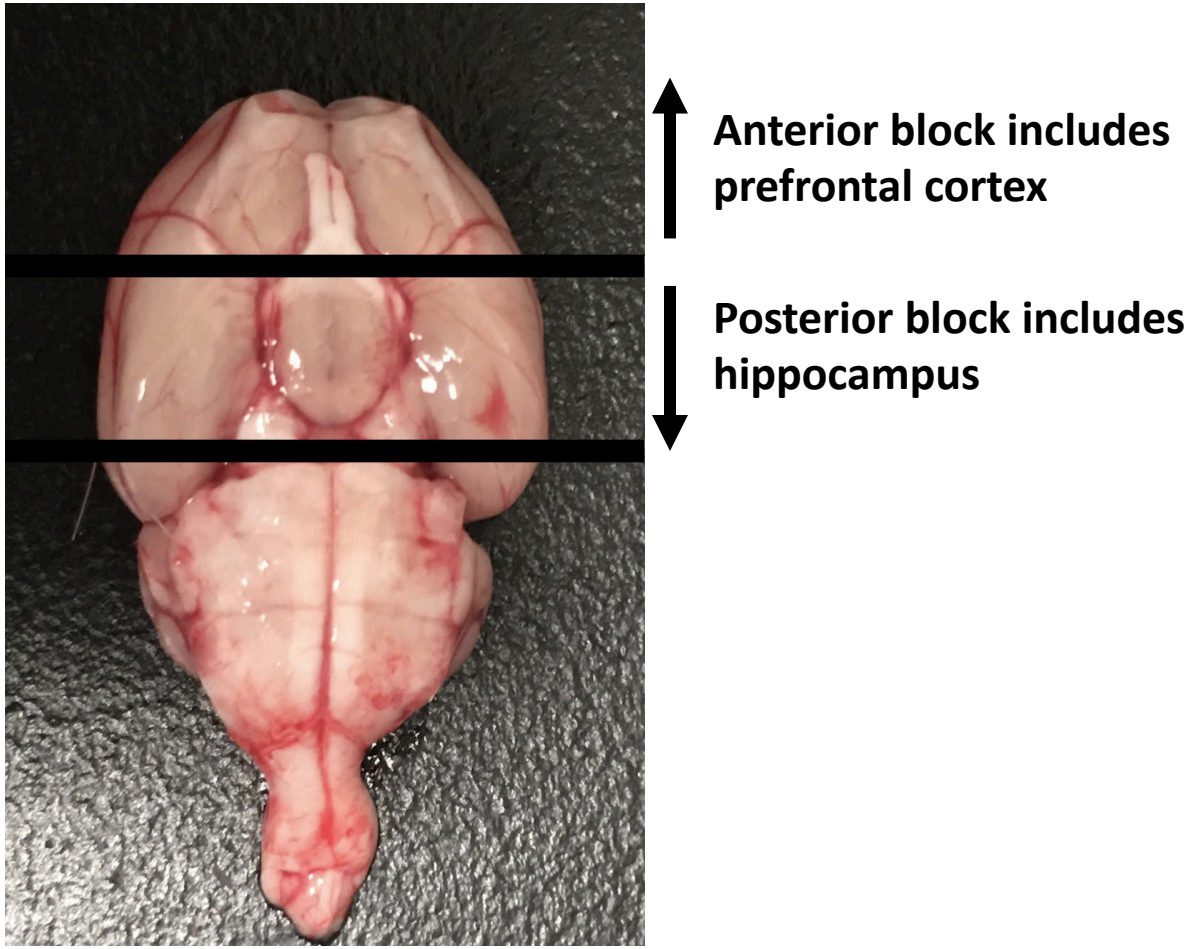
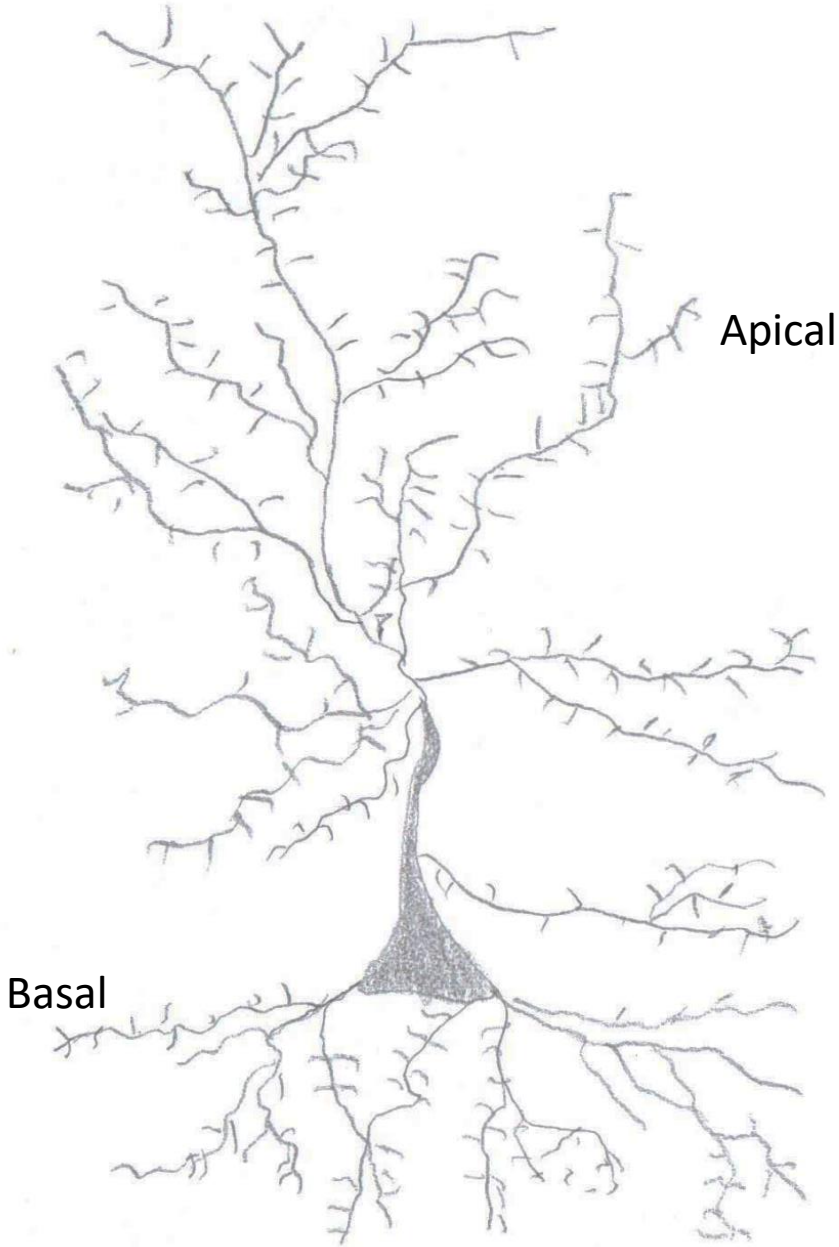
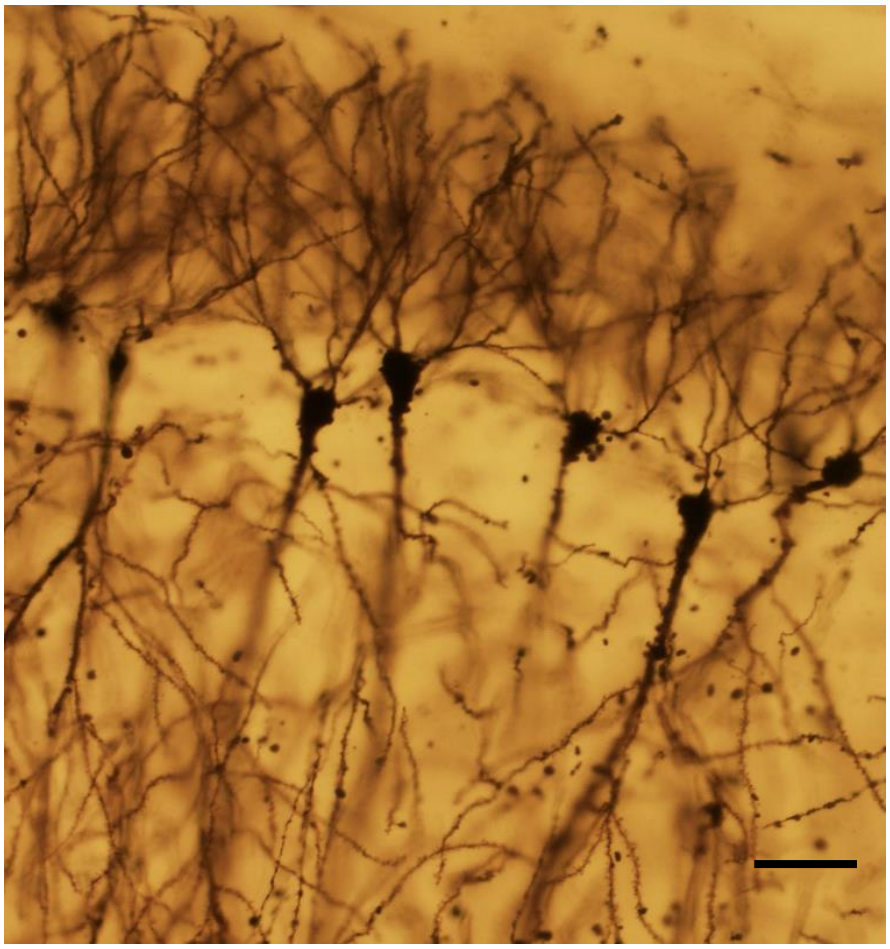


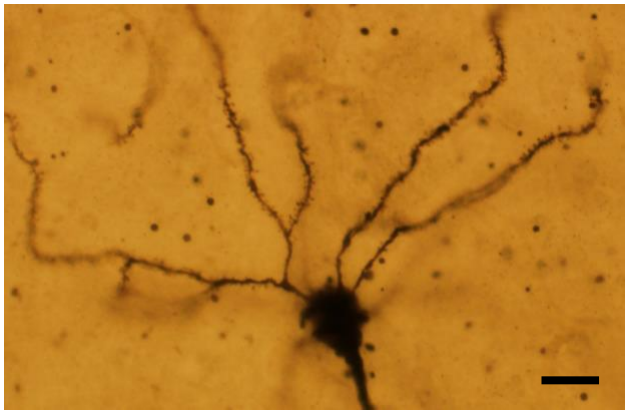
Figure 2



Photos of Golgi impregnated Cells

Click here to access/download;Figure;Figure 3.pdf





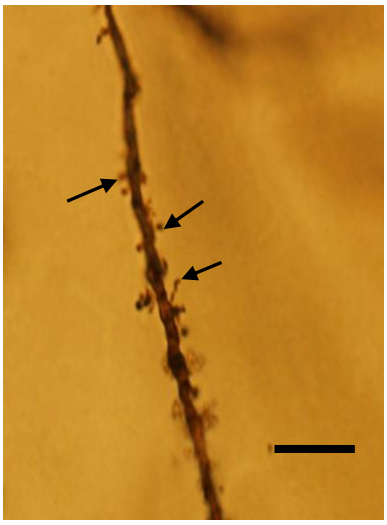
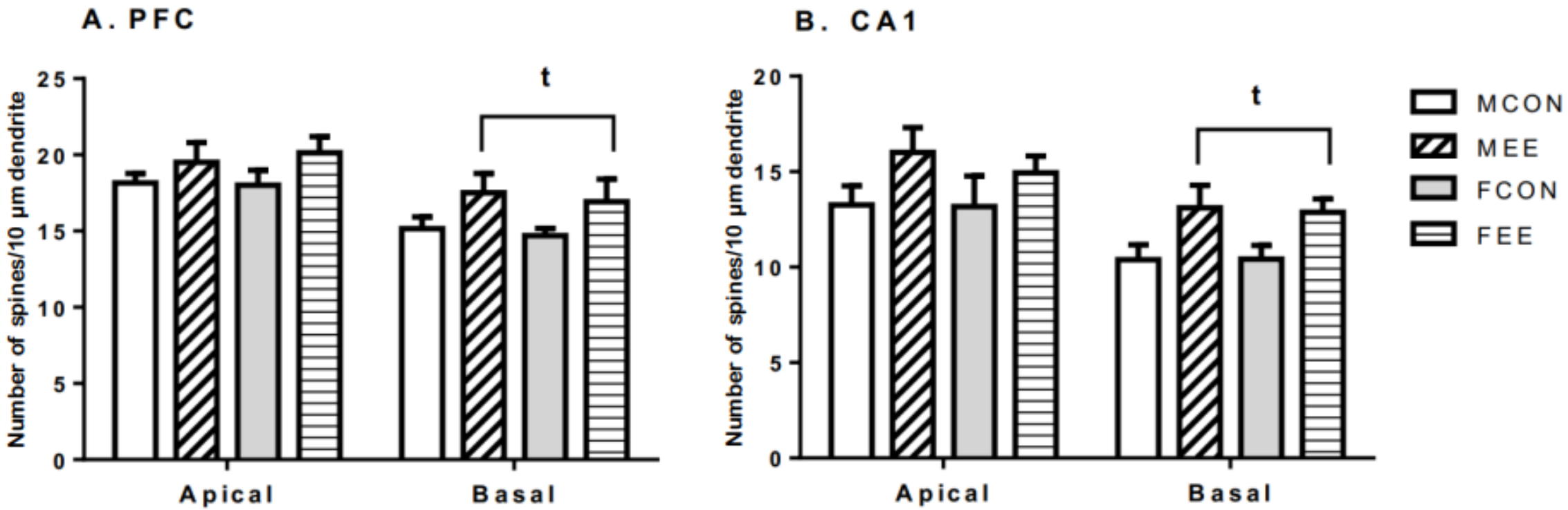


Figure 4



To the editor: I have reviewed and made the corrections that I felt appropriate and responded to every point. I have a personal emergency which limits my ability to add figures. I completely understand if you feel that the manuscript requires these and want to stop the process here but I simply have no choice.

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2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: FD NeuroTechnologies, Kimwipes. Reference to commercial products has been removed.

3. Please include the ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution. Done
4. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Done
5. Please add more details to your protocol steps:
Line 72: Please provide an approximate estimation of the amount of solution to be prepared. Done
Line 77: please mention how the rats are sacrificed. Done
Line 95: Solution C comes with the Golgi staining kit? If not, then please include the details in the Table of Materials. It does come with the kit and this has been added to the text.
Line 188: Please include citations for the determination of spine count and density. These have been added.
18. Please include one line space between the protocol steps and highlight that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader. Done
6. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next and also is in-line with the Title of the manscript. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. However, the NOTEs cannot be filmed, so please do not highlight. Done

7. Please modify the Result section to include all the observations and conclusions you can derive from the Figures. The Results section should focus on the effectiveness of your technique backed up with data. DONE
8. Each Figure/Table Legend should relevant include a title and a short description of the data presented in the Figure and symbols.
9. Figure 1: Please include scale bar if possible. I cannot at this point, Sorry for that.
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11. Please do not abbreviate journal names in the References. The referecnes are done according to JOVE syle quidelines in endnote

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Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The authors made a proposal of a modification of the Golgi stain.

Major Concerns:
Line 45
The three-dimensional visualization of neurons is not dependent on the Golgi impregnation. This type of visualization is related to the microscopy technique. What does erratic mean in this context? I have removed the word erratic which was a poor choice but I feel that the fact that the entire neuronis impregnated and, therefore, it appears three dimensional. The fact tht this is under the microscope in implied. However I added that the visualization is undera light microscope.
Line 75 - Line 79
How is the staining prevented from being contaminated with blood? Not sure but there isn’t an issue with not rinsing the sections.
Line 137
How are the sections on the slide prevented from being damaged if it's necessary for them to be completely dry before staining? Drying is an issue for other procedures, such as immunohistochemistry but here it is not and, importantly, the sections fall off during stainng if not dry.
Line 169
Which is the technique to remove the coverslip without damaging the tissue? A description has been added here.
Line 171
The information is not clear. What do the authors want to say? Statement has been

clarified. Coverslips tend to stick together and separating them in advance precvents putting more than one coverslip on a section.
Line 181
In relation to "six cells per region", is it six cells per hemisphere? Statement has been amended. Six cells/region/brain.
Line 218
It is not necessary to include this information. The results observed in the graphs are not related to the objective of the project. I suggest to remove this part of the information. I defer to the editor here. It seems off to show a data set with no information as to where it came from.
Line 230
Other versions of the Golgi stain also allow processing many sections simultaneously. The modification proposed by the authors is not actually innovating. I disagree with this as I have used other Golgi methods where fewer sections are stained at once.
Line 231-232
This proposal is more laborious than the Golgi-Cox method, for example, and the results are similar, which is related to the information in line 234. I respectfully disagree. No point is made regarding this being the best method. It is simply described as a rapid, useful one.
Line 242
This information is inconsistent with the objective of the project. This is a blank line and I don't know what the reviewer is referring to here.

Minor Concerns:
Line 28
Golgi impregnation is not only is used to impregnate dendritic spines, but can be used to impregnate the whole neuron, which is related to line 30. Absolutely true however in keeping within the guidelines of JOVE, the purpose here was do label and count spines. Moreover, the fact that Golgi canbe used for all neurons is stated in the summary (A modification of the rapid Golgi method, which can be adapted to any part of the nervous system, is described for staining neurons in the hippocampus and medial prefrontal cortex of the rat.)

Line 65
What does safe mean? Is it safe for the researcher? Why? This has been amended. Less exposure to the chmeicals is safer for the researcher.
Line 91
The bottles must be airtight, otherwise the solution's vapors will destroy the foil (aluminum). We use bottles with caps, and they are airtight. The foil was to keep light out if brown bottles were not used.

What does RT mean? I didn't find the meaning before this line. RT = Room temperature? Yes, and this has been amended.
Line 142

What does mls mean? Are the authors referring to milliliters? I think ml is correct. This has been corrected.
Line 152
Which staining solution is referred to in this line? Golgi impregnation. This has been amended.
Line 175
What do the authors consider "a few weeks"? We need specific information. More specific times have been added.
Line 188
The authors have to mention that dendritic spines must be observed at 1000x magnification, which corresponds to the total magnification when combining a 10x eyepiece with an 100x objective lens. True and this has been added.
Line 203
The figures are not labelled with their corresponding number. I assume Figure 1 is the first one after the References, but this may confuse the reader . The figures were uploaded according to JOVE instructions.
Line 238
What do the authors mean by "middle of CA1"? Middle has been removed. I meant well within but this is not needed.
Line 246
The small number of cells stained is more of an advantage than a disadvantage because, if the Golgi impregnation stained all the cells in the brain, the microscope field of view would look completely dark (black). Agreed and the text has been amended.

Reviewer #2:
Manuscript Summary:
This work provides an advancement for the Golgi method usage. The fact that it can be performed in brains that were kept frozen is excellent.
The steps needed to develop this technique are clearly described and the results are shown accordingly. I would like to ask the authors to provide some additional information, as follows:

1) I think that it is important to describe the substances and proportions that compose each solution in the "Rapid Golgi Stain Kit". This can allow the reader to know exactly what are the substances that compose this specific commercial kit. For example, do you use mercure chloride or osmium tetroxide? Excellent point. However, the manufacturer does not disclose this.

2) Please include more information on safety precautions and how to handle waste material. This has been added to the text. Solutions are treated as hazardous waste.

3) Please describe how rats are sacrificed and include a critical comment on the time elapsed between brain removal and the beginning of the Golgi technique. It would be interesting if you could discuss the possibility of structural changes in the nervous system related to this ischemic and excitotoxic period, which might affect specific

neuronal and glial morphological parameters. Rats are sacrificed by guillotine following CO2 euthanasia. This has been added to the text. Seconds later the brains are removed to the Golgi solutions. I agree that there may be changes in the tissue resulting from excitotoxicity/ischemia in this time, but we treat all the brains in the same way which ought to eliminiate this as a issue for a given experiment. It is worth studing but I have not done so (yet).

4) Do you have data from sections > 100 micrometers? Would you comment what could be the critical limit for the section thickness in your experience? I have added that you could use 150 um. The issue is that the sections fall off the slides when they are so thick. So you need to balance the thickness with getting through the staining process.

5) Please discuss the possibility that the actual dendritic spine density can be underrepresented by the procedure for counting spines. I mean, would you include a comment on the limitation of counting spines depending on the dendritic shaft thickness and the possibility of having more representative values when obtaining data from a thin dendrite? Otherwise, it would also be recommended to the reader to look for other approaches to visualize spines along the dendritic circumference, as mentioned for studying spine subtypes. Using the same branch of a given dendritic tree decreases this likelihood but it is hard to be sure and the text has been amended to reflect this.

6) Let me suggest to include an additional figure showing a pyramidal neuron from the prefrontal cortex, indicating the specific dendritic segment sampled and the visible dendritic spines at the highest magnification.
This figure would complement the present report nicely. I agree but as I will address the editor I cannot do this now. .

I am looking forward to the possibility of reading the authors´responses. This is an important contribution to the field.

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