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Using the Parafilm-assisted Microdissection (PAM) Method to Sample Rodent Nucleus Accumbens

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[Abstract] Microdissection techniques are very important for anatomical and functional studies focused on neuroscience, where it is often necessary microdissect specific brain areas to perform molecular or anatomical analyses. The parafilm®-assisted microdissection (PAM) was previously described and involves the microdissection of tissue sections mounted on parafilm-covered glass slides. In this work, we describe the use of the PAM method to microdissect rodent nucleus accumbens (NAc). (1) We first describe the best way to perform the mouse euthanasia and how to remove the brain. (2) Next, we describe how to prepare the slides with parafilm® that will be used to receive the brain slices. (3) Following, we describe how to handle the brain in the cryostat, how to align the hemispheres and how to identify the NAc antero-posterior limits. (4) We also describe how to perform the staining and dehydration of the slices, a critical step to facilitate the microdissection and preserve macromolecules. (5) In the final step, we describe how to identify the dorso-ventral and latero-medial limits of the NAc and, finally, how to perform the manual microdissection of the area. This is a low-cost technique that allows the researcher to specifically microdissect any brain region, from which intact RNA and proteins can be extracted to perform several molecular analyses (*e.g.*, real-time PCR, Western blot, and RNA-seq).

Keywords: Microdissection, Nucleus accumbens, Neuroanatomy

[Background] Nucleus accumbens (NAc) is part of the basal ganglia located in the rostroventral part of the striatum. Specifically, NAc is part of a structure that we call ventral striatum, which also comprises the olfactory tubercle. It is well known the role of NAc in the dopaminergic mesolimbic pathway, which regulates motivational behavior and is responsible for emotional and contextual behaviors (Baik, 2013). NAc has already been implicated in psychiatric disorders (*e.g.*, depression), drug addiction, obesity, and chronic pain, which show the relevance in studying the neurobiology of this brain structure (Nestler and Carlezon, 2006; Volkow *et al.*, 2011; Volkow and Morales, 2015; Brandão *et al.*, 2019; Serafini *et al.*, 2020).

Based on this, it is important to have a precise technique describing how to microdissect the NAc, sampling this structure for molecular analysis. The punch technique (Palkovits, 1983), for example, allow us to microdissect brain sections (including NAc) with a hollow needle and is widely used in

neurobiological laboratories. Advantages of punch technique are low cost and fast sampling. Disadvantage of using this technique is the limited accuracy, once the experimenter is restricted to the cylindrical shape of the punch tool and because the experimenter must punch fresh tissue, which is hard to identify some surrounding neuroanatomic structures of the NAc, such as the ventral pallidum.

Because of this, depending on the molecular analysis and the objective, it is preferable using a more accurate technique, such as laser-assisted microdissection (or laser capture microdissection). Laser-assisted microdissection technique is a method to microdissect tissue under direct microscopic visualization (Espina *et al.*, 2006). Advantages of using this technique are the high accuracy on delineation of the NAc and the capacity of sampling smaller areas (*e.g.*, NAc core or shell) or even cell populations. Disadvantages of this technique are the long time until sampling the tissue and the high cost to perform, requiring an entire facility with microscope and laser equipment.

Interestingly, Franck *et al.* (2013) described the parafilm-assisted microdissection (PAM) as a sampling method, a technique which involves the microdissection of tissue sections mounted on parafilm-covered glass slides. This group described the application of this technique for prostate cancer (Quanico *et al.*, 2015), ovarian tissue (Delcourt *et al.*, 2017) and for specific brain areas such as cerebellum, hypothalamus, and hippocampus (Franck *et al.*, 2013; Quanico *et al.*, 2017a and 2017b; Delcourt *et al.*, 2018). The PAM technique can be easily performed, once it uses low-cost materials and allows high accuracy on delineating the target brain area. Disadvantage of this technique is the limitation in microdissection smaller structures, such as subthalamic nucleus, for example.

In this manuscript, we will describe the whole process, from the brain storage after euthanasia to molecular analysis, of using the PAM technique to specifically microdissect the rodent NAc, also describing how to stain the brain slices to easily identify the anatomical limits of the NAc. It is worth mentioning that, as above-mentioned, this protocol can be used to microdissect a great range of brain regions, depending on the size and shape. Tissue obtained from this technique can be used for molecular analyzes such as real-time PCR, Western Blot, and RNA-seq.

Materials and Reagents

1. Common microscope slides 25 mm x 75 mm (*e.g.*, Sigma, catalog number: S8902)
2. Plastic paraffin or laboratory film (Parafilm®, Bemis Company, catalog number: P7543)
3. Cryostat blade (*e.g.*, Leica, catalog number: 14035838383)
4. Scalpel n° 11 (*e.g.*, Sigma, catalog number: S2771)
5. Adhesive tape (*e.g.*, 3M, catalog number: 2214)
6. Aluminum foil (*e.g.*, Sigma, catalog number: Z185140)
7. Staining racks (*e.g.*, Electron Microscopy Science, catalog number: 70315)
8. 50 ml conical tube (*e.g.*, Fisher Scientific, catalog number: 14-432-22)
9. Needle 18G (*e.g.*, GIME Professional Medical Products, catalog number: 23750)
10. 1.5 ml microtube (*e.g.*, Sigma, Eppendorf, catalog number: Z606340; preferably RNAse free)
11. Tissue freezing medium (*e.g.*, Tissue-Tek® O.C.T. Compound, Sakura, catalog number: 4583)

12. Ethanol 100% (room temperature – 18 °C to 25 °C) (*e.g.*, Sigma, catalog number: E7023)
13. Ethanol 100% (4 °C) (*e.g.*, Sigma, catalog number: E7023)
14. Cresyl violet (*e.g.*, Sigma, catalog number: C5042)
15. Methylbuthane (*e.g.*, Sigma, catalog number: 277258) – room temperature
16. Dry ice
17. Cool box
18. Metallic can
19. 2 solutions of Ethanol 70% (4 °C; diluted from ethanol 100%) (*e.g.*, Sigma, catalog number: E7023 (see Recipes)
20. Ethanol 50% (4 °C; diluted from ethanol 100%) (*e.g.*, Sigma, catalog number: E7023) (see Recipes)
21. Cresyl Violet 1% (see Recipes)

Equipment

1. Cryostat (*e.g.*, Leica, model: CM1950)
2. Tissue holder *or* cryostat chucks (*e.g.*, MarketLab, PolarChuck™, catalog number: ML39130)
3. (Optional) Lupe (*e.g.*, ZEISS, model: Stemi 508)

Procedure

A. Euthanizing the mouse and handling the brain
   1. It is recommended to euthanize the mouse through guillotine decapitation or cervical dislocation, being careful to not damage the brain. Chemical techniques (both injectable and inhalational) of euthanasia are not indicated because they can cause biochemical changes in the brain (Leary *et al.*, 2013), such as isoflurane even at low doses (Bekhbat *et al.*, 2016). However, which euthanasia technique to be used is the researcher's choice, although the above-mentioned arguments must be considered.
   2. Remove the brain from the skull using the technique described by Spijker (2011) or any other of your choice. It is important to remove fast and with no damage to the tissue.
   3. Freeze the brain immersing it in methylbutane at -50 °C (using dry ice).
      CRITICAL STEP: For this, you can put dry ice in a cool box and the methylbutane in a metallic recipient (*e.g.* metallic can). Place the methylbutane-containing metallic recipient on the dry ice to cool down. It's important to mention that the rapid freezing of the brain, which happens in temperatures below -50 °C, may cause cracks in the tissue when slicing in the cryostat. Therefore, keep the methylbutane temperature above -50 °C (you can use an infrared thermometer for greater accuracy) during the procedure.
   4. After freezing the brain, it is important to store it individually in the freezer at -80 °C in a package that will preserve the organ structure. You can store the brain at -80 °C up to one year (we did

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not test more than this period in our laboratory). In our laboratory we use low cost materials to stock brain, such as that shown in **Figure 1** which is made with one-layer aluminum foil and adhesive tape.

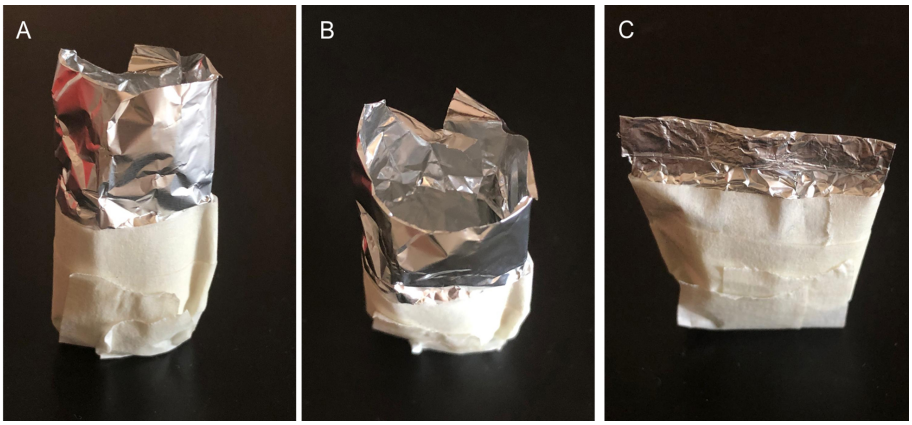

Figure 1. Stocking the brain. A. Front view of the package to stock the brain in the freezer (-80 °C), produced in aluminum foil and adhesive tape. B. Upper view of the package to stock the brain in the freezer, note the top opening to place the brain inside the package. C. After placing the brain, seal the package by folding tightly the top of the aluminum foil.

B. Preparing the glass slides

1. Cut pieces of Parafilm® that fits to cover the glass slide surface (25 mm x 75 mm).
   Note: We recommend cutting the Parafilm® slightly smaller than the glass slide, this way is easier to perform the staining using the staining racks.
2. Attach the Parafilm® to the glass slide using adhesive tape, leaving free the surface on which the slices of the brain will be positioned (**Figure 2**).
   Note: Do not stretch the piece of Parafilm®.

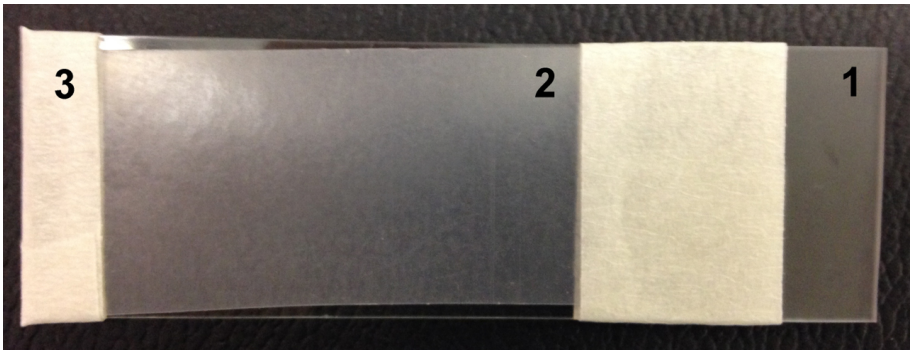

Figure 2. Microscope slide prepared to PAM protocol. Detail of the microscopy slide (1) covered with a not stretched small piece of Parafilm® (2) fixed with adhesive tape (3).

C. Processing the brain in the cryostat

1. Place the brain in the tissue holder with the anterior region facing upwards (**Figure 3**), that is,

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immersing in the tissue freezing medium the posterior side of the brain (*i.e.*, the side containing the medulla oblongata). This way it will be easier to find reference points to align the hemispheres.

CRITICAL STEP: If you need to microdissect structures located in the brainstem is recommended positioning the brain in the tissue holder inversely, immersing the anterior region of the brain (*i.e.*, the side containing the olfactory bulb) in the tissue freezing medium. This is necessary because the way the brain is frozen (using methyl butane at -50 °C) makes the brain stem fragile, breaking easily due to the pressure of the cryostat blade.

*Note: It is also recommended positioning the brain vertically in the holder of the tissue holder, this also prevents the brainstem from breaking (see **Figure 3C**).*

Figure 3. Placing the brain in the cryostat apparatus. A. Anatomical directions of the mouse brain in a top view (scale in cm). B. Placement of the brain in the tissue holder (or cryostat chucks), note the anterior region directed upwards (C).

Note: How we recommend placing the brain in the holder of the tissue holder: vertically, hampering the brainstem break due to the pressure of the cryostat blade.

2. Align the hemispheres so the cryostat blade will cut the brain perpendicularly. To align the two hemispheres (when they are both in the same position - or very similar - in relation to bregma) of the brain the experimenter must slice the brain until it is divided into four quadrants, separated by the rhinal sulcus and longitudinal fissure (see **Figure 4**). The experimenter should use these depressions as neuroanatomic reference to align the hemispheres, by moving the holder of the tissue holder.

   CRITICAL STEP: During slicing, maintain the cryostat temperature at -15 °C, above this temperature the slice will unfreeze, and below this temperature the slice will crack.

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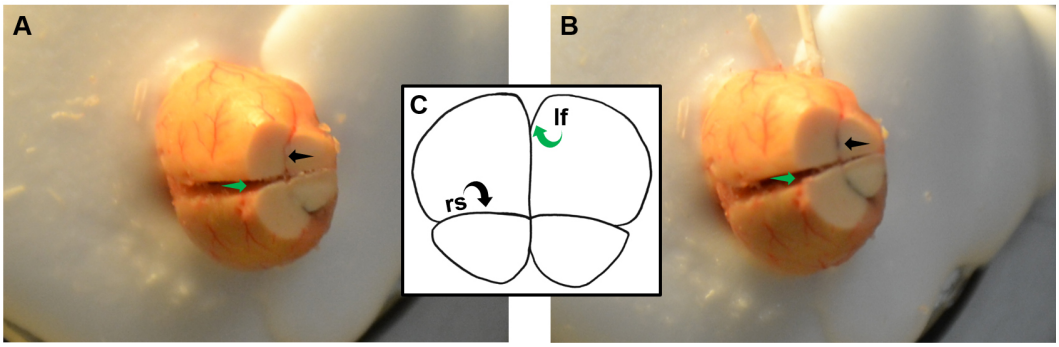

Figure 4. Neuroanatomic references to align the hemispheres. Note that in the image (A) the hemispheres are not aligned, and, in the image (B), the hemispheres are aligned. C. Insert showing a graphical scheme of the neuroanatomic references (rhinal sulcus (rs) = black arrow; longitudinal fissure (lf) = green arrow).

3. Identify the beginning of the NAc and start collecting the slices on the slide with Parafilm®. The NAc begins when the experimenter is able to identify the corpus callosum and anterior commissure by the naked eye (see **Figure 5A**). From this moment, the experimenter should place the slices in one of the slides prepared with Parafilm® (see an important hint on how to perform this step in **Figure 6**) until the anterior commissure cross the median plane of the brain (commissurated region, where there is passage of fibers from one hemisphere to another), as shown in **Figure 5B**. This commissurated region indicates the NAc posterior limit. Carefully place the slices on the top of the Parafilm® covering the slide. After collecting all the slices from the brain, the slide should look like the one shown in **Figure 7**. If you slice with a thickness of 60µm you will collect approximately 24 slices until you see the anterior commissure crossing the median plane of the brain.
   CRITICAL STEP: After placing the slices on the slide do not remove from the cryostat until start the next step (staining and dehydration), otherwise the slices will unfreeze.
   Note: If it is difficult to visualize by naked eye, the experimenter can collect a slice on a common slide (with no Parafilm®), stain it with a drop of Cresyl Violet 1% (diluted in 50% alcohol) and visualize in a microscope.

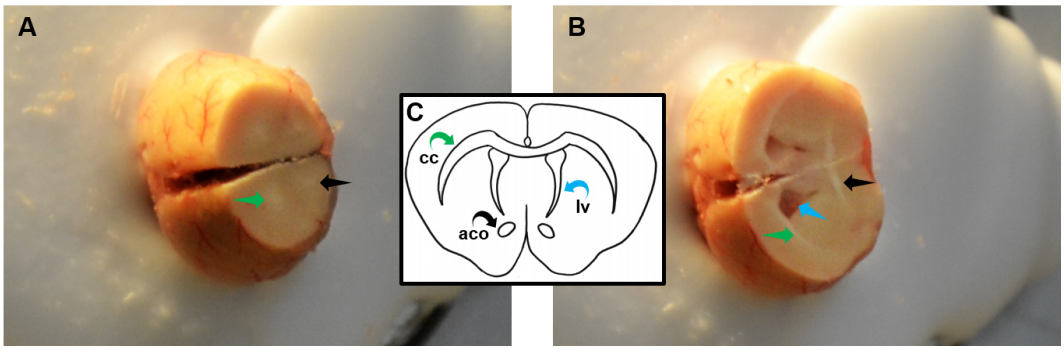

Figure 5. Identifying anterior and posteriors limits of the NAc. A. Reference points to identify the beginning of the NAc (anterior anatomical limit) and start collecting slices in the

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slides prepared with Parafilm®. B. Reference points to locate the end of the NAc (posterior anatomical limit) and stop collecting slices, note the commissurated region. C. Insert showing a graphical scheme of the neuroanatomic references (corpus callosum (cc) = green arrow; anterior commissure (aco) = black arrow; lateral ventricle (lv) = blue arrow).

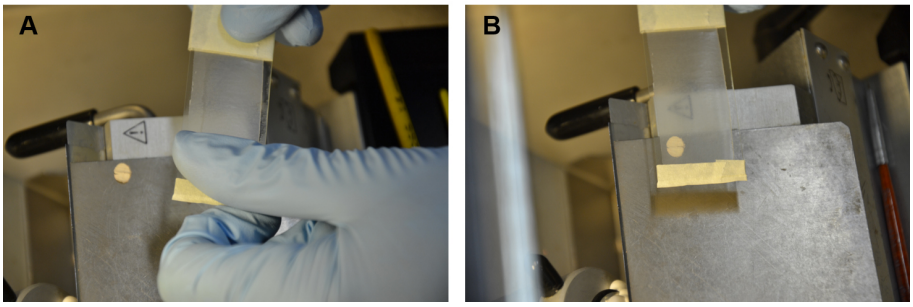

Figure 6. How to position the slices on the slide with Parafilm®. First, warm the back of the slide (which has no Parafilm®) with your finger (A) then approach the slide to the slice, which will attach to the Parafilm® (B).

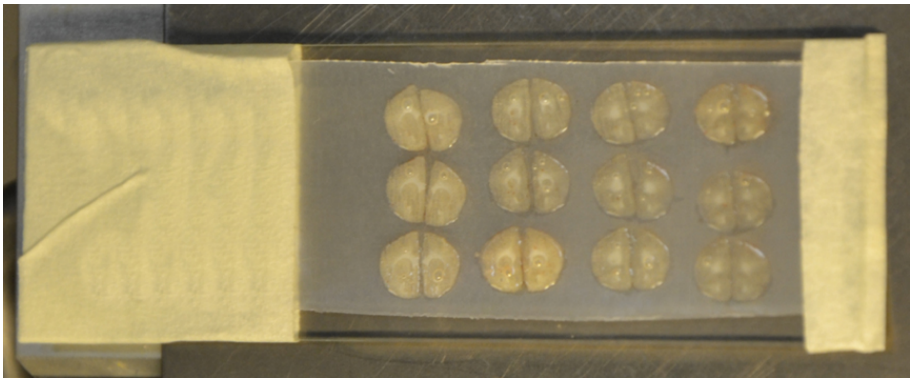

Figure 7. Slide with NAc slices. Detail of the slide with the NAc slices placed on the top of the Parafilm®.

D. Staining the slices

1. Stain and dehydrate the slices (which facilitates microdissection and preserves macromolecules), using the bath sequence described below, by immersing the slices-containing slides into the staining racks. It is worth mentioning that the slices do not fall from the slide after staining, once the Parafilm® helps in the slice adhesion. After staining the slices, the slide should look like the one shown in **Figure 8**.
   a. 120 s in alcohol 70% (4 °C);
   b. 90 s in Cresyl Violet 1% (diluted in 50% alcohol) (4 °C);
   c. 2x Fast wash (2s) in alcohol 70% (4 °C);
   d. 120 s in 100% alcohol (4 °C);
   e. 120 s in 100% alcohol (room temperature);
   f. Leave to dry for 15 min (optional: use a lab hood).

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CRITICAL STEP: Renew all alcoholic solutions after 20 uses or after 30 days of the first use. This does not apply to Cresyl Violet 1% solution, which can be used up to 60 times or after 3 months of the first use.

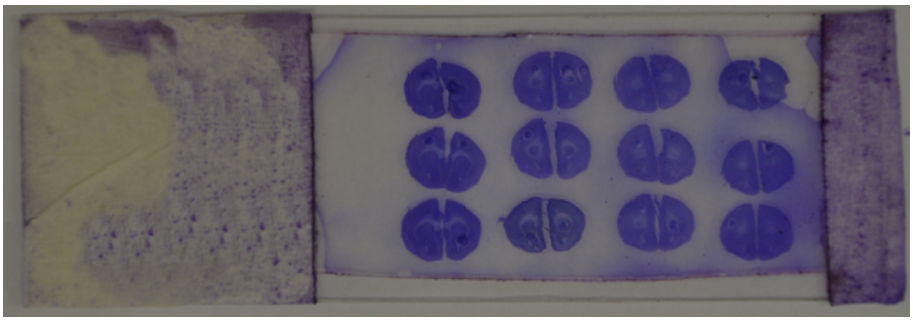

Figure 8. Slide with stained NAc slices. Detail of the slide with stained NAc slices placed on the top of the Parafilm®.

2. Stock the slides in pairs, one with the back to another, inside a 50 ml conical tube (**Figure 9**).
   CRITICAL STEP: To preserve tissue dehydration, seal the conical tube with Parafilm®. This way the slide with the slices can be frozen at -20 °C for several months with no damage to the tissue.

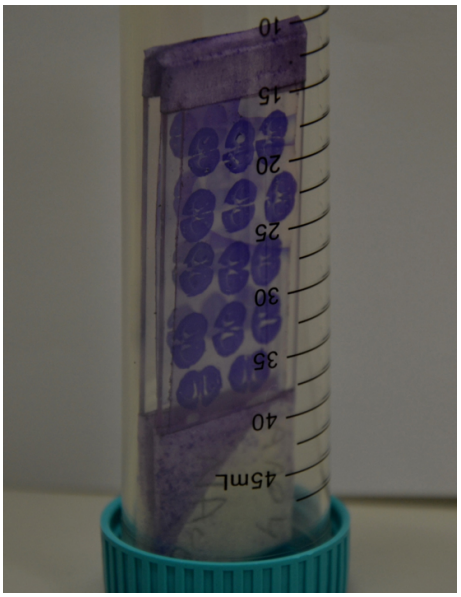

Figure 9. Stocking the slides. Stock the slides in pairs inside a 50 ml conical tube, one with the back to another.

E. NAc manual microdissection
   1. Remove the conical tubes containing the slides from the freezer and left at room temperature for, at least, 15 min to unfreeze.
      CRITICAL STEP: Just open the conical tube after total thawing.
   2. Position the slide under a magnifying glass (optional), which can facilitate to visualize the brain

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areas. At this point, the experimenter must identify the anterior commissure (a whitish circular structure). Assisted by a scalpel n° 11 scrape all the tissue belonging to the anterior commissure, removing it. After performing this scraping, make a cut around the NAc (also cutting the Parafilm®). At this moment we recommend the experimenter to consult an atlas (*e.g.*, Franklin and Paxinos, 2013), confirming that only NAc tissue is being removed. **Figure 10** shows in sequence the location of the NAc and how the cuts around the NAc should be performed using the scalpel. After this step, remove the tissues with assistance of a needle, placing it in a 1.5 ml microtube (see **Figure 11**). Seal the tube with Parafilm® and store it at -80 °C for molecular biology procedures.

Note: To facilitate the NAc removal with the needle, the experimenter can leave a slightly raised portion of the Parafilm® when cutting with the scalpel.

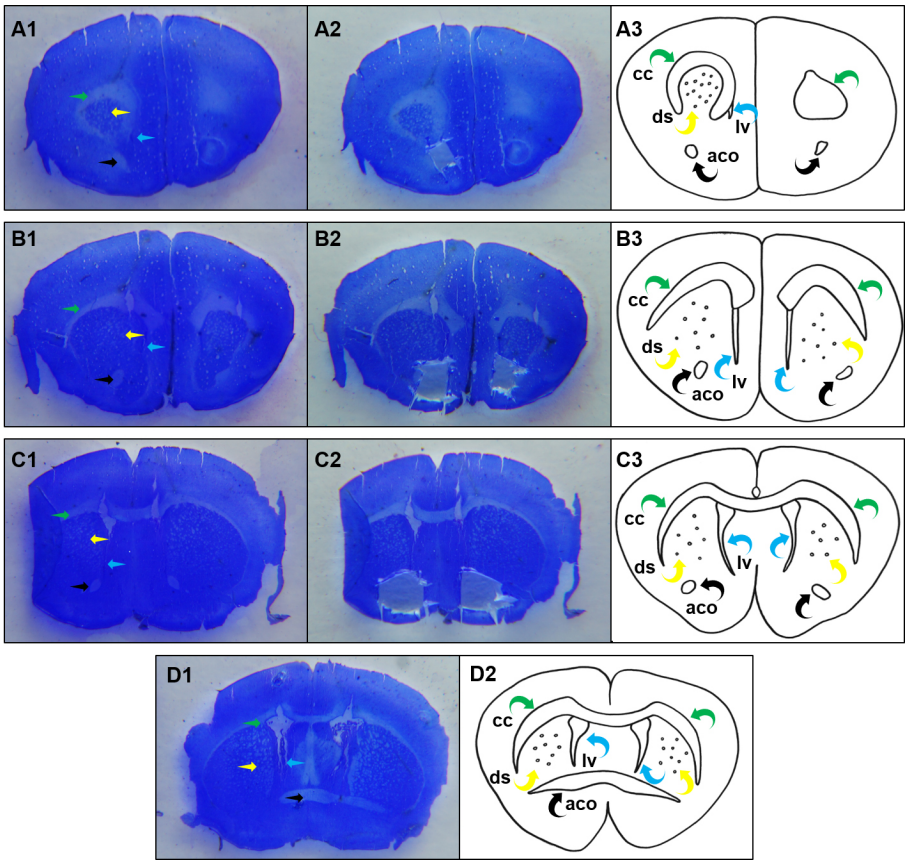

Figure 10. NAc manual microdissection. (A1), (B1), and (C1) show the representative sequence of the beginning (anterior limit), middle and end portion (posterior limit) of the NAc, respectively. (A2), (B2), and (C2) show these same representative slices after NAc been cutted by a scalpel (note that NAc is removed along with the Parafilm®). (A3), (B3), (C3), and (D2) show a graphical scheme of the neuroanatomic references. (D1) shows a slice without NAc, note that the anterior commissure is crossing the median plane of the brain, indicating the end of the NAc. (corpus callosum (cc) = green arrow; anterior commissure (aco) = black arrow; lateral ventricle (lv) = blue arrow; dorsal striatum yellow arrow).

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Note: In most of the slices you can consider the superior limit of the NAc as the dorsal striatum, inferior limit as ventral pallidum, medial limit as medial septal complex and the end of the lateral ventricle. Lateral limit is harder to identify, but you can consider the beginning of the cortex.

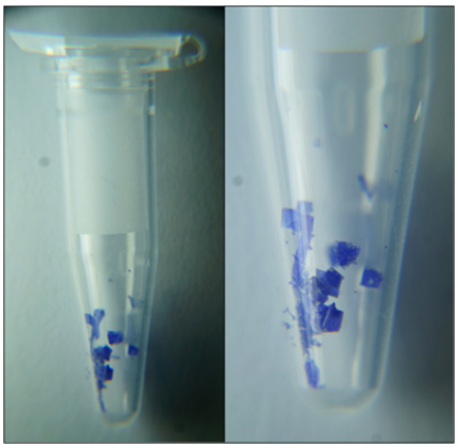

Figure 11. Using microtubes to stock NAc tissue. 1.5 ml microtube used to stock NAc tissue taken from the mice's brain along with the Parafilm®.

Data analysis

Microdissected tissue can be processed with TRIzol® reagent for the macromolecules extraction. Microdissection can yield low amount of tissue, because of this, we recommend using 0.5 ml of TRIzol® reagent per sample. For the other reagents used in the TRIzol® reagent protocol, we recommend using the proportions suggested in the reagent data sheet (see TRIzol® Reagent User Guide here).

Note: The pieces of Parafilm® remain on the surface of the aqueous phase after the first centrifugation of the protocol (phase separation), at this moment they can be easily removed.

In the next steps, some changes in the TRIzol® reagent data sheet can be performed. In the RNA precipitation step, it is recommended to add to the aqueous phase, besides 0.25 ml of 100% isopropanol, 5 µg of RNase-free glycogen or 2 µl of linear acrylamide as carriers (they will co-precipitate with the RNA, increasing the extraction efficiency). In this RNA precipitation step, we also recommend incubating the sample at -20 °C overnight (to maximize RNA precipitation) and then centrifuging at 12,000 *x g* for 10 min. In the next step of the protocol (RNA wash), we recommend performing one or two RNA washes, more than this may cause excessive RNA loss. Finally, the RNA can be resuspended using 10 µl of RNase-free water or 0.5% SDS solution.

It is important to mention that TRIzol® reagent protocol may not have high proteins extraction efficiency. Therefore, we suggest an alternative protein extraction protocol using 8 M urea. For this suggested protocol, add 500 µl of 8 M urea to the microdissected sample, incubate for 20 min at room temperature (the experimenter can perform 3 fast pulses in a sonicator), and then centrifuge at 12,000 *x g* for 5 min. Use the supernatant. The pieces of Parafilm® will also be suspended after

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this centrifugation and can be easily removed.

A. RNA analyses

The RNA extracted by TRIzol® reagent protocol yields material in quality and quantity sufficient for real-time PCR and RNA-seq techniques. These techniques have already been successfully performed in our laboratory with samples obtained from the PAM protocol (see Pagliusi Jr. *et al.*, 2018 and Brandão *et al.*, 2019). **Table 1** shows the RNA quantification and purity of 5 different samples microdissected through the PAM protocol and processed using above-mentioned TRIzol® reagent protocol.

Table 1. RNA analyses. RNA quantification and purity of 5 different NAc samples microdissected through the PAM protocol and processed using the TRIzol® reagent protocol.

Sample	Quantification ng/µl	Ratio 260/280
1	700.01	1.971
2	796.67	1.864
3	770.84	1.903
4	649.02	1.818
5	859.31	1.905

B. Protein analyses

Although we believe that the protein extracted by the TRIzol® reagent protocol yields material in quality and quantity sufficient for Western-Blot (WB) and ELISA techniques (in samples microdissected through the PAM protocol), only WB was tested in our laboratory. Also, Franck *et al.* (2013) performed mass spectrometry using samples from the PAM protocol. **Figure 12** shows a WB for β-actin and a ponceau performed in our laboratory using 5 different samples microdissected through the PAM protocol (same samples used for the above-mentioned RNA analyses) and processed using the TRIzol® reagent protocol.

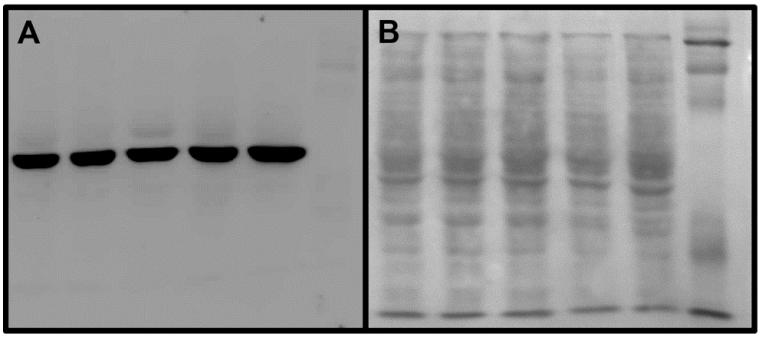

Figure 12. Protein analyses. (A) Western blot for β-actin and (B) ponceau staining membrane performed in our laboratory using NAc samples from the PAM protocol and processed using the TRIzol® reagent protocol.

Recipes

1. Cresyl Violet 1% (stock temperature: 4 °C)
   2.5 g Cresyl violet
   250 ml ethanol 50%
2. Ethanol 70% (stock temperature: 4 °C)
   60 ml ethanol 100%
   140 ml distilled water
3. Ethanol 50% (for Cresyl Violet 1% preparation):
   100 ml ethanol 100%
   100 ml distilled water

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Competing interests

We declare no competing interests.

Ethics

All experiments presented here were approved by the Ethics Committee on the Use of Animals at the Biology Institute of the University of Campinas - CEUA/UNICAMP (protocols 3849- 1 and 4249-1).

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