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We use this protocol and it's working

Preparation of tissue for Ribo-Tag/RNAseq analysis

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ABSTRACT

This protocols describes the RiboTag virus vectors injected into mouse brain, tissue dissection, immunoprecipitation and RNA extraction for RNAseq.

ATTACHMENTS

RiboTagRNAseq.docx

GUIDELINES

Follow institutional guidelines and protocols.

MATERIALS

Stereotactic injection of RiboTag virus in the brain

Skip this section if using an alternative method to express RiboTag in the cells of interest (e.g.: generation of a specific transgenic mouse).

Different viral preparations might be required for specific cell types. In this specific case, a combination of cre-dependent viral construct (e.g: AAV9-EF1a-DIO-Rpl22l1-Myc-DDK-2A-tdTomato-WPRE) and mice expressing cre under the DAT promoter (B6.SJL-*Slc6a3*$^{tm1.1(cre)Bkmn}$/J) were used to achieve expression in midbrain dopaminergic neurons.

Materials:

- Anesthetic: isoflurane

- Anesthesia machine (Smiths Medical) with connector tubing, induction chamber and filter canisters for isoflurane waste

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- Stereotaxic surgery frame and scope (David Kopf Instruments)
- Sterile surgery tools (forceps, fine scissors, needle holder as needed)
- Sterile drape
- Heating pad and temperature probe
- Non-steroidal analgesic (e.g. Metacam)
- Ophthalmic ointment
- Sterile 0.9% saline
- Antiseptics: povidone-iodine swabs and 70% ethanol swabs
- Scale to measure the weight of the mouse
- Hair clipper
- Drill with foot petal and sterilized drill bit
- Sterile cotton swabs
- Viral stock solution
- Suture material
- EMLA cream or bupivacaine line block

- Antibiotic ointment

- Glass micropipettes (Drummond Scientific) pulled with P-97 glass puller (Sutter Instruments). It is recommended to add some volumetric references on the pipettes based on their specifics.

- Post-surgery care: clean empty mouse cage on heating pad for recovery; clean mouse cage with extra gel food for post-surgery holding.

Recommended PPE:

- Disposable gown

- Face mask

- Face shield/goggles

- Nitrile gloves

- Sterile gloves

Depending on the biosafety level (BSL) recommended for type of virus injected, an appropriate biosafety cabinet might be required.

Tissue dissection

Materials:

- Anesthetic (ketamine 50 mg/Kg and xylazine 4.5 mg/Kg – varies according to institutional protocols)

- Vibratome (VTS1200S Leica microsystems) with removable ice tray and cutting chamber and vibro-check tool

- 2 large ice trays

- Large beaker

- 2 glass petri dishes (one of the two can be substituted with a medium weighing boat)

- 1 circular filter paper

- Peristaltic pump (Gilson) with tubing and connectors

- Blood-gas mixture (95% O2, 5% CO2) tank connected to bubblers.

- Dissection tools (scissors, fine scissors, spring scissors, tweezers, spatula, according to preferences)

- Double-edged razor blades

- Single-edge razor blades

- artificial cerebro-spinal fluid (aCSF), pre-chilled

- Freshly made “slicing solution”: modified aCSF.

- Pre-frozen slicing solution

- Immersion blender

- Perfusion needle (preferred: 27 gauge ½ inch)

- Holding chamber for slices, filled with pre-chilled aCSF and kept in ice

- Pre-solidified 2% agarose

- Superglue

- Precision wipes

- Water wash bottle

- Extra needles

- Carcass bag

- Plastic or glass transfer pipette

- Dissection microscope

- Disposable scalpels

- Petri dishes for dissection with a small volume of ice cold aCSF (recommended: use petri dishes of different sizes and place the petri dish with aCSF inside the larger one, filling the space between with ice. This will help keeping the dissection solution cold).

- Dry ice or liquid nitrogen

- 1.5ml Eppendorf tubes to collect samples

Recommended PPE:

- Lab gown/disposable gown

- Face mask

- Face shield/goggles

- Examination gloves (cut-resistant gloves are recommended)

- Cryo-gloves to handle dry-ice/liquid nitrogen

Solutions:

Different types of aCSF are adopted by different groups and are optimized for different for different preparations. For tissue dissection, it is recommended to use the solution normally used for experimental purposes, but pre-chilled.

The aCSF adopted for experiments on SNc neurons has the following composition: 135.75 mM NaCl, 2.5mM KCl, 1.25mM NaH_2PO_4, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 3.5 mM glucose.

The slicing solution for SNc neurons has the following composition: 49.14 mM NaCl, 2.5 mM KCl, 1.43 mM NaH_2PO_4, 25 mM $NaHCO_3$, 25 mM glucose, 99.32 mM sucrose, 10 mM $MgCl_2$ and 0.5 mM $CaCl_2$.

All the solutions are continuously bubbled with blood-gas mixture before and during the procedure. This will maintain the proper oxygenation and pH.

Immunoprecipitation and RNA extraction for RNAseq analysis

Materials:

- Magnet (Dyna-Mag2, Thermo Fisher Scientific)
- Refrigerated minicentrifuge (Beckman Coulter 20R)
- 1 large ice tray
- Dry ice
- RNase-free 1.5-ml microcentrifuge tubes (Axygen)
- RNase-free filtered tips
- RNase-free pestles for 1.5-ml microcentrifuge tubes (USA Scientific)
- Tube rotator (Thermo Fisher Scientific)
- Pierce Protein A/G Magnetic Beads (Thermo Fisher Scientific)
- Anti-FlagM2 Magnetic Beads (Sigma-Aldrich)
- SUPERase In RNase Inhibitor (Thermo Fisher Scientific)
- Protease inhibitors (Sigma-Aldrich)
- Cyclohexamide (Sigma-Aldrich)

- DL-DTT (Sigma-Aldrich)

- Nonident P-40 Substitute (Sigma-Aldrich)

- UltraPure DNase/RNase-Free Distilled Water (Thermo Fisher Scientific)

- Ethanol (Sigma-Aldrich)

- RNAeasy Micro kit (Qiagen)

- RNaseZap (Thermo Fisher Scientific)

Solutions:

Tissue homogenization buffer has the following composition: 50mM Tris-HCl (pH 7.4), 125 mM KCl, 12 mM MgCl2, 1% NP-40, 1mM DTT, 1X Protease inhibitors, 200 units/ml RNase inhibitor, 100ug/ml cycloheximide.

Washing buffer has the following composition: 50mM Tris-HCl (pH 7.4), 325mM KCl, 12 mM MgCl2, 1% NP-40, 1mM DTT, 100ug/ml cycloheximide.

Recommended PPE:

- Disposable gown

- Face mask

- Goggles

- Nitrile gloves

Stereotactic injection of RiboTag virus in the brain - Procedure

1 Prepare a clean empty mouse cage on a heating pad and a clean mouse cage with gel food for post-operatory care.

2 Set up sterile working area including stereotaxic frame

3 Measure the weight of the mouse

4 Anesthetize mouse in induction chamber (recommended: 2.5% isoflurane, 200ml/min flow rate)

5 Hair over surgery area can be quickly clipped before transferring the mouse onto the stereotaxic frame.

6 Once the mouse is deeply anesthetized (~5 min), stop anesthesia and move the mouse to the stereotaxic frame over the heating pad with the temperature probe and secure the mouse mouth on the nose cone. Restart anesthesia (directed towards the nose cone).

7 The heating pad settings should be adjusted so that the temperature probe placed under the mouse should read a body temperature between 33-37C.

8 Apply ophthalmic ointment over eyes

9 Inject appropriate volume (based on mouse weight and desired dosage) of analgesic; an appropriate amount of saline can also be injected to prevent dehydration during the procedure.

10 Carefully insert and secure the ear-bars. The position of the mouse head will be verified and adjusted once the skull is exposed, but it is recommended to make sure that the head is not visibly
tilted.

11 Clean the area of the incision with the povidone-iodine swab followed by the ethanol swab. Repeat 3 times.

12 It is preferred to apply line-block anesthetic (0.15% bupivacaine) under the skull skin before starting the procedure rather than applying EMLA cream on the sutured skin at the end of the surgery.

13 With the fine scissor, expose the skull by making an anterior-posterior incision

14 Visually identify bregma and lambda.

15 Insert a glass pipette (a small volume of non-toxic food dye can be used to help marking the relevant spots) on the stereotaxic arm holder and lower it onto the skull.

16 Mark bregma by gently touching the intersection of the coronal/sagittal sutures with the pipette tip, and zero the coordinates on the reader.

17 Move to lambda (intersection of lambdoid and sagittal sutures) and measure its position relative to bregma.

18 Minimize the deviation of dorso/ventral (D/V) and medio/lateral (M/L) distance between lambda and bregma by adjusting the position of the head.

19 Re-zero the coordinates at bregma and repeat bregma/lambda measurements until satisfactory.

20 Once the head is in the correct position, it is possible to identify the desired injection spot.

21 It is recommended to use the measured anterior/posterior (A/P) distance between bregma and lambda to calculate an adjustment factor for the final coordinates: the measured B-L distance will be divided by the reference distance of 4.21. For an adult mouse, the obtained value ("adjustment ratio") should be close to 1, and in this case no coordinates adjustment is required (but still optional). For smaller mice, the reference coordinates should be multiplied by the calculated adjustment ratio to obtain the final coordinates for the specific mouse.

22 Move the pipette to the spot indicated by the adjusted A/P and M/L coordinates and mark it.

23 Whether performing uni-lateral or bi-lateral injections, it is recommended to mark the spots on both sides of the skull, and to measure their relative dorso-ventral position. Their relative D/V deviation should be minimized by adjusting the position of the head.

24 Once the desired spot has been marked, the marker pipette can be removed, and a hole is drilled in the skull at the indicated position.

25 Blood and debris are cleaned with sterile saline and sterile cotton swabs.

26 Insert micropipette with volumetric references in the holder and connect it to a syringe to apply positive/negative pressure.

27 Draw up desired volume of viral solution in the syringe by applying negative pressure.

28 Lower pipette loaded with the viral solution into the hole until the tip touches the dura. Zero the dorso-ventral coordinate.

29 Gradually lower the pipette tip into the brain until the desired dorso-ventral coordinate is reached.

30 Slowly inject the desired volume of viral solution (recommended: ~150/200nl/min) by gently and gradually applying positive pressure.

31 Release pressure and leave the pipette in position for ~5-10min so that the viral solution can spread and be absorbed by the tissue.

32 Slowly retract viral injection pipette.

33 Suture skin.

34 Optional: repeat saline injection to prevent dehydration

Post-surgery care:

35 Remove animal from stereotaxic frame and place it in the clean, empty cage on heating pad until deambulatory (~10-15min).

36 Once awake and deambulatory, mouse can be moved to the clean cage with gel food, also on heating pad.

37 24 hours after surgery, a second dose of Metacam is administered and antibiotic ointment is applied on the sutured skin.

38 The health status of the mouse is monitored over the following days. If needed, additional doses of Metacam or saline can be administered.

39 Mouse is normally kept in a cage on heating pad for at least 4 days and is then moved to standard housing.

40 Mice are sacrificed for tissue collection 4 weeks after surgeries.

Tissue dissection - Before the procedure:

41 A few hours/day before the procedure it is recommended to prepare some slicing solution and freeze it (~200ml). Alternatively, freshly made slicing solution can be kept at 4C until the moment of the procedure. Prepare also fresh aCSF and pre-chill it at 4C.

42 Right before the procedure, prepare fresh slicing solution.

43 Break the pre-frozen slicing solution into a large beaker and add freshly-made slicing solution. With the help of the immersion blender transform the frozen/fresh mix of slicing solution into a slushie.

44 Keep bubbling the slushie and start running it through the tubing connected to the peristaltic pump, with a needle attached to the connector on the other end of the tubing. Place the needle back into the slushie so that the ice-cold solution will keep recirculating until the moment of the procedure.

45 Pour some cold aCSF into the holding chamber where the slices will be collected and place it into a container with ice, under continuous bubbling.

46 Fill the two large trays with ice.

47 In one of the trays, model the ice to create an inclined area where the mouse will be placed during the perfusion. Pre-chill the dissection tools in the remaining ice.

48 In the other tray, place a petri-dish upside-down, in contact with the ice, and cover it with the filter paper. Dig the other dish into the ice, leaving it empty. Later slicing slushie will be poured in this

dish.

49 Lay a precision wipe over the ice and place the razor blades, a 0.5x0.5in block of agar and the holding stage of the vibratome chamber over it, so that they stay cold.

50 Turn on the vibratome, pre-adjust the settings: for midbrain coronal slices a thickness of 220um, a speed of 0.06 mm/s, and an oscillation amplitude of of 1mm are recommended.

51 Attach the vibro-check tool, carefully insert the double-edge blade into the holder and perform the vibration check. Follow instruction to minimize the vibration.

52 Lift the blade holder, remove the vibro-check and leave blade in (vibration check should be performed each time a blade is inserted in the holder).

53 Place the vibratome slicing chamber in the vibratome ice tray, and fill the the area of the ice tray outside of chamber with ice.

54 Prepare an area for tissue dissection, with a dissection microscope, the dissection petri dish (see above), clean pre-chilled dissection tools and scalpels.

55 A contained with a small amount of dry ice or liquid nitrogen should be nearby.

56 Pre-chill Eppendorf tubes to collect tissue samples in ice.

Procedure:

57 Terminally anesthetize the mouse according to institutional protocols.

58 Bring the anesthetized mouse to the dissection area and verify that the mouse is fully anesthetized. This can be performed by pinching one of the posterior paws and observing the presence (or lack of) pain reflex. The mouse must be fully anesthetized before starting the transcardiac perfusion.

59 Once full anesthesia is achieved, the mouse can be positioned on the dissection area and needles inserted in its paws can be used to avoid movement.

60 The mouse should be positioned in a supine position, with the head oriented away from the operator. If the operation area is inclined, the mouse should be oriented so that the head is facing down-ward.

61 Holding the skin just below the sternum with a tweezer, cut the skin just below the tweezer tips, exposing the peritoneal cavity and the rib cage. The diaphragm is intact.

62 Expand the cut and with the scissors cut the fascia connecting the skin to the rib cage.

63 Once the rib cage is clearly visible, carefully cut the diaphragm without damaging the beating heart. Cut the chest cage and lift it toward the head. A needle can be used to hold it in position while operating. The liver should be visible in the abdominal cavity.

64 Carefully insert the needle connected to the peristaltic pump (where the slicing solution slushie is still circulating) in the left ventricle of the heart, and rapidly pinch the right atrium with the spring scissor. Dark-red blood should start flowing out of it immediately. Hold the needle in position, while the solution washes out the blood from the mouse. The heart should still be beating. A

wash water bottle can be used to remove excess blood and see more clearly.

65 Within 10-20s the liver should start looking paler, and less blood should be flowing from the right atrium.

66 When the liver looks clear of blood, remove the needle, and quickly decapitate the mouse with the scissor.

67 Transfer the head into the empty petri dish on ice and fill it with some of the slicing slushie remaining from the perfusion. Don't discard the rest. The head should be submerged in the solution.

68 With a fine scissor, cut the skin and expose the median line of the skull. Cut off the posterior part of the skull. Then, carefully cut along the median line, towards the rostral part of the head. Past bregma, apply two diagonal cuts toward the eyes, and two other later cuts along the lambdoidal sutures. During this stage, keep the head in the slicing slushie or remove it only briefly and put it back in to maintain the brain cold.

69 Carefully open the skull with the help of the tweezers and expose the brain. With the spatula, gently remove the brain and slide it into the slicing slushie in the petri.

70 Discard the skull into the carcass bag. Remember to remove any ear-tag.

71 Wet the filter paper on top of the other petri dish with slicing slushie.

72 Take the vibratome holding stage from the ice, wipe it dry with a precision wipe and apply super glue to the center of it.

73 Gently move the brain from the slushie to the filter paper. With the single edge blade, carefully remove the rostral part of the striatum (creating a uniform flat surface) and the cerebellum (for midbrain coronal slices. Obtaining slices from other regions will require different orientation and preparation of the brain).

74 With a spatula, move the brain from the filter paper to the vibratome holding stage, on the glue. Place the frontal part of the brain should be facing down. Position the agarose block on the glue, in contact with the dorsal part of the brain. Make sure that the brain and the agarose block are properly set by gently touching them with the spatula.

75 Move the holding stage into the slicing chamber, adjusting its orientation so that the ventral part of the brain is facing the blade, and the agarose in facing the operator.

76 Quickly pour slicing slushie into the chamber, covering the brain. Set the chamber on the vibratome. Add a small bubbler to the chamber.

77 With the spatula, gently move the residual icy part of the slushie toward the operator, leaving the brain visible.

78 Lower the blade into the solution, adjust its position/inclination, set the start/end point of the slicing cycle and manually lower the blade closer to the region where you expect to start collecting slices.

79 Start the vibratome.

80 Discard fragments of brain and slices from undesired area, until the region of interest is reached.

81 Immediately transfer each slice containing the region of interest from the slicing chamber into the holding chamber. Alternatively, depending on the number of slices to be collected and on how fast is the operator, the slice(s) can be directly transferred to the dissection petri dish.

After slicing:

82 Quickly transfer slices one by one on the dissection petri dish and with the help of the dissection microscope dissect the area of interest.

83 Immediately collect the tissue of interest in the pre-chilled tube. Keep the volume of solution transferred to the tube with the tissue to a minimum.

84 Discard remaining tissue.

85 Repeat for each slice of interest.

86 Once all the tissue of interest is collected, label the tube, and quickly freeze it by putting the tube in dry ice or liquid nitrogen.

87 Once all the slices of interested are dissected, wash the apparatus and the tools and clean the working station.

88 Remember to collect a fragment of the tail in case genotyping will be needed.

89 Discard the mouse carcass and extra tissue in the designated bag.

90 Carefully dispose of all the sharps according to institutional guidelines.

91 Store tubes containing the frozen samples at -80C until processing.

Immunoprecipitation and RNA extraction for RNAseq analysis...

92 Transfer tubes containing the frozen samples from -80C freezer to dry ice. Place one tube containing the sample to ice, add 100ul of prechilled tissue homogenization solution and homogenize the tissue. Add 700ul of tissue homogenization solution

93 Proceed with other tubes one by one

94 Centrifuge the tubes at 4^0 C for 10 min at 12,000g

95 Transfer the supernatant into new prechilled tubes, add 40ul of prewashed Pierce Protein A/G Magnetic Beads

96 Rotate gently the tubes at 4°C for 1 hour

97 Collect beads with a magnet (well-chilled in an ice bucket), transfer the supernatant into new prechilled tubes, add 40ul of prewashed Anti-Flag Magnetic Beads

98 Rotate gently the tubes at 4°C for 16-18 h

99 Collect the beads with a magnet and resuspend them in 800ul of washing buffer, rotate gently the tubes at 4°C for 15-20 min

100 Repeat the wash three times

101 Resuspend the beads in 300ul RLT Lyses buffer from RNAeasy Micro kit at room temperature, vortex the mixture, incubate it for 10 min at room temperature

102 Remove the beads with magnet and proceed to RNA purification according to the kit manufacture instructions

After RNA extraction:

103 Samples are transferred to NUseq core for RNAseq analysis.