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Extraction of brain tissue with fluorogold-labelled neurons for transmission electron-microscopy imaging

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We use this protocol and it's working

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Abstract

Protocol for injection of Fluorogold particles for retrograde labelling or neurons, perfusion, fixation, and tissue extraction for Transmission Electron Microscopy observation.

This protocol is intended for the preparation of samples to be processed and imaged at the Northwestern University Center for Advanced Microscopy (NU CAM; https://www.feinberg.northwestern.edu/sites/cam/index.html).

Guidelines

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Materials

Material for Fluorogold stereotaxic injections

- Fluorogold (Santa Cruz)

- Anesthetic: isoflurane

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

- 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

- 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.

Materials for perfusion/fixation:

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

- Peristaltic pump (Gilson) with tubing and connectors

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

- Dissection tray

- 50ml falcon tubes

- PFA stock solution (recommended: 16% PFA solution, Electron Microscopy Science)
- Glutaraldehyde (GA) stock solution (recommended 25% Sigma-Aldrich)
- Sodium cacodylate buffer stock solution (recommended 0.4M, Electron Microscopy Sciences)
- 10X PBS
- pHmeter and related reagents/tools
- Perfusion needle (preferred: 27 gauge ½ inch)
- Extra needles
- Solid fixative waste collection bin
- Liquid fixative waste collection bin
- Vacuum-trap jar and connector tubing
- Water wash bottle
- Carcass bag

- working solution of PBS can be prepared from 10X concentrated solution

- Fixative solution: 2% PFA, 1.25% GA in cacodylate buffer 0.1M. For better results, it is recommended to prepare a fresh fixative solution right before the procedure.

- For all solutions, adjust pH of PBS and fixative solutions to 7.3-7.4

Material for tissue extraction:

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

- Dissection tools (tweezers, spatula, according to preferences)

- Double-edged razor blades

- Single-edge razor blades

- PBS

- Medium weighing bowl or petri dish

- Pre-solidified 2% agarose

- Superglue

- Precision wipes

- Water wash bottle

- Plastic or glass transfer pipette/small brush

- 6/12/24 wells cell culture plates (according to preference) to collect slices

- Fluorescence microscope with low magnification objective, light source and UV filter set compatible with Fluorogold observation per manufacturer's specifications.

- Disposable fine scalpel.

- Hinged plastic vials with a small amount of fixative solution (see above)

- Paper/pen to sketch the exact anatomical position of the cells labelled with fluorogold.

Material for miscroscopy:

- Transmission Electron Microscope - Technai Spirit G2 (FEI Company)

- Imaging software

- Liquid nitrogen

- Precision tweezers

- Precision wipes

Troubleshooting

Safety warnings

Follow general and institutional guidelines and safety warnings.
Use appropriate PPE (lab gown, face mask, face shield/goggles, examination gloves (cut-resistant are recommended), cryo-gloves.
Handle fixatives under a chemical hood.

Stereotactic injection of fluorogold particles in the brain

1 Prepare the fluorogold working solution by dissolving fluorogold powder in the appropriate volume of sterile NaCl 0.9% saline. Recommended working concentration: 1% fluorogold

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

3 Set up sterile working area including stereotaxic frame

4 Measure the weight of the mouse

5 Anesthetize mouse in induction chamber (recommended: 2.5% isoflurane, 200ml/min flow rate) 5m

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

7 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).

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

9 Apply ophthalmic ointment over eyes

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

11 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.

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

13 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.

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

15 Visually identify bregma and lambda.

16 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.

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

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

19 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.

20 Zero the coordinates at bregma and repeat bregma/lambda measurements until satisfactory.

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

22 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.

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

24 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 of the right and left spots should be minimized by adjusting the position of the head.

25 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.

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

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

28 Draw up desired volume of fluorogold solution in the syringe by applying negative pressure.

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

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

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

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

33 Slowly retract injection pipette.

34 Suture skin.

35 Optional: repeat saline injection to prevent dehydration

Post-surgery care 5d 0h 15m

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

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

38 24 hours after surgery, a second dose of Metacam is administered and antibiotic ointment is applied on the sutured skin. 1d

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

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

41 Mice are sacrificed for tissue extraction 5-6 days after fluorogold injection.

Perfusion and fixation 12h

42 Under the hood, pour PBS and fixative solutionsin 50ml tubes.

43 Attach the perfusion needle to the connector atthe end of the tubing

44 Start running PBS through the tubing.

45 If possible, it is recommended to have aconnector system with a switch that allows to pre-load the fixative solutionand the PBS solution in the respective collection tubing and quickly switchfrom one to the other avoiding the need to move a single collection tube fromone solution to the other, interrupting the procedure.

46 A tubing connected to a vacuum-trap can be usedto immediately collect waste solution during the procedure.

47 Terminally anesthetize the mouse according to institutional protocols.

48 Bring the anesthetized mouse to the dissection tray 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 trans-cardiac perfusion.

49 Once full anesthesia is achieved, the mouse can be positioned and secured on the dissection tray.

50 The mouse should be positioned in a supine position, with the head oriented away from the operator. If possible, the dissection tray should be slightly inclined, with the mouse oriented so that the head is slightly pointing down-ward.

51 Holding the skin just below the sternum with a tweezer, cut the skin just below, exposing the peritoneal cavity and the rib cage. The diaphragm should remain intact.

52 Expand the cut and with the scissors cut the fascia connecting the skin to the rib cage, toward the head.

53 Once the rib cage is clearly visible, carefully cut the diaphragm without damaging the beating heart. Cut the rib 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.

54 Carefully insert the needle connected to the peristaltic pump (where PBS is 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.

55 As soon as the liver start to look depleted of blood, shift the perfusing solution to fixative.

56 Maintain the needle in position and rinse with water.

57 In general, we recommend perfusing each mouse with a volume of fixative approximately corresponding to their weight in grams (e.g.: 20 ml of fixative solution for a 20g mouse).

58 As the fixative reaches the tissues, some appendages of the mouse might start to move or contract. This normally indicates that the fixation is working. If the perfusion is done correctly, this should start shortly after changing the perfusion solution to fixative.

59 Once the desired volume of fixative has run through, stop the perfusion, remove the needle and release the mouse. The carcass should be very stiff.

60 Decapitate the mouse with the scissor.

61 With the fine scissors, 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.

62 Carefully open the skull with the help of the tweezers and expose the brain.

63 With the spatula, gently remove the brain and slide it into the remaining fixative solution in the falcon tube. Close the falcon tube and gently shake it.

64 Properly dispose of the mouse carcass in the carcass bag.

65 Dispose of all the fixative waste (liquid and solid) and sharps according to institutional guidelines.

66 Clean/wash all the tools/equipment.

67 Leave the brain in fixative overnight at 4C. 12h

Tissue extraction and isolation

68 Shortly before the slices collection, remove fixative solution where the brain has been kept overnight and rinse 3 times for 10 minutes each with PBS. Keep the brain in a clean tube with fresh PBS.

69 Cut a 0.5×0.5in rectangle of agarose

70 The recommended settings for slicing are: 400um thickness, 1mm oscillation amplitude, speed ~0.2-0.3mm/s

71 Obtain fixed thick slices:

71.1 Remove the fixed brain from PBS and place it on the weighing bowl/petri dish.

71.2 With the single edge blade, cut the brain according to the desired slicing orientation. For coronal midbrain slices, it is recommended to cut off the more rostral part of the brain, creating a flat surface that will be used to glue the brain to the slicing chamber stage, and to remove the cerebellum.

71.3 Apply super glue to the slicing chamber stage.

71.4 Gently glue the brain onto the stage, in the desired position. For coronal midbrain slices, it is recommended to position the ventral part of the brain facing the blade, with the rostral side at the bottom. If desired, glue also the piece of agarose on the side of the brain that will not be facing the blade.

71.5 Place the cutting stage in the chamber, set the chamber in the vibratome tray and attach it to the vibratome.

71.6 Fill the cutting chamber with PBS.

71.7 Lower the blade holder (with blade already inserted) into the chamber and adjust the position/settings of the blade.

71.8 Start cutting.

71.9 While still far from the region of interest, it is possible to manually move the blade closer to the target area rather than cutting thin slices throughout the entire tissue, unless desired.

71.10 Discard debris/excess tissue.

71.11 When at the target area, cut 400-500um slices, gently collect them with a transfer pipette or a tweezer and move them to one of the wells of the collection plate. It is recommended to keep track of the collection order for anatomical accuracy. It is also recommended to keep each slice in a separate well in a plate, so that it will be easier to identify the one with the best fluorogold signal.

71.12 Once all the slices are collected, stop the vibratome, return the blade holder to a safe position, discard extra solution and what remains of the brain tissue accordingly.

72 Isolation of region containing Fluorogold-labelled cell

72.1 Move the plate with the slices to the microscope station.

72.2 Turn on the microscope and observe slices with the appropriate fluorogold-compatible filter set.

72.3 Identify the slice with the best fluorogold signal and sketch on some paper the exact anatomical location of the area of interest. Accurately tracking the position of the fluorogold labelled cells will be very important for the following steps.

72.4 Move the selected slice to a petri dish with some PBS and using the tweezers and the disposable scalpel dissect a 1mm by 1mm square of tissue containing the area of interest. It is recommended to carefully sketch the exact distribution of the fluorogold-labelled area also within the tissue square.

72.5 Under a chemical hood, transfer the selected block of tissue to the hinged plastic vial containing fixative solution.

72.6 The tissue block can be stored in the vial with fixative at 4C.

73 Carefully dispose of all the waste and sharps according to institutional guidelines. Wash the apparatus and the tools and clean the working station.

74 This protocol is intended for the preparation of samples to be processed and imaged at the Northwestern University Center for Advanced Microscopy (NU CAM) The vial containing the selected tissue will be transferred to the Electron Microscopy core for further processing. Provide copies of the sketches identifying the exact position of the region of interest to the Core personnel.

75 Electron Microscopy core will proceed with processing the tissue for electron microscopy observation according to their protocol (https://www.feinberg.northwestern.edu/sites/cam/docs/protocols/tem-methods.pdf).

75.1 This includes post-fixation in buffered 2% OsO4, staining in 3% uranyl acetate; dehydration in ascending ethanol; transition to propylene oxide and embedding in Embed 812 (Electron Microscopy Sciences).

75.2 The sample will be then sectioned using an ultramicrotome (Leica Ultracut UC6). 1um thick sections are stained with Toluidine Blue O and 70 nm sections are collected on 200

mesh copper grids and stained with uranyl acetate and Reynolds lead citrate.

75.3 The 70nm are now ready for electron microscopy imaging.

Transmission Electron Microscopy

76 The operation of the transmission electron microscopy station requires prior training, observation, and approval by the NU CAM Core personnel. Please refer to the training module/instructions for all the operations.

77 Image acquisition

77.1 Full microscope apparatus is turned on an operated according to specific instructions

77.2 Liquid nitrogen is used as a cooling agent, as instructed (handle with cryo-gloves)

77.3 Carefully insert one sample grid in the microscope apparatus according to instructions.

77.4 The transmission electron microscope is operated at 80kHz

77.5 Different levels of magnification can be adopted.

77.6 At lower magnification, identify cell bodies with morphological features corresponding to the cells of interests.

77.7 Identify retrogradely-labelled cells by the presence of cytological structures that indicate Fluorogold uptake (e.g.electron-dense vesicular bodies, multilamellar electron-dense vesicles).

77.8 Collect high magnification/high resolution images.

77.9 Export and save the collected images.

78 Carefully shut off microscope station according to instructions.

78.1 Carefully remove grid from microscopy apparatus according to instructions.

78.2 Collect residual liquid nitrogen (handle with cryo-gloves).

78.3 Carefully clean the work station.