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Protocol status: Working
 We use this protocol and it's working

🌐 Immunofluorescence for confocal imaging

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ABSTRACT

This protocol describes the steps for tissue preparation for immunostaining and confocal images acquisition. It also describes the steps for immunostaining and confocal imaging.

ATTACHMENTS

[Immunofluorescence for confocal imaging.docx](#)

MATERIALS

Perfusion and fixation

Materials:

- 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

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- 10X PBS
- pHmeter and related reagents/tools
- Perfusion needle (preferred: 27 gauge ½ inch)
- Extra needles
- Solid PFA waste collection bin
- Liquid PFA waste collection bin
- Vacuum-trap jar and connector tubing
- Water wash bottle
- Carcass bag

Recommended PPE:

- Performing the procedure and handling PFA under a fume hood is strongly recommended.
- Lab gown/disposable gown
- Face mask
- Face shield/goggles

- Examination gloves
(cut-resistant gloves are recommended)

Solutions:

- PBS can be prepared from 10X concentrated solution
- 4% PFA solution is prepared by diluting the concentrated PFA stock and PBS 10X stock in water. For better results, it is recommended to prepare a fresh 4% PFA solution in PBS right before the procedure. Adjust pH of PBS and PFA solutions to 7.3-7.4

Slice preparation

Materials:

- 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 petridish
- 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

Recommended PPE:

- Lab gown/disposable gown
- Face mask
- Face shield/goggles
- Examination gloves (cut-resistant gloves are recommended)

Immunofluorescence

Materials:

- Pre-cut slices
- Orbital shaker

- PBS
- Blocking reagent, e.g.: Normal Goat Serum (NGS) – the correct blocking reagent/blocking solution should be established based on the characteristics of the antibodies used.
- Triton-X100 (detergent)
- Primary antibody(s)
- Secondary antibody(s)
- Hard-drying mounting medium (recommended ProLong Diamond, ThermoFisher Scientific)
- Microscopy slides
- Glass coverslips (recommended #1.5, VWR)

Recommended PPE:

- Lab coat/disposable gown
- Examination gloves

Confocal imaging

Materials:

- Pre-mounted microscopy slides
- Confocal laser scanning microscope with appropriate objectives (recommended: 10x/0.4 or a 60x/1.35 immersion) connected to a computer with the appropriate imaging software
- Immersion oil
- Precision wipes
- 70% Ethanol/lens cleaning solution
- Image processing software (recommended: FIJI).

Perfusion and fixation - Before the procedure:

- 1 Under the hood, pour PBS and PFA solutions in 50ml tubes.
- 2 Attach the perfusion needle to the connector at the end of the tubing
- 3 Start running PBS through the tubing.
- 4 If possible, it is recommended to have a connector system with a switch that allows to pre-load the PFA solution and the PBS solution in the respective collection tubing and quickly switch from one to the other avoiding the need to move a single collection tube from one solution to the other, interrupting the procedure.

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

Procedure:

- 6 Terminally anesthetize the mouse according to institutional protocols.
- 7 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.
- 8 Once full anesthesia is achieved, the mouse can be positioned on the dissection area and needles can be inserted in its paws to avoid movement.
- 9 The mouse should be positioned in a supine position, with the head oriented away from the operator. If the operation area is slightly inclined, the mouse should be oriented so that the head is facing down-ward.
- 10 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.
- 11 Expand the cut and with the scissors cut the fascia connecting the skin to the rib cage.
- 12 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.

- 13 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.
- 14 As soon as the liver should start looking whiter, shift the perfusing solution to PFA.
- 15 Maintain the needle in position and rinse with water.
- 16 In general, we recommend perfusing each mouse with a volume of PFA approximately corresponding to their weight in grams (e.g.: 20 ml of PFA solution for a 20g mouse).
- 17 As PFA reaches the tissues, some appendages of the mouse might appear to be moving or contracting. This normally indicates that the fixation is working. If the perfusion is done correctly, this should start shortly after changing the perfusion solution to PFA.
- 18 Once the desired amount of PFA has run through, stop the perfusion, remove the needle and release the mouse. The carcass should be very stiff.
- 19 Decapitate the mouse with the scissor.
- 20 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.

- 21 Carefully open the skull with the help of the tweezers and expose the brain.
- 22 With the spatula, gently remove the brain and slide it into the remaining PFA solution in the falcon tube. Close the falcon tube and gently shake it.

After the procedure:

- 23 Properly dispose of the mouse carcass in the carcass bag.
- 24 Dispose of all the PFA waste (liquid and solid) and sharps according to institutional guidelines.
- 25 Clean/wash all the tools/equipment.
- 26 The brain can remain in PFA for post-fixation for a variable time depending on experimental needs. We recommend keeping it in PFA for 1-2 hours.
- 27 Once the post-fixation time has passed, remove PFA solution, substitute with PBS and agitate.
- 28 Rinse with PBS for 10 minutes.

- 29 Repeat the rinse step with fresh PBS for a total of 3 times.
- 30 Move the brain to a new clean tube with PBS and label it appropriately.
- 31 Store the brain in PBS at 4C until further processing.

Slice preparation - Before the procedure:

- 32 Prepare PBS
- 33 Add appropriate volume of PBS to each well of the cell culture plate where slices will be collected.
- 34 Cut a 0.5x0.5in rectangle of agarose
- 35 Turn on the vibratome. If desired, insert the double edge blade and perform vibration check.
- 36 The recommended settings for slicing are: 50-60um thickness, 1mm oscillation amplitude, speed ~0.2-0.3mm/s

Procedure:

- 37 Remove the fixed brain from PBS and place it on the weighing bowl/petri dish.
- 38 With the single edge blade, cut the brain according to the desired slicing orientation. For coronal midbrain slices, it is recommended to cut 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.
- 39 Apply super glue to the slicing chamber stage.
- 40 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.
- 41 Place the cutting stage in the chamber, set the chamber in the vibratome tray and attach the to the vibratome.
- 42 Fill the cutting chamber with PBS.
- 43 Lower the blade holder (with blade already inserted) into the chamber and adjust the position/settings of the blade.
- 44 Start cutting.

- 45 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.
- 46 Discard debris/excess tissue.
- 47 When at the target area, cut 50-60um slices, gently collect them with a transfer pipette or a brush 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.

After the procedure:

- 48 Once all the slices of interest are collected, stop the vibratome, return the blade holder to a safe position, discard extra solution and what remains of the brain tissue accordingly.
- 49 Carefully dispose of all the sharps according to institutional guidelines.
- 50 Wash the apparatus and the tools and clean the working station.
- 51 Slices can be stored in the plate with PBS at 4C. It is recommended to wrap cell plate and its lid with parafilm to avoid PBS evaporation.
- 52 For longer storage, a preservative (e.g. sodium azide) can be added to the PBS.

Immunofluorescence - Before the procedure:

- 53 Prepare PBS
- 54 If needed, prepare aliquots of NGS. Store at -20C and thaw immediately before use.
- 55 If needed, reconstitute/aliquot antibodies and store them according to manufacturer's instructions. Thaw them on ice immediately before use.
- 56 It is recommended to prepare the other solutions as needed

Procedure:

- 57 Select the slices of interest and transfer them in a new plate with PBS.
- 58 For better results when performing an immunostaining targeting an epitope contained in subcellular organelles, we had better results performing a short permeabilization stage with a higher concentration of detergent. This step is however optional.
- 59 Prepare permeabilization solution: 0.5% Triton in PBS. The desired volume of permeabilization solution depends on the number of slices to process, the size of the wells/volume of solution needed to cover the slices. For a 24 wells plate, we recommend 1 ml/well of solution for permeabilization/PBS wash/blocking steps, but this can be decreased to ~300ul/well for incubation with antibodies.

- 60 Remove PBS from each well containing a slice and substitute with permeabilization solution.
- 61 Incubate for 15 mins with constant gentle movement over orbital shaker.
- 62 While the slices are incubating with permeabilizing solution, prepare blocking solution (e.g.: 10% NGS, 0.25% Triton-X100 in PBS). This is the solution where the primary antibody/antibodies will be diluted, so when calculating the required volume account for the volume required for both steps (e.g.: 1ml + ~300ul per well).
- 63 After the 15 minutes, remove permeabilization solution, rinse with PBS, and cover slices with blocking solution.
- 64 Incubate for 30 mins with constant gentle movement over orbital shaker.
- 65 During the incubation, dilute the primary antibodies in blocking solution according to recommended dilution.
- 66 After the 30 mins incubation, remove blocking solution and apply diluted primary antibody/antibodies.
- 67 Incubate over gentle agitation. Depending on the quality of the antibody used, it can be recommended to incubate the slices with the primary antibody solution over-night at 4°C, with gentle agitation.
- 68 After primary antibody incubation, remove primary solution and cover slices with PBS.

- 69 Wash for 10 mins over gentle agitation.
- 70 Repeat wash step for a total of 3 times.
- 71 During the washes, prepare the blocking solution for the secondary antibody/antibodies (e.g.: 10% NGS in PBS). Calculate needed volume based on the number of slices/size of wells and dilute secondary antibody/antibodies according to manufacturer's instructions.
- 72 After the PBS washes are completed, remove PBS and cover slices with the secondary antibody solution.
- 73 Incubate at room temperature with constant gentle agitation on orbital shaker for 60 mins.
- 74 After incubation, discard secondary antibody solution and cover with PBS.
- 75 Repeat 3 10 mins PBS washes as above.
- 76 After PBS washes, with a fine brush carefully mount slices on clean and labelled microscopy slides.

- 77 Gently remove excess solution.
- 78 Let dry in the dark (recommended: overnight).
- 79 Following day/when the slices have dried on the slide: apply a small amount of hard-drying mounting medium sufficient to cover the slices. Carefully avoid the formation of air bubbles. Gently apply a coverslip over the slices and the mounting medium
- 80 Let cure overnight in the dark.

After the procedure:

- 81 Dispose of waste and excess reagents/solution according to institutional guidelines.
- 82 Clean tools/working station.
- 83 Once the mounting medium is cured, slides are ready for observation.
- 84 Fully mounted microscopy slides should be stored in a designated container at 4C until time of observation.

Confocal imaging - Procedure:

- 85 Turn on laser/microscope/computer according to specific manuals/instructions
- 86 Access imaging software
- 87 Load microscopy slides in the designated stage
- 88 Proceed with observation/image acquisition according to the specific microscope/software manuals

After the procedure:

- 89 Clean immersion objectives with a lens wipe and the appropriate cleaning solution
- 90 Export/save images
- 91 Turn off software/microscope/laser according to specific instructions
- 92 Images can be observed and appropriately adjusted using an imaging software (e.g. FIJI).

93 Copies of the original unprocessed images acquired should be stored.