Perfusion-fixation procedure for adult rhesus monkeys

Solutions to prepare

1. Pentobarbital (25 mg/ml solution).
2. Ringer's solution (see recipe below).
3. Fixative 2.5 L [4% paraformaldehyde (Fisher Scientific Cat AC416785000), 0.1% EM grade glutaraldehyde (Electron Microscopy Sciences Cat 16220), 0.1M phosphate buffer, pH 7.4]. Note that glutaraldehyde must be added to the fixative solution only at the time of perfusion (see recipe below).

Equipment and room needed

1. Necropsy room equipped with proper exhaust and ventilation to reduce exposure to aldehydes.
2. Peristaltic pump (recommended model Fisher brand GP1000).
3. Oxygen tank (95% O_2, 5%CO_2).
4. Surgical instruments (scalpels, hemostats of various sizes, bone cutter, scissors, bone saw, forceps, rongeur)

Before beginning

1. Wear PPE before entering the perfusion room, i.e., gloves, coat, hair net, safety glasses, face mask and face shield or goggles.
2. Make sure the Hazardous Waste container is not full. Replace if full.

Procedure

1. Add glutaraldehyde to the fixative solution
2. Start oxygenation of the Ringer's solution
3. Anesthetize the animal with Ketamine (10 mg/kg, i.m.) or Telazol (3-5 mg/kg, i.m.) in its home cage and bring to the necropsy room
4. Place an i.v. catheter
5. Perform a tracheal intubation
6. Inject 1 ml of heparin (i.v.)
7. Inject an overdose of pentobarbital (25 mg/kg i.v.)
8. Ensure the absence of all reflexes, including toe pinch reflexes and brainstem reflexes, such as corneal reflexes
9. Cut the thoracic cage to provide access to the heart, clamp the descending aorta, open the pericardium and expose the heart muscle, make an incision of the right atrium and insert the perfusion needle in the left ventricle.
10. Artificially ventilate the animal through the tracheal tube during the Ringer's solution perfusion
11. Infuse transcardially ~300-400 ml of cold oxygenated Ringer's solution at a rate of 80-90 ml/min through a needle (14G; 1.5 inch long) inserted in the left ventricle. Let the infused solution exit the vascular system through a hole in the right atrium
12. Perfuse 2.5 liters of fixative, starting at a rate of 80-90 ml/min for the first liter and then reduce the rate to 50 ml/min for the remaining solution.

13. After perfusion, fix the animal's head in a stereotaxic frame and open the skull using a bone saw and rongeur.
14. Cut the brain in 10 mm-thick blocks in the coronal stereotaxic plane, remove the resulting blocks of tissue from the skull and post-fix them for 24 hours in 4% paraformaldehyde solution in PB (0.1M, pH 7.4) at 4^0C.
15. Transfer the tissue into a phosphate-buffered saline (PBS, 0.01M, pH 7.4) solution.
16. Cut the brain in 50 um-thick sections using a vibrating microtome or a freezing microtome and store in a -20^0C freezer in an anti-freeze solution (see receipe below) until further processing.

RINGER'S SOLUTION

Dissolve the following reagents, in order, in 1000 ml of distillled water

- 0.60 g HEPES
- 11.86 g NaCl
- 0.223 g KCl
- 0.353 g $CaCl_2$
- 2.18 g $NaHCO_3$
- 0.177 g KH_2PO_4
- 0.32 g $MgSO_4$
- 1.8 g D-glucose

FIXATIVE SOLUTION

CAUTION: PREPARE THIS SOLUTION UNDER A HOOD

Final volume: 1 liter

Concentration: 4% paraformaldehyde

0.1% glutaraldehyde

- Heat 500 ml distilled water until the temperature reaches 60˚C.
- Dissolve 40 g paraformaldehyde with strong stirring action for 20 min.
- Add NaOH drop by drop until the solution is almost clear.
- Let the solution cool for one hour.
- Filter the solution in another beaker and add 500 ml PB (0.2M pH 7.4)

KEEP IN THE FRIDGE

Just before the perfusion, substitute 4 ml of fixative by 4 ml of glutaraldehyde 25%.

ANTI-FREEZE SOLUTION

Prepare the following solution in the same order:

For 1000 ml solution	
13.8 g	Sodium phosphate monobasic, $NaH_2PO_4 \cdot H_2O$
25.8 g	Sodium phosphate dibasic heptahydrate, $Na_2HPO_4 \cdot 7H_2O$
400 ml	Distilled water
300 ml	Ethylene glycol
300 ml	Glycerol

Sections can be stored in this solution at -20C freezer for many months without loss of ultrastructural preservation and tissue antigenicity.