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# APPENDIX

I. Preparation of Tris buffer (pH 8.6)

| | | |
|---|---|---|
| NaCl | 7.2 | g |
| KCl | 0.37 | g |
| $MgSO_4 \cdot 7\ H_2O$ | 0.25 | g |
| Tris | 4.48 | g |
| Dist. $H_2O$ | 1000 | ml |

(Adjust pH to 8.6)

II. A perfusion-fixation method for Procedure I and II

(a) Preparation of phosphate buffer 0.1 M. pH 7.4 for Procedure II.

Stock solution

| | | | |
|---|---|---|---|
| Solution A : | $NaH_2PO_4$ | 13.801 | g |
| | Dist. $H_2O$ | 1000 | ml |
| Solution B : | $Na_2HPO_4$ | 14.198 | g |
| | Dist. $H_2O$ | 1000 | ml |

Working phosphate buffer

| | | |
|---|---|---|
| Solution A | 160 | ml |
| Solution B | 840 | ml |

(Adjust pH to 7.4)

(b) Preparation of phosphate buffer 0.05 M. pH 7.4 for procedure I

Mix volumes of the 0.1 M phosphate buffer with equivalent volumes of distilled water and adjust pH to 7.4

(c) Saline for procedure II

0.09% NaCl in distilled water. Bring pH up to 7.4 with NaOH.

(d) The fixative for procedure I. 1% paraformaldehyde, 1.5% glutaraldehyde, 4% sucrose in 0.05 M phosphate buffer.

| | |
|---|---|
| 2% stock paraformaldehyde | 1000 ml |
| 30% glutaraldehyde | 100 ml |
| 80% sucrose in 0.05 M phosphate buffer | 100 ml |
| 0.05 M phosphate buffer | 800 ml |

2% Stock paraformaldehyde

| | |
|---|---|
| paraformaldehyde | 20 g |
| 0.1 M phosphate buffer | 950 ml |

heat untill paraformaldehyde dissolve on a hot plate then filter and add buffer to adjust the volume to 1000 ml store in bottle untill used.

(e) The fixative for procedure II 1% paraformaldehyde, 1.25% glutaraldehyde in 0.1 M phosphate buffer.

| | |
|---|---|
| 2% stock paraformaldehyde | 1000 ml |
| 50% glutaraldehyde | 50 ml |
| 0.1 M phosphate buffer | 950 ml |

III. The reaction process

(a) The Procedure I

Following, OLMOS, HARDY AND HEIMER (1978)

The TMB-procedure

Stock solutions

(A) Gelatin-buffer solution

| | | |
|---|---|---|
| Absolute ethanol | 100 | ml |
| Dimethyl sulfoxide (DMSO) | 10 | ml |
| 0.05 M (acetate buffer. pH 4.3) | 20 | ml |
| Gelatin (Fisher. 270 Bloom) | 5 | gm |
| Distilled water | 870 | ml |

(B) TMB solution

| | | |
|---|---|---|
| TMB | 0.36 | gm |
| Absolute ethanol | 270.00 | ml |

It is practical to store the stock solution in the refrigerator.

Staining protocol

1. Rinse a maximum of 40 sections in distilled water.

2. Wash in 5% nickel ammonium sulfate (NAS) for five minutes.

3. Rinse briefly in distilled water.

4. Incubate at $0^{\circ}C$ (use an ice bath or a refrigerator) with frequent agitation in the following solution

Gelation buffer sol'n (A) 76 ml

TMB sol'n (B) 4 ml

5% sodium nitroprusside (fresh) 1.35 ml

(2 ml for poorly fixed material)

0.75% $H_2O_2$ every 20 minutes up to a total of on hour. Start examining the sections under the microscope for crystalization. If the sections are free of crystals, they can usually be incubated for 20 more minutes if necessary.

5. Rinse in ice cold distilled water (fading night occur if the water is not cold).

6. Mount onto gelatin coated slides and let them air dry in room temperature.

7. Dehydrate in graded alcohol solutions and xylene as usual and enclose in permount

(b) The procedure II Following Mesulam (1978)

1) Saline wash

Transcardial perfusion is initiated by a rapid bolus of physiological saline at room temperature

2) Fixative

Saline perfusion is immediately followed by the fixative at room temperature in volume of 250 ml for one rat. The rate flow is adjusted so that half the volume flows as rapidly as possible while the other half is administered more slowly in order to keep the total time of perfusion with fixative at 20-30 minutes.

3) Buffer-sucrose post-fixation

The fixative is immediately followed by perfusion with 10% buffer-sucrose.

4) Storage of the brain

The brain can be removed immediately and stored overnight in 30% sucrose in 0 1 M phosphate buffer at $4^{o}C$.

5) Cutting

The brain is frozen with dry pee on the stage of a freezing microtome and cut in transverse sections at 50 micrometer thick.

6) Storage of tissue sections

The cut sections are collected in the working phosphate buffer (0.1 M. pH 7.4).

A Tetramethylbenzidine method (From Mesulam 1978)

Perparation of solution

a) Acetate buffer at pH 3.3

| | |
|---|---|
| 0.1 M Sodium acetate | 200 ml |
| Dist. $H_2O$ | 200 ml |
| 1.0 M HCl | 190 ml |

Make up the volume to 1000 ml with distilled water. Check the final pH and titrate with concentrated acetic acid or sodium hydroxide in order to bring the final pH to 3.3.

b) Solution A

| | | |
|---|---|---|
| Dist. $H_2O$ | 185 | ml |
| Acetate buffer pH 3.3 | 10 | ml |
| Sodium nitroferricyanide | 100 | mg |

Multiples of these quanities may be used for larger volumes.

c) Solution B

| | | |
|---|---|---|
| 3.3'.5.5'-tetramethylbenzidine | 10 | mg |
| Absolute ethanol | 5 | ml |

This may be heated to $37^{o}$-$40^{o}$C to dissolve the TMB.

d) Incubation solution

| | | |
|---|---|---|
| Solution A | 195 | ml |
| Solution B | 5 | ml |

Neither solution A nor solution B should be older than 2 hours.

e) Peroxide solution

This should have a concentration of 0.3% of $H_2O_2$ in water.

f) Neutral red solution

Add 40 ml of pH 4.8 acetate buffer to each liter of a 1% aqueous neutral red solution. To prepare the buffer, add 500 ml of 0.1 N acetic acid to 750 ml of 0.1 N sodium acetate. Filter before using.

g) Postreaction storage and ringing solution

| | |
|---|---|
| Acetate buffer pH 3.3 | 10 ml |
| Dist. $H_2O$ | 190 ml |

The Procedure

1) Rinse

Rinse in six changes of distilled water (10-15 seconds each).

2) Pre-reaction soak for 20 minutes at room temperature.

Sections are immersed in the incubation solution is prepared by mixing solutions A and B as described above. The actual mixing should be done within the reaction vessel and only seconds before the sections are introduced. This solution should maintain a clear amberemerald color during the 20 minutes otherwise. chemical contamination must have occured.

3) Enzymatic reaction for 20 minutes at room temperature

Adding 5 ml of 0.3% $H_2O_2$ solution to each 100 ml of the incubation solution. The sections are removed, the $H_2O_2$ rapidly stirred in sections until 20 minutes.

4) Rinse

After enzymatic reaction rinse the sections three times with 'postreaction storage and rinsing solution' prepared as described above. The sections are left in this solution until they are mounted. (They can

be left there for up to 4 hours at 0-4$^{o}$C without appreciable loss of reaction product.

5) Mounting

The sections are mounted at room temperature from the 'postreaction storage and rinsing solution' on to glass slides subbed with chrome-alum. These are left to air dry for 4 hours to 7 days at room temperature.

6) Counterstain

Counterstain for 15 minutes in the 1% buffered neutral red solution

7) Dehydration

Expose all sections to distilled water (10 seconds), 70% ethanol (10 seconds each) and then to two baths of xylene (2-5 minutes each).

# BIOGRAPHY

Mrs. Jiamjit Sangsuwan was born on October, 9th 1954, in Khon Kean province, Thailand. She graduated Bachelor of Science in Nursing from Faculty of Nursing, Khon Kean University in 1976 and started to work as instructor at Department of Medical-Surgical Nursing, Faculty of Nursing, Khon Kean University from 1977 up to present.