GALLYAS SILVER STAIN FOR MYELIN

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Adapted from Gallyas F (1979)*

This protocol has been optimised for use with frozen primate brain tissue (marmoset and macaque). It uses mounted slides (other versions are available for free floating slides but in our experience these give inferior results particularly with respect to high shrinkage).

Tissue preparation steps

1. Transcardial perfusion with heparinised 0.1M phosphate buffered saline followed by 4% buffered paraformaldehyde.
2. Overnight post-fixation in 4% buffered paraformaldehyde.
3. Remove brain from skull and block for sectioning.
4. Cryoprotect block with 10%, 20%, 30% sucrose in buffered paraformaldehyde.
5. Tissue block frozen and sections cut at 40 or 50µm.
6. Tissue sections post-fixed in 4% buffered formalin for minimum 2 weeks (general rule 1 week per 10µm tissue thickness) then mounted on double gel-subbed slides out of warm buffered 0.3% gelatine solution. Air dry for 5-7 days before staining.

(Note: This procedure has a high risk of sections detaching from slides during staining. Mounting and drying as above reduces this risk)

Solutions to be prepared the day before staining

Ensure that you add chemicals **in the order listed** and use a high quality water source such as RO or milliQ, if dH_2O is not available)

SOLN A

Sodium Carbonate anhydrous 'A.R.'	50g/L
dH_2O	1L

SOLN B – (wrap bottle in foil)

Ammonium Nitrate	2g/L
Silver Nitrate	2g/L
Dodeca-Tungsto-silicic acid	10g/L
dH_2O	1000ml

SOLN C – (wrap bottle in foil)

	Ammonium Nitrate	2g/L
	Silver Nitrate	2g/L
	Dodeca-Tungsto-silicic acid	10g/L
	38 – 40% Formaldehyde	6ml/L
	dH_2O	1000ml

0.2%	Potassium ferricyanide	2g/L (dH_2O)
0.5%	Sodium Thiosulfate	5g/L (dH_2O)
0.5%	Acetic acid (stock solution)	25ml/5L (dH_2O)
0.1%	Acetic acid	100ml of 0.5% stock + 400ml dH_2O
0.05%	Acetic acid	50ml of 0.5% stock + 450ml dH_2O
50%	Ethanol (EtOH)	250ml EtOH + 250ml dH_2O
30%	EtOH	150ml EtOH + 350ml dH_2O

Solutions to prepare on the day of staining:

- Pyridine (2 parts – 300ml) + Acetic anhydride (1 part – 150ml)

- Silver Nitrate – (wrap bottle in foil)

dH_2O	450ml
Ammmonium Nitrate	0.9g
Silver Nitrate	1g
4% (1M) NaOH	2.7ml
(add NaOH dropwise, whilst stirring solution)	

- Solutions A + B + C

Add B slowly to A whilst stirring in a foil-covered dish, then add C slowly. If a precipitate forms, throw solution away. To be mixed just before required. Solutions added in proportion according to ambient temp (see table).

Temp ^oC	Soln A	Soln B	Soln C
15 ± 2	10	0	10
20 ± 2	10	3	7
22 ± 2	10	4	6
25 ± 2	10	5	5
30 ± 2	10	7	3
eg. 22^oC	250	100	150

- Only use glass or plastic staining racks and dishes (no metal)
- It is advisable to keep a set of glassware specifically for solution preparations and staining for this protocol. We use Schott bottles, glass measuring cylinders and 1.5L pyrex loaf dishes. Ensure that the slides are adequately covered with solution at all steps (450 - 500ml per wash for 50 standard slides and 900 - 1000ml per wash for 50 3’ x 2’ slides with the dish described). A standard 25 slide staining dish will require about 300ml per wash/solution used.

- All staining steps are carried out in the fume cupboard on an orbital shaker.
- It is also advisable to turn the first slide in each rack around to avoid over-staining of that slide.

Troubleshooting

Sections should not be over-developed in the A + B + C solution. If development occurs in under 3 mins, slides should be removed from this solution and go to the next step.

Slides should be left in Potassium ferricyanide until any signs of muddiness or rusty-looking deposits have disappeared.

If sections begin to lift, fix the stain in sodium thiosulfate, rinse in dH_2O and allow slide to dry in air. Re-flattening of the section can be assisted by brushing the section dry. Slide can be dry-coverslipped (attach a coverslip with DPX) 1 – 2 days later.

NB. Pyridine/acetic anhydride solution can be put aside for **same-day** re-use **only**

Staining method – Gallyas Silver Stain for Myelin

REAGENT	TIME (min)	COMMENTS
dH_2O	5	Blot before next step
Pyridine and acetic anhydride	60	Do not re-use dish
50% EtOH	3	
30% EtOH	3	
0.05% acetic acid	3	
0.1% acetic acid	3	
0.5% acetic acid	10	
Silver Nitrate Solution	60	Wrap dish in foil
0.5% acetic acid	10	
Solution A+B+C	3 – 5	Mix just prior to use, wrap dish in foil
0.5% acetic acid	1	
Potassium ferricyanide	20 – 40	May be left longer if necessary
Sodium thiosulfate	2	
0.5% acetic acid	1	
0.5% acetic acid	1	
Solution A+B+C	3 – 5	Mix just prior to use, wrap dish in foil
0.5% acetic acid	1	
0.5% acetic acid	1	
Potassium ferricyanide	20 – 40	May be left longer if necessary
Sodium thiosulfate	2	
0.5% acetic acid	1	
0.5% acetic acid	1	
Solution A+B+C	3 – 5	Mix just prior to use, wrap dish in foil
0.5% acetic acid	1	
Potassium ferricyanide	20 – 40	May be left longer if necessary
Sodium thiosulfate	2	
dH_2O	5	
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Dehydrate and coverslip		Suggested protocol - see below
50% EtOH	2	
70% EtOH	2	
95% EtOH	2	
100% EtOH	2	
100% EtOH	2	
Xylene 1	2	
Xylene 2	2	
Attach coverslip with DPX		

* Silver staining of myelin by means of physical development.
Gallyas F, 1979, Neurol Res 1:203-209.