CLEARING PROTOCOL

This is an adapted protocol from articles (PMID: 22198277, PMID: 22479475 PMCID: PMC3316521)

PERFUSION AND POST-FIXATION OF TISSUE

- Perfuse the animals with 4% paraformaldahyde pH 7.3-4.
- Postfix ON in 4% Paraforaldehyde at 4^0 Celsius and rinse in PBS pH 7.3-4, two to three times 15 minutes each.
- Store in PBS at 4^0 C. If you are planning long term storage add Sodium azide to 0.01%.

Do not add sucrose at any step.

CHEMICALS

There are two types of Tetrahydrofuran (THF)

- **THF** w/ stabilizer (SigmaAldrich: #186562-1L)
- **THF** w/o stabilizer (SigmaAldrich: #401757) We use this one.
- **BABB** is aa mix at a ratio of 1:2 with **Benzyl alcohol and Benzyl benzoate**
  1 volume **Benzyl alcohol (BA)** (SigmaAldrich: #305197-100ML)
  2 volumes B**enzyl benzoate (BB)** (SigmaAldrich: # B6630-250ML)
- **Aluminum oxide** (SigmaAldrich: ##199443-1Kg)

Whether you use THF with or without stabilizer you need to eliminate the peroxides using aluminum oxide in a chromatography column. For every 100-120 ml of THF use 30 grams of silica. Around 15% of the THF remains in the silica. So when you calculate how much THF you need take into account this loss. Instructions for the various parts to set up for the chromatography column are found bellow.

TIPS FOR HANDLING THE CHEMICALS

Might look intimidating and discouraged from using this protocol but I can attest once you have it done 2-3 times should be a “walk in the park”.

Precautions for handling THF, BABB components.

- ☑ -Avoid breathing them. Use gloves and goggles.
- ☑ -Consider them all “nasty” chemicals and therefore absolutely don’t dump down the drain. Empty all solutions in glass bottles and keep separate the various chemicals, don’t mix. Every few months to avoid build up excess of peroxides call your University’s EHS department or responsible department to dispose these chemicals. The aluminum oxide in the column is hard to dislodge unless you let open the column in the chemical hood for 2 days. Once dried you can dispose properly.
- ☑ -Don’t use plastic pipettes to handle the chemicals. Use Pasteur glass pipettes and FALCON transfer pipet #357575. For incubations of the specimen and handling of the solutions use borosilicate cylinders and vials.

INCUBATION TIMES

All dilutions of THF are done in “bi-distiled” water

Mouse Spinal Cord.

Solution volume for each step, 3-4 ml

1) 50 vol% for 2- 2.5 hrs
2) 80 vol% for 2- 2.5 hrs
3) 100 vol% for 2- 2.5 hrs
4) 100 vol% for 2- 2.5 hrs or overnight if you want to do the final steps and imaging the next day. We do ON.
5) BABB, 2-3 ml for 1 hr (until transparent, usually within an 1 hour)

Rat Spinal cord.

Solution volume for each step, 4 ml

1) 50 vol% for 3 hrs
2) 80 vol% for 3 hrs
3) 100 vol% for 3 hrs
4) 100 vol% overnight
5) BABB, 3-4 ml for 1 hr or until transparent

Mouse Brain

Solution volume for each step, 15 ml

1) 50 vol% for 12 hrs
2) 80 vol% for 12 hrs
3) 100 vol% for 12 hrs
4) 100 vol% overnight
5) BABB, 9-10 ml for 2-3hrs (until transparent)

Rat Brain

Solution volume for each step, 20-25 ml

1) 50 vol% for 24 hrs
2) 80 vol% for 24 hrs
3) 100 vol% for 24 hrs
4) 100 vol% overnight
5) BABB, ~15ml for 2-3hrs (until transparent)

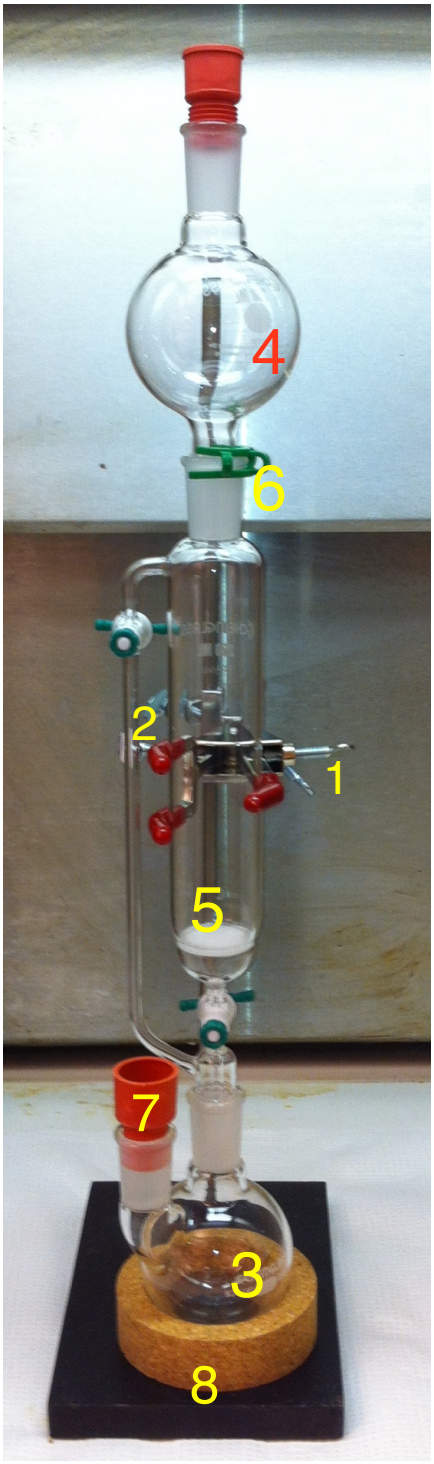
All vials are incubated on a shaking platform at relatively low speed. Protect from light. After clearing we image the same day. So when you plan make sure you can do all your imaging the same day.

All items are purchased from **Chemglass**

You need a **stand** for setting everything up under a chemical hood

Bellow is the ideal setup and how a chemist will do it.

In my experience you don't need 3, 4, 6, 7 and 8. For 3 you can just use bottle with wide opening. You just need a funnel on the top for pouring reagents into the column.



1) Clamp three prong extension # CG-9205-06 (https://chemglass.com/clamps-three-prong-extension?AspxAutoDetectCookieSupport=1)

2) Clamp holder # CG-9252-01 (https://chemglass.com/clamp-holders-large?sku=CG-9252-01)

3) Flask heavy wall round bottom, 250ml 2 neck RBF # CG-1518-03 (https://chemglass.com/flasks-heavy-wall-round-bottom-2-necks?sku=CG-1518-03)

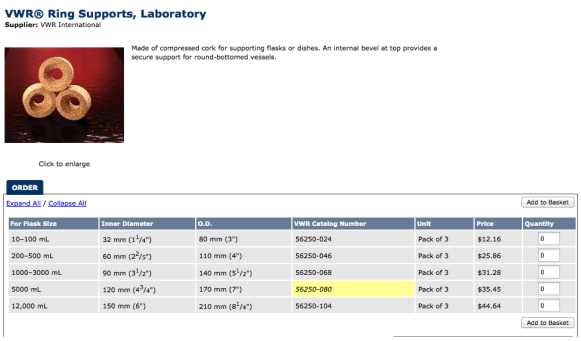
4) RESERVOIR # CG-1190-11 (https://chemglass.com/reserviors-chromatography-standard-taper-joints?sku=CG-1190-110

5) FUNNELS, ADDITION, GRADUATED, PTFE STOPCOCKS # CG-1734-03 (https://chemglass.com/funnels-separatory-graduated-ptfe-stopcocks-1)
For the funnel you need to ask for a quote of a FRITTED FUNNEL 24/40 lower inner 250 ml
MAKE SURE YOU ASK FOR THE FRITTED QUOTE.

6) Clamp, Keck #CG-145-05 (https://chemglass.com/clamps-keck-standard-taper-joints?sku=CG-145-05)

7) Septum stopper CG-3024-05 (https://chemglass.com/septum-stoppers-suba-seal-septa?sku=CG-3024-05**)**

8) Ring Support for the round wall flask.
This is from VWR. #56250-04



VWR® Ring Supports, Laboratory

Supplier: VWR International

Made of compressed cork for supporting flasks or dishes. An internal bevel at top provides a secure support for round-bottomed vessels.

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For Flask Size	Inner Diameter	O.D.	VWR Catalog Number	Unit	Price	Quantity
10–100 mL	32 mm (1 1/4")	80 mm (3")	56250-024	Pack of 3	$12.16	0
200–500 mL	60 mm (2 2/3")	110 mm (4")	56250-046	Pack of 3	$25.86	0
1000–3000 mL	90 mm (3 1/2")	140 mm (5 1/2")	56250-068	Pack of 3	$31.28	0
5000 mL	120 mm (4 7/4")	170 mm (7")	*56250-080*	Pack of 3	$35.45	0
12,000 mL	150 mm (6")	210 mm (8 1/4")	56250-104	Pack of 3	$44.64	0

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