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Sample Preparation for Serial 2-Photon Tomography

May 9, 2017

The following is the protocol for embedding a sample in agar in preparation for serial two-photon tomography. This follows the methods of Ragan, *et al.*, 2011. The protocol describes preparation of solutions, embedding the sample in agar, covalently bonding the sample to the agar, and preparation for imaging of the sample. The purpose of the covalent bonding is to cause the agar to adhere to the surface of the sample via a redox reaction between $NaBH_4$ and $NaIO_4$. This results in improved sectioning quality and makes it more likely that sectioned slices remain attached to the agar sheet after removal from the block.

1 MATERIAL LIST

1. Agarose (Sigma, Type 1, #A6013 or #A0169).
2. $NaIO_4$ (Sigma, #S1878, MW 213.89 g/mol); light-sensitive, hygroscopic, toxic.
3. 100 mM Phosphate buffer (4.8g/L Monobasic sodium phosphate monohydrate, 17.2g/L Dibasic sodium phosphate heptahydrate[1]. You can also make up the solution in a 2 L flask and work at a concentration of 50 mM (we usually do this). It should come to about pH= 6.8. Keep at room temperature until needed. Refrigerated PB will out-gas as it warms and bubbles may form on the objective.
4. Borax ($Na_2B_4O_7$ * $10H_2O$; CAS: 1303-96-4, MW 381.37 g/mol); 0.05 M solution = 19 g/L.
5. Boric Acid (H_3BO_3 , Sigma #B6768, MW 61.83 g/mol); 0.05 M = 3 g/L.
6. $NaBH_4$ (Sigma #452882, MW 37.83 g/mol); toxic, use in chemical hood.

[1] Values for anhydrous salts are 4.2 g and 9.2 g respectively.

7. Stir plate/stir bars.
8. Vacuum suction with sub-micron filters.
9. Aluminum foil or box to protect solutions from light.
10. Large forceps for holding the sample.
11. Neodymium block magnets for adhering the slide to the steel plate in water bath. We use either 20 mm x 10 mm x 2 mm (Q-20-10-02-N) or 22 mm x 8.5 mm x 1.4 mm (Q-22-8.5-1.4-SHN) from www.supermagnete.ch These magnets have a pull force of about 2.1 and 1.3 kg respectively. Don't go stronger than this: it can pull the blade downwards towards the end of the run if you get close to the magnet.
12. Glass microscope slides with coarse writing surface on one end
13. Epoxy (5 minute Araldite or 5 minute Henkel 1365868).
14. Superglue (water resistant, LOCTITE 1647358 and LOCTITE 454 both work).

2 MAKE OXIDISED AGAROSE

Make (4.5%) agarose solution in 10 mM $NaIO_4$, by mixing the following in a 250 mL beaker. You will not melt the agar during this stage. The purpose of this stage is to generate oxidised agarose that will later undergo a redox reaction with the surface of the sample.

- 2.25 g agarose of the type specified above (these are known polymerize after priming).
- 0.21 g $NaIO_4$.
- 100 ml of PB.
- Gently stir for 2-3 hours at RT, protect from light. If go over three hours the agarose may polymerize poorly and become brittle.
- Filter the solution with vacuum suction and a sub-micron filter.
- Wash out all remaining $NaIO_4$ with PB. We use three or four washes x 50 mL per wash and wait for all the PB to pass through the filter before applying the next wash.
- Remove the filter from suction, then re-suspend the agarose in 50 mL of PB.
- Store the oxidized agarose in the fridge in a light-protected container for up to about two or three weeks.

3 PREPARE BORATE/BOROHYDRIDE SOLUTION

First create a stock of borate buffer by adding 19 g borax and 3 g boric acid to 1 liter of water. Stir until dissolved then adjust the pHto 9.0–9.5 with 1 M NaOH. You can keep the solution indefinitely at room temperature and use it for the following steps. To make the borate/borohydride solution you will:

- Heat 100 ml of borate buffer to 40°C.
- Add 0.2 g $NaBH_4$ in fume hood. CO_2 will be released.
- Stir for 15–30 min and protect the bottle from light.
- Leave the bottle in the hood overnight with the cap over the bottle but not tightened due to gas formation. Tighten cap next morning. Avoid using the solution the same day as it is made as gas pockets can form inside the agarose, creating a spongy structure that sections poorly.
- The borate/borohydride solution can be stored up to 1 week, but fresher solution will be better for bonding the sample to the agar.

4 EMBED THE SAMPLE IN AGAR

The sample will now be embedded in an agar block to allow it to be cut. There are various ways of conducting this process. We employ a reusable metal mould and support the sample in the middle of it using two thin retractable metal supports. We do this because we work with brains and this makes it easy to maintain consistent positioning of the sample across experiments. Our procedure is as follows:

- Maintain the sample in 50 mM PB (or 100 mM, if you prefer) for 24 hours in order for it to equilibrate with the osmolarity of the cutting medium. This must be done before embedding.
- Remove the fixed sample from the PB and rest on a folded KimWipe, gently drying the sample.
- Now might be a good time to remove any external membranes still present on the sample. The embedding will be more effective if the agar bonds directly to the sample rather than surrounding sheath tissue. Membranous sheath often fails to be cut by the blade and ends up between the objective and the sample surface, causing pronounced imaging artifacts.
- Support the ventral brain on two metal supports. Place metal mould around the brain. (PHOTOS TO COME)
- Shake the oxidised agarose suspension and pour out about 10 mL into a small beaker.

- Heat agarose in a microwave until it boils. This usually occurs quickly. You can stir with a thermometer: about 80°C is normally sufficient.
- Use a thermomemter to monitor the temperature of the agarose. When the agarose reaches about 60°C, slowly fill the embedding mould. Fill steadily and keep a support, such as forceps, above the sample to stop it from floating away from the supports.
- Wait for the agar to set.
- If necessary, you can trim away agar with a razor blade to ensure that the blade will be parallel to the coronal plane during sectioning. Note that for obtaining coronal sections from brains it works best to cut from cerebellum to olfactory bulb.
- In principle the embedded brain can be stored in PB at 4°C for some time, however we generally use embedded brains immediately.

5 Bonding of the sample to the agar

In this step we perform the redox reaction to covalently bind the oxidised agar block to the surface of the sample. If the following procedure is successful, the agarose-tissue sections will curl as they are sliced.

- Place the embedded brain in a 50 mL beaker.
- Cover the sample with the borohydride/borate solution.
- Leave in solution **either** overnight at 4°C **or** at room temperature for 2–3 hours. You should not shake the sample during this time.
- Once bonded, treat the sample gently.

6 Mount block on imaging slides

Making a magnetic slide

- Thoroughly mix the two-part epoxy on a weighing boat. The epoxy used should be water resistant (not all epoxy is). Use 5 minute epoxy. Waiting hours for powerful magnets to glue to slides is annoying.
- The sample will eventually be glued to the rough writing surface. You will glue the magnet to the smooth surface.
- Using a plastic tool, apply epoxy to the reverse side of the writing surface.
- Place the magnet onto the epoxy and press down gently.

- We generally make several slides at once, but the magnets are very powerful so the slides should be prepared a few cm away from each other to make sure the magnets don't drift as the epoxy dries.
- Despite using 5 minute epoxy, you should ideally wait at least an hour (of not overnight) for the glue to thoroughly dry. This decreases the likelihood of the epoxy softening and coming away during the long imaging procedure.

Attaching the agarose block to the slide

- Remove the block from the borohydride solution, and rinse with PB to remove excess solution on the sides of the block.
- Dry the block with a KimWipe or similar.
- Apply a liberal drop of superglue to the writing (rough) surface of the magnetic slide, and place the block on the drop. *Caution:* avoid the super-glue spilling over the edge of the block and spreading up the side. If the blade hits superglue it will fail to cut and likely push the sample off the slide.
- Allow the superglue to dry for 5 to 10 minutes.
- Do not leave the block out of solution for more than about an hour. The agarose is mostly water so will evaporate and shrink.

Setting up the sample for imaging

- Place the slide on the metal block at the bottom of the imaging tray. Do this carefully with two hands, one hand on either side of the slide. The the magnets are strong enough to snap the slide hard into position and snap it in the process.
- Once at the microscope, fill the sectioning tray with PB. The correct quantity of fluid is when the water level reaches the mid-point of the short upper section of the original cutting bath that came wit the system. i.e. to within about 1 cm of the top of the bath. If the tray is not filled sufficiently you will be unable to see the position of the laser beam on the sample surface from the left hand face of the sample bath. This is necessary to set up the acquisition.
- Remember to use PB stored at room temperature.
- Lower microscope z-stage and carefully place the filled tray in the center of the holder. If securing the bath in place with a thumb-screw then tighten until you just start to feel mild resistance. Don't over-tighten.
- Be careful about re-using slides. Water has a tendency to break down the glue and so it can fail on later re-use even if the slide has been allowed time to dry.