Lugol's Iodine Staining Protocol

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Background

Lugol's iodine solution renders soft-tissues radiopaque under CT imaging. It achieves this through diffusion of iodine into soft-tissue. Because different types of soft-tissues (neurons, muscles, vessels) incorporate iodine at different rates, this staining results in contrast among structures when CT scanned. This technique, called diffusible-iodine contrast-enhanced (dice)CT, has become a powerful method to image and reconstruct biological structures. One caveat to iodine staining is that it may lead to severe tissue shrinkage, especially for embryos, which can be mitigated using the STABILITY protocol.

Key References

Gignac PM, et al. 2016. Diffusible iodine-based contrast-enhanced computed tomography (diceCT): an emerging tool for rapid, high-resolution, 3-D imaging of metazoan soft tissues. *J Anat* 228: 889–909.

Gignac PM, Kley NJ. 2014. Iodine-enhanced micro-CT imaging: methodological refinements for the study of the soft-tissue anatomy of post-embryonic vertebrates. *J Exp Zool B Mol Dev Evol* 322: 166–176.

Metcher BD. 2009. MicroCT for comparative morphology: simple staining methods allow high-contrast 3D imaging of diverse non-mineralized animal tissues. *BMC Physiol* 9: 1–14.

Metcher BD. MicroCT for developmental biology: a versatile tool for high-contrast 3D imaging at histological resolutions. *Dev Dyn* 238: 632–640.

Wong MD, Spring S, Henkelman RM. 2013. Structural stabilization of tissue for embryo phenotyping using micro-CT with iodine staining. *PLoS ONE* 8(12): e84321.

Warnings

- Keep any compound used in iodine staining away from ammonium-based substance. They are highly reactive and explosive.
- Lugol's iodine solution is highly corrosive. Do NOT dispose it down the sink. Neutralize the solution (see below) and then dispose it in proper laboratory waste containers.

Specimens

- Specimens should be freshly fixed in 10% neutral-buffered formalin for at least 2 weeks but 2 months if possible (Weisbecker 2012 *Brain Struct Fuct*). Because iodine binds to lipids, which are soluble in alcohol, iodine staining is less effective for ethanol-fixed specimens.
- If staining embryos, then use of hydrogel-based STABILITY protocol (Wong et al. 2012) is highly recommended to minimize tissue shrinkage artifact. Two-day submersion in 20% w/v sucrose solution will also reduce shrinkage artifact (Morhardt & Witmer, abstract).

- Record any morphometric data prior to staining because the staining will alter the size and weight of the specimen.

Sodium Thiosulfate Solution

- Materials needed: sodium thiosulfate (solid), de-ionized water, jug, scale, liquid measure
- Before making Lugol's iodine solution, it is a good idea to first prepare its neutralizing solution in case of accidental spills.
- To make 10% w/v sodium thiosulfate solution, mix 200g solid sodium thiosulfate with 1882 mL de-ionized water in a large jug. Keep it in the container at room temperature until needed.
- In case of a spill, wipe with paper towels soaked in sodium thiosulfate. The iodine solution should become clear, which is a visual cue that it has been neutralized.

Lugol's Iodine Solution

Materials Needed: iodine (solid), potassium iodide (solid), de-ionized water, scale, liquid measure, shaker, sealable box.

1. Determine how much iodine (I_2), potassium iodide (KI), and water is needed. Consult PI for concentration and duration of iodine stain. Useful references are Gignac et al. 2016 and Watanabe et al. 2019. If not pressed for time, it is generally better to use lower concentration over longer duration of staining to reduce any shrinkage artifact.

   To calculate amount for **100mL** solution:

   Sum of I_2 and KI weights should be the concentration, where amount of KI is twice that of I_2. For 7.5% solution, you would need 2.5g I_2 and 5.0g KI. Likewise, for 3% solution, you would need 1.0g I_2 and 2.0g KI. To determine the amount of water for 7.5% solution:

   (2.5g I_2 / 4.9335 g/mL) + (5.0g KI / 3.123 g/mL) + X mL H_2O = 100 mL
   0.506 mL I_2 + 1.6 mL KI + X mL H_2O = 100 mL
   X mL H_2O = 97.894

   Therefore, you will need 2.5g I_2, 5.0g KI, 97.9 mL H_2O to make 100mL of 7.5% Lugol's iodine solution.

2. Weigh iodine crystals into one small container and potassium iodide powder into a separate small container.
3. Measure DiH2O into a graduated cylinder.
4. Using a funnel, add chemicals to mixing container (jug with cap), then add water, rinsing out chemical containers and adding them to the bottle.
5. Cover with cap tightly and shake vigorously until chemicals are dissolved (at least few minutes).
6. Add specimen to appropriate container and cover with iodine solution. Use either an amber container or place into a carboard box as iodine solution is light sensitive. Place on shaker and leave for determined duration. Staining time will depend on sample.

7. To clean containers, use 10% sodium thiosulfate and dump into chemical waste container – do not put down sink! After neutralizing, thoroughly rinse containers with water to ensure that sodium thiosulfate is washed off for future use.

CT Imaging

- When specimen is ready to be scanned, transfer the specimen from the container and use a vacuum sealer to package the specimen. Remove excess iodine solution as much as possible to prevent imaging artifacts. This package can be placed in a container for the scanner. For best results, prevent the stained specimen from coming into contact with light (e.g., could use polystyrene cup and lid to place and secure the specimen).
- Package the specimen securely for CT at least 2 hours prior to scanning so that the specimen has time to settle into position.
- Upon CT imaging, if soft-tissue contrast is inadequate, then consult with PI to determine the duration and concentration of additional staining.

De-Staining Specimens

- Submerge specimen in 1% sodium thiosulfate solution and place on rocker. Check daily and replace with fresh 1% sodium thiosulfate solution every day until the specimen is back to its natural color. Use of higher concentration of sodium thiosulfate will lead to build up of precipitate on the specimens.
- After de-staining is complete, submerge specimen in 10% neutral buffered formalin for a day on the rocker. Then, dispose the formalin and replace with fresh formalin solution.

Hydrogel STABILITY

This protocol is largely based on steps developed by Vera Weisbecker's lab.

To make 400mL hydrogel solution (the solution can be kept in -20 freezer indefinitely):

- 350mL ice-cold 4% PFA
- 40mL 40% w/v acrylamide solution
- 10mL 2% w/v bis-acrylamide solution
- 1.0g VA-044 initiator
- 0.2g saponin (Note: the saponin apparently helps the hydrogel solution to spread throughout the specimen. It does create bubbles in the hydrogel (you will notice them after polymerisation but apparently these bubbles don't occur inside the specimen – only outside (according to the CLARITY wiki and forum).

Step 1: Mix the solution
I mixed the above ingredients together, keeping them on ice at all times to prevent any early polymerisation.

Step 2: Immerse specimens in hydrogel solution.

Because my specimens were all previously fixed with formalin, I left them in the hydrogel solution for one and a half to two weeks (typically, 3 days is good for embryos). This is because the hydrogel binds to certain sites (amine groups) on biomolecules within the specimen, and many of these sites will have been used up by the original fixing of the specimen. The prolonged incubation in hydrogel solution allows the solution to 'find' and bind to any unused sites. This will ensure an even spread of hydrogel monomers throughout your specimen. The hydrogel solution must be kept cold at all times (doesn't have to be ice-cold – in a fridge at 4 degrees is fine). So basically, I put my specimens in hydrogel solution for about 2 weeks in the fridge. If you are using fresh specimens, the 3 day immersion (according to Wong et al) in hydrogel solution should be fine.

Note: best to keep the amount of hydrogel for each specimen constant. This way, the degree of polymerization will be the same given the same temperature and duration. Larger volumes will take longer time to polymerize.

Step 3: Prevent air from getting to surface of gel (just before gel polymerisation).
The original protocols say (as you know) to use a desiccation chamber to replace any air in your container (which contains your specimen in the hydrogel solution) with nitrogen. I actually used oil instead, and found that it worked just fine! Basically, just before putting the specimens in hydrogel solution into the hot water bath for polymerisation, I carefully pipetted a fairly thick layer of oil (vegetable, canola, or peanut – they all work fine) onto the surface of the hydrogel. However, if you already have the setup for the desiccation, you might just want to go with that!

Step 4: Polymerisation of gel
I put the containers (specimen in hydrogel solution + oil layer) in a hot water bath at 37 degrees for three hours. Depending on the volume of the hydrogel and desired diffusibility of subsequent staining (e.g., Lugol's iodine), may want to lessen to 2.5 hours. As long as the hydrogel does not flow when turned upside down (although do it slowly because oil), then it's polymerized enough.

Step 5: Removal of excess gel
I found removing the gel was pretty easy (although a little harder for specimens that had hair/fur). I just used dry gloves and Kim wipes (working in a fume hood), and most of the gel just peeled away from the specimen.

Step 6: Iodine staining
After removing the excess gel I put my specimens straight into iodine solution. I found that staining times were a little longer than for specimens without hydrogel stabilisation. My specimens were between 0.8cm – 5.5cm in length.

NOTE: you might notice after the iodine staining that your specimen has a strange texture or lumps of strange texture/discoloration to it – don't worry! This is just some excess gel that hasn't been removed on the outside of the specimen. It can be carefully scraped off if need.
NOTE: may want to stain in a box in a fridge to prevent further polymerization of hydrogel.

Step 7. Agarose gel for physical security of specimen during scanning
- 1% agarose solution
- For 50mL solution, mix 0.5g agarose with 50mL water (TBE buffer).
- Microwave for 1 minute.
- Swirl, then microwave again for 1 minute.

- Swirl, and make sure that agarose is fully dissolved (no clear flakes).
- Pour some in container to build a “platform” the specimens and allow gel to solidify for 20-25 minutes.
- Repeat to submerge the specimen in agarose.