**Evans blue (EB) perfusion and tissue clearing protocol**

1. Induction of Anesthesia

Note: Tail vein injection can be utilized as an alternative EB administration route and does not require anesthesia. However, precise injection often requires significant training and practice to master. While our method requires anesthesia, it may be easier for users to perform injections successfully.

1. Prior to starting, prepare a solution of EB (Sigma, E2129). EB is mixed at 2% w/v in 0.9% NaCl. Filter with a 0.22mm filter to remove any particulates. A larger stock can be prepared and stored at room temp for use over several months.

2. Weigh the mouse. This will be needed to determine the amount of EB to inject.

3. Place animal in induction chamber and seal/close top.

4. Anesthetize the mouse with isoflurane vaporizer set to 5% in 100% oxygen, ~5 min.

5. Ensure that animal has reached a surgical plane of anesthesia via toe pinch.

6. Switch system flow to nosecone. Remove animal from chamber and position in the nosecone.

7. Adjust gas flow with vaporizer to 2-3% and flush air. If animal has started responding, gently restrain in nosecone until fully anesthetized. Perform toe pinch again prior to injection.

1. Injection of Evans blue (EB)

8. Draw up the appropriate volume of EB into a 1ml syringe capped with a 27-31g needle. A range of 1-6ml/g body weight is injected, with 3ml/g being optimal for adult mice (i.e. for 3 ml/g, a 25g mouse gets 75ml of EB). Expel any air bubbles.

9. Wipe the surgical area with 70% ethanol.

10. Carefully cut up the midline of the animal until reaching the bottom of the rib cage. Make a “T” at the top of the incision and gently lay skin over to the side, exposing the diaphragm.

11. There are two options for left ventricle (LV) injection:

i) Using forceps, grab the sternum and gently pull towards the head of the animal, pressing the heart against the diaphragm until easily visible. Insert the needle through the diaphragm into the LV and inject EB.

ii) If the above method proves difficult, make a small incision in the diaphragm avoiding cutting or nicking the heart or any pleural cavity organs. If you cut the heart or any large blood vessels the procedure will not work. Open up the diaphragm until the heart is exposed. Insert needle into the left ventricle and inject EB.

12. Wait approximately 5 minutes to allow the EB to circulate. The snout, paws, and tail will turn blue, indicating successful administration. Exposed organs will also turn blue.

13. Decapitate the animal with scissors while still under anesthesia to euthanize and proceed to tissue harvest.

15. Fix samples in 4% PFA (usually O/N at 4C degree for most whole organs) and process the tissue for imaging. This will vary for the tissue collected and the preferred imaging method.

C. For whole tissue clearing with modified iDISCO

16. After appropriate tissue fixation, wash in 1X phosphate buffered saline (PBS) with shaking at room temp for 3 x 30 minutes.

17. Dehydrate with methanol/H2O series: 20%, 40%, 60%, 80%, 100%; 1 hour each.

18. Wash further with 100% methanol for 1 hour

19. Incubate for 3 hours, with shaking, in 66% DCM (Dichloromethane, Sigma 270997) / 33% methanol at room temp

20. Incubate in 100% DCM 2 x 15 minutes with shaking.

21. Incubate in 100% DBE (Dibenzyl ether, Sigma 108014) until tissue is clear, no shaking necessary. The tube should be filled almost completely with DBE to prevent the air from oxidizing the sample.

 22. Proceed with imaging