Table S1 − Clearing and staining protocol modified from C.S. Rose (James Madison University, 1999). Original protocol modified from D. Cannatella, L. Trueb (unpubl.) and G. Dingerkus and L. Uhler, 1977, Journal of Stain Technology 52:229-232

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Ethanol Gradation

1. Fix specimen in 10% neutral buffered formalin (2 days minimum)
2. Rinse in distilled or reverse-osmosis (RO) water (H2O) three times for 5-10 minutes each
3. Place specimen in 75% ethanol (EtOH) for 2-12 hours
4. Eviscerate specimen, making sure to remove liver and eyes; if specimen is too small, make a sagittal incision into the body cavity and perforate eyes
5. Place in 90% EtOH for 2-12 hours
6. Place in 95% EtOH for 2-12 hours
7. Place in 100% EtOH for 2-12 hours or repeat step 5

\*\* Do not reuse EtOH ~ all but 100% can be reused once if necessary \*\*

Alcian Blue Treatment

Recipe for 100 mL of solution: 30+ mg Alcian blue + 80 mL 95% EtOH + 20 mL glacial acetic acid; stir supersaturated solution for up to an hour; pour solution through a coffee filter 1-3 times to remove un-dissolved Alcian grains.

\*\* Alcian solution will permanently stain glassware \*\*

\*\* Solution can be reused if pH is ≤ 3 \*\*

1. Place specimen in Alcian blue solution for 24-36 hours depending on size. A dark

blue film should be apparent on the specimens.

1. Place in 100% EtOH for 1-2 hours or do step 9 twice
2. Place in 95% EtOH for 1-2 hours
3. Place in 90% EtOH for 1-2 hours
4. Place in 75% EtOH for 1-2 hours
5. Place in distilled or RO H2O for 1-2 hours

Trypsin Treatment

Recipe for 100 mL of solution: 1 g trypsin powder + 30 mL saturated sodium borate (=Borax) + 70 mL distilled or RO H2O \*\* Mix well\*\*

1. Place specimen in trypsin solution for 12-24 hours depending on size

\*\* Discard used trypsin solution after use \*\*

\*\* Repeat trypsin step with fresh solution if specimens are not limp or if tissues are not largely transparent after 24 hours \*\*

Bleach Treatment

Recipe for 100 mL of solution: 100 mL 0.5% potassium hydroxide (KOH) + 3 drops 3% hydrogen peroxide (H2O2)

1. Place specimen in bleach solution for 12-24 hours depending on size

\*\* Discard after use \*\*

\*\* Stop treatment if bubbles form in the tissues and switch to a lower concentration \*\*

\*\* If tissues are still not transparent or pigment remains after 24 hours, add 1-3 more drops H2O2 to solution and allow to sit for another 12-24 hours \*\*

Alizarin Treatment

Recipe for 100 mL of solution: 100 mL 0.5% KOH + miniscule amount of Alizarin grains to make solution dark, but still transparent purple

\*\* Swirl solution; let sit for 10 minutes at a time before adding more Alizarin \*\*

1. Place specimen in Alizarin solution for 12-24 hours depending on size

\*\* Discard after use \*\*

Glycerin Gradations and Storage

Recipe for 100 mL of solution: 1) [3:1] 75 mL 0.5% KOH + 25 mL glycerin, 2)

[1:1] 50 mL KOH + 50 mL glycerin (=glycerol), 3) [1:3] 25 mL KOH + 75 mL glycerin

1. Place specimen in 3:1 solution for 12-24 hours
2. Place in 1:1 solution for 12-24 hours
3. Place in 1:3 solution for 12-24 hours

\*\*Solutions can be reused once \*\*

1. Place specimen in 100% glycerin + grains of thymol (preservative)

END

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\*\* For most steps (except Alizarin staining) you can go longer by 12-24 hours, but check specimen frequently \*\*