# A Whole-Mount Method for Trypsin Clearing and Collagen Type II Antibody Staining

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# A Whole-Mount Method for Trypsin Clearing and Collagen Type II Antibody Staining

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**Although cartilage and bone are often stained to study development in fishes, collagen forms before either of these tissues during skeletal ontogeny. We describe a new method that combines conventional trypsin clearing of whole-mount specimens with staining of Collagen Type II using antibodies. Specimens were fixed briefly in paraformaldehyde, digested in a trypsin solution, bleached with hydrogen peroxide, permeabilized with Proteinase K, antigen labeled with primary and secondary antibodies, and stored in glycerol. This method makes both cellular and acellular collagen visible under ultraviolet light with limited background staining. Specimens showed no signs of damage from any of the solutions used for staining, but the length of time for fixation appears to be important. This method permits visualization of collagen condensation prior to cartilage formation in endochondral bone and can be used to study evolution of skeletal ontogeny and to develop new skeletal characters for phylogenetic analysis.**

CLEARING and staining of bone with Alizarin Red S is an important method to study skeletal differences and development among taxa of vertebrates. In 1967, Taylor introduced the use of trypsin to digest most proteins, rendering specimens transparent in glycerol to observe the skeleton (see also Taylor and Van Dyke, 1985). Dingerkus and Uhler (1977) added use of Alcian Blue to produce specimens that are cleared and double stained for cartilage (blue) and bone (red), and Potthoff (1984) summarized the protocol to clear and double stain larval fishes. Unfortunately, conventional Alcian staining employs an acidic solution that may dissolve bone, but Walker and Kimmel (2007) developed a non-acid staining method to stain cartilage with Alcian. Later, Filipski and Wilson (1984) presented a simple method using Sudan Black B to stain nerves in cleared fishes, and Song and Parenti (1995) demonstrated triple Alcian, Alizarin, and Sudan staining for bone, cartilage, and nerves.

Antibody staining is an important method to visualize the products of genes expressed during development and has been used to investigate a wide range of problems. For example, proteins that control limb development have been stained to acquire characters to study evolutionary relationships (Panganiban et al., 1995), and antibody staining has been used to study the function of the genes that encode specific proteins (Diederich et al., 1991). The methods for antibody staining are well established (Ramos-Vara, 2005) and can be used to stain archival formalin-fixed samples (Shi et al., 2011). Thus, antibody staining methods can add important tools for use in ichthyology and herpetology. In this paper, we describe a method to stain Collagen Type II (Col II) in cleared whole-mount fish using antibodies. Col II is an extracellular protein that is involved in endochondral bone formation, and its expression should precede the formation of cartilage during bone development (Hall and Miyake, 1995). Established methods have produced excellent staining of cartilage through purely chemical means (Walker and Kimmel, 2007). However, earlier visualization of the formation of precursors to endochondral elements of the skeleton before cartilage formation will advance our understanding of ontogeny and provide additional characters that are expressed earlier in development than cartilage formation. As part of an ongoing study of skeletal ontogeny in Threespine Stickleback (*Gasterosteus aculeatus*), we developed a method, which we describe here, to clear specimens with trypsin and stain them using an antibody to Col II.

## MATERIALS AND METHODS

***Sample acquisition.***—Gravid females and sexually mature males of Threespine Stickleback were collected from Rabbit Slough (61°32.155′N, 149°15.178′W), Matanuska-Susitna Borough, Alaska on 12 June 2011. This population is anadromous and has a robust skeleton, including a continuous row of ~33 bony lateral armor plates per side, which characterizes anadromous populations of *G. aculeatus* (e.g., Bell, 1981; Aguirre et al., 2008).

Parents were transported in aerated coolers of ambient water to the University of Alaska Anchorage. One mass cross, using nine males and nine females, was performed within 24 h of capture using Hagen's (1967, 1973) *in vitro* method. The parents were euthanized with MS 222 (Tricane), and sperm was obtained by dissecting the testes out of males and chopping them finely with a razor blade in a few drops of diluted (3.5 ppt) artificial sea water (''fish water''). Ova were squeezed from females into a few drops of fish water in a Petri dish. Sperm and ova were mixed separately, and the suspended, mixed sperm was spread over the ova with a pipette, taking care to minimize transfer of testicular debris. The mixture of sperm and ova was allowed to sit for 20 min, until the chorions separated from the membranes of the ova, indicating fertilization. Fertilized eggs were washed repeatedly in fish water to remove testicular debris and ovarian mucous.

The fertilized eggs were incubated in Petri dishes at 19°C with fish water changes one or two times per day. At 15.5 h post fertilization (hpf), the cohering eggs were separated from each other by gently forcing forceps between them and treated with methylene blue (1–2 drops/3.8 L) and furan-2 (2.24 mg/L) for 5 h. At 69.5 hpf, eggs were transferred to 50 ml tubes (~150 eggs/tube) and cooled on ice to 5°C. After aerating the chilled fish water, the tubes were nearly filled, leaving ~1 ml of air, and sealed for transport to Stony Brook University. Upon arrival, the eggs were gradually warmed,

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**Table 1.** Cleared Whole-Mount Antibody Stain Procedure. See text for further details.

| Step | Solution | Time |
|---|---|---|
| 1 | Remove visceral organs | |
| 2 | Trypsin (0.1% trypsin in 30% saturated sodium borate solution) | 12 h |
| 3 | Potassium hydroxide 1% solution | 2 × 5 min |
| 4 | Bleach-fresh (15% hydrogen peroxide:85% potassium hydroxide solution) | 3–12 h |
| 5 | Potassium hydroxide 1% solution | 2 × 5 min |
| 6 | Proteinase K (10 μg/ml) | 1.3 min/mm SL |
| 7 | PBT (Phosphate buffered saline + 0.1% Tween 20) | 10 min |
| 8 | Blocking solution (5% normal rabbit serum in PBT) | 1 h |
| 9 | PBT | Quick wash |
| 10 | Primary antibody (1:100 dilution in PBT) | 10 h |
| 11 | PBT | 2 × 10 min |
| 12 | Blocking solution | 1 h |
| 13 | PBT | Quick wash |
| 14 | Secondary antibody (1:200 dilution in PBT) | 10 h 4°C |
| 15 | PBT | 5 × 5 min |
| 16 | PBT:glycerol series (3:1, 1:1, 1:3) | 2 h ea. |
| 17 | Glycerol 100% | |

returned to Petri dishes with fresh fish water, and kept in a constant-temperature incubator at 19°C until transfer to aquaria, 11 days later. They were reared in aquaria with recirculating, filtered fish water at an average temperature of 18.9°C and 14:10 h (light:dark) photoperiod for the remainder of the experiment. They were fed brine shrimp nauplii starting on the fourth day after hatching, after the yolk sack had been resorbed.

***Staining.***—Specimens were stained as follows (Table 1): Live specimens were anesthetized with MS-222 (Tricane) and fixed at room temperature (23°C) in a pH-neutral, isotonic, phosphate-buffered saline solution (PBS; Sambrook and Russell, 2001) with 4% paraformaldehyde (PFA) for 2 h in a 1.5 ml microfuge tube. They were washed briefly with 100% methanol to remove excess PFA and stored in methanol at −20°C. Storage immediately after fixing maintains specimens in good condition for at least two months. Prior to trypsin digestion, the visceral organs of larger specimens can be removed with micro-dissection scissors or a pointed number 11 scalpel. Specimens were digested in a solution made up of 0.1% trypsin (wt/vol) in 30% saturated aqueous solution of sodium borate (Taylor, 1967) for 12 h. Next, specimens were washed twice with 1% potassium hydroxide (KOH) for five min each. A fresh bleach solution with a 15:85 ratio of 3% $H_2O_2$:1% KOH (Westerfield, 2007) was then added to the microfuge tubes, which were left open at room temperature for 3 to 12 hours or until skin pigments were bleached. The bleaching solution was removed with two washes of 1% KOH for five min each. Cellular membranes were permeabilized by immersion in a solution of Proteinase K (Roche) at 10 μg/ml for 1.3 min per mm standard length (SL; distance from the tip of the upper jaw to the posterior end of the last vertebra). Proteinase K was removed with two washes of PBS with 0.1% Tween 20 (PBT) for 10 min each. Specimens were treated with blocking solution of 5% normal rabbit serum in PBT for 1 h at room temperature. Excess blocking solution was removed with a quick wash of PBT before application of primary Col II antibody (II-II6B3, Developmental Studies Hybridoma Bank; Linsenmayer and Hendrix, 1980) at a 1:100 dilution in PBT, with rocking for 10 h at room temperature. Unbound primary antibody was removed with two washes of PBT for 10 min each. Blocking solution was applied again for 1 h at room temperature. Secondary antibody (DyLight 488-conjugated AffiniPure Rabbit Anti-Mouse IgG [H+L], Jackson ImmunoResearch) was applied using a 1:200 dilution in PBT for 10 h at 4°C. Unbound secondary antibody was removed with five washes of PBT for 5 min each. Specimens were then transferred through a PBT:glycerol series in four steps ending in 100% glycerol for viewing and storage.

Antibody-stained specimens were immersed in glycerol for viewing under UV light from an EXFO X-Cite Series 120 Q with a Zeiss Discovery V20 Stereoscope using a GFP (FITC 535) filter. Photographs were taken using the AxioCam HR and AxioVision software. Alcian–Alizarin double stained specimens were processed according to Walker and Kimmel (2007), immersed in glycerol and photographed under visible light using a Leica S6D stereomicroscope and attached Cannon PowerShot S45 camera with RemoteCapture Software. Adobe Photoshop CS was used to resize photographs. Measurements were taken using NIH ImageJ 1.44p software. SL was used as a basis of comparison instead of age, as previous studies on fish development found it to be a better predictor of developmental stage than age (Parichy et al., 2009). Osteological terminology for Threespine Stickleback is used according to Bowne (1994).

## RESULTS

The fixing, clearing, and staining protocol described above and summarized in Table 1 produces excellent staining for Col II in developing Threespine Stickleback. All major elements of the endoskeleton are shown, including the chondrocranium, splanchnocranium, the shoulder and pelvic girdles, pterygiophores, vertebrae (including the centra, and neural and haemal spines of the caudal region), supraneurals, the hypural plate, and the epurals (Fig. 1A–E). In particular, the pelvic girdle stains with antibodies for Col II at a smaller size (7.496 mm SL) than with Alcian for cartilage

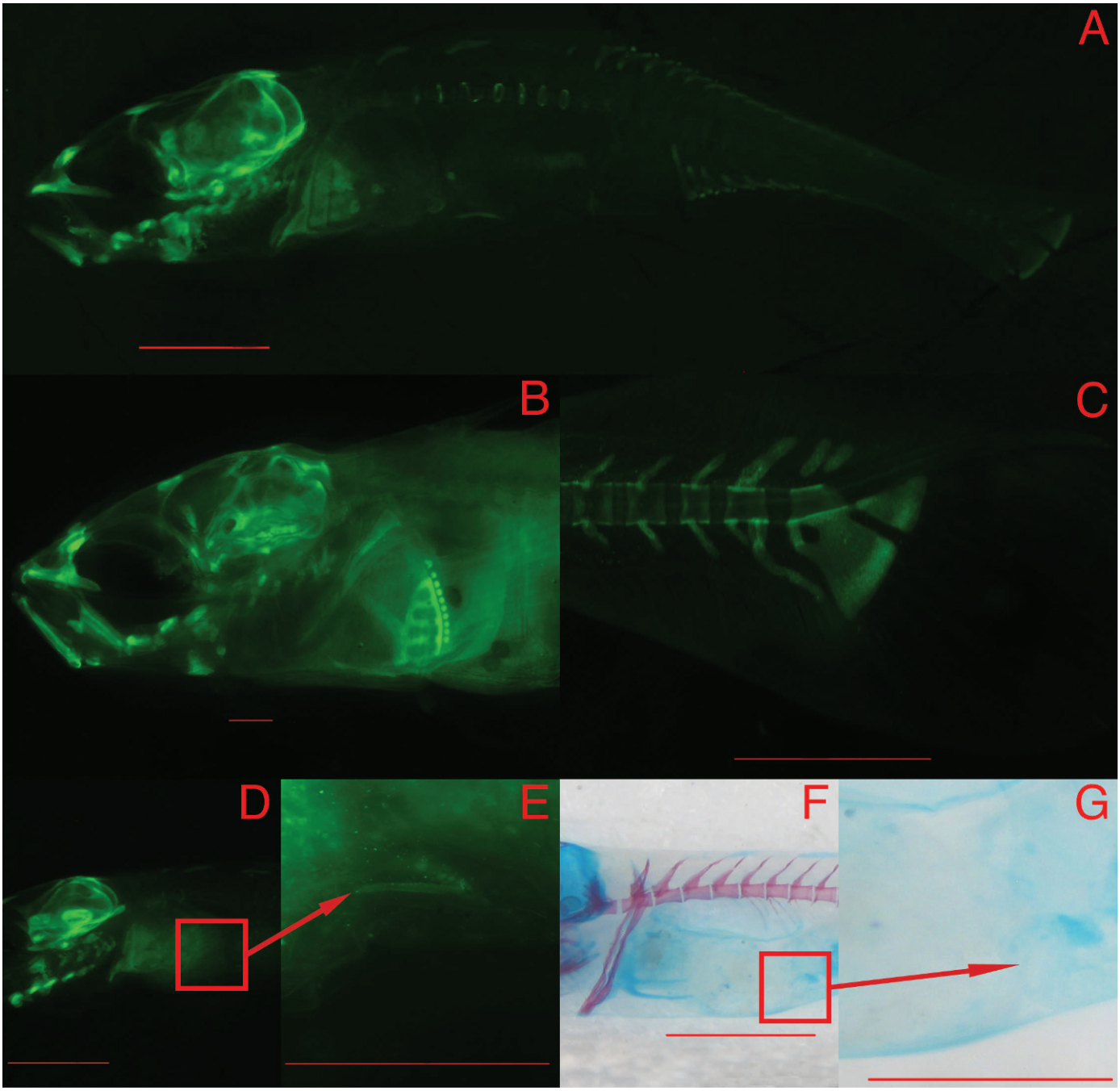


**Fig. 1.** Whole-mount specimens cleared and antibody stained for Collagen Type II or double stained with Alcian and Alizarin. (A) Col II staining throughout the skeleton at 662.5 hours post fertilization (hpf), 8.176 mm standard length (SL). Scale bar: 1 mm. (B) Cranial region showing intense staining in the skull (e.g., lateral ethmoid, suborbitals, dentary) and pectoral skeleton (e.g., posterior to actinosts, proximal ends of fin rays) at 1382.5 hpf, 13.138 mm SL. Scale bar: 0.5 mm. (C) Caudal region showing vertebral centra and neural and haemal spines, epurals, hypurals, and extracellular collagen fibers at 386.5 hpf, 6.649 mm SL. Scale bar: 0.5 mm. (D) Pharyngeal and shoulder regions showing splanchnocranium staining at 554.5 hpf, 7.547 mm SL. Scale bar: 1 mm. (E) Enlargement of area in box in D showing pelvic rudiment (arrow) stained for Col II. Scale bar: 0.5 mm. (F) Shoulder and abdominal region stained with Alcian and Alizarin at 590.5 hpf, 7.58 mm SL. Scale bar: 1 mm. (G) Enlargement of area in box in F showing that pelvis does not stain with Alcian even in an older, larger specimen than in E, in which the pelvis stained for Col II. Scale bar: 0.5 mm.

(7.926 mm SL). Very little background staining occurred in younger fish, but seems to increase in older fish (Fig. 1B). The concentration of secondary antibody (Table 1, step 14) may be reduced or wash times (Table 1, step 15) after its application may be increased to compensate for overstaining, if necessary. In skeletal structures which are made up of more than one type of bone, such as the paired-fin girdles, this method will only stain endoskeletal components.

## DISCUSSION

There are several important distinctions between our protocol and established methods such as those of Walker and Kimmel (2007). First, our method stains for Collagen Type II (Linsenmayer and Hendrix, 1980), while Walker and Kimmel's stains for cartilage, specifically mucopolysaccharides (Scott, 1996). Though both of these extracellular

**Table 2.** Visualization of Pelvic Collagen and Cartilage during Development of Stickleback. SL is standard length, HPF is hours post fertilization, and +/− refers to presence or absence of staining.

| Collagen | | | Cartilage | | |
|---|---|---|---|---|---|
| SL (mm) | HPF | +/− | SL (mm) | HPF | +/− |
| 6.947 | 590.5 | − | 7.164 | 554.5 | − |
| 7.496 | 626.5 | + | 7.58 | 590.5 | − |
| 7.547 | 554.5 | + | 7.662 | 578.5 | − |
| 7.971 | 602.5 | + | 7.719 | 626.5 | − |
| 8.089 | 638.5 | + | 7.926 | 602.5 | + |
| 8.104 | 650.5 | + | 8.151 | 638.5 | + |
| 8.157 | 566.5 | + | 8.32 | 614.5 | + |

proteins are precursors to bone, their appearance need not overlap either spatially or temporally during development. Taking advantage of this difference, our method detects the formation of precursors to endochondral bone earlier than conventional Alcian staining. For example, staining for Col II revealed development of the pelvic girdle earlier than staining for cartilage (Table 2). However, this result may be specific for endochondral bone. Hernandez et al. (2005) assumed overlap in spatial and temporal expression of Col II and cartilage by using the II-II6B3 antibody (Col II antigen) to stain for cartilage associated with mesenchymal bone in the cranial region of Zebrafish (*Danio rerio*).

Some of the solutions used in this protocol may reduce the quality of the final result of the staining process. Therefore we tested each solution in turn to examine its possible effect on the quality of preparations. Fixation time was the most important step in the staining process. Specimens fixed for 12 and 24 h showed greatly increased background staining compared to those fixed for 2 h (data not shown). However, although trypsin is a protease, extended digestion time (24 h) in trypsin did not appear to reduce the quality of preparations for visualization of collagen (data not shown). Increasing the time specimens were in the bleaching solution up to 24 h did not damage the sample, despite warnings by Walker and Kimmel (2007) that it could (data not shown). Finally, we used Proteinase K to increase membrane permeability and penetration of the antibodies. Even though it seems plausible that Proteinase K could damage the Col II antigen, there was no evidence that samples exposed to Proteinase K for 24 hours were damaged (data not shown). Our final concern is that materials in the gut also stain with secondary antibody, and when possible the gut should be removed from specimens before staining.

This method is complementary to Alcian staining and reveals Col II condensation as a precursor to formation of bones earlier than established methods for cartilage staining with Alcian. Although it has been demonstrated here only with Threespine Stickleback, we believe it could be used successfully with other species as well, because similar methods have been used with Zebrafish (*Danio rerio*; Hernandez et al., 2005).

Analysis of the timing and rate of development is important to understand ontogeny. Evolution of the onset, offset, and rate of development (i.e., heterochrony) can provide important insights into the developmental changes responsible for differences in adult morphology (Gould, 1977; Alberch et al., 1979). Since collagen condensation is an earlier process in skeletal ontogeny than chondrification or ossification, this method also could provide insights from earlier events in skeletal ontogeny into deeper phylogenetic relationships.

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