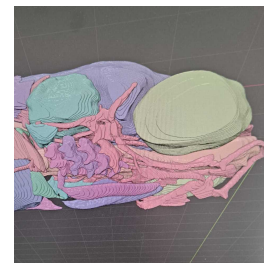


May 21, 2025

Labeling of Zebrafish Organs and Substructures in Light Data in Amira 3D Classic Segmentation Workroom

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We use this protocol and it's working

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Abstract

Intended for use on light microscopy datasets of larval zebrafish ubiquitously expressing soma- and membrane-based fluorescent proteins. Methods are subject to change based on ultimate segmentation goals and expansion into variable light microscopy datasets.

Segmentation Summary

1. Amira Project and Segmentation Setup
2. Mask Segmentation
3. Overview of Class Types
4. Glossary and Segmentation Techniques

See protocol in References for general Amira segmentation tips.

Materials

Amira 3D 2022.2

Amira Project and Segmentation Setup

1

After opening a new Amira project, select **File > Open Data**, or click+drag the raw data tiff to the empty space under the **Project** tab. Confirm coordinates and voxel size in the **Image Read Parameters** window.

For higher label resolution: Select the arrow of the raw data object in the **Project** tab. In the window search bar, type 'resample'. Select the **Resample** object. With the **Resample** object selected, adjust **Voxel Size:** under **Properties** to half of the original resolution value. Select **Apply**, and select the newly generated raw data object as the new working raw data object.

Select the **Segmentation** tab to automatically generate a label field. Confirm **Image:** dropdown under **Segmentation Editor** is the raw data. Under **Materials**, ensure the **Exterior (Not Assigned)** material remains unlocked. The existing **Inside** material may be renamed and used as the first cell segmentation material.

Mask Segmentation

2

Before segmenting organ classes, creating a mask of the zebrafish sample will aid in determining organ segmentation boundaries and later mesh visualization.

2.1

Under the Segmentation tab, adjust the 2D data contrast range to the data histogram by selecting the 'Data Histogram' option in the Display Control window (Figure 1). This will present the general shape of the sample, which can be roughly segmented in XY, interpolated through the Z plane, and globally smoothed (Figure 2). The final fish mask should include all signal in the data (Figure 3).

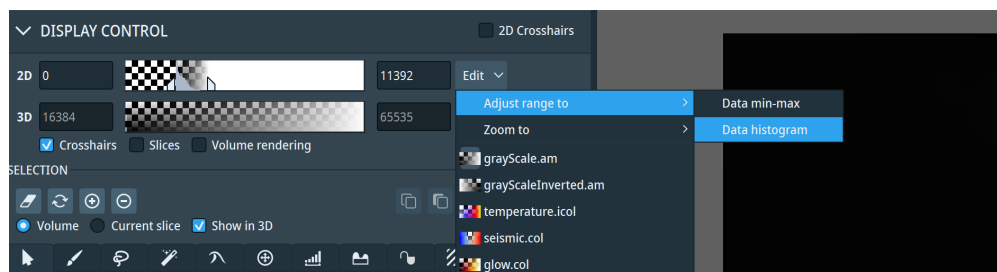


Figure 1.

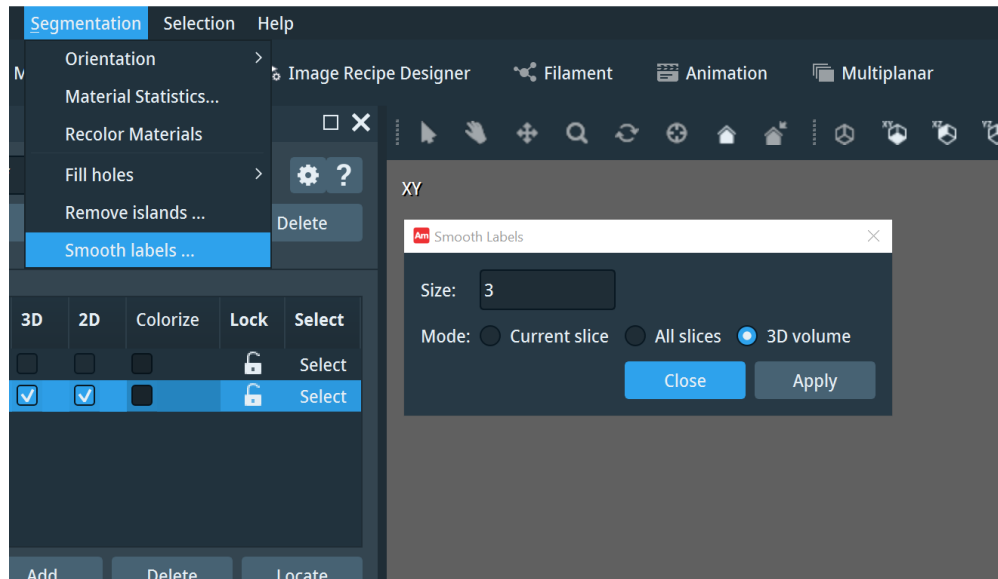


Figure 2.

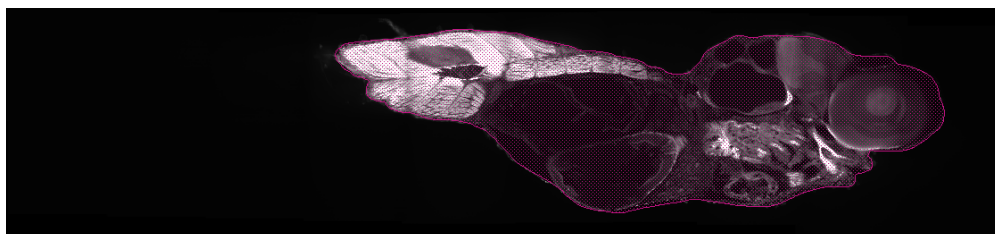


Figure 3.

- 2.2 Create a new label field for each class type (see Overview of Organ Class Types) to organize each class and avoid overwriting segmentations.

Note: Since class types have a range of signal across multiple light channels, multiple channel datasets were used across class types. The best channel dataset was chosen for each class type segmentation, and segmentations in different channels were overlaid after Amira export.

Overview of Class Types

- 3 All substructure and organ classes fall under a class type. Amira label fields may be organized by class type to track classes with similar patterns in the data.

Muscle

Trunk Muscles

The fish contains two main truck muscles, the epaxial and hypaxial muscles separated by the transverse septum.

Pectoral Girdle Muscles

Three muscles take their origin at the ventral tip of the posterior cleithrum, the first bony structure to emerge. This is just lateral to the caudal edge of the heart.

Abdominal muscle

The abdominal muscle (in the literature called posterior hypaxial muscle) sends long muscle fibers caudo-dorsally to join the ventral boundary of the hypaxial muscle where there is a fascial thickening. It attaches to the ventral side of the epaxial myotomes at the level of myotomes 4, 5, 6.

Sternohyoid muscle

The sternohyoid muscle courses anteriorly and is formed of two consecutive myotomes reaching the urohyal cartilage.

Sternobranchial muscle

Sternobranchial muscle (my name). The third muscle of the group courses dorsally and attaches to the base of the skull at the level of the base of 5th ceratobranchial arch.

Branchial Muscles

Branchial Levator 5.

This muscle attaches at the caudal rim of the ear bone and project ventro-medially to attach to the fifth branchial arch. This is one of the muscles which keep together the cranium and the jaw.

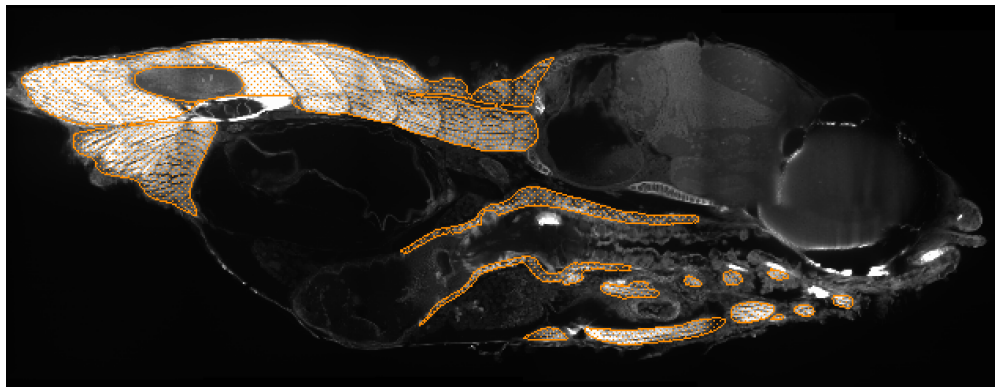


Figure 4. All muscle subtypes in a single XY slice.

Cartilage

Neurocranium

The cranium is formed of different cartilaginous structures. The base of the brain can be divided into the prechordal elements and the para and postchordal elements. The ethmoid plate connects to the basal plate (also parachordal cartilage) via trabeculae. This basal plate abuts the notochord. The basicapsule emanates from the basal plate encircling the base of the developing ear. It comprises the anterior, lateral and posterior basicapsular commissure. This is also sometimes referred to as the auditory capsule. At the anterior portion of the basal plate a perforated fossa is present formed by lateral commissure through which vessels run. The basal plate extends caudal to merge with the occipital plate which extends two dorsal elements, the occipital arches. The cleithrum, a prominent vertical structure, acts as a central element of the zebrafish skeletal system.



Figure 5. All cartilage fragments in a single XY slice.

Notochord.

A structural and signaling element located directly under the spinal cord containing large vacuolated cells surrounded by notochord sheath cells.

The **pharyngeal teeth pad** is situated at the upper region of the pharynx.

Gills are protected by the operculum, a covering that shields the delicate structures underneath. The gills themselves are individually labeled.

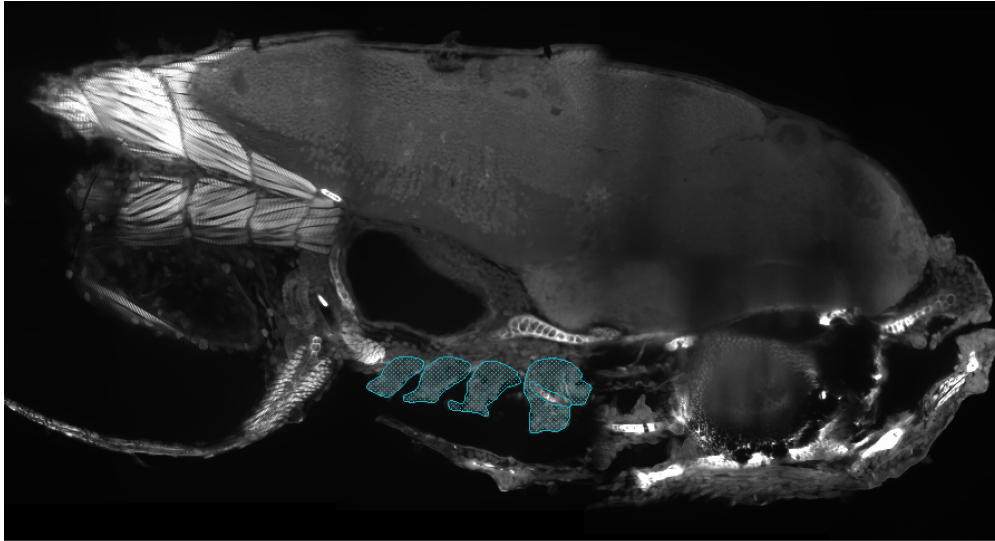


Figure 6. All four gills in a single XY slice.

The pectoral fins are anterior limbs situated on each side of the fish.

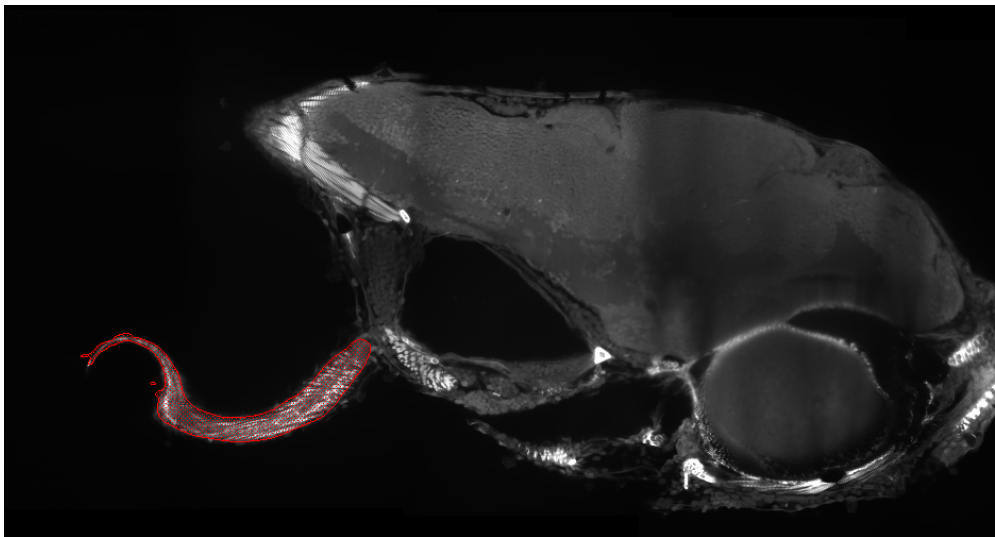


Figure 7. One pectoral fin in a single XY slice.

Sensory organs include the eyes, with distinct labels for structures such as the iris and exterior, and the ears, which encompass the entire organ along with its internal compartments. Hair cells line the base of the ear in uniform lines.

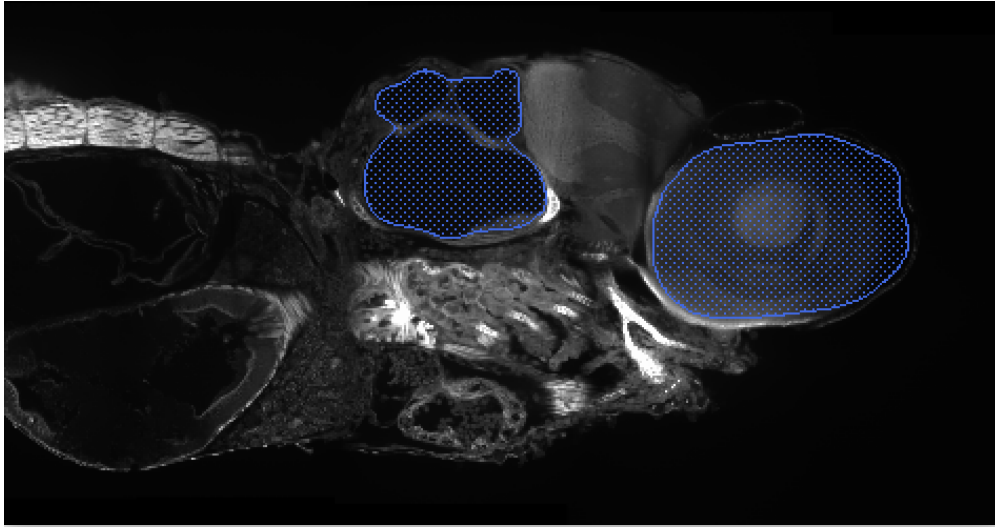


Figure 8. Ear (left) and eye (right) in a single XY slice.

Organs include:

- **Gastrointestinal tract:** starts at the mouth, extending along the body cavity.
- **Heart:** includes the **ventricle**, a large, heavily lined cavity, the **atrium**, the more dorsal/caudal, thin cavity, and **ductus arteriosus**, the muscular outlet.
- **Kidney:** looping structure located in the midline and extending along the body cavity.
- **Swim Bladder:** characterized by its large size and dark interior pixels.
- **Gallbladder:** notable for its contrast between the muted interior and bright surrounding membrane.
- **Pancreas:** divided into **exocrine** regions, featuring wiry tubules near the swim bladder, and **endocrine** clusters of cells adjacent to the exocrine area.
- **Liver:** identified by its dark border and unique pattern, distinct from the surrounding muscle.

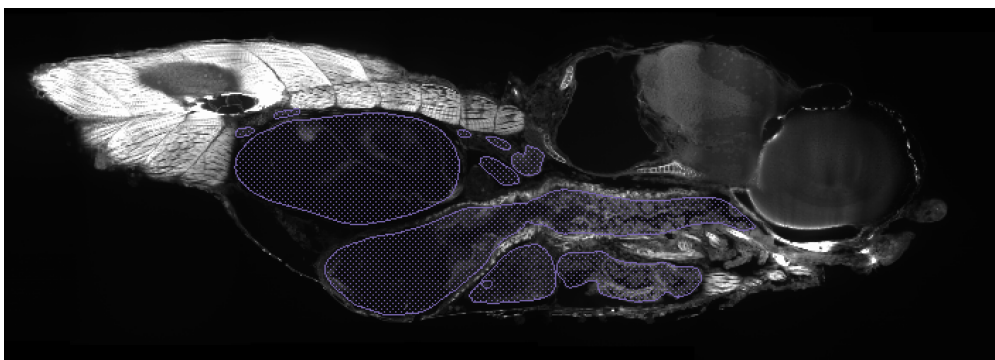


Figure 9. All organs in single XY slice.

Nervous system includes:

- **Spinal Cord:** Extends out from the brain, surrounded by epaxial muscle and dorsal to the notochord.
- **Pineal gland:** Located dorsally in the midline of the brain.
- **Habenulae:** A paired structure situated lateral to the pineal gland.
- **Brain:** Includes internal features while excluding surrounding muscle and nearby ears.
- **Posterior lateral line ganglion:** A dorsal ganglion directly behind the ear.
- **Sympathetic Nervous System/Adrenals:** Bright clusters of cells located near blood vessels.

Glossary and Segmentation Techniques

- 4 For all classes, carefully annotate intermittent slices of objects down the XY plane, then interpolate (ctrl+i) and add selection to the appropriate class material. Check the object segmentation in XZ and YZ planes to ensure segmentation does not overlap with any other data patterns relating to a different class.

Note: Smoothing may cause excessive material shrinking in datasets where the Z dimension is relatively short.

In many instances, multiple class patterns will have signal overlap in the same channel (Figure 12). Visible muscle fibers and cartilage should always be segmented in instances of overlap with other classes.

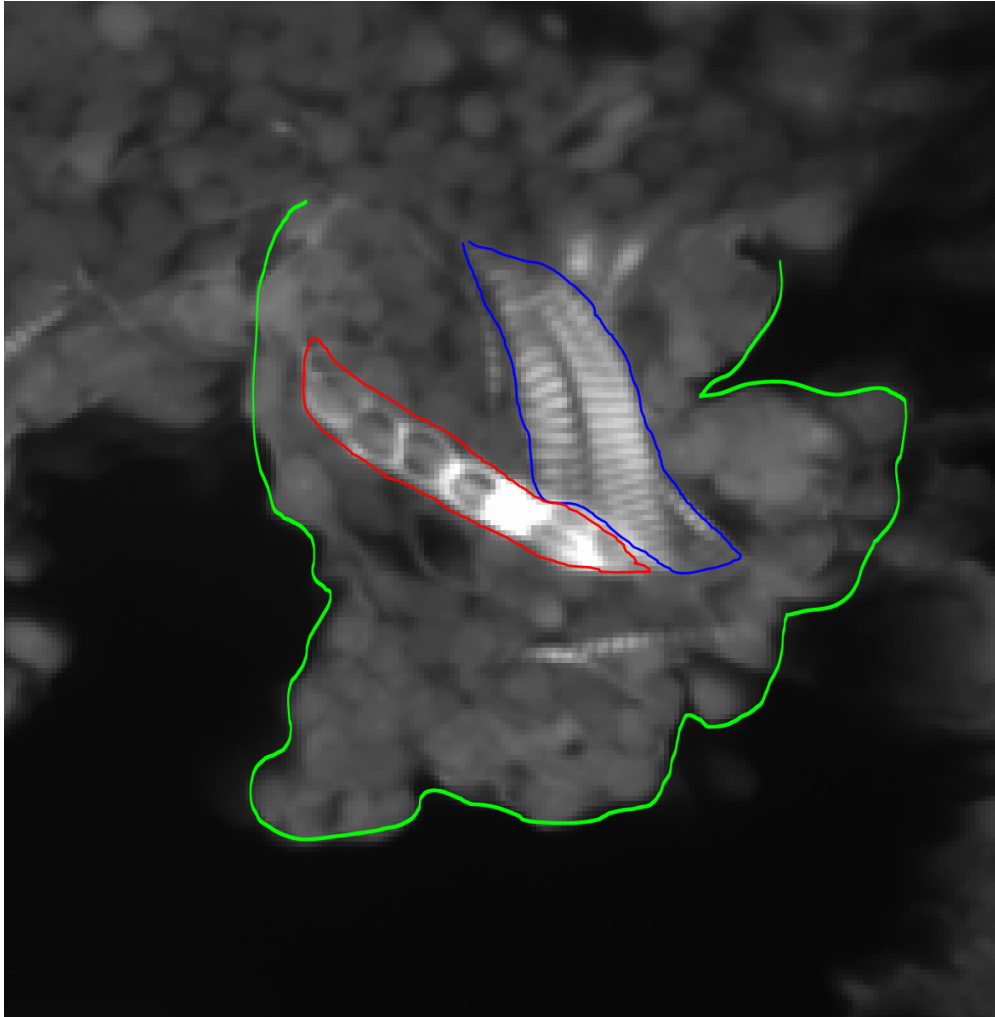


Figure 12. Gill class (green) contains some muscle fibers (blue) and cartilage (red) in an XY slice.

4.1 Muscle

Epaxial and hypaxial muscles

Clearly defined dorsal and ventral bundles (top and bottom) are segmented separately and avoid the spinal cord and notochord, respectively.

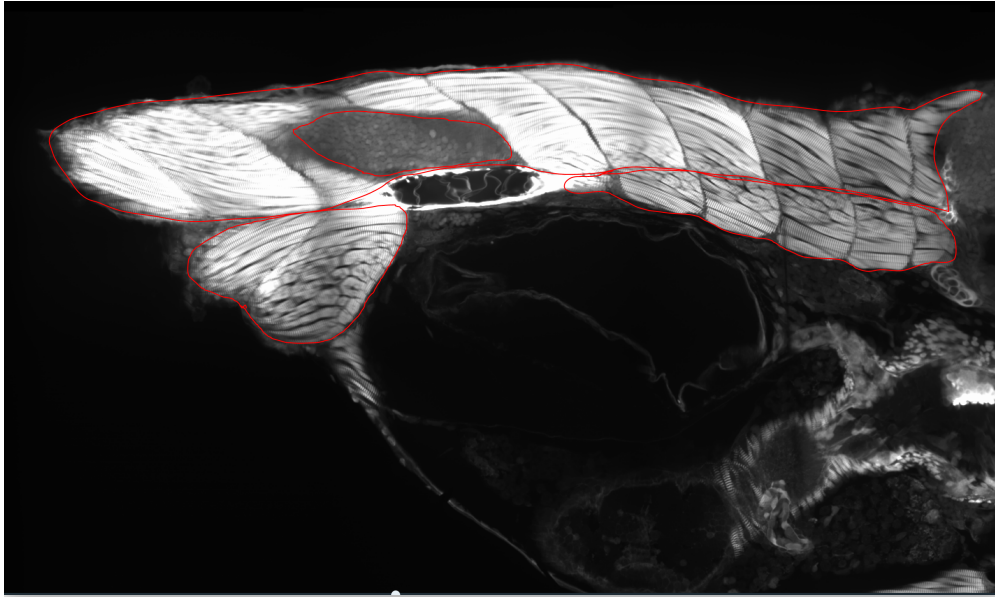


Figure 13.

Abdominal muscle

More dispersed, streaking pattern extends across the ventral surface up towards the hypaxial muscles. Small, bright nerve signals can be included in the more sparse muscle patterns.

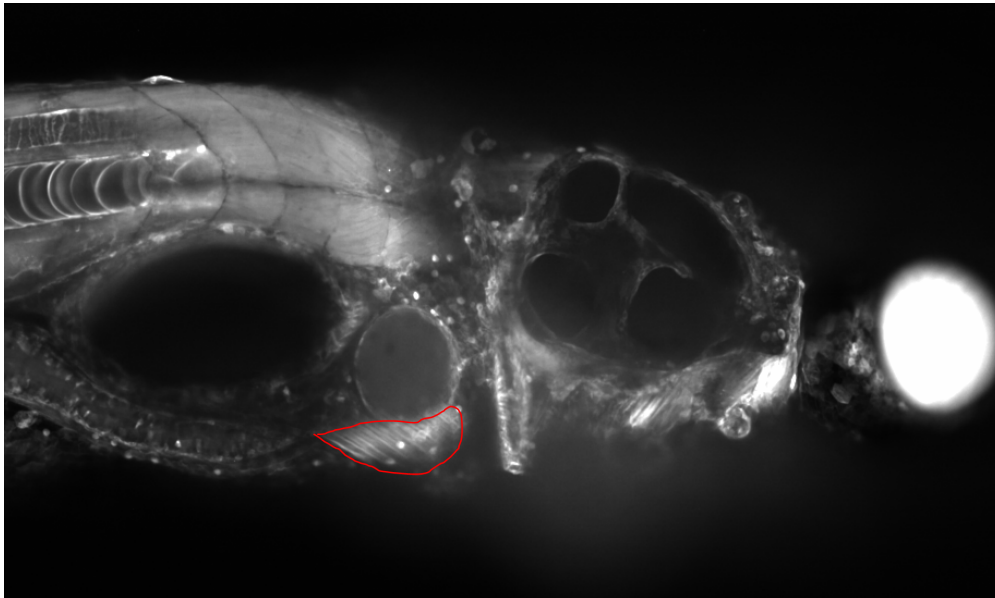


Figure 14.

Pectoral girdle muscles and cranial muscles

Short, small muscle bundles that wrap around organs like the gut and surround the gills, heart, and mouth.

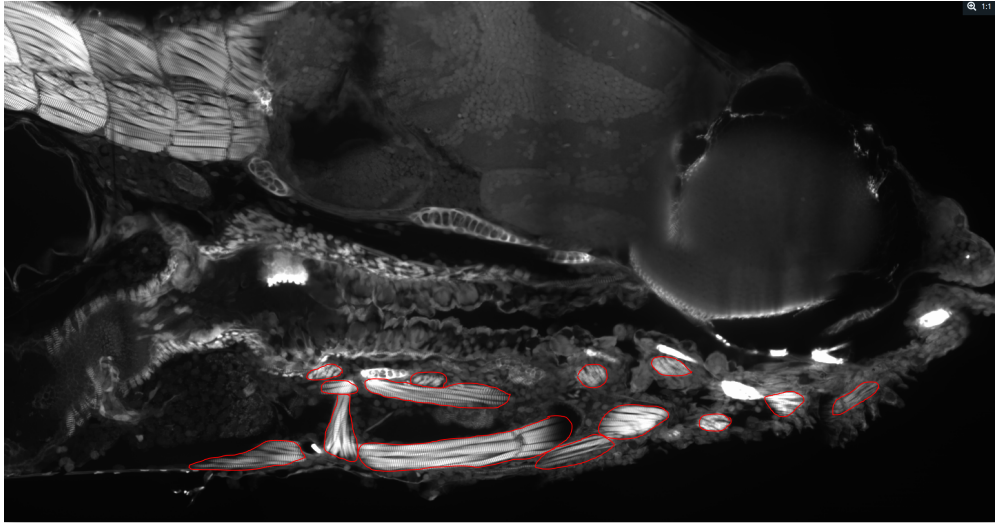


Figure 15.

4.2 Cartilage

Fragments run throughout the body, primarily surrounding the ears, brain, and gills. Texture pattern is hollow, porous, and bright.

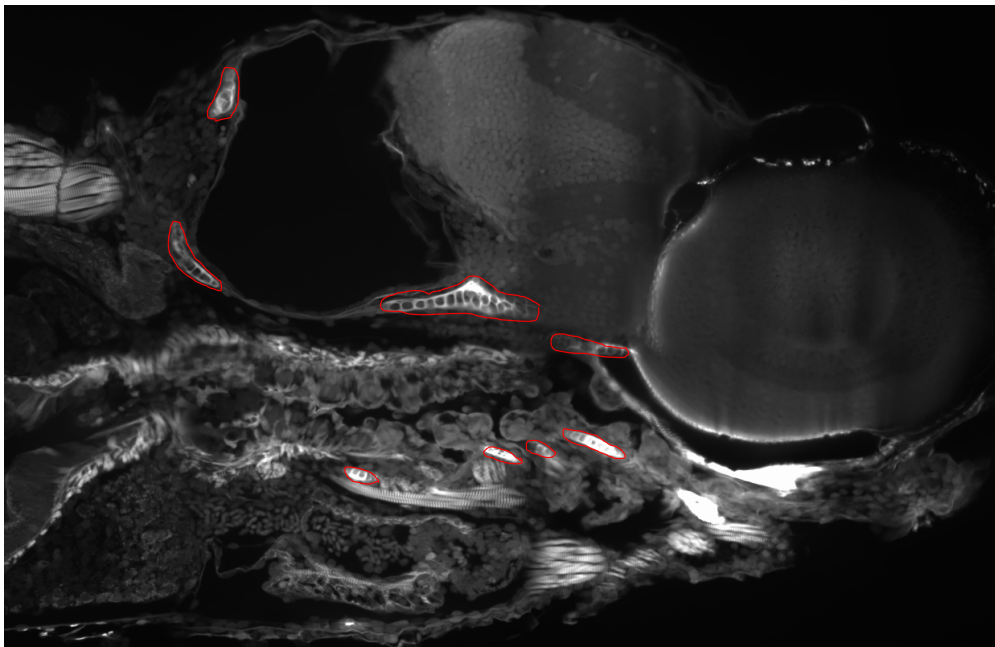


Figure 16.

Cleithrum

Small, bright bars pass behind the ears and gills, extending towards pectoral fins. The cleithrum segments do not have a distinct cartilage signal pattern.

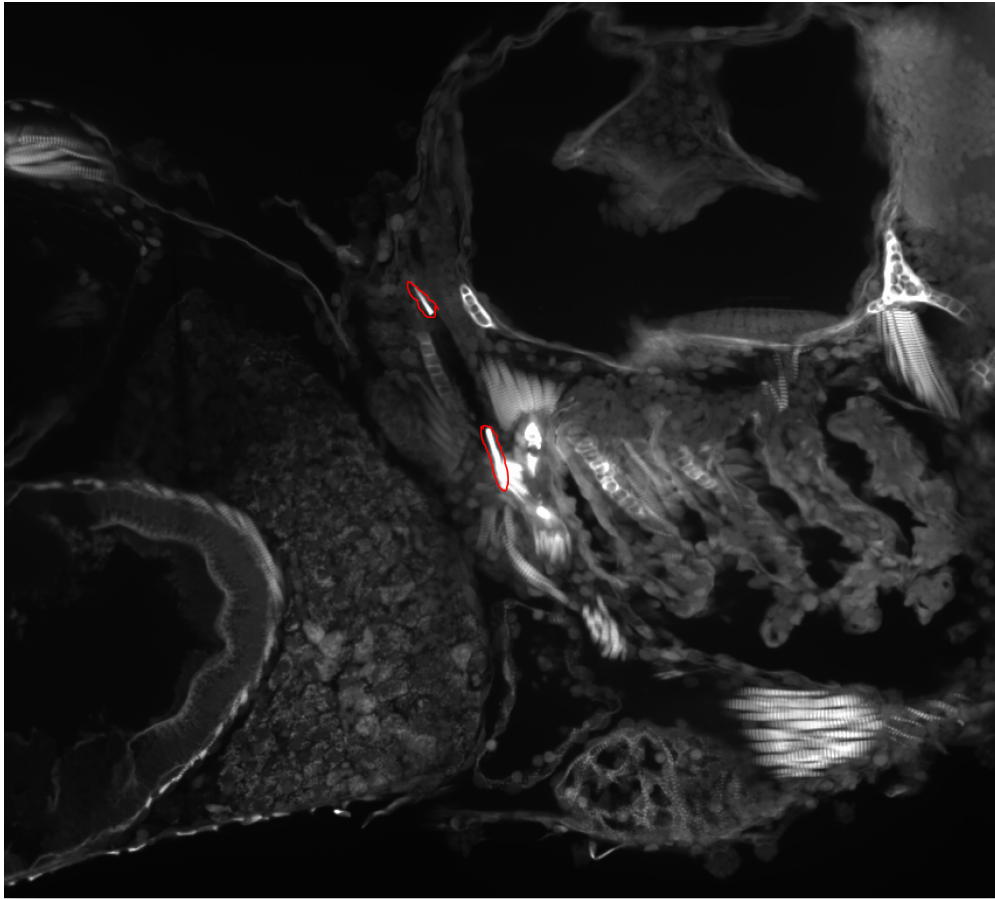


Figure 17.

Pharyngeal teeth pad

Packed, bright, and webbed signal pattern towards the back of the pharynx, distinct from surrounding gut folds.

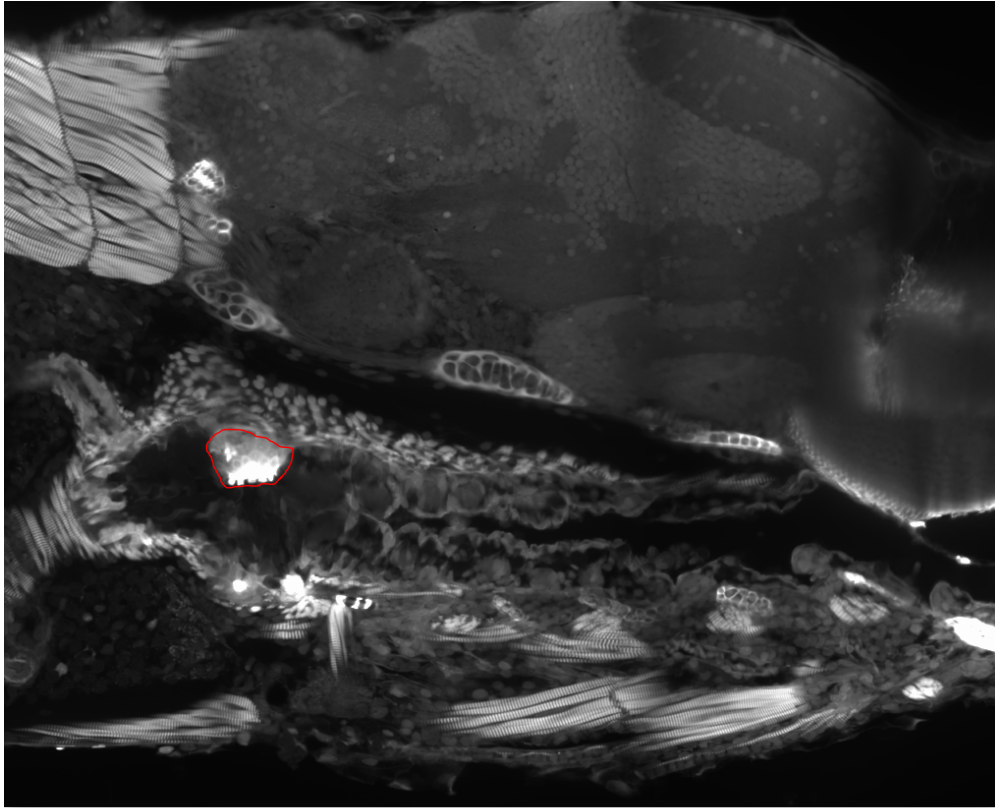


Figure 18.

4.3 Gills

Two sets of four gills have a soft, gray dotted signal pattern, with gill 4 (yellow) being closest to the cleithrum and gill 1 (green) being closest to the eye.

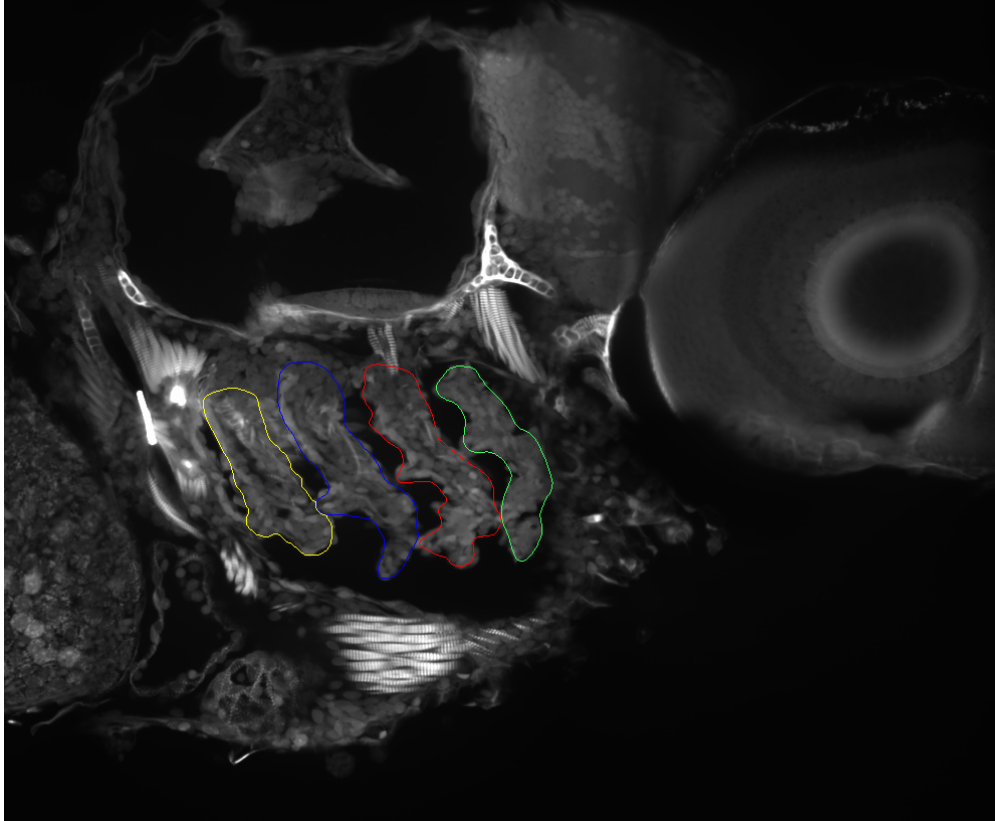


Figure 19.

4.4 Pectoral Fins

The pectoral fins have small muscle fiber and cartilage signal pattern. Muscle and cartilage may be segmented separately and combined to create the total pectoral fin segmentation.

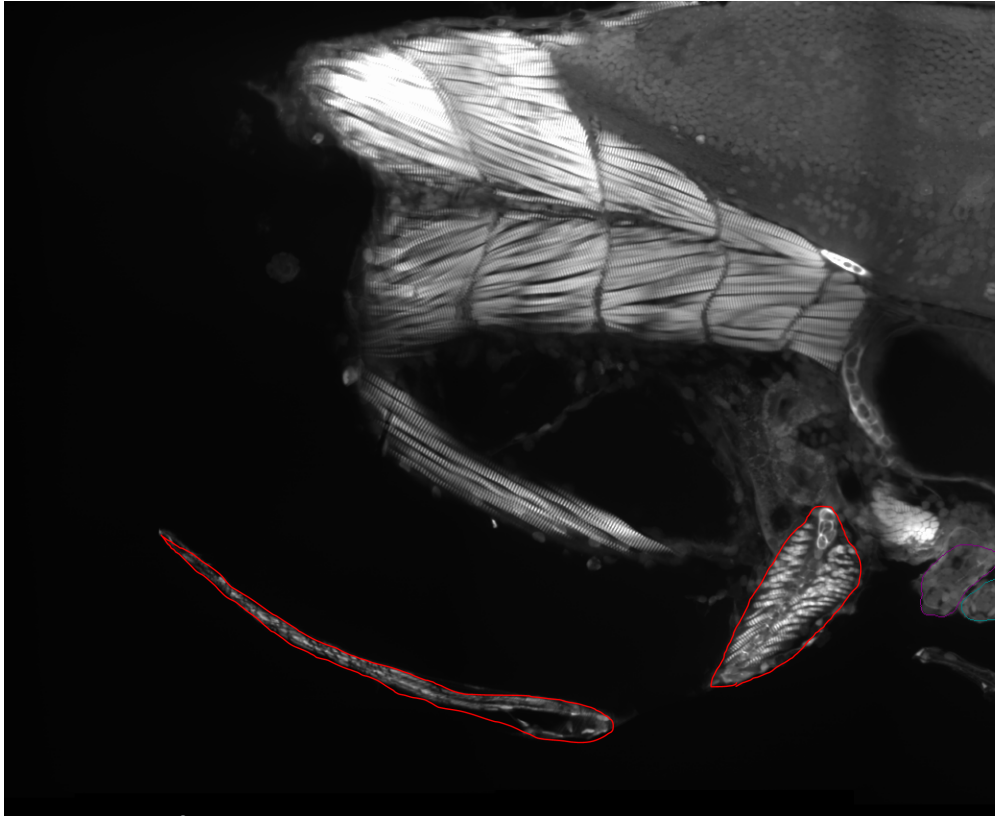


Figure 20.

4.5 Sensory Organs

Eye

The most anterior organ, the eye has a soft, radial signal pattern with a thin, bright membrane.

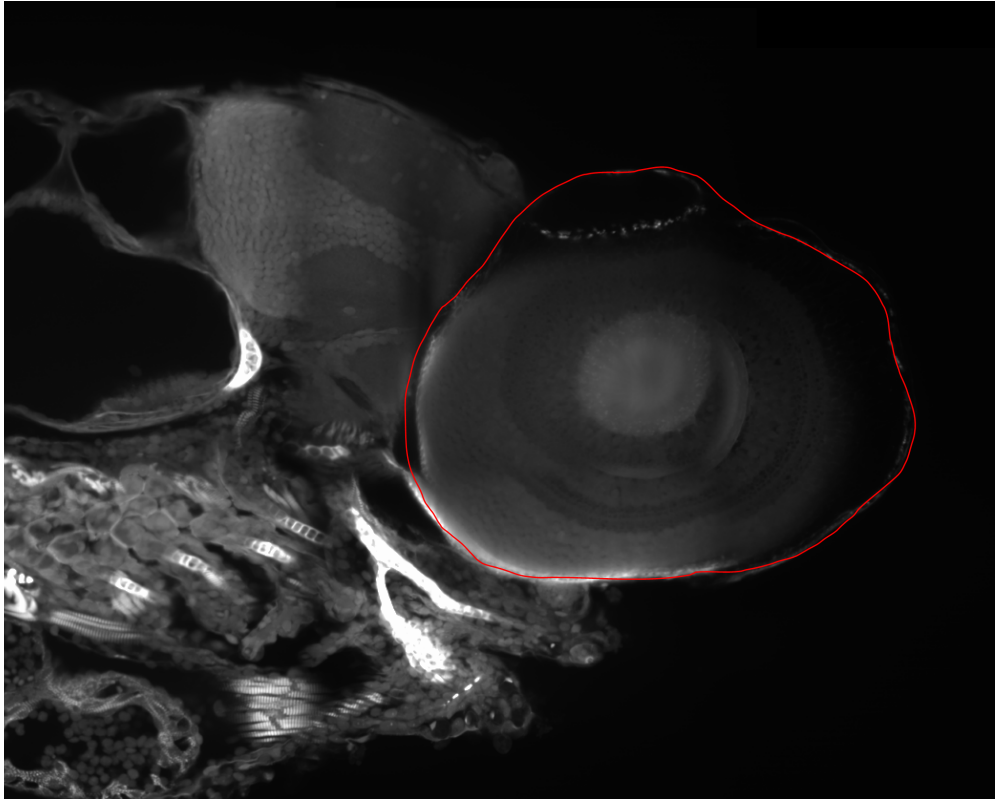


Figure 21.

Ear and auditory hair cells

Ear signal pattern is primarily empty compartments with hair cells (blue) lining the base of the ear.

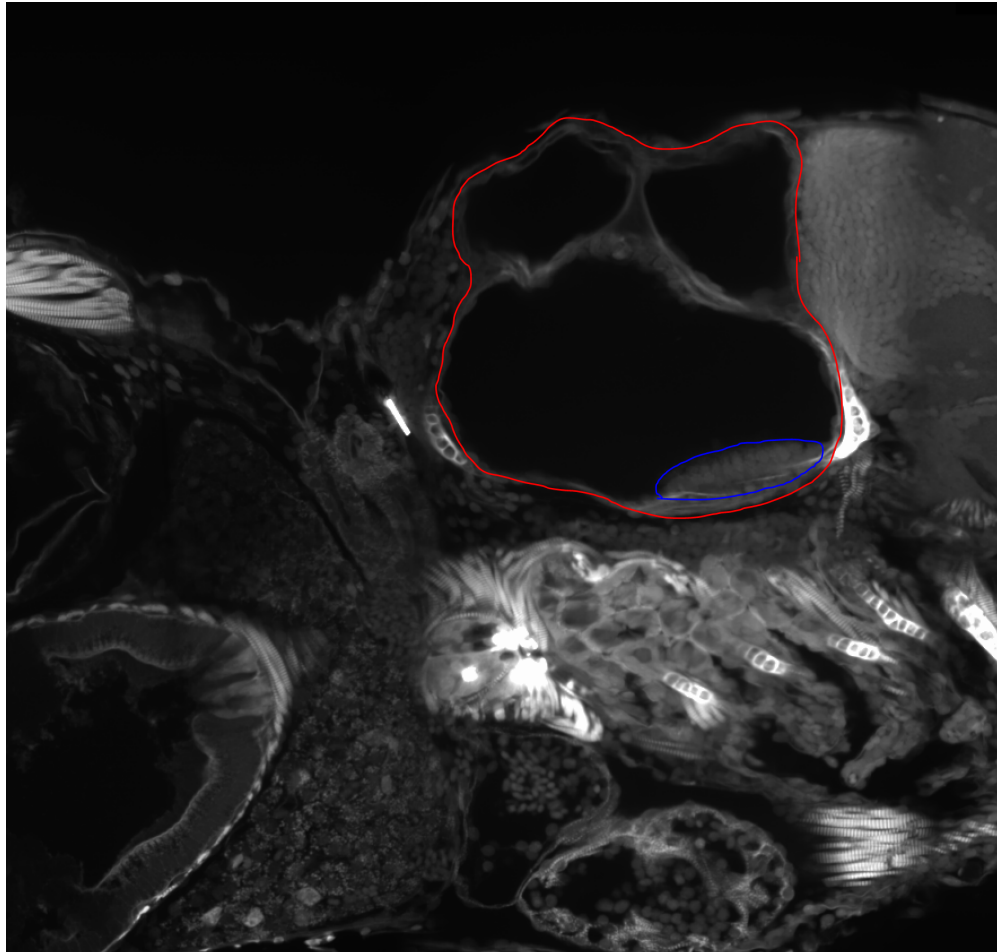


Figure 22.

4.6 Organs

Gastrointestinal tract

Extending from mouth to anus, the gut texture is hollow with irregular folds of soft, gray dots.

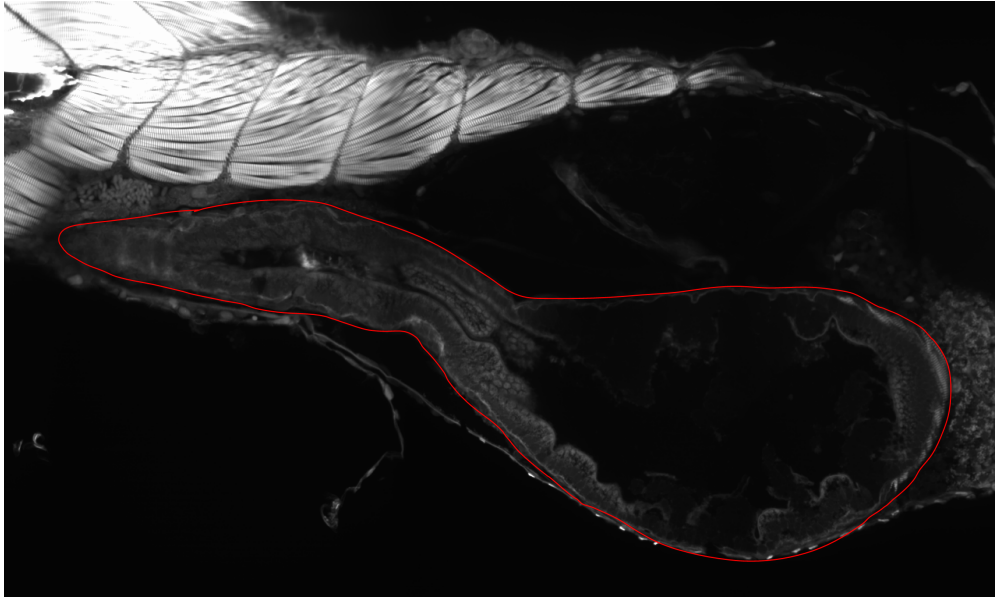


Figure 23.

Heart

The ductus arteriosus (red) is the smallest compartment and closest to the eye with a signal pattern of soft gray dots throughout, the ventricle (blue) is most heavily lined with a visible valve separating the ventricle from the atrium (green), which is thinly lined with visible blood cells dispersing out of the compartment.

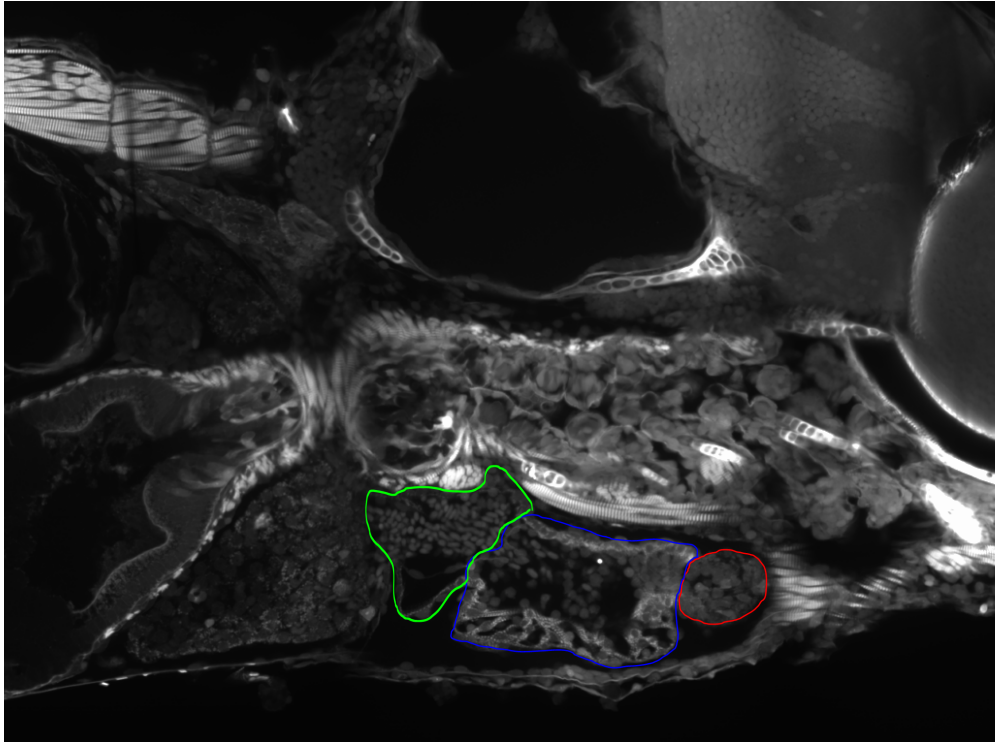


Figure 24.

Kidney nephron

The nephron and glomerulus with a thick, soft gray signal pattern that bundle dorsal to the liver, caudal to the ears, and dorsal to the gallbladder. Ducts are segmented separately to generally track nephric structure.

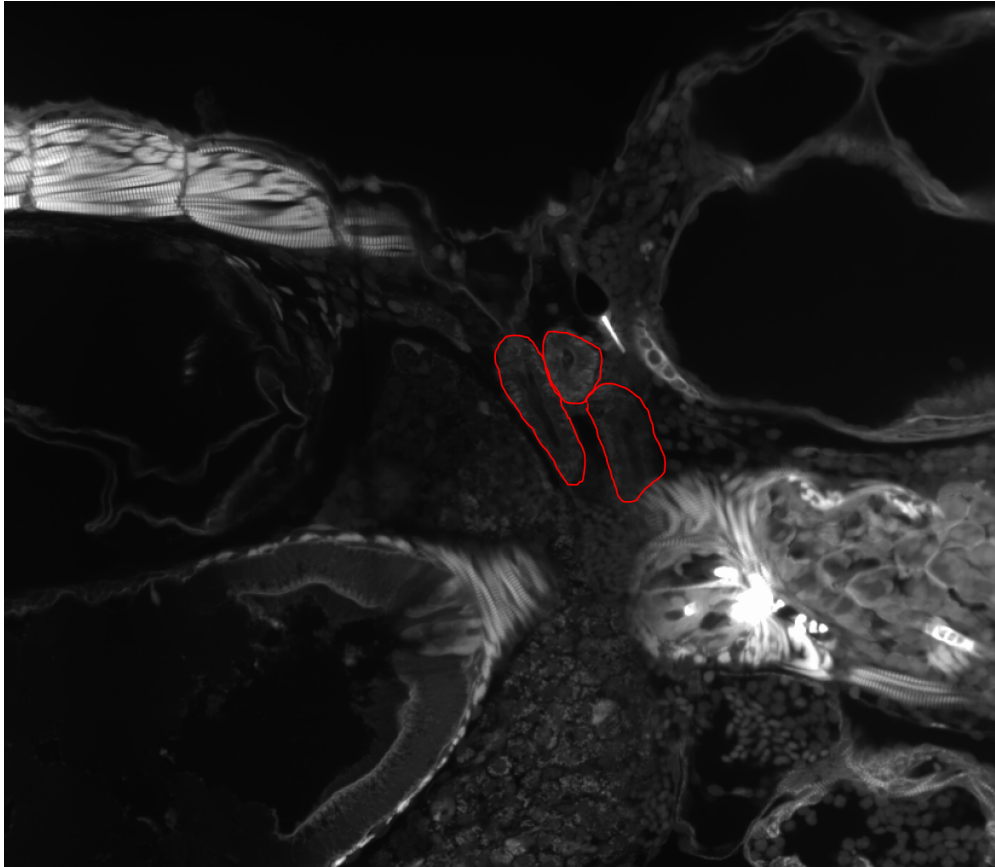


Figure 25.

Swim bladder

A mainly empty compartment with minimal signal, the swim bladder takes up a large portion of the body cavity and is directly above the gut.

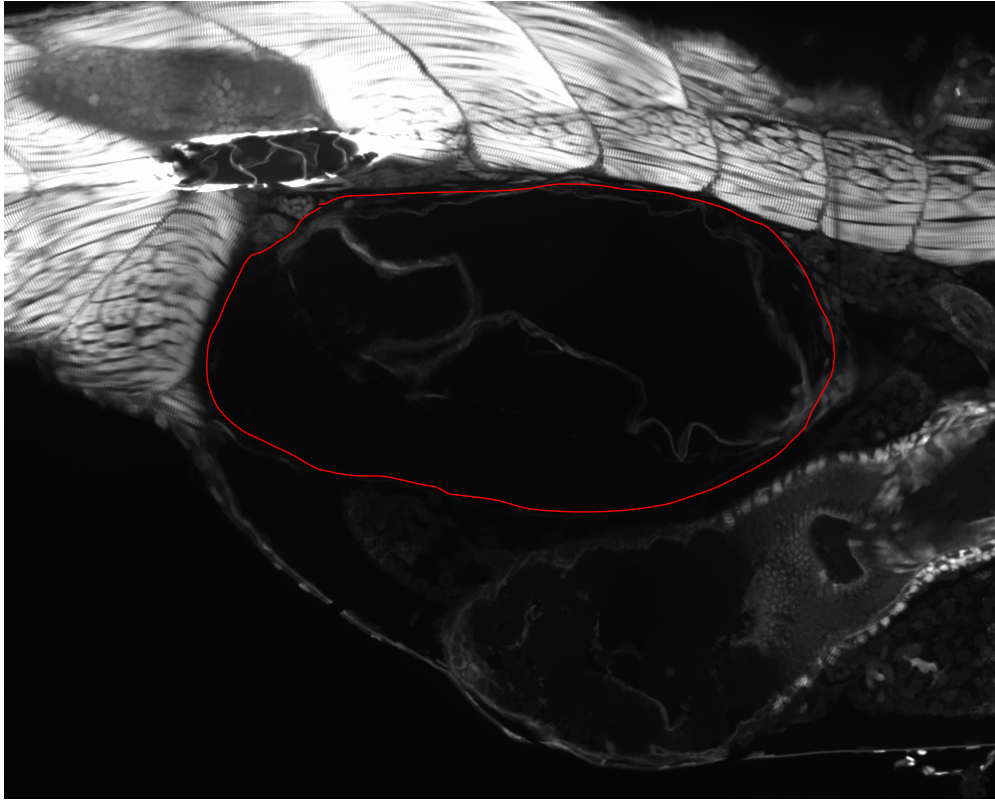


Figure 26.

Gallbladder

Surrounded by the liver, the gallbladder often has a dark, soft interior signal pattern, lined with brighter, sparse dotted signal. While generally spherical, some invagination may be present.

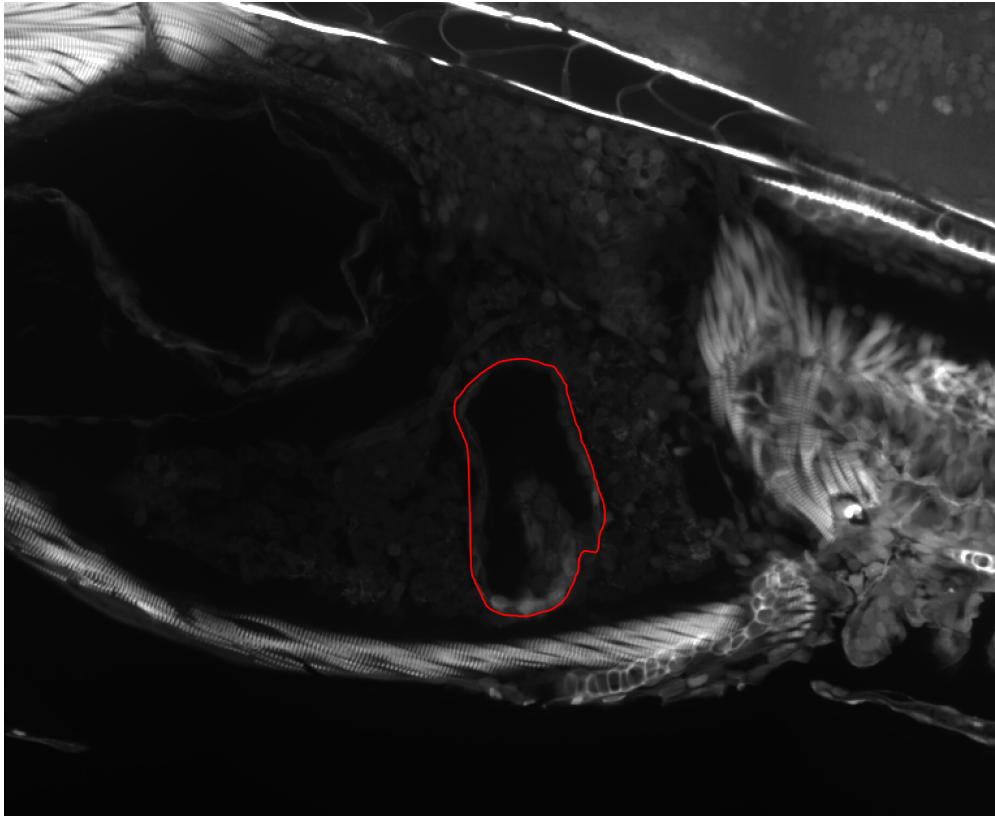


Figure 27.

Swim-bladder duct and gall bladder duct



Figure 28.

Liver

Wedge between the gut and heart, the unique liver signal texture appears as a gradient of intensity and surrounds the gallbladder. Adjust data contrast as needed in order to include all liver signal.

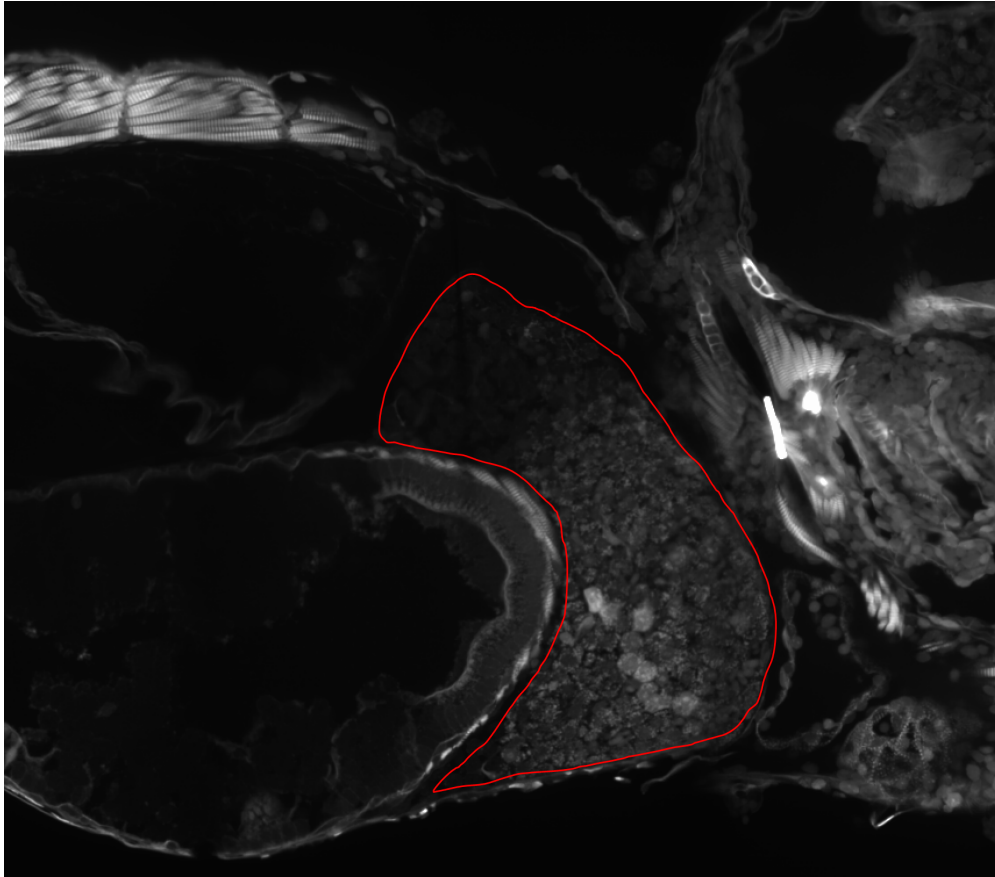


Figure 29.

4.7 Nervous System and Notochord

Notochord

Surrounded by bright nerve signal, the notochord passes between epaxial and hypaxial muscles and into the main body cavity. Signal pattern is porous with thin bright strands passing vertically through a cross-section.

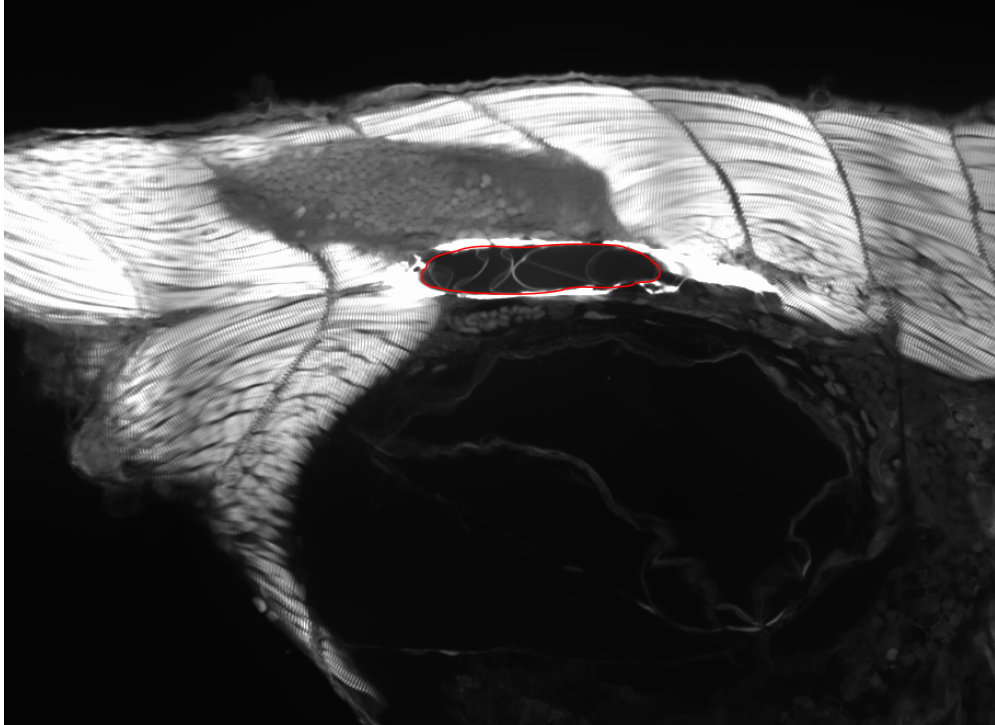


Figure 30.

Spinal cord

Directly dorsal and parallel to the notochord, the spinal cord has a varied soft gray dotted signal pattern with some bright nerve signal dispersed throughout.

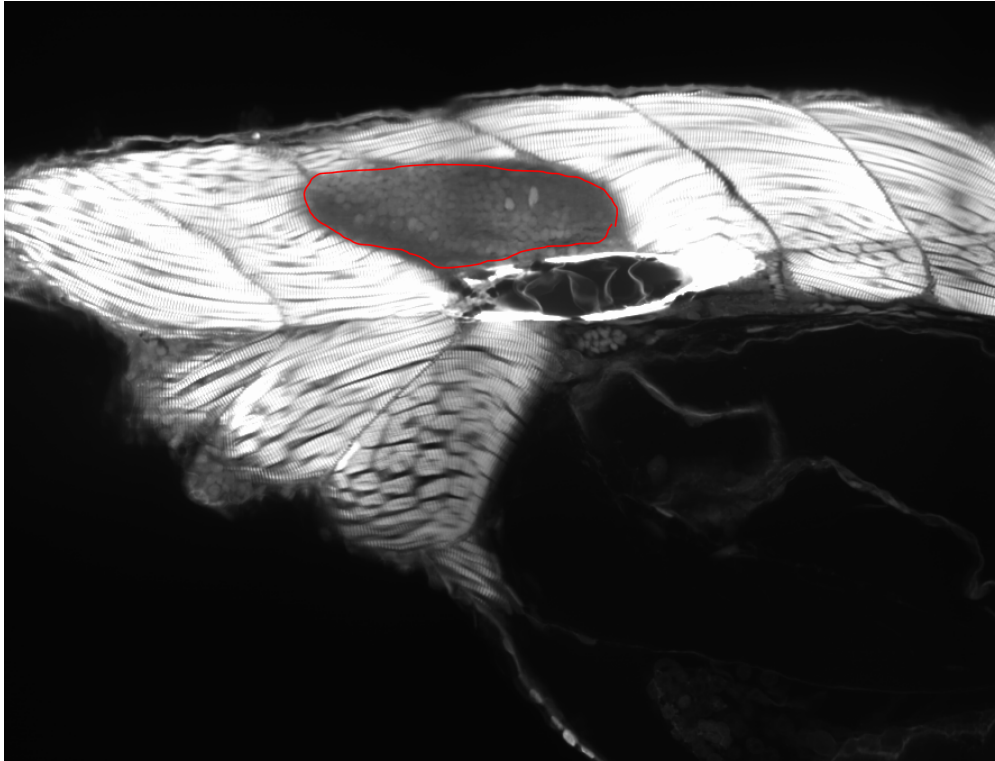


Figure 31.

Pineal gland

Smaller, granular signal pattern is surrounded by slightly large gray cells located dorsally, between the eyes. The gland is physically separate from other brain regions.

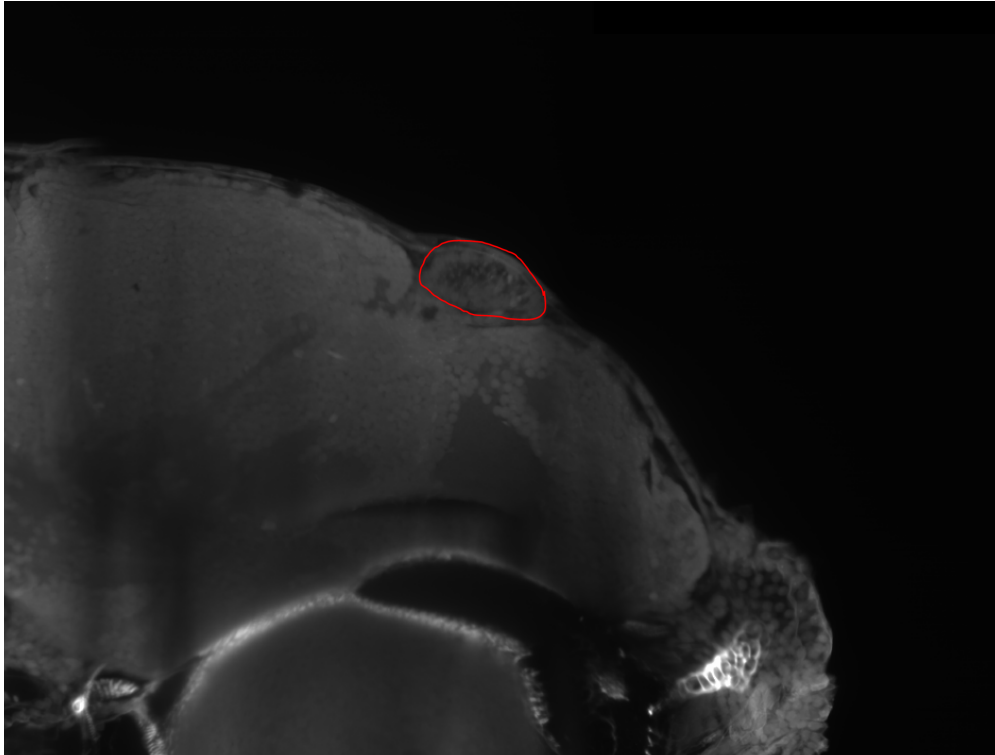


Figure 32.

Habenula

Both lobes flank the pineal gland, with identical signal patterns to one another; dark soft interior is lined with slightly lighter gray cells.



Figure 33.

Brain

Composed of multiple signal patterns, the brain includes most gray signal in the dorsal cephalic region of the dataset. The brain connects directly to the spinal cord, which shares a similar signal pattern, and includes the pineal gland and habenula.

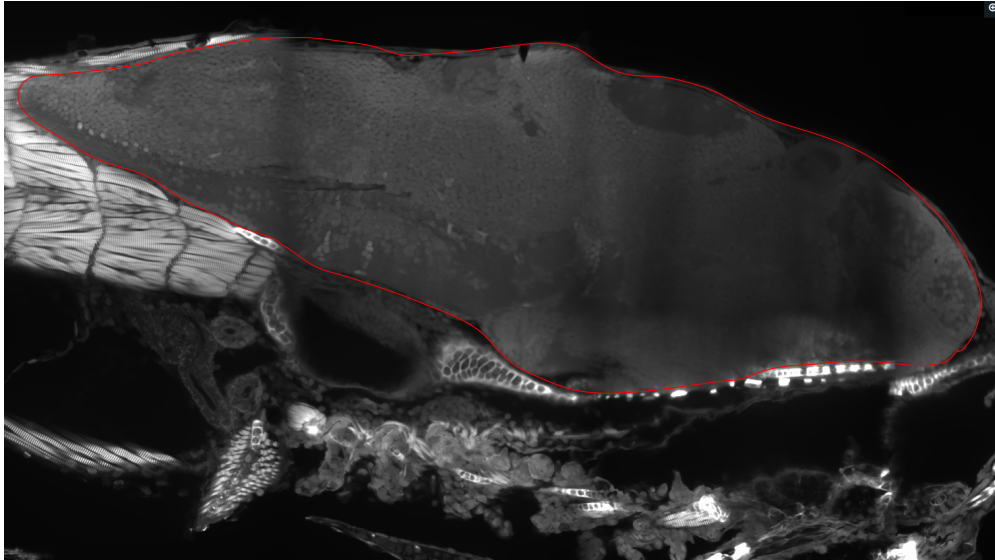


Figure 34.

Nodose Ganglion

Bundle of soft, gray dots clustered behind the ear, forming an upside down triangle shape.

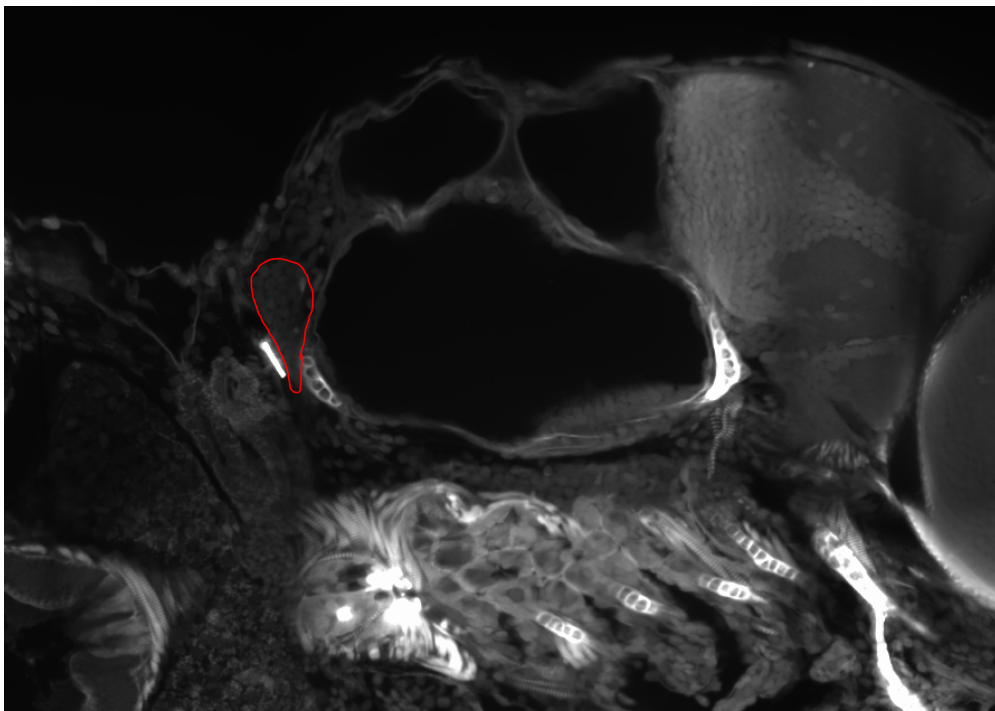


Figure 35.

Autonomic Nervous System

Saturated signal is thresholded out using the Magic Wand tool (3) under the Segmentation tab. Once selection is added to a material, noise and excess signal is removed from the segmentation and significant nerve signals are refined.

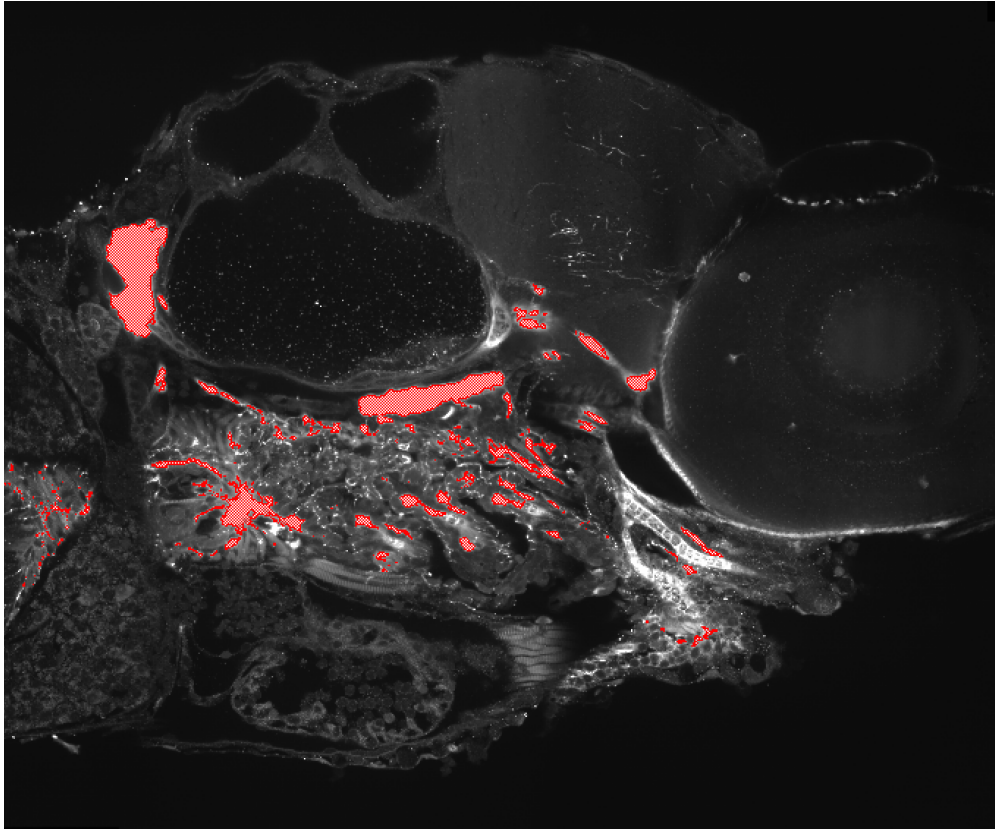


Figure 36.

Figure 31.

Protocol references

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<https://zebrafishucl.org/>