Figure 1

Adult Axolotls, Female (Left, Leucistic , 23 cm) and Male (Right, Wild Type, 26 cm)

Osteoclasts/Giant Cells in Regenerating Axolotl (*Ambystoma mexicanum*) Spinal Cord Meninges. An Unexpected Presence.

An article by Ellen A.G. Chernoff, Teri Belecky-Adams, Hai V. Nguyen-Salfity, Sarah T. Scott and Hidehito Takenaka (IUPUI-Department of Biology)

The adult spinal cord of the axolotl (Ambystoma mexicanum, **Figures 1,2**) resembles that of mammals in that it has white matter and gray matter, is surrounded by three layers of connective tissue meninges and has an ependymal cell lined central canal (**Figure 3**). After a penetrating wound, the connective tissue meninges around the cord invade the lesion, but a permanent scar does not form. To accomplish this, a large amount of fibrotic material, full of collagen, must be removed. The ependymal cells found around the central canal are known to be activated, grow out and have a role in removing the fibrotic material.

More surprisingly, osteoclast-like cells, classically involved in remodeling bone, appear throughout the reactive meninges and produce matrix-degrading enzymes, as well, acting on the fibrotic material. This is an unusual case of action of these large cells multinucleated on a “normal” soft tissue.

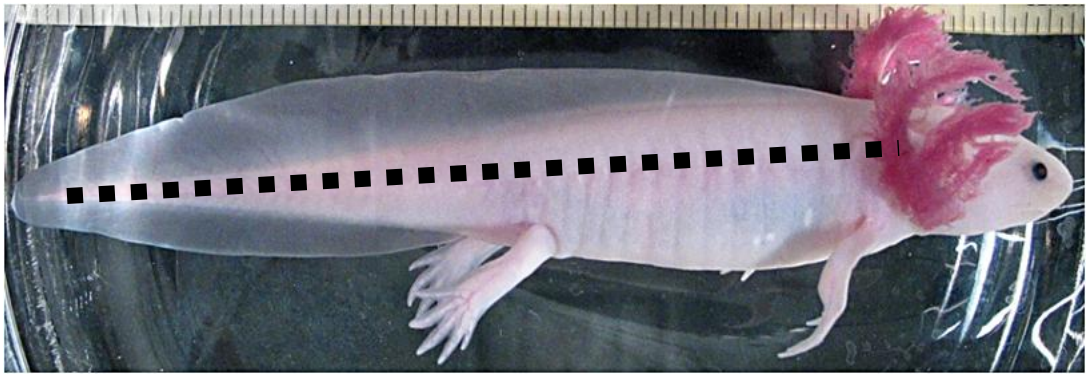


Figure 2. Axolotl lightly anesthetized for side picture (they have a strong "righting reflex"). Dashed line shows rough location of spinal column

Osteoclast-like cells are found in bone and other mineralized tissues and are part of the remodeling process in bone, cartilage and teeth. They are known to be involved in the bone-remodeling of salamander limb regeneration and tooth shedding. Osteoclasts are "fried egg/flying saucer" shaped cells (**Figure 4**) that seal themselves to bone or matrix and break it down (**Figure 5**). They are also found in soft tissues as relatively rare benign or malignant giant cell tumors and in granulomas induced by infection and foreign bodies. Here it is seen that they appear on the reactive meninges during adult axolotl spinal cord regeneration. They are absent in control/intact axolotl meninges, so some signal from the injury attracts them in. The single-nucleus precursor cells that form them are osteoclast precursors or macrophages, which, in turn come from cells called monocytes from bone marrow. **This bone marrow origin was first described in salamanders**.

Axolotl osteoclasts/giant cells produce the matrix degrading enzymes Cathepsin K and Matrix Metalloproteinase-9, as in bone, **This observation is the first that finds these multinucleated cells involved in remodeling regenerating soft tissue instead of infected or tumorous soft tissue, bone and cartilage.**

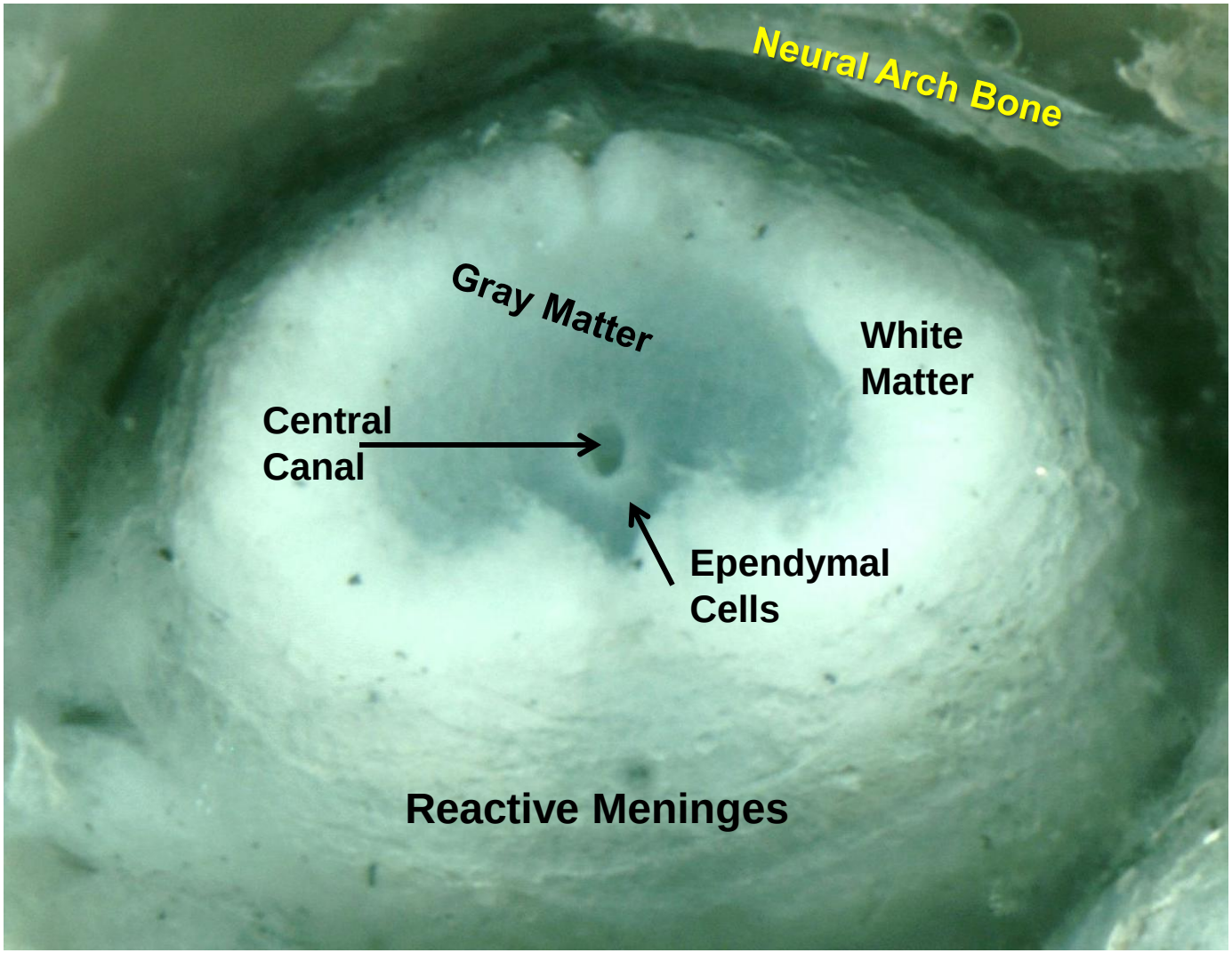


Figure 3. Whole mount cross-section of the cranial stump region from an 18day regenerating adult axolotl cord in spinal column. This cord tissue is intact. This stereo microscope image shows the central canal, white and gray matter and thickened. The injury-reactive meninges surrounding the cord (glassy appearance) is greatly thickened with excess matrix material. Stereomicroscope image. It is a pretty typical tetrapod spinal cord.

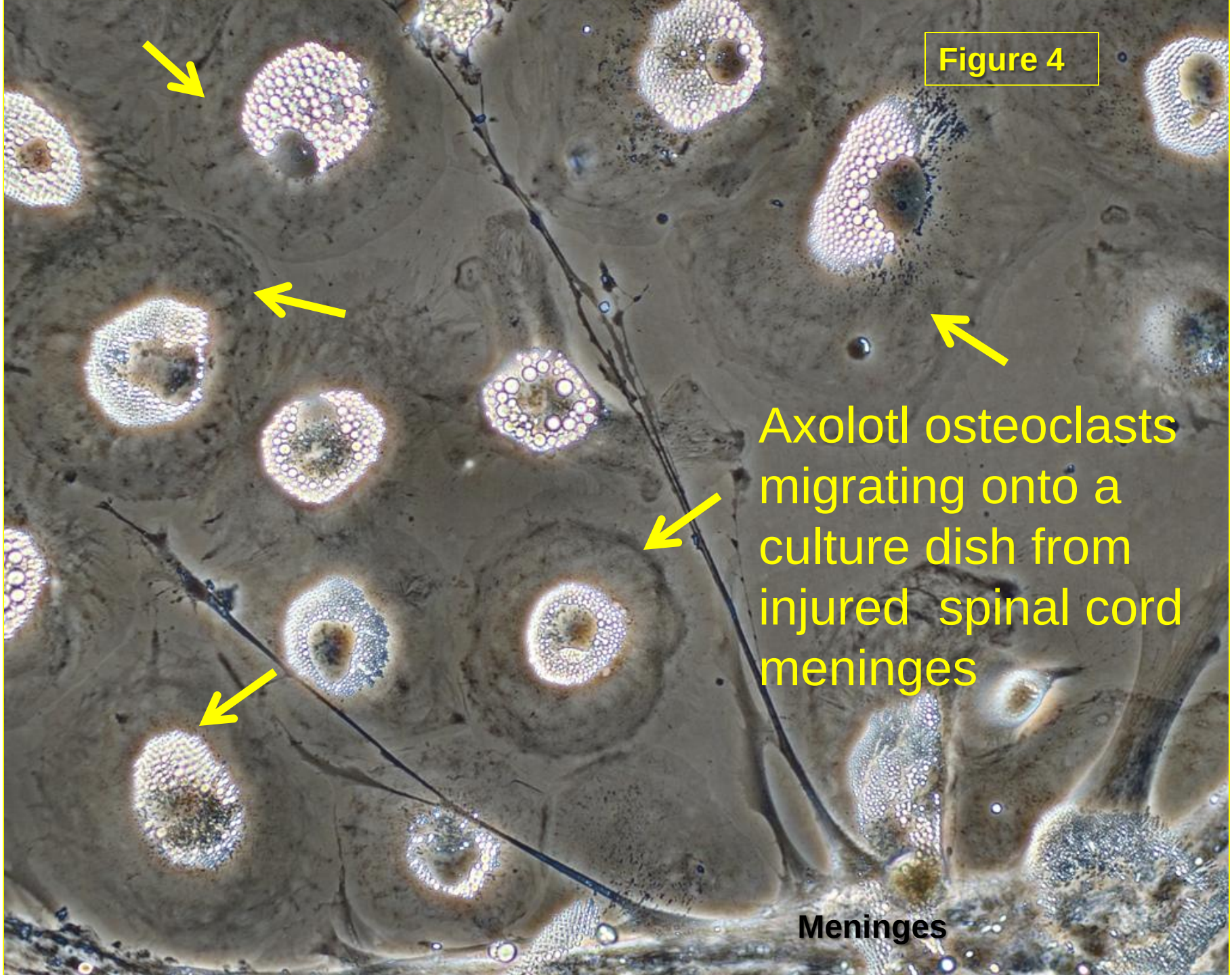
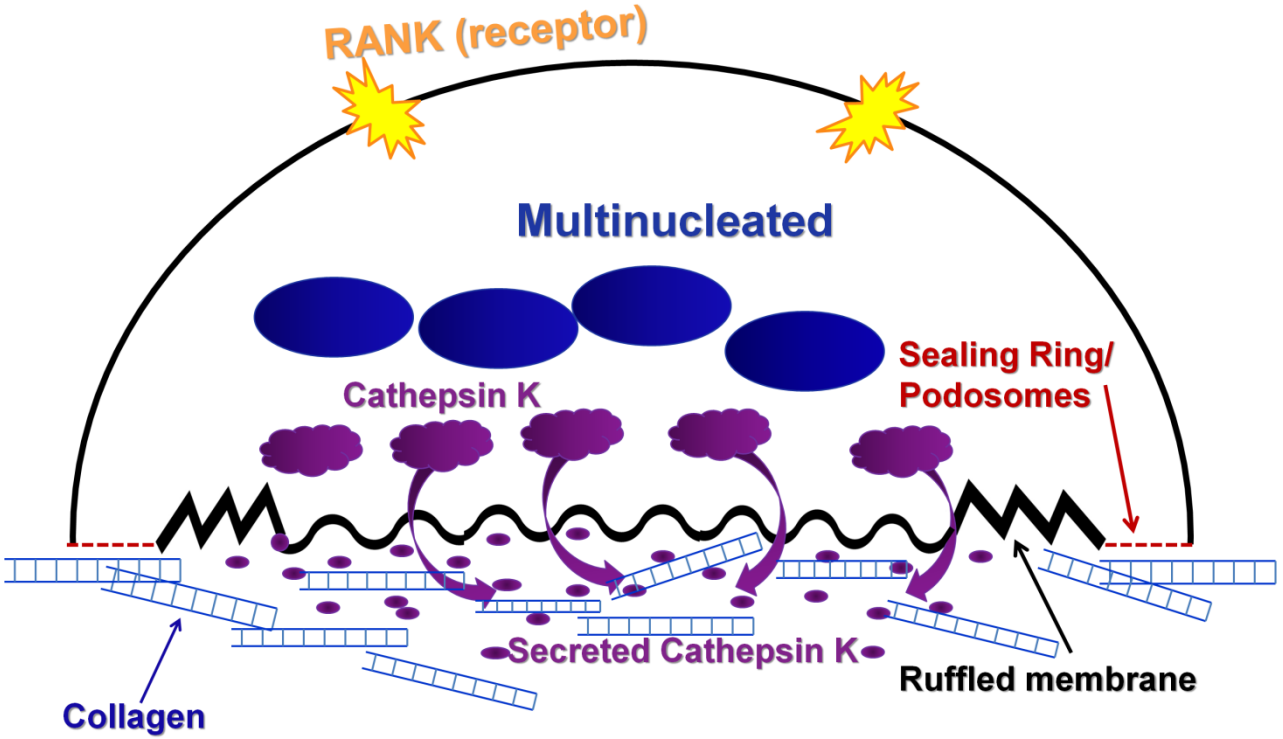


Figure 4
Axolotl osteoclasts migrating onto a culture dish from injured spinal cord meninges
Meninges

Free Side of Osteoclast/Giant Cell

Figure 5



Substratum-Attached Side of Osteoclast/Giant Cell

A mature osteoclast is a multinucleated cell that forms from the fusion of pre-osteoclasts and/or macrophages formed from bone marrow monocytes. It seals to its substrate with a ring of podosomes and ruffled membrane and digests it. In bone it secretes acid material to demineralize, then breaks down matrix, including collagen. In the meninges it just breaks down matrix using specialized digestive enzymes including Cathepsin K, a cysteine proteinase.

Diagram: Chernoff lab

Axolotl Cord Meningeal Reaction

The normal cord from the lumbar region is shown exposed (**Figure 6**). The cord is cut completely under anesthesia (full transection) and allowed to regenerate. Analgesia and antibiotics are administered. 14-18 days, depending on size of the animal, brings the wound to the stage where ependymal cells are growing out of the cord and meninges has invaded (**Figure 7**).

A thickened mass of fibrotic meninges surrounds the regenerating cord ends and fills the space between them (**Figure 8**).

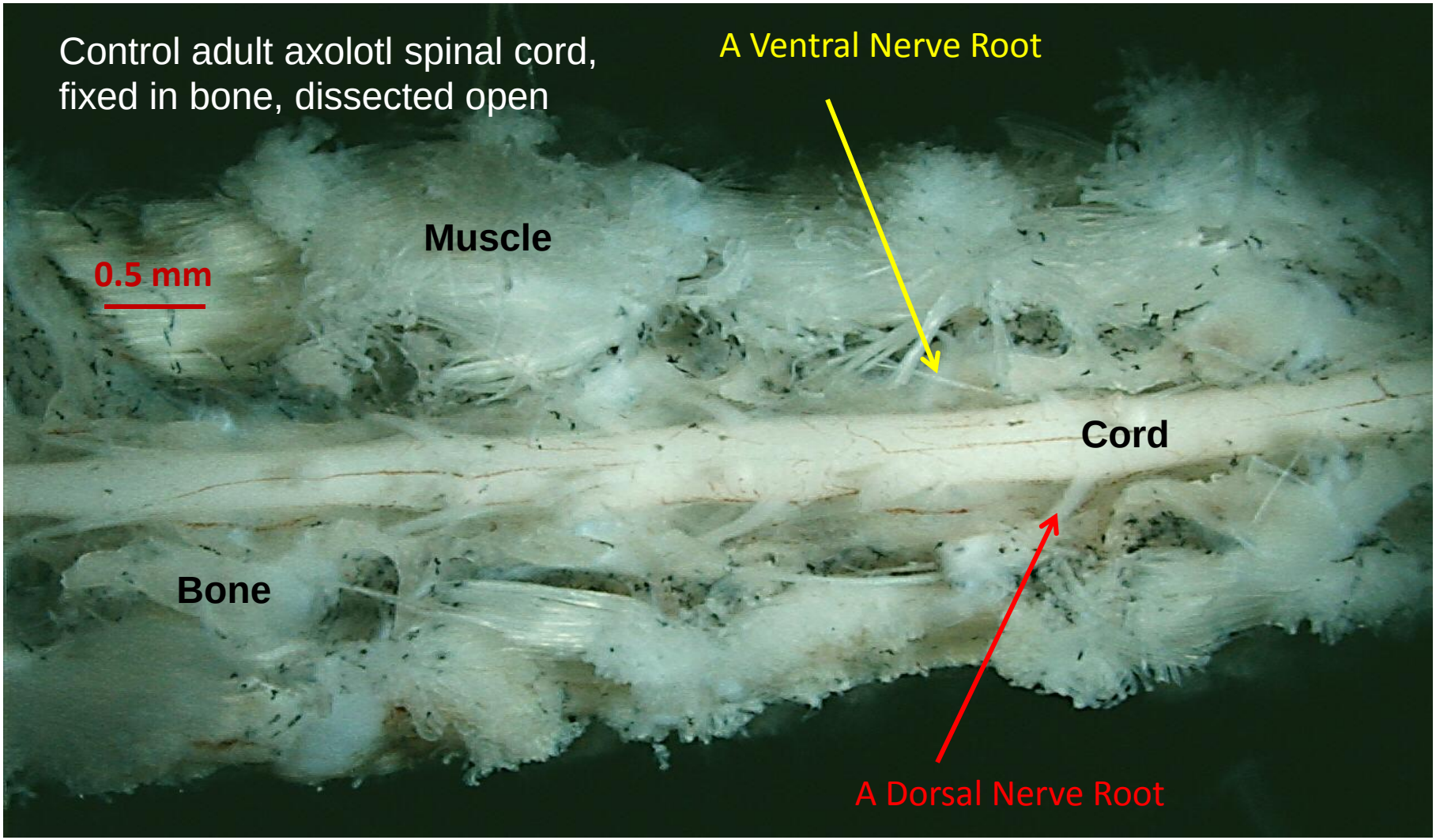


Figure 6. Whole mount preparation of intact (control) adult lumbar region spinal cord. Fixed in bone, whole spinal column removed and neural arch dissected away. Photographed using a stereomicroscope/camera system. The dorsal spinal artery is visible on the surface and nerve roots are present. Encased in thin meningeal layers. No osteoclasts.

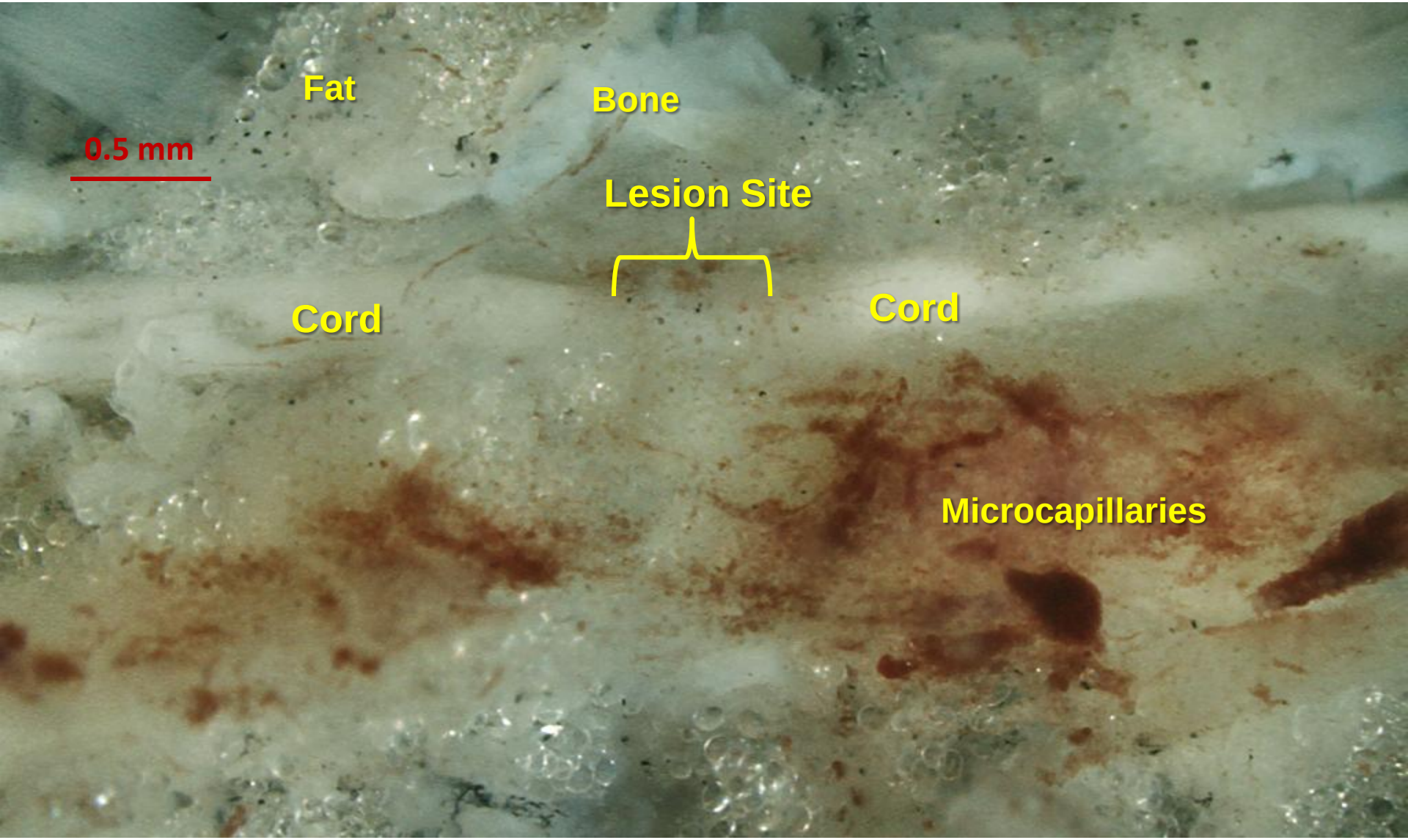


Figure 7. Exposed 18Day regenerating adult Axolotl cord *in situ.* The cord was completely transected and allowed to regenerate. A mass of meningeal extracellular matrix material and cells occupies the space between the cranial and caudal outgrowth and surrounds it. The meningeal material has a”frothy appearance. Steromicroscope image.

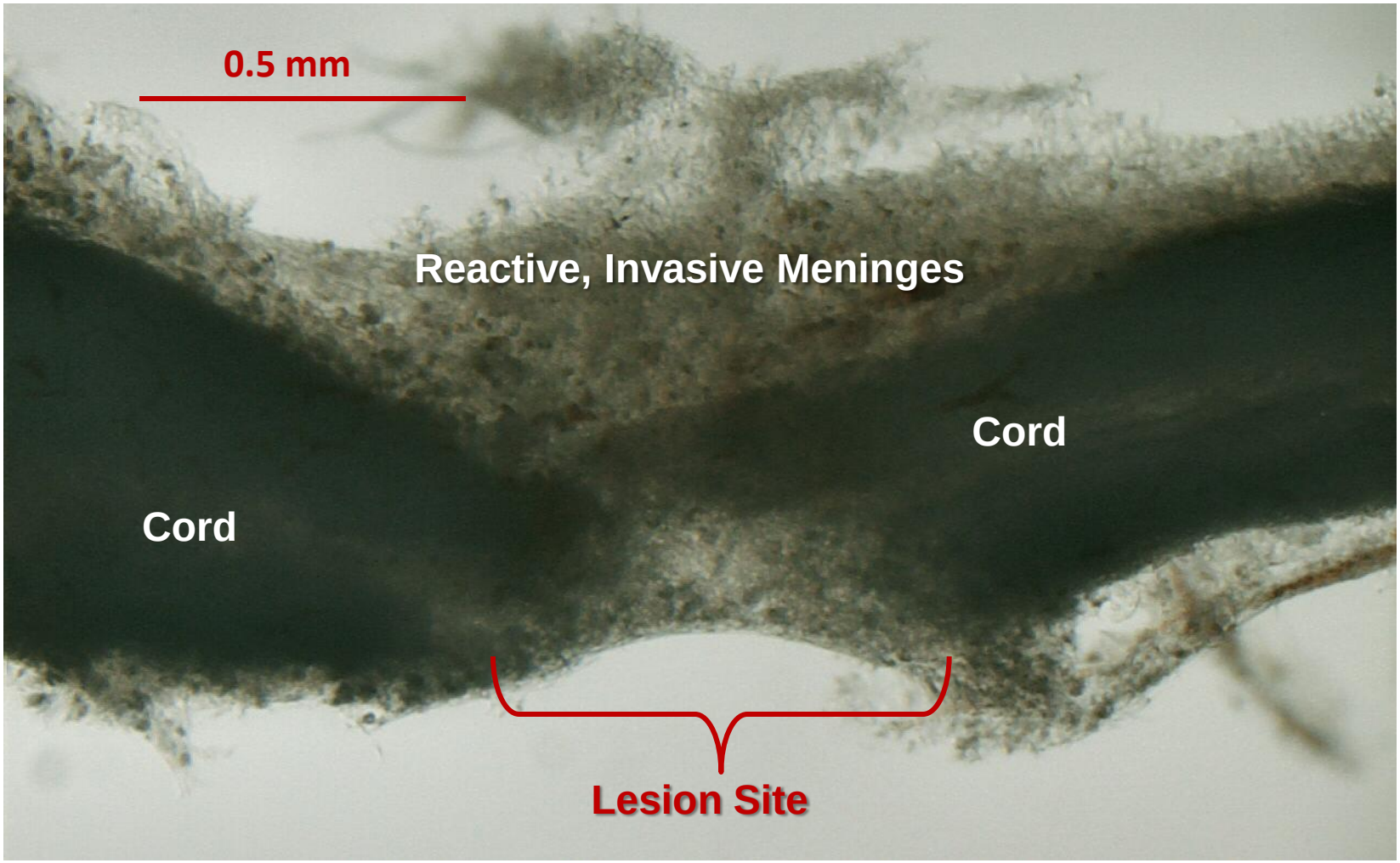


Figure 8. The 18Day regenerating cord is dissected free of the bone. The mass of meningeal material around and between the cranial and caudal stumps is visible. Whole mount preparation, stereomicroscope image, backlit to show meningeal matrix

Meningeal Matrix Components

Fibrillar collagen fills tissue spaces and is a large component of this material produced by meningeal cells; see the blue stained material in the sectioned lesion site (**Figure 9**). Meningeal cells grow into the lesion site using this material and ependymal cells grow into it from the spinal cord. A simplified diagram is shown in **Figure 10**.

A heavy layer of collagen and proteoglycan (extracellular matrix) covers the surface of the cord for about 0.5 cm from the lesion site and covers the cord cells growing out from the stump (**Figure 11**). Yellow stained collagen is seen mixed with blue stained sulfated proteoglycan. Osteoclasts/Giant Cells are seen on the reactive meninges enveloping the regenerating end, close to the lesion site (orange/reddish cells).

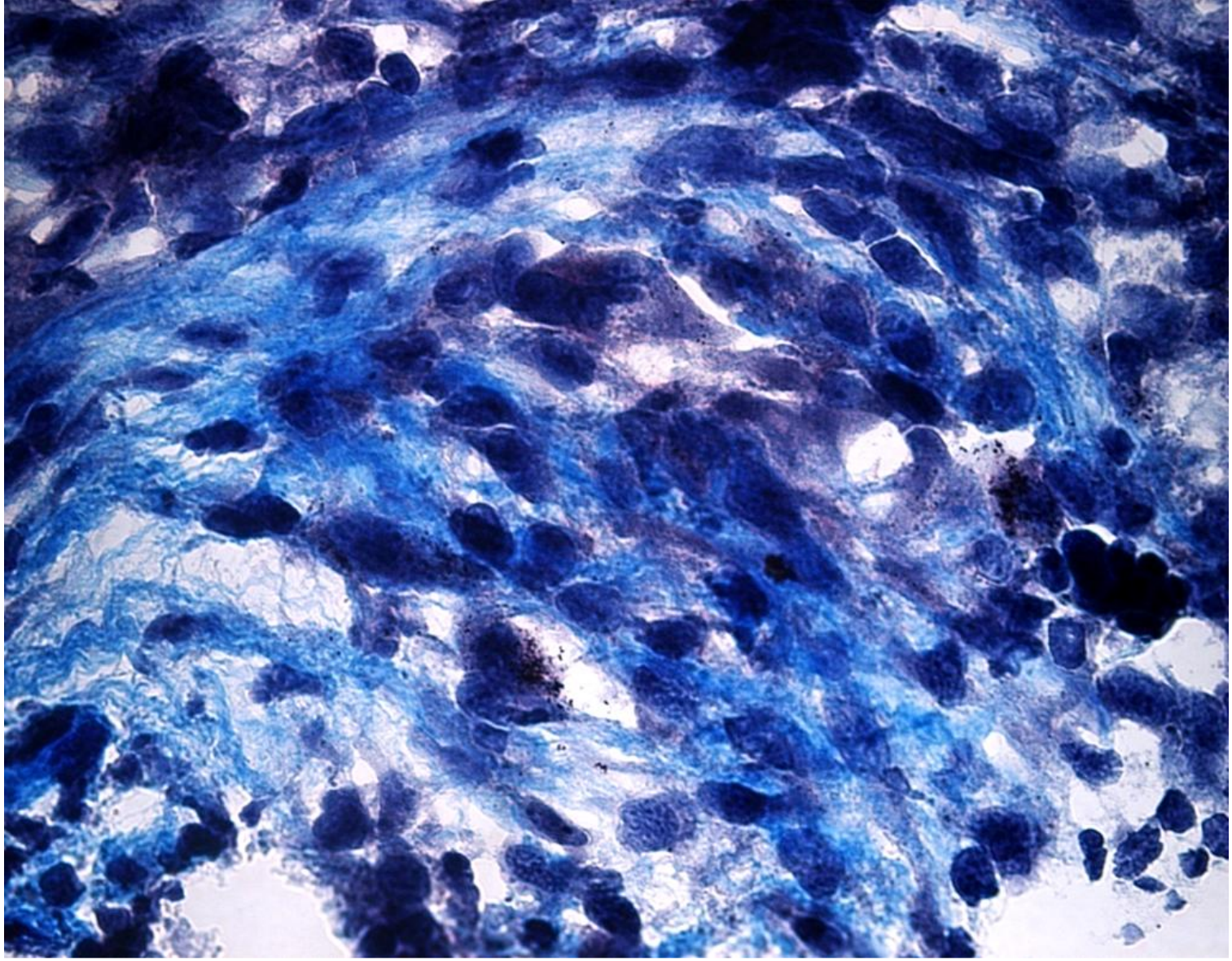


Figure 9. 15 micron parasagittal section through the ependymal outgrowth and meningeal invasion in a 14Day regenerate adult axolotl cord. Masson Trichrome stained: Aniline Blue show collagen accumulation. Purple shows cell cytoplasm/nuclei

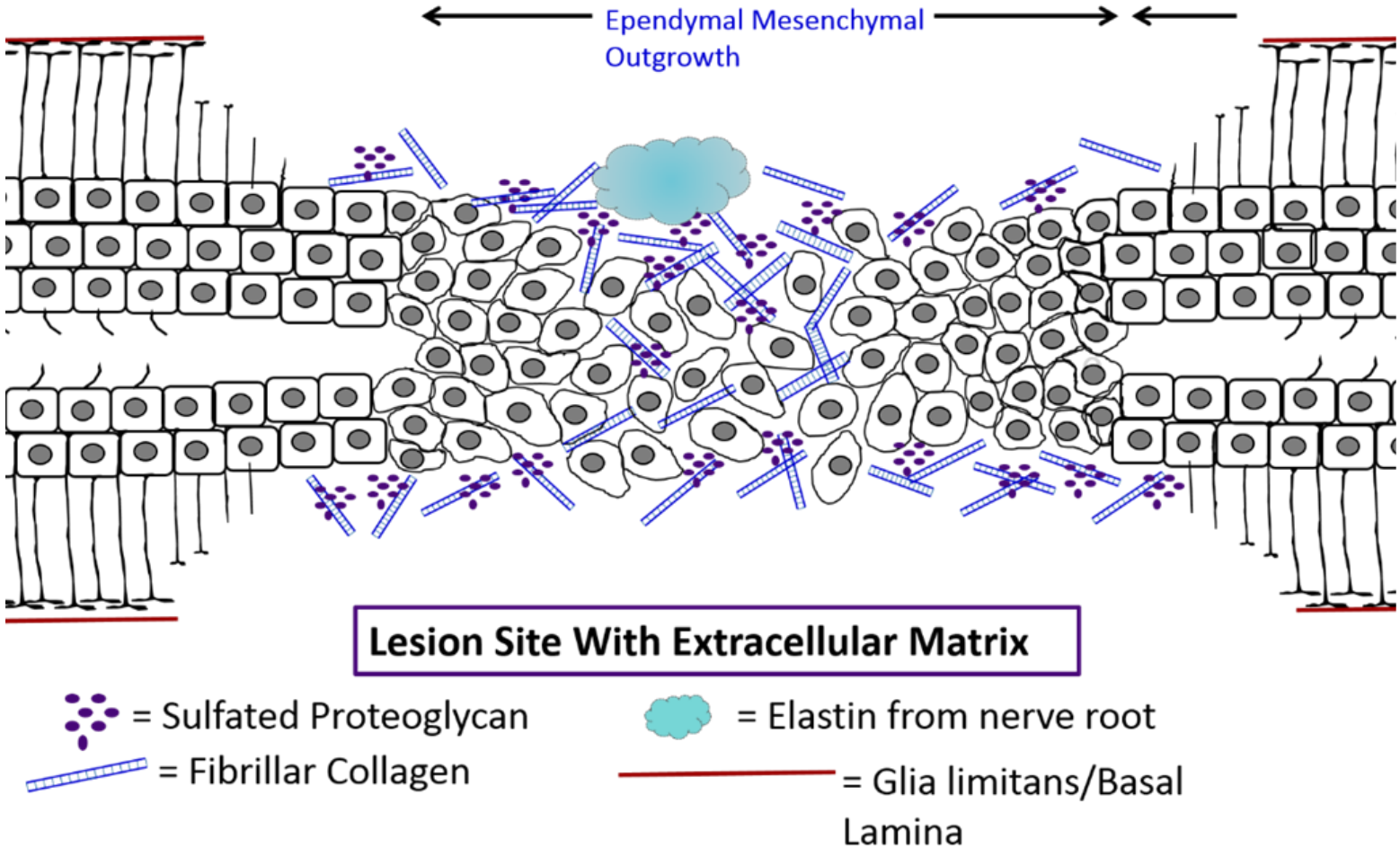


Figure 10. Shows a cartoon of ependymal outgrowth among fibrillar collagen and proteoglycan within the lesion site. Compare with tissue in **Figure 9**.

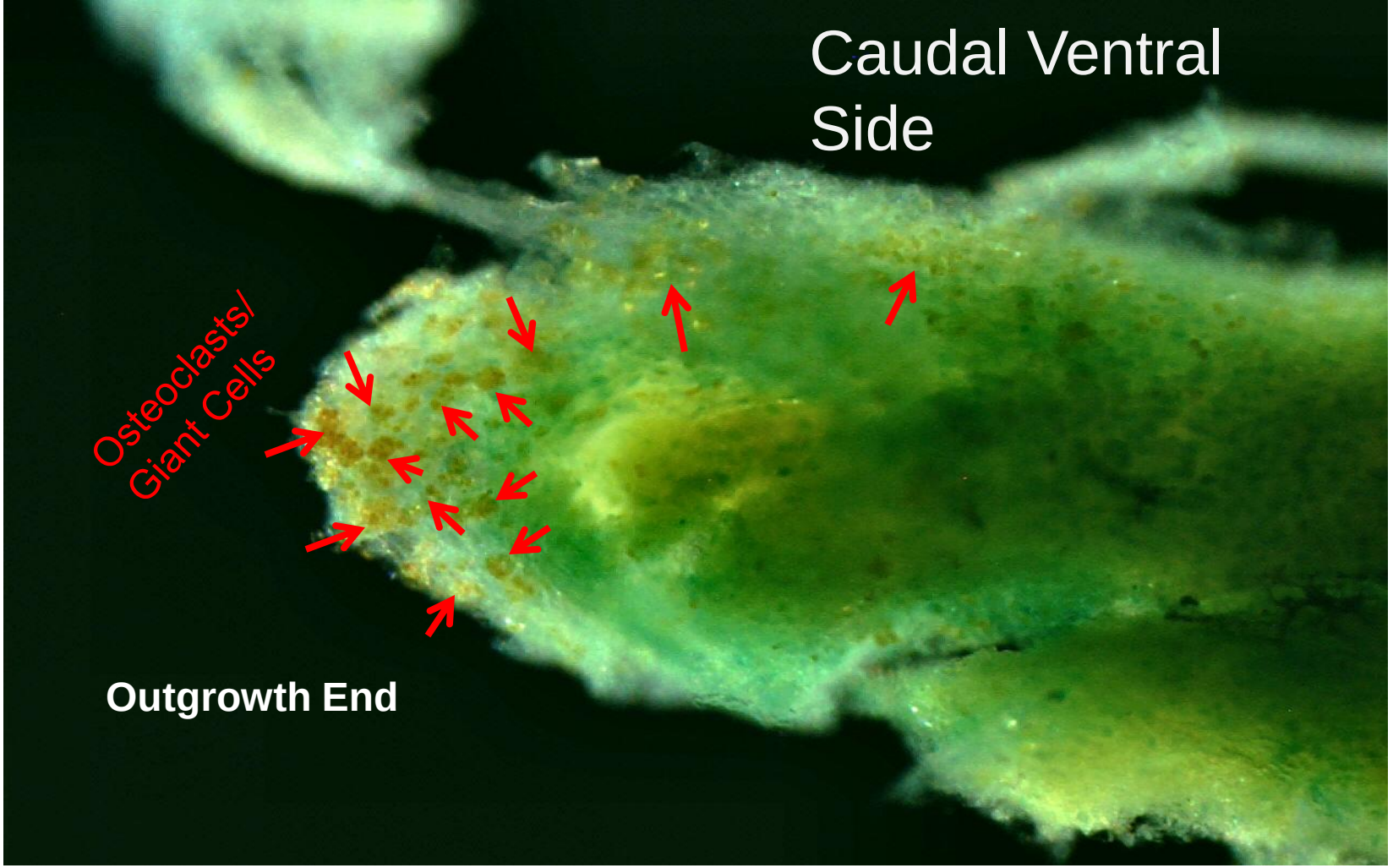


Figure 11. Regenerating end of cord from 18 days post-lesioning. The surface is covered by meningeal matrix. Metanil yellow shows the presence of fibrillar collage (yellow), Alcian Blue stain shows the presence of sulfated proteoglycan (blue, or green in conjunction with the collagen stain). The red arrows show the presence of osteoclasts, which appear orange/reddish due to lipid content and the staining procedure (red arrows)

Are These True Osteoclasts??

The cells are found on/in the meninges, clustered within ~1.5 mm of the lesion site. Their lipid content makes them easy to spot. (**Figures 12,13**). The morphology is seen as osteoclast like as the cells migrate from the meninges in culture (**Figure 12**). The multinucleated state is easiest to see with a nuclear stain (**Figure 12 inset**)

Osteoclast and Giant Cells have a characteristic cytoskeletal structure that is essential to their ability to seal to their substrate. The use their close adhesion to deliver a characteristic set of proteolytic enzymes. We show that the characteristic podosome ring is present (**Figure15**) and that the characteristic cysteine protease, Cathepsin K is produced by osteoclasts sealed to the reactive axolotl meninges (**Figure 15**).

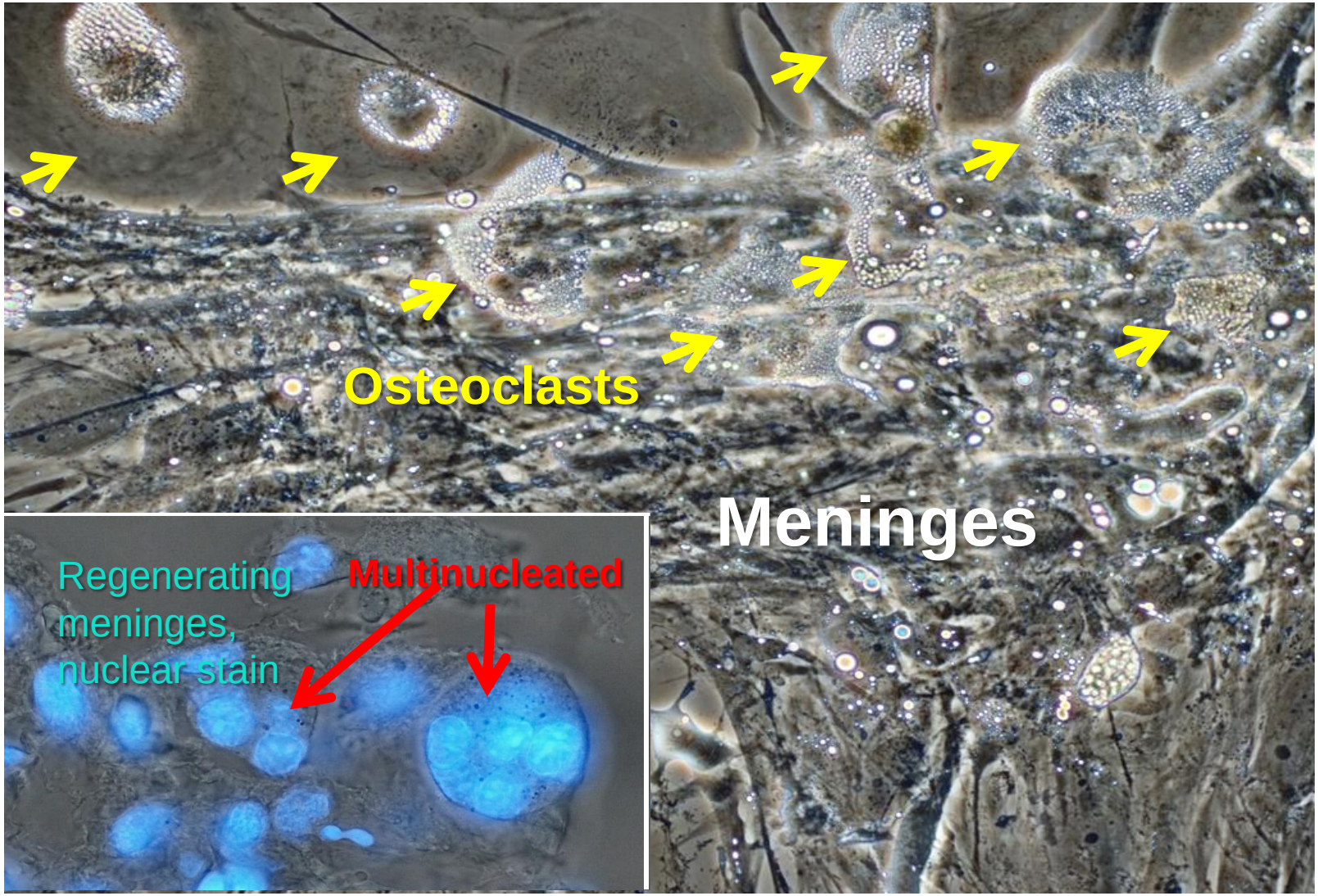


Figure 12. Reactive meninges was removed and cultured on a fibronectin-coated dish. Osteoclasts migrate from the meningeal explant (yellow arrows). Note the characteristic accumulation of lipid droplets around the nucleus/nuclei. Phase contrast image, inverted optics compound microscope. The inset image shows a combined DAPI DNA/nuclear stain (blue fluorescence) combined with a DIC image. Fluorescence/DIC microscope.

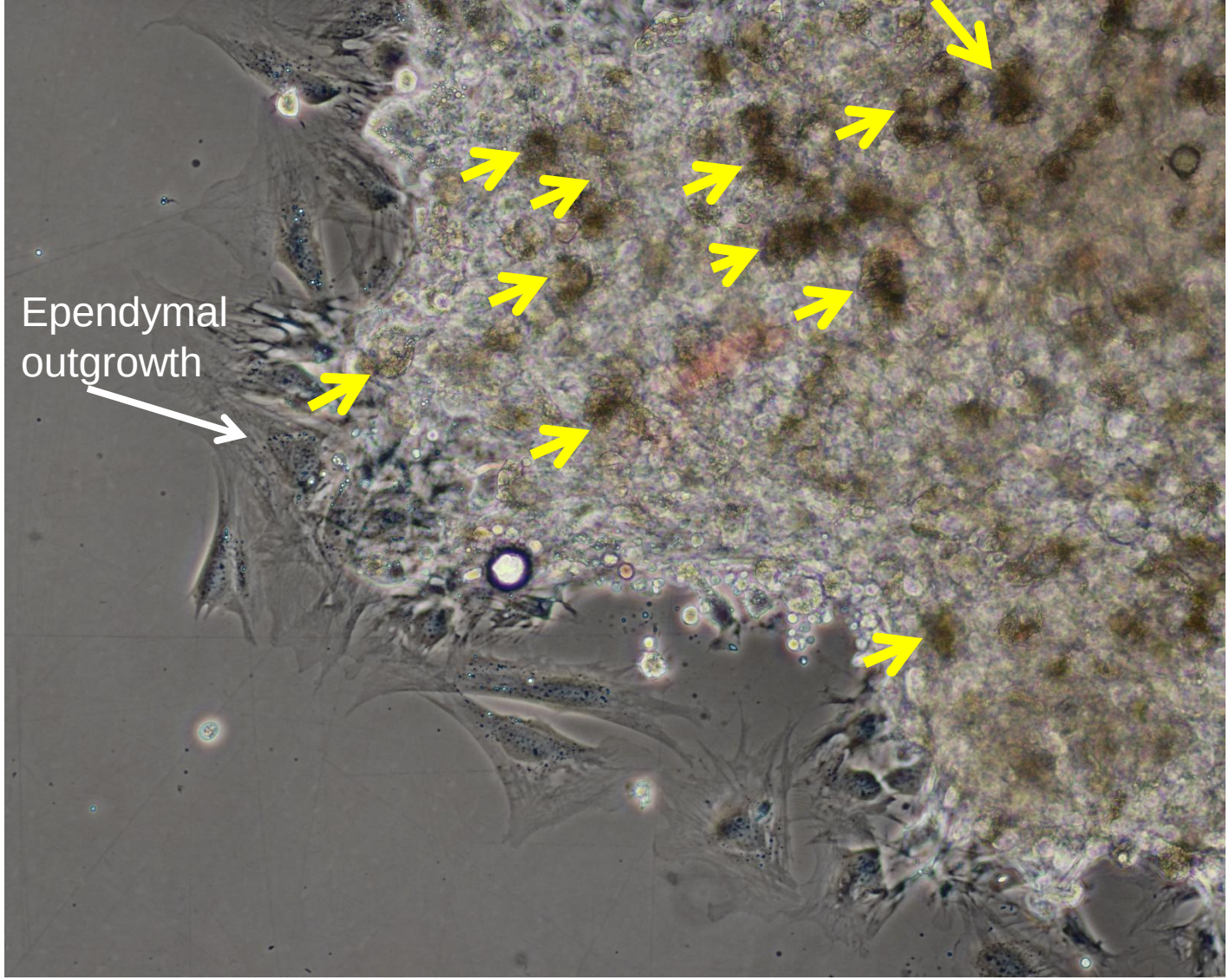


Figure 13. A piece of the regenerating outrowth was harvested 14Days after surgery and placed in culture. This explant has mixed ependymal cells and meninges. Yellowish osteoclasts are present on the meninges, clustered near the lesion site outgrowth/ingrowth. Yellow Arrows

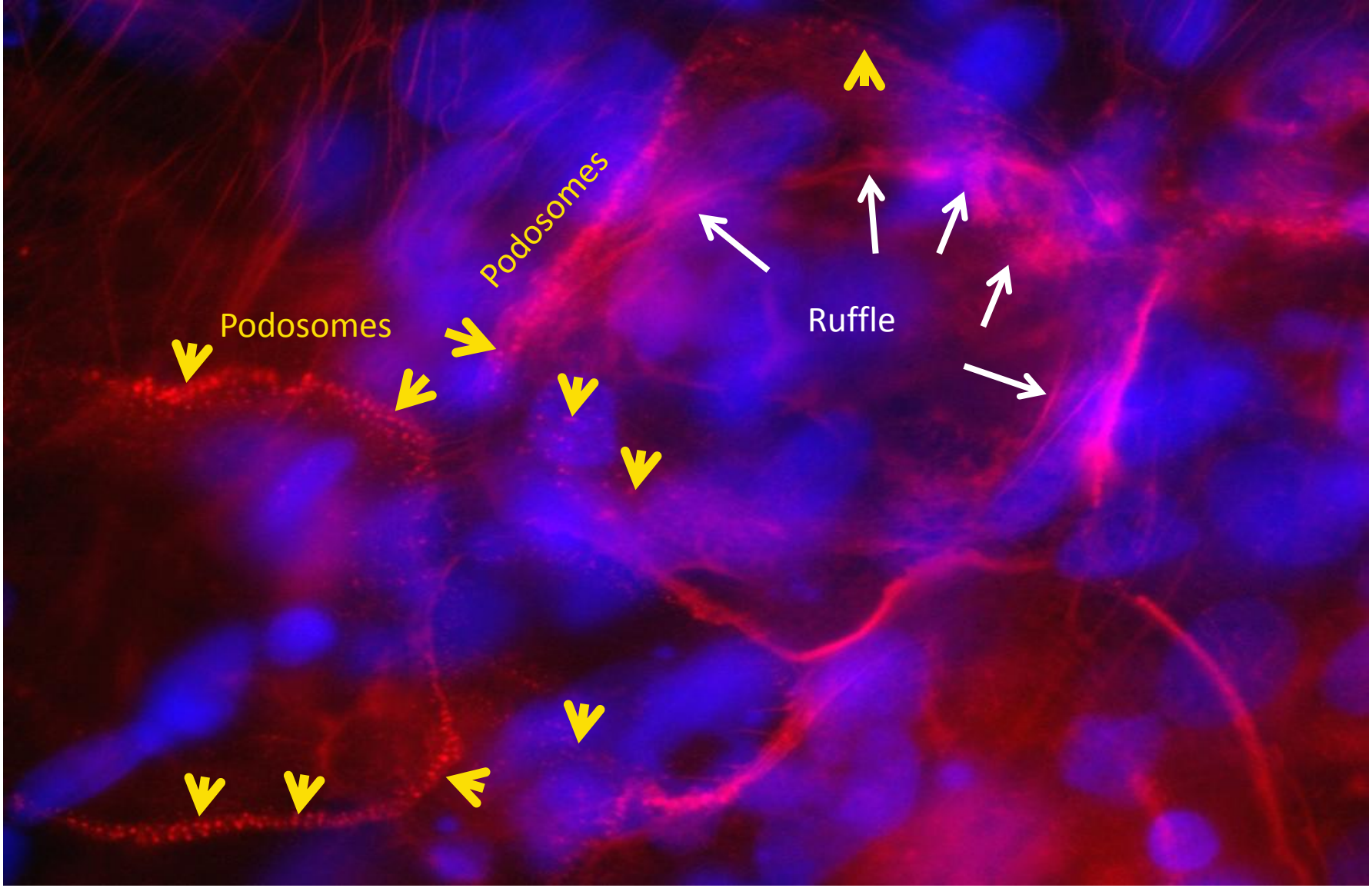


Figure 14. Whole mount Rhodamine-Phalloidin, filamentous actin (cytoskeletal protein, red) fluorescent stain of meninges on a cultured 2 week regenerate meningeal explant. Group of osteoclasts with actin-containing podosomes (groups of dots), sealing ring with actin-containing ruffled membrane. A group of several osteoclast/giant cells is seen. DAPI stain (blue) shows groups of nuclei. Non-confocal image, inverted 'scope.

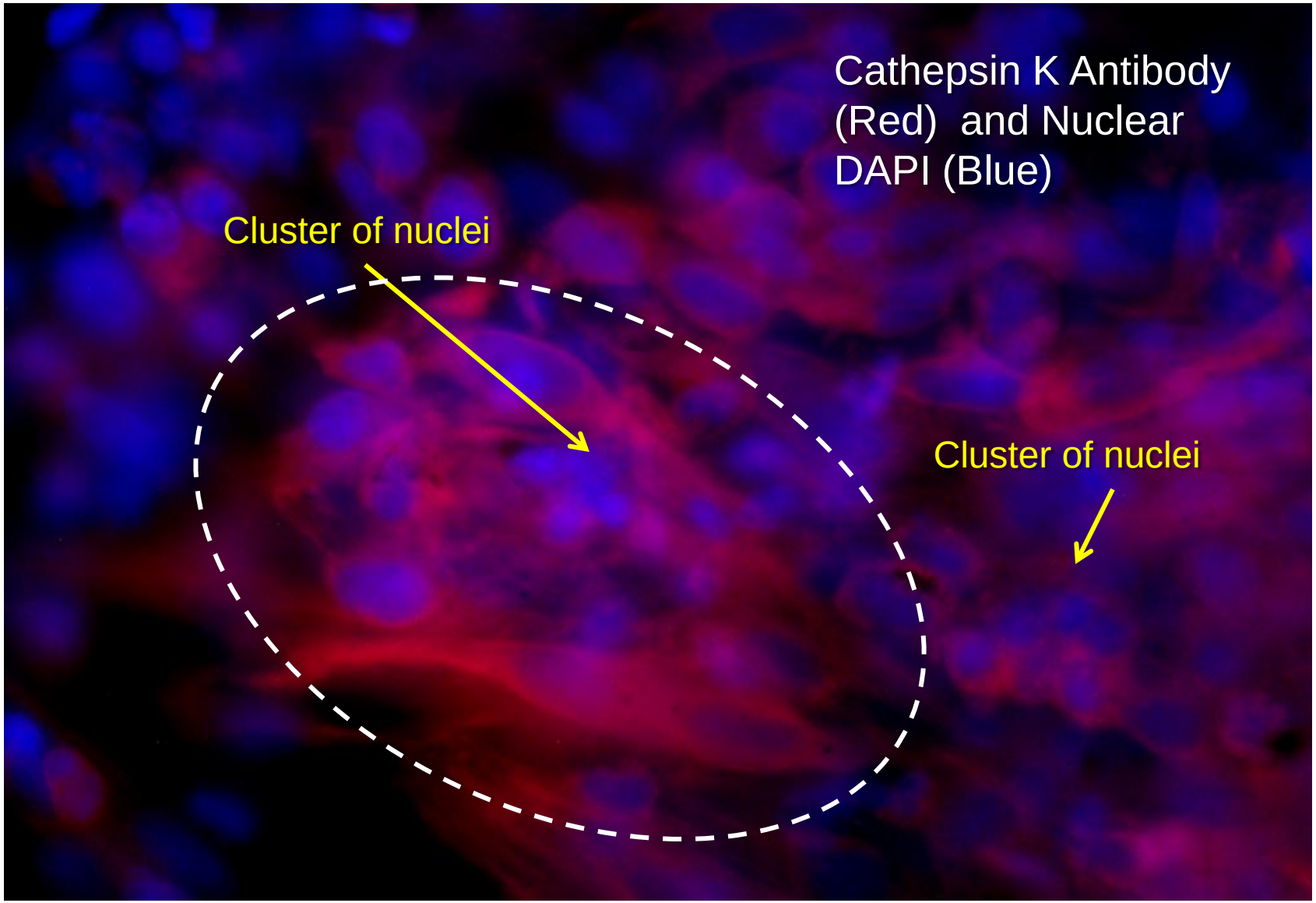


Figure 15. Whole mount fluorescence image of 16Day regenerating cord meninges. Stained with anti-cathepsin K antibody for osteoclast/giant cell cysteine protease(red). A large cathepsin K-positve cell cluster is circled. Counterstained with nuclear stain DAPI (blue).

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