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THE DEVELOPMENTAL EXPRESSION PATTERN OF TUJ1 AND TENASCIN IN THE UTRICLE AND SACCULE OF JAPANESE QUAIL

by

Emily Elizabeth Bradof

An Independent study submitted in partial
fulfillment of the requirements for the degree of:

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J. David Dickman, Ph.D., Independent Study Supervisor

Introduction

Vestibular System Overview

The vestibular system is the portion of the ear responsible for maintaining balance. The vestibular system is comprised of three semi-circular canals (horizontal, posterior, and superior) and two otolithic organs called the utricle and saccule. Other animals, such as birds and reptiles have a third otolith organ called the lagena. The three semi-circular canals sense rotational head movements, whereas the otolith organs sense linear acceleration and head orientation relative to gravity (Stach, 1998). The vestibular labyrinth is located in the temporal bone alongside and posterior to the cochlea (Dickman, 2002). The outer portion of the labyrinth is called the bony labyrinth and it functions as a protective structure that encloses the delicate vestibular components within the membranous labyrinth. The bony labyrinth is filled with perilymph, which serves to bathe the exterior surfaces of the vestibular and auditory organs. The membranous labyrinth is filled with endolymph, which covers the vestibular receptors as well as the auditory receptors in the cochlea (Dickman, 2002). The otolith organs, called the utricle and saccule, and the ampulla of each semicircular canal have specialized sensory epithelia (Stach, 1998). The vestibular sensory system allows humans and other animals to maintain their balance and stabilize their gaze on a fixed object while moving. Sensory information from the vestibular system is also sent to the cerebral cortex and cerebellum. These areas of the brain incorporate many types of sensory information such as motor, visual, somatosensory, vestibular and visceromotor to coordinate necessary

motor reflexes (Dickman, 2002). Patients in otolaryngology clinics with disorders of the vestibular system often experience dizziness, vertigo, and nausea.

Otolith Organs

The otolith organs detect linear motion, using gravitational and inertial cues, and send the information to the brain via neural signals. Each otolithic organ is an arrangement of hair cells that makes up the neuroepithelial transduction mechanism for the vestibular end organs (Katz, 2002). The sensory receptors of the otolith organs consist of an epithelial layer of cells. This layer of cells is made up of hair cells and support cells. The sensory epithelium is covered by a gelatinous matrix called the otoconial membrane. On top of this lies a layer of calcium carbonate crystals referred to as otoconia. Otoconia are displaced by linear accelerations of the head due to inertial forces. When otoconia are displaced, they move the otolith membrane and pull the hair cell stereocilia, which stimulates the hair cells and sends an electrical impulse to the brain. The hair and support cells in the sensory epithelia of the otolith end organs are arranged in a highly organized way in adult animals.

Hair Cells

Stereocilia project from the top of the hair cells into the otoconial membrane and are very sensitive in detecting movement. There are about 60 to 100 stereocilia arranged hexagonally on each hair cell as well as a single kinocilium. Small filaments connect each stereocilia together, so that the bending of one stereocilia directly affects its neighbor.

There are two types of hair cells, type I and type II, and they have different innervation patterns by eighth cranial nerve fibers. Type I hair cells are shaped like a

chalice while type II hair cells are cylinder-shaped. Type I hair cells are ensconced in a nerve calyx, which is an afferent terminal while type II hair cells are innervated by synaptic boutons (Dickman, 2002). In adult animals, type I and type II hair cells are located in different places on the sensory epithelia of the otolith organs. Within the utricle and the saccule, there is a narrow strip of epithelium called the striola, which encompasses the polarization reversal line. In the utricle, hair cells are organized with the kinocilium on the same side as the striola; however, hair cells in the saccule are polarized with the kinocilium away from the striola. Hair cells are oriented in these different directions along the vestibular organs in order to detect specific kinds of movement (Dickman, 2002). Type I hair cells are clustered along the striola. Type II cells make up the majority of the hair cells in the extra-striolar portion of the epithelium. The striola line is curved and runs near the edge of the macula, so only specific hair cells are deflected as a result of linear motion along a specific direction. Unfortunately, little is known concerning how and when this highly specific pattern of type I and type II cells develops.

Goals

The development of the otolith organs was the primary focus of my independent study. The goal of my independent study project was to identify a protein that is expressed only in type I hair cells that can serve as a marker for these unique cells. This specific marker was used to establish the temporal and spatial distribution of type I hair cells during the development of the Japanese quail. Two questions were posed: 1) When do type I hair cells first appear in the otolithic vestibular epithelia of Japanese quail and 2) What is the regional distribution of type I hair cells in the saccule and utricle of

Japanese quail throughout development? The calyces of the type I hair cells are one of the structures that will be marked for developmental patterns in my independent study. Since the calyces surround only type I cells, they were used them to identify the presence of type I hair cells in the vestibular otolith sensory epithelia.

Materials and Methods

The method used to identify calyceal terminals and type I cells was whole-mount fluorescent light immunohistochemistry. Whole-mount immunohistochemistry utilizes the natural antigen-antibody reaction within an intact piece of tissue to label particular proteins with a chemical that emits light when stimulated. This emitted light is then detected using both standard fluorescent microscopy and confocal microscopy. The purpose of this experiment was to see how, when and where type I hair cells appear in the utricle and saccule by observing patterns under fluorescent microscopy. The developmental expression patterns of two different proteins were followed in this study.

TUJ1

The TUJI antibody, which binds to a neuron specific class III beta tubulin, has been widely used to study developing neurons and their precursors (Molea et al, 1999). Beta tubulin is a protein that makes up the structural constituents of microtubules, which provide cell structure and support along with locomotion and cellular transport. Beta tubulin (TUJI) is expressed in neuronal axons of all age groups and, because it labels calyx units, is an indirect marker for the presence of type I hair cells. Using this antibody, neuronal cell types can be observed through their differentiation, migration and distribution.

Tenascin

"Tenascin is a large, oligomeric glycoprotein that has also been called tenascin-C, and cytotactin" (Swartz and Santi, *1998*). Tenascin is found in many places such as the mammary gland, kidney, intestine, skin and nervous tissue and in tissues undergoing embryogenesis and oncogenesis. Tenascin is linked to cell migration, differentiation, proliferation, attachment and detachment and is considered a homeostatic factor in tissue repair. "Tenascin-C is an extracellular matrix gycoprotein found in the embryo at sites of epithelial-mesenchymal interactions, cell motility and tissue modeling" (Tucker, 1998). In the chinchilla vestibular system, tenascin was observed (using immunoreactity) in the calyces at the base of hair cells in the saccule and utricle maculae and crista ampularis. Tenascin was present "between the VIII nerve calyces and type I vestibular hair cell body" (Shwartz and Santi, 1998). However, the regions of the hair cell containing tenascin were only half way up the nerve calyx. Tenascin may act as a structural component and help regulate the shape of hair cells and perhaps be linked to guiding neural innervations of hair cells.

Animals

There are several reasons why Japanese quail (*Coturnix coturnix japonica*) were used in this study. Avian eggs are ideally suited for developmental microgravity research. The incubation period for quail is only 16 days and once hatched the young birds require no parental care. Also, quail finish their vestibular development before they hatch, unlike mammalian models. Because of their small size, Japanese quail thrive in small cages, are inexpensive to keep and mature in about 6 weeks (Dickman et al, 2003).

Protocol

The eggs were incubated in a commercial forced air incubator that was set at 37.5 degrees Celsius with humidity at 55%. In the incubator, the eggs were rotated once every hour. Tissue was harvested at seven different developmental time-points ranging from embryonic day 6 (E6) to adult. In dissection, the embryo was removed from the egg and anesthetized by cold narcosis in 4 degrees Celsius saline. When sacrificing an adult quail or hatchling, Carbon dioxide gas was used. Next, the embryo or hatchling/adult was weighed and then decapitated. The head was bisected and each half was placed into 0.1M phosphate buffered saline (PBS). The brain was removed to expose the vestibular system in the temporal bone. The otoconia above the utricle and saccule were removed using PBS. The temporal bones were placed in 4% paraformaldehyde in phosphate buffer for 30 minutes. Next, the utricles and saccules were dissected from the temporal bone and placed in 0.1 M PBS at 4 degrees Celsius overnight (Huss and Dickman, 2003).

The next day, non-specific binding sites in the utricles and saccules were blocked for 90-120 minutes in 2% Normal Horse Serum (NHS), 2% Normal Goat Serum (NGS), 1% Bovine serum Albumin (BSA) and 0.2% Triton-X-100 (in PBS). Titron-X-100 is a detergent that allows the antibodies to reach their target proteins. Primary antibodies were diluted by adding 2 microliters of TUJ-1 antibody (Covance, 1:500) and 5 microliters of anti tenascin antibody (Chemicon, 1:200) to 1 ml PBS/0.2% Triton-X-100. Then, 20 microliters of NHS was added and mixed in the vortex. The tissue was incubated overnight at 4 degrees Celsius in primary antibodies. Next, the tissue was rinsed five times in PBS over 15 minutes. It was then incubated for two hours in

secondary antibodies which consisted of a 1:150 dilution of biotinylated anti-mouse IgG (Vector), and a 1:500 dilution of Cy-3 conjugated anti-rabbit IgG (Amersham) in PBS with 0.2% Triton X-100. This was rinsed five times over 15 minutes and incubated for one hour in streptavidin-conjugated Alexa 488, diluted 1:500 at room temperature, on a shaker. Cy-3 and Alexa are chemical fluorophores that give off light at two different wavelengths when excited by the microscope's high intensity light source. Streptavidin has a high binding affinity to biotin and because it attaches at a 5:1 ratio (respectively), it serves as an amplification technique. Next, the tissue was rinsed five times in PBS over 15 minutes. Then, the tissue was bathed in a 1:1000 dilution of bisbenzimide that reacts with DNA chromosomes and the nucleus of cells (nuclear stain). This is a chemical bond, not immunological and gives off a blue light, which helps to find the location of the cell and aids in description. Negative controls were used (no primary antibodies) as well as positive controls (chick lagena). The tissue was then mounted on slides in glycerol/PBS (9:1) and coverslipped. It was made certain that the hair cells were facing up when mounted on the slide. The utricles and saccules were then digitally photographed using an inverted fluorescent microscope or a laser confocal microscope. Using the Image Pro software, false color was added to the original black and white images. In some cases, separate images of the two markers were merged together into a single picture.

Results

Our experiments show that TUJ1 strongly labels the axons of the nerve fibers in utricles and saccules of all ages. An example of this labeling is Fig. 1, which shows the neural axon fibers of an E12 saccule marked by TUJ1. Figures 2-5 are images of the

utricle and saccule with TUJ1 at early, middle and adult stages. TUJ1 will label the calyces of Type I hair cells at different developmental stages. At E8, TUJ1 shows very little specific staining in the sensory epithelium (Fig.2) at this early stage. In Fig. 3, embryonic stage E10, TUJ1 stains distinct round areas in the epithelium, which are in close proximity to each other. Fig. 4 displays the saccule at E15, which shows how the stained calyces are clustering around the semicircular striola region (marked by arrows in image). Fig. 5 is an image of P7 saccule, which shows clear calyces stained green by TUJ1. A clear differentiation line in the middle of the striola is shown in Fig. 6 of the adult utricle where TUJ1 positive calyces do not appear. This area marks the line of polarity reversal where only type II hair cells exist.

Figures 7-10 are images of the utricle and saccule labeled with tenascin at early, middle and adult stages. Tenascin will preferentially stain receptor proteins at the base of the type I hair cell. In fig. 7, embryonic stage E8, a definite outline of many hair cells can be observed, but the location of these cells is disperse and they are less defined, suggesting early formation. By the middle stage E10, Fig. 8, the labeled hair cells are beginning to cluster in a more definitive pattern and the tenascin staining is more complete for some cells. However, other cells in this image show only punctate labeling which suggest early development of newly forming, or immature, cells. By E15, Fig. 9 and adult, Fig. 10, the tenascin positive cells are cleary visible. These cells surround the striola line in the utricle and mark the fully formed type II band (delineated by arrows in Fig. 10). The type I cells are completely developed with fully labeled bases. The extrastriolar regions of the epithelium show very little staining.

The Image Pro Software was used to merge images of TUJ1 and tenascin together. Fig. 11 reveals a merged image at E10, where we see clear calyces stained green by TUJ1 encompassing the tenascin stained hair cell base, which are the fine red rings. Fig. 12 and 13 show TUJ1 merged with tenascin in the E12 utricle and saccule respectively. A clear illustration of the calyx and type I hair cell base is shown in Fig. 14 for an adult utricle, where there are several red stained type I hair cells encompassed by the green calyces. Fig. 15 displays a confocal microscope image of an adult utricle with an excellent view of the striola region and type II band (delineated by arrows) as well as the base of the hair cells (red rings) with surrounding calyces (green spheres).

Discussion

Conclusions

In this study, we observed that embryonic day 10 is the earliest point when distinct type I hair cells are forming and their calyx nerve endings are beginning to develop in the distinct region of the epithelium known as the striola line. This area runs parallel to the lateral edge of the utricular epithelium and in a semicircular pattern in the saccular epithelium of Japanese quails. Once it is established at E10 during embryonic development, the spatial pattern of type I hair cells does not radically change as the animal ages. In our study, we noted that TUJI is expressed in calyx nerve endings starting at E10, which, in adult quail utricles, will mark the outline of calyceal terminals in the striola region. Calyceal terminals are seen as spherical clusters of type I hair cells under fluorescent microscopy. Tenascin is expressed in type I hair cells near the interface with the calyx nerve ending starting at E10, although mostly immature cells were observed.

Tenascin seemed to mark the lower portion of the type I hair cell while TUJ1 marked the calyx terminal surrounding the type I cell. In merged images and confocal microscopy, tenascine (red circles) marked the base of the type I hair cells while TUJ1 (green centers) labeled the calyx structures deeper into the tissue. These antibodies allowed us to follow the cellular maturation of the quail otolith sensory epithelia as the animals developed form early embryos to adults. In summary, at E10, the type I cells were only beginning to develop and calyx formations were immature. At E12, the type II band and striola region is observed, but was not well defined. At E15, type I cells were more complete and the type II band and striola region was clearly formed, and by adult, was fully developed.

Future Research

Future research will use on-section immunohistochemistry to accurately define the expression pattern of Tenascin in relation to the calyx nerve ending. Also, researchers will use the current antibodies to compare the type I hair cell/calyx patterns in quail raised at 2 G with those raised in a 1 G environment to see if gravity is a necessary factor for the normal development of the vestibular sensory epithelia.

Acknowledgements

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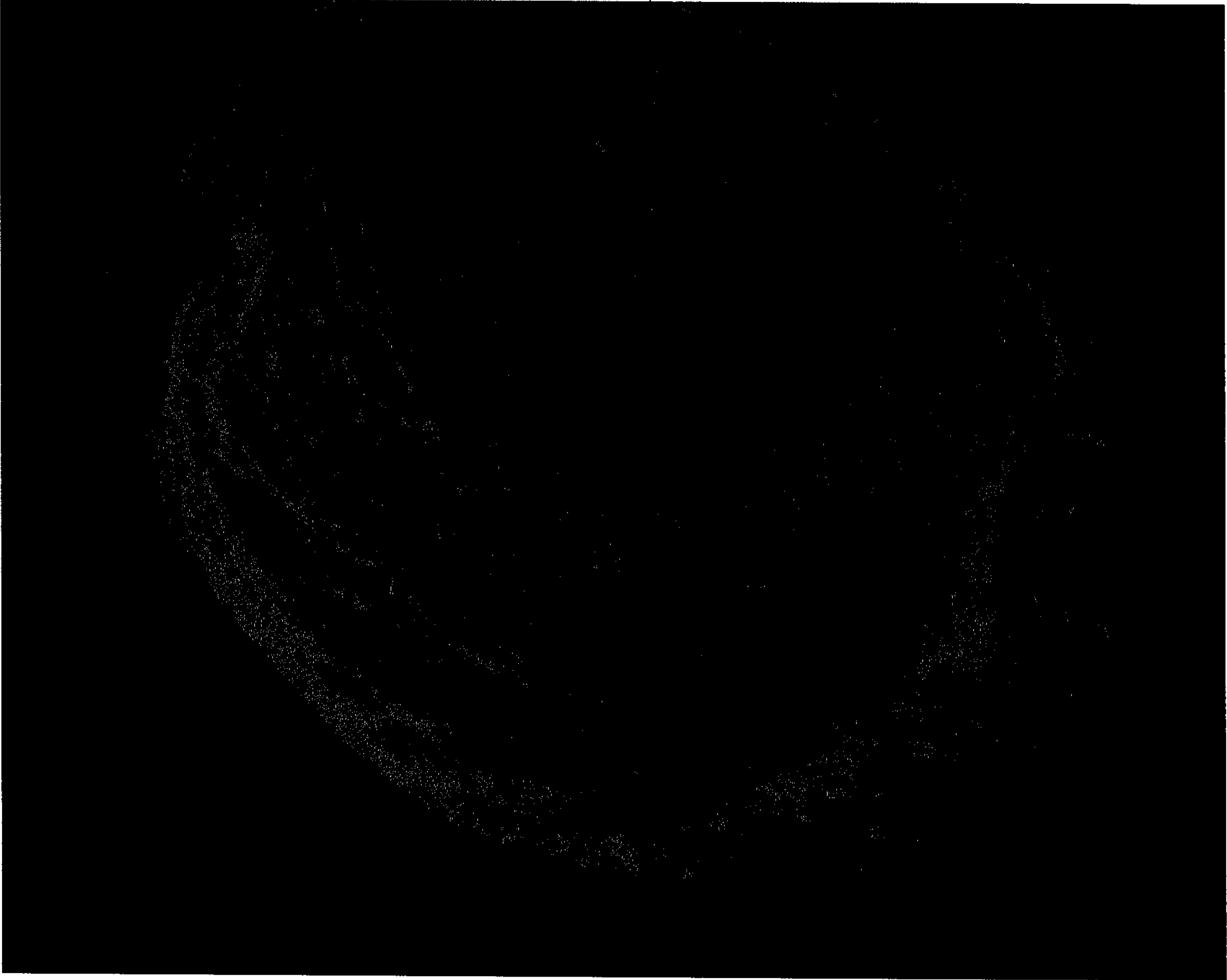

Fig. 1. E12 Saccule TUJ1 10x

Fig. 2. E8 Utricle TUJ1 20x

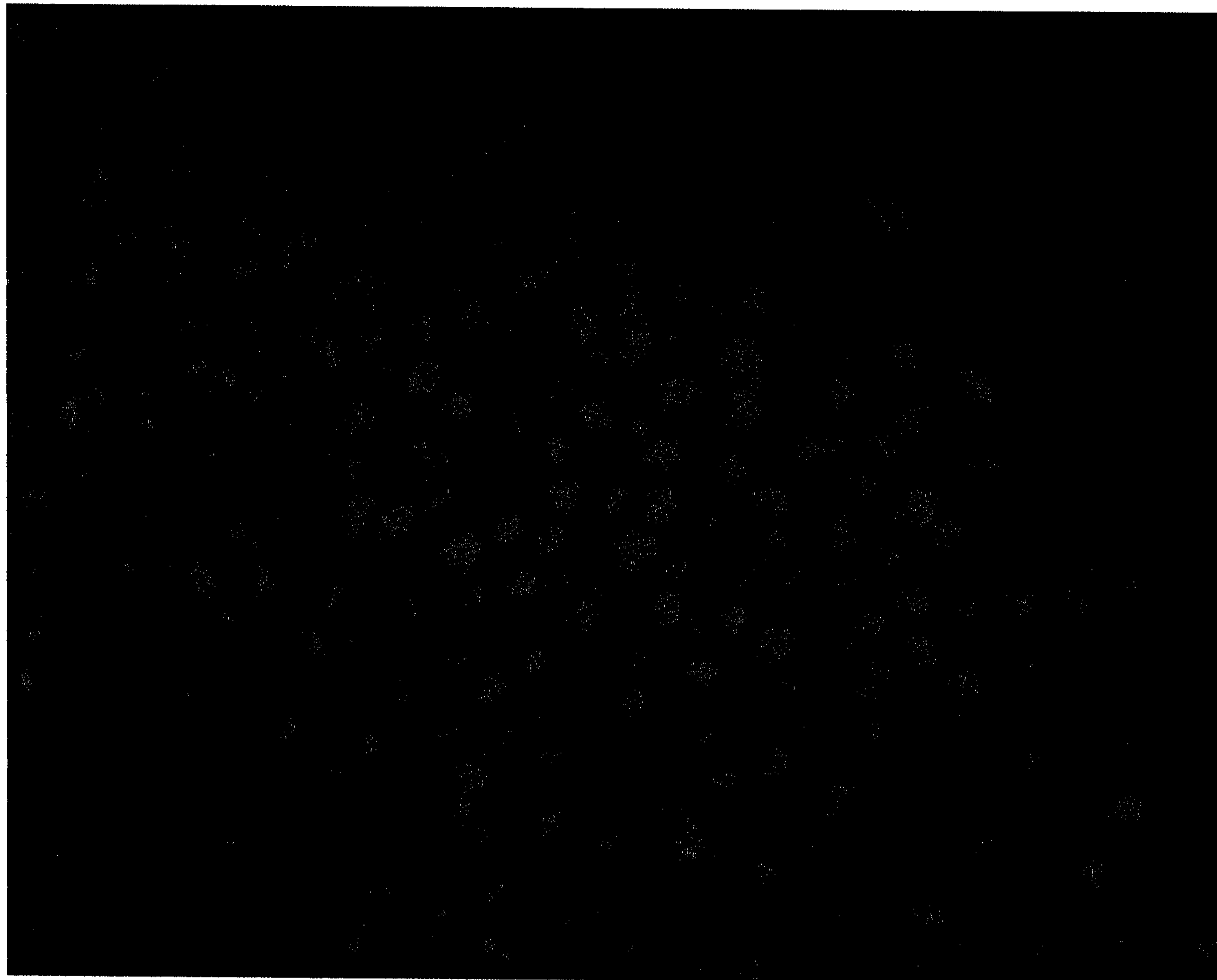

Fig. 3. E10 Utricle TUJ1 60x

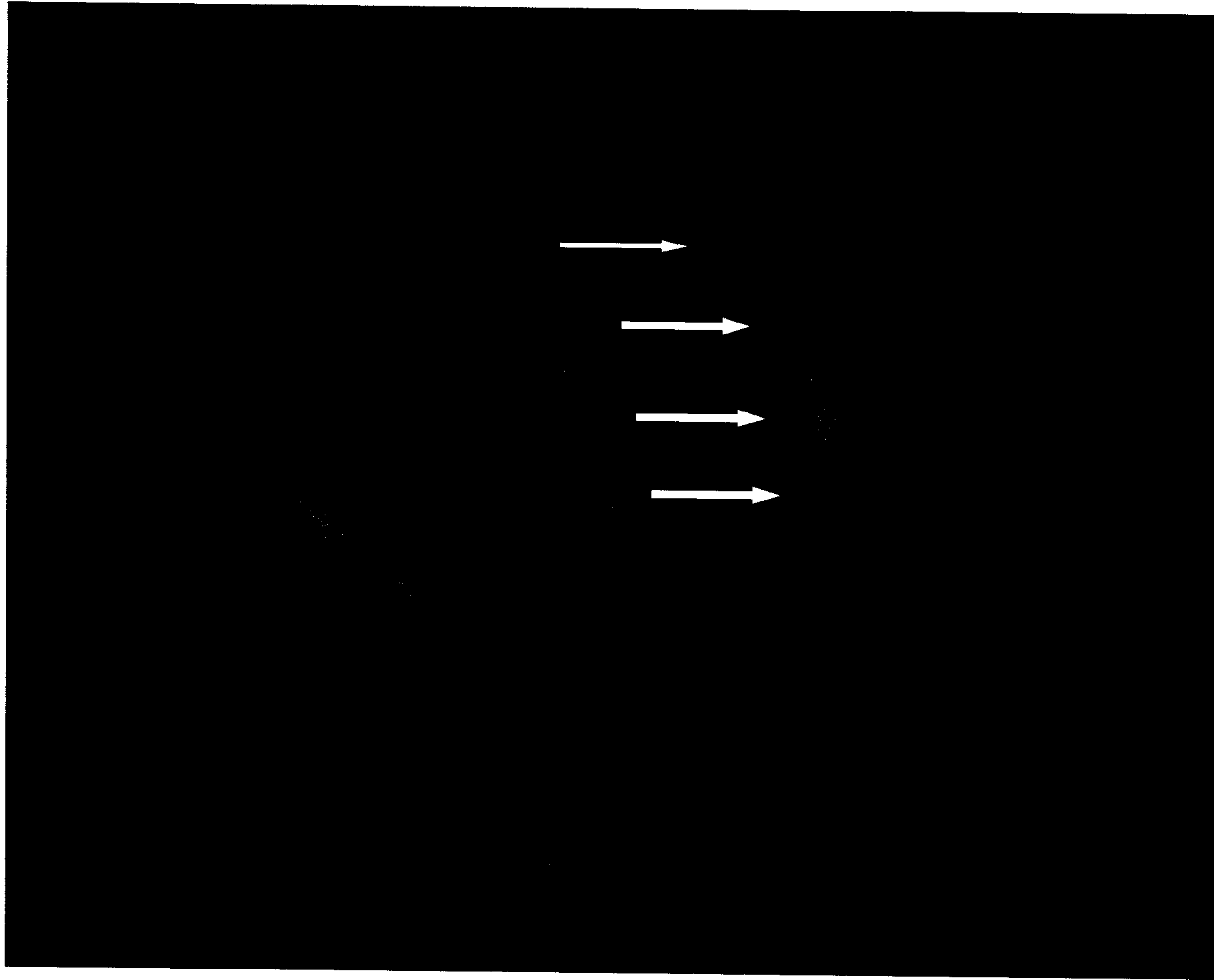

Fig. 4. E15 Saccule TUJ1 10x

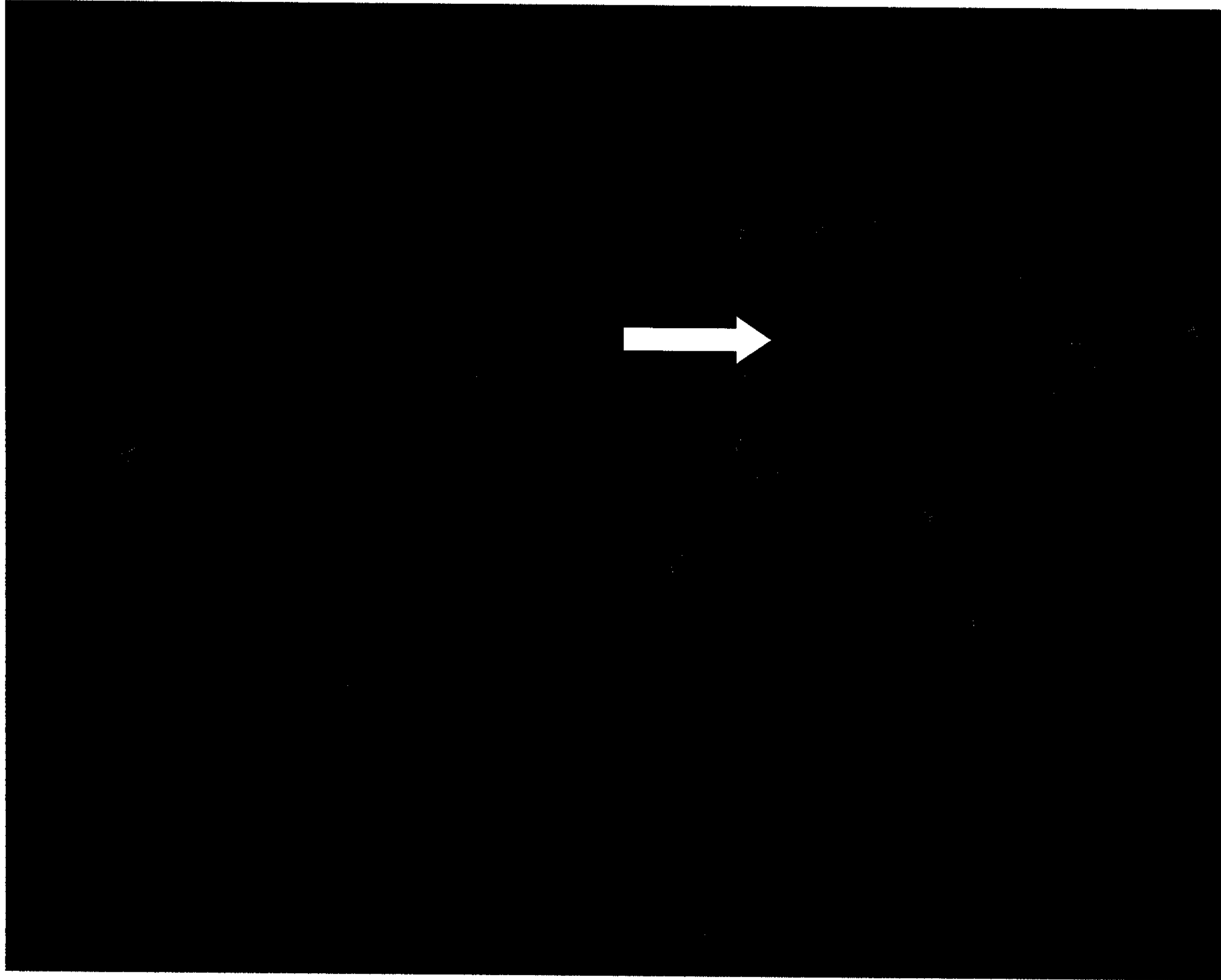

Fig. 5. P7 Saccule TUJ1 60x

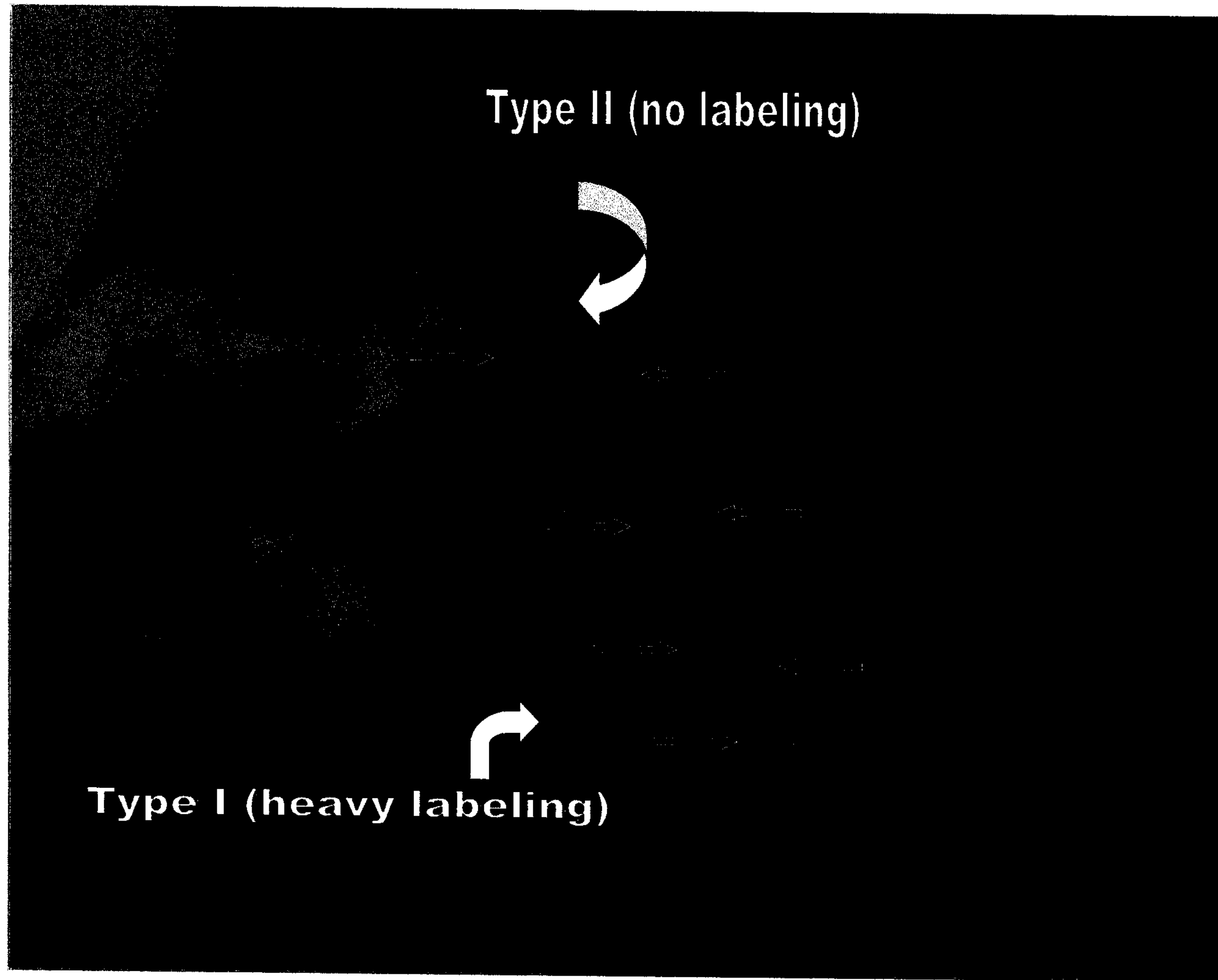

Fig. 6. Adult Utricle TUJ1 10x

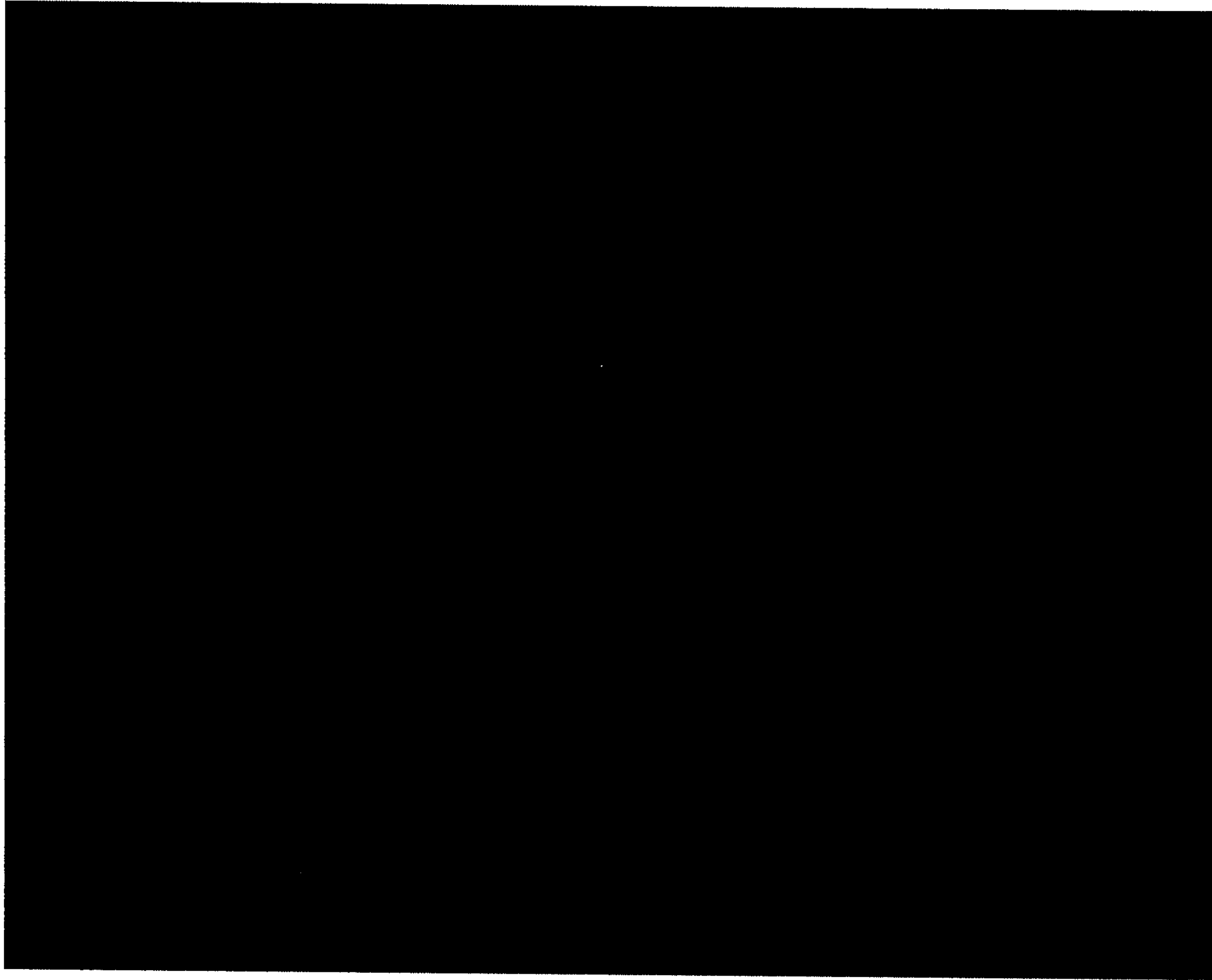

Fig. 7. E8 UtricleTenascin 60x

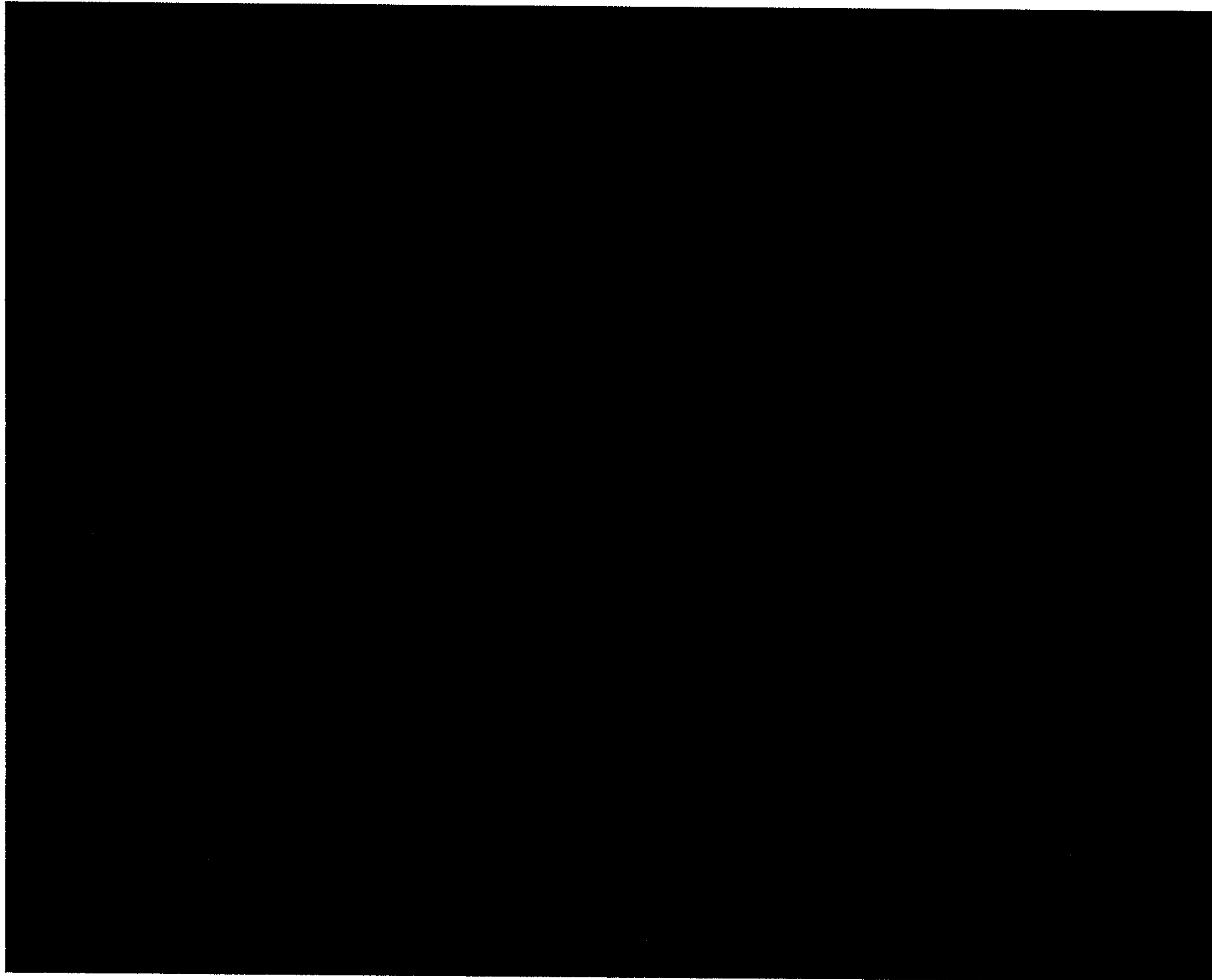

Fig. 8. E10 Utricle Tenascin
60x

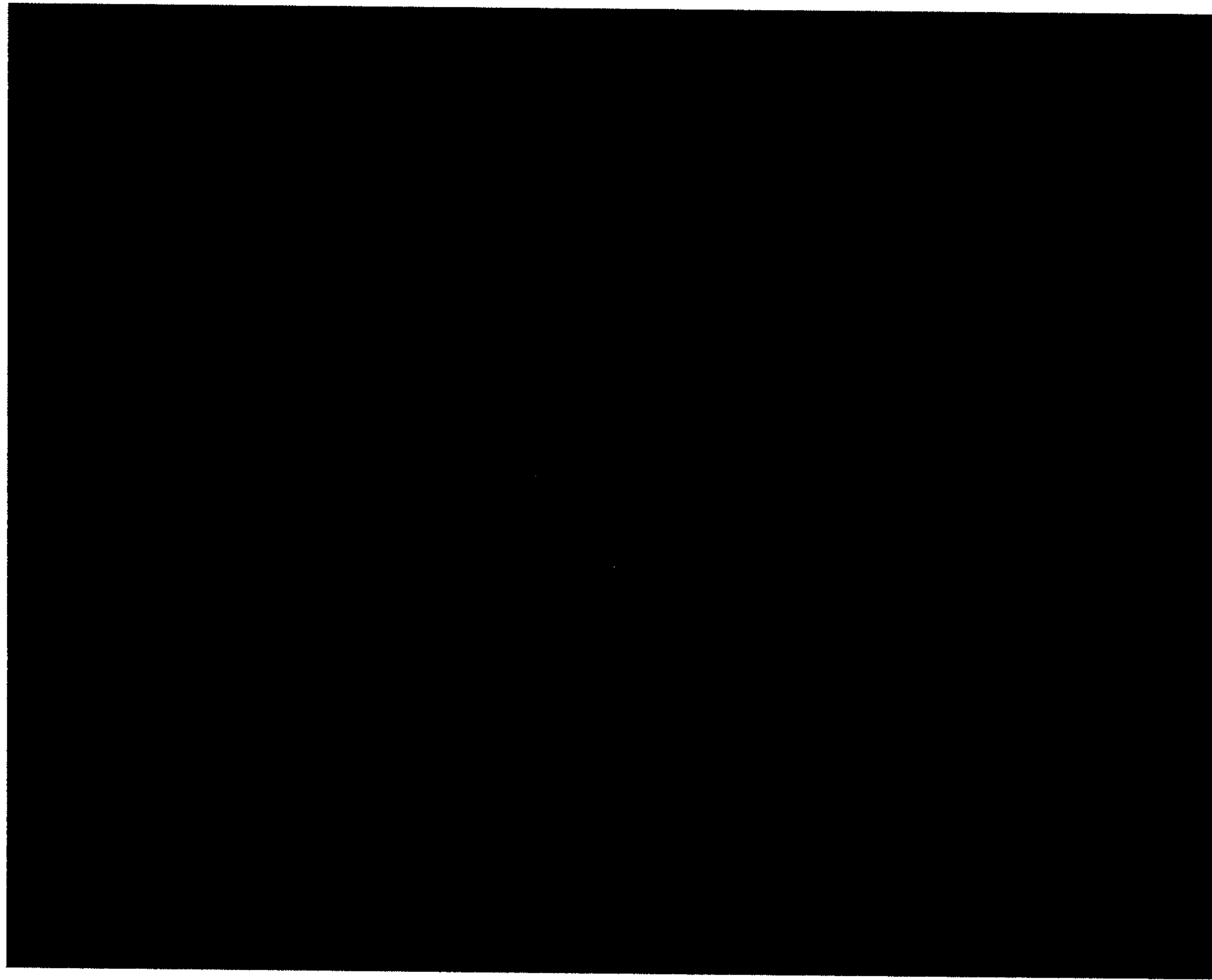

Fig. 9. E15 Utricle Tenascin 60x

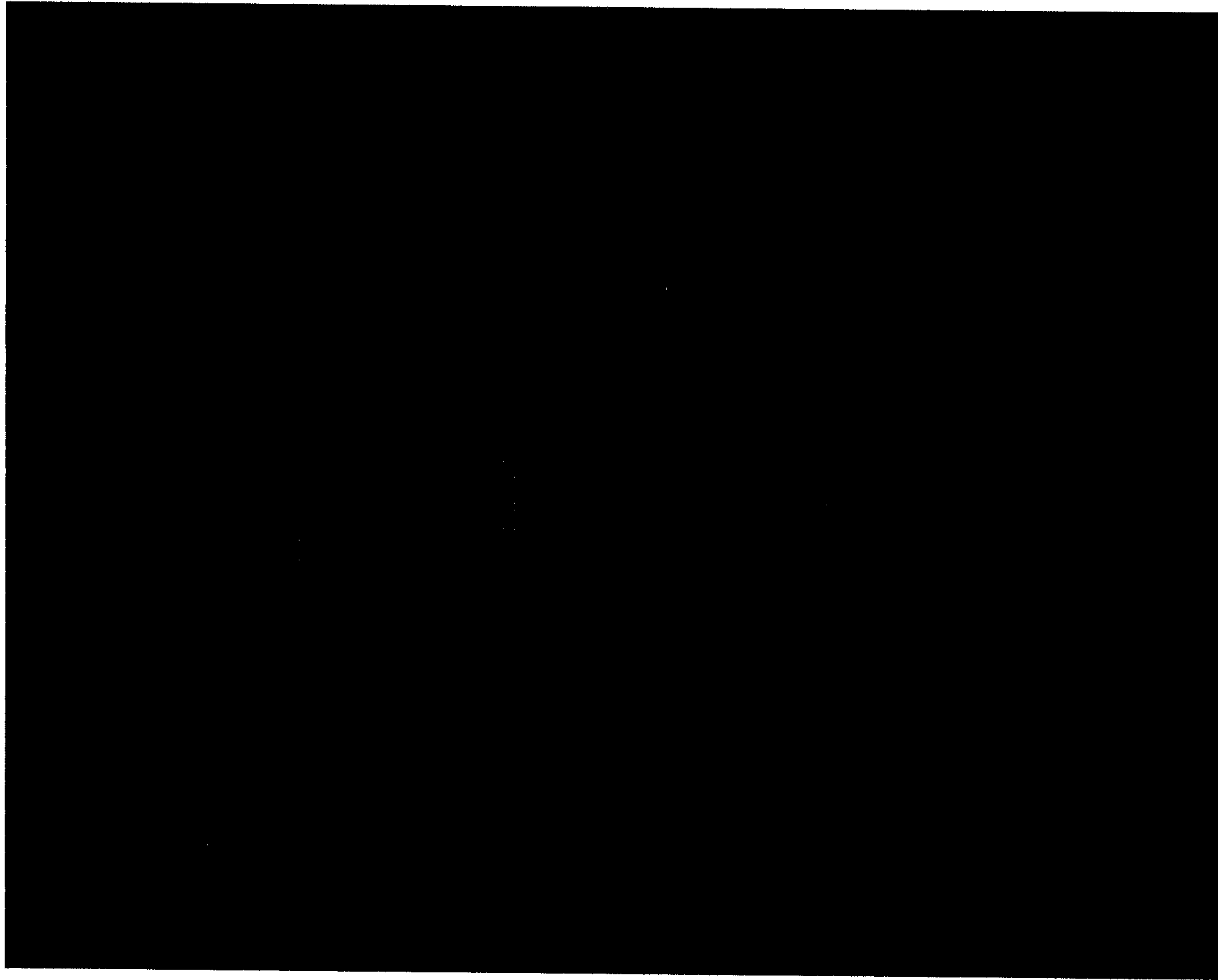

Fig. 10.Adult Utricle Tenascin
60x

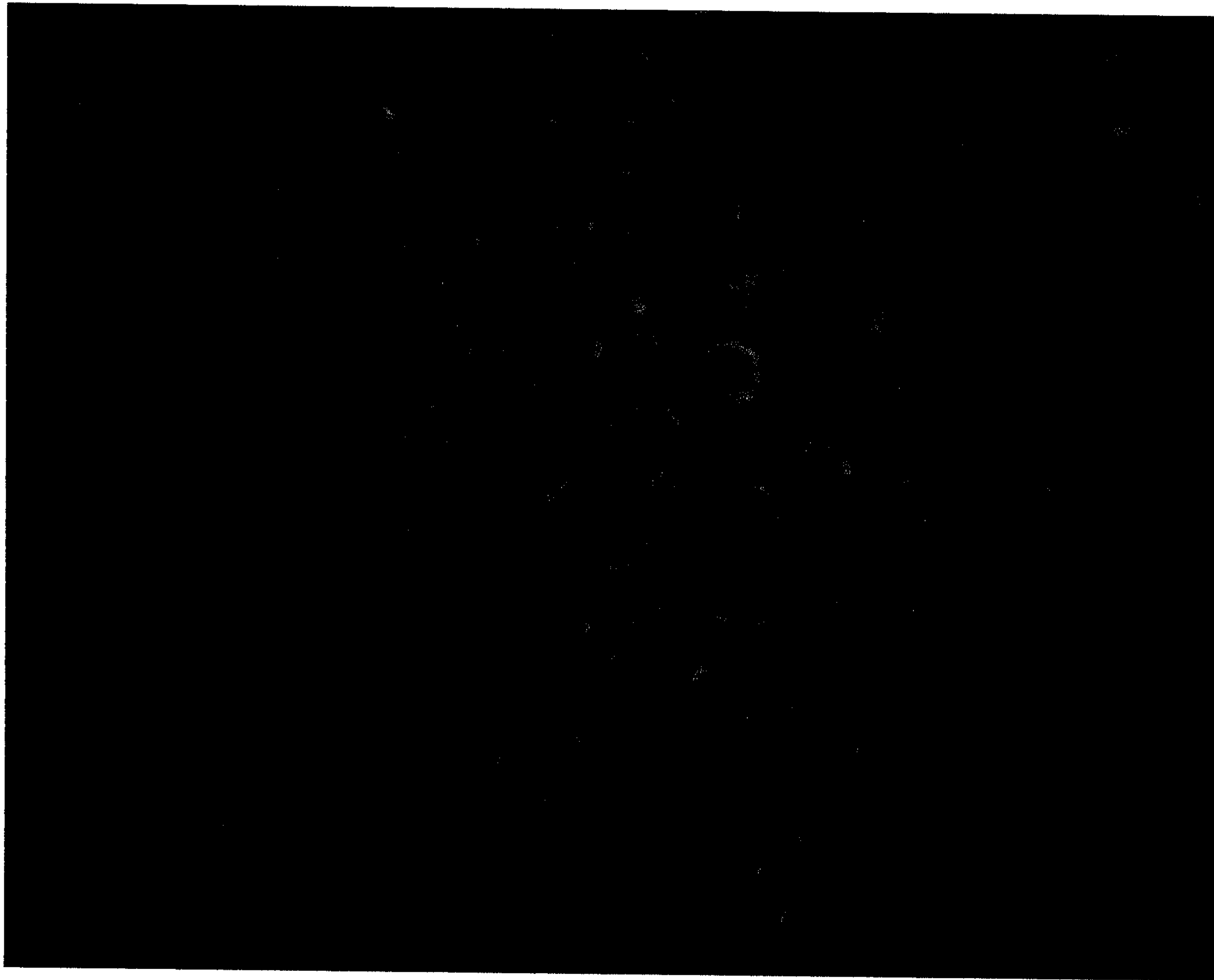

Fig. 11. E10 Utricle TUJ1 and Tenascin merged 60x

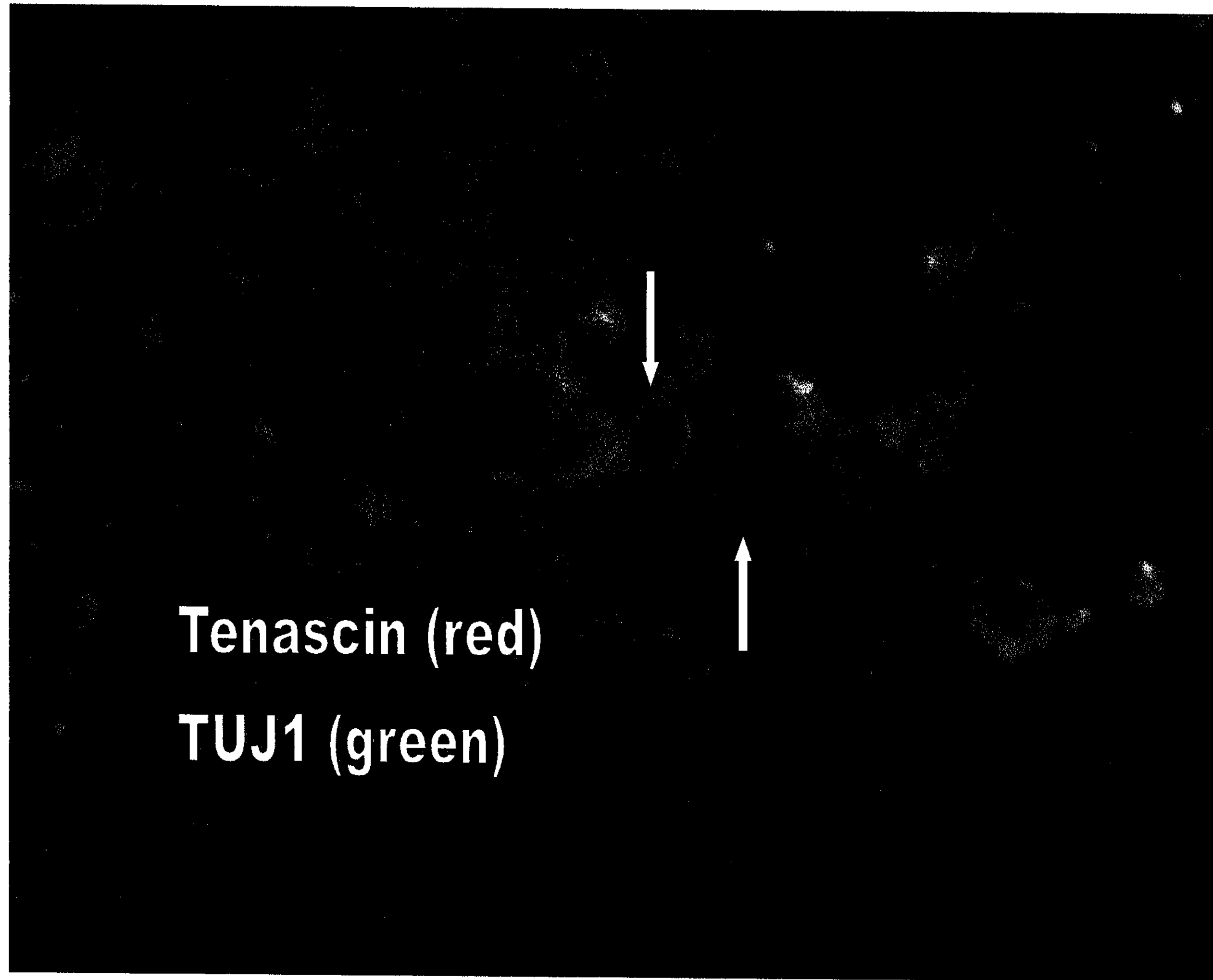

Fig. 12. E12 Utricle TUJ1 and Tenascin merged 60x

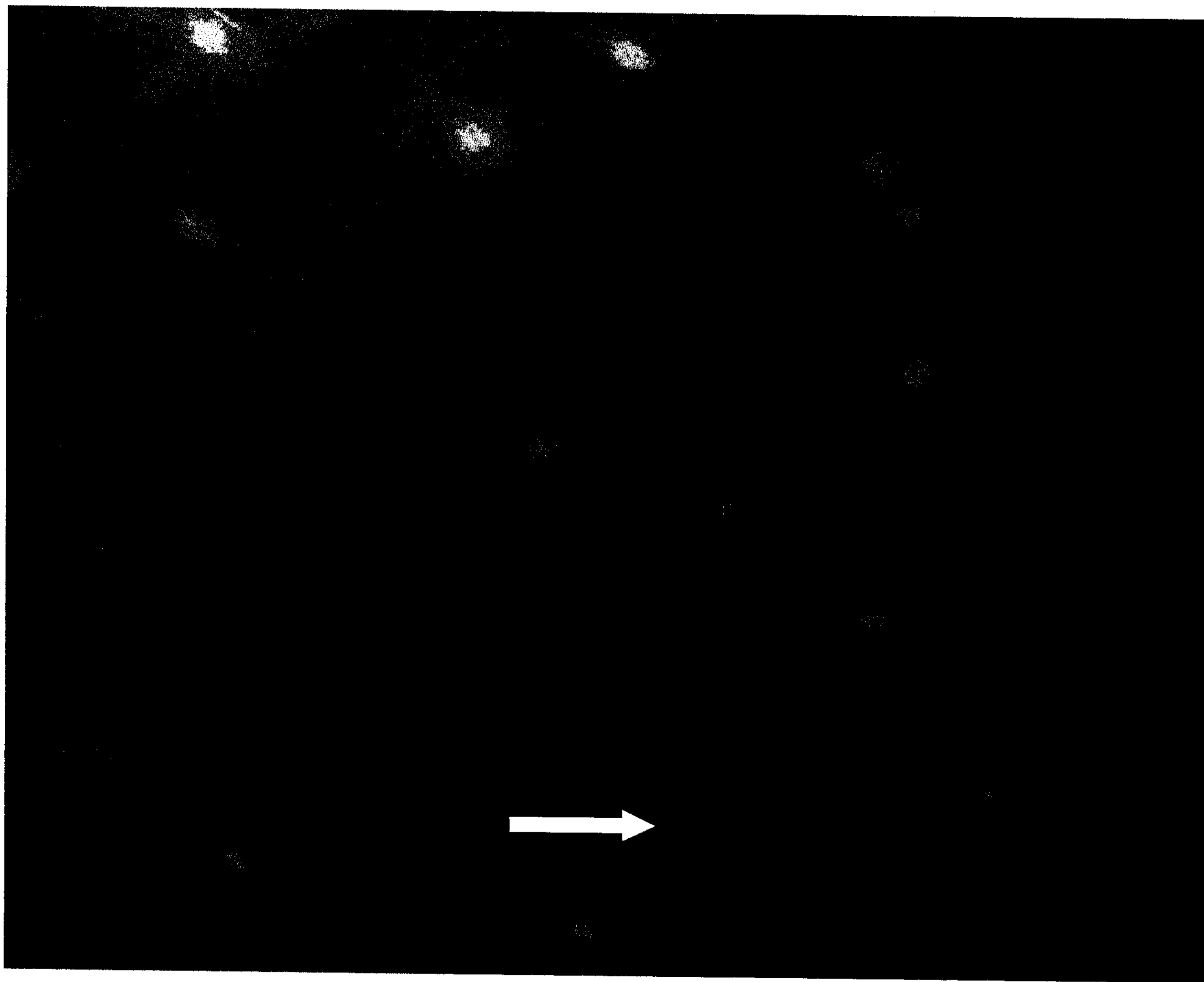

Fig. 13. E12 Saccule TUJ1 and Tenascin merged 60x

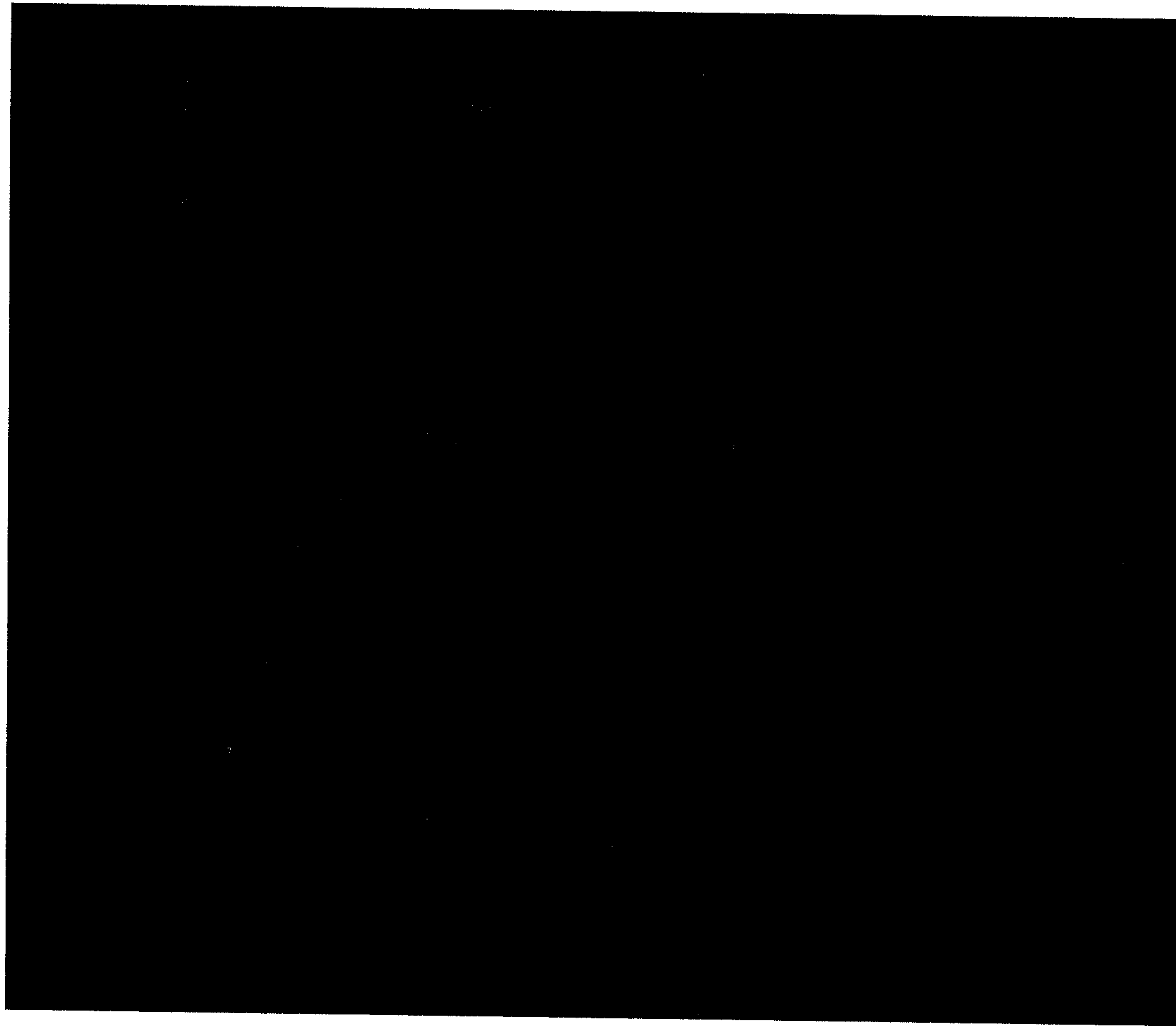

Fig. 14. Adult Utricle TUJ1 and Tenascin merged 60x

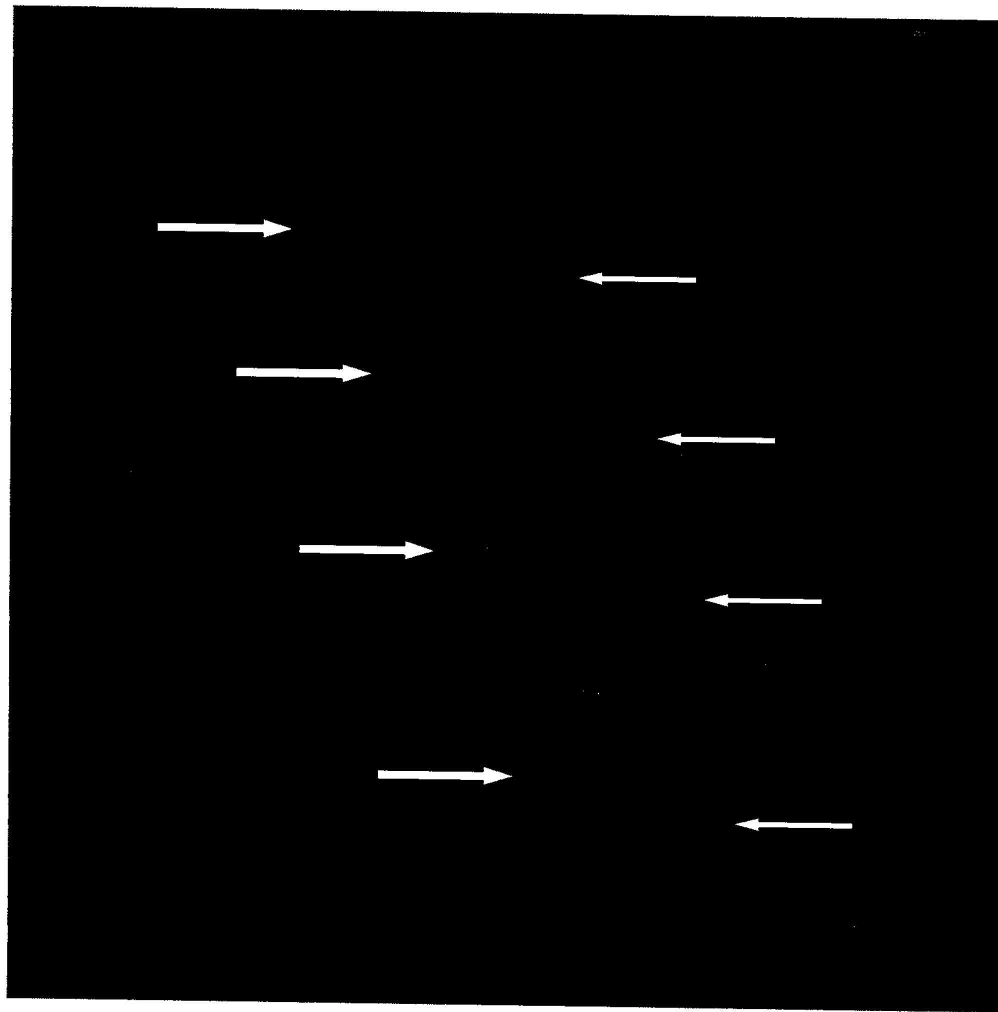

Fig. 15. Adult Utricle TUJ1 and Tenascin merged 60x