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A NEW MODEL SPECIES FOR CULTURED TELEOST RETINAL CELL STUDIES: LIGHT AND SCANNING ELECTRÓN MICROSCOPY

by

Lori Ann Steinfeldt, B.S.

Thesis submitted in partial fulfillment of the requirements for the degree of

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A NEW MODEL SPECIES FOR CULTURED TELEOST RETINAL CELL STUDIES: LIGHT AND SCANNING ELECTRON MICROSCOPY

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Purpose: To examine retinal horizontal and bipolar cells cultured from goldfish (*Carassius auratus*), black crappie (*Pomoxis nigromaculatus*) and bluegill (*Lepomis macrochirus*) on the light and electron microscope levels; and to compare them with cells from a more established model, the wild white bass (*Roccus chrysops*). Methods: Retinas from all species were dissociated by methods developed with wild white bass (Dowling et al., 1985). Cell type was identified within the first 3 hr and cells were cultured for up to 2 wk in L-15 medium. At various intervals, cells were fixed in glutaraldehyde, photographed with phase LM and processed for SEM by either the ethanol dehydration method or the OTOTO method (Thompson et al., 1992 and modified Gabriel, 1982). Specimens were viewed with an Hitachi SEM. Each technique was evaluated for safety, simplicity, and image quality. Results: Bluegill and goldfish retina yielded few cells of inferior quality. Neurite outgrowth was limited. In contrast, crappie retina yielded adequate number of recognizable cell morphology, including: rods, cones, horizontal cells (H1s, H2s, H3s, H4s), and bipolar cells (BPs). These cells closely resembled the same cell types obtained from white bass (Vaughan & Lasater, 1990). The most numerous cells were H2s and H4s. Neurite outgrowth was acceptable and long-term survival of HCs and BPs was routine. H1s were observed still in contact with one another and presumably still connected by gap junctions. Neither of the two SEM techniques applied yielded superior results; rather both resulted in distracting artifacts. Conclusion: Crappie are easier to capture and maintain in the laboratory than wild white bass and their retinal cells perform in culture more like those from wild white bass than the other species tested. SEM observations confirm some axon-like and dendrite-like process growth of the bipolar cells and marginal growth in the H1s. The ethanol dehydration method for preparing cells for SEM is quick, simple and cost effective although it lacks the contrast and high resolution provided by the OTOTO method. Supported by NIH grant EYO9038. None.
INTRODUCTION

Cultured retina cells from adult animals are useful for the elucidation of biochemical, biophysical and pharmacological properties of individual cell types (Dowling, et al., 1985). The isolation and primary culturing of retinal neurons from the teleost has been used as a model for cultured mammal retinal cells. Horizontal cells of the teleost have been studied for many years because the cells are large and easy to maintain in culture. A species used for its horizontal cells in this lab is the wild white bass (*Roccus chrysops*). Indeed it has become an established model for retina cells in culture (Vaughan & Lasater, 1990). However, obtaining this species from the wild and maintaining it in captivity may present some difficulties. The wild white bass must be collected from local ponds and lakes, thereby requiring the researcher to obtain a state permit for gathering this species live. Furthermore, many animals may suffer damage or death from the collection and transportation process. The equipment necessary for the capture and maintenance of these animals is expensive. Finally, many animals die in captivity due to the high stress of leaving their natural environment and food sources to be moved to a captive environment. In search of a more easily obtained species which yields an equivalent quality of retina cells, other teleosts have been tried.

The following species were selected as an alternative to the wild white bass, for a variety of reasons. The goldfish (*Carassius auratus*), was considered an appropriate alternative because it is commercially obtainable, accustomed to captivity and is often used in vision studies. The goldfish, however, does not use its visual system in a manner similar to the white bass. Where vision is important to the bass to hunt and capture its food, the olfactory system is important to the goldfish to merely locate its food supply. For this reason, two other species with visual needs similar to the bass are being tried. The bluegill (*Lepomis macrochirus*), and black crappie (*Pomoxis nigromaculatus*), belong to the Centrarchid or sunfish family. Adult sunfish are piscivorous. The white bass has similar feeding habits. For these animals, the visual system is an important part of their survival. The sunfish are considered an appropriate alternative for this study because they are easily obtained from local ponds or lakes and
because their lifestyle is more similar to that of the white bass. Unfortunately, as with the white bass, the sunfish are wild and therefore stressed under captivity.

Although bass horizontal cells have been closely examined using light microscopy techniques, (Vaughan, 1990a) these retina cells have never been examined using scanning electron microscopy (SEM). SEM allows us the opportunity to view cells at ultra high magnification while maintaining good resolution. It is of interest to examine the retina cells of the teleost using SEM to identify the ultrastructure of these cells and perhaps get a better understanding of relationships between these cells. Of particular interest to this lab is the relationship between coupled horizontal type one cells (H1s). It is possible that SEM will provide the magnification necessary to examine these cells.

Non-teleost retina cells have been examined using SEM. Perlman, *et al.* (1989) examined isolated turtle retina using SEM and Thompson and Norby (1992) examined artificial retina-muscle synapses using SEM. However, SEM has not been reported in the literature for any of the teleost species mentioned above. Although bass retina cells have been examined using TEM, SEM gives the researcher the acuity and detail of TEM with the 3-dimensional advantage of SEM. With this tool, we should be more able to characterize cell sizes, shapes and growth patterns. In an attempt to find an effective method for preparing cells for SEM, two methods will be compared, the OTOTO method (Gabriel, 1982) and the ethanol dehydration method (Perlman, *et al.* (1989).

The purpose of this paper is to first, examine retina horizontal and bipolar cells cultured from goldfish, black crappie, and bluegill on the LM and SEM levels; and then to compare their cells with the more established model, the wild white bass. Secondly, two methods for preparing cells for SEM will be evaluated according to simplicity of technique, safety of procedures and quality of image.
MATERIALS & METHODS

ANIMALS

Adult black crappie (*Pomoxis nigromaculatus*) and bluegill (*Lepomis macrochirus*) were collected locally from the wild and maintained in aquaria on the ambient light/dark cycle before use. Adult goldfish (*Carassius auratus*) were obtained commercially (Carolina Biological, Burlington, N.C.). All fish were held under a 12:12 light:dark cycle before use.

CELL CULTURE

Methods used have been previously published (Dowling, Pak, Lasater, 1985; Vaughan and Lasater, 1990). Before dissection, fish were dark-adapted for at least 2 hr. Dissections were performed under dim red light at room temperature. Animals were anaesthetized by chilling for 10 to 15 min. and euthanized via cervical transection. All eyes were dipped in 95% ethanol and hemisected under sterile conditions which were maintained thereafter. The anterior segment, lens and most of the vitreous were discarded. The posterior eye cups were rinsed in L-15 culture medium (Sigma). The retina was gently pulled away from the pigmented epithelium with sterile forceps and incubated for 40-60 min. at room temperature in 10 ml of L-15 medium supplemented with 0.16 mg of cysteine-activated papain (Worthington Biochemical). Tissue was then briefly rinsed in DNAase-supplemented L-15. Triturated tissue, using a sterile wide-bore glass pipette, resulted in supernate containing isolated cells. Cells were incubated in 2 ml L-15 medium in Primaria tissue culture dishes (Falcon) and stored at 17°C. Cells were fed, observed and photographed every 2-3 days. Cell type was identified within the first 3 hr and cells were fixed with 2.5% glutaraldehyde in phosphate buffer for 15 min at various intervals for up to 2 wk. All fixed dishes were stored at 4°C until used.
LIGHT MICROSCOPY (LM)

Cells were observed using an Olympus BH light microscope equipped with phase optics and photographed with Kodak Tmax 100 film. Cell types were identified according to published criteria for the white bass (Dowling, Pak, Lasater, 1985; Lasater, 1988; Vaughan and Lasater, 1990).

SCANNING ELECTRON MICROSCOPY (SEM)

Cells were prepared for SEM by the OTOTO method (Gabriel, 1982) or the ethanol dehydration (EtOH) method (Thompson and Norby, 1992). The two methods were evaluated for simplicity of technique, safety of procedures and quality of image.

Briefly, the OTOTO method was as follows. Cells were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (PB) for 30 min and then fixed for an additional 30 min with 2% glutaraldehyde plus 2% osmium tetraoxide (OsO₄) in PB for 1 hr. After rinsing thoroughly in double-distilled water, the cells were reduced with 1% aqueous thiocarbohydrazide (TCH) for 30 min and rinsed again in water. Cells were again oxidized with 1% aqueous OsO₄ for 30 min; rinsed; reduced; rinsed and oxidized with TCH and OsO₄ respectively. Following a thorough rinse in water, cells were dehydrated stepwise in ethanol (30% to 100%) and then critical-point dried using Freon 113. Cells were mounted on an aluminum stub and sputter coated with iridium using an ion beam sputterer VCR Group Inc. IBS/TM 200S. Cells were viewed using an Hitachi S4000 FESEM and photographed with Kodak Tmax 100.

Briefly, the ethanol method was as follows. Cells were fixed overnight in 2.5% glutaraldehyde in 0.1 M phosphate buffer saline (PBS). Following a thorough rinse in PBS, cells were dehydrated stepwise in ethanol and processed from that point as described above.

Neither SEM method was tried on the bluegill or goldfish due to the dramatically poor yield of cultured cells. Scanning EM data shown are for cultured black crappie retinal neurons only.
RESULTS

CELL YIELD BY SPECIES

In our hands, bluegill and goldfish retinas yielded few cells of inferior quality with limited neurite outgrowth. Cell survival from both species was poor, with most cells dying by day 3. Due to poor performance in culture, further phase LM and SEM evaluation of these species was not conducted. In contrast, black crappie retina yielded an adequate number of cells of recognizable morphology, including: rods, cones, horizontal cells (H1s, H2s, H3s, H4s), and bipolar cells (BPs). Survival of crappie cells in culture was sufficiently consistent for further evaluation with phase LM and SEM.

PHASE LIGHT MICROSCOPY

Black Crappie Retina - General Observations

Following dissociation, freshly-isolated cells may take several hours of incubation (3 to 5 hrs) to firmly attach to the dish. Many of the highly damaged cells die and are discarded with the first change of culture medium. After 24 hrs, healthy cells take on a more rounded appearance. Cell types from most layers of the retina can be identified in culture including: rod and cone photoreceptors, bipolar cells (BP), and each of the four types of horizontal cell (H1, H2, H3, H4).

Photoreceptors, specifically rods (Fig. 1) and cones (not shown), were isolated in fresh cultures and occasionally with the axon still intact. Most photoreceptors are discarded with the first change in culture, although sometimes these cells, especially the outer segment of the cones, remain in culture for several days. Photoreceptors, however, do not appear to thrive and grow in culture medium as do the HCs.

Isolated black crappie cells were shown to be alive and healthy in culture for up to 12 days (Fig. 5.D). Although it was
observed that the quantity of live cells diminished over time, cell death was not qualitatively measured. Cells held in culture for several days showed growth of axon-like neurites for H1s (Fig. 2.B; 2.C) and fine spine-like neurites (Fig. 3.B; 3.C) for all HCs and BPs.

Black Crappie: Horizontal Cells

_Distinguishing features of horizontal cell types_

All four identified horizontal cell types isolated in the wild white bass were also identified in black crappie cell cultures (Fig. 1-4). Contained in these cultures were the smaller round H1s, both coupled and singular (Fig. 1; 2); as well as the stellar H2, typical of the H2s seen in the wild bass. Although the soma size of H1s and H2s tend to be similar, H2s contain more space due to their larger dendritic spread. For example, the H2 in Figure 1.A has a soma diameter of approximately 25 µm, which is only 5 µm larger than the mean soma diameter of H1 cells in the same figure. However, when comparing dendritic spread, there is a measurable size difference. The dendritic spread of the H2 is 67 µm and the mean dendritic spread of the H1s is about 35 µm. As an additional example, in Figure 1.B, the mean soma size and dendritic spread for the H1s is 14 µm and 45.3 µm respectively; and for the H2 shown, 14 µm and 75 µm. In effect, the dendritic spread is significantly greater in the H2 than in the H1 although the soma diameter is nearly the same. Therefore, the soma diameter, as a means to compare H1s and H2s is of little significance.

In general, H3s are larger than H2 cells. For example, in Figure 3, the labeled H3 has a soma diameter and dendritic spread of 100 µm and 125 µm compared to the H2's 60 µm and 90 µm, respectively. The H3 is distinguished from the H2 by its larger size and elongated cell body (Fig. 3.C). Neither the H2 nor the H3 shows evidence for regeneration of axon-like neurites, unlike H1s which frequently show axon-like process growth. H4s are frequently obscured by cellular debris (Fig. 4.A), although occasionally, H4s will be isolated without distracting debris, rendering the nucleus more visible (Fig. 4.C). This stellate cell type is distinguished from the stellate H2 by its more cylindrical dendrites and generally larger dendritic spread. The H4 dendrites also often have an almost curly appearance (Fig. 4) though this is not as pronounced in the black crappie as it is in the wild white bass. In contrast with
H1s, H2s, and H3s, H4s are axonless in the intact retina. Figure 4.B beautifully illustrates the size difference and other distinctive features between the H1, H2 and H4 cell types.

H1s are often found coupled in cell cultures (Fig. 1.B; 2.B; 2.C). In Figure 1.B, a triplet has settled out of the initial retinal dissociate still attached to one another and presumably coupled by gap junctions. Cells which settle out of the initial retinal dissociated coupled, remain as coupled cells as the culture ages (Fig. 2.B; 2.C).

Regeneration of axon-like or dendrite-like processes

The only horizontal cell type that appeared to show axon-like neurite regeneration in the black crappie is the H1 cell. Through the vigorous dissociation procedure, the fragile axon is detached from the cell body. Frequently fragments of apparent detached axons can be seen in the culture fluid of freshly isolated cells (Fig. 1.A). As a freshly isolated H1, a few stout dendrites remain, giving the cell a stellate appearance (Fig. 2.A). After a few days, H1s which have lost their axon due to dissociation, exhibit evidence of neurite outgrowth (Fig. 2.B; 2.C). Small, medium and large neurites, similar to those distinguished by Vaughan and Lasater (1990) in the wild white bass, are observed (Fig. 2.B). As the cell ages, if conditions permit, the length of the axon increases. Axon length is expressed in soma sizes to standardize the expression of growth between different cell sizes. Figure 2.C shows six-day-old cells which have lost their axons during dissociation. The top cell has regenerated a large, axon-like neurite (three soma sizes long), whereas neurite outgrowth from the two bottom cells appears restricted to small and medium neurites. Vigorous regeneration of axon-like processes is not consistently observed in the black crappie.

Although other HCs show no evidence for regeneration of an axon-like neurites, there is frequent growth of spike-like fine neurites generally elongating from the main dendrites (Fig. 3; 4).

Black Crappie Bipolar Cells

Black crappie cell cultures are filled with a variety of bipolar cells (Fig. 5). These cells are easy to identify by their highly arborous dendritic spines and long narrow axon. BPs survive the dissociation process and thrive in culture for many days. Shown are cells at 4 days, 6 days, 7 days, and 12 days (Fig.
5). Freshly-isolated bipolar cells are more difficult to identify due to the tremendous amount of cellular debris. None are shown. The axons of BPs generally survive the dissociation procedure intact. As defined by Lasater (1988), BP cells of both the large and small class were identified (Fig. 5). BPs in culture grow very fine neurites from dendrites and axon terminals that are barely resolvable with LM at this magnification. Use of SEM greatly increased the resolution of these cells and allows for increased magnification.

**SCANNING ELECTRON MICROSCOPY**

Cells were prepared for SEM via one of the two methods as described above. One method involved heavy metal impregnation and is referred to as the OTOTO method (Gabriel, 1982). The other method primarily involves the use of ethanol and is referred to as the EtOH method (Perlman, *et al.* (1989). Since no one has previously documented teleost cells with SEM, identification of these cells was based on LM studies of gross morphology.

**Black Crappie - General Observations**

Cells from several time points (freshly isolated (FIC), 5 days and 6 days) were examined. Although several images were unidentifiable, many cell types (H1, H2, H3, H4, bipolar, Muller cells, rods and cones) were photographed. Figures 11.A and 11.B show the size and shape differences between the first three types of horizontal cells. These figures also provide quick visual information about the differences used to process these cells.

In general, processing artifacts were associated with both methods, rendering neither method superior. An obvious artifact associated with the OTOTO method, in our hands, was the number and variety of fractures. Some cells had multiple fracture sites (Fig. 8; 9). In general, the larger the cell, the higher the incidence of fracturing. Since H1s and non-horizontal cells had few fractures, it was difficult to distinguish these types of cells processed by either the OTOTO or EtOH method. The most dramatic artifact associated with the EtOH method is the ghost-like appearance of the cells. Overall, EtOH cells
appear almost flat, whereas the OTOTO cells have a fuller look. Several examples of this artifact are shown (Fig. 8-11). This absence of contrast or "fingerprint" effect can be illustrated throughout the use of this method. Figure 11.C is perhaps the most dramatic example. The outline of this fingerprint is typical of those of other Hls. But, strangely enough, the cell body does not seem to be present. This artifact alone makes it easy to separate those cells treated by the OTOTO method versus those treated by the EtOH method. Where the OTOTO cells seem rough and irregular (Fig. 11.A), the dehydrated cells look smooth (Fig. 11.B). Another artifact associated with both methods, although most commonly associated with the EtOH method, is the shrunken-look of the cell body seen in many cells. This effect is most prevalent in the BPs and Hls (Fig. 7; 10).

**Black Crappie: Other Cell Types**

*Bipolar Cells*

In a blind comparison, it is difficult to determine which non-horizontal cells were processed using the OTOTO or EtOH method. When examining BPs (Fig. 6), it would be difficult to determine that Figures 6.A and 6.B are six-day-old cells processed via the OTOTO method and Figure 6.C is a five-day-old cell processed via EtOH. In other words, there is no significant difference between either method, except that fracturing of thin processes is more frequently seen in cells where the OTOTO method was used (arrowhead). All cells have characteristics of bipolar cells as previously described and shown in phase LM (Fig. 5). However, in contrast to light microscopy, SEM details the arborous dendritic neurites and lamella.

*Photoreceptors*

Rods and cones were also located using SEM. As with bipolar cells, distinguishing one method from the other was difficult for the cones (Fig. 7.A; 7.C). Although single and double cones were frequently identified, usually only the inner segment remained. Even as freshly isolated cells, the cones are highly damaged (Fig. 7.B). The image obtained after processing may represent the densely packed mitochondria found in this part of the cone when the plasma membrane is somehow stripped away during the dissociation procedure. Rods, although slightly more difficult to locate, were also identified (Fig. 2.B)
and remained fairly intact. Rods sustain far less damage from the dissociation procedure when compared to cones.

Other non-horizontal cell types were photographed, but many were difficult to identify. Figure 6.D is a five-day-old cell tentatively identified as a Muller cell. It was so identified by its bifurcated foot-like processes (Muller end-feet). Morphological identification of this cell type is based on previous identification of isolated rabbit retina cells with LM (Vaughan and Fisher, 1987). No other cell of this type was seen. These glial cells do not generally survive in culture.

**Black Crappie Horizontal Cells**

*Distinction features of horizontal cell types using SEM*

The general appearance of black crappie HCs prepared with SEM parallels that seen with phase LM (cf. Fig. 2, 3, & 4). Tiny neurites from the main dendrites have survived the dissociation procedure intact and are more easily observed using SEM. In general, many more such neurites are observed after several days, presumably reflecting growth in culture. Again, such neurite growth detail as well as lamella elaboration is easily observed using SEM. For the H4s, little neurite growth is observed after five days in culture (Fig. 8.A; 8.B; 8.C; 8.E). This supports light microscopy observations.

*Differences between the SEM processing methods for horizontal cells*

Fractures were a significant artifact associated with SEM processing; most frequently with the OTOTO method. Cell fracturing can be discussed as involving three areas: fractures of the soma, proximal fractures of the neurites, and distal fractures of the neurites. These fracture sites are defined as follows. Any fracture of the soma is a complete separation within the cell body. Any proximal fracture of the neurites is a complete separation of the soma and neurite origin. Any distal fracture of the neurites is a complete separation within the neurite. Figures 8.C, 8.D and 9.B provide good examples of the three types of fractures. Incidence of fractures were not quantitatively measured in this study.

In general, more fractures of all types were seen in the H4, H2 and H3 cells (see Fig. 8, and 9 respectively). For the non-horizontal cells as well as H1s, very little cell fracturing was observed. Similarly, very few fractures were observed for cells processed with the EtOH method.
The EtOH method yielded a flat, almost ghost-like image, but with better preservation of lamellar structures compared to OTOTO cells (Fig. 8-11). In some images, the cells are so flattened as to suggest that they are mere footprints of membrane preserved by this method, as if the cells body has been removed (Fig. 11.B; 11.C). The "footprint" effect was observable in all cell types.
DISCUSSION

In an attempt to find an alternative model to the wild white bass (Roccus chrysops) for cultured retinal cell studies, cultured cells of the goldfish (Carassius auratus), black crappie (Pomoxis nigromaculatus), and bluegill (Lepomis macrochirus) were examined. Since the retina cultures of both the goldfish and the bluegill yielded few cells of inferior quality with limited neurite outgrowth, the use of these species for cell cultures is not recommended.

In contrast, black crappie retina yielded an adequate number of cells of similar morphology to the wild white bass. Black crappie are easier to capture and maintain in the laboratory than wild white bass and their retinal cells perform in culture more like those from bass than the other species tested. Cultures of these cells were examined further with phase LM and SEM. Scanning EM observations confirm some dendrite-like neurite growth of all HCs and BPs and marginal axon-like neurite growth of the H1s.

Neither method tried for preparing cells for SEM proved to be superior to the other. Both methods were associated with distracting artifacts. The OTOTO method is labor intensive, requires the use of highly toxic chemicals, and most frequently results in fractures of the cells. The EtOH method is quick, simple, and cost effective although all cell types look flat and H1s often become severely shrunken. It is suggested that a combination of both methods might yield the best results.

Cell Morphology - A Comparison To Wild White Bass

Black crappie cells survive as well in culture as the white bass. Furthermore, using phase LM all four horizontal cell types, BPs and photoreceptors can be easily identified. Morphologically these cells closely resemble those of the wild white bass in both size and structure with only a few subtle differences. Single cones, double cones and rods are all present in freshly isolated cultures. As with the bass, photoreceptors are highly damaged by the dissociation process and lie dormant throughout the culture period. BPs closely resembled those of the bass. Additionally, both small and large class BPs, as defined by Lasater (1988) for the wild white bass, were identified. All the HCs closely resembled the corresponding cells in the bass with respect to both size and shape. The H4s, however, differed slightly
from those in the bass in that the secondary and tertiary dendrites which tend to give bass H4s a very curly appearance were less pronounced in the black crappie. As in the wild white bass, H1s are often found coupled in cultures, presumably by gap junctions. With regards to cell morphology, the black crappie is an acceptable alternative model to the wild white bass.

**Cell Growth - A Comparison To Wild White Bass**

As with the wild white bass, HCs held in culture for several days showed possible growth of axon-like neurites for H1s and fine spine-like neurites for all HCs and BPs. Dendritic arborization of BPs was consistent and vigorous. In contrast to the wild white bass, vigorous regeneration of axon-like processes is inconsistent in the black crappie. With regards to HCs regeneration of dendrite-like neurites, the black crappie is an adequate alternative model to the wild white bass. With regards to H1s regeneration of axon-like neurites, the black crappie is an unacceptable alternative to the wild white bass, the H1s from which demonstrate more consistent regeneration. With regards to BP regeneration of dendrite-like neurites, the black crappie is a good alternative model to the wild white bass.

**SEM - General Comments**

Although the OTOTO method and the EtOH method yielded satisfactory results, neither method was exemplary. SEM does provide an avenue for closer examination of these cells. With either method, secondary growth of the delicate lamellae and fine neurites was better magnified and resolved. However, the EtOH method does accentuate the lamella better than the OTOTO method. SEM also allows for closer examination of BPs and the photoreceptors which were of good quality by both methods. Finally, either preparatory method is adequate when examining growth cones and other forms of neurite regeneration at higher magnification than allowed for using LM.

**OTOTO Method**

The OTOTO method involves a great deal of preparation, making it a very time consuming procedure, specifically with respect to the oxidation/reduction steps. Approximated time involved in preparing the cells to critical point drying is 11 hrs. The total time involved for the entire procedure from
fix to scanning is approximately 24 hrs. The overall cost of the OTOTO method is significantly higher than the dehydration method. Aside from the added chemicals and lab time, use of OsO₄ rapidly adds to the projects costs. Because OsO₄ is highly toxic, it is expensive to ship, store and discard, not to mention the increased safety risks involved when working with this chemical. OsO₄ is used in three steps at low concentrations (1% and 2%). To process one dish, approximately 5 ml of diluted osmium waste is generated. The OTOTO method is significantly more complicated than the dehydration method. More steps, more chemicals, and more time is involved. By increasing the complexity of the method, there is also more room for error on the part of the technician.

The OTOTO method has positive and negative features associated with it. The most obvious artifact associated with the OTOTO method, in my hands, is the number and variety of fractures. An overwhelming number of the cells identified contained some type of fracture. Several points may account for the number of fractures. First, heavy metal impregnation via OsO₄ may tend to make these cells brittle. Therefore, by decreasing the flexibility of the cell, the tendency to fracture may be increased. Additionally, the freshness of the cells before processing and the quality of handling may also relate to the number of fractures. For example, if the cells are not fresh or if at any point during the processing they have dried out, then the cells may fracture for reasons unrelated to processing method alone.

One other negative artifact although not as distracting as the fractures, is the apparent shrinkage of the cell body of many cells. This is especially obvious in the BPs and neurites of some of the other cells. OTOTO does however, provide high resolution detail for ultra structure examination. Even at 15 thousand times the magnification, resolution is good. This provides a valuable tool for the interested researcher.

Ethanol Dehydration Method

By far the most simple and cost effective method employed, the EtOH method is basically the final ethanol dehydration steps used in the OTOTO method before critical point drying begins. Following overnight soak in 2.5% glutaraldehyde, the EtOH method to critical point drying can be completed in less than 3 hrs. Time needed for this procedure from fix to scanning is about 22 hrs. Cost is minimal, especially since OsO₄ and other chemicals are not used. Furthermore, since the oxidation and reduction
steps have been removed, personal and environmental exposure to toxins is limited to Freon 113 (for critical point drying). Therefore, this method is fast, safe, simple and inexpensive.

Some problems are associated with this procedure. Cells processed using the EtOH method look different from those processed using the OTOTO method. Overall, the dehydrated cells frequently appear more two-dimensional, almost flat; whereas the OTOTO cells have a much greater contour or depth. This artifact may be accounted for by the following reasoning. First, since glutaraldehyde only fixes protein, and since ethanol removes all the available fat and water from the cells, then with all the water removed, structurally nothing is left to hold the cell in place. Therefore the turgidity of the cell is lost causing it to collapse. The OTOTO method preserves the cell from shrinking, the ethanol dehydration method does not. This reasoning may also account for the degree of shrinkage seen in the H1s and BPs processed by the EtOH method.

The EtOH method is quick, simple and cost effective. Although flatness and shrinkage of the cell, when compared to the traditional OTOTO method, is a sacrifice in quality, overall, this procedure is the effective for getting preliminary results. If size and shape are necessary, than the OTOTO method may be employed, inspite of the distracting fractures. Perhaps a less intensive heavy metal impregnation procedure will provide the most desirable results.
FIGURE 1

Phase light microscopy of freshly-isolated black crappie retinal cells illustrating a typical yield of cell types. Scale bars = 50 µm.

A) Shown are a typical large H2 and two smaller H1s, with scattered rod photoreceptors (arrowheads). Both types of HC have lost their axons. The filamentous structure to the right of the H2 may be fragments of broken HC axon (*). The mean soma size and dendritic spread for the H1s shown is 20 µm and 35 µm, respectively; and for the H2 shown, 25 µm and 67 µm.

B) Shown at higher magnification are a single H2 and H1, and to the left, a triplet of H1s which appear to have settled out of the initial retinal dissociate still attached to one another and presumably coupled by gap junctions. The mean soma size and dendritic spread for the H1s shown is 14 µm and about 45 µm, respectively; and for the H2 shown, 14 µm and 75 µm.
FIGURE 2

Phase light microscopy of black crappie H1s at different days in culture. Scale bars = 50 µm.

A) One freshly-isolated H1 which has lost its axon through the dissociation procedure. A few stout dendrites remain, giving the cell a stellate appearance.

B) Three 4-day old coupled H1s which lost their axons during dissociation but exhibit evidence of neurite outgrowth. Small (s), medium (m), and large (l) neurites are observed.

C) Three 6-day old coupled H1s which lost their axons during dissociation. The top cell has regenerated a large, axon-like neurite (arrow) which is approximately three soma sizes long. Neurite outgrowth from the two bottom cells appears restricted to small and medium neurites at this point.
Phase light microscopy of black crappie H2s and H3s at different days in culture. Scale bars = 50 µm.

A) Freshly-isolated H2 of typical appearance and size which has lost its axon during dissociation. Six main dendrites remain and the nucleus is visible.

B) Three-day-old H2. No evidence for regeneration of an axon-like neurite is seen, but spike-like fine neurites have elongated from the main dendrites.

C) Six-day-old H3, H2, and bipolar (BP) cells. The H3 cell type, as in the white bass, is distinguished from the H2 by its size and elongated cell body. No evidence for regeneration of axon-like like neurites is seen in either type of HC. The BP has a short axon (arrowhead) which probably survived the dissociation procedure intact. According to criteria established by Lasater (1988), this is probably a large class type BP.
FIGURE 4

Phase light microscopy of black crappie H4s at different days in culture. Scale bars = 50 µm.

A) Freshly-isolated H4 partially obscured by cellular debris. This stellate cell type is distinguished from the stellate H2 by its more cylindrical dendrites and generally larger dendritic spread. The H4 dendrites also often have an almost curly appearance though this is not as pronounced in the crappie as it is in the white bass. In contrast with H1s, H2s, and H3s, H4s are axonless in the intact retina.

B) Three-day-old H4, H1, and H2. The difference in cell size and shape between these types is evident.

C) Eight-day-old H4. The nucleus is visible. A few fine neurites have extended from the main dendrites of this cell.
Phase light microscopy of black crappie bipolar (BP) cells at different days in culture. The axons of BPs generally survive the dissociation procedure intact. All four BPs shown here are oriented with apical dendrites upward and axons downward. BPs in culture grow very fine neurites from dendrites and axon terminals that are barely resolvable with light microscopy at this magnification. Scale bar = 50 µm.

A) Four-day-old BP cells. Large class BP in the center of the photo with a small class BP in the upper left-hand corner (see arrowhead).

B) Six-day-old large class BP.

C) Seven-day-old BP cells. Large class BP in the center of the photo with a small BP above (see arrowhead). It is not known if this cell had a short axon to begin with or if it is regenerating an axon lost during dissociation.

D) Twelve-day-old BP.
FIGURE 6

Scanning electron microscopy (SEM) of black crappie BPs at different days in culture, prepared by the OTOTO or EtOH method (see text for explanation). Scale bars as shown. The growth of new lamellipodia from dendrites and tiny neurites from both dendrites and axon terminals is evident using SEM (cf. Fig. 5). No significant difference in cell morphology is seen in BPs processed by either method, except that fracturing of thin processes is more frequently seen in cells processed by the OTOTO method.

A) Six-day-old large class BP processed with OTOTO method. Note the fractured axon (arrowhead).

B) Six-day-old large class BP processed with OTOTO method.

C) Five-day-old large class BP processed with EtOH method.

D) Five-day-old cell tentatively identified as a Muller cell processed with EtOH method. These glial cells do not generally survive in culture.
Figure 7

Scanning electron microscopy (SEM) of freshly-isolated black crappie photoreceptors processed by the OTOTO or EtOH method (see text for explanation). Scale bars as shown.

A) Double cone inner segment processed by OTOTO method. This image may represent the densely packed mitochondria found in this part of the cone when the plasma membrane is stripped away during the dissociation procedure.

B) Two rods processed by OTOTO method, oriented with the inner segments upward and the long, thin axons downward. Arrowheads indicate the cell bodies. Rods sustain far less damage from the dissociation procedure compared to cones.

C) Double cone inner segment processed by EtOH method.
FIGURE 8

SEM of black crappie H4s at different days in culture processed by OTOTO or EtOH methods. Scale bars as shown. The general appearance of these H4s parallels that seen with phase LM (cf. Fig. 4). Tiny neurites from the main dendrites have survived the dissociation procedure intact (arrowheads; A, B). Fracture artifacts are pronounced in the OTOTO cells (A, C, D). The EtOH method yielded a flat, almost ghost-like image (B, E). Little neurite growth occurs in H4s during five days in culture (cf. A, B with C, E).

A) Freshly-isolated H4 by OTOTO method.
B) Freshly-isolated H4 by EtOH method.
C) Five-day-old H4 by OTOTO method.
D) Six-day-old H4 by OTOTO method.
E) Five-day-old H4 by EtOH method.
FIGURE 9

SEM of black crappie H2s or H3s at different days in culture, processed by OTOTO or EtOH methods. Scale bars as shown. The general appearance of these H2s and H3s parallels that seen with phase LM (cf. Fig. 3). Few tiny neurites from the main dendrites have survived the dissociation procedure intact (arrowheads; A, B, C, D). Many more such neurites are observed after several days (E-I), presumably reflecting growth in culture. Fracture artifacts are pronounced in the OTOTO cells (A, B, E, F). The EtOH method yielded a flat, almost ghost-like image (C, D, G, H, I), but with better preservation of lamellar structures compared to OTOTO cells.

A) Freshly-isolated H2 with OTOTO method.
B) Freshly-isolated H3 with OTOTO method.
C) Freshly-isolated H2 with EtOH method.
D) Freshly-isolated H3 with EtOH method.
E) Five-day-old H2 with OTOTO method. Note the growth cone-like process (arrow) despite the rounded-up appearance of this cell which usually signifies damage.
F) Six-day-old H2 with OTOTO method.
G) Five-day-old H2 with EtOH method.
H) Five-day-old H3 with EtOH method.
I) Five-day-old H3 with EtOH method.
FIGURE 10

SEM of black crappie H1s at different days in culture processed with OTOTO or EtOH methods. Scale bars as shown. The general appearance of these H1s parallels that seen with phase LM (cf. Fig. 2). Multiple H1s that are contacting one another may be electrically coupled via gap junctions (A, B, C, G). Fracture artifacts associated with the OTOTO method are infrequent in this cell type. The EtOH method yielded a flat, almost ghost-like image (C, D, E, G). A frequent H1 feature with both methods is the apparent shrinkage of the cytoplasm down around the nucleus (B, D, G).

A) Freshly-isolated H1s processed with OTOTO method.

B) Freshly-isolated H1s processed with OTOTO method.

C) Freshly-isolated H1s processed with EtOH method.

D) Freshly-isolated H1 processed with EtOH method.

E) Freshly-isolated H1 processed with EtOH method.

F) Five-day-old H1 processed with OTOTO method. Limited neurite outgrowth has occurred; the rounded appearance of the cell body may signify damage.

G) Five-day-old H1s processed with EtOH method. Neurite outgrowth has occurred in the form of medium neurites and tiny neurites.
FIGURE 11

SEM of black crappie HCs at different days in culture processed with OTOTO or EtOH methods. Scale bars as shown.

A) Freshly-isolated H1 and H2 processed with OTOTO method. The large H2 bears a dramatic fracture artifact. The size difference between these two cell types is evident.

B) Freshly-isolated H1, H2, and H3 processed with EtOH method. These cells are so flattened as to suggest that they are mere footprints of membrane preserved by this method.

C) Five-day-old H1 processed with EtOH method. Note the elaborate formation of filopodia and the ghost-like or footprint appearance of the cell body.
LITERATURE CITED


APPLICATION FOR GRADUATION WITH HONORS DEGREE

Date: April 1, 1993
Name: Jon Ann Steinelt
Social Security Number: 529-53-0187

I hereby apply to graduate with
- University Honors
- University Honors with Departmental Honors in
- Departmental Honors in

List Honors classes completed:
1. Econ 200
2. Chem 222
3. Psy 350
4. Psy 351
5. Honor 390

List Honors classes to be completed:

Thesis title (Completed thesis must be submitted to the Honors Office before approval can be given for graduation):
A New Model Species For Cultured Teleost Retinal Cell Studies

Light And Scanning Electron Microscopy

Date of presentation of thesis:
April 22, 1994

Approved:

Director of Honors Program

Department Advisor
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**Note:** The table includes courses and their corresponding credit hours and grades for an honors degree. The table is incomplete as some courses and grades are not fully visible or legible.