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SCANNING ELECTRON MICROSCOPIC STUDY OF ROCKFISH PRESERVED AT EITHER AMBIENT TEMPERATURE OR BY ISOTHERMAL FREEZE-FIXATION

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Abstract

Fresh rockfish (Bocaccio, Sebastus paucispinis) fillets were blast frozen and stored at either -5°C or -20°C for 60 days. At defined sampling intervals, specimens were removed and chemically fixed at either ambient temperature or isothermally, at the respective storage temperatures (-5°C or -20°C). The results indicated that isothermally freeze-fixed specimens showed a degree of crushing and/or distortion of the muscle fiber, the deposition of presumed ice crystals in the cell membrane, the multi-dimensionality of connective tissue and the preservation of fine detail. In specimens fixed at ambient temperatures, structural components had relaxed back to a state similar to their native conformation. Isothermal freeze fixation appears to be an excellent means to preserve frozen muscle tissue for the study of the effects of freezing and frozen storage.

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Introduction

Post-mortem fish flesh deteriorates rapidly even when chilled, due to microbiologic and biochemical factors. As a consequence, fish shipped to landlocked areas may be frozen for preservation. Similarly, fish, such as tuna, is routinely brine frozen at sea and may be preserved in this way for periods of several months before it is processed at a commercial cannery. The biochemical deterioration of fresh and frozen fish has been well documented (Sikorski et al., 1976). The occurrence of physical changes which may be caused by the ice alone is controversial.

Although transmission electron microscopy (TEM), scanning electron microscopy (SEM) and light microscopy (LM) have all been used to determine structural changes in fish flesh, TEM micrographs of fish muscle are far more commonly published than are either LM micrographs or SEM micrographs. SEM, unlike TEM, produces micrographs which show considerable depth of field - a distinct advantage in visualizing the three-dimensional quality of a sample.

Fixation of specimens for any microscopic method requires precision. Geissinger and Stanley (1981) reviewed the implication of high (25 to 37°C) versus low (0 to 4°C) temperature fixation. It was noted that although fine structures were better preserved at higher temperatures, lower fixation temperatures might be desirable to prevent autolytic changes in the tissue during fixation. Frozen tissue presents a greater problem to the microscopist because one frequently needs to determine beforehand the importance of the location and size of ice crystals in frozen tissue as well as how tissue will be affected after thawing. Preservation of structure with ice crystals intact compounds the problem. Fixing thawed specimens or thawing during fixation not only may fail to preserve distortions caused by ice crystal formation and ice crystal growth but may also allow tissue to return to or near its natural state, thereby causing information on the size of ice crystals to be lost.

One method of fixation which does not disturb ice crystals within tissue is Isothermal Freeze Fixation. In this method, the fixative is pre-equilibrated with ice at the temperature of fixation [added freezing point depressing solutes perform this function]. Since the fixative is thermodynamically at equilibrium with ice, it should not disturb ice within tissue. Glutaraldehyde, or other fixatives, crosslink the tissue matrix between ice crystals, so that when the tissue is thawed, holes remain in the original locations of the crystals. If no fixative were present, the tissue matrix might relax and fill the cavities previously occupied by ice.

Richardson and Scherubel (1908) first reported observations on what may have been isothermally freeze-fixed beef. Initially, these researchers used ethanol in serial concentrations to substitute for the aqueous phase in the muscle tissue. Specimens were viewed with the light microscope after ethanol substitution, embedding and staining. It was noted that mixtures of NaCl and formaldehyde would be suitable for freeze-fixation. MacKenzie et al. (1975) later utilized designed mixtures of a combination of compounds consisting of glutaraldehyde, formaldehyde, osmium tetroxide and NaCl. The proportions of each chemical varied with the fixation temperature used, in the range of -2°C to -10°C. Observations on rat hearts and frozen frog skeletal muscle indicated the placement and configuration of ice crystals. Asquith and Reid (1980) reported on the preparation of frozen specimens for LM and TEM by isothermal freeze-fixation at temperatures to -20°C. Freeze fixed longissmus dorsi was examined by LM and TEM which elucidated the location and size of ice crystals within the muscle structure.

Drying is another variable associated with specimen preparation. Freeze dried specimens can exhibit artifacts caused by extra ice crystal formation at the lower temperatures necessary for this procedure (Chabot, 1979) (freeze drying of unfixed tissue should not be performed above the collapse temperature). It is not clear whether previously fixed tissue could be damaged by freeze drying. Critical point drying might result in fewer artifacts (Humphreys et al., 1974) and less distortion (Boyde, 1978).

The objective of this study was to compare conventional (ambient temperature) fixation to isothermal freeze-fixation of rockfish and to test the suitability of this method for scanning electron microscopy. A particular objective was the location of ice crystals, and potential ice crystal damage within the frozen tissue.

Materials and Methods

Rockfish (Bocaccio, Sebastus paucispinis) were caught off the coast of Northern California and were shipped on ice to the Institute of Marine Resources. Upon arrival the fish were ca. 36 hr old. Each fish was immediately eviscerated, skinned and fillets (ca. 200g each) were removed for microscopic and biochemical testing. Fillets were placed into aluminum pans (10x5.6 x4.8cm) and were blast frozen (to -20°C). Thermocouples inserted into the center of representative fillets were used to monitor the rate of freezing. When fillets had reached an internal temperature of -20°C, which took 3-4 hrs, samples were divided into treatments to be stored at either -5°C or -20°C. The evaluation schedule for the fillets was at 0, 1, 10, 18 and 60 days of storage. At each scheduled interval, specimens were either isothermally freeze-fixed, or thawed (for ca 12 hrs at 4°C) prior to fixation at ambient temperature (ca 22°C).

Fixation

The conventionally fixed specimens were placed into a 0.1M cacodylate buffer (pH 6.8) containing 2% glutaraldehyde. Specimens to be isothermally freeze-fixed were placed (in the appropriate freezer) into the same cacodylate-glutaraldehyde buffer which contained up to ca 40% dimethylsulfoxide (DMSO). The concentration of DMSO was such as to allow the formation of a small quantity of ice crystals in the fixation solution at either -5 or -20°C, respectively (Asquith and Reid, 1980).

At each scheduled sampling interval, $3mm^3$ specimens were removed from the thawed fillets and placed into fixative and held for 24 hours at 22°C. After 24 hours the specimens in fixative were transferred to storage at 4°C for 6 days. After using a chilled oil-free razor blade to remove $3mm^3$ sections of fillet, isothermal freeze-fixation was conducted on sections which had been stored at either -5°C or -20°C by placing them into isothermal freeze fixatives containing ice at those temperatures. Specimens in fixative were maintained at each respective temperature for 7 days.

After 7 days, specimens were washed for 30 mins in a 0.1M cacodylate buffer containing 5% sucrose. Serial dehydration of specimens in ethanol followed. Specimens were then immersed into liquid Freon and cryofractured. The ethanol was then replaced with amyl acetate and the specimens were dried to critical point. The dried specimens were then mounted onto aluminum stubs; pulse-sputter-coated with gold and viewed with either a Philips PSEM Model 400 (tungsten filament) or an ISI DS 130 scanning electron microscope equipped with a LaB_6 cathode.

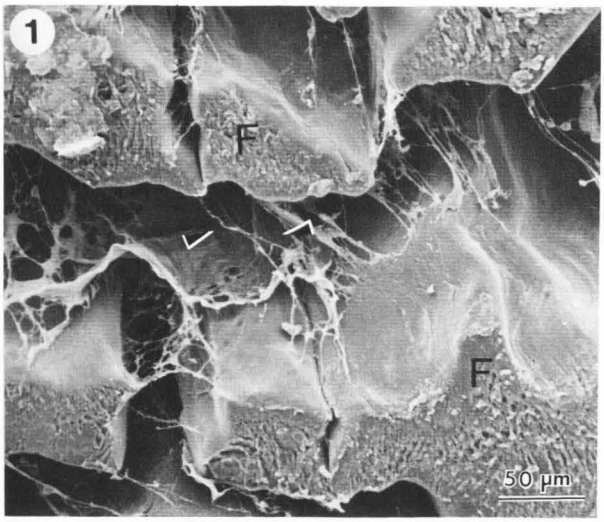
Results and Discussion

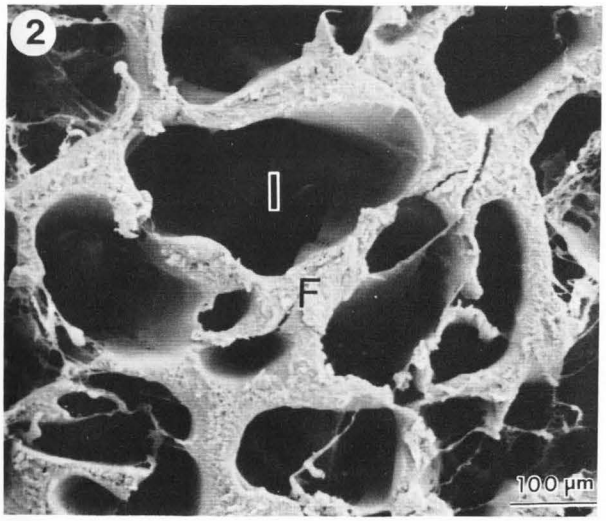
Observation of the specimens has included study of the size and shape of the muscle fiber, spaces and their arrangement between muscle fibers, integrity of the sarcomere and detail of the surface structure.

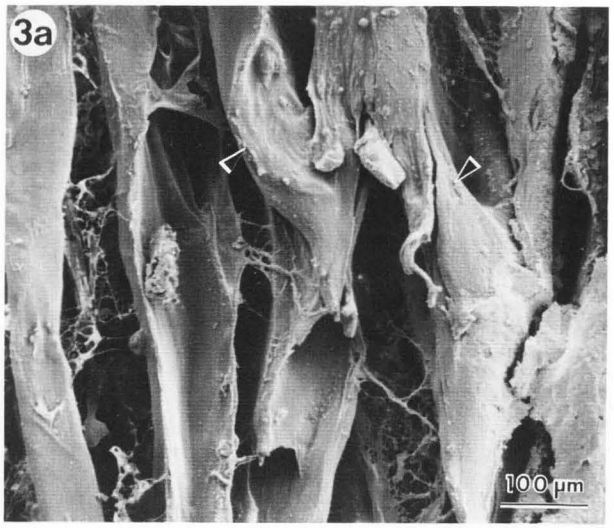
The difference in spacing between cross-sectional views of muscle fibers and the dimensions of individual muscle fibers is evident when comparing specimens preserved by either the conventional (ambient temperature) fixation or the isothermal freeze-fixation technique. Photomicrographs of the conventionally fixed specimen show larger fascicles and smaller spaces in the

interstices between muscle fiber bundles (Figure 1). The isothermal freeze-fixed specimen shows evidence of larger spaces between fascicles than the conventionally fixed specimens (Figure 2). These areas are presumably sites of ice crystal deposition/formation. The fascicles appear in a denser, more compact configuration, possibly a result of the osmotic dehydration of the muscle bundle. Lilejemark (1969) previously reported distortions of the normally hexagonal (in cross section) myofibril of cod when held at - 40°C for periods as long as 40 months. Bello and co-workers (1982) similarly found crushed myofibrils in goldfish frozen slowly (-10°C).

Longitudinal views of the freeze-fixed muscle specimens further indicate the occurrence of fascicles distortion in the frozen state (Figure 3). In this figure, the distortion is evident by the sinuous nature of the fascicular grouping. Further, a gaping has occurred between the muscle bundle and the basal lamina. A shifting or gaping of the cod sarcoplasmic reticulum has been







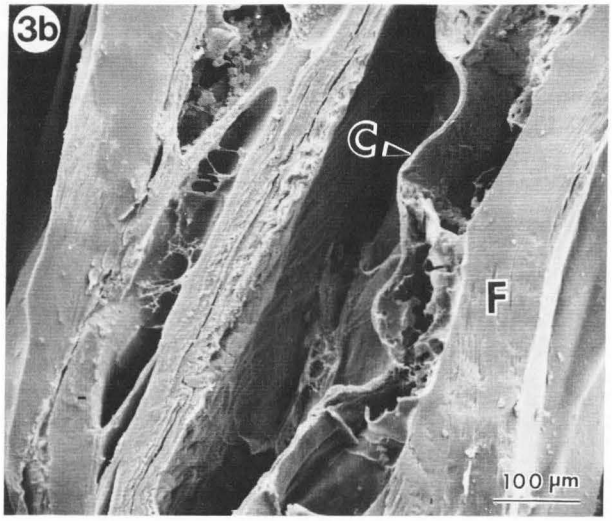


Figure 1. Cross section of rockfish muscle held at -20°C for 10 days. This specimen was thawed for ca. 12 h at 4°C before fixation at ambient temperatures. There are spaces (arrows) between bundles of muscle fibers (F).

Figure 2. Cross section of isothermally freeze-fixed rockfish muscle held at -20°C for 10 days. There are large spaces (I) between dense bundles of muscle fibers (F).

Figure 3. Longitudinal sections of isothermally freeze-fixed rockfish muscle held at -5°C for 18 days. a) The fiber bundles become distorted (arrows) from the formation of ice crystals. b) Gaping has occurred between the muscle bundles (F) and the cell membrane or basal lamina (C).

previously reported (Jarenback and Liljemark, 1975a, b, c; Liljemark, 1969). The cell membrane may further be damaged by either penetration of ice crystals or their growth at its surface (Figure 4). Cell membranes are more prone to ice crystal damage than is the pliable collagen sheath. It has been previously reported that, to a limited extent, large ice crystals can rupture cell walls thus initiating the outward movement of intracellular constituents (Giddings and Hill, 1978; Hamm, 1960; Hamm and Deatherage, 1960).

Muscle bundles thawed prior to fixing at ambient temperatures have normally assumed their native configuration and orientation (Figure 5). Thus ice crystal conformations in the frozen tissue may go undetected because of migration of some water back into the cell upon thawing. It is interesting to note the low relief and closely knit/dense spatial configurations between areas of connective tissue overlying muscle bundles (Figure 6). In freeze-fixed material, however, the connective tissues show a dimensionality and a loose spatial configuration (Figure 7). Smaller circular structures were more easily observed in the freeze-fixed specimens rather than those in tissues fixed at ambient temperature. The dimensionality or high relief of isothermally freeze-fixed tissues demonstrates the pliability/extensibility of the connective tissue and indicates the areas of ice crystal deposition/formation.

In specimens fixed either at ambient or at low temperatures (-5 or -20°C), the sarcomere is visible at higher magnifications (5,500x). The integrity of the sarcomere is evident in those specimens isothermally freeze-fixed (Figure 8). After 10 days of frozen storage, Z discs or possibly transverse bands overlying the same, and A- and I- bands can be seen. However, specimens thawed prior to fixing at ambient temperature have globular bodies adhering to the surface of the myofibrils and there is a loss of visible banding characteristic of the sarcomere (Figure 9). Transverse bands overlying the Z-disc are evident.

Magnifications in excess of 20,000x are at the lower level of high resolution electron microscopy. Geissinger and Stanley (1981) found that the fine detail of muscle specimens was best preserved at higher fixation temperatures (25 to 37°C). In this study, fine detail of frozen connective tissue was well preserved in isothermal freeze-fixation. Artifact formation does not seem to present a major problem with isothermal freeze-fixation. Preliminary studies in which samples maintained in a specially constructed low temperature storage microscope have first been examined by optical microscopy prior to isothermal freeze-fixation, and then reexamination showed no gross changes to occur in ice crystal size and location. Results from previous electron microscopic study of tuna and bonito in this laboratory have demonstrated the good preservation of fine detail of specimens fixed at ambient temperatures (Lampila and Brown, unpublished). This study demonstrates that, with the isothermal freeze-fixed cod, the detailed structure of the frozen system can be preserved. Isothermal freeze-fixation may be a useful method for best preserving, fish muscle for subsequent SEM study, without ice crystals altering muscle structures.

Acknowledgements

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The authors wish to acknowledge Mr. Jack Pangborn and Mr. Bob Munn for their technical assistance.

Figure 4. Longitudinal section of isothermally freeze-fixed rockfish muscle held at -5°C for 18 days. The cell membrane (basal lamina) has been damaged by either penetration of ice crystals or their formation and growth at its surface (arrows).

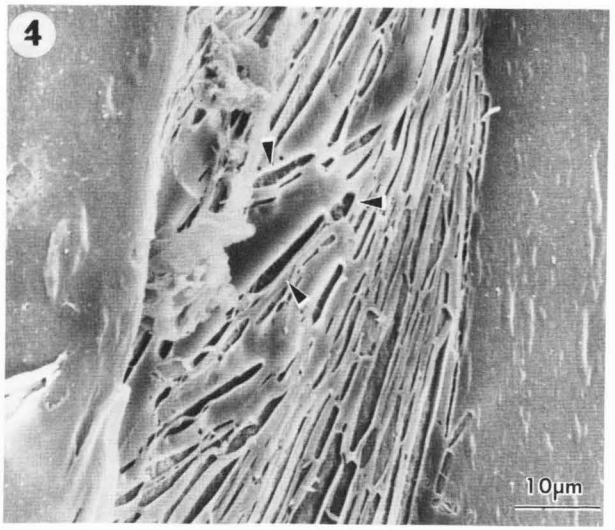
Figure 5. Longitudinal section of rockfish muscle held at -20°C for 18 days. These specimens were thawed for ca. 12 h at 4°C prior to fixation at ambient temperature. Fascicles (F) have assumed their native configuration during thawing. There is low relief of the muscle specimen.

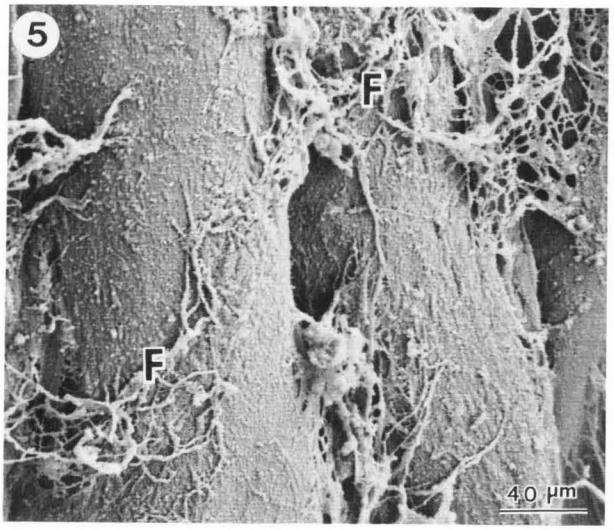
Figure 6. Longitudinal section of rockfish muscle held at -20°C for 18 days. These specimens were thawed for ca. 12 h at 4°C prior to fixation at ambient temperature. There is low relief and a dense configuration of connective tissues between adjacent muscle bundles.

Figure 7. Longitudinal section of isothermally freeze-fixed rockfish muscle held at -20°C for 18 days. There is high relief or depth of the extensible/pliable connective tissues between adjacent muscle bundles.

Figure 8. Longitudinal section of isothermally freeze-fixed rockfish muscle held at -20°C for 10 days. The integrity of the sarcomere (S), and structures interpreted as, Z-discs (Z), A-bands (A) and I-bands (I) is evident.

Figure 9. Longitudinal section of rockfish muscle held at -20°C for 18 days. These specimens were thawed for ca. 12 h at 4°C prior to fixation at ambient temperature. The only visible striations characteristic of the sarcomere (S) are the transverse bands overlying the Z-disc (Z).


4
10μm


5
F
F
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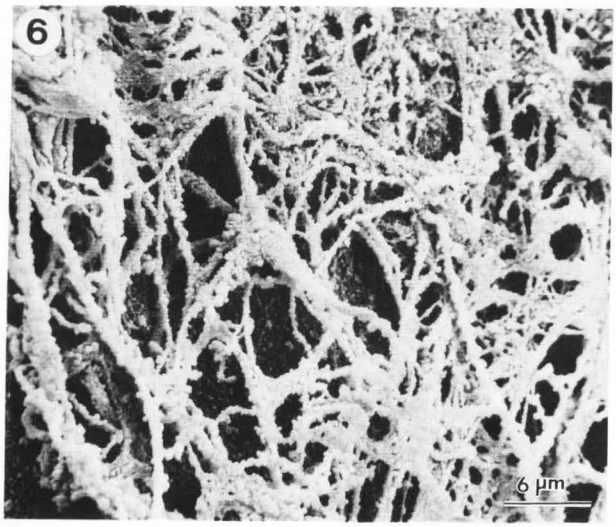
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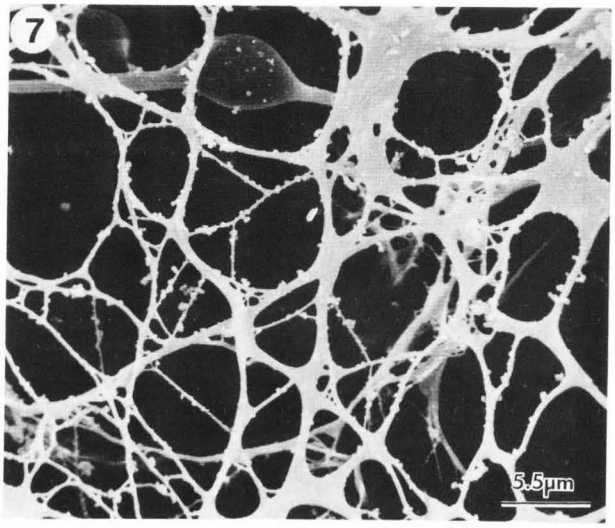
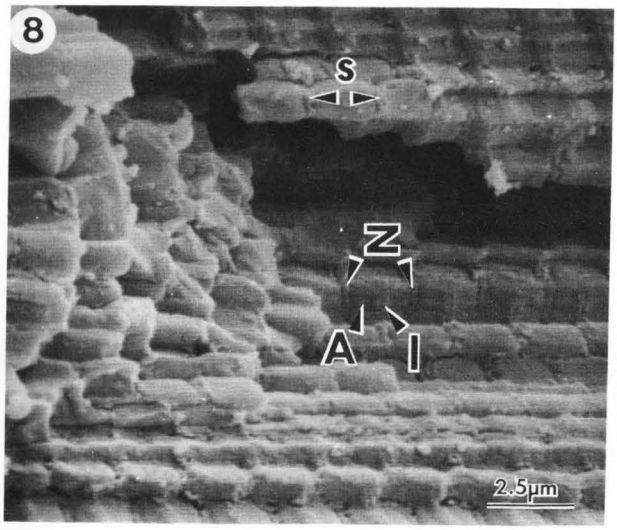
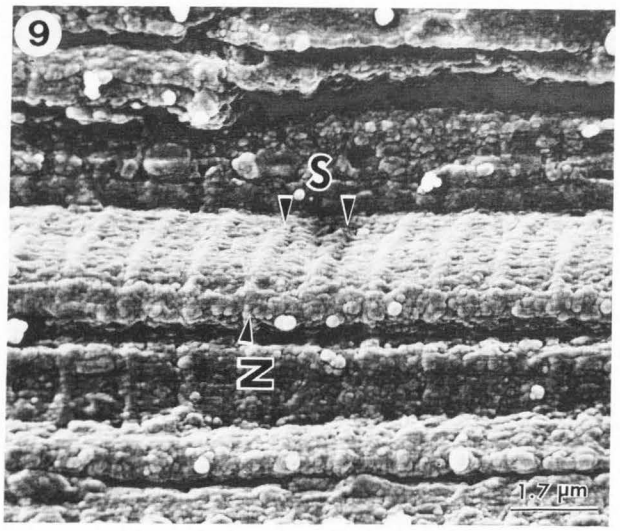
6 μm


7
5.5μm


8
S
Z
A
I
2.5μm


9
S
Z
1.7 μm

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Discussion with Reviewers

Reviewer: The authors mention "low relief" and "high relief" in describing appearance of structures i.e., captions of Figures 5, 6 and 7. Please explain.

Authors: The term "relief" pertains to the projection of figures from their respective background. In this work, the degree of relief has been designated as either "high", projecting greater dimension or distance from the plane or "low", as being more flat in appearance.

Reviewer: What is the nature of the unmarked globules in Figure 9?

Authors: The exact identity of these globules is undetermined. Previous reports in the literature have tentatively identified these globular bodies as mitochondria. These structures do appear to be consistent with the surface morphology of muscle as published by other researchers. Similarly, since the ambient temperature fixative has been widely used, it is not expected that these globules are artifacts.