HIGH RESOLUTION SCANNING ELECTRON MICROSCOPY OF
ISOLATED AND IN SITU CYTOSKELETAL ELEMENTS

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ABSTRACT
Evidence is presented that cytoskeletal structures (actin filaments, intermediate
filaments, and microtubules) can be resolved by scanning electron microscopy
after osmium impregnation of biological material, using thiocarbohydrazide as a
ligand, followed by critical-point drying. These different classes of filaments or
tubules can be identified both as purified protein polymers and as structured
organelles within cryofractured or detergent-extracted cells.

The three-dimensional organization of cytoskeletal elements has become a topic of wide interest in
the field of cell biology. Structural studies in this
field have relied principally on three approaches:
(a) high-voltage transmission electron microscopy
(TEM) with serial sectioning (3) or negative
staining (1, 3, 22); (b) conventional transmission electron mi-
croscopy (TEM) with serial sectioning (3) or negative
staining (1, 3, 22); (c) immunohistochemical
techniques, mainly at the light microscopic level
(5, 6, 16). Although three-dimensional information
is readily obtained by secondary emission, scan-
ing electron microscopy (SEM), this method has
not been exploited for studies of cytoskeletal struc-
ture, largely because of inadequate resolution. Sev-
eral commercial instruments achieve resolutions
of 3–5 nm, yet routine specimen coating tech-
niques generally add a 15–25 nm layer of metal,
which limits the use of this approach for high
resolution of most subcellular materials.

As part of a investigation relating cytoskeletal
elements to the assembly of myofibrils within em-
bryonic chick muscle, we have compared several
coating techniques used in preparing specimens
for SEM. In this paper, we present evidence that
at least one of these methods permits the resolution
by SEM of microtubules, intermediate filaments,
and microfilaments, both as purified polymers and
as structured organelles within cells. When used
in combination with either cryofracture or deter-
gent extraction, it is now feasible to visualize cy-
tofilamentous structures by SEM and to take ad-
vantage of the great depth of field intrinsic to the
instrument.

MATERIALS AND METHODS
Preparation of Proteins
Rabbit skeletal muscle F-actin was a gift from Dr.
Roger Lucas, and was used in a buffer containing 0.1 M
KCl, 2 mM MgCl₂, 2 mM EGTA, 10 mM Tris-maleate,
pH 7.0. The preparation exhibited a single band of
~43,000 daltons when analyzed by sodium dodecyl sul-
fate-polyacrylamide gel electrophoresis (SDS-PAGE)
(15).

Porcine brain microtubule proteins were a gift from
Dr. David Soifer; microtubules were obtained from this
preparation by two cycles of polymerization-depolym-
erization (20) and finally assembled in 0.5 M MgCl₂,
1 mM EGTA, 1 mM GTP, 4 M glycerol, 50 mM mor-
pholinoethane sulfonic acid (MES), pH 6.9. This sample
consisted primarily of α- and β-tubulins with a small
amount of several microtubule-associated proteins.

Intermediate filament protein ("skeletin") was puri-
fied from chicken gizzard smooth muscle according to
the method of Small and Sobieszek (22) and polymerized
into filaments by dialysis against 10 mM sodium acetate,
1 mM cysteine, pH 4.0. By SDS-PAGE, it consisted of
primarily a 55,000-dalton peptide with a small amount
of actin.

Preparation of Samples for Scanning
Electron Microscopy
After appropriate dilution, a drop of the filament
preparation was placed on a gelatin-coated Thermanox
plastic coverslip (Lux Scientific Corp., Newbury Park, Calif.), allowed to adhere for ~30 s, and thoroughly rinsed with the sample buffer. Each coverslip was then immersed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, for 15 min, rinsed, and further immersed in 1% OsO₄ in the same buffer for 15 min, at ambient temperature.

Further osmium impregnation was carried out according to the Malick and Wilson modification (18) of the method of Kelley et al. (12), with further adjustments to accommodate thin specimens and cell monolayers on coverslips. The filament preparations on coverslips were rinsed for 15 min with five to seven changes of distilled water, and then treated with a freshly prepared and filtered, saturated solution of thiocarbohydrazide (TCH, Polysciences, Inc., Warrington, Penn.) in water. After rinsing in water as described above, samples were reacted again for 15 min with 1% OsO₄ in water. The TCH-osmium cycle was repeated once more, and the samples were then dehydrated in a series of acetone (25, 50, 70, 90, and 100%) and critical-point dried from liquid CO₂.

Whole cell preparations were derived from embryonic chick muscle cultures (4) and processed for SEM by a method developed in this laboratory (Ip and Fischman, manuscript in preparation). Briefly, cultures grown on plastic coverslips were fixed in glutaraldehyde, OsO₄-TCH-treated as described above, and dehydrated through a series of acetone followed by a series of amyl acetate in acetone. A coverslip was then removed from 100% amyl acetate, inverted but not drained, and carefully placed, cell-side down, onto a clean copper plate. The entire “sandwich” was plunged into liquid nitrogen, and allowed to equilibrate for a few minutes. The coverslip was then peeled off under liquid nitrogen and transferred quickly into 100% amyl acetate at room temperature. Critical point drying was then performed conventionally from liquid CO₂.

Scanning Electron Microscopy

Scanning electron microscopy was performed with a JEM 100C instrument equipped with a high resolution scanning device (ASID-4D) and a side-entry goniometer. An accelerating voltage of 40 kV was used. Images were recorded at an instrumental magnification of 20,000-50,000 by SEM (Fig. 1), the filaments exhibit a diameter of 14–16 nm, which is approximately twice the width of actin filaments examined by negative stain TEM (Fig. 1, inset). The filaments tend to be straight or gently curved, unbranched, and often appear beaded (arrows, Fig. 1). Studies are in progress to determine whether this beaded-ness is related to the helical substructure of the F-actin filament.

Polymerized microtubules are also resolved by SEM after the osmium-TCH procedure (Fig. 2). The diameter of these structures is 32–37 nm as compared to a diameter of 25–28 nm after uranyl acetate negative staining (Fig. 2, inset). By SEM, the microtubules exhibit an electron-transparent core, presumably the result of beam penetration through the upper layer of protofilaments (arrow, Fig. 2). Some microtubules appear rough-surfaced but no obvious periodicity has been detected. The granular texture of the background in both SEM and TEM images may correspond to intermediates of microtubule assembly (21) which have attached to the gelatin-coated support.

Intermediate filaments which have been reconstituted from chicken gizzard “skeletin” are shown in Fig. 3. In agreement with previous reports (3, 22), these filaments have a complex, branched appearance in which considerable variability of
filament diameters is evident. Presumably, this variability reflects extensive side-to-side aggregation of individual filaments. In areas where single filaments are resolved, their diameters measure 10-12 nm by negative-stain TEM and 18-22 nm by SEM.

Having satisfied ourselves that the osmium-TCH procedure permitted the visualization of individual cytofilaments in the SEM, we proceeded to examine cultured embryonic chick muscle cells, the interiors of which had been exposed by cryofracturing in liquid nitrogen-frozen amyl acetate following fixation and osmium impregnation. Within myotubes (Fig. 4), it is possible to resolve microfilaments, microtubules and intermediate filaments, based on their orientation and individual diameters. Large vesicular structures (arrowheads, Fig. 4), which we assume to be membrane-enclosed, may represent immature elements of the sarcoplasmic reticulum. Thin-section studies of this material are in progress.

We have demonstrated that, with suitable specimen preparation, it is feasible to resolve and identify the various classes of filamentous structures within a cell or cytoskeleton by SEM, and thus obtain direct three-dimensional information regarding their organization in situ. Previous studies have pointed to this possibility (13, 14, 17) by showing that isolated organelles are amenable to high-resolution SEM observation. However, except for studies of the erythrocyte ghost (7), there have been few reports on high-resolution SEM analysis of cytoplasmic filament arrays.

In our experience, routine sputter coating severely hampers the use of SEM at magnifications necessary for the resolution of cytoskeletal elements. Indeed, several authors have pointed out that some of the features observed in sputter coated specimens at high magnifications are, in fact, artifactual (8, 10). In this report, we demon-

**Figure 3** Scanning electron micrograph of intermediate filaments assembled from partially purified "skeletin." The filaments tend to be aggregated and branched, making diameter measurement difficult. The apparently single filament shown by the arrow has a diameter of 18.7 nm. Bar, 0.2 μm. x 40,000. (Inset) In vitro assembled intermediate filaments as seen by negative-stain TEM. Bar, 0.2 μm. x 50,000.
FIGURE 4  Scanning electron micrograph of a myotube from a 1-wk-old chick skeletal muscle culture, processed as described in Materials and Methods. The long axis of the myotube runs from top to bottom in the micrograph. A portion of the myotube was removed during the fracturing procedure, exposing a number of cytoskeletal structures: (MT) microtubules; (IF) filament 18.7 nm in diameter, presumed to be intermediate filament; (MF) filament of 13.8 nm, believed to be actin microfilament. The vesicular bodies (arrowheads) may be elements of the sarcoplasmic reticulum. No myofibrillar components are seen in this particular field. Bar, 0.5 μm. X 80,000.
strate that the osmium-TCH technique provides a reasonable alternative to conventional metal coating. We recognize that a metal thickness of 3–4 nm can also be obtained by high vacuum evaporation. However, we have consistently found it difficult to obtain uniform metal coats by this technique with highly contoured specimens such as cytoskeletons, even with the use of a rotary tilt stage.

Assuming that the "native" diameter of actin filaments, as measured in negatively stained TEM images, is 7 nm (9) and that the osmium-TCH layer is distributed uniformly over the filaments, and further, that there is no significant shrinkage of specimen or coating as a result of dehydration and critical point drying, we calculated the thickness of the coating to be 3–4 nm. If shrinkage is significant (14), this value may have to be revised upward. Transmission electron microscopy of osmium-TCH-coated, dried, re-embedded and thin-sectioned material also confirmed that a coat of this thickness is routinely obtained (reference 11; Ip and Fischman, manuscript in preparation).

Fig. 4 represents our initial attempt at "looking inside the cell" by SEM using the osmium-TCH technique. The results so far have been encouraging. In cultured chick embryo fibroblasts, skeletal, and cardiac muscle cells, we have been able to demonstrate, after various extraction procedures, an insoluble cytoskeletal network composed primarily of actin and 10-nm filaments (11), similar to those seen by others using immunofluorescence and TEM techniques (1, 6, 24, 25). Pudney and Singer (19) have reported similar results. We conclude, in the osmium-TCH technique, particularly when used in conjunction with specific localization techniques (e.g., antibody labeling), should provide a valuable alternative to conventional or high-voltage TEM where three-dimensional information is required.

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