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Lula L. Hilenski
University of South Carolina

Louis Terracio
University of South Carolina

Roger Sawyer
University of South Carolina

Thomas K. Borg
University of South Carolina

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EFFECTS OF EXTRACELLULAR MATRIX ON CYTOSKELETAL AND MYOFIBRILLAR ORGANIZATION IN VITRO

Lula L. Hilenski[1]*, Louis Terracio[2], Roger Sawyer[3] and Thomas K. Borg[1]

1)Department of Pathology, 2)Department of Anatomy, 3)Department of Biology, University of South Carolina, Columbia, SC 29208.

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Abstract

The distribution and three-dimensional relationship of myofibrillar and cytoskeletal components during myofibrillogenesis were examined in preparations of neonatal rat cardiac myocytes processed in parallel for scanning electron microscopy (SEM), intermediate voltage transmission electron microscopy (IVEM) and immunofluorescence (IF). Of the various methods used for processing, optimal results were achieved by pre-extraction with Triton X-100 in an actin-stabilizing buffer. This procedure effectively removed the surface membrane, as viewed by SEM images, while preserving myofibrillar and cytoskeletal structure, as evidenced by IF for actin, α-actinin and vinculin. Cytoskeletons in SEM images consisted of a cortex of anastomosing filaments through which ran parallel filament bundles oriented in the long axis of the cell and attached along their length to the substrate by numerous fine filaments. In IVEM images, myofibrils were laterally connected at the level of the Z bands. Myocytes grown on different extracellular matrices showed different patterns and distributions of both striated myofibrils and focal adhesions, as determined by IF for α-actinin and vinculin, respectively. Cells on collagen I and III contained striated myofibrils which extended to the cell perimeters where focal adhesions were predominately located. Cells on laminin and fibronectin matrices exhibited myofibrils and focal adhesions more centrally located. In addition, cells on laminin contained circumferential arcs of filaments near the cell periphery.

KEY WORDS: myofibrillogenesis, cardiac myocytes, extracellular matrix, cytoskeleton, focal adhesions, actin, α-actinin, vinculin, stabilizing buffer, Triton X-100.

*Address for correspondence:
Lula L. Hilenski, Department of Pathology, University of South Carolina, School of Medicine, Columbia, South Carolina, USA 29208
Phone No. (803) 733-3393

Introduction

Regularly aligned sarcomeres within myofibrils of terminally differentiated striated muscle cells are specialized for coordinated contractile activity. Cytoskeletal and contractile proteins assemble and become oriented into these highly organized three-dimensional arrays of myofibrils during myogenesis. Although ultrastructural and immunofluorescence studies have described the structural organization of major myofibrillar components (Ishikawa, 1983; Wang, 1985), the mechanisms for the sequential assembly and temporal alignment of these proteins and their three-dimensional interactions during myofibrillogenesis are poorly understood. Models for the in vivo assembly and alignment have proposed that: a) myofibril assembly is initiated at Z bodies or plaques (Hill and Lemanski, 1985; Markwald, 1973) or at minisarcomeres containing α-actinin (Sanger et al., 1984, 1986a, 1986b); b) transitory stress-fiber-like structures (SFLS) serve as scaffolds for nascent myofibrils (Dlugosz et al., 1984); c) titin aggregates are formed in synchrony with myofibrils (Tokuyasu and Maher, 1987a, 1987b) and may function in alignment (Hill et al., 1986; Tokuyasu, 1983; Wang et al., 1988) or d) desmin intermediate filaments, which connect Z bands, may align myofibrils (Lazarides, 1980).

During heart development in vivo, myocytes are exposed to changing extracellular matrix (ECM) components which are believed to be important in regulating cell behavior during heart morphogenesis (Borg et al., 1989; Borg and Terracio, 1989). Myocytes have transmembrane receptors for ECM components that interact with ECM molecules on the outside of the cell and cytoskeletal proteins in the cytoplasm. This intimate association between myocytes and ECM suggests that matrix components might also influence internal cytoarchitecture within cells. Two central questions implicit in this association are: a) how external signals, if any, from the environment affect myofibrillogenesis and b) how various cytoskeletal and contractile components interact in the three-dimensional filamentous networks. The first question has been recently investigated in neonatal rat cardiac myocytes grown in vitro on different extracellular matrix components (Borg et al., 1983, 1984, 1989; Borg and Terracio, 1989; Lundgren et al., 1985; Terracio et al., 1989). In

this neonatal cell model, initial stages of myofibril assembly do not appear to require new synthesis but merely the assembly of existing proteins already present in the cell. In contrast, the embryonic cell, upon which most studies of myofibrillogenesis are based, may depend on de novo synthesis of myofibrillar and cytoskeletal proteins. The experiments on neonatal cardiac myocytes showed that: a) assembly and patterns of myofibrils are different on different ECM components; b) the degree of cell spreading on specific ECM components is receptor dependent; and c) cytoskeletal structures such as costameres, which are vinculin-containing rib-like structures located between myofibrils and the sarcolemma (Pardo et al., 1983), and focal adhesions containing vinculin are formed in association with assembly of myofibrils and co-localize with ECM receptors (Borg et al., 1989). These patterns and associations were demonstrated by analysis of data from immunofluorescence and interference reflection microscopy. Both of these methodologies are essentially two-dimensional imaging systems.

Since the ECM-cell surface receptor-cytoskeleton continuum exists in three-dimensional networks of filaments and associated proteins, scanning electron microscopy (SEM), with its relatively large depth of field, would be particularly useful in visualizing these interactions. A major limitation to SEM analysis of cytoskeletons is the need to solubilize the surface membrane of living myocytes without concomitantly destroying myofibrillar structure. Various protocols for processing of cytoskeletons have been evaluated and reviewed by Bell and co-workers (Bell, 1981; Bell and Stark-Vancs, 1983; Bell et al., 1988). In the paper presented here, results from various published protocols for preparing cytoskeletons for SEM and the methodologies which gave optimal results are described. Evidence from immunofluorescence (IF), intermediate voltage microscopy (IVEM) of whole cell mounts and SEM shows that different ECM matrices influence the distribution of cytoskeletal components.

Materials and Methods

Cell Isolation and Culture

Myocytes from 4-5 day neonatal rats were isolated and separated from fibroblasts by a selective attachment method (Borg et al., 1984). Cells were cultured on Formvar-coated gold grids attached to 22-mm round glass coverslips coated with purified ECM components such as laminin (LN), fibronectin (FN), and collagen I + III (Coll I + III). Before coating with ECM components and plating with cells, grids and coverslips were sterilized by ultraviolet light. Coverslips were placed in 35 mm plastic Petri dishes containing F12K culture medium supplemented with 8% horse serum, 3% fetal bovine serum, penicillin (100 U/ml), streptomycin (50 μg/ml), fungizone (0.25 μg/ml) and cytosine arabinoside (10 μg/ml). Cells were maintained at 37°C in an atmosphere of 5% CO_2 in air. Time in culture ranged from 12 hours to 6 days. Cells attached to the gold grids were processed for whole mount IVEM while cells on coverslips were used for IF and SEM.

Specimen Preparation

Use of SF buffer as extraction vehicle. Cells were rinsed in phosphate buffered saline (PBS) + 50 mM KCl at 37°C, extracted with 0.5% Triton X-100 in SF buffer (Sucrose, Fluoride) (100 mM NaF, 50 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 10 mM K-phosphate, pH 6.8, 0.2 mM dithioerythritol, 1 M sucrose) (Blikstad and Carlsson, 1982) for 5 min at 37°C, fixed in 2% paraformaldehyde in SF buffer, pH 6.8, for 10 min, rinsed in 0.1 M glycine in HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (70 mM KCl, 5 mM $MgCl_2$, 3 mM EGTA, 30 mM HEPES, pH 7.0) (Isobe et al., 1988), exposed to primary antibody and then to secondary antibody as described below for immunofluorescence. At this point, cells for IF were mounted in PBS:glycerine (1:3) for observation. Cells for whole mount IVEM and SEM were processed further by fixation in 2% glutaraldehyde in HEPES buffer, treated with 0.2% tannic acid in HEPES buffer, then postfixed in 0.5% osmium tetroxide in HEPES buffer. After dehydration in a graded ethanol series, cells were critical point dried with liquid CO_2 according to the method of Ris (1985). Grids were removed from the coverslips and coated with carbon in a Denton vacuum evaporator. Whole mounts were examined in a JEOL JEM 200-CX transmission electron microscope operated at 200 kV. Coverslips coated with gold by vacuum evaporation were examined in a JEOL JSM-35 scanning electron microscope operated at 15 kV.

Metal coating of specimens. To compare methods of coating specimens for SEM, parallel cultures of unextracted control cells were coated with gold in either: (1) a Bio-Rad Polaron Division E5100 Series II 'Cool' Sputter Coater operated with a cooled specimen stage at 2.4 kV and 20 mA for 120 seconds at 0.02 mbar or (2) in a Denton DV-502 vacuum evaporator.

Physical rupture of cells. Cells were ruptured with a jet of standard breaking solution (SBS) (150 mM KCl, 2 mM $MgCl_2$ in 20 mM Na_2PO_4-$NaHPO_4$, pH 7.4) (Boyles and Bainton, 1979) delivered by a 25 gauge needle and then immediately immersed in a 1:1 SBS-4% glutaraldehyde solution in 20 mM sodium phosphate buffer, pH 7.4, for 60 min at 37°C. Cells were then osmicated in 1% osmium tetroxide in 0.1 M sodium phosphate + 4% sucrose for 60 min at room temperature, treated with aqueous 0.5% uranyl acetate for 60 min, dehydrated in an ethanol series, critical point dried and coated with gold as described above.

Four-step fixation procedure. Some unextracted control cells and cytoskeletons were processed by a four-step fixation procedure (Schroeter et al., 1984) as follows: (1) 2% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4, containing 0.1 M sucrose for 30 min, followed by three rinses in 0.1 M cacodylate buffer for 10 min each; (2) 1% osmium tetroxide in 0.1 M cacodylate buffer for 30 min, followed by three rinses in 0.1 M cacodylate buffer for 10 min each; (3) 1% tannic acid in 0.1 M cacodylate buffer for 1 hour, followed by three rinses in 0.1 M cacodylate and a 1 min distilled water rinse. Dehydration was effected through a graded acetone series for 10 min each up to 70% acetone; (4) specimens were then left in 3.5% uranyl acetate in 70% acetone overnight in the dark, followed by 3-5 changes in

Table 1. SUMMARY OF METHODS FOR PREPARING CYTOSKELETONS OF RAT HEART CELLS

Fixation/extraction methods for immunofluorescence[1]	Sarcomere structure maintained[2]	Surface membrane removed[3]	Reference
I. Pre-extraction			
a. 0.5% Triton in PBS, pH 7.2, 5 min, 37°C; fix 2% PF in PBS, pH 7.2, 10 min, RT	-	+	-
b. 0.5% Triton in KMP, pH 7.5, 5 min, 37°C; fix 2% PF in KMP, pH 7.5, 10 min, RT	Variable	+	Isobe and Shimada, 1983
c. 0.5% Triton in SF, pH 6.8, 5 min, 37°C; fix 2% PF in SF, pH 6.8, 10 min, RT	+	+	Blikstad and Carlsson, 1982
II. Prefixation			
a. 2% PF in PBS, pH 7.2, 10 min, 37°C; 0.5% Triton in PBS, pH 7.2, 10 min, 37°C	+	-	Bell, 1981
b. DTSP (0.2 mg/ml in DMSO) in D-PBS + 50 mM KCl, pH 7.4, 10 min, 37°C; 0.5% Triton in same buffer, 10 min, 37°C	-	Variable	Bell, 1981
c. DTBP (4 mg/ml) in D-PBS + 50 mM KCl, pH 7.4, 4 min, 37°C; 0.5% Triton in same buffer, 10 min, 37°C	-	Variable	Bell, 1981; Toyoshima et al., 1986
III. Simultaneous fixation/extraction			
a. 2% PF + 0.5% Triton in PBS + 10 mM $MgCl_2$ + 1 mM EGTA, pH 7.2, 30 min, 37°C	+	-	Bell, 1981
b. DTSP (0.2 mg/ml in DMSO) + 0.5% Triton in D-PBS + 50 mM KCl, pH 7.4, 30 min, 37°C	+	Variable	Bell, 1981
c. DTBP (4 mg/ml) + 0.5% Triton in D-PBS + 50 mM KCl, pH 7.4, 10 min, 37°C	+	Variable	Bell, 1981
IV. Physical rupture			
a. SBS, pH 7.4, from buffer jet quickly; 2% PF in PBS, pH 7.4, 10 min, RT	+	+	Boyles and Bainton, 1979

ABBREVIATIONS: PF (paraformaldehyde);PBS (2 mM NaH_2PO_4, 7 mM Na_2HPO_4, 137 mM NaCl, pH 7.2);D-PBS (Dulbecco's PBS: 137 mM NaCl, 3 mM KCl, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, 0.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4); KMP (50 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA and 10 mM K-phosphate buffer, pH 7.5); SF (100 mM NaF, 50 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 10 mM K-phosphate, pH 6.8, 0.2 mM dithioerythritol, 1 M sucrose); DTSP [dithiobis (succinimidyl) propionate]; DTBP (dimethyl 3,3' dithiobispropionimidate); SBS (150 mM KCl, 2 mM $MgCl_2$, 20 mM Na_2PO_4-$NaHPO_4$, pH 7.4); DMSO (dimethylsulfoxide); RT (room temperature).

[1] Only the initial steps for each procedure are given. Processing for SEM required additional fixation steps in glutaraldehyde and osmium tetroxide (optional treatments with tannic acid and uranyl acetate) to stabilize the samples during subsequent dehydration, critical point drying and metal coating.

[2] Sarcomere spacing (2-2.3 μm) was determined by indirect immunofluorescence for actin and for α-actinin which labels Z bands.

[3] Surface membrane removal was determined by scanning electron microscopy of critical point dried and gold-coated specimens.

70% acetone. Dehydration was continued in 90% acetone, followed by three changes of 100% acetone. Cells were critical point dried and coated with gold as above.

Immunofluorescence

Cells fixed by the various protocols listed in Table 1 were incubated for 30-60 min with a 1:20 dilution of rabbit anti-α-actinin (Transformation Research, Framingham, MA) in PBS [137 mM NaCl, 2 mM NaH_2PO_4, 7 mM Na_2HPO_4, pH 7.2, containing 0.5% bovine serum albumin (BSA)] or a 1:10 dilution of anti-actin (Miles Laboratories, Elkhart, IN) in PBS + 0.5% BSA at 37°C in a moist chamber. Control cultures were incubated in rabbit preimmune serum in PBS + 0.5% BSA. After three buffer washings in PBS + 0.5% BSA for 10 min each, samples were incubated in a 1:30 dilution of fluorescein isothiocyanate (FITC) conjugated

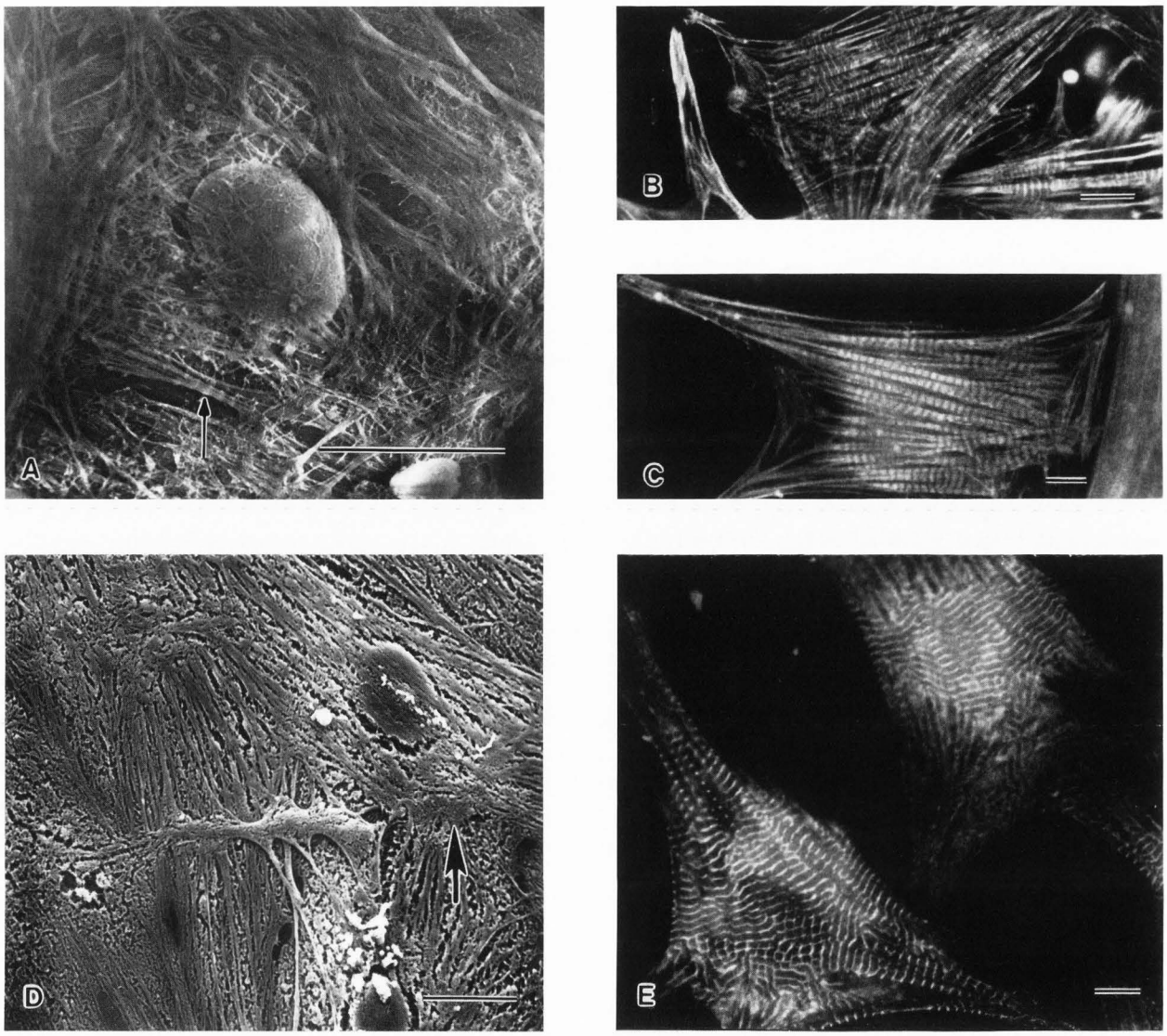


Figure 1. SEM and IF images of cytoskeletons in cardiac myocytes extracted with 0.5% Triton X-100 in PBS either prior to fixation (A + B), after fixation (C), or simultaneously during fixation (D + E) in aldehyde buffers. A. SEM of myocyte pre-extracted in PBS. The surface membrane has been completely solubilized revealing branching myofibrils in a meshwork of filaments. Note the striations in one filament bundle (arrow). B. Indirect IF for actin in myocytes pre-extracted in PBS shows loss of sarcomeric structure. Compare with sarcomeres in cell prefixed in paraformaldehyde before detergent extraction (C). D. SEM of myocytes simultaneously fixed and extracted. Surface membrane (arrow) has not been solubilized. E. Indirect IF for α-actinin in myocytes simultaneously fixed and extracted shows Z bands in sarcomeres. Bars=10 μm.

to goat anti-rabbit IgG (Organon Teknika-Cappel, West Chester, PA) in PBS + 0.5% BSA as above. For labeling of focal adhesions and costameres, cells processed by Protocol Ic were incubated in 1:20 dilution of mouse monoclonal anti-vinculin (ICN ImmunoBiologicals, Lisle, IL) in PBS + 0.5% BSA. Controls were incubated in PBS + 0.5% BSA. For secondary antibody labeling, cells were incubated in 1:30 dilution of FITC affinity purified antibody conjugated to mouse Fab (ICN) in PBS + 0.5% BSA. For labeling of F actin, cells were incubated in a 1:50 dilution of rhodamine-labeled phalloidin (Molecular Probes, Eugene, OR) in PBS + 0.5% BSA for 30 min at 37°C. Fluorescence was observed with a 63X objective on a Zeiss IM-35 microscope equipped with epifluorescence. Photographs were taken with Kodak Tri-X Pan film (400 ASA) with an exposure time of 60 seconds.

SDS Gel Electrophoresis

Gel electrophoresis (SDS-PAGE) was used to

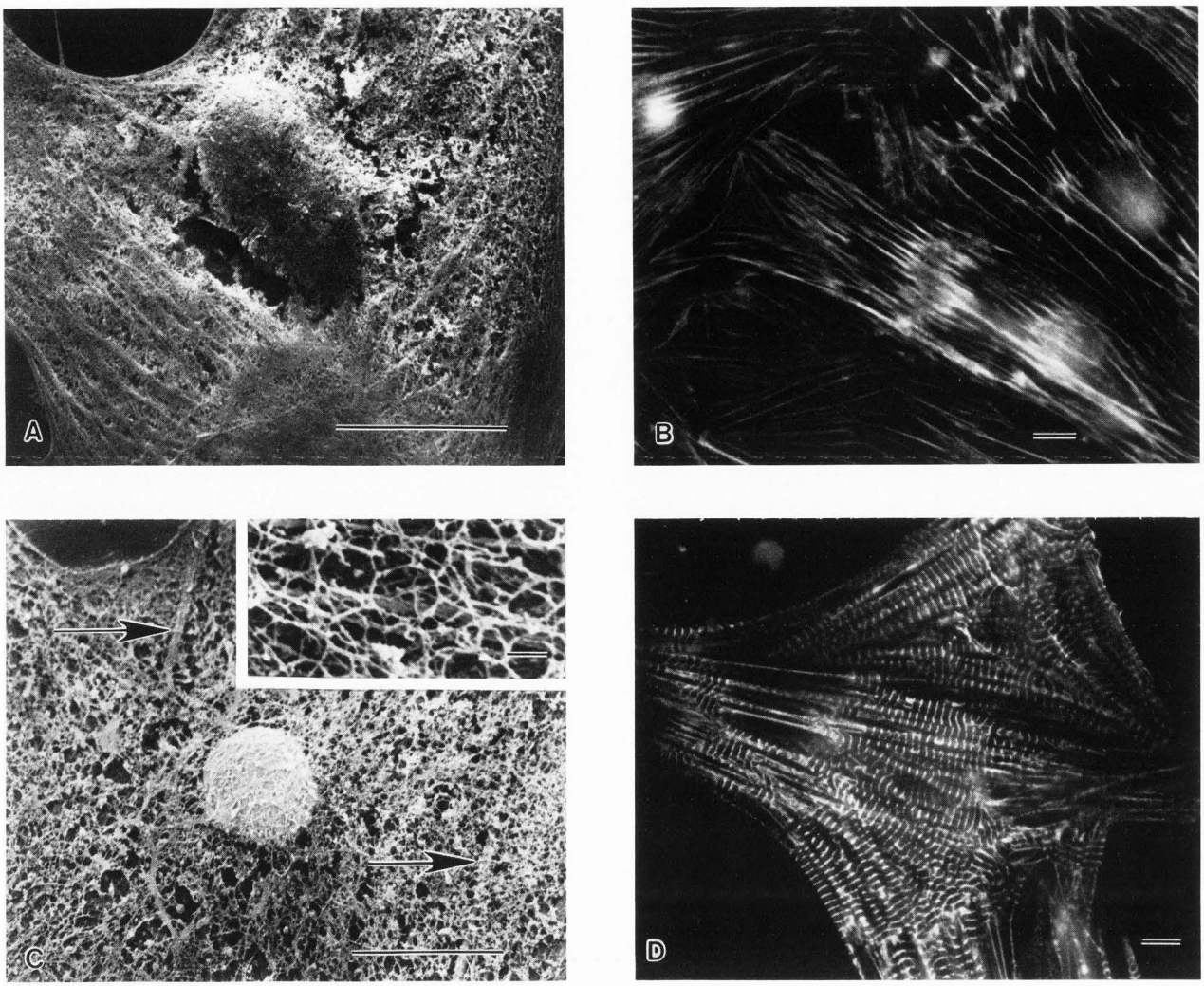


Figure 2. SEM and IF of myocytes crosslinked with DTBP before (A and B) or simultaneously (C and D) during Triton X-100 extraction. A. SEM of myocytes crosslinked in DTBP before extraction shows areas of intact membrane which obscures underlying cytoskeleton. B. Indirect IF for actin in myocytes prefixed in DTBP shows that cells have lost their sarcomeric structure. Compare with Fig. 1C which shows prefixed cell with obvious striated patterns indicative of intact sarcomeres. C. SEM of myocyte treated with DTBP and Triton X-100 simultaneously shows cortical network of anastomosing filaments through which run filament bundles (arrows). Inset is high magnification of honey-combed network of cortical filaments. D. α-actinin IF in myocytes treated with DTBP + detergent shows sarcomeric structure remains intact. Bars=10 μm (A,B,C,D). Bar= 1 μm (C-inset).

determine whether contractile proteins were extracted during the permeabilization process. Extraction buffer was freeze dried, mixed with sample buffer and analyzed by SDS-PAGE according to the procedures of Laemmli (1970). The identification of actin and myosin was made by comparison to standards exhibiting the same relative molecular weight (M_r).

Results

Table 1 is a summary of the various protocols that were used to process myocytes for SEM; only the initial steps are given. The main objective in specimen preparation was to establish one methodology that would allow cells to be processed in parallel for IF, whole mount IVEM and SEM. The two main criteria for evaluating the protocols were: a) maintenance of sarcomeric structure as determined by indirect IF staining of actin or α-actinin; and b) removal of surface membrane as determined by SEM images. Initial attempts to extract myocytes in Triton X-100 in PBS before fixation effectively removed the surface membrane and revealed underlying filament bundles by SEM. A few of the filament bundles showed periodic

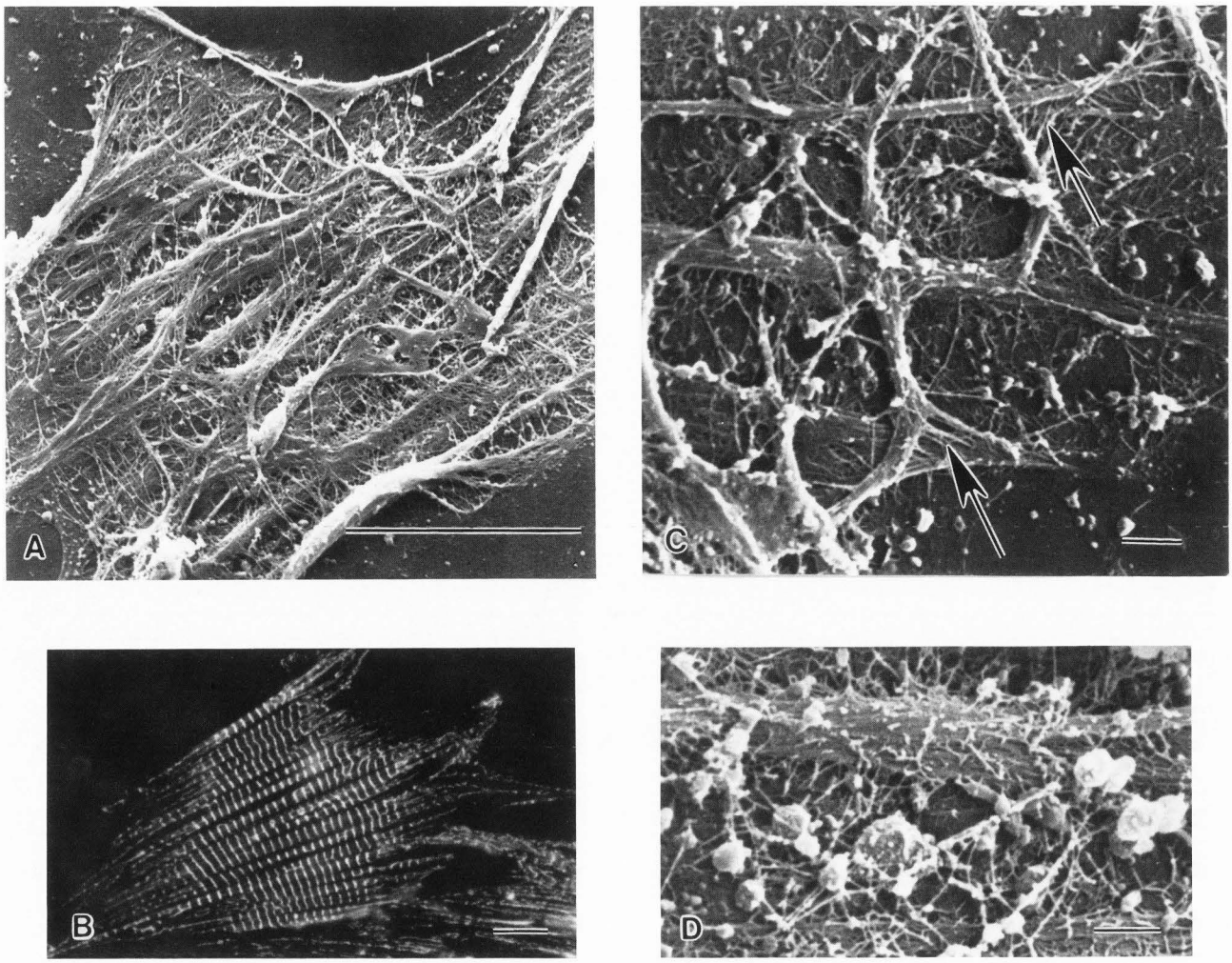


Figure 3. Substrate-attached material remaining after myocytes were physically ruptured with buffer jet of SBS (Protocol IVa in Table 1). A. SEM of ruptured cells shows several layers of filament bundles most of which are parallel to the long axis of cell; transverse or diagonal bundles overlie the longitudinal arrays. B. Indirect IF for α-actinin in a ruptured cell shows parallel rows of striated myofibrils remaining on substrate. Bars=10 μm (A,B). C. Longitudinal filament bundles terminate at the cell margins where other bundles oriented at right angles overlie and connect the parallel arrays. Fine filaments appear to anchor the bundles to each other and to the substrate (arrows). D. High magnification of a filament bundle shows the networks of numerous fine filaments projecting from the bundle surface. These fine filaments appear to anchor the bundle along its length to the substrate. Bars=1 μm (C,D).

densities suggestive of sarcomeric patterns (Fig. 1A). However, indirect IF labeling for actin in these cells revealed that sarcomeric patterns were either lost or severely distorted (Fig. 1B). For comparison, the regular sarcomeric pattern seen in cells that were fixed in paraformaldehyde first before extraction is shown in Fig. 1C. Analysis of the medium after pre-extraction showed by SDS-PAGE that both actin and myosin were released by the detergent treatment (data not shown).

When cells were simultaneously fixed and extracted in Triton X-100 and 2% paraformaldehyde, the surface membrane remained partially intact, obscuring the underlying cytoskeleton when viewed by SEM (Fig. 1D). Myofibrillar structure, as determined by Z band fluorescence labeling with anti-α-actinin, was maintained (Fig. 1E). Thus, the use of aldehyde fixatives either before or during detergent extraction prevented the membranes from being solubilized. Alternatively, fixation after extraction resulted in loss of myofibrillar integrity in most cells.

The use of less effective fixatives such as the bifunctional protein cross-linking reagents dithiobis (succinimidyl) propionate (DTSP) or dimethyl 3,3′ dithiobispropionimidate (DTBP) (Pierce Chemical Co. Rockford, IL) (Bell, 1981) gave similar results. Cells prefixed in either fixative could be partially extracted with detergent (Fig. 2A), but myofibril structure was

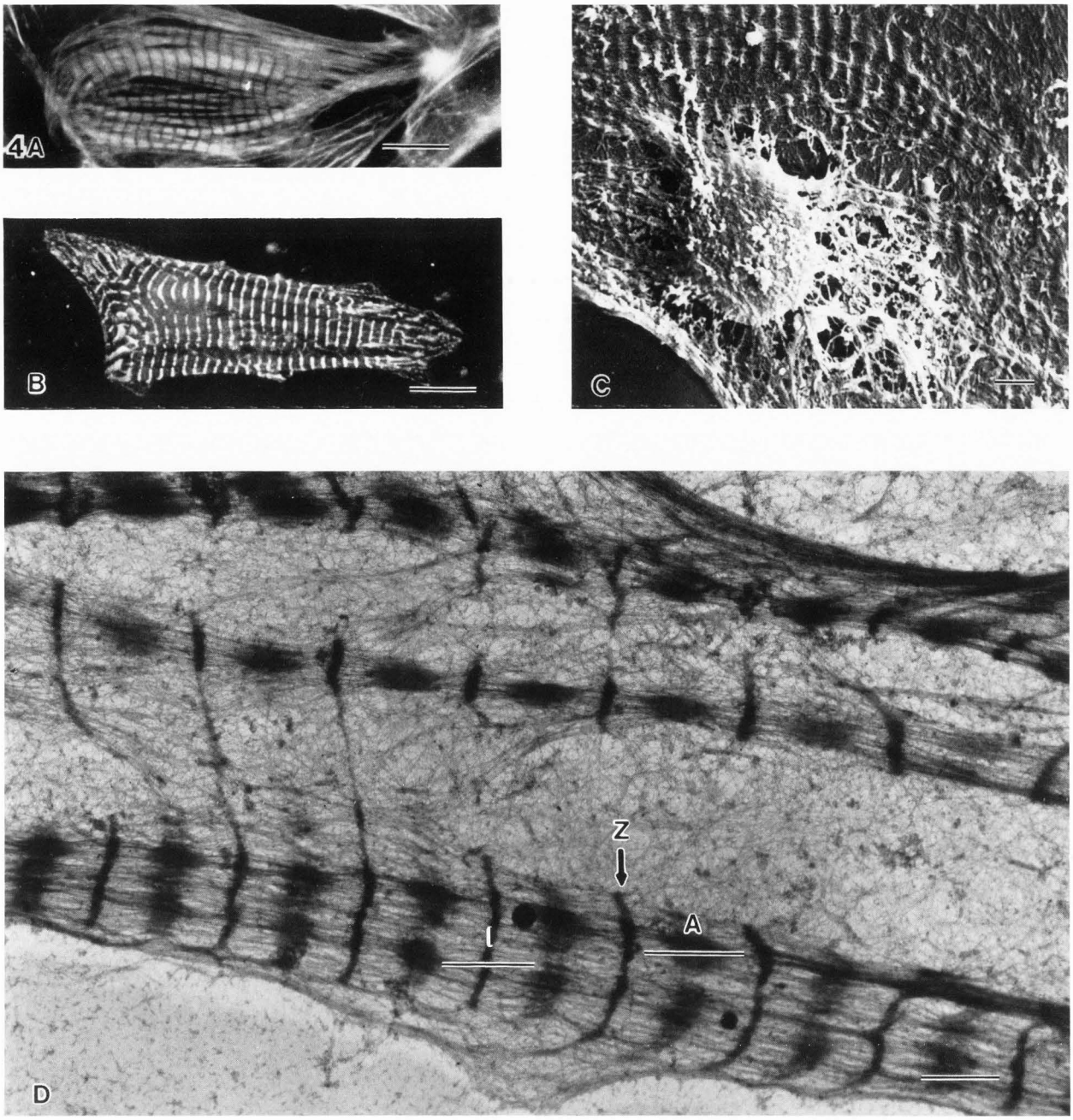
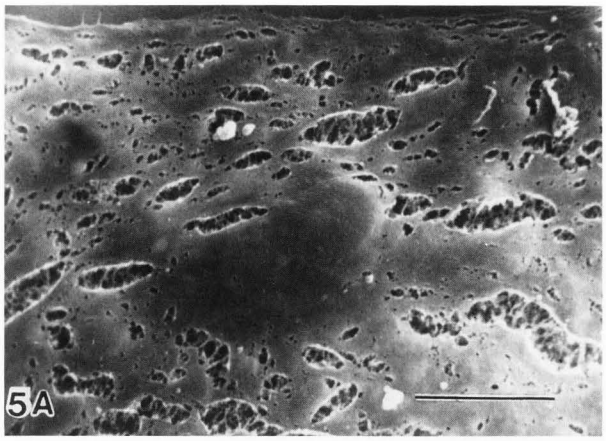


Figure 4. Myocytes pre-extracted with Triton-X 100 + SF buffer (Protocol Ic in Table 1) and prepared for IF (A, B), SEM (C) and IVEM (D). A and B. Indirect IF of actin (A) and α-actinin (B) shows sarcomeric banding patterns are maintained. C. SEM shows presumptive Z band densities in parallel rows of myofibrils. D. Whole mount IVEM shows myofibrils with definitive Z, I and A bands. Z lines of various widths are laterally connected. Note that the myofibril at the cell margin is wider than the more interior myofibrils. Bars=10 μm (A,B). Bars= 1 μm (C,D).

distorted (Fig. 2B). Simultaneous extraction and fixation gave somewhat better results at both the IF and SEM levels (Figs. 2C and 2D). However, there were always some cells in the samples with partially extracted membranes and disrupted myofibrils.

When the surface membrane was removed, an underlying cortical network of anastomosing filaments (Fig. 2C inset) obscured the filament bundles attached to the substrate. In order to view this substrate-attached material, a method for shearing off the upper portions of the myocytes was adapted (Boyles and Bainton, 1979).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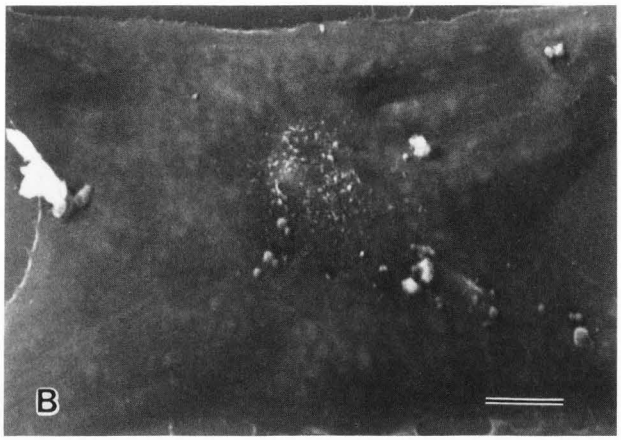
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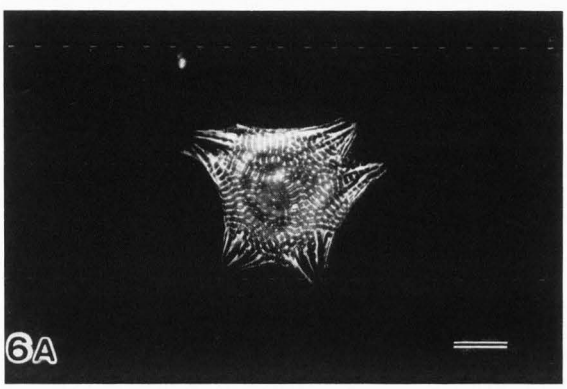
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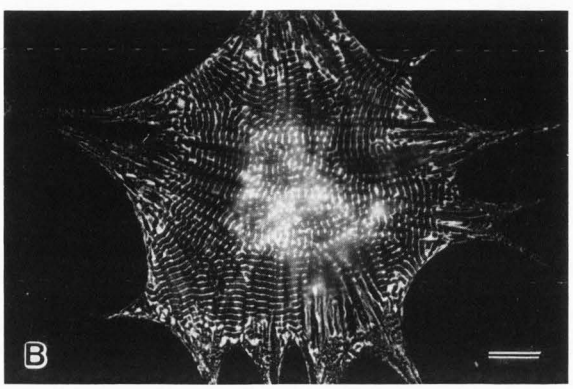
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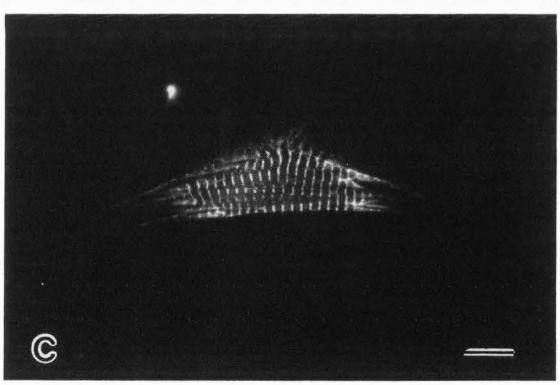
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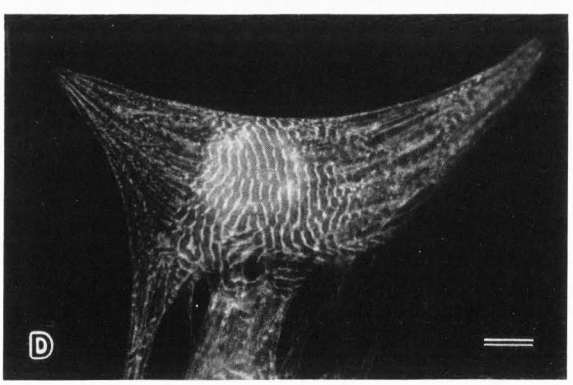
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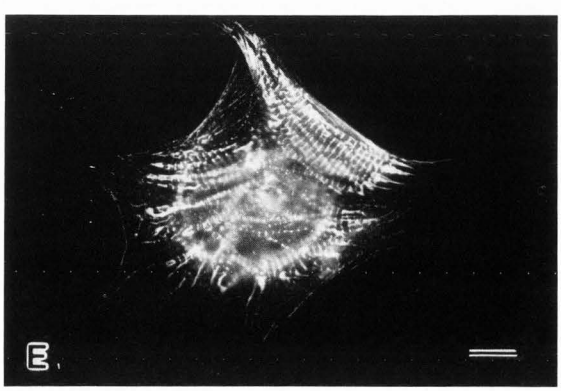
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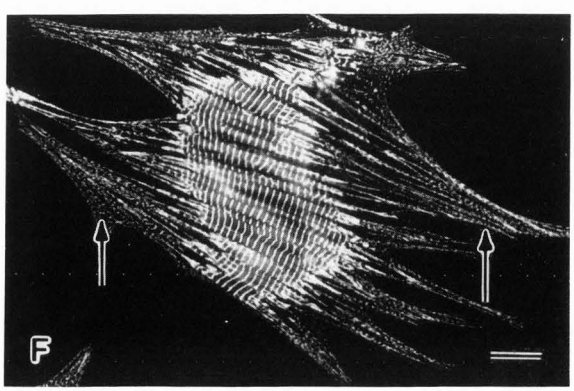
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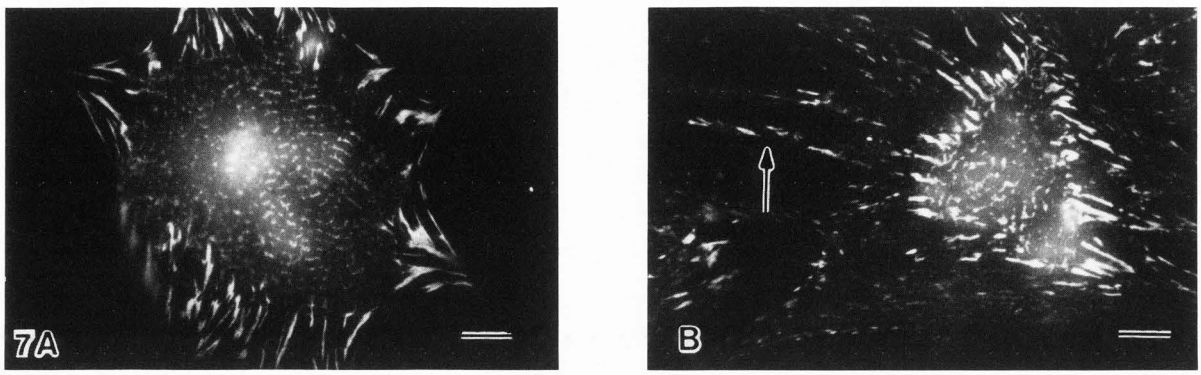


Figure 7. Indirect IF localization of vinculin in focal adhesions and in the rib-like pattern known as costameres. A. Cells on Coll I + III show focal adhesions concentrated at the cell periphery. B. Cells on LN exhibit intense fluorescence in the focal adhesions more centrally located, radiating from the nuclear area. Focal adhesions are sparse in the cellular processes (arrow). Bars=10 μm.

Figure 5. SEM of unextracted cells comparing sputter coating (A) with vacuum evaporation (B) of gold onto the specimens. Cells were processed in parallel by fixation in 2% glutaraldehyde followed by postosmication, dehydration in acetone and critical-point drying. The cell that was sputter coated (A) has large cracks and holes in the surface membrane, while the cell that was coated by vacuum evaporation of gold shows an intact membrane (B). Bars = 10 µm.

Figure 6. Indirect IF localization of α-actinin in neonatal cells grown on Coll I + III (A-18 hours, B-4 days), on LN (C-18 hours, D-4 days), or on FN (E-18 hours, F-4 days). Myofibrils, visible by fluorescent Z bands, extend to the cellular outlines of the stellate cells on Coll I + III (A and B) but do not extend into the linear processes in cells grown on LN (C and D) or on FN (E and F). F. In cellular processes, α-actinin stains in a punctate pattern (arrows). Bars=10 μm.

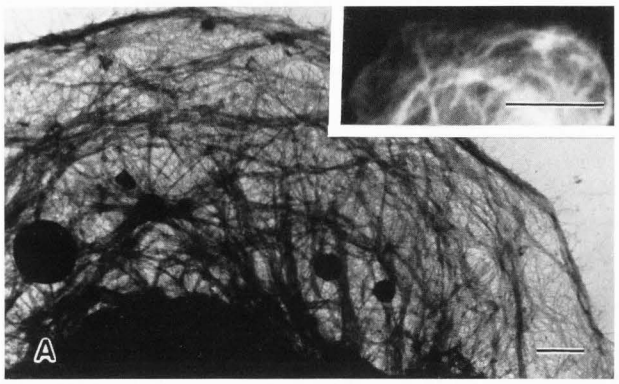
In the ruptured cells, large bundles of filaments were aligned parallel to the long axis of the cell (Fig. 3A). Overlying these parallel bundles were smaller arrays and individual fine filaments which ran perpendicularly or diagonally and connected the large bundles to each other and to the substrate. Indirect IF labeling of α-actinin indicated that at least some of these parallel filament bundles were myofibrils (Fig. 3B); however, myofibrils and stress-fiber-like bundles could not be differentiated at the SEM level using these techniques. Neither individual filaments nor banding patterns indicative of sarcomeres could be detected in these cells. Bundles of filaments which terminated at the cell perimeters were laterally connected (Fig. 3C). Filament bundles were anchored to the substrate along their length by numerous fine filaments (Fig. 3D).

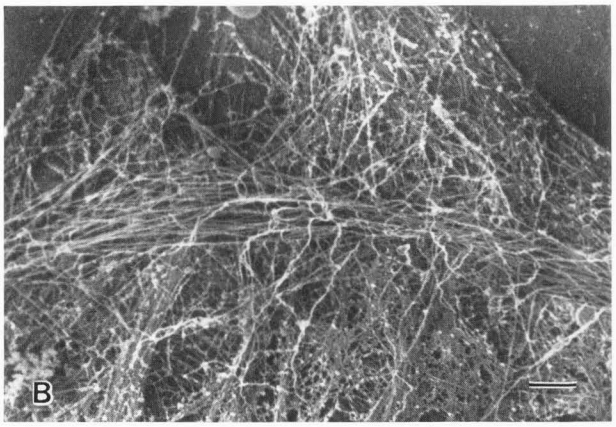
Of all the variables tested for obtaining good cytoskeletal preservation, the composition of the extraction buffer proved to be the most critical. The best visualization of myocyte cytoskeletons by SEM and the best morphological preservation of myofibrils in IF were achieved with an actin-stabilizing buffer (Blikstad and Carlsson, 1982). Cells extracted and processed according to this procedure exhibited sarcomeric patterns at the IF level when labeled for actin (Fig. 4A) or for α-actinin (Fig. 4B). Z band densities were visible even at the SEM level (Fig. 4C). Whole mount IVEM of these cells (Fig. 4D) revealed definitive Z, I and A bands. Other details were also observed. Filaments splaying out from myofibrils appeared to be continuous with nascent myofibrils. Z bands of various widths were laterally connected and also appeared to be continuous with filaments in the cellular perimeters.

Another important parameter for optimal preservation of structure in SEM was the method of metal coating of specimens. Unextracted control cells were processed in parallel by fixation in glutaraldehyde followed by osmium, dehydrated in an ethanol series, critical point dried and coated with gold in either a sputter coater or a vacuum evaporator. Cells that were sputtered coated under conditions as described in the Materials and Methods had large cracks and holes in the surface (Fig. 5A), while cells that were coated by vacuum evaporation showed intact surfaces (Fig. 5B).

ECM Effects on Myofibrillogenesis

Cells on LN, FN and Coll I + III allowed to spread in culture showed different patterns of both myofibrils and focal adhesions as determined by indirect IF labeling of α-actinin and vinculin, respectively. After 18 hours, polygonal cells on Coll I + III contained striated myofibrils in multidirectional arrays that extended to the periphery of the cell (Fig. 6A). Well-spread cells on Coll I + III for 4 days contained myofibrils oriented predominantly in two axes which overlapped in perpendicular arrays in the cell center and which extended into small cellular processes





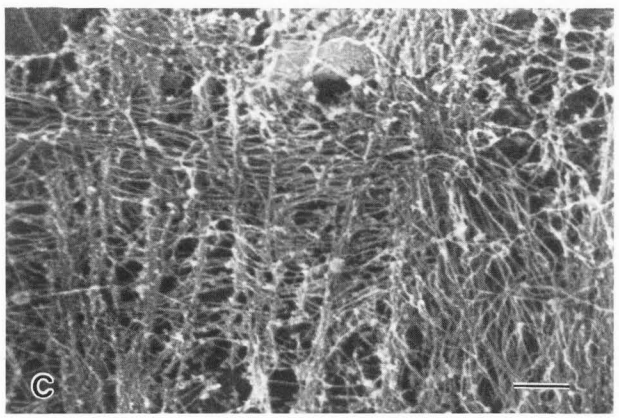


Figure 8. Circumferential filaments of myocytes grown on LN substrate. A. Whole mount IVEM of a myocyte grown on LN for 12 hours shows two concentric arcs of filaments. Radial filament bundles extend from the nuclear area and intersect with the arcs at an angle. This cell was pre-extracted with Triton-X 100 in SF buffer. Inset shows rhodamine-labeled phalloidin localization of F actin in circumferential arcs in cell spread on LN for 8 hours. B. SEM of the cytoskeleton of a cell perimeter shows a circumferential arc of filaments. Parallel filament bundles (presumptive myofibrils) terminate near the arc. This cell was grown on LN for 4 days. C. SEM showing numerous lateral connections between filament bundles at cell perimeter. Bars=1 μm. Bar= 10 μm (A-inset).

replete with punctate α-actinin staining (Fig. 6B). The terminal ends of the striated fibrils had short nonstriated sections. In contrast, myofibrils in cells grown either on LN (Figs. 6C and 6D) or on FN (Figs. 6E and 6F) demonstrated a different distribution to that seen on Coll I + III. Myofibrils in cells grown on both LN and FN were arranged in laterally aligned parallel arrays which did not extend into the cellular processes (Figs. 6C, 6D, 6E and 6F). Instead, these areas contained parallel linear rows of punctate α-actinin concentrations which extended to the cell perimeters (Fig. 6D and 6F). Well-spread cells contained unidirectional parallel myofibrils oriented in the long axis of the cell (Fig. 6D and 6F). Some of the linear rows seemed to be continuous with striated myofibrils (Fig. 6F).

Anti-vinculin labeling in cells grown for 4 days on Coll I + III and on LN also showed different distributions. Vinculin fluorescence in cells on Coll I + III was localized in elongate strands and "chevron" shaped patterns at the cell margins and in costameres (Pardo et al., 1983) in the central portions of the cells (Fig. 7A). On LN, vinculin-labeled focal adhesions were more centrally located and appeared to radiate from the nuclear area (Fig. 7B). The elongate cellular processes exhibited small randomly scattered areas of vinculin labeling.

Circumferential Bundles of Actin Filaments

A unique feature of cells grown on LN was the appearance of circumferential arcs of filaments that were evident in IF (Fig. 8A inset), whole mount IVEM (Fig. 8A) and in SEM (Figs. 8B and 8C). These circumferential filaments were found both in the early stages of cell spreading and in well-spread cells. Filament bundles terminated in the vicinity of the arcs (Figs. 8A and 8B) and often appeared to be laterally connected by filaments in the arcs (Fig. 8C).

Discussion

Specimen preparation for SEM requires that the surface membrane be removed or the cell broken open to reveal the underlying cytoskeleton. This removal is usually achieved with non-ionic detergents such as Triton X-100, or by physical rupture by buffer jet (Boyles and Bainton, 1979), or a "sandwich technique" (Batten et al., 1980; Isobe and Shimada, 1983). Both detergent-extracted and physically ruptured cells were used in the present studies to examine the spatial organization of the cytoskeleton and myofibrils in neonatal rat cardiac myocytes. The ultimate goal is to visualize the three-dimensional cytoskeletal interrelationships as cells adhere and adapt to culture on different ECM components. Since the intention was to correlate SEM images with stereo-pair whole mount IVEM and with IF of cells labeled with antibodies to various cytoskeletal and contractile proteins, a preparative procedure for SEM that would allow cells to be processed in parallel for all three procedures was sought and selected.

Although good results for IF and whole mount IVEM could be achieved by prefixation in aldehyde

fixatives before extraction, this prefixation step prevented subsequent removal of the surface membrane. Use of less effective fixatives such as the bifunctional protein crosslinking reagents DTSP and DTBP in the prefixation step led to disruption of sarcomeric structure. This may have been due to the fact that they are relatively poor fixatives (Bell et al., 1988). The fixative properties were improved when detergent was added together with these fixatives. Sarcomeric patterns were retained, suggesting that the detergent was a vehicle for entry of these fixatives into the cells. SEM of cytoskeletons either prefixed or simultaneously fixed and extracted with the fixatives gave variable results. Although there were areas in which cytoskeletal components could be viewed, there were also large expanses of intact membranes. Recently, Bell and co-workers have reported using a two-step procedure with sulfonated crosslinkers added to the detergent solution (Bell et al., 1988). This method was not attempted in this study, but may be effective in stabilizing cytoskeletal structures without preventing membrane solubilization with detergent.

Although prefixation gave variable results in SEM, pre-extraction with Triton X-100 in PBS before fixation was effective in removing the membrane, but destroyed myofibrillar structure and released solubilized actin and myosin into the medium. A similar loss of actin, tubulin and α-actinin was reported in HeLa cells (Bravo et al., 1982). When the ionic composition of the pre-extraction buffer was altered and included EGTA (KMP buffer, Protocol Ib in Table 1) (Isobe and Shimada, 1983), the myofibrillar structure was improved in most cells, but preservation was not ideal. Many cells in the preparation showed loss of sarcomeric structure. Only when the actin-stabilizing SF buffer (Protocol Ic) (Blikstad and Carlsson, 1982) was used as the vehicle for pre-extraction were both maintenance of myofibrillar structure and membrane removal achieved (Figs. 4C, 6 and 7). This SF buffer was originally developed to stabilize pools of unpolymerized and filamentous actin during Triton X-100 cell lysis (Blikstad and Carlsson, 1982). This procedure appeared to stabilize filaments even after additional processing for IVEM and SEM in osmium tetroxide as evidenced by whole mount IVEM images (Figs. 4D and 8A). Filaments, presumably actin, are preserved both in myofibrillar bands and in circumferential arcs. Although osmium tetroxide is known to disrupt actin filaments (Maupin-Szamier and Pollard, 1978; Small, 1981), the use of tannic acid before osmication prevents this disruption (Begg et al., 1978). In addition to actin, the SF buffer appeared to stabilize α-actinin and vinculin as well (Figs. 6 and 7). Thus the critical variable for achieving optimal cytoskeletons in neonatal rat myocytes is the composition of the detergent extraction buffer, a finding in agreement with Bell and co-workers (1988).

An additional criterion that was important in SEM processing of cytoskeletons was the coating of specimens by vacuum evaporation instead of sputter coating. Although a magnetron sputter coater with a cooled specimen stage was used initially in the present studies, both intact cells and cytoskeletons showed large cracks in their structure due presumably to heating of the specimen during coating (Bell, 1981). This specimen destruction was evident even when the glass coverslips containing the critical-point-dried cells were placed in direct contact with the cooled specimen stage. Thus, metal coating by vacuum evaporation was the method of choice in subsequent work.

Having established the preparative methods which gave optimal results for viewing cytoskeletons for SEM, studies on myofibrillogenesis in neonatal rat myocytes grown on different ECM components were initiated. IF data show that cells grown on FN, LN and Coll I + III exhibit different patterns and distributions of both striated myofibrils and focal adhesions. Cells on collagen matrices both at 18 hours and 4 days after plating contained striated myofibrils which terminated near the cell periphery where focal adhesions were predominantly located. In contrast, striated myofibrils in cells both on LN and on FN were located in the center of the cells and exhibited nonstriated regions in the periphery which appeared to be continuous with rows of punctate α-actinin concentrations.

A similar staining pattern in which striated myofibrils terminated in nonstriated regions or in punctate α-actinin spots has been observed in other cardiac myocytes in culture. (Atherton et al., 1986; Atherton and Behnke, 1988; Dlugosz et al., 1984; Sanger et al., 1986a, 1986b). In embryonic chick cardiac myocytes, these nonstriated regions determined by rhodamine phalloidin staining for actin were interpreted to be stress-fiber-like structures (SFLS) which served as preexisting scaffolds upon which proteins assembled into striated myofibrils (Dlugosz et al., 1984). In another interpretation, the punctate localization of α-actinin in the terminal regions of mature myofibrils was thought to be nascent Z bands in minisarcomeres. In elegant studies in which fluorescently labeled α-actinin was microinjected into living cells (Sanger et al., 1986a, 1986b), punctate α-actinin spacings were shown to increase in length from 0.9-1.3 μm in nascent noncontractile myofibrils to 1.6-2.3 μm in mature contractile myofibrils. Myosin was demonstrated in both nascent minisarcomeres and in fully formed sarcomeres in living cells (Mittal et al., 1987) and in fixed cells (Sanger et al., 1986a). Both of these models using embryonic cardiac myocytes interpreted the terminal portions of myofibrils to be areas of assembly. In contrast, studies using neonatal cardiac myocytes interpreted these termini to be areas of disassembly or remodeling (Atherton et al., 1986; Atherton and Behnke, 1988). These authors found that nonstriated terminals were associated only with free edges of cells not in contact with other cells. These nonstriated termini corresponded with focal adhesions determined by vinculin staining and interference reflection microscopy. Thus myofibrils that were associated with cell-cell junctions were more stable than those associated with cell-substratum junctions (Atherton et al., 1986; Atherton and Behnke, 1988). Studies presented in this paper suggest that the focal adhesions and myofibril distributions may reflect

different responses to matrix substrate. It is also worth noting that vinculin staining in costameric patterns is in the central portion of the cells where the Z lines of the mature myofibrils are located on all matrices. The costameres which attach to the sarcolemma and overlie Z bands (Pardo et al., 1983) are areas in which collagen receptors are also located (Borg et al., 1989; Terracio et al., 1989). There are putative physical connections among the Z lines, costameres, sarcolemma and ECM receptors (Borg and Terracio, 1989; Terracio et al., 1989). This myofibril-membrane association, perhaps via vinculin, could reinforce this region and mechanically couple the membrane to the contractile apparatus during myofibril contraction. Removing the membrane by pre-extraction in a buffer such as PBS, which does not stabilize cytoskeletal proteins, leads to disruption of the membrane-myofibril connection and consequently to loss of myofibril structure.

Networks similar to the subsarcolemmal cortical meshwork of filaments evident in Fig. 2C were shown to be networks of actin filaments in cytoskeletons of physically ruptured cardiac myocytes of the neonatal hamster (Isobe et al., 1988). These authors, by combining freeze-drying and rotary-replication methods with immunogold TEM, were also able to resolve 2-5 nm filaments. These filaments interconnected S1-decorated actin filaments and were also attached to the membrane. Although these structures were not resolved with the SEM instrumentation used in the present studies, numerous fine filaments that anchored filament bundles along their length were identified (Fig. 3D). These anchoring filaments could maintain myofibrils in position during contraction.

Myofibrils could also be maintained in position by lateral connections between Z bands (Fig. 4D). These filamentous connections were shown to be desmin intermediate filaments in skeletal (Lazarides, 1980; Tokuyasu et al., 1985) and cardiac myocytes (Isobe et al., 1988; Tokuyasu et al., 1983). Although a structural role for desmin in connecting myofibrils in mature skeletal muscle cells has been demonstrated (Lazarides et al., 1982), desmin filaments are not thought to participate in the alignment process itself; lateral registry of myofibrils is seen in embryonic myotubes before desmin connections are identified (Hill et al., 1986; Tokuyasu et al., 1985). The interaction of nascent myofibrils with the cell membrane via vinculin-containing structures has been demonstrated in the earliest stages of heart formation in vivo (Tokuyasu, 1989), suggesting that cell membranes are important in directing myofibrillogenesis. Studies of the sequential assembly and alignment of myofibrils would be facilitated by analysis of the three-dimensional interrelationships among labeled cytoskeletal and contractile proteins.

The circumferential arcs, which were most prominent in myocytes grown on LN substrates (Fig. 8A), resembled actin filament arcs in spreading fibroblasts (Soranno and Bell, 1982) and the parallel arcs of α-actinin aggregates in cardiac myocytes (Sanger et al., 1984). The presence of these arcs in cells on LN suggests, among other reasons, that cells are more motile on this substrate.

In summary, the studies presented in this paper show that immunohistochemical localization of antibodies to both myofibrillar and cytoskeletal proteins can be achieved in cells that have been extracted with detergent in an actin-stabilizing buffer prior to aldehyde fixation. Future studies utilizing immunoelectron SEM could provide insights into how ECM components influence three-dimensional myofibril assembly as myocytes adhere and spread in response to external signals from the changing environment in the matrix.

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Discussion with Reviewers

P.B. Bell: The work reported in this paper is a beautiful example of the importance of the extraction medium for determining the outcome of extracting cells with detergents. It also demonstrates the importance of selecting extraction media that are appropriate for the cells or tissues with which one is working. Would you please discuss why the SF buffer helps to preserve the sarcomeric structure so well. Have you tried any other buffers, such as the microtubule stabilizing buffer that we have found to give good results with stabilizing the cytoskeleton of fibroblast-like cells?
Authors: Most of the buffers that are reported to stabilize microtubules or other cytoskeletal proteins are hypertonic buffers containing high osmotic concentrations (> 500 mOsmoles) of sucrose or polyethylene glycol (PEG). Clearly, high osmolarity alone is not sufficient for good preservation. Poor results have been reported for 300 mM and 500 mM sucrose (Bell and Stark-Vancs, 1983) and for 4% PEG (Blikstad and Carlsson, 1982). We have not tried all types of microtubule stabilizing buffers such as the one you cite (Bell et al., 1988) which, in addition to 4% PEG, contains 1 mM EGTA, 100 mM PIPES (piperazine-N,N'-bis (2-ethanesulfonic acid) and 0.0015% Phenol Red. Systematic studies are needed to determine the effects of addition of mono- and divalent cations, reducing agents, chelating agents, and protease inhibitors included in extraction buffers.

P.B. Bell: The damage to the surfaces of cells that you report with sputter coating demonstrates that you cannot have blind faith in the efficacy of particular methods or machines. However, I am still of the opinion, which is supported by data, that sputter coating is a superior method for coating biological samples with thin metal films suitable for SEM as well as STEM and TEM (Bell et al., 1987, J. Electron Microsc. Technique 7:149-159; Lindroth et al., 1987, Scanning 9:47-56; Bell et al., 1988, text reference). However, as you have shown, it is also possible to get unacceptable poor results with sputter coating. Unfortunately, most of the commercially available sputter coaters do not provide enough control over the coating conditions to permit one to optimalize the results. Is cracking of the samples a general phenomenon with your coater, or do the results vary with the type of sample being coated? Have you tried using metals other than gold, varying the time and current, or changing the distance between the target and the sample?
Authors: The specimen surface damage with sputter coating is not a general phenomenon and does indeed depend upon cell type. Although the damage is routinely seen on cultured neonatal myocytes, which are flat and well-spread cells, freshly isolated adult myocytes, which are cylindrical cells, do not exhibit such destruction. Investigators in several other laboratories have personally communicated similar difficulties with sputter coating of well-spread myocytes. Whether the specimen destruction occurs in other well-spread cells or instead is related to the contractile cytoskeleton of cultured myocytes is not known. As a result of these communications and because we achieved satisfactory results with vacuum evaporation during metal coating, we chose because of time constraints not to conduct systematic studies of various parameters during sputter coating.

P.B. Bell: Did you try to rupture cells by shearing with SF medium instead of SBS medium? Might not this approach optimalize preservation and visualization of the structure of the myofibrils?
Authors: We did not use SF medium in the shearing buffer because myofibril integrity, as judged by immunofluorescence of anti-α-actinin labeling of Z bands, was maintained by shearing with SBS medium, followed quickly by fixation. As we are particularly interested in examining this substrate-attached material after the upper cytoskeletal cortex has been removed, we will continue to experiment with various protocols such as the one you mentioned.

P.B. Bell: I agree that, of the methods you tested, extracting in Triton in SF buffer was **optimal** for preserving and visualizing the structure of myofibrils by IF, IVEM, and SEM. But would you not agree (as shown in Fig. 2, this paper) that you obtained **maximum** preservation of cytoskeletal/cytomatrix structures by simultaneously crosslinking and extracting?
Authors: The major problem with either fixing first or fixing simultaneously with any of the fixatives we used (paraformaldehyde, DTSP or DTBP) was that a great number of the myocytes as viewed by SEM had large areas of intact, unextracted membrane obscuring the underlying cytoskeleton. We do not rule out the possibility that, by manipulating various parameters in the extraction/fixation protocol, we could achieve optimal preservation of cytoskeletal structure with simultaneously crosslinking and extracting.

R.S. Decker: Demonstration of actin and myosin extraction by SDS-PAGE needs documentation.
Authors: We have chosen not to show data on the SDS-PAGE gel electrophoresis of actin and myosin as the relative molecular weights (M_r) of these two proteins are well-documented in the literature. The inclusion of photographs of the gels in this report thus seemed redundant.