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CRYOULTRAMICROTOMY AND IMMUNOCYTOCHEMISTRY IN THE ANALYSIS OF MUSCLE FINE STRUCTURE

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Abstract

Cryoultramicrotomy, which avoids the use of harsh fixation procedures, deleterious dehydration and plastic embedding can be combined with immunocytochemistry to determine the ultrastructural localization of cellular proteins. Our attempts to use the cryosectioning technique in combination with immunolabelling to bridge the gap between light and electron microscopic analysis of muscle morphology have enabled us to obtain new information on fibre typing at the ultrastructural level. Furthermore, we have obtained a marked improvement in the resolution of myofibrillar structures by using semithin cryosections for fluorescence microscopy. Data are also presented on correlated light and electron microscope immunocytochemistry of myocardial intermediate filaments confirming the presence of longitudinally oriented intermediate filaments of desmin in the region of the intercalated discs of mammalian cardiac myocytes, whereas elsewhere in the myocyte the bulk of intermediate filaments of desmin is concentrated in the intermyofibrillar space at the level of the Z disc.

KEY WORDS: Cryoultramicrotomy, immunocytochemistry, immunoelectronmicroscopy, fluorescence microscopy, muscle fibre types, myofibrillar M-band structure and composition, M-protein, myomesin, heart, intercalated discs, intermediate filaments.

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Introduction

Cardiac and skeletal muscles are very heterogeneous tissues containing fibres which are capable of performing a wide range of functional properties. Several classification schemes to separate muscle fibres into fibre types on the basis of various characteristics have been proposed (for review see Schmalbruch 1985). However, it is difficult to unify the concept of fibre types, as it is difficult to compare the classification schemes based on physiological properties with those based on biochemical or morphological criteria. Undoubtedly, some physiological parameters like contraction time and fatigability are clearly correlated to enzymatic activities which can be measured either with enzyme-histochemical or biochemical methods (see reviews Burke 1981, Pette and Vrbova 1985, Schmalbruch 1985). However, to transfer such information to the ultrastructural level has often been difficult, as current conventional methods of tissue preparation do not, in general, allow a direct comparison of different properties (Eisenberg 1983). For example, conventional fixation, dehydration, and embedding of muscle tissue destroy the enzymes whose activities are the basis for enzyme-histochemical fibre typing. Some correlative results to support the concept of fibre typing have been obtained by comparing unfixed serial cryostat sections, alternately processed for enzyme histochemical stains and for electron microscopy (Thornell et al. 1976, Eisenberg and Kuda 1977, Sjöström et al. 1982). However, the ultrastructural morphology of such sections has naturally been of low quality.

Immunological and biochemical analyses have now shown that a number of isoforms of myofibrillar proteins that can influence fibre characteristics may be present in one and the same fibre (Billeter et al. 1980, Thornell et al. 1984a,b). Consequently the need and importance of immunocytochemical methods for studies of the structure and composition of tissues and cells both at the light and electron microscopic levels have

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become apparent.

Since 1972 we have, in our laboratory, used cryoultramicrotomy in the analysis of muscle fine structure. This technique has proven to be superior to conventional plastic embedding, sectioning and staining of muscle tissue, since it is possible to avoid harsh fixation procedures and the deleterious effects of dehydration and plastic embedding (see also Tokuyasu 1980). As a result considerable new morphological information has been obtained on myofibrillar components (e.g., Sjöström et al. 1973, Sjöström and Squire 1977a,b, Carlsson et al. 1982, Thornell and Carlsson 1984). A new nomenclature for the fine structure of the myofibrillar M-band in the center of the A-band is based on observations of negatively stained cryosections (Sjöström and Squire, 1977a,b). Up to nine symmetrical striations designated M1-M9,9' running across the thick filaments in longitudinally sectioned myofibrils were resolved. The major striations M1, M4,4', and M6,6' were interpreted to represent the so called M-bridges (Knappeis and Carlsen, 1968), whereas the weak striations were related to the presence of additional unknown proteins involved in the packing of the myosin molecules in the backbone of the filaments. By combining immunolabelling of myofibrils using antibodies against the muscle form of creatine kinase (MM-CK) and high molecular weight M-band proteins with ultrathin cryosectioning and negative staining, we have been able to show that MM-creatine kinase makes up the M-bridges M4,4' and possibly also M1, while M6,6' have a different composition (Strehler et al. 1983). However, it was not possible to clarify whether the high Mr M-band proteins constituted the M-filaments running parallel to the thick filaments interconnecting the M-bridges (Knappeis and Carlsen, 1968) or ensheathed the thick filaments in the M-band (Strehler et al. 1983).

Another important observation to come out of negatively stained ultrathin cryosections of muscle tissue is that the M-band structure varies between different fibres (Sjöström and Squire 1977a,b, Sjöström et al. 1982, Thornell and Carlsson 1984, Carlsson and Thornell 1987). Since fibres with different M-band structures are likely to belong to different motor units with different functional properties, the M-band structure could serve as an ultrastructural marker for fibre typing. Much of our research over the past years has been devoted to obtain confirmation for this by correlative studies and to further elucidate the structure and composition of the M-band (Thornell et al. 1985a, Carlsson 1985).

In the present study we elaborate on our attempts to combine the cryosectioning technique with immunocytochemistry in order to bridge the gap between light microscopic and electron microscopic analysis of muscle morphology paying special attention to the M-band structure and its composition and as well as to intermediate filaments.

Materials and Methods

Guinea pigs were anaesthetized with pentobarbital. The soleus and the gastrocnemius muscles were excised, immediately put into cold (+4 °C) relaxing buffer containing 0.1 M KCl, 1 mM EGTA, 5 mM EDTA, 1 mM dithiothreitol pH 7.0 and pinned onto a piece of cork. Heart moderator bands (trabecula septomarginalis) of adult cows were excised immediately after slaughter and put in the relaxing buffer. Adult hens were anaesthetized with pentobarbital. The white pectoralis muscles were excised and pinned onto cork as described previously (Strehler et al. 1983).

Fixatives

A variety of fixatives has been used. For preparations to be used for conventional cryosectioning, 2.5% glutaraldehyde in phosphate buffered saline (PBS) for 1 to 24 hours was used. For preparations to be used for immunocytochemistry, fixation was carried out in aldehyde detergent mixtures in the cytoskeleton buffer (solution 1) described by Small (1981; see also Small et al. 1986). The most successful combinations with regard to the preservation of antigenicity and visualization of structure were fixation for 1 to 4 hours in a mixture of 0.5% glutaraldehyde and 0.5% Triton X-100 or 2% paraformaldehyde, 0.2% glutaraldehyde and 0.2% Triton X-100 (Sigma Chemical Co). After fixation and two washes in solution 1 (2 x 15 min) to remove the fixative, muscle strips were treated with sodium borohydride (0.5 mg/ml) freshly dissolved in ice-cold solution 1, 3 x for 15 min to reduce free aldehyde groups (Weber et al. 1978).

Antibodies

The preparation and characterization of monoclonal antibodies (mAbs) against M-protein of Mr 165,000 and myomesin of Mr 185,000, both from chicken pectoral muscle, have been described in detail (Grove et al. 1984, 1985). The antibodies against myosin slow and fast heavy chains were prepared and characterized as described in Butler-Brown et al. (1982) and Butler-Brown and Whalen (1984). Antibodies against the Purkinje fibre intermediate filament protein of Mr 55,000 were prepared and characterized according to Thornell et al. (1985b). Fluorescein (FITC) or tetramethyl rhodamine isothiocyanate (TRITC) conjugated sheep antirabbit IgG or rabbit antimouse IgG were purchased from Dakopatts (Copenhagen, Denmark). Five or 10 nm gold-conjugated goat antirabbit IgG or rabbit antimouse IgG were obtained from Janssen Chimica, Belgium.

Freezing

For making cryostat sections, pieces of unfixed tissue of either cardiac or skeletal muscles were placed on a drop of embedding medium (OCT Compound, Miles Laboratories, Naperville, Illinois). All blocks were rapidly frozen by immersion in isopentane precooled with liquid nitrogen. For cryoultramicrotomy, small pieces of fixed tissue were treated with glycerol (10%, 20%, and 30% in PBS) for 30 min or 2.3 M sucrose for 30 min to 2 hours. The pieces were mounted

on metal holders, rapidly immersed in Freon chilled with liquid nitrogen, and stored frozen in liquid nitrogen until use.

Cryoultramicrotomy

Cryosections of varying thickness, or of alternating thick and thin thickness, were cut with glass knives on an LKB Ultrotome 8801 A, equipped with the LKB cryo-attachment and cryo-kit control unit 14803 or with an LKB CryoNova. Sections were cut as described in detail by Carlsson et al. (1982), floated on the surface of a mixture of DMSO/H_2O (1:1), transferred to a water surface and picked up on glass slides or Formvar-coated grids. Alternatively the specimens were sectioned dry and the sections were picked up on a drop of sucrose and further treated as described by Tokuyasu (1980). For visualization of general morphology the sections were negatively stained with ammonium molybdate (2%, pH 7.3).

Immunohistochemistry

For visualization of immunolabelling on cryostat sections standard procedures were used (Polak and Varndell, 1984). Essentially the same protocols were used for both the immunolabelling of sections cut on the cryoultramicrotome and for visualization in the fluorescence microscope. After a short rinse in PBS the sections were incubated at room temperature for 15 to 30 min with a drop of a solution containing specific antisera or control serum diluted 50-fold in PBS followed by several changes of PBS. The sections were then incubated in the same manner with the appropriate fluorescence labelled secondary antibody (diluted 1:40 to 1:100 in PBS). Extensive washing in PBS for 30-60 minutes with several changes of buffer was followed by mounting a coverslip onto the sections with a drop of glycerol/PBS 2:1 or Mowiol 4-88, pH 8.5 (Hoechst, Frankfurt, West Germany). For immunoelectronmicroscopy grids were incubated in 5% normal goat serum with 0.1% BSA in gold buffer (GB) (Small et al. 1986) for 15-30 min at room temperature. The grids were then transferred to the primary antibody dissolved in 2% normal goat serum and 1% BSA in GB for 60 min at room temperature. After three washes of 15 min each in 0.1% BSA/GB, the grids were transferred to the second gold conjugated antibody and incubated for 1h at room temperature. The gold conjugated antibody was subjected to 5-10 min centrifugation before use to remove any possible aggregates. After two washes in GB the grids were negatively stained with ammonium molybdate (2% pH 7.3).

Light and electron microscopy

Light microscopy was carried out using a Leitz Wetzlar Orthoplane microscope equipped with epifluorescence optics. For fluorescein fluorescence, a selective excitation filter (BP 485/20 nm) was used. Electron microscopy was performed on a Philips EM 300 electron microscope operating at 80 kV and equipped with an anti-contaminating device.

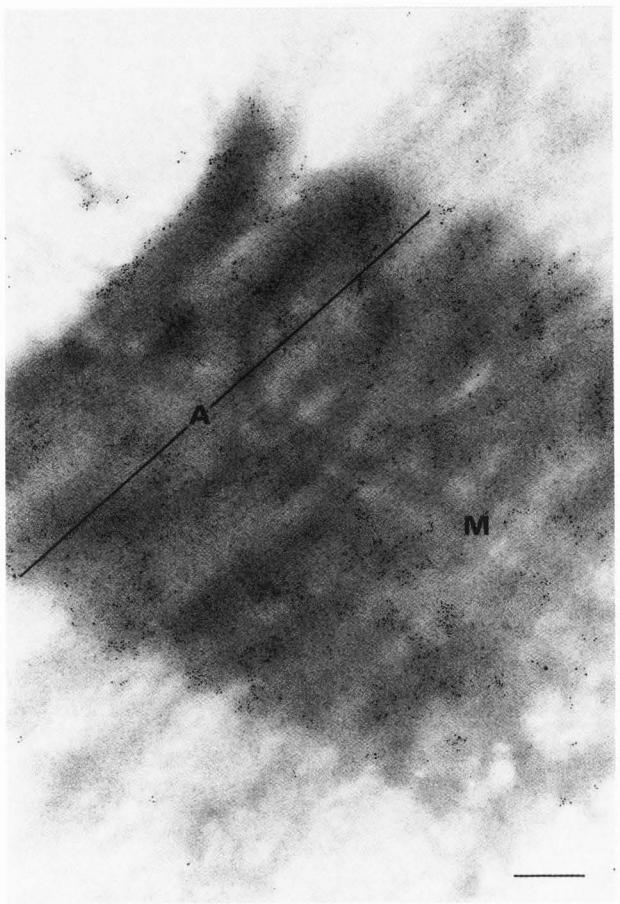


Fig 1: Longitudinal cryosection of myofibril from guinea pig gastrocnemius muscle. The muscle was freeze protected with glycerol, cut on DMSO/H_2O, labelled with antibodies against fast myosin and a gold tagged secondary antibody stained lightly with ammonium molybdate. As the A-band (A) is labelled, the myofibril belongs to a fast twitch motor unit, however, the resolution of the M-band (M) is insufficient for interpretation of its fine sturcture. Bar = 200 nm.

Results

Relationship between M-band structure and muscle fibre types

It is well-known that muscle fibres of slow twitch and fast twitch motor units contain different isomyosins (Pette and Vrbova 1985). These isomyosins in fact give rise to the differences in ATPase activity, utilized for enzyme-histochemical fibre typing. Thus, a direct method for obtaining an ultrastructural correlation of M-band structure to fibre type would be to visualize the type of isomyosin in the A-band by immunolabelling with specific antibodies to slow or fast myosin and then determine the corresponding M-band structure. By this approach we have obtained good immunolabelling of the A-band in ultrathin cryosections of different guinea pig muscles, but the resolution of the M-band structure has so far been unacceptable

(Fig 1). Therefore we have proceeded in another way to achieve the same goal. Cryosections were collected on formvar carbon-coated grids followed by immunolabelling with antislow or antifast myosin as the primary antibody and FITC-labelled antibody as the second one. Thereafter we examined the sections in the light microscope in the fluorescent mode (Figs 2A and 3A). After taking micrographs the sections were washed, negatively stained and examined in the electron microscope (Figs 2B, 2C and 3B). The use of grids with numbered squares helped to identify the same regions in the light and in the electron microscope. This method has permitted us to show that fast muscle fibres in guinea pig skeletal muscle have a three- (strong M1, M4,4' weak M6,6') or a five-line (strong M1, M4 4' and M6 6') transverse pattern in the M-region (Figs 2D, 4B and C), whereas slow twitch fibres contain a four-line pattern (strong M4,4' and M6,6') with the central M-line (M1) being very weak or missing (Figs 3C and 4A). At high magnification it was also obvious that the different striations within the M-bands of different fast and slow twitch units varied in appearance. There seemed to be a correlation between thin myofibrillar Z discs and the three-line M-band pattern and between broad Z discs and a five-line pattern, however, this was not an obligate correlation.

Structure and composition of the myofibrillar M-band

So far three proteins, creatine kinase (MM-CK), myomesin (Mr 185,000) and M-protein (Mr 165,000), plus the myosin molecules forming the thick filaments, have been shown to participate in the formation of the M-band. Monoclonal antibodies (mAbs) have now been prepared against M-protein and myomesin (Grove et al. 1984). Thereby it became clear that the polyclonal antibodies we used previously (Strehler et al. 1983) thought to be against a Mr 165,000 protein, in fact reacted with both the Mr 165,000 and the Mr 185,000 proteins. To further elucidate to which structures in the M-band M-protein and myomesin contribute, we have repeated the experiments as described in Strehler et al. (1983),using the mAbs to M-protein and myomesin. In sections of myofibrils incubated with polyclonal antibodies there was never any doubt as to whether the M-region was labelled or not, however, with the mAbs such a distinct difference was not seen. To be sure that we had successful penetration and labelling of the M-region, we cut alternating ultrathin and semithin sections with the cryoultramicrotome. The semithin sections were put on glass slides, stained with FITC-coupled secondary antibodies and examined in the light microscope. In this way we could verify that immunolabelling had occurred, although no dramatic shift in density was obtained ultrastructurally (Fig 5).

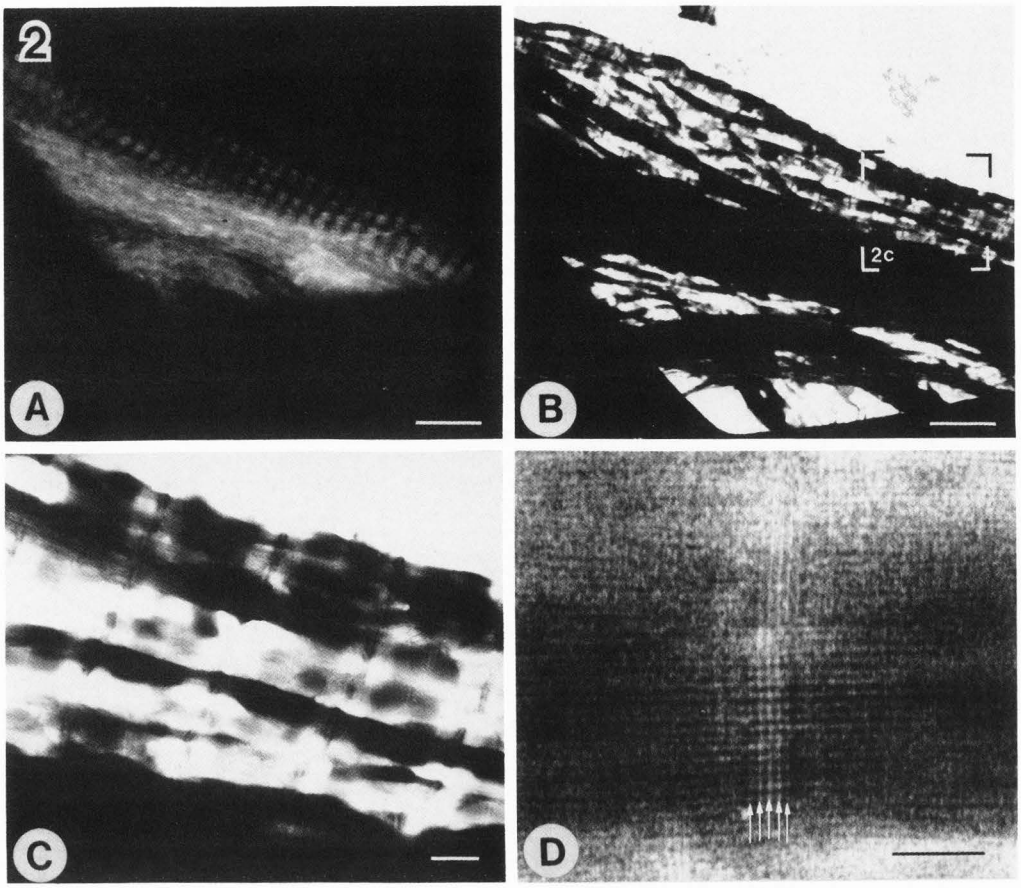
A direct result of these trials was the observation of a marked improvement in resolution which could be obtained by using sections cut with the cryoultramicrotome instead of an ordinary cryostat. In cryostat sections of longitudinally cut muscle fibres stained with any of the antibodies against the three M-band proteins, it was difficult to distinguish precisely to which of the myofibrillar bands the labelling corresponded. However, in the thin sections, which are easily obtained with a cryoultramicrotome, the improved resolution is apparent (Fig 6). In fact, shifts in the organization of the sarcomeres are easily seen. Furthermore, such sections can also be used for double immunolabelling. Still another feature of these sections is the autofluorescence of mitochondria which can be used to distinguish fibre types on the basis of the mitochondrial content (Schmalbruch,1985).

Fig 2: A: Cryosection of guinea pig gastrocnemius muscle on a grid labelled with antibodies against fast myosin followed by a FITC labelled secondary antibody and viewed in the fluorescence microscope. Note fluorescence of the myofibrillar A-bands. In B the same area is shown in the electron microscope after the grid had been negatively stained with ammonium molybdate. In C the enclosed region is viewed at higher magnification and in D at still higher magnification over an M-band in the center of a myofibrillar A-band. Note that an M-band with five lines (arrows) can be observed. Bar = A-B 10 µm; C 1 µm; D 200 nm.

Fig 3: A: Cryosection of guinea pig soleus viewed in the fluorescence microscope after labelling with antibodies against slow myosin. In B the same area is shown in the electron microscope at low magnification. In C at higher magnification it is obvious that the M-band of this type of fiber contains four strong lines (arrows) with the central line being absent or very weak. Bar = A 20 µm; B 10 µm; C 200 nm.

Intermediate filaments in myocardial cells

Another example which illustrates the marked improvement in the resolution of immunolabelled semithin sections viewed in the light microscope is the visualization of intermediate filaments in cardiac muscle. Using ordinary cryostat sections, 4-8 µm thick, a transverse pattern is typically seen across the muscle fibres after labelling with anti-desmin. Intense staining is also seen at cell to cell borders and at the intercalated discs (Fig 7A). In 0.2-0.5 µm thick sections, however, a dramatic improvement in resolution was seen. In such sections the fluorescence was confined to short transverse strips or dots in between myofibrils and to a double band and longitudinal strings in the intercalated disc region (Fig 7B). The information from such sections directly correlated to that obtained from ultrathin cryosections labelled with anti-desmin followed by gold conjugated secondary antibodies (Fig 7C). In such sections the myofibrillar Z discs are unlabelled, but are interconnected by strands of intermediate filaments in transverse register. At intercalated discs large numbers of gold particles were seen at transverse and longitudinal cell borders as well as in strands running parallel to the myofibrils (Fig 7D).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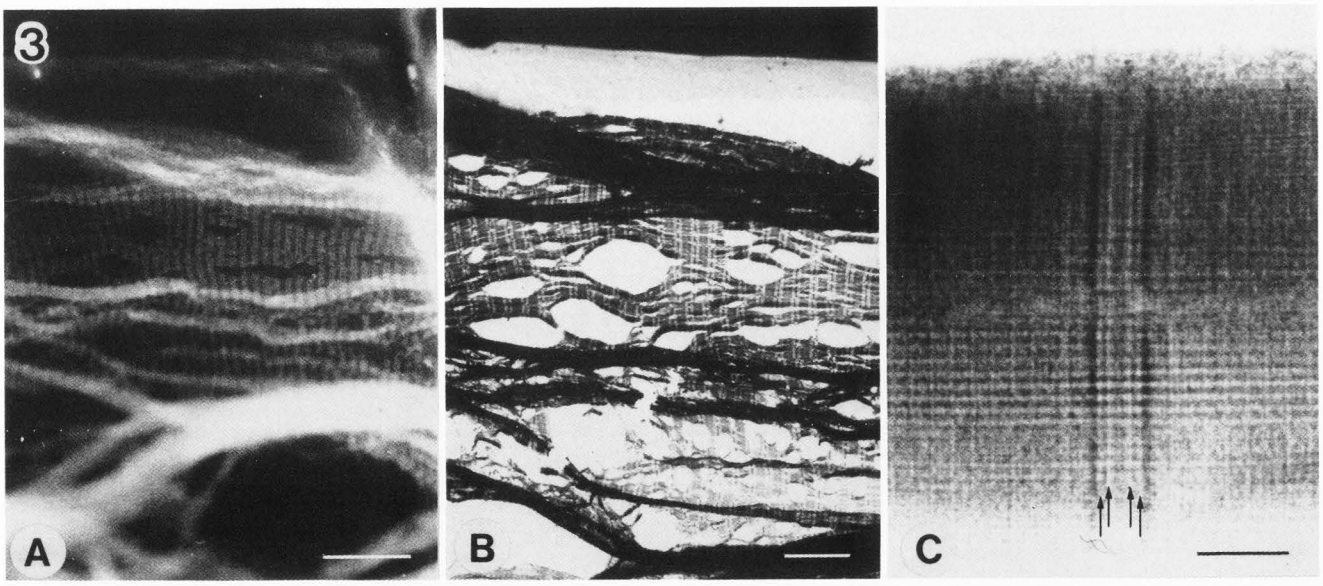
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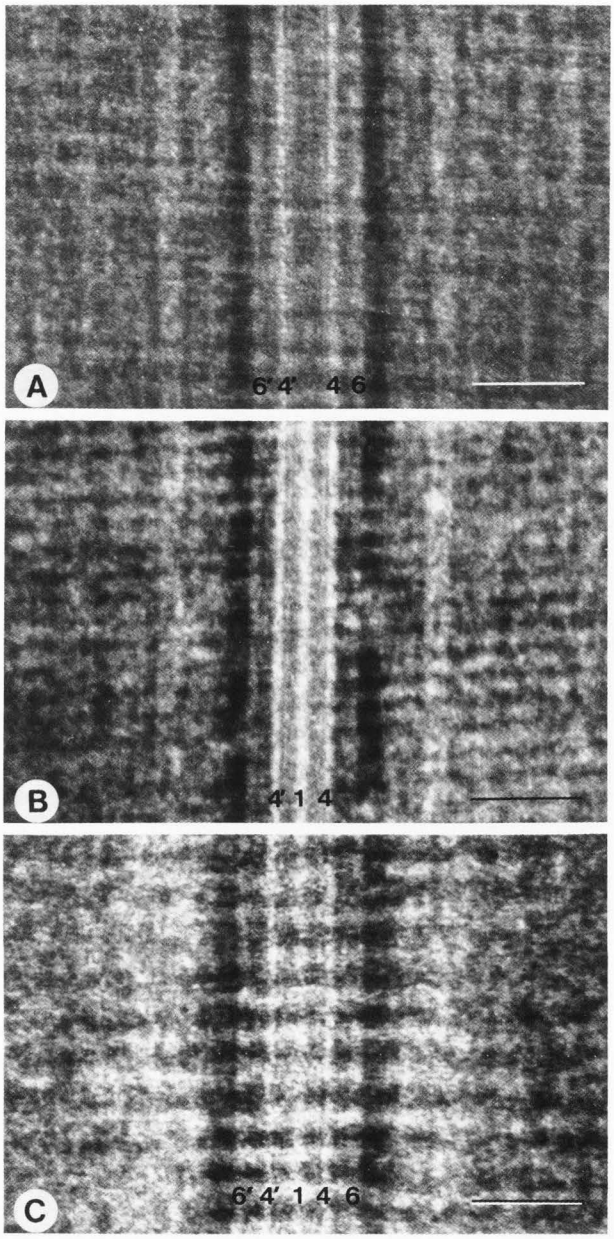


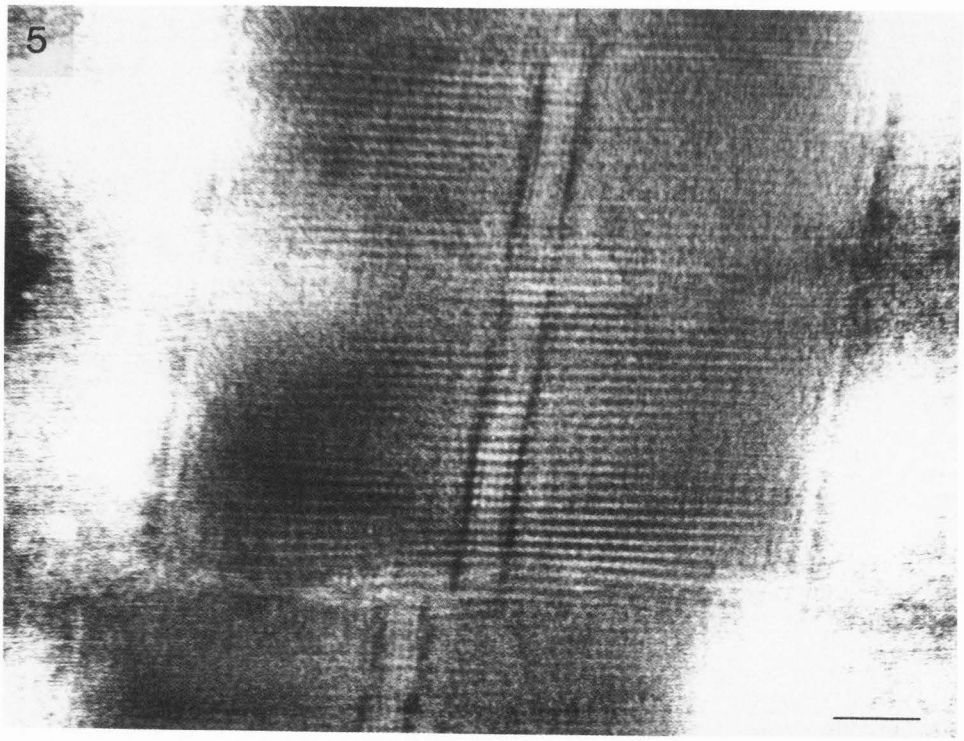
Fig 4: Comparison of the M-band patterns seen in guinea pig gastrocnemius and soleus muscles. A: Four-line pattern (4,4', 6,6') of slow twitch motor unit. M4,4' and M6,6' are the strongest lines, whereas M1 is very weak. B and C: Fibres of fast twitch units with three (1, 4,4') and five (1, 4,4', 6,6') strong M-lines in the M-band respectively. Note additional faint lines. Bar = 80 nm.

Discussion

The new possibilities in cell biology of combining visualization of structure together with molecular identity have given rise to the development of a number of methods for immunoelectron microscopy which have been reviewed by Polak and Varndell (1984). Cryoultramicrotomy together with immunocytochemistry is one possible way to reveal the structure and composition of cells and cell organelles. Its use has also been popularized by others (see Tokuyasu 1980, 1983a, Griffiths et al. 1984). The major problem with immunoelectron microscopy is the extent of tissue fixation which is necessary to preserve antigenic determinants and yet allow antibody penetration and good visualization of structure. Therefore for each antibody and corresponding structural component to be visualized, careful determination of reactivity and tissue preservation has to be carried out (see e.g., Tokuyasu et al. 1981 and Griffiths et al. 1984).

Here we have presented new information about the M-band structure in relation to fibre types of slow and fast twitch units of guinea pig gastrocnemius and soleus muscles. These muscles have previously been extensively studied by enzyme histochemistry, biochemistry, and physiology (Barnard et al. 1970a,b, 1971) as well as by electron microscopy combined with morphometrical analysis (Eisenberg and Kuda 1976, 1977, Eisenberg 1983). On the basis of Z disc width combined with volume density of mitochondria, 89% of the fibres were calculated to be accurately typed in relation to the enzyme histochemical composition of specific portions of the muscles. However, when a mixed muscle was examined, the scattergram of Z disc width and volume density of mitochondria would have been interpreted as a single cluster of fibres representing one fibre type indicating that these parameters are not discriminative enough for distinguishing fibre types (Eisenberg and Kuda 1977). As shown here a four-line M-band pattern distinguishes all fibres containing the slow myosin isoform, whereas fast twitch fibres had either a three- or a five-line pattern. As the four-line pattern was always seen in fibres stained with slow myosin and the three- and five-line pattern in fibres stained with fast myosin, we conclude that the M-band pattern can be used as an ultrastructural marker for slow and fast twitch fibres. Similar results have been obtained for rat slow and fast muscle fibres where we have mapped motor units electrophysiologically and then examined their M-band pattern of mapped fibres (Thornell et al. 1985a, 1987). From this study we also conclude that subtyping of fast twitch fibres on the basis of the M-band pattern is arbitrary since we do not know to which physiological parameter the different M-band patterns are correlated.

From our attempts to analyse the M-band structure and composition it is clear that the high Mr M-band polyclonal antibodies showed a higher avidity for their chemically fixed antigens than the mAbs against myomesin and M-protein. Since the mAbs detected just one antigenic site, insufficient labelling of molecules below levels of detectability at the ultrastructural level might be a problem, especially if the antibodies are untagged. However, if a secondary tagged antibody is used,



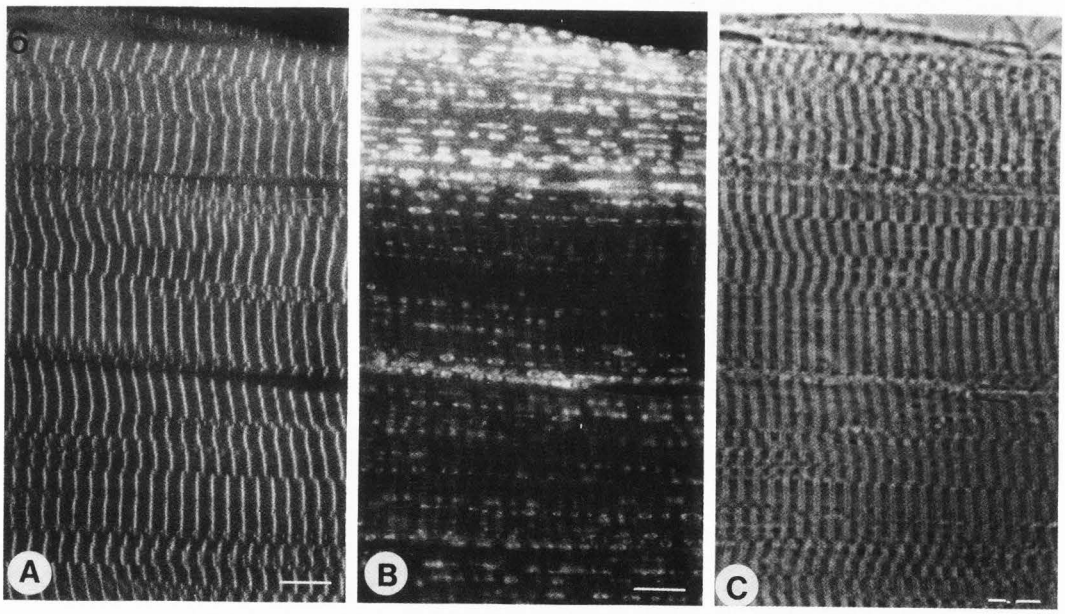


Fig 5: Ultrathin cryosection of chicken pectoralis muscle fibre immunolabelled with mAbs against M protein and negatively stained with ammonium molybdate. No increased density of the M-lines or of the area in between the thick filaments in the M-band is seen, although positive labelling was confirmed at the light microscopic level. Bar = 200 nm.

Fig 6: Approximately 0.5 µm section cut with a cryoultramicrotome and labelled with mAbs against myomesin followed by a FITC labelled secondary antibody.

A: Viewed in the fluorescence microscope with filter set for FITC. Strongly fluorescent lines across the myofibres are observed.
B: The same area as in A but viewed with filter set for a wave length of 550 nm. Fluorescent dots are now observed.
C: Phase contrast image of the same area. By comparison of the images in A and B with C it is clear that the upper fibre represents a mitochondrial rich fibre type but that all myofibrils contain myomesin in the M-band. Bar = 10 µm.

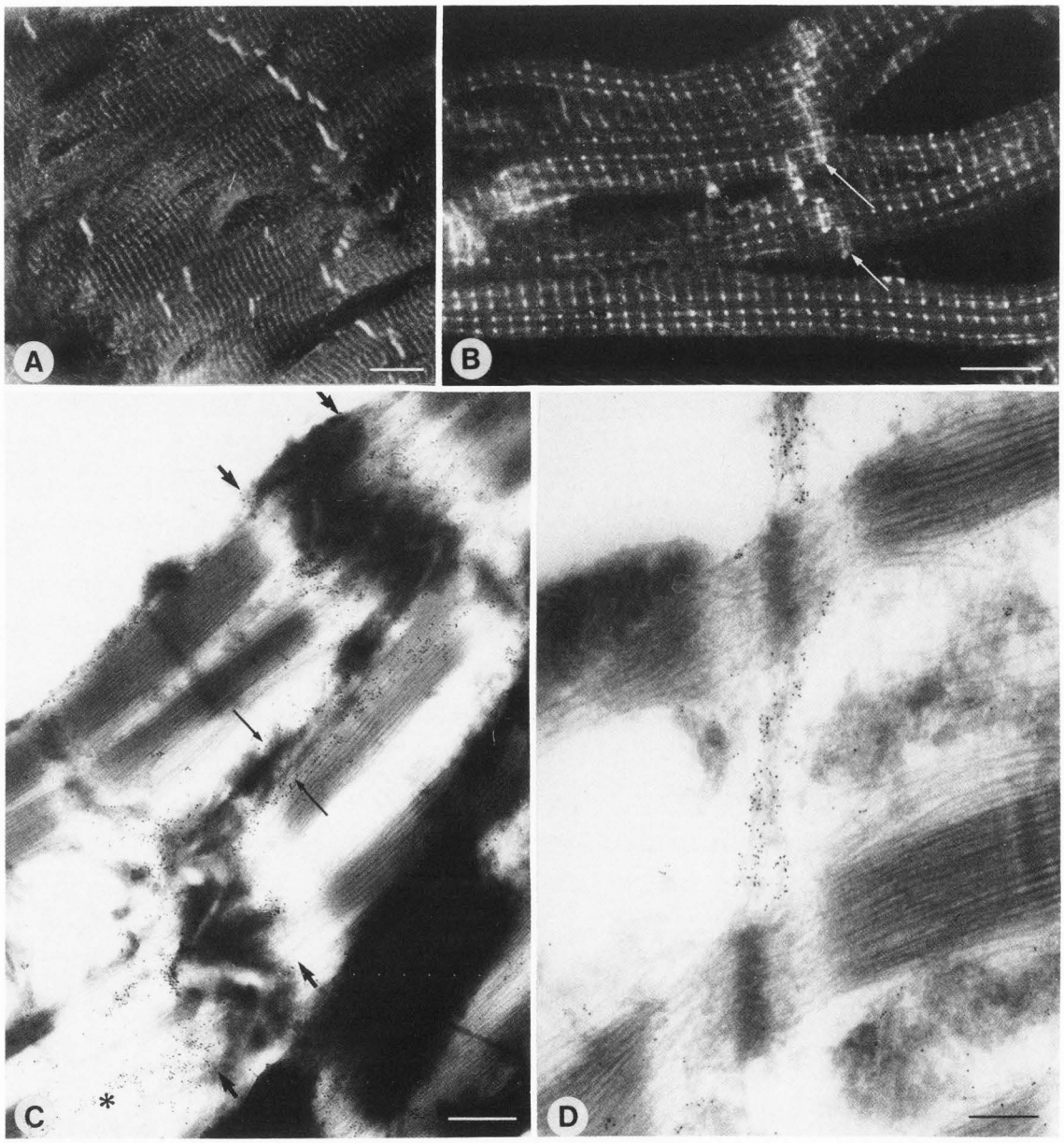


Fig 7: A: Cryostat section (6 µm) of ordinary ventricular bovine myocardium labelled with anti-desmin antibodies followed by a FITC labelled secondary antibody and photographed in the fluorescence microscope. Note the transverse striations across the muscle cells and the strong fluorescence at cell to cell borders of the intercalated discs.
B: Cryoultramicrotome section (0.2-0.5 µm) of myocardial cells labelled and viewed as in A. Note that fluorescence now is confined to short strips or dots in between the myofibrils and that the intercalated disc shows up as a double band structure (arrows) to which longitudinal running fluorescent strings are attached.
C and D: Immunoelectron micrographs of ordinary bovine myocardial cell. Ultrathin cryosections labelled with anti-desmin and secondary gold-tagged antibody, protected with methyl cellulose and stained with uranyl acetate.
C: Intercalated disc area with strong labelling at both sides of the transverse (large arrows) and the longitudinal (small arrows) cell to cell attachment region. Furthermore, strings of gold particles are also seen running parallel to the myofibrils (asterisk).
D: Note labelling of filaments interconnecting the myofibrillar Z discs.
Bar = A 25 µm; B 10 µm; C 400 nm; D 200 nm.

the volume occupied by the antibodies will reduce the spatial resolution which is necessary for positive identification of labelling of closely spaced lines such as those in the M-band. Here the use of directly gold-labelled primary mAbs or mAb Fab fragments might be the way to improve ultrastructural high resolution immunocytochemistry of the M-band. However, from the present data we conclude that it is unlikely that M-protein and myosin make up the M-filaments in the M-region, since no increase in density could be detected between the thick filaments. The present results favour that both M-protein and myomesin ensheath the thick filaments as suggested by model number B in Strehler et al. (1983).

Another major advantage of cryoultramicrotomy is that it provides thin sections that show a marked improvement in resolution of labelling in the light microscope as demonstrated here for immunolabelling of the M-band in skeletal muscle and of intermediate filaments in cardiac muscle (see also Tokuyasu et al. 1984). With respect to the intermediate filaments we confirm here the presence of longitudinally oriented intermediate filaments of desmin in the region of the intercalated discs of mammalian cardiac myocytes (Thornell et al. 1978), whereas elsewhere in the myocyte the bulk of intermediate filaments of desmin is concentrated in the intermyofibrillar space at the level of the Z disc.

In chicken cardiac muscle, on the other hand, longitudinally oriented networks of intermediate filaments have been demonstrated in the interfibrillar spaces, in addition to the transversely oriented networks that surround myofibrils at the level of the Z-band (Tokuyasu 1983a,b). This suggests that the cytoskeleton in cardiac myocytes differs from that of chicken and that the cytoskeleton is enforced in mammalian cardiac myocytes in the intercalated disc region.

In conclusion, we are convinced that the use of cryoultramicrotomy in combination with light and electron microscope immunocytochemistry have a broad field of application in other areas of cell biology.

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References

Barnard RJ, Edgerton VR, Peter JB (1970a) Effect of exercise on skeletal muscle. I. Biochemical and histochemical properties. J Appl Physiol 28, 762-766.

Barnard RJ, Edgerton VR, Peter JB (1970b) Effect of exercise on skeletal muscle. II. Contractile properties. J Appl Physiol 28, 767-770.

Barnard RJ, Edgerton VR, Furukawa T, Peter JB (1971) Histochemical, biochemical, and contractile properties of red, white, and intermediate fibers. Am J Physiol 220, 410-414.

Billeter R, Weber H, Lutz H, Howald H, Eppenberger HM, Jenny E (1980) Myosin types in human skeletal muscle fibres. Histochemistry 65, 249-259.

Burke RE (1981) Motor units: Anatomy, physiology and functional organization. In: Handbook of Physiology. Section 1. The nervous system. Vol II, Motor control. VB Brooks (ed) Amer Phys Soc, Washington, pp 345-422.

Butler-Browne GS, Bugaisky LB, Cuenoud S, Schwartz K, Whalen RG (1982) Denervation of newborn rat muscles does not block the appearance of adult fast myosin heavy chain. Nature (Lond) 299, 830-833.

Butler-Browne GS, Whalen RG (1984) Myosin isozyme transitions occurring during the postnatal development of the rat soleus muscle. Develop Biol 102, 324-334.

Carlsson E (1985). Ultrastructure and composition of the myofibrillar M-band in relation to muscle specialization. Umeå University Medical Dissertation. New Series No 148.

Carlsson E, Kjörell U, Thornell LE, Strehler EE, Lambertsson A (1982) Differentiation of the myofibrils and the intermediate filament system during postnatal development of the rat hearts. Eur J Cell Biol 27, 62-73.

Carlsson E, Thornell LE (1987) Diversification of the myofibrillar M-band in rat skeletal muscle during postnatal development - An ultrastructural study. Cell Tiss Res, in press.

Eisenberg BR, Kuda AM (1976) Discrimination between fiber populations in mammalian skeletal muscle by using ultrastructural parameters. J Ultrastruct Res 54, 76-88.

Eisenberg BR, Kuda AM (1977) Retrieval of cryostat sections for comparison of histochemistry and quantitative electron microscopy in a muscle fiber. J Histochem Cytochem 25, 1169-1177.

Eisenberg BR (1983) Quantitative ultrastructure of mammalian skeletal muscle. In: Handbook of Physiology - Skeletal Muscle. LD Peachy, RH Adrian, SR Geiger (eds), Amer Phys Soc, Bethesda, Maryland, 73-112.

Griffiths G, McDowall A, Back R, Dubochet J (1984) On the preparation of cryosections for immunocytochemistry. J Ultrastruct Res 89, 65-78.

Grove BK, Kurer V, Lehner C, Doetschman TC, Perriard JC, Eppenberger HM (1984) A new 185,000-dalton skeletal muscle protein detected by monoclonal antibodies. J Cell Biol 98, 518-524.

Grove BK, Cerny L, Perriard JC, Eppenberger HM (1985) Myomesin and M-protein: Expression of two M-band proteins in pectoral muscle and heart during development. J Cell Biol 101, 1413-1421.

Knappeis GG, Carlsen F (1968) The ultrastructure of the M line in skeletal muscle. J Cell Biol 38, 202-211.

Pette D, Vrbova G (1985) Invited review: Neural control of phenotypic expression in mammalian muscle fibers. Muscle & Nerve 8, 676-689.

Polak JM, Varndell IM (1984) Immunolabelling for electron microscopy. Elsevier, Oxford.

Schmalbruch H (1985) Handbook of Microscopic Anatomy, Vol II/6, Skeletal Muscle, Springer-Verlag.

Sjöström M, Squire J (1977a) Fine structure of the A-band in cryosections. The structure of the A-band of human skeletal muscle fibers from ultrathin cryosections negatively stained. J Mol Biol 109, 49-68.

Sjöström M, Squire J (1977b) Cryo-ultramicrotomy and myofibrillar fine structure: a review. J Microsc 111, 239-278.

Sjöström M, Thornell LE, Cedergren E (1973) The application of cryoultramicrotomy in the study of the fine structure of myofilaments. J Microsc 99, 193-204.

Sjöström M, Kidman S, Henriksson-Larsén K, Ängquist KA (1982) Z- and M-band appearance in different histochemically defined types of human skeletal muscle fibers. J Histochem Cytochem 30, 1-11.

Small JV (1981) Organization of actin in the leading edge of cultured cells: influence of osmium tetroxide and dehydration on the ultrastructure of actin meshwork. J Cell Biol 91, 695-705.

Small JV, Fürst DO, De Mey J (1986) Localization of filamin in smooth muscle. J Cell Biol 102, 210-220.

Strehler EE, Carlsson E, Eppenberger HM, Thornell LE (1983) Ultrastructural localization of M-band proteins in chicken breast muscle as revealed by combined immunocytochemistry and ultramicrotomy. J Mol Biol 166, 141-158.

Thornell LE, Carlsson E (1984) Differentiation of myofibrils and the M-band structure in developing cardiac tissues and skeletal muscles. Expl Biol Med 9, 141-147.

Thornell LE, Sjöström M, Ringqvist M (1976) Attempts to correlate histochemical and ultrastructural features of individual skeletal muscle fibers. J Ultrastruct Res 57, 224.

Thornell LE, Eriksson A, Stigbrand T, Sjöström M (1978) Structural proteins in cow Purkinje and ordinary ventricular fibres - a marked difference. J Mol Cell Cardiol 10, 605-616.

Thornell LE, Edström L, Billeter R, Butler-Browne GS, Kjörell U, Whalen RG (1984a) Muscle fibre type composition in dystal myopathy (Welander). J Neurol Sci 65, 269-292.

Thornell LE, Billeter R, Butler-Browne GS, Eriksson PO, Ringqvist M, Whalen RG (1984b) Development of fiber types in human fetal muscle. An immunocytochemical study. J Neurol Sci 66, 107-115.

Thornell LE, Carlsson E, Kugelberg E, Grove BK, Eppenberger HM (1985a) New aspects on the structure and composition on the myofibrillar M-band. J Muscle Res Cell Motil 6, 81.

Thornell LE, Eriksson A, Johansson B, Kjörell U, Franke WW, Virtanen I, Lehto VP (1985b) Intermediate filament and associated proteins in heart Purkinje fibers: A membrane-myofibril anchored cytoskeletal system. Ann NY Acad Sci 455, 213-240.

Thornell LE, Carlsson E, Kugelberg E, Grove BK (1987) Myofibrillar M-band structure and composition of physiologically defined rat motor units. Dev Biol, in press.

Tokuyasu KT (1980) Immunochemistry on ultrathin frozen sections. Histochem J 12, 381-403.

Tokuyasu KT (1983a) Present state of immuno-cryoultramicrotomy. J Histochem Cytochem 31, 164-167.

Tokuyasu KT (1983b) Visualization of longitudinally oriented intermediate filaments in frozen sections of chicken cardiac muscle by a new staining method. J Cell Biol 97, 562-565.

Tokuyasu KT, Geiger B, Dutton AH, Singer SJ (1981) Ultrastructure of chicken cardiac muscle as studied by double immunolabelling in electron microscopy, Proc Natl Acad Sci USA 78, 7619-7623.

Tokuyasu KT, Maher PA, Singer SJ (1984) Distribution of vimentin and desmin in developing chick myotubes in vivo. I. Immunofluorescence study. J Cell Biol 98, 1961-1972.

Weber K, Rathke PC, Osborn M (1978) Cytoplasmic microtubular images in glutaraldehyde-fixed tissue culture cells by electron microscopy and by immunofluorescence microscopy. Proc Natl Acad Sci USA 75, 1820-1824.

Discussion with Reviewers

B.A. Afzelius: Did you try the immunohistochemical protocol also on unfixed muscle tissue? I presume that muscle cells are so solidly built that there is a chance that unfixed (but glycerinated) tissue might give some information.

Authors: We routinely use unfixed muscle tissue for immunohistochemical analysis of cryostat

sections, but not for sections cut with the cryoultramicrotome. However, we are sure that at least semithin sections of chemically unfixed tissue can be cut for and retained during the immunohistochemical procedures.

G.M. Roomans: The use of fixed material necessarily limits the number of different physiological or contraction states of the muscle that can be studied. Is this a serious disadvantage? Could you envisage a method using rapid freezing of unfixed tissue in a defined physiological state (Nassar et al. 1986) followed by cryosectioning on a dry knife, and vapor fixation of the section prior to immunocytochemistry (Frederik et al. 1984)?
Authors: For our aims presented in this paper chemical fixation has not been disadvantageous. However, in order to study morphological features related to different physiological contraction states of a muscle fibre, the muscle fibres have, of course, to be preserved at the states in question. So far the only way to get rapid enough preservation is to use quick freezing. We have made some attempts to achieve this ourselves (Sjöström et al. 1973); however, at that time we were primarily interested in elemental analysis of muscle fibres (Sjöström and Thornell 1975). At present we are collaborating with P Edman at the Department of Pharmacology, University of Lund, who has developed methods to achieve rapid freezing of single intact frog skeletal muscles at known intervals following electrical stimulation. Analyses of electron microscopical images of such fibres after cryosectioning and negative staining with the aim to reveal the features of myosin crossbridges at various state of contraction are in progress.

J. and R. Wroblewski: Please state the time period tissue was allowed to relax after excision and prior to fixation.
Authors: We have used a number of various formulas for processing muscle samples. The time period for tissue relaxation in buffer has been from 0 to 60 min. For our aims the time period of relaxation has not been critical.

J. and R. Wroblewski: Is treatment with glycerol before cryofixation better than with sucrose? Would you please comment on that.
Authors: The rationale for using glycerol in our early cryosectioning studies was that the molecular organization of myofibrillar proteins was known not to be affected by such a treatment, whereas the effects of sucrose were unknown. However, we nowadays obtain good cryoprotection and preservation with sucrose but we have not studied systematically whether treatment with glycerol or sucrose gives any differences in morphological preservation.

B. Forslind: Could you explain what differences in the morphology you induce by the different glycerol concentrations?
Authors: A glycerol concentration of 30% is needed for adequate freeze protection of muscle fibres (Sjöström 1975). If not used, mechanical damage, because of ice crystal formation, will affect the morphology of the muscle fibres. For studies of myofibrillar fine structure glycerol treatment is permissible as the myofibrils are still functioning and able to contract; however, the membranes will be permeabilized and destroyed. The aims of the prefixation with formaldehyde and a low concentration of glutaraldehyde are to balance between preservation of fine structure and antigenic determinants of muscle fibres and protection against disruption during subsequent steps of glycerol treatment, sectioning and staining.

B.A. Afzelius: The low level of labelling seems to be a problem when using monoclonal antibodies, evidently because each monoclonal antibody against a certain antigenic molecule reacts with one of its many antigenic sites only. Would it be realistic to plan for an experiment where several monoclonal antigens against for instance fast myosin would be prepared and then run an experiment using a mixture of these monoclonal antibodies?
Authors: Yes, in fact we have tried to use a mixture of different monoclonal antibodies against M-protein and myomesin. However, the amount of labelling has still not been enough for a reliable evaluation of where the two M-band proteins are localized within the M-band.

J. and R. Wroblewski: As one aim of your study was to find a way of fibre typing at the EM level by using labelling of different proteins within A- and M-band, the most direct way would be as you tried (Fig 1), to use antibodies to slow or fast myosin labelling the A-band and correlate the results to M-band pattern. When comparing Fig 1, where the positive stain seems to have been used, with negatively stained muscles (Figs 2B and 3B), it is obvious that the cutting plane in Fig 1 is not the same as in the negatively stained muscles. The morphology of muscle positively stained with uranyl acetate will improve upon embedding the sections in methylcellulose or LR-white after uranyl staining procedure, prior to drying. The positively stained sections do reveal several morphological structures better (even if not optimal for M-bands), than the negatively stained sections do.
Authors: We agree that depending on the aims sometimes positive staining procedures are more advantageous than negative staining. We are currently trying to improve our procedures for immunoelectron microscopy which includes the use of other embedding media and stains. Still in our hands negative staining is preferable.

B. Forslind: Have you tried to analyze the repeating patterns that you obtain by using light diffraction methods on the photographic negative material and if that is not the case do you think you could extract additional information from your material with this technique?
Authors: Yes, we have. Undoubtedly any type of objective analysis gives the possibility of additional information. Images of cryosectioned

muscle fibres are very suitable for objective analysis either by optical diffraction or other methods for averaging of periodic images (see e.g., Squire et al. 1986).

Additional References

Frederik PM, Bomans PHH, Busing WM, Odselius R, Hax WMH (1984) Vapor fixation for immunocytochemistry and X-ray microanalysis on cryoultramicrotome sections. J Histochem Cytochem 32, 636-642.

Nassar R, Wallace NR, Taylor I, Sommer JR (1986) The quick freezing of single intact skeletal muscle fibers at known time intervals following electrical stimulation. Scanning Electron Microsc 1986; I: 309-328.

Sjöström M (1975) Ice crystal growth in skeletal muscle fibres. J Microsc 105, 67-80.

Sjöström M, Thornell L-E (1975) Preparing sections of skeletal muscle for transmission electron analytical microscopy (TEAM) of diffusible elements. J Microsc 103, 101-112.

Sjöström M, Johansson R, Thornell L-E (1973) Cryoultramicrotomy of muscle in defined functional states. Methodological aspects. In: Electron Microscopy and Cytochemistry. E Wisse, W Th Daems, I Molenaar, P van Duijn (eds), North Holland Publ Co, Amsterdam, pp 387-391.

Squire JM, Luther PK, Agnew GD (1986) Averaging of periodic images using a microcomputer. J Microsc 142, 289-300.