# Correlated Confocal and Intermediate Voltage Electron Microscopy Imaging of the Same Cells Using Sequential Fluorescence Labeling, Fixation, and Critical Point Dehydration

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# CORRELATED CONFOCAL AND INTERMEDIATE VOLTAGE ELECTRON MICROSCOPY IMAGING OF THE SAME CELLS USING SEQUENTIAL FLUORESCENCE LABELING, FIXATION, AND CRITICAL POINT DEHYDRATION

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## Abstract

Confocal laser scanning microscopy (CLSM) and intermediate voltage transmission electron microscopy (IVEM) each has its own particular advantages. CLSM can examine living cells, but is particularly useful when applied to cells that have been lightly fixed, permeabilized, and stained with fluorescent-labeled antibodies for localization of specific molecular species at the resolution of the light microscope while still in the hydrated state. IVEM provides much higher resolution images, but requires more drastic preparation procedures, including dehydration. This paper presents methods for combining these complementary approaches to examine exactly the same cells sequentially by CLSM and IVEM. Cells are grown in culture on sterile formvar films spread over gold index grids on cover glasses, which are mounted on larger cover glasses or microscope slides with spacers to prevent compression of the cells. Light and epifluorescence microscopy, and CLSM are performed concentrating on cells in grid openings. Then the grids are fixed with aldehydes followed by $OsO_4$, dehydrated and critical point dried (CPD) from liquid $CO_2$. Immediately following CPD, the grids are ready for examination in the IVEM. Low magnification (300-600x) survey images allow correlation of the IVEM images with the light microscopic images. In higher power images, structures that are fluorescent labeled can be related to corresponding regions in the IVEM images.

**Key Words**: Confocal microscopy, high voltage electron microscopy, correlated microscopy, specimen preparation, cell cultures, fluorescence immunocytochemistry, fluorescence labeling.

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## Introduction

The light microscope has a singularly important advantage over most other kinds of microscopes in that it can be used to visualize dynamic processes in living cells. Such live preparations produce images that can be both beautiful and informative, within the limits of resolution of the light microscope and the investigator's abilities to stain cellular components while still maintaining the cells in the living state with normal physiological functions. Movies of cells in action are possible and often necessary for adequate description of dynamic cellular activities.

At the other end of the spectrum, with respect to specimen preparation, is the sequence of preparative steps that a cell usually is subjected to prior to imaging with transmission electron microscopy (TEM) as a thin section. For TEM, biological specimens may be subjected to fixation with aldehydes and osmium tetroxide, alcohol dehydration, embedding in epoxy resins, and microtomy. Exquisite in their detail, the resulting images have much higher resolution of fine structure than is possible in light microscopy. Such images also potentially are very revealing about the mechanisms underlying physiological activities in these cells, provided that the effects of specimen preparation procedures can be evaluated, and also provided that the detailed information present in the TEM images can be understood in relation to the living cell images. One major difficulty in understanding the relationship between light and electron images is to account for possible effects on cellular structure of the preparation procedures used. Another difficulty arises because one is required to relate images of one cell observed in the light microscope to images of a different cell seen in the TEM. While this may be straightforward for larger, well-formed structures with easily recognized forms (mitochondria or striated myofibrils, for example), the correlation can be difficult at best for smaller structures, structures that are found only infrequently, and structures with more subtle forms. Thus electron microscopists and cell biologists are presented with a real

challenge when they wish to bridge the gap between light and electron microscopy in a precise way, in order to combine the special information each type of microscope provides. This is the bridge this paper will focus on.

One important plank in the bridge between light and electron microscopy of biological cells has been available for 20 years or more: the high (or intermediate) voltage TEM (see Peachey *et al.*, 1974 for several early examples). These instruments, few in number but powerful in their penetration of thick specimens, are readily available thanks to the wisdom and funding of the Biomedical Research Technology Program of the National Institutes of Health (Bethesda, MD). Whole cells, grown on or otherwise mounted on electron microscope specimen grids, can be imaged intact in such higher voltage electron microscopes, avoiding some of the more drastic preparation steps required in thin-section electron microscopy, in particular the embedding and sectioning steps. Thus one can examine whole, intact cells with all the resolution available in the TEM with minimal mechanical disturbances, and with reduced, if not exactly minimal, chemical treatment.

This paper describes some methods and results from an effort to provide a direct correlation between light and electron microscopy, by imaging the same cells sequentially in microscopes of both types.

## Materials and Methods

All of the examples shown are from cells grown in culture. The procedure followed was similar to that in use in several laboratories for preparing cells grown in culture for high voltage (HVEM) or intermediate voltage electron microscopy (IVEM) imaging, in which cells are released from their host tissues using chemical or enzymatic treatment and then plated on formvar-coated electron microscope (EM) specimen grids for culture (Wolosewick and Porter, 1976; Murakami *et al.*, 1993).

The overall goal was to examine the same cells, first without any form of treatment, by light microscopy, and finally, after fixation and dehydration, by TEM. Phase microscopy was often used first, and was useful for judging the state of the cultures and the maturity of the cells. Fluorescence microscopy then followed, using fluorescent probes or fluorescent labels conjugated to specific antibodies to identify and locate specific proteins in cells, and brief fixation and permeabilization of the cells to allow penetration of the labels. Confocal fluorescence scanning laser microscopy provided greater 3D resolution of the distribution of labels and provides images free of background, out of focus fluorescence. Then the cells were ready for the more drastic specimen preparation procedures, including dehydration, required

for transmission electron microscopy.

In order to facilitate examination of cells using various forms of microscopy at various stages in a sequence of specimen preparation steps, and especially in order to provide for localization and imaging of the exact same cells through all these steps, several modifications were introduced into the usual procedures for electron microscopy of whole mounts of cultured cells. These procedures, including the modifications, will now be described in some detail. This description will include some procedures not used for the illustrations presented here, in order to provide a broader picture of the specimen preparation methods available for sequential confocal and electron microscopy.

### Cell culture

Cells were grown on glass coverslips prepared with gold EM grids, as follows. Clean glass slides were dipped in an approximately 1% solution of formvar in ethylene dichloride, and dried in air. The plastic film was floated off on a clean water surface, and the slide was discarded. Three or four gold EM specimen grids, previously cleaned with dilute acid and rinsed, were placed on top of the floating plastic film, in an asymmetric pattern so that individual grids could be identified uniquely later, even when the film and grids had been inverted. Gold grids do not poison or otherwise contaminate the cultures, and the use of "finder grids" with index marks to identify the grid openings is useful in tracking individual cells through the various microscopic examinations. Next, a square cover glass was pressed down on the plastic film carrying the grids, pushed through the water surface, inverted under water, and brought back into air, with care not to wrinkle the film or lose the grids. Air drying and sterilization overnight under UV light completed the preparation of the culture substrates.

All of the cells illustrated here are from cultures of primary heart explants from 8-day chick embryos. Cells were dispersed by treatment with 0.05-0.1% trypsin in calcium- and magnesium-free Hank's balanced salt solution at 37°C for 10-20 min followed by filtration to remove undissociated tissue. The resulting mixed cardiac myocytes and fibroblasts were cultured for 3-7 days in Eagle's minimum essential medium (Flow Laboratories, McLean, VA) plus 10% fetal bovine serum (Sigma, St. Louis, MO) at a density of about $2.5 \times 10^4$ cells per dish containing one cover glass. Cells begin to attach and spread within one day, and begin to take on characteristic shapes: flat and multipolar for fibroblasts and more rectangular and usually bipolar for myocytes. By day three, some myocytes form cell attachments, or fuse to form myotubes and start to contract.

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## Phase microscopy

Phase microscopy was done directly on cells growing over holes in the EM grids, while the cells continued to grow in the culture medium. Cells obscured by grid bars or not growing over grids can be examined at this stage, but these cells cannot be examined later by electron microscopy. When the desired stage of growth had been achieved, cover glasses were removed from the culture dishes and the preparation for sequential microscopy started.

## Epifluorescence microscopy

Typically the cells, still on the grids and cover glasses, were fixed briefly (e.g., 10 min at room temperature) in 4% paraformaldehyde in PBS (phosphate buffered saline). The cells were permeabilized by immersion in 0.1% Triton-X-100 in PBS at room temperature for about 10 min, followed by a brief rinse in PBS. Labeling with fluorescent probes followed. This could be a direct fluorescent label, such as fluorescein-phalloidin, or a sequence of primary and fluorescent-labeled secondary antibodies, such as rabbit anti-myosin followed by goat anti-rabbit antibody conjugated to fluorescein. The incubation times and concentrations of probes or antibodies used depended on the particular probes and antibodies. Fluorescein isothiocyanate (FITC)-phalloidin (Sigma), used for the cells illustrated in this paper, was utilized by adding 3.3 $\mu$l of a 100 $\mu$g/ml stock in ethanol to 400 $\mu$l of PBS, with incubation at room temperature for 20 min. After a 10 minute rinse in PBS, the cover glasses were mounted on larger glass coverslips, with the grids and cells facing the larger coverslip and the two coverslips held apart with thin plastic spacers or otherwise held in place so the grids and the cells would not be crushed. Various other configurations of slides and cover glasses are possible, with the important points being not to crush the preparations, to have the cells as close as possible to the cover glass through which images are made, and to be able to disassemble the mount in order to recover the grids for further processing. The mounting medium used at this stage was a glycerol-water mixture, with the addition of anti fading agents. A standard fluorescence microscope, either inverted or upright, is suitable for visual examination of the preparations and photography. Images were recorded on black and white or color film, with records being kept of the locations of the cells photographed in relation to the index marks on the grids and the grid location on the cover glass. Epifluorescence images shown here were obtained using a 35 mm camera on the microscope contained in the confocal microscopy setup, using the standard epifluorescence filter set and a Zeiss (Oberkochen, Germany) PlanApo oil immersion objective, numerical aperture (n.a.) 1.3. The instrument was then switched to the confocal scanner for confocal microscopy, without moving the specimen.

## Confocal laser scanning microscopy

We used both a Biorad MRC-600 confocal scanner with a Zeiss Axiophot upright microscope and an argon-ion laser and a Leica upright confocal microscope with an argon-krypton laser. The mounting of the specimens was not changed from that used for epifluorescence microscopy. The same cells were located and, typically, one or more Z-series scans were made and stored for future analysis and comparison to the IVEM images. Details are provided in the figure legends for the images shown here.

## Intermediate voltage electron microscopy

Following all steps of light microscopy, the slides and/or cover glass sandwiches containing the specimen grids and cells were opened so that the grids could be processed further for electron microscopy. One method used involved immersing the assembly under water, and gently lifting the cover glass carrying the grids away from the opposing slide or cover glass. In an alternate method, the assembly was set, with the cover glass carrying the grids downward, on pedestals in a glass container, such that the pedestals contacted only the upper, larger glass slide or cover glass. Water was then added to the container until the bottom surface of the assembly was immersed. After some time, the lower cover glass floated away from the upper one, and the cover glass with the grids attached floated to the bottom of the container. These methods eliminated surface tension effects, and the separation of the assembly without damage to the preparations was facilitated. Gentleness is paramount at this stage, as the thin support films on the grids can easily broken be or dislodged.

The cover glasses and attached grids with cells were fixed with 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 at room temperature for 20 minutes, rinsed with buffer alone, and post fixed in 1% osmium tetroxide in the same buffer at 4°C. The preparations are then rinsed briefly with distilled water, and optionally stained with 2% uranyl acetate in water for 10 minutes followed by another rinse in distilled water. Next, the grids were gently detached from the cover glass by cutting around them, through the plastic film, while under water. The grids were then placed in a grid holder designed to fit into the critical point dryer (CPD) for the remainder of the processing.

Dehydration was done in a graded series of ethanol-water mixtures, about 3 minutes in each concentration, using a series of 15%, 30%, 50%, 75%, 95%, and 100% ethanol (3 times in 100%). CPD was carried out starting with the chamber filled with just enough 100% ethanol to cover the grids in the holder, and with

approximately 5 cycles of introduction of liquid carbon dioxide and bleeding, with 5 minute soaks between. The chamber was slowly bled to atmospheric pressure after warming to about 60°C. Finally, the grids were lightly coated with carbon in a vacuum evaporator to stabilize the films.

Intermediate voltage electron microscopy was done on JEOL (Tokyo, Japan) 4000-EX instruments either at Chiba University, Japan or at the University of Pennsylvania in Philadelphia, at 300-400 kV.

## Results

Figs. 1-4 show portions of the same two early myoblasts in a 4-day cultured heart explant from an 8-day old chick embryo. The preparation was stained with FITC-labeled phalloidin to stain f-actin. Fig. 1 is the epifluorescence image, and shows arrays of actin-containing stress fibers. The arrowhead points toward a group of such actin-containing fibers, just inside the cell membrane of one of the cell processes. Some of these actin-containing fibers are seen to lie close to the lateral cell membranes of the cell processes, while some extend across the cell processes, in a pattern that is typical for these cells. Fig. 2 is a confocal projection along the Z-axis of a series of 16 images of the same specimen, taken at a Z-spacing of 0.48 $\mu$m between scans. The total vertical thickness included in this projection is approximately 8 $\mu$m, and covers the entire thickness of the distal portions of these cell processes. Thus all the same structures that appear in the non-confocal, epifluorescence image also appear in the confocal image. However, the pattern of stress fibers is shown more clearly in the extended focus confocal projection than in the epifluorescence image because of the removal of out of focus light by the confocal aperture. For the same reason, evidence of very early myofibril formation, not seen in the epifluorescence image, is visible in the confocal projection as linear arrays of fluorescent dots in the region indicated by the asterisk. The arrowhead outside the cell in Fig. 2 has the same placement with respect to the cell processes as in Fig. 1. In addition, the arrow in Fig. 2 points to a small, lateral extension from an internal stress fiber, which is not as clearly seen in Fig. 1. Such fine details often become apparent in the epifluorescence image only after they have been seen in the confocal images.

Fig. 3 is an IVEM image from the tip region of the upper-left cell process in Figs. 1 and 2, at higher magnification that the earlier images, and rotated slightly in the counter-clockwise direction. For orientation, an arrowhead points in the same direction and to the same region of the cell as the arrowhead in the earlier figures. It should be noted that, unlike in the fluorescence

(*Figures 1-4 on facing page*)

**Figure 1.** Epifluorescence image of portions of two attached myoblastic cells in a 4-day culture of an heart explant from an 8-day old chick embryo. This preparation was stained with FITC-phalloidin. Each cell has a cytoplasmic process extending upward from the bottom of the figure. The arrowhead points toward a group of actin-containing fibers near the end of one of these processes. Bar indicates 10 $\mu$m.

**Figure 2.** Confocal projection, shown as a negative, along the optic axis of a series of 16 fluorescence confocal images of the same cell as seen in Fig. 1, taken with a focal change of 0.48 micrometers between scans. The arrowhead is positioned in the same location as in Fig. 1. The arrow points to a fine lateral projection from one of the stress fibers. This projection is only barely visible in the epifluorescence image, but very clear in the confocal projection. Pixel size 0.17 micrometers. Bar indicates 10 $\mu$m.

**Figure 3.** IVEM image of the same cell at low magnification. Again, the arrowhead points at the equivalent position marked in the previous figures. The arrow points to the same lateral projection as indicated in Fig. 2. Bar indicates 5 $\mu$m.

**Figure 4.** Higher magnification IVEM image of the same cell. The arrow points to the same lateral projection indicated earlier. The arrowheads point to other structures, presumed to be mitochondria from their morphology, that are similar in size and density to the lateral projection indicated. These clearly are not actin-containing structures, since they are not visible in the epifluorescence and confocal fluorescence images, demonstrating the importance of having the specificity of the fluorescence labels and the ability to identify the same structures in the two kinds of images. Line indicates 1 $\mu$m.

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images, all structures are visible in the IVEM image, since specificity of staining with the phalloidin stain is lost in these images. The resolution of structure is, of course, much better in the IVEM. The arrow in Fig. 3 points to the same extension of a stress fiber as in Fig. 2. One can clearly see this extension in the IVEM image. One can also conclude that nearby structures, similar in appearance in the IVEM to this extension, do not contain f-actin, since they are not stained in the confocal fluorescence image. Examples of such structures are the two dense objects indicated by the arrowheads in Fig. 4, which do not appear at all in the fluorescence images, and which are tentatively identified by size and shape as mitochondria. Fig. 4 is at higher magnification, and shows more clearly the structures

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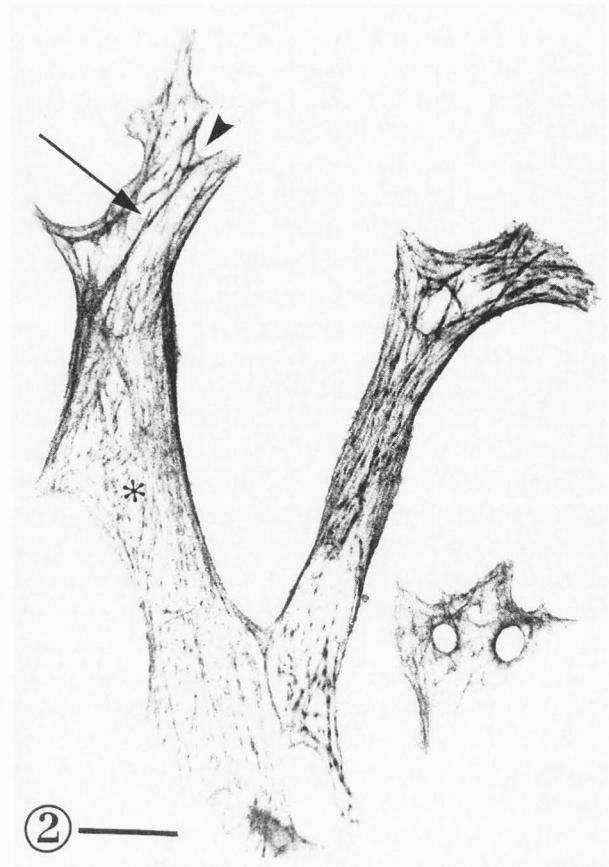
indicated.

The next set of images, Figs. 5-8, is from a fibro-blastic cell prepared in the same way. These images illustrate that, under certain conditions, identification and correlation of even fairly fine structures easily can be achieved by visual inspection of corresponding images from epifluorescence, confocal, and IVEM microscopes. Four arrows mark the tips of actin stress fibers project-ing from the main body of the cell toward a tapering cell process, which points toward the left edge of each figure. From the epifluorescence image (Fig. 5), one could describe the four processes, from top to bottom, as #1 long and narrow, #2 shorter and broader, #3 long and narrow, with a forked tip, and #4 broad and possi-bly representing a bundle of several individual and separate stress fibers. In Fig. 6, the confocal image, these fibers are easily identified and their descriptions, as given above, are confirmed. Additionally, variations in fluorescence along the length of #1, and the composi-tion of #4 as consisting of multiple fibers, are consider-ably more apparent because of the greater clarity of the confocal image compared to the epifluorescence image. The IVEM images (Figs. 7 and 8) provide additional resolution of these features, e.g., the branching of fiber #2 near its end, and details of the subfiber composition of #4.

Part of the reason we have been able unambiguously to identify the corresponding structures in the light and electron micrographs in these figures is that the selected structures have characteristic shapes and are relatively free from closeby and superimposed other structures. The next example will show how information from the fluorescence images can provide information and structural identification not readily available in the IVEM images because of additional material obscuring this information.

Fig. 9 is a confocal projection of a cell in the same type of culture that yielded the cells shown in Figs. 1-8. In this cell, the fluorescence image reveals circular, banded structures with a periodicity of approximately two micrometers (arrow). We identify these periodic structures as myofibrils early in development. In the corresponding locations of the IVEM image (Fig. 10, arrow), dense, curved structures are found around the nucleus, but these do not appear obviously banded, and would not have been thought to be myofibrils on the basis of the IVEM images alone. Without the correlated image from the confocal microscope, with its fluorescent antibody identification of actin-containing striated fibrils, this cell could have been misidentified in the IVEM as a fibroblast rather that a developing myoblast. The confocal fluorescence image, in this case, provides a useful map to lead the investigator to a specific subset of structures of interest in the often more cluttered and

*(Figures 5-8 on facing page)*

**Figure 5**. Epifluorescence image of portion of a fibroblastic cell in a 4-day culture of an heart explant from an 8-day old chick embryo. A tapered cell process extends downward at the bottom of the figure. Arrows indicate the distal tips of 4 stress fibers extending toward and into this cell process. Bar indicates 10 μm.

**Figure 6**. Confocal projection along the optic axis of a series of 11 fluorescence confocal images of the same cell as seen in Fig. 5, taken with a focal change of 0.48 micrometers between scans. The same four stress fibers are indicated by arrows. Pixel size 0.14 micrometers. Bar indicates 10 μm.

**Figure 7**. IVEM image of the same cell at low magnifi-cation, with arrows indicating the same four stress fibers. Greater detail of fine structure of the fibers is available in this image, including branching near the tip of the third fiber from the left and the subfibrillar nature of the fiber on the right. Bar indicates 10 μm.

**Figure 8**. Higher magnification IVEM image of the same cell as in Figures 5-7. The fiber on the right now is clearly resolved into several finer fibers. Bar indi-cates 5 μm.

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confusing image from the electron microscope.

## Discussion

We have demonstrated that a single cell can be imaged through several forms of microscopy, including fluorescence confocal microscopy and 400 kV IVEM, and that intracellular structures can be identified in the corresponding images. One potential value of this kind of correlated microscopy on the same cells is the possibility of identifying structures early in their forma-tion in the cell, as during cell differentiation. For example, striated myofibrils are easy to identify in either light or electron microscope images when they are fully formed (Ishikawa *et al.*, 1990) by virtue of their charac-teristic and stereotyped pattern of striations. Prior to the emergence of this pattern, however, it can be very difficult to identify forming structures in the absence of information on their protein content, which information may be easily available using fluorescent antibodies. Correlation, in a spatially precise way, between fluores-cent images using labeled antibodies and IVEM images of the same cells allows this chemical identification to be carried over to the high resolution images of the electron microscope, where very small and perhaps irregularly shaped and incompletely formed structures can be sorted out from a maze of similar structures and then be studied at high resolution.
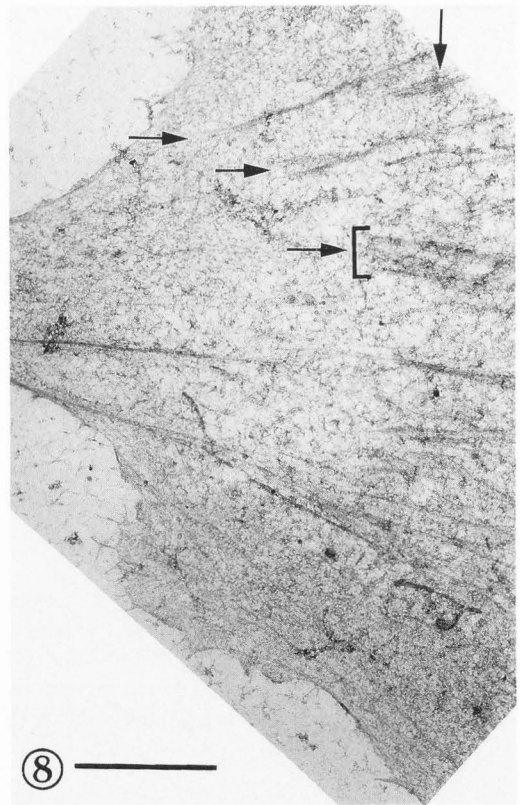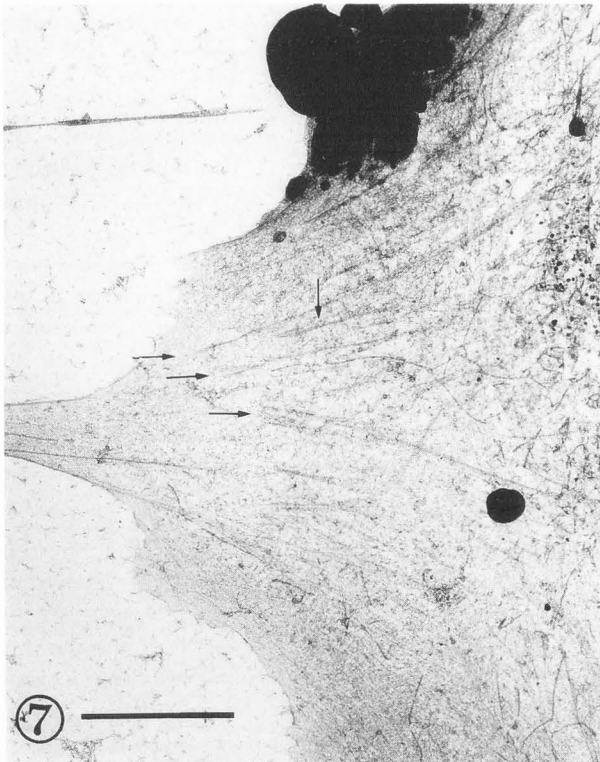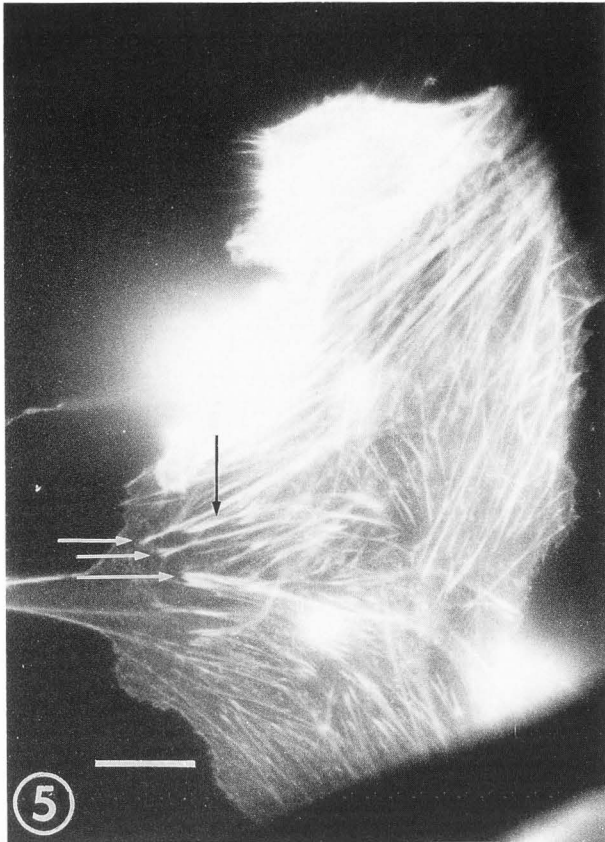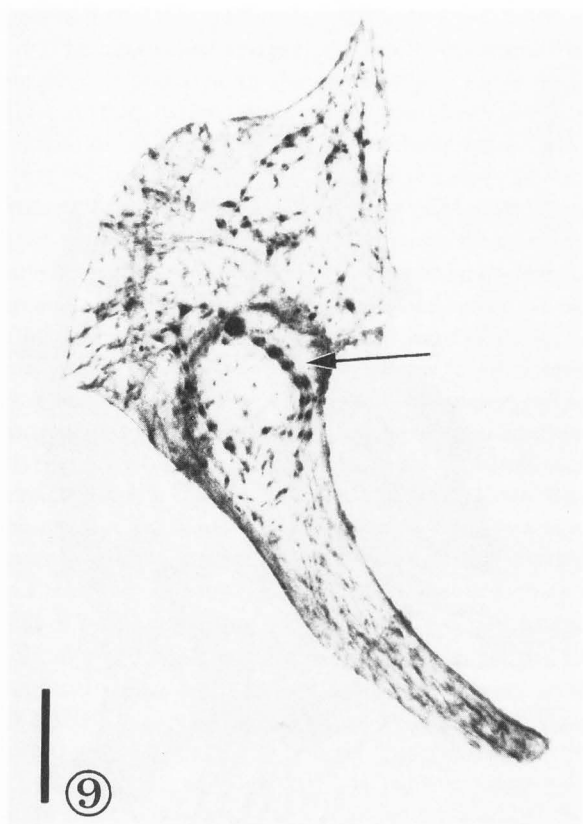
**Figure 9**. Confocal projection along the optic axis of a series of 13 fluorescence confocal images of a cell in a similar preparation, taken with a focal change of 0.48 $\mu$m between scans. Several curved structures are seen, with periodic fluorescence at a spacing of about 2 micrometers (arrow). These are identified as developing myofibrils. Pixel size 0.10 $\mu$m. Bar indicates 10 $\mu$m.

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A significant factor in our ability to relate images of the same cells taken using different microscopes to each other, and visually to identify with certainty the same structures in the different images, is the high contrast of the structures with respect to their immediate background as well as the relative absence of superimposed structure in the IVEM images, where all material shows up, not only that which has been stained with the fluorescent tag. We have shown one example (Figs. 9 and 10) where details of the correspondence was not clear, based on appearance in the two images, but the structures still could be identified by overall shape and by location in the cell. We have found many examples, in our studies, where the structural field was to complex and too cluttered with different classes of structures, especially in the IVEM images, to be able to identify with certainty which structures in the IVEM corresponded to fluorescent structures in the light micrographs.



**Figure 10**. IVEM image of the same cell as in Fig. 9 at the same magnification. While the curved structure indicated with the arrow in Fig. 9 can be found, and while it can be seen to curve around a nucleus in the cell, this structure has no apparent periodicity in this electron image. Bar indicates 10 $\mu$m.

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Clearly it would be valuable, in such cases, to exactly superimpose the two images, so that structures in the fluorescence image would lie directly over the structures in the same regions of the IVEM image, allowing for their identification. We have started to do this, but have not advanced far enough at this time to present the results. We need to precisely match images with respect to magnification, position, and orientation, and to compensate for shrinkage or other spatial distortions in one or another image, before this approach will bear fruit.

We are not unaware, of course, of the possibility of using antibodies tagged with, for example, gold particles, which can be imaged directly in the electron microscope and which can potentially provide the sort of chemical identification we need without the necessity of combining light and electron images. We consider our approach and the immuno-EM approach as complementary, and we plan to make comparisons between the two

approaches in the future. As is usually the case, we expect that having two solutions to the same problem will prove valuable, and that there will be specific instances in which each method proves to be superior to the other.

## Acknowledgments

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## Discussion with Reviewers

**M. Malecki:** Did you estimate the degree of shrinkage of cells exposed to dehydration and critical point drying? How is this going to affect attempts to superimpose images from LSCM and IVEM described in your future plans?

**Authors:** We have started to address the question of spatial distortions between confocal and EM images by comparing test specimens with easily identified and located fiducial objects, such as fluorescent-stained fluorescent beads, either alone on a film or combined with cell preparations. Shrinkage during dehydration is one possible source of such distortion, and would take place after confocal and before IVEM imaging. Optical distortion in one or the other microscope is the another. So far we have only evaluated the magnitude and nature

of the distortion, without trying to distinguish between distortion during preparative procedures and optical distortion, though we expect to do that in the more distant future. While the results are still preliminary, they are somewhat encouraging in that it appears that the residual differences between images after adjusting for magnification and orientation are small, and they may be correctable with a simple, affine transformation. However, we wish to leave the possibility of needing higher-order transformations as an open possibility for the future.

**M. Malecki:** In this project you were using the transmission electron microscope operating at 300-400 kV. Can you share your experience on using lower accelerating voltages to study structure of cell whole-mounts? Particularly interesting would be your comments concerning the beam damage of cell whole-mounts observed at various accelerating voltages.

**Authors:** We, and others, have in fact studied whole-cell mounts at lower voltages, as low as 100 kV. In general terms, the results in terms of image quality are good in the thinner parts of well-spread cells, and not as good where the cells are thicker, as one would expect. We have not systematically studied beam damage at different voltages, though the expectation would be that there would be less damage at higher voltages because of reduced inelastic scattering.

**M. Malecki:** In your other paper (Heath and Peachey, 1987) you were successful in imaging of muscles within 0.75 $\mu$m thick sections using an energy filtering transmission electron microscope (EFTEM) operating at 80-100 kV. What is your experience in studying cell whole-mounts with EFTEM?

**Authors:** Energy filtering electron microscopy at lower accelerating voltages could also be used in studies of whole cells such as we have reported here, though we have not tried it. As you point out, we have used an 80 kV energy filtering microscope to visualize sections of embedded cells up to 0.75 $\mu$m thick with good success (Peachey *et al.*, 1987), and this should work on at least the thinner parts of whole cell mounts. The limit on thickness that we encountered with sections in the EFTEM was that beyond about half micrometer in thickness, there was so much inelastic scattering at 80 kV that there were not enough electrons within a reasonable width of energy window to be able to form an image in an acceptable exposure time. Whole mounts of cells do not have embedding material, so thicker specimens should be acceptable, but probably not the region of a tissue culture cell close to the nucleus, which can be 10 micrometers or more in thickness.

**M. Malecki:** You provide an example of studies on myofibril assembly as the justification for this project. In such a case preservation of filaments is of utmost importance. Can you include images demonstrating preservation of filaments e.g. actin and myosin prepared according to the protocols you describe or provide comments concerning that preservation?

**Authors:** We have published some images, both from confocal microscopy and IVEM, of more mature cardiac myocytes prepared using the same protocol (Ishikawa *et al.*, 1990). These images show well-preserved myofibrils with essentially the adult form of banding and the usual arrangement of thick and thin filaments, indicating that the preservation of these filaments is satisfactory.

**J. Turner:** The critical point drying method has been largely abandoned, I believe for the wrong reasons, and it is nice to see it revived here. However, to ignore the fact that it is a controversial method does not serve the readers. If nothing else, more detail concerning the CPD method is essential and comments related to these details would be helpful.

**Authors:** We took the following precautions to keep the residual water in the specimens low during and after the CPD procedure. The absolute alcohol used was stored over molecular sieve, to remove water. The minimum amount of ethanol needed to cover the specimens was used in the CPD chamber at the start of the drying procedure, making its removal easier. The liquid carbon dioxide was of the "bone dry" classification from the supplier, but was not further dried by us. When carbon dioxide was introduced into the chamber, we watched (through a window in the top of the chamber) the swirling of the liquid and ran as much carbon dioxide through the chamber on each cycle as was consistent with not damaging the grids. The grids were held in a special holder that allowed good circulation of liquid around the grids, while still preventing them from becoming lost. This active, rinsing and stirring procedure lasted at least 5 minutes on each cycle, and was followed by a minimum of a 5 minute soak before the next cycle was initiated. In our experience, this protocol results in cells with good three dimensionality, which we check by stereo imaging of small cell processes such as ruffles or microspikes from the cell surface. Occasionally we do not see such good preservation of three dimensionality, and we suspect that this most often is due to a poor batch of liquid carbon dioxide, since a new tank usually solves the problem. It also is important to note that even though we immediately transfer the dried grids to a vacuum desiccator with phosphorous pentoxide for storage, and are careful not to expose the grids to the atmosphere any longer than necessary when loading them into the IVEM, within a couple of days the cells begin to have an altered appearance in which very fine structures are not crisply delineated. It is difficult to describe the appearance in a very distinct way, but is easily recognized with experience. We say that the cells have a "mushy" look, and we believe that this may be due to a small amount of water picked up by the specimens, since the problem seems to be worse in the summer than in the winter.

**J. Turner:** Do the authors have any experience or comments about a comparison of CPD with freezing methods?

**Authors:** No, I am afraid we don't.

**Reviewer III:** Does using a mounting medium of glycerol-water, and then removing the specimens in water, then fixing in 0.1 M cacodylate buffer have an effect on the structural integrity of the sample? In the removal process how do you avoid getting objective oil in the water mixture?

**Authors:** We do not see any obvious swelling of these cells, which previously have been permeabilized with detergent and fixed with glutaraldehyde, when we remove the cover glass using water after light microscopy and further fix in buffer. We conclude that these cells are no longer osmotically active. Still, this removal procedure must be carried out very slowly and carefully, or there can be mechanical damage to the cells and/or the support films. We clean the cover glasses with ether-ethanol or another solvent mixture to remove all the immersion oil before carrying out the cover glass removal procedure.

**Reviewer III:** The formvar film is highly hydrophobic and deteriorates with prolonged exposure to UV. Could you discuss alternative methods available for sterilization of the formvar film and how to make the film a better substrate for cell adhesion?

**Authors:** Our formvar films are thicker than what one usually uses for electron microscopy at 100 kV. The higher accelerating voltages that we use allow us to use thicker support films, and this makes the preparations more likely to survive the multiple handling steps we subject them to. This also may help to reduce the effects of UV sterilization, though we have not specifically investigated this point. Presumably one could sterilize these grids prior to plating the cells using chemicals, but we have not found it necessary to use any method other than UV irradiation. Contamination of cultures has not been a problem in these studies. We do modify the procedure for making the films when we have trouble with cell spreading or adhesion. If the cells do not spread well or do not adhere to the film, then we try to make the films more hydrophilic. First, films cast

on a water surface, rather than on glass, and then picked up so that the cells will be plated on the side of the film that was facing the water when the film was cast seem to aid cell spreading and adhesion. Second, glow discharge treatment of the grids also makes them more hydrophilic and thus improves cell adhesion and spreading. Occasionally, for some types of cells, the problem seems to the opposite, that the films are too hydrophilic, and in this case we plate the cells on the air-contact surface of water surface-spread films, which we believe to be more hydrophobic.

**Reviewer III:** Correlative microscopy is extremely powerful by the ability of gaining direct ultrastructural information on previously dynamic processes, however to date the limitations are the labor intensive manual process and the ability to unequivocally superimpose two images. Elaborate on the obstacles that must be overcome for computerized image analysis of correlative microscopy and the future of correlating 3-D data from CSLM and whole mount EM.

**Authors:** A complete discussion of these important points would more than double the length of this paper, and will have to wait for another occasion. We will try to respond briefly, however, and provide some references to other publications from our laboratory. Our initial approach to 3-D analysis of electron micrographs of thick specimens has been to accept that considerable human labor will be necessary, at least at first. We like to put a more positive spin on this by saying that the human eye-brain system is very powerful at 3-D recognition of classes of objects when seen in 3-D, as in the case of stereo pairs of EM images made by specimen tilting between exposures. Therefore we use this powerful recognition capability rather that to try to develop entirely computer-based recognition algorithms and programs (in fact, however, we also are doing the latter in parallel). In our initial approach to this, we use the computer to display multiple digitized IVEM stereo images and to generate 3-D cursors, which the operator can use to point to or trace structures that he/she has identified visually (Ishikawa *et al.*, 1990; Peachey *et al.*, 1995). The computer then uses information obtained from the placement of these cursors by the operator to calculate 3-D positions, sizes, and shapes of the selected objects, and further can generate graphic depictions of the objects so selected, located, and traced. The reviewer's request emphasizes the correlation, in 3-D, of this information from IVEM with parallel information obtained by confocal microscopy, and this is exactly the emphasis of our work at present. The confocal microscope gets 3-D information not as stereo pairs of images, but as serial optical slices through the volume of the specimen. 2-D projections can be calculated through these 3-D data sets, and software is provided with confocal microscopes to do just that. Pairs of 2-D confocal projections can be viewed stereoscopically, and then treated the same way we treat stereo pairs from the IVEM of the same specimens. Additionally, there are programs available for extracting 3-D models from serial section images (e.g., Young *et al.*, 1987). The future of correlating these two kinds of 3-D data sets from the two kinds of microscopes will depend on finding ways to merge these 3-D representations of the same structures extracted from the two kinds of images. It is relatively easy to think of ways of doing this by returning to 2-D representations, e.g. by making projections in the equivalent directions through the two extracted data sets (models, reconstructions, etc.), and then superimposing these projections for comparison. The two projections can be color-coded, and the merging, or lack of merging, of the colors will reveal what matches and what does not. Ideally, however, one would prefer to stay in the 3-D world of the reconstructions, and to merge these in some 3-D equivalent of 2-D superposition. Whether this can be done in a useful way, using color or some other form of coding to separate the two data sets in the mind of the observer, remains to be seen in the future.

### Additional References

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