Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 5251–5257, June 1995

Review

Rethinking cell structure

(electron microscopy/cytoskeleton/nuclear matrix/mitosis/cell membranes)

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ABSTRACT **Cell structure, emerging from behind the veil of conventional electron microscopy, appears far more complex than formerly realized. The standard plastic-embedded, ultrathin section can image only what is on the section surface and masks the elaborate networks of the cytoplasm and nucleus. Embedment-free electron microscopy gives clear, high-contrast micrographs of cell structure when combined with removal of obscuring material such as soluble proteins. The resinless ultrathin section is the technique of choice; it is simple and inexpensive, and it uses ordinary electron microscopes. The resulting pictures reveal a world of complex cell structure and function. These images necessarily change our conception of the cytoskeleton, nuclear matrix, mitosis, and the relation of membranes to cytostructure.**

The observational instruments of an experimental science often delineate its most fundamental ideas. In astronomy, every pivotal innovation in teloscopy literally recasts the cosmos: the simple Galilean telescope's discovery of Jupiter's moons was an unparalleled liberation from static, heliocentric heavens of crystalline spheres. Today, radio and γ-ray telescopes give us an unsettled universe of neutron stars and black holes. In like manner, biology owes many of its basic concepts to a single class of instruments that reveal the invisible. The microscopes of Leuwenhoeck and Hooke first showed cells, and our notions of cell structure have evolved with improvements in light and electron optical instruments. Given the profound importance of microscopy to cell biology thought, it is quite surprising that the most powerful magnifying instrument of all, the transmission EM, affords a very truncated view of reality.

Cell biology has long labored under a serious misperception of cell structure since conventional electron micrographs do not show most of the cell's architectural components. This is not an intrinsic technical weakness of electron imaging, however, and recent, very simple EM techniques give a far more accurate and detailed picture of cell structure. This review briefly explains the problem with conventional EM and how it is solved. Examples of the resulting EM images show that they differ profoundly from the customary picture of internal cell architecture.

Introduction of a more accurate means of visualizing cell structure is timely since the perceived roles of cell architecture are evolving rapidly. I believe the resulting conceptual transformation will prove apposite and, in Kuhn's wonderfully apt, if now lamentably clichéd, phrase, a "paradigm shift." An emerging school of research looks beyond current interests, largely grounded in solution biochemistry, to focus on cell structure, its design and organization, and, most important, its crucial roles in biochemical and developmental functions. As described here, EM can fully and accurately image cell structure, establish new concepts, and serve as a powerful adjunct to structure research.

Unfortunately, at times the study of cell structure has been demeaned as of merely observational interest and unfavorably compared with a supposedly more rigorous and reductionist biochemistry. Even if such denigration were ever justified, it is increasingly inappropriate and irrelevant; contemporary studies of cell structure are as disciplined and reductionist as any. Moreover, cell architecture is proving critical to understanding many differentiated cell functions (1–3), cellular form (4), cellular growth controls, neoplastic transformation, and a wide spectrum of gene regulation events (5, 6). Such thoroughly rigorous studies add cellular mechanics and plumbing to the more usual emphasis on chemistry. Only such research can discover the mechanisms of cell collaboration in constructing tissue patterns and organism form, subjects recalcitrant to the more conventional chemical approach. Ultimately, structure research will illuminate that most vexing and refractory of puzzles—the nature and location of the genomic instructions dictating the form of cells, tissue, and, ultimately, organisms.

A major impediment to the study of cell structure has been the understandable perception that there was simply not very much of it. The conventional electron micrograph of cells shows cells with little more of a framework than a marshmallow. The largely featureless interior strongly discouraged searching for organizing principles that, instructed by the genome, determine cell, tissue, and organism form. Despite its universality, the conventional EM image was realized as seriously incomplete. Paradoxically, optical microscopy could display elaborate, often dramatic cytoskeletal architecture (7–9) but at relatively low optical resolution. The far more powerful EM could show the cell interior only as formless "plasm" with organelles seemingly scattered about. There appeared no cell framework for determining cell shape, cell polarity, and the obviously nonrandom morphology and distribution of organelles. The nucleus showed no scaffold or matrix for organizing enormous lengths of chromatin. Also, the chromatin appeared only as dots rather than as the expected thick chromatin solenoids. Mitosis seemed a largely inexplicable, almost magical process. Nevertheless, despite concerns about accuracy, the embedded thin-section electron micrograph continued to be the norm. They do show some things extremely well—e.g., membrane profiles and cross-sections of organelle interiors.

Failure to image internal cell structure is not a fault of the EM. The "marshmallow" image is entirely due to the plastic embedding used in traditional EM samples to allow cutting ultrathin sections. The plastic-embedded section originated in the earliest days of EM when its shortcomings were not obvious and cells were considered extremely fragile. Today, embedding is often an obstacle to understanding.

EM of embedment-free samples reveals the cell interior comprising an astonishing array of complex structures. These elaborate and dynamic structural networks are present throughout the cytoplasm and nuclei in all cells and, I believe, should be

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Abbreviation: DGD, diethylene glycol distearate.

considered the true "cytoskeleton." Cytoskeleton, as a term, is often considered roughly synonymous with the three filaments most often imaged by fluorescence microscopy (microfilaments, intermediate filaments, and microtubules). These filaments are but a small part of the newly revealed cytostructure (perhaps 10–15% by biochemical measurement). Removing obscuring plastic and soluble proteins allows strikingly clear EM of the intricate filament armatures in the cytoplasm and nucleus. Research into cell structure need no longer struggle with the featureless marshmallow cell.

Why the Conventional Embedded EM Section Conceals Most of the Cell's Interior Structure

A brief reflection on elementary optical principles shows why the usual embedded section shows so little of reality. All microscopy requires that a sample differ optically (or electron optically) from its surroundings. Both light microscopes and EMs form a magnified image by focusing perturbations in the illuminating rays. The images in a bright-field microscope result from differential absorption affected by selective stains. Similarly, phase-sensitive optical instruments use interference to image the differential retardation of light by the sample compared with the surrounding milieu. Such interference instruments can be quite sensitive to subtle optical properties of the sample. Although biology textbooks often incorrectly state otherwise, the EM is primarily a phase-contrast instrument. Although not often thought so, it is an extremely sensitive instrument that can easily image relatively electron lucent objects, such as small caliber protein fibers, and needs no heavy metal staining.

The extreme sensitivity and resolution of the EM are completely subverted by embedding samples in a plastic resin that matches their electron optical properties. The atomic composition of plastics, which determines their electron index of refraction, is almost identical to that of biological samples. To an electron, the sample volume is now essentially homogeneous and it experiences little phase difference between the sample and its surroundings that could serve to form an EM image. Anyone who has, by oversight, tried to view an EM section without poststaining knows how blank the viewing screen will be.

Producing an image with an embedded sample requires "staining" the section so that heavy metal atoms bind to bits of sample protruding through the section surface. Only these surface atoms form the familiar EM image; nothing in the section interior is portrayed. Most organelles and fibers lie below the actual section surface where they remain invisible. Filaments in particular are difficult to image since they must lie in the plane of the section surface or else appear as a mere dot. Apart from the image of stain bound to those dense organelles and membranes penetrating the section surface, the cell interior appears largely formless. This technique of dense plastic embedding might strike us today as curious, but 40 years ago it served to make some sense from images otherwise hopelessly confused by the unexpected appearance of soluble proteins. The sections showed membranes very well, which were then of primary interest. The price exacted for this seeming clarity was the masking of what we now consider critical internal cell structures.

Embedment-free EM

About 15 years ago, Keith Porter (10) reintroduced a much older EM technique, one that viewed whole mounts of cells without any embedding material. The resulting images showed a dense, complex, polymorphic mesh that he termed the "microtrabecular lattice." The images seemed mysterious and could not be related to anything in orthodox micrographs; "artifact," used in its pejorative sense, was heard. Repeating Porter's studies was initially difficult since few had access to the necessary 1-MeV EM. The high voltage served to penetrate the dense microtrabecular lattice. However, a simple technical development has made a high-voltage microscope unnecessary; the ultrathin, resinless section allows viewing cells with a garden variety 80-kV microscope and achieves results equivalent to those of Porter.

Resinless sections are made by embedding the sample in a temporary medium and

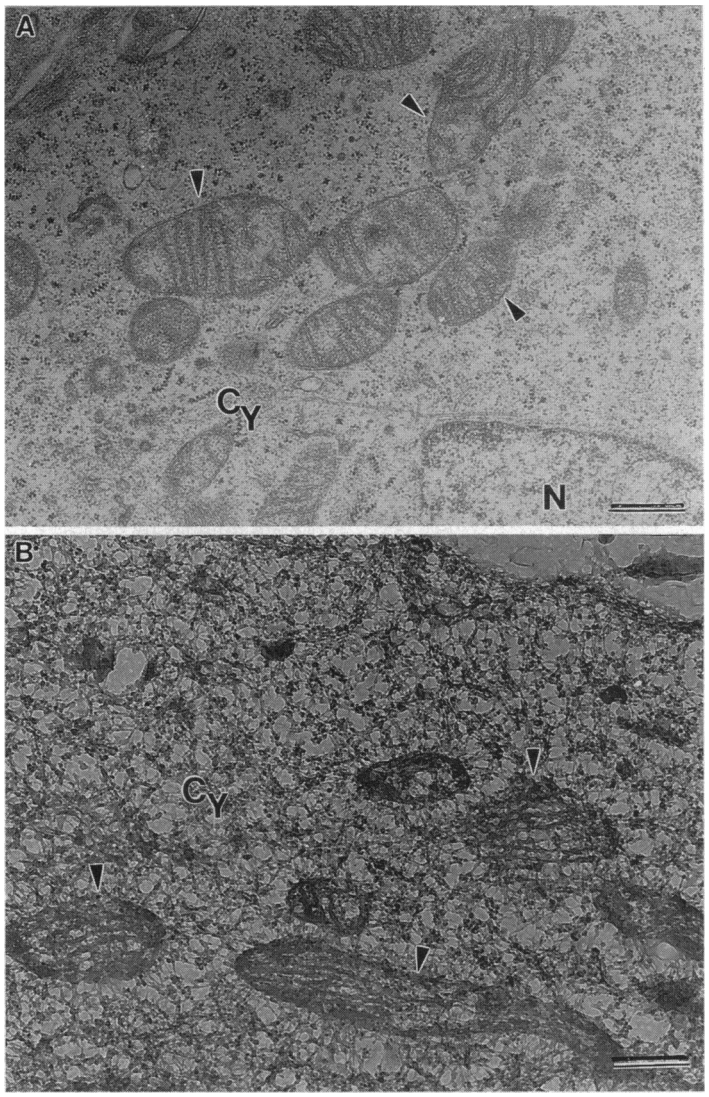

FIG. 1. Comparison of transmission electron micrographs of whole HeLa cell sections prepared through the conventional Epon technique (*A*) and the DGD resinless method (*B*). (*A*) Mitochondria (arrowheads) are seen "floating" in the cytoplasm (C_Y) of a HeLa cell that was embedded in Epon, sectioned, and conventionally poststained. N, nucleus. (*B*) Resinless section of a fixed HeLa cell that was initially embedded in DGD shows mitochondria (arrowheads) enmeshed in the cytoplasmic microtrabecular lattice that is revealed through this technique. (Bar = 100 nm.)

cutting sections on the ultramicrotome. The resulting ultrathin sections are placed on a grid, extracted with solvent to remove the temporary embedding medium, and then dried through the CO_2 critical point. Glutaraldehyde-fixed samples, even when extensively extracted, are remarkably tough and appear to maintain their form even when undergoing considerable manipulation. The resinless section technique was first developed by Wolosewick (11) using polyethylene glycol (PEG) as the provisional embedment. We found using PEG technically difficult and developed an alternative technology based on using diethylene glycol distearate (DGD) for interim embedding (12). Most find the DGD cuts far more easily than PEG and, since it is not water soluble, it can be floated in the usual water-filled microtome trough. Recently, we have adopted a modified form of DGD (Antibed; the kind gift of the EMCorp, Chestnut Hill, MA) that avoids some problems with brittleness.

Coping with the Newly Visible Soluble Proteins

Creating embedment-free sections is but the first step in unveiling cytoarchitecture. Now the soluble proteins, conveniently hidden within embedded sections for a half century, are visible. The contrast with conventional micrographs is striking but not very informative. Fig. 1*A* shows HeLa cell cytoplasm in a familiar, Epon-embedded, poststained section and Fig. 1*B* shows an unstained, resinless section of the same cell material. The ghostly trabecular network in Fig. 1*B*, present but invisible in Fig. 1*A*, consists of the soluble proteins cross-linked by the fixative into a stable structure. The soluble proteins are most of the cell mass and obscure organelles, such as mitochondria, which can be only dimly glimpsed. Their presence surely discouraged the early microscopists since, once visible, they effectively hid everything else. The soluble protein problem very likely led to adoption of the embedded section, because it at least showed membrane outlines.

With our increased knowledge of cell structure, we can now do much better than enshroud the soluble proteins in embedding plastic, thus covering up most of the cell's interior. Instead, we can selectively and completely remove the soluble proteins by extraction with nonionic detergent. The mechanism of the detergent extraction is often misunderstood; nonionic (in contrast to ionic) detergents do not affect proteins and so they do not "extract" in the usual sense of that term. Instead, the detergent simply dissolves membrane phospholipids, destroying the plasma membrane barrier and allowing soluble proteins to passively diffuse away. If the extraction buffer salts and pH are close to the intact cell's original interior milieu, the procedure leaves cytoskeletal structures intact and with nearly native morphology.

The detergent-extracted cytoskeleton, free of soluble proteins, is potentially ideal for both EM and biochemical studies. However, EM using conventional embedded sections is particularly poor for viewing the detergent-extracted cytoskeleton. The three-dimensional filament networks of the cytoskeleton are precisely the type of material that images most poorly in conventional sections. Fig. 2*A* shows the feeble picture of detergent-prepared cytoskeletal structures afforded by an Epon-embedded section. Portions of both the cytoplasmic region (upper right) and of the nucleus (lower left) are visible. Ribosomes are the most visible component in the cytoplasm due to the avidity of their RNA for poststaining uranyl ions. The conspicuous, nonrandom clusters of the polyribosomes reflect their binding to the cytoskeleton that, of course, is not visible here. Some ribosomes are associated with what may be remnants of the rough endoplasmic reticulum. The image of the nucleus is also uninformative, showing chromatin, mostly clumped, and little else.

Fig. 2*B* shows the striking, high-contrast cytoskeleton image afforded by an unstained, resinless section. The microtrabecular lattice of soluble proteins is, of course, gone, leaving an unobstructed view of the cytoskeleton. The profusion of cytoskeletal structures includes micro- and intermediate filaments, polyribosomes attached to the cytoskeleton, and myriad small structures not yet identified. The nuclear space shows thick, knobby

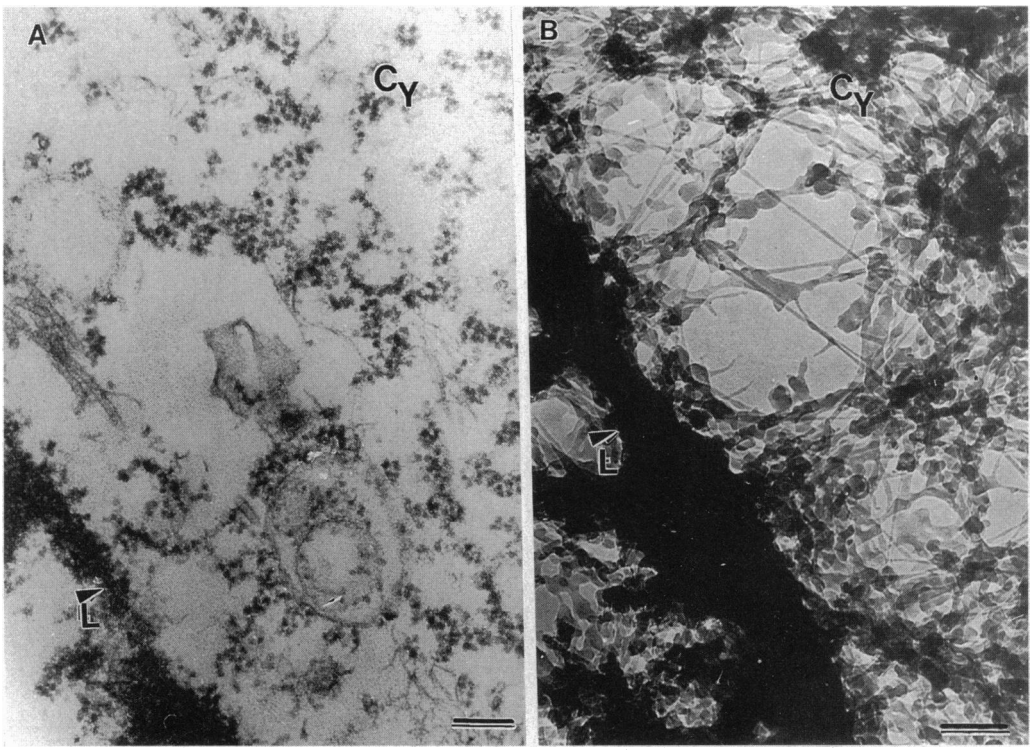

FIG. 2. HeLa cell cytoskeletal preparations as seen by conventional (*A*) and unembedded (*B*) EM. The resinless section shows, in contrast to the conventional one, the interconnected cytoskeletal filaments that span throughout the cytoplasm (C_Y) and are anchored on the nuclear lamina. (Bar = 100 nm.)

agglomerates of chromatin and thin fibers, previously unseen, which are nuclear matrix filaments.

The simple and effective nonionic detergent extraction reveals a highly intricate cytostructure that, unlike the structureless cell, appears suitable for carrying out complex architectural instructions. The resinless sections are three-dimensional objects and yield true stereoscopic images when viewed with a tilting stage. In three dimensions, the cell networks resemble "tensegrity" structures—i.e., built of elements that experience tension and compression but no bending. However, the detergent-extraction procedure does exact a serious price: solubilization of all cell membranes. We will see later that saponin extraction that selectively removes cholesterol from membranes, leaving much of the phospholipid, preserves a significant portion of the internal cell membranes.

The Core Filaments of the Nuclear Matrix

Selective extraction can do more than simply remove soluble proteins. With suitable modification, extraction can be used to peel away cell structures, enabling us to peer more deeply into the cell interior. This has been particularly useful in resolving the controversies surrounding the existence of the nuclear matrix. Few biological subjects in recent times have aroused such passionate dispute. Exactly why the idea of nonchromatin nuclear structure engendered so much contention will probably remain a puzzle. The existence of an organizing scaffold would surely seem compelled by the complexities of nuclear organization and behavior that hardly appear due to soluble components. Very likely the complete invisibility of the purported nuclear matrix in contemporary micrographs, even after chromatin depletion, helped incite apprehensions as to its reality (13).

The nuclear matrix is hidden under overwhelming amounts of electron-dense chromatin that must be removed before microscopy or biochemical analysis. Berezney and Coffey (14, 15) first detected the nuclear matrix biochemically as the nonchromatin nuclear material remaining after vigorous extraction of chromatin with DNase and high salt. This and similar preparations served in much important research and established the matrix as a participant in important nuclear events (16–23). The preparations were particularly useful for the early specific hormone receptor studies (18, 25, 26). However, the nuclear matrix remained invisible in the conventional electron micrographs, provoking further skepticism concerning whether the matrix really existed. Of course, invisibility was also a serious obstacle to further research.

The embedded EM section cannot image the nuclear matrix for the same reason that it cannot show the cytoskeleton; resinless sections are far better for this purpose. The need to remove the overwhelming amounts of electron-opaque chromatin is an added complication. Chromatin is bound tenaciously to the nuclear matrix and removing it requires relatively harsh procedures. Effecting an essentially complete elimination of chromatin while minimizing distortion of the matrix has been a long-standing technical goal. Fig. 3 shows resinless section images of our most recent preparation that we believe is the most native produced to date.

Fig. 3*A* shows the nuclear matrix filament network while Fig. 3*B* is a higher-magnification view. The matrix consists of a dense, anastomosing network of 10- to 13-nm filaments. We have suggested these be called "core filaments" since they are the innermost structures of the cell. Although they superficially resemble intermediate filaments, they share no epitopes with either lamins or intermediate filaments and so appear unrelated. Enmeshed in the filaments are many dense bodies, some of which are rich in RNA splicing components.

The identification, localization, and characterization of nuclear matrix components are just beginning. An important impetus comes from finding nuclear matrix proteins that are cell-type specific (27–29) and others that are markers of malignancy (30–32). Direct measurements of transcription show nuclear matrix participation in its regulation (5). These results suggest a role for matrix proteins in determining gene expression.

Many antibodies have been created that specifically immunostain nuclear matrix proteins. Many, although not all, of these antibodies give a speckled immunofluorescence staining pattern as shown in Fig. 4*A*. Fig. 4*B* shows that immunogold staining with the same antibody decorates the dense bodies enmeshed in the core filament network. The "speckles" clearly correspond to some but not all dense bodies. Several anti-nuclear matrix antibodies, such as B1C8 shown here, bind to proteins in spliceosomes, suggesting that the dense bodies are related to RNA splicing.

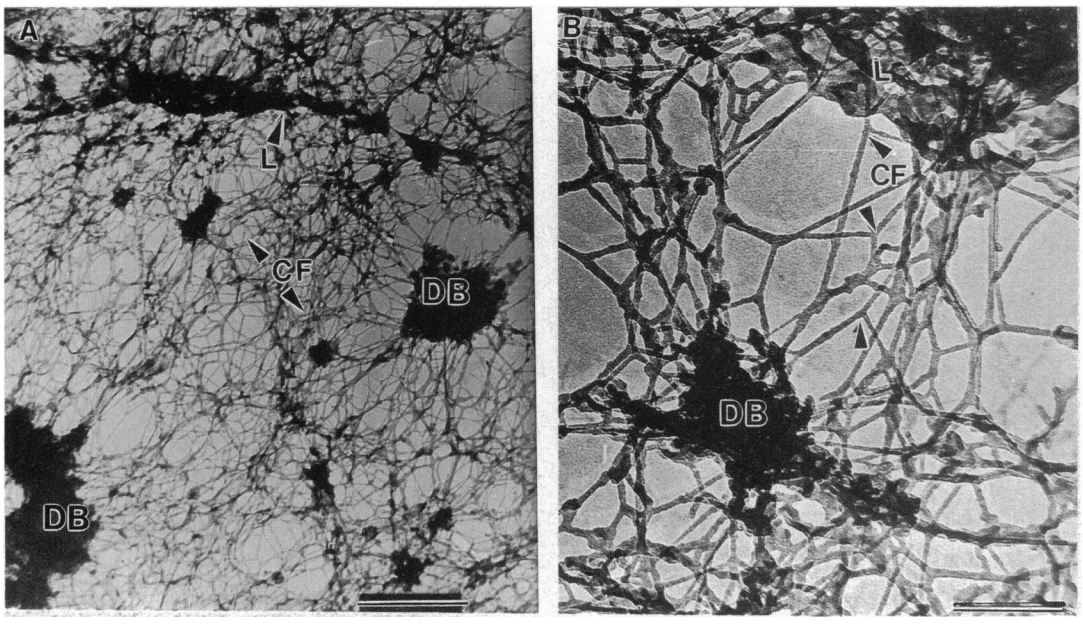

FIG. 3. Embedment-free ultrastructure of nuclear matrix core filaments. Micrographs show at low (*A*) and high (*B*) magnification the interrelations between the nuclear matrix components. The anastomosing network of the core filaments (CF) enmeshes electron-dense aggregates that form the dense bodies (DB) and connects with the nuclear lamina (L). (*A*, bar = 500 nm; *B*, bar = 200 nm.)

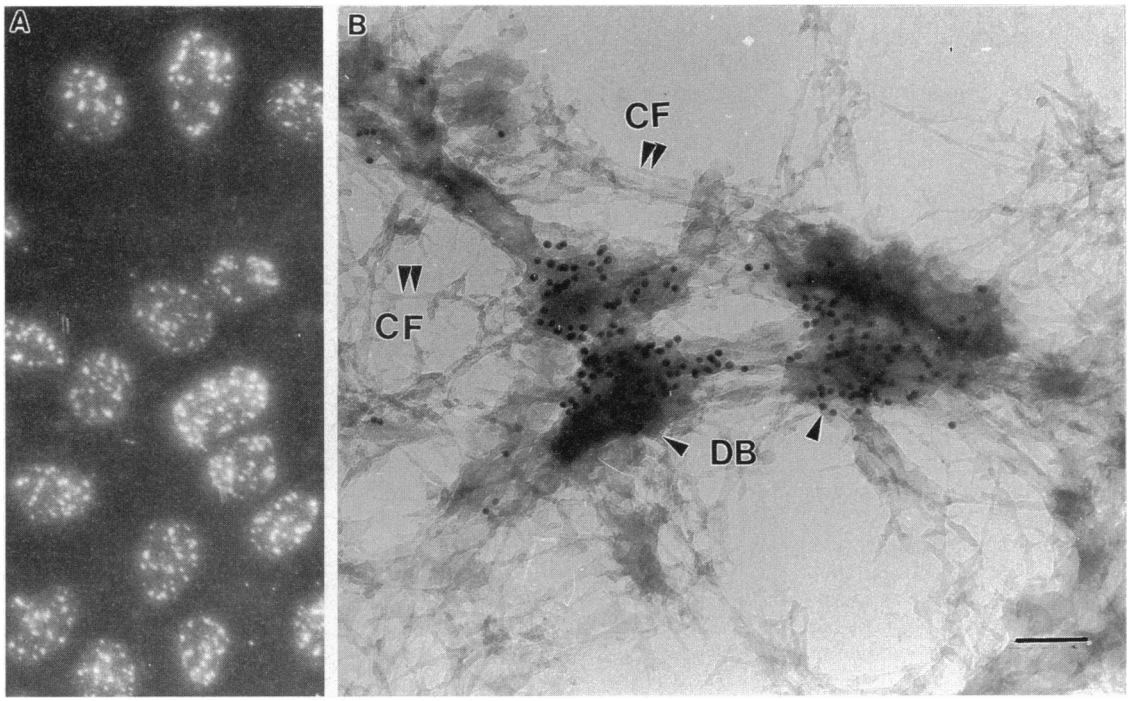

FIG. 4. Immunostaining of core filament preparations. (*A*) Immunofluorescent image of Caski cell nuclei stained with B4A11 monoclonal antibody. Speckled staining pattern is further revealed by immunogold resinless EM of the core filament nuclear matrix (*B*). Dense bodies (DB) are decorated with 10-nm immunogold and are enmeshed in core filament (CF) matrix. (Bar = 100 nm.)

Cell Structure at Mitosis

The rearrangements during cell division are surely the most profound in a cell's repertoire. While the biochemistry unique to mitosis has been an active and fruitful area of research, little has been adduced concerning cell architecture at mitosis. Apart from studies of microtubule functioning, most important questions about mitotic cytostructure remain. These puzzles include the mysterious agents effecting chromosome movement before the spindle forms, the machinery directing spindle fibers precisely to the kinetochores, the mechanisms for nuclear dissolution and reformation, etc.

Conventional embedded-section EM can only hint at the profound rearrangements of the cytoskeleton and nuclear matrix at mitosis. When the nuclear lamina disappears at late prophase, the cytoskeleton and nuclear matrix meld into a new, cell-wide structure until now mostly unseen. At best, the embedded section suggests only a few large components of the mitotic apparatus such as the chromosomes, short stretches of spindle fibers, and the occasional centriole.

The resinless section displays a new world of mitotic cell cytostructure. Only the soluble proteins need be extracted; there is no longer a need to extract chromatin since it has been compacted into chromosomes. Space limitations prevent a comprehensive presentation here but the metaphase plate in Fig. 5 shows the unique information the technique affords. Compared with the vague, shadowy suggestions of conventional micrographs, the filament and spindle fiber networks emerge here as sharp, well defined, and far more complex than previously suspected.

A complex filament network enmeshes the metaphase chromosomes in Fig. 5, filaments that are mostly invisible in conventional electron micrographs. The dense filament bundles extending horizontally from the chromosomes are the spindle fibers that, when lying in a thick-section plane as here, appear in their entirety. Especially arresting are the many thin filaments interconnecting chromosomes and coupling spindle fibers. These may originate in the core filaments of the interphase cell but firm identification awaits the development of core filament-specific antibodies. The major import of these pictures is that, rather than floating in an amorphous sea, the major elements of

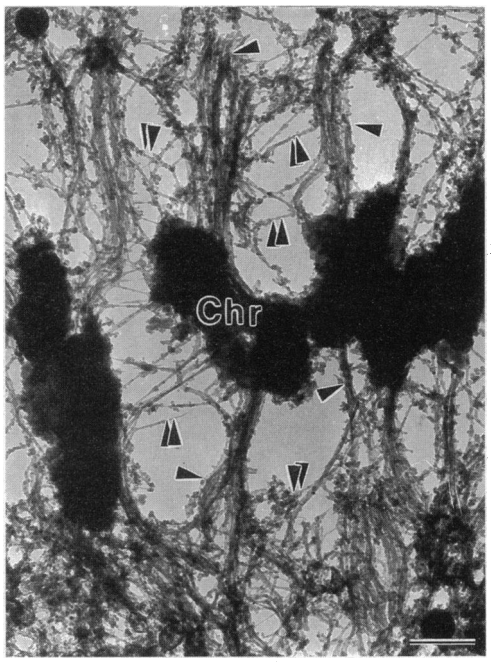

FIG. 5. Resinless electron micrograph of a mitotic HeLa cell. Chromosomes (Chr) are shown "suspended" on spindle fibers (arrowheads), which are interconnected through numerous thin filaments (double arrowheads). (Bar = 500 nm.)

the mitotic cell are part of a complex skeletal network that underlies the mechanical events of mitosis.

Membranes in Resinless Sections: Extraction with Saponin

The absence of cell membranes is a shortcoming of the preparations for resinless sections shown so far. This is not an intrinsic weakness of the technique but results from the detergent extraction of all phospholipids to remove soluble proteins. Saponin, substituted for Triton X-100, punctures the plasma membrane by preferentially extracting cholesterol (24). This weak detergent leaves much of the plasma membrane phospholipid bilayer intact while allowing the soluble proteins to diffuse away from the cell.

Fig. 6 shows mitochondria and other membrane-bounded cellular organelles enmeshed in the cytoskeleton in saponin-extracted cells. The mitochondrial cristae appear as dark zebra stripes inside the organelle. Cytoskeletal filaments anastomose with the membrane surfaces of the organelles. This picture suggests that the usual view of membrane-enveloped organelles as structurally autonomous entities is incomplete. The resinless section micrographs intimate that cytoskeleton filaments may serve as organelle frameworks enveloped by the lipid bilayers as canvas covers the framework of a tent. In this view, the cytoskeleton plays a more central role in organelle structure than has been appreciated. Such a role could explain the often complex morphologies of membranes and organelles.

Rethinking Cell Structure

Resinless microscopy affords images that lead to some radical conclusions. First, and probably most important, the resinless section can be enormously powerful but only if combined with removing obscuring material. No single method is completely satisfactory for all purposes. The soluble proteins, which confounded early embedment-free microscopy, are most easily extracted with nonionic detergent but at the cost of losing all internal membranes. Extraction with saponin effects the same removal while leaving much but not all of the internal membrane structures. The saponin preparation offers unique possibilities for deeper study of membrane cytoskeleton interactions. Removing tenaciously bound chromatin while preserving a nearly native nuclear matrix has been far more challenging. The core filament nuclear matrix preparations have been our most successful effort to date.

A second important point is that cell structures are remarkably tough. They can retain their detailed morphology through extraction, embedding and deembedding, sectioning, and critical point drying. I suspect one reason for originally adopting embedded sections was a belief in the ephemerality of cells once removed from tissue. The concern was doubtless justified in the early days before autolysis was appreciated and controlled.

Finally, the resinless section techniques are especially simple; most microscopists find them far less difficult than preparing the conventional ultrathin Epon sections. They should not be a technical obstacle to those interested in adopting these procedures.

The images revealed by the techniques described here compel serious reexamination of what we believe a cell to be. The old view, which might be oversimplified as a "fluid" model, is of moderately viscous "plasm" bounded and contained by a plasma membrane composed of a lipid bilayer into which proteins are inserted. The new idea is that the cytoskeleton serves as a solid framework or armature with an outer boundary of a protein sheet with lipids inserted. This framework is anchored on a second protein sheet or lamina that is the surface of the nucleus and bounds a separate framework, the nuclear matrix. Both nuclear and cytoplasmic frameworks probably conform to the definition of tensegrity (33) since most biological fibers can be strong in tension and even in compression but very weak in bending.

Many specific questions of cytoskeleton and nuclear matrix function can now be examined in light of our deeper insight into cell structure. In addition, more pro-

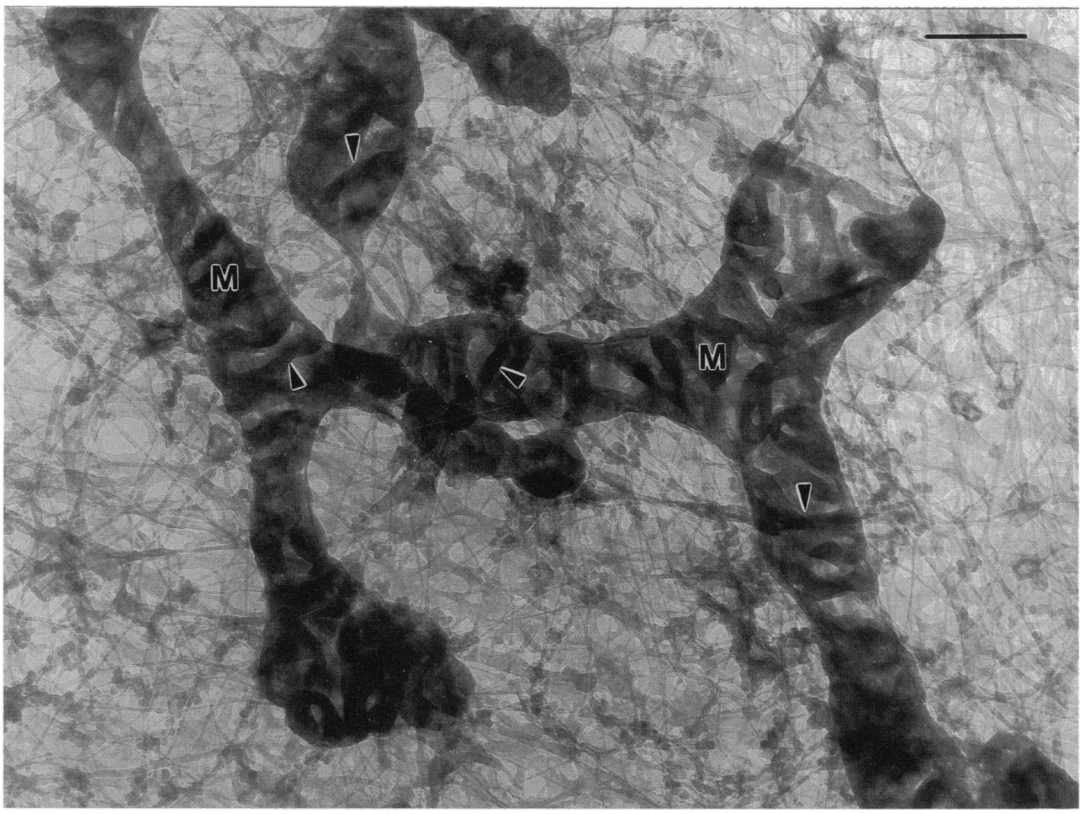

FIG. 6. Ultrastructure of a smooth muscle cell from rat aorta after saponin extraction. Whole-mount electron micrograph shows mitochondria (M) intimately connected with the cytoskeletal framework. Arrowheads point to mitochondrial cristae. (Bar = 200 nm.)

found intellectual questions are posed by the intricate structural networks now seen in cells—e.g., where are the complex instructions organizing the elaborate networks that underlie architecture? Like astronomical dark matter, the coding for architectural information seems invisible and largely ignored although its effects are everywhere evident. The subject could be disregarded when cells appeared lacking much structure. Once we add a wholly new spectrum of architectural complexity to genome encoding, we can no longer ignore the >90% of the vertebrate genome that does not directly instruct protein synthesis. This "extra" DNA has long been vexatious and seemingly illogical in properties. Despite its often intense transcriptional activity, it is frequently dismissed as "junk," a designation that may be intellectually soothing but hardly helpful scientifically. Molecular cell biology can scarcely aspire to conceptual completeness while ignoring the vast majority of organismic DNA.

Form and structure are not natural subjects for biochemistry that, in the macroscopic world, deals with scalar quantities—i.e., amounts, rates, etc. Building the complex designs glimpsed in any anatomy or physiology text requires, at the very least, instructions that are vectorial—i.e., that specify direction and place. These instructions are encoded somewhere—it seems very likely that they reside in the heavily transcribed but "non-protein coding" DNA. Building staggeringly complex organs—e.g., brains or kidneys—by simply specifying the constituent protein components (as suggested by the more extreme formulations of molecular biology that genes are simply proteins) is unlikely. Such a strategy would be tantamount to trying to specify a bridge or an edifice by merely giving a list of parts. Indeed, *Gray's Anatomy*, seen with an engineer's eye, suggests that the complexity of the instruction sets for mammalian morphology require large regions of the genome: very likely much or most of the currently ignored, non-protein coding, 90% (or more) of the genome. I suspect that future cell scientists will marvel at the density and ingenuity of genome instructions for structure while wondering how we could overlook them for so long.

EM was made possible by the remarkable technical skills of Gabrielle Krockmalnic. Katherine Wan conducted much of the difficult biochemical measurements. Jeffery Nickerson conducted several of the important studies but, most importantly, has been a constant source of stimulation and wise reflection. It is not possible to credit all who have contributed to these thoughts but among them are Alice Fulton, Gary Stein, Laura Manelides, Don Coffee, and many others. This work was supported by grants from the National Institutes of Health (CA45480-08, CA67628-28, and AR42262-01).

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