Recent structural insight into mitochondria gained by microscopy

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Abstract

Novel applications of microscopy have recently provided new insights into mitochondrial structures. Diverse techniques such as high resolution scanning electron microscopy, transmission electron microscopy, electron microscope tomography and light microscopy have contributed a better understanding of mitochondrial compartmentalization, dynamic networks of mitochondria, intermembrane bridges, segregation of mitochondrial DNA and contacts with the endoplasmic reticulum among other aspects. This review focuses on advances reported in the last five years concerning aspects of mitochondrial substructure or dynamics gained through new techniques, whether they be novel microscope methods or new ways to prepare or label specimens. Sometimes these advances have produced surprising results and more often than not, they have challenged current conceptions of how mitochondria work. © 1999 Published by Elsevier Science Ltd. All rights reserved.

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1. Introduction

1.1. Mitochondria: more than just the cell’s power plant

One of the most significant differences between prokaryotes and eukaryotes is the possession of mitochondria. Indeed, mitochondria are believed to be derived from bacteria engulfed by primitive eukaryotes hundreds of millions of years ago. The major function of
these organelles (although by no means the only one) is the synthesis of ATP (adenosine triphosphate) from ADP (adenosine diphosphate) and inorganic phosphate by the process of oxidative phosphorylation. In eukaryotic cells, oxidative phosphorylation occurs only in mitochondria. Every cell in humans contains between 5 and 2000 mitochondria. Their overall shape is variable. In many instances, they have a fairly regular shape, either spherical or cylindrical. Spherical mitochondria, for example found in liver, generally vary from 0.5–5 μm in diameter. Simple cylindrical mitochondria tend to be at least 0.2 μm in diameter and up to 20 μm long. However, highly branched mitochondria and mitochondria exhibiting specialized shapes, such as annuli, discs or cup-shapes have been documented (Munn, 1974). Such shapes increase the surface area to volume ratio, and may enhance the exchange of metabolites between mitochondria and the cytosol. Mitochondria consume over 80% of the oxygen we breathe and make over 90% of the energy our cells use. They use oxygen to obtain energy by oxidizing hydrogen rich molecules in food. This process converts food into ATP, water and carbon dioxide. ATP is the energy currency used universally by life on earth. Without ATP, muscles could not contract and neurons could not fire. Mitochondria literally make it possible to move and to think. Clearly, an understanding of the structure and operation of mitochondria and their interaction with other cellular components is essential to appreciate the success of eukaryotes, from one-celled organisms to the complexity that defines humans.

In order to appreciate how mitochondria are more than just the cell’s power plant, it is important to grasp the diversity of mitochondrial function, and how defects in function can lead to disease. Mitochondria in different organisms and tissues differ dramatically in their consumption of oxygen and production of ATP. For example, heart mitochondria consume 50 times the oxygen that liver mitochondria consume because of the high-energy demand in the continuous pumping of blood. However, liver mitochondria have adopted a different task and are specialized to detoxify ammonia, a waste product of protein metabolism, by enzymatic action. Mitochondria also differ in the fuels they can utilize. For example, heart mitochondria depend primarily on fatty acids to meet their energy needs. In contrast, liver mitochondria use both sugars and fats. It is this diversity of tasks, which reflects the observed diversity of mitochondrial structure that makes mitochondria integral to cell activity.

The motivation to study how mitochondrial function relates to structure has recently intensified because defects in function have now been linked to many of the most common diseases of aging. Among these are Alzheimer dementia, Parkinson disease, type II diabetes mellitus, stroke, atherosclerotic heart disease and cancer. Even such autoimmune diseases as rheumatoid arthritis, multiple sclerosis and systemic lupus erythematosus manifest mitochondrial components. While it cannot yet be stated that mitochondrial dysfunction causes these diseases, it is clear that mitochondria are intimately involved because their function is measurably disturbed, and sometimes gross morphological changes are observed. Even though, the causative link of mitochondria in these diseases has not been substantiated, the role of mitochondria as an orchestrator of apoptosis (programmed cell death) has now been firmly established (Green and Reed, 1998).

1.2. Mitochondria and cell death

Not only are mitochondria pivotal in fueling cell life, but they are also pivotal in controlling cell death. The role of mitochondria in apoptosis has generated a flurry of recent activity utilizing microscopy to endeavor to detect structural alterations that may provide a glimpse at the mechanisms of apoptosis triggered by mitochondria. How do these organelles initiate programmed cell death? At least three general mechanisms have been proposed, and their effects may be interrelated (Green and Reed, 1998). They are: (1) release of proteins, such as cytochrome c, that trigger activation of caspase family proteases; (2) disruption of electron transport, oxidative phosphorylation and ATP production; and (3) change in cellular redox potential. Participation of pro- and antiapoptotic Bcl-2 family proteins in these mechanisms remains a fertile ground for discovery, including the use of immunomicroscopy to complement the biochemical assays. In certain apoptosis scenarios, the potential across the mitochondrial inner membrane (∆Ψm) collapses; this indicates that the permeability transition (PT) pore, a nonspecific high conductance channel, has opened. However, cytochrome c release and activation of caspases can occur before any detectable loss of ∆Ψm, implying that PT pore opening may occur downstream of caspase activation. Alterations in mitochondrial ultrastructure may accompany the release of cytochrome c, and perhaps other activators such as apoptosis-inducing factor and intramitochondrial caspases. Two mechanisms have been proposed. One involves osmotic disequilibrium, which would sequentially expand the matrix space, rupture the outer membrane and release these caspase activators. The other envisions opening of channels or transient holes in the outer membrane that would release cytochrome c without concomitant swelling. Electron microscopy may provide clues as to how general these two mechanisms are, and whether only a few mitochondria need to release their caspase activators to cause cell death, or whether the great majority is involved.

1.3. Thrust of mitochondrial structural research

In this decade, the thrust of mitochondrial research involving microscopy has progressed along two lines. One, motivated by observations described above, is to attempt to understand the different signals that converge on mitochondria, whether in normal processes such as apoptosis, or in diseases. Tried-and-true microscopic techniques are employed in these endeavors where the novelty is in probing
structural changes based on biochemical stimuli. The other thrust is the use of novel microscopy on “normal” mitochondria to try to tease out structural features that were either not visualized or not fully appreciated with the techniques available to early investigators. These novel techniques, which include three-dimensional (3D) deconvolution light microscopy, high-resolution scanning electron microscopy (HRSEM) and electron microscope tomography, are providing new and sometimes surprising answers to the questions of mitochondrial compartmentalization and interactions with their nearest neighbors. This review focuses on the latter thrust, which was aptly described by one contributor to the field as “new views of an old organelle” (Mannella et al., 1997).

2. Historical perspective

2.1. Baffles or septa?

To understand what constitutes a “new view”, a brief recapitulation of the controversy concerning mitochondrial structure that occurred during the formative years of cell biology as a field may provide perspective as to how the currently displayed models of mitochondria found their way into modern textbooks (Rasmussen, 1997). Models of the structure of mitochondria came principally from the transmission electron microscope (TEM). Views of the internal structure originated in the 1950s with Palade (1952) and Sjostrand (1953) and their colleagues, who interpreted the 3D architecture of the multiple, membrane-bound compartments from electron micrographs of sectioned tissue. They found that mitochondria have two distinct membrane systems, the outer membrane and the inner membrane. According to Palade, the inner membrane protrudes into the mitochondrion interior in a “baffle-like” manner, but does not go all the way across and connect to the opposite periphery; i.e. it does not form septa. The leaves or lamellae that form the baffle were dubbed cristae, and were aligned more or less orthogonally to the long axis. The baffle arrangement meant that there was a large opening on the side of the lamellae facing the periphery. Fig. 1 shows the salient features. This model of cristae fit well with the opinion of contemporary biochemists that many of the mitochondrial enzymes involved in oxidative reactions must be supported in some definite spatial relationship. Palade thought that the inner membrane baffles might bear these enzymes in the proper order in linear chains analogous to an assembly line. This view is consistent with observations that mitochondria from highly active tissues, e.g. bird flight muscle, typically have large numbers of tightly packed cristae, whereas mitochondria in organisms living in an almost anaerobic environment, e.g. tapeworms, contain few cristae. In contrast to Palade’s model was Sjostrand’s view, that the cristae are actually a stack of independent membranous lamellae, which were referred to as “septa”. This view held that with few exceptions, there was no continuity between the cristae and peripheral membranes.

How could two such disparate models be proposed from similar electron micrographs? Part of the difference arose...
because of the necessity of inferring 3D organization from TEM images of thin sections, which provide projection images that are inherently 2D. The early debate centered on who had superior-quality micrographs and on the issue of specimen preservation. Both groups acknowledged that Sjostrand’s pictures were better, but a debate ensued over the best preservation techniques. Sjostrand believed that it was safest to maintain tissue in a physiological state until the moment of fixation. Thus, he injected live animals with fixatives, excised tissue rapidly and immersed it in fixative prepared in phosphate-buffered saline that was adjusted to physiological pH and salt content. Palade made no attempt to employ a physiologically compatible vehicle for preserving tissue because he did not think it was important. It is an interesting aside that Sjostrand’s and not Palade’s views on tissue type examined, with the exception of brown fat. They interpreted from biochemistry to morphology; for Sjostrand, interpretation flowed the other way. Naturally, Palade’s approach appealed to the biochemists of the time. Also, Sjostrand (1956) conceded a little ground in 1956 by admitting that in “isolated cases” cristae are continuous with the inner bounding membrane. By the late 1950s, Palade had moved on to other interests, but Sjostrand directed a meticulous set of 3D reconstructions made by stacking projection images of serial thin-sections through mitochondria. From these, it was noted that narrow stalks might connect cristae to the bounding membrane. However, Sjostrand qualified this feature by stating that these stalks rarely appeared in sections of good preparations, and that there was no fluid in them. For biochemists of the era, whether Palade’s folds or Sjostrand’s septa accurately described cristae was less important than the conceptualization of a membranous framework to support ordered chains of enzymes. Both models support this framework concept and it was not until the acceptance of Mitchell’s chemiosmotic theory of oxidative phosphorylation, proposed in 1961 (Mitchell, 1961), that Palade’s model prevailed. It was easier to accommodate Palade’s model with chemiosmosis, although the wide-held notion that Sjostrand’s model is incompatible with Mitchell’s theory was never substantiated.

Daems and Wisse (1966) reported that cristae attach to the inner boundary membrane by small tubes, which were called pediculi. This study involved careful serial sectioning of well-preserved and stained mitochondria, and the resulting model could be thought of as an improvement on Sjostrand’s model. The pediculi model received attention in books focusing exclusively on mitochondria in the 1960s and 1970s, but was generally ignored in textbooks. Pediculi seemed to have been forgotten until 1994, when two seminal papers were published in a special issue of Microscopy Research and Technique (Lea et al., 1994; Mannella et al., 1994). These papers focused on new views of mitochondrial structures afforded by recent technical advances in HRSEM and electron tomography, respectively. The HRSEM work showed that tubular cristae, and not lamellar cristae, predominate in many tissue types in higher animals, while the electron tomography work confirmed the pediculi concept, and extended the topological mapping of two mitochondrial membrane systems. Key to these new views was the improved 3D resolution provided by techniques not available to earlier investigators. The remainder of this review highlights new insights on mitochondrial structure obtained by novel applications of microscopy.

2.2. Which model prevailed?

To understand why Palade’s model gained acceptance, instead of Sjostrand’s, one need look no further than the manner used to interpret micrographs. Both agreed almost completely about what constitutes a good micrograph. However, for Palade, interpretation flowed from biochemistry to morphology; for Sjostrand, interpretation flowed the other way. Naturally, Palade’s approach appealed to the biochemists of the time. Also, Sjostrand (1956) conceded a little ground in 1956 by admitting that in “isolated cases” cristae are continuous with the inner bounding membrane. By the late 1950s, Palade had moved on to other interests, but Sjostrand directed a meticulous set of 3D reconstructions made by stacking projection images of serial thin-sections through mitochondria. From these, it was noted that narrow stalks might connect cristae to the bounding membrane. However, Sjostrand qualified this feature by stating that these stalks rarely appeared in sections of good preparations, and that there was no fluid in them. For biochemists of the era, whether Palade’s folds or Sjostrand’s septa accurately described cristae was less important than the conceptualization of a membranous framework to support ordered chains of enzymes. Both models support this framework concept and it was not until the acceptance of Mitchell’s chemiosmotic theory of oxidative phosphorylation, proposed in 1961 (Mitchell, 1961), that Palade’s model prevailed. It was easier to accommodate Palade’s model with chemiosmosis, although the wide-held notion that Sjostrand’s model is incompatible with Mitchell’s theory was never substantiated.

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3. What have we learned lately?

New insights into mitochondrial structure have been realized with such diverse techniques as HRSEM, electron tomography, wide-field light microscopy and confocal microscopy (see Table 1). The excitement of discovering new facets of mitochondrial structure has initiated a renaissance in imaging mitochondria, leading a new group of structural biologists to re-examine a question which was thought to have been answered. Only new results from “normal” mitochondria will be treated here. The structural alterations found in diseased mitochondria or through the action of drugs are beyond the scope of this review.

3.1. HRSEM

When describing mitochondria, one usually emphasizes the structural features that are common among all or most mitochondria along with a brief mention of important variations. However, recent HRSEM work emphasized the importance of structural variety. Lea and Hollenberg (1989) and Lea et al. (1994) challenged the prevailing view that cristae in higher animals are invariably lamellar. By using 3D stereo HRSEM, they showed the existence of tubular cristae about 30 nm in diameter in every mammalian tissue type examined, with the exception of brown fat. They were one of the pioneers to point out the inadequacy of serial-section reconstructions in delimiting cristae morphology, because the limited z-resolution does not allow for a reliable mapping of the convoluted membrane topology. The discovery that tubular cristae are common in higher animals was a significant link to the bacterial origin of mitochondria. The main argument against this link had been the observation that tubular cristae predominate in lower eukaryotes, which were thought to correspond to similar tubes found in purple bacterium, believed to be the organism from which mitochondria evolved. Lamellar cristae were believed to be the only form in higher animals, and
hence, likely did not develop from the tubular form. However, the existence of tubular cristae in mitochondria of higher animals (see Fig. 2) removed this argument against the link between mitochondria and purple bacteria. Furthermore, the observation that striated muscle mitochondria contain cristae tubes and lamellae in varying proportions, and that brown fat mitochondria contain only lamellar mitochondria led to the hypothesis that cristae conformation is a direct consequence of the specialized function of the respective tissue. For example, the lamellar cristae may be a consequence of the heat-producing aspect of brown fat.

A recent technical advance in HRSEM allowed new insight into the diversity of the structure of human muscle fiber mitochondria that was previously unimagined. The novel technique was to couple osmium-hydrazine impregnation with a well-known aldehyde-osmium-DMSO-osmium (A-ODO) procedure (Ogata and Yamasaki, 1997). The A-ODO exposed large areas of mitochondria, and the impregnation permitted examination without metal coating with the potential for increased resolution. Using ultra-HRSEM, it was found that mitochondrial morphology varied significantly between white, red, and intermediate muscle fibers. Mitochondrial shape and configuration were simplest in white fibers; paired long thin mitochondria encircled myofibrils at the level of the I-band. In contrast, mitochondria in red fibers were stubby, and often were connected by a slender stalk across the A-band to the next row of mitochondria. Mitochondria in intermediate fibers resembled those in red fibers, but were thinner and longer and possessed a slenderer stalk. This architecture of mitochondria in human muscle fiber types was markedly different from that found in rat muscle types, which was considered the architypal mammalian muscle. The take-home-lesson from these recent HRSEM studies is that the diversity of mitochondrial shape and placement may permit these organelles to respond to different cellular environments. We might take this suggestion one step further and ask, “can mitochondria in different regions of the same cell show diversity of structure that is physiologically relevant, such as might occur when a calcium wave propagates through a cell?” One can imagine that a calcium wave might induce a change of inner membrane conformation, since calcium can affect the energized state of this membrane. A transition from condensed (shrunken or condensed matrix volume) to orthodox (expanded matrix volume) conformation, as defined by Hackenbrock (1968),...
and used subsequently to delimit inner membrane conformation in relation to energy states of mitochondria described in literature, might be instigated by such a wave.

Using HRSEM, Senda and Yoshinaga-Hirabayashi (1998) showed that three kinds of intermembrane bridges exist within the mitochondria that may play a role in the structural integrity of the two-membrane systems. One bridge connects the outer and inner boundary membranes. The other two bridges link adjacent cristae membranes, either across the intracristal space or across the matrix. They used a quick-freeze, deep-etch technique with a wide variety of tissue types and with both chemically fixed and fresh specimens to demonstrate that these bridges are a consistent feature that are likely to occur in vivo. It was proposed that the bridges across the outer and inner boundary membranes might keep these membranes apart and thus maintain the intermembrane space, or perhaps to prevent its dilation. The intercristae bridges might help support the orientation of the cristae.

3.2. Electron tomography

Electron tomography is a powerful technique that is closely related to computerized axial tomography used by CAT-scanners in radiological imaging, in that computational methods are used to calculate a 3D structure from many 2D images or projections recorded over a wide range of tilt angles. The first use of electron tomography with thick-sections of mitochondria described improvements in the 3D visualization of the interior of rat liver mitochondria (Mannella et al., 1994) over serial-section reconstructions and HRSEM. It was emphasized that because HRSEM can only image-exposed surfaces, full 3D information about individual mitochondria could not be obtained. The limitation with serial-section reconstructions from thin sections is the low z-resolution, which corresponds to the section thickness, typically 30–100 nm, and with this resolution certain important membranous structures, such as contact sites and crista junctions, cannot be resolved along this dimension. Crista junctions are the narrow, tubular openings that connect the cristae membranes to the inner boundary membrane, and contact sites are defined as regions where the outer and inner boundary membranes come in such close apposition that they cannot be separately resolved (Perkins et al., 1997a). Mannella and coworkers found that to accurately map the convoluted inner membrane topography, the z-resolution should be at least 5–10 nm. This resolution is routinely attained with electron tomography.

Electron tomography presently is the imaging technique
that provides the highest 3D resolution of the internal structure of mitochondria in thick sections. In contrast to serial-section techniques, where the specimen is sliced as thin as possible, electron tomography employs sections cut thick enough, typically 0.25–1.0 μm, to contain a large portion of the mitochondrial volume. By tilting the section in the microscope and recording images from the same mitochondrion, a full 3D density distribution can be reconstructed by computational means (see Fig. 3); hence one is not obliged to draw inferences from partial surface views or thin slices. The tilting is performed around one or more axes over an angular range of typically ±60° in tilt increments of 1–2°. Because thick sections are used, it is necessary to use higher-voltage electron microscopes, ranging from 400 to 3000 kV, in order to obtain suitable images without high background from inelastically scattered electrons.

Mannella et al. (1994, 1997) showed that the commonly accepted concept that liver mitochondria have cristae composed of extended sheet-like, parallel involutions of the inner membrane was incorrect. Instead, they showed that intracisternal spaces in mitochondria with a condensed matrix (Hackenbrock, 1968) connected both to the mitochondrial periphery and to each other by narrow tubular extensions, and that these interconnected cristae possess multiple crista junctions (openings) at the inner boundary membrane. In contrast, their orthodox mitochondria (expanded matrix; Hackenbrock, 1968) had cristae with few interconnections and each had a single, narrow junction, also tubular, to the inner boundary membrane. Perkins et al. (1997a,b) obtained similar results from neuronal mitochondrial imaged in situ, but with significant differences. The neuronal mitochondria were in the orthodox conformation and contained both tubular and lamellar cristae. Some cristae remained tubular throughout while others merged to form large or small lamellar cristae. Thus, the lamellar cristae joined the inner boundary membrane only via multiple crista junctions; the larger the crista the more crista junctions they possessed (see Fig. 3). Perkins and coworkers found that crista junctions are relatively narrow and uniform in size (28 ± 6 nm diameter), and they never observed connections between lamellar cristae. On the basis of insights gained from electron tomographic reconstructions of liver (Mannella et al., 1994, 1997) and neuronal (Perkins et al., 1997a,b) mitochondria, it was suggested that crista junctions might be a uniform structural principle. In order to test the hypothesis that this biomembrane junction is the governing architecture among all higher animals, Perkins et al. (1998) examined the structure of brown fat mitochondria by electron tomography, because HRSEM indicated that brown fat mitochondria contain only lamellar cristae. They found that both chemically fixed and cryofixed brown fat mitochondria exhibited no tubular cristae, but instead had only large lamellar cristae (Perkins et al., 1998). However, all connections between the inner boundary and cristae membranes were through tubular crista junctions virtually identical to those found in neuronal mitochondria. Furthermore, it was found that the basic cristae architecture of cryofixed mitochondria was the same as that found in chemically fixed mitochondria suggesting that while no fixation procedure is completely free of artifacts, this architectural feature is likely found in vivo.

Mannella et al. (1994, 1997) hypothesized that crista junctions might represent a barrier to the free diffusion of ions between the intracisternal space and the intermembrane space. If diffusion is limited, then microcompartmentation of protons may result in greater pH gradients locally across the cristae membranes. This hypothesis is of great interest to bioenergeticists because of Mitchell’s chemiosmotic theory of ATP generation. The argument is presented as follows. If instead of crista junctions there were large openings into the intracisternal space, a salient feature of the baffle model, then protons ejected from the matrix should rapidly diffuse into the cytosol through the numerous pores in the outer membrane. So, the pH component of the chemiosmotic potential would be simply proportional to pH_{matrix} − pH_{cytosol}.
In contrast, if a proton diffusion barrier existed at crista junctions, then a greater chemiosmotic potential could be established locally. The diameters of crista junctions, ca. 30 nm, are very large compared to protons, however, and if they are fully open, they would not present a barrier to diffusion of protons. On the other hand, crista junctions might be a barrier to diffusion of membrane proteins. This would allow compartmentalization of inner membrane function by segregating different inner membrane proteins between the inner boundary membrane and the crista membrane.

As electron tomography has become a more routine tool in mitochondrial research, a number of technical improvements have been implemented to improve the quality of reconstructions. One such advance is double-tilt electron tomography (Penczek et al., 1995). Double-tilt electron tomography simply consists of collecting a second single-tilt series after rotating the specimen by 90° around an axis perpendicular to the section plane, i.e., the z-axis. Both series are aligned with respect to each other and the 3D information is merged to more completely fill the sampled Fourier space. Since the addition of a second tilt series increases radiation damage to the specimen, a number of tilt selection schemes have been investigated in which fewer projections at low tilt angles and more projections at higher tilt angles were taken, leaving the total number of projections constant. It was found that a more faithful reconstruction could be obtained with the double-tilt technique without using a greater number of tilt images than were used with the conventional single-tilt technique. Fig. 4 is a side-by-side comparison of a double-tilt tomogram with a single-tilt tomogram showing improved visualization of the inner and outer membranes in the vicinity of the crista junctions.

Technical improvements in computational tools have recently been made to further the capability to extract quantitative information from tomograms, such as membrane surface areas, compartment volumes and the distribution of contact sites (Perkins et al., 1997a, 1998). The interpretation and measurement of the substructures of mitochondria depend critically on the use of tools for segmenting and classifying the features of interest, software for making 3D measurements, and programs for interactively visualizing components of the structure. For example, the capability to segment and classify features of the tomographic volume was essential for the interpretation of mitochondrial architecture, and led to the full visualization of crista junctions (Mannella et al., 1994, 1997; Perkins et al., 1997a). Li et al. (1997) developed another tool for interactive segmentation that has been successfully applied to mitochondria. The advantage of their tool is the 3D cursor, which is used to define and isolate features of interest displayed, and maybe hidden, inside the volume. Once isolated, these features can be viewed from any angle, from which measurements can be made and relationships analyzed.

Computational tools were also recently developed in order to perform automated tomography. Computer control of the relevant microscope functions and on-line auto-focus, cross-correlation and alignment have been implemented to automate the collection of a tilt series and can achieve low total electron exposure (Koster et al., 1992, 1997). Automated tomography is especially useful when dealing with cryo-specimens (Frank, 1995) that are embedded in vitreous ice and are extremely sensitive to exposure to the electron beam. The attractiveness of cryo-techniques is the ability to maintain the sample in physiological states and to monitor dynamic events through very rapid freezing. By using a CCD-based imaging system for automated tomography, Mannella et al. (1998) showed that unfixed, unstained mitochondria quick-frozen in vitreous ice had the same crista junction morphology found in chemically fixed and stained mitochondria embedded in plastic.

Because of the relatively high 3D resolution afforded by electron tomography, new insight into contact sites were gained (Mannella et al., 1996a; Perkins et al., 1997a, 1998). The outer and inner boundary membranes come close together with no discernible space between them at numerous locations called contact sites (Hackenbrock, 1966). These sites are believed to be the main locations at which proteins imported from the cytosol are transported across both membranes to the matrix through protein translocation pores (reviewed by Schatz and Dobberstein, 1996).
The number of sites of peptide translocation is highly dependent on the energy state of the mitochondrion, because an energized inner membrane is required to complete the passage of the precursor peptide through the outer membrane (Pfanner and Neupert, 1990; Baker and Schatz, 1991). It is not clear which components are responsible for the formation of contact sites and whether there exist two types of contact sites: stable and labile (van der Klei et al., 1994). Contact sites may be held together by creatine kinase octomers (Schnyder et al., 1988) that reside in the intermembrane space and/or by the protein translocation complexes, MOM and MIM (now called TOM and TIM; Berthold et al., 1995) that would act as protein "grommets" anchored in the outer and inner membranes.

Perkins et al. (1997a,b) characterized the sizes and distributions of contact sites in orthodox mitochondria observed in chemically fixed and embedded neuronal tissue by electron tomography. The width of contact sites (~14 ± 2 nm) implied a very close association of the two membranes, but did not support the "semi-fusion" model (Venetie (1982)). Since the contact site width was not less than twice the width of a single membrane (~7 nm), there was no basis to invoke partial membrane fusion to account for them. The extent of contact sites parallel to the membranes was similar to their width, 14 ± 7 nm, suggesting that they may be formed by a single complex of proteins. By examining projection images, it was previously hypothesized that contact sites might be proximal to crista junctions in order to facilitate transport of proteins destined for the cristae. However, measurements on tomographic volumes indicate that contact sites are randomly located with respect to these junctions. The implications of this result must be reexamined in light of results from cryofixed mitochondria. The only significant structural difference observed between cryofixed and chemically fixed brown fat mitochondria is that in the cryofixed mitochondria, almost all the outer membranes were close to the inner boundary membrane with no visible space observed between them (Perkins et al., 1998). The average width from the outer surface of the outer membrane to the inner surface of the inner boundary membrane in cryofixed mitochondria was 17 nm, 5 nm narrower than the comparable measurement in chemically fixed mitochondria and close to the 14 nm width measured for contact sites. The close apposition of the outer membrane and inner boundary membrane essentially over all the periphery in cryofixed mitochondria indicates that this entire area is dimensionally competent for protein import, and that there may be different classes of contact sites, e.g. stable and labile, with different functions.

In comparing the structures of chemically fixed liver, neuronal and BAT mitochondria, a number of similarities stand out, which may be significant for mitochondrial biogenesis. The outer and inner boundary membranes were far enough apart to reveal discrete stable contact sites holding the two together. In all three types of mitochondria, the inner boundary membrane invaginated at numerous sites, the crista junctions, which were tubular and relatively uniform in diameter. In brown fat mitochondria, the crista junctions were tubular for only a short extent before broadening into lamellar compartments. One important point to note is that this increase in cristae surface area did not cause an increase in the diameter of the crista junctions. In liver and neuronal mitochondria, however, the tubular structure of the crista junctions often persisted to the extent that some crista were entirely tubular while other tubular cristae merged to form lamellar cristae of various sizes. The uniform size and shape of crista junctions between mitochondria types with different cristae morphologies suggested that there might be some components that cause their formation and stabilize their structure. This led to the hypothesis that cristae begin by a budding of the inner boundary membrane at crista junctions and grow by the addition of membrane components, perhaps at the junction site, causing tubular cristae to grow longer and eventually merge with other cristae to form large lamellar compartments. In contrast to the network of merging tubular cristae observed in neuronal mitochondria, no such network was observed in brown fat mitochondria.

Another intriguing feature observed in tomographic reconstructions is an association between the outer mitochondrial membrane and the endoplasmic reticulum (ER) membrane (Mannella et al., 1996b, 1998; Perkins et al., 1997a). These contacts are punctate and about 15 nm in diameter. Vance and Shiao (1996) showed that the newly synthesized phosphatidylserine was imported into the mitochondria through contacts with an "endoplasmic reticulum-like mitochondria-associated membrane." The views of punctate contacts between ER and mitochondrial membranes could be a “snapshot” of lipid transfer to the mitochondrion.

A novel technique, at least as applied to mitochondria, is to embed unfixed and unstained isolated mitochondria in vitreous ice (Mannella et al., 1998). These "naked" organelles were rapidly frozen in solution (frozen-hydrated) and imaged with a cryo-electron microscope at low temperature. The frozen-hydrated specimens are very sensitive to the electron beam, and so fewer images in a tilt series could be collected. Hence, the 3D resolution was necessarily lower. An automated CCD-based tomography system facilitated data collection. The advantage of this technique is that direct visualization of mitochondrial substructure close to the native state can be achieved. This method confirmed the presence of crista junctions found previously in chemically fixed and cryofixed mitochondria. The inner boundary membrane and the outer membrane were observed to be close together as observed in cryofixed and freeze-substituted specimens, but there was a space between them that was not observed in the freeze-substituted specimens. The latter were stained, however, which might have obscured any space between the two membranes.

Double-tilt electron tomography (Deng et al., 1998) as well as mathematical modeling (Deng and Mieczkowski, 1994) and
A part of the mitochondrial division involves the partitioning of DNA. Mitochondria in some organisms have a nucleoid, which can be visualized by DAPI staining, where the DNA is organized by proteins. During mitochondriokinesis, the DNA molecules, which are ~1/100 the size of bacterial DNA, were separated equally between daughter mitochondria. The mechanism of separation appeared to be under different control than the MD ring constriction. The MD ring actually consists of two rings, an outer ring and an inner ring. The outer ring is in the cytoplasm and the inner ring is in the matrix adjacent to the inner boundary membrane at the constricted isthmus of dividing mitochondria. During constriction, the outer ring thickened while the inner ring remained unchanged. It was suggested that the outer ring generates the motive force during division and the inner ring may simply be a remnant of the bacterial contractile ring (FtsZ ring). The MD ring was observed to be a bundle of fine filaments. Interestingly, this ring was not labeled by anti-actin antibody or by phalloidin. Furthermore, division was not inhibited by cytochalasin B. Hence, it does not appear that actin is a component of the MD ring and its composition remains unsure. However, it was determined that the MD ring proteins are encoded by genes in the nuclear genome, and that the growth of this ring is independent from nuclear DNA synthesis. In summary, the identification of the MD ring by conventional electron microscopy has added insight into the division mechanism of mitochondria that is similar in principle to the division of bacteria, plastids and eukaryotic cells.

On a smaller scale, electron microscopy has provided views of plasmid-like DNA in mitochondria in the animal kingdom (Endoh et al., 1994). Numerous kinds of mitochondrial plasmids have been reported in fungi and plants. However, until recently such plasmids were not known among mitochondria of animals. Mitochondrial DNA from
Paramecium caudatum was isolated by electrophoresis into bands of three distinct types. Mitochondrial genomic DNA was found in a high molecular weight band and was designated as mtDNA-type. The plasmid-like DNAs were designated either type I or type II DNAs depending on the molecular weight of the bands. Remarkable features of type II DNA strands were visualized in electron micrographs. One observed feature was a hairpin structure that had a loop at one terminus and a protruding segment of single-stranded DNA at the other terminus. Another feature was the presence of stable dimer structures. The monomers paired with base pairing in opposite directions suggest the presence of inverted repeats. It was hypothesized that these unusual dimers may aid DNA replication by having the constituent monomers serve as a primer for each other. Another viewpoint is that, type II DNAs may not be plasmids, but rather parasitic DNAs. In either case, the observation of plasmid-like DNA in mitochondria is intriguing because of how they might have been inserted, the implication for replication mechanism, and whether they can replicate autonomously or are under the control of host mitochondria.

While tissue-dependent differences in mitochondrial morphology have been documented ever since thin sections of tissue were first examined in electron microscope, it has been generally assumed that mitochondrial function and response to stimuli are similar across tissues. The notable exception to functional homogeneity is brown fat mitochondria, which produce heat by uncoupling ATP production from the electron transport chain. A new study of brain and liver mitochondria provided evidence that mitochondrial response to stimuli is not as homogeneous as assumed previously. Interest in the PT pore has been increasing because of its putative role in the response of brain cells to ischemia and excitotoxic agents. The PT pore has been studied mostly in isolated liver mitochondria, and it has been assumed that the properties discovered in this model system would be the same in brain mitochondria. However, through the use of electron microscopy and biochemical tools, it was found that those chemicals which open the PT pore and cause swelling and loss of glutathione in liver mitochondria, did not produce the same effect in isolated brain mitochondria (Hastings et al., 1998). The implication is that brain mitochondria are more resistant to the induction of permeability transition. It can be argued from these results that properties found in mitochondria of one tissue type may not translate easily to mitochondria of other tissue types.

While fusion of adjacent mitochondria has been observed for many years at the level of light microscopy, only recently have fusion events been visualized at the level of electron microscopy. Mitochondria do not form de novo, but assemble from pre-existing mitochondria by fission and fusion events. By using phase-contrast light microscopy, Bereiter-Hahn and Voth (1994) were able to record video sequences of fusing mitochondria. Fixation was used to arrest these mitochondria in various stages of fusion, and thin-section electron microscopy provided high-resolution images of this process. By employing correlative light and electron microscopies, it was possible to differentiate between fusing mitochondria and mitochondria that were transiently close to one another. Fusion does not appear to depend on physiological condition, but rather on mitochondrial motility. It was seen that the peripheral mitochondrial membranes in the contact zone, where early fusion events take place, exhibited a high electron density. This high density might reflect clustering of membrane proteins involved with fusing the outer and inner boundary membranes of the apposed mitochondria. It was found that the fusing regions were commonly devoid of cristae, which also argues for the localized presence of specialized fusion machinery in the regions of the merging peripheral membranes. While fusion of the peripheral membranes occurred on a relatively fast time scale (1–3 s), rearrangement of cristae progressed on a longer time scale (>20 s). Rearrangement in the transition region between two mitochondria occurs via the elongation of cristae. Everywhere else, the cristae maintain the same arrangement that existed in the separate mitochondria before merging. De novo cristae, hypothesized to bud from crista junctions, have yet to be documented in the transition region or for that matter anywhere else.

Electron microscopic evidence that mitochondria may be capable of propagating by means other than fission was recently published (Dai et al., 1998). A slow-sedimenting (ss-) mitochondrion was identified in mitochondrial fractions and observed in situ within rapidly growing mung bean seedlings. Besides possessing cristae (in the larger, but undetected in smaller ss-mitochondria), these mitochondria had cytochrome oxidase subunit III (detected in situ with immunoelectron microscopy) and mitochondrial DNA (mtDNA). Smaller than their neighbors (70–300 nm in size), ss-mitochondria lacked demonstrable respiratory capability, and were almost always in contact with a filament-aligned membrane-like structure. The correlation of size with the appearance of sparse cristae suggests that these mitochondria may not be static, but rather intermediates in a developmental pathway leading to respiratory-competent mitochondria. Further, despite several unique mitochondrial characteristics, ss-mitochondria appeared to be immature structurally as well as functionally. Binary fission of mitochondria with more classical structure was also detected in mung bean seedlings. These observations imply that small ss-mitochondria may be “nascent” mitochondria from a yet unidentified mitochondria-propagation scheme, and that mitochondria may be capable of propagating by more than one pathway.

3.4. Light microscopy

A novel technique for imaging mtDNA has shown that heterogeneity of mitochondrial ultrastructure extends to
Mitochondria and the endoplasmic reticulum (ER) are in close proximity to each other, and their network is constantly rearranging. (b) 3D image of mitochondria taken with a 100× objective lens and computationally deblurred. Note the mitochondrial reticulum. (c) Recovery of mtGFP fluorescence after photobleaching. The first and second images were recorded immediately before and after photobleaching. The following three images were recorded at 2 min intervals after and the final image 30 min after photobleaching. The rapid recovery indicates an interconnected mitochondrial network with luminal continuity. (d) Combined 3D imaging of mitochondria and ER in a HeLa cell transiently expressing mtGFP and erGFP. The two 3D images are superimposed. Mitochondria are in red, ER in green and the overlaps of mitochondria and ER result in a white color. The mitochondrial reticulum in close proximity to the ER was estimated to be ~5–20% of the total. (Reproduced by permission from Rizzuto et al., 1998. Close contacts with the endoplasmic reticulum as determinants of mitochondrial calcium responses. Science, 280, 1763–1766.)

DNA molecules as well (Bendich, 1996). Molecules of mtDNA in the 50–150 kb size region from various fungi and plants were obtained from gel bands and after soaking in ethidium bromide to make fluorescent were analyzed as moving pictures during electrophoresis with video fluorescence microscopy. The lengths of mtDNA in fields of between 10 and 100 molecules were measured and their structures were noted (circular or linear) by examining frames of videotape. The longest molecules were measured and compared to a size standard (lambda DNA, 48.5 kb) after encounters with agarose fibers had extended the strands to their maximum length. It was found that most of the fractionated mtDNA were linear and ranged in size from 50 to 150 kb. Very little of the mtDNA was in the conformation of large circular molecules. This finding is important because nearly all mtDNA extracted from metazoan animal cells has been in the form of genome-sized circular molecules. It was expected that circular mitochondrial genomes would also be the norm in other organisms. Circular molecules are universally depicted in reviews and textbooks describing mtDNA of fungi and plants. Moreover, recent models of recombination, rearrangement and loss of plant mtDNA sequences were based on the assumption of circular genomes. Since the existence of circular mtDNA molecules has been established in only a subset of eukaryotes, the conformation from a diverse sampling of organisms needs to be demonstrated, and more realistic models need to be constructed for those genomes with known linear conformation.

A recent application of wide-field fluorescence microscopy has revealed that the structure of mitochondria in yeast has direct consequences for cytoplasmic inheritance (Nunnari et al., 1997). In haploid cells of opposite mating type, mtDNA and mitochondrial proteins were labeled with different fluorescent dyes. After mating, it was found that parental mitochondrial protein markers were redistributed rapidly throughout the zygotes, providing evidence that parental mitochondria fuse and their constituent proteins intermix. In fact, it appears that yeast mitochondria form a single dynamic network of compartments, and its interconnectedness is maintained by a balance of fusion and fission events. The generation of such a mitochondrial reticulum is thought to facilitate signaling, and passage of metabolites and energy throughout cells (Ichas et al., 1997). One of the fluorescent markers was a matrix-targeted GFP. Its complete mixing in the zygote indicated that fusion of both outer and inner membranes occurred. Of necessity, fusion of separate membranes requires distinct fusion steps. It is likely that both steps involve a unique fusion apparatus operating on the respective membranes. In contrast to the mixing of proteins after fusion, the mtDNA remained separately localized to each half of the zygote. The lack of diffusion through the mitochondrial network implied that daughter cells could have three different mitochondrial genotypes. Those that bud from either end are likely to contain mtDNA from only one of the two parents, but those that bud from the middle might contain mtDNA from both parents. The precise mechanism for mtDNA transmission during mating is unknown; however, lack of diffusion might be an important vehicle for controlling the accurate inheritance of the mitochondrial genome. This further suggests that active mechanisms for maintaining separate mtDNA are involved, which may include anchoring of mtDNA molecules to specific locations within the mitochondrion. Active mtDNA segregation offers a structural explanation for the nonrandom transmission patterns that have been observed genetically for years now.

A dynamic network of largely interconnected mitochondria was also observed in normal cultured fibroblasts (Handran et al., 1997) and in an immortalized human cancer cell line (HeLa cells; Rizzuto et al., 1998) (Fig. 6). The vitality of fibroblast mitochondria was monitored with the vital dyes JC-1 and TMRE. Confocal light microscopy was used in this study to collect 0.4 μm optical sections. Each optical section was the average of 64 frames recorded at a rate of 30 frames/s. The stack of sections was deconvolved and rendered into an image that simulates 3D space. These images displayed a reticulum of mitochondrial...
compartments. By using two differently colored green fluorescent proteins specifically targeted to either mitochondria or ER in HeLa cells, the spatial relation between these organelles was analyzed by Rizzuto and coworkers with a high-speed wide-field fluorescence imaging system (Fig. 6c,d). 3D reconstructions were made by optical sectioning and were computationally deblurred to reveal that the mitochondrial network was mostly tubular and underwent continuous rearrangement (Fig. 6b). The imaging system used laser illumination, which allowed 128 × 128 pixel images to be collected in 30 ms using a low-noise CCD camera. With this high-speed system, 41 optical sections with a z-step of 0.25 μm were collected in ~1 s. Stacks of optical sections were taken 30 s apart. 3D reconstructions of mitochondria from these stacks demonstrated that growth, retraction, fission and fusion had occurred within 1 min of observation indicating a high structural plasticity in agreement with cinematic recordings using phase contrast microscopy (Bereiter-Hahn and Voth, 1994). The interconnectedness of mitochondrial networks was confirmed by the recovery of fluorescence within 30 min after photobleaching a portion of the network.

The binding of calcium to aequorin produces luminescence. Rizzuto et al. (1998) used aequorin chimeras specifically targeted to the cytosol, mitochondrial intermembrane space, or matrix to monitor the calcium concentrations sensed by the mitochondrial calcium uptake systems. Through the opening of IP3-gated channels, calcium is released from the ER, the intracellular calcium storehouse. It was shown that the areas of close apposition between the ER and mitochondria were also the areas where an increase in calcium concentration saturated the binding of calcium to aequorin in the mitochondrial intermembrane space. It thus appeared that at contacts between the ER and mitochondria, microdomains of high calcium concentration were generated, which could allow for a rapid uptake of a relatively large amount of calcium by mitochondria. After uptake, there appeared to be a rapid diffusion of calcium within the mitochondrial matrix, because a large portion of the matrix aequorin discharged its luminescence. This rapid diffusion might be a mechanism for quickly tuning mitochondrial metabolism to the cell’s needs for ATP. The microheterogeneity of the calcium signal emphasizes the importance of the cellular distribution of mitochondria in relation to the ER.

Mitochondrial morphology in yeast may be linked to inheritance, i.e. to the mechanism of mitochondrial fission. Coupling light and electron microscopy with genetic analyses, it has been shown that mutations in genes required for normal inheritance and distribution alter mitochondrial morphology (Berger and Yaffe, 1996). These genes encode proteins found in either the outer or inner boundary membranes. Instead of the normal mitochondrial reticulum, these mutant yeast exhibited either small round mitochondria (Fisk and Yaffe, 1997), or giant round mitochondria (Berger et al., 1997) depending on the mutation.

Using light microscopy, Visser et al. (1995) showed that the number of mitochondria in yeast can vary from one to more than 40 depending on the growth phase, the oxygen availability and the carbon source. When yeast are grown on a carbon source that represses genes for respiratory proteins, 2–3 long, branched mitochondria are observed that lie near the cell periphery. However, when a carbon source is used that does not repress these genes, many small mitochondria are seen dispersed throughout the cell. More recently, Church and Poyton (1998) used light and electron microscopy to show that neither assembled cytochrome oxidase, nor cellular respiration is required for normal mitochondrial morphology or volume in yeast. This work suggests that the morphological changes documented by Visser and coworkers are not a consequence of respiratory capacity, but rather to another, as yet undetermined factor. It was pointed out that although respiration is not involved in the maintenance of morphology, oxygen availability and glucose repression might influence other metabolic processes, which in turn may affect mitochondrial morphology. An interesting question is whether mitochondrial morphology can be governed by the principle of symmorphosis (Weibel et al., 1991), which has as its tenet that “no more structure is formed and maintained than is required to satisfy functional needs”. If symmorphosis can be applied to yeast mitochondria, then the observation that mitochondrial morphology is normal in the absence of respiration implies that mitochondrial structure is maintained for purposes other than respiration. It is important to note that in addition to being the site for oxidative phosphorylation, mitochondria also synthesize certain amino acids and precursors for the synthesis of pyrimidines and heme. This capability makes mitochondria essential to the normal operation of eukaryotic cells, whether they are grown aerobically or anaerobically.

4. Perspectives

What has allowed the advances in our understanding of mitochondrial structures reviewed here? First and foremost, the capabilities of microscope technology have been improving. This is most evident in electron tomography and SEM, where improvement in technique in the latter has added the superlatives “ultra high-resolution” to SEM. It should be emphasized again that electron tomography increased the resolution attained in 3D reconstructions of whole mitochondria by about an order of magnitude over that attained by the technique previously used, serial-section reconstruction. It is no wonder then that with this increased resolution new facets of membrane topography were revealed. As with most scientific endeavors that utilize computers, microscopy has benefited from the revolution in computer technology experienced this decade. Nowhere is this more apparent than in the resurgence of light microscopy, where the capability to monitor dynamic events by fast recording media is made possible by fast computers and
CCD cameras. Computers that can handle the analysis and storage of large volumes of data have also been instrumental in the growth of electron tomography. Moreover, the availability of new software tools for viewing and analyzing segmented volumes have been invaluable for extracting hidden features and understanding spatial relationships. Finally, the advent of a greater number and more sensitive biochemical probes that can be used in conjunction with various microscopies has broadened how we can dissect working mitochondria.

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