

Organization of the Sea Urchin Egg Endoplasmic Reticulum and Its Reorganization at Fertilization

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Abstract. The ER of eggs of the sea urchin *Lytechinus pictus* was stained by microinjecting a saturated solution of the fluorescent dicarbocyanine DiI₁₈(3) (DiI) in soybean oil; the dye spread from the oil drop into ER membranes throughout the egg but not into other organelles. Confocal microscopy revealed large cisternae extending throughout the interior of the egg and a tubular membrane network at the cortex. Since diffusion of DiI is confined to continuous bilayers, the spread of the dye supports the concept that the ER is a cell-wide, interconnected compartment. In time lapse observations, the internal cisternae were seen to be in continuous motion, while the cortical ER was stationary. After fertilization, the internal ER appeared to become more finely divided, beginning as a wave apparently coincident with the calcium wave and becoming

most marked by 2–3 min. By 5–8 min the ER returned to an organization similar to that of the unfertilized egg. The cortical network also changed at fertilization; it became disrupted and eventually recovered. DiI labeling allowed continuous observations of the ER during pronuclear migration and mitosis. DiI-stained membranes accumulated in the region of the microtubule array surrounding the sperm nucleus and centriole (the sperm aster) as it migrated to the center of the egg; this accumulation persisted near the centrosomes and zygote nucleus throughout pronuclear fusion and the first two mitotic cycles. We have used a new method to observe the spatial and temporal organization of the ER in a living cell, and we have demonstrated a striking reorganization of the ER at fertilization.

THE ER is the site of protein synthesis (Palade and Siekevitz, 1956) and lipid synthesis (Wilgram and Kennedy, 1963) and it is one of the intracellular compartments involved in calcium regulation (Streb et al., 1984; Ross et al., 1989). The ER has distinct functional domains, such as the nuclear envelope (Watson, 1955), and rough and smooth ER (Palade, 1955); there are other morphologically identifiable domains that may have functional significance, such as cisternae and tubules (Palade and Porter, 1954), and cortical and noncortical ER (Porter, 1961). ER membranes participate in membrane traffic with the Golgi apparatus (Palade, 1975), and have structural interactions with microtubules (Terasaki et al., 1986) and actin filaments (Kachar and Reese, 1988). In view of all of these characteristics, the ER may be comparable to the plasma membrane in complexity of function and perhaps exceeds it in morphological complexity.

New information about the distribution of the ER has been obtained with the fluorescent dicarbocyanine dye DiOC₆(3) (Terasaki et al., 1984). This dye permeates the plasma mem-

brane and stains many if not all intracellular membranes; it has been primarily useful in the periphery of cultured cells where the ER is a single two dimensional layer that can be easily distinguished from other membranes (Terasaki, 1989). To investigate the three dimensional distribution of ER in living cells, we have adapted a method that has been used for visualizing the complex form of neurons in intact tissues. This method for staining the plasma membrane involves introducing a longer alkyl chain fluorescent dicarbocyanine dye "DiI" (DiI₁₈(3) or DiI₁₆(3)) into contact with the cells; the lipophilic dye transfers into the plasma membrane and diffuses within the membrane bilayer throughout the neuronal processes (Honig and Hume, 1986, 1989; see also Haugland, 1989). This dye has recently been used to stain the ER and sarcoplasmic reticulum in broken cell preparations where DiI aggregates contact and incorporate in the membrane bilayer and then diffuse within it (Henson et al., 1989; Baumann et al., 1990; Terasaki, M., J. H. Henson, D. A. Begg, B. Kaminer, and C. Sardet, unpublished results). To apply a source of DiI to the membrane of the ER in an intact living cell, we dissolved the dye in soybean oil (Wesson cooking oil) and microinjected the dye-saturated oil into the cytoplasm of sea urchin eggs. Using confocal microscopy (White et al., 1987), we found that the

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dye spread through the ER, but not into other organelles, thus allowing us to visualize the structure of the ER in the egg.

Previous studies in fixed preparations have provided evidence for transformations in ER organization that accompany physiological changes in oocytes and eggs. A structural reorganization of the ER occurs during oocyte maturation of the frog *Xenopus* (Gardiner and Grey, 1983; Campanella et al., 1984; Charbonneau and Grey, 1984; Larabell and Chandler, 1988); the ER cisternae come to surround the cortical granules, establishing the ER in a form and location that may serve to release calcium and cause cortical granule exocytosis at fertilization. A similar development of tubular ER around the cortical granules occurs between the vitellogenic oocyte and the mature egg in the sea urchin (Henson et al., 1990). Indications of changes in ER structure at fertilization include the finding that the ER appears to be disrupted on cortices derived from recently fertilized sea urchin eggs (Sardet, 1984; Henson et al., 1989); a question remains however whether this is a change in the ER or a change in the cortex that causes it to shear differently when the cortex is prepared after fertilization. In *Xenopus*, electron microscopy has shown that junctions between the cortical ER and plasma membrane decrease at fertilization (Gardiner and Grey, 1983) and in another frog, *Discoglossus*, fertilization results in a rearrangement of the subcortical ER near the animal pole (Campanella et al., 1988). Using the DiI-oil drop injection technique, we have now made continuous observations of the rearrangements of the ER in the living sea urchin egg during fertilization. We find that within the first 20 min after fertilization, the ER throughout the egg cytoplasm undergoes a dramatic sequence of structural changes.

Materials and Methods

Sea urchins (*Lytechinus pictus*) were obtained from Marinus, Inc. (Venice, CA); this species was used because of the optical clarity of its eggs. Eggs and sperm were obtained by injection of 0.5 M KCl into the coelomic cavity. The gametes were suspended in artificial sea water. Experiments were performed at 22–24°C.

DiI₁₈ (3): 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI)¹ was obtained from Molecular Probes (Eugene, OR). A saturated solution of DiI in oil was made by mixing several crystals of DiI in 100 μ l of soybean oil (Wesson oil; obtained from Food Buoy, Woods Hole, MA, and Sutton Place Gourmet, Bethesda, MD; lots from both sources behaved similarly). The solution was kept at room temperature, and was used over a period of several days.

The DiI solution was microinjected into eggs held between parallel coverslips of number zero thickness (Kiehart, 1982). The eggs were observed during injection using a standard Zeiss upright microscope with a 10 $\times$ phase contrast objective. The oil solution was microinjected with a constriction pipette connected to a micrometer syringe (Hiramoto, 1974; Kishimoto, 1986). After injection, the chamber holding the eggs was transferred to the stage of the confocal microscope. Insemination was accomplished by introducing a sperm suspension through the open side of the injection chamber.

Eggs were observed using a laser scanning confocal microscope (Model 600; Bio-Rad Laboratories, Oxnard, CA) with an argon laser for illumination and coupled with a Zeiss Axioplan or a Nikon Optiphot microscope. Observations were made using a Zeiss Planapo 63 $\times$ N.A. 1.4 objective lens or a Nikon Planapo 60 $\times$ N.A. 1.4 objective lens. For the observations, the laser was used at full power with a 1 or 3% neutral density filter, and with the confocal aperture between 3 and 5. A stepper motor was used for collecting Z-series images; this motor had a minimum step size of 0.18 μ m for the Zeiss microscope and 0.1 μ m for the Nikon microscope.

Images were stored on a Panasonic 3031F optical memory disk recorder

1. Abbreviation used in this paper: DiI, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate.

(OMDR). The OMDR was operated in its on line mode, using macro programs run by the BioRad confocal microscope software. Scale bar calibrations were obtained by recording images of a stage micrometer for each zoom setting and scanning speed used, for both x and y directions. To make the figures, the video monitor was photographed using 35-mm film (TMAX 100; Eastman Kodak Co., Rochester, NY). A monitor with an adjustable video image height was used to obtain identical magnification in x and y directions.

Results

Internal and Cortical ER of the Unfertilized Egg

After injection into a sea urchin (*Lytechinus pictus*) egg of

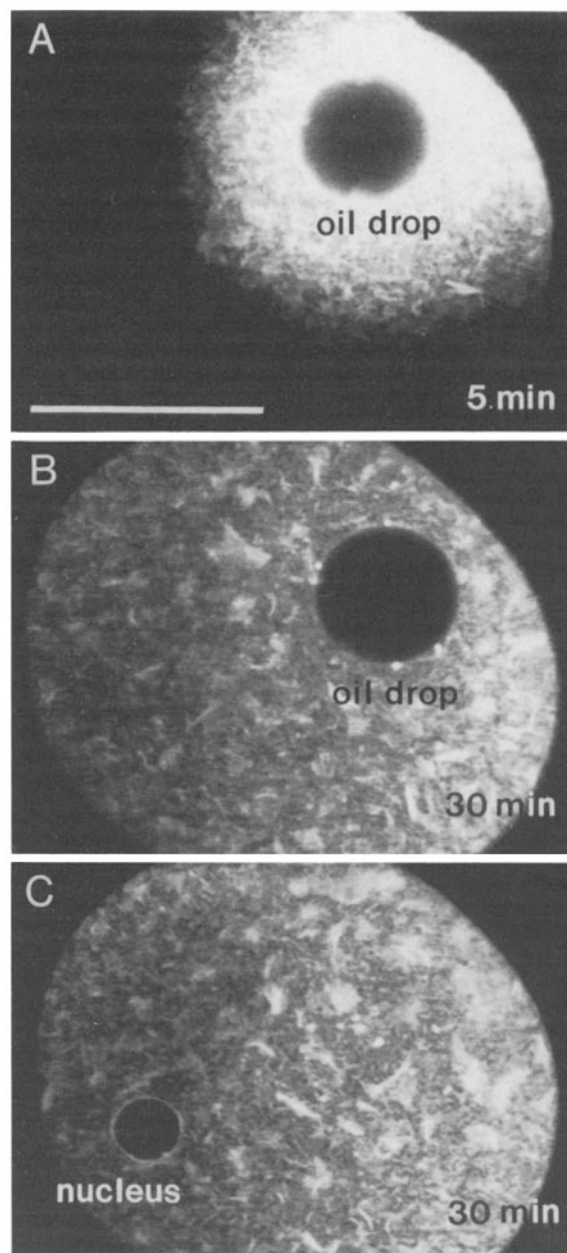


Figure 1. Spreading of DiI from an injected oil drop into the ER of an unfertilized egg. (A) 5 min after injection. The dye has moved from the oil drop into the nearby cytoplasm but has not yet spread across the egg. (B) 30 min after injection. The dye has spread throughout the cytoplasm but is still more concentrated near the oil drop. (C) 30 min after injection. An optical section showing the nucleus. Bar, 50 μ m.

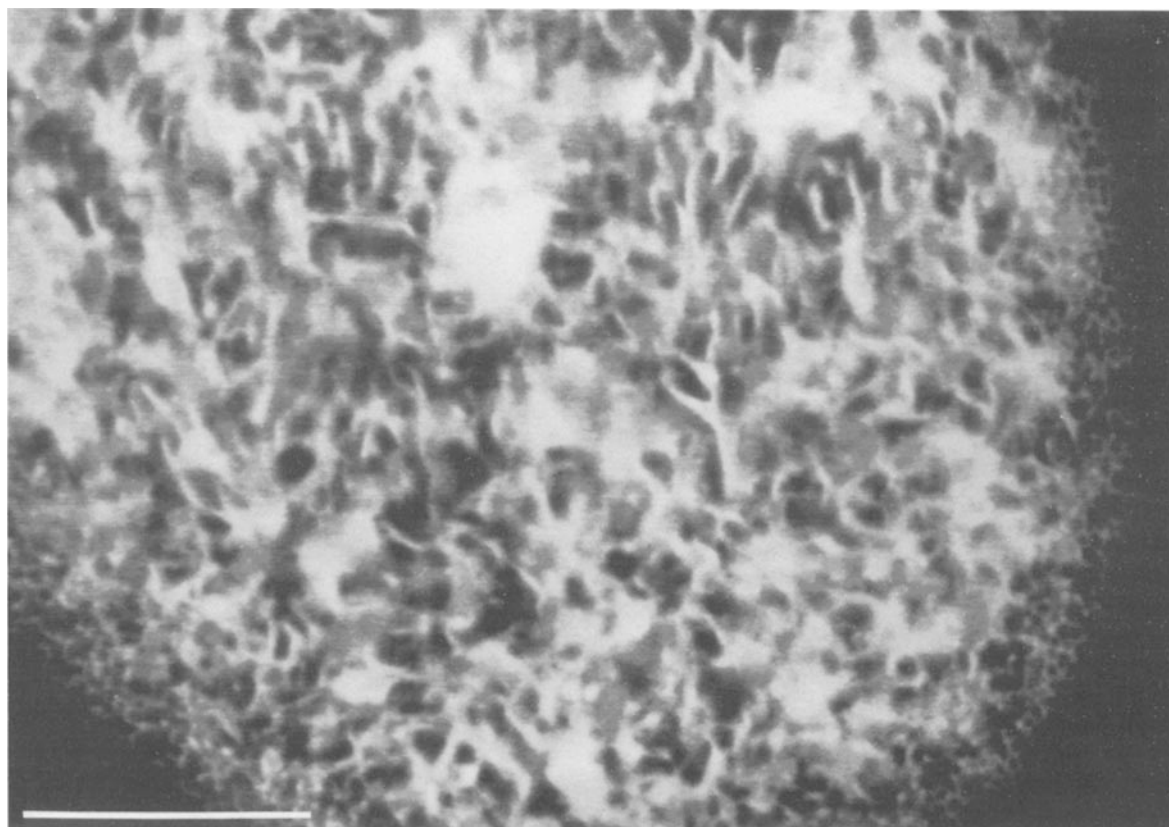


Figure 2. ER of the unfertilized egg. An optical section slightly below the top surface of the egg. Cisternae (lamellar sheets) are seen in the central region, with the tubular network at the periphery. Bar, 10 μm .

a drop of soybean oil saturated with DiI (4–20 μl , 0.5–3% of the egg volume), the dye spread throughout the egg (110 μm diameter) over a period of ~ 30 min (Fig. 1, *A* and *B*). Although little detail could be discerned by conventional fluorescence microscopy, observations with the confocal microscope allowed visualization of a system of cisternae (lamellar sheets) and tubules that we identify, as will be considered in the Discussion, as the ER.

The fluorescent image appeared to indicate that the dye had labeled interconnected sheets of membrane arranged in apparently random orientations (Figs. 1, 2, 5 *D*, 6 *A*, and 7 *A*). The lamellar sheets were seen both in cross-section and en face, with dimensions of ~ 1 –10 μm . The sheet-like nature of these structures was further demonstrated by taking serial optical sections at 1- μm intervals (Fig. 3 *A*). Similar patterns of variously oriented sheets of membrane were observed throughout the cytoplasm, at depths of 2 to 50 μm ; however, at levels deeper than ~ 20 μm , the quality of the images was poorer, such that individual cisternae were less distinct. The cell nucleus was visible as a dark, dye-free area ~ 20 –30 μm below the surface and was surrounded by a continuous bright boundary (Fig. 1 *C*).

Movement of ER membranes was seen in images taken in the same focal plane at 1- or 2.6-s intervals (Fig. 3 *B*). When observed in time-lapse video, all of the membranes were in motion, in apparently random directions. One component of this motion was translation of cisternae in the plane of the optical section; observations of four successive frames showed individual cisternae moving 0.7–1.6 μm in 7.8 s (aver-

age = 1.0 μm , $n = 11$). Because observations were limited to a single focal plane and were not faster than 1 per s, we could not characterize the movements of the ER in greater detail.

The staining pattern in the cortex of the egg (the region of the egg adjacent to the plasma membrane) appeared as a network of interconnected tubules. In an optical section slightly below the cortex (Fig. 2), the network was visible around the periphery. In an optical section at the cortex level, only the network was visible (Figs. 4, 5 *A*, 8 *A*). The areas enclosed by the interconnecting tubules had dimensions of ~ 1 μm . In contrast to the internal cisternae, the cortical network did not move when observed in time lapse sequences. In serial optical sections at 0.3- μm intervals (Fig. 5), the cortical network appeared clearly in only one section (Fig. 5 *A*). At 0.9 μm beyond the cortex level, the staining pattern showed randomly oriented sheets of membranes like those throughout the deeper cytoplasm (Fig. 5 *D*). Between the cortex and the internal region was a transition zone which seemed to consist of tubules oriented perpendicular to the plasma membrane.

Except in the region directly adjacent to the oil drop, where small stained objects were sometimes observed (not shown), and at the nuclear envelope, the only structures stained were the interconnecting sheets in the interior of the egg and the tubular network at the cortex. DiI did not stain the cortical granules or the yolk granules; these are abundant, membrane bound organelles ~ 1 μm in diameter located respectively at the cortex (see Henson et al., 1989) and

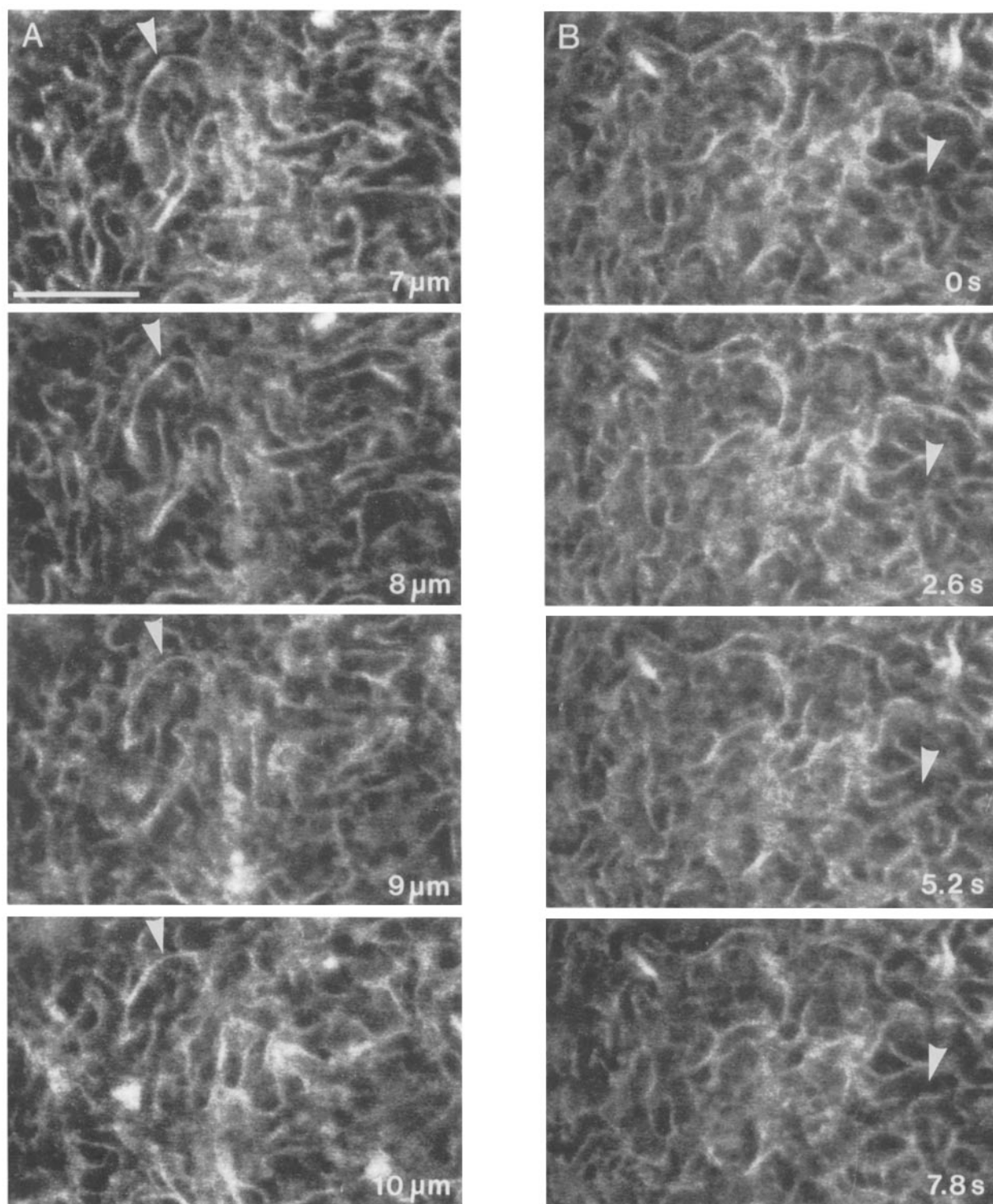


Figure 3. ER of the unfertilized egg. (A) Serial optical sections in 1- μ m steps 7–10 μ m from the surface. Arrowheads point to a long curved line that is present in each section, indicating that it is a membrane sheet seen in cross section. (B) Sequential scans of an optical section 7 μ m from the surface, taken at intervals of 2.6 s. Scanning time per frame = 2 s. Arrowheads indicate a region in which changes can be seen. When viewed in time-lapse video, all of the membranes are seen to be moving. Bar, 10 μ m.

throughout the interior (see Franklin, 1965; Summers and Hylander, 1974). These observations support the interpretation that DiI diffuses from the oil droplet through contacts with the interconnected membranes of the ER. If DiI was instead spreading through the aqueous regions of the cytoplasm and then partitioning into membranes, the cortical

granule and yolk granule membranes would be expected to stain.

Changes of the ER at Fertilization

At fertilization, both the internal and cortical ER underwent a rapid change in structure, followed by a slower return to

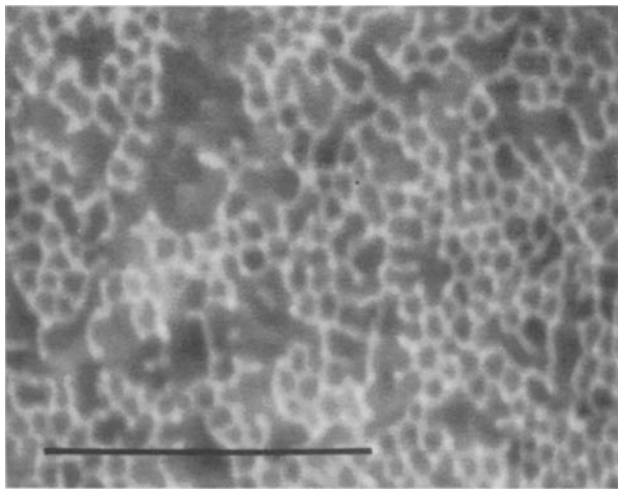


Figure 4. ER of the unfertilized egg. An optical section through the cortex of the egg, showing the tubular network. Bar, 10 μm .

a configuration similar to that of the unfertilized egg. The internal ER appeared to change to a more finely divided state; subsequently, the large sheets and spaces between the sheets reappeared (Figs. 6 and 7). This phenomenon was most clearly observed at levels $<20\ \mu\text{m}$ from the surface ($10\ \mu\text{m}$ in Figs. 6 and 7). Deeper in the cytoplasm, similar changes in the configuration of the ER could be detected, but the optical images at these levels were relatively poor. By changing the focus during the observation period, we excluded the possibility that the observed changes were due to an axial shift in the region of the egg that was in the focal plane. A transformation of the cortical ER also occurred; soon after fertilization, the cortical layer of tubular membranes was disrupted (Fig. 8 B). Later, a network of cortical membranes was reestablished (Fig. 8 C). These changes in the structure of the ER were observed in 12 out of 12 eggs (from 8 different animals) that were observed at various depths from the surface during fertilization. Observations with transmitted light microscopy showed that these eggs elevated their fertilization envelopes normally, indicating that normal cortical granule exocytosis occurred.

The initial changes in the ER, observed at depths 10–20 μm from the surface, began within 1 min after fertilization, as determined by the observation that they occurred at about the same time as the egg underwent a contraction of the cytoplasm (compare the shape of the egg in Fig 6, A and B) ($n = 5$ eggs). This contraction is known to occur within the first minute after fertilization (Schatten, 1981; Eisen et al., 1984; Hafner et al., 1988). The changes in the ER began as a wave of altered morphology that traveled across the egg (Fig. 6 B). In eggs oriented such that the fertilization cone

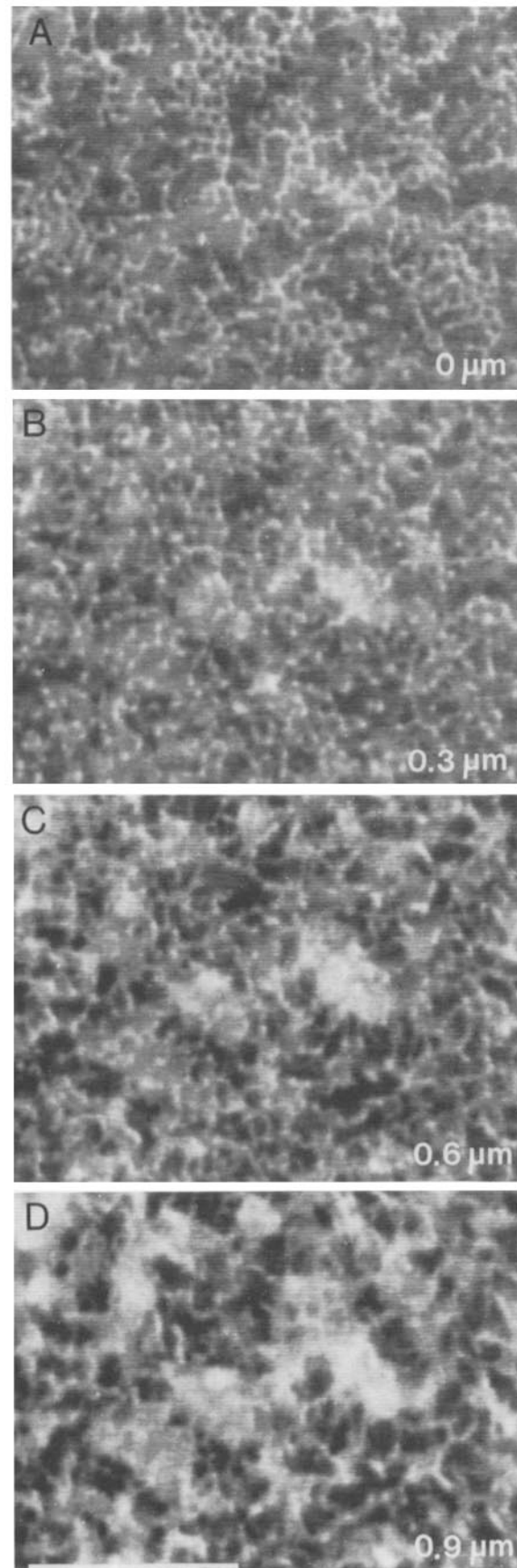


Figure 5. ER of the unfertilized egg. Serial optical sections in 0.3- μm steps, moving from the cortex deeper into the cytoplasm. (A) The network of tubular membranes in the cortex. (B) 0.3 μm beyond the cortical layer of ER. Bright dots may represent cross sections of ER tubules oriented perpendicular to the cell surface. (C) 0.6 μm beyond the cortical layer. (D) 0.9 μm beyond the cortical layer. Bar, 10 μm .

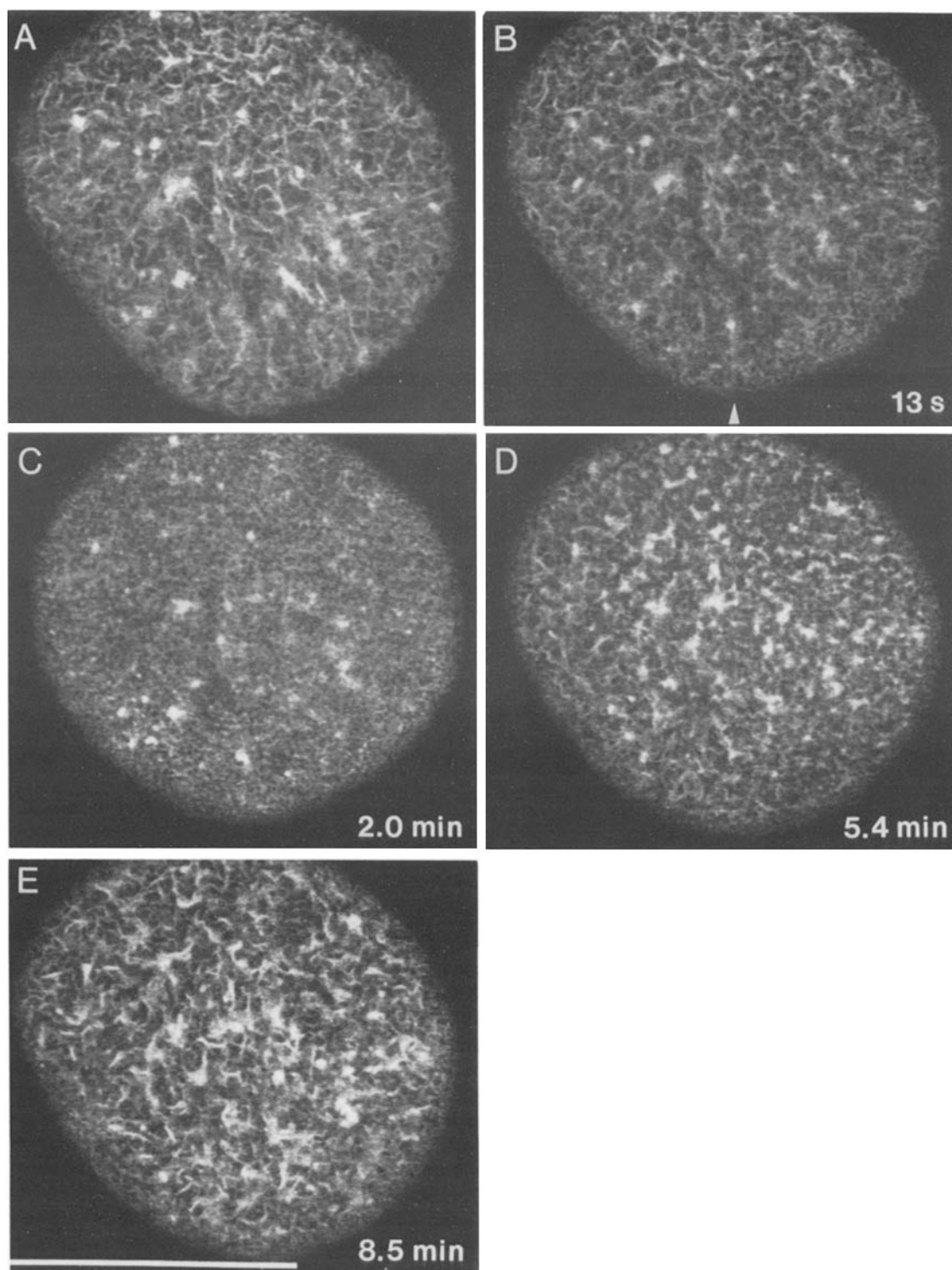


Figure 6. Change in the ER structure at fertilization. Optical sections 10 μm below the egg surface. (A) Before fertilization. (B) 13 s after the change in the ER structure was first detected. In comparison with A, a change in structure can be seen in the lower half of the egg but not in the upper half. The arrow marks the region where sperm entry was subsequently observed. (C) 2.0 min after the ER change began. Large cisternae seen in A are no longer visible. (D) 5.4 min after the ER change began. Large lamellar sheets are reappearing. (E) 8.5 min after the ER change began. The structure of the ER is similar to that before fertilization. Bar, 50 μm .

marking the point of sperm entry could be seen, the wave was observed to start in that area ($n = 3$ eggs). Because the wave did not have a sharp front, it was not possible to measure precisely the time required for the wave to cross the egg; however, this time was estimated to be $\sim 15\text{--}30$ s ($n = 4$ eggs).

After the initial wave crossed the egg, the dimensions of the sheets of membrane continued to decrease over the next 1–3 min (Figs. 6 C and 7 B). At this time, the sizes of the ER components were so small that their organization could not be clearly discerned with the light microscope, but bright

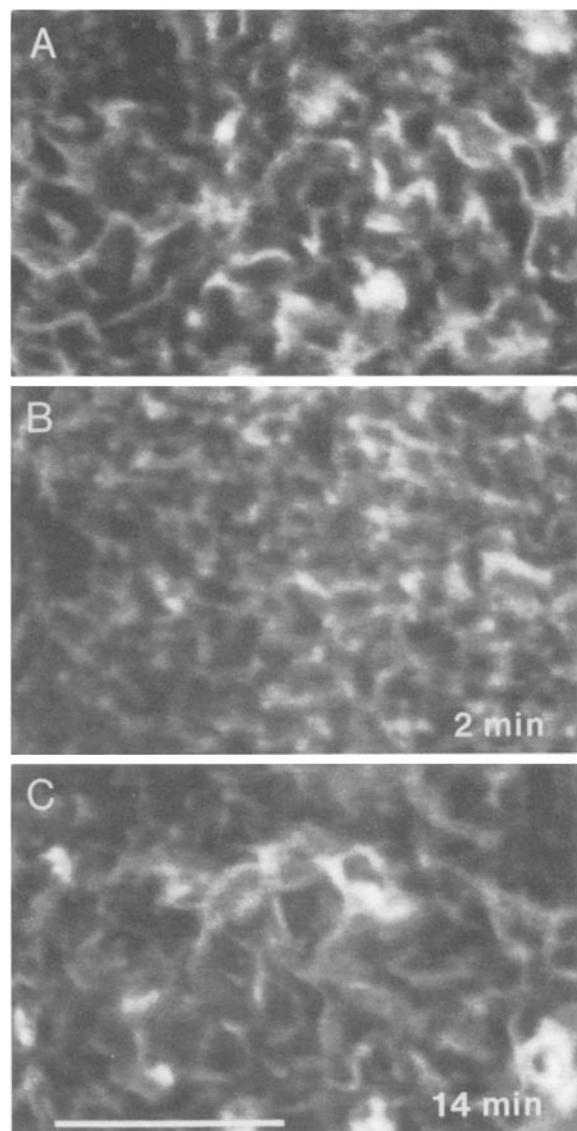


Figure 7. Change in the ER structure at fertilization. Optical sections 10 μm below the egg surface. Higher magnification views from another egg like that shown in Fig. 6. (A) Before fertilization. (B) 2 min after the ER change began. (C) 14 min after the ER change began. Bar, 10 μm .

dots in the image suggested the presence of tubular ER (Fig. 7 B). Bright dots which might be interpreted as optical sections through tubular ER were also seen in the thin transitional layer between cortical and internal ER in the unfertilized egg (Fig. 5 B). However, except in this region, bright dots of comparable size were not generally seen in the ER of unfertilized eggs or in fertilized eggs after 10 min. At 3–5 min after the initial change, the internal ER began to reassemble into larger sheets (Fig. 6 D). By 5–8 min, the internal ER had returned to an organization similar to that of the unfertilized egg (Fig. 6 E and 7 C), except in the region of the developing sperm aster, as will be described below. These measurements of the kinetics of the division and reformation of the ER structure were made from continuous recordings of 4 eggs that were viewed at 10–20 μm from the surface.

Observations at the cortex showed that the disruption of the cortical network occurred within 1–2 min after sperm

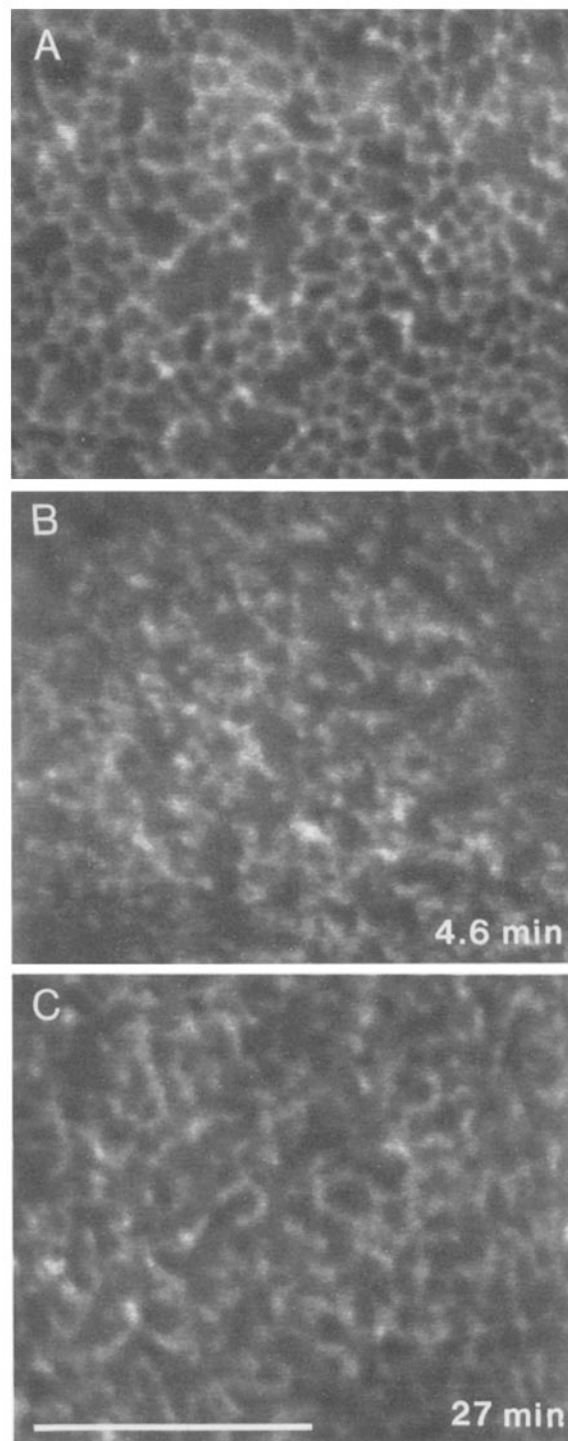


Figure 8. Change in cortical ER structure at fertilization. (A) Before fertilization. (B) 4.6 min after the ER change began. The cortical ER network has been disrupted. (C) 27 min after the ER change began. The cortical ER network is reforming. Bar, 10 μm .

were added to the observation chamber. At $\sim$ 10–30 min after fertilization, the network pattern of the cortical ER began to reappear (Fig. 8 C). By the time of cleavage, the ER in the cortex closely resembled the ER in the cortex of the unfertilized egg (not shown). These changes in the cortical ER network were seen in three out of three eggs examined, but their time course was not determined precisely.

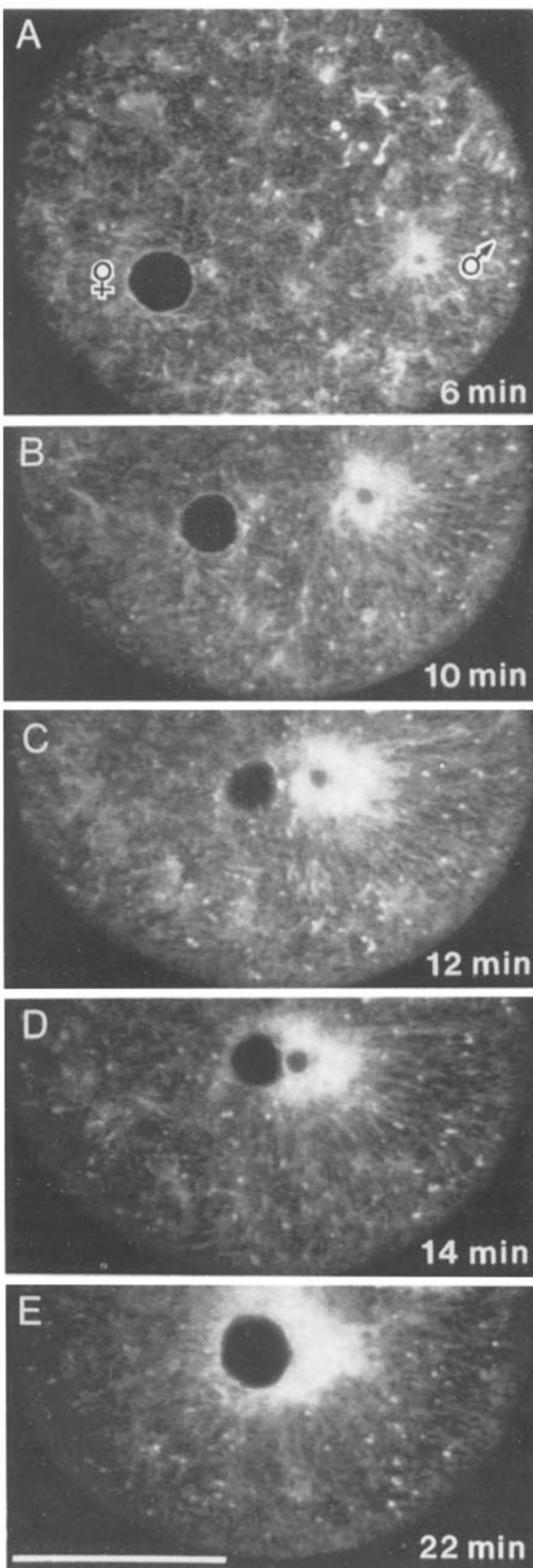


Figure 9. Accumulation of DiI-stained membranes during pronuclear migration. Times indicate minutes after fertilization. (A) 6 min. Symbols indicate the female and male pronuclei. Membrane accumulation around the male pronucleus is already occurring. Increased accumulation is seen at subsequent time points. (B) 10 min. (C) 12 min. (D) 14 min. (E) 22 min. Pronuclear fusion has occurred. Bar, 50 μ m.

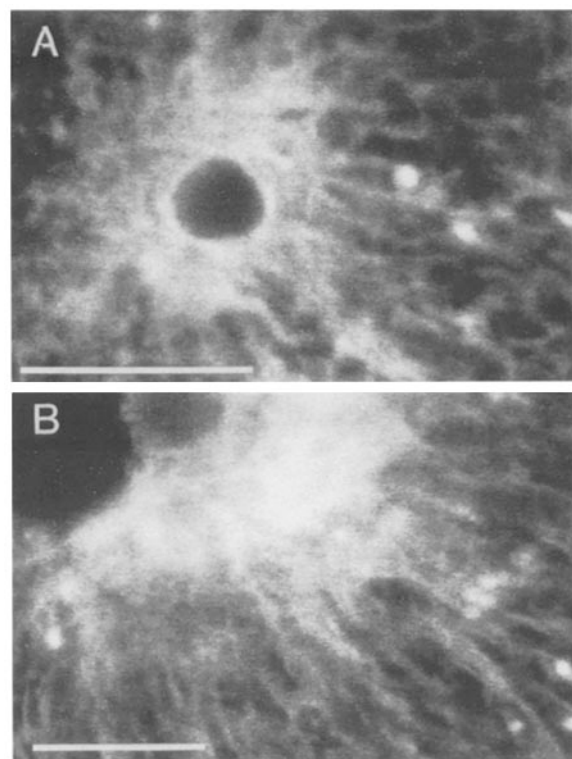


Figure 10. Accumulation of DiI-stained membranes during pronuclear migration. Same embryo as Fig. 9. (A) 7 min after fertilization. (B) 15 min. The female and male pronuclei are now in contact. The image brightness was set to optimize the image of the membrane array several microns from the nuclei, although this resulted in saturation of the fluorescence image closer to the nuclei. At lower image brightness or smaller confocal aperture, structural detail in the more central region still could not be resolved, for reasons as discussed in the text. Bars, 10 μ m.

Accumulation of DiI-stained Membranes around the Male Pronucleus and Zygote Nucleus

During the period of pronuclear migration, DiI-stained membranes accumulated around the male pronucleus (Figs. 9 and 10) (observations in five eggs). No such accumulation was seen around the female pronucleus; however, when the female pronucleus reached the male pronucleus, it became engulfed in the membranes associated with the male pronucleus. The membranes around the male pronucleus were seen in the same location as the microtubules that surround the male pronucleus during its migration (Longo and Anderson, 1968; Bestor and Schatten, 1981; Hamaguchi et al., 1985), and were aligned in a radial pattern like that of the microtubule aster. Within the mass of DiI stained membranes, the two pronuclei fused and formed the zygote nucleus (Fig. 9).

The membranes remained accumulated around the zygote nucleus throughout the $\sim$ 30 min between pronuclear fusion and the beginning of mitosis (continuous observations of four embryos). At the time of mitosis (Fig. 11), the membranes around the nucleus became arranged in a pattern similar to that of the microtubule arrays (Harris, 1975; Bestor and Schatten, 1981; Hamaguchi et al., 1985; Henson et al., 1989). Components of the membranes in the mitotic apparatus were aligned like the astral microtubules as well as the spindle microtubules (Fig. 11, A, B, and C). No obvious breakdown of the DiI-stained membranes in the mitotic ap-

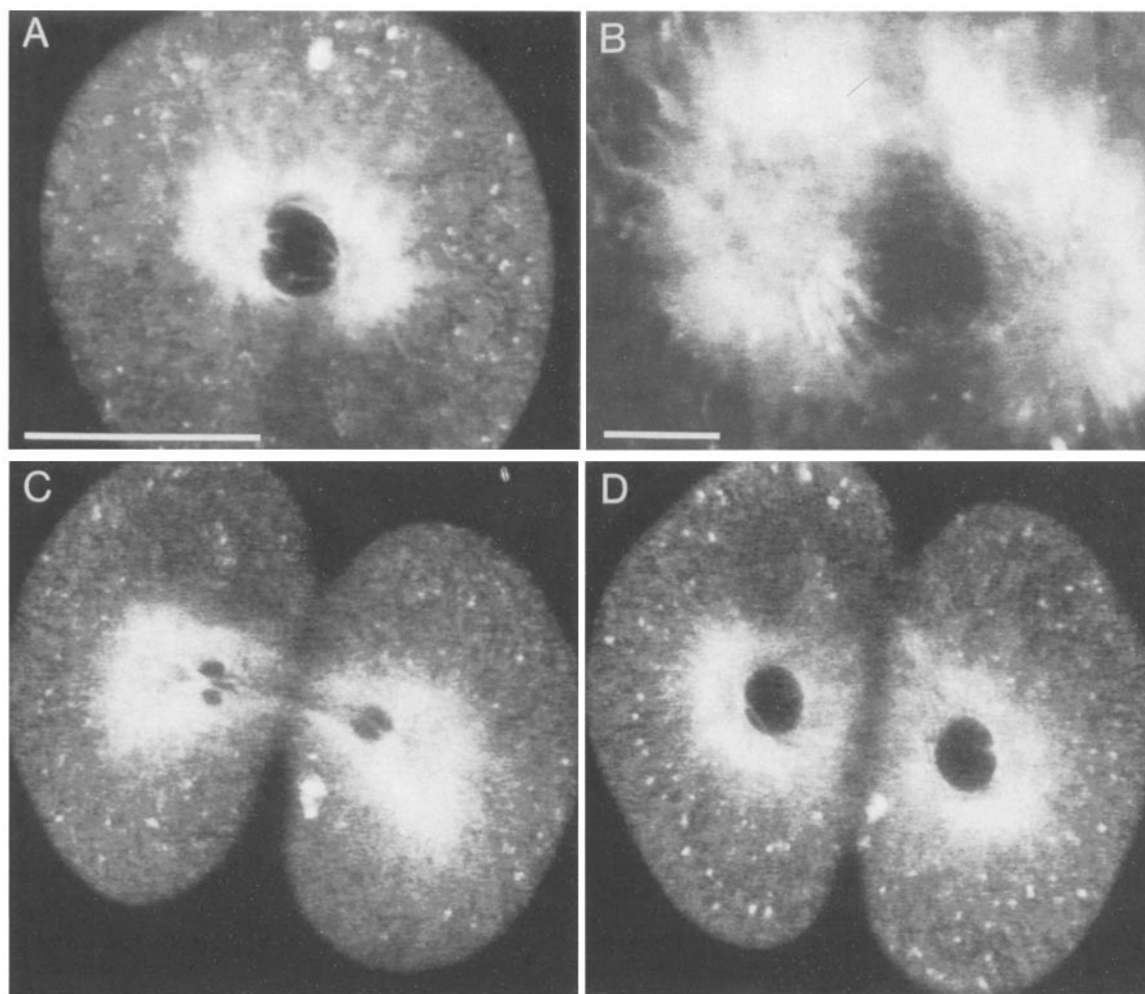


Figure 11. Distribution of DiI-stained membranes during mitosis. (A) Late prophase. (B) Metaphase or early anaphase. (C) Telophase. (D) Interphase. As described both here and in the text, we were unable to obtain better resolution of the membranes in the dense central region. Bars: (A, C, and D) 50 μm ; (B) 10 μm .

paratus or in the surrounding cytoplasm was seen at the time of the nuclear envelope breakdown (Fig. 11 B). DiI-labeled embryos proceeded through normal first and second cleavage. Throughout this period, DiI-stained membranes remained accumulated around the nucleus, even during interphase (Fig. 11 D). Membrane accumulation in the cell center throughout the mitotic cycle was observed in continuous recordings from four embryos. Although later development was not observed systematically, two embryos were seen to develop to normal mesenchyme blastulae.

Attempts to resolve detail in the central regions of the sperm aster and mitotic asters where the membranes were most dense were unsuccessful, probably in part because the membranes were separated by distances smaller than the resolution of the light microscope (~ 200 nm; see Longo and Anderson (1968) and Harris (1975) for electron micrographs of these regions). Also, these structures were beyond the ~ 10 - μm region from the surface where the best images could be obtained. The dye appeared, nevertheless, to be confined primarily to the ER, since there was no evidence in regions at the edge of the dense masses (Figs. 10 and 11 C) of staining of the yolk platelets, membrane-bound organelles of sufficient size to be seen if dye was incorporated (Franklin, 1965; Summers and Hylander, 1974). However, we cannot

exclude the possibility that membrane-bound organelles derived from the DiI-stained ER by subsequent membrane budding were also stained. The presence of small bright patches in the cytoplasm (see Figs. 9–11) suggested that with time, this may be occurring.

Discussion

Staining of the ER by Intracellular Injection of a DiI-saturated Oil Drop

DiI is a lipophilic molecule that has two 18 carbon alkyl chains; these chains intercalate into the bilayer of red blood cell membranes (Axelrod, 1979). In many other cells as well, DiI has been found to stain only membranes (see Haugland, 1989). DiI diffuses within bilayers (Fahey et al., 1977), but does not transfer at appreciable rates to adjacent membranes (Dragsten et al., 1981; Honig and Hume, 1986), presumably because of its strong association with bilayers via its long carbon chains. Thus, in the egg, DiI is very likely to spread in a continuous membrane bilayer, though it could also stain other membrane compartments through dye transfer by membrane traffic.

An antibody to a calsequestrin-like protein has been

shown to label ER membranes in sea urchin eggs (Henson et al., 1989). Staining by DiI resembles this labeling very closely. The calsequestrin-like protein is seen in ER cisternae by electron microscopic observations of immunoperoxidase stained sections; it is also seen by immunofluorescence microscopy of semi-thin frozen sections as a "tubuloreticular network" throughout the egg (Henson et al., 1989). The pattern of antibody staining appears very much like DiI staining, but which the better resolution afforded by serial confocal images reveals to be a system of interconnected lamellar sheets. The staining by both techniques corresponds with conventional electron microscopy, which shows profiles of ER cisternae throughout the egg cytoplasm (see Longo and Anderson, 1968; Summers and Hylander, 1974; Luttmner and Longo, 1985; Henson et al., 1989). Both the antibody and DiI also label a cortical network that corresponds to rough ER seen on isolated egg cortices by electron microscopic methods (Sardet, 1984; Chandler, 1984) and by staining with fluorescent dyes (Henson et al., 1989; Terasaki, M., J. H. Henson, D. A. Begg, B. Kaminer, and C. Sardet, unpublished results). A continuous boundary around the nucleus is labeled by both methods; correspondingly, electron microscopic studies have shown that the outer membrane of the nuclear envelope often has ribosomes bound to it and is in continuity with other elements of rough ER (Watson, 1955). Lastly, two other organelles, the cortical granules and the yolk granules, are not stained by either technique. Thus, both techniques specifically stain at least major parts of the ER of the sea urchin egg.

It is not known, however, if these techniques stain all of the ER. It is possible that some ER membranes do not contain the calsequestrin-like protein or that some ER membranes are discontinuous with the DiI-stained ER membranes. It is also possible that the methods label other compartments besides the ER. Both techniques show a concentration of membranes in the mitotic apparatus; profiles of smooth tubular membranes in this region have been observed by electron microscopy (Harris, 1975). Although these membranes may be smooth ER, some of them may be parts of other, recently discovered organelles, such as the *trans*-Golgi network (Griffiths and Simons, 1986), an "intermediate compartment" between the ER and Golgi apparatus (Lippincott-Schwartz et al., 1990), or tubular lysosomes (Swanson et al., 1987); these other organelles could conceivably contain the calsequestrin-like protein or be stained by DiI through membrane traffic.

Regardless of these considerations, the observed staining by DiI, within the time frame of the present experiments, is of a membrane system that corresponds with known characteristics of the ER. The novelty of the staining by DiI is that it allows temporal observations of the three dimensional organization of the ER in a living cell. Sea urchin embryos injected with DiI-saturated oil drops develop normally, undergoing normal cortical granule exocytosis and early cleavage, and in preliminary observations, developing to blastulae. The occurrence of these normal physiological processes indicates that DiI is not perturbing cellular functions. With this technique, fixation damage is avoided, and the use of a living cell allows observation of ER motility and structural changes. This enables us to add new information to that known from previous studies of fixed eggs.

Characterization of the ER of Unfertilized Eggs

The DiI staining method shows two major forms of the ER in the unfertilized egg: a stationary tubular network at the cortex, and a motile, uniformly distributed system of interconnected lamellar sheets (cisternae) in the interior. The movements of the interior ER are apparently independent of microtubules, because few if any microtubules are present in the unfertilized egg (Harris et al., 1980). In contrast, the two-dimensional network of tubular ER in the thin periphery of cultured cells and in growth cones of sympathetic neurons undergoes constant rearrangements, including tubule extensions, retractions, branchings, and fusions, which are closely related to microtubules (Terasaki et al., 1986; Lee and Chen, 1988; Dailey and Bridgman, 1989; Sanger et al., 1989; Lee et al., 1989).

The staining by DiI provides evidence for extensive continuity of sea urchin egg ER. Since DiI diffuses only in continuous bilayers, a simple explanation of its staining is that the cortical network and interior cisternae are completely interconnected and comprise all of the ER. However, the staining does not definitively prove this, because as noted above, there may be discontinuous ER membranes that are not stained by dye diffusion, and it is conceivable that discontinuous ER membranes could be stained through dye transfer by membrane traffic, or that discontinuous ER compartments are stained through multiple contacts with the oil droplet. The continuity of ER membranes is of interest because a cell-wide interconnected membrane could serve to coordinate processes throughout the cytoplasm (e.g., Palade, 1956; Porter, 1961); the spread of DiI in sea urchin eggs suggests that at least part of the ER forms such a compartment.

Reorganization of the ER at Fertilization

The structural change in the ER at fertilization begins as a wave that passes across the egg within ≤ 30 s, like the wave of Ca release (Eisen et al., 1984; Swann and Whitaker, 1986; Hafner et al., 1988; Hamaguchi and Hamaguchi, 1990). Then the organization of the ER returns to an arrangement similar to that of the unfertilized egg; this is completed by 5–8 min after fertilization. In *Lytechinus pictus*, cytoplasmic Ca returns towards its prefertilization level with a roughly similar time course (Crossley et al., 1991), although an exact comparison of kinetics cannot be made because our measurements and those of Crossley et al. were made at different temperatures. The approximate temporal correlation of the rise and fall in intracellular Ca and the structural changes in the ER suggests that these events are related, particularly since the source of Ca that is released at fertilization is probably the ER (Eisen and Reynolds, 1985; Han and Nuccitelli, 1990; Terasaki, M., and C. Sardet, unpublished results).

The initial change in the ER structure occurs throughout the cytoplasm. The uniform, apparently nonpolarized distribution of the ER begins to change at about the time microtubules first appear in the cytoplasm as an astral array around the sperm nucleus (see Longo and Anderson, 1968; Bestor and Schatten, 1981; Hamaguchi et al., 1985). At this time, DiI-stained membranes accumulate near the sperm pronucleus as it migrates to the center of the egg. The presence of tubular membranes in the sperm aster was previously de-

scribed by Longo and Anderson (1968), who proposed that these membranes were ER. Using confocal microscopy of living eggs stained with DiI, we have provided additional evidence that these membranes are ER, and that they are concentrated in the sperm aster relative to adjacent regions of the cytoplasm. This accumulation of membranes probably accounts for the accumulation of clear cytoplasm in this region, described by Chambers (1939) as "the lake." Since ER moves along microtubules in other cells (reviewed in Terasaki, 1990), and since the movement of the female pronucleus into the sperm aster is microtubule mediated (Zimmerman and Zimmerman, 1967; Hamaguchi and Hiramoto, 1986), it is possible that the accumulation of membranes within the sperm aster is due to minus-end-directed motors associated with ER moving along microtubules of the sperm aster. Once accumulated in the cell center, the membranes remain concentrated around the nucleus during mitotic stages (Harris, 1975; Henson et al., 1989), as well as during interphase.

Possible Functions of the ER Changes at Fertilization

The structural changes in the ER are likely to have functional significance with respect to other events occurring at fertilization. One such possible function concerns the breakdown of the sperm nuclear envelope and its reformation after decondensation of the sperm chromatin. The breakdown of the sperm nuclear envelope begins at ~1–2 min after insemination (Longo and Anderson, 1968; *Arbacia punctulata*, 20–23°C), at about the same time that the ER cisternae throughout the egg cytoplasm are becoming more finely partitioned. Reformation of the male pronuclear envelope is complete by ~6 min after insemination (Longo, 1976; *Arbacia punctulata* 20°C), at about the same time that the ER cisternae throughout the egg cytoplasm regain their original structure. Since the nuclear envelope is continuous with the ER, and since experimental evidence indicates that the male pronuclear envelope is derived from the egg ER (Longo, 1976), it is possible that the breakdown and reformation of the sperm nuclear envelope are a special case of the general ER changes. Thus one function of the ER changes could be to allow breakdown and reformation of the sperm nuclear envelope, events that are necessary to allow sperm chromatin to decondense (see Longo and Anderson, 1968). Although we did not observe corresponding changes in the egg nuclear envelope, this part of the ER could be resistant to the changes occurring elsewhere. Likewise, it has been observed that the nuclear envelope at the anterior and posterior ends of the sperm does not undergo breakdown (Longo and Anderson, 1968; Longo, 1976). Another possible function of the ER changes within the first few minutes after fertilization could be to facilitate the initial movement of the sperm nucleus into the egg cytoplasm, since the partitioning of the ER cisternae might change the mechanical properties of the cytoplasm (see Hiramoto, 1969a,b). The accumulation of membranes in the center of the fertilized egg (see also Harris, 1975; Henson et al., 1989) could be of functional significance in relation to processes that occur during mitosis (Scholey et al., 1985; Petzelt and Hafner, 1986; Silver, 1986).

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References

- Axelrod, D. 1979. Carbocyanine dye orientation in red blood cell membrane studied by microscopic fluorescence polarization. *Biophys. J.* 26:557–574.
- Baumann, O., T. Kitazama, and A. P. Somlyo. 1990. Laser confocal scanning microscopy of the surface membrane/t-tubular system and the sarcoplasmic reticulum in insect striated muscle stained with DiI_{Ca}(3). *J. Struct. Biol.* 105:154–161.
- Bestor, T. H., and G. Schatten. 1981. Anti-tubulin immunofluorescence microscopy of microtubules present during the pronuclear movements of sea urchin fertilization. *Dev. Biol.* 88:80–91.
- Campanella, C., P. Andreuccetti, C. Taddel, and R. Talevi. 1984. The modifications of cortical endoplasmic reticulum during in vitro maturation of *Xenopus laevis* oocytes and its involvement in cortical granule exocytosis. *J. Exp. Zool.* 229:283–293.
- Campanella, C., R. Talevi, D. Kline, and R. Nuccitelli. 1988. The cortical reaction in the egg of *Discoglossus pictus*: a study of the changes in the endoplasmic reticulum at activation. *Dev. Biol.* 130:108–119.
- Chambers, E. L. 1939. The movement of the egg nucleus in relation to the sperm aster in the echinoderm egg. *J. Exp. Biol.* 16:409–424.
- Chandler, D. E., and J. Heuser. 1979. Membrane fusion during secretion. Cortical granule exocytosis in sea urchin eggs as studied by quick-freezing and freeze fracture. *J. Cell Biol.* 83:91–108.
- Charbonneau, M., and R. D. Grey. 1984. The onset of activation responsiveness during maturation coincides with the formation of the cortical endoplasmic reticulum in oocytes of *Xenopus laevis*. *Dev. Biol.* 102:90–97.
- Crossley, I., T. Whalley, and M. Whitaker. 1991. Guanosine 5'-thiotriphosphate may stimulate phosphoinositide messenger production in sea urchin eggs by a different route than the fertilizing sperm. *Cell Reg.* 2:121–133.
- Dailey, M. E., and P. C. Bridgman. 1989. Dynamics of the endoplasmic reticulum and other membranous organelles in growth cones of cultured neurons. *J. Neurosci.* 9:1897–1909.
- Dragsten, P. R., R. Blumenthal, and J. S. Handler. 1981. Membrane asymmetry in epithelia: is the tight junction a barrier to diffusion in the plasma membrane? *Nature (Lond.)* 294:718–722.
- Eisen, A., and G. T. Reynolds. 1985. Source and sinks for the calcium released during fertilization of single sea urchin eggs. *J. Cell Biol.* 100:1522–1527.
- Eisen, A., D. P. Kiehart, S. J. Wieland, and G. T. Reynolds. 1984. Temporal sequence and spatial distribution of early events of fertilization in single sea urchin eggs. *J. Cell Biol.* 99:1647–1654.
- Fahey, P. F., D. E. Koppel, L. S. Barak, D. E. Wolf, E. L. Elson, and W. W. Webb. 1977. Lateral diffusion in planar lipid bilayers. *Science (Wash. DC)* 195:305–306.
- Franklin, L. E. 1965. Morphology of gamete membrane fusion and of sperm entry into oocytes of the sea urchin. *J. Cell Biol.* 25:81–100.
- Gardiner, D. M., and R. D. Grey. 1983. Membrane junctions in *Xenopus* eggs: their distribution suggests a role in calcium regulation. *J. Cell Biol.* 96:1159–1163.
- Griffiths, G., and K. Simons. 1986. The trans-Golgi network: sorting at the exit site of the Golgi complex. *Science (Wash. DC)* 234:438–443.
- Hafner, M., C. Petzelt, R. Nobiling, J. B. Pawley, D. Kramp, and G. Schatten. 1988. Wave of free calcium at fertilization in the sea urchin egg visualized with fura-2. *Cell Motil. Cytoskel.* 9:271–277.
- Hamaguchi, M. S., and Y. Hiramoto. 1986. Analysis of the role of astral rays in pronuclear migration in sand dollar eggs by the colcemid-UV method. *Dev. Growth Diff.* 28:143–156.
- Hamaguchi, Y., and M. S. Hamaguchi. 1990. Simultaneous investigation of intracellular Ca²⁺ increase and morphological events upon fertilization in the sand dollar egg. *Cell Struct. Funct.* 15:159–162.
- Hamaguchi, Y., M. Toriyama, H. Sakai, and Y. Hiramoto. 1985. Distribution of fluorescently labeled tubulin injected into sand dollar eggs from fertilization through cleavage. *J. Cell Biol.* 100:1262–1272.
- Han, J. K., and R. Nuccitelli. 1990. Inositol 1,4,5-trisphosphate-induced calcium release in the organelle layers of the stratified, intact egg of *Xenopus laevis*. *J. Cell Biol.* 110:1103–1110.
- Harris, P. 1975. The role of membranes in the organization of the mitotic apparatus. *Exp. Cell Res.* 94:409–425.
- Harris, P., M. Osborn, and K. Weber. 1980. Distribution of tubulin-containing structures in the egg of the sea urchin *Strongylocentrotus purpuratus*. *J. Cell Biol.* 84:668–679.
- Haugland, R. P. 1989. Handbook of Fluorescent Probes and Research Chemicals. Molecular Probes, Inc., Eugene OR. 234 pp.
- Henson, J. H., D. A. Begg, S. M. Beaulieu, D. J. Fishkind, E. M. Bonder, M. Terasaki, D. Lebeche, and B. Kaminer. 1989. A calsequestrin-like protein in the endoplasmic reticulum of the sea urchin: localization and dy-

- namics in the egg and first cell cycle embryo. *J. Cell Biol.* 109:149–161.
- Henson, J. H., S. M. Beaulieu, B. Kaminer, and D. A. Begg. 1990. Differentiation of a calsequestrin-containing endoplasmic reticulum during sea urchin oogenesis. *Dev. Biol.* 142:255–269.
- Hiramoto, Y. 1969a. Mechanical properties of the protoplasm of the sea urchin egg. I. Unfertilized egg. *Exp. Cell Res.* 56:201–208.
- Hiramoto, Y. 1969b. Mechanical properties of the protoplasm of the sea urchin egg. II. Fertilized egg. *Exp. Cell Res.* 56:209–218.
- Hiramoto, Y. 1974. A method of microinjection. *Exp. Cell Res.* 87:403–406.
- Honig, M. G., and R. I. Hume. 1986. Fluorescent carbocyanine dyes allow living neurons of identified origin to be studied in long-term cultures. *J. Cell Biol.* 103:171–187.
- Honig, M. G., and R. I. Hume. 1989. DiI and DiO: versatile fluorescent dyes for neuronal labeling and pathway tracing. *Trends Neurosci.* 12:333–341.
- Kachar, B., and T. S. Reese. 1988. The mechanism of cytoplasmic streaming in characean algal cells: sliding of endoplasmic reticulum along actin filaments. *J. Cell Biol.* 106:1545–1552.
- Kiehart, D. P. 1982. Microinjection of echinoderm eggs: apparatus and procedures. *Methods Cell Biol.* 25:13–31.
- Kishimoto, T. 1986. Microinjection and cytoplasmic transfer in starfish oocytes. *Methods Cell Biol.* 27:379–394.
- Larabell, C. A., and D. E. Chandler. 1988. Freeze-fracture analysis of structural reorganization during meiotic maturation in oocytes of *Xenopus laevis*. *Cell & Tissue Res.* 251:129–136.
- Lee, C., and L. B. Chen. 1988. Behavior of endoplasmic reticulum in living cells. *Cell.* 54:37–46.
- Lee, C., M. Ferguson, and L. B. Chen. 1989. Construction of the endoplasmic reticulum. *J. Cell Biol.* 109:2045–2055.
- Lippincott-Schwartz, J., J. G. Donaldson, A. Schweizer, E. G. Berger, H.-P. Hauri, L. C. Yuan, and R. D. Klausner. 1990. Microtubule-dependent retrograde transport of proteins into the ER in the presence of brefeldin A suggests an ER recycling pathway. *Cell.* 60:821–836.
- Longo, F. J. 1976. Derivation of the membrane comprising the male pronuclear envelope in inseminated sea urchin eggs. *Dev. Biol.* 49:347–368.
- Longo, F. J., and E. Anderson. 1968. The fine structure of pronuclear development and fusion in the sea urchin, *Arbacia punctulata*. *J. Cell Biol.* 39:339–368.
- Luttmner, S., and F. J. Longo. 1985. Ultrastructural and morphometric observations of cortical endoplasmic reticulum in *Arbacia*, *Spisula* and mouse eggs. *Dev. Growth Diff.* 27:349–359.
- Palade, G. E. 1955. A small particulate component of the cytoplasm. *J. Biophys. Biochem. Cytol.* 1:59–67.
- Palade, G. E. 1956. The endoplasmic reticulum. *J. Biophys. Biochem. Cytol.* 2:85–98.
- Palade, G. E. 1975. Intracellular aspects of the process of protein secretion. *Science (Wash. DC)*. 189:347–358.
- Palade, G. E., and K. R. Porter. 1954. Studies on the endoplasmic reticulum. I. Its identification in cells in situ. *J. Exp. Med.* 100:641–656.
- Palade, G. E., and P. Siekevitz. 1956. Liver microsomes. An integrated morphological and biochemical study. *J. Biophys. Biochem. Cytol.* 2:171–200.
- Petzelt, C., and M. Hafner. 1986. Visualization of the Ca^{2+} transport system of the mitotic apparatus of sea urchin eggs with a monoclonal antibody. *Proc. Natl. Acad. Sci. USA.* 83:1719–1722.
- Porter, K. R. 1961. The ground substance: observations from electron microscopy. In *The Cell*. D. J. Brachet and A. E. Mirsky, editors. Chapter 9. Vol. II. Academic Press, New York. 621–675.
- Ross, C. A., J. Meldolesi, T. A. Milner, T. Satoh, S. Supattapone, and S. H. Snyder. 1989. Inositol 1,4,5-trisphosphate receptor localized to endoplasmic reticulum in cerebellar Purkinje cells. *Nature (Lond.)*. 339:468–470.
- Sanger, J. M., J. S. Dome, B. Mittal, A. V. Somlyo, and J. W. Somlyo. 1989. Dynamics of the endoplasmic reticulum in living non-muscle and muscle cells. *Cell Motil. Cytoskel.* 13:301–319.
- Sardet, C. 1984. The ultrastructure of the sea urchin egg cortex isolated before and after fertilization. *Dev. Biol.* 105:196–210.
- Schatten, G. 1981. Sperm incorporation, the pronuclear migrations, and their relation to the establishment of the first embryonic axis: time-lapse video microscopy of the movements during fertilization of the sea urchin *Lytechinus variegatus*. *Dev. Biol.* 86:426–437.
- Scholey, J. M., M. E. Porter, P. M. Grissom, and J. R. McIntosh. 1985. Identification of kinesin in sea urchin eggs, and evidence for its localization in the mitotic spindle. *Nature (Lond.)*. 318:483–486.
- Silver, R. B. 1986. Mitosis in sand dollar embryos is inhibited by antibodies directed against the calcium transport enzyme of muscle. *Proc. Natl. Acad. Sci. USA.* 83:4302–4306.
- Streb, H., E. Bayerdorffer, W. Haase, R. F. Irvine, and I. Schulz. 1984. Effect of inositol-1,4,5-trisphosphate on isolated subcellular fractions of rat pancreas. *J. Membr. Biol.* 81:241–253.
- Summers, R. G., and B. L. Hylander. 1974. An ultrastructural analysis of early fertilization in the sand dollar, *Echinarchinus parma*. *Cell & Tissue Res.* 150:343–368.
- Swann, K. S., and M. J. Whitaker. 1986. The part played by inositol trisphosphate and calcium in the propagation of the fertilisation wave in sea urchin eggs. *J. Cell Biol.* 103:2333–2342.
- Swanson, J., A. Bushnell, and S. C. Silverstein. 1987. Tubular lysosome morphology and distribution within macrophages depends on integrity of cytoplasmic microtubules. *Proc. Natl. Acad. Sci. USA.* 84:1921–1925.
- Terasaki, M. 1989. Fluorescent labeling of endoplasmic reticulum. *Methods Cell Biol.* 29:125–135.
- Terasaki, M. 1990. Recent progress on structural interactions of the endoplasmic reticulum. *Cell Motil. Cytoskel.* 15:71–75.
- Terasaki, M., J. D. Song, J. R. Wong, M. J. Weiss, and L. B. Chen. 1984. Localization of endoplasmic reticulum in living and glutaraldehyde fixed cells with fluorescent dyes. *Cell.* 38:101–108.
- Terasaki, M., L. B. Chen, and K. Fujiwara. 1986. Microtubules and the endoplasmic reticulum are highly interdependent structures. *J. Cell Biol.* 103:1557–1568.
- Watson, M. L. 1955. The nuclear envelope. Its structure and relation to cytoplasmic membranes. *J. Biophys. Biochem. Cytol.* 1:257–270.
- White, J. G., W. B. Amos, and M. Fordham. 1987. An evaluation of confocal versus conventional imaging of biological structures by fluorescence light microscopy. *J. Cell Biol.* 105:41–48.
- Wilgram, G. F., and E. P. Kennedy. 1963. Intracellular distribution of some enzymes catalyzing reactions in the biosynthesis of complex lipids. *J. Biol. Chem.* 238:2615–2619.
- Zimmerman, A. M., and S. Zimmerman. 1967. Action of colcemid in sea urchin eggs. *J. Cell Biol.* 34:483–488.