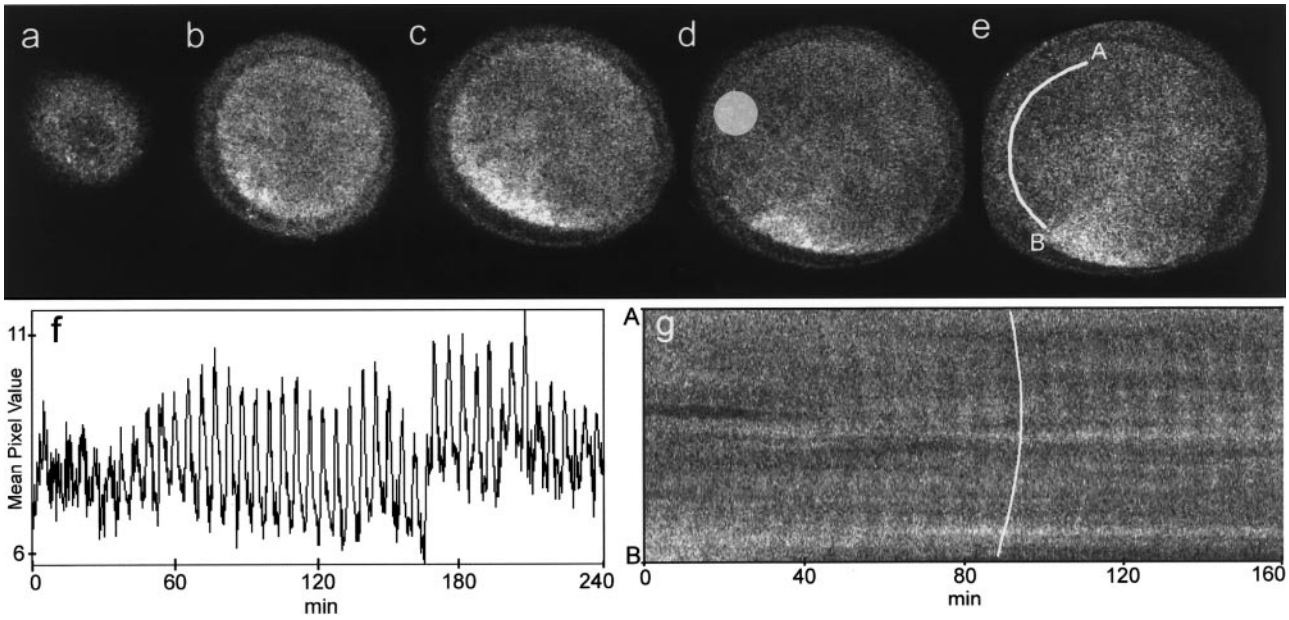

Figure 1. *Lateral views of a 5-somite zebrafish embryo showing 5 parasagittal imaging planes (a–e). The planes are separated by 40 μm and span a total of 200 μm in depth. Plane (a) is nearest the surface of the embryo while plane (e) is nearest the midline. The time interval represented by (a–e) is near 200 min in (f). The circle in (d) shows a sampling area whose pixel intensity profile is shown in (f). The curved line in (e) indicates the path in the X-Y plane along which the data stack was re-sliced, as shown in (g). The white line in (g) shows the approximate time profile of one wave.*

through the embryo towards the tail bud. Other waves showed the inverse pattern, *i.e.*, they started near the tail bud and traveled rostrally. The three-dimensional structure of the calcium waves hinted at by these initial experiments are far more complex than could be inferred from sequences imaged at single optical planes. To completely reconstruct the waves through space and time, the temporal phase of wave peak values must be quantified for all X-Y positions in all optical planes through the time series. Such analyses may provide further clues to the developmental role of the panembryonic calcium signaling system.

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Squid Sperm to Clam Eggs: Imaging Wet Samples in a Scanning Electron Microscope

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We introduce Wet SEM, a new imaging technology that allows electron microscopy of wet samples. The samples are placed in sealed specimen capsules and are separated from the vacuum in the microscope chamber by an impermeable, electron-transparent membrane. Imaging is performed in a standard scanning electron microscope (SEM) using a backscattered electron detector (BSE) (Fig. 1A). The technique is described in a pending patent application (1).

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The Wet SEM technique presents, in many respects, a new imaging modality. First, the complete separation of the sample from the vacuum allows direct imaging of fully hydrated, whole-mount samples in an electron microscope operating at moderate beam energies. Such samples include primary or cultured animal cells, micro-organisms, plants, animal and human tissues, and a variety of fluid samples. Second, the use of backscattered electron detection and the elimination of charging allow the internal structure of cells to be visualized by scanning electron microscopy. This is in contrast with customary SEM imaging (using secondary electron detection), which is restricted to the surface. The depth of

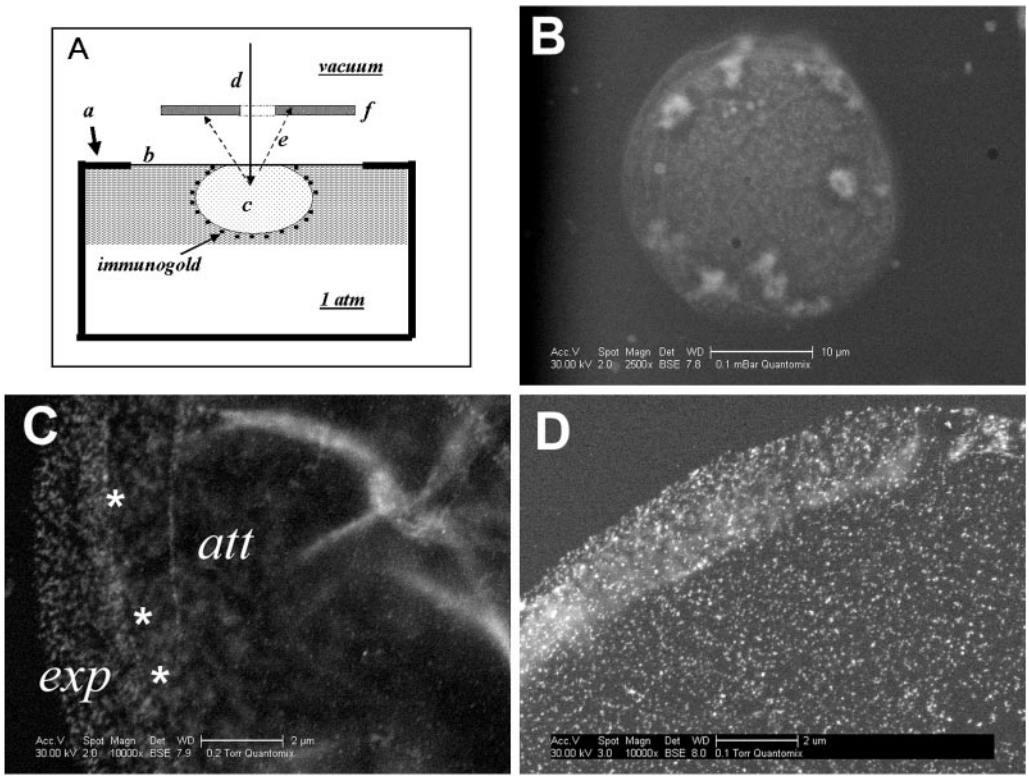

Figure 1. *Wet SEM imaging of clam egg nuclei. (A) Schematic cross-sectional view of the capsule enclosing a sample. The generally rigid capsule (a) is topped by a window, covered by an electron-transparent partition membrane (b). The sample (c) is in close contact with the partition membrane. When placed in the evacuated chamber of a SEM, the sample is maintained in a wet state at atmospheric pressure. The microscopic image is obtained when the scanning electron beam (d) penetrates the partition membrane and interacts with the sample, and backscattered electrons (BSE) (e) are detected by a BSE detector (f). (B) Clam egg nucleus, adhering to the capsule's partition membrane, was stained with uranyl acetate and imaged in a wet state in an scanning electron microscope (FEI XL-30 ESEM-FEG). Bar = 10 μm. (C) Clam egg nucleus, adhering to the capsule's partition membrane, labeled with anti-nuclear pore complex antibodies and 0.8-nm gold secondary antibodies, followed by silver enhancement. Bar = 2 μm. (D) Clam egg nucleus, labeled as in (C) but with anti-lamin antibodies. Bar = 2 μm.*

the imaged layer is limited by the penetration of the beam electrons, and is estimated to be 2–3 μm. Furthermore, the depth can be varied by changing the acceleration voltage of the electron beam (typically within the range of 10–30 keV), yielding three-dimensional information.

The method presents some additional beneficial features, some of which were not wholly anticipated. We obtain resolutions between 10–100 nm, an order of magnitude smaller than could be predicted from the volume of interaction of the electron beam with an aqueous sample. Contrast between materials of low atomic number, such as carbon and oxygen, can be readily detected. Thick samples, such as tissue biopsies, can be imaged without thin sectioning: only the top layer of up to 3 μm is seen. Both the global and high-resolution distribution of colloidal gold labels on cells can be readily determined.

The Wet SEM is uniquely suitable for samples, including lipid-rich structures and hydrated gels, which are adversely affected by standard processes of dehydration that use organic solvents. The method has several practical advantages over standard EM techniques that derive from the simplicity of sample preparation; these advantages include the ability to process and image numerous samples, the ability to look at whole cells, the imaging of tissue specimens (especially of epithelial tissues), and the imaging of myelin sheaths in neural tissue.

During our 2-month stay at the Marine Biological Laboratory, we have explored several applications of the technology with scientists and visitors. One example is shown in Figure 1. Clam egg nuclei were fixed in formaldehyde, then placed in the imaging capsule. A brief centrifugation (500 × *g*, 5 min) caused the nuclei to adhere stably to the poly-L-lysine-coated partition membrane, and all subsequent treatments were performed in the capsule.

Figure 1B shows a nucleus stained with uranyl acetate; the condensed chromosomes are clearly visible, as are the outline of the nucleus and diffusely stained nuclear proteins. The chromosomes seem to be surrounded by a dark "halo," the significance of which is not yet clear.

Figure 1C shows a portion of a nucleus stained with antibodies to the nuclear pore complex, followed by secondary antibodies that are conjugated to 0.8-nm colloidal gold particles. The silver-enhanced gold particles are visible as bright dots. Note the edge (asterisk) between the region of the nuclear membrane that is attached to the partition membrane of the capsule (*att*) and the region that is exposed to the solution (*exp*); the immunolabel, which recognizes the outer aspect of the nuclear pore, has bound

only the exposed region (as depicted in the schematic distribution of gold labels in Fig. 1A).

Figure 1D shows a portion of a nucleus stained with antibodies to lamin. Note that, in contrast to Figure 1C, the immunolabeling extends through the entire visible region of the nucleus. We attribute the difference to the location of lamin inside the nucleus, so access by labeling antibodies is not blocked by adhesion to the capsule's partition membrane.

These results show that the Wet SEM technique can derive meaningful information at high resolution from samples that were subjected to treatments comparable to those used to prepare for light microscopy.

This work was performed in collaboration with Yosef Gruenbaum of the Hebrew University and Robert Goldman of Northwestern University. The XL-30 scanning electron microscope was a generous loan from FEI Company, Peabody, MA.

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Formation of the Blastoderm and Yolk Syncytial Layer in Early Squid Development

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You can observe a lot by watching.
—Yogi Berra

Nearly all of what we know of cephalopod development has been drawn from live or fixed embryos collected from naturally laid jelly capsules (1, 2, 3). Many aspects of development—including pronuclear migration and cleavage (4), cytoplasmic and cellular movements (5), differentiation and organogenesis—would be better understood and more effectively compared to other embryos if examined in the living state. To begin this work, we have used time-lapse video microscopy to record the development of *in vitro*-fertilized squid embryos, *Loligo pealeii,* from early cleavage through the formation of the external yolk syncytial layer (YSL) and the early phase of epiboly. Adding the dimension of time to our analysis revealed new and intriguing elements in the development of this organism.

In vitro-fertilized squid embryos were prepared (6) and oriented for imaging in depressions made in 0.2% agarose (Sigma)-lined plastic petri dishes (Falcon) filled with Millipore (0.22 μm) filtered seawater. Dishes of embryos were placed on a universal transmitted light illuminator with an adjustable reflector that allowed for bright field or oblique illumination, or a combination of the two. To minimize heat transfer, a KL 1500 constant-color-temperature fiber-optic source with an infrared filter was used to illuminate the specimen. A Zeiss Stemi SV 11 stereomicroscope with a 1.6× Planapochromat lens was used for time-lapse imaging. An intermediate mount was placed between the lens and microscope body to align the light path with the center of the front lens, right eyepiece, and camera. A computer-controlled Axiovision software program was used for image acquisition from an MRc5 Zeiss digital camera set to optimal resolution (2584 × 1936). Digital images were collected at 5- or 7-min intervals at 21 °C for 2–12.5 h. Throughout these periods, embryos appeared to cleave and develop normally.

Ten separate time-lapse sessions were carried out and images from two are presented. In the first session, images were collected at 5-min intervals, from first cleavage through blastoderm formation; three of these images are presented in Fig. 1a–c. The first image was taken 8 h post-fertilization (hpf) after the fourth cleavage (Fig. 1a). Each cleavage furrow is numbered in the order of its occurrence, and the polar bodies (pb and arrow) are visible resting in the first cleavage furrow. The larger blastomeres, formed by the unequal third cleavage furrow characteristic of cephalopod embryos (1), identify the future anterior midline of the embryo. The second image (Fig. 1b) was taken 9.6 hpf at sixth cleavage (arrowhead). This cleavage separates the central blastomeres from the outer syncytial layer of the embryo, which is continuous with the yolk cell. The final image (Fig. 1c) was taken 16.25 hpf and reveals a well-defined blastoderm surrounded by radiating clusters of cells, the outermost of which are continuous with the yolk cell. The boundary created at sixth cleavage (arrowhead) remains well preserved.

In the second session, images were collected at 7-min intervals, from blastoderm formation to the onset of epiboly, 26–27.5 hpf; three of these images are presented in Fig. 1d–f. At 26 hpf, the boundary formed at sixth cleavage has become more distinct, as cells have moved into the blastoderm; and pairs or small clusters of sister blastomeres formed during earlier cleavages radiate

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