With my research on determination and cell sorting drifting to a dead end and the turmoil of my personal life settled, I was ready and eager in many ways to begin a new life. In retrospect the last years of my marriage with Galya and the few years of my ecstatic courtship with Madeleine were considerable distractions from the planning and progress of my research. Now, cosily married to Madeleine, and having made a decision to remain at Yale, my mind was finally cleared and I was free to contemplate and plan seriously what direction my subsequent research should take. An added event egged me on. Right after I assumed my professorship I was invited to present one of the principal papers at the 25th Symposium of the Society for Developmental Biology on *Major Problems in Developmental Biology* to take place at Haverford College in 1965. This was an honor to be taken seriously and I did so. I chose *Morphogenetic Cell Movements* for my title, for this was what was on my mind and where I decided my major research efforts would be henceforth. Following on my readings of Holtfreter's stimulating papers on gastrulation at the end of my graduate work, my own studies of epiboly in the *Fundulus* embryo, and my abrupt realization of the motile capacities of many normally stable tissue cells from the aggregation work, I decided to concentrate my research on cell movements or, more generally, cell motility in the Metazoa, multicellular animals, with emphasis on the morphogenetic cell movements of gastrulation and early development.

Curiously, once again at an important juncture of my research career I encountered my friend Dietrich Bodenstein. To my delight, he was the chair of my session at the Symposium and gave me a brief but generous and humorous introduction. By then, Dietrich was also in good shape personally. He finally had an academic position commensurate with his distinguished scientific and human qualities—Professor of Biology at the University of Virginia (where he quickly became a fan of Mr. Jefferson). Ordinarily I am reasonably relaxed when lecturing, but this time I was distinctly nervous. The audience was very distinguished, including Dietrich, C.H. Waddington, Paul Weiss and others of similar stature. But once I got going, my well-honed speaking abilities took over.
In order to try to understand the motility of ordinary tissue cells, i.e., cells bereft of specialized motile organs like cilia and flagella, I had to turn myself into a cell biologist, both intellectually and technically. Fortunately, with the advent of spectacular technical advances like electron microscopy, phase-contrast and Nomarski optics, and autoradiography, to name a few, the field of cytoplasmic cell biology had become light-years more sophisticated than it was when I was a graduate student at Columbia (taking the Cytology Course 25 years previously). With these new armaments and vastly increased understanding of the cytoplasm, the subject was so changed it was no longer called "cytology." A first step in my new program of research was to reinvigorate my study of epiboly in *Fundulus* with the benefit of some of the new technology. For this I needed to become proficient in electron microscopy and time-lapse cinemicrography. The latter was easy, the former difficult. With good luck, the husband of my devoted research assistant, Judy Lentz, was a cell biologist in the Anatomy Department of the Yale Medical School, an expert electron microscopist, a fine, careful scientist and a person eager to teach me. So I moved myself over to the Medical School a few days a week to learn some electron microscopy with Thomas Lentz and the Anatomy Department’s electron microscope technician (a position we lacked in the Biology Department). It was great fun being exposed to a most powerful and elegant but very subtle technique with infinitely patient instructors. My work over there in Anatomy with Tom Lentz soon led to a very profitable collaboration in Woods Hole on the fine-structure of the *Fundulus* blastoderm during cleavage, blastulation, and gastrulation. Now that I was in a position to evaluate the quality and the meaning of electron micrographs, I decided that my most productive research effort with electron microscopy would be collaborative. I know a lot about fish development and could thus define the problem and with my surgical skill prepare the material for fixation; my collaborator would do the electron microscopy. We would then evaluate the results and write the papers together. This worked out very well with Tom and the result was three papers eventually published in the *Journal of Cell Biology*, representing my formal entry into the field of cell biology.

To renew my research on *Fundulus* meant a return to Woods Hole where I had not worked since before that great year in France. It was the summer just before my marriage to Madeleine at Yale. Madeleine loved Woods Hole. The beaches and sun brought back fond memories of her many vacations in La Ciotat on la Côte d’Azur and she reveled in the immediate, warm acceptance and hospitality of my Woods Hole friends. She especially enjoyed the relaxed social life of the laboratory, something she had never been exposed to before. We spent frequent evenings in the Cap’n Kidd with various friends.
REJUVENATION
OF RESEARCH

and at small cocktail parties. Also, this was her first total immersion in the English language. Although her command of English was still pretty rudimentary, she managed well with her desire, vigor, vivacity and, of course, her marvelous Gallic charm. I naturally took great pride in displaying my warm, beautiful woman. Along with the sun, the beaches, and our friends, she also adored our unpretentious little shack in the woods, now her own summer home, and in no time, with the little money we had, transformed and endowed it with a decorative charm it had never had before.

In that first summer at Woods Hole for Madeleine, we had a number of attractive friends but certain ones stood out. They were, of course, Mike and Ruth Bennett, and we met Allison and Marie Burnett at what turned out to be sort of a coming-out cocktail party thrown by Red and Lil Saunders in their Devil’s Lane cottage. Al was an embryologist like me and a member of the staff of the Embryology Course. Madeleine and I immediately found Al and Marie sympathique—wonderful, vivacious company.

Al was strikingly handsome, even beautiful, like a Greek god in a Michelangelo statue. He had a trim, well-proportioned body, a finely crafted chin, sparkling eyes, and flamboyant mane of curly, prematurely graying hair. He was full of all kinds of funny stories, a truly gifted raconteur, and even a poet. The four of us quickly struck it off and spent much time together throughout the languid summer evenings, laughing at Al’s elaborate stories. He would always start a story by saying that every word of his tale was true, no matter how weird and hilarious the stories were that he unfolded. He delivered his convoluted tales in an elaborate and colorful style, at a rapid, entertaining pace, all the while laughing, moistening his lips, and looking about for encouragement. He was a wonderful companion.

Al was also an avid and sophisticated bird-watcher, which led to a memorable expedition one early morning in July. He and Phil Dunham, another jolly friend, arrived to pick me up at 5:30 A.M. in the Burnett station wagon, properly equipped with binoculars and a couple of 6-packs of cold beer. With the windows wide open, Al would identify birds for us from their songs and then we tried to find them with the car and see them with the binoculars. After the beer had flowed and our senses sharpened, we heard and saw more and more birds (without even leaving the car). By 8:00 A.M., the birds stopped singing and there were fewer of them available for sighting by car. So we all went home and went back to bed.

Phil was a cell physiologist and some years after the birding adventure, we did a neat little experiment on Fundulus eggs with our mutual friend Mike Bennett. As I look back on it, Phil and Al were two of the most fun-loving people Madeleine and I have ever known. Mirth and outright laughter came so easily with both of them. Regretfully, however, we haven’t seen either of them now for
many years. Phil got divorced and remarried. His new bride unexpectedly did not like Woods Hole, particularly, I think, some of Phil’s partners in crime. So he stopped coming.

Al Burnett later on slowly, tragically, and inexorably drank himself out of his excellent faculty position at Northwestern, out of his marriage, and, finally out of his life. His decline and ultimately his premature death were apparently beyond his control. What a sad, heartbreaking end that was for everybody who knew him. How could someone who enjoyed life so much end it so tragically? Obviously, some of the joy with which he enlivened so much the daily lives of his many friends must have been a façade. Years ago, alcoholism was viewed as a character flaw. Al could be the poster boy for the notion that alcoholism is a disease like diabetes mellitus or schizophrenia. As far as I know, Al didn’t have a bad bone in his body. Al Burnett was brilliant, handsome, generous, apparently tortured by unseen demons, but now he is simply gone. I’m getting depressed just writing about it. I wish he were here to lift my spirits with a funny story.

In the laboratory, my plan was to study the surface activity and contact relations of the four entities of the Fundulus embryo during cleavage, blastulation and gastrulation—namely, the monolayered enveloping layer (EVL) at the surface, the deep cells and the yolk syncytial layer (YSL) beneath the EVL, and the yolk cytoplasmic layer (YCL) containing the yolk (see Figure 6.1). For this study we used electron microscopy and were particularly interested to learn what changes occur during gastrulation, particularly epiboly, at the level of fine structure. It was quite exhilarating, a fascinating old problem with a new technique and a new collaborator. An unknown world was about to open up in familiar territory. And it did. I refer the reader to Chapter 6, for detailed description of the developmental anatomy of a fish embryo during these early stages.

We found that the cells of the epithelial enveloping layer were joined together by circumferential, occluding tight junctions as early as the cleavage stages, coupling each cell tightly to its immediate neighbors. Then, as development proceeds and epiboly begins, these tight junctions increase in extent and other junctions appear, in particular desmosomes, which are fiber-anchored cytoplasmic plaques that firmly attach many epithelial cells to their neighboring cells. During epiboly, the EVL is subjected to ever-increasing tension, as the blastoderm expands to cover the large yolk sphere, and its cells need to be firmly attached. The circumferential tight junctions and desmosomes serve this function and prevent the cells from being torn apart by the tension created by traction forces generated by spreading at the margin of the blastoderm and transmitted across the continuous epithelial sheet of the superficial cells of the blastoderm.

Gap junctions are also present. These are so called because although their plasma membranes are closely apposed to one another, there is a narrow intercellular gap clearly visible between them. Gap junctions have another interesting feature. They consist of arrays of six protein subunits, embedded in
and spanning the cell membrane, creating aqueous channels between adjacent cells. Gap junctions are of great importance physiologically because they allow passage of small molecules such as water, ions, and even small regulatory substances between cells. They provide the structural basis for electrical coupling between cells, perhaps allowing groups of coupled cells to communicate with one another. Mike Bennett, my old friend from his undergraduate days at Yale, who was then, after his Rhodes Scholarship years at Oxford, a Professor of Neuroscience at the Albert Einstein School of Medicine, was just down the hall from me at the MBL. We were both interested in studying electrical coupling in Fundulus, he because of his involvement in the electrophysiology of cell coupling and I because, as an embryologist, I am always interested in cell communication. Mike, as an old alumnus of Zoo 23, was also damned interested in embryos. The collaborative research project was easy for me. I would provide Mike with nice, clean dechorionated (remember, few can accomplish this difficult microsurgical maneuver without trashing the embryo) Fundulus embryos at the right stage of development and he would study their electrical properties with his sophisticated electrophysiological apparatus and delicately controlled microelectrodes. Mike sported a rather luxurious beard at the time and I was always worried that he would drag it through the medium as he hunched over the microscope. He found that the cells of the EVL are indeed electrically coupled by the gap junctions and by extracellular space as well. He also found that the outer surfaces of the EVL and the yolk cytoplasmic layer have extraordinarily high electrical resistance, that is, they are virtually impermeable. Both of these discoveries are of great interest embryologically—the coupling to provide a means of communication between cells and the low permeability of the egg surface to protect the egg from the variations in salinity of its capricious, intertidal aqueous environment. While Mike, Phil Dunham, and I were making electrophysiological observations, we also exposed Fundulus eggs to radioactive water for an hour or so and then checked them for uptake of radioactivity. Only a minute quantity of radioactive water entered the embryos and very slowly at that. The small amount of labeled water that did get in also took a long time to get out. We found that the embryo is even impermeable to water! So, the Fundulus embryo doesn’t develop in brackish seawater at all; it develops inside in its own internal environment, its own milieu interieur, in the phrase of the great nineteenth-century French physiologist, Claude Bernard. Its impermeable surface and its tough chorion protect the Fundulus embryo satisfactorily from the vicissitudes of its tumultuous and variable environment.

Cell Rearrangement

An arresting feature of Fundulus gastrulation is that once the EVL is formed in the blastula stage, its cells essentially cease dividing. How then does
this cellular monolayer handle its great expansion during epiboly with only about 5,000 cells? In two ways. First, its individual cells expand in area enormously, under the tension, becoming exceedingly thin and flat; its array of junctions preventing the cells from pulling apart. This explains the integrity of the EVL during the great expansion of epiboly but does not address another feature—the topographic changes within the EVL as its margin expands approaching the equator of the egg and then diminishes, with a decrease in the number of cells at the margin, as it moves past the equator toward the vegetal pole. Second, Raymond Keller (then at Berkeley) and I decided later on that individual cells of the EVL must somehow change position, i.e., rearrange, to make for these topographic changes. Ray had already shown, during his postdoctoral stay in our lab at Yale, before he joined the Berkeley faculty, that equally tightly joined epiblast cells of the frog, *Xenopus*, rearrange extensively during gastrulation, as the cell sheet moves toward the blastopore to undergo involution. So why not *Fundulus* EVL cells? In an important way the *Fundulus* cells were more interesting because we had considerably more electron microscopic and electrophysiological information about them.

I would fix and stain the surface of the EVL with the old-fashioned silver nitrate technique to reveal its cell boundaries, and Ray would record the detailed process of rearrangement of the very thin, highly transparent living cells with time-lapse cinemicrography, using Nomarski differential interference contrast optics. Because of the thinness of the cells, this presented an exceedingly challenging focusing problem. We found, however, in spite of these technical difficulties, that cells of the EVL do indeed rearrange during epiboly and appear to do so without disrupting the tight permeability barrier. For example, individual cells recede from the margin during the last half of epiboly and cells in the deep interior of the sheet move and rearrange also. With this, we established that the cells of the EVL adjust to the topographic changes in the sheet imposed by epiboly by active rearrangement. Indeed, these militarily precise rearrangements are integral active players in the mechanism of epiboly. We had affirmed for *Fundulus* epiboly a new class of morphogenetic cell movement.

Exciting and important though this discovery was for helping understand gastrulation, it raised an intriguing and mystifying cell biological question. Given their junctional complexes with large tight junctions, lots of desmosomes and gap junctions, how do EVL cells rearrange with no leakage? They must either break and reform their intercellular junctions rapidly or somehow slide along the surfaces of their immediate neighbors without breaking the junctions, much as jostling arrays of soap bubbles can rearrange, exchanging neighbors, without separating. The answer is not known. Clearly these neatly defined, structurally complex junctions that appear to be so stable in static electron micrographs are in fact very dynamic. This presents a fundamental
problem for the cell biologist that has not yet attracted appropriate attention. And, finally, there is another unanswered question. What forces control the wondrous precision of these rearrangements?

Among my collaborators, a few people stand out. Raymond Keller is one of these. He came to do postdoctoral research with me on the recommendation of a dear friend from my student days at Woods Hole, Ray Watterson. Keller had just completed his doctoral research at the University of Illinois, a masterly study of the normal morphogenetic movements of gastrulation in *Xenopus*, when he came to our lab to begin studies of the cellular mechanisms underlying gastrular movements. Thus began one of my closest friendships. Stocky in stature, keen of intellect, deft of hand, funny, and absolutely nuts about gastrulation, Ray is a distinct personage and fabulous to work with side-by-side, hour-by-hour. With our often idiotic ways and our running jokes, he and I have always had a marvelously good time together, at work or play. Also, Madeleine's comely daughter, Anne-Laure, just loved Ray's eyes—"*Quels beaux yeux*," she said one evening! First thing I did the next morning was take a look. Ray and I had a lot of fun together at Woods Hole, not only in scientific collaboration, but also in informal social settings (Figure 9.1).

Figure 9.1. The author shucking clams for dinner on the deck of his home in Woods Hole. Ray Keller took the photograph.
Programmed Endocytosis

In my first studies of epiboly, I had established that the underlying yolk syncytial layer has the capacity to undergo epiboly by itself, bereft of the rest of the blastoderm (see Chapter 6). This observation suggested that the YSL could be the motive force of epiboly and that the epiboly of the cellular blastoderm is therefore passive, except for the active cell rearrangements within the EVL sheet. Since only the marginal cells of the EVL are attached to the YSL, the attachments between them must be especially firm. The electron microscope revealed that they are indeed firm. Where each marginal cell of the EVL is in contact with the YSL, it forms an especially extensive tight junction. As epiboly progresses and the pull on the EVL by the independently expanding YSL increases, the already large tight junctions increase further, and the marginal surface of each EVL cell becomes deeply embedded in the underlying YSL. This contact remains unbroken as epiboly advances and the EVL is put under more and more tension.

There is additional reason to believe that the YSL is the motive force in epiboly. The YSL completes epiboly independently of the EVL and the deep cells. Indeed, removal of the EVL and deep cells leads to an acceleration of YSL epiboly, as if it is normally held back by the burden of pulling the cellular blastoderm. So, given the central role of the YSL in blastoderm epiboly, its own epiboly deserved close scrutiny. After all, we are talking about a truly spectacular developmental process—during epiboly, the surface area of this syncytium and its attached EVL expands more than tenfold!

As the YSL expands, the yolk cytoplasmic layer (YCL) (with which it is confluent) diminishes. Does the YCL surface contribute to the YSL surface during this process? When carbon marks are placed on the YCL during epiboly, they remain immobile until approached by the expanding YSL; then they simply remain in place at the margin of the YSL and are carried with it to the vegetal pole. The YCL surface seems to disappear at or in the immediate vicinity of the border of the YSL. It does not contribute to the expansion of the YSL surface.

Carbon marking experiments answered one question but raised others. How does the surface of the YCL disappear at the margin of the YSL and where does it go? Being dissuaded that it does not simply evaporate into thin air we looked more carefully. There were a myriad of vesicles in the marginal cytoplasm of the YSL viewed in the electron micrographs. This observation suggested to my then collaborator, Teiichi Betchaku, that these vesicles may be derived from endocytosis or engulfment of the surface of the YCL, and, if so, this would explain the disappearance of YCL surface in that region. To test for endocytosis, we placed eggs undergoing epiboly in a solution of the fluorescent dye lucifer yellow for a few minutes and then washed and observed them in a microscope suitably equipped to detect the fluorescent tracer. What we saw was absolutely stunning! The thin band of the external YSL at the junction of the YSL
with the YCL was jam-packed with tiny fluorescent yellow vesicles, indicating rapid endocytosis of cell surface. Moreover, the endocytosis was confined to this narrow strip, forming a thin, bright, fluorescent ring around the egg. No other part of the egg surface was fluorescent. Our previous studies of the surface of the E-YSL with SEM showed it to be highly folded, suggesting that this folding might be somehow involved in endocytosis at this precise locale. We checked this by exposing eggs in mid-epiboly to ferritin, an electron-dense protein. Sure enough, when viewed in the electron microscope, the ferritin was engulfed in the valleys between the folds, indicating that this highly local endocytosis is somehow related to this highly local folding of the E-YSL. This explains the disappearance of the YCL at this juncture. We found that the YCL is internalized and gets out of the way, instead of standing in the way of the expanding YSL. Since this endocytosis occurs in Holtfreter solution or even distilled water, it is not mediated by macromolecules in the medium. In short, we are dealing with a programmed ring of internalization of cell surface that passes as a slow wave of precisely localized endocytosis down the surface of the egg only during epiboly. There is no endocytosis before epiboly begins, none after it ends, and none elsewhere on the egg surface. Since this internalization accounts for the disappearance of the YCL as it is replaced by the YSL, it represents a crucial facet in the mechanism of Fundulus epiboly and is therefore a prime example of programmed endocytosis serving as a morphogenetic mechanism. Moreover, it may well operate in other embryonic systems where cell surface diminishes. Incidentally, the endocytotic vesicles derived from the surface of the YCL do not join the surface of the YSL from beneath. They move down into the lower cytoplasm of the YSL.

**Microvilli and the Storage of Cell Surface**

The YCL does not contribute surface to the YSL. Where does the increasing surface of the YSL come from? It could come from the insertion of newly synthesized surface material from the cytoplasm beneath or from unfolding of surface already there at the onset of epiboly. One of my students, Carol Erickson, and I had already shown with the scanning electron microscope that stationary fibroblasts have numerous minute, finger-like projections called microvilli. Then, when the cells spread and begin to move, these microvilli are replaced by lamellipodia. Since the surface area of the microvilli of the rounded cell approximately equals that of the flat surface area of a spread cell, we concluded that the expanded surface of a spreading cell is derived from surface already there in the microvilli of a rounded cell. Insertion of new surface is not needed. When Teiichi Betchaku and I examined the surface of the internal YSL of the Fundulus egg at the onset of epiboly, we were amazed to find a strikingly similar situation, a forest of long, branched microvilli much
like those of a rounded fibroblast (Figure 9.2). Then, in more advanced stages of epiboly, these long microvilli were gradually replaced by fewer shorter microvilli. When we made a rough estimate of the total surface of the YSL at the beginning of epiboly, taking microvilli into account, we found there to be enough surface in storage to provide for almost all the spreading of surface during subsequent epiboly. An expanding YSL does not need insertion of new surface material. It has its own reserve store of ready-made surface in its microvilli before epiboly begins. The change in surface contour during the spreading of the arcane syncytial layer of a fish embryo in vivo and of the ubiquitous fibroblast in vitro is certainly striking and surely suggestive of how other cell surfaces spread during embryogenesis. How these microvilli redistribute their surface in both the fish YSL and the mammalian fibroblast is still a mystery. However, the presence of contractile microfilaments in both suggests a possible mechanism.

Figure 9.2. Scanning electron micrograph showing surface details of unspread stationary (left) and spread motile (right) deep cells of the Fundulus gastrula on the surface of the YSL. Reprinted with permission of John Wiley & Sons, Inc.

Similar to its surface, the rest of the protoplasm of the YSL also redistributes during the progress of epiboly. The thickness of the YSL at the beginning of epiboly, 27-45 μm, diminishes to 2.5-4.0 μm at the end. This large initial amount of cytoplasm clearly suffices for the subsequent expansion. The cytoplasm of the slowly replaced YCL probably also contributes. But not much. The YCL is only about 2.0 μm thick.
Since much of this fine structural analysis of the normal relations of the various layers of the *Fundulus* embryo required fixation of the whole egg, with its large viscous yolk mass, the collaboration of someone extremely skilled and creative in electron microscopy was a necessity. Teiichi Betchaku was just such a person. He was meticulous to a fault. His skills as a photographer and microscopist were so great and his standards so unreasonably high, that he was not sufficiently productive. While he worked in my lab as a postdoctoral fellow, several of my graduate students picked his brain on photography and microscopy to great advantage, increasing their technical skills as a result of his expert instruction, but being careful to take his advice with a grain of salt. He was like Ansel Adams in the scientific darkroom with his attention to achieving perfection in printing negatives. Unfortunately, he was not as prolific as Ansel Adams. He moved on to my lab from a position as our departmental electron microscopist. He was fired as department microscopist because he insisted too often that the microscopes were not yet fine-tuned enough for routine usage. However, what was a fault for the department was for me exactly what I needed for the *Fundulus* research. After many trials his persistency and meticulousness triumphed and provided us with the beautiful and convincing structural detail I have just been describing. He was a strange man and we had a strange relationship, marred frequently by misunderstandings stemming in part from our different cultural backgrounds and no doubt also from our mutual stubbornness. Me stubborn? As an old-fashioned (and patriotic) Japanese, he found it extremely difficult to express any disagreements with me verbally. It took me in my naiveté quite a while to realize that when he said humbly “Yes, but...” he really meant, “No, not at all!” Well, we had many misunderstandings, but since we were both filled with curiosity about how epiboly works and both basically are warm human beings, we produced some good stuff together. Mind you, Teiichi was by no means merely a superb technician; he was a very bright, indeed creative man. For example, it was his idea to look for endocytosis in the external YSL to explain the disappearance of the YCL surface, one of our major discoveries. However keen his intelligence, it was unfortunately quite disorganized, rendering him incapable of composing an acceptable scientific paper. In sum, in spite of misunderstandings and various aggravations, we complemented each other and, given patience, were actually a good team. Our published papers testify to this. Our *magnum opus*, covering epiboly of the EVL, YSL, and YCL, was so long that one journal wanted us to split in two. Split epiboly? Never! So we published it in the good old JEZ. Fortunately the JEZ had just gone to larger pages and smaller print, otherwise our big paper, with its 51 illustrations, would have filled a full single issue!

Teiichi loved Woods Hole, its scientific life and the swimming, and I am sure that that helped him to tolerate his frequent impatience with me. Curiously,
this very intelligent man never became fluent in English, spoken or written, in contrast to his lovely wife, Yasuko, who spoke fluent, unaccented English. (She incidentally was quite a considerable person professionally—head of the Japanese section of the Metropolitan Museum of Art.) In some ways, Teiichi was a caricature of the occidental image of a Japanese tourist. He actually bragged about possessing fifty (50) cameras. Well, when we weren’t annoyed with each other for one thing or another we genuinely enjoyed each other’s company. We had a bond; we were both entranced by epiboly in Fundulus and its potential as a model for other developmental systems.

With this brief discourse, I have tried to present in an understandable way some of the highlights of our research on how epiboly takes place during Fundulus gastrulation and how our results relate to certain problems of development generally. Now I must point out that although this research on epiboly required considerable effort on my part and that of my collaborators, it occupied me full-time only intermittently over a span of over twenty years. This is a frequent situation in scientific research. Many investigators succeed or fail in one or several lines of research fairly quickly if the problem is discrete. However, where the research problem or program entails a global system, like the development of a whole embryo, as in the case of epiboly, the research may extend over many years, with sustained attention during some years and complete neglect in others. Delays along the way may be caused in many ways, such as a sudden paucity of new fruitful ideas, the absence of eye-opening technology not yet invented or perfected, like transmission and scanning electron microscopy, and Nomarski differential interference contrast microscopy, and, finally, the distraction of new, exciting ideas in other lines of research that invited pursuit. All of these were involved in my many years of intermittent attention to epiboly. The pursuit of a scientific problem is often like that.

The Origin and History of the YSL and the Onset of Gastrulation

After the really captivating research on cell rearrangement, the storage of cell surface, and programmed endocytosis, I personally had no further good ideas for a while on how to continue attacking epiboly, and also Betchaku had disappeared, with no notification, never to be seen again. Nevertheless, my fascination with the process and my long devotion to my beautiful, faithful and sometimes cooperative companion, the Fundulus egg, never flagged; and one day I got to thinking. Here I’ve been spending a sizeable chunk of my life working on the incredible epibolic spreading of the YSL without ever taking a good look at how the egg forms this layer in the first place. Indeed, we knew little more about how this syncytium forms in teleosts generally than what was described over a hundred years ago in famous papers by Alexander Agassiz (at
Harvard and son of Louis Agassiz) and C.O. Whitman at Chicago (1884) and H.V. Wilson at the University of North Carolina (1889). My friend and colleague Charles (Chuck) Kimmel and R.D. Law at the University of Oregon had confirmed some of the old work in a small paper in the 1980s on the zebra fish but, although they didn’t carry their study on to the beginning of gastrulation, their nice study with modern optics was a stimulus for me. How about a really full study of the origin and early history of the syncytial layer in Fundulus? Why, yes, indeed. By then I was an old man in my seventies, but, being emeritus and thus relieved of teaching and committee work, I could devote full-time to it. Moreover, since the study would require mainly the use of time-lapse videomicrography, at which I was expert, I could do it all by myself. By the way, by then we were no longer making old-fashioned movies but had switched to time-lapse video. It had the immense advantage over movies in that we were able to run the tape right off and see immediately if we had gotten it right, instead of waiting for days for the film to be processed in Boston.

I knew already from the literature and my work with Tom Lentz that the cytoplasm of the YSL of Fundulus is derived from the uncleaved cytoplasm of the yolk cell at the base of the cleaving blastodisc. I soon confirmed earlier work of others, namely that it becomes nucleated by the collapse of certain marginal blastomeres during late cleavage. One day early on as I had just observed numerous nuclei in the YSL, I stepped out of the room for a few minutes. On returning, the nuclei were no longer visible! What a shock! I couldn’t believe my eyes. Where had they gone? After some frantic intense watching, however, they reappeared, smaller and twice as numerous. They had disappeared while undergoing mitosis. I felt like the fool I was. I’m sure glad nobody else was in the room.

The first YSL nuclei engage in mitosis in waves that pass through the cytoplasm from right to left or from left to right. Then, the next nuclei to invade from the blastoderm undergo mitosis, and the next, and so on. The most gripping feature of these orderly mitoses is that the first nuclei to invade divide just five times and the next nuclei to invade divide just four times and the next just three times and then they all stop mitosis simultaneously. The number of mitoses for each nucleus in the yolk syncytium is obviously controlled by their time of entry into the YSL cytoplasm. The most reasonable hypothesis to explain this regimen of mitoses is an increase in the nucleocytoplasmic ratio. There is a great increase in the number of nuclei in the forming YSL with little or no increase in cytoplasm. Just because an hypothesis is reasonable doesn’t mean it is valid. It needs to be tested by an experiment. This cessation of mitotic activity takes place gradually, with the duration of each mitosis (and especially of each interphase between mitoses) increasing steadily with each succeeding mitosis. This deceleration and cessation of the nuclear cycle in the formation of YSL was of interest to me not only as a widespread phenomenon of early development in
many organisms, as in *Drosophila*, but particularly for its special consequences in *Fundulus*. Soon after mitosis ceases in the YSL, an entirely new process begins. The now wider YSL with its numerous, evenly spaced nuclei (Figure 9.3) begins to contract.

![Figure 9.3. Evenly spaced nuclei (arrows) in the YSL of the *Fundulus* embryo. The animal pole is at the top of the photograph. Reprinted with permission of John Wiley & Sons, Inc.](image)

This contraction has several important consequences. First, the broad expanse of the nucleated YSL ringing the blastoderm narrows. With this, its nuclei become increasingly crowded and one by one slip beneath the blastoderm and nucleate the unnucleated cytoplasm of the yolk cell, forming the internal YSL. As contraction of the YSL continues, its surface is thrown increasingly into folds, laying the basis for endocytosis, which now begins precisely in the valleys between the folds. The internal YSL and the attached blastoderm, i.e., the enveloping layer, begin to be pulled over the yolk by the contracting E-YSL. This is how epiboly begins. Very soon after the onset of epiboly, other movements of gastrulation begin. Deep cells within the blastoderm undergo ingression and begin their convergent movement toward the embryonic shield, where they will contribute massively to the formation of the embryo proper. You can imagine my
wonder and excitement as I watched this sequence, so fundamental for formation of the embryo, unfold before my very eyes (accelerated and dramatized by time-lapse video).

Since these events, commencing with cessation of mitosis in the YSL, eventually result in a transition from one phase of development (cleavage and the formation of the YSL) to another phase of development (gastrulation), I have termed it the YSL transition. This transition is similar to another transition that has been intensively studied in other embryos, such as sea urchins and amphibians. Since there is no cell growth during cleavage, i.e., no increase in cytoplasm, the nucleocytoplasmic ratio is about doubled each time a blastomere divides. As the nucleocytoplasmic ratio increases, the cell cycle becomes more and more prolonged, with irregular mitoses and longer interphases, until it finally stops. The cessation of cleavage is then followed by a new developmental phase. Cells become motile and new messenger RNA (mRNA) is synthesized for the first time. Molecular biologists speak of mRNA synthesis from DNA as transcription, i.e., the genetic code of deoxyribonucleotides is transcribed into a related intermediate code of ribonucleotides in mRNA. Next, by translation, the code of mRNA is translated into an amino acid sequence that forms the backbone of a protein molecule. I confirmed this series of events in the cleavage of the Fundulus blastoderm, observing the onset of motility, but not mRNA synthesis. Chuck Kimmel and others confirmed both of these observations for the zebrafish embryo. This important transition at the end of cleavage has been named the mid-blastula transition. It precedes the YSL transition and marks the beginning of cellular differentiation. The YSL transition marks the beginning of gastrulation and morphogenesis.

This research on the formation of the YSL and the beginning of epiboly and other cell movements of gastrulation, which I count among my most important, was performed alone. I was solitary, my only company being the Fundulus embryo, my then state-of-the-art optical and video equipment, and my full view of the Hole, Nonameset Island and the flag of the Fisheries, which gave me the wind direction. I had a very good time. Collaborative research for me has been by and large profitable scientifically and enjoyable socially but there is no substitute for working by yourself with beautiful, familiar material on a problem that you know will yield significant results and new insights, given proper attention.

Zebra Fish

During the later phases of our research on Fundulus, work on the embryogenesis of the zebra fish (Brachydanio rerio) came on the scene in a rush. Warren Lewis had already appreciated the lucidity of its egg and the ease of breeding it in the laboratory in the 1940s but it was not until George Streisinger
at the University of Oregon demonstrated how its genetics could be manipulated. Did the zebra fish embryo become a major subject of research on development. Here was a vetebrate that could be easily bred in the laboratory, like the mouse; and, whose genes, like those of the mouse, could be studied for their role in development. But the zebra fish has a big advantage over the viviparous (producing live young after internal incubation in adults) mouse. It is oviparous like Fundulus. Its fertilization and development occur outside the female. For these important attributes, research on zebra fish development soon became popular, even fashionable in recent years, contributing much to our understanding of vertebrate embryogenesis.

It has been quite satisfying and exhilarating for me to watch this beautiful teleost embryonic material so widely and often wisely exploited. It has also been rewarding for my ever-voracious ego to suddenly see our work and that of Jane Oppenheimer on Fundulus and of Bill Ballard on the trout and other fish recognized by a large group of investigators and cited frequently in their publications. Unfortunately, to this day, although the importance of our work on Fundulus is duly cited and recognized, little of it has been repeated on the zebra fish embryo. This is a shame because the zebra fish egg is smaller than that of Fundulus and consequently completes its epiboly at an earlier stage of gastrulation. There may be important and interesting developmental differences between these two piscine species. To try to stir interest in repeating some of the Fundulus work on zebra fish, I invited myself to lecture on Fundulus epiboly at the biannual meeting on zebra fish genetics and development in Cold Spring Harbor in 1996 (the first time, in contrast to many others, that I invited myself to present a lecture). My invitation was accepted and I gave the George Streisinger Memorial Lecture titled Lessons from Fundulus. For a while it was to no avail. I simply did not understand why. Wasn’t anybody curious? A friend afterward complimented me on my “splendid lecture” but intoned that it was perhaps a little long. I responded like Senator Hubert Humphrey. “Was it? Well, I enjoyed every minute of it!” Incidentally, I dedicated this lecture to my long-time friend and close embryological colleague, Chuck Kimmel, for he more than anyone else stimulated research on the development of zebra fish. It is rare that a whole field of research gets started primarily from the steady, enthusiastic, and productive investigative activity and personality of one person.

So, did my lecture to a large assemblage of zebra fish embryologists have any effect? It is hard to gauge these things but maybe it did. In the last few years, for example, considerable attention has been paid to the YSL of zebra fish, particularly to patterns of movement of its nuclei and the importance of the YSL in early induction and determination of the blastoderm. One substantial paper on nuclear movement in the zebra fish YSL, published in 2001, was even dedicated to “Professor J.P. Trinkaus on the 50th anniversary of his discovery that the Yolk Syncytical Layer of the Fundulus heteroclitus embryo is capable of undergoing
autonomous epiboly after removal of the embryonic blastoderm." What a gift it
is to be so old that you have the opportunity of seeing in print appreciation of
some of your earliest work!

**Individual Cell Movement**

During those years of on and off research on the mass movement of
epiboly, I also wished to learn something about how *individual* tissue cells and
groups of them move *in vivo*, within the living organism, especially in directional
ways that lead to the formation of tissues and organs. One of the several reasons
for my fascination with this problem and my persistent interest in it was the work
by Weston on the neural crest, taking place right under my nose. Neural crest
cells move directionally as individuals and in groups, and, in many cases, park
themselves at great distances (on the embryonic scale) from their source. Neural
crest cells and a myriad of other motile tissue cells wend their way mysteriously
with only the usual equipment of virtually all tissue cells; namely, a plasma
membrane at the surface with variable adhesiveness, a highly structured
cytoplasm with microtubules and contractile microfilaments. My consuming
interest, of course, was to gain some insight into the motile activities of
embryonic cells during the massive movements of gastrulation. I soon learned
that an interest in the directional movements of cells required a broad, catholic
approach, viz., study of both normal and cancerous cells in culture. You see, in
many kinds of cancers, cells become increasingly mobile, often breaking away
from their primary site of origin, say in the breast or prostate gland, and invading
other organs such as the brain, lungs, and liver. I also became interested in all
kinds of moving cells in adults, such as wound epithelia and white blood cells,
as well as during embryogenesis. This meant not limiting myself or my
laboratory to one or another organism or tissue or to any particular stage of
development. The object was to discover something about how tissue cells move
and how they often do so directionally. Some cells are more convenient material
for this kind of research. But the emphasis was always on the problem, not the
precise material or techniques. This broad approach needed many minds and
hands and was possible for me only because during those years I had an
increased number of graduate students and postdoctoral fellows, some of whom
were outstanding. They would follow up on one or another of my ideas and
quickly generate new ideas and approaches of their own (which is what one
expects of advanced students).

Though much of our work on the movements of individual cells and
groups of cells is fascinating and of general interest, it is much too big a subject
to recount at this point. It needs a full chapter of its own and will get it after I
deviate to retrace our steps and delve into some of the memorable aspects of our
lives around and outside my research during those many years.