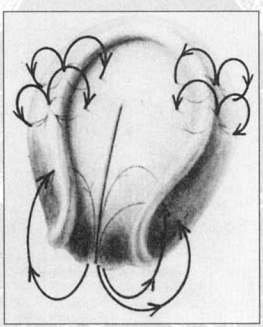
THE ELECTRIC EMBRYO

How Electric Fields Mold the Embryo's Growth Pattern And Shape



by Colin Lowry

Contrary to the prevailing view in biology today, embryonic development is controlled by electric fields, whose very existence is ignored by the molecular reductionists.

Background: Selected stages in the embryonic development of the axolotl. Foreground: Schematic of ionic currents traversing early amphibian embryo.

In the early 1970s, biophysicist Lionel Jaffe was pondering the question of how an embryo creates the body axis which determines the head from the tail, or the top from the bottom. The single-cell embryo appears to be symmetrical. As it develops, the embryo must create asymmetries and singularities leading to a body plan, and eventually to the formation of specialized cell types. How does the embryo establish its own polarity? These important questions guided the scientists of the 19th century in their investigations into the organization of living systems, but have been largely ignored in the 20th century.

Working at Purdue University, Jaffe was studying the fertilized eggs of the seaweed-like plant known as fucus to try to answer these questions. Fucus, unlike many animal species, does not have a pre-set axis in the egg, and does not create the first axis until after fertilization. Jaffe was steeped in the literature and experiments having to do with electric potentials and living systems going back into the 19th century. He recalled that in the 1920s, Elmer Lund had done experiments with fucus, and found that external factors influence the creation of the body axis. Normally, exposure to light can set the direction of the body axis in fucus, resulting in an unequal first cell division. Lund also grew fucus embryos in the dark, and by applying a weak electric current through the surrounding water, found that this current could also set the body axis.

Thinking through Lund's result, Jaffe wondered if the fucus embryos themselves created an electrical current which could establish a polarity and set the body axis. He then designed a series of experiments to test the hypothesis that the embryos created electric currents that flowed in and around themselves. To be able to measure the strength and direction of these electric currents, Jaffe and his student, Richard Nuccitelli, developed an ultrasensitive vibrating-tip microprobe in 1974. With the new probe, Jaffe measured minute ionic currents flowing in the aqueous medium around the fucus embryos, and found that these currents were driven in a loop through the embryos, establishing the body axis.

Jaffe's results re-opened investigations into electric fields in directing embryonic development, and the related process of limb regeneration. Now, armed with superior modern equipment for measuring bioelectric currents, Jaffe's students could attempt to answer questions about how electric fields could influence cell migration, wound healing, and pattern formation in the embryo, and how cells could "know" their position within an embryo. This approach was in direct opposition to the radical reductionist views that were increasingly dominating biology at that time—and still do today. Instrumental in Jaffe's unique ability to design these crucial experiments were his intellectual roots in the works of the early 20th century biologists, which remain mostly unknown to scientists today.

Two scientists, Richard Borgens and Kenneth Robinson, who were graduate students at Purdue University trained by Jaffe, have continued and expanded the investigations into electric fields and living processes up to the present day. Borgens and Robinson were part of a closely knit group of students who matured as researchers in a scientific culture very different from that found today.

With Jaffe as their mentor, there was a strong emphasis on the historical issues of science. Jaffe kept the fundamental questions in embryology of the 19th and early 20th century fresh in the minds of his students. The students were required to learn the history of science, and the contributions and crucial experiments of individual scientists stretching back to the 19th century. Three times a week the students would meet as a group with Jaffe and his colleague Joseph Vanable, to discuss projects that were ongoing in the laboratory. Richard Borgens described the importance of these meetings in providing a "cross-fertilization of ideas," which influenced his own scientific approach.

Do Currents Control Limb Regeneration?

Richard Borgens had come to Jaffe's laboratory with an established interest in limb formation during development. He had done his master's degree project on the formation of the bones of the limb in the embryonic chick. Limb development in the embryo has many features in common with limb regeneration in adult amphibians. After the development of the vibrating tip probe, the first thing Borgens examined, using this new tool, was the process of limb regeneration in adult salamanders.

Experiments from the early 1940s had shown that there are electrical changes at the surface of the limb after amputation in regenerating amphibians. In the first experiments in 1977, Borgens found that an immediate response to amputation of the limb in salamanders was the production of an intense electric current, driven out of the tip of the limb stump. This current followed a path out of the stump tip, and returned in a loop through the water, or the moisture on the skin, to the undamaged area of the limb behind the amputation. The density of the current ranged from 20 to more than 100 micro-amps (μA) per square centimeter just after amputation, and fell steadily over time to a level of just a few $\mu A/cm^2$ within a few days.

The decrease in the current flowing out from the stump coincides with the formation of the wound epithelium (skin), which creates increased resistance in the circuit path. All of the animals that naturally regenerate their limbs as adults, which includes newts, salamanders, and axolotls (all members of the urodele order, tailed amphibians) produce a strong electric current running through the tip of the amputated limb stump.

What is known to occur at the cellular level during regeneration? After amputation, the wound surface of the limb is covered by a thin wound epithelium in a matter of hours. Beneath this wound epithelium, cellular debris is transported away, and uninjured cells begin the process of de-differentiation. These cells lose their specific characteristics that identify them as muscle, bone, or cartilage cells, and transform themselves into a mesenchyme-like cell, called a blastemal cell. These blastemal cells have properties similar to embryonic cells, in that they can differentiate into any cell type. It is this group of cells, known as the blastema, that will grow and form the regenerating limb.

Experiments have shown that two tissues are essential for the initiation and control of limb regeneration. First, limb regeneration requires the formation of a wound epithelium, and cannot proceed in the absence of it. Also, the wound epithelium gives a directionality to the outgrowth of the blastemal cells below it. If the wound epithelium is surgically moved, and placed in an eccentric position upon the stump, the regenerating limb will grow in this new direction.

The second essential tissue for regeneration is the presence of intact peripheral nerves. If the nerves leading into a regener-

ating limb are removed, no regeneration can proceed. The dependence on the presence of nervous tissue has led to the search for growth factors produced by nerve cells that could help explain this phenomenon. So far, many growth factors have been identified, but none of them can substitute for the presence of the intact nerve in the limb stump.

More Questions

The electric currents that Borgens had measured at the stump tip raised several questions. What was responsible for generating this current? Did the current and the resulting electric field influence the behavior of the cells involved in regeneration? Borgens set out to find the source and the character of the electric current. It was well known that skin in amphibians and all other vertebrates produces a voltage difference across its surface. This "skin battery" maintains an inwardly positive potential ranging from 40 to 80 millivolts by actively transporting sodium ions (Na+) and other positively charged ions from outside to inside. Because the epithelial cells are themselves polarized in structure, and are tightly joined, they create a resistant barrier to the flow of positive ions in the opposite direction. However, any break in the epithelium would create a low-resistance pathway, leading to the immediate flow of positively charged ions outward.

To see if the skin battery was responsible for the generation of the currents leaving the limb stump, Borgens manipulated the concentration of sodium ions in the pond water surrounding salamanders. When the sodium ion concentration was increased five-fold, the currents that were measured leaving the limb stump were also found to increase approximately five-fold. If the salamanders were kept in water that was deficient in sodium, the stump currents were found to be reduced by 90 percent. These effects could be reversed by returning the animal to normal pond water.

Now that the currents leaving the limb stump in salamanders had been roughly defined, Jaffe, Borgens, and Vanable were curious to see if the application of these electric fields upon a non-regenerating amphibian, (anurans) such as the frog, could induce limb regeneration. They chose the grassfrog (genus *Rana*), and constructed an electric stimulator which could be implanted under the skin of the back, with two electrodes emanating from it. In one group of animals, the negative electrode was routed through the core tissues of the limb stump, with the uninsulated portion in contact with the stump tip tissue. The positive electrode was implanted beneath the skin of the back. This setup matches the polarity of the current flow that was measured in the salamander. In the second experimental group, the electrodes were reversed, with the positive electrode routed into the limb stump tip. A control group had electrodes implanted with an inactive electric stimulator.

The results in the control group of grassfrogs showed no regenerative response, with the limb stump healed over with thick skin and scar tissue. However, after weeks of stimulation with the negative electrode at the stump tip, the second group of animals showed varying degrees of regeneration of the limb. These limbs were abnormal in their external appearance, and upon examination their internal tissues were disorganized and undifferentiated. This was a striking result, even though the regenerated limbs were incomplete and hypomorphic. The third group of animals, which had a positive electrode implanted at the stump tip, resulted in the degeneration of the stump tip tissues, and no regenerative response. These experiments demonstrated that by artificially imposing electric currents of the same polarity and similar field intensity as found in the salamander, regeneration could be initiated in the normally non-regenerative adult frog.

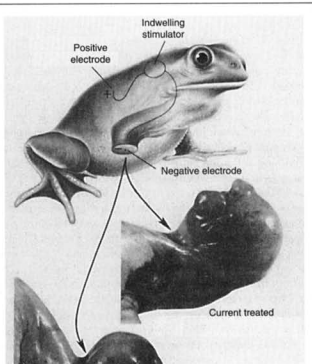


Figure 1
ELECTRICAL STIMULATION OF FORELIMB REGENERATION IN ADULT FROGS

The negative electrode is routed into the limb stump tip, and the positive electrode under the skin of the back, as depicted in the drawing of the frog. The photographs below show actual responses of current-treated and sham-treated limbs. The arrows point to the plane of amputation in the limbs.

Source: R. Borgens, *J. Exp. Zool.*, Vol. 200, pp. 403-416, 1977. Reprinted by permission of Wiley-Liss, Inc.

Experiments with Frogs after Amputation

The initial experiments were followed up two years later, in 1979, with an examination of the currents in non-regenerating frogs after amputation. Although frogs (anurans) and salamanders (urodeles) are closely related, there are differences in the anatomy of their limbs which may help to explain why adult frogs do not normally regenerate. Frog larvae are able to regenerate portions of their tails and limbs until the stage in metamorphosis when the subepidermal lymph spaces begin to develop in the limbs. This subepidermal lymph space in the adult frog limb could act as a low-resistance pathway for shunting

Vincent P. Walter, Purdue University

Dr. Richard Borgens and a student working in his laboratory at Purdue University. Borgens is using the microscope to photograph specimens.

current generated by the skin battery away from the core tissues of the limb stump. Salamanders and other urodeles do not have this subepidermal lymph space.

Borgens measured the stump currents in adult frogs, and found that the highest density of current was found leaving the subepidermal lymph space. The current running through the core tissues was found to be four to five times lower in intensity than those in the lymph space. Also, the peak currents leaving the stumps of adult frogs were weaker than those found in newts. These differences suggested that the lack of sufficient electrical currents running through the core tissues of the limb, the area which gives rise to the blastemal cells, results in diminished regenerative ability.

Because the adult frog does produce a stump current, if the topography of the current could be made similar to the urodele, perhaps regeneration in the adult frog would be more complete. Borgens refined the experiment using electrical stimulators in frogs, this time using the African clawed frog *Xenopus laevis*. *Xenopus* is known to naturally produce a minor regenerative response to amputation, which usually consists of a short spike of cartilage covered by skin. Using internally implanted electric stimulators, with the negative electrodes running through the core tissue of the limb stump, Borgens observed that, after two months, these animals had grown limbs that were similar to the "paddle" stage of limb found in salamanders before the digits form.

The control group of animals had spikes of cartilage surrounded by skin typical of the natural response. Curiously, many of the electrically treated limbs appeared almost normal, externally, but the internal structure was quite disorganized and abnormal. These regenerative structures were called "pseudolimbs," and had large amounts of nervous tissue running through the cartilage in a disorganized pattern.

The conventional understanding of developing limbs is that the internal cellular organization determines the external form. However, the pseudolimbs are a challenge to this notion, and point to the importance of electric fields in determining morphology.

In his next experiments, also in 1979, Borgens examined what the effect of specifically blocking the sodium ion channels of the skin would have on regeneration in salamanders. He had shown previously that the stump currents seemed to depend on sodium ions as their charge carrier. Salamanders and newts had their forelimb stumps treated daily with the sodium channel blocker amiloride. At first, all of the treated animals were blocked from regenerating their limbs, but after a period of time about half of the animals began to regenerate. Measurements of currents emanating from the stump tips in these animals showed that they indeed generated strong currents in the absence of sodium.

Amazingly, some of the animals which had been inhibited from regenerating for weeks by the amiloride treatment, recovered and began to regrow their limbs at a greatly accelerated rate. This was a startling result, which again brought up new questions. When these animals were examined during the period of inhibition, their limb stumps were covered by full thickness skin, and scar tissue, which is indicative of non-regenerating species or permanently arrested regeneration. However, these animals escaped this inhibition, and then regrew at accelerated rates, sometimes producing limbs that were more fully developed than those of the control animals.

Was there something building up in the inhibited limb stumps that produced this accelerated growth? This question could not be answered by the experiments then, and remains a mystery today. However, the fact that salamanders and newts could produce stump currents after recovering from the inhibition, meant that these animals had adapted by using other positively charged ions, such as calcium and potassium, when the sodium channels were blocked. In later experiments, it was shown that if the animals were kept in an environment that lacked calcium, potassium, and sodium, stump currents could not be generated and regeneration was blocked.

Field Model of Regeneration

Borgens's experiments leave no doubt as to the importance of the electric field in initiating and controlling regeneration. But what are the targets of this field? What are some of the possible effects an electric field produces in cells? We know from earlier experiments that the nerves and the wound epithelium are the two crucial tissues required for regeneration to occur, so could these be targets of the electric field?

From experimental measurement, the electric field is strongest at the wound epithelium, where the current flows out from the limb stump. The flow of current establishes polarity in

the limb, and creates voltage gradients, which could provide cells with a way of sensing their position. Also, the current flow provides a directionality for the cells to grow outward. Electric fields have been shown to influence the direction of cell migration, so this could also be one of its effects in the developing limb.

From the experimental results, it seems that the immediate flow of current after amputation is crucial in initiating the regenerative response. Thinking about this problem from the standpoint of responses to injury, the first thing that changes after injury to the skin, or to the membrane of a single cell, is a flow of electric current. Cells must have evolved injury responses that detect this current flow as a signal, which initiates the healing process. In the case of amphibian regeneration, the de-differentiation of cells in the blastema may be a result, directly or indirectly, of the current flowing out of the limb stump.

The other primary target of the electric field, logically, is the nervous tissue, which is required for regeneration to proceed. *In vitro* experiments have shown that neurons grown in culture will extend and grow neurites (precursors to axons and dendrites) preferentially toward the cathode (negative pole) in an electric field. Given the formation of the pseudolimb in the adult frogs, it is curious that so much of the internal tissue was nerve. With the negative electrode at the stump tip in the frog, a hypomorphic regeneration proceeds, and the polarity of the applied field is the same as that which neurites grow toward in culture.

Borgens suggests that the field polarity helps to direct peripheral nerve regrowth into the limb stump, which supports limb regeneration. However, the situation in the regenerating limb with regards to the relationship between the nerves and the wound epithelium still leaves many questions unanswered. How does the field coordinate the actions of these two essential tissues during regeneration? Do the cells of the regenerating limb grow according to a global electric field, which has already defined the shape and orientation of the limb? To address this tantalizing question, Borgens directed his next experiments to the process of limb formation in the embryo.

Does the Embryo Use Fields to Drive Its Development?

Richard Borgens's interest in embryonic limb development overlapped with the work of his friend and colleague Kenneth Robinson. In 1983, they both published research papers on the role of the electric field in predicting the location of the emerging limb bud in two different animal embryos. Dr. Robinson had been studying the effects of electric fields on the growth and behavior of cells for several years. Robinson was Lionel Jaffe's first graduate student, and was interested in discovering how electric fields in an embryo might control the behavior of cells.

The experimental history of electric fields and their influence upon cells, especially neurons, left the issue unresolved between claims of the fields guiding nerve growth, and a complete denial of any effect on nerve growth. Robinson was aware of the work of S. Ingvar in 1920, who was the first to demonstrate that an applied current could orient the direction of neurite outgrowth along the lines of the electric field. His report also suggested that there was a different growth response toward the cathode (-) than toward the anode (+) of the applied field. However, in 1934, Paul Weiss, a scientist at Rockefeller University, published his experimental results claiming unequivocally that electric fields had *no* effect upon the orientation and growth of nerve fibers. The description of the experiments in Weiss's report was sketchy, and suggested serious flaws in the results. Yet, Weiss's work established what became the dominant belief on the matter.

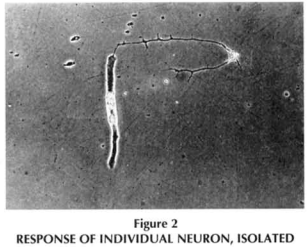


Figure 2
RESPONSE OF INDIVIDUAL NEURON, ISOLATED FROM THE NEURAL TUBE, TO ELECTRIC FIELD
The neuron (top right) extends neurites toward the negative (cathode) pole of the field (left). The long neurite is just touching the myoblast cell.

Source: K.R. Robinson, Purdue University

Robinson's mentor, Lionel Jaffe, had published a study of the response of dorsal root ganglion cells to an applied electric field in 1979, showing a preferential growth of neurites toward the cathode. However, the effect upon individual cells could not easily be seen, as these cultures contained hundreds or thousands of nerve fibers. Robinson was sure that Weiss's report was wrong, but he also wanted to be able to quantify the electric field strength required to influence the growth of an individual neuron.

Robinson's solution was to isolate the developing neurons from the neural tube of *Xenopus laevis* embryos, and grow them in culture in the presence of an electric field. In this way, individual cells could be studied, and the electric field threshold for an effect on neurite growth could be defined. In experiments in 1981, working with postdoctoral fellow Colin McCaig, Robinson found that the *Xenopus* neurons grew more neurites toward the cathode pole, and that neurites would make several turns orienting toward this pole. They found that the threshold for this effect was very low, a field strength of 7 millivolts per millimeter (mV/mm) could influence the cells.

They also tested the response of isolated myoblasts (muscle precursors) to the applied electric field in culture. The myoblasts had a higher threshold for response, of 36 mV/mm, and grew with their long axis of growth perpendicular to the poles of the electric field. These experiments established that different cell types responded in distinctive ways to an electric field, which would be important within an embryo whose cells are differentiating into specific cell types.

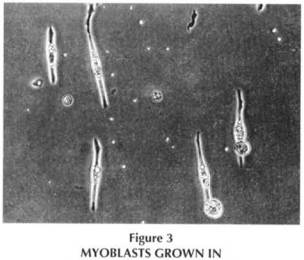


Figure 3
MYOBLASTS GROWN IN PRESENCE OF ELECTRIC FIELD

Myoblasts respond to the electric field by orienting their long axes perpendicular to the poles of the field (left negative, right positive). These cells are the precursors of muscle.

Source: K.R. Robinson, Purdue University

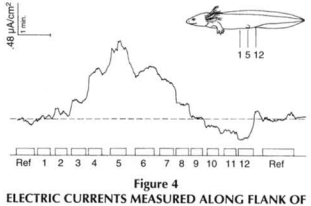


Figure 4
ELECTRIC CURRENTS MEASURED ALONG FLANK OF AXOLOTL DURING LIMB BUD FORMATION

Peaks above the dotted line indicate outcurrents, and below the dotted line indicate incurrents. The numbers represent the location on the axolotl where the measurements were taken. Note the strong outward current at position 5, which is the site of the emerging limb bud on the axolotl.

Source: R. Borgens, *J. Exp. Zool.*, Vol. 228, pp. 491-503, 1983. Reprinted by permission of Wiley-Liss, Inc.

Now that the fields required to influence the behavior of cells *in vitro* had been defined, the next step was to see if the embryo possessed fields of similar strength. McCaig and Robinson chose to study the *Xenopus* embryo during the process of neurulation, when the neural tube and the layout of the nervous system is first established. In experiments in 1982, they measured the electric potential generated by the ectoderm (outer layer) of the embryo, and found that it increased to 60mV (internally positive) or higher as neurulation proceeded. This voltage was much higher than those that they had previously found to be sufficient to effect the behavior of cells in culture. However, in this initial experiment they were not able to measure the direction or pattern of current flowing in the embryo.

During neurulation, cells must migrate from the area surrounding the neural tube to many locations in the body of the embryo. Could the electric fields be the cause of this migration, and do these fields guide the direction of the cell movements? An important group of cells that form around the neural tube and migrate to various distant locations is the neural crest cells. These cells differentiate into a wide variety of tissues, including ganglions of the autonomic nervous system, glands, skin, and even bone. It is not well understood what makes these cells migrate, and how they are directed to their destination.

Robinson isolated neural crest cells from *Xenopus* embryos, and exposed them to an applied electric field in culture. Individual cells and groups of cells migrated toward the cathode of the field at strengths of 10mV/mm or greater. This corresponds to a voltage drop of less than 1mV across the diameter of each cell. Fields of this magnitude could easily exist within the embryo, making electrical current a vector which could guide neural crest cell migration.

In 1984, Robinson used the vibrating-tip microprobe to try to detect the pattern of the currents near the surface of the *Xenopus* embryo. He found that positive currents were directed inward over most of the embryo surface, but that there was a strong outcurrent at the blastopore, which is a small hole in the ectoderm left from an earlier invagination of cells. This crucial experiment demonstrated that there was a global pattern of currents running through the embryo.

Embryonic Limb Development

What happens to embryonic cells in the region of the developing limb is fairly well defined anatomically in amphibians. Robinson and Borgens were eager to discover what role electric fields played in the events of limb formation in the embryo. Along the flank of the amphibian embryo, where the limb will form, one of the first things that happens is that the epithelial cells loosen their tight intercellular junctions. This creates a slightly leaky epithelium, which continues to degrade through the programmed death of some of the epithelial cells in the area of the limb bud. Beneath the epithelium, large quantities of mesenchyme cells accumulate, and later they start to migrate and grow outward. The migration of many cell types is involved in the process, including neural crest cells, fibroblasts, and, later, the growth of neurites extending into the growing limb bud.

Robinson examined the *Xenopus* embryo as it begins to form its hind limbs, and found that the area of the limb bud was the site of a strong outflow of current. Currents ranging from 2 to 12 $\mu A/cm^2$ left the limb bud, and returned in a loop to the surrounding areas of the flank. Because hind limb development occurs first in *Xenopus* and other frogs when the embryos are still quite small, and the time required for the process is rather short, it was difficult to make measurements of the current throughout the process of limb development.

Borgens took advantage of the larger size of the larvae, and the longer time frame for the development of the hind limbs,

in the axolotl to study the currents in limb development. The axolotl is rather well differentiated by this point in development, as its hind limbs develop after its fore limbs (Figure 4). Using the vibrating-tip microprobe, Borgens found, that for the week prior to the visible formation of the limb bud, the epithelial flank of the axolotl was an area of diffuse outcurrent, which became focussed and increased in intensity at the site of the limb bud. In fact, peak outcurrents of 2 to 3 $\mu A/cm^2$ occur at the site of limb bud formation, just before the limb becomes visible. The measurements of the current outflow could predict the exact site of the limb bud 4 to 6 days before it actually formed! The current path leaves the limb bud region and returns in loops to the areas of the flank, a short distance from the bud.

Borgens continued measuring the currents at the limb bud, finding that as the limb bud grows out from the epithelial flank, the current intensity decreases slowly, and in some cases, the current later reverses polarity and flows into the tip of large limb buds, approximately 0.5 mm in length.

From the experiments of Robinson and Borgens, a new understanding of limb formation has emerged. A developmentally programmed loosening of the tight junctions between the cells of the epidermis allows current to begin to leak out of the area where the limb bud will form. This disruption of the transepidermal potential leads to greater changes in the anatomy of the flank epidermis, such as the dying and sloughing off of epidermal cells in the region. The voltage gradient created by this current would be negative in the area of the leak.

The accumulation of mesenchyme cells, and the migration of other cells to the limb bud may be driven by the electric field. The limb bud area would act like the cathode of an electric field, which has been shown to direct cell migrations. Neurites also grow preferentially in this direction, which would innervate the developing limb.

Global Embryonic Electric Fields

The process of pattern formation within a developing embyro has fascinated scientists for decades. Robinson wanted to discover if the changes in the pattern of current flow could be correlated with physical changes in the arrangement of cells and tissues in the embryo. He and his student Kevin Hotary examined the embryonic chicken in 1990, measuring external current flows and voltage potentials in 2.5- to 4-day old chicks. They measured the currents surrounding the posterior intestinal portal (PIP), an area where the developing gut, near the tail, creates a break in the epithelium as it remodels. (See Figure 5.)

Measurements using the vibrating-tip microprobe were done on chicks from developmental stages 14-22. At stage 14, only weak currents flowing into the PIP could be detected, but by stage 16, the current reversed direction and flowed out from the PIP. The strong current flow outward from the PIP ranged from 50 μA/cm2 to a peak of 110 μA/cm2 at stage 17, and corresponded to the formation of the break in the epithelium of the gut.

Robinson and Hotary also measured the trans-epidermal potential of the chick embryos from the same stages of development, by inserting glass microelectrodes through the skin of the embryo. The average voltage potential across the skin was 16 mV, but this varied with the location on the embryo, and the stage of development. By taking many measurements of the change in voltage from head to tail along the embryo, they found that there was an internal voltage gradient averaging 10mV/mm toward the tail, which was the most negative. In regions close to the tail, the gradient was found to be as steep as 21mV/mm.

Neural crest cells begin to migrate in the chick at the stage where the first currents flowing out from the PIP are detected. Also, since neural crest cells were previously shown to migrate toward the cathode *in vitro* at field strengths of 10mV/mm or more, the voltage gradients measured in the chick would be more than sufficient to guide their migration toward the tail end, which acts as a cathode. As a result of these experiments, Robinson proposed that the major role of electric fields may be to guide the migration of cells in the embryo. But what else could the fields control?

The formation of the neural tube from the folding of the ectoderm, and subsequent detachment from the overlying layer of new ectoderm, lays the basis for the development of the central nervous system. After the neural tube forms, its presence induces the differentiation and patterning of other structures and organ systems. Undifferentiated cells in the vicinity of the neural tube will develop into specific cell types according to their position relative to it. Hans Driesch proposed nearly 90 years ago, that the developmental fate of an individual cell is a function of its position within the embryo as a whole.

Considering the neural tube's importance in determining the fate of the cells in the embyro, Robinson asked if the neural tube itself generated an electric field. To answer this question, he and Hotary inserted microelectrodes into the neural tubes of *Xenopus* embyros. By recording the electric potential across the wall of the neural tube, they found that there was a voltage of −23 mV through the dorsal (back) side of the tube, with the interior lumen being negative. The polarity is the opposite of that found at the ectoderm of the embryo, which maintains an inwardly positive potential; but this is not surprising, because

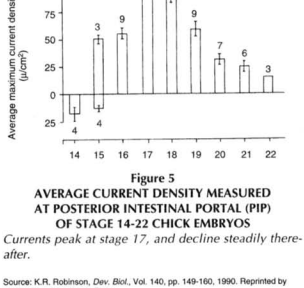


Figure 5
AVERAGE CURRENT DENSITY MEASURED AT POSTERIOR INTESTINAL PORTAL (PIP) OF STAGE 14-22 CHICK EMBRYOS
Currents peak at stage 17, and decline steadily thereafter.

Source: K.R. Robinson, *Dev. Biol.*, Vol. 140, pp. 149-160, 1990. Reprinted by permission of Academic Press.

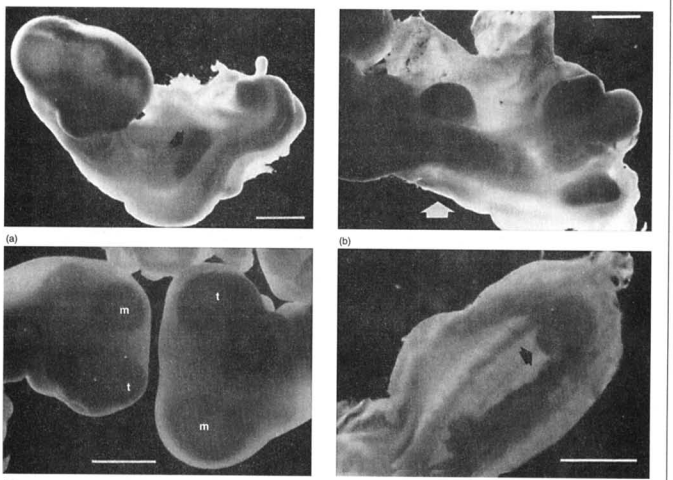


Figure 6

ABNORMALITIES IN LIMB, HEAD, AND GUT DEVELOPMENT IN CURRENT-SHUNTED CHICK EMBYROS

(a) Embryo has a duplicated wing bud on the side opposite the site of shunt implantation (arrow). The tail is also abnormal. (b) Wing bud is completely absent (arrow), while the leg buds are enlarged and flattened (asterisk). (c) Brain development is severely retarded in the current-shunted embryo on the left (asterisk). Brain divisions are small and abnormal, compared to the embryo on the right. The brain regions mesencephalon (m) and telencephalon (t) are labelled. (d) Ventral side of a current shunted embryo showing an abnormal outgrowth from the PIP (arrow).

Source: K.R. Robinson, *Development*, Vol. 114, pp. 985-996, 1992. Reprinted by permission of Company of Biologists, Ltd.

the neural tube interior is the same as the exterior of the embryo ectoderm, as a result of folding.

When the ventral side of the neural tube potential was measured, it was found to be more positive than the dorsal side. Surprisingly, areas just lateral to the ventral side of the tube differed in voltage by an average of 5mV. The pattern of electric potentials shows that the electric field generated around the neural tube is not radially uniform. Also, measurements of potentials around the neural tube in *Xenopus* embryos of various stages of development showed that the field intensity varied from stage to stage.

From the measurements, it was clear that the neural tube drives current primarily in a dorsal to ventral loop, creating strong fields that vary in magnitude from 50 to as much as 500 mV/mm. The cells near the lateral surfaces of the neural tube exterior would be exposed to very strong fields that would certainly influence their development. The somites that differentiate into muscle are found near the lateral walls of the neural tube, and *in vitro* experiments have already shown the response of myoblasts (which develop from the somites) to fields of only 36mV/mm. The neural tube electric field also coincides with the dorsal-ventral spatial patterning in the central nervous system.

Now that two major sources of electric fields have been discovered in the embryo, what is the importance of the interaction between the two fields during the embryo's differentiation?

Changing the Natural Current Topography

If the normal path of current flow were altered during the development of the embryo, what kind of effect would this have?

Robinson returned to the chick embryo, and devised experiments in 1992 which conclusively proved the importance of the electric field in development. Robinson and Hotary implanted tiny hollow glass shunts filled with an ionically conductive solution into the flank ectoderm of chick embryos between developmental stages 11-15. As a control, they implanted solid glass shunts that are not electrically conductive, in the same area in another group of embryos. The shunts were

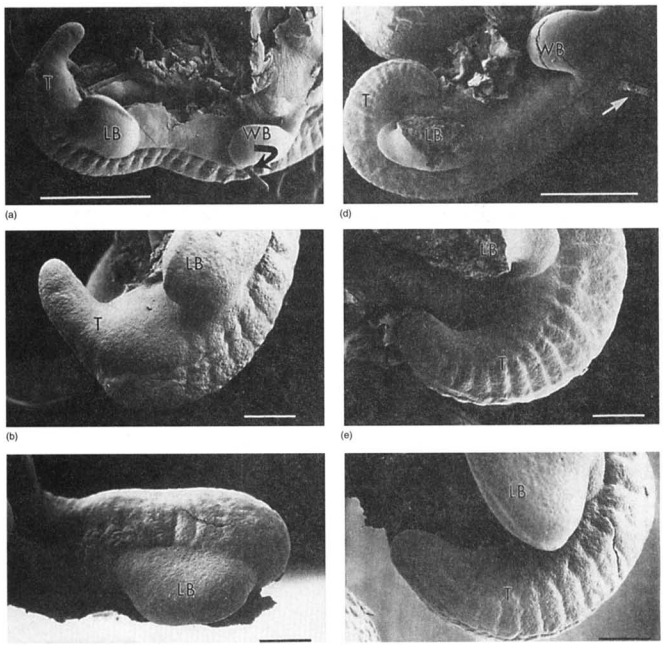


Figure 7
SCANNING ELECTRON MICROGRAPHS OF CURRENT-SHUNTED, SOLID-GLASS-IMPLANTED, AND CONTROL EMBRYO CHICKS

(a) Current-shunted embryo showing defective tail development. Arrow indicates the location of the implanted current shunt. (b) Closeup of the tail of embryo shown in (a). The end of the tail lacks a neural tube and other internal structures. (c) Current-treated embryo that failed to develop any tail. (d) Solid-glass non-conductive implant results in normal-looking structures in this embryo. (e) Closeup of tail region of embryo in (d), showing normal tail structure. Leg bud has been partially removed to allow better examination of the tail. (f) Normal chick embryo tail. Labels used are WB for wing bud, LB for leg bud, T for tail.

Source: K.R. Robinson, *Development*, Vol. 114, pp. 985-996, 1992. Reprinted by permission of Company of Biologists, Ltd.

implanted just before the appearance of strong outward currents from the PIP near the tail of the chick. Robinson expected his conductive shunts to reduce the natural current emanating from the PIP, and to direct the current out of the embryo in a different path.

After allowing the embryos to continue developing for a period up to three days, the embryos were collected and examined at stage 18 or 20. (See Figure 6.) The results in the current-shunted embryos displayed a staggering array of defects in structures throughout the body. About 92 percent of the current-shunted embryos showed some kind of defect, while only 11 percent of the solid-glass-shunt controls showed minor defects in development. Measurements of the current leaving the conductive shunts showed an average of 18 $\mu A/cm^2$ flowing out of the embryo, while no current could be detected flowing out of the solid glass shunts. Vibrating probe measurements of the PIP current in the conductive-shunt chicks showed a reduction of the current by an average of 30 percent, compared with a reduction of only 1 to 7 percent with the solid-glass shunts.

In the embryos whose current had been short-circuited by the conductive shunts, the most common defects were found in the tail region, which is consistent with the reduction of the PIP current. Tails were sometimes completely lacking in these embryos, and shortened tails were also found (Figure 7). In the few embryos that produced a tail of normal length, the internal tissue lacked normal structures, such as a complete neural tube, notochord, or somites. These embryos had abnormal gut structures, including sacs filled with undifferentiated mesenchyme cells.

The current-shunted embryos also displayed altered head development, including lack of brain divisions, and defective eye development, although at lesser frequencies. Defects in limb bud formation were also present, with missing limb buds or duplicated limb buds occurring on both sides of the embryo. None of the control embryos had altered limb buds.

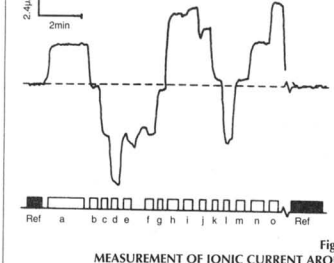
Changing the path of the current flow had global effects on the embryo, and challenged the earlier notion that the electric field's primary influence on development was only through the guidance of cell migrations. Most of the events in embryo development do not depend on cell migration, and instead depend on the interpretation of position to direct differentiation.

Also, areas above the current shunts in the chick, such as the head, still showed a wide range of defects in development. Only a *field concept* of the control of development could explain these startling results. The disruption of the global electric field pattern in the embryo by the shunts may have altered cell recognition of position, which would help explain some of the results. The disruption also changes the interaction between the field produced by the ectoderm and the internal field produced by the developing spinal cord.

Embryonic Field Manipulation

In 1994, Robinson and Borgens each designed experiments to interrupt the endogenous electric field of early amphibian embryos through the use of an externally applied field. Before disrupting the field however, Borgens examined the embryos of *Xenopus* and the axolotl at the stage when the ectoderm is folding itself to form the neural tube. Using the vibrating-tip microprobe, he found strong ionic currents flowing out from the lip of the neural folds (Figure 8). Measuring laterally down the flank away from the lip of the folds, the outward currents decreased in intensity, and were found flowing inward at a distance from the folds. These outward current loops disappeared in this area once the neural tube was completely fused (Figure 9).

Trans-epithelial potentials were mapped in the larger axolotl embryos during the stages of neural tube formation. By insert-



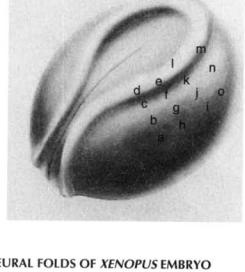


Figure 8

MEASUREMENT OF IONIC CURRENT AROUND NEURAL FOLDS OF *XENOPUS* EMBRYO

Graph (left) shows current flowing out of the embryo (below dotted line) and flowing into embyro (above dotted line). Letters below the graph correspond to the position on the drawing of the embryo (right). Outward currents are strongest near the lip of the neural folds; currents down the flank flow inward.

Source: R. Borgens, *J. Exp. Zool.*, Vol. 268, pp. 307-322, 1994. Reprinted by permission of Wiley-Liss, Inc.

ing glass microelectrodes into the area beneath the developing neural tube, measurements were made of potentials ranging from 18 to 64 mV. A voltage gradient from head to tail was found, with the tail being negative. The voltage differences between any two measurement points varied considerably, from 5 mV/mm to as high as 63 mV/mm. Again, the voltage gradient changed as the embryo developed.

Borgens then tested the effect of an applied field on the development of axolotl embryos. Embryos undergoing neurulation were oriented within a chamber, where they could be exposed to electric field strengths predicted to be similar or slightly higher than the natural fields. The embryos were oriented relative to the poles of the field in three ways: head towards the cathode, tail towards the cathode, or perpendicular to the cathode.

Severe abnormalities in body structures occurred in all three groups. However, the end of the embryo nearest the cathode displayed the most frequent and severe malformations (Figure 10). When the tail end faced the cathode of the field, tail and abdomen defects were the most common, and in many cases the head structures appeared normal in these embryos. In the opposite orientation, head defects predominated, with the tail structure appearing normal. The embryos placed perpendicular to the cathode showed an even distribution of defects to the head and tail regions.

Borgens also measured the trans-epithelial potential (TEP) of the embryos while they were exposed to the external field. With the tail facing the cathode, the TEP of the tail ectoderm underwent hyperpolarization, and increased in a range of 16 to 56 mV. The head ectoderm was depolarized, and often reversed polarity, with a change of 30 to 80 mV negative. The threshold field strength required to alter the TEP was found to be between 6 mV/mm and 25 mV/mm.

Robinson and his student Hotary took a different approach to altering the electric field in the *Xenopus* embryo. First, they re-examined the current flowing out of the blastopore, and found it to be much larger than their earlier measurements.

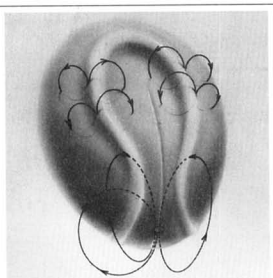


Figure 9
ARTIST'S DRAWING OF CURRENT LOOPS AROUND NEURAL FOLDS IN *XENOPUS* EMBRYO
The drawing is based on measurements done by R. Borgens. The loops at the bottom of the embryo depict the current leaving the blastopore. These currents drive the process of neural tube formation.

Source: R. Borgens, *Dev. Dynamics*, Vol. 202, pp. 101-114, 1995. Reprinted by permission of Wiley-Liss, Inc.

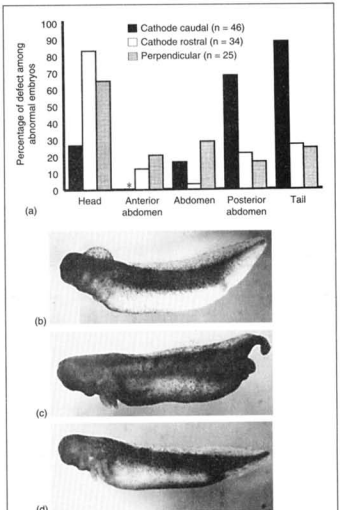


Figure 10
EFFECT OF 50MV/MM ELECTRIC FIELD ON AXOLOTL EMBRYOS ORIENTED IN THREE POSITIONS RELATIVE TO CATHODE OF FIELD
(a) The graph shows the percentage of defects in body regions in the three different orientations: cathode caudal (facing tail), cathode rostral (facing head) and perpendicular. (b) Embryo oriented with its head toward the cathode shows a large bulge on the dorsal surface of the head, as well as defects in other cranial structures. (c) Embryo oriented with its tail toward the cathode shows abnormal tail development, and bloated abdomen. (d) Unexposed control embryo showing normal structures.

Source: R. Borgens, *J. Exp. Zool.*, Vol. 268, pp. 323-338, 1994. Reprinted by permission of Wiley-Liss, Inc.

Currents were first detected flowing out from the blastopore at stage 14, peaking at stage 22, which coincides with the embryo's process of neurulation. The endogenous currents range from 2 to a peak of 115 $\mu A/cm^2$ over this period (Figure 11).

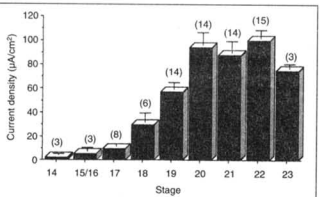


Figure 11
AVERAGE OUTWARD CURRENT DENSITIES MEASURED AT BLASTOPORE OF STAGE 14-23 *XENOPUS* EMBYROS

Numbers in parentheses above the bars indicate how many embryos were examined at that stage.

Source: K.R. Robinson, *Dev. Biol.*, Vol. 166, pp. 789-800, 1994. Reprinted by permission of Academic Press.

What if these natural currents could be nullified, or even reversed in polarity? What would be the effect on the development of the embyro? Robinson chose to use a microelectrode impaled just under the ectoderm of the embryo to disrupt the natural current flow. First, however, he had to determine what strength of current would be required to interfere with the blastopore current. By measuring the current flowing from the blastopore with the vibrating-tip microprobe, simultaneous with the application of current from the microelectrode, he found that an applied current of 100 nano-amps (nA) eliminated outward flow of current. Applied current of 500 nA effectively reversed the polarity of current flow at the blastopore, resulting in strong inward currents.

Xenopus embryos were impaled between stages 14 and 16, and the current was applied for up to 11 hours. One group of embyros exposed to 100 nA of inward current, nullifying the blastopore current, showed significantly abnormal structures in 20 out of 23 embryos. Common defects were found in head structure, absent eye development, and the failure of the neural tube to fuse at the anterior end. In many cases bulges appeared on the ventral surface of the embryo, which sometimes ruptured, spilling cells into the surrounding water (Figure 12). At higher levels of applied current, 250 nA or 500 nA, the abnormalities became more severe—Figure 12(f).

An additional experiment took two groups of embryos, and applied 100 nA of current in opposite polarities. In five embryos with inward current, all developed abnormalities. In the five in which an outward flowing current was applied, aug-

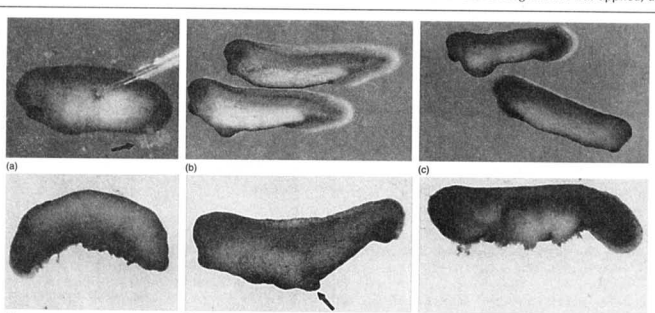


Figure 12
INTERNALLY APPLIED ELECTRIC CURRENT EFFECTS IN *XENOPUS* EMBRYOS

(a) A 100-nA inward current applied through the electrode nulls the natural current and causes cells to be ejected out of the blastopore. (b) Treated embryo shown at a later stage (at bottom), with an untreated control (above). Note the ventral bulge and bloated shape, as compared to the control. (c) Embryo treated with 100 nA (above) and control (below). (d) Embryo treated with 250 nA of current results in reduced head structures. (e) Embryo treated with 250 nA current, showing reduced head structure, abnormal tail, and an ectopic cement gland (arrow). (f) Embryo treated with 500 nA, which completely reverses the polarity of the natural current. It is disintegrating along its ventral side.

Source: K.R. Robinson, *Dev. Biol.*, Vol. 166, pp. 789-800, 1994. Reprinted by permission of Academic Press.

menting the endogenous current, only one of the five developed abnormally. The results reinforced the view that the disruption of the polarity of the global electric field in the embryo has serious effects on its development.

Can External Form Exist without Internal Differentiation?

In 1995, Borgens and postdoctoral fellow Riyi Shi did a series of experiments that demonstrated the crucial role played by the electric field of the neural tube in directing the differentiation of the internal structure of the embryo. The results also challenged the long held notion that internal differentiation of cells produces the external form of the embryo.

Axolotl embryos complete the formation and fusion of the neural tube by about stage 20 in their development. Borgens had previously measured the electric potential across the walls of the neural tube in the axolotl, and found that it was usually 80 mV to 90 mV, which produces strong electric fields that are not radially uniform in the surrounding cells. The ectoderm and the neural tube are both generators of electric fields in the embryo. Borgens wanted to test what would happen to the embryo if only the internal field generated by the neural tube were disrupted.

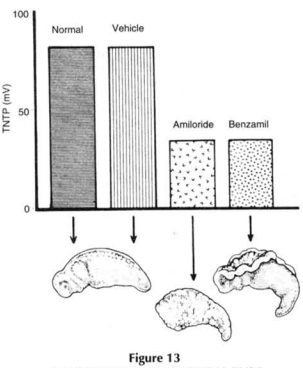


Figure 13
EFFECT OF REDUCING NEURAL TUBE ELECTRIC FIELD ON DEVELOPMENT

Bar graph shows the neural tube electric potential (TNTP) in embryos treated with the sodium channel blockers amiloride and benzamil, compared to normal embryo. Drawings below depict the severely abnormal morphology of treated embryos. The amiloride-treated embryo lacks all head structures. The benzamil-treated embryo's neural tube dissociates and exvaginates through the overlying ectoderm of the dorsal surface.

Source: R. Borgens, *Dev. Dynamics*, Vol. 203, pp. 456-467, 1995. Reprinted by permission of Wiley-Liss, Inc.

The electric potential of the neural tube depends largely on the transport of sodium ions and other positively charged ions out of the internal lumen of the tube. Borgens and Shi took advantage of this, by using the sodium channel blockers, benzamil and amiloride, to reduce the trans-neural tube potential

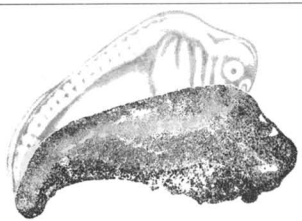


Figure 14
PSEUDOEMBRYO

A mid-sagittal section through a pseudoembryo, showing the complete lack of internal structure, with only undifferentiated clusters of cells. The normal internal structure of an axolotl embryo is superimposed (gray) just above the pseudoembryo. Note that the external shape of the pseudoembryo is basically normal.

Source: R. Borgens, *Dev. Dynamics*, Vol. 203, pp. 456-467, 1995. Reprinted by permission of Wiley-Liss, Inc.

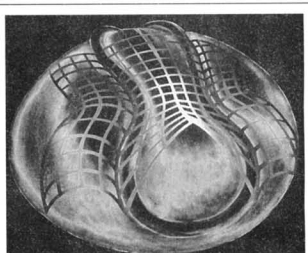


Figure 15
TOPOGRAPHY OF ELECTRIC FIELD ON SURFACE OF AMPHIBIAN EMBRYO

Artist's reconstruction of topography of electric field on the surface of an amphibian embryo. The graph depicts the intensity of voltage potential at the embryo's surface during neurulation.

Source: R. Borgens, *Dev. Dynamics*, Vol. 202, pp. 101-114, 1995. Reprinted by permission of Wiley-Liss, Inc.

(TNTP) in axolotl embryos at stage 21 to 23. When benzamil or amiloride was introduced into the lumen of the neural tube, the TNTP was found to be reduced by 50 percent or more (Figure 13). Treated embryos were then allowed to develop for 36 to 52 hours longer, when they were collected and compared to control embryos between stages 31 to 34.

In all of the embryos where the TNTP had collapsed as a result of treatment with benzamil or amiloride, there were severe abnormalities, of two different types. The first, was the formation of embryos which had completely chaotic internal organization, and grossly malformed external morphology. These lacked eye, ear, or neural tube structures, and did not resemble the control embryo's normal structure in any way. It was impossible to determine dorsal from ventral, or head from tail, because the major body axes were not present. Many of these embryos did not survive to stage 34.

The second group appeared as relatively normal in external form, with abnormal and undifferentiated internal structure (Figure 14). These were called "pseudoembryos." The pseudoembryos showed the disaggregation of the cells of the neural tube. They lacked all normal internal structures, including the gut. In embryos where groups of cells had started to form the primordia of the notochord and somites before treatment, these cells were found to be disassociated. Most of the body was filled with unorganized masses of undifferentiated cells. In some of these embryos, as the neural tube cells lost their polarity, these cells exvaginated in a sheet, and erupted through the overlying ectoderm, leading to the death of the embryo. It was determined that in the surviving pseudoembryos, the cells of the neural tube were not killed by the treatment with the sodium channel blockers; they just lost their ability to generate the electric field, which seems to determine their physical association.

The formation of these pseudoembryos challenges the concept that internal tissue differentiation determines the external form (morphology). In this case, disrupting the internal field of the neural tube led to a lack of differentiation of internal structure, yet the overall form of the embryo still developed in an almost normal fashion. Borgens describes this as the uncoupling of the global control of pattern formation from the local controls of tissue differentiation.

This may mean that the global external field produced by the ectoderm, which was not directly affected in the experiments, continues to guide the formation of the overall shape of the embryo, in the absence of the neural tube field. An analogous situation was also found in the formation of pseudolimbs in the electrically stimulated regeneration in adult frogs. The internal structure of the limb was abnormal and disorganized, yet the limb appeared almost normal externally.

These results cannot be explained by the traditional understanding of the relationship between internal tissue structure and external form.

A Field Theory of Development

There is an inherent paradox in the decades of research on electric fields in living systems. The cells are the source of the electric field, yet their behavior is subordinate to the field. Furthermore, the existence of the electric field may precede the movement or differentiation of the cells. This can be seen clearly in embryonic limb formation, where the current is flowing out of the area that will form the limb bud 4 to 6 days prior to its physical emergence. In many cases in embryonic development, the cells appear to "grow into" the shape and direction defined by the electric field.

This problem was studied by the great Russian biologist Alexander Gurwitsch, who in his 1922 paper "A Concept of Embryonal Fields," proposed that cells create a field that determines their future migration and growth patterns as an organism develops (Gurwitsch's work is discussed in the Spring, Summer, and Winter 1998 issues of *21st Century*.) Gurwitsch's concept was that the field was vectorial in nature, which is certainly true of the electric fields studied by Borgens and Robinson.

The work of Borgens and Robinson has produced a new field concept of development. Borgens proposed in 1995, that electric fields create three-dimensional voltage gradients that establish the coordinates of position, create the pattern, and influence the development of the embryo. From measurements of the field, it was possible to construct a topographical map of

Electric Fields Used to Treat Spinal Cord Injury

Dr. Richard Borgens is the head of the Center for Paralysis Research at Purdue University, where he has applied his interest in electric fields to treating spinal cord injury. After years of studying how electric fields can influence the growth of nerve cells, in 1992, Borgens investigated whether an applied electric field could help repair spinal cord injury in paralyzed dogs. He designed electric stimulators, whose electrodes could be implanted near the site of the spinal cord injury, to deliver an applied current of 200 micro-amps which creates a field strength of 135 to 210 micro-volts/mm.

In the mammalian spinal cord, axons extend from nerve cell bodies in both directions, so it was necessary to reverse the polarity of the field every 15 minutes, because axons grow preferentially toward the cathode of the field. In a clinical trial with injured dogs, the electric field stimulators produced a significant improvement in neurological function after six months. It was found that for the stimulators to be effective, the treatment must begin within two weeks after the injury.

Borgens and his colleague, Dr. Riyi Shi, have—for the first time—restored electrical impulse transmission in a severed, isolated mammalian spinal cord. Working with spinal cords isolated from guinea pigs, they used the polymer polyethylene glycol (PEG) to fuse the severed spinal cords. PEG fuses cell membranes, and when applied to the area of the break in the spinal cord, acts like cellular glue. When the PEG treated spinal cords were tested for electrical impulse transmission, it was found that 5 to 58 percent of impulses were restored. To be effective, the PEG treatment must be done within 24 to 36 hours.

This treatment is now being investigated in live animal models, and it is hoped it will eventually be used in clinical trials on humans. If successful, it could change the way spinal cord trauma is treated.

the electric field pattern on the surface of the embryo (see illustration on back cover). Currents flowing in the early embryo are aligned with the major body axes: head to tail, and dorsal to ventral. Voltage gradients, such as that found along the head to tail axis, offer a way for cells to know the coordinates of their position, and influence the direction and destination of migrating cells. The resulting fields within an embryo, such as the neural tube field, are not uniform in all directions.

Also, different areas of the embryo may have different resistance to current flows, creating a very complex and variable field pattern, which would be needed to create distinct tissues and structures. Cell types have different responses to an electric field, and different thresholds for an effect. Muscle cells orient themselves perpendicular to the poles of an electric field, while nerve cells grow their extensions preferentially toward the negative pole. The electric field allows the embryo to create singularities within itself, even though the field is global.

Robinson describes the embryo as creating electric current leaks and flows in a stage-specific and developmentally regulated manner. The embryo uses the flow of current and the electric field to drive the formation of the major structures and establish polarity of form. This is seen in the current flows associated with the folding of the neural tube, the early formation of the gut, and in limb bud development.

The pattern of the current flows and voltage gradients changes from stage to stage in development, and varies from species to species. However, *the pattern at any stage for a given species is invariant.* This concept directly echoes that of Gurwitsch's invariant principle. Gurwitsch also proposed that the embryonic field varied throughout development, but that this pattern was invariant for a given species. It is interesting to note the similarities in the ideas of Borgens and Robinson to those of Gurwitsch's field concept, although they were not familiar with the work of Gurwitsch.

Borgens and Robinson are currently working to bridge the gap between the vast amount of information about what happens at the molecular level, and their discoveries of the crucial role of the electric field in the development of the embryo. Scientists trained only in reductionist molecular biology would never be able to discover the *global* role the electric field plays in development. It is not possible to study all of the minute details of events that occur at the molecular level, and from that, determine that something larger must be controlling these varied processes. In fact, Borgens and Robinson were able to make these discoveries because they were trained in an historical perspective of science by mentors who were not trapped in reductionist ideology.

Commenting on the limitations imposed on today's scientists by the prevailing reductionist molecular ideology, Richard Borgens said, "It's taking the biology out of the biologist." The problem is that the reductionist approach has tried to reduce living processes to nothing more than molecular ping-pong, while avoiding the fundamental questions about the unique character of living systems. Kenneth Robinson amusingly said that he "would have dozens more grants if he could find one gene, or one ion channel" that was responsible for all of the electric fields he has found in decades of research!

The discoveries of Borgens and Robinson have laid the basis for a revolution in understanding how living organisms create electric fields to direct their growth and development. The next breakthroughs in biology will likely be made by those scientists who apply the field concept to solve problems at the molecular, cellular, and organismal levels.

Colin Lowry, a cell biologist, is an associate editor of 21st Century *magazine.*

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