Published in final edited form as:

Annu Rev Biomed Eng. 2012 ; 14: 295–323. doi:10.1146/annurev-bioeng-071811-150114.

Regulation of Cell Behavior and Tissue Patterning by Bioelectrical Signals: Challenges and Opportunities for Biomedical Engineering

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Abstract

Achieving control over cell behavior and pattern formation requires molecular-level understanding of regulatory mechanisms. Alongside transcriptional networks and biochemical gradients, there functions an important system of cellular communication and control: transmembrane voltage gradients (V_{mem}). Bioelectrical signals encoded in spatiotemporal changes of V_{mem} control cell proliferation, migration, and differentiation. Moreover, endogenous bioelectrical gradients serve as instructive cues mediating anatomical polarity and other organ-level aspects of morphogenesis. In the past decade, significant advances in molecular physiology have enabled the development of new genetic and biophysical tools for the investigation and functional manipulation of bioelectric cues. Recent data implicate V_{mem} as a crucial epigenetic regulator of patterning events in embryogenesis, regeneration, and cancer. We review new conceptual and methodological developments in this fascinating field. Bioelectricity offers a novel way of quantitatively understanding regulation of growth and form in vivo, and it reveals tractable, powerful control points that will enable truly transformative applications in bioengineering, regenerative medicine, and synthetic biology.

Keywords

ion flow; transmembrane potential; electric field; morphogenesis; embryogenesis; cancer; regeneration

INTRODUCTION

Definitions and Scope

Embryonic patterning, regenerative repair, and suppression of cancerous disorganization all require continuous signal exchange among cells, tissues, and organ systems within the body. Alongside well-known biochemical cues, there exists an important and fascinating system of

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DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

bioelectrical communication. The segregation of charges achieved by ion fluxes through ion channel and pump proteins gives rise to a transmembrane voltage potential (usually on the order of −50 mV, inside negative) across every cell membrane, and the parallel arrangement of cells with transporters localized to specific domains results in epithelial batteries driving transepithelial potentials (1, 2). All cells, not just excitable neurons and muscle, generate and receive bioelectrical signals encoded in changes in transmembrane potential and ion fluxes that change on a timescale of minutes to days. These are DC potentials; specifically excluded from discussion here are action potentials in excitable cells (millisecond-scale spiking), AC electric and magnetic fields, and the effects of exogenous electromagnetic field exposure, as well as the interesting literature on developmental roles of the geomagnetic field, charge transfer in DNA, static charges, and electromagnetic communication among cells via ultraweak photon emission (see Supplement 1; follow the Supplemental Materials **link** on the Annual Reviews home page at http://www.annualreviews.org).

Endogenous electric fields allow long-range communication, whereas gap-junctional (GJ) connections between cells establish local domains of isoelectric and iso-pH cell groups (3) and encode cell identity (4). Bioelectrical signals are one component of the host's morphogenetic field of instructive information in which all body cells are embedded. Recent reviews have covered the progress made on the molecular mechanisms of electric-field-based guidance of cell motility and orientation in the context of wound healing (5-10), implicating Rho-, β-adrenergic receptors–, and PI(3)Kγ-dependent pathways. Here, we focus specifically on endogenous voltage gradients across the plasma membrane (V_{mem}). This potential is mainly due to the movement of chloride, sodium, potassium, and hydrogen ions; despite the large literature, calcium signaling (11, 12) is not discussed here because it signals at very low concentrations—its effects are mediated by its chemical nature, not its contribution to transmembrane potential.

V_{mem} not only is a key mediator of differentiation and proliferation state on a single-cell level (Figure 1), but also plays an important role in coordinating cellular activities toward the large-scale patterning needs of the host during in vivo morphogenesis. Exciting recent developments of new tools and data sets highlight the importance of V_{mem} as an instructive signal in vivo and reveal novel opportunities for biomedical intervention as well as insight into fundamental aspects of complex biological regulation.

A Brief History of Bioelectricity

Early discoveries of "animal electricity" can be traced to Luigi Galvani in the late 1700s. E.J. Lund, in the 1920s and 1930s, focused on currents and showed that anatomical polarity was predicted and controlled by the bioelectric polarity of ion flows in vivo (13). H.S. Burr, in the 1930s and 1940s, focused on measuring and correlating voltage gradients with future developmental pattern in a wide range of species (14, 15); his measurements showed that voltage gradients are quantitatively predictive of morphology, suggesting that the fields carried patterning information. Some of the best early functional results were obtained by Marsh & Beams (16-18), who controlled anterior-posterior polarity specifically in planarian regeneration via external electrical stimulation of worm fragments. Enormously influential for the field was the work from the 1970s by Lionel Jaffe and coworkers, including

Richard Nuccitelli, Ken Robinson, and Richard Borgens (19-26), driving investigations of bioelectric controls of limb regeneration (27, 28), tail development (29), cell migration and orientation through the embryo (10, 30), oogenesis (31), and coordination of morphogenesis with histological differentiation (32) in amphibian, avian, and invertebrate model systems. Likewise, V_{mem} was suggested to be a key parameter mediating proliferation control, differentiation state, and neoplastic transformation (33-35) in a wide range of cell types (Figure 1).

The Age of Molecular Bioelectricity

Strong functional data over the past 50 years (Table 1) have shown that some bioelectric events are not merely physiological correlates of housekeeping processes but rather provide specific signals regulating cell behavior during embryonic development and regenerative repair (18, 21, 26, 36-38). Data indicated a role of endogenous bioelectrical signals controlling limb and spinal cord regeneration (39-41), cell and embryonic polarity (42-44), growth control (45, 46), and migration guidance of numerous cell types (2). Yet, the field as a whole remains unknown to several generations of modern cell and developmental biologists. This is largely due to the fact that the rise of molecular biology focused attention on the development of tools, protocols, and concepts most suited to the study of biochemical signals, making it extremely difficult to dissect the genetic basis and downstream targets of discrete bioelectric events. The past decade has seen a resurgence of the field of bioelectricity as new techniques in molecular physiology have enabled high-resolution functional approaches. This work has implicated voltage gradients as instructive signals in novel aspects of patterning, allowed the mechanistic connection of biophysical events with canonical upstream and downstream genetic pathways, and revealed bioelectric parameters as convenient “control knobs” on cells and tissues that can be exploited for important advances in biomedical intervention.

Though many modern workers are unaware of bioelectrical signaling as a cohesive field, new data are forging connections between molecular genetic pathways and ion flows, such as ion channel genes as hits in microarray and network analyses (47) and voltage-modulating drugs found in small-molecule screens (48). A superb example of such a convergence is the recent elegant study implicating sodium/hydrogen exchange in planar polarity in *Drosophila* (49), a relationship that was predicted by data on the role of bioelectrical signals during left-right patterning in the plane of embryonic epithelia (50). Several channelopathies with morphogenetic phenotypes have now been discovered by unbiased approaches (Table 2), though ion transporters are usually deprioritized for analysis in comparative microarray experiments because cell and molecular biologists are not yet accustomed to thinking of voltage as an instructive signal. By highlighting the techniques and tools now available, and illustrating strategies for integrating bioelectrical signals with mainstream pathways, it is hoped that workers in multiple subfields will consider that modulation of ion flows, currents, and voltages may be at the root of their favorite patterning or mispatterning problem.

MOLECULAR TOOLS AND APPROACHES

A variety of new reagents and methodologies have been developed for molecular analysis of bioelectrical signals in vivo (51, 52). These are described below.

Screens: Identifying the Targets, Implicating Bioelectricity

Using an inverse drug screen, researchers can determine whether ion flow may be a causal factor in a particular context as well as inexpensively and rapidly implicate specific endogenous ion transporter proteins for further molecular validation (53). This is a chemical genetics approach that capitalizes on a tiered (least-specific to more-specific) tree-based distribution of channel and pump-blocker compounds that enables an efficient binary search for likely candidates. Usually no more than 5–10 compounds need to be tried in the chosen assay to home in on an ion translocator type. Such screens probing native bioelectrical mechanisms have resulted in the identification of channels and pumps as novel components of left-right patterning (54, 55), anterior-posterior polarity determination in planarian regeneration (56, 57), tail regeneration in *Xenopus* tadpoles (58, 59), and mammalian stem-cell regulation (48, 60, 61). Robust development of novel compounds that target ion channels will further improve the power and efficiency of these screens. Proliferation of such compounds also drives the need for databases coupled to “expert systems”—software that can guide researchers when choosing which reagent to use after each node in the transporter family tree has been probed, removing the need for broad pharmacological expertise before embarking on loss-of-function bioelectric screens.

Characterization of Bioelectrical Properties In Vivo

To dissect a bioelectrical signal, researchers must first characterize the spatiotemporal distributions of the ionic parameters in vivo and then determine how the distributions correlate with anatomical and genetic patterning events. Unlike mRNA and protein levels revealed by in situ hybridization and immunohistochemistry, physiological properties cannot be studied in fixed samples: Reporters in the living state must be used (Figure 2). Tools for characterizing bioelectric events now include highly sensitive ion-selective extracellular electrode probes (62, 63) that reveal ion flux, microelectrode arrays (64), and techniques that report the content of individual ion species such as protons (65) and sodium (59). Voltage gradients can now be visualized in three-dimensional time-lapse, using fluorescent reporters of transmembrane potential such as the cell-permeable dyes CC2-DMPE and $DiSBAC_2(3)$ (54, 66, 67), proteins (68-71), and more exotic nanoscale materials (72) suitable for use in any optically accessible tissue (73, 74). These are a significant improvement on traditional electrophysiology because whole organs can be imaged at once and the data can be collected noninvasively over long time periods in moving samples. Crucially, the use of fluorescent reporters avoids a key artifact of electrophysiological measurements: Use of a single impaling electrode led to the erroneous concept of one V_{mem} value per cell. In fact, cell membranes are often a patchwork of many microdomains with distinct V_{mem} values, thus potentially storing and communicating a rich information stream to internal events as well as adjacent neighboring cells.

Although many of these reagents have been optimized for rapid (neuronal) electrical signals (75, 76), they are adaptable to the slower dynamics of patterning events because of their modular nature. Versatile, genetically encoded voltage sensors are now available; these include FRET-based proteins with plasma membrane targeting as well as red-shifted fluorescence (for better penetration of longer-wavelength light), e.g., VSFP2.3 (77-79). Several studies have now explored the endogenous patterns of ion flows during morphogenetic events (67) or the behavior of stem cells (80). As a way of obtaining minimally invasive diagnoses, this technique is also being introduced into medical practices. It can be used to identify cells with abnormal growth potential, which is revealed by their specific physiological signature (81).

These quantitative data are analyzed using differential equations (82, 83), particle-tracking simulations (84), and object-oriented software tools for physiological modeling (85, 86), thus revealing the complex dynamic behavior and autocatalytic order within biophysical gradients. Molecular-level detection of ion flows and their resulting voltage gradients, together with computational integration of physiological and anatomical data, reveal and explain the boundaries of physiological domains within tissues [such as the borders of hyperpolarized cell groups that define gene-expression compartments during craniofacial patterning (87)], identify which ion-concentration gradients contribute to voltage changes, and allow correlations of alterations in bioelectric properties with subsequent changes in cell behavior in situ—an important component of generating testable hypotheses for functional experiments.

Much opportunity remains for the development of specific, bright, ratiometric dyes that localize exclusively to the desired subcellular locale (e.g., plasma membrane or intracellular vesicles). Especially exciting will be the use of multiple physiological dyes in FACS experiments to identify subpopulations within “pure” stem and other cell types that differ in key bioelectric properties, as has been observed for HUVEC cells (88). Such experiments will clearly highlight physiological properties that are cell autonomous (maintained in dissociated cells) versus bioelectric states that can be maintained only within connected cell groups.

Targeted Functional Experiments

Ascertaining instructive roles for bioelectric events requires that changes in V_{mem} be inducible on demand in vivo and in a spatiotemporally controlled manner. Researchers must then link these changes to cell- and tissue-level outcomes, as is routinely done in knockdown and overexpression experiments for biochemical signals. Once a screen or microarray approach indicates a specific target, loss-of-function experiments can be done by inhibiting or knocking down a specific channel or pump that underlies a given ion flow. For example, a hyperpolarizing pump can be inhibited to lower the transmembrane potential in target cells, as was done to identify roles for V_{mem} in embryonic left-right asymmetry (54, 89), tadpole tail regeneration (58, 59), and muscle cell differentiation (90, 91). The data obtained shed light on the endogenous roles of ion transport events within any given developmental or regenerative context.

Direct application of electric fields is a technique well suited to the study of cellular responses to physiological-strength electrical signals in vitro (6, 92); however, the complex impedance of living tissues makes it difficult to use external electric fields to precisely modulate voltage gradients in vivo. Highly targeted gain-of-function experiments, such as the misexpression of a K^+ channel or single-subunit proton pump to hyperpolarize cells, can now be performed using well-characterized ion transporter proteins to induce known changes in ion content and transmembrane potential in specific cells. These reagents, derived from the work of gut and kidney physiologists, form a rich tool kit for molecular investigations of bioelectricity. Misexpression of the P-type (single-protein) proton pump was recently shown to be sufficient to induce regeneration of the tadpole tail when the native V-ATPase (a 13-subunit V-type H^+ pump complex normally required for regenerative response) had been inhibited (58). This illustrates an important principle: Hyperpolarization produced by the pump activity was the crucial factor for initiating tail regeneration, not the specific structure or sequence of the gene product. Likewise, manipulation of a regulated chloride channel allowed the experimental control of V_{mem} in frog embryo cells, revealing a role for transmembrane potential in triggering a neoplastic-like transition in neural-crest (stem-cell) derivatives (93, 94).

Although genetic perturbation can provide high-resolution information about bioelectric patterning control, the uncertainties of gene therapy require that pharmacological techniques also be developed for use in biomedical strategies; these take advantage of natively expressed channels and pumps to effect the necessary changes in transmembrane potential (95). One recent example is the design of a sodium ionophore cocktail, which induced full regeneration of the tadpole tail (a complex neuromuscular appendage including spinal cord) in a range of nonregenerative conditions after just one hour of exposure (59); another is the use of proton/potassium exchanger and chloride channel modulators to control the regenerative polarity of planaria: Fragments that normally develop a head and tail at their appropriate locations could be induced at will to reprogram their blastema growth, resulting in double-head or no-head regenerated animals (57).

Isolating the Information-Bearing Component of Bioelectrical Signaling

The activity of ion transporters results in several distinct biophysical events. For example, a plasma membrane V-ATPase proton pump simultaneously hyperpolarizes the cell (changing V_{mem}) and acidifies the extracellular milieu. It is critical to be able to distinguish which of these bears instructive information for downstream effects on cell behavior. This can be done by using the rich tool kit of currently available, well-characterized transporters to isolate each component in a given assay, enabling researchers to target rescue experiments to distinguish among voltage, specific ion concentration, or nonionic roles. For example, in the case of the induction of tadpole tail regeneration by a V-ATPase (58), it was first shown that a P-type ATPase could substitute for the multiunit complex (ruling out scaffolding or binding roles dependent on protein structure and implicating proton pumping as a requirement). Then, electroneutral transporters (such as NHE, the sodium-hydrogen exchanger) were used in the rescue assay to distinguish between pH and voltage roles. Pore mutants of channels (in which a single mutation abolishes ion conduction but leaves other structural roles intact) are useful to test for nonelectrical signaling functions of ion

transporter proteins. Gating channel mutants and pumps with altered kinetics can be used to reveal upstream signals controlling the bioelectric events and the temporal properties of the signal, respectively. Identifying specifically which biophysical event is necessary and sufficient for a given response is critical to link bioelectrical signals to downstream mechanisms, as doing so suggests candidate mechanisms for transduction into second-messenger pathways.

Connecting to Canonical Signaling Pathways

Understanding the role of bioelectricity in pattern regulation requires the identification of both the molecular source of an ion flow (expression and function of a given transporter) and its consequences (amplification by second-messenger systems into biochemical and genetic cascades). Dissection of the upstream and downstream processes for any bioelectrical signal allows a mechanistic understanding of cells as dynamical systems cycling between biophysical and biochemical regulatory events (Figure 3).

Some bioelectrical signals are mediated by levels of specific ions; for example, sodium levels are sensed by salt-inducible kinases in tail regeneration (59), whereas potassium and other cation levels can affect DNA structure (96, 97) and gene expression (98) directly. However, in many cases, the voltage change is crucial, regardless of which transporter and whatever movement by an ion species generates it. This is the case, for example, in the neoplastic-like transformation of melanocytes, where depolarization of the instructor cells has the exact same outcome whether it is achieved with chloride, potassium, or protons (93).

How do changes in membrane potential couple to transcriptional responses and alter cell behavior? Transduction mechanisms (for a detailed review, see 99) that convert V_{mem} levels into changes of gene expression include voltage regulation of the movement of small signaling molecules across gap junctions and through voltage-powered transporters such as the serotonin transporter SERT, conformational changes in integrins, tyrosine phosphorylation (100), and electrophoretic separation of protein complex subunits in the membrane plane. One particularly interesting mechanism downstream of V_{mem} involves voltage-sensitive phosphatases that hydrolyze phosphoinositides upon depolarization of the membrane potential (101, 102). By allowing voltage changes to reversibly switch the enzymatic activity of the tumor suppressor PTEN, these modular proteins illustrate another way in which electrical activity can be transduced into an important and well-studied biochemical signal. The function of the tumor suppressor SLC5A8 (a sodium/butyrate transporter) can transduce voltage changes into chromatin modification (103), as it links V_{mem} changes into an influx of butyrate—a histone-acetylating pathway. Indeed, bioelectric linkage to histone modifications has now been shown in dopamine neuron differentiation (104) and left-right embryonic patterning (105). Table 3 summarizes the transduction mechanisms implicated in several known cases of bioelectrical signaling in patterning; although the known set of transduction modes should be tested for any bioelectrically guided process, it is likely that additional ways of coupling ionic signaling to nuclear effectors remain to be discovered.

BIOELECTRIC EVENTS FUNCTION IN CELLULAR REGULATION

Coherent regenerative responses, embryonic self-assembly, and tumor suppression throughout the life span require integration of cell movement, differentiation, and proliferation coordinated into the morphogenetic plan of the host. Bioelectrical signals are important regulators on two levels: controlling individual cell behavior and carrying information for higher-level (tissue or organ) patterning cues.

Bioelectric Control of Cell-Level Properties

The movement of progenitor cells toward wounds has been observed in planaria (106), zebrafish brain (107), and mammalian stem-cell homing (108). Some effects of electric fields applied to cells include a change of orientation (parallel or perpendicular to field lines), growth (extension of processes), or migration (toward the anode or cathode) (109, 110). Modern protocols (111) used to study galvanotropism avoid polarization of substratum molecules and release of electrode products into medium—factors that confound cell responses to the field. Despite some controversy (112) over which cell types respond to physiological-strength electric fields (usually on the order of 50 mV mm^{-1} and as high as 500 mV mm^{-1} within the neural tube) (113, 114), it is clear that a large variety of embryonic and somatic cells exhibit galvanotaxis in electric fields of the magnitude often found in vivo (115-117). In embryos, such voltage-gradient patterns may be sensed by motile cells as positional coordinates guiding cell movement in vivo (30). Interestingly, electric-field cues tend to override biochemical ones (6). Electric guidance also occurs in several types of tumors (118); recently, voltage-gated sodium channels have been strongly implicated in this phenomenon (119) suggesting that endogenous bioelectric states may be a factor in metastatic invasion. Bioelectric events are also important not only for the generation of guidance signals, but also for cell-autonomous responses to fields during migration (120) when channels such as $K_{Ca}3.1$ provide instructive signals for the direction of cell movement (121).

Early links between ion flow and differentiation came from the observation that ventral ectoderm explants could be differentiated into a variety of different cell types by careful modulation of extracellular-medium ion content (122, 123). Bioelectrical signals apply to the differentiation of embryonic as well as stem cells, the latter of which have unique profiles with respect to ion channel expression and physiological state (124-128). Moreover, functional experiments have recently shown that membrane voltage controls differentiation of human mesenchymal stem cells: Undifferentiated mesenchymal stem cells are depolarized and increase their polarization during differentiation into fat or bone. Artificial depolarization can keep them in a stem-cell-like state, indeed overriding the presence of chemical differentiating agents (60). V_{mem} is also a regulator of neural progenitor cell differentiation in the mouse embryonic brain cortex (61), whereas Kir2.1 (KCNJ2) channel-mediated hyperpolarization controls differentiation in human myoblasts via a calcineurin pathway (90, 129). Importantly, a degree of dedifferentiation can be induced by ionic modulation (34, 130), and long-term depolarization can coax mature neurons to re-enter the cell cycle. This raises the possibility that bioelectrical signals can

induce a degree of stem-cell-like plasticity in terminally differentiated somatic cells (34, 45, 131).

Bioelectrical signals can control the rate of mitosis, which is closely linked to differentiation, as plastic cells tend to proliferate more than most terminally differentiated somatic cells. Indeed, a comparative analysis of membrane-voltage properties of various kinds of cells (Figure 1) reveals a striking relationship between depolarization and the control of differentiation and proliferation (132). Numerous studies have implicated K^+ currents as protagonists of proliferation and cell-cycle progression (133, 134; also reviewed in 33, 135). Cell proliferation appears to be controlled mostly by membrane potential (136-138), such as occurs in endothelial progenitors (139), although the effect is not always cell autonomous: In the frog embryo, depolarized cells can induce distant neural-crest derivatives to overproliferate (94). This is an important component of the regenerative response, as, for example, only astrocytes with depolarized V_{mem} (lacking inward rectifier K^+ channels) display active proliferation in response to injury (140, 141).

A considerable literature now exists on the role of specific native ion transporters, including the sodium-hydrogen exchanger and a variety of K^+ and Cl^- channels in cell-cycle progression, although many questions remain about the associated mechanistic details (133, 142-145). H^+ efflux is a particularly relevant transporter for efforts to control regenerative growth: In the zebrafish eye, V-ATPase is required for retinoblast proliferation (146), whereas proton fluxes control the elongation of the tadpole tail (58) and the growth dynamics of pollen tubes (147-149). Roles for bioelectrical signals are now beginning to be implicated in stem-cell regulation (150), including within embryonic stem cells, induced pluripotent stem cells, and cardiomyocyte progenitors (151-153).

Removal of specific cells through programmed cell death is a part of tissue sculpting in a variety of systems utilizing stem cells (154), tissue renewal (155), and transdifferentiation (156). Apoptosis is regulated by hyperpolarization via a set of K^+ and other channels (157, 158); for example, inhibition of K^+ channels can promote apoptosis (159), whereas activation of K^+ channels can inhibit it (160). Surprisingly, programmed cell death has recently been shown to be required for regeneration (161), suggesting that tight control over programmed cell death (by bioelectric means as well as via biochemical pathways) may be an important aspect of regenerative interventions.

Taken together, the data indicate that transmembrane potentials function as widely applicable regulators of key cell behaviors. This largely untapped but powerful set of cellular “control knobs” is of particular relevance for bioengineers and those seeking to transition findings from developmental biology into therapeutic strategies.

Bioelectrical Signals Mediate Global Patterning Cues

In the case of cancer suppression, researchers use ion modulators to target patterning disturbances at the level of individual cells (162). However, long-range coordination of cell activity, necessary for pattern formation at the level of organs and the entire body plan, also involves bioelectrical signals mediated by voltage gradients and electric fields. Gap junctions (163,164) not only augment cells’ ability to sense extracellular electrical

signals from their neighbors (165), but also partition cell fields into functional domains, for example, when delimiting regions of neurogenic precursors in the spinal cord (166).

The simplest examples of the roles of ionic signals in multicellular systems involve healing epithelial layers, where the fields resulting from disruption of the integrity of the polarized layer provide guidance cues for growth of migratory cells that repair the wound. Much molecular data are now available about the alveolar epithelium (167) and the cornea in particular, where electric fields (168-171) and cell-autonomous changes in transmembrane potential (172,173) are involved. Other tissues where bioelectric cues contribute to repair include the spinal cord (174-176); indeed, this modality is now used in human clinical trials with paralyzed patients (177).

A more complex example of morphogenetic control by bioelectric cues is revealed by the role of currents during vertebrate appendage regeneration. Thorough reviews of the early work of bioelectric effects on regeneration (augmentation of innervation, control of polarity, and alteration of differentiation) are given in References 26, 178, and 179. Amputated amphibian limbs maintain a current of injury—a direct-current signal that is very different in regenerating and nonregenerating animals. In salamanders and newt limbs, which have superb regenerative ability, several hours after amputation, the density of the stump current reaches 10–100 mA cm^{-2} and the electric field is on the order of 50 mV mm^{-1} (180). Studies of regeneration gradients using electrical isolation, shunting, ion channel blockers, or exogenous reversal of the gradient inhibited regeneration (25, 28, 29, 44) demonstrate that these biophysical events are factors necessary to regulate regeneration. Guided by measurements of field density, voltage gradient, and direction in endogenous regenerating systems, several labs (181, 182) have shown that application of exogenous fields (with physiological parameters) can induce limb regeneration in species that normally do not regenerate, including amphibia (183-185) and aves (186). Recently, molecular details have been uncovered about the guidance of regenerative events in vertebrate appendages. The tail of *Xenopus* tadpoles contains spinal cord, muscle, vasculature, and epidermal components. A combination of pharmacological and molecular-genetic analyses using dominant-negative and constitutively active ion transporters implicated strong H^+ pumping from the wound as an instructive factor in regeneration (58), controlling the appearance of proliferative cells and required for the correct pattern of innervation. Thus, tadpoles normally rely on the V-ATPase hydrogen pump to drive regeneration during early stages. More importantly, during later stages when tadpoles cannot regenerate, the entire regenerative cascade can be reproduced by artificially driving H^+ efflux via misexpression of a heterologous (yeast) pump protein (187).

One of the more remarkable findings over the past 10 years has been that these bioelectric cues are distinct from the metabolic gradients proposed by Child (188), because it is usually possible to dissociate experimentally the housekeeping functions of bioelectric properties from their more subtle patterning roles. Much as modulation can transmit information on top of a strong carrier wave, targeted artificial perturbation of V_{mem} usually results not in toxicity, death, or uninterpretable dysmorphias, but in specific, coherent changes of large-scale patterning (57, 58, 93).

V_{mem} changes and ion flows are components of long-range, cellular signaling pathways that occur during embryogenesis and regeneration. They can regulate the transport of diffusible signaling molecules into and out of cells, as occurs for the electrophoretic transport of maternal serotonin among early embryonic blastomeres during left-right patterning (189). They can also cause the release of diffusible secondary messengers from specific cells (93). V_{mem} changes in adjacent cells can propagate over long distances via conventional gap-junctional paths (190). Propagation can also be enabled using more exotic nanotubes—narrow cytoplasmic structures with gap junctions at their base that can conduct electrical signals between cells as a kind of nanowire (191)—which remain to be investigated in complex tissues. V_{mem} levels of key cell groups carry instructive information mediating large-scale polarity along major body axes, including head to tail (planarian regeneration) (57), left to right (embryogenesis) (50), and base to tip (pollen tube outgrowth) (147, 149). V_{mem} levels also carry positional information for migratory cell types (30) and master-regulator-like signals that initiate complete, highly orchestrated, self-limiting downstream patterning cascades such as tail regeneration (58, 59).

More than 50 years ago, researchers observed that spatial patterns of bioelectric parameters (e.g., voltage difference between specific locations) quantitatively predicted anatomical features that developed at much later time points and, thus, may control morphogenesis as a kind of subtle scaffold (13, 192, 193). However, only recently has it become possible to probe the instructive nature of such physiological gradients with molecular resolution. Using voltage-reporter dyes and time-lapse microscopy, a noninvasive map was made of the bioelectrical gradients during the formation of the vertebrate face (87). A complex regionalization of the voltage gradient demarcates the interior of the neural tube and the future mouth, while thin bilateral crescents on the edge of the face (Figure 2b) mark the position of the first pharyngeal pouch. These bioelectrically unique regions match the expression patterns of key genes that regulate differentiation and migration of tissues in the face. These gradients are natively driven by differences in the activity of the V-ATPase proton pump. Artificially perturbing the pattern of the voltage domains results in changes in the expression of important patterning genes such as *Slug*, *Mitf*, and *Frizzled3* and in the subsequent characteristic defects in the morphology of craniofacial structures. Such spatiotemporal profiling of the native physiology, combined with a detailed characterization of the anatomical and molecular-genetic perturbations of the boundaries of the hyperpolarization domains, revealed a superb example of how physiology can serve as a subtle prepattern for regions of gene expression, much as transcriptional domains act as prepatterns for subsequent anatomy (e.g., the Hox code). A convergence of modeling and molecular physiology data will be required to define intervention protocols able to alter such patterns at will and, thus, potentially repair a variety of craniofacial birth defects.

UNIQUE ASPECTS: A DIFFERENT PARADIGM OF SIGNALING

Bioelectrical signals are epigenetic, in the sense of Waddington’s epigenetic landscape; they underlie physiological heterogeneity (194) and are a component of the system-level stable state that directly reflects Waddington’s original meaning (195). One of the most interesting areas of future research is the incorporation of physiologically generated information with

the function of transcriptional networks to understand how biophysics and genetics interplay in the creation and maintenance of large-scale shape.

An interesting and important consequence of multiscale control of bioelectrical signals is their ability to act as “master regulators”: to trigger coherent, self-limiting, downstream morphogenetic cascades. For example, the activation of tail regeneration by a proton transporter illustrates that signals of extremely low information content can induce responses in the host that are far too complex for us to bioengineer directly, as the micromanagement of the construction of a limb or an eye from individual cell types is currently beyond our reach. This is an extremely attractive property for biomedicine, because it suggests that desirable tissue outcomes could be induced long before we know everything there is to know about how a complex organ or appendage is assembled. However, fully capitalizing on the promise of bioelectric controls for bioengineering applications requires a number of important changes in the way these pathways are probed because their unique properties have important implications regarding the design of experiments and interpretation of data.

Bioelectric networks exhibit nonlinear behavior, often with surprising dynamics, e.g., “virtual electrodes” (196). Changes in membrane voltage gradients affect the function of voltage-sensitive ion channels, which in turn alters membrane potentials further, thereby implementing a dynamical system with potentially multiple attractor states (197, 198). Likewise, gap junctions shape the electrical properties of cell groups and are sensitive to changes in transmembrane potential and pH. These scenarios offer very rich opportunities for cell groups to use ion flows to implement both positive- and negative-feedback mechanisms. The former, such as those created by the hydrogen/potassium exchanger regulation via potassium-sensitive NF-κB (199), can be used to amplify small physiological signals, whereas the latter, such as those created by depolarization-induced activation of the hyperpolarizing V-ATPase pump (200), can be used to ensure robustness of patterning against perturbations.

Ion transporters are gated posttranslationally; thus, bioelectrical signals derive some of their behavior from the intrinsic physics governing the movement of charged molecules in electric fields. While such physiological dynamics ultimately feed into transcriptional programs, it is important to note that much order and heterogeneity can be generated among cells with identical levels of protein expression in the absence of changes in mRNA and protein levels. Much as action potentials and calcium fertilization waves in eggs drive wave fronts of moving signals in the absence of transcription or translation, multicellular networks implementing voltage-sensitive gap junctions and ion channels can establish complex regionalizations of V_{mem} and GJ coupling as a kind of autocatalytic process [perhaps supporting Turing-Child dynamics (82)]. This is likely to be an evolutionarily ancient example of living systems capitalizing upon “order for free” (201), derived from fundamental physics.

A difficulty of current approaches to signal pathways is that physiologically derived patterning is largely invisible to widespread protein or mRNA-profiling techniques. Cells with the exact same complement of channel proteins can be in different physiological states (because channels can be gated by other physiological signals), and cells with very different

channel gene profiles can be in the same physiological state (because the same V_{mem} and pH range can be achieved by many different ion fluxes). For example, screens based on knockouts, morpholinos, RNAi, or protein or mRNA profiling would have entirely missed all the early events of left-right patterning in the frog because this system uses a voltage gradient to distribute small-molecule morphogens and is driven entirely by maternal proteins and the physics of electrophoresis for many hours before zygotic transcription begins (55, 202). Likewise, isolated or sorted cell populations that appear to be pure by molecular markers turn out to include several discrete groups with distinct physiological properties (88). Studies are beginning to identify specific signatures, such as those belonging to tumors (203), on the basis of ionic properties.

The above-mentioned aspects of bioelectrical pathways are important for multiple reasons. They occur in vivo and must be incorporated into our understanding of endogenous pattern formation mechanisms. They must also be dealt with (or indeed, exploited) in bioengineering approaches seeking to extend beyond what already can be found in nature.

FUTURE DIRECTIONS AND OPPORTUNITIES: CAPITALIZING ON BIOELECTRICITY

The lack of any necessary 1:1 mapping between real-time physiology and ion transporter expression profile has a positive side. For example, in the tadpole, a yeast H^+ pump (which does not occur in vertebrates) could induce regeneration during nonregenerative conditions (58); thus, biomedical applications could potentially use the most convenient of many channels or pumps to achieve the desired change in cell physiology and tissue outcome. The most transformative applications of this technology will require a mechanistic understanding of how patterning information is derived from the real-time dynamics of physiological networks. Similar to the way early computer memories stored bits as the directions of current flow within individual coils of wire, morphogenetic information can readily be encoded in the paths of ion flow within and among cells. Ion channel circuits with hysteresis (memory) properties (197, 198, 204, 205) as well as the use of gap junctions and ion channels by non-neuronal cells to form nerve-like computational networks (206, 207) have been described. Brief changes of physiological polarity have long been known to cause permanent reversal of anatomical polarity (e.g., in the chick blastoderm) (208-210). Recent molecular data have shown that transient perturbation of the physiological state in a flatworm permanently and radically alters its large-scale anatomy even though the animal's DNA sequence is unchanged (211). Upon future rounds of subsequent injury without any further manipulation, such worms regenerate to the new pattern, illustrating that real-time manipulation of the physiological state is a powerful entry point toward the manipulation of "target morphology" in biological systems that regulate their shape.

A full understanding of the capabilities of such biophysical systems will require an entirely new high-resolution data set: a complete physiomic profile that can be mined to discern the mapping between bioelectric properties and cell behavior (Figure 4)—the bioelectric code (akin to the genetic and epigenetic codes). One new technology that will facilitate the gathering of such data sets is the creation of transgenic model systems that constitutively

express reporters of V_{mem} and other bioelectric parameters (68, 212). *Xenopus*, zebrafish, and mice in which every cell natively reports in vivo measurements of pH, voltage, ion content (Figure 5) and conductance (213) will allow workers in many fields to flesh out the physiological state-space concept and to analyze enough quantitative data to develop predictive, physiological models that encompass the feedback loops and synthesize molecular-genetic and bioelectric data (52, 190, 214).

In addition to profiling, the bioelectric state of any cell/tissue of interest must be controlled within functional experiments. Optogenetic techniques (215-217), in which ion flow through light-sensitive channels can be controlled with extremely tight spatiotemporal specificity, offer new opportunities for mechanistic insight into novel bioelectric events. Although these reagents are currently optimized to control rapid spiking in nerve and muscle (218), it is likely that constructs such as the step-function opsins can be made to regulate V_{mem} in any cell of interest. Indeed, optogenetic lines in tractable organisms such as zebrafish (219) and mouse (220) already exist; several labs are working to create transgenic *Xenopus laevis* lines in which any cell or tissue of interest will express light-sensitive hyperpolarizing/depolarizing channels (Figure 6), allowing immediate access to bioelectrical experiments in cell, developmental, or regenerative biology using LED arrays (221) or other patterned-light sources. A promising new chemical approach confers photoregulation upon existing potassium channels (222, 223), making it ideal both for probing endogenous channel roles and for biomedical applications to control growth bioelectrically without the need for transgenesis.

Together, biochemical, physiological, genetic, and computer modeling techniques are converging to provide an extremely powerful set of approaches to probe functionally the roles of ion flows in pattern regulation. One last component to enable the implementation of these new insights as biomedical interventions in vivo is a delivery technology. Once our models become predictive enough to suggest precisely which biophysical changes need to be made in target cells, improved techniques will be required to provide the necessary reagents. One such technology involves the construction of regenerative sleeves—bioreactors applied directly to wounds (e.g., limb amputations)—in which the physiological state of wound cells can be precisely controlled by pharmacological, optical, electrical, and genetic means to trigger regeneration and to control patterning.

Importantly, the new field of molecular bioelectricity has transformative potential not only for morphogenesis within basic developmental biology, but also for biomedical applications to induce regeneration and treat cancer. The bioelectrical mechanisms being uncovered are profoundly powerful new “building blocks” for efforts in synthetic biology. Alongside the chemical and transcriptional modules found in the tool kit of researchers currently working in this field (224, 225), the addition of bioelectrical signaling modules—DNA cassettes encoding ion channels/pumps and transducers of bioelectrical signals to and from genetic end points—will allow these workers to exploit the transmembrane potential, ion flows, and self-generated electric fields to expand radically the capabilities of hybrid biological-engineered constructs. For example, rational control of growth and form among somatic cells (in contrast to soups of bacteria or yeast) can take advantage of bioelectric regulation of proliferation, orientation, and migration to allow the self-assembly of complex three-

dimensional patterns. Moreover, bioelectrical implementation of information-processing, memory, and signaling in non-neural cells grown in a sheet promises the creation of highly parallelized, robust computational tissues, as has been suggested by studies of electrically mediated memory in plants and bone (226, 227) that support algorithms far different from those possible via the Von-Neumann architectures employed by current computers. The unique properties of electrical networks with complex feedback offer remarkably interesting tools for the bioengineer; the use of bioelectric components in ways that go beyond the developmental programs made possible through evolution will be an essential part of the field of synthetic morphology (228), whose capabilities we have barely begun to explore.

The widely conserved, multiscale, instructive capacity of bioelectric events makes ion flows and voltage gradients a powerful control modality. Recent discoveries have begun to shed light on the interplay between genetic and biophysical signals. The development of specific strategies for analysis and control of physiological information storage is sure to open exciting new vistas in regenerative medicine, bioengineering, and synthetic biology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This review is dedicated to the memory of H.S. Burr, an early pioneer of the investigation of the biological importance of voltage gradients. M.L. thanks members of the Levin lab, Richard Nuccitelli, Ken Robinson, Richard Borgens, and the late Lionel Jaffe for numerous useful discussions. M.L. is grateful for support by the National Institutes of Health (EY018168, AR061988, GM078484, AR055993), the G. Harold and Leila Y. Mathers Charitable Foundation, and the Telemedicine and Advanced Technology Research Center at the US Army Medical Research and Materiel Command through award W81XWH-10-2-0058.

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SUMMARY POINTS

1. Steady-state voltage gradients in nonexcitable cells are important regulators of proliferation, migration, differentiation, and apoptosis. They also bear instructive positional and anatomical polarity cues in pattern formation in the context of embryonic development, regeneration, and cancer.
2. Together with targeted pharmacological strategies, genetic misexpression of well-characterized channels and pumps as well as their mutants can be used to perform screens for novel patterning roles and to functionally alter V_{mem} and thereby achieve remarkable changes of large-scale patterning, such as respecification of organ identity in regeneration blastemas and induction of complete appendage regeneration.
3. Fluorescent reporters of physiological state are available for real-time bioelectric analyses of patterning, and physiomic profiling is needed for high-resolution comprehensive data sets to dissect out the bioelectric code (mapping of biophysical properties to tissue outcomes).
4. Channels and pumps are gated posttranslationally; thus, confounding factors such as redundancy and compensation must be avoided when designing screens and interpreting phenotypes; it is crucial to think of membrane voltage as an information-carrying signal, not only specific gene products.
5. The “necessary and sufficient” instructive morphogenetic signal is often V_{mem}, not a specific channel gene, because cells with the same protein profile can be in different physiological states and cells expressing different complements of ion channel and pump proteins can achieve the same membrane potential range.
6. Ion flow networks composed of multiple voltage- and pH-gated ion transporters can possess complex behaviors and drive feedback loops, establishing patterned physiological inhomogeneities among genetically identical cells that serve as important epigenetic prepatterns that guide complex growth and form.
7. Patterning information can be encoded in the dynamical state of a physiological network, enabling bioengineered systems in which spatially distributed computation and memory are implemented in non-neural cell constructs.
8. Bioelectric control modules are an important new set of tools for synthetic biology, regenerative medicine, and bioengineering.

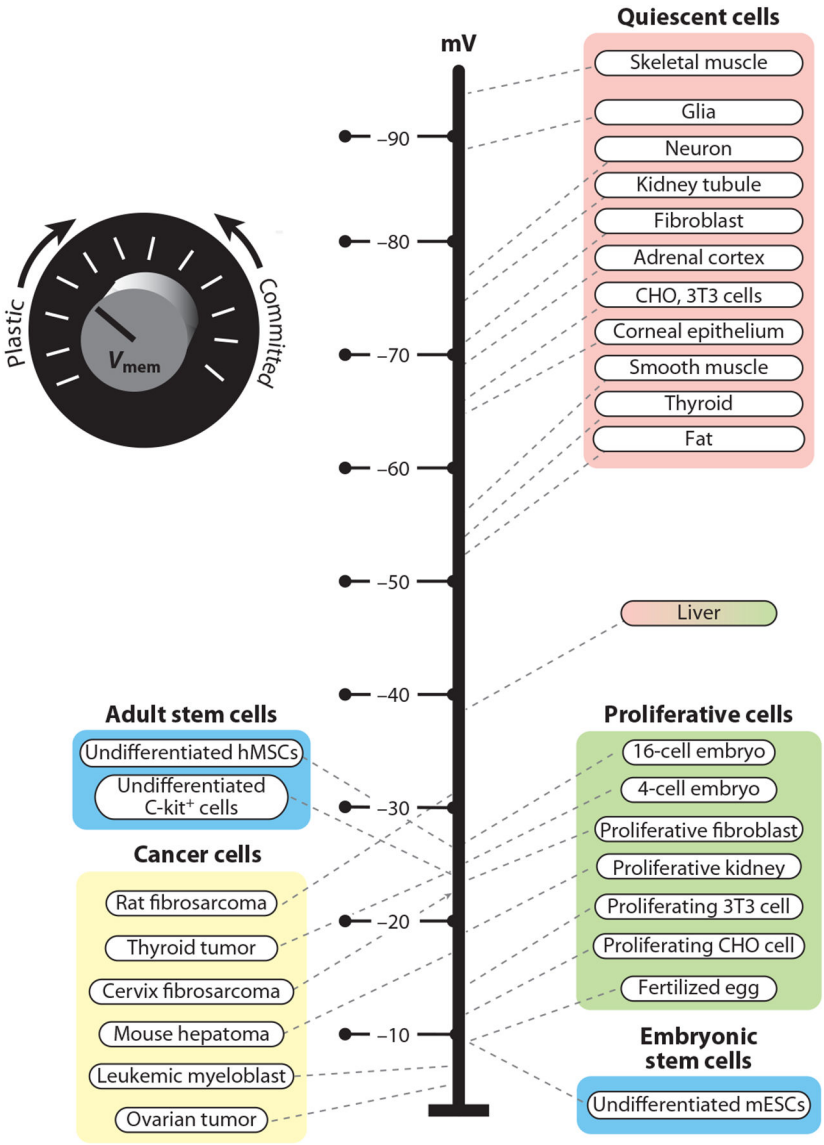

Figure 1.

Membrane voltage is a key parameter regulating cell properties. A small sample of a much larger data set (taken after Reference 132) reveals the striking partitioning of cell types along the depolarized → polarized axis. Cells that are highly plastic (able to proliferate rapidly, undifferentiated) tend to be depolarized. Cells that are mature, terminally differentiated, and quiescent tend to be hyperpolarized. The mammalian liver is an interesting example—an adult tissue that exists close to the depolarized range and has unique regeneration potential. Importantly, V_{mem} is not simply a reflection of cell state but an instructive parameter: Artificial depolarization can confer neoplastic-like properties on somatic cells and prevent stem-cell differentiation, whereas artificial hyperpolarization can induce differentiation and suppress proliferation. Abbreviations: CHO, Chinese hamster ovary; hMSC, human mesenchymal stem cell; mESC, mouse embryonic stem cell.

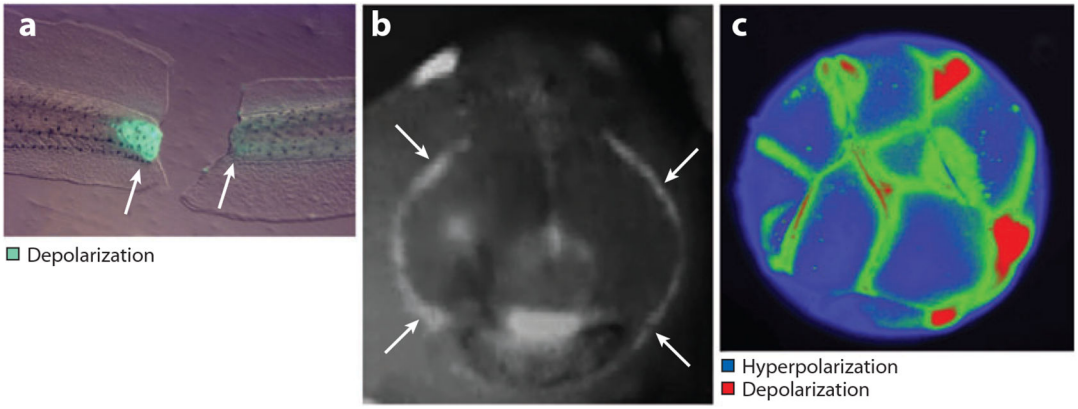

Figure 2.

V_{mem} gradients can be imaged in vivo. Voltage-sensitive fluorescent dyes (66) reveal a bioelectric map within complex tissues in vivo. This can be used to profile noninvasively the physiology of the tadpole tail during regenerative and nonregenerative conditions (*a*) (green indicates depolarization), the assembly of the tadpole face (*b*) (from Reference 87) (white arrows indicate hyperpolarized cell groups), and early embryogenesis (*c*) (frog embryo; red indicates depolarization, whereas blue indicates hyperpolarization).

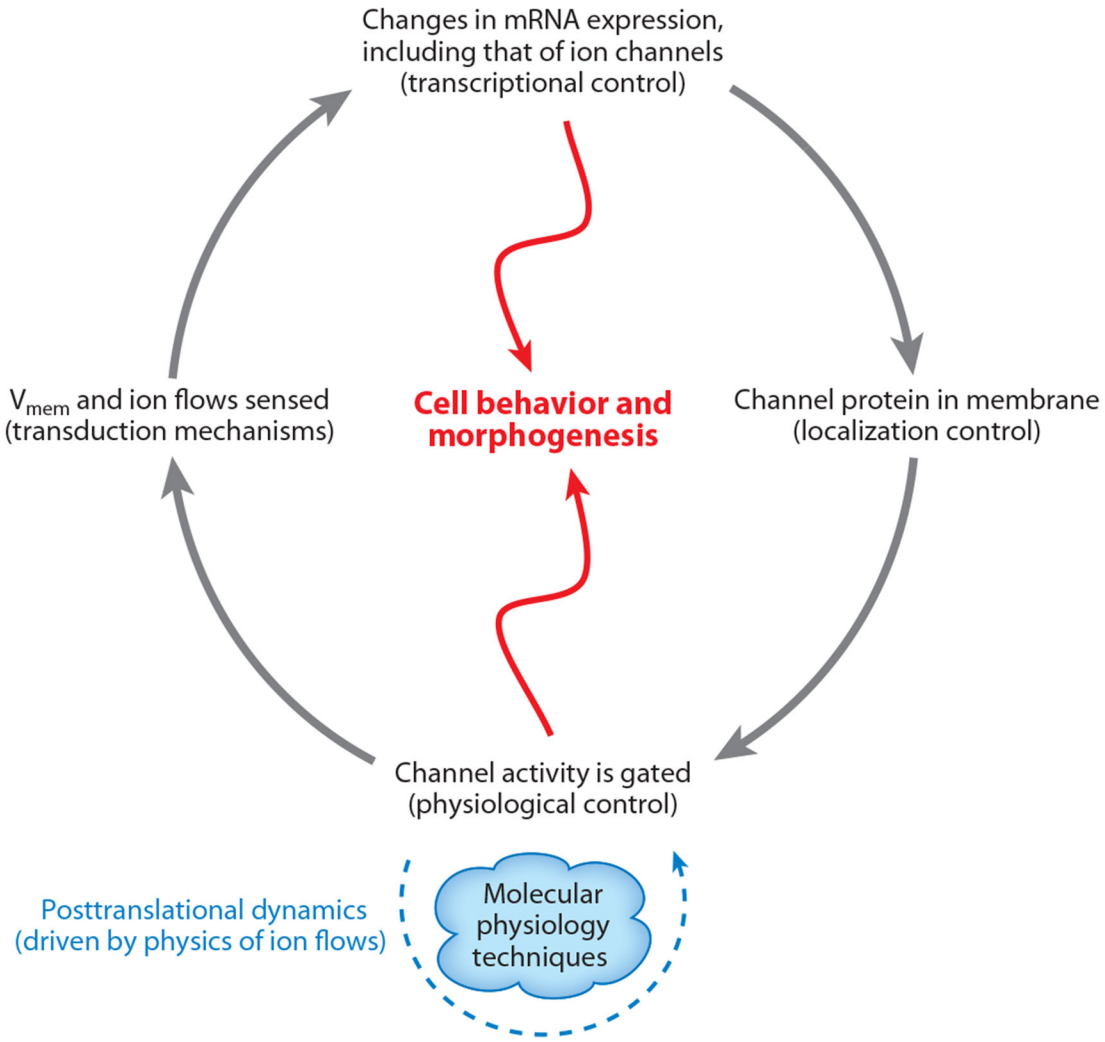

Figure 3.

Bioelectrical and genetic pathways form a cyclical dynamical system. The continuous interplay between biophysical and genetic mechanisms form a dynamical system: ion flows both control and are controlled by biochemical signals. Transcriptional events set up the expression of ion transporters, which regulate each other's activity through physiological (posttranslational) mechanisms such as voltage gating of K^+ channels. Transduction mechanisms (e.g., voltage-dependent regulation of entry of small signaling molecules through gap junctions) convert these signals into changes of gene expression. Changes in cell behavior and patterning can be driven not only by the well-known genetic cascades, but also directly by bioelectric cues that do not require changes in transcription or translation (such as movement and alignment of cells in electric fields as well as voltage-controlled movement of small signaling molecules through cell-membrane transporters).

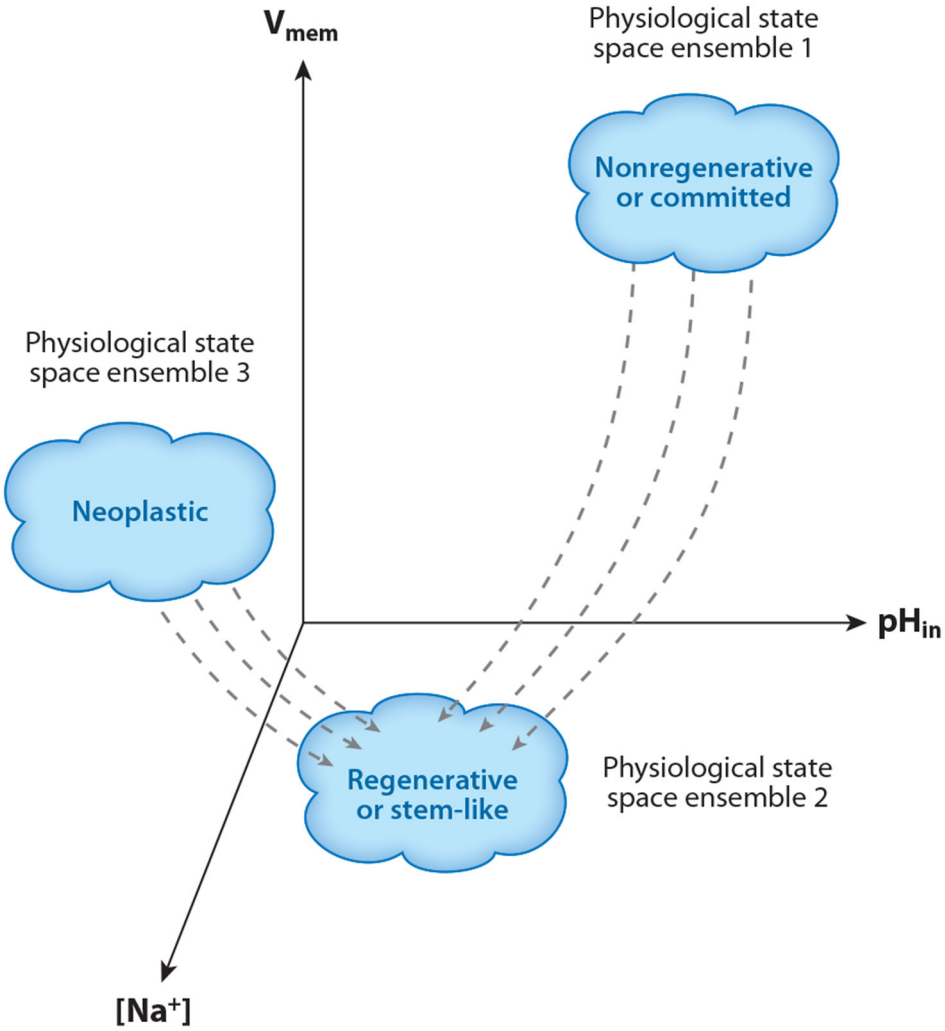

Figure 4.

The state-space hypothesis of the bioelectric code. Membrane voltage is a powerful determinant of cellular state (94, 229), but a single parameter such as V_{mem} (Figure 1) is likely to be only a primitive approximation to the true richness of bioelectric control. Cell and tissue properties can be localized within a multidimensional physiological state space containing a number of orthogonal dimensions indicating membrane voltage, intracellular pH, K^+ content, nuclear potential, Cl^- content, surface charge, etc. One hypothesis is that cells are grouped in distinct regions of this state space, corresponding to stem cells, tumor cells, somatic cells, and other interesting categories. This hypothesis implies that, given the necessary quantitative data, it will be possible to drive the desired changes in cell behavior (using pharmacologically targeting native channels/pumps and misexpression of well-characterized channel/pump constructs) by moving cell states into desired regions of this state space. For example, some cells may need to be depolarized by 20 mV and their internal pH acidified, to induce proliferation.

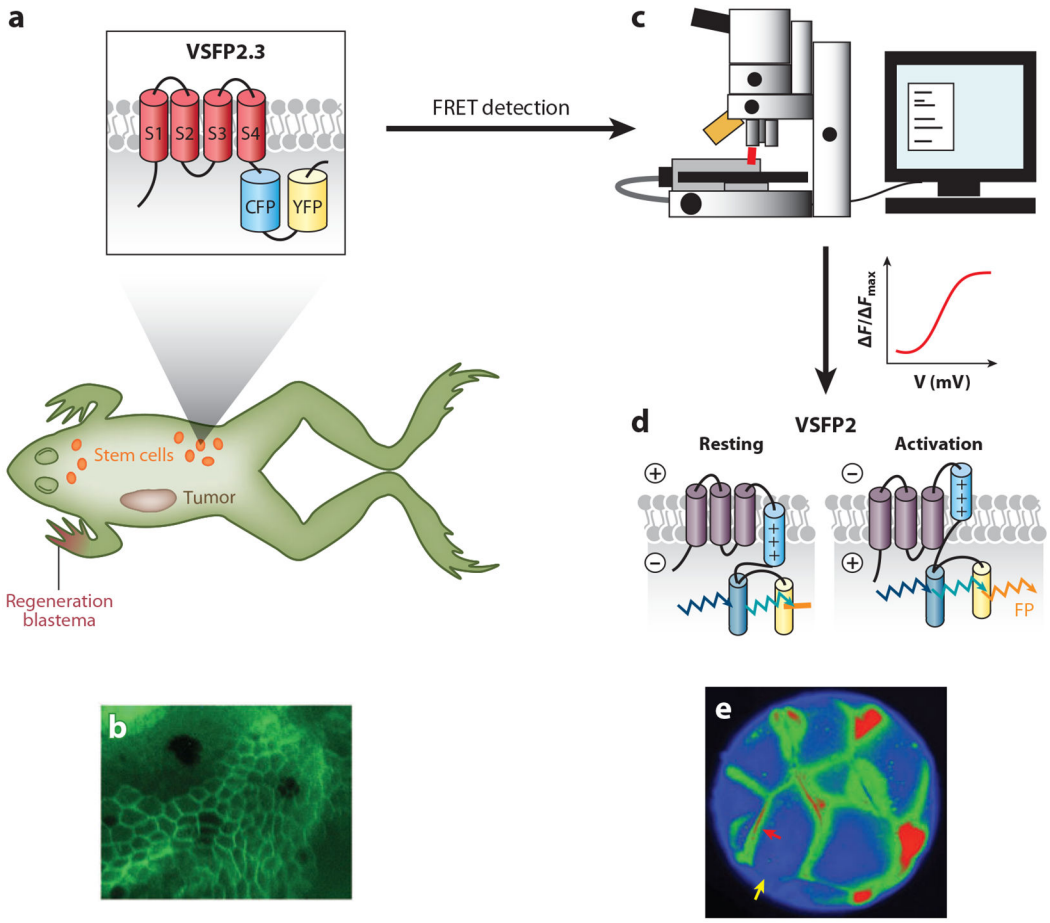

Figure 5.

A model system for comprehensive physiomic profiling. The physiomic data set needed to flesh out the state-space hypothesis (Figure 4) requires high-resolution physiological data on multiple cell types from different organs and disease conditions. The merger of existing x,y,z,t,g (real-time three-dimensional anatomy and gene expression) data sets with physiological measurements will require that researchers develop transgenic model systems in which any desired cell/tissue of interest can be imaged in vivo. For example, transgenic frogs expressing the voltage reporter protein VSFP2.3 (schematized in panel *a*); (*b*) cell surface expression will allow microscopy approaches (*c*) to observe transmembrane potential data as a fluorescence resonance energy transfer (FRET) signal (*d*) in any tissue/organ/stage of interest, such as the sample data on the gradients among cells during embryonic development (*e*). Red arrow indicates a depolarized region of the cell membrane; yellow arrow indicates a polarized region of the cell membrane.

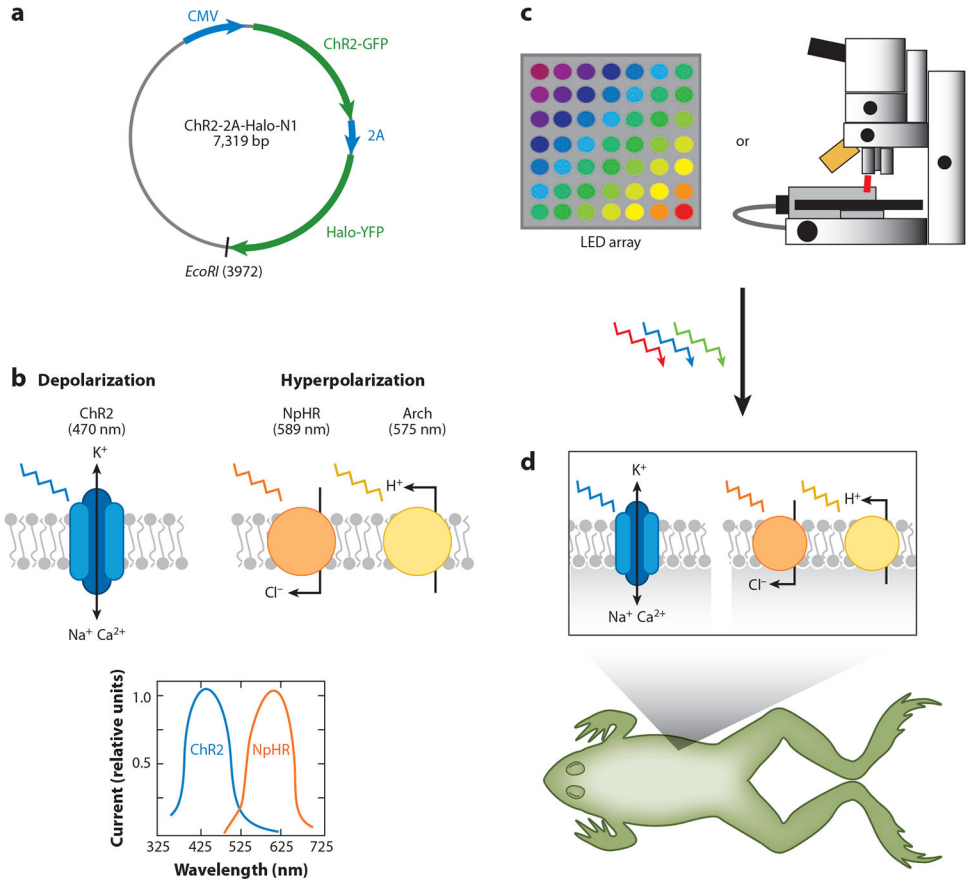

Figure 6.

A model system for functional experiments in bioelectricity. (*a*) A vector can carry an insert encoding a depolarizing channel such as channelrhodopsin (ChR2) and one encoding a hyperpolarizing channel such as halorhodopsin, separated by the viral 2A sequence, thus allowing both proteins to be made from the same mRNA. Such protein variants (*b*) allow cells to be depolarized or hyperpolarized by exposure to light of a specific wavelength. Optical stimulation can be achieved by standard laser microscopy or special LEDs arranged in an array (*c*) that can, thus, provide patterned (tightly controlled with respect to the spatiotemporal pattern of imposed V_{mem}) control of the voltage gradient in any cell or tissue of a transgenic animal that constitutively expresses such a construct (*d*).

Table 1

Physiological data on endogenous bioelectrical signals' roles in morphogenesis[a]

Role	Species/system	Reference(s)
Cellular polarization (anatomical asymmetry of cell or epithelium)	*Alga fucus*, yeast	21, 230
Patterning in gastrulation, neurulation, and organogenesis	Chick, axolotl, frog	29, 30, 32, 54, 89, 231
Directional transport of maternal components into the oocyte	Moth, *Drosophila*	232
Growth control and size determination	Segmented worms	233
Neural differentiation	*Xenopus* embryo	61, 234
Polarity during regeneration	Planaria and annelids	16-18, 38, 57

[a]Studies in which bioelectric parameters have been functionally shown to have an instructive patterning role.

Table 2

Genetic data identifying patterning roles for ion channels or gap junctions[a]

Protein	Morphogenetic role	Species	Reference
TMEM16A chloride channel	Tracheal morphogenesis	Mouse	235
Kir7.1 potassium channel	Melanosome development	Zebrafish	236
KCNH2 potassium channel	Cardiac morphology	Mouse	237
Cx41.8 gap junction	Pigmentation pattern	Zebrafish	238
Cx43 gap junction	Fin regeneration	Zebrafish	239
Cx43 gap junction	Fin-size regulation	Zebrafish	240
Kir2.1 potassium channel	Craniofacial morphogenesis (Andersen-Tawil syndrome)	Mouse	241

[a]Although single-gene mutation approaches are not well suited to uncovering roles for V_{mem} (because of the high degree of compensation and redundancy among ion channel family members), a number of ion transport regulators have been identified in unbiased screens for morphogenetic mutants. Comprehensive screens for bioelectrical signaling in development will require methods in which V_{mem} is systematically altered in distinct cell types, among discrete ranges of voltage, for example, by using tight physiological or optical control of genetically misexpressed transporters.

Table 3

Known transduction mechanisms by which ion flows impact cell behavior[a]

Developmental role	Key biophysical event	Transduction mechanism	Reference(s)
Tail regeneration in *Xenopus*: first step	Voltage change (repolarization)	Guidance of neural growth	58
Tail regeneration in *Xenopus*: second step	Intracellular sodium content	SIK2 (salt-inducible kinase)	59
Neoplastic conversion of melanocytes in *Xenopus* tadpoles	Voltage change (depolarization)	Serotonin movement	93, 94
Polarity determination in planarian regeneration	Voltage change	Ca^{2+} flux through voltage-gated calcium channel	57
Left-right patterning in *Xenopus* embryos	Voltage change	Serotonin movement	54, 89, 189, 242
Trachea-size control in *Drosophila*	Ion-independent function	Planar polarity, septate junction structure	243

[a]Studies in which the links between bioelectrical signals and downstream genetic responses during morphogenesis have been identified.