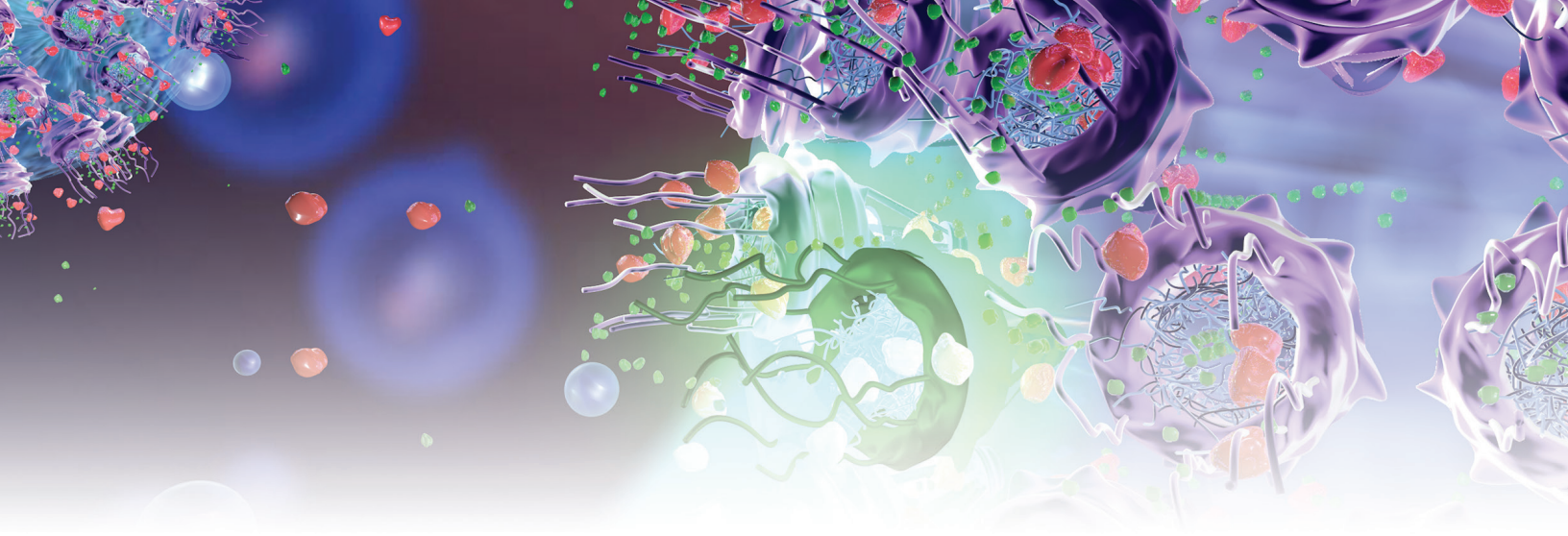
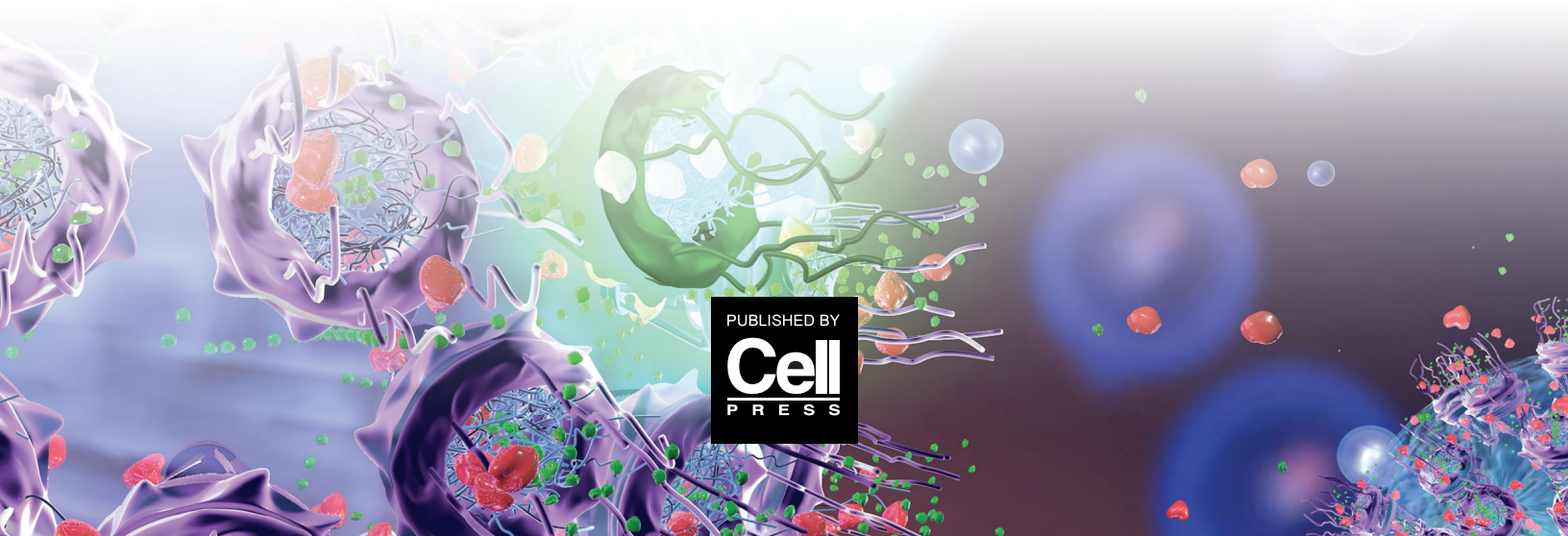


The State of Biophysics

Biophysical Society

Biophysical *Journal*



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Introduction to Biophysics Week: What is Biophysics?

Biophysics is a thriving discipline, as is evident by the breadth and depth of the science that is being presented at the Biophysical Society Annual Meetings and published in *Biophysical Journal*. Yet, biophysics also has an identity problem—due to the wide range of research topics that properly fall under the general rubric of biophysics—and biophysicists often find themselves challenged when asked to describe what the term actually represents.

Biophysics, as a distinct discipline, can be traced to a "gang of four": Emil du Bois-Reymond, Ernst von Brücke, Hermann von Helmholtz, and Carl Ludwig—all four being physicians and the former three being students of the great German physiologist Johannes Müller, who, in 1847, got together to develop a research program based on the rejection of the, at the time, prevailing notion that living animals depend on special biological laws and vital forces would differ from those that operate in the domain of inorganic nature. In contrast, the group sought to explain biological function using the same laws as are applicable in the case of physical and chemical phenomena. As stated by Ludwig and quoted from Cranefield (1) "We four imagined that we should constitute physiology on a chemico-physical foundation, and give it equal scientific rank with Physics." They coined the term "organic physics," and du Bois-Reymond stated, in the introduction to his seminal work *Untersuchungen über thierische Elektrizität* (http://vlp.mpiwg-berlin.mpg.de/library/data/lit28623/index_html?pn=1&ws=1.5), that (translation by Cranefield (1)) "it cannot fail that … physiology … will entirely dissolve into organic physics and chemistry."

It did not quite work out that way and, despite the scientific accomplishments of these four, in particular Helmholtz and Ludwig, the program faltered. In 1982, when Karl Pearson introduced the term "Bio-Physics" in *The Grammar of Science* (2) to describe the science that links the physical and biological sciences, he also noted "This branch of science does not appear to have advanced very far at present, but it not improbably has an important future."

Indeed, more or less as Pearson wrote these pithy comments, Julius Bernstein (3) published his description of a possible mechanistic basis for the development of transmembrane potential differences based on studies by Nernst and Planck on electrodiffusion. A few years later, Archibald V. Hill published his seminal work on the Hill equation (4). Both studies are reminiscent of the 1847 group's program and serve as prototypical examples of biophysics as the quantitative study of biological phenomena.

The mainstay of biophysical research in the early part of the twentieth century was neuro- and muscle physiology, disciplines that lend themselves to quantitative analysis and in which most of the investigators had trained in biology or medicine. In the latter half of the century, an increasing number of biophysicists were trained in chemistry, physics, or mathematics, which led to the development of the modern generation of optical and electron microscopes, fluorescent probes (whether small molecules or genetically encoded proteins), synthetic oligonucleotides, magnetic resonance and diffraction methods, as well as the computational methods that, by now, have become indispensable tools in biophysical research. Yet, we continue to face the question, "What is biophysics?" Maybe the best way out of this conundrum is to heed the advice of A.V. Hill, who long ago noted that "the employment of physical instruments in a biological laboratory does not make one a biophysicist," rather it is "the study of biological function, organization, and structure by physical and physicochemical ideas and methods" (5). It is the mindset—the focus on the importance of providing a quantitative, theoretically based, analysis of the problem under study—that is important! This emphasis on theory and quantitation is central to the methodological developments that provide the foundation for current biophysical research. It also leads to a possible answer to question in the title—biophysics is the quantitative approach to the study of biological problems.

Indeed, we are beginnning to fulfill the vision of the "gang of four" in 1847, based in large part on the emerging convergence of increasingly sophisticated quantitative experimental approaches together with computational studies, such as molecular dynamics simulations that use classical and statistical mechanics to explore protein function. Some of these developments are summarized in the following series of articles which has been compiled by the Biophysical Society's Publications Committee in conjunction with Biophysics Week to provide an overview of the state of biophysical studies and to heighten the awareness of the importance of biophysics as a central discipline in modern biological research.

One of the driving forces in current biophysical research has been the development of novel microscopes that make it possible to visualize structures at spatial resolutions that transcend the diffraction barrier. The diffraction barrier limits the ability of optical microscopes to distinguish among points that are separated by (lateral) distances less than one-half the wavelength of the light that is used to

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visualize the specimen of interest. Another force in biophysical research has been the development of (usually) fluorescent probes that make it possible to visualize living cells, including cells deeply embedded in tissues and even live animals. Many of the exciting advances in optical microscopy are summarized in the contribution by Rick Horwitz "Cellular Biophysics."

Genetically encoded fluorescent probes have proven to be particularly powerful tools because they can be targeted to specific cells and intracellular organelles, thereby facilitating exploration of problems that were beyond the capability of chemical probes. Targeting probes to specific cell types in a tissue means that investigators can study living cells and tissues at high spatial and temporal resolution and can use focused light impulses to manipulate genetically encoded targets, thereby manipulating cell function at the whole organism level. In this latter approach, the role of the microscope has changed fundamentally from being a tool to observe biological function to becoming a tool to manipulate biological function. The discoveries that led this important and novel field, coined optogenetics, are discussed in the contribution by Adam E. Cohen "Optogenetics: Turning the Microscope on Its Head."

The power of optical microscopy also enables researchers to probe the forces that underlie macromolecular function at the single-molecule level. The ability to visualize the function and motions of individual molecules leads to qualitatively different studies than are possible using measurements on ensembles of molecules, which only report on the average behavior of the ensemble. For example, if you want to elucidate the mechanics of human locomotion, it would not be very helpful to observe the movement of marathon runners across the Verrazano-Narrows Bridge in the New York Marathon; you would need to focus on the motion of individual runners to understand the sequence of events. Proteins and nucleic acids similarly undergo complex motions that best are examined at the single-molecular level, and Taekjip Ha's article "Probing Nature's Nanomachines One Molecule at a Time" describes some of the exciting developments in this rapidly expanding field.

The advances described by Horwitz, Cohen, and Ha build on developments in optical microscopy; equally important advances have taken place in electron microscopy. A new generation of cryo-electron microscopes with direct electron detectors enables atomic resolution studies on macromolecular structures based on images of thousands of individual molecules. This approach differs fundamentally from the analysis of crystal diffraction patterns or distance constraints obtained in nuclear magnetic resonance studies, and the novel developments allow researchers to determine the atomic resolution structures of macromolecules that cannot be crystallized, which has led to a revolution in structural biology. Edward H. Egelman's "The Current Revolution in Cryo-EM" traces the key methodological advances that underlie the current revolution, which depend not only on the advances in the hardware, but also on advances in the software that is required to process the large amount of data needed for the elucidation of atomic resolution structures.

As noted in Taekjip Ha's contribution, proteins are nanoscale machines that underlie much of what we consider to be characteristic of life. Because normal life (health) is so critically dependent on the proper function of these nanoscale machines, it often has been assumed that proteins need to be folded into well-defined structures to accomplish their intended functions; this turns out to be incorrect! Over the last 20 years or so, it has become apparent that many important proteins have amino acid sequences that cannot fold into conventional folded structures. This has led to a fundamental revision of the relation between protein sequence, structure, and function. These developments are summarized by H. Jane Dyson in "Intrinsically Disordered Proteins." Somewhat surprisingly, many intrinsically disordered proteins, or disordered regions within otherwise well-folded proteins, turn out to function as key elements in protein interaction networks. Moreover, because these sequences are disordered, they are susceptible to chemical modification that often is critical for normal (as well as abnormal) function, which makes them useful for designing targeted interventions.

Many human diseases result from mutations that alter the sequence and thus the function of important proteins. In some cases, the changes in function can be understood "simply" from how a given mutation alters the function of the cells that host the protein; in other cases, it becomes necessary to understand how the mutation alters the function of systems of interacting cells. This change in thinking, from focusing on the intrinsic properties of, for example, proteins or cells in isolation, to exploring the complex interactions that occur at the molecular, cellular, and system levels becomes important for understanding not only how normal body function is maintained, but also how human disease develops. In their article "Inherited Arrhythmias: Of Channels, Currents, and Swimming" Maura M. Zylla and Dierk Thomas discuss how inherited arrhythmias are best understood through such multiscale approaches, using the family of diseases that are lumped under the rubric, the long QT syndrome, which may cause sudden cardiac death due to the development of fatal arrhythmias. Most cases of sudden cardiac death are due to degenerative changes in the coronary vessels. A small fraction, however, results from changes in the function of a family of membrane proteins, the ion channels, that are responsible for normal cardiac rhythmicity. These changes in rhythmicity can lead to sudden loss of consciousness and even death—often in young people. As noted by Zylla and Thomas, an abnormal increase in the duration of the electrical impulses (the action potentials) that drive the heart and pump blood throughout the body may paradoxically lead to an, often sudden, increase in heart rate that may

compromise the heart's ability to move blood through the body. Reduced blood flow to the brain may lead to the sudden unconsciousness and death that are characteristic of this class of diseases. This is a situation where it becomes important to be able to sort through the underlying problems at many different levels of complexity, ranging from isolated channels to cells to how the cells are organized and interact in the tissues of the heart. Once these interactions are understood through biophysical analysis, it becomes possible to develop rational therapies.

The importance of multiscale approaches to understand both normal and abnormal body function is developed further by Andrew D. McCulloch in "Systems Biophysics: Multiscale Biophysical Modeling of Organ Systems." Focusing again on the heart, McCulloch emphasizes how it becomes important to understand the system at many different, mutually interacting, levels of complexity. The electrical system triggers the contractions of the cardiac cells that make the heart an efficient pump; however, to fully understand the heart's mechanical performance, it is necessary to delineate the coupling between the atria and the ventricles as well as the dynamics of the heart valves and the blood flow through the coronary circulation. Problems must be approached from the molecular to the tissue level and then coupled with the electrical and mechanical performance to develop an understanding of overall heart function, which can be accomplished through multiscale computational modeling.

The final contribution in this series, "How Viruses Invade Cells," is by Fred Cohen, who describes the mechanism(s) by which important viruses, such as influenza, HIV, and Ebola, are able to infect cells and "highjack" cellular processes. These cellular processes would normally support the regulated turnover of membrane components as well as cell division, but they are diverted to produce proteins encoded by the virus genome, which is necessary for viral replication and exit from the cells, leading to the infection of other cells. A key first step in viral infection is to insert the viral genome into the cell that is being attacked. This often happens through a series of processes that begin with viral uptake into lysosomes that normally are charged with hydrolyzing ingested materials. Once in the lysosome, the viral envelope fuses with the lysosomal membrane, a process that is activated by the very acid environment in the lysosome, and the viral genetic enters into the host cell's cytoplasm. As noted by Cohen, the most reliable way to prevent infection is to eliminate viral entry. To do so, however, requires understanding the underlying mechanisms of this process, which depends on the sophisticated methods that have been described in other contributions in this collection.

The contributions in this collection are not intended to provide a comprehensive overview of the excitement and importance of biophysical research. Rather, they provide examples of how one can use the power of the biophysical approach—the methods and analysis, the emphasis on quantitation, and the conceptual approach to problem solving—to understand important questions related to both normal and abnormal biological function, including human disease.

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Cellular Biophysics

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Cellular biophysics is the branch of biophysics that studies cells from the perspective of a physicist or physical chemist by applying physical methods to interrogate cell structure and function, and developing models of cells using physics and physical-chemical principles. Early on, biophysics was usually practiced by physicists or other researchers with physics-based training who had changed fields, but by the 1960s many PhD programs in biophysics had been developed for undergraduate physics and physical-chemistry majors wanting to study biology.

After World War II, biophysics in general got a lift from the field of radiation physics, which was trying to understand the effects of radiation on life and genetic mutations. This came in the wake of H.J. Muller's Nobel Prize studies showing that x rays induced mutations in *Drosophila*. Another major area of biophysics research focused on emerging structural methods such as x-ray diffraction, and spectroscopic methods such as fluorescence and magnetic resonance. This was the advent of the field of molecular biophysics, and these methods were used to determine the structures and functions of individual molecules and contributed to the molecular biology revolution.

On the cellular side, however, there was great interest in the physiology of nerve and muscle cells, and understanding how molecular components drive cell function. Forces and electrical activity are topics of great interest to physicists, and biophysicists have played a major role in understanding them in biological systems. Nerve cells propagate spikes in electrical potential, called action potentials, across an individual cell, and these signals transmit information from one nerve cell to another nerve or muscle cell. These spikes can be initiated by electrical or chemical stimuli and are measured using electrodes. This research culminated in a Nobel Prize to Alan Hodgkin, Andrew Huxley, and John Eccles in 1963 (1).

Muscles generate force through a mechanism involving contraction of individual muscle cells. Our understanding of this process has been greatly enhanced by detailed structural studies of the organization of muscle cells using electron and light microscopy. Muscle cells form highly organized repetitive filamentous structures, and changes in the spacing of these repetitive structures during contraction form the basis of the sliding-filament hypothesis, which holds that the molecular components found periodically along the muscle fiber slide to affect contraction (2).

Microscopy: a major theme in cellular biophysics

The organization and activities of cells are major themes in cellular biophysics, and studies have focused on observing complex structures inside cells, detecting cellular activities, and extending methods developed to study purified biological molecules to microscope-based cellular measurements. Microscopy, which functions across multiple scales of time and spatial resolution, is at the center of these studies. The highly localized and often transient nature of cellular activities is an overarching theme that has emerged from live-cell microscopy and drives contemporary cellular biophysics. For example, some cellular receptors come together to form small bimolecular complexes when they become functionally active. Analogously, many signals that regulate cellular processes are generated from large molecular complexes that form transiently on scaffolds residing in specific locations. These molecular interactions produce new structures that change conformations, produce new functions, or create more efficient organizations resulting in enhanced activity (3). On a larger spatial scale, cellular components are often organized into discrete, readily visible structures, often referred to as organelles or molecular machines. These large, identifiable molecular machines make proteins (ribosomes) generate energy (mitochondria), protect and regulate genetic material (nucleus), and cause cells to contract (actomyosin filaments) (4). They also appear to occupy specific regions and act transiently.

One goal of contemporary cellular biophysics is to understand the molecular details of how cellular components organize to generate and regulate specific activities. Another goal is to determine how all of these diverse cellular activities and structures work together to produce characteristic and specialized cellular behaviors. Biophysicists are also developing mathematical and computational models that describe these cellular functions.

At present, microscopy is at center stage in the world of cellular biophysics, driven by the development of new microscopic methods and fluorescence reagents specialized for cellular imaging. Recent advances in light microscopy now allow us to view structures at previously unattainable spatial and temporal resolutions, image live cells in tissues and animals, and visualize many colors (and thus different molecules) in the same measurement. Amazingly, new

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highly sensitive cameras even allow for the visualization of individual molecules (5–9). Similar to advances in light microscopy, improvements in electron microscope tomography now allow us to see the molecular architecture of organelles in cells at a higher level of detail (10). Finally, measurements can be made in living cells of molecular attributes, including concentration, binding affinities, and diffusion and flow, which were previously studied by using purified components in test tubes (11). All of these measurements provide data that can be used to develop and test theories and mathematical models for complex cellular phenomena.

New fluorescence reagents complement advances in microscopy. They include genetically encoded tags that can be attached to biological molecules, as well as dyes and other fluorescence reagents that localize to specific cellular structures or sense biological activities, telling not only where a molecule or organelle is but also what it is doing at that time. These reagents allow us to localize and measure the positions and dynamics of molecules and the complexes in which they reside, as well as when and where cellular activities occur.

Biosensors are another useful tool that was developed to measure alterations driven by cellular processes. Fluorescence changes are induced in biosensors when molecules come into close proximity or undergo a conformational change (12,13). Similarly, optogenetic reagents allow perturbations of cellular function with great spatial and temporal resolution (14). In contrast, other microscopic methods probe the interaction of the cell with its exterior, sensing the forces that cells exert though their contacts with other cells or connective tissue components (15).

Looking ahead

These are exciting times in cellular biophysics, and this era of breathtaking progress and newly developed technologies points to a bright future for the field. We now have tools to address questions that have lingered for decades, and recent findings are raising new questions that are moving science down important and unexpected paths. Imaging in particular has benefited from significant advances, and our understanding of cellular organization and activities is becoming ever more refined and providing new insights into cellular processes such as cell differentiation. The development of biosensors that report the activities of various cellular machines and processes is still young, but these devices have already revealed how the machinery of the cell is integrated, coordinated, and regulated (16). New genome-editing technologies are being used to introduce genetic tags to specific proteins using the cell's own genome and regulatory apparatus, enabling researchers to obtain highly quantitative measurements regarding the numbers of proteins and organelles (17) present in a cell. Our ability to detect single molecules has already provided highly detailed insights into the mechanisms of specific cellular processes, such as cargo transport along microtubules (18). One goal is to develop mathematical or computational models for individual cellular processes. These models could be based on detailed physical-chemical principles, as has been done for some highly complex and integrated processes, such as membrane protrusion and cytokinesis in vitro (19). They could also be integrated whole-cell models, such as that described for the life cycle of a bacterium (20). The promise is that new methods in quantitative microscopy will provide better data, leading to models that are increasingly realistic and predictive.

The complexity of cells in terms of the numbers of different molecular machines and regulatory complexes, and the numbers of molecules that comprise them, has forced us to look at cells from the point of view of a single or small group of molecules at a time. This approach has been enormously productive, as each protein and complex of proteins becomes a source of fascinating new information as we learn more. However, each molecular machine or complex is comprised of many molecules, each cell has many different organelles and complexes, and there are many different kinds of cells, each exhibiting specialized behavior. In addition, tissues are comprised of many different cell types working together. This integrative behavior of cells is highly challenging and therefore largely uncharted territory.

The ever-growing complexity of understanding how cells work at a molecular level is driving researchers to work more collaboratively and form multidisciplinary teams. Many areas of specialization are needed to understand cellular functions and how they are altered by genetic and environmental factors. New multidisciplinary groups and institutes are being formed, and arguably the largest effort along this line is the Allen Institute for Cell Science, cofounded and supported by Paul Allen, the cofounder of Microsoft.

The Allen Institute aims to develop predictive computational models of cell behaviors and how they respond to environmental and genetic alterations. In its initial project, the Institute is focusing on live-cell imaging and using genome editing of induced pluripotent stem cells (iPSCs) to measure the locations and relative organization of cellular machinery, regulatory complexes, and activities, as well as the concentrations and dynamics of key molecules. iPSCs proliferate and can be induced to differentiate into different kinds of cells, including muscle, nerve, gut, and skin (Fig. 1). Using genome editing, investigators can inactivate a gene or change it by inserting either a mutation that mimics a disease or a fluorescence protein tag, which allows quantitative estimates of molecular number. Once developed and characterized, these cells will become a launch pad for the study of many different cell types by members of the Institute and the greater scientific community, to whom they will be distributed freely. The goal is to measure the changes that occur when cells execute their various activities, as well as when

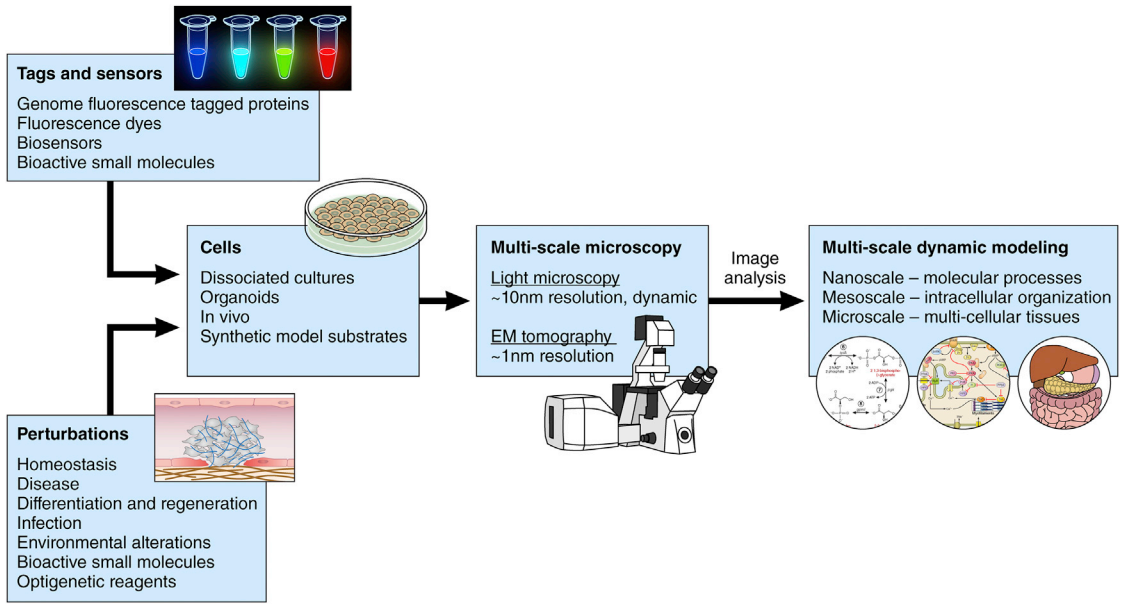

FIGURE 1 A systems approach to meso- and nanoscale imaging and modeling. To see this figure in color, go online.

they differentiate into specialized cells and respond to genetic and environmental alterations, including drug intervention. Investigators could then combine these measurements with other cellular data to develop computational models that predict cellular states and behaviors during homeostasis, regeneration, and disease.

To execute this program, the Institute will foster multidisciplinary research, with a strong focus on physical methods and approaches. This research will have a major biological component that includes the processing, genome editing, and differentiation of iPSCs. These cells will be used to understand different cell states and how these states change as the cells execute their characteristic behaviors and respond to different environments. The Institute will incorporate engineering aspects by bringing its activities to large-scale, automating, and integrating methodologies, and undertaking systems-level approaches. Physical science approaches will take center stage in the state-of-the-art microscopic methods and biosensors that will be employed. Computational and mathematical modeling will benefit from theoretical physics, computer science, and applied math and engineering approaches, as both systems- and physicochemical-level models will be employed. A novel product of the project, an animated cell, will be a visual output designed to integrate image data and existing structural data, and will show the dynamic inner organization and workings of a cell in unprecedented detail. It is also designed to integrate quantitative data on subcellular structures that can be visualized together, allowing the viewer to see, both en groupe and selectively, the relative positions of cellular structures and activities.

The Institute's model for research is defined by attributes that can be applied to similar ventures. These characteristics include the use of large-scale and integrative approaches, such as looking at effects on several cellular components rather than a specialized one. The Institute will share data, reagents, models, and tools openly with the community, and will focus on interdisciplinary team science with clear objectives and milestones.

Cellular biophysicists are working toward understanding cells as individuals and collectives, and how this drives tissue, organ, and organism functions. Such knowledge would help satisfy our innate human curiosity about how life works, and would also contribute significantly to regenerative medicine and disease therapies by elucidating tissue formation and identifying new therapeutic targets.

Cellular biophysics also drives innovation and economic growth. Efforts to understand the biology of the cell have driven the development of new technologies, including two-photon, confocal, light-sheet, and superresolution microscopies. These technologies will greatly impact the pharmaceutical industry by advancing drug discovery and improving diagnostic methodologies. Finally, the ability to model the cell and its regulatory pathways, connecting genomic, epigenetic, environmental, and other data with quantitative cellular data of the kind discussed here, holds enormous predictive promise, leading to a computational "cell clinic" where one can query what the effects of different alterations will be on cell and tissue function, building on the premise that most disease originates from alterations in cell function.

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Optogenetics: Turning the Microscope on Its Head

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Look outside your window. You will likely see green plants, perhaps some yellow, pink, or white flowers, maybe a bird with blue, brown, or red in its feathers and eyes. The world is full of living color, and life has evolved a dizzying variety of chromophores for signaling and photoreceptors for sensing the dynamically changing photic environment.

Scientists are now identifying these chromophores, tweaking them, and then reinserting the genes responsible for them under control of cell-type-specific promoters into species separated by up to two billion years of evolution (1) (Fig. 1). This molecular mix-and-match has led to mice whose neurons are multicolored like an electronics ribbon cable, fish in which brain activity-induced changes in calcium concentration cause active brain regions to light up, and recently, molecular tools by which one can use light to turn on or off the expression of nearly any gene in the genome.

Equally important has been a radical change in how scientists use the microscope. Since the time of Leeuwenhoek, microscopes conveyed light from a sample, greatly magnified, to a viewer. Now microscopes are also used to illuminate a sample with light in precisely sculpted patterns of space, time, color, and polarization. The light tickles molecular actuators, leading to activation of cellular processes in patterns of space and time determined at the whim of the experimenter.

This review describes how scientists are identifying, modifying, and applying optically active proteins, the instrumentation being developed for precisely targeted illumination, and open challenges that a bright student might solve in the next few years.

The fluorescent protein palette

The term optogenetics was coined in 2006 to describe genetic targeting of optically responsive proteins to particular cells, combined with spatially or temporally precise optical actuation of these proteins (2). The field actually started more than a decade before, with the discovery that the gene for green fluorescent protein (GFP) could be transferred from the jellyfish A*equorea Victoria* to the worm *Caenorhabditis elegans* (3), lighting up that worm's neurons.

Triggered by this discovery, scientists adopted a twofold approach to finding fluorescent proteins (FPs) with more colors and better optical properties. Some tweaked the protein scaffold, looking for mutations that increased brightness, photostability, or folding speed or changed the color. Others swam around coral reefs with fluorescence spectrometers, identifying fluorescent creatures and cloning out genes for new FP scaffolds. Both approaches have been spectacularly successful (4). The GFP-derived palette ranges from far blue (emission peaked at 424 nm) to yellow-green (emission peaked at 530 nm).

One of the motivations for these explorations was to develop red-shifted FPs because of the relatively greater transparency and lower background fluorescence of tissue in the near infrared range compared with visible wavelengths (5). One source of red-shifted FPs is the bacterium, *Rhodopseudomonas palustris*, found, among other places, in swine waste lagoons, which produces bacteriophytochrome-based FPs that require a biliverdin chromophore to fluoresce. These proteins enable one to peer deep into the body of a mouse, watching, for instance, a tumor grow under the skin (6).

Perhaps the most dramatic application of the FP palette is in the so-called "Brainbow" mouse (7) (Fig. 2 *A*). Through a clever combination of random genetic rearrangements, each neuron in this mouse produces a distinct set of fluorescent markers derived from a coral, a jellyfish, and a sea anemone. The beautiful multi-hued labeling permits scientists to track the delicate axons and dendrites of individual cells, which otherwise would appear as an impenetrable monochrome tangle.

Blinking, highlighting, and binding

Rainbow-colored mice are visually appealing and scientifically useful, but the capabilities of FPs go far beyond simply tagging structures. Many of these proteins fluoresce to different degrees and in different colors depending on the local environment around the chromophore. This property has found a dizzying array of applications.

Blinking

At the single-molecule level, many FPs spontaneously blink on and off. Some colors of illumination favor the dark state and others the bright state, and proteins can be coaxed in and out of the fluorescent state under optical control. In photoactivation light microscopy, individual FP molecules are turned on sparsely, localized with subdiffraction precision,

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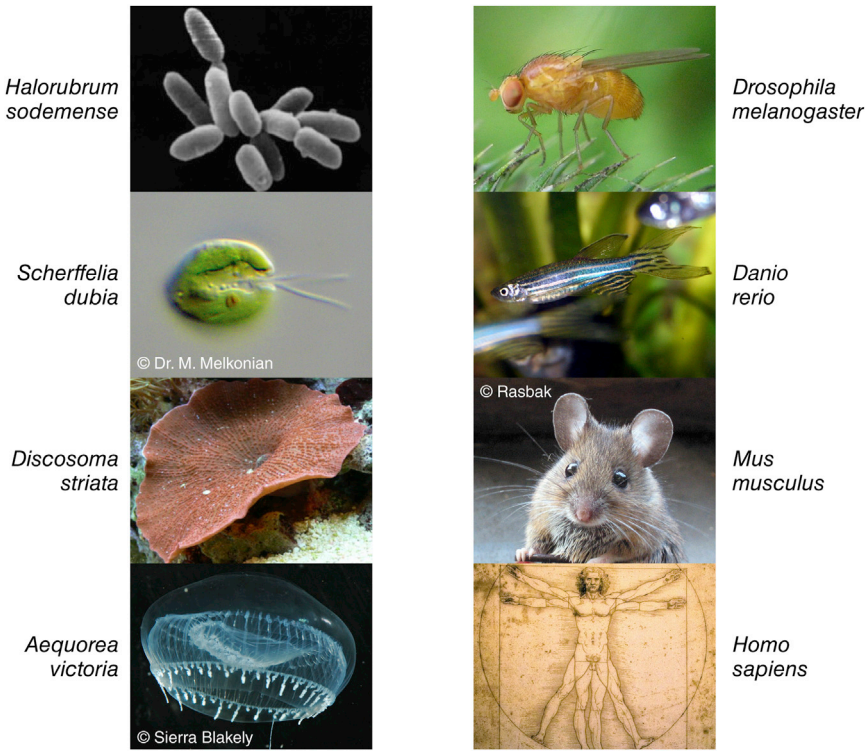

FIGURE 1 Optogenetic mix-and-match. (*Left*) Organisms whose genes have yielded new optogenetic tools. (*Right*) Organisms into which scientists have transferred these genes. To see this figure in color, go online.

and then turned off. Iterating this process hundreds of times builds up a pointillist image of the sample, with resolution far below the diffraction limit (8). This advance was recognized in the 2014 Nobel Prize in Chemistry.

Highlighting

Photoactivatable and photoswitchable FPs have served as optical highlighters for tracking the flow of matter in a cell. One can tag a cellular structure with a flash of light and then follow the motion of that structure through the cell. This enables one to probe how mitochondria move through neurons and track the assembly and disassembly of microtubules.

Binding

Many nonfluorescent proteins change shape when they bind a ligand or a partner. The chromophore in most FPs must pack snugly among surrounding amino acids to fluoresce. Crack open the protein barrel or expose the chromophore to water, and the fluorescence goes away. This combination of features has been exploited by constructing circularly permuted FPs in which the two ends of the amino acid chain are linked, and a new break is introduced near the chromophore. A slight tug on the new ends of the chain can reversibly disrupt the fluorescence and, by fusing nonfluorescent sensor domains to circularly permuted FPs, one can make fluorescent sensors that report ATP, calcium, membrane voltage, and ligand binding to G protein-coupled receptors.

The most dramatic applications of fluorescent sensor proteins come from the GCaMP family of Ca^{2+} indicators (Fig. 2 *B*). The concentration of this ion blips upward every time a neuron fires. Expression of GCaMP-based reporters in the brains of worms, flies, fish, and mice has led to spectacular movies of the coordinated activation patterns of thousands of neurons.

Within the last year, scientists have started to engineer more complex combinations of functions into GFP-based optogenetic tools. For instance, the calcium-modulated photoactivatable ratiometric integrator (CaMPARI) protein starts life as a fluorescent calcium indicator, and, in the simultaneous presence of neural activity and violet illumination, converts from green to red (9). This behavior lets one record a photochemical imprint of the calcium level in a large volume of tissue at a defined moment in time. One can then image the tissue at leisure, with high resolution in space, to map this snapshot of activity.

Many new types of sensors are still needed. A fluorescent reporter for glutamate has been described (10), but reporters for many other neurotransmitters (gamma-aminobutyric acid, dopamine, serotonin, and acetylcholine) are still in development. It also is challenging to sense physical forces. Fluorescent reporters for membrane voltage (11) and cytoskeletal tension (12) have been developed, but we lack voltage indicators that perform well enough to be used in vivo or that can be targeted to intracellular membranes (mitochondria, vesicles, and endoplasmic reticulum). We also lack fluorescent reporters for many of the subtle, but

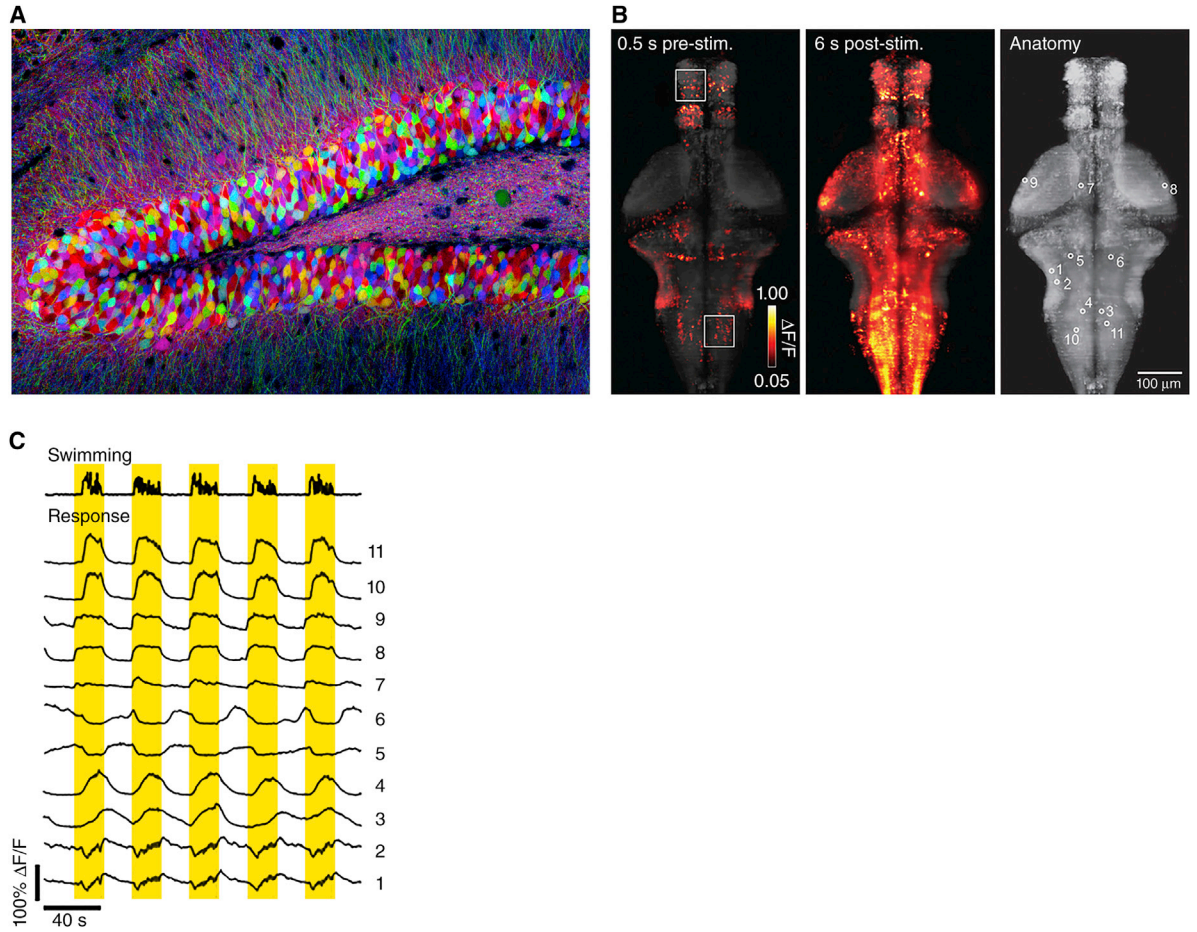

FIGURE 2 Fluorescent protein-based sensors light up the brain. (*A*) Brainbow mouse hippocampus. Random genetic recombination events turn on a different subset of fluorescent proteins in each neuron, giving each one a unique hue. (*B*) Imaging neural activity in the brain of a zebrafish via the calcium indicator GCaMP6s. The fish was immobilized over an image of a drifting grating. When the grating started to move (*stim*), the fish tried to swim to maintain its position within the visual field. The central image shows the brain regions that activated when the fish swam. (*C*) Activity of neurons indicated in the right panel of (*B*) during swimming. (*A* is from Jeff Lichtman; *B* and *C* are from (23).) To see this figure in color, go online.

likely important, physical forces in biology: plasma membrane tension, osmotic pressure, or stresses between cells and their neighbors or the surrounding extracellular matrix.

In the applications described above, light interacts with the FPs—eliciting fluorescence, changing the brightness, or changing the color—but the light does not fundamentally change the underlying biological process (at least not intentionally; phototoxicity is a constant concern for these experiments). The true power of optogenetics emerged when scientists started to use light to perturb the underlying biology in a precise way.

Microbial rhodopsins bring light to the membrane

Most living things sense and respond to changes in light. A diverse set of transducers has evolved to couple light into biochemical signals. Here, we focus on the microbial rhodopsins as a paradigmatic example. The first microbial rhodopsin was discovered in the early 1970s in a halophilic archaeon, *Halobacterium salinarum*, in the salt marshes of San Francisco Bay. The protein has seven transmembrane α helices and a retinal chromophore covalently bound in its core. Upon illumination, the retinal undergoes a trans-to-*cis* isomerization, which induces a series of shape changes in the protein that lead to pumping of a proton from inside the cell to the outside. The protons return back into the cell through the ATP synthase, powering the metabolism of the host.

More than 5000 types of microbial rhodopsins have been identified by metagenomic sequencing. They are found in archaea, prokaryotes, and eukaryotes. Most are uncharacterized, and these proteins mediate a huge variety of interactions between sunlight and biochemistry. Some act as light-driven proton pumps (e.g., bacteriorhodopsin, proteorhodopsins, and archaerhodopsins) and others act as light-driven chloride pumps (e.g., halorhodopsin), light-activated signaling molecules (sensory rhodopsins), or light-gated cation channels (channelrhodopsins).

The discovery that channelrhodopsin 2, derived from the green alga *Chlamydomonas reinhardtii*, functioned as a

light-gated cation channel triggered a race to apply this protein to control neural firing with light. A first-person historical account has been written by one of the chief protagonists, Ed Boyden (13), and a thorough review of the early literature has also been written by Karl Deisseroth, another key protagonist, and his colleagues (14). Early demonstrations in cultured neurons were quickly followed by demonstrations in mouse brain slice, chick spinal cord, worms, flies, and zebrafish. Control of rodent behavior started with simple whisker movements, but then quickly expanded to control of locomotion, sleep, feeding, aggression, memory, and social interactions (Fig. 3 *A*).

Recent work on pup rearing demonstrates the sophistication and precision that optogenetic stimulation has reached (15). In male or female mice showing parenting behavior, a subpopulation of neurons became active in the medial preoptic area. These cells were genetically targeted with a Cre-dependent channelrhodopsin construct and, in virgin male mice that normally show aggression toward pups, optogenetic actuation reversibly switched the animals into a grooming mode. These and many other optogenetic experiments demonstrate that seemingly complex rodent behaviors can be elicited by precise actuation of relatively small numbers of neurons in genetically defined circuits.

Converting microbial rhodopsins into reporters

Efforts to engineer better optogenetic neural modulators relied heavily on mechanistic insights obtained from decades of detailed biophysical studies of the photocycle of bacteriorhodopsin and its homologs. A standard technique in this arena was transient absorption spectroscopy, which triggers the photocycle with a flash of light and subsequently records absorption spectra as a function of time. Motion of the proton through the protein core was accompanied by shifts in the absorption spectrum.

My lab discovered that microbial rhodopsin proton pumps are weakly fluorescent and that this fluorescence varies depending on the location of a proton in the core of the protein. Changes in membrane voltage could reposition the proton, thus changing the fluorescence. We realized that this phenomenon might provide a novel route toward one of the longest-standing challenges in neuroscience: to develop a fast and sensitive optical reporter of membrane voltage.

The initial proteorhodopsin-based voltage indicator (called PROPS) functioned only in bacteria and led to the discovery that *Escherichia coli* generate spontaneous electrical spikes (16). Neither the underlying mechanism nor the biological function of this spiking is well understood. Of the millions of species of bacteria in the world, we know almost nothing about the electrophysiology of any of them.

PROPS did not work in mammalian cells because the protein did not traffic to the plasma membrane. After an unsuccessful year-long effort to engineer membrane trafficking into PROPS, we switched to Archaerhodopsin 3 (Arch), a protein derived from a Dead Sea microorganism, *Halorubrum sodomense*, which was discovered in the early 1980s by an Israeli microbiologist. Arch immediately showed voltage-sensitive fluorescence in mammalian cells (17). Further protein engineering eliminated the photocurrent and improved the sensitivity and speed of the protein, leading to the QuasAr family of voltage indicators (18).

By good fortune, the fluorescence of microbial rhodopsins is excited by red light and emits in the near infrared.

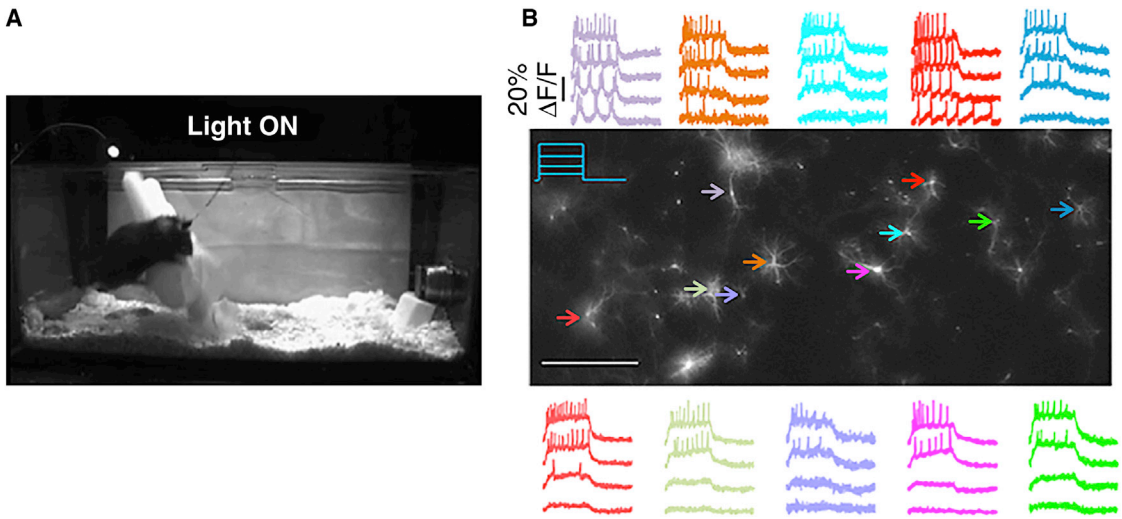

FIGURE 3 Optogenetic control and readout of neural activity. (*A*) Optogenetic control of aggression. In this still from a movie, the mouse is expressing Channelrhodopsin 2 in its ventromedial hypothalamus, ventrolateral subdivision (VMHv1). Illumination of this region through an optical fiber causes the animal to attack an inflated rubber glove, which it would otherwise ignore. (*B*) All-optical electrophysiology. These rat hippocampal neurons express the Optopatch constructs, comprising a blue light-activated channelrhodopsin variant (CheRiff) and a red light-activated voltage indicator (QuasAr2). Illumination with 500 ms pulses of blue light triggers intensity dependent neural activity. Scale bar, 0.5 mm. (*A* is from (24); *B* is from (25).) To see this figure in color, go online.

This feature leaves the rest of the visible spectrum open for other applications: combination with other GFP-based reporters or pairing with optogenetic actuators. We developed a system for all-optical electrophysiology based on a combination of the QuasAr voltage indicators with a new optogenetic actuator derived from a freshwater alga from a pond in the south of England. With this Optopatch construct, one could stimulate a neuron to fire with a flash of blue light and record the response with red excitation and near infrared fluorescence (Fig. 3 *B*). Optopatch has enabled high-throughput functional phenotyping of neurons in culture. It is now being applied to the study of human induced pluripotent stem cell-derived neurons with mutations associated with ALS, epilepsy, and schizophrenia. A key challenge with the microbial rhodopsins is to increase the brightness of their fluorescence so they can be used in tissue and in vivo.

Optogenetic control inside the cell

In recent years, the toolchest of optogenetic actuators has grown dramatically. For nearly any cellular process, someone is working to bring it under optical control. See the article by Zhou et al. (19) for an excellent recent review. Animal rhodopsins have been engineered to control signaling by G proteins, opioid pathways, and serotonin pathways. For control in the cytoplasm, proteins have been developed to regulate enzyme activity and trigger signaling cascades as well as optical control of organelle trafficking (20) (Fig. 4, *A* and *B*).

Developments around optical control over the processes of DNA editing and transcription have been particularly exciting. Transcription activator-like effectors and, more recently, the CRISPR/Cas9 system enable targeting of proteins to arbitrary DNA sequences. Once there, depending on the effector domain, the protein can cut the DNA or turn transcription on or off. Light-activated variants have been made that enable optical control of the activity of nearly any gene in the genome (21,22) (Fig. 4, *C* and *D*).

The microscope as a two-way tool

Much activity in the optogenetics world has focused on molecular transducers. Innovations in the targeted delivery of light are equally important. The concept of the microscope as a passive observation tool is being replaced by the idea that light provides precise handles for tugging and pushing on molecular machines.

Advances in video projector technology have been a key driver of the instrumentation. Digital micromirror devices

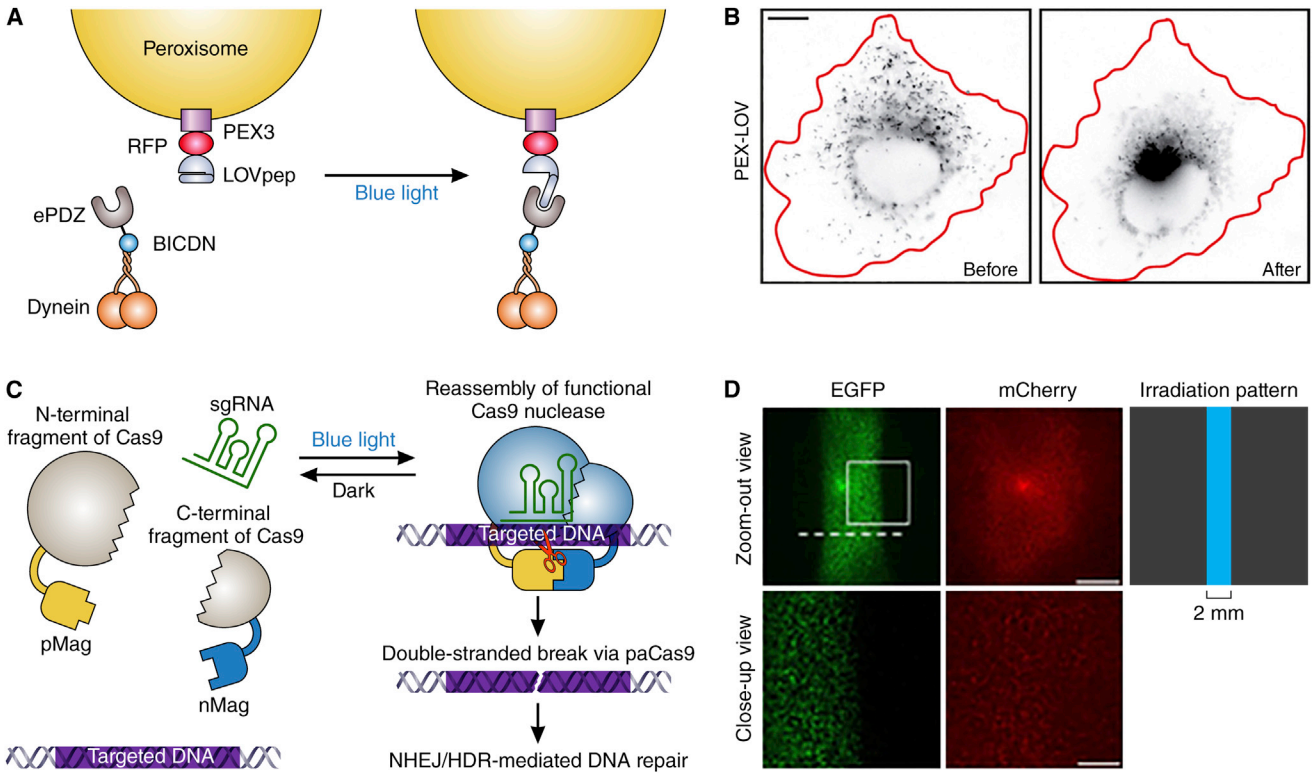

FIGURE 4 Optogenetic control of intracellular processes. (*A*) Control of trafficking. Light-induced association between the LOVpep and ePDZ domains tethers the peroxisome to the motor protein dynein. (*B*) Upon illumination, the dynein drags the peroxisomes toward the nucleus. (*C*) Control of gene editing. Light-induced association of the pMag and nMag domains brings together the two pieces of a split Cas9 nuclease, restoring its ability to cut DNA. The Cas9 cuts at the location determined by the sequence of the guide sgRNA. (*D*) In cells expressing a genetic construct where DNA cleavage led to expression of eGFP, eGFP fluorescence was seen only in the illuminated region. (*A* and *B* are from (26); *C* and *D* are from (27).) To see this figure in color, go online.

(DMDs) comprise an array of typically ~10^6 microfabricated mirrors, each of which can be electrostatically deflected between two orientations. One orientation reflects light to the sample, the other to a beam dump. If one places a DMD in the image plane of a microscope, then each micromirror maps to a single spot on the sample. Thus, one can have ~10^6 points of light, each individually controllable at up to ~10 kHz. The optical path between a DMD and the sample is identical to the path between the sample and the camera, only the arrows on the light rays are reversed.

Another important advance is the development of liquid crystal spatial light modulators (SLMs), which modulate the phase, rather than the amplitude, of the light. This capability can be used to focus or diffract light into user-specified patterns. The SLM has the advantage over the DMD that it can achieve higher illumination intensity at specified points; the SLM redirects light from regions that should be dark to regions that should be bright, whereas the DMD simply blocks light from reaching the dark regions. However, the SLM is not as fast as the DMD, and it is more complex to control because there is not a simple relationship between the pattern on its pixels and the pattern on the sample.

Future opportunities

For almost any cellular function or biochemical process, one can imagine using optogenetics to gain control. For instance, one would like to use light to tag RNA molecules for subsequent pulldown and sequencing, to tag protein molecules for subsequent analysis by mass spectrometry, or to control the cell-cell interactions in a developing embryo or a healing wound. These capabilities have not yet been developed, but they are readily envisioned.

To achieve maximum flexibility, one would need robust two-photon-activated optogenetic constructs. Then one could turn on or off any endogenous or exogenous gene in intact tissue on the basis of an arbitrary measurement. A challenge here is the low efficiency of two-photon photochemistry. The use of two-photon excitation to trigger autocatalytic amplification cascades may provide a route.

There are many instrumentation challenges. We need better ways to localize optical excitation at greater depths and with greater spatial precision in highly scattering tissues or to image fluorescence emission in three dimensions in scattering tissues. Structured illumination or optical coherence techniques may help bypass optical scattering, and the integration of imaging with computation represents one of the forefronts of optogenetics.

With the right combinations of genes and optical hardware, one can imagine exciting new directions in biology. If one could control cell-cell interactions optically, one could perhaps optically sculpt tissues with novel or unusual shapes and functions. Optogenetic stimuli might mimic the spatially patterned gradients of morphogen signaling that guide embryonic development, but with the much greater flexibility of light compared to diffusion, one might coax cells to grow into multicellular structures that could not arise by natural means.

Optogenetics will likely find applications in humans. Companies are currently working to develop channelrhodopsins for vision restoration. Light-controlled proteases may one day provide an ultra-precise surgical tool or an optically triggered viral infection could enable spatially targeted gene therapy. Tattoos with reporter proteins (or the genes encoding them) could provide simple diagnostics, allowing the facile transdermal readout of physiological state.

CONCLUSIONS

Optogenetics as a field cuts cleanly across traditional disciplinary boundaries. Ecology and genetics provide a source of proteins; advanced spectroscopy and structural biology elucidate molecular mechanisms; molecular biology and biochemistry are used to engineer proteins; sophisticated instrumentation delivers light. An understanding of cell biology or neuroscience is needed to develop reasonable biological questions, and rigorous computation is essential to process the torrents of data that often result. The development of optically instrumented life forms promises to continue for the decades ahead.

Optogenetics also illustrates the difficulty in predicting where basic science will lead. The Optopatch constructs combine genes from an archaeon from the Dead Sea, an alga from England, an FP from a coral, an FP from a jellyfish, and a peptide from a pig virus. The discoverers of these individual genes likely never suspected that they would be combined one day and used in human neurons to study a cell-based model of neurodegeneration in amyotrophic lateral sclerosis.

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Probing Nature's Nanomachines One Molecule at a Time

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Did you know that proteins are nanoscale machines that help us think, dance, and keep the threat of cancer at bay? Did you know that biology is a new research frontier for physicists? Here, I will discuss how biophysicists are using light-based tools to poke and examine nature's nanomachines, one molecule at a time, uncovering the amazing acrobatic abilities that are essential for all forms of life.

Proteins as nanomachines

DNA is our genetic material that stores all the information necessary to build our cells and body. Proteins are made based on genetic information encoded in the DNA, and they are the central players that perform nearly all chemical reactions that make life possible. Because of proteins, our nerves can fire, our eyes can sense colors, and we can move our muscles. Proteins are often called *nano*machines because they are unimaginably small, just a few nanometers across. How small is a nanometer? Human hair is about the thinnest object our naked eyes can see and is ~80,000 nm thick. That is, we can fit 20,000 proteins across the width of a single hair! If your body is expanded to the size of the earth, a single cell in your body would be about as big as a good-sized city, and a single protein molecule would be about as tall as a person. Many proteins are also called nano*machines* because they work as molecular motors that convert chemical energy into mechanical energy and they do so with precision and robustness that would make the best engineers cry in envy. A great example of molecular motors is a DNA packaging motor that can push thousands of basepairs of DNA into the very small volume of a virus particle, eventually reaching a pressure like that found inside a champagne bottle (1). Another example is a protein called kinesin that carries cargoes inside the cell. Just as cars move on the highway using gasoline as the fuel, kinesins move on cellular highways called microtubules using the chemical energy released from the burning of ATP, the cellular currency of energy (2). These cargo-carrying motor proteins move directionally, some moving toward the cell center and others moving away from the cell center, and by selectively turning on and off a subset of motors, cells can move pigments around, allowing animals such as chameleons to change their skin color to match their surroundings. Such directional movements are also crucial for delivering cargoes to different parts of a nerve cell, which cannot rely on random, diffusional movement due to their substantial length. A better understanding of these molecular motors may help us detect and treat human diseases that are caused by defects in these proteins and may lead to the design and synthesis of artificial nanoscale machines. To study these tiny machines, we need tools that can examine biological motions at the nanoscale.

Single-molecule measurement technologies

In the last 20-plus years, breathtaking technological developments, often coming out of the laboratories of physicists, have allowed researchers to study nature's nanomachines at the level of single molecules, establishing the field of single-molecule biophysics.

Why are single-molecule measurements necessary and powerful? If all of the molecules under observation move in lock step, as in a marching band, averaging their signals would not obscure their movements. But this is generally not the case, and in most cases, you cannot forcibly synchronize their motion. Just imagine trying to achieve that for college students on campus! For such non-synchronizable situations, single-molecule measurements can reveal complex dynamics that are hidden in ensemble experiments. In addition, molecules can have personalities; that is, nominally identical molecules can behave differently due to their complex composition built from thousands of atoms, and they may even be moody, changing their characters over time. Averaging over a heterogeneous population, therefore, can be misleading. Steven Chu, a Nobel laureate, famously joked that on average, one person on this planet has one ovary and one testicle. Finally, single-molecule measurements allow us to make correlations between molecular properties. Think of Americans' views on climate change and the safety of genetically-modified-organism crops. Only when we survey individuals do we realize that conservatives tend to dismiss scientific consensus on climate change and that liberals often ignore scientific evidence supporting the safety of genetically-modified-organism crops. For these reasons,

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single-molecule measurement techniques that use the ability to detect and manipulate single molecules have become widely adopted by the researchers. Here, I will discuss two major fluorescence-based technologies used for single-molecule measurements.

Single-molecule localization and tracking stoichiometry

Imagine a soccer field being imaged from a faraway planet. If you attach an LED lamp to your favorite soccer player, because of the fundamental phenomenon called light diffraction, that lamp may appear to be about the size of the soccer field to that distant observer. Nevertheless, using a simple mathematical trick, we can determine the position of the lamp, or "localize" it, with great precision, say, down to the size of the player's shoe. In principle, we can track the movements of the player and his feet with precision limited only by how many photons are being detected to form the image. For example, we can determine the size of his gait during a run, or his "step size" if you were to use the jargon of biophysicists. The same trick can be used to localize individual protein nanomachines and track their positions over time.

Using this single-molecule localization-and-tracking approach, researchers have shown that myosin V, which carries a cargo along a track called the actin filament, moves in steps of 37 nm in length (3). Furthermore, by labeling the "foot" of myosin V with a single dye, a 72-nm step length was observed, which is double the center-of-mass step length. The latter finding conclusively showed that myosin V walks like a human adult, with the two feet taking the leading position alternatingly, instead of crawling like an inchworm (4). These types of studies also showed that proteins can slide along the length of DNA and determined the rapidity of their motion and whether the motion was unidirectional or not. As I will discuss below, a protein's motion on DNA in search of a target can be very important in keeping the threat of cancer at bay.

In addition, if we label a single protein with one dye and one dye only, we can deduce how many proteins are working together at a given time by determining the brightness of the protein assembly; that is, if it is four times as bright as a single dye, we can conclude that four copies of the same protein are needed for a certain function (5). If multiple dyes of different colors are used, we can also determine how many different kinds of proteins are functioning in the same biological process. This type of "stoichiometric" information is often difficult or tedious to obtain using other approaches.

However, single-molecule localization alone cannot follow the internal motion of the molecule. We can see how quickly a soccer player can change direction and how fast he runs with a ball, but this alone does not tell us what makes Maradona a great player instead of a merely average player. We need to increase the number of degrees of freedom that are being observed.

Single-molecule FRET: conformational changes and molecular interactions

One powerful technique that can follow the conformational changes of a molecule is fluorescence resonance energy transfer (FRET). "Conformation" is jargon used by biologists to denote the shape of a molecule. Just as a soccer player needs to change his bodily shape or posture to run and handle a ball, proteins need to change their conformations repeatedly to carry out their duties. In FRET, dyes of two different colors, say green and red, are attached to two sites of a protein. Normally, when we excite the green dye with a laser, we would see only green photons coming out. But when the two are very close to each other, within a few nanometers, the two dyes communicate with each other and the excitation energy is transferred from the green dye to the red dye such that we now see red photons come out. The relative ratio of the two colors is then used as a measure of their distance from each other, and if we know where the dyes are attached on the protein, we can deduce conformational changes of the molecule. Fig. 1 shows a cartoon of a rap musician dancing, undergoing conformational changes between different postures that are detected as anticorrelated changes in the intensities of green and red signals.

Let me illustrate the use of FRET to study DNA repair. Inside every cell of our body there are two meters of DNA. Because there are ~10^{14} human cells in our body, with the estimated renewal of 100 times for an average cell during our lifetime, our body would need to make about one light year length of DNA. DNA is under constant threat of damage. For example, sunlight and smoking can cause DNA damage that can accumulate and eventually lead to cancer. If DNA repair did not exist when we need to make so much DNA, we would die of cancer at a far younger age than is usually the case.

One major mechanism of DNA repair is called homologous recombination. When a segment of DNA is broken, the cell uses another copy of the same DNA as a template to repair the breakage. To aid this process, a protein filament is formed around the broken DNA, and this filament then searches for a matching DNA sequence in a sea of millions of basepairs of DNA. This is no easy task and is often compared to finding a needle in a haystack.

How does the cell accomplish this feat rapidly yet accurately? One possibility is to perform a three-dimensional search. Let the filament land on a random location of the target DNA, and if there is no sequence match, then dissociate and repeat until a match is found. It is equivalent to dating random people on the street until a soul mate is found, which would be exceedingly time consuming if you live in a big city. Another possibility is to perform

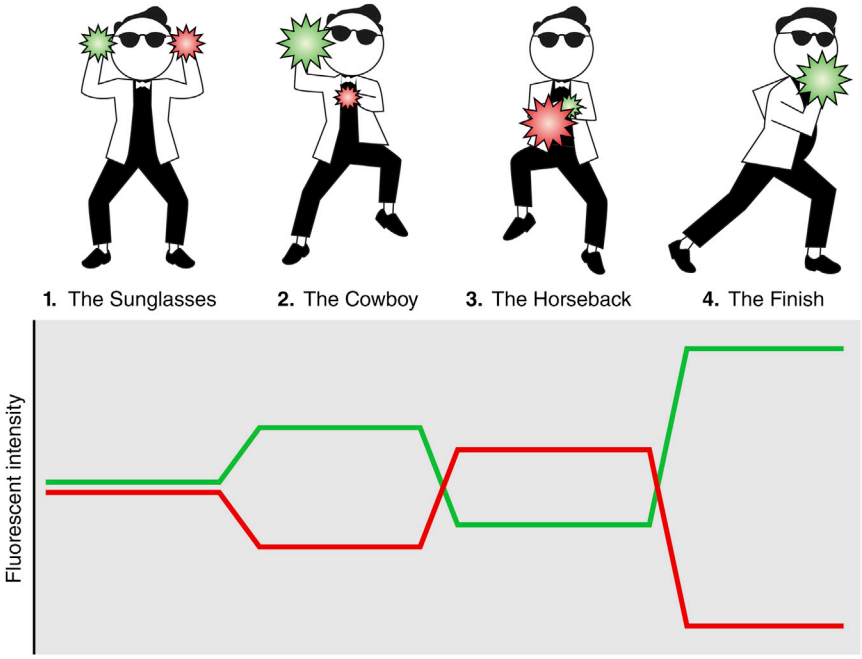

FIGURE 1 How to record the dancing moves of a rap musician. Labeling the right hand with a green dye and the left hand with a red dye makes it possible to follow the distance changes between the two hands as a function of time. When the two hands are very close to each other (horseback posture), fluorescence excitation energy is transferred from the green dye to the red dye, in a process called FRET, so that the red signal is stronger than the green. When the two hands are far away from each other (cowboy), there is little energy transfer and the green signal is stronger. Once the dance finishes and the red dye is released from the musician, only green signal is observed. In the case of protein nanomachines, FRET occurs over a length scale of a few nanometers. Because of close proximity, the two dyes would not appear as two separate objects in single-molecule imaging. Instead, one sees a single object that changes color over time. This illustration was created by Dr. Jingyi Fei at the University of Chicago. To see this figure in color, go online.

one-dimensional (1D) sliding. This is akin to joining a book club to sample a few dozen of its members in search of a potential mate. The filament around broken DNA can bind to a random DNA sequence but slides along the DNA back and forth over hundreds of basepairs. This local search can be much more effective when combined with a three-dimensional search. If the local search is unsuccessful, the filament can dissociate and bind another region of DNA, which would be equivalent to joining a different club, such as a knitting club.

Using FRET, one can probe for such 1D sliding activity. A green dye on the filament and a red dye on the target DNA would result in fluctuations in FRET, anticorrelated changes in the intensities of green and red signals, providing evidence for 1D sliding (6). But can the filament really find the target sequence through sliding? To address this question, we can put two near-matching sequences on the target DNA. The filament would spend some time on one near-matching sequence and after it realizes that the match is not perfect, it will leave and slide, and will land on the other near-matching sequence, and this can continue back and forth. This would be like dating two twin brothers, each of whom is not a perfect match. Overall, single-molecule FRET measurements suggest that such 1D sliding may accelerate the finding of the matching sequence by as much as 250 times, possibly aiding DNA repair greatly.

Marriage of single-molecule FRET and optical tweezers

Another single-molecule technique, called optical tweezers, or what I call "chopsticks made of light," can apply very small forces, down to 10^{-12} N of force, and measure the response mechanically at the nanometer level. For example, optical tweezers have been used to measure the step size of many molecular motors (2). Recently, the precision of optical tweezers has improved to the angstrom scale, almost the size of a water molecule (7).

In optical-tweezers measurements, it is as if you are closing your eyes and using your hands to manipulate and measure the response of an object, whereas in fluorescence measurements, you have your hands tied in back and make passive observations with your eyes. By combining the two, we can hope to sample the best of both worlds.

For example, we can use optical tweezers to measure the activities of single proteins such as helicases, which unwind DNA into single strands using the energy of ATP molecules and at the same time measure the conformational changes of the protein using FRET. Using such a hybrid instrument, it was shown that a helicase called UvrD unwinds DNA when it takes the "closed" conformation and rezips DNA when it takes the "open" conformation (8). When a related helicase was forced to maintain the closed form, it became a superhelicase that can unwind thousands of basepairs without falling off, even against a very strong opposing force (9). This superhelicase may be useful for various biotechnological amplifications, such as rapid pathogenic DNA detection and sequencing in developing countries or during surgery in hospitals.

Future outlook

Single-molecule measurements have revealed the amazingly complex but elegant abilities of nature's nanomachines to

perform life's central functions. Yet these molecules by themselves are not alive, and they function only when they work in the cellular milieu. How living cells come alive through the concerted actions of individual molecules is a major challenge for future research. Researchers are making progress on multiple fronts.

On one front we might call "extreme in vitro," we can measure multiple properties from single-molecular assemblies. FRET can be extended up to four colors, allowing us to measure six distances simultaneously. This can also be combined with multiaxis optical tweezers to study, for example, DNA replication, which is an amazingly rapid and accurate process that requires more than a dozen different proteins, with single basepair resolution, while at the same time probing how many proteins of which kind are present at each step of the reaction. On another front, we can push the technology to the cellular level, and even to the tissue level, ultimately hoping to view single-molecule activities in living cells in full glorious detail. Finally, we might find a third way, where protein complexes can be captured from freshly sacrificed cells or animals, or patient tissues, for detailed single-molecule analysis.

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The Current Revolution in Cryo-EM

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Structural biology is the study of the molecular architecture of proteins and nucleic acids, which are the basis for all life forms. Structural biology came into its own as a field during the 1950s when the atomic structures of DNA (1) and several globular proteins (2) were solved. Knowledge of these structures alone is not enough to understand their functions, but it has become clear that a detailed mechanistic picture of function is not possible without structural information. Studying structure can reveal how molecules have evolved, and this type of insight would otherwise be lost by looking at only the molecule's sequence.

X-ray crystallography has been the primary technique responsible for determining atomic models of macromolecules and macromolecular complexes during the past 60 years. X-ray crystallography begins with the crystallization of a purified molecule or molecular complex. An x-ray beam is directed at the crystal, and a unique pattern results from the diffraction of x-ray beams in different directions after encountering atoms in the crystal. An atomic model is generated from this diffraction pattern data that satisfies stereochemical constraints and whose diffraction matches the observed pattern within experimental error. This approach has been enormously powerful but is limited by the fact that the molecule or complex of interest must be crystallized, which is not always possible. Polymers, such as protein or nucleoprotein filaments, typically must have exactly two, three, four, or six subunits per turn, or multiples of these numbers, to pack into a crystal.

Nuclear magnetic resonance techniques emerged in the 1980s and allowed people to determine the three-dimensional (3D) structure of macromolecules in solution (3). These solution techniques can be quite laborious and are very difficult to use for large complexes.

In this review, I will discuss how cryo-EM (electron cryomicroscopy to some in the field, cryo-electron microscopy to others) has rapidly (Fig. 1) emerged as one of the main techniques for determining the structures of many macromolecular complexes at near-atomic resolution.

The "hardware"

The first electron microscope was built in the 1930s by Ernst Ruska, but it was not until ~1945 that the first applications to biological samples were made by Keith Porter and colleagues. Electron microscopy (EM) has been a valuable imaging tool for biological specimens but is limited by both hardware constraints and the nature of biological samples. In contrast to light, electrons scatter strongly from air, which requires that the column in an electron microscope be under a vacuum. This generates a problem in imaging biological material because a specimen, which may be found naturally in an aqueous environment, must be dried before it can be introduced into the vacuum of the electron microscope. Another major problem of the electron microscope arises from the fact that the contrast generated by thin biological samples is quite weak, much as the contrast generated by a cell in solution is quite weak in the conventional light microscope. Different staining methods have been developed to generate contrast for dried biological samples in the electron microscope, just as histochemists have worked for more than a century to create such stains that can be used in the light microscope. Since the focus of this review is on EM as a tool for macromolecular structure determination, I will not deal with the very rich history of using EM to look at whole cells and tissues, or with the recent impressive advances in electron tomography (4).

The negative staining method for EM emerged in the 1960s and was used extensively in the 1970s and 1980s for looking at isolated macromolecules and macromolecular complexes. In light microscopy, positive stains typically bind to a particular substance and this provides the contrast between what is stained and readily observed and the unstained remainder. The negative stain technique, developed by Hugh Huxley and colleagues (5), involves surrounding a sample with an electron-dense solution, such as uranyl acetate. This solution is excluded by the molecule or complex of interest and can be dried to form a glass that is placed in the vacuum of the electron microscope. Negative staining reveals the molecule due to its stain exclusion properties, and only the shape of the region where stain has been excluded can be distinguished. The negative stain technique might be able to show whether a protein was globular or elongated, but does not elucidate characteristics of a molecule's secondary structure, such as the presence of α-helices and β-sheets, which determine the overall folding of the protein.

A great advance came in 1975 when Richard Henderson and Nigel Unwin demonstrated that unstained two-dimensional protein crystals of arrays of bacteriorhodopsin, imaged by EM at a resolution of ~7 Å, could show the presence of α-helices (6). These arrays were embedded in glucose, which preserved the array's structure even when dry. The contrast was exceedingly weak in glucose-embedded samples, and the use of crystals was still needed to generate a visible

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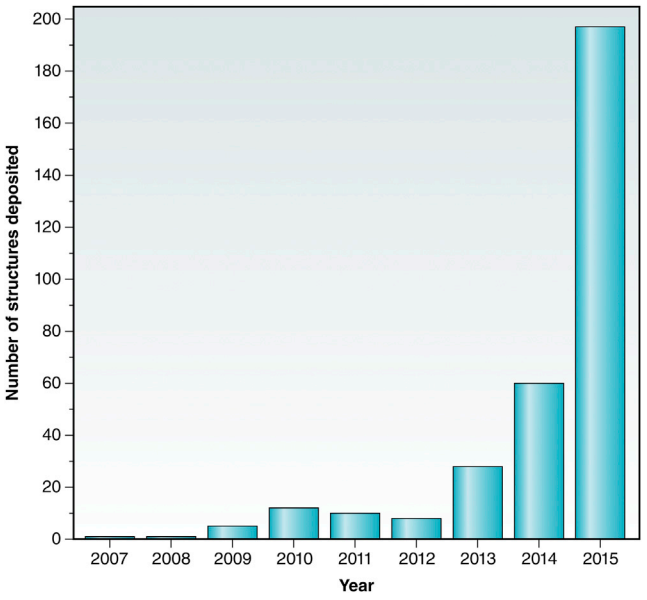

FIGURE 1 The number per year of 3D cryo-EM reconstructions deposited to the Electron Microscopy Data Bank (EMDB) at better than 5.0 Å resolution. To see this figure in color, go online.

structure. At around the same time, Ken Taylor and Bob Glaeser demonstrated that frozen and fully hydrated samples could be imaged by EM (7) using a cryo-stage that kept the specimen near liquid nitrogen temperatures while it was in the vacuum of the electron microscope. This surmounted the fundamental problem plaguing biological EM, which was the previous inability to image hydrated samples in the microscope. But Taylor and Glaeser froze samples conventionally, which meant that the water froze into crystalline ice and caused irreversible damage to biological samples due to changes in water volume. The field of cryo-EM took an enormous leap forward when Jacques Dubochet and colleagues developed a method for the routine vitrification of EM samples (8). When water is frozen extremely quickly, it undergoes vitrification and forms an amorphous solid phase, a glass that is not crystalline (9). Given the relative molecular simplicity of water, this was a surprising observation because such a phase was never predicted theoretically. This transition to a vitreous glass does not disrupt macromolecular structures. Rather, molecules become frozen in whatever state they exist in solution, which is called cryofixation. The thin vitrified film containing the molecules of interest can be maintained at liquid nitrogen temperatures for many days in a vacuum with negligible sublimation. An entertaining personal account of the development of vitrification for EM samples by Jacques Dubochet has appeared recently in *Biophysical Journal* (10).

The contrast of macromolecules embedded in vitreous ice was much greater than when embedded in glucose, but these are still weakly scattering objects that can best be viewed by a phase-contrast method, similar to phase-contrast in light microscopes. The phase-contrast technique used in cryo-EM involves defocusing the microscope but requires a coherent source. Conventional electron microscopes use a simple filament as an electron source much like that found in an incandescent light bulb, but these sources lack the coherence needed for high-resolution phase-contrast imaging. A field emission gun for the electron microscope had been developed (11), and the combination of this source, commercial cryo-stages, and a vitrification method that could be used reproducibly and reliably meant that cryo-EM started to be used in many laboratories around the world in the 1990s.

EM can be quite labor intensive, as an experienced microscopist might need to spend weeks or even months on the microscope to collect the large amount of images needed for the image analysis and reconstruction discussed below. The fully automated electron microscope was developed by Carragher and colleagues (12), which was a significant advance that allows current microscopes to work in an unattended manner 24 h a day, 7 days a week.

The traditional means of recording an image in the electron microscope, dating back to the time of Ruska, involved photographic film. But using film is very tedious, it must be developed and then scanned for subsequent digital image processing, and it limits how many images can be acquired in a day. For many applications, charge-coupled device (CCD) detectors were used to surmount these problems, but CCD detectors were worse than film in terms of sensitivity and resolution. The current revolution in cryo-EM is due directly to the adaptation of complementary metal oxide semiconductor chips (13), hardened to prevent damage from electrons, which have a resolution and sensitivity greater than film and a readout rate much faster than CCD detectors.

The "software"

The images obtained by cryo-EM are projections of a 3D structure onto a two dimensional film or detector. Like a medical chest x-ray, these projections can be rich in information but can be hard to interpret due to the superposition of all structure onto a single plane. At about the same time that computed tomography was being developed in medical radiology, it was also realized that one could recover the 3D information from EM specimens. David DeRosier and Aaron Klug generated the first 3D EM reconstruction (14). Rather than using multiple images as would be done in medical tomography, they took advantage of the helical symmetry present in the tails of an icosahedral bacteriophage. The helical symmetry means that identical copies of a protein are related to each other by just a rotation and translation in the tail, so a single projection image of the tail provides all of the information to generate a 3D reconstruction. A vast number of assemblies in biology are helical, but many other types of structures exist. Single particle methods in EM began with Joachim Frank and colleagues (15), and took advantage of the fact that when a large ensemble of molecules is imaged by EM, all possible

orientations may be found. Thus, a 3D reconstruction can be generated by determining the orientations of individual particles. Single-particle approaches have been applied to helical structures (16), overcoming the need in the original DeRosier and Klug method (14) for long-range helical order in a structure to achieve a reasonable resolution. In a crystal, long-range order is maintained by space group symmetry but a helical polymer does not maintain such long-range order. Deviations from an ideal or average symmetry accumulate, and a segment that is far from a reference segment may show little correlation in terms of the expected positions of subunits. This is similar to the bending of a polymer in solution or a strand of spaghetti in boiling water. The conformation of a polymer having the minimum energy is typically a straight rod, but thermal fluctuations accumulate, thereby knowing the orientation of the polymer at one point does not allow one to predict the orientation of a segment a long distance away. The helical viruses shown in Fig. 2 are rather rigid compared to flexible filamentous plant viruses, such as bamboo mosaic virus (17), but an analysis of the helical symmetry in such viruses shows that long-range helical order does not exist. With single particle methods in cryo-EM, a reconstruction at ~3.8 Å resolution (Fig. 3) was obtained for *Sulfolobus islandicus* rod-shaped virus 2 (SIRV2) (18), something that would not have been possible at any reasonable resolution just 3 to 4 years ago, before direct electron detectors were commercially available.

The number of single particles used in 3D reconstructions by cryo-EM can easily exceed hundreds of thousands, something that would have been computationally impossible back in 1968 when 3D EM began. Just as progress in DNA sequencing (due to the highly computational nature of current sequencing) has roughly followed Moore's Law, stating that the number of transistors in an integrated circuit doubles every 2 years, progress in cryo-EM has also followed the advances in computing power. Many images are needed to overcome the inherently poor signal/noise ratio in cryo-EM. Fig. 2 shows an example of a typical micrograph. Most of the power in the image, which is proportional to the variance, or the sum of the squared deviations from the average intensity, in the image is actually due to noise. A significant part of this noise arises from the electron counting statistics or shot noise because a low enough electron dose is used, which can cause random fluctuations in electron counts in neighboring pixels to become significant. This noise is more negligible as many particle images are aligned and averaged together, and a much greater problem becomes the conformational heterogeneity that exists for many molecules and complexes when they are not packed tightly in a crystal.

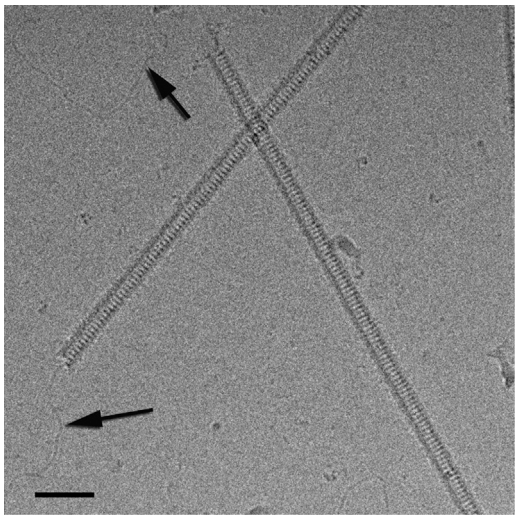

FIGURE 2 A cryo-EM showing two *Sulfolobus islandicus* rod-shaped virus 2 (SIRV2) virions (18). SIRV2 is a virus that infects a hyperthermophile living in nearly boiling acid. Almost all of the long capsid seen is formed by several thousand identical copies of a coat protein that wraps around and protects the DNA. The arrows point to double-stranded DNA that can be seen emerging from the ends of the virions. The scale bar is 500 Å.

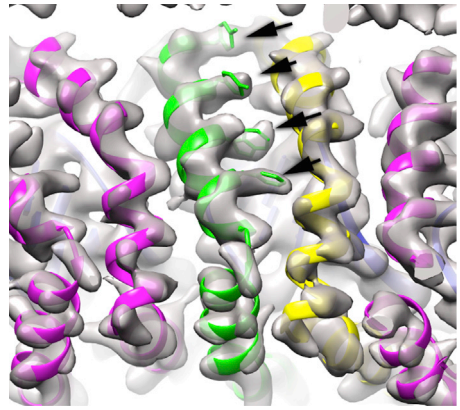

FIGURE 3 The interior of a 3D reconstruction from SIRV2 (18). Most of the capsid protein in the virus is found in the form of α-helices, and at a resolution of ~3.8 Å the pitch and right-handed twist of these α-helices are readily visible in the density map (*transparent gray surface*). Every capsid protein is identical, and the building block of this assembly is a symmetrical dimer formed by two copies of the capsid protein. Thus, the yellow and green helices come from two different subunits and form antiparallel helices because the polypeptide chains are oriented in opposite directions from each. At the available resolution, bulky aromatic side chains can be visualized (*arrows*). Because the sequence of the capsid protein is known, this allows for an unambiguous threading of the sequence through these helices. To see this figure in color, go online.

The approach to this heterogeneity in solution NMR is to generate an ensemble of structures, all consistent with the constraints observed, whereas in cryo-EM one might generate a few structures that clearly correspond to different conformational states or only a single structure, excluding those particles that do not match this structure. For the ribosome, a recent study (19) described starting with >1.6 million particles, which were reduced to a more homogeneous subset of ~400,000 particles, yielding a resolution of better than 2.9 Å. We simply do not know the limit of resolution obtainable by cryo-EM for macromolecules. A recent structure (20) of a bacterial enzyme, β-galactosidase, reached 2.2 Å (Fig. 4)

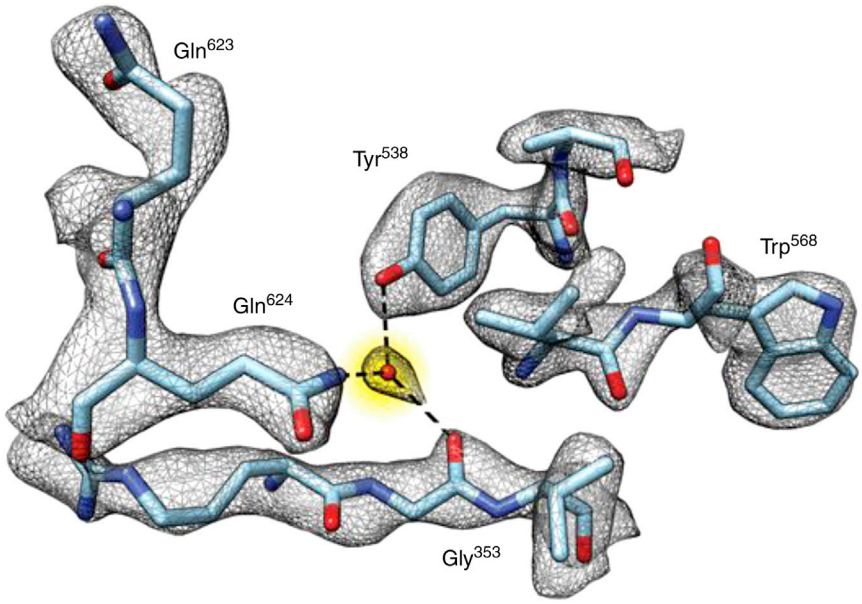

FIGURE 4 A small region from the 2.2 Å resolution cryo-EM reconstruction of β-galactosidase (20). The resolution is high enough to see an ordered and bound water molecule in the center (*yellow*). To see this figure in color, go online.

and the authors suggested that the main limitation on resolution that we now face may be simply the intrinsic flexibility of proteins and not the hardware or software that we use. Nevertheless, it is clear that further improvements in microscopes, detectors, and image processing software will lead to many more macromolecular complexes that can be solved at resolutions approaching 2.0 Å. The growing field of super-resolution light microscopy has been aimed at surmounting the fundamental limitation on resolution in light microscopy, the wavelength of light (~0.5 μm or 5000 Å). In cryo-EM, the wavelength of the electrons is typically <0.02 Å, therefore the wavelength of the illumination does not set the physical limit on resolution.

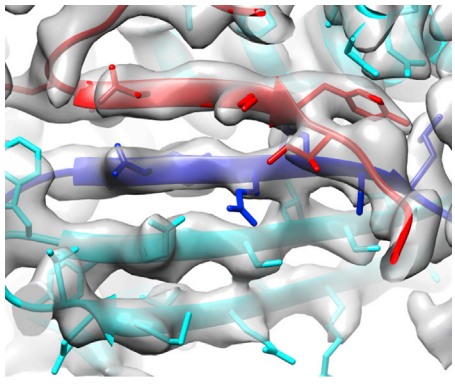

FIGURE 5 A region from the type VI secretion system sheath of *Vibrio cholerae* (21), reconstructed by cryo-EM at ~3.2 Å resolution. The atomic model that was built into the reconstruction (*transparent gray surface*) has three different molecules shown in cyan, red, and blue. At a resolution worse than ~4 Å, one might have ambiguities in tracing the individual polypeptide chains present in each molecule. The resolution that was achieved prevented any such ambiguities, and it was clear that the β-sheet shown involved two strands from one molecule (*cyan*), and one strand from each of two different molecules (*red* and *blue*). To see this figure in color, go online.

What biological insight does one gain from higher resolution? Consider Fig. 5, which shows a β-sheet from the sheath of a type VI secretion system in *Vibrio cholerae*. The resolution of this cryo-EM reconstruction (21) was ~3.2 Å, high enough to allow a complete chain trace of ~600 amino acids in the asymmetric unit of the sheath. Because the sequences of the two proteins in this asymmetric unit were known, it was possible to thread this sequence through the density placing the large and bulky side chains (as in Fig. 3) into their corresponding density. If one had only 5 Å resolution, portions of this structure might have been built correctly but ambiguities would have existed, such as in the β-sheet shown, and it would be unlikely that the correct connectivity could be established. Or consider Fig. 4, where ordered water molecules are visualized by cryo-EM reconstruction, and suggest that the resolution is high enough to understand enzymatic reactions or design drugs.

CONCLUSIONS

The rapid advances in cryo-EM over the past several years make it nearly impossible to predict where the field will be in several years. It is reasonable to expect that the exponential growth of near-atomic resolution structures determined by cryo-EM (Fig. 1) will continue, but we still do not know possible limits in resolution or how large a complex must be to reach near-atomic resolution. The future of cryo-EM is certain to be exciting, with new biological insights gained from this powerful technique.

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Making Sense of Intrinsically Disordered Proteins

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Proteins form the molecular scaffolding of life and are essential to catalyzing the chemical reactions that sustain living systems. These characteristics have led us to think that proteins function only when folded into the right structure. The central dogma of molecular biology states that genetic information encoded in the DNA sequence is transcribed into messenger RNA and then translated into a sequence of amino acids, which folds into a protein. The mechanisms that govern how a linear sequence of amino acids folds into the correct three-dimensional structure are still not well understood. Biophysical techniques have been indispensable to unraveling how protein structures fold, and many of the major factors that determine how the amino-acid sequence codes for the folded protein structure are beginning to be understood.

The genomic era that began at the end of the 20th century gave scientists access to complete genome sequences. Scientists observed that some of the predicted protein sequences derived from genomes were not expected to fold into normal globular protein structures (1). At the same time, experimental studies began to uncover examples of important protein molecules and domains that were incompletely structured or completely disordered in solution yet remained perfectly functional (2,3) (Fig. 1). In the following years, an explosion of experimental data and genome annotation studies mapped the extent of this intrinsic disorder phenomenon and explored the possible biological reasons for its widespread occurrence. Answers to the question of why a particular domain would need to be unstructured are as varied as the systems where such domains are found.

One of the hallmarks of intrinsically disordered proteins (IDPs) is a marked bias in the amino-acid composition, including a relatively low proportion of hydrophobic and aromatic residues, and a relatively high proportion of charged and polar residues (Fig. 2). The high frequency of small hydrophilic amino acids renders these sequences as unlikely candidates for membrane or scaffolding proteins. Yet many of the proteins identified in surveys, as well as in concurrent NMR experiments, showed that these proteins were involved in important cellular processes such as control of the cell cycle, transcriptional activation, and signaling (4,5), and they frequently interacted with or functioned as central hubs in protein interaction networks (6). The amounts of various IDPs in the cell are tightly regulated to ensure fidelity in signaling. Altered abundance of IDPs is associated with disease (7).

Disordered sequences can also be found in proteins that contain ordered, structured domains, and these disordered sequences are termed intrinsically disordered regions (IDRs). Some IDRs function as linkers between interaction domains (Fig. 2), and in some cases, their properties as polymers contribute to their function (8). Many IDRs contain sequence elements that interact with partners and frequently fold upon binding. For example, the intrinsically disordered interaction domain of the transcription factor STAT2 folds upon binding to its partner, the TAZ1 domain of CREB-binding protein (CBP) (Fig. 3) (9). Backbone flexibility of an IDR in its free state enables it to bind to multiple targets, which increases its potential repertoire of responses, as exemplified in the binding of the hypoxia-inducible factor HIF-1α. The transactivation domain of HIF-1α binds to its partner TAZ1 as a helix (10), whereas the same HIF-1α sequence binds to the hydroxylating enzyme FIH as a β-strand (Fig. 4) (11).

Disorder makes IDR sequences accessible to posttranslational modification and IDRs are rich in modification sites. IDRs facilitate efficient protein-protein interactions using only a small number of residues. A folded protein would need to be much larger to provide an interaction surface area equivalent to that seen with IDRs, as illustrated in Fig. 4. This efficiency is important in signaling, as it translates into the ability to bind with high specificity but only modest affinity, enabling dissociation of the IDR after signaling is complete. Signaling can be turned off by competition between IDRs for a particular physiological partner, mediated by slightly different binding sites (Fig. 5). The reaction of cells to hypoxia (low oxygen) is a good example of this phenomenon (Fig. 6). Under normal conditions, the HIF protein is synthesized in the cell, but is degraded upon hydroxylation of two prolines. Interaction with the transcriptional coactivator CBP is further interdicted by the hydroxylation of an asparagine in the C-terminal activation domain (CTAD). Under hypoxic conditions, the hydroxylation reactions no longer occur, so the protein is stable to degradation and the CTAD can interact with the TAZ1 domain of CBP, leading to transcription of hypoxia-response genes such as VEGF, which promotes growth of blood vessels (12). Such a response is dangerous if not constrained, however, and the signal must be turned off before adverse physiological effects occur. One of the genes

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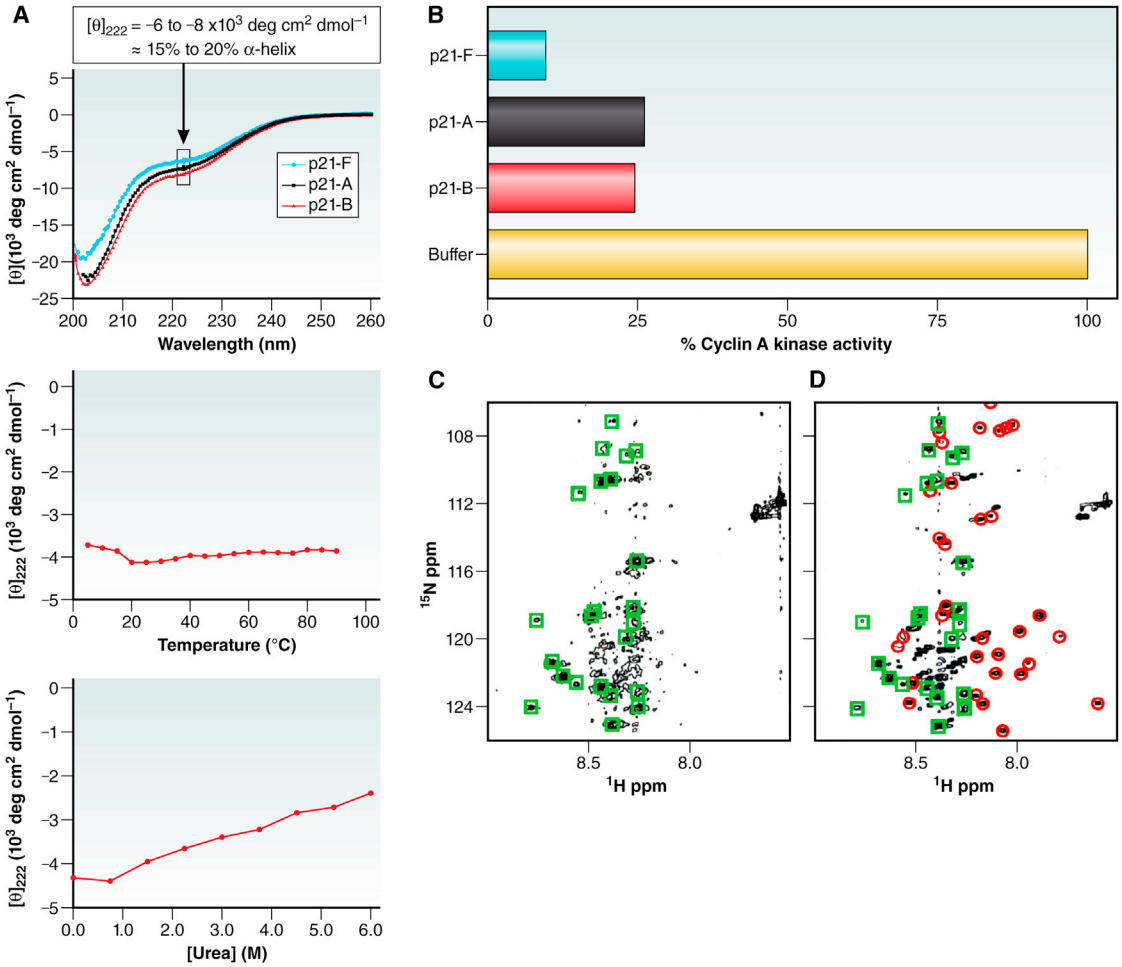

FIGURE 1 (*A*) The cyclin-dependent kinase inhibitor p21 does not form recognizable structure in solution, indicated by the far-UV circular dichroism spectrum of constructs of various lengths (*top panel*), by the absence of temperature-induced (*middle panel*), or urea-induced (*bottom panel*) unfolding transitions. (*B*) All of the p21 constructs are active in the inhibition of cyclin-A kinase activity. (*C* and *D*) ^{1}H-^{15}N HSQC NMR spectra of free p21 (*C*) and p21 bound to cyclin-dependent kinase-2. (*D*) Squares indicate cross peaks present in the same place in the two spectra, and circles denote new cross peaks at positions that indicate that folding has occurred (adapted from Kriwacki et al. (2) with permission, © 1996 National Academy of Sciences, USA). To see this figure in color, go online.

transcribed in response to the hypoxia signal encodes an inhibitor, CITED2, which removes the HIF CTAD from the CBP TAZ1 domain, turning off the hypoxia response. Several anti-cancer treatments include inhibition of the hypoxia response, which prevents vascularization of tumors, thus restricting their growth (12).

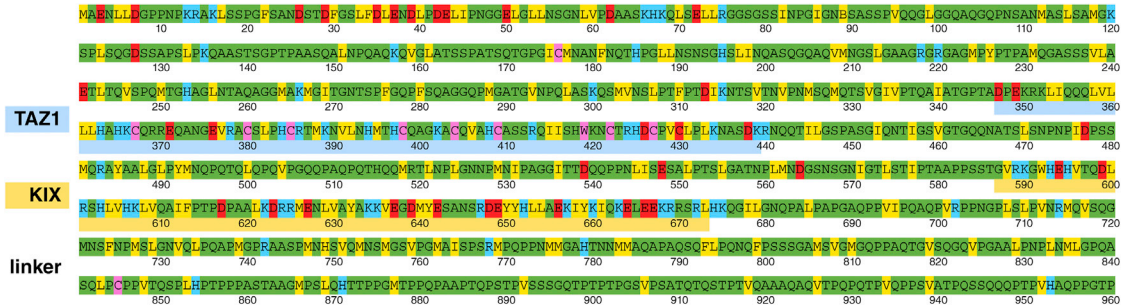

FIGURE 2 Illustration of the sequence bias found in disordered sequences. The amino-acid sequence of a portion of the multidomain transcriptional coactivator CBP is classified by amino-acid type: green, small hydrophilic amino acids (G, A, S, T, N, Q, P); yellow, hydrophobic amino acids (V, L, I, M, F, Y, W); red, acidic amino acids (D, E); blue, basic amino acids (K, R, H); pink, cysteine (C). The sequences of two folded domains, TAZ1 (*blue*) and KIX (*yellow*), show much greater sequence diversity than the disordered flanking and linker domains, which are predominantly green, indicating a heavy bias toward small hydrophilic amino acids (adapted from Dyson and Wright (4)).

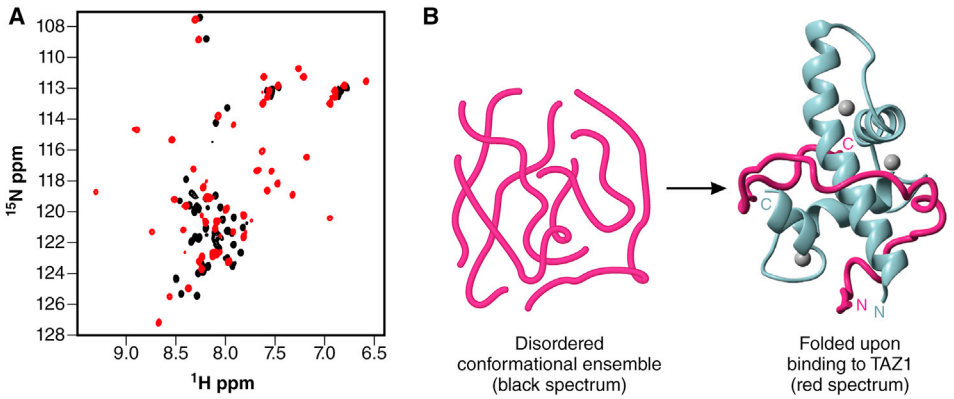

FIGURE 3 Coupled folding and binding of the transcription factor STAT2 on the TAZ1 domain of CBP. (*A*) Disorder in the free STAT2 is shown in the small resonance dispersion in the ^{1}H dimension of the black ^{1}H-^{15}N HSQC spectrum. The structured nature of the bound STAT2 is shown by the increased ^{1}H dispersion of the gray spectrum. (*B*) Schematic diagram illustrating the conversion of the disordered conformational ensemble of free STAT2 into a structured form on the TAZ1 (adapted from Wojciak et al. (9)). To see this figure in color, go online.

IDPs offer novel advantages as therapeutic targets. Their central role in key cellular signaling pathways (5), their frequent association with disease (7), and the reversible nature of their intermolecular interactions, by which they bind with high specificity but modest affinity, makes them extremely attractive targets for small molecule drugs or stapled peptide mimetics (13,14). Indeed, many viruses hijack the host cell by using their own viral IDPs, e.g., the adenovirus E1A or papillomavirus E7 oncoproteins (15,16), to compete with cellular IDPs for binding to key regulatory proteins (17). IDPs commonly bind to concave grooves in the surface of their target proteins; the interactions are predominantly hydrophobic and the fit is more intimate than to their globular protein counterparts. Finally, it may prove possible to design drugs targeted against the IDP itself, rather than its globular target.

Disordered regions of proteins provide a uniquely versatile and useful toolbox for reactions in the cell. Interestingly, the majority of IDRs so far characterized are from eukaryotic systems, in which they are intimately involved with the signaling and physiological control required for multicellular organisms. IDRs are found in prokaryotes, but they tend to be associated with unusual functions in particular bacteria, for example the toxin-antitoxin systems of phage-infected *Escherichia coli* (18).

Protein molecules are rarely, if ever, completely rigid. Dynamic motions of backbone and side chains, independent of the tumbling of the whole molecule, can be estimated by various spectroscopic means, and are frequently associated with the function of enzymes. Disordered regions and fully disordered proteins can be thought of as a continuation of this characteristic, by which functional disorder, an extreme form of local protein dynamics, is functional through the particular advantages bestowed by the disordered state. The continuum between rigidity and complete disorder provides an expanded proteome, allowing proteins to perform multiple tasks through interactions with different partners or under different conditions. Disorder occupies an important biological niche that promises

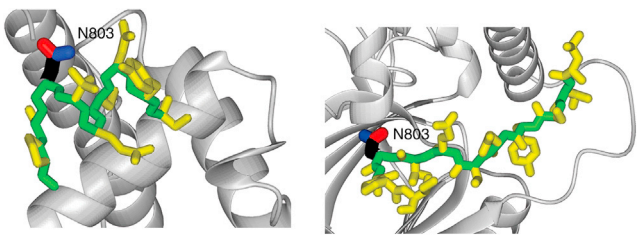

FIGURE 4 Structural differences between the transactivation domain of HIF-1α bound to the TAZ1 domain of CBP (10) (*left panel*), where the sequence containing the regulatory asparagines appears as an α-helix, and the same sequence bound to the hydroxylating enzyme FIH (11) (*right panel*), where it appears as a β-strand. To see this figure in color, go online.

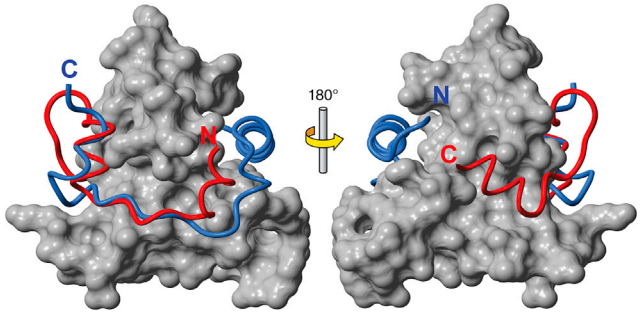

FIGURE 5 Comparison of the structures of ligands bound to the CBP TAZ1 domain. The surface of TAZ1 (almost identical in both complexes) is shown in gray, with the backbone of HIF-1α-C-terminal activation domain (10) in red (labeled N in the *left image* and C in the *right image*) and the CITED2-*trans*-activation domain (19) in blue (labeled C in the *left image* and N in the *right image*). (*Left* and *right panels*) 180° rotation around the vertical axis in the plane of the page (adapted from Wojciak et al. (9)). To see this figure in color, go online.

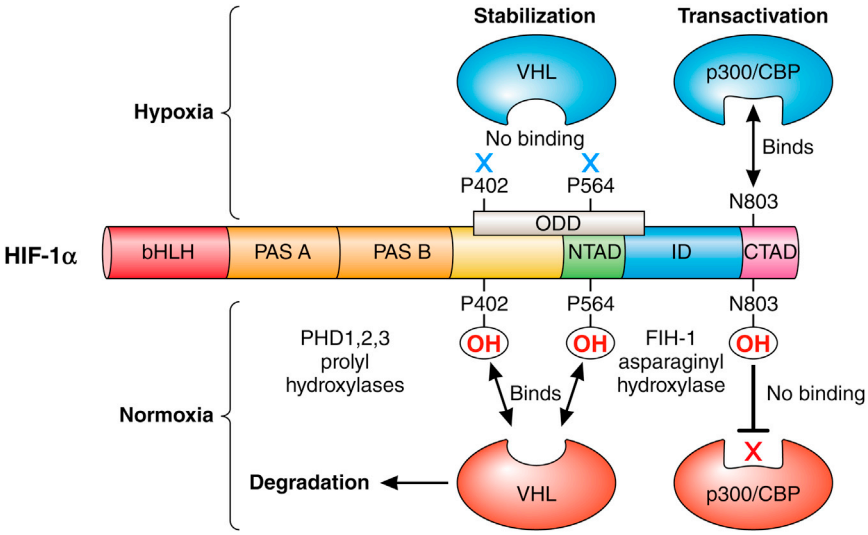

FIGURE 6 Schematic diagram showing the regulation of the HIF-1α transcription factor under normal oxygenation conditions (*bottom*), where proline hydroxylation in the central oxygen-dependent degradation domain recruits the von Hippel-Lindau factor, leading to degradation, and asparagine hydroxylation in the C-terminal activation domain lowers the affinity for transcriptional activators. In hypoxic conditions (*top*), neither the prolines nor the asparagines are hydroxylated, with the result that HIF-1α is stabilized and binds to CBP/p300 to promote transcription of hypoxia-response genes. (Reproduced from Dyson (20) with permission.) To see this figure in color, go online.

to yield important new insights into how biological systems operate.

ACKNOWLEDGMENTS

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Inherited Arrhythmias: Of Channels, Currents, and Swimming

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Inherited arrhythmia syndromes may predispose individuals to life-threatening arrhythmias and sudden cardiac death (SCD). Affected patients are usually young and their everyday life may be impaired by recurrent loss of consciousness, palpitations, or dizziness. They might have even been saved from SCD by successful cardiopulmonary resuscitation, which may be the first manifestation of their disease.

Making the diagnosis is often difficult, as signs and symptoms may by unspecific, variable in time, and depend on external influences or situations. Biophysical studies, however, have contributed significantly to characterizing specific inherited arrhythmias, analyzing their underlying cause(s) and mechanism(s), identifying risk factors for the development of life-threatening arrhythmias, as well as developing specific treatment strategies.

Many inherited arrhythmias result from dysfunctional ion channels, which are proteins at the cell surface dedicated to enable movement of ions across the cell membrane. These diseases are therefore named "channelopathies." Biophysical methodology is particularly suitable for the investigation of ion channel function to deduce potential therapeutic targets. Different experimental models can be employed, ranging from the expression and characterization of mutation-derived ion channels on single cells to observations in affected patients during diagnostic clinical procedures. Additionally, therapeutic agents are tested in laboratory experiments to confirm their efficacy in restoring coordinated electrical activity.

The field of inherited arrhythmias is one example of how biophysical research contributes to elucidating disease mechanisms and developing specific therapeutic strategies. As a consequence, evidence derived from biophysical studies can be transferred to clinical practice to the benefit of patients affected by inherited arrhythmia syndromes.

Clinical case: the unexpected end of a swimming trip

On a summer day, a 7-year-old girl suddenly becomes unconscious during swimming in a public pool and is rescued out of the water by her father. Several months ago, she already had to spend 1 week in the hospital after a concussion due to loss of consciousness while riding her bike. When she was 2 years old, she received cochlear implants due to inner ear hearing loss. Both parents are healthy. Her 5-year-old brother experienced loss of consciousness for the first time 2 days ago. She is taken to the outpatient clinic of the local hospital by her parents.

As a first step, an electrocardiogram (ECG) is obtained (Fig.1 *A*). The pathological finding becomes apparent when comparing the ECG signal to the signal of a healthy person (Fig. 1 *B*). The time period marked by the blue line is strikingly prolonged in this young patient. This time period reflects the time between the start of electrical activation of the ventricular muscle (seen as the *Q-Wave* in the ECG, Fig. 1 *A*), and the time at which the ventricular muscle enters the resting state again (marked by the end of the *T-wave*, Fig. 1 *A*). The disease that is suspected in this girl is called long-QT syndrome (LQTS).

Inherited arrhythmias and sudden cardiac death

The LQTS is an inherited arrhythmia that is associated with loss of consciousness and may be a cause of sudden cardiac death. SCD refers to death by cardiovascular cause within 1 h of acute onset of symptoms. SCD rates are estimated between 50 and 100 per 100,000 deaths in the general population and 180,000–450,000 cases in the United States annually (1). The prominent underlying reason for SCD is arrhythmia affecting the lower chambers of the heart, the ventricles, which are responsible for pumping blood into the circulatory system of the body and lungs. In most cases, acquired structural heart disease, e.g., coronary artery disease or thickening of the heart muscle due to longstanding untreated hypertension or heart valve dysfunction, predisposes for the development of ventricular arrhythmias (1,2). However, ~5% of SCD cases are estimated to result from inherited arrhythmias (3). Additionally, in many cases of an undiscovered cause of SCD, an inherited arrhythmia may have been the predisposing factor.

Ion channels and cellular electrical activity

The underlying mechanisms for inherited arrhythmias were comprehensively investigated in genetic and biophysical studies. Many inherited arrhythmias have been found to result from dysfunctional ion channels on the cardiac

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Maura M. Zylla and Dierk Thomas contributed equally to this work.

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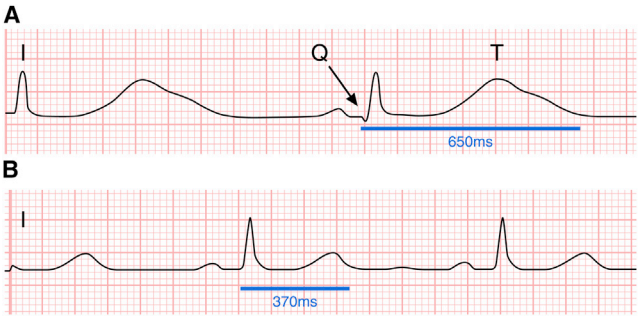

FIGURE 1 ECG trace of a patient with LQTS and a healthy individual. (*A*) ECG trace from patient with LQTS (*lead I*). The QT interval (*blue line*), from the beginning of the Q-wave (*arrow*, Q), marking of the start of electrical activation of the ventricular muscle, to the end of the T-wave (T), representing regression of electrical activation, is severely prolonged. (*B*) ECG trace from a healthy individual (*lead I*). The QT interval is within the normal range of <440 ms. Note the difference between the QT interval in the ECG from the patient in (*A*) and that of the healthy individual. To see this figure in color, go online.

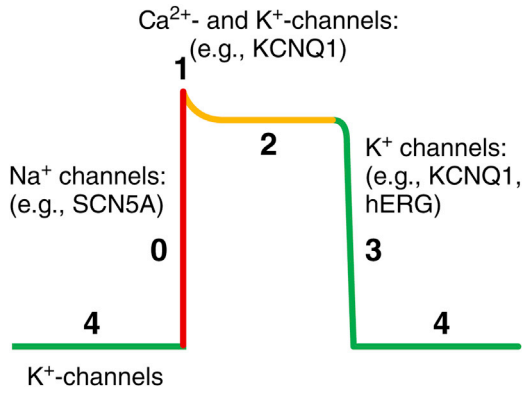

FIGURE 2 The action potential of a ventricular cardiomyocyte. The different phases of the action potential and the respective ion channels involved are depicted. The respective genes encoding for the ion channels are shown in parentheses. Note the KCNQ1- and hERG-channels (mainly active during repolarization), which are affected in certain subtypes of LQTS. Also note the SCN5A-channels, which play a role in Brugada syndrome and familial sick sinus syndrome (active during depolarization). Red (phase 0), depolarization period; yellow (phase 2), plateau-phase; green (phases 3 and 4), repolarization period and resting membrane potential. To see this figure in color, go online.

muscle cells called cardiomyocytes. Each cardiomyocyte expresses a variety of ion channels on its cell membrane. Different types of ion channels are permeable for specific ions and have distinct biophysical properties. Ion channels control the coordinated electrical activation of cardiomyocytes as the ion currents they pass lead to changes in the electrical potential across the membrane of the cell, called the membrane potential. The membrane potential is determined by a difference in distribution of ions between the inside and the outside of the cell. Ion channels can open and close depending on specific triggers. Many are voltage gated and open or close depending on changes of the membrane potential. Also, some ion channels are directly or indirectly, via intracellular molecular processes, influenced by the binding of certain signaling molecules or hormones.

At rest, the membrane potential is negative because it is dominated by potassium conductance. An activation of the cell by electrical excitation is seen as a deviation from this baseline potential in the form of the so-called action potential. Researchers assess the action potential (Fig. 2) by measuring electrical activity of an electrically excitable cell. In cardiomyocytes, the action potential begins with a phase of activation (depolarization) that is seen in an upstroke of the action potential caused by an influx of sodium ions into the cell. Subsequently, a plateau-phase is predominantly determined by calcium entering the cell. In this phase, calcium initiates molecular processes leading to contraction of the cardiomyocyte and beating of the heart. Afterward, the phase of regression of electrical activation (repolarization) and relaxation is initialized by a delayed activation of potassium channels and an efflux of potassium out of the cell, which finally restores the negative resting membrane potential (Fig. 2).

Electricity and cellular orchestration

Just like in an orchestra, the performance of the whole system of the heart muscle depends on the reliable function of each single part. Following electrical activation, mechanical cellular contraction is initiated. Cardiomyocytes are connected to each other and are able to communicate via electrical activity. However, electrical activity has to spread among the cell assemblies in a coordinated manner to guarantee efficient mechanical contraction of the ventricle as a whole. Only if all cardiomyocytes are orchestrated in a coordinated manner, the ventricles can sufficiently fulfill their main function of pumping blood to support the central nervous system and the peripheral organs with oxygen. The natural pacemaker of the heart is the sinus node, which is located at the right atrium and controls the heart rate in normal rhythm (Fig. 3). From there, the electrical activity spreads to the atria. After atrial excitation is completed, electrical activity is transferred to the ventricles via a switch point at the junction between the atria and the ventricles, called the AV node (Fig. 3). This coordinated spread of activity is ensured by the fact that, when one part of the myocardium has been activated and has contracted, it enters a resting state and becomes inert. This phase of temporary inactivation is an intrinsic property of ion channels. On a cellular basis, the cardiomyocyte can only enter the next phase of depolarization after the membrane potential has returned to baseline. During the plateau phase of the cardiac action potential (phase 2, Fig. 2) and period of repolarization (phase 3, Fig. 2) the cell is not sufficiently excitable. This contributes to a sufficient delay until the next cycle of activation can be initiated. Consequently, the

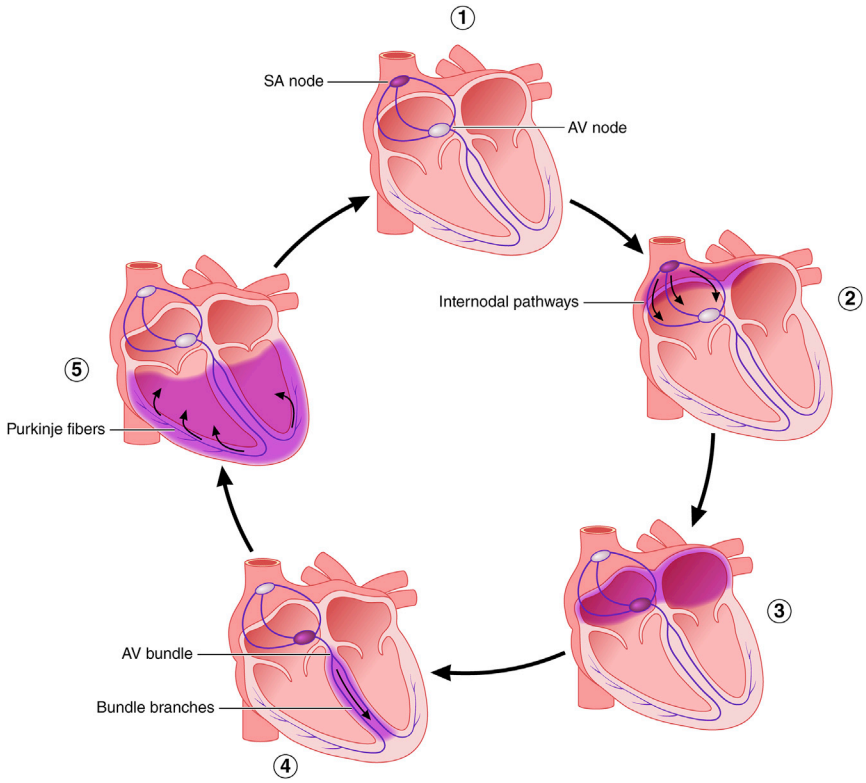

FIGURE 3 Conduction system of the heart. An electrical impulse is generated by the sinoatrial (SA) node or sinus node (*1*). Electrical activity then spreads across the atria, which causes contraction of atrial myocardium (*2* and *3*). After passing the AV node, electrical activation is propagated to excite the ventricular myocardium via bundle branches and Purkinje fibers (*4* and *5*). To see this figure in color, go online.

electrical activity can proceed to the neighboring myocardium in only one direction. Of importance, this intricate coordination depends on the length of the action potential, which ensures a balance between heart rate and excitation properties of the cardiomyocytes. The length of the action potential, however, is dependent on the coordinated interplay of ion channels. Therefore, if one type of ion channel is dysfunctional, the electrical activation of the single cell is altered, which, in turn, results in a disturbance of the electrical properties and the contraction process of the whole ventricular muscle.

LQTS: how delay causes acceleration

One example for impaired ventricular function due to a dysfunction of ion channels is the LQTS, which was diagnosed in the girl from our clinical case example.

Most patients with LQTS present at a young age with recurrent loss of consciousness. Depending on the subtype of LQTS, sudden loss of consciousness often occurs during physical activity (particularly during swimming), in response to startling sudden noises or to emotional stress. The reason for loss of consciousness in these patients is the development of a fast ventricular arrhythmia. Due to its characteristic morphology of twisting spikes around an isoelectric baseline, this arrhythmia is called "torsades-de-pointes"(TdP) tachycardia (Fig. 4). As a result of severely elevated heart rates and a disturbed spread of electrical activity through the ventricles during the arrhythmia, the heart fails to supply the central nervous system with sufficient amounts of oxygenated blood. If these arrhythmias are self-terminating, the patient experiences dizziness or loss of consciousness for a short period of time. However, TdP tachycardia may degenerate into ventricular fibrillation, which is often the cause of SCD in these patients.

Genetic analyses of patients with LQTS have, in the majority of cases, revealed mutations in genes encoding for cardiac potassium channels. Prominent subtypes of potassium channels that may be affected are KCNQ1-channels or hERG-channels (4–6). These potassium channels are both involved in the repolarization phase of cardiomyocytes (phase 3, Fig. 2). Biophysics enters at this stage. Biophysical studies were designed to assess mechanistic consequences of these identified gene mutations for the function of the respective ion channel. For this purpose, genetic information from long-QT patients was transferred to nonexcitable cells, which are easy to examine and do not express other endogenous ion channels, or other noncardiac cell lines. These cells consecutively expressed the defective potassium channels, encoded by the transferred genetic information, on their cell membrane. Thus, they constituted an experimental model in which cellular electrical activity could be measured and biophysical properties of defined ion channels could be analyzed. These experiments revealed that the changes in protein structure lead to altered biophysical properties of these potassium channels and, as a result, a reduction of the potassium

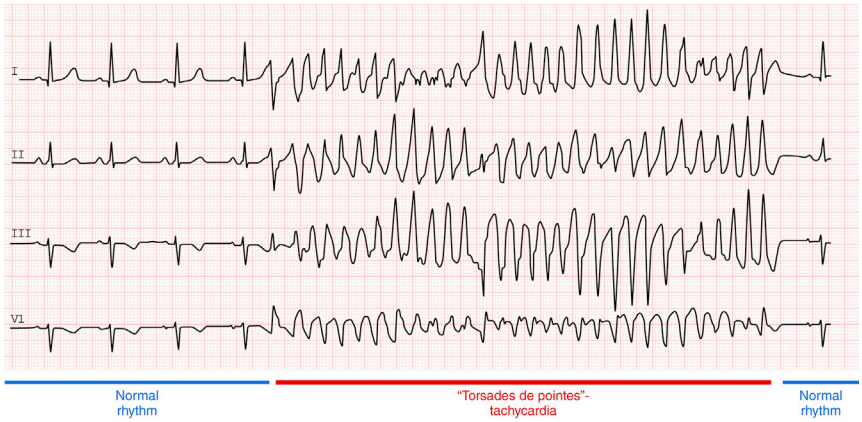

FIGURE 4 Example of self-terminating TdP tachycardia. Normal sinus rhythm (marked by *blue line*) is disrupted by a short period of TdP tachycardia" (*red line*), which is self-terminating and followed, again, by normal sinus rhythm (*blue line*). To see this figure in color, go online.

current (7). The effect on a cellular level is a delay in repolarization.

However, the cell lines and the experimental setup that were used do not represent the native environment of cardiomyocytes. To confirm this finding in circumstances more analogous to the human myocardium, insights from stem cell biology and studies on cell differentiation were employed. Various differentiated cells, such as human skin cells from a patient's small skin biopsy, can be reprogrammed into pluripotent stem cells. These have the ability to differentiate into various kinds of tissue and are called "induced pluripotent stem cells" (iPSC). To investigate underlying mechanisms in LQTS, iPSCs generated from affected patients' tissue were differentiated into beating cardiomyocytes that were genetically homologous to the cardiomyocytes of the patients. Thus, these cells also expressed the respective dysfunctional ion channels. Similarly, in the iPSC model, investigations regarding the biophysical properties of the affected potassium channels confirmed the reduced potassium current and the resulting loss of function of these ion channels (8,9).

Different mutations have been found in genetic screening of affected families since the LQTS was first described in the 1960s by Romano and Ward. Experimental data have elucidated different molecular mechanisms that lead to a dysfunction of potassium channels. In general, these can affect either the quantitative expression of channels on the cell membrane or the speed and extent of channel closing and opening (10). The resulting effect in both cases is a delay in repolarization.

A prolonged repolarization period influences recovery of calcium channels, which operate during the active contraction period of the cardiomyocyte and the plateau phase of the action potential. After the plateau phase and during repolarization, calcium channels should remain inactivated until the subsequent activation cycle. Premature reactivation results in so-called afterdepolarizations, resulting in a prematurely triggered action potential and reactivation of the cell (10). Furthermore, alterations in potassium channel function lead to a spatial dispersion of repolarization within different parts and layers of the ventricular muscle. Instead of proceeding in only one direction due to well-distinguished areas of excitable and inert myocardium, the electrical activity can spread in multiple directions, initiate reentry mechanisms or degenerate into a chaotic pattern (10). These aspects are thought to form the basis for potentially life-threatening TdP tachycardias in these patients.

The girl in our case example additionally suffered from inner ear hearing loss. Although affecting two entirely different organs her cardiac arrhythmias and her hearing problem have a common cause: KCNQ1-channels are expressed both in cardiomyocytes and in cells responsible for sensory transduction in the inner ear. Thus, a dysfunction of these channels affects not only cardiac but also inner ear function in a subtype of LQTS, called Jervell-Lange-Nielsen syndrome. This is one example in which biophysical studies on ion channels deliver important findings to different medical fields.

Stress as trigger for TdP tachycardia

Driven by observations from clinical practice (11), biophysical studies also helped to elucidate the mechanisms by which certain triggers, like physical activity, drive the development of the arrhythmia. Stress hormones are able to influence biophysical properties and trafficking of potassium channels (12–14). Well-known examples of stress hormones are catecholamines such as adrenaline and noradrenaline that are produced in the adrenal gland. They bind to specific receptors on different cell types of the heart to adapt heart rate and contractility in response to increased demand for peripheral oxygen supply. This is required during physical activity and in situations of mental or emotional stress, such as being startled due to sudden noises or from nervousness before an exam or a performance on stage. However, as long-QT patients often display arrhythmias during physical exercise or emotional stress, catecholamines have been suspected to exceed this typical physiological effect and may contribute to the

risk for arrhythmias in these patients. In healthy subjects, stress hormones cause a compensatory decrease in repolarization duration with increasing heart rates, e.g., during sports or emotional stress. As a result, the QT interval on the ECG becomes shorter and, thus, the action potential duration decreases with increasing heart rate. Consequently, in healthy subjects the optimal balance of pacing rate by the sinus node and action potential duration is maintained to guarantee a coordinated spread of electrical activity. In contrast, patients with LQTS often display an even more enhanced prolongation of the QT interval upon increases in heart rate or during the recovery period after exercise (15,16). This mismatch between action potential duration and heart rate increases susceptibility for arrhythmia. The potential underlying mechanism appears to be a disturbed interaction with molecular pathways initiated by stress hormones that would normally lead to an increase in potassium current through these channels (12,17). In contrast to healthy individuals, exercise in long-QT patients seems to exacerbate the effect of the dysfunctional biophysical properties. This demonstrates a loss of compensatory response to the influence of stress hormones.

The role of biophysical evidence in drug herapy development for inherited arrhythmias

Biophysical studies have not only helped to characterize defective ion channel function, findings have also guided medical therapy in patients with LQTS. The ventricular myocardium in these patients is particularly susceptible to arrhythmia development under the influence of stress hormones, such as catecholamines; therefore, beta blockers were evaluated for arrhythmia prevention. Beta blockers inhibit cardiac receptors for catecholamines, so that these cannot exert their effect on intracellular molecular processes. In the clinical setting, beta blockers have been shown to decrease exercise or stress-related QT abnormalities in patients with LQTS (16). Most important, beta blocker therapy decreases the rate of serious cardiac events and may offer protection from sudden cardiac death (11).

The evidence generated from biophysical studies helped not only to identify beneficial substances for arrhythmia prevention but also drugs that affected patients should avoid. A number of drugs commonly used for various indications interfere with ion channels, e.g., certain antibiotics and antidepressants. In individuals without impaired ion channel function, these effects can be compensated for or do not reach a critical level. In individuals with already impaired function of certain ion channels, the defects are aggravated, enhancing the susceptibility for life-threatening arrhythmias. Therefore, patients with LQTS are advised on which substances they should avoid, offering additional potential for arrhythmia prevention (18).

The role of biophysics in other channelopathies

The LQTS is only one example in which biophysical studies have helped elucidate the mechanisms behind inherited arrhythmias. Various ion channels contributing to the cardiac action potential may, when dysfunctional, cause a disruption in normal activation patterns. Another example is the relatively rare Brugada syndrome that is often associated with defective sodium channels (SCN5A-channels). Similar to LQTS, it also predisposes for loss of consciousness and sudden cardiac death due to increased susceptibility for ventricular arrhythmias. However, patients do not present with a prolonged QT interval but rather may show conduction abnormalities predominantly in ECG leads that represent the right ventricle.

Genetic variants found in families affected by Brugada syndrome were expressed in transgenic mice to serve as a model for biophysical characterization. Electrophysiological measurements in mouse models with impaired SCN5A-function have revealed that electrical activity spreads more slowly through the right ventricle in these mice, a phenomenon called “conduction slowing” (19). These features were enhanced by the application of flecainide, a sodium-channel blocker, underlining the role of a reduced sodium current in the development of this abnormal spread of electrical activity. Conduction slowing also results in a spatial dispersion of electrical activity and, thus, in increased vulnerability for arrhythmias.

Furthermore, not only fast ventricular arrhythmias are caused by ion channel defects. Channelopathies may also affect the natural pacemaker of the heart, the sinus node, which in a healthy heart causes the heart rate to adapt depending on the demand for oxygen supply in the periphery, such as during exercise. If the sinus node or the transmission of electrical impulses from the sinus node to the atria is impaired, the heart rate can become too slow for sufficient blood supply of the central nervous system and the periphery, leading to dizziness or loss of consciousness. These patients often require pacemaker implantation to compensate for the dysfunction of the sinus node. The underlying disease is called sick sinus syndrome and can also be the result of a gene mutation. This is the case in familial sick sinus syndrome, in which also SCN5A-channels have been shown to be involved (20). However, the functional result of the SCN5A-mutation in familial sick sinus syndrome is different from that in Brugada syndrome. When the gene mutation found in familial sick sinus syndrome was investigated in a mouse model, an impaired conduction of electrical activity from the sinus node to the atria could be identified as a possible underlying mechanism (21).

Therefore, different mutations in the same gene encoding for the same cardiac ion channel can lead to entirely different rhythm disorders with different clinical manifestations. This underlines the fact that not only the type of affected ion channel is relevant but also the effect an

alteration in channel structure has on its function in the context of cardiac activity. Furthermore, mutations in genes encoding for ion channels are in certain cases associated with structural alterations of the heart muscle, which may directly affect the strength of the heart (22). These intricacies can be well distinguished by biophysical methods in cellular experiments, animal models, computer simulations, and clinical observations in patients affected by channelopathies.

In summary, biophysical studies have contributed significantly to the discovery and understanding of inherited arrhythmias. Additionally, they help to identify prospective drug targets, as well as potential risk factors for cardiac arrhythmias. Furthermore, because many diseases affect biophysical properties of cells in different organs, evidence derived from biophysical studies may help patients and physicians on a multidisciplinary level.

Advice for the young swimmer: staying on the safe side (of the pool)

After careful evaluation of the case and genetic testing, the girl and her parents are informed about the diagnosis of Jervell-Lange-Nielsen syndrome and its potential risks. She receives a low-dose beta blocker for prevention of arrhythmias and avoidance of strenuous exercise, particularly swimming, is recommended. Her younger brother turned out to have developed a respiratory infection, which led to the episode of loss of consciousness but, apart from that, is entirely healthy.

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Systems Biophysics: Multiscale Biophysical Modeling of Organ Systems

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The reductionist movement of twentieth century biological science successfully used the tools of biochemistry, molecular biology, and structural biology to provide us with an increasingly detailed parts list of living systems. As the troves of molecular data grew, the advent of bioinformatics brought to bear information technologies that allowed biological scientists to annotate, query, search, and integrate these data with relative ease. This gave birth to systems biology, which seeks to reconstruct networks of the molecular interactions that give rise to the essential biochemical, biophysical, and regulatory functions of cells, and that give the different cell types the unique properties they need to build specialized organ systems such as the central nervous system, the musculoskeletal system, and the cardiovascular system. With these increasingly detailed, yet invariably still incomplete, molecular network reconstructions, the foundation has been laid for systems models of biological functions at all scales that simulate the dynamic physiology of living systems, especially cells, as large circuit diagrams of functional interactions. Great promise is held by these new quantitative, computer-driven approaches that can provide a new level of integrative scientific insight and identify promising new pharmacologic therapies.

Biological systems are exquisitely structured and depend critically on their dynamic three-dimensional organization to achieve their physiological functions. The challenge of building models that integrate structurally across physical scales of biological organization from molecule to cell to organ system and organism is a defining problem of modern biophysics. Like systems biology, this field of multiscale modeling is data-intensive. We depend on structural biology, microscopy, and medical imaging technologies to build high-quality, high-resolution data sets on molecular, cellular, tissue, and organ structures. But multiscale modeling also relies heavily on physics to define and constrain that ways that molecular and cellular processes can scale up to produce tissue and organ-scale physiology.

The need for multiscale modeling of organ systems is readily apparent when we put ourselves in the shoes of the physician. Patients present with symptoms and diseases that manifest at the tissue, organ, and whole body scales. But medical therapies target specific molecules. How can we diagnose and effectively treat illnesses without understanding the multiscale relationships between molecules and the whole body?

Take the case of the heart diseases. Cardiac arrhythmias are disorders of the heart's electrical system. Many of them can be traced to alterations in specific ion channels in the heart cell membrane, yet all arrhythmias are organ-level phenomena. Their manifestation depends on electrical interactions between cells and are commonly associated with altered coupling between cells or structural changes in the extracellular matrix that organizes the cells into cardiac muscle tissue. Similarly, the engines for muscle contraction are molecular motors in the cardiac muscle cells. The efficiency with which the heart converts contractile forces into ventricular pumping is critically dependent on the arrangement of the motors within the cell, their regulation by electrical excitation, the architecture of muscle cells and matrix in the tissue, the size and shape of the ventricular walls, and the coupling via the heart valves of the ventricles of the atria and the vasculature. Any number of these properties can change in congenital or acquired heart diseases. Understanding how the many molecular, cellular, tissue, and organ scale alterations give rise to cardiac electrical and mechanical dysfunction has been a primary motivation for the development of multiscale biophysics models of the heart.

There is no single paradigm or recipe for multiscale modeling. Different organs are specialized for different functions involving different physics. At the same time, the same physical principles are exploited by many living systems for different purposes, so approaches developed for one system can often be applied to others. The heart has electrical, mechanical, and transport functions all linked together. Fig. 1 summarizes some of the popular paradigms that have been used to develop and integrate multiscale models of cardiac physiology. On the top, specialized proteins form channels in the cell membrane for specific ions to cross, carrying electrical charge with them, which in combination with each other can change the voltage across the whole cell membrane and allow electrical impulses to propagate through the heart tissue, giving rise to electric fields in the torso that are detected by electrodes on the body surface as electrocardiograms. Similarly, molecular motors are organized into contractile filaments in the muscle cells. Electrical depolarization of the muscle cell triggers brief releases of calcium, causing a rise in filament tension that is distributed in three

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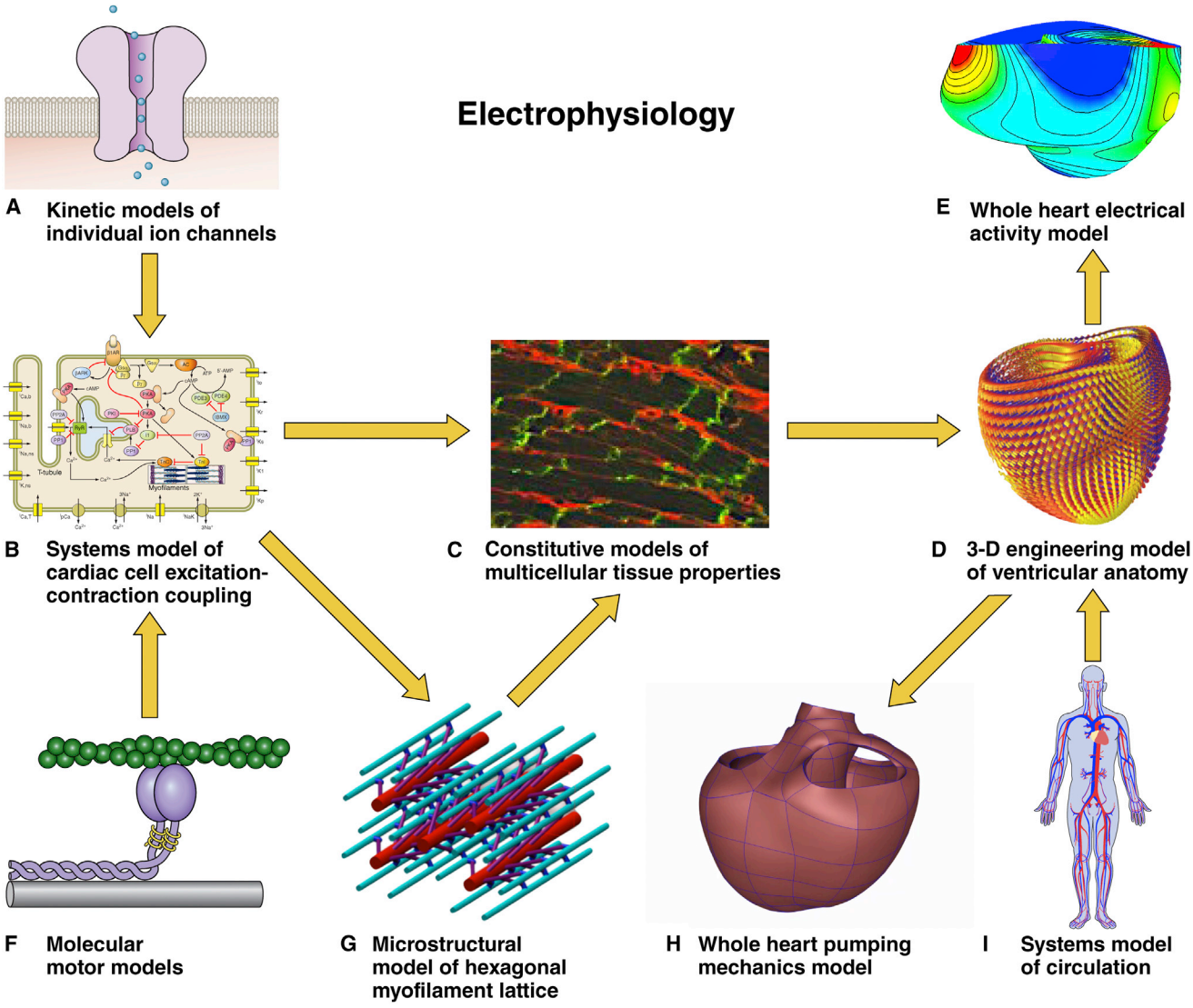

FIGURE 1 (*A–I*) Multiscale models of cardiac electrophysiology and biomechanics can be combined to model cardiac electromechanical function in health and disease. To see this figure in color, go online.

dimensions through the hexagonal myofilament lattice and into the tissue. The chambers contract against the load of blood pressure in the circulation, pressure that was generated originally by the heart itself. This description shows that the top scale of our cardiac mechanical models is a model of pressures, flows, and resistances of the circulation. These electrical and mechanical systems are coupled through the intracellular calcium release system, and models of intracellular calcium fluxes provide a link between these systems, allowing us to build coupled models of cardiac electromechanics and hemodynamics. Because these systems all require energy, models of cell metabolism and oxygen delivery to the heart muscle also find a natural fit in this modeling framework. We explore each subsystem below with an emphasis on the governing physics and the driving scientific questions and clinical applications.

The cardiac electrical system

It has long been known that the heart generates electrical current, a phenomenon that Dutch physiologist Willem Einthoven (1860–1927) successfully exploited in his invention of the electrocardiogram (ECG or EKG) in 1903, for which he received the Nobel Prize for Medicine in 1924. The origin of the electrical activity detected in the ECG is the flow of ions across the membranes of cardiac muscle cells. At rest, the muscle cells (myocytes) have negative electrical potential with respect to the outside. Each heartbeat is triggered by pacemaker cells that cause a wave of

electrical excitation to propagate through the atria and then the ventricles. This excitation is a transient period of electrical depolarization carried by sodium ions rushing into cells, followed by a repolarization due mainly to the outward flux of potassium ions. The dynamics of this process is made possible by specialized ion channels in the cell membrane that can open and close as a function of the membrane potential itself.

Modern multiscale models of cardiac electrical activity take into account the voltage-dependent kinetics of dozens of different ion channels, pumps, and transporters that carry sodium, potassium, calcium, and chloride ions (Fig. 1 *A*). They can account for detailed knowledge of the numerous different states the channels can occupy, made possible by detailed single-channel recordings and even the specific effects of many drugs and gene mutations. They include the capacitance of the membranes in a whole cell model (Fig. 1 *B*) and the resistive electrical coupling between neighboring muscle cells at the tissue scale (Fig. 1 *C*) as well as the three-dimensional anatomy of the cardiac chambers and the complex spiral-wound laminar organization of the muscle fibers in the heart walls (Fig. 1 *D*). The most important underlying physics for these sophisticated integrated models of whole heart electrical activity (Fig. 1 *E*) is well established: Ohm's law is used to relate the ion channel and intracellular resistances to the membrane voltage. Kirchhoff's current law provides the other key physical principle that Alan Hodgkin and Andrew Huxley famously used in their 1952 mathematical model that explained the ionic mechanisms of the electrical impulse conduction along a nerve, work for which they received the Nobel Prize in 1963.

Today, sophisticated multiscale systems models of cardiac electrical activity are not only helping to elucidate basic scientific mechanisms, they are increasingly helping us to understand human cardiac arrhythmias, and they may soon become part of the cardiologist's tool kit. Important ongoing questions being addressed include: How do cellular instabilities lead to arrhythmias and under what conditions? How important are the molecular alterations in the cell compared with the structural changes associated with heart disease at the tissue and organ scales? How can we design smarter and more reliable pacemakers and defibrillators? Will drugs be effective at terminating or preventing specific arrhythmias, and can we identify potentially dangerous proarrhythmic drugs before they reach the clinic? Finally, can we identify who is most at risk and most likely to benefit from therapies such as implantable cardioverter defibrillators?

The cardiac mechanical system

The basic function of the heart to pump blood through the body has been recognized since William Harvey's publication in 1628 of *Exercitatio Anatomica de Motu Cordis et Sanguinis in Animalibus* in which he clearly established the concept of blood circulation and the central importance of the heart as a pump. The German physician and physiologist Otto Frank (1865–1944) and English physiologist Ernest Starling (1866–1927) separately performed the ground-breaking experiments on the pumping mechanics of the heart that established what is now known as the Frank-Starling law of the heart. This important law states that the more the heart fills and the longer the muscle fibers are stretched the more strongly the ventricular pumps contract. The most important applicable physics are again well established and originally due to Isaac Newton, namely the conservation of linear momentum. Modern multiscale models of cardiac mechanics solve Newton's laws for the heart walls as continua subject to the additional constraints of mass and energy conservation.

The challenge is to link the pumping mechanics of the cardiac chambers (Fig. 1 *H*) both up in scale to explain the interactions between the filling and contraction of the cardiac chambers and the pressures and flows in the circulatory system (Fig. 1 *I*), and down in scale to the level of the molecular motors (Fig. 1 *F*) in the cardiac myocytes that convert biochemical energy to mechanical work. A critical intermediate mesoscale is the complex three-dimensional organization of the cells and matrix of the heart into a three-dimensional continuum capable of withstanding cycles of very large shape changes every second, uninterrupted, a billion times throughout a lifetime. Until recently, most computational models of cardiac tissue-scale mechanical properties were largely descriptive engineering models, but as quantitative three-dimensional microscopy techniques improve in resolution and molecular specificity (Fig. 1 *C*), we are starting to see new microstructural models of cardiac tissue mechanics that will replace these more traditional formulations. At the molecular level, Huxley's famous 1957 model of muscle contraction (Fig. 1 *F*), which has been revised and extended many times, still forms the core of cardiac mechanical models. Recent work has focused on incorporating detailed models of the effects of the hexagonal myofilament lattice structure inside the myocytes (Fig. 1 *G*), the biochemistry of chemomechanical energy conversion, and the regulation of the strength of cardiac muscle, especially by calcium ions, which mediate the process known as excitation-contraction coupling (Fig. 1 *B*). Intracellular calcium transients triggered by the electrical action potential are the key link between cardiac electrical excitation and contraction. By including intracellular calcium dynamics in multiscale models of the heart, we now have fully coupled electromechanical models of the heart. As such, these models are both multiscale and multiphysics.

Some of the important scientific and clinical problems being addressed by modern multiscale multiphysics cardiac mechanical and electromechanical models include: How do specific drugs or defects in single genes lead to substantially

altered whole organ pumping? When a patient has disease, how reversible is it and how much of the dysfunction is due to an initial insult or genetic defect versus subsequent alterations in the natural history of the disease? How do the three-dimensional microstructure of the heart walls and the changes associated with diseases such as myocardial infarction and ventricular hypertrophy affect the mechanical pumping performance of the heart in vivo? Can we use models based on clinical data to design clinical trials or predict outcomes of therapy?

Hemodynamics, oxygen delivery, and metabolism

Cardiac wall mechanics is a problem of solid mechanics, but blood flow through the cardiac chambers and coronary vessels is a fluid mechanics problem. Modern techniques in computational fluid dynamics (CFD) have made detailed analysis of blood flow through the coronary arteries, inside the atrial and ventricular chambers, across the heart valves, and in the great vessels highly practical when coupled with accurate anatomic reconstructions from cardiac computed tomography (CT) or magnetic resonance imaging (MRI). Again, the governing physics come from conservation of momentum, mass, and energy. The most important governing equations are the Navier-Stokes equations (named after Claude-Louis Navier, 1785–1836, and Sir George Stokes, 1819–1903), which express Newton's second law for fluid flows. What makes blood flow, especially through the heart and valves? An interesting CFD problem is that the walls are moving, and in the case of the cardiac chambers, the motion of the walls is driving the flow itself. This is where the development recently of robust algorithms for modeling fluid-structure interactions (FSI) has had a great impact. It is now possible to make patient-specific models of blood flows through the heart, valves, and vessels and to use them to predict the effects of surgical procedures. Nowhere is the potential clinical impact of this computational modeling technology more promising than in the development of better surgical procedures for infants and toddlers born with congenital heart defects (the most common class of birth defect).

Models of blood flow in the coronary circulation must take into account the mechanical effects on the coronary blood vessels of the squeezing of the heart walls during each heartbeat. In every other circulation in the body, blood flow is highest during systole when the blood pressure is highest. This is the phase in the heart when the stresses in the wall are greatest, thereby squeezing the coronary blood vessels and restricting systolic flow. This defines another especially challenging problem that couples heart wall mechanics with regional coronary blood flow. The demand for blood is driven by the need for oxygenation of the cardiac myocytes, which is in turn driven by the regional mechanical work demand on the muscle cells. Current efforts are linking models of wall mechanics, contraction, and energy metabolism to models of coronary blood flow and oxygen transport. Many cases of heart disease are associated with ischemia and metabolic stress that the need for such new models is pressing.

Future prospects

More than 50 years of cardiac computational modeling starting with Denis Noble's 1961 cardiac cell model, together with a great deal of experimental testing and validation, have laid the foundations for exciting progress in understanding the integrative mechanisms of human heart diseases, improving diagnosis and therapy planning, and discovering new therapeutics. Some of the developments that we expect to see in the near future include patient-specific computational cardiac modeling, augmented medical imaging technologies, new drug target identification and repurposing of existing drugs, the discovery of new combinatorial drug therapies, and the development of models that span the longer timescales of cardiac development, disease progression, and aging. We will also continue to see the growth of modeling closely connected with basic research as we develop more comprehensive models of animal cardiac cells and disease and new models of cardiac progenitor cells derived from human stem cells.

Among the most mature multiscale cardiac systems models are models of ventricular electrophysiology and mechanics. Excellent progress in this field shows promise of clinical impact in the not-too-distant future. Currently, to help protect patients at risk of sudden cardiac death, implantable cardioverter defibrillators (ICDs) are being used. ICDs are life-saving but also expensive and not without risk of complications. The consequences of shocks inappropriately delivered by ICDs can be harmful, and to avoid missing those patients who might need an ICD, many are implanted but never needed. The latest multiscale models of ventricular fibrillation can be customized to patient anatomy and myocardial infarct morphology and show exciting promise to better discriminate those patients at highest risk from those who may not need an ICD without the need for lengthy invasive clinical testing in the cardiac electrophysiology lab. Similarly, pacemaker implantation in patients with dyssynchronous heart failure can improve cardiac pumping performance by resynchronizing the electrical activation of the left and right ventricles. A significant fraction of patients receiving this cardiac resynchronization therapy (CRT) do not improve significantly. New patient-specific models customized with clinical measurements from cardiac imaging, electrocardiography, and cardiac catheterization are showing the potential to better predict CRT outcomes and optimize the performance of the therapy in those who receive it. These patient-specific models have been made possible by improved four-dimensional medical imaging technologies such as cardiac CT and MRI. As modeling based on these images becomes easier and more

common, we can expect to see some of these model simulations built into the computers that process scanner images to allow more detailed functional properties of the heart to be visualized. Drugs target specific molecules, but their biological impacts are dependent on effects that propagate through large cellular networks of molecular interactions. The short- and long-term therapeutic and adverse effects of these molecular perturbations are dependent on responses at the level of the tissue, organ, and whole body. Multi-scale systems models will help to identify adverse drug effects earlier in the expensive drug development pipeline, identify promising drug candidates that might not have been found with more traditional approaches, and find potential new applications for existing drugs alone or in combination.

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How Viruses Invade Cells

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Every so often news about a viral outbreak goes viral and catches widespread public attention in the media. Human immunodeficiency virus (HIV), West Nile virus, avian influenza (bird flu), Ebola, Middle East respiratory virus, and Zika virus have each become, in a flurry of headlines and broadcasts and interviews, the focus of the media's spotlight. And then, like other crises, they fade from view leaving the public with a new health concern to worry about but little knowledge of the actual factors involved in the problem. Behind the scenes, however, scientists are continually at work trying to understand and defend against these insidious infectious agents.

Viruses are perfect parasites. It has been known for decades that once a virus gets inside a cell, it hijacks the cellular processes to produce virally encoded protein that will replicate the virus's genetic material. Viral mechanisms are capable of translocating proteins and genetic material from the cell and assembling them into new virus particles. Contemporary research has revealed specific mechanisms viruses use to get inside cells and infect them.

An individual viral particle, called a virion, is a far simpler structure than a bacterium. It has often been questioned whether a virus is alive. It is certainly not living in the everyday sense of the word. Virions consist of genetic material—DNA or RNA enclosed in a protein coating. Many viruses, called enveloped viruses, have an additional outer membrane that encloses the protein coat. This membrane envelope is material co-opted from the cell's own membrane. As the new virion buds out from an infected host cell, it is wrapped by the cell's bilayer membrane and carries with it any protein that happens to be embedded in the membrane at the budding site. Enveloped viruses are then free to begin a new cycle of infection by fusing their cell-derived envelope with the cellular membrane of an uninfected cell.

Some types of enveloped virus fuse directly to the cell's outer (plasma) membrane, whereas others are engulfed whole by endocytosis or similar processes and then fuse their envelope with the membrane of the engulfing internal organelle (e.g., an endosome) to gain access to the interior of the cell. In either case, the genetic material of the virus has invaded the cell through the barrier of its membrane, and infection will inevitably follow (Fig. 1). Infection can be prevented if fusion of the viral envelope with the cell or endosomal membrane can be blocked. Similarly, if a vaccine can be directed against the viral fusion protein, infection can be prevented. Vaccines against the influenza virus, for example, target the fusion proteins of the virus.

Viral genetic material is relatively small, encoding only a few proteins. All enveloped viruses contain fusion proteins, which are the molecules responsible for fusing the envelope to a cellular membrane. These proteins are derived from the virion's genetic sequence. The precise genetic material, the amino acid sequence, and details in structure of a fusion protein are unique for each type of virus. Consequently, broad-spectrum antiviral drugs do not exist, and specific vaccines and drugs typically need to be developed for each virus type. The viral surface of an individual virion contains multiple copies of its fusion protein. Influenza virus, for example, typically contains 500–1000 copies, whereas HIV contains only about a dozen copies (1,2). A virion's machinery is so efficient that each cell infected by even a single virion can produce about a million new virions. Because enveloped viruses use similar mechanisms for delivery of genetic material into cells, there may be ways to prevent infection before viral entry that would be effective for large numbers of different viruses.

The membrane that is the skin of a cell and an enveloped virion, and is the gateway of viral entry, consists of lipids and proteins. Lipids are roughly linear molecules of fat that are attached at one end to a water-soluble headgroup. Lipids provide the cohesion that keeps biological membranes intact. They spontaneously arrange themselves into a lipid bilayer because oily fat does not mix with water. The headgroups of one monolayer face an external aqueous solution, whereas the headgroups of the other monolayer face the interior of the cell. Integral membrane proteins, such as viral fusion proteins, are inserted into the bilayer and project out from the lipid surface into the external solution-like icebergs. Membranes are generally 50% lipids and 50% proteins by weight, but proteins are much heavier than lipids, and so there are about a hundred times more lipids than proteins in a membrane. Membranes are able to fuse to each other because they are fluid (3), and the lipids provide fluidity to the membrane.

Viruses initially stick to cell membranes through interactions unrelated to fusion proteins. The virus surfs along the fluid surface of the cell and eventually the viral fusion proteins bind to receptor molecules on the cell membrane (4). If only binding occurred, the two membranes would remain distinct. Fusion does not happen spontaneously because

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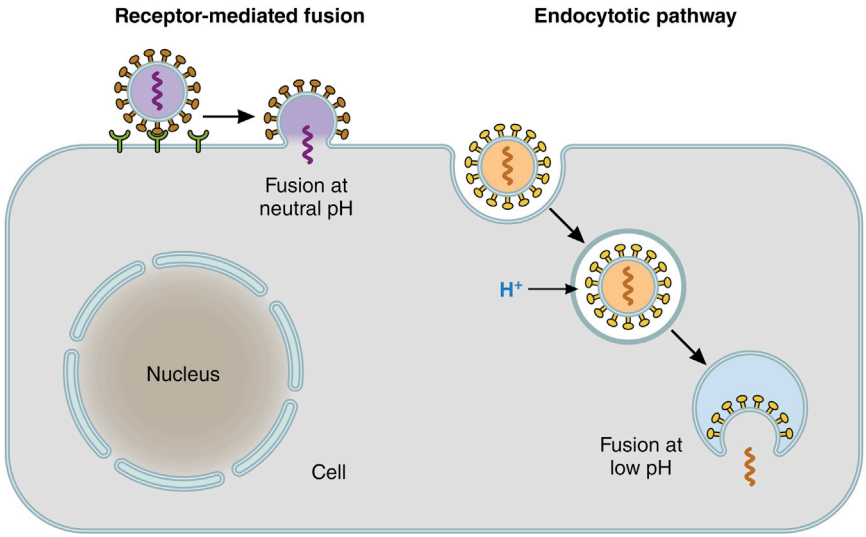

FIGURE 1 Viral entry pathways. Virus can fuse either directly to the plasma membrane (receptor-mediated fusion) or after being swallowed into an endosome. Which of these routes is followed depends on the type of virus. In fusion with the plasma membrane, the virus binds to a protein in the cell membrane. The function of this cellular protein (a receptor for the virus, shown in *green*) is perverted to induce a conformational change in the viral fusion protein, leading to fusion. For virus that is triggered within an endosome, the endosome's acidic conditions induce fusion. In either case, the viral genome passes through a fusion pore into cytosol, and infection is initiated. To see this figure in color, go online.

bilayers are stable. Fusion proteins do the work of prodding lipids from their initial bilayer configuration. These proteins cause discontinuities in the bilayers that induce the lipids of one membrane (e.g., the viral envelope) to connect with lipids of another (e.g., a cellular membrane), converting two bilayers into one.

Fusion proceeds in two major steps (Fig. 2). First, the two monolayers from opposite membranes that touch each other merge, a process known as "hemifusion." The two unmerged monolayers collapse onto each other to create a single bilayer, known as a hemifusion diaphragm, which continues to prevent the viral genome from entering cytosol. In the second step, the fusion proteins disrupt this single bilayer to create a pore that provides an aqueous pathway between the virus and the cell interior. It is through this fusion pore that the viral genome gains entry into a cell and begins infection.

Hemifusion and pore formation appear to require comparable amounts of work, but the exact amount of energy needed for each step is not yet known (5). These energetic details may be important because the more work required to achieve a step, the easier it may be to pharmacologically block that step. These energies are supplied by the viral fusion proteins, which are essentially molecular machines. Some of their parts move long distances during the steps of fusion. Fusion proteins can be thought of as a complex assembly of wrenches, pliers, drills, and other mechanical tools.

Because fusion is not spontaneous, discontinuities must be transiently created within the bilayer that allows water to reach the fatty, oily interior of the membrane. Even a short-lived exposure of a small patch of the fatty interior to water is energetically costly. Similarly, creating a pore in a hemifusion diaphragm requires exposure of the bilayer interior to water (6). In contrast, pore enlargement needs no such exposure. Nevertheless, pore enlargement requires the most amount of work in the fusion process.

Energy is also needed because of another fundamental property of bilayer membranes. Though bilayers are fluid, they don't entirely behave like water or oil, in that they do not assume the shape of their container. Biological membranes have shapes that are determined by their precise lipids and the proteins associated with them (7). Work is required to force membranes out of their spontaneous shape, which is the shape of lowest energy. The fusion pore that connects the virus and cell is roughly an hourglass shape (8). The wall of a fusion pore is a membrane with components that are a mixture of the two original membranes. An hourglass shape deviates significantly from the spontaneous shape of the initial membranes that constitute the pore. The greater the diameter of the pore, the greater is the area of the lining membrane, and so pore expansion is a highly energy consuming process. Viral genetic material, the genome, is rather large, on the order of ~100 nm. The initial fusion pore is only ~1 nm, so considerably more membrane must line a pore as it enlarges to a size sufficient to allow passage of a viral genome from a virus to a cell interior. In fact, it appears that more energy is required for pore expansion than for hemifusion or pore formation.

All viral fusion proteins contain a greasy segment of amino acids, referred to as a fusion peptide or fusion loop. Soon after activation of the fusion protein, the fusion peptide inserts into the target membrane (either plasma or endosomal). At this point, two extended segments of amino acids are anchored to the membranes: the fusion peptides in the target membrane and the membrane-spanning domains of the fusion proteins in the viral envelope (Fig. 2). The fusion proteins continue to reconfigure, causing the two membrane-anchored domains to come toward each other. This pulls the viral envelope and cellular membrane closely together (9). The fusion proteins exert additional forces, but exactly what these forces are and how they promote fusion remains unknown.

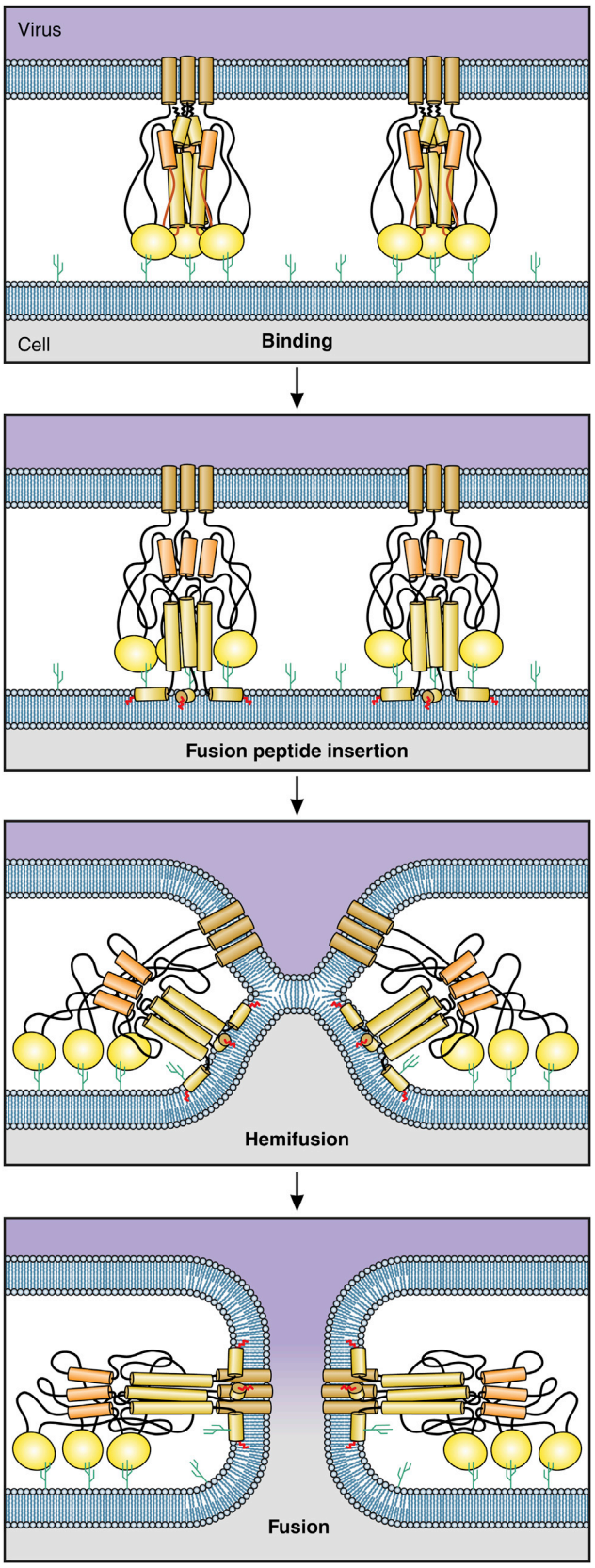

FIGURE 2 The steps of fusion. Virus binds to specific receptors (each illustrated as a small *cactus*) on a cell membrane. Initially, four monolayers (in *blue*) separate the two interior aqueous compartments. After fusion peptides insert into the target membrane, monolayers that face each other merge and clear from the merged region. The noncontacting monolayers bend into the cleared region and come into contact with each other, forming a new bilayer membrane known as a hemifusion diaphragm. At this point (hemifusion), only two monolayers separate the compartments. The fusion protein acts as a nutcracker to force the formation of a pore within the hemifusion diaphragm. This establishes continuity between the two aqueous compartments and fusion is complete. To see this figure in color, go online.

An initial step in a cell's digestive system is to internalize extracellular materials through engulfment by endosomes. A virion engulfed into an endosome is like a Trojan horse, because the cell perceives the virus particle as food. Endosomes become increasingly acidified as they move from the cell surface further into the cell's interior. Fusion of viruses within endosomes depends critically on the acidic environment. By breaking molecular bonds, acid triggers the conformational changes in the fusion protein that lead to the sequential steps of membrane fusion.

The hemifusion diaphragm is a bilayer membrane that is unusual in that each of its lipid monolayers is derived from different membranes, and it does not contain any membrane-spanning proteins (10). Several copies of the fusion protein within a virus are required to induce both hemifusion and pore formation. During hemifusion, the proteins form a ring just outside the diaphragm and act cooperatively to create stresses that lead to a local rupture in the diaphragm, thereby creating the initial fusion pore. The universality of this mechanism is remarkable when one considers that the primary amino acid sequences and structures of fusion proteins are quite diverse.

Influenza, HIV, and Ebola are enveloped viruses of significant public health concern. Each virus encodes a unique fusion protein: hemagglutinin (HA) for influenza, envelope glycoproteins for HIV (Env), and glycoprotein for Ebola (GP).

The earliest descriptions of an illness that was likely influenza were written in the 1500s and were called "catarrhal fever" (11). The flu pandemic of 1918 resulted in the deaths of some 20 million people and arguably accelerated the end of World War I (12). Flu pandemics have continued to occur periodically, as they did in 1947, 1957, 1968, and 2009, but were far less deadly.

Influenza virus is not free to infect other cells upon budding because HA binding to specific sugars, sialic acids, that protrude from cell surfaces prevents a virus from freeing itself from the cell. Another envelope protein, neuraminidase (NA), cleaves sialic acids off the cell, setting the influenza free. Drugs that are NA inhibitors, such as the well-known Tamiflu (oseltamivir), stop further infection within an individual by eliminating the cleavage of sialic acids (13).

Research efforts for influenza, HIV, and Ebola virus have focused on targeting their fusion proteins. But particular properties of the viruses and their proteins have hindered

the successful development of vaccines that protect against infection.

Standard vaccines against envelope viruses prime the immune system to generate antibodies (Abs) against the envelope proteins. In the case of influenza, Ab binding is mainly to HA, and secondarily to NA. Abs bind to exposed outer portions of envelope proteins and are large, thereby hindering close engagement of the virus with a cell membrane. Some antigenic sites surround an indented pocket within the surface of HA that is responsible for binding sialic acids on cell surfaces. Abs thus block the binding of HA to plasma membranes, eliminating the membrane fusion that leads to infection. HA readily mutates, and although the accumulated individual mutations lead to only small changes in the conformation of HA, these mutations greatly reduce binding of Abs to HA. Hence, a new vaccine must be developed each year (14).

Influenza presents another problem: its genome is not one continuous strand of RNA, like most viruses, but is segmented into multiple strands. Segmentation allows the genes for HA and NA to reassort: the RNA strands of different flu viruses—such as genes from an avian flu virus and a mammalian flu virus—combine to make what is essentially a new virus. Reassorted viruses are described in terms of HA and NA types and are termed H1N1, H3N2, H5N1, and so forth. Some reassortments cause periodic influenza pandemics that are characterized by an unusually large number of severe, and sometimes fatal, infections (15).

HIV-1 is clinically, to date, the most important retrovirus. Retroviruses transcribe RNA into DNA in a process called reverse transcription, and the viral DNA is incorporated into the genome of the host cell. HIV is a relatively recent emerging virus, appearing in the last 70 years or so. It has independently jumped to humans at least four times, probably due to the bush meat trade of gorillas and chimpanzees, and from chimps kept as pets (16). Currently, ~35 million people are infected, with about two-thirds of them living in Sub-Saharan Africa. Viruses not only cause diseases, but have also been important in evolution. Retroviruses can move large gene segments from one organism to another, and some 100,000 pieces of retroviral DNA make up ~8% of the human genome (17).

The traditional approach of using attenuated or inactivated virus, and by extension, envelope proteins, as vaccines has been ineffective against HIV-1 for a number of reasons. The fidelity of the reverse transcriptase of HIV-1 is low and therefore mutations in the viral protein occur frequently. As a result, HIV-1 Env mutates so rapidly that it quickly evades a static vaccine. Furthermore, Env is highly glycosylated, effectively sugarcoating the exposed portion of the protein, and Abs do not bind well to sugars. There is a small unglycosylated region on the surface of Env, and efforts were directed against this bald spot but did not lead to clinically effective approaches. Many nontraditional vaccine approaches have been developed and tested and these efforts continue, but none have yet been sufficiently successful. Modern biology and public health measures have combined to develop positive methods to prevent and treat the acquired immunodeficiency syndrome.

Antiretroviral therapies have largely eliminated the progression of viral infection to AIDS in individuals for whom these therapies have been available. Relatively soon after HIV-1 was identified, blood supplies were able to be accurately screened for HIV contamination. This was achieved only because prior advancements in the biological sciences allowed the development of new diagnostic methods that were sensitive enough to detect HIV. More recently it has been shown that HIV infection can be eliminated from the body: the Berlin Patient infected with HIV (and suffering from leukemia) received a stem cell transplant and was thereafter free of the virus (18).

The recent Ebola outbreak was caused by the deadliest of the three types of Ebola virus strains known to infect humans, with a fatality rate exceeding 50%. It typically takes ~4–10 days from the time of infection to the appearance of symptoms, but symptoms can manifest in as little as 2 days or as long as 3 weeks. It appears that with Ebola, unlike influenza, infected individuals do not become contagious until they exhibit symptoms. Trial vaccines using virus inactivated by traditional methods have proven unsuccessful, but viruses using recombinant technologies are showing considerable promise. Several other approaches may also be effective, including a cocktail of humanized murine monoclonal Abs, which have been shown to be statistically effective in protecting nonhuman primates.

Acidification of endosomes causes Ebola fusion in an unusual manner. Influenza HA, HIV-1 Env, and Ebola GP are cleaved into two subunits before viral-cell binding. This cleavage confers to HA and Env the full ability to induce fusion. In contrast, Ebola GP must be cleaved at an additional site to cause fusion. This cleavage occurs within endosomes by a protease (cathepsin) that is effective at low pH (19). A conformational change ensues, allowing Ebola GP to bind to an endosomal receptor, Niemann-Pick type C1. Binding activates GP, and a merger between the viral and endosomal membranes then proceeds. The identification of Niemann-Pick type C1 as a receptor opens up a new potential target for a small molecule drug to block binding and prevent infection (20).

The most reliable way to prevent infection caused by any virus is to eliminate entry in the first place. Intellectual and technological progress has been great, but recurrent viral outbreaks highlight the need for more innovative approaches. In addition to the proteins responsible for viral entry, many other targets are being explored, including genetic variations that increase susceptibility to infection, proteins that bind to viral proteins, and host immunity proteins. Genomic and proteomic analysis of cellular factors and their interactions, manipulation of experimental animals, live cell

and molecular imaging, and analysis and integration of protein and gene data sets will identify host factors that viruses exploit in their life cycle. Because viruses make use of cellular machinery—and invariably do so in a streamlined and robust manner—future viral studies will provide new understandings that will apply not only to virally induced diseases but to other diseases as well. Biophysics has been an integral part of understanding viral entry mechanisms, which have brought new insights and discoveries that just a few years ago could not have been imagined.

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