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A bright future: optogenetics to dissect the spatiotemporal control of cell behavior

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Cells sense, process, and respond to extracellular information using signaling networks: collections of proteins that act as precise biochemical sensors. These protein networks are characterized by both complex temporal organization, such as pulses of signaling activity, and by complex spatial organization, where proteins assemble structures at particular locations and times within the cell. Yet despite their ubiquity, studying these spatial and temporal properties has remained challenging because they emerge from the entire protein network rather than a single node, and cannot be easily tuned by drugs or mutations. These challenges are being met by a new generation of optogenetic tools capable of directly controlling the activity of individual signaling nodes over time and the assembly of protein complexes in space. Here, we outline how these recent innovations are being used in conjunction with engineering-influenced experimental design to address longstanding questions in signaling biology.

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Introduction

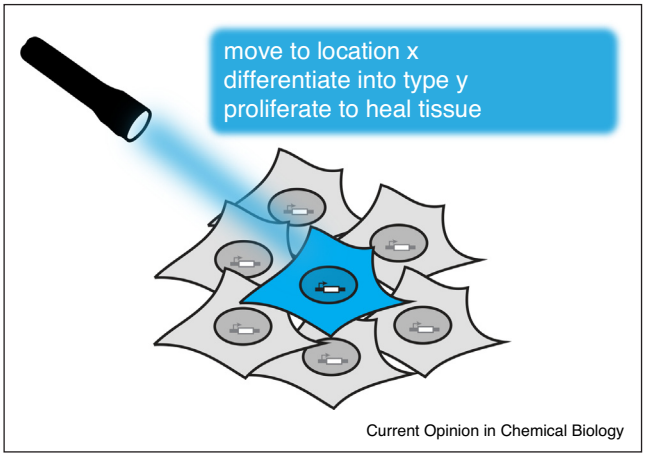
Modern biologists and bio-engineers often draw an analogy between the cell and a computer. In one variant of this analogy, genes are 'programs' that can be executed, and the machinery of transcription and translation serves as the computer's hardware to execute the program of interest. But cells are equally analog robots, with a broad array of complex sensors and actuators that allow them to actively gain information about their environment and respond accordingly. And if the cell is a robot, then cell signaling — the biomolecular circuitry that operates between the cell's exterior and its nucleus — comprises the robot's senses of sight, touch, and smell. This idea of signaling pathways as complex, analog circuits that enable cellular perception was explored in a seminal perspective twenty years ago [1]. It and subsequent work ushered in an era in which we describe the functional performance of cell signaling systems using terms loaned from engineering disciplines, such as robustness, sensitivity, and gain. (Indeed, annual use of the word 'robustness' in PubMed manuscripts has increased by 20-fold in as many years, from 179 in 1997 to 3279 in 2017.)

If anything, these early perspectives may have actually *underestimated* the extent of complex information processing carried out by signaling networks. Recently-developed fluorescent biosensors have revealed that individual cells naturally exhibit rich dynamics and transitions between signaling states that would have been impossible to predict from the single-timepoint, cell-averaged studies of the 20th century. Virtually every core eukaryotic signaling pathway has now been observed to undergo complex, non-monotonic dynamics in living cells, including pulses, oscillations, and even traveling waves [2–6]. These dynamics are no artifact of cell culture, as they persist *in vivo* and at the tissue scale: for instance, the mitogen-activated protein kinase (MAPK) Erk exhibits waves of activity that propagate across the skin of living mice [7•]. Complexity is also apparent in the spatial assembly of protein modules. A growing number of cell surface receptors, intracellular kinases, scaffold proteins, and even metabolic enzymes have been observed to assemble into higher-order complexes, rather than simple protein complexes with defined stoichiometry, and with material properties that range from liquid-like droplets to solid aggregates [8–10]. Yet despite their ubiquity, the roles played by inducible protein clustering are still poorly defined.

This exquisite spatial and temporal regulation is evidence of an intracellular signaling 'code' that we have not yet cracked. What collections of molecular interactions generate these emergent behaviors? What features carry essential information about the cell's sensory experiences? To achieve the ultimate goals of systems and synthetic biology — accurately predicting and programming cell behavior — we must first define the instruction set that is accessible through cell signaling, and then develop new methods to deliver these instructions to a particular cell and at a particular time (Figure 1).

Although there has long been considerable interest in questions of signal transmission, the field has largely

Figure 1



Programming cell behavior with optogenetics.
Modern optogenetic tools enable the delivery of user-defined inputs to cells with spatiotemporal precision, allowing us to begin defining the programming language cells use to send and receive information.

lacked experimental approaches to directly control individual signal features (e.g. the timing or duration of a pulse of signaling, or the specific collection of proteins in an aggregate or droplet) and to assess their effect on downstream processes. Cellular optogenetics is ideally suited to meet this challenge, offering a modular and generalizable set of tools for controlling specific biochemical reactions in space and time. In this review, we outline how a new generation of optogenetic techniques is enabling scientists to address this frontier of cell signaling. Much like the pioneering biochemistry that defined the biomolecular parts list necessary to generate a cell response, optogenetics is poised to decode the minimum *instruction set* required to direct cell behaviors.

Optogenetic approaches for multiscale control of protein function

While light-controlled ion channels have matured into a tool in the arsenal of nearly every neurobiologist, light-gated protein tools for cell biology (the tools of so-called 'cellular optogenetics') are still under active development, and the field is undergoing advances in both the degree and kind of control that is achievable. Here, we focus on a select set of recent approaches that we believe will be of particular importance for the next generation of precise perturbative experiments exploring the language of cell signaling.

Single-protein approaches: reversible uncaging of arbitrary linear motifs

Linear motifs play a dominant role in eukaryotic protein organization [11]. In contrast to well-folded protein domains, linear motifs obey a simpler set of rules, as a given motif's function is defined solely by its primary amino acid sequence. These functions can be diverse, from protein binding (e.g. MAPK-docking peptides or proline-rich motifs) [12,13], degradation tags [14], or signals that alter subcellular localization [15]. A series of recent studies established systems for light-switchable presentation of a wide range of linear motifs, making this class of protein-based switches arguably the first to be generally photoswitchable in living cells.

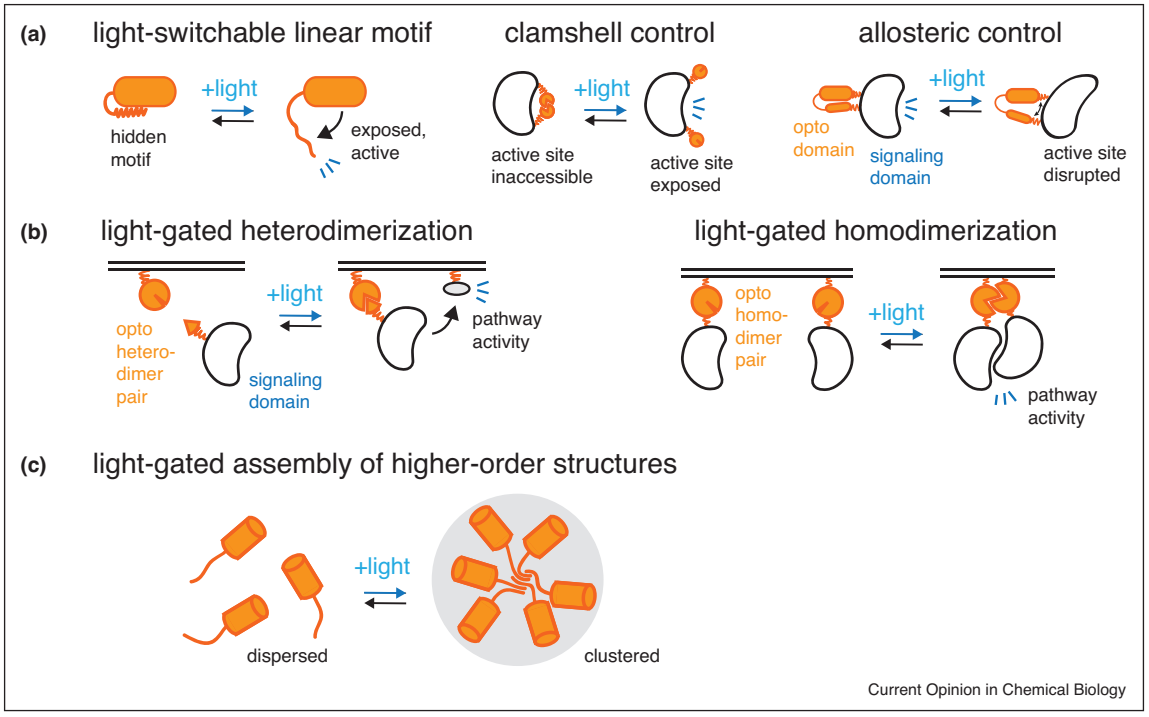
Linear motif photo-switching was enabled by engineering the LOV2 domain from *Avena sativa* Phototropin 1 (AsLOV2). Blue light stimulation of AsLOV2 causes its 20 amino acid C-terminal helix (termed the 'Jα helix') to become unstructured and un-dock from the photosensitive LOV domain [16], a process that reverses within minutes in the dark. Strickland *et al.* reasoned that, by altering Jα helix residues that are not required for docking, a novel linear motif could be encoded in the sequence and then exposed in a light-switchable manner [17•] (Figure 2a, left). This discovery led to an explosion of innovative LOV-based tools, including photoswitchable nuclear import [18,19], nuclear export [20,21], protein-protein binding [17•,22••], protein degradation [23], and presentation of MAPK docking peptides [24]. These studies demonstrate this approach's high degree of generality: simply fusing an engineered LOV domain to a protein of interest enables light-gated control over its subcellular localization, stability, and even interactions with specific targets.

Single-protein approaches: steric and allosteric modulation of protein activity

If LOV domains enable control over linear motifs, what about direct optogenetic control over folded domains? Two recent approaches aim to address this longstanding challenge using distinct optogenetic strategies. One is based on the idea of a 'clamshell' protein, where a domain of interest is flanked on both sides by sequences that can bind one another, sterically preventing the domain from associating with downstream targets (Figure 2a, middle). Chemically regulating the association between such N- and C-terminal sequences proved sufficient to regulate protein activity [25]. To adapt this design to light-based control, Lin and colleagues engineered variants of the fluorescent protein Dronpa, whose homo-dimerization can be dissociated with light. By fusing copies of Dronpa to the N and C termini of various target proteins, they established photoswitchable control over multiple guanine nucleotide exchange factors (GEFs), a viral protease, and a series of mammalian kinases [26••].

A second method relies on engineering allosteric control, rather than direct steric occlusion of an active site. Allosteric control can be achieved by inserting a 'hairpin' protein domain into a solvent-exposed loop, and then altering the physical distance between the hairpin's N and C termini [27]. Such a system thus acts as a stimulus-

Figure 2



Optogenetic techniques to control protein behavior.
(a) Three prominent approaches have been developed for engineering optogenetic control over single protein activity: (left) an optogenetic switch can be used to establish light-dependent caging/uncaging of a functional linear motif; (middle) light-gated binding partners can be placed on either side of a protein's active site to establish 'clamshell'-based occlusion/exposure of the active site; and (right) a light-switchable hairpin domain can be engineered into a protein of interest such that light-dependent opening/closing of the hairpin allosterically alters the target protein's active site.
(b) Two-protein optogenetic systems: (left) light-dependent heterodimerization partners, shown here controlling the subcellular localization of a target signaling domain; (right) light-dependent homodimerization partners, shown here controlling the interaction between two membrane-bound signaling domains.
(c) Multi-protein optogenetics: recent developments have enabled the light-dependent assembly of protein clusters and higher-order structures by attaching intrinsically disordered protein regions (which tend to self-aggregate) to optogenetic multimerization domains.

switchable hairpin, opening and closing to alter a target protein's binding or catalytic activity, resulting in a novel form of light-controlled allostery.

Hahn *et al.* realized that the same AsLOV2 domain described earlier has N and C termini that lie roughly 10 Å apart in the dark, and that this distance is increased by light-induced unfolding of the Jα helix [28••] (Figure 2a, right). In this seminal study, the authors used structural analyses to identify insertion positions whose motion is coupled to the protein's active site, enabling them to design light-controlled allosteric switches into a number of kinases, Rho family GTPases and GEFs. Similar approaches have now been extended to additional proteins, including the apoptotic protease caspase-3 [29].

Two-protein approaches: light-gated hetero-dimerization and homo-dimerization

Many optogenetic tools come as a two-protein package, where a photoswitchable domain only binds to a target protein in one illumination condition (either in the dark or lit state). Such systems were among the first non-neuronal optogenetic tools to be developed [30–33] and have emerged as the most widely-used in signaling biology (Figure 2b). These tools are primarily implemented to either control protein localization (i.e. by tethering an optogenetic domain at a subcellular location and linking its binding partner to an effector protein); or to drive the association between two proteins of interest. These are particularly powerful strategies for probing cell signaling, where altering protein localization (e.g. dimerization of cell surface receptors; membrane translocation of signaling effectors) is often sufficient to control biological function (Figure 2b).

Notable recent advances include a series of 'iLID' proteins with lit-state affinities that range from nanomolar to millimolar [22••,34] and the 'ZDark' proteins which invert the usual polarity and bind LOV domains only in the dark [35•]. By taking advantage of optogenetic

dimerization systems, it has been possible to create light-switchable receptor tyrosine kinases (RTKs) [36•], TGFβ receptors [37], transcription factors [38,39], and membrane-recruited signaling effectors [31,32].

'N'-protein approaches: light-dependent oligomerization and mesoscale protein clustering

Recent studies have revealed the widespread spatial organization of proteins into 'membraneless organelles' on a previously-unappreciated scale. Higher-order protein complexes are now thought to regulate diverse intracellular processes ranging from metabolic flux [40] to receptor activation [41•,42], intracellular signaling [8], and gene expression [43]. The properties of these mesoscale protein assemblies can be highly variable, ranging from gel-like aggregates to liquid-like protein droplets, but the field has lacked tools to drive transitions between these states on demand. A new suite of optogenetic tools has emerged to meet this challenge by providing precise control over the timing, location, and material properties of light-dependent protein clusters.

Photoswitchable protein clustering was first observed using the Cryptochrome 2 (Cry2) protein from *Arabidopsis thaliana*, a phenomenon that can be enhanced by mutations that increase its oligomerization affinity [44•,45]. Cry2 clustering was quickly put to use to control receptor clustering and activation in Wnt and RTK signaling [44•,46] (Figure 2c). However, the extent and kinetics of protein clustering were found to be highly variable, as clustering occurs more readily at certain intracellular locations, particularly in the nucleus or on the plasma membrane.

This heterogeneity was largely solved by the inclusion of intrinsically disordered protein regions (IDRs) in optogenetic oligomerization systems. IDRs are known to phase-separate *in vitro* and at high intracellular concentrations [47], and in work with the Brangwynne laboratory, we reasoned that we could use optogenetic clustering as a molecular switch to regulate IDR-dependent phase separation. Indeed, we found that IDR-Cry2 fusion proteins clustered in a light-dependent manner, forming liquid-like droplets or gels depending on the IDR and Cry2 variant used [48]. Subsequent studies have demonstrated this approach's generality to additional IDRs and light-dependent oligomerization systems, such as the PixELL system in which light can be used to dissociate liquid droplets [49••].

The emerging story of photoswitchable aggregation is not yet finished, and other exciting approaches continue to emerge. For instance, Inoue *et al.* recently reported that repeated arrays of light-gated heterodimerizers can form large-scale polymer networks [50•]. These and other innovations will continue to expand the toolbox for forming mesoscale assemblies of proteins and nucleic acids.

Connecting biological form with function using precisely-defined light stimuli

A growing number of studies are now beginning to approach signaling biology as an engineering discipline, uncovering how biochemical signaling networks encode, filter, and store information. The tools of cellular optogenetics are poised to play a key role, probing signaling pathways with both natural and un-natural stimuli to establish how spatial and temporal signals are interpreted by the cell. Here, we outline recent work that has pioneered the use of optogenetics for dissecting cell decision-making.

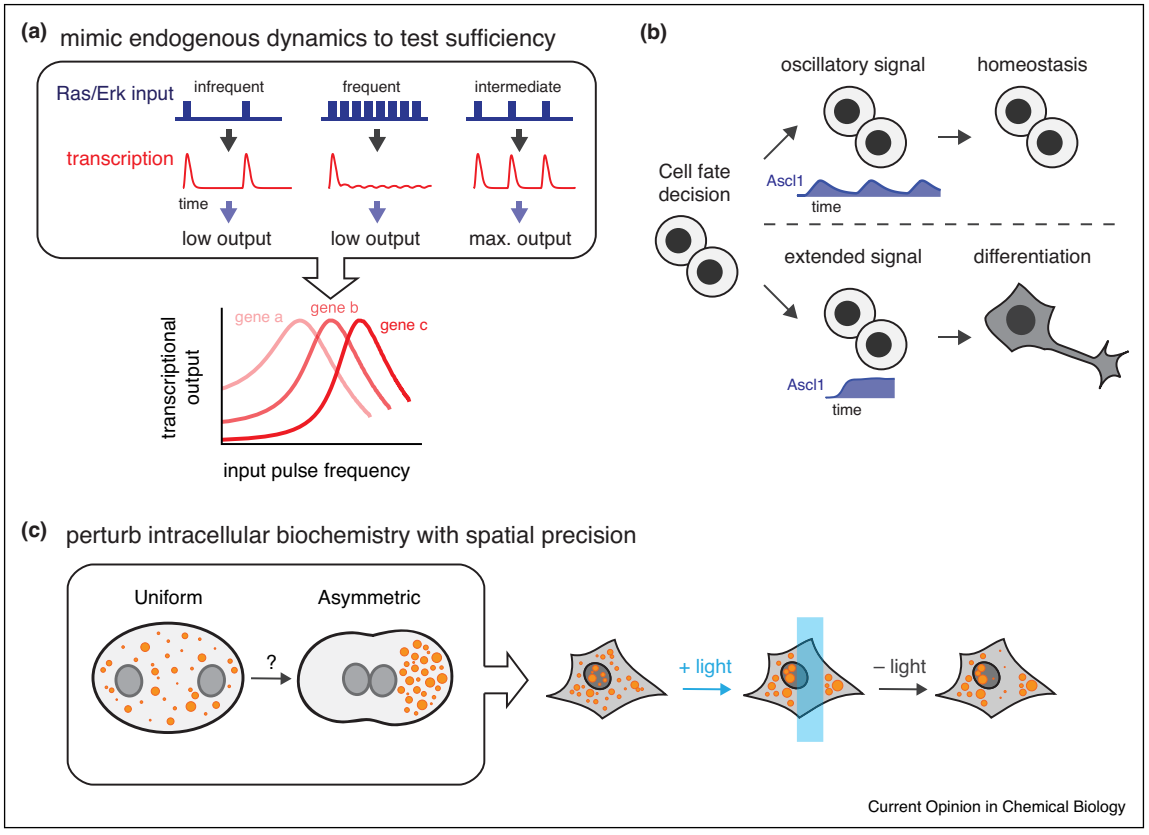
Optogenetics for cracking the code of cell signaling dynamics

Signaling dynamics are widespread, but what features of pathway activity (e.g. pulse amplitude, duration, or frequency) encode the essential information to trigger a cellular response? A classic example of this challenge is found in studies of the Ras/Erk MAPK pathway. Pioneering work in rat PC12 cells [51] suggested that these cells could be selectively driven to make one of two cell fate decisions simply by varying Ras/Erk activation dynamics. Transient, pulsatile inputs were linked to cell division, while sustained signaling led to differentiation, a correlation that has largely held up in subsequent experiments [52]. Optogenetics is ideally suited to tease apart questions of *how* signaling dynamics are interpreted into cellular responses; because light intensity can be tuned over time, we can envision directly altering the dynamics of pathway activation while monitoring live-cell reporters of cellular responses (e.g. gene expression or the acquisition of a differentiated morphology).

Recent work from our group demonstrates exactly this sort of all-optical input/output analysis, providing an initial toehold into how dynamic Erk signaling is interpreted by mammalian cells [53•]. We traced the flow of information from Ras inputs to Erk activation, target gene transcription, and target protein accumulation by coupling optogenetic Ras control with live-cell fluorescent biosensors at each of these nodes (Figure 3a). We found that repeated, optimally-spaced pulses of light-driven Erk activity could be used to maximize target gene expression output, and that the optimal frequency for gene expression coincides with that observed from cells grown in highly proliferative conditions [54]. Our findings thus suggest that the frequency of Erk activity pulses, not just Erk's steady-state level, can be a determining factor driving target gene expression.

Although the above study links signaling dynamics to gene expression, there are still a scarcity of examples where such dynamics have been shown to control actual all-or-none cell fate decisions. However, in a landmark study, Imayoshi *et al.* used precise optogenetic control of temporal signaling activity to demonstrate that neural

Figure 3



Interrogating the temporal and spatial properties of biological systems using optogenetic inputs.
(a) Transcriptional dynamics of Erk target genes in response to time-varying Ras/Erk inputs. For a set of five immediate early genes, maximum transcriptional output can be achieved by stimulating cells with an optimal, intermediate pulse frequency.
(b) Dynamics of the naturally oscillatory transcription factor Ascl1 during neural progenitor cell (NPC) fate decision making. Using optogenetic control over Ascl1, NPCs can be driven either to differentiate with sustained signaling input or to maintain a multipotent progenitor state with oscillatory inputs.
(c) Protein clusters can maintain spatial asymmetry within cells. Optogenetic control over the dissolution of phase-separated protein clusters can be used to establish light-defined spatial asymmetries that are retained long after the removal of stimulus.

progenitor cell (NPC) differentiation is dynamically gated by the naturally oscillatory transcription factor Ascl1 [55••] (Figure 3b). The authors inserted a light-dependent Ascl1 transgene into primary NPCs in which both endogenous copies of Ascl1 had been knocked out, thereby removing any complication from underlying endogenous oscillations. They exposed these cells to either oscillatory or sustained light inputs and found that, while oscillatory Ascl1 dynamics maintained a multipotent progenitor state, sustained Ascl1 activity promoted neuronal differentiation. This approach represents an important experimental paradigm: one can directly test the sufficiency of a specific signaling feature (e.g. oscillations) by creating a feedback-less 'open-loop' system in which user-defined inputs are the only source of dynamics. In doing so, the authors definitively showed that dynamic signaling at a single node is, by itself, sufficient to direct cell fate.

Patterned optogenetic inputs to unravel the sensing and encoding of spatial information

Although dynamic regulation is widespread, spatial cues can be equally important in driving all-or-none cellular transitions. Classical examples of spatial regulation include the interpretation of continuous morphogen gradients to discrete domains of gene expression during embryogenesis [56], the asymmetric condensation of P granules in the *C. elegans* embryo [57•], and the front-back polarization of migrating cells [58,59]. Traditional chemical-biology approaches are ill-suited to alter these complex spatiotemporal patterns, largely because slow binding kinetics and fast diffusion limit their precision. Because light can be patterned with high spatial precision, optogenetics is ideal to probe how spatially-restricted signals are sensed and interpreted by cells. This precision has enabled innovative studies in which spatially-defined optogenetic inputs were used to

interrogate polarized collective epithelial cell movement [60•], the local effects of cytoskeletal transport on axon outgrowth [61], and the impact of local cell contractility on global tissue organization during embryogenesis [62•,63].

Another illustrative example of spatial control emerged from our work developing the PixELL system, which forms phase-separated liquid droplets that can be instantly dissolved with light [49••]. Illuminating one region of a cell resulted in the local dissolution of droplets and diffusion of monomeric protein to the dark side of the cell, setting up an asymmetric pattern of liquid droplets within minutes. Strikingly, this pattern was retained for hours after light was removed, demonstrating that protein condensates and aggregates possess a form of long-term spatial memory that does not require any additional biochemical positive or negative feedback loops (Figure 3c). The future appears bright for studies of spatial information, as one can imagine how spatial light patterns might be applied to dissect the establishment of spatial patterns in directed cell migration or the interpretation of morphogen gradients in embryogenesis or regenerating tissues.

Future challenges

Cellular optogenetics has accomplished a great deal in its nearly ten years of existence, but a number of challenges still lie ahead as this field matures into adolescence. With a few notable exceptions, we still lack the tools to plug in light as the sole source of protein activity at particular signaling nodes. Light-induced activity is often summed with activity from the endogenous pathway, and gain-of-function optogenetic systems cannot 'carve into' or disrupt this endogenous pattern. A possible solution is to combine optogenetic control with genetic replacement by exchanging endogenous proteins for light-controllable variants or expressing optogenetic variants in a genetically null background [55••]. With the increasing ease of CRISPR-based gene modification, such techniques may be more broadly applied in the coming years.

Multi-color optogenetics represents a second emerging frontier, as it would enable real-time control over stimulus combinations, not just dynamics. However, such applications have been challenging because most of the tools of cellular optogenetics (including all Cry-, LOV-, and BLUF domain-containing proteins) respond to blue light with broad, highly-overlapping excitation spectra. One solution may come from a different family of photosensitive proteins, the phytochromes. Many phytochromes are red-light-sensitive (thus immediately providing a second stimulus wavelength); moreover, recent studies have identified phytochrome family members that respond to many additional wavelengths [64]. Phytochrome-based systems have earned a reputation as difficult to use because they typically require addition of the small-molecule chromophores phytochromobilin or phycocyanobilin [65], which can be cumbersome to purify and add, especially *in vivo* where delivery and clearance may be limiting. Recent methodologies for chromophore production in mammalian cells [66], as well as the development of phytochrome-based tools using biliverdin as a chromophore [67•], suggest that these difficulties may soon be overcome.

In sum, optogenetic inputscoupled with downstream live-cell reporters are now enabling a new generation of engineering-informed signaling biologists to investigate howspecific spatiotemporal signals dictate cell decision-making. As the field of cellular optogenetics enters its second decade, we look forward to continued innovations that will provide fundamental insights into how cells encode/decode information, how this information is integrated to inform cell fate decisions, and how pathologic alterations in these dynamic networks contribute to disease.

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