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The structure of dynamic GPCR signaling networks

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Abstract

G protein coupled receptors (GPCRs) stimulate signaling networks that control a variety of critical physiological processes. Static information on the map of interacting signaling molecules at the basis of many cellular processes exists, but little is known about the dynamic operation of these networks. Here we focus on two questions. Firstly, is the network architecture underlying GPCR activated cellular processes unique in comparison to others such as transcriptional networks? We discuss how spatially localized GPCR signaling requires uniquely organized networks to execute polarized cell responses. Secondly, what approaches overcome challenges in deciphering spatiotemporally dynamic networks that govern cell behavior? We focus on recently developed microfluidic and optical approaches that allow GPCR signaling pathways to be triggered and perturbed with spatially and temporally variant input while simultaneously visualizing molecular and cellular responses. When integrated with mathematical modeling, these approaches can help identify design principles that govern cell responses to extracellular signals. We outline why optical approaches that allow the behavior of a selected cell to be orchestrated continually are particularly well suited for probing network organization in single cells.

Initial knowledge about GPCR activated pathways was acquired predominantly using biochemical methods. These approaches have helped generate static network diagrams of molecules involved in several GPCR controlled cellular responses. GPCR activation by an external stimulus, however, does not merely lead to the progressive activation of molecules along a single linear pathway. Rather, it leads to the modulation of a circuit of molecules wired to execute different types of feedforward and feedback loops that generate unique cellular outputs such as polarization, adaptation, oscillations and contraction. Further complexity is inherent in physiological processes regulated by GPCRs, because the output exhibits changes in amplitude and frequency and involves changes in cell shape. This suggests that the GPCR activated signaling networks that regulate these processes are spatially and temporally dynamic.

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Approaches to understand the operation of GPCR activated signaling have focused at one end on the interactions and kinetics of signaling protein activities, and at the other end on elucidating the design principles of signaling networks regulating cellular behavior. These two areas are individually discussed below. The review focuses mainly on two model systems -- cell migration and calcium oscillations -- where the GPCR networks control stereotypical but complex cellular output.

Kinetics of GPCR signaling

Upon heterotrimeric G protein activation by a GPCR, both the G protein α and βγ subunits are capable of interacting with numerous signaling proteins. G protein activation is the first event in the signaling cascade, so the kinetics of the G protein activation cycle can strongly influence network dynamics. Computational modeling has been used to predict the kinetic properties of GPCR activated signaling [1–4]. Modeling has been used in combination with biochemical characterization of pathway components to suggest that GAP activity in G protein signaling can significantly accelerate deactivation in the absence of an agonist but provide high output when the receptor is active [5]. Mathematical modeling combined with experimental analysis of the G protein mediated pheromone receptor pathway has been used to estimate the rate of G protein activation and deactivation in a yeast cell [6].

While these studies have been valuable, they provide limited information about how signaling circuits dynamically execute specific cell behaviors. To progress towards such a goal, mathematical modeling has to be combined with experimental approaches that provide information about the molecular and cellular response to GPCR activation. Such approaches have provided clues about the modular activity that regulates cell migration and Ca^{2+} oscillations and are discussed below. Instead of collating all motifs that are known to underlie GPCR regulated responses, we focus on the conservation of GPCR network architecture that dictates similar cell behavior across cell types and species. We discuss whether these pathways operate in distinctly different ways compared to other networks such as transcriptional networks.

A challenge in this area has been to obtain quantitative information about cell behavior and underlying signaling network activity while continually varying input to single cells spatially and temporally. Such information is critical for ensuring that the molecular and cellular response is fully and accurately represented and to develop more complete models. Further on below, we elaborate on newer approaches that may be able to help obtain this data and the potential impact they can make on our understanding of GPCR systems control of cell behavior.

GPCR network structure that controls similar cell behaviors is conserved across cell types and species

Gradients of chemoattractants that activate specific GPCRs induce immune cell movement, morphogenetic movement and cancer cell metastasis. Migration has been studied most extensively in the social amoeba *Dictyostelium discoideum* and in immune cells, mainly neutrophils [7]. A migratory cell demonstrates a number of stereotypical properties such as

directional sensing, adaptation, decisive switch-like responses, amplification of shallow gradients and spontaneous basal level polarization.

The asymmetric GPCR activation across a migratory *Dictyostelium* or immune cell creates a gradient of a second messenger, PIP3 and actin cytoskeleton remodeling. The cells sense and migrate in the direction of higher GPCR activity. Both *Dictyostelium* and immune cells adapt to uniform GPCR activation by terminating the migratory response. Adaptation allows the cell to respond to increases in the intensity of a signal over a wide range of background signal intensities. The directional sensing and adaptation requirements can both be satisfied by a model that invokes an activator that is localized to the site of signal input, and an inhibitor that can diffuse away to distant regions of the cell (local-excitation and global-inhibition – LEGI) (Box 1) [8]. The capability for adaptation results from the underlying incoherent feedforward loop (IFFL) (Box 2), in which the activator and inhibitor act on the same downstream effector with differential kinetics (fast activator and slow inhibitor). The differential movement of the activator and inhibitor allows the maintenance of intracellular gradients in response to stimulus gradients.

To explain the different properties of migration, different models have been proposed [9–17]. Amplification has been explained by different motifs -- a LEGI mechanism coupled with a module that generates a threshold dependent switch-like step downstream [13], and a LEGI mechanism combined with balanced inactivation or an excitable network [8, 17]. Ultrasensitivity and switch-like response have been explained by a modified LEGI model in which the activator and inhibitor additionally act antagonistically on each other [12]. Spontaneous basal level polarization has been explained by a FitzHugh-Nagumo model [18], and by incorporating an additional polarization module consisting of a local positive feedback and a global negative feedback to the excitable network [14]. Global positive feedback has been invoked to account for buffering of activity at the back of a cell [15].

Our aim is not to provide an exhaustive list of the network topologies that underlie migratory behavior above. In the sections below we discuss how regardless of the cell type for which the models were originally developed, they can essentially explain the migratory behavior of both *Dictyostelium* and immune cells. This is remarkable and suggests that the GPCR network structure at the basis of migratory responses has been retained over vast evolutionarily distance. One possible reason for this conservation may be that it incorporates energetic efficiency, flexibility and robustness. Flexibility allows different properties such as directional sensing, adaptation and amplification that constitute migratory behavior to be supported by the same structure. Robustness provides the expected output despite changes in parameter values. One prediction from this is that in other GPCR controlled processes such as contraction, secretion and adhesion, network structure will be conserved evolutionarily. This suggests that the best approach towards an overall understanding of network control of cell behavior will be to identify signaling organization in the most experimentally accessible model system and use this knowledge to probe other systems.

Unique properties of GPCR network structure compared to other networks

A variety of network motifs are found impregnated in the architecture of biomolecular networks that have been characterized [19]. A question of interest is whether the motifs present in the structure of GPCR networks have evolved in a distinct fashion compared to transcriptional or metabolic networks. We examine this question using two model systems -- Ca^{2+} oscillations and cell migration.

Ca^{2+} oscillations induced by GPCRs are thought to encode information that regulates contraction, secretion and cell differentiation [20]. The central pathway involves G protein activation of PLC and the generation of IP3, which triggers calcium release from the endoplasmic reticulum *via* IP3 receptors. Signaling modules that govern the calcium response have been identified [21–23]. Models capable of generating calcium oscillations have commonly employed a fast positive feedback loop coupled with a delayed negative feedback loop (Box 3). Direct actions of G protein subunits on an effector can also function as a logic gate. For instance, αq and βγ subunits synergistically activate PLCβ3, a key effector in the calcium response network [24, 25]. A model incorporating differential specificity of αq and βγ subunits for PLCβ isoforms and negative feedback of calcium on the Gi-coupled receptor can account for both specificity and robustness of the calcium response [26].

Inspection of the network motifs that govern Ca^{2+} oscillations suggests that they are likely to be found in other oscillatory systems such as those that are at the basis of the cell cycle [27]. Similarly, the signaling motifs involved in cell migration described above are known to occur in other biomolecular networks [28]. However, the structures of GPCR stimulated networks do possess unique characteristics because they demonstrate spatial complexity (Fig. 1).

GPCRs activate polarized cell behaviors such as cell motility, yeast budding and neurite initiation [29, 30] which require communication across a cell. Although signaling motifs at the front and back of a cell may be similarly engineered, they need to communicate with each other to execute differential behavior at the front where there is increased receptor activity and the back where receptor activity is lower. The network architecture of cells capable of GPCR activated polarized behavior has evolved to include specific modes of communication between network motifs that are separated in space. Mathematical models that explain migratory behavior have included such dynamic links. Biologically they may be diffusible molecules, cytoskeletal elements [15] or the plasma membrane [31].

Spatial complexity in network architecture may not be restricted to the more obvious polarized cell behaviors like migration. Most native ligands of GPCRs act in a paracrine mode, so many different cell types are likely to experience spatial variations in GPCR activation that result from spatially varying ligand concentrations at the cell surface. These spatial variations in activation may trigger differential responses. Thus spatiotemporally dynamic links between motifs may be part of the network architecture of some other cell types also that respond specifically to localized GPCR stimulation. Traditional therapeutics may have side effects that can be overcome by pharmacologically targeting a network. It

will therefore be valuable to identify any unique facets of the network structure of GPCR activated cellular behaviors so that they can be targeted with specificity.

Network topology in single cells

As methods to examine the proteome, genome and transcriptome of individual cells have advanced, it has become obvious that there is cell to cell variation in these constituents of cell populations [32, 33]. However, there have been limited studies that focus on signaling network structure in single cells. The development and application of microfluidic devices and optical methods to study single cells has suggested that there is cell to cell variation in signaling responses [12, 13, 34].

Studies of GPCR signaling networks have traditionally measured a population of cells responding to receptor activation. These experiments can identify the components that are required to produce a given response, and in many cases identify causal connections within the network. Some aspects of network dynamics can be understood with biochemical methods in cell populations [35]. However, the dynamics are often masked at the population level, so single cell perturbation and imaging are valuable. Below we highlight recent examples where single cell imaging has been combined with microfluidics to generate variations in agonist concentration in order to distinguish between different network structures comprising GPCR signaling.

One example is adaptation in the GPCR network that controls chemotaxis. The incoherent feedforward loop, as described above, is capable of providing perfect adaptation. However, a negative feedback loop with an integrator can also provide perfect adaptation, and has been implicated in several biological systems [36, 37]. To generate conditions capable of distinguishing between these two network motifs, single cell imaging of *Dictyostelium* was combined with microfluidics to generate stepwise increases in agonist over a wide range of concentrations [11]. Experimentally it was determined that Ras activity adapts with similar kinetics over a wide concentration range. Simulations revealed that only the IFFL was capable of producing similar results (Fig. 2A). Similarly, a “ramp” input was able to distinguish between these two motifs, and suggested that the IFFL is responsible for adaptation of PIP3 levels in *Dictyostelium* [13].

A second example is a phase locking method combining single cell imaging of calcium oscillations and periodic microfluidic GPCR stimulation [23]. Multiple models are capable of producing calcium oscillations in response to a step increase in agonist, but they differ in their responses to more complex temporal patterns of agonist concentration (Fig. 2B). When cells were treated with a train of periodic pulses of an agonist, their calcium responses were found to vary with the time delay between pulses. When the delay between input pulses was sufficiently small, cells exhibited alternating full and subthreshold calcium spikes. Only one of the models, when appropriately modified, was able to generate the subthreshold spikes, indicating that it most accurately represents the true network.

Orchestrating and perturbing GPCR networks

Information gleaned from the model systems above suggests that the molecules that constitute a signaling network are often constantly shifting in their location and activity over time within a cell. To understand how a potentially spatiotemporally dynamic GPCR network drives equally complex cellular output, experimental approaches that possess several critical capabilities are required. They should be able to deliver measured signaling inputs to a single cell and control signaling activity. In the same cell, they should also enable measurement of the spatiotemporal changes in the activity of molecules in the GPCR pathway and the cellular response.

Traditional pharmacological approaches relying on the introduction of agonists, antagonists, activators and inhibitors have been valuable in probing signaling pathways but are limited in terms of spatial targeting and temporal confinement of signaling activity in a single living cell. Substrate patterning has been used to overcome this hurdle and provide spatial control over the delivery of pharmacological agents that regulate signaling [38]. However, this approach does not provide dynamic spatial and temporal control over signaling. Microfluidic devices provide more dynamic control [39, 40], but cells have to be used in devices that require appropriate development and engineering. Additionally, since small molecules in solution modulate signaling, GPCR activation and deactivation are limited by diffusion dependent exchange inside the device.

A color opsin from the visual system has been shown to be capable of activating GPCR signaling networks when exogenously expressed in other cell types [12, 30]. It provides temporal and spatial control over signaling and is capable of orchestrating cell behavior with variable inputs (Fig. 3). It allows quantitation of both the molecular and cellular responses during activation of the entire network. It facilitates single cell studies. The same cell can be exposed to varying input. It can be observed over long periods of time in a culture dish. Other cells in the same dish can be similarly studied for comparison in an identical environment.

Other optical approaches allow a single protein to be targeted rather than a network. Optically activated phytochromes and cryptochromes bind to specific protein partners. This interaction has been exploited to optically control protein movement and activity [41, 42]. For instance, PIP3 production can be localized by optically targeting PI3K to a selected region of a cell [43]. This approach can be used to perturb specific motifs in a GPCR network and examine its role.

Conclusion

There are essentially two ways of trying to understand network dynamics. One is to take into account the rate constants for all the known reactions and any known spatial movement of the proteins, assemble this information together, and infer how the entire system responds. This approach is limited because not all of the proteins in the network are known and the information about the kinetics of the individual reactions and protein movement is still sparse. Additionally, while this approach provides information about how a particular

protein influences the kinetics of signaling activity that is constrained to part of the network, it usually does not provide insight into the structure and dynamics of the whole network.

The complementary approach queries what kinds of networks can produce a particular cell behavioral response. This approach has the advantage that it can identify how the signaling circuitry is engineered. However since it does not depend on the specific assignment of known molecules to roles conceived in a model, there remains the need to connect abstract models or network motifs with actual molecules. Our personal bias is that optical approaches are especially well suited to attain this goal. The opsin based approach is not circuit disruptive and can activate a signaling network in its entirety. This approach can be used to perturb signaling pathways spatially and temporally. It can activate specific second messengers and corresponding effectors with specificity. Approaches based on light sensitive protein binding domains such as those from cryptochrome and phytochrome can optically target specific molecules in a cell for perturbation. Iterative cycles of experiments with these two approaches combined with innovative modeling can thus provide a picture of not only the wiring of signaling pathways, but also the specific molecules that constitute this wiring and the dynamic alterations in their communication patterns. Such a picture is needed in order to gain an understanding of how an external signal can mold the physiological state of cells in desired directions.

An approach of this nature can be expanded to identify the organization of signaling pathways that are activated when more than one receptor in a cell is stimulated to provide an integrated cellular output. Imaging of responses will allow spatial changes in the localization of signaling molecules such as G protein subunits and their differential kinetics to be included in a comprehensive model of GPCR control of dynamic cellular processes. GPCRs today are the single most important target of beneficial drugs. Complete models of these signaling networks and their control of dynamic cell responses can facilitate systems therapeutics.

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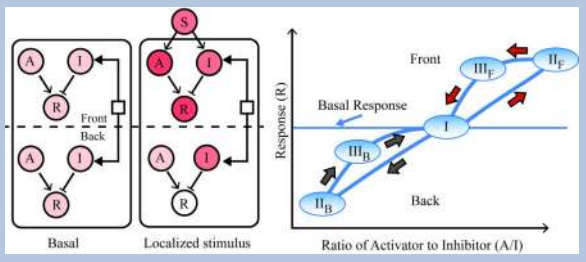
Box 1. Cell migration mediated by a LEGI mechanism

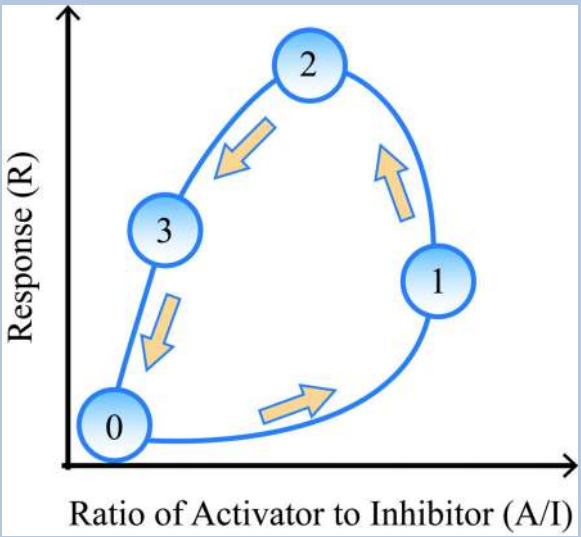
The IFFL in combination with diffusion of the inhibitor inside the cell is capable of explaining the phenomena of polarization of output response across the cell in presence of an asymmetric input stimulus (S) [10]. In the absence of the stimulus, the response is at the basal level throughout the cell. The application of the stimulus to a particular region of the cell (front) triggers up-regulation of both the activator and the inhibitor, with inhibitor being slow and diffusible to the un-exposed region of the cell (back) while the activator remains local and dynamically fast in the front. This is the main basis of the Local Excitation Global Inhibition (LEGI) model [7, 8, 10, 11], illustrated by the diffusion of only inhibitor, in the middle diagram above. In cell migration, the asymmetric response generated inside the cell can be in the form of the second messenger PIP3. Different proteins are targeted to the front and back of the cell by this asymmetry to control extension of the front and retraction of the back.

The dynamics of the response in each compartment is dependent on the local Activator to Inhibitor ratio (A/I) (i.e., A/I in front or back). On application of the stimulus, the concentration of activator (A) increases in the front, leading to a rapid increase in the ratio A/I and the response (R) (path I–II_F in the right most diagram). In the back, the activator remains at the basal level and the inhibitor produced in the front diffuses to the back, thereby reducing the response from the basal level (path I–II_B) leading to polarization. On removal of the stimulus at II_F, the response in the front and back returns to the basal level by way of intermediate states III_F and III_B due to reduction in the activator and inhibitor concentrations through the cell. Thus, the LEGI model incorporating an IFFL with a fast local activator and a slow global inhibitor provides directional sensing and adaptation, on application and removal of an asymmetric input.



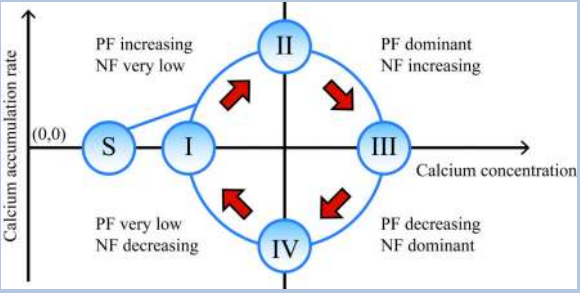
Box 2. Adaptation Mediated by an Incoherent Feed Forward Loop (IFFL)

Output response from an IFFL motif demonstrates adaptation[11] through the dynamics of a fast activator (A) and a slow inhibitor (I), which both act on the downstream response (R) (see Box 1). The realization of a transient response in R through the dynamics of A and I is shown in the diagram. On exposure to a uniform increase in the stimulus (S), both activator and inhibitor are up-regulated (see path 0–1), however, due to faster dynamics of the activator relative to the inhibitor, the ratio A/I increases rapidly (along path 0–1). The Response R is slightly slower compared to the dynamics of the ratio A/I and reaches a maximum value after A/I attains a maxima (along path 1–2). During this time the inhibitor concentration increases marginally, thereby decreasing the ratio A/I from the maximum. In the dynamic phase 2–3, as the inhibition level is far from saturation, its concentration keeps on increasing, albeit with a slower rate. However, the activator level reaches saturation, and thereby decreases the ratio A/I in this phase. In the final phase (3–0), the inhibitor also reaches saturation leading to the response (R) attaining pre-stimulus levels. It should be noted that although the ratio A/I is the same as that existing before the application of the stimulus, the concentrations of A and I are higher at the end of the adaptation. Further, for a subsequent higher stimulus, the sensitivity of the response decreases due to the presence of higher concentration of inhibitor. This network structure can explain how, for example, the second messenger PIP3 displays an adaptive response to a sustained, uniform increase in GPCR activation.



Box 3. Calcium Oscillations mediated by coupled fast positive and slow negative feedback

A network incorporating a fast positive feedback (PF) along with a slow/delayed negative feedback (NF) is shown to impart oscillatory behavior [21, 22]. The diagram above captures dynamics of calcium oscillations by such a network. On application of the stimulus (at point S), the fast PF rapidly increases the calcium accumulation rate (path S-II). During this phase the effect of NF is not felt due to its slower dynamics. After a certain delay, the action of NF results in decrease of the calcium accumulation rate, however it remains positive for a certain time (path II–III). On further increase in the NF, the two feedback effects cancel out at point III and the calcium accumulation rate becomes zero. This does not result in a steady state, because the slow negative feedback is out of phase and continues to reduce the calcium accumulation rate along path III–IV, resulting in a drop in calcium concentration. Beyond point IV, the effect of reduced calcium concentration nullifies the effect of NF, resulting in a drop in the calcium reduction rate along path (IV–I). As the calcium concentration ceases to drop at point I, the effect of PF now again starts dominating, resulting in continuation of the cycle leading to sustained oscillations. Models of calcium stimulation by Gq coupled GPCRs commonly incorporate this motif. Proposed models have differed in terms of where these feedback loops are positioned within the G protein-PLC-IP3-Ca^{2+} pathway.



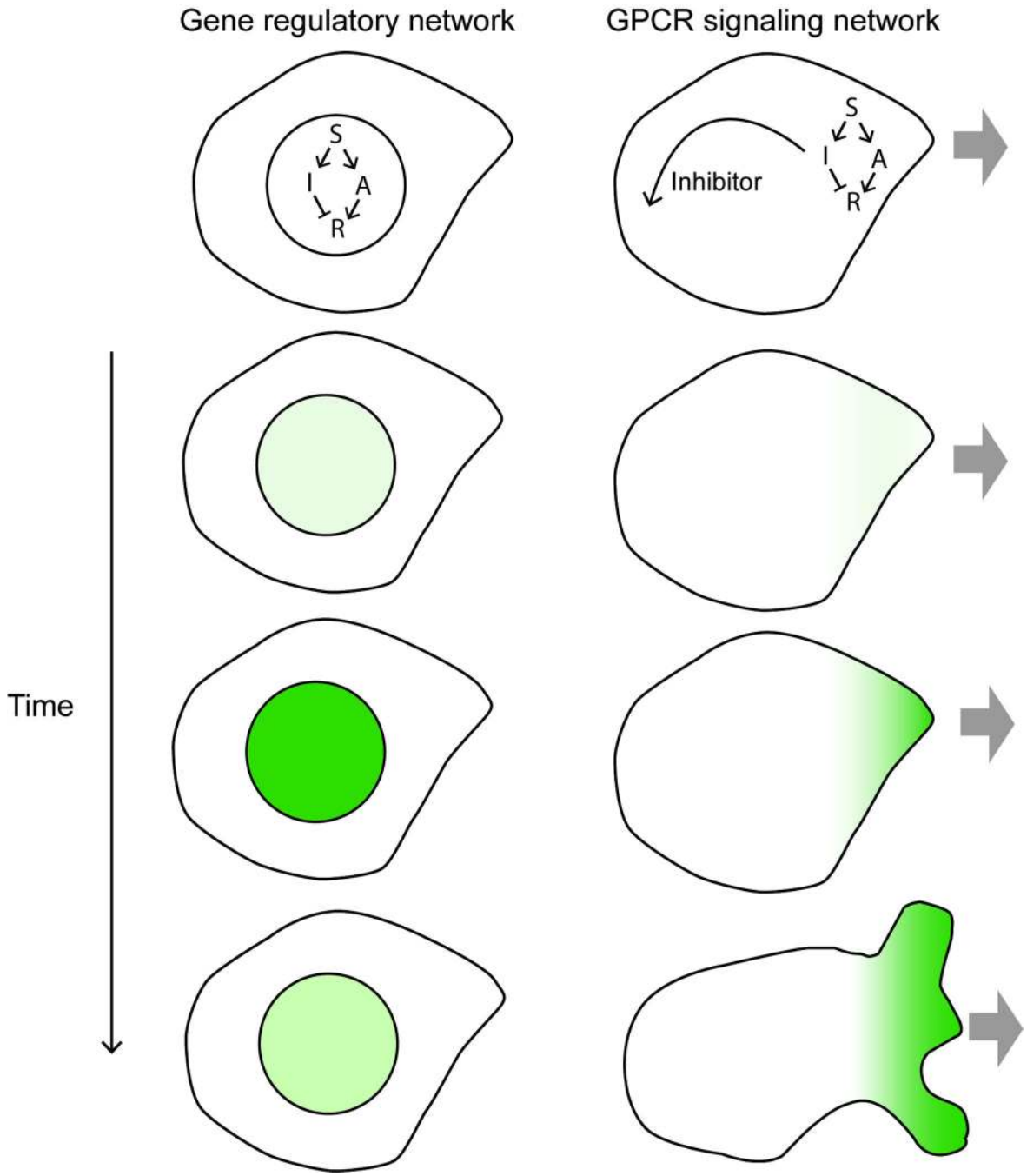


Figure 1.

Comparison of gene regulatory networks with a GPCR network that regulates polarized cell behavior. The GPCR network structure includes dynamic links between network motifs that guide spatial response. The network motif shown is the incoherent feedforward loop (Box 2) which becomes the LEGI motif (Box 1) in the case of the migrating cell due to diffusion of the inhibitor to the back of the cell. Grey arrow denotes direction of chemoattractant gradient.

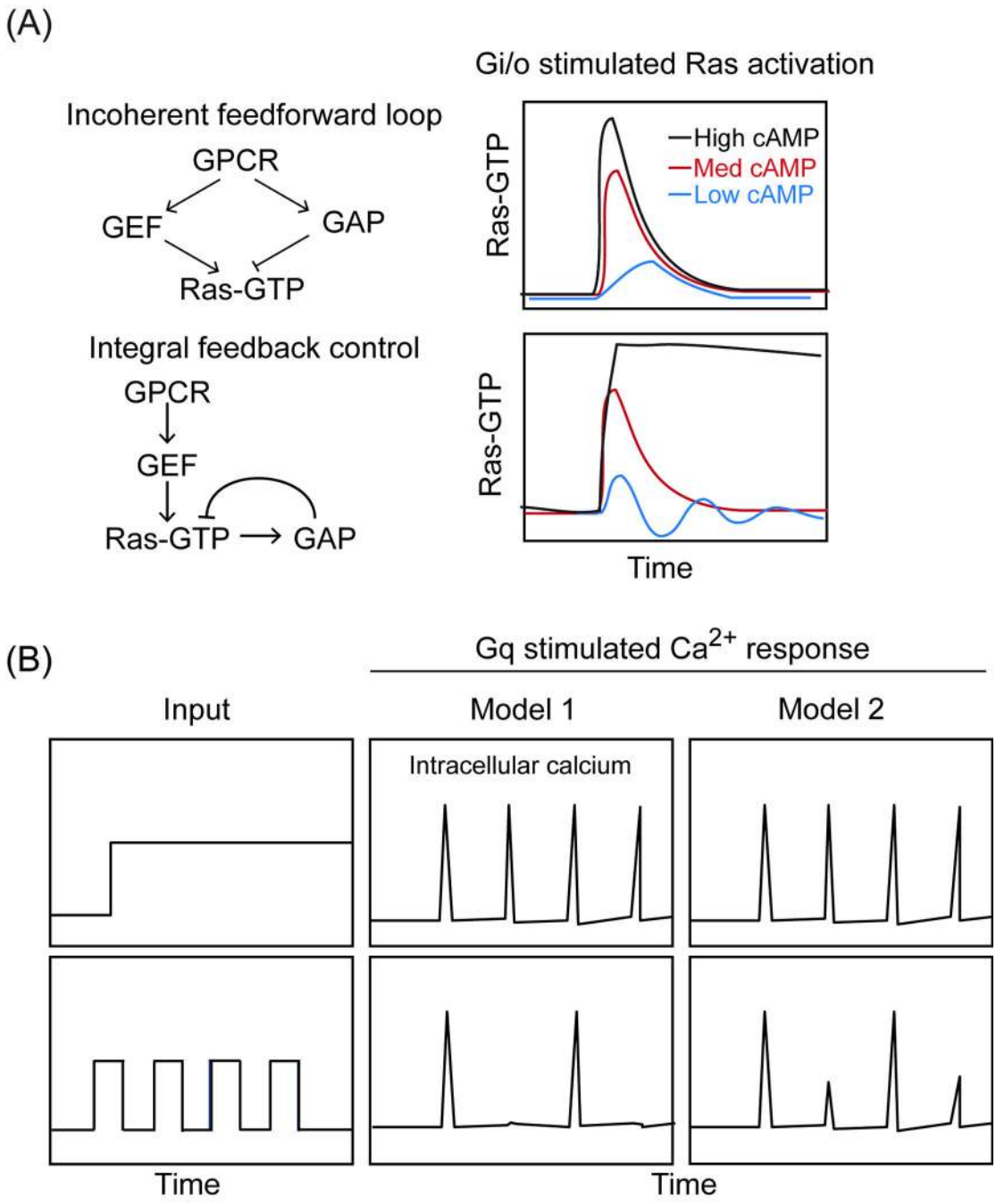


Figure 2.

Imaging single cell responses to dynamic inputs helps to determine network structure. (A) Single cell imaging of GPCR stimulated Ras activation was used to determine the network motif that provides adaptation [11]. The incoherent feedforward loop (IFFL) and the integral feedback control motifs are both capable of perfect adaptation, but only IFFL responds to a wide range of agonist concentrations with similar adaptation kinetics as was found experimentally. (B) A phase locking method was used to test different network models for the Gq stimulated calcium response [23]. Subthreshold calcium spikes observed by the phase locking method helped to differentiate between models that could not be distinguished in experiments that used a step increase in agonist concentration.

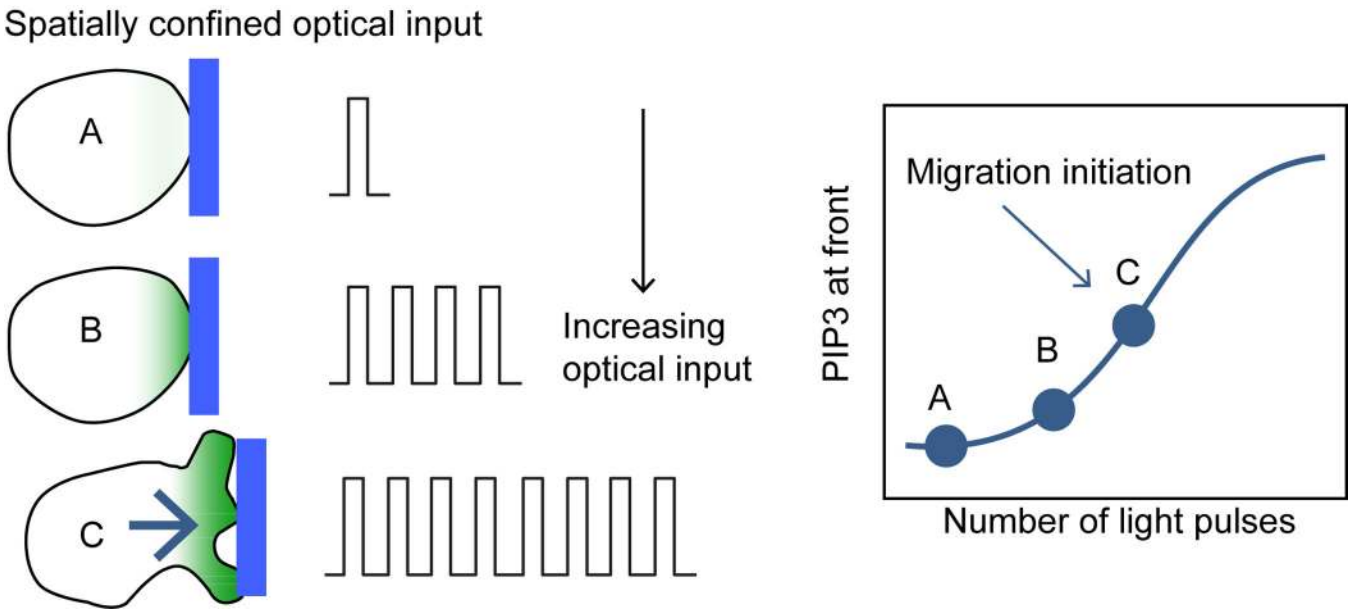


Figure 3.

Activating G protein signaling with spatially localized discrete optical inputs [30]. Macrophage-like cells expressing blue opsin were optically activated at one end of a cell. The accumulation of PIP3 at the front of the cell exhibited a switch like behavior with increasing number of light pulses, and cell migration correlated with the steepest region of the response [12].