Fluorescence imaging of signaling networks

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Receptor-triggered signaling processes exhibit complex cross-talk and feedback interactions, with many signaling proteins and second messengers acting locally within the cell. The flow of information in this input–output system can only be understood by tracking where and when local signaling activities are induced. Systematic strategies are therefore needed to measure the localization and translocation of all signaling proteins, and to develop fluorescent biosensors that can track local signaling activities in individual cells. Such a biosensor tool chest can be based on two types of green fluorescent protein constructs that either translocate or undergo fluorescence-resonance-energy transfer when local signaling occurs. Broad strategies to measure quantitative, dynamic parameters in signaling networks, together with perturbation approaches, are needed to develop comprehensive models of signaling networks*.

Recent technical developments have made it possible to ask how information flows from many different cellular-receptor inputs to a diverse set of physiological cell functions (outputs). Expression profiling [1], proteomics [2] and evolutionary comparison with known signaling proteins [3] can now be used to identify all predicted signaling proteins in a particular cell type. Although studies in yeast and other model organisms have been leading the way, these approaches can now be applied to obtain a list of relevant signaling proteins in a particular mouse or human cell. Given our current knowledge, one can expect a typical mammalian cell to contain somewhere between several hundred and a few thousand different signaling proteins, and more than ten second messengers. These signaling proteins and second messengers are organized in a network-like fashion, and it is likely that some of the principles of metabolic networks [4] and transcriptional networks [5,6] also apply to mammalian signaling networks.

To discuss some of the unique features of the cell signaling problem, an idealized signaling network is shown in Fig. 1. As a working hypothesis, the internal structure of this input–output system can be described using three concepts: signaling pathways, signaling modules and signaling nodes. For signaling pathways, the main flow of information is sequential and goes

through a linear chain of intermediate steps from the receptor to a particular cell function. This idea has been extremely powerful and has shaped our current view of signal transduction. For example, tyrosine kinase and other receptor stimuli turn on a multistep mitogen-activated-protein-kinase pathway to activate specific genes that upregulate cell growth [7].

The second concept of signaling modules can be subdivided into modules that are connected by feedback involving a diffusion step (diffusible feedback systems) and modules that are physically linked complexes of signaling proteins and/or scaffolding proteins [8,9]. An example of a diffusible feedback module can be seen in the positive and negative feedback loops by which calcium and the inositol-trisphosphate receptor regulate cytosolic calcium concentration [10]. One example of a signaling scaffold module is the adaptor protein AKAP79, which can bind multiple signaling proteins and thereby provides a functional link for the coordinated activity of protein kinases and other signaling proteins [9]. Signaling nodes, the third concept, are molecules that are activated by many signaling inputs and, in turn, have many downstream targets [4]. Examples of signaling nodes include the second messengers, Ca^{2+} and phosphatidylinositol(3,4,5)triphosphate, as well as the small GTPase Ras and protein kinase C. Nodes might play a key role in coordinating signaling networks because they can connect signaling pathways and modules that seem at first glance not to be related to each other.

Because subcellular localization and translocation are increasingly known to be crucial for cellular information processing [11], systematic microscopy efforts to measure the subcellular localization and translocation of all signaling proteins have become an essential part of the quest to understand a particular signaling network. To attack the problem of information flow, one has then to measure where and when these signaling proteins are active. This is a big experimental challenge that might be solved by using many fluorescent activity reporters (biosensors) that can track local signaling activities over time. A quantitative understanding of signaling networks could then be obtained by using these biosensors to measure how combinations of different receptor inputs control the amplitude and timing of intermediate signaling steps and how these intermediate signaling steps control the different physiological outputs. This approach would also require systematic perturbation of the signaling network in order to

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Fig. 1. Three concepts that are useful for describing signaling networks. Cell signaling is initiated by receptor stimuli. Each connection point reflects a signaling protein or second messenger, with lines indicating functional interactions. (a) Linear signaling pathways. (b) Modular structures within the network. (c) Nodes, which can be proteins or second messengers. Nodal points are regulated by many upstream events and/or regulate many downstream events.

obtain delay times between signaling events and to determine the strength of cross-talk between nodes, modules and pathways.

Although an understanding of dynamic properties of cellular signaling networks will require both fluorescence microscopy and perturbation approaches, this article only touches on the latter and mainly focuses on the usefulness of different types of fluorescent biosensors and microscopy techniques to dissect signaling processes and networks.

Localization and receptor-triggered translocation of all signaling proteins

Powerful strategies based on genetic approaches, protein–protein interaction maps, *in silico* predictions, synthetic lethality screens and clustering strategies using microarray data [12–17] have initially been developed in yeast and other model organisms to predict modules and pathways systematically. Similar strategies for particular human and mouse cell types are currently under way in several laboratories. Even though subcellular localization has not yet been used as a major technique to identify modularity, the first inroads into this challenging experimental problem have been made from datasets that were created using tagged yeast proteins [18,19] and a small subset of green-fluorescent-protein (GFP)-conjugated human proteins with unknown function [20]. A systematic live- and fixed-cell microscopy effort to measure the localization of all signaling proteins in a mouse B-cell model is currently under way in our laboratory under the umbrella of the Alliance for Cell Signaling (<http://www.afcs.org/>).

Eukaryotic cells contain a significant number of subcellular structures that are relevant for signaling and can be distinguished using various fluorescent markers. Although the main signaling sites are the plasma membrane, cytosol and nucleus, other relevant structures include the nuclear membrane, nucleoli, centrosomes, endoplasmic reticulum, recycling endosomes, lysosomes, Golgi, mitochondria, secretory vesicles and various cytoskeletal and other scaffolding structures. An overall understanding of the organization of signaling systems requires an analysis that measures which proportion of

each signaling protein localizes to the different subcellular structures. Next, one has to add the time dimension and to measure whether fractions of these signaling proteins change their localization in response to different receptor inputs. Marked changes in localization (translocation) have been observed for many soluble proteins [e.g. protein kinases, lipases and GDP–GTP exchange factors (GEFs)] as well as for membrane-bound or transmembrane proteins (e.g. lipid-modified signaling proteins and channel proteins, respectively) [10].

An experimental strategy to investigate protein localization and translocation in fixed cells is to use antibodies against native proteins and to compare subcellular localization against marker antibodies before and after stimulation. Some of the problems with the fixed-cell approach are that high-quality antibodies are difficult to create for all signaling proteins, that it is not possible to obtain same-cell time-course measurement of localization, that fixation protocols have to be adapted for different antibodies and that fixation is often found to alter the localization of the native protein. This currently limits the usefulness of this strategy to a subset of signaling proteins for which there are high-quality antibodies and that are bound to cytoskeletal and other structures that preserve well during fixation.

A second strategy to obtain protein-localization data is to use expressed proteins that are tagged with GFP or other tags [21]. Although functional tagged proteins have been obtained for most signaling proteins that have been tried so far, some tinkering was often needed to retain intact activity. GFP fusion tags were often found to require ‘inert’ linker sequences that had to be placed specifically at either the C- or the N-terminus of proteins or at internal sites [20]. A main concern for this approach is that transient expression introduces additional tagged protein on top of the native protein. If the number of physiological docking sites for a signaling protein is limiting, the observed localization of the tagged protein might differ from the localization of the native protein. This approach provides useful localization data if the signaling proteins have a targeting mechanism that cannot readily be

saturated or if the local concentration of docking sites is high compared with the concentration of the expressed proteins. In some cases, subcellular localization is only apparent in cells that express low concentrations of the tagged proteins. Fixation and immunolocalization of tagged proteins can be used in some cases to increase sensitivity and to wash out weakly docked proteins [22].

An important use of GFP-conjugated signaling proteins is measuring time courses of protein translocation in response to receptor stimulation. Systematic studies of the translocation of signaling proteins can be performed by taking sequential fluorescence-microscopy images during and after cell stimulation [11]. In an additional use of these constructs, fluorescence-recovery-after-photobleaching measurements can be used to measure the mobility of signaling proteins [23,24]. Important parameters that can be measured for large sets of proteins include the apparent diffusion coefficient and the proportion of tightly bound or immobile protein. For cases in which a protein is tightly bound to a signaling complex, the measured recovery reflects the exchange rate (which is approximately equal to the dissociation time constant, k_{off}).

In terms of the goal of identifying the relevant pathways, modules and nodes in signaling networks, knowledge of the subcellular localization, translocation and fluorescence-recovery-after-photobleaching mobility can be used to group signaling proteins according to location as well as to measure time courses and rates of a subset of the internal signaling processes.

Translocation biosensors as tools to track local signaling processes over time

The important problem in understanding the flow of information in the network is to know where and when signaling proteins are actually activated. Currently, the most useful techniques to look at local activation kinetics are based on fluorescence microscopy and involve the imaging of small-molecule- or protein-based fluorescence-signaling reporters. This fluorescence biosensor strategy was first introduced by Roger Tsien's design of a fluorescent calcium indicator [25], which became a powerful tool to track calcium signals in individual cells. A subsequent study introduced the idea of imaging fluorescent biosensors that are localized to distinct subcellular structures within a cell [26], an approach that had previously been used for cell ensemble measurements using bioluminescence reporters [27].

For cases in which the translocation of a fluorescently conjugated signaling construct can be related to a molecular activation state, translocation itself can be used as a means of tracking the local concentration of second messengers, protein phosphorylation or the local activation state of a signaling protein. Signaling proteins that undergo translocation as part of their activation process include Akt, protein kinases A and C, calmodulin (CaM), Syk, and CaMKII (Fig. 2a). Minimal protein domains for translocation were identified in many of these cases and were used as more-specific tools to measure a particular signaling process. Such translocation biosensors have been developed by our and other laboratories during the past few years. They include SH2 domains from phospholipase C and Syk to measure local tyrosine phosphorylation [28], a PH domain

Fig. 2. Many signaling proteins translocate as part of their activation process. (a) Change in the distribution of a GFP-tagged protein kinase C molecule in tumor mast cells stably transfected with the platelet-activating-factor receptor before and after stimulation with platelet-activating factor [64]. (b) Total internal reflection microscopy measurement of the platelet-derived growth factor (PDGF)-induced translocation of green-fluorescent-protein-Akt(PH) domains in a fibroblast cell line [36]. The left panels show the cell before and the right panels after stimulation.

from phospholipase C δ to measure phosphatidylinositol (4,5)-bisphosphate [29], PH domains from ARNO and Akt for measuring phosphatidylinositol (3,4,5)-trisphosphate [30,31], as well as C1, C2 and many other domains to measure various signaling responses [11,31,32].

Many signaling processes are organized at the plasma membrane and involve an activation step that leads to the translocation of signaling proteins from the cytosol to the plasma membrane or from the plasma membrane to the cytosol and nucleus. Commercially available automated image analyses are well suited to measuring nuclear translocation but have more difficulty measuring translocation to other cellular sites. Our recent studies suggest that total internal reflection (TIRF) microscopy, which was first developed by Axelrod and co-workers for biological applications [33] and later by Almer's group for vesicle-fusion studies [34], is a powerful technique for quantitatively measuring the plasma membrane translocation and dissociation of signaling proteins [35–37]. The advantages of this TIRF approach to monitoring plasma membrane signaling processes are that the translocation is reduced to a simple intensity increase (Fig. 2b), that the signal-to-noise ratio is improved, that large cell numbers can be measured at the same time and that the phototoxicity and photobleaching are minimal, which enables measurements over long periods of time.

Using FRET to measure local signaling processes

Fluorescent biosensors based on fluorescence-resonance-energy transfer (FRET) [38–40] typically involve elegant

designs that reflect our knowledge of molecular activation mechanisms. In the first GFP-based examples of this approach, Persechini and co-workers made a biosensor that measures the binding of Ca^{2+} -CaM to a CaM-binding peptide flanked by C- and N-terminal blue fluorescent protein and GFP [41], whereas Miyawaki, Tsien and co-workers made a biosensor that measures the binding of Ca^{2+} ions to a similar construct that included a CaM-binding peptide as well as CaM itself [42]. The first construct functions as an indicator of the free Ca^{2+} -CaM concentration and the second as an indicator of the free Ca^{2+} concentration.

There are also a few recently developed alternative strategies. Jovins, Bastiaens and co-workers developed a method based on measuring the changes in fluorescence lifetime (as a more-sensitive measure than steady-state energy transfer) that led to new insights into receptor activation and phosphorylation mechanisms [43]. FRET measurements have also been combined with translocation to obtain a collision FRET signal in the plasma membrane [44]. In another approach, protein-protein interactions were monitored locally in cells [45,46]. Currently, the most feasible FRET-based approach to tracking the flow of information in signaling networks is to use biosensors that have both cyan and yellow fluorescent proteins (CFP and YFP) [or GFP and red fluorescent protein (RFP)] linked to the same construct. This type of FRET biosensor can be targeted to different intracellular sites and has been shown to work for two large protein families: small GTPases [47] and protein kinases [48–50] (Fig. 3).

Although the current developments are very encouraging, a main limitation of the FRET strategy is the often-small signal, which makes it difficult to measure partial activation. Because two GFP variants (e.g. CFP and YFP) are needed for FRET measurements, only one parameter can typically be measured in a cell. By contrast, up to three of the translocation biosensors can be used in the same cell using three GFP variants (CFP, YFP and RFP [51]). For both translocation and FRET biosensors, one has to consider that the expression of a particular biosensor might alter the amplitude and time course of the overall

signaling response. For example, biosensors such as the Ca^{2+} -CaM FRET biosensor and the SH2-domain translocation biosensors interfere with or block downstream signaling [22]. Nevertheless, the FRET Ca^{2+} indicators and the PH domains and other lipid-interacting translocation biosensors described above seem to have only a minimal effect on downstream signaling if they are produced at moderate levels [36]. Some of the concerns about the use of FRET and translocation biosensors can only be eliminated by doing a genome-wide analysis of the signaling-network kinetics and then assessing whether the obtained results are internally consistent and can be used to model the signaling network.

Single-cell measurements versus ensemble measurements

Experience with studying calcium signals and gene expression in single cells has shown that even homogeneous cell populations have a high degree of cell-to-cell variability. This might reflect different states of a cell (e.g. stage of the cell cycle), different subpopulations or stochastic variability in the expression of different genes. More importantly, key parameters that define the dynamic properties of cellular signaling networks cannot be extracted from measurements of cell ensembles. Such parameters include delay-time constants between signaling steps, co-operativity or bistability in the activation process, ranges of time constant in positive and negative feedback loops, and timing patterns such as oscillations. For example, it is now clear that there are calcium oscillations in a wide range of cell types and that, by varying such parameters as oscillation frequency, cells can trigger selective physiological responses, such as the expression of particular genes [52,53]. The existence of these oscillations only became apparent when single-cell measurement methods became available [54]. Another example for the need for single-cell measurements is the all-or-none activation of mitogen-activated-protein kinases in *Xenopus* oocytes, which appears to be a graded response in experiments that measure averaged responses from an ensemble of cells [55].

Many single-cell recordings are often needed to obtain

Fig. 3. Cellular response of a FRET indicator that reports Abl tyrosine-kinase activity [49] in an NIH 3T3 cell before and after stimulation with platelet-derived growth factor (PDGF). (a) Time course showing the distribution of the phosphorylated Abl biosensor. False-color image of the ratio of the yellow- and cyan-fluorescent-protein images. The FRET signal is dramatically elevated in the membrane ruffles, as is the concentration of the indicator (b).

Fig. 4. Example of a large-area imaging experiment, which can be used to track thousands of signaling responses from individual cells as a function of time [37]. Simultaneous measurement of plasma membrane translocation events in individual tumor mast cells transfected with the C2 domain of protein kinase C γ conjugated to yellow fluorescent protein after receptor stimulation. Each bright spot is a transfected cell whose surface plasma membrane is illuminated by total internal reflection excitation. A representative time course of translocation is shown. Bar in (a) 1 mm; bar in (c) 50 s. Abbreviation: PAF, platelet-activating-factor.

the necessary statistics to analyze the temporal response patterns (Fig. 4). This requires low-magnification fluorescence microscopy and parallel measurements with at least a few hundred cells. Such large cell numbers can readily be monitored by low-magnification imaging of cells that express FRET biosensors [56] or contain calcium indicators and other biosensors. We recently introduced a method to measure plasma membrane translocation simultaneously in thousands of individual cells using a large-area TIRF system [37], and nuclear and other translocation studies in large numbers of cells can be made using automated commercial microscope systems. This suggests that the equipment is in place to perform a parallel analysis of signaling networks using many receptor stimuli, fluorescent biosensors and functional readouts.

Inputs, outputs and perturbations: identifying timing and cross-talk

A key condition for setting up cellular assays to investigate signaling networks is the identification of the physiologically relevant inputs and outputs. Although the relevant receptor stimuli are known in many cases and are easy to apply, it is more difficult to develop cell-based assays for known physiological outputs. Nevertheless, several useful single-cell strategies have been developed to measure physiological responses such as apoptosis, motility, secretion [34], cell depolarization [57], protease activity [40], the activation of particular transcriptional promoters [56] or the translocation of glucose transporters after insulin stimulation [58]. In combination with biosensors that can measure intermediate signaling steps, such assays to measure physiological responses in single cells offer a powerful basis for perturbation screens of the signaling network.

The ultimate goal of a cell-wide perturbation strategy of a particular signaling network is to create a dataset that provides sufficient quantitative and dynamic detail for a model of the signaling network. The ideal perturbation strategy is based on the rapid activation or inhibition of all intermediate signaling steps in a signaling networks while different intermediate signaling steps are monitored over time using FRET or translocation biosensors. Such systematic rapid perturbations are currently only possible using a limited set of known small-molecule inhibitors and activators. A promising strategy that is currently being developed by the Shokat laboratory is the expression of different protein kinases that can be rapidly switched on or

off using small molecule activators [59]. Other interesting chemical perturbation strategies are based on the immunosuppressant drug FK506 [60] and on cell-based small-molecule screens to identify selective cellular inhibitors or activators [61]. This is an exciting field with much potential for the creation of new tools to dissect signaling networks.

As a less-ideal but more feasible approach, a promising perturbation strategy that alters the signaling system on timescales of hours to days is RNA interference, first demonstrated to specifically silence genes in *Caenorhabditis elegans* [62]. This technique has now been shown to reduce the expression of specific proteins in mammalian cells [63]. An equally feasible technique is based on the transient expression of large sets of dominant negative and constitutively active signaling constructs that can interfere with different parts of the cellular signaling network. Both these 'slow' perturbation techniques can be combined with biosensor measurements and functional readouts, and will contribute to the identification of the functionally relevant modules and pathways in signaling networks.

Concluding remarks

Recent developments with fluorescent probes for signal transduction and imaging technologies have made it possible to systematically explore the localization and translocation of all signaling proteins in a given cell. Fluorescent biosensors based on translocation and FRET offer many possibilities for tracking the flow of information in a cellular signaling system. Perturbation strategies can now be performed that span an entire signaling network and give insight into its modular structure. In the short term, such projects will provide valuable databases for all signaling researchers to generate hypotheses about molecular interactions and the importance of particular signaling events. In the longer term, the collection of quantitative data on the feedback time constants and cross-talk between different signaling steps will create a basis for the quantitative modeling of signal-transduction networks.

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