Analyzing Cell Fate Control by Cytokines Through Continuous Single Cell Biochemistry

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ABSTRACT

Cytokines are important regulators of cell fates with high clinical and commercial relevance. However, despite decades of intense academic and industrial research, it proved surprisingly difficult to describe the biological functions of cytokines in a precise and comprehensive manner. The exact analysis of cytokine biology is complicated by the fact that individual cytokines control many different cell fates and activate a multitude of intracellular signaling pathways. Moreover, although activating different molecular programs, different cytokines can be redundant in their biological effects. In addition, cytokines with different biological effects can activate overlapping signaling pathways. This prospect article will outline the necessity of continuous single cell biochemistry to unravel the biological functions of molecular cytokine signaling. It focuses on potentials and limitations of recent technical developments in fluorescent time-lapse imaging and single cell tracking allowing constant long-term observation of molecules and behavior of single cells. J. Cell. Biochem. 108: 343–352, 2009.   © 2009 Wiley-Liss, Inc.

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A century ago scientists realized that blood of anemic animals is able to stimulate red blood cell development in recipient animals. The evidence for a soluble factor in blood responsible for enhanced red blood cell production was finally found in 1953 and the factor was called erythropoietin (Epo). It took until 1977 to first isolate Epo, which is mainly produced in the kidneys. Finally, the cloning of the *erythropoietin* gene and cDNA in the 1980s enabled the recombinant expression in bacteria and mammalian cells and allowed production of Epo in industrial dimensions [Jelkmann, 1986]. Today, Epo and many other cytokines are crucial for clinical therapy. Over decades, several product generations with improved modifications were engineered by companies around the world and cytokines became a multi-billion dollar market for the pharmaceutical industry.

However, answers for many of the long-standing basic questions concerning cytokine function remain elusive. To date, it is usually unknown how exactly different molecular changes induced by cytokines lead to specific cell fates in different cell types.

In this review we will discuss the general concepts, technical necessities, and limitations of cytokine research using the extensively analyzed and clinically important hematopoietic cytokines as an example. However, the general concepts, disputes, and experimental necessities to analyze their function also hold true for other cytokines and cell types.

Hematopoietic cytokines influence the generation of blood cell lineages from hematopoietic stem and progenitor cells. They include stem cell factor (SCF), interleukin (IL) 3, 5, 6, 7, and 11, thrombopoietin (Tpo), Epo, fms-like tyrosine kinase 3 ligand (Flt3L), and macrophage (M)-, granulocyte (G)-, and granulocyte-macrophage (GM)-colony-stimulating factors (CSFs) [Metcalf, 2008].

Cytokines are a diverse group of soluble proteins and peptides that act as humoral regulators of cell fates. Most cytokines are glycoproteins that are secreted by cells using classical secretory pathways. Some cytokines are produced only by specialized cells of a particular organ, while others are secreted by many different cell types of the body [Metcalf, 2008]. They either circulate in the blood and act as hormones, or are more locally restricted as local soluble mediators. Moreover, cell membrane bound forms of cytokines have been described. Their expression is strictly regulated and often cannot be substituted by their soluble counterparts [Anderson et al., 1990; Stein et al., 1990]. The membrane-associated forms and soluble forms that have been immobilized by binding to the extracellular matrix are of particular interest to the concept of localized niches that control the fate of specific cell types by short range signals from their microenvironment.

Cytokines bind to their specific receptors on the cell surface, leading to multimerization of the receptors inducing the activation

of intracellular signaling pathways. Most cytokines have a very high specificity for their receptors and usually only bind to one receptor. Cytokine receptors belong to different protein families: receptors like the G-CSF, GM-CSF, Epo, and Tpo receptors lack intrinsic protein kinase activity, and require intracellular signaling molecules with kinase activity for signal transduction. In contrast, some hematopoietic cytokine receptors have kinase activity that is activated upon ligand binding. For example, the receptors for SCF (c-KIT), M-CSF (c-Fms), and Flt3 ligand (Flt3) belong to the tyrosine kinase receptor family.

Of note, for some cytokines, soluble decoy receptors also exist that bind and can block free cytokine molecules, opening an additional possibility of modulating cytokine activity [Levine, 2008].

MULTI-FUNCTIONALITY AND REDUNDANCY OF CYTOKINES

Despite their clinical and commercial relevance and decades of intense academic and industrial research, it proved surprisingly difficult to describe the biological functions and molecular control of cell fates by cytokines in a precise and comprehensive manner. As summarized in Figure 1, several properties of cytokine biology make the analysis of their functions and molecular signaling mechanisms very difficult: As described in more detail below, cytokines usually have diverse biological effects, but may also overlap with effects from other cytokines. At the same time, each cytokine activates a multitude of signaling pathways to induce transcriptional and epigenetic programs, but these signal pathways are also activated by many other cytokines with different cellular fate outcomes.

Often, several different cytokines have the same effects on a particular cell type. Therefore, the loss of one cytokine or its receptor can often be compensated by other cytokines (Fig. 1A). One example for this phenomenon of redundancy is the effect of the colony-stimulating factors M-CSF, GM-CSF, or IL3. All three individually support the development of myeloid progenitors into macrophages. The same holds true for G-CSF, GM-CSF, or IL3 for neutrophil granulocytic development. Loss of function studies addressing cytokine function in vivo by using either cytokine or cytokine receptor knock-out animals are often hampered by compensatory effects of redundant cytokines, even in knock-out models lacking several different cytokines or their receptors [Hibbs et al., 2007]. Other cytokines may be dysregulated upon loss of the activity of a cytokine as a compensatory mechanism [Fievez et al., 2007]. This redundancy of cytokine effects is likely to be an important natural backup system for the vital need of constant hematopoiesis, but it makes conclusions from in vivo studies very difficult. To study the function of an individual cytokine, defined cytokine conditions are essential, which are impossible to assess in vivo using existing technologies. Hematopoiesis takes place at specific sites, of which the cellular and molecular composition still remains largely unknown. It is not even clear whether these niches consist of many or just single cells. Our current knowledge of the exact molecular composition of these niches thus is restricted to a few ill-defined candidate molecules, and the concentration of cytokines at hematopoietic sites in vivo will remain extremely difficult to

assess. At present, it is therefore necessary to analyze function and molecular mechanisms of cytokine signaling under defined conditions in vitro at first, and then confirm and complement newly gained insights by in vivo studies.

In addition to the redundancy of different cytokines for a specific biological effect, activation of a single cytokine can influence a wide range of cell fates (Fig. 1B). After the identification of different cytokines, it was widely thought that they mainly regulate the proliferation and survival of individual cell types. However, intensive research then revealed that individual cytokines also can initiate maturation, and control various functions of mature cells (Fig. 1B) [Pixley and Stanley, 2004; Metcalf, 2008]. In addition, the influence of cytokines on the lineage choice of multipotent cells had long been postulated but remained disputed for decades (see below).

THE NEED FOR CONTINUOUS SINGLE CELL FATE OBSERVATION

The generation of mature cells from their progenitors, which can take several days or even weeks, will be influenced both by different cytokines, and by different functions of one cytokine, in a simultaneous and/or sequential fashion (Fig. 2). Some of these cytokine functions like survival are essential during the whole differentiation process, whereas others may only act at specific narrow time windows during differentiation. Additionally, variations of the strength of the cytokine signal as well as the interplay with other cytokines or intracellular factors might modulate the functional outcome of a cytokine signal over time.

In order to understand the biological effect of cytokines, it is therefore crucial to be able to observe the cells' behavior constantly and at the single cell level. Since different combinations of cellular behaviors can lead to identical population outputs over time [Schroeder, 2005, 2008; Rieger and Schroeder, 2008], discontinuous fate analysis of complex cell populations does not allow non-ambiguous interpretation of experimental data. Only continuous analysis of cell fates at the single cell level enables valid conclusions about how the individual cells in an observed cell population actually behave over time—which is the basis for understanding the control of cell fate decisions by cytokine signaling.

The decades long uncertainty about a potential lineage instructive effect of cytokines is a good example for the necessities for continuous cell fate quantification when analyzing cytokine function. It is clear that lineage-specific cytokines can increase the production of cells of a specific lineage. However, it remained disputed whether these cytokines can influence lineage choice by instructing multipotent cells to differentiate into this lineage [Metcalf, 1998]. Alternatively, cytokines may merely allow the survival, proliferation and/or maturation of cells that had already independently decided for this lineage. In the latter case, the cytokines' function would then only be to select cells that have been committed to this lineage by cytokine-independent mechanisms [Enver et al., 1998]. It is well known that in the presence of only one lineage-specific cytokine (such as M-CSF), only cells
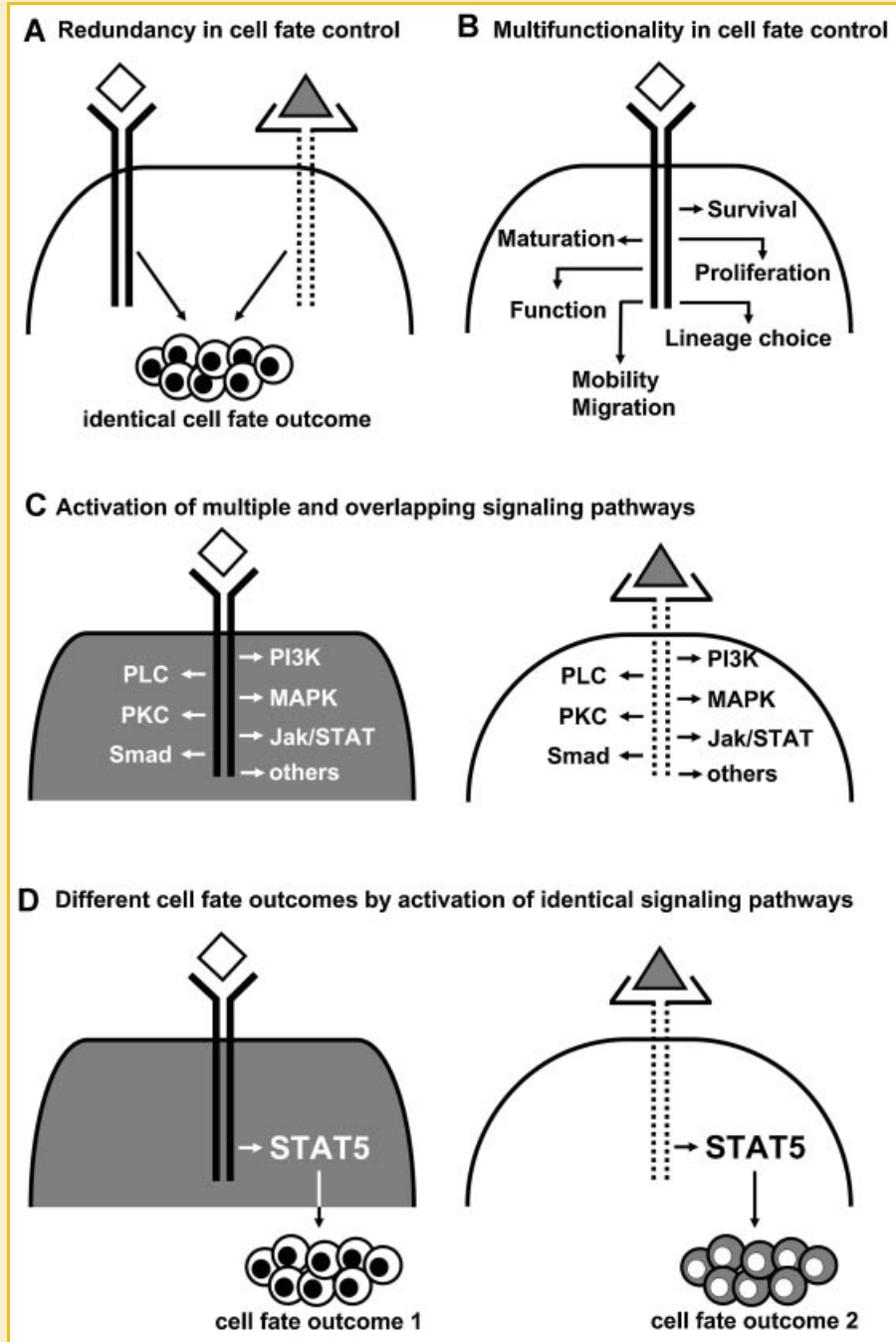
Fig. 1. Complexity of cytokine signaling and cell fate control. A: Different cytokines can have the same cell fate effects. B: Individual cytokines control many different cell behaviors. C: Different cytokines often activate common signaling pathways. D: Despite activating identical signaling pathways, different cytokine receptors can induce different cellular functions. PLC, phospholipase C; PKC, protein kinase C; MAPK, mitogen–activated protein kinase; PI3K, phosphoinositide 3 kinase; JAK, Janus kinase; STAT, signal transducer and activator of transcription.

of a particular lineage (i.e., monocytic cells) will appear in cultures from multipotent progenitors. Both instructive and selective cytokine functions are able to yield this result. In such a situation, the occurrence of even a single cell death during the differentiation would allow explanation of unilineage cell appearance by a pure selective effect of cytokines on progenitor survival—the one dying cell could have been a cell that committed to a different lineage and died because of the lack of this lineage's supporting cytokine. Existing cell fate observation technologies never allowed excluding the potential death of a few individual cells during days long colony development. The exclusive selective effect of cytokines on lineage choice could therefore never be excluded and a potential lineage instructive cytokine function remained disputed.
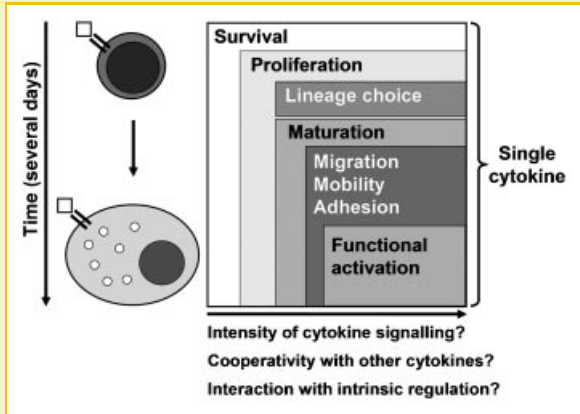
Fig. 2. Synchronous and sequential cytokine functions during differentiation. Most cytokines have multiple functions during the development from immature progenitors to functional mature cells. The different functions of a cytokine are well timed, and some of them last the whole differentiation process, whereas others might only act during short time windows. During the development of a lineage, cells in certain differentiation states can have different responsiveness to a cytokine. In addition, different cytokine signal strengths can induce different cell fates. Only the constant observation of cells during the whole differentiation process will allow to understand and to dissect the interplay and timing of individual effects of a cytokine.

To address this and other long-standing fundamental questions in cytokine biology, we developed and used bioimaging approaches allowing the continuous long-term observation of differentiation processes at the single cell level [Schroeder, 2005, 2008; Eilken et al., 2009; Rieger et al., 2009]. This technology allows the constant observation of cell behavior of all cells in a culture over up to several weeks, including the reliable detection of cell division, morphology, and cell death. This approach allowed us to continuously observe identity and fate of each single cell of a colony developing from granulocyte-macrophage progenitors (GMPs) into mature macrophages or granulocytes in the presence of only M-CSF and G-CSF, respectively. A pure selective function of these cytokines in GMP lineage choice could be excluded by the lack of single cell death events during the differentiation process, proving that M-CSF and G-CSF instruct GMP lineage choice [Rieger et al., 2009]. This example well illustrates the need for constant observation of cell behavior when trying to understand the biological effects of cytokines.

In conclusion, the control of cell fates by cytokine signaling can only be reliably analyzed if cell fates can be assessed continuously, quantitatively, and at the single cell level.

CONTEXT DEPENDENT CELL FATE OUTCOMES OF OVERLAPPING CYTOKINE SIGNALING PATHWAYS

The diverse biological effects of individual cytokines raised the question of how activation of a single receptor can induce these different functions. An important step was the identification of specific regions of intracellular receptor domains of cytokine receptors that could be linked to distinct functions in cell fate

control. Specific domains for cell proliferation, survival, and maturation could be defined by receptor mutant studies [Fukunaga et al., 1993; Miyajima et al., 1993]. Later, it became clear that these regions contained docking sites for adaptor molecules linking to signaling pathways, which are often activated by phosphorylation of, for example, a tyrosine residue. This allowed identification of different signaling pathways emanating from different cytokine receptors. Common signaling pathways such as the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, the mitogen-activated protein kinase (MAPK) pathway, the phosphoinositide 3 kinase (PI3K) pathway, the protein kinase C (PKC) pathways, the phospholipase C (PLC) pathways, and the Smad pathway have been associated with various cytokine receptors with high overlap (Fig. 1C). The attribution of specific cell fate effects to the activation of specific signaling pathways by cytokine receptors allows an explanation for the polyfunctionality of cytokines.

However, although different cytokine receptors were found to induce the same signaling pathways, they can induce different biological effects (Fig. 1D). For instance, while the activation of the GM-CSF and Epo receptors has different cellular consequences, both signal via STAT5 [Hennighausen and Robinson, 2008]. Similarly, although the receptors for IL3, IL5, and GM-CSF all contain the common beta chain and share many signaling pathways, they permit distinct functions in different cell types [Miyajima et al., 1993].

The exact combination of intracellular signaling pathways, activated at a specific strength by different cytokines, could lead to unique biological outcomes. In addition, different biological effects of identical signaling pathways in different cell types could be caused by differences in accessibility of responsive target gene regions due to different chromatin states, by the availability of adaptor molecules for larger transcription activating or modulating complexes, or by availability of molecules for inhibition or co-activation of signaling intermediates.

While it is not yet clear how most cell type specific effects of cytokine signals are controlled, it is very important to note that the cell fate effect of a cytokine will be highly context dependent. Specific effects will only be induced if the cytokine signaling is induced in a cell type with the right composition of molecular components to allow, inhibit, or modulate the signal transduction. The biological function of cytokines can therefore only be analyzed in cell types that physiologically respond to these cytokines, and generalizations of results from one cell type have to be taken with care. In particular, conclusions from studies activating cytokine signaling in cell types not physiologically responding to this cytokine, for example by ectopic expression of the cytokine receptor, might therefore be of limited value.

THE NEED FOR CONTINUOUS MOLECULAR SINGLE CELL ANALYSIS OF CYTOKINE SIGNALING

Molecular analysis in primary stem and progenitor cells suffers from severe technical limitations. The activation of many signaling pathways is marked by phosphorylation events which are often

detected by phosphorylation-specific antibodies in western blots of whole cell lysates or after immunoprecipitation. Although the sensitivity of these methods has increased over the years, they still require large numbers of cells, and are only suited for homogenous cell populations. However, in stem and progenitor cell research, the low number of available cells hinders the usage of classical protein detection methods. Moreover, even after multicolor flow cytometry purification, primary hematopoietic cells are still rather heterogeneous [Rieger and Schroeder, 2008]. For these reasons, methodologies allowing molecular read outs at the single cell level are important for the analysis of heterogeneous populations such as stem and progenitor cells. For RNA quantification, several methods have been established to investigate up to the whole transcriptome of single cells: multiplex single cell reverse transcription-polymerase chain reaction (RT-PCR) and quantitative RT-PCR, as well as efficient in vitro mRNA and miRNA amplification methods for single cell cDNA libraries and microarray analyses [Billia et al., 2001; Miyamoto et al., 2002; Warren et al., 2006]. However, these methodologies kill the analyzed cells and therefore do not allow observation of dynamic molecular behavior over time. In addition, it will ultimately be necessary to observe protein levels and/or activation at the single cell level. Since in contrast to nucleic acids the amplification of the proteome is not possible, and although proteome analysis methods have dramatically increased in sensitivity, the protein amount from tens of thousands of cells is still required. Therefore, alternative methods are essential to follow protein mediated signaling in living single cells. Flow cytometry is a powerful method to quantify protein levels in single cells, provided suitable antibodies are available. Extracellular proteins can be analyzed on living cells, and used to sort and further cultivate living cells (Fig. 3A). Surprisingly, there is still a shortage of suitable antibodies against most murine cytokine receptors. Alternatively, fluorescent labeling of cytokines allows the simultaneous stimulation and detection of cytokine receptors (Fig. 3A) [Lidke et al., 2004]. In addition, robust methods have been developed to detect phosphorylated molecules by intracellular phosphoflow cytometry. Increasing numbers of applicable antibodies now allow analysis of most signaling pathways. Combined detection of activated signaling molecules together with cell type specific antigens allows investigation of cytokine signaling in subpopulations of heterogeneous cell populations [Perez and Nolan, 2006]. The lack of information of subcellular protein localization in flow cytometry could be overcome by developments such as the combination of a flow cytometer and microscope, commercialized as FlowCAM [Sieracki et al., 1998]. However, intracellular detection of antigens with specific antibodies requires cell fixation, preventing observation of the future fate of these cells.

Nevertheless, while the aforementioned approaches allow molecular analysis at the single cell level, they still lack the ability to observe the molecular dynamics of signaling in individual cells over time. Since the sequence and duration of activation of different signaling pathway components is tightly regulated, only continuous observation of these events will allow a full understanding of how combinations of molecular signals control specific cell fates. The transient phosphorylation of STAT factors only minutes after cytokine receptor activation serves as an example of the importance of continuous measurements. In this regard, continuous imaging approaches have the potential to allow the constant observation of both molecular dynamics and cellular behaviors. However, it is important to note that although automated systems have drastically increased the throughput of microscopy, it still is far from the speed of flow cytometry analysis.

MOLECULAR SINGLE CELL TIME–LAPSE IMAGING

Signaling pathways often consist of a cascade of molecular events, regulated in a tightly controlled temporal and spatial order. Individual molecules within these cascades may change their subcellular location, their binding to other proteins and/or their activity. All these features can be detected by fluorescent imaging in real-time, and combinations of different biosensors can enable the visualization of the whole process from the stimulated receptor at the cell surface to the transcribed target gene in the nucleus (Fig. 3). This prospect article aims at illustrating the necessity and the potential of continuous single cell imaging methods to unravel cytokine signaling events at the molecular level. General concepts and some of these biosensors are described in more detail below. Animated illustrations of concepts for monitoring signaling processes in living cells can be found elsewhere [Hahn, 2003].

DETECTING PROTEIN LOCALIZATION

Observing the location of signaling components is an easy way of detecting molecular activation. The activity of enzymes as well as metabolite concentrations can be measured using protein fusions that change subcellular location in response to a signal (Fig. 3E). Phosphorylation of STAT molecules leads to their dimerization and translocation from the cytosol to the nucleus for transcriptional activation. The accumulation of fluorescent-labeled STAT molecules in the nucleus can be used as a measurement of JAK/STAT activation [Weijer, 2003]. Similarly, labeled components of the Wnt signaling pathway, shuttling from the cytosol to the nucleus, have been used to assess Wnt signaling activity in real-time [Bienz, 2002]. Other molecules relocate from the cytosol to the plasma membrane upon receiving their signals: One of the best characterized group of translocation sensors detect various phosphoinositide intermediates. These important signaling mediators lead to the recruitment of a number of downstream signaling molecules to the plasma membrane through their ability to bind these intermediates. The sensors consist of fluorescent protein fusions with binding domains for specific phosphoinositide intermediates such as the pleckstrin homology domains of various enzymes (e.g., PLC, Akt) [Varnai and Balla, 2008]. In a similar approach, the protein kinase Cγ (PKCγ) domain C1 has been used to measure diacylglycerol (DAG) levels at the plasma membrane [Oancea et al., 1998]. The activity of small GTPases Ras, Rap1, Rac1, and Cdc42 could be determined in real-time by tracing their subcellular location [Hodgson et al., 2008]. Upon PI3K activation, protein kinase B (PKB) translocates to the plasma membrane and GFP-tagged PKB has been used to demonstrate this translocation in the response to insulin, platelet derived growth factor, epidermal growth factor, and serum [Andjelkovic et al., 1997]. For these translocation studies, a clear
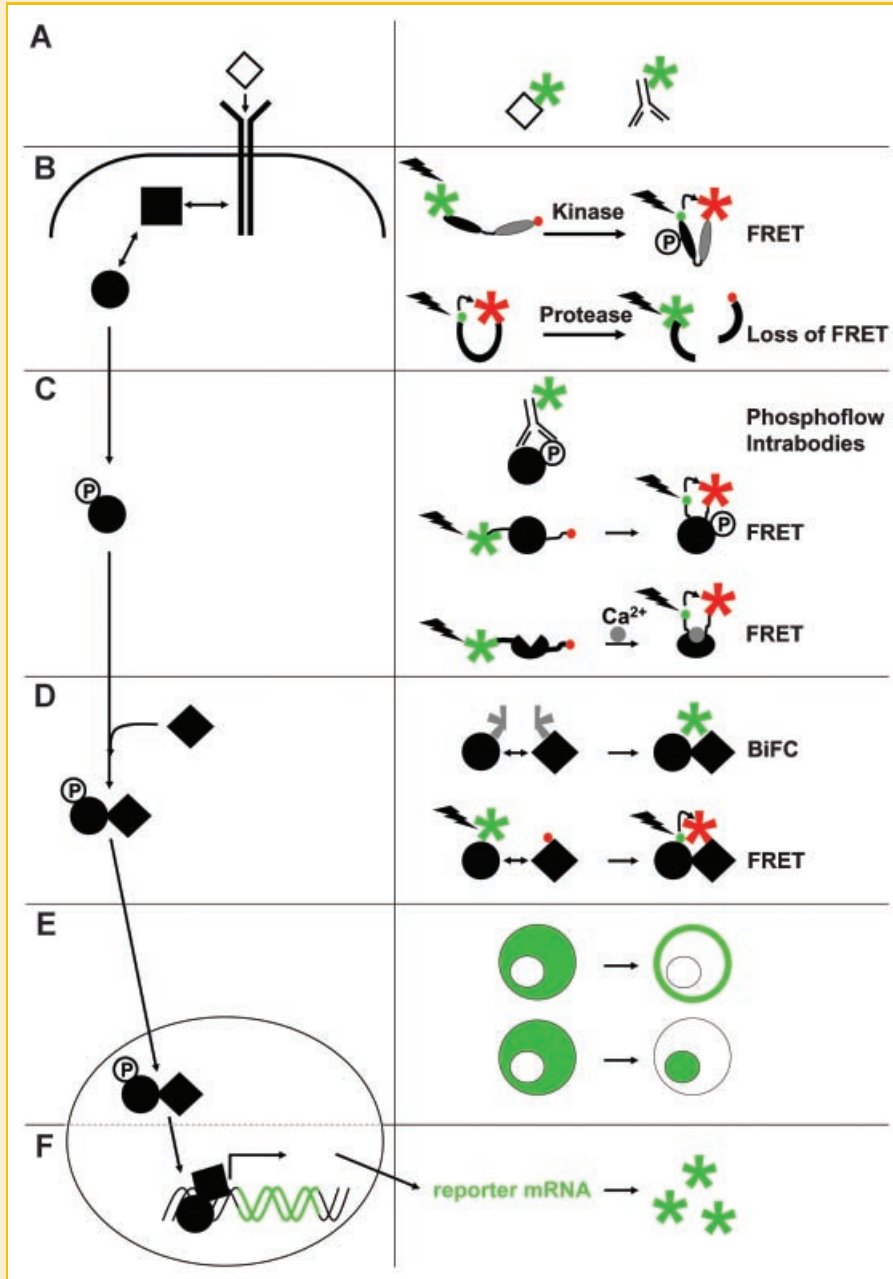
Fig. 3. Approaches for continuous analysis of signaling molecules in single cells. Most of these methods are based on fluorescent molecules analyzed by time–lapse imaging. Individual steps in signal transduction from the receptor binding of the cytokine (A) to the transcriptional activation of target genes (F) can be quantitatively scored with the appearance/disappearance or change of location or intensity of fluorescent signals. (A) Receptor expression and binding, (B) enzymatic activity of signaling components (kinase, phosphatase, protease), (C) protein modification and/or metabolite concentration, (D) protein–protein interaction, (E) subcellular translocation, (F) target gene transcriptional activation.

separation of the fluorescence in the cytoplasm and the membrane must be provided by high enough optical resolution and might require, for example, confocal or total internal reflection fluorescence microscopy.

FLUORESCENT REPORTERS FOR GENE TRANSCRIPTION
Another comparably simple way of detecting the activity of a specific signaling pathway by imaging approaches is the labeling of pathway specific target genes. The expression of fluorescent proteins has been utilized as a real-time reporter for the binding and initiation of transcription of certain transcription factors (Fig. 3F). To facilitate high specificity and high levels of reporter expression a cassette of multiple transcription factor binding sites is usually cloned into a known target gene promoter region, which drives the expression of a fluorescent reporter. Nowadays, constructs for the detection of the most prominent transcription

factors are commercially available. Of note, this method measures the binding of certain transcription factors upon stimulation, but conclusions about the exact signaling pathway may be hard to draw, because different pathways might activate the same transcription factors. Moreover, detection of transcription is delayed by the necessary translation and maturation of visible fluorescent protein. In addition, the use of destabilized forms of fluorescent proteins might be necessary to detect the inactivation of signaling pathways without long temporal delays—with the drawback of decreasing sensitivity of the reporter to levels that may prevent efficient detection.

PROTEIN–PROTEIN INTERACTIONS

Direct contact of proteins facilitates the transduction of signals, mostly in cascades, from the receptor to the cell nucleus where target gene transcription takes place. The binding of proteins can be visualized by bimolecular fluorescence complementation (BiFC) approaches (Fig. 3D). Here, two binding partners are fused with fragments of fluorescent proteins, which by themselves are unable to emit light. Only upon binding of both fragments is their fluorescent ability restored. This approach has also worked successfully with enzymes such as β-galactosidase [Rossi et al., 1997] or dihydrofolate reductase [Pelletier et al., 1998]. BiFC has been used to visualize many different protein interactions, including transcription factor complexes such as Jun and Fos [Hu and Kerppola, 2003] or Myc/Mad/Max [Grinberg et al., 2004], or JAK binding to stimulated erythropoietin receptor [Remy et al., 1999]. In the later example a new mechanism for JAK activation upon cytokine stimulation by conformational change of the preformed dimerized receptor chains was demonstrated. By using combinations of different fluorescent protein fragments with different spectral properties, the interactions between different proteins can be distinguished by specific emitting colors in the same cell simultaneously, and complex formations among alternative interaction partners can be addressed [Hu and Kerppola, 2003].

Protein binding can also be visualized by fluorescence resonance energy transfer (FRET) technology and related methods such as fluorescence lifetime imaging microscopy (FLIM) or fluorescence recovery after photobleaching (FRAP) [reviewed in Okumoto et al., 2008] (Fig. 3D). In FRET, a donor fluorophore fused to one binding partner is excited and the resulting emitted energy is transferred to a suitable acceptor fluorescent molecule on another binding partner, then emitting detectable light of longer wavelength. Only when both binding partners are in close proximity (<10 nm) can FRET occur. FRET has been utilized to visualize, for example, the binding of the EGF receptor and its downstream signal transducing molecule Grb2 [Sorkin et al., 2000], G-protein coupled receptor association [Azpiazu and Gautam, 2004], or interaction of the transcription factors Erg and Jun [Camuzeaux et al., 2005].

DETECTION OF PHOSPHORYLATION

The use of antibodies only detecting specific phosphorylated proteins in combination with live cell imaging initiated the hunt for tools to study protein phosphorylation in real-time (Fig. 3C). In a hallmark study, Bastiaens and coworkers investigated the activation of GFP-tagged epidermal growth factor receptor with Cy3-labeled phosphotyrosine-specific antibodies by FLIM, based on the principle of FRET [Verveer et al., 2000]. Ng et al. [1999] quantitatively measured phosphorylated PKC in living cells expressing GFP-PKCα. After the microinjection of a fluorescently labeled anti-phospho-PKCα antibody the phosphorylation status of PKCs could be correlated to its activation by FRET-based imaging. However, microinjection of phospho-specific antibodies is laborious and invasive. Ideally, information of the phosphorylation status of a protein should be permitted by genetically encoded tools. The expression of fluorescent proteins fused to intrabodies, small antibody molecules often generated from naturally occurring single chain antibodies from camelidae, could be an exciting future optimization of this approach (Fig. 3C) [Rothbauer et al., 2006].

Genetically encoded detection of protein phosphorylation in real-time at the single cell level has been achieved by unimolecular biosensors that are specific for protein kinase/phosphatase activity. These biosensors consist of a specific kinase substrate domain, and a phosphorylation recognition detection domain, both sandwiched between a suitable fluorescent protein pair for FRET (Fig. 3B). Phosphorylation of the biosensor leads to a conformational change allowing efficient FRET activity. The balance between kinase and phosphatase activity in single cells can be visualized with this system. Biosensors have been developed allowing real-time studies of many important kinases such as the insulin receptor, Akt, Src, the epidermal growth factor receptor, and protein kinases A, B, C, and D among other protein kinases [reviewed in Zhang and Allen, 2007]. The ability to target biosensors to a particular subcellular region has revealed valuable information of subcellular-regional differences in PKC behavior [Gallegos et al., 2006].

A different approach uses peptides that bind metal ions in a phosphorylation status-dependent manner. Using chelation-sensitve fluorophores with these peptides allow the detection of altered fluorescence upon phosphorylation and metal incorporation [Turk, 2008].

CONFORMATION SENSING

Changes in protein activity or interaction with binding partners often lead to a change in protein conformation. These changes in conformation can be detected in live cell imaging by different FRET methods. Conformation changes of a protein can be measured by placing the protein of interest in the middle of a FRET fluorescent protein pair. Kinase biosensors have been constructed utilizing the conformational change of the enzyme upon activation (Fig. 3C). As one example, inactive PKC contains a so-called pseudosubstrate, which is released from the enzymatic center upon PKC activation. The enzyme changes from a closed inactive to an open active conformation, which can be observed by changes of FRET activity with appropriate PKC biosensor constructs [Verbeek et al., 2008]. Other examples are biosensors for MAPK, extracellular signal-regulated protein kinase 2 (ERK2), PKB/Akt, or MAP kinase-activated protein kinase 2 (MK2) [reviewed in Zhang and Allen, 2007]. Concentrations of small signaling molecules such as inositol-triphosphate (IP3), cGMP, or calcium ions could be quantified in living cells by conformational changes of their specific binding proteins, which have been modified as efficient FRET biosensors (Fig. 3C) [Nikolaev et al., 2006; Remus et al., 2006; Shcherbo et al.,

2009]. Moreover, real-time quantification of metabolites such as glucose, maltose or glutamate among others can be applied using metabolite-dependent conformation biosensors in living cells [Okumoto et al., 2008].

Circular permutated fluorescent proteins have been engineered by swapping the N- and C-portions of the parental fluorescent protein changing the fluorescent properties of the new molecule. Cloning a calcium sensitive motive within permutated fluorescent proteins resulted in a highly sensitive biosensor for changes in intracellular Ca^{2+}, which regain fluorescence upon Ca^{2+} binding [Nagai et al., 2004].

One of the first fluorescent biosensors that has been invented could determine protease activity (Fig. 3B). Since then several different sensors for protease activity in apoptosis research (determining the activity of certain caspases) and for calpain proteolytic activity in muscle tissue of living animals have been reported [Stockholm et al., 2005].

CONSIDERATIONS WHEN WORKING WITH BIOSENSORS

Biosensors should elucidate intracellular biological processes with minimal disturbance of the system. The amount of biosensor molecules within a cell must be high enough to signal in a high dynamic range for reliable detection, but should ideally be diluted by an excess amount of endogenous products. Substrate indicators as biosensors might be more neutral than other forms of biosensors. However, researchers have to take possible side effects caused by the presence of the biosensor carefully into consideration. Enzymatic reporters as biosensors can overwhelm the pathway by additional signaling activity. Although substrate indicators are less prone for perturbation of the system, they might buffer the level of endogenous substrates. Perturbation of one pathway by a biosensor might lead to artificial enhancement or suppression of related pathways in the cell due to compensatory effects. Another important point is the integrity of the fluorescent indicator. First, the sensor must be expressed in a sufficient amount in the cell. Different promoters with various expression strengths in combination with inducible systems can be utilized to tune the biosensors expression. Structural misfolding of the biosensor can either lead to a non-fluorescent molecule with full enzymatic activity, or vice versa, to a fluorescent molecule with lost enzymatic activity. In the first case, the detected fluorescent signal under-represents the real amount of biosensor stimulation. The non-florescent biosensor population quenches the real signal strength. In the second case, the background fluorescent would be significantly increased, which will lead to a less sensitive detection of real signal in the system. Misfolding can often be altered by using different linker sequences and length. The development of new fluorescent proteins with even shorter maturation times will significantly help to find more suitable fluorescent protein pairs for FRET.

Additionally, more red and infrared fluorescent proteins with longer excitation and emission wavelengths (IFR1.4, Kate, Katushka, Plum, Cherry, taqRFP) will increase the sensitivity for FRET microscopy, and will significantly enhance in vivo applications [Shcherbo et al., 2009; Shu et al., 2009]. The simultaneous use of multiple biosensors covering different signaling pathways will unravel the interplay of complex receptor signaling in high temporal and spatial resolution over time. The combination of multiple fluorescent protein-tagged proteins that naturally interact with each other might allow the quantification of competing protein complexes within a system [Grinberg et al., 2004].

CONCLUSION

Cytokines act in a multifunctional fashion on primary stem and progenitor cells. They influence different aspects of cellular behavior in a context dependent, simultaneous, and sequential fashion throughout differentiation processes. Therefore, cytokine effects on cell fates must be observed continuously in cells in which these cytokines play a physiological role. Modern prospective purification protocols of stem and progenitor populations allow the enrichment of relatively pure populations. However, the remaining heterogeneity within these populations has to be counteracted by analyses at the single cell level which are still challenging at the protein level. Time-lapse molecular imaging in combination with long-term single cell tracking offers one excellent possibility to investigate the influence of molecular signals on cell fates. Signaling cascades from the cytokine receptor binding to the activation of target gene transcription can be observed by fluorescent microscopy based methods using a growing number of available biosensors.

In combination, continuous quantitative observation of cell and molecular behavior at the single cell level will significantly improve our understanding of the intriguing puzzle how cytokines control cell fates at the molecular level.

REFERENCES

Anderson DM, Lyman SD, Baird A, Wignall JM, Eisenman J, Rauch C, March CJ, Boswell HS, Gimpel SD, Cosman D, Williams DE. 1990. Molecular cloning of mast cell growth factor, a hematopoietin that is active in both membrane bound and soluble forms. Cell 63:235–243.

Andjelkovic M, Alessi DR, Meier R, Fernandez A, Lamb NJ, Frech M, Cron P, Cohen P, Lucocq JM, Hemmings BA. 1997. Role of translocation in the activation and function of protein kinase B. J Biol Chem 272:31515–31524.

Azpiazu I, Gautam N. 2004. A fluorescence resonance energy transfer-based sensor indicates that receptor access to a G protein is unrestricted in a living mammalian cell. J Biol Chem 279:27709–27718.

Bienz M. 2002. The subcellular destinations of APC proteins. Nat Rev Mol Cell Biol 3:328–338.

Billia F, Barbara M, McEwen J, Trevisan M, Iscove NN. 2001. Resolution of pluripotential intermediates in murine hematopoietic differentiation by global complementary DNA amplification from single cells: Confirmation of assignments by expression profiling of cytokine receptor transcripts. Blood 97:2257–2268.

Camuzeaux B, Spriet C, Heliot L, Coll J, Duterque-Coquillaud M. 2005. Imaging Erg and Jun transcription factor interaction in living cells using fluorescence resonance energy transfer analyses. Biochem Biophys Res Commun 332:1107–1114.

Eilken HM, Nishikawa S, Schroeder T. 2009. Continuous single-cell imaging of blood generation from haemogenic endothelium. Nature 457:896–900.

Enver T, Heyworth CM, Dexter TM. 1998. Do stem cells play dice? Blood 92:348–351; discussion 352.

Fievez L, Desmet C, Henry E, Pajak B, Hegenbarth S, Garze V, Bex F, Jaspar F, Boutet P, Gillet L, Vanderplasschen A, Knolle PA, Leo O, Moser M, Lekeux P, Bureau F. 2007. STAT5 is an ambivalent regulator of neutrophil homeostasis. PLoS ONE 2:e727.

Fukunaga R, Ishizaka-Ikeda E, Nagata S. 1993. Growth and differentiation signals mediated by different regions in the cytoplasmic domain of granulocyte colony-stimulating factor receptor. Cell 74:1079–1087.

Gallegos LL, Kunkel MT, Newton AC. 2006. Targeting protein kinase C activity reporter to discrete intracellular regions reveals spatiotemporal differences in agonist-dependent signaling. J Biol Chem 281:30947–30956.

Grinberg AV, Hu CD, Kerppola TK. 2004. Visualization of Myc/Max/Mad family dimers and the competition for dimerization in living cells. Mol Cell Biol 24:4294–4308.

Hahn K. 2003. Monitoring signaling processes in living cells using biosensors. Sci STKE 205:tr5.

Hennighausen L, Robinson GW. 2008. Interpretation of cytokine signaling through the transcription factors STAT5A and STAT5B. Genes Dev 22:711–721.

Hibbs ML, Quilici C, Kountouri N, Seymour JF, Armes JE, Burgess AW, Dunn AR. 2007. Mice lacking three myeloid colony-stimulating factors (G-CSF, GM-CSF, and M-CSF) still produce macrophages and granulocytes and mount an inflammatory response in a sterile model of peritonitis. J Immunol 178:6435–6443.

Hodgson L, Pertz O, Hahn KM. 2008. Design and optimization of genetically encoded fluorescent biosensors: GTPase biosensors. Methods Cell Biol 85:63–81.

Hu CD, Kerppola TK. 2003. Simultaneous visualization of multiple protein interactions in living cells using multicolor fluorescence complementation analysis. Nat Biotechnol 21:539–545.

Jelkmann W. 1986. Erythropoietin research, 80 years after the initial studies by Carnot and Deflandre. Respir Physiol 63:257–266.

Levine SJ. 2008. Molecular mechanisms of soluble cytokine receptor generation. J Biol Chem 283:14177–14181.

Lidke DS, Nagy P, Heintzmann R, Arndt-Jovin DJ, Post JN, Grecco HE, Jares-Erijman EA, Jovin TM. 2004. Quantum dot ligands provide new insights into erbB/HER receptor-mediated signal transduction. Nat Biotechnol 22:198–203.

Metcalf D. 1998. Lineage commitment and maturation in hematopoietic cells: The case for extrinsic regulation. Blood 92:345–347; discussion 352.

Metcalf D. 2008. Hematopoietic cytokines. Blood 111:485–491.

Miyajima A, Mui AL, Ogorochi T, Sakamaki K. 1993. Receptors for granulocyte-macrophage colony-stimulating factor, interleukin-3, and interleukin-5. Blood 82:1960–1974.

Miyamoto T, Iwasaki H, Reizis B, Ye M, Graf T, Weissman IL, Akashi K. 2002. Myeloid or lymphoid promiscuity as a critical step in hematopoietic lineage commitment. Dev Cell 3:137–147.

Nagai T, Yamada S, Tominaga T, Ichikawa M, Miyawaki A. 2004. Expanded dynamic range of fluorescent indicators for Ca(2+) by circularly permuted yellow fluorescent proteins. Proc Natl Acad Sci USA 101:10554–10559.

Ng T, Squire A, Hansra G, Bornancin F, Prevostel C, Hanby A, Harris W, Barnes D, Schmidt S, Mellor H, Bastiaens PI, Parker PJ. 1999. Imaging protein kinase Calpha activation in cells. Science 283:2085–2089.

Nikolaev VO, Gambaryan S, Lohse MJ. 2006. Fluorescent sensors for rapid monitoring of intracellular cGMP. Nat Methods 3:23–25.

Oancea E, Teruel MN, Quest AF, Meyer T. 1998. Green fluorescent protein (GFP)-tagged cysteine-rich domains from protein kinase C as fluorescent indicators for diacylglycerol signaling in living cells. J Cell Biol 140:485–498.

Okumoto S, Takanaga H, Frommer WB. 2008. Quantitative imaging for discovery and assembly of the metabo-regulome. New Phytol 180:271–295.

Pelletier JN, Campbell-Valois FX, Michnick SW. 1998. Oligomerization domain-directed reassembly of active dihydrofolate reductase from rationally designed fragments. Proc Natl Acad Sci USA 95:12141–12146.

Perez OD, Nolan GP. 2006. Phospho-proteomic immune analysis by flow cytometry: From mechanism to translational medicine at the single-cell level. Immunol Rev 210:208–228.

Pixley FJ, Stanley ER. 2004. CSF-1 regulation of the wandering macrophage: Complexity in action. Trends Cell Biol 14:628–638.

Remus TP, Zima AV, Bossuyt J, Bare DJ, Martin JL, Blatter LA, Bers DM, Mignery GA. 2006. Biosensors to measure inositol 1,4,5-trisphosphate concentration in living cells with spatiotemporal resolution. J Biol Chem 281:608–616.

Remy I, Wilson IA, Michnick SW. 1999. Erythropoietin receptor activation by a ligand-induced conformation change. Science 283:990–993.

Rieger MA, Schroeder T. 2008. Exploring hematopoiesis at single cell resolution. Cells Tissues Organs 188:139–149.

Rieger MA, Hoppe PS, Smejkal BM, Eitelhuber AC, Schroeder T. 2009. Hematopoietic cytokines can instruct lineage choice. Science 325:217–218.

Rossi F, Charlton CA, Blau HM. 1997. Monitoring protein-protein interactions in intact eukaryotic cells by beta-galactosidase complementation. Proc Natl Acad Sci USA 94:8405–8410.

Rothbauer U, Zolghadr K, Tillib S, Nowak D, Schermelleh L, Gahl A, Backmann N, Conrath K, Muyldermans S, Cardoso MC, Leonhardt H. 2006. Targeting and tracing antigens in live cells with fluorescent nanobodies. Nat Methods 3:887–889.

Schroeder T. 2005. Tracking hematopoiesis at the single cell level. Ann NY Acad Sci 1044:201–209.

Schroeder T. 2008. Imaging stem-cell-driven regeneration in mammals. Nature 453:345–351.

Shcherbo D, Murphy CS, Ermakova GV, Solovieva EA, Chepurnykh TV, Shcheglov AS, Verkhusha VV, Pletnev VZ, Hazelwood KL, Roche PM, Lukyanov S, Zaraisky AG, Davidson MW, Chudakov DM. 2009. Far-red fluorescent tags for protein imaging in living tissues. Biochem J 418:567–574.

Shu X, Royant A, Lin MZ, Aguilera TA, Lev-Ram V, Steinbach PA, Tsien RY. 2009. Mammalian expression of infrared fluorescent proteins engineered from a bacterial phytochrome. Science 324:804–807.

Sieracki CK, Sieracki ME, Yentsch CS. 1998. An imaging-in-flow system for automated analysis of marine microplankton. Mar Ecol Proy Ser 168:285–296.

Sorkin A, McClure M, Huang F, Carter R. 2000. Interaction of EGF receptor and grb2 in living cells visualized by fluorescence resonance energy transfer (FRET) microscopy. Curr Biol 10:1395–1398.

Stein J, Borzillo GV, Rettenmier CW. 1990. Direct stimulation of cells expressing receptors for macrophage colony-stimulating factor (CSF-1) by a plasma membrane-bound precursor of human CSF-1. Blood 76:1308–1314.

Stockholm D, Bartoli M, Sillon G, Bourg N, Davoust J, Richard I. 2005. Imaging calpain protease activity by multiphoton FRET in living mice. J Mol Biol 346:215–222.

Turk BE. 2008. Understanding and exploiting substrate recognition by protein kinases. Curr Opin Chem Biol 12:4–10.

Varnai P, Balla T. 2008. Live cell imaging of phosphoinositides with expressed inositide binding protein domains. Methods 46:167–176.

Verbeek DS, Goedhart J, Bruinsma L, Sinke RJ, Reits EA. 2008. PKC gamma mutations in spinocerebellar ataxia type 14 affect C1 domain accessibility and kinase activity leading to aberrant MAPK signaling. J Cell Sci 121:2339–2349.

Verveer PJ, Wouters FS, Reynolds AR, Bastiaens PI. 2000. Quantitative imaging of lateral ErbB1 receptor signal propagation in the plasma membrane. Science 290:1567–1570.

Warren L, Bryder D, Weissman IL, Quake SR. 2006. Transcription factor profiling in individual hematopoietic progenitors by digital RT-PCR. Proc Natl Acad Sci USA 103:17807–17812.

Weijer CJ. 2003. Visualizing signals moving in cells. Science 300:96–100.

Zhang J, Allen MD. 2007. FRET-based biosensors for protein kinases: Illuminating the kinome. Mol Biosyst 3:759–765.