Multiparameter Imaging for the Analysis of Intracellular Signaling

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In biological experimentation and especially in drug discovery there is a trend towards more complex test systems. Cell-based assays are replacing conventional binding or enzyme assays more and more. This development is strongly driven by novel fluorescent probes that give insight into cellular processes. Target proteins are studied in their natural environment; this gives much more realistic test results, especially with respect to enzyme location and kinetics. However, in the complex environment of cells, many parameters contribute to the performance of the protein of interest. Therefore, it would be desirable to monitor simultaneously as many of the relevant cellular processes as possible. Here, we discuss the possibilities and limitations provided by multiparameter monitoring of cellular events with fluorescent probes. Some novel examples of the use of fluorescent probes and multiparameter imaging are shown.

Introduction

Most of our knowledge in cell biology is based on results obtained from in vitro experiments. The commonly used enzyme and binding assays can be performed with good accuracy and reproducibility. Among other parameters, it is crucial that the protein preparations are sufficiently pure. Then the determination of thermodynamic and kinetic parameters permits direct comparison of results from related proteins or among different laboratories. Nevertheless, monitoring protein performance in the natural environment of living cells could provide a much more realistic look at protein activity. Especially, kinetic parameters might differ largely among different living cells and in particular in different cell lines. Therefore, single-cell analysis allows cellular diversity to be addressed, which is not possible in experiments with larger cell ensembles or under in vitro conditions. Following two parameters in more than one cell or even in two separate experiments ignores cell heterogeneity. For instance, the synchronized oscillation of intracellular calcium levels and protein kinase C (PKC) activity could only be demonstrated by simultaneous measurements in the same cell.[1] Cell-to-cell variability also prevents superposition of transient translocation events due to the individual shape of cells. Therefore, in order to reach temporal and spatial resolution of more than one event simultaneously, experiments within the same cell are mandatory. Other techniques, like flow cytometry, deal with large cell populations and produce little temporal and no spatial resolution.

Fluorescence-based methods are definitely among those most widely used to monitor cellular events.[2,3] The combination of two or more fluorescent methods employed in one single-cell experiment is called a multiparameter or multiplexing experiment.

Early applications of fluorescent probes had to rely on ex vivo labeled proteins that were then microinjected into the cells of interest.[4] However, these days, most detection molecules are genetically encoded fusion proteins based on green fluorescent protein (GFP) or one of its many relatives.[5,6] The diversity of these coral- or jellyfish-derived proteins enables the use of most of the visible spectrum (Figure 1); this is an important prerequisite for multiparameter imaging (see below). Ideally, these proteins should be inert with respect to intracellular events. Intrinsically, however, GFPs tend to form dimers and some of the novel red fluorescent proteins are associated in tetramers. In addition, some varieties are sensitive to pH and anions (e.g., the yellow fluorescent protein (YFP)). A tremendous amount of work has gone into the optimization of fluorescent proteins, resulting in almost neutral constructs with respect to the above-mentioned caveats.[7] The longer end of the spectrum, namely above 600 nm, is still difficult to use with fluorescent proteins, although significant steps have been taken to convert chromoproteins to fluorescent proteins by introducing mutations.[8,9] A disadvantage of GFPs is their size of about 28 kDa, which makes fusions problematic in applications in which steric factors need to be considered.[10]

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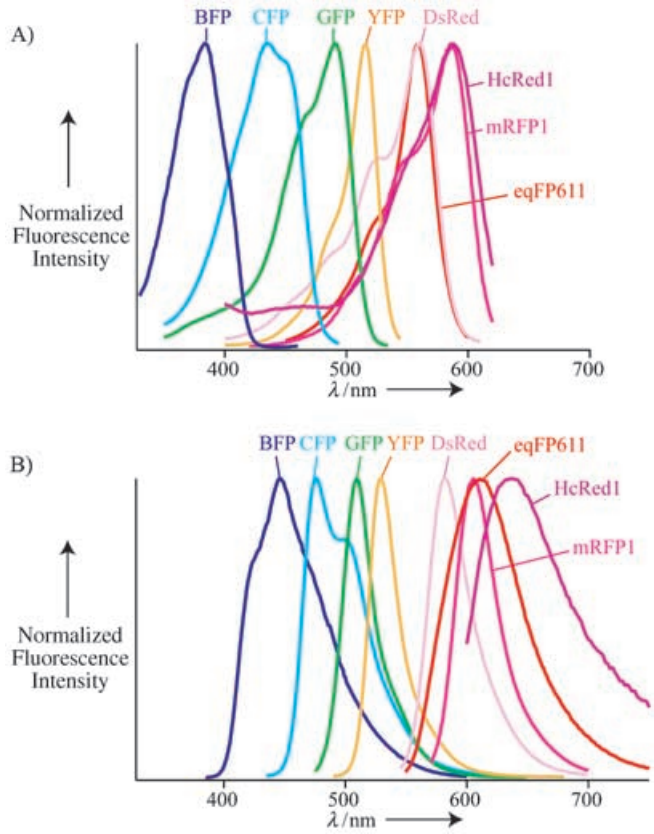

Figure 1. A) Excitation and B) emission spectra of some commonly used fluorescent proteins including the blue fluorescent protein (BFP), cyan fluorescent protein (CFP), GFP, and YFP derived from the jellyfish *Aequorea victoria* GFP,[11] the (tetrameric) red fluorescent protein from the coral *Discosoma* sp. (DsRed),[12] the far red fluorescent protein from the sea anemone *Entacmaea quadricolora* (eqFP611),[13] the monomeric red fluorescent protein derived from DsRed (mRFP1),[7] and the (dimeric) far red fluorescent protein derived from the purple chromoprotein of *Heteractis crispa* (HcRed1).[8] Very recently, the number of available fluorescent proteins has vastly increased.[14–16] Figure adapted from ref. [3] with permission.

FRET Probes

The enormous advantage of most fluorescent probes used in living cells is the high degree of spatial and temporal dynamics that the readout provides. One of the favored designs is based on the relative conformational change of two fluorophores that exhibit fluorescence resonance energy transfer (FRET), for instance, induced by an enzymatic reaction (Figure 2).

Since FRET is very sensitive to changes in distance and orientation, relatively small alterations in the part of the molecule where fluorophores are attached will result in a more or less substantial FRET change, usually in the range of 10 to 100%, in some cases significantly higher (up to 600%).[17,18] Due to the ratiometric nature of the measurement, probe performance should be independent of probe concentration, provided that the two fluorophores are in a fixed stoichiometry, as is the case when two fluorescent proteins are attached to one sensor unit. This ensures that measurements can be performed reliably and that results are comparable between laboratories.

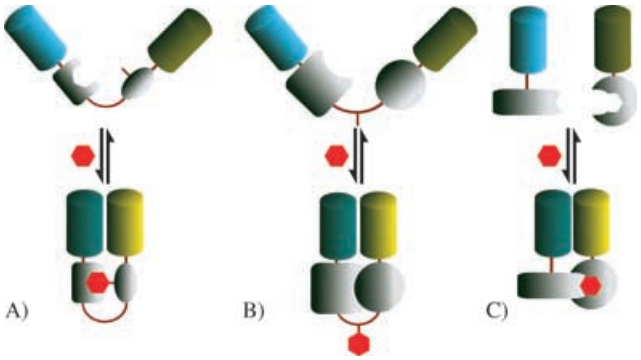

Figure 2. Models of three widely used designs of FRET probes based on conformational changes of the sensor unit. Cyan and yellow barrels depict the FRET donor and acceptor dyes, respectively. A) A substrate unit is attached to a sensor unit. Upon modification of the substrate region, the substrate–sensor interaction is altered; this leads to increased or decreased FRET. B) Two interacting domains change their relative conformations when a substrate loop is modified. C) Bimolecular FRET probes based on two labeled interacting partners. The latter could be employed for protein–protein and protein–ligand interactions. Note that all three designs permit the monitoring of biochemical reactions or binding events in both directions.

Dynamic probes based on FRET (Figure 2) have been developed for phosphorylation/dephosphorylation events (Figure 3),[1,19–25] methylation,[26] heterotrimeric G protein activity,[27,28] receptor occupation,[29] changes in lipid concentrations,[30] ion concentrations,[31,32] second messengers such as inositol 1,4,5-trisphosphate,[33] calcium ions,[34] cAMP,[4,22,35] cGMP,[36] and nitric oxide,[37] as well as protein interaction[38] and oncogene activity,[39] just to name some of the most prominent examples. It is conceivable that there will be FRET probes for most biochemical events in living cells in the future.

What are the limitations? Despite its broad range of applications, standard FRET probes are somewhat limiting when it comes to multiparameter imaging. The regular CFP/YFP FRET pair requires excitation at 440 nm and emission data collection from 470 to 530 nm. This covers roughly 100 nm of the visible spectrum. There is certainly the possibility to add another FRET pair with excitation at 543 nm and emission in the red range, but this would leave us with only two parameters available. At this point, it should be mentioned that the emission spectrum of CFP has a large and undesired overlap with the emission spectrum of YFP.[11] This prohibits easy visual discrimination between direct donor excitation and sensitized emission due to FRET. Furthermore, both CFP and YFP are relatively sensitive to photobleaching. Novel fluorescent proteins with slimmer excitation/emission spectra and higher photostability would be highly desirable. Another solution to cellular imaging might be quantum dots, which have exceptionally slim emission bands and vast photostability.[40] However, due to the preparation of quantum-dot conjugates, microinjection of the structures for intracellular live-cell imaging has up to now been unavoidable. Lower wavelengths in the UV region require fairly expensive lasers and radiation generates substantial cell toxicity. Measurements below 360 nm are usually limited by the lack of light transmittance of the optical equipment. New laser diodes and two-photon excitation are currently replacing old-fashioned UV

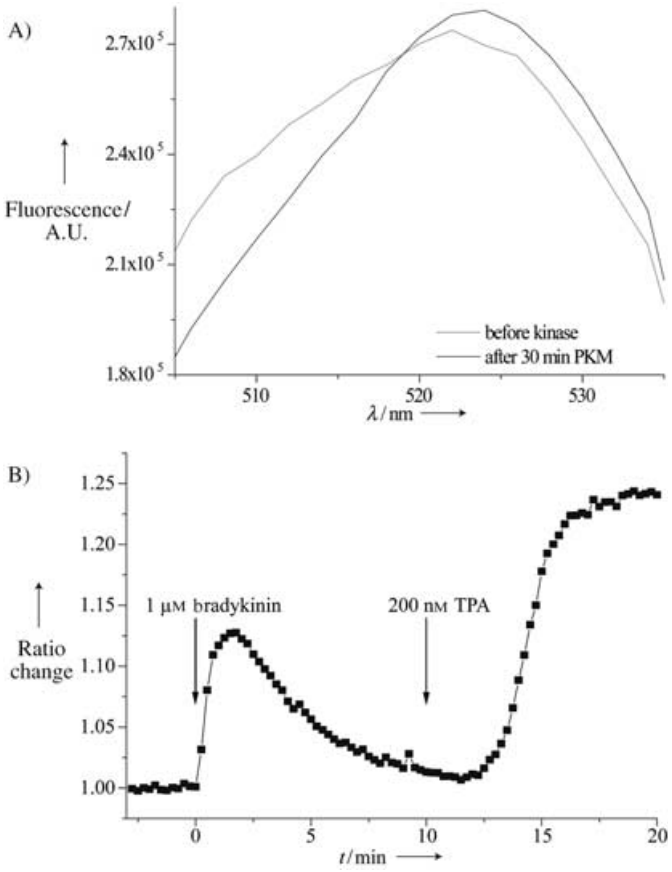

Figure 3. A) Spectra of the recombinant PKC-FRET probe, KCP-1, before and after phosphorylation by a soluble catalytic subunit of PKC (PKM).[19] KCP-1 is constructed as shown in Figure 2B. Specific phosphorylation of the substrate loop by PKC leads to an increase in FRET. The donor is GFP^2; the acceptor is enhanced YFP (EYFP). B) PKC activity monitored over time in N1E-115 cells that are transiently transfected with KCP-1 and stimulated with bradykinin and phorbol ester (TPA).

lasers. A larger reservoir that could extend the usable spectrum is available above 600 nm. In this respect, the recent developments providing a second generation of red fluorescent proteins with emission maxima extending to 648 nm is of great interest, although further red shifts are desirable.

One option for attaching fluorophores to proteins in living cells without bulky additions are biarsenical-tetracysteine labeling systems, which were recently also employed for FRET probes.[41,42] Additional labeling techniques that work in living cells will soon be generally available and will allow for the introduction of small-molecule fluorophores, including those that fluoresce above 600 nm. Again fusion proteins are essential for this technology. One of the best-studied examples involves fusing the 28 kDa alkylguanine transferase (AGT) to the protein of interest.[43–45] When expressed in cells and provided with a suitable substrate, usually 6-*O*-alkylated guanine, AGT alkylates itself by transferring the alkyl group to its own cysteine. When used in FRET experiments with enhanced GFP (EGFP), several acceptor fluorophores were introduced and tested successfully.[46] For FRET applications, it would be particularly interesting to have two independent enzyme-fusion techniques available that enable orthogonal labeling of proteins in living cells. The AGT technology requires significant chemical work for the preparation of guanine-linked fluorophores. Especially linker type and length could vary significantly between fluorophores. Furthermore, the compounds need to be sufficiently permeant to cell membranes so that it is possible to apply the reagents extracellularly and wash away excess dye after the intracellular labeling event. Accordingly, negatively charged dyes often need to be masked by enzymatically removable groups like acetates or other esters.

This technology provides a multitude of applications. For instance, a genetically encoded protein unit could serve as a spatially targeted and defined anchor. Covalent binding of fluorescent probes that monitor intracellular events, such as pH changes or ion fluctuations, would give indicators that perform with largely improved spatial resolution. However, chemistry is required to provide the technical solutions.

Adding Parameters by Including Other Spectroscopic Modalities

One way of establishing multicomponent readouts is to further exploit spectroscopic axes in fluorescence microscopy. One can, for instance, look at multimerization of components by using homo-FRET approaches that require only one detection channel. The contrast for multimerization is then provided by recording the reduction in polarization of the fluorescence emission due to homo-FRET.[47] Also, energy transfer between a fluorescent donor and a nonfluorescing, absorbing acceptor (or quencher) can be used. This approach has found widespread application in molecular-beacon technology, for example, for measuring transcriptional activation or in situ hybridization.[48] Nonfluorescent acceptor dyes for FRET are dabcyl[49] and QSY[50] quenchers, and both have been chemically introduced into proteins. For live-cell experiments, genetically encoded chromoproteins[9] with high absorption but no fluorescence would be similarly useful. Donor quenching due to FRET can be imaged independently from donor concentration or donor/acceptor ratios by using fluorescence lifetime imaging microscopy (FLIM, for reviews on FRET-FLIM see refs. [51–53]). Excited-state decay dynamics in FRET can also be used to discriminate between sensitized emission and direct acceptor excitation. For instance, if a donor is used with delayed fluorescence or phosphorescence (lifetime 100 ns–100 μs) in combination with a short-lived acceptor (lifetime 1–5 ns), FRET induces a sensitized acceptor lifetime of several orders of magnitude higher than the regular acceptor fluorescence lifetime.[54–56] Hence, by using the spectroscopic time axis or polarization axis it is possible to generate contrast within one spectroscopic channel. A third, but not spectroscopically determined option for monitoring stable interactions and using only one spectroscopic signal is the bimolecular fluorescent-complementation approach. This method employs split GFP molecules that have one half fused to one interacting partner and the other half to the other partner. Only interacting species will produce single-channel fluorescence.[57] Even multiple interactions can be visualized with this method by employing

split cyan, green, or yellow proteins.[58] Furthermore, the color of the complemented fluorescent protein reports which interaction is occurring. However, complementation requires a specific orientation of the split GFPs in the complex; this limits the number of interactions that can be monitored. Furthermore, if a fluorophore is formed from the split GFPs, its maturation can take several hours, and after complementation the partners can no longer dissociate. These factors limit the applicability of the complementation technique in monitoring dynamic protein interactions and signaling events.

Full exploitation of the spectroscopic modalities of microscopes in conjunction with the above-mentioned genetically encoded chemical cell labeling together with the arsenal of fluorescent proteins, will further enhance the possibilities for multiparameter imaging. In principle, the design of single-live-cell assays for imaging three or four interaction pairs with little spectroscopic bleed-through, should be possible.

Translocation-Based Probes

Translocation of enzymes is often a crucial part of intracellular signaling. A simple approach to measuring enzyme activity or concentration changes of a particular compound is, therefore, based on fluorescent-protein fusion with proteins that translocate, for instance, from the plasma membrane to the cytosol or vice versa. Similarly, translocation from and to the nucleus can easily be studied with such proteins. Events that have been investigated include phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) breakdown,[59,60] diacylglycerol (DAG) build-up in the plasma membrane (Figure 4),[61] phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3) formation,[62] and changes in intracellular calcium levels.[63] The advantage of this approach for multiparameter imaging is that only a single fluorophore is used per probe. On the other hand, the amount of probe in a given cell might differ significantly from that in another cell. For instance, in cases of high expression levels, the cytosol might not be cleared of a membrane-binding probe because all binding sites are occupied.

Multiparameter Measurements

As soon as the many variants of GFP became available,[64] protein-expression experiments of two (or more) orthogonally labeled proteins were successfully performed.[65,66] More recently, the color palette of fluorescent proteins has been significantly expanded[14–16] thus allowing larger ensembles of different proteins to be monitored simultaneously.

In signal transduction, the advantage of multiparameter measurements would be the immediate recognition of cross-talk within a single or between several signaling pathways. Up to now, there have been few examples of multiparameter experiments in the same cell. An elegant way of combining measurements of PtdIns(3,4,5)P_3 formation at the plasma membrane, with insertion of the GLUT4 glucose transporter into the plasma membrane, was achieved by using dual wavelength evanescent wave microscopy.[67] Very recently, two Ras isoforms were monitored simultaneously by Philippe Bastiaens' laboratory.[68] Three intracellular parameters have also been visualized simultaneously by Sawano and co-workers.[69] Using a novel epifluorescence microscope, Ca^{2+}–calmodulin (Ca^{2+}–CaM), PKC, and their common target, the myristoylated alanine-rich PKC substrate (MARCKS), were observed in single live HeLa cells. In addition, evidence for the interaction of MARCKS and Ca^{2+}–CaM was obtained by FRET measurements.[69] Probes included Fura-2 for calcium measurements and MARCKS–GFP and PKCγ–DsRed fusion proteins for monitoring translocation to and from the plasma membrane. In another study, the connection between epidermal growth factor (EGF) signaling and its effector Ras and tyrosine phosphorylation was investigated.[70] EGF receptor (EGFR) occupation and EGF–EGFR internalization was followed with the help of rhodamine-tagged EGF. Membrane-bound Ras activation was monitored in COS cells that express Raichu–Ras, a reporter that distinguishes between the GDP- and GTP-bound forms of Ras.[39] Alternatively, protein phosphorylation was followed in the same cells by using Picchu-X, a FRET sensor for tyrosine phosphorylation.[71] Both fusion-protein FRET probes function through an altered interaction between a substrate unit and a recognition domain (Figure 2A).

The question arises: how many parameters need to be monitored in order to increase our understanding of intracellular signaling? Surely, we will not be able to monitor all the events that we would like to observe. Therefore, we should investigate certain closely intertwined signaling modules as they

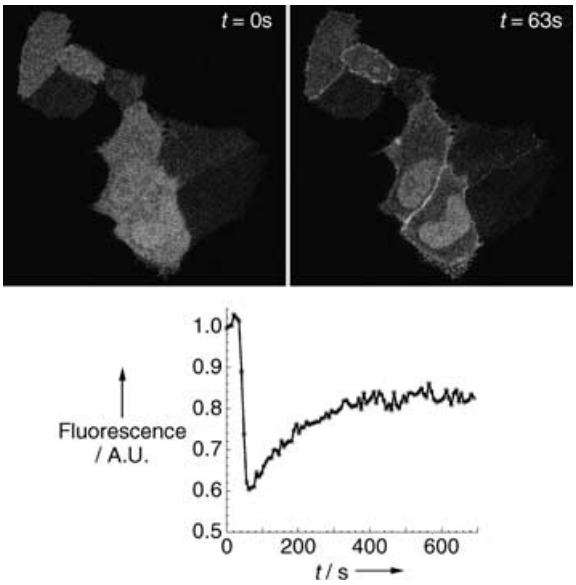

Figure 4. Translocation probes permit monitoring of concentration changes of molecules of interest in different subcellular compartments. Commonly used probes attach to the plasma membrane by recognizing a lipid species, in this case diacylglycerol (DAG), by using the C1a domain of PKCγ (a generous gift from Tobias Meyer). HeLa cells were transfected with YFP–C1a and the bradykinin receptor (BK2), and were stimulated with 1 μM bradykinin. This activated phospholipase C (PLC) as indicated by the translocation of the DAG-binding domain from the cytosol ($t = 0$ s) to the plasma membrane ($t = 63$ s). The formation of DAG by PLC was analyzed over time by quantifying cytosolic fluorescence (see graph). A fast increase in DAG was followed by a slow turnover of the DAG signal.

were mentioned by Tobias Meyer.[72,73] An example would be receptor-mediated PtdIns(4,5)P_2 breakdown and its immediate consequences, such as, DAG formation, calcium release, and protein kinase activation.[61] Two examples where two or three of these parameters are measured simultaneously are shown in Figure 5 and Figure 6. For the first time, translocation sensors are used in combination with a cytosolic FRET probe for PKC. This could serve as a demonstration of how a signaling module can be analyzed in real time. The number of events that can be viewed will be crucial to the effectiveness of the approach. Up to now, we have been able to transiently express a maximum of four fluorescent indicators at about even levels (data not shown). Of course, stably transfected cell lines would help in this respect.

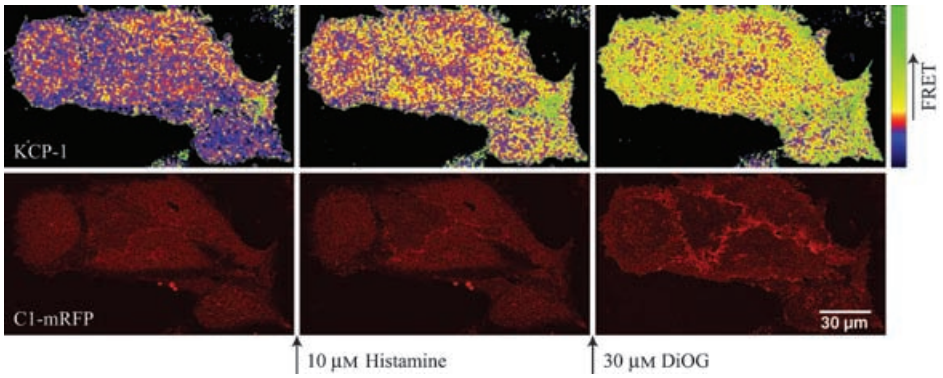

Figure 5. Activation of PKC, measured with the KCP-1 sensor, was combined with the use of a translocation probe that monitors DAG accumulation in the plasma membrane of HeLa cells. Addition of histamine led to a change in the emission ratio of KCP-1, which is depicted by false color (upper panel). A fusion construct of the C1 domain of PKC with monomeric RFP,[7] translocated from the cytosol to the plasma membrane when histamine was added. Subsequent addition of di-*O*-octanoyl glycerol (DiOG), a soluble DAG derivative, resulted in a maximum response of PKC activity and mRFP translocation.

Formulation of Modules and Picking of Markers

The definition of a module largely depends on the questions asked. Usually, a module should be a cluster of events which are closely intertwined, predominantly in the form of tightly coupled reactions.[73,74] For a given signaling pathway, a number of modules will be involved. For instance, receptor occupation and signaling across the plasma membrane will be followed by modules that describe the generation and performance of the primary diffusible components. These include second messengers generated at the plasma membrane and proteins translocating from the cytosol that recognize substituents at the plasma membrane (e.g., phosphorylated receptors or certain lipid species). Subsequently, modules describing the respective effectors, such as kinase or phosphatase cascades or other posttranslational modifiers, small GTPases and their regulators, translocation and activation of transcription factors etc., have to be constituted. Finally, modules are formulated that include cellular responses. Examples for these modules are gene transcription, cytoskeleton remodeling (morphogenesis), cell division, apoptosis, secretion, or cell differentiation. Due to the limited number of parameters that can be followed simultaneously, ideally it would suffice to monitor the performance of a single dominant component within each module. In order to determine this dominant component, each module needs to be scrutinized by fluorescent probes in a multiparameter setup. This includes the determination of kinetic parameters and detailed subcellular localization. Events that are too transient, such as the formation/removal of an intermediate, are usually less suitable for reflecting the performance of a module. On the other hand, components which exhibit low resting levels are particularly suitable as markers because the dynamic range would be sufficiently large to monitor even subtle changes.

After picking markers for each relevant module, their multiparameter imaging should then permit the analysis of entire signaling pathways, ideally from the receptor to the final physiological response in real-time and in one cell. We expect that this kind of biochemical analysis will vastly improve our understanding of complex cellular processes. At the same time, the number of descriptors will remain small enough to easily handle the results. We also expect that this technique will be used in drug discovery processes in the future.

Physiological Readouts

As can be deducted from the above, it is crucial to combine the investigation of signaling networks with studies that address physiological questions. Therefore, relevant readouts need to be provided for physiological responses. Several approaches that have been developed for measuring secretion,[75] changes in membrane potential,[76,77] transcription,[48,78] translocation and trafficking of gene products,[67,79] apoptosis,[80,81] and other events are available. The combination of intracellular monitoring with physiological setups that allow work with patient tissue will be even more challenging.

Quantitative Aspects

Besides generating multiparameter images that provide contrast reports on physiological activities, the challenge will be to generate quantitative readouts of molecular states from these images. For instance, for a translocating biosensor reporting on the presence of a signaling lipid in the membrane, one should be able to quantitatively relate the extent of translocation to the surface concentration of this lipid. Similarly, when using FRET assays the extent of protein phosphorylation

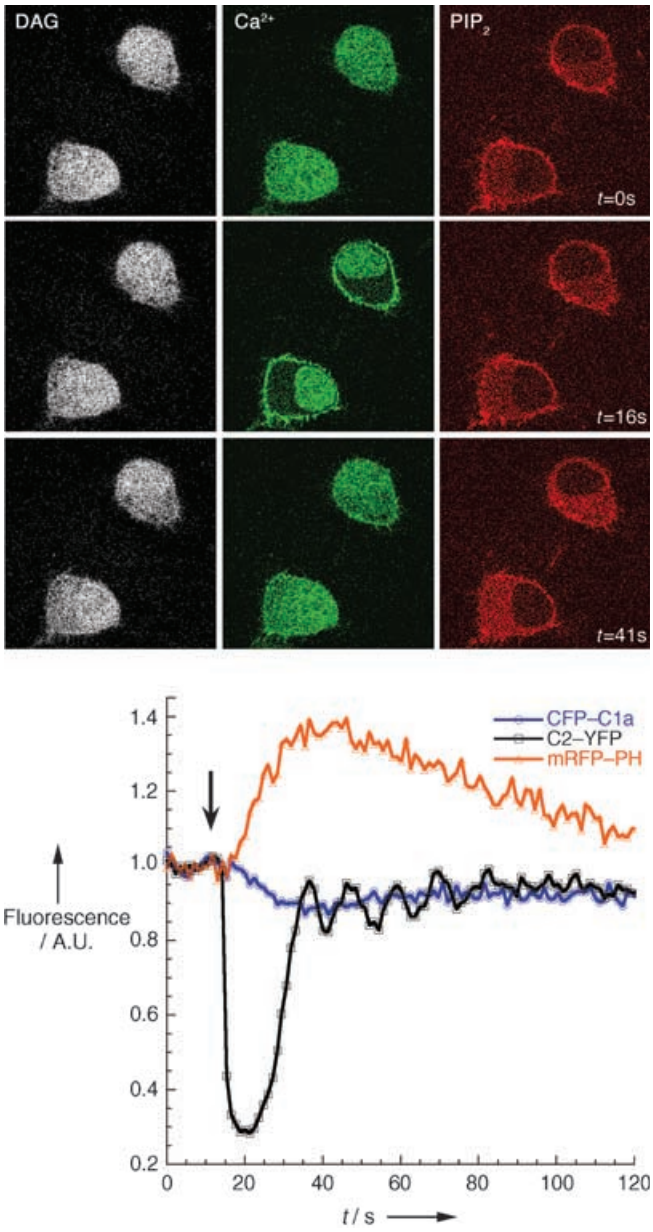

Figure 6. Activation of PLC measured by three parameters in a single living cell. N1E-115 cells were transfected with CFP–C1a, the calcium-sensitive C2 domain of PKC fused to YFP (C2–YFP), and the pleckstrin homology domain of PLCδ1 fused to mRFP (mRFP–PH); DAG, calcium, and PtdIns(4,5)P_2 were then measured. The response of these domains to PLC activation, which was achieved by the addition of 1 μM bradykinin (marked by the arrow), was quantified over time by plotting the cytosolic fluorescence. Clearly, the hydrolysis of PtdIns(4,5)P_2 is paralleled by the formation of DAG. However, the increase of intracellular free calcium released by Ins(1,4,5)P_3 is terminated much faster than the DAG response.

should be quantified in a way that the FRET or ratio image can be converted to reflect the degree of protein phosphorylation in live cells. Besides the obvious demands for simultaneous acquisition of fluorescent signals in multiple spectroscopic channels, the quantitative approach needs calibration of the readouts. For a number of ratiometric sensors such calibrations have been successfully reported (e.g., the calcium sensor cameleon,[34] cAMP sensor,[22] etc.). For nonratiometric sensors like translocating molecules or bimolecular (interaction) FRET sensors, the calibration is much more difficult. Such calibrations will turn out to be especially complicated in multiparameter setups. Yet, we think this multiparameter quantitative data analysis (in 3D: *xy* plus time, or 4D: *xyz* plus time) will enable novel ways to fully understand signaling events or physiological modules in living cells. The data can be used as an input for spatiotemporal in silico models that describe these modules.[82,83] If indeed four or five parameters are imaged simultaneously, whole-cell measurements of spatiotemporal dynamics, which consist of millions of data-points in space and time, will put enormous constraints on the models. We anticipate that these constraints will be instrumental in identifying missing or superfluous components in the models for each of the modules. Systematic experimental perturbation of the modules (e.g., by inhibitors, modulators, RNAi, or photoactivation approaches) could further enhance the constraints on the models.[84]

Conclusions and Outlook

Although a large number of fluorescent probes have been prepared up to now, we are still in the early phase of visualizing intracellular events. However, we can imagine now what could be achieved with upcoming developments. It will, for instance, be possible to look at many more probes and the set of fluorophores in use will be largely extended. The various probes will inevitably be used in combination and it would be advantageous if future probe design could make room for multiparameter imaging.

Will this be suitable for imaging in living animals? As for any imaging that involves "thicker" specimens, longer-wavelength dyes are essential for overcoming limitations by absorbance, light scattering, and tissue autofluorescence. Up to now, large structures like tumors stained with fluorescent proteins or quantum dots have been imaged.[85–89] It will be interesting to see how much of this technology can be adapted to single-cell detection in tissue. Effective measurements will also require improved hardware. In particular, improvements in speed, parallel acquisition, flexibility in usable excitation/emission wavelengths, multimodal acquisition (wavelengths, polarization, and lifetime), automation, standardization of protocols, and ease of programming of complex measurements are very important. Promising developments include imaging devices that provide spectrally resolved data collection and deconvolution of the output.[90,91] At the same time novel visualization tools and data processing tools will be required in order to quantitatively analyze the complex multidimensional image data. It will be interesting to see, how signaling modules will be defined and analyzed. To describe and predict the performance of the multitude of cellular events computational modeling approaches will be essential.[82,83] Multiparameter experiments could validate the resulting models. One challenge in this respect will be the integration of cell heterogeneity and the acquisition of quantitative data. There will probably be scientific dispute about which marker best reflects the performance of a subset of signaling events. Finally, it will be very important to supple-

ment multiparameter imaging with a quantitative readout of the final cellular and physiological response. When the physiological parameters cannot be monitored by using fluorescent probes, new hybrid technology will be required which is currently only partially available. On the other hand, such hybrid technology will even further increase the overall number of monitored events. Patch-clamp rigs attached to fluorescence microscopes are certainly good examples. Other equipment will be needed to measure intracellular and physiological events from patient tissue.

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