Imaging biochemistry inside cells

Fred S. Wouters, Peter J. Verveer and Philippe I.H. Bastiaens

Proteins provide the building blocks for multicomponent molecular units, or pathways, from which higher cellular functions emerge. These units consist of either assemblies of physically interacting proteins or dispersed biochemical activities connected by rapidly diffusing second messengers, metabolic intermediates, ions or other proteins. It will probably remain within the realm of genetics to identify the ensemble of proteins that constitute these functional units and to establish the first-order connectivity. The dynamics of interactions within these protein machines can be assessed in living cells by the application of fluorescence spectroscopy on a microscopic level, using fluorescent proteins that are introduced within these functional units. Fluorescence is sensitive, specific and non-invasive, and the spectroscopic properties of a fluorescent probe can be analysed to obtain information on its molecular environment. The development and use of sensors based on the genetically encoded variants of green–fluorescent proteins has facilitated the observation of ‘live’ biochemistry on a microscopic level, with the advantage of preserving the cellular context of biochemical connectivity, compartmentalization and spatial organization. Protein activities and interactions can be imaged and localized within a single cell, allowing correlation with phenomena such as the cell cycle, migration and morphogenesis.

Fluorescence spectroscopy approaches, previously confined to cuvette-based measurements, are progressively making their way into the field of cell biology. This novel development adds an aspect other than spatial resolution to microscopy – detection of protein activity in the cell. Mainly because of the availability of an ever-increasing range of intrinsically fluorescent proteins that can be genetically fused to virtually any protein of interest, the area of their application as fluorescent biosensors has reached the inner workings of the living cell. The most basic use of fluorescent biosensors is the collection of photons from a cell or tissue to detect the occurrence of a process with temporal resolution. Spatial information expands the usability of biosensors by adding subcellular and supracellular information. On the subcellular level, the read-out of the biosensor can be sampled with spatio-temporal resolution, enabling the morphological dissection of the studied process: for example, what are the organelles or compartments participating and is the process polarized? On the supracellular level, fluorescence imaging of biosensors in a collection of cells allows the determination of cell-to-cell variation. Many studies show biosensor images of multiple cells to demonstrate the repeatability or homogeneity of the process. However, heterogeneity of a process in a cell population can be of considerable biological interest, and the simultaneous imaging of multiple cells has been used to assess the extent of variation in cellular responses. Apart from cell cultures, biosensors can also be used to map processes in multicellular tissues. For example, cathepsin D proteolytic activity was imaged in tumors in situ by the use of probes emitting in the near-infrared, and the localization of Zn²⁺ release was imaged with anatomical resolution in organotypical hippocampal cultures after electrical stimulation. Another payoff of spatial resolution is the possibility to integrate data from different biosensors or other cell-state parameters to gain additional information – for example, on causal connections. The biological machinery inside cells can be investigated by various microscopic techniques and biosensors.

Here, we divide these approaches into three sections: first, the redistribution of biosensors in cells detected by their fluorescence intensity; second, fluorescent indicators that change their intrinsic fluorescence properties as a function of a physiological parameter or chemical reaction state; and finally, sensors for protein interactions.

Imaging fluorescence patterns

A simple but useful design of a biosensor consists of a minimal protein domain fused to green–fluorescent protein (GFP) that interacts specifically with molecules that are transiently generated at specific sites in cells. This allows monitoring of second-messenger generation by imaging translocation of the fluorescent protein molecule. Examples of this type of sensor are those based on lipid-binding domains such as the C1 domains of protein kinase C (PKC), the PA domain of Raf and the pleckstrin-homology (PH) domain of several proteins. After cell stimulation, these three types of sensors translocate to the plasma membrane upon an increase in diacylglycerol (DAG), phosphatidic acid or 3'-phosphoinositide content, respectively. Lipid metabolite production can be quantified by determining the ratio of cytosolic to membrane fluorescence intensity. This approach was pioneered in an elegant study by Oancea and Meyer that showed how signal-transduction processes use the same signaling molecules to achieve different cellular responses. The kinetics of translocation of the PKC DAG-binding C1 domain and Ca²⁺-binding C2 domain were compared with those of full-length PKC. It was shown that signal specificity could be achieved by temporal coordination of Ca²⁺ and DAG signals in PKC activation. Sustained Ca²⁺ oscillations were found to be necessary for the release of the inaccessible ‘clamped’ C1 binding site at the plasma membrane to allow a prolonged activation by DAG binding. A further example where unique insight was obtained with a fluorescent biosensor approach is a study where a minimal PH domain was used to image the generation and distribution of 3'-phosphoinositides in the plasma membrane by...
Box 1. Fluorescence intensity measurements of FRET in a microscope

Interactions between two proteins can be imaged by detecting fluorescence resonance energy transfer (FRET) between donor and acceptor fluorophores (Fig. I). FRET is a photophysical phenomenon where energy is transferred non-radiatively from a donor fluorophore to an acceptor fluorophore with an efficiency defined by:

$$E = rac{R_0^6}{R_0^6 + r^6}$$  \[I\]

where $r$ is the distance between the two fluorophores, and $R_0$ is the distance at which 50% energy transfer takes place (typically 2-6 nm). There are a number of ways to measure FRET in a microscope that can be broadly divided into intensity-based methods and fluorescence decay-kinetics-based methods (see Table 1).

Excitation of a donor fluorophore in a FRET pair leads to quenching of donor emission and in an increased, sensitized, acceptor emission. Intensity-based FRET detection techniques make use of these effects. Because it is possible to measure the donor fluorescence emission specifically without the contaminating leak-through of the acceptor emission, a good way to detect FRET is to compare the quenched with the unquenched donor emission after specific photobleaching of the acceptor fluorophore. An apparent energy transfer efficiency $E_D(i)$ can be defined in each position $i$ of an image, which is equal to the true FRET efficiency in the complex $E$ multiplied by the fraction of donor-tagged molecules, $\alpha_D(i)$, that are in a complex at position $i$:

$$E_D(i) = 1 - \frac{F_D^D(i)}{F_D^p(i)} = E \cdot \alpha_D(i)$$  \[II\]

where $F_D^D(i)$ and $F_D^p(i)$ are the donor emission images before and after photobleaching. The detection of donor quenching is experimentally straightforward because of the intrinsic specificity of the donor fluorescence detection and acceptor photo-destruction. However, this method has limited applicability in living cells because photobleaching requires prolonged illumination during which relocation of the donor-tagged molecules can occur.

Detection of FRET through the sensitized acceptor emission presents a more complex situation than donor-only detection because the measured image must be corrected for leak-through of the donor emission and for direct excitation of the acceptor. The acceptor emission upon specific acceptor excitation, $F_A^A(i)$, and the fluorescence through the donor filter upon donor excitation, $F_D^D(i)$, are measured at each position $i$ of an image. These are used to correct the acceptor emission upon donor excitation, $F_A^D(i)$, to obtain the sensitized emission. Apparent FRET efficiencies, $E_A(i)$, can then be calculated, analogous to Eqn II:

$$E_A(i) = \frac{F_A^D(i) - F_A^p(i)}{F_A^A(i)} = \frac{R_D - R_0 - F_A^p(i) \cdot R_E}{F_A^A(i)} = C \cdot E \cdot \alpha_A(i)$$  \[III\]

where the constant $C$ is the ratio of donor and acceptor brightness, and $\alpha_A(i)$ is the fraction of acceptor-tagged molecules that are in a complex at position $i$. $R_D$ is the ratio of the detection efficiencies of the acceptor filter set. It can be obtained from a sample that has only donor molecules by exciting at a single wavelength and dividing the total intensities detected through the acceptor and donor filters. $R_E$ is the ratio of the extinction coefficients of the acceptor when excited at the donor and acceptor wavelengths. It can be obtained by exciting a sample with only acceptor molecules at the donor and acceptor wavelengths, and dividing the total intensities detected through the acceptor filter. These two ratios are constant factors that are assumed to be spatially invariant in the image. A possible pitfall with this approach is that the quantum yields of donor and acceptor might vary in the sample owing to environmental factors such as pH, compromising the scalar correction factors $R_A$ and $R_E$. In the case where relative concentrations of donor and acceptor are constant at each measurable position in a sample, a ratio measurement of $F_A^D(i)$ and $F_A^D(i)$ can be used to detect FRET qualitatively. This approach should be avoided unless a constant stoichiometry of the donor–acceptor pair can be ensured, as is the case in chimeric constructs where donor and acceptor are attached to the same molecule and changes in FRET occur owing to changes in conformation (e.g. cameleons).

References

f Wouters, F.S. et al. (1988) FRET microscopy demonstrates molecular association of non-specific lipid transfer protein (nsL-TP) with fatty acid oxidation enzymes in peroxisomes. EMBO J 7, 7179–7189
g Bastiaens, P.I.H. et al. (1996) Imaging the intracellular trafficking and state of the AB5 quaternary structure of cholera toxin. EMBO J. 15, 4246–4253
induced chemotaxis of cells. This type of sensor direction of platelet-derived growth factor (PDGF)-
sensors is based on a change in the efficiency of
of membrane 3 compartments by incorporating a specific localization
indicators can be targeted to specific cellular
sequences can be incorporated into the fluorescent
strongly coupled to the bulk pH so that it can be used
as a ratiometric probe. One class of compound
variants, which can be quantified by ratio imaging.
by judicious choice of mutations, a pH-sensing probe
at low pH because of chromophore protonation. Their
different pKa values, ranging from 6.1 to 7.2, lead to
a difference of the relative intensity of the two GFP
variants, which can be quantified by ratio imaging.
By judicious choice of mutations, a pH-sensing probe
consisting of a single GFP mutant has been created
whose spectral properties, unlike native GFP, are
strongly coupled to the bulk pH so that it can be used
as a ratiometric probe. One class of compound
sensors is based on a change in the efficiency of
fluorescence resonance energy transfer (FRET) between a donor and acceptor GFP variant (Box 1)
fused to protein domains or subunits that change
their interaction upon ligand binding. Ca2+-sensing
‘cameleons’ consisting of blue/cyan GFP donor molecules separated from green/yellow GFP acceptor molecules by calmodulin and the M13 calmodulin-binding domain units, are probably the best-known examples of this type of sensor. Ca2+ causes the
connecting units to interact, resulting in compression
of the construct and therefore an increase in the
efficiency of FRET. Since the donor–acceptor stoichiometry of these kinds of constructs is constant,
this change can be reliably detected by the change in
fluorescence emission ratio of the two GFPs (see
Box 1). Similar probes have been designed to detect small organic compounds such as ATP, GTP or
adenosine 3′,5′-cyclic monophosphate (cAMP). Back
in 1991, an indicator for the signaling molecule cAMP
was reported. This sensor was based on a
reconstituted fluorescent enzyme, consisting of donor-labeled regulatory and acceptor-labeled catalytic
subunits of protein kinase A, that was microinjected
into cells. cAMP production leads to dissociation of
these subunits, which is detected by the corresponding reduction in FRET efficiency between the
donor and acceptor fluorophores. Nowadays, a
genetically encoded sensor for cAMP is available were the fluorophores are introduced by fusing the cyan
and yellow variants of GFP. Recently, generic
fluorescent reporters were developed that are based
on the insertion of foreign peptide sequences into an
internal GFP loop. Alternatively, these peptides
can be fused to the new C- and N-termini after
forming a circular permutation of GFP. These
foreign grafts can be either two protein domains that
interact or sequences that, upon ligand binding,
drive a conformational change. Thus, a structural
change in GFP is effected that decreases fluorescence emission from the chromophore, possibly by
decreasing its pKa. So far, this has only been applied
to the detection of Ca2+ ions, but it will not take
long for other single GFP sensors to be developed that
report on the concentration of small molecules such as cAMP or detect changes in phosphorylation or
proteolytic processing.
Complementary to the detection of small
compounds, fluorescent sensors have been developed

---

**Table 1. Different approaches for measuring FRET**

<table>
<thead>
<tr>
<th>Intensity based</th>
<th>Refs</th>
<th>Fluorescence-decay-kinetics based</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor intensity with acceptor photobleaching</td>
<td>34,39,45,48–52,54</td>
<td>Donor photobleaching</td>
<td>36,48–50,54,55,57,58</td>
</tr>
<tr>
<td>Donor intensity</td>
<td>1,56</td>
<td>Donor fluorescence lifetime</td>
<td>26–28,40,50,54,59,60</td>
</tr>
<tr>
<td>Sensitized emission</td>
<td>22,29,35–37,41–45,51</td>
<td>Acceptor lifetime in-growth</td>
<td>23</td>
</tr>
<tr>
<td>Donor–acceptor intensity ratios</td>
<td>16,17,19,24,25,38</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The listed references refer to research that used the approaches given in each row.*

Abbreviation: FRET, fluorescence resonance energy transfer.

---

using GFP fused to PH domains derived from the
ADP-ribosylation factor nucleotide-binding-site opener (ARNO) and the general receptor for
3′-phosphoinositides (GRP1). Spatial gradients of membrane 3′-phosphoinositides correlated with the
direction of platelet-derived growth factor (PDGF)-
induced chemotaxis of cells. This type of sensor
design will certainly be applied to other protein modules – for example to detect protein
phosphorylation by using Src-homology 2 (SH2) or
phosphotyrosine-binding (PTB) domains fused to
GFP variants. It will be interesting to see whether proteins containing multiple phosphoamino-acid-
binding domains are also able to integrate or decode
temporal patterns of protein phosphorylation in
signalling pathways.

**Fluorescence indicators of physiological state or for biochemical reactions**

Chemical sensors for Ca2+ or other ions were probably
the first to use a change of fluorescence property, such
as quantum yield or spectral profile, upon chelation of
the ion. These probes allow the quantitative imaging
of the ion concentration inside cells after calibration
of their fluorescence response in a separate in vitro
experiment. Nowadays, a plethora of molecular
physiological indicators, often based on GFP, are available for measuring H+ concentration,
Zn2+ concentration, Cl− concentration and Ca2+
concentration. In addition, biologically active
sequences can be incorporated into the fluorescent
probe. For example, GFP-based physiological indicators can be targeted to specific cellular
compartments by incorporating a specific localization
signal in the fusion construct.

Fusion constructs containing two GFP variants
allow the determination of pH inside cells and
organelles. The GFP variants lose their emission
at low pH because of chromophore protonation. Their
different pKa values, ranging from 6.1 to 7.2, lead to
a difference of the relative intensity of the two GFP
variants, which can be quantified by ratio imaging.

---

http://tcb.trends.com
activity22–24 is based on a sequence of two variants caspase activities that regulate apoptosis in cells. originating from the interest in the cascade of monitoring proteolytic activity in cells, probably or covalent state. There is a great deal of interest in to report on cellular protein activity or conformational or covalent state. There is a great deal of interest in the cascade of caspase activities that regulate apoptosis in cells. A genetically encoded fluorescent probe for caspase activity22–24 is based on a sequence of two variants of GFP linked by a sequence that is recognized and cleaved by a caspase, thereby abolishing FRET between the molecules. Protein phosphorylation is another important biological activity that has been studied by FRET measurements. To monitor PKA activity in cells, a conformational phosphorylation sensor was made by fusing two GFP variants with the kinase-inducible domain of the transcription factor cAMP-responsive-element binding protein (CREB). Phosphorylation of the sensor by PKA resulted in a reduction of FRET between the GFPs that could be followed by ratiometric imaging in cells25 (Box 1). Because these activity probes are based on genetic fusion of the donor–acceptor pair, this design allows straightforward ratio measurements to be used.

To examine situations where activity is detected by, or arises from, interactions of initially dispersed compounds, a different approach must be followed. Examples are the measurements of the activity of GFP-tagged PKC26 or ErbB127,28, as judged by their phosphorylation state, by detecting FRET upon binding of Cy3-labeled phosphoamino-acid-specific antibodies. In cases such as these, ratio measurements cannot be applied because a fixed donor–acceptor stoichiometry cannot be assumed. However, alternative fluorescence-microscopic methods are available to detect quantitatively the

References


Another FRET-based activity measurement using the diagnostic binding of a reporter molecule to detect the active state of a protein was used for the GTPase Rac. In this study, an acceptor-labeled minimal binding domain derived from the PAK1 kinase (PBD) – that is known to bind to Rac exclusively when Rac is in the GTP-bound (active) state – was used in combination with a Rac–GFP chimera donor. In these experiments, high levels of activated Rac were observed in membrane ruffles, and a gradient of activated protein was seen inside motile cells that coincided with the direction of movement. This implies that a graded, rather than localized, distribution of Rac activation is responsible for directional persistence of cellular motion. This experimental set-up represents an elegant fluorescent analog of a classical biochemical binding study. Here, binding was detected by FRET-induced sensitized acceptor-emission (see Box 1). In this study, an excess of acceptor-PBD is needed to detect all activated Rac proteins. To compare the concentration of active Rac relative to that of total Rac, the sensitized emission would need to be compared with the total amount of fluorescence originating from GFP–Rac in the absence of FRET, which is difficult to achieve with this type of measurement. Furthermore, it would have been possible to use a donor-based method, such as acceptor-photobleaching, or FLIM, to overcome the problems associated with excess acceptor-tagged probe.

**Probes for detecting protein interactions**

As entire sequences of genomes from several organisms, most notably *Homo sapiens* [30,31], are becoming increasingly available, uncovering the functional connectivity of the proteome has become a main effort of modern biology. Interactions are being mapped by large-scale approaches [32] to identify clusters of proteins that perform specific functions. Optical approaches in live cells will be instrumental in this effort, but, mainly due to technological difficulties, they have been applied primarily to detect functional interactions between selected proteins of interest. FRET measurements provide a very useful tool to detect molecular associations of fluorescently tagged proteins as they occur in the living cell. This sets the use of FRET in cells apart from the numerous analytical biochemical and genetic approaches that are currently in use. Accordingly, a clear increase in the use of FRET-based interaction detection is apparent in the recent body of scientific literature. An overview of recent publications, classified by the approach taken, can be found in Table 1. Here, we will discuss the application of FRET approaches in these studies.

Detection of FRET could potentially be used in a proteomics strategy to identify novel binding partners or to establish an interaction matrix between sets of (related) proteins. The obvious attraction of live cell-based interaction screening is that the measurements...
Box 3. Quantitative detection of protein interactions by FLIM

Fluorescence resonance energy transfer (FRET) can be used to image protein interactions (see Boxes 1 and 2). In each resolvable volume element, a mixture of states (bound or unbound) exists (Fig. 1a). The total intensity at each point of an image is a sum of the intensities of both states, weighted by the relative amounts \( \alpha \) (populations) of bound and unbound molecules. In fluorescence lifetime imaging microscopy (FLIM), the measured lifetime is a nonlinear weighted function of the true lifetimes and the populations of each state. A quantitative approach is desirable, where the populations of each species are calculated, along with the true energy-transfer efficiency in the complex. This is feasible with FLIM as the donor fluorophore has different fluorescence kinetics in the bound or unbound states. Recently, instruments have become available that enable the measurement of the individual decay kinetics of bound and unbound species along with their populations\(^{13}\). Until now, these have not been used to determine populations of bound and unbound molecules by FRET, because signal-to-noise issues complicate the measurements. This can be solved using advanced data analysis methods such as global analysis\(^{a}\) that use a priori knowledge of the biochemical system. The fluorescence decay kinetics of each state must be correctly modeled – that is, the correct decay model (single exponential or multi-exponential) for each state, and the influence of the environment in the specimen, must be taken into account in the analysis. In the simplest case, the fluorescence of each state can be assumed to be a single exponential, and the influences of the environment are negligible\(^{1}\). Global analysis then enables estimation of the lifetime of each state along with the relative concentrations in each resolvable volume element (Fig. 1b), without further reference measurements. This allows calculation of the true FRET efficiency in the complex, using the following expression:

\[
E = 1 - \frac{\tau_F}{\tau_D} \tag{1}
\]

where \( \tau_D \) is the lifetime of the donor without FRET (unbound state) and \( \tau_F \) is the lifetime of the donor when FRET occurs (bound state).

References

A smaller-scale approach to study the function of a collection of proteins that constitute a given pathway has been successful. This approach has been applied to map the pathways of nuclear transport factors through the nuclear pore complex. In this study, an interaction matrix between a CFP-tagged importin or exportin receptor with a panel of YFP-tagged nucleoporins was established in living yeast cells. Powerful genetic manipulation possibilities in yeast, including chromosomal gene insertion to prevent overexpression artifacts and the use of a genetic background where function of the fusion protein is required for viability, can make this organism an attractive vehicle for screening purposes. Indeed, two previously reported interactions were detected in the FRET screening, and a novel interaction was verified by co-immunoprecipitation. The connectivity uncovered in this study showed the existence of a substantial overlap, with distinct contact points for the two receptor translocation pathways. In these experiments, FRET was quantified by a variant of the donor/acceptor intensity ratio method (see Box 1), where sensitized acceptor emission and total donor emission are used. This method is only appropriate under conditions where the stoichiometry of the interacting proteins is fixed and known. The validity of protein interactions that were identified in this FRET screen therefore needs to be verified by rigorous correction for non-FRET fluorescence contributions (Box 1) or by using an alternative concentration-independent FRET detection technique.

A number of studies have been designed to prove the existence of expected protein interactions or confirm them in the setting of the living cell—for example, to exclude false-positive or indirect interactions from biochemical approaches. This is essentially a low-throughput proteomics approach. Of course, detection of an unconditional—that is, constitutive—interaction merely establishes the architecture of a multi-molecular particle, even though it provides mechanistic insight. Constitutively oligomerized states were observed for the Fas receptor, where a qualitative sensitized emission-based cell-sorting assay was complemented by microscopic quantitation, and for the EGF receptor as judged by quantitative donor bleaching kinetics and FLIM (Box 2). Constitutive homo-oligomerization was also found for SNARE complexes, showing that significant amounts of stable SNARE complexes exist before synaptic vesicle fusion and that these complexes are assembled during the preceding vesicle docking and priming phase. Interactions between different proteins were detected between the Four-and-a-half LIM-only FHL2 and FHL3 proteins and the apoptosis-regulating proteins Bcl2 and Bax in mitochondria and between Pit-1 and Ets-1 transcription factors in the nucleus. These studies rely on the detection of sensitized emission, but only in the Bcl2/Bax and SNARE studies was the sensitized emission corrected for direct fluorescence contributions (Box 1).

Physiological, biochemical and FRET imaging evidence for the functional light-induced interaction between phyB and cry2 proteins has been presented. Notably, in this study, FRET between the green- and red-emitting spectral variants of GFP was used. Sensitized emission measurements were reported without correction for direct fluorescence contributions, but, because these measurements are susceptible to misinterpretation, the study was complemented by acceptor photobleaching measurements. The use of living cells in this study implies that rapid acceptor-photobleaching was used as, otherwise, labeled proteins would be able to relocate before acquisition of the unquenched donor fluorescence image. In general, care should be taken when using DsRed as an acceptor because it suffers from two properties that limit its use. First, the native form of DsRed is a tetramer. Even though the stoichiometry in fusion constructs is possibly different, specific residues have been identified that mediate homo-association. Second, DsRed matures through a green-emitting intermediate. This emission is generally not observed owing to efficient FRET to mature molecules in the multimeric complex. All DsRed-based FRET observations—intensity- and lifetime-based—will be complicated by this occurrence of intrinsic FRET. The recent publication of the crystal structure of DsRed will hopefully lead to the availability of monomeric DsRed variants.

The association of the nsL-TP lipid carrier with a number of peroxisomal fatty acid β-oxidation enzymes has been monitored by FRET, suggesting that there is an enzyme complex in which nsL-TP performs a substrate-presentation role. FRET imaging between antibodies against the cholera toxin A-subunit and directly labeled pentameric B-subunits has implied that the Golgi serves as the intracellular target and site of toxin disassembly. Separate detection of the holotoxin and its subunits by FRET indicated that the A-subunit is redirected to the plasma membrane, whereas the B-subunit persists in the Golgi after disassembly.

The detection of an inducible interaction can offer a higher level of information, giving more insight into function. The proteolysis of PKCδ induced by phorbol myristate acetate (PMA) in the nucleus represents a signaling event that is part of the PKC-mediated signaling pathway. Recruitment of Gbg to activated EGF receptors can be used as a measure of EGF-induced receptor activation, analogous to the determination of its phosphorylation status. In this case, the sensitized emission should be compared with the unquenched donor fluorescence, which, with the labeling strategy used, would be more easily obtained by acceptor photobleaching.

Monitoring the change of a conditional interaction can be used as a diagnostic read-out for (part of) the activity of a pathway. Many papers have used knowledge derived from analytical biochemistry
approaches to develop a cell-based FRET approach. The Rac activity sensor described earlier is a good example of such a measure of a conditional interaction as a read-out. Additionally, the activation of nuclear hormone-mediated transcription was detected by the interaction of the nuclear hormone receptor with co-activator proteins. Similarly, the interaction of the YFP-tagged co-activators peroxisome proliferator-activated receptor binding protein (PPBP) and steroid receptor coactivator-1 (SRC1) with GFP-tagged retinoic acid receptor (RAR) and estrogen receptor (ER) was used to demonstrate ligand-dependent binding. Agonist and antagonist-dependent FRET effects can be discerned, validating the use of this assay to measure the regulation of the transcription process and to screen for new regulators or ligands.

Outlook
Considering the central biological question of the physiological relevance of observed chemical reactions and the increasing collection of ‘traditional’ analytical assays being translated into cell-based biosensor approaches, it is to be expected that the functional imaging field has yet to reach its final potential. The possibilities for the quantitative and selective detection of biochemical states and reactions of proteins by the various FRET techniques should provide a wealth of data. FRET allows the detailed workings of the protein machines underlying the various cellular processes to be investigated, which would otherwise be compromised by the preparative steps unavoidable in biochemical approaches. Furthermore, visualization of intracellular gradients in enzymatic activities, such as phosphorylation and GTPase activity, can now be related to morphogenetic processes, where the distribution of activity shapes the cellular response. Multiple approaches are at the disposal of the researcher that utilize the different spectroscopic consequences of FRET and will prove to be instrumental in the challenge to uncover the functional connectivity of the proteome. It will be important to analyze the individual cell states defined by multiple biochemical activities as measured by fluorescence imaging and to correlate this with the cellular phenotype or response.

Potential interactions can be predicted from genomic sequences by using computational methods that apply evolutionary criteria to proteins or proteomes. Consequently, the tools for the study of pathways and processes of ever-increasing magnitude and complexity are now becoming available. At the same time, computer simulation of interactions within functional units of the proteome will become important because the increasingly complex physiochemical network will exhibit properties that are not intuitively apparent. Ultimately, cells can be studied as nonlinear systems where variations in responsiveness to the same stimulus are expected within a cell population dependent on the ‘internal protein setting’ of the individual cell. A way of coping with this biological complexity is to improve and extend on the current technology. For instance, simultaneous detection of multiple protein interactions is in principle possible using FLIM. Thus multicomponent analysis of protein processes in cells by fluorescence microscopy will complement classical genetics, bioinformatics and protein biochemistry in order to obtain the full picture of how information is processed in a cell.

References
4. Rizzo, M.A. et al. (2000) The recruitment of Raf-1 to membranes is mediated by direct interaction with phosphatidic acid and is independent of association with Ras. J. Biol. Chem. 275, 23911–23918
This Review by Fred Wouters, Peter Verveer and Philippe Bastiaens is the first in a series of articles on the uses of GFP in a variety of applications and technologies. The next issue of Trends in Cell Biology features a review by Andrew Belmont, ‘Visualizing chromosome dynamics with GFP’. Look out for other articles in the series to follow in subsequent issues.

GFP in Motion 2

All Trends in Cell Biology subscribers will receive a free copy of the ‘GFP in Motion 2’ CD with this issue of the journal. The CD contains a selection of movies of live-cell imaging using GFP-labeled proteins. It has been compiled by Beat Ludin and Andrew Matus and is sponsored by Biolmage A/S (with APBiotech), Chroma Technology Corporation, Clontech, Life Imaging Services, Perkin Elmer and Universal Imaging Corporation. If you are not a subscriber to Trends in Cell Biology and would like to purchase a copy of the CD, please contact: stacey.sheeky@bmn.com

http://tcb.trends.com