REVIEW

Fluorescence and bioluminescence procedures for functional proteomics

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This review aims to provide an overview of current optical procedures used in functional proteomics, investigating protein localization, protein–protein interaction, intracellular signaling events, and second messenger generation in living cells. Reporter assays using proteins tagged with fluorescent or bioluminescent moieties are discussed. Recently, intracellular biosensor assays, flow cytometry-based techniques (fluorescent cell barcoding), as well as transfected cell microarray assays involving RNA interference coupled with automated imaging were introduced and have been adopted as screening platforms for annotating small molecules, investigating signaling events, or in phenotype analysis. These novel methodological advances include improved image acquisition and processing techniques and help linking *in vitro* observations to *in vivo* processes. In addition, the acquired data are increasingly quantitative in nature and will therefore pave the way for modeling of signaling cascades and other complex cellular events, an important step toward systems biology.

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Abbreviations: **ACP,** acyl carrier protein; **AGT,** O^6-alkylguanine transferase; **BRET,** bioluminescence RET; **cAMP,** cyclic adenosine monophosphate; **cGMP,** cyclic guanosine monophosphate; **(E)GFP,** (enhanced) green fluorescent protein; **EGFR,** epidermal growth factor receptor; **(E/M)YFP,** (enhanced/monomeric) yellow fluorescent protein; **(E/M)CFP,** (enhanced/monomeric) cyan fluorescent protein; **Epac,** exchange protein directly activated by cAMP; **ES complex,** enzyme–substrate complex; **FlAsH,** fluorescein arsenical hairpin binder; **FLIM,** fluorescence lifetime imaging microscopy; **Fluc,** firefly luciferase; **FP,** fluorescent protein; **FRET,** fluorescence RET; **GPCR,** G-protein-coupled receptors; **HTP,** high-throughput; **IGF,** insulin-like-growth factor; **NK-1R,** neurokinin-1 receptor; **NO,** nitric oxide; **PCA,** protein fragment complementation; **PIP_2,** phosphatidylinositol-4,5-bisphosphate; **PKA,** protein kinase A; **PTP1B,** phosphotyrosine phosphatase 1B; **RET,** resonance energy transfer; **Rluc,** *Renilla* luciferase; **RNAi,** RNA interference

1 Introduction

As proteins are intricate elements in all aspects of life, discovering their function, determining their expression levels at a given time, and performing quantitative interaction analyses are essential for understanding biology at a systems level. The experimental approaches for fulfilling these tasks vary greatly. Lately, genome-wide experiments opened up the possibility of generating a more complete picture of protein function, but so far mostly on a qualitative basis. Only a small fraction of the proteins encoded by the human genome have been characterized biochemically with respect to their actual function. Moreover, the biological functions of proteins are not only determined by their intrinsic characteristics such as catalytic activities or PTMs, but also by their interaction with other proteins, their regulation as well as their subcellular localization. In particular, the (transient) binding to partner proteins, metabolites or cellular compartments greatly influences protein function and activity. In the case of signal transduction cascades, it has become evident that signal propagation depends on the spatially and tempo-

rally controlled assembly of protein complexes. Disruption of cells during sample preparation often leads to the disruption of signaling units, which is likely to interfere with the signaling output. In addition, also low affinity interactions that can be meaningful at high local concentrations of the interactants inside cells are often missed by *in vitro* methods. On the other hand, after cell lysis proteins might bind to other proteins that they usually will never encounter in the cell under physiological conditions. From the above, it is apparent that *in vitro* and *in vivo* approaches for studying protein function are highly complementary. Thus, the main focuses of this review are examples of optical methods and their applications for addressing spatial and temporal proteomics aspects.

The cellular expression of proteins with different fluorescent tags made it possible to visualize proteins, protein-complex assemblies, second messengers and even enzyme activity in real time and with spatial resolution [1]. Resonance energy transfer (RET) approaches are providing information on the 1–10 nm scale. Imaging by standard fluorescence microscopy is usually limited by the diffraction limits (~200 nm in *xy* dimensions, ~700 nm in the *z* axis), but novel techniques such as stimulated emission depletion (STED), ground state depletion (GSD) microscopy, and related techniques are now closing the gap between the molecular and the optical approaches [2]. High throughput (HTP) microscopy and flow cytometry give insight into how cells manage to regulate complex processes like cell division, motility, migration, or differentiation, often in response to external stimuli.

A major future challenge will be the integration and correlation of the massive data sets obtained by proteomics or imaging approaches. Successful integration of these approaches will ultimately lead to advancements in our fundamental understanding of biological systems as well as drug target identification and treatment of disease.

2 Light emitting probes

2.1 Fluorescent proteins (FPs)

The broad use of fluorescence imaging in cell biology was initiated by cloning of the green fluorescent protein (GFP) from the jellyfish *Aequoria victoria* [3]. Since then, a large number of GFP-related FPs has been used as genetic fusions with proteins of interest to investigate protein expression, localization, and protein–protein interactions. The discovery of other GFP-like proteins (reviewed in: [1, 4–6]) and the application of rational and/or random mutation techniques have lead to an impressive range of FPs with improved spectral properties and a reduced tendency to dimerize or oligomerize, an undesired feature of wild-type FPs [7–9]. Important applications for FPs are genetically encoded RET (see below) and translocation probes. Recently, photoactivatable FPs were developed, providing new possibilities for protein tracking [10–15]. They are especially useful for studying localization dynamics when combined with state-of-the-art microscopic techniques, such as photoactivatable localization microscopy (PALM) in combination with total internal reflection (TIRF) microscopy and by using an electron-multiplying CCD (EMCD) camera capable of detecting single photons [2, 16, 17]. Due to background autofluorescence of living cells, this has been performed only with fixed specimen so far.

2.2 Bioluminescent proteins

Luciferases catalyze the light emitting reactions that produce bioluminescence. The key step is the oxygenation of a substrate molecule (luciferin), whereupon energy-rich peroxidic intermediates are generated. Spontaneous decomposition of these intermediates generates electronically singlet state products that subsequently decay emitting a photon of visible light (reviewed in: [18–21]). Widely utilized luciferases are derived from firefly (*Photinus pyralis*; Fluc; 60 kDa) and sea pansy (*Renilla reniformis*; Rluc; 36 kDa). *Gaussia princeps* luciferase, because of its relatively small size (19.9 kDa) and its higher bioluminescent signal is gaining increasing popularity [22–25]. Fluorescent signals are generally brighter and allow for a better spatial resolution in cells than bioluminescent signals, which greatly limits the use of luciferases for imaging applications. However, the very low autoluminescence of cells and tissues usually results in higher S/Ns for bioluminescent assays, particularly compared with fluorescent imaging in the green to red part of the spectrum. Far-red-emitting luminescent (and fluorescent) proteins [26], improved versions of existing bioluminescent reporters [27] as well as their substrates [28] will allow for more sensitive assays. Importantly, as the photon emitting luciferin molecule is nested in the luciferase enzyme, the catalyzed light emission can lead to a nearly 100% emission quantum yield, which is advantageous for downstream applications like bioluminescence RET (BRET) or genetic reporter assays.

2.3 Chemical labeling approaches

A range of chemical labeling strategies for the coupling of fluorophores to protein tags *in vivo* are available (reviewed in ref. [29, 30]). These tags have in common that they can be labeled post-translationally with a variety of synthetic fluorescent probes. One approach based on human O^6-alkylguanine transferase (hAGT, SNAP-tag™, 23 kDa) that is used to covalently label proteins of interest tagged with the enzyme. AGT catalyses the transfer of a fluorescently labeled benzyl derivative from the O^6-position of guanine to a cysteine to form a stable thioether [31]. Background labeling of endogenous AGT in mammalian cells can be prevented by using AGT-deficient cell lines. Selective labeling of extracellular protein moieties can also be achieved by the acyl carrier protein (ACP [32]) or the related peptide carrier (PCP [33]) method. Both tags are relatively small (8–10 kDa), and have

excellent labeling specificity, however, labeling can only be performed outside of cells, due to the impermeability of the involved phosphopantheine transferase (PPTase) and its substrates, fluorescent coenzyme A conjugates, which are catalytically transferred to a serine residue of ACP or PCP [34]. The HaloTag™ labeling technology was introduced as a tool for imaging cells expressing the HaloTag reporter (33 kDa) fused to a protein of interest. A covalent bond between the fusion protein and a specific haloalkane ligand carrying a fluorescent label is formed rapidly under physiological conditions [35]. Another method exploits the activity of dihydrofolate reductase (DHFR, 18 kDa) to noncovalently bind methotrexate or trimethoprim linked chemical probes [36, 37]. Intein-mediated protein splicing, a self-catalytic process where a intervening intein sequence is excised from a protein to form a native peptide bond between extein sequences, was successfully used to deliver a variety of cargo molecules to a cysteine at the C-terminus of target proteins [38–40], or to reconstitute a full reporter protein, *e.g.*, a fluorescent protein (FP), upon intein-assisted complementation concomitant with the interaction of interest [41, 42].

For some applications, the addition of a large reporter protein to the protein of interest might interfere with its correct targeting and/or function [43]. Small genetic tags, such as the tetracysteine motive Cys-Cys-Xaa-Xaa-Cys-Cys, where Xaa can be any amino acid but cysteine, and optimally proline, and glycine, can be useful in this case. The sequence selectively binds membrane-permeant fluorescent biarsenicals, the fluorescein and resorufin derivatives fluorescein arsenical hairpin binder, green fluorescence (FlAsH), or resorufin arsenical hairpin binder, red fluorescence (ReAsH), respectively, and can be used to observe protein trafficking, assembly, and degradation [44, 45]. The technology and its applications were recently reviewed by Machleidt *et al.* [46]. The commonly used nickel-nitriloacetic acid (Ni-NTA)-His-tag (0.7 kDa) can be used for *in vivo* labeling of recombinant proteins [47, 48]. Ni-NTA derivatized fluorophores reversibly bind to His-tagged proteins, however, cell entry, specificity of labeling, and signal stability may be of concern when applying this method in living cells.

3 RET procedures

The most established homogenous assays to visualize protein–protein interactions, protein conformational changes, or cellular signaling events in living cells are based on RET of fluorescence (FRET) or bioluminescence (BRET). In principle, RET is the phenomenon of energy transfer from a fluorescent or a luminescent donor molecule to a fluorescent or nonfluorescent acceptor in close proximity (~1–10 nm, depending upon absorbance and quantum yield of the light emitting probes) without the involvement of photons [49–51]. This results in an enhanced fluorescence emission of the acceptor fluorophore due to excitation by the donor molecule, accompanied by a reduction of light emission of the fluorescent or bioluminescent donor (Fig. 1). Providing a sufficient overlap of the donor fluorescence emission spectrum with the absorption spectrum of the acceptor molecule, the efficiency of RET is dependent on the molecular distance at an inverse sixth power [50, 51].

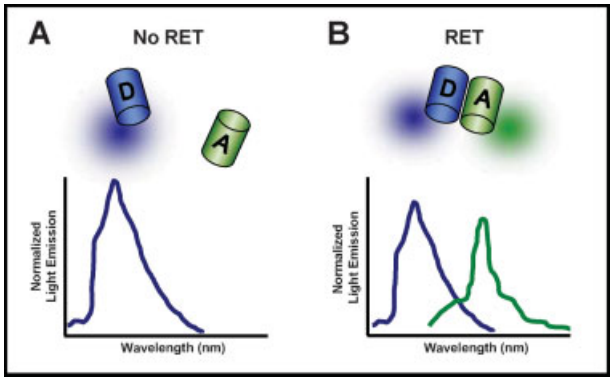

Figure 1. The principle of resonance energy transfer (RET) used for the fluorescence (FRET) and bioluminescence (BRET) methodology. RET requires the spatial proximity (1–10 nm) of a suitable donor-acceptor system with a substantial spectral overlap between donor emission and the acceptor absorption. A, if the distance between a donor (D) and an acceptor molecule (A) is not sufficient for energy transfer to occur, ideally, only the light emission of the donor molecule can be detected (blue spectrum/curve). B, close proximity, *i.e.*, mediated by a specific protein–protein interaction, leads to an energy transfer from the donor to the acceptor molecule that in turn emits light of a higher wavelength (green curve). The energy transfer causes a loss in donor and a gain in acceptor light emission intensity.

Traditional FRET [30, 52–54] and BRET [55, 56] assays monitor the changes in the ratio of the acceptor and donor light emission. FRET requires excitation by an external light source and a fluorescent imaging microscopy setup, whereas the BRET experiments are mainly employed as microplate assays using a multilabel reader with the appropriate filter setup for bioluminescence and fluorescence light emission readout. FRET has a superior spatial and temporal resolution, whereas BRET is easier adaptable to throughput assays. Provided that sufficiently stable luciferase substrates and automated liquid handling systems are used, BRET also allows for temporal resolution. In general, BRET provides a robust, relatively easy to establish assay with a high S/N. The FRET method, to the contrary, may require extensive internal controls to reduce problems associated with fluorescence bleed-through and autofluorescence which vary with the reporters used as well as with the detection method [57]. To circumvent problems of conventional FRET imaging, Elangovan *et al.* [58] published a method for one- and two-photon excitation microscopy implementing a software correction algorithm that allows for the removal of spectral bleed-through and variation in the fluorophore expression level for the donor and acceptor molecules in the same cell the FRET signal is measured, whereas a traditional setup requires signal measurements from several control cells transfected with

donor and acceptor only. Other techniques, such as fluorescence lifetime imaging microscopy (FLIM), which allows to monitor the reduction of donor lifetime upon FRET to another fluorophore or to a nonfluorescent acceptor molecule [59, 60], bypass some of the problems that can be associated with filter-based detection methods. RET can also occur between two GFP molecules of the same kind (termed homo-FRET) and can therefore be exploited to detect the transition of monomeric to higher order complexes inside the cell. This phenomenon is measured by time-resolved fluorescence anisotropy microscopy, which detects the reduction in fluorescence depolarization upon RET [61–63]. Advantages of FLIM, sensitized emission and anisotropy measurements are that only one fluorescence channel is needed for their readout and that they are independent of the concentration of the fluorescent probe. This should enable, in principle, the parallel determination of multiple cellular parameters. A method to track the movements and speed of fluorescently labeled probes inside the cell is fluorescence recovery after photobleaching (FRAP), where intense illumination with monochromatic light is applied to locally destroy fluorophores, which are then replaced by intact molecules diffusing into this area of the cell. Photobleaching is also exploited in photobleaching digital imaging microscopy (pbDIM) which detects the time required to bleach a fluorophore upon irradiation. This technology can be used to detect RET, but so far only in fixed specimen. For further information concerning FRET imaging, we refer to reviews by Suhling *et al.* [64] and Jares-Erijman and Jovin [30], and references cited therein.

RET-based indicators can be subdivided into two types, intra- and intermolecular probes [65]. In intramolecular probes, two molecules that constitute a RET pair are expressed as a fusion protein separated for example by two binding domains, which change their interaction pattern upon stimulation, or by a protease recognition site. As the molar ratio of the two light emitting proteins is fixed, a simple ratiometric measurement is sufficient to determine the RET efficiency. Intramolecular probes are therefore particularly suitable for investigating protein activity, or quantification of a second messenger (*e.g.*, see Fig. 6). Unfortunately, most current labeling and imaging technologies still require high expression levels of the fluorescent or luminescent indicators, which might lead to intracellular crowding or displacement effects [66, 67]. Therefore, the perturbation of cellular events is less likely when employing a monomolecular sensor [65]. However, if for example regulation, scaffolding, or targeting of the proteins of interest is to be studied in more detail, intermolecular reporters – even comprised of full-length proteins – may be desirable (see Figs. 2 and 3B). However, the construction of such a probe can be difficult to perform and the background correction measures that have to be undertaken are more elaborate than for monomolecular RET sensors.

4 RET and protein fragment complementation (PCA) probes for functional proteomics

4.1 Protein–protein interactions

A wealth of RET-based studies investigate the function of G-protein-coupled receptors (GPCR), elucidating their oligomerization, their interaction with β-arrestins and adenylyl cyclases, or G-proteins (Fig. 2), to name a few [68]. BRET was successfully used to study tyrosine kinase receptors (insulin and insulin-like-growth factor-1 (IGF-1) receptors) [69]. The ligand-induced conformational changes of the homodimeric insulin and IGF-1 receptors, the heterodimeric insulin/IGF-1 hybrid receptors as well as the regulation of tyrosine kinase receptors by protein tyrosine phosphatases and molecular adaptors was studied employing BRET. The method was also adapted for HTP screening assays to identify small molecules influencing the signaling of the receptors [69]. Taking advantage of the extracellular ACP-labeling method, Meyer *et al.* [70] could demonstrate that the neurokinin-1 receptor (NK-1R) is present as a monomer at physiological receptor concentrations, by posttranslational labeling of heterologously expressed ACP-NK-1R fusion proteins with different donor/acceptor ratios. FRET was only observed at high expression levels of the receptor, yet too low for stochastic encounters if the receptors were homogeneously distributed throughout the plasma membrane. Using mathematical modeling, it was concluded that the NK-1R monomers reside individually in small microdomains of about 10 nm.

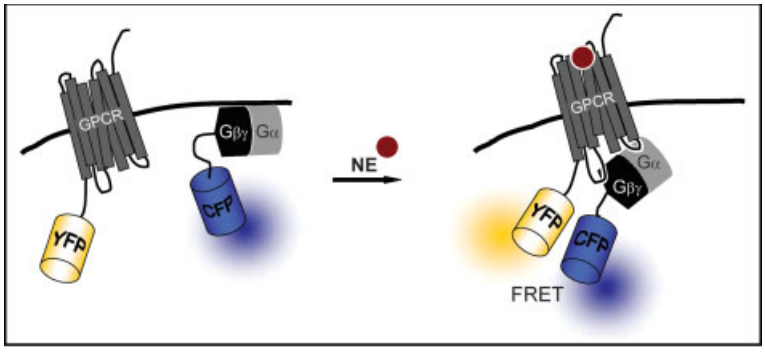

Figure 2. Visualization of protein–protein interactions in living cells exemplified using GPCR [193]. The YFP-labeled GPCR and the CFP-labeled trimeric G-protein co-localize at the plasma membrane, but only upon agonist binding (NE, norephinephrine), protein–protein interaction occurs, which can be monitored by FRET as increase in emission ratio.

In functional studies investigating protein kinase A (PKA) isoforms, Prinz *et al.* [71] and Diskar *et al.* [72] were able to demonstrate that the high S/N of BRET indicators comprised of the full-length PKA subunits is advantageous for investigating the specific regulation of type I and type II PKA holoenzyme dissociation mediated by cyclic adenosine monophosphate (cAMP, Figs. 3A and B, Table 1). Previous attempts to design a FRET sensor based on the PKA type I holoenzyme had failed (M. Zaccolo, personal communication).

An example of how live cell imaging can be used to understand important aspects of cellular events is mitosis. Here, FRET probes of the small guanosine triphosphatase Ran and importin-β [73], as well as tubulin and a regulator of microtubule dynamics, stathmin were used to measure the spatial activity of spindle assembly proteins, allowing to predict their effect on microtubule function [74].

In PCA assays, the N- and C-terminal halves of the reporter proteins are genetically fused to the proteins of interest and reconstitute to form a functional bioluminescent or fluorescent reporter upon the target protein–protein interaction, providing high sensitivity and thus a superior S/N [75]. They are particularly well suited for visualization of protein–protein interactions [76, 77], and for medium to HTP pharmacological screening approaches [115, 116] (Figs. 3C and D). Most of these approaches do not allow analysis of interaction kinetics, but they enable multiparameter investigations and even live animal imaging, as only one light emitting probe *per* interaction event is monitored [78–80]. Recently, the first reversible PCA assay employing Rluc was developed using PKA as a model system, which can be used to detect the dynamic of a PKA holoenzyme in response to pharmacological stimulation [81] (Table 1). A PCA assay based on the 29 kDa TEM-1 β-lactamase of *Escherichia coli* is also circumventing the problem of irreversible reassembly of FPs [82]. The enzyme accepts a variety of substrates, *e.g.*, the chromogenic substrate nitrocefin [83] or the fluorescent substrate CCF2/AM [84]. The conversion of both substrates by the reconstituted β-lactamase leads to an amplified light signal which can be easily detected in living cells and *in vitro*.

4.2 Protein function

The work of Nakamura *et al.* illustrates the practical and rational considerations that need to be undertaken during the construction and optimization of a FRET sensor, here: a probe visualizing the spatial and temporal control of the Ras and Rho family of GTPases (Raichu-probe) [85, 86].

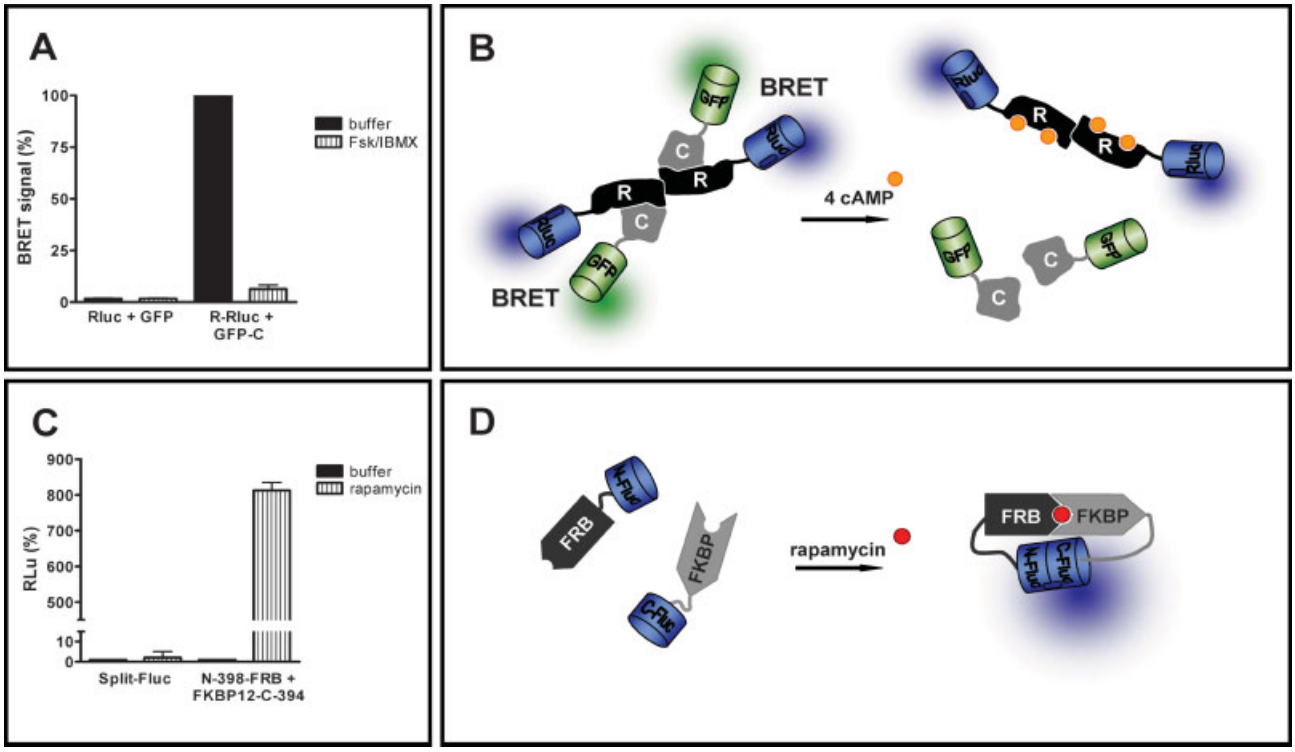

Figure 3. Indicators containing bioluminescent proteins. A and B, Depicted is a BRET-indicator for cAMP-induced holoenzyme dissociation. The bimolecular sensor comprised of RII-Rluc and GFP-C exhibits BRET due to PKA holoenzyme formation when intracellular cAMP concentration is low. Upon stimulation with the adenylyl cyclase activator forskolin (Fsk) and inhibition of phosphodiesterases by 3-isobutyl-1-methylxanthin (IBMX), the cAMP-concentration rises, leading to a complete holoenzyme dissociation in case of the PKA-II sensor [71]. C and D, A PCA assay based on fragmented Fluc allows the detection of rapamycin-induced interaction of FK506-binding protein 12 fused to the C-terminal half (FKBP12-C-Fluc) and FKBP12-rapamycin-binding protein fused to the N-terminal half of Fluc (N-Fluc-FRB) [78].

Table 1. Examples of genetically encoded reporters for cAMP generation

Sensor	Binding-domain	Notes	EC_{50} (cAMP)[a)]	RET/PCA-pair	Ref.
PKA-IIβ	RIIβ-CNBD-A/B	Holoenzyme dissociation leads to loss in FRET	~0.3–0.5 μM	EBFP/GFP CFP/YFP	[121, 122, 192]
PKA-IIβ	RIIβ-CNBD-A/B	Holoenzyme dissociation leads to loss in Rluc PCA	n.d.	Rluc-F1/Rluc-F2	[81]
PKA-Iα	RIα-CNBD-A/B	Holoenzyme dissociation leads to loss in BRET	n.d.	Rluc/GFP^2	[56, 71]
PKA-IIα	RIIα-CNBD-A/B	Holoenzyme dissociation leads to loss in BRET	n.d.	Rluc/GFP^2	[71]
Epac1	Epac-CNBD	Conformational change leads to loss in FRET	~50 μM	ECFP/EYFP	[123]
Epac1	(Mutant) Epac-CNBD	Conformational change leads to loss in FRET	~12–50 μM	CFP/YFP	[124, 125]
Epac1-camps Epac2-camps PKAcamps	Isolated CNBD	Conformational change leads to loss in FRET	~1–2.5 μM	CFP/YFP	[126]

a) Abbreviations: cAMP, cyclic adenosine monophosphate; CNBD, cyclic nucleotide binding domain(s); Epac, exchange proteins directly activated by cAMP; PKA, protein kinase A; R, regulatory subunit; Rluc-F, Rluc fragment; n.d., not determined.

Hoffman *et al*. demonstrate in a comparative study with human adenosine A2a receptors that the receptor downstream signaling (here: coupling to adenylyl cyclase and subsequent cAMP generation) was only possible by employing a FlAsH-CFP FRET pair. The conformation sensitive probe was prepared by inserting a tetracysteine tag, capable of recruiting FlAsH, in the third intracellular loop of the receptor in combination with cyan fluorescent protein (CFP) fused to the C-terminus. Agonist binding to the receptor resulted in loss of RET, concomitant with a conformation change upon ligand binding. When yellow fluorescent protein (YFP) was inserted instead of the tetracysteine tag, FRET changes occurred upon ligand binding, albeit with a much lower S/N and, importantly, without a correct pharmacological response of the receptor [87]. This study impressively demonstrated that introduction of a (large) fluorescent tag can negatively influence downstream signaling or activity of an investigated protein.

4.3 Protein phosphorylation

A variety of FRET-based sensors have been created and were used successfully to monitor kinase specific phosphorylation reactions in living cells [88, 89]. The genetically encoded reporters are monomolecular probes containing a specific phosphorylation site for the target kinase sandwiched between two variants of GFP (see Table 2, and references cited herein). The FRET change (10–50% increase or decrease, depending on the construct) is either induced by binding of a neighboring phosphorylation recognition domain, inserted in frame with the phosphorylation site, or by a conformational change elicited upon phosphorylation of the substrate sequence. Improved S/Ns can be achieved with assays based on synthetic, caged compounds, or peptide substrates with attached ion-chelating fluorophores [89], however, membrane permeability would make these sensors much more valuable. Recently, development of the pleckstrin-based PKC sensor KCP-1 [90] resulted in an intracellular dual parameter reporter (KCAP-1), which is capable of monitoring PKC and/or PKA activity in one experiment, sensed by independent phosphorylation sites. PKC activity is followed by an increase whereas PKA activity results in a decrease in FRET efficiency [91]. An example of successfully applying short peptide fluorescent substrates for PKA in living cells was presented by Higashi *et al*. [92].

4.4 Protein degradation

Proteases are involved in important processes such as protein maturation, remodeling of the extracellular matrix, blood clotting, and the immune system. They have been implicated in numerous pathologies, for example, cardiovascular diseases, cancer, neurodegenerative diseases, diabetes type II, inflammation, cystic fibrosis, and infectious diseases, such as AIDS and hepatitis C, and they are recognized as important drug targets. Sensors for intracellular protease activity display a simple readout particularly suitable for screening approaches because they are composed of a specific protease recognition motif flanked by two fluorophores; thus protease activity is followed by a sharp decrease in the FRET or BRET signal [93–96]. Other sensors include substrates for matrix metalloproteases, caspases, and cathepsins [97, 98]. Most proteases are extracellular acting proteins. However, some sensors for caspases [99, 100] and papain family cysteine proteases [101] were designed to act in living cells. Some probes even work in whole animals [102, 103].

Table 2. Genetically encoded kinase-activity sensors employing FRET or circular permutated FPs

Kinase	Sensor	Phospho site(s)	Binding domain	FRET pair or cpFP	Ref.
PKA	AKAR	LRRASLP	14-3-3τ	ECFP/EYFP	[177]
PKA	AKAR2	LRRATLVD	FHA1	ECFP/EYFP	[178]
PKA	AKAR3	LRRATLVD	FHA1	CyPeT/YPeT	[179]
PKA	ART	EILSRRPSYRKILNDLSSD (CREB)	–	BGFP/RGFP	[180]
PKA	KAP-1	Mutant-PH-DEP (Pleckstrin) RKSLRRASLG	–	GFP^2/EYFP	[91]
PKB (Akt)	Aktus	RGRSRSAP	14-3-3η	ECFP/EYFP	[181]
PKB (Akt)	BKAR	RKRDRLGTLGI	FHA2	MCFP/MYFP	[182]
PKC	CKAR	RFRRFQTLKIKAKA	FHA2	MCFP/MYFP	[109]
PKC	KCP-1	PH-DEP (Pleckstrin)	–	GFP^2/EYFP	[90]
PKC/PKA	KCAP-1	PH-DEP (Pleckstrin) RKSLRRASLG	–	GFP^2/EYFP	[91]
PKD	DKAR	LSRQLTAAVSE	FHA2	MCFP/MYFP	[183]
Abl/EGFR	Picchu	PYAQP (CrK)	SH2 (Crk)	CFP/YFP	[184]
Abl	Crk-based	PYAQP (CrK)	SH2 (Crk)	ECFP/EYFP	[185]
IR	Sinphos	ETGTEEYMKMDLG	SH2 (PI3-K)	cpECFP cpEGFP cpEYFP	[186]
IR	Phocus-2pp	ETGTEEYMKMDLG	SH2 (PI3-K)	CFP/YFP	[187]
ERK	Erkus	KRELVEPLTPSIEAPNQALLR	FHA2	MCFP/MYFP	[188]
ATM	ATOMIC	LETVSTQELYSI	FHA2	MCFP/MYFP	[189]
EGFR	EGFR-rep.	EEAEYMNMAPQ	SH2 (Shc)	ECFP/EYFP	[185]
EGFR	FLAME	EGFR autophosphorylation	PTB	ECFP/EYFP	[190]
Src	Srcus	EIYGEF	SH2 (Src)	ECFP/EYFP	[185]
Msk-1	Histone-P	ARTKQTARKSTGGKAPRKQ-LATKAARKSAP (Histone 3)	14-3-3τ	CFP/YFP	[191]

Abbreviations: Abl, abelson tyrosine kinase; ATM, ataxia telengiectasia mutated; CREB, cAMP response element-binding protein; cpFP, circular permutated FP; EGFR, epidermal growth factor receptor tyrosine kinase; ERK, extracellular signal-regulated protein kinase; FHA, forkhead-associated domain; IR, insulin receptor tyrosine kinase; Msk-1, mitogen-activated stress kinase 1; PH-DEP, pleckstrin homology, disheveled, Egl-10, pleckstrin domain; PTB, phosphotyrosine-binding; Src, SH2, Src-homology 2; PKA, protein kinase A; PKB (Akt), protein kinase B; PKC, protein kinase C; PKD, protein kinase D.

FRET-capable small organic molecules have been used as enzyme-sensitive probes to measure for example phosphorylation or esterase activity *in vitro* [104]. Only a few not genetically encoded but membrane-permeable reporters for studies in living cells have been introduced, including a β-lactamase probe for reporter gene assays [105], peptide-based caspase sensors [100], and probes that measure phospholipase A2 activity [106, 107].

4.5 Local protein activity

After being able to determine the number of proteins and possibly even locating the protein to certain areas within a cell, it is still not clear, whether these proteins are in an active state. Even if the state of PTMs is known, this will only be a hint toward the activity status of the protein of interest. A safe answer would be provided if we knew about the ability to form enzyme–substrate (ES) complexes. Very recently, a probe able to monitor ES complexes was introduced for the phosphotyrosine phosphatase 1B (PTP1B) [108] (Fig. 4). The technique is based on FLIM with the GFP- or YFP-labeled PTP1B serving as a FRET donor and an artificial cytosolic phosphopeptide carrying the FRET acceptor. In the cellular setup chosen, the peptide was dephosphorylated by the phosphatase resulting in a decrease of ES complex formation. However, the peptide was also a substrate for a receptor tyrosine kinase (here: the epidermal growth factor receptor, EGFR). This rephosphorylation resulted in a steady state of phosphorylated *versus* dephosphorylated peptide, which showed local differences. In areas where the probe exhibited short fluorescence lifetimes, corresponding to higher amounts of ES complex; little phosphatase activity may be assumed. Long fluorescence lifetimes corresponded to less ES complexes and hence higher enzyme activity. An alternative to this brand new approach would be to stably locate enzyme sensors (or any other sensor) at various intracellular areas of the cell [109, 110]. Simultaneous monitoring of enzyme performance would then provide a map of spatially resolved activities.

4.6 Protein translocation probes

Protein translocation is an important factor in cell physiology, because many molecules need to be targeted to a certain area of the cell in order to exhibit their specific function. For instance, transcription factors need to enter the nucleus for

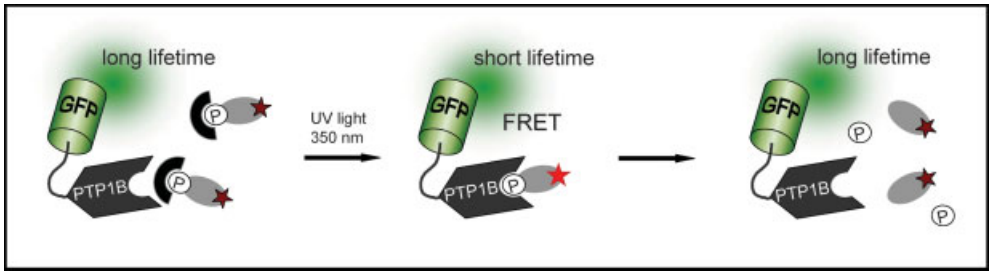

Figure 4. Monitoring local enzyme activity by spatially resolved detection of ES complex formation [108]. Protein tyrosine phosphatase PTP1B terminates growth factor signal transduction by dephosphorylation of receptor tyrosine kinases. PTP1B was fused to a donor chromophore (EGFP). A synthetic, photoactivatable phosphotyrosine peptide substrate was equipped with an acceptor fluorophore. The photolabile chemical protection group of the substrate hinders the substrate to bind to the active site of the enzyme until its photolytic removal induces protein-peptide interaction, resulting in a drop of EGFP emission lifetime. After dephosphorylation, dissociation of reaction products occurs, and FRET is impossible.

initiating mRNA synthesis. This change in location is frequently used to monitor the initiation of signaling pathways by simply expressing a fluorescent fusion protein with transcription factors such as signal transducer and activator of transcription (STAT) 3 [111, 112], or nuclear factor kappa B (NFκB) [113]. Similar applications were developed for Ran GTPases, offering the possibility to study nuclear import and export [114]. One caveat of this method is that additional protein activity is introduced to cells, unless a nonfunctional mutant is employed. For other applications of translocation probes, single nonfunctional domains are used. These often recognize ions or lipids and translocate to and from membranes if changes in the ligand concentration occur (Fig. 5). A prominent example is the C1 domain of protein kinase C which translocates to the plasma membrane when diacylglycerol (DAG) is present [74]. DAG levels rise transiently after hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) or activation of phospholipase D plus lipid phosphate phosphatases (LPP) or phosphatidic acid phosphohydrolase (PAP). Apart from single domains, entire enzymes fused to FPs are frequently used as sensors, too. In fact, the kinetic of full-length PKCα translocation was shown to be independent of expression levels ([115]; Reither and Lipp, unpublished results), demonstrating that enzyme and domain may both be equally used. The advantage of using translocation probes is that a single fluorophore suffices as readout of the dynamic protein movement. This is especially desirable in multiparameter imaging, when multiple sensors are used in the same cell [116]. The majority of lipid binding domains employed so far have been directed to recognize higher phosphorylated phosphoinositides such as (PIP_2) and phosphatidylinositol-3,4,5-trisphosphate (PIP_3) [117, 118]. In some cases, cotranslocation of several different proteins was used in FRET approaches to demonstrate protein–protein interaction. For instance, annexin A4 forms stable and nondynamic assemblies at the inner leaflet of the plasma membrane when intracellular calcium concentrations reach pathologically high levels (Fig. 5). This assembly is readily monitored by simultaneously expressing ECFP and EYFP fusions of annexin A4 [119]. In summary, translocation probes are excellent alternatives to RET probes, provided that the fraction of translocating molecules is high. Especially for imaging changes in lipid concentrations, fluorescently tagged lipid binding domains are the only means currently available. Quantitative data can be obtained, if the overall number of fluorescent molecules is known and rationing of fluorescent intensities in different areas of the cell such as nucleus versus cytoplasm is possible.

4.7 Quantification of second messenger molecules

The proteins arguably enjoying the most attention are kinases, while on the small molecule level these are the second messengers. Various inter- and intramolecular sensors probing second messenger levels in intact cells have been introduced in the past two decades, pioneered by Adams *et al.* [120], who used chemically labeled full-length PKA subunits to visualize the intracellular generation of cAMP after microinjection. Later, full-length PKA subunits were genetically fused to variants of GFP and Rluc for FRET [121, 122] and BRET analysis [71] (Figs. 3A and B, Table 1), respectively. These RET-based sensors visualized for the first time cAMP microdomains with high local concentrations of the second messenger in simple fibroblasts and cardiac myocytes. Targeted disruption of PKA binding to A-kinase anchoring proteins abolished the sensitivity of the sensors, indicating the importance of correct subcellular positioning of the kinase. However, for sole determination of cAMP levels in intact cells, the above-mentioned indicators suffer limitations in sensitivity and temporal resolution as they display complex activation kinetics due to the cooperative activation mechanism of the heterotetrameric holoenzyme of PKA upon cAMP binding. Thus, monomolecular sensors comprised of the full-length sequence of the protein exchange proteins directly activated by cAMP (Epac) sandwiched between CFP and YFP were developed [123–125] (see Fig. 6, Table 1). Epac consists of a cAMP binding domain that regulates its functional activity, the GTP loading of the small G protein Rap1.

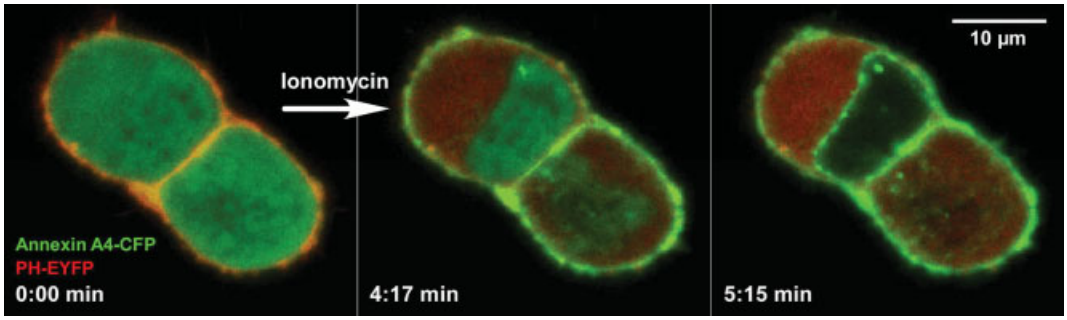

Figure 5. Two translocation probes working simultaneously. After elevating intracellular calcium levels in N1E neuroblastoma cells with the ionophore ionomycin, calcium-triggered phospholipase C activity leads to PIP_2 breakdown. This is monitored by the release of the PIP_2-binding EYFP-tagged PH domain from the plasma membrane (orange pseudocolor). At the same time, Ca^{2+}-sensitive translocation of annexin A4-CFP to the plasma membrane is visualized by the complete clearing of the cytosol (green pseudocolor). Note that annexin A4 also translocates from the nucleosol to the inner leaflet of the nuclear membrane, however, with a significant time delay [119]. Confocal images were taken on a Leica SP2 with a 63 × objective by Alen Piljić, EMBL Heidelberg.

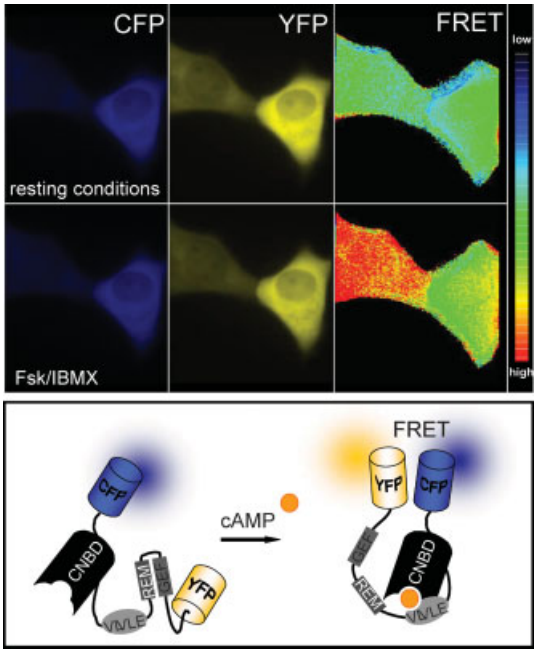

Figure 6. Visualization of second messenger generation using an Epac1-derived cAMP sensor [124]. The intramolecular FRET sensor CFP–Epac1(δDEP-CD)–YFP (blue and yellow pseudocolor) based on the Epac1 protein can be used to quantify the increase in intracellular cAMP upon stimulation of adenylyl cyclases by forskolin (Fsk) and inhibition of phosphodiesterases by IBMX. Depicted in the bottom panel is a schematic drawing of the sensor, which exhibits FRET upon binding of cAMP, due to a conformational rearrangement. FRET imaging was performed as published previously [125]. The images were kindly provided by M. Zaccolo and M. Berrera.

Epac-derived indicators display a fast activation kinetic, albeit at the expense of some sensitivity due to the lower cAMP binding affinity. Two groups independently demonstrated that hormone-induced cAMP oscillations in the physiological concentration range can be monitored with high temporal resolution using sensors that are comprised of isolated cAMP binding domains of Epac or PKA solely [126, 127]. To image cAMP and PKA activity oscillations in rat retinal explants, Dunn *et al.* [128] employed the various types of FRET sensors detecting cAMP generation and phosphorylation by PKA [128].

An evolution of genetically encoded indicators has also taken place for the second messenger cyclic guanosine monophosphate (cGMP), where the first two sensors were based on almost complete cGMP-dependent protein kinase (PKG) lacking only the dimerization domain [129–131]. More recently, isolated domains of either PKG or cGMP-dependent phosphodiesterase 5 were employed for the construction of cGMP-sensitive FRET probes [132].

A variety of synthetic small fluorescent probes and genetically encoded fluorescent indicators for Ca^{2+} have been developed in the past. For a detailed comparison, please refer to [86, 133, 134]. Reiff *et al.* [135] tested the usability of ten different genetically encoded Ca^{2+} indicators for non-destructive investigation of neural activity in living *Drosophila melanogaster* larvae. Eight reliable genetic Ca^{2+} indicators for the imaging of neural activity in presynaptic terminals of the larval neuromuscular junction were identified and tested for overall fluorescence signal amplitude, signal kinetics, and S/N levels. New and improved sensors were identified: GCaMP1.6 (based on calmodulin as the Ca^{2+} sensor and circular permutated EGFP) gave highest fluorescence signals under resting conditions and sensed high neural activity with the largest and fastest fluorescence changes. TN-L15, an indicator using troponin C as the Ca^{2+} binding element sandwiched between CFP and Citrine, showed the fastest rise of all indicators at lower rates of nerve activity and had simpler signal kinetics compared to the calmodulin-based indicators employed in this study [135, 136]. Troponin, a specialized Ca^{2+} binding protein, is not involved in signal transduction processes like calmodulin and thus should only minimally interfere with other cell components. An improved version of TN-L15, TN-XL was developed by

reducing the contaminating affinity of the sensor to Mg^{2+} and by substituting Citrine by a permuted variant of the FP [137]. Recently, an Ca^{2+} indicator called CaGF (calcium green coupled to FlAsH) was published, combining the advantages of a targeted small tetracysteine tag with a small molecule Ca^{2+} chelator, which generally exhibit faster Ca^{2+}off-rates than genetically encoded sensors based on Ca^{2+} binding proteins [138]. It should therefore have superior temporal and spatial resolution necessary to monitor localized spikes of the ion.

Sato *et al.* [131] developed an amplifier-coupled FRET-based indicator for nitric oxide (NO), with a detection limit down to the picomolar level. In this elegant approach, a cGMP sensor developed earlier [131] was covalently coupled to soluble guanylyl cyclase, which generates up to 6000 molecules cGMP *per* minute upon binding of one NO molecule in vascular endothelial cells for example. A related approach with endogenously expressed soluble guanylyl cyclase and the ectopically expressed cGMP sensor, a cell line was developed, capable of sensing oscillatory release of picomolar concentrations of NO from neighboring hippocampal neurons [140], which were preceded by Ca^{2+} oscillations, as imaged simultaneously using a yellow cameleon FRET sensor [141]. In general, one can expect to see more studies investigating the concerted action of signaling molecules in complex cellular regulation networks (*e.g.*, circadian rhythms, heart rhythm) in the future [116].

5 Imaging-based HTP assays

In contrast to the more functionally centered imaging methods exemplified above, the investigation of physiological and pharmacological perturbations in living cells has led to the development of experimental setups allowing for quantitative phenotyping of single cells or cell populations. The parameters of interest include cell shape and size, cell cycle stage, protein expression profiling, PTM of proteins, protein localization, and protein trafficking. These assays require sophisticated equipment for automated sample preparation, automated fluorescence microscopy and image analysis as well as high performance computing setups, because the collected data sets are usually large and the phenotypic variations can be too subtle to be discerned by human personnel.

The field of HTP imaging evolves rapidly, and some of the pioneering studies will be detailed here. At first, phenotype analyses evolved from typical genetic systems, including the global analysis of protein localization in budding yeast [142–144]. Recently, whole-genome phenotyping was performed using HTP RNA interference (RNAi) in early embryogenesis in *Caenorhabditis elegans* [145], in mammalian cell culture [146–149] creating powerful tools for functional genomics approaches, and multivariate profiling in drug screening efforts [149]. An important improvement was the introduction of automated time-lapse imaging instead of endpoint assays for HTP (Fig. 7). In a proof of principle approach, Neumann *et al.* spotted 50 transfection-ready small interfering RNAs onto glass coverslips and overlaid each spot with approximately 50 HeLa cells yielding a live cell microarray. To check for mitosis, the HeLa cells stably expressed the core histone 2B tagged with GFP to assay for chromosome segregation and characterization of resulting phenotypes as they emerged at different time points after transfection [147]. By imaging the cells every 30 min for an entire period of 2 days the approach allows the discerning of primary (early) and secondary effects of gene knockdown in living cells. However, the poor time resolution is still the limiting factor for real HTP screening applications, which requires technological advancements regarding image acquisition, image analysis, and microscope-control hardware [150, 151]. These automated microscopy assays could be run in medium to HTP format to screen libraries of cDNA or RNAi, aptamers, proteins, antibodies, or alike in target discovery efforts.

Another imaging technology successfully applied for multiplexed analysis of antigens at the single cell level is flow cytometry. The current instrumentation allows for the simultaneous analysis of ten or more individual parameters [152], including intracellular proteins, protein phosphorylation, cytokines, nucleic acids, extracellular surface markers, and other antigens [153, 154]. Flow cytometry involves the use of a beam of laser light projected through a liquid stream containing the cells, or other small particles like color-coded beads for example [155], which when struck by the focused light, give out signals which can be picked up by a series of detectors at a rate of up to 100 000 cells *per* second [151]. One major advantage is that a mixture of cells can be investigated, and that rare cells of interest can be sorted out for further analysis. The cells are usually stained with fluorescent dyes, often coupled to antibodies, which specifically recognize cellular constituents. This enables large-scale profiling experiments of cellular signatures, and the comparative analyses of normal and diseased cell stages, as well as testing drug efficacy in patients during clinical monitoring. In an extension of this method, termed fluorescent cell barcoding, the

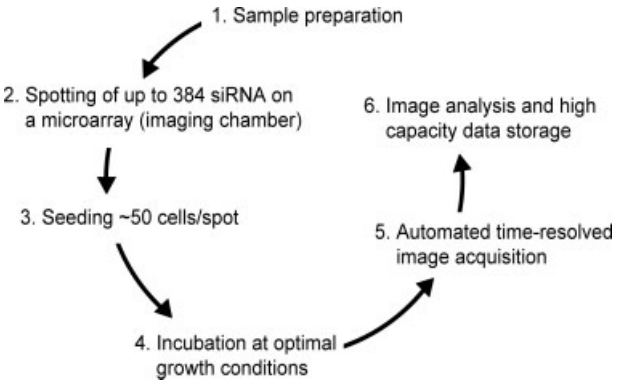

Figure 7. Flow chart of the step-by-step setup for an HTP RNAi phenotyping experiment [147]. For details, see Section 7.

combinatorial use of different intensities of fluorescent markers, by incubating cells with different concentrations of them, produces an array of signature intensities. This was used to obtain signaling profiles of a heterogeneous primary cell line (mouse splenocytes) stimulated with interferon-γ investigated in 96-well format, using three different colors in a combinatorial fashion. The setup for example allowed for the sorting of different cell types (B-cells, $CDC4^+$ T cells, $CDC4^-$ T cells, CD11b-hi cells) and subsequent analysis of phospho-STAT1 levels elicited by different doses of the cytokine [156]. The simultaneous identification of subpopulations of cells with flow cytometry combined with multiplexed imaging can yield a vast amount of data that can be used for biomarker development, compound screening and lead optimization.

6 From modeling to simulation

Imaging and proteomics techniques can provide information about cellular networks at different levels of detail. A more complete understanding of the complex regulation of cellular "behavior" therefore requires the merge of (HTP) proteomics and imaging data with genetic and expression profiling data for example [157, 158]. Top-down approaches generate a vast amount of systems-level data; however, our understanding of complex systems is still rather limited due to the difficulties to integrate the data into relevant models [159]. The comparison of the experimental data from the investigation of the global protein–protein interaction network of *Saccharomyces cerevisiae* by yeast two-hybrid and MS-based strategies revealed a surprisingly small overlap of only 3% of the two methods [160]. This underscores the importance of hypothesis driven research and detailed biochemical analyses to verify interactions and to put confidence in HTP data. Since many imaging approaches generate data of specific protein–protein interactions or changes in molecule numbers, one should be able to draw a detailed map of for example signaling networks after activation by an extracellular stimulus. Theoretical approaches like modeling or simulations will help to manage the complexity and dynamics connected with this task [161]. This implies the directed reduction of the multidimensionality and increasing the degree of transparency of the system. In addition, we are able to reduce uncertainty and instability in the systems reactions.

There is a considerable interest in understanding signaling networks not only in basic research, but also in the biomedical field [162], because most pathologies are not dependent on one single critical factor but can rather be seen as a simultaneous change of activity patterns of a large number of factors. By modeling a complex network like a cell, these multiparameter dependencies can be addressed and suitable drug or diagnostic targets may be identified.

For quantitative simulation approaches, exact data of concentrations and binding constants are required [163, 164]. Up to now, these data have only been provided in a few cases utilizing imaging techniques [165]. Herein lies the challenge: (i) data obtained should have quantitative character, and (ii) data from different experimental setups with different measures need to be combined into one consistent model or simulation. A method particularly amenable to quantitative modeling is flow cytometry, as it produces protein expression data or protein modifications, such as phosphorylation from thousands of individual cells. Here, Bayesian network computation can be applied to model experimental data derived from flow cytometry data. The data gain particular relevance, as stimulatory cues (*e.g.*, activation of T cells by anti-CD3/CD28 antibody) or specific perturbations (*e.g.*, activation of PKA by a membrane permeable cAMP analog or inhibition of phosphatidylinositol 3-kinase by LY294002) are included in the analysis [166].

Data derived from conventional live cell imaging are usually qualitative or partially qualitative in nature. Consequently, qualitative modeling is a helpful starting point for installing a system and will reflect structural interaction characteristics of data. By imaging up or down regulation of a specific protein or the (local) change in the concentration of a specific signaling molecule is visualized. With these data, a typical approach for analyzing signaling networks is to apply sensitivity or structural analysis in simple Boolean networks [167, 168]. In addition, there is a number modeling tools available that provide the possibility to generate or to modify signaling networks [169–171].

In our group, an alternative method based on complexity management utilized in industrial and economic environments is employed to reflect the structural properties of complex signaling networks. In contrast to Boolean networks, this approach opens the possibility to include a large number of elements and dimensions with strong interconnectivity – both in a linear and nonlinear, in a continuous and discontinuous fashion. When generating a structure representing a signaling network, the basic idea is to connect single elements (proteins, small signaling molecules, ions, *etc.*) with direct qualitative interaction terms. These input/output relations are usually characterized by differential equations. By using qualitative relations – instead of defined functions – we get a very flexible instrument able to cover even highly complex concepts. This allows us to integrate data from imaging experiments as well as from any conventional proteomics approach. An existing experimentally derived network can be extended by specific and systematic hypotheses. The resulting model can in turn be verified by experiments and hence its coherence can be tested. Such a process is exceedingly helpful to detect gaps and inconsistencies in the construction of complex systems. In an iterative process, the model may subsequently be developed and refined. Dynamic sampling of this model structure will reveal a multitude of features which are characteristic for complex systems in general and especially for cells which otherwise are not available with static

or function-limited analyses. In particular, we are able to more precisely localize where the system acts chaotically showing volatile and dramatic changes of state. Even more importantly, with this approach we can determine and test arrays of model conditions with respect to stability and viability of the entire system.

Combining theoretical and experimental setups makes it possible to generate and validate models. Therefore, modeling does not only support the understanding of a dynamic and complex system, it also provides the possibility to systematically evaluate new modulators and their targets with respect to their performance in the context of a complex system. This will streamline research efforts and should ultimately lead to acceleration in biomedical research.

7 Outlook

The greatest advantage that imaging provides over conventional proteomics procedures is that it enables time-resolved data acquisition of cellular events. Hereby, a cell needs to be recognized as an individual entity with a qualitative and quantitative distinct dynamic ensemble of responses due to an individual pattern of gene expression. Of course, standard proteomics approaches such as MS, CE, microarrays, or real-time Western blotting continue to be indispensable. The development of analytical platforms using highly sensitive fluorescence detection methods in conjunction with nanofluidic sample preparation, high-performance chromatography or CE will allow profiling the fingerprint of single cell proteomes in the future [172]. Combined with the increasingly quantitative nature of imaging data generated on automated microscopes, these approaches will significantly contribute to establishing a complete data set for cellular events.

Another major contribution will arise as soon as systems biology has produced simulations, *e.g.*, signaling cascades, cell motility, or endocytosis. Imaging will then be the method of choice to verify these models and to identify situations were the simulation needs to be extended or modified. This step of systems analysis will strongly rely on multiparameter imaging where many different events are monitored simultaneously in one cell. Thus, a major challenge remains the development of fluorescent/luminescent probes and donor-acceptor pairs, in case of RET applications, that are suitable for multicomponent imaging. Here, quantum dots might hold great potential due to their brightness and stable fluorescence or luminescence light emission, however, targeted intracellular labeling of proteins has not been achieved yet [173, 174]. Furthermore, the ultra-fast response pattern of biological networks requires high-performance molecular imaging setups capable of imaging molecular events, at even higher temporal and spatial resolution than achievable to date. However, the acquisition of quantitative data in cultured cells might only provide a distorted picture. Therefore, imaging of events in functional organs or living animals employing tissue-specific promoters and targeting signals [175, 176] would be greatly preferred. Taken together, crossdisciplinary efforts of proteomics, cell biology, optical imaging, nanotechnology, medicine, and biostatistics are imperative to comprehensively describing cellular systems.

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