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REVIEW

Imaging the boundaries—innovative tools for microscopy of living cells and real-time imaging

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Abstract Recently, light microscopy moved back into the spotlight, which is mainly due to the development of revolutionary technologies for imaging real-time events in living cells. It is truly fascinating to see enzymes "at work" and optically acquired images certainly help us to understand biological processes better than any abstract measurements. This review aims to point out elegant examples of recent cell-biological imaging applications that have been developed with a chemical approach. The discussed technologies include nanoscale fluorescence microscopy, imaging of model membranes, automated high-throughput microscopy control and analysis, and fluorescent probes with a special focus on visualizing enzyme activity, free radicals, and protein–protein interaction designed for use in living cells.

Keywords Fluorescence microscopy · Live-cell imaging · Fluorescent probes · Real-time imaging

Introduction

Where conventional approaches hit the boundaries, a merging of distinct yet overlapping disciplines is more likely to bring the desired progress in scientific discoveries, e.g., chemical tools have the power to enlighten complex cell biology. This chemical biology approach has gained popularity with the development of isoform-specific small molecule inhibitors that help to dissect pathways where conventional knockout experiments fail. Nowadays, chemical biology has an established role in the advancing of cell biology and drug discovery. Apart from target identification of small molecules, advances in imaging methods comprise another area in which chemical biology is having an impact on the study of cellular processes. Especially live-cell imaging with optical microscopy techniques are very powerful because they enable us to see in real time what is going on and they shape invaluably our understanding of cellular processes.

This review will shed some light on the currently available tools and techniques that are used to monitor protein–protein interactions, conserve and access lipids in membranes, record enzymatic activities, measure metabolites as well as image analysis software developments for high-throughput screening and technical advances to improve the imaging resolution (Table 1).

Optical reporters for intra- and intermolecular interactions—a solution to the problem of bulky fluorescent proteins

A powerful technique to monitor protein dynamics and interactions offering high spatial resolution is Förster resonance energy transfer (FRET). However, protein–protein interactions can be missed, as FRET strongly depends on the relative orientations of the donor and acceptor chromophores [1]. Moreover, the bulky fluorescent proteins (donor and acceptor) can interfere with protein folding and interaction (Fig. 1a) [2].

Griffin et al. [3] designed and synthesized a small (<700 Da), membrane-permeant ligand for a small binding domain containing a tetracysteine motif (CCXXCC) which can be genetically incorporated into proteins of interest. The biarsenic ligand can be linked to various spectroscopic

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Table 1 Overview of recently developed imaging tools and methods for imaging and live-cell imaging in response to a specific cell-biological question

Cell-biological question	Biophysical/chemical approach	Example of methods and tools developed	Example of successful application in microscopy	Selected references
Bulky fluorescent proteins interfere often with protein–protein interaction and scaffolding studies using FRET	Cell-permeable small biarsenical multi-use affinity probes (MAPs) that bind a tetracysteine motif	FlAsH, ReAsH, HoXAsH, ChoXAsH fluorescent probes In combinations with pulse chase EM fluorescence photooxidation	Surveying protein domain conformation, association and translocation Protein trafficking Direct correlation of live-cell images with EM detection	[8, 11, 12]
Imaging curvature sensing and lipid–protein interaction	Model membranes	GUVs Membranes on solid supports	Lipid–protein interactions Fig. 2	[18, 21, 23]
Direct non-invasive imaging of enzyme activity	Reporter substrate that increases fluorescence upon cleavage Probes that bind the active site of an enzyme and fluoresce upon covalent modification	Smart probes Caged compounds Fluorescent activity-based probes (ABPs)	Imaging of enzyme activity in live cells and testing of small-molecule inhibitors (proteases, kinases)	[38–40]
Sensing-specific molecules	Biosensor Metal-based probes Boronate-based probes	FRET on a biosensor carbonic anhydrase Guanylate cyclase Cu(II) fluorescein-based compound (CuFL) PG1 and PC1	Zinc imaging NO sensing in vivo in real time H_2O_2 detection in neurons	[52, 53, 57, 58, 66]
High-throughput imaging screens—problem of rigidity and specialized approach of commercial systems	Controlling microscope hardware Automated image analysis	μManager http://www.micro-manager.org Image J http://rsb.info.nih.gov/ij/	High-throughput image-based chemical compound screen	[80]
Resolution barrier of optical lenses	Photoswitchable fluorophores	STED STORM RESOLFT 4Pi	Nanoscale fluorescence microscopy in whole (live) cells, e.g., mitochondria	[95, 97, 101, 104]

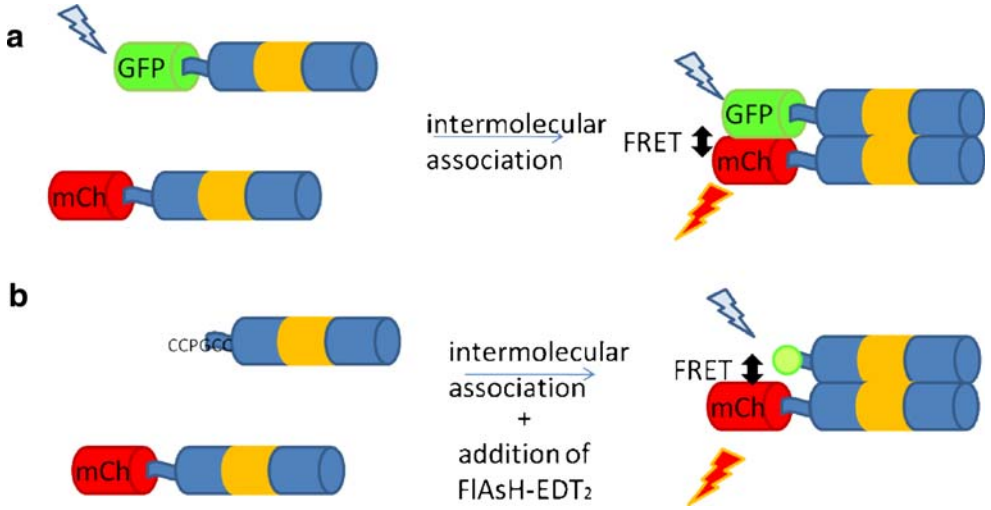

Fig. 1 Strategy for detection of protein–protein interaction. **a** Two potentially interacting proteins are genetically engineered, so that one of them is expressed with a donor fluorochrome (e.g., GFP) and the other with an acceptor (e.g., mCherry) in the same cell. If the two proteins physically interact upon stimulus, increased intensity at the acceptor emission maximum will be observed when the complex is excited at the maximum absorbance wavelength of the donor. Failure of the proteins to form a complex results in no acceptor fluorescence emission. The bulky fluorescent proteins often interfere with correct protein folding and consequently with interaction of proteins. **b** Instead of a bulky GFP, the protein of interest was tagged with the short the tetracysteine motif. The ligand FlAsH, which becomes strongly green fluorescent upon binding, can then act as a donor and can now participate in FRET molecular interactions as explained in (**a**)

probes or crosslinks and has only few other binding sites in non-transfected mammalian cells (Fig. 1b).

The first successful ligand for tetracysteine-tagged proteins was a fluorescein derivative with two As(III) substituents called fluorescein arsenical helix binder (FlAsH), which binds with high affinity and specificity to proteins tagged with the tetracysteine motif and thereby becomes strongly green fluorescent [3]. Meanwhile, many analogues of FlAsH have been synthesized including ReAsH, a resorufin derivative excitable at 590 nm and fluorescing in the red, and blue-fluorescing biarsenicals such as HoXAsH and ChoXAsH [4].

As binding to endogenous cysteine pairs would cause toxicity and non-specific labeling, antidotes such as 1,2-ethanedithiol (EDT) or 2,3-dimercaptopropanol are usually added in micromolar levels to outcompete endogenous pairs of thiols for FlAsH binding. Millimolar concentrations of these antidotes can outcompete the tetracysteine motifs and thereby strip FlAsH off the target proteins if desired [5]. However, one group reports unspecific binding of FlAsH in HeLa-S3 cells to endogenous, cysteine-containing proteins and recommends FlAsH-EDT2 labeling for recombinant proteins that express at very high levels [5]. Therefore, further development of these probes is necessary. Recently, improved biarsenical–tetracysteine motifs have been developed. This is promising, as they have been shown to enable the detection of a much broader spectrum of cellular proteins [6].

All these properties make biarsenical affinity probes a very useful tool as an alternative to bulky fluorescent protein tags. This approach has been successfully employed to study G protein-coupled receptor activation [7], protein kinase (PKA) translocation [8], β-tubulin dynamics [9], and the conformational change of the β_2 adrenergic receptor [10]. Recently, there are advances to further develop this method in order to study discrete conformational states of a protein. Luedtke et al. [11] employed bipartite tetracysteine motifs to study intra- and intermolecular interactions of two polypeptides or two basic zinc finger domains, respectively, each containing half of the motif. Their findings strongly suggest that this method could be used in vivo in order to study discrete conformational states of proteins. Furthermore, biarsenical probes have been employed in pulse-chase studies, where Gaietta et al. [12] have elegantly elucidated protein traffic of connexin-43 with successive labeling of different protein pools using FlAsH and ReAsH to distinguish younger and older proteins. They have also used biarsenicals as selective electron microscopy (EM) stain. Fluorescent photooxidation of ReAsH is used as a coupling step to allow direct correlation of live-cell imaging with EM.

Interestingly, ReAsH can also act as a singlet oxygen generator which, in combination with singlet oxygen-sensing green fluorescent protein (GFP), has been used for the detection of protein–protein interaction over a 25-nm distance, whereas conventional FRET is limited to distances up to about 8 nm [12].

Conservation and detection of membrane lipids

The cellular functions of the variety of lipids are much less understood than protein functions. This is partly due to the difficulties one faces when trying to image them. Probing

lipids for microscopy has the big disadvantage that each small molecule binding to the lipid of interest interferes with the whole lipid signalling and the integrity or composition of the affected membrane. Firstly, if the probe recognizes and binds the headgroup, it may compete with endogenous proteins that act on the headgroup (e.g., pleckstrin homology (PH) domain-containing proteins acting on phosphoinositides). Secondly, if a fluorophore is targeted to the fatty acid tail of the lipid, the curvature stress of the membrane in which the lipid is located might be altered and cause the lipid composition to change.

Moreover, unlike in the detection of unmodified proteins in fixed cells where endogenous proteins can be detected with antibodies to give an image of the state of the protein–protein interaction or protein localization in a cell at a certain time point, lipids cannot be easily crosslinked for preservation during processing. Popular fixatives for protein networks, aldehydes, do not react with most lipids and fixatives used in EM fixation act only on unsaturated lipids and crosslink with proteins via their carbon–carbon double bonds in the acyl chains [13].

Furthermore, in order to probe cytosolic and nuclear proteins with antibodies, the cells need to be permeabilized with detergents. This is usually done with 0.1% Triton X-100 or other non-ionic surfactants. However, even short treatment with these detergents can cause the extraction of soluble lipids and the selective retention of insoluble lipid domains which could probably induce the formation of artificial lipid microdomains.

Taken together, all this makes the observation of lipid–protein interactions extremely difficult to image because there are no tools that will allow for lipid and protein fixing at the same time. However, for the moment, biophysicists can use membrane models in order to study lipid behavior and interaction with proteins.

One possibility is the supported lipid bilayer, whereby vesicles or fragments from biological samples are fused to the surface of a suitable solid support. With this method, co-existing gel and liquid phases have been distinguished with a high spatial resolution and compositions have been obtained using secondary ion mass spectrometry [14]. Cellular membrane fragments on solid supports have been imaged with atomic force microscopy [15, 16] and EM [17]. Interestingly, Perez et al. [18] use a simpler fluorescence imaging method of membrane proteins to investigate their organization in supported cell-membrane sheets. They demonstrated how micrometer-sized membrane sheets can be detached from a plasma membrane of a living cell by pressing poly-L-lysine-coated coverslips on the cells' apical sides. One can obtain planar membrane sheets with endogenous lipid and protein composition, displaying unrestricted lateral mobility of the lipids in the two leaflets and preserved functionality of the proteins [18, 19]. The method has many advantages. Firstly, the samples have a low background fluorescence due to the removal of the cytosolic components that usually confer autofluorescence. Secondly, the mechanically stable membrane sheets retain their natural composition and functioning membrane receptors are still able to bind their ligands (Fig. 2). Therefore, this method displays an ideal solution for studying lipid–protein interaction and lipid localization on the plasma membrane in vitro. However, for relative quantification such as detection of phosphoinositide species, an alternative method might be more accurate. For instance, lipids from fixed whole cells could be heat transferred onto a silanized coverslip. This is more like a single cell "lipid extract" in which the cell shape is preserved and the selective loss of lipid species during staining is minimized in comparison to other methods (manuscript in preparation) (Fig. 2c) [20].

Another model system for membranes that has recently seen great progress includes bilayers in form of vesicles made from well-defined mixtures of pure lipids. Small unilamellar vesicles have diameters in the nanometer range and giant unilamellar vesicles (GUVs) in the micrometer range. In combination with fluorescent probes that accumulate in either the liquid-ordered or liquid-disordered phase, GUVs allow imaging of phase separation. This setup has been used to study the role of membrane composition in regulating the function and activity of peripheral and integral membrane proteins [21, 22].

Furthermore, proteins may drive lipid membranes to adopt levels of curvature that do not correspond to the minimum energy state for that particular bilayer region. For instance, a number of trafficking proteins, including amphiphysin and dynamin, are implicated in the budding of membrane vesicles, a process mediated by the bin/amphiphysin/Rvs161/167 domain [23–25].

Variation in membrane curvature may regulate the localization of effector proteins such as actin filaments or scaffold proteins. In vitro systems that allow measurement of the curvature elastic stress and simultaneous observation of protein localization, activity, and lipid behavior would be useful in exploring these processes. A disadvantage is the lack of complexity as compared to physiological membranes. However, this model system has not yet been exploited to its full extent and it could give us more insights into essential biological processes such as membrane fusion, trafficking, signalling, cell–cell recognition, lipid–enzyme interaction, etc.

Live imaging of enzyme activity—smart probes, caged compounds, and fluorescently quenched activity-based probes (qABPs)

Common biochemical methods to monitor enzyme activity barely assess the functional state of endogenous enzymes as

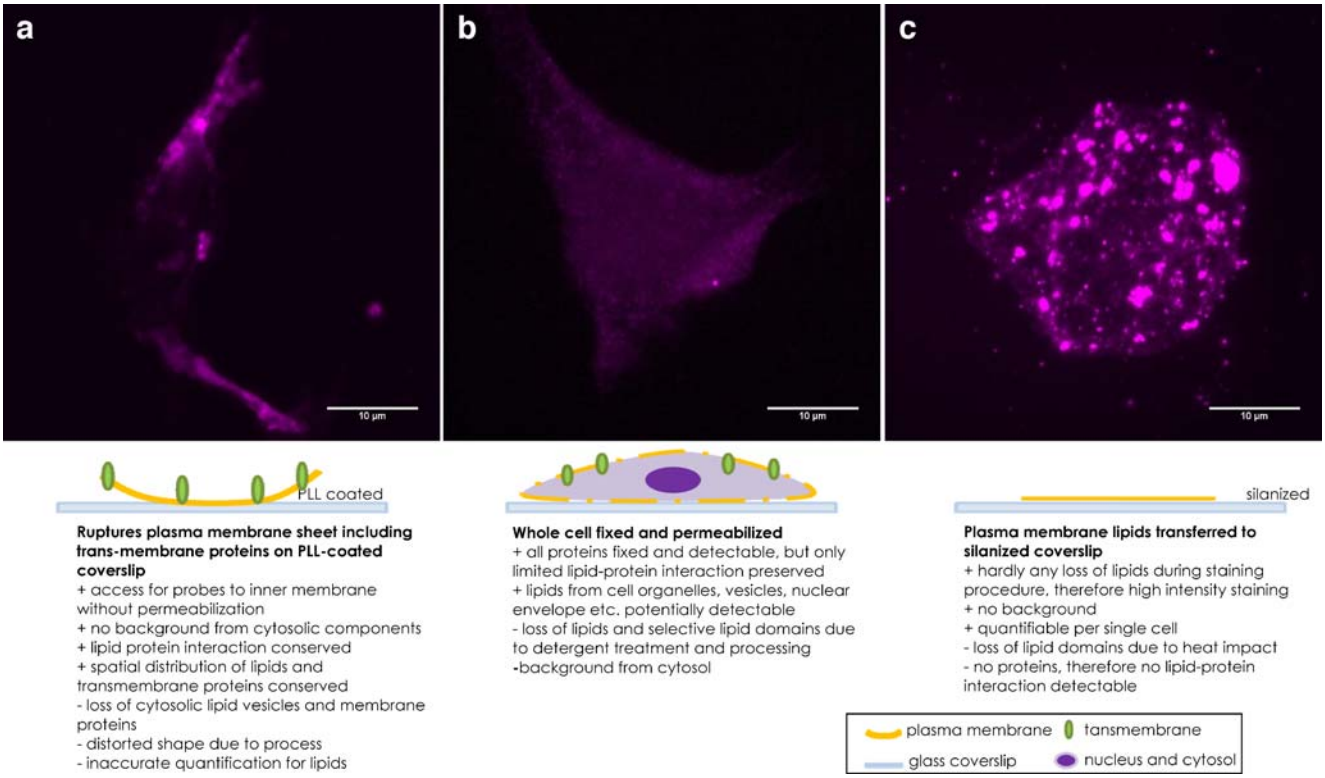

Fig. 2 **a** A membrane sheet of a NIH 3T3 fibroblast with the extracellular leaflet in contact with the glass and the cytoplasmic leaflet directed to the solution (mounting medium). The coverslip was probed for phosphatidylinositol(3,4,5)P3 and phosphatidylinositol (3,4)P2 with a labeled Akt-PH-domain as described in [20]. **b** In comparison, a whole cell, fixed with 4% para-formaldehyde (PFA) and permeabilized with digitonin, was subjected to the same labelling procedure. **c** "Lipid print" of a fixed cell. After fixation with PFA, the membrane lipids of the cell were transferred with heat and pressure to a silanized coverslip and labeled as above. All pictures were acquired using the same settings so that their intensities are directly comparable

they often use recombinantly expressed proteins, which are tested in an artificial environment. To address this issue, a number of elegant imaging methods have been developed that enable the monitoring of enzyme activity on specific substrates in a cell in real time.

Fluorescent probes can be physically quenched to minimize their emission signal in the non-activated state while becoming brightly fluorescent after specific molecular conformational changes or reactions. This can be achieved by using reporter substrates that, when processed by a given enzyme target, produce a signal that can be imaged. The first generation of these so-called smart probes were designed to detect caspase activity using a peptide linked to a fluorophore, which would be released following substrate cleavage resulting in an increased fluorescence intensity of the free fluorophore (Fig. 3a) [26]. Other methods were FRET-based probes where cleavage of the linker that separates the FRET pair would result in an increase of the donor and reduction of the acceptor emission (Fig. 3b) [27]. The specificity for individual enzymes is conferred by the fluorescent peptide sequence in combination with an enzyme-specific inhibitor. The change in fluorescence would correlate with enzyme activity and a ratiometric readout corrects for the variations in probe concentration and cell thickness [28]. This method has been employed successfully for measuring protease activity, including extracellular proteolysis of matrix metalloproteinases and cathepsin K [28–31].

Kinases constitute the largest enzyme class in mammalian proteomes and are fundamental for the function of signal transduction cascades [32]. However, the design of reporter probes for in vivo monitoring of kinase activity is challenging because the addition of a relatively small entity, the phosphate group, is difficult to detect. The discrimination between a non-phosphorylated and phosphorylated peptide substrate can be made when conjugated to the fluorochrome 6-acryloyl-2-dimethylaminonaphthalene (acrylodan) that dramatically decreases its fluorescence intensity upon phosphorylation of the peptide [33]. This approach has been used to measure autophosphorylation of cAMP-dependent PKA in living cells [34]. However, there is a need for fluorophores that are less dependent on the substrate they are conjugated to in order to gain specificity for other kinases or phosphatases.

Another interesting development is the application of "photoactivable" (caged) compounds. Similar to "smart

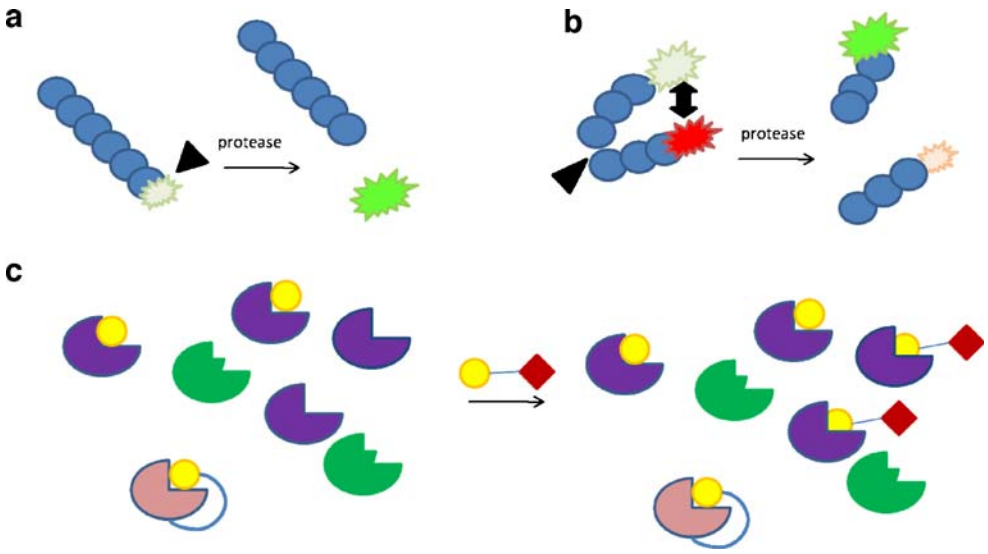

Fig. 3 **a** A target peptide sequence linked to a fluorophore, which is released following substrate cleavage, is employed as a "smart probe". The free fluorophore displays increased fluorescence intensity as compared to the peptide-linked one. **b** FRET-based "smart probes" show an increase of the donor and reduction of the acceptor emission where cleavage of the linker separates the FRET pair. **c** ABP profiling: only enzymes whose active site is not occupied by an inhibitor or autoinhibition and who have the complementary binding sites will be labeled with the tag of the ABP probe

probes", caged compounds are biologically inactive until modified. In this case, the controlled release of the active compounds is subject to a brief pulse of light. There is a huge variety of caged compounds such as adenosine triphosphate, calcium, nucleotides, amino acids, glucose, and phosphate esters [35]. However, their application for imaging enzyme activity in real time seems to be limited. Wang et al. [36] have characterized cell-permeable caged substrates for protein tyrosine phosphatases such as PTP in order identify inhibitors of tyrosine phosphatases. 1-(2-Nitrophenyl)ethyl-protected fluorescein diphosphate (NPE-FDP) undergoes rapid photolysis to release FDP upon irradiation with UV light inside cells. The generated FDP can be used by phosphatases to produce fluorescein monophosphate and subsequently fluorescein, which can be detected and quantified. However, the detection of the product was only successful in cells with highly overexpressed phosphatases (e.g., alkaline phosphatase). Further developments are needed to make caged compounds applicable to imaging. Very promising is the design of two-photon excitation caged compounds and photoreversible caged compounds [37].

The downside of the methods described above is that they are all based on a reporter substrate that has to be specific for the enzyme of interest. This is rather difficult to achieve as most signal peptide sequences are usually targeted by a whole family of proteases and also phosphorylation sites are highly conserved sequences that are recognized by a whole class of kinases. Additionally, it is elaborate to genetically engineer bulky reporter probes into an organism and, more importantly, genetic modifications often have the disadvantage of toxic side effects and counter-regulatory effects in response to the abundance of an artificial protein. Therefore, small molecule probes that covalently attach to an enzyme target to monitor enzyme activity have been developed as an alternative. So-called ABPs make use of the chemical interactions, usually a covalent modification, specific to the enzyme of interest and provide an indirect readout for enzyme activity. ABPs contain a reactive group ("warhead") that covalently modifies the active site residue of an enzyme, thereby controlling the selectivity (Fig. 3c). The reactive group is conjugated via a linker region, which can further contribute to the enzyme-specific recognition, to the tag for visualization [38]. This method was successfully employed in in vivo imaging of mostly proteolytic enzymes (for reviews see [38–42]). However, the major limitation of these probes is their general fluorescence both when bound to an enzyme target and when free in solution. To overcome this limitation, Blum et al. [43] designed quenched probes (qABPs) that become fluorescent only after covalent modification of a protease target. They used acyloxy-methyl ketone as reactive group that had a quenching molecule attached which would be removed upon covalent modification by a cysteine protease through the loss of the acyloxy group. With this tool, they successfully imaged cysteine protease activity in NIH 3T3 cells in real time. The same group also reported recently the design of quenched near-infrared ABPs for cysteine proteases, which can be imaged in tumors of living mice and in the explanted tumor [44].

In conclusion, "smart probes" and ABPs have made it possible to visualize enzyme activity in vivo with the use of common imaging modalities. Such tools promise to facilitate the identification and validation of new drug targets and will elucidate many signaling cascades. However, further developments into probes for monitoring enzyme activity are urgently needed.

Sensing-specific molecules

As mentioned above, probes for biomolecules, which detect the substrate or product of an enzyme of interest, have become invaluable tools for the characterization of cellular

processes. However, those probes are mostly designed for peptides and large organic biomolecules. Imaging of inorganic molecules moved only recently into the spotlight. Probes designed for these molecules could provide important information about various cellular processes because bivalent cations, nitric oxide (NO), and oxygen radicals often act as second messengers. Herein, a few examples of recently developed sensing tools will be discussed.

Calcium has been recognized as an important second messenger for over a hundred years [45] and there are many established methods for calcium imaging, which are described elsewhere [46, 47]. But another bivalent cation, zinc, has been shown to be of great importance. Zinc is a co-factor of many enzymes [48] and plays an important role in synaptic transmission [49], mitochondrial function and apoptosis [50], ageing, and disease [51]. Although zinc is the second most abundant transition metal cation in biology, the majority of cellular zinc is bound and must therefore be distinguished from free zinc (or rapidly exchangeable zinc) [52]. It was thought that the cytosolic free zinc concentrations are in the femtomolar range, but recently Bozym et al. [53] have imaged intracellular zinc concentrations in a ratiometric FRET approach and detected surprisingly free zinc levels of approximately 5 pM in PC-12 (pheochromocytoma) and Chinese hamster ovary cells. Their approach is based on FRET from a zinc-bound aryl sulfonamide to a fluorescent label on the carbonic anhydrase, a biosensor for zinc.

A variety of approaches with different sensor molecules have also led to new findings about the role of zinc in living systems (see [52] for review) and there is no doubt that these developments will lead to more important answers in the relatively new field of zinc biology. This shall encourage the development of sensors for molecules which initially were thought to be insignificant.

Another, however well established, messenger for cellular signalling is NO. The diverse biological functions of this molecule have been extensively described [54] but, due to its rapid diffusion and reactivity, the detection and visualization in live cells is very difficult. The downside of most small-molecule-based fluorogenic probes for NO such as *o*-diaminofluoresceins is that their signal is only generated by the reaction with oxidized NO products to form triazole species, rather than with NO itself. This means that NO-related bioevents cannot be detected in real time with those organic molecule sensors. Lim and Lippard [55] recently developed metal-based fluorescent NO sensors that enable real-time life imaging with a good spatial resolution. They describe a number of complexes with different advantages such as iron(II) complexes, cobalt(II) complexes, ruthenium(II) porphyrins, and dirhodium(II) tetracarboxylates, which fluoresce upon NO interaction with their metal centers. A copper(II) of a fluorescein-based ligand has already been successfully employed for in vivo imaging in live cells [56, 57] and demonstrates NO production in macrophages in response to lipopolysaccharide and interferon-γ. This so-called turn-on probe for NO is very exciting as it is highly specific for NO over other reactive nitrogen and oxygen species at physiological pH. It works on the basis of reductive nitrosylation of copper(II), displacing an attached fluorophore and releasing the metal, thereby turning on the fluorescence, as copper(II), which is an effective fluorescence quencher, is reduced to copper(I) and released. Another promising approach is based on the amplified generation of guanosine-3′,5′-cyclic monophosphate (cGMP) by soluble guanylate cyclase upon NO binding [58, 59]. It can be detected by a cell-based genetically encoded indicator for cGMP that has a donor (CFP) and acceptor (YFP) for FRET and emits cGMP-dependent FRET signals. A particular advantage of this method is the enhanced reversibility and high sensitivity (20-pM detection limit) [58, 59].

The NO sensors discussed seem to be very promising and have the advantage of sensing NO itself with high specificity and not its reaction products. Nevertheless, it should be pointed out that there is a vast selection of NO-sensing tools, which have been discussed in detail in an excellent review by Wardman [60].

Chemical probes for other free radicals such as reactive oxygen species (ROS) are also important. Among those, H_2O_2 is emerging as a newly recognized messenger in growth factor response and signal transduction [61–64]. However, probing this molecule with specificity has been challenging due the lack of selective probes and the problem of photoactivation at excitation wavelength in the UV range [65]. While the commonly used reduced fluorescein dyes have the advantage to be non-fluorescent until they are oxidized, they have major drawbacks and, if not used correctly, are very likely to produce artifacts (see [60] for an excellent review). Recently, Miller et al. [66] reported a new approach for the detection of H_2O_2 in live cells. They developed boronate-based fluorescent H_2O_2 probes with visible excitation and emission wavelength and directly visualize and track H_2O_2 signalling in primary neurons. The specificity of this probe is partly due to the fact that, as opposed to conventional ROS probes, catalysts are not required for the boronate-based probe response. The downside is the low reactivity with H_2O_2 which was reported to be approximately 0.5–1 M^{-1} s^{-1} [67]. This might limit the application of boronate dyes to certain cell types or certain stimuli causing oxidative bursts.

While the monitoring of specific free radicals in a spatiotemporal manner is aimed high, imaging overall redox changes might open new avenues for oxidation biology research. Changes in the redox equilibrium influence major cell functions including cell cycle, migration,

differentiation, and apoptosis [68–72]. Alterations in the redox equilibrium are due to changes in the ratios of glutathione/glutathione-disulfide or reduced/oxidized thioredoxin [73, 74], but measuring these concentrations is problematic and the assays are not suitable for imaging. Redox-sensitive GFP species, however, allow real-time observation of the species' oxidation state, which is indicative of the redox equilibrium of a cell or cell compartment [75]. roGFP1 (GFP with mutations S147C/Q204C) and roGFP2 (S147C/Q204C/S65T) respond to a variety of oxidants when expressed in the cytoplasm or targeted to organelles. The reversible response can be measured ratiometrically, canceling out differences in the probe concentration. Redox changes in macrophages, growth factor-stimulated H_2O_2 production, and hypoxic conditions have been imaged using these probes [76].

More interestingly, but not directly related to measuring ROS, redox-sensitive GFP species allow the detection of protein–protein interactions over tens of nanometers through cells, well beyond the range of FRET. Diffusion of singlet oxygen from a photosensitizer to an acceptor sensor can detect proximities up to 70–100 nm without any orientation dependence. For instance, the detection of protein–protein interaction over a 25-nm distance has been reported by Gaietta et al. [12]. They used connexin-43 gap junctions as scaffolds in order to separate ReAsH, a singlet oxygen generator in the cytosol of one cell, from the singlet oxygen-sensing GFP in the cytosol of a neighboring cell. This illustrates how "thinking outside the box" can result in the discovery of a tool for a purpose completely unrelated to that which it was designed for.

High-throughput screening on the microscope

Drug discovery is not only a major focus for large pharmaceutical companies but gains also more and more attention in academic research. Compound libraries are screened for the effects of a drug on a variety of molecular and cellular targets. While taking into account cell-type specificity, cell permeability, accessibility to subcellular compartments, biostability, toxicity, and off-target effects, ideally, all the responses of the interconnected normal or diseased cellular-pathway networks should be explored.

These parameters are crucial to be determined at an early stage within the drug discovery process but in vitro biochemical methods usually test only a single target neglecting the intercellular structural and functional networks. Charge-coupled device imaging microscopy provides one opportunity to measure subcellular structures providing even spatial and quantitative information of the intact living cell and enables a multiparametric characterization of the effects of a drug or other treatments. However, visual assays are usually low-throughput, as they traditionally require manual image acquisition, visual inspection, and individual analysis. Performing automated functional microscope-based assays on a large set of proteins in cells is presently still a challenge. However, they have not only the advantage of high throughput; automated image acquisition and analysis also help to minimize the bias that is often the problem with manual microscopy techniques.

An image-based screen requires the following steps: assay development, automation and coordination of sample preparation, image acquisition and data storage, analysis of image data, and lastly integration of the results with existing knowledge (e.g., bioinformatics databases).

Some automated screening microscopes are commercially available, but those systems have been designed and optimized for special applications, which restricts the possibilities of adaptation to new assays. And systems with ultrahigh-throughput capacities are often lacking the single cell or subcellular resolution.

To avoid the drawbacks of commercial instruments, some groups have designed their own image acquisition systems that produce high-quality images for large-scale functional screening. Liebel et al. [77] describe a modular microscope-based screening platform and its application to the development of two cell-based assays addressing protein secretion and Golgi integrity.

Further, high-resolution screening applications for several research areas including screening of chemical compound libraries for their effect on cell adhesion, discovery of novel cytoskeletal genes, discovery of cell migration-related genes, and a siRNA screen for perturbation of cell adhesion have been developed by Paran et al. [78, 79].

Apart from the construction of the actual screening microscope, software that controls the microscope hardware is needed. μManager (http://www.micro-manager.org) is an open source software package for imaging and control of automated microscopes on multiple platforms (Windows, Mac, and Linux). It enables flexible protocols such as 3D image acquisition and complex analysis and is a good alternative to costly off-the-shelf software that is often designed for only one specific application [80].

Ultimately, the knowledge extracted from all the acquired information should be used for deciding on which potential targets to pursue and which screening leads are qualified for further development. But extracting quantitative measurements from the images is extremely challenging if it exceeds simple cell counting or particle size measurements that only require a threshold to be defined for the software. However, with all the sophisticated tools available, some of them mentioned above, the analysis software also needs to be further developed to recognize these structures and activities and distinguish them from

background noise in automated screenings. A simple example of that problem is the automated analysis of focal adhesion structures. These are the sites in the cell where the actin cytoskeleton is connected to sites of extracellular matrix adhesion sites [81]. Focal adhesions are signaling complexes of special interest in tumor biology since they are involved in cell adhesion, hence metastasis and aggressiveness of the tumor [82, 83]. Many of its components such as focal adhesion kinase, Src, and paxillin can get tyrosine phosphorylated on several residues which reflects the activity and affects the integrity of the complex [84–86]. This can be visualized by simple staining with an anti-phosphotyrosine antibody linked to a fluorophore (Fig. 4). However, although these structures are easy to identify for a cell biologist, a "virtual eye" might fail because it is difficult and laborious for a researcher to express the morphology of those structures in numbers to define densitometry parameters, shape, and position and intensity thresholds in the software for it to recognize the structure in any given image. Fortunately, another open source software, ImageJ (http://rsb.info.nih.gov/ij/), has been successfully used for automated quantification of different phenotypes such as focal adhesions, wound healing, apoptosis and fluorescent labeling of cell organelles, etc. [87–90]. Researchers can upload their scripts on this website where they are available as a plug-in to download for everyone else with a similar approach.

This is only the beginning of dramatic advances enabled by new fluorescent reagents designed for use in living cells and analysis with sophisticated but easy-to-use open source software tools that are constantly improved by researchers and made available online.

Breaking the diffraction resolution barrier

Due to its wave nature, light cannot be focused to an infinitely small spot [91]. According to Abbe's theory, the smallest focal spot could be a third of a wavelength in diameter, with the size of the illuminated area limited to approximately 250 nm in the focal plane and 500 nm in the direction of the optical axis. Recently, a number of novel microscopy techniques have been shown to overcome the Abbe limit and achieve resolution of up to 20 nm (Table 1) [92–94]. Such a technique is stimulated emission depletion microscopy (STED), which is based on the following principle: In order to make the fluorescence spot smaller, one has to inhibit the fluorescence from its rim. This is achieved by two beams of ultrashort laser pulses of different colors. The first one excites the fluorophore and the second one, a slightly red-shifted beam directed to the rim of the fluorescing spot, forces the fluorophore into the ground state, i.e., it depletes the emission without destroying it. As a result, the fluorescence is quenched everywhere in the focal spot except in the center. By increasing the intensity of the beam, the fluorescent spot can be progressively narrowed down, in theory, even to the size of a molecule. With this technique, a much smaller fluorescence spot than Abbe would have predicted can be produced. Donnert et al. [95] present evidence that fluorescence microscopy in a nanoscale range is indeed possible. They used STED microscopy to show nanoscale protein patterns on endosomes, the punctuated structures of intermediate filaments in neurons, nuclear protein speckles in mammalian cells with conventional optics, and nanoclusters of a mitochondrial outer membrane protein, Tom20 [93].

A similar method involves reversible photoswitching of a marker protein between a fluorescence-activated and a non-activated state [96, 97]. These reversible saturable optical fluorescence transitions (RESOLFT) and multiple imaging cycles to reconstruct the image allow the precise location of individual fluorophores to be determined limited only by the photokinetics of the protein [97, 98].

A less technically demanding approach, which does not require high-intensity pulsed lasers, is stochastic optical reconstruction microscopy (STORM). It requires specialized microscope control and data processing in combination with a commonly available microscope. The process consists of a series of imaging cycles, whereby in each cycle only a fraction of the fluorophores in the field of view

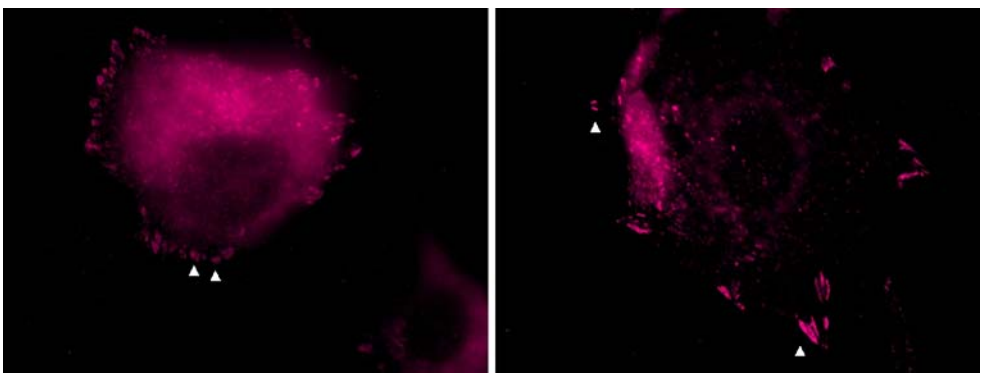

Fig. 4 NIH 3T3 cells were fixed with PFA and stained with a fluorophore-labeled phosphotyrosine mouse antibody (4G10). Fluorescent microscopy reveals heavy tyrosine phosphorylation of proteins in focal adhesion patterns (*white arrows*). Unspecific staining and/or background from other tyrosine phosphorylated proteins renders parameter definition for automated quantification difficult

are switched on. Providing their images are not overlapping, each of the active fluorophores is optically resolvable from the rest and the position of these fluorophores can be determined with high accuracy. Over the course of multiple activation cycles, the positions of numerous fluorophores are determined and used to construct a high-resolution STORM image. With this approach, an imaging resolution of approximately 20 nm has been reached using a total internal reflection fluorescence microscope, low-power continuous-wave lasers and a photoswitchable cyanine dye [99–101]. By using activator–reporter-labeled secondary antibodies, even multicolor STORM imaging becomes possible. Each probe consists of a photoswitchable "reporter" fluorophore that can be cycled between fluorescent and dark states and an "activator" that facilitates photoactivation of the reporter.

Bates et al. [102] simultaneously imaged microtubules and clathrin-coated pits with Cy2-Alexa 647 for microtubules and Cy3-Alexa 647 for clathrin. Their two-color STORM images reveal ultrastructural information not discernable in conventional fluorescence images. To further develop this promising approach, new activator–reporter pair probes will be a great advantage for multicolor STORM. Also, the reactivation efficiency of these photoswitchable "reporters" is crucial in order to perform 3D STORM imaging [103].

A different approach is to use a combination of two objectives to optimize light gathering. For example, in 4Pi microscopy, two opposing objective lenses are used coherently so that the two wavefronts add up and produce a main focal spot that is sharper in the *z*-direction by about three to four times (4Pi of type A). A similar improvement is obtained if the lenses add their collected fluorescence wavefronts in a common point detector (4Pi of type B). A combination of both leads to a fivefold to sevenfold improvement of resolution along the *z*-axis (4Pi of type C) [104]. This approach has been applied for 3D imaging of microtubules of a mouse fibroblast cell and yielded an axial resolution of approximately 100 nm [105].

There are a number of other subdiffraction limit microscopy techniques available such as "structured illumination", "non-linear structured illumination", and "near field" techniques, which are described in detail elsewhere [106, 107].

All of these recent approaches have made significant progress in resolution increase. Now, the challenge lies in finding techniques that enable or facilitate nanoscale microscopy on living samples.

Apart from the examples listed above, there are countless other cell-biological questions that could be answered with the development and application of ingenious probes for fluorescence imaging in real time. Such probes will be very distinct from each other and might range from small molecules to long peptide chains, but they also will have to have some common features to fulfill this aim. Box 1 summarizes the characteristics that are required for a probe to be eligible for biological applications in live cells. They might sound very basic, but are essential to have in mind when developing such tools.

The techniques and tools presented in this review represent only a small selection of the creativity delivered through chemical biology. Each method has its advantages and disadvantages, which might have been mentioned but were not the subject of this review (please refer to the reviews and articles referenced in Table 1). This review merely gives examples of a broad variety of common limitations cell biologists encounter with imaging methods and where chemical biology delivered a solution or at least a promising input that will drive further advances. Those elegant examples of fruitful interactions between biologists and chemists or biophysicists are meant as an invitation to cross the boundaries and to look into areas where a solution is urgently needed. Only the crosstalk between chemists and biologists will bring us a step further.

Box 1: "Essentials" and "desirables" of fluorescent probes for live-cell imaging

Fluorescent probes for imaging in living cells should be…

✓	stable and functional at cellular pH (usually pH=7.4, but dependent on compartment) and temperature (up to 37°C)
✓	cell permeable (or amenable to transfection)
✓	non-toxic
✓	Specific and selective (enzyme families, classes of molecules, single proteins, phosphorylation sites, etc.)
✓	fast (binding, turnover)
✓	reversible or irreversible depending on application

Fluorescent probes for imaging in living cells should…

✓	excite and emit in the visible or near infrared (to avoid interference and cellular damage by UV light)
✓	deliver spatial resolution (low background)
✓	not be prone to photoactivation or photobleaching
✓	ideally increase fluorescence as a readout (instead of quenching)

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