Enzyme activity – it's all about image

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Unraveling the functional roles of proteins is a major challenge facing the postgenome researcher. Advances towards this goal have been made through the development of both chemical and biochemical tools for monitoring protein activity. Recently, a myriad of fluorescence-based imaging tools have emerged for *in vitro, in vivo* and whole animal applications. These tools have provided methods to monitor the spatial and temporal distribution of proteins and bioorganic molecules dynamically. Here, recent advances in chemical and biochemical techniques that allow the detection of enzymatic activity within intact cells and *in vivo* are reviewed. Such technologies have the potential to be integrated into drug-development programs to facilitate both the functional validation of pharmaceutical targets and the treatment of human disease.

Understanding the function of proteins within the context of their natural cellular environment is perhaps one of the greatest challenges facing disciplines such as biochemistry, cell biology and animal physiology. Visualizing enzymatic activation and regulation within a cell or an animal in real-time provides a foundation for understanding how a protein functions in a multifaceted milieu of molecules [1]. In contrast to simple gene expression profiling, which can provide information about the regulation of a given process at the level of changes in bulk RNA messages, protein expression and enzyme activation are often difficult to measure and are controlled by a complex set of mechanisms that are independent of transcription. Furthermore, a protein can exert its function in many ways, for example through interactions with other proteins, nucleic acids and small-molecule binding partners. This daunting level of complexity in posttranslational regulation has motivated investigators to design methods for monitoring enzyme activity dynamically within the physiologically relevant environment of cells and whole organisms.

Most enzymes harbor a set of controls that tightly regulate their activity within the cell. Enzymes can be regulated by multiple mechanisms such as their spatial and temporal expression, binding to small-molecule or protein cofactors and posttranslational modification. Thus, attempts to understand functional regulation of an enzyme using an *in vitro* approach is often misleading. One reason for the irrelevance of *in vitro* data is the disruption of organelles and fine compartments, which lead to the release of activators or inhibitors that artificially affect enzymatic activity. Furthermore, the use of fixed *in vitro* assay conditions might not accurately imitate the conditions found *in vivo*. Hence, tools for determining enzymatic activity in the context of an intact cell or preferably within a whole organism have great value. Although strategies for monitoring enzyme activity have been developed for a wide range of enzyme families, proteases and kinases have received most attention because of the large size of these families, the understanding of the relevant substrate–enzyme interactions and the potential for identification of new small-molecule drug targets. Attempts to study the cellular and physiological role of these enzymes have focused on the identification of downstream substrates [2–4]. However, the process of substrate identification can often be difficult and provides no information about the temporal and spatial regulation of an enzyme. Therefore, new technologies have been developed allowing direct visualization of kinase, phosphatase and protease activities. These technologies involve both the design of 'smart' imaging reagents and the development of techniques and optical instrumentation that allow a sensitive, rapid and high-resolution detection of enzyme activity within cells and whole organisms [5]. Because it is impossible to describe all technologies that can be used to address the question of protein function in a single review, in this article the focus will only be on technologies that provide a direct readout of enzyme activity. In particular, recent advances in the design of novel cleavable substrates, protein reporters and small molecule activity-based probes will be discussed. These tools will surely have a dramatic impact on the study of proteins at the level of physiological function.

Small-molecule substrate reporters of enzymatic activity

Fluorogenic substrates have long been used for detailed kinetic measurement of enzyme activity *in vitro*. These reagents carry fluorescent groups, and thus energy emission upon their enzymatic conversion to product can be monitored over time (Figure 1). Although the majority of basic fluorogenic probes cannot be directly applied to complex cellular environments, some fluorescence-based substrates have been designed to measure enzyme activity *in vivo*. The primary challenge in using a substrate-based imaging approach lies in the ability to generate probes that are specific for an individual enzyme. The most successful substrate-based probes are those designed to study the regulation and distribution of protease and kinase activity. Examples of these reporter probes are outlined below.

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 0962-8924/$ - see front matter doi:10.1016/j.tcb.2003.11.002

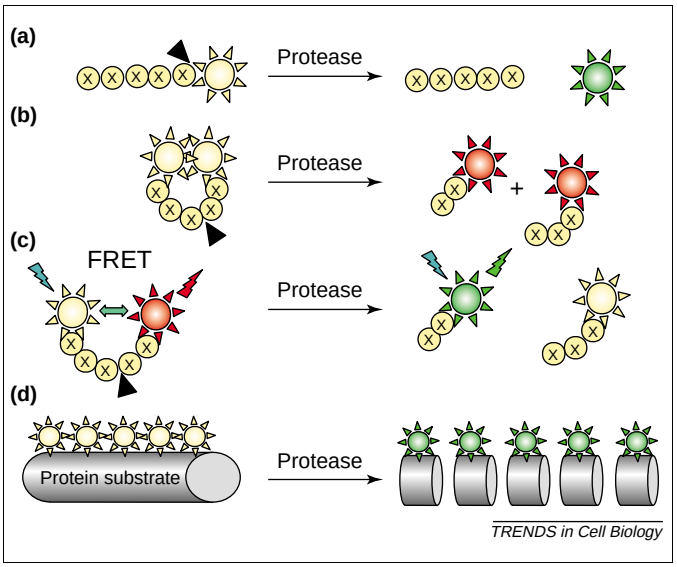

Figure 1. Fluorogenic substrate-reporters for the imaging of protease activity. **(a)** A simple peptide-based reporter of protease activity. This class of reagents is typically used for *in vitro* application and uses a quenched peptide-bound fluorochrome that provides a fluorescent signal upon protease cleavage [6]. **(b)** A reporter substrate in which the fluorophore is quenched by proximity to another molecule of the same fluorophore on the substrate. Fluorescence is detected upon hydrolysis of the substrate by a protease. For example, the xanthenes family of fluorophores forms a quenched-fluorescent dimer that can be disrupted to release the fluorescent monomers upon substrate cleavage [7–9]. **(c)** Fluorescence resonance energy transfer (FRET)-based probes work on the basis of the incorporation of donor and acceptor fluorochromes in proximity to each other. Proteolytic cleavage of the linker releases FRET [11]. **(d)** The release of fluorescence from a quenched fluorescent protein substrate is driven by protease-mediated degradation of the substrate [13]. Black arrowheads designate putative cleavage site; 'X' indicates amino acid or specificity elements incorporated into the peptide substrate.

Reporters for proteases

Virtually all of the protease-targeted fluorogenic substrates described function by a mechanism in which fluorescence of the reporter molecule increases and/or changes following cleavage within the peptidic scaffold of the probe. Many of the first generation fluorogenic and colorimetric imaging substrates were designed to detect caspase activity *in situ* using a synthetic tetrapeptide (e.g. Asp-Glu-Val-Asp as caspase-3 substrate) attached to a fluorescent (e.g. 7-amino-4-trifluoromethyl coumarin) or colorimetric (e.g. p-nitroanilide) molecule. In apoptotic cells, caspase activity can be imaged by detecting free fluorochrome or dye molecules that are released following substrate cleavage (Figure 1a) [6]. A major drawback of using UV-excitable fluorochromes is the high background that results from the illumination of cells with UV light. To avoid this problem, probes have been developed that take advantage of the unique ability of xanthenes fluorophores to form dimers leading to fluorescence quenching [7]. Using these fluorophores, cell-permeable substrates have been developed, in which xanthene fluorophores are linked to a caspase-specific cleavable linker. Disruption of the xanthene dimers following cleavage by the protease generates an absorption spectral shift, as well as a marked increase in fluorescence intensity, which can be used as a direct readout of caspase activity in live cells (Figure 1b) [8,9]. Other methods use quenched fluorogenic substrates in accordance with the principle of fluorescence resonance energy transfer (FRET). A typical FRET-based probe contains two fluorochromes situated less than 100 Å apart; the emission wavelength of the donor fluorochrome overlaps with the excitation wavelength of acceptor fluorochrome such that the former can transfer energy nonradioactively to the latter [10]. Cleavage of the linker separating the two fluorochromes suppresses the energy transfer resulting in an increase in the emission intensity of the donor and a reduction or elimination of the acceptor emission (Figure 1c) [11]. This change in fluorescence intensity can be monitored and correlates with protease activity. It should be noted that ratiometric probes, such as FRET-based reporters, are better than the simple intensity-based probes because they eliminate the artifacts caused by the variations in probe concentration and cell thickness.

The detection of extracellular proteolytic activity alleviates the need for cell-permeable probes and allows the use of intact proteins as substrates. Several studies used full-length proteins modified with small organic fluorophores as molecular beacons for proteolytic activity. Extracellular matrix components, such as gelatin and collagen, have been labeled with fluorescein in which fluorescence is quenched. Upon degradation of these labeled matrix-proteins by extracellular proteases, fluorescence is restored and can be measured (Figure 1d). Using this methodology, Sameni *et al.* [12,13] determined real-time degradation of basement-membrane components and localized proteolytic events within live cells. The activity of matrix-degrading enzymes, such as cathepsin B [13] and matrix metalloproteinases [14], have also been visualized *in situ* using collagen and gelatin labeled with fluorescent probes, respectively. Monitoring the ability of a cell to degrade the components of extracellular matrix has proven to be important for understanding cellular processes such as migration and invasion. Furthermore, integrating the use of specific inhibitors with fluorescent protein substrates can help to define the role of individual proteases in these processes.

Reporters for kinases

Designing reporter probes for *in vivo* and *in situ* monitoring of kinase activity has proven to be very challenging. This is primarily because kinases modify their substrates through the addition of a relatively small phosphate group. Thus, measuring enzyme activity requires a method that can distinguish between phosphorylated and nonphosphorylated substrate. To do this, two different approaches have been developed. The first approach, pioneered by Lee *et al.* [15], involves the use of a fluorescent peptide substrate for a kinase that can be introduced into an oocyte, sampled over time and analyzed by capillary zone electrophoresis. This method allows the separation of extremely small volumes of substrate and product peptides based on differences in their electrophoretic mobility through the capillary. Direct quantification using a laser-induced fluorescence detector allows the measurement of the kinetic rate of substrate turnover by a given kinase. This technique has been used to measure the activity of protein kinase C (PKC), cdc2K and protein kinase A (PKA) in *Xenopus* oocytes following treatment with pharmacological and physiological stimuli. The same

method has been applied to other systems that use rat basophilic leukemia and mouse Swiss 3T3 cells [16]. A second method directly measures protein kinase activity *in situ* using a kinase-specific probe that changes its fluorescence properties upon phosphorylation [17]. When conjugated to a short peptide-substrate, the fluorochrome acrylodan shows a dramatic decrease in its fluorescence intensity following phosphorylation. Using this type of reporter probe, PKC activity following L-glutamate activation was measured in primary hippocampal neurons [17]. The advantage of this reagent is that it allows dynamic measurement of changes in substrate fluorescence without the need for taking samples and/or measurements outside the cell. However, substrates with optimal quenching properties might be difficult to design for all members of the kinase family.

Near infrared (NIR) fluorogenic substrates

The recent development of NIR-conjugated imaging reagents has allowed the noninvasive visualization of enzyme activity in whole organisms [18]. The high penetration of photons emitted by NIR fluorochromes, together with the low autofluorescence of nontarget tissues in the NIR spectrum, has made NIR-based reagents ideal for *in vivo* imaging. Furthermore, because NIR fluorochromes undergo endogenous quenching when positioned in close proximity to one another, it is possible to use these molecules as molecular beacons for protease activity [18]. Such protease-responsive reagents are made of a polylysine scaffold in which multiple lysine side-chains are modified with the cyanine-based dye Cy5.5. Protease-mediated cleavage of the amide bond, which positions the Cy5.5 molecules in proximity of one another, rescues quenching and results in a marked increase in NIR fluorescence intensity (Figure 2). To direct the specificity of probes towards individual proteases, peptide substrates have been incorporated between the polylysine side-chains and the fluorochrome. Protease-sensing NIR probes have been generated to monitor proteases such as lysosomal cathepsins [19,20], metalloproteases [21] and coagulation enzymes [22,23]. In addition, the new generations of NIR detectors allow tomographic imaging of protease activity with high positional accuracy [24,25]. Because this method has relatively high resolution (1–2 mm) and allows quantitative measurements of signals, it can be integrated with *in vivo* trials of inhibitors to provide direct pharmacodynamic information for lead compounds [26,27].

Protein–based reporters of enzyme activity

Fluorescent reporters

The green fluorescent protein (GFP) from *Aequorea victoria* has become a key element of virtually all protein-based probes designed to image molecular and cellular events [28]. GFP can be engineered through mutagenesis to yield multiple fluorescent protein tools with various physical properties. GFP variants have been generated that harbor improved fluorescence properties and possess different absorbance and emission spectra [29]. Fluorescent proteins have been extensively used as markers for gene expression, as spatial and temporal protein reporters and as tools to detect protein–protein interactions [30]. The latter application takes advantage of the FRET. The most commonly used FRET reagents are based on the cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) pair, although the recent development of stable red fluorescent proteins (RFPs) has expanded the applications of FRET-based reagents [31].

Recently, the use of FRET has been extended further to design biochemical tools for monitoring enzymatic activity inside cells. Incorporation of a cleavable linker between two GFP variants provides a construct that can be used for

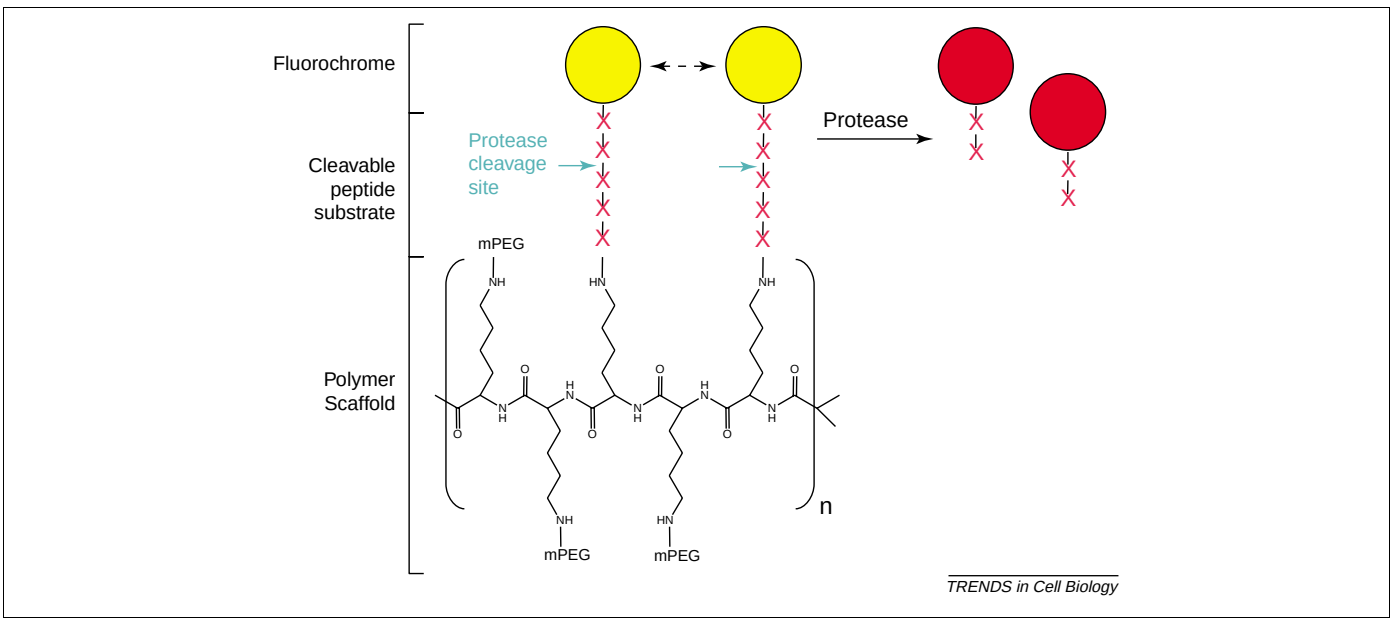

Figure 2. Near infrared (NIR) fluorogenic reporters for *in vivo* imaging. The structure of a NIR-imaging reporter for monitoring MMP-2 activity *in vivo* is depicted. NIR fluorochromes are grafted onto a copolymer (methoxy-polyethylene-glycol-derivatized poly-L-lysine) through a protease-sensitive peptide linker. The release of quenched fluorescence occurs following protease-mediated cleavage of the linker substrate [27]. 'X' designates amino acid or specificity elements that are incorporated into the peptide substrate.

monitoring protease activity (Figure 3a) [32–35]. In this way, it is possible to visualize the execution of apoptosis in a single cell by monitoring the caspase-mediated cleavage of the substrate. The major drawback associated with the use of CFP and YFP is their relatively weak fluorescence compared with the wild-type GFP. In addition, cells show stronger autofluorescence when excited by the blue wavelength of light. To address these problems, investigators have developed a novel FRET-based fluorescent readout that makes use of spectrally similar GFP molecules. This technique, called fluorescence lifetime imaging microscopy (FLIM) [36], detects changes in fluorescence that occur on a nanosecond time scale by measuring changes in the phase and modulation of fluorescence lifetime. In this method the excitation light is modulated in a sinusoidal fashion. The fluorescence intensity shows a delay or phase-shift (τ_Φ) and a modulation depth (τ_m) that depend on the decay constant of the fluorescence material and the modulation frequency. This method could be used in combination with FRET so that changes in τ_Φ and τ_m fluorescence lifetime can predict FRET efficiency for each pixel of the image. These measurements can be spatially determined to generate an image that will reflect the proximity of the donor and acceptor at defined locations within a cell. This method was applied for measuring caspase-3 activity within cells in which apoptosis was induced [37].

A FRET-based reporter probe was also designed to detect changes in kinase and phosphatase activity within cells. In the first approach, a peptide harboring a phosphorylation consensus sequence was inserted between the donor and acceptor fluorescent proteins. The conformational change induced by phosphorylation results in a spectral shift of the FRET chimera (Figure 3b). Using this approach, Nagai *et al.* [38] developed a PKA-activity reporter that utilizes red-shifted and blue-shifted GFP variants (RGFP and BGFP) linked through a peptide substrate from the kinase-inducible domain of the CREB-binding protein (Figure 3a). A second approach utilizes a CFP–YFP FRET linker that contains a kinase substrate fused to a specific phosphopeptide-binding domain. Phosphorylation of the peptide promotes the interaction of the two domains leading to a shift in the fluorescence properties of the FRET construct (Figure 3c). Similar FRET-based fluorescent reporters have been generated for both serine [39,40] and tyrosine [41] kinases. Because the dephosphorylation of a substrate can revert the fluorescence spectrum of the kinase reporter, readout of activity of both kinases and phosphatases can be achieved using these substrates.

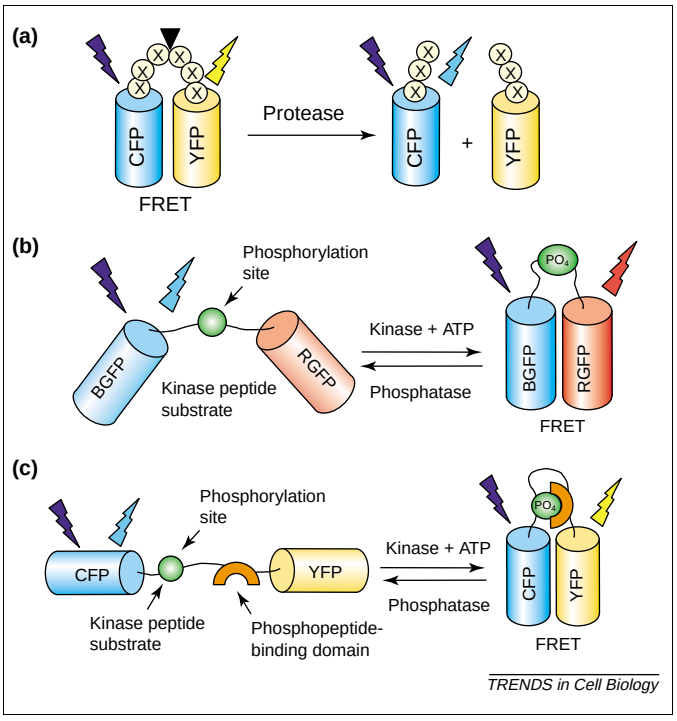

Figure 3. Cell-based imaging reporters using mutant variants of the *Aequera victoria* GFP. **(a)** A FRET-based reporter used for the detection of protease activity in live cells [32–34]. FRET between a donor CFP and an acceptor YFP can be perturbed by protease-mediated cleavage of the peptide linker. **(b)** A FRET-based reporter of kinase activity. Modulation of FRET as a consequence of phosphorylation is achieved by a conformational change of the fluorescent fusion protein. **(c)** FRET-inducing conformational change of a CFP–YFP chimera as a result of the binding of the phosphorylation site of the kinase-substrate to a recombinant phosphopeptide domain following phosphorylation. 'X' designates amino acid or specificity elements incorporated into the peptide substrate. BGFP, blue-shifted GFP; CFP, cyan fluorescent protein; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; RGFP, red-shifted GFP; YFP, yellow fluorescent protein.

Bioluminescent imaging reporter probes

Most recently, new imaging techniques have emerged that use the bioluminescent firefly protein, luciferase. The major advantage of using bioluminescence reporters is their high sensitivity, combined with their minimal background. Laxman *et al.* [42] have designed a novel luciferase-based reporter probe for the imaging of caspase-3 activity. This probe is comprised of a luciferase protein flanked by two estrogen receptor (ER) regulatory domains that are linked through a caspase-3-peptide substrate. In the intact fusion protein, luciferase activity is silenced because of the steric inhibition of luciferase luminescence mediated by the binding of heat-shock proteins to the

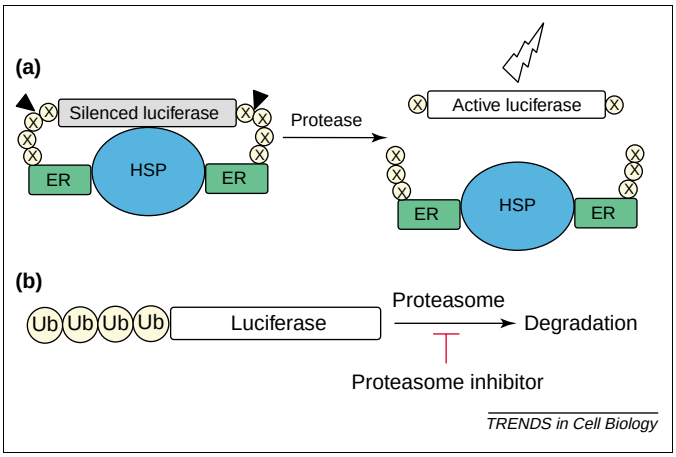

Figure 4. Bioluminescent reporters for *in vivo* imaging. **(a)** A luciferase fusion protein flanked by estrogen receptor regulatory domain (ER) through a cleavable linker, which is silenced because of the contact inhibition; the inhibition is mediated by the binding of a heat shock protein (HSP) to the fusion protein [42]. 'X' designates amino acid or specificity elements incorporated into the peptide substrate. **(b)** The structure of a ubiquitin–luciferase fusion protein designed to monitor proteasome activity and inhibition [43].

ER domains of the reporter (Figure 4a). The cleavage of the ER silencing domains by caspase-3 results in a 10-fold increase in luminescence. Importantly, this technology was used for real-time imaging of caspase activity in reporter-expressing tumors implanted into mice [42]. The induction of apoptosis resulted in a threefold increase in bioluminescence compared with the uninduced control animals. In another study, Luker *et al.* [43] designed a bioluminescent ubiquitin–luciferase (Ub–Luc) fusion protein reporter to image the activity of the 26S proteasome. In cells transfected with this probe, a rapid attenuation of bioluminescent signal was observed owing to the rapid proteasome-mediated degradation of Ub–Luc (Figure 4b). *In vivo* imaging of proteasome activity was performed by xenografting cells expressing Ub–Luc into nude mice. A comparison of the signal of construct-expressing cells with xenografts of cells expressing wild-type luciferase provided a readout of signal loss caused by proteasome activity. Interestingly, the efficacy of the proteasome inhibitor bortezomid could be determined using this imaging approach, suggesting that it can be a powerful method for monitoring the efficacy of small-molecule drugs in a rapid and noninvasive manner.

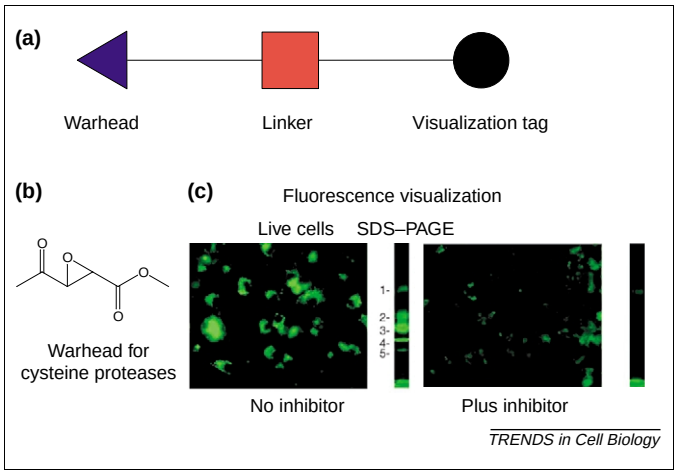

Figure 5. Visualizing enzyme activities in live cells using activity-based probes (ABPs). **(a)** General structure of an ABP. **(b)** Chemical structure of a cysteine-protease-reactive warhead. The epoxide warhead is derived from the natural product E-64, a potent inhibitor of cysteine proteases. **(c)** Images of the activities of cysteine protease in live cells [50]. Cells from the dendritic cell line DC2.4 were incubated with a fluorescently tagged ABP containing the epoxide warhead in the absence or presence of a cysteine-protease inhibitor. Fluorescence was visualized in live cells by either microscopy or lysing cells and separating proteins by SDS–PAGE. At least five different cysteine proteases are enzymatically active in this cell line.

Activity-based enzyme modifiers

Virtually all of the methods described above use reporter substrates for imaging of enzyme activity. Although many of these methods are useful for activity-based imaging, they all suffer from the selectivity of probes for a specific enzyme target (i.e. a peptide has the potential to function as a substrate for more than one class of proteolytic enzymes). Furthermore, these methods require both the use of bulky cell-impermeable molecules and the introduction of reporter probes through genetic manipulation of cells or organisms. An alternative method of visualizing enzyme activities uses small-molecule probes that covalently attach to an enzyme-target using chemical interactions that are specific for the target enzyme(s). Thus, covalent modification serves as an indirect readout of activity. These probes are called activity-based probes (ABPs). In contrast to fluorogenic substrate reporters, ABP-labeled proteomes can be analyzed using biochemical methods, thus allowing the specific identification of the individual enzymes that are active within a given protein milieu. Furthermore, selectivity for individual or subset of enzymes can be controlled by the nature and type of the reactive group on the probes [44–46].

ABPs generally contain three main functional groups: the chemically reactive group or warhead, which covalently modifies an active-site residue of the enzyme of interest; a linker region, which can be specific for different enzymes; and a tag, which is used to visualize the modified enzyme (Figure 5a). ABPs have been developed to target many enzyme families including cysteine and serine proteases, kinases and phosphatases. The visualization of labeled enzymes involves the use of SDS–PAGE to separate target proteins followed by direct visualization of the tag on the ABP [44–46].

Although most of the applications of ABPs have been *in vitro*, there are several examples of enzymes that have been labeled *in situ* and *in vivo*. In general, the use of ABPs *in vivo* has been difficult because most ABPs are large multifunctional molecules that carry cell-impermeable tags. However, in some cases ABPs have been developed that are freely cell permeable because of the general hydrophobic nature of the molecules. Darzynkiewicz and colleagues [47,48] have used fluorescent ABPs to monitor the increase in caspase activity after the induction of apoptosis in a variety of cell lines; probes for this application are now commercially available. These probes can be used for relatively high-throughput quantitative assay of apoptosis and the results correlate well with the more classical readouts of apoptosis such as DNA fragmentation. Additionally, ABPs that target the papain family of cysteine proteases have been used to label these proteases *in situ* (Figure 5b). In these experiments, either a fluorescent or biotinylated ABP was used to localize enzyme activity to the lysosomes in live cells or to identify enzymes that are associated with the development of cataracts in mouse lens tissue [49,50].

Two groups have reported methods of using small untagged ABPs that are functionalized and thus accept chemical ligation of a visualization tag following enzyme labeling [51,52]. In the first example, Cravatt and colleagues [51] gave the only report of the use of an ABP for labeling enzymes *in vivo*. An ABP was developed that contained a small chemical group to facilitate the attachment of a fluorophore. A chemical ligation reaction developed by Sharpless and colleagues [53] was used to couple a fluorescent tag to the probe. In this experiment, the untagged ABP was injected into mice, tissues were removed and chemical ligation of the fluorophore tag was performed *in vitro*. The specific activity of heart enoyl CoA hydratase was visualized using this method. In the second example, Ovaa *et al.* [52] used a similar technique to label proteasome subunits with an irreversible inhibitor, using chemical ligation to attach a biotin tag for visualization. These experiments used a chemical ligation method

developed by Bertozzi and colleagues [54,55] and were performed in tissue culture cells, not live animals. The development and utilization of such novel capturing techniques are significant steps towards making ABPs smaller and more cell-permeable – two parameters that are crucial for imaging enzyme activity *in situ* and *in vivo* using ABPs.

Concluding remarks

Recent developments in the design of novel reporters for enzymatic activity have made it possible to visualize enzyme function in a physiologically relevant setting. Imaging probes have been designed that use both substrates and small-molecule covalent labels for tracking the activation of enzymes. The use of probes for noninvasive optical imaging of mouse models of disease has the potential to make a major contribution to the process of validating pharmacological models and assessing therapeutic efficacy of drug candidates. Although there are still relatively few examples of probes that can be used for direct readout of enzyme activity, many other modalities for imaging exist that can be applied to generate new probes for this purpose. Techniques such as positron emission tomography and magnetic resonance imaging have already begun to incorporate the use of 'smart probes' to detect gene expression and molecular interactions [56–58]. Furthermore, multifunctional reagents that can be used with several imaging modalities are also becoming available [59,60]. Finally, the recently emerged field of functional proteomics is evolving rapidly and providing better tools for 'mining' the proteome and for the detection of enzyme activity *in vivo* [51]. Such techniques will facilitate the identification and validation of new drug targets and the assessment of issues related to bioavailability and potency *in vivo* by direct noninvasive measurement of pharmacokinetic and pharmacodynamic parameters.

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