Title: Caught green-handed: methods for *in vivo* detection and visualization of protease activity.

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Highlights: This review collects the existing methods and strategies available to detect and visualize proteolysis *in vivo*, focusing on the most relevant techniques that can be implemented in a lab.

Abstract:

Proteases are enzymes that cleave peptide bonds of other proteins. Their omnipresence and diverse activities make them important players in protein homeostasis and turnover of the total cell proteome as well as in signal transduction in plant stress response and development. To fully understand protease function, it is of paramount importance to assess when and where a specific protease is active. Here, we review the existing methods to detect *in vivo* protease activity by means of imaging chemical activity-based probes and genetically encoded sensors. We focus on the diverse fluorescent and luminescent sensors at the researcher's disposal and evaluate the potential of imaging techniques to deliver *in vivo* spatiotemporal detail of protease activity. We predict that in the coming years, revised techniques will help to elucidate plant protease activity, functions and hence expand the current status of the field.

Key words: activity-based probes, *in vivo* imaging, fluorescent, luminescent, protease, plants, proteolysis, sensor, reporter.

Abbreviations:

ABP: Activity-based Probes.

BRET: Bioluminescent Resonance Energy Transfer.

CA-GFP: Caspase Activatable GFP.

CFP: Cyan Fluorescent Protein.

DEVD: minimal consensus cleavage site for caspase-3 activity composed by aspartate-glutamate-valine-aspartate, processed after the C-terminal aspartate of the motif.

eGFP: Enhanced variant of GFP containing double point mutations (F64L and S65T) which enhances photostability and folding efficiency.

FRET: Förster Resonance Energy Transfer.

GAL4: Galactose 4 transcription factor.

GFP: Green Fluorescent Protein derived from jellyfish *Aequorea victoria.*

HCV: Hepatitis C Virus.

HIV: Human Immunodeficiency Virus.

mRFP / RFP: monomeric Red Fluorescent Protein; Red Fluorescent Protein.

NLS: Nuclear Localization Signal.

PCD: Programmed Cell Death.

TEVp: Tobacco Etch Virus protease.

UAS: Upstream Activating Sequence.

UV-C: Ultraviolet C radiation which can act as germicidal.

VC3AI: Venus Caspase-3 Activation Indicator.

YFP: Yellow Fluorescent Protein with a T203Y mutation related to GFP. Improved versions of YFP include Citrine and Venus.

1. Introduction

Proteases exert a tight control on cellular functions by breaking the polypeptide chain of their substrate proteins. Substrate cleavage is the result of the recognition between the target amino acid sequence of a substrate and the binding pocket of a protease. This action can lead to changes in the localization, biomolecular interactions, turnover or enzymatic activity of their substrates. The importance of proteases across biological kingdoms and viruses cannot be overstated as they intervene in most developmental processes and responses to environmental cues (Turk, 2006; van der Hoorn, 2008). Proteases are also quite numerous, for example *Arabidopsis thaliana* has a reviewed number around 600 proteases, totalling to 2% of the protein coding genes, similar in number and family conservation to other plant species like rice and poplar (García-Lorenzo *et al.*, 2006; van der Hoorn, 2008; Lallemand *et al.*, 2015).

Proteases are classified based on the catalytic amino acid inside their binding pocket, being cysteine, serine, aspartic acid, threonine or glutamic acid, whereas metalloproteases use a coordinated metal ion to catalyze peptide bond hydrolysis. In cysteine, serine and threonine proteases, the peptide bond is broken after nucleophilic attack of their respective catalytic amino acid aided by a molecule of water on the carbonyl group of the peptide bond. For metallo-proteases, aspartic and glutamic acid proteases, cleavage occurs by activation of a water molecule which then performs the nucleophilic attack. Asparagine peptide lyases, are also capable of peptide bond cleavage by an elimination reaction, and their classification as proteases is still under debate given the differences in their catalytic mechanism (Rawlings *et al.*, 2011). Until the date, such enzymes have not been identified in plants.

The actual protease recognition site in the substrate is generally defined by the amino acids surrounding the scissile peptide bond and these amino acids are denoted as P and P' ($P_4P_3P_2P_1{\downarrow}P'_1P'_2P'_3P'_4$) where the downward arrow indicates the peptide bond cleavage (Schechter and Berger, 1968). Proteases can show narrow substrate selectivity, like thrombin and its minimal substrate LVPR↓GS (Gallwitz *et al.*, 2012), to have a broader substrate selectivity such as trypsin that

cleaves after arginine and lysine (Olsen *et al.*, 2004). Many proteases have a certain degree of permissibility in their substrate sites such as the Tobacco Etch Virus protease, which recognizes the optimal substrate sequence ENLYFQ↓S but also recognizes EXLYXQ↓X sequence where X denotes amino acids with similar properties to those found in the optimal substrate sequence (Boulware *et al.*, 2010; Sandersjöö *et al.*, 2017). Considering protease specificity and activity redundancy over a common substrate are of main importance when designing a probe for *in vivo* applications. In general, most part of the substrates should be as specific as possible for a single protease and in some particular cases the detection of a processed substrate can confirm entrance in specific biological processes. This is well exemplified by caspase-3 and caspase-7 which are cysteine proteases that cleave after aspartic acid (D) and are considered triggers of apoptosis.

To understand the *in vivo* relevance of protease activities, it is important to know their spatiotemporal activity profiles. Gene expression and protein distribution patterns in plant tissues can certainly offer some clues to the role of a particular protease. However, their activity is often strictly restrained following their synthesis. For most proteases, initial protein synthesis produces an inactive or zymogenic proteoform. Subsequently, a first layer of control is exerted by zymogen activation during which for instance an inhibitory propeptide is catalytically removed, as it is the case for subtilisin-like serine proteases or subtilases in plants (Meyer *et al.*, 2016). Other proteases lack clear inhibitory propeptide signals and instead require cleavage of internal sites for their activation. One example is the cleavage of the linker region between the p20 and p10 domains of metacaspases and caspases (Salvesen *et al.*, 2016). Additional internal cleavages or maturation of proteases can further affect their activity (Gu *et al.*, 2012). Protease activation can also occur following a switch in the microenvironment such as altered pH, elevated calcium levels or other activating conditions, such as proximity induced multimerization (Lam and Zhang, 2012). Post-translational modifications (PTMs) may further determine a protease's activation state. For example, the catalytic cysteine of Arabidopsis metacaspase 9 (AtMC9) is reversibly regulated by S-nitrosylation that blocks its activity (Belenghi *et al.*, 2007). Other proteases in Arabidopsis such as ClpP1, Deg2 and ClpC2 were also found to be S-nitrosylated, but the importance of this modification is not clear

(Romero-Puertas *et al.*, 2008; Hu *et al.*, 2015). Proteases are also regulated by proteinaceous inhibitors (protease inhibitors or PIs) that bind the catalytic site, thereby blocking the capability to process their substrates (Grosse-Holz and van der Hoorn, 2016). Such interactions can be reversible, as it occurs for certain cysteine proteases and their inhibitors, cystatins (Benchabane *et al.*, 2010), or irreversible, for example SERPIN1 that traps both AtMC9 and RD21A (Vercammen *et al.*, 2006; Lampl *et al.*, 2013; Grosse-Holz and van der Hoorn, 2016). PIs themselves are subject to regulation under various stress or developmental conditions, and a given protease can be regulated by multiple PIs (Rustgi *et al.*, 2018), thereby further increasing the repertoire to fine-tune regulation of protease activities. Lastly, proteases localize to all sub-compartments in cells (van Wijk, 2015) and protease activity can depend on relocation of proteases in cells. For example, phytaspase delocalizes from the extracellular to the intracellular space (Chichkova *et al.*, 2010) and vacuolar processing enzymes first need to be released into the cytosol during programmed cell death (Hatsugai *et al.*, 2015).

Researchers studying proteases often use peptidic probes that report proteolytic activity. Ideally, such probes are used *in vivo* to evaluate protease activities in their natural cell environment, while delivering spatiotemporal resolution. Over the years, increasing knowledge of protease substrate specificity has allowed to develop better tools for studying protease activity. Here, we review the use of chemical probes and genetically encoded sensors in plants and future expectations. We then widen the horizon towards techniques available for *in vivo* spatiotemporal protease activity detection in the mammalian protease field, which are expected to be implemented in plant cells.

2. Chemical probes

2.1. Chemical activity-based probes

Activity-based probes (ABPs) are chemical probes capable of detecting the catalytic enzymatic site of an enzyme (Heal *et al.*, 2011). ABP can be synthesized from a known chemical inhibitor which is generally termed warhead, followed by a linker that

can resemble an amino acidic sequence allowing a certain degree of specificity in the design and finally a tag that can be used with different purposes such as pull-down or imaging. These chemical probes mimic a substrate cleavage/scissile bond and lock the proteolytic mechanism in a covalent intermediate stage (Sanman and Bogyo, 2014). Inherently, ABPs only exist for proteases that react with their substrate through a covalent intermediate like for cysteine, serine and threonine proteases, but not for aspartic, glutamic acid and metallo-proteases (van der Hoorn, 2008). Probes for the latter classes are photo-affinity probes based on reversible inhibitors and require UV exposure to establish a covalent bond (Li *et al.*, 2000). ABPs entail a reporter tag to facilitate detection. Fluorescent reporter tags are practical tools for cell biology studies, usually permit cell entry, but do not report on the identity of the labelled proteases. By contrast, biotinylated probes can be readily used to purify and identify the labelled proteases by means of mass spectrometry, but the entry of biotinylated probes into the cell is often problematic. To overcome this issue, chemical moieties called “minitags” can be coupled to e.g. fluorophores or biotin using a bio-orthogonal chemical coupling reaction, such as click chemistry between alkynes (C≡C) and azides (N_3) (Speers and Cravatt, 2004; Kaschani *et al.*, 2009b). Minitagged probes are efficient to cross cellular membranes, reason why they are frequently used for *in vivo* labelling. However, minitags detection usually involves a Cu^+-catalysed coupling reaction in the protein extraction (*ex vivo*). Other two-step labelling processes include the Staudinger-Bertozzi ligation and the Diels-Alder ligation (Verdoes and Verhelst, 2016). Using specific coupling protocols and reagents, it is possible to perform the coupling reaction *in situ* like in fixed tissues (Paper *et al.*, 2018), or even *in vivo* (Chang *et al.*, 2010).

Fluorescently labelled proteins can be visualised in protein gels after electrophoresis (profiling) or by microscopic imaging (Fig. 1A). Although nearly all fluorescent ABPs can be used for *in vivo* labelling and profiling, imaging makes little sense if the probes are not sufficiently specific. For instance, active serine proteases can be efficiently profiled *in vivo* with fluorophosphonate probes (Liu *et al.*, 1999; Kaschani *et al.*, 2009a). However, these probes label over 50 different proteins, making life-cell imaging with such probes not very informative due to redundancy of activity over one ABP. In fact, more selective and specific probes are better suited for activity-based imaging. For instance, three fluorescent ABPs targeting the proteasome revealed

quick labelling from 1 minute for subunits β2 and β5, while subunit β1 was labelled in a timeframe of around one hour (Kolodziejek *et al.*, 2011).The probes were designed based on known proteasome inhibitors from vinyl sulfones, epoxomicin and syringolin A. Another probe designed for detection of vacuolar processing enzyme probes displayed fluorescence in the vacuole of plant cell cultures and leaves while this signal was absent in vacuolar processing enzyme null mutants (Misas-Villamil *et al.*, 2013).

An important technical limitation of fluorescently labelled probes is that they fluoresce also when not bound to a target and may thus cause background signals that obscure detection of labelled proteins. This problem is often solved by washing out non-reacted probes or by using low probe doses such that eventually all probes are immobilized on their targets. An elegant alternative solution are quenched probes, which have been described for various cysteine proteases but were mostly used in mammalian systems (Blum *et al.*, 2005; Edgington *et al.*, 2009; Verdoes *et al.*, 2012; Edgington *et al.*, 2013). These probes contain a quenching group that suppresses the fluorophore emission of light until the probe interacts with the protease (Edgington-Mitchell *et al.*, 2017).

Although activity-based imaging also has limitations because these probes inactivate the labelled proteases, and that some labelling time (up to one hour) is needed to achieve labelling, the great advantage of these probes is that after imaging, the labelled proteins can also be detected upon separation of extracts on protein gels to identity the labelled proteins.

2.2. Chemical protease substrate probes

Together with ABPs, the utilization of fluorescent chemical reagents that react to protease activity can be of interest for the plant field. For example, DEVD-NucView488 (Fig. 1B) can be used to visualize caspase by nuclei labelling (Cen *et al.*, 2008). It contains a DNA intercalating reagent similar to thiazole orange that react with a negatively charged group of DEVD hiding fluorescence. This motif is cleaved by caspases after the second aspartic acid (DEVD↓) leading to subsequent DNA binding of the reagent and thereby inducing detectable fluorescence. When indole-3-carbinol is applied to trigger apoptosis to cell cultures, the nuclei of these cells are thereby fluorescently labelled. In plants, caspase-3 and -7-like activities

have been detected in the cytosol, generative cell and vegetative nuclei of *Papaver roheas* pollen (Bosch and Franklin-Tong, 2007, 2008) using CR-$(DEVD)_2$. In this probe, two DEVD peptides were added to cresyl violet, which quenches its fluorescence (Fig. 1C). DEVDases and proteases with caspase-3/7-like activities release the DEVD peptides, uncovering the compound's fluorescence. With this probe, caspase-3-like activity was detected in the vegetative nucleus, cytosol and generative cells of pollen tubes of poppy plants during induction of self-incompatibility. Despite the fact that other proteases are known to be involved in pollen programmed cell death (PCD) in Arabidopsis (Zhang *et al.*, 2014), the actual poppy plant proteases involved remain unidentified. These probes were further developed to include infrared fluorophores that can be used to image in deeper tissues of life animals and are now developed to image malignant tumours during surgery (Blum *et al.*, 2007; Edgington *et al.*, 2009; Verdoes *et al.*, 2012).

3. Direct-fluorescent sensors of proteolytic activity

Most of the existing proteolytic sensors are based on modifications of eGFP (Fig. 2A and 2B) and its derived fluorescent proteins. The use of these proteins can be advantageous due to their modifications in emission and excitation parameters. It is also a point to consider that other proteins can outperform eGFP in specific environments like super folding versions of GFP, Cherry2, or split mNeonGreen2 (Feng *et al.*, 2017) and that researchers can profit of them to engineer protease activity sensors in the future.

3.1. FRET sensors

Förster Resonance Energy Transfer (FRET) applications are nowadays employed in almost every field of cell biology and have the longest history as genetic protease reporters (Weiss, 2000). FRET is based on energy transfer between two proteins or particles in a spatial contiguity by a linker containing a specific amino acid sequence. Upon excitation, the donor transmits its energy to the acceptor which is then excited and emits fluorescence. Once the proximal position is lost, the acceptor signal decreases and the donor signal enhances (Fig. 2C). FRET proteolytic biosensors include detection of Xa protease (Mitra *et al.*, 1996), thrombin (Zhang, 2004), trypsin (Heim and Tsien, 1996), caspase-1(Mahajan *et al.*, 1999), caspase-3 (Xu *et al.*,

1998; Luo *et al.*, 2001) and caspase-8 (Luo *et al.*, 2003), anthrax lethal factor protease (Kimura *et al.*, 2007), matrix metalloprotease (MMP) 1 (Ouyang *et al.*, 2008), MMP-2 (Yang *et al.*, 2007) and MMP-9 (Stawarski *et al.*, 2014); ADAM 17 protease (Chapnick *et al.*, 2015), neutrophil elastase (Schulenburg *et al.*, 2016), Hepatitis C Virus (HCV) NS3 protease (Sabariegos *et al.*, 2009) and calpain (Vanderklish *et al.*, 2000; Stockholm *et al.*, 2005).

FRET reporters are made of two fluorescent proteins with complementing features in emission and absorbance. Some improvements were made aimed at increasing FRET efficiency. For example, two mutations in the surface between the interaction of a YFP variant and a Cyan Fluorescent Protein (CFP) increases FRET 4-fold compared to the original versions of these proteins (Vinkenborg *et al.*, 2007). More recently, novel FRET sensors included the use of weak interactor peptides at the fluorescent proteins, bringing them spatially together and enhancing energy transfer (Grunberg *et al.*, 2013). This set of sensors was named helper-interaction FRET and proteolytic sensors were arranged to recognise caspase activity using LDEVD as linker between mTurquoise2, and mCitrine, which are improved versions of CFP and YFP respectively. The position of the weak interactor peptides was tested at the N- and C-termini of the sensor or included in structural loops of the fluorescent proteins. Upon caspase-3 activity, the linker is cleaved, and despite the helpers, the proximity is lost and the protease acceptor loses its fluorescence in higher ratios than common FRET sensors that did not include the interaction helpers.

In plants, FRET sensors are routinely used for the detection of protein-protein interactions and intracellular signals such as calcium levels, abscisic acid, pH levels or ATP concentration and have been extensively reviewed in (Grossmann *et al.*, 2018). However, reports on successful use of genetically encoded sensors for plant proteases are scarce. Zhang and colleagues used FRET based sensor with an amino acid linker, separating the two chromophores, containing a DEVD sequence for the detection of caspase-3-like activity in Arabidopsis protoplasts when exposed to UV-C light (Zhang *et al.*, 2009). Exposure to UV-C light for less than half an hour resulted in a reduced FRET signal with a maximum at 1 hour after treatment, indicating a relatively high activation by caspase-3-like proteases. The reduction in signal observed was not detected in the negative control reporter that used the DEVG linker sequence that is not recognized by caspase-3-like protease. Caspase-

3-like activity is a recurrent topic in the field of plant proteases although there are no genuine caspase genes found in plant genomes, and though inhibitors of caspases generally block plant PCD (Sueldo and van der Hoorn, 2017). A FRET based biosensor was also used in plant protoplasts for the detection of a latent peptidase activity of the ubiquitin-activated peptidase DA1 (Dong *et al.*, 2017). The approach used eGFP and mCherry flanking the whole sequence of DA1 substrate, Big Brother instead of the conventional YFP/CFP pair. An increase in the green fluorescence lifetime, which in this case corresponded to the donor eGFP, was shown when DA1 was co-expressed, indicating that mCherry part is distant and that FRET decreased.
Apart from reporting protease activity *in vivo* and *in vitro,* FRET reporters can serve as markers of certain pathways like apoptosis and to screen for inhibitors of enzymatic activities (Jones *et al.*, 2000; Zhang, 2004). Another FRET application is the generation of randomized substrate libraries to detect protease specificity (Fretwell *et al.*, 2008) as a way of designing fluorescent probes that are as specific as possible for the protease of interest.

3.2. Fluorescence complementation

The newest generation of genetic fluorescent protease reporters use fluorescence complementation. This mechanism relies on split versions of fluorescent proteins with null or decreased fluorescence emission, which in proximity can complement and reconstitute fluorescence (Ohad *et al.*, 2007). Although this technique has been mainly exploited for the detection of protein-protein interaction such as bimolecular fluorescence complementation (BiFC) it can be tuned for the detection of proteolytic activity. Here we include designs where both parts are designed as separated segments to recompose fluorescence and rearrangements in the amino acid composition that disturb protein structure or destabilizes chromophore formation. To detect protease activity, it is necessary that these reporters include a protease recognition sequence that partially reconstitutes or enhances fluorescence after processing (Fig. 3). Some of these sensors are conceived as individual sensors (Fig. 3A-C and 3E), others have a stable additional fluorescent signal, independent of proteolysis, to obtain a ratiometric signal (Fig. 3D). Structural loops are candidate regions in which protease cleavage sites can be introduced, though probe design has to be carefully considered and success can depend on both the protease and

the cleavage site used. For instance, eGFP loops 7, 8 and 9 were modified to include a motif that is known to be cleaved by trypsin (Chen *et al.*, 2009b). The design including grafting at several locations such as the glutamic acid in the loop 9 at position 172 of the fluorescent protein by introducing a linker of 31 amino acids, showing a better response after incubation with trypsin *in vitro* and after induction of trypsin formation with caerulein in pancreatic cell culture with increased levels of trypsinogen, which is the trypsin precursor. The same design showed positive results when studying caspase-3 activity by crafting DEVD-motifs, overall indicating that previous mentioned glutamic acid at loop 9 might be well suited to develop new protease reporters upon implementing minimal substrates (Chen *et al.*, 2009a) (Fig. 3A).

More recently, Callahan and colleagues modified a split GFP protein reporter system (Kamiyama *et al.*, 2016) by partially caging a GFP11 β-strand that has a high affinity for the remaining GFP1-10 β-strands (Callahan *et al.*, 2010). In this reporter named Pro-GFP, the GFP11 β-strand replaces the exposed loop of the protease inhibitor eglin c with a proteolytic site at the C-terminal part of this β-strand. Both inhibitory domains are highly stable and remain together even after cleavage however, cleavage concedes enough flexibility for GFP11 to complement GFP1-10 (Fig. 3B). This sensor was also tailored for the detection of thrombin, caspase-3 and HIV proteolytic activity, showing a 6-fold increasing in signal when the proteases were present.

VC3AI is a single protein biosensor for caspases 3 and 7 (Zhang *et al.*, 2013). VC3AI stands for Venus Caspase-3 Activity Indicator shaped by a circularly permuted YFP protein with a F46L mutation called Venus. In its design, the original design of N- and C-halves of Venus are inverted and ligated using a typical caspase cleavage site. VC3AI is flanked by inteins (Mills *et al.*, 2014), which are peptides that can self-excise leading to association of the remaining adjacent sequences, in this case both ends of the permuted sensor which is then circularized. The structure of the circular protein constricts the Venus moieties in a format with decreased fluorescence, which, after cleavage, is loosen up and shows higher levels of fluorescence (Fig. 3C). Detection of apoptotic events in tumour cell culture showed a 10-fold fluorescence increase within 8 hours after induction of apoptosis. Here, DEVD

processing was also observed in non-apoptotic cells, which may lead to exploring new functions of caspases apart from cell death, and exemplifies the power of generating new biological hypotheses with these sorts of probes. Versions of this sensor using blue, green and red fluorescent proteins, termed respectively, CC3AI, GC3AI and RC3AI, also successfully reconstituted fluorescence upon cell death induction. This extended colour palette opens diverse possibilities to combine with several cell dyes or markers indicating cell viability and to exploit the intrinsic characteristics of each fluorescent protein. In a further study, such different probes for detecting caspase-3 activity in fruit flies were reported, thereby increasing the applications of the caspase reporter toolkit (Schott *et al.*, 2017).

iProteases are a group of sensors in which the two domains of an Infrared Fluorescent Protein are truncated and fused both N- and C-terminal to self-complementing split eGFP which here acts as the ratiometric signal (To *et al.*, 2015). The domains in the truncated version keep a crucial cysteine distant enough from the site of chromophore formation, that is mediated by biliverdin. This cysteine displacement avoids thioether formation between biliverdin in the binding site and the cysteine, and therefore blocks fluorescence. Using a linker holding specific substrates for TEVp, caspase-3/7 and HCV protease, respective iSensors (iTEV, iCasper and iHCV) were obtained, showing how a single sensor design can be turned into a potent detector of dynamics for different proteases. The analysis of the cleaved purified sensor showed an increase in fluorescence ten seconds after biliverdin addition, detecting immediate response which can be used for quick readout of proteolysis when biliverdin is available. Results *in vivo* indicated detection of apoptosis events in Drosophila in a frame time of 1 hour after staurosporine addition, which is a protein kinase inhibitor which induces apoptosis including caspase activation. The presence of a ratiometric permanent green signal allowed to detect transfected cells independently of protease activity.

Another ratiometric sensor, dubbed ZipGFP was developed for detection of proteases action, where eGFP was first split and interaction of the separate parts was blocked by zipping together the C- and N-terminal ends of β-strands 1 to 10 and β-strand 11 with heterodimers of E5 and K5 coils (To *et al.*, 2016). Ratiometry was achieved by N-terminal fusion of eGFP β-strands 1-10 to mCherry with a T2A

peptide for self-cleavage by ribosomal skipping (Szymczak *et al.*, 2004), producing equimolar amount of protease reporter and mCherry (Fig. 3D). Cleavage of TEVp and caspase-3 substrate sites liberated the E5/K5 heterodimers allowing the cavity of the main structure to be complemented both *in vitro* and during apoptosis of zebrafish embryos. ZipGFP outperformed previously described FRET sensors given a 10-fold increase of eGFP signal, which rarely could be detected by energy transfer (To *et al.*, 2016).

3.3. Single fluorescently labelled sensors

Various sensors use translational fusions to proteins or protein domains that hamper GFP folding, and therefore fluorescence, or reduce lifetime of the sensor due to their instability in a particular cellular environment. For instance, Caspase Activatable GFP sensors (CA-GFP) are based on this concept (Nicholls *et al.*, 2011). Here, eGFP is fused to a 27 amino acid stretch of the monomer of the homotetrameric influenza M2 proton channel protein, bridged by a DEVD sequence. The M2 domain also tetramerized in CA-GFP, thereby hindering correct maturation of the eGFP chromophore and reducing its fluorescence (Fig. 3E). Caspase cleavage freed this quenching domain, thereby enhancing fluorescence 45-fold in bacteria, while only 3-fold in mammalian cell culture. These results illustrate that protease reporters can be highly system-dependent and that they need to be tested and optimized for each organism or cellular context. Interestingly, N-terminal fusions of the M2 domain did not quench eGFP fluorescence while C-terminal fusion gave 40-fold increases for caspase-3/7 (Nicholls and Hardy, 2013). YFP, Cerulean and mNeptune versions were later developed with varying results, probably because of the variable spatial conformation and protein maturation of the different fluorophores (Wu *et al.*, 2013). Similarly, the fusion of GFP to a C-terminal bacterial degron ssrA (AANDENYNYALAA) through different substrates of TEVp showed preference for ENLYFQ↓G over other minimal substrates in *Escherichia coli* (Kostallas and Samuelson, 2010; Kostallas *et al.*, 2011). Here, ssrA is an optimized peptide that induces fast protein turnover of the sensor via the bacterial degradation system shaped by the caseinolytic proteinase XP complex. This knowledge about GFP structure was exploited to permute the last β-strand at the beginning of the sensor that includes proteolytic cleavable sites at the loops between the original β-strands 6 and 7 or β-strands 7 and 8. The circularly permuted GFP reported 50 times faster degradation of Lon bacterial substrates *in vivo* than native GFP, possibly this is due to the decreased thermal stability of the permuted sensors (Wohlever *et al.*, 2013).

3.4. Proteolysis-mediated delocalization sensors

This class of sensors exploits protein localization (and re-localization) to read out cleavage by proteases. The simplest design come from the observation of changes

in cellular compartmentalization using single fluorescently fusion proteins. However, some minimal conditions are required: the fluorescent tag needs to be attached to the mobile component and this tag may not interfere with specific signals or transit peptides present in the protein addressing correct localization. In the cell content, some transcriptional effectors are targeted to other location than nucleus in a state that requires to undergo PTMs for transport to the nucleus where they can exert their function (Andréasson *et al.*, 2006; Iwata *et al.*, 2017). In Arabidopsis, NAC Transmembrane Motif 1 is processed by an intramembrane protease, likely phytocalpain, loses its transmembrane domain and relocates to the nucleus (Kim *et al.*, 2006). The processing was visualized by GFP fused to whole NAC factor and in the truncated versions of the protein, mimicking cleavage site but blocked when the full protein tagged is incubated with specific phytocalpain inhibitors.

Dual-tagged substrate proteins provide information for both parts of a cleaved construct. An example in *Arabidopsis thaliana* is mCherry/GFP-tagged NAC017 at its N- and C-site respectively. Proteolysis occurs after application of antimycin A, an inducer of mitochondrial retrograde signalling. Initially, both GFP/mCherry reside at the endoplasmic reticulum membrane, but the mCherry signal moves to the nucleus after treatment, while GFP remains at the original position. Although no additional experiments to determine the identity of the responsible protease have been performed, the readout can be used as retrograde signalling markers when antimycin A is used (Ng *et al.*, 2013).

A clear example of de-localization based reporters are caspase biosensors containing an N-terminal Nuclear Export signal (NES) sequence followed by a caspase recognition site, YFP and a C-terminal Nuclear Localization Signal (NLS) C-terminal sequence (NES-DEVD-YFP-NLS), being the NES detachable by caspases (Tang *et al.*, 2012). YFP is present in the cytosol and nuclei in normal conditions but turns mainly nuclear after treatment with apoptotic inducers. Something to take into consideration with these sensors is that they only work when the protease and the sensor can meet each other in the same subcellular space, in this case cytosol and/or nucleus. An elegant application of dual fluorescent sensors is the demonstration of elusive separase activity in cohesin cleavage, necessary for sister chromatid disengagement. A cohesin domain was doubly tagged with mCherry and

eGFP at its N- and C-terminus respectively. Additionally, to ensure co-localization with separase to its active cell cycle phase, the sensor was cloned in frame with a centromeric located protein (CENP-B) or histone marker for chromosomal localization (H2B). Separase processing of its substrate led to chromosomal red labelling, while green signal diffused to the rest of the cell. This sensor helped to elucidate how separase regulation occurs in HeLa cells and to detect protease activity in a specific cell cycle phase in a concrete and controlled subcellular location (Shindo *et al.*, 2012).

A successful reporter denoted apoptotic processes in different organisms such as Drosophila and chicken embryos (Bardet *et al.*, 2008). This reporter named apoliner, depends on membrane anchoring using mCD8 followed by mRFP, a BIR1 domain and a NLS-eGFP. Caspase activity was detected both by fluorescence microscopy and immunoblots in a period shorter than one hour in fruitfly cell lines after induction of apoptosis. Initially, in healthy cells membrane co-localization of mRFP and eGFP was found, while the latter transited to the nucleus once caspases were activated. At the final stage of apoptosis, eGFP was likely degraded by additional proteases and mRFP remained as a unique signal at membranes. Apoliner showed no impact on developmental cell death measured by the TUNEL assay in Drosophila and could detect caspase activity in chick embryos faster (6 to7 hours) than by immunohistochemistry (17 hours). Another similar probe used reporters as molecules that change localization after protease cleavage (Kim *et al.*, 2013). Here, both single- and dual-colour fluorescent reporters were used that determined hepatitis C virus NS3 protease activity by miss-localization of one or both of their components. In an appealing approach for the production of multiple proteins in *Escherichia coli* and mammalian cells, a single open reading frame alternating proteins with TEVp cleavage sites showed efficient delivery of fluorescently tagged sub-products (Chen *et al.*, 2010). A correct cleavage and quantification of the different proteins in bacteria was detected on immunoblot. One of the mammalian cell lines included diverse localization proteins to nuclei, membranes and cytosol with masked target peptides which, after processing, label this location with the different fluorescent protein markers, showing flexibility for designing delocalization sensors.

4. Transcriptional reporters

Protease cleavage of membrane-bound transcriptional activators can result in reporter gene activation. Examples of setups relying on this mechanism include CaspaseTracker and CasExpress (Tang *et al.*, 2015; Ding *et al.*, 2016) that study caspase activity during apoptosis and its evasion by anastasis. The term anastasis refers to a Greek word for resurrection. In molecular biology, it indicates the mechanism by which cells experiencing caspase activation are capable to exit apoptosis (Sun *et al.*, 2017). Although caspase activation has been thought to be a point of no return in apoptosis, anastasis is showing the potential of organisms to evade cell death. Both systems exploit the fusion of a membrane-bound mCD8 protein to the yeast transcription activator protein galactose transcription factor (GAL4) through a caspase-3-like recognition sequence (DQVD) and a BIR1 domain for enhanced caspase recognition. Membrane tethering by mCD8 prevents GAL4 to translocate to the nucleus which only occurs upon cleavage at the DQVD site. Once in the nucleus, GAL4 binds to the transcription enhancer Upstream Activation Sequence (UAS) and induces both nuclear targeted RFP expression and expression of a recombinase named flippase. A third component of the sensor, is a cassette that contains a transcriptional stop sequence flanked by recombinase sites followed by an initial untranscribed GFP. Transcription arrest is reverted through recombinase activity, excising the fragment of DNA which is subsequently inherited by the cell-line lineage (Fig. 4A). This system presents a captivating feature by which transient caspase activity and developmental caspase activity can be discriminated. Here, RFP expression reflects relatively fast apoptotic activity, GFP expression accounts for caspase activities during physiological events and development. The main difference between both systems is that with CaspaseTracker GFP is expressed in the cytosol, while with CasExpress, both signals are found in the nucleus.

Splitting transcriptional inductors using a cleavable linker between their DNA binding site and the transcriptional effector also allows to detect proteolysis (Smith and Kohorn, 1991). Recently, a series of new transcriptional reporters of activity has been developed to screen for Hepatitis C Virus NS3 protease inhibitors (Fig. 4B and C) (Tague *et al.*, 2018). NS3 is a protease that cleaves in *Cis*, meaning that it cleaves from its containing sequence, which can be exploited for conditional degradation (Lin *et al.*, 2008; Chung *et al.*, 2015). To study drug-conditioning

proteolysis, Tague and colleagues split Gal4 into its DNA binding domain and its transcriptional activation and translationally fused to a NS3 protease through a linker containing the protease minimal substrates at both sides. In normal conditions the protease can free itself by cleavage, and the spatial contiguity of the components are lost. On the contrary, when the protease activity is inactivated by a specific inhibitor, the whole complex remained intact and moves together to the nucleus, where it recognizes the UAS region leading to H2B-Citrine expression which resulted in nuclear fluorescence (Fig. 4B). Another system uses sequestering of the Gal4 protein by a translational fusion with a protein that localizes in the membrane and NS3 protease as dock. The system is robust enough to keep Gal4 away from the nuclei if there is presence of inhibitor, but on the contrary to the previous system, proteolysis leads to fully active Gal4 to translocate and induce expression of the reporting signal (Fig. 4C). Those are vey elegant examples of both on-to off and off-to on induction and repression of a reporting system based on proteolysis and controlled by inhibitors. In the same study, an inactive Cas9 version serving as location system and transcriptional activation domain was also used. Here, by changing the guide RNA design, any reporter or product of an endogenous gene can be used as readout. In addition, an “inhibitor-off” system was developed by sequestering a full transcriptional activator into the membrane. This system is switched on upon protease activation, allowing the transcription factor to relocate to the nucleus.

5. Bioluminescent sensors

Besides fluorescence means to detect protease activities, luminescent reporters can also be used. Bioluminescence resonance energy transfer (BRET) are based in the same principle than FRET, but generally the protein acting as donor is a luciferase which needs a substrate such as coelenterazine and ATP or oxygen to emit luminescence (Subramanian *et al.*, 2006). Depending on the range of emission of the luciferase different fluorescent proteins can be used as acceptors (Fig. 5A). In plants BRET has been used to detect processing of several versions of ubiquitin-like proteins AtAtg8 (a-i) by the Arabidopsis cysteine protease AtAtg4 in a study linking proteolysis, trafficking and autophagy (Woo *et al.*, 2014). The different versions of Atg8 were cloned into a reporter consisting of a N-terminal fusion to Citrine and a C-

terminal fusion to an optimized version of Renilla luciferase (Woo and von Arnim, 2008). Interestingly, BRET ratios showed substantial differences between wild-type plants and double *Atg4a4b* mutants. BRET independent Citrine signal was detected in wild-type vacuoles, mainly as autophagosomes, indicating that the cleaved sensor behaves as the naturally occurring protein whereas the punctuated signal is lost in the mutant lines, likely due to the absence of proteolytic processing but is detected in the cytosol. Furthermore, luciferases retain activity in native gels after addition of luciferin allowing direct detection of cleaved reporter fragments containing intact luciferase. Other BRET sensors were developed for detecting caspase-3 activity using click beetle green luciferase and tandem dimer Tomato (Gammon *et al.*, 2009). One of the newest sensors uses NanoLuc (Hall *et al.*, 2012), an engineered luciferase with optimized characteristics, with mNeonGreen for the individual detection of caspase-3, -8 and -9 (den Hamer *et al.*, 2017). A 10-fold decrease of BRET signal *in vitro* after staurosporine addition was reported.

A different design was followed for creating iGLuc, initially consisting of pro-interleukin fused to Gaussia luciferase (Bartok *et al.*, 2013). Similar to the CA-GFP probe, multimerization of the protein via pro-interleukin inactivated luciferase luminesce, which increased more than 500-fold upon caspase-1 activation (Fig. 5B). iGluc could also be used when the cleavage site was changed for a caspase-3 or a TEVp recognition site and addition of its respective proteases (Bartok *et al.*, 2013). This design also allowed the generation of specific and functional luciferase reporters for *in vitro* detection and *in vivo* imaging of other proteases such as the enterovirus 3C protease (Zhang *et al.*, 2017).

Similar to the fluorescence complementation probes, structural destabilization of luciferase reporters that can be reconstituted by proteolysis was also explored. By cyclic permuting the N- and C-terminal parts of firefly luciferase, luminescence is decreased. Both regions can be linked together by a protease cleavage site that allows enough structural flexibility after cleavage to enhance luciferase activity (Fig. 5C). This model was demonstrated for caspase-3/7/8, enterokinase and TEVp among others (Fan *et al.*, 2008). Another luciferase reporter benefits from a split firefly luciferase reporter system by addition of coil-coiled domains that hamper luciferase reconstitution by spatial limitation. The linker region can be detached by caspase-3 or TEVp cleavage, both luciferase parts can then come together and

perform mono-oxygenation of luciferin, producing luminescence (Shekhawat *et al.*, 2009). This was also used for reporting proteolysis by applying the split β-lactamase system, which shows potential applications for other split reporter versions.

One of the main drawbacks of luminescent sensors is the necessity to deliver/provide co-factors for the emission of luminescence. The addition of supplementary substances might modify plant endogenous responses and requires of proper controls. Moreover, detection of luminescence can decrease over time, affecting experiments where long time tracking is necessary. On the other hand, luminescence-based sensors are more sensitive than fluorescent ones and generally display very low background signals, which improves quantitation.

Conclusions and perspectives

In the last decade, the implementation and development of novel protease activity probes for use in plants has led to significant biological insight on processes ranging from development and senescence to biotic stress (Morimoto and van der Hoorn, 2016). Some of these studies leveraged the potential of imaging spatiotemporal protease activity, as addressed in the beginning of this review, together with an increased use of chemicals and dyes for imaging. ABPs in the plant field have mainly been used for the identification of active proteases *in vivo* and *in vitro*. The diversity of established protocols in plants have greatly aided in the pipeline for protease identification using mass spectrometry. However, not many probes have been used for imaging, mostly because they label many proteases from the same class. One of the main drawbacks of chemical probes is that ABPs work as inhibitors of proteolytic activity, thus inactivating enzymatic activity and potentially blocking downstream processes and disturbing cell biology. Possibly though, only a part of the total pool of ABP-targeted proteases is labelled, allowing the other fraction of the pool to perform their biological role. In addition, the membrane-permeability and bioavailability might be limited in some cases, although many fluorescent probes can be used for labelling *in vivo*. Therefore, to study *in vivo* processes one should use the minimal amount of ABP that permits the tracking and visualization of the target protease while minimizing the effect on plant physiology.

More recently, the development of proteinaceous probes to visualize protease activity has evolved. Genetically encoded probes include fluorescent, luminescent and transcriptional detectors of proteolysis. Genetic reporters are relatively cheap and easy to tune for specific protease requirements by standard cloning techniques available in most molecular biology labs. They also allow versatility when specific tissues are the area of interest where a protease is expressed. Genetic probes do not inhibit proteases, but might compete for genuine substrates. Therefore, it is important to test that such reporters do not have a significant influence on the system to which they are applied. For instance, adding a substrate of caspases may speed up apoptosis generating internal bias in the experiment. Different genetic reporters allow fast detection of proteolysis without additional steps needed for labelling or washing of reagents. In terms of speed, FRET reporters are well-suited for real time and fast processes, because their readout depends on protein proximity, which is lost immediately upon processing. Fluorescent complementation follows a gradual activation, but reporting the activity is delayed in time. Luminescent sensors have as an advantage that they allow very precise measurements, but, on the other hand, they require expensive co-factors. Lastly, transcriptional sensors are interesting tools that mark activity from parental cells in which proteolysis occurs. However, they are without any doubt the slowest to report activity due to their mechanism of translocation, transcription and maturation of newly synthesized proteins.

Genetic protease reporters have been underexplored in plant research, maybe due to the lack of known protease activation mechanisms and the poor number of validated substrates. Minimal knowledge on protease specificity is generally required to design such tools, meaning the capacity to identify real or synthetic substrates by some of the existing proteomic techniques used in N-terminomics (Demir *et al.*, 2018). However, these arguments also hold true for chemical probes. Nevertheless, probes could be a useful tool to determine specificity of the cleavage motifs in cases where N-terminomics are not suitable to identify specific peptides. For instance, some of peptides resulted from proteases activity can contain physical and chemical properties which made them undetectable using mass spectrometry, but which can be inserted in sensors to determine action of proteases with the proper controls. A

considerable effort will be required to establish trusted protocols and protease-specific probes in plant research. Except for the limited availability of biliverdin in all plants cellular compartments (Kohchi *et al.*, 2001) which would limit the use of iProteases sensors (To *et al.*, 2015), there seem no significant biological barriers to adapt existing genetic probes to plant research. In mammalian studies, caspases were mostly targeted by various genetic probes, primary because of their importance in disease and inflammation, but perhaps also because they are a prime example of an inducible protease with a well-known activation mechanism with very distinctive cleavage profiles and therefore easy to investigate (Salvesen *et al.*, 2016). Apart from reconfirming previous observations, genetic protease probes could serve to discover new biology, as exemplified in the case of anastasis (Sun *et al.*, 2017). As plant proteases are generally less studied than mammalian proteases, this provides a clear incentive for translation of the concepts of genetic probes to plants and the discovery of new biology.

Box

Considerations when working with or translating chemical and genetic probes to plants:

- Occam's razor principle states that the simplest solution is most likely the best. The most uncomplicated sensors and the least number of additional components introduced in the experiments might help to produce results. Also probes with less sophisticated equipment requirements for readout will facilitate the work of the researchers (excitation/emission).
- Probe design. There is not a clear rule on how long a linker can be, this might depend on the probe and on the nature of the amino acids and the final spatial conformation of its ends. Some examples in this review indicate that it is possible to detect cleavage of some full proteins labelled at the C- and N-termini as is the case for DA1 and separase substrates using FRET or delocalization sensors. Additionally, there exists a lot of variability between organisms and efficiency on the reporters. We suggest to test reporters with known proteases, such as TEVp, can help to optimize the system. Furthermore, we encourage to use uncleavable linker sequences containing flexible linkers such as repetitions of glycine-glycine-serine or known proteolysis resistant sequences that can have mutagenized version of key residues in the recognition sequence as negative controls. Additional controls using mutant lines where proteases are knock-out or are inactive is highly recommended.
- The selection of the fluorophores for a concrete application need to be considered (Evers *et al.*, 2006; Bajar *et al.*, 2016). Far red probes developed specifically for imaging deeper in mammalian tissues are not particularly needed for plant protease research. For example, using red shifted chemicals or proteins in the aerial parts of plants might overlap with chlorophyll auto-fluorescence, thereby complicating imaging.
- Cell permeability. Chemical probes are very diverse and presence of hydrogen bond donors and acceptors can reduce membrane permeability (Verdoes and Verhelst, 2016). This is exemplified with some biotinylated and carboxylate containing probes. This problem can be circumvented by

conjugation of cell-penetrating peptides or addition of a two-step labelling protocol. Carboxylic acid can also be masked in the shape of ethyl ester, which increases its permeability.

- Subcellular localization. Genetic probes can be targeted via targeting signals, whereas chemicals are generally untargeted. Targeting might be a way to increase a probe's specificity. Most genetic probes so far have been applied in the cytosol, although there should be no problem targeting them to organelles. A point of concern might be the effect of pH variation for example in the secretory pathway (decreasing pH along trafficking from the ER to the extracellular space or vacuole).
- Kinetics. Looking at fast processes is probably most efficient using FRET probes. Slower folding of complementation type probes or incubation times for chemical labelling better serve a static and macroscopic overview of protease activity.
- Availability of protease substrate specificities is a bottleneck for both chemical (that frequently employ peptide chemical bonds in their structure) and genetic probes. However, these are increasingly becoming available for plant proteases (Demir *et al.*, 2018). Protease specificity of genetic probes might benefit here from the added information on P' site signatures, which are mostly not present in chemical probes because of their make-up having a functional group (warhead) in the P1' position.

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Sensor	Readout	Mechanism	Protease(s) targeted	Uses	Substrates	Maximal Substrate coverage	Response	References
Activity Based Probes (ABP)	Fluorescence	Catalytic site binding	+++	*in vitro/ in vivo*	+++	P4-P1'	Fast (1)	(Morimoto and van der Hoorn, 2016)
DEVD-NucView488	Fluorescence	Cleavage activation	Casp-3	*in vitro/ in vivo*	DEVD	P4-P1	Fast	(Cen *et al.*, 2008)
CR-(DEVD)2	Fluorescence	Cleavage activation	Casp-3	*in vitro/ in vivo*	DEVD	P4-P1	Fast	(Bosch and Franklin-Tong, 2007)
FRET	rFluorescence	Cleavage reduces signal	+++	*in vitro/ in vivo*	+++	P5-P5' (2)	Fast	+++
Grafted GFP	Fluorescence	Fluorescence Complementation	Trypsin, Casp-3	*in vitro/ in vivo*	32 aa linker, DEVD	NA	Medium	(Chen *et al.*, 2009a; Chen *et al.*, 2009b)
Pro-GFP	Fluorescence	Fluorescence Complementation	Thrombin, Casp-3, HIV protease	*in vivo*	+++	+++	Medium	(Callahan *et al.*, 2010)
VC3AI	Fluorescence	Fluorescence Complementation	Casp-3	*in vitro/ in vivo*	DEVD	P4-P1'	Medium	(Zhang *et al.*, 2013)
iProteases	rFluorescence	Fluorescence Complementation	Casp-3, TEVp, HCV	*in vitro/ in vivo*	+++	9-15 aa consensus sequence	Fast-Medium	(To *et al.*, 2015)
ZipGFP	rFluorescence	Fluorescence Complementation	Casp-3, TEVp	*in vitro/ in vivo*	DEVD/ENLYFQ	+++	Medium	(To *et al.*, 2016)
Caspase Activatable-GFP	Fluorescence	Fluorescence Gain	Casp-3, Casp-7, TEVp	*in vitro/ in vivo*	+++	P6-P4'	Medium	(Nicholls *et al.*, 2011; Nicholls

(CA-GFP)								and Hardy, 2013; Wu *et al.*, 2013)
GFP-ssrA	Fluorescence	Fluorescence Gain	TEVp	*in vitro/ in vivo*	ENLYFQX variants	P6-P1'	Medium	(Kostallas and Samuelson, 2010; Kostallas *et al.*, 2011)
NES-DEVD-YFP-NLS	Fluorescence change	Miss-localization	Casp-3	*in vivo*	DEVD	P4-P1	Fast	(Tang *et al.*, 2012)
Separase sensor	Fluorescence change	Miss-localization	Separase	*in vivo*	Cohesin	Full protein	Fast	(Shindo *et al.*, 2012)
Apoliner	Fluorescence change	Miss-localization	Casp-3	*in vivo*	DEVD	P4-P1	Medium-Late	(Bardet *et al.*, 2008)
Caspase Tracker	Fluorescence	Fluorescence transcription	Casp-3	*in vivo*	DQVD	P4-P1'	Late	(Tang *et al.*, 2015; Tang *et al.*, 2018)
CasExpress	Fluorescence	Fluorescence transcription	Casp-3	*in vivo*	DQVD	P4-P1	Late	(Ding *et al.*, 2016)
Chemogenetic probes	Fluorescence	Fluorescence transcription	HCV	*in vivo*	NA	NA	Late	(Tague *et al.*, 2018)
BRET	rLuminescent	Cleavage reduces signal	+++	*in vitro/ in vivo*	+++	+++	Fast	(Gammon *et al.*, 2009; Hall *et al.*, 2012; Woo *et al.*,

								2014)
iGluc	Luminescent	Fluorescence Gain	+++	*in vitro/ in vivo*	+++	NA	Medium	(Bartok *et al.*, 2013)
CP-Luc	Luminescent	Luminescence Complementation	+++	*in vitro/ in vivo*	+++	NA	Medium	(Fan *et al.*, 2008)
Coiled-coil Luciferase	Luminescent	Luminescence Complementation	Casp-3, TEVp	*in vitro/ in vivo*	DEVD/ENLYFQ	P6-P1'	Medium	(Shekhawat *et al.*, 2009)

Table 1. Main protease reporters mentioned in this review and their characteristics

(1) Dependent on cell permeability.

(2) Linkage length highly variable depending on design.

+++ Indicates more than 3 counts on this cell value.

Aa: amino acids.

rFluorescence: ratiometric Fluorescence.

Casp: Caspase

NA: Not available

Figure Legends

Figure 1. Representation of chemical probes used for protease detection.

A) Different uses of ABPs and several possibilities to apply for protease detection combined with MS, gel detection of active proteases and imaging techniques using fluorescence microscopes. **B)** DEVD-NucView488 mode of action. A carboxyl derivative of thiazole orange is used to synthesize the probe that contains an acetylated tetrapeptide which hampers the dye reaction with DNA. Once it is released in can report signal, corresponding to caspase-3 activity. **C)** Representation of Rationale for CR-$(DEVD)_2$ for the detection of caspase-3 like activity. The figure depicts quenched fluorescence of cresyl violet (CR) by two DEVD caspase-3 substrates which are bound to. After processing by activated caspases, the peptides are released and cresyl violet turns fluorescent serving as a readout of protease activity.

Figure2. Fluorescent proteins at the rescue of proteolytic activity.

A) Schematic representation of the β-barrel fold of eGFP. β-barrel numbers are indicated over the structure. Important residues including in different experiments for the development of proteolysis-sensitive version of eGFP are indicated with a circle. When the specific residues are behind a strand in the spatial dimension the circle is displaced with a discontinuous line. Asparagine at position 144 (N144), glutamine at position 157 (Q157) and glutamic acid at position 172 (E172) were used for the grafting experiments including processing sites in the loop 7, 8 and 9 of eGFP respectively. A154 and D155 are commonly used for splitting GFP in both C- and N-terminal and used in BiFC assays and VC3AI and derivate proteolytic sensors. Lysine 214 (K214) is generally used to delimitate GFP1-10. Its complementing part GFP11 starts at arginine at site 215 (R215) and lacks the last 8 amino acids being a threonine (T230) the last of them as used in ZipGFP and ProGFP. NH_2 and COOH indicates the N- and C-terminal ends of eGFP. **B)** Cartoon representing the secondary structure of GFP. The corresponding amino to the design of protease sensitive eGFP are also indicated in this cartoon. **C)** Example of a FRET sensor

using CFP and YFP. Both proteins are part of a single molecule joined by a linker containing a cleavable peptide. Generally, only the donor fluorescent protein is excited which uses energy to transmit to the acceptor. When proteins are not in a spatial proximity, this energy transfer is lost, the donor fluorescence increases and the acceptor fluorescence decreases as indicated by the background stars. The maximal indicated distance to emit fluorescence is generally accepted as 10 nm of distance between fluorescent proteins as indicated in the figure.

Figure 3. A palette of genetically encoded fluorescent reporters for the detection of proteolytic activity. Representation of the different mechanisms employed by genetically encoded sensors of protease activity based on fluorescent proteins. Fluorescent proteins are depicted in the colour of their maxima spectra emission and cleavage sites are indicated by discontinuous thick black lines with scissor indicating cleavage and by open dotted lines after processing. **A)** Representation of a grafted version of eGFP**.** By addition of a cleavable recognition site in a eGFP loop after a glutamic acid in position 172, one of the β-strands of eGFP is misplaced affecting the overall final protein conformation. The position of the amino acids indispensable for chromophore formation is in this manner sufficiently distant to lower eGFP efficiency. Once the loop constringency is loosened up by protease activity, the β-strand can relocate to its natural position. Note that other two amino acids in previous loops were used for this experiment as indicated in figure 2. **B)** The Pro-GFP sensor. Pro-GFP is based on the enclosure of the β-11 strand of eGFP using a proteolytic resistant protein domain of eglin c containing two alpha-helices (indicated as grey cylinders) and a cleavable site. While the protein containing β-strands 1-10 is not capable of producing fluorescence, complementation reconstitutes eGFP conformation and fluorescence. **C)** Venus Caspase 3 Activity Indicator (VC3AI). VC3AI is based on the constriction and modification of the structure of a YFP variant. The fluorescent protein is permuted by generating new N- and C-terminal ends at A154 and D55 and the original termini are linked using a protease recognition site showed by the black dotted line. To circularize the sensor Dc and Dn fragment of *Npu* DnaE intein are added to the new termini which can self-release in a process known as protein splicing and lead to

circularization of the sensor (indicated by the horizontal grey arrow). In this conformation the fluorescent protein is not capable to efficiently reassemble with itself or other permuted circularize proteins. Once that the linker is processed complementation of the molecule occurs and the fluorescence is reconstituted as indicated by the black arrow. **D)** ZipGFP ratiometric protease detector. ZipGFP contains the β-strands 1-10 of eGFP linked by a 2A peptide to mCherry. Those sequences are efficiently processed by ribosomal skipping of the 2A-like peptide resulting in a constitutive and continuous ratiometric red signal. The β-strands 1-10 and 11 of eGFP contain additional extensions incorporating binding peptides that knot the structure impeding unspecific reconstitution by both parts. The peptide position can be relaxed by cleavage of one of the linkers re-establishing GFP as a full functional reporter. **E)** Caspase Activatable GFP (CA-GFP). C-terminal fusion of eGFP to the 28 amino acids of the intermembrane domain of influenza matrix protein 2 leads to tetramerization of the sensor hampering maturation of the chromophore. Upon proteolysis, the multimeric dependence of GFP is broken and four molecules of GFP can mature and emit fluorescence.

Figure 4. Transcriptional based reporters for the detection of proteolysis in a cell type. A) Scheme of the functioning mechanism of CaspaseTracker. A transcriptional activator Gal4 containing a cleavage site for caspase-3, DEQD, is translationally fused to a membrane anchored protein, which blocks unspecific transcription. Proteases cleaving the site liberate the activator, which then trans-localizes to the nucleus, binds the UAS sequence and transiently expresses nuclear RFP and a recombinase such as flippase. The last part of the reporters contains a constitutive promoter followed by flippase recognition targets (yellow triangles) containing signals for transcription termination and an external eGFP coding sequence which initially is not transcribed. Once the recombinase is expressed, it excises the DNA fragment bordering the flippase recognition targets sites allowing permanent cytosolic GFP expression in the cell and in the cell lineage originated from this cell. **B)** An off-to-on proteolytic drug-inducible transcriptional reporter where both parts of Gal4 necessary for transcription are divided by adding a NS3 protease. Under normal conditions, the activation domain is cleaved off and even if the binding

domain can bind to the reporter gene, histone H2B labelled with Citrine, the signal is not present. When a NS3 inhibitor is present the continuity of the structure of the protein is maintained, shifting all together to nuclei position and inducing gene expression. **C)** Example of a proteolytic on-to-off drug-repressed transcriptional system, where Gal4 is confined to the membrane by a peptide with a NS3 protease in between. The system is initially active by action of NS3 which liberates Gal4 to report its activity. Once the drug is present, Gal4 is maintained in the membrane and the signal vanishes.

Figure 5. Luminescent reporters for the detection of protease activity. A) Schematic drawing to depict BRET rationale. The different BRET systems are based on the proximity transfer of energy of luciferases, usually blue light to a proximal fluorescence protein, in this case eGFP. The ratio between emission and absorption at different wavelengths serves as a readout for processing of the linker peptide containing a minimal substrate. **B)** Representation of iGLuc system of Gaussia luciferase based on the multimerization of pro-interleukins diminishing luciferase action. The action of protease serves as element to allow release of the conformation of the multimers resulting in a luminescence enhancement. **C)** Drawing of the cyclic permuted Luciferase (CP-Luc) used for the detection of proteolysis. N- and C-terminal domains have been swapped and connected using a linker containing a site for proteolysis. Keeping the luciferase domains separate avoids enzymatic activity when co-factors are added, but it is enhanced after freeing the components. **D)** Mechanism of the auto-inhibited coil-coiled reporter Luciferase. In this system, complementation of the component parts is blocked by coil-coiled element addition to the split luciferase structures, blocking the interaction of the halves. When this appendages are proteolytically removed, both parts of firefly luciferase can reconstitute the structure, which in presence of luciferin and ATP, will emit luminescence.

Figures