The term ‘zymography’ denotes the visualization of enzymatic activity by substrate conversion. Zymography was introduced in 1962, when Gross and Lapière developed an assay for detecting collagen degradation in tadpole tissue and described a matrix metalloproteinase (MMP) for the first time Fig. 1. With zymography, the conversion of the enzyme substrate into an altered reaction product by hydrolysis can literally be visualized. The biochemical reaction is measured by detection methods specific for either the appearance of the reaction product or the disappearance of the substrate. Zymography techniques can thus be developed for any hydrolase acting on any biological substrate: proteins and peptides, oligosaccharides and polysaccharides, lipids and even nucleic acids. Any kind of biological sample can be analyzed, including cell and tissue extracts, secretomes of cells or tissues appearing in culture fluids, whole blood, plasma, serum and other complex body or lavage fluids, tissue sections and whole organisms, using one of the three main zymography techniques described here.

In contrast with solely quantitative test tube or microtiter plate detection methods for substrate conversion, zymography yields quantitative and qualitative information: for instance, about the molecular weight and the presence of different enzymes or variant forms of the hydrolases present in a sample. In contrast with enzyme-linked immunosorbent assays (ELISAs), which do not discriminate between intact molecules, complexes and degradation products, zymography allows specific forms of an enzyme to be studied. In contrast with immunohistochemistry (IHC) techniques, with in situ and in vivo zymography local net activity may be observed. Several types of zymography approaches exist for specific applications, and each type yields specific information.

The now widely used technique of in gel zymography (IGZ) was introduced in 1978 to study plasminogen activators. In the original form of IGZ (named overlay zymography or indirect zymography), an SDS-PAGE gel was used to separate protease-containing samples, which were then overlaid on an indicator gel, thereby enabling substrate degradation and detection. The detection of plasminogen activators was based on the conversion of plasminogen to plasmin followed by the degradation of fibrin films in the indicator gel. The method was later optimized by incorporating the protein substrate directly into the SDS-PAGE gel, thus improving the resolution of this technique. With the gel copolymerized with both gelatin and plasminogen,
plasminogen activators locally converted plasminogen into plasmin, which degraded gelatins. This improved method was also termed ‘secondary direct zymography’, which refers to a one-gel system (direct) and substrate conversion by the second enzyme in a chain. Successively, a ‘primary direct zymography’ technique was introduced and later optimized into a (semi-)quantitative assay. With direct incorporation of gelatins, gelatinolytic enzymes such as gelatinsase A (MMP-2) and gelatinase B (MMP-9) can be detected and purified, and their expression, regulation, inhibition, processing and degradation can be studied.

In situ zymography (ISZ) became widely applicable after the introduction of fluorescently labeled substrates and appropriate equipment for visualizing fluorescence. ISZ is a technique that yields localization information about active enzymes in tissue sections. In ISZ samples the enzymes must be kept in an active form. This implies that harsh fixation methods, often used in classical histochemistry (and IHC) techniques, must be avoided. Consequently, ISZ is performed by using either fresh or frozen tissue sections. An advantage of ISZ over other histochemical and IFIC techniques is that it visualizes endogenous enzymes in their biological contexts.

The introduction of whole-body imaging techniques for small animals enabled in vivo zymography (IVZ). This technique relies on the use of protease-activated fluorogenic probes. In the early days of the technique, the substrates were not specific and were useful to detect only broad proteolytic activities. More specific probes have since been developed containing different fluorophores, which allow simultaneous detection of various enzymes. IVZ has allowed the mapping of MMP activity patterns in an intact organism.

IGZ, ISZ and IVZ are complementary techniques. When correctly executed and interpreted, they provide information that cannot be obtained by conventional immunoassays or other techniques. For example, protease gene expression data are less informative than generally appreciated because these enzymes are often encoded as inactive precursors and are subject to various forms of post-translational regulation. Therefore, the development of activity-based assays is highly desirable.

In gel zymography: characterization of proteases

In an IGZ experiment, hydrolytic enzymes are separated by their molecular weights and detected by their ability to degrade a substrate. This technique lends itself to the analysis of any cell or tissue lysate, cell culture or body fluid. Major substrate classes include proteins and sugars, such as gelatin, caseins, chondroitin sulfates, fibrin, hyaluronan and xylans (see Supplementary Table 1 for a complete list). The standard IGZ method requires the copolymerization of the substrate with acrylamide in the separating gel of SDS-PAGE. After sample separation by nonreducing SDS-PAGE, the SDS is replaced by a non-ionic detergent that has a lower critical micelle concentration (see Supplementary Fig. 1 for guidance on choosing the right detergent). This step allows the enzymes to partially refold to their active conformation. Next, the gel is incubated in a buffer with essential cofactors, which allows the enzymes to degrade the copolymerized substrate. Proteolytic zones on this ‘zymogram’ become visible following staining procedures. A detailed technical description of substrate selection, sample collection, post-run procedures and troubleshooting is provided in Supplementary Note.

With IGZ, information can be obtained about levels of specific enzyme forms or on potential proteolytic activity, molecular weights and the presence of covalent complexes or potentially active enzyme fragments in complex biological samples. IGZ does not yield information about the net proteolytic activity in samples, although that is a commonly made error in interpretation. Several proteinases (for example, proMMPs) are secreted as inactive zymogens with an inhibitory propeptide domain. Whereas in nature these proteinases are activated by proteolytic removal of the propeptide, during electrophoresis the propeptide is unfolded and its inhibitory segment is pushed away from the enzyme catalytic site by SDS. After electrophoresis and post-run treatment of the zymogram, the proteases are only partially refolded, which results in a catalytically active enzyme and visualization of the originally inactive enzyme proforms. This is therefore an artifact of the assay and does not represent true biological activity. Because these proteases are covalently anchored to the hydrolytic enzyme proforms, after electrophoresis the proforms are detected at higher molecular weights than the activated enzyme forms from which the propeptides are cleaved off. Furthermore, noncovalently bound complexes, such as tissue inhibitor of metalloproteinases (TIMP)-MMP complexes, are dissociated by the presence of SDS during

Figure 1 | Development of zymography over five decades. Since the introduction of plasminogen-fibrin overlay zymography, IGZ has been widely used for the analysis of proteases and their inhibitors. Recent variations of this technique have been developed in 1962, has been more widely applied since the development of fluorescently labeled and highly quenched substrates. Advances in substrate development of fluorescently labeled and highly quenched substrates have since been developed containing different fluorophores, which allow simultaneous detection of various enzymes. IVZ has allowed the mapping of MMP activity patterns in an intact organism.
the electrophoresis step\textsuperscript{5,16–18}. Hence, the zymolytic bands of the zymogram are not a measure of the net enzyme activity present in the sample but should rather be seen as a measure of potential enzymatic activity\textsuperscript{9,16,18}. Unfortunately, results from gel zymography experiments are often incorrectly interpreted and stated as ‘enzyme activity’, even when only the proform of the enzyme is detected or when the biological samples contain an excess of inhibitors. To determine the net enzyme activity in a sample, researchers must use a complementary technique\textsuperscript{18,19}. Tests to measure total enzyme activity include the use of fluorogenic\textsuperscript{20}, biotinylated\textsuperscript{6}, radiolabeled\textsuperscript{21} or other\textsuperscript{22} substrates.

Both qualitative and quantitative aspects are essential in performing a successful IGZ experiment. The identification of relative molecular weights is ideally done by using a standard set of substrate-converting enzymes with known molecular weights. Furthermore, quantification of proteolytic bands requires multipoint calibration experiments and accurate densitometry measurements (Supplementary Note).

IGZ for the analysis of complex biological samples. Many studies have demonstrated the use of IGZ, particularly gelatin IGZ\textsuperscript{4,5,23,24}, for the analysis of biological samples. IGZ has been used to discover\textsuperscript{4} and identify\textsuperscript{6} new enzymes, and it complements microtiter-based activity assays for studying enzyme inhibitors\textsuperscript{8}. It is a key technique for studying regulation of expression and post-translational modifications of proteinases\textsuperscript{9}. In addition to having basic research applications, IGZ is also used in clinical contexts (Supplementary Table 2). Proteases have been analyzed by IGZ for several types of cancer\textsuperscript{24–27}, heart and vascular\textsuperscript{28–31}, inflammatory\textsuperscript{32,33} and neurological diseases\textsuperscript{33}. IGZ has also been applied to identify proteases present in plants\textsuperscript{35}, animals\textsuperscript{36–38} or bacteria\textsuperscript{29,39,40}. Gelatin zymography in particular detects other MMPs.

Figure 2 | IGZ, ISZ and IVZ readouts. (a) IGZ zymogram with two standard ladders (240 and 600 picograms (pg) total material) of recombinant human MMP-9 variants and a sample of prepurified human serum. The standard preparation includes multimeric MMP-9, monomeric MMP-9 and an MMP-9 domain deletion mutant. The deletion mutant is used as an internal reference for quantification of the human serum sample. ProMMP-9 and proMMP-2 are the enzyme precursors or zymogens of MMP-9 and MMP-2. NGAL represents neutrophil gelatinase B–associated lipocalin, covalently linked to MMP-9. Recombinant MMP-9 is less glycosylated and has a lower molecular weight than natural MMP-9. (b) ISZ on a cerebellum tissue section of an MMP-9 knockout mouse with cerebral malaria, of MMP-9. Zones of gelatinolytic activity are shown as bright green on a black background. Arrow indicates a transverse section of a blood vessel\textsuperscript{52}. Blood vessels indicate the presence of gelatinase activity, different from that with a highly quenched fluorogenic gelatin substrate. Fluorescence staining of a cerebellum tissue section of an MMP-9 knockout mouse with cerebral malaria, of MMP-9. Zones of gelatinolytic activity are shown as bright green on a black background. Arrow indicates a transverse section of a blood vessel\textsuperscript{52}. (c) Differential IVZ in a developing zebrafish embryo with two fluorogenic MMP substrates, as described in ref. 58. The embryo (24 h post fertilization) was injected at the site indicated by the arrow with a mixture of MMP substrates A580 (AnaSpec cat. no. 60585) (red fluorescence) and G520 (no. 60580) (green fluorescence), allowed to recover for 1 h, fixed and imaged; maximum projections of four confocal data sets were assembled using Fiji. Image courtesy of J. Keow and B. Crawford.
**Variations on IGZ.** In recent years, variations on conventional IGZ have been developed, including further tweaks on transfer-gel zymography (TGZ) techniques (Table 1). ‘Antibody zymography’ has been used to examine the inhibitory potential of antibody-based antivenoms on venom protein gelatinolytic activity.41 ‘Microfluidic gradient-gel zymography’ was developed for rapid and automated analysis of heterogeneous protein samples and for inhibitor screening. The technique reveals molecular weights, quantities, and activities of the enzymes simultaneously42.

Conventional SDS-PAGE IGZ can also be complemented with an additional isoelectric focusing dimension (two-dimensional (2D) zymography)35,43. This extra dimension, based on the pI point of individual proteins, is particularly useful for highly complex samples, such as organ and tissue extracts and blood samples. Proteins with charge variants, such as those glycosylated with glucuronic acid or sialic acid, or those phosphorylated or citrullinated, are readily separated in the first dimension, after which classical substrate zymography is used as the second dimension. Individual

### Table 1 | Overview of variations on conventional gel zymography

<table>
<thead>
<tr>
<th>Technique</th>
<th>Goal or present use</th>
<th>Advantages (+) and disadvantages (−)</th>
<th>Comments</th>
<th>Ref(s).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody zymography</td>
<td>Examination of the inhibitory potential of antibody-based antivenoms on venom protein gelatinolytic activity.</td>
<td>+ Optimized for antibodies + Useful for inhibitor testing</td>
<td>Useful in studies to evaluate the efficacy of inhibitory antibodies of protease activity.</td>
<td>41</td>
</tr>
<tr>
<td>Caseogram or caseogram print</td>
<td>Detection of acid proteases. Based on an overlay gel containing skim milk. Protease activity is visible as opaque zones.</td>
<td>− Intensity does not correlate with activity</td>
<td>Has been used to quantify pepsinogen.</td>
<td>71,72</td>
</tr>
<tr>
<td>2D zymography</td>
<td>An extra dimension of protein separation by isoelectric focusing.</td>
<td>+ Useful for heterogeneous samples + Improved separation of molecules with similar MW + Better suited for subsequent mass spectrometry analysis</td>
<td>Especially useful for samples containing proteases with similar MW. Because an additional separation by isoelectric focusing is applied, it is easier to perform subsequent mass spectrometric analysis.</td>
<td>35,43</td>
</tr>
<tr>
<td>Synthesis activity–based zymography</td>
<td>Detection of lipases and esterases.</td>
<td>+ High resolution + Short incubation times</td>
<td>Based on the esterification reaction between fatty acids and alcohols.</td>
<td>73</td>
</tr>
<tr>
<td>Transfer zymography</td>
<td>Enables accurate MW determination and proteomic analysis after a zymography experiment by use of classical SDS-PAGE for protein separation.</td>
<td>+ Accurate $M_\text{r}$ estimation + Semiquantitative − Time consuming − Less sensitive than direct IGZ − Difficult when proteins are highly different in size</td>
<td>Same procedure and equipment as for western blotting. The nitrocellulose or PVDF membrane is replaced by a receiving gel that is a polycrylamide- or agarose-based gel containing the (protein) substrate.</td>
<td>2,44</td>
</tr>
<tr>
<td>Microfluidic gradient-gel zymography</td>
<td>Rapid, automated analysis of heterogeneous protein samples and simultaneous determination of MW, amount and activity ($k_{\text{cat}}$ and $K_\text{m}$).</td>
<td>+ Fast + Automated + Quantitative + Determination of $k_{\text{cat}}$, $K_\text{m}$ and MW</td>
<td>Optimized for calf intestinal alkaline phosphatase and horseradish peroxidase.</td>
<td>42</td>
</tr>
<tr>
<td>Mixed-substrate zymography</td>
<td>Detection of different enzymes in a single secondary substrate gel. Was developed for accelerated screening of enzymes produced by microorganisms.</td>
<td>+ Fast + Simultaneous detection of different enzymes in one secondary substrate gel</td>
<td>After conventional SDS-PAGE, proteins are electrotransferred to a second mixed-substrate gel. Optimized for the detection of lipase and protease activity in a single gel.</td>
<td>45</td>
</tr>
<tr>
<td>Multiple-layer zymography</td>
<td>Simultaneous detection of multiple types of enzyme.</td>
<td>+ Fast + Simultaneous detection of different enzymes</td>
<td>After conventional SDS-PAGE, proteins are electrotransferred to a stack of different substrate gels. Optimized for the detection of cellulase, lipase and protease activity from single samples.</td>
<td>46,45</td>
</tr>
<tr>
<td>Optical zymography</td>
<td>Detection of PA levels with a copolymerized probe of a repeating selective uPA substrate motif ([GGSGRSANAKC-NH$_2$]$_x$) terminally capped with fluorescent dye (Cy5.5) and a PEGylated polyl-lysine graft copolymer.</td>
<td>+ uPA specific + Fast − Low sensitivity</td>
<td>Sensitivity of a conventional casein-plasminogen zymography (3 pg uPA) was still higher than that of optical zymography (40 pg uPA).</td>
<td>74</td>
</tr>
<tr>
<td>Real-time zymography and real-time reversed zymography</td>
<td>Real-time monitoring of the zymolytic reactions after electrophoresis.</td>
<td>+ Easy optimization of incubation times + Higher sensitivity with low amounts of substrate + Semiquantitative + Yields kinetic parameters</td>
<td>FITC-labeled substrates are used, and the reaction is monitored with a transilluminator.</td>
<td>75</td>
</tr>
</tbody>
</table>

FITC, fluorescein isothiocyanate; $k_{\text{cat}}$, catalytic constant; $K_\text{m}$, Michaelis constant; $M_\text{r}$, apparent molecular mass; MW, molecular weight; PVDF, poly(vinylidene difluoride); PA, plasminogen activator; SDS-PAGE, sodium dodecyl sulfate PAGE; uPA, urokinase-type plasminogen activator.
Figure 4 | Three different approaches for ISZ. In the photographic emulsion–based approach (left), the tissue section is first placed on a glass slide and then immersed in a photographic emulsion containing the substrate. The section is visualized by light microscopy, and proteolytic activity is detected by light zones on a dark background. In the fluorescently labeled substrate–based approach (middle), the glass slide is first covered by a layer of fluorescent substrate. With a fluorescence microscope, zones of proteolysis are detected as black on a fluorescent background. The highly quenched substrate approach (right) relies on a fluorogenic substrate. This substrate is highly quenched, and fluorescence will emerge when the substrate is cleaved. With a fluorochrome microscope, proteolytic activity is seen as fluorescence on a dark background.

Protein bands in the gels can be identified after excision followed by Edman degradation or in-gel trypsin digestion and mass spectrometry analysis. However, because an IGZ gel also contains substrate fragments, the identification of protease bands becomes more challenging in 2D zymography. This problem can be solved by running a conventional SDS-PAGE gel in parallel with IGZ.44,40

In TGZ, the enzymes are transferred to one or more separate substrate gel(s) after electrophoretic separation (Fig. 3b). This technique has recently regained attention since the finding that multiple secondary substrate gels can be stacked to gain simultaneous information on multiple enzymes. These tweaks have been called electrophoretic TGZ, mixed-substrate zymography and multiple-secondary substrate gels can be stacked to gain simultaneous information on multiple enzymes. These tweaks have been called electrophoretic TGZ, mixed-substrate zymography and multiple-layer zymography.

Reverse zymography enables the detection of protease inhibitors in biological samples. In this technique, both the substrate and enzyme are copolymerized within the whole gel. The substrate is uniformly degraded except where an inhibitor present in the sample blocks the enzyme activity. After staining procedures, the position of inhibitors can be seen as a band containing intact substrate.19, Oliver et al. optimized this technique for the analysis of MMP inhibitors by copolymerizing gelatin, MMP-2 and MMP-9 in acrylamide gels.47

**In situ zymography: 2D snapshots of enzyme activity**

ISZ is able to detect and localize particular protease activities in tissue sections. Unlike IGZ, ISZ is able to determine net enzymatic activity because inactive proforms and enzyme–inhibitor complexes are not detected. The widely used technique of IHC detects immunoreactivity but often cannot discriminate between active and inactive forms of an enzyme, whereas ISZ confirms net functional activity. Hence, the combination of ISZ and IHC represents a powerful approach to study proteases in tissue samples. Supplementary Table 3 lists studies in which ISZ was applied to sections from various organs. With ISZ, a 2D map of proteolysis in vivo can be generated. With the use of serial section analysis, a 3D picture may be reconstructed, potentially allowing validation of IVZ results.

**Different ISZ approaches.** Three different approaches exist for ISZ: the photographic emulsion–based method, the fluorescently labeled substrate technique and highly quenched fluorescently labeled substrate approach (Fig. 4). In the photographic emulsion–based approach, tissue sections on glass slides are immersed in a photographic emulsion containing silver-coated substrates; proteolysis is then visualized by light microscopy. When the substrate is cleaved, the silver concentration diminishes accordingly. Hence, zones of proteolysis are seen as white spots on a black background. In the fluorescently labeled substrate approach, glass slides are first coated with a fluorescent substrate. Subsequently, the tissue sections are mounted onto the slides, and, after an incubation period, proteolysis is visualized by fluorescence microscopy. Proteolysis is seen as black holes on a fluorescent background.48 In the highly quenched substrate approach, tissue sections on glass slides are incubated with a highly quenched fluorescently labeled substrate. Owing to the loss of quenching after proteolysis, zones of proteolytic activity appear fluorescent against a darker background (Fig. 2b).

When performing ISZ, it is important to understand the composition and topology of the tissue. With the photographic emulsion–based approach, it is difficult to apply a uniform substrate layer. In contrast, the fluorescently labeled substrate approach enables evaluation of the uniformity of the substrate layer before mounting of the tissue. However, the sensitivity of the latter method depends on the efficiency of the substrate labeling and substrate cleavage because activity is localized according to the disappearance of the fluorescent signal. The highly quenched fluorescently labeled substrate approach has high sensitivity and is easier to standardize, and it is therefore the preferred method.

**Interpretation of ISZ data.** ISZ measures the net activity of proteases that degrade the chosen substrate. Therefore, the measured activity is strongly dependent on the enzyme specificity toward the substrate. Because most substrates are degraded, with greater or lesser efficiency, by a variety of proteases, it is difficult to determine exactly which protease is responsible. For example, trypsin, widely
used to generate tryptic fragments in proteomics analysis, will generate strong signals in gelatin ISZ of exocrine pancreas tissue. For det... probes are responsible for the ISZ signals, ISZ is often combined with other techniques, such as IHC or in situ hybridization. Another approach is to incorporate inhibitors in control experiments (Supplementary Table 4 and Supplementary Fig. 1). Inhibitors of a class of proteases can be added, such as EDTA, which chelates bivalent ions and therefore inhibits the activity of Zn$^{2+}$-dependent MMPs. Other inhibitors include specific inhibitory peptides, TIMPs, small-molecule inhibitors and monoclonal antibodies. To determine whether the observed activity is due to a specific hydrolase, one may compare wild-type and knockout animals.

When examining a certain disease pattern, keep in mind that not all effects of a protease are related to substrate catalysis. For example, although proteolysis is an important activity of MMP-9, this protease also has functions that do not require enzyme activity, such as the induction of signal transduction by binding to surface receptors. Therefore, it remains critical to monitor the total amount of protease present by IGZ. It is also important to determine optimal incubation times when performingzymography experiments. A short incubation time may result in poor reactivity, whereas long incubation times may result in complete substrate conversion when large amounts of enzyme are present. Finally, we stress that the detection of net enzymatic activity by ISZ is valid only if thezymogens are not artificially activated during the ISZ protocol.

Combining ISZ with other techniques. Valuable information can be extracted by combining ISZ with IHC. A variety of specific antibodies are available, including primary antibodies that target ECM molecules, cellular receptors and more. With a combination of ISZ, IGZ and IHC, selective in situ proteolytic cleavage of β-dystroglycan, by MMP-2 or gelatinase A in combination with MMP-9 or gelatinase B, was demonstrated at the site of leukocyte migration through the blood-brain barrier in a model for experimental autoimmune encephalomyelitis. ISZ and IHC were recently combined to study Huntington's disease. In that work, MMP-2, MMP-9, laminin, EBA (a blood-brain barrier endothelial cell antigen), NeuN (neuron marker) and GFAP (astrocyte marker) were first detected by IHC. After the MMP-2 and MMP-9 expression pattern was acquired, ISZ was performed. Net gelatinolytic activity was colocalized with laminin and EBA, suggesting that in Huntington's disease the basal lamina of blood vessels is digested by gelatinases and, more specifically, by MMP-9. Other studies have also demonstrated the value of combining IHC and ISZ.

In vivo zymography: real-time 3D imaging of enzyme activity IVZ enables the localization of proteolytic activity in an intact organism. The technique has been applied to detect net MMP activity in living tissue and in whole organisms such as developing embryos of zebrafish or mice. From such studies on zebrafish, it became clear that valuable information is lost in IGZ and ISZ because of sample preparation procedures. For example, homogenization of tissue samples in IGZ results in the overall distribution of enzyme activators. Enzymes and inhibitors may be artificially mixed by tissue sectioning in ISZ. In contrast, IVZ has the power to yield insights in the spatial and temporal (real-time 3D) distribution of proteolytic activity in a whole organism.

IVZ requires a biocompatible fluorogenic substrate that is detectable upon disintegration by proteolytic cleavage. The substrate peptide can be selected on the basis of known consensus sequences for either a specific protease or a protease class. Currently, several approaches exist. One is the use of Förster resonance energy transfer (FRET)-quenched peptides, which consist of a quencher group and a fluorophore on opposite ends. When these groups are in close contact on the intact peptide, fluorescence is minimal because of energy exchange between the fluorophore and quencher. When the peptide is cleaved, the fluorophore and quencher diffuse and fluorescence is detected. Different fluorophore and quencher pairs with different fluorescence spectra are available. Hence, a mixture of different FRET-quenched peptides with different sequences can be used for simultaneous detection of different enzymes. With growing numbers of FRET-quenched peptides becoming commercially available, the application of IVZ is also growing, especially for studies on the dynamics of ECM remodeling. Recently, a multiplex FRET-quenched peptide-based assay named ‘differential IVZ’ was developed. With this assay, several ‘hot spots’ of proteolytic activity in developing zebrafish embryos were detected by using MMP-specific FRET-quenched peptides (Fig. 2c). Substantial hydrolysis was observed in the developing brain, specific cells of the spinal cord and trigeminal ganglion, intersomitic boundaries and individual myofibrils in the tissue.

Another approach is to use substrates that are heavily labeled with NIR fluorophores. Because of the close positioning of the fluorophores on the substrate, fluorescence is quenched; upon proteolytic cleavage of the substrate, the fluorophores are dispersed, which results in fluorescence. NIR fluorescence has the advantages of low absorption by tissue and reduced scattering.

Specialized NIR probes have been developed. For example, the rhodamine-tagged hydroxamate benzophenone probe for the detection of active MMPs contains a hydroxamate group that functions as a high-affinity inhibitor of MMPs by binding to the conserved Zn$^{2+}$ ion in the active site. A photoactivable benzophenone group is placed at a nonbinding part of the molecule so that the probes can be photo–cross-linked only to MMPs with a free active site. Although initially used for the in vitro detection of active proteases, these probes are also applicable in vivo. Many of the currently existing probes were developed and used for the detection of tumor-related proteolysis or metastatic niches. Nanoparticles, such as poly(l-lysine) polymers coated with multiple methoxy poly(ethylene glycol) groups, are often used as vehicles for delivering quenched NIR fluorochromes to highly proliferating cells to detect proteolytic activity associated with tumor cells. Although IVZ experiments are difficult to standardize and quantify, the use of nanoparticles offers the opportunity to attach an internal reference fluorophore next to a quenched substrate. This enables the detection of both cleaved and uncleaved probes. A ‘proteolytic nanobeacon’, PB-M7NIR, was successfully developed for monitoring in vivo MMP-7 activity that colocalized with malignant lesions. MMP-7 activity in the tumor microenvironment was further investigated in histological sections that clearly colocalized MMP-7 activity with the ECM. ‘MMPSense’ probes are also commercially available. A review on whole-body optical imaging techniques with animal models was written by Kaijzel et al.

Presently, the availability of specialized IVZ probes is limited, and custom-made probes are expensive. However, available
fluorophores, quenchers and corresponding laser excitation and detector hardware will enhance the expansion of this methodology. Another limitation relates to the size of the studied species. Because of signal absorption and scattering in tissue, NIR fluorescence is preferred over FRET probes. Multiplex FRET-quenched probes are limited to IVZ in transparent species and small embryos. The development of substrate-coated nanoparticles that provide both signals for intracellular uptake and labels for in vivo imaging—magnetic resonance imaging, for example—should make it possible to perform IVZ in larger animals.

### Zymography as a reverse degradomics technique

The concept of ‘degradomics’ was introduced 10 years ago. This approach seeks to define the complete substrate repertoire of an enzyme. Since then, new high-throughput screening strategies have been developed and have enabled the discovery of various new metalloproteinase substrates, including intracellular substrates for MMPs, as well as plant protease substrates. However, in vivo enzymatic activity is dependent on parameters such as spatial distribution, zymogen activation, natural inhibitors, post-translational modifications and so on. Therefore, efficient substrates are not necessarily biologically significant substrates.

#### Table 2 | Comparisons of IGZ, ISZ and IVZ

<table>
<thead>
<tr>
<th></th>
<th>In gel zymography (IGZ)</th>
<th>In situ zymography (ISZ)</th>
<th>In vivo zymography (IVZ)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>Fluids (body fluids, tissue extracts, etc.)*</td>
<td>Tissue sections (brain, liver, lung, etc.)**</td>
<td>Whole intact organism (zebrafish, mouse, etc.)</td>
<td>*Prepurification may be required for complex biological samples ** Reveals the composition and topology of the tissue</td>
</tr>
<tr>
<td>Special substrate requirements</td>
<td>High molecular weight, cross-linking</td>
<td>Fluorogenic substrates, radiolabeled substrates</td>
<td>Fluorogenic substrates, FRET peptides, specialized probes, NIR fluorescence</td>
<td></td>
</tr>
<tr>
<td>Visualization</td>
<td>Coomassie blue, PhAST Blue, silver stain, Congo red, amido black*</td>
<td>Light microscopy, fluorescence microscopy</td>
<td>Optical imaging techniques</td>
<td>*Dependent on the substrate</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>Substrate and method dependent*</td>
<td>Substrate and method dependent*</td>
<td>Substrate and method dependent*</td>
<td>*Highly dependent on the substrate, detection method, protocol and the equipment available for detection</td>
</tr>
<tr>
<td>Resolution</td>
<td>Substrate and method dependent*</td>
<td>Substrate and method dependent*</td>
<td>Substrate and method dependent*</td>
<td>*IGZ has a higher resolution than overlay zymography</td>
</tr>
<tr>
<td>Cost</td>
<td>Inexpensive (&lt;ELISA)</td>
<td>ISZ &gt; IGZ</td>
<td>IVZ &gt; ISZ</td>
<td></td>
</tr>
</tbody>
</table>

### Enzyme forms detected

- **Zymogens**: Yes, discrimination between proforms and activated forms
- **Enzyme fragments**: Yes, when they contain an intact catalytic site
- **Covalent complexes**: Yes
- **Noncovalent complexes**: No, noncovalent complexes dissociate during experiment
- **Inhibited species**: Yes, no discrimination between active species and species inhibited by a noncovalently bound inhibitor

### Type of activity detected

- **Net activity**: No*<sup>†</sup>
- **Potential activity**: Yes
- **Extra information obtained**: Molecular weight Yes, Localization in tissue Yes (2D)*, Quantitative Semi*

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*ELISA, enzyme-linked immunosorbent assay; FRET, Förster resonance energy transfer; IHC, immunohistochemistry; NIR, near infrared.*
in vivo. Moreover, a single substrate can often be processed by several different proteases, resulting in its stepwise degradation with different outcomes for the functionality of the substrate. To study this, the concept of ‘reverse degradomics’ was introduced, which seeks to measure the total impact of a complex biological sample on a particular substrate. Fluorescently labeled substrates are incubated with biological samples, and at different time points the degree of degradation is assessed in a high-throughput manner. This enables one to predict and compare the total impact of complex samples on, for example, key disease-related substrates, in a setting close to the in vivo substrate environment.

Zymography is fundamentally a ‘reverse degradomics’ technique. Zymography techniques provide additional information on the spatial distribution of activity, an important parameter that cannot be obtained with the currently available high-throughput (reverse) degradomics platforms. Because this is a key parameter for protease activity, linking (reverse) degradomics patterns to specific locations in vivo is indispensable for studying biology and medicine. Although efforts have been made toward developing high-throughput IGZ screening systems, standard ISZ and IVZ protocols remain too time consuming for large-scale reverse degradomics studies. Ideally, a high-throughput reverse degradomics system should be linked to in situ techniques to simultaneously determine the degree and the location of proteolytic activity.

Conclusions and future perspectives

We reiterate the importance of correctly interpreting results obtained with IGZ, ISZ and IVZ. In particular, misinterpretation of IGZ data as net enzyme activities must be avoided. When ISZ and IVZ experiments are performed, it is important to include appropriate control conditions to confirm which proteases are degrading the substrate. Table 2 compares the different types of zymography experiments in terms of sample, substrate requirements, readout, resolution and so on. Understanding the advantages, disadvantages and limitations of zymography methods is critical for choosing the appropriate technique for specific biological questions. A good insight into the biochemistry behind different zymography methods is obligatory for correct interpretation of the data.

We believe that new technological developments will contribute to a better understanding of protease biology. Zymography technology has played an important role in studies of proteases, particularly MMPs and plasminogen activators. Zymography methods can also be applied using many other substrates, including glycans, lipids and nucleic acids.

We recommend that in future studies, relative abundances of both active and inactive forms of proteases in situ or in vivo should be stated to generate a biologically relevant snapshot of the role of the studied protease. Standardized, user-friendly and correctly interpretable zymography tools for non-experts need to be developed and commercialized. With the introduction of fluorescent probes, miniaturization of biochemical analytical tools and advances in microscopy, we believe that zymography techniques will become more widespread. The combination of mass spectrometry techniques and zymography will ultimately allow the exact trimming pattern of individual substrates by proteases to be determined in situ and in vivo.

Note: Supplementary information is available in the online version of the paper.

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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37. This paper illustrates the power of combining IG2, IS2 and IHC.
43. An example of how nanoparticles are useful tools for IVZ with the use of near-infrared fluorophores with low tissue absorption.
48. A critical review of degradomics as a methodology to study the substrate repertoires of single proteases.


Definition of reverse degradomics as a substrate-based methodology to study the protease repertoire of single substrates. In fact, substrate zymography is a reverse degradomics method.


