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Activity-based Protein Profiling

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Advanced article

Article Contents

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Activity-based protein profiling is a method to study a subset of the enzymatically active proteome. This method uses chemical probes that covalently react with active enzymes. These labelled proteins can subsequently be analysed by means of a detection tag on the probe. A diverse set of probes has been developed for many enzyme classes, such as serine hydrolases, proteases, glycosidases and kinases. Different analytical techniques are currently available to visualise, identify and quantify probe-labelled proteins with high efficiency. Activity-based protein profiling has well-developed applications in discovering new drug targets and in profiling inhibitors for potency and selectivity. Activity-based protein profiling will, therefore, continue to aid research both in fundamental biology and drug discovery.

Introduction

Activity-based protein profiling (ABPP) is a method to study the abundance of active enzymes in complex proteomes. ABPP uses chemical tools, termed activity-based probes (ABPs), which covalently and irreversibly react with a nucleophile in the active site of targeted proteins. Because only active enzymes are labelled by a probe, ABPP measures the abundance of active enzymes. This can differ from the total abundance of an enzyme, considering the activity of enzymes is regulated by posttranslational modifications **See also**: **Proteins: Post-synthetic Modification – Function and Physical Analysis**.

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This makes ABPP a unique and powerful method. Increasingly, ABPP is called activity-based or chemical proteomics (Simon and Cravatt, 2010), complementing abundance-based proteomics **See also**: **Shotgun Proteomics**. ABPP can be used to compare activity of certain enzymes between different proteomes, for example between healthy and diseased tissue, which enables drug target discovery. Furthermore, ABPP can be applied to characterise inhibitors and drug candidates for both potency and selectivity in a native physiological context, aiding the selection of therapeutically relevant compounds.

Every ABPP experiment consists of two parts: an activity-dependent labelling part and an analytical part to visualise and characterise this labelling event. This general view of ABPP shows it is a multidisciplinary endeavour: organic chemistry is needed to synthesise and characterise ABPs, analytical chemistry to provide the read-out of the labelling event, and biology to understand the proteomes being studied.

In this article, we will start with the first part: the labelling of active proteins using an ABP. The design of an ABP will be explained and several examples of probes and their enzyme targets will be discussed. In the second section, an overview is provided of the analytical platforms available to visualise the labelled proteome. Finally, in the third section, the applications of ABPP will be reviewed, focussing on comparative ABPP and competitive ABPP experiments.

Labelling

An ABP generally consists of three main parts (**Figure 1a**): the first part is the trap, also called warhead, which is able to form a covalent bond with the target enzyme. Usually, the trap is an electrophilic group (Shannon and Weerapana, 2015), as is the case for the fluorophosphonate probe shown in **Figure 1a**, which forms a covalent bond with nucleophilic serine residues. The second part is the linker, which can be changed to fine-tune chemical properties of the probe such as cell permeability, solubility, affinity and selectivity towards specific enzymes. The third part of the probe is the tag, which enables the detection of enzyme(s) labelled by the probe. This tag can be a fluorophore for visualisation, an affinity tag (often biotin, as shown in **Figure 1a**) that

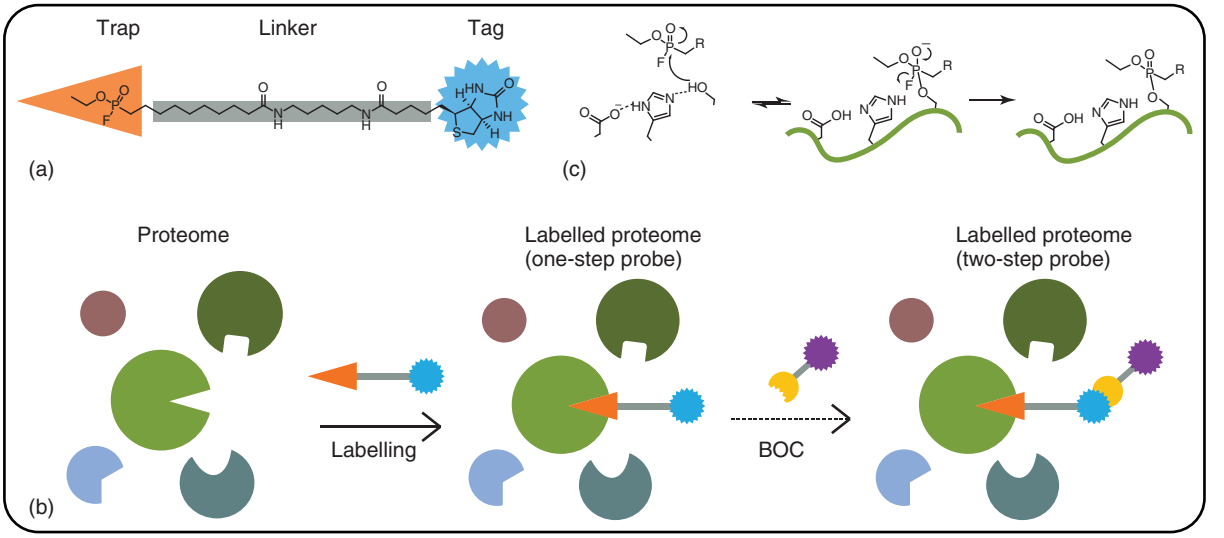


Figure 1 **Labelling enzymes with an activity-based probe**. (a) General activity-based probe design, with fluorophosphonate-biotin as example. (b) Probe labelling cartoon: two-step labelling using bioorthogonal chemistry (BOC) is optional for probes equipped with a suitable tag. (c) Mechanism of serine hydrolase labelling: catalytic triad reacting with the fluorophosphonate trap.

is used to enrich or purify probe-labelled enzymes (pulldown), a radioactive label or a ligation handle for a two-step labelling procedure (Speers *et al*., 2003).

In the labelling part (**Figure 1b**), the ABP binds covalently to the target enzyme. This labelling event can take place in lysates, intact cells, tissues or living organisms (Blum *et al*., 2007). There are two types of probes for the detection of active proteins (**Figure 1b**): (1) one-step probes make use of a compound with a detection tag already installed and (2) two-step probes rely on a ligation handle, which can be used to install the detection tag after the probe has reacted with the protein. One-step labelling is fast and efficient, but the large tag can decrease the affinity and selectivity of the probe for the target enzymes and/or may interfere with cell permeability. Two-step probes may circumvent these issues, but are less efficient in the workflow. Key is that the ligation handle and the detection tag react in a bioorthogonal manner, which means that the biological system does not interfere with the coupling reaction (Willems *et al*., 2011). The most commonly used bioorthogonal reaction is the 'click' reaction where an alkyne moiety reacts with an azide moiety in a copper(I)-catalysed cyclisation (Tornøe *et al*., 2002). For an extensive review on different types of bioorthogonal chemistry, see Patterson *et al*. (2014).

In **Table 1**, several examples of ABPs for different enzyme classes are depicted. For a comprehensive overview, the reader is referred to excellent reviews (Evans and Cravatt, 2006; Nodwell and Sieber, 2012). Here, predominantly ABP design will be discussed using enzyme class specific examples to explain the different methods of probe design.

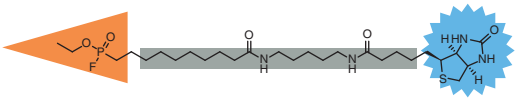
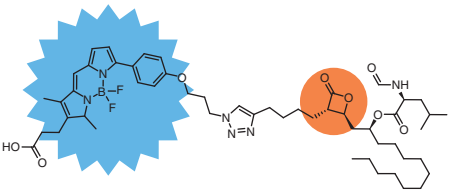
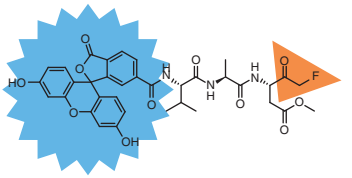
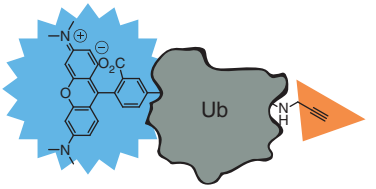
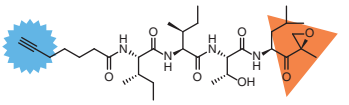
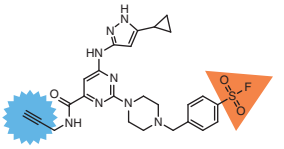
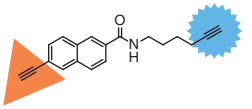
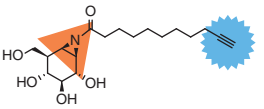
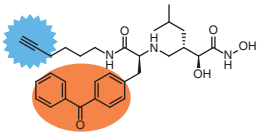
Serine hydrolases

Probe **1** (**Table 1**) is a broad-spectrum probe, which is designed to react with any serine hydrolase. The hydrophobic linker between the electrophilic trap and the biotin group does not contain any side chains that can provide extra interactions with selected members of the hydrolases, thus providing no specificity for a particular serine hydrolase. The mechanism of covalent bond formation between a fluorophosphonate probe and the catalytic triad of a serine hydrolase is depicted in **Figure 1c** (Liu *et al*., 1999). The aspartic acid and histidine residues form a charge relay system with the serine, increasing its nucleophilicity. The catalytically active serine nucleophile of the hydrolase attacks the electrophilic fluorophosphonate, which results in expulsion of a fluoride ion and concurrent covalent binding of the enzyme with the probe. The formed covalent bond is stable and the active site is occupied, rendering the enzyme inactive. Probe **2** is an example of a tailored probe, used for profiling of the lipase DAGL-α (diacylglycerol lipase alpha) and other related proteins (Baggelaar *et al*., 2013). The design of this probe is based on the anti-obesity drug Orlistat, which has an irreversible covalent binding mechanism, with a lactone as electrophilic trap. This example highlights one method of ABP design: using a known covalent inhibitor as a template. The tag used for probe **2** is a fluorophore.

Cysteine proteases

ABPs for the family of cysteine proteases have also been extensively described (Kato *et al*., 2005). For example, Probes **3** and **4** are based on the natural substrates of their target enzymes (a peptide for caspases and ubiquitin for the deubiquitinases) and have an electrophilic trap. Cysteine proteases use a catalytic cysteine residue, and owing to the soft nature of the nucleophile, can be trapped by soft electrophiles. These traps include reactive groups such as vinyl sulfones, iodoacetamides and epoxides. Cysteine proteases ignore harder electrophilic traps such as fluorophosphonates and sulfonyl fluorides. Caspases, a subfamily of cysteine proteases, can be labelled selectively and efficiently by using a low-reactive fluoromethylketone trap (probe **3**, **Table 1**). The peptidic linker element is required for

Table 1 Enzyme classes and reported activity-based probes specific to that class (orange trap and blue tag as in **Figure 1**)

Entry	Target enzymes	Probe structure	Reference
1	Serine hydrolases		Liu *et al.* (1999)
2	Lipases		Baggelaar *et al.* (2013)
3	Caspases		Bedner *et al.* (2000)
4	Deubiquitinases		Ekkebus *et al.* (2013)
5	Proteasome		Li *et al.* (2013)
6	Kinases		Zhao *et al.* (2017)
7	Cytochrome P450		Wright and Cravatt (2007)
8	Glycosidases		Kallemeijn *et al.* (2012)
9	Metallohydrolases		Saghatelian *et al.* (2004)

selective caspase-specific recognition (Bedner *et al.*, 2000). The reaction of a terminal alkyne trap with the active site cysteines in deubiquinating enzymes is an example of the importance of the recognition element in the activity profile of an ABP (Ekkebus *et al.*, 2013). Normally, alkyne moieties are considered unreactive towards nucleophiles, however, when attached to the protein ubiquitin (Ub, probe **4**, **Table 1**), the alkyne is able to function as electrophilic trap.

Threonine proteases

In threonine proteases, an *N*-terminal threonine acts as the catalytic nucleophile. The secondary alcohol of the threonine is activated by the basic *N*-terminal amine, via an ordered water molecule in the active site. The proteasome is a multisubunit protein complex containing several active sites. The natural product epoxomicin is a covalent inhibitor for each of these

subunits. Probe **5** (**Table 1**) is based on epoxomicin, containing an epoxyketone electrophilic trap, which reacts with both the threonine nucleophile and the *N*-terminal amine base in the active site. Probe **5** is equipped with an alkyne tag, which can be used for two-step labelling.

Kinases

Kinases comprise one of the largest enzyme families and are a common target for cancer drugs as well. Generally, kinases catalyse the phosphorylation of their substrate using ATP (adenosine triphosphate). These enzymes lack a nucleophilic catalytic residue and, therefore, development of probes for kinases has been challenging. Recently, probe **6** (**Table 1**) was reported as a broad-spectrum kinase ABP (Zhao *et al*., 2017). This probe contains a sulfonyl fluoride trap that targets a conserved lysine residue in the ATP-binding site of kinases.

Cytochrome P450s

Cytochrome P450s are a family of enzymes that metabolise a wide variety of substrates, including drug molecules. For this enzyme family alkyne-containing probes have been developed (probe **7**, **Table 1**) (Wright and Cravatt, 2007). P450 enzymes oxidise the alkyne to a highly reactive ketene species, which forms a covalent bond in the active site. Interestingly, probe **7** contains two alkynes, and the enzyme will only oxidise the conjugated alkyne group, leaving the other alkyne group available as a ligation handle.

Glycosidases

Glycosidases catalyse the hydrolysis of glycosidic bonds and thereby this enzyme family degrades a wide variety of substrates: saccharides, glycolipids and glycoproteins.

For glycosidases, ABPs have been developed based on the natural product cyclophellitol, an irreversible inhibitor with an epoxide electrophilic trap. Probe **8** is an example of these cyclophellitol-inspired probes, with an aziridine trap and an alkyne tag and is used to profile the retaining β-exoglucosidase subfamily of glycosidases (Kallemeijn *et al*., 2012).

Photoaffinity probes

Not all enzymes have a suitable nucleophile in the active site that can be targeted with an electrophilic trap. These enzymes can sometimes be labelled with probes bearing a photoreactive trap (Geurink *et al*., 2012). These photoaffinity probes form covalent bonds by UV (ultraviolet) irradiation of the photoreactive group. For example, metallohydrolases have been targeted using probe **9** (**Table 1**) (Saghatelian *et al*., 2004). A metal ion in the active site is chelated to the hydroxamine group of the probe and covalent linkage is induced upon UV irradiation of the benzophenone as photoreactive group.

In summary, both the choice of trap and the linker determine the type of enzymes that will be labelled by the probe. The nature of the tag determines the means of detection, which will be discussed in the following sections.

Analytical Platforms

The purpose of the second analytical part of an ABPP experiment is to visualise the labelling event (Sieber and Cravatt, 2006). Of note, ABPP does not measure catalytic activity, meaning the turnover of substrate(s) to product(s) in a certain amount of time. Instead, ABPP measures the amount of available active sites of a certain enzyme and thereby reports on the functional state of this protein. In general, the tag of the probe determines the read-out technology to be used (**Tables 2** and **3**). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and liquid chromatography-mass spectrometry (LC-MS) are the most used analytical orthogonal platforms. In the following section, the advantages and disadvantages of these analytical platforms will be discussed (**Figure 2**).

In gel-based experiments, the labelled proteins are separated and characterised by molecular weight. First, proteins are denatured using the detergent SDS, loaded on a polyacrylamide gel and subsequently separated using gel electrophoresis (SDS-PAGE). Proteins labelled by one-step fluorescent ABPs are visualised with in-gel fluorescence scanning. Alternatively, ABPs with a biotin can be visualised using streptavidin-horseradish peroxidase (HRP) in a western blot experiment. This technique is robust, simple, has a high throughput and can be performed directly using lysates. To assign the identity of the fluorescently labelled proteins, specific inhibitors or genetic deletion of the gene is required. Disadvantages of the gel-based ABPP include a limited resolution and sensitivity. Also, the identity of the measured proteins sometimes remains ambiguous and the possibility for automation is limited (Patricelli *et al*., 2001).

For LC-MS-based ABPP experiments, proteins are labelled with a biotinylated ABP, enriched using (strept)avidin chromatography (pulldown) and digested with a protease. The

Table 2 Comparison of ABPP analytical platforms

Analytical platform	Protein (μg)/measurement	Throughput	Sensitivity	Identification	Site of labelling	Native proteome
SDS-PAGE	10	+	−	−	−	+
LC-MS	100	−−−	+	+	+	+
CE-LIF	0.1	++	++	−	−	+
FluoPol	0.1	+++	−	−	−	−
EnPlex	0.001	++++	+	−	−	−
Microarray	1	++	+	+	−	+

Table 3 Main advantages and disadvantages of each ABPP analytical platform

Analytical platform	Advantages	Disadvantages
SDS-PAGE	Robust, simple, low sample requirements	Limited resolution, sensitivity, no identification, no automation
LC-MS	High information content, high resolution and sensitivity	High sample requirements, cost of instrument
CE-LIF	High resolution, sensitivity, automation possible	No identification
FluoPol	High throughput, kinetics	*In vitro*, enzyme amount required
EnPlex	High throughput, multiplexed	Requires immobilised purified enzymes
Microarray	Identification, sensitivity, throughput	Dependent on high quality antibodies

resulting peptides are separated with liquid chromatography and measured using mass spectrometry (Li *et al*., 2013). The measured peptides will allow the identification of the labelled proteins. The peptides are sequenced using MS/MS experiments, and these peptide sequences are searched against a database of protein sequences. If a cleavable linker is used, the site of modification can be identified by releasing the probe-labelled peptide from the avidin bead and measuring the specific probe-peptide conjugate (Weerapana *et al*., 2007; Yang *et al*., 2013). This provides direct evidence that a probe has covalently labelled a protein. LC-MS-based ABPP has high resolution, sensitivity and information content. However, the throughput is low, elaborate sample preparation is needed and pulldown experiments commonly suffer from high background of abundant unlabelled proteins.

To improve the resolution, sensitivity and automation possibilities for SDS-PAGE, capillary electrophoresis coupled to laser-induced fluorescence scanning (CE-LIF) has been developed (Okerberg *et al*., 2005). Proteomes labelled with a fluorescent probe are digested with a protease and the resulting peptides separated using capillary electrophoresis. The fluorescence signal arising from probe-labelled peptides is measured. This distinguishes proteins with similar molecular weight, which comigrate on an SDS-PAGE gel.

Fluorescence polarisation (FluoPol)-ABPP has been developed to perform high-throughput screens and to assess inhibitor kinetics (Bachovchin *et al*., 2009; Lahav *et al*., 2017). FluoPol measures the apparent size of a molecule, because a small fluorescent probe rotates quickly in solution resulting in low polarisation of light, while a large probe-protein adduct rotates slowly giving rise to a high polarisation signal. The advantage of FluoPol compared to substrate assays is that it can be used to find inhibitors for poorly characterised enzymes of which the substrate is unknown. Recently, FluoPol has also been applied in cellular imaging where free and bound probe could be distinguished, thereby separating the background signal from free fluorescent probes (Dubach *et al*., 2014). Interestingly, FluoPol can also be performed with noncovalent probes. A potential disadvantage of FluoPol is the requirement of purified or overexpressed enzyme. Typically, FluoPol assays only measure the potency of inhibitors against one enzyme. Recently, EnPlex was developed, a technique that makes it possible to assess both potency and selectivity of inhibitors (Bachovchin *et al*., 2014). Multiple purified enzymes are immobilised on coloured Luminex beads, with a different colour for each enzyme. These beads are mixed, incubated with inhibitor and subsequently labelled with a biotinylated ABP, which is stained with coloured streptavidin. The bead mixture is measured by flow cytometry, detecting both the identity (bead colour) and activity (streptavidin colour) of each enzyme. Owing to the requirement of multiple purified enzymes, this platform is elaborate to set up, but once available has the highest throughput.

A technique that has the identification advantage of LC-MS but with higher throughput is microarray ABPP (Sieber *et al*., 2004). The probe-labelled proteome is incubated with an antibody microarray and a fluorescence signal is measured for the probe-labelled proteins. This technique is dependent on high-quality antibodies and prior knowledge of the probe targets is required (there is no discovery possibility as with LC-MS).

Figure 2 and **Tables 2** and **3** summarise the analytical platforms that can be coupled to ABPP. Various techniques can be combined with each other, such as SDS-PAGE and CE-LIF, which can be coupled to LC-MS to identify the tagged proteins (Bachovchin *et al*., 2010). In short, protein bands from SDS-PAGE can be excised and digested with a protease or using an in-gel digestion and the resulting peptides will be measured by LC-MS. The probe-labelled peptides from CE-LIF can be enriched using anti-fluorophore antibodies and also identified with LC-MS.

Applications

Over the last two decades, ABPP has been developed into a mature method. The labelling methods and analytical platforms have become well established. Therefore, ABPP is increasingly applied to answer biological questions by exploiting the unique ability of ABPP to directly report on enzyme activity in living biological systems. Two types of experimental setups have been widely used: comparative and competitive ABPP (Cravatt *et al*., 2008).

In comparative ABPP, the active enzyme levels in (at least) two different proteomes are analysed. These different proteomes can for instance be of two samples of a tissue in which one is in a healthy and the other is in a diseased state (**Figure 3a**). Alternatively, comparative ABPP can be used to study the effects of pharmacological intervention on the enzyme activity. The goal of comparative ABPP is to highlight any differences or similarities in active protein levels between different biological samples. This information can be used to identify metabolic pathways that are affected in disease states. This may lead to the identification

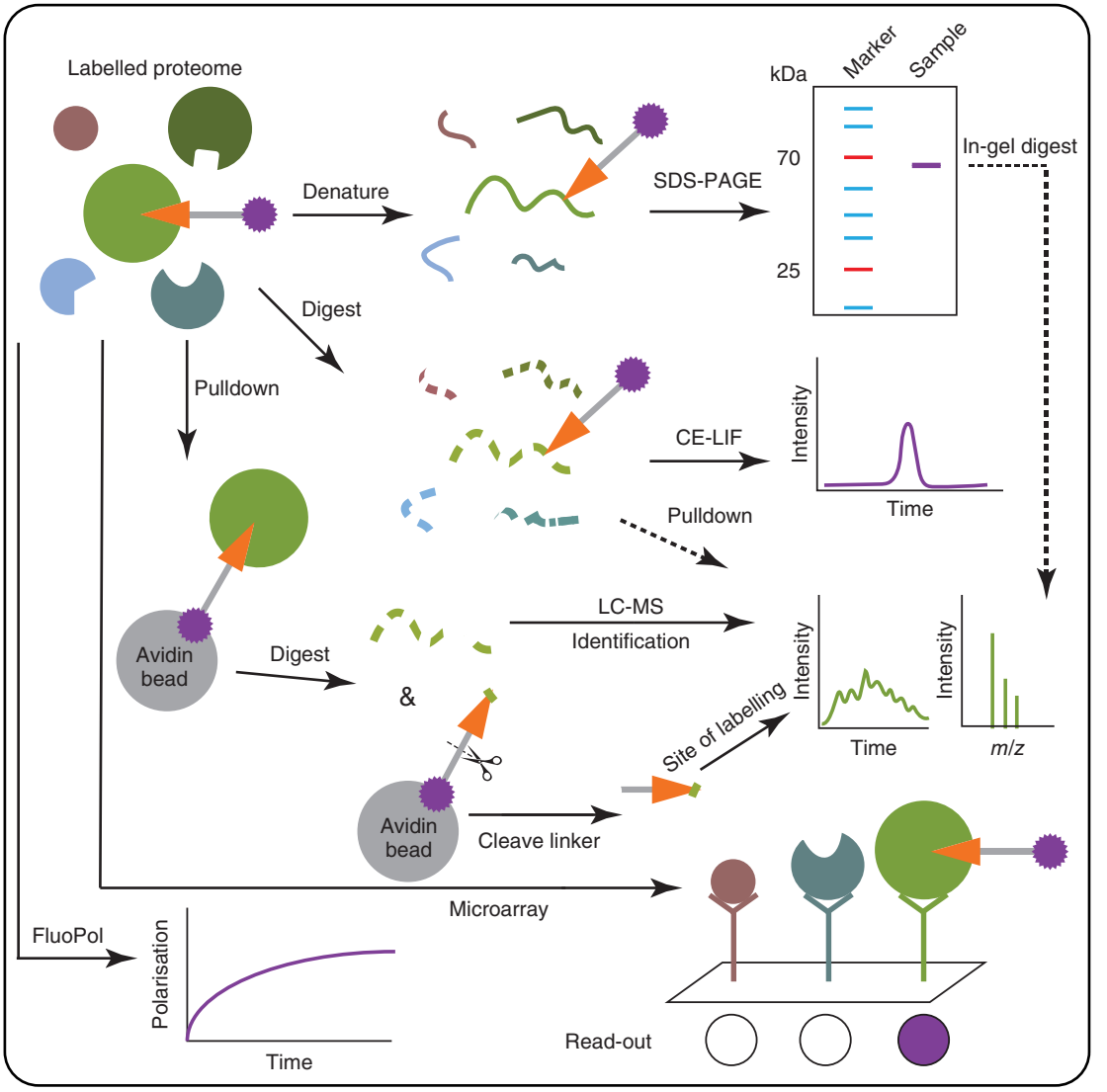


Figure 2 Visualisation of ABPP analytical platforms: SDS-PAGE, CE-LIF, LC-MS, microarray and FluoPol.

of potential new drug targets. For example, monoacylglycerol lipase was found to more active in aggressive versus nonaggressive human cancer cell lines, thereby nominating this enzyme as a potential pharmacological target for cancer therapy (Nomura *et al*., 2010a,b). Comparative ABPP has been used in many biological processes, such as host–virus interactions (Blais *et al*., 2010, 2012), microbial virulence factors (Puri *et al*., 2010) and diet-induced obesity (Sadler *et al*., 2012). Furthermore, ABPP can be used to identify novel enzymes, such as PLA2G4E as a calcium-dependent *N*-acyltransferase (Ogura *et al*., 2016).

Inhibitor potency and selectivity can be simultaneously evaluated in a competitive ABPP experiment using broad-spectrum ABPs (**Figure 3b**) (Leung *et al*., 2003). ABPP efficiently guides the hit and lead optimisation process, thereby shortening the drug discovery process. Interestingly, there is also a chance for serendipitous discoveries, such as identifying novel hits for other enzymes. In competitive ABPP a sample is pretreated with an inhibitor before the ABP is added to label residual enzyme activities. A decrease in fluorescence intensity of the bands will indicate whether the compound interacted with a protein. Competitive ABPP is also an excellent way to confirm target engagement of an enzyme in a cellular or animal model. For example, probe **1** (**Table 1**) was used to screen a library of compounds against a library of enzymes to identify inhibitors for a diverse set of serine hydrolases (Bachovchin *et al*., 2010). Competitive ABPP was also used to guide the discovery and optimisation of CNS (central nervous system)-active DAGL inhibitors (Ogasawara et al., PNAS, 2016). Recently, ABPP was used to profile the protein interaction landscape in human brain and cortical neurons of BIA 10-2474, an experimental drug that caused the death of volunteer in a phase 1 clinical trial (van Esbroeck *et al*., 2017). It was found that BIA 10-2474 inhibited several lipase off-targets,

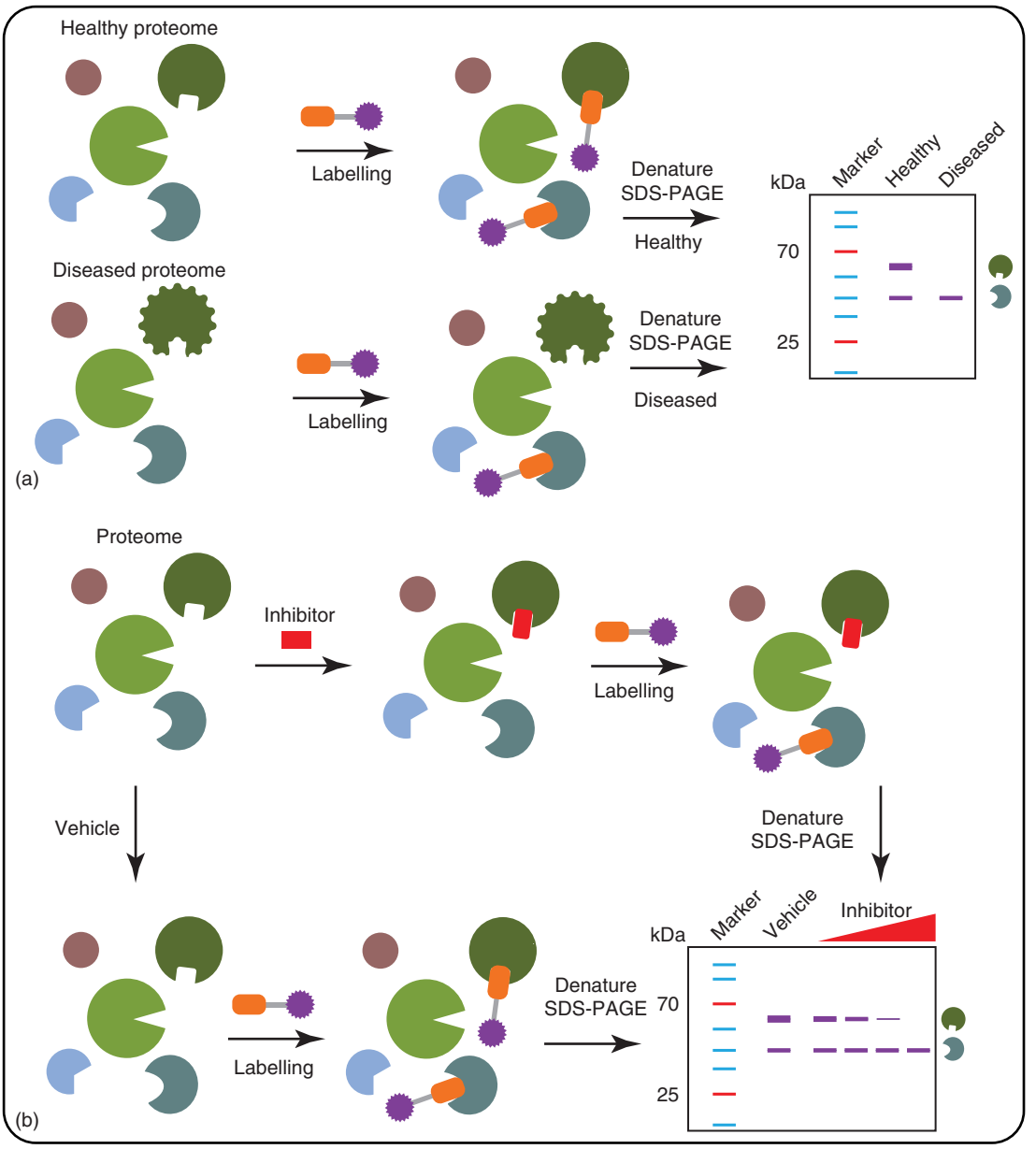


Figure 3 **ABPP experiments**. (a) Comparative ABPP. (b) Competitive ABPP.

which were not identified by the classical selectivity screening assays. It is, therefore, recommended that preclinical drug discovery should include (competitive) ABPP to profile the drug candidate on human tissues and cells.

Competitive ABPP is, however, restricted to profiling enzyme activities identified by the probe. For an ideal drug target profiling study, the drug candidate itself should be converted into an ABP (Kallemeijn *et al*., 2012). This is, however, difficult to realise if the inhibitor does not contain a protein reactive functionality. A combination of broad-spectrum ABPs targeting various enzyme families would therefore be ideal to get a broad overview of the selectivity profile of the drug candidate. Other chemical proteomics techniques such as cellular thermal shift assays (CETSA) (Reinhard *et al*., 2015) and drug affinity responsive target stability (DARTS) (Lomenick *et al*., 2009) are used to get a proteome-wide selectivity profile; however, these are not necessarily activity-based and should be used only as complementary techniques.

Future Prospects

ABPP is a powerful method to study enzyme function in a native biological setting. In the future, novel probes will be required

to enable further exploration of the enzymatically active subset of the proteome. Furthermore, new analytical platforms should be developed to enhance the sensitivity and resolution of the ABPP technique to detect low abundant enzymes and to study the effects of posttranslational modifications on the proteins. Increasing the throughput of ABPP experiments by using automation is another desired feature. Organic chemists should develop novel probes to target novel enzyme classes and further develop cleavable linkers to identify the site of modification with novel fragmentation techniques such as electron transfer dissociation (Syka *et al*., 2004). Importantly, biologists could benefit a lot from the current ABPP toolbox. Recent examples of online, searchable databases, such as chemicalprobes.org and probes-drugs.org (Skuta *et al*., 2017; Arrowsmith *et al*., 2015), aid scientists in selecting the optimal probes. The ABPP-field could benefit from adding the best probes to these open data resources and making well-characterised probes available. ABPP will continue to play an important role in elucidating the function of proteins and the discovery and development of novel drugs.

Glossary

Covalent bond A bond that is based on the sharing of electrons and forms a stable chemical linkage.

Enzyme A protein that catalyses a chemical reaction in a biological setting.

Inhibitor A compound that blocks the activity of an enzyme.

Pulldown Assay to pull certain proteins out of a solution.

Proteome All the proteins expressed in a cell at a certain moment in time.

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Further Reading

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