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Tagging and detection strategies for activity-based proteomics

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The field of activity-based proteomics is a relatively new discipline that makes use of small molecules, termed activity-based probes (ABPs), to tag and monitor distinct sets of proteins within a complex proteome. These activity-dependant labels facilitate analysis of systems-wide changes at the level of enzyme activity rather than simple protein abundance. While the use of small molecule inhibitors to label enzyme targets is not a new concept, the past ten years have seen a rapid expansion in the diversity of probe families that have been developed. In addition to increasing the number and types of enzymes that can be targeted by this method, there has also been an increase in the number of methods used to visualize probes once they are bound to target enzymes. In particular, the use of small organic fluorophores has created a wealth of applications for ABPs that range from biochemical profiling of diverse proteomes to direct imaging of active enzymes in live cells and even whole animals. In addition, the advent of new bioorthogonal coupling chemistries now enables a diverse array of tags to be added after targets are labeled with an ABP. This strategy has opened the door to new *in vivo* applications for activity-based proteomic methods.

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Introduction

One of the primary goals of the field of proteomics is to develop methods to quantify global differences in protein expression levels with the ultimate goal of assigning function to networks of proteins in normal and pathological processes. However, there are several factors that complicate both the systems-based analysis of protein expression levels and the assignment of function to specific protein targets. The primary difficulty stems from the overwhelmingly large and dynamic range of protein abundance in a given proteome that complicates the use of simple analytical separation methods to resolve proteins [1]. Second, many proteins, such as enzymes, are functionally regulated by a series of post-translational mechanisms, leading to a lack of correlation between activity and expression levels. To address these limitations, chemical or activity-based proteomics makes use of small molecule probes that enable specific enrichment of enzyme targets as well as direct profiling of enzyme activity levels. Importantly, this technique enables the specific monitoring of the regulation of enzyme activity and, therefore, provides a link to function.

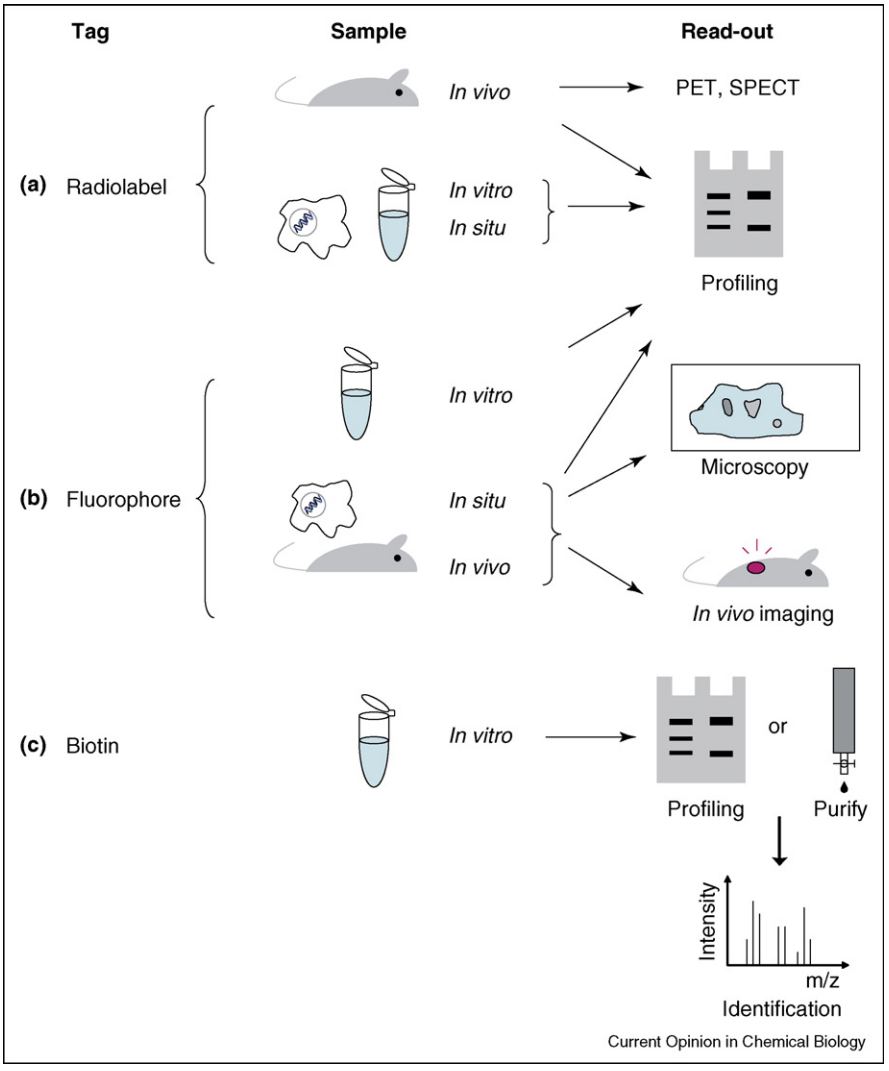
At the heart of the activity-based proteomics technology are the small molecules known as activity-based probes (ABPs) that are designed to interact with active-site residues of enzymes resulting in the formation of a stable covalent bond. These reagents can be engineered to bind a specific target or entire families of related targets. In addition, there are multiple ways in which probes can be designed to interact within active sites to effect covalent attachment. Mechanism-based ABPs, which are mainly based on irreversible enzyme inhibitors, use electrophilic 'warheads' that specifically react with catalytic residues in the enzyme's active site. Alternatively, photocrosslinkers can be conjugated to molecules that tightly bind within an active site, resulting in modification of potentially non-catalytic residues upon irradiation with UV light. In both cases, probes only bind to active forms of the target enzymes and measurement of probe modification can therefore be used as an indirect measure of enzyme activity. Regardless of the mechanism of probe attachment, all ABPs require a tag that provides a means to measure or observe targets. Because an extensive number of reviews have appeared over the past few years that outline the specifics of current ABPs as well as their applications to biological systems [2••,3–5], we chose to focus this review on addressing the scope and limitations of different tagging methods, and how these strategies can be used for diverse applications of ABPs both *in vitro* and *in vivo*. In particular, we focus on the use of isotope, fluorescent, affinity and tandem tags and discuss their applications to biological samples and methods for read-out of activity (Figure 1).

Isotope tags

Radioisotopes

Radioactive isotopes are commonly used in biological applications for the detection of a variety of biomolecules and have also proven to be useful in activity-based proteomics. In particular, ^{125}I has been incorporated into many classes of ABPs because it can be readily introduced by simple iodination methods designed for proteins and peptides (for specific protocols for iodination of ABPs see

Figure 1



A general schematic diagram of the primary labeling methods used in activity-based proteomics and their potential applications. **(a)** Radiolabels are attractive owing to their high sensitivity and broad sample applicability. The read-out is limited to gel-based detection of enzymatic activities, although *in vivo* imaging through PET or SPECT is a possible future application. **(b)** ABPs tagged with fluorophores can be applied for profiling in a wide range of samples and read-outs including *in vitro* and *in vivo* applications. **(c)** Biotinylated probes are generally limited to *in vitro* profiling applications and are primarily used for enrichment and identification of target enzymes.

[6•]). In addition, several examples of probes have been designed to incorporate ^{3}H as it can be used to replace a hydrogen in a probe without altering its structure [7]. However, the use of tritium is limited by its overall low specific activity, thereby requiring long exposure times to analyze labeling patterns.

In principle, any covalent inhibitor having a phenol function in its structure can be converted to an ABP. One of the most common methods for the introduction of the ^{125}I at the *ortho* position of a phenol group is to use radical initiators as a solid-phase catalysis in aqueous solutions. An alternative method requires the conversion of the hydroxyl into a trimethylstannyl group before replacement by a radioactive iodine under the influence of chloramine-T (Figure 2). The primary advantage of radioactive tracers is that they require minimal equipment for production, provide a highly sensitive signal with low background and are easy to detect using simple autoradiography methods. Moreover, the relatively small size of the iodine atom compared with biotin or fluorescent tags, results in minimal modification to the parent inhibitor. However, the relatively short half-life of ^{125}I means that radio-iodinated probes cannot be stored for prolonged periods. In addition, these reagents require more care in handling and use in a laboratory setting. Furthermore, the radiolabels provide no direct means to isolate and identify labeled targets. Thus, most radio-labeled ABPs are used for profiling known

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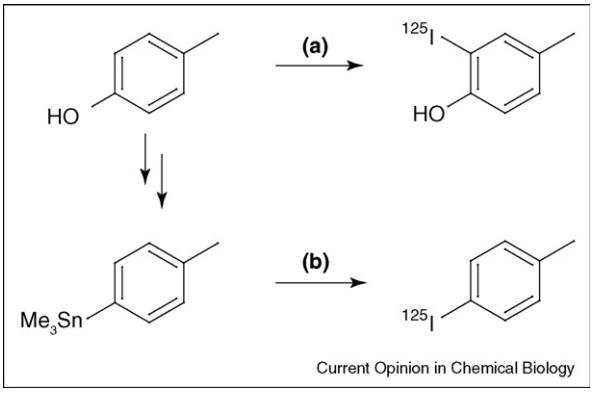
Figure 2



Methods for iodination of phenolic functions facilitated by **(a)** Iodo-Gen®, Na^{125}I, and **(b)** chloramine-T, Na^{125}I.

targets or when specific antibodies can be used to confirm the identity of the target enzymes.

Radio-labeled probes have mainly been used to profile changes in enzyme activity by analysis of labeled samples by 1D or 2D gel electrophoresis. Because cell permeability is generally comparable to the parent inhibitor form, probes can be used in diverse samples, including cell or tissue lysates, intact cells or whole organisms. There have been several examples of *in vitro* applications of radio-labeled probes for profiling changes in activity of cysteine protease targets, including the cathepsins [8,9], the proteasome [10,11] and caspases [9]. For these studies probes are added directly to a lysate *in vitro*, followed by visualization of labeled targets by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

Alternatively, some classes of radio-labeled probes have been used to label active enzymes in intact cells [8,10]. This enables the activity of a target to be monitored within its native cellular environment and prevents complications that result from the release of endogenous inhibitors of enzymes when cells or tissues are disrupted. In a recent example, Percival and coworkers [12•] synthesized a cell-permeable ^{125}I-labeled diazomethyl ketone probe that labels cathepsins B, L, K and S after treatment of live cell cultures. They demonstrate the utility of this reagent for profiling the potency and selectivity of small molecule cathepsin inhibitors in whole cells.

Taking the application of radio-labeled ABPs one step further, Méthot *et al.* [13•] developed a cell permeable ^{125}I-labeled caspase probe that could be used in cell extracts, intact cells and even in whole animals. Administration of this radio-labeled fluoromethyl ketone probe into septic mice enabled direct *in vivo* measurement of the fraction of caspase inhibition required to block apoptosis-induced DNA fragmentation. The high degree of selectivity and sensitivity of the radio-labeled probe enabled the development of this *in vivo* assay.

Given the recent success of radio-labeled and fluorescently labeled (see fluorescent label section) probes for direct *in vivo* applications, it might be possible to extend the use of ABPs to non-invasive imaging techniques using positron emission tomography (PET) or single photon emission calculated tomography (SPECT) [14]. Conjugation of isotopes such as ^{18}F or ^{64}Cu to ABPs should result in probes that report on enzyme activity in live subjects. Even with the relatively short half-lives of the isotopes causing some complications in the generation of the imaging agent, extension of ABPs to non-invasive imaging methods is likely to be developed in the near future.

Stable isotopes

In addition to radio-isotopes, stable isotopes have the potential to be of value for the field of activity-based proteomics. Stable isotope tags have been extensively used in quantitative proteomics applications and have become a major focus of attention of the proteomics community in the past 5–10 years. Methods such as isotope-coded affinity tags (ICAT) [15], stable isotope labeling by amino acids in cell culture (SILAC) [16] and absolute quantification (AQUA) [17] have been developed using probes, peptide standards or amino acids labeled with heavy and light versions of hydrogen or carbon atoms. These heavy and light reporters can be distinguished by differences in mass upon analysis by mass spectrometry. For the ICAT technique, a general cysteine reactive probe is used in heavy- and light-labeled form to covalently modify cysteine-containing proteins in two samples of different origin. After combination and digestion, relative abundance can be quantified by measuring the abundance of heavy- and light-labeled peptides by mass spectrometry. This method has proven extremely valuable for the bulk quantification of changes in protein abundance and is suitable for direct translation to ABPs. A first example of an isotope-coded ABP has been reported by Overkleeft and co-workers [18], who synthesized DCG-04, a general papain probe, in a light and heavy version. However, mass spectrometry validation of this method has not yet been established. It should also be noted that recent studies using spectral counting methods suggest that relative quantification might be possible without the need for isotope labels [19]. In fact, Cravatt and co-workers [20••] have recently demonstrated the use of non-isotopically labeled ABPs to quantify changes in enzyme activity levels by direct mass spectrometry methods, suggesting that the use of stable isotope labels might only be required for monitoring subtle changes in enzyme activity.

Affinity tags

One of the primary applications for ABPs is their use as a handle to isolate labeled target enzymes. Biotin is the

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most commonly used affinity tag for activity-based proteomics, and has been used in numerous studies. Its diffusion-limited binding to streptavidin makes it a powerful tool for both detection of probe-labeled enzymes by affinity blotting methods and purification of ABP–enzyme complexes using avidin-coated resins. For this reason, biotin still remains the affinity label of choice for isolation and identification of targets of ABPs. However, there are several limitations to the use of biotin as a tag for activity-based proteomics. First, biotin has low cell permeability properties, which prevents its use for *in situ* and *in vivo* applications. As a result, biotin-labeled probes are mostly used *in vitro* to isolate targets from cell or tissue extracts. Second, once a biotin-labeled protein is bound to an avidin resin, elution of labeled protein requires harsh conditions such as detergents or denaturants that release nonspecific, unlabeled background proteins. In addition, most cells and tissue extracts contain a significant number of endogenously biotinylated proteins that are also purified by the avidin affinity resin. Interestingly, these shortcomings have recently been addressed with the advent of bioorthogonal labeling methods that enable biotin to be added after cell lysis (see tandem labeling section) and with the development of cleavable linkers that facilitate specific release of probe-labeled proteins [21•].

As an alternative to biotin, short peptide sequences that are recognized by specific antibodies can also serve as detection tags. The first example of this type of tag was reported for probes that target deubiquitinating (DUB) enzymes [22,23]. In this example, a full-length ubiquitin protein was equipped with a hemagglutinin (HA) tag and converted to an ABP by chemical modification of the C-terminus with a covalent warhead. Target proteases could be detected by SDS-PAGE analysis followed by western blotting using an anti-HA antibody. Furthermore, the identity of the target DUBs could be determined by immunoprecipitation using the HA antibody followed by mass spectrometry-based identification. Since the publication of this first study, several related classes of HA-tagged probes for other ubiquitin-like proteins have been reported [24].

Although the use of peptide and protein tags that can be recognized by an antibody has advantages in terms of selective detection and ease of isolation, it also has disadvantages. In particular, peptide tags are large, difficult to incorporate into synthetic small molecule scaffolds and generally block cell permeability of probes. To get around these issues, Patricelli and co-workers [25•] have developed monoclonal antibodies that specifically recognize the small organic fluorophore rhodamine. These antibodies were used to isolate serine hydrolase targets that had been labeled by a rhodamine conjugated ABP, enabling subsequent analysis of fluorescent peptides by mass spectrometry and capillary electrophoresis. Although this method shows great promise, the antibodies used for this application are not currently commercially available. Thus, efforts to develop antibodies that recognize additional small molecule fluorophores will be required before this technique can find widespread applications in activity-based proteomics.

In addition, other more complex and creative methods have been developed to tag ABPs to facilitate their isolation by affinity methods. In one particularly innovative method, Winssinger and Harris [26,27] synthesized libraries of ABPs that were labeled with peptide nucleic acid (PNA) tags that could be used for isolation of labeled targets on oligonucleotide microarrays. In addition, the sequence of the tag served as a reporter that identified the structure of the probe to which it was attached. Thus, pools of probes could be used to label complex mixtures, followed by isolation of target enzymes and identification of optimal probe structures for each target.

Finally, an alternative affinity isolation approach completely removes the tag and instead makes use of an ABP directly bound to a resin. Examples include the work of Hesek *et al.* [28,29] in which resin-bound inhibitors were used to monitor changes in matrix metalloproteinase activity. Although this method alleviates the need for specific covalent modification of targets, it suffers from the limitation that it can only be used with cell or tissue extracts. Furthermore, target binding might be limited in some cases by accessibility to the resin-bound probe.

Fluorophores

Perhaps the greatest advance in tagging methods for activity-based proteomics has been the use of small molecule fluorophores. The great diversity of commercially available fluorophores, combined with their broad range of structural properties and absorbance and emission spectra, have dramatically increased the scope of applications for ABPs. Initial applications of fluorescently tagged ABPs were reported over four years ago [30,31] and the number of new fluorescent probes has expanded rapidly ever since. Some of the most significant advantages of fluorescently labeled ABPs are their use for direct imaging of labeled targets using fluorescence microscopy as well as their ease of detection in gel-based read-outs.

A wide variety of fluorophores have now been used for the development of ABPs. The most commonly used classes include fluorescein and rhodamine [30], dansyl [32], NBD (nitrobenz-2-oxa-1,3-diazole) [33], BODIPY (dipyrromethene boron difluoride) [31] and the cyanine (Cy)-dyes [34] (see Table 1). The first class of fluorescein and rhodamine is relatively inexpensive, but suffers from rapid photobleaching, making them less suitable for most imaging applications. BODIPY and Cy-dyes display high absorption coefficients, high quantum yields, narrow absorption peaks and relatively large stoke shifts.

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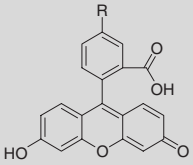
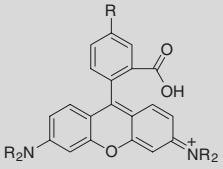
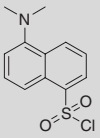
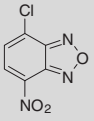
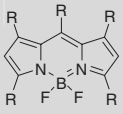
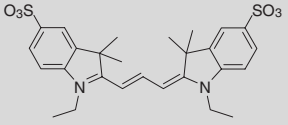
Table 1

Structures of commonly used fluorophores and advantages and disadvantages of their use for activity-based proteomics.

Fluorophore	Structure	Advantage	Disadvantage
Fluorescein		Relative high absorptivity; excellent quantum yield; high water solubility; inexpensive; stable to solid-phase peptide synthesis methods.	Fast photobleaching; pH sensitive below 7; relative broad emission; self-quenching; lack of cell permeability.
Rhodamine		Readily excited — more photostable than fluorescein.	Low quantum yield (1/4 of fluorescein); complex absorption complex; lack of cell permeability.
DNS		Good FRET acceptors; fluorescent as dansyl amide and not chloride; large stokes shifts; inexpensive.	Environmental-dependent quantum yields and emission maxima.
NBD		Can react with secondary amine; inexpensive.	Solvent-dependent absorption and emission; quantum yield and extinction coefficients; non-fluorescent adduct of aromatic amines.
BODIPY		High extinction coefficients (>60 000 $cm^{-1}M^{-1}$); excellent photostability; high fluroscence quantum yields; narrow emission bandwidth; good photostability; pH insensitive; no ionic charge; non-polar; minimal effect on electrophoresis mobility; cell permeable.	Unstable under most standard solid-phase peptide chemistries; expensive.
Cyanine		pH insensitive (3–10); DMSO tolerance; good aqueous solubility; superior photostability; stable to standard peptide synthesis.	Expensive.

Abbreviation: DNS, 5-dimethylaminonaphthalene-1-sulfonamide.

Furthermore, these fluorescent tags are hydrophobic and freely penetrate cell membranes. These combined properties make them suitable for a variety of biological applications. However, the commercially available activated ester forms of these fluorophores are extremely expensive and, therefore, have prevented large scale production of fluorescent ABPs.

Most new probe classes make use of commercially available fluorophores because they enable direct read-out of probe labeling in SDS-PAGE gels using a laser scanner. This provides a significant advantage over biotin and radiolabeled probes, as gels can be scanned while still in the glass plates used during electrophoresis, rather than requiring elaborate gel processing procedures to make quantitative measures of probe-labeled proteomes. In addition, several of the fluorophores are highly hydrophobic and can help to enhance the cell permeability of labeled probes. For example, the general papain family protease probe DCG-04, which contains a biotin tag, can only be used in extracts or in cells that actively uptake the probe, whereas BODIPY labeled forms of this probe freely penetrate live cells and can be used for biochemical profiling and live cell imaging [31]. In addition BODIPY–DCG-04 analogs have been used for *in vivo* labeling of cysteine cathepsins in whole animals. Administration of the fluorescent probe by intravenous injection into transgenic mice bearing pancreatic tumors resulted in labeling of active cysteine cathepsins in diverse tissues including tumors [35••]. This *in vivo* activity could be monitored by fluorescence microscopy of histological tissue sections. These studies enabled the direct imaging of enzyme activity and provided insight into the distribution of protease activity within the complex microenvironment of a tumor.

Although fluorescent ABPs have found applications in live cell and *in vivo* imaging, the intrinsic fluoresence of the unbound probes precludes their use in real-time

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imaging because free probe must be removed by washing before specific signals can be observed. Recently, Blum *et al.* [36•] have overcome this problem by developing ABPs that contain a fluorescent quencher that is lost upon activity-dependent binding to an enzyme target. In the first example of a fluorescently quenched ABP (qABP), a peptide acyloxymethyl ketone containing a BODIPY fluorophore and quenching group was used to image cathepsin activity in whole cells without the need to wash away free probe [36•]. Extension of this technology to other probe classes is likely to yield additional new cell-based and *in vivo* imaging agents that have superior signal to noise ratios.

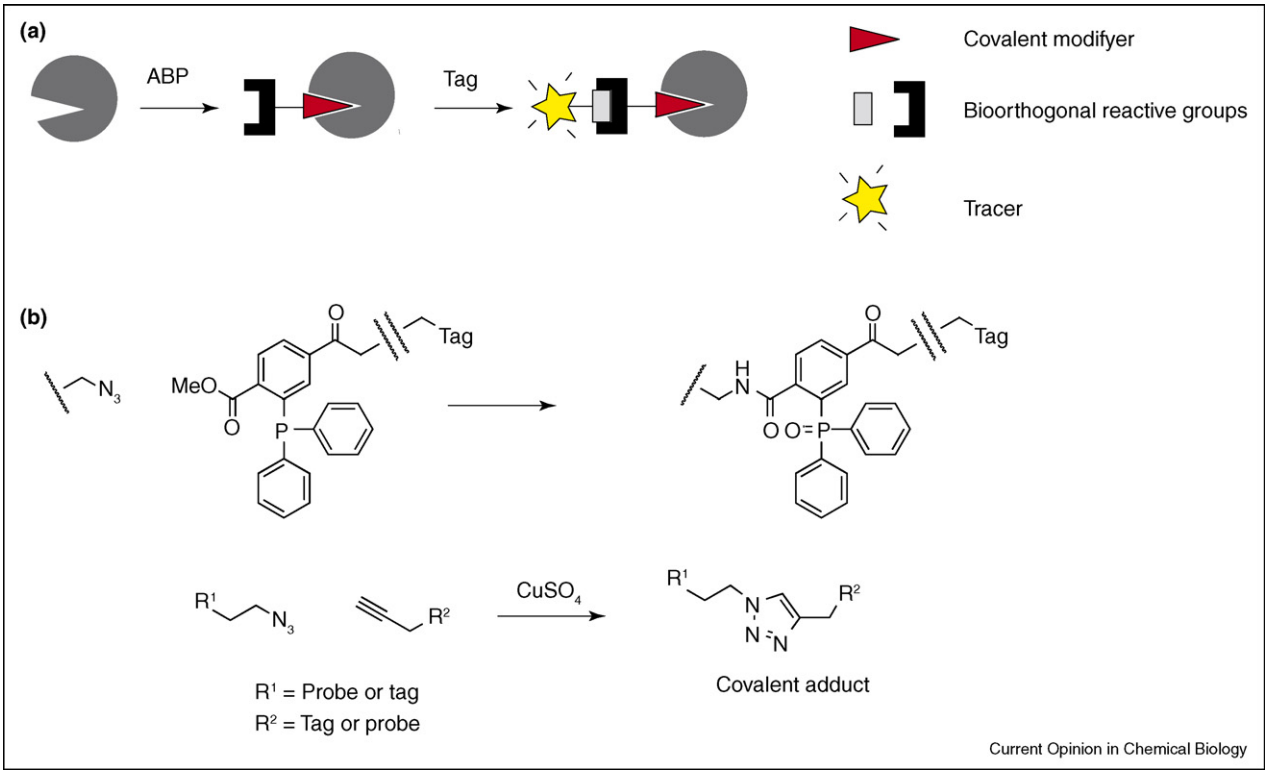
Looking forward, it seems possible that the initial successes using fluorescent ABPs for *in vivo* applications could be translated into new therapeutic imaging agents for use in human patients. There have been several recent advances in the development of fluorescent reporters of enzyme activity. In particular, synthetic protease substrates that carry quenched near-infrared fluorescent (NIRF) tags that become fluorescent upon proteolytic processing have been developed and show promise in animal models of human disease [37–39]. The use of similar NIRF tags on ABPs should facilitate their use for non-invasive imaging. Importantly, NIRF–ABPs would enable signals observed in whole body images to be linked to specific proteomic labeling profiles that can be used to identify enzyme targets. Such applications to non-invasive fluorescent imaging, such as those using radiological agents, are likely to be a future direction for activity-based proteomics.

Tandem labeling strategies

An additional significant advance in tagging methods for ABPs has been the development of small surrogate tags that facilitate the attachment of reporter tags to a probe by using highly specific bioorthogonal ligation chemistry (Figure 3). The main advantage of this method is the ability to use small tags that have minimal effects on target binding and cell permeability and that can be later used for chemical modification with any of several diverse labels (e.g. biotin and fluorophore). This so-called tandem labeling process has been developed for ABPs in the last couple of years and has been successfully demonstrated using two different ligation chemistries.

The first method makes use of a modified Staudinger reaction in which a probe containing an azide functional group is reduced and subsequently reacts to form a stable

Figure 3



Tandem labeling strategies for activity-based proteomics. **(a)** Schematic of the tandem labeling approach in which a latent ABP carrying a surrogate tag is used to label targets in a complex proteome. Tags or tracers are then attached by a specific bioorthogonal ligation chemistry, resulting in labeled protein targets that can be visualized. **(b)** Specific ligation chemistries used for ABPs include a modified Staudinger ligation (top) or a copper-mediated cycloaddition or click reaction (bottom).

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Table 2

General summary of advantages and disadvantages of the primary tagging methods used in activity-based proteomics.

Method	Advantage	Disadvantage
Isotope:		
Radioactive	Small size; easy detection; chemical stability; *in vivo* activity; high sensitivity.	Harmful; fast degradation; gel-based read-outs.
Stable	Easy insertion; chemical stability; *in vivo* activity.	Expensive equipment for analysis; complex data analysis.
Affinity tags:		
Biotin	Easy enrichment and isolation; strong binding; easy detection.	Relatively large; non-cell-permeable; difficult to elute from affinity resins.
HA	Antibody detected; easily blotted; easy elution from antibody resins.	Non-synthetic; time consuming development; large size and peptidic; non-cell-permeable.
PNA	High-throughput analysis; easy detection and isolation; oligonucleotide binding; potential for coding.	Relatively large; non-trivial chemistry.
Resin-tethered	No need for covalent modification of targets.	Cannot be used for *in vivo* applications; limited access to probes by target enzymes.
Fluorophore	Easy detection; quenchable; some cell permeable; *in vivo* activity; imaging applications.	Large size; some instability; background signal.
Tandem	Secondary attachment of tags; small size; biologically compatible; tag flexibility; high-throughput analysis; *in vivo* activity.	Chemical coupling yields fluctuation in different systems; metal catalysts used.

amide with an appropriately derivatized phosphine reporter tag. This strategy was first developed by Bertozzi [40] to tag modified carbohydrates on the cell surface and also proved to be useful for ABPs. In particular, this two-step labeling strategy has been employed for detection of active proteasomes in intact cells using an azide containing probe, which was then labeled with a biotin-derivitized phosphine [41]. Interestingly, recent work by the same group has demonstrated that direct *in vivo* labeling of proteasomes is possible using cell permeable fluorescent probes, thus obviating the need for the tandem labeling reaction [32].

A second and more common tandem labeling strategy makes use of the so-called 'click' chemistry, in which the [2+3] cycloaddition of an alkyne and azide functional group is facilitated by the addition of a copper catalyst. Originally developed by Sharpless and co-workers [42], this strategy was modified by Cravatt and co-workers [43] for use in activity-based proteomics. The two reaction partners are both fully biocompatible and cyclizations can be initiated in aqueous solutions that even contain strong denaturants such as SDS and urea. Thus, this has become the method of choice for tandem labeling applications and several probes containing either an azide or alkyne tag have been recently reported [21•,34,44]. Overall, both of these tandem labeling methods have had a significant impact on the field of activity-based proteomics and will probably be extensively used in future generations of ABPs.

Conclusions

The field of activity-based proteomics has seen significant technological advances in the recent past that have helped to vastly increase the number of applications of this methodology for addressing important biological questions. With the development of many new classes of ABPs has come an ever increasing diversity of targets that can be monitored at the level of regulation of enzyme activity. In addition, there has been a rapid advance in the methods used to tag and detect probe-labeled targets. Here, we have outlined some of the more significant advances in tagging methods as well as pointed out some of the strengths and weaknesses of each approach. A summary of these issues is outlined in Table 2 to provide some degree of guidance when deciding which labeling strategy is best suited for a particular proteomic application. Undoubtedly the field will continue to grow in the coming years and additional labeling methods will be added to the list.

Acknowledgements

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