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An overview of activity-based probes for glycosidases

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Abstract

As the scope of modern genomics technologies increases, so does the need for informative chemical tools to study functional biology. Activity-based probes (ABPs) provide a powerful suite of reagents to probe the biochemistry of living organisms. These probes, featuring a specificity motif, a reactive chemical group and a reporter tag, are opening-up large swathes of protein chemistry to investigation *in vitro*, as well as in cellular extracts, cells and living organisms *in vivo*. Glycoside hydrolases, by virtue of their prominent biological and applied roles, provide a broad canvas on which ABPs may illustrate their functions. Here we provide an overview of glycosidase ABP mechanisms, and review recent ABP work in the glycoside hydrolase field, encompassing their use in medical diagnosis, their application for generating chemical genetic disease models, their fine-tuning through conformational and reactivity insight, their use for high-throughput inhibitor discovery, and their deployment for enzyme discovery and dynamic characterization.

Highlights

- Glycosidases carry out many essential functions across all domains of life
- Activity-based probes can interrogate complex biological samples for glycosidase activity
- Glycosidase probes can characterize enzymes of biomedical/biotechnological interest
- Analysis of conformational itineraries may inform the design of future probes.
- Activity-based probes assist high-throughput discovery of inhibitors

1. Introduction and History

The development of modern genomic technologies has provided us with the ability to sequence (and even edit) the genetic code of essentially any organism. However, our understanding of how this wealth of genetic information links to biological function *in vivo* is still limited. The majority of the biochemical reactions that underpin life are catalyzed by protein enzymes, whose primary sequences are encoded by genes, but whose functions are often difficult to deconvolute from the genetic code. Enzyme structures and functions are hard to predict from primary sequence alone, and their activities may be further modulated by post-translational modifications, processing, intermolecular interactions and subcellular localization.

Activity-based protein profiling (ABPP) has gained prominence as a powerful tool for the functional annotation of enzymes within complex biological milieu. ABPP relies on the availability of suitable activity-based probes (ABPs) that react with broad classes of enzymes, allowing investigation of their identity and functions. ABPs are typically comprised of 3 main components: 1) a specificity endowing motif that directs the ABP towards a particular group of enzymes through key-interactions to the enzyme active-site, 2) a chemical 'warhead' that reacts (often irreversibly) with catalytic residues within the active-site, 3) a reporter group that enables detection and/or capture of the ABP after reaction with the enzyme (**Figure 1a**).

ABPs are intimately linked with the concept of mechanism-based inhibitors. The first ABPs were derived from linking mechanism-based inhibitors of particular enzyme classes to reporter moieties that could be exploited for analysis. Early work by Mason *et al.* showed that cathepsin B and L specific ABPs could be developed by simple modification of existing inhibitors to incorporate a ^{125}I radioisotope[1]. Tuning the 'peptide' sequence of these ABPs enabled targeting of different classes of proteases, such as the profiling of proteasomal β-subunits by ^{125}I-nitrophenyl-$(Leu)_3$-vinylsulfone as demonstrated by Bogyo, Ploegh and coworkers[2]. This strategy of converting inhibitor to ABP was also used by Cravatt and coworkers in their seminal 1999 report, showing that reactive fluorophosphonates could be derivatized to produce broad spectrum serine hydrolase ABPs, with a biotin reporter group allowing for detection by peroxidase conjugated avidin[3].

ABPs have now been developed for many enzyme classes, including methyltransferases[4], kinases[5], phosphatases[6], ubiquitin ligases[7], and glycosidases[8-11] (**Figure 1b**). This review summarizes recent work in the development of glycosidase targeting ABPs, with some comments regarding possible future directions for the field. For a more historical perspective, the reader is invited to consult the comprehensive review of Rempel and Withers[12], and references therein.

2. ABPs for glycoside hydrolases

Carbohydrate containing biomolecules (oligosaccharides, polysaccharides, small and macro-molecular glycoconjugates) play important metabolic, signaling and structural roles across all domains of life. Glycoside hydrolases (GHs; also known as glycosidases) are a major class of enzymes responsible for hydrolytic breakdown of carbohydrates, and are essential for diverse biological processes. ABPP of glycosidases can provide insights into the role these enzymes play in complex biological systems, as well as enabling the identification of previously unknown glycosidases.

Mechanistically, glycosidases are classified as inverting or retaining, depending on whether the enzymatic reaction proceeds with net inversion or retention of stereochemistry at the cleaved glycoside anomeric center[13]. Inverting glycosidases typically catalyze hydrolysis *via* a single step nucleophilic displacement at the substrate anomeric center. In contrast, most retaining glycosidases utilize a two-step mechanism mediated by two key catalytic residues: a nucleophile and a general acid/base, and proceed *via* a covalent glycosyl-enzyme intermediate (**Figure 2a**). Trapping this covalent intermediate is the conceptual basis for most mechanism-based inhibitors and ABPs that target retaining glycosidases.

2.1. Glycosidase ABPs that stabilize the covalent intermediate lifetime

A major group of retaining glycosidase mechanism-based inhibitors function by extending the lifetime of the covalent 'glycosyl'-enzyme intermediate, which can be achieved using activated fluorosugar[14-16] (**Figure 2b**) or carbasugar[17] based molecules. In their seminal work, Vocadlo and Bertozzi reported the use of 6-azido-2,6-dideoxy-2-fluoro-β-D-galactosyl fluoride ABPs to label β-galactosidases in crude cell lysates. The modified enzymes were detected by means of Staudinger

ligation to a phosphine-FLAG tag, followed by immunoblot[9]. Fluorosugar based ABPs have since been synthesized for the labeling of β-glucosaminidases[18], β-xylanases[19,20] and acid β-glucocerebrosidase (GBA)[21]. Although fluorosugars work well against many glycosidase classes, they are rapidly turned over in some cases (*e.g.* 2-deoxy-2-fluoro-α-D-glucosides by α-glucosidases)[15], precluding the use of these compounds as general probes for all retaining glycosidases.

Cyclopropyl and vinyl carbasugar inhibitors operate in a conceptually similar manner to fluorosugars, by forming a stable 'glycosyl' intermediate that accumulates on enzyme[22]. However, these molecules also suffer from similar drawbacks to fluorosugars, being turned over too rapidly in some cases to be of use as probes. To ameliorate problems of rapid turnover, fluorosugar and carbasugar moieties can be combined. Thus cyclopropyl and vinyl 2-deoxy-2-fluoro-carbagalactose inhibitors have been observed to form longer lived α-galactosidase-conjugates compared to both their 2-deoxy-2-fluoro-galactose and carbagalactose congeners[23,24].

2.2. *Electrophilic glycosidase ABPs*

Cyclitol epoxides, such as conduritol B epoxide (CBE; **Figure 2c**), employ an electrophilic trap mechanism to label their target. Pioneering work by Legler in the 1960s provided a possible route into glycosidase ABPs through the use of ^{14}C radiolabeled CBE[25,26]. However, the intrinsic promiscuity of CBE (partially related to its internal symmetry) renders it a poor tool for the study of glycosidase and cellular biology. A marked improvement on CBE was realized with the discovery of the natural product cyclophellitol. First isolated from *Phellinus sp[27]*, cyclophellitol resembles β-D-glucose in configuration, but with a highly electrophilic epoxide instead of an aglycone. Engagement of cyclophellitol by retaining β-glucosidases results in attack of the epoxide by the β-glucosidase catalytic nucleophile, leading to irreversible alkylation of this residue[28] (**Figure 2c**).

Since its discovery, multiple synthetic routes towards cyclophellitol have been reported[29], however these typically required multi-step synthetic transformations and expensive starting materials. The facile synthesis of cyclophellitol from D-xylose by Madsen and coworkers in 2005 paved the way for exploitation of this scaffold for the development of ABPs[30]. In 2010 Witte *et al.* reported the first cyclophellitol derived ABPs, in which a reporter group was appended to the C6 position of the cyclophellitol 'glucosidic' ring[10]. This produced highly potent (nanomolar K_I) and selective ABPs for lysosomal glucocerebrosidase (GBA), enabling detection of this enzyme in lysates from various biological samples. Although useful for investigating GBA activity, the bulky C6 substituent of first generation cyclophellitol-derived ABPs precluded their activity against other retaining β-glucosidases. This was addressed by the development of *N*-acylated and *N*-alkylated cyclophellitol aziridines, in which the reporter moiety is projected towards the direction normally occupied by the substrate aglycon, leading to broad spectrum ABPs that label all retaining β-glucosidases[31]. Modulation of substitution and stereochemistry around the 'glycosidic' ring of the cyclophellitol scaffold has now provided ABPs for a wide range of different retaining glycosidases[32-35].

Haloketone (typically bromoketone) glycosides are another example of electrophilic glycosidase ABP, which inhibit retaining glycosidases *via* alkylation of nucleophilic residues within the enzyme active site (**Figure 2d**). C-bromoketone glycosides and *N*-bromoacetyl-glycosylamines have been developed for the inhibition of β-glucosidases, β-glucanases[36], and endo-xyloglucanases[37]. Although there are relatively few reports of ABPs based upon haloketone type inhibitors, Keresztessy *et al.* reported the use of ^{14}C radiolabeled *N*-bromoacetyl-β-D-glucosylamine to identify the catalytic acid/base residue of cyanogenic β-glucosidase (linamarase) from *Manihot esculenta Crantz* (cassava)[8]. Anderson *et al.* also reported the use of an azido N-iodoacetyl-glycosylamine probe to profile secreted cellulolytic enzymes of several *Trichoderma reesei* strains[38]. It should be noted that whilst there

seems no obvious reason why haloketone glycosides should not also label inverting glycosidases, to our knowledge only inhibition of retaining glycosidases has been observed to date.

2.3. Glycosidase ABPs that generate reactive intermediates in situ

Photoactivatable affinity-based probes (AfBPs) have been explored as potential tools for glycosidase profiling (**Figure 2e**). A 1992 study by Kuhn *et al* showed that thioglycosides functionalized with a photoactive diazirine moiety could label the active site of human lysosomal β–hexosaminidase A[39]. Similarly, Orth *et al* demonstrated that benzophenone containing GlcNAc configured AfBPs label the active site of recombinant bacterial *N*-acetylglucosaminidase NagZ[40]. Gandy *et al* utilized a suite of iminosugars functionalized with photoactivatable aromatic azides to profile both recombinant glycosidases and glycosidases in crude *E. coli* lysates[41]. Notably, the authors of this study reported labeling of both retaining and inverting glycosidases by their probes.

One general 'disadvantage' of photoactive AfBP profiling is that these molecules do not only target enzymes, but can in principle label any carbohydrate binding protein of appropriate specificity, as demonstrated by the labeling of a GM2-activator protein by a ganglioside based diazirine AfBP[42]. Furthermore, photoactivation of probes in free solution can contribute to off target labeling of even non-carbohydrate binding proteins. A true ABP based strategy for labeling glycosidases has been to employ probes that only release a reactive group upon enzymatic attack, which can then proceed to label nearby residues around the enzyme active site. The glycosylmethyltriazines pioneered by Sinnott and coworkers are early examples in this class[43], which fragment upon enzyme mediated protonation to form a reactive carbocation that alkylates nearby nucleophilic amino acid sidechains. However, glycosylmethyltriazines are prone to non-enzymatic degradation at acidic pHs, making the study of glycosidases (which typically require acidic pH for optimal activity) using these molecules problematic.

Quinone methide based molecules are now predominant amongst the class of reactive group generating ABPs, and have been used to study glycosidases in both biomedical and biotechnological contexts[44-46] (**Figure 2f**). Although useful against both retaining and inverting glycosidases, the use of quinone methide ABPs can also lead to unspecific protein labeling, due to diffusion of activated quinone methide electrophiles out of the enzyme active sites of their initial formation. Such off-target labeling suggests that quinone methide ABPs are better suited for broad labeling of macromolecular complexes or cellular structures[11,44,45], rather than precision labeling of individual glycosidases.

3. Glycosidase conformational itineraries and ABP reactivity

One of the defining features of glycosidase mediated substrate cleavage is the complex series of conformational distortions undergone by the substrate glycone ring during the catalytic cycle. These distortions are necessary to accommodate the oxocarbenium ion-like transition state of glycoside hydrolysis, in which at least 4 atoms of the glycoside ring (including the endocyclic oxygen) must be coplanar[47]. The sequence of conformational distortions employed by a glycosidase to process its substrates is known as the conformational itinerary. These itineraries are typically conserved between related glycosidase families (**Figure 3a**).

Recent years have seen an increasing use of conformational free energy landscape (FEL) analysis as a tool to predict the conformational itineraries employed by different glycosidases[47]. FELs can also be used to calculate conformations favored by glycosidase ABPs and mechanism-based inhibitors, which can shed light on differences in labeling efficiency between different classes of glycosidase ABPs, and inform the design of new probes with improved potency and/or specificity. FEL analysis of cyclophellitol-derived inhibitors/ABPs has shown that these molecules typically adopt a $^{4}H_{3}$ half-chair

ground state conformation, due to the stereochemical constraints placed on the cyclitol ring by the strained epoxide/aziridine moiety[48]. Significantly, many families of retaining glycosidases process their substrates via a $^4H_3$ transition state complex[47], and thus cyclophellitol-derived ABPs act as transition state mimics for these enzymes, typically showing low μM to nM equilibrium constants for initial binding (K_I = ~15 μM for cyclophellitol against the *Tm*GH1 β-glucosidase from *Thermotoga maritima*)[28]. Indeed, even neutral non-electrophilic carba-cyclophellitols, containing a cyclopropyl in place of the epoxide, are competent reversible glycosidase inhibitors due to conformational mimicry (**Figure 3b**)[48].

The Michaelis complex conformation, adopted by substrates upon initial enzyme binding, can also be exploited for the design of mechanism-based glycosidase inhibitors. Cyclophellitol cyclosulfate inhibitors were designed based on this principle, with the first (glucose configured) inhibitors of this class showing exquisite selectivity for inhibition of α-glucosidases compared to β-glucosidases. The ground state conformation of cyclophellitol cyclosulfates is a $^4C_1$ chair, which closely resembles the Michaelis complex conformation utilized by α-glucosidases, but not the $^1S_3$ Michaelis complex conformation utilized by β-glucosidases. Thus α-cyclophellitol cyclosulfates rapidly inactivate α-glucosidases, whilst their β-configured congeners are only marginal β-glucosidase inhibitors[49] (**Figure 3c**).

Interestingly, mechanism-based inhibitors/probes that employ non-native conformational itineraries are well tolerated by some glycosidases. The α-D-galacto configured cyclopropane-carbasugars pioneered by Bennet and coworkers adopt an unusual $^2H_3$ Michaelis complex conformation in the active site of α-galactosidases, owing to conformational restriction across the C5–O5 equivalent bond (the O5 position being occupied by a tertiary carbon in these molecules)[24]. Despite their non-native Michaelis conformation, these molecules react efficiently with GH36 α-galactosidases, although their ability to label GH27 α-galactosidases is more limited[17]. Taken together, it appears that mechanism-based inhibitors employing non-native conformational itineraries can selectively target some classes of glycosidases, hinting at a strategy for the development of family specific inhibitors/probes.

4. Recent developments in the glycosidase ABP field

4.1. Glycosidase ABPP for biomedical diagnostics

Several pathophysiological states, including mucopolysaccharoidoses and various lysosomal storage diseases (LSDs), arise from insufficient levels of glycosidase activity. Diagnosis of these conditions typically involves the use of activity assays to determine residual glycosidase activities in patient tissues. Such assays can be non-trivial, with specific conditions or inhibitors required to deconvolute activity of the enzyme of interest from overlapping enzymes in the tissue. ABPP is uniquely suited to address this challenge, and ABPs against various classes of glycosidases have demonstrated promise as possible diagnostic tools (**Figure 4a**).

GBA is perhaps the most well-studied glycosidase from an ABPP-centric perspective; GBA deficiency is the causal driver of Gaucher's disease, caused by abnormal buildup of the GBA substrate glucosylceramide in cells. Symptoms of Gaucher's disease include thrombocytopenia, hepatosplenomegaly, and osteoporosis, with the type II variant also involving severe neuropathy with patients typically succumbing in infancy. ABPP using cyclophellitol-derived probes allows for rapid evaluation GBA activity in patient tissues, and can identify GBA deficiency in Gaucher's disease samples with sensitivity comparable to immunoblotting (a considerably more laborious technique)[10]. Use of suitably configured ABPs has also demonstrated that lysosomal α-glucosidase, lysosomal β-galactocerebrosidase, and lysosomal α-galactosidase are all amenable to ABPP profiling,

opening up avenues for the rapid diagnosis of Pompe[34], Krabbe[50] and Fabry[32] diseases respectively. Interestingly, the irreversible inhibitory nature of many ABPs can also be used to chemically 'knock out' enzyme activity *in vivo*. Zebrafish treated with selective cyclophellitol derivatives accumulate glucosylsphingosine (derived from acid ceramidase mediated conversion of glucosylceramide), indicating 'knock out' of GBA activity and recapitulation of a Gaucher's disease phenotype[51].

Glycosidase overexpression can also be linked to pathophysiologies. Heparanase is an endo-β-glucuronidase principally responsible for heparan sulfate (HS) breakdown in the body. Aberrant HPSE overexpression results in excessive HS degradation within the extracellular matrix surrounding cells, and releases of growth factors stored by extracellular HS networks. These factors increase cell motility and growth, and thus heparanase drives metastasis in a wide range of cancers. β-D-glucuronide configured cyclophellitol-derived ABPs detect heparanase in human tissue lysates, and may be useful diagnostic tools to track aberrant heparanase upregulation during cancer development[35].

4.2. Glycosidase ABPP for imaging applications

Many glycosidase ABPs are cell permeable, allowing for their use in cells, tissues, and even whole organisms, enabling a variety of imaging applications (**Figure 4a**). van Smeden *et al* have shown that epidermal GBA in intact skin sections can be visualized by confocal microscopy following application of fluorescent cyclophellitol-derived ABPs[52]. Similar GBA imaging in human fibroblasts was demonstrated by van Meel *et al*, who also applied correlated light and electron microscopy (CLEM) to confirm specific staining of the lysosomal compartment[53].

In situ imaging to visualize cellular uptake and trafficking may be a useful technique to assess the efficacy of therapeutic enzyme uptake following enzyme replacement therapy regimens. Phenix *et al.* demonstrated that ^{18}F-fluorosugar pre-labeled recombinant GBA could be tracked in mice by PET imaging, allowing for the uptake of GBA into organs following enzyme administration to be quantitated[21]. Higher resolution imaging of enzyme trafficking into cellular organelles has also been reported, albeit in cell culture systems. van Meel *et al.* used CLEM to visualize lysosomal accumulation of recombinant ABP pre-labeled GBA by human fibroblasts following its addition into culture media. The authors found that lysosomal accumulation of recombinant GBA occurred even in the absence of LIMP-II (the protein responsible for lysosomal trafficking of endogenous GBA) suggesting that lysosomal trafficking of recombinant GBA is a LIMP-II independent process[53]. Similarly, Artola *et al.* tracked ABP pre-labeled α-L-iduronidase (IDUA) uptake by mucopolysaccharidosis (MPD)I and mucolipidosis II (MD)II fibroblasts. In this case, application of mannose-6-phosphate abrogated uptake of recombinant IDUA, confirming that IDUA uptake was dependant on the mannose-6-phosphate pathway[54].

4.3. Glycosidase ABPP for inhibitor discovery

Fluorescent glycosidase ABPs can be exploited to screen for inhibitors in compound libraries through the use of the high-throughput fluorescence polarization (FluoPol) assay format. When excited with plane polarized light, enzyme bound fluorescent ABPs will retain a greater degree of polarization in their emitted light compared to free ABPs in solution. Thus, incubation of enzyme-ABP mixtures with compounds that reduce ABP binding will lead to reduced fluorescence polarization (**Figure 4b**). Lahav *et al.* used cyclophellitol aziridine equipped with a TAMRA-fluorophore in a FluoPol assay to identify inhibitors of non-lysosomal glucocerebrosidase (GBA2), which displayed 100,000 fold selectivity for GBA2 over GBA[55]. GBA2 is involved in the degradation of glucosylceramide and is a potential therapeutic target in Niemann-Pick type C disease[56]. FluoPol-ABPP may provide a general strategy

to identify inhibitors of retaining GHs, without the requirement for screening assays based on fluorogenic or chromogenic substrates.

4.4. Glycosidase ABPP for biotechnology

Although many early glycosidase ABPP studies were biomedical in focus, recent years have seen an increasing drive to employ ABPP techniques towards biotechnological applications (**Figure 4c**). One early study in this arena, by Hekmat and coworkers, showed the promise of ABPP for the profiling of bacterial secretomes for biomass degrading enzymes[19]. The authors used 2-deoxy-2-fluoro-disaccharides to profile the secretome of *Cellulomonas fimi* for cellobiohydrolase and xylobiohydrolase activity. Coupling isotopically tagged and biotinylated versions of these probes to mass spectrometry, the authors quantified active enzymes under variable growth conditions and also identified a previously unknown xylanase[57]. Schröder *et al.* recently carried a similar analysis of β-xylosidases and β-xylanases in *Aspergillus niger* secretomes using cyclophellitol-derived ABPs. The authors demonstrated by both fluorescent labeling and proteomic analyses that *A. niger* secreted distinct catabolic enzymes depending on the carbon source utilized during its growth[58].

A comprehensive ABPP study of biomass degradation carried out by Chauvigné-Hines *et al.* utilized a suite of glucose and cellobiose configured ABPs to study the *Clostridium thermocellum* secretome[44]. A wide range of ABP chemistries were used in this study, encompassing fluorosugar, bromoketone and quinone methide based probes. Impressively, the authors were able to identify a large subset of the *C. thermocellum* glycosidases involved in biomass breakdown, including many proteins of the macromolecular cellulosome complex. The use of such 'suites' of ABPs may represent a useful strategy to overcome limitations inherent to each class of glycosidase ABP (see section 3).

ABPP has also been used as a tool to study intestinal microbiota. Whidbey and coworkers recently used quinone methide generating β-glucuronidase ABPs for labeling of whole cells derived from mouse gut microbiomes[59]. The authors sorted labeled cells using fluorescence activated cell sorting (FACS) to produce both labeled and unlabeled subpopulations. The identities of the β-glucuronidase positive community members were subsequently revealed by 16S rRNA sequencing of subpopulations. β-glucuronidase active taxa were compared between hosts and under antibiotic treatment conditions, revealing distinct taxa active in different hosts. This is a powerful technique as it clearly links function to taxa without the need for genomic context.

5. Future challenges for glycosidase ABPP

Several decades after the development of the first mechanism-based glycosidase inhibitors, the field of activity-based glycosidase profiling is now reaching maturity, at least with respect to retaining glycosidases. The field has seen the development of several different warhead chemistries[10,14,36], as well probes tailored to react with specific retaining glycosidase families[10,17,49]. Our experience suggests that, in principle, any retaining glycosidase that employs the Koshland double displacement mechanism is amenable to ABP profiling. Whilst 'monosaccharide' ABPs that target retaining *exo*-glycosidases have been relatively well explored, profiling of retaining *endo*-glycosidases will require more complex ABPs structures to mimic the length and/or branching of their natural substrates. Given past and recent successes in the glycosylation of fluoroglycoside[20,60], cyclophellitol-derived[58,61], and bromoketone[37] warheads, the general profiling of retaining endo-glycosidases appears to be within reach.

In contrast to fluorophosphonate-type ABPs, which react with large numbers of serine hydrolases, the development of truly broad-spectrum retaining glycosidase ABPs has remained elusive. Removal of hydroxyls from an ABP scaffold, as was recently done for cyclophellitol-derived ABPs[62], yields

broader spectrum probes at a cost of markedly reduced potency. The use of multiple ABPs in multiplexed labeling experiments provides a possible strategy to circumvent this problem, which also offers an attractive bonus: the relative specificity rendered by the configuration of the probes translates to the specificity of the glycosidases that are labeled. Interrogation of *e.g.* a microbial secretome derived from biomass growth using a set of differently fluorescent glycosidase ABPs will yield a 'multicolored' fingerprint of the possible glycosidases present. First forays in activity-based secretome profiling indicate the huge potential of glycosidase ABPs in the field of biotechnology, in addition to their more developed applications in biomedicine (inhibitor discovery, target engagement, diagnostics).

The enzymatic mechanism of retaining glycosidases proceeds through a covalent enzyme-substrate intermediate, which has provided us with a fortuitous route towards the design of ABPs for this enzyme class. The extensive choice of probe chemistries now on offer for retaining glycosidases may present a daunting choice for researchers when selecting suitable ABPs for their studies. In our experience, cyclophellitol-derived ABPs are excellent first choice molecules for a wide range of *in vitro*, *in cellulo* and even *in vivo* applications. Cyclophellitol-derived ABPs typically adopt a ground state $^{4}H_{3}$ conformation that mimics the substrate transition state conformation for many glycosidases[48,58], meaning these probes often label with high potency, producing stable enzyme-cyclitol linkages suitable for various downstream analyses. Conversely, glycosidases that do not employ a $^{4}H_{3}$ transition state conformation can be less reactive towards cyclophellitol-derived ABPs, as has recently been observed with GH11 β-xylanases (proposed to utilize a $^{2,5}B$ transition state conformation[58]). In such cases, fluorosugars may be more suitable, as they more closely resemble natural substrate glycones[63]. For β-glucosidase, β-galactosidase, and related glycosidase classes, fluorosugar inhibitors typically form stable, long-lived intermediates well suited for ABP derivation. However, in some cases (notably with 2-deoxy-2-fluoro-α-glycosides and α-glycosidases), fluorosugar inhibitors are too rapidly turned over to be of use as probes. In such cases, combining fluoro- and carbasugar chemistries may help to stabilize the covalent enzyme-probe intermediate[23,24]. Alternatively, controlled turnover of fluorosugar inhibitors can be useful in time-dependent labeling applications, where reactivation of the enzyme is desired.

In contrast to the wide range of ABPs available for typical retaining glycosidases, ABPs that efficiently and specifically label other glycoprocessing enzymes (atypical retaining glycosidases utilizing oxidative or substrate assisted mechanisms, inverting glycosidases, glycosyl transferases, polysaccharde lyases, oxygenases) remain elusive. Opening up these enzyme classes to ABPP has huge potential in both the biomedical and biotechnological arenas. Some progress towards ABPP of inverting glycosidases has been made with photo-affinity and quinone methide based probes, which are indiscriminate with respect to catalytic mechanism[41]. However, these labeling chemistries also suffer from a relative lack of efficiency and selectivity. We postulate that widespread adoption of ABPP for other glycoprocessing enzyme classes will only take place after effective irreversible enzyme active site binders are identified. The development of such molecules will require the efforts of high-level synthetic chemistry, coupled to computation and structural analysis of the reaction mechanisms and conformational itineraries utilized by these diverse enzyme classes.

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Declaration of Interest

The authors declare no conflict of interest.

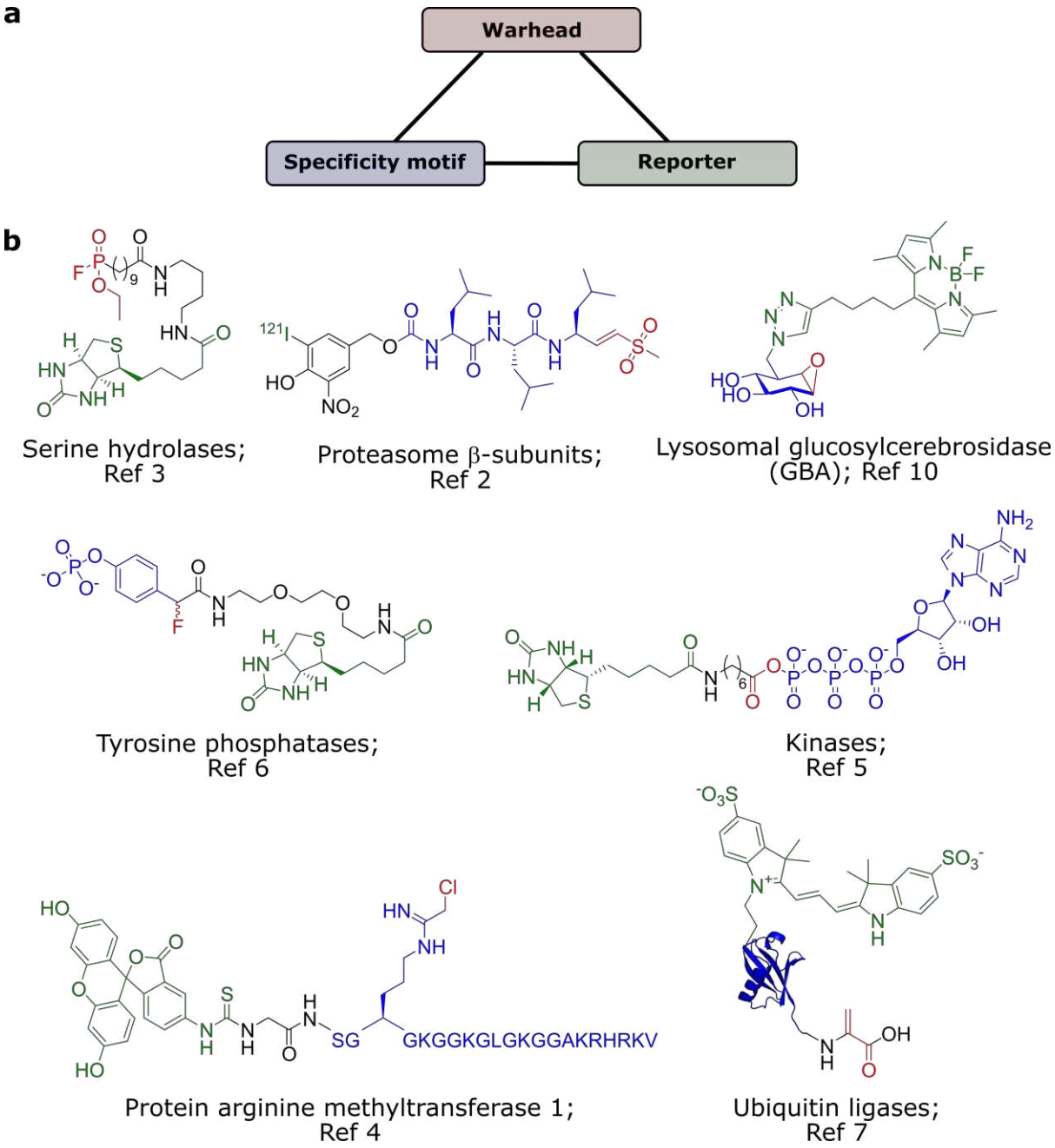

Figure 1 Conceptual basis for ABP design. **a** The three fundamental components of an ABP. Note - some probes may lack a specificity motif, endowing them with broad spectrum activity against a range of enzymes e.g. serine hydrolase probes in Ref [3]. **b** Examples of ABPs designed to target various enzymes and enzyme classes. Colors correspond to ABP components as outlined in **a**.

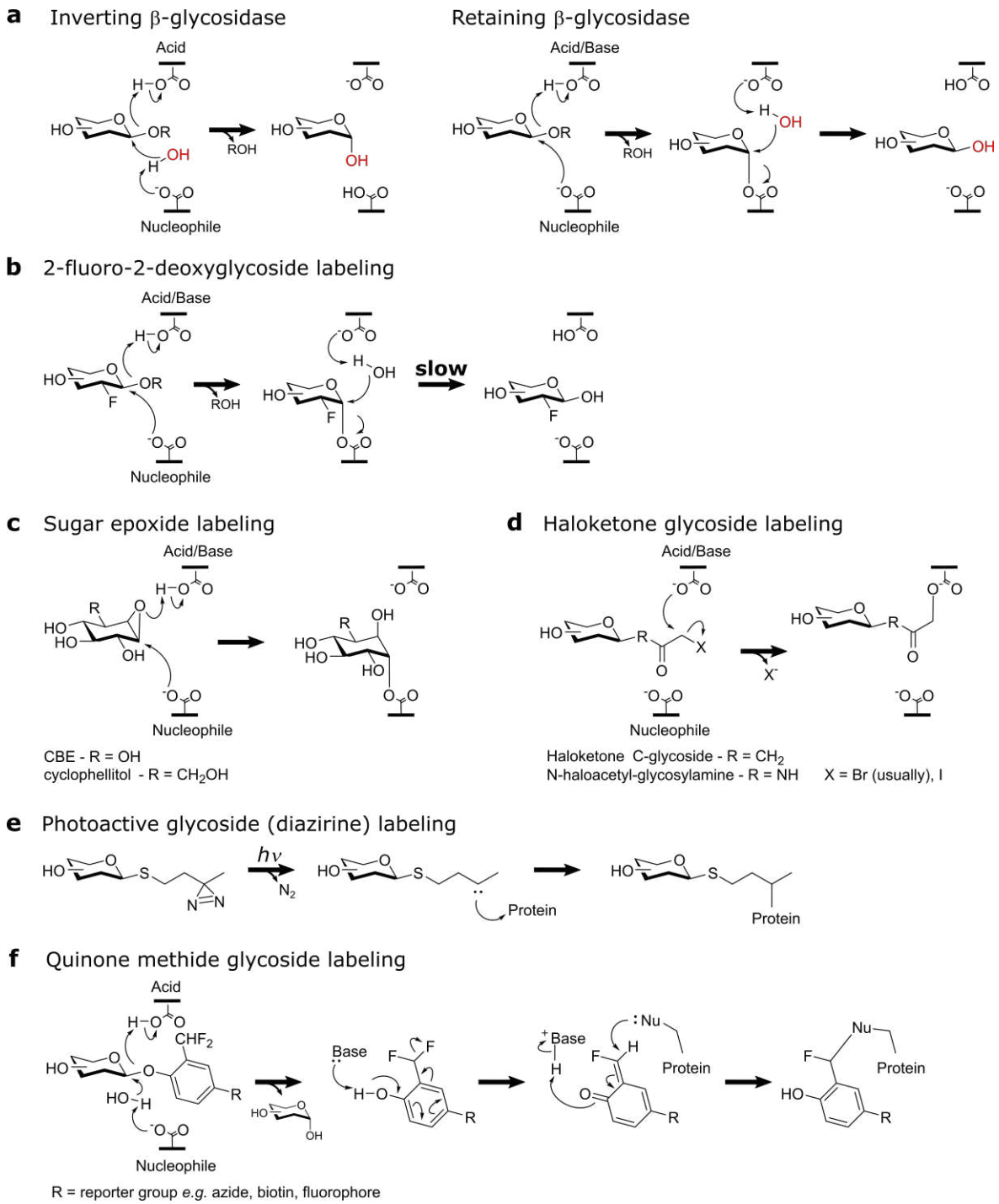

Figure 2 Mechanisms of glycoside hydrolases and inhibitor action. **a** Mechanism of glycoside cleavage by inverting (one step) and retaining (two step) glycosidases **b** Fluorosugar inhibitors (2-deoxy-2-fluoro-glycoside shown here) are processed by retaining glycosidases, but the slow deglycosylation step results in inactivated enzyme. **c** Sugar epoxides (e.g. CBE or cyclophellitol) are attacked at their pseudo-anomeric center by glycosidase catalytic nucleophiles, leading to irreversible alkylation of this residue. **d** Haloketone glycosides employ an electrophilic trap, which can irreversibly alkylate residues within an enzyme active site (alkylation of the catalytic acid/base residue is shown here). **e** Photoactive glycoside AfBPs form a reactive species upon photoexcitation (carbene formation from a diazirine shown here), which reacts with neighboring protein residues to form irreversible linkages. **f** Quinone methide glycosides release a reactive species when cleaved, which can irreversibly alkylate a nearby nucleophilic protein residue.

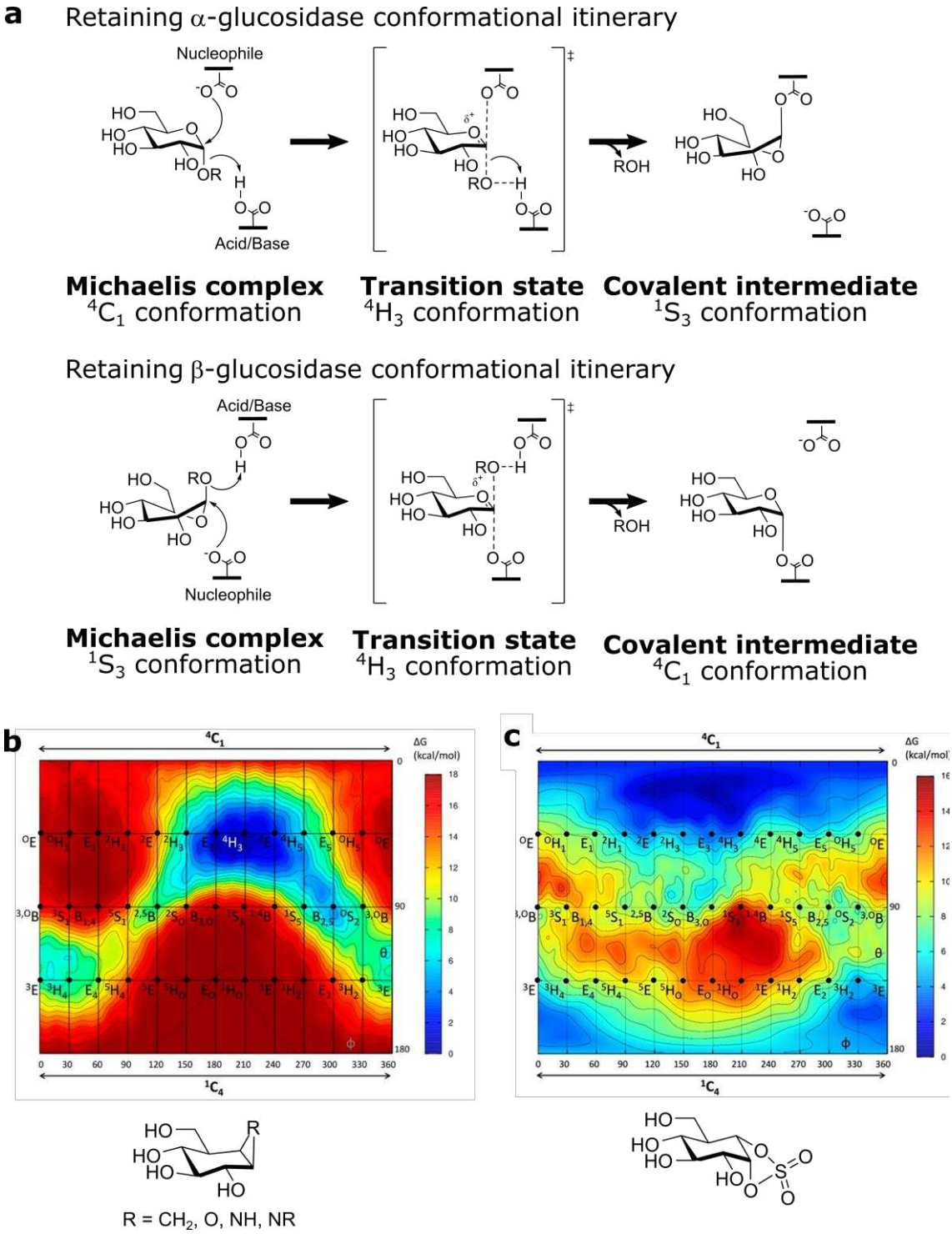

Figure 3 Conformational aspects of glycosidase ABP reactivity. **a** Conformational itineraries for half-reactions (initial substrate binding to covalent intermediate formation) catalyzed by typical retaining α- and β- glucosidases. Both classes of enzymes proceed *via* a $^{4}H_{3}$ transition state. **b** Conformational FEL for a cyclophellitol derived molecule (carba-cyclophellitol, R=CH_2), showing a ground state conformation centered around $^{4}H_{3}$. Cyclophellitol-derived ABPs are transition state mimics and potent inactivators of many retaining classes of retaining glycosidases. **c** Conformational FEL for α-cyclophellitol cyclosulfate, showing a ground state conformation centered around $^{4}C_{1}$. This conformation matches the typical α-glucosidase Michaelis complex conformation, rendering α-cyclophellitol cyclosulfates potent selective inactivators of α-glucosidases.

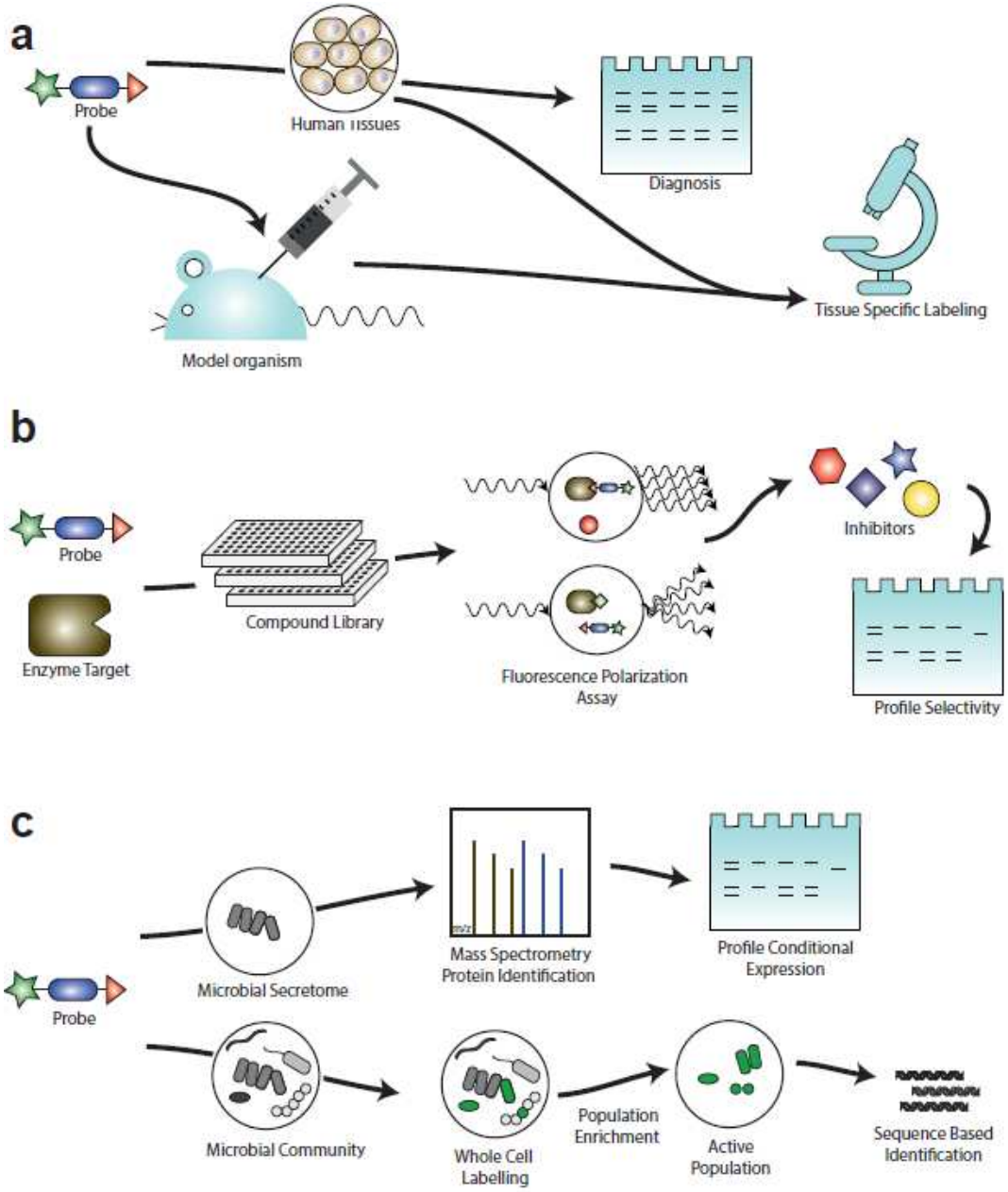

Figure 4 Recent applications of Glycosidase ABPs for protein profiling. **a** Glycosidase specific probes can detect the absence of enzyme expression in genetic diseases. ABPs have also been used to detect the presence of glycosidase activity in tissue samples, as well as in whole organisms *in vivo*. **b** FluoPol assays for glycosidases involves the use of a fluorescent ABP, the enzyme of interest and compound libraries. Glycosidase labeling by the ABP results in a maintenance of fluorescence polarization, whilst competition by inhibitors results in a loss of polarization. The specificity of identified hits can then be interrogated through SDS-PAGE and other downstream assays. **c** Glycosidase ABPs can be used to profile biomass degrading enzymes and microbiota community members. Active enzymes present in microbial secretomes can be profiled by SDS-PAGE and active proteins identified using pulldowns and mass spectrometry. Whole cells can be labeled and sorted with the use of FACS. Communities of either active or inactive populations can then be investigated through sequence-based identification of microbial taxa.

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