Insider information on successful covalent protein coupling with help from SpyBank

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Abstract

New biological properties can stem from the freedom to link, multimerize or multiplex protein building-blocks. The peptide SpyTag on one protein irreversibly reacts with SpyCatcher on another protein, through spontaneous isopeptide bond formation. Reaction is specific in a wide range of cellular environments and all components are genetically-encoded, making this chemistry accessible to molecular biologists. SpyTag/SpyCatcher has been widely used for enzyme immobilization, colocalization of different enzymatic activities, and increasing enzyme resilience. Here we present routes and advice for efficient design, expression and purification of SpyTag/SpyCatcher constructs in bacterial and eukaryotic environments, including the latest 002 variants, and how to analyze reaction efficiency. The SpyInfo webpage collates the different publications and patents using SpyTag/SpyCatcher, while the SpyBank database lists their sequences and expression routes. The ability of SpyTag/SpyCatcher to react in a broad range of situations creates diverse opportunities for augmenting the function of enzymes and other biomolecules.

Keywords: protein-protein interaction, synthetic biology, nanobiotechnology, nanoassembly, protein superglue, metabolite channeling, protein engineering, plug-and-display.

1. Introduction

Covalent coupling enables the construction of protein assemblies beyond nature or conventional genetic fusion. The SpyTag/SpyCatcher system has been termed a genetically-encoded click chemistry, without the complication of using any unnatural amino acids (Wang & Zhang, 2018). SpyCatcher fused to one protein irreversibly reacts with the SpyTag on another protein to form a spontaneous isopeptide bond (Figure 1A). The reaction is irreversible and specific in a range of cellular environments (Zakeri, Fierer, Celik, Chittock, Schwarz-Linek, Moy, et al., 2012; Keeble, Banerjee, Ferla, Reddington, Anuar, & Howarth, 2017;

Bedbrook, Kato, Ravindra Kumar, Lakshmanan, Nath, Sun, et al., 2015). In this chapter we describe methods and principles to design and generate successful SpyTag/SpyCatcher constructs. There are various other approaches for covalent coupling of proteins with other proteins, which we have recently summarized (Banerjee & Howarth, 2018).

The CnaB2 domain of the *Streptococcus pyogenes* fibronectin-binding adhesin FbaB spontaneously forms an intramolecular isopeptide bond to stabilize the protein (Hagan, Björnsson, McMahon, Schomburg, Braithwaite, Bühl, et al., 2010). Our group split this domain into two parts: the SpyCatcher protein and the SpyTag peptide (Figure 1B) (Zakeri et al., 2012). Upon mixing together, the amide bond between Lys and Asp spontaneously reconstitutes, enabling irreversible linking of proteins fused to the SpyCatcher and SpyTag (Figure 2A). Subsequently, rational design and directed evolution produced SpyCatcher002 and SpyTag002 (Figure 2B), with an order of magnitude faster reactivity, so reacting to completion even at low (100 nM) protein concentrations (Figure 2B) (Keeble et al., 2017). The sequence relationships between the most common versions of SpyTag/SpyTag002 and SpyCatcher/SpyCatcher002 in the published literature are shown in Figure 2C, including SpyCatcher versions with truncations at each terminus (Li, Fierer, Rapoport, & Howarth, 2014).

SpyTag/SpyCatcher can produce constructs of non-linear and unprecedented topologies (Sun, Zhang, Mahdavi, Arnold, & Tirrell, 2014; Zhang, Sun, Tirrell, & Arnold, 2013; Wang, & Zhang, 2016) and can be used orthogonally with other coupling technologies, such as HaloTag or streptavidin (Peschke, Rabe, & Niemeyer, 2017; Fairhead, Veggiani, Lever, Yan, Mesner, Robinson, et al., 2014). SpyTag/SpyCatcher reactions can couple proteins together with high specificity in biological environments including bacterial outer-membranes (Keeble et al. 2017; Peschke et al., 2017), the mammalian cytosol (Zakeri et al., 2012; Hinrichsen, Lenz, Edwards, Miller, Mochrie, Swain, et al., 2017), biofilms (Nguyen, Botyanszki, Tay, & Joshi, 2014), the mammalian plasma membrane (Zakeri et al., 2012), and living *Caenorhabditis elegans* (Bedbrook et al., 2015).

SpyTag/SpyCatcher reactions have a wide-spread use in assembling complexes, enabling the combination of properties of one protein with the protein to which it is covalently coupled (Figure 3). Libraries of antigen-specific binding reagents (DARPins, nanobodies etc.) fused to SpyTag and libraries of detection proteins (fluorescent proteins, reporter enzymes, or toxins) fused to SpyCatcher (Figure 3A) have been created as part of a "Portable, On-Demand Biomolecular Manufacturing" platform (Pardee, Slomovic, Nguyen, Lee, Donghia, Burrill, et al. 2016). Virus-like particles (VLPs) fused to SpyCatcher have been used to create "Plug-and-display" immunogens by multivalently displaying SpyTagged vaccine candidates, to accelerate vaccine generation (Figure 3B) (Brune & Howarth, 2018). Intracellular super-resolution imaging reagents for mammalian cell microscopy have been generated: Cys-SpyCatcher was conjugated with maleimide-Alexa Fluor 647 and added to fixed HEK293T cells expressing cellular components fused to SpyTag (Figure 3C) (Pessino, Citron, Feng, & Huang, 2017). SpyTag-fusions were also detected with high sensitivity in Western blot by Alexa Fluor 647-SpyCatcher, since the low affinity of antibodies detecting conventional peptide tags can often be limiting (Figure 3D) (Dovala, Sawyer, Rath, & Metzger, 2016).

Improving the coordination of enzyme function has been a long-standing challenge in biotechnology and synthetic biology. Genetic fusions linking together different enzymes can be successful but can often lead to misfolding and are restricted in the relative spatial orientation of the active sites (Pröschel, Detsch, Boccaccini, & Sonnewald, 2015). Many peptide:protein interactions have been employed to connect enzymes, but stability and specificity of these contacts has often been limiting (Pröschel et al., 2015). Therefore, the simple and irreversible linkage between SpyTag and SpyCatcher can make a contribution to enzyme organization. Particular applications so far include cyclizing enzymes to improve their thermal resilience (Figure 4A). This was initially demonstrated for β-lactamase, where

SpyTag-β-lactamase-SpyCatcher retained solubility and activity following boiling (Schoene, Fierer, Bennett, & Howarth, 2014). This "SpyRing" approach has since been extended to luciferase, phytase, glucanase and trehalose synthase (Si, Xu, Jiang, & Huang, 2016; Schoene, Bennett, & Howarth, 2016a; Gilbert, Howarth, Harwood, & Ellis, 2017; Schoene, Bennett, & Howarth, 2016b; Wang, Wang, Wang, Zhang, Wu, & Zhang, 2016; Xu, Xu, Huang, & Jiang, 2018). SpyTag has also been used in covalent modification of biofilms to make "living materials" with controlled mechanical and catalytic functions (Figure 4B) (Nguyen et al. 2014; Botyanszki, Tay, Nguyen, Nussbaumer, & Joshi, 2015). SpyTag was applied to the intracellular delivery of enzymes by modular linkage to cell penetrating peptides (Figure 4C) (Hoffman, Milech, Juraja, Cunningham, Stone, Francis, et al., 2018; Stone, Heinrich, Juraja, Satiaputra, Hall, Anastasas, et al., 2018). SpyTag also facilitated construction of multimerized enzyme assemblies either using VLPs (Röder, Fischer, & Commandeur, 2017) (as in Figure 3B) or other biological nanoreactors (Figure 4D) (Alves, Turner, Daniele, Oh, Medintz, & Walper, 2017; Pröschel, et al., 2015; Giessen, & Silver, 2016; Yin, Guo, Liu, Zhang, & Feng, 2018). Such complexes also enable a scaffolding function, organizing proteases for amplification towards cancer diagnosis (Stein, Nabi, & Alexandrov, 2017), as well as immobilizing enzymes for robust nanopore DNA sequencing devices (Stranges, Palla, Kalachikov, Nivala, Dorwart, Trans, et al., 2016).

We have assembled a more complete list of publications and patents using SpyTag/SpyCatcher technology at the SpyInfo webpage (www.bioch.ox.ac.uk/howarth/info.htm). The webpage also contains the papers applying other spontaneous isopeptide bond-forming systems (Zakeri et al., 2012; Abe, Wakabayashi, Yonemura, Yamada, Goto, & Kamiya, 2013). The first split pair was isopeptag/Pilin-C, but pilin-C is much larger than SpyCatcher and reacts much slower (Zakeri & Howarth, 2010). SnoopTag/SnoopCatcher results from the engineering of a D4 domain of the RrgA protein from *Streptococcus pneumoniae*, reacting orthogonally (with no cross-reactivity) to SpyTag/SpyCatcher (Veggiani, Nakamura, Brenner, Gayet, Yan, Robinson, et al., 2016). Further engineering of the CnaB2 domain produced SpyLigase, while SnoopLigase was generated from the RrgA D4 domain (Fierer, Veggiani, & Howarth, 2014; Buldun, Jean, Bedford, & Howarth, 2018). These ligases join two peptides (formed from parts of the original domains) together via isopeptide bonds. However, the speed of reaction of these ligases and their activity with low concentration of target protein is worse than for SpyTag/SpyCatcher ligation.

The procedures outlined below should help to avoid potential pitfalls, as well as integrating the insight that is now available from the validation of a wide range of SpyTag and SpyCatcher fusions in different organisms, compartments and protein contexts.

2. SpyDesign – construction of successful SpyTag/SpyCatcher reagents

2.1 SpyBank database:

SpyBank is an online database that we have compiled of amino acid sequences of SpyTag- or SpyCatcher-fusions available from published papers or patents by different academics or companies. Example entries are shown in Table 1. As of September 2018, there are more than 400 sequence entries, available for download from our webpage (www.bioch.ox.ac.uk/howarth/info.htm). In addition to listing corresponding author and citation, SpyBank contains important experimental design information on the expression host, cellular compartment, where the protein was fused, which version of SpyCatcher or SpyTag was used, as well as the amino acid sequence of the constructs used (where available). The plasmids for basic SpyCatcher and SpyTag constructs are available from the Addgene plasmid repository (www.addgene.org): SpyCatcher (#35044); ΔN1ΔC2SpyCatcher (#87376);

SpyTag-maltose binding protein (MBP) (#35050); AviTag-SpyCatcher (#72326); SpyCatcher002 (#102827); SpyTag002-MBP (#102831).

SpyBank reveals that *Escherichia coli* is by far the most common system for expression of SpyTag/SpyCatcher fusions. However, a range of other species have been successfully used. Other bacteria used for expression were *Bacillus subtilis*, *Lactococcus lactis,* and *Salmonella Typhimurium*. Among eukaryotes, expression has been performed in *Saccharomyces cerevisiae*, *C. elegans*, insect cells (ExpresS2, Sf9, *Trichoplusia ni*), human cell-lines (HeLa, HEK293) and the plant *Nicotiana benthamiana*. Expression has also been performed in cell-free systems (Pardee, et al., 2016). This diversity supports the wide-spread biological compatibility of SpyCatcher and SpyTag.

In addition to cytosolic protein expression, SpyTagged proteins have been secreted from HEK293T cells, using signal peptides at the N-terminus of the construct to target for endoplasmic reticulum translocation. Some signal peptides are useful for many proteins, but others are more specific (Kober, Zehe, & Bode, 2013), so trials with different signal peptides may be useful for the highest expression levels. These cells can also be used to express proteins targeted to the membrane. Alternative signal peptides compatible with membrane display of SpyTagged proteins are listed in SpyBank.

2.2 Construct design:

When designing constructs involving SpyTag and SpyCatcher variants, the following considerations should be taken into account:

(i) Choice of SpyCatcher and SpyTag variant.

For N- or C-terminal fusion of SpyCatcher, we would recommend the ΔN1SpyCatcher construct (Figure 2C) (Li et al., 2014), which gave us the best results in the challenging situation of VLP fusion (Brune, Leneghan, Brian, Ishizuka, Bachmann, Draper, et al. 2016). If the SpyCatcher is to be inserted in an internal protein loop, we suggest ΔN1ΔC2SpyCatcher, which was successfully applied for bacterial microcompartments (Hagen, Sutter, Sloan, & Kerfeld, 2018). If reaction speed is limiting, we recommend switching to SpyCatcher002 to accelerate the reaction with SpyTag, especially at low (< 100 nM) protein concentrations (Keeble et al. 2017). We have found that the N-terminal sequence of SpyCatcher expression constructs starting with GAMVD result in a trace amount of side-reaction with the reactive lysine of another SpyCatcher molecule, since this sequence partially resembles the SpyTag sequence (Keeble et al. 2017). During the development of the faster reacting SpyCatcher002, we found that this off-pathway reaction was enhanced, requiring the mutation of the sequence to GAMVT (Keeble et al. 2017). Thus, we recommend also including this mutation in SpyCatcher constructs, as well as ensuring that sequences like this are not used in linker regions.

SpyBank shows many examples of SpyTag being used at the N- or C-terminus. SpyTag may also be used in exposed linker regions between protein domains (Zakeri et al., 2012). There are fewer examples of SpyTag being used in a loop within a folded domain (Hagen et al., 2018; Kasaraneni, Chamoun-Emanuelli, Wright, & Chen, 2017; Moon, Bae, Kim, & Kang, 2016); optimization may sometimes be required, because SpyTag reacts in an elongated conformation with SpyCatcher (Figure 1B) (Li et al., 2014). SpyTag and SpyTag002 can be used interchangeably but reaction with any SpyCatcher variant will be faster with SpyTag002 (Keeble et al., 2017).

(ii) Choice of fusion site.

SpyCatcher and SpyTag can be used equally well on the N- or C-terminus, since the reaction is mediated through side-chains. SpyBank also contains several constructs with more than one

SpyTag or more than one SpyCatcher moiety (Zhang et al., 2013; Wieduwild & Howarth, 2018). One may prefer a particular terminus because it is distant from a binding site or active site, because previous fusion to other tags has been successful (e.g. to a His_6-tag or fluorescent protein), or because there is lower sequence conservation at that terminus (Chen, Zaro, & Shen, 2013). If an initial construct shows sub-optimal activity, it is worth moving SpyCatcher or SpyTag to the opposite terminus, to see if activity is improved (Si et al., 2016).

SpyCatcher and SpyTag must be sterically accessible to one another for reaction to occur. This consideration is of particular importance when using large multimeric proteins, where either the N- or C-terminus may point into the core of the protein (Hagen et al., 2018; Brune & Howarth, 2018).

(iii) Choice of linker.

We always insert a linker between SpyTag or SpyCatcher and the protein of interest. Initially we usually try GSGESGSG (Veggiani et al., 2016). Gly/Ser linkers show a good balance of flexibility, solubility and protease resistance (Chen et al. 2013) and we often include a Glu in the spacer to increase hydrophilicity. The linker increases the accessibility of SpyTag/SpyCatcher for faster and higher yielding reaction. The linker also reduces the chance of interference in the structure or function of the fused protein. If a linker has been used on a similar protein in SpyBank, we would suggest copying that linker. If we find slow or incomplete reaction, we will often move to a 9-residue linker, such as GGGGSGGGGS (Veggiani et al., 2016). When using ΔN1ΔC2SpyCatcher in an internal loop, GGGSGGS was used on each side (Hagen et al., 2018). With SpyTag in an internal loop, linkers such as GGGS on both sides have been used successfully in SpyBank (Alves et al., 2017).

We suggest avoiding linkers containing Asp or Asn close to the C-terminal side of SpyCatcher. For example, a sequence at the C-terminus of SpyCatcher like ...AHIGSG**D**G... could promote self-reaction of the Asp in bold with the reactive lysine of SpyCatcher (C-terminus of SpyCatcher is underlined). Also, we avoid negatively-charged peptides (e.g. myc tag or C-tag) immediately adjacent to SpyTag, where electrostatic complementarity may slow reaction with SpyCatcher.

(iv) Codon usage.

We have nearly always used the original codons for SpyTag and SpyCatcher from *S. pyogenes* and this usage has not caused problems for *E. coli* expression. Gene design companies or online servers are able to suggest optimized codon usage for any species, but sometimes unpredicted effects can lead to worse expression. For expression in other organisms, it is worth looking in SpyBank to identify previous constructs expressed in that species. For simplicity, SpyBank only shows amino acid sequences, but SpyBank should help users to go to the primary source and find the DNA sequence. An exception that we found is the expression of SpyTag at the N-terminus in *E. coli*, where the issue is not codon frequency but codon complementarity. Secondary structure near the Shine-Dalgarno sequence can greatly reduce protein expression yield. Thus, the sequence needs to be optimized using a Ribosome Binding Calculator (score 10,000 or above) to maximize expression levels (Salis 2011). We give here SpyTag DNA sequences for two common promoter systems for bacterial expression:

T7 vector `5′-gcacacatagtaatggtagacgcctacaagccgacgaag-3′`

T5 vector `5′-gctcatatcgtcatggttgacgcgtataaaccgaccaaa-3′`

`A H I V M V D A Y K P T K`

Our constructs will typically have a glycine immediately after the initiating methionine/formyl methionine, which can help expression, consistent with the N-end rule for protein stability (Varshavsky, 2011).

2.3 Cellular expression and purification:

After creation of the desired expression construct, the plasmid is next transformed into an expression strain. We will not go into detail here on protocols that are standard for any protein but focus on issues specific to SpyTag/SpyCatcher. Our standard Ni-NTA purification protocol is described in detail previously (Howarth & Ting, 2008).

(i) Bacterial expression. SpyBank reveals that a wide-range of different *E. coli* strains can be used for expression of SpyTag/SpyCatcher fusions. Hence, if you find low expression in one strain, you should try others. Two *E. coli* strains we recommend are BL21 (DE3) RIPL (Agilent) (helping to express proteins with rare codons) and C41 (DE3) (helping expression of toxic proteins) (Miroux & Walker, 1996). *E. coli* BL21 (DE3) transformed with a plasmid encoding Erv1p and DsbC allowed us to express efficiently proteins containing a disulfide bond in the cytosol (Veggiani et al., 2016). Supplementing LB media with 0.8% glucose may reduce leaky expression before induction, which aids growth for toxic proteins (Grossman, Kawasaki, Punreddy, & Osburne, 1998). Alternative growth media such as 2×TY or auto-induction media can also be used with SpyTag/SpyCatcher constructs. After inducing with IPTG at OD_{600} 0.5, we typically grow the cells for a further 4 hours at 30 °C. For most constructs we can clearly see induction by SDS-PAGE with Coomassie staining on the whole cell lysate (Figure 2A).

If expression has failed, we will first try an alternative induction temperature (18 °C for 16 hours) or a different *E. coli* strain. Purification of His_6-tagged fusions by Ni-NTA is carried out by standard methods (Zakeri et al., 2012) ensuring protease inhibitors are present during cell disruption and initial purification. After elution from affinity chromatography, proteins can be dialysed into the buffer of choice (typically phosphate-buffered saline, PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 pH 7.5). Dialysis is especially important after Ni-NTA purification, since high concentrations of imidazole slow down the SpyTag/SpyCatcher reaction. If there is substantial aggregation upon dialysis, we will try a buffer with a pH more than 1 unit from the isoelectric point (pI) of the fusion protein (e.g. 50 mM Tris base with the pH adjusted using boric acid, or 50 mM glycine with 25 mM sodium citrate with the pH adjusted using HCl or NaOH).

A further round of purification such as gel filtration can be used if required, but many SpyCatcher- or SpyTag-linked proteins are typically pure enough to couple to the binding partner after one step purification (Figure 2A). C-tag purification is also efficient for SpyTag-fusions and may be preferred for vaccine studies (Bruun, Andersson, Draper, & Howarth, 2018). For the highest purity, we typically combine Ni-NTA purification with gel filtration chromatography or C-tag purification (Brune et al., 2016).

Expression yields of 15-30 mg per liter of culture are typically achieved with SpyCatcher- or SpyTag-fusions, although VLP fusion was more challenging (Bruun et al., 2018). Proteins can be stored at 4 °C overnight or indefinitely in aliquots at -80 °C. Certain fusions are likely to be sensitive to freeze-thaw, but where we have tested SpyCatcher-fusions on vaccine platforms, we found good resilience to freezing or lyophilization (Brune, Buldun, Li, Taylor, Brod, Biswas, et al., 2017; Bruun et al., 2018).

(ii) Mammalian secretion. Three related mammalian cell-lines have been widely used in SpyBank – HEK293T (adherent), HEK293 FreeStyle and HEK293Expi. Although several SpyTag-containing constructs are reported (with SpyTag at either N- or C-terminus), there are fewer examples in SpyBank of secretion of a SpyCatcher-fusion. The dynamic structure of

SpyCatcher may sometimes reduce the efficiency of secretion. Successful published examples include SpyCatcher at the N-terminus of the IgG Fc domain (Alam, Gonzalez, Hill, El-Sayed, Fonge, Barreto et al., 2017) and SpyCatcher linked to a range of antigens for immunization (Thrane, Janitzek, Matondo, Resende, Gustavsson, de Jongh, et al., 2016). It appears more difficult to express SpyCatcher-fusions than SpyTag-fusions in mammalian cells.

Expression constructs can be transiently transfected into the cells at mid-confluency using polyethylene imine (PEI) (HEK293T and HEK293 FreeStyle) or ExpiFectamine 293 (HEK293Expi). After secretion of the protein into the media for the desired time (typically 3-7 days), the media is removed from the cells and protease inhibitors are added to prevent proteolysis. After a repeated centrifugation to decrease the amount of cell debris, the supernatant should be syringe-filtered (0.45 μm). Binding buffer should now be added to the filtered supernatant as required for purification: 50 mM Tris•HCl, 300 mM NaCl, pH 7.8 to ¼ of total volume. This is important for pH buffering, since the growth medium does not buffer effectively without high CO_2. Given the large volume of cell supernatant produced (100-1,000 mL typically), affinity purification (His_6-tag or C-tag) is essential to concentrate the protein and can now proceed as required for the standard protocols.

3. Analysis of SpyTag/SpyCatcher covalent coupling reactions

3.1 Reaction of components:

SpyTag/SpyCatcher reaction readily occurs without requiring any specific anions or cations, at a range of temperatures (4-37 $^{\circ}$C), at pH from 4 to 8, in the presence of various detergents (Tween-20, Triton X-100, Nonidet P-40, CHAPS, but not SDS), and even under chaotropic conditions such as 4 M urea (Keeble et al., 2017). This flexibility makes SpyTag/SpyCatcher coupling suited to a wide range of coupling tasks. We typically mix the SpyCatcher- and SpyTag-proteins in a buffer such as PBS pH 7.5 with each partner at 10 μM and incubate for 2 hours at room temperature. Reaction is slower, for example, on the crowded surface of VLPs, where we usually incubate for 16 hours (Brune et al., 2016; Bruun et al., 2018). To ensure one partner is fully reacted, we recommend incubating with one component in 1.5-3× excess. Given that the reaction follows second-order kinetics (Zakeri et al., 2012; Keeble et al., 2017), it is not just the ratio but also the absolute concentration which is important for reaction speed. Given the broad tolerance of SpyTag/SpyCatcher reaction described above, it is worth including additives (e.g. Ca^{2+}, glycerol, cofactors) likely to stabilize the proteins of interest.

When testing new constructs, we recommend using purified SpyCatcher or SpyTag-MBP proteins as positive controls under the same conditions. These positive controls are very helpful for troubleshooting, to determine whether the buffer or protein constructs may be at fault, if the novel protein partners do not react as desired. Negative control constructs are also helpful to validate that any observed effects depend on covalent reaction. Such controls bear a mutation in the reactive residue of SpyTag (SpyTag002 DA-MBP Addgene # 102832) or SpyCatcher (SpyCatcher EQ Addgene # 35045 or SpyCatcher002 EQ Addgene # 102830) (Zakeri et al., 2012; Keeble et al., 2017).

3.2 Analysis of reaction:

(i) SDS-PAGE

Formation of covalent SpyTag/SpyCatcher reaction products *in vitro* can be observed and quantified by SDS-polyacrylamide gel electrophoresis (Figure 2A), since the isopeptide bond is stable to boiling in SDS (Zakeri et al., 2012; Keeble et al., 2017). This approach enables convenient quantitation by densitometry, based on either formation of the SpyTag:SpyCatcher conjugate or the depletion of SpyTag or SpyCatcher starting material. Note that the mobility of the SpyTag:SpyCatcher conjugate may be slightly different from the molecular weight

because of its branched nature (Zakeri et al., 2012; Schoene et al., 2016) and a second band may be present on SpyCatcher-fusions, as discussed in section 3.2 (ii).

(ii) Mass spectrometry

Mass spectrometry is helpful to confirm the exact product of SpyTag/SpyCatcher reaction. Isopeptide bond formation for this pair results in loss of H_2O (18 Da). For analysis of the reaction of purified samples, we dialyze into 10 mM ammonium acetate, since the low ionic strength and volatile buffer components assist in obtaining a clean spectrum. A common impurity after *E. coli* expression is a gluconylated adduct (Geoghegan, Dixon, Rosner, Hoth, Lanzetti, Borzilleri et al., 1999), but we have never experienced functional consequences of this impurity.

(iii) Fluorescence microscopy

The specificity and efficiency of *in vivo* reaction of SpyTag/SpyCatcher can be examined by microscopy. The first step is labeling with fluorescent proteins fused to the SpyCatcher or SpyTag construct and using microscopy to detect the cellular distribution of the target protein. This approach was used to demonstrate that SpyTag-Channelrhodopsin-mCherry constructs can react with SpyCatcher-GFP in *C. elegans* (Bedbrook et al., 2015).

(iv) Western blotting

Western blotting was performed on lysate from cells expressing SpyCatcher or SpyTagged proteins, with reaction occurring upon staining of the transferred membrane (Dovala et al., 2016). Western blotting can also be used to follow reaction occurring on living cells, such as to show that biotinylated-AviTag-SpyTag002-MBP reacted specifically with the bacterial outer membrane-displayed Intimin-myc tag-SpyCatcher002, using Streptavidin-Horse Radish Peroxidase (to detect the biotinylated protein) or an anti-myc antibody (followed by secondary antibody-HRP) to follow the total pool of cell-surface Intimin (Keeble et al., 2017).

3.3 Conjugate purification:

After the desired reaction time, unreacted SpyTag or SpyCatcher components may be purified from the covalent complexes, using size-exclusion chromatography, spin-filtration or dialysis (Brune et al., 2016). When there is a large size difference between the two partners (e.g. SpyTag-antigen separation from antigen-decorated VLP), we found dialysis to be a simple and efficient approach, leading to minimal dilution of the sample (Brune et al., 2016).

3. Concluding Remarks

We have described here guidelines on how to design and purify constructs to use SpyTag/SpyCatcher for covalent coupling of proteins, based on our own experience and the results of many other groups, as compiled in SpyBank. It is relatively simple to generate functional SpyTag or SpyCatcher constructs in a range of cellular systems. Reaction is typically efficient and selective either *in vitro* or at the surface of cells. There are few examples of SpyTag/SpyCatcher reaction inside living cells, so it will be important to validate this application in future work, perhaps with new iterations of Tag/Catcher pairs. Future development should also seek to establish the principles for efficient use of SpyTag/SpyCatcher in protein loops and efficient secretion of SpyCatcher-fusions from mammalian cells. Combining SpyTag/SpyCatcher with related technologies such as SnoopCatcher (Veggiani et al., 2016) and SnoopLigase (Buldun et al., 2018) will also provide further opportunities for creating novel protein architectures, signaling teams (Veggiani et al., 2016) and immune stimulants (Brune et al., 2017).

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Conflicts of interest

M.H. and A.H.K. are authors on a patent application covering sequences for enhanced isopeptide bond formation (UK Intellectual Property Office 1706430.4). M.H. is an author on a patent for isopeptide bond formation (EP2534484) and a SpyBiotech co-founder, shareholder and consultant.

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Figure legends

Figure 1: Principle of SpyTag/SpyCatcher system. **A** Scheme depicting the spontaneous amidation reaction of a lysine side-chain on SpyCatcher to the side-chain of an aspartic acid on SpyTag to form an isopeptide bond. **B** Cartoon illustrating how the CnaB2 domain of FbaB was split and engineered to generate a protein partner, SpyCatcher, reactive with the SpyTag peptide.

Figure 2: SpyTag/SpyCatcher purification and reactivity. **A** SpyTag/SpyCatcher purification. 16% SDS-PAGE with Coomassie staining showing post-induction cell lysate for SpyCatcher002 (lane 2) and SpyTag002-MBP (lane 3) expressed in *E. coli*, illustrating high expression levels. Also shown are purified SpyCatcher002 (lane 4), purified SpyTag002-MBP (lane 5), and SpyCatcher002 mixed with SpyTag002-MBP (lane 6); each at 5 μM in PBS pH 7.5 at 25 ˚C for 1 hr. **B** Increased rate of SpyTag002/SpyCatcher002 reaction. Densitometry from SDS-PAGE for isopeptide bond formation between SpyTag002-MBP and SpyCatcher002 (black) or SpyTag-MBP and SpyCatcher (gray) at 0.1 μM (top) or 10 μM (bottom) in succinate-phosphate-glycine buffer at 25 ˚C. Error bars represent mean ± SD from triplicates (some error bars are too small to be visible). Data adapted from (Keeble et al., 2017). **C** Amino acid sequence of variants of SpyTag (top) or SpyCatcher (bottom).

Figure 3: Examples of non-enzymatic uses of SpyTag/SpyCatcher. **A** On-Demand Biomolecular Manufacturing platform, using *in vitro* transcription/translation and SpyTag/SpyCatcher to multiplex binding and detection reagents. **B** Plug-and-Display vaccination platform to facilitate decoration of VLPs. **C** Super-resolution cellular imaging with dye-labeled SpyCatcher. **D** Schematic of Western blot detection using Alexa Fluor 647 dye coupled to SpyCatcher.

Figure 4: Examples of enzymatic uses of SpyTag/SpyCatcher. **A** Cyclization of enzymes to improve thermal resilience, showing a cartoon of SpyTag-β-lactamase-SpyCatcher. **B** Production of enzymatic bacterial biofilms. **C** Attachment of chemically-synthesized cell penetrating peptides to enzymes, promoting delivery of functional enzyme intracellularly. CCF2-AM is a membrane-permeable dye which is de-esterified by esterases in the cytosol to give CCF2. Cleavage by cytosolic β-lactamase changes CCF2's fluorescent spectrum. **D** Biological nanoreactors through formation of a covalently-linked enzyme network from a dimeric cytochrome P450 monooxygenase and a tetrameric glucose dehydrogenase, adapted from (Yin et al., 2018).

Table 1: SpyBank sample entries.

Figure 1

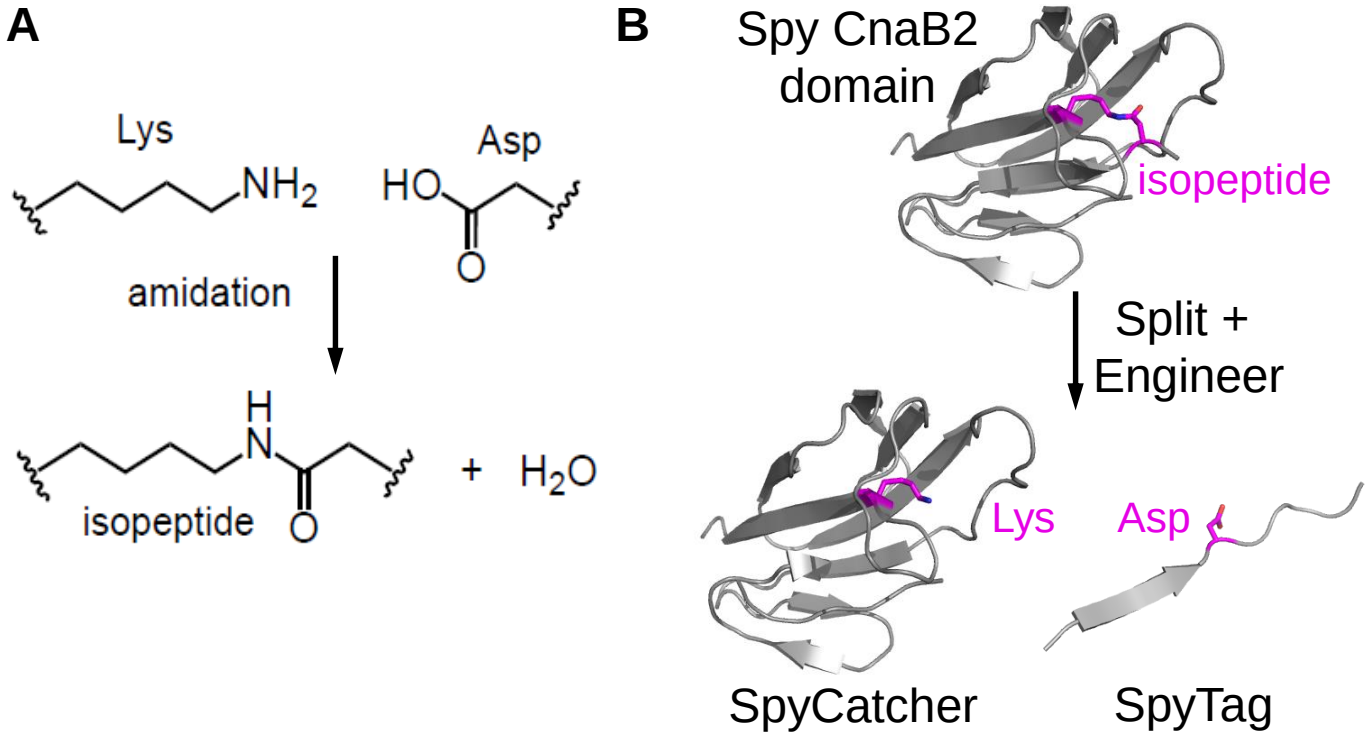
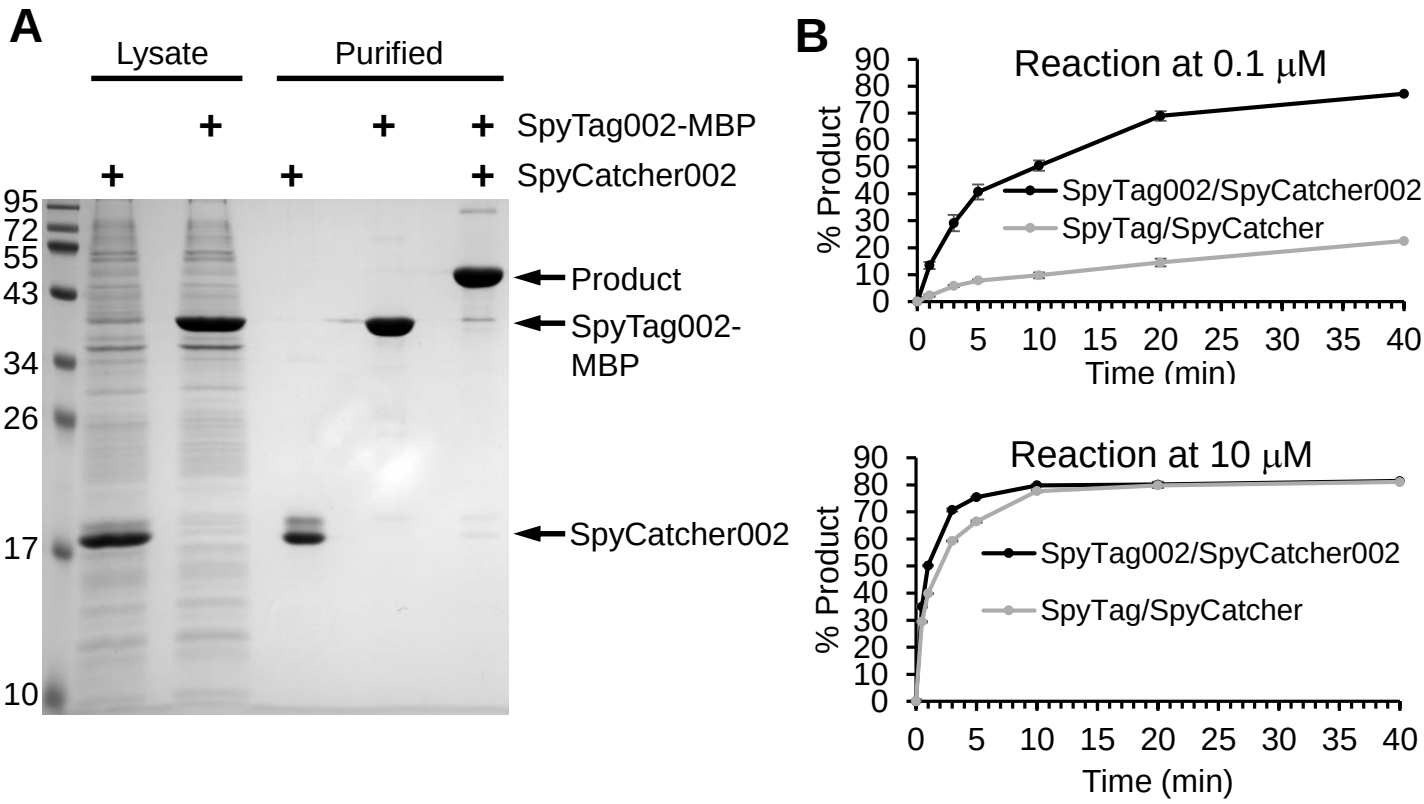

A
Lys
NH2
Asp
HO
O
amidation
H
N
isopeptide
O
+ H2O
B
Spy CnaB2
domain
isopeptide
Split +
Engineer
Lys
Asp
SpyCatcher
SpyTag

Figure 2



C

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SpyTag              -AHIVMVDAYKPTK
SpyTag002           VPTIVMVDAYKRYK

SpyCatcher          VDTLSGLSSEQGQSGDMTIEEDSATHIKFSKRDEDGKELAGATMELRDSSGKTISTWISD
SpyCatcher002       VTTLSGLSGEQGPSGDMTTEEDSATHIKFSKRDEDGRELAGATMELRDSSGKTISTWISD
ΔN1SpyCatcher       ----------------------DSATHIKFSKRDEDGKELAGATMELRDSSGKTISTWISD
ΔN1ΔC2SpyCatcher    ----------------------DSATHIKFSKRDEDGKELAGATMELRDSSGKTISTWISD

SpyCatcher          GQVKDFYLYPGKYTFVETAAPDGYEVATAITFTVNEQGQVTVNGKATKGDAHI
SpyCatcher002       GHVKDFYLYPGKYTFVETAAPDGYEVATAITFTVNEQGQVTVNGEATKGDAHT
ΔN1SpyCatcher       GQVKDFYLYPGKYTFVETAAPDGYEVATAITFTVNEQGQVTVNGKATKGDAHI
ΔN1ΔC2SpyCatcher    GQVKDFYLYPGKYTFVETAAPDGYEVATAITFTVNEQGQVTVNG---------
```

Figure 3

A Multiplexed detection

Mix cell-free reactions

Library of SpyTag-DARPins or antibodies

Covalent coupling

Library of SpyCatcher-detection proteins e.g. alkaline phosphatase

B Vaccination

SpyCatcher-virus-like particle

+ SpyTag-Antigen

Immunogenic assembly

C Super-resolution localization of mammalian intracellular proteins

Dye-SpyCatcher

Nuc

SpyTagged protein bound to intracellular target

D Western blot detection

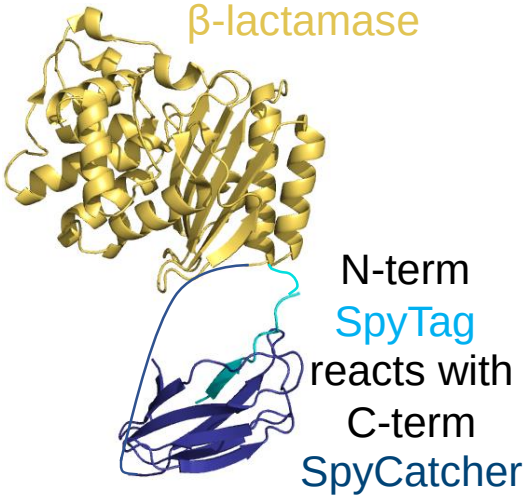
Lysate of SpyTagged protein

kDa
198
98
62
49
38
28
17
14

Protein detected by incubating membrane with SpyCatcher-dye

Figure 4

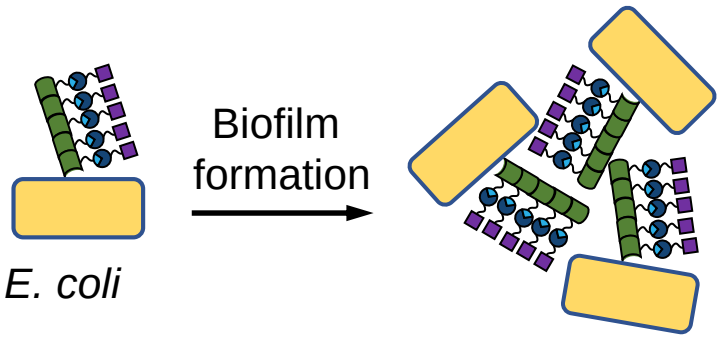
A Enzyme cyclization for resilience



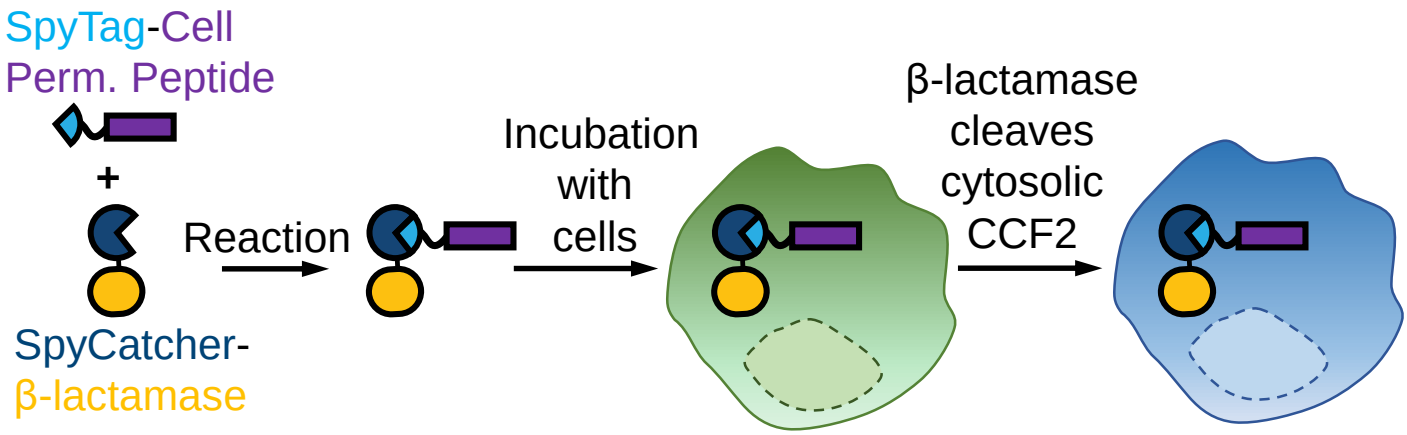
B Catalytic biofilm formation

SpyTag-CsgA form curli that react with added SpyCatcher-Enzyme

Biofilm with stabilized enzyme activity



C Enzyme delivery to intracellular sites



D Multimeric-enzyme reactor assembly

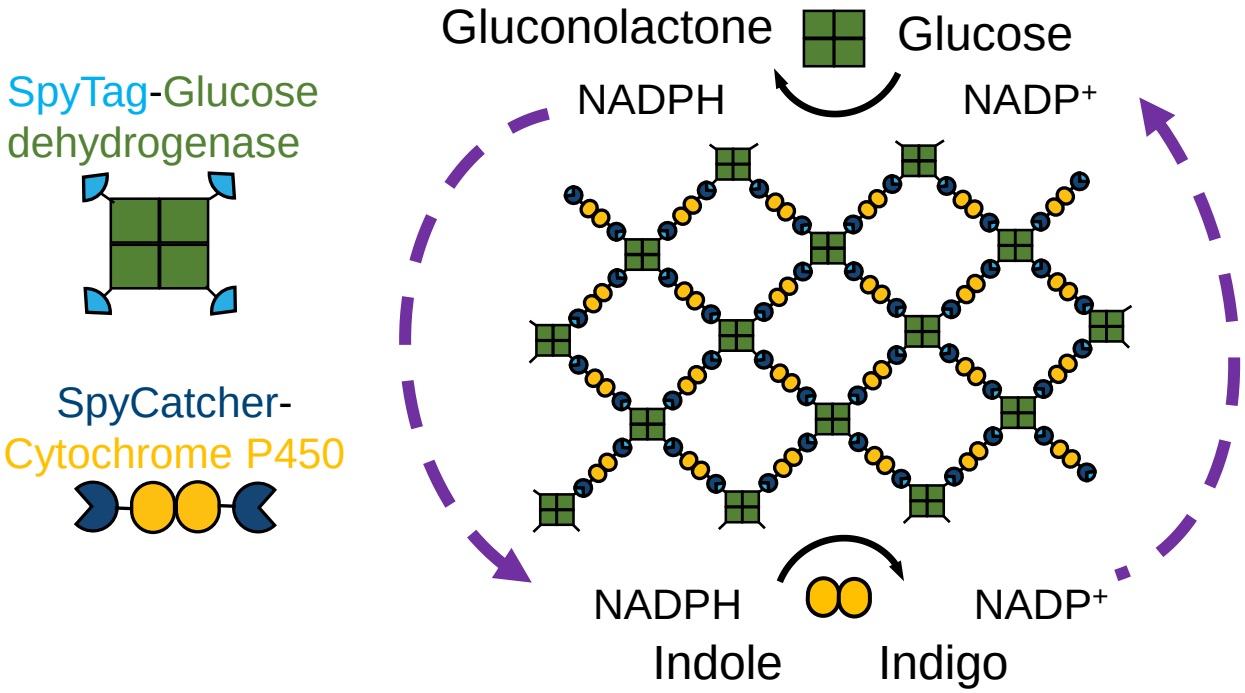


Table 1

Publication title	Journal name or Patent	Corresponding author	doi or patent code	Publication or priority date YEAR MONTH DAY)	Construct name (and Seq ID from patent)	Expression host	Compartment expressed (cytosol, periplasm, membrane, secreted, in vitro)	N-term, C-term, internal	AA sequence (Tag or Catcher bold red, linker where annotated in blue and underlined, * stop codon)
Plug-and-Display: decoration of Virus-Like Particles via isopeptide bonds for modular immunization.	Sci Rep.	Mark Howarth	10.1038/srep19234	2016 01 19	SpyCatcher-VLP (aka ΔN1-SpyCatcher-AP205CP3)	E. coli C41	cytosol	N-term SpyCatcher	MGSSHHHHHHGSG**DSATHIKFSKRDEDGKELAGATMELRDSSGKTISTWISDGQVKDFYLYPGKYTFVETAAPDGYEVATAITFTVNEQGQVTVNGKATKGDAHI**GSGGSGGSGANKPMQPITSTANKIVWSDPTRLSTTFSASLLRQRVKVGIAELNNVSGQYVSVYKRPAPKPEGCADACVIMPNENQSIRTVISGSAENLATLKAEWETHKRNVDTLFASGNAGLGFLDPTAAIVSSDTTA*
Programmed loading and rapid purification of engineered bacterial microcompartment shells	Nat Commun	Cheryl A. Kerfeld	10.1038/s41467-018-05162-z	2018 07 23	pET11n::HTST	E. coli BL21 (DE3)	cytosol	Internal SpyTag	MDHAPERFDATPPAGEPDRPALGVLELTSIARGITVADAALKRAPSLLLMSRPVSSGKHLLMMRGQVAEVEESMIAAREIAGAGGGSGGS**AHIVMVDAYKPTK**GGSGGSGALLDELELPYAHEQLWRFLDAPVVADAWEEDTESVIIVETATVCAAIDSADAALKTAPVVLRDMRLAIGIAGKAFFTLTGELADVEAAAEVVRERCGARLLELACIARPVDELRGRLFF*