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Rapid analysis of protein expression and solubility with the SpyTag–SpyCatcher system

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ABSTRACT

Successful isolation of well-folded and active protein often first requires the creation of many constructs. These are needed to assess the effects of truncations, insertions, mutations, and the presence and position of different affinity tags. Determining which constructs yield the highest expression and solubility requires the investigator to express and partially purify each construct, and, in the case of low-expressing proteins, to follow the protein using time-consuming Western blots. Even then, many proteins form soluble aggregates, which may only be apparent after more extensive purification via size exclusion chromatography. In this work, we have utilized a covalent bond-forming tag/domain pair, known as SpyTag/SpyCatcher, to rapidly and specifically attach a fluorescent label to proteins of interest in cellular lysates. Once labeled, tagged proteins can easily be followed via SDS–PAGE and fluorescence size exclusion chromatography (F-SEC) to assess expression levels, solubility, and monodispersity without the need for purification. These techniques enable rapid and facile analysis of proteins, which may greatly facilitate optimization of protein expression constructs.

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1. Introduction

SpyCatcher is an engineered protein domain which specifically recognizes and covalently bonds to a thirteen amino acid tag (SpyTag) [1,2]. The protein was engineered from a single domain of the fibronectin-binding protein FbaB from *Streptococcus pyogenes*, which naturally forms an internal isopeptide bond between a lysine and aspartate [3,4]. Such bonds are well studied [5,6], and are likely common in nature – specifically amongst Gram-positive bacteria. These isopeptide bonds have proven to be stable over a wide range of pH, temperatures, redox environments, and detergents; thus, the SpyCatcher technology has found great utility in a number of applications, including the generation of bioactive hydrogels [7], catalytic biofilms [8], and thermostable proteins [9].

One application that has not yet been explored for the SpyCatcher system is to facilitate protein detection. Because SpyCatcher recognizes a thirteen residue tag, it has the potential to be extremely specific, akin to an antibody, but with the added benefit of forming a covalent linkage to a tagged ligand. Attachment of a bright fluorophore to SpyCatcher could thus create a fluorescent "antibody-like" reagent that would specifically interact only with tagged proteins – facilitating their detection even in the context of whole cell lysate or other heterogeneous populations.

We therefore engineered a fluorescently-labeled SpyCatcher protein, and assessed its utility for protein detection via three techniques: Western blot analysis, direct detection of tagged analyte proteins after SDS–PAGE separation, and by F-SEC. Briefly, we found that fluorescent SpyCatcher can be used as a one-step Western blot detection reagent because it can readily form isopeptide bonds with tagged proteins adhered to a nitrocellulose membrane. Furthermore, pre-incubation of fluorescent SpyCatcher prior to SDS–PAGE, and subsequent direct fluorescence imaging of the gel, enabled Western blot-like detection and exquisite specificity without the need for any membrane transfer, antibody incubation, or washing. Finally, the use of fluorescent SpyCatcher for F-SEC enabled rapid determination of aggregation and dispersity of our tagged protein construct, without prior purification. Overall, the fluorescent SpyCatcher system provides useful tools that may enable the screening of constructs for yield and for solubility much more rapidly, and with less analyte, than other methods.

2. Materials and methods

2.1. Intact LC–MS

Intact protein mass spectra were acquired on an Agilent 6530 QToF coupled to an Agilent 1290 UHPLC using the Dual Electrospray

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Ionization source. The column used was a 2.1 × 50 mm Agilent PLRP-S 5 μm 1000 Å reverse-phase HPLC column, and the solvent system was 0.1% formic acid in water (Buffer A) and 0.1% formic acid in acetonitrile (Buffer B), with the column equilibrated with 2% Solvent B. Samples were loaded directly onto the column with the flow-through diverted to waste, then eluted with a 90 s 2–50% gradient. The MS instrument was tuned in the 3200 Da *m*/*z* range and data were acquired in the range from *m*/*z* 400–2000, with the capillary voltage set at 5500 V and the fragmentor and skimmer set at 175 and 65 V respectively. Peaks in the total ion chromatogram were selected automatically and an average *m*/*z* spectrum extracted. From each *m*/*z* spectrum a zero charge mass deconvolution was generated over a wide mass range, typically 10–110 kDa, using maximum entropy deconvolution and a step mass of 0.5 Da.

2.2. Cloning, expression, and purification of SpyCatcher(S49C)

The SpyCatcher protein as published by [2] was initially synthesized as an *Escherichia coli* codon optimized gene by Life Technologies (Grand Island, NY) with the sequence:

gatagcgcaacccacatcaaattcagcaaacgtgatgaagatggtaaagaactggcagg
cgcaaccatggaactgcgtgatagcagcggtaaaaccattagcacctggattagtgatg
gtcaggtgaaagatttttatctgtaccctggcaaatacacctttgttgaaaccgcagcacc
ggatggttatgaagttgcaaccgcaattacctttaccgttaatgaacagggccaggttac
cgtgaatggt

The gene was amplified from the synthetic construct with primers containing 20 bp of homology to the pRham N-term His vector (Lucigen, Middleton, WI).

Forward:catcatcaccaccatcaccatcacctggaggtgttatttcaaggacctgata
gcgcaacccacatcaa
Reverse:gtggcggccgctctattactcgtgccactcgatcttctgggcttcgaaaatgtc
gttcagtccaccagagccaccattcacggtaacctggc

The forward primer also added an additional two histidine residues (to create an 8xHis tag). The reverse primer included nucleotides encoding 3′ biotin acceptor peptide (GLNDIFEAQKIEWHE) for potential biotinylation experiments. Finally, the PCR product was assembled into the vector using Gibson Assembly (NEB Gibson Assembly 2x Master Mix), and then used as a substrate for transformation of *E. cloni* 10G chemically competent cells (Lucigen, Middleton, WI). Colonies resistant to kanamycin (Kan) were grown, and the plasmid isolated via Miniprep (Qiagen). This plasmid DNA was used as a template for site-directed mutagenesis using primer 1 (actgcgtgattgcagcggtaaaac) and primer 2 (tccatggttgcgcctgcc) and the Q5 Site-directed Mutagenesis Kit (NEB). These primers mutate serine 49 (S49) to cysteine, enabling maleimide chemistry. Importantly, S49 is surface exposed and is far from the SpyCatcher active site residues (based on the crystal structure PDBID: 4MLI [2]).

C43(DE3) [10] chemically competent cells (Lucigen, Middleton, WI) were transformed with sequence-verified vector. A single colony from this transformation was picked and used to inoculate a 100 mL overnight culture in lysogeny broth (LB) [11] supplemented with 50 μg/mL kanamycin which was grown in a 500 mL Erlenmyer flask at 37 °C while shaking at 250 rpm. This culture was used to inoculate two 1 L cultures in Terrific Broth [12] supplemented with 50 mM 3-(N-morpholino)propanesulfonic acid (MOPS) pH 7.5, 10 mM sodium phosphate buffer pH 7.0, and 50 μg/mL kanamycin. These cultures were grown until their OD_{600} reached ~1.0, at which point the temperature was decreased to 22 °C and the cultures were induced with 0.1% (w/v) L-rhamnose monohydrate. Induced cultures were grown in 2.8 L Fernbach flasks overnight at 22 °C while shaking at 250 rpm.

Cells were harvested via centrifugation at 6000×*g* for 20 min, and then re-suspended in lysis buffer (25 mM MOPS pH 7.5, 400 mM NaCl, 10 mM imidazole, 2 mM tris(2-carboxyethyl) phosphine (TCEP), and protease inhibitors (Roche, Basel, Switzerland)). The cells were then lysed by passing them three times through a microfluidics cell homogenizer (Microfluidics model M-110P cell homogenizer) at 18,000 psi. The lysate was cleared via centrifugation at 40,000×*g* for 60 min at 4 °C.

Cleared lysate was loaded onto a 5 mL HisTrap FF column (GE Healthcare Life Sciences, Pittsburgh, PA) at 3 mL/min flow rate, washed with 5 column volumes of lysis buffer, and eluted with a linear gradient of lysis buffer supplemented with 0–500 mM imidazole over 18 column volumes. Of note, the resin became darkly colored upon washing, and prior to elution, possibly due to overloading of the column. Eluted fractions were analyzed by LC–MS to ensure the protein was full length and otherwise unmodified. Fractions were pooled and concentrated using a 3500 MWCO concentrator (EMD Millipore, Billerica, MA), and loaded onto a Superdex 200 26/60 column (GE Healthcare Life Sciences, Pittsburgh, PA) pre-equilibrated with 50 mM Tris pH 7.0, 150 mM NaCl, and 1 mM TCEP. The protein was flowed through this column at 2.5 mL/min, and the peak fractions were concentrated and then used for the labeling reaction.

2.3. Labeling of SpyCatcher(S49C) with AlexaFluor647

Alexa Fluor 647 C_2 Maleimide (AF647, Life Technologies, Grand Island, NY) was prepared by dissolving 1 mg of dye into 77 μL dimethyl sulfoxide (DMSO) to create a 10 mM stock solution, which was protected from light by wrapping it in aluminum foil. 444 μL of SpyCatcher(S49C), corresponding to approximately 2 mg, was added to the 10 mM stock solution of AF647 to achieve a 1:5 M ratio of protein to dye. The reaction tube was covered with foil and rocked gently for 1 h at room temperature. Completion of the reaction was confirmed by LC–MS (as described above) by detecting the mass shift of the protein from 13,198 to 14,179 Da. Upon completion of the reaction, excess maleimide dye was quenched by the addition of 1 mM dithiothreitol (DTT). Removal of excess dye was achieved by binding the labeled protein to a 1 mL HisTrap FF column, washing with lysis buffer, and eluting with lysis buffer supplemented to 500 mM imidazole (step elution).

2.4. Cloning and purification of 8xHis–SpyTag–GST–FLAG–AVI

GST was PCR-amplified from pDEST15 (Life Technologies, Grand Island, NY), and cloned into a pRham (Lucigen, Middleton, WI) vector flanked by an 8xHis–SpyTag on the N-terminus, and a FLAG-AVI tag on the C-terminus. BL21(DE3) cells (Lucigen, Middleton, WI) were then transformed with this construct, and plated on LB/agar plates supplemented with 50 μg/ml kanamycin. A single colony was used to inoculate an overnight starter culture in LB as described above. The starter culture was used to inoculate 6 × 1 L cultures in Terrific Broth supplemented with 50 mM MOPS pH 7.5, 10 mM sodium phosphate monobasic pH 7.0, and 50 μg/mL kanamycin, which were grown in 2.8 L Fernbach flasks at 37 °C while shaking at 250 rpm. When the cells reached approximately OD_{600} ~ 1.0, the temperature was reduced to 22 °C, and each culture was supplemented with 0.2% w/v L-rhamnose monohydrate to induce expression. The cells were centrifuged and harvested the next day (~16 h later).

Unless otherwise stated, purification steps were performed at room temperature. The cell paste from 2 L of growth was re-suspended in lysis buffer (100 mM sodium phosphate buffer pH 7.0, 2 mM TCEP, protease inhibitors (cOmplete Protease Inhibitors, Roche, 1 tablet per 50 mL) and lysed via three passages

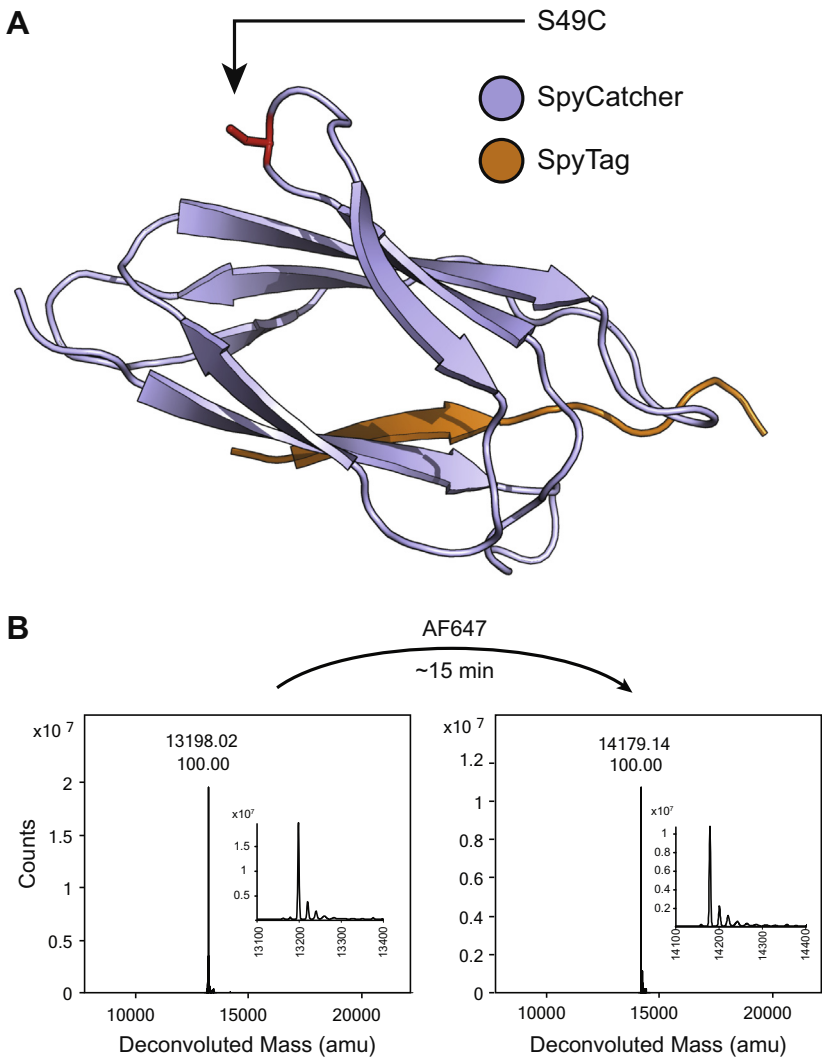

Fig. 1. Creation of fluorescent SpyCatcher. (A) Structure of the SpyCatcher protein (purple) bound to a SpyTag peptide (orange), with the S49 residue highlighted in red. (B) LC–MS analysis of SpyCatcher(S49C) pre- and post-addition of AlexaFluor 647. Note that the reaction goes to completion rapidly. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

through the cell homogenizer at 18,000 psi, as described above. A sample of whole cell lysate (WCL) was collected at this point. The WCL was then centrifuged at ~160,000×*g* for 40 min at 10 °C, and the supernatant collected. A sample of the supernatant ("Cleared lysate") was collected at this point for F-SEC studies.

The cleared lysate was then supplemented with 10 mM imidazole and loaded onto a 5 mL HisTrap FF column at 4 mL/min (using an AKTA Pure, GE Healthcare Life Sciences, Pittsburgh, PA). The column was pre-equilibrated with IMAC Buffer (25 mM MOPS pH 7.5, 400 mM NaCl, 10 mM imidazole, 2 mM TCEP). The protein was then eluted from the column with a continuous gradient from IMAC buffer supplemented from 10 mM imidazole to 500 mM imidazole. A sample of both early and late eluting protein peaks ("Wash" and "Eluate") was collected. Eluted protein was cleaved overnight with 3C protease at 4 °C, while dialyzing against Dialysis Buffer (25 mM MOPS pH 7.5, 400 mM NaCl, 40 mM imidazole, 2 mM TCEP). Cleavage was confirmed the next day by LC–MS (performed as described above) by detecting the mass shift from 33,143 to 31,185 Da corresponding to loss of the N-terminal tag. The protein was then flowed through a 1 mL HisTrap FF column (also pre-equilibrated with IMAC Buffer). The flow-through was collected and concentrated to ~0.4 mL using an Amicon Ultra 15 centrifugal filter unit (EMD Millipore, Billerica, MA) at 4 °C, then loaded onto a Superdex 200 26/60 SEC (GE Healthcare Life Sciences, Pittsburgh, PA). Most of the protein eluted at the column's void volume and as apparent oligomers (possibly due to intermolecular disulfide bonds having formed among GST's 4 cysteines). However, a smaller peak corresponded to soluble protein; this was pooled and collected.

2.5. Western blots of 8xHis–SpyTag–GST–FLAG–AVI

Cleared lysate from both an empty vector control (pRham from Lucigen with no insert) and GST-expressing cells were run next to each other on a 4–12% NuPAGE Bis-Tris SDS–PAGE gel (Life Technologies, Grand Island, NY) at 200 V for 45 min – after which the protein was transferred to a nitrocellulose membrane using the iBlot system (Life Technologies, Grand Island, NY). The membrane was blocked for 1 h with 3% bovine serum albumin (BSA)/PBS and then probed with either anti-His (1:5000, Qiagen Catalog # 34660), anti-FLAG (1:1000, Agilent Catalog # 200472), IR-Streptavidin (1:25,000, LI-COR Catalog # 32230), or AF647-SpyCatcher (1:500) and nutated at 4 °C overnight. The membranes were then washed five times in PBS with 0.1% Tween 20 (PBS-T) for five minutes. The anti-His- and anti-FLAG-bound membranes were then probed with goat-anti-mouse 680 (1:10,000, LI-COR Catalog # 68020) or

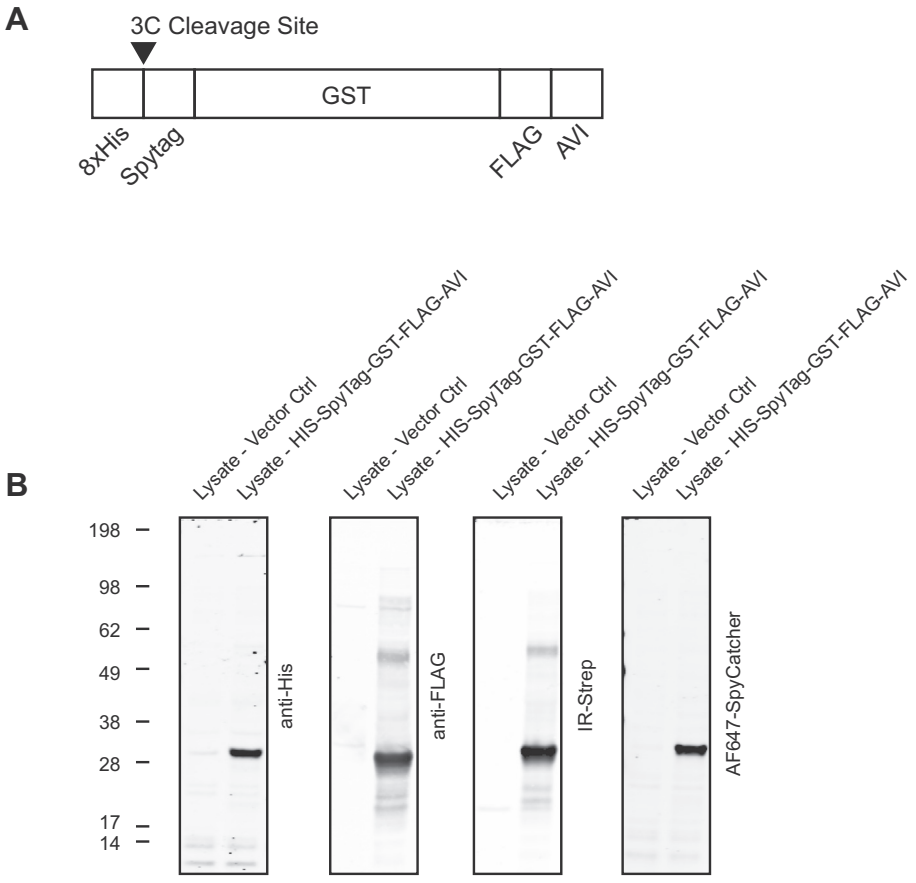

Fig. 2. Fluorescent SpyCatcher can be used as a Western blot probe. (A) Schematic of the test protein used for the analyses in this study. (B) Western blot analysis using either anti-His, anti-FLAG, IR-streptavidin, or AF647-SpyCatcher. The "Vector Ctrl" lysates are from *E. coli* expressing an empty pRham vector (Lucigen). GST: Glutathione-S-Transferase; 8xHis: octahistidine tag; SpyTag: a tag consisting of AHIVMVDAYKPTK; FLAG: a tag consisting of DYKDDDDK; AVI: the BirA recognition site for biotin conjugation, with the sequence GLNDIFEAQKIEWHE; AF647: AlexaFluor 647.

donkey-anti-mouse 800 (1:10,000, LI-COR Catalog # 32212) secondary antibodies, respectively. The membranes were then washed five times with PBS-T for five minutes, with the final wash taking place overnight at room temperature. The membranes were scanned on a LI-COR infrared scanner at both 700 and 800 nm wavelengths.

2.6. Direct detection of proteins following PAGE

Samples of WCL, cleared lysate, IMAC flow through, IMAC wash, and IMAC eluate from the preceding GST purification were collected, and were compared to the WCL and cleared lysate from the empty vector control. For each reaction, 2 μL of sample was added to 1 μL of fluorescent SpyCatcher reagent (~1 μg); these were allowed to incubate for 1 h in the dark. After incubation, 5 μL of 2x SDS load buffer were added to each reaction, and 5 μL of the resultant solution were loaded into each lane of a 4–12% Bis-Tris PAGE gel (Life Technologies, Grand Island, NY). The gel was run at 200 V for 45 min under foil, and then scanned using an Odyssey infrared scanner without washing or additional handling steps (LI-COR, Lincoln, NE) at 700 nm (using the default intensity setting 5).

2.7. Limit of detection

To assess limit of detection, fully purified and cleaved GST was serially diluted from a starting reaction concentration of 10 μM to 20 nM (dilution buffer: 25 mM MOPS pH 7.5, 150 mM NaCl, 2 mM TCEP). Each dilution was reacted with ~1 μg of AF647-conjugated SpyCatcher for 2 h in the dark, before loading one half of the reaction onto a 4–12% Bis-Tris gel (Life Technologies, Grand Island, NY). The gel was run at 120 V for 105 min, and then scanned without washing or additional handling steps on a LI-COR infrared imager at 700 nm.

2.8. Fluorescence SEC

Cleared lysates from either empty vector control (pRham, Lucigen, Middleton, WI) cells or GST-expressing *E. coli* cells were reacted with AF647-conjugated SpyCatcher (approximately 2 μg/10 μL lysate), followed by dilution to 100 μL with the mobile phase buffer (100 mM sodium phosphate monobasic pH 7, 0.05% w/v n-Dodecyl-β-D-Maltopyranoside (DDM), 2 mM TCEP). Samples and standards were analyzed using an Agilent 1100/1200 HPLC (G1312A binary pump, G1367A autosampler with chiller, and G1315B diode array detector, ChemStation Rev. B.03.02-SR2). A Jasco fluorescence detector (X-LC 3120FP with monochromator and 150 W Xenon lamp) was connected in-line with the HPLC between the auto-sampler and diode array detector. Samples and standards were stored at 4 °C in the auto-sampler prior to injection (10 μL). Chromatography was performed on a Sepax Zenix SEC-300 column (4.6 × 150 mm, 3 μm particle, 300 Å pore, part number 213300-4615) with a Sepax Zenix SEC-300 guard column (4.6 × 50 mm, 3 μm particle, 300 Å pore, part number 213300-4605). The mobile phase consisting of 100 mM sodium phosphate, pH 7, 0.05% w/v DDM, 2 mM TCEP was run through the system at

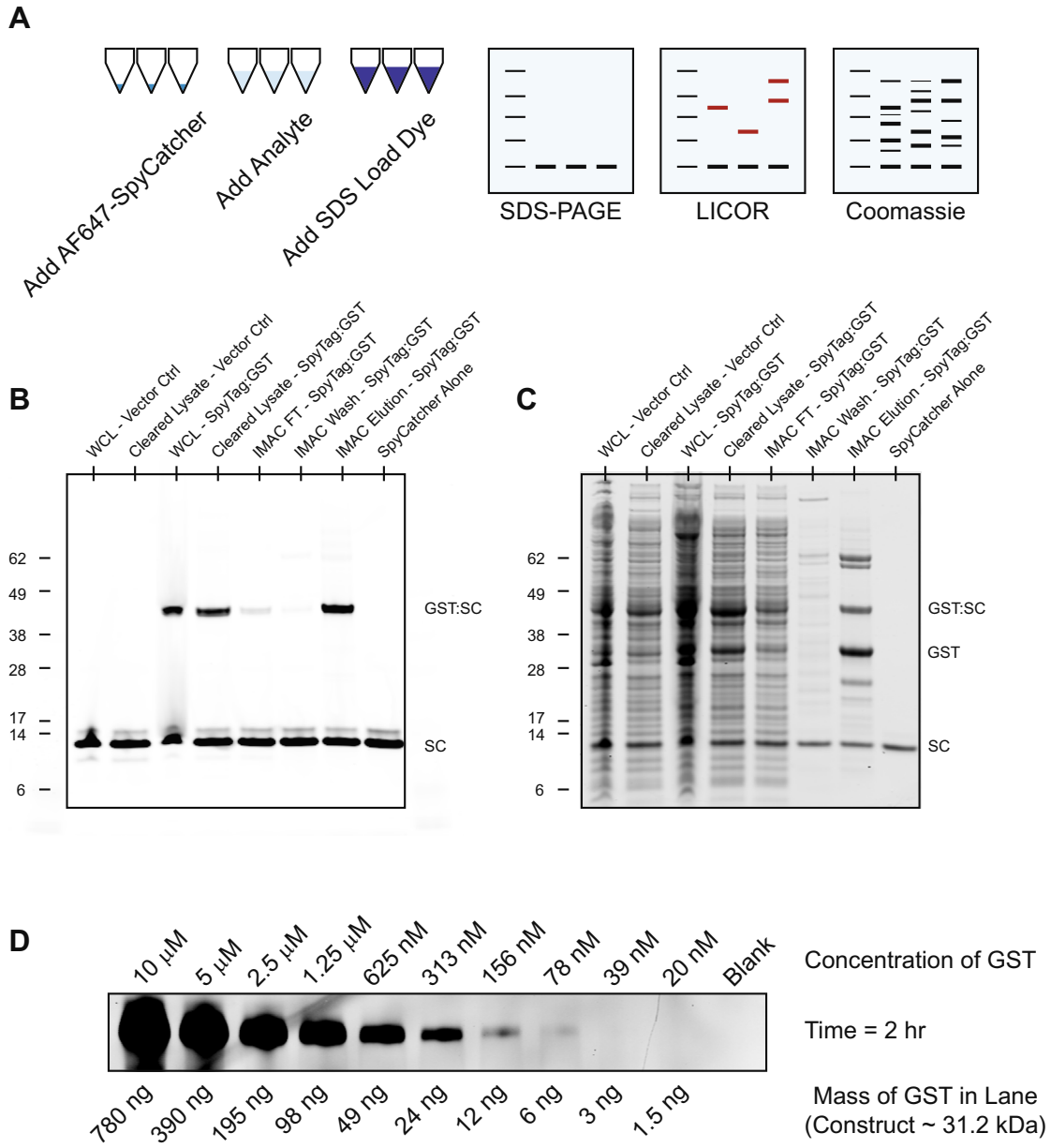

Fig. 3. Fluorescent SpyCatcher can be used to detect proteins directly following SDS–PAGE. (A) Schematic depicting the procedure for direct detection of tagged analytes. (B) Imaging of fluorescent SpyCatcher that has been pre-incubated with analytes collected at different points during an IMAC purification of SpyTagged GST (see Fig. 2A). Left two lanes: negative control lysates from cells expressing an empty pRham vector. (C) The same gel as in (B) stained with coomassie to visualize total protein. (D) Limit of detection of the direct detection method utilizing AF647-conjugated SpyCatcher. WCL: whole cell lysate; SC: SpyCatcher.

0.5 mL/min for 12 min per injection. Detection on the diode array was recorded at 220 and 260 nm. Detection on the fluorescence detector was performed at 647 nm excitation and 670 nm emission wavelengths with an 18 nm bandwidth to detect the Alexa Fluor 647 dye (gain set to 10,000, attenuation set to 128, with 1 V corresponding to 240,841.7 mAU.

3. Results and discussion

3.1. Generation of fluorescent SpyCatcher

We created a fluorescent version of SpyCatcher by a two-step process. First, we introduced a cysteine to a solvent-exposed loop in SpyCatcher, far from the active site of the protein (Fig. 1A). After expression and purification of SpyCatcher(S49C), we conjugated a maleimide-coupled fluorophore (AlexaFluor 647) to create our fluorescent SpyCatcher reagent (Fig. 1B).

One important feature of this reagent was its ease of production. The SpyCatcher(S49C) protein expressed at very high levels (>50 mg/L) and purified to 99% homogeneity after standard purification steps (IMAC and SEC). The rational placement of the cysteine ensured that coupling the fluorophore would be fast and complete; the SpyCatcher(S49C) was fully conjugated to AlexaFluor 647 within 15 min, as determined by LC–MS (Fig. 1C). Another important feature of SpyCatcher(S49C) is its variety of potential uses. Theoretically, any maleimide can be coupled to the cysteine residue, enabling many applications that require multiple different fluorophores (such as Förster Resonance Energy Transfer), and indeed any applications that require the conjugation of functional groups or molecules to a protein of interest.

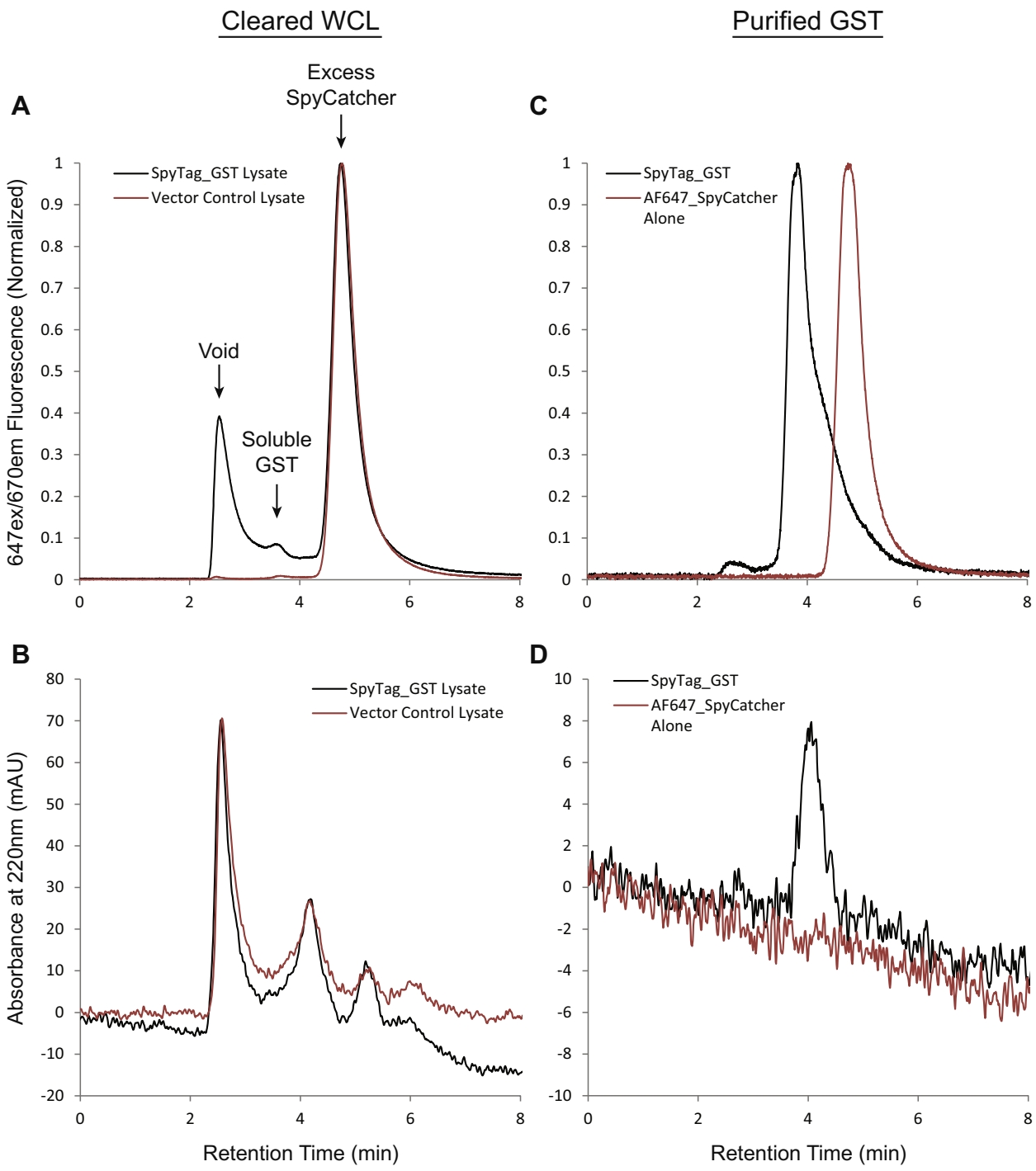

Fig. 4. F-SEC analysis of a test protein using AF647-conjugated SpyCatcher. (A) Analysis of cleared lysate from either cells expressing an empty pRham vector (red) or cells expressing the GST test construct (see Fig. 2A; black). Note there are three peaks in the GST-expressing cell lysate, corresponding to excess SpyCatcher (also present in the negative control), soluble GST, and aggregated GST (the void volume). (B) The same SEC analysis as (A), but showing the UV absorbance at 220 nm. (C) F-SEC analysis of the purified soluble fraction of SpyTagged-GST (black), compared to the fluorescent SpyCatcher reagent alone (red). (D) The same SEC analysis as (C), but showing the UV absorbance at 220 nm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Fluorescent SpyCatcher can be used as a 1-step Western blot reagent

To test SpyCatcher's utility and specificity as a detection reagent for proteins containing SpyTag, we used fluorescent SpyCatcher as a probe for western blot analysis. We collected lysate from cells expressing an empty vector as well as from cells expressing GST fused to a number of tags (8xHis, SpyTag, FLAG, and AVI; Fig. 2A). We then probed with either anti-His, anti-FLAG, fluorescent Streptavidin, or fluorescent SpyCatcher, and compared the specificity of each reagent (Fig. 2B). We found that SpyCatcher was able to react with denatured protein adhered to a nitrocellulose membrane, and that this reaction was highly specific – as evidenced by the presence of only a single band in the lane

corresponding to *E. coli* lysate wherein SpyTagged-GST was expressed, and no visible bands in the lane corresponding to cells expressing an empty vector control (pRham, Lucigen) (Fig. 2B).

3.3. SpyCatcher enables direct and specific detection of protein following SDS–PAGE

We next wished to see if we could take advantage of the covalent linkage between SpyCatcher and SpyTag-containing proteins by pre-incubating fluorescent SpyCatcher with our various analytes prior to SDS–PAGE. Because the linkage is covalent, the fluorophore is still bound to the tagged protein even after denaturation by SDS. In this way, the SDS–PAGE gel may then be directly imaged by a fluorescence scanner, and there would be no need for time consuming Western blots. Protein expression levels and solubility could then be assessed by comparing the amount of detected protein in the whole cell lysate and cleared lysate, respectively.

To test this technology, we pre-incubated fluorescent SpyCatcher with samples taken throughout a standard immobilized metal affinity chromatography (IMAC) purification (whole cell lysate, cleared lysate, IMAC flow-through, IMAC wash, and IMAC elution) using cells expressing the aforementioned multi-tagged GST construct, and then performed SDS–PAGE (Fig. 3A). As a negative control, we pre-incubated the fluorescent SpyCatcher with whole cell lysate and cleared lysate from cells expressing an empty pRham vector. Immediately after SDS–PAGE separation, we scanned the gel using a LI-COR infrared scanner.

As seen in Fig. 3B, we were able to specifically image the tagged GST throughout the purification. The specificity and detection limits of the fluorescent SpyCatcher-bound analytes were comparable to traditional Western blots (Fig. 2), but this technique did not require any transfer of protein to membranes, blocking steps, antibody incubations, or washes – greatly reducing the amount of time, effort, and reagents necessary to complete the analysis. Importantly, the fluorescent SpyCatcher did not bind to any endogenous proteins found in *E. coli*, as seen by the lack of additional bands in the vector control lanes. This could be a concern for specific bacteria such as *S. pyogenes*, which contains the endogenous protein from which SpyCatcher is engineered.

There are two main caveats to this method. First, the apparent size of the construct is expected to increase by the size of the SpyCatcher protein (approximately 14 kDa in our analyses, but as low as 9 kDa when lacking tags). One result of this is that when comparing to coomassie stained gels, there may be both bound and unbound protein shifted by the size of SpyCatcher (this can be seen in Fig. 3C). Second, each lane is expected to have a band corresponding to excess SpyCatcher, running near the bottom of the gel, which may be very bright. Running a buffer control is necessary to determine if certain bands are the protein of interest or if they are minor contaminants from the SpyCatcher purification. Nevertheless, pre-incubation with fluorescent SpyCatcher seems to be a quick and effective alternative to Western blot analysis.

To determine the limit of detection when using fluorescent SpyCatcher with SDS–PAGE, we utilized fully purified GST containing a SpyTag and made serial dilutions ranging from 10 μM to 20 nM. Each dilution was incubated with fluorescent SpyCatcher for two hours prior to performing SDS–PAGE. We found that we could begin to detect protein at concentrations as low as 78 nM, corresponding to approximately 6 ng of GST (Fig. 3D). Importantly, due to the irreversibility of the SpyCatcher/SpyTag interaction, it is likely that the limit of detection is a function of kinetics. At lower concentrations, the velocity of the reaction decreases such that less protein binds to SpyCatcher [1]. It may thus be possible to improve the limit of detection with longer incubation periods, if very low levels of detection are necessary. However, 78 nM roughly corresponds to ~50 protein molecules per *E. coli* cell, and is thus suitable for the majority of detection applications.

3.4. Fluorescence SEC can assess aggregation state of tagged proteins from cell lysates

While comparing protein levels in whole cell lysates versus cleared lysates can provide some information about solubility, proteins often form soluble aggregates which are not apparent using any denaturing technique (such as SDS–PAGE). To accurately assess protein constructs' dispersity and aggregation states, one must purify the protein of interest and examine its retention time during size exclusion chromatography – specifically the amount of protein that elutes in the void volume versus that which elutes in the included volume. When examining large numbers of protein constructs, these multiple rounds of purification can be extremely time consuming, even when performed on a small scale.

Fluorescence size exclusion chromatography (F-SEC) provides a means to generate a SEC trace specifically for a tagged protein of interest, without having to perform prior purification [13]. In an F-SEC analysis, the protein of interest is specifically tagged with a fluorophore, and followed using fluorescence signal during size exclusion chromatography. The benefit of this technique is that by tracking protein with fluorescence instead of UV absorbance, it can be detected specifically even in the context of a highly mixed population (such as cell lysate) because native *E. coli*/expression host proteins are not photo-active in the wavelengths utilized by the fluorophore. Current F-SEC labeling strategies typically involve expressing fusions with GFP or other fluorescent proteins [13,14]. However, while these fusion proteins are effectively visualized, the addition of such large domains can have substantial effects on expression and solubility, confounding analysis. Creation of GFP fusions also requires additional cloning and purification steps.

Another reagent that has been used for F-SEC is dye-conjugated Ni^{2+}-NTA, which interacts reversibly with poly-histidine tags [15]. Because the probe is added post-translationally, it avoids many of the potentially confounding issues of using fluorescent protein fusions. However, despite its utility, dye-conjugated Ni^{2+}-NTA has some shortcomings. Because the probe is non-covalent, it can dissociate during SEC, and it may be especially sensitive to buffer composition (for example, buffers containing EDTA would chelate the Ni^{2+}, causing the probe to dissociate from the analyte protein's poly-histidine tag). Furthermore, dye-conjugated Ni^{2+}-NTA is not trivial to produce, requiring fluor-coupled peptide synthesis, addition of maleimide NTA molecules, and charging of the NTA moieties with nickel.

We performed F-SEC utilizing the fluorescent SpyCatcher reagent. The SpyCatcher reagent has the benefits of being a post-translational and covalent modification, as well as being extremely easy to produce – combining the advantages of fluorescent protein fusions with the fluorescent Ni^{2+}-NTA probe, while avoiding many of their shortcomings. As a proof of concept experiment, we examined cleared lysate from cells expressing SpyTag-GST alongside lysate from *E. coli* cells expressing an empty vector control. We found that when incubated with the empty vector-control lysate, only one fluorescent peak was observed, corresponding to unbound SpyCatcher – again highlighting the specificity of the SpyCatcher/SpyTag interaction. However, when F-SEC was performed on lysate from the SpyTag–GST-expressing *E. coli*, two additional peaks were observed – corresponding to soluble GST and to aggregated GST (the latter eluting at the void volume of the SEC column) (Fig. 4A). In this case, despite robust expression as evidenced by Western blot (Fig. 2B) and direct detection using SpyCatcher (Fig. 3B), most of the protein eluted at the column's void volume, suggesting it existed primarily as soluble aggregates. As expected,

examination of the cell lysate by UV absorbance did not yield useful information about the expressed protein (Fig. 4B).

We also tested F-SEC with purified SpyTag-GST (Fig. 4C). With fully purified protein present at higher concentrations, the SpyCatcher–SpyTag reaction proceeded nearly to completion, leaving only a small peak of unreacted SpyCatcher. Importantly, we found that the fluorescence signal was much more robust compared to the UV absorbance; the latter approached the noise floor of the detector (Fig. 4D). These data suggest that conjugation of a bright fluorophore via a SpyCatcher adaptor to a SpyTagged analyte may enable the development of assays to assess protein complex formation, to determine protein-detergent complex size, or to track any other changes in hydrodynamic radius, while utilizing only very small quantities of protein. Additionally, these data show that addition of the fluorescent SpyCatcher reagent does not induce aggregation.

4. Conclusions

One of the most time consuming steps in projects involving protein biochemistry is screening large numbers of constructs for high expression and solubility. It is often necessary to perform Western blot analysis to identify the heterologously-expressed protein from the milieu of the whole cell lysate. These analyses take many hours, require expensive antibodies, and often lead to ambiguous results due to non-specific antibody binding. In this work, we have confirmed that covalent binding of SpyCatcher is highly specific to the SpyTag sequence, and when conjugated to a fluorophore, produces a reagent that can be used to detect tagged proteins. Fluorescent SpyCatcher can be treated much like an antibody for Western blot analysis, but more efficiently, it can simply be pre-incubated with SpyTagged analyte proteins prior to SDS–PAGE separation and imaging. Direct imaging of the gel following electrophoresis provides the same information as that generated by a Western blot, but in a fraction of the time, and using fewer reagents and procedures. Furthermore, the fluorescent SpyCatcher reagent can be used for F-SEC analysis – providing important information about the aggregation state and monodispersity of tagged proteins prior to any purification. Without F-SEC, this information can only be obtained after purification (leading to size exclusion chromatography), and from scaled-up cellular expression trials. Importantly, while in this work we have demonstrated the utility of the SpyCatcher system using an N-terminally SpyTagged test protein, we have found equal success when placing the tag at the C-terminus or in internal solvent exposed loops (data not shown). Overall, fluorescent SpyCatcher has proven a useful tool to enable the rapid assessment of expression and solubility of tagged proteins.

Author Contributions

DD, CMR, and LEM conceived the research. DD performed experiments. WSS, CMR, and LEM constructed the F-SEC instrument. DD and LEM wrote the manuscript.

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