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Chemical modification of the tryptophan residue in a recombinant Ca2+-ATPase N-domain for Studying Tryptophan-ANS FRET

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TITLE:

Chemical Modification of the Tryptophan Residue in a Recombinant Ca^{2+}-ATPase N-domain for Studying Tryptophan-ANS FRET

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SUMMARY:

ANS binds to the Ca^{2+}-ATPase recombinant N-domain. Fluorescence spectra display a FRET-like pattern upon excitation at a wavelength of 295 nm. NBS-mediated chemical modification of Trp quenches the fluorescence of the N-domain, which leads to the absence of energy transfer (FRET) between the Trp residue and ANS.

ABSTRACT:

The sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) is a P-type ATPase Ca^{2+} that has been crystallized in various conformations. Detailed functional information may nonetheless be obtained from isolated recombinant domains. The engineered (Trp552Leu and Tyr587Trp) recombinant nucleotide-binding domain (N-domain) displays fluorescence quenching upon ligand binding. An extrinsic fluorophore, namely, 8-anilino-1-naphthalene sulfonate (ANS), binds to the nucleotide-binding site via electrostatic and hydrophobic interactions with Arg, His, Ala, Leu, and Phe residues. ANS binding is evidenced by the increase in fluorescence intensity when excited at a wavelength (λ) of 370 nm. However, when excited at λ of 295 nm, the increase in fluorescence intensity seems to be coupled to the quenching of the N-domain intrinsic fluorescence. Fluorescence spectra display a Föster resonance energy transfer (FRET)-like pattern, thereby suggesting the presence of a Trp-ANS FRET pair, which appears to be supported by the short distance (~20 Å) between Tyr587Trp and ANS. This study describes an analysis of the Trp-ANS FRET pair by Trp chemical modification (and fluorescence quenching) that is mediated by *N*-bromosuccinimide (NBS). In the chemically modified N-domain, ANS fluorescence increased when excited at a λ of 295 nm, similar to when excited at a λ of 370 nm. Hence, the NBS-mediated chemical modification of the Trp residue can be used to probe the absence of FRET between Trp and ANS. In the absence of Trp fluorescence, one should not observe an increase in ANS fluorescence. The chemical modification of Trp residues in proteins by NBS may be useful for examining FRET between Trp residues that are close to the bound ANS. This assay will likely also be useful when using other fluorophores.

INTRODUCTION:

Föster resonance energy transfer (FRET) has become a standard technique for determining the distance between molecular structures after binding or interaction in protein structure and function studies[1–4]. In P-type ATPases, FRET has been used to investigate the structure and function of the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA)[2,5–8], e. g., structural fluctuations during the catalytic cycle have been analyzed in the whole protein by FRET[7].

FRET donors are diverse, and range from small fluorescent (extrinsic) molecules to fluorescent proteins[9,10]. Tryptophan (Trp) residues (due to their fluorescence) are useful for identifying structural changes in protein amino acid sequences[11,12]. The fluorescence intensity of Trp depends substantially on the polarity of its surrounding environment[13,14]. Therefore, ligand binding usually generates structural rearrangements in proteins/enzymes[15,16]. If Trp is present at or located close to the protein binding site, structural fluctuations frequently affect the degree of Trp exposure to aqueous media[13,14]; thus, the change in polarity results in quenching of the Trp fluorescence intensity[13,14]. Hence, the fluorescent property of Trp is useful for performing ligand binding studies for enzymes. Other physical phenomena may also lead to Trp fluorescence quenching[17–20], e. g., FRET and changes in medium polarity. Energy transfer from the excited state of Trp to a fluorophore also has potential applications, e. g., affinity determination of small ligands in proteins[21]. Indeed, Trp has been primarily used as a fluorescence donor in FRET studies in proteins[22–24], e. g., in terbium (Tb^{3+}) FRET studies, a Trp residue is used frequently as an antenna for energy transfer to Tb^{3+} [25–27]. Trp displays various advantages over other FRET donors due to its inherent constitutive character in the protein structure, which eliminates the need for preparative processes that may affect the function/structure of the studied protein[24]. Thus, the identification of radiative decays (energy transfer and changes in the medium polarity that are induced by protein structural rearrangements) is important for drawing accurate conclusions regarding ligand binding in protein structural studies[13,14,19,28].

In protein structural studies, an extrinsic fluorophore, namely, 8-anilino-1-naphthalene sulfonate (ANS), has been primarily used in experiments related to protein folding/unfolding[28,29]. ANS binds to proteins/enzymes in the native state, usually in the binding sites of substrates[31–33]; an increase in ANS fluorescence quantum yield (ΦF) (namely, an increase in fluorescence intensity) is induced by exciting the protein at λ=370 nm when suitable interactions of ANS with Arg and His residues in hydrophobic pockets occur[34–37]. In various studies, the occurrence of FRET (when exciting at λ within 280–295 nm) between Trp residues (donors) and ANS (acceptor) has been reported, which is based on the following: 1) overlap of the fluorescence emission spectrum of Trp and excitation spectrum of ANS, 2) identification of a suitable distance between one or more Trp residue(s) and ANS for energy transfer, 3) high ANS quantum yield when bound in protein pockets, and 4) characteristic FRET pattern in the fluorescence spectra of the protein in the presence of ANS[3,17,27,37,38].

Recently, ligand binding to the nucleotide-binding domain (N-domain) in SERCA and other P-type ATPases have been investigated using engineered recombinant N-domains[40–46]. Molecular engineering of the SERCA N-domain has been used to move the sole Trp residue (Trp552Leu) to

a more dynamic structure (Tyr587Trp) that is close to the nucleotide-binding site, where fluorescence variations (quenching) may be used to monitor structural changes upon ligand binding[34]. Experimental results have demonstrated that ANS binds (as ATP) to the nucleotide-binding site in the purified recombinant SERCA N-domain[34]. Interestingly, the ANS fluorescence increases upon binding to the N-domain upon excitation at a λ of 295 nm, while the intrinsic fluorescence of the N-domain decreases[34], thereby producing a FRET pattern that suggests the formation of a Trp-ANS FRET pair.

The use of NBS has been proposed to determine the content of Trp residues in proteins[47] by absorbance assay of modified proteins. NBS modifies the highly absorbing indole group of Trp to the less absorbent oxindole[47, 48]. This results in the loss (quenching) of the Trp fluorescent property[40]. Hence, NBS-mediated chemical modification of Trp residues may be used as an assay to define the role of Trp (as a donor) when FRET is hypothesized.

This protocol describes the chemical modification of the sole Trp residue by NBS in the engineered recombinant N-domain of SERCA as a protein model. Experimental results demonstrate that the ANS fluorescence intensity still increases in the chemically NBS-modified N-domain[34], which lacks intrinsic fluorescence. Therefore, the assay is useful for demonstrating the absence of FRET between the Trp residue and ANS when bound to the N-domain[34, 40, 49]. Hence, this assay (NBS chemical modification of Trp) is useful in proving the presence of the Trp–ANS FRET pair in proteins.

PROTOCOL:

1. Determination (*in silico*) of the ANS and SERCA N-domain interaction

1.1. Generate a three-dimensional (3D) structure of the protein (SERCA N-domain) by molecular modeling using the preferred protein modeling software[50].

1.2. Identify the amino acid residues that form the nucleotide-binding site using the preferred molecular structure software[51], and determine the presence of Arg and Lys residues[35]; these are required for ANS binding and to increase the fluorescence intensity (quantum yield).

1.3. Perform molecular docking (using the preferred docking software)[52–54] to determine the interactions of ATP, fluorescein isothiocyanate (FITC) (which forms a covalent bond with Lys515 labeling the nucleotide-binding site), and ANS with amino acids residues in the nucleotide-binding site (**Figure 1**).

1.4. Calculate the molecular distance (Å) between Trp residue and bound ANS using the measurement tool in the preferred software.

1.5. Perform molecular dynamics simulation of ANS-N-domain complex to determine the stability of the interaction[52,54]. Then, perform the *in vitro* experiments when the stability of the complex has been confirmed.

2. Expression and purification of the recombinant N-domain

2.1. Synthesize the gene coding for N-domain[40].

2.2. Design and construct the plasmid that contains the synthetic gene that codes for the N-domain[40].

2.3. Express and purify by affinity chromatography (Ni-NTA), the engineered recombinant N-domain. Perform an SDS-PAGE of the purified protein to determine the purity (**Figure 2**)[40].

2.4. Determine the protein concentration by studying the absorbance at λ of 280 nm with the N-domain extinction coefficient (ε= 11,960 $M^{-1} \cdot cm^{-1}$)[40].

3. Monito the formation of the ANS-N-domain complex based on ANS and N-domain fluorescence intensity changes.

3.1. Prepare an ANS stock solution in *N*,*N*-dimethylformamide.

3.1.1. Weigh a small amount (1-5 mg) of ANS, and dissolve it in 1 mL of the final volume of *N*,*N*-dimethylformamide, e. g., 3.2 mg (10.69 mM final concentration).

3.1.2. Prepare a 100 µM ANS aqueous stock solution using the ANS solution in *N*,*N*-dimethylformamide, e. g., add 9.4 µL of the 10.69 mM ANS solution to 990.6 µL of 50 mM phosphate buffer with pH 8.0 to obtain a final volume of 1 mL.

3.1.3. Mix the solutions by vortexing 3 - 5 times for 15 s.

NOTE: In the following experiment, use only the ANS aqueous stock solution. Freshly prepare the ANS aqueous stock solution before initiating the experiments.

3.2. Prepare the NBS stock solution in *N*,*N*-dimethylformamide.

3.2.1. Weigh a small amount (1-5 mg) of NBS, and dissolve it in 1 mL of *N*,*N*-dimethylformamide, e. g., 5.3 mg in 1 mL (29.78 mM final concentration).

3.2.2. Prepare a 1 mM NBS aqueous stock solution using the NBS solution in *N*,*N*-dimethylformamide, e. g., add 3.36 µL of the 29.78 mM NBS solution to 96.64 µL of 50 mM phosphate buffer with pH 8.0 to obtain a final volume 0.1 mL.

3.2.3. Mix the solutions by vortexing 3 - 5 times for 15 s.

NOTE: Freshly prepare the NBS aqueous stock solution before starting the experiments.

3.3. Titrate the N-domain with ANS, and record the fluorescence spectra by excitation at λ=295 nm at 25 °C.

3.3.1. Obtain the fluorescence spectrum baseline.

3.3.1.1. Place 1 mL of 50 mM phosphate buffer with pH 8.0 in a 1 mL fluorescence quartz cuvette.

3.3.1.2. Position the cell in the thermo-stated cell chamber (25 °C) of the spectrofluorometer and set the excitation λ to 295 nm.

3.3.1.3. Record the fluorescence spectrum (305 - 550 nm).

NOTE: The fluorescence spectrum of the 50 mM phosphate buffer with pH 8.0, which serves as the blank sample, is subtracted from all obtained fluorescence spectra.

3.3.2. Obtain the intrinsic fluorescence spectrum of the N-domain.

3.3.2.1. Place 900 µL of 50 mM phosphate buffer with pH 8.0 in a fluorescence quartz cuvette.

3.3.2.2. Add 100 µL of N-domain (10 µM) suspension to obtain a 1 µM N-domain final concentration in a 1 mL final volume.

3.3.2.3. Gently homogenize using a micropipette ~20 times to ensure the homogeneity of the solution.

NOTE: The protein should be freshly purified to obtain high-quality intrinsic fluorescence spectra, e. g., the purified recombinant N-domain may only be used for a week after purification.

3.3.2.4. Position the cell in the thermo-stated cell chamber (25 °C) of the spectrofluorometer and set the excitation λ to 295 nm.

3.3.2.5 Record the N-domain intrinsic fluorescence spectrum (305–550 nm).

3.3.3. Add ANS, and obtain the fluorescence spectrum by excitation at λ=295 nm.

3.3.3.1. Add a 2 µL aliquot of 100 µM ANS aqueous stock solution to the suspended N-domain (1 µM) to obtain a 0.2 µM ANS final concentration.

3.3.3.2. Gently homogenize using a micropipette ~20 times to ensure the homogeneity of the solution.

3.3.3.3. Position the cell in the thermo-stable cell chamber (25 °C) of the spectrofluorometer and set the excitation λ to 295 nm.

3.3.3.4. Record the fluorescence spectrum (305–550 nm).

3.3.3.5. Repeat the ANS additions and fluorescence spectra recording above 1:1 molar relationship ANS:N-domain.

3.3.3.6 Subtract the blank spectrum from each spectrum using suitable software.

3.3.3.7. Plot all the spectra in a single graph.

3.3.3.8. Determine whether the spectra form a FRET-like pattern. The ANS-N-domain fluorescence spectra form a FRET-like pattern (**Figure 3A**).

4. N-domain intrinsic fluorescence titration by Trp chemical modification with NBS.

4.1. Repeat steps 3.3.1 and 3.3.2.

4.2. Add a 1 µL aliquot of 1 mM NBS aqueous stock solution to the suspended N-domain (1 µM) to obtain a final concentration of 1 µM NBS.

4.3. Gently homogenize by using a micropipette ~20 times to ensure the homogeneity of the solution.

4.4. Position the cell in the thermo-stable cell chamber (25 °C) of the spectrofluorometer and set the excitation λ to 295 nm.

4.5. Record the fluorescence spectrum (305–550 nm) (**Figure 3B**).

4.6. Repeat the NBS addition and fluorescence spectra recording until minimal N-domain intrinsic fluorescence quenching is observed[40]. In the N-domain, this usually occurs at a molar ratio of ~5–6 NBS/N-domain[40].

NOTE: NBS rapidly quenches (<5 s) the intrinsic fluorescence of the N-domain; a decrease in fluorescence intensity is observed. Proceed immediately to the next step, as NBS may also react with other amino acid residues[47].

4.7. Subtract the blank spectrum from each spectrum using suitable software.

4.8. Plot all spectra in a single graph (**Figure 3B**).

5. Titrate the NBS-modified N-domain with ANS by recording fluorescence spectra at 25 °C.

5.1. Perform Step 3.3.3 using the NBS modified N-domain that was generated in Step 4.

5.2. Subtract the blank spectrum from each spectrum using suitable software.

5.3. Plot all spectra in a single graph (**Figure 3C**).

5.4. The generated fluorescence spectra (**Figure 3C**) support or refute the occurrence of FRET, i.e., when FRET occurs, the ANS fluorescence does not increase and vice-versa.

6. Evidence of ANS binding to the chemically modified N-domain by excitation at λ=370 nm.

6.1. Perform Step 3.3.3 using the NBS modified N-domain that was generated in Step 4 but changing the excitation λ to 370 nm.

6.2. Subtract the blank spectrum from each spectrum using suitable software.

6.3. Plot all spectra in a single graph (**Figure 3D**).

6.4. Confirm ANS binding to the N-domain by observing the increase in ANS fluorescence intensity. ANS binding to the N-domain shows a fluorescence increase when excited at λ=370 nm (**Figure 3D**). As a control, the fluorescence spectrum of ANS (alone) in 50 mM phosphate buffer with pH 8.0 was obtained exciting at λ of 295 and 370 nm (Figure 4, not shown in video).

NOTE: The stoichiometric relationship of NBS:Trp that is required for chemical modification depends on the degree of burying of the Trp residue(s) in the protein under study[46,47,55,56]. Therefore, it is recommended to determine the NBS:protein/(Trp) molar ratio, beforehand.

REPRESENTATIVE RESULTS:

Molecular docking shows the binding of ANS to the nucleotide-binding site of the N-domain via electrostatic as well as hydrophobic interactions (**Figure 1**). Molecular distance (~20 Å) between the Trp residue and ANS (bound to the nucleotide-binding site) supports the occurrence of FRET (**Figure 1**). The designed (engineered) recombinant N-domain was obtained at high purity by affinity chromatography (**Figure 2**) and was suitable for fluorescence experiments. Fluorescence spectra of the ANS-N-domain complex displayed a FRET-like pattern upon excitation at λ=295 nm (**Figure 3A**). Chemical modification of the Trp residue by NBS led to quenching of the intrinsic fluorescence of the N-domain (**Figure 3B**). In the chemically NBS-modified N-domain, the experimental results demonstrate that ANS fluorescence increased upon excitation at λ=295 nm (**Figure 3C**), similar to that observed in the nonmodified N-domain (**Figure 3A**). Therefore, direct excitation of ANS at λ=295 nm provides the most energy for ANS fluorescence (**Figure 3C**), as suggested previously[28]. ANS binding to the chemically modified N-domain is evidenced by an increase in its fluorescence when excited at λ=370 nm (**Figure 3D**). Therefore, FRET does not occur between the Trp residue and ANS that is bound to the nucleotide-binding site.

FIGURE AND TABLE LEGENDS:

Figure 1: Molecular docking of ANS to the nucleotide-binding site of the Ca^{2+}-ATPase N-domain.

ANS molecular docking was performed using AutoDock Vina software (http://vina.scripps.edu/) and a generated 3D model of the N-domain[40]. The engineered N-domain contains mutations Trp552Leu and Tyr587Trp (shown in blue). Amino acid residues that form the nucleotide-binding site are represented as balls and sticks and highlighted in orange. This figure has been modified with permission from Springer Nature: Springer, Journal of Fluorescence. Copyright (2020)[34].

Figure 2: SDS−PAGE of the engineered recombinant Ca^{2+}-ATPase N-domain. The N-domain was subjected to affinity purification using a chromatographic column. Fractions that corresponded to absorption (at λ=280 nm) peaks were subjected to SDS−PAGE and visualized by Coomassie blue staining. The ∼30 kDa His-tagged N-domain is formed by 27 kDa of N-domain Ca^{2+}-ATPase and 3 kDa of poly-His tag. The Ca^{2+}-ATPase N- domain purity was determined to be ≥95% by densitometry using the ImageJ software (https://imagej.nih.gov/ij/download.html).

Figure 3: NBS-mediated chemical modification of the Trp residue in the N-domain disproves FRET between Trp and ANS that is bound to the nucleotide-binding site. A. FRET pattern of the ANS–N-domain complex upon excitation at λ=295 nm. ANS was added (final concentration in μM: Spectra a, 0; b, 0.2; c, 0.4; d, 0.6; e, 0.8; f, 1.0; g, 1.2; and h, 1.4) to the suspended N-domain (1 μM). **B.** Fluorescence quenching of the N-domain by NBS (NBS concentration in μM: a, 0; b, 1; c, 2; d, 3; e, 4; and f, 6). NBS mediates chemical modification of the Trp residue. N-domain intrinsic fluorescence was observed upon excitation at λ=295 nm. **C.** Fluorescence spectra of ANS that is bound to the chemically modified N-domain upon excitation at λ=295 nm. The experimental conditions are as in **A**. Figures A, B, and C have been modified with permission from Springer Nature: Springer, Journal of Fluorescence. Copyright (2020) [34]. **D.** Fluorescence spectra of ANS that is bound to the chemically modified N-domain upon excitation at λ=370 nm. The N-domain was suspended in 1 ml of 50 mM phosphate buffer (pH 8.0) and aliquots of NBS, and ANS was added accordingly, as described in **A** (ANS) and **B** (NBS).

Figure 4: ANS fluorescence spectra. ANS (1.4 μM) in 50 mM phosphate buffer with pH 8.0 was excited at λ of 295 and 370 nm; the spectra are presented in black and blue, respectively.

DISCUSSION:

Fluorescence spectra of the ANS-N-domain complex display a FRET-like pattern when excited at a λ of 295 nm, while the molecular distance (∼20 Å) between the Trp residue and ANS seems to support the occurrence of FRET (**Figure 1**). Trp chemical modification by NBS results in a less fluorescent N-domain (**Figure 3B**, Spectrum f); hence, energy transfer is not possible. The ANS fluorescence spectra are similar to that of the nonmodified N-domain when excited at a λ of 295 nm (**Figure 3A and C**).

Therefore, direct excitation of ANS at a λ of 295 nm is the main source of ANS fluorescence when it is bound to the ATP binding site (**Figure 3C)**, which is in agreement with the mechanism that was proposed by other authors[28]. Therefore, FRET from the Trp residue to bound ANS does not occur in the N-domain-ANS complex. Nonetheless, NBS-mediated chemical modification of Trp residues in other proteins supports FRET between Trp and ANS, e. g., in the enzymes xylose

reductase from *Neurospora crassa*[49], the α-subunit of F1-ATPase from yeast mitochondria[58], and thermolysin[59].

The assay would perform well in proteins/enzymes with hydrophobic pockets (binding sites) that contain His and Arg residues, as these contribute to the stabilization of the ANS interaction. Additionally, such proteins should ideally contain a sole Trp residue that is located at the protein surface, namely, accessible for rapid reaction with NBS[40, 41, 49].

Alternatively, to analyze the Trp-ANS FRET pair in proteins, chemical modification of His residues by acetylation and succinylation may be used to hamper the ANS interaction in the protein/enzyme binding site[60]. Deletion of the Trp residue by mutation is another strategy for analyzing FRET. However, this might be time-consuming, and the constructs may exhibit structural differences, thereby affecting ligand binding[61]. Similarly, mutation of Arg and His residues at the ligand-binding site may generate unforeseen structural changes, thereby rendering the mutated protein unsuitable for experiments[62].

With regards to the Trp residue, the performance of the NBS-chemical modification assay would be limited in the following cases: 1) if the Trp residue is buried deeply at the core of a well folded and compact protein; since the NBS moiety would be unable to access the Trp residue due to the absence of large cavities[41,48,63], 2) if Trp residues is located in a membrane-embedded structures (transmembrane α-helix), as the aqueous character of NBS will prevent it from entering the hydrophobic medium[32,56,64], 3) if the protein structure contains multiple Trp residues; as the variations in accessibility and physicochemical environment may be large, thereby rendering difficult the assignment of a fluorescence signal change to a Trp residue[32,41,56], 4) if ANS binding to proteins is due mainly to hydrophobic interaction, as the ANS fluorescence increase is due mainly to electrostatic interactions[32,65–67], and e) if static quenching of Trp occurs, e. g., in the presence of oxygen[68].

NBS mediated chemical modification of Trp residues appears to be a rapid and easy assay for studying FRET between Trp and ANS that is bound to proteins/enzymes. Other Trp-modifying reagents may be used instead of NBS, e. g., hydroxy-5-nitrobenzyl bromide (HNB)[69,70]. Finally, the assay may be applicable to the detection of proposed FRET pairs of Trp with other flurophores[21].

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DISCLOSURES:

The authors declare that they have no competing financial interests.

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Figure 1. Molecular docking of ANS to the nucleotide binding site of Ca2+-ATPase N-domain

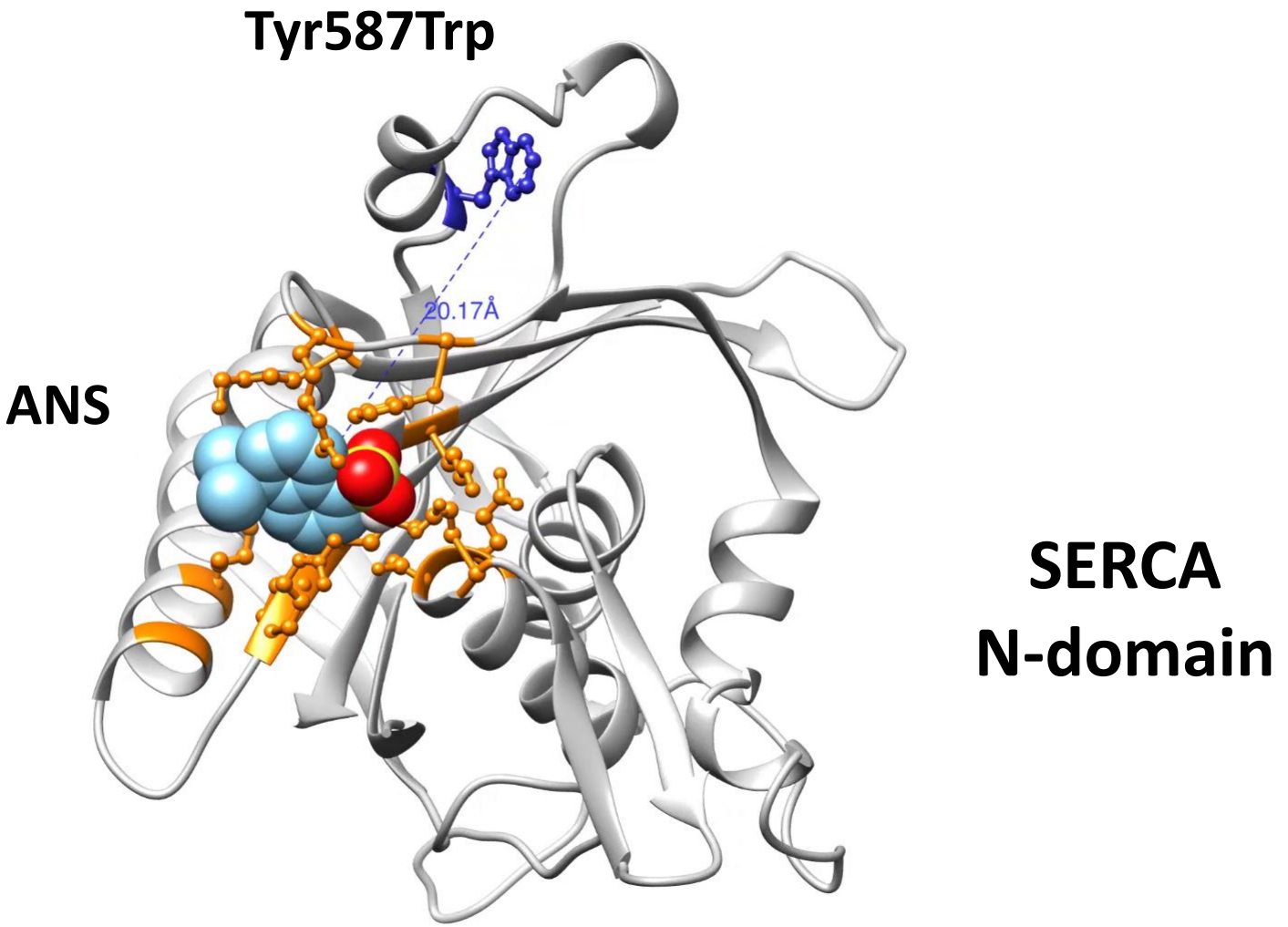

Figure 1. J.G. Sampedro and Y. Cataño

Figure 2. SDS−PAGE of the engineered recombinant Ca2+-ATPase N-domain

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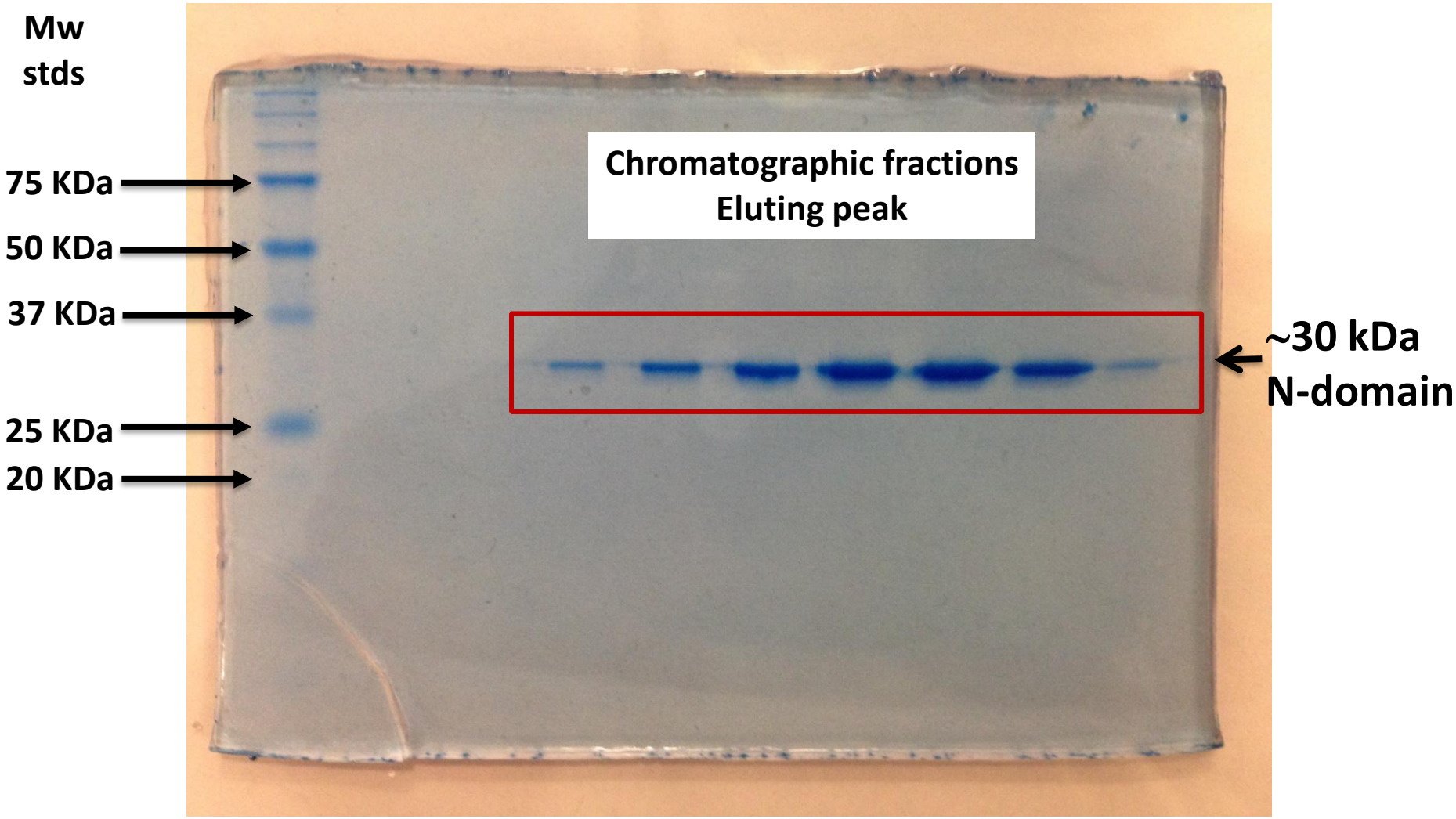

Figure 2. J.G. Sampedro and Y. Cataño

Figure 4. ANS fluorescence spectra

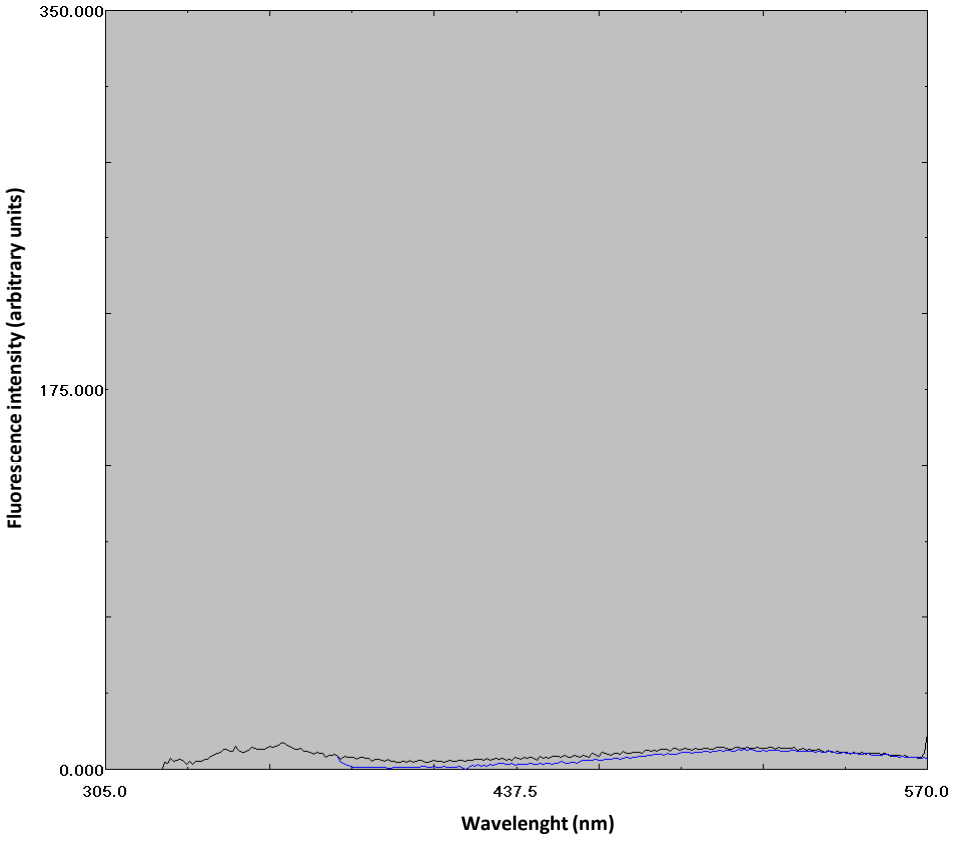

Figure 4. J.G. Sampedro and Y. Cataño

Figure 3. NBS mediated chemical modification of Trp residue in N-domain disprove FRET between Trp and ANS

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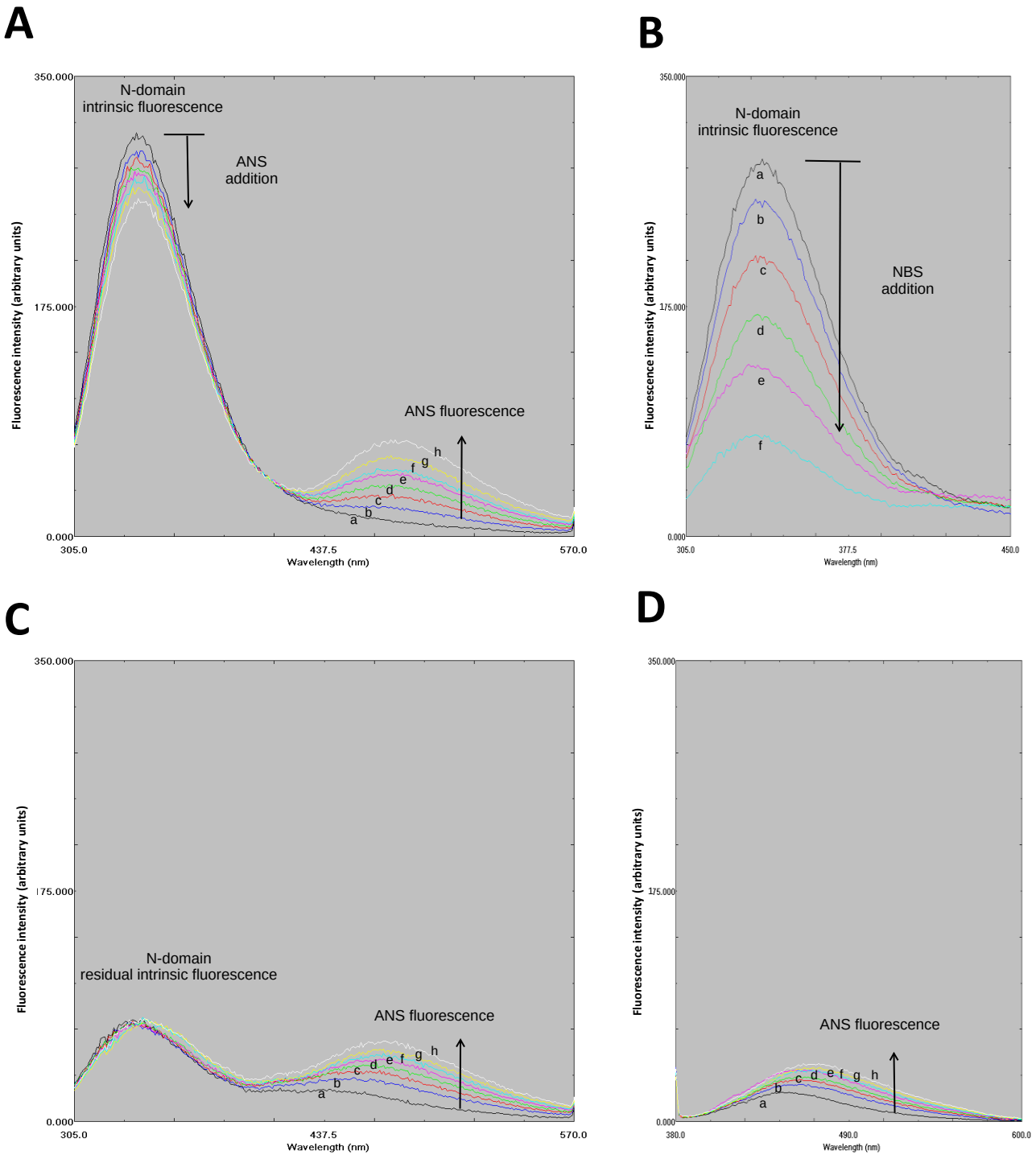

Figure 3. J.G. Sampedro and Y. Cataño

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Table of Materials

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August 12th, 2021

Dr. Lena von Voithenberg
DKFZ (German Cancer Research Center)
Guest Editor JoVE

Collection of video articles on Förster Resonance Energy Transfer (FRET): Methods and Applications.

Dear Dr. von Voithenberg

Manuscript JoVE62770_R1

Response to reviewers

Please enclosed find the **response to the Editor request** regarding the modified manuscript and video now entitled **"Chemical modification of the tryptophan residue in a recombinant Ca^{2+}-ATPase N-domain for studying tryptophan ANS FRET"**. The title was modified as suggested by Editorial office and a professional copyediting service from the original manuscript and video "**Chemical modification of the tryptophan residue in a recombinant SERCA N-domain elucidates FRET between tryptophan and 8-anilinonaphthalene sulfonate**" by José G. Sampedro and Yolanda Cataño. Therefore, this is a modified manuscript and video being submitted for review and eventual publication in Journal of Visualized Experiments (JoVE).

The manuscript was modified as requested in the word formatted manuscript provided. After that, the manuscript was proofread (American English) by the ACS Copyediting Services (only few modifications were rejected); a certificate of the service is attached. In regard to the video, please note that this was also modified as requested. Importantly, in the protocol section, new videos were included aiming to describe and visualize in detail each step of the protocol and to homogenize the text in the manuscript with that in the video, e.g., materials, reagents, volumes of liquids, as well as the action sequence now are clearly visualized. Further, it was included a Results section (as requested) that summarize the relevant data obtained, this leads to easily drawn a conclusion and makes

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clear the importance of the protocol.

We hope the paper and video content now may fulfill the requirements for publication in JoVE.

My suggested reviewers with expertise in the field are:

1. Dr. Andrea Hawe – Leiden University.
   E-mail: ahawe@chem.leidenuniv.nl. Fax: +31 71 527 4565

2. Dr. Jaroslava Miksovska – Florida International University.
   E-mail: miksovsk@fiu.ed. Tel.: +1 305-3487406

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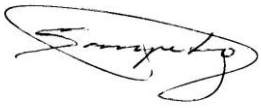

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RESPONSE TO REVIEWERS

Editorial comments:

Manuscript:

1. The editor has formatted the manuscript to match the journal's style. Please retain.

> **Re:** The Journal style in the formatted manuscript was not modified.

2. The manuscript needs thorough proofreading. Please employ professional copyediting services.

> **Re:** The manuscript was proofread by the ACS copyediting services. A certificate of the service is attached.

3. Please address specific comments marked in the manuscript.

> **Re:** The requested modifications marked in the manuscript were addressed; a list of the performed changes is provided below.

Video:

1. Please ensure that the narration in the video is homogenous to the text manuscript.

> **Re:** Now the narration in the video is homogeneous and mostly matches to the text in the manuscript.

2. Please include a separate representative result section and include and describe all the results figure there.

> **Re:** A separate representative result section now is included and briefly described (10:33).

3. Please ensure that the narration matches the text being shown in the video. When saying add this solution… show the tube with the label what is being added to the cuvette.

Here are a few examples:
4:07: The narration talks about vortexing but the video is not showing it. Please ensure that the narration and the video matches throughout.
5:06 the narration says 100 microliters of the solution to be added but the added solution is 1 mL. Please check.
5:20: The narration says to gently homogenize but the talent is wiping the cuvette here.

> **Re:** In the video, now the narration and scenes match closely the text of the protocol. Please note that new videos were included in order to homogenize the text in the manuscript with that in the video. Materials, reagents, volumes of the liquids and materials now match well to those described in the protocol.

4. Title Cards:
• Please remove the Periods i.e. after the word "Domain".
• Please remove the Periods i.e. after "NBS".
• Please remove the Periods i.e. after "25 C"
• Please add a standalone Conclusion chapter title card to the video.
• Please capitalize the first letter of every important word in your all Chapter title card.

> **Re:** The requested modifications were performed, e.g. deletion of periods and capitalizations of all important words. A standalone conclusion chapter title card now is included (11:31).

• There doesn't appear to be a Results. Please add a Results section and Results chapter title card to the video.

> **Re:** Now, both a Result section and a Result chapter title card is included (10:33).

5. Video Editing Content:

• 03:05 Please complete the narration before going to a new shot or Chapter.

Re: The narration now is complete.

6. Audio Editing and Pacing:

• Audio levels are low. Please increase the audio levels and ensure that the audio level peak average around -9 dB.

Re: Audio levels were increased and now peak around -9dB.

Editor comments in the manuscript:

A1: Title made concise. Please check and change in the video as well. The manuscript needs a thorough proofreading. Please employ professional copyediting services.

> **Re:** The title was changed in the video as well, as suggested. A professional copyediting service (ACS authoring services) was used to proofread the manuscript; a certificate of proofreading is included. Few modifications were rejected.
>
> https://secure.authoringservices.acs.org/en/certificate
>
> use the verification code **5474-2F79-C906-325C-4A2C**

A2: Please bring out clarity on the goal of the protocol being presented.

> **Re:** The abstract was slightly rewritten in order to provide clarity on the goal of the protocol presented.

A3: The pump function is the objective of current study? Please reword.

> **Re:** The phrase "which function is a matter of current study" was deleted to avoid confusion about the subject of the present study.

A4: Any amino acids or only Tryp?

> **Re:** All the interacting amino acids with ANS at the nucleotide binding site now are mentioned (lines 29-30).

A5: Please reword for clarity. ANS binds Tryp in the above situation? Please clarify that part.

> **Re:** The sentences were rewritten in order to provide clarity as suggested (lines 34-36).

A6: Is the modification mediated by NBS or is it in the NBS domain. Please clarify.

> **Re:** Clarification is provided now; the sentences were rewrite (lines 39-40).

A7: How is this probed? This needs clarity.

Re: To clarify, it was included the following sentence (lines 40-41): i.e. in the absence of Trp fluorescence no increase in ANS fluorescence should be observed.

A8: Like?

Re: An example is provided as follows (lines 62-63) e.g., affinity determination of small ligands in proteins.

A9: Please introduce this as well before taking about energy transfer.

Re: Done, please see the inclusion in line 61.

A10: What kind?

Re: The kind of conclusion now is stated in lines 70-71 as follows: for drawing accurate conclusions regarding ligand binding in protein structural studies.

A11: Please expand during the first time use.

Re: complete description of the acronym ANS now is included at first time of appearance (lines 73-74).

A12: How does proper distance relate to tryp binding. Please clarify.

Re: The sentence refers to proper distance for energy transfer not for ANS binding. To clarify the above the sentence was rewrite as follows (line 82-83):

> 2) the identification of a proper distance between Trp residue(s) and ANS for energy transfer,

A13: So protein pockets contain Tryp and not the aqueous solution?

Re: Clarification is provided by stating that ANS displays high quantum yield when bound to proteins as follows (line 83):

> 3) the high ANS quantum yield when bound in protein pockets,

A14: How does this related to Tryp? In line 77 you said that ANS interacts with Arg and His.

Re: The statement described in line 77 about ANS interaction with Arg and His residues as the source of ANS fluorescence is maintained. Hence part of the sentence was deleted to avoid confusion about the role of Trp on ANS fluorescence at this stage. The deleted sentence was the following:

> "the intrinsic fluorescence intensity of protein decreases, while the fluorescence intensity of ANS increases"

A15: Please explain this with respect the tryp position.

Re: Explanation is provided by including the words "(as ATP)" in line 92. That is, ANS binds similarly as ATP, hence the distance to Trp and more important the structural movements induced (is) are the same regardless of the bound ligand (ATP or ANS).

A16: Please make the narration and the text more homogenous. There can be steps in.

Re: Now, the narration in the video and the text in manuscript are more homogeneous.

A17: Please italicize *in silico* in the video.

Re: Now in the video *in silico* is italicized.

A18: How is this done?

Re: Now it is described how it is done, as follows (lines 128-129):

> 1.4. Calculate the molecular distance (Å) between Trp residue and bound ANS using the measurement tool in the preferred software.

A19: Something is not right here. Please check.

Re: missing letters were corrected.

A20: In the video please use the term "obtain" instead.

Re: The term obtain now is used in the video.

A21: Please check.

Re: same as in **A19.**

A22: Please check.

Re: same as in **A19.**

A23: Please include the actual permission obtained with the revision and upload it as supplementary file.

Re: The obtained permission now is included as supplementary file.

A24: This part needs to be in the introduction to clarify the rationale.

Re: The paragraph was moved to the introduction section and combined with the last two paragraphs to clarify the rationale as suggested (lines 98-102).

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