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Genetically Encoded and Boolean-Responsive FRET Biosensors

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Abstract

Genetically Encoded Boolean-Responsive FRET Biosensors

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The development of genetically encoded FRET-based biosensors incorporating Boolean logic operators offers enhanced signal detection abilities within biological complex systems. In this work, we take advantage of autonomously compiled molecular topology, linking FRET donor proteins to acceptor species through a series of protease recognition sites with defined connectivity. When intact, the species exhibit FRET; however, when proteolytically separated following user-specified protease input combinations, FRET decreases. Using these methods, we demonstrate a YES-gated reporter sensitive to Sortase 2A9 protease, as well as an AND-gated species that requires both 2A9 and Tobacco Etch Virus (TEV) proteases for complete response. Operation-matched proteolytic cleavage led to a preprogrammed decrease in FRET signaling, confirming input-specific responses. These findings establish a fundamental framework for next-

generation biosensors that integrate protein topologies and Boolean logic operators to enhance signal detection with high specificity.

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DEDICATION

To my wonderful family, extended family, and friends

Chapter 1. INTRODUCTION

1.1 BIOSENSORS

Biosensors are analytical tools that detect biomolecule properties and interactions within an environment which subsequently transduce into quantifiable signals for various applications, such as diagnostics, chemical biology, and environmental monitoring¹. The first biosensor, known as the Clark electrode, was developed by Leland C. Clark to monitor oxygen levels during surgery². Common biorecognition interactions such as antigen-antibody binding and enzyme-substrate recognition are used in modern biosensors to report disease states through distinct signal outputs. For example, the prostate-specific antigen (PSA) diagnostic test is used to detect prostate cancer. This test implements a sandwich-type enzyme-linked immunosorbent assay (ELISA) technique to identify these antigen-antibody interactions, producing a measurable signal characterized by a color change³.

1.2 FRET AND FRET-BASED BIOSENSOR APPLICATIONS

The implementation of Förster Resonance Energy Transfer (FRET)-based biosensors has become a commonly adopted approach to enhance the sensitivity and dynamic range of biosensing platforms. These advancements support the development of more effective diagnostic tools and a deeper understanding of disease mechanisms.

FRET is a nonradiative energy transfer process that occurs between a donor and acceptor fluorophores with partially overlapping absorption/emission that are in close proximity (typically 1-10 nm). The FRET efficiency (E) quantifies the proportion of energy successfully transferred from a donor to an acceptor. FRET efficiency is inversely proportional to the sixth power of the

distance between the donor and acceptor.

Furthermore, other factors including the spectral overlap of donor emissions and acceptor absorption (**Figure 1**), the orientation of their respective dipoles (κ^2), and the donor's quantum yield and acceptor's extinction coefficient also influence the FRET efficiency⁴.

FRET-based detection methods have been widely used across diverse biomolecular studies. Due to the requirement of nanometer scaled proximity between fluorophores, FRET enables the useful analysis for probing the structural and functional dynamics of biomolecules⁴.

A notable example is the development of transient time-resolved FRET [(TR)²FRET], which provided a new perspective into the actin-myosin contraction cycle. This approach revealed that actin initiates the power stroke prior to phosphate dissociation, challenging previous muscle contraction models⁵.

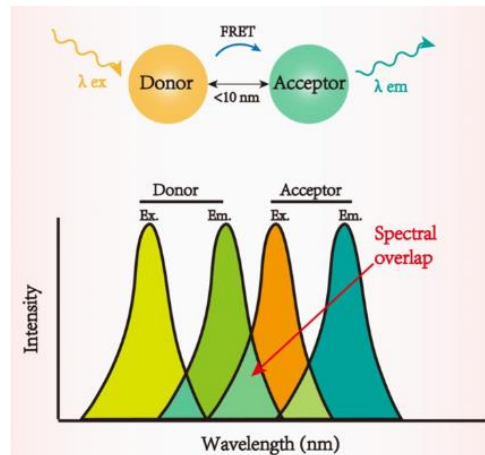


Figure 1. General Principles of FRET⁴

1.3 FRET BIOSENSOR LIMITATIONS

Despite their versatility, conventional FRET-based biosensors have several limitations. Biological environments are complex as numerous nonspecific molecular interactions can produce false-positive signals. Additionally, conventional FRET-based biosensors often lack the sensitivity of detecting target molecules in very small concentrations. FRET-based biosensors are commonly categorized as intramolecular and intermolecular, classically called Phogemon systems⁶.

Intramolecular FRET-based biosensors contain both donor and acceptor fluorophores within the same molecule, enabling conformational changes to induce a FRET response. In contrast, intermolecular biosensors place the donor and acceptor molecules on separate molecules, and the FRET signal arises only when these independent molecules come into close proximity⁷⁻⁸. Intramolecular FRET-based biosensors have been beneficial in live-cell applications due to their high signal-to-noise ratio and ratiometric imaging analytical properties⁹. However, the design of intramolecular and intermolecular biosensors is capable of single-input sensing only restricting their ability to detect multiple and more complex biological inputs⁸. Similarly, protease-based FRET biosensors, such as those that detect caspase-3 activity, also follow single-input architectures, further limiting detection versatility¹⁰.

To address these current limitations in single-input designs, our lab previously applied Boolean logical operators to manipulate and control molecular behaviors through the engineering of spatial arrangement of reactive elements within a macromolecule. This strategy allows the construction of biological structures that respond to a diverse range of environmental stimuli, including enzymes¹¹⁻¹², oxidative & reductive environments¹³, and light¹⁴⁻¹⁷.

With these logical-circuit based properties, we recently demonstrated the use of Boolean logic operators to enable biomolecular systems to respond within complex environments¹⁸⁻²⁰. Three common types of programmable logic gates are commonly used in designing such molecular circuits. The YES-gate is a linear construct that requires a single input to trigger a response. The multi-input AND-gate (denoted as $\wedge$) incorporates two inputs arranged in parallel, requiring both to be present for an effective response. In contrast, the OR-gate is designed for either of the two inputs to be present to produce a response (**Figure 2a-c**).

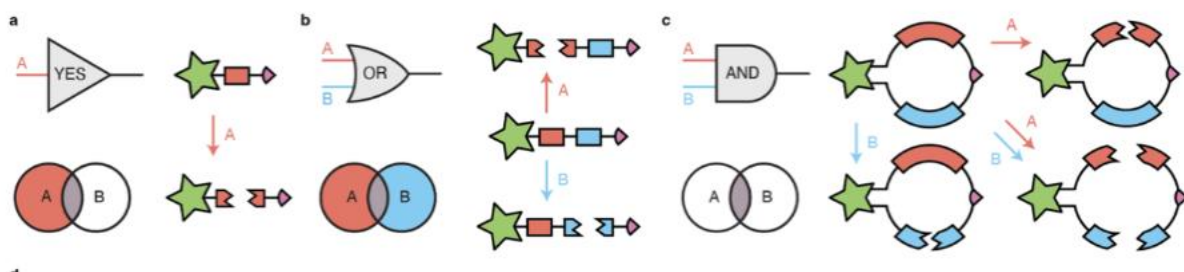


Figure 2. Schematic illustration of the YES-gate (a), OR-gate (b), and the AND-gate (c). Each logical circuit requires different inputs for a favorable response.

1.4 ADDRESSING CHALLENGES

In this work, we report the development of protein-based FRET biosensors capable of exercising YES and AND-type Boolean logic in response to precise protease inputs. The single-input YES gate is a linear protein construct in which EGFP and mScarlet3 are connected by flexible linkers and the Sortase 2A9 recognition site (**Figure 5a**). Upon exposure to Sortase 2A9, the peptide will be cleaved. As a result, the increased separation between the fluorophores will result in a decrease in FRET efficiency.

The multi-input AND gate follow similar design principles but requires the addition of Sortase 2A9 and TEV proteases to modulate signaling. This protein construct is generated using Split-Intein Circular Ligation of Peptides and Proteins (SICLOPPS²¹⁻²²), resulting in a covalently bonded cyclic protein (**Figure 3**). EGFP and mScarlet3 are connected by flexible linkers that contain the protease sites positioned in parallel (**Figure 6a**). This unique design enables single and multiple input proteolytic events to occur, allowing functional versatility of FRET biosensors in multi-faceted environments.

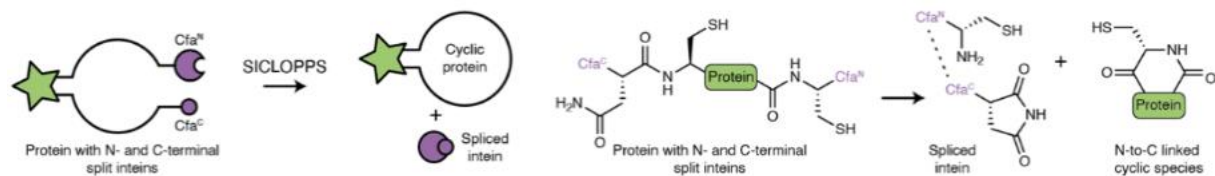


Figure 3. SICLOPPS mechanism in designing cyclic proteins

Chapter 2. EXPERIMENTAL METHODS

2.1 PLASMID CONSTRUCTION

The desired DNA sequences were ordered from GenScript (Piscataway, NJ) and cloned into the pET-29b(+) plasmid vector between the NcoI and BlnI restriction sites. To enable purification of both proteins, we installed a 6x-Histidine tag to facilitate immobilization via Ni-NTA-immobilized metal affinity chromatography (IMAC). The open reading frame (ORF) of each construct is shown below, with color-coded portions highlighting specific regions of the plasmid and their corresponding genetic sequences.

EGFP-mScarlet3 - A YES

MGSS-EGFP-GGS(x5)-2A9-GGS(x5)-mScarlet3-GGS(x5)-6xHis

ATGGGAAGTTCTATGGTATCAAAAGGGGAGGAATTGTTTACCGGCGTAGTTCCGATT
CTGGTCGAGCTGGATGGCGACGTGAACGGTCACAAATTCAGCGTGAGCGGTGAGGG
CGAGGGCGATGCAACCTACGGTAAATTGACCTTGAAGTTCATTTGTACCACCGGCAA
ACTGCCGGTGCCGTGGCCGACCCTCGTGACCACGTTGACCTACGGTGTTCAAGTGCTT
TAGCCGTTACCCGGACCACATGAAACAACATGATTTCTTCAAGAGCGCTATGCCGGA
AGGTTATGTTCAAGAACGCACCATTTTTTTTCAAGGACGATGGAACTACAAGACGCG
TGCGGAAGTTAAGTTTGAGGGCGACACTCTGGTAAATCGTATTGAGCTGAAGGGCA
TCGATTTCAAGGAGGACGGCAACATCCTGGGTCACAAGCTGGAGTATAACTATAAT
AGCCACAACGTGTATATCATGGCCGACAAGCAAAAAACGGTATCAAAGTTAACTT
CAAGATTCGCCATAATATTGAAGACGGCTCGGTGCAACTGGCGGACCACTACCAGC
AGAATACCCCGATCGGTGACGGTCCGGTCCCTGCGGATAACCATTACCTGTCCA
CGCAAAGCGCTCTGTGCAAAGATCCGAATGAAAAACGTGATCACATGGTTCTGTTG
GAGTTCGTGACTGCCGCAGGTATCACCCCTGGGTATGGATGAATTGTACAAGGGTGGC
AGCGGTGGATCTGGTGGCTCTGGTGGTAGTGGTGGCTCTCTGGCGGAAACCGGTGGC
GGTTCAGGCGGTTTCAGGTGGTTCGGGTGGCTCCGGAGGAAGCATGGATAGCACCGA
AGCTGTGATTAAAGAGTTCATGCGTTTTAAGGTTACATGGAAGGTTCCATGAACGG
CCACGAATTCGAGATCGAGGGTGAGGGGGAGGGTTCGTCGGTATGAAGGCACCCAGA
CGGCCAAATTGCGTGTTACCAAAGGTGGCCCTCTGCCGTTTAGCTGGGATATTCTGT
CCCCGCAGTTTATGTATGGCTCTAGAGCGTTACCAAACATCCGGCAGACATCCCGG
ACTACTGGAACAGTCCTTTCCGGAAGGCTTTAAGTGGGAACGTGTTATGAATTCG
AGGACGGCGGCGCGGTTAGCGTCGCGCAGGATAACCAGCCTGGAAGACGGCACCCCTT
ATCTATAAGGTGAACTGCGCGGTACGAACTTCCACCGGATGGCCCAGTTATGCAG
AAAAAACCATGGGTTGGGAAGCGAGCACTGAGCGCCTGTACCCGGAGGACGTTGT

CCTGAAGGGTGACATCAAGATGGCTTTGCGGCTGAAGGACGGTGGGCGTTACCTGG
CCGACTTCAAGACTACGTACCGCGCGAAAAAGCCGGTTCAAATGCCGGGTGCGTTT
AACATTGATCGTAAATTAGATATCACCTCTCACAACGAGGACTATACCGTCGTGGAA
CAGTATGAGCGCAGCGTGGCGCGTCATAGCACAGGTGGCAGCGGTGGCTCAGGTGG
TAGCGGTGGTTCCGGTGGGTCCCTCGAGCACCACCACCACCACCCTGAG

EGFP-mScarlet3 - A A T

MGSS-Cfa^c-Ext^c-GGS(x5)-EGFP-GGS(x5)-2A9-GGS(x5)-mScarlet3-GGS(x5)-TEVc-
GGS(x5)-6xHis-GGS(x5)-Ext^N-Cfa^N-SsrA-6xHis

ATGGGAAGTTCA GTAAAAATTATATCTAGGAAGAGTCTGGGTACGCAAAACGTTTA
CGACATCGGGGTCGAGAAGGACCACAACCTTCCTGCTGAAGAACGGCCTGGTCGCGA
GCAACTGCTTCAACGGTGGTTCCGGTGGTAGCGGTGGTCCGGTGGTAGCGGCGGTT
CCATGGTGAGCAAGGGTGAGGAGCTGTTTACCGGTGTGGTGCCGATCCTGGTCGAG
CTGGATGGCGATGTTAACGGCCACAAATTCTCCGTGAGCGGTGAGGGCGAAGGTGA
TGCAACGTACGGCAAACCTCACCTCAAGTTCATTTGTACCACCGGCAAACCTGCCGGT
TCCGTGGCCGACTCTGGTCACCACATTGACTTATGGTGTTCAATGTTTTAGTCGTTAT
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CAAGAACGTACCATTTTTTTTCAAAGATGATGGTAATTACAAGACCCGTGCTGAGGTG
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TGTACATCATGGCGGACAAGCAAAAAAATGGCATCAAAGTAACTTCAAGATCCGC
CATAACATTGAGGACGGTAGTGTCCAACCTGGCGGACCACTATCAGCAAAATACCCC
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CTTGAGCAAAGACCCGAATGAAAAACGTGATCACATGGTTCTCTTGAGTTTCGTGAC
TGCGGCCGGTATTACCTTGGGCATGGATGAATTATATAAAGGCGGTTCGGGCGGAA
GCGGTGGCTCTGGCGGTTCAAGGTGGTAGCCTGGCGGAGACGGGCGGCGGTAGCGGG
GGTTCCGGCGGCAGCGGGGGTTCCGGCGGGCTCGATGGACAGCACCGAAGCAGTTAT
TAAAGAATTCATGCGTTTTTAAAGTGCACATGGAAGGTTCAATGAACGGTCATGAATT
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TGCGCGTTACCAAGGGGGGTCCGCTGCCGTTAGCTGGGATATCCTAAGCCCGCAGT
TTATGTATGGTTCTCGTGCGTTTACCAAACATCCGGCAGATATCCCGGACTATTGGA
AACAGAGCTTTCGGAGGGGCTTCAAGTGGGAACGCGTGATGAATTTTGAGGACGGC
GGTGCGGTGTCCGTGGCCCAGGATACGAGCCTTGAGGACGGTACTCTAATTTATAAG
GTGAACTTAGAGGTACGAACTTCCCGCCTGATGGTCCGGTTATGCAGAAGAAAAC
CATGGGTGGGAAGCGTCCACTGAGCGTCTGTACCCGGAAGACGTGGTACTGAAGG
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AAGACCACGTATCGTGCGAAAAAACCAGGTGCAAATGCCGGGTGCATTTAATATTGA
TCGTAAACTTGACATTACTAGCCACAACGAAGACTACACGGTTGTCGAGCAGTATGA
ACGTAGCGTAGCCCGTCATAGCACCGGTGGCTCCGGCGGCTCTGGGGGCTCTGGTG
GTAGCGGCGGTAGCGGTGGTTCCGGCGGTTCTGAAAACCTGTATTTCCAGTCGGGCG
GGTCCGGTGGCTCTGGTGGTAGCGGCGGGAGCGGGGGTAGCCATCACCACCACCAC
CACGGCGGCTCCGGCGGCTCTGGTGGTAGCGGCGGTTCCGGCGGCTCTGCGGAATA
CTGCTTGAGCTATGATAACGAAATTCTGACCGTGGAATACGGTTTTCCTGCCGATCGG
TAAATTTGTTGAGGAACGCATTGAGTGCACCGTTTACACCGTTGATAAAAACGGCTT

TGTTTACACCCAACCGATCGCCCAATGGCACAATCGTGGTGAACAGGAGGTTTTTGA
GTACTGCTTGGAGGACGGCAGCATCATTCGTGCGACGAAGGACCACAAATTTATGA
CCACCGATGGTCAGATGCTGCCGATCGACGAGATCTTTGAACGCGGTCTGGATCTGA
AGCAGGTTGACGGGTTGCCGGCTGCTAATGATGAGAACTACGCACTGGCAGCCTAA
CTCGAGCACCACCACCACCACCT

2.2 PROTEIN EXPRESSION AND PURIFICATION

Plasmids were chemically transformed into Escherichia Coli BL21 competent cells (New England Biolabs, C2530H). Overnight small-scale cultures were prepared in 10mL LB media and 10 μ L of kanamycin. For large scale bacterial expressions, cultures were expanded in 500mL LB media at 37 °C in the presence of kanamycin until an optical density (600 nm) of 0.6-0.7 was reached. Protein expression was induced by addition of 0.5 mM isopropyl β -D-thiogalactopyranoside (IPTG) and incubated overnight at 18 °C.

Post-induction samples were centrifuged (4000 x g for 20 minutes at 4 °C). Pellets were resuspended in 40mL lysis buffer, 400 μ L of 100mM phenylmethanesulfonyl fluoride (PMSF) and sonicated on ice (30% amplitude and 33% duty cycle, 1s pulse on, 2s pulse off for 6 minutes). The total lysate bacterial samples were centrifuged (10,000 x g for 20 minutes at 4 °C) and filtered through a 0.20 μ m filter.

Protein purification occurred using Nickel-NTA-based IMAC on an ÄKTA Pure (Cytiva). The AND gate construct was further purified via size exclusion chromatography (SEC) to isolate cyclical constructs from SICLOPPS-related multimers. Purified proteins were spin-concentrated, flash frozen in liquid nitrogen, and stored at -80 °C. Based on visual inspection of band intensity, the target protein appeared to be >90% pure, with minor protein impurities likely resulting from conformational changes during protein folding and maturation. A linearized product was observed for the AND gate construct, likely resulting from N- to C- terminal cleavage rather than intein mediated trans-splicing.

2.3 PROTEIN VERIFICATION

Protein identities were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and liquid chromatography mass spectrometry (LC-MS) analysis (S2 and S4).

2.4 PROTEASE TREATMENT

Both protein constructs were incubated under identical buffer conditions (20 mM Tris, 50 mM NaCl, pH = 7.5, 1 mM DTT, 1 mM CaCl₂, 18 mM GGG) and treated with Sortase 2A9 and TEV protease at a 1:10 protease:operator molar ratio. Proteolytic cleavage reactions were carried out at 30 °C for 4 hours and confirmed with SDS-PAGE and LC-MS analysis (S3 and S5).

2.5 FRET ANALYSIS

Three technical replicates of 10-15 µL from each input condition of both constructs were pipetted into a Costar 96-well black polystyrene plate with an opaque bottom. Fluorescence measurements were acquired using a BioTek Synergy Microplate Reader (BioTek; Winooski, VT) with excitation and emission settings optimized for EGFP (donor fluorophore) and mScarlet3 (acceptor fluorophore). Excitation was performed at 479 nm, and emission was measured at 587 nm to determine the FRET signal. FRET efficiency was calculated as the ratio of EGFP to mScarlet3 fluorescence intensities and normalized to the average FRET efficiency of the untreated condition (S6).

Chapter 3. RESULTS

3.1 YES-GATE

A one-way ANOVA revealed no statistically significant difference in FRET efficiency between the 2A9-only condition and the 2A9 with TEV condition ($p > 0.05$), with average FRET efficiencies of 0.68 and 0.67, respectively. Both of these conditions were significantly different compared to the control group and TEV-only group ($p < 0.001$). No significant difference was observed between the control and TEV-only group ($p > 0.05$), with an average FRET efficiency of 1.00 ($p > 0.05$). These results support the hypothesis that the YES-gate construct selectively responds to Sortase 2A9 alone.

Complete FRET efficiency data for all input conditions are summarized in Table **(S6)** and shown in Figure **(5d)**. A schematic Illustration of Sortase 2A9-mediated cleavage is provided in Figure **(5a)**. Protein cleavage was further confirmed via LC-MS and SDS-page analysis **(Figure 5b, 5c, and S5a)**.

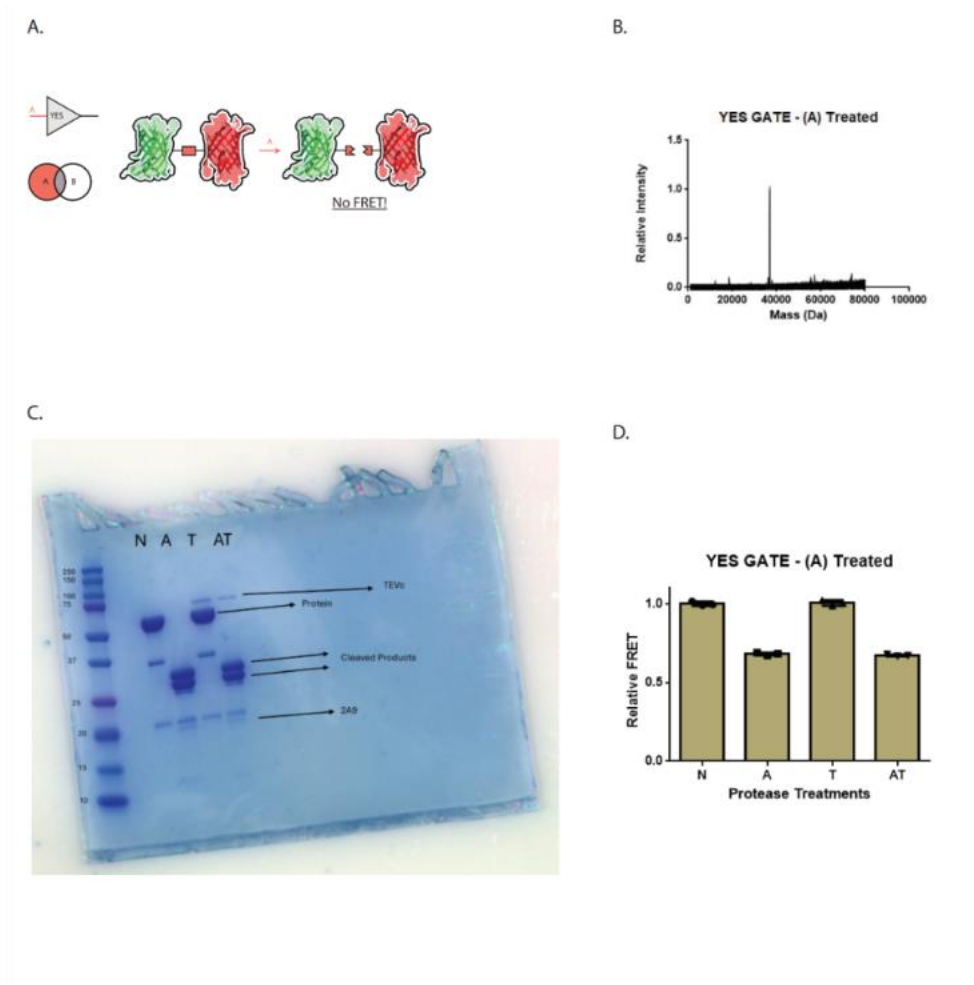


Figure 5. (A) Schematic illustration of the YES-GATE construct. No FRET signal occurs upon Sortase 2A9 treatment. (B) Mass spectrometry analysis of the YES-GATE construct confirming mass after protease treatment. (C) SDS-PAGE analysis of protease cleavage conditions: lanes A, T, A&T correspond to the Sortase 2A9 only, TEV only, and the combination of Sortase 2A9 and TEV. (D) Relative FRET efficiency across various protease treatments, showing selective response towards Sortase 2A9.

3.2 AND-GATE

A one-way ANOVA revealed statistically significant differences between all groups ($p < 0.0001$). The 2A9 with TEV condition had the lowest average FRET efficiency of 0.64, while the TEV-only condition had the highest average FRET efficiency, of 0.91, among the treated groups ($p < 0.0001$). The 2A9-only group showed an average FRET efficiency of 0.77 ($p < 0.001$). The results suggest complete cyclic protein cleavage in the 2A9 with the TEV group, as indicated by its lowest average FRET efficiency compared to the other conditions.

Complete FRET efficiency data for all input conditions are summarized in Table **(S6)** and shown in Figure **(6d)**. A schematic Illustration of Sortase 2A9-mediated cleavage is provided in Figure **(6a)**. Protein cleavage was further confirmed via LC-MS and SDS-page analysis (**Figure 6b, Figure 6c, and S5b**).

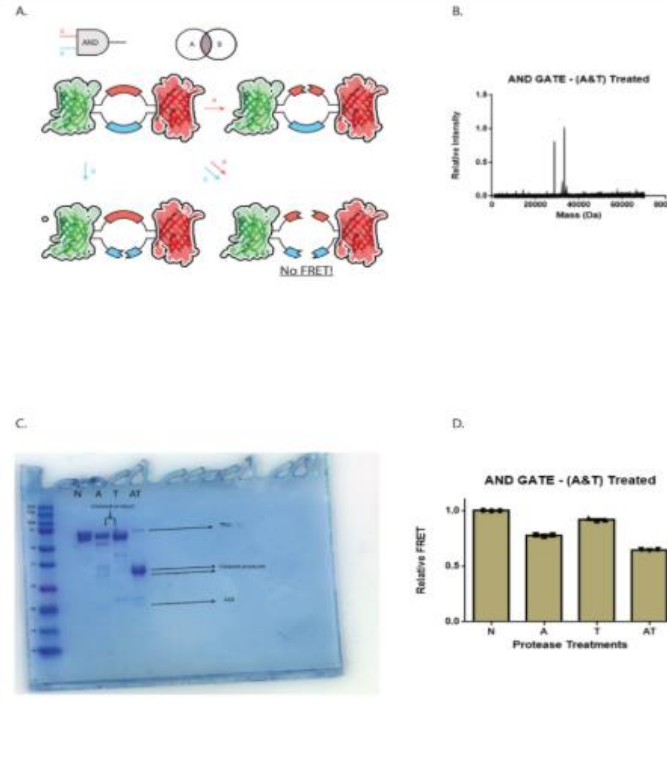


Figure 6. (A) Schematic illustration of the AND-GATE construct, which modulates FRET signals in response to different protease inputs. (B) Mass spectrometry analysis of the AND-GATE construct following treatment with both confirming mass after protease treatment Sortase 2A9 and TEV. (C) SDS-PAGE analysis of protease cleavage conditions: lanes A, T, A&T correspond to the Sortase 2A9 only, TEV only, and the combination of Sortase 2A9 and TEV. (D) Relative FRET efficiency across various protease treatments, demonstrating various responses depending on input conditions.

Chapter 4. DISCUSSION

This paper demonstrates the possibility of designing highly sensitive and multiplexed FRET-based biosensors through Boolean logic implementation. By embedding logical operators into protein constructs, our approach allowed selective responses to both single and combinatorial protease inputs. Specifically, the YES-gate construct exclusively responded to Sortase 2A9, while the AND-gate design required the presence of both Sortase 2A9 and TEV. Noticeable decreases in FRET efficiency were observed, laying the groundwork for development of next-generation biosensors with programmable topologies and a heightened input specificity.

Future work will explore the expansion of diversifying various logical circuit architectures to further advance detection strategies. Incorporating gates such as OR and NOR gates can improve the ability to suppress undesired signals. Furthermore, the combination of nested Boolean gates, such as YES-AND or AND-OR, may offer more robust and sophisticated protein circuit architectures that can yield expanded capabilities in signal production and versatility across complex biological environments. However, increasing design complexity comes with limitations. As the molecular architecture of the biosensor grows, the distance between fluorophores may increase, potentially reducing FRET efficiency in the absence of the intended stimulus.

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