

Genetically Encoded FRET Biosensor Detects the Enzymatic Activity of Prostate-Specific Antigen

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Abstract: Prostate cancer is the most common cancer among men beyond 50 years old, and ranked the second in mortality. The level of Prostate-specific antigen (PSA) in serum has been a routine biomarker for clinical assessment of the cancer development, which is detected mostly by antibody-based immunoassays. The proteolytic activity of PSA also has important functions. Here a genetically encoded biosensor based on fluorescence resonance energy transfer (FRET) technology was developed to measure PSA activity. In vitro assay showed that the biosensor containing a substrate peptide 'RLSSYYSGAG' had 400% FRET change in response to 1 µg/ml PSA within 90 min, and could detect PSA activity at 25 ng/ml. PSA didn't show enzymatic activity toward the biosensor in serum solution, likely reflecting the existence of other inhibitory factors besides Zn²⁺. By expressing the biosensor on cell plasma membrane, the FRET responses were significant, but couldn't distinguish well the cultured prostate cancer cells from non-prostate cancer cells under microscopy imaging, indicating insufficient specificity to PSA. The biosensor with the previously known 'HSSKLQ' substrate showed little response to PSA in solution. In summary, we developed a genetically encoded FRET biosensor to detect PSA activity, which may serve as a useful tool for relevant applications, such as screening PSA activation substrates or inhibitors; the purified biosensor protein can also be an alternative choice for measuring PSA activity besides currently commercialized Mu-HSSKLQ-AMC substrate from chemical synthesis.

Keywords: Prostate-specific antigen; fluorescence resonance energy transfer; serine protease; biosensor; prostate cancer

1 Introduction

Prostate cancer has exerted big challenge to the health of men, ranked as the first in incidence and second in mortality among all cancers [1]. To meet the clinical demands, there have been accumulating efforts in developing approaches for screening, diagnosis, and prognosis of this cancer [2]. Prostate-specific antigen



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(PSA) is secreted by the prostate gland which concentration in seminal fluid can reach about 0.5–2 mg/ml, and the serine protease activity of PSA can also cleave proteins to help regulate cell migration [3–6]. Since 1980s, PSA concentration in serum has been applied as a biomarker for assessing prostate cancer [7,8], possibly due to the weakened prostate architecture by the cancer to cause PSA leakage into the circulation system [9]. Based on data from American Cancer Association in 2016, men have about a 1 in 4 chance of having prostate cancer with serum PSA level between 4 and 10 ng/ml, and an over 50% chance above 10 ng/ml [10].

The current major measurement for PSA concentrations is antibody-based immunoassays which can provide specificity and sensitivity [11–13]. At the same time, clinical feedbacks indicated that PSA index alone has its limitation, such as in over-diagnosis and over-treatment, and lacking of telling the aggressive prostate cancers from localized ones [14,15]. As a serine protease, the proteolytic activity of PSA has important function in liquefying the semen and releasing the motile spermatozoa [16,17]. From studies, PSA activity also showed a surprising role in anti-angiogenesis and anti-tumorigenesis [18–21], which has led to the development of substrate peptides to stimulate PSA activity for prostate cancer treatments [20,22–24]. Among inhibitors, Zn^{2+} has been shown to have a high potent in inhibiting PSA protease activity in semen and *in vitro* [16,25].

Methods have been applied to measure PSA proteolytic activity, such as fragmentation of substrate proteins from cleavage, and fluorometry using 4-morpholinecarbonyl-HSSKLQ-AMC as a substrate [26]. Here we added a new fluorescence assay for measuring PSA activity based on fluorescence resonance energy transfer (FRET) technology. Non-genetically-encoded biosensors based on FRET technology have been reported to detect PSA protein concentrations, which were mostly based on combinatory applications of nanoparticles or quantum dots with antibody immunoassay [27–29], or aptamers for selective binding of PSA protein [30,31]. Hybrid microarray chips with FRET technology have been reported to measure PSA activity, which were double conjugated with polymers as energy donor and dye-labeled PSA substrate peptides as energy acceptor [32].

FRET technology has been well applied in studies of biomechanics and immunology [33,34]. In this work, the genetically encoded FRET biosensor for PSA activity could easily be produced from bacterial culture in the lab [35], which contains a substrate peptide ‘RLSSYYSGAG’ previously characterized through substrate phage display selection [36]. The FRET assay can be done conveniently at one step by scanning the sample under a fluorescent plate reader. The biosensor showed 400% FRET change in response to 1 μ g/ml PSA in solution, and could detect PSA activity at 25 ng/ml. This genetically encoded biosensor can detect the activity on live cells as well, although the FRET response of the current version couldn’t distinguish cultured prostate cancer cells from non-prostate cancer cells due to insufficient serine-protease specificity. Our tests with serum also suggested the existence of other inhibitory factors in serum (likely the reported α 1-antichymotrypsin and α 2-macroglobulin) for PSA activity besides Zn^{2+} [17]. This work provided an alternative approach to measure PSA activity or study PSA activity-relevant biology. We just noted that a FRET-based PSA biosensor had been reported in 2005 (unavailable for the English version) with similar design strategy [Qi, X., Wang, Z., Cai, Z., et al. Chinese Journal of Pharmacology and Toxicology, 2005(3)].

2 Results

2.1 Development of a Sensitive FRET Biosensor to Measure PSA Activity

PSA is a serine protease whose activity has important biological functions *in vivo*. We designed a genetically encoded biosensor based on FRET technology to measure PSA activity. As shown in Fig. 1A, the biosensor protein consists of a cyan fluorescent protein (ECFP), or sfCyPet (a mutant of ECFP, not suitable for mammalian expression) at the N-terminus followed with a PSA substrate peptide ‘RLSSYYSGAG’, and a yellow fluorescent protein YPet at the C-terminus. As demonstrated in the literature [36], the cleavage site in the substrate by PSA enzymatic activity is after SSYY residues

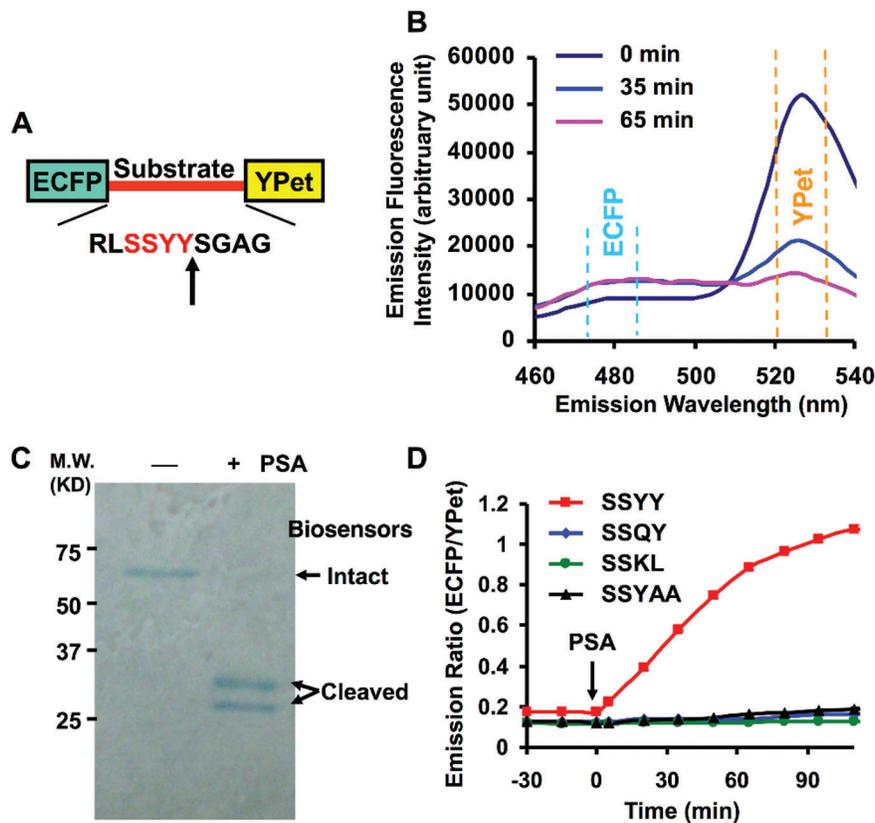


Figure 1: *In vitro* characterization of the PSA biosensor. (A) The design of the biosensor consisting of ECFP (or sfCyPet), a substrate ‘RLSSYYSGAG’, and YPet. The arrow indicates the cleavage site in the substrate by PSA. (B) The emission spectrum changes of the biosensor (1 μ M) excited at 427 nm when incubated with 1 μ g/ml PSA in solution. The two emission peaks are labeled as ECFP and YPet. (C) The biosensor protein was resolved on SDS-PAGE gel after incubation with or without 1 μ g/ml PSA in solution. The intact biosensor was cleaved into two fragments by PSA. (D) The time courses for the FRET responses of the biosensor with different substrates (SSYY, SSQY, SSKL, or SSYAA) when incubating with 1 μ g/ml PSA in solution

(indicated by the arrow in Fig. 1A). The biosensor was subcloned into the bacterial expression vector pRSETb (Invitrogen) for protein expression and purification.

In vitro characterization of the biosensor demonstrated a rapid and dramatic FRET change to commercially purchased PSA protein in solution (Fig. 1B). The biosensor displayed a dominant YPet emission around 526 ± 10 nm when excitation of ECFP at 427 nm, which indicated a highly efficient energy transfer between the two fluorophores. Within 35 min after addition of 1 μ g/ml PSA protein in solution, a big decrease of YPet emission occurred along with an increase of ECFP emission, indicating reduced FRET efficiency upon PSA cleavage of the biosensor protein. This was confirmed by resolving the biosensor protein on SDS-PAGE gel, which showed the cleavage of the intact biosensor into two similar-size fragments after incubation with PSA (Fig. 1C). These results demonstrated that the designed biosensor had high FRET response to PSA activity.

As quantification shown in Fig. 1D, upon with addition of 1 μ g/ml PSA, the biosensor containing the SSYY substrate had FRET ratio (ECFP/YPet) shift from ~ 0.2 to 1.0 in 90 min, indicating $\sim 400\%$ change in FRET response. Once the ‘RLSSYYSGAG’ substrate (SSYY) was changed to ‘RGGISSQYIV’ (SSQY) or

‘RLSSYAAGAG’ (SSYAA), there was almost no FRET response during the *in vitro* assay (Fig. 1D), indicating the preference of PSA activity to the SSYY substrate. When replacing the SSYY substrate with previously reported substrate ‘HSSKLQ’ plus a short linker ‘GGG’ behind, the SSKL version didn’t show FRET response to PSA enzymatic activity (Fig. 1D). Taken together, the SSYY peptide is a sensitive substrate of PSA activity for construction of the FRET biosensor.

We further characterized the biosensor sensitivity in detecting different PSA concentrations. As shown in Fig. 2A, the biosensor displayed significant FRET responses to PSA at the concentrations of 100–400 ng/ml,

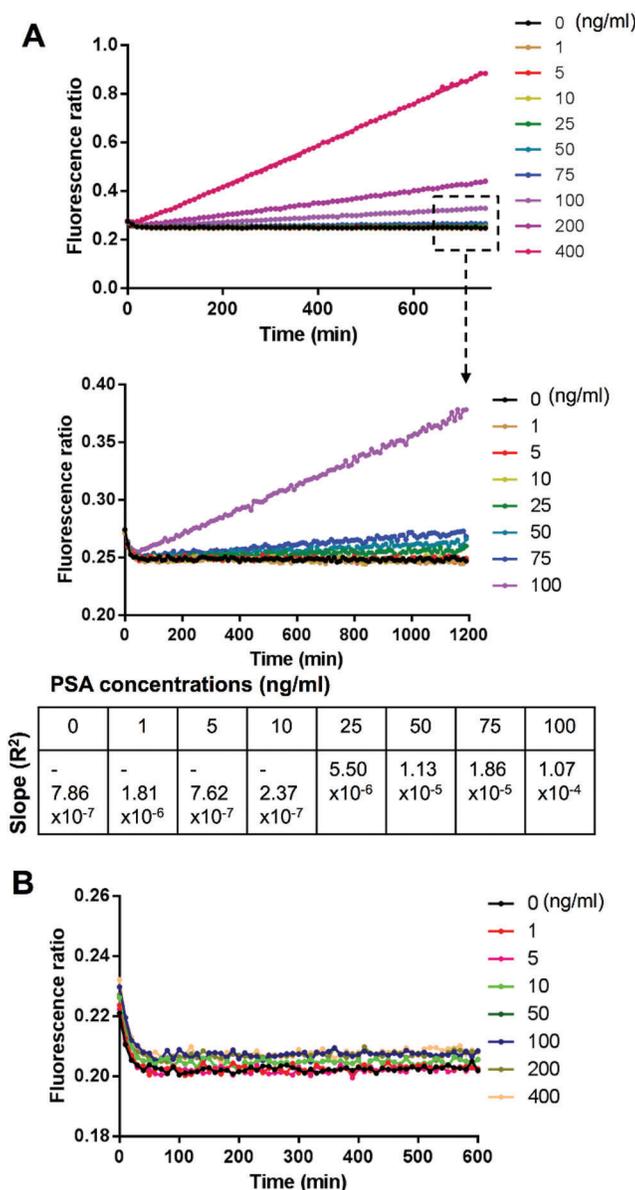


Figure 2: The sensitivity check of the biosensor to different PSA concentrations. (A) The FRET responses (emission ratio of ECFP/YPet) of the ‘SSYY’ biosensor to different PSA concentrations (1–400 ng/ml) in solution. The curves of FRET responses to 1–100 ng/ml PSA were further enlarged to indicate the less significant changes, and the corresponding slope factor R^2 of the curves were listed in the table. (B) The non-response of the ‘SSKL’ biosensor to the different PSA concentrations (ng/ml) in solution

and still reserved detectable reaction to 25 ng/ml PSA, as measured by the calculated slope factors (R^2) on the FRET change curves. Consistently with the result in Fig. 1D, the SSKL version didn't have FRET responses to different concentrations of PSA in solution (Fig. 2B).

2.2 Inhibited PSA Activity in Serum

In considering that serum PSA is an important biomarker in clinical applications, we tested the FRET response of the biosensor in serum environment. In commercially purchased fetal bovine serum (FBS), the biosensor didn't have obvious FRET response to the added PSA at the concentrations of 1–400 ng/ml (Fig. 3A), in contrast to the responsive results in non-serum solution (Fig. 2A). Since Zn^{2+} has been reported to inhibit PSA activity, 1 or 10 mM EDTA was added to chelate Zn^{2+} in FBS, but there was still no obvious FRET response to 1 μ g/ml PSA (Fig. 3B). Similar tests were done in healthy men serum supplemented with 1 μ g/ml PSA with or without adding 10 mM heavy-metal chelator EDTA, and no obvious FRET response was recorded (Fig. 3C). These results suggest the existence of other strong inhibitory factors besides Zn^{2+} in serum for PSA activity, maybe like the reported α 1-antichymotrypsin and α 2-macroglobulin [17].

2.3 FRET Responses of the Biosensor in Prostate and Non-prostate Cancer Cells

PSA is produced mostly by prostate cells, and also found at low-level expression in some other tissues [37]. We tried to test whether the developed biosensor was able to detect the cultured prostate cancer cells. The biosensor was positioned onto the outer surface of cell plasma member, which allowed the biosensor to be accessible by the extracellular proteolysis (Fig. 4A). LNCaP and DU145 are two human prostate cancer cells and well applied into research studies [38]; HeLa is human cervical epithelial cancer cells lacking PSA expression, which was examined as non-prostate cancer cells [39].

LNCaP and DU145 cells expressing the biosensor on the surface had significantly higher FRET levels than those cells expressing the biosensor in the cytosol (Figs. 4B and 4C), indicating high proteolytic activity on the cells. However, HeLa as non-prostate cancer cells also showed similar high FRET response as LNCaP and DU145 (Figs. 4B and 4D). Regarding the possible effect from the serum-containing medium, the FRET responses were also recorded from cells cultured in low-serum medium (0.5% FBS), which didn't showed big difference to those in 10% FBS medium (Fig. 4D). In considering the high FRET response in HeLa cells and reportedly lacking PSA expression in DU145 cells [40], the current version of the biosensor didn't have sufficient specificity to PSA activity besides its high sensitivity.

3 Discussion

Prostate cancer occurs at high frequency in incidences among men population [1]. The studies on prostate cancer-relevant projects have also been intensive in the past decades, for instance, scientific publications which titles contain 'prostate-specific antigen' or 'prostate-specific membrane antigen' are counted in the number of ~three hundred every year from 2016 to 2019 in the database of Pubmed, among which ~220 papers are from 'prostate-specific antigen'. PSA level in blood has been a routine clinical biomarker to evaluate prostate health and the cancer progress. To well understand the multiple aspects of PSA biology can be of help for both clinical and academic merits.

Here we developed a genetically encoded FRET biosensor to measure PSA protease activity. The biosensor displayed high sensitivity with 400% FRET change within 90 min in response to 1 μ g/ml PSA, and could detect 25 ng/ml PSA (Figs. 1D and 2A). PSA also showed selective activity toward the 'SSYY' substrate, and change of the peptide to 'SSQY' or 'SSYAA' resulted in almost no response to PSA (Fig. 1D). The biosensor displayed gradually enhanced FRET reaction speeds along with the increase of PSA concentrations in solution from 25 to 400 ng/ml (Fig. 2A). Hence, this biosensor is

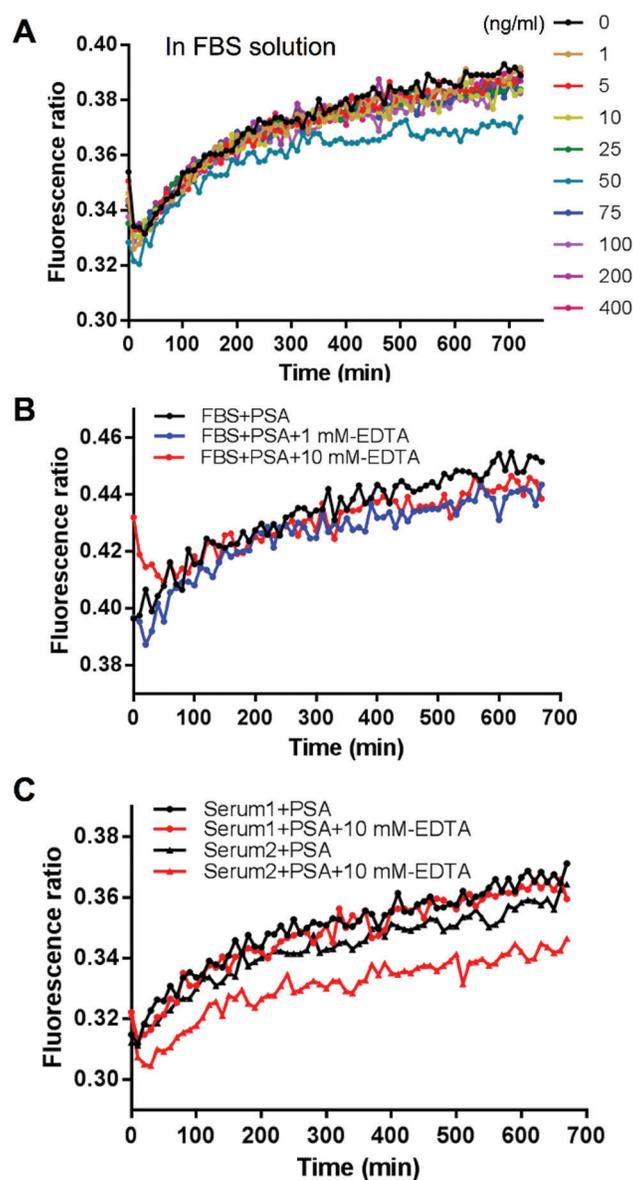


Figure 3: The non-response of the biosensor to PSA in serum environments. (A) The FRET responses (emission ratio of ECFP/YPet) of the biosensor to PSA (1–400 ng/ml) in fetal bovine serum (FBS) solution. The slight response might be due to other enzymatic activity from serum contents. (B) The FRET responses of the biosensor to 1 µg/ml PSA in FBS with or without adding the heavy-metal chelator EDTA (1 mM or 10 mM). (C) The FRET responses of the biosensor to 1 µg/ml PSA in serum samples from two healthy men with or without adding EDTA (10 mM)

capable of detecting PSA activity in solution and also measuring the relative level of PSA activity based on the speed of FRET response.

Although this biosensor had an impressive FRET response (with 400% change within 90 min) to 1 µg/ml PSA, the detectable PSA concentration at 25 ng/ml is far higher than the pg/ml-measurable scale by the antibody-based immuno-assays [11]. However, the activity-based assay can still be valuable, for example, this FRET technology-based method provides a convenient way for studying PSA activity-relevant topics,

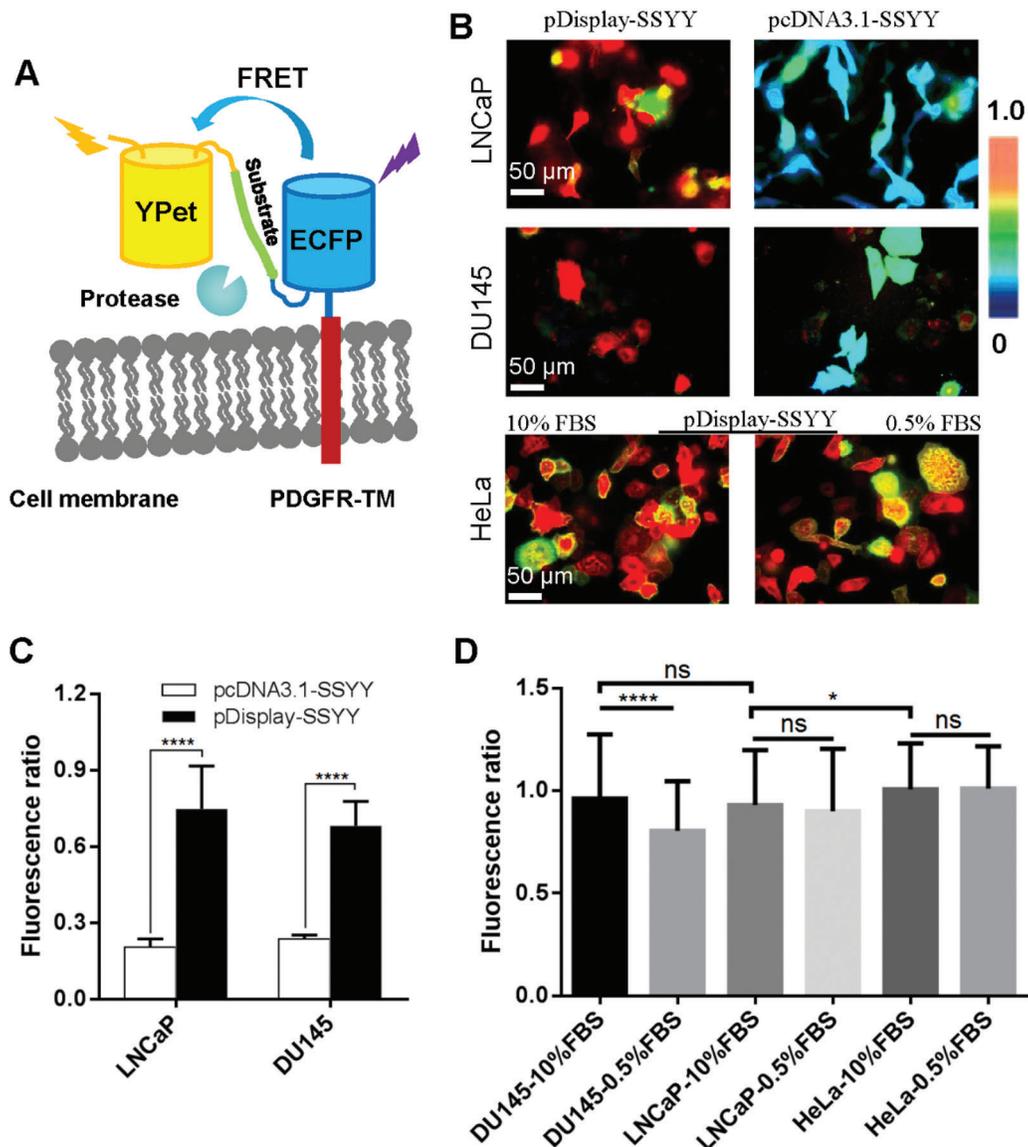


Figure 4: The FRET responses of the PSA biosensor in prostate cancer cells. (A) The diagram illustrates the PSA biosensor located on cell membrane and accessible to extracellular protease activity. The biosensor is positioned on the membrane by PDGFR-TM, and cleavage of the substrate peptide by protease would cause separation of ECFP and YPet, and result in a decrease of FRET efficiency. (B) The representative ratiometric (ECFP/FRET) images of PSA biosensor (SSYY) on different types of cells. The biosensor in pDisplay vector was expressed on the cell membranes, and pcDNA3.1 version within the cell cytosol. LNCaP and DU145 are human prostate cancer cells, and HeLa is human cervical cancer cells. (C) The quantified FRET ratio (ECFP/FRET) of the SSYY biosensor (in pDisplay or pcDNA3.1 vector) in the different cell types. For sample sizes, N = 99, 120 (pDisplay version), and 122, 100 (pcDNA3.1 version) for LNCaP, DU145 groups respectively. (D) The quantified FRET ratio (ECFP/FRET) of the SSYY biosensor expressed on the cell membrane in the different cell types, which were cultured in 10% FBS or 0.5% FBS medium for ~24 h before imaging. For sample sizes, N = 120, 182, 137, 102, 62, 43 for DU145, LNCaP, and HeLa groups in 10% and 0.5% FBS culture medium respectively. Due to the weak fluorescence expression in DU145 cells, the excitation light was stronger for DU145 than for LNCaP and HeLa cells during imaging. The data on these bar graphs are expressed as mean ± S.D. * and **** refer to *p*-value < 0.05 and 0.0001, and ‘ns’ refers to *p*-value > 0.05 in statistical analysis from Student’s *t*-test

such as screening activation substrates or inhibitors of PSA, and peptide designs in regulating PSA activity for anti-angiogenesis and anti-tumorigenesis.

This genetically encoded FRET biosensor protein can easily be produced from bacterial culture, and the protease activity can be read out from FRET response at one step under the fluorescent plate reader [41]. Hence, this biosensor protein provides an alternative choice for detecting PSA activity besides the currently commercialized Mu-HSSKLQ-AMC or Mu-HSSKLQ-AFC substrate (Sigma) for fluorometry measurement, which requires multi-step chemical synthesis.

Studies at the cellular level indicated that the FRET responses of the biosensor couldn't well distinguish the cultured prostate cancer cells from non-prostate cancer cells. The insufficient specificity of this biosensor didn't reach one wished possibility to detect free prostate cancer cells in the circulation system by flow cytometry technology. Actually, some lab studies have tried to establish the technique for attachment of fluorescent biosensor to cells for screening of cancer cells [42–44]. Further improvement of the biosensor specificity for PSA activity at cellular levels would be demanded for the detection of prostate cancer cells.

In summary, we have developed a genetically encoded FRET biosensor to measure PSA activity, which can be a useful tool for studying PSA-relevant biology or for applications in the field of prostate health. Regarding the relatively easy way to produce the biosensor protein and to measure the FRET response, this biosensor development provides an alternative choice besides the conventional Mu-HSSKLQ-AMC substrate for PSA activity measurement.

4 Materials and Methods

4.1 Reagents, Cell Culture and Transfection

The PSA protein for *in vitro* assays was purchased from Sigma. Human cervical epithelial cells (HeLa) and human prostate cancer cells LNCaP and DU145 were cultured in 90% basal culture medium (Gibco) with 10% fetal bovine serum (Gibco) in an incubator maintaining 5% CO₂ and a temperature at 37°C. HeLa cells were cultured in DMEM-high glucose medium (supplemented with NaHCO₃ 1.5 g/L), and LNCaP and DU145 in RPMI-1640 medium (supplemented with NaHCO₃ 1.5 g/L, glucose 2.5 g/L, and Sodium Pyruvate 0.11 g/L). The biosensor DNA was transfected into the cells by Lipofectamine 3000 reagent (Thermo Fisher Scientific). After transfection for 36 h, cells were detached by Accutase solution (Sigma) in PBS, and transferred to confocal glass-bottom dishes (Corning) coated with 10 µg/mL Fibronectin, and the cell imaging experiments were performed 12–16 h later.

4.2 Constructions of the FRET Biosensors

The PSA FRET biosensor was constructed by ligation of ECFP fragment, the substrate-YPet fragment into pRSETb vector by using BamHI, SacI and EcoRI sites for bacterial expression. To anchor the biosensor on the surface of cell plasma membrane, YPet fragment and the substrate-ECFP fragment were ligated into pDisplay vector by using BglII, SacI and PstI sites. The synthesized biosensor protein from pDisplay vector consists of an Ig k-chain leader sequence at the N-terminal to lead the protein into the secretory pathway in cells, and a PDGFR (platelet-derived growth factor receptor) transmembrane domain (PDGFR-TM) at the C-terminal to anchor the biosensor on cell membrane (Invitrogen). To express the biosensor in cell cytosol, the 'ECFP-substrate-YPet' fragment was subcloned into pcDNA3.1 vector using BamHI and EcoRI sites. Four substrates were applied in the DNA constructions, including 'RLSSYYSGAG', 'HSSKLQGGG', 'RGGISSQYIV', and 'RLSSYAAGAG' [26,36].

4.3 Purification of the FRET Biosensor and *in vitro* Assay of PSA Protease Activity

The method for expression and purification of the biosensor proteins, and the protocol for *in vitro* assay were described in our previous works [35,41]. Briefly, the biosensor DNA in pRSETb vector was transformed into the *E. coli* bacteria BL21, and the protein expression was induced by 0.4 mM IPTG

(Isopropyl beta-d-1-thiogalactopyranoside, Sigma) in LB medium cultured at room temperature for 16–20 h. The collected bacteria were lysed into B-PER Bacterial Protein Extraction Reagent (Thermo Scientific). The biosensor protein containing a 6xHis tag at the N-terminal was purified by nickel chelating chromatography (HisPur Ni-NTA agarose resin from Thermo Scientific). The biosensor protein was further dialyzed into phosphate-buffered saline (PBS) solution, and the BCA protein assay kit (Takara) was used to measure the final protein concentrations.

In vitro characterization of the biosensor in response to PSA protease activity was conducted with a fluorescence plate reader (TECAN) at 37°C. The biosensor protein was diluted as 1 μM into the buffer 50 mM Tris-HCl and 0.1 M NaCl (pH 7.8) supplemented with PSA protein at designed concentrations. Under the fluorescence plate reader, ECFP in the biosensor was excited at 427 nm, and the emission spectrum between 460 and 540 nm was recorded every 5–10 min along the time courses. The emission ratio at 478/528 nm in corresponding to the ECFP and YPet emission peaks was quantified as FRET efficiency of the biosensor.

4.4 Microscopy Imaging and FRET Quantification in Cells

The FRET microscopy (Zeiss) was equipped with X-Y-Z control stage for multiple-position imaging, fine auto-focusing function for time-lapse imaging, and temperature-CO₂ control chamber for maintaining cell culture conditions. Specifically, for FRET imaging, the ECFP and FRET channels were switched automatically by the software-controlled dichroic rotator. The parameters for the excitation filter and dichroic mirror of both channels, emission filter of ECFP channel, and emission filter of FRET channel are 436 ± 20 nm, 455 nm, 480 ± 40 nm, and 535 ± 30 nm respectively. The 40x oil objective (Zeiss) was used for acquiring the FRET images. The intensity from the X-Cite light source (Lumen Dynamics) was reduced to 1/16 for sample excitation during the imaging, so as to largely avoid photo-bleaching of the fluorescent proteins.

For FRET quantification, the ECFP and FTET images were processed and quantified by the image analysis software package Fluocell, which was developed in MATLAB and is available for downloading from Github (<http://github.com/lu6007/fluocell>). The statistical analysis software GraphPad Prism 6 was used for data analysis, and the FRET quantification from a group of cells was expressed as mean ± S.D. In this work, the Student's t-test was applied to measure statistical difference between groups with *p*-value < 0.05 as significant difference.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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