A Genetically encoded BRET-based SARS-CoV-2 Mpro protease activity sensor

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Abstract

The SARS-CoV-2 main protease, M^{pro}, is critical for its replication and is an appealing target for designing anti-SARS-CoV-2 agents. In this regard, a number of assays have been developed based on its cleavage sequence preferences to monitor its activity. These include the usage of Fluorescence Resonance Energy Transfer (FRET)-based substrates in vitro and a FlipGFP reporter, one which fluoresces after M^{pro}-mediated cleavage, in live cells. Here, we have engineered a pair of genetically encoded, Bioluminescence Resonance Energy Transfer (BRET)-based sensors for detecting SARS-CoV-2 M^{pro} proteolytic activity in living host cells as well as in vitro assays. The sensors were generated by sandwiching M^{pro} N-terminal autocleavage sites, either AVLQSGFR (short) or KTS AVLQSGFRKME (long), in between the mNeonGreen and nanoLuc proteins. Co-expression of the sensor with the M^{pro} in live cells resulted in its cleavage in a dose- and time-dependent manner while mutation of the critical C145 residue (C145A) in M^{pro} completely abrogated the sensor cleavage. Importantly, the BRET-based sensors displayed increased sensitivities and specificities as compared to the recently developed FlipGFP-based M^{pro} sensor. Additionally, the sensors recapitulated the inhibition of M^{pro} by the well-characterized pharmacological agent GC376. Further, in vitro assays with the BRET-based M^{pro} sensors revealed a molecular crowding-mediated increase in the rate of M^{pro} activity and a decrease in the inhibitory potential of GC376. The sensor developed here will find direct utility in studies related to drug discovery targeting the SARS-CoV-2 M^{pro} and functional genomics application to determine the effect of sequence variation in M^{pro}.

Introduction

COVID-19 has become a global health threat with more than 300 million infections and more than 5 million deaths as of January 2022. The causative agent, Severe Acute Respiratory Syndrome Corona Virus 2 (SARS-CoV-2) of the *beta-coronavirus* family shares 79% similarity with SARS-CoV and 50% similarity with MERS-CoV (Middle East respiratory syndrome coronavirus)^{1, 2}. SARS-CoV-2 infection cycle is initiated by the processing of two polypeptides, pp1a and pp1ab, bearing the non-structural proteins³ by the auto-catalytically released viral proteases, 3-chymotrypsin-like cysteine protease (3CL^{pro}) or main protease (M^{pro})^{4, 5}, and papain-like protease (PL^{pro})⁶⁻⁸. M^{pro} functions as a homodimer with each monomer containing an active site formed by a conserved catalytic dyad of Cys-His^{7, 9}, and cleaves the large polyprotein pp1ab at 11 sites⁷. Specifically, M^{pro} recognizes a highly conserved core sequence with a critical Gln residue for cleavage¹⁰⁻¹³. Importantly, M^{pro} cleavage sequences are not known to be recognized by human proteases, thus making M^{pro} an attractive target for anti-SARS-CoV-2 therapy¹⁴.

Given the critical role played by M^{pro} in SARS-CoV-2 infection and the cleavage specificity, a number of assays have been developed to monitor the proteolytic activity of M^{pro}. Genetic reporter assays based on fluorescence and bioluminescence provide sensitive and effective systems to assess the cellular functions including cell signaling, protein dimerization, conformational changes of proteins and protein-protein interactions in live cells¹⁵⁻¹⁸. Researchers have developed fluorescence and bioluminescence-based reporter assays for screening antiviral molecules against various coronaviruses (e.g. FRET¹⁹, split-luciferase^{20, 21} assays). Specifically, a number of studies have utilized fluorescence resonance energy transfer (FRET)-based in vitro assays, wherein peptide substrates containing the M^{pro} cleavage sequences are used as reporter, for the identification of antivirals against SARS-CoV-2 M^{pro}²²⁻²⁶. Additionally, a FRET-based assay was utilized for the identification of Boceprevir, GC376, and calpain inhibitors II, XII as potent inhibitors of SARS-CoV-2 M^{pro}²⁷. On the other hand, a FlipGFP-based construct containing the M^{pro} N-terminal autocleavage site has been developed to screen the antivirals against SARS-CoV-2^{28, 29}. In such a construct, M^{pro}-mediated cleavage of FlipGFP in live cells results in the generation of the fluorescent form of GFP from the non-fluorescent form. A later report by Drayman *et al.*,

Figure 1. Genetically encoded, BRET-based live cell SARS-CoV-2 M^{pro} protease activity sensor. A schematic representation of the genetically encoded, BRET-based SARS-CoV-2 M^{pro} protease activity sensor expressed in live cells. Close positioning of the NLuc and mNG proteins result in a significant resonance energy transfer in the absence of the SARS-CoV-2 M^{pro} protease activity. Activity of the SARS-CoV-2 M^{pro} results in the cleavage of the sensor resulting in a decrease in the resonance energy transfer between NLuc and mNG leading to a decrease in the green fluorescence of the sensor. Shown in grey is the surface representation of M^{pro} structure (SARS-CoV; PDB: 2Q6G⁶⁵) highlighting the critical C145 residue (green) required for proteolytic activity and the peptide substrate peptide (TSAVLQSGFRK; red).

have combined FlipGFP and luciferase assays to successfully identify additional M^{pro} inhibitors³⁰.

In addition to the above, Bioluminescence Resonance Energy Transfer (BRET) has been used in developing a range of genetically encoded, live cell sensors³¹⁻³⁴. BRET relies on the non-radiative resonance energy transfer from a light emitting luciferase protein (donor) upon oxidation of its substrate to a fluorescent protein (acceptor) with an excitation spectrum overlapping with the luciferase emission spectra. In addition to the spectral overlap, BRET also depends on the physical distance and relative orientation of the donor and the acceptor proteins.^{17, 18, 35, 36} The latter has been successfully utilized in generating a variety of molecular sensors including detecting small molecules³⁷⁻⁴⁰, structural changes in proteins^{38,41}. While a number of donor-acceptor pairs with distinct spectral and energy transfer efficiencies have been utilized for BRET-based sensor development⁴², the combination of mNeonGreen (mNG)⁴³⁻⁴⁶, a bright green fluorescent protein, and nanoLuc (NLuc)^{47, 48}, a small bright and stable luciferase have gained significant usage in the recent times including proteolytic cleavage sensors^{39,49} due to excellent spectral overlap and light emission characteristics⁵⁰⁻⁵⁶.

In the present study, we have engineered BRET-based M^{pro} proteolytic activity sensors by inserting the M^{pro} N-terminal autocleavage sequences (either the short AVLQSGFR^{4, 26} or the long KTS AVLQSGFRKME^{5, 26} in between the mNeonGreen (mNG; acceptor) and the nanoLuc luciferase (NLuc; donor) in a single fusion construct. The sensor constructs showed robust cleavage activity in live cells when coexpressed with the wild type M^{pro}, both in a dose-dependent and time-dependent manner, but not in the presence of the catalytically dead C145A mutant M^{pro57-59}, and with a faster kinetics and higher specificity compared to the FlipGFP-based M^{pro} sensor. We have further determined the utility of the sensors in pharmacological inhibition of the M^{pro} using the well-established M^{pro} inhibitor, GC376^{27, 60-62}. Additionally, in vitro assays showed a molecular crowding-mediated proteolytic cleavage rate enhancement and inhibitor potency decrease.

Materials & Methods

M^{pro} N-terminal autocleavage sequence analysis

A total of 1984 sequences for the SARS-CoV-2 pp1a polyprotein available at the NCBI Virus database (<https://www.ncbi.nlm.nih.gov/genome/viruses/>) were downloaded and aligned using MAFFT server (<https://mafft.cbrc.jp/alignment/server/>)^{63, 64}. The aligned sequences of the pp1a polyprotein were analyzed for the conservation of the M^{pro} N-terminal autocleavage positions (AVLQSGFR) (Fig. 1).

Structural modeling of M^{pro} N-terminal autocleavage peptide sequences

The crystal structure of the N-terminal peptide substrate complexed with SARS-CoV main protease H41A mutant (PDB: 2Q6G⁶⁵, Chain D, aa seq: TSAVLQSGFRK) was used as a template for generating the 3D models for the short and long M^{pro} cleavage peptides, including the linker region, of the M^{pro} sensor (short cleavage peptide aa seq: EFGTENLYAVLQSGFRGSGGS, long cleavage peptide aa seq: EFGTENLYKTS AVLQSGFRKMEGSGGS). Models were generated using MODELLER (10.1 release, Mar. 18, 2021)⁶⁶. Briefly, the short and long sequences were aligned with the template in PIR format. For each peptide, 100 models were initially generated using “Automodel” function and “very-slow” MD refining mode. Scoring functions such as modpdf, DOPE, and GA34, were used to assess the generated models. The model with the lowest DOPE score was further refined by

loop modelling using very-slow loop MD refining mode to generate 100 refined models. The same scoring functions were used to assess the refined models. The stereochemical quality of the final model was assessed with PROCHECK⁶⁷.

Molecular dynamics simulation

To neutralize the positive and negative charges on the peptide's termini, the N- and C-termini were capped with N-acetyl and N-methyl amide capping groups, respectively. Topology and parameter files were generated using CHARMM-GUI webserver⁶⁸. The biomolecular simulation systems included the peptide model, with all hydrogens added, solvated in TIP3P (transferable intermolecular potential with 3 points)⁶⁹ cubic water box with 10 Å minimum distance between edge of box and any of the peptide atoms. Charges were neutralized by adding 0.15 M NaCl to the solvated system. The total number of atoms was 15480 and 18233 for the short and long peptide simulation systems, respectively. In silico molecular dynamics simulations were performed using Nanoscale Molecular Dynamics (NAMD) software⁷⁰ version 2.13 with the CHARMM36(m) force field⁷¹. A 2 fs time-step of integration was set for all simulations performed. First, energy minimization was performed on each system for 1000 steps (2 ps). Following energy minimization, the system was slowly heated from 60 K to 310 K at 1 K interval to reach the 310 K equilibrium temperature using a temperature ramp that runs 500 steps after each temperature increment. Following thermalization, temperature was maintained at 310 K using Langevin temperature control and at 1.0 atm using Nose-Hoover Langevin piston pressure control⁷². The system was then equilibrated with 500000 steps (1 ns) using Periodic Boundary Conditions. The NAMD output structure was then used as an input for Gaussian accelerated molecular dynamics (GaMD) simulation utilizing the integrated GaMD module in NAMD and its default parameters^{73, 74} which included 2 ns of conventional molecular dynamics (cMD) equilibration run in GaMD, to collect potential statistics required for calculating the GaMD acceleration parameters, and another 50 ns equilibration run in GaMD after adding the boost potential^{74, 75}, and finally GaMD production runs for 1000 ns. Both equilibration steps in GaMD were preceded by 0.4 ns preparatory runs. All GaMD simulations were run at the "dual-boost" level by setting the reference energy to the lower bound, i.e., $E = V_{\max}$ ⁷⁶. One boost potential is applied to the dihedral energetic term and the other to the total potential energetic term. The details for calculating the boost potentials including the equations used have been described previously^{73, 76, 77}. The upper limits of standard deviation (SD) of the dihedral and total potential boosts in GaMD were set to 6.0 kcal/mol. All GaMD simulations were performed using similar and constant temperature and pressure parameters. For all simulations, short-range non-bonded interactions were defined at 12 Å cut-off with 10 Å switching distance, while Particle-mesh Ewald (PME) scheme was used to handle long-range electrostatic interactions at 1 Å PME grid spacing. Trajectory frames were saved every 10,000 steps (20 ps) and trajectory analysis was performed using the available tools in Visual Molecular Dynamics (VMD) software⁷⁸. Trajectory movies were compiled based on 1000 frames using Videomach (<http://gromada.com/videomach/>) to generate 41 s movies (24 fps) in AVI format.

M^{pro} BRET sensor plasmid construct generation

The BRET-based M^{pro} activity sensors were developed based on previously reported M^{pro} N-terminal autocleavage peptides, namely AVLQSGFR⁴ (nucleotide sequence 5' GCA GTG CTC CAA AGC GGA TTT CGC 3') and KTS AVLQSGFRKME^{5, 26} (nucleotide sequence 5' AAA ACG AGT GCC GTA TTG CAG AGT GGG TTT CGG AAA ATG GAA 3'), referred to as mNG-M^{pro}-Nter-auto-NLuc and mNG-M^{pro}-Nter-auto-L-NLuc, respectively. For this, fragments BstXI-mNG-M^{pro}-Nter-auto-NLuc-XhoI and BstXI-mNG-M^{pro}-Nter-auto-L-NLuc-XhoI were synthesized (Integrated DNA Technologies, IDT; Iowa, USA) and inserted into pIDTSmart (Kan) vectors to

generate the plasmid constructs pIDT-mNG-M^{pro}-Nter-auto-NLuc and pIDT-mNG-M^{pro}-Nter-auto-L-NLuc, respectively. Both vectors were transformed into *E. coli* for amplification and purified using Qiagen mini-prep kit. Restriction enzymes BstX-I and XhoI were used to excise the two DNA fragments of interests from entry clones pIDT-mNG-M^{pro}-Nter-auto-NLuc and pIDT-mNG-M^{pro}-Nter-auto-L-NLuc and ligated into similarly digested destination plasmid pmNeonGreen-DEVD-NLuc [Addgene: 98287]⁴⁹ and further confirmed by Sanger sequencing. One Shot TOP10 Competent *E. coli* cells were transformed with 2 µL of the ligation reaction and plated in LB agar plates with 100 µg/ml ampicillin. Sequences were confirmed by Sanger sequencing⁷⁹ using a pair of forward and reverse primers, 5' GCACAGCCAGAACCACATATACCTT 3' and 5' CACCACCTTGAAGATCTTCTCGATCT 3', respectively. For bacterial expression and purification of the M^{pro} sensor, the mNG-M^{pro}-Nter-auto-NLuc plasmid construct was digested with *Hind*III and *Xho*I and the mNG-M^{pro}-Nter-auto-NLuc fragment was subcloned into similarly digested pET-28b(+) plasmid.

Cell culture and transfection

All experiments reported in the manuscript were performed with HEK 293T cells, which were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, and 1% penicillin-streptomycin and grown at 37 °C in 5% CO₂^{18, 38, 39, 41, 80-84}. Transfections were performed with polyethyleneimine (PEI) lipid according to manufacturers' protocol. Briefly, HEK 293T cells were seeded onto 96-well white plates before 24 h of transfection. The plasmid DNA (sensor and M^{pro}), Opti-MEM (Invitrogen; 31985088) and 1.25 µg/well of PEI lipid (Sigma-Aldrich; 408727-100mL) were combined using pipetting and incubated at room temperature for 30 min before being added to cells by droplet. The PEI stock solution of 2 mg/mL was prepared by diluting in sterile Milli-Q water and stored at -80 °C.

Live cell, BRET-based M^{pro} proteolytic cleavage activity assays

Live cell M^{pro} proteolytic cleavage activity assays were performed by co-transfecting HEK 293T cells with either the pmNG-M^{pro}-Nter-auto-NLuc or the pmNG-M^{pro}-Nter-auto-L-NLuc M^{pro} sensor plasmid constructs along with either pLVX-EF1alpha-SARS-CoV-2-nsp5-2xStrep-IRES-Puro (M^{pro} WT) (a gift from Nevan Krogan (Addgene plasmid # 141370; <http://n2t.net/addgene:141370> ; RRID:Addgene_141370)⁸⁵ or pLVX-EF1alpha-SARS-CoV-2-nsp5-C145A-2xStrep-IRES-Puro (C145A mutant M^{pro}) plasmid (a gift from Nevan Krogan (Addgene plasmid # 141371 ; <http://n2t.net/addgene:141371> ; RRID:Addgene_141371)⁸⁵ in 96-well white flat bottom plates (Nunc; 136101). For dose-response experiments, the filler plasmid (a pcDNA3.1-based plasmid) is also co-transfected. In case of time-course experiment, a pcDNA3.1-based plasmid was used as a control (No M^{pro}). The time-course experiments were carried out at 1:5 reporter-to-protease ratio. Post 48 h (or otherwise indicated) of transfection, BRET measurements were performed by the addition of furimazine (Promega, Wisconsin, USA) at a dilution of 1:200. In time-course experiments, BRET was measured at the indicated time points. Experiments were performed in triplicates and repeated a minimum of two times.

Western blot analysis

HEK 293T cells co-transfected with the M^{pro} sensor and the M^{pro} (wild-type or mutant) plasmids were lysed in 200 µl of 2x Laemmli sample buffer (50 mM Tris-Cl pH 6.8, 1.6% SDS, 8% glycerol, 4% β-mercaptoethanol and 0.04% bromophenol blue) (heated to 85°C and sonicated prior to addition). Equal volumes of the cell lysates (30 mL) were separated by 10% SDS-PAGE using running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) at a constant voltage of 100 V for 1.5 h following which proteins were transferred onto PVDF (Polyvinylidene fluoride) membranes. Membranes were blocked in Tris-buffered saline containing 0.1% Tween-20 (TBS-T)

with skimmed milk (5%) for 1 h at room temperature. Blots were incubated either with anti-His antibody (6x-His Tag Monoclonal Antibody (HIS.H8), Alexa Fluor 488; ThermoFisher Scientific-MA1-21315-A488; 1:5000) or with anti-Strep-tag mouse monoclonal antibody (anti-Strep-tag mouse monoclonal, C23.21; PROGEN- 910STR; 1:5000) overnight at 4°C in dilution buffer (TBS-T containing 5% bovine serum albumin (BSA). Secondary anti-mouse IgG HRP (Anti-Mouse Ig:HRP Donkey pAb; ECM biosciences- MS3001; 1:10000 diluted in TBS-T) was used to detect M^{pro} and the cleaved M^{pro} sensor proteins.

Live cell M^{pro} proteolytic cleavage inhibitor assay

HEK 293T cells were co-transfected with either pmNG-M^{pro}-Nter-auto-NLuc or pmNG-M^{pro}-Nter-auto-L-NLuc plasmid along with either pLVX-EF1alpha-SARS-CoV-2-nsp5-2xStrep-IRES-Puro (M^{pro} WT) (Addgene plasmid # 141370) or pLVX-EF1alpha-SARS-CoV-2-nsp5-C145A-2xStrep-IRES-Puro (M^{pro} C145A) (Addgene plasmid # 141371) plasmid in 96-well white flat bottom plates. We have used 1:5 reporter-to-protease ratio for the assay. Eight hours post-transfection, GC376 (GC376 Sodium; AOBIOUS- AOB36447; stock solution prepared in 50% DMSO at a concentration of 10 mM) was added to the cells at different concentrations. After 24 h of incubation with the inhibitor, BRET measurements were performed by the addition of furimazine (Promega, Wisconsin, USA) at a dilution of 1:200. The percentage activity was calculated by normalizing the BRET ratio with the negative control (No M^{pro}). Two independent experiments were performed in triplicates for each sensor construct.

Live cell, FlipGFP-based M^{pro} proteolytic assay

For live cell FlipGFP-based M^{pro} proteolytic activity assays, HEK 293T cells were seeded onto 24-well plates and co-transfected with the FlipGFP sensor plasmid (pcDNA3 FlipGFP(Mpro) T2A mCherry; a gift from Xiaokun Shu; Addgene plasmid # 163078)²⁹ and either the WT or the C145A mutant M^{pro} expressing plasmid DNA (1.25 µg/well) using PEI lipid after 24 h of cell seeding. For transfection, cells were imaged using a EVOS FL microscope (Life Technologies; 4' objective) at the indicated time in the red (to monitor mCherry expression to determine transfected cells) and the green (to monitor conversion of non-fluorescent FlipGFP into the fluorescent GFP form after M^{pro}-mediated cleavage) channels. Images were analyzed for percentage GFP positive (GFP⁺) cells, number of transfected cells and total number of analyzed cells for each time point using Fiji⁸⁶. The ImageJ macro script used for the analysis is provided in the Supporting Text. For determining number of GFP⁺ cells, GFP intensities obtained for each cell was background corrected and threshold was applied.

Cell lysate preparation for in vitro BRET assays

To prepare cell lysates containing the M^{pro} sensors, HEK 293T cells were transfected with either the mNG-M^{pro}-Nter-auto-NLuc or the mNG-M^{pro}-Nter-auto-L-NLuc M^{pro} BRET sensor and washed with chilled Dulbecco's Phosphate-Buffered Saline (DBPS) 48 h post transfection. Cells were lysed in a buffer containing 50 mM HEPES (pH 7.5), 50 mM NaCl, 0.1% Triton-X 100, 1 mM Dithiothreitol (DTT) & 1 mM ethylenediamine tetraacetic acid (EDTA)⁸⁷ on ice. Cell lysates were collected in a 1.5 mL Eppendorf tube and centrifuged at 4 °C for 1 h at 14,000 rotations per min (RPM) following which supernatant were collected and stored at -80 °C until further usage.

In vitro, BRET-based M^{pro} proteolytic cleavage activity assays

In vitro BRET-based M^{pro} proteolytic cleavage activity assays were performed by incubating cell lysates containing the short, BRET-based M^{pro} sensor with different concentrations (0.5, 5, 50 and 500 nM) of recombinantly purified SARS-CoV M^{pro} (SARS coronavirus, 3CL Protease, Recombinant from E. coli; NR-700; BEI

Resources, NIAID, NIH; stock solution of the protein was prepared by dissolving the lyophilized protein in 50 μ M in Tris-buffered saline (TBS) containing 10% glycerol and BRET monitored through luminescence scans. The effect of molecular crowding was monitored by incubating the sensor and the protease in the absence or presence of 25% (w/v) of polyethylene glycol (PEG) of a range (0.4, 2, 4, 8, 20 or 35 kDa) of molecular weights (Sigma-Aldrich). GC376 (GC376 Sodium; AOBIOUS - AOB36447; stock solution prepared in 50% DMSO at a concentration of 10 mM) inhibition of M^{pro} (50 nM) activity was monitored under a range of the inhibitor concentrations in the absence or presence of 25% (w/v) PEG 8K. BRET measurements were performed at 37 °C by the addition of furimazine (Promega, Wisconsin, USA) at a dilution of 1:200. The bioluminescence (467 nm) and fluorescence (533 nm) readings were recorded using Tecan SPARK multimode microplate reader and used to calculate the BRET ratios (533 nm/467 nm). Total mNG fluorescence in cell lysates containing the short, BRET-based M^{pro} sensor was measured by exciting the samples at 480 nm and emission acquired at a wavelength of 530 nm.

BRET and fluorescence measurements

BRET measurements were performed using a Tecan SPARK® multimode microplate reader. Bioluminescence spectral scan was performed from 380 to 664 nm wavelengths with an acquisition time of 400 ms for each wavelength to determine relative emissions from NLuc (donor) and mNG (acceptor) and quantify BRET, which is expressed as a ratio of emissions at 533 nm and 467 nm. In some experiments, BRET measurements were performed by measuring emission only at 533 and 467 nm. Total mNG fluorescence in the sensor expressing cells was measured by exciting the samples at 480 nm and emission acquired at a wavelength of 530 nm.

Data analysis and figure preparation

GraphPad Prism (version 9 for macOS, GraphPad Software, La Jolla California USA; www.graphpad.com), in combination with Microsoft Excel, was used for data analysis and graph preparation. Figures were assembled using Adobe Illustrator.

Results and Discussion

BRET-based M^{pro} proteolytic cleavage activity sensor design

In order to develop a live cell, BRET-based specific reporter to monitor M^{pro} proteolytic cleavage activity, we generated fusion proteins containing the M^{pro} N-

terminal autocleavage sequence sandwiched between mNG (acceptor) and NLuc (donor) proteins (Fig. 1). The mNG and NLuc pair (acceptor and donor, respectively) has been used in a number of BRET-based sensor and show efficient energy transfer from NLuc to mNG. Thus, in the absence of any proteolytic cleavage, the sensor constructs are expected to display significant emission in the green channel. However, upon proteolytic cleavage of the sandwiched autocleavage peptide, the sensor constructs will display reduced emission in the green channel with a concomitant increase in the emission in the blue channel (Fig. 1).

Both SARS-CoV-2¹⁰ and SARS-CoV-1⁸⁷ M^{pro} show a significant preference for the N-terminal autocleavage sequence (AVLQSGFR; short sensor; Fig. 1,2A; see Supporting Text for complete sequence of the sensor constructs) as a substrate compared to other cleavage sequences in the pp1a polyprotein in terms of catalytic efficiency, and has been widely utilized in FRET-based, in vitro assays^{22, 26, 28, 88} as well as in a FlipGFP-based, live cell assay²⁸. Additionally, we analyzed all available pp1a polyprotein sequences reported for SARS-CoV-2 isolates at the NCBI Virus database for any variation in the cleavage sequence. This indicated that the N-terminal autocleavage sequence is invariable in all isolates reported and therefore, the sensor construct designed here will serve as a reliable reporter for M^{pro} proteolytic cleavage activity.

We note that while BRET comes with several advantages including a higher signal-to-noise ratio and an extended dynamic range compared to some other methods⁸⁹, the presence of the acceptor and

donor proteins i.e. mNG and NLuc at the N- and C-termini, respectively could potentially affect the interaction of the cleavage peptide with the M^{pro} dimer and thus, in turn, affect the cleavage efficiency of the peptide. This is especially relevant given that the binding of the peptide substrate has been reported to allosterically activate the SARS-CoV-1 M^{pro} dimer. Therefore, we generated a second, extended M^{pro} sensor construct the KTSAVLQSGFRKME peptide sequence (containing additional three residues on each sides of the AVLQSGFR core sequence; long sensor; Fig. 3B; see Supporting Text for complete sequence of the sensor construct), which has also been used in several previous reports^{5, 22, 26}. A key requirement for efficient cleavage of peptide substrates by M^{pro} is their structural flexibility as previous studies have reported the formation of secondary structural element can alter cleavage activity⁹⁰⁻⁹², especially given that secondary structure prediction indicated α -helical propensity by both the short as well as the long peptide (Supp. Fig. 1). In order to assess structural flexibility and secondary structure formation by the two peptides, we generated structural models of the peptides using the substrate peptide co-crystallized with the H41A mutant SARS-CoV-1 M^{pro65} and performed all-atom, explicit solvent, GaMD simulations that allow enhanced sampling of protein conformational states by applying harmonic boost potential that follows Gaussian distribution to accelerate conformational transitions^{73, 74}. Structural models were generated using Modeler⁶⁶ (Fig. 2A,B) and MD simulations were performed using the NAMD software⁹³ for a total duration of 1 ms for each peptide (Supp. Movies 1,2). These simulations indicated significant structural fluctuations in the two peptides as revealed by relatively large root-mean-squared-deviation (RMSD) and root-mean-squared-fluctuations (RMSF) (Fig. 2C,D). Further, radius of gyration (R_g) measurements of the peptides over the course of simulation also revealed structural fluctuations of the peptides with an appreciably greater fluctuations observed for the short peptide compared to the long one (Fig. 2G,H). Additionally, this analysis also revealed a greater R_g for the longer peptide compared to the short peptide (median R_g values of 9.4 vs. 10.2 Å) as expected. Finally, secondary structure analysis of the peptides over the course of the 1 ms long simulation trajectory revealed that the peptides largely show a propensity to form turns (Fig. 2I, J). Notably, certain central residues in the shorter peptide may form α -helix that was not seen with the longer peptide leading to the possibility of a differential cleavage efficiency of the peptides by M^{pro}. These results were in agreement with dynamic light scattering (DLS) measurements that showed an average size of 6.2 ± 1.2 nm for the short M^{pro} biosensor (Fig. S1C), suggesting a compact structure of the sensor construct, which may show high BRET signal that is critical for detecting sensor cleavage. In the following, we report experimental results with both the sensor constructs in order to provide a comparative analysis and determine the one that serves as a better substrate and thus, provide a superior evaluation of M^{pro} proteolytic cleavage activity in live cells.

BRET-based M^{pro} proteolytic cleavage activity sensor characterization in live cells

In order to test the functionality of the BRET-based M^{pro} proteolytic cleavage activity sensors, we transfected HEK 293T cells with the short and long sensors either alone or along with the M^{pro} expressing plasmid in a 1:5 sensor-to-protease plasmid ratio (Fig. 3). Additionally, we utilized the catalytic dead C145A mutant M^{pro} as a negative control in these experiments since Cys145 is essential for the proteolytic activity of M^{pro57-59}. The transfection efficiency and expression of the sensor constructs was monitored by imaging live cells for mNG fluorescence using an epifluorescence microscope, which showed an efficient transfection and expression of the sensor constructs after 24 h of transfection (Supp. Fig. S2).

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Figure 3. Efficient cleavage of the M^{pro} sensor constructs in live cells. (A,B) Schematic showing the M^{pro} sensor constructs - short (A) and long (B) – with SARS-CoV-2 M^{pro} N-terminal autocleavage sequence. (C,D) Graph showing a representative bioluminescence spectra of the short (C) and long (D) M^{pro} sensor constructs either in control cells or in cells expressing the WT or C145A mutant M^{pro}. Data were fit to a two Gaussian model reflecting mNG fluorescence and NLuc bioluminescence peaks. Note the reduction in the mNG peak (533 nm) of both the short and the long sensors when coexpressed with the wild type M^{pro} while no reduction was observed when coexpressed with the C145A mutant M^{pro}. (E,F) Graphs showing total mNG fluorescence (measured prior to substrate addition) in cells expressing the short (E) and the long (F) sensors. (G) Graph showing BRET ratio (ratio emission at 533 nm and 467 nm) of the short (left side) and the long (right side) M^{pro} protease activity sensors in either control cells or when coexpressed with the wild type or the C145A mutant M^{pro}. Inset: graph showing percentage change in BRET of the short (left side) and the long (right side) when co-expressed with the wild type or the C145A mutant M^{pro}. Data shown are mean ± S.D. from a representative of independent experiments performed multiple times. (H) Top panel: anti-His tag blot showing cleavage of the short (left side) and the long (right side) M^{pro} sensor constructs in either control cells or in cells co-expressing the wild type or the C145A mutant M^{pro}. Note the release of an approximately 30 kDa, His₆-tagged-mNG fragment in cells expressing the wild type but not in the C145A mutant M^{pro}. Bottom panel: anti-Strep-tag blot showing expression of the M^{pro} in the respectively transfected cells.

We then determined the spectral properties of the two sensors in live cells. For this, sensor construct transfected cells in adherent conditions were incubated with NLuc substrate and emission in the range of 380 and 664 nm wavelength were detected using a microplate reader. In the absence of coexpression of M^{pro}, both the short and the long sensors showed two peaks corresponding to NLuc (467 nm) and mNG (533 nm), respectively, as determined from two Gaussian fitting of the spectral data (Fig. 3C,D; top panels). Coexpression of the wild type M^{pro} resulted in a decrease in the mNG emission peak in cells expressing either of the sensor constructs (Fig. 3C, D; middle panel) while no such decrease was observed when the C145A mutant M^{pro} was coexpressed with the sensor constructs (Fig. 3C,D; bottom panel). We note that the coexpression of either the wild type or the C145A mutant M^{pro} 57-59 did not result in any significant change in the intracellular levels of the sensor constructs as determined from mNG fluorescence at 530 nm under excitation with 480 nm light (Fig. 3E,F).

We then determined the BRET ratio of the sensor constructs in live cells under different M^{pro} coexpression conditions as a ratio of emission at 533 nm and 467 nm. Basal BRET ratio of the short and long sensors were found to be 2.37 ± 0.17 vs 1.79 ± 0.06 (mean $\pm$ standard deviation; $N = 6$ each; independent experiments performed in triplicates; $p < 0.0001$), respectively, indicating that the additional 6 residues in the long sensor resulted in a $24 (\pm 2)\%$ decrease in the BRET ratio. Coexpression of the wild type M^{pro} resulted in a significant decrease in the BRET ratio while no significant decrease was observed in the presence of the C145A mutant M^{pro} (Fig. 3G). Importantly, both the short and the long sensor expressing cells showed $\sim 75\%$ reduction in the BRET ratio in the presence of the wild type M^{pro} (Fig. 3G, inset), indicating that these sensors will provide a wide dynamic range for monitoring M^{pro} proteolytic cleavage activity in live cells. Importantly, no change in the BRET ratio in cells expressing either of the sensors was observed in presence of the C145A mutant M^{pro} indicating high specificity of the BRET signals of these sensors (Fig. 3G, inset).

In order to confirm that the reductions in the BRET observed upon coexpression with M^{pro}, we performed western blot analysis of cell lysates prepared from the M^{pro} sensor transfected cells. For this, we utilized the N-terminal His₆-tag in the M^{pro} sensor constructs, which will be retained in the N-terminal, mNG protein containing fragment upon proteolytic cleavage, and C-terminal 2x Strep-tag in the M^{pro} protein for detecting cleavage of the M^{pro} sensor constructs and the expression of M^{pro}, respectively. Cells transfected with only the M^{pro} sensor constructs showed a band of the expected molecular weight of ~ 50 kDa (as predicted from the amino acid sequence of the sensor constructs) (Fig. 3H and Supp. Fig. S3). Indeed, cells coexpressing the wild type M^{pro}, as assessed from the anti-Strep-tag blot, showed a band of ~ 30 kDa corresponding to the predicted molecular weight of the cleaved N-terminal fragment containing the His₆-tag and the mNG protein with a concomitant loss of the full-length sensor constructs (Fig. 3H). However, cells coexpressing the C145A mutant M^{pro}, as assessed from the anti-Strep-tag blot, did not show the cleaved sensor fragment (Fig. 3H). Agreement of these results with the BRET measurements shown above establishes that the reduction in the BRET ratio observed in the presence of the wild type M^{pro} is due to the proteolytic cleavage of the sensor constructs, and therefore, live cell BRET ratio measurements can be reliably used as a measure of M^{pro} proteolytic activity.

M^{pro} dose-dependent cleavage of the sensor in live cells

Having established that the BRET ratio could be used to detect M^{pro} proteolytic activity of the sensor constructs, we aimed to determine the M^{pro} dose-dependent cleavage of the sensor constructs in live cells. For this, we cotransfected the cells with the 25 ng/well sensor constructs and a range of M^{pro} plasmid concentrations (0, 0.0125, 0.125, 1.25, 12.5 and 125 ng/well) and monitored bioluminescence spectra in adherent cells after 48 h. This revealed a M^{pro} plasmid dose-dependent shift in the bioluminescence spectra (Supp. Fig. S4) and the BRET ratio (Fig. 4A,B) of both the short and the long sensor in the presence of the wild type M^{pro} but not in the presence of the C145A mutant M^{pro}. Discernable decreases in the BRET ratio could be observed at a minimum amount of 1.25 ng of M^{pro} plasmid DNA and a maximum decrease in the BRET ratio of ~80% at the highest concentration of 125 ng for both sensor constructs (Fig. 4A,B; insets). The analysis has also showed that the EC₅₀ values are 1.09 ± 0.09 ng/well and 0.91 ± 0.89 ng/well for the short and the long sensors, respectively. These data demonstrate the functional potency of M^{pro} expressed in these cells.

Monitoring the temporal dynamics of M^{pro} proteolytic activity in live cells

We then monitored the temporal dynamics of M^{pro} proteolytic activity in live cells. Towards this, we transfected the cells with the M^{pro} sensor constructs either in the absence or in the presence of the wild type or the C145A mutant M^{pro} plasmid and monitored the bioluminescence spectra from 4 h until 48 h post transfection (Supp. Fig. S5). Analysis of the bioluminescence spectra obtained from cells expressing either of the M^{pro} sensors indicated a lower BRET ratio after 4 h of transfection, which increased with time and plateaued after 16 h of transfection in the absence of M^{pro} (Fig. 4C,D). Although mNG shows a relatively fast maturation time compared to several other fluorescent proteins⁹⁴, these data likely indicate a relatively slower intracellular maturation of mNG compared to NLuc^{48, 95} (Supp. Fig. 6). Importantly, a significant decrease in the BRET ratio of cells expressing either of the M^{pro} sensors could be observed in the presence of the wild type M^{pro} after 8 h of transfection (Fig. 4C,D). BRET ratio of the cells continued to decrease in the presence of the wild type M^{pro} until 48 h of transfection while no

such decrease was observed in the presence of the C145A mutant M^{pro} (Fig. 4C,D; insets). The half-life of the protease was found to be 13.22 ± 3.22 h and 9.81 ± 2.54 h for the short and the long sensors, respectively. These data suggests that the BRET-based M^{pro} sensor developed here could report M^{pro} proteolytic activity as early as 8 h of infection, although this may vary depending on the actual expression of the protease in host cells.

Comparison with the FlipGFP-based M^{pro} proteolytic sensor in live cells

Having established the monitoring of expression-dependent proteolytic activity of M^{pro} in live cells, we then performed similar experiments with the recently reported FlipGFP-based M^{pro} proteolytic activity reporter²⁹ to compare their performance of the biosensors in reporting M^{pro} proteolytic activity in live cells. For this, we transfected HEK 293T cells with the FlipGFP M^{pro} sensor expression plasmid along with either the WT or C145A M^{pro} expression plasmid and monitored GFP expression in the cells to ascertain

conversion of the non-fluorescent protein to a fluorescent one while mCherry expression in the cells was used for detecting transfected cells. Epifluorescence imaging of the cells post 4 h of transfection revealed the appearance of GFP fluorescence in the transfected cells, as ascertained from mCherry fluorescence, in the presence of WT M^{pro} after 24 h of transfection ($48 \pm 2\%$) while more cells showed GFP fluorescence after 48 h of transfection ($70 \pm 4\%$) (Fig. 5A,B). These data indicate a delayed response of the FlipGFP sensor to M^{pro} proteolytic activity in comparison to the BRET-based sensor. Additionally, a significant number of cells were found to be GFP positive after 24 h ($9 \pm 1\%$) and 48 h ($20 \pm 1\%$) of FlipGFP transfection in the presence of the C145A mutant M^{pro} (Fig. 5C,D). This is contrast to the observations made with the BRET-based sensor in the presence of the mutant M^{pro} (Fig. 4C,D).

Monitoring pharmacological inhibition of M^{pro} proteolytic activity in live cells

Finally, we determined the utility of the BRET-based M^{pro} sensors in pharmacological inhibition of M^{pro} proteolytic activity in live cells. Towards this, we treated cells coexpressing the M^{pro} sensors and M^{pro} with various concentrations of GC376, which has been shown to inhibit M^{pro} in live cells^{27, 60-62}, after 8 h of transfection based on the results reported above, and determined bioluminescence spectra of the cells after an additional 24 h (Supp. Fig. S7). A GC376 dose-dependent increase in the BRET ratio of cells coexpressing either the short or the long sensor and the wild type M^{pro} was observed, while no sensor cleavage was observed in the presence of the C145A mutant M^{pro} (Fig. 6A,B). Percentage proteolytic cleavage activity determined from the BRET ratio indicate that GC376 starts to inhibit M^{pro} at $33.3 \mu\text{M}$ concentration and continued to do so until a concentration of $333 \mu\text{M}$ (Fig. 6A,B; insets). The data has showed that the IC_{50} values for the short sensor is $127.4 \pm 23.33 \mu\text{M}$ and that for long

sensor is $194.7 \pm 7.49 \mu\text{M}$. The lower efficacy of GC376 observed here compared to previous reports^{27, 28, 60-62, 27, 58} perhaps indicates a cell type- or M^{pro} expression-dependent effect. Taken together, these data indicate that the BRET-based M^{pro} proteolytic activity sensors developed here can be utilized for screening antivirals targeted against M^{pro} .

Monitoring M^{pro} proteolytic cleavage activity in vitro

Having established the utility of the BRET-based M^{pro} sensor in live cell studies, we then focused on determining their utility in vitro using a recombinantly purified M^{pro} . For this, we prepared lysates from HEK 293T expressing either the short or the long M^{pro} sensor construct, incubated equivalent amounts of the lysates with three different concentrations (5 μM , 500 nM and 50 nM) of the recombinantly purified M^{pro} and monitored BRET following addition of the NLuc substrate (Fig. 7A,B). This assay revealed a M^{pro} concentration-dependent proteolytic processing of the M^{pro} sensors as ascertained from the decreasing BRET ratios. Importantly, the assay also indicated that a minimum of 500 nM of the recombinantly purified M^{pro} is required for a discernable proteolytic cleavage of the sensors as the BRET ratio of the sensors decreased to a lesser extent in the presence of 50 nM M^{pro} protein while a substantially higher rate of cleavage was observed under 500 nM M^{pro} .

Enzyme kinetics assays performed using a range of the short M^{pro} sensor concentrations 200 nM of M^{pro} revealed a low k' value of $3.09 \pm 0.02 \mu\text{M}$, which is lower than the K_m value of 17⁹⁶ or 56⁹⁷ μM determined using a FRET-based peptide substrate, and Hill coefficient of 1.58 (Supp. Fig. S8), suggesting a significant

cooperativity in the protein. Further, an increase in M^{pro} concentration resulted in a decrease in the Hill coefficient to 1.16 (Supp. Fig. S8), suggesting a role for protein dimerization in the observed cooperativity in M^{pro} .⁹⁸ We then performed the assays in the presence of GC376 to determine the pharmacological inhibition of M^{pro} activity in vitro. For this, 500 nM M^{pro} was preincubated with a range of concentrations of GC376 (10^{-4} to 10^{-9} M) for 30 minutes at 37 °C and cleavage activity was monitored after addition of lysates prepared from cells expressing either the short or the long M^{pro} sensor. Incubation with GC376 resulted in a decrease in the rate of proteolytic cleavage of both the short and the long M^{pro} sensor (Fig. 7C,D) with IC_{50} values of 73.1 ± 7.4 and 86.9 ± 11.0 nM for the short and the long sensor, respectively (Fig. 7G,H).

In vitro assays reveal molecular crowding-mediated increase in M^{pro} proteolytic activity and a decrease in inhibitor efficacy

We took advantage of the slow rate of the M^{pro} sensor cleavage under 500 nM M^{pro} to determine the effect of molecular crowding on the proteolytic activity of the protein. Molecular crowding in the intracellular environment caused by the presence of soluble and insoluble macromolecules such as proteins, nucleic acids, ribosomes and carbohydrates has been shown to impact both structure and stability of proteins in cells as well as enzyme kinetics⁹⁹⁻¹⁰⁴ including a decrease in the activity HCV NS3/4A protease¹⁰⁵ and an increase in the proteolytic activity of SARS-CoV M^{pro} ¹⁰³. For this, we included 25% (weight/volume; w/v) of 20000 Da (20K) polyethylene glycol (PEG), which is a non-toxic, hydrophilic polyether that serves as a crowding agent and has been extensively utilized to simulate the molecular crowding in vitro^{106, 107}, in the assays and monitored cleavage of the M^{pro} sensors under 500 nM M^{pro} under varying concentrations of GC376. Inclusion of 25% PEG 20K resulted in a substantial increase in the rate of proteolytic cleavage of the M^{pro} sensors in the absence of GC376 (Fig. 7E,F). This data suggests that molecular crowding caused by PEG 20K is likely effective in causing increased dimerization of M^{pro} , a feature critical for its catalytic activity, through an increase in the effective concentration of the protein due to excluded volume effects, and thus increases the rate of proteolytic cleavage of the M^{pro} sensor. Importantly, while GC376 could inhibit M^{pro} activity, the IC_{50} values as obtained from BRET ratios after 2 hours of incubation with both the short as well as the long sensor indicated a large shift (IC_{50} values of 2623 ± 760 and 10260 ± 3280 nM, respectively) (Fig. 7G,H). Together, these data indicate that M^{pro} could be more active in the crowded environment of an infected host cell compared to in vitro conditions, and may require higher concentrations of pharmacological inhibitors for effective inhibitions of its catalytic activity than those determined from in vitro assays.

Conclusion

To conclude, we have developed genetically encoded, BRET-based M^{pro} protease activity sensors for use in live cells as well as in vitro assays and have validated their utility for antiviral drug discovery using GC376 as a proof of principle. The use of BRET, with NLuc as the bioluminescence donor and mNG as the resonance energy acceptor, enabled highly sensitive detection of M^{pro} proteolytic activity in live cells. Additionally, the sensors developed here did not show any cleavage, either in the absence of M^{pro} or in the presence of the catalytically dead, C145A mutant M^{pro} , thus, displaying high specificity. The BRET-based sensors developed here appears to possess better sensitivity and specificity compared to the FlipGFP-based M^{pro} with respect to. The BRET-based sensor allowed detection of molecular crowding-mediated increased activity of M^{pro} enabling the discovery of reduced inhibitory efficacy of GC376 under a crowded condition. We believe that these sensors will find utility in both detecting active SARS-CoV-2 infection as well in screening antivirals developed for targeting M^{pro} proteolytic cleavage activity in live cells. Additionally,

they can be utilized for determining effects of genetic variation in the M^{pro} amino acid sequence that may arise during the evolution of the virus.

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Author Contributions

K.H.B. conceived the experiments. A.M.G, W.S.A, S.R, A.G.N.F, S.M.N.U, M.A. and K.H.B. performed experiments, analyzed data, prepared figures, and wrote the manuscript. All authors reviewed the manuscript.

Competing interests

K.H.B. & A.M.G. are inventors on a US Provisional Patent application number 63/275217, assigned to Qatar Foundation for Education, Science and Community Development, entitled “BRET-based coronavirus M^{pro} protease sensor and uses thereof” relating to the development and uses of the M^{pro} sensor. Other authors declare no competing interests.

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