Supporting Information

Amplification-Free SARS-CoV-2 Detection Using Nanoyeast-scFv and Ultrasensitive Plasmonic Nanobox-Integrated Nanomixing Microassay

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| Figure | Description |
|--------|-------------|
| S1     | Effect of ac-EHD (i.e., nanomixing) for SARS-CoV-2 detection. |
| S2     | Flow cytometry analysis of S309 scFv display level and RBD binding on yeast cells. |
| S3     | Nanoflow cytometry measurement of the size distribution of S309 nanoyeast-scFvs. |
| S4     | The reproducibility study of microelectrode surface functionalization. |
| S5     | The reproducibility study of nanobox surface functionalization. |
| S6     | Comparison of the performance between S309 nanoyeast-scFvs and S309 monoclonal antibodies. |
| S7     | Specificity study for the detection of soluble RBD. |
| S8     | Specificity study for the detection of SARS-CoV-2. |
Chemicals and Materials: Hydrogen tetrachloroaurate (III) trihydrate (HAuCl₄·3H₂O), silver nitrate (AgNO₃), MMC, 11-mercaptoundecanoic acid (MUA), dithiobis (succinimidyl propionate) (DSP) were purchased from Sigma Aldrich. Ascorbic acid of analytical grade was obtained from MP Biomedicals, Australia. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (Sulfo-NHS) were bought from ThermoFisher Scientific. SARS-CoV-2 spike RBD antibody (Cat: 40592-T62) was ordered from Sino Biological. Anti-HA tag antibody (Cat: ab26228) was purchased from abcam. Ultrapure water generated from Milli-Q water system with an electrical resistance of 18.2 MΩ cm was employed through the nanoparticle preparation process.

Design and cloning of gene for SARS-CoV-2 RBD-mNeonGreen fusion protein: The protein sequences of SARS-CoV-2 RBD (GenBank: QHD43416.1 319-541) and mNeonGreen (GenBank: AGG56535.1) were fused via a gly-ser linker (GGGGS), signal sequence and Twin-strep-tag sequence were added at the N and C-terminal respectively for secretion and purification of the expressed fusion protein. The designed fusion protein sequence was optimized for *Cricetulus griseus* (Hamster) codon usage and Kozak consensus sequence was added at 5'-end of the gene sequence. The resulting synthetic gene was commercially synthesized and cloned into a mammalian cell expression vector pcDNA™3.1(+) (Gene Universal, China). Transfection-quality DNA was produced in-house using the Qiagen Plasmid Plus Maxi Kit per the manufacturer's protocols.

Cell culture and transient transfection for RBD-mNeonGreen fusion protein: The SARS-CoV-2 RBD-mNeonGreen fusion protein was expressed in ExpiCHO-S cells according to manufacturer's instructions (Thermo Fisher Scientific). In brief, ExpiCHO-S cells were cultured in ExpiCHO Expression medium (Thermo Fisher Scientific, Cat: A2910001) in a humidified 8% CO₂ incubator at 37 °C, 110 rpm. The day prior to transfection, cells were split to 3–4 x 10⁶ cells/mL. After 24 h, stocks were 7–10 x 10⁶ cells/mL and were diluted to 6 x 10⁶ cells/mL with the addition of fresh medium.

ExpiCHO transfections were performed using the ExpiCHO Expression System Kit (Thermo Fisher Scientific, Cat: A29133) according to the manufacturer's protocol. Briefly, ExpiFectamine CHO transfection reagent and filtered plasmid DNA were separately diluted in OptiPRO SFM (Thermo Fisher Scientific, Cat: 12309019). ExpiFectamine CHO and DNA mixtures were immediately combined and incubated for 1-5 min. The ExpiFectamine CHO-DNA-OptiPRO mixture was then added to cells. For ExpiCHO Max Titer Protocol transfections, Enhancer and 16% v/v feed were added 18–22 h post-transfections; cultures were then temperature shifted to 32 °C and an additional 16% v/v feed was added on day 5 post-transfection. ExpiCHO Max Titer Protocol transfections were harvested on day 12 post-transfection. Cell cultures were centrifuged at 1900×g for 15 min at room temperature and the supernatants were filtered through a 0.2 μm PES filter (FisherScientific Scientific, Cat: 09-741-02).

Purification of SARS-CoV-2 RBD-mNeonGreen fusion protein: The filtered supernatant was loaded on a StrepTactin-4Flow column (IBA Lifesciences, Germany) equilibrated with the purification buffer (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA). The column was then washed with 50 column volumes of purification buffer. The fusion protein was eluted by purification buffer containing 50 mM biotin (IBA Lifesciences, Germany).
**Protein yield and purity determination:** SDS-PAGE was performed with the 12.5% acrylamide in the separating gel under reducing conditions, with the PageRuler prestained marker, 5 μL/lane (Thermo Fisher Scientific). Gel was stained by the Simply Blue Safe Stain (Thermo Fisher Scientific) and Gel image was processed with ImageLab software (Bio-Rad, Hercules, USA) to determine protein purity. Amicon centrifugal concentrator, with a 10 kDa molecular weight cutoff (MWCO) (MilliporeSigma, Burlington, USA) was used to concentrate as well as to remove the biotin from the elution pool (3000 g at 4°C). The fusion protein concentration was determined by measuring the A280 using a Nanodrop One spectrophotometer (Thermo Fisher Scientific, MA, USA). Final protein was dispensed as 0.5 mL or 0.05 mL aliquots, snap frozen in liquid nitrogen, and stored at −80°C.

**Flow Cytometry Profiling of Yeast Surface Display:** 100 μL of expressed yeast cells (10^7 cells per mL) were washed with 500 μL cold 0.1% BSA in PBS and centrifuged at 4000 rpm for 4 min at 4 °C. The pellet was resuspended in 100 μL of 0.1% BSA in PBS containing DyLight650 labelled anti-Myc antibody (1:100 dilution) (Abcam ab117487) and indicated concentration of RBD-mNeonGreen and incubated in the dark at 4 °C for 1 h on rotation. Cells were then collected by centrifugation at 4000 rpm for 4 minutes and washed with 0.1% BSA in PBS and the pellet was resuspended in a final volume of 500 μL 0.1% BSA in PBS. Control samples without antibody simultaneously prepared and used as a background reference. Flow cytometry analysis was performed by using CytoFLEX Platform (Beckman Coulter) equipped with 488 and 633 nm lasers and 525/40 and 660/20 nm band-pass filters. Data were analyzed in CytExpert software.

**RT-qPCR based Quantification of gamma irradiated SARS-CoV-2:** The RT-qPCR based quantification of gamma irradiated SARS-CoV-2 was performed by researchers at the Australian Centre for Disease Preparedness (ACDP), CSIRO, Geelong. The viral RNA was extracted from the gamma irradiated virus sample using the MagMAX™-96 Viral RNA Isolation Kit (QA/23-2-14). The calibration curve of copy number quantification was generated with E gene standards. The gBlock synthetic DNA standard was purchased IDT and prepared to have different concentrations with nuclease-free water. The assay utilized the AgPath-ID One-Step RT-PCR master mix (ThermoFisher Scientific) based on the following primers: CoV-E-fwd (5’-AGT ACG AAC TTA TGT ACT CAT TCG TT-3’), CoV-E-R2 (5’-ATA TTG CAG CAG TAC GCA CAC A-3’) and TaqMan probe: CoV E Probe 5’-6-FAM-ACA CTA GCC ATC CTT ACT GCG CTT CG-MGB-3’). RT-qPCR for the detection of SARS-CoV-2 was performed in duplicates with the use of the mean from duplicate reactions for calculation on the Applied Biosystems (ThermoFisher Scientific). The performed cycling conditions were as follows: 45 oC for 10 min, 95 oC for 10 min, followed by 45 cycles of 95 oC for 15 s and 60 oC for 45 s.

**Instrumentations:** SEM and TEM images of nanoboxes were taken from a JEOL-7100 field emission SEM microscope and a JEOL-2100 microscope, respectively. Flow cytometry characterizations of yeast cells were acquired on a Beckman Coulter CytoFLEX Flow Cytometer. NTA of the nanobox and nanoyeast size distribution was measured on a Malvern NanoSight NS300. Nanoflow cytometry measurement was performed on a Flow NanoAnalyzer (NanoFCM Inc.). SERS mapping images were scanned on a WITec alpha 300 R spectrometer with the use of 632.8 nm He-Ne laser as the excitation source, 20 × air objective, 0.05 s integration time, and an EMCCD camera. Each scanning image had the size of 60 μm × 60 μm with 1 μm step size.
**Figure S1.** Effect of ac-EHD (i.e., nanomixing) for SARS-CoV-2 detection. The measurement of RBD (500 ng/mL) on the microelectrodes with EHD (+) and without EHD (-), respectively. Error bars indicate the standard error of three replicates.

**Figure S2.** Flow cytometry analysis of S309 scFv display level and RBD binding on yeast cells. Yeast cells were labelled with anti-myc-Dy650 and RBD-mNeon Green with concentrations of (a) 0 µM; (b) 0.01 µM; (c) 0.1 µM; (d) 0.5 µM; (e) 1 µM; and (f) 10 µM.
**Figure S3.** Nanoflow cytometry measurement of the size distribution of S309 nanoyeast-scFv5s.

**Figure S4.** The reproducibility study of microelectrode surface functionalization. SERS signals of RBD detection (500 ng/mL) on five different electrodes.
Figure S5. The reproducibility study of nanobox surface functionalization. SERS signals of five batches of nanoboxes covalently bound with antibodies.

Figure S6. Comparison of the performance between S309 nanoyeast-scFvs and S309 monoclonal antibodies. The Raman intensity achieved by using S309 nanoyeast-scFvs and S309 monoclonal antibodies as capture reagents for RBD detection, respectively.
Figure S7. Specificity study for the detection of soluble RBD. (a) SERS mapping images of the different negative controls; (b) the extracted average Raman spectra from (a); and (c) corresponding Raman intensity at 1603 cm$^{-1}$.

Figure S8. Specificity study for the detection of SARS-CoV-2. (a) SERS mapping images of the positive sample and different negative controls; (b) the extracted average Raman spectra in (a); and (c) the Raman intensity at 1603 cm$^{-1}$ from the spectra.