

## **SARS-COV-2 Spike binding to ACE2 in living cells monitored by TR-FRET**

Erika Cecon<sup>1</sup>, Matilda Burridge<sup>1</sup>, Longxing Cao<sup>2</sup>, Lauren Carter<sup>2</sup>, Rashmi Ravichandran<sup>2</sup>,  
Julie Dam<sup>1,3,4</sup>, Ralf Jockers<sup>1,3,4,5</sup>

<sup>1</sup> Université de Paris, Institut Cochin, INSERM, CNRS, F-75014 PARIS, France

<sup>2</sup> Institute for Protein Design, University of Washington, Seattle, WA 98195

<sup>3</sup>Co-senior authors

<sup>4</sup>corresponding authors

<sup>5</sup>Lead Contact

**Running title:** Cellular RBD-ACE2 binding assay

## **ABSTRACT**

Targeting the interaction between the SARS-CoV-2 Spike protein and human ACE2, its primary cell membrane receptor, is a promising therapeutic strategy to prevent viral entry. *In vitro* assays using purified components have revealed the prominent role of the receptor binding domain (RBD) of the Spike protein for ACE2 binding. A simple and quantitative assay for Spike binding to ACE2 in a cellular environment is lacking. We developed here an RBD-ACE2 binding assay based on time-resolved FRET that reliably monitors the interaction in a physiologically relevant, cellular context. The assay is modular and has a broad range of applications as it can monitor the impact of cellular components such as heparan sulfate, lipids and membrane proteins on the RBD-ACE2 interaction and can be extended to the full-length Spike protein. The assay is HTS-compatible and can detect small-molecule competitive and allosteric modulators of the RBD-ACE2 interaction with high relevance as SARS-CoV-2 therapeutics.

**Word count:** 150

**KEYWORDS:** SARS-CoV, spike protein, ACE2, TR-FRET, HTRF, COVID-19, 2019-nCoV, HTS

## INTRODUCTION

The worldwide SARS-CoV-2 pandemic provoked an urgent need for effective therapeutic solutions to prevent and treat the resulting COVID-19 disease. Rapid progress was made on understanding the molecular basis of viral infection and inhibition of the initial steps of viral-host cell recognition was identified as a promising strategy for therapeutic intervention. The SARS-CoV-2 Spike (S) protein is required for virus attachment and entry into target cells through binding to the human Angiotensin-Converting Enzyme 2 (ACE2), the primary SARS-CoV-2 entry receptor (Zhou et al., 2020). Cryo electron microscopy studies suggest that two S protein trimers bind simultaneously to an ACE2 homodimer (Yan et al., 2020). The S protein is composed of S1 and S2 domains with the ACE2 receptor binding domain (RBD) located in the S1 region. Successful viral entry requires proteolytic cleavage of the S protein between S1 and S2 domains by the transmembrane protease serine 2 (TMPRSS2) expressed by the host cell (Hoffmann et al., 2020). Other components of the host cell have been suggested to participate in this core SARS-CoV-2/ACE2 complex such as heparan sulfate proteoglycans (HSPG) (Clausen et al., 2020) (Zhang et al., 2020) or CD4 (Davanzo et al., 2020). These auxiliary components are likely to confer cell type specificity but their precise role remains to be determined.

Interfering with the RBD/ACE2 interaction has been proposed as an attractive therapeutic strategy as demonstrated by the inhibitory effect of neutralizing anti-ACE2 antibodies (Baum et al., 2020) and nanobodies (Huo et al., 2020), de-novo designed miniprotein inhibitors based on the ACE2 helix interacting with RBD (Cao et al., 2020) or small-molecule FDA-approved drugs (Fu et al., 2020). Small-molecules are particularly attractive because they are generally cost-effective, show good stability and a high rate of penetration over biological barriers to easily reach their targets. Identification of such molecules currently relies mainly on acellular

*in vitro* assays suitable for high-throughput screening (HTS) purposes. However, these assays do not take into account the role of the cellular environment, which is likely to have a major impact on the formation of the SARS-CoV-2/ACE2 complex. We developed here a time-resolved Fluorescence Resonance Energy Transfer (TR-FRET) assay that probes the molecular proximity and conformational changes of the RBD/ACE2 complex in a cellular context. The assay successfully detects competitive and allosteric modulators of the RBD/ACE2 complex, is suitable for HTS applications and allows validation of candidate compounds identified in *in vitro* assays. The assay can be easily customized by co-expressing auxiliary components thus providing new mechanistic insights in the modulation of the RBD/ACE2 core complex, enabling the design of the most relevant cellular environment for tailored inhibitor screening.

## RESULTS

### **Binding of RBD of the SARS-CoV-2 Spike protein to ACE2 monitored by TR-FRET**

TR-FRET assays are increasingly used to monitor molecular interactions at the nanometer scale with high signal-to-noise ratio due to the temporal separation between sample excitation and energy transfer measurements (Degorce et al., 2009) (Figure 1A). The assay is based on the energy transfer between an energy donor (N-terminal SNAP-tagged human ACE2 labelled with terbium (Tb), SNAP-ACE2 in our case) and an energy acceptor (RBD of the SARS-CoV-2 Spike protein labelled with the d2 fluorophore, RBD-d2 in our case), which occurs only if both are in close proximity to each other (<10 nm) (Figure 1B). SNAP is an O6-alkylguanine-DNA alkyl transferase, that catalyzes its own covalent binding to fluorescent derivatives of benzylguanine such as Lumi4-Tb (Keppler et al., 2003). For the binding assay the SNAP-ACE2 was expressed in HEK293 cells and labelled with the cell-impermeant Lumi4-Tb. The SNAP-ACE2 fusion protein migrated at an apparent molecular weight of 180kD in SDS-PAGE

experiments upon expression in HEK293 cells (Figure S1). Binding of the RBD-d2 tracer to Lumi4-Tb-labelled SNAP-ACE2 was saturable at equilibrium and with nanomolar affinity ( $K_d=14.6 \pm 2.5$  nM;  $n=10$ ) (Figure 1C). Non-specific binding was defined in the presence of an excess of unlabelled RBD (1  $\mu$ M) and resulted in a signal-to-noise ratio higher than 15 (Figure 1C). RBD-d2 association and dissociation was observed in a time-dependent manner (Figure 1D-E), with  $k_{on}$  and  $k_{off}$  values of  $1.3 \cdot 10^6$   $M^{-1} s^{-1}$  and  $2.93 \cdot 10^{-3}$   $s^{-1}$ , respectively and a calculated  $K_d$  of 2.3 nM (Table 1). The  $k_{off}$  was similar to and the  $k_{on}$  6.6 times higher than the mean  $k_{on}$  of previous *in vitro* studies (Table S1). No specific binding of RBD-d2 was observed in cells expressing similar amounts of the Lumi4-Tb-labelled SNAP-tagged VEGF receptor 2, a single-transmembrane control receptor of similar size and topology (Figure 1F). Several hormones, cytokines, chemokines and lectins (100nM) found in the extracellular milieu were unable to displace RBD-d2 binding to Lumi4-Tb-labelled SNAP-ACE2 demonstrating the high specificity of the assay (Figure 1G). Immunofluorescence experiments showed that RBD-d2 only binds to cells expressing Lumi4-Tb-labelled SNAP-ACE2 but not the control SNAP-LepR (leptin receptor), a single transmembrane protein with a large extracellular domain (Figure 1H). The Lumi4-Tb-label was not observed in the RBD-d2 channel, evidence that there was no leakage of the fluorescence signal between the two channels (Figure 1H). Taken together, these results show that fluorescently labelled RBD-d2 binds with high (nanomolar) affinity and high specificity to ACE2 expressed in HEK293 cells.

### **Inhibition of SARS-CoV-2 Spike RBD binding to ACE2**

Identification of molecules interfering with RBD binding to ACE2 constitutes a major application of our TR-FRET assay. In order to validate this aspect of the assay we performed competition binding experiments with unlabelled RBD. Competition of the RBD-d2 tracer to ACE2 occurred at the nanomolar range ( $pK_i=7.70 \pm 0.03$ ) confirming the high-affinity binding

of RBD which is identical to that of the RBD-d2 tracer (Figure 2A). We then tested the recently developed high-affinity miniprotein LCB1v3, which is based on the ACE2 helix that interacts with RBD (Cao et al., 2020) and we observed a full competition of the RBD-d2 tracer in the subnanomolar range ( $pK_i=9.42 \pm 0.1$ ;  $n=5$ ). To demonstrate the suitability of the assay to identify new inhibitory compounds in a HTS mode, we determined the  $Z'$  that estimates the robustness and reliability of HTS assays (Zhang et al., 1999). A  $Z'$  value of 0.83 was obtained for a decrease in TR-FRET signal of 84% at 5 nM of RBD-d2 and 300 nM of RBD competitor (Figure 2B), which is in the upper range (0.7 to 1) considered as excellent performance for HTS. Taken together, these results demonstrate the HTS-suitability of our TR-FRET assay in the competition mode.

Several small-molecule compounds have been described to interfere with the RBD-ACE2 interaction in *in vitro* assays. This is the case of the AlphaLISA proximity assay that was applied to screen 3,384 small-molecule drugs and pre-clinical compounds for drug repurposing (Hanson et al., 2020). We tested five out of the 25 high-quality hits identified in this *in vitro* assay with reported apparent  $IC_{50}$  values in the low micromolar range. Three compounds, Cangrelor, Elaidic acid and Fenbendazole with reported *in vitro*  $IC_{50}$  around 10-15  $\mu$ M (Hanson et al., 2020), were unable to compete RBD-d2 at concentrations up to 100 or 1000  $\mu$ M (Figure 2C-E). Enalapril maleate showed a tendency of inhibition, while Corilagin (reported *in vitro*  $IC_{50}$  of 7.5 and 0.1  $\mu$ M, respectively (Hanson et al., 2020) showed a statistically significant 30% inhibition at 100  $\mu$ M (Figure 2F-G). Collectively, these data suggest a significant difference in the apparent  $IC_{50}$  of RBD-ACE2 interaction inhibitors between the *in vitro* and cellular binding assays, with an estimated loss of affinity of 1 to 2

logs. Information obtained with the cellular TR-FRET assay will be crucial to select the most promising compounds to move forward in the drug development process.

### **Modularity of the SARS-CoV-2 Spike RBD/ACE2 binding**

The binding of SARS-CoV-2 to ACE2 and cellular entry of the virus occurs in a complex cellular environment that should be taken into consideration when characterizing the Spike/ACE2 interaction and when searching for potential inhibitors. To evaluate whether our TR-FRET assay is able to fulfil this requirement we studied the impact of modulating the cellular environment on our binding assay.

We first explored the possibility to detect competitors of the RBD/ACE2 binding in a transcellular mode. HEK293 cells expressing fluorescently Lumi4-Tb-labelled SNAP-ACE2 were either mixed with mock-transfected cells or cells expressing unlabelled ACE2 (1:1 ratio of cells) and the RBD-d2 tracer ([Figure 3A](#)). Only unlabelled ACE2 cells inhibited the TR-FRET signal (~50%) demonstrating the possibility of detecting trans-cellular inhibitors with the TR-FRET assay.

TMPRSS2 is known to promote the cellular entry of SARS-CoV-2 by cleaving the spike protein of SARS-CoV-2, a necessary step for viral entry (Hoffmann et al., 2020). Such a mechanism would indicate a molecular proximity between ACE2 and TMPRSS2. To elucidate the impact of TMPRSS2 on the RBD/ACE2 interaction, we co-expressed TMPRSS2 and SNAP-ACE2. The presence of TMPRSS2 reduced the TR-FRET signal by 50% at a saturating concentration of the RBD-d2 tracer ([Figure 3B](#)) and constant amounts of Lumi4-Tb-labelled SNAP-ACE2 ([Figure 3B, insert](#)), indicating that TMPRSS2 induces a conformational change in the RBD/ACE2 complex modifying the distance between the two fluorophores. As the TMPRSS2 cleavage site on the Spike protein is located outside of the RBD, between the Spike's S1 and the S2 domain, we used the d2-labelled Spike S1-S2 as tracer. Binding of Spike S1-S2-d2 to

Lumi4-Tb-labelled SNAP-ACE2 was readily observed and competed by unlabelled Spike S1-S2 (200 nM) (Figure 3C). Reduction of the TR-FRET signal was once again observed at a constant amount of Lumi4-Tb-labelled SNAP-ACE2 indicating that the effect is independent of the binding of TMPRSS2 to its Spike substrate binding site and suggesting a direct effect of TMPRSS2 on ACE2.

HSPG are known modulators of SARS-CoV and SARS-CoV-2 entry and exogenous heparin has been shown to mitigate the binding of RBD to ACE2 (Clausen et al., 2020) (Zhang et al., 2020). Consistently, we observed an inhibition of RBD-d2 binding to ACE2 ( $IC_{50}=96\pm 23.4$   $\mu\text{g/ml}$ ;  $n=4$ ) in our TR-FRET assay (Figure 3D). The inhibition was partial (~50%), which is consistent with the proposed allosteric, rather than competitive, binding mode (Figure 3D). Indeed, heparan sulfate/heparin has been shown to bind to the RBD at a site that is distinct from the ACE2 binding site (Clausen et al., 2020). Binding of heparin is suspected to induce conformational changes in RBD and to stabilize its open conformation prone to ACE2 binding (Clausen et al., 2020). Equilibrium binding experiments show significantly lower affinity in the presence of heparin (Figure 3E; Table 1) and kinetic studies showed a 3.4 times slower association rate ( $k_{on}$ ) and comparable dissociation rate ( $k_{off}$ ) than in the absence of heparin (Figure 3F-G; Table 1). Collectively, the results show that our RBD/ACE2 assay captures interaction inhibitors with allosteric binding mode and clarifies the effect of proximal heparan sulfates present in the extracellular matrix on the RBD/ACE2 interaction, *i.e.* increasing the affinity due to increased association rate.

Another potential modulator of RBD/ACE2 interaction in the cell surface is CD4, which has been recently described as essential for viral entry into CD4<sup>+</sup> T helper lymphocytes (Davano et al., 2020). Molecular modeling predicted binding of the extracellular N-terminal domain of CD4 to SARS-CoV-2 Spike RBD at a site overlapping with ACE2 binding suggesting a competition of CD4 and ACE2 for RBD binding. Further functional observations suggest rather

a concerted action of ACE2, TMPRSS2 and CD4 to allow the infection of CD4<sup>+</sup> T cells by SARS-CoV-2. To clarify this issue, the co-expression of full-length CD4 and SNAP-ACE2 showed a significantly decreased affinity and maximal binding of the RDB-d2 tracer to ACE2 (Figure 3H, Table 1), suggesting a competition between CD4 and ACE2 for RBD. Kinetic measurements showed a 3.3 times slower association rate ( $k_{on}$ ) and comparable dissociation rate ( $k_{off}$ ) than without CD4 (Table 1). These results suggest additional modulation of the binding properties of the RBD/ACE2 interaction by CD4 through conformational changes.

## DISCUSSION

In this study, we have developed a novel binding assay that probes the interaction of ACE2 with the SARS-CoV-2 Spike protein at the plasma membrane of living cells. The assay is based on the proximity between ACE2 and RBD on cells and displays multiple advantages: rapid and easy to perform, homogeneous without washing steps, compatible with HTS in a physiologically relevant cellular context. The assay will be of valuable importance in the pandemic as a reliable foundation/starting point for drug discovery, being compatible with HTS and as a research tool for understanding the mechanism of action and pharmacology of the RBD/ACE2 interaction.

*In vitro* RBD/ACE2 binding assays rely on the immobilization of one of the interacting partners, typically purified ACE2, to a support or sensor chip. Among those assays, the ELISA technique is commonly used to assess RBD binding to purified ACE2 in the presence of putative competitors. However, such assays are not homogenous, require washing steps and can suffer from detection limits, large variability and non-negligible false positives as detailed recently in the context of COVID-19 (GeurtsvanKessel et al., 2020). Other more sophisticated *in vitro* binding techniques include surface plasmon resonance (SPR) and bilayer interferometry (BLI). The range of  $K_d$  values of the RBD/ACE2 complex determined by SPR and BLI varied widely

from 1.2 to 117 nM (mean  $42.5 \pm 41.3$  nM) across reported *in vitro* binding studies (Table S1). Likewise, corresponding  $k_{on}$  and  $k_{off}$  values vary widely with mean values of  $1.8 \pm 1.1 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$  and  $4.9 \pm 3.3 \times 10^{-3}\text{s}^{-1}$ , respectively. Kinetic parameters from cellular binding assay were lacking. The  $k_{on}$  and  $k_{off}$  values derived from our TR-FRET assay were  $1.3 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$  and  $2.93 \times 10^{-3}\text{s}^{-1}$ , respectively. Interestingly, the TR-FRET  $k_{on}$  was 6.6 times higher compared to *in vitro* studies indicating that cellular components significantly enhance the association rate of RBD to ACE2.

Available *in vitro* assays lack the complexity of cellular context including participation of auxiliary cell surface proteins or cellular elements. Viral entry-associated membrane fusion requires a priming step of the spike protein mediated by host proteases including TMPRSS2 that cleaves the spike protein at a site between the S1 and S2 domains to activate plasma membrane fusion (Hoffmann et al., 2020). Previous studies showed that TMPRSS2 interacts with ACE2 leading to the proteolytic cleavage of ACE2, which was proposed to augment viral infectivity of SARS-CoV-1 (Shulla et al., 2011) (Heurich et al., 2014) and potentially of SARS-CoV-2 (Mohammad et al., 2020). In our binding assay, expression of TMPRSS2 decreases the TR-FRET signal between RBD or full-length spike (S1-S2) with ACE2 suggesting a conformational change within the RBD/ACE2 complex. TMPRSS2-induced cleavage of ACE2 is unlikely to account for the decreased TR-FRET signal as the assay is homogenous and the soluble ACE2 domain remains fully functional in terms of RBD binding as demonstrated by others (Shang et al., 2020) (Toelzer et al., 2020).

HSPG are expressed at the cell surface of many cells and have been shown to be essential for SARS-CoV-2 entry in combination with ACE2, adding a further cell context-dependent component to the RBD/ACE2 interaction (Clausen et al., 2020). HSPGs contain negatively charged heparan sulfates that promote interactions with a variety of positively charged cargos

(Christianson & Belting, 2014). The RBD harbors a positively charged cleft suspected to accommodate heparan sulfate in a ternary complex composed of heparan sulfate/RBD/ACE2 (Clausen et al., 2020). Addition of exogenous heparin competes with the heparan sulfate binding to RBD (Clausen et al., 2020) (Zhang et al., 2020). By probing the proximity of RBD to ACE2 at the plasma membrane, our TR-FRET assay revealed a 50% decrease of the TR-FRET by heparin and a significant increase in the  $k_d$  of RBD binding to ACE2 due to a decreased  $k_{on}$ . Of note, the  $k_{on}$  of  $3.8 \cdot 10^5 \text{ M}^{-1} \text{ s}^{-1}$  in the presence of heparin was close to the mean  $k_{on}$  of *in vitro* studies of  $1.8 \cdot 10^5 \text{ M}^{-1} \text{ s}^{-1}$  as compared to the  $k_{on}$  of  $13 \cdot 10^5 \text{ M}^{-1} \text{ s}^{-1}$  in our cellular TR-FRET assay in absence of any competitors. These data are compatible with the suspected function of HSPGs, present in a cellular context, to accelerate and facilitate the interaction of RBD with ACE2 by increasing the affinity (decreased  $K_d$  and increased  $k_{on}$ ) and the residential time. The partial displacement of the RBD-d2 tracer by heparin suggests an allosteric effect of heparin on the RBD/ACE2 interaction. Displacement of HSPGs by heparin most likely induces conformational changes within RBD that alter the affinity of RBD for ACE2 in an allosteric manner. This would be in favor of the model in which RBD would adopt an “up/open orientation” facilitating endocytosis and viral infection (Wrapp et al., 2020) (Walls et al., 2020). Alternatively, ACE2 might exist in two conformers, one being heparan sulfate-dependent and the other heparan sulfate-independent. Heparin would inhibit binding of RBD to one ACE2 conformer and RBD can still bind ACE2 in the second ACE2 conformation, but with a lower affinity. In conclusion, the important impact of HSPG on the RBD/ACE2 interaction nicely illustrates the importance of the cellular environment for the RBD/ACE2 interaction.

Intensive research on SARS-COV-2 has given rise to the hypothesis that, beside ACE2, other cellular components including heparan sulfate (Clausen et al., 2020) and alternative secondary receptors like neuropilin1 (Cantuti-Castelvetri et al., 2020), CD4 (Davanzo et al., 2020), CD147

(Wang et al., 2020) (Ulrich & Pillat, 2020) and GRP78 (Ibrahim et al., 2020) could actively participate in viral binding and entry. Here we observed that CD4 co-expression with ACE2 systematically decreases the plateau of TR-FRET signal, indicating CD4-mediated modulation of RBD/ACE2 complex. Cellular components such as membrane proteins can influence and modulate the RBD/ACE2 interaction, reinforcing the importance of performing the binding studies in cells either for mechanistic investigations, pharmacological characterization or screening campaigns.

An easy, robust, sensitive and reproducible biochemical interaction assay is essential for implementing HTS campaigns. Our TR-FRET RBD/ACE2 binding assay fulfills these criteria with its homogenous format, a robust TR-FRET signal (10-30 fold over background), an excellent  $Z'$  score of 0.83 and its high sensitivity detecting inhibitors in the subnanomolar range. A recent study aimed at identifying inhibitors of the RBD/ACE2 interaction in a drug repurposing strategy through an *in vitro* AlphaLISA proximity-based assay (Hanson et al., 2020). We picked 5 out of the 25 top hits identified by this *in vitro* assay for validation in our cellular TR-FRET assay. Only 1 out of 5 compounds, corilagin, the most active compound in the AlphaLISA, showed a partial inhibition at 100 $\mu$ M, a concentration more than 100 times higher than the  $IC_{50}$  reported in the AlphaLISA assay. The recently described LCB1v3 mini-protein targeting the RBD/ACE2 interaction showed an  $IC_{50}$  in the subnanomolar range (Cao et al., 2020) validating our assay for the screening of RBD/ACE2 interaction inhibitors with high affinity. Collectively, these data show that *in vitro* assays can overestimate the inhibitory capacity of compounds and highlight the need to perform screening campaigns with cellular assays.

Our TR-FRET assay is highly suitable for the characterization of therapeutic antibodies in terms of epitope specificity, binding properties, and in structural/functional studies

(DeFrancesco, 2020). In addition, performing the TR-FRET assay in a physiological context with cellular complexity can improve the assessment of vaccine efficacy by evaluating whether RBD-specific antisera might block the interaction between RBD to ACE2 at the level of the cell rather than in a restrictive *in vitro* setting.

In conclusion, the novel TR-FRET assay allows studying of the interaction of RBD with ACE2 on living cells in a physiological context. Unlike an *in vitro* binding assay, an additional layer of complexity is observed biological membranes containing further relevant components such TMPRSS2, HSPG, CD4, etc. Hence the assay can be adapted for a specific purpose via co-expression of membrane receptors. We expect, therefore, that our TR-FRET assay can be applied to boost drug development programs for COVID-19, to characterize neutralizing antibodies or optimize vaccine efficacy, as well as for mechanistic studies of viral Spike binding to cells.

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## **AUTHOR CONTRIBUTIONS**

Conceptualization, E.C., J.D., and R.J.; Biochemical investigation, E.C., M.B., and J.D.; L.C., R.R., and L.C. contributed unique reagents; Writing – Original Draft, J.D., and R.J.; Writing –

Review and Editing, E.C., J.D., and R.J.; Funding Acquisition, R.J; Supervision, E.C., J.D. and R.J.

### **COMPETING INTERESTS**

The authors declare no competing interest.

**Table 1. Binding constants of SARS-CoV-2 Spike RBD binding to ACE2 determined in TR-FRET assay.**

Condition	Equilibrium $K_d$ (nM)	$K_{on}$ ( $M^{-1} \cdot s^{-1}$ )	$K_{off}$ ( $s^{-1}$ )	Kinetically derived $K_d = K_{off}/K_{on}$ (nM)	RT = $1/K_{off}$ (min)
Control	$14.6 \pm 2.5$	$1.3 \cdot 10^6$	$2.93 \cdot 10^{-3}$	2.3	4.1
+ Heparin	$25.2 \pm 8.7^*$	$3.8 \cdot 10^5$	$2.78 \cdot 10^{-3}$	7.3	7.0
+ CD4 expression	$28.9 \pm 9.5^*$	$3.9 \cdot 10^5$	$2.31 \cdot 10^{-3}$	6.0	7.2

Data are expressed as mean  $\pm$  SEM of 3 to 10 independent experiments. \* $p < 0.05$  compared to the 'Control' condition by paired  $t$ -test. RT, residence time.

## FIGURE LEGENDS

**Figure 1. Development of SARS-CoV-2 Spike protein/ACE2 TR-FRET binding assay.** **A)** Principle of time-resolved TR-FRET assay. **B)** Scheme illustrating the TR-FRET-based RBD-d2 binding assay to SNAP-tagged ACE2 labelled with Lumi4-Tb. **C)** Saturation binding curve of RBD-d2 to Lumi4-Tb-labelled SNAP-ACE2 expressed in HEK293 cells (n=10). Non-specific binding was defined in the presence of an excess of unlabelled RBD (1  $\mu$ M). **D-E)** Association (**D**) and dissociation (**E**) kinetics of RBD-d2 binding to Lumi4-Tb-SNAP-ACE2 expressed in HEK293 cells (representative curve, n=4). Dissociation was initiated by adding unlabelled RBD (1  $\mu$ M). **F)** Binding of RBD-d2 to Lumi4-Tb-SNAP-ACE2 but not Lumi4-Tb-SNAP-VEGFR2; insert: expression level of Lumi4-Tb-labelled SNAP-ACE2 and SNAP-VEGFR2. Data are expressed as mean  $\pm$  SEM of at least 3 independent experiments, each performed in triplicate. **G)** Competition of RBD-d2 binding to Lumi4-Tb-SNAP-ACE2 by non-labelled RBD (1  $\mu$ M), leptin, IL6, MCP1, Gal3, insulin, EGF, VEGF (each at 100 nM); data are expressed as mean  $\pm$  SEM of 3 independent experiments, each performed in triplicate; \*\*\*\*p=0.0001 by one-way ANOVA. **H)** RBD-d2 interaction with HEK293 cells expressing either SNAP-ACE2 or SNAP-LepR monitored by immunofluorescence microscopy. See also Figure S1.

**Figure 2. Detection of competitors of the RBD/ACE2 interaction using the TR-FRET-based assay.** **A)** Competition of RBD-d2 binding to Lumi4-Tb-SNAP-ACE2 in HEK293 cells by unlabelled RBD or LCB1v3 miniprotein; data are expressed as mean  $\pm$  SEM of at least 4 independent experiments, each performed in triplicate. **B)** Determination of Z' by collecting TR-FRET signals for Lumi4-Tb-labelled SNAP-ACE2 expressing HEK293 cells in the absence or presence of RBD (300nM) from 50 different wells each. **C-G)** Competition of RBD-d2

binding to Lumi4-Tb-SNAP-ACE2 by Cangrelor, Elaidic acid, Fenbendazole, Enalapril Maleate or Corilagin; data are expressed as mean  $\pm$  SEM of 3-6 independent experiments, each performed in triplicate. \*\*\*\*p<0.0001 by one-way ANOVA.

**Figure 3. Modularity of SARS-CoV-2 Spike RBD/ACE2 binding.** **A)** Competition of RBD-d2 binding to Lumi4-Tb-SNAP-ACE2 by HEK293 cells expressing unlabelled SNAP-ACE2 (transcellular mode) or RBD (1 $\mu$ M). **B-C)** Binding of RBD-d2 (**B**) or Spike S1-S2-d2 (**C**) to Lumi4-Tb-labelled SNAP-ACE2 in HEK293 cells with and without TMPRSS2 co-expression. Insert: expression level of Lumi4-Tb-labelled SNAP-ACE2 in both conditions. Unlabelled RBD (1 $\mu$ M), unlabelled S1-S2 (200 nM). **D)** Competition of RBD-d2 binding to Lumi4-Tb-SNAP-ACE2 by heparin. **(A-C)** Data are expressed as mean  $\pm$  SEM of 3 independent experiments, each performed in triplicate; \*\*\*\*p<0.0001 by one-way ANOVA, or two-way ANOVA when comparing the effect of the presence of TMPRSS2; n.s. = not-significant. **E)** Saturation binding curve of RBD-d2 to Lumi4-Tb-SNAP-ACE2 in the absence and presence of heparin (3 mg/mL; n=4). Non-specific binding was defined by an excess of unlabelled RBD (1  $\mu$ M). HSGP, heparan sulfate glycoprotein. **F-G)** Association (**F**) and dissociation (**G**) kinetics of RBD-d2 binding to Lumi4-Tb-SNAP-ACE2 in the absence or presence of heparin (3 mg/mL; n=3). Dissociation was initiated by adding unlabelled RBD (1  $\mu$ M). **H)** Saturation binding curve of RBD-d2 to Lumi4-Tb-labelled SNAP-ACE2 in HEK293 cells with and without CD4 co-expression (representative curve, n=5). Non-specific binding was defined by an excess of unlabelled RBD (1  $\mu$ M).

## STAR \*METHODS

### RESOURCE AVAILABILITY

#### *Lead Contact*

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ralf Jockers (ralf.jockers@inserm.fr).

#### *Materials Availability*

This study did not generate new unique reagents / Plasmids generated in this study have been deposited to [Addgene, name and catalog number or unique identifier].

#### *Data and Code Availability*

This study did not generate/analyze any datasets or code.

### EXPERIMENTAL MODEL AND SUBJECT DETAILS

#### *RBD-d2 and S1-S2-d2*

RBD and full-length Spike (S1-S2) were purchased from SinoBiological and labelled with d2 by Cisbio Bioassay on lysines with a N-hydroxysuccinimide activated d2 dye in 100mM PO<sub>4</sub> buffer (pH8). Molar ratio of d2/protein is calculated according to protein concentration measured at 280nm and d2 concentration determined at 665nm.

#### *LCB1.v3 miniprotein*

The LCB1.v3 protein was designed using computational methods and produced using standard IPTG expression in BL21pLysS cells (Novagen). The protein was lysed using a microfluidizer and purified via Immobilized Metal Affinity Chromatography (IMAC) using Cytiva IMAC Sepharose FF resin charged with NiSO<sub>3</sub> and eluted using imidazole. The protein was polished using Size Exclusion Chromatography (SEC) on a Cytiva Superdex 75 Increase column. Binding of the purified protein to RBD was confirmed via Bilayer Interferometry (BLI) using Sartorius Octet96.

#### *Cell lines*

HEK293T (RRID:CVCL 0063) cells were obtained from Sigma-Aldrich and authenticated by the provider. Cell cultures were maintained in Dulbecco's Modified Eagle's Medium (DMEM) Glutamax (Invitrogen) supplemented with 10% fetal bovine serum and 1% streptomycin-penicillin, at 37°C (95% O<sub>2</sub>, 5% CO<sub>2</sub>). Cell lines were checked regularly for any mycoplasma contamination.

### METHOD DETAILS

#### *Expression vectors and cell transfection*

Plasmids encoding for SNAP-tagged proteins (human VEGFR2, human LepR) were obtained from Cisbio Bioassays (Codolet, France). The SNAP-tagged human ACE2 construct was obtained by introducing the ACE2 sequence into the SNAP vector through restriction enzyme cloning. The pCEP4-myc-ACE2 vector was a gift from Erik Procko (Addgene plasmid # 141185 ; <http://n2t.net/addgene:141185>; RRID:Addgene\_141185) (Chan et al., 2020). The SNAP protein is also fused to a FLAG tag. The TMPRSS2 vector was a gift from Roger Reeves (Addgene plasmid # 53887; <http://n2t.net/addgene:53887>; RRID:Addgene\_53887) (Edie et al., 2018). The CD4 vector was a gift from Dr. Stefano Marullo (Achour et al., 2009). Cells were transfected with 1 µg of SNAP-ACE2 or other SNAP-tagged proteins (when indicated) in 6-

well plate using jetPEI reagent according to the supplier's instructions (Polyplus-transfection, New York, NY, USA), and assay was performed 48h post-transfection.

### ***TR-FRET binding assay***

SNAP-tagged ACE2 is fluorescently labelled by incubating the cells with a SNAP suicide substrate conjugated to the long-lived fluorophore Terbium cryptate (Tb; Lumi4-Tb, 100 nM; Cisbio Bioassays) in Tag-lite labelling medium (1h, on ice) (Keppler et al., 2003). After several washes, cells are collected using enzyme-free cell dissociation buffer (Sigma-Aldrich), resuspended in Tag-lite buffer and distributed into a 384-well plate. Efficient fluorescent labelling of SNAP is verified by reading fluorescence signal at 620nm. Cells are then incubated with vehicle or test compounds, followed by addition of RBD-d2, all diluted in Tag-lite buffer (final reaction volume of 14  $\mu$ l). After 2h incubation (at room temperature or 37°C, as indicated), TR-FRET signal is detected using a plate reader (Tecan F500; Tecan, Männedorf, Switzerland) with the following settings: excitation at 340 nm (Tb, energy donor), emission at 665 nm (d2, acceptor) and 620 nm (donor); delay of 150  $\mu$ s; and integration time of 500  $\mu$ s. Data are expressed as the acceptor/donor ratio or normalized as % when indicated (maximal TR-FRET ratio = 100%, basal without d2 = 0%). Association kinetics were determined by continuously monitoring TR-FRET signal during 60 min after addition of RBD-d2 (5nM). For dissociation kinetics, cells were incubated for 2h with RBD-d2 (5 nM) to reach the equilibrium, followed by the addition of saturating concentrations of non-labelled RBD. TR-FRET signals were then immediately and continuously monitored for 60 min. For the competition curves, competitors were pre-incubated 1h before adding RBD-d2. Obtained IC<sub>50</sub> values were converted into K<sub>i</sub> using the Cheng-Prusoff equation (Cheng & Prusoff, 1973). Z' value (Zhang et al., 1999) of the TR-FRET assay was determined using total and non-specific (excess of unlabelled RBD) as positive and negative controls, respectively, as follow:

$$\text{Z-factor} = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}$$

where  $\sigma_p$ = standard deviation of positive control;  $\sigma_n$ = standard deviation of negative control;  $\mu_p$ =mean of positive control;  $\mu_n$ =mean of negative control

### ***SDS-PAGE / Western Blot***

Lysates from cells transfected with SNAP-ACE2 were resolved in SDS-PAGE gel (10%), followed by protein transfer to nitrocellulose membranes. Membranes were blocked in 5% non-fat dried milk in TBS (10 mM Tris-HCl, pH 8, 150 mM NaCl), and immunoblotted with primary antibodies against the FLAG tag (1:1,000; F7425, Sigma-Aldrich), diluted in 0.3% BSA in TBS (overnight, 4°C). Immunoreactivity was revealed using secondary antibodies coupled to 680 or 800 nm fluorophores (1:15,000, diluted in 0.3% non-fat dried milk in TBST; LI-COR Biosciences, Lincoln, NE, USA), and readings were performed with the Odyssey LI-COR IR fluorescent scanner (LI-COR Biosciences).

### ***Immunofluorescence***

HEK293T cells expressing SNAP-ACE2 were plated in coverslips, labelled with green fluorophore-fused SNAP substrate, and incubated with RBD-d2 (20 nM, 2h). After several washes, cells were fixed with paraformaldehyde 4% solution (15 min), incubated with DAPI (Sigma-Aldrich) to stain cell nuclei, and the slides were analyzed under Zeiss Observer.Z1 microscope). Images were analysed using the ImageJ software.

## QUANTIFICATION AND STATISTICAL ANALYSIS

Data are presented as means  $\pm$  SEM of the indicated  $n$  (number of independent experiments), each performed at least in triplicate to ensure the reliability of single values. TR-FRET data are expressed as the acceptor/donor ratio or normalized to % of maximal binding. Bmax was defined as the maximal TR-FRET ratio obtained in equilibrium, while 0% is defined as the TR-FRET ratio measured in the absence of RBD-d2. Non-specific binding is determined in the presence of an excess of unlabelled RBD (1  $\mu$ M). Value of  $K_d$  was determined by plotting TR-FRET ratio against RBD-d2 concentration (saturation curves), and data were fitted to the non-linear regression “one-site-total and non-specific binding” (GraphPad Prism software version 6, RRID:SCR\_002798). Values of  $pIC_{50}$  ( $-\log IC_{50}$ ) and  $I_{max}$  (maximal inhibition as %, with 100% set as the competition obtained with non-labelled RBD) were obtained following non-linear regression of data from a minimum of eight different concentrations per experiment, repeated at least three times independently using the log (inhibitor) versus response (three parameters) fitting equation (GraphPad Prism). The  $k_{on}$  and  $k_{off}$  values were calculated from the association and dissociation kinetic experiments by fitting the data to the association kinetic model equation or one phase exponential decay equations, respectively, using the GraphPad Prism software. Statistical analysis was performed using GraphPad Prism software version 6. Comparisons between two groups were performed using Student's  $t$  test, while multiple groups comparisons were performed through ordinary one-way or two-way analysis of variance (ANOVA), followed by Tukey's or Dunnett's multiple comparison post hoc test in the cases where the differences between group means were significantly different ( $p < 0.05$ , one-way ANOVA) or when the main effects and interaction effects were detected as statistically significant ( $p < 0.05$ , two-way ANOVA). Values of  $p < 0.05$  were considered statistically significant.

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