nature research

Peer Review File

Manuscript Title: De novo design of modular and tunable protein biosensors

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referee expertise:

Referee #1: sensors, allostery

Referee #2: protein design and sensors

Referees' comments:

Referee #1 (Remarks to the Author):

The manuscript "De novo design of modular and tunable allosteric sensors", by Quijano-Rubio et al. describes the "application" of a design approach pioneered by the Baker lab to develop what is now (as a result of this paper) the new standard for biosensor design. To be clear, this is not derivative. In other words, even though I describe this as an application, it is certainly not an already described technology or approach focused on a new problem. In fact, the "approach" in this paper is based on a design strategy described earlier by the Baker group (Langan et al., Nature, 2019), which was the de novo design of allosteric proteins. The approach (i.e., designing from parts) utilizes expertise that is essentially unique to the Baker lab (i.e. the Rosetta algorithm and the associated design platform that has been developed over the past 20 years). The approach taken in this manuscript is to develop a two-component system consisting of a Cage and a Key molecule, with the cage molecule able to occupy either an open or a closed conformation, with the open conformation able to make interactions and bind with the key (which also is detectable through a complementation via a two-part luciferase interaction that is facilitated when the key and cage binding). The quintessential element to the approach is that the key can bind a ligand of choice (which can be designed) but only in the open conformation (Fig. 1). Thus, the principle is straightforward. The binding of target can under the right conditions provide enough energy to switch the equilibrium form favoring the closed to favoring the open conformation, and the luciferase signal provides the ability to quantify the amount of target present by detecting the fraction in the open conformation. Although this is theoretically possible, and the principle straightforward, doing it has been THE barrier to success since the field was started. Indeed, most successful design approached to date come not from designing the protein but from designing clever experimental selection mechanisms that allow the researchers to choose the variants that may work, even if they don't understand why, which quite expectedly necessarily requires much simpler architecture, and which does not allow rational tuning. Thus, the ability to design a biosensor system to: 1) have the equilibria poised at the correct relative energies, 2) have the interaction energy designed, and 3) be able tune them to meet the needs of the system, is what makes these designs work, separates this approach from anything done to date, and opens a whole new avenue of design. Indeed, this work effectively demonstrates how one can modularize protein domains from nature, repurpose them for new goals, and design in the appropriate tuning to make them effective. The result is pico-molar detection and a dynamic range of more than 1700%, which I believe can even be improved upon with further design and optimization. The authors applied this approach to several systems including Bcl-2, hIgG1 Fc domain, the Her2 receptor, botulinum neurotoxin B, troponin I and the HBV antibody to demonstrate their scope and utility. The performance is fantastic.

I do however have three questions that may be related but need clarification.

1) What are the curves through the data-points in figures 2,3, and 4? Are they fits of the data?

2) There are apparent changes in the Hill coefficient (i.e. the apparent cooperativity of the system). This is likely due to the changing of the regime where one free energy dominates at low ligand and, as the equilibrium is shifted, the steepness changes. This should be explained both in the simulations and in the test cases, where the behavior seems more complex.

3) The model articulated in Fig. 1 is essentially a thermodynamic cycle where the terms for delGLT, delGCK and delGR are independent from the other processes (in other words, it is assumed that there is not (unintentional) allosteric coupling between sites and tghus the terms are equal in both processes in which these terms are shown in Fig. 1A). As the effectiveness of a biosensor is predicated on precision, it would be useful to explain how the authors can evaluate the lack of coupling in their design systems. I bring this up because the Hill coefficients noted in 2 (above) will be affected by coupling between the free energy terms noted above.

In short, this is an outstanding, original and transformative paper that establishes a new paradigm for biosensor design. The data are very convincing and the conclusions appear sound. Aside from the minor concerns noted above, I am convinced this work will have an immediate impact on the field. In addition, I find the manuscript very well written and accessible to a broad scientific audience. In my opinion, it should be published in Nature.

Referee #2 (Remarks to the Author):

The manuscript by Quijano-Rubio and coworkers reports on the development of a new, modular biosensor platform that allows bioluminescent detection of a wide range of protein biomarkers. The platform is based on the Latching Orthogonal Cage-Key Proteins (LOCKR) platform of helical bundle proteins in which binding of input proteins opens up a latch that actuates a downstream process (ref 2). Here the system is used to control complementation of a split luciferase. The sensor system consists of two protein components: the LucCage, which contains a latch that includes both the small-bit luciferase peptide and an analyte binding motif which is designed such that key residues that are known to interact with the target analyte are interacting with the Cage scaffold and not available for binding to the analyte. The second component is the lucKey, which contains a helix that can compete with the latch domain in LucCage fused to LargeBiT. Analyte binding to the analyte binding motif destabilized the intramolecular interaction of the latch with LucCage, which allows intermolecular binding of the LucKey and reconstitution of luciferase activity. The system is modular to the extend that the same LucKey and basic LucCage scaffold can be used, although the introduction of the analyte binding domain in LucCage does require de novo design and some optimization for each new analyte. In this respect the concept is not more modular than previously reported bioluminescent switches such as the LUCID and LUMAB platforms, although the platform reported here is more suitable for protein targets, whereas LUCIDs and LUMAB target small molecules and antibodies, respectively. Another novel aspect is the integration of de novo protein design and biosensor development. The potential of the technology to be broadly applicable is clearly demonstrated by reporting sensor systems for a broad variety of protein biomarkers, including anti-SARS-Cov-2 antibodies and the SARS-CoV-2 spike RBD protein. The latter uses a recently developed high-affinity de novo binding domain for the RBD, and was apparently integrated in the sensor system in only 3 weeks.
While the latter is impressive and a testament of the ease by which the platform can be adapted to new and emerging target analytes (at least by a protein engineering powerhouse), I also feel that the analytical possibilities and limitations of the new sensor system have not been extensively explored/characterized. The thermodynamic modeling/simulations depicted in Figure 1 shows that the system provides multiple opportunities to tune the systems performance in terms of sensitivity

and dynamic range, including tuning the affinity of latch-cage interaction, the analyte-binding domain interaction, the cage-key interaction and, because it is a two component system, the concentration of the two sensor components. In most cases, only the effect of concentration is studied, which allows one to tune the responsive concentration range. However, the increase in luminescence is relatively modest in many cases, in particular for a split luciferase system, where a >300-fold increase in luminescence should in principle be possible when the SBiT is effectively caged and full complementation is reached upon analyte binding. Little insight is provided why in many cases the increase is modest, e.g. whether this is due to inefficient caging, or a limited thermodynamic driving force for activation upon analyte binding. Another important issue that is not addressed but important for understanding the system and its potential for point-of-care applications, is the increasing background signal that is often observed. E.g. Fig 2b/c/d and Fig 3e all show an increase in luminescence even in the absence of analyte. What is the origin of this effect, does it mean that also in the absence of the analyte the key is able to bind to the cage but that the binding is kinetically hampered in the absence of the analyte? Finally, a potential drawback of the system seems to be its slow kinetics (except for the antibody sensors), sometimes taking several hours to reach equilibrium even at a relatively high analyte concentration. These relatively slow kinetics may be governed by the kinetics of latch dissociation, and require more characterization and discussion in the manuscript. Finally, as the system is designed for homogeneous biosensing in point-of-care diagnostics, it would be useful to test the performance of the system in complex samples such as (diluted) serum.

Other questions and comments:
- the system was introduced from the perspective of the previous work of these authors on the LOCKR system, arguing in a rather abstract terms that in the present system 'the flow of information is reversed' and the 'key and analyte' become equivalent. I am afraid that this will make it harder for the average reader to understand the sensor principle than a more direct introduction and explanation of the system and its components.

- It would be helpful if the authors could discuss whether there are any restrictions on the structure of the analyte binding domain for it to be compatible with the Cage, e.g. all domains seem to have an N-terminal alpha-helix.
- ref 6-8 do not all refer to semi-synthetic systems, ref 38 (line 813) is not the first example of an antibody sensor based on the beta-lactamase-BLIP system (-> https://pubs.acs.org/doi/10.1021/cb400406x)
- one could argue whether the target is allosterically regulating luciferase activity (line 88-89), I think it is better describes as competitive displacement
- The interaction between the LBiT and SBiT can be tuned between sub-nM and 190 microM Kd. The authors should clearly mention which LBiT variant they used, and whether they varied this affinity.
- Many of the titration data in the main figures lack error bars, while they typically are present in the figures in the extended data. Please add them here as well. This is particularly important in Fig 4D, as the standard deviation is necessary to calculate the LOD.
- The specificity of the various sensors is studied by monitoring the response to all other analytes in time (Figure 5). These traces show variation in the luminescent signal for the blank and in the presence of non-target analytes, sometimes increasing, sometimes decreasing. What is the origin of these time-dependent effects? Also. why does the signal in some cases in the presence of the analyte first show an increase and then a decrease in luminescent signal?
- Line 265-267 'Most of these platforms depend on the specific geometry of target-sensor interaction to trigger a conformational change..' -> this may be true for some systems, but it does not apply to most of the examples referred to in ref 20, 35, 7, 34 and 6
- line 297: ref 36 does not report on the incorporation of designed binders
- Table S3: the sequence of the LCB1 domain is missing in this table. The same is true for the sequences of the lucCageRBD variants in Table S5.
- Table S4: the potential to photobleach is presented as a drawback of the LUMABS and CLASH

systems, but this seems farfetched for a bioluminescent system.

A Response to Referees letter

De novo design of modular and tunable protein biosensors

Referees' comments:

Referee #1 (Remarks to the Author):

The manuscript “De novo design of modular and tunable allosteric sensors”, by Quijano-Rubio et al. describes the “application” of a design approach pioneered by the Baker lab to develop what is now (as a result of this paper) the new standard for biosensor design. To be clear, this is not derivative. In other words, even though I describe this as an application, it is certainly not an already described technology or approach focused on a new problem. In fact, the “approach” in this paper is based on a design strategy described earlier by the Baker group (Langan et al., Nature, 2019), which was the de novo design of allosteric proteins. The approach (i.e., designing from parts) utilizes expertise that is essentially unique to the Baker lab (i.e. the Rosetta algorithm and the associated design platform that has been developed over the past 20 years). The approach taken in this manuscript is to develop a two-component system consisting of a Cage and a Key molecule, with the cage molecule able to occupy either an open or a closed conformation, with the open conformation able to make interactions and bind with the key (which also is detectable through a complementation via a two-part luciferase interaction that is facilitated when the key and cage binding). The quintessential element to the approach is that the key can bind a ligand of choice (which can be designed) but only in the open conformation (Fig. 1). Thus, the principle is straightforward. The binding of target can under the right conditions provide enough energy to switch the equilibrium form favoring the closed to favoring the open conformation, and the luciferase signal provides the ability to quantify the amount of target present by detecting the fraction in the open conformation. Although this is theoretically possible, and the principle straightforward, doing it has been THE barrier to success since the field was started. Indeed, most successful design approached to date come not from designing the protein but from designing clever experimental selection mechanisms that allow the researchers to choose the variants that may work, even if they don’t understand why, which quite expectedly necessarily requires much simpler architecture, and which does not allow rational tuning. Thus, the ability to design a biosensor system to: 1) have the equilibria poised at the correct relative energies, 2) have the interaction energy designed, and 3) be able tune them to meet the needs of the system, is what makes these designs work, separates this approach from anything done to date, and opens a whole new avenue of design. Indeed, this work effectively demonstrates how one can modularize protein domains from nature, repurpose them for new goals, and design in the appropriate tuning to make them effective. The result is pico-molar detection and a dynamic range of more than 1700%, which I believe can even be improved upon with further design and optimization. The authors applied this approach to several systems including Bcl-2, hIgG1 Fc domain, the Her2 receptor, botulinum neurotoxin B, troponin I and the HBV antibody to demonstrate their scope and utility. The performance is fantastic.

I do however have three questions that may be related but need clarification.

1) *What are the curves through the data-points in figures 2,3, and 4? Are they fits of the data?*

Response: These curves were generated by sigmoidal 4PL non-linear regression for EC50 calculation. This information is included in the methods section.

2) *There are apparent changes in the Hill coefficient (i.e. the apparent cooperativity of the system). This is likely due to the changing of the regime where one free energy dominates at low ligand and, as the equilibrium is shifted, the steepness changes. This should be explained both in the simulations and in the test cases, where the behavior seems more complex.*

Response: This is an excellent point. The steepness of the sensor response to increasing target concentrations in the simulations is determined by the Kd of the target binding interaction and the concentration of the sensor components. When the concentration of the sensor components is greater than the Kd of the binding interaction, added target becomes fully complexed with the sensor, and the response is effectively linear in the target concentration. When the concentration of sensor components is close to the Kd of the target-binding interaction, the response is closer to a standard sigmoid binding curve. In the test cases, this same effect contributes. As the reviewer points out, couplings between the binding free energies could also affect the steepness of the transition, but as noted in our response to the following question, such couplings if they exist appear to be quite weak.

We have added these points to Extended Data Fig 1, in which we include a version of Figure 1f with a linear x axis which makes the titration behaviour at high sensor concentrations more evident.

3) The model articulated in Fig. 1 is essentially a thermodynamic cycle where the terms for delGLT, delGCK and delGR are independent from the other processes (in other words, it is assumed that there is not (unintentional) allosteric coupling between sites and thus the terms are equal in both processes in which these terms are shown in Fig. 1A). As the effectiveness of a biosensor is predicated on precision, it would be useful to explain how the authors can evaluate the lack of coupling in their design systems. I bring this up because the Hill coefficients noted in 2 (above) will be affected by coupling between the free energy terms noted above.

Response: The reviewer is absolutely correct; our thermodynamic model assumes that the ΔG_{LT}, ΔG_{CK} and ΔG_{open} are independent from each other. It is formally possible that the sites are allosterically coupled. As suggested by the reviewer in question 2, and noted above, such coupling could alter the steepness of the sensor response to target. Such couplings could exist, but given the similarity of the transitions (taking into the sensor concentration dependence, as noted in the response to the previous question) they are not likely to be large. In principle, it should be possible to obtain quantitative estimates for the magnitudes of such couplings by explicit fits to the experimental data, but we have not been able to do so robustly given the number of fit parameters and the limited dataset.

In short, this is an outstanding, original and transformative paper that establishes a new paradigm for biosensor design. The data are very convincing and the conclusions appear sound. Aside from the minor concerns noted above, I am convinced this work will have an immediate impact on the field. In addition, I find the manuscript very well written and accessible to a broad scientific audience. In my opinion, it should be published in Nature.

We are very glad the reviewer shares our enthusiasm for the approach!

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Response: We agree with the reviewer; LUCID and LUMAB are modular platforms for small molecule and antibody sensors. The challenge with a general protein sensor is the much greater diversity of binding interactions--this is clear when compared to LUMAB, which takes advantage of the common bivalent and geometrically spaced interaction between Fabs and their targets. Our system here makes protein detection almost as modular as small molecule and antibody detection are with LUCID and LUMAB by coupling target binding interactions, which are very different in each individual case, to a common overall conformational change. While bivalent antibody binding directly determines the nature of the conformational change in LUMAB (separating the flanking reporter domains), target binding in the lucCage sensor contributes thermodynamically but not structurally to the opening of the switch and the binding of the key. We have clarified these points in the revised manuscript.

Another novel aspect is the integration of de novo protein design and biosensor development. The potential of the technology to be broadly applicable is clearly demonstrated by reporting sensor systems for a broad variety of protein biomarkers, including anti-SARS-Cov-2 antibodies and the SARS-CoV-2 spike RBD protein. The latter uses a recently developed high-affinity de novo binding domain for the RBD, and was apparently integrated in the sensor system in only 3 weeks.
While the latter is impressive and a testament of the ease by which the platform can be adapted to new and emerging target analytes (at least by a protein engineering powerhouse), *I also feel that the analytical possibilities and limitations of the new sensor system have not been extensively explored/characterized.* The thermodynamic modeling/simulations depicted in Figure 1 shows that the system provides multiple opportunities to tune the systems performance in terms of sensitivity and dynamic range, including tuning the affinity of latch-cage interaction, the analyte-binding domain interaction, the cage-key interaction and, because it is a two component system, the concentration of the two sensor components. *In most cases,*

only the effect of concentration is studied, which allows one to tune the responsive concentration range.

Response: We agree with the reviewer; our platform offers multiple opportunities for tuning system performance, and we did not fully explore these in the original manuscript. In the revised manuscript, we have added supplementary data with simulation (Extended Data Fig. 1) and experimental data (Extended Data Fig. 13) showing how the lucCageRBD sensor can be tuned by varying the lucKey affinity for lucCage (K_{CK}) and the lucKey concentration. We prepared a truncated lucKey, which is 14 residue shorter than the full-length lucKey, for experimental evaluation of the effect of K_{CK} on the dynamic range of lucCageRBD. The truncated lucKey exhibited 2.8-fold higher dynamic range than the full-length lucKey owing to reduced background signal, which is consistent with simulation data (we carried out simulations over a broader range of parameter values than in Fig. 1B in the original manuscript to make the contributions of varying the different thermodynamic parameters clearer). Further consistent with the simulation data, we find that decreasing lucKey concentration increases the dynamic range of lucCageRBD owing to reduced background signal, but with concomitant reduction of the saturated signal. These results are now shown as Extended Data Fig. 13.

However, the increase in luminescence is relatively modest in many cases, in particular for a split luciferase system, where a >300-fold increase in luminescence should in principle be possible when the SBiT is effectively caged and full complementation is reached upon analyte binding. Little insight is provided why in many cases the increase is modest, e.g. whether this is due to inefficient caging, or a limited thermodynamic driving force for activation upon analyte binding.

Response: We agree with the reviewer that the increase in luminescence is lower than that expected for full luciferase reconstitution for many of the sensors. In the revised manuscript, as suggested by the reviewer, we discuss possible origins of this sub-optimality. The simple thermodynamic model suggests that the dynamic range at saturating target concentration is determined solely by the background signal in the absence of target which can be tuned through ΔG_{open} and ΔG_{CK} (Now shown in Extended Data Fig. 1 and 13). The dynamic range of sensors with high background should in theory be able to be improved just by tuning those two parameters. However, sub-maximal activation at saturating target concentration also contributes to the limited dynamic range; this effect is not captured by the simple thermodynamic model. We have quantified the contributions to the overall dynamic range in Supplemental Table 4. Both the background signal in the absence of target, and the extent of activation at saturating target concentrations varies considerably between the different sensors, and are clear areas for improvement. We also provide in Extended data Fig. 14 an evaluation of the effect of key concentration on the extent of activation.

Another important issue that is not addressed but important for understanding the system and its potential for point-of-care applications, is the increasing background signal that is often observed. E.g. Fig 2b/c/d and Fig 3e all show an increase in luminescence even in the absence of analyte. What is the origin of this effect, does it mean that also in the absence of the analyte the key is able to bind to the cage but that the binding is kinetically hampered in the absence of the analyte?

Response: The increase in luminescence in the absence of analyte in the figures in the original manuscript reflects a lack of equilibration of the system prior to addition of

analyte and was due to suboptimal experimental design. In an actual use case, the components would be pre-mixed and so lack of equilibration would not be an issue. In the revised manuscript, we have extended the equilibration period prior to analyte addition, which leads to a flattening of the baseline. For example, in the revised specificity comparison, which now includes the RBD sensor, the components were allowed to fully equilibrate, and the baselines are quite flat. We apologize for the confusion this caused in the original manuscript and we have updated the experimental protocol in the method section of the manuscript to reflect this.

Finally, a potential drawback of the system seems to be its slow kinetics (except for the antibody sensors), sometimes taking several hours to reach equilibrium even at a relatively high analyte concentration. These relatively slow kinetics may be governed by the kinetics of latch dissociation, and require more characterization and discussion in the manuscript.

Response: We agree with the reviewer, the slow kinetics likely reflects slow dissociation of the latch from the cage. Future work will aim for more rapid opening and closing of the switch to allow more rapid binding to analyte when it is present (keeping ΔG_{open} close to its current value to avoid increasing background or decreasing sensitivity). We have incorporated this point in the revised manuscript.

Finally, as the system is designed for homogeneous biosensing in point-of-care diagnostics, it would be useful to test the performance of the system in complex samples such as (diluted) serum.

Response: We tested the lucCageRBD sensor for SARS-CoV-2 RBD detection in simulated nasal matrix and serum. The luminescence intensity decreases in these media in comparison with that in a buffer solution. We found that sensor responses to RBD spiked in to four different patients' sera varied in luminescence intensity. We overcame the challenge in quantifying analyte levels posed by this variability guided by an excellent suggestion from Prof. Maarten Merkx-- we calibrated the system by ratiometric sensing of the RBD sensor in different matrices. We show that, by internal referencing with the CyOFP1-teLuc-CyOFP1 BRET system, the matrix effects on the bioluminescence intensity can be controlled for, and thus the variations of the intensiometric bioluminescence can be minimized. These results are now shown as Extended Data Fig. 12.

Other questions and comments:
- the system was introduced from the perspective of the previous work of these authors on the LOCKR system, arguing in a rather abstract terms that in the present system 'the flow of information is reversed' and the 'key and analyte' become equivalent. I am afraid that this will make it harder for the average reader to understand the sensor principle than a more direct introduction and explanation of the system and its components.

Response: We agree with the reviewer, and have considerably simplified and shortened the sensor description.

- It would be helpful if the authors could discuss whether there are any restrictions on the structure of the analyte binding domain for it to be compatible with the Cage, e.g. all domains seem to have an N-terminal alpha-helix.

Response: We have added an extra paragraph to the Supplementary design methods including details about what features make an analyte binding domain compatible with lucCage.

- ref 6-8 do not all refer to semi-synthetic systems, ref 38 (line 813) is not the first example of an antibody sensor based on the beta-lactamase-BLIP system (-> https://pubs.acs.org/doi/10.1021/cb400406x)

Response: we corrected and added the references suggested by Referee #2.

- one could argue whether the target is allosterically regulating luciferase activity (line 88-89), I think it is better describes as competitive displacement

Response: We agee that "allostery" is a loaded term here, and to avoid argument, we have removed it from the revised manuscript.

- The interaction between the LBiT and SBiT can be tuned between sub-nM and 190 microM Kd. The authors should clearly mention which LBiT variant they used, and whether they varied this affinity.

Response: We have clarified this ambiguity in the revised manuscript. We used the SmBit and LgBit pair originally described by Dixon et al. 2016, designated as 114 (lowest affinity peptide, 190 microM) and 11S, respectively.

- Many of the titration data in the main figures lack error bars, while they typically are present in the figures in the extended data. Please add them here as well. This is particularly important in Fig 4D, as the standard deviation is necessary to calculate the LOD.

Response: The titration graphs shown in the main figures represent data collected in triplicate and have errors bars. However, in the cases where there is little variation between data points, the error bars are covered by the symbol (e.g., Fig. 2c, right panel) while in other cases, the error bars are visible (e.g., Fig. 2d, right panel or Fig. 3b, right panel).

- The specificity of the various sensors is studied by monitoring the response to all other analytes in time (Figure 5). These traces show variation in the luminescent signal for the blank and in the presence of non-target analytes, sometimes increasing, sometimes decreasing. What is the origin of these time-dependent effects? Also. why does the signal in some cases in the presence of the analyte first show an increase and then a decrease in luminescent signal?

Response: Figure 5 has now been updated to add the lucCageRBD sensor. The variation in luminescence in the presence of non-target analytes is mainly due to variability in the buffer conditions in which the target is stored, which can slightly affect the absolute luminescence intensity. In the previous experimental set up, the target volume constituted 10% of the total reaction volume, whereas now, we have reduced the added volume to 4% of the total reaction, which significantly reduced the non-target variability. The decrease of signal in the presence of target in the previous figure version was due to substrate depletion. The new Figure 5 is with experimental conditions with higher substrate concentration to prevent substrate depletion.

- Line 265-267 ‘Most of these platforms depend on the specific geometry of target-sensor interaction to trigger a conformational change..’ -> this may be true for some systems, but it does not apply to most of the examples referred to in ref 20, 35, 7, 34 and 6

Response: In this statement, we are mainly making reference to sensors that detect proteins targets through a protein-protein interaction. As Referee #2 mentions, small molecule sensors are usually not constrained by a specific geometry of target-sensor interaction. To make this more clear to the reader, we have updated the main text accordingly.

- line 297: ref 36 does not report on the incorporation of designed binders

Response: Thank you for noticing this mistake. We meant to cite the recent preprint describing a collaboration between the Correia Lab and the Merkx Lab: BioRxiv preprint doi: https://doi.org/10.1101/2020.03.11.988071. This has been corrected in the manuscript.

- Table S3: the sequence of the LCB1 domain is missing in this table. The same is true for the sequences of the lucCageRBD variants in Table S5.

Response: the LCB1 sequence and all the sequences of the screened lucCageRBD variants have now been added to Table S3 and Table S6 (previously Table S5).

- Table S4: the potential to photobleach is presented as a drawback of the LUMABS and CLASH systems, but this seems farfetched for a bioluminescent system.

Response: We agree--what we meant here was to point out the stability issue of fluorescent protein being photobleached by ambient light. To clear the confusion, we decided to remove this row from the new Table S5 (previously Table S4).

ADDITIONAL REQUEST
In addition, we sent the work informally to an epidemiologist to look at the SARS-CoV2 data, who noted "My only question on reading the section on SARS-CoV2 (in particular, figure 4) is why they didn’t test the RBD, which is the epitope most commonly used in conventional antibody detection tests (the second most common is the N protein). I haven’t previously seen any antibody detection tests based on the M protein. Perhaps they could clarify this point." Please answer this (i.e. benchmark to RBD assays) in addition to the other reviewer queries.

Response: This is an excellent point given current understanding of SARS-CoV2 antibody responses. However, when we designed lucCage sensors comprising linear SARS-Cov-2 epitopes in January 2020, the number of studies evaluating the immunogenicity of SARS-Cov-2 linear epitopes was very limited. Many of the papers on the subject (cited in this manuscript) strongly indicated that linear epitopes from the SARS-CoV and SARS-CoV-2 nucleocapsid and membrane proteins had strong (and specific) reactivity with patient samples and could have potential use in diagnostics (thus, the decision to use these epitopes as a proof of principle of this technology). As indicated in the epidemiologist comment, the full RBD is currently being used as the antigen of preference in serological testing. Current literature suggests that most of the epitopes responsible for these interactions are non-linear, which complicates their incorporation into the lucCage platform (https://www.medrxiv.org/content/10.1101/2020.07.13.20152587v1.full.pdf). We could insert the entire RBD directly into the cage, but the resultant much more complicated

fusion protein would almost certainly have to be expressed in mammalian cells. We searched for immunogenic linear-epitopes in the Spike protein outside the RBD domain. Some articles point to some promising linear epitopes that have strong reactivity with patient sera (https://www.nature.com/articles/s41467-020-16638-2), but lack of easily-accessible pure antibodies that specifically recognize these regions has delayed our efforts to develop sensors against these epitopes. A more straightforward way to detect anti-RBD antibodies would be to use lucCageRBD, in a similar fashion as the HBV PreS1 detection demonstrated in Figure 3. In order for this to work, patient-derived antibodies would need to outcompete the lucCageRBD-RBD interaction, While possible, as demonstrated with the PreS1 case, such antibodies would need to have a binding site on RBD that overlaps with the designed RBD binder (LCB1) and be able to outcompete it (LCB1 has picomolar affinity). Because of these complications, we consider this beyond the scope of the current manuscript.

uthor Rebuttals to Initial Comments:

Reviewer Reports on the First Revision:

Referees' comments:

Referee #2 (Remarks to the Author):

The authors have addressed essentially all my comments and questions adequately. I was also pleased with the additional experimental data provided, in particular the demonstration of the lucCageRBD sensor for detection of SARS-CoV-2 spike protein in serum samples. I recommend publication in Nature, but have two minor suggestions for improvement:
- In figur4 4d, 4th panel the concentration of the RBD protein that was spiked in was not mentioned. I think this is important to mention, also to avoid the impression that the +/- samples in patient samples actually represent serum from patients infected with SARS-Cov-2. The term ‘patients’ may lead to confusion in this case
- I noticed that the ratiometric detection method of the Merkx group that was adapted by the authors has been reported in a BioRxiv manuscript (https://www.biorxiv.org/content/10.1101/2020.10.31.363044v1).It would be good to acknowledge this work, as it provides more context of the method