Reviewers' comments:
Reviewer #1:

Manuscript Summary:
The manuscript describes a method for sensing intracellular proteins and linking this to a transcriptional output.

Major Concerns:
-A mathematical model is mentioned, but not really used in optimization or presented. It might be better to just omit this statement since it currently reads as if it is an afterthought. Otherwise, please clarify from where it is available and how to use it to guide the development of new devices.
We thank the reviewer for the feedback. Indeed, a mathematical model was used and described in the original manuscript but including it in this protocol would probably be out of the scope of this paper. We then decided to remove the reference to it to avoid confusion.

Minor Concerns:
-It may be necessary to explain what TEVp is in the abstract so that those who read it to decide if the protocol is of use can understand it.
We have now included the description of TEVp in the abstract (lines 40-42)

-Not clear what 'an interactor' is, perhaps a concrete example would help, though I am not sure if this is a real word or not.
We have replaced the word “interactor” with a deeper description of “molecules that interact with” throughout the manuscript

-Lines 75-77. Not clear if no solutions were proposed or if there were solutions proposed but these required bespoke engineering
The main novelty of this system is that it is a unique framework that can be easily adapted for theoretically any cytosolic protein. This is different from before where devices were built for a specific protein. We have provided a better description of this uniqueness in the revised version of the manuscript.

-The protocol describes transfection of HEK and Jurkat cells. This may lead readers to assume they must use one of these types of cells, but this is not the case. Perhaps clarify by including a Note. Also, do the authors feel that these exact protocols for transfection are necessary or could one transfect by another means or using a different optimized protocol?

We thank the reviewer for pointing this out. We have now included notes to explain that both cell lines and transfection methods can be chosen according to applications and preferences of the researchers.

-Lines 354-6, given that LD0 gives higher functionality, would you still recommend trying linkers? Or can this step be omitted?
Indeed, we observed better performances with LD0 (along with other conditions mentioned in the discussion). We would suggest to try it (but not limiting it to it) as first option, and included it in

the revised discussion.

Reviewer #2:

Manuscript Summary:

This manuscript is interesting for the researches that are working on monitoring protein expression intracellularly. However, it is a very complex co-expression system. Every system needs at least two fusion proteins for co-transfection and each fusion protein 1 needs two to four components. The efficiency and sensitivity can be effect by many factors in this system, such as the efficiency of the TEVp, the introbody epitope overlap, the efficiency of the TF and the downstream output protein transcription and translation.

The overall of this manuscript is not well written, the quality of the figures and tables should be improved.
We thank the reviewer for the feedback. We have worked to substantially increase the quality of language as well as of the figures. The latter were generated with Adobe Illustrator and converted in PDF with the maximal quality in the set-up.

Major Concerns:

1. How sensitive is this system? The authors should suggest the expression level of the POI, which fits into this system.
We thank the reviewer for the comment. Indeed, sensitivity is a critical parameter to indicate the potential use of our devices. In our original manuscript we have tested as proof of concept the response of the device to increasing NS3 concentration. Although for format constraints reasons we have not added the data in this manuscript, we have mentioned their importance in the discussion (line 361-364) providing the reference to the original paper. In addition, we have tested the response to HIV infection which were concentrated to titers typically used in the immunology labs (performed by our collaborator at Ragon Institute).

2. The authors suggested that to optimize the sensing performance was to avoid background activation of this system. Therefore, the author should include 'in the absence of the protein of interest-POI' controls such as POI knock out cell line or blocking antibody treatment to set the background.
Our experiments and output quantification were performed using a Negative Control condition that is output quantification in the absence of the POI. We clarified this method including a note for each device we tested (Lines: 209-210,252-253)

3. How to control the expression efficiency of these two fusion proteins?
One fusion protein (fusion protein 1) included a fluorescent protein (mKate), which allowed membrane visualization and to quantify transfection efficiency). The second fusion protein (fusion protein 2) did not include fluorescent marker, but for each test we ran a control in which we maximize TEVp expression to induce reporter expression. This control is a proxy of transfection efficiency of our systems.

4. Is the membrane tag is needed for the fusion protein1? The author should show member localized imaging data in the representative result.

We performed membrane localization imaging and showed it in the original manuscript. We now include these images in Figure 3d.

5. Why fusion protein 2 does not include a fluorescent marker to test the fusion protein2 expression and cell transfection.
We thank the reviewer for pointing this out. As we explain in the previous comment, we followed TEVp activity by output activation. Therefore, the reporter gene functions as a proxy of TEVp expression.

6. The author should show representative results for FACS and microscopy
Flow cytometry data are presented in Figure 2 and 4 and plotted as ratio of reporter fold induction, calculated as the normalized fluorescence in the presence of the POI divided by the normalized florescence in the absence of the POI. For the normalization we used the mKate as transfection marker. We now included in Fig.2 microscope images of the reporter activation in the presence of NS3.

7. The author did not explain to the reader how to select between LD0 or LD15, TCS(S) or TCS(L), and who to choose the position of TEVp.
We thank the reviewer for the useful comment. The selection of all parameters was made to increase the likelihood to achieve a protein-specific induction of readout. Because of the complex architecture, we sought to build variants that allow the chimeric proteins to be more or less flexible, and to render the TCS accessible to TEVp upon interaction of the intrabodies with the target protein. All the features are included in Table 1 and mentioned in a note of the revised manuscript (lines 119-122). In the discussion we comment on the best results achieved as setting rules for future protein sensing-actuating devices.

8. The author did not maintain the affinity of the intrabodies.
We are not sure to understand the comment. Since the intrabodies were previously published, the affinity to the proteins were already measured.

9. Only Figure 4 c has a statistic, but not other figures? We cannot get the conclusion for the effective range for the readout.
We could calculate the statistics for all the experiments performed, for which instead we plot the fold induction and relative standard deviation. However, we believe that since the scope of this manuscript is to describe clear protocols, we focused our effort to provide more useful information to the readers.

Minor Concerns:

1. Table 2 should be better organized and proved more information to make it correlated to the different domains of figure 1
Figure 1 provides the general structure of the framework. In Table 2 we opted to specify the single parts of the architecture that resulted in better sensing devices. Also, to avoid confusion we removed the HTT and Tat devices that are not described in this manuscript as well as the extended name of the plasmid that do not give additional information about the characteristics of the system. We hope that these modifications make table 2 more suitable to the readers.

2. Line 155. "Optional:.." the On and OFF models should be better defined. Same for line 176.

We specified what On and Off modes refer to.

3. The FACS method and the microscopy method to observe the output should write separate and clearer.
We have now created a protocol section of the FACS method (Protocol 2). We also explained better the microscopy method used for observation (Protocol 1.6) although since it was purely used for observations, we did not create a new section for it.

Reviewer #3:

Manuscript Summary:
The authors have described a protocol for engineering a modular genetic platform that sense intracellular proteins and activate a specific cellular response. The device operates on intracellular antibodies or small peptides to sense with high specificity the protein of interest. They have described the detection of proteins such as mutated huntingtin, NS3 serine-protease, Tat and Nef proteins to detect Huntington's disease, hepatitis C virus (HCV) and human immunodeficiency virus (HIV) infections respectively.

Major Concerns:
I did not notice any major issues in this submission.

Minor Concerns:

There are no images of the fluorescence detection of these proteins. If the authors could have taken microscopic images of the cells with fluorescence responses and the control cells without fluorescence responses, it would have helped the readers of the article immensely. Then, the readers would know what they should expect to see, when they try to repeat this reported protocol in their own labs. I am sure the apoptotic cells would have been very interesting to see in this study. If these images are provided in the video version of this article, then, this minor comment can be ignored.

In view of my comments above, I would recommend a minor revision of the manuscript.

We thank the reviewer for the positive comments. We have now included the microscopy images in the new figure 2e-f of the revised manuscript.