nature portfolio

Peer Review File

Fast and real-time electrical transistor assay for quantifying SARS-CoV-2 neutralizing antibodies

Web links to the author's journal account have been redacted from the decision letters as indicated to maintain confidentiality.

8th Oct 21

Dear Professor Fraboni,

Thank you for submitting your manuscript, "Next Generation Serology: a fast and real-time electrical assay to quantify SARS-CoV-2 neutralizing antibodies", to Communications Materials. It has now been seen by 3 referees, whose comments are appended below. You will see that while they find your work of potential interest, they have raised concerns that must be addressed.

In particular, you will see that Reviewer 3 seeks clarification regarding how your technology compares to other papers they mention. Please add a discussion of this in the revised manuscript. Furthermore, Reviewer 2 asks for reproducibility measurements and statistical analysis, and Reviewer 1 also asks for a description of the device design and the sensitivity compared to the PCR method. In light of these comments, we cannot accept the manuscript for publication, but are interested in considering a revised version that addresses these serious concerns.

We hope you will find the referees' comments useful as you decide how to proceed. Should further experimental data or analysis allow you to address these criticisms, we would be happy to look at a substantially revised manuscript. However, please bear in mind that we will be reluctant to approach the referees again in the absence of major revisions. If the revision process takes significantly longer than three months, we will be happy to reconsider your paper at a later date, as long as nothing similar has been accepted for publication at Communications Materials or published elsewhere in the meantime.

We are committed to providing a fair and constructive peer-review process. Please don't hesitate to contact us if you wish to discuss the revision in more detail.

When submitting your revised manuscript, please include the following:

-A response letter with a point-by-point reply to each of the referee comments and a description of changes made. Please include the complete referee report in the response letter. Please note that the response letter must be separate to the cover letter to the editors.

-A marked-up version of the manuscript with all changes to the text in a different colored font. Please do not include tracked changes or comments. Please select the file type ‘Revised Manuscript - Marked Up’ when uploading the manuscript file to our online system.

-A clean version of the manuscript. Please select the file type ‘Article File’.

-An updated Editorial Policy checklist, uploaded as a ‘Related Manuscript File’ type. This checklist is to ensure your paper complies with all relevant editorial policies. If needed, please revise your manuscript in response to these points. Please note that this form is a dynamic ‘smart pdf’ and must therefore be downloaded and completed in Adobe Reader. Clicking this link will download a zip file containing the pdf.

-Life Sciences: Reporting

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We understand that due to the current global situation, the time required for revision may be longer than usual. We would appreciate it if you could keep us informed about an estimated timescale for resubmission, to facilitate our planning. Of course, if you are unable to estimate, we are happy to accommodate necessary extensions nevertheless.

Please do not hesitate to contact me if you have any questions or would like to discuss the required revisions further. Thank you for the opportunity to review your work.

Best regards,

Rona Chandrawati, PhD
Editorial Board Member
Communications Materials
orcid.org/0000-0002-9780-8844

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The manuscript by Decataldo et al demonstrates the use of OECTs for monitoring the effect of neutralising antibodies on SARS-CoV-2 infected cells. The work is motivated by the need of fast and high throughput assays for evaluating antibodies efficiency. Indeed, they demonstrate that OECTs can be used to differentiate between cells infected with the virus with and without neutralising antibodies. They performed the necessary controls and evaluated the reproducibility of their devices as well as their reusability.
I recommend publication to Communications Materials after the authors have addressed my comments below.

1. Figure 1a shows the schematic of the device with two channels and one gate within the same PDMS well. Why the use two channels and not one channel per device?
2. Figure 2b in the legend says: TECH-OECT with 6 devices inserted and electrically connected. From the photograph it is not clear how the electrical wiring is designed and also what is the novelty on the design of TECH-OECT from other OECT arrays with PDMS wells. The authors should show a more descriptive photograph that highlights the novelty of their design or use a schematic.
3. Figure 1c: This panel corresponds to the expected response of the device. Y-axis is labeled only as device output, but they should say that this is the time response of the device, and it will be nice to correlated it with the schematic on the right. So the reader can understand the concept that when

the cell layer is disrupted, the time response of the device decreases.
4. The authors name the “non neutralising serum” as “serum N” and the “neutralising serum” as “serum P” but this is counterintuitive because Neutralising also starts with N. Is this a standard terminology or is it chosen by the authors?
5. The threshold of determining if the antibodies are neutralising effectively the virus is set at 1.2 AU. This value is reached after 44h. How consistent is this? There is a clear trend between the different cases, but the threshold is barely reached. Do the author expect that the response of the device will decrease further if the experiment is run for longer time?
6. Figure 2c what does the PB450-A means as a label for x-axis? Also what do the two different histograms (red and black) correspond to?
7. Figure 3a. why the Serum P curve decreases over time?
8. In Figure 4 they compare the OECT response with the PCR test and they conclude that the OECT shows that the 1:160 dilution is not effective for neutralising the virus while the PCR test fails to show that. But which method is the most sensitive, the OECT or the PRC? could it be that the OECT fails to detect that this concentration is effective? Is there any other method to use to verify that the OECT gives the correct conclusion?
9. In general, for the PCR results it will be good to include a short description in the main text as well on what does the graph shows as the paper is meant for scientists from a variety of disciplines
10. In the discussion the authors claim that their method is quantitative. Their results do not show that. How they can be quantitative? Do they show how many cells are healthy? Or efficiency on the virus neutralisation in a quantitative manner?
11. Figures S3, S4 can be colourful

Reviewer #2 (Remarks to the Author):

The manuscript “Next Generation Serology: a fast and real-time electrical assay to quantify 1 SARS-CoV-2 neutralizing antibodies” demonstrates electrical monitoring of the CytoPathic Effect induced by SARS-28 CoV-2 on VeroE6 using Organic Electrochemical Transistors (OECTs).
The utilized methods are described in detail and the manuscript is well written. The work is of topical interest and relevant to a broader community and therefore fits the scope of the journal well. Relevant work is cited and the newly introduced method is referenced to state-of-the-art methods (PCR and Cytofluorimeter analysis).

I can mainly evaluate the work from the device perspective and have the following comments and questions:

1) Three measurements is a very low quantity to evaluate the reproducibility or for any statistical analysis.
I am further missing a more detailed description on the devices that were used for the reproducibility and reliability measurements. Were the respective three devices fabricated in one batch? (Were batch-to-batch variations considered?) If using one substrate: Were neighboring devices tested for each category or were the analytes/categories well distributed over the whole substrate?
2) A Photograph of the set-up would be helpful.
3) How crucial is the read out after 48h? It seems that the Serum P nevertheless leads to an increase in the output signal in the long-term?

4) How can the different kinks in the electrical output be explained? (e.g. Figure 4a)
5) I am missing a graph (in the SI) with the original OECT data (transfer and output curves). How are the devices parameter changing (e.g. threshold voltage and mobility)? Could a detailed analysis of these parameter explain overlapping trends e.g. in Figure 3a (green curves) and Figure 4 (kinks).
6) Minor: line 352: There is no figure 5d in the manuscript

Reviewer #3 (Remarks to the Author):

Reviewer comments
There is an urgent need for live-virus based neutralizing antibody assays for SARS CoV-2. Demand exceeds availability due to the requirement for BSL-3 laboratory facilities. Current methods involve visual enumeration of immobilized plaques in virus-infected cultures (PRNT), or staining of virus-induced cytopathic effects (CPE) in the cell culture (that can be quantitated by plate reading instruments). A growing number of surrogate neutralization assays that can be performed outside of BSL-3 laboratories are also available, but those correlate with live-virus assays only qualitatively.
The authors of the current manuscript have previously developed a method in which transistors (OECTs) in the bottom of a culture well produce an electrical signal which is measurably influenced by the cytopathic effects of virus infection of cell growing in those wells. The manuscript is a proof-of-principle using sera from healthy blood donors known to be positive and negative for antibodies to SARS CoV-2. Data are compared to PRNT data on the same samples, but details of that PRNT, including where it was performed, are missing.
The first part of the Introduction, describing the importance of neutralizing antibody testing, could be shortened.
The long paragraph starting on line 64 is poorly written. It confuses descriptions of the PRNT with description of determining neutralization by staining of overall CPE, sometimes referring to both as "PRNT". The labor-saving advantages of electrical read-out without staining are described, but a major advantage over PRNT is not mentioned – namely elimination of subjective judgement in enumerating plaques.
The investigators previously published a proof-of principle of the OECT read-out using toxic chemicals or particles on cultured cells; the current report extends this to a study of SARS-CoV-2 infected cells in a BSL-3 laboratory.
The novelty is diminished by publication of several other similar systems in which CPE is monitored by changes in electrical signal detected by electrodes on the surface of the culture wells. In addition to Guo, et al. (reference # 36, cited by the investigators), see, for example:
L Hong, et al. "Ultrafast, sensitive, and portable detection of COVID-19 IgG using flexible organic electrochemical transistors". Science Advances, 15 September 2021, 7:eabg8387.
K Akhil, et al., "A rapid detection of COVID-19 Viral RNA in human saliva using electrical double layer-gated field-effect transistor-based biosensors". Advanced Material Technologies 2021; 2100842.
K Ditte et al., "Rapid detection of SARS-CoV-2 antigens and antibodies using OFET biosensors based on a soft and stretchable semiconducting polymer. ACS Biomaterial Science and Engineering, Sept. 14, 2021, https://doi.org/10.1021/acsbiomaterials.1c00727.
"Maestro Z" commercially available from Axion Biosystems, www.axionbio.com: 96-well electrical impedance-based instrument for monitoring virus-induced CPE (validated for automated SARS CoV-2 viral titration).
Some strengths of the work include good attention to detail in the Results section and use of controls, including the effects of antibody in the absence of virus, and comparison with optical

images and correlation with PCR (line 310).
The consequences of uneven distribution of cells and CPE were initially of concern to this reviewer, but are adequately addressed starting on line 394.
Regarding practical use, the technology is at an early stage: The large culture well with incorporated transistors is complicated to manufacture and not compatible with common multiwall plate geometries. It is difficult to foresee large-scale manufacturing and an affordable price, as would be required to impact the current needs for neutralizing antibody work. The proposed re-use of the culture wells, although demonstrated by the investigators, would not be practical in everyday use.
The reviewer has attached proposed additional edits and suggestions in an attached copy of the draft manuscript.
Review by:
Carl V. Hanson
California Department of Public Health
Richmond, CA 94703

Authors response letter to the Referees

Reviewer #1 (Remarks to the Author):

The manuscript by Decataldo et al demonstrates the use of OECTs for monitoring the effect of neutralising antibodies on SARS-CoV-2 infected cells. The work is motivated by the need of fast and high throughput assays for evaluating antibodies efficiency. Indeed, they demonstrate that OECTs can be used to differentiate between cells infected with the virus with and without neutralising antibodies. They performed the necessary controls and evaluated the reproducibility of their devices as well as their reusability. I recommend publication to Communications Materials after the authors have addressed my comments below.

We thank the Reviewer #1 for his/her comments and suggestions, which give us the possibility to further clarify, improve and complete our work.

1. Figure 1a shows the schematic of the device with two channels and one gate within the same PDMS well. Why the use two channels and not one channel per device?

The Reviewer is right OECT is composed by one channel and one gate at fixed distance. We patterned this configuration to double up device monitoring of the same well in order to average the extracted signals, since cell layer dis-homogeneous distributions after seedings could conceal the correct outcome.

We thus add in the Materials & Methods, "OECT Device Fabrication" sub-section, line 180:

"The dual channel configuration doubled up the device monitoring of the same well, thus taking into account possible cell layer dis-homogeneity after seeding, that may conceal the correct outcome."

2. Figure 2b in the legend says: TECH-OECT with 6 devices inserted and electrically connected. From the photograph it is not clear how the electrical wiring is designed and also what is the novelty on the design of TECH-OECT from other OECT arrays with PDMS wells. The authors should show a more descriptive photograph that highlights the novelty of their design or use a schematic.

As suggested by Reviewer 1, we introduced the experimental set up schematic and the wiring connections and a picture of the TECH-OECT during seeding, adding Figure S2. Furthermore, it was added in the main text, line 258:

"A schematic representation of the experimental setup and wiring connections is reported in Fig. S2a, while Fig.S2b shows TECH-OECT during seeding at the beginning of an experiment."

And a brief description of the experimental set up was written in the Supporting Information to explain Figure S2.

"Fig. S2a shows the schematic circuitry of the electrical connections of the experimental setup. TECH-OECT platform allows for the monitoring of up to 6 cell cultures, placed on three rows. A flat multicable is used as electrical connector, extracting the signal from the OECTs in the incubator towards the multiplexing system, which is controlled by the Keysight Source Meter Unit (SMU) for channel and gate voltage polarization and device selection. All the system has a common ground and is controlled by a PC laptop software, afterwards used for data analysis. Fig. S2b reports a picture of the TECH-OECT during an experiment: dimensions are

similar to standard petri multiwell plates and the wiring with the multicable could be done after the seeding, facilitating operator's work."

3. Figure 1c: This panel corresponds to the expected response of the device. Y-axis is labeled only as device output, but they should say that this is the time response of the device, and it will be nice to correlated it with the schematic on the right. So the reader can understand the concept that when the cell layer is disrupted, the time response of the device decreases.

We agree with the Reviewer suggestion, the colours of the picture borders are not enough to highlight the correlation between the ideal signal output graph and the schematic representation of the cell cultures. Thus, we introduced coloured lines to match the final points of the graph with the corresponding picture in Figure 1c, we changed the Y-axis name into "OECT Time Response" and we slightly modify Fig.1c to avoid arrows/virus/antibodies superimposition in the schematic.

4. The authors name the "non neutralising serum" as "serum N" and the "neutralising serum" as "serum P" but this is counterintuitive because Neutralising also starts with N. Is this a standard terminology or is it chosen by the authors?

The reviewer is right, the labels were chosen by the authors to indicate the non-neutralizing serum so defined as "negative" (N) compared to the neutralizing or positive serum (P).

We thus add in the main text in line 282-283:

..neutralizing (positive serum= serum P) and non-neutralizing antibodies (negative serum = serum N).

5. The threshold of determining if the antibodies are neutralising effectively the virus is set at 1.2 AU. This value is reached after 44h. How consistent is this? There is a clear trend between the different cases, but the threshold is barely reached. Do the author expect that the response of the device will decrease further if the experiment is run for longer time?

The Reviewer is right that the selected threshold of 1.2 a.u. is barely reached but we found this value consistent with the averaged experimental trends on viral-infected cells, carried out in two different laboratories. Indeed, Figure 3a reports an average response over three devices for the different "incubation mix". We expect that longer time would lead to lower OECT time responses in the infected cultures, according to the observed decreasing trend. However, the signal is already robust and reproducible with the selected threshold and it allows to avoid eventual cell overpopulation stress in the healthy growing cultures and to have a faster screening: owing to the real-time feature of the assay, the researcher could set 44h as the "first reading time" to assess the strongest responses, while keeping the uncertain cultures under observation for longer time (for example 24h more, if needed).

We thus add in Materials and Methods, "OECT data analysis" sub-section, line 204:

"We then choose 1.2 a.u as threshold value at the end of the experiment to separate viral-infected cultures (< 1.2 a.u.) from healthy ones (> 1.2 a.u.), since it was consistent with the averaged experimental trends carried out in two different laboratories, on three devices and for the different "incubation mix"."

6. Figure 2c what does the PB450-A means as a label for x-axis? Also what do the two different histograms (red and black) correspond to?

PB450 is the wavelength used to detect DAPI fluorescence. The black histogram corresponds to unstained healthy cells whereas red histogram represents suffering cells characterized by membrane alterations that allow the DAPI to enter.

To be clearer, we add in the caption of the cytofluorimeter figures both in the main text (line 321) and in the Supporting Information:

"The black histogram corresponds to unstained healthy cells whereas red histogram represents suffering cells characterized by membrane alterations that allow the DAPI to enter. "

7. Figure 3a. why the Serum P curve decreases over time?

We hypothesize that cell growth is faster in the Serum P curve, owing to the boost and nutrients received from the serum addition (without any viral infection stressing the culture), as could be also interpreted focusing on the first 20h from seeding, where the green curve has always higher time response values than the other cultures. According to this idea, at the end of the experiment a slight stress due to cell overpopulation could be present in healthy, serum P-boosted cell cultures, with a small decrease in the device response. Similarly, the same trend can be observed in panel "a" of Figure 2 for both positive (Serum P) and negative serum (Serum N).

We added in the description of Fig.3a in the "Results" Section, line 331:

[..], " in which the slight decrease in the last 12h was ascribed to cell overpopulation (the overabundant serum probably boosted cell proliferation and growth [Refs])."

In order to avoid cell stress due to overpopulation, the assay duration was set at 44h.

8. In Figure 4 they compare the OECT response with the PCR test and they conclude that the OECT shows that the 1:160 dilution is not effective for neutralising the virus while the PCR test fails to show that. But which method is the most sensitive, the OECT or the PRC? could it be that the OECT fails to detect that this concentration is effective? Is there any other method to use to verify that the OECT gives the correct conclusion?

We have probably misleaded Reviewer 1 with our explanation, since we wanted to highlight that the viral neutralization was present also for the 1:160 dilution, in accordance both with the PCR and with the OECT response (above the 1.2 a.u. threshold). However, we thought that this dilution was a borderline one (according also to the previous neutralizing serum titer PRNT tests) owing to the viral-induced cell stress, which is visible in the OECT curve trend (low time response values).

Moreover, it is crucial to point out that PCR and OECT assays are not exactly comparable since they are based on different principles. While OECT provides a measure of cellular integrity and therefore the ability of the virus to replicate and determine CPE, PCR instead detect the viral genome independently of viral replication and virus vitality.

In order to be clearer, we changed the main text (line 351) from:

"This response may be ascribed to the fact that 1:160 serum dilution represents a borderline antibodies concentration, which is not completely effective in viral neutralization, but still decelerate viral proliferation. It is indeed worth to note that PCR analysis for 1:160 positive serum dilution reports a "standard" viral stop after 48 h, thus failing in the identification of this borderline concentration."

to:

"This response may be ascribed to the fact that 1:160 serum dilution represents a borderline antibodies concentration, which decelerate viral proliferation but still allows for a strong, viral-induced cell stress highlighted by the low-increasing curve trend and the final value. It is worth to note that PCR analysis for 1:160 positive serum dilution reports a "standard" viral stop after 48 h, hindering the identification of this borderline concentration, which may be interesting for more accurate analysis on the virus-cell interactions. Indeed, it is crucial to point out that OECT and PCR are not exactly comparable since the former provides measurements of cell layer integrity (thus evaluating potential viral replication and CPEs on the cell culture), while the latter detect the viral genome, indipendently on viral activity or replication."

We also changed Figure 4b, adding the error bars in the PCR graph.

9. In general, for the PCR results it will be good to include a short description in the main text as well on what does the graph shows as the paper is meant for scientists from a variety of disciplines

We agree with the Reviewer suggestion, and we thus add in the Materials & Methods section, "Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)" sub-section the PCR description and how we calculated the fold change (line 227):
"In a real-time PCR assay a positive reaction is detected by accumulation of a fluorescent signal. The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold. Ct levels are inversely proportional to the amount of target nucleic acid in the sample (i.e. the lower the Ct level the greater the amount of target nucleic acid in the sample). The fold change reported in our graph represents the Ct level at the end of the experiment (T48h), normalized with respect to its initial value at the beginning of the experiment (T0h)."

In the main text, we add the reference to the complete PCR description when we first introduced a PCR graph (Fig. 2a), line 284:

"(as described in the Materials & Methods section)"

10. In the discussion the authors claim that their method is quantitative. Their results do not show that. How they can be quantitative? Do they show how many cells are healthy? Or efficiency on the virus neutralisation in a quantitative manner?

Using "quantitative" in the Discussion, we meant that a quantitative titer of the neutralizing serum could be provided with our method, as in standard PRNT assays.

Even though optical-electrical correlations may be carried out to provide the number of healthy cells (or, more likely, the coverage of the active surface area, as observed in our previous work "Organic Electrochemical Transistors: Smart Devices for Real-Time Monitoring of Cellular Vitality"), this was not the scope of our work, since we needed to quantify the serum dilution capable of neutralizing the viral infection.
Since we understood that the text may be misleading, we moved the word quantitative close to serum neutralization assay (line 390), since the quantification provided by our system refers to the serum dilution.

11. Figures S3, S4 can be colourful

We agree with the Reviewer 1 that a colorful image would be easier to comprehend, and we introduced three colours for the first, second and third time tests.

Reviewer #2 (Remarks to the Author):

The manuscript "Next Generation Serology: a fast and real-time electrical assay to quantify 1 SARS-CoV-2 neutralizing antibodies" demonstrates electrical monitoring of the CytoPathic Effect induced by SARS-28 CoV-2 on VeroE6 using Organic Electrochemical Transistors (OECTs).The utilized methods are described in detail and the manuscript is well written. The work is of topical interest and relevant to a broader community and therefore fits the scope of the journal well. Relevant work is cited and the newly introduced method is referenced to state-of-the-art methods (PCR and Cytofluorimeter analysis).

We acknowledge the Reviewer #2 for his positive remarks as well for the comments and work done, and we report the answers to his concerns below.

I can mainly evaluate the work from the device perspective and have the following comments and questions:
1) Three measurements is a very low quantity to evaluate the reproducibility or for any statistical analysis. I am further missing a more detailed description on the devices that were used for the reproducibility and reliability measurements. Were the respective three devices fabricated in one batch? (Were batch-to-batch variations considered?) If using one substrate: Were neighboring devices tested for each category or were the analytes/categories well distributed over the whole substrate?

We perfectly agree with the Reviewer 2 that three devices are not a huge number for a complete statistical analysis. Being our prototype a proof-of-concept assay for serum-neutralization, still far from commercial distribution, we tested reproducibility with three devices. However, we would like to highlight that the three devices used for the weighted average trends are sensors from different batches, differently placed on the TECH-OECT board and the results were obtained from two different laboratories (namely, the BSL-3 laboratories respectively at Unit of Microbiology, Great Romagna Hub Laboratory, Cesena, Italy and at the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna (IZSLER), Brescia, Italy).

We understand that this information could be important for the sake of the article, and we thus changed in the main text, line 324:

"The reproducibility and reliability of our devices has been assessed replicating the experiment three times (Fig 3)."

In:

"The reproducibility and reliability of our devices has been assessed replicating the experiment three times (Fig 3), using devices fabricated in different batches and carrying out the assays in two different laboratories (see Materials and Methods)."

We also changed in the Discussion, line 397:

"This is a proof-of-concept research of a novel technology which could potentially replace (or work in parallel with) subjective optical evaluation or expensive laboratory equipment."

2) A Photograph of the set-up would be helpful.

Aiming at a better understanding of our work and complying the request from both Reviewer 1 and Reviewer 2, we introduced a schematic representation of the TECH-OECT connections towards the experimental set-up and a picture of the TECH-OECT during seeding, adding Figure S2 and a brief description on the Supporting Information.

It was added in the main text, line 258:

"A schematic representation of the experimental setup and wiring connections is reported in Fig. S2a, while Fig.S2b shows TECH-OECT during seeding at the beginning of an experiment."

And a brief description of the experimental set up was written in the Supporting Information to explain Figure S2.

"Fig. S2a shows the schematic circuitry of the electrical connections of the experimental setup. TECH-OECT platform allows for the monitoring of up to 6 cell cultures, placed on three rows. A flat multicable is used as electrical connector, extracting the signal from the OECTs in the incubator towards the multiplexing system, which is controlled by the Keysight Source Meter Unit (SMU) for channel and gate voltage polarization and device selection. All the system has a common ground and is controlled by a PC laptop software, afterwards used for data analysis. Fig. S2b reports a picture of the TECH-OECT during an experiment: dimensions are similar to standard petri multiwell plates and the wiring with the multicable could be done after the seeding, facilitating operator's work."

3) How crucial is the read out after 48h? It seems that the Serum P nevertheless leads to an increase in the output signal in the long-term?

We chose 44/48h for the reading as a compromise: first, we needed a reliable assay in which the viral-replicating cells were strongly stressed/killed by the Sars-CoV-2 as showed by optical images, cytofluorimeter analysis and OECT data (time response below 1.2. a.u); second, we wanted a fast test (within two days) in which cells reached a confluent layer while avoiding overpopulation stress in healthy cell controls (which starts to be visible in the green curve of Serum P in Fig 3a, where we hypothesize that cell growth is faster in the Serum P curve, owing to the boost and nutrients received from the serum addition, thus leading to a slight cell stress due to overpopulation at the end of the assay).and our hypothesis is reported in the answer to question 7 of Reviewer 1).

4) How can the different kinks in the electrical output be explained? (e.g. Figure 4a)

We can not exclude *a priori* that transitory biological events may be the cause of the kinks in electrical outputs. However, we hypothesized that vibrations and external noises coming from the lab (eventually higher when the incubator is opened for other experiments) would introduce these fluctuations and kinks in the extracted signal. It is thus important to analyse the complete trend of the curve. Furthermore, the real-time monitoring allows to have several consecutive points which can be mediated (or data can be rejected if too far from the trend) to extract the correct outcome. To highlight this, we add in the "Results" section, line 296:

"Kinks and fluctuations in the curves are likely induced by transitory biological events and/or external electrical noises/mechanical vibrations and are irrelevant for the assay outcome since the real-time monitoring allows for complete trend analyses, extracting the correct results."

5) I am missing a graph (in the SI) with the original OECT data (transfer and output curves). How are the devices parameter changing (e.g. threshold voltage and mobility)? Could a detailed analysis of these parameter explain overlapping trends e.g. in Figure 3a (green curves) and Figure 4 (kinks).

We agree with the reviewer and we added the OECT transfer and output characterization in Fig.S1, changing the main text, line 249, in:

The device schematic and electrical characterization are shown in Fig.1a and Fig. S1, respectively, [..]

Moreover, in order to prove that our devices are not affected by the *in-vitro* culture experiment, we added the device characterization after the revitalization protocol (Fig. S5), showing a slight reduction in the current/conducibility of the OECT, which is typical after their prolonged use (Kim, SM., Kim, CH., Kim, Y. *et al.* Influence of PEDOT:PSS crystallinity and composition on electrochemical transistor performance and long-term stability. *Nat Commun* **9,** 3858 (2018). https://doi.org/10.1038/s41467-018-06084-6). However, current modulation upon gating is still maintained as well as the device time response, which degrades only at the third use (Fig. S8), again not affecting the output of the serum-neutralization test (Fig. 5, S6 and S7). We thus added Fig. S5 and in the main text, line 373:

The device characterization after the revitalization protocol is reported in Fig.S5, displaying a reduction in the OECT conducibility but still confirming the device current modulation upon gating.

It is finally worth noting that the device measurement protocol was optimized in our previous works (doi: 10.1002/admt.201900207 and doi: 10.1002/adbi.201900204) and relies on the continuous monitoring of the OECT time response upon a positive voltage pulse on the gate, during the whole time of the incubation, aiming at the extraction of the correct outcome of the test owing to the evaluation the complete trend.

6) Minor: line 352: There is no figure 5d in the manuscript

Reviewer 2 is correct, it was a refuse in the main text, and we deleted “and 5d”.

Reviewer #3 (Remarks to the Author):

Reviewer comments:

There is an urgent need for live-virus based neutralizing antibody assays for SARS CoV-2. Demand exceeds availability due to the requirement for BSL-3 laboratory facilities. Current methods involve visual enumeration of immobilized plaques in virus-infected cultures (PRNT), or staining of virus-induced cytopathic effects (CPE) in the cell culture (that can be quantitated by plate reading instruments). A growing number of surrogate neutralization assays that can be performed outside of BSL-3 laboratories are also available, but those correlate with live-virus assays only qualitatively.

The authors of the current manuscript have previously developed a method in which transistors (OECTs) in the bottom of a culture well produce an electrical signal which is measurably influenced by the cytopathic effects of virus infection of cell growing in those wells. The manuscript is a proof-of-principle using sera from healthy blood donors known to be positive and negative for antibodies to SARS CoV-2. Data are compared to PRNT data on the same samples, but details of that PRNT, including where it was performed, are missing.

We thank the Reviewer 3 for his/her suggestion and careful reading of our paper, and below we report the suggested modifications and answers to this open question. To improve the description of the PRNT method and where it was performed, we added in the Materials & Methods section, “Sera samples and virus infection” sub-section, line 135:

“A PRNT assay requires the formation of a virus-antibody (in an equal volume) complex *in vitro* during an incubation for 30 minutes at 37 °C in thermo shaker and subsequent seeding on cells susceptible to viral infection. After incubation (typically 72 h), the CytoPathic Effect (CPE) is evaluated by fixing and staining the cells with formaldehyde in Crystal violet and, subsequently, reading the absorbance using the

spectrophotometer at 560 nm wavelength. The highest serum dilution that neutralizes 90% of viral replication and thus its CPE is reported as the neutralizing titer."

And (line 144):

"PRNT assays were performed at the BSL-3 laboratory at Unit of Microbiology, Great Romagna Hub Laboratory, Cesena, Italy."

As for the PCR, we also add in the main text where we first cite the PRNT test (namely when we start the description of Fig. 4 in the "Results" section, line 345):

as described in Materials and Methods section

To refer the reader to the complete description of the PRNT assay employed.

The first part of the Introduction, describing the importance of neutralizing antibody testing, could be shortened. The long paragraph starting on line 64 is poorly written. It confuses descriptions of the PRNT with description of determining neutralization by staining of overall CPE, sometimes referring to both as "PRNT".

We reduced the paragraph length, moving the description of the PRNT to the Materials and Methods Section, aiming at complying both the Reviewer suggestions and having a complete description of the performed PRNT assay on the Materials Section, including where it was carried out. Furthermore, we change the paragraph in the "Introduction", line 62, in:

"There are several methods for determining the antibody titer, but the Serum Neutralizaiton (SN) is considered the gold standard for measuring the presence and levels of neutralizing antibodies in tested sera. In addition to the conventional SN, to obtain a precise quantification of the titer of neutralizing antibody for a virus, an assay called PRNT (Plaque Reduction Neutralization Test) was developed for studies of inactivation (or neutralization) of animal viruses, and modified to measure serum antibody neutralization titer.[8,9] The PRNT assay has been already used to test the level of protection of the population in countries affected by other zoonotic coronaviruses, such as MERS.[10]"

The labor-saving advantages of electrical read-out without staining are described, but a major advantage over PRNT is not mentioned- namely elimination of subjective judgment in enumerating plaques.

This is a correct observation. Thus, in order to stress this advantage and to support both comments, we added in the "Discussion", line 397:

"This is a proof-of-concept research of a novel technology which could potentially replace (or work in parallel with) subjective optical evaluation or expensive laboratory equipment."

The investigators previously published a proof-of principle of the OECT read-out using toxic chemicals or *particles on cultured cells; the current report extends this to a study of SARS-CoV-2 infected cells in a BSL-3 laboratory. The novelty is diminished by publication of several other similar systems in which CPE is monitored by changes in electrical signal detected by electrodes on the surface of the culture wells. In addition to Guo, et al. (reference # 36, cited by the investigators), see, for example:*
L Hong, et al. "Ultrafast, sensitive, and portable detection of COVID-19 IgG using flexible organic electrochemical transistors". Science Advances, 15 September 2021, 7:eabg8387.
K Akhil, et al., "A rapid detection of COVID-19 Viral RNA in human saliva using electrical double layer-gated field-effect transistor-based biosensors". Advanced Material Technologies 2021; 2100842.
K Ditte et al., "Rapid detection of SARS-CoV-2 antigens and antibodies using OFET biosensors based on a soft and stretchable semiconducting polymer. ACS Biomaterial Science and Engineering, Sept. 14, 2021,

https://doi.org/10.1021/acsbiomaterials.1c00727. "Maestro Z" commercially available from Axion Biosystems, www.axionbio.com: 96-well electrical impedance-based instrument for monitoring virus-induced CPE (validated for automated SARS CoV-2 viral titration).

We thank the Reviewer for informing us of these research articles, released slightly after our submission, on biosensors for viral RNA and antibodies/antigens detection, which we therefore cited in the "Discussion" by adding in line 449:

"Similarly, biosensors for the detection of SARS-CoV-2 viral RNA, antigens and antibodies (e.g. type G Immunoglobulins) have been developed, exploiting the transistor geometry and a surface functionalization for signal amplification and selectivity, respectively. [citations]"

Owing to the aspecific nature of our assay, we also added in line 454:

"Furthermore, differently from the previous works, our assay is not Sars-CoV-2 specific but could be translated towards several viruses and cell lines, accordingly setting the health/infection threshold and normalized time response ranges."

Finally, we add the commercial instrument citation in the main text in line 458: "even using commercially available instruments [citation]"

Some strengths of the work include good attention to detail in the Results section and use of controls, including the effects of antibody in the absence of virus, and comparison with optical images and correlation with PCR (line 310). The consequences of uneven distribution of cells and CPE were initially of concern to this reviewer, but are adequately addressed starting on line 394. Regarding practical use, the technology is at an early stage: The large culture well with incorporated transistors is complicated to manufacture and not compatible with common multiwall plate geometries. It is difficult to foresee large-scale manufacturing and an affordable price, as would be required to impact the current needs for neutralizing antibody work. The proposed re-use of the culture wells, although demonstrated by the investigators, would not be practical in everyday use. The reviewer has attached proposed additional edits and suggestions in an attached copy of the draft manuscript.

We agree with the Reviewer that our technology is still at an early stage of development, and we just demonstrated it as a proof-of-concept. Scale up of OECT devices, as well as of multiple readout electronics and software, is well known and could be at low cost. Actual OECT dimensions are compatible with a well of 11 mm diameter corresponding to those of an existing 24 multiwell plate. However, using standard lithography fabrication, OECTs can be miniaturized, reducing costs and well dimensions, and multiwell designs up to 48 wells are easily envisaged. We are presently working on the technology scalability and re-use protocol targeting to increase the number of cultures studied in parallel and boosting operator's efficiency, however this engineering improvements are beyond the aim of this work. In order to be clearer on the main text, we added in "Discussion" section, line 464:

"Our electrical system is still at an early stage of development, and we just demonstrated it as a proof-of-concept. Scale up of OECT devices, as well as of multiple reader and software analysis, is well known and could be at low cost. Actual OECT dimensions are compatible with a well of 11 mm diameter corresponding to those of an existing 24 multiwell plate. However, using standard lithography fabrication, OECTs can be miniaturized, reducing costs and well dimensions, and multiwell designs up to 48 wells are easily envisaged."

We have seen the Reviewer 3 editing, and we changed "merged" with "immersed".

28th Nov 21

Dear Professor Fraboni,

Your manuscript titled "Next Generation Serology: a fast and real-time electrical assay to quantify SARS-CoV-2 neutralizing antibodies" has now been seen again by our referees, whose comments appear below. In light of their advice I am delighted to say that we are happy, in principle, to publish a suitably revised version in Communications Materials under the open access CC BY license (Creative Commons Attribution v4.0 International License).

We therefore invite you to revise your paper one last time to address the remaining comment of Reviewer 3. At the same time we ask that you edit your manuscript to comply with our journal policies and formatting style in order to maximise the accessibility and therefore the impact of your work.

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Best regards,

Rona Chandrawati, PhD
Editorial Board Member
Communications Materials
orcid.org/0000-0002-9780-8844

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

The authors have addressed all my comments therefore I recommend publication.

Reviewer #2 (Remarks to the Author):

Thank you for clarification and addressing the comments.
I recommend the publication of the manuscript.

Reviewer #3 (Remarks to the Author):

Thank you for improving the manuscript in response to the initial review. I recommend publication.
I have attached a copy of the revised manuscript with a few minor suggested edits.
One criticism remains from the earlier review and is highlighted in my "Comment" in the edited version that I have attached:
It is a strength that your novel assay was compared to a gold-standard live-virus neutralizing antibody assay in a BSL-3 lab. Live-virus assays can be done by scoring the inhibition of viral CPE by

antibodies either visually or colorimetrically after staining. A "PRNT" assay, on the other hand, is done by immobilizing foci of infected cells ("plaques") under a soft agarose layer and enumerating them. Thus, the PRNT description in the manuscript does not describe an actual PRNT.