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Point-of-care test system for detection of immunoglobulin-G and -M against nucleocapsid protein and spike glycoprotein of SARS-CoV-2

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ABSTRACT

The coronavirus disease 2019 (COVID-19) epidemic continues to ravage the world. In epidemic control, dealing with a large number of samples is a huge challenge. In this study, a point-of-care test (POCT) system was successfully developed and applied for rapid and accurate detection of immunoglobulin-G and -M against nucleocapsid protein (anti-N IgG/IgM) and receptor-binding domain in spike glycoprotein (anti-S-RBD IgG/IgM) of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Any one of the IgG/IgM found in a sample was identified as positive. The POCT system contains colloidal gold-based lateral flow immunoassay test strips, homemade portable reader, and certified reference materials, which detected anti-N and anti-S-RBD IgG/IgM objectively in serum within 15 min. Receiver operating characteristic curve analysis was used to determine the optimal cutoff values, sensitivity, and specificity. It exhibited equal to or better performances than four approved commercial kits. Results of the system and chemiluminescence immunoassay kit detecting 108 suspicious samples had high consistency with kappa coefficient at 0.804 (P < 0.001). Besides, the levels and alterations of the IgG/IgM in an inpatient were primarily investigated by the POCT system. Those results suggested the POCT system possess the potential to contribute to rapid and accurate serological diagnosis and epidemiological survey of COVID-19.

1. Introduction

Coronavirus disease 2019 (COVID-19) has spread rapidly around the world; the cumulative number of confirmed cases worldwide has exceeded 35 million, and the global death toll is more than 1.0 million. The unexpected COVID-19 epidemic brought not only changes in the lifestyle of people but also unpredictable negative effects on the political and economic situation worldwide. The real villain of the human pandemic is a novel coronavirus that is closely related to severe acute respiratory syndrome coronavirus identified in 2003; the novel coronavirus has been officially named as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) by the International Committee on Taxonomy of Viruses [1,2]. To cope with the aggressive and long-lasting COVID-19 epidemic caused by SARS-CoV-2, researchers around the world are sparing no effort to advance the development of available vaccines and therapeutic drugs. Although the relevant work is progressing rapidly, people still need to wait patiently for the verification results of the clinical trial. Thus, prevention and control of the COVID-19 epidemic is the main measure until available vaccines and drugs are developed; timely diagnosis and treatment are important to curb the transmission of SARS-CoV-2, and many methods have been developed and applied [3,4].

Nucleic acid detection and antibody detection are the common approaches for COVID-19 diagnosis. The prevalence of SARS-CoV-2 infection can be accurately estimated by antibody detection, because the results of antibody detection will be positive if the volunteers have been infected but are recovering even have been recovered. Nucleic acid detection manifest the subjects are or not being infected with SARS-CoV-2, which is the reference standard method for COVID-19 diagnosis, but its use is limited due to intensive labor requirement, time-consuming...
In the early days of the outbreaks, the number of infections worldwide is thought to be substantially underestimated owing to the shortage of detection kits and high percentage of mild and asymptomatic patients [6–9]. Combined antibody and nucleic acid detection, the sensitivity for COVID-19 diagnosis increased from 67.1 % to 99.4 % [10]. Hence, antibody detection is a beneficial complement to nucleic acid detection and an effective approach to investigate the epidemiology. Huang group [11] have investigated the antibody responses of patients with COVID-19 to SARS-CoV-2; the immunoglobulin-G (IgG) and immunoglobulin-M (IgM) titers plateaued within 6 days after seroconversion. The spike (S) glycoprotein and nucleocapsid (N) protein are expressed in the body after infection with SARS-CoV-2, thereby stimulating the immune system to produce corresponding antibodies against the N protein and the S glycoprotein (anti-N and anti-S). Antibodies against the receptor-binding domain in the S glycoprotein (anti-S-RBD) can block the interaction between the virus and receptor and are called neutralizing antibodies [12]. The detection of anti-N and anti-S-RBD antibodies based on immunoassays has been widely employed for clinical and auxiliary diagnosis [13].

Immunoassays developed for anti-SARS-CoV-2 antibody (including IgG and IgM) detection can be classified into lateral flow immunoassay (LFIA) [14,15], enzyme-linked immunosorbent assays (ELISA) [16,17], and chemiluminescent immunoassays (CLIA) [18]. A number of anti-SARS-CoV-2 antibody detection kits have been approved by the Food and Drug Administration, the National Medical Products Administration (NMPA), and Conformite Europeenne. CLIA is available on an automatic detection platform and has high sensitivity; this assay has been considered as the authoritative method in immunological detection, but it is not conducive to point-of-care test (POCT) because of the requirements for special and expensive instruments and reagents. LFIA is considered as the most attractive POCT device and exhibits several advantages including simple technical requirement, rapid detection capability, portability, affordability, and high detection accuracy and efficiency [19]. Colloidal gold nanoparticles (CGNPs), lanthanide-doped polystyrene nanoparticles, selenium nanoparticles, and Eu(III) fluorescent microspheres have been used as labels for developing LFIA to sensitively and specifically detect anti-SARS-CoV-2 antibodies [20–23]; among which, CGNP-based LFIA (CG-LFIA) is always the most popular and commonly used tool. However, existing CG-LFIAs for anti-SARS-CoV-2 antibody detection are restricted by detection with the naked eye. Results would be subjectively misjudged if investigators work long hours, especially in cases of either low-light environment or low-level anti-SARS-CoV-2 antibodies. Therefore, several small portable devices that can read and record the signal intensity of test lines on CG-LFIA have been developed and applied to meet the objective and accurate detection capability of CG-LFIA [24].

In March, the National Institute of Metrology has developed and issued the certified reference materials (CRM) of humanized anti-S IgG (specific to RBD) and anti-N IgG solutions (https://www.ncrm.org.cn/). Based on the CRM, the current study aims to develop a POCT system for investigation of anti-N IgG/IgM and anti-S-RBD IgG/IgM levels and alterations in patients or cured population who suffered from COVID-19. A POCT system, including the CG-LFIA based on capture method principle...
for detection, homemade portable reader for signal acquiring and the CRM for evaluation, was successfully established to objectively and sensitively detect anti-N IgG/IgM and anti-S-RBD IgG/IgM in human serum (Fig. 1). As schemed in Fig. 1A, the CG-LFIA was constituted with sample pad, conjugated pad with detection probes, nitrocellulose membrane with G/M test lines and control line, absorbent pad and PVC pad. In theory, when the human serum sample is free of anti-N IgG/IgM and anti-S-RBD IgG/IgM, the detection probes can not be captured and migrate the nitrocellulose membrane without color appearing on G and M test lines under the capillary force of the absorbent pad. By contrast, anti-N IgG/IgM and anti-S-RBD IgG/IgM were specially bound to the detection probes and captured by mouse anti-human IgG and IgM antibodies coated on the G and M test lines with red color appearing, but the result was invalid in the case of control line was no color appearing (the results were graphed in Fig. 1A). The red color of the test lines changed from weak to strong with increasing IgG/IgM concentration. The optical signal intensity of the test line was recorded and outputted in digital form by using a homemade reader to improve the sensitivity and avoid the influences of subjective and environmental factors on the detection results (Fig. 1D). The developed POCT system was predicted to contribute to precise control and prevention of the COVID-19 epidemic.

2. Materials and methods

2.1. Materials, reagents and samples

Chloroaucric acid (HAuCl₄·3H₂O) was purchased from Sigma–Aldrich Chemical Corporation (St. Louis, Mo, USA). Trisodium citrate, bovine serum albumin (BSA), and Tween-20 were purchased from Aladdin Reagent Co. (Shanghai, China). Recombinant N protein, recombinant S-RBD protein, and mouse anti-human IgG and IgM were provided by Beijing WDK Biotechnology Co. Ltd. (Beijing, China). Human monoclonal antibodies solution CRM [GBW(E)091110] and [GBW(E)091109] were provided by the National Institute of Metrology (Beijing, China). The sample pad, glass-fiber membrane, PVC pad, and absorbent pad were obtained from Kinbio Tech Co., Ltd. (Shanghai, China). Nitrocellulose (NC) membrane was purchased from Sartorius (Göttingen, Germany). All solvents and other chemicals were of analytical reagent grade.

Human serum samples including blank serum samples from healthy persons, positive serum samples, and continuous clinical course samples from patients with COVID-19 and clinically suspicious serum samples from patients whose nucleic acid of SARS-CoV-2 was positive by RT-PCR but did not have any clinical symptoms were obtained from Wuhan Jinyintan Hospital, inactivated at 56 °C for 30 min, and stored at −80 °C until use. This study was approved by the Medical Ethics Committee of Wuhan Infectious Disease Hospital (KY-2020-75.01) during May, 2020 to May, 2021 (Wuhan, China).

2.2. Instrumentation

ZX1000 Dispensing Platform and CM4000 Guillotine Cutting Module were supplied by Kinbio Tech Co., Ltd. (Shanghai, China). Ultrapure water was purified with Milli-Q system from Millipore Corp. (Bedford, MA, USA).

2.3. Preparation of CGNP-recombinant protein conjugations

CGNPs were prepared according to a previous report [25]. The conjugation of CGNP-recombinant N protein was prepared as follows: 10 mL of the CGNP solution was adjusted to pH 8.0 by adding 0.2 M K₂CO₃. About 1 mL of the recombinant N protein was added dropwise to the CGNP solution to a final concentration of 0.6 mg mL⁻¹ under gentle stirring. The mixture was agitated for 30 min at room temperature and added with 0.5 mL of BSA (20 %, w/v) for blocking for 20 min. The mixture was centrifuged with 12,000 rpm at 4 °C for 30 min to remove the unconjugated free recombinant protein. The resulting precipitate was dissolved in 2.5 mL of 1.0 M Tris-HCl containing polyvinylpyrrolidone K30, sucrose, BSA, and ProClin300. The colloidal gold conjugated with recombinant S glycoprotein was prepared with similar method.

2.4. Preparation of CG-LFIA test strips

The structure of the CG-LFIA test strip is shown in Fig. 1A. The sample pad was treated with 50 mL borate buffer (pH 7.4, containing 1% BSA, 0.5 % Tween-20, and 0.05 % sodium azide) and dried at 60 °C for 2 h. The conjugations of the CGNPs-recombinant protein used as detection probes were sprayed to an untreated glass fiber membrane with 3.0 μl/cm and completely dried at 35 °C with a vacuum dryer for 2.5 h. Mouse anti-human IgG and anti-human IgM were dispensed with 1.0 μl/cm onto the NC membrane as the G and M test lines and dried at 45 °C for 2 h. The control probes and lines on NC membrane were CGNP-tylosin-BSA and mouse anti-tylosin antibody respectively. The CG-LFIA test strip was assembled by attaching the sample pad, conjugated pad, NC membrane, and absorbent pad onto the PVC pad with overlapping 2.0 mm on top of each other. The fabricated CG-LFIA test strip was cut into 4.0 mm wide strips, stored at room temperature, and kept dry.

2.5. Assay procedures

In brief, 10 μL of human serum and 80 μL of PBS buffer (containing 1 % BSA and 0.5 % Tween-20) were added successively to the sample well of the CG-LFIA test strip. Within 15 min, the CG-LFIA test strip was scanned with the homemade portable reader to record the optical signal intensity of the test line. The qualitative result would be outputted on the LED screen of the portable reader.

2.6. Application of the POCT system

According to the assay procedures, the developed POCT system was used to supplementary diagnose 108 clinically suspicious serum samples. Sixty-three of the positive serum samples and 6 of the continuous clinical course samples from an inpatient with COVID-19 were measured by our POCT system to investigate the levels and alterations of anti-N IgG/IgM and anti-S-RBD IgG/IgM. The results were graphed with GraphPad Prism version 5.0.

2.7. Statistical analysis

IBM SPSS Statistics 26.0 software was used for statistical analysis. The value of Pearson Chi-Square (χ²), P-value of McNemar test, and kappa coefficient were calculated. P < 0.05 was considered to be statistically significant.

3. Results and discussion

3.1. Parameters of the homemade portable reader

A homemade portable reader (illustrated in Fig. 1B) was developed based on modern photoelectric technology to read and record the optical signal intensity of the test line on the GC-LFIA test strip. In the portable reader, high-performance LED with the wavelength of 500–520 nm was used as light source, and the light was focused on the GC-LFIA test strip by optical lens. According to reflectance spectroscopy, the CGNPs on the test strip would produce optical reflection signal due to the strong absorbance of CGNPs in the green region [26]. The produced reflective optical signal was converted into photoelectrical signals by conversion module and calculated as peak area outputted on the LED screen. The optical signal intensity of the test line increased with increasing amount of CGNPs. The absorbance accuracy, repeatability, and linearity error of
the portable reader were 2.00 %, 0.50 %, and 1.00 %, respectively. The portable reader has the functions of automatic diagnosis and wavelength calibration.

3.2. Performances of the POCT system

The immunoreaction on the CG-LFIA test strip was related to the signal intensity and detection result. The immunological kinetics analysis of the CG-LFIA test strip was carried out by detecting the negative and positive human serum samples. The signal alteration of the test line was recorded by portable reader every one minutes within 45 min. The optical signal intensity of the positive sample increased with the reaction time but trended to balance and remained basically the same from 15 min to 45 min; meanwhile, that of the negative sample altered insignificantly (Fig. 1S). The detection of anti-SARS-CoV-2 IgG/IgM with the developed POCT system was completed within 15 min.

The serum samples from different people exhibited a great disparity because the component and content in serum often vary with sex, age, and physiological and nutritional conditions of the donors. The matrix effect is inevitable. Receiver operating characteristic (ROC) curve analysis and Youden index calculation were performed to determine the optimal cutoff value of the POCT system for anti-N IgG/IgM and anti-S-RBD IgG/IgM detection; these methods have been widely used across many disciplines [27]. A total of 196 of human serum samples (145 negative and 51 positive) were detected by the developed POCT system. The ROC curves plotted according to the optical signal intensities obtained by IBM SPSS Statistics 26.0 software are shown in Fig. 2. The values of the area under curve (AUC) for anti-N IgG/IgM and anti-S-RBD IgG/IgM were 0.974, 1.000 and 0.987, 1.000, indicating the high accuracy of the POCT system to detect anti-SARS-CoV-2 IgG/IgM. Overall, based on the analysis of Youden index and practical application situation, the optimal cutoff values for anti-N IgG and IgM detection were

Fig. 2. ROC analysis for the optical signal intensities of anti-N IgG (A), anti-S-RBD IgG (B), anti-N IgM (C) and anti-S-RBD IgM (D) obtained by the developed POCT system.

Table 1
The precision of the developed POCT system (n = 3).

| Sample | anti-N IgG | anti-S-RBD IgG | anti-N IgM | anti-S-RBD IgM |
|--------|-----------|---------------|-----------|---------------|
|        | mean  | RSD%| mean  | RSD%| mean  | RSD%| mean  | RSD%| mean  | RSD%| mean  | RSD%|
| NO.1   | 0.000 | –   | 1.907 | 21.3| 0.000 | –   | 0.475 | 25.6| 0.000 | –   | 0.498 | 18.3|
| NO.2   | 6.295 | 15.1| 5.027 | 18.5| 2.887 | 9.0 | 1.333 | 12.3| 5.288 | 17.3| 0.987 | 1.000|
| NO.3   | 28.872| 12.1| 5.062 | 17.4| 10.564| 11.4| 0.727 | 13.5| 3.021 | 13.7| 1.000 | 1.250|
| NO.4   | 1.680 | 4.2 | 1.972 | 21.4| 1.394 | 5.1 | 0.491 | 19.0| 2.318 | 24.44| 1.433 | 12.5|
| NO.5   | 1.799 | 17.3| 18.531| 10.7| 1.038 | 22.7| 4.185 | 20.5| 1.797 | 17.75| 1.718 | 20.9|
| NO.6   | 6.311 | 19.1| 18.525| 6.0 | 2.771 | 11.7| 4.512 | 6.9 | 2.771 | 11.7| 4.512 | 6.9|

Table 1 continued

| Sample | anti-N IgG | anti-S-RBD IgG | anti-N IgM | anti-S-RBD IgM |
|--------|-----------|---------------|-----------|---------------|
|        | mean  | RSD%| mean  | RSD%| mean  | RSD%| mean  | RSD%|
| NO.1   | 0.000 | –   | 1.907 | 21.3| 0.000 | –   | 0.475 | 25.6| 0.000 | –   | 0.498 | 18.3|
| NO.2   | 6.295 | 15.1| 5.027 | 18.5| 2.887 | 9.0 | 1.333 | 12.3| 5.288 | 17.3| 0.987 | 1.000|
| NO.3   | 28.872| 12.1| 5.062 | 17.4| 10.564| 11.4| 0.727 | 13.5| 3.021 | 13.7| 1.000 | 1.250|
| NO.4   | 1.680 | 4.2 | 1.972 | 21.4| 1.394 | 5.1 | 0.491 | 19.0| 2.318 | 24.44| 1.433 | 12.5|
| NO.5   | 1.799 | 17.3| 18.531| 10.7| 1.038 | 22.7| 4.185 | 20.5| 1.797 | 17.75| 1.718 | 20.9|
| NO.6   | 6.311 | 19.1| 18.525| 6.0 | 2.771 | 11.7| 4.512 | 6.9 | 2.771 | 11.7| 4.512 | 6.9|

* Serum samples were tested three times a day.
* Six of serum samples were tested three times per day for five consecutive days.
1.870 with sensitivity of 95.9 %, 96.6 % and specificity of 96.1 %, 100 % (Table 1S). These results demonstrated that the developed POCT system exhibited good performances in detection of anti-SARS-CoV-2 IgG/IgM. The limitation of the POCT system was that the specificity has not been evaluated because of the unobtainable human antibodies against relative virus, and it would be completed in our subsequent work.

The precision of the developed POCT system was evaluated based on intra-assay and inter-assay variations. Six human serum samples were tested three times a day for five consecutive days, and relative standard deviation (RSD) was calculated. As presented in Table 1, the intra-assay and inter-assay RSDs for anti-N IgG/IgM detection were less than 22.65 % and 25.98 %, and those for anti-S-RBD IgG/IgM detection were 6.01 %–25.63 % and 10.73 %–23.97 %, respectively. These results indicated that the developed POCT system can detect anti-SARS-CoV-2 IgG/IgM in human serum precisely.

### 3.3. Method comparison

Four commercial kits approved by NMPA (A-, B-, C-, and D-Kit) were used to compared with our developed POCT system. Among them, the B-Kit and D-Kit can detect IgG and IgM with two separate test lines, but the A-Kit detect IgG and IgM with only one test line, and the C-Kit can only detect IgG. The method comparisons were carried out by detecting 24 serum samples of healthy people and serum samples from 23 patients with COVID-19 confirmed by clinical diagnosis. Results suggested that 24 serum samples of healthy people were negative tested by the five methods, which indicated that the negative coincidence rate was 100 %, but the positive tests were different (Table 2S). For example, sample No. 15 was tested with IgG and IgM negative by A-Kit, but anti-S-RBD IgM was positive detected by our developed POCT system. IgM in sample No. 15 and No. 23 qualitatively detected by C-Kit were inconsistent with what the developed POCT system detected. As shown in Table 3S, the results obtained by our developed POCT system and A-Kit, C-Kit were compared by χ² test, the P-values of McNemar test were 1.000, 1.000, and the coefficients of kappa test were 0.957 and 0.915 (P < 0.001), which suggested high consistency of the POCT system, A-Kit and C-Kit. According to the detection results, the IgM detection showed low consistency between our developed POCT system and B-Kit and D-Kit with the kappa coefficients at 0.449 and 0.377. But the IgG detection exhibited with sublime consistency, the χ² test showed that the coefficients of kappa test were 0.871 and 0.957, the P-values of McNemar test were both 1.000 (Table 4S). Either IgG or IgM was positive, the sample would be defined as positive, the 24 negative samples and 23 positive samples were detected accurately by our developed POCT system, B-Kit and D-Kit (Table 2S). Thus, the developed POCT system exhibited equal to or better performances than four approved commercial kits. The proposed POCT system was successfully developed and can be used to objectively detect anti-SARS-CoV-2 IgG/IgM in serum with the advantages of simple operation, convenience, rapid procedure, and inexpensiveness.

### 3.4. Applications of the developed POCT system

Clinically suspicious serum samples were collected from 108 patients whose nucleic acid of SARS-CoV-2 were positive as determined by RT-PCR but did not have any clinical symptoms. These suspicious samples were tested by our developed POCT system and CLIA kit (provided by Shenzhen Mindray Bio-medical Electronics Co. Ltd.) to ensure the accuracy of the diagnostic tests. The CLIA kit did not distinguish anti-N or anti-S-RBD, the sample would be considered as IgG positive when the detection data was greater than 10 U/L, and considered as IgM positive when the data was greater than 1.000 COI. For the developed POCT system, the sample detected with any one of anti-N IgG/IgM and anti-S-RBD IgG/IgM should be defined as positive. The detection results were displayed in Table 5S, the positive results were marked with red. It indicated that the positive rate of IgG was more than that of IgM, both of IgG and IgM were detected as positive in seven serum samples. As shown in Table 2, the χ² test showed that the coefficient of kappa test was 0.804, P-value of McNemar test was 0.125, which indicated that the developed POCT system has high degree of consistency with the CLIA kit. Hence, the proposed POCT system can be employed as supplementary diagnostic methods in prevention and control of the COVID-19 epidemic.

No study has been conducted and no products have been developed for quantitative detection of anti-SARS-CoV-2 IgG/IgM in human serum; as such, the levels of IgG/IgM in patients are unknown. Based on the principle of the CG-LFIA, the levels of IgG/IgM were positively associated with the signal intensities on the G and M lines. Sixty-three positive serum samples were measured by the developed POCT system to primarily investigate anti-N IgG/IgM and anti-S-RBD IgG/IgM levels. It found that five of the serum samples contained all of the anti-N IgG/IgM and anti-S-RBD IgG/IgM. The IgG levels are shown in

| Table 2 |
| --- |
| The χ² test of the results obtained by the developed POCT system and CLIA kit. |
| The developed POCT system | Positive | Negative | Total |
| --- | --- | --- | --- |
| CLIA Kit | 82 | 6 | 88 |
| Positive | 1 | 19 | 20 |
| Total | 83 | 25 | 108 |
| Pearson Chi-Square | 71.232 |
| Degree of freedom | 1 |
| P-value of McNemar test | 0.125 |
| Kappa coefficient | 0.804 (P < 0.001) |

Fig. 3. Levels of anti-N and anti-S-RBD IgG (A) and anti-N and anti-S-RBD IgM (B) in serum samples of 63 COVID-19 patients. Error bars represent the standard deviation of three measurements.
Fig. 4. The anti-N and anti-S-RBD IgG/IgM level alterations in a hospital patient with COVID-19 from 0 to 21 days. Error bars represent the standard deviation of three measurements.

Fig. 3A, all of the serum samples were detected with one or both of anti-N IgG and anti-S-RBD IgG positive, consistent with the clinical diagnosis. The Fig. 3B displays the levels of anti-N IgM and anti-S-RBD IgM, the amount of IgM positive was less than that of IgG positive, and the positive rate of anti-S-RBD IgM was higher than that of anti-N IgM.

Our developed POCT system was also applied to monitor the IgG/IgM level alterations in the patients with COVID-19. The serum samples were collected from a hospital patient from 0 to 21 days, and the results obtained by the POCT system were shown in Fig. 4, the anti-N IgG/IgM and anti-S-RBD IgG/IgM were tested with negative within two days, but all of the four antibodies were detected at the seventh day. The levels of IgM decreased from 7 to 21 days, the anti-N and anti-S-RBD IgG kept at a high level in the observation period, which indicated that the patient was gradually recovering. In the future, additional comprehensive studies and sophisticated technologies are required for accurate measurement of IgG and IgM concentrations in the body; this approach would be a landmark for antibody detection.

4. Conclusions

A rapid and simple POCT system was developed successfully for detection of anti-N IgG/IgM and anti-S-RBD IgG/IgM in human serum to confirm and exclude SARS-CoV-2 infectors as an assistant approach of nucleic acid testing. The system has a high degree of consistency with the CLIA kit for detection of clinically suspicious serum samples. Based on the portable reader and the CRM, the POCT system is expected to be employed in primarily to evaluate the infection rate of SARS-CoV-2 in the population and determine the levels and alterations of anti-SARS-CoV-2 IgG/IgM. The proposed system provides more options for anti-SARS-CoV-2 IgG/IgM detection, which is helpful for the control of the COVID-19 epidemic.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.snb.2020.129415.

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