Cell-Free Synthetic Biosensors Based on CRISPR/Cas Mediated Cascade Signal Amplification for Precise RNA Detection

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Article

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Abstract

Synthetic biology has been harnessed to create new diagnostic technologies. However, most synthetic biosensors involve error-prone amplification steps and limitations of accuracy in RNA detection. Here, we report a cell-free synthetic biosensing platform, termed as SHARK (Synthetic Ezyme Shift RNA Signal Amplifier Related Cas13a Knockdown Reaction), to efficiently and accurately amplify RNA signal by leveraging the collateral cleavage of activated Cas13a to regulate cell-free enzyme synthesis. Based on cascade amplification and customized enzyme output, SHARK behaves a broad compatibility in different scenarios. Using a personal glucose meter, we detected 50 copies/ μ l SARS-CoV-2 on a SHARK-loaded paper. In addition, when combined with machine learning, SHARK can perform bio-computations and thus provide miRNA patterns for cancer diagnosis and staging. SHARK shows characteristics of precise recognition, cascade amplification and customizable signal outputting in one pot comparisons with established assays based on 64 clinical samples, presenting great potential in developing next-generation RNA detection technology.

Introduction

Rapid, accurate, and cost-effective detection of RNA biomarkers plays important roles in aiding pandemic prevention, medical treatment, and prognostic management¹⁻⁴. Generally, quantitative reverse transcription PCR (qRT-PCR) has been engaged as the “gold standard” method for measuring gene strands in clinic⁵, but some challenges remain when detecting RNA species, due to their low abundance, easy degradation, short length, and single nucleotide mutations, *etc*^{6, 7}. Inadequate access to the qRT-PCR equipment has also limited the application for RNA detection in resource limited settings. Recently, various strategies, such as isothermal amplification methods⁸ and nanoprobe techniques⁹, have been developed to sense RNA targets and output fluorescent or colorimetric signals for readout. However, these strategies involve multiple amplification reactions that may cause nonspecific amplification and false positive errors, yielding signals with poor accuracy and reliability¹⁰.

Synthetic biology based on cell-free gene expression (CFE) system has been harnessed to create new diagnostic technologies to detect a variety of pathogens and disease biomarkers¹¹. The RNA detection process of synthetic biosensors is programmed to recognize the gene targets through base pairing, amplify signals *via* biosynthetic reactions, and express reporter proteins (*e.g.*, fluorescent proteins or various enzymes that can further augment the signals by catalyzing substrates), outputting tailor-made signals for different detection scenarios^{12, 13}. Usually, these synthetic sensors generate limited increase in signal ($<10^3$ fold), and thus in most cases a separate gene amplification step is needed to augment and convert the target signal¹³⁻¹⁵, leading to issues of poor accuracy and tedious operation. CRISPR/Cas13a can accurately identify and cleave specific RNA sequences and further augment the signals by means of collateral nonspecific catalytic cleavage, presenting excellent capabilities in accurate nucleic acid recognition and signal amplification¹⁶. For example, Cas13a can recognize and cleave single-stranded RNA targets, and its collateral activity is subsequently activated to cleave almost all reporter RNA,

generating a fluorescent signal as readout¹⁷. Although the dynamic range is over seven orders of magnitude of activated Cas13a-catalyzed cleavage¹⁸, these strategies still need to be combined with additional amplification steps to meet the detection needs. Considering that activated Cas13a may carry out unspecific cleavage of mRNA in CFE process^{19,20}, it is convincing that coupling the cell-free synthetic biosensor with CRISPR/Cas13a will solve the issues of target recognition accuracy and signal amplification integration, and realize portable and easy-to-use platform for RNA detection with customized signals.

In this study, we developed an RNA detection platform by coupling CRISPR/Cas13a with CFE system for precise recognition and cascade amplification of different RNA species, termed as **SHARK** (Synthetic Enzyme Shift RNA Signal Amplifier Related Cas13a Knockdown Reaction). In brief, trace RNA targets in tested samples were first recognized by the CRISPR RNA (crRNA), and then activated the Cas13a to digest the mRNA in CFE system, reducing the expression of reporter enzymes and outputting electrochemical, colorimetric, or bioluminescent signals with a “turn-off” behavior. Integrating the SHARK into different point-of-care devices, we rapidly detected the virus RNA of SARS-CoV-2 *via* a portable glucose meter, and sensitively operated bio-computations with multiple microRNAs as inputs for cancer diagnosis in paper analytical devices and digital-chips, indicating that our SHARK platform holds a great potential for developing rapid, accurate, and multi-module strategies for disease diagnosis.

Results

SHARK enables accurate and sensitive RNA detection in a single reaction. In synthetic biosensors, the plasmid is transcribed into mRNA to initiate the protein expression, and the degradation of mRNA will obviously influence the output signals. Here, in SHARK platform, CRISPR/Cas13a can precisely recognize the RNA targets and activate its collateral cleavage activity, which is then used to regulate the mRNA levels to generate different concentrations of enzymes, finally outputting different modules of signals (Fig. 1a). To testify our hypothesis, the activation of CRISPR/Cas13a was investigated by incubating the target RNA with unspecific mRNA molecules produced by a HiScribe T7 High Yield RNA Synthesis Kit. With increasing target concentrations, most transcribed mRNA strands were digested into fragments, indicating that the target RNA recognized the crRNA and activated the catalytic cleavage (Fig. 1b). Then, we coupled the CFE system with CRISPR/Cas13a to build a series of SHARK platforms, which generated different kinds of enzymes that could be detected by many commercial kits, including sucrose invertase (slnv), glucose-6-phosphate dehydrogenase (G6PD) and luciferase (Luc) (**Supplementary Fig. 1a**). The gel results showed that compared with nonsense control RNA, the target RNA selectively activated the SHARK, and significantly repressed the enzyme expression (Fig. 1c). The kinetics of SHARK was also determined across a range of different concentrations of target RNA. For example, the activation of the SHARK sensor generating G6PD was easily visualized by naked eyes, and it exhibited much faster kinetics compared with that expressing enhanced green fluorescent protein (eGFP) as reporters (Fig. 1d), suggesting that the cascade amplification of signals *via* mRNA translation and enzymatic reaction greatly benefited the detection. The experimental (**Supplementary Fig. 2a**) and simulation results

(**Supplementary Fig. 2b,c**) confirmed that as low as 10 fM RNA targets could trigger significant signal distinction with high reliability (CV = 5.51%). Taken together, SHARK could sensitively detect trace RNA at femtomolar range, behaving a better performance compared with other strategies, such as Cas13a-based detection¹⁷ and toehold switch synthetic gene networks¹⁴ (Fig. 1e).

SHARK output different modules of signals. The great advantage of CFE system is the versatile capability in rational design and integration of different reporter proteins. However, so far, only limited enzymes have been explored as reporters in the synthetic bio-sensors, such as eGFP and β -galactosidase^{14, 21}. Here, to facilitate the signal readout, we explored to introduce several enzymes (*i.e.*, slnV, G6PD and Luc) that can be easily detected using commercial kits into SHARK as reporters (**Supplementary Note 1**). First, we used slnV as reporters to build a biosensor (slnV-SHARK). The addition of target RNA activated SHARK, degraded the mRNA, and reduced the slnV expression, which catalyzed the conversion of sucrose to glucose (Fig. 2a). After loading the samples, we conveniently and sensitively detected trace RNA through measuring the glucose levels using a personal glucose meter (PGM, Accu-Chek Performa) with a limit of detection (LOD) at 11.72 fM (Fig. 2b). In contrast, we also developed a G6PD-SHARK sensor that converted the RNA signal to G6PD, and finally to the production of formazan, outputting colorimetric signals that can be visually measured without need for any instruments (Fig. 2d) with LOD at 9.50 fM (Fig. 2e). Differently, in a sensor of Luc-SHARK, the target RNA reduced the expression of luciferase, which catalyzed the bioluminescence reaction (Fig. 2g) with high activity, low background noise, and fast response time, benefiting the RNA detection with a large dynamic range (10^{-4} - 10^2 nM) and LOD at 6.55 fM (Fig. 2h).

Then we investigated the specificity of SHARK. Most isothermal exponential amplification methods show poor performance in distinguishing gene strands with high sequence homology^{10, 22, 23}. As designed, CRISPR/Cas13a system has the capability to discriminate single nucleotide mutations since that it requires strict crRNA-target pairing to activate the collateral cleavage^{18, 24} (**Supplementary Fig. 3a**). To testify our hypothesis, we synthesized three RNAs analogs with single-base mutation. As expected, in all three kinds of sensors of SHARK, the RNA targets strictly recognized the cognate crRNA and yielded reduced signals, whereas those with one-base mismatch barely activated CRISPR/Cas13a system, and generated strong signals similar to those from scrambled RNA (Fig. 2c,f,i), indicating that SHARK possessed high specificity in discriminating single nucleotide alterations. Further, the interference of human total RNA in samples was also investigated, and the biosensors outputted reliable signals even with a high molar ratio of target RNA to total RNA at 1:1000 (**Supplementary Fig. 3b-d**). Thus, our SHARK has provided customizable signals with high sensitivity, specificity, and reliability, inspiring us to apply it in point-of-care testing of various RNA species at different scenarios.

Paper-loaded slnV-SHARK sensor facilitates rapid and reliable detection of SARS-CoV-2 using PGM.

Infectious diseases caused by different viruses exert great threats to human health, and to date, the COVID-19 coronavirus pandemic has caused more than two million deaths²⁵. Similar to other deadly respiratory syndromes (*e.g.*, Severe acute respiratory syndrome coronavirus (SARS), Middle East

Respiratory Syndrome Coronavirus (MERS)), rapid and accurate detection of SARS-CoV-2 will greatly benefit the contact tracing, treatment options and isolation requirements, meaning the urgent need to develop point-of-care technologies available at a variety of scenarios^{26,27}. To favor the testing of SARS-CoV-2 at home, we designed a paper-loaded freeze-drying slnv-SHARK sensor with rapid readout by a PGM, which is easily accessible on the market (Fig. 3a). First, we constructed contrived SARS-Cov-2 and synthesized three crRNAs targeting the genes of replicase polyprotein 1ab (Orf1ab), nucleocapsid (N) and envelope protein (E) in SARS-CoV-2, respectively (**Supplementary Fig. 4**)²⁷. Here, the combination of multiple crRNAs could activate multiple nuclease-inactive ribonucleoprotein complex²³, and thus effectively increase the active Cas13a concentrations so as to improve sensitivity. When mixing the three crRNAs in the detecting solution containing contrived SARS-Cov-2 samples, the slnv-SHARK sensor accurately identified virus concentrations as low as 1 fM, outputting a signal value of 21.5 ± 0.9 with high reliability (CV = 4.18%, Fig. 3b). Considering the actual viral loads in clinic samples²⁶, we serially diluted the pseudovirus on throat swab (from 10 to 7×10^8 copies per swab) with scrambled RNA as controls. The slnv-SHARK sensor outputted a decreased signal between 29.7 and 1.4, with a dose-dependent response from 20.2 to 5.3 at a concentration range of 10^{-5} - 10^2 nM (*i.e.*, $\sim 10^3$ to 10^7 copies/ μ L) with high accuracy and reliability, setting a cut-off value at 22.5 (~ 50 copies/ μ L) (Fig. 3c).

To facilitate the detection at home, we integrated slnv-SHARK with a PGM into a disposable device previously developed in our lab²⁸, in which the users only need to drop the samples on the loading paper and press the start button, outputting a digital result within 1.5 h (Fig. 3d, **Supplementary Fig. 5**). We used qRT-PCR method to calibrate the slnv-SHARK-based detection, and several dilutions of pseudovirus at a range of 1.5×10^2 to 10^8 copies/ μ L were measured with three negative swabs as controls. The slnv-SHARK device generated reliable and easy-to-readout results between 5.3 and 22.5 that was highly consistent with those from the Ct values of qRT-PCR between 14 and 37 (Pearson r coefficient = 0.96) (Fig. 3e), meaning that the cut-off value of 22.5 was equivalent to Ct = 37 (the standard qRT-qPCR value from Disease Control and Prevention (CDC) of China). Further randomized double blind tests in laboratory confirmed that both the slnv-SHARK and qRT-PCR methods showed high coincidence in detecting positive samples (**Supplementary Fig. 6**), as well as in discriminating negative control samples (**Supplementary Table 1**).

Finally, we investigated the cross-reactivity between SARS-CoV-2 and other respiratory diseases related pathogens (*e.g.*, MERS, human coronavirus HKU1, Influenza A virus (IVA), *etc.*). The heat map (Fig. 3f) reflected that the SHARK device could easily discriminate the pathogen RNA from others with the same concentration (1 nM). In turn, by designing cognate crRNA, our device could also detect the RNA targets from different pathogens (Fig. 3g, h). Taken together, slnv-SHARK-based device could sensitively and accurately sense the SARS-CoV-2 RNA and output digital signals with high reliability and robustness against matrix effect and interference (**Supplementary Fig. 7a, b**), presenting as a convenient and reliable strategy for rapid detection of SARS-CoV-2 at resource limited settings such as home or community.

Paper-based G6PD-SHARK sensor enables multiple miRNA detection for cancer staging. miRNA is a class of endogenous small noncoding RNA molecules, which serve as promising clinical biomarkers in various human diseases, including cancer²⁹. However, the characteristics of miRNAs, such as short length, high sequence homology, and low abundance, make it a great challenge to develop sensitive and specific sensors³⁰. CRISPR/Cas systems possess special capability in recognizing short gene targets (20–40 nucleotides) with high specificity and reliability, making it possible to sensitively detect miRNAs. Considering the flexibility of our SHARK platform, we designed a pattern pad to detect multiple miRNA targets in serum from the patients of non-small cell lung cancer (NSCLC), offering abundant information to evaluate the cancer staging.

We first built a data mining model to select biomarkers *via* bioinformatics tool, and seven miRNAs highly related to NSCLC (Fig. 4a) were selected with four upregulated (*i.e.*, miR141, miR-30a, miR-20a, Let-7b) and three downregulated (*i.e.*, miR-126, miR-328, miR-19b) compared with healthy people by analyzing the miRNA expression database of 915 NSCLC and 105 healthy individuals from The Cancer Genome Atlas (TCGA). Then we designed a G6PD-SHARK based paper origami device with two detection zone respectively for loading testing sample (T) and control standard sample (C). The G6PD-SHARK sensor was pre-packed in the top layer through a freeze-drying procedure, which was folded with the base layer together to assemble the device for sample loading and signal amplification, generating an easy-to-read visual output (Fig. 4b, **Supplementary Fig. 8a, b**). To easily extract the results, we quantified the colorimetric signals *via* Image J software and used the ratio of T to C (T/C) to reflect the miRNA concentrations (Fig. 4c). Using a synthetic miR-20a sample, we verified that the T/C signal was proportional to the miRNA concentration ranging from 10^{-5} and 10^{-1} nM with LOD values as low as 7.56 fM, and thus the sensor could be used to detect most miRNAs in serum (10^{-5} ~10 nM)^{31,32}. Meantime, the sensor also showed excellent capability in discriminating different miRNA targets (Fig. 4d) even in the presence of 10-fold interferences with the signal deviation less than 5.21%. Especially, in the sensor targeting let-7b, which is highly overexpressed in most cancers³³, we found other members of let-7 family (*i.e.*, let-7a, let-7c, let-7d) with few mismatched bases had little interference (less than 4.97%) at the concentrations of 10, 50, and 100 pM (**Supplementary Fig. 9**), suggesting that the G6PD-SHARK platform guaranteed the detection of miRNAs with high sensitivity and specificity to distinguish single nucleotide mutations.

In clinic, more than 70% of NSCLC patients are diagnosed at advanced stages with a poor five-year survival rate (~ 19.40%) reported by the Surveillance, Epidemiology, and End Results program, and early diagnosis will greatly benefit the treatments³⁴. We next used our G6PD-SHARK-based sensor to detect the serum samples from lung cancer patients (**Supplementary Table 2**). We observed the up-regulation of miR-30a (2.91 ± 0.12), miR-20a (2.82 ± 0.15), Let-7f (3.13 ± 0.15), and down-regulation of miR-126 (0.61 ± 0.05), miR-328 (0.24 ± 0.01) in the patients with moderate sensitivity (40.25%-70.71%) and specificity (29.28%-71.25%), showing good correlation to the bioinformatics profiles. ROC curve analysis showed

that five miRNAs among them could be promising biomarkers for NSCLC, with AUC values from 0.64 to 0.76. Further, logistic regression analysis was used to construct combined models to evaluate these 5 candidates to verify the NSCLC signatures. Combining the five miRNAs (*i.e.*, miR-30a (+), miR-20a (+), Let-7f (+), miR-126 (-), miR-328 (-)) enhanced the AUC to 0.88 (sensitivity = 84.24%, specificity = 86.51%), indicating that multiple miRNA patterns are more reliable in cancer diagnosis than the single biomarker (Fig. 4e). Considering that the miRNA signatures may vary with cancer progress, we sought to find the relationship between the miRNA profiles and TNM (tumor, nodes, and metastasis) stage of NSCLC. The miRNA signatures of different patients at different stages (I, II, III, IV) diagnosed by pathological section were profiled in Fig. 4f using G6PD-SHARK-based sensor and qRT-PCR. The miRNA profiles showed a high coincidence between our SHARK sensor and qRT-PCR method (Pearson r coefficient = 0.84) (**Supplementary Fig. 10a**). The patients at the same stage shares similar miRNA signatures, which also dynamically changed along with the cancer progression. That is, miR-126, miR-328, and miR-30a were positive at advanced stage, but negative at stage I (**Supplementary Fig. 10b-d**), while miR-20a exhibited higher expression at all stages compared with healthy people (**Supplementary Fig. 10e**). Overall, our G6PD-SHARK-based sensor showed high sensitivity and specificity in detecting femtomolar levels of short miRNAs in the serum of lung cancer patients, and provided unique miRNA patterns for cancer diagnosis and staging.

G6PD-SHARK-CPU sensor enables miRNA computation for cancer diagnosis. To realize early cancer diagnosis at home, we sought to optimize G6PD-SHARK sensor by outputting one simple diagnostic result from multiple miRNA detection through bio-computation (Fig. 5a). First, we built a computational classifier with a support-vector-machine (SVM) model according to the published work³⁵, and trained the system using the miRNA profiles of NSCLC patient and healthy individuals from TCGA. To avoid over-fitting of machine learning, different models were evaluated to improve the classification performance (**Supplementary Fig. 11, Supplementary Note 2**) with a training database (105 NSCLC and 60 healthy samples), and generated an optimized model including seven miRNA inputs (miR-761 (-), Let-7f (-), miR-126 (-), miR-20a (+), miR-515 (+), Let-7b (+), miR-5688 (+)) associated with weights ranging from inter values of -5 to + 9, achieving identification accuracy of 85.72%, sensitivity of 87.11%, and specificity of 78.12% (Fig. 5b, **Supplementary Table 3**).

To facilitate the computation using the up-regulated and down-regulated miRNAs, we integrated a Cas9 system with the G6PD-SHARK to develop central processing unit (G6PD-SHARK-CPU) to transform miRNAs information. We combined the transcriptional and translational regulation systems together to tune the signal by using the up-regulated miRNAs related NSCLC to turn off the signal *via* G6PD-SHARK (OFF) and using the down-regulated miRNAs to turn on the signal *via* the Cas9-RsgRNA (ON) (**Supplementary Fig. 12a, Supplementary Note 3**). To test the ON/OFF system, we introduced G6PD reporter plasmids and measured the colorimetric signal as an output (Absorbance < 0.5 as TRUE). We observed 30-fold activation with high sensitivity (0.1 pM) and specificity when the target miRNA present in the ON system (Fig. 5c **left, Supplementary Fig. 13**), and also significant suppression in the OFF system (Fig. 5c **right**). Next, we designed an A NIMPLY B gate with miR-126 (-) and miR-5688 (+) as inputs, and

only the input of (0, 1) could output a TRUE signal (1) (Fig. 5d). Finally, we tested G6PD-SHARK-CPU system with clinical serum samples from NSCLC and healthy individuals. The optimal diagnostic point was calculated at the probability score of 0.55 as cut-off value (Fig. 5e) from the receiver operating characteristic (ROC) curves (Fig. 5f). Through bio-computation, 28 of 38 NSCLC patients were identified with a sensitivity of 77.78% (95% CI: 64.92%, 88.28%), while 1 of 26 healthy samples was misclassified with specificity of 96.15% (95% CI: 81.11%, 99.81%), and accuracy of 86.42% (95% CI: 65.82%, 96.14%) (Fig. 5g). Despite that we simplified the miRNA transformation, G6PD-SHARK-CPU sensor still presented as a promising user-friendly platform to favor the routine early cancer screening.

Digital-Luc-SHARK device achieves absolute quantification of miRNA. Usually, the fold change of miRNAs is subtle at early stage of cancer. Inspired by the high sensitivity and specificity of SHARK in distinguishing miRNAs, we next integrated QuantStudio™ chip (20K data points) to design a digital-Luc-SHARK device with luciferase generating bioluminescence as reporters (Fig. 6a). To improve the signal, we constructed a fusion protein consisting of luciferase and yellow fluorescent protein (YFP), exhibiting a 7.45-fold enhancement over native luciferase *via* bioluminescence resonance energy transfer (BRET)³⁶ (Fig. 6b). Then we designed a portable luminescence detection device consisting of an intensified CCD camera, a mirror and a filter with magnifier (Fig. 6c, **Supplementary Fig. 14**). After mixing the miRNA samples with the luc-SHARK reagents and dispersing it in the picoliter-scale reactors, we observed the increase of the positive spots on the chip with the increase of target miRNAs, which was consistent with Poisson distribution equation (**Supplementary Fig. 15**), presenting a dynamic range from 100 to 5×10^4 copies/ μ L (2 to 1000 aM) (Fig. 6d), and showing over 10^4 -fold enhancement in sensitivity compared with the tube-based SHARK process. Further, by employing an IC Capture software, the full view of the chip was collected and analyzed, generating a detection linear range of 200 – 20,000 copies/ μ L (**Supplementary Fig. 16a, b**). By virtue of the strict recognition, the digital-Luc-SHARK device also showed high specificity in discriminating the members of Let-7 family with interference less than 0.51% (Fig. 6e).

We next evaluated the accuracy of digital-Luc-SHARK, digital PCR, and qRT-PCR in quantifying miR-20a in clinic serum samples from NSCLC patients. All tests generated positive results, and the relative expression results from digital SHARK showed similar variations to those measured with digital PCR (Fig. 6f, **Supplementary Fig. 17**). Finally, we used this handheld digital-Luc-SHARK device to precisely detect the miRNA markers for early diagnosis of NSCLC. Four miRNAs (*i.e.*, let-7b, miR-126p, miR-20a and miR-30a), previously tested as NSCLC biomarkers, were picked for validation. Figure 6g reflected that the up-regulation of let-7b, miR-30a, and miR-20a was highly related to the early-stage patients (Stage I, II) with Let-7b and miR-30a four times higher than those in the healthy people. The accurate and reliable quantification of miRNAs using our handheld digital-Luc-SHARK device makes it promising to achieve convenient early NSCLC diagnosis at home.

Discussion

In the present study, we developed a cell-free synthetic biosensor platform, *i.e.*, SHARK, for detection of a variety of RNA species. By leveraging the collateral cleavage of activated Cas13a to regulate the mRNA

translation, we achieved strict recognition, cascade amplification, and customizable signal output in one pot. This SHARK platform shows excellent compatibility in integrating with various point-of-care devices, and outputs different modules of signals, enabling the diagnosis of different infectious and chronic diseases with high sensitivity and reliability at resource limited settings.

Synthetic biology has provided a powerful tool to develop diagnostic technologies to sense various targets (*e.g.*, gene sequences¹⁵, biomarkers³⁷, chemical contaminants³⁸), regulate the amplification *via* transcription and translation, express a series of enzymes, and output diverse detectable signals. One limitation of our SHARK platform is that it needs 1.5-3 hours to complete the entire detection process. Through optimizing components in SHARK, it is promising to further optimize the amplification processes, and select enzymes with high catalytic kinetics to realize rapid detection³⁹. Another challenge is how to linearize the amplification response of the SHARK to fit the bio-computation with multiple inputs⁴⁰. For broad clinical use, mathematical models and programmable automations should be developed for user-defined diagnosis. Taken together, we envision that our SHARK will be a versatile plug-and-play platform to develop sensitive, low-cost and easy-to-use diagnosis technologies.

Methods

Plasmids design and preparation. The open reading frame of slnv (EC 3.2.1.26), G6PD (EC 1.1.1.49), Luc (EC 1.13.12.7), RLuc-YFP were amplified from commercial plasmids (Addgene) and cloned into the vectors of pET23a, pIX, pFN6A, and pET30 backbone, respectively⁴¹. The plasmids would be used as the templates for CFE reactions. All plasmids were assembled by T7 promoter, ribosome binding site (RBS), N-terminator as additional coding parts.

Cell-free enzyme expression. Cell-free enzyme expression reactions were performed following the instructions of the manufacturer. Briefly, 20 μ L of reaction mixture contains 14 μ L of S30T7 reagents (Promega, L1110), 1 μ L of RNase inhibitor (ThermoFisher Scientific AM2694, 20U/L), 5 μ L of enzyme expression plasmid in nuclease-free ddH₂O. The enzymatic kinetic curves were evaluated to determine the appropriate experiments condition at different plasmid concentrations and reaction time (**Supplementary Note 1**). For most experiments, we selected plasmid concentrations that allowed readouts in less than 90 min and maximized the dynamic range (**Supplementary Fig. 18**). The concentrations of plasmids were as follows: 1, 6 nM for pET23a-G6PD plasmid DNA; 2, 10 nM for pIX-Invertase plasmid DNA; 3, 2 nM for pFN6A-Luciferase plasmid DNA; 4, 0.1 nM for pET30-RLuc-YFP plasmid DNA.

LbuCas13a non-specific cleavage of transcribed mRNA. Cleavage assay was performed with 50 nM purified LbuCas13a (BIO-LIFESCICO, M20201), 1 μ M transcribed mRNA (**Supplementary Table 4**, NEB, E2040S), 50 nM crRNA (**Supplementary Table 5**) in nuclease cleavage buffer (50 mM Tris-HCl, 60 mM NaCl, 1.5mM MgCl₂, pH 8.5 in ddH₂O), and then varied target RNA in different assay was added to each 20 μ L reaction. The reaction was incubated at 37°C for 25 minutes. The cleavage ability was analyzed through the gel electrophoresis.

One pot SHARK assay for RNA detection. One pot SHARK assay was performed using LbuCas13a for the trans-cleavage assay and cell free enzyme expression for signal transduction of RNA targets. The final concentration of each reagent in the system was optimized (**Supplementary Note 1**). In brief, 11 µL of cell free enzyme expression reaction system was mixed with 4 µL of a CRISPR reaction buffer, and then we added 5 µL of target RNA and reaction buffer. After incubation at 37 °C for 90 min, the corresponding enzyme substrates were added for signal readout.

Colorimetric assay readout. The colorimetric assay was performed in 10 µL Tris buffer (50 mM, pH 8.5) containing 0.5 mM NADP⁺, 10 mM WST-8, 10 mM mPMS. After signal amplification in G6PD-SHARK assay, these mixtures were added and incubated for 5 min at 37 °C to produce orange yellow formazan. The absorbance at 450 nm was measured with a multimode microplate reader (Tecan Spark 10M).

PGM assay readout. Sucrose was hydrolyzed to glucose by slnV at 37 °C, which was then detected by PGM. After signal amplification in slnV-SHARK assay, 10 µL 0.5 M sucrose was added and then incubated for 1 min. Finally, 5 µL of the reaction solution was tested by a PGM.

Bioluminescence assay readout. After signal amplification in Luc-SHARK assay, 20 µL of bioluminescent substrate (Beyotime, RG051M) was added, and the bioluminescent signal was measured using a bioluminescence reader after 5 min at 37 °C in dark.

Mathematical modeling. Rate equation model was established based on the transcription, translation and collateral cleaving process for SHARK assay as follows: (1–4).

$$\frac{d}{dt} \text{mRNA} = \frac{k_{ts} \times \text{TsR} \times \text{DNA}}{K_S + \text{DNA}} - \frac{k_{cas} \times \text{mRNA} \times \text{Protein}}{K_m + \text{mRNA}} \quad (1)$$

$$\frac{d}{dt} \text{Protein} = \frac{k_{tl} \times \text{TIR} \times \text{mRNA}}{K_I + \text{mRNA}} - k_{mat} \times \text{Protein} \quad (2)$$

$$\frac{d}{dt} \text{TsR} = - \frac{k_{cs} \times \text{TSR} \times \text{DNA}}{K_S + \text{DNA}} \quad (3)$$

$$\frac{d}{dt} \text{TIR} = - \frac{\delta_{\text{TIR}} \times \text{TIR}}{K_{\text{TIR}} + \text{TIR}} \quad (4)$$

The Michaelis-Menten constants and parameter values were depicted in **Supplementary Table 6**.

Preparation of contrived SARS-CoV-2. The contrived SARS-CoV-2 contains partial ORF1ab gene sequence, E Gene and N Gene coding region sequences of SARS-CoV-2. These target fragments were synthesized and cloned into a retroviral vector to perform as pseudovirus. The pseudovirus was synthesized and

inactivated by Beijing Tsingke Biotechnology Co., Ltd.. We spiked inactivated virus into human plasma/serum (IRMM, ERM-DA470K), Saliva (Solarbio A7990) or simulated lung fluid (Tegent, 1700 – 0800) and diluted to simulate clinical samples. Then we followed the instructions of RNA extraction kit (Qiagen, 52904) to extract pseudovirus RNA.

Fabrication of integrated device for SARS-CoV-2 detection. The integrated device was composed of three modules, *i.e.*, paper-based nucleic acid extraction module, SHARK module and PGM detection module. The whole device was designed by SolidWorks²⁸. The detection module contains one PGM and one test strip.

Multi-channel paper-based device fabrication. Paper Analytical Device (PAD) for multiple miRNAs detection consisted of Whatman paper and two layer doubled-stick tapes. The detection pattern of PAD was designed with CorelDRAW and printed with laser engraving machine. The two layer doubled-stick tapes were enabled to isolate different channel area and form a liquid reservoir for sample loading.

Bioinformatics analysis. NSCLC related miRNA expression data come from TCGA. The screening search formula was follows, (NSCLC OR Non-small cell lung cancer OR Lung squamous cell carcinoma OR Lung adenocarcinoma) AND (Serum OR Plasma OR Free nucleic acid) AND (miRNA OR microRNA OR miR_). The adjusted *P* value was less than 0.05, and differential expression fold change was > 2 or < -2 . R language package (edgeR) was used to screen differentially expressed miRNAs.

Cas9 cleavage assay. *In vitro* Cas9 cleavage experiment was conducted using 10 μ L 1X cleavage buffer (10 mM Tris-HCl, 10 mM $(\text{NH}_4)_2\text{SO}_4$, 50 mM KCl, 2 mM MgSO_4 , pH 8.5) containing 1 μ M target dsDNA, 40 nM Cas9, 50 nM RgRNA (**Supplementary Table 7**). The mixture was pre-incubated for 10 min at 37 °C, and then different concentrations of target RNA (10^{-5} - 10^3 nM) were added. The Reaction was incubated for 30 min at 37°C. The cleavage ability was analyzed by gel electrophoresis.

SVM training and validation. To train the SVM classifier, we obtained the miRNA profile data of GSE40738 from TCGA, which was used to identify the up or down-regulated miRNAs in NSCLC compared with healthy individuals (The process details in **Supplementary Note 2**). Then the selected miRNAs were validated with associated weights. To get simple result by G6PD-SHARK-CPU, the up-regulated miRNAs (+) associated with NSCLC were responded to the SHARK assay, and the down-regulated miRNAs (-) were responded to the Cas9 assay. The concentrations of crRNAs and RsgRNAs were tuned to the relevant weights.

qRT-PCR. We reversed transcribed contrived SARS-CoV-2 RNA using Goldenstar RT6 cDNA synthesis kit (TsingKe, TSK301). miRNAs were reversely transcribed using reagents from the TaqMan miRNA Reverse Transcription Kit (Applied BioSystems, A25576) together with the stem-loop primers (**Supplementary Table 8**). The reverse transcription thermal-cycling procedure was performed at 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min. And then PCR experiments were performed on an Applied Biosystems 7500fast instrument with the ChamQ universal qPCR master system (Vazyme, Q711-02).

Digital RT-PCR. Digital RT-PCR reaction was prepared by combining target RNA and QuantStudio 3D Digital PCR Master Mix (PN A26358) according to the manufacturer's instructions. Then the reaction solution was loaded in one QuantStudio 3D Digital PCR Chip (PN 100027736). End-point fluorescence data were collected on the QuantStudio 3D Digital PCR instrument and analyzed using the QuantStudio 3D Analysis Suite software (Thermo Fisher Scientific).

Digital SHARK assay. The digital SHARK reaction mixture (10 μ L) was prepared by mixing the following reagents: 5.5 μ L of CFE system mixed with 2 μ L of a Cas13a reaction mixture, the volume was then brought up with target miRNA and nuclease free water. The optimized plasmid concentration in microwell was 0.1 nM^{42, 43}. The chips (Biosystems™ QuantStudio™: diameter = 60 μ m, volume $\approx$ 1 nL) were incubated for 2 h before bioluminescent imaging.

Portable Bioluminescence Detection Instrumentation. The handheld bioluminescence detection device includes a 10 $\times$ microscopic lens (Kase), a mirror and a charge coupled device (CCD, Mshot). The handheld detection device was designed using SolidWorks. The image of chip was obtained by CCD and analysed by IC capture software. The quantitative results were calculated based on the equations, and the relationship between the proportion of positive units (f_0) with the copy of miRNA concentration (c_0) and the dilution factor (x_{dil}) was as follows,

$$f_0 = 1 - e^{-c_0 \cdot x_{dil}} \quad (5)$$

Transform the equation: $-\ln(1 - f_0) = c_0 \cdot x_{dil} \quad (6)$

The result of linear regression analysis showed that the output of digital-Luc-SHARK corresponded to the model of the Poisson distribution. The initial copy number concentration (c_0) of miRNA can be calculated using this equation.

Study population collection and serum samples preparation. We obtained serum samples from 38 NSCLC patients (diagnosis confirmed by histopathology) at the First Affiliated Hospital, Xi'an Jiaotong University (Xi'an, Shaanxi, China). All cases were staged according to the revised TNM classification schema⁴⁴. In addition, serum samples from 26 healthy individuals of the First Affiliated Hospital were collected and used as controls. The age and sex were matched with NSCLC patients. This study was approved by the Ethics Committee at the First Affiliated Hospital of Xi'an Jiaotong University. The subject information was anonymized prior to analysis. All methods were performed in accordance with the approved guidelines in full adherence to the Declaration of Helsinki. Total miRNAs were extracted and isolated from plasma samples using miRNeasy Serum/Plasma Kit (Qiagen, USA) according to the manufacturer's protocol.

Statistical analysis. Data were shown as mean $\pm$ s.d.. Statistical analysis was performed using a two-tailed Student's *t*-test or one-way ANOVA with post hoc tests. The *P* value less than 0.05 was considered to be of statistical significance. The software SPSS and GraphPad Prism 8.0 were applied for data analysis and graph drawing. Limit of detection (LOD) of the platform was determined by 10-fold dilution

of target RNA. For validation purposes, the “3s” approach was applied to approximate LOD, in which the adjusted standard deviation is multiplied by three.

Declarations

Data availability

The authors declare that all of the data supporting the findings of this study are available within the paper and its supplementary information file.

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Author contributions

F.X., P.H.Z and C.Z. conceived the project. C.Z., P.H.Z., F.X. and H.R. designed the experiments. C.Z. H.R. P.P.J., L.C., J.C.J., P.W.Y., Y.X.L. J.L. and X.M.D performed the experiments and analysed the results. All authors wrote, reviewed and approved the final version of the manuscript.

Competing interests

All authors declare no conflicts of interest.

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Figures

Figure 1

SHARK-based signal sensing and amplification. **a** Workflow of the SHARK platform. **b** Collateral cleavage activity of LbuCas13a in the buffers containing different concentrations of target RNA (1 fM, 1 pM, 1 nM, 1 μM). **c** Expression profiles of SHARK generating different enzymes (slnv, G6PD, Luc). * indicates the band of the target proteins. **d** Kinetics of SHARK reactions with G6PD and eGFP as reporters. Shading indicates the data presented as mean ± s.d. (n = 3 independent experiments). **e** Comparison of detection

performance for trace RNA using different strategies. All signal ratio were calibrated and compared by those detected when the concentration of target RNA was 10 μ M (100%). The data are presented as mean $\pm$ s.d. (n= 4 independent experiments).

Figure 2

SHARK platform outputting different modules of signals. a The slnv-SHARK sensor outputs an electrochemical signal. The slnv-SHARK is activated by the target RNA to reduce the expression of invertase, which can catalyze the conversion of sucrose to glucose, outputting a turn-off electrochemical signal detected by a PGM. b Electrochemical signals collected by a PGM in the presence of diluted target RNA. c Specificity of the slnv-SHARK. d G6PD-SHARK sensor outputs a colorimetric signal. The G6PD-SHARK is activated by the target RNA to reduce the expression of G6PD, which can catalyze the conversion of glucose-6-phosphate (G6P) to 6-phosphogluconate (6-PG), outputting a turn-off colorimetric signal (Formazan) detected by naked eyes. e Colorimetric signals in the presence of diluted target RNA. f Specificity of the G6PD-SHARK. g Luc-SHARK sensor outputs a bioluminescent signal. The Luc-SHARK is activated by the target RNA to reduce the expression of luciferase, which can catalyze the conversion of D-luciferin to oxyluciferin, outputting a turn-off bioluminescent signal detected by a spectrometer. h Bioluminescent signals collected by a spectrometer in the presence of diluted target RNA. i Specificity of the Luc-SHARK. All tests were performed with 4 biological replicates.

Figure 3

Point-of-care testing of SARS-CoV-2 with paper-based slnc-SHARK using PGM. **a** Workflow for analysis of paper-based slnc-SHARK sensor. The spiked pseudoviruses were extracted from the samples by the fully integrated paper-based, and then captured by three different crRNAs, which activates the Cas13a to cleave the mRNA and thus reduce the slnv expression, outputting an electrochemical signal collected by PGM. **b** The optimization of crRNA numbers in SHARK. SHARK with N gene crRNA, E gene crRNA and Orf1ab gene crRNA individually and combinedly were tested against 5 fM of pseudovirus RNA. **c** Detection of viral RNA with different concentrations using SHARK. **d** Disposable and integrated paper-based SHARK with PGM as readout. The reagents of SHARK were freeze-dried onto paper to create stable and portable module. **e** Correlation of the detection results from SHARK-based PGM and RT-qPCR. The boxes indicate positive (green tick) or negative (red cross) of the PGM-based detection. The threshold value of 22.5 was set as the cutoff of a positive result. The results are fitted with a logistic fit. **f** Specificity of the SHARK sensor for detecting various pathogens RNA related to similar respiratory symptoms. Each of targeting RNA were 10 nM (MERS, Middle East Respiratory Syndrome Coronavirus, HKU1, human coronavirus, IVA, Influenza A virus). **g** SHARK assay for IVA detection. **h** SHARK assay for E. coli 16S rRNA detection. All data in **b**, **c**, **e**, **g**, **h** are represented as mean $\pm$ s.d. (n=3 independent experiments). * P<0.05.

Figure 4

Paper-based G6PD-SHARK outputting colorimetric signal for multiplex miRNAs analysis. **a** Bioinformatics analysis of miRNA expression profiles to predict target miRNAs for NSCLC diagnosis. **b** Schematics of the paper-based G6PD-SHARK for multiplexed detection of miRNAs (Top). Images of the paper-based colorimetric output of patient and reference sample (Down). **c** Colorimetric detection of different concentrations of RNA inputs. T/C values indicate the ratios of the signals from testing sample (T) to that from control buffers (C). **d** Orthogonality of the device for highly specific detection of target miRNAs. **e** Expression levels of miRNAs in cancer (Orange) and normal serum (White). Two downregulated and three upregulated miRNAs were all significant between NSCLC with non-NSCLC. The AUC scores were identified and compared with logistic regression model. All values were quantitatively measured using ImageJ. All data are represented as mean $\pm$ s.d.. * $P < 0.05$ (two-tailed Student's t-test). **f** Post-normalization heat map of miRNA expression from NSCLC patients' serum confirmed by RT-qPCR and G6PD-SHARK. RT-PCR were performed in triplicate and Ct values were averaged. The heat map is scaled versus the control group with the up-regulated miRNAs shown as red (>1) and the down-regulated as blue (<1).

Figure 5

G6PD-SHARK-CPU system for miRNA bio-computation. **a** Workflow for NSCLC diagnosis with SHARK computation platform. In silico training was performed using publicly available database to extract miRNA information related to NSCLC, and specific miRNA patterns and their associated weights were selected. Then G6PD-SHARK-CPU combined the transcriptional and translational regulation systems by G6PD-SHARK (OFF) and Cas9-RsgRNA (ON), finally outputting simple colorimetric signals for cancer diagnosis. **b** t-SNE plot determined by using the most significant miRNAs. **c** ON/OFF system of the G6PD-SHARK-CPU. In the ON system, negatively related miRNAs (-) bind with RsgRNA and Cas9 to repress reporter gene, and produce enzymes. In the OFF system (i.e., SHARK), the positively related miRNAs (+) reduce the enzyme concentrations through activating the Cas13a. **d** An A NIMPLY B gate. G6PD expression is inhibited in the presence of miR-126 (-) by the activated Cas9, whereas, in the presence of miR-5688(+), G6PD is produced. **e** Selected miRNAs patterns and their associated weights for the classifier model. The score of 0.55 indicates good performance for NSCLC diagnosis. * $P < 0.05$. **f** G6PD-SHARK-CPU achieved a classification model with an AUC value of 0.92. **g** Analysis of 64 clinical samples with 38 NSCLC samples and 26 healthy samples using G6PD-SHARK-CPU.

Figure 6

Absolute quantification of miRNA using digital-Luc-SHARK. **a** Schematic of the digital-Luc-SHARK assay. Target miRNA together with SHARK mixture are emulsified with oil into the QuantStudio™ chip. If one droplet doesn't contain miRNA, the enzyme can be expressed and generate bioluminescent signal (Termed "0"). In turn, the miRNAs activate the Cas13a to digest the mRNA, thus yielding a positive droplet (Termed "1"). By coupling a handheld device, the signals on the digital SHARK chip can be easily collected. **b** Improvement of the signal intensity in microwell by using RLuc-YFP via BRET. Expression plasmids encoding RLuc-YFP or Luc were added in CFE for 90 min and the bioluminescent spectra were measured. **c** The diagram of the light path diagram in the handheld instrument. **d** Representative end-point bioluminescent images in the presence of serially diluted miR-20a (10× magnification, scale bar: 200 μm). **e** Specificity of the digital SHARK. crRNAs were designed according to the four members of the let-7 family. **f** Comparison of digital SHARK and digital RT-PCR. **g** The absolute expression of miR-126, Let-7f, miR-30a, and miR-20a for healthy people (blue) and early-stage (red) NSCLC patients detected using digital SHARK.

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