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Note (Invited)

SATORI: Amplification-free digital RNA detection method for the diagnosis of viral infections

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With the recent global outbreak of COVID-19, there is an urgent need to establish a versatile diagnostic method for viral infections. Gene amplification test or antigen test are widely used to diagnose viral infections; however, these methods generally have technical drawbacks either in terms of sensitivity, accuracy, or throughput. To address this issue, we recently developed an amplification-free digital RNA detection method (SATORI), which can identify and detect viral genes at the single-molecule level in approximately 9 min, satisfying almost all detection performance requirements for the diagnosis of viral infections. In addition, we also developed practical platforms for SATORI, such as an automated platform (opn-SATORI) and a low-cost compact fluorescence imaging system (COWFISH), with the aim of application in clinical settings. Our latest technologies can be inherently applied to diagnose a variety of RNA viral infections, such as COVID-19 and Influenza A/B, and therefore, we expect that SATORI will be established as a versatile platform for point-of-care testing of a wide range of infectious diseases, thus contributing to the prevention of future epidemics. This article is an extended version of the Japanese article published in the SEIBUTSU BUTSURI Vol. 63, p. 115-118 (2023).

Key words: digital bioanalysis, genetic test, automation, CRISPR-Cas13a, RNA virus

Significance

SATORI can identify and detect viral RNA at the single-molecule level with a high sensitivity of 6.5 aM (3.9 copies/μL), equivalent to the RT-qPCR, and rapid detection in 9 min, similar to the antigen test. In addition, SATORI has been implemented on an automated platform (opn-SATORI) and a low-cost and compact platform (COWFISH), which is expected to enable rapid and accurate diagnosis of various viral infections as a point-of-care test in the future.

Introduction

The SARS-CoV-2 pandemic has reinforced the importance of rapid and accurate diagnosis of infectious diseases. These methods are effective in preventing further spread of infection and reducing the risk of exacerbation [1]. Currently, antigen and genetic testing are the most commonly used diagnostic methods for infectious diseases. Antigen testing is a simple and rapid diagnostic method that targets antigenic proteins; however, its sensitivity and accuracy are relatively low. In contrast, genetic testing targets viral genes and generally amplifies them for detection with high sensitivity and accuracy; however, it takes approximately an hour for detection. Recently, through extensive research and development around the

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world, genetic tests have been developed that can detect viruses in approximately 30 min [2]. Further reduction in detection time remains a challenge. Accordingly, the existing diagnostic methods for viral infections have technical trade-offs in sensitivity, accuracy, and throughput, and the development of a new technology that meets all these requirements is necessary.

To address this issue, we recently developed SATORI, a novel detection method for viral genes based on digital bioanalysis by combining CRISPR-Cas13a (RNA-guided RNase) and single-molecule analysis of enzymatic reactions using a micro-chamber array [3] (Figure 1). SATORI is a highly sensitive and accurate method that can identify and detect viral genes at the single-molecule level in approximately 9 min without amplification process, satisfying almost all detection performance requirements for the diagnosis of viral infections. In addition, we have continued to develop other platforms, such as an automated platform (opn-SATORI) [4] and a low-cost compact fluorescence imaging system (COWFISH) [5] for SATORI, with the aim of practical application in clinical settings. Here, we present the current status of our latest technologies and their future prospects for the diagnosis of infectious diseases.

Digital Bioanalysis

Digital bioanalysis is a method of analyzing target biomolecules in a state of limiting dilution. The method is called "digital" because analytical signals of the molecule are represented in binary system (present = 1, and absent = 0), and is inherently sensitive and quantitative due to single-molecule analysis [6]. Recent developments in microchip technology have significantly improved throughput, making it a versatile platform for the analysis of biomolecules ranging from nucleic acids to proteins. Consequently, digital bioanalysis using microchips has become a key technology in a variety of instruments. For example, digital PCR [7] and ELISA [8] are representatives based on digital bioanalysis that are commercially available and adopted for drug discovery and medicine. Digital bioanalysis using microchips is an emerging technology that paves the way for the next generation of bioanalyses.

SATORI: A Genetic Test Based on Digital Bioanalysis Using CRISPR-Cas13a

Recently, CRISPR-Cas has attracted attention as a new genetic test for the detection of viral genomes [9,10]. Cas12a and Cas13a, which are widely used in genetic testing, form a binary complex with CRISPR-RNA (crRNA), which consequently form a specific ternary complex with the target DNA or RNA, having complementary sequences to crRNA. This ternary complex formation activates Cas12a/13a, which nonspecifically cleaves the surrounding single-stranded DNA/RNA (ssDNA/RNA). When a fluorescent reporter, ssDNA/RNA labeled with a fluorophore and quencher, is added, it is cleaved by activated Cas12a/13a, resulting in physical dissociation of the fluorophore and quencher to produce a fluorescence signal. The fluorescence signal increases over time as the Cas12a/13a reaction proceeds, and the presence of the target DNA/RNA in the sample can be determined by detecting the fluorescence signal [11-13].

Conventional methods for the detection of viral RNA by Cas12a/13a are not sensitive enough to detect low copies of viral RNA genes in a sample, and are mainly used in combination with nucleic acid amplification methods such as PCR. Two representative methods using Cas12a/13a have been reported: i) DETECTR: viral RNA is reverse-transcribed into DNA, which is amplified and subsequently detected by Cas12a [13], and ii) SHERLOCK: the amplified DNA product is transcribed back into RNA and subsequently detected by Cas13a [12]. Because both methods use an amplification step, they achieve high sensitivity (~2–20 aM (~1–10 copies/μL)) equivalent to RT-qPCR; however, they require more than 30 min for detection [14]. Therefore, even with the use of Cas12a/13a, achieving both high sensitivity and rapid detection of viral genomes is not possible.

To address this issue, a variety of amplification-free, digital assay-based genetic tests have been developed worldwide (Table1). Among them, we have recently developed SATORI (CRI**S**PR-b**a**sed amplifica**t**i**o**n-free digital **R**NA detect**i**on) [3], a genetic test based on digital bioanalysis of CRISPR-Cas13a, which enables amplification-free viral RNA detection with high sensitivity and throughput. The detection process of viral RNA by SATORI is shown in Figure 1B.

STEP1: Mix samples containing viral RNA with a reaction solution containing binary Cas13a-crRNA complexes and fluorescent reporters to form ternary complexes.

STEP2: Drop the above reaction solution onto a microchip and capture and fractionate individual ternary complexes in a microchamber.

STEP3: The fluorescent reporter is cleaved by the ternary complex, resulting in a rapid increase in fluorescence signal in a short time (within a few minutes). Fluorescence images of several hundred thousand of microchambers were acquired by fluorescence microscopy, and the number of microchambers showing fluorescence signals (e.g., positive chambers) was measured by image analysis.

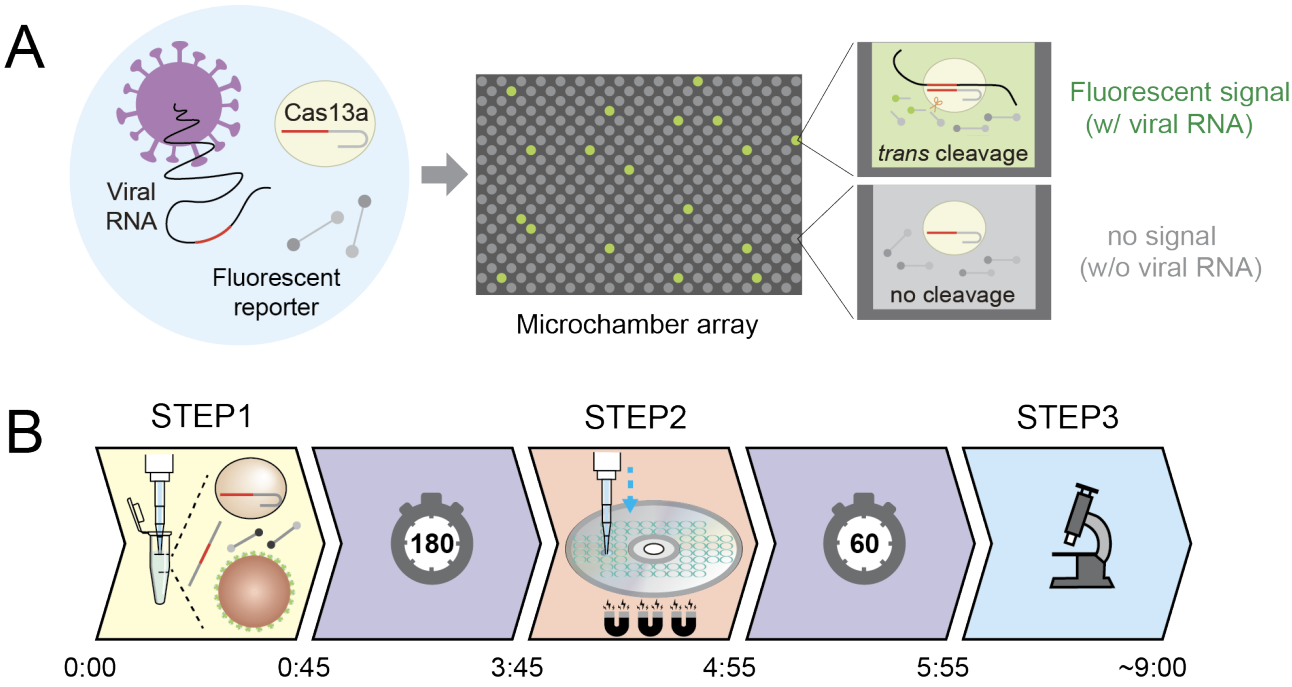

Figure 1 (A) Schematic illustration of SATORI. (B) Timeline of amplification-free digital RNA detection with SATORI. The entire process is automated and completed within 9 min.

The number of positive chambers increased in proportion with the concentration of viral RNA in the sample, because digital bioanalysis ensures high accuracy of viral RNA detection, and the advantage of the small feature of microchambers enabled rapid detection. Therefore, SATORI required only approximately 5 min per sample (the world's fastest) with a detection sensitivity of 720 aM (430 copies/μL) for SARS-CoV-2 RNA, even without the use of an amplification step [4]. The sensitivity of RT-qPCR was equivalent to a Ct value of approximately 30, suggesting that a further 100-fold improvement in sensitivity is required to be equivalent to that of RT-qPCR.

Although digital bioanalysis can identify and detect biomolecules at the single-molecule level, it has not achieved sufficient detection sensitivity owing to the conventional problem of low capture efficiency of target molecules in microchambers. To solve this problem, we developed a method that dramatically improves the capture efficiency of viral RNA using magnetic beads. Specifically, mixing samples and biotin-labeled Cas13a in STEP1 and adding avidin-coated magnetic beads, Cas13a, which forms a ternary complex with viral RNA, is immobilized and concentrated on the surface of the magnetic beads. In STEP2, a magnet was placed near the bottom of the microchambers, forcing the magnetic beads to be captured and concentrated in the microchamber, which significantly improved the capture efficiency of the ternary complex. The magnetic bead manipulation increased the total detection time to approximately 9 min, but improved the detection sensitivity to 6.5 aM (3.9 copies/μL: equivalent to Ct 38) in combination with a screening of a new Cas13a ortholog, i.e. LtrCas13a, which has a higher activation efficiency upon viral RNA binding. Validation using clinical specimens showed that a positive diagnosis of COVID-19 was possible with 98% accuracy [4].

In addition to the above solutions, the conditions for higher detection sensitivity of the digital bioassay using CRISPR-Cas are summarized as follows: 1) a larger number of microchambers to be analyzed, 2) a smaller number of false-positive chambers when there is no target molecule, 3) a higher activation efficiency of CRISPR-Cas upon target RNA/DNA binding, and 4) a higher capture efficiency of target molecules to the chambers by using enrichment methods, e.g. magnetic beads. Although the analysis of a larger number of microchambers increases the detection time, SATORI satisfies points 1) - 4) in a balanced manner, resulting in both higher sensitivity and the fastest detection of viral RNA among amplification-free digital RNA/DNA detection methods using CRISPR-Cas (Table 1).

Table 1 Comparison of amplification-free digital DNA/RNA detection methods using CRISPR-Cas

Method	Cas type	Target gene	LOD	Detection time	Type of microchamber/ droplet	Size/ Number of microchamber/ droplet	Enrichment process	Ref.
SATORI (1st ver.)	LwaCas13a	SARS-CoV-2 N-gene/ genome	5 fM/ 13 fM	<5 min	glass-based microchamber	3 fL/ 120,000	No	[3]
SATORI (2nd ver.)	LtrCas13a	SARS-CoV-2 N-gene/ genome	220 aM/ 280 aM	7 min	CD-based microchamber	30 fL/ 500,000	No	[4]
SATORI (COWFISH)	LtrCas13a	SARS-CoV-2 N-gene/ genome	N.D./ 480 aM	6 min	glass-based microchamber	13 fL/ 650,000	No	[5]
opn-SATORI	LtrCas13a	SARS-CoV-2 N-gene/ genome	2.4 aM/ 6.5 aM	9 min	CD-based microchamber	30 fL/ 500,000	magnetic beads	[4]
DEX droplet system	LwaCas13a	SARS-CoV-2 S-gene	89 aM	>40 min	glass-based microchamber	50 fL/ >1,000,000	aqueous two-phase system	[15]
dCRISPR	LwaCas13a	SARS-CoV-2 genome	~ 2 aM	50 min	PDMS-based microchamber	50 fL/ 1,000,000	magnetic beads	[16]
STAMP-dCRISPR	LwaCas13a	synthetic HIV-1 RNA	~ 1 fM	30 min	track-etched polycarbonate membrane	~10 pL/ 10,000	No	[17]
Ultralocalized Cas13a assay	LbuCas13a	SARS-CoV-2 genome	~ 10 aM	N.S.	droplet microfluidics	~10 pL/ >20,000	No	[18]
Droplet-based Cas13a assay	LbuCas13a	SARS-CoV-2 genome	~ 2 aM	20 min	polydisperse droplets	~10 pL/ 160,000	No	[19]
PddCas	LbCas12a LbuCas13a	HPV/ SARS-CoV-2	~100 aM/ ~10 aM	N.S.	polydisperse droplets	<70 pL/ N.S.	No	[20]

N.D.: not determined, N.S.: not stated.

Automation of SATORI

In the diagnosis of infectious diseases in clinical settings, full automation of SATORI is expected to reduce the risk of infection to the operator and eliminate human error, as well as having various advantages such as ensuring reproducibility and effective use of time. Therefore, we attempted to develop opn-SATORI (an aut**o**mated **p**latform o**n** **SATORI**) for practical use in clinical settings [4] (Figure 2).

SATORI consists of two main processes: solution manipulation using a pipette and fluorescence imaging and analysis using a microscope. We have developed opn-SATORI, consisting of a fluorescence microscope and an automated dispensing robot skilled in solution manipulation, which can consistently and automatically complete the entire process of SATORI through intercommunication. The fluorescence microscope, a main component of opn-SATORI, is equipped with dedicated software (NIS-Elements, Nikon) that enables full automation of fluorescence imaging and analysis to control external devices through macros. Therefore, an automated dispensing robot optimized for the microscope was developed in collaboration with BIOTECH Co., Ltd., a dispensing machine manufacturer in Japan, and integrated with a microscope system to establish a fully automated detection platform for viral RNA. Although the development of the platform has been a smooth process, many difficulties occurred in the optimization of dispensing protocols. In some cases, reproducing the operations that humans casually perform by hand was not an easy task, many areas existed where experimental procedures have been reviewed to ensure that they can be reproducibly controlled by machine operation.

For example, commercially available platforms for digital bioanalysis generally used microfluidic channels; however, many problems have been reported, such as clogging of channels during sample filling. Therefore, we have developed an open-type microchamber array that can be filled with a sample using a simple pipette movement of an automated dispensing robot. After repeated trial and error, we have succeeded in establishing a simple protocol that allows SATORI to be performed only by dropping or aspirating the solution. In addition, for magnetic bead enrichment, an objective lens-type magnet holder was attached to the motorized turret of the microscope, allowing reproducible control of the distance to the bottom side of the microchamber array using the autofocus adjustment mechanism originally installed in the microscope. By automating SATORI, the detection of SARS-CoV-2 RNA can now be performed continuously and unattended, and a large number of detection results can be obtained by simply setting up the samples and reagents and pressing the start button of the operating program. In addition, once a protocol is established, stable results can be obtained even if the operator changes, demonstrating the potential for use in both basic research and medical diagnostics.

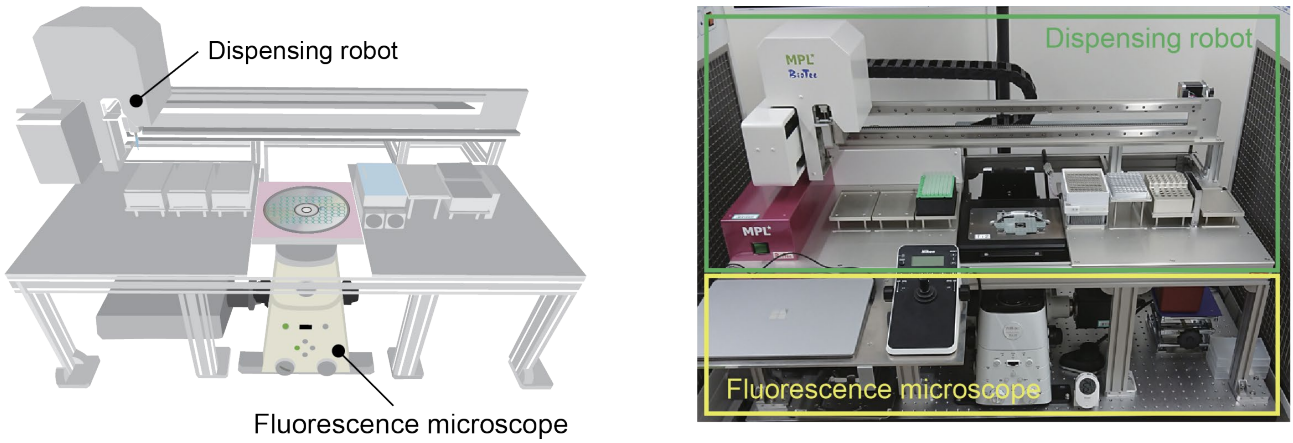

Figure 2 Photograph and illustration of opn-SATORI

Toward Point-of-Care Testing

opn-SATORI is large, measuring 160-cm wide, 70-cm deep, and 160-cm high, and is expensive because the detection system consisted of a confocal microscope. Therefore, opn-SATORI will be difficult to install in clinics and quarantine stations because of its large size and high cost. Consequently, the development of a compact and low-cost platform for SATORI is highly anticipated. To address this issue, we developed a compact and low-cost alternative detection system to the confocal microscope, **Co**mpact **w**ide-field **f**emtoliter-chamber **i**maging **s**ystem for **h**igh-speed digital bioanalysis (COWFISH) [5]. The core of COWFISH is a Nikon SLR camera and a telecentric lens for machine vision (Myutron) (Figure 3A), which is also used in AMATERAS [21], a trans-scale scope capable of observing a large field of view of 150 mm^2 with a spatial resolution of 2.5 µm. In addition, COWFISH was assembled using commercially available parts, e.g., LEDs, excitation filters, and aspherical condenser lenses were purchased from Thorlabs, at a total cost of approximately $8,700, which is much less expensive than a confocal microscope. COWFISH is 35-cm wide, 45-cm deep, and 30-cm high, which is less than one-fifth of the footprint of opn-SATORI. Although COWFISH does not have a sub-µm spatial resolution like a microscope, it can easily observe the fluorescence of microchambers with a diameter of 3.5 µm at 8 µm intervals. All parts and their prices are listed in our previous paper as supplementary material [5], hence, the platform may be rebuilt and utilized in other areas.

To evaluate the performance, SATORI was performed using COWFISH and compared to the performance of a confocal microscope. Using a simplified method without magnetic bead enrichment, the detection sensitivity of SARS-CoV-2 RNA was evaluated to be 480 aM (290 copies/µL) in 6 min of measurement with COWFISH, showing that the performance is comparable to that of a confocal microscope. In addition, because the microchamber array, which is approximately 7 mm in diameter, fits completely into a single field of view of the COWFISH (11.8 mm in width and 7.9 mm in height), the fluorescence image of approximately 650,000 microchambers can be acquired at once (Figure 3B), resulting in one-tenth of the acquisition time required by a confocal microscope, which requires 64 sections of the same area. In the future, we intend to develop a compact dispensing robot incorporating COWFISH as a small and automated platform for SATORI for practical applications in biomedical fields. The platform to be developed will be compact and low-cost, and will also allow for parallel processing, making rapid diagnosis for multiple viruses and samples possible simultaneously.

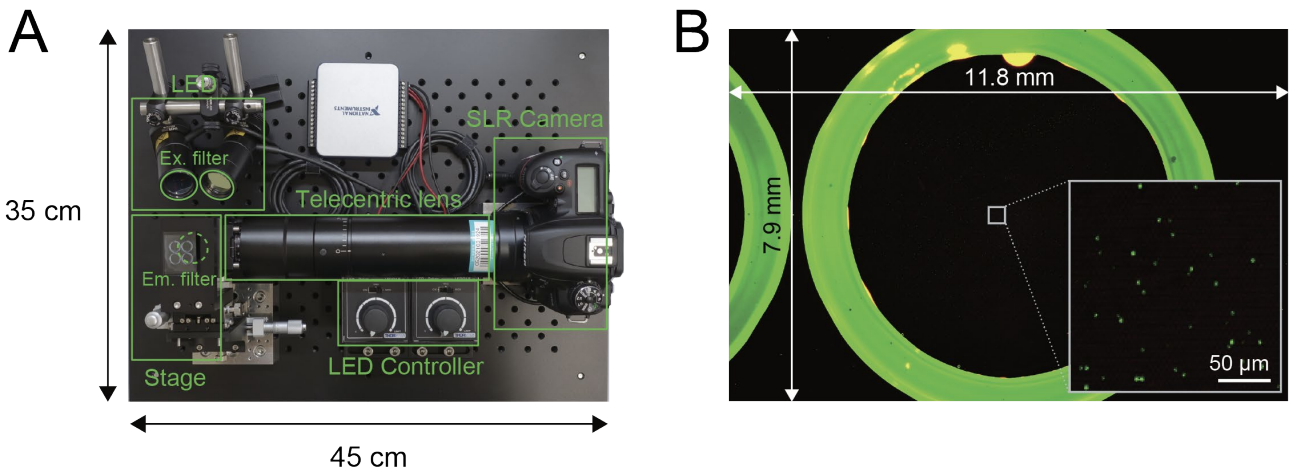

Figure 3 (A) Photograph of COWFISH. (B) Fluorescence image acquired by COWFISH

Conclusion

With the COVID-19 outbreak, developing a diagnostic platform was urgently needed, which combines the high detection sensitivity and accuracy of genetic tests with the rapid detection of an antigen test. We recently developed SATORI, which identifies and detects SARS-CoV-2 viral RNA at the single-molecule level with a high sensitivity of 6.5 aM (3.9 copies/μL), equivalent to the RT-qPCR, and rapid detection in 9 min, similar to the antigen test. In addition, we have developed a fully automated or low-cost and compact platform of SATORI for practical applications, which will need further miniaturization and cost reduction. SATORI can be inherently applied to diagnose a variety of respiratory viral infections, such as influenza A/B and RS viruses, allowing rapid and accurate diagnosis. In the future, we anticipate that SATORI will be established as a versatile platform for point-of-care testing of a wide range of infectious diseases (Figure 4).

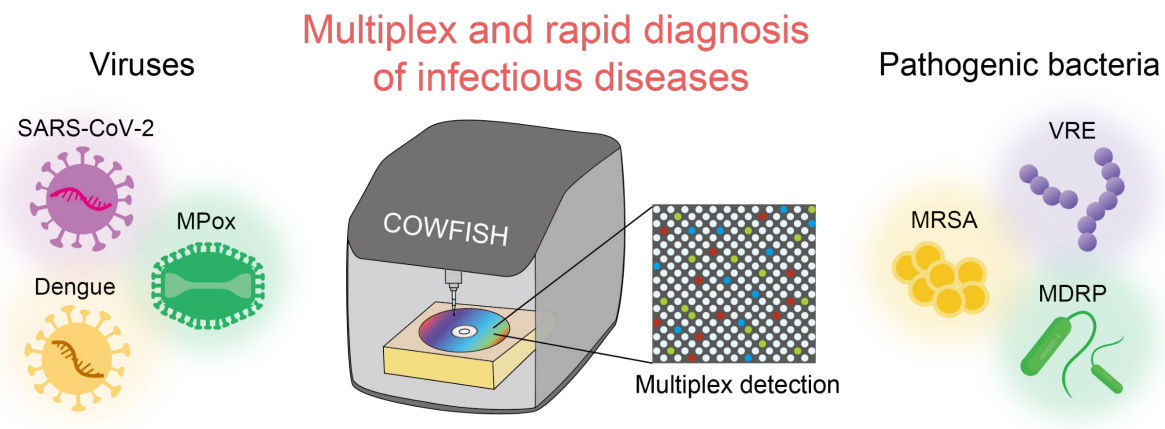

Figure 4 Future prospects of SATORI

Conflict of Interest

The authors declare no competing interests.

Author Contributions

T.I., H.S., and R.W. wrote the manuscript.

Data Availability

The data are available from the corresponding author upon reasonable request.

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