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Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA
RIVERSIDE

Integrated Microfluidic Devices Coupled with RPA/CRISPR Assays for Rapid
Detection of Infectious Disease

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Chemical and Environmental Engineering

by

Ruonan Peng

June 2026

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ABSTRACT OF THE DISSERTATION

Integrated Microfluidic Devices Coupled with RPA/CRISPR Assays for Rapid Detection of Infectious Disease

by

Ruonan Peng

Doctor of Philosophy, Graduate Program in Chemical and Environmental Engineering
University of California, Riverside, June 2026
Dr. Ke Du, Chairperson

Rapid, sensitive, and accessible pathogen diagnostics are urgently needed to address the global health burden of infectious diseases and the growing threat of antimicrobial resistance. Conventional methods, such as culture-based assays and PCR, remain limited by long turnaround times, sophisticated instrumentation, and restricted multiplexing capabilities, which make them impractical for deployment in resource-limited or point-of-care (POC) settings.

This dissertation presents the development of integrated microfluidic platforms coupled with biochemical assays based on recombinase polymerase amplification (RPA) and CRISPR/Cas12a detection for rapid, portable, and instrument-free diagnostics. A series of microdevices were engineered to overcome limitations in fluid transport, sample processing, and assay integration. First, a Rotation-Chip was designed as a low-cost, reusable platform that enables high-throughput multiplexing through manual fluid transfer. Leveraging superhydrophobic coatings and simple rotational mechanics, the device achieves uniform reagent distribution across 60 wells and supports accurate pathogen detection without

pumps or valves. Second, a nano-sieve microdevice incorporating deformable bead stacks was developed to selectively concentrate bacteria from complex samples, increasing sensitivity when paired with RPA/CRISPR assays. Third, an instrument-free duplex microfluidic system was created for simultaneous detection of *Leishmania* species, demonstrating robustness, leak prevention, and compatibility with lyophilized reagents for field deployment. Finally, a silicon micropillar platform was engineered for one-pot RPA/CRISPR assays, where micropillar surface chemistry and structure enhanced probe immobilization, reduced premature cleavage, and improved assay signal.

Together, these platforms highlight a systematic effort to address the challenges of multiplexing, portability, reagent stability, and assay sensitivity. The work demonstrates that combining biochemical assays with engineered microstructures enables diagnostic solutions that meet the World Health Organization's ASSURED criteria (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable). This dissertation not only provides new insights into integrating CRISPR-based detection with microfluidics but also establishes versatile device designs adaptable for diverse infectious pathogens, offering significant promise for the future of point-of-care diagnostics.

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Chapter 1

Introduction

1.1 The Need for Decentralized Molecular Diagnostics

Rapid and accurate diagnosis is essential for the effective management of infectious diseases, enabling timely treatment, limiting transmission, and supporting public health surveillance [10, 11]. Conventional diagnostic methods, including culture-based assays and polymerase chain reaction (PCR), have long served as the clinical gold standard due to their reliability and high analytical performance [12]. However, these approaches are fundamentally constrained by their reliance on centralized laboratory infrastructure.

Culture-based techniques, while robust, typically require 24–72 hours to yield results and are labor-intensive, making them unsuitable for time-sensitive clinical decisions [13]. PCR-based diagnostics significantly improve sensitivity and reduce assay time; however, they depend on thermocycling, precise temperature control, and specialized instrumentation, limiting their accessibility outside well-equipped laboratory settings [14]. In practice, diagnostic workflows often involve sample transport, batching, and delayed processing,

extending the effective time-to-result to several days, particularly in resource-limited or decentralized environments [15].

This discrepancy highlights a critical gap between diagnostic capability and accessibility. While laboratory-based methods can deliver high-quality results within controlled settings, their deployment in real-world scenarios remains limited. For point-of-care (POC) applications, where rapid decision-making is essential, delays in diagnosis can lead to inappropriate treatment, increased transmission, and poorer patient outcomes [16, 17, 18].

To address these challenges, the World Health Organization introduced the AS-SURED criteria—Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable—as guiding principles for the development of point-of-care diagnostics[19]. While these criteria have driven the advancement of many portable testing platforms, the expectations for modern diagnostics have evolved. Increasingly, there is a demand for POC systems that not only meet these operational criteria but also achieve analytical performance comparable to laboratory-based molecular diagnostics, particularly in the context of emerging infectious diseases and global health threats [15, 20].

This shift reflects a transition from qualitative or “good-enough” testing toward high-sensitivity, multiplexed molecular diagnostics capable of supporting precision medicine and antimicrobial stewardship [21]. The COVID-19 pandemic further emphasized this need, demonstrating that while highly sensitive molecular assays exist, their dependence on centralized infrastructure can limit scalability and timely deployment during critical periods [16, 22].

Consequently, the central challenge in modern diagnostics is no longer the ability to detect pathogens, but the ability to deploy high-performance detection systems in decentralized and resource-limited settings. Achieving this requires integrating advanced molecular sensing technologies with engineering solutions that enable rapid, robust, and instrument-free operation at the point of care. In this context, the problem is no longer detection, it is deployment.

1.2 Molecular Detection Technologies: Capabilities and Constraints

1.2.1 Isothermal Amplification

Isothermal nucleic acid amplification technologies have emerged as attractive alternatives to polymerase chain reaction (PCR) for point-of-care (POC) diagnostics, as they eliminate the need for thermocycling and enable amplification at a constant temperature[23]. Among these approaches, recombinase polymerase amplification (RPA) is strategically selected in this dissertation due to its unique alignment with the physical and operational constraints of POC microfluidic systems. While multiple isothermal methods exist, RPA provides a distinct combination of biochemical, kinetic, and integration advantages that directly address the challenges associated with developing rapid, portable, and instrument-free diagnostic platforms[24].

Unlike PCR, which relies on thermal denaturation at high temperatures (≥ 95 °C), RPA operates at near-physiological temperatures (37–42 °C) through a coordinated enzymatic mechanism involving recombinase proteins, single-stranded DNA-binding (SSB) pro-

teins, and strand-displacing DNA polymerases. Recombinases form nucleoprotein filaments with primers, enabling strand invasion into double-stranded DNA at homologous regions, while SSB proteins stabilize the displaced strands and polymerases drive continuous DNA synthesis(**Figure 1.1**)[1]. This mechanism enables exponential amplification without thermal cycling, eliminating the need for bulky instrumentation. Critically, the low operating temperature also mitigates issues such as bubble formation, evaporation, and thermal instability that are commonly encountered in high-temperature amplification systems, thereby enhancing compatibility with enclosed microfluidic environments[25].

A key advantage of RPA is its rapid reaction kinetics. Amplification can typically be achieved within 10–20 minutes, often from very low copy numbers of target nucleic acids, making it highly suitable for time-sensitive diagnostic applications. This rapid turnaround directly supports the goal of real-time or near-real-time detection in POC settings. In addition, RPA requires only a simple two-primer system, which significantly reduces assay complexity compared to other isothermal methods that rely on multiple primers. This simplicity not only facilitates assay design and optimization but also minimizes nonspecific amplification and primer–dimer formation, which is particularly important for multiplexed detection strategies implemented in later chapters of this work[1]. Beyond its intrinsic amplification efficiency, RPA is selected for its strong compatibility with downstream CRISPR-based detection systems. The operating temperature of RPA closely matches the optimal activity range of CRISPR-associated nucleases such as Cas12a, enabling seamless integration into one-pot or sequential workflows without temperature transitions[26]. RPA-generated amplicons serve as effective substrates for CRISPR-mediated detection, where recombinase-

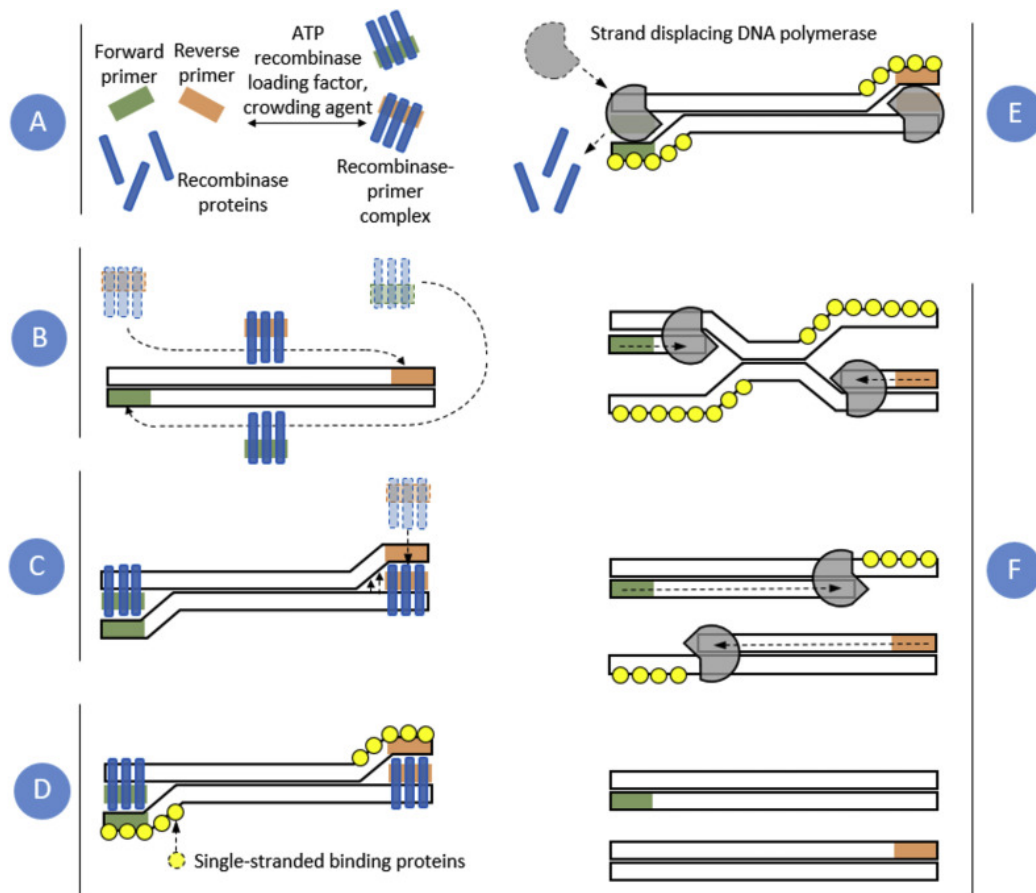


Figure 1.1: RPA amplification scheme. Recombinase proteins form complexes with each primer (A), which scans DNA for homologous sequences (B). The primers are then inserted at the cognate site by the strand-displacement activity of the recombinase (C) and single stranded binding proteins stabilise the displaced DNA chain (D). The recombinase then disassembles leaving the 3'-end of the primers accessible to a strand displacing DNA polymerase (E), which elongates the primer (F). Exponential amplification is achieved by cyclic repetition of this process. Reproduced from [1] with permission. Copyright License Number: 6245610642887.

assisted amplification produces sufficient target DNA to activate Cas nucleases and generate amplified fluorescence signals. Importantly, RPA produces relatively short and discrete amplicons, which facilitate efficient target recognition by CRISPR systems, in contrast to more complex amplification products that may hinder nuclease access.

Furthermore, RPA demonstrates strong tolerance to inhibitors present in complex biological samples, reducing the need for extensive sample purification and simplifying overall workflow. This robustness is essential for real-world applications where sample preparation must be minimized. Combined with its low energy requirements, rapid kinetics, and ease of integration, RPA serves not only as an amplification method but as a central enabling technology for achieving sensitive, rapid, and field-deployable diagnostics.

Taken together, the selection of RPA in this dissertation is driven by its ability to overcome key limitations associated with conventional and alternative amplification strategies. Its low-temperature operation, fast amplification, minimal primer requirements, and seamless compatibility with CRISPR-based detection and microfluidic platforms make it uniquely suited for integrated POC diagnostic systems, forming the biochemical foundation for the technologies developed in this work.

1.2.2 CRISPR-Based Detection

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) systems, originally identified as adaptive immune mechanisms in bacteria and archaea, have been repurposed into powerful platforms for nucleic acid detection[27]. The defining feature of CRISPR-based diagnostics is their programmability, which enables precise recognition

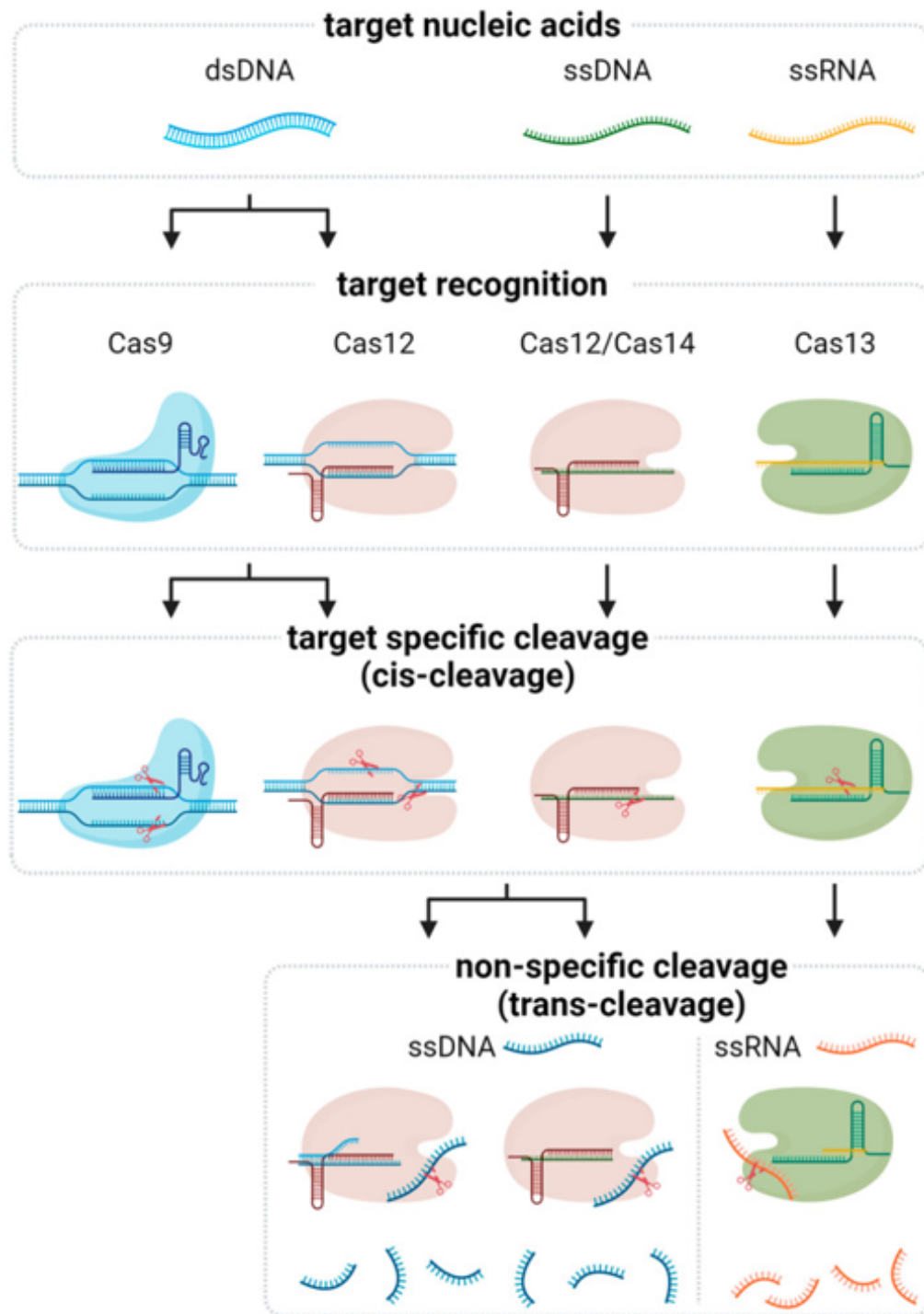


Figure 1.2: Molecular mechanism of CRISPR-associated nucleic acid detection. Reproduced from [2]. Licensed under Creative Commons Attribution 4.0 International (CC BY 4.0).

of target nucleic acid sequences through a guide RNA (gRNA) that directs a CRISPR-associated (Cas) nuclease to a complementary sequence. Upon target binding, the Cas enzyme is activated and initiates cleavage, providing a highly specific mechanism for molecular detection [2, 28].

A major advantage of CRISPR-based detection lies in the diversity of Cas effectors, each offering distinct sensing capabilities (textbfFigure 1.2)[2]. Cas9, guided by a single guide RNA (sgRNA), introduces site-specific double-stranded DNA breaks and is particularly useful for applications requiring single-nucleotide specificity, such as single nucleotide polymorphism (SNP) discrimination [29]. In contrast, Cas12a and Cas13 exhibit a unique collateral (trans) cleavage activity, which forms the basis for signal amplification in diagnostic assays. Upon recognition of a specific target sequence, these nucleases become activated and nonspecifically cleave nearby reporter molecules, generating amplified fluorescent or colorimetric signals. Cas13 targets RNA and underpins platforms such as SHERLOCK for direct RNA detection [30], while Cas12a targets DNA and is widely used for detecting DNA pathogens or amplification products [31]. More recently, compact nucleases such as Cas14 have expanded the CRISPR diagnostic toolkit by enabling detection of single-stranded DNA without strict protospacer adjacent motif (PAM) requirements, offering additional flexibility for detecting short or fragmented nucleic acids [32].

Among these systems, Cas12a has emerged as a leading candidate for diagnostic applications, particularly in combination with isothermal amplification strategies. Cas12a exhibits both sequence-specific cleavage of double-stranded DNA and a robust collateral cleavage activity against single-stranded DNA reporters once activated. This dual function-

ality enables intrinsic signal amplification without the need for additional enzymatic steps, significantly enhancing detection sensitivity. Another key advantage is its relatively simple PAM requirement (TTTV, where V = A, C, or G), which expands its targetable sequence space across diverse genomes [33]. Furthermore, Cas12a generates staggered (sticky) ends upon cleavage, providing additional flexibility for assay design and downstream engineering [34].

Beyond sensitivity, the programmability of CRISPR systems enables rapid reconfiguration of detection targets through simple modification of the guide RNA sequence, without the need to redesign complex probe systems. This feature allows CRISPR-based diagnostics to be readily adapted for emerging pathogens, genetic variants, and multiplexed detection schemes. When combined with upstream amplification methods such as RPA, CRISPR-based detection can achieve extremely high sensitivity, often reaching attomolar levels, while maintaining high specificity[35].

Taken together, CRISPR-based detection systems provide a powerful combination of programmability, specificity, and signal amplification, making them ideally suited for next-generation molecular diagnostics. In this dissertation, CRISPR/Cas12a is specifically leveraged as the detection module due to its compatibility with isothermal amplification, robust collateral cleavage activity, and suitability for integration into microfluidic platforms, forming a critical component of the overall diagnostic framework.

1.2.3 Integration Challenge in RPA–CRISPR Diagnostic Systems

While RPA and CRISPR are biochemically compatible, their integration into a unified, field-deployable platform presents a significant engineering coordination challenge.

The transition from a multi-step laboratory protocol to a "one-pot" microfluidic device is not a trivial scaling exercise; it is a complex optimization of reaction-diffusion kinetics and surface science[36].

A primary challenge is the kinetic mismatch between amplification and detection. RPA is a continuous exponential process, whereas CRISPR-Cas12a activation depends on the accumulation of amplicons. Premature nuclease activation can lead to the degradation of primers or amplicons before they reach detectable thresholds, thereby quenching the reaction. Conversely, delayed activation extends the time-to-result, undermining the utility of POC testing[37, 38].

Furthermore, micro-scale confinement introduces new variables: reduced reaction volumes increase the system's sensitivity to concentration fluctuations and surface-mediated inhibition. Achieving an instrument-free, "sample-to-answer" system requires the microfluidic architecture to manage these biochemical interactions without external intervention. Overcoming this integration bottleneck is the central theme of this dissertation, addressed through the development of controlled reaction environments in Chapters 4 and 5.

1.2.4 Signal readout methods for CRISPR-Based nucleic acid detection

Accurate signal readout is a critical component of CRISPR-based nucleic acid detection, as it bridges the molecular recognition of target sequences with practical diagnostic interpretation. Without an effective readout mechanism, even the most sensitive biochemical assays cannot be applied in clinical or field settings. Several approaches have been developed to capture and quantify the collateral cleavage activity of Cas nucleases. Among them, fluorescence-based systems are the most widely adopted, while colorimetric assays and

electrochemical platforms provide alternative solutions depending on diagnostic needs[39]. Fluorescence-based detection remains the benchmark readout method in CRISPR diagnostics due to its superior sensitivity and quantitative capability [40]. In these systems, fluorophore–quencher reporters are cleaved upon Cas activation, producing strong signals that can be monitored in real time. Compared with colorimetric methods, which are simple and equipment-free but largely qualitative[41], fluorescence assays enable precise and quantitative measurement of nucleic acid levels with much lower limits of detection, reaching attomolar sensitivity[42]. A further advantage is kinetic monitoring, which allows reaction dynamics to be tracked and assay conditions optimized. Although fluorescence typically requires dedicated instruments such as plate readers or fluorometers, recent advances in portable and low-cost devices have broadened its applicability for point-of-care use.

Figure 1.3 illustrates a digitization-enhanced CRISPR/Cas-assisted one-pot virus detection (deCOViD) chip partitions a one-step, fluorescence-based Cas12a-assisted reverse transcription recombinase polymerase amplification assay into sub-nanoliter wells on a commercial digital microfluidic platform.. This approach enabled quantitative detection of as little as 1 genome equivalent μL^{-1} of SARS-CoV-2 RNA in under 30 minutes, representing one of the fastest and most sensitive CRISPR/Cas-based assays reported to date [3]. By combining high sensitivity, rapid response, and quantitative output, fluorescence readouts continue to serve as the reference standard for evaluating and advancing CRISPR-based diagnostic platforms [43].

Colorimetric methods translate CRISPR-mediated cleavage events into visible color changes, offering a simple and equipment-free readout that is particularly suited

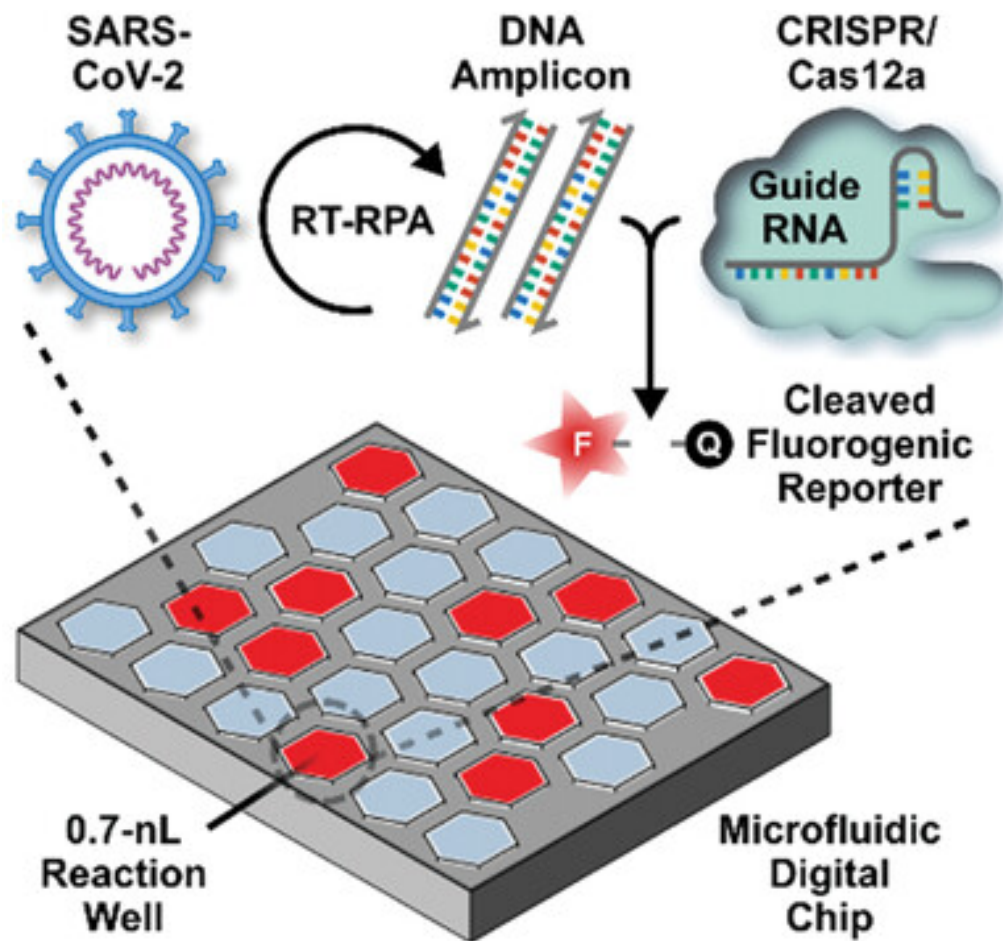


Figure 1.3: Overview of digitization-enhanced CRISPR/Cas-assisted one-pot virus detection (deCOVID). Reproduced from[3]. Licensed under Creative Commons Attribution 4.0 International (CC BY 4.0).

for point-of-care applications. Typically, these assays employ lateral flow strips or gold nanoparticle aggregation systems, in which the Cas nuclease’s collateral cleavage disrupts a reporter molecule that controls the presence or absence of a colored band or solution change[44, 45, 46]. Because results can be interpreted by the naked eye, colorimetric detection eliminates the need for specialized instruments and enables use in resource-limited settings or even at-home testing. While generally less sensitive than fluorescence-based approaches, colorimetric systems strike a practical balance between accessibility, cost-effectiveness, and diagnostic utility. Their rapid readout and ease of use have led to widespread exploration in applications ranging from viral detection, such as SARS-CoV-2, to bacterial and parasitic pathogens[41, 47]. Broughton *et al.* demonstrated the DETECTR system for SARS-CoV-2 detection using a Cas12a-based assay combined with lateral flow readout, enabling results to be interpreted by the naked eye within 40 minutes[48]. In addition, Li *et al.* developed an assay employing DNA-modified AuNPs as a colorimetric probe, enabling naked-eye detection with a sensitivity of 100 copies/ μL or 10^{-8} ng/ μL of *F. temperatum* DNA, as shown in **Figure 1.4**[4].

Electrochemical detection leverages the collateral cleavage activity of Cas nucleases to generate electrical signals that can be monitored with high sensitivity. In these assays, nucleic acid probes immobilized on electrode surfaces are cleaved by activated Cas enzymes, resulting in measurable changes in current, voltage, or impedance. This approach enables CRISPR diagnostics to be implemented in miniaturized, chip-based formats with low power consumption, facilitating integration into portable electronic systems[49, 50]. For example, He *et al.* developed a PCR-assisted E-CRISPR biosensor with a limit of detec-

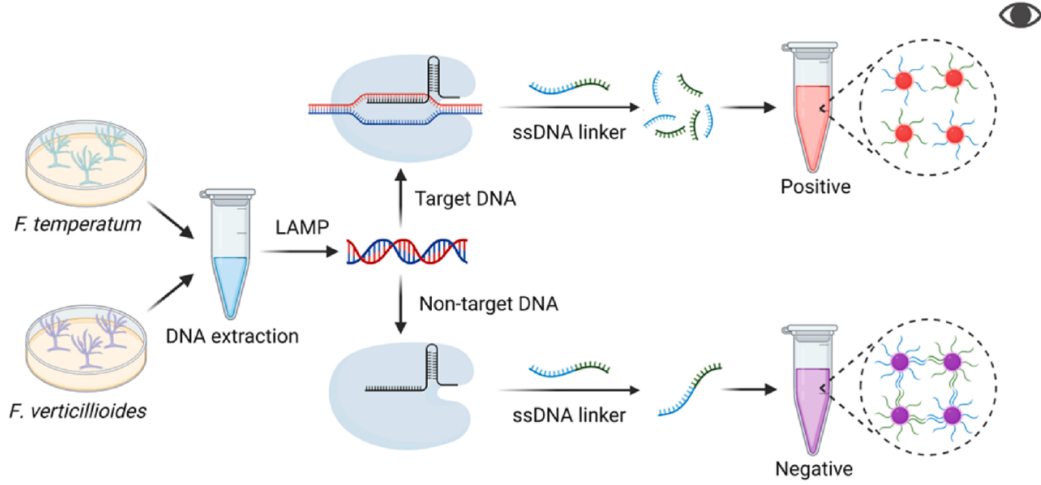


Figure 1.4: Scheme of the Au-CRISPR for visual detection of *F. temperatum*.. Reproduced from[4]. Licensed under Creative Commons Attribution 4.0 International (CC BY 4.0).

tion of 55 CFU/mL in PBS. In this system, hairpin DNA (hpDNA) probes labeled with methylene blue (MB, 3'-terminal) and a thiol group (5'-terminal) were immobilized on a gold electrode surface. The stem-loop structure of the hpDNA positioned MB close to the electrode, producing a strong baseline electrochemical signal that was disrupted upon Cas-mediated cleavage[5], as shown in **Figure 1.5**. While electrochemical detection broadens the versatility of CRISPR assays and holds promise for point-of-care applications, its sensitivity and quantitative accuracy generally remain lower than fluorescence-based systems.

Beyond infectious disease detection, readout strategies have been adapted for broader applications in research and biomarker discovery. For example, optical imaging platforms enable high-resolution tracking of CRISPR activity in cellular contexts, while sensor-based systems—such as field-effect transistors and nanomaterial-based detectors—demonstrate the potential for ultra-sensitive and real-time monitoring[51]. Collectively, these diverse approaches highlight the adaptability of CRISPR diagnostics to different testing environments.

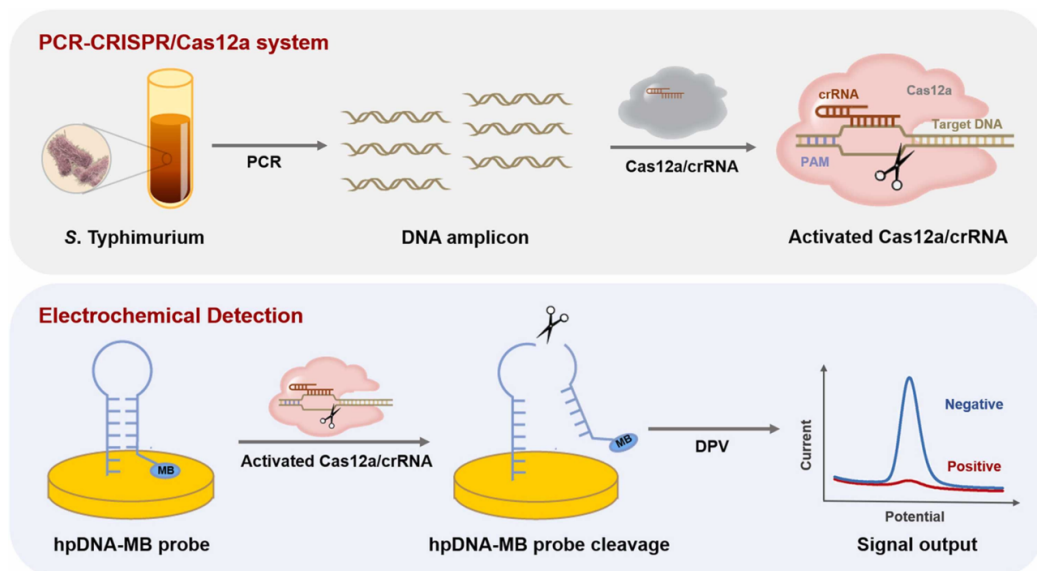


Figure 1.5: illustration of PCR-based E-CRISPR biosensor for the detection of *S. Typhimurium*.. Reproduced from[5] with permission. © 2022 Elsevier B.V. All rights reserved. License Number 6252381076971.

By aligning readout methods with specific applications, researchers can tailor CRISPR-based assays for centralized laboratories, point-of-care clinics, or even at-home testing, underscoring the pivotal role of signal detection in advancing molecular diagnostics.

Despite these advantages, CRISPR-based diagnostics still face several challenges that limit their translation into widespread clinical and point-of-care applications. One key limitation is the difficulty of integrating amplification and detection into a single “one-pot” reaction; premature activation of the nuclease can degrade amplicons before sufficient signal is generated, reducing assay sensitivity. Multiplexing also remains a challenge, as simultaneous detection of multiple targets requires careful optimization to prevent cross-reactivity and signal overlap. In addition, the stability of CRISPR reagents, particularly when stored outside controlled laboratory conditions, poses practical concerns for deployment in low-resource environments.

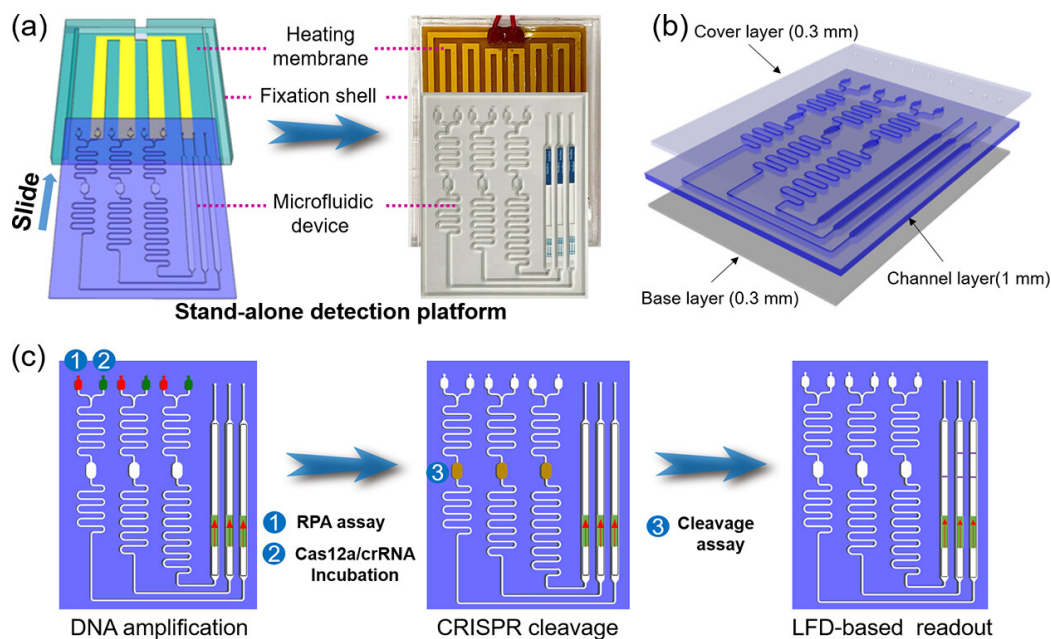


Figure 1.6: Scheme of the M3-CRISPR detection platform. (a) A cartoon drawing (left) and the photograph (right) of the heating membrane-based multiplexed microfluidics CRISPR (M3-CRISPR) system. (b) Scheme showing the microfluidic device composed of the cover layer, the channel layer, and the base layer. (c) A brief on-chip detection protocol. The RPA assay and the Cas12a/crRNA incubation were first carried out, followed by the cleavage assay and the readout. Reproduced from [6] with permission. Copyright © 2023, American Chemical Society. License Number: 6252340609446.

1.3 Microfluidic Engineering for Molecular Sensing

1.3.1 Physical and Operational Advantages of Microfluidics

Microfluidic technologies represent a fundamental paradigm shift in molecular diagnostics by enabling the precise manipulation of fluids at the microliter to nanoliter scale. The inherent miniaturization of these systems significantly reduces reagent consumption and lowers operational costs, while simultaneously accelerating reaction kinetics through a drastic increase in the surface-area-to-volume ratio [52, 53, 54, 55]. In the context of the RPA and CRISPR assays discussed in Section 1.2, these features are essential for achieving the rapid turnaround times required for point-of-care (POC) clinical decision-making.

A key strength of microfluidics lies in its ability to integrate multiple steps of the diagnostic workflow within a single, compact device. Processes such as sample preparation, nucleic acid amplification, and signal detection can be spatially organized and automated on-chip, reducing manual intervention and minimizing the risk of contamination. This level of integration enables the development of self-contained diagnostic systems suitable for decentralized settings[56, 57]. For example, integrated platforms combining amplification and CRISPR-based detection have demonstrated the feasibility of on-chip molecular diagnostics. As shown in **Figure 1.6**, the M3-CRISPR system incorporates multiple functional layers to enable multiplexed detection within a single microfluidic device, illustrating how fluid handling, reaction control, and signal readout can be coordinated in a compact format[6].

Microfluidic platforms also offer strong potential for scalable multiplexing, allowing simultaneous detection of multiple targets within a limited footprint. By leveraging compartmentalization strategies, such as droplet-based systems, microfluidics enables high-throughput analysis without significantly increasing device complexity. As illustrated in **Figure 1.7**, droplet microfluidics encapsulates amplification and CRISPR reactions within discrete microreactors, allowing parallel detection of multiple targets with high sensitivity[7]. This approach is particularly valuable for applications requiring detection of diverse pathogens or multiple genetic markers.

In addition to chip-based systems, microfluidics can be adapted into low-cost and instrument-free formats, such as paper-based devices. As shown in **Figure 1.8**, μ PAD-based platforms integrate RPA and CRISPR detection in a simple, portable format, enabling rapid diagnostics with minimal equipment requirements[8]. These systems highlight the versatility

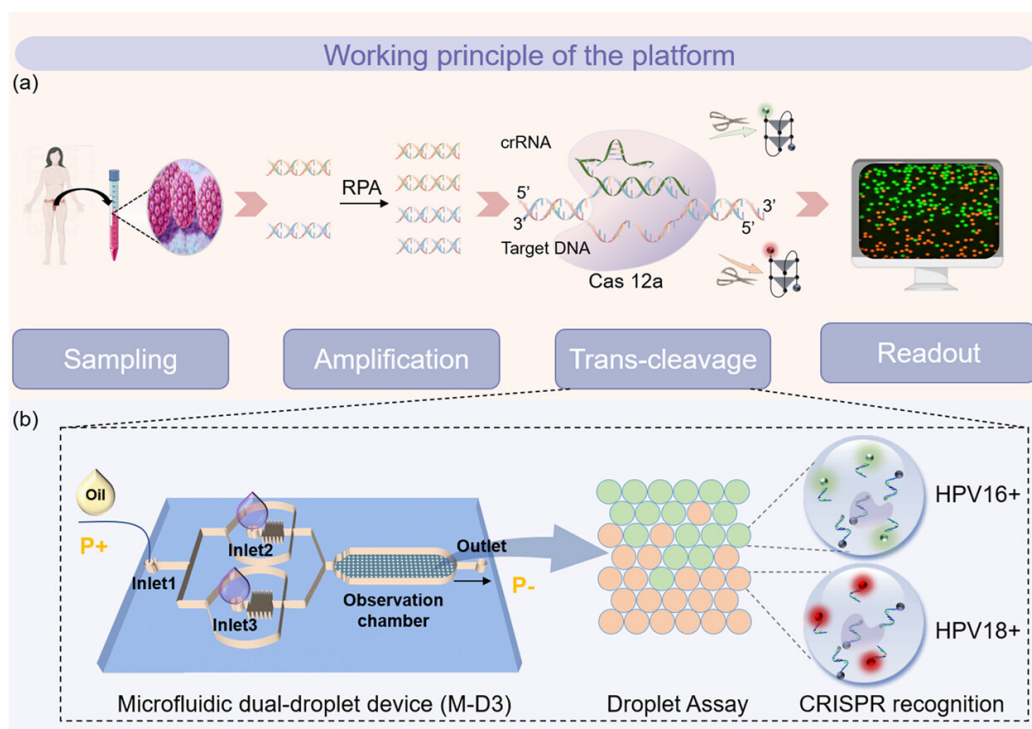


Figure 1.7: Working principle of droplet-based microfluidics platform. (a) Overview of the steps involved in the detection process in M-D3. (b) On-chip assay. The RPA amplicons and CRISPR-associated reagents were encapsulated in the droplets. The trans-cleavage assay occurred in the droplets and resulted in different fluorescence signals depending on the HPV subtypes of the tested sample. Reproduced from[7] with permission. *Copyright* © 2023, American Chemical Society. License Number: 6252350073171.

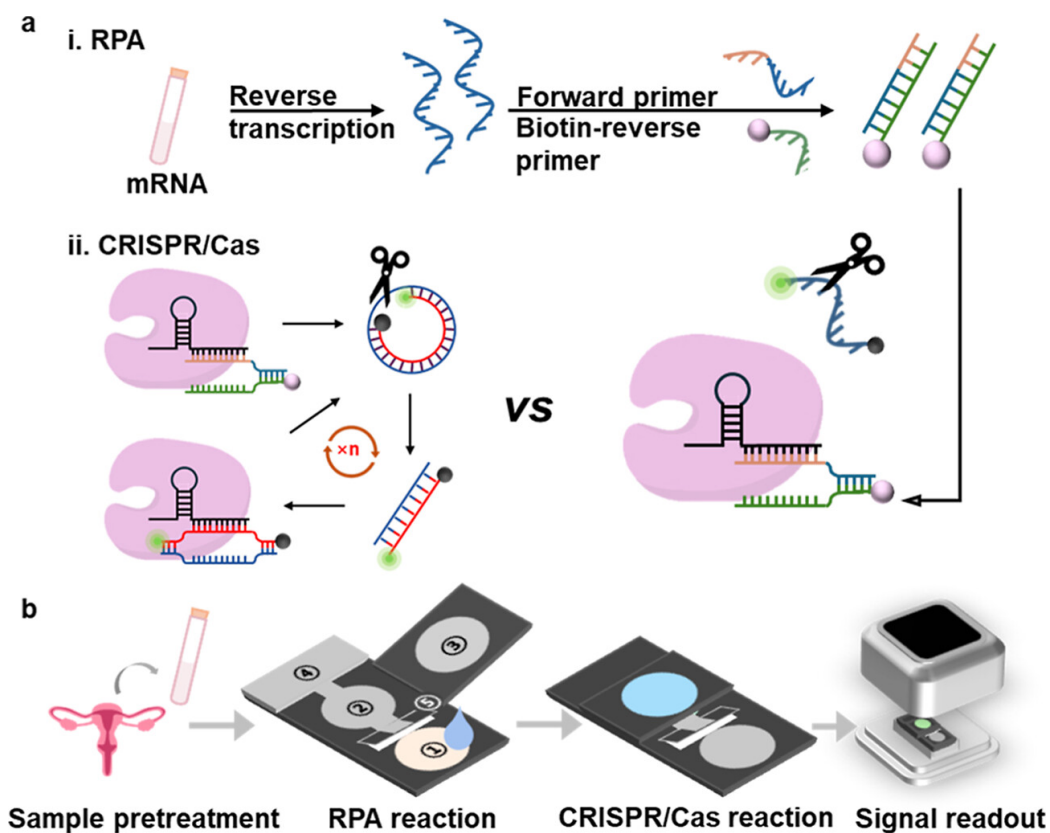


Figure 1.8: Schematic of RPA-CRISPR/Cas12a biosensors on μ PADs. (a) Working principles of RPA-CRISPR/Cas12a biosensors (i. RPA-mediated ssDNA trigger labeling, ii. Linear vs circular reporter-assisted CRISPR/Cas amplification). (b) The detection process of μ PADs (the structure of μ PADs. Reproduced from [8] with permission. Copyright © 2025, American Chemical Society. License Number: 6252350811966.

of microfluidics in addressing different operational needs, from high-performance laboratory devices to accessible field-deployable tools.

By confining reactions within these micro-scale geometries, these platforms enhance mass transport and local concentration of target amplicons, enabling the attomolar detection limits reported in the literature [6, 7].

1.3.2 The Engineering Gap: Current Limitations and Challenges

Despite the theoretical advantages of microfluidics and the high sensitivity of RPA/CRISPR assays, the development of practical point-of-care (POC) systems remains hindered by unresolved system-level bottlenecks. To bridge the gap between laboratory prototypes and real-world deployment, the diagnostic workflow must be evaluated as a coupled engineering system. In this dissertation, four primary bottlenecks are identified which define the technical limits of current diagnostic technologies and form the structural foundation for the research objectives pursued in this work.

Autonomous Fluid Handling and Actuation

The first bottleneck involves the precise manipulation of fluids without relying on laboratory infrastructure. A significant proportion of existing microfluidic systems remain "tethered" to bulky external hardware, such as syringe pumps or pressure controllers, which compromises portability and increases the likelihood of system failure in resource-limited settings [58]. Furthermore, in multiplexed platforms, achieving uniform volumetric distribution across parallel reaction chambers is critical to ensure diagnostic reproducibility [56]. Variations in flow resistance or surface energy can lead to uneven reagent partitioning. Overcoming this requires robust, self-actuating fluid control strategies that operate independently of external instrumentation, a central focus of the rotational microfluidic platform developed in Chapter 2.

Sample Preparation and Enrichment

The second bottleneck occurs at the transition between raw biological matrices and analytical detection, widely known as the "world-to-chip" problem [59]. While microfluidics excels at processing nanoliter volumes, infectious disease diagnosis often necessitates the analysis of milliliter-scale clinical samples to detect low-abundance pathogens, particularly in early-stage infections [60]. Most current "integrated" platforms bypass this primary step, assuming the input is already purified DNA or RNA [12]. This massive volume mismatch (often exceeding 1,000,000:1) creates a significant sampling bias that results in high false-negative rates. Addressing this requires on-chip architectures capable of high-efficiency target enrichment, an engineering challenge investigated through the micropillar-based enrichment strategies in Chapter 3.

Scalable Multiplexing and Signal Specificity

The third bottleneck relates to the capacity for simultaneous multi-target detection. Multiplexing is clinically essential for differential diagnosis of pathogens with overlapping symptoms or identifying antimicrobial resistance markers. However, transitioning from single-target to multiplexed CRISPR sensing introduces significant cross-reactivity risks and signal interference [61]. The engineering challenge lies in maintaining signal integrity and high specificity when multiple primer sets and guide RNAs (gRNAs) coexist in a shared or partitioned reaction space. Achieving robust multiplexing requires a co-design of assay chemistry and device architecture to manage these complex molecular interactions, as explored in the duplex sensing systems of Chapter 4.

Kinetic Harmonization in "One-Pot" Systems

The final bottleneck involves the condensation of amplification and detection into a unified "one-pot" environment. While performing RPA and CRISPR simultaneously minimizes aerosol contamination and user intervention, it triggers a state of kinetic competition. Because both enzymatic systems share the same thermal window, the Cas12a nuclease may prematurely cleave primers or nascent amplicons, thereby quenching the amplification reaction before it reaches the analytical threshold [37, 38]. Current designs struggle to balance amplification velocity with detection sensitivity without complex chemical additives. This work addresses this optimization problem in Chapter 5 by using surface-engineered scaffolds to stabilize and harmonize one-pot kinetics.

Collectively, these four bottlenecks highlight that the primary challenge in modern molecular diagnostics is not the performance of individual components, but the lack of coordinated integration across the entire diagnostic workflow. Existing technologies often address these challenges in isolation, resulting in fragmented solutions that are difficult to translate into practical POC systems.

This dissertation addresses these challenges through a modular engineering approach, in which each bottleneck is targeted by a specific microfluidic innovation. By systematically addressing sample preparation, fluid handling, multiplexing, and reaction integration, this work aims to establish a framework for developing integrated, scalable, and deployable molecular diagnostic platforms.

1.4 Objective and Outline

This dissertation establishes that the successful transition of CRISPR-based molecular diagnostics from the laboratory to the point-of-care requires the engineering of integrated microfluidic architectures that resolve the bottlenecks of sample concentration, multiplexed fluid transport, and reagent stability through tailored surface-liquid interactions.

This introduction in Chapter 1 reviews the background, current status, and significance of diagnostic technologies for infectious diseases. It highlights the limitations of traditional approaches such as culture and PCR, and introduces emerging biochemical assays, including CRISPR-based detection and isothermal amplification methods. CRISPR/Cas-based assays provide excellent specificity, rapid detection within minutes to hours, and programmability for multiplexed pathogen detection, making them particularly suitable for resource-limited settings. Amplification methods such as RPA offer high sensitivity and rapid nucleic acid amplification at constant temperatures, eliminating the need for thermal cycling. When combined with microfluidic platforms, these assays hold strong potential for developing practical, low-cost, and portable diagnostic devices.

Chapter 2 presents the design and validation of the Rotation-Chip, a pocket-sized, manually operated microfluidic device for high-throughput multiplexed pathogen detection. Using a shearing-driven channel design and superhydrophobic coatings, the chip enables uniform reagent mixing and parallel processing of up to 60 reactions without pumps or valves. Integrated with intracellular fluorescence assays, the Rotation-Chip demonstrates sensitive detection of ampicillin-resistant *E. coli*, offering a simple, reusable, and instrument-free platform suitable for point-of-care (POC) applications.

Chapter 3 introduces a nano-sieve microdevice designed for bacterial enrichment and sensitivity enhancement. By concentrating target pathogens prior to detection, the device improves assay performance for low-abundance samples. Coupled with CRISPR-Cas12a fluorescence readout, the nano-sieve system enables reliable detection of *Staphylococcus aureus*, demonstrating how microfluidic pre-processing can overcome the limitations of conventional sample preparation.

Chapter 4 describes a duplex microfluidic assay for the detection of *Leishmania* species. Leveraging RPA and CRISPR/Cas12a in an array-based platform, this system enables simultaneous identification of multiple species in a single test. The duplex design reduces assay time and reagent consumption while improving diagnostic accuracy, highlighting the value of multiplexed detection in addressing parasitic diseases with overlapping clinical symptoms.

Chapter 5 presents the development of a silicon micropillar chip for stable one-pot integration of RPA and CRISPR/Cas12a detection. Through surface functionalization and micropillar array engineering, the platform overcomes challenges of premature cleavage and poor reagent stability, enabling efficient one-pot amplification and detection. This chapter demonstrates how microfabrication and biochemical optimization can be combined to advance portable nucleic acid diagnostics.

Finally, Chapter 6 summarizes the contributions of this dissertation, reflecting on how the integration of microfluidics with RPA/CRISPR assays addresses key barriers in pathogen detection. Future directions are proposed, including further multiplexing strategies, reagent stabilization for field deployment, and the incorporation of digital microfluidics

and artificial intelligence for automated data analysis. Together, these advances outline a roadmap toward next-generation diagnostic platforms that are sensitive, affordable, and deployable at the point of care.

Chapter 2

Building an Instrument-Free Platform for Multiplexed Pathogen Analysis

This chapter is adapted from the following publication: Jiacheng He, Ruonan Peng, Henry Yuqing, Rafi Karim, Juhong Chen, Guoyu Lu, and Ke Du. Superhydrophobic rotation-chip for computer-vision identification of drug-resistant bacteria. *ACS Applied Materials & Interfaces*, 15(23):27732–27741, 2023[62].

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2.1 Abstract

The transport, distribution, and mixing of microfluidics often require additional instruments, such as pumps and valves, which are not feasible when operated in point-of-care (POC) settings. Here, we present a simple microfluidic pathogen detection system known as Rotation-Chip that transfers the reagents between wells by manually rotating two concentric layers without using external instruments. The Rotation-Chip is fabricated by a simple computer numerical control (CNC) machining process and is capable of carrying out 60 multiplexed reactions with a simple 30 or 60° rotation. Leveraging superhydrophobic coating, a high fluid transport efficiency of 92.78% is achieved without observable leaking. Integrated with an intracellular fluorescence assay, an on-chip detection limit of 1.8×10^6 CFU/mL is achieved for ampicillin-resistant *Escherichia coli* (*E. coli*), which is similar to our off-chip results. We also develop a computer vision method to automatically distinguish positive and negative samples on the chip, showing 100% accuracy. Our Rotation-Chip is simple, low-cost, high-throughput, and can display test results with a single chip image, making it ideal for various multiplexing POC applications in resource-limited settings.

2.2 Introduction

Food- and water-borne infections, including diarrhea, gastrointestinal ailments, and systemic illnesses, present a major health threat to individuals and healthcare facilities[63]. Many pathogens, such as *Escherichia coli* (*E. coli*), could lead to food- and water-borne illnesses and have been reported to cause considerable morbidity and mortality[64]. Early detection of bacteria not only increases the likelihood that patients receive appropriate medical

treatment but also prevents the spread of these pathogens and reduces the risk of antimicrobial resistance[60, 65]. The conventional method for bacteria detection is plate culturing[66]. Despite being relatively inexpensive and reliable, plate culturing is time-consuming, commonly 2 to 3 days[67], and each positive culture is labor-intensive[68]. Moreover, injured or nonculturable microorganisms could result in insufficient detection capacity of plate culturing[69]. Polymerase chain reaction (PCR) is a widely used molecular-based method for bacterial detection[70]; however, PCR involves thermocyclers and sophisticated bulky equipment, which hardly make it readily conducive to Point-of-Care (POC) diagnostics[71]. Besides, it has limited multiplexing capability due to the design requirement for primer sets to be non-complementarity and to anneal at similar temperatures[72]. Typically, only three to four target sequences are detected simultaneously in a single reaction[73]. Therefore, multiplexed high-throughput POC detection, which could screen various analytes such as pathogens simultaneously, would be highly desirable because it allows for a rapid, affordable, and reliable qualitative readout.

Following the World Health Organization’s ASSURED Challenge (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable)[74], recent research efforts have been focusing on culture-independent POC approaches. Economically, the market demands high throughput to maximize time and cost efficiency. Integrated biological systems like microfluidic chips can meet these criteria[67]. With a high degree of automation, many of these portable devices can analyze a large number of samples at once and output detection signals qualitatively[75]. Qualitative biosensors can reduce the cost of detection even more by not relying on sophisticated instruments such as a spectrometer[76]

or a smartphone-based microscope[75]. For example, three-dimensional microfluidic paper-based analytical devices (3D- μ PADs) offer an affordable solution for analyzing multiplexed assays[77]. However, one major challenge with μ PADs and all different kinds of microfluidics is distribution homogeneity, where channels must have the same hydrodynamic resistance for every branch in a certain region to prevent a bias in the final output. Additionally, the use of adhesive makes them irreversibly bound devices that cannot be reused[77]. For multiplexed detection on a microliter or smaller scale, microfluidic systems often require pumps and valves to transport the sample to many reaction wells for the reaction to occur[78, 79]. The requirement for hydraulic control and gas-permeable materials can make on-chip reagent storage difficult and increase the cost and complexity of operation[9].

A notable example of a qualitative microfluidic detection device is the SlipChip[9, 80], as shown in **Figure 2.1**. By using two pieces of etched soda-lime glass with chromium and photoresist coating, the SlipChip mechanically transports precise volumes of sample to mix with preloaded reagents without pumps or valves[9]. This device is designed to analyze very small volumes as it delivers samples in wells of only about 5 nL and a throughput of 48 individual reactions. However, evaporation prevention using a layer of fluorocarbon lubrication is necessary for the operation of the SlipChip, and reagent preloading has to be carefully carried out with volume-controlled droplets in specifically angled Teflon tubing. Mechanically, the device assembly lacks structural robustness since the two halves of the device are secured by nothing but two small binder clips. In addition, the SlipChip is fabricated via standard photolithography and glass wet etching, which is costly and requires a cleanroom setting[9].

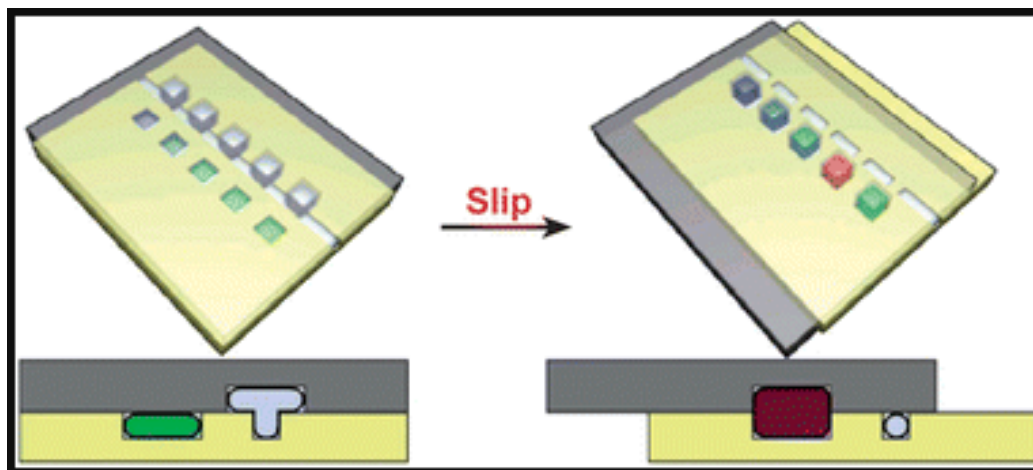


Figure 2.1: Schematic of Slipchip (Reproduced from [9]) with permission. Copyright license number: 1720772-1

2.3 Objectives

The transport, distribution, and mixing of fluids in microfluidic systems often rely on external pumps and valves, which increase complexity and limit their use in point-of-care (POC) settings. Existing devices also face challenges such as inhomogeneous reagent distribution, leaking, and tedious manual pipetting, all of which reduce practicality for high-throughput diagnostics. The objective of this study is to develop a simple and portable microfluidic platform for high-throughput, multiplexed pathogen detection suitable for POC applications. To achieve this, we designed and fabricated a pocket-sized Rotation-Chip, a two-layer polycarbonate device with superhydrophobic coating produced by CNC machining. By manually rotating the chip, reagents can be efficiently distributed and mixed across 60 reaction wells without the need for external pumps, valves, or pipetting, which are impractical in resource-limited settings. The device aims to address key challenges of existing microfluidic systems by incorporating a shearing-driven channel design for uniform

distribution, reversible and reusable assembly, superhydrophobic surface chemistry, and low-cost fabrication. Furthermore, the platform is integrated with an intracellular fluorescence assay for detecting drug-resistant *E. coli* and coupled with a computer vision algorithm to automatically analyze fluorescence intensity, providing accurate readouts without user bias. Together, these objectives seek to establish a robust, low-cost, and instrument-free diagnostic solution that advances point-of-care pathogen detection.

2.4 Materials and Methods

2.4.1 Chip Fabrication

Measuring 80 mm in diameter, the top and bottom layers of the chip body were machined out of two pieces of 3-mm thick clear polycarbonate sheets by an automated CNC machine (ProtoTrak DPM5). On the one-step design variant, each well is 2.5 mm in diameter and 1 mm deep. The channels connecting the reagent array are of the same depth as the wells and 0.5 mm wide. Since a slight overfill is important for distribution homogeneity, these dimensions yield an effective fill volume of 5 μL per half well, 10 μL per assembled well, and 100 μL per assembled reagent array.

2.4.2 Surface Treatment and Transport Efficiency

Surface treatments using two materials, Teflon (No. D14896684) and NeverWet (No. 274232), were conducted individually to evaluate and compare their hydrophobic performance. Before each surface treatment, the inner surface of each chip layer was cleaned in an ultrasonic bath of deionized (DI) water for 5 min and dried with an air gun. For surface

treatment using Teflon, the Teflon solution was poured into a 100-mm Petri dish to form a pool about 3 mm deep. With its inner surface facing down, each layer was gently dipped into the Teflon solution until all features were covered. The layer was then lifted from the solution to let excess solution drip off while being slowly roasted by hand to achieve a reasonably even coating. After coating, both layers were placed, coated surface up, in their glass Petri dishes, and baked overnight at 80 °C. The surface treatment using NeverWet was performed per the product manual. The “base coat” was sprayed onto the inner surface of each layer from approximately 20 cm away, then it was air-dried at room temperature for 30 min. The “top coat” was then applied in the same manner twice, 2 min apart. The coatings were allowed to cure at room temperature overnight before use.

2.4.3 Assay and detection limit

Fluorescein di- β -D-galactopyranoside (FDG) was purchased from Abcam. To prepare a 10 nM stock solution, 5 mg of FDG was dissolved in 761.6 μ L of H₂O: DMSO: EtOH (8:1:1) and stored at -20 °C in the dark. β -galactosidase (β -gal) powder (Sigma-Aldrich) with an activity of 1,000 U was dissolved in 1 mL of PBS. The solution was serially diluted by a factor of 10 into 0.001, 0.01, 0.1, and 1 U/mL for further use. For the one-step assay, FDG was used to measure the intracellular activity of β -gal in LacZ strains, and ampicillin-resistant *E. coli* BL21 (DE3) (New England BioLabs, Inc.) with pUC19 plasmid (New England BioLabs, Inc.) was selected as the model stain. After the pUC19 vector was transformed into the *E. coli* strain by heat shock, LB-agar plates which contained 100 μ g/mL ampicillin (Sigma-Aldrich) were used to grow *E. coli*. After the plates were incubated overnight at 37 °C, a single colony of *E. coli* harboring pUC19 plasmid was then inoculated

into LB broth media supplemented with 10 $\mu\text{g/mL}$ ampicillin, followed by overnight incubation at 37 °C under 200 rpm. After that, 1 mL of the bacterial cells were pelleted in a centrifuge at 10,000 g for 5 min and resuspended in 1 mL of PBS. This step was then repeated twice to completely remove the culture media. Next, the optical density (OD600) of the bacterial cells was measured to calculate the concentration, and serial dilution was performed in PBS for the harvested bacterial cells for further use.

Based on the volume of the one-step chip design, 5 μL of either β -gal or *E. coli* samples were added into 5 μL of FDG to perform off-chip experiments, and 5 μL of PBS was added instead as the negative group. The 10 μL mixture was vortexed and placed in a 37 °C water bath for 30 min under dark conditions to avoid the photobleaching of FDG. After the incubation, the mixture was excited by a blue light transilluminator (brand: SmartBlueTM, Part number: NEB-E4100, excitation wavelength of 465 nm) for naked-eye observation. Finally, 60 μL of nuclease-free water (Thermofisher) was added to each tube, which was then characterized by a commercial spectrofluorometer (JASCO FP-8500, USA).

Prior to starting the on-chip experiment, the chip was sterilized with ultraviolet (UV) light for 30 min. Then, 100 μL of FDG was loaded into each reagent channel, and 5 μL of the sample was loaded into each sample well, along with 5 μL of PBS as a negative group. The top layer was slowly rotated 30° while holding the bottom layer steady; then the chip was placed into a Ziploc bag for waterproofing and incubated in a 37 °C water bath for 30 min in the dark. Finally, the rotated chip was placed onto the transilluminator to check the fluorescence.

2.4.4 Image processing

Test sample circles were segmented by mean-shift algorithm using intensity as the segmentation criteria[81]. Once the test sample circles were segmented, we applied Hough transform to detect each sample[82]. To quantify the fluorescence intensity, we extracted the color intensity histogram feature to represent each segmented sample and classified the samples based on each sample’s color intensity histogram feature[83]. To remove the background influence, 0 values in the histogram was removed, as 0 represents the black color, which can increase the difference between positive and negative color intensity histogram features. For decision tree classification, the input data was represented as a set of feature vectors and each one has a set of attributes or features. To classify a new sample using a decision tree, the sample was passed down the tree from the root node to a leaf node, based on the values of its input features. The leaf node represents the predicted class label for the sample.

2.5 Results

Figure 2.2a shows the end of an *E. coli* detection assay, where qualitative detection results can be observed in a Rotation-Chip under the UV light of a transilluminator. **Figure 2.2b** shows the Rotation-Chip as an assembly of a pair of CNC machined polycarbonate disks with varying features. The top layer is designed to rotate clockwise relative to a static bottom layer about the central post. A guide pin and a guide rail system ensure the rotation is constrained to a fixed path with a physically defined initial position, final position, and rotational freedom of 30 degrees. An interference fit between the post and

the central top layer hole ensures precise detuning and prevents the top layer from falling out of the assembly with ease. When assembled, the two layers of the one-step chip form 6 groups of radially symmetrical test groups, each capable of running 10 tests. Each test group contains a reagent array and a sample array positioned along the same rotational path. The reagent array contains 10 reagent wells of 10 μL whose bottom halves are connected by a continuous channel on the bottom layer. The channel inlet to the left of the channel is designed to fit most micropipette tip sizes during loading, and the channel outlet is through a small hole in the top layer for pressure equalization. Together, these features enable the user to fill all reagent wells at once regardless of the number of wells in the array, avoiding repetitive pipetting and saving operation time. On the other hand, the sample array contains wells of the same number and size as the reagent array but is kept separate to avoid contamination across different tests. To make sure the assembly process takes place before the loading process, the top halves of the sample wells are through holes so the user can individually load samples post-assembly. **Figure 2.3a** and its accessory cross-

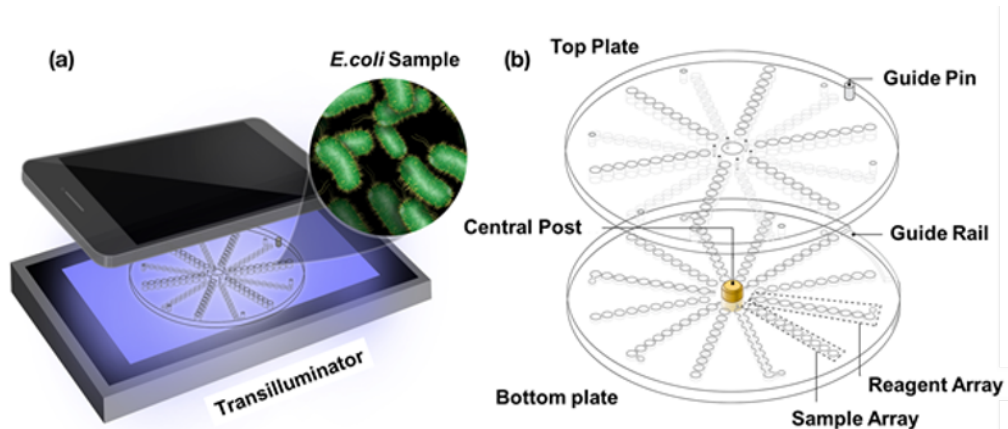


Figure 2.2: Rotation-Chip design. (a) Fluorescence is excited by a transilluminator and the results are read by either naked dye or a smartphone. (b) Exploded view showing the mechanical components of the one-step chip design.

sectional views show the operation mechanics of running a test group in an assay, where the blue and the red fluids represent the reagent and sample, respectively. To start the fluid transportation process, the user fills both layers of the reagent array with the reagent before rotating the top layer while holding the bottom layer still. During the rotation, this shearing motion carries discrete volumes of the reagent fluid and carries them along the rotational path. The rotation operation is complete when the guide pin touches the end of the guide rail as the reagent fluid makes contact with the sample in each sample well. In **Figure 2.3b**, the two-step chip executes the same fluid dynamics, but the addition of the secondary reagent produces a mixture of the two reagents that mixes the sample at the end of a 60-degree rotation, shown in red, purple, and yellow, respectively. **Figure 2.4a** shows

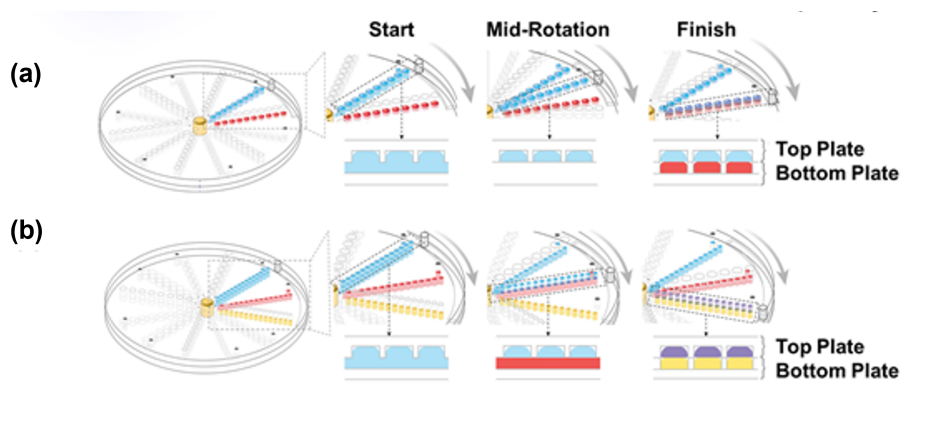


Figure 2.3: Operational schematic of the fluid transportation of the (c) one-step chip design and (d) two-step chip design.

the contact angles of Rotation-Chip with no coating, Teflon coating, and NeverWet coating, respectively (from left to right). Among them, NeverWet coating processes the most hydrophobic property, of which the static contact angle is 156.8 degrees. The SEM images of the Rotation-Chip before and after the NeverWet coating are shown in **Figure 2.4b**. The

NeverWet coating consists of fluorocarbon-coated silica particles to produce surface roughness and native superhydrophobicity, as shown in the zoom-in view, while the uncoated chip shows a clean surface. To measure the number of wells leaked during the rotation process and to determine the coating on which side of the chip would provide the best transport efficiency, food dye was used as a model for reagents and samples. After the food dye was loaded on the Rotation-Chip, the chip was rotated 30 degrees clockwise and the number of leaked wells was counted by the naked eye. The experiment for each coating situation was repeated 5 times to minimize the error. **Figure 2.4c** shows the transport efficiency results for uncoated, Teflon-coated, and NeverWet coated Rotation-Chip. The NeverWet coated chip transports 92.78% of the reagent while that of Teflon coating is only 86.02%. For the chip without any coating, all the food dye leaked out of the wells during the rotation, resulting in a transport efficiency of 0. The leak count for the NeverWet coated Rotation-Chip under different conditions is shown in **Figure 2.4d**. No coating was represented by ‘-’ while NeverWet coating was represented by ‘+’. The first symbol represented the coating situation for the top layer, and the second symbol represented the coating situation for the bottom layer. When both layers were coated with NeverWet, the leakage of Rotation-Chip was significantly minimized. The transport efficiency of the Rotation-Chip was tested with a dedicated chip for testing, where there was only one well on the top layer of the Rotation-Chip. The weight of 10 μ L of the food dye was measured with the high precision laboratory balance (Intelligent PM-300), then 10 μ L of the food dye was added into the well and rotated for 5 circles. After that, the remaining food dye in the well was removed by a pipette, which was then weighed with the same balance to calculate the

transport efficiency of the Rotation-Chip. The experiment for each coating situation was repeated 5 times to minimize the error. To test the stability of the coating against shear, we measured the static contact angle of the NeverWet coated chip after 5 rotations (each rotation is 30 degrees). As shown in **Figure 2.4e**, the static contact angle on both plates remains above 150 degrees, indicating the superhydrophobicity remains effective after five rotations. **Figure 2.5** is the food dye demonstration on the two-step chip before and after the NeverWet coating. Before the NeverWet coating (top row), there was liquid leakage during both reagent loading and chip rotation. After the NeverWet coating (bottom row), the liquid leakage was significantly minimized, and this avoided sample contamination and improved the reagent utilization, which provided a more accurate results readout. Also, the variety of well sizes demonstrates the morphological multiplexing ability of a two-step chip. With appropriate well dimension adjustment, the Rotation-Chip may be easily compatible with some two-step assays that require multiple mixing steps.

Escherichia coli (*E. coli*) can produce the enzyme β -galactosidase (β -gal) to break lactose into galactose and glucose[84]. Fluorescein di- β -D-galactopyranoside (FDG) is one of the most sensitive and versatile fluorogenic substrates for the β -gal[85]. **Figure 2.6a** shows the two-step sequential hydrolysis of non-fluorescent FDG by β -gal, first converting it to mono-beta-D-galactosidase (FMG) and then to highly fluorescent fluorescein (Excitation/Emission, 488/512), which can be detected by an LED transilluminator[86]. The fluorescence intensity (512 nm) of off-chip results of the assay in response to purified β -gal with different activities (0 - 1 U) is shown in **Figure 2.6b**. After a 30 min reaction, the fluorescence intensity was evaluated by a commercial spectrofluorometer (JASCO FP-8500,

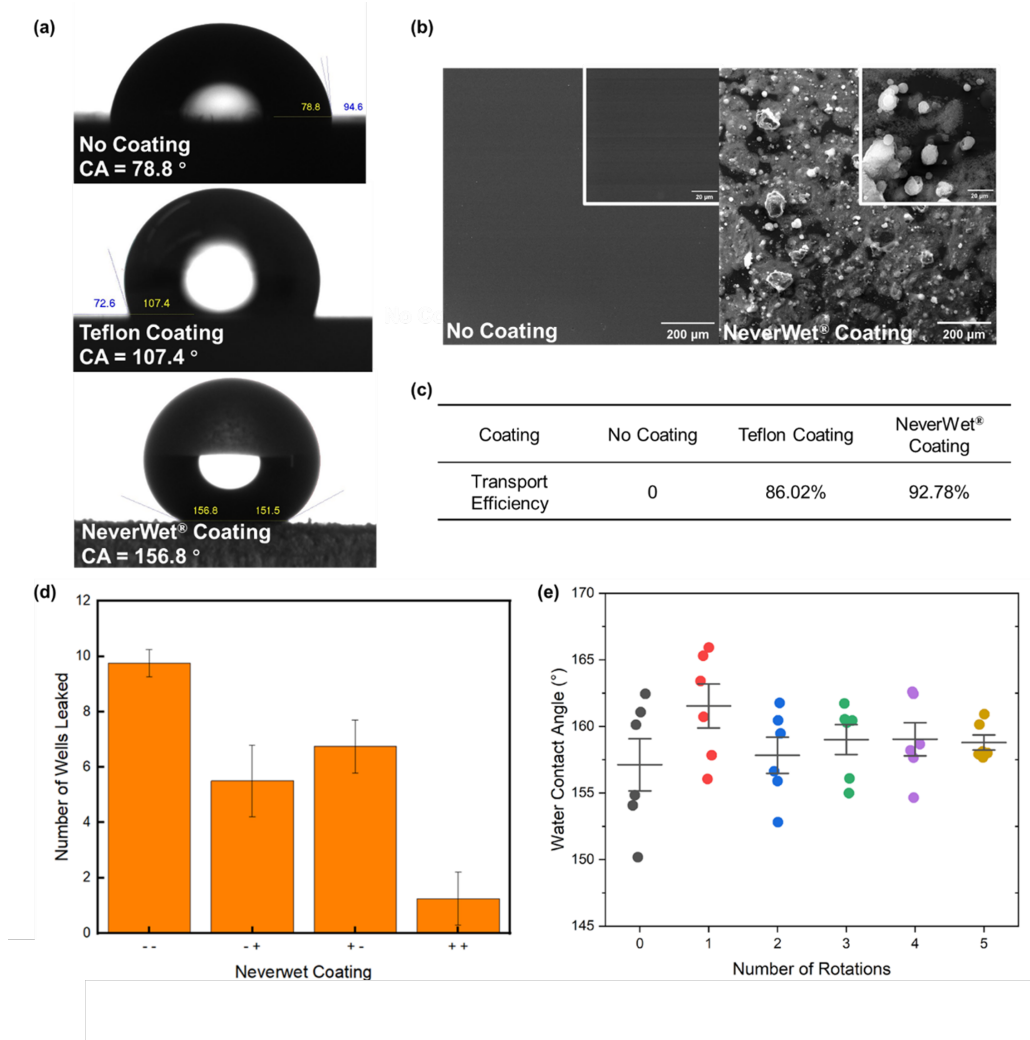


Figure 2.4: Surface treatment and transport efficiency of Rotation-Chip. (a) The static contact angles of Rotation-Chip with no coating, Teflon coating, and NeverWet coating (from left to right). (b) SEM images of Rotation-Chip before and after the NeverWet coating. (c) Transport efficiency of the Rotation-Chip with Teflon and NeverWet coating. (d) Rotation-Chip leak count for NeverWet coatings under different conditions. No coating is represented by ‘-’ while NeverWet coating is represented by ‘+’. The first symbol represents the coating situation for the top layer, and the second symbol represents the coating situation for the bottom layer. (e) Water contact angle of NeverWet coated surface after 30-degree rotation (six randomly selected spots for each rotation).

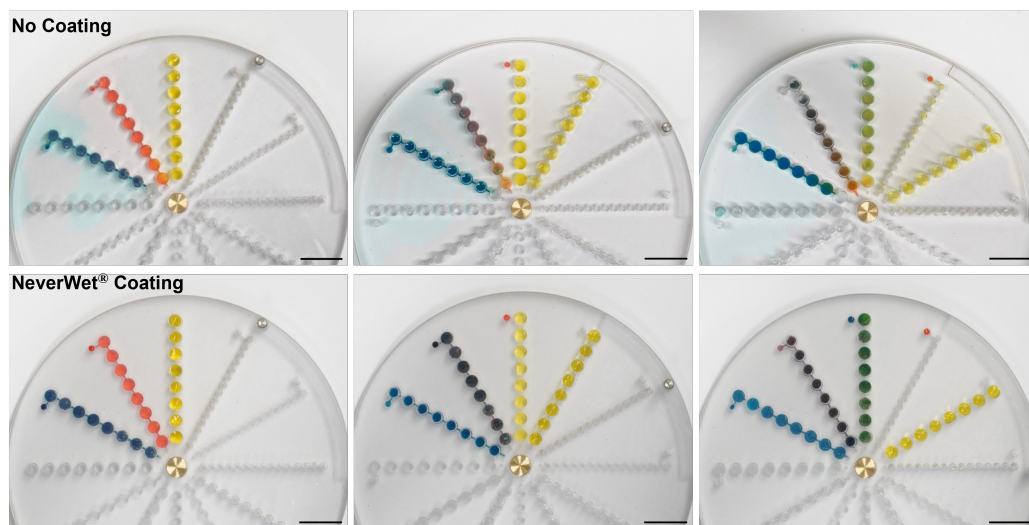


Figure 2.5: The food dye demonstration on the two-step chip before and after the NeverWet coating (scale bar: 10 mm).

USA). As shown in **Figure 2.6c**, more fluorescein is produced by FDG hydrolysis with the increasing β -gal concentration, and the fluorescence intensity is increased. For the on-chip experiment, 100 μ L of FDG was added to the reagent array, and 5 μ L of β -gal was added sequentially in each well. After rotation and incubation, the chip was excited from the bottom with the LED illuminator, and the results are shown in **Figure 2.6d**. When the β -gal activity is greater than 0.01 U, the fluorescence differences between positive and negative groups can be observed on the transilluminator by the naked eye. This detection sensitivity is comparable to the off-chip results.

We then optimized the *E. coli* detection by varying the FDG concentration ranging from 0.01 to 0.25 nM. As shown in **Figure 2.7a**, when *E.coli* concentration is higher than 1.8×10^7 CFU/mL, the fluorescence differences between positive and negative groups can be easily observed by the naked eye. The fluorescence intensity of each group was then

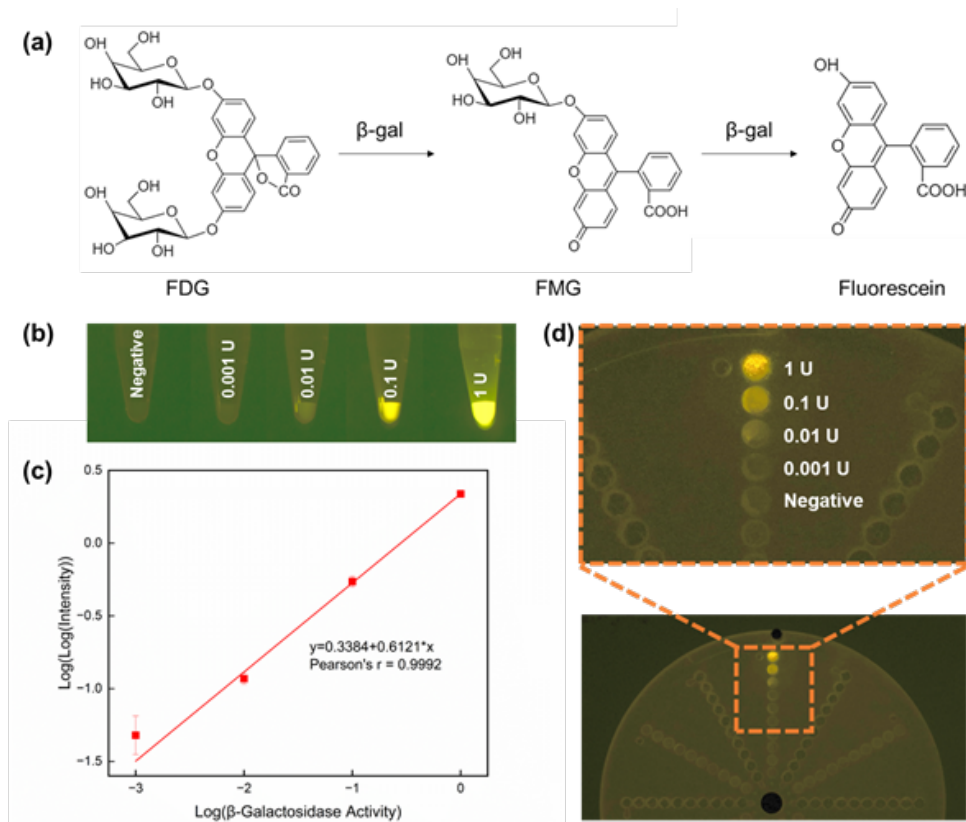


Figure 2.6: (a) Schematic of β -gal sequential hydrolyzation of non-fluorescent FDG. (b) Fluorescence intensity (512 nm) of off-chip results in response to β -gal with different activities (from 0–1 U). (c) Off-chip results of fluorescence intensity (512 nm) correspond to various concentrations of the β -gal. The linear region of the Log versus [Log (fluorescence intensity at 512 nm)] plotted as a function of the logarithmic concentration of β -gal (from 0.001–1 U) is highlighted (Pearson's $r = 0.9992$). (d) On-chip detection results of β -gal (from 0–1 U) on One-Step Chip coated with NeverWet.

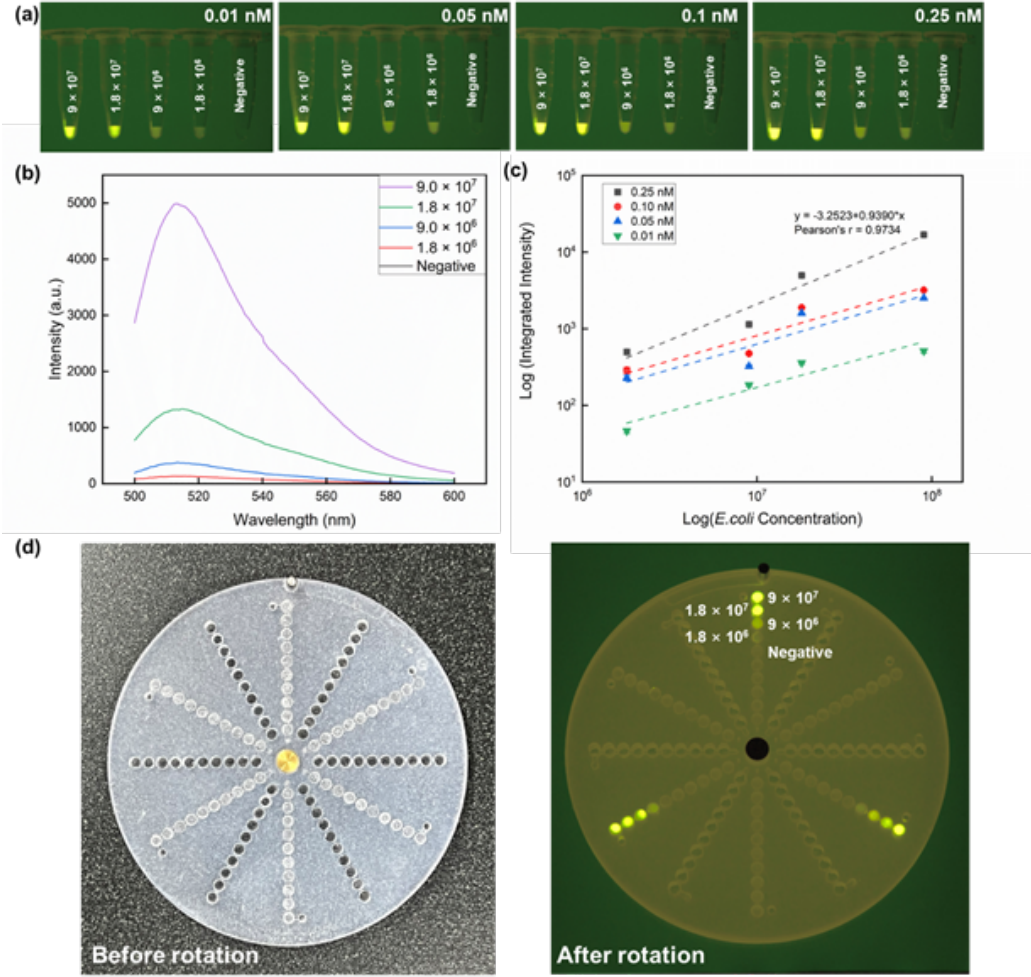


Figure 2.7: (a) Off-chip detection of *E. coli* (1.8×10^6 – 9×10^7 CFU/mL) with FDG of 0.01, 0.05, 0.1, and 0.25 nM (from top to bottom). (b) Off-chip results of fluorescence intensity corresponding to various concentrations of the *E. coli* (1.8×10^6 – 9×10^7 CFU/mL) at an FDG concentration of 0.25 nM. (c) Off-chip results of integrated fluorescence intensity (560–580 nm) corresponding to various concentrations of the *E. coli* (1.8×10^6 – 9×10^7 CFU/mL) on a logarithmic scale (Pearson's $r = 0.9734$). (d) Detection of *E. coli* at different concentrations (1.8×10^6 – 9×10^7 CFU/mL) with 0.25 nM FDG on One-Step Chip coated with NeverWet.

quantified by the spectrofluorometer, and the results at an FDG concentration of 0.25 nM are shown in **Figure 2.7b**. When the FDG concentration is 0.25 nM, the integrated fluorescence signal is highest with the best log-log linear fitting (Pearson's $r = 0.9734$) among the four groups, as shown in **Figure 2.7c**. Thus, we selected 0.25 nM of the FDG for on-chip experiments. The FDG and E.coli loaded chip before incubation is shown in **Figure 2.7d** (left), with E.coli concentration ranging from 1.8×10^6 to 9×10^7 CFU/mL. When the E.coli concentration is higher than 1.8×10^6 CFU/mL, the fluorescence signal is easily observable, as shown in **Figure 2.7e**, which is comparable to the off-chip results. In addition, three independent sets of on-chip experiments (a total of 12 samples) were performed simultaneously by a single rotation, and the fluorescence readings are consistent among the three sets.

On-chip automatic recognition can be considered as a binary classification problem[87], either positive or negative. We randomly selected the same number of negative samples as positive samples to train the system, which could help remove the bias caused by the imbalanced number of class samples. Due to the limited number of training samples, we applied 5-fold cross-validation to train the decision tree. In each round, 4 folds were used for training and 1 fold was utilized for testing. Therefore, all the samples can be used for testing. From the testing result, all the positive and negative samples can be correctly identified by our system. The entire pipeline for processing images and classifying the testing samples is shown in **Figure 2.8**. Eventually, a 100% classification accuracy to separate the detected positive samples from the negative samples is achieved.

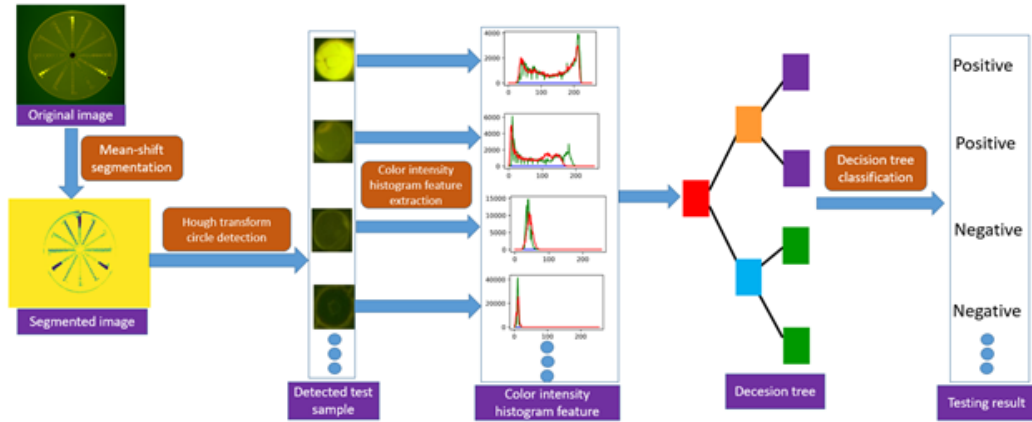


Figure 2.8: Image processing and testing result classification pipeline of computer vision enabled *E. coli* sensing on the superhydrophobic Rotation-Chip.

2.6 Discussion

Due to its design, operation, and manufacturability, the Rotation-Chip has many advantages compared to other POC systems for pathogen detection. First, the design of the Rotation-Chip results in good portability, throughput, and reliability. The motion in which a top plate slides across a bottom plate has been the traditional method to mechanically manipulate fluids through wells and channels. However, this linear motion disrupts the overall shape of the device and limits throughput per unit area by extending the top plate outside the boundary of the preoperative chip, exposing inner features or treated surfaces like in the case of the SlipChip[9, 80]. By adopting a rotational design, the Rotation-Chip can deliver the same fluidic manipulation while keeping all sensitive features in a covered state and staying mechanically intact. Such a design lowers the chip’s spatial footprint and increases its maximum feature density for more portability and throughput. To ensure a reasonably easy rotation effort, the top and bottom plates of the Rotation-Chip are intentionally unclamped, with the assembly held together by only gravity and the friction from the mating

between the central post and the central top plate hole. This design makes leakage prevention a crucial challenge to overcome for the fluid transport process to work with meaningful transport efficiency. Specifically, we found that surface treatment for hydrophobicity to be an effective leakage prevention solution without compromising mechanical integrity or needing dedicated sealing. Notably, we can achieve a superhydrophobic surface using a commercially available spray coating, which is inexpensive and easy to apply. The low surface energy from the superhydrophobicity contributes positively to the Rotation-Chip’s ability to transport discrete volumes of fluid efficiently by keeping the intra-droplet surface tension high enough to withstand the shear stress from the rotation and minimize inter-plate leaking.

Second, the simplistic design of the Rotation-Chip results in an easy manual operation of a simple four-step process: load, rotate, incubate, and read. The user can follow the protocol described above and obtain a qualitative result without any expertise in using complicated laboratory equipment or interpreting fluorescence curves. In addition, all 120 wells can be screened at the same time with our computer vision method, making our Rotation-Chip a powerful POC system. Working together with a simple two-part assembly, the superhydrophobic surfaces of the Rotation-Chip allow for easy cleaning and reusability of at least five times as of this study. Another way to reduce operational complexity and cost is through system integration. Thanks to its constant size, the bottom of the Rotation-Chip can be equipped with a custom microhot plate that can be configured to the temperature requirement of the assay, inexpensive and plug-and-play[88]. This reduces process complexity and further decreases cost by removing the need to use a water bath

and its accessory supply, such as a sealable bag, to keep water from entering the chip during incubation.

Lastly, several popular methods of microfluidic chip fabrication are available to produce the Rotation-Chip, including polydimethylsiloxane (PDMS) molding[89], glass etching[90], CNC machining[91], and injection molding[92]. Though conventional and inexpensive, PDMS molding has properties that were unsuitable for the shear strength and overall rigidity requirements of our application. For the prototypes shown in this study, CNC machining was chosen to be our method of fabrication. Compared to lithographic methods such as wet or dry etching, which are done on brittle material and are time-consuming[92], CNC machining has a much faster lead time and facilitates the dimensional customizability of the design by allowing for the agile production of several design variants. A downside of CNC machining is the surface finish. Since the machined surface is always the inner functional surface in subtractive manufacturing, micro-marring caused by the CNC tool was unavoidable. This poor surface finish increased the surface shear stress, which contributed negatively to the transport efficiency of the chip, as indicated by a satisfactory but improvable experimental value of 92.78%. Another drawback of CNC machining lies in how small the chip features could be made, an uncommon constraint in microfluidic device manufacturing. For future applications where better surface finish and more unit-area throughput (the maximum number of wells in a certain chip area reasonably visible to the naked eye) are needed, high-resolution 3D printing[93] and injection molding[92] can be good alternatives to the aforementioned manufacturing methods for future production of the Rotation-Chip. For example, injection molding could be an efficient and cost-effective alternative for large-

scale fabrication. Using the same clear polycarbonate material or cheaper substitutes like acrylic, an injection molded chip could accommodate smaller microfluidic features to even the nanoliter scale and provide a smoother surface finish[9] for better unit-area throughput and transport efficiency.

While the commercial FDG- β -gal assay applied to the Rotation-Chip is lysis-free and sensitive, various other qualitative intracellular assays with even higher sensitivity comparable to PCR can be incorporated into the Rotation-Chip[94]. For example, by exploiting the redox pathway of *E. coli*, p-benzoquinone was used to mediate a sensitive and economical bioassay to detect *E. coli* concentrations as low as 1.0×10^4 CFU/mL by providing a colorimetric readout in 1 h[95]. Su *et al.* also developed a sensitive colorimetric method for *E. coli* detection that can be applied to the two-step chip with appropriate dimension customization. Mercaptoethylamine (MEA) could bind to *E. coli* through electrostatic adhesion, and it is also easily conjugated to gold nanoparticles (AuNPs) via the -SH group. When presented with *E. coli* under a certain pH condition, the MEA-AuNPs complex leads to a color change from blue to red in less than 5 min, which is observed by the naked eye[96]. Moreover, the chip can be employed for antimicrobial susceptibility testing assays[97]. These assays were developed to rapidly detect drug resistance caused by β -lactamase to aid in the treatment of urinary tract infections, which provides a sensitivity of 90.9% and specificity of 97.6% within a timeframe of 30 min. The caged enzyme amplifier (papain) is combined with a β -lactamase-targeting probe. When exposed to β -lactamase, the papain is activated, and this could further produce a colorimetric signal output via the hydrolysis of N α -Benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPA)[98].

Noteworthy, a structurally similar device that uses a rotational motion was developed for the detection of viral nucleic acids. RT-PCR was performed in the microfabricated glass reservoirs[99]. Our Rotation-Chip, on the other hand, is made by a low-cost CNC machining process, which is desirable for disposable and inexpensive POC applications and is more dimensionally tolerant and less prone to damage than glass. In addition, the round chip design ensures user safety and ergonomics, in contrast to the square design[99].

Besides healthcare, the Rotation-Chip has applications in a variety of other important areas, including food quality and environmental safety[100]. In this work, centrifuge was used to remove the culture media, which can also be achieved by a small microcentrifuge at the point of care. To process raw food samples, processes such as separation, pre-enrichment, and enrichment can be included before inputting the samples into the Rotation-Chip[101]. Due to its portability, our Rotation-Chip also makes a good candidate for testing environmental pollution from ambient samples[102]. In the future, a smartphone app can be developed by using our robust computer vision method and directly working with the Rotation-Chip for accurate, automatic, and high-throughput detection applications[103].

2.7 Conclusion

In conclusion, we have developed a microarray-based rotational microfluidic device for high-throughput multiplexed pathogen detection assays. This pocket-sized device offers several advantages over existing microfluidic systems, including distribution homogeneity, reversibility, superhydrophobic surface chemistry, and ease of fabrication and operation. We

demonstrated the efficacy of the Rotation-Chip by using it to detect ampicillin-resistant *E. coli* through an intracellular fluorescence assay. The chip showed a detection limit similar to off-chip results but without the need for tedious manual pipetting. Furthermore, we developed a mean-shift algorithm to evaluate the fluorescence intensity of all the samples on the rotational chip, achieving 100% accuracy in classification. Future research can focus on expanding the capabilities of the Rotation-Chip by incorporating additional assays for the detection of different pathogens or biomarkers. Optimization of the chip's design and surface treatments can further enhance its performance and reliability. Additionally, efforts can be made to integrate automated sample handling and data analysis systems to streamline the entire diagnostic process. Overall, the Rotation-Chip presents a promising platform for POC diagnostics, offering a rapid, affordable, and reliable solution for the detection of food- and water-borne pathogens and other infectious agents. Its potential impact in healthcare settings and resource-limited environments is significant, as it can enable early detection and appropriate medical treatment while minimizing the spread of pathogens and the development of antimicrobial resistance.

Chapter 3

Harnessing Nano-Sieve

Microdevices for Sensitive

Bacterial Detection

This chapter is adapted from the following publication: Ruonan Peng, Xinye Chen, Fengjun Xu, Richard Hailstone, Yujie Men, and Ke Du. Pneumatic nano-sieve for crispr-based detection of drug-resistant bacteria. *Nanoscale Horizons*, 8(12):1677–1685, 2023[104].

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Minor modifications have been made to conform to dissertation formatting.

3.1 Abstract

The increasing prevalence of antibiotic-resistant bacterial infections, particularly methicillin-resistant *Staphylococcus aureus* (MRSA), presents a significant public health concern. Timely detection of MRSA is crucial to enable prompt medical intervention, limit its spread, and reduce antimicrobial resistance. Here, we introduce a miniaturized nano-sieve device featuring a pneumatically-regulated chamber for highly efficient MRSA purification from human plasma samples. By using packed magnetic beads as a filter and leveraging the deformability of the nano-sieve channel, we achieved an on-chip concentration factor of ~ 15 -fold for MRSA. We integrated this device with recombinase polymerase amplification (RPA) and clustered regularly interspaced short palindromic repeats (CRISPR)-Cas detection system, resulting in an on-chip limit of detection (LOD) of approximately 100 CFU/mL. This developed approach provides a rapid, precise, and centrifuge-free solution suitable for point-of-care diagnostics, with the potential to significantly improve patient outcomes in resource-limited medical conditions.

3.2 Introduction

The prevalence of antibiotic-resistant bacterial infections has become a major concern for both individuals and healthcare facilities, causing an estimated 1.27 million fatalities globally and contributing to nearly 5 million deaths in 2019[105]. One particular pathogen, methicillin-resistant *Staphylococcus aureus* (MRSA), stands out as a prominent multidrug-resistant (MDR) bacterium that presents a serious challenge[106]. MRSA can cause skin infections[107], pneumonia[108] and even sepsis[109], and it exhibits resistance

to beta-lactam antibiotics, including methicillin, penicillin, amoxicillin, and oxacillin, which are commonly used in the treatment of bacterial infections[110]. Consequently, infections caused by MRSA are associated with high morbidity and mortality rates, making it a significant public health issue[111].

Early detection of MRSA is crucial as it enables timely and appropriate medical intervention, prevents the spread of these pathogens, and reduces the risk of antimicrobial resistance[112, 113]. For detection, it is essential to isolate bacteria from the samples collected from nose[114], blood[115] or urine[116]. Membrane-based filtration is a widely used and advantageous method for capturing bacteria due to its cost-effectiveness, simplicity, and rapidness[117]. Furthermore, a significant challenge associated with this technique is the extensive volume of iterative washing buffer required to retrieve the captured bacteria from the membrane[118]. This washing process can inadvertently lead to dilution of the captured bacteria, reducing their concentration to levels that may fall below the detection limit of downstream detection processes. Alternatively, microfluidic platforms have emerged as a powerful tool in the field of bacterial purification and concentration[119, 120, 121]. Those platforms could be functionalized to rapidly and efficiently separate and concentrate target bacteria, depending on various working principles, including inertial force[122, 123], hydrodynamics[124, 125], electrophoresis[126, 127], and acoustics[128, 129]. However, these techniques require either complicated fabrication processes or extra laboratory-based instruments, increasing the complexity of microfluidic platforms for delicate operations. Therefore, it is important to develop a simple and direct process of fabricating a microfluidic platform while ensuring it can effectively separate the target bacteria[130].

Traditional MRSA detection methods, such as cultured-based techniques, are time-consuming and labor-intensive. Molecular methods like polymerase chain reaction (PCR) require thermocyclers and sophisticated bulky equipment, which renders them unsuitable for resource-limited point-of-care (POC) environments[62]. Clustered regularly interspaced short palindromic repeats (CRISPR)-Cas (CRISPR-associated) systems, particularly the Cas12 and Cas13 nucleases, have gained significant attention in the field of in vitro diagnostics[131]. Among them, Cas12a relies solely on a complementary crRNA for targeting specific DNA sequences and utilizes a single RuvC domain to cut the target DNA, a process known as cis-cleavage[132, 31]. Moreover, Cas12a exhibits collateral activity, referred to as trans-cleavage, which allows it to non-specifically cleave neighboring single-stranded DNAs (ssDNA) following target binding[133]. To exploit this feature for detection purposes, ssDNA can be labeled with a fluorophore-quencher, and upon Cas12a activation through target binding, the cleavage of ssDNA generates an increase in fluorescence signal[134]. CRISPR-Cas12a system operates at 37°C, making it more suitable for POC detection than traditional PCR methods[135]. Additionally, by combining the CRISPR-Cas system with isothermal amplification methods such as recombinase polymerase amplification (RPA)[136], rolling circle amplification[137] (RCA), and loop-mediated isothermal amplification (LAMP)[138], the specificity and sensitivity of CRISPR-Cas detection can be further enhanced in POC settings[139]. Notably, RPA stands out as a widely adopted amplification method due to its simplicity, rapidity, and compatibility with the same temperature requirements as CRISPR assays.

We introduce a miniaturized and versatile nano-sieve device with a pneumatically-regulated chamber that allows for rapid purification and highly concentrated isolation of MRSA from plasma samples. To achieve this, we developed a simplified, direct, and cost-efficient fabrication process for this nano-sieve device, incorporating multiple channels. With a three-dimensional (3-D) magnetic beads-stacked microstructure within the channel, the highly efficient bacteria capture was preceded by precisely controlling the applied flow rate. Leveraging the deformability of the nano-sieve channel, a remarkable concentration (around 15-fold) of captured bacteria was achieved by adjusting the volume ratio of initial sample solution and retrieved buffer solution. This unique functionality of nano-sieve significantly enhances the limit of detection (LOD) when combined with the developed RPA and CRISPR-Cas assay, ultimately achieving an on-chip LOD of approximately 100 CFU/mL. Importantly, the entire process can be completed in less than 4 hours under physiological temperature and room temperature, without the need of centrifugation. Therefore, our approach of integrating the microfluidic-based multiplexing purification and a rapid and precise molecular detection could potentially improve the sensitivity and specificity of MRSA detection.

3.3 Materials and Methods

3.3.1 Device fabrication

The pneumatically-regulated nano-sieve device consists of a channel-patterned glass substrate and a PDMS topping with a pneumatic chamber. Briefly, a cleaned 4-inch glass wafer (University wafer, D263, 550 μm , double side polished) was deposited by a

layer of TEOS (PECVD, AME P5000) with a thickness of 200 nm. After spin-coating a thin layer (1 μm in thickness) of positive photoresist (AZ Mir 701), a plastic photomask (Fineline Imaging, CO, USA) was applied to transfer the channel pattern onto the photoresist layer. Followed by buffer oxide etching (BOE), the nano-sieve channel was created on the glass substrate, with a width of 5 mm and a length of 15 mm. To fabricate a pneumatic chamber of 2 mm in height and 2 mm in width, a PDMS mixture with one part of curing agent and ten parts of base polymer (SYLGARDTM 184, Krayden Inc., CO, USA) was poured into a three-dimensional (3-D) printed mold (Fictiv, CA, USA). After the curing process of PDMS in the oven at 60 °C overnight, the chamber layer was punched by a 1 mm puncher (INTEGRATTM MiltexTM), for the pneumatic regulation by the air pump (Precigenome LLC, CA, USA). This chamber layer was bonded onto a thin layer of cured PDMS thin film (200 μm in thickness) via plasma treatment (Electro-Technic Products, IL, USA). Then the entire part was baked on the hot plate at 100 °C for 2 hrs to obtain the robust bonding in between. To finalize the fabrication of a functional device, the nano-sieve channel on the substrate was sealed by bonding with the pneumatic layer via plasma treatment and baking process on a hot plate. Before using the device for experiments, the holes of inlet and outlet were punched by a 1 mm puncher, so that the microfluidic tubing (Scientific Commodities, Inc., BB31695-PE/3) can connect the device to the sample sources in a syringe (BD 1 mL, NJ, USA).

3.3.2 Beads stacking functionalization

A strategy of creating the 3-D microstructure of stacked beads (Alpha Nanotech Inc., NPSSMX1, 10 mg/mL, 10 μm , 10 mL) was applied to physically capture the target

bacteria from the initial sample. In this study, the nano-sieve channel was first rinsed and cleaned by isopropyl alcohol (IPA) solution. Before introducing the magnetic beads into the channel, the air pump was used for ensuring the pressure applied in the pneumatic layer consistently at 12 Psi. Then, a 50 μL of 10 μm magnetic beads with a concentration of 10 mg/mL was injected into the channel to form a “coarse filter” by employing a syringe pump with a flow rate of 30 $\mu\text{L}/\text{min}$. The injection of another 50 μL of 5 μm magnetic beads with a concentration of 10 mg/mL into the channel was followed, to form a “fine filter” under a flow rate of 20 $\mu\text{L}/\text{min}$. Finally, this functionalized nano-sieve device is ready for separating the target bacteria from the introduced sample solution.

3.3.3 Bacterial culture

Methicillin-resistant *Staphylococcus aureus* (ATCC 43300) and *Staphylococcus aureus* (ATCC 25923) were purchased from Fisher Scientific, Kanamycin-resistant *E. coli* 10798 and *E. coli* MG1655 were from lab stock. The bacteria were cultured in tryptic soy broth medium (MilliporeSigma) and maintained on the tryptic soy agar plate. After the overnight culture at 37 °C under 200 rpm, 1 mL of bacterial cells was centrifuged at 8000g for 5 min to form a pellet. The pellet was then resuspended in 1 mL of phosphate buffer saline (PBS) or a mixture of 1:4 of human plasma (MilliporeSigma) and PBS. This process was repeated twice to completely remove culture media. The concentration of the bacterial cells was determined by counting the colony-forming unit (CFU) on standard TSA plates. The harvested cells were diluted in PBS or a 1:4 mixture of plasma and PBS using a 10-fold dilution for future use.

3.3.4 Bacterial staining

Two microliters of *BacLight* green dye (MilliporeSigma) was mixed with 1 mL PBS-based bacteria solution, and the mixture was incubated at room temperature for 20 min by following the manufacturer's instruction. Then the solution was centrifuged at 8000g for 5 min to discard the PBS that consists of extra dye. The bacteria were resuspended in 1 mL fresh PBS for the further use.

3.3.5 Bacteria capture and concentration

The 600 μ L of prepared sample solutions, in which the bacteria with a certain concentration were spiked in 1:4 diluted plasma solution, was first loaded into several 1 mm sterile syringes. A multi-channel syringe pump was used to simultaneously introduce the sample solutions into the functionalized nano-sieve channels through the microfluidic tubing, under a flow rate of 4 μ L/min. After completing the separation process, the air pump was stopped and a 30 μ L of fresh PBS solution was applied for rinsing the entire channel to retrieve the magnetic beads and the captured targets, from the channel to a sterile centrifuge tube. A simple and direct separation with an external magnet was used for extracting the bacteria-involved PBS solution, which is ready for the further detection based on RPA/CRISPR technique. The concentration factor in this case is expected to be 20-fold.

3.3.6 Fluorescence microscopy imaging

After the completion of bacteria separation, 10 μ L of each retrieved sample and initial sample were placed onto a glass slide for the measurement of cell density. Under

the microscope equipped with a high-speed camera and the fluorescent light resource, the fluorescent images were captured and analyzed by using Leica LAS X software.

3.3.7 TEM Characterization

A JEOL 2100 transmission electron microscope (TEM) operating at 200 kV was used to image the samples. An aqueous solution of magnetic beads +/- DNA was deposited onto a carbon-coated copper TEM grid and left to allow evaporation of the water. Then, the dried grids were dipped into 18 Mega Ohm water for 30 s to remove excess salts. The images were then captured with a Gatan Orius camera.

3.3.8 Nucleic acid extraction

The nucleic acid preparation involves bacterial lysis and DNA purification, which was carried out at room temperature and completed in less than 30 min. To prepare the enzymatic lysis buffer (20 mM Tris-HCl, 2 mM EDTA, and 1.2% Triton X-100), lysozyme (Thermo Fisher) was added at a concentration of 20 mg/mL immediately before use. Next, 18 μ L of the enzymatic lysis buffer was added to 10 μ L of the pathogens and incubated for 10 min. Following this, 2 μ L of proteinase K (Thermo Fisher) was added, and the mixture was incubated for 5 min. For DNA purification, AMPure XP beads (Beckman Coulter) were used following the vendor's instruction with slight modification. Briefly, 54 μ L of magnet beads were added to the lysate and incubated for 5 min. The tube was then placed on the magnet for 2 min to separate beads from the solution. Next, the beads were washed twice with 70 μ L of 5 M Guanidinium chloride (MilliporeSigma), and the DNA was eluted with 20 μ L of nuclease-free water (Thermo Fisher).

3.3.9 RPA amplification and CRISPR-Cas12a detection

TwistAmp[®] Basic kit was purchased from TwistDx[™]. The RPA primers, crRNA, AsCas12a, and fluorophore-quencher probes were all obtained from Integrated DNA Technologies, and detailed information about the synthetic oligonucleotides are listed in Table 3.1. The RPA primer sets were designed using PrimerQuest[™] Tool. Additionally, NEBuffer[™] r2.1 was purchased from New England Biolabs. The RPA reaction was conducted based on the instructions: A mixture of 29.5 μ L of rehydration buffer, 11.2 μ L of nuclease-free water, and 2.4 μ L each of forward and reverse primers (10 μ M) was added to the enzyme pellet. Then, 2 μ L of purified DNA and 2.5 μ L of MgOAc (280 mM) were added and mixed to achieve a total volume of 50 μ L. The mixture was incubated at 37 °C for 20 min. Following the incubation, 2 μ L of RPA amplicons were added to a pre-assembled CRISPR-Cas12a mixture comprising 50 nM of AsCas12a, 62.5 nM of crRNA , 10 $\times$ buffer, and 2.5 μ M of ssDNA-FQ probe, resulting in a final reaction volume of 20 μ L. The reaction solution was incubated at 37 °C for 30 min. After the incubation, the mixture was excited by a blue light transilluminator (brand: SmartBlue, Part number: NEB-E4100, excitation wavelength of 465 nm) for naked-eye observation. Finally, 20 μ L of nuclease-free water was added to 5 μ L of the mixture, which was then characterized by an Agilent BioTek Cytation 5 imaging reader.

Table 3.1: List of synthetic oligos sequence used in this study

Name	Sequence (5'-3')
crRNA 1	UAAUUUCUACUAAGUGUAGAUAGUUCUGCAGUACCGGAUUUG
Target 1	CAAATCCGGTACTGCAGAACT
F1	GATTAACCCAGTACAGATCCTTTCAATCTA
R1	ATAGCCATCATCATGTTTGGATTATCTTTATC
F2	TATGCAACAAGTCGTAAATAAAACACATAAAG
R2	TCATATGATATAAACCACCCAATTTGTCTGCC
crRNA 2	UAAUUUCUACUAAGUGUAGAUCUAGAGUAGCACUCGAAUUAG
Target 2	CTAGAGTAGCACTCGAATTAG
F3	AAACAAGCAATAGAATCATCAGATAACATTT
R3	TATAGATTGAAAGGATCTGTACTGGGTTAAT
F4	AAACAAGCAATAGAATCATCAGATAACATTT
R4	AAGGATCTGTACTGGGTTAATCAGTATTTC
ssDNA-FQ Probe	/56-FAM/TTATT/3IABkFQ

3.4 Results

The schematic of the whole system is presented in **Figure 3.1a**, where multiple nano-sieve channels are designed for multiplexing separation of bacteria from initial samples. **Figure 3.1b** shows the picture of a practical nano-sieve device, including the beads-stacked channel filled with blue food dye and the pneumatic layer filled with red

food dye. This indicates the device can be successfully fabricated without any leaking issues. In **Figure 3.1c**, the fabrication flow of a pneumatically-regulated nano-sieve device is exhibited. It started with a thin layer of TEOS (200 nm in thickness) deposited onto a pre-cleaned glass wafer, which was followed by a spin-coated layer of positive photoresist. After that, a pattern of nano-sieve channel was transferred from a plastic photomask to the photoresist layer by photolithography technique, then defined on the layer of TEOS by BOE process. The patterned channel with a thickness of 200 nm was created, and finally covered by a thin layer of positive photoresist as a sacrificial support for PDMS bonding procedure. This coated photoresist can eliminate the technical issue of collapsed PDMS roof[140], significantly enhancing the fabrication of nano-fluidic channels. The pneumatic chamber was made by employing a 3-D printed mold, and a thin film of uncured PDMS was sandwiched by glass slides, with a supporting ORACAL film to define the thickness of this PDMS film to be created. Then the pneumatic chamber and the cured PDMS thin film were bonded through the plasma treatment. The fabrication of the nano-sieve device was subsequently completed by bonding the pneumatic chamber layer and the glass substrate patterned with nano-sieve channels via plasma treatment. Both treatments (marked by the red dashed rectangles) were followed by the baking process on a hot plate to achieve the strong bonding in between.

The pre-loaded stacked beads array within the half section of the nano-sieve channel is displayed in the optical micrograph in **Figure 3.2a**, which are well secured by the positive pressure applied in the pneumatic chamber. Another half section per channel was connected to the outlets for collecting the waste liquids. **Figure 3.2b** presents the experi-

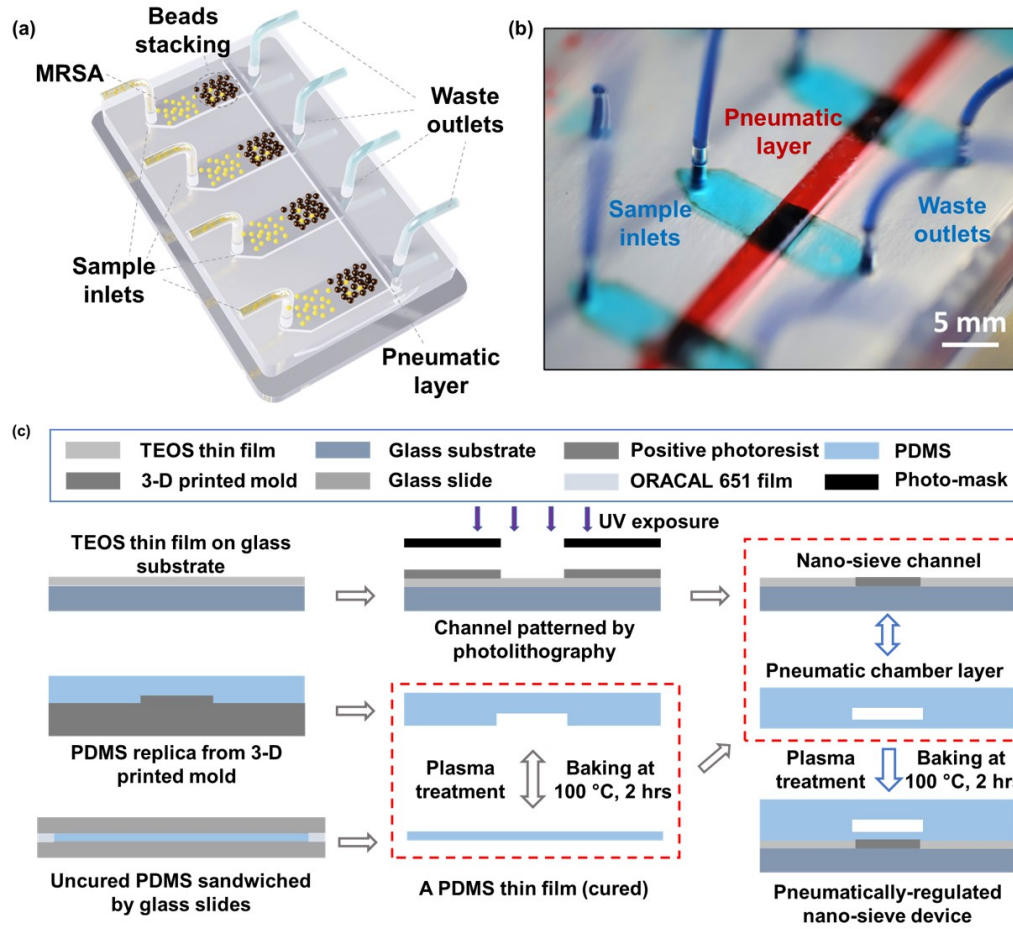


Figure 3.1: (a) Schematic of nano-sieve device. The MRSA sample is injected through sample inlets, and subsequently, bacteria become entrapped within the beads stacking region of the microfluidic channel, allowing for effective MRSA concentration. Meanwhile, the waste liquid, consisting of plasma and phosphate-buffered saline (PBS), exits the device through designated waste outlets. (b) Image of realized multi-channel nano-sieve with food dye. (c) Fabrication process of nano-sieve device.

mental setup, regarding a multiplexing separation of target bacteria under the observation of fluorescence microscopy. Within these nano-sieve channels as shown in **Figure 3.2c**, only the target bacteria stained by the green dye can display the green fluorescent signals. During the flow condition, the bacteria were carried by flowing fluid, moving forward to the area of stacked beads, where they were physically captured by the array of 5 μm beads. It is noticed that the ratio of MRSA bacteria to the magnetic beads is very low without saturation problems. As shown in **Figure 3.2d** and **Figure 3.2e**, the original bacterial sample and retrieved bacterial sample were compared to highlight the on-chip concentration capability of this powerful nano-sieve system. The retrieved bacteria sample shows higher concentration of target bacteria than the original bacteria sample that has a lower concentration. Due to the pure physical process, there is no on-chip culturing involved through the MRSA purification.

After successfully concentrating MRSA using nano-sieve, standard plate count was employed to quantify the concentration ability of nano-sieve. Table 3.2 displays the concentration factors achieved with various inlet concentrations using the nano-sieve device. The concentration factor was determined by dividing the inlet concentration by the outlet concentration. A total of 600 μL of MRSA was injected into inlets, while 30 μL of PBS was used to retrieve the MRSA, resulting in a theoretical concentration factor of 20. However, as the MRSA concentration increased, the experimental concentration factor slightly decreased. One possible reason for this is that some bacteria may have leaked out through the waste outlets due to the increase in MRSA concentration. This suggests that the nano-sieve is more suitable for concentrating low-concentration bacteria.

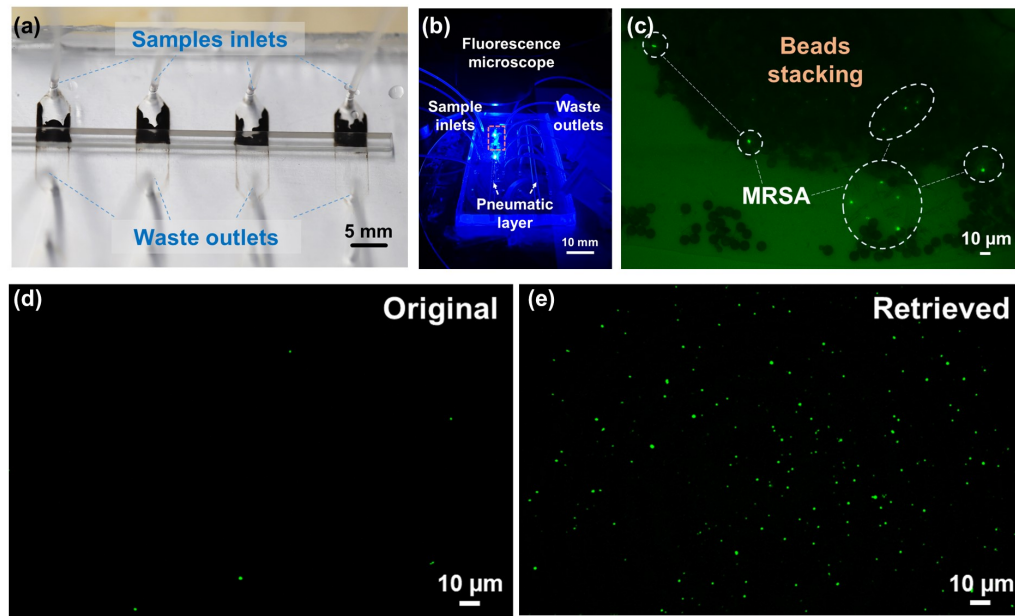


Figure 3.2: (a) Magnet beads pattern in the channel without MRSA. (b) Experimental setup of multiplexing separation of target bacteria under the fluorescence microscope. (c) Beads stacking with MRSA under fluorescence microscope. (d) The original bacterial sample and (e) retrieved bacterial sample were compared to indicate the on-chip concentration capability of the nano-sieve device.

Table 3.2: Concentration factor of nano-sieve

Channel	Inlet	Outlet	Experimental	Theoretical
	Concentration	Concentration	Concentration	Concentration
	(CFU/mL)	(CFU/mL)	Factor	Factor
1	4.00×10^2	$(6.00 \pm 2.94) \times 10^3$	15.00	20.00
2	5.93×10^3	$(8.47 \pm 1.07) \times 10^4$	14.27	20.00
3	7.43×10^4	$(8.37 \pm 1.40) \times 10^5$	11.26	20.00
4	3.43×10^5	$(4.07 \pm 0.60) \times 10^6$	11.84	20.00
5	3.80×10^6	$(4.07 \pm 0.60) \times 10^7$	10.79	20.00

The nucleic acid purification process using magnet beads after the MRSA lysis is shown in **Figure 3.3a**. In this process, magnet beads are introduced into a solution containing DNA, wherein a substantial amount of salt and polyethylene glycol (PEG) is present[141]. The DNA molecules become crowded out and bind to the surface of the beads through electrostatic interactions[142] and molecular crowding[143]. Magnet fields are then applied to collect DNA-bound beads, effectively removing unwanted debris such as membrane lipids and proteins[144]. Then, guanidinium chloride is utilized to wash DNA as it disrupts protein-DNA interactions and aids in solubilizing and denaturing proteins[145]. Finally, purified DNA is eluted using nuclease-free water. **Figure 3.3b** shows the mechanism of RPA amplification. This process relies on the coordinated activities of recombinases, single-stranded DNA-binding proteins, and DNA polymerases to achieve isothermal amplification of the target DNA. The reaction is initiated by recombinases facilitating the binding

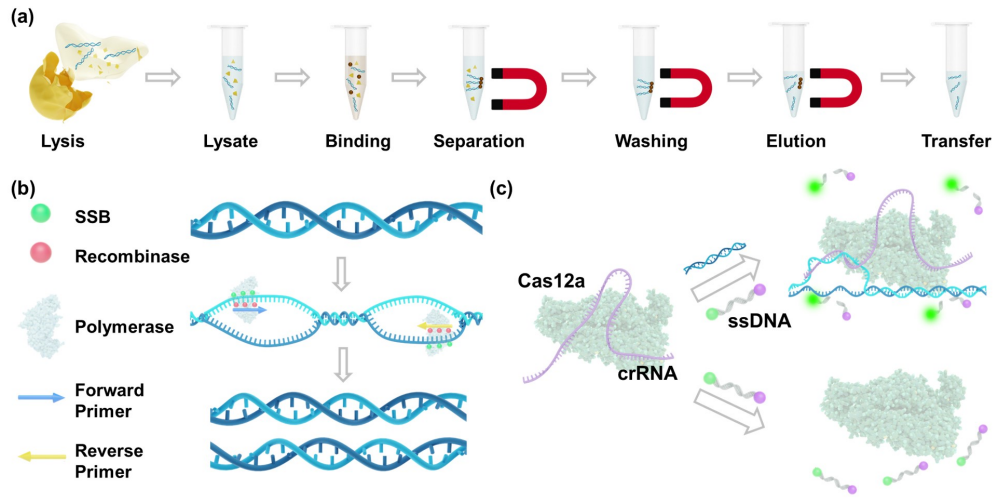


Figure 3.3: (a) Nucleic acid purification process using magnet beads after MRSA lysis. (b) Mechanism of RPA amplification. Recombinases assist in primer binding to target DNA, while SSB proteins stabilize the strands. Polymerases then use building blocks (dNTPs) to form new DNA strands. (c) Mechanism of CRISPR-Cas12a detection involving the formation of a Cas12a-crRNA-DNA complex and cleavage of the fluorophore-quencher probe.

of primers to the target DNA sequence. Single-stranded DNA-binding proteins stabilize the displaced DNA strands, allowing DNA polymerases to efficiently extend the primers along the DNA template in the presence of deoxynucleotide triphosphates (dNTPs), resulting in the synthesis of new DNA strands. Following this, the RPA amplicons are introduced into the CRISPR-Cas12a reaction. As shown in **Figure 3.3c**, in positive samples, when the Cas12a-crRNA complex encounters the complementary target DNA, it undergoes a conformational change, leading to the activation of its nuclease activity. Cas12a then cleaves the target DNA at a specific site (cis-cleavage) as well as the collateral ssDNA probes (trans-cleavage), leading to the release of fluorescence signals from the fluorophore. In negative samples lacking the target DNA, the nuclease activity of Cas12a remains inactivated, preventing the cleavage of the probe and the generation of fluorescence signals.

The assay development started with primer screening. Two CRISPR RNA and four primers sets were chosen, and their sequences are listed in Table 3.1. The input DNA was extracted from 10^8 CFU/mL MRSA and purified using magnet beads. The excitation and emission wavelengths were set at 480 nm and 520 nm, respectively. The results of the primer screening are depicted in **Figure 3.4a**. With the preceding RPA amplification, the fluorescence signal exhibited a substantial increase in comparison to CRISPR-Cas12a detection performed without RPA. Among the groups, the combination of crRNA2 and primer set 4 demonstrated the highest fluorescence signal, thus being chosen for subsequent experiments. To further evaluate the assay performance, a comparison was made between the magnet beads purification and a scenario without such purification. The results revealed a significant reduction in fluorescence signal without magnet beads purification, as illustrated in **Figure 3.4b**. One possible explanation is that the presence of EDTA, lysosome, and proteinase K, which were introduced during MRSA lysis, might have disrupted the enzyme system, ultimately leading to the failure of DNA amplification and detection. On the other hand, magnetic beads purification effectively eliminated unwanted debris, including lysosome and proteinase K, thereby ensuring the successful amplification and detection of the target DNA. **Figure 3.4c** displays the TEM images of magnet beads only (top) and magnet beads plus DNA (bottom). In the top image, aggregation and clustering of the beads can be observed, while the bottom image demonstrates the binding of DNA molecules to the magnet beads through electrostatic interactions and molecular crowding. Furthermore, the specificity performance of the assay was evaluated using three additional strains: wild-type *E. coli* K12 (**Figure 3.4d**), kanamycin-resistant *E. coli* K12 (**Figure 3.4e**), and wild-type

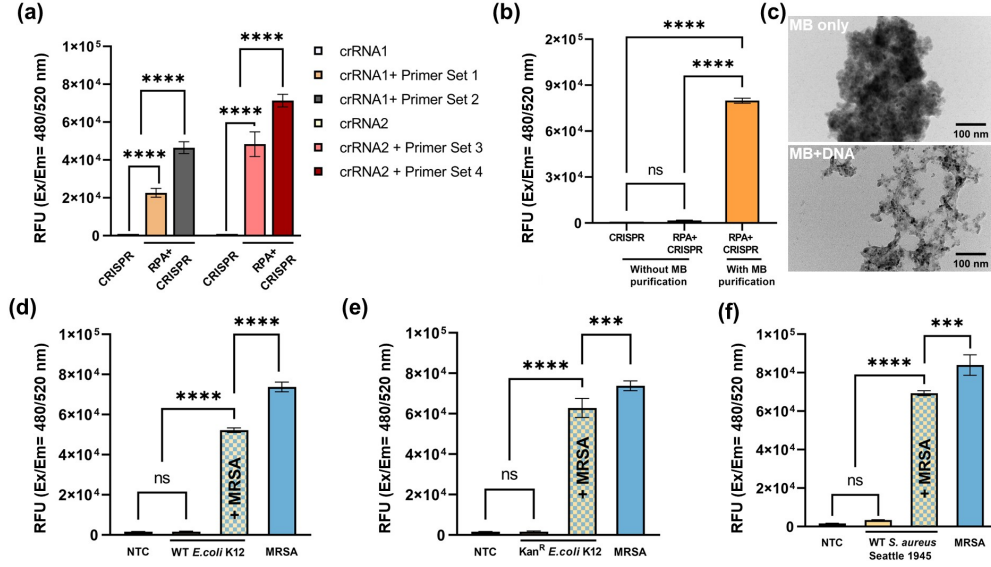


Figure 3.4: (a) Primer screening for RPA amplification. (b) MRSA detection with/without purification using magnet beads. (c) TEM images of magnet beads only (top) and magnet beads plus DNA (bottom), with a scale bar of 100 nm. (d), (e), and (f) represent the specificity tests of this assay using wild-type *E. coli* K12, kanamycin-resistant *E. coli* K12, and wild-type *S. aureus*, respectively. “NTC” refers to no template control. The data are represented as mean $\pm$ standard deviation ($n = 3$). For statistical analysis, *ns*, not significant = $p > 0.05$; *** = $0.0001 < p \leq 0.001$; **** = $p \leq 0.0001$.

S. aureus (**Figure 3.4f**). The input DNA was extracted from bacterial cultures with a concentration of 10^8 CFU/mL. The PBS buffer solution lacking bacteria was served as no template control (NTC). The results show that the fluorescence signals generated by these bacteria were comparable to that of the NTC group. However, upon mixing these bacteria with MRSA at a ratio of 1:1, the fluorescence signal of the mixture significantly increased. Particularly, the fluorescence signal produced by wild-type *S. aureus* reached nearly the same level as that in pure MRSA. These results not only confirm the specificity of the assay but also suggest that the presence of other bacteria does not interfere with MRSA detection results.

Figure 3.5a presents a comparison of fluorescence signal obtained from off-chip and on-chip detection, encompassing various inlet MRSA concentrations ranging from 10^4 CFU/mL to 10^6 CFU/mL. When the MRSA concentration reached 10^6 CFU/mL, both the on-chip and off-chip results exhibited saturation in fluorescence signal. As the MRSA concentrations decreased, the off-chip results displayed a decline in fluorescence signal, whereas the on-chip results remained saturated. Notably, at an MRSA concentration of 10^4 CFU/mL, a discernible difference in fluorescence signal between the on-chip and off-chip results became evident, as depicted in **Figure 3.5b**. Subsequently, the on-chip detection limit was determined by further decreasing the MRSA concentration. **Figure 3.5c** presents the fluorescence signal acquired from on-chip detection using inlet MRSA concentrations ranging from 10 CFU/mL to 10^3 CFU/mL, accompanied by the corresponding endpoint images displayed in **Figure 3.5d**. At an MRSA concentration of 100 CFU/mL, the naked eye easily discerned the fluorescence differences between the positive and negative groups, which were further validated through one-way ANOVA analysis of quantified characterization[146]. The presence of two asterisks between the 100 CFU/mL group and NTC indicates a statistically significant difference, stating that our assay can reliably detect MRSA at a concentration of 100 CFU/mL. On the other hand, the 'ns' (non-significant) result between the 10 CFU/mL group and NTC explains that the fluorescence signals from these two groups are not significantly different, which states that detection limit of our assay lies somewhere between 10 CFU/mL and 100 CFU/mL. Therefore, a detection limit of 100 CFU/mL was established for MRSA detection using the nano-sieve device.

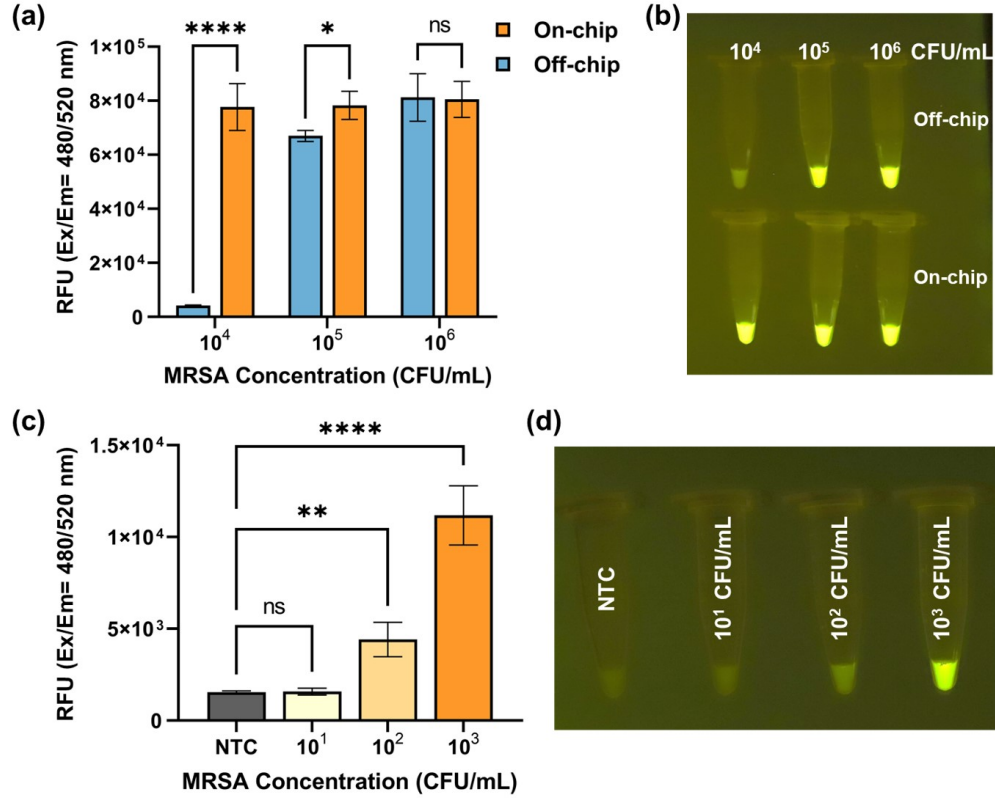


Figure 3.5: (a) Quantified fluorescence signal of off-chip and on-chip detection with varying inlet MRSA concentrations ranging from 10^4 CFU/mL to 10^6 CFU/mL (Ex/Em = 480/520 nm). (b) Endpoint images of the reactions excited by a transilluminator (wavelength: 465 nm) in response to different inlet MRSA concentrations ranging from 10^4 CFU/mL to 10^6 CFU/mL (excitation wavelength: 465 nm). (c) Quantified fluorescence signal of on-chip detection with inlet MRSA concentration ranging from 10 CFU/mL to 10^3 CFU/mL (Ex/Em = 480/520 nm). (d) Endpoint images of the reactions excited by a transilluminator (wavelength: 465 nm) in response to different inlet MRSA concentrations ranging from 10 CFU/mL to 10^3 CFU/mL. “NTC” refers to no template control. The data are represented as mean $\pm$ standard deviation ($n = 3$). For statistical analysis, *ns*, not significant = $p > 0.05$; * = $0.01 < p \leq 0.05$; ** = $0.001 < p \leq 0.01$; **** = $p \leq 0.0001$.

3.5 Discussion

The approach of rapidly purifying and highly concentrating pathogens from a large volume of bodily fluids could be crucial for disease diagnostics, such as sepsis, at the early stage[147, 148]. Compared to the surface chemistry technique, pathogen separation based on the physical structure of microfluidic platforms could be simpler and more efficient, while minimizing contamination issues. Compared to filter membranes, our device only needs a small amount of buffer solution to collect and concentrate the trapped bacteria to extend the detection limit. The concentration factor could be further improved by adjusting the volume ratio of initial sample solution and buffer solution[149, 150]. Our nano-sieve system has been functionalized via a 3-D beads-stacked microstructure that can be precisely tuned by applied flow rate[151, 152], and optimized via a pneumatic layer that can counterbalance the hydrodynamic pressure during the flow condition[153, 154]. Moreover, our pneumatically-regulated nano-sieve has been developed with an extremely low aspect ratio of 1:25,000, significantly reducing the hydrodynamic pressure that may affect the mechanically-driven separation process. This flexible pneumatic layer enhances the adaptability of our nano-sieve channel compared to a rigid nanofluidic channel. Therefore, this deformable pneumatic layer enables a more reliable bead-stacking by applying the positive air pressure during the purification process and an easier target release by offering the negative air pressure during the retrieval process. In addition, we recently showed that the pneumatic-controlled nano-sieve with a patterned microstructure on the bottom of the substrate could efficiently enhance the capture of nanoscale targets at a higher flow rate[153]. In the future, the development of 3-D spaced beads array, such as a combination of various sized beads, could be

beneficial to a higher capture efficiency of target bacteria, aiming to improve the detection limit by combining with our optimized molecular detection technique. While our current study involved spiking MRSA into plasma samples, future research will focus on testing clinical samples to validate the applicability of our approach in a real-world clinical setting. Currently, our group is engaged in the development of a rapid and efficient method for purifying and identifying antibiotic-resistant bacteria (ARB) from human blood samples. Through an immunomagnetic assay, highly concentrated red blood cells (RBCs) could be removed from bacteria-spiked samples, while effectively retaining the target bacteria for subsequent purification using the optimized nano-sieve device. Presently, our system only processes up to six samples simultaneously, but by introducing more channels during the chip fabrication process, we can achieve the simultaneous detection of hundreds of clinical samples within a 6-inch wafer using a simple equipment setup comprising a multipump, pipetting system, heat block, magnet, transilluminator, and necessary reagents. To prevent the samples from being contaminated, the initial samples were loaded in the single-used and sterilized syringe, then pumped through the sterilized microfluidic tubing into each independent channel that was filled with the beads stacking. Consequently, the entire environment is closed and sterilized for target bacteria purification, and there is no contact between the equipment and the applied sample. Such high-throughput capability will significantly reduce the turnaround time for patients and healthcare providers to obtain the test results, which is crucial for expediting disease diagnosis, facilitating prompt medical interventions, and ensuring timely implementation of appropriate treatment strategies[155]. This pneumatically-regulated nano-sieve device was designed and fabricated as a cost-effective

and disposable system for POC applications without recycling concerns. The MRSA purification process is entirely based on silica microbeads stacking within the channels of device. This entire chip could be stored in environmental conditions for an extended duration, making it well-suited for sample preparation for POC setting. Additionally, one novelty of our approach is centrifuge-free DNA extraction by using the immunomagnetic beads. This method not only facilitated our work with spiked plasma samples but also eliminated the need for centrifugation, which is superior to commercially available spin column extraction kits, which rely on complicated and expensive centrifuge processes. Our centrifuge-free method is more affordable and further cost reductions could be achieved by synthesizing our own DNA extraction beads. On the other hand, by designing different primers and CRISPR RNA, this microfluidic device enables multiplexing for different pathogens. This feature holds significant promise in the diagnosis of diseases potentially caused by multiple pathogens, such as sepsis[156]. While our detection assay is currently performed in tubes after the nano-sieve concentration, requiring manual pipetting, our future work will focus on the incorporation of a platform for the detection assay, such as the funnel-adapted sensing tube (FAST)[157] or a digital multiplex dRPA chip[158]. Also, we could simplify the operation process by introducing a one-pot RPA and CRISPR assay[159]. By continuously optimizing our nano-sieve device and CRISPR assays, a higher sensitivity of our designed system could be achieved for a rapid and multiplexing detection of bloodborne and urine traction diseases.

3.6 Conclusion

In conclusion, we have introduced a miniaturized and versatile nano-sieve device with a pneumatically-regulated chamber for the rapid purification and highly concentrated isolation of MRSA from plasma samples. Our simplified and cost-efficient fabrication process, incorporating multiple channels and a 3D beads-stacked microstructure, enables highly efficient capture of MRSA by precisely controlling the flow rate, resulting in a significant concentration of captured bacteria. The integration of this device with RPA and CRISPR-Cas12 assay enhances the detection sensitivity, achieving a lower on-chip detection limit of 100 CFU/mL compared to the off-chip limit of 10^4 CFU/mL. Our sensitive detection method can be completed within a short timeframe of 4 hours under physiological temperature conditions, eliminating the need for centrifugation. The scalability of the nano-sieve device allows for the simultaneous processing of multiple clinical samples and multiplexing detection of different pathogens. By improving the sensitivity and specificity of MRSA detection, our approach holds promise in contributing to better patient outcomes and addressing the challenges posed by antibiotic-resistant bacterial infections.

Chapter 4

Instrument-free Method for Duplex Leishmania Detection

This chapter is adapted from the following publication: Ruonan Peng, FNU Yuqing, Taralyn J Wiggins, Negin Bahadori, Joshua S Rogers, Jacob Waitkus, Yun Chen, Gregory J Tobin, Stephen J Dollery, and Ke Du. Multiple- channel funnel adapted sensing tube (mfast) for the simple and duplex detection of parasites. *ACS sensors*, 10(4):2678–2688, 2025[38].

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4.1 Abstract

Leishmaniasis poses a significant global health threat, infecting millions of people annually, particularly in tropical and subtropical regions. Timely and accurate detection of *Leishmania* species is crucial for effective treatment and control of this debilitating disease. This study introduces the Multichannel Funnel Adapted Sensing Tube (MFAST) chip, a simple diagnostic tool designed for the rapid detection of *Leishmania panamensis*. MFAST is fabricated through 3D printing and sacrifice butadiene styrene (ABS) and the reagents are transported between reservoirs by gravity. We combine experiments and finite element analysis to facilitate smoother fluid flow, improving the overall performance of the device. Highly sensitive and specific RPA-CRISPR/Cas12a assay is utilized in the chip, achieving a detection limit as low as 1,000 parasites/mL (detecting as few as 5 parasites per reaction). The multi-channel design enables duplex detection, allowing for simultaneous identification of both *L. braziliensis* and *L. panamensis* through distinct channels. Furthermore, stability tests indicate that lyophilized reagents retain functionality for up to 15 days when stored at 4 °C, underscoring the potential of this chip for practical diagnostic applications in low resource settings.

4.2 Introduction

Leishmaniasis ranks second only to malaria among parasitic diseases in terms of mortality[160]. It is caused by the *Leishmania* parasite, a protozoan transmitted to humans through the bite of infected phlebotomine sandflies. The transmission occurs when a sandfly bites an infected person or animal and then bites another individual, injecting

them with the *Leishmania* parasite[161, 162]. *Leishmania* is a genus in the family Trypanosomatidae, comprising over 20 species known to cause disease in humans[163, 164]. The disease disproportionately affects vulnerable populations, particularly in areas with malnutrition[165], poor housing[166], weakened immune systems[167], and limited health-care access[168]. There are three main forms of leishmaniasis: cutaneous leishmaniasis (CL), visceral leishmaniasis (VL), also known as kala-azar, and mucocutaneous leishmaniasis (ML)[169]. While VL is the most deadly form, ML is more prevalent in the Americas and can cause severe complications, particularly through ulceration of the mucous membranes of the nose, mouth, or throat[170, 171, 172]. Mucocutaneous leishmaniasis rarely heals on its own and, if left untreated, can lead to life-threatening complications. Significant facial disfigurement can occur due to the destruction of the oronasopharyngeal mucosa and cartilage in later stages. In severe cases, it can affect the larynx, leading to aspiration pneumonia[173].

Early detection is crucial, especially in resource-limited settings, to enable timely medical intervention and reduce the risk of disease spread[104]. While VL is the most dangerous form of the disease, it can be diagnosed using a combination of clinical signs and parasitological or serological tests[174]. However, serological tests cannot distinguish between active and prior infection[169] and have limited value in diagnosing CL and ML due to poor antibody responses in these cases[175]. For CL and ML, diagnosis largely relies on clinical symptoms and parasitological tests, which confirm the presence of the parasite in affected tissues[176]. These tests, however, often face challenges due to limited specificity or sensitivity[177]. Nucleic acid-based approaches, such as polymerase chain

reaction (PCR), offer high sensitivity but require advanced equipment and precise thermal cycling, making them less viable for point-of-care (POC) diagnostics, especially in resource-limited settings[62].

In recent years, clustered regularly interspaced short palindromic repeats (CRISPR)-Cas 12a system has gained recognition as a promising tool for *in vitro* diagnostics, combining precision and simplicity in the detection of specific DNA sequences[178]. This system employs a guide RNA (crRNA) to recognize and cleave target DNA, a process known as *cis-cleavage*. Upon activation, it also engages in *trans-cleavage*, where it indiscriminately cuts nearby single-stranded DNA (ssDNA)[179]. This property is utilized in diagnostic assays by using ssDNA reporters tagged with a fluorophore and quencher. When Cas12a cleaves the reporter, fluorescence is generated, indicating the presence of the target DNA[180]. Unlike traditional PCR, which relies on precise thermal cycling, Cas12a operates at a constant temperature of 37 °C[181], making it ideal for point-of-care (POC) applications where rapid, cost-effective, and portable diagnostics are crucial[182, 183].

To enhance sensitivity, CRISPR-Cas12a is often coupled with isothermal amplification methods such as recombinase polymerase amplification (RPA), which can be performed at the same temperature[1]. RPA enables the rapid amplification of target DNA through the concerted actions of recombinases, single strand binding (SSB) proteins, and DNA polymerases. The process begins when recombinases guide primers to the target DNA sequence. SSB proteins stabilize the unwound DNA strands, while DNA polymerases extend the primers along the template DNA in the presence of deoxynucleotide triphosphates (dNTPs), facilitating the synthesis of new DNA strands[184]. The RPA-CRISPR two-step

process requires sequential handling to prevent premature degradation of amplified DNA and it presents practical challenges. The requirement for manual pipetting between steps increases the risk of aerosol contamination, which can lead to false positives and complicate the workflow[185]. Our lab previously developed a funnel adapted sensing tube (FAST) chip that combines RPA and CRISPR reaction for power-free, pipette-free, and sensitive detection of SARS-CoV-2[157]. In the FAST chip, RPA and CRISPR reagents are stored in separate chambers, isolated by simple valves, with the reaction initiated by simply pulling the rods. This system achieved a high sensitivity of approximately 10 fM; however, the original FAST chip was single-channel did not include any negative controls on the same chip.

In this study, we introduce an all-in-one Multi-channel Funnel Adapted Sensing Tube (MFAST) chip for the duplex detection of *Leishmania* species and specific detection of species that cause mucocutaneous leishmaniasis, such as *L. panamensis*, without cross-contamination. To achieve this, we perform vapor smoothing of a 3D-printed Acrylonitrile butadiene styrene (ABS) core, which increases liquid transport efficiency by approximately 10%, and the application of NeverWet coating on the valves, which reduces leakage rates by over 20%. Additionally, plasma treatment of the inner surfaces further enhances hydrophilicity, allowing smoother fluid transport through gravity. The multi-channel design also enables simultaneous negative and positive control reactions within the same chip, reducing the likelihood of false positives and improving diagnostic reliability. The chip demonstrates the ability to detect *Leishmania* species with a sensitivity as low as 1,000 parasites/mL (detecting as few as 5 parasites per reaction), enabled by a highly specific

RPA-CRISPR/Cas12a assay. By integrating a portable blue light transilluminator, this chip is operated without using pipettes, significantly simplifying on-site diagnostics. The duplex testing capability further enhances efficiency by minimizing reagent consumption and enabling the simultaneous detection of multiple species. This versatile, low-cost platform is well-suited for point-of-care (POC) diagnosis of parasites, particularly in low-resource settings.

4.3 Materials and Methods

4.3.1 Fabrication of MFAST chip

The 3D model of the MFAST chip core was designed using SolidWorks. It was fabricated using fused deposition modeling X1 Carbon 3D printer (Bambu Lab) with PolyLite™ ABS (Polymaker) material. The printed core was then suspended in the vapor smoothing chamber with a copper wire for two and a half min to remove the layer lines. The vapor smoothing chamber consists of a tall glass jar with approximately 1 cm of 100% acetone covering the bottom and was heated at 90 °C. During this period, the vaped acetone would dissolve the surface of the printed ABS core therefore removing the layer line and creating a glossy finish, as shown in Figure 4.2a. The hooks, designed for hanging by the wire, were snipped off after the smoothing process. It was then glued on the 60 x 15 mm petri dish with super glue (Loctite). The 10:1 ratio mixture of the Polydimethylsiloxane (SYLGARD™ 184 Silicone Elastomer Kit base) and curing agent was poured into the petri dish and filled to the top. It was then placed at room temperature for 2 days to degas before being placed into an oven at 70 °C for 4 hours. After curing, the MFAST chip was

submerged with acetone in an ultrasonic cleaner until the ABS core was fully dissolved. The valves of the MFAST chip were 3D printed using UltraCraft Reflex 3D Printer combo (HeyGears) with UltraPrint-Modeling PAT10 Transparent Resin (HeyGears). Then, the surface of the valve was coated with NeverWet (Rust-Oleum, No. 274232). The valves were left to dry for 30 min in between the two coating steps. Both steps were applied from approximately 20 cm away, and then air dried at room temperature overnight before use.

4.3.2 Surface treatment and quantification

The transport efficiency of the chip was measured by adding 100 μL of food dye into the chambers in the second row and then extracting the food dye from the third row after it had been transported and repeated 6 times. The weight of the food dye was measured by using an electronic balance. The leak test was performed by adding 100 μL of food dye into the second row, closing all the valves and then shaking it 5 times by hand. Then, the liquid remaining in the second row was extracted and measured. The water contact angle of the inner chamber was measured by creating a large flat cavity surface using the same process as the MFAST chip with ABS sacrificial molding. It was then cut into size for the water contact angle testing. The plasma treatment of the MFAST chip was performed by placing it into an oxygen plasma chamber for 10 min with 100 W RF power, 20 sccm O_2 , and 0.6 mBar pressure.

4.3.3 Two-phase flow simulation

The simulation investigates a two-phase flow involving water as the continuous phase and air as the dispersed phase, using COMSOL Multiphysics. The primary objective

is to model the interaction between the dispersed air phase and the continuous water phase in a controlled geometry. The Laminar Flow interface uses the Navier-Stokes equation for incompressible fluid flow, ensuring that the velocity and pressure fields of the continuous water phase are accurately simulated. The Phase Transport interface models the behavior of the dispersed air phase, starting from a closed valve configuration filled with air and transitioning to an open valve, allowing water to flow. Boundary conditions include an inlet velocity defined by a step function, allowing a gradual increase in velocity from 0 to 0.01 m/s to simulate valve opening. The initial conditions set the water as uniformly distributed, with air present at a volume fraction of 0.8, representing the initial state with closed valves. The simulation explores different contact angles (30°, 100°, and 150°) to observe changes in the volume fraction and velocity fields.

4.3.4 Parasite culture and lysis

The following *Leishmania* species were purchased from BEI Resources:

- *L. panamensis*, strain PSC-1 (MHOM/PA/94/PSC 1), NR-50162;
- *L. braziliensis*, strain HOM/BR/75/M2903, NR 50608;
- *L. gerbelli*, strain RHO/CN/62/20, NR-50601;
- *L. infantum*, strain HOM/CN/93/KXG-LIU, NR 50605;
- *L. tropica*, strain HOM/TR/99/EP41, NR-51828; *L. donovani*, strain HOM/IN/83/AG83, NR-50602;
- *L. venezuelensis*, strain MHOM/VE/80/H-16, NR-29184.

The parasites were cultivated in tissue culture by inoculating thawed samples into T-25 flasks containing Modified M199 medium (Gibco, Ref: 12350-039) supplemented with

10% heat-inactivated fetal bovine serum (Neuromonics, Cat. No. FBS006) and 10 µg/mL hemin chloride (Millipore Sigma, Ref: 3741-5GM) at 25°C, and subsequently expanded into T-182 flasks. Parasite density was determined using a hemocytometer and adjusted to 5×10^6 parasites/mL, followed by pelleting at $2,000 \times g$ and washing with $1 \times$ PBS. Next, 1 mL of 0.1% Triton X-100 in PBS was added, and the mixture was incubated at 56°C for 10 minutes.

4.3.5 RPA amplification and CRISPR-Cas12a detection

The TwistAmp® Basic kit was obtained from TwistDx Ltd. NEBuffer™ r2.1 was purchased from New England Biolabs. The RPA primers, fluorophore-quencher (F-Q) probe, crRNA and AsCas12a (Alt-R™ A.s. Cas12a Ultra) were purchased from Integrated DNA Technologies, with detailed information about the synthetic oligonucleotides provided in Table S1. The DNA sequence of different *Leishmania* species were aligned using clustalW and Bioedit, and the RPA primer sets were designed using the Primer 3 program. The crRNAs were designed using CRISPOR.

The target mixture was prepared by combining 5 µL of target with 21.25 µL nuclease-free water and 3.75 µL of magnesium acetate (280 mM). To prepare RPA mixture, 4.8 µL of RPA primers (10 µM) were combined with 59 µL of primer free rehydration buffer, and the TwistAmp® Basic reaction pellet was dissolved in the mixture. For the CRISPR mixture, 7.5 µL of crRNA (1 µM) was mixed with 6 µL of Cas12a (1 µM), and the complex was then equilibrated at room temperature for 10 min. Subsequently, 12 µL of NEBuffer™ r2.1 (10×) and 1 µL of ssDNA probe (100 µM) were added to complete the CRISPR mixture.

The target mixture was added to RPA mixture, where the magnesium acetate contained in the target mixture initiated the RPA reaction. The reaction was incubated at 37 °C for 20 min. Following this, the mixture was added to the pre-assembled CRISPR mixture and incubated at 37 °C for 30 min.

4.3.6 *Leishmania* detection with MFAST

The CRISPR mixture was pipetted into the bottom chamber, and valves were inserted into bottom gaps to seal the CRISPR reagents. Subsequently, the RPA mixture was introduced to the second chamber, and the valves were inserted into the corresponding gaps to seal the RPA reagents. The target mixture was then introduced to the top chamber and sealed with valves. For negatives controls, either the RPA primers or the crRNAs were replaced with nuclease-free water. To initiate the reaction, the second row of valves was pulled to allow the target mixture to flow into the RPA chamber. The MFAST chip was then incubated on a heating pad at 37 °C for 20 min. Following this incubation, the bottom valves were pulled to allow the mixture to flow into the CRISPR chamber, which was incubated at 37 °C for an additional 30 min. A blue light transilluminator (SmartBlue, Part No: NEB-E4100, excitation wavelength of 465 nm) was subsequently used to excite the mixture for naked-eye observation. Finally, 20 µL of nuclease-free water was added to 5 µL of the mixture, and the resulting solution was characterized using an Agilent BioTek Cytation 5 imaging reader.

4.3.7 Lyophilization

For the off-chip assay, given that the RPA enzymatic pellets were provided in a lyophilized form, the lyoprotectant dextran and/or sucrose was incorporated into the CRISPR reaction prior to transferring the mixture to PCR strip tubes, each with a small hole punctured in the cap. When using a 384-well plate, a PCR sealing film (Thermo Fisher Scientific) was applied over the wells, with a small hole punctured in each well. The RPA and CRISPR reactions were subsequently pre-frozen and freeze-dried in a freeze dryer (Harvest Right, Model No: HRFD-SML-BK) overnight. For the on-chip lyophilization, following the loading of reagents into the respective chambers, the chip was laid flat with the valves slightly open to leave a gap, and then pre-frozen and freeze-dried in a freeze dryer overnight.

4.4 Results

The fabrication process of the MFAST chip is illustrated in **Figure 4.1a**. The chip core was 3D-printed using ABS material. To create a smooth surface, the printed core was suspended in a vapor chamber with heated acetone, which dissolved surface imperfections and produced a glossy finish, enhancing the clarity of virtualization. The smoothed core was then attached to a petri dish, and a mixture of polydimethylsiloxane (PDMS) and curing agent was poured into the mold. Once cured, the chip was submerged in acetone, dissolving the ABS core and leaving behind the final microfluidic structure. **Figure 4.1b** shows a photograph of a fabricated MFAST chip filled with food dye, which is distinctly

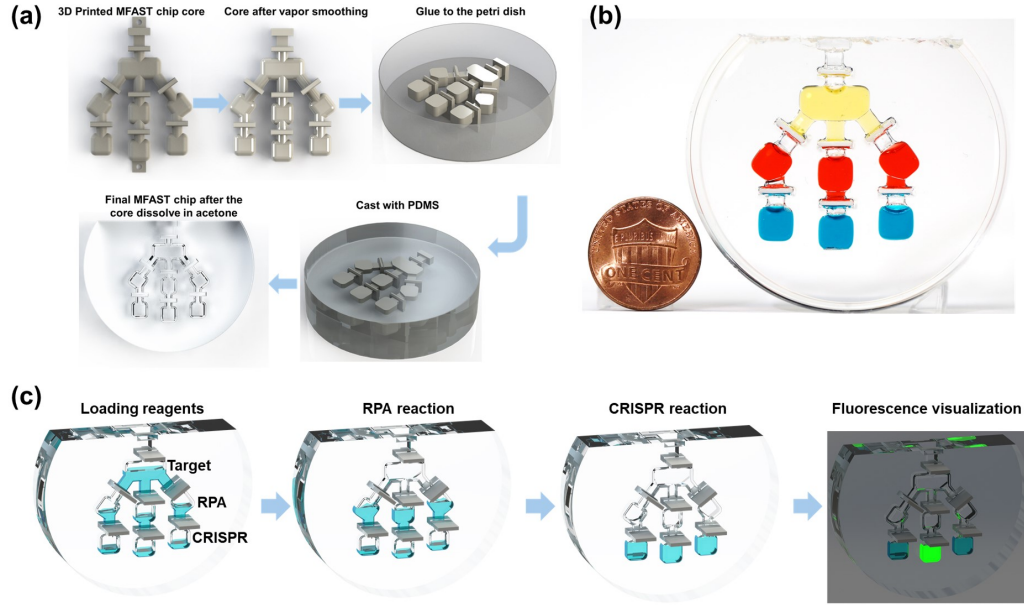


Figure 4.1: Schematic of the MFAST chip. (a) Fabrication process of MFAST chip. (b) Photograph of MFAST filled with food dye with a US one-cent coin for scale. (c) Working principle of the MFAST for visual detection of *Leishmania*.

observable to the naked eye. The chambers are delineated by 3D-printed rectangle piece that serve as functional valves. The volumes of the chambers, from top to bottom, are around 338 μL , 160 μL and 160 μL , respectively. The corners of the chambers are rounded to minimize dead volume, thereby enhancing the efficiency of reagent flow. This design modification facilitates smoother transport of reagents, ultimately improving the overall operational efficiency of the chip.

Figure 4.1c illustrates the visual detection process employed by the self-contained MFAST chip. Prior to initiating the reaction, RPA and CRISPR reagents were systematically loaded into the designated chambers of the MFAST chip. Specifically, 26.5 μL of the CRISPR-Cas12a complex was introduced into the bottom chambers, while a total of 68.6 μL of a mixture containing primers and a rehydrated TwistAmp[®] basic reaction pellet

was loaded into the second row of the chamber. The top chamber was filled with 75 μL of a nuclease-free water and magnesium acetate mixture, which is used to activate the RPA reaction. Once 15 μL of the target was added to the top chamber, the chip was prepared for visual detection. The amplification reaction commenced by pulling the top valves, enabling the target to flow downwards and mix with the RPA reagents. After a 20-minute incubation at 37°C on a heating pad, the resulting amplicons were transferred to the CRISPR reaction chamber for an additional incubation of 30 minutes at the same temperature. Following this, a blue light transilluminator was utilized to excite the fluorophore, allowing for naked-eye observation of the reaction results.

After fabricating the MFAST chip, surface treatments were made to optimize its performance, including the application of a NeverWet coating on the valves and vapor smoothing of the 3D-printed core. To demonstrate the leak-prevention capabilities of the NeverWet coating, **Figure 4.2a** shows a food dye test. In the left image, the MFAST chip is loaded with yellow, red, and blue dyes in the top, middle, and bottom chambers, respectively. The valves in the left and middle channels were coated with NeverWet, while the right channel remained uncoated. In the middle image, after opening the second row of side valves and shaking the chip, the yellow and red dyes mixed to form orange color in the coated left channel. In contrast, in the uncoated right channel, the yellow dye leaked into the red, followed by mixing with the green dye. In the right image, after pulling the bottom side valves, the orange dye mixed with the green in the coated left channel. Meanwhile, in the uncoated right channel, all the dyes mixed together. The valves in the middle channel, coated with the NeverWet, prevented any leakage or mixing.

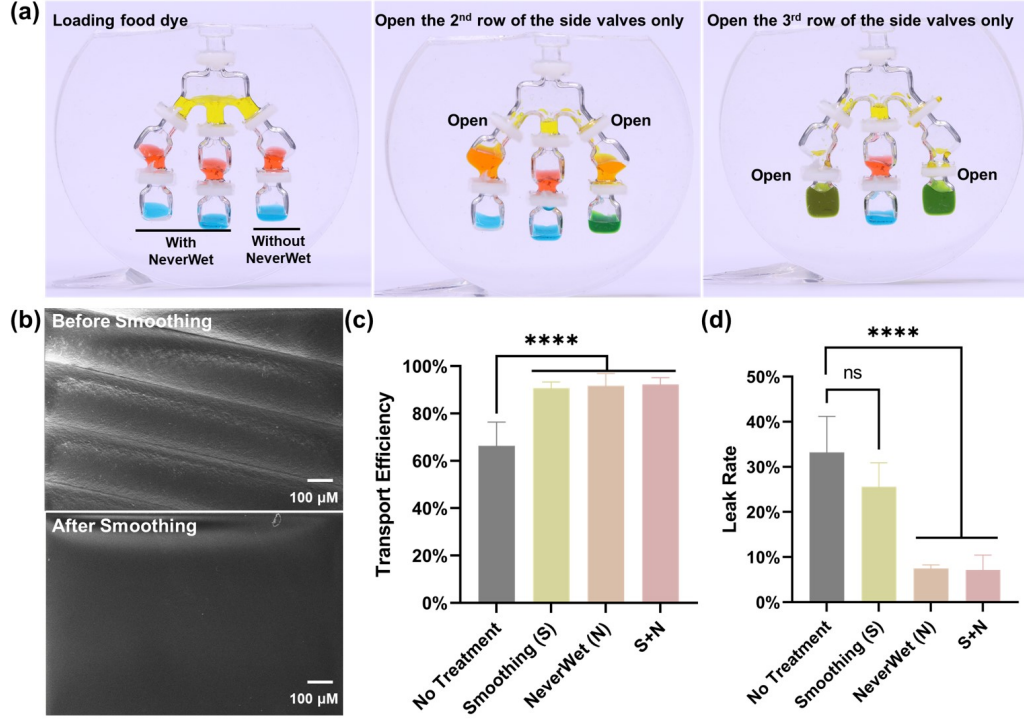


Figure 4.2: Surface treatment and quantification of MFAST chip. (a) Food dye test evaluating the leak-prevention capabilities of the NeverWet coating. The left photo illustrates the food dye loading process with all valves closed. The valves in the left and middle channels were coated with NeverWet, while the right channel remained uncoated. The middle photo depicts the mixing of food dye after opening only the second row of side valves while keeping the valves of the middle channel closed. The right photo shows the mixing of food dye after opening only the bottom row of side valves while keeping the valves of the middle channel closed. (b) SEM images of the ABS core before (top) and after (bottom) vapor smoothing. (c) Transport efficiency of the MFAST chip without treatment, with vapor smoothing of the ABS core, with NeverWet coating on the valves, and with both treatments combined. (d) Leak rate of the MFAST chip without treatment, with vapor smoothing of the ABS core, with NeverWet coating on the valves, and with both treatments combined. Statistical analyses were performed using one-way ANOVA analysis. The data are represented as mean $\pm$ standard deviation ($n = 3$). *ns*, not significant = $p > 0.05$; * = $0.01 < p \leq 0.05$; ** = $0.001 < p \leq 0.01$; **** = $p \leq 0.0001$.

Clear visualization of the reactions is critical for accurate result interpretation, as any surface imperfections can scatter light or cause distortions, ultimately reducing the clarity of the output. The vapor smoothing eliminates the layer lines formed during the 3D printing process and result in a glossy finish on the core surface. This process transformed the replicated PDMS surface from translucent to transparent, which improved visibility for users to observe the status of the chambers. As shown in **Figure 4.2b**, evaluated under SEM, vapor smoothing not only improved the visual appearance but also enhanced the performance of the MFAST chip. **Figure 4.2c** shows that this process increased the transport efficiency of liquids by reducing the layer lines that could trap fluid during transport. The efficiency of liquid transport was tested by measuring the amount of food dye transferred from the second row of the MFAST chip to the third row. Results demonstrated that vapor smoothing improved transport efficiency by approximately 10%. The NeverWet coating, by creating a superhydrophobic surface, reduced liquid adhesion to the valve and repelled fluid trapped in the intricate design of the valves. In this test, food dye was added to the second row of the chip, and the amount of remaining dye was measured after shaking the chip five times with all valves closed. The results, shown in **Figure 4.2d**, revealed that the NeverWet coating reduced leakage by more than 20%, providing significant protection against leaks. While vapor smoothing had a less substantial effect on leak prevention, it still provided slight improvements in the absence of NeverWet.

To improve liquid transfer, the water contact angle of the inner MFAST chip chamber was altered. Our simulation, as shown in **Figure 4.3a**, illustrates that making the inner surface of the MFAST chip more hydrophilic, by reducing the contact angle

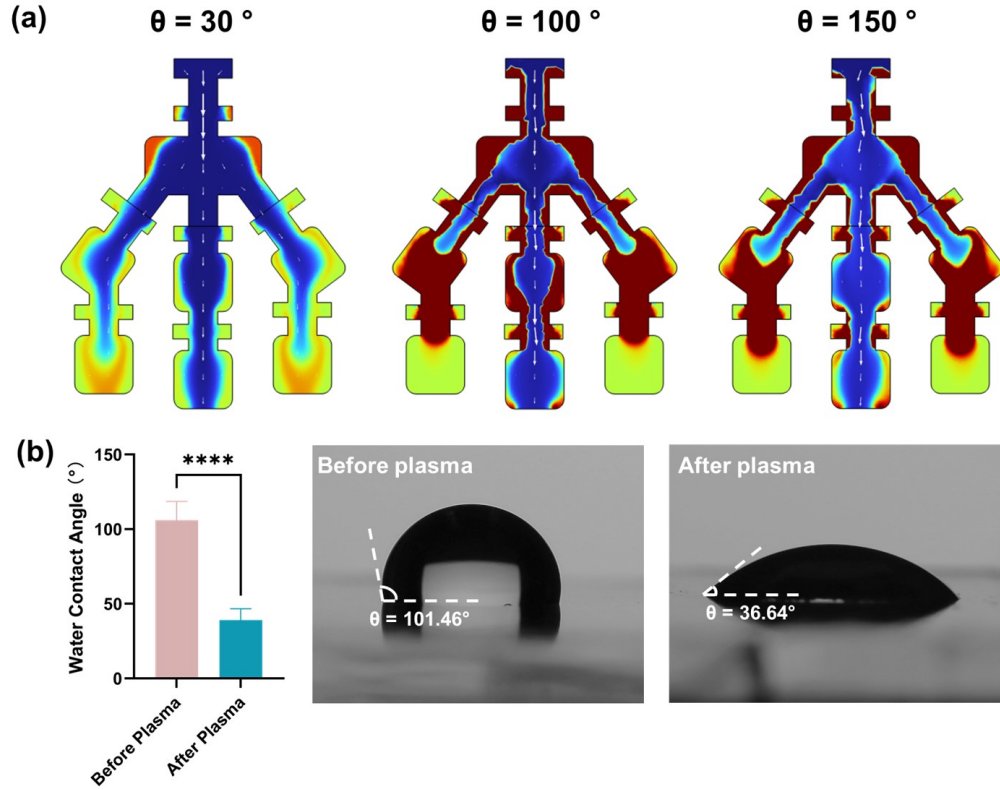


Figure 4.3: Liquid flow simulation and effect of plasma treatment on wettability. (a) Simulation results of volume fraction showing the effect of varying surface wettability at time = 6 s. (b) Static contact angles of MFAST chip before (left) and after (right) plasma treatment. Statistical analyses were performed using unpaired t -test analysis. The data are represented as mean $\pm$ standard deviation ($n = 3$). ns , not significant = $p > 0.05$; $*$ = $0.01 < p \leq 0.05$; $**$ = $0.001 < p \leq 0.01$; $****$ = $p \leq 0.0001$.

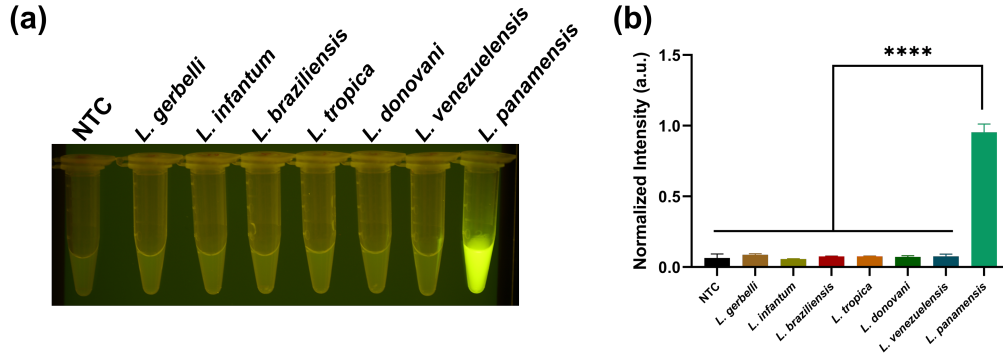


Figure 4.4: (a) Endpoint images of the reactions with 1×10^6 parasites/mL of different *Leishmania* strains. Fluorescence was excited by a transilluminator (wavelength: 465 nm) (b) Normalized fluorescence signals of the reactions with 1×10^6 parasites/mL of different *Leishmania* strains. Statistical analyses were performed using one-way ANOVA analysis.

with water to 30 degrees, results in a more efficient flow pattern. With that in mind, oxygen plasma treatment was used to decrease the water contact angle of the MFAST chip's inner surface[186]. As shown in **Figure 4.3b**, the water contact angle of the inner cavity surface dropped from 106.05° to 39.09° after the plasma treatment. We found that the transportation of the fluid to the subsequent chamber became much easier after the plasma treatment where only a little tap was required.

The specificity and sensitivity of the RPA-CRISPR assay for the detection of *L. panamensis* were subsequently evaluated. To assess specificity, fluorescence was compared between *L. panamensis* at a concentration of 1×10^6 parasites/mL and six other *Leishmania* strains, namely *L. braziliensis*, *L. gerbelli*, *L. infantum*, *L. tropica*, *L. donovani*, and *L. venezuelensis*, as well as a non-template control (NTC). The endpoint fluorescence images of the reactions are shown in **Figure 4.4a**. Upon excitation with a blue light transilluminator, the fluorescence differences between *L. panamensis* and the other groups were

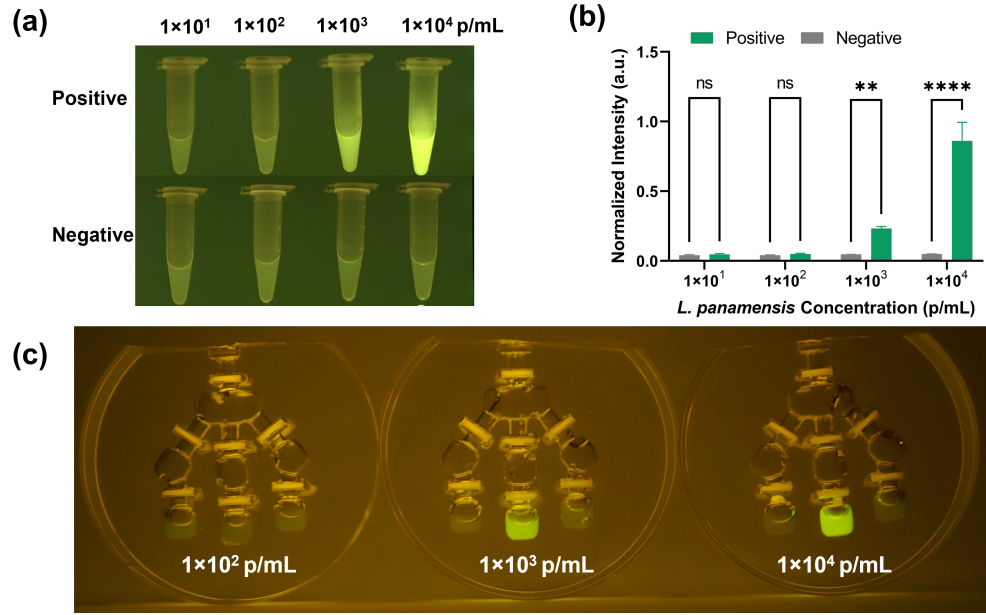


Figure 4.5: (a) Endpoint images of the reactions excited by a transilluminator (excitation wavelength: 465 nm) in response to different *L. panamensis* concentrations ranging from 10 to 1×10^4 parasites/mL. (b) Normalized fluorescence signals of the reactions with different *L. panamensis* concentrations ranging from 10 to 1×10^4 parasites/mL. Statistical analyses were performed using two-way ANOVA analysis. (c) Endpoint images of the on-chip reactions excited by a transilluminator (excitation wavelength: 465 nm) in response to different *L. panamensis* concentrations ranging from 100 to 1×10^4 parasites/mL. “NTC” refers to no template control. The data are represented as mean $\pm$ standard deviation ($n = 3$). ns, not significant = $p > 0.05$; * = $0.01 < p \leq 0.05$; ** = $0.001 < p \leq 0.01$; **** = $p \leq 0.0001$.

clearly distinguishable by the naked eye. The normalized fluorescence signal is presented in **Figure 4.4b**, indicating that the fluorescence intensity of the six non-target strains and the NTC group was significantly lower than that of *L. panamensis*, demonstrating the high specificity of the RPA-CRISPR assay. To determine the detection limit, different concentrations of *L. panamensis* were used as targets and added to the RPA mixture. Following the RPA reaction, the amplicons were introduced into a pre-mixed CRISPR reaction and incubated at 37°C for 30 minutes. For the negative control groups, either the RPA primers or the crRNAs were replaced with nuclease-free water. The endpoint fluorescence images of the reaction are shown in **Figure 4.5a**, with the corresponding normalized fluorescence signals presented in **Figure 4.5b**. Distinct fluorescence differences were observed at concentrations of 1×10^3 parasites/mL (corresponding to DNA from 5 parasites) and 1×10^4 parasites/mL (corresponding to DNA from 50 parasites). However, no significant fluorescence difference between positive and negative groups was detected at a concentration of 1×10^2 parasites/mL (corresponding to DNA from 0.5 parasites) or lower. The on-chip detection results are presented in **Figure 4.5c**. The left channel represents the negative group without RPA primers, while the right channel represents the positive group without crRNAs. Compared to the negative groups, the positive group exhibited a significantly stronger fluorescence signal at a concentration of 1×10^3 parasites/mL (as few as 5 parasites per reaction), demonstrating consistency with the off-chip results.

Since the chip was designed with multiple channels, it can be applied for duplex detection by loading two sets of RPA primers and crRNAs targeting different *Leishmania* species. One channel was used for the detection of all seven *Leishmania* species, while another channel was specific for *L. panamensis*, which is associated with mucocutaneous

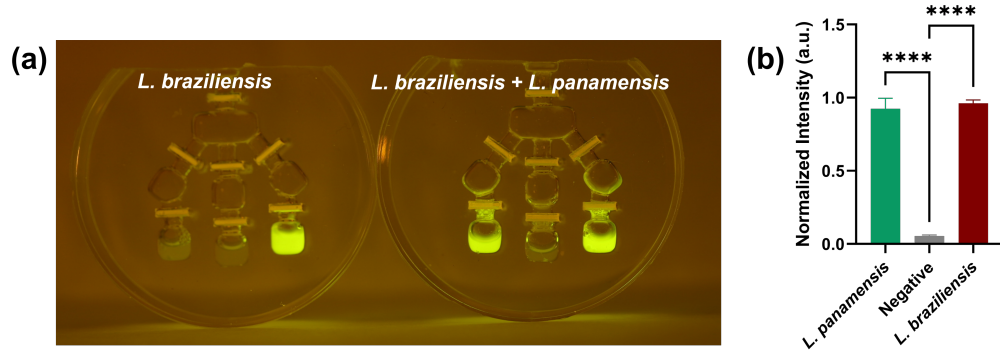


Figure 4.6: (a) Endpoint images of the on-chip reaction with 1×10^6 parasites/mL of *L. braziliensis* (left) and a 1:1 mixture of *L. panamensis* and *L. braziliensis*. In the left image, the two left channels represent negative controls. In the right image, the middle channel serves as a negative control. Fluorescence was excited by a transilluminator (wavelength: 465 nm) (b) Normalized fluorescence signals from the reactions with 1×10^6 parasites/mL of a 1:1 mixture of *L. panamensis* and *L. braziliensis*. Statistical analyses were performed using one-way ANOVA analysis. The data are represented as mean $\pm$ standard deviation ($n = 3$). **** = $p \leq 0.0001$.

diseases. The third channel served as a negative control without RPA primers. Among the seven species, *L. braziliensis* was used as the sole target, with the CRISPR and RPA reagents for detecting all seven species loaded into the right channel, while the left two channels served as negative controls. The results presented in **Figure 4.6a** (left) indicate that the positive group generated noticeably stronger fluorescence compared to the negative controls, demonstrating effective detection of the target without cross-contamination and false-positive results. When a 1:1 mixture of *L. panamensis* and *L. braziliensis* was analyzed, with the RPA and CRISPR reagents specific to *L. panamensis* loaded into the left channel of the MFAST chip. As shown in **Figure 4.6a** (right), both the left and right channels displayed strong green fluorescence, whereas the middle negative group did not, thereby confirming the successful duplex detection of both species. The quantitative normalized fluorescence intensity in **Figure 4.6b** further supports these findings, showing that the

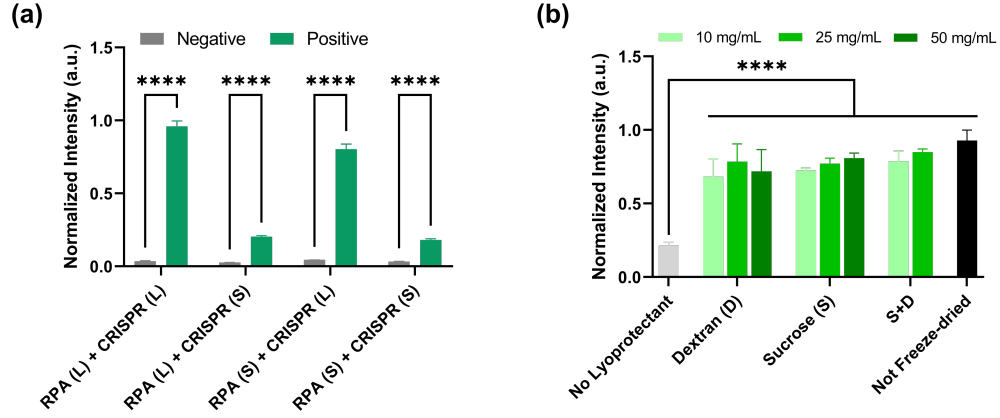


Figure 4.7: (a) Impact of lyophilization on the performance of RPA and CRISPR reactions. (b) Performance of lyophilized reactions supplemented with dextran and/or sucrose at concentrations of 10, 25, and 50 mg/mL. Statistical analyses for lyophilization results were performed using two-way ANOVA analysis. The data are represented as mean $\pm$ standard deviation ($n = 3$). **** = $p \leq 0.0001$.

Leishmania groups exhibited considerably higher fluorescence intensity compared to the negative control.

To enhance the storage stability[187], ease of transportation[188], and overall usability of the MFAST chip, the reagents were subjected to lyophilization. This process involves initially freezing the reagents, followed by sublimation of water during the primary drying phase. Secondary drying is then conducted to eliminate residual moisture and achieve the target final moisture content[189]. The RPA and CRISPR reagents were lyophilized independently, and their performance was subsequently evaluated and compared. The enzyme pellet in the RPA kit, which was originally supplied in lyophilized form, was added after the RPA reagents underwent the freeze-drying process. The input target was 1:1 mixture of *L. panamensis* and *L. braziliensis* with the concentration of 1×10^6 parasites/mL. The results, as shown in **Figure 4.7a**, demonstrate that the fluorescence

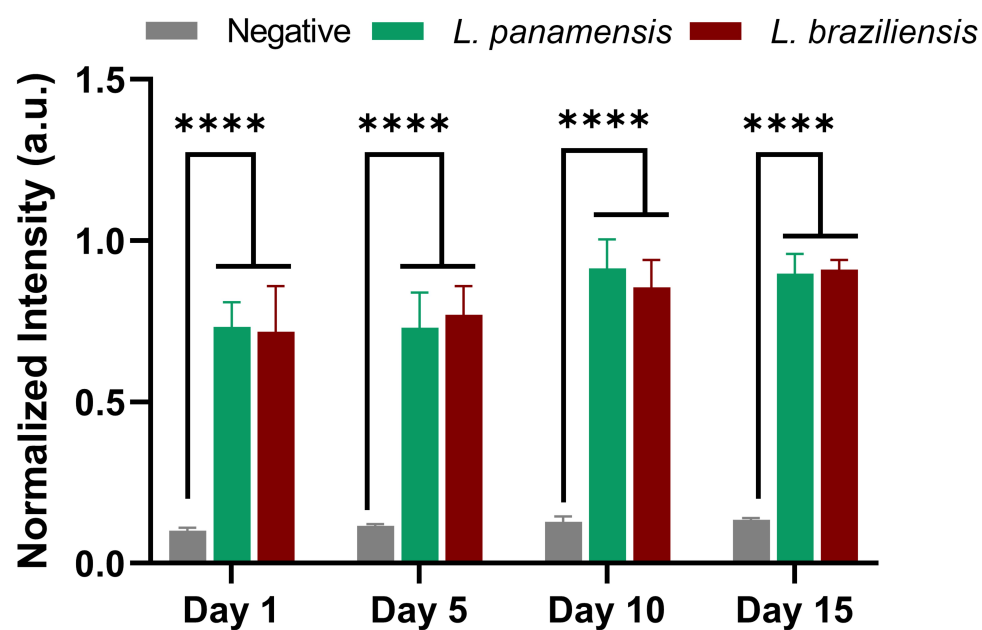


Figure 4.8: Stability test of lyophilized reactions stored at 4 °C. Statistical analyses for lyophilization results were performed using two-way ANOVA analysis. The data are represented as mean $\pm$ standard deviation ($n = 3$). **** = $p \leq 0.0001$.

intensity generated by the lyophilized solid form (S) was significantly lower than that of the liquid reagents (L), indicating a reduction in assay performance. To protect Cas12a during freeze-drying, stabilizing agents like dextran and/or sucrose were added to maintain its structure and functionality[190]. As shown in **Figure 4.7b**, we found that the activity of reactions increased with sucrose concentration, and dextran exhibited a concentration optimum of 25 mg/mL in the final reaction. Combining 25 mg/mL each of sucrose and dextran exhibited approximately 85% recovery of the prelyophilization signal. Additionally, we assessed the stability of the lyophilized reagents stored at 4 °C. As depicted in **Figure 4.8**, the lyophilized reagents remained functional and stable for up to 15 days.

4.5 Discussion

The freeze-drying process negatively affected the efficiency of both RPA and CRISPR reactions, with a more pronounced reduction observed for CRISPR, likely due to the loss of Cas12a activity during lyophilization. This loss can be attributed to stresses induced during the lyophilization process, including cold denaturation, exposure to ice-water interfaces, and freeze concentration, which can lead to protein unfolding or aggregation. During the drying phases, the removal of water also affects the its structural integrity, potentially causing irreversible denaturation[191]. Excipients such as dextran and sucrose form hydrogen bonds with specific sites on the protein surface, effectively replacing the thermodynamic stabilization role of water that is lost during drying. This process increases the free energy of protein unfolding, thereby preventing denaturation and preserving the native structure, ultimately enhancing storage stability[192].

Compared to previous FAST chips[157], the chip design has changed from one column to three columns for duplex detection. The valve design was changed from a carbon fiber rod to a 3D-printed flat valve with NeverWet coating to enhance the sealing between the chambers so that the FAST chip no longer needs to be cut into two pieces. The material for the MFAST sacrificial core has changed from polyvinyl alcohol (PVA) to acrylonitrile butadiene styrene (ABS) due to its better printability, cheaper material cost, and faster dissolving time[193, 194, 195]. Our new process also improves the visibility of the chip by using the vapor smoothing process to remove the layer lines created from the 3D printing process, which has been proven to be an effective method to polish the surface of the 3D-printed parts[196]. Plasma treatment of the chamber was added to improve the fluid flow between the chambers. Overall, the vapor smoothing, NeverWet coating, and plasma treatment were to improve the usability and reliability of the MFAST chip, which is an important step toward real-world applications.

This multi-channel setup enables both positive and negative results to be observed simultaneously on a single chip by simply adding the sample into the reagent-preloaded system, streamlining the detection process of ML-causing species such as *L. panamensis* and minimizing the risk of false positives. By integrating both positive and negative reactions into a closed system, the chip significantly reduces contamination risks, simplifies handling, and enhances its suitability for rapid, point-of-care diagnostics. Moreover, the duplex detection design allows for the simultaneous detection of different *Leishmania* species within a single run, increasing throughput and shortening assay time compared to sequential testing. This not only conserves reagents by running multiple assays on the same chip, lowering the

overall costs, but also improves result reliability through integrated negative controls for real-time comparison, further reducing the chances of false positives.

Other detection and quantification methods of the parasitic such as microscopy[197], raman spectrometry[198], and quartz crystal microbalance[199] would require trained technicians and expensive equipment. On the other hand, the MFAST device only requires a heating device and a UV flashlight and does not require a lab environment to perform the test allowing it to be used in point-of-care applications. Its lightweight, compact design also makes it ideal for POCT use, while being advantageous for packing and shipping, conserving space, and helping to keep the overall diagnostic cost low. Compared to the traditional optical-based technique, the user can quickly determine the result based on the fluorescence signal without the need for a detection device or training[200, 201].

Using the 3D printing method to produce the sacrificial mold could give the designer more flexibility when designing the mold and a faster turnout time for smaller test batches. The 3D-printed coarse surface could be compensated with the vapor smoothing process, but the dimension accuracy would be compromised. However, this process is capable of switching the 3D-printed sacrificial core with an injection-molded sacrificial core which would dramatically decrease the production time of the core and eliminate the need for a vapor smoothing process while maintaining the dimension accuracy. Because it is using the sacrificial molding method, it would be difficult to scale up the production to be comparable to the injection molding method. However, the advantage of this method was that it could create features that the injection molding cannot produce, no limitation of draft angles, or cannot produce in single piece parts, which would require additional processes to

assemble. By altering the water contact angle of the inner surface of the MFAST chamber with plasma treatment, the flow property was changed. However, the plasma treatment is not permanent. Its effect degrades over time and would cause issues for long-term storage. In the future, the inner surface would include microstructures, such as the super hydrophilic honeycomb structure[202], to achieve long lasting hydrophilicity to give the FAST chip a longer shelf life. During the testing of the chip, it was observed that the distribution of the liquid when transporting from the top row of the chamber to the middle row was not even, where the middle column would have more liquid than the other two columns. In the future, the diameter of the channel could be changed with the help of simulation to produce an even distribution of the liquid across all the chambers and allowing it for more than three columns.

4.6 Conclusion

We developed an all-in-one Multi-channel Funnel Adapted Sensing Tube (MFAST) chip using sacrificial molding for *in vitro* detection of species that cause mucosal leishmaniasis such as *L. panamensis*. Several surface treatments were applied to enhance its performance. Vapor smoothing of the ABS core improved liquid transport efficiency by 10%, while coating the valves with NeverWet reduced leakage by over 20%. Additionally, plasma treatment was performed on the inner surfaces of the MFAST chip to increase hydrophilicity, facilitating smoother liquid flow, as demonstrated in simulation results. For detection, we employed the RPA-CRISPR/Cas12a assay, which showed high specificity for *L. panamensis* compared to other *Leishmania* strains, along with a sensitivity of 1,000 par-

asites/mL (as few as 5 parasites per reaction). To expand its diagnostic capabilities, the multi-channel design of MFAST enabled duplex detection by incorporating distinct primers and crRNAs in separate channels. This successfully achieved simultaneous detection of *L. braziliensis* and *L. panamensis* without cross-contamination. Furthermore, we tested the stability of lyophilized reagents preloaded in the chip. With excipients of 25 mg/mL of sucrose and dextran, the lyophilized reagents remained functional and stable for up to 15 days. The MFAST chip demonstrates a promising platform for leishmaniasis diagnosis, offering potential for multiplexing and reagent stability in field settings.

Chapter 5

Advancing One-Pot CRISPR Diagnostics with Engineered Microstructures

This chapter is based on a preprint:

Ruonan Peng, Zhenxuan Yuan, Demis D. John, Biljana Stamenic, Fatt Foong, Bhaskar Sharma, FNU Yuqing, Chuqing Zheng, Jacob Waitkus, Ke Du. Silicon Micropillar-Enhanced CRISPR Biosensor for Rapid and Sensitive Detection of Drug-Resistant Bacteria. *bioRxiv* 2025.12.15.694528. doi: 10.64898/2025.12.15.694528

5.1 Abstract

The growing threat of antibiotic-resistant pathogens, such as methicillin-resistant *Staphylococcus aureus* (MRSA), underscores the urgent need for rapid, sensitive, and field-deployable diagnostic technologies. Here, we present a silicon micropillar-enhanced CRISPR biosensor that integrates high-aspect-ratio microstructures with a one-pot RPA/CRISPR-Cas12a assay for ultrasensitive and specific detection of MRSA. Micropillar arrays with fixed diameters and varying heights (100 μm , 300 μm , and 500 μm) were fabricated via deep reactive ion etching and functionalized for surface probe immobilization. Suboptimal crRNA design was employed to modify Cas12a activation kinetics, enabling declined trans-cleavage and enhanced end-point signal accumulation. The 500 μm micropillar configuration demonstrated a tenfold improvement in sensitivity compared to the 100 μm array, with a limit of detection reaching 10^3 CFU mL^{-1} . The platform also showed high specificity against non-target bacterial strains. These findings highlight the potential of combining microstructured chips with one-pot CRISPR diagnostics to advance next-generation point-of-care tools for infectious disease monitoring.

5.2 Introduction

The global rise of antibiotic resistance represents an urgent threat to public health, undermining the effectiveness of standard treatments and increasing the incidence of severe and persistent infections[203]. In the United States alone, antibiotic-resistant infections are responsible for an estimated 23,000 deaths annually and contribute to over \$20 billion in additional healthcare costs[204]. Among these drug-resistance pathogens,

methicillin-resistant *Staphylococcus aureus* (MRSA) is particularly concerning due to its resistance to beta-lactam antibiotics and widespread occurrence in both healthcare and community settings[205]. MRSA can cause a variety of infections, including skin and soft tissue infections[206], pneumonia[207], bloodstream infections[208], and surgical site infections[209], many of which can become life-threatening if not promptly diagnosed and treated[210]. Traditional detection methods, such as bacterial culture and PCR-based assays, often suffer from limitations including long turnaround times, susceptibility to contamination[211], reliance on thermal cycling and specialized laboratory equipment[62]. These challenges have driven growing interest in developing point-of-care diagnostic platforms that are rapid, sensitive, and easy to deploy.

Clustered regularly interspaced short palindromic repeats (CRISPR) and associated (Cas) nucleases, particularly Cas12 and Cas13 nucleases, have emerged as powerful tools for *in vitro* molecular diagnostics due to their programmability[212], high specificity[213], and collateral cleavage activity[214]. Among them, Cas12a has attracted significant attention for DNA detection. It uses a single CRISPR RNA (crRNA) to recognize double-stranded DNA targets adjacent to a specific Protospacer Adjacent Motif (PAM), typically 5'-TTTV-3' (where V = A, C, or G)[215]. Upon target recognition, Cas12a becomes activated and initiates two types of nuclease activity: *cis*-cleavage, which cuts the target DNA at the distal end of PAM[44], and *trans*-cleavage, which enables indiscriminate cleavage of surrounding single-stranded DNA (ssDNA)[216]. This *trans*-cleavage activity forms the foundation of signal amplification in CRISPR-based biosensors, in which cleavage of fluorophore-quencher (FQ) probes releases fluorescence upon activation[217, 104]. Addi-

tionally, Cas12a operates efficiently at 37 °C, making it more compatible with point-of-care (POC) applications than thermal cycling-based PCR methods[135].

To further improve detection sensitivity, the CRISPR-Cas12a system is often coupled with isothermal amplification techniques such as recombinase polymerase amplification (RPA)[38]. RPA amplifies target DNA at a constant temperature through the combined action of recombinases, single-stranded DNA-binding proteins (SSBs), and strand-displacing DNA polymerases. In this process, recombinases facilitate the pairing of primers with complementary target sequences, SSBs stabilize the displaced DNA strands, and DNA polymerases extend the primers in the presence of deoxynucleotide triphosphates (dNTPs), resulting in rapid and efficient amplification[184]. While the combination of RPA and CRISPR enables rapid and sensitive detection, conventional two-step workflows, where amplification and detection occur in separate stages, require manual reagent transfers, increasing procedural complexity and the risk of cross-contamination[185]. In attempts to merge the two reactions into a single one-pot assay introduces new challenges. Cas12a may be activated prematurely, leading to early cleavage of RPA amplicons before sufficient amplification occurs. This reduces the overall template concentration and limits signal output[218, 219]. Moreover, FQ probes inherently exhibit background fluorescence due to incomplete quenching. Excess probe concentrations can increase this baseline signal, reducing signal-to-noise ratio and ultimately limiting detection sensitivity[220].

Microfabricated platforms with micro- and nanostructured features have played a pivotal role in advancing biosensing and biological applications by enabling enhanced sensitivity[221], reduced sample volumes[222, 223, 224], and overall device miniaturization[225,

226, 227, 228, 229]. Among these, micropillar arrays have attracted considerable interest due to their ability to significantly increase the available surface area for probe immobilization, thereby enhancing target capture efficiency and reaction kinetics[230]. Several recent studies have implemented micropillar-based diagnostics for nucleic acid detection utilizing fluorescence[231], electrochemical[232], or colorimetric readouts[233]. Yet, few of these systems incorporate CRISPR, and even fewer combine CRISPR with upstream isothermal amplification in a one-pot format. Integration of one-pot RPA/CRISPR assays into microstructured platforms remains underexplored, primarily due to challenges such as synchronizing amplification and detection kinetics within confined microenvironments, achieving stable probe immobilization, and maintaining reagent compatibility. Overcoming these hurdles would unlock the full potential of CRISPR diagnostics on chip-based systems, enabling portable, sensitive, and field-deployable biosensors.

In this study, we present a silicon micropillar-enhanced CRISPR biosensor designed for the rapid, sensitive, and specific detection of MRSA. The platform leverages high-density surface probe immobilization combined with a one-pot RPA/CRISPR-Cas12a reaction to streamline assay workflows and minimize contamination risk. To enhance the probe binding capacity and improve signal generation, we fabricated high-aspect-ratio silicon micropillars using deep reactive ion etching, with structures featuring fixed diameters and heights of 100 μm , 300 μm , and 500 μm . We demonstrate that increased pillar height correlates with significantly higher probe immobilization, enabling more efficient detection. Furthermore, by designing suboptimal crRNAs to modulate Cas12a activation kinetics, we achieved improved signal amplification and specificity. The optimized 500 μm micropillar

chip demonstrated a tenfold improvement in sensitivity compared to the 100 μm configuration, highlighting the strong link between device geometry and assay performance. These findings underscore the promise of integrating microfabricated platforms with CRISPR-Cas diagnostics to advance next-generation molecular detection systems for infectious disease monitoring.

5.3 Materials and Methods

5.3.1 Device fabrication

< 100 > silicon wafers with an initial thickness of 1025 μm (University Wafer; dopant type and resistivity intentionally unspecified) were used for chip fabrication. To enable deep silicon etching, a dual-layer hardmask consisting of 150 nm of Al_2O_3 and a 2–3 nm SiO_2 capping layer was deposited using a Veeco Nexus IBD-O Ion Beam-Assisted Sputter Deposition (IBD) system. Prior to deposition, wafers were cleaned with acetone and isopropanol. Photolithography was performed using around 1.7 μm of nLOF2020 negative photoresist patterned on an I-Line Heidelberg MLA150 direct-write lithography tool. The oxide hardmask layers were subsequently etched using a BCl_3 -based inductively coupled plasma (ICP) process (0.5 Pa chamber pressure, 500 W ICP power, 50 W RF bias, 30 sccm BCl_3 , and 15 °C helium backside cooling). Residual photoresist was removed using sequential NMP treatment at 80 °C, followed by piranha etching and O_2 plasma ashing.

Deep silicon etching was carried out using a three-step Bosch process on a Plasmatherm DSE-III ICP etcher. Etch parameters were optimized via design-of-experiments (DOE) to vary aspect ratios while maintaining consistent pillar diameters. The lithographic

design was fixed at 20 μm pillar diameter with 10 μm spacing. Etch depth was tuned by adjusting the dry etching time to achieve final depths of 100 μm , 300 μm , and 500 μm . Test structures were included on each wafer to characterize depth and sidewall profiles using stylus profilometry and tilted SEM imaging.

After etching, the oxide hardmasks were removed using a combination of buffered HF (BHF) and piranha solution etches. Wafers were then dry oxidized in a Tystar 8300 tube furnace to produce a conformal around 100 nm SiO_2 layer on all exposed silicon surfaces, passivating the micropillar structures. Finally, individual chips were singulated using an ADT 7100 dicing saw. To prevent mechanical damage to the high-aspect-ratio structures during dicing and drying, precautions were taken including reduced water flow and a final isopropyl alcohol rinse to enable gentle air-drying at room temperature.

The container was designed using SolidWorks and 3D printed with a HeyGears Relex 3D printer using Modeling PAT10 Transparent Resin. The printed part was cured in the HeyGears UltraCraft Cure system for 55 minutes at 46 $^{\circ}\text{C}$.

The soft cap of the container was also designed in SolidWorks and fabricated using a syringe-based injection molding process. A Platinum 15A silicone mixture from BBDINO was loaded into a syringe and injected into a 3D-printed mold. The mold was then placed in an oven and cured overnight at 50 $^{\circ}\text{C}$

5.3.2 Surface treatment

The device was immersed and incubated in 10% APTES (MP Biomedicals) dissolved in ethanol for 30 minutes with gentle shaking at 80 rpm. After incubation, it was rinsed three times with ethanol to remove excess APTES, followed by baking on a heating

pad at 120 °C for 30 minutes. The device was then immersed in 2.5% glutaraldehyde (GA) solution (Thermo Scientific, prepared in PBS) and incubated for 1 hour. Afterward, it was rinsed three times with PBS to remove unbound GA and dried using an air gun. Subsequently, 20 μ L of 50 μ M ssDNA probe was added to the device and incubated overnight at 4 °C. The ssDNA-functionalized chip was then washed using PBS for three times, with each wash lasting 10 minutes at 80 rpm. Finally, the chip was dried with an air gun and was ready for one-pot RPA/CRISPR-Cas12a detection.

5.3.3 Bacterial culture and lysis

Methicillin-resistant *Staphylococcus aureus* (ATCC 43300) and *Staphylococcus aureus* (ATCC 25923) were purchased from Fisher Scientific. Drug-resistant *E. coli* DH5 α (Addgene 46012, carrying plasmid pCM184 conferring resistance to ampicillin, tetracycline, and kanamycin) and *E. coli* K12 (ATCC 10798) were sourced from lab stock. All bacterial strains were cultured in tryptic soy broth (MilliporeSigma) and maintained on tryptic soy agar plates. Following overnight incubation at 37 °C with shaking at 200 rpm, 1 mL of culture was centrifuged at 8000 g for 5 min to pellet the cells. The pellet was resuspended in 1 mL of phosphate-buffered saline (PBS), and the washing step was repeated twice to completely remove residual media. Bacterial concentrations were determined by counting colony-forming units (CFU) on standard TSA plates. The washed bacteria were subsequently diluted in PBS. For DNA release, MRSA cells with different concentrations were lysed by heating at 95 °C for 10 minutes.

5.3.4 DNase characterization

RNase-free DNase I (1 U/ μ L) was purchased from Thermo Scientific. The silicon chip was manually diced into four smaller pieces prior to use. The DNase treatment followed the manufacturer's protocol. Each reaction had a total volume of 300 μ L, consisting of 30 μ L of 10 $\times$ reaction buffer with MgCl₂, 30 μ L of DNase I, and 240 μ L of nuclease-free water. For negative group, the DNase I was replaced by nuclease-free water. The chip fragment and the prepared reaction mixture were placed in a 0.6 mL centrifuge tube and incubated at 37 °C for 1 hour. After incubation, the chip was removed, and the fluorescence of the remaining solution was measured using an Agilent BioTek Cytation 5 imaging reader (Ex/Em = 495/520 nm).

Table 5.1: List of synthetic oligos sequence used in this study

Name	Sequence (5'-3')
Forward primer	AAACAAGCAATAGAATCATCAGATAACATTT
Reverse primer	AAGGATCTGTACTGGGTTAATCAGTATTTC
crRNA 1	UAAUUUCUACUAAGUGUAGAUUUGCUAGAGUAGCACUCGAAU
Target 1	AACGATCTCATCGTGAGCTTA
crRNA 2	UAAUUUCUACUAAGUGUAGAUGUUUUUCAUGCCUUUUUCAA
Target 2	CAAAAAAGTACGGAAAAAGTT
FQ probe	/56-FAM/TTATT/3IABkFQ
Cy3-labeled probe	/5AmMC6/iSp18/TTATTGCGTGAAGTTATT/3Cy3Sp/
FAM-labeled probe	/5AmMC6/iSp18/TTATTGCGTGAAGTTATT/36-FAM/

5.3.5 RPA amplification and CRISPR-Cas12a detection

TwistAmp[®] Basic kit was purchased from TwistDx[™]. The RPA primers, crRNA, LbCas12a, and single strand DNA(ssDNA) probes were obtained from Integrated DNA Technologies. Detailed sequences of all synthetic oligonucleotides are listed in **Table. 5.1**. RPA primers were designed using the PrimerQuest[™] Tool. NEBuffer[™] r2.1 was purchased from New England Biolabs. To prepare the CRISPR-Cas12a mixture, 3 μ L of 1 μ M crRNA and 3 μ L of 1 μ M Cas12a were combined and incubated at room temperature for 10 minutes. Subsequently, 3 μ L of 10 $\times$ NEBuffer r2.1 was added. For off-chip assays, 2 μ L of 10 μ M ssDNA-FQ probe was included in the reaction; for on-chip assays, the probe was replaced with nuclease-free water. The RPA reaction was prepared according to the manufacturer's instructions: 29.5 μ L of rehydration buffer, 13.2 μ L of nuclease-free water, and 2.4 μ L each of 10 μ M forward and reverse primers were added to the lyophilized enzyme pellet. Then, 2.5 μ L of 280 mM MgOAc was added to initiate the reaction. For one-pot reactions, 17 μ L of the RPA mixture was added to the prepared CRISPR-Cas12a mixture, followed by the addition of 2 μ L of MRSA lysate, resulting in a final reaction volume of 30 μ L. The reaction was incubated at 37 °C or 42 °C for 1 hour. After incubation, fluorescence was measured using an Agilent BioTek Cytation 5 imaging reader (Ex/Em = 495/520 nm).

5.4 Results

In this study, we used silicon micropillar-enhanced CRISPR biosensor for rapid and sensitive detection of drug-resistant bacteria. **Figure. 5.1a** is a photograph of the physical

device, both the top and bottom sides of the chip are designed with triangular features to facilitate reagent distribution. A schematic overview of the fully assembled device is shown in **Figure. 5.1b**, showing the chip enclosed within the container and sealed with the cap. The silicon chip is first placed into a custom-designed container, and the container is demonstrated by filling with the red food dye. Pre-mixed RPA/CRISPR reagents are then dispensed onto the micropillar side of the chip. To minimize evaporation and prevent cross-contamination during isothermal amplification at 42 °C, the container is sealed with a soft silicone cap.

Figure. 5.1c presents a schematic of the microfabrication process used to construct the micropillar array. To ensure mechanical integrity after 500 µm-deep etching, <100> silicon wafers were used for high-aspect-ratio deep reactive ion etching (DRIE). Following solvent cleaning with acetone and isopropanol, a dual-layer hardmask consisting of 150 nm Al₂O₃ and a ~2–3 nm SiO₂ cap was deposited using Ion Beam-Assisted Sputter Deposition (IBD). This system was selected for its automated operation and relatively high deposition rates, making it well-suited for this process. Lithography was performed using ~1.7 µm of nLOF2020 negative photoresist on an I-Line Heidelberg MLA150 direct-write tool. Al₂O₃ served as the primary hardmask due to its high etch selectivity in Bosch processes [234]. However, a known challenge with Al₂O₃ is its susceptibility to etching in TMAH-based developers, which can cause photoresist delamination during development [235, 236]. This issue was mitigated by adding a thin SiO₂ cap layer: thick enough to protect the underlying alumina during development, yet thin enough (~2–3 nm) to be fully removed during the subsequent BCl₃-based ICP etch without requiring an additional

step. While other deposition techniques such as ALD or sputtering are also compatible with this hardmask system, IBD was chosen for its ease of use and process reliability [237]. After patterning, the oxide hardmask was etched using a BCl_3 ICP process optimized for Al_2O_3 , which simultaneously removed the thin SiO_2 cap layer. The underlying silicon was etched using a modified three-step Bosch process. Process parameters were optimized via design of experiments (DOE) to achieve aspect ratios of $\sim 50:1$. Following DRIE, the hardmask was removed, and a conformal SiO_2 passivation layer was produced via thermal oxidation. Finally, the wafers were diced into individual chips, with special precautions, such as reduced water flow and isopropyl alcohol rinsing, taken to protect the delicate high-aspect-ratio micropillars during dicing and drying. **Figure. 5.1d** are SEM images of the fabricated micropillar arrays with varying heights of 100 to 500 μm . Image (i) shows a top-down view highlighting the uniform spacing and circular arrangement of the micropillars. Images (ii–iv) display tilted side views of the arrays, revealing consistent vertical alignment and well-defined cylindrical structures across different heights. These features ensure a high surface-to-volume ratio and reproducible fluid interactions critical for downstream biochemical assays.

Figure. 5.2a presents a schematic illustrating the surface functionalization process for a silicon micropillar array and its application in a surface-immobilized CRISPR-Cas12a detection assay. The process begins with plasma treatment to hydroxylate the silicon surface, introducing reactive hydroxyl groups. Then, (3-aminopropyl)triethoxysilane (APTES) is used for silanization by forming covalent siloxane bonds with the surface and introduces terminal amine ($-\text{NH}_2$) groups [238]. The amine-functionalized surface is subse-

quently treated with glutaraldehyde (GA), a bifunctional aldehyde, which reacts with surface amines to form imine (Schiff base) linkages, leaving free aldehyde groups available for further conjugation [239]. A fluorophore-labeled, amine-modified single-stranded DNA (ss-DNA) probe is then covalently immobilized onto the surface via reaction with the exposed aldehyde groups [240]. Upon introduction of the target DNA, a one-pot RPA/CRISPR-Cas12a reaction is initiated. The target sequence is first amplified by RPA, then recognized by the Cas12a, which is activated and exhibits nonspecific trans-cleavage activity [241]. This results in the cleavage of the immobilized DNA probes, releasing the fluorophores into the surrounding solution. After the chip is removed, the released fluorescence signal in the solution serves as a readout for the presence of the target sequence.

Figure. 5.2b presents the water contact angle measurements following plasma and APTES treatments for both the flat silicon chip (left) and the micropillar chip (right). Following plasma treatment, both surfaces exhibited a contact angle of 0° , indicating highly hydrophilic surfaces due to the formation of surface hydroxyl groups. After APTES treatment, the contact angle increased to 59.15° for the flat chip and 109.12° for the micropillar chip. This increase reflects the introduction of amine-functionalized alkyl chains from APTES, which impart moderate hydrophobicity. The much higher contact angle on the micropillar chip is attributed to the surface roughness amplifying the hydrophobic effect through the Cassie-Baxter wetting regime, where air pockets become trapped between the pillars, reducing liquid–solid contact [242]. Following GA treatment, the flat surface exhibited a slight increase in contact angle to 67.74° , likely due to additional aldehyde functionalization contributing limited changes in surface energy. In contrast, the micropillar surface showed

a substantial decrease in contact angle to 25.53° , indicating a wetting regime transition from Cassie-Baxter to Wenzel [243]. This shift suggests that GA increased the surface energy sufficiently for water to infiltrate the microstructures, resulting in full wetting and enhanced apparent hydrophilicity. **Figure. 5.2c** presents fluorescence microscopy images that confirm successful surface functionalization and probe immobilization on 300 μm -tall micropillars. The images were acquired after incubation with Cy3-labeled ssDNA probes. In **Figure. 5.2c**, Panel (i) shows a top-view image obtained using a FITC filter, where green fluorescence indicates the presence of glutaraldehyde. This signal arises from the intrinsic fluorescence of GA due to Schiff base formation, confirming that the aldehyde layer remains intact on the surface [244]. Panel (ii) is a top-view image captured with a TRITC filter, showing red fluorescence corresponding to the Cy3 label. The strong, uniform fluorescence signal confirms successful immobilization of the Cy3-labeled probes onto the functionalized surface. Panel (iii) shows a side-view image under bright-field illumination, which reveals the physical structure and vertical alignment of the micropillars. Panel (iv) displays a side-view TRITC fluorescence image, demonstrating a consistent distribution of Cy3 fluorescence along the entire height of the pillars. This uniform signal suggests effective and homogeneous probe attachment throughout the 3D microstructure, validating the robustness of the surface chemistry and the accessibility of the reactive groups even at the base of the pillars.

Figure. 5.3a displays the sequences of the target DNA and corresponding crRNAs. The target site is located within the *mecA* gene, which encodes methicillin resistance by encoding penicillin-binding protein 2a (PBP2a). This protein exhibits low affinity

for β -lactam antibiotics, allowing bacterial cell wall synthesis to continue despite antibiotic treatment and thereby conferring methicillin resistance [245]. A key element in CRISPR-Cas12a targeting is the Protospacer Adjacent Motif (PAM), which dictates where Cas12a binds and cleaves. The canonical PAM recognized by Cas12a is 5'-TTTV-3', where V represents A, C, or G [215]. In this study, two crRNAs were designed for the one-pot RPA/CRISPR-Cas12a assay: crRNA1 (5'-TTCT-3') and crRNA2 (5'-CCTA-3') target sequences adjacent to suboptimal PAMs. **Figure. 5.3b** illustrates the workflow of the one-pot RPA/CRISPR-Cas12a reaction, emphasizing the interaction between target amplification and Cas12a-mediated trans-cleavage. In the presence of a canonical PAM, Cas12a is rapidly activated, leading to early cleavage of RPA amplicons. This depletes the template pool, limiting further amplification and reducing overall signal accumulation. In contrast, suboptimal PAMs attenuate Cas12a activation, allowing RPA to continue generating more amplicons. The resulting higher template yield leads to delayed but intensified trans-cleavage activity, ultimately producing a stronger fluorescence signal.

To systematically assess the contribution of each assay component and confirm that the fluorescence signal originates from the intended biochemical reaction, a panel of eight reactions (labeled #A through #H) was designed, each containing a different combination of key reagents (see table in **Figure. 5.4a**). The presence or absence of individual components was denoted by “+” and “-”, respectively. Among them, only the reaction #H containing all the necessary elements (including target DNA, RPA reagents, CRISPR-Cas12a complex and fluorescent probe) produced a significantly increased normalized fluorescence signal. In contrast, reactions missing one component yielded minimal to no fluorescence. These results

confirm that each component is essential for assay functionality and, critically, that the fluorescence signal arises from the specific CRISPR-Cas12a-mediated trans-cleavage reaction following target recognition, rather than from any nonspecific fluorescence contributed by individual reagents. This systematic validation underscores the specificity and integrity of the one-pot assay for MRSA detection.

Figure. 5.4b presents the results of temperature screening for the one-pot RPA/CRISPR-Cas12a assay. Incubation at 42 °C resulted in a significantly stronger fluorescence signal compared to 37 °C, indicating enhanced assay performance. RPA exhibits optimal activity in the range of 37–42 °C, while Cas12a typically functions best near 37 °C. At 42 °C, the elevated RPA activity promotes rapid and efficient target amplification, while the slightly reduced Cas12a activity delays trans-cleavage initiation. This temporal separation allows for sufficient amplicon accumulation prior to Cas12a-mediated probe cleavage, ultimately improving signal intensity and assay sensitivity. **Figure. 5.4c** evaluates the performance of two different crRNAs. Among the candidates, crRNA2, targeting a site adjacent to a suboptimal PAM, produced the highest fluorescence signal. This supports the hypothesis that reduced Cas12a activation due to suboptimal PAMs delays trans-cleavage activity, allowing more time for RPA to generate amplicons. The increased amplicon yield results in a stronger downstream fluorescence signal once Cas12a is eventually activated. crRNA1, which also targets a suboptimal PAM, generated a moderate increase in fluorescence relative to the negative control, though not as pronounced as crRNA2. Additionally, co-delivery of crRNA1 and crRNA2 resulted in a reduced fluorescence signal compared to crRNA2 alone. Based on these results, crRNA2 was selected for subsequent experiments

due to its superior signal output, optimal kinetics for delayed Cas12a activation, and its ability to reduce reagent cost by eliminating the need for crRNA combinations.

Following overnight incubation with the FAM-labeled ssDNA probe, the chip was washed four times with PBS to remove excess unbound probes. After each wash, the fluorescence of the wash solution was measured, as shown in **Figure. 5.5a**. A strong fluorescence signal was observed after the first wash, consistent with the high concentration of free probe applied during the labeling step. Subsequent washes led to a substantial decrease in fluorescence, and the signal plateaued after the 3rd and 4th wash, indicating the effective removal of unbound probes. This ensured that any fluorescence observed in downstream assays would originate from specific probe cleavage rather than residual unbound fluorophores. To evaluate the relationship between probe binding capacity and micropillar height, DNase was used to enzymatically cleave the immobilized ssDNA probes, and the released fluorescence was measured (**Figure. 5.5b**). The fluorescence intensity increased with increasing micropillar height of 100 μm , 300 μm , and 500 μm , suggesting a greater number of cleaved probes. Since the pillar diameter was kept constant, taller micropillars corresponded to higher aspect ratios, providing more surface area for probe immobilization, resulting in enhanced binding capacity. **Figure. 5.5c** shows the specificity of the on-chip one-pot assay using genomic DNA from three non-target bacterial strains: wild type *E. coli*, kanamycin-resistant *E. coli* DH5 α , and wild type *S. aureus*. Bacterial DNA was extracted from bacterial cultures at a concentration of 10^8 CFU mL⁻¹. A PBS buffer solution lacking bacteria served as the no template control (NTC). The fluorescence signals generated by all bacterial samples were comparable to that of the NTC, demonstrating that the assay specif-

ically detects the *mecA* gene and does not cross-react with non-target bacterial genomes. Sensitivity testing results for micropillars with heights of 100 μm and 500 μm are shown in **Figure. 5.5d** and **Figure. 5.5e**, respectively. The limit of detection (LOD) for the 100 μm pillars was 10^4 CFU mL^{-1} , while the 500 μm pillars achieved a tenfold improvement, with a LOD of 10^3 CFU mL^{-1} . This enhanced sensitivity with increased pillar height can be explained by several synergistic effects.

First, taller micropillars present a larger surface area due to their higher aspect ratio, which allows for greater immobilization of ssDNA probes. A higher density of surface-bound probes increases the likelihood of interaction with activated Cas12a complexes, thereby amplifying the overall fluorescence signal. Second, the increased surface area enables higher signal accumulation per target event, which improves the signal-to-noise ratio and allows for the detection of lower concentrations of target DNA. Third, the 3D structure of taller pillars may promote more efficient diffusion and interaction of reaction components within the chip microenvironment, enhancing hybridization and cleavage kinetics. Finally, since the FAM-labeled probe lacks a quencher, background fluorescence is minimal, enabling even small changes in signal to be readily distinguished. Together, these factors contribute to the improved detection performance observed with taller micropillar structures.

5.5 Discussion

We demonstrated that integrating a one-pot RPA/CRISPR-Cas12a assay with high-aspect-ratio silicon micropillar arrays enhances the sensitivity of MRSA detection. By precisely fabricating micropillar structures with identical diameters but varying heights

(100, 300, and 500 μm), probe binding efficiency is shown that directly scales with surface area, as validated by DNase cleavage assays. The increased three-dimensional surface provided by taller micropillars facilitated higher probe loading, resulting in improved signal output upon target recognition. Additionally, fluorescence imaging confirmed uniform probe distribution across the micropillar height, validating the effective functionalization of the entire vertical surface. These architectural advantages, combined with controlled surface chemistry (plasma treatment, APTES silanization, and glutaraldehyde coupling), established a robust and reproducible platform for surface-immobilized CRISPR detection.

When integrated with isothermal amplification like RPA, a key challenge in CRISPR-based diagnostics is the risk of premature Cas12a activation, which can result in amplicon consumption before sufficient signal accumulates. To overcome this, we designed crRNAs adjacent to suboptimal PAM sequences to delay Cas12a trans-cleavage kinetics. The crRNA screening demonstrated that suboptimal PAMs, such as CCTA, provided better assay performance than canonical sequences, likely due to reduced cleavage efficiency early in the reaction. This strategy helped temporally decouple amplification and detection phases within a one-pot reaction, maintaining simplicity while enhancing signal output. Furthermore, temperature screening indicated that 42 °C was optimal for balancing RPA efficiency with moderated Cas12a activation. These findings emphasize the importance of crRNA design and temperature tuning for optimizing one-pot CRISPR systems. Importantly, our platform achieved a detection limit of 10^3 CFU mL^{-1} for MRSA using 500 μm micropillars—tenfold more sensitive than the 100 μm condition, while maintaining excellent specificity, as no cross-reactivity was observed with wild-type *E. coli* or *S. aureus* strains.

Despite relying on benchtop fluorescence imaging in this proof-of-concept study, the underlying design of our system is well-suited for POC diagnostics. The one-pot reaction format eliminates complex multi-step protocols and minimizes contamination risk, which are two essential features for use in decentralized or resource-limited settings. The small reagent volumes, isothermal conditions, and chip-based containment make the platform inherently portable and user-friendly. Future iterations could integrate the device with low-cost, compact detection modules such as smartphone-based fluorescence readers or colorimetric outputs, further enhancing its field deployability. While silicon substrates offer excellent reproducibility and microstructural precision, transitioning to lower-cost polymers via scalable fabrication methods (e.g., injection molding or soft lithography) could support large-scale manufacturing and commercial translation. Importantly, although this study focused on MRSA detection, the modular nature of the surface functionalization and CRISPR assay design enables easy reconfiguration for a wide range of pathogens and nucleic acid targets. In addition, the use of standard microfabrication techniques enables high-throughput production, with a single silicon wafer yielding up to 40 individual chips. This scalability supports multiplexing by enabling multiple chips, each dedicated to a distinct target, to be processed in parallel for comprehensive pathogen screening within a unified workflow. With continued development, this micropillar-enhanced CRISPR biosensor presents a promising foundation for rapid, sensitive, and accessible diagnostics at the point of care.

5.6 Conclusion

In summary, we developed a micropillar-structured silicon chip that significantly enhances CRISPR-based detection through improved probe immobilization and optimized reaction kinetics. By integrating a one-pot RPA/CRISPR-Cas12a assay with a micro-fabricated platform, we achieved rapid, sensitive, and specific detection of MRSA. Taller micropillars provided increased surface area, leading to greater probe density and stronger fluorescence signals, while suboptimal crRNA design enabled better control over Cas12a activation timing. The platform demonstrated tenfold sensitivity improvement and high specificity, highlighting its promise for future application in portable, contamination-free, and cost-effective diagnostic systems. This work provides a foundation for further development of microstructured, CRISPR-integrated biosensors for clinical and field diagnostics.

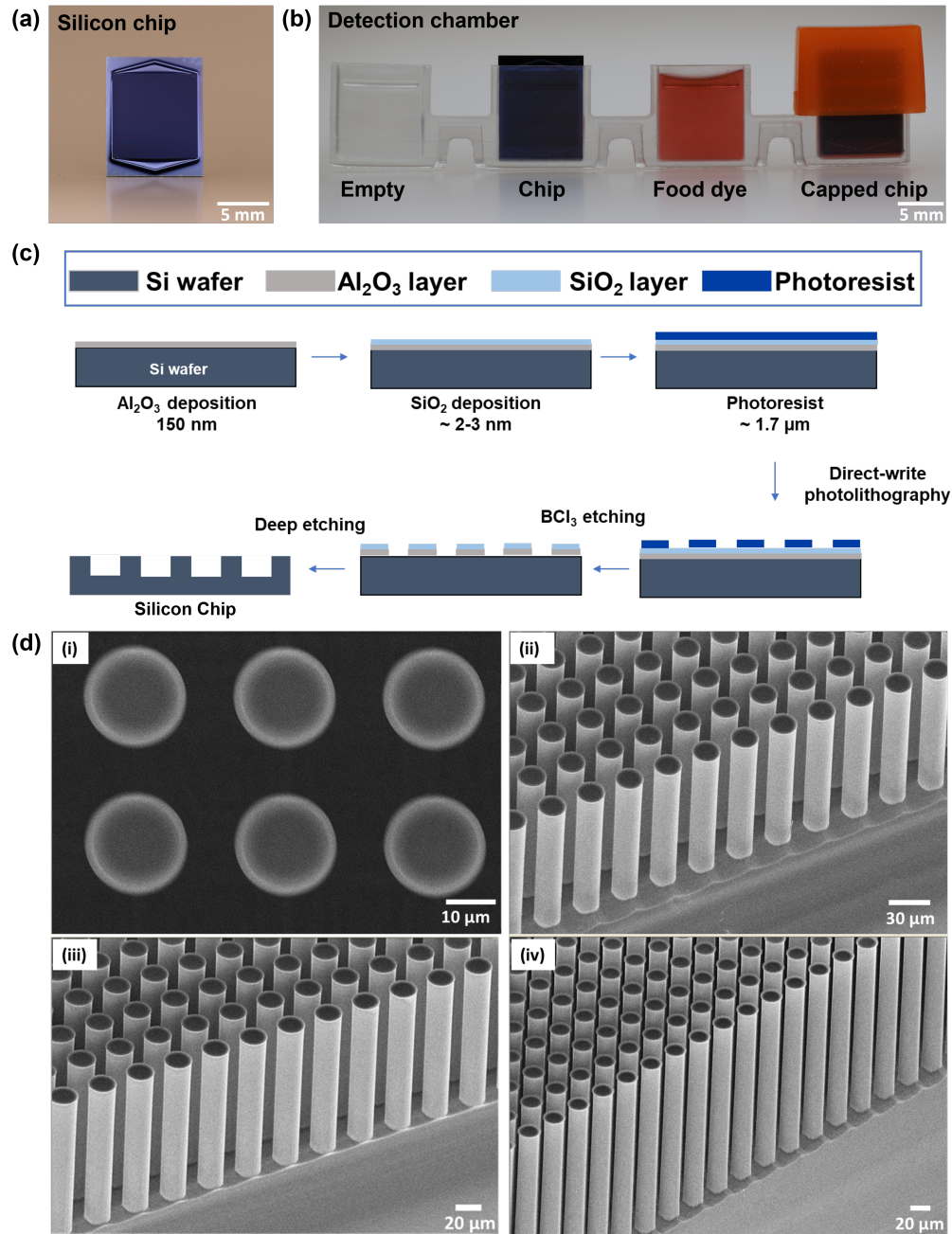


Figure 5.1: Illustration of the design, fabrication, and structural characterization of the micropillar chip. (a) Photograph of the silicon chip. (b) Photograph of the fabricated silicon chip integrated into the detection chamber. (c) Fabrication process of silicon chip. (d) SEM images of micropillars. (i) Top view of micropillars. (ii), (iii), and (iv) side view of micropillars with the height of 150 μm , 300 μm and 500 μm , respectively.

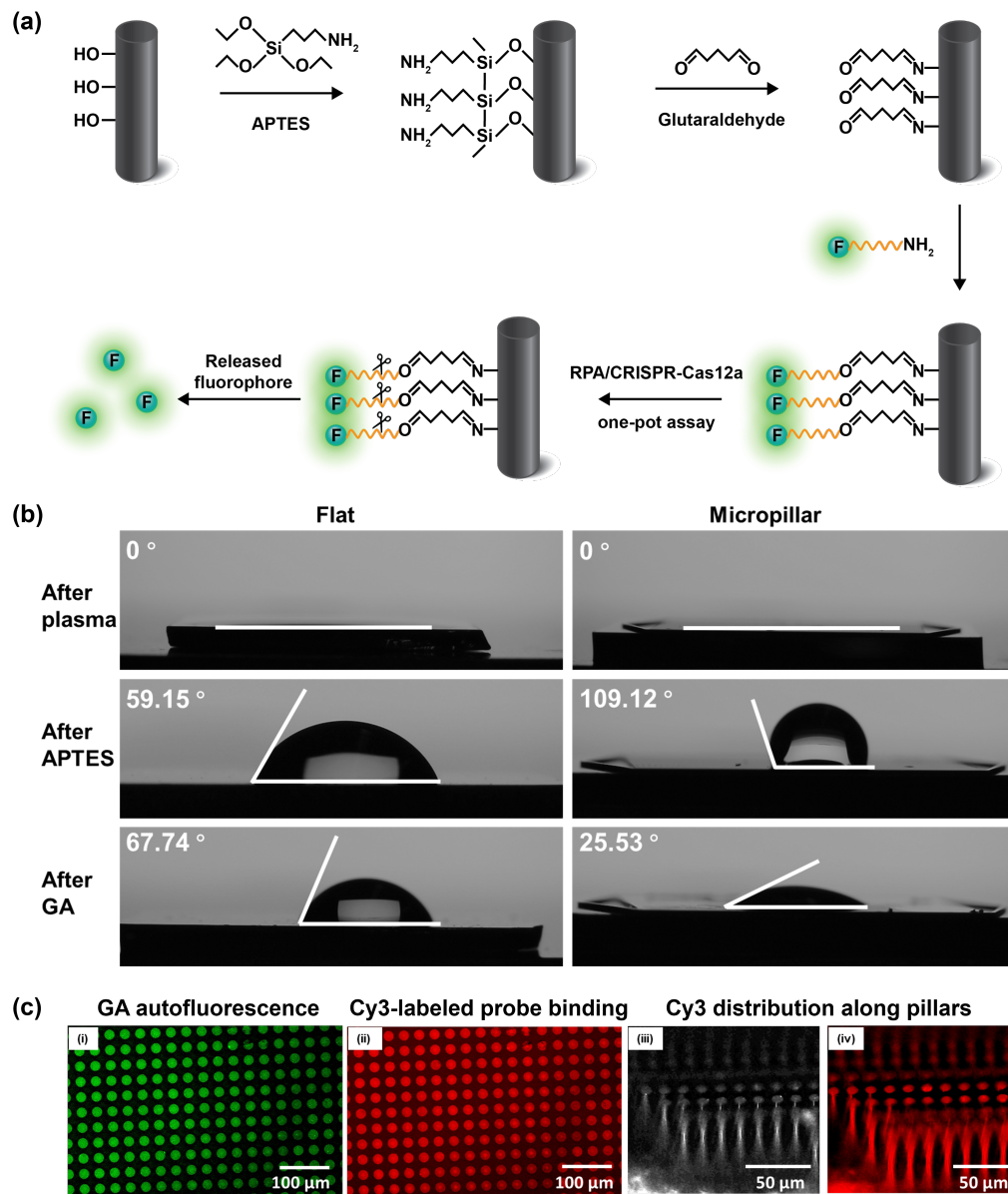


Figure 5.2: Surface treatment and characterization of the micropillars. (a) Schematic illustration of the surface functionalization process, single-stranded DNA (ssDNA) probe immobilization, and the one-pot RPA/CRISPR-Cas12a detection workflow. (b) Water contact angle measurements of flat and micropillar chips following plasma and APTES treatments. (c) Fluorescence images of 300 μm -tall micropillars after Cy3-labeled probe binding. (i) Top view under FITC filter: green fluorescence indicates GA presence. (ii) Top view under TRITC filter: red fluorescence corresponds to Cy3-labeled probes. (iii) Side view under bright field microscopy. (iv) Side view under TRITC filter showing Cy3 fluorescence distribution.

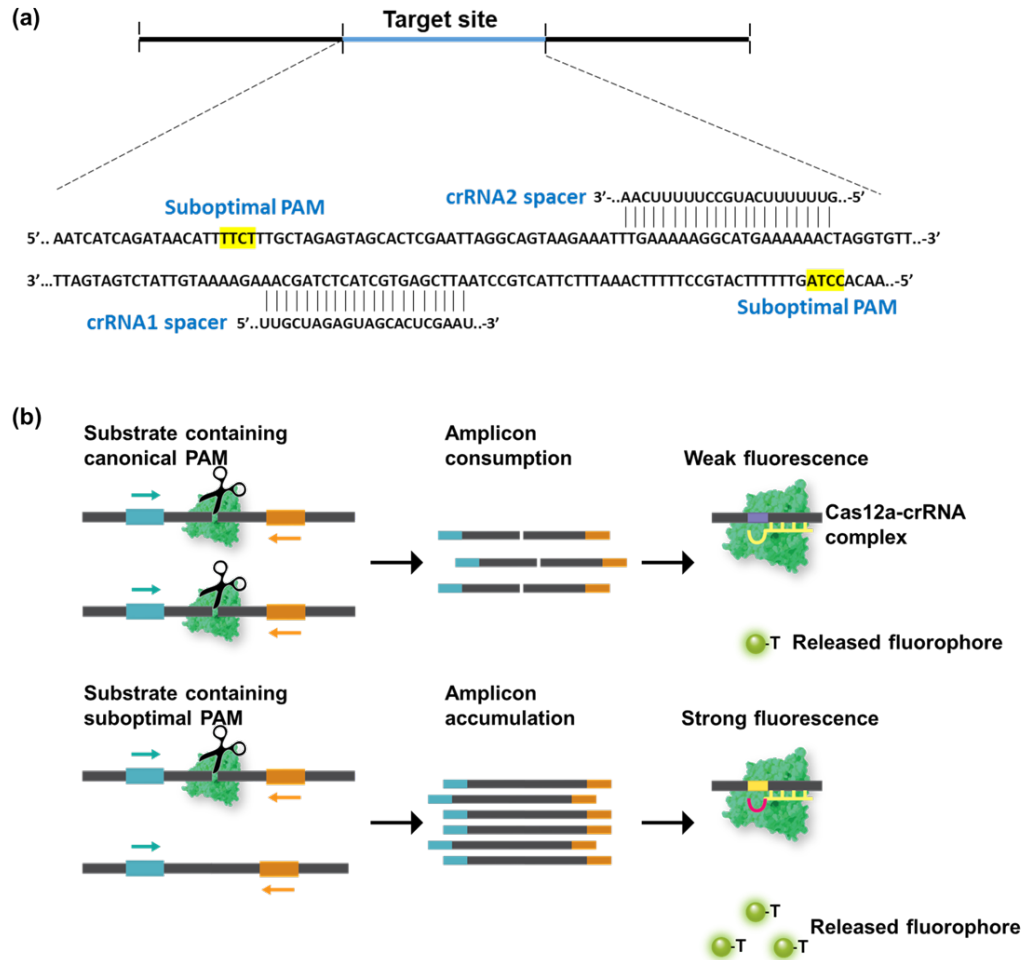


Figure 5.3: Assay design and mechanism. (a) MRSA target DNA sequence and corresponding crRNA designs featuring suboptimal PAM sequences. (b) Schematic illustration of the one-pot amplification and cleavage workflow. In the presence of a canonical PAM, Cas12a is rapidly activated, leading to early cleavage of RPA amplicons. This depletes the template pool, limits further amplification, and reduces overall signal accumulation. In contrast, suboptimal PAMs attenuate Cas12a activation, allowing RPA to continue amplifying the target sequence, resulting in greater amplicon yield and enhanced downstream fluorescence signal.

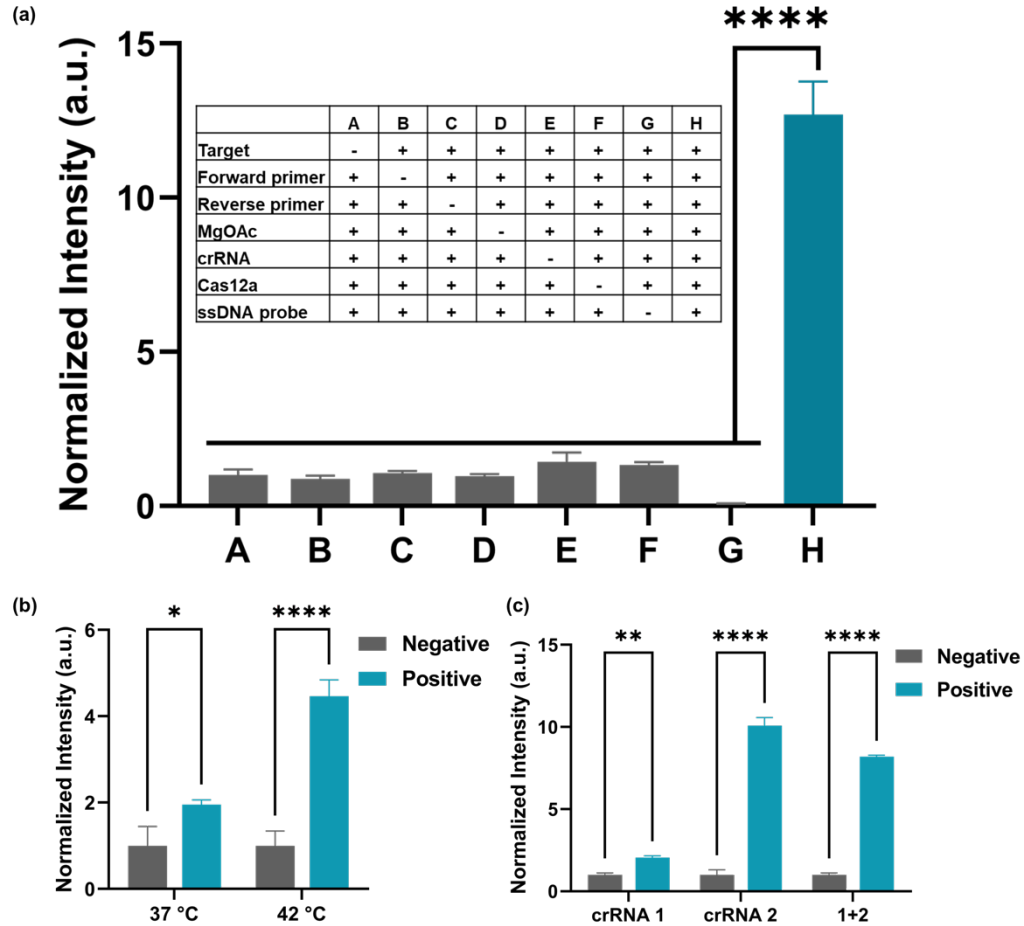


Figure 5.4: Off-chip assay evaluation. (a) Evaluation of crRNA designs for MRSA detection using fluorophore-quencher (F-Q) probes. The input target concentration is 10^7 CFU mL⁻¹. Normalized fluorescence signals are shown for each reaction. Statistical analysis was performed using one-way ANOVA. Inset: eight test reactions evaluating various combinations of assay components. Reaction #H includes all necessary components for the one-pot RPA/CRISPR-Cas12a assay, while other reactions omit specific elements to assess their contributions to signal generation. (b) Optimization of incubation temperature for the one-pot assay. The input target concentration is 10^5 CFU mL⁻¹. Statistical analysis were performed using two-way ANOVA. (c) crRNA screening for improved performance in the one-pot assay. The input target concentration is 10^6 CFU mL⁻¹. Statistical analysis were performed using one-way ANOVA. The data are represented as mean $\pm$ standard deviation ($n = 3$). For statistical analysis, *ns*, not significant = $p > 0.05$; * = $0.01 < p \leq 0.05$; ** = $0.001 < p \leq 0.01$; *** = $0.0001 < p \leq 0.001$; **** = $p \leq 0.0001$.

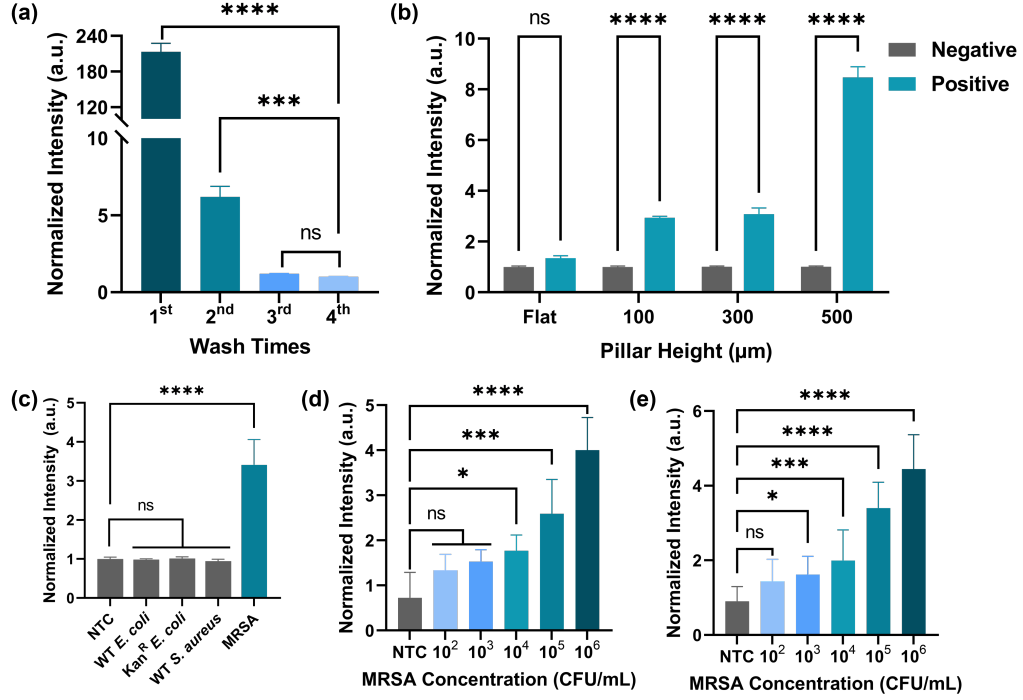


Figure 5.5: On-chip assay evaluation. (a) Comparison of normalized fluorescence intensity in the wash solution after rinsing the chip with 200 μ L of PBS following probe binding. Statistical analysis was conducted using unpaired t -test analysis. (b) Normalized fluorescence intensity reflecting the amount of ssDNA probe bound to micropillars of varying heights. Statistical analysis were performed using two-way ANOVA. (c) On-chip specificity test using wild-type *E. coli* K12, kanamycin-resistant *E. coli* DH5 α , and wild-type *S. aureus*, respectively. “NTC” refers to no template control. (d) Sensitivity evaluation of micropillars with a height of 100 μ m. (e) Sensitivity evaluation of micropillars with a height of 500 μ m. Statistical analysis were performed using one-way ANOVA. The data are represented as mean $\pm$ standard deviation ($n = 3$). For statistical analysis, ns , not significant = $p > 0.05$; * = $0.01 < p \leq 0.05$; ** = $0.001 < p \leq 0.01$; *** = $0.0001 < p \leq 0.001$; **** = $p \leq 0.0001$.

Chapter 6

Conclusions and Future Work

6.0.1 Conclusions

This dissertation demonstrates the potential of integrating CRISPR/Cas12a- and RPA-based biochemical assays with innovative microfluidic platforms to advance infectious disease diagnostics. By systematically designing and evaluating four complementary devices—the Rotation-Chip, nano-sieve microdevice, duplex *Leishmania* detection system, and silicon micropillar one-pot platform—this work addresses multiple barriers that have traditionally limited the translation of molecular assays into point-of-care (POC) applications.

Across these platforms, several important contributions were achieved. First, instrument-free fluid transport and multiplexing were realized through mechanical innovations such as rotational alignment and shearing-driven channels, eliminating the need for bulky pumps and valves while maintaining high distribution efficiency. Second, sample enrichment and preparation were successfully incorporated into the microfluidic workflow,

as demonstrated by the nano-sieve system, which improved detection limits by selectively concentrating low-abundance bacteria from complex samples. Third, structural and surface engineering strategies, such as superhydrophobic coatings and silicon micropillar arrays, provided precise control over reagent handling and assay kinetics, enabling robust one-pot integration of RPA and CRISPR detection. Finally, stability and portability were enhanced through the use of lyophilized reagents and reusable device architectures, further aligning the platforms with the practical demands of field deployment.

Collectively, these advances contribute to fulfilling the World Health Organization’s ASSURED criteria (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable to end-users). The ability to perform high-throughput, multiplexed, and sensitive detection without complex instrumentation brings molecular diagnostics closer to true accessibility in resource-limited environments. Moreover, the incorporation of computational tools, such as automated image analysis, demonstrates how biochemical assays can be paired with digital technologies to deliver accurate, unbiased results at the POC.

Beyond infectious diseases, the design principles established here are broadly applicable. The integration of biochemical assays with microfabricated devices offers a generalizable framework for diagnostics in environmental monitoring, food safety, agricultural testing, and personalized medicine. By tailoring device geometry, surface chemistry, and assay chemistry, the approaches demonstrated in this dissertation can be readily adapted to detect a wide range of nucleic acid biomarkers.

Looking forward, future work should focus on scaling up fabrication methods such as injection molding or high-resolution 3D printing to enable mass production, expanding multiplexing capacity to detect panels of clinically relevant pathogens simultaneously, and further leveraging smartphone-based imaging and machine learning algorithms for automated data interpretation. Additionally, regulatory validation and field testing will be critical steps in translating these technologies from laboratory prototypes into clinically approved diagnostic tools.

In summary, this dissertation establishes a cohesive roadmap for bridging biochemical assay innovation with device engineering to achieve rapid, reliable, and widely deployable diagnostics. By advancing CRISPR/Cas12a and RPA detection within versatile microfluidic platforms, this work contributes to the ongoing global effort to make molecular diagnostics faster, more affordable, and more accessible, ultimately strengthening public health preparedness against both current and emerging infectious threats.

6.0.2 Future Work

Following the completion of this dissertation, my research will extend toward the development of biosensing assays based on Giant Magnetoresistance (GMR) technology at the University of California, San Diego. GMR sensors enable highly sensitive detection of biomolecular interactions through magnetic nanoparticle labeling, converting binding events into measurable electrical signals with low background noise and high signal stability. Compared to optical detection methods, GMR-based sensing offers advantages in scalability, multiplexing, and compatibility with compact electronic readout systems, making it particularly attractive for next-generation diagnostic platforms.

Building on the microfluidic integration and assay development strategies established in this work, future research will focus on designing GMR-based assays that support rapid, quantitative, and multiplexed detection of nucleic acids and protein biomarkers. In particular, efforts will be directed toward integrating GMR sensing with microfluidic sample processing and amplification workflows, enabling streamlined “sample-to-answer” systems. This includes optimizing surface functionalization for efficient target capture, improving magnetic nanoparticle labeling strategies for enhanced signal amplification, and developing robust assay architectures that maintain sensitivity while minimizing nonspecific binding.

Additionally, future work will explore the potential for combining GMR sensing with isothermal amplification and CRISPR-based detection to create hybrid diagnostic platforms that leverage both biochemical specificity and electronic readout. Such systems could enable real-time monitoring, digital quantification, and high-throughput multiplexing in a portable format. Ultimately, this research aims to advance the development of integrated diagnostic technologies that are scalable, instrument-compact, and suitable for clinical and point-of-care applications, extending the system-level engineering principles established in this dissertation to emerging biosensing modalities.

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