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Primer

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Single-cell pathogen diagnostics for combating antibiotic resistance

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Abstract

Bacterial infections and antimicrobial resistance are a major cause for morbidity and mortality worldwide. Antimicrobial resistance often arises from antimicrobial misuse, where physicians empirically treat suspected bacterial infections with broad-spectrum antibiotics until standard culture-based diagnostic tests can be completed. There has been a tremendous effort to develop rapid diagnostics in support of the transition from empirical treatment of bacterial infections towards a more precise and personalized approach. Single-cell pathogen diagnostics hold particular promise, enabling unprecedented quantitative precision and rapid turnaround times. This Primer provides a guide for assessing, designing, implementing and applying single-cell pathogen diagnostics. First, single-cell pathogen diagnostic platforms are introduced based on three essential capabilities: cell isolation, detection assay and output measurement. Representative results, common analysis methods and key applications are highlighted, with an emphasis on initial screening of bacterial infection, bacterial species identification and antimicrobial susceptibility testing. Finally, the limitations of existing platforms are discussed, with perspectives offered and an outlook towards clinical deployment. This Primer hopes to inspire and propel new platforms that can realize the vision of precise and personalized bacterial infection treatments in the near future.

Sections

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Introduction

The emergence and spread of multidrug-resistant bacterial strains poses a worldwide health-care challenge[1–3]. These pathogens cause more than 2.8 million antibiotic-resistant infections per year, resulting in 35,000 deaths and up to $20 billion in health-care costs in the United States alone[4,5]. A driving cause of this global threat is the widespread misuse of antibiotics, which dramatically accelerates the frequency of antibiotic resistance[6,7]. Precise antibiotic prescription and antibiotic stewardship are largely hindered by time-consuming diagnostic procedures, which involve sample transportation, lengthy culture-based pathogen identification and antimicrobial susceptibility testing (AST)[8]. During this period, typically 3–5 days, empirical antibiotics are prescribed according to the worst-case scenario. This practice leads to unnecessary and improper treatments, poor clinical outcomes and emergence of multidrug-resistant bacteria. Compounding the problem is the limited development of new antibiotics and fewer available treatments for multidrug-resistant bacteria[9–11]. To address this health-care crisis, advanced pathogen diagnostic technologies are urgently needed to deliver diagnostic results to physicians within hours instead of days, to help personalize antibiotic treatments, precisely manage bacterial infections and combat antibiotic resistance.

Current diagnosis of bacterial infection begins with sample collection and transport to a centralized clinical microbiology laboratory. Once there, the samples are initially screened for bacterial infection through enrichment culture, also known as clinical isolation, before further analysis by Gram staining, pathogen identification to assist the selection of antibiotic candidates and AST to decide the appropriate prescription[12] (Fig. 1a). This approach is highly dependent on culture-based procedures that hinder the timely delivery of definitive diagnosis. For clinical samples, such as blood, which typically have low concentrations of bacteria, enrichment culture can take several days to reach a detectable level of bacteria. In response, there has been an effort to accelerate pathogen identification and AST. Nucleic acid amplification-based techniques, which can increase and detect bacterial nucleic acids within a few hours, have contributed to pathogen identification and detection of gene markers for antibiotic resistance[13–19]. Developments in biosensing methods — for example, optics[20–29], electrochemistry[30–35] and Raman spectroscopy[36–40] — have also accelerated bacteria detection and AST. Furthermore, the advent of microfluidics demonstrates that miniaturization of conventional assays to the submicrolitre scale offers a powerful strategy for achieving rapid turnaround times[41–56]. These techniques and their potential clinical merits have been reviewed elsewhere[57–70]. Recently, single-cell pathogen diagnostics — assays and devices capable of detecting bacteria at, or originating from, the single-cell level — have attracted significant attention as a promising emerging solution for pathogen identification and AST, with unique advantages (Fig. 1b). Single-cell pathogen diagnostics detect individual bacterial cells and their growth within a few replication cycles, meaning diagnosis can be significantly shortened (Box 1). Moreover, single-cell pathogen diagnostics can identify rare bacteria in populations, allowing rapid diagnosis for heteroresistance, polymicrobial infections and diagnosis directly from minimally processed samples. Such timely and precise diagnostic results collectively provide actionable information for physicians to prescribe personalized medicines, rather than resorting to empirical treatments.

Numerous research reports and several recent review articles[71–76] are evidence of the growing interest in single-cell pathogen diagnostics. The wide range of reported platforms gives researchers a broad foundation to build on. However, the many options also present difficulties for assessing, adopting or developing platforms for an intended use case. As a result, there is a critical need for an end-to-end guide for researchers who wish to work with single-cell pathogen diagnostic platforms or gain holistic understanding of existing research. This Primer aims to provide such a guide by introducing principles and considerations for designing and implementing single-cell pathogen diagnostic platforms, including the need to incorporate capabilities of single-cell isolation, detection assay and output measurement. Each of these capabilities can be realized through a range of methods whose working principles, context of use, strengths, weaknesses and compatibility with other methods are discussed in detail. Representative results towards pathogen diagnosis are presented and potential clinical applications for bacterial detection, pathogen identification and AST are highlighted. Solutions to enhance the reproducibility of replicating experimental conditions and data acquisition are described and the challenges of deploying single-cell pathogen diagnosis in clinical settings are outlined. Finally, the Primer provides insights into these challenges and an outlook of future developments towards clinical applications.

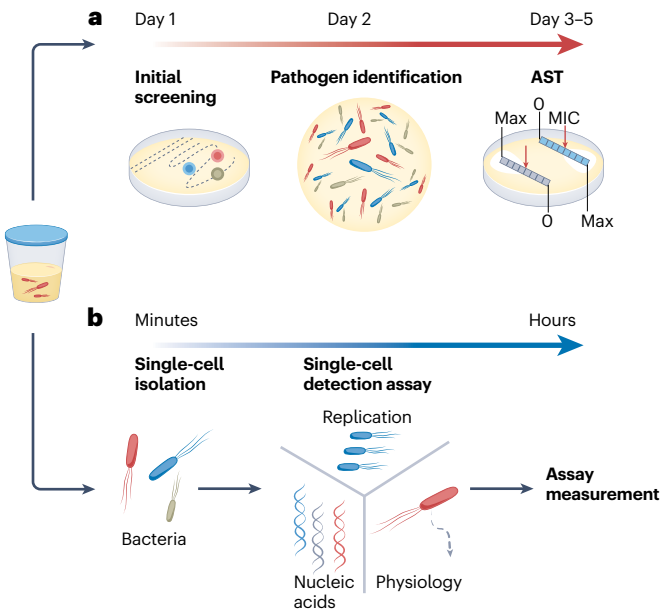


Fig. 1 | Workflow of pathogen diagnostics using standard clinical microbiology procedures and single-cell pathogen diagnostics. a, Current practice for pathogen diagnostics consists of initial screening to examine the presence of bacteria and subsequent lengthy culture-based procedures for pathogen identification and antimicrobial susceptibility testing (AST). **b**, Single-cell analysis implements rapid pathogen diagnostics by sampling and testing a bacterium at the single-cell level. MIC, minimum inhibitory concentration.

Experimentation

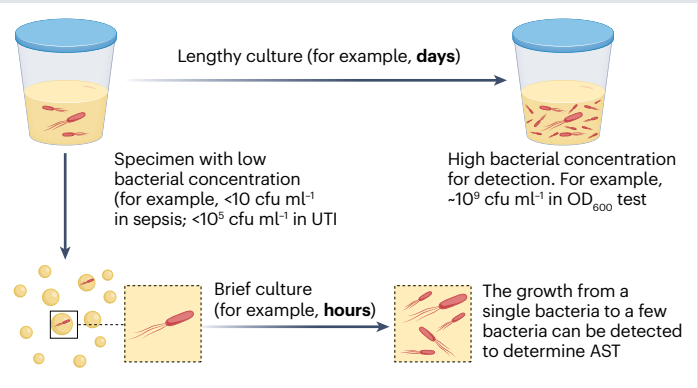
The first experimental step is to consider the sample input type for the intended application and to set up appropriate samples. After considering the sample, it is important to design and implement a single-cell pathogen diagnostic platform with three essential capabilities: isolating single bacterial cells, performing an assay for detecting the isolated cells and measuring the assay output. Each of these capabilities can be accomplished through numerous methods (Fig. 2), but every method has a unique working principle, design, implementation, context of use and compatibility with other techniques. This section introduces each

Box 1

How a single-cell growth assay speeds up diagnosis

Standard microbiology diagnosis

Bacterial concentrations can be low in clinical specimens, meaning lengthy enrichment culture may be needed to accumulate sufficient bacteria quantities and ensure that they can be detected using standard instrumentation in clinical settings. The turnaround time of this step depends on the initial bacterial concentration in the specimen and the species' growth rate. For example, enrichment culture for *Escherichia coli*, a common species in urinary tract infections (UTIs), usually takes 1 day, whereas enrichment culture for tuberculosis-associated *Mycobacterium tuberculosis* can be as long as a few weeks due to the species' slow growth (see the figure).



Single bacteria growth assay

To shorten the detection time required in diagnosis, single bacteria are isolated in a confined, usually small, volume. In the confined volume, each bacterium is individually monitored. Only a brief culture, comparable with the timescale of bacterial replication, is needed to determine bacterial growth. The small volume creates high local concentrations of the bacteria and potential molecular markers for detection. Taken together, such single-cell assays enable rapid detection of bacterial growth, and when different antibiotic conditions are administered, they also enable rapid antimicrobial susceptibility testing (AST).

cfu, colony forming unit.

capability and the various methods within them, providing guidance for the design and implementation of a single-cell pathogen diagnostic platform.

Sample preparation

At the start of each experiment, sample matrices, prevalent bacteria species and typical bacterial loads should be considered. Clinical specimens vary in sterility; for instance, cerebrospinal fluid is sterile, whereas vaginal specimens often contain background bacteria and flora. Blood contains a large number of human cells and urine may contain flora and human cells. All human cells and flora should ideally be separated from bacterial cells. Urine samples can also have a large pH range, which may affect assays. As a result, appropriate sample processing steps may be needed depending on the sample type. Prevalent bacterial species responsible for infectious diseases and antimicrobial resistance can be found from the research literature — for example, for bloodstream[77,78], urinary tract[79] or hospital-acquired infections[80] — or, alternatively, through governmental organizations, such as the World Health Organization (WHO) and the US Centers for Disease Control and Prevention (CDC). Clinical specimens may contain multiple species of bacteria, referred to as polymicrobial, and typical bacterial loads can vary. For example, in sepsis, a low bacterial load of 1–10 colony forming units (cfu) ml^{-1} in blood specimens is common[81], whereas for urinary tract infections (UTIs) a higher bacterial load of ≥10^5 cfu ml^{-1} in urine specimens is considered positive[82,83].

After becoming familiar with the sample matrices, prevalent bacteria species and typical bacterial loads, the next step is to locate reference strains of common bacterial species and their respective antibiotic-resistant strains. Many of these strains can be acquired from biorepositories, such as the American Type Culture Collection (ATCC) of the United States and the National Collection of Type Cultures of the United Kingdom. For the development of new single-cell pathogen diagnostic platforms, *Escherichia coli* is the most common starting point because it is one of the most prevalent bacterial causal agents of infectious diseases. Other important pathogens include *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Enterococcus faecium*. For *E. coli*, the ATCC provides a widely used reference strain (ATCC 25922), although non-pathogenic *E. coli* strains, such as K-12 or those incorporating fluorescence proteins, have also been used[84–87].

Progressing from contrived samples to clinical samples can take multiple steps. At the initial development stage, it is common to suspend bacteria in a buffer, for example phosphate buffered saline, or a culture broth, such as Luria–Bertani broth. If clinical applications are pursued, then ATCC reference strains and the standard cation-adjusted Mueller–Hinton broth are highly recommended. If accessible, clinically isolated bacteria provided by clinical microbiology laboratories, known as clinical isolates, suspended in a buffer or a culture broth represent a reasonable next step after testing reference strains. Subsequently, clinical isolates spiked into clinical specimens, such as commercially available artificial urine samples and whole blood samples verified as negative via standard culture-based methods, can serve as an appropriate intermediate step before testing patient-derived clinical specimens[13,88–90]. In this intermediate step, the bacterial species, bacterial concentration range and mimicking specimens to be spiked should be aligned with the overall research aims. Ideally, the test should be verified with multiple strains and samples to capture the heterogeneities of the species and sample type. Additionally, when AST is a focus, standard testing conditions, such as those described in the Clinical Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines, should be

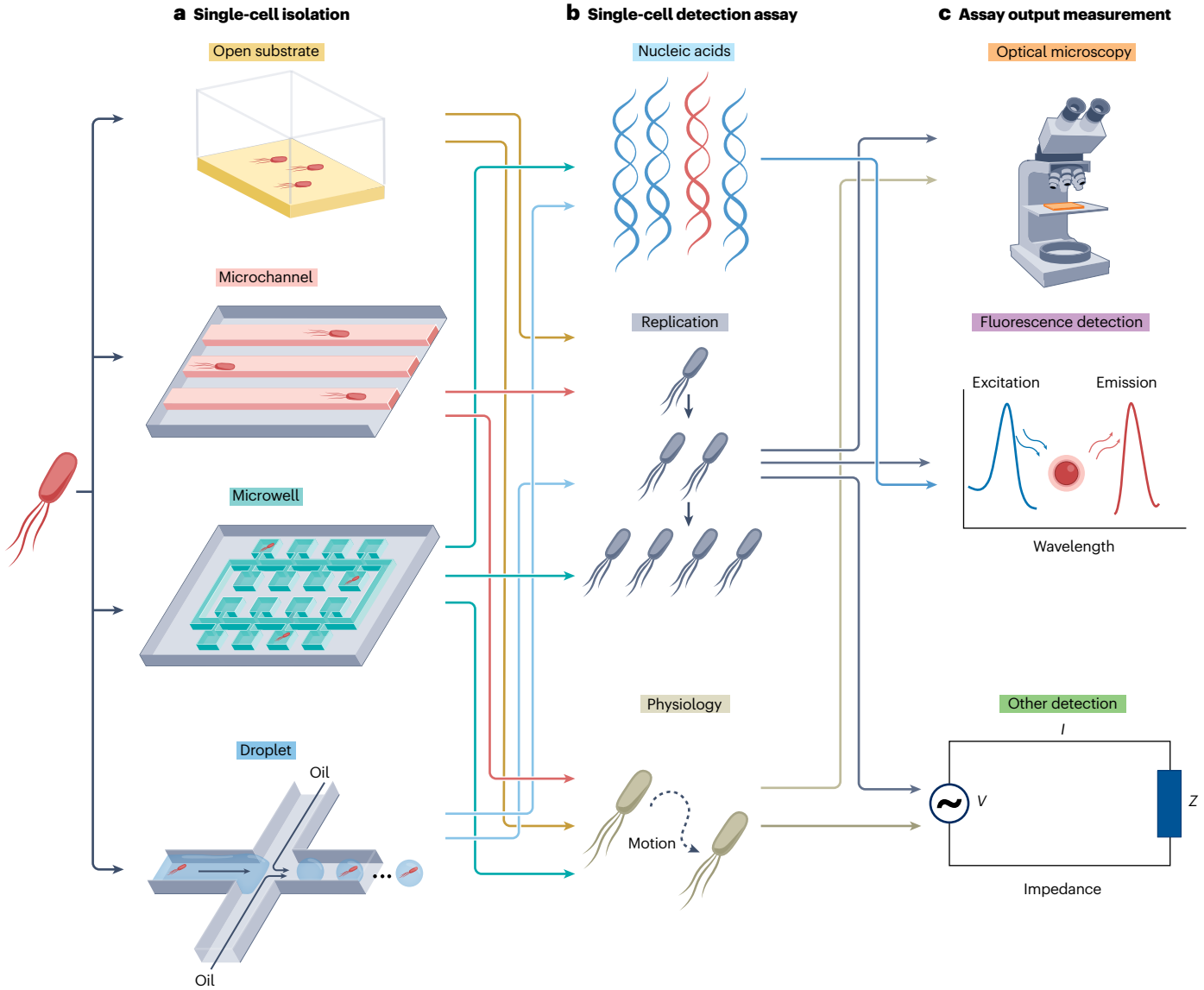


Fig. 2 | Essential capabilities of single-cell pathogen diagnostic platforms, including single-cell isolation, detection and measurement. a, Single bacterial cells are first isolated in a confined space using micro/nano engineering tools such as open substrates, microchannels, microwells and droplets. **b**, These individual bacteria are subsequently detected by investigating bacterial replication, nucleic acid detection and cellular physiology. **c**, The detection assays are measured and evaluated by imaging analysis, fluorescence read out and bioelectronics measurement. Single-cell pathogen diagnosis is realized by integrating these methods (indicated by the arrows).

considered and adopted as necessary. For example, broth microdilution is a standard AST method, and cation-adjusted Mueller–Hinton broth is the recommended medium for broth microdilution. It is therefore advisable to use the appropriate medium and avoid introducing an additional difference from the standard method.

Single-cell isolation

The first essential capability of single-cell pathogen diagnostic platforms is to fix the position of, or compartmentalize, single bacterial cells. Considerable advances in microscale and nanoscale engineering have led to the development of numerous highly efficient devices that can isolate single bacterial cells[91]. This section generalizes these devices based on their representative structure and working principle into four categories: open substrate, microchannels, microwells and droplets (Fig. 2a). For each device category, the discussion describes their structure and working principle, design considerations, implementation requirements, context of use and compatibility with other methods.

Open substrate. Perhaps the most straightforward strategy for isolating single cells is to capture them on a substrate and detect individual cells with powerful detection modalities that have single-cell resolution, such as high magnification microscopy. An effective approach for

open capture is to trap bacteria in agarose, where cell fractions near the surface of the agarose substrate can be easily detected when the bacterial distribution is uniform[92,93]. Another means to capture bacterial cells on a substrate is by biochemically functionalizing the substrate. For example, APTES ((3-aminopropyl) triethoxysilane)[94], chitosan[95] or an antibody[96] may be used to physically tether bacteria at the single-cell level. This surface functionalization can be accomplished by standard biochemical procedures. For instance, IgG, a common antibody capable of attaching to the bacterial cell membrane, has been coated by bonding its primary amine groups with *N*-hydroxysulfosuccinimide ester receptors on a surface to immobilize bacteria[97]. Such functionalized substrates can rapidly capture bacteria upon contact. Importantly, these capturing methods maintain bacterial viability, and also support detection of phenotypic changes under antibiotics.

Advantages and disadvantages of this open substrate strategy should be considered. On one hand, implementation of this strategy is simpler than most other single-cell isolation approaches. The devices mostly avoid intricate microfluidic structures, which is an important advantage if the required microfabrication facilities are unavailable. On the other hand, high concentrations of bacteria (10^7–10^9 cfu ml^{-1} (refs. [98–101])) are typically needed to ensure sufficient bacterial cells are captured for analysis. Isogenic bacteria can still have heterogeneous phenotypes[102,103]. Consequently, phenotypic detection of single bacteria requires sufficient cells for a reliable diagnosis. However, high bacterial concentrations can often only be attained through culture. This requirement means that open substrate is best suited for AST of cultured positive samples and clinically isolated bacteria, rather than screening bacteria from clinical samples or identifying bacterial species. Finally, the single-cell detection modalities coupled to open substrate often operate at a low throughput. For example, high magnification microscopy typically detects a few single bacterial cells within its small field of view. Scanning across large areas of the substrate is possible, but at the expense of more sophisticated hardware, for example a motorized stage for microscopy, and requiring greater computing power. These issues have motivated researchers to pursue single-cell isolation strategies using microchannels, microwells and droplets.

Microchannels. Microchannels with dimensions comparable with bacterial cells have been used to confine individual bacteria for pathogen diagnosis[104]. As bacterial sizes are on the micrometre scale, the microchannels are a few micrometres in width and depth (0.5–2 µm (refs. [104–106])), and are typically fabricated using precise manufacturing tools, such as soft lithography[91,107–109] (see Supplementary Note 1). These microchannels can be loaded with bacteria by a capillary effect or syringe pump, where the bacteria stochastically enter and distribute in the channels. To enhance the loading efficiency, the microchannels are designed in a parallelized and high-throughput manner. To concentrate bacteria in a designated detection region, the microchannels typically integrate with additional modules to trap the bacteria in place. For instance, a physically reduced channel size can trap bacteria when the channel dimensions are no larger than the bacterial cells[104,110–112]. Additionally, an electrokinetic force exerted on bacteria can immobilize them in situ[105,113]. These mechanisms allow the channels to capture bacteria at extremely low concentrations by continuously loading and manipulating large volumes of samples. However, the sample loading rate is typically low due to the high flow resistance – calculated by the Hagen–Poiseuille equation $R = (8\eta L)/(\pi r^4)$, where η, L and r represent the dynamic viscosity of the fluid, equivalent length and radius of the channel, respectively – in the microchannels, limiting the sample volume for detection in a given time. Consequently, the sample volume can be tested using microchannels typically in the order of microlitres. The limit of detection is around 10^3 cfu ml^{-1}, although this may be improved by optimizing the sample loading technique. The volume that can be analysed is dynamic. To detect low-concentration bacteria, samples need to flow through the device for a longer time to increase analysis volume. If no bacteria are detected after a predetermined time, then the sample may be free of bacteria or below a known concentration. This approach is straightforward and also maintains cell viability for monitoring bacterial replication.

A useful feature of the micron-size channel is that it behaves as a physical filter for minimizing the matrix components. As a bacterium significantly increases the fluid resistance of the channel, a channel is typically loaded with only one cell. Traps with tuneable heights, using membrane microvalves that can partially close due to applied pressure, can be engineered to selectively trap bacteria based on their shape and size, offering a new means for pathogen identification[111]. There are, however, some considerations and potential concerns for the use of microchannels. For example, microchannels are susceptible to clogging from particulates in complex clinical samples, especially with high cellularity. This may limit use with direct samples to answer clinical questions as it requires additional sample preparation steps. Densely spaced microchannels may be incompletely isolated from adjacent channels, making them incompatible with assays that include diffusible molecules such as dyes as cross-contamination that can lead to false positive results. Additionally, microfabrication requirements for fine features are stringent. A further limitation of all the microfluidic devices discussed is that they are susceptible to air bubbles during operation and reagent evaporation during any incubation step. As a result, it is critical to use care when working with these devices. Techniques such as immersing microfluidic devices in the liquid phase and engineering waterproof materials in situ may be needed to mitigate evaporation[106,114–116].

Microwells. Individual bacteria can be captured in microwells or microchambers that are physically separated or separated by an immiscible fluid. Compared with open substrates and microchannels, microwells offer a distinct advantage of isolating individual bacterial cells (namely discretization), as well as nucleic acids and reaction products derived from them. These advantages enable precise quantification of bacteria. Moreover, the small and identical volume of every microwell results in high and uniform local bacteria concentration across the entire device, enabling sensitive detection while avoiding the inoculum effect[117]. Compared with droplets, microwells offer simpler experimental procedures and better compatibility with time-lapse detection due to their fixed spatial positions. Despite these advantages, typical microwell devices are designed with a fixed, often low, total sample volume due to the number (<50,000) and volume (100 pl–10 nl) of the wells. This limitation results in trade-offs between the total sample volume, well volume and device footprint. This trade-off determines the overall performance characteristics, including the detection limit (~10^3 cfu ml^{-1}) and assay time. When designing microwells to implement culture-based assays, it is important to consider the volume and aspect ratio to avoid limiting diffusion, which would deplete nutrients or enzymatic substrates[118,119]. Another consideration is that, except for a few recent microwell devices[120–122], current microwell devices only support assays that can be performed in a single step. Consequently, the selection of microwells as the single-cell isolation

method must be paired with an appropriate single-step single-cell detection assay.

Multiple microwell device architectures have been developed, but they have comparable discretization efficiencies and similar dexterity requirements. The architectures can be broadly classified as microvalve-enclosed microwells[123], immiscible oil-separated microwells[124–128], SlipChips[129] and membrane-based microwells[130] (see Supplementary Note 2). Although each device class has unique experimental parameters for optimization, which are beyond the scope of this Primer and discussed in refs. [123–130], the devices share an objective to isolate microwells and discretize bacterial cells, resulting in similar characterization experiments. Typically, microwell devices are initially characterized using a bacteria-free mock sample, preferably using the same reaction buffer or culture broth as the sample, supplemented with a food dye or a fluorescent dye to facilitate microscopy observation. This initial characterization aims to quantify the number of properly loaded and isolated microwells, monitor evaporation of isolated samples within microwells during incubation and adjust device design and experimental parameters as needed, before bacterial samples can be tested.

Droplets. Discretization of a bacterial sample into numerous tiny droplets in an oil phase, with each droplet containing at most one bacterium, facilitates high sensitivity and high-throughput detection. The small volume of each droplet results in a high effective bacterial concentration, enabling rapid detection. The ability to detect individual single cell-containing droplets allows absolute quantification of the bacterial load with a high accuracy[90,131,132]. Moreover, droplet devices typically operate in a continuous flow, which has the advantage of being able to process large sample volumes and giving a wide detection dynamic range (1–10^9 cfu ml^{-1}). Compared with other single-cell isolation methods, droplets are particularly useful for quantitatively analysing samples with unknown bacterial loads. Finally, droplets allow detection of rare targets that are undetectable by a bulk-based detection method. This capability is useful for detecting rare resistant bacterial cells in a largely susceptible population, a scenario known as heteroresistance[133]. Although useful for high sensitivity and high-throughput detection, there are important considerations for using droplets to develop single-cell pathogen diagnostics. First, such detection has been predominantly achieved with an accompanying high sensitivity and high-throughput droplet detector built in-house. Although microscopy can be used to image droplets, its field of view can limit the number of droplets detected per image, which can limit the throughput and, in turn, the sensitivity. Additionally, compared with other single-cell isolation methods, operating a droplet device is a more intricate procedure, requiring a steeper learning curve.

Implementation of droplet devices begins with selecting the most appropriate droplet device from a wide range of device architectures (see Supplementary Note 3), which involves extensive design, operation and characterization considerations. Currently, active droplet generation devices are sometimes employed. Active devices are multilayer devices that incorporate programmable microfluidic membrane valves, such as Quake valves[134], to precisely inject various assay reagents into an oil phase and create droplets of various sizes (typically nanolitre scale) and contents, for instance different antibiotic types and concentrations[135–138], at the expense of low throughput (<1 Hz) and in-house instrumentation. More commonly, passive droplet generation devices are used and connected to commercial syringe pumps for control. Passive devices are single-layer devices that use static microconstrictions with pre-designed cross-sectional widths and heights, for example flow-focusing junctions, to perturb the interfacial tension between co-flowing streams of an aqueous sample and an oil phase to generate aqueous droplets surrounded by a continuous oil phase[139] at high throughput (>100 Hz). After microfabricating the droplet device, often by soft lithography (see Supplementary Note 1), extensive characterization is performed to ensure efficient discretization of bacteria. The sample must be directly tested in the droplet device to evaluate whether it can be discretized into droplets. The composition of the oil phase — the oil and surfactant — must be optimized to ensure reliable droplet generation without droplet merging. The flow rates of both the sample and the oil phase should be tuned to navigate the different droplet generation regimes[139,140] and to optimize droplet size and frequency. The size uniformity of the droplets, the monodispersity, is often evaluated with bright-field microscopy and a haemocytometer. To perform downstream assays, generated droplets containing discretized target bacteria are often collected in reaction tubes, incubated in an external temperature-controlled instrument and reinjected into another device that facilitates droplet detection. This manual workflow is needed for lengthy incubation and can help optimize conditions, with an eventual aim of developing an integrated device that directly appends incubation and detection to droplet generation. Once developed, this integrated device removes the need for manual operation and can facilitate automation. However, incubation conditions within the device become fixed. In both cases, it is important to make sure that false positive droplets, which are empty droplets with spurious signals, in the no-bacteria controls occur as infrequently as possible, as this is key to achieving highly sensitive detection. It is also important to check that negative, empty droplets in all samples are detectable because accurate bacterial load quantification requires the total number of droplets in each experiment.

Single-cell detection assays

The second essential feature of single-cell pathogen diagnostic platforms is the capability to perform an assay to detect the isolated cells. This section also streamlines the discussion by categorizing available detection assays as nucleic acid detection assays, replication assays and physiology assays (Fig. 2b). For each assay category a brief description is given of the detection mechanism, experimental considerations, context of use and compatibility with other methods.

Bacterial nucleic acid detection assays. Detection of bacterial nucleic acids is a widely used method to determine the presence of bacteria, quantify the bacterial load, identify bacterial species and, in some cases, perform AST. Bacterial nucleic acid detection assays typically use species-specific and/or resistance-specific — for example, *mecA* and *mecC* for methicillin-resistant markers[141], *vanA* and *vanB* for vancomycin-resistant markers[142,143] — primers or probes. The assays can often be completed in a few hours directly from clinical specimens without the time-consuming culture enrichment step. Although different nucleic acids (DNA or RNA) can be targeted, single-cell pathogen diagnostic platforms have focused on detecting genomic DNA via nucleic acid amplification methods, such as polymerase chain reaction (PCR), or ribosomal RNA (rRNA) via probe hybridization such as fluorescence in situ hybridization[144]. Notably, bacterial nucleic acid detection assays are predominantly detected using fluorescence and are most often implemented in microwells and droplets (Fig. 2b, arrows towards nucleic acid detection). When compared with open substrate and microchannels, microwells and droplets can more effectively localize diffusive fluorescence signals around the isolated single cells

to retain digital quantification. Assay temperatures and reaction optimization are key parameters. High assay temperatures, such as the 95 °C hot start and denaturation steps in PCR, can cause considerable evaporation in devices. Comprehensive reaction optimization, for example buffer composition and polymerase, is needed to ensure that bacterial nucleic acids can be amplified from clinical specimens containing potential reaction inhibitors[145,146] in microwells and droplets in single-step assays. Typically, before conducting a single-cell nucleic acid detection assay in a device, the assay methodology is optimized using a benchtop instrument at bacterial concentrations comparable with those in the device.

Bacterial DNA — either gene sequences that specify bacterial species or gene markers that confer antimicrobial resistance — is the most common target for bacterial nucleic acid detection assays. In the context of single-cell detection, this strategy is particularly useful for quantifying the bacterial load and identifying individual bacterial species within a sample that contains multiple bacterial species. Because there are typically only one to a few copies of the target gene per bacterial cell, nucleic acid amplification techniques such as PCR are needed. Here, PCR is most commonly conducted, with the addition of an intercalating dye or TaqMan probe that enables fluorescence-based end point detection of microwells or droplets containing bacteria with the target gene. In addition to optimizing polymerase, primers and probe concentrations or the thermocycling conditions, selecting an inhibitor-resistant polymerase and adding PCR-enhancing additives[147] are necessary for direct detection from complex sample matrices such as blood. To achieve one-step operation, the 95 °C hot start step in PCR can double as thermal lysis. However, thermal lysis is only applicable to Gram-negative bacteria, which can partly explain the scarcity of platforms for detecting Gram-positive bacteria. In addition to PCR, loop-mediated isothermal amplification (LAMP) has been used to detect Gram-negative bacteria such as *E. coli* and *Salmonella* by their DNA and mRNA, respectively[130]. LAMP has become a mature method with commercially available reagents and several strategies for fluorescence detection, such as Calcein and SYBR Green. The constant reaction temperature (typically 60–65 °C) removes the need for a thermocycling instrument, the main advantage of LAMP. However, even for direct detection of *E. coli* and *Salmonella*, additional lysis reagents such as sodium fluoride (NaF) and lysozyme to the LAMP reagents are necessary[130].

Bacterial 16S rRNA is an attractive target for bacterial identification due to several reasons. First, it is a well-established method to analyse 16S rRNA sequences for phylogenetic classification of bacteria[148,149]. Second, there are large and curated databases of rRNA sequences — including the Ribosomal Database Project[150] and GenBank — that can facilitate probe design to detect bacterial 16S rRNA. Third, there are abundant rRNA molecules (~10^3–10^5 copies[151–153]) per cell. The abundance of rRNA enables amplification-free detection of single cells. Such amplification-free detection is most commonly accomplished through brief thermal lysis followed by hybridization between fluorogenic probes, such as molecular beacons[154] or displacement-based duplex probes[155–157], and species-specific sequences in 16S rRNA within picolitre-scale droplets that create high local concentrations of 16S rRNA. This hybridization-based detection method eliminates the need for nucleic acid extraction, minimizing sample preparation and enabling single-step, sample to answer operation. Recently, researchers have used 16S rRNA abundance as a surrogate marker for bacterial response to antibiotic exposures. It was shown that rapid AST can be accomplished through droplet-based quantitative measurements of single-cell 16S rRNA abundance following brief exposure to antibiotics, as short as 10 min (ref. [157]). Despite these advantages, the requirement of a small reaction volume and high-throughput detection means this strategy is almost exclusively used within droplets and the feasibility of detecting Gram-positive bacteria remains to be investigated.

Bacterial replication assays. Bacterial replication is the most direct way to reveal the phenotypic response of bacteria to antibiotics, making it a widely used method for single-cell AST[125,158–161]. This strategy is attractive because it is applicable for both Gram-negative and Gram-positive bacteria and is independent of the resistance mechanism. Measuring replication at the single-cell scale can potentially reduce the assay time to one replication, or the doubling time of the bacterial species (Box 1). As a result, bacterial replication has been coupled to all four single-cell isolation methods (Fig. 2b, arrows towards bacterial replication). The bacterial concentrations in microdevices are typically higher than standard bacterial concentrations in conventional broth microdilution AST, resulting in an inoculum effect[162], which may lead to inaccurate AST and minimum inhibitory concentration (MIC) results. Thus, it is critical to optimize the assay conditions such as the incubation time. Bacterial replication assays generally do not classify or identify bacterial species. Consequently, bacterial replication assays should be applied along with a single-cell identification assay. Finally, the assay times could be long for bacteria with long doubling times such as *Mycobacterium tuberculosis*, whose doubling time is >24 h. The ability to identify slow growing bacteria in contrast to susceptible strains is critical, as a resistant bacterium that is falsely classified as susceptible is a major error in AST. These considerations should be assessed prior to choosing a bacterial replication assay when developing a single-cell pathogen diagnostic platform.

Bacterial replication assays can be direct or indirect. For AST, both should adhere to standard conditions as often as possible, such as using cation-adjusted Mueller–Hinton broth and 37 °C as the culture broth and incubation temperature. Direct bacterial replication assays only require the target bacteria, a culture broth and antibiotics, which subject bacteria to the simplest environment for replication without additional, potentially inhibitory, reagents. Bacterial replication can then be readily detected though optical microscopy — by counting individual cells or measuring the length or area occupied by the growing cells — or through alternative means such as changes in electrical impedance[98,99,104,105,110–112,163]. Indirect bacterial replication assays use an additional surrogate molecule, usually an enzymatic substrate, that produces a detectable signal, such as fluorescence or colour change, to indicate bacterial growth[125,158,164–166]. These molecules can diffuse, and are best suited for completely isolated reaction compartments, such as microwells and droplets. The diffusive surrogate molecules can produce strong signals spanning entire microwells or droplets that contain replicating bacteria. Such strong signals can be readily detected in parallel, facilitating high-throughput detection of many cells per sample and, thereby, enhancing experimental reliability. Currently, resazurin is perhaps the most widely used surrogate molecule[125,158,161]. Bacterial metabolites from replicating bacteria, such as nicotinamide adenine dinucleotide hydrogen, can reduce resazurin into resorufin, which is strongly fluorescent. However, addition of a surrogate molecule can inhibit bacterial replication. This concern is usually circumvented by setting the no-antibiotic control as the reference for relative comparison with antibiotic samples and experimenting with several surrogate molecule concentrations to ensure only minor inhibition to replication. For both assays, time-course measurements with appropriate antibiotics, including no-antibiotic controls, should be performed to determine

the minimum incubation time that can reliably differentiate bacterial replication, minimizing false negative and false positive results.

Bacterial physiology assays. In addition to replication, other physiological features of bacteria, such as size, morphology, enzyme activity and motion, can be used to classify some bacteria species or to perform AST[113,167–172]. Size and morphology have long been used to classify bacteria, for example cocci, bacilli, diplococci and tetrads[111]. Enzyme activity of bacteria can also be used for identification, through addition of chromogenic media that change colour in the presence of a specific bacteria[173]. Importantly, when performed at the single-cell scale, rapid physiological changes occur in bacteria in response to antibiotic exposure, offering an attractive strategy for rapid AST. However, these physiological features are often species-specific, with little to no standardization. Therefore, the bacterial physiology strategy is best suited to specific scenarios, where the pathogens of interest are known.

Motion is the most frequently used physiological feature for performing AST. For instance, motion of individual bacteria confined on an open substrate has been shown to reflect antibiotic susceptibility of bacteria[94,174] (Fig. 2b, arrows towards bacterial physiology). In this scenario, although long-range motion (swimming) of bacteria is limited, short-range motion (wiggling) exists due to the molecular motor and bacterial flagella activities, which is closely associated with bacterial viability[174]. Bacteria move actively and contribute to large short-range motion in the absence of antibiotics or when they are resistant to tested antibiotics. By contrast, this motion dramatically reduces when bacteria are susceptible to an applied antibiotic. Detection of short-range motion under various antibiotic conditions can quickly reveal antibiotic susceptibility. This single-cell motion can be directly monitored using advanced imaging techniques, such as plasmonic imaging and tracking[175], surface-enhanced Raman scattering[37–40] and subcellular fluctuation imaging[174,176]. It can also be detected using ultrasensitive biosensors that capture mechanical fluctuations from the bacterial motion[94,177]. Using this mechanism, AST results can be detected within seconds to minutes[174,175,177].

Single-cell assay output measurement

The third essential capability of a single-cell pathogen diagnostic platform is to measure the output from single-cell detection assays. This section consolidates three prevalent techniques for measuring single-cell diagnosis results, including image analysis, fluorescence read out and bioelectronics measurement (Fig. 2c). There is a focus on fundamental mechanisms, core parameters and the major applications of these technologies.

Optical microscopy. Optical microscopy is the most established and accessible measurement method for single-cell detection assays. A microscope equipped with a 20× (or larger) objective and a charged-coupled device or complementary metal–oxide–semiconductor camera is readily capable of visualizing single bacterial cells in bright-field mode and phase contrast mode[99,104,105,178]. Time-lapse optical microscopy is especially useful for bacterial replication assays and some bacterial physiology assays (Fig. 2c, arrows towards bright-field microscopy), as the relative change in bacteria can be revealed from time-lapse images. Time-lapse images can be analysed using widely accessible software such as ImageJ[179]. Custom software tools have also been developed. For example, the computational tool ChipSeg segments a bacterial population, which is cultured in a microfluidic device and imaged by time-lapse microscopy, into individual cells[180]. The main advantage of optical microscopy, especially under bright-field microscopy, is accessibility and simplicity, as most research laboratories have access to standard microscopes that require minimal optimization. Notably, some researchers use phase contrast microscopy to better visualize bacteria[102,163]. Although bacteria can be observed more clearly using phase contrast microscopy, the required phase contrast condensers and phase contrast objective lenses add considerable cost to a microscope. An important consideration for optical microscopy is that the high magnification required to visualize individual bacterial cells restricts the field of view to tens or low hundreds of bacterial cells. Finally, when viewed through the lens of portable diagnostic tools that can be used outside centralized laboratories, in resource-limited settings, there has seen a push for developing cell phone-based microscopes and lensless imaging that can detect single bacterial cells[181,182]. However, further investigation is needed to attain comparable detection performance with standard microscopes.

Fluorescence imaging, microscopy and spectroscopy. Fluorescence-based read out is the most widely used read-out for single-cell detection assays, especially nucleic acid detection assays and replication assays (Fig. 2c, arrows towards fluorescence). Various fluorogenic reagents — including fluorescent dyes (EvaGreen and resazurin), fluorophores (fluorescein) and fluorescent proteins (green fluorescence protein) — with unique excitation and emission ranges can be used to create fluorescence-based single-cell detection assays. It is essential to ensure matching excitation and emission wavelengths between the fluorogenic reagents and the read-out instruments.

Fluorescence-based read out can be commonly implemented as imagers, microscopes and spectroscopes, with each instrument having a unique compatibility with various single-cell isolation devices and single-cell detection assays. In-depth discussion of different implementations and their associated instrumentation is beyond the scope of this Primer and can be found elsewhere[183–186]. Fluorescence imaging is typically performed with either a commercial image scanner or a custom camera-based imager with the necessary excitation light source, an optical filter and optional temperature-controlled heating apparatus[128,187,188]. These imaging systems can capture a large field of view (>5 cm × 5 cm) that exceeds the footprint of most single-cell isolation devices discussed previously. However, there are trade-offs between the field of view, resolution and scanning time. The imagers are best suited for imaging assay output within large microwells (~100 μm × 100 μm × 100 μm). Commercial fluorescence microscopes with charged-coupled device cameras are compatible with all previously discussed single-cell isolation devices and detection assays, from dye-stained or fluorescent protein expressing bacteria to resazurin assay output in microwells and droplets[189]. Despite this versatility, fluorescence microscopes typically have a smaller field of view (<1 mm × 1 mm, depending on objectives). Fluorescence spectroscopes are commonly custom-built, including an excitation source (laser or light-emitting diode), a detector (photomultiplier tube or avalanche photodiode), filters, mirrors and other optical components as needed. They are most often used to perform end point measurements of droplets that sequentially flow through the spatially fixed detection volume of the fluorescence spectroscope, which results in a time trace of fluorescence signals that correspond to individual droplets. Regardless of implementation, optimization of fluorescence detection should include an adjustment of the excitation light source intensity and the detector's detection time interval, alongside comparisons of the fluorescence signals with and without bacteria at each condition.

Other detection methods. The high diversity of bacterial physiology has enabled it to be detected by various methods at the single-cell level to determine pathogen diagnostics. For instance, measurement of cell density or mass of a single bacterium has been demonstrated for measuring antibiotic resistance using a suspended microchannel resonator[168,190]. The suspended microchannel resonator is a delicate biosensor capable of measuring the buoyant mass of individual bacteria and differentiating resistant or susceptible strains by their osmotic shock response after antibiotic exposure[168]. Measurement of the bacterial cells' physical profile, including length, width and morphology, has been demonstrated at nanometre resolution using pioneering imaging analysis that can identify cell deformation in the presence of an antibiotic, which can determine antibiotic susceptibility[172,191]. Single-cell assays of bioelectrical properties have made substantial contributions to single-cell pathogen diagnosis due to the simplicity and flexibility for read-out acquisition. For example, the increase of bacterial cells and/or the accumulation of metabolism products from a single bacterium during growth could cause considerable change in the electrical features of a confined small volume, such as microchannels and microwells, resulting in a detectable electrical signal[166,169,192] (Fig. 2c, arrows towards bioelectronics). Among others, the impedance signal represents a typical measuring parameter that can be quantitatively captured using an electrical circuit in situ, such as a Wheatstone bridge circuit. This signal can be acquired and recorded in hardware for analysis. This experimental set-up reduces the dependence on delicate instrumentation. However, to implement this methodology for measuring single-cell assays, it is necessary to first calibrate the measurement in the designated medium, as electrical conductivity varies with the medium, potentially interacting with the bioelectrical signal and incapacitating the measurement.

Results

In this section, representative results from single-cell pathogen diagnostics are provided. First, the section briefly introduces common tools for data acquisition and analysis. As similar assays generate similar data, the discussion is categorized based on nucleic acid detection assays, replication assays and physiology assays.

Data acquisition and analysis tools

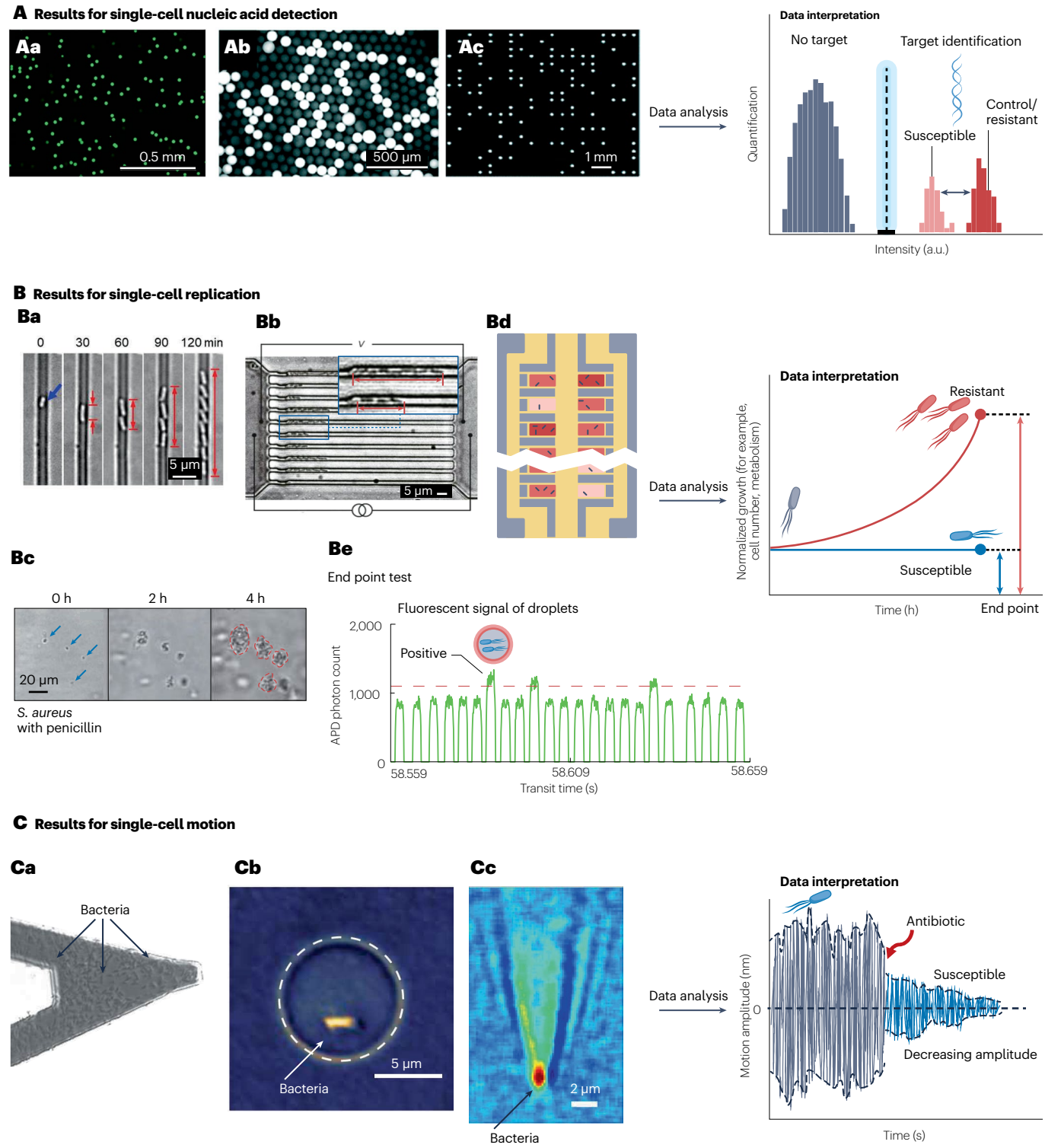
Common tools for data acquisition and analysis include ImageJ, MATLAB and LabVIEW (Laboratory Virtual Instrument Engineering Workbench) (Table 1). ImageJ, with built-in or custom plug-ins[179], has been the go-to software for analysing image-based data generated from nucleic acid detection assays and replication assays through optical microscopy and fluorescence. Image analysis can also be accomplished using other software packages, such as MATLAB, which is capable of automating and scaling up the analysis of large data sets. MATLAB can be used to analyse non-image data, such as the fluorescence signal of droplets in a time trace acquired through another software package, such as LabVIEW. LabVIEW, which uses a data acquisition card, can achieve versatile data acquisition ranging from electrical impedance across microchannels, fluorescence intensity in droplets and nanoscale motion of single bacteria[94,112,154,193].

Bacterial nucleic acid detection assays

Nucleic acids in individual bacteria can be detected to identify bacteria at the single-cell level and, simultaneously, quantify bacterial load. It can be performed by discretizing the bacterial cells in confined volumes followed by a fluorogenic detection process (Fig. 3A, left). Commonly, after bacteria are individually isolated and lysed, the bacterial nucleic acids from a single bacterium are released, amplified and detected with fluorescence read out[130] (Fig. 3Aa). This basic scheme is expanded towards various configurations with modifications on demand. A representative configuration is to detect rare bacteria in blood and quantify the bacteria at the single-cell resolution. It has been demonstrated by encapsulating bacteria together with blood cells in picolitre droplets, releasing and amplifying 16S rDNA from each individual bacterium, and detecting the 16S rDNA with fluorogenic complementary probes[147] (Fig. 3Ab). Importantly, the assay conditions are carefully tuned to enable PCR amplification in the presence of inhibitors in blood, such as haemoglobin. Overall, these assays successfully demonstrate detection of nucleic acids at the single-cell level in a one-pot format, which eases detection. However, this may hinder the detection of broad-spectrum bacteria requiring multiple reaction steps[132,194,195]. In response, a microwell device has been developed for single-cell level detection

Table 1 | Data acquisition and analysis for results from single-cell detection

Software	Capability	Advantages	Limitations	Representative practices
ImageJ and its derivatives, such as Fuji and ImageJ2	Quantitatively analyse bacterial cells, cell morphology and cell counting in images Measure fluorescence signal by pixels in images	Easy to use via a graphical user interface An open-source software with built-in plugs and manual scripts, programmable by Python, R and JavaScript, for customized functionalities	Limited for image analysis Challenging to identify targets in an automatic means, especially in complex matrices, which may hinder applications for analysing big data sets	Evaluate antibiotic resistance by measuring bacterial growth[98,100,111] Quantify bacterial load by testing fluorescence signal from discretized bacteria[125,126]
MATLAB	Perform image analysis for bacterial recognition, quantification and fluorescence signal evaluation Analyse data in other formats, such as signal from discrete droplets	Programable (by MATLAB language) for customized functionalities Scalable for batch processing in an automatic manner Allows machine learning and deep learning using built-in toolboxes, such as Neural Networks	Requires expertise in programming May require a high volume of computing resources The license is not free	Scenarios that analyse a large set of data[110,163] Identify/classify pathogens by their biological features[206,238–240]
LabVIEW (Laboratory Virtual Instrument Engineering Workbench)	Simulate laboratory instruments for gauging signal from designated sensors, which are typically read using a data acquisition card	Graphical programming of virtual instruments (the G language) with high flexibility Reduced need for hardware Straightforward electrical read out	Requires expertise in electronics and circuitry Compatibility between versions can be an issue Limited access for free open-source libraries	Detect electrical and/or photonic properties of a single bacterium[112,157] Examine antibiotic resistance by testing bacterial motion[94,174]



of *S. aureus*[121] (Fig. 3Ac), a Gram-positive strain that requires enzymatic lysis[196]. As this lysis step is not compatible with PCR amplifications[197], the detection is achieved by implementing bacteria partitioning, cell lysis, PCR amplification and fluorescence detection step by step in each individual microwell.

The fluorescence read-out results from single-cell nucleic acid detection assays (Fig. 3A, left) are often visualized as histograms (Fig. 3A, right). Statistically, samples with the target nucleic acids possess a higher intensity than samples with no target nucleic acids. As a result, the presence of the target nucleic acids can be differentiated by

Fig. 3 | Representative results from single-cell pathogen diagnostics and corresponding analysis and interpretation. A, Single-cell nucleic acid detection is performed by identifying DNA/RNA markers associated with pathogen identification and/or resistance. This detection is typically implemented in confined volumes, such as microwells (panels **Aa**[130] and **Ac**[121]) and droplets (panel **Ab**[147]), to localize nucleic acids released from individual bacteria. The results are typically presented in fluorescence format with single-cell resolution that can be analysed to identify and quantify the target of interest. **B**, Single-cell growth is implemented in microchannels (panels **Ba**[111] and **Bb**[112]), open substrates (panel **Bc**[163]), microwells (panel **Bd**[158]) and droplets (panel **Be**[201]), with various detection mechanisms and measurement approaches that can be quantitatively analysed to determine the relative growth and, thereby, interpret the antimicrobial resistant profiles. **C**, Single-cell motion is captured by nanomechanical biosensors (panels **Ca**[94] and **Cb**[174]) and advanced imaging techniques (panel **Cc**[96]). As the cell motion varies with antibiotic conditions, it has been investigated to reveal antimicrobial resistant profiles. *S. aureus*, *Staphylococcus aureus*. Part **Aa** adapted from ref. [130], CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/). Part **Ab** adapted with permission from ref. [147], Royal Society of Chemistry. Part **Ac** adapted with permission from ref. [121], Royal Society of Chemistry. Part **Ba** reprinted with permission from ref. [111], PNAS. Part **Bb** adapted with permission from ref. [112], PNAS. Part **Bc** adapted with permission from ref. [163], AAAS. Part **Bd** reprinted with permission from ref. [158], PNAS. Part **Be** reprinted with permission from ref. [201], Wiley. Part **Ca** adapted from ref. [94], Springer Nature Limited. Part **Cb** adapted from ref. [174], Springer Nature Limited. Part **Cc** adapted with permission from ref. [96]. Copyright 2016 American Chemical Society.

setting an upper threshold above the mean for samples with no target nucleic acid (Fig. 3A, right). This threshold can be determined based on the confidence interval of the negative sample (Fig. 3A, right, dotted line). The confidence interval is generally set to 95% or above, indicating roughly two times the standard deviation above the mean. The shape of the droplet may also be used to identify droplets, complementing the threshold approach[90,198]. Consequently, pathogen identification is revealed by correct identification of associated nucleic acids. Moreover, as positive microwells or droplets are counted, the bacteria can be quantified at the single-cell resolution. Alongside counting, it is also possible to differentiate the fluorescence intensities within the positive microwell or droplet, which provides an additional parameter for AST. This strategy is particularly applicable for droplet-based and 16S rRNA-based pheno-molecular AST[157], where the no-antibiotic control can have stronger fluorescence signals in positive droplets than positive droplets of the antibiotic-treated sample, reflecting a greater quantity of 16S rRNA (Fig. 3B, right, intensity shift in coloured groups).

Before quantifying an unknown concentration of bacteria, it is customary to calibrate the system using samples spiked with various known concentrations of bacteria or samples with known bacterial loads. A linear relationship between the input concentrations and the number of detected bacteria should be obtained. The most important parameter is the average number of bacteria per microwell (λ), which is determined by the product of the input concentration of bacteria and the microwell volume. The values used to calculate the probability (P) of detecting k bacteria discretized in a microwell are based on the Poisson distribution ($P(X = k; \lambda) = (\lambda^k e^{-\lambda})/k!$). Testing three input concentrations of bacteria such that $\lambda = 0.01$, $\lambda = 0.1$ and $\lambda = 1$ should provide sufficient initial validation. Importantly, based on the Poisson distribution, at $\lambda = 0.01$ essentially 1% of all microwells in the device would contain a single bacterial cell. The microwell occupancy with single bacterial cells at $\lambda = 0.1$ would be ~9%, instead of 10%, and at $\lambda = 1$ occupancy is only ~37%, rather than 100% (Box 2). This is because as λ increases, the probability of a microwell containing more than a single bacterial cell also increases.

Bacterial replication

Bacterial replication under antibiotics has been studied at the single-cell level to determine antibiotic susceptibility[104,105,110–112,199,200]. To trace a single bacterium, cells are first captured individually in a microchannel, microwell, droplet and open substrate, allowing bacterial growth in situ under the designated antibiotic condition (Fig. 3B, left). The growth of the isolated bacteria is monitored by imaging the bacterial cells, where the single-cell morphological changes, such as the bacterial size, are recorded. For instance, the bacterial cell number increases exponentially over time when there is no antibiotic applied or the bacterium is resistant to the antibiotic[111,163] (Fig. 3Ba,Bc). Alternatively, the replication of a bacterium may change the electrical impedance/resistance of the microenvironment and that change can be measured to detect bacterial growth[112,192]. A representative result is that the electrical resistance of the microchannel proportionally increases with the bacteria number because bacterial cells growing along the microchannels can block the ionic current across the channels and enhance the electrical resistance[112] (Fig. 3Bb). Besides these approaches, which interface directly with bacterial cells, the metabolic activity of individual bacteria has been researched to evaluate bacterial growth[125,158–161]. As bacteria grow in small confined volumes, such as picolitre droplets and microwells, metabolic products generated from the bacteria accumulate gradually, quickly resulting in high concentrations that can be detected by a fluorogenic resazurin assay[158,201] (Fig. 3Bd,Be). The fluorescence signal is quantitatively measured to assess bacterial growth in response to different antibiotic conditions. Of note, the fluorescence signal in droplets is individually detected to ensure high detection sensitivity and the detection is generally performed at the end time point of the assay[201] (Fig. 3Be). Statistical analysis of the signal, such as the ratio of droplets with high fluorescence intensity, can reflect the metabolism and therefore bacterial growth.

By interpreting the bacterial growth in the presence of corresponding antibiotics, the antimicrobial susceptibility profile can be determined (Fig. 3B, right). The detected signals are normalized to estimate the relative change in the growth. This relative change can include a series of time-lapse data points, which can be fit to an exponential model to generate a curve. This curve may be further analysed to determine the growth rate and the fastest time for differentiating growth in various conditions. Growth of bacteria above the CLSI breakpoints suggests that the bacteria are resistant to the antibiotic. Otherwise, the bacteria are susceptible to the antibiotic (Fig. 3B, right). Similarly, the resistance or susceptibility can be determined by investigating the relative change of the growth at the end point (Fig. 3B), such as the fluorescence intensity in droplets. Generally, the determination of resistance or susceptibility can be accomplished within a few hours (one or a few replications) using the single-cell growth test, compared with the days required for standard procedures.

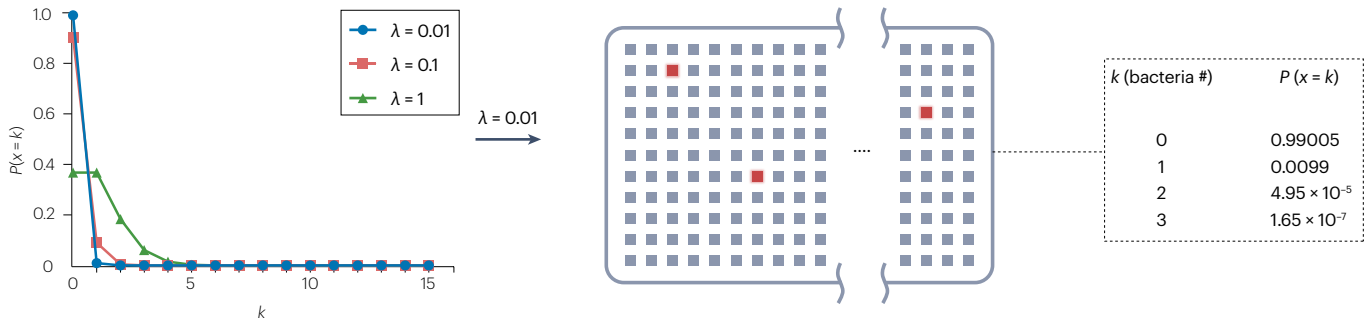
Bacterial physiology

Detecting the cellular motion of a captured bacterium — for instance, in situ wiggling — can rapidly reveal bacterial response to an antibiotic. The motion of the bacterium creates nanoscale mechanical fluctuations

Box 2

Poisson distribution and its application for single-cell isolation and quantification

The Poisson distribution is a discrete probability distribution that describes the possibility of a given number of events happening in a given time or space. Each event should be independent of the previous event and the frequency of events should follow a constant mean rate. The probability mass function at the given number of events is $P(X = k) = (\lambda^k \cdot e^{-\lambda})/k!$, where λ, k and e are the designated mean rate of the occurrence, the number of occurrences and Euler's number, respectively. High probability is observed when the number of occurrences is around the designated mean rate. Online Poisson distribution calculators can be helpful to visualize distributions at various λ values. In scenarios for sampling bacteria in a fixed space, small λ is generally designed. For instance, as λ=0.01, the majority of the space, for example microwells and droplets, are blank, whereas a very small portion (0.999%) of the space contains 1 bacterium (see the figure). In these bacteria-containing spaces, the bacterial concentration elevates dramatically, supporting high sensitivity detection and short turnaround detection time.



of the substrate. By capturing a bacterium on an atomic force microscope cantilever, the bacterial motion and fluctuations can be measured by detecting the motion of the cantilever with the reflection of a laser beam[20,94] (Fig. 3Ca). This nanomechanical sensing mechanism can be further developed to improve the detection sensitivity and multiplexity by capturing bacteria on an array of graphene drums. Each drum consists of an ultrathin (<1 nm) graphene substrate suspended on top of a circular cavity. Owing to the small mass, high stiffness and micrometre-sized area of the graphene substrate, the motion from a single bacterium causes a detectable fluctuation in the substrate that can be measured using laser interferometry[174] (Fig. 3Cb). Alternatively, advances in imaging techniques have enabled direct monitoring of a single bacterium's motion. Surface plasmon resonance imaging is a representative method for monitoring this motion[22,23]. In this method, polarized light is directed to create surface plasmons on the gold surface, and the reflected light is detected. When resonance occurs between the surface plasmon and the reflection light, the reflected light is absorbed, leading to a weak signal. As bacterial motion can change the surface plasmons, the motion from a single bacterium could be recorded by the reflected light[96] (Fig. 3Cc).

The bacterial motion reflects the bacterial viability under antibiotics and therefore indicates antibiotic resistance (Fig. 3C, right). As bacteria move in a favourable condition, such as the culture medium, the motion has a large range, which can be quantitatively analysed by outlining the amplitude, namely $\sigma = \sqrt{z(t)^2}$, where σ and $z(t)$ represent the equivalent distance and trajectory of the motion, respectively (Fig. 3C, right, dotted lines). The amplitude of the motion could be dramatically attenuated when an antibiotic is applied, suggesting that the bacteria are susceptible to the antibiotic. By contrast, the motion will not be attenuated if the bacterium is resistant to the antibiotic. Notably, motion inhibition caused by the antibiotic occurs within a few minutes, if not seconds, allowing the potential for rapid AST.

Applications

Single-cell pathogen diagnostics were developed to speed up traditional diagnosis, guide antibiotic use in a timely manner and help combat antibiotic resistance. This section reviews applications of single-cell pathogen diagnosis platforms for accelerating pathogen detection and identification, and AST. As single-cell pathogen diagnosis is at an early stage, applications are selected by considering their clinical potential, namely the capability of testing clinically relevant samples, including clinical patient samples, spiked samples and clinical isolates.

Pathogen detection and identification

Single-cell pathogen diagnosis serves as a valuable tool to facilitate rapid detection of bacteria with high sensitivity[202]. It has been applied to detect the presence of bacteria in clinically relevant samples within a few hours. The samples could contain bacteria at low concentrations and involve complex matrices. More specifically, bacteria spiked in culture medium at concentrations below 10^5 cfu ml^{-1}, a clinical cutoff for UTIs, have been detected by examining the bacterial metabolic activity in a microwell device[125] (Fig. 4a). This device successfully demonstrated

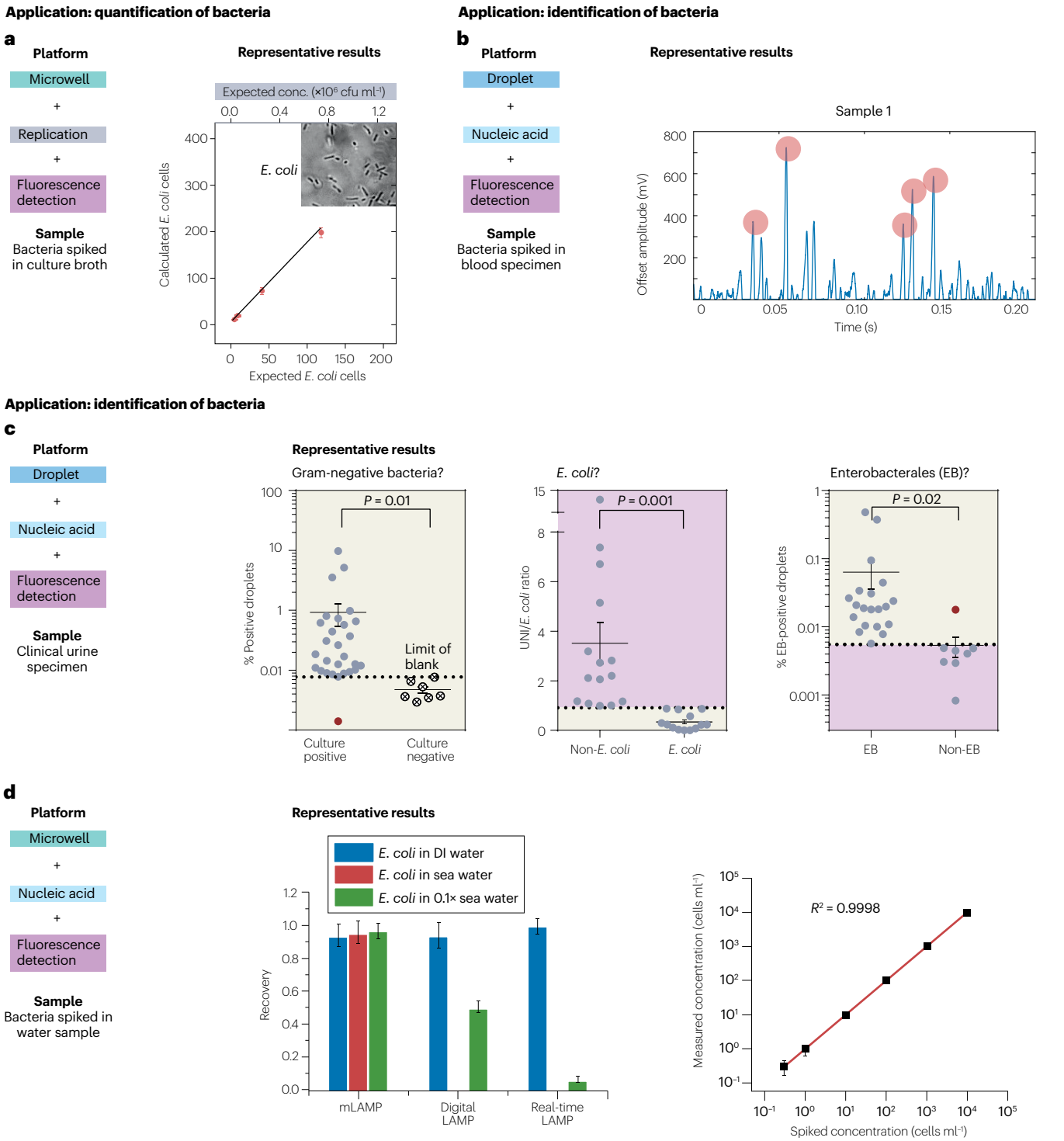


Fig. 4 | Applications of single-cell pathogen diagnostics for pathogen detection and identification. a, The presence of rare bacteria is quantitatively detected by measuring the metabolism product from individual bacteria captured in microwells[125]. **b**, Antibiotic-resistant bacteria spiked in whole blood are identified by molecularly probing the resistant DNA markers released from a single bacterium in droplets[147]. **c**, Bacteria in clinical urine samples are identified by examining 16S ribosomal RNA (rRNA) markers from individual bacteria to determine Enterobacterales and *Escherichia coli* strains[157]. **d**, Bacteria spiked in seawater are identified by detecting mRNA and DNA from a single bacterium captured in microwells[130]. cfu, colony forming unit; LAMP, loop-mediated isothermal amplification. Part **a** reprinted with permission from ref. [125]. Copyright 2018 American Chemical Society. Part **b** reprinted with permission from ref. [147], Royal Society of Chemistry. Part **c** reprinted from ref. [157], CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/). Part **d** reprinted from ref. [130], CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/).

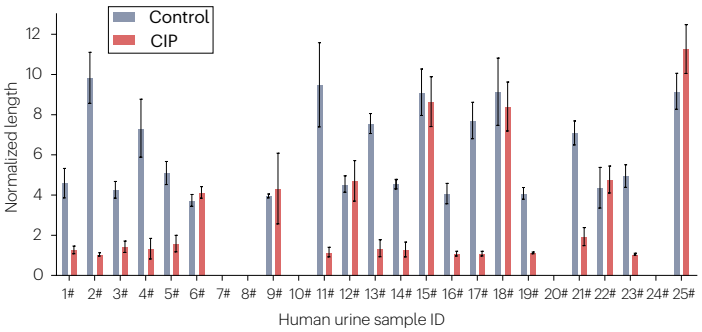
Application: AST (binary call for susceptibility/resistance)

a

Platform
Microchannel + Replication + Optical microscopy

Sample
Clinical urine specimen

Representative results

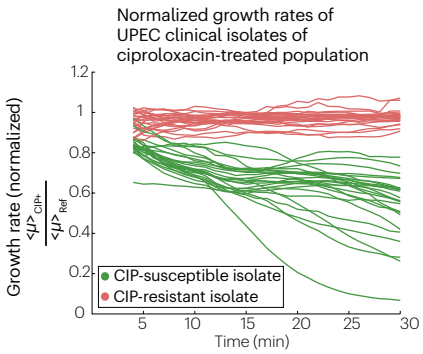


b

Platform
Microchannel + Replication + Optical microscopy

Sample
Clinical isolate spiked in culture broth

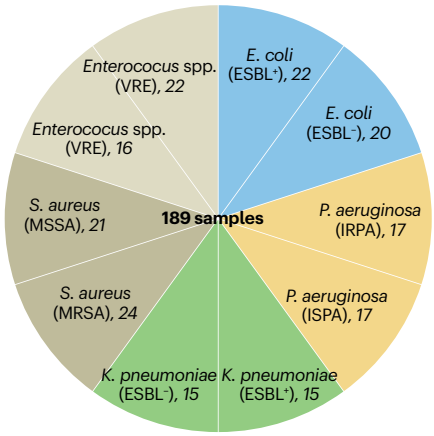
Representative results



c

Platform
Open substrate + Replication + Optical microscopy

Sample
Clinical isolate spiked in culture broth



Representative results

Strain name, number of samples

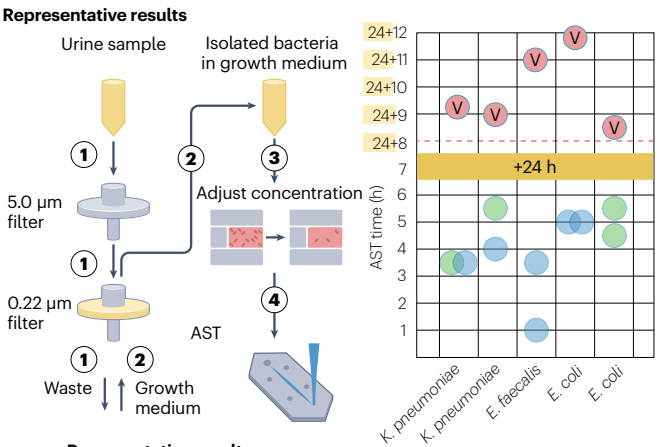
Bacteria	*n* tests	BMD test			Discrepancy number			Discrepancy rate (%)				Average TTR (hours)
		S	I	R	mE	ME	VME	mE	ME	VME	CA	
Gram-negative	1,423	860	80	478	117	26	9	8.15	3.25	1.88	89.3	3
Gram-positive	975	547	31	397	39	10	4	4.00	1.82	1.01	94.6	4
Total	2,398	1,407	116	875	156	36	13	6.51	2.56	1.49	91.5	3–4

d

Platform
Microwell + Replication + Flourescence detection

Sample
Clinical urine specimen

Representative results

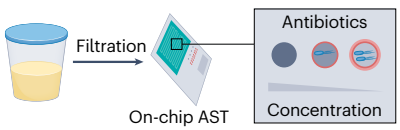


e

Platform
Droplet + Replication + Flourescence detection

Sample
Clinical urine specimen

Application: AST (MIC measurement)



		SXT	CIP	GEN	TOB
Urine 1	**Clinical MIC/**μg ml^{-1}	≤2/38	≤1	≤4	≤4
	INTRP	S/S	S/S	S/S	S/S
Urine 2	**Clinical MIC/**μg ml^{-1}	≤2/38	≤1	≤4	≤4
	INTRP	S/S	S/S	S/S	S/S
Urine 3	**Clinical MIC/**μg ml^{-1}	≤2/38	≤1	≤4	≤4
	INTRP	S/S	S/S	S/S	S/S
Urine 4	**Clinical MIC/**μg ml^{-1}	≥4/76	≤1	≤4	≤4
	INTRP	R/R	S/S	S/S	S/S
Urine 5	**Clinical MIC/**μg ml^{-1}	≤2/38	≤1	≤4	≤4
	INTRP	S/S	S/S	S/S	S/S
Urine 6	**Clinical MIC/** μg ml^{-1}	≥4/76	≥4	≤4	≤4
	INTRP	R/R	R/R	S/S	S/S
Urine 7	**Clinical MIC/** μg ml^{-1}	≥4/76	≤1	≤4	≤4
	INTRP	R/S	S/S	S/S	S/S
Urine 8	**Clinical MIC/**μg ml^{-1}	≥4/76	2	≥32	≤4
	INTRP	R/R	I/I	R/R	S/I

f

Platform
Droplet + Replication + Flourescence detection

Sample
Bacteria spiked in culture broth

Representative results

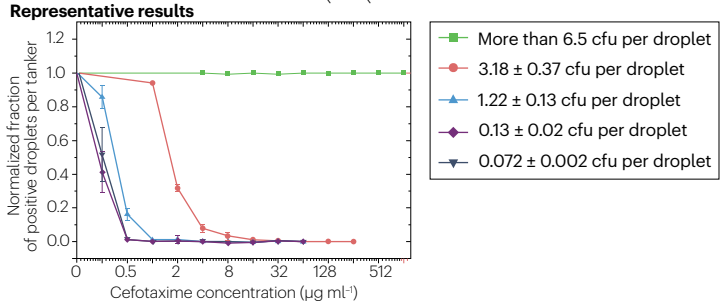


Fig. 5 | Applications of single-cell pathogen diagnostics for AST. a, Pathogen classification was performed by examining the shape and size of bacteria at the single-cell level. Bacterial growth of individual bacteria was monitored to disclose the antimicrobial susceptibility testing (AST) profiles for 25 clinical urine samples with blinded pathogens[111]. **b**, AST profiles of 49 clinical isolates were obtained by measuring bacterial growth of individual bacterial cells captured in microchannels in the presence of antibiotics[110]. **c**, AST of 189 clinical isolates was performed by testing bacterial growth captured at the single-cell level on an open substrate[163]. **d**, AST was implemented for five clinical urine samples across two antibiotics by testing bacterial metabolism confined in microwells[158]. **e**, Multiplex AST profiles of eight clinical urine samples were demonstrated by examining individual bacterial metabolism against four antibiotics with eight concentrations in a picolitre droplet system[201]. **f**, Heteroresistance profiles of a bacterial population were studied by detecting phenotypic growth of individual bacteria under antibiotic conditions in droplets[204]. BMD, broth microdilution; cfu, colony forming unit; CIP, ciprofloxacin; *E. faecalis*, *Enterococcus faecalis*; ESBL, extended-spectrum β-lactamase; IRPA, imipenem-resistant *Pseudomonas aeruginosa*; ISPA, imipenem-susceptible *Pseudomonas aeruginosa*; *K. pneumoniae*, *Klebsiella pneumoniae*; MIC, minimum inhibitory concentration; MRSA, methicillin-resistant *Staphylococcus aureus*;MSSA, methicillin-susceptible *Staphylococcus aureus*; TTR, time to results; UPEC, clinical uropathogenic *Escherichia coli*; VRE, vancomycin-resistant *Enterococcus*. Part **a** reproduced with permission from ref. [111]. Copyright 2019 PNAS. Part **b** reproduced with permission from ref. [110]. Copyright 2017 PNAS. Part **c** reprinted with permission from ref. [163], AAAS. Part **d** reproduced with permission from ref. [158]. Copyright 2017 PNAS. Part **e** reprinted with permission from ref. [201], Wiley. Part **f** reprinted with permission from ref. [204], Royal Society of Chemistry.

detection for *E. coli* and *S. aureus* within 3 h. Importantly, the device quantitatively determines the bacterial concentration by counting the positive wells, supporting the evaluation of the severity of the infection.

For positive samples, the next step is to identify the bacterial species. Identification informs the selection of antibiotics for AST, as the recommended panel of antibiotics to assay varies with bacterial species. Compared with chromogenic agar and mass spectroscopy, which require lengthy culture to obtain mono isolates for detection, single-cell pathogen diagnosis has been applied to rapidly identify pathogens by testing specific gene markers (Fig. 4b–d). Taking this underlying principle, single-cell pathogen identification has been demonstrated for identifying antibiotic-resistant bacteria spiked in blood, where the blood was diluted ten times, with 100% sensitivity and specificity[147]. The resistant gene marker ($bla_{CTX\text{-}M\text{-}14}$) from a single bacterium is released, amplified and detected in individual picolitre droplets[147] (Fig. 4b). This method results in a sensitivity of 10 cfu ml^{-1}. Bacterial load is quantified at the single-cell resolution, with a sample to answer time below 1 h. Similarly, single-cell pathogen identification has been applied to identify bacteria from clinical urine samples, where two fluorogenic probes examined 16S rRNA markers from individual bacteria[157]. Enterobacterales and *E. coli* strains were determined in parallel within 30 min (Fig. 4c). In a clinical study of 50 de-identified patient urine specimens, this approach demonstrated excellent sensitivity and specificity compared with clinical standard methods, suggested by high areas under curves (>0.95) in a receiver-operating curve analysis. Although not a clinical application, a device formed by asymmetric filter membranes has demonstrated a 1-h identification of rare bacteria in a large volume of unprocessed environmental samples (10 ml seawater). This device removes complex chemical and biological components, such as large particles, sand, plankton and small inhibitory molecules, while concentrating bacteria in microwells on the device for single-cell level nucleic acid-based identification. This device not only achieved an exceptional bacteria recovery rate, close to 100%, but also allowed for a wide dynamic range and single-cell sensitivity (0.3–10,000 cells ml^{-1})[130] (Fig. 4d), indicating the potential for adaptation in clinical diagnostics.

Antimicrobial susceptibility testing

Another key application of single-cell pathogen diagnostic platforms is AST. Evaluation of the bacterial growth in the presence of an antibiotic at concentrations defined by CLSI guidelines is used to determine antibiotic susceptibility and the MIC of antibiotic.

A single-cell diagnostic platform has been applied to test the antibiotic susceptibility of bacteria in clinical urine samples to ciprofloxacin within 2 h (ref. [111]). This AST is achieved by directly imaging bacterial growth in microchannels. In a clinical comparison study, this system implemented AST for 25 clinical urine samples with blinded pathogens and achieved 100% agreement with broth dilution methods[111] (Fig. 5a). Similarly, by imaging bacterial growth in microchannels, the susceptibility of clinical uropathogenic *Escherichia coli* (UPEC) isolates towards ciprofloxacin could be uncovered in less than 30 min. This is demonstrated by the testing of 49 clinical UPEC isolates, with 100% agreement with gold-standard disc diffusion measurements[110] (Fig. 5b). Single-cell AST has been demonstrated in a high-throughput manner, where individual bacterial cells are immobilized on the surface of 96-well plates using agarose[163]. The single-cell growth in each well is automatically imaged using time-lapse bright-field microscopy. This automated system performs AST within 4 h and has been applied for testing MICs of 43 antibiotics towards 189 clinical isolates, with 91.5% categorical agreement and 6.51% minor, 2.56% major and 1.49% very major discrepancies[163] (Fig. 5c). An alternative to direct imaging of single-cell growth, metabolic products from bacterial growth have been demonstrated to accomplish AST for five clinical urine samples across two antibiotics in 1–5.5 h (ref. [158]). The antimicrobial resistant profiles were correctly determined for eight out of ten bacteria–antibiotic combinations[158] (Fig. 5d). This scheme has also been developed for multiplex detections of multiple antibiotics across various concentrations by programmable encapsulation of bacteria with on-demand antibiotics in picolitre droplets. The bacterial metabolism of the encapsulated individual bacteria is tested in various antibiotic conditions in ~90 min. This system was applied to test the MIC of four antibiotics for eight clinical urine samples, with 93.8% concordance with the clinical report[201] (Fig. 5e).

In addition to rapid AST, single-cell pathogen diagnostic platforms can also detect heteroresistance, where rare resistant bacteria exist in numerous susceptible bacteria and may predominate and spread after an improper antibiotic course[133,203]. In response, single bacterial growth under a certain antibiotic condition is individually tested in an extremely high-throughput manner in droplets. For example, each concentration of cefotaxime was tested in 1,900 droplets in parallel[204], which captured the heterogeneity in antibiotic response among the population[204] (Fig. 5f).

Notably, emerging methods of combining pathogen identification with AST in one test have shown great merit in promptly delivering comprehensive diagnostic results. Compared with direct AST, a parallelized

pathogen identification test could quantitatively determine the bacterial concentration[205] and its relative changes[206] to complement and assist AST. Furthermore, bacteria captured in adaptable microchannels could be classified according to their physical shape and size[111]. This initial classification guides the selection for proper antibiotic candidates for AST. Alternatively, pathogen identification of single bacteria discretized in picolitre droplets can be detected by its nucleic acids. Meanwhile, quantitative analysis of these nucleic acids, which varies with bacterial growth during antibiotic exposure, has been demonstrated to resolve antibiotic resistance profiles[157]. These techniques have been applied to conduct pathogen identification and AST for clinical urine samples and derive results on timescales as short as tens of minutes.

Reproducibility and data deposition

There are significant variabilities in the design, implementation and application of single-cell pathogen diagnostic platforms. Additionally, there are significant variabilities in microfluidic devices, including manufacturing, assembly, packaging, interconnections, component integration and flow control[207]. Although there is recognition of the importance of standardization in microfluidic devices[208], the research community has been slow to adopt this practice[207]. Nevertheless, as the research community becomes increasingly aware of the need for and benefits of research reproducibility, transparency and standardization, there is optimism that standardized single-cell pathogen diagnostic platforms will become typical in the near future. In the meantime, perspectives are offered here towards achieving reproducible and standardized single-cell pathogen diagnostic platforms.

Although there are currently no broadly agreed reporting standards for single-cell pathogen diagnostic platforms, for further progress and development it is essential that experimental details are published completely and transparently. These details should include, but are not limited to, device design and fabrication, including dimensions of microfluidic structures within devices, photolithography masks in appropriate files and fabrication protocols; device operation, such as surface treatment, oil composition, sample and oil flow rates, and droplet size; single-cell detection assay parameters and conditions, for example DNA/RNA extraction, amplification and substrate concentrations; single-cell assay output measurement parameter and conditions, including microscopy magnification, fluorescence excitation source power, camera exposure time and bias voltage; and data analysis, for example the number of analysed cells, curve fitting functions and parameters, number of replicates per condition, number of tested and replicated devices, and statistical analysis methods. It is helpful to provide schematics for external instrumentation, especially if the instrumentation is custom-built. It is also helpful to describe data acquisition hardware and software and deposit software codes that are used for data analysis, for example by using a repository such as GitHub.

An alternative to standardizing microfluidic devices, external instruments and experimental procedures is to standardize benchmark experiments for specific application areas and ensure that new single-cell pathogen diagnostic platforms acquire the appropriate benchmark results. For initial screening of bacterial infection and bacterial load quantification, single-cell pathogen diagnostic platforms should have a linear relationship between input quantities of bacteria and detected quantities of bacteria across a reasonable dynamic range, with a clearly defined limit of detection. In practice, however, bacteria can be viable but non-culturable[209,210] and can be lost in devices, for example at interconnections. These systemic causes of inaccurate quantification can increase or decrease the slope of the linear relationship but should not distort the linearity. The limit of detection should be clearly defined based on the no-bacteria control, using the threshold of mean + three standard deviations. Only 99.7% of data occur within three standard deviations of the mean, in a normal distribution, so if the number of bacterial cells, microwells or droplets to be detected is large, a higher threshold such as four to six standard deviations should be used. For bacterial species identification, the specificity should be verified against non-target bacterial species, preferably using reference strains in addition to clinical isolates. For AST, the reference strains, culture broth, determining MIC for reference strains and categorizing susceptibility/resistance should be standardized. Essential agreements between MICs measured by new single-cell pathogen diagnostic platforms and MICs measured by a standard method, such as broth microdilution, or reference MICs provided by the CLSI guide should be reported. Moreover, categorical agreement between new single-cell pathogen diagnostic platforms and standard methods must be quantified and reported. For pilot clinical studies, details should explain the number of specimens, whether the specimens are pre-screened and with which standard diagnostic method. The sensitivity and specificity with respect to the standard diagnostic method should be reported. Additional context for sensitivity and specificity values, such as using pre-screened specimens for proof of feasibility, should be welcomed rather than discouraged.

To improve reproducibility, transparency and standardization, deposition of experimental data into a central repository such as GitHub or publication of experimental protocols and videos in peer-reviewed journals should be encouraged. Both raw and curated data — images, fluorescence signals, electrical signals — along with applicable software tools and data analysis protocols, could be shared. Alternatively, detailed experimental protocols could be submitted to peer-reviewed protocol journals, including video-based protocols, such as the protocol on microfluidic picolitre bioreactor for microbial single-cell analysis[211]. The community could advocate for more video-based protocols in peer-reviewed journals, and gradually view them as a requirement for research.

Limitations and optimizations

Although single-cell pathogen diagnostics creates new opportunities to address limitations in conventional diagnostic methods, it presents its own limitations and challenges. When a single-cell technique is implemented to bypass the culture step, fundamental issues associated with the clinical samples can present limitations in the analysis. These issues create challenges, alongside various steps of the single-cell diagnosis workflow, which require careful optimization to ensure assay reliability. Notably, these limitations are interrelated and should be considered as a whole when integrating procedures. This section highlights notable limitations and discusses areas of optimization required for translating the single-cell diagnosis platforms towards managing infectious diseases.

Sample complexity

A major challenge for single-cell pathogen diagnostics stems from the complexity of clinical samples. For example, it is difficult to perform confirmatory diagnosis of different samples, from a blood sample with 1–100 cfu ml^{-1} pathogen for sepsis, a urine sample with more than 10^5 cfu ml^{-1} of a single bacterial species for a UTI or a vaginal swab with mixed flora of bacteria for bacterial vaginosis. The complex matrices in clinical samples may hinder assay performance. Host cells can clog devices, variable concentrations of proteins and salts can inhibit detection methods and a large range of pH values in urine samples

may affect the detection assays. Simple dilution/buffer exchange and filtration methods are often included in assays to reduce the matrix effect[158]. However, the increased volume and bacterial loss during sample preparation can decrease assay sensitivity, making it necessary to couple these approaches with enrichment or sample preparation steps. The mismatch between large sample volumes from clinical samples and the small sample volumes that can be analysed adds to the challenge. In single-cell pathogen diagnostic platforms, each bacterium is confined in a picolitre to nanolitre volume, and most single-cell capturing techniques only handle a sample volume in the order of microlitres. This means platforms typically analyse only a fraction of the clinical specimens. If only a small number of bacteria are analysed, the sensitivity requirement becomes critical. To ensure a sufficient number of bacteria are captured for the analysis, single-cell pathogen diagnostic platforms often require a bacterial concentration larger than 10^3 cfu ml^{-1} (or 1 cfu μl^{-1} per microlitre), determined by the total analysis volumes of single-cell isolation techniques. This limitation is worsened by the large surface to volume ratio of microfluidic devices, which are prone to biofouling and loss of the target cells[212]. The sampling issue is particularly challenging for infections that have a low bacterial count, such as bloodstream infections, or a limited sample volume, for example neonatal infections. The combination of these issues demands a high bacterial load and sampling efficiency for a reliable clinical diagnosis. Microchannels and droplets have the capacity to process samples continuously and can potentially alleviate this sample volume limitation, albeit at the expense of assay time. As an addendum to addressing this limitation, a sample pre-processing step that enriches the bacterial sample should be introduced for a robust single-cell analysis. Several biochemical or physical enrichment methods have been developed and can be integrated into the diagnosis workflow to prevent undersampling and minimize false negatives[213–215].

Testing throughput

Low testing throughput represents another limitation for single-cell pathogen diagnostic platforms. For example, most single-cell AST platforms to date can only test one antibiotic condition per device[110–112,157,164]. They are unable to provide additional AST information beyond the binary call of susceptibility or resistance. On the other hand, both CLSI and EUCAST guidelines recommend generating an antibiogram by testing multiple antibiotics at predefined, clinically relevant titrations against the causative bacteria. The antibiogram is essential for selecting appropriate antibiotics, ensuring effective treatments, facilitating antibiotic stewardship and monitoring MICs towards resistance surveillance. Among the single-cell pathogen diagnostic platforms discussed in this Primer, a few platforms increase testing throughput by incorporating multiple identical units in a single device[125,216]. Although practical, this strategy can only test a few conditions. Other strategies for further increasing the testing throughput have been demonstrated via parallel processing with an automated instrument[163] or with colour-coded beads[217] and via sequential analysis in droplet devices that can generate multiple groups of droplets, similar to an assembly line[201].

Comprehensive and accurate diagnosis

To date, few single-cell pathogen diagnostic platforms have comprehensively integrated initial screening of bacterial infection, quantification of bacterial load, identification of bacterial species and AST capabilities. Most single-cell pathogen diagnostics can only provide partial diagnosis. For example, platforms based on replication and physiology generally cannot identify the species of bacteria. Although they enable rapid AST, these single-cell platforms are restricted to testing clinical isolates, as they are unable to process clinical samples that contain background bacteria and flora. As a result, it is critical to couple compatible assays that can perform bacterial identification. For example, this can be done by combining a nucleic acid hybridization assay with a cell imaging assay[155] or resazurin assay. On the other hand, for nucleic acid assays, typical amplification and hybridization approaches cannot distinguish between live and dead cells. A false positive pathogen call based on dead cells may result in unnecessary antibiotic usage, which can disrupt the patient's microbiota and lead to secondary infections. Finally, it should be noted that detection of Gram-positive bacteria is currently less frequent than for Gram-negative bacteria.

Clinical translation

The ultimate goal for the development of single-cell pathogen diagnostic platforms is clinical deployment. For clinical translation of single-cell diagnostics, biological samples, disease parameters and potential test sites — for example, clinical laboratories, outpatient clinics or hospital bedsides — must be considered during development. Unfortunately, existing platforms are often complex and expensive, jeopardizing their clinical utility. One critical consideration is the simplicity in practice. An ideal device implements a simple sample to result diagnostic model and integrates core functions with user-friendly interfaces[218,219]. Also, it is important to design a device that requires little to no training to fit both in central microbiology laboratories and non-tradition settings, such as clinics[220]. It would be more useful to fully diagnose bacterial infections at the point of care to offer actionable treatment advice, which may dramatically improve clinical outcomes[221]. In addition, reducing cost is a critical factor in facilitating the widespread adoption of a device[219]. These limitations could be systematically addressed through collaboration between research and clinical laboratories, which can serve as the first step in the translational pathway. In summary, it is envisioned that single-cell analysis techniques may transform pathogen diagnostics, with future use in personalized medicine and precise infection management[222].

Outlook

The limitations of existing single-cell pathogen diagnostic platforms offer opportunities for future advances. This section highlights research opportunities, emerging research trends and potential research directions. Collectively, these discussions form an outlook for the future development of single-cell pathogen diagnostics.

Development of new technologies

A research priority for single-cell pathogen diagnostics is developing platforms that can comprehensively integrate initial screening, bacterial load quantification, species identification and AST, directly from clinical samples with minimal or no sample preparation. Additionally, where the test will be deployed dictates the allowable complexity of the testing procedure. Although single-cell pathogen diagnostic platforms are not expected to fully meet the WHO ASSURED criteria — affordable, sensitive, specific, user-friendly, rapid, robust, equipment-free and delivered to those who need it[223–225] — for point-of-care diagnostic testing, they should aim to achieve these criteria. Currently, however, most single-cell pathogen diagnostics are too complex and delicate, as they require dedicated instrumentations and skilled technicians to run the assays. During development, efforts should be made to simplify the

Glossary

Categorical agreement
A metric for evaluating single-cell antimicrobial susceptibility testing platforms, defined as the percentage of clinical isolates classified in the same susceptibility category as the reference method.

Capillary effect
A physical phenomenon where liquid flows in a narrow space due to capillary force. This flow action is accomplished autonomously without the assistance of an external instrument.

Discretization
The compartmentalization of a bulk sample into numerous discrete and isolated volumes, typically using microfluidic technology, that enables single cells to be encapsulated and spatially constrained.

Essential agreements
A metric for evaluating single-cell antimicrobial susceptibility testing platforms, defined as the percentage of measured minimum inhibitory concentrations (MICs) within a single doubling dilution of the reference MICs.

Heteroresistance
A phenotype in which subpopulations of bacterial cells have higher antibiotic resistance compared with the susceptible main population.

Inoculum effect
The effect where the minimum inhibitory concentration of an antimicrobial reagent for inhibiting bacterial growth increases with the bacterial concentrations in the test.

Limit of detection
The minimum amount of a target of interest, such as bacteria, that can be distinguished from the absence of the target at a specified confidence level.

Soft lithography
A technique for fabricating microfluidic devices by conformably replicating elastic structures from a rigid mould. The fabrication is highly repeatable. It is called soft as this technique fabricates elastic materials.

Wheatstone bridge circuit
An electrical circuit that is able to measure the electrical resistance of a variable target with high accuracy. This circuit consists of two parallelized branches and each branch includes two electrical resistors. The electrical potential between the electrical resistors is measured on each branch and the potential difference between the two points is used to monitor the change of the target resistor on one branch.

system and automate implementation. Simple imaging systems, such as those found in cell phone cameras, can have remarkable resolution, are adequate for detection, are user-friendly and are cost-efficient compared with dedicated microscope-based systems[182].

Research and development of new single-cell pathogen diagnostic platforms would benefit from incorporating emerging technologies and testing alternative antibacterial strategies. Notable examples of emerging technologies include machine learning and deep learning, which have already proved useful for enhancing the performance of several single-cell pathogen diagnostic platforms[14,28,206,217,226–228]. Nanopore sequencing has enhanced the ability to study complex microbial samples through the possibility of sequencing long reads in real time using inexpensive and portable technologies. It has been shown that nanopore sequencing can be used to monitor the human microbiome and in infectious disease diagnostics[229]. Marriage between single-cell pathogen diagnostic platforms and nanopore sequencing is worth exploring and may catalyse new diagnostic capabilities. Finally, the application of single-cell pathogen diagnostics can be broadened to evaluate various antibacterial strategies, such as antibiotic combinations[135,230,231], nanoparticles[232] and antimicrobial peptides[233], that may generate new insights through single-cell analysis.

Detection of fastidious bacteria

Beyond detection of common pathogens, which are easily cultured in clinical laboratories, single-cell pathogen diagnostics has the potential to improve diagnosis of fastidious bacteria, which are difficult to cultivate and not routinely considered by standard clinical analysis. For example, AST for *M. tuberculosis*, the causative agent of tuberculosis, can take weeks to months. Rapid PCR tests such as Cepheid's MTB/RIF provide rapid identification and rifampicin susceptibility, but are limited in the scope of antimicrobials and resistance mechanisms. Analysis of single cells in an agarose matrix under a gradient of antibiotics has been demonstrated to provide a phenotypic AST for *M. tuberculosis* in days[178,234]. Similarly, the growth rate of *Borrelia* spirochaetes, the causative agent of Lyme disease, also limits clinical detection. Indirect serological methods are typically used to diagnose Lyme disease. However, single-cell biosensing assays may enable direct pathogen detection, improving early diagnosis and treatment[235].

Analysis of mixed samples

Single-cell pathogen diagnostics have a unique capacity to detect mixed samples, with the potential to analyse microbiota. A recent single-cell pathogen diagnostic platform — combining microchannels, bacterial replication assays, nucleic acid detection assays, optical microscopy and fluorescence microscopy — demonstrated the determination of susceptibility profiles for up to four antibiotics for individual bacterial species in mixed samples, with combinations of up to seven species, in 2 h (ref. [236]). This capability can remove the need for culture-based isolation of individual bacterial species prior to AST. Such single-cell AST against multiple bacterial species may represent a new frontier for AST, although it is critical to carefully evaluate the results against standard AST. Understanding the complexity of the gut microbiota can help understand the influence of bacteria on health and disease[132]. Single-cell culture has been shown to achieve a larger representation of the microorganisms in stool than conventional culture, specifically enhancing detection of rare and slow growing species[237]. Further, single-cell analysis could identify antimicrobial resistant species not detected by agar plate cultivation. Having a more complete picture of the microbiota and the presence of antibiotic-resistant microorganisms could aid in understanding several diseases and treatment responses.

Application to diseases

Single-cell pathogen diagnostics can be broadly applied to diagnose various infectious diseases. Among them, UTIs remain a priority. UTIs are among the most common infections in both community (uncomplicated UTI) and health-care-associated (catheter-associated UTI) settings. Rapid diagnostics, providing identification and AST present an opportunity for targeted treatment, reducing the use of broad-spectrum antibiotics and promoting better antibiotic stewardship. In addition to their prevalence and significance, UTIs present a less severe challenge when compared with bloodstream infections because bacterial concentrations in infected urine are high ($\geq 10^5$ cfu ml^{-1}) and the time frame from sample collection to initiation of treatment can be longer. UTIs can be viewed as an important milestone for the development of single-cell pathogen diagnostic platforms. By contrast, bloodstream infections may be viewed as a long-term challenge that single-cell

pathogen diagnostics has the potential to address. This is because bloodstream infections may be life threatening and timely treatment is essential. If sepsis is suspected, broad-spectrum antimicrobial treatment is initiated without waiting for pathogen identification. Single-cell pathogen diagnostics that can provide rapid pathogen identification, resolve polymicrobial infection and correlate pathogen quantity with disease severity can improve patient care by rapidly narrowing in on the most effective, targeted treatment before antibiotics are administered. Directly testing blood samples would significantly reduce the time to results and would have the greatest clinical impact. However, diagnostic platforms that can shorten the time from positive blood culture to diagnosis would still be useful. Assays under development using 3D particle sorting, such as the integrated comprehensive droplet digital detection platform, enrich the target pathogen for direct detection[90].

Demonstration of clinical utility

A priority for translation of single-cell pathogen diagnostics is to demonstrate the clinical reliability and utility. Deployment in clinical laboratories will likely be the first step in the translational pathway. Correlating single-cell assay parameters with existing guidelines to provide comparable information is a key step for translation of some devices. Conversely, if a device only provides partial information, such as pathogen classification and susceptibility for a limited number and concentration of antibiotics, clinical testing to show utility for improving patient care and outcomes becomes critical. During such a study, user feedback should be collected to improve efforts towards simplifying the platform and automating implementation.

Conclusions

This Primer provides an end-to-end guide for assessing, designing, implementing and applying single-cell pathogen diagnostic platforms, with the vision that, in the near future, such platforms can screen bacterial infection, identify bacterial species and perform comprehensive AST within the clinically relevant time frame for enabling personalized antibiotic treatments and precise infection management. Although existing platforms have yet to realize this vision, the current trajectory of advancement, combined with the emergence of new tools, offers a promising outlook. It is hoped that this Primer can inspire and propel further advances in single-cell pathogen diagnostic platforms. There is optimism that precise and personalized bacterial infection treatments, enabled by effective single-cell pathogen diagnostics, will become a reality in the near future.

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References

1. Molton, J. S., Tambyah, P. A., Ang, B. S. P., Ling, M. L. & Fisher, D. A. The global spread of healthcare-associated multidrug-resistant bacteria: a perspective from Asia. *Clin. Infect. Dis.* **56**, 1310–1318 (2013).
2. World Health Organization. *Global Antimicrobial Resistance Surveillance System — Manual for Early Implementation* (World Health Organization, 2015).
3. Zowawi, H. M. et al. The emerging threat of multidrug-resistant Gram-negative bacteria in urology. *Nat. Rev. Urol.* **12**, 570–584 (2015).
4. US Centers for Disease Control and Prevention. *Antibiotic Resistance Threats in the United States* (CDC, 2013).
5. US Centers for Disease Control and Prevention. *Antibiotic Resistance Threats in the United States, 2019* (CDC, 2019).
6. Chait, R., Vetsigian, K. & Kishony, R. What counters antibiotic resistance in nature? *Nat. Chem. Biol.* **8**, 2–5 (2012).
7. Blair, J. M. A., Webber, M. A., Baylay, A. J., Ogbolu, D. O. & Piddock, L. J. V. Molecular mechanisms of antibiotic resistance. *Nat. Rev. Microbiol.* **13**, 42–51 (2015).
8. Brook, I., Wexler, H. M. & Goldstein, E. J. C. Antianaerobic antimicrobials: spectrum and susceptibility testing. *Clin. Microbiol. Rev.* **26**, 526–546 (2013).
9. O'Connell, K. M. G. et al. Combating multidrug-resistant bacteria: current strategies for the discovery of novel antibacterials. *Angew. Chem. Int. Ed.* **52**, 10706–10733 (2013).
10. Lewis, K. Platforms for antibiotic discovery. *Nat. Rev. Drug Discov.* **12**, 371–387 (2013).
11. Kinch, M. S., Patridge, E., Plummer, M. & Hoyer, D. An analysis of FDA-approved drugs for infectious disease: antibacterial agents. *Drug Discov. Today* **19**, 1283–1287 (2014).
12. Clinical and Laboratory Standards Institute. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically* 11th edn (Clinical and Laboratory Standards Institute, 2018).
13. Chen, L. et al. Direct-qPCR assay for coupled identification and antimicrobial susceptibility testing of *Neisseria gonorrhoeae*. *ACS Infect. Dis.* **4**, 1377–1384 (2018).
14. Athamanolap, P., Hsieh, K., Chen, L., Yang, S. & Wang, T.-H. Integrated bacterial identification and antimicrobial susceptibility testing using PCR and high-resolution melt. *Anal. Chem.* **89**, 11529–11536 (2017).
15. Poritz, M. A. et al. FilmArray, an automated nested multiplex PCR system for multi-pathogen detection: development and application to respiratory tract infection. *PLoS ONE* **6**, e26047 (2011).
16. Lee, J.-G. et al. Microchip-based one step DNA extraction and real-time PCR in one chamber for rapid pathogen identification. *Lab Chip* **6**, 886–895 (2006).
17. Vora, G. J., Meador, C. E., Stenger, D. A. & Andreadis, J. D. Nucleic acid amplification strategies for DNA microarray-based pathogen detection. *Appl. Env. Microbiol.* **70**, 3047–3054 (2004).
18. Kodani, M. & Winchell, J. M. Engineered combined-positive-control template for real-time reverse transcription-PCR in multiple-pathogen-detection assays. *J. Clin. Microbiol.* **50**, 1057–1060 (2012).
19. Gootenberg, J. S. et al. Multiplexed and portable nucleic acid detection platform with Cas13, Cas12a, and Csm6. *Science* **360**, 439–444 (2018).
20. Bian, K. et al. Scanning probe microscopy. *Nat. Rev. Methods Primers* **1**, 36 (2021).
21. Skinner, J. P. et al. Simplified confocal microscope for counting particles at low concentrations. *Rev. Sci. Instrum.* **84**, 074301 (2013).
22. Wang, S. et al. Label-free imaging, detection, and mass measurement of single viruses by surface plasmon resonance. *Proc. Natl Acad. Sci. USA* **107**, 16028–16032 (2010).
23. Homola, J. Surface plasmon resonance sensors for detection of chemical and biological species. *Chem. Rev.* **108**, 462–493 (2008).
24. Wang, R. et al. cAST: capillary-based platform for real-time phenotypic antimicrobial susceptibility testing. *Anal. Chem.* **92**, 2731–2738 (2020).
25. Cansizoglu, M. F., Tamer, Y. T., Farid, M., Koh, A. Y. & Toprak, E. Rapid ultrasensitive detection platform for antimicrobial susceptibility testing. *PLoS Biol.* **17**, e3000291 (2019).
26. Volbers, D. et al. Interference disturbance analysis enables single-cell level growth and mobility characterization for rapid antimicrobial susceptibility testing. *Nano Lett.* **19**, 643–651 (2019).
27. Leonard, H., Halachmi, S., Ben-Dov, N., Nativ, O. & Segal, E. Unraveling antimicrobial susceptibility of bacterial networks on micropillar architectures using intrinsic phase-shift spectroscopy. *ACS Nano* **11**, 6167–6177 (2017).
28. Zhou, K. et al. Dynamic laser speckle imaging meets machine learning to enable rapid antibacterial susceptibility testing (DyRAST). *ACS Sens.* **5**, 3140–3149 (2020).
29. Mo, M. et al. Rapid antimicrobial susceptibility testing of patient urine samples using large volume free-solution light scattering microscopy. *Anal. Chem.* **91**, 10164–10171 (2019).
30. Sin, M. L. Y. et al. In situ electrokinetic enhancement for self-assembled-monolayer-based electrochemical biosensing. *Anal. Chem.* **84**, 2702–2707 (2012).
31. Sin, M. L., Gau, V., Liao, J. C. & Wong, P. Integrated microfluidic systems for molecular diagnostics: a universal electrode platform for rapid diagnosis of urinary tract infections. *IEEE Nanotechnol. Mag.* **7**, 31–37 (2013).
32. Liu, T. et al. Electrokinetic stringency control in self-assembled monolayer-based biosensors for multiplex urinary tract infection diagnosis. *Nanomed. Nanotechnol. Biol. Med.* **10**, 159–166 (2014).
33. Gao, J. et al. A multiplex electrochemical biosensor for bloodstream infection diagnosis. *SLAS Technol. Translating Life Sci. Innov.* **22**, 466–474 (2017).
34. Altobelli, E. et al. Integrated biosensor assay for rapid uropathogen identification and phenotypic antimicrobial susceptibility testing. *Eur. Urol. Focus* **3**, 293–299 (2016).
35. Mach, K. E. et al. Development of a biosensor-based rapid urine test for detection of urogenital schistosomiasis. *PLoS Negl. Trop. Dis.* **9**, e0003845 (2015).
36. Zhang, M. et al. Rapid determination of antimicrobial susceptibility by stimulated Raman scattering imaging of D_2O metabolic incorporation in a single bacterium. *Adv. Sci.* **7**, 2001452 (2020).
37. Yang, K. et al. Rapid antibiotic susceptibility testing of pathogenic bacteria using heavy-water-labeled single-cell Raman spectroscopy in clinical samples. *Anal. Chem.* **91**, 6296–6303 (2019).
38. Hong, W. et al. Antibiotic susceptibility determination within one cell cycle at single-bacterium level by stimulated Raman metabolic imaging. *Anal. Chem.* **90**, 3737–3743 (2018).
39. Wang, H. et al. Simultaneous capture, detection, and inactivation of bacteria as enabled by a surface-enhanced Raman scattering multifunctional chip. *Angew. Chem. Int. Ed.* **54**, 5132–5136 (2015).

40. Cheng, I. F., Chang, H.-C., Chen, T.-Y., Hu, C. & Yang, F.-L. Rapid (<5 min) identification of pathogen in human blood by electrokinetic concentration and surface-enhanced Raman spectroscopy. *Sci. Rep.* **3**, 2365 (2013).
41. Czilwik, G. et al. Rapid and fully automated bacterial pathogen detection on a centrifugal-microfluidic LabDisk using highly sensitive nested PCR with integrated sample preparation. *Lab Chip* **15**, 3749–3759 (2015).
42. Kalsi, S. et al. Rapid and sensitive detection of antibiotic resistance on a programmable digital microfluidic platform. *Lab Chip* **15**, 3065–3075 (2015).
43. Dou, M., Dominguez, D. C., Li, X., Sanchez, J. & Scott, G. A versatile PDMS/paper hybrid microfluidic platform for sensitive infectious disease diagnosis. *Anal. Chem.* **86**, 7978–7986 (2014).
44. Fernández-Carballo, B. L. et al. Low-cost, real-time, continuous flow PCR system for pathogen detection. *Biomed. Microdevices* **18**, 34 (2016).
45. Hou, H. W., Bhattacharyya, R. P., Hung, D. T. & Han, J. Direct detection and drug-resistance profiling of bacteremias using inertial microfluidics. *Lab Chip* **15**, 2297–2307 (2015).
46. Schwartz, O. & Bercovici, M. Microfluidic assay for continuous bacteria detection using antimicrobial peptides and isotachophoresis. *Anal. Chem.* **86**, 10106–10113 (2014).
47. Tsou, P.-H. et al. Rapid antibiotic efficacy screening with aluminum oxide nanoporous membrane filter-chip and optical detection system. *Biosens. Bioelectron.* **26**, 289–294 (2010).
48. Wang, C.-H., Lien, K.-Y., Wu, J.-J. & Lee, G.-B. A magnetic bead-based assay for the rapid detection of methicillin-resistant *Staphylococcus aureus* by using a microfluidic system with integrated loop-mediated isothermal amplification. *Lab Chip* **11**, 1521–1531 (2011).
49. Tu, H. et al. Profiling of immune–cancer interactions at the single cell level using microfluidic well array. *Analyst* https://doi.org/10.1039/D0AN00110D (2020).
50. Li, H., Garner, T., Diaz, F. & Wong, P. K. A multiwell microfluidic device for analyzing and screening nonhormonal contraceptive agents. *Small* **15**, 1901910 (2019).
51. Takagi, R. et al. A microfluidic microbial culture device for rapid determination of the minimum inhibitory concentration of antibiotics. *Analyst* **138**, 1000–1003 (2013).
52. Mohan, R. et al. A multiplexed microfluidic platform for rapid antibiotic susceptibility testing. *Biosens. Bioelectron.* **49**, 118–125 (2013).
53. He, J. et al. A novel microbead-based microfluidic device for rapid bacterial identification and antibiotic susceptibility testing. *Eur. J. Clin. Microbiol. Infect. Dis.* **33**, 2223–2230 (2014).
54. Chen, C. H. et al. Rapid antimicrobial susceptibility testing using high surface-to-volume ratio microchannels. *Anal. Chem.* **82**, 1012 (2010).
55. Yi, Q. et al. Direct antimicrobial susceptibility testing of bloodstream infection on SlipChip. *Biosens. Bioelectron.* **135**, 200–207 (2019).
56. Goel, M., Verma, A. & Gupta, S. Electric-field driven assembly of live bacterial cell microarrays for rapid phenotypic assessment and cell viability testing. *Biosens. Bioelectron.* **111**, 159–165 (2018).
57. Davenport, M. et al. New and developing diagnostic technologies for urinary tract infections. *Nat. Rev. Urol.* **14**, 296–310 (2017).
This review provides vision of emerging diagnostic tools towards UTIs.
58. Tay, A., Pavesi, A., Yazdi, S. R., Lim, C. T. & Warkiani, M. E. Advances in microfluidics in combating infectious diseases. *Biotechnol. Adv.* **34**, 404–421 (2016).
59. Bauer, K. A., Perez, K. K., Forrest, G. N. & Goff, D. A. Review of rapid diagnostic tests used by Antimicrobial Stewardship Programs. *Clin. Infect. Dis.* **59**, S134–S145 (2014).
60. Sin, M. L. Y., Mach, K. E., Wong, P. K. & Liao, J. C. Advances and challenges in biosensor-based diagnosis of infectious diseases. *Expert. Rev. Mol. Diagn.* **14**, 225–244 (2014).
61. Shin, D. J., Andini, N., Hsieh, K., Yang, S. & Wang, T.-H. Emerging analytical techniques for rapid pathogen identification and susceptibility testing. *Annu. Rev. Anal. Chem.* **12**, 41–67 (2019).
62. Surrette, C. et al. Rapid microbiology screening in pharmaceutical workflows. *SLAS Technol. Translating Life Sci. Innov.* **23**, 387–394 (2018).
63. Li, Y., Yang, X. & Zhao, W. Emerging microtechnologies and automated systems for rapid bacterial identification and antibiotic susceptibility testing. *SLAS Technol. Translating Life Sci. Innov.* **22**, 585–608 (2017).
64. Dietvorst, J., Vilaplana, L., Uria, N., Marco, M.-P. & Muñoz-Berbel, X. Current and near-future technologies for antibiotic susceptibility testing and resistant bacteria detection. *TrAC. Trends Anal. Chem.* **127**, 115891 (2020).
65. Idelevich, E. A. & Becker, K. How to accelerate antimicrobial susceptibility testing. *Clin. Microbiol. Infect.* **25**, 1347–1355 (2019).
66. van Belkum, A. et al. Innovative and rapid antimicrobial susceptibility testing systems. *Nat. Rev. Microbiol.* **18**, 299–311 (2020).
This review overviews detailed technical progress for rapid AST.
67. Zhang, K., Qin, S., Wu, S., Liang, Y. & Li, J. Microfluidic systems for rapid antibiotic susceptibility tests (ASTs) at the single-cell level. *Chem. Sci.* https://doi.org/10.1039/D0SC01353F (2020).
68. Li, H., Morowitz, M., Thomas, N. & Wong, P. K. Rapid single-cell microbiological analysis: toward precision management of infections and dysbiosis. *SLAS Technol. Translating Life Sci. Innov.* **24**, 603–605 (2019).
69. Trotter, A. J., Aydin, A., Strinden, M. J. & O'Grady, J. Recent and emerging technologies for the rapid diagnosis of infection and antimicrobial resistance. *Curr. Opin. Microbiol.* **51**, 39–45 (2019).
70. Bard, J. D. & Lee, F. Why can't we just use PCR? The role of genotypic versus phenotypic testing for antimicrobial resistance testing. *Clin. Microbiol. Newsl.* **40**, 87–95 (2018).
71. Hsieh, K., Mach, K. E., Zhang, P., Liao, J. C. & Wang, T.-H. Combating antimicrobial resistance via single-cell diagnostic technologies powered by droplet microfluidics. *Acc. Chem. Res.* **55**, 123–133 (2022).
72. Postek, W. & Garstecki, P. Droplet microfluidics for high-throughput analysis of antibiotic susceptibility in bacterial cells and populations. *Acc. Chem. Res.* **55**, 605–615 (2022).
73. Qin, N., Zhao, P., Ho, E. A., Xin, G. & Ren, C. L. Microfluidic technology for antibacterial resistance study and antibiotic susceptibility testing: review and perspective. *ACS Sens.* **6**, 3–21 (2021).
74. Khan, Z. A., Siddiqui, M. F. & Park, S. Progress in antibiotic susceptibility tests: a comparative review with special emphasis on microfluidic methods. *Biotechnol. Lett.* **41**, 221–230 (2019).
75. Wu, F. & Dekker, C. Nanofabricated structures and microfluidic devices for bacteria: from techniques to biology. *Chem. Soc. Rev.* **45**, 268–280 (2016).
76. Ruszczak, A., Bartkova, S., Zapotoczna, M., Scheler, O. & Garstecki, P. Droplet-based methods for tackling antimicrobial resistance. *Curr. Opin. Biotechnol.* **76**, 102755 (2022).
77. Rhee, C. et al. Prevalence of antibiotic-resistant pathogens in culture-proven sepsis and outcomes associated with inadequate and broad-spectrum empiric antibiotic use. *JAMA Netw. Open* **3**, e202899 (2020).
78. Umemura, Y. et al. Current spectrum of causative pathogens in sepsis: a prospective nationwide cohort study in Japan. *Int. J. Infect. Dis.* **103**, 343–351 (2021).
79. Ronald, A. The etiology of urinary tract infection: traditional and emerging pathogens. *Disease-a-Month* **49**, 71–82 (2003).
80. Jones, R. N. Microbial etiologies of hospital-acquired bacterial pneumonia and ventilator-associated bacterial pneumonia. *Clin. Infect. Dis.* **51**, S81–S87 (2010).
81. Wain, J. et al. Quantitation of bacteria in blood of typhoid fever patients and relationship between counts and clinical features, transmissibility, and antibiotic resistance. *J. Clin. Microbiol.* **36**, 1683–1687 (1998).
82. US Centers for Disease Control and Prevention. *Urinary Tract Infection* (*Catheter-Associated Urinary Tract Infection [CAUTI] and Non-Catheter-Associated Urinary Tract Infection [UTI]*) *Events* (CDC, 2022).
83. Schmiemann, G., Kniehl, E., Gebhardt, K., Matejczyk, M. M. & Hummers-Pradier, E. The diagnosis of urinary tract infection: a systematic review. *Dtsch. Arzteblatt Int.* **107**, 361–367 (2010).
84. Dobrindt, U. et al. Analysis of genome plasticity in pathogenic and commensal *Escherichia coli* isolates by use of DNA arrays. *J. Bacteriol.* **185**, 1831–1840 (2003).
85. Tokel, O. et al. Portable microfluidic integrated plasmonic platform for pathogen detection. *Sci. Rep.* **5**, 9152 (2015).
86. Clermont, O., Bonacorsi, S. & Bingen, E. Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Appl. Environ. Microbiol.* **66**, 4555–4558 (2000).
87. Benserhir, Y. et al. Silicon nanowires-based biosensors for the electrical detection of *Escherichia coli*. *Biosens. Bioelectron.* **216**, 114625 (2022).
88. Chung, H. J., Castro, C. M., Im, H., Lee, H. & Weissleder, R. A magneto-DNA nanoparticle system for rapid detection and phenotyping of bacteria. *Nat. Nanotechnol.* **8**, 369–375 (2013).
89. Choi, J. et al. Direct, rapid antimicrobial susceptibility test from positive blood cultures based on microscopic imaging analysis. *Sci. Rep.* **7**, 1148 (2017).
90. Kang, D.-K. et al. Rapid detection of single bacteria in unprocessed blood using integrated comprehensive droplet digital detection. *Nat. Commun.* **5**, 5427 (2014).
This study presents an integrated comprehensive droplet digital detection system that can detect bacteria in diluted blood at the single-cell resolution.
91. Weibel, D. B., DiLuzio, W. R. & Whitesides, G. M. Microfabrication meets microbiology. *Nat. Rev. Microbiol.* **5**, 209–218 (2007).
This review provides vision at the interdisciplinary level between microfabrication and microbiology.
92. Sun, P. et al. High-throughput microfluidic system for long-term bacterial colony monitoring and antibiotic testing in zero-flow environments. *Biosens. Bioelectron.* **26**, 1993–1999 (2011).
93. Choi, J. et al. Rapid antibiotic susceptibility testing by tracking single cell growth in a microfluidic agarose channel system. *Lab Chip* **13**, 280–287 (2013).
94. Longo, G. et al. Rapid detection of bacterial resistance to antibiotics using AFM cantilevers as nanomechanical sensors. *Nat. Nanotechnol.* **8**, 522–526 (2013).
This study demonstrates an atomic force microscope cantilever-based system to detect bacterial motion in the presence of antibiotics and, thus, determine the antibiotic susceptibility.
95. Tréguier, J. et al. Chitosan films for microfluidic studies of single bacteria and perspectives for antibiotic susceptibility testing. *mBio* **10**, e01375-19 (2019).
96. Syal, K. et al. Antimicrobial susceptibility test with plasmonic imaging and tracking of single bacterial motions on nanometer scale. *ACS Nano* **10**, 845–852 (2016).
This study demonstrates a plasmonic imaging technique to measure nanometre motion of bacterial cells under antibiotic conditions and, thereby, determine the antimicrobial susceptibility.
97. Syal, K. et al. Rapid antibiotic susceptibility testing of uropathogenic *E. coli* by tracking submicron scale motion of single bacterial cells. *ACS Sens.* **2**, 1231–1239 (2017).
98. Kong, T. et al. Adhesive tape microfluidics with an autofocusing module that incorporates CRISPR interference: applications to long-term bacterial antibiotic studies. *ACS Sens.* **4**, 2638–2645 (2019).
99. Li, B. et al. Gradient microfluidics enables rapid bacterial growth inhibition testing. *Anal. Chem.* **86**, 3131–3137 (2014).

100. Liu, Y.-N., Chen, H.-B. & Liu, X.-W. Rapid assessment of water toxicity by plasmonic nanomechanical sensing. *Anal. Chem.* **92**, 1309–1315 (2020).
101. Kohler, A. C., Venturelli, L., Longo, G., Dietler, G. & Kasas, S. Nanomotion detection based on atomic force microscopy cantilevers. *Cell Surf.* **5**, 100021 (2019).
102. Pitruzzello, G., Baumann, C. G., Johnson, S. & Krauss, T. F. Single-cell motility rapidly quantifying heteroresistance in populations of *Escherichia coli* and *Salmonella typhimurium*. *Small Sci.* **2**, 2100123 (2022).
103. Lidstrom, M. E. & Konopka, M. C. The role of physiological heterogeneity in microbial population behavior. *Nat. Chem. Biol.* **6**, 705–712 (2010).
104. Wang, P. et al. Robust growth of *Escherichia coli*. *Curr. Biol.* **20**, 1099–1103 (2010).
105. Lu, Y. et al. Single cell antimicrobial susceptibility testing by confined microchannels and electrokinetic loading. *Anal. Chem.* **85**, 3971–3976 (2013).
106. Long, Z. et al. Microfluidic chemostat for measuring single cell dynamics in bacteria. *Lab Chip* **13**, 947–954 (2013).
107. Xia, Y. & Whitesides, G. M. Soft lithography. *Angew. Chem. Int. Ed.* **37**, 550–575 (1998).
108. Rogers, J. A. & Nuzzo, R. G. Recent progress in soft lithography. *Mater. Today* **8**, 50–56 (2005).
109. Quake, S. R. & Scherer, A. From micro- to nanofabrication with soft materials. *Science* **290**, 1536–1540 (2000).
110. Baltekin, Ö., Boucharin, A., Tano, E., Andersson, D. I. & Elf, J. Antibiotic susceptibility testing in less than 30 min using direct single-cell imaging. *Proc. Natl Acad. Sci. USA* **114**, 9170–9175 (2017).
111. Li, H. et al. Adaptable microfluidic system for single-cell pathogen classification and antimicrobial susceptibility testing. *Proc. Natl Acad. Sci. USA* **116**, 10270–10279 (2019).
This study presents an adaptable microfluidic device that isolates individual bacterial cells in channels, performs pathogen classification based on bacterial shape and size, and conducts phenotypic AST at the single-cell level.
112. Yang, Y., Gupta, K. & Ekinci, K. L. All-electrical monitoring of bacterial antibiotic susceptibility in a microfluidic device. *Proc. Natl Acad. Sci. USA* https://doi.org/10.1073/pnas.1922172117 (2020).
This study demonstrates a microfluidic device that allows for sensitive detection of single bacterial cell growth via all-electrical means.
113. Peitz, I. & van Leeuwen, R. Single-cell bacteria growth monitoring by automated DEP-facilitated image analysis. *Lab Chip* **10**, 2944–2951 (2010).
114. Zhu, X. et al. Arrays of horizontally-oriented mini-reservoirs generate steady microfluidic flows for continuous perfusion cell culture and gradient generation. *Analyst* **129**, 1026–1031 (2004).
115. Heo, Y. S. et al. Characterization and resolution of evaporation-mediated osmolality shifts that constrain microfluidic cell culture in poly(dimethylsiloxane) devices. *Anal. Chem.* **79**, 1126–1134 (2007).
116. Flueckiger, J., Bazargan, V., Stoeber, B. & Cheung, K. C. Characterization of postfabricated parylene C coatings inside PDMS microdevices. *Sens. Actuators B Chem.* **160**, 864–874 (2011).
117. Lenhard, J. R. & Bulman, Z. P. Inoculum effect of β-lactam antibiotics. *J. Antimicrob. Chemother.* **74**, 2825–2843 (2019).
118. Li, H., Lu, Y. & Wong, P. K. Diffusion–reaction kinetics of microfluidic amperometric biosensors. *Lab Chip* **18**, 3086–3089 (2018).
119. Hai, P. et al. High-throughput, label-free, single-cell photoacoustic microscopy of intratumoral metabolic heterogeneity. *Nat. Biomed. Eng.* **3**, 381–391 (2019).
120. Hu, J., Xu, Y., Gou, T., Zhou, S. & Mu, Y. High throughput single cell separation and identification using a self-priming isometric and Equant screw valve-based (SIES) microfluidic chip. *Analyst* **143**, 5792–5798 (2018).
121. Hu, J. et al. A vacuum-assisted, highly parallelized microfluidic array for performing multi-step digital assays. *Lab Chip* **21**, 4716–4724 (2021).
122. Zhukov, D. V. et al. Microfluidic SlipChip device for multistep multiplexed biochemistry on a nanoliter scale. *Lab Chip* **19**, 3200–3211 (2019).
123. Ottesen Elizabeth, A., Hong Jong, W., Quake Stephen, R. & Leadbetter Jared, R. Microfluidic digital PCR enables multigene analysis of individual environmental bacteria. *Science* **314**, 1464–1467 (2006).
124. Zhu, Q. et al. A scalable self-priming fractal branching microchannel net chip for digital PCR. *Lab Chip* **17**, 1655–1665 (2017).
125. Hsieh, K. et al. Simple and precise counting of viable bacteria by resazurin-amplified picoarray detection. *Anal. Chem.* **90**, 9449–9456 (2018).
126. Yeh, E.-C. et al. Self-powered integrated microfluidic point-of-care low-cost enabling (SIMPLE) chip. *Sci. Adv.* **3**, e1501645 (2017).
127. Cohen, D. E., Schneider, T., Wang, M. & Chiu, D. T. Self-digitization of sample volumes. *Anal. Chem.* **82**, 5707–5717 (2010).
128. Gansen, A., Herrick, A. M., Dimov, I. K., Lee, L. P. & Chiu, D. T. Digital LAMP in a sample self-digitization (SD) chip. *Lab Chip* **12**, 2247–2254 (2012).
129. Du, W., Li, L., Nichols, K. P. & Ismagilov, R. F. SlipChip. *Lab Chip* **9**, 2286–2292 (2009).
130. Lin, X. et al. Asymmetric membrane for digital detection of single bacteria in milliliters of complex water samples. *ACS Nano* **12**, 10281–10290 (2018).
131. Kao, Y.-T. et al. Microfluidic one-pot digital droplet FISH using LNA/DNA molecular beacons for bacteria detection and absolute quantification. *Biosensors* https://doi.org/10.3390/bios12040237 (2022).
132. Zheng, W. et al. High-throughput, single-microbe genomics with strain resolution, applied to a human gut microbiome. *Science* **376**, eabm1483 (2022).
133. Andersson, D. I., Nicoloff, H. & Hjort, K. Mechanisms and clinical relevance of bacterial heteroresistance. *Nat. Rev. Microbiol.* **17**, 479–496 (2019).
134. Unger, M. A., Chou, H.-P., Thorsen, T., Scherer, A. & Quake, S. R. Monolithic microfabricated valves and pumps by multilayer soft lithography. *Science* **288**, 113–116 (2000).
This study demonstrates the active microfluidic on–off valves via soft lithography.
135. Li, H., Zhang, P., Hsieh, K. & Wang, T.-H. Combinatorial nanodroplet platform for screening antibiotic combinations. *Lab Chip* https://doi.org/10.1039/D1LC00865J (2022).
136. Zhang, P., Kaushik, A., Hsieh, K. & Wang, T.-H. Customizing droplet contents and dynamic ranges via integrated programmable picodroplet assembler. *Microsyst. Nanoeng.* **5**, 22 (2019).
137. Gu, W., Zhu, X., Futai, N., Cho Brenda, S. & Takayama, S. Computerized microfluidic cell culture using elastomeric channels and Braille displays. *Proc. Natl Acad. Sci. USA* **101**, 15861–15866 (2004).
138. Eduati, F. et al. A microfluidics platform for combinatorial drug screening on cancer biopsies. *Nat. Commun.* **9**, 2434 (2018).
139. Zhu, P. & Wang, L. Passive and active droplet generation with microfluidics: a review. *Lab Chip* **17**, 34–75 (2017).
140. Rosenfeld, L., Lin, T., Derda, R. & Tang, S. K. Y. Review and analysis of performance metrics of droplet microfluidics systems. *Microfluid. Nanofluidics* **16**, 921–939 (2014).
141. Becker, K. et al. Detection of *mecA*- and *mecC*-positive methicillin-resistant *Staphylococcus aureus* (MRSA) isolates by the new Xpert MRSA Gen 3 PCR assay. *J. Clin. Microbiol.* **54**, 180–184 (2016).
142. He, Y.-H. et al. Real-time PCR for the rapid detection of *vanA*, *vanB* and *vanM* genes. *J. Microbiol. Immunol. Infect.* **53**, 746–750 (2019).
143. Courvalin, P. Vancomycin resistance in Gram-positive cocci. *Clin. Infect. Dis.* **42**, S25–S34 (2006).
144. Frickmann, H. et al. Fluorescence in situ hybridization (FISH) in the microbiological diagnostic routine laboratory: a review. *Crit. Rev. Microbiol.* **43**, 263–293 (2017).
145. Zhang, Z., Kermekchiev, M. B. & Barnes, W. M. Direct DNA amplification from crude clinical samples using a PCR enhancer cocktail and novel mutants of Taq. *J. Mol. Diagn.* **12**, 152–161 (2010).
146. Kermekchiev, M. B., Kirilova, L. I., Vail, E. E. & Barnes, W. M. Mutants of Taq DNA polymerase resistant to PCR inhibitors allow DNA amplification from whole blood and crude soil samples. *Nucleic Acids Res.* **37**, e40 (2009).
147. Abram, T. J. et al. Rapid bacterial detection and antibiotic susceptibility testing in whole blood using one-step, high throughput blood digital PCR. *Lab Chip* **20**, 477–489 (2020).
148. Fox, G. E. et al. The phylogeny of prokaryotes. *Science* **209**, 457–463 (1980).
149. Olsen, G. J. & Woese, C. R. Ribosomal RNA: a key to phylogeny. *FASEB J.* **7**, 113–123 (1993).
150. Cole, J. R. et al. Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res.* **42**, D633–D642 (2014).
151. Amann, R. & Fuchs, B. M. Single-cell identification in microbial communities by improved fluorescence in situ hybridization techniques. *Nat. Rev. Microbiol.* **6**, 339–348 (2008).
152. Zwirglmaier, K., Ludwig, W. & Schleifer, K. H. Recognition of individual genes in a single bacterial cell by fluorescence in situ hybridization — RING-FISH. *Mol. Microbiol.* **51**, 89–96 (2004).
153. Amann, R. & Ludwig, W. Ribosomal RNA-targeted nucleic acid probes for studies in microbial ecology. *FEMS Microbiol. Rev.* **24**, 555–565 (2000).
154. Rane, T. D., Zec, H. C., Puleo, C., Lee, A. P. & Wang, T.-H. Droplet microfluidics for amplification-free genetic detection of single cells. *Lab Chip* **12**, 3341–3347 (2012).
155. Gao, J. et al. Nanotube assisted microwave electroporation for single cell pathogen identification and antimicrobial susceptibility testing. *Nanomedicine* **17**, 246–253 (2019).
156. Mach, K. E. et al. Optimizing peptide nucleic acid probes for hybridization-based detection and identification of bacterial pathogens. *Analyst* **144**, 1565–1574 (2019).
157. Kaushik, A. M. et al. Droplet-based single-cell measurements of 16S rRNA enable integrated bacteria identification and pheno-molecular antimicrobial susceptibility testing from clinical samples in 30 min. *Adv. Sci.* https://doi.org/10.1002/advs.202003419 (2021).
This study demonstrates a droplet-based system that can isolate a single bacterium in droplets, probe 16S rRNA for pathogen identification and quantify the concentration of the 16S rRNA in response to antibiotics to determine AST results.
158. Avesar, J. et al. Rapid phenotypic antimicrobial susceptibility testing using nanoliter arrays. *Proc. Natl Acad. Sci. USA* **114**, E5787–E5795 (2017).
This study presents a simple yet efficient device for rapid AST by testing bacterial metabolism products in an array of nanolitre wells.
159. Kim, K. P. et al. In situ monitoring of antibiotic susceptibility of bacterial biofilms in a microfluidic device. *Lab Chip* **10**, 3296–3299 (2010).
160. Wang, Y., Ran, M., Wang, J., Ouyang, Q. & Luo, C. Studies of antibiotic resistance of β-lactamase bacteria under different nutrition limitations at the single-cell level. *PLoS ONE* **10**, e0127115 (2015).
161. Azizi, M. et al. Nanoliter-sized microchamber/microarray microfluidic platform for antibiotic susceptibility testing. *Anal. Chem.* **90**, 14137–14144 (2018).
162. Brook, I. Inoculum effect. *Rev. Infect. Dis.* **11**, 361–368 (1989).
163. Choi, J. et al. A rapid antimicrobial susceptibility test based on single-cell morphological analysis. *Sci. Transl. Med.* **6**, 267ra174 (2014).
This study demonstrates rapid AST by capturing bacteria in agarose and, subsequently, monitoring individual bacterial growth in the presence of antibiotics.
164. Kaushik, A. M. et al. Accelerating bacterial growth detection and antimicrobial susceptibility assessment in integrated picoliter droplet platform. *Biosens. Bioelectron.* **97**, 260–266 (2017).

165. Kao, Y.-T. et al. Gravity-driven microfluidic assay for digital enumeration of bacteria and for antibiotic susceptibility testing. *Lab Chip* **20**, 54–63 (2020).
166. Scherer, B. et al. Digital electrical impedance analysis for single bacterium sensing and antimicrobial susceptibility testing. *Lab Chip* https://doi.org/10.1039/D0LC00937G (2021).
167. Alafeef, M., Dighe, K. & Pan, D. Label-free pathogen detection based on yttrium-doped carbon nanoparticles up to single-cell resolution. *ACS Appl. Mater. Interfaces* **11**, 42943–42955 (2019).
168. Knudsen, S. M., von Muhlen, M. G., Schauer, D. B. & Manalis, S. R. Determination of bacterial antibiotic resistance based on osmotic shock response. *Anal. Chem.* **81**, 7087–7090 (2009).
169. Spencer, D. C. et al. A fast impedance-based antimicrobial susceptibility test. *Nat. Commun.* **11**, 5328 (2020).
This study demonstrates a microfluidic channel device that performs rapid AST by examining the impedance signal of the channel caused by individual bacteria passing through the channel.
170. Chung, C.-C., Cheng, I.-F., Yang, W.-H. & Chang, H.-C. Antibiotic susceptibility test based on the dielectrophoretic behavior of elongated *Escherichia coli* with cephalexin treatment. *Biomicrofluidics* **5**, 021102 (2011).
171. Kalashnikov, M., Lee, J. C., Campbell, J., Sharon, A. & Sauer-Budge, A. F. A microfluidic platform for rapid, stress-induced antibiotic susceptibility testing of *Staphylococcus aureus*. *Lab Chip* **12**, 4523–4532 (2012).
172. Iriya, R. et al. Real-time detection of antibiotic activity by measuring nanometer-scale bacterial deformation. *J. Biomed. Opt.* **22**, 126002 (2017).
173. Perry John, D. A decade of development of chromogenic culture media for clinical microbiology in an era of molecular diagnostics. *Clin. Microbiol. Rev.* **30**, 449–479 (2017).
174. Rosłoń, I. E., Japaridze, A., Steeneken, P. G., Dekker, C. & Alijani, F. Probing nanomotion of single bacteria with graphene drums. *Nat. Nanotechnol.* **17**, 637–642 (2022).
This study improves the detection sensitivity of bacterial motion and demonstrates AST at single-cell sensitivity.
175. Chiang, Y.-L. et al. Innovative antimicrobial susceptibility testing method using surface plasmon resonance. *Biosens. Bioelectron.* **24**, 1905–1910 (2009).
176. Bermingham, C. R. et al. Imaging of sub-cellular fluctuations provides a rapid way to observe bacterial viability and response to antibiotics. Preprint at *bioRxiv* https://doi.org/10.1101/460139 (2018).
177. Bennett, I., Pyne, A. L. B. & McKendry, R. A. Cantilever sensors for rapid optical antimicrobial sensitivity testing. *ACS Sens.* **5**, 3133–3139 (2020).
178. Choi, J. et al. Rapid drug susceptibility test of *Mycobacterium tuberculosis* using microscopic time-lapse imaging in an agarose matrix. *Appl. Microbiol. Biotechnol.* **100**, 2355–2365 (2016).
179. Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH image to ImageJ: 25 years of image analysis. *Nat. Methods* **9**, 671–675 (2012).
180. de Cesare, I. et al. ChipSeg: an automatic tool to segment bacterial and mammalian cells cultured in microfluidic devices. *ACS Omega* **6**, 2473–2476 (2021).
181. Banik, S. et al. Recent trends in smartphone-based detection for biomedical applications: a review. *Anal. Bioanal. Chem.* **413**, 2389–2406 (2021).
182. Ong, D. S. Y. & Poljak, M. Smartphones as mobile microbiological laboratories. *Clin. Microbiol. Infect.* **26**, 421–424 (2020).
183. Herman, B. *Fluorescence Microscopy* (Garland Science, 2020).
184. Sanderson, M. J., Smith, I., Parker, I. & Bootman, M. D. J. C. S. H. P. Fluorescence microscopy. *Cold Spring Harbor. Protocols* **2014**, pdb. top071795 (2014).
185. Lichtman, J. W. & Conchello, J.-A. Fluorescence microscopy. *Nat. Methods* **2**, 910–919 (2005).
186. Lakowicz, J. R. (Ed.) *Principles of Fluorescence Spectroscopy* 27–61 (Springer, 2006).
187. Hatch, A. C. et al. 1-Million droplet array with wide-field fluorescence imaging for digital PCR. *Lab Chip* **11**, 3838–3845 (2011).
188. O'Keefe, C. M. et al. Facile profiling of molecular heterogeneity by microfluidic digital melt. *Sci. Adv.* **4**, eaat6459 (2018).
189. Scheler, O., Kaminski, T. S., Ruszczak, A. & Garstecki, P. Dodecylresorufin (C12R) outperforms resorufin in microdroplet bacterial assays. *ACS Appl. Mater. Interfaces* **8**, 11318–11325 (2016).
190. Burg, T. P. et al. Weighing of biomolecules, single cells and single nanoparticles in fluid. *Nature* **446**, 1066–1069 (2007).
191. Guan, Y. et al. Kinetics of small molecule interactions with membrane proteins in single cells measured with mechanical amplification. *Sci. Adv.* **1**, e1500633 (2015).
192. Kara, V. et al. Microfluidic detection of movements of *Escherichia coli* for rapid antibiotic susceptibility testing. *Lab Chip* **18**, 743–753 (2018).
193. Scheler, O. et al. Droplet-based digital antibiotic susceptibility screen reveals single-cell clonal heteroresistance in an isogenic bacterial population. *Sci. Rep.* **10**, 3282 (2020).
194. Chijiiwa, R. et al. Single-cell genomics of uncultured bacteria reveals dietary fiber responders in the mouse gut microbiota. *Microbiome* **8**, 5 (2020).
195. Lan, F., Demaree, B., Ahmed, N. & Abate, A. R. Single-cell genome sequencing at ultra-high-throughput with microfluidic droplet barcoding. *Nat. Biotechnol.* **35**, 640–646 (2017).
196. Gill, C., van de Wijgert, J. H. H. M., Blow, F. & Darby, A. C. Evaluation of lysis methods for the extraction of bacterial DNA for analysis of the vaginal microbiota. *PLoS ONE* **11**, e0163148 (2016).
197. Shehadul Islam, M., Aryasomayajula, A. & Selvaganapathy, P. R. A review on macroscale and microscale cell lysis methods. *Micromachines* https://doi.org/10.3390/mi8030083 (2017).
198. Altamore, I., Lanzano, L. & Gratton, E. Dual channel detection of ultra low concentration of bacteria in real time by scanning fluorescence correlation spectroscopy. *Meas. Sci. Technol.* **24**, 065702 (2013).
199. Matsumoto, Y. et al. A microfluidic channel method for rapid drug-susceptibility testing of *Pseudomonas aeruginosa*. *PLoS ONE* **11**, e0148797 (2016).
200. Tong, W. et al. Gradient functionalization of various quaternized polyethylenimines on microfluidic chips for the rapid appraisal of antibacterial potencies. *Langmuir* **36**, 354–361 (2020).
201. Zhang, P. et al. A cascaded droplet microfluidic platform enables high-throughput single cell antibiotic susceptibility testing at scale. *Small Methods* **6**, 2101254 (2022).
This study demonstrates a droplet device for rapid and multiplex phenotypic AST.
202. Hare, P. J., LaGree, T. J., Byrd, B. A., DeMarco, A. M. & Mok, W. W. K. Single-cell technologies to study phenotypic heterogeneity and bacterial persisters. *Microorganisms* https://doi.org/10.3390/microorganisms9112277 (2021).
203. Lyu, F. et al. Phenotyping antibiotic resistance with single-cell resolution for the detection of heteroresistance. *Sens. Actuators B Chem.* **270**, 396–404 (2018).
204. Postek, W., Gargulinski, P., Scheler, O., Kaminski, T. S. & Garstecki, P. Microfluidic screening of antibiotic susceptibility at a single-cell level shows the inoculum effect of cefotaxime on *E. coli*. *Lab Chip* **18**, 3668–3677 (2018).
This study presents a droplet device that is able to study the inoculum effect by performing parallelized single-cell AST at high throughput.
205. Schoepp, N. G. et al. Rapid pathogen-specific phenotypic antibiotic susceptibility testing using digital LAMP quantification in clinical samples. *Sci. Transl. Med.* **9**, eaal3693 (2017).
This study demonstrates a digital LAMP quantification method, which quantifies bacterial growth with high sensitivity for rapid phenotypic AST.
206. Athamanolap, P. et al. Nanoarray digital polymerase chain reaction with high-resolution melt for enabling broad bacteria identification and pheno-molecular antimicrobial susceptibility test. *Anal. Chem.* **91**, 12784–12792 (2019).
207. Reyes, D. R. et al. Accelerating innovation and commercialization through standardization of microfluidic-based medical devices. *Lab Chip* **21**, 9–21 (2021).
208. Klapperich, C. M. Microfluidic diagnostics: time for industry standards. *Expert. Rev. Med. Devices* **6**, 211–213 (2009).
209. Roszak, D. B. & Colwell, R. R. Metabolic activity of bacterial cells enumerated by direct viable count. *Appl. Environ. Microbiol.* **53**, 2889–2893 (1987).
210. Tian, D. et al. Significance of viable but nonculturable *Escherichia coli*: induction, detection, and control. *J. Microbiol. Biotechnol.* **27**, 417–428 (2017).
211. Gruenberger, A. et al. Microfluidic picoliter bioreactor for microbial single-cell analysis: fabrication, system setup, and operation. *JoVE* https://doi.org/10.3791/50560 (2013).
212. Schoenitz, M., Grundemann, L., Augustin, W. & Scholl, S. Fouling in microstructured devices: a review. *Chem. Commun.* **51**, 8213–8228 (2015).
213. Tjandra, K. C. et al. Diagnosis of bloodstream infections: an evolution of technologies towards accurate and rapid identification and antibiotic susceptibility testing. *Antibiotics* https://doi.org/10.3390/antibiotics11040511 (2022).
214. Forsyth, B. et al. A rapid single-cell antimicrobial susceptibility testing workflow for bloodstream infections. *Biosensors* https://doi.org/10.3390/bios11080288 (2021).
215. Fang, Y. L. et al. An integrated microfluidic system for early detection of sepsis-inducing bacteria. *Lab Chip* **21**, 113–121 (2021).
216. Kang, W., Sarkar, S., Lin, Z. S., McKenney, S. & Konry, T. Ultrafast parallelized microfluidic platform for antimicrobial susceptibility testing of Gram positive and negative bacteria. *Anal. Chem.* **91**, 6242–6249 (2019).
217. Svensson, C.-M. et al. Coding of experimental conditions in microfluidic droplet assays using colored beads and machine learning supported image analysis. *Small* **15**, 1802384 (2019).
218. Deiss, F., Funes-Huacca, M. E., Bal, J., Tjhung, K. F. & Derda, R. Antimicrobial susceptibility assays in paper-based portable culture devices. *Lab Chip* **14**, 167–171 (2014).
219. Trick, A. Y. et al. A portable magnetofluidic platform for detecting sexually transmitted infections and antimicrobial susceptibility. *Sci. Transl. Med.* **13**, eabf6356 (2021).
220. Chen, F.-E. et al. Toward decentralizing antibiotic susceptibility testing via ready-to-use microwell array and resazurin-aided colorimetric readout. *Anal. Chem.* **93**, 1260–1265 (2021).
221. Carleton, P. F. et al. National institute of biomedical imaging and bioengineering point-of-care technology research network: advancing precision medicine. *IEEE J. Transl. Eng. Health Med.* **4**, 1–14 (2016).
222. Ho, D. et al. Enabling technologies for personalized and precision medicine. *Trends Biotechnol.* **38**, 497–518 (2020).
223. Kettler, H. et al. *Mapping the landscape of diagnostics for sexually transmitted infections : key findings and recommendations* (World Health Organization, 2004).
224. Land, K. J., Boeras, D. I., Chen, X.-S., Ramsay, A. R. & Peeling, R. W. REASSURED diagnostics to inform disease control strategies, strengthen health systems and improve patient outcomes. *Nat. Microbiol.* **4**, 46–54 (2019).
225. Mabey, D., Peeling, R. W., Ustianowski, A. & Perkins, M. D. Diagnostics for the developing world. *Nat. Rev. Microbiol.* **2**, 231–240 (2004).
226. Yu, H. et al. Phenotypic antimicrobial susceptibility testing with deep learning video microscopy. *Anal. Chem.* **90**, 6314–6322 (2018).
227. Iriya, R. et al. Rapid antibiotic susceptibility testing based on bacterial motion patterns with long short-term memory neural networks. *IEEE Sens. J.* **20**, 4940–4950 (2020).

228. Nguyen, M. et al. Using machine learning to predict antimicrobial MICs and associated genomic features for nontyphoidal *Salmonella*. *J. Clin. Microbiol.* **57**, e01260-18 (2019).
229. Ciuffreda, L., Rodríguez-Pérez, H. & Flores, C. Nanopore sequencing and its application to the study of microbial communities. *Comput. Struct. Biotechnol. J.* **19**, 1497–1511 (2021).
230. Brochado, A. R. et al. Species-specific activity of antibacterial drug combinations. *Nature* **559**, 259–263 (2018).
231. Kulesa, A., Kehe, J., Hurtado, J. E., Tawde, P. & Blainey, P. C. Combinatorial drug discovery in nanoliter droplets. *Proc. Natl Acad. Sci. USA* **115**, 6685–6690 (2018).
232. Sánchez-López, E. et al. Metal-based nanoparticles as antimicrobial agents: an overview. *Nanomaterials* **10**, 292 (2020).
233. Zhang, L.-j & Gallo, R. L. Antimicrobial peptides. *Curr. Biol.* **26**, R14–R19 (2016).
234. Golchin, S. A., Stratford, J., Curry, R. J. & McFadden, J. A microfluidic system for long-term time-lapse microscopy studies of mycobacteria. *Tuberculosis* **92**, 489–496 (2012).
235. Flynn, C. & Ignaszak, A. Lyme disease biosensors: a potential solution to a diagnostic dilemma. *Biosensors* https://doi.org/10.3390/bios10100137 (2020).
236. Kandavalli, V., Karempudi, P., Larsson, J. & Elf, J. Rapid antibiotic susceptibility testing and species identification for mixed samples. *Nat. Commun.* **13**, 6215 (2022).
237. Watterson, W. J. et al. Droplet-based high-throughput cultivation for accurate screening of antibiotic resistant gut microbes. *eLife* **9**, e56998 (2020).
238. Rho, E. et al. Separation-free bacterial identification in arbitrary media via deep neural network-based SERS analysis. *Biosens. Bioelectron.* **202**, 113991 (2022).
239. Yang, M. et al. Machine learning-enabled non-destructive paper chromogenic array detection of multiplexed viable pathogens on food. *Nat. Food* **2**, 110–117 (2021).
240. Zhang, Y., Jiang, H., Ye, T. & Juhas, M. Deep learning for imaging and detection of microorganisms. *Trends Microbiol.* **29**, 569–572 (2021).

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All authors declare no competing interests.

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