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Review Article

Recent Developments of Chip-based Phenotypic Antibiotic Susceptibility Testing

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Abstract Antibiotic susceptibility testing (AST), the screening of effective types and dosages of antibiotics, has become significantly important in the antimicrobial-resistance era over the last few decades. In order to overcome the limitations of conventional AST methods, several recent studies have developed AST platforms which exhibit the advantages of microfluidics. They demonstrated the performance of the platforms by determining effective antimicrobials for bacterial strains and their minimum inhibitory concentrations within hours. In this review, we cover recent developments of on-chip approaches for measurements of bacterial growth as well as for dilutions of antibiotic concentrations. We also discuss Point-of-Care AST devices that employ inexpensive materials and simple working principles to operate screenings near the site of care, which can potentially bring current laboratory-limited assays to clinical standards. All thing considered, emerging microfluidic AST devices have the potential to be decent alternatives to commonplace macro-scale AST methods.

Keywords: Microfluidic chips, Antibiotic susceptibility testing, Drug screening platforms, Minimum inhibitory concentrations, Point-of-Care devices

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Introduction

Antibiotic resistance of pathogenic bacteria has been recognized as a significant challenge in recent decades[1,2]. In particular, problems associated with antibiotics, such as inappropriate prescriptions, prolonged treatment, and excessive consumptions, are exacerbating the severity of this challenge[3,4]. Thus, *in vitro* antibiotic susceptibility testing (AST) is becoming increasingly important in this post-antibiotic era, since they provide medical information for finding suitable types of antibiotics as well as their effective dosages, which inhibit bacterial growth and retard resistance emergence[5].

Conventional phenotypic AST methods mostly fulfill their role in screening proper antibiotic medications, particularly complemented with genotypic AST methods (e.g. polymerase chain reaction-based techniques and DNA microarrays)[6,7]. The traditional phenotypic assays such as micro-dilution on 96-well plates and disk diffusion on agar plates provide feasibility measurements without the need of biochemistry tools[8,9]. While these standard methods have become the staple of *in vitro* phenotypic ASTs, they nevertheless have several limitations. These macro-scale methods are labor-intensive to prepare the wide range of dosages, and are time-consuming to differentiate drug-susceptible cells from healthy cells (16-20 hours)[10]. They also require large specimen samples which don't allow them to be used routinely. These drawbacks may hinder the ideal implementation of AST, which potentially results in weakened surveillance of resistance trends[11].

In recent years, microfluidic AST platforms for phenotypic screening have emerged, with superior per-

formance in certain aspects compared to the conventional methods[12,13]. The advantages are namely: lower cost, smaller amount of resources, reduced turnaround time, possible automation, more rapid evaluation, and higher sensitivity, comparatively[12,13]. Although most of the AST platforms have tested common types of antibiotics (e.g. Ampicillin, Gentamicin, and Ciprofloxacin, etc) against wild-type strains (e.g. *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus,* etc.), these novel chips demonstrated their improved performance and validated their technical characteristics[14]. Here, we review the on-chip approaches for AST purposes, which are categorized into methods of observing growth levels and methods of diluting antibiotic concentrations. We also cover applications of Point-of-Care (POC) devices in AST, since the promising POC assays have demonstrated improved simplicity and utility over other laboratory-based microfluidic AST chips.

Measurement Methods of Cell Growth Level and Susceptibility in the Microfluidic Chips

Information about the growth level of bacteria, both in the presence and absence of antibiotics, should be provided for determining the baseline growth and susceptibility. In a microfluidic platform, while small volumes of samples could allow the entire duration of testing to be reduced to a matter of hours, they conversely make the measurements of cell growth level to be technically more difficult. Due to these small volumes and their consequent low accessibility of the samples loaded on the chip, on-chip growth level cannot be precisely measured using simple equipment, or using the naked eyes as in the conventional macroscale methods. Thus, most microfluidic studies have employed specific equipment, assays, or original techniques for obtaining the on-chip growth levels of bacteria. In this section, we review the chip-based measurements in four main categories: fluorescence imaging, metabolic activity indicators, optical imaging without labeling, and magnetic beads rotation measurement (Table 1). All the aforementioned categories are highlighted with their general characteristics and example on-chip studies showing their benefits over conventional methods.

Fluorescence Imaging for Cell Density and Viability

To monitor bacterial cell growth, many microbiologists have utilized bacteria strains that constitutively express plasmid-encoded green fluorescence protein (GFP)[15]. However, this approach involves a preliminary process of genetic medication at the molecular level, which may be complex for some strains and interferes with rapid AST[16]. In addition, GFP plasmid that is transformed to bacteria confers resistance to certain antibiotics and requires the antibiotic- supplemented media for constitutively expressing GFP. Despite these drawbacks, fluorescence imaging has been frequently utilized even in the microfluidic phenotypic AST (Table 1), because a significant linear correlation between fluorescence intensities and cell densities is stably presented for GFP-expressing strains, which allows for real-time monitoring of on-chip cell growth[17]. As a representative example, Golchin *et al*. monitored the on-chip growth and viability of GFP-expressing *Mycobacterium smegmatis*, whose susceptibility to Rifampicin was highlighted by the uptake of the nucleic acid stain such as propidium iodide, using a confocal laser microscope with high resolution (Figure 1a)[18].

Metabolic Activity Indicators

Metabolic activity is involved in the life cycle of bacteria cells, and the magnitude of the activity is significantly correlated to cell growth or cell susceptibility to antibiotics. Resazurin is one of the most commonly used metabolism markers, and when it is irreversibly metabolized by bacteria, it is converted to resorufin, which exhibits strong fluorescence that can be observed using fluorescence microscopes even in the microfluidic chips (Table 1)[19]. For instance, Azizi *et al*. measured the fluorescence change from the metabolic product of resazurin and confirmed the inhibitory effect of Kanamycin on *E. coli* that was confined within nanoliter-sized chambers (Figure 1b)[20].

The adenosine triphosphate (ATP) bioluminescence assay, another common indicator of metabolism, estimates the metabolic activity of bacteria by measuring photons (550-570 nm) from which luciferin substrate is catalyzed by luciferase in the presence of ATP and oxygen[21]. However, since the intensities of bioluminescence signals are significantly weaker than fluorescence intensities, the assay generally require more sensitive microplate readers that can record photons. For that reason, bioluminescence assays have not been employed frequently in microfluidic AST studies. Exceptionally, Dong *et al*. utilized them to quantify the bacterial growth in a multi-layered device that could be compatible with standard microplate readers[22].

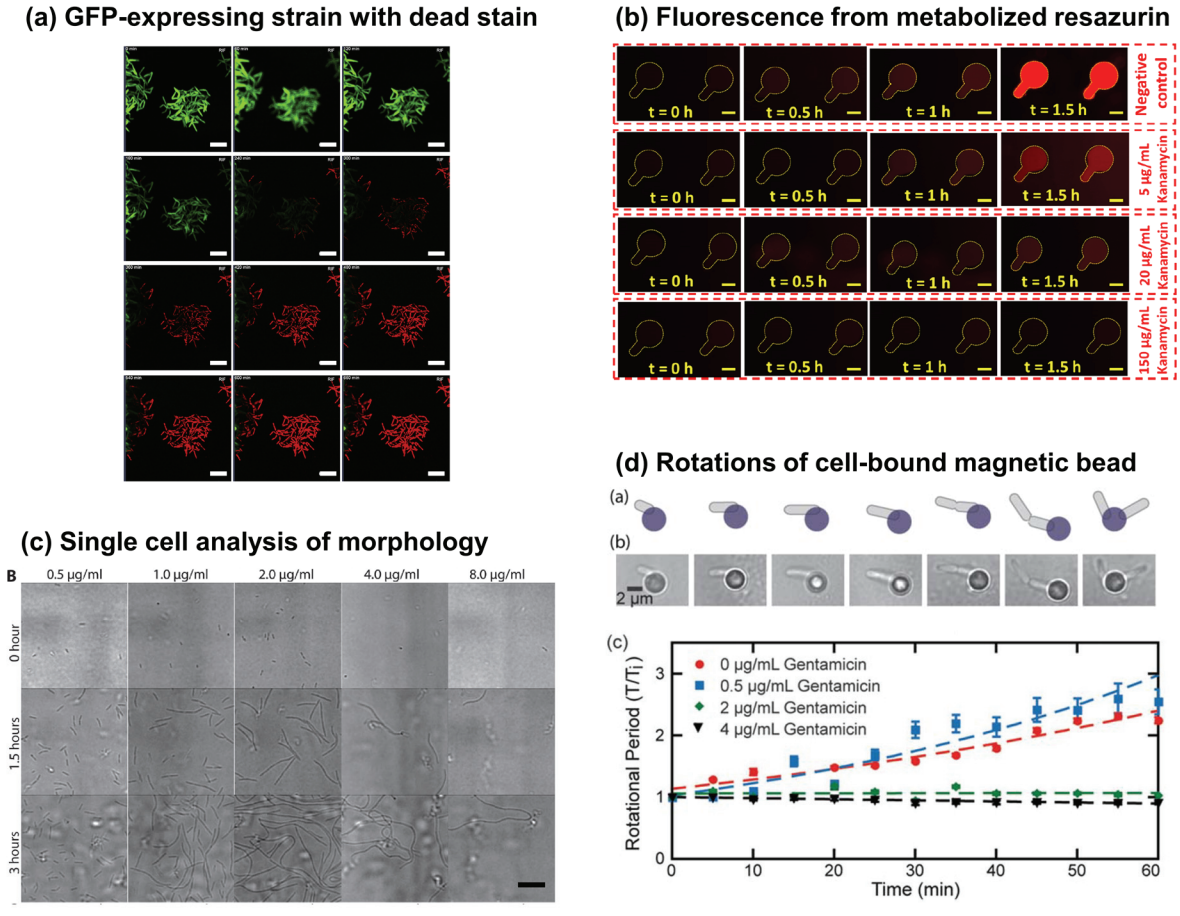

Figure 1. Representative on-chip bacterial growth measurement methods. (a) During 11 hours of Rifampicin treatment, GFP- expressing *M. smegmatis* gradually lost the GFP signal (green) and showed propodium iodide signal (red)[18]. Scale bars 10 μm. (b) Decreasing intensity of the fluorescence signal (red) from the metabolic product of resazurin indicates inhibited metabolism of *E. coli* by Kanamycin in a dose-dependent manner[20]. Scale bars 100 μm. (c) Brightfield microscope images show the filamentary formation of single cells of *P. aeruginosa* induced by Ceftazidime treatment[23]. Scale bar 25 μm. (d) Rotational periods of *E. coli*-bound magnetic beads gradually increase due to cell division, but increasing concentration of Gentamicin brings about constant rotational periods of the cell-bound bead[25]. Scale bar 2 μm. Images reproduced from the references with permission.

They could measure the ATP bioluminescence signal generated from the metabolism of a pathogen sample with high sensitivity, and verified antimicrobial effects of eight drugs in 3-6 hours.

Optical Imaging Without Labeling

Several microfluidic-based studies have utilized brightfield microscope for single cell analysis or grayscale-based analysis without engineered strains and pre-staining process, demonstrating their applicability and simplicity (Table 1). As a representative study of single cell imaging, Choi *et al*. mixed agarose gel with the bacterial sample and injected the mixture into the microchannel, resulting in fixed bacteria in the same location, without altering the properties of the cell (Figure 1c)[23]. They determined the minimum inhibitory concentrations (MICs) of antibiotics by screening changes in morphology (e.g. filamentary formation, swelling, dividing) and numbers of individual cells using a brightfield microscope. In the research by Hou *et al*., which employed grayscale- based analysis, they also used agarose gel to immobilize the bacteria cells[24]. They obtained bacterial densities from grayscale intensity changes of the images (from black to white) originated from the cell growth inside the gel, which is similar to measuring the turbidity of bacteria samples in optical density measurements. Although this approach requires a relatively higher initial cell density than in the single cell analysis method, the simple working principle enables a wide range of researchers to easily measure the growth level of a broad spectrum of strains.

Table 1. Monitoring methods for bacterial growth utilized in the microfluidic ASTs**.**

Types of methods	Descriptions of methods	Advantages/Disadvantages	Refs.
Fluorescence imaging	Fluorescence protein reporter	Real-time imaging, Stability of fluorescence, Pre-engineered strains required	17, 18, 47-53
	Fluorescence viability staining	Incubation for labeling required	54-58
Metabolic activity indicators	Fluorescence signal from the product of resazurin	Applicable to the patient sample, High sensitivity	20, 31, 59-61
	ATP bioluminescence	High sensitivity, Difficult for optical imaging, Occasionally pre-engineered strains required	22
	pH changes	Applicable to the patient sample, No microscope required	34, 39, 62
Optical imaging without labeling	Number and/or morphology of cells	Applicable to the patient sample, Single cell analysis, Image processing required	23, 33, 63-67
	Grayscale of images due to cell growth	Applicable to the patient sample, High concentrations of cells required	24, 27, 28, 68
Magnetic beads rotation measurement	Rotational period of magnetic beads correlated with cell growth	High sensitivity, Immunoassay required, External equipment required	25, 26, 69

Magnetic Beads Rotation Measurement for Cell Growth Level

The Kopelman group developed a biosensor that quantifies antibiotic toxicity in 15 - 30 minutes by measuring the rotation rate of cell-bound magnetic beads (Figure 1d, Table 1)[25,26]. They coated a single or small population of bacteria on an 8-μm-diameter magnetic bead by an immunoassay (off-chip), and generated nanoliter-volume droplets encapsulating the bacteria-bound beads in a microfluidic chip[23,24]. Under an external magnetic field, the rotational rate of the beads changed due to the growth and division of the bacteria, which was observed via a microscope. Although there were drawbacks, such as the immunoassay process and the time-consuming pre-incubation, their approach exhibited high sensitivity and rapid evaluation in the growth measurements.

Microfluidic Approaches for Diluting Antibiotic Concentrations

Some studies have employed microfluidic chips that enable the dilution of concentrations of an antibiotic, taking advantage of the precise manipulation of micro-volume fluids. On-chip dilution of antibiotic concentrations allows automation, reproducibility, and reduced turn-around time of preparing dilutions, in spite of employing complex designs of chips and requiring external equipment. In this section, we review on-chip dilution of antibiotics by three main methods: diffusion-based gradient formation, micro-droplet, and micro-array (Table 2).

Diffusion-based Formation of Concentration Gradients

Several studies have developed microfluidic devices which can generate a continuous and linear concentration gradient of the drug onto bacteria-trapped hydrogel between parallel channels by using a syringe pump (Table 2). Due to the constant flow in the channels by the pump, molecules of the drug collectively diffuse from the drug-supplemented channel to the drug-free channel. The complete formation of concentration gradient takes 30 to 90 minutes depending on the characteristic length of diffusion of the chip, and the gradient is maintained during the AST using the pump. For instance, Kim *et al*. injected a mixture of an agar gel and bacteria suspensions into a 1300 μm-wide channel and then applied the antibiotic gradient using an external pump for 6 hours to determine the MIC against *P. aeruginosa* (Figure 2a)[27]. They obtained the bacterial susceptibility to specific concentrations of the antibiotic by dividing the gel region into twenty-one portions along the gradient, and compared the MICs of the antibiotics tested in the chips with the standard MICs presented by the Clinical Laboratory Standards Institute. Furthermore, for on-chip combinatory AST in particular, Kim *et al*. utilized a microfluidic platform enabling formation of two orthogonal

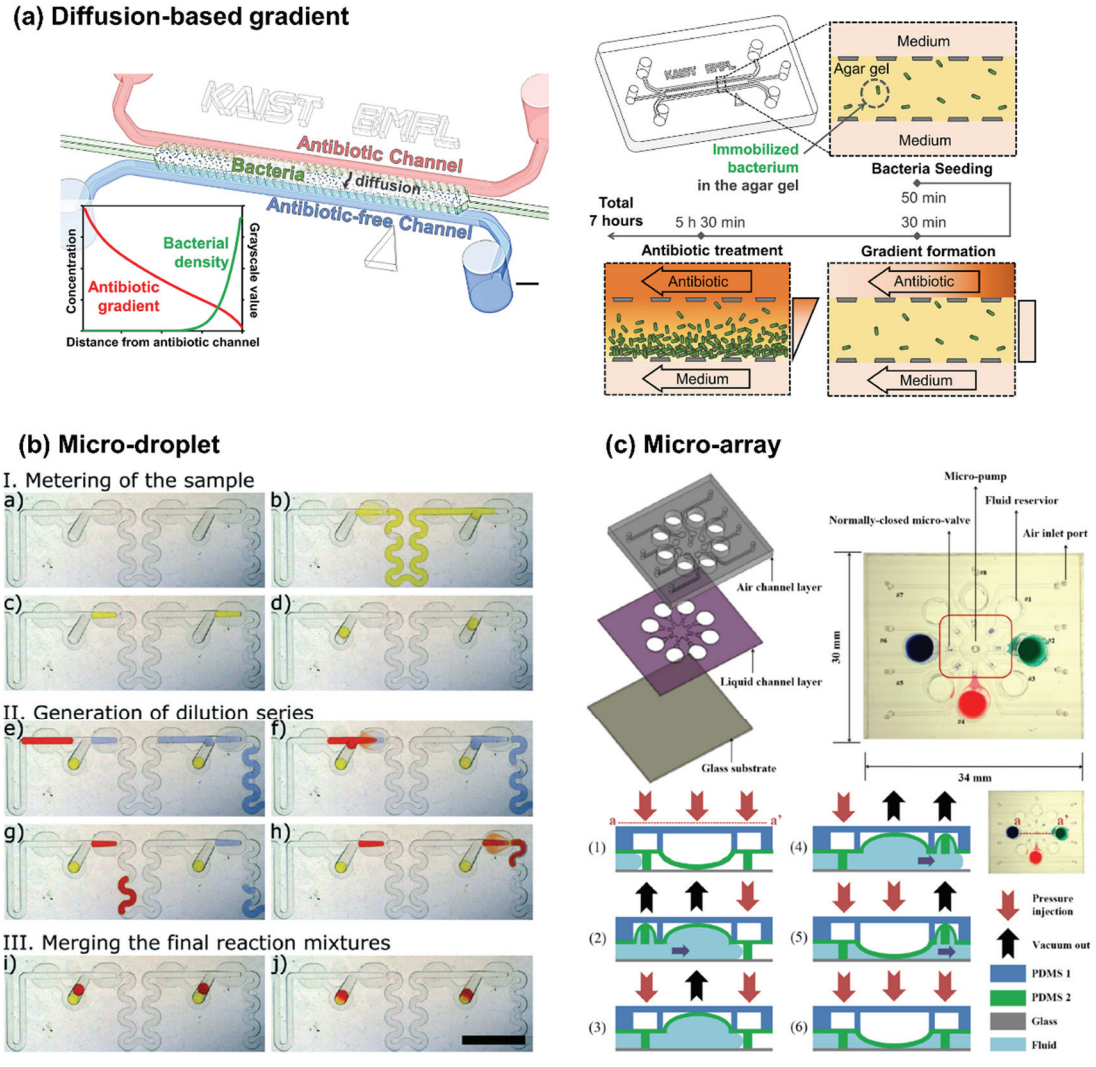

Figure 2. Representative on-chip antibiotic concentration diluting methods. (a) The flowing media both with and without an antibiotic in two parallel channels creates a linear concentration gradient of the antibiotic in the region of bacteria-trapped gel[27]. Scale bar 1.2 mm. (b) The mixing module passively generates a series of droplets with dilutions of a drug and mixes them with the bacteria suspension. Scale bar 5 mm. Reproduced from ref. 31 – Published by The Royal Society of Chemistry. (c) The valve-integrated micro-array automatically operates dilution functions of a drug within 9 minutes using an external pneumatic pump[34]. Images reproduced from the references with permission.

concentration gradients onto the bacteria-trapping gel within 35 minutes[28]. They tested the combinatory effects of several antibiotic pairs against *P. aeruginosa* on the chips, and they categorized the pairs into synergy or antagonism in 7 hours after injecting the antibiotic-supplemented media.

Dilution Using Micro-droplets

Micro-droplet systems automatically enable the creation of water-in-oil droplets whose antibiotic concentrations are serially diluted[29,30]. Individual droplets (volume of pL to mL) act as miniaturized incubators for the encapsulated target cells, which could additionally include drugs or viability-indicating dyes. In spite of having the disadvantages such as the addition of a surfactant to prevent coalescence, a potential risk of cross-contamination, and evaporation of the plug, this system is capable of precise control with small volumes of reagents and bacteria suspensions, enabling rapid AST against pathogens (Table 2). Interestingly, Derzsi *et al*. developed a passive-diluting platform that could make droplets using five manual pipettes, and then screen antibiotic toxicity in 5 hours

Table 2. On-chip diluting methods of antibiotic concentrations.

Types of methods	Initial cell density (CFU/mL)	Time to assay readout (hours)	Advantages/Disadvantages	Refs.
Diffusion-based gradient formation	$10^6 – 10^8$	3 – 5	Fine-tuning of dosage, Single cell analysis, Limited dosage range, External actuator required	24, 27, 28, 47, 63, 68, 70
Micro-droplet	$5 \times 10^4 – 10^6$	1 – 7	Wide dosage range, Small volume of reagent and sample, High-throughput, Multiplexing, Complicated operations, External actuator required, Oil and surfactant required,	31, 48, 53, 60, 61
Micro-array	$5 \times 10^5 – 3 \times 10^8$	3 – 24	Wide dosage range, Small volume of reagent and sample, High-throughput, Single cell analysis, Limited mixing ratio, External actuator required,	17, 33, 34, 58

(Figure 2b)[31]. The mixing module of the chip successively made aliquots of injected bacteria suspension, antibiotics, and pure medium (containing resazurin) with pre-defined volumes using the Rayleigh-Plateau instability. The measurement of fluorescence intensity confirmed that the final eleven droplets have serially diluted concentrations of an agent (approximately 160-fold between the lowest and highest concentrations). After incubating the droplets for 4 hours at 37 °C, they measured the resofurin fluorescence intensity of individual droplets and obtained the dose-response profile of Ampicillin against *E. coli*, consequently determining the MIC.

Dilution on the Micro-array Chips

Other systems featuring micro-arrays were also employed for generating various sets of antibiotic concentrations (Table 2)[32]. For instance, a tree-shape array causes the injected drugs-supplemented media to be separated and merged repeatedly, which in turn results in the drug concentrations to be gradually diluted, eventually generating a continuous profile of drug concentration onto a chamber that contains the bacteria suspension[33]. Another example is a valve-integrated array which operates the loading and mixing of the reagents by controlling the flow in the channels using a pneumatic pump (Figure 2c)[17]. Lee *et al*. utilized the valve-integrated array to treat various concentrations of Vancomycin against wild-type or Vancomycin-resistant *Enterococcus faecalis* in the chamber, and determined the MICs through pH-dependent colorimetric changes of the broth[34]. The system automatically mixed and diluted the bacteria suspension and antibiotic broth in 9 minutes, using the pump to press membrane-shape valves onto the channels.

Point-of-Care Microfluidics for AST

Point-of-Care tests, or POC tests in short, refer to the experiments carried outside the laboratory at or near the site of patient care, using equipment that can be easily delivered and that produce instantaneous (or at the very least, quick) results[35-37]. POC tests have found major applications in diagnostics[38] and antimicrobial resistance measurement[39-42], areas in which microfluidic devices are widely employed due to their patent advantages, such as being small, portable, and being able to manipulate small volumes of reagents. A representative example is the research conducted by Cira *et al*., where they introduced a poly-dimethylsiloxane (PDMS) microfluidic chip in which sample liquids were loaded and isolated in individual chambers by the presence of air or oil (Figure 3a)[39]. The device was particularly useful in determining the MIC of antibiotics since it isolated the bacteria chamber from the experimenter. Bacterial growth was easily identified by the change of color using a pH indicator. In addition to the usual advantages that microfluidic devices provide, this device was highly portable, avoiding nonessential equipment.

Most microfluidic AST devices are fabricated using cheap and accessible soft polymers (including PDMS) or glass, but these have certain disadvantages, such as being complex to operate and requiring oxygen plasma treatment to make channels. Furthermore, PDMS requires the careful consideration of uncured oligomers leaching into media, as well as the rapid partitioning of small hydrophobic molecules, including drug molecules, into the bulk, both of which adversely affect the data collected[43]. To overcome these inconvenient barriers, a market for a different category of microfluidic devices has emerged; this is where pa-

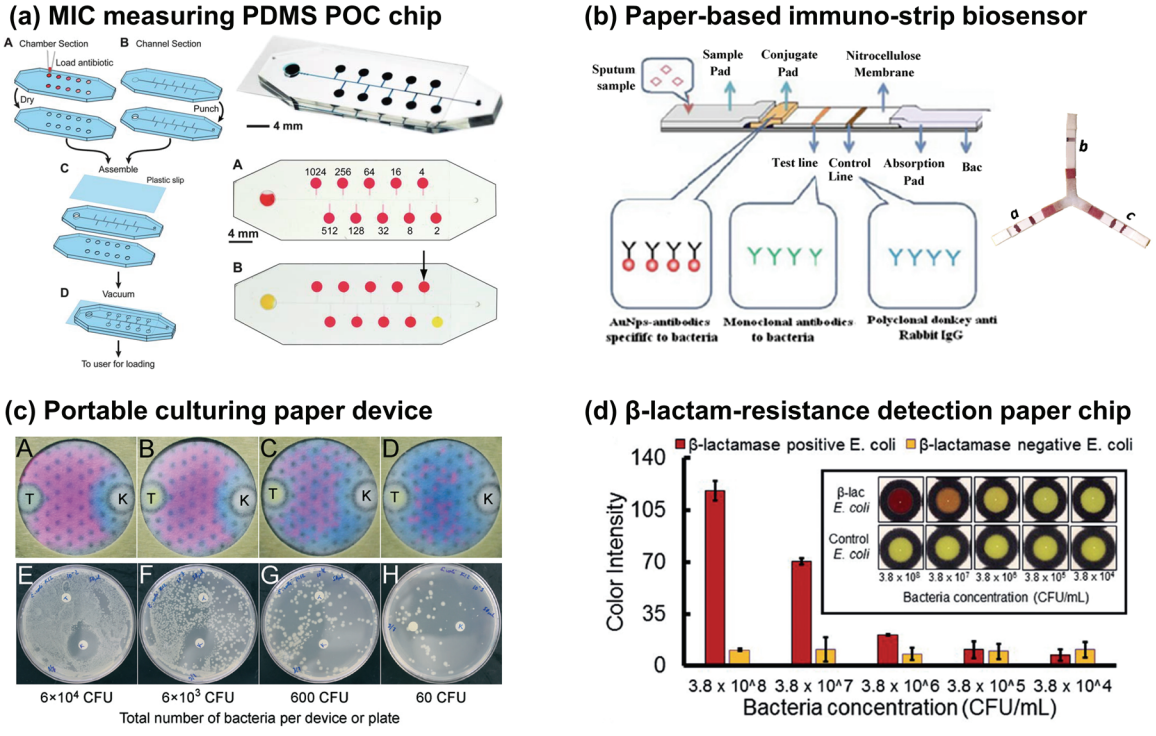

Figure 3. Representative Point-of-Care (POC) microfluidic devices for antibiotic research. (a) POC chip is assembled by connecting the channels to the chambers with aligning the microfluidic channels in one layer of PDMS and the chambers in another. The two top-view images show the determination of MIC values of wild-type *E. coli* and Kanamycin-resistant *E. coli* in two parallel devices[39]. (b) Immuno-strip biosensor is composed of numerous pads and a membrane. The device with 3 branches was used for testing specificity using *S. aureus*, *P. aeruginosa*, and *S. aureus* + *P. aeruginosa*[40]. (c) The device was used to compare results of Tetracycline and Kanamycin AST with those of AST on agar-filled Petri dishes using different concentrations of *E. coli*[41]. (d) Serial dilutions tests were conducted of *E. coli* that were both positive and negative (control) for expressing β-lactamase in order to exhibit specificity. Only β-lactamase expressing *E. coli* resulted in color change[42]. Images reproduced from the references with permission.

per-based microfluidic chips enter the fray. The paper-based devices have recently undergone a soar in popularity due to their low-cost, low weight, and easy disposability while overcoming the limitations of the previously mentioned polymer materials[38,44,45]. For instance, in the research undertaken by Li *et al*., they used a multiplex immuno-sensing paper-disc for the detection using capillary action based antibody conjugated gold nanoparticles, and the subsequent analysis of whole cell bacteria, namely *P. aeruginosa* and *S. aureus* (Figure 3b)[40]. The detection was rapid, highly specific, and had a considerable detection range. In addition, Deiss *et al*. introduced a paper-based AST device in which the susceptibility of *E. coli* and *Salmonella typhimurium* were measured using the changing of colors from blue to pink to indicate growth (Figure 3c)[41]. The device was cheap and the fabrication and use were easy to follow. Furthermore, it produced results comparable to that of the conventional Kirby-Bauer AST, which is done with antibiotic-permeated paper disks atop a slab of agar[46]. Moreover, Boehle *et al*. also used low-cost paper-based devices to detect β-lactamase mediated resistance in *E. coli* by observing color changes in the colorimetric assay (Figure 3d)[42]. In all representative examples, the devices were cheap and easy to fabricate and use while needing a small volume of samples and reagents in relatively short amounts of time.

Conclusions and Future Perspectives

In this paper, we cover four prevalent methods of determining cell growth level for baseline and susceptibility of microfluidic phenotypic AST studies, and also review three on-chip methods of diluting antibiotic concentrations for alleviating burdens of experimental preparations. Lastly, we focus on the POC microfluidic chips for their use in AST because these devices have many advantages over alternative macro-

or micro-scale AST platforms, such as low use of resources, low cost of materials, user-friendly handling, production of rapid results, and tailoring for specific user needs. All things considered, while most of the studies for microfluidic AST have screened general types of antibiotics against wild-type bacteria, focusing on the technical validation of their developed devices, those technologies have provided promising results which could make us consider them as standard methods applicable to clinical cases in the near future. Thus, it can be concluded that multifarious approaches are being used for different facets of microfluidic AST research.

Microfluidics for AST, and microfluidics in general, is an ever-growing field, with the gap between the conventional testing platforms and microfluidic chips getting smaller by the day. More novel antibiotic growth level measurements and antibiotic dilution methods are being studied at this very moment. In order to bring microfluidic chips for AST into the mainstream, collaborations are needed between the engineers who make the chips and the clinicians who would use them commercially. As microfluidic systems become simpler to use, and superfluous components such as active pumps are replaced by passive ones, the world would be more conducive to the aforementioned collaboration. As the microfluidic-based AST chips continue to be developed, we expect them to be widely utilized for their convenience and versatility in the near future.

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Conflict of Interests The authors declare no competing financial interests.

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