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Microfluidics for Rapid Detection of Live Pathogens

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Rapid, sensitive, and selective detection of live pathogens remains a key priority for quality control and risk assessment. While conventional methods often require complicated workflows, costly reagents, lab equipment, and are time-consuming, rendering them inadequate for field testing and low-resource settings. Increased attention has been drawn to developing alternative low-cost and rapid methods to detect on-site live pathogens in different environmental matrices. Among them, microfluidic devices that integrate various laboratory functions in a miniaturized manner have proven to be a promising tool for the rapid and sensitive detection of pathogens. Herein, the development of microfluidic devices specifically designed for the detection of live pathogens is discussed along a concise summary of novel microfluidics systems recently developed, contrasted to conventional methods regarding assay time, the limit of detection, and target organisms. These include a variety of micro total analysis systems (μTAS) and microfluidic paper-based analytical devices (μPADs) in combination with molecular methods and traditional live cell detection techniques, such as cell culture, DNA intercalating dyes, resazurin, and immobilized bioreceptors (e.g., aptamers and capture antibodies). Furthermore, insights on the future perspectives of microfluidics for live pathogen detection with a highlight on the rapid and low-cost method development for field testing are provided.

1. Introduction

Infectious diseases are placed in the top ten leading causes of death worldwide, accounting for over four million deaths each year (WHO, 2020). Rapid, sensitive, and species-specific detection of pathogens is a key component for preventing the spread of common diseases. Traditional methods for pathogen detection include cell culture methods and metabolic assays, and molecular techniques such as polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA).[1,2] These techniques are well-established and used as routine methods in laboratories worldwide; however, they have significant drawbacks with respects to their practicality and functionality (**Table 1**).

Culture techniques and metabolic assays are considered the golden standard for the detection of pathogens and diagnosis; nevertheless, these assays can take days or even weeks to obtain conclusive results and are regarded as time-consuming and labour-intensive methods.[3] Furthermore, only a very small percentage of organisms are culturable in laboratory conditions whilst the vast majority enter an unculturable state, leading to false-positive results that can have grave implications in relation to public health.[4,5] On the other hand, molecular techniques such as PCR and ELISA provide fast test results and species-specific diagnosis but are incapable of distinguishing live from dead pathogens; a major limitation in many fields such as water quality monitoring, food safety, and medical diagnostics.

For instance, the detection of live pathogens is crucial for the monitoring of drinking water quality and ensuring the safety of a water source. Due to insufficient water networks, millions of people worldwide lack access to clean potable water. It is, therefore, crucial to have fast and sensitive techniques that can selectively detect viable organisms and identify potential pathogens. Only live microorganisms pose a threat to human health, thus selective detection of viable organisms is essential to assess the safety of a water source and avoid false positive results. To this end, culturing assays are carried out on a regular basis to identify possible microbial contaminants in water.[6,7]

In the food industry, both production surfaces and food samples are routinely analyzed in order to assess the production hygiene and safety of the food products. Approximately 250 foodborne diseases have been identified so far and it is estimated by the Center for Disease Control (CDC) that each year 48 million people suffer foodborne illnesses. Microbial testing in every step of food production is therefore a major concern for the food industry and an essential component in reducing and eliminating foodborne illnesses. Methods that offer rapid,

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DOI: 10.1002/adfm.202212081

Table 1. Comparisons of traditional cell culturing techniques, metabolic assays, PCR, and ELISA for pathogen detection.

	Live cell detection	Specificity	Sensitivity	Assay cost	Assay time	Assay procedure
Cell culture	Yes	Can be genus- or species-specific, subject to the selective culture medium used	High (1 CFU mL^{-1}, dependant on incubation time)	High	24 h–5 days, subject to the organisms generation time and any further assays to confirm organism's genus or species	Complex
Metabolic assays	Yes	Can be genus or species specific, subject to the selective culture medium used	Moderate (10–10^6 CFU mL^{-1}, dependant on previous enrichment step)	Moderate	1–24 h (typically 4 h of incubation with the reagent of choice)	Simple
PCR	No	Can be genus- or species-specific	High (1–10 CFU mL^{-1})	Moderate	2 h	Simple
ELISA	No	Can be genus- or species-specific	Moderate (10–10^6 CFU mL^{-1}, dependant on previous enrichment step)	Moderate	2 h	Simple

sensitive, and specific detection of microbial pathogens are in high demand, as conventional cell culturing techniques take days to weeks to complete. More importantly, only live microorganisms pose a threat to human health, thus selective detection of viable organisms is essential for providing an accurate assessment of food products and hygiene standards.

In the medical field, detection of live pathogens is also essential whilst carrying out regular hygiene tests to comply with surface cleanliness standards[8,9] and during drug susceptibility testing for diagnostic purposes. With regard to clinical diagnostics, it is of the utmost importance to utilize a technique that can accurately and selectively detect viable pathogens to provide an accurate diagnosis, allowing medical professionals to correctly prescribe and administer the necessary medical treatment. Due to the emergence and rapid spread of antimicrobialresistant bacteria originating from overuse and misuse of antibiotics worldwide, accurate diagnosis through drug susceptibility testing has become imperative to prevent the spread of these dangerous organisms.[10] Furthermore, as only viable organisms are able to multiply and spread in the environment and through populations, it is necessary to develop techniques that can specifically detect live pathogens to control disease outbreaks and avoid large-scale epidemics.

An overestimation of microbial contamination can lead to unnecessary wastage of food and drinking water, incorrect assessments of production and surface cleanliness, and erroneous clinical diagnosis. It is thus essential to employ a technique that can selectively detect live pathogens, as opposed to the total number of cells, presence of DNA molecules, or protein detection that are not specific indicators of viable organisms.

To accurately distinguish live from dead microorganisms, biomarkers such as cell membrane integrity, metabolic activity, and RNA synthesis have proven to be effective for cell viability testing.[11] Many culture-based and molecular-based techniques are currently available for live cell detection. Nevertheless, there is still a need to develop new techniques that can provide automation of the workflow, low assay costs, and have the potential to be utilized for field testing or employed in locations where there is no access to laboratory infrastructure. Whether for food and water quality analysis, early warning systems for disease outbreaks, or clinical analysis and drug susceptibility testing, a highly sensitive and specific technique is of the utmost importance to replace current methodologies.

As an alternative to conventional techniques, microfluidic devices have been developed that incorporate many of the traditional detection mechanisms such as cell culture, DNA intercalating dyes and metabolic reactions, and that integrate the key assay steps into a single operational chip.[12] In recent years, microfluidics has quickly gained momentum for point-of-care (POC) applications, due to its ability to miniaturize and significantly simplify a large number of laboratory processes. Furthermore, the easy and cost-effective production of microfluidic devices, particularly noticeable by paper-based microfluidics, has proven a key advantange in their application for field testing in resource-limited settings.

In this review, we will provide a summary of the microfluidic sensors that have been developed so far for live pathogen detection and critically discuss how they compare to conventional methods with regard to assay time, limit of detection, and target organisms. To the best of our knowledge, this is the first review paper on sensors for live pathogen detection, which is focused specifically on microfluidic devices.

2. Conventional Techniques used for Detection of Live Pathogens

2.1. Culture-Dependant Techniques

Despite several drawbacks, such as the inability to detect non-culturable bacteria as well as time-consuming and labour-intensive protocols, culture-based microbiological assays remain the golden standard for live pathogen detection due to their high sensitivity, specificity, and quantitative capabilities. Several commercially available tests that detect microbial indicators, such as *Escherichia coli* (*E. coli*), are based on cell culturing methods. They employ colony-counting techniques, most probable number (MPN), and Presence/Absence assays.[13] These techniques rely on the use of specific growth media to selectively culture and isolate pathogens of interest. The detection of live cells is carried out by growing colonies on solid media, measuring the optical density of a liquid culture, or directly counting cells using microscopy.

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These methods require days or even weeks to produce results, depending on the ability of the pathogen to form visible colonies, and while a positive culturing result can be used as conclusive evidence of living organisms, an unsuccessful culturing cannot be used as proof of the absence of live cells in a sample.[11] The problem of detecting viable but non-culturable bacteria (VNCB) remains one of the most significant challenges, which may cause false-negative results and dangerous consequences in clinical diagnostics (e.g., drug susceptibility testing). Despite being common practice worldwide, very few of these culturing tests are suitable for low-resource settings as they require expensive laboratory facilities, and trained personnel can take days or even weeks to produce results, making them impractical for specific tasks such as routine water quality monitoring or for rapidly tracing disease outbreaks.[14]

2.2. Culture-Independent Techniques

Aside from traditional cell culturing techniques, there are also numerous other molecular-based methodologies that are termed culture-independent methods, which are commonly used to differentiate live from dead cells. They rely primarily on assessing cellular integrity, measuring metabolic activity, and detecting RNA.[15] Due to the wide range of cell types and sample matrices, there is not a single universal technique that can be applied to all cases. In terms of practicality, cost-effectiveness, sensitivity, and selectivity, each method has its advantages and limitations, and therefore particular attention should be taken when deciding which method to utilize.[16]

2.2.1. Membrane Integrity

Membrane integrity is a biomarker commonly used to determine the viability of cells in a sample. Cells that have lost the integrity of their membrane and allow passage of otherwise non-permeable molecules are considered dead or non-viable. Fluorescent dyes are widely utilized in various ways to evaluate the movement of molecules through membranes and assess cell viability.[16] Two types of fluorescent dyes are used for cell membrane integrity determination: a) dyes that can only permeate compromised cell membranes or b) dyes that can permeate both intact and degraded cell membranes. The discrimination of viable cells in a sample can be accomplished using dual-staining kits such as the LIVE/DEAD BacLight Bacterial Viability kit that uses two fluorophores; one that can permeate viable cells and another that can only permeate cells with compromised cell membranes.[17,18]

The use of fluorescent DNA-intercalating dyes coupled with a DNA-amplification method such as PCR or loop-mediated isothermal amplification (LAMP), has also proven to be an effective method for detecting live or dead organisms.[19] The method can be applied to detect multiple types of pathogens, such as bacteria, viruses, protozoa, and fungi. The technique consists of using DNA-intercalating dyes that can only permeate compromised cell membranes and bind to the DNA. The most commonly used intercalating dyes are propidium monoazide (PMA) and ethidium monoazide (EMA).[20] The technique is based on the entry of the dyes in cells with compromised membranes, light activation of the intercalating dyes, and subsequent irreversible binding of the dyes to the DNA molecules. This technology can be coupled with amplification techniques such as PCR, where the DNA bound to the dyes can no longer be amplified by DNA polymerase, thus enabling exclusive detection of DNA contained in cells with intact membranes.

2.2.2. Metabolic Activity

In metabolically active cells, a number of chemical reactions occur that can be detected through fluorescent, luminescent, or colorimetric means. Adenosine triphosphate (ATP) is a biomarker that is commonly used for viability testing. ATP synthesis ceases upon cell death and can therefore be used to determine the presence of live cells in a sample. Luciferase enzymes use ATP as a substrate to produce bioluminescence that can be detected and measured to quantify the number of live cells in a sample.[21] This method is fast and effective; however, exogenous, or non-microbial sources of ATP can lead to the overestimation of live cells and species-specific detection is not possible. With regard to practical considerations, bioluminescence detection requires expensive equipment, costly reagents, and trained personnel, and is consequently not well-suited for resource-limited settings. Other methods to detect cell respiration include the use of tetrazolium dyes to measure dehydrogenase activity, and resazurin dye to measure cellular oxidoreductase activity. These techniques detect metabolic activity of the cell and thus do not allow the specific identification of the live organisms in a complex environmental sample, they only act as indicators of total live cells.

The ability of a cell to multiply is also used as a marker for live cells. Isotopes such as ^{13}C, ^{15}N, and $H_2{}^{18}O$ can be incorporated into DNA by metabolically active cells and detected in a method termed Stable Isotope Probing (SIP).[22] DNA-SIP is the technique most commonly used, although depending on the application and the phylogenetic information one is looking for, DNA, RNA, phospholipid fatty acid, and proteins can be used as targets of SIP labelling.[23] Since only metabolically active cells that are replicating their DNA are capable of incorporating the labelled isotopes, live microbes can be separated from dead and unlabeled microbes. Labelled DNA is separated from unlabelled DNA by density gradient centrifugation, providing an idea of the amount of metabolically active cells in the sample.[24] DNA and RNA SIP is often followed by a sequencing approach to identify the live cells in the sample. Asides from labelling DNA, proteins can also be labelled by using artificial amino acids in a technique called bioorthogonal noncanonical amino acid tagging (BONCAT). These artificial amino acids can later be tagged with different reporters, such as fluorophores and used to detect protein synthesis, a process that can only occur in metabolically active cells. BONCAT followed by rRNA-targeted fluorescence in situ hybridization (FISH),[25] fluorescence-activated cell sorting (FACS),[26] and 16S rRNA gene sequencing is used to identify live cells in a sample. This method has been used to measure metabolically active cells in heterogeneous matrixes such as soils[27] and marine sediments.[26,28]

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In addition to the techniques mentioned above, metabolic activity of a cell can also be detected by measuring heat flow. Physical, biological, or chemical processes in the cell are accompanied by heat production or consumption, making heat flow a good biomarker for viable cells. Isothermal microcalorimetry (IMC) using commercial isothermal microcalorimeters can be used to measure heat production.

2.2.3. RNA Detection

Cellular RNAs are very unstable molecules that are rapidly degraded once outside the cell, making them a more appropriate biomarker for viable cells than DNA.[29] RNA-based methods have been used in combination with a variety of different techniques, such as qPCR, sequencing, transcriptomics and. In recent years, the isothermal amplification method, nucleic acid sequence-based amplification (NASBA), has become a popular alternative to RT-qPCR for RNA analysis. It has been used to determine the viability of pathogens such as *E. coli* and *Vibrio cholerae* in water sources.[30–32] The major advantage of NASBA compared to RT-PCR is that genomic DNA present in the sample does not lead to false positive results; as single-stranded RNA is specifically targeted, DNA is not involved in the cycle and there is no need add include DNases to reduce DNA contamination, or include an RNA extraction step.[33] This is particularly important in low biomass samples, as DNase treatments often degrade RNA due to contamination with RNases.[34]

In recent years a technique termed Molecular Viability testing (MVT) was developed that uses nutritional stimulation to induce pre-rRNA synthesis, followed by an RT-qPCR amplification step to determine the presence of live bacteria in a given sample.[16] MVT can be conducted by dividing a sample into two aliquots stimulating one of the aliquots by adding growth media and comparing the synthesis of pre-rRNA molecules to a control aliquot that has not been nutritionally stimulated. If an increase in pre-rRNA molecules is seen compared to a control sample with no nutrition stimulation, then one can infer that there are live bacteria in the sample. This technique was first used to detect viable bacteria in water by ratio metric detection[35] and was later adopted for studies of complex human samples such as serum[36] and milk.[37]

3. Microfluidic Platforms for Detection of Live Microorganisms

Microfluidics refers to the handling of small amounts of fluids that are constrained to sub-millimetre structures. Capillary forces allow the passive flow of liquid through channels, thus enabling the transport of substances through a device, and can be used to process, separate, or mix fluids, depending on the application. It has proven to be a useful tool for the miniaturization of various laboratory processes in a single chip, reducing sample size, reagent volumes and greatly simplifying workflows and reducing costs.[38] Microfluidic devices can be grouped into two main categories: μTAS and μPADs. They differ in their manufacturing method as well as in the sampling and detection components that can be integrated into each type of device.

The concept of μTAS was first introduced in 1990, which consisted of a liquid chromatograph on a silicon chip that integrated sample pre-treatment, separation, and detection.[39] Currently, polydimethylsiloxane (PDSM) is the most widely used substrate for μTAS, however, they can be made using several different materials such as silicon, glass, quartz, and other polymers, and the microfluidic channels are typically fabricated by etching and photolithography. The main body of the device that contains the microfluid channels is connected to external units such as pumps, injectors, droplet generators, and microheaters to achieve sampling and detection.[40] μTAS have gained increased popularity in recent years and many lab-on-a-chip (LOC) devices have been developed to scale down one or various lab processes in a single chip format by creating 3D structures in PDMS substrates. The involved detection and readout systems can vary from electrical signals using screen-printed electrodes, to fluorescent and colorimetric visual detection. They can be fabricated to contain different materials in a single device and etched or 3D printed with channels of various shapes and sizes that can precisely control the flow of picolitre to microlitre sample and reagent volumes. Due to these capabilities, μTAS have been used primarily for biological and chemical analysis, as they enable cell transport, confinement, and culture.[41] Their applications include cellomics, medical diagnosis, and environmental monitoring and have also been applied as microreactors for pharmaceutics. Some of the advantages of these devices include the use of low sample and reagent volumes, decreasing assay costs and assay time, simplified operations, and increased functionality compared to traditional assays. Many efforts have been placed to develop microfluidic chips that can replace current laboratory-based testing techniques and can be used as POC testing devices to be applied in low-resource settings.[42]

μPADS on the other hand, are manufactured using paper and wax to create microfluidic channels and contain all of the sampling and detection functions within the main body of the device. They provide cost-effective fabrication, with paper being accessible globally and at low cost, quickly becoming an attractive choice for developing POC biosensors for pathogen detection. Paper-based devices are lightweight and portable, can be easily stored, transported, and disposed of by incineration, leaving behind no contaminants and making them ideal for testing in the field. These devices present good biocompatibility and can contain stored reagents within the paper matrix, minimizing sample and reagent pipetting steps during the operational procedure and greatly reducing the technical training requirements.[43]

Paper-based microfluidic systems consist of nitrocellulose or cellulose supports that are treated with hydrophobic substances such as wax, PDMS, and ink to contain hydrophilic channels to control the flow of liquid within the devices. This provides a passive flow of liquid without the requirement of pumps or valves that more complex microfluidic devices often employ.[44] The use of capillary force to move liquid from one section of the device to another offers a power-free fluidic transport, ideal for POC testing. μPADs have been developed as 3D or 2D structures, depending on the requirement to conduct

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liquid flow through different reaction zones and achieve an integrated detection system. To increase the functionality of paper-based microfluidic devices while maintaining a reduced size, 3D μPADs have been developed. They are manufactured by stacking pieces of paper one on top of the other or by folding sections of the paper device in a specific order following the practice of origami.[45] These devices provide multiplexing capabilities; a single inlet can lead to multiple detection areas where each different target is analysed. Splitting pads and connecting pads that contain specific channels embedded in the paper allow for a vertical and horizontal flow of liquid that can be guided through the different layers of the device and into the detection zone where colorimetric, fluorescent, or electrical detection is carried out. Screen-printed electrodes can be embedded in the paper, and fluorescent probes or dyes are often stored in the paper matrix or added to produce a detectable signal. To quantify results, naked eye analysis is being replaced by smartphone-based technology coupled with an image processing system that can accurately measure the concentration of the target in a sample.

Many μPADs and μTAS have been developed to detect pathogens from a wide range of matrices, including food sources, wastewater, drinking water, and human serum and blood amongst others, and have been used for a variety of applications such as food and drinking water quality control, environmental monitoring, and clinical analysis.[46–52] However, the detection mechanism typically employed by these devices is often based on DNA amplification or protein detection that are not suitable biomarkers for cell viability, and therefore cannot be used for live pathogen sensing.

There is an urgent need to develop innovative microfluidic devices that can quickly and sensitively distinguish live from dead pathogens. In the following sections, we will discuss novel microfluidic systems that incorporate traditional live cell detection techniques for the identification of live pathogens. Similarly, to conventional methods, these devices rely on whole-cell detection and cell membrane integrity, as well as utilizing metabolic biomarkers and cell culturing to assess cell viability. They show potential for miniaturization and field testing and have proven to be useful to detect a range of clinically relevant live bacteria, such as *E. coli*, *Salmonella* spp., and *Campylobacter* spp., whilst greatly reducing detection times compared to conventional techniques, from weeks or days to up < 1 h in some cases (**Table 2**).

3.1. Membrane Integrity

As previously mentioned, current techniques for live bacteria detection heavily rely on assessing the integrity of the cell membrane. This can be performed by employing DNA-intercalating dyes such as PMA and EMA that selectively permeate cells with compromised cell membranes, typically followed by qPCR to amplify DNA originating from live cells whose membranes remain intact. Membrane integrity can also be assessed with the use of antibodies and aptamers selected to specifically bind to antigens present in cell membranes, followed by electrochemical detection of the binding event. If the proteins on the membrane have been denatured, they will no longer be able to bind to the active site of the antibodies or aptamers and thus dead cells will not generate a detectable signal, allowing for selective identification of live cells. In the past decade, several microfluidic devices have been developed to detect live cells by assessing membrane integrity through DNA-intercalating dyes and immobilized bioreceptors such as antibodies or aptamers.

3.1.1. DNA-Intercalating Dyes

One of the first microfluidic devices that included the use of an intercalating dye, was developed in 2012 to detect live MRSA cells.[53] A PDMS and glass microfluidic chip was designed that included EMA pre-treatment, cell lysis, DNA denaturation, and on-chip PCR (**Figure 1**A). The workflow consisted of incubating EMA, the sample, and specific probe-conjugated beads on the loading chamber of the chip. EMA would penetrate cells with compromised cell walls and covalently bind to dsDNA, rendering it incapable of being amplified by PCR. Using a pump, the contents of the loading chip were transported to the PCR reaction chamber where on-chip heaters enabled cell lysis and posterior denaturation of DNA. ssDNA strands were then able to hybridize with the specific probe-conjugated beads that were then isolated using a magnet under the chip. PCR reagents were added to the chip and on-chip amplification was carried out followed by fluorescence measurements. To carry out the on-chip PCR reaction, a temperature sensor and microheaters were employed as additional components of the μTAS. With this technique, they were able to detect 10^2 CFU mL^{-1} in only 2.5 h.

In line with this mechanism of live cell detection, other devices were later developed that involved on-chip PMA or EMA treatment coupled with PCR. A PDMS and glass chip was developed capable of detecting viable bacteria from human joint samples in < 1 h and with an LOD of 10^4 CFU mL^{-1}, showing promising potential for clinical applications.[54] The chip contained a wash buffer chamber, a PCR reagent chamber, a reaction chamber, and a series of valves and transport units to enable the flow of liquid from one chamber to the next. The system functioned by preloading the chip with vancomycin-coated magnetic beads (these were used as a visual probe due to their ability to bind to bacterial cell walls), washing buffer, and PCR reagents. The sample that had previously been incubated with EMA was then added to the reaction chamber where, following a brief period of light exposure to activate the dye, the vancomycin beads captured the bacteria. After 10 min of incubation period, bacteria-bead complexes were captured by placing a magnet underneath the chip and PCR inhibitor were eliminated by pumping the washing buffer into the chamber. Finally, PCR was carried out in the reaction chamber where only DNA from live cells (DNA not bound to EMA) was amplified, with the fluorescent signal measured to determine the presence of viable cells.

Further optimizations of this chip were done to eliminate the PCR step and instead carry out rapid optical detection using gold nanoparticles.[55] Bacteria-bead complexes were captured on the chip, followed by cell lysis and denaturing of dsDNA that was carried out by increasing the temperature to 95 °C. 16S probe-coated gold nanoparticles were then introduced into the

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Table 2. Summary of microfluidic devices developed for live pathogen detection, including the detection technique, the target organism, the limit of detection (LOD) and the assay time.

	Technloigies	Targets	LOD	Assay time	References
Membrane integrity	PDMS and glass substrate chip integrating EMA treatment and on-chip PCR	Methicillin-resistant *Staphylococcus aureus* (MRSA)	10^2 CFU mL^{-1}	2.5 h	[53]
	PDMS and glass substrate chip integrating EMA treatment and on-chip PCR	Gram+ and Gram- bacteria found in periprosthetic joint infection	10^4 CFU mL^{-1}	55 min	[54]
	PDMS and glass substrate chip integrating EMA treatment and gold nanoparticle probe or on-chip PCR	*Pseudomonas syringae*, *Staphylococcus aureus (S. aureus)*, *E. coli*, *Enterococcus sp.*, MRSA	10^2 CFU mL^{-1}	30 min	[55]
	Microfluic droplet platform and fluorescein isothiocyanate (FITC)-labeled aptamers	*E. coli* and *Salmonella*	N/A	N/A	[56]
	PMA pre-treatment and LAMP amplification on µPAD	*E. coli*	10^3 CFU mL^{-1}	2 h	[57]
	PMAxx pre-treatment and LAMP amplification on a portable chip	*Candida. albicans*	10^4 CFU mL^{-1}	<1 h	[58]
	Bacterial capture and on-chip PMA treatment	*Mycobacterium tuberculosis (M. tuberculosis)*	100 CFU	90 min	[59]
	PMA pre treatment and nucleic acid lateral flow strip	*E. coli*	8.1×10^2 CFU mL^{-1} or 81 CFU g^{-1}	2 h	[60]
	Impendance sensor on glass substrate with immobalized antibodies	*E. coli* and *Salmonella* spp.	10 CFU mL^{-1}	<1 h	[61]
	Impendance sensor on glass substrate with immobilized antibodies	2 *Salmonella* serotypes	300 cells mL^{-1}	<1 h	[48]
	Impendance sensor on glass substrate with immobilized antibodies	3 *Salmonella* serotypes	7 cells mL^{-1}	<1 h	[62]
Metabolic activity	lacZ T4 bacteriophage and paper portable culture device	*E. coli*	<10 CFU mL^{-1}	5.5 h	[63]
	ATP detection on µPAD	*Salmonella*	2.6×10^7 CFU mL^{-1}	35 min	[64]
	Chromogenic reaction and detection on µPAD	*Campylobacter spp.*	10 CFU cm^{-2}	10 h	[65]
	Picoarray device with rezasurin dye	*E. coli* and *Staphylococcus aureus*	Not provided	3 h	[66]
	Chromogenic reaction and detection on µPAD	Coliforms	10^4 CFU ml^{-1}	6 h	[67]
	Microfluidic droplet device and labeled antibodies.	*Salmonella typhimurium (S. typhimurium)*	10^6 CFU mL^{-1} (1 bacterial cell per two-to-ten droplets)	<5 h	[68]
	Microfluidic droplets containing resazurin on silicon chip .	*S. typhimurium*	50 CFU mL^{-1}	5 h	[69]
	Dip and test array with rezasurin dye	Gram- and Gram+ bacteria	10 CFU mL^{-1}	12 h	[70]
	Chromogenic reaction and detection on µPAD	Coliforms	3 CFU mL^{-1}	24 h	[71]
	Phage-based bioluminescence	*E. coli*	4.1 CFU 100 mL^{-1}	5.5 h	[72]
	Microfluidic droplets containing resazurin	*E. coli*	2×10^8 CFU mL^{-1}	2 h	[73]
	Chemiluminescence detection in microwell array chip	*E. coli*	560 CFU mL^{-1}	2–4 h	[74]
	Dipstick and bacteriophage	*E. coli and S. aureus*	250 CFU mL^{-1}	4–8 h	[75]
	On-chip culture and resazurin dye reaction	*E. coli*	2 CFU 100 mL^{-1}	12 h	[76]
	Pico-droplet array chip and resazurin dye reaction	*E. coli*	500 CFU mL^{-1}	4 h	[77]

reaction chamber where they bound to the ssDNA, generating a measurable colour change after the addition of hydrochloric acid (Figure 1B). During the denaturing step, EMA-bound DNA remained a double-stranded molecule and could not bind to the gold nanoparticles. Therefore, the visible colour change was due specifically to viable cells in the sample. The whole process was completed in 30 min and was able to detect as little as 10^2 CFU mL^{-1}. If more information regarding the bacterial species present or antibiotic characteristics of the bacteria is needed, an additional on-chip PCR step was also developed to produce results in a subsequent 40 min.

Recent advances in the use of intercalating dyes have been made by coupling PMA with isothermal amplification methods such as LAMP andrecombinase polymerase amplification (RPA), thus avoiding the temperature cycling requirements of PCR, simplifying workflows and increasing its potential for low resource setting and field testing applications.[78,79] An origami style µPAD was designed that was able to detect only live

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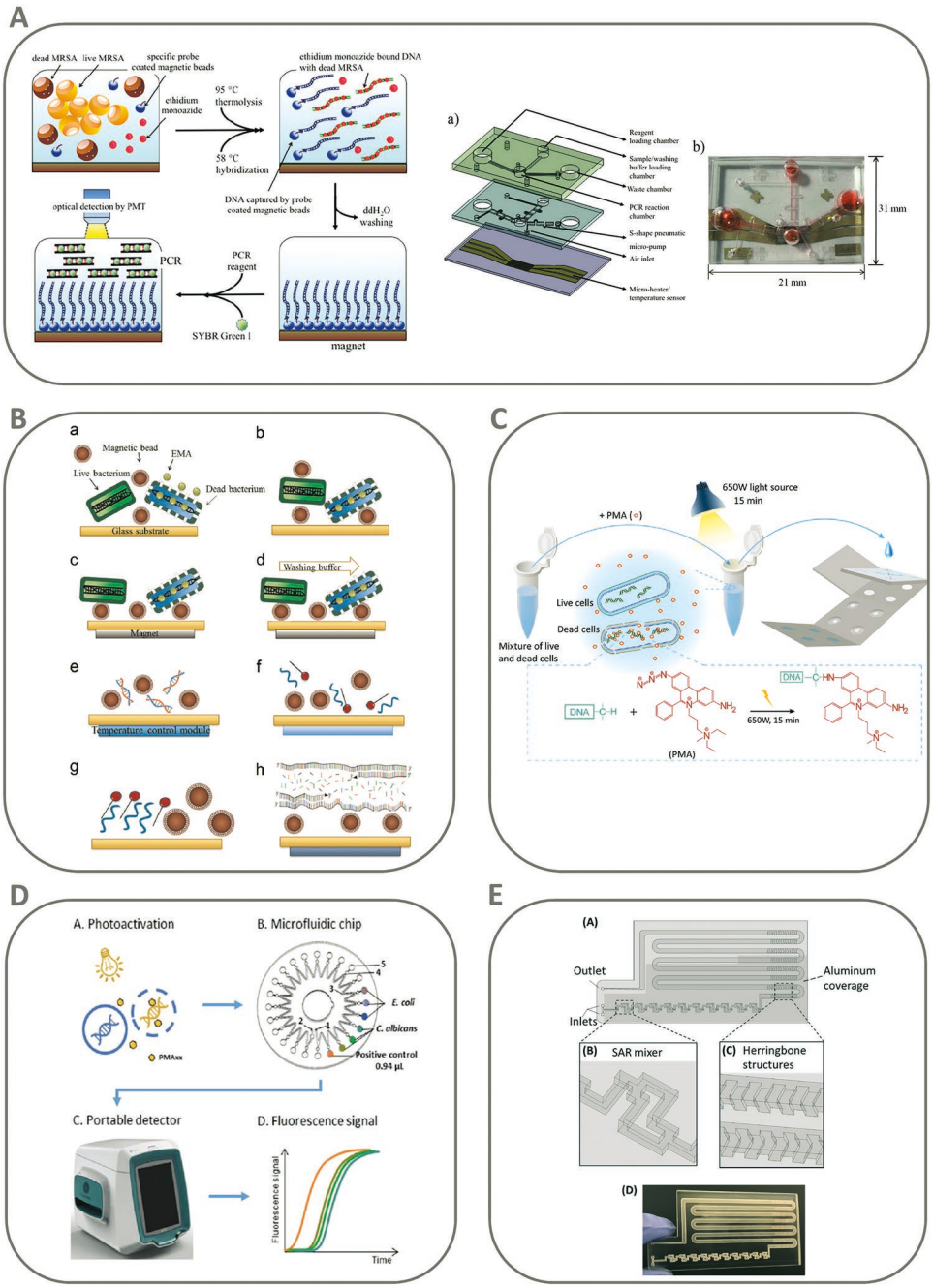


Figure 1. A) Schematic illustration of the detection mechanism and a layered view of the microfluidic chip. Reproduced with permission.[53] Copyright 2012, AIP Publishing. B) Schematic illustration of the microfluidic chip with its different components and chambers, as well as photographs from the bottom and upper layers of the device. Reproduced with permission.[55] Copyright 2015, Elsevier. C) Schematic illustration of sample preparation prior to PMA-LAMP assay on the paper origami device. Reproduced with permission.[57] Copyright 2019, Americal Chemical Society. D) Schematic illustration of detection procedure using a portable microfluidic chip system. Reproduced with permission.[58] Copyright 2021, Royal Society of Chemistry. E) Illustration and photograph of the plastic 3D printed chip with SAR mixers and serpentine incubation channel for on-chip PMA sample treatment. Reproduced with permission.[80] Copyright 2018, Royal Society of Chemistry.

E. coli and *Salmonella* cells by carrying out an in-tube sample pre-treatment step with PMA dye followed by LAMP amplification on the paper device.[57] Following in-tube incubation of the sample with PMA and photoactivation of the dye, the sample was introduced into the paper device. After successive folding steps that enabled the sample to flow through the hydrophilic paper channels and into a compartment where the LAMP reagents were added, DNA amplification was carried out at a constant temperature of 63 °C and the colorimetric results observed by the naked eye (Figure 1C).

Lin et al. developed a microfluidic chip for multiplexed detection of pathogens, using in-tube pre-treatment of the sample

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with PMAxx dye, followed by on-chip LAMP amplification and real-time fluorescence detection on a portable detector (Figure 1D).[58] The total assay time, from sample pre-treatment to fluorescent detection, was just under 1 h (30 min for PMAxx treatment and 25 min for LAMP reaction), rendering this assay rapid and suitable for on-site testing. Furthermore, the chip consisted of 24 reaction chambers, with species-specific LAMP primers embedded in each reaction well, potentially enabling extensive multiplexing capabilities.

Wang et al. developed a PDMS chip that was able to detect a total of 100 CFU in 90 min, incorporating on-chip capture of *M. tuberculosis* cells using a heparin-binding hemagglutinin antibody and live cell detection via PMA treatment.[59] The chip contained 12 PCR reaction chambers to further carry out on-chip PCR and the fluorescence signal was detected with a laser-induced fluorescent module. In order to automate the process, micromixers as well as micropumps were used as external components. Most recently, Wen et al. developed a nucleic acid lateral flow strip (NALFS) in combination with PMAxx sample pre-treatment and immunomagnetic separation to detect *E. coli* in lettuce samples.[60] The NALF assay worked by attaching molecules such as fluorescein amidite (FAM) and biotin to species-specific primers, carrying out LAMP amplification and capturing the amplicons with antibodies on the test line. A total assay time of 2 h was achieved, and the limit of detection was 81 CFU g^{-1} of contaminated lettuce.

PMA/EMA sample treatment is traditionally performed in a tube and involves many pipetting steps, UV light exposure and cold incubation periods. The workflow not only requires experienced personnel but also increases the detection time by ≈ 1 h, rendering it impractical for rapid on-site detection. In an effort to integrate PMA treatment into a microfluidic device, Zhu et al. proposed detecting live microbial cells by using a 3D printed chip to perform on-chip PMA treatment followed by real-time DNA amplification.[80] The chip was made from clear plastic 3D printing material and consisted of ten split and recombine (SAR) mixers and an incubation channel in serpentine shape (Figure 1E) The PMA alongside the sample was injected into the device in the inlets and later mixed in the SAR mixers until they enter the incubation channel. Following incubation, the sample was collected and used for real-time PCR. This technique shows great potential for eliminating in-tube pre-treatment with PMA/EMA. If current microfluidic devices that use DNA intercalating can successfully integrate this serpentine structure into their design to perform on-chip PMA/EMA treatment, the workflow might be further simplified.

Microfluidic devices have proven useful in coupling the use of DNA-intercalating dyes into a miniaturized and automated workflow to detect viable bacteria, reducing the time for results from days or weeks to a few minutes or hours compared to traditional culturing techniques. Furthermore, DNA intercalating dye-based live cell detection in comparison to culturing methods has the major advantage of being able to detect viable but non-culturable bacteria. Since there is no cell culturing involved in the process, the uncertainty of reporting false negative results due to VNCB is greatly reduced.[81–83]

However, DNA-intercalating dyes are not devoid of limitations; whilst it has shown to be useful for distinguishing live bacteria from heat-inactivated cells with disrupted cell membranes, further work must be done to determine its sensitivity to cell viability following other inactivation methods such as UV light exposure, as well as chlorine and antibiotic treatment. In terms of practical applications, techniques that use fluorescent dyes have many limitations; The dyes require cold storage, and the incubation periods are also carried out on ice. For intercalating dyes such as PMA and EMA, photoactivation of the dyes and a centrifugation step are also necessary, adding to the complexity of the technique. Furthermore, the duration and temperature of the incubation and the concentration of the dye have to be optimized for each bacterial species and sample matrix tested. This can result in difficulties when multiplexing and generating reproducible results.[84]

3.1.2. Antibodies and Aptamers

Bacterial cells present several different surface antigens such as lipopolysaccharides, carbohydrates and proteins in their membranes that act as biorecognition molecules for antibodies. Monoclonal antibodies synthesised to specifically bind to these regions can therefore be used to detect whole bacterial cells, either with optical or electrical sensors. In general, optical sensors utilize the ELISA principle, consisting of immobilized antibodies that bind to the analyte and the addition of a secondary antibody, labelled with a fluorophore, or conjugated with an enzyme, that also binds to the analyte and produces an optical signal. Electrochemical biosensors for whole cells include amperometric, potentiometric, and impedimetric types of detection, with impedimetric being the most popular sensing technique.

For the detection of whole viable bacterial cells, impedance biosensors offer a miniaturized, low-cost, and sensitive detection mechanism.[85] They consist of glass chips with sets of fluid inlets/outlets and microchannels that contain a focusing region to concentrate bacteria using dielectrophoresis, and a cell-sensing region with integrated electrode arrays (IDEAs). In the IDEA, specific antibodies are immobilized that target antigens found on cell surfaces. Live cells contain intact antigens that can bind to the antibodies and generate an impedance measurement; however, in dead cells these surface antigens are damaged and can no longer be recognized by the antibodies, producing lower impedance measurements.

In recent years, a handful of microfluidic impedance biosensors have been developed to detect viable whole bacterial cells. Recently, Liu et al. developed an impedance biosensor for the detection of two different serotypes of *Salmonella* cells in turkey food samples.[48] The biosensor consisted of a glass chip with two detection regions fabricated using microchannels. The detection regions were made of micro-gaped interdigitated electrode (IDE) arrays containing immobilized anti-*Salmonella* antibodies for impedance measurements to detect the presence/absence of cells (**Figure 2**A). The device contained two sensing IDE arrays, each with a different antibody for a specific *Salmonella* serotype. The sample was introduced through the antigen inlet using a syringe pump, after that it was left for half an hour to allow binding of antibodies to *Salmonella* cells and a subsequent washing step was performed. The impedance was then measured using an impedance analyser and compared to the

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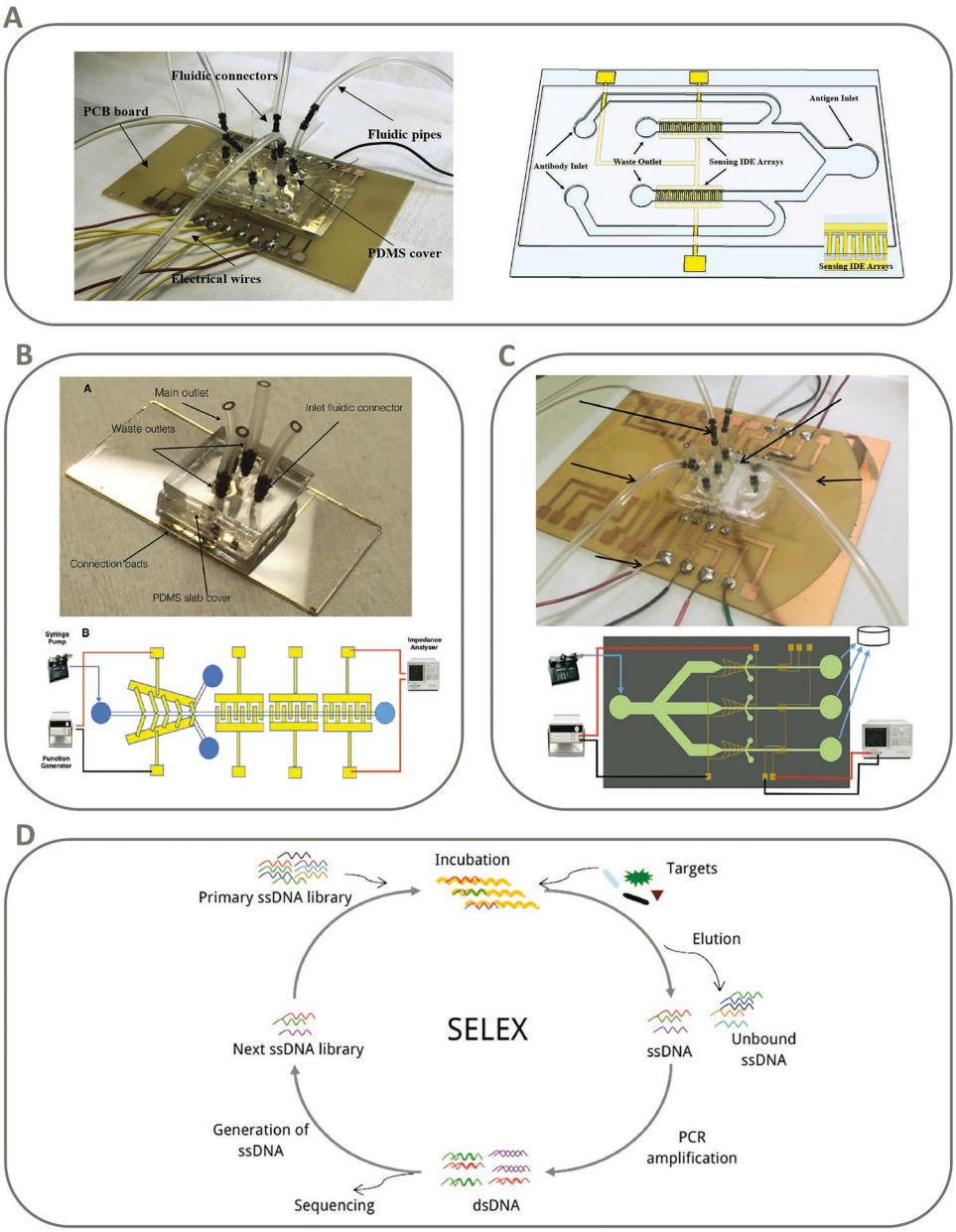


Figure 2. A) Photograph and schematic illustration of the microfluidic chip and its components. Reproduced with permission.[48] Copyright 2019, PLOS ONE. B) Photograph and schematic illustration of the microfluidic chip and its components. Reproduced with permission.[61] Copyright 2018, Wiley-VCH. C) Photograph and schematic illustration of the microfluidic chip and its components. Reproduced with permission.[62] Copyright 2019, Elsevier. D) Schematic illustration of the SELEX process. Reproduced with permission.[86] Copyright 2022, Wiley-VCH.

measurement obtained prior to sample injection. In this way, the difference in the impedance measurement corresponds to the concentration of cells present in the sample. This device was used to test live and heat-killed *Salmonella* cells, showing a considerable difference between the impedance measurement of live and dead cells.

Likewise, another microfluidic impedance biosensor was developed that contained three channels with IDEAs and was able to detect three *Salmonella* serotypes with a limit of detection of 7 cells mL^{-1} in under 1 h (Figure 2C).[62] In a similar manner, Abdullah et al. developed a device manufactured by surface micromachining on a glass substrate.[61] The chip integrated various electrode arrays used to concentrate the bacterial cells and direct them to the detection channel where impedance measurements were taken on an array of detection electrodes (Figure 2B). Specific detection of *Salmonella* and *E. coli* cells was achieved by utilizing immobilized antibodies in the detection zone, demonstrating its potential to detect different targets with good specificity. They were able to differentiate live from dead cells by comparing impedance measurements. Dead cells with damaged cell surfaces were unable to bind to the immobilized antibodies and failed to produce an impedance measurement

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compared to viable cells with intact cell walls. The device was used to detect as little as 10 CFU mL^{-1} in under 1 h.

In terms of sensitivity and speed, impedimetric biosensors offer a good alternative for viable cell detection, capable of detecting 300 CFU mL^{-1} or less in under 1 h. This is considerably faster and more sensitive than techniques based on DNA intercalating dyes. Moreover, microfluidic chips offer great multiplexing capabilities compared to other sensing technologies as a single sample inlet can be later subdivided into multiple channels depending on the number of targets, offering a wider sensing capacity, crucial for pathogen detection applications and simultaneous identification of multiple bacterial species. However, their fabrication is more complicated than simpler devices such as paper-based sensors and the use of antibodies also increases their cost and complexity. Advances in aptamer technology show promise for further reducing the costs and increasing the flexibility of binding sites of impedance biosensors.

In the last few decades the use of aptamers in biosensor technology has quickly gained popularity as an alternative to conventional antibodies. Nucleic acid aptamers are short single-stranded DNA or RNA molecules that are selected in vitro to specifically bind to a target of interest. Compared to antibodies, aptamers offer several advantages such as low cost, easy synthesis, unlimited shelf life, they can be easily modified to add functionalities and can be selected to bind to almost any target. To obtain these highly specific molecules, large libraries of oligonucleotides go through a process termed Sequential Evolution of Ligands by Exponential Enrichment (SELEX). It that involves multiples rounds of positive and negative selection of the molecules to the target of interest followed by an enrichment step of the selected aptamers (Figure 2D).[86] Aptamer targets range from small molecules such as carbohydrates and toxins, to peptides and proteins and even whole cells. The ability of aptamers to bind to cell surface structures has proved particularly useful in detecting live bacteria. Through multiple rounds of selection, aptamers can be obtained that specifically bind to cell wall structures of bacteria that are not present in dead microorganisms whose cell wall has been damaged.[87]

Based on whole-cell detection and employing electrochemical measurements, several different devices have been developed to detect live pathogens[88–90] showing potential for numerous applications from environmental monitoring to clinical diagnosis. Zhang et al. developed a PDMS and glass microfluidic device with immobilized aptamers to selectively bind live *E. coli* cells and subsequently detect the fluorescent signal of FITC-stained cells.[56] A microfluidic channel was carved using soft lithography onto the PDMS slide and contained an input and outlet where the syringe pump was connected to introduce the sample. Once the sample was introduced, the FITC-stained bacteria entered the microchannel where they bound to the anti-*E. Coli* aptamers and, after washing steps, the fluorescence signal was detected using a fluorescent microscope.

The ability to synthesise aptamers that can selectively bind to surface antigens of live cells offers a great alternative to conventional antibodies. These aptamers can be carefully selected by running successive screening rounds using dead cells as targets to eliminate non-specific binding to dead cells. Furthermore, the concept could also be transferred to differentiating infectious from non-infectious viral particles, widening the scope of its application.[88] More work needs to be carried out in this field to develop more practical and cost-effective sensing technologies capable of viability testing. As with any technique that is based on assessing cell membrane integrity, it must not be overlooked, that false positive results may arise from dead cells that retain an intact cell membrane, overestimating the level of contamination.

3.2. Metabolic Activity and Cell Culture

There are many commercially available assays that reply on colorimetric, fluorescent and bioluminescent detection of metabolic activity or cell division to determine the presence of live pathogens in a sample.[91–93] These methods require long assay times and complicated workflows, therefore, microfluidic devices that greatly miniaturize and automize the protocols could potentially be a useful approach to improve live cell detection.

ATP is one of the most used biomarkers for cell viability; however, little work has been done to incorporate ATP measurements into microfluidic devices. One of the first attempts was carried out by Lee et al. who developed a biosensor for real-time detection of ATP from aerosols.[94] The biosensor consisted of an aerosol condensation system, coupled to a silicon microfluidic chip and a bioluminescence sensor. The condensation process allowed the aerosol samples to be quickly concentrated and hydrolysed and then inserted into a microfluidic channel where lysis buffer and D-luciferin-luciferase reagents were introduced through separate inlets. The microfluidic channel served to mix the reagents with the bacterial cells enabling the reaction of free ATP with D-luciferin-luciferase and transport the mixture into the detection area where an electric circuit was used to measure the bioluminescence intensity (**Figure 3**A).

Jin et al. developed a 3D μPAD to detect the ATP of live bacteria.[64] The paper microfluidic device consisted of two zones, a reagent zone, and a testing zone. ATP aptamers were immobilized on the reagent zone, followed by the addition of horseradish peroxidase (HRP)-labeled oligonucleotide, complementary to the ATP aptamer. Meanwhile, the testing zone was treated with 3-amino-9-ethylcarbazole (AEC) and polyethylene glycol (PEG). The device functioned by inserting the sample solution into the reagent zone where free ATP from lysed cells would bind to the ATP aptamer, releasing the HRP-tagged DNA. The μPAD was then folded to produce the flow of the HRP-tagged DNA into the testing zone. H_2O_2 was added to the test zone where AEC was oxidated by HRP/H_2O_2. This reaction produced a colour change (from light yellow to red-brown) that was used to determine the concentration of ATP and thus the presence of live bacteria in the sample (Figure 3B). This process involved minimum handling of reagents and rapid detection, showing potential for POC applications. However, the limit of detection reported was 2.6×10^7 CFU mL^{-1}, very high compared to other microfluidic devices such as all ATP-based assays, it was not a species-specific technique.

Exploiting the use of genus-specific chromatographic reactions, a 2D μPAD device was developed to selectively detect live *Campylobacter* bacteria by employing a chromogenic substrate

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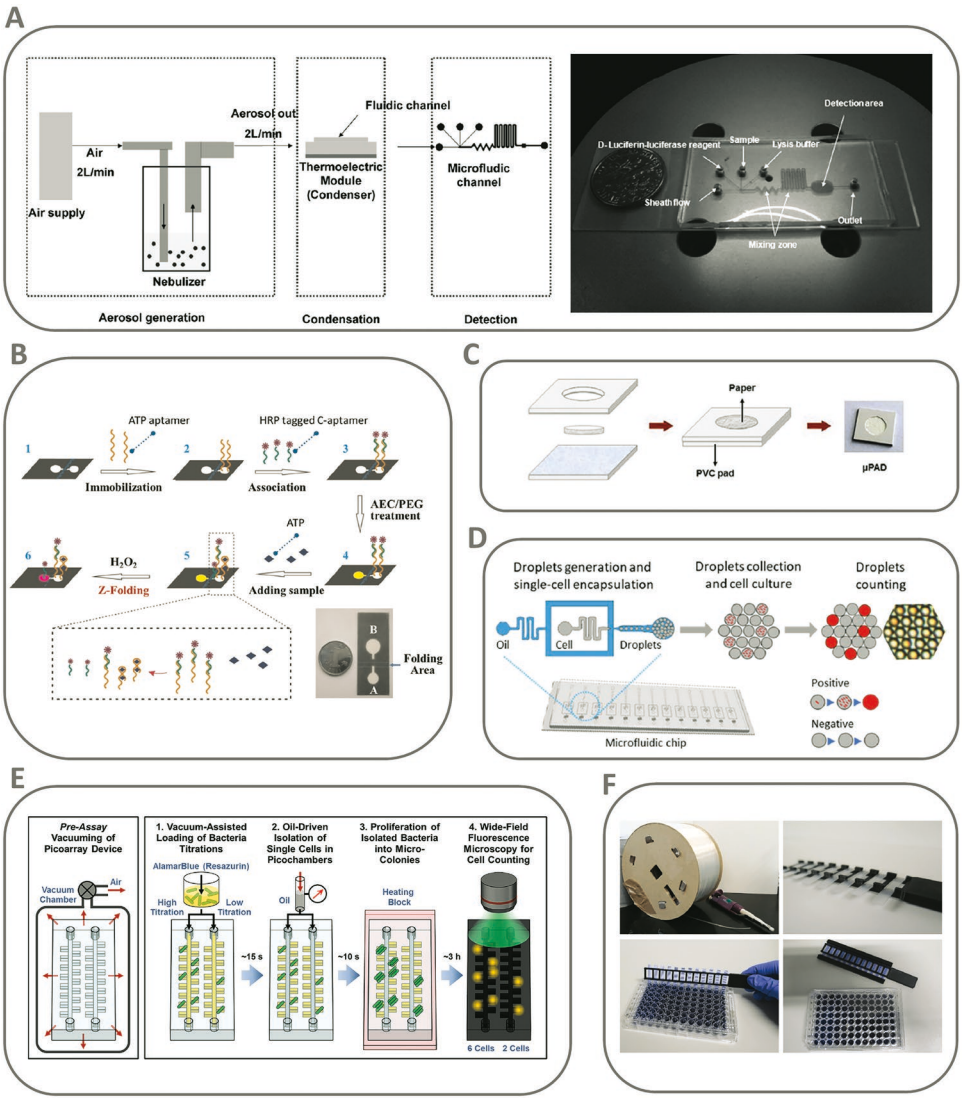


Figure 3. A) Diagram of the experimental setup, including aerosol generation, condensation and detection, and a photograph of the microfluidic device.[94] B) Schematic illustration of the paper device for ATP detection. Reproduced with permission.[64] Copyright 2015, Elsevier. C) Schematic diagram of the paper device for colorimetric detection of cells. Reproduced with permission.[65] Copyright 2018, Elsevier. D) Schematic Illustration of droplet microfluidic chip, droplet collection and cell culture, and counting of fluorescnet droplets. Reproduced with permission.[69] Copyright 2020, Elsevier. E) Diagram of the workflow for detecting live cells using a picoarray device with resazurin. Reproduced with permission.[66] Copyright 2018, American Chemical Society. F) Photographs of the dip and test device and loaded with sample and resazurin dye. Reproduced with permission.[70] Copyright 2021, Elsevier.

specific to the campylobacter enzyme α glucosidase.[65] The microfluidic paper device consisted of two polyvinyl chloride (PVC) pads pressed together with a filter paper centre where the sample was introduced and came into contact with the dried substrate (Figure 3C). Once rehydrated, the substrate reacted with the enzymes from the live bacteria to produce a colorimetric reaction that could be detected by the naked eye or using an image processing software to obtain quantitative results. Unlike commonly used metabolic dyes such as resazurin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), this type of enzymatic reaction has the major advantage of being genus- or species-specific depending in the combination of enzyme and substrate used and can be easily adapted for detection of different pathogens of interest. The same detection mechanism was employed by Bisha et al. to detect *E. coli, Salmonella* spp., and *Listeria monocytogenes* in agricultural water.[95] Detection of total coliforms, a commonly used indicator of water contamination, has also been integrated into 2D µPADs that employ β-galactosidase, a coliform-specific enzyme to generate a chromogenic reaction.[67,71,96]

Bacteriophages, viruses capable of infecting and replicating in host bacteria, are commonly used as a tool for the detection and identification of bacterial populations. A range of existing bacteriophages can be engineered with synthetic biology to contain a reporter gene, rendering them a promising tool for pathogen detection. In recent years increased efforts have been made to develop bacteriophage-based assays for clinical diagnostics and drug susceptibility testing. Due to the ability of phages to only infect live cells, bacteriophage-based assays enable the specific identification of viable bacteria. Once the

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phage infects its host organism, viable host organisms can be identified by detecting a fluorescent or luminescent signal produced by the reporter phage, or by the production of lysis plaques produced during cell lysis. For example, Alonzo et al. developed a microfluidic chip able to detect as little as 4.1 CFU 100 mL^{-1} in 5.5 h.[72] The chip contained inlet and outlet ports for sample, phage, and growth media injection, and outlet ports connected to a waste container. Water samples were first introduced into the chip and filtered via an in-chip polyvinylidene difluoride (PVDF) filter that traps the bacteria present in the sample. Culture media and reporter phages containing the NanoLuc gene are then added to induce the synthesis of the NanoLuc enzyme by viable bacteria. The reporter enzyme is then transported to a different chamber of the chip containing a nitrocellulose (NT) membrane that traps the reporter protein, and following the addition of substrate and the consequent production of luminescence, viable cells can be detected by placing the chip on a separate detection instrument.

Dönmez et al. also utilized bacteriophages for live bacteria detection but instead of using a reporter phage, they exploited the phage's natural ability to produce lytic plaques.[75] Plaque forming units (PFU) are commonly produced in solid agar plates and require a laborious procedure and overnight incubation. In this study a microfluidic dipstick was developed for fast and simple PFU measurements, achieving a limit of detection of 250 CFU mL^{-1} in 4–8 h of incubation depending on the target organism. A major advantage of phage-based assays is their ability to infect bacteria and replicate at a faster rate than its host, producing large quantities of progeny, reporter proteins and/or lytic plaques, often rendering bacteriophage-base detection of viable bacteria, faster, more specific, and more sensitive than traditional culturing techniques.

Dyes such as Resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) are commonly used as indicators in cell viability assays, these assays are based on an enzymatic reaction that produces an irreversible colour change or production of fluorescence. Resazurin is a phenoxazine dye that is cell-permeable, it's non-toxic to cells, weakly fluorescent, nontoxic, and redox-sensitive. It has a blue colour that when reduced by metabolically active cells turns to resorufin that is pink and highly fluorescent. Rodoplu et al. developed a microfluidic chip consisting of a PDMS layer constructed on a glass coverslip with a culture dish at the bottom (diameter of 20 mm).[76] Water samples containing target bacteria were mixed with antibody-bound magnetic particles in Eppendorf tubes and the was mixture introduced into the chip via the sample inlet. The inlet chamber contained a magnet that served to concentrate the bacteria in the chip without the need for a vacuum or syringe pump. Culture media with Resazurin dye was then introduced into the device and incubated at 37 °C for 12 h. Following the incubation period, the colorimetric and fluorescent signal was recorded with a portable mobile-phone platform.

Recent advances in droplet microfluidics have enabled the miniaturization of liquid cell culturing techniques. Contrary to traditional cell culturing in agar plates, droplet microfluidic devices are capable of creating a monodispersed layer of microdroplets, that encapsulate single cells with growth medium and a metabolic indicator such as the resazurin dye or labeled bioreceptors to quantify cell division. An et al. recently developed a device that could detect viable Salmonella cells directly from food samples, achieving detection limits as low as 50 CFU mL^{-1} within 5 h.[69] Monodispersed microdroplets were utilized for encapsulating *Salmonella* cells, enabling single-cell culture. Each droplet contained a culture medium and resazurin as a metabolic indicator for live cells. Aerobic respiration in metabolicaly active cells produced the reduction of resazurin into the highly fluorescent resorufin, allowing for quick identification of viable cells. The silicon microfluidic chip consisted of a bacterial solution inlet, a mineral oil inlet, and an outlet for droplet collection. Droplets that exited the collection outlet were then placed into a 24-well plate for subsequent 5 h culture and analysis using a fluorescent microscope (Figure 3D).

Similarly, Hsieh et al. developed a device that isolated single cells in separate chambers and utilized Rsaezurin dye as an indicator of cell viability.[66] The device consisted of a PDMS-based picoarray device (Figure 3E) where the samples were loaded alongside the Mueller–Hinton broth and the resazurin dye. Partitioning oil was injected into the device, flowing through the channels and separating the sample into picochambers, thus isolating single cells in each picochamber. Viable cells then reduced the resazurin dye and the subsequent fluorescent signal was detected using a fluorescent microscope.

Harmon et al. utilized droplet microfluidics to develop a miniaturized bioreactor to detect viable *S. typhimurium* cells in under 5 h.[68] Bacterial cells were separated into droplets that contained growth medium and FITC-labeled anti-*S. typhimurium* antibody. Following 5 h of incubation, fluorescence intensity was measured using a fluorescent microscope. The sample was separated so as to encapsulate one bacterial cell every two to ten droplets generated. Since the fluorescence signal produced by a single cell was not sufficient to be detected, they established an optimum incubation period of 5 h to allow viable cells to multiply and thus generate a measurable signal. By carrying out a 5 h incubation step, they were able to selectively detect viable cells in the sample.

Further work on droplet microfluidics was carried out by Akuoko et al. They recently developed a microfluidic setup capable of encapsulating single bacteria together with a resazurin dye to detect live cells within just 2 h of incubation time.[73] The PDSM chip was fabricated using soft lithography and plasma bonding to create the microfluidic channels, and was connected to a fluid pump system for droplet generation.

More recently, Needs, Osborn and Edwards developed a "dip and test" device consisting of arrays of microcapillaries that functions similarly to droplet microfluidics to separate samples into multiple compartments and enumerate live bacteria (Figure 3F).[70] The strips of the device are dipped into a 96-well plate containing the sample and resazurin dye. These were then taken out and left incubating overnight for end-point colorimetric detection or fluorescent measurements using a fluorescent microscope.

Wu et al. developed a chemiluminescence digital microwell array chip for live *E. coli* detection.[74] 6-Chloro-4-methylumbelliferyl-β-D-glucuronide (6c-MUG) was chosen as the probe that can be cleaved by the β-D-glucuronidase in *E. coli* to produce a fluorescent signal and achieve fast single bacterial

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detection. The chip was fabricated with soft-lithography and PDMS moulding to contain 38 400 microwells of the following dimension: 30 × 30 × 50 µm. The sample containing *E. coli* and the probe was introduced in the microwell array followed by thermosetting oil to encapsulate single bacteria in the wells. If the microwell contained live bacteria, they would multiply during the incubation period and produce β-D-glucuronidase that would then cleave the probe, resulting in a detectable fluorescent signal. In a similar manner, Suo et al. used thermosetting oil to encapsulate *E. coli* cells in individual droplets within a PDMS chip and detect viable cells via resazurin dye reaction.[77]

The major advantage of using devices that enable single-cell cultures, such as microfluidic droplet chips and microfluidic picoarrays, lies in the ability to quantify live bacteria. These techniques use the Poisson distribution to enumerate the bacteria present in the sample. Moreover, due to reduced contaminants, smaller volumes and more efficient mixing, growth rates of bacteria in droplets have proven to be quicker than in traditional liquid culture, reducing enrichment times and improving overall assay time.[97] As with all metabolic dyes, however there is no certainty with regard to the identity of the bacteria present in the sample as all viable cells are capable of reducing resazurin.

Overall, very few microfluidic devices have been developed to detect live pathogens using metabolic activity as a viability biomarker. Current attempts exploit ATP production, Resazurin and MTT reduction, and glucosidase activity. Other metabolic biomarkers such as RNA and protein synthesis or heat production could still be explored for possible viability testing solutions in combination with microfluidics. In many cases, one of the greatest limitations of using metabolic activity as a biomarker for cell viability is the inability to perform species-specific detection. Further research has yet to be conducted in this field to identify possible biomarkers such as proteins and RNA sequences that confer specific information about an organism's identity. With regard to assay time, culturing and metabolic assays are inherently restricted by each organism's duplication time, rendering these assays considerably longer than techniques based on membrane integrity. Whilst incubation times may be longer, the sample enrichment step allows the limit of detection of these techniques to be lower than membrane integrity-based methods, with some devices having reported the ability to detect < 50 CFU mL^{-1}.

In terms of sensitivity and assay time, droplet-based microfluidics shows promising results, having achieved low limits of detection and greatly reducing the assay time compared to traditional culturing techniques. Nonetheless, the incubation time compared to traditional resazurin dye assays carried out in a 96-well plate does not differ. Due to the small droplet volumes, special consideration has to be taken when optimizing the volume of culture medium and regulating the pH within the device to promote cell activity. Droplet microfluidics enables the use of smaller sample and dye volumes, therefore reducing assay costs. Droplet generation simultaneously encapsulates both sample and indicator dye which eliminates tedious pipetting steps, greatly automating the workflow. Furthermore, due to the number of microfluidic droplets generated, it has great potential for high-hroughput screening assays.[98]

4. Conclusion

Methods for live pathogen detection are essential in many applications ranging from food and water monitoring to disease outbreak detection. Existing laboratory-based techniques for live pathogen detection, such as cell culturing methods, offer great sensitivity and quantitification capabilities, however, are lacking in species-specific identification, ease-of-use, quick assay times, and POC testing applications. Meanwhile, traditional molecular-based techniques such as PCR, count with species-specific identification of pathogens and quick assays times, however, are unable to distinguish between live and dead cells. To overcome the limitations of traditional technologies, the field of microfluidic sensing platforms has widened its scope in the last two decades with the objective of enabling rapid, specific and on-site live pathogen detection systems.

µPADs and µTAS are miniaturized all-in-one sensing platforms that allow sample processing and detection within a single operational device. To develop a technique that can simultaneously possess all the advantages of cell culturing such as live cell detection and high sensitivity, and of molecular-based methods such as species-specific detection and quick assay times, microfluidics has been combined with cell culturing, metabolic and molecular-based assays to develop innovative techniques that can overcome the shortcomings of traditional methods. However, the challenges associated with the miniaturization of the operational steps whilst still retaining high sensitivity and specificity have resulted difficult to overcome, greatly hindering rapid advances in microfluidic technology for live cell detection.

One of the many obstacles faced during miniaturization is that microfluidic devices are inherently small and can retain reduced volumes of liquid, therefore, the addition of external pumps and sample enrichment via filtering techniques has to be incorporated for large sample volume analysis. Moreover, for the use of microfluidic devices in a lab-free setting, innovations involving isothermal amplification techniques must also be incorporated to migrate from PCR-based methods. At present, most devices are still at an early stage of proof of concept where more validation is required for their widespread application. Many µTAS still require the use of laboratory infrastructure and expensive equipment such as fluorescent microscopes, therefore, there is still a need to further simplify these devices and their detection mechanism to enable rapid pathogen testing in the field.

Microfluidic platforms have the potential to provide a rapid, low-cost and easy-to-use species-specific method for sensitive live pathogen detection. Advantages of microfluidic devices include cost-effective fabrication and low reagent costs, integration of multiple functions in an intergrated system, and faster response times. Despite their advantages, microfluidic systems are still lacking in sensitivity and have limited capacity for large sample volumes. For the use of microfluidics in low resource settings, many limitations such as ease of use, cold storage and electricity free operations still need to be overcomed to make them suitable for field testing. When developing new techniques, it is therefore crucial to take into account their potential application; for instance, in clinical analysis, having a short assay time might be more important than achieving very low

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Table 3. Summary of advantages and limitations of conventional pathogen testing techniques and microfluidic sensors for live pathogen detection.

Technique		Aadvantages	Limitations
Conventional techniques for pathogen detection	Cell culture/metabolic assays	High sensitivity High sample volume capacity Quantitative Live cell detection	Experienced personnel Laboratory infrastructure High cost Long assay times Partial specificity
	PCR/ELISA	High sensitivity High specificity High sample volume capacity Quantitative Quick assay times Highly specific	Experienced personnel Laboratory infrastructure High cost No live cell detection
Microfluidic sensors for live pathogen detection	µTAS	Minimally trained personnel Reduced sample and reagent volumes Low cost Quick assay times	Laboratory infrastructure Use of external components Low sensitivity; many at a proof-of-concept level Few are quantitative Complex manufacture
	µPADS	Minimally trained personnel No laboratory infrastructure; suitable for field testing Simple manufacture Reduced sample and reagent volumes Low costQuick assay times	Low sensitivity; many at a proof-of-concept level Not quantitative

limits of detection, whilst for drinking water monitoring, a high sensitivity is crucial for compliying with strict hygene standard. Consequently, the trade-offs between assay time, sensitivity, and complexity in relation to their application must be taken into account when comparing these devices' potential and contrasting them with traditional methods.

We have summarized the advantages and limitations of traditional pathogen testing techniques and novel microfluidic sensors developed to date for live pathogen detection (**Table 3**).

In the last few years, there has been a surge in the development of microfluidic systems, particularly for *E. coli* and total coliform detection for their application in water quality monitoring. These systems have exploited the use of microarrays and droplet microfluidics in combination with metabolic dyes to rapidly and specifically detect live cells whilst still retaining the quantifying capabilities of traditional culturing techniques. Droplet-based or microarray-based techniques have shown the greatest advances in the field and demonstrated the potential to accurately detect live pathogens in a miniaturized manner.

Recent work in µPADs has rendered them the best option for point of care applications, as they contain all of the detection functions within a single device (also namely lab-on-a-paper), providing quicker, cheaper, easier operations, enabling ultra-sensitive and selective multiplexed detection of pathogens for broad-spectrum applications. Dry reagents that improve the ease of use and increase shelf life are particularly important to develop devices for POC testing. Paper-based devices have shown great potential for POC testing as they can integrate all functions within the single device and can be manufactured to contain dry reagents on the paper substrate, thus improving shelf life and usability.

Regarding live cell detection, current work on microfluidic platforms is very restricted, focusing mainly on DNA intercalating dyes, impedance sensors, resazurin dye, ATP detection, and droplet cell cultures. However, there are still several different techniques, such as RNA detection protein synthesis, and heat flow monitoring, whose potential to be integrated into a microfluidic platform could still be explored. With the recent outbreak of SARS-COV-2, a global effort to improve diagnostic techniques for viruses has quickly increased. The use of impedance-based devices that differentiate infectious from non-infectious viral particles based on the integrity of the viral envelope, is an area that requires further investigation and could potentially be of great use in clinical and epidemiological studies.

Acknowledgements

The authors acknowledge support from a Royal Academy of Engineering Frontier Follow-up grant (FF\1920\1\36) and Z.Y. thanks UK NERC Personal Fellowship grant (NE/R013349/2). The graphical abstract was made BioRender.com.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

cell viability, microfluidics, pathogen detection, sensors

Received: October 18, 2022
Revised: February 2, 2023
Published online: March 3, 2023

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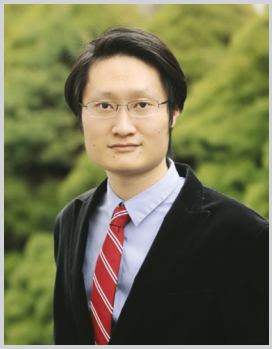
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Microfluidics for rapid detection of live pathogens

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2023-05-17
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https://doi.org/10.1002/adfm.202212081
Spatola Rossi C, Coulon F, Ma S, et al., (2023) Microfluidics for rapid detection of live pathogens. Advanced Functional Materials, Volume 33, Issue 21, May 2023, Article Number 2212081

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