Microfluidic tools for probing micro-culprits

Opportunities and challenges in microfluidic diagnostics

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Early diagnosis is one of the most important factors for successfully treating a disease—be it cancer or an infection—but it continues to present a challenge for physicians. Imaging tools such as X-ray, computed tomography, magnetic resonance imaging, or ultrasound are often used to look for solid tumors in cancer diagnosis. Agar plates, flow cytometry, and PCR are typically used to identify pathogens for monitoring and managing infectious diseases. These “classical” diagnostic tools, however, often have low sensitivity, require sophisticated medical facilities, have long turnaround times, and are costly. More simple, accurate, quicker, cheaper, and, in particular, personalized *in vitro* diagnostics are therefore needed for the rapid screening and detection of diseases.

Microfluidic devices are characterized by microchannels with width ranging from submicrons to few millimeters. They can shuttle picoliters or even smaller volumes of biological fluids into functional compartments with high precision. The possibility of using different materials—polymers, paper, or gels—for creating microfluidic devices further reduces overall developmental and production costs. On the whole, miniaturized diagnostic tools have immense advantages over the conventional technologies, particularly in the clinical setting, in terms of sample size, speed of analysis, portability, throughput, multiplexing, and parallelization. This has great potential for cheap and disposable diagnostics, which can make a big impact for patients in both developed and developing countries. Microfluidics is

thus an ideal candidate for developing point-of-care (POC) platforms that fit the bill for the “ASSURED” (Affordable, Sensitive, Specific, User friendly, Rapid and robust, Equipment-free and Deliverable to end-users) criteria set by the WHO.

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Since the early 1990s, microfluidics has been a very promising technology in biomedical engineering for designing sensitive biosensors leveraging on fluid dynamics in the micro-scale. The applications of microfluidics have gradually diversified into sample preparation and delivery, *in vitro* assay development, and sensors and detectors, yet the number of microfluidic technologies that have been translated from “bench to bedside” is minimal. There are many possible reasons for the low translation rate: from limited improvements over existing technology to government policies and regulations. Here, we discuss the origins of microfluidics in diagnostics for infectious diseases and cancer, with a focus on cell-sorting applications for liquid biopsy, from rare cell capture to bulk and single-cell analysis to biomarker discovery. We next discuss efforts and challenges to clinical translation and commercialization, including

lessons learned from our own experience in Biolidics Limited (formerly known as Clearbridge BioMedics). We also provide a prospectus of how to overcome these barriers and for future directions.

Detecting pathogens

Despite global efforts and enormous progress to fight infectious diseases, they continue to pose a serious threat throughout the world, with more than 30 previously unknown diseases emerging since 1970. Many of these have a high mortality because of cross-species transmissibility of pathogens, antimicrobial resistance, or a lack of effective medical countermeasures, such as vaccines or antimicrobials. Newly emerging and known pathogens including HIV/AIDS (1960-present), cholera (1961-present), smallpox (1974), plague (1994 and 2017), SARS (2002–2003), Ebola (2014–2016), Zika (2015-present), and dengue (2016) have caused anything from small, isolated outbreaks to full-scale pandemics during the past century.

An effective means to contain and control such epidemics involve accurate and timely diagnosis to identify patients for treatment and to disrupt further transmission. Using POC devices makes the most sense for providing rapid and actionable information at the time and site of an encounter. A recent observational study in eight sub-Saharan African countries found that POC testing for early HIV diagnosis resulted in improved accuracy (98.3% vs. 18.7%), reduced turnaround time for test results (0

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vs. 55 days), earlier therapy initiation (0 vs. 49 days), and lower costs (US \$27.24 vs. \$131.02) compared to conventional HIV testing (source: *The Lancet HIV*).

A widely used microfluidic-based POC platform is the immunochromatographic strip or lateral flow device that is used for a multitude of applications, including *in situ* detection and rapid screening of bacterial, viral, and pathogenic antigens [1]. Another approach involves miniaturization of key detection technologies such as real-time PCR for on-chip nucleic acid analysis, or enzyme-linked immunosorbent assay, or ELISA for on-chip immunoassays. The “laboratory-on-a-chip” from the HandyLab is an exemplary microfluidic platform for real-time PCR: It provides various hands-off fluidic operations: nucleic acid extraction, clean-up, reagent dissolution, mixing, amplification, and detection. The mChip test is a miniaturized ELISA immunoassay that demonstrated rapid (less than 20 min) and accurate detection of biomarkers for HIV and syphilis with low sample requirements (1 µl of unprocessed whole blood).

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Yet, despite technological advancements toward miniaturization and integration of sensors and detectors to facilitate automation and user-friendly operation, only a few microfluidic POC tools are capable of processing complex fluids such as whole blood. One such example is the work by Hou *et al* [2], which uses microfluidic enrichment of infected red blood cells from unprocessed whole blood as a biomarker to diagnose malaria (Fig 1, top left). Another challenging aspect of the POC platforms is their shelf-life, as detection reagents are required to be stored on-chip. While a controlled environment provides long-term shelf-life and stability for the reagents, changes to the surrounding conditions, such as temperature and humidity, may affect their reactivity, resulting in failed tests and poor reliability.

Given that many infections have similar symptoms and biomarkers for identification—for instance, malaria and sickle cell disease—the integration of multiple sensing modalities into a single POC microfluidic chip may provide more systematic and reliable diagnosis of infectious diseases. Furthermore, it is imperative to evaluate and correlate the diagnostic and therapeutic outcomes of POC devices that are currently being used in developing countries with conventional techniques to gather a comprehensive understanding of the clinical and economic impact of these platforms. Using techniques of deep learning and artificial intelligence in such correlational studies may further enable the development of algorithms to predict the medication and its dosage required to achieve a specific control or suppression of viral replication and evolution.

Detecting circulating tumor cells

Cancer is the second leading cause of death worldwide. From each gram of tumor, approximately 1 million cancer cells can passively and actively enter the bloodstream every day [3]. Due to a combination of physical and oxidative stress, however, only a tiny minority of about 0.02% of all circulating tumor cells (CTC) is able to resist programmed cell death, escape the immune response, travel to a distant organ, and ultimately grow into a metastatic lesion. As such, only several tens of CTCs are present in millions to billions of blood cells, which make their detection and isolation extremely challenging. Since the first report on CTC isolation technique in 1960, various platforms, based on either biochemical or biophysical properties of CTCs, has been gradually developed to detect and isolate patient-derived CTCs, some of the leading technologies are poised for translation to clinical practice.

About 90% of cancers develop from epithelial tissues, and the first-generation CTC enrichment platforms utilized antibodies targeting specific epithelial cell surface receptors (e.g., EpCAM and CK)—a technique referred to as immunoaffinity enrichment. Single or cocktails of antibodies can be attached to microposts or surfaces that serve as filters for positive selection of CTCs and/or for depletion of nonspecific background cells, such as leukocytes. Some of these technologies have been successfully pushed into commercialization throughout

the past decade, including CellSearch®, AdnaTest®, MACS®, MagSweeper®, IsoFlux™, BioFluidica CTC Detection System®, EasySep™, and Dynabeads®.

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While these systems have demonstrated prognostic significance of CTC enumeration across various cancer types and made major progress, they are limited by a lack of robust CTC-specific biomarkers to isolate subpopulations of CTCs depending on the antibody used (e.g., EpCAM or CK expressing cells). Molecular characterization confounded by the resulting false positives—circulating normal epithelial cells expressing cell surface markers or activated leukocytes—or false negatives (mesenchymally shifted or metastatic CTCs) can be further complicated by the inherent cellular heterogeneity of CTCs. The development of label-free approaches as second-generation platforms for accurate and high-throughput enrichment of CTCs has been fueled in part to address these limitations.

Label-free technology

Microfluidics has come to the fore as a powerful tool (Fig 1) [2,4–8] owing to its ability to leverage on the biophysical qualities of the cells such as size, deformability, density, dielectric property, and optical or image characteristics for various on-chip operations from simple manipulation of fluids and particles to single-cell analysis. Exemplary devices for CTC isolation may include inertial focusing (ClearCell® FX, Vortex HT chip), dielectrophoretic (ApoStream™, DEPArray™), and acoustophoretic microfluidic systems. A key benefit of applying such marker-independent techniques for CTC enrichment is the ability to detect tumor cells in circulation without making any prior assumption about the type of cancer cells and surface marker expression—which may vary depending on disease status—thereby providing specific biomarkers associated with clinical outcomes.

Figure 1. Label-free microfluidics for CTCs: from rare cell capture to cell expansion and single-cell analysis.

Top left: Stiffer malaria-infected RBCs (iRBCs) migrate toward the sidewalls of the microchannel, with reported recovery rate of ~ 75% and > 90% for early-stage and late-stage iRBCs, respectively [2]. Right: Larger and stiffer CTCs can be separated from smaller blood cells, with reported recovery rate of > 85% [4]. Enriched patient-derived CTCs can be used for CDX generation, cell counting, and single-cell isolation. Molecular analyses of single CTCs have been successfully done for mutation detection [5], patient stratification [6], and tumor typing [8] across diverse solid cancers. Bottom left: CTCs can be expanded in the absence of growth factor supplements without requiring prior enrichment using a microfluidic device, allowing high-throughput drug screening in a patient-specific manner [7].

One of our efforts to apply inertial microfluidics to liquid biopsy was the utilization of a spiral microchannel (Fig 1, top right). With optimized fluid flows, larger CTCs with a diameter of 8–22 µm could be efficiently separated from the smaller blood cells (RBCs ~ 8 µm; leukocytes ~ 8–14 µm), achieving > 85% cancer cell recovery, and 99.99% depletion of leukocytes in whole blood [4].

This phenomenon is termed Dean Flow Fractionation (DFF), whereby suspended cells in curved microchannels experience hydrodynamic lift force and dean drag force induced by secondary flow, both of which depend on cell size. By modulating the dimension of the spiral microchannel in depth and width, microparticles of different sizes in complex mixtures can be sorted and concentrated.

Single-cell analysis

With the advent of next-generation sequencing (NGS) technology, emerging data have provided solid evidence for the existence and prevalence of specific CTC subpopulations with differing metastatic potential at the molecular level. This has stimulated studies to identify and characterize these

putative cellular precursors of metastasis. Owing to technical challenges, however, these studies have been limited to analyzing bulk-cell CTC populations, missing out on information about cellular heterogeneity, which constitutes an important determinant of treatment resistance. Furthermore, the analysis and interpretation of molecular data derived from bulk samples have not always been straightforward owing to spurious or confounding effects of contaminating leukocytes, which are pronounced particularly in transcriptomic studies.

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To address the challenges of CTC heterogeneity and inevitable leukocyte contamination, single-cell technologies have emerged as a third-generation approach. The past decade has seen a dramatic increase in single-cell sequencing and expression profiling platforms—scRNA-seq, scPCR, Western blotting, whole genome, or exome sequencing—that are being applied to study mammalian organogenesis, embryo development, neuronal diversity, and intratumoral heterogeneity. Their application to probing patient-derived CTCs, however, has been comparatively limited, largely owing to the extreme rarity of detectable and intact CTCs and the associated technical challenges. There is currently no standard workflow encompassing all steps required for single-CTC analysis including single-cell isolation, preamplification, quality control, sequencing, and profiling.

Despite these technical problems, several recent studies have successfully characterized CTCs at the single-cell resolution. They have identified clinically useful genomic alterations, including those that are private, that is, mutations that are not present in matched tumor tissue, therapeutically targetable mutations—functions of mutations that can be inhibited with existing, available FDA-approved drugs—and newly discovered mutations that are not found in public domain databases. At the transcriptomic level, studies revealed highly complex and

heterogeneous expression patterns, providing a comprehensive window into the evolving landscape of cancer transcriptomes while accounting for cellular heterogeneity. Clearly, the potential of single-CTC-based liquid biopsies is huge.

In the years since the transition from bulk to single-cell analysis, our group has been developing a suite of microfluidic devices and integrated platforms, to reach single-cell level and further reveal clinically significant CTC-derived biomarkers (Fig 1, bottom right). In 2016, Yeo *et al* [5] demonstrated the use of a cell-mechanic-based microfluidic device in isolating single lung CTCs harboring key mutations in the EGFR gene (i.e., T790M and L858R). The same microfluidic approaches have been applied to transcriptomic analysis in subsequent years and have revealed a set of specific genes consistently associated with early recurrence and metastatic spread in NSCLC patients [6]. To the best of our knowledge, this study was the first to leverage single-CTC-derived signatures to better inform patient stratification.

From “bench to bedside”

Despite a massive surge of interest in microfluidics, conventional fluorescence-assisted cell sorting (FACS) and magnetic-assisted cell sorting (MACS) remain the choice of cell-sorting technology in clinical settings. This is largely because they have been subjected to years of refinement through product development, automation, optimization, and standardization, and achieve superior performance in terms of throughput, sorting speed, processing volume, purity, and reliability [9]. In contrast, only a small handful of microfluidic technologies have recently made it to the market, very few of which are fully automated and can be applied to rare cells in biological fluids while achieving sorting performance comparable to FACS and MACS devices.

An early example is the ClearCell[®] FX system (Biolidics, Singapore), which utilizes DFF principles to enrich intact and viable CTCs from whole blood of a cancer patient in a fully automated, label-free manner (Fig 1, top right; [10]). Since its official launch in 2014 at the American Society of Clinical Oncology Meeting in Chicago, the system has been deployed worldwide, with a reported sensitivity and specificity of > 80% in detecting CTCs from clinical

samples, and has obtained CE-IVD, US FDA Class I and CFDA Class I registration, as of today.

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Translation of such micro-technologies to mass production to clinical use, however, is not an easy task. It involves instrumentation, platform development, refinement, clinical validation, manufacturing, and distribution, among other steps, and requires multiple expertise. Microfluidics is still a nascent field where systems are developed, often by engineers, initially for basic research purposes. Poor communication between the engineers and the end users—biologists and clinicians—makes the resulting platforms difficult to handle and operate for routine practice. It is often not made clear how the technology can address the unmet need in the clinical context, or if it is, at least, comparable to well-established diagnostic technologies in terms of practicality and device performance, provided it has a competitive advantage in manufacturing costs at the production scale. Most of the microfluidic platforms for biomedical applications have been miniaturized macro-scale instruments, with minimal, or even no incremental value, creating high levels of apprehension among clinicians to branch away from the conventional approaches.

The resulting ambiguity in marketing strategies further leads to limited funding opportunities, which is arguably the most critical factor for successful translation of microfluidic diagnostic products. Regulatory affairs add another layer of complexity to the process; in the USA, the approval of medical device should take place through either the 510(k) pathway, claiming that the present technology is comparable to the existing FDA-approved technology, or premarket approval, which requires more in-depth evidence supporting that the technology is safe and effective. As a result, receiving regulatory clearance itself takes considerable time, and large investments. Thus, CellSearch[®] still remains, to date, the

only CTC assay approved by the FDA for use in diagnosis for breast, prostate, and colorectal cancer.

Regulation and accreditation

Another deterrent to translation is the accreditation from regulatory bodies and acceptance of the technology by the end users. Despite the suitability of microfluidics and its accompanying advantages for liquid biopsy, the FDA has so far only approved one blood test (cobas® EGFR Mutation Test) for cancer diagnosis. Without regulatory approval, oncologists will continue to regard blood tests as an add-on to standard-of-care tissue analysis, and not as a replacement, although their use is now recommended by the European Society for Medical Oncology and the National Comprehensive Cancer Network guidelines in cases where there is insufficient tumor material or tumors are not easily accessible. While more time and data will be required to make blood tests a mainstream tool, molecular evidence is mounting that liquid biopsies not only have excellent concordance with tissue biopsies, but also have higher detection rates of actionable mutations across diverse cancer types.

“Despite the suitability of microfluidics and its accompanying advantages for liquid biopsy, the FDA has so far only approved one blood test [...] for cancer diagnosis”

Analysts tend to agree that oncologists are slowly but surely becoming more comfortable with blood tests for metastatic cancer, where the less invasive attributes of liquid biopsy become particularly handy, with excitement for commercially available NGS tests, such as InVisionFirst®-Lung, FoundationOne®Liquid, Guardant360™, and Lunar test (source: Evaluate). Genomic testing solely based on tissue biopsy may indeed miss out guideline-recommended cancer biomarkers in up to 50% of cancer patients, as is in the case for NSCLC (source: Guardant Health). A regulatory green light from the FDA would substantially facilitate the widespread adoption of liquid biopsies. Microfluidic diagnostics successfully translated into daily clinical practice will provide

clinicians with readily understandable and reliable results in a short turnaround time. Although it takes a few days to derive clinically relevant information from single CTCs in current laboratory settings, we foresee that the time will only get shorter with the continual development and optimization of fully automated, multi-modular microfluidic devices.

Concluding remarks

The microfluidic market is projected to generate a revenue of US\$12.45 billion by 2025 from US\$3.6 billion in 2018. A significant portion of this will come from medical devices such as *in vitro* diagnostic platforms (glucometers and pregnancy strips) and POC devices. Upcoming techniques of 3D printing and newer or cheaper materials, such as paper, coupled with automation, and machine learning is expected to accelerate the growth of the microfluidic market (www.grandviewresearch.com/industry-analysis/microfluidics-market). Despite its slow market growth during the past two decades, the adaptability of microfluidics to target different applications has enabled them to stay relevant and remain synchronized with the changing demands.

In cancer diagnostics, a lack of understanding of the prognostic significance of circulating biomarkers could hinder the development of cell-enrichment technologies and success in related diagnostics. For example, there is still no consensus on which specific markers to use for CTC capture in immunoaffinity-based technology and whether CTCs or CTC-derived gene signatures *per se* could serve as an indicator of tumor burden. As such, it is imperative that larger clinical studies validate the predictive value of CTCs and their genetic material in a prospective manner. Once the utility of CTCs and specific biomarkers is established, integrated frameworks for microfluidic platforms should be developed and generalized to avoid any discrepancy resulting from different enrichment or isolation technologies, which is pronounced particularly in CTC studies.

The promises of microfluidics far outweigh the setbacks in its translation. As a “platform enabling technology,” microfluidics can be used for applications in the pharmaceutical industry—for drug development and testing or disease diagnosis through POC test platforms. The realization

of the full commercial potential of this technology will occur only if we, biomedical engineers and developers, remain in tune and updated with the present-day market and users’ demands and requirements.

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Conflict of interest

C.T.L. is an advisor and shareholder of Biolidics Ltd. The authors declare that they have no conflict of interest.

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