Chapter 5
Single-Cell Microfluidic Cytometry for Next-Generation High-Throughput Biology and Drug Discovery

Westbrook M. Weaver and Robert Damoiseaux

Abstract The cell is the smallest unit of life. Commensurate with its importance in biology and medicine, it has traditionally been the focus of technologies seeking to add to our understanding of physiological processes relating to life, death, and disease. Over the past decade, our understanding of cellular complexity has been bolstered by the advent of increasingly precise techniques for the investigation of cellular phenomena. Microfluidic cell cytometry combines analysis on the single cell level with integrated separation and processing techniques and has emerged as one of the most powerful techniques in this context. This chapter will encompass an overview of the current landscape and novel trends as well as challenges facing the technologies in this new field.

Abbreviations

CCD	Charge-coupled device
CD	Cluster of differentiation
CDR	Complementarity determining region
CMOS	Complementary metal-oxide semiconductor
CTC	Circulating tumor cell
DC	Deformability cytometry
DEAL	DNA-encoded antibody libraries

W.M. Weaver
Department of Bioengineering, University of California, Los Angeles, CA, USA

R. Damoiseaux (✉)
Molecular Screening Shared Resources, California NanoSystems Institute,
570 Westwood Plaza, Room 2145, UCLA Receiving, Los Angeles, CA 90095, USA
e-mail: rdamoiseaux@mednet.ucla.edu

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DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial to mesenchymal transition
EpCAM	Epithelial cell adhesion molecule
FOV	Field of view
HAI	Hospital-acquired infection (also healthcare-associated infection)
HCS	High-content screening
HIV	Human immunodeficiency virus
HTS	High-throughput screening
IFNγ	Interferon gamma
ISAAC	Immuno-spot array assay chip
MCF7	Michigan Cancer Foundation-7
MET	Mesenchymal to epithelial transition
MITOMI	Mechanically induced trapping of molecular interaction
PCR	Polymerase chain reaction
PMA	Phorbol 12-myristate 13-acetate
POC	Point-of-care
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SCBC	Single-cell barcode chip
Simoa	Single molecule array
STEAM	Serial time-encoded amplified microscopy
TF	Transcription factor
TNFα	Tumor necrosis factor alpha

5.1 Introduction

Undoubtedly, as the length of scale of interrogation decreases from tissues to single cells, we find that the complexity of multicellular entities is much greater than the sum of their parts one would have assumed. One of the main problems in translational research is the fact that an aggregate measurement of cells (e.g., in a microtiter plate) does not reflect the uniqueness of cell subpopulations, let alone single cells, which might have unique or rare phenotypes. However, these rare cells can often play a crucial role in biology and are quite different from overall phenotypes present in a population of cells. Furthermore, cells behave very differently when they are embedded in an interactive structure such as tissue, where cells are stimulated by their microenvironment and in turn also contribute to the mechano-biological and overall state of the microenvironment. Environmental cues such as tissue mechanics, soluble factors, and fluidic forces have been implicated in the initiation and/or progression of normal physiology such as tissue development [1–3] as well as a number of disease states, e.g., tumorigenesis [4,5] or even instances of bacterial pathogenesis [6].

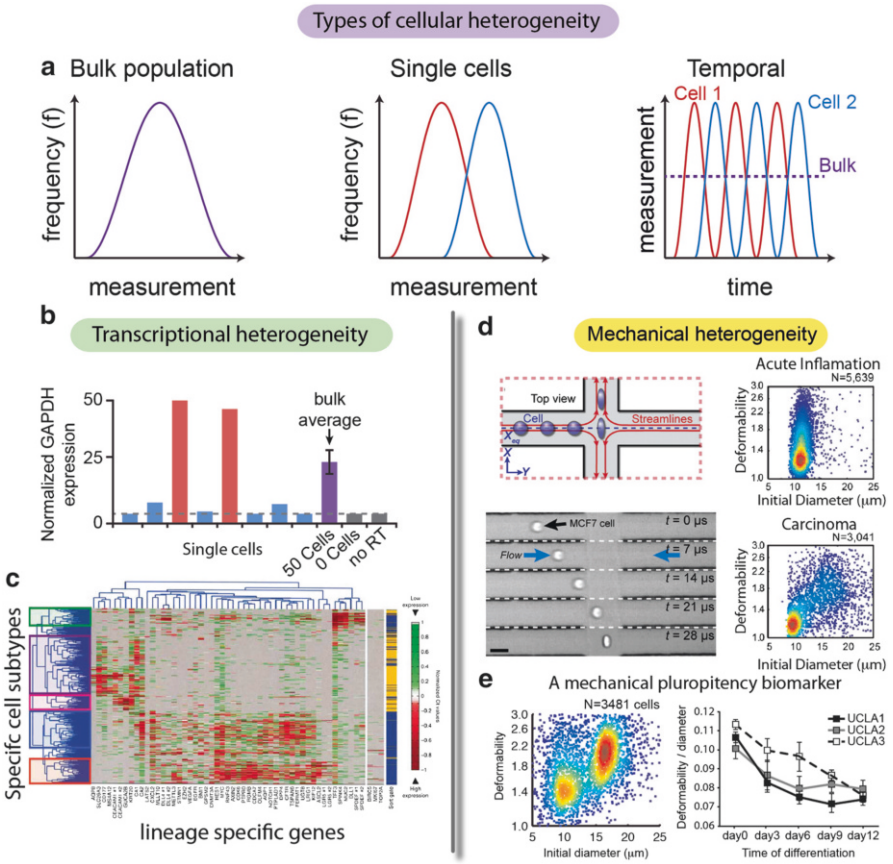

Fig. 5.1 (**a**) Potential ways in which cellular heterogeneity can be manifested in the measurement of a bulk cell population. (**b**) Single cell RT-PCR using microfluidics has shown directly that previously described "incomplete" siRNA knockdown is the result of pooling the heterogeneous response of a cell population in which some cells display complete knockdown, whereas others display only ~50 %. (**c**) Transcriptional heterogeneity within a colon tumor biopsy at the single cell level. Delineations between subtypes are illustrated in this heat map representation. (**d**) Mechanical biomarkers measured in high throughput are indicative of cell subtype. Measurement of pleural fluid effusions from human patients delineates between inflammation and carcinoma prognoses. (**e**) Pluripotency is also linked to cellular deformability, and quantitation of deformability can predict differentiation state

5.1.1 Single Cells Drive Population Phenotypes

The influence of single cells on a large population can be seen in many instances of biology (Fig. 5.1). For example, a normal functional human immune system contains, at any given moment, millions of B-cells, producing discrete subsets of antibodies

that comprise human-acquired immune recognition [7]. Further, upon infection, T-cells secrete a suite of interleukins and cytokines in a polymorphic and dynamic fashion, where each step is required for a proper immune response. Transcriptional profiles of bulk cells have been found to be the sum of digitized "on/off" states of individual cells (Fig. 5.1b, c). Even bacterial cells, typically thought of as purely single-celled organisms, often come together in communities of multiple species and act as a pseudo tissue [8,9], secreting extracellular matrix and modulating metabolic profiles to increase fitness and survival [10,11]. This process requires seemingly clonal populations to develop into distinct heterogeneous phenotypic subsets of cells.

Therefore, an understanding of the heterogeneity of a cell population is paramount for our full understanding of any given biological process. Moreover, resolution down to the single cell level is important for diagnostic purposes and also to increase our understanding of the underlying disease to better inform treatments and identify therapeutic targets. In this vein, the development of high throughput, quantitative methods of investigation on the single cell level is a necessity, due both in part to the need for identification of small subsets of cell populations, and biological variation between patient samples frequently encountered in the clinic. At the same time, the rareness of a cell population can make it difficult to obtain statistically significant results and gain insight into, e.g., regulatory pathways involved in physiology. High throughput microfluidic cell cytometry introduces a new paradigm of measurement, moving from standard well plate formats toward serial flow-through processing as well as miniaturization and parallelization of the well plate concept on chip (Fig. 5.2).

5.1.2 Microfluidics Break the Fundamental Limitations of HTS

Of course, microtiter plate screening methods and conventional high throughput and high content screening (HCS) methods have been very useful for drug discovery and basic research over the past few decades, leading to numerous improvements in medicine and significant additions to our biological understanding. These techniques are useful in this context because of their robust and simple nature, as well as the fact that the high throughput screening (HTS) community has evolved around the microtiter plate, resulting in technologies tailored to their shape, size, and functionality. The limitations of the microtiter plate format become evident when we consider the detection of rare subpopulations and rare cellular events: in biological measurement, statistics are extremely important due to biological variation, stemming both from the inability of these experiments to control for all variables and the heterogeneity of cell populations due to cell cycle state, stem cell populations, and other distinct subpopulations even within clonal cell samples. Simple calculations regarding the frequency of a rare cell (perhaps 1 % of the whole population: a generous number) in a confluent 384 well plate (about 5,000 mammalian cells total) lead us to the realization that, in each well, we could only hope to capture the phenotype of 50 cells in a best-case scenario. In HTS applications where hundreds of thousands

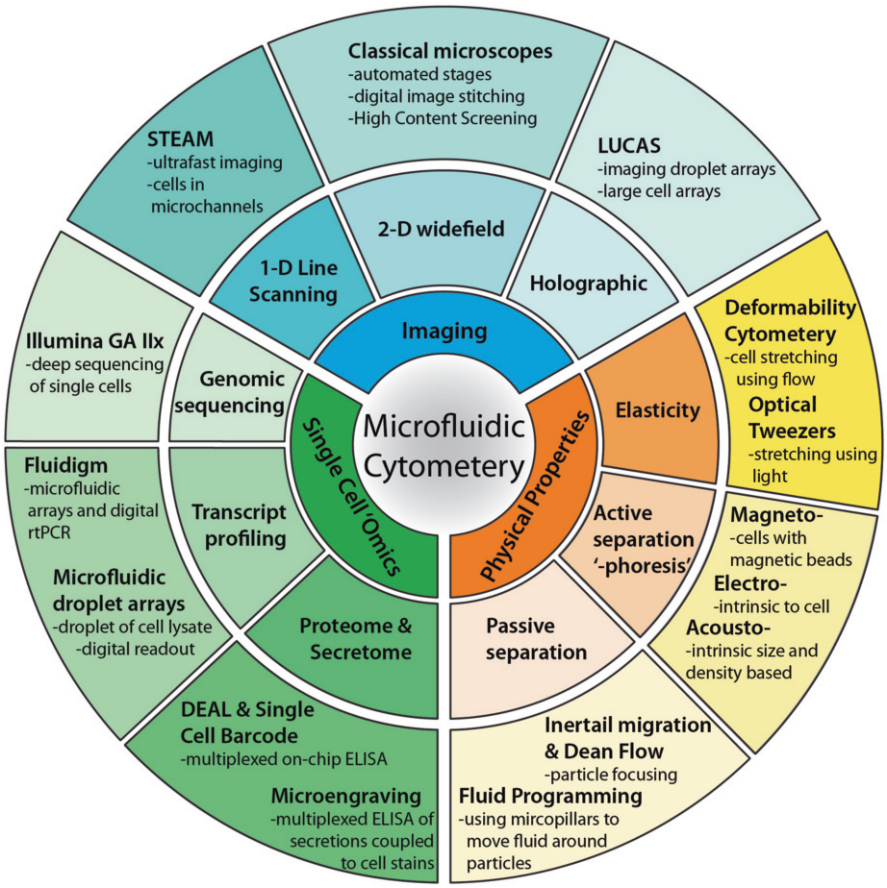

Fig. 5.2 The components of microfluidic cytometry. Technical components such as microfluidic liquid handling and measurement, coupled with novel-imaging techniques are enabling this emerging field. Significant advancement has been made in the fields of proteome profiling of individual cells isolated from carcinomas, secretome profiling of immune cells, transcriptional and genomic profiling of individual cells, as well as the development of mechanical biomarkers for cell state and function

of compounds are to be screened, an enlargement of sample size is not practical and we are left with a dangerously low sample size for reliable measurement. This issue can be remedied to some extent by HCS approaches (allowing multiparameters analysis) and replicate wells (increased sample size), but the fundamental statistical problem of small sample size persists in HTS and also diagnostics, where cell populations of interest might be even much rarer than 1 % of the total cell. Two possible solutions to this problem are either a switch to serial processing of a large number of single cells, or combining enrichment of rare cells with miniaturization of surface assays using, e.g., cell-based microarrays. Microfluidic cell cytometry platforms offer potential methodologies for both of these approaches.

In fact, several powerful methods of single cell analysis have emerged over the past few years, building on classical methods of molecular and cellular analysis techniques. These microfluidic cytometry technologies can be organized based on the measurement type of (1) classical fluorescent and brightfield imaging, (2) physical characterization of single cells, and (3) single cell genomic transcriptomic and proteomic profiling, as depicted in Fig. 5.2. Single cell deep sequencing and reverse transcription-PCR (RT-PCR), whole blood and single cell proteome profiling, secretome arrays, and microfluidic high-speed serial processing and separation of cell types have brought the scientific community a deeper understanding of the importance of cell heterogeneity. New biomarkers based on cellular mechanics have been identified as potential prognostic and diagnostic measurements. In this chapter we will focus primarily on these recent technologies in the context of their biological question, and how they have enabled potential diagnostic tools and furthered scientific understanding of complex biology. We will conclude this chapter with a closer look at platform technologies, translational applications, and future trends of this exiting technology.

5.2 Tumorigenesis and Cancer Biology

Cancer itself cannot be defined as a single disease, but is rather an umbrella term, describing an ever-increasing set of characteristics and cellular aberrations that ultimately lead to tissue neoplasia, metaplasia, and tumor formation [12]. It is this tumor formation that results in dysregulation of the normal tissue equilibrium and eventually organ shutdown.

Differences aside, there is an underlying theme between many cancer types, which is the existence of a stem-cell like subpopulation [13,14], giving rise to new progeny and may lead to the neoplastic behavior, as well as the circulating tumor cell (CTC), ultimately responsible for tumor metastasis by detaching from the original tumor, travelling through the vasculature and exiting into the interstitium at a new site. The stem-cell like population is thought to be formed by dedifferentiation of existing tumor cells as well as the activation of epithelial–mesenchymal transition (EMT), and the reverse mesenchymal–epithelial (MET) transformation [15]. These processes are not yet fully understood, but the current paradigm suggests that EMT occurs both in the tumor mass and during the processes of intravasation into the blood stream [16], most likely leading to a dedifferentiated state similar to a Twist 1-mediated stemness [17]. Conversely, MET, a redifferentiation process, is thought to occur downstream during extravasation to a metastatic site and may be required for cellular proliferation and metastatic formation [18].

Both cancer stem cells and CTCs play a pivotal role in disease progression. Cancer stem cells (perhaps having undergone an EMT) residing in the tumor mass may contribute significantly to drug resistance and relapse after treatment regimes [19,20], and CTCs are implicated in metastasis and hence disease progression [21]. Both of these cell types are small fractions of the cancer cell population. In the case

of the CTC, once in the blood, it is an extremely rare cell—sometimes as low as 1 cell in 1 mL of patient blood containing approximately 10^9 normal red blood cells and white blood cells.

5.2.1 Single-Cell Genotyping and Mechanophenotyping

Microfluidic cell cytometry-based detection and profiling of cell types from the primary tumor mass, as well as the CTCs in the bloodstream, offers a very powerful tool for monitoring the progression event as well as dissecting the mechanisms of progression itself. For example, our understanding of the tumorigenic process is enhanced by single cell transcriptional analysis. This microfluidic cytometry technique has shown that colon tumors exhibit the transcriptional diversity of different cellular lineages in a normal colon (Fig. 5.1c). Further, monoclonal tumors from a single ($n = 1$) cell xenograft, still exhibit this transcriptional pattern of multiple cellular lineages, giving strong evidence for the hypothesis of a stem-like population within the tumor mass and retention of this property within this cell subpopulation [22]. This single cell transcription profiling technique utilizes integrated microfluidic circuits of pressurized valves and individually addressable chambers.

When moving away from the primary tumor mass, cellular populations residing in blood or pleural cavity effusions have to be considered. In this context, high throughput microfluidic flow-through techniques have proven to be very useful. Among these techniques is deformability cytometry, a technique in which whole cells are serially deformed in an extensional flow region at a rate of 1,000s per second (Fig. 5.1d) [23]. This method has indicated that deformability may be an effective biomarker for identification of acute immune responses, as well as indication of prognosis for varying cancer types. Complementation of conventional cytological analysis of cell smears and pathology performed on tissue blocks with a quantitative method such as deformability cytometry may help significantly reduce the instance of "atypical" cytological cases in the clinic. These "atypical" cases often result from the lack of clear indicators of malignancy such as large nuclear to cytoplasmic ratio and abnormal nuclear morphology [24]. These cases are notoriously hard to diagnose in early stages, often resulting in tumor progression before confirmed diagnosis, leading to a poor prognosis before treatment can even begin. In fact, deformability cytometry uses mechanophenotyping of pleural cavity effusions to quantitatively diagnose these "atypical" cases as either malignant or benign prior to a conventional cytological follow-up visit, which involves expensive molecular tests and secondary tissue biopsies [25].

5.2.2 Capture and Analysis of CTCs from Blood

Moreover, the isolation of rare CTCs has been accomplished using flow-through microfluidics. The methodologies employed include surface marker-based methods

utilizing separation based on magnetic beads [26–28] or posts [29]. These shaped objects can be coated with capture antibodies against cells of epithelial origin, which are not normally found in blood. Another approach utilizes nanomaterial-based capture that exploits the aberrant cell surface structure of a CTC. Here, the cell surface interacts directly with the nanomaterial in a Velcro-like fashion [30]. Magnetic methods are specific; however, somewhat lower throughput (15 min for 200 μL of blood) than other methodologies as high flow rates impart fluidic forces on the cells that can overcome the magnetic forces holding the magnetic beads in place. Posts coated with antibodies have proven to be effective for capturing cells; however, the subsequent release has proven nontrivial. A high flow rate CTC capture technique by micro-vortices in sudden expansion and contraction chambers has shown promise for high throughput CTC isolation, using inertial forces for size-based separation in flow [31,32]. This method also allows easy release of concentrated CTCs in high purity (>90 % purity) for downstream analysis [33]. The ability of captured cells to be easily released for downstream processing will enable both scientific discovery and clinical diagnosis. Combination of these capture techniques with single cell sequencing, RT-PCR and immunocytological staining on chip will enable extremely accurate determination of CTC state, and perhaps (when combined with knowledge of EMT and MET and their effect on tumor progression) will enable better diagnosis and more effective treatment regimes.

While not strictly cytometry based, we would like to mention as well other detection methodologies which we expect to have an impact on this field in the near future. Proteomic profiling of both whole blood and, more recently, single cells has shed tremendous light on the cellular states that define certain subpopulations of cells in tumorigenesis. The single cell barcode chip (SCBC) [34] utilizes DNA-encoded antibody library (DEAL) [35] arrays, combined with sandwich ELISA on the channel surface for multiplexed detection of up to 12 plasma proteins for whole blood, or cellular proteins (both cytosolic and membrane bound) in the case of the single (up to 5) cell chip. The SCBC has enabled a more comprehensive understanding of altered signaling states under treatment with erlotinib and emphasized heterogeneity with respect to this response between single glioblastoma cells. This insight could enable a better understanding of drug treatment options specific to cell state, novel targets, and pathways of interest for the cancer cell. Moreover, these data could be used to better direct the usage of combination therapy such as drug cocktails, even rational design, or at least selection of future drugs for use in the patient.

5.3 Bacterial and Viral Pathogenesis

Bacterial pathogenesis is a complex problem to approach due to the wide variety of important infectious organisms, and the myriad of mechanisms utilized by various bacterial species for infectivity. The opportunistic microbes responsible for hospital-acquired infections (HAIs) resulting from implanted medical devices are

of particular mention in part because of the significant impact they have on the healthcare system today [36,37] and the increasing difficulty in treatment because of antibiotic resistance [38,39].

5.3.1 Nanoliter Encapsulation of Bacterial Cells

The question of how antibiotic resistance arises in a population of bacteria has been recently addressed utilizing microfluidic two-phase plug-based systems. These are flow-through, serial systems, where large volumes (up to liters) of bacterial solutions, or even raw ecological samples such as pond water, are in turn compartmentalized into nanoliter droplets in microfluidic channels [40]. The tenability of these systems has been further increased by the introduction of secondary and tertiary solutions to the plug flows by mixing of the miscible stock aqueous solutions from multiple fluidic inlets into combined plugs-in-oil flows (see Fig. 5.3), allowing bacteria to be compartmentalized with varying concentrations and types of antibiotics, as well as the readout for measurement: typically a DNA-intercalating dye (Fig. 5.3). Subsequently, downstream these plugs are quantified using fluorescence microscopy [41]. These systems have directly shown evidence for the founder effect, in which a single bacterium from a clonal population acquires resistance to an antibiotic and creates a new clonally distinct population (Fig. 5.3b) [42].

The ability of certain bacteria to form, and exist in, both mono and multispecies biofilms have long been considered a virulence determinant, especially when considering implantable device-related infections [37]. Further, quorum sensing, the ability of bacteria to "sense" the local density of their inter and intraspecies partners, is thought to control the biofilm forming capability in many of these organisms by genetic regulation which often includes toxin production [43,44]. Microfluidic systems have been well suited for investigation of these phenomena as next-generation high throughput culture systems, incorporate fluid flow with well-defined shear, and spatially organized compartmentalization of bacteria. Using high throughput measurements on microwell arrays confining bacteria spatially while maintaining paracrine-signaling capability, "quorum acting" has been utilized to directly observe such phenomena in B. cereus. Here, high local density (i.e., small distances between microwells) induced transcriptional changes leading to increased clotting capabilities when exposed to whole blood [45].

5.3.2 Viral Load Detection in Fluids

Detecting the presence and amount of viral particles in both patient blood and ecological samples will be paramount for effective diagnosis and monitoring of antiviral treatments. Classically, the enzyme-linked immune-sorbent assay (ELISA),

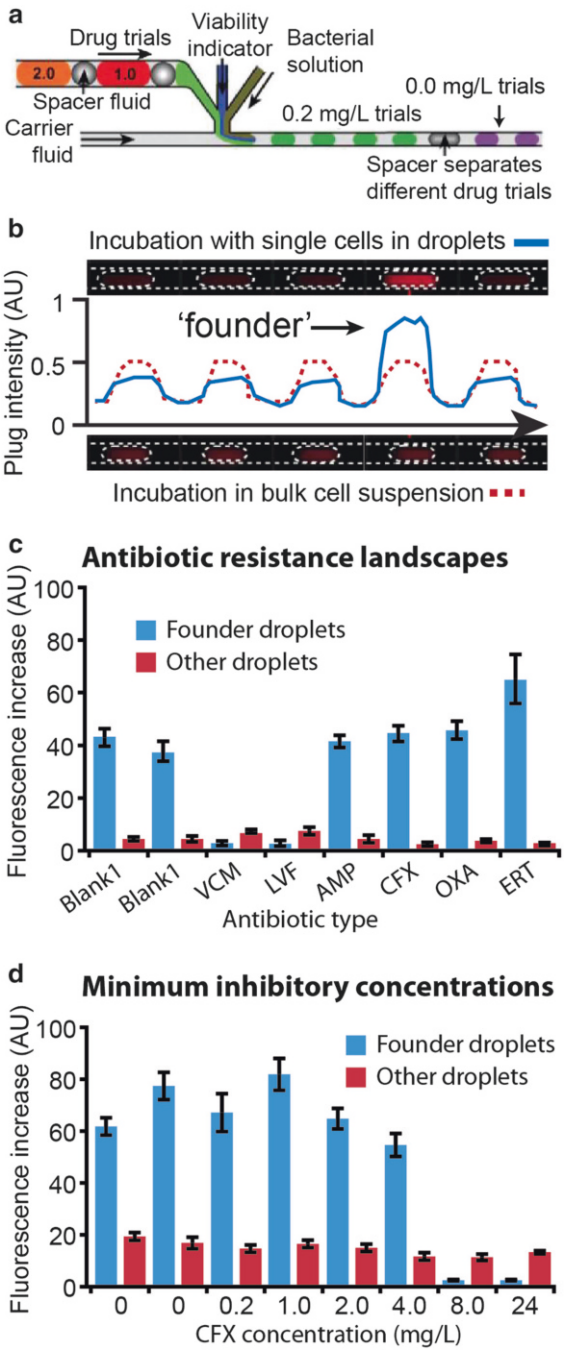

Fig. 5.3 (**a**) Stochastic confinement coupled with plug-based microfluidic handling allows for the interrogation of single bacterial responses to antibiotic treatments. (**b**) Direct observation of the founder effect is seen when bacteria are individually compartmentalized with antibiotic before coming in contact with the drug. (**c**, **d**) This system can be used to generate (**c**) antibiotic resistance and (**d**) minimum inhibitory concentration profiles for any bacterium, in this case MRSA

combined with novel PCR methods such as the T7 polymerase technique, rolling circle amplification, and isothermal PCR, have been indispensable tools in detecting, with high sensitivity and specificity, the presence of proteins in solution. The recent advances in high throughput droplet microfluidics and microwell arrays have enabled an even lower limit of detection (approaching the sub-femtomolar concentration regime) while simplifying the readout, through the use of digital ELISA assays [46]. The readout concept is similar to that of digitized PCR and RT-PCR, relying on extremely low dilutions of target molecules, and use of Poisson distributions and statistics. Typically these platforms use beads as the solid phase, where these are then compartmentalized in wells or droplets [47–49]. Further, some point-of-care (POC) devices have been developed using these "ELISA on chip" methods. Specifically, the mChip combines the ease of fluid handing in a lab-on-chip context, with gold nanoparticle-linked ELISA [50]. The readout for this platform is quite clever, in that the assay relies on the growth of silver layers on the gold nanoparticles. If there are gold nanoparticles on the surface (i.e., antigen present), then the absorbance of the silver is measured using inexpensive light-emitting diodes and detectors. This is an excellent example of the miniaturization of a sensitive technique for an affordable and effective POC platform.

5.4 Single-Cell Immunotyping

The human immune system is immensely complex, composed of multiple distinct cell types, residing in different tissues. B cells producing antibodies are responsible for the hosts-acquired immunity and are the mechanisms by which we can become vaccinated against infection from ecological pathogens. Estimations are that at any given point, there are 10^{10}–10^{11} B cells producing at least ~10^{10} distinct antibodies, as measured by deep sequencing of their complementarity determining region $(CDR)_3$ [51]. Further, the pattern of communication between cell types through the secretion of interleukins and cytokines forms a complex network, much of which can be monitored through analysis of blood with the right toolset.

5.4.1 Nanowells and Microengraving for Secretome Analysis

For example, functional cellular immunotyping is a potentially powerful tool for patient monitoring and scientific discovery, enabled primarily by a handful of technologies employing massively parallel arrays of nanowells etched in silicon termed "microengraving" (Fig. 5.4c, d) [52]. This technique combines both the microwell technology with ELISA-like surfaces upon which the wells are "stamped," leaving the imprint of the secretome from the cell in that well. These experiments are typically carried out in two parts: First, cells are seeded on surfaces with

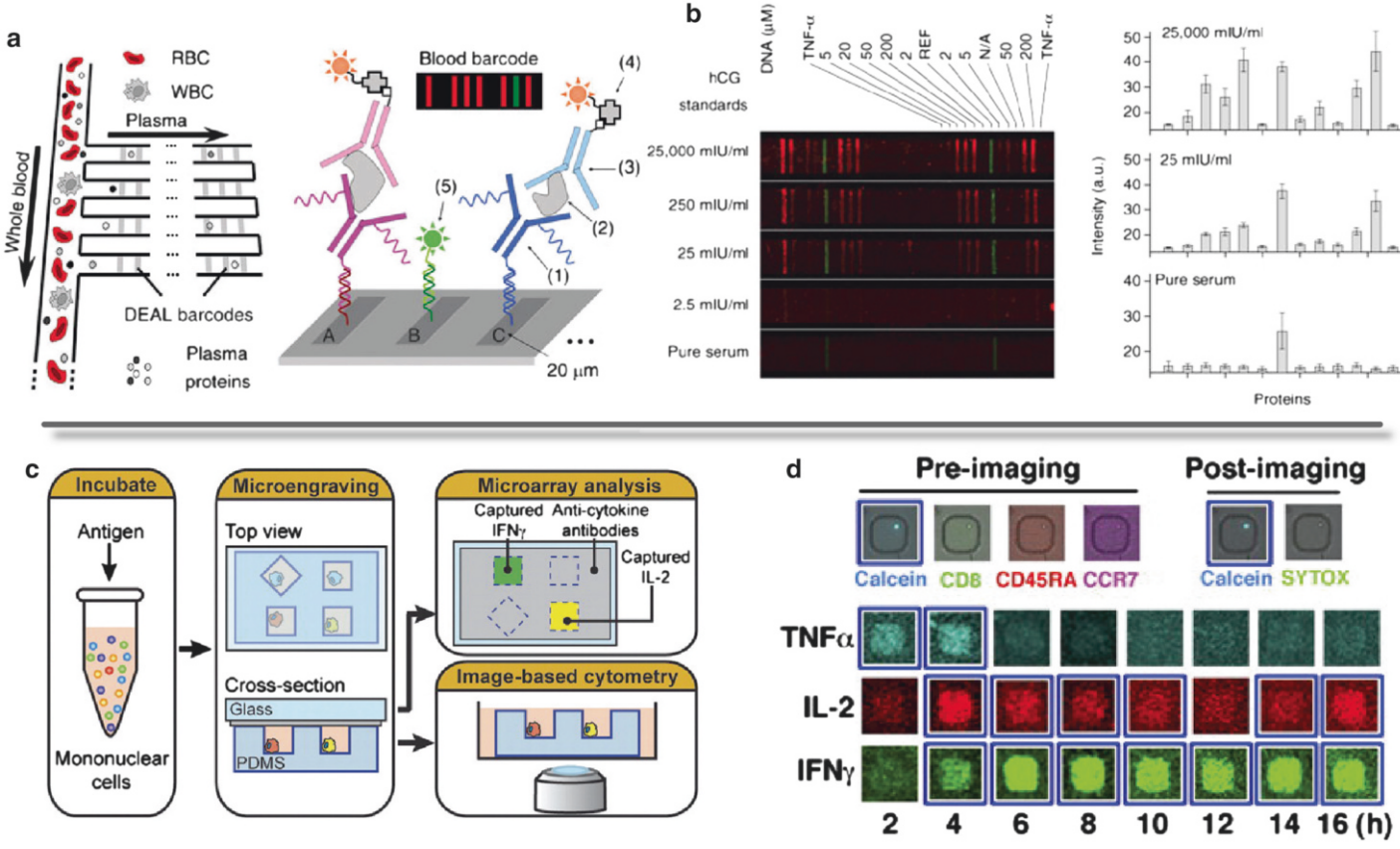

Fig. 5.4 (**a**) Proteomic profiling of whole blood plasma or single cells has been accomplished using a combination of DNA-encoded antibody libraries (DEAL) and valving microfluidics. (**b**) Readout is similar to a barcode, using ELISA to detect captured target protein patterns on the surface. Up to 12 proteins can be profiled simultaneously. (**c**) Secretome profiling of immune cells using a technique termed microengraving has shed tremendous light on the dynamic responses of single T and B cells to stimulation. (**d**) Temporal responses can be easily measured using this technique, coupled with cell staining of identifying CD markers and live/dead staining

highly parallelized etched single cell wells using passive gravity sedimentation. After cell seeding, the substrates can be gently washed and aspirated without removing the cells from their respective well. Subsequently, the cell type of interest (typically T cells) are stimulated by the addition of a small molecule, washed and a glass slide functionalized with antibodies against known secreted factors such as TNFα, various interleukins, and interferons are placed face down over the wells. After a given incubation time of interest, the slide is removed and the cells are fixed and stained for various surface markers such as CD4, CD8, and can even be used with pre-stained cell barcodes for multiplexing [53]. Simultaneously, the cover slide is used as an ELISA substrate to identify components of the secretome of each single cell well where the number of individual components that can be assayed in a single experiment is limited only by the existence of an antibody directed against it, the number of mutually exclusive fluorescent readouts on the imaging system being used, and of course the physical space allowed for the microengraved array. This tool has been extremely powerful in immunophenotyping, as well as uncovering a never before seen phenomenon of the polyfunctional dynamic T cell response to infection challenge: In this scenario, T cells respond asynchronously to activation ex vivo with phorbol 12-myristate 13-acetate (PMA) and ionomycin. Their production of IL-2, TNFα, and IFN-γ, is sequential, and the program repeatable as well as dependent upon differentiation state [54]. Microengraving has also been used to quantitatively characterize the dynamic recovery of HIV-specific CD8+ T cells [55].

Secretions from immune cells have been characterized also by other ELISA-based nanowell technologies. Notably, the immunospot array assay on a chip (ISAAC) utilizes a nanowell array in which the top surface (from which the wells are etched) is coated with antibodies against IFNγ and TNFα [56]. This allows for detection of CD4+ T cells (via cell surface staining) multiplexed with assessment of secretion of one or more cytokines. This technique measures similar analytes/phenotypes as the previously mentioned technique but requires only one substrate. However, the resulting patterns formed on the chips require significant image analysis in order to determine the original well from which the analyte was secreted, as ELISA signals from neighboring wells can overlap (Fig. 5.4d).

5.4.2 Real-Time Detection of Transcriptional Changes in Immune Cells

Other techniques utilizing integrated microfluidic culture systems coupled with fluorescence microscopy and RT-PCR on chip have shed light on how information processing occurs in response to TNF-α [57]. This technique illustrates that NF-kappaB is digitally activated on a cell-by-cell basis in response to a homogenous TNF-α dosage. This observation was previously unobtainable without the use of a high throughput, quantitative single cell analysis using these microfluidic systems.

5.5 Platform Functionalities and Components

Sample image capture and subsequent analysis are classic bottlenecks in HTS system flows, and novel imaging modalities enable many of these next-generation screening platforms to be practically implemented. Imaging techniques can be grossly categorized into three subsets: (1) classical lens-based imaging coupled with automated stages and capture, (2) wide field of view (FOV) holographic techniques, and (3) superfast serial methods. Typical lens-based systems are widely used in conjunction with commercial automated xyz stages for printed microarrays, as well as HCS of both 384 and 1,536 well plate formats. These techniques are well described and will not be the focus here.

5.5.1 Novel Imaging Modalities

Novel lens-free, wide FOV systems make use of a CMOS or CCD pixel array, resulting in overall small instrument size while maintaining large fields of view, up to 10 mm × 10 mm [58,59]. This is a very powerful technique as it allows for capture of all z planes in the FOV because there are no lenses, only direct collection of transmitted light from the sample on the CMOS array. The absence of lenses both enables the wide FOV and lowers the cost. However heavy image postprocessing and reconstruction from the "holographic" image collected is required. Typically, these systems have found use in cell imaging for using brightfield and darkfield imaging especially for counting purposes as well as cell motility and morphology measurements [60,61]. There have been recent efforts toward the development of fluorescent-imaging modalities using lens-free systems, where success has been made and demonstrated with capabilities for resolving single *C. elegans* [62]. These systems will be very well suited for imaging of digitized ELISA and PCR arrays both in droplets and discrete wells, especially since reconstruction is not necessary for data acquisition in these cases. Further, these imagers are easily coupled with microfluidic systems because the chips can simply rest directly on the CMOS array, making integration with droplet generation technologies very simple [63].

Ultrafast serial imaging is utilized in a number of microfluidic platforms relying on fast serial processing of single cells in flow. Commercial high-speed CCD cameras are utilized for a number of applications, including deformability cytometry and most inertial microfluidic techniques; however, these produce a significant cost burden on the researcher or end user. Serial time-encoded amplified microscopy (STEAM) is a novel imaging method using ultrafast line scanning to detect particles and cells in flow [64]. This technique has been used to detect specific cell types coated with beads containing anti-EpCAM antibodies, as well as to identify morphological parameters unique to MCF7 breast cancer cells as compared to normal white blood cells in flow [65]. Coupled with simple straight microfluidic channels designed for particle focusing, this technique has unparalleled throughput, as the speed of the imaging is in the GHz range, allowing throughputs approaching 100,000 cells/s, and extremely low false positive rates of one in a million.

5.5.2 *Microfluidic Interrogation and Separation Techniques*

Microfluidic technology enables such a wide range of novel processes from liquid handling and sample processing to the ability to address single cells in high throughput that it deserves special mention. These technologies utilize a range of force fields to manipulate and address single cells in flow. Magnetic, electric, and even optical fields can be used to manipulate single cells in three dimensions. Magnetic forces are typically used in conjunction with pre-labeling of cells with magnetic particles coated with antibodies directed against a surface marker of interest, followed by passing the cell-bead conjugates through microchannels with embedded magnetic elements [28]. The fields augment the trajectories of cells dependent upon labeling, and then these cells can be siphoned off from channel outlets. Electroosmosis works in a similar fashion, however pre-labeling of cells is not necessary, as the electromobility of cells is an intrinsic property, shown to be dependent upon membrane composition and cell size, thus allowing discrimination between cell types [66,67]. Optical forces have also been employed as a single cell interrogation technique, termed "optical tweezers" [68]. This method traps a cell in a focused optical field, relying on the difference in refractive index between the cell and its surrounding solution. The field can then be tuned to physically stretch the cell, measuring its strain response to a stress field, inferring mechanical properties of the cells such as elastic modulus. These platforms are very precise using highly tunable field forces and gradients; however, their throughput is somewhat limited to about 10 cells per minute at maximum, although a number of massively parallelized optical trap methods are being developed [69].

Converse to the addition of external force fields in microfluidics, the use of the flow fields generated simply by fluid moving through the channels is also a highly effective method for ordering and addressing cells serially in microchannels. Inertial focusing of particles and cells to specific locations within a channel cross-section is a novel technique being adopted by an increasing number of research groups [70]. Briefly, the balance of the shear gradient lift force (stemming from the parabolic velocity profile in a channel) and a wall effect force (possibly from the reflection of viscous wakes generated by the particles themselves) [71] ultimately leads to deterministic positioning of objects in the channel cross-section. Further, this location is dependent on the size, shape, and deformability of the particles in flow. This phenomenon requires the presence of non-negligible inertial forces, which was initially thought to be unobtainable in microflows simply due to the velocity of flow required to reach Reynolds numbers greater than 1 (less than 1 is referred to as Stokes flow, where only viscous forces are considered to be of consequence). Practically speaking, Reynolds numbers of 10 or greater are the operating regimes for inertial microfluidics. This technique offers a very powerful method of passive ordering for imaging and/or separation of particles in flow. One type of flow field, the extensional flow, has been used in conjunction with upstream inertial focusing of cells to the channel center, to deliver cells to a region of extremely high fluid shear, imparting large (~10–100 nN) forces on cells. Deformability cytometry is a microfluidic technique utilizing this very force field, coupled with high-speed brightfield

imaging to measure cell deformation [23]. This technique can process cells on the order of 1,000s per second, and generate quantitative plots of deformability, size, as well as a number of cellular characteristics including membrane ruffling and dynamic responses to stretch.

The use of inertial forces in microchannels can also be employed to move both fluid and particles within the cross-section of a channel. It has recently been shown that the presence of non-moving obstacles in flow (as compared to particles or cells, which are moving in flow) coupled with inertial flow ($Re > 10$), results in nonreversible fluid parcel migration in a channel cross-section [72,73]. This is extremely useful for separation of particles (or cells) from an incoming stream that also contains analysis reagent (such as an antibody stain or small molecule). This effectively washes the particles or cells in flow without the addition of any external force field, and the operation range is at high flow rate, allowing 1,000s of cells per second processing rate, assuming concentrations of 10,000s of cells per ml in the incoming solution.

Together these microfluidic techniques have the ability to transform HTS platforms. Many of these technologies are still in the development stage, although some are in the process of moving to market. The obvious limitation at hand is the integration of these microscale fluidic platforms with current HTS workflows, involving large robotics, microtiter plate storage of solutions and chemicals, as well as current macroscale liquid handling techniques. The true transformative power of the microscale technologies cannot be realized until (1) a standard protocol for integration of macro and micro is set and (2) these research and development level technologies, mostly utilizing PDMS on glass chips, are moved to materials and platforms that are more amenable to scaled up production, such as injection molding of hard polymers and premade integrated circuits.

5.5.3 Macro- and Micro-Integration

The development of true next generation, functional high throughput and HCS methods will be hinged on the proper integration of macroscale automation tools used in screening today, with the highly precise microscale methodologies for measurement reviewed here. This realm has been mostly untouched thus far, aside from one example of integration that has led to an extremely powerful tool.

Microfluidic mechanically induced trapping of molecular interactions (MITOMI) combines the power of macroscale robotics for printing DNA microarray libraries with the highly controlled binding microenvironments of microfluidic large-scale integration [74,75]. Microfluidic large-scale integration utilizes a two-layer channel design built in elastic PDMS to achieve valving capabilities in microfluidics. In this technique, the top channel (or control channel) resides above the bottom channel, separated by a thin PDMS membrane [76]. When the top channel is pressurized with either air or liquid, the PDMS membrane deforms and closes the bottom channel. This valve can be released simply by relieving the pressure applied to the top layer.

In the MITOMI technique, synthetic DNA library arrays are printed and immobilized on epoxy surfaces, followed by channel alignment and bonding. Using the valving techniques offered with large-scale integration, a solution containing transcription factor (TF) proteins is injected into the channels, hydrating the DNA and allowing for interaction between TF and DNA. After incubation, button valves are actuated, trapping the DNA and TF in their equilibrium ratios on an antibody-coated spot on the channel surface (targeting the TF). Subsequently, channels are washed with clean buffer, removing any molecules that were not trapped under the button valve. Measurement of fluorescence of the bound TF (pre-labeled with Bodipy) and the DNA (pre-labeled with Cy5) allows for direct, quantitative measurement of equilibrium conditions, specifically the ratio of the number of TF molecules and DNA molecules by comparing surface fluorescence against a premade calibration curve. This technology has the potential for direct measurement of the dissociation constant (K_D) of the TF and that sequence of DNA and may be applied to any interaction based on a single binding site model.

This methodology has been automated using robotics and microfluidics which lead to insights of the "binding fingerprints" of transcription factors. Further, this platform has been applied to protein recognition of the secondary structures of RNA molecules [77], and one could easily imagine this techniques applicability in any molecular interaction study. These insights will be very useful both in more quantitative understanding of genetic circuit regulation mechanisms and in rationally designing our own synthetic biology circuits.

5.6 Translational Applications

Although research and development of novel technologies in the academic sector is a major driving force for discovery and understanding, their implementation and scale-up to the industrial sector is what brings the technology to the bedside. In order for these "future" technology platforms to really have an effect on treatment efficacy and patient outcome, a compartmentalized, marketable platform must be realized.

Currently, there are a number of examples of commercialized versions of these technologies, including digital PCR and ELISA methods, as well as microfluidic processing and cell handling. These technologies appear to be successful for two main reasons: (1) they are not over-complicated (although there are high levels of functionality in the systems) leading to robust and reproducible behaviors and (2) they have been converted to a "black box" type system, where there are only two main components that the user has to worry about: a measurement box and a disposable cassette. These two aspects allow for relatively easy integration into current workflows of HTS and HCS systems. Further, many companies have sized their cassettes to match the microtiter plate format, allowing for direct integration with current robotic handling systems.

There are a few companies of note that have successfully commercialized a microfluidic technology, imaging technology, or a combination of the two. Both digital ELISA and digital PCR methods have become readily available to researchers over the past few years. Quanterix's Simoa (single molecule array) technology, utilizing nanowells for molecule capture, sealed with an oil layer flowed over the substrate, has been successful in the areas of viral and bacterial pathogen detection, resulting mainly from the extremely low level of detection the system offers. Commercialized digital PCR methods include Fluidigm [78], using integrated microfluidic valves and digital PCR readout, and Raindance [79] as well as QuantaLife [80] (which was recently acquired by Bio-Rad) both employing droplet-based microfluidic systems for compartmentalization of the sample solutions.

There are also many companies that are still in the start-up stages of development. Companies utilizing microfluidic methods include Cytovale [23], one of the first companies harnessing inertial microfluidics in a commercialized product for cellular deformability evaluation. This company aims to translate the deformability cytometry platform discussed in the tumorigenesis and cancer biology section of this chapter. Imaging-based technologies are also being commercialized, most notably Holombic [58], commercializing the LUCAS holographic imaging system, as well as Nantwork, who has acquired the STEAM [64] ultrafast line-scanning imaging technology. Although both of these companies are commercializing novel imaging modalities, they are pursuing very different markets, where Holombic is geared toward the simple, POC tool for diagnostics in the field and the STEAM technology may be more fitting for a research or core lab setting.

5.7 Conclusion and Future Directions

The burgeoning field of single-cell microfluidic cytometry has not only provided the scientific community with a new tool set for quantitative biological investigation, but has already led to novel insight such as the discovery of rare cell phenotypes previously unresolvable. The ability of these techniques to miniaturize and parallelize well plate techniques using microarrays or serially process large numbers of single cells in high throughput has been an enabler for this push forward in scientific discovery.

A key challenge for the future for these microfluidic-based assays is their integration with existing HTS workflows. Microfluidic cytometry devices can empower HTS and HCS by adding novel readouts including single cell biomechanical measurements, biomarker development and detection as well as single cell genomics and proteomics. These measurements could transform our understanding of the effects of small molecule libraries on cell function, and greatly increase the efficacy of treatments derived from these screens.

It is likely that, in the next decade, both basic science and drug discovery pipelines will see some dramatic changes with functional integration of these novel technologies into the workflows. If microfluidic cell cytometry toolsets are thoughtfully developed, with HTS and HCS integration in mind, then it is reasonable to

predict an exponential increase in data throughput and content, creating a near future where understanding the connectivity and dynamics of every component of a system as complex as a multicellular eukaryotic structure is possible.

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