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Advances in High-Throughput Single-Cell Microtechnologies

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Abstract

Micro-scale biological tools that have allowed probing of individual cells - from the genetic, to proteomic, to phenotypic level - have revealed important contributions of single cells to direct normal and diseased body processes. In analyzing single cells, sample heterogeneity between and within specific cell types drives the need for high-throughput and quantitative measurement of cellular parameters. In recent years, high-throughput single-cell analysis platforms have revealed rare genetic subpopulations in growing tumors, begun to uncover the mechanisms of antibiotic resistance in bacteria, and described the cell-to-cell variations in stem cell differentiation and immune cell response to activation by pathogens. This review surveys these recent technologies, presenting their strengths and contributions to the field, and identifies needs still unmet toward the development of high-throughput single-cell analysis tools to benefit life science research and clinical diagnostics.

Introduction

With the advent of technologies that allow detailed investigation of individual cells – from the genomic to phenotypic level, it is now clear that such a single-cell approach is essential in understanding cellular heterogeneity and its biomedical importance. In particular, the ability to isolate subpopulations of cells resistant to certain drugs in cancer treatment and microbial pathogenesis, has lead to the understanding that cells comprising less than 1% of the total population can, in fact, be the most important cells to eradicate during treatment. Further, the development of next-generation immunologic therapeutics will require the isolation of subpopulations of antigen-presenting and cytokine-producing cells, sometimes comprising less that 0.2% of the total population of CD8+ cells in the blood. The differentiation process of pluripotent stem cells, as well as induction of pluripotency from somatic cells results in significant cell subpopulations, and, if better understood, this process could be used to create complex tissues or cell-based therapies for implantation and tissue regeneration.

This review will focus specifically on high throughput technologies recently developed for the purpose of analysis and isolation of single cells from heterogeneous populations. The goal of these technologies is two-fold: to increase the understanding of the biological processes mentioned previously, as well as to develop improved clinical diagnostics and

more effective therapeutics that can target rare cell populations. The technologies reviewed here range from photolithographically patterned 3D microwell technologies and 2D adhesive substrates, to continuous flow technologies and miniaturization of conventional techniques to an automated, on-chip format.

Cancer Biology

Cancer is a complex, dynamic and heterogeneous disease, which requires an array of new technologies to tackle. An invasive malignant phenotype can develop due to a variety of genetic and epigenetic changes, resulting in significant heterogeneity of cancer cells both within a single tumor and between 'distinct' tumors [1–4], ultimately affecting responses to cancer therapeutics [5] and clinical outcome. Understanding the underlying cellular heterogeneity, manifested as dysfunctional molecular pathways, holistic biophysical differences, and differential response to therapies at the single-cell level will provide insights to improve diagnostic and therapeutic strategies. An alternative source of single cells for such analysis is available in the extremely rare population of circulating tumor cells (CTCs) in peripheral blood of cancer patients, and recent work has focused on isolation and analysis of these cells and their roles in metastasis [6,7]. Micro-scale technologies have been developed to perform single-cell analysis to better understand the complexity of cancer and achieve improved diagnostics through understanding genetic differences, resulting protein expression, and overall drug susceptibility.

Advances in genomics and proteomics at the single-cell level can provide insights into aberrant molecular pathways that contribute to the significant heterogeneity in cancer cells. Single-cell genomic sequencing has identified rare, single copy mutations associated with tumorigenesis [8,9]. These methods, however, are still low-throughput (tens to hundreds of cells) and require significant manual effort. Single-cell PCR methods make use of integrated fluidic circuits [10–13] or droplet-based digital-PCR [14,15] to analyze transcripts that vary from cell-to-cell and can easily be masked by bulk measurements. These methods have also been used to identify the cause of radiation treatment resistance of certain cancer cells [16]. Proteomic methods that can report protein levels down to the single-cell level have been recently developed [17]. Future integration of proteomic methods with genomic methods at the level of single cells would also expand our understanding of the heterogeneity in genetic lesions and the associated protein pathways affected.

Mutations and protein expression differences result in whole cell biophysicial changes that are linked to an invasive phenotype [18,19]. Hydrodynamic and optical methods have been developed to assay cell mechanical properties - primarily the ability of cells to change shape with an applied load [20,21]. Tools for analyzing cell dielectric characteristics have also been developed [22]. Additionally, single-cell technologies to assay cell mass, cell cycle progression, deformability and surface friction make use of the suspended microchannel resonator (SMR) [23,24]. These label-free biophysical approaches have potential to achieve low-cost diagnostic analysis of cancer, while maintaining the ability to sample large heterogeneous populations and identify important outliers. Other label-free properties include the migration of single cells, which can be assayed in an automated fashion to uncover cell-cell interactions [25]. Measurements and throughputs of these approaches vary substantially and will likely have separate application niches in diagnostics and in identifying invasive phenotypes for research, quickly and inexpensively. Alternate methods of applying stress to single cancer cells – using compressed microchannels [26] or magnetic nanoparticles [27] – have begun to reveal the role of the mechanical environment in cancer cell mitosis and polarization.

Single-cell analysis tools are just beginning to be applied to determine drug response, with future applications in determining the differential response to therapies at the single-cell level. Droplet-based techniques have shown promise for drug-screening on single-cells by creating arrays of encapsulated cells with various drugs and drug concentrations in a high-throughput manner [28]. High-throughput single-cell image cytometry techniques, as well as imaging based on ultrafast spectral imaging could also be applied for morphological analysis of cell response to drugs [29,30].

With the development of such a wide suite of methods for the characterization of cancer cell genetics, proteomics, and subsequent variations in biophysical properties at the fundamental single-cell level of the disease, our understanding of tumor biology can be rapidly expanded. The application of these methods to understanding fundamental processes of tumorigenesis, metastasis and potential therapeutics will be critical, and should open up synergistic diagnostic opportunities.

Stem cells and regenerative medicine

Stem cells encompass a subset of cells that display both differentiation and self-renewal capabilities, and are often heralded for their potential to revolutionize medicine and bioengineering [31,32]. Various intrinsic properties and signals from the cell microenvironment contribute to stem cell fate and function. High-throughput, single-cell analytical and isolation techniques are able to address core issues in the field, including the biology behind individual stem cell fates by allowing the systematic probing of cell response to different factors, and the isolation and purification of differentiated cell populations essential for their application in regenerative medicine. Of particular note is the ability of single-cell analysis to answer two major concerns in stem cell biology: 1) the importance of the heterogeneity that naturally arises in stem cell populations and how it influences cell fate [33,34], and 2) the ability to isolate and present cues to individual stem cells to better understand and control differentiation[35,36].

Genetic expression patterns of stem cells are a unique marker by which their state is determined. A number of single cell techniques enable the study of individual cell expression, the most notable being single-cell RT-PCR [37]. This approach, in conjunction with FACS to isolate cells has been used to generate expression data from large sets of individual stem cells, and has been used to determine the heterogeneity and fate of stem cell populations [38]. An alternative approach includes the use of fluorescence in situ hybridization (FISH) and its variants to image genes directly [39,40]. These approaches, although incapable of monitoring transcript number as in RT-PCR, can give spatial information not possible in RT-PCR, and are an alternative to costly GFP cell lines.

Cellular microarrays and microwell technologies have been used to control the cell microenvironment and explore the combinatorial effect of microenvironmental factors including matrix and cell-cell contacts [41–44]. Despite the capability of these platforms to screen the effect of complex combinations of cell microenvironment signaling cues, they are usually static and do not allow continuous manipulation of cell microenvironments, unlike microfluidic-based approaches[45]. Microfluidic technologies allow for analyzing hundreds of cells in parallel and are used for a variety of applications from automated tracking of dividing hematopoietic stem cells (HSC) to high-throughput detection of cell cycle phases in individual HSCs [46,47]. Because of their precise morphogen delivery capability, microfluidic approaches are ideal for probing the effect of morphogen concentration on stem cell differentiation while simultaneously controlling microenvironment factors [48,49], and can be parallelized to perform multiplexed assays [49]. In addition to microfluidic platforms, cell patterning is conventionally used to control the shape of multicellular constructs,

thereby inducing differentiation down specific lineages, although it is usually limited to 2D manipulation of the cell microenvironment [50]. To overcome the limitation of 2D patterning, hydrogels of various chemistries can be used to isolate and study single cells, capable of providing controlled environmental chemical and mechanical cues [51].

Microbiology and pathogenesis

Heterogeneity within bacterial cell populations is of increasing interest when considering the emergence of antibiotic resistance, as well as cell-to-cell quorum sensing in communal developments such as biofilm. Single-cell and species heterogeneity is also involved in the development and equilibrium of the human gut microbiota, which cannot be investigated as a blended parameter. Biofilm formation on implanted medical devices are the most significant cause of hospital acquired infections, resulting in ~1 million cases, and $10 billion each year, and microbiota imbalance can lead to gastrointestinal pathophysiology and improper acquired immunity development. Understanding the mechanisms by which single or small populations of cells in a mixed population can dominate disease processes is of utmost importance to develop rational treatments for infection, with long lasting effectiveness, i.e. minimizing resistance emergence and controlling biofilm formation and ecology.

Technologies enabling high throughput single cell analysis of bacterial cells separate and compartmentalize individual cells for future nucleic acid, proteomic, secretion, or phenotypic analysis and rely on plug based two-phase systems and stochastic confinement into femtoliter compartments. The Chemistrode [52], and variations on this capillary plug based technology, have enabled direct observation of single cell 'founder' phenomena in which rare individual cells compartmentalized into single cell plugs with the antibiotic of interest show marked resistance although the majority of the population is susceptible [53]. Further, this technique has been implemented to isolate rare single cells from multispecies mixtures, and identify them downstream via 16sRNA probes [54]. Stochastic confinement using micro-scale SU-8 wells has indicated that a single cell can 'self' quorum-sense, and that quorum-sensing is highly variable in small clonal populations of cells; both previously unobserved phenomena[55]. Confinement in a honeycomb array with connected environments was also used to allow bacterial cells to travel through and sample each environment [56]. This technology has shown that large gradients in antibiotic and niche environments lead to accelerated emergence of antibiotic resistant cells. An alternate method for confinement of single-cells using microfluidic valving was used for gene analysis of environmental bacteria to study symbiotic relationships [57,58], and a technique combining large scale integration of microfluidic valving and water-in-oil two-phase systems may prove to be very useful in downstream applications of sequencing and molecular techniques after single-cell confinement [59].

Automated imaging and computational analysis-based techniques have also proved useful for analysis of single cell near-surface motility mechanisms. Motility via flagellar movement has previously been postulated as a mediator of biofilm morphological development, and Conrad et al. have employed an automated optical tracking method to demonstrate this directly. Their method also allows quantitation and classification of rare subtypes of movement, previously unobserved by the microbiology community, and has also been used to characterize early biofilm development and show that single cell surface trajectories can lead to enriched cell subpopulations [60].

Both unique phenotypic analysis and isolation of molecules at high concentration in small microfluidic compartments have significantly moved the field forward. There is still much to learn about cell-cell communication and motility in the development of prokaryotic tissues.

Neuroscience

The patch clamp technique is widely used to investigate cellular behavior of excitatory neurons at the single-cell level *in vitro*. Initially, patch clamping – applying a fixed voltage and measuring the current across the cell membrane using a pipette - allowed researchers to study electrophysiology in one single cell at a time. To achieve more statistically robust datasets, parallelized multi-patch clamp setups and chip-based planar patch clamp systems with multiple addressable pores were developed[61], allowing higher throughput for drug screening and the investigation of rare defects in ion channels related to neurodegenerative diseases[62]. Cellular phenotypes are highly susceptible to a complex extracellular environment, comprised of cell-neighbors and topological and mechanical ques. To understand their contributions to physiology, micro and nano engineered cell culture tools are necessary, where microfluidic platforms[63–69], micro- and nano-structured and patterned surfaces[70–72], and multi cell arrays[73,74] have found application areas in neuroscience research.

Microfluidic platforms compartmentalize neural cell structures in different dimension channels [64,65,75], allowing the separation of the cell body from its neurites and local chemical treatments. While most microfluidic platforms separate two or more cell populations, controllable single neural cell alignment was realized by Dinh et al. [63] or by Takayama et al. [69] through a combination of cell cages and fluid flow.

Methods to shape and control the extracellular topography precisely around neural cells have advanced from simple stripe and dot shaped protein patterns towards complex combinations of shorter and longer patterns [76]. To polarize the cytoarchitecture of single dissociated neurons a hexagonal star pattern with one continuous and multiple stepped pattern generated a long versus multiple short neurites in more than 60% of seeded cells [72]. This pattern technique yields highly controllable cell arrays with hetero-directional stage 1 polarized neurons in a culture [77], suitable for pharmaceutical screens.

While the patterning techniques target early developmental questions, synapse formation is the next critical step towards functional neural circuits. A large-scale synapse assay called synapse microarray has been developed by Shi et al. to quantitatively screen drugs involved in synaptogenesis [74].

Single-cell culture platforms have already been employed by the neuroscience field and have provided initial results in manipulating both single-cell architecture and neural networks with the ability to control cell and network polarity. Researchers have now started to combine single-cell tools with co-cultures of neurons and non-neural cells, however the role of non-neural cells in cell polarization and neural development, especially related to cell models of mental disorders remains an open topic. In the future, cellular disease models should be more strongly integrated into current single-cell techniques. Questions concerning how polarity and guidance impact neural development, or in a later stage, the degeneration of neurite networks still remain.

Immunology

The human immunological network is complex, and known to play roles in a number of disease states including bacterial and viral pathogenesis, tumorigenesis and metastasis[78–80], as well as autoimmune disorders. The development of acquired immunity is driven via the presentation of antigens on the major histocompatibility complex (MHC) types I and II by a variety of cell types and subsequent recognition by T cells. Antibodies are produced by B cells after successful antigen presentation on MHCII molecules and subsequent T cell recognition. Immune cells also secrete factors including many types of interleukins that

modulate coordinated immune response, interferon gamma (IFN-γ), known to regulate viral replication, and tumor necrosis factor alpha (TNF-α) which is thought to inhibit tumorigenic growth and modulate both acute and chronic inflammatory responses.

At any given point, there is thought to be 10^6 to 10^8 different types of MHC-antigen complexes being presented, with the potential for a similar number of unique antibodies to be produced. This poses a fundamental problem of cellular heterogeneity when sampling immune cells for isolation of therapeutic human monoclonal antibodies (mAb), for monitoring the dynamics of the immune system in a pathological state, and for the isolation of single cells to characterize active molecular pathways and phenotype. In order to further understand the immune system's role in controlling cancer as well as bacterial and viral infections, isolation and characterization of the diverse set of specialized single immune cells is necessary. Both flow through systems characterizing gradient effects on immunological phenotypes [81], as well as microwell technologies for single cell analysis have proven effective in furthering our understanding of immune system function.

Compartmentalization technologies have been employed as effective tools for proteome and secretome analysis for immunophenotyping. In particular, the 'micro-engraving' process allows both time dependent, high throughput analysis of secreted factors via immunofluorescence while simultaneously detecting multiple cell types from cellular surface markers[82–84]. Similarly, Jin et al have created a complementary immunospot array based method for isolation of antibody secreting cells (ASCs) called 'ISAAC', in which microwells are etched into silicon and coated with 'catching' antibodies to fluorescently detect secreted antibodies of interest[85]. Zhu et al have employed similar microwell techniqes, where instead PEG hydrogels are used as the structural component and detection of secretions occurs via integrated aptamer-on-gold electrode sensors [86,87]. These microwell based technologies make use of the fundamental concepts of ELISA and ELISPOT, the current gold standard approaches for secretion and proteome analysis[88]. However the compartmentalization of single cells using microfabrication, coupled with simultaneous detection of cells and their individual secretions is what has allowed new powerful insights into the heterogeneity of the immune system, such as the discovery that T-cells programmatically, sequentially release cytokines, although this occurs asynchronously in a population [84]. Recent results also indicate that an increasing fraction of cells is digitally activated in response to increasing TNF- α concentration, but are capable of analogue information processing after stimulation, producing unique classes of NF-κB signals[89].

Conclusion

The advent of single-cell analysis has brought both an increase in understanding of cellular heterogeneity, but also revealed that our understanding of how individual cells contribute to tissue phenotypes and pathology is limited. In order to further our understanding of important disease states manifested at the tissue and organismal level, such as tumorigenesis and metastasis, neurological disorders, compromised immunity and auto-immune disorders, and tissue regeneration, the development of high-throughput single-cell analysis approaches have been and will continue to be necessary. These technologies increase statistical significance, as biological variance is often high at the individual-cell level, while simultaneously empowering multiplexed analyses incorporating control over multiple environmental factors and stimuli. Successful, next-generation technologies will combine previous technology fundamentals to make direct comparisons between cellular biophysics (e.g. response to force, migration in gradients, growth under fluid shear), genomics, and phenotype, as well as further multiplex stimuli and quantify outputs. Besides aiding in answering fundamental questions concerning cell control, such correlations can enable

future low-cost biophysical diagnostic readouts, backed by extensive molecular data. Finally, new approaches to identify epigenetic changes within single-cells (e.g. chromatin methylation, acetylation, and structure) is also fundamentally missing and will provide additional key insights in the near future.

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was able to identify single-tumor cells that were able to recapitulate a heterogeneous tumor population, indicative of a cancer stem cell. Application of this process to other tumor types will prove useful for cancer therapeutic methods. [PubMed: 22081019]

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Resonator (SMR) is a powerful tool in the biophysical characterization of single-cells. As cells pass through the SMR, the resonant frequency changes. The resonant frequency is detected by measuring the deflection of a laser beam. The change in resonant frequency is depended on buoyant mass and position of the cells, which allows for precise characterization of mass and cell growth. This method is also able to track division times of single-cells over several generations. This will valuable in understanding biophysical properties of cancer cells, but is extremely limited by throughput. Integration of this system with methods of trapping and releasing large number of cells will be critical. [PubMed: 21826361]

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Highlights

- Single-cell analysis is necessary to understand complex tissue-scale biology.

- Biological variance and heterogeneity require high-throughput, quantitative methods.

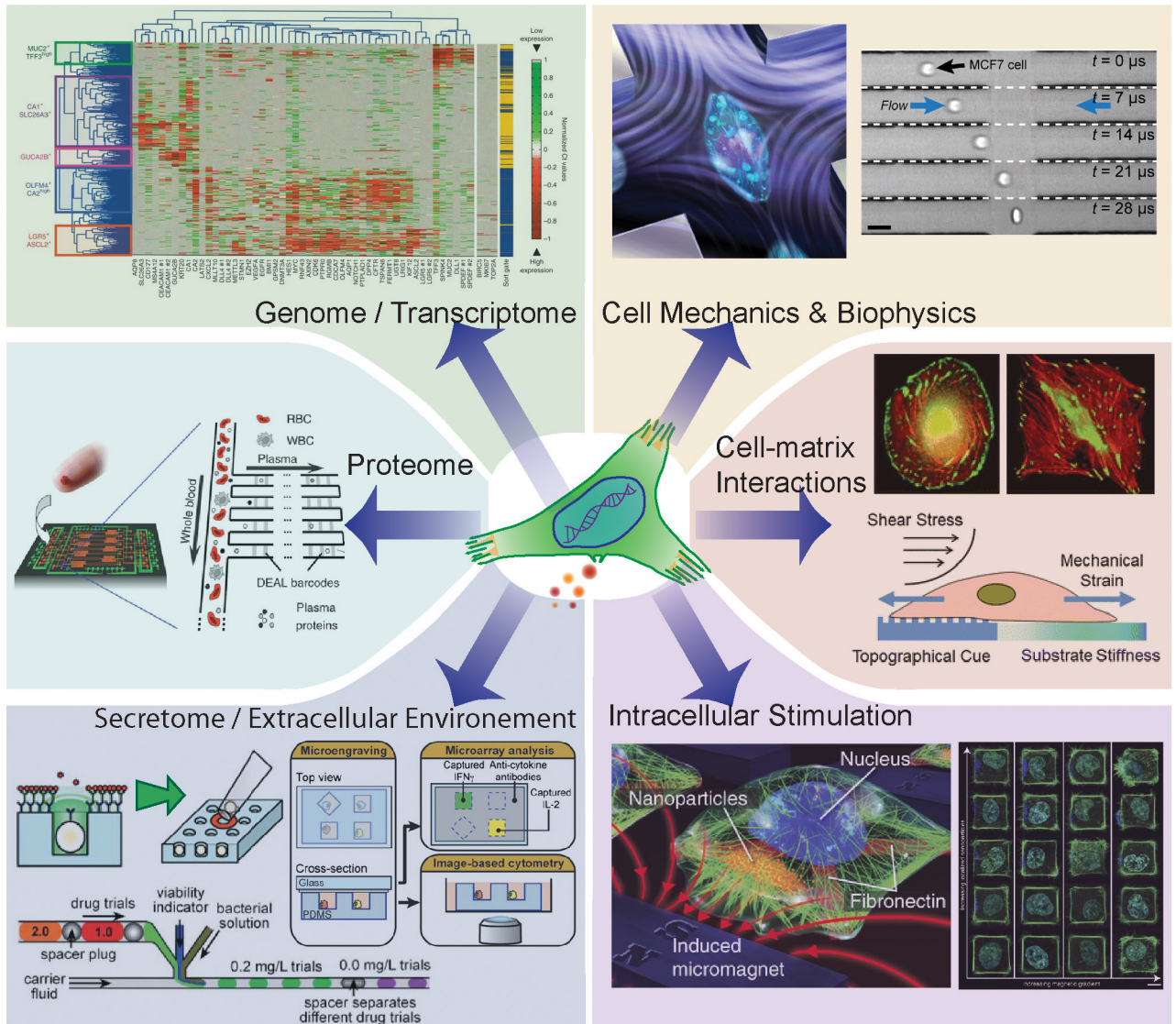- Single-cell platforms will enable novel diagnostics for tissue-scale analysis.
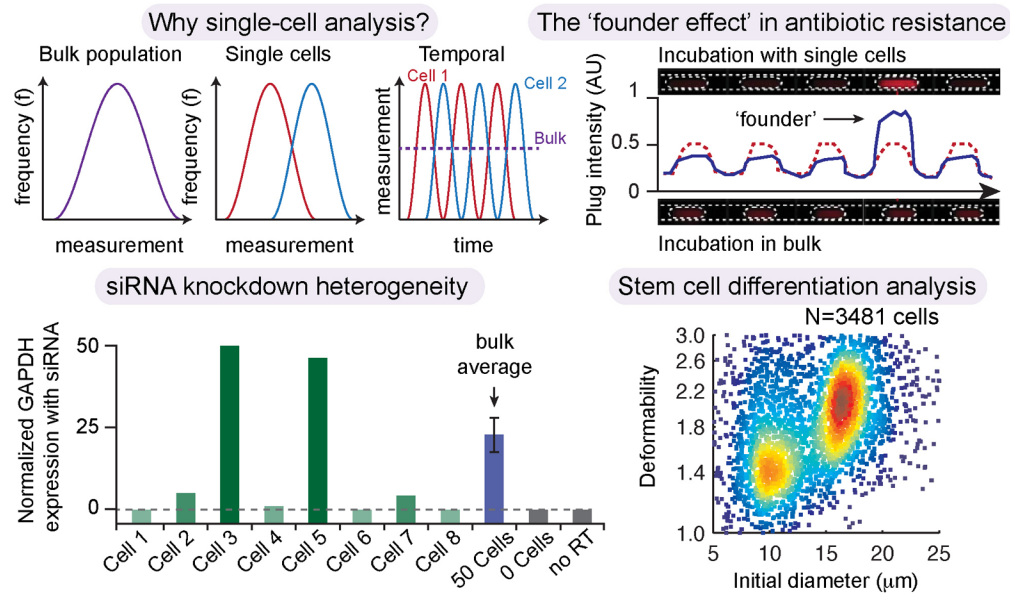
Figure 1.
High throughput, single cell analysis tools grouped by the cellular property which they quantify. Single cell techniques range from phenotypic characterization of antibiotic resistance in plug-based systems (bottom left) to proteome, genome, and transcriptome analysis using bar-codes and integrated valving microfluidics. Continuous flow microfluidic systems are currently being developed to measure whole cell deformability in high throughput, towards real-time patient diagnosis and new regenerative medicine tools. Massively parallel cellular surface patterns are used to probe cell-matrix interactions, as well as force generation within cells when coupled with magnetic nanoparticles.

Figure 2.
(A) Important cell population subsets and dynamics can be easily masked by conventional bulk analysis. (B) Confinement of single cells into two-phase plug systems has allowed for direct observation of the 'founder' phenomenon in bacterial antibiotic resistance. When cells are pre-incubated then segmented, all plugs have a low baseline fluorescence, but when each cell is segmented individually and exposed to antibiotic only in the plug, cells either die (dark), or proliferate because they are resistant (red). (C) siRNA knockdown of a housekeeping gene GAPDH shows high variability cell to cell, where in some cases knockdown is ~100%, and in others is only ~50% effective, giving the typical 25% activity bulk measurement of an 'effective' knockdown. (D) Single cell analysis of differentiating stem cells shows a distinct difference between differentiated and pluripotent cells.

Table 1

Summary of the technologies discussed here to perform high throughput single cell analysis

Technology	Biological applications	Methodology	Throughput	Metric output/measurement
Single-cell sequencing, FISH, RT-PCR	Genome, transcriptome	Microfluidic large-scale integration	96 cells/array	• DNA sequences • Flour images, analogs & binary
Deformability cytometry	Continuous flow label-free biomarker measurement	Continuous extensional flow in PDMS microchannel	~1000 cells/sec	• Whole-cell deformability • Cell optical parameters
Micro-cantilever	Cell mass & growth rates	Etched channels in silicon	1 cell/sec	Resonant requency of cantilever
DEAL barcoding	Proteome profiling and secretome profiling	ELISA on surface patterned antibody/aptamer barcodes	1 cell/barcode	Multiplexed surface fluorescence
Optical tracking	High resolution near-surface motility	Automated, high-restracking of cells	~100 cells/assay	Kinetic optical brightfield images
2D surface patterning	Cell - matrix interactions	Adhesive patterns on glass, hydrogel, or PDMS	~10^5 cells/arrays	Fluorescence and brightfield end-point or real time imaging
Chemistrode/plugs	Single cell isolation for downstream applications	Segmentation of cell pop, in two phase systems	~500 cells/min	• Plug fluorescence (viability) • Live cells from those plugs
FACS	Surface markers and cytosolic markers	Fluorescence-based cell separation in electric field	~1000 cells/sec	Fluorescence staining of cells
STEAM imaging	Cellular surface marker identification	Imaging cells bound to particles via antibody	~1000 cells/sec	Presence of surface markers
Microwells and microengraving	Protein secretion	PDMS microwells and ELISA antibody capture slides	~10^5 Cells/array	• Cellular secreted factors • Cellular surface proteins
Robotic printing	Functional cellular phenotypes	Print cells or adhesive patterns on 2D surfaces	~10^9 cells/array	Fluorescence and brightfield end-point or real time imaging
Microfluidic cell traps	Functional cellular phenotypes	PDMS u-wells for storage under continuous flow	~10^5 Cells/array	Fluorescence and brightfield end-point or real time imaging
Nanoparticle stimulation	Point stimulation of intracellular space	Micromagnet arrays and adhesive patterns on glass	~10^5 Cells/array	Fluorescence and brightfield end-point or real time imaging