Genomic Analysis at the Single-Cell Level

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Abstract

Studying complex biological systems such as a developing embryo, a tumor, or a microbial ecosystem often involves understanding the behavior and heterogeneity of the individual cells that constitute the system and their interactions. In this review, we discuss a variety of approaches to single-cell genomic analysis.

INTRODUCTION: WHY SINGLE CELLS?

The genomic era has enabled a wide range of technologies for measuring high-throughput quantitative biological data. These technologies, ranging from fluorescence microscopy and polymerase chain reaction (PCR) to microarrays and sequencing, have successfully been applied to bulk samples containing many thousands of cells. Furthermore, the ability to dissect heterogeneity with single-cell resolution has become a powerful approach to study complex phenomena such as cancer, development, microbial ecology, and noise in biological systems.

For example, a developing embryo, tissue, or tumor consists of many types of cells that may be spatially organized in intricate structures. The cell types that are responsible for renewing the tissue may consist of a vanishingly small fraction of the entire tissue. In order to understand the development and cellular hierarchies of these complex systems the various cell types have to be classified and analyzed. Another example is in microbiology: In most environmental samples, only one percent of the species can be grown in pure culture. Therefore, the ability to isolate individual microbes from a complex environmental sample and study their genomes provides a powerful analytical tool to probe biological "dark matter."

It is also becoming clear that gene expression is rather noisy at the single-cell level, both because of the small number of molecules controlling biochemical processes in the cell and the changing microenvironment (19, 24, 61). Given this constraint, one might ask how living organisms elicit the desired outcome to a given condition. Levsky et al. (46) discussed possible solutions to this problem: It might be that many genes are functionally redundant and that the differences in possible biological outcomes from variations in the expression of individual genes are less significant. Another possibility they proposed is that the variability in messenger RNA (mRNA) numbers is "averaged out" over time such that all cells express the necessary genes at some time point, resulting in similar protein levels. Finally, it is likely that the levels of certain critical cellular components are tightly regulated by biochemical mechanisms, such as feedback loops. Recently, Shinar et al. (73) found general structural attributes in biochemical networks that confer robustness to concentrations of certain chemical components in the network.

In order to test these various hypotheses, precise high-throughput single-cell genomic measurements must be performed. In this review, we examine the main single-cell genomic technologies and discuss their capabilities, throughput, and applications.

MICROSCOPY-BASED METHODS

Since the invention of the microscope, much of the progress in cell biology depended on finding the best way to view proteins, organelles, and other cellular components. Pathologists use a wide variety of antibodies to stain specific proteins in fixed cells and tissue sections (12). Cell imaging has been revolutionized with the development of genetically encoded fluorescent proteins, which have enabled unprecedented single-cell analysis of protein location and trafficking within live cells (11, 74).

For example, in a recent work Livet et al. (48, 50) genetically labeled neurons with multiple distinct colors by means of stochastic Cre/lox recombination of fluorescent proteins and were able to visualize hundreds of neighboring axons and synaptic contacts in brains of transgenic mice. Viollier et al. (92) studied the spatial organization of the chromosome of the bacterium *Caulobacter crescentus*. By expressing the fluorescently tagged DNA-binding proteins LacI-CFP (cyan fluorescent protein) and TetR-YFP (yellow fluorescent protein) in combination with a transposition strategy for inserting the binding sites lacO and tetO for these proteins into random sites in the bacterial DNA, they were able to label the cellular position of 112 individual loci dispersed over the circular chromosome of individual living bacteria. They found that these loci are arrayed in linear order along the long axis of the cell and

that this organization is being established during cell division while DNA replication is still in progress.

Microscopy approaches have also been used to directly visualize nucleic acids. RNA fluorescent in situ hybridization (FISH) uses fluorescently tagged oligonucleotide probes to mark expressed mRNA molecules in fixed cells (22, 66). A probe can be designed for any gene with a known sequence, and cells can be studied in the context of their surrounding tissues, which is important for studying tissues and tumors. For example, Raj et al. (64) used RNA-FISH to count single mRNA molecules in single cells, and by stochastic modeling of the cell-cell variability, calculated biochemical parameters involved in gene transcription. Topalidou et al. (88) used single-cell mRNA counting to show that the gene *alr-1* in *Caenorhabditis elegans* is essential to control the cell-cell variability of the gene *mec-3*, a key regulator in the differentiation of the nematode's touch receptor neurons. In another work, Raj et al. (65, 80) used mRNA counting in *C. elegans* embryos to study incomplete penetrance—the phenomenon by which only a fraction of a population of organisms that harbors a mutation at a particular genetic locus develops the corresponding mutant characteristics. They showed that in mutants for the gene *skn-1*—a maternally supplied regulator for intestinal development in *C. elegans*—the embryos had a larger variability in an intermediate downstream transcription factor *end-1* than the wild type. Intestinal differentiation occurred only in the small fraction of embryos whose *end-1* expression exceeded a certain threshold.

It is also possible to follow mRNA molecules in live cells that were engineered for this purpose. Bertrand et al. (5) constructed a system with two components: (*a*) a reporter mRNA designed to contain multiple binding sites (stem loop structures) for the coat protein of the bacterial phage MS2, and (*b*) a GFP-MS2 fusion protein that is constitutively expressed. This method, also known as "MS2 tagging," enabled them to visualize real time localization of the mRNA in living yeast cells. Golding et al. (27, 28) used a similar approach to track single mRNA molecules in living *Escherichia coli*, along with the levels of their associated protein.

The strength of microscopy is that it preserves the geometry of the tissue or cell that one is studying, and one can use this to understand the spatial relationships between various cells or cellular components and how it corresponds to their gene expression properties. The limitations of microscopy-based methods mainly relate to their throughput and the ability to automate and parallelize genomic measurements. These limitations can sometimes be circumvented by partitioning the sample into many microchambers in combination with automated microscopy. Neumann et al. (58, 59) used transfected cell microarrays, in which cells are directly seeded on a microarray spotted with small interfering RNA (siRNA) transfection mixes. In conjunction with live cell imaging and automated identification of single-cell phenotypes by digital image processing, they were able to perform genome-wide phenotypic profiling for siRNAs, targeting each of the approximately 21,000 protein-coding genes in the human genome. A different approach was used by Gomez-Sjoberg et al. (29), who designed a microfluidic device with 96 chambers, each allowing cells to grow under separate time-dependent controlled conditions. Tay et al. (86) used this device to study the response of the transcription factor nuclear factor (NF)-κB to different concentrations of the signaling molecule tumor-necrosis factor (TNF)-α in thousands of live cells, and found, in contrast to population-level studies with bulk assays, that the activation is a heterogeneous "digital" process at the single-cell level with fewer cells responding at lower doses. Falconnet et al. (20) used a similar device to study cell-cell variability in yeast response to pheromone signaling across various genotypes and conditions. Taniguchi et al. (85) created a library of more than 1,000 strains of *E. coli*, with each strain having a particular gene tagged with the YFP coding sequence. Using a combination of fluorescence microscopy and mRNA FISH (targeting the

FISH: fluorescent in situ hybridization

siRNA: small interfering RNA

YFP coding sequence), they simultaneously measured protein and mRNA levels in thousands of single cells. These high-throughput measurements were facilitated by an automated microfluidic device. Four thousand cells of each strain were injected into each of the 96 separate lanes and immobilized on a polylysine-coated coverslip for automated fluorescence imaging with single-molecule sensitivity.

Fluorescence-activated cell sorting (FACS): the act of sorting cells based on fluorescent properties measured by flow cytometry

HSCs: hematopoietic stem cells

FLUORESCENCE-ACTIVATED CELL SORTING

In the 1970s, flow cytometry and fluorescence-activated cell sorting (FACS) were developed in order to automate the process of single-cell characterization (72). A FACS machine works by flowing a cell suspension through a narrow stream of fluid. The cells are passed through a laser beam, and the scattered light is collected by detectors that can process it to measure cell characteristics, such as size and granularity. The stream is then passed through a nozzle and broken into small drops, each drop containing a single cell. The drops acquire an electric charge and are then electrostatically deflected into different containers according to the measured cell characteristics. By staining the cells with fluorescent antibodies specific to proteins that are bound to the cell membrane—also called surface markers—it is possible to sort different cell types according to the expression levels of these markers.

FACS machines can process tens of thousands of cells per hour and can measure up to approximately 18 surface markers at a time. The cells remain alive at the end of the process and can be used for functional biological experiments, such as transplantation assays in stem cell research. FACS has become a core technology tool in biomedicine especially in characterizing different types of cells in the blood and immune systems. For example, in the 1980s FACS technology was used to identify the hematopoietic stem cells (HSCs) (75). Similarly, stem cells have been identified for solid tissues [e.g., breast (71, 78)] and tumors (1, 13). The ability of FACS to perform multiparametric measurements is also useful as a computational tool for investigating biochemical networks. Sachs et al. (69) used FACS to measure the phosphorylation levels of 11 phosphoproteins and phospholipids in human primary naive $CD4^+$ T cells. They first perturbed the cells with different stimuli, fixed and stained them with antibodies specific to phosphorylated states of signaling proteins, and then used FACS to measure the levels of all 11 markers in thousands of individual cells. Using Bayesian inference they reconstructed the signal transduction network and found previously unknown causal regulatory relationships. Recently, Bendall et al. (2, 4) combined flow cytometry with mass spectrometry: Rather than using fluorescent tags, they used transition element isotopes not normally found in biological systems as chelated antibody tags, which allowed them to overcome the limitations of background noise and spectral overlap between fluorescent tags. Using this technique they created a single-cell profile of the healthy primary human hematopoietic system with 34 simultaneously measured cellular parameters and identified new cell populations involved in hematopoietic differentiation.

Using DNA binding dyes (e.g., DAPI, Hoechst), FACS can be used to study the DNA content of single cells. One application is flow karyotyping—the quantitative analysis and sorting of individual human chromosomes in order to detect and localize genetic abnormalities such as chromosomal deletions and aneuploidy (89, 90). Another application is to discern between different stages of the cell cycle in single cells: DNA binding dyes in conjunction with pulse labeling of the DNA-replicating cells with a synthetic nucleoside (BrdU) can allow one to discern between the G1, S, and G2/M phases in single cells. These methods can be used to study tumors and genetically inherited diseases. FACS can also be combined with FISH. For example, Kalyuzhnaya et al. (38) used FISH with probes targeting the 16S rRNA genes of type I and type II methanotrophs—two types of methane metabolizing bacteria. Subsequent FACS sorting was used to separate and enrich these two

populations, followed by PCR amplification of specific sequences for phylogenetic analysis.

The main limitations of FACS machines are related to the number of different fluorescent markers that can be measured simultaneously, the cost of the instruments, and the skill required to operate and maintain them. It is also challenging to detect genes with low expression levels and the spatial context of the cells within their original tissue is lost.

SINGLE-CELL QUANTITATIVE POLYMERASE CHAIN REACTION

PCR was invented in the 1980s and is used for amplification, detection, and quantification of nucleic acids. Quantitative reverse transcription PCR (qRT-PCR or RT-PCR) incorporates reverse transcription and a fluorescent probe (35) or dsDNA binding dye (e.g., SYB Green I) that allow sensitive measurement and detection of starting material down to single molecule range. The reverse transcription can be performed with gene specific primers or with random hexamers and oligo-dTs. PCR can be multiplexed to allow targeted amplification and quantification of many genes from the same sample.

PCR has been used to amplify DNA (47) and mRNA (43) from single cells for purposes of genetic testing, immunology studies, and gene expression. Bengtsson et al. (3) measured gene expression of five genes from 169 individual mouse pancreatic cells under conditions of high and low glucose and found that mRNA levels can be approximated by a log-normal distribution and that some genes correlate well with each other and others do not. Warren et al. (94) used similar methods to measure cell-cell variability in mouse HSCs, granulocytes, naive B cells, and naive T cells, and showed that cell-cell heterogeneity does not increase with mouse age. This study also demonstrated that multigene measurements can be used to distinguish between the different cell populations within a tissue because different cell types appear as distinct clouds in multidimensional gene space. In a different work (93), they used the more precise approach of digital PCR (dPCR) to make absolute single cell mRNA measurements of the transcription factor PU.1 and the housekeeping gene GAPDH in various hematopoietic cell populations [HSCs, common myeloid progenitors (CMPs), common lymphoid progenitors (CLPs), etc.]. They found divergent levels of PU.1 expression within the CMP's. Stahlberg et al. (77) studied multigene profiles of single astrocytes—star-shaped glial cells that are the majority cell type in the central nervous system—and used hierarchical clustering, principal component analysis (PCA), and self-organizing maps (SOM) to identify two subpopulations with distinct gene expression profiles.

Despite these successes, mutliplex quantitative polymerase chain reaction (qPCR) is limited in the number of reactions. For example, if we wish to measure 100 genes from 100 cells we need 10,000 reactions. Microfluidic chips can be used to overcome these limitations by combinatorially mixing the samples and gene detectors and by performing thousands of reactions in parallel on a single chip (49, 76). In a recent work with our collaborator Michael Clarke, we used qPCR on microfluidic devices to compare the expression levels of genes responsible for scavenging reactive oxygen species (ROS) in tumorigenic and non-tumorigenic single cells taken from a mammary tumor induced in transgenic mice (15). We found that some ROS genes were overexpressed in the tumorigenic cells and that this may explain their resilience to radiation treatment. Guo et al. (31) measured the expression levels of 48 genes in hundreds of cells in order to study the differentiation process in embryogenesis. They found that 64 cell-stage mouse blastocysts contain three distinct cell types, which can be distinguished by their gene expression profiles, but that at earlier stages the blastomeres coexpress transcription factors specific to different lineages. They also identified two anticorrelated genes that can be used as early markers for differentiation. Petriv et al. (62) measured the expression profiles of microRNA (miRNA) molecules for phenotypically distinct cell populations isolated from the

RT: reverse transcription

Digital PCR (dPCR): a method for counting DNA molecules in a sample. The sample is diluted and partitioned into hundreds of chambers such that each chamber contains no more than one molecule, whose presence or absence is detected by qPCR

CMP: common myeloid progenitors

CLP: common lymphoid progenitors

qPCR: quantitative polymerase chain reaction

miRNA: microRNA

LCM: laser capture microdissection

RNAseq: RNA sequencing

mouse hematopoietic system and used these profiles to infer lineage relations and functional similarity. Single-cell miRNA measurements were used to check the heterogeneity of representative populations. Flatz et al. (23) studied the immune response to different prime-boost vector combinations encoding HIV Env, a gene encoding the viral envelope. Although all vaccines stimulated antigen-specific CD8 T-cell populations of similar magnitude, phenotype, and functionality, single-cell gene expression profiling showed that different subsets of CD8 T cells were differentially induced by different vaccines.

Microfluidic chips are also useful for automated single-cell isolation and allow for more efficient RNA purification and amplification. Marcus et al. (52) designed a microfluidic chip that employed microscopic bead columns for extracting total mRNA from individual single cells and for synthesizing cDNA. This approach provides high mRNA-to-cDNA efficiency and decreases the risk of contamination, and was later used to profile gene expression in single human embryonic stem cells (ESCs) (102). Bontoux et al. (8) used a similar microfluidic chip to profile single neuronal progenitors using on-chip RT followed by template-switching PCR (TS-PCR) amplification (54). Kralj et al. (40) demonstrated T7 amplification of cDNA from single-cell quantities using bead columns constructed in microfluidic chips. Recently, White et al. (96) designed a microfluidic device capable of performing single cell capture, cell lysis, reverse transcription, and qPCR for hundreds of individual cells per run. Using this device, they were able to measure mRNA levels and miRNA levels, and perform single nucleotide variant detection in thousands of single cells.

SINGLE-CELL MICROARRAYS AND RNA SEQUENCING ANALYSIS

Microarrays enable measurement of thousands of genes at once by hybridization of a fluorescently labelled biological sample to an array consisting with thousands of synthetic oligonucleotide probes. Modern tiling microarrays can be used for high resolution genomic measurements and whole transcriptome measurements (55), and can also detect noncoding transcripts and miRNAs (79). Microarrays typically require 1–2 μg of mRNA (45, 70), which corresponds to 10^6–10^7 cells [assuming 0.1–1 pg of mRNA per cell (17)]. To apply this approach to single cells, the mRNA from each single cell has to be amplified using PCR (16, 30, 37), T7 (17, 51, 91), or isothermal (42) amplification.

Chiang et al. (9) isolated 60 individual cells from a morphologically uniform pancreatic epithelium of a mouse embryo, amplified their transcriptomes using PCR-based amplification, and used microarray analysis to identify distinct cell types. They used these findings to propose a pathway for pancreatic development. Tietjen et al. (87) used laser capture microdissection (LCM) to isolate single cells from the mouse olfactory epithelium and by similar methods found unique expression profiles for olfactory progenitors and neurons. Kamme et al. (39) used LCM, T7-based amplification, and microarray analysis to study heterogeneity in individual CA1 neurons in the rat hippocampus. In a more recent work, Kurimoto et al. (41) used a combination of PCR and T7-based amplification to amplify mRNA for more than 80 high-density microarray hybridizations from a single mammalian cell.

Next-generation sequencing technologies are creating a revolution in the sequencing of whole genomes and transcriptomes. Sequencing provides information that cannot be accessible by other methods (56) since there is no a priori choice of gene products to be probed. DNA sequencing is used to characterize mutations and variability in the genome (e.g., copy number variations) for both coding and noncoding regions. RNA sequencing (RNAseq) can be used to estimate gene expression by counting the number of reads that align to the coding region of every gene. Additionally, it can detect miRNAs and splice variants, and discover new uncharacterized transcripts and markers.

Similar to microarrays, sequencing RNA or DNA from single cells requires amplification by PCR (30, 82, 83) or isothermal amplification (42). Tang et al. (83) devised a PCR-based amplification protocol to amplify total mRNA from a single mouse blastomere for mRNA-sequencing and identified 75% more reads than microarrays and more than a thousand previously unknown splice junctions. In another work (81), they used this technique to study changes in the transcriptome during the transition from inner cell mass (ICM) cells of blastocysts to ESCs and were able to identify changes in transcript variants and miRNAs.

In general, 20 to 40 million sequencing reads are needed for detection of new genes, splicing variants, and exons (84). The amplification from single-cell quantities introduces bias and non-specific products. For this reason, most sequencing and microarray findings require validation by qPCR, in situ hybridization, or staining. One way that amplification bias may be overcome is by randomly attaching a diverse set of bar codes in order to convert a population of identical DNA molecules into a population of distinct DNA molecules suitable for threshold detection. The absolute number of molecules of each species can then be found by performing amplification and using microarrays or sequencing to identify and count the absolute numbers of distinct target sequences (26).

Historically, the main limitations of microarrays have been cost and low throughput in terms of number of samples. However, the costs of microarray analysis have dropped dramatically and today they are competitive with sequencing-based methods to analyze the transcriptome, thereby having kept pace with the Moore's law advances in sequencing throughput. It has been argued that microarrays represent a more economic and more practical workflow for high-throughput studies (99). In the coming years, we expect that microarray- and sequencing-based approaches will continue to compete and each will have certain technical advantages.

SINGLE-CELL GENOMIC SEQUENCING

Sequencing has provided innumerable insights into the activities of microbes, the ebb and flow of their populations, and their interactions with one another, with multicellular organisms, and with the physical environment. Historically, this work focused on cloned DNA fragments and pure samples of genomic DNA obtained from axenic cultures. Thus, whole-genome studies were generally limited to the minority of species for which laboratory culture conditions had been established. Single-cell genome sequencing has emerged as a new route to access the uncultivated majority directly, without the need for laboratory growth or indeed a viable sample. The approach has been used to obtain the draft genome of TM7, a candidate phylum with environmental and clinical relevance (53). Other examples include marine microbes (68, 98), an insect symbiont (97), organisms from complex microbial communities such as cattle rumen (36), and the first single-cell archaeal genome, also the first sequence of a wild and free-living ammonium-oxidizing archaeon, *Nitrosoarchaeum limnia* (6).

Single-cell sequencing is useful not only to access the genomes of uncultivated organisms, but also for comparing the genetic sequences of individual cells sequenced from a population. Consortia of cells, be they populations of cells in a tissue, microbial communities of the human microbiome, or environmental microorganisms, contain genomic variation, which provides a window into biologic and ecologic processes of interest. Indeed, genomic heterogeneity drives some aspects of phenotypic heterogeneity observed among closely related cells.

There exist two principal technical requirements for single-cell sequencing, (*a*) the physical isolation of a single cell, and (*b*) the preparation of that material for DNA sequencing. With respect to the first challenge, several approaches have been successfully implemented, including microfluidic flow (53), flow cytometry (68, 98), micromanipulation (97), and

MDA: multiple displacement amplification

dMDA: digital multiple displacement amplification

optical tweezing (6). The femtogram quantities of genomic DNA present in individual microbial cells are insufficient to directly sequence, even with the advent of third generation single molecule sequencing approaches (10, 18, 34, 63, 67). As a result, single-cell genome sequencing depends critically on whole-genome amplification, which has typically been accomplished by multiple displacement amplification (MDA) (14). MDA amplifies a denatured DNA sample isothermally by random priming and the strong strand displacement activity of phi29 DNA polymerase. The reaction can achieve high amplification fold from the contents of a single microbial cell and yield the nanograms to micrograms of DNA typically required for next-generation sequencing (95). For sequencing, the high molecular weight MDA product is transformed into smaller pieces and appended with sequencing platform-specific adaptor sequences.

DNA contaminants pose a particular challenge to de novo genome sequencing projects, as they cannot be identified by comparison with a reference sequence. Contaminants can be introduced at any stage of the workflow, although the steps before amplification are the most sensitive. Any stray DNA molecules introduced spuriously from the microbial sample, laboratory environment/apparatus, or reagents have a strong potential to make up a significant portion of the sequencing library and negatively impact the construction of an accurate sequence assembly. Process engineering can effectively address sample-borne, environmental, and apparatus contamination, but not reagent contamination. Two approaches have emerged to suppress the impact of reagent contamination: UV light treatment of reagents (100), and reduction of the amplification volume to the nanoliter scale (6, 53).

Discussion on the occurrence of a primer-derived or template-independent background in MDA has called into question the possibility of eliminating contaminants from MDA reactions (32, 44, 60, 68, 101). Recently, we introduced an assay called digital MDA (dMDA) for counting contaminating DNA fragments with improved sensitivity (7). dMDA established the absence of a template-independent product under commonly used MDA conditions and was used to validate the absence of contamination at the femtogram per ml level in custom-prepared MDA reagents. The adoption of dMDA as a quality control measure by reagent manufacturers would have a dramatic effect in accelerating the adoption of MDA for a variety of applications, including single-cell genome sequencing.

Informatics techniques for assembling and analyzing single-cell genome data are also rapidly advancing (33), and converging with methods used for assembly and analysis of metagenomic datasets due to analogous challenges (e.g., widely variable coverage depth). The combination of single-cell and metagenomic data has proven synergistic at a technical level (6, 36) and promises to forge new conceptual links between single genomes and populations of genomes. The future for single cell–based genomic analyses of microbes is exceptionally bright.

Single-cell genomic analysis has also been used to reconstruct lineages of tumors and tissues in multicellular organisms. Shapiro and colleagues (25) used microsatellite sequence data—short noncoding tandem repeats in the genome—to build lineage trees of tissues and tumors. More recently, Navin et al. (57) used FACS to isolate, amplify, and sequence DNA from approximately 100 single nuclei from different regions of a human primary breast tumor and its associated liver metastasis. From low resolution phylogenetic genomic analysis, they concluded that a single clonal expansion formed the primary tumor and seeded the metastasis.

It is also possible to isolate and amplify single chromosomes from a single cell. Fan et al. (21) developed a microfluidic device capable of separating and amplifying homologous copies of each chromosome from a single human metaphase cell in independent chambers. This enabled them to study the two alleles (or haplotypes) of each chromosome independently. This method can be used

to obtain accurate haplotype information in personal genome sequences, to understand meiotic recombination and to directly study the human leukocyte antigen haplotypes of an individual.

One of the main limitations on applying sequencing to single-cell genomes relates to artifacts of the amplification process. This process creates an uncontrolled bias, chimeras, and non-specific products. We believe that these limitations will be overcome to some extent in the near future by development of better amplification methods and by spatially isolating individual chromosomes, and this remains an area of active research.

SUMMARY: GENERAL CHARACTERISTICS OF SINGLE-CELL GENOMIC TECHNOLOGIES

In this review, we presented the main technologies used today for single-cell genomic studies. These technologies are used for diverse applications ranging from understanding tissue development and cancer to identifying genetic disease, discovering new microbes, and reconstructing lineages and haplotypes.

Single-cell genomic technologies can be characterized by the following parameters: (*a*) throughput in number of cells; (*b*) throughput in number of genes; (*c*) what does the

Table 1 A summary of the main genomic technologies for single cell analysis. Each technology is characterized by its throughput and capabilities, and is suitable for different applications. Throughput in terms of number of cells or genes is approximate

Technology	Number of cells	Number of genes	What does the technique detect?	Capabilities
Dynamic proteomics or MS2 tagging	100	1–2	Fluorescently tagged proteins	Live cells Time resolution Engineered cell lines
mRNA fluorescent in situ hybridization (FISH)	100	3–4	Fluorescently tagged mRNA molecules	Fixed tissues or cells
Flow cytometry	Thousands per hour	~18	General cell characteristics and fluorescently tagged antibodies bound to proteins in cell	Live cells Dissociated tissue Membrane bound proteins (mainly)
Single cell quantitative polymerase chain reaction (qPCR)	1–100	3–4	qPCR threshold cycles that reflect the original amount of target mRNA/DNA in sample	Lysed cells Long time per reaction Many reactions required (number of reactions = number of cells × number of genes)
Multiplex qPCR on microfluidic chips	Few hundreds to 1,000	96 at a time	qPCR threshold cycles that reflect the original amount of target mRNA/DNA in sample	Lysed cells Many reactions multiplexed on chip
Single cell microarray	1–10	20,000	Hybridization of sample to oligonucleotide probes representing genes	Lysed cells Uncontrolled amplification bias Tiling arrays can detect noncoding regions and splice variants.
Single cell sequencing	1–10	20,000	Assembled sequence reads	Lysed cells Can detect non-coding regions and splice variants Uncontrolled amplification bias Multiplexing samples with barcodes

technique actually measure? (e.g., does it provide time resolution? Does it measure analog or digital quantities?); and (*d*) what is the measurement precision and bias? Given that these characteristics usually compromise each other, different technologies are used for different applications. Sometimes a combination of technologies is needed. For example, FACS is used to isolate single cells that are enriched for stemness markers, followed by single-cell qPCR or sequencing to analyze the enriched population. In **Table 1**, we summarize the main characteristics of each technology discussed.

Tools enabling the study of cells' genomic contents and expression empower discovery and analysis across a broad swath of bioscience. Genetic and phenotypic heterogeneity among cells is the rule, not the exception, in tissues and natural populations of microbes, and single-cell techniques are the most powerful methods for resolving such cell-to-cell variation. Increasingly, this heterogeneity is being linked to the development, dynamics, function, and dysfunction of tissues and microbial communities. Discovery of the genomic sequences of uncultivated microbes benefits tremendously from single-cell techniques, as does elucidation of the sequence and structure of human genomes from complex primary tissue. Signatures of rare diseased cells and clues to unravel molecular etiology are emerging from single-cell gene expression data. The most informative future studies will link genomic data from single cells to their microscopic physical and biological context.

SUMMARY POINTS

1. The cell is the basic quantum of life. Understanding complex biological systems such as a developing embryo, a tumor, or a microbial ecosystem requires understanding the behavior and heterogeneity of the individual cells that constitute the system and their interactions.
2. Advanced imaging techniques in combination with fluorescent antibodies, genetically encoded fluorescent proteins, and molecular probes enable visualization of cells, proteins, and nucleic acids in live and fixed cells.
3. FACS allows characterization of tens of thousands of single cells and isolation of different cell populations according to their characteristics.
4. qPCR can amplify, detect, and accurately measure nucleic acids in single cells down to single molecule resolution.
5. Microarrays and sequencing technologies enable measurement of thousands of genes at once from single cells and can detect noncoding regions and splice variants. For single-cell quantities amplification is required, which may introduce bias and non-specific products.
6. DNA contaminants pose a particular challenge to single-cell de novo genome sequencing, as they cannot be identified by comparison with a reference sequence.
7. Microfluidic devices can be used for combinatorial mixing of thousands of qPCR reactions in parallel and can provide high mRNA-to-cDNA efficiency and decreased risk of contamination.

FUTURE ISSUES

1. There is currently a need to improve the throughput of single-cell gene expression methods with respect to number of cells and number of genes that can be measured at once.

2. Existing techniques for whole-genome/trancriptome amplification prior to sequencing suffer from bias and non-specific products that have to be characterized and eliminated. In particular, there is a need to develop methods for multiplexing samples and precise unbiased counting of molecules.
3. Devices for rapid isolation and characterization of single cells from small biological specimens and biopsies have to be improved in throughput and simplicity of use.
4. Future challenges include devising sensitive high-throughput assays for characterizing proteins, signaling, epigenetic, and metabolic states in single cells, and correlating these measurements with physiological characteristics.
5. There is a need to develop computational tools for understanding the biology that will become accessible through high-throughput single-cell data. These tools will most likely involve complex networks.

DISCLOSURE STATEMENT

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Contents

Errata

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