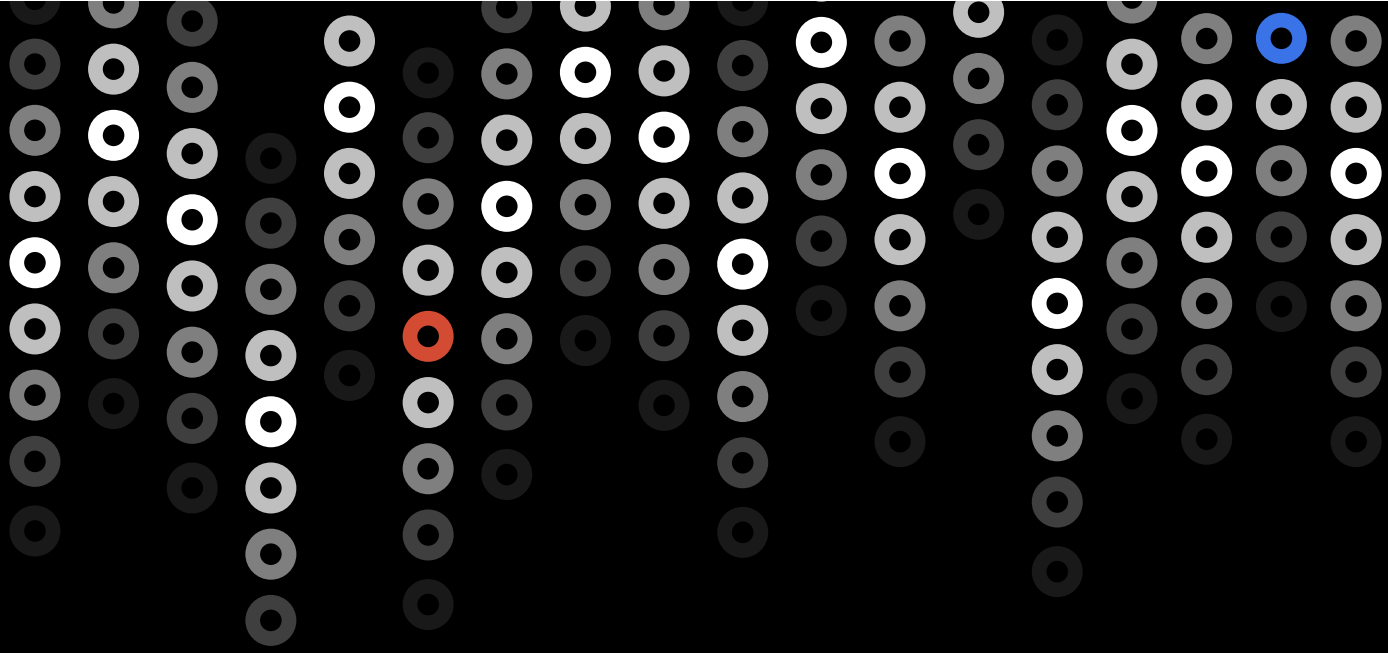


YOUR GROUND CONTROL IN HIGH-THROUGHPUT BIOLOGY

WHITE PAPER

DG-WP0002- Rev A - White Paper

SINGLE-CELL SEQUENCING POWERED BY DROPLET MICROFLUIDICS

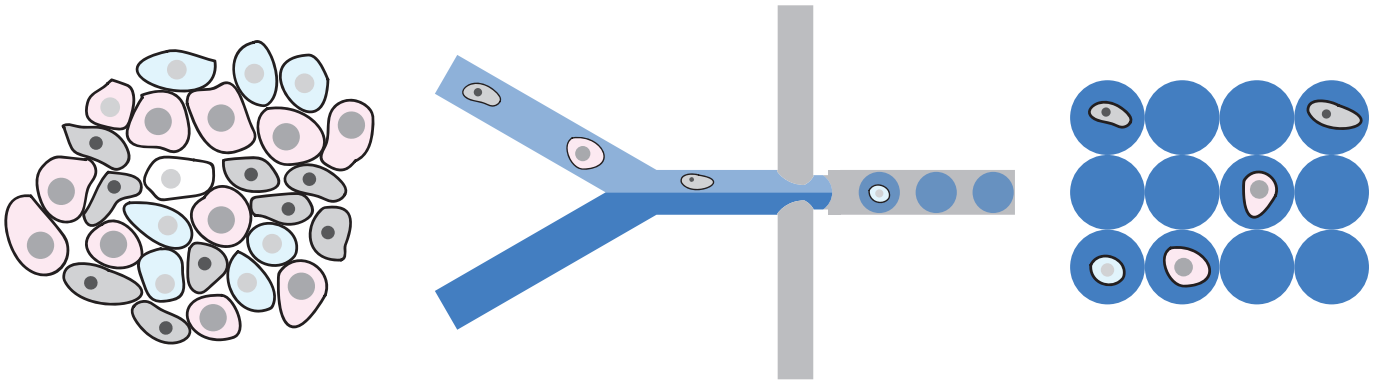
Droplet Genomics

DROPLETGENOMICS.COM

Single-cell sequencing powered by droplet microfluidics

SUMMARY:

- Single-cell sequencing allows studying cellular heterogeneity that cannot be captured by conventional bulk techniques.
- Using micrometer-sized droplets as surrogate reaction chambers dramatically reduces the reagent demand, increases throughput, and improves sensitivity.
- Sequencing readout might be used to interrogate multiple cellular modalities.



INTRODUCTION

Cells may form independent organisms or be part of complex multicellular structures, e.g., an average human body consists of 3×10^{13} cells[1]. Many diseases start from single malfunctioning cells; therefore, technologies able to study cellular biology are of great importance. Numerous conventional methods are focused on cell populations rather than individual cells. However, isogenic populations exhibit functional heterogeneity and multi-modal distributions that cannot be adequately captured by bulk techniques.

Since the first reports on gene expression profiling of single cells[2], the field of single-cell sequencing has been advancing rapidly. Single-cell sequencing, as well as single-cell multi-omics, were named Methods of the Year in 2013 and 2019, respectively, by Nature Methods in recognition of their importance for revealing biological information at the unitary resolution of life.

The main steps of a single-cell sequencing workflow include cell isolation, lysis, nucleic acid (NA) processing, amplification, library preparation, high-throughput sequencing, and data analysis. Isolation of cells is the key step in single-cell sequencing, which preferably should be fast, effective, and sufficiently gentle to

maintain the original gene expression profiles. Currently available approaches of cell separation include limiting dilution, micromanipulation, laser capture microdissection (LCM), fluorescence-activated cell sorting (FACS), and microfluidics. The characteristics and outcomes of these methods are rather different, with limiting dilution, micromanipulation, and LCM being laborious and of severely limited throughput. FACS is a high-throughput approach, however capable only to sort the cells in the wells of conventional microtiter plates. In contrast, microfluidics empowers rapid compartmentalization of individual cells together with appropriate reagents into individual water-in-oil chambers compatible with a cellular scale. Droplet-based microfluidic devices create monodispersed micrometer-sized emulsions that result in substantially reduced reagent demand and increased sensitivity of molecular assays[3].

Multiple layers of genetic and epigenetic regulatory mechanisms act in concert to shape the developmental trajectory of cells. Single-cell analysis technologies that use sequencing readout are not restricted to widely adopted RNA sequencing (RNA-seq), but can also interrogate DNA, chromatin conformation, and protein expression (Table 1).

Single-cell DNA sequencing (scDNA-seq) reveals mutations and structural changes of cellular genomes and helps to analyze somatic clonal structures. Single-cell epigenome sequencing focuses on phenotypic changes that are not related to variations in the DNA sequence but rather to epigenetic modifications, such as DNA methylation, chromatin accessibility, histone modifications, and others. Single-cell transcriptome sequencing, or scRNA-seq, measures gene expression at a cellular resolution to identify biologically meaningful differences in cell clusters. Protein expression profiling is also possible using DNA barcodes linked to antibodies. Ultimately, multi-omic integration of several layers of biological information at single-cell resolution will dramatically enhance our ability to understand the complex machinery of the cell[4].

PRACTICAL CONSIDERATIONS

Variations of experimental design and sample preparation methodologies dramatically affect the outcome of single-cell sequencing experiments. Currently, there is no gold standard, however, large consortia, such as Human Cell Atlas, are working on the identification of optimal sample preparation conditions and generating high-quality standardized datasets that will serve as a reference[4]. Several practical considerations related to droplet-based experiments are discussed below.

Table 1. Examples of microfluidics-assisted sequencing library preparation methods to analyze single cells.

Modality	Method
Genome	SiC-seq[5], targeted scDNA-seq[6]
Epigenome	dsciATAC-seq[7], Drop-ChIP[8]
Transcriptome	inDrop[9], Drop-seq[10]
Proteome	AbSeq[11]
Genetic screening	Perturb-seq[12], TAP-seq[13]
Multi-omics	CITE-seq[14], REAP-seq[15], QuRIE-seq[16], ASAP-seq[17], ECCITE-seq[23]

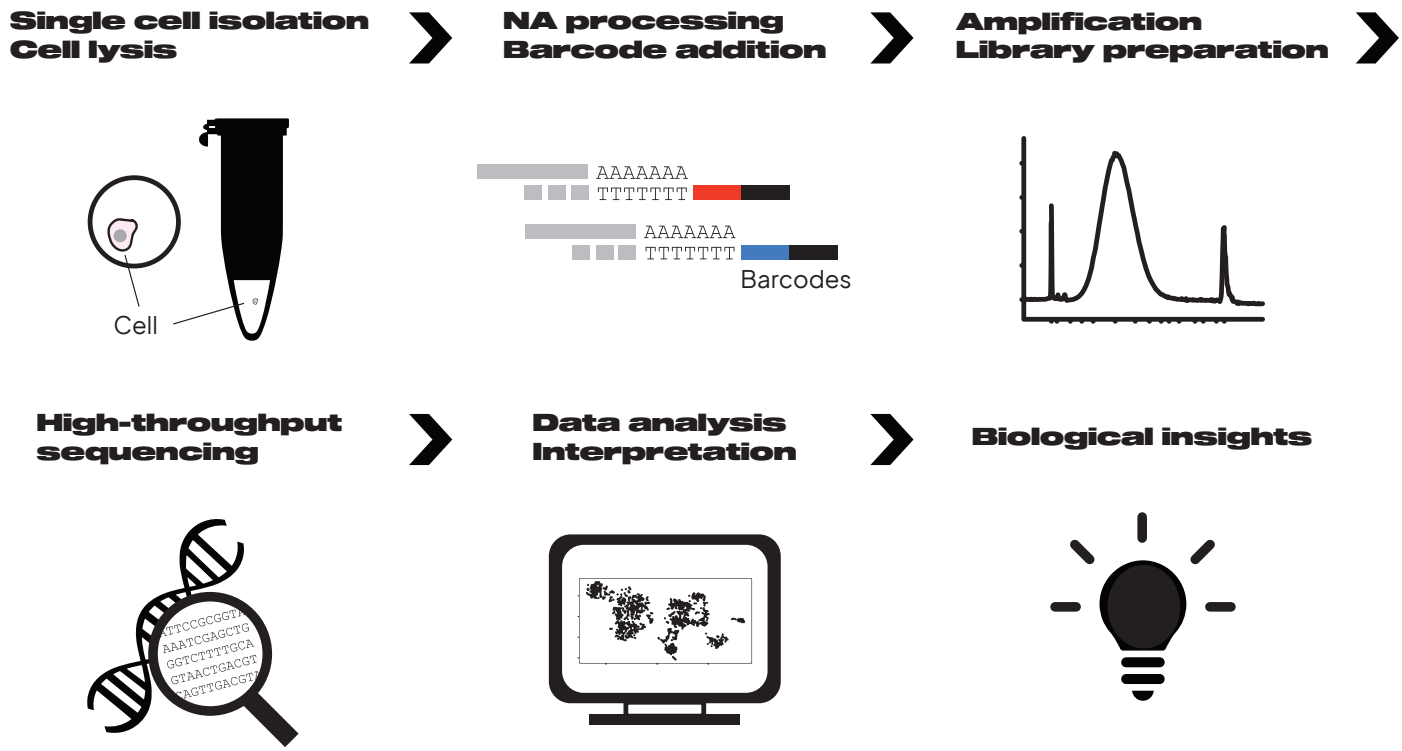
Throughput

The experimental throughput is always a major concern in the design of single-cell studies. Because of tissue complexity, dynamicity of the cell cycle, and other intrinsic variations, as well as technical noise, the data from a small number of cells are typically inadequate to reflect the state of a biological sample[18]. On the other hand, an increase in the number of available cells with the help of droplet-based encapsulation poses a practical limitation to the number of reads that can be sequenced per cell. For scRNA-seq studies, the recent mathematical estimation proposes to sequence as many cells as possible at approximately one read per cell per gene to extract the maximum amount of information from the experiment with a given budget[19].

Droplet microfluidics allows rapid compartmentalization at a frequency of thousands of droplets per second. Furthermore, droplets are intrinsically scalable as the number of produced droplets is not limited by the physical dimensions of the microfluidic chip. Importantly, encapsulation is a random process that relies on Poisson statistics. Productive droplets should contain both the cell and the carrier of barcodes (usually beads) to label its nucleic acids. When cells and beads are delivered by double Poisson distribution, the fraction of the resulting productive droplets is 2–4%. Close packing of barcoding beads that allows for synchronized delivery enables near-perfect loading of droplets and, hence, cell capture efficiency of >75%. The latter approach is most suitable when the total amount of cells in the starting sample is limited[20].

Sensitivity

The sensitivity of nucleic acid detection is a fundamental indicator of the performance of single-cell sequencing experiments, especially scRNA-seq. It reflects the overall efficiency of a method for capturing target molecules and converting them into a sequencing-ready library



through several steps of enzymatic processing. High sensitivity permits the detection of lowly expressed genes or other rare events.

While higher sensitivity can be achieved by increasing sequencing depth, optimization of enzymatic reactions might increase library complexity to improve sensitivity without inflating the cost. Among the droplet-based methods the best currently achievable sensitivity is the detection of ~17,000 transcripts from ~3,000 genes on average with ~36,000 reads per cell barcode[18].

Barcoding

To trace sequencing information back to individual cells and individual molecules, barcodes are attached to nucleic acids early in the workflow. Compartment, or cell, barcodes are synthetic sequences that are identical for all molecules deriving from the same cell but differ between the cells. Another layer of barcoding is the labeling of individual molecules with unique molecular identifiers (UMIs) that allow to correct amplification biases and reduce the technical noise. This is especially important for quantitative applications, such as gene expression profiling.
Barcode addition is usually achieved by using beads, which provides the advantage of efficient pairing with cells in the case of packed delivery during encapsulation. Moreover, efficient synthesis of oligonucleotides on beads ensures sufficient barcode abundance to tag target molecules. Bead-free barcoding methods were also reported, e.g., using single semi-randomized oligonucleotides that are clonally amplified in droplets before being attached to target molecules[21], however, such techniques have not been widely adopted.

MULTI-OMIC APPROACHES

The initial efforts in single-cell sequencing developments were focused on scRNA-seq, however, it was quickly realized that the combination of transcriptomic profiling with the characterization of other biomolecules would provide information that is more than the sum of its parts[22].

Using droplet-based microfluidics, high-throughput analyses of different modalities have enabled (i) linking of protein profiling and transcriptome measurements to integrate proteins into a molecular model of dynamic cellular responses; (ii) pairing changes in chromatin states with transcriptional profiles or protein levels; (iii) measuring transcriptional responses to genetic perturbations, and more. Interrogating multiple modalities essentially relies on modifying molecular assays so that all biomolecules of interest deriving from the same cell would be able to capture the same cell barcode. As an impressive example, Mimitou et al. have proposed a modular ECCITE-seq approach that is able to characterize at least five modalities from individual cells[23].

Single-cell multi-omic methods have already expanded the toolkit for the analysis of complex molecular and cellular networks. Nevertheless, technological advances that add additional layers of information and allow to perform even more complex manipulations with isolated cells are of great interest. Notably, further improvements are not a prerogative of assay optimization and might also arise through the advancement of microfluidic compartmentalization.

To make droplet microfluidics more accessible to researchers, several commercial systems were launched with only little room for customization. The emergence of flexible platforms with the ability to fine-tune conditions and continuously monitor the experiment will empower the further development of single-cell sequencing technologies.

CONCLUSIONS

- Single-cell sequencing technologies are instrumental in the identification of cell composition of tissues, understanding cellular responses to genetic manipulations, deciphering complex diseases, and many other exciting applications.
- Droplet microfluidics has substantially contributed to the single-cell sequencing field by reducing the reagent cost and increasing cellular throughput.
- Barcode addition at the early stages of the workflow simplifies sample handling by allowing to pool cells into batches without losing cellular identity.
- Sequencing readout can be applied to profile several characteristics of the cell: RNA, DNA, chromatin accessibility, protein expression. An integrated analysis of these modalities offers dramatic potential for discoveries and deeper understanding of cellular biology.

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