Single Cell Technologies: Beyond Microfluidics

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Abstract
Single cell RNA-sequencing (scRNA-seq) has been widely adopted in recent years due to standardized protocols and automation, reliability and standardized bioinformatic pipelines. The most widely adopted platform is the 10X Genomics solution. While powerful, this system is also limited by its high cost, moderate throughput and the inability to customize due to fixed kit components. This review will cover new approaches that do not rely on microfluidics and thus have low entry costs, are highly customizable and are within the reach of any lab possessing molecular biology expertise.

Introduction
Breakthroughs in single-cell omics are providing unprecedented opportunities to investigate diverse biological questions including tissue and tumor heterogeneity, cell activity dynamics, fate determination and cellular responses to environmental variations. Since first being described in 2009 (1), single-cell technologies have developed rapidly, and currently most laboratories are performing single-cell RNA sequencing (scRNA-seq) using equipment from 10X Genomics based on droplet microfluidics (2,3). The wide adoption of this technology reflects its reliability and ease of use. However, alternative single-cell methods based on different technologies are now emerging. These novel scRNA-seq and single-cell (multi-) omics offer researchers higher throughput, dimensionality and cost efficiency. This article will review current single-cell technologies beyond droplet-based microfluidic scRNA-seq, and review key concepts, advantages, and applications of these novel methods.

Droplet-based microfluidics
1. Method overview
Droplet microfluidics technology combines accurate manipulation of flow rates of individual cells and chemical particles with a cell partitioning system allowing single cell capture in aqueous microdroplets (Figure 1A) (4). Each cell-encapsulated droplet contains a unique barcode used for molecular indexing in subsequent reactions (2). This technology is mature and exhibits high throughput, excellent gene detection sensitivity and time efficiency. For example, the Chromium system provided by 10X Genomics can process tens of thousands of cells within a one-day workflow.

Droplet microfluidics requires generation of a high-quality single-cell suspension. However, in many cases, cell dissociation from solid tissues (e.g. kidney) remains a challenge because some cell types exhibit poor viability following enzymatic disassociation treatment, and other cells are more resistant to disassociation in the context of tissue collagen matrix (5). Therefore a growing number of studies use isolated single nucleus suspensions (i.e. snRNA-seq) instead of cells. Results indicate that snRNA-seq presents gene detection sensitivity and clustering visualization comparable to scRNA-seq and could further improve identification of rare cell types (5–7). In snRNA-seq, RNA reads are usually enriched for intronic genes. These nascent transcripts represent the earliest steps in transcription and can be used to study temporal effects in single cells (8). Single nucleus-based approaches also
eliminate disassociation-induced transcriptional stress responses and are fully compatible with frozen clinical specimens. Potential concerns on snRNA-seq include incomplete characterization of genes that have uneven distribution between nucleus and cytoplasm such as some cellular state-defining genes in human microglia (9) and the challenge of adapting nuclear isolation protocols to different tissues. Since most single-cell technologies are compatible with either single-cell or single-nucleus isolation, we will refer to cells alone in this article unless otherwise specified.

2. Applications in single-cell (multi-)omics

Most scRNA-seq approaches utilize poly-thymidine (polyT)-tailed oligonucleotides to capture polyadenylated mRNA and synthesize cDNA by reverse transcription for subsequent library preparation (Figure 1B). But the same droplet microfluidic platform can also be modified to study other cell parameters, such as DNA or protein, by changing reaction chemistry and device parameters. The single-cell assay for transposase-accessible chromatin using sequencing (scATAC-seq) (10,11) provides readouts of epigenetic regulation by probing for accessible chromatin with a transposase (Figure 1C). The transposase has a high DNA affinity and inserts an oligonucleotide whenever it binds accessible double stranded DNA in a process called tagmentation. By preloading the transposase with barcoded oligonucleotides that also contain PCR primers, these regions of open chromatin can be amplified to create libraries for downstream next generation sequencing. Other epigenomic readouts include measuring histone modifications by chromatin immunoprecipitation with sequencing (ChIP-seq) (12), DNA methylation by bisulfite sequencing (13) and chromosome structure by conformation capture (Hi-C) (14) at single-cell resolution.

A growing number of studies now profile multimodal information from single cells to obtain a more comprehensive understanding of cellular events (15). For example, simultaneous measurement of gene expression and open chromatin (scRNA-seq + scATAC-seq) on the 10X droplet microfluidics platform device has been developed (16) and is commercially available. In such assays, transposase-induced open chromatin tagging is performed in bulk and these transposed cells are then loaded onto the microfluidics device with modified chemistry to capture both mRNA and probed DNA in a cell. Another recent assay also successfully identified transcriptome and transcription factor-binding sites concurrently at single-cell resolution (17). In addition, joint profiling of single-cell transcriptome and targeted proteome is now possible, in which the bulk sample is first treated with either oligonucleotide-conjugated antibodies (18,19) or affinity-optimized aptamers (20) so that protein signals are transformed to DNA readouts and can be processed to each partitioned droplet.

All aforementioned methods can provide a snapshot of the current cell state of a biological system. On the other hand, a few recent methods focus on parallel profiling of transcriptome and lineage history in same cells (21). Single-cell lineage tracing can be achieved by inducing the expression of a CRISPR-Cas9 system, so that inducible genome editing can accumulate as time goes and be identified by scRNA-seq on the droplet microfluidics platform (22–25). Another approach is to transduce cells with
heritable barcodes at timepoints of interest and then deconvolute the lineage tree across these timepoints (26). Single-cell lineage mapping could present promising opportunities to depict clonal history of cells in development and cellular plasticity in response to various environmental changes.

Split-pool barcoding
1. Method overview

A new single-cell manipulation method called split-pool barcoding (also termed single-cell combinatorial indexing) has emerged in recent years (27–32). Unlike droplet microfluidics-based approaches in which each cell is barcoded with one unique oligonucleotide, this method achieves single-cell resolution by marking each cell with unique combinations of several oligonucleotides. This method does not require physically isolated single cells in a reaction chamber (e.g. a droplet).

In split-pool barcoding, a group of cells is placed in each well of a multiwell plate. Each well contains a unique barcode that is incorporated into each cell in the well. After these barcodes are incorporated, the cells from all wells are pooled and then redistributed to wells in another multiwell plate with different barcodes in each well. The process is typically repeated a third time, again with a new set of unique barcodes. In this way, nearly all cells will be indexed with a unique combination of three different oligos (Figure 2A). Different ways to incorporate barcodes into cells are presented in Figure 2B.

To further clarify the principle of split-pool barcoding, here is an example of 3-round barcoding using 384-well plates (in practice, four different 96-well plates), in which there are a total of 384 different Round 1 oligo barcodes (R1), 384 R2 and 384 R3 barcodes. First, in each of the 384 wells of the first-round plate, a certain number of cells will be loaded, supplemented with one well-specific R1 oligo. Then, all cells in the first plate are pooled together and redistributed to 384 wells of the second-round plate, where each well contains one unique R2 oligo. After three rounds of barcoding, each cell will be indexed with a combination of three barcodes (R1-R2-R3). The total number of barcode combinations is $384^3$ or ~56 million unique combinations. Ultimately, we can assign reads originating from the same cell in sequencing data by discriminating the $384^3$ barcode combinations.

Of note, in a split-pool barcoding experiment, the number of final recovered cells must be far lower than the total number of barcode combinations to avoid barcode collisions (i.e. that multiple cells may be indexed with the same barcode by chance). This can be explained by a mathematics concept called the Birthday Problem (8) (Equation 1) – the probability that in a group of $n$ randomly chosen people that two of them will share the same birthday (or in our case, the same barcode). It turns out that the probability of a shared birthday is 50% in a group of just 23 people. In a randomly selected group of 365 people, about 37% of them will share the same birthdays with someone else in the group. By extension, if $384^3$ cells are recovered from the $384^3$ barcode combinations, ~37% of the cells will share cell barcodes complicating downstream analysis. But reducing total cell number reduces these “collision rates.” By starting with just 1 million cells, the collision rate is <1%, which is actually
substantially lower than collision rates from microfluidic scRNA-seq which are typically ~5%. Understanding the effect of barcode collisions can help researchers to estimate the final throughput based on their split-pool experimental design.

Equation 1

In the event of a total of \( N \) cells achieved from a split-pool barcoding experiment with a total of \( D \) barcode combinations, the collision rate \( P \) is:

\[
P = \frac{N - D + D \left( \frac{D - 1}{D} \right)^N}{N}
\]

2. Advantages of split-pool barcoding

One major advantage of split-pool barcoding technology is its scalability for ultra-throughput sequencing. In the example stated above, we can profile several million cells in one single experiment using the 3-round 384-well indexing strategy. The unprecedented throughput has enabled researchers to study molecular variations of a highly heterogenous tissue at multiple developmental stages, or even decipher single-cell omics of a whole organism. For example, one split-pool barcoding scRNA-seq method has successfully provided a 2-million-cell transcriptomic landscape of mouse embryo organogenesis (33) and a human fetal atlas covering 4 million cells across 15 organs (34). Another scRNA-seq method, conceived with a similar spilt-pool barcoding strategy, was used to profile mouse brain and spinal cord at different developmental stages with high throughput (27).

This technology also significantly reduces reagent waste and therefore offers much lower per-cell costs (< $0.02) compared with other methods (33). The experiment can be performed on common multiwell plates without upfront investment in a microfluidic controller, which makes the technology more accessible to laboratories. By comparison, generating a 1 million scRNA-seq dataset using the 10X Chromium system would cost ~$250,000 in kit costs alone which is at least ten fold more expensive than split-pool barcoding. In addition, split-pool barcoding enables sample multiplexing (i.e. processing distinct samples in one experiment and demultiplexing them from sequencing data), because in the first-round indexing, each well is deposited with a unique barcode (R1) and cells from a certain sample, and therefore, R1 identifies the sample type of origin and can be used to demultiplex samples in data processing (Figure 2A). This reduces batch effect since many different samples can be processed at the same time. Batch effects represent a major challenge to the in integration of single-cell data from multiple experiments using standard workflows (35).

There are three main limitations of split-pool barcoding. One is its limited sensitivity in gene detection per cell compared to the 10X Chromium. Split-pool approaches can currently identify only ~25% of genes typically detected with 10X Chromium. Partially offsetting this is the much higher number of cells processed, and the fact that cell types can still be readily distinguished with a few hundred gene counts
per cell (33,36). Also, this method is expected to achieve higher gene detection sensitivity in the future as the advancement of sequencing power and further protocol optimization. A second major limitation of this technology is the absence of validated bioinformatic workflows such as exist for 10X Chromium data, such as CellRanger and Seurat. In practice this means that only labs with moderate informatic skills, including coding in both Python and R, will be able to analyze data generated by this method. Finally, split-pool barcoding is laborious. While a library of 1 million cells can be created in one week, this is a busy week filled with thousands of pipetting steps. A comparison of droplet microfluidic vs. split-pool barcoding approaches is summarized in Tables 1 and 2.

3. Applications in single-cell (multi-)omics

An appealing feature of split-pool barcoding is that it is highly customizable. In addition to scRNA-seq using this approach, first described in 2017 (28), split-pool barcoding has been successfully adapted to study single-cell genome sequencing (37), ATAC-seq (31), DNA methylation (38) and Hi-C (39) with improved throughput and sensitivity. One group recently optimized the barcoding strategy and chemistry of ATAC-seq in order to profile the chromatin accessibility networks of nearly one million human fetal cells (40). Another study developed a split-pool barcoding protocol for studying dynamics of single-cell transcription by labelling newly synthesized mRNA (41), and successfully characterized the dynamics of cell cycle regulation and receptor activation after cortisol stimulation.

Fewer single cell multiomics approaches based on split-pool barcoding have been developed. The first assay for joint profiling of chromatin accessibility and transcriptome was described in 2018 (32), in which both reverse transcription (RNA-seq indexing) and transposase-induced transposition (ATAC-seq indexing) are performed on cell-containing wells, which enables parallel preparation of both libraries in subsequent steps. This study successfully identified the relationship between epigenetic landscapes and underlying gene expression programs including in adult mouse kidney. More recently improved protocols for measuring both single cell RNA and chromatin accessibility have been developed with gene detection sensitivity comparable to droplet microfluidics methods and with improved throughput (8,30).

We note that many single-cell omics technologies built on droplet microfluidics may also be expanded to split-pool barcoding approaches. For example, joint profiling of single-cell transcriptomes and lineage history may also be adapted to split-pool barcoding profiling, using the same genetically engineered models mentioned above. Although the current number of studies leveraging split-pool barcoding is relatively limited, we envision future growth in these areas to leverage the high throughput, low cost and flexibility of this platform.

Other single-cell methods

Beyond the two methods mentioned above that are currently widely used, several other techniques exist to manipulate single cells (42,43). Methods developed at the early stage of single-cell era, such as limiting dilution and micromanipulation, are
usually low-throughput and require laborious pipetting and will not be reviewed in this article. Instead, we review a few single-cell manipulation methods that are still actively used, including circuit microfluidics, microwell(nanowell)-based assays, flow cytometry and mass cytometry (Figure 3). We highlight the importance of these methods since they are mostly compatible with full-length transcript identification protocols such as SMART-seq (44), and therefore, more illustrative of studying gene alternative splicing events such as exon skipping and intron retention, which are usually underrepresented in droplet microfluidics methods due to their 3’ end bias (45). In the end, we briefly review the newly emerged spatial transcriptomics technologies which can promote our understanding of cell identity in the tissue context.

1. Circuit microfluidics
   Besides droplet-based microfluidics approaches, there exist other types of microfluidics devices for single-cell manipulation. A comprehensive review of microfluidics technologies can be found elsewhere (4,46). One actively used approach is circuit microfluidics, which enables automatic isolation and capture of hundreds of single cells from a cell suspension. This microfluidics system harnesses a microvalve to achieve accurate single-cell fluid control and the microchannel structure is distinct from droplet microfluidics (47). A commercialized circuit microfluidics platform (48) uses an integrated microfluidic chip to capture cells with a specific size range, in which the quality of loaded cells can be evaluated under a microscope. In addition to scRNA-seq and scATAC-seq, this platform can be used to study single-cell multiomics including co-assay for scRNA-seq and scATAC-seq (49) and joint profiling of single-cell transcriptome and targeted proteome (50).

2. Microwell(nanowell)-based assays
   In microwell(nanowell)-based assays, the cell suspension is dispensed into a microarray containing many microwells (nanowells) which are sized to capture single cells. For single-cell indexing, beads bearing well-specific barcodes can be added to each well. Recent work has improved the throughput of these assays and reduced experimental costs by either improving the microarray fabrication technique (51) or developing automated platforms (52). Using this method, two studies generated a single cell Mouse Cell Atlas and a Human Cell Landscape, respectively by scRNA-seq (53,54). An assay for scATAC-seq (55) was also described and showed robust characterization of distinct types of hematopoietic cells. Compared to other high-throughput technologies, microwell(nanowell)-based assays also have advantages in reduced cell doublet rates and improved viability of captured cells, because it enables examining the morphology of deposited cells in each well under a microscope and removal of potential doublets.

3. Flow cytometry & mass cytometry
   There is a long history of using flow cytometry to quantitatively measure features (e.g. protein expression) of individual cells. Flow cytometry is still used to deliver single
cells to a microchamber or multiwell plate containing cell-specific barcodes. Although low-throughput, these methods allow researchers to extract rare cells of interest (e.g. expressing certain cell markers) from a bulk cell suspension just before cells are lysed and single-cell reactions occur. Simultaneous measurements of RNA and chromatin accessibility (56), or RNA and proteins (57), in sorted single cells have also been described.

In addition, a fusion technology of flow cytometry and mass spectrometry, called mass cytometry, is playing an important role in the field of single-cell proteomics (58). Compared to conventional flow cytometry that usually couples antibodies to fluorophores and is therefore limited in the number of identifiable features per cell, mass cytometry leverages antibodies conjugated with heavy-metal isotopes that can be quantified by mass spectrometry. This enables characterization of a broader repertoire of features with high specificity and throughput. Mass cytometry has been successfully applied in single-cell immunology and hematology studies (59,60), where well-defined cell surface markers can be harnessed as antigen targets to discriminate different cell types.

4. Spatial transcriptomics

With all single cell modalities described in this review so far, positional information is lost during preparation of single cell or nucleus suspensions. A very exciting emerging area is spatial transcriptomics in which gene expression profiles are linked to the locations of a cell or group of cells in a tissue section. A full description of spatial transcriptomic technologies is beyond the scope of this review, but they can be broadly divided into two categories. The first is fluorescence in situ hybridization (FISH)-based methods, in which mRNA transcripts are directly labeled in a section. Examples of this approach include sequential FISH (61) and multiplexed error-robust FISH (MERFISH) (62). The second approach is based on next generation sequencing methods and typically involves the coupling of mRNA with a molecular barcode which records the location of that mRNA on a tissue section (63). The most common example of this approach is the Visium Spatial Gene Expression solution from 10X Genomics.

Perspective and discussion

Single-cell omics has already enhanced our molecular understanding of cellular events in heterogenous tissue in both health and disease. The growing diversity of technologies that enable these studies offer enhanced scale, multimodal capability and decreased cost. On the other hand, the emergence of diverse single-cell methods also raises potential challenges in integrating data from distinct platforms since they display different library complexity and varied performance depending on the biological samples analyzed (64,65). One solution to this problem is adoption of technologies with massive scale and multiplexing flexibility, such as split-pool barcoding, by generating a library containing many different samples in a single experiment.
Choosing the best single cell approach for an investigator’s needs depends on a variety of factors including budget, informatic expertise, sample number, desired per cell detection sensitivity and more. Familiarity with the increasing diversity of single cell solutions will allow investigators to design their optimal experiment.

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Table 1. Comparison of droplet microfluidics and split-pool barcoding.

| Feature                      | Droplet Microfluidics | Split-pool Barcoding |
|------------------------------|-----------------------|----------------------|
| Throughput                   | High                  | Ultra high           |
| Sample multiplexing          | Limited compatibility#| High compatibility   |
| Dependence on advanced equipments | Yes                  | No                   |
| Gene detection sensitivity   | High                  | Moderate             |
| Multiomics compatibility     | Yes                   | Yes                  |
| Library generation pipeline  | Well established      | Less optimized       |
| Bioinformatics resources     | Rich                  | Limited              |
| Representative platforms     | 10X Chromium (2); Drop-seq (3) etc. | sci-RNA-seq3 (33); SPLiT-seq (27); sci-CAR (32); SHARE-seq (8) etc. |

#Sample multiplexing can only be achieved with additional technologies such as cell hashing (66).

Table 2. Comparison of two common scRNA-seq platforms for droplet microfluidics and split-pool barcoding: 10X Chromium and sci-RNA-seq3.

| Feature                      | 10X Chromium (per lane) | sci-RNA-seq3 (per experiment) |
|------------------------------|-------------------------|-------------------------------|
| Throughput                   | <10^4 cells             | 10^6 cells                    |
| Costs per cell*              | $0.2                    | $0.01                         |
| Sequencing depth required (per cell) | >30,000 raw reads | >5,000 raw reads |
| Number of detected genes per cell | >2,000                | >500                          |
| Labor to generate library    | Moderate (1-2 Days)     | High (1-2 Weeks)              |
| Data pre-processing methods  | User-friendly software (e.g. CellRanger) | Customized pipeline required |

*Estimated costs for reagents are presented. Illumina sequencing costs are not included.
Figure Legends

Figure 1. Single-cell omics based on droplet microfluidics.
(A) In droplet-based microfluidics platforms, each cell is encapsulated in a droplet. Cells are then lysed and molecules of interest (e.g. mRNA or open chromatin) are captured by uniquely barcoded beads.
(B) In scRNA-seq, mRNAs are captured by oligos (usually conjugated on beads) containing a polyT segment, a unique cell barcode, a unique molecular identifier (UMI) and other adapter sequences. Then, cDNAs are synthesized by reverse transcription. Library modification (e.g. via template switching) is performed to enable further library amplification.
(C) scATAC-seq utilizes a transposase (e.g. Tn5) to recognize regions of open chromatin. The transposase is pre-loaded with adapter oligos which will be annealed to the ends of probed gDNA. The modified gDNA can be captured by oligos (usually conjugated on beads) containing a complement segment, a unique cell barcode and other adapter sequences.

Figure 2. Overview of split-pool barcoding.
(A) Concepts of split-pool barcoding. First, cells are distributed to a multiwell plate and each well contains a unique oligo barcode. Then, cells are pooled and redistributed to another multiwell plate for molecular indexing. Split-pool barcoding enables high scalability, throughput and sample multiplexity in an experiment.
(B) Different strategies of molecular indexing: cells can be indexed by either mRNA reverse transcription, DNA ligation, DNA PCR reaction or chromatin transposition.

Figure 3. Overview of other single-cell methods.
In circuit microfluidics, individual cells are isolated in a microchannel and collected into a microchamber. In microwell (nanowell)-based assays, each microwell contains a uniquely barcoded bead and cell suspension is loaded onto the microarray. In flow cytometry, cells are sorted into a multiwell plate for subsequent reactions. In mass cytometry, cells are probed with metal-conjugated antibodies allowing accurate quantification of features of interest.
Figure 2

(A)  

1. Split
2. 1st Round Barcoding
3. Pool

High Throughput
- Cell 1
- Cell 2
- Cell 3
- Cell 4

Multiplexity
- Sample 1
- Sample 2
- Sample 3
- Sample 4

Scalability

Library Generation → Next Generation Sequencing

(B)  

1) Reverse transcription (for mRNA)
2) Ligation (for DNA products)
3) PCR (for DNA products)
4) Transposition (for chromatin)

Reverse transcription
- mRNA
- polyA

Ligation
- Ligation Linker Adaptor
- Linker-adapted DNA
- Barcode

PCR
- PCR Primer Adaptor
- Primer-adapted DNA
- Barcode

Transposition
- gDNA
- Barcode

Barcode-loaded Transposase
Figure 3

Circuit microfluidics

Microwell (nanowell)

Flow cytometry

Mass cytometry