# Dissecting biological systems at the level of single cells

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A major focus of technology development over the past years has been on increasing sensitivity to work with low cell numbers. The ultimate goal for many cell biologists is to assay cells individually. The desire to do so is in part fueled by the increasing appreciation of extensive heterogeneity in many tissues. For example, tumors are very heterogeneous, and often include not only different tumor cell clones, but also differentiated cells, infiltrating T-cells, macrophages, and fibroblasts. In the hematopoietic system, it has become apparent that even the most sophisticated flow cytometry sorting scheme has limits: highly purified hematopoietic stem cells still exhibit heterogeneous behaviors when assessed using single-cell transplantation assays (Dykstra et al., 2007; Kiel et al., 2005). In this blog, we will outline some of the most exciting developments and state-of-the-art technologies that stand to transform our understanding of tissue organization.

**Next Generation Sequencing of single cells**

Many single-cell assays start with isolation of individual cells. There are several ways to approach this. The most widely used method is fluorescence-activated cell sorting (FACS), in which single cells can be deposited in 96- or 384-well plates using a flow cytometer (Figure 1, panel A). For example, Smart-Seq2 deposits cells directly into lysis buffer, followed by enzymatic reactions to reverse transcribe and amplify the mRNA (Picelli et al., 2014). Smart-Seq was one of the first single-cell RNA-seq protocols and has the distinct advantage of capturing whole transcripts, as opposed to other technologies that are 3’ biased.

Microfluidics can also be used to capture cells in tiny droplets, followed by molecular barcoding of molecules in the droplet (Klein et al., 2015; Macosko et al., 2015) (Figure 1, panel B). Microfluidics enables processing of thousands of cells per experiment, resulting in much lower cost and labor; however, current protocols only capture the 3’ end of the transcript. For example, the companies Fluidigm, 10X Genomics and 1CellBio all offer single-cell RNA-seq technologies based on microfluidics. Recently, researchers at MIT have isolated single cells in slides with ~86,000 subnanoliter wells (Gierahn et al., 2017) (Figure 1, panel C). Single cells are captured together with beads, followed by sealing using semipermeable membranes, cell lysis and hybridization of the mRNA to barcoded oligos on the beads. This technology has the potential to further reduce cost and increase accessibility of single-cell RNA-seq, but is currently not offered by a commercially available platform.

Following cell isolation and capturing of nucleic acids of interest, the material needs to be amplified using enzymatic reactions. RNA-seq generally starts with reverse transcription of RNA into DNA, whereas genomic DNA protocols, such as ATAC-seq and ChIP-seq, begin with amplification using PCR or T7 RNA polymerase (Buenrostro et al., 2017; van Galen et al., 2016; Rotem et al., 2015). Illumina adapter ligation, Nextera or PCR are the most common approaches to prepare the DNA for Next Generation Sequencing.

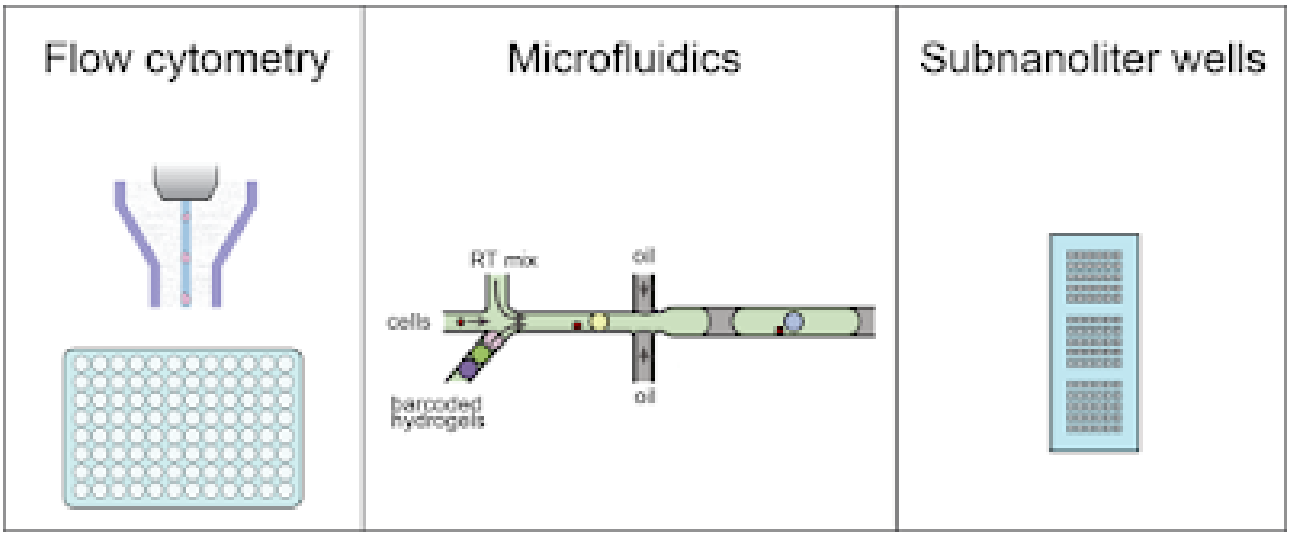


Figure 1: Single cell isolation for nucleic acid sequencing can be performed using flow cytometry (A), microfluidics (B) or subnanoliter wells (C).

**Data analysis**

With single-cell sequencing technologies, vast amounts of data are generated, and data analysis presents a formidable challenge. Demultiplexing, alignment, quality checks and duplicate filters can influence all downstream steps of the analysis. Some technologies incorporate unique molecular identifiers (UMIs), which tells you whether two similar sequencing reads were derived from the same or different starting molecules. Some protocols include linear amplification by T7 RNA polymerase, which can increase sensitivity, but can affect how duplicate sequencing reads should be collapsed. Setting a minimum number of detected transcripts is a common method to filter low-quality cells, but may inadvertently exclude cells that have less mRNA (such as hematopoietic stem cells). After quality filtering, cells are often clustered using dimensionality reduction methods such as Principal Component Analysis (PCA) or t-Distributed Stochastic Neighbor Embedding (t-SNE). These methods can be influenced by artifacts such as read depth and batch effects, that have to be carefully controlled. Considering these variables before starting a single-cell RNA-seq project is essential (Grün and van Oudenaarden, 2015). Many laboratories are working on the computational challenges in analyzing single-cell data, and investigators such as Dana Pe'er, Peter Kharchenko and John Marioni have published packages that can help with analysis.

**Single-cell imaging**

Since measuring averages of heterogeneous populations often mask unique properties of rare cell types, such as adult hematopoietic stem cells, it is evident that single-cell analysis is a prerequisite for unbiased understanding of cellular and molecular behavior (Schroeder, 2011). The single-cell sequencing approaches mentioned above have significantly improved our understanding of cellular and molecular heterogeneity. However, there is another layer when studying biological processes lasting days/weeks (i.e. lineage commitment of embryonic or adult stem cells); the temporal dynamics. Over a given timeframe, both

developmental and cell-cycle stage might influence the profile of individual cells. Since high-throughput sequencing requires lysing cells prior to downstream analysis, such methods are limited to a static picture of cell's properties, and therefore lack temporal resolution. Live-cell imaging, ideally in an in vivo setting, could provide such data. Advances in non-invasive in vivo imaging allowed observation of entire zebrafish embryos for periods up to 2.5 days (Keller et al., 2010). Unfortunately, technical challenges such as optical tissue properties (most embryos are less transparent than zebrafish), size, accessibility to relevant structures (i.e. bone marrow imaging) and inability to long-term immobilize living animals pose significant obstacles. This limits in vivo imaging to few compatible tissues and to short time frames, and thus to events with rapid kinetics. In vitro time-lapse imaging offers an attractive, but technically challenging alternative, requiring expertise in a number of hardware and software components. It enables monitoring fates and dynamic molecular properties of individual cells and their progeny before, during and after a certain change occurs for periods up to 2 weeks (Kokkaliaris et al., 2016). Specialized software can then be used to attribute specific properties to individual cells and reconstruct the kinship within a colony in multidimensional lineage trees post-acquisition (Skylaki et al., 2016). When coupled with endpoint gene expression methods, it can retrospectively identify cell-state transitions (Hormoz et al., 2016). However, in vitro time-lapse imaging is currently limited to tracking few proteins simultaneously and cannot substitute the need for observing biological phenomena in their physiological environment. Although its usability depends on the biological question, in vitro time-lapse imaging can be a powerful approach for high throughput screenings or monitoring signaling dynamics over time.

**Conclusions**

Recent advances in single-cell analysis have significantly improved our understanding of cell behavior in homeostasis and disease. Sequencing RNA or DNA from single cells poses great engineering and computer science challenges. The innovations in this field are fast-paced, and future breakthroughs will enable higher capture efficiencies of molecules within cells, with robust protocols that are accessible to more investigators. Such technologies are already contributing to a reconsideration of the hematopoietic hierarchy (Nestorowa et al., 2016; Paul et al., 2015; Villani et al., 2017; etc.). Coupling gene-expression assays with in situ live-cell imaging adds another dimension enabling detection of local differences between similar or anatomically distinct regions of the same tissue (Silberstein et al. 2016). As methods become more sophisticated and multiplexed, unraveling complex tissues at the level of cellular resolution will make a lasting contribution to our understanding of biological systems in health and disease.

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