VIP *Very Important Paper*

Opportunities for Single-Cell Sequencing in Synthetic Biology

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Single-cell sequencing describes the ability to extract information from a single cell among a heterogeneous mix of cell types by sequencing its DNA or RNA. The development of this technology created tremendous excitement in biology. Akin to gaining higher resolution on a microscope, single-cell sequencing opened our eyes towards previously unknown cellular heterogeneity and the underlying molecular mechanisms. This technology, however, could in principle be applied to any non-living particle capable of carrying nucleotides. This article proposes the application of single-cell sequencing in synthetic biology in order to analyze large numbers of artificially-generated or re-engineered synthetic cells simultaneously. The high-throughput nature of this technology provides the opportunity to test multiple hypotheses in parallel and thus accelerate the pace of discovery. Synthetic biology serves as a particularly exciting example of many overlooked non-canonical applications of single-cell sequencing.

1. Advantages of Single-Cell Sequencing

DNA sequencing describes a method to determine the sequence of nucleotides that constitute DNA. The information obtained from sequencing of DNA (or RNA) extracted from thousands of cells informed many aspects of biology. In particular, sequencing the collection of all mRNAs (the transcriptome) was of interest as it harbors not only information about the cellular composition of a population of cells but also provides insight about which genes are expressed, which is a major determinant of the cell's molecular state.[1] Hence, sequencing the transcriptome revealed valuable details about the cells they were isolated from. This information can for instance be used to unbiasedly discover most important genes or pathways during development of organs.[2] Further, the transcriptomes of healthy and pathogenic tissues can be compared in order to learn about the mechanisms of malignant transformation of cells.[3]

Despite the rich information gain obtained from RNA-sequencing and its facilitation of major discoveries, one critical shortcoming remained: the ability to look at differences on the single-cell level. Without this gain in resolution, many insights would remain hidden in the heterogeneous complexity of the cell population. Technological advances starting from the first demonstration of single-cell sequencing[4] took the field of biology by storm.[5] Technically, this was achieved by collecting single cells into separate reaction tubes and converting their RNA into cDNA which could then be amplified. The nucleotides of the amplified cDNA were then optically read out using next-generation sequencing.[6] These days, the labor-intensive step of handling many test tubes is avoided by encapsulating cells into lipid droplets which contain unique barcodes.[7] This allows for profiling millions of nucleotides from tens of thousands of cells in a single reaction tube. Gaining large amounts of transcriptomic information on the single-cell level helped to unravel previously unknown cell types[8] and allowed the molecular characterization of almost all cell types in a given tissue.[9] But the rapid methodological advances quickly went beyond building cellular reference catalogs. Creative new computational tools allowed for the reconstruction of developmental trajectories, inference of spatial information within organs as well as regulatory mechanisms.[10] Furthermore, even though RNA sequencing was continuously at the forefront of the technological development, single-cell information can now also be obtained from other molecules and their modifications such as DNA, histones and the methylation of both.[11]

Given that a cell represents the basic building block of life, it is not surprising that cell- and developmental-biologists were most interested in applying this technology. Yet, looking at this technology from more abstract point-of-view, it becomes clear that this technology might also be useful in other scientific disciplines. After all, this method reads out information in form of nucleotide sequences from small, compartmentalized volumes. These 'volumes', however, do not necessarily have to be naturally occurring cells. This article aims to highlight opportunities for single-cell sequencing beyond classic cell- and developmental biology which were overlooked for a surprisingly long time[4] (Figure 1). I will particularly focus on synthetic biology applications as examples of non-canonical usages of single-cell sequencing primarily because of its multidisciplinary nature and the exciting opportunities in this discipline. However, the ideas presented here might be of interest to many other fields, some of which might have never encountered this powerful technology.

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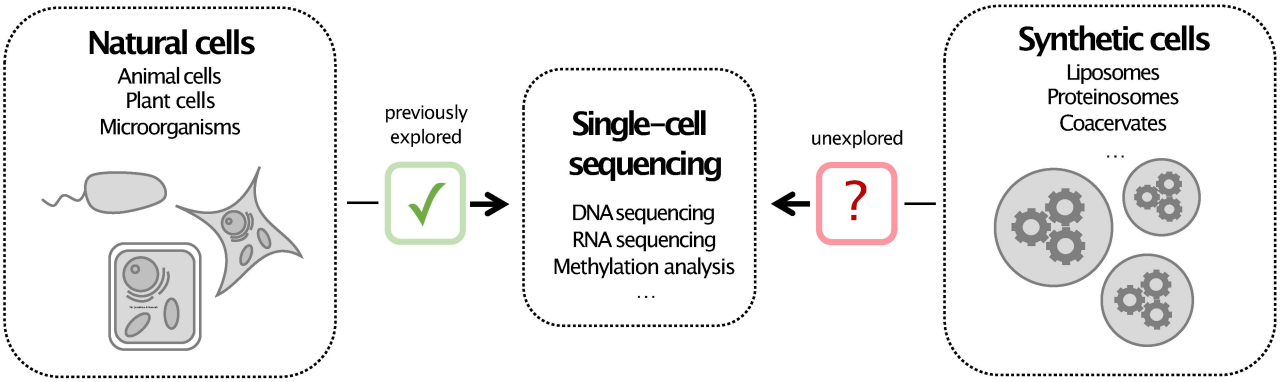

Figure 1. Summary scheme illustrating the current focus of the single cell sequencing field on naturally occurring cells and the currently unexplored "opportunity space" in synthetic biology.

2. Applications of Single-Cell Sequencing in Synthetic Biology

Synthetic biology has matured from a niche field to one of the most promising areas of science with particularly high expectations for the private sector.[12] Although synthetic biology has become complex and multifaceted, this article will focus on benefits of single-cell sequencing of artificial cells and crudely split the field into bottom-up and top-down biology. Yet, I hope that the reader will be able to extrapolate aspects covered here towards other facets of synthetic biology and beyond.

2.1. Bottom-Up Synthetic Biology

2.1.1. Nucleic Acid Compartmentalization in Self-Assembling Systems

Bottom-up synthetic biology aims to build cell-like structures and ultimately autonomous cells from scratch.[13] The starting-points are building blocks that self-assemble into closed or semi closed compartments. Many different building blocks have been proposed for compartmentalization such as lipids, proteins or inorganic polymers.[14] Despite the fact that these molecules are chemically diverse, the common denominator is their ability to self-assemble in confined compartments which is one of the cornerstones of artificial cell generation. Self-assembly is in itself a large field of research that is of interest to many different disciplines (engineering, chemistry, physics) and has thus been extensively reviewed.[15] Naturally, biologists are also interested in the self-assembly given that it is a fundamental principle of life itself. One striking example of self-assembly with medical relevance is the formation of viruses.[16] Virus capsids self-assemble and thereby encapsulate RNA/DNA for further propagation. Many aspects of this process such as nucleic acid-encapsulation efficiency could be understood more deeply by sequencing the DNA/RNA content of single viral particles. Using nucleotide-barcoded viral capsid libraries could be used to understand how capsid variants influence virus assembly and their effect on transduction efficiency as previously described for chimeric AAV capsid generation.[17] Further, these results could be used to select interesting variants for more extensive structural analysis.

Drug delivery also frequently takes advantage of self-assembling to encapsulate relevant molecules. Studying self-assembled drug vehicles on the single particle level by leveraging single-cell technology could greatly advance our understanding of the underlying dynamics of encapsulation. Clinical relevance for reading out RNA from self-assembled drug vehicles was exemplified by a recent FDA approval of the first RNA interference (RNAi) drug for treatment of familial amyloidotic polyneuropathy.[18]

2.1.2. Characterizing Artificial Expression Systems in Synthetic Cells

Cell-free expression systems use cell-extracts (or recombinant proteins) to harness the transcription/translation machinery without the necessity of sustaining living cells.[19] These systems allow for more controlled bioengineering since the components are determined by the experimental setup. This freedom creates a great opportunity to prototype individual parts or genetic circuits which could provide well-characterized components for synthetic cells once characterized in isolation. On the other hand, it also opens a large variable space, meaning that many components (or variants thereof) could be combined in many different ways to gain any optimal outcome the researcher strives to achieve. Extracting useful information from this large space of opportunities/variables is, however, exactly what single-cell sequencing aims to achieve. Given that transcription is at the heart of cell-free expression systems, single-cell sequencing would be a straightforward approach to read out RNA molecules produced by encapsulated artificial expression systems. In this context, the droplet formation as part of the standard single-cell sequencing protocol would generate a synthetic cell instead of encapsulating one. One important advantage of single-cell genomics in this context is that the experiments are multiplexed. This means that hundreds or

thousands of different conditions could be analyzed in a single experiment which would be difficult if not unfeasible using more traditional experimental approaches.

One example that illustrates this advantage is the investigation of gene-expression elements from intractable microorganisms.[20] Here the authors elegantly demonstrate the power of multiplexing analysis of many variables by automated testing of gene expression elements from non-model microbial hosts which are difficult or impossible to culture. However, with fluorescence as the final readout a lot of information would be missed that could be easily obtained from sequencing all nucleotides of every reaction mixture as commonplace in the field of single-cell sequencing.

2.1.3. Characterization of Membrane-Free Compartmentalization via Liquid-Liquid Phase Separation or Coacervation

Usually, artificial cells are thought of as lipid-encapsulated entities. This is unsurprising given that life as we know it is lipid-encapsulated, as well. However, other artificial cell models exist which are devoid of lipid membranes. Systems such as colloidosomes, proteinosomes or coacervates form through self-assembly and circumvent many disadvantages of lipid membrane-bound systems such as low permeability or instability of fatty acid to multi-valent cations and temperature.[14a] Further, due to the ease of self-assembly and the dynamic nature of membrane-free systems, it was speculated that these protocell systems could serve as a protocell model for the origin of life on earth.[21] In general, the origin of life field provides attractive questions to tackle for the RNA sequencing community, given that it currently stands most firmly on the shoulders of the RNA world hypothesis. This hypothesis was popularized as a consequence of RNA's intriguing dual ability to store information and catalyze reactions.[22] Several studies have already investigated the behavior of ribozymes in coacervates.[23] Yet, questions such as differences of ribozyme performance across single coacervates as a function of coacervate properties and content remain currently unanswered. This question could be addressed using single-cell sequencing.

In general, many basic aspects of the nucleotide-related properties of membrane-less protocells are not yet fully characterized using state-of-the art technology. For instance, the diversity of nucleotide sequences in coacervates and how they differ from one coacervate to the other is still unknown. Membrane-less protocells can be generated using a variety of different molecules which are characterized by different chemical properties.[14a] Another question is how RNA composition differs not just within a homogenous pool of membrane-free protocells, but also across a variety of chemically diverse protocells. Here, differences in RNA uptake dynamics as well as 'RNA communication' across membrane-less compartments would be exciting avenues to explore for single-cell sequencing.

2.2. Top-Down Synthetic Biology

2.2.1. Minimal Cell Generation

Top-down synthetic biology represents the second big branch which aims to build artificial cells. The goal here is to generate artificial cells by taking existing cells as a starting point for diverse modifications.[24] The two branches of artificial cell generation might arguably be still far away from 'meeting in the middle'. Yet, both approaches might similarly benefit from single-cell technology in order to answer relevant questions for their field.

In many ways the top-down approach appears more accessible to current single-cell sequencing approaches. This is mainly because the starting point here is equivalent to the starting point of generic single-cell sequencing experiments: namely a living cell. These experiments could be approached as simply characterizing a 'novel', previously unknown, cell type and hence would be straightforward from a technical point of view.

In the last decade, a number of exciting breakthrough studies exemplified what fascinating insights can be gained by top-down synthetic biology. The starting point was the attempt to synthesize and assemble the *Mycoplasma genitalium* genome.[25] This study demonstrated that a complete genome could be synthesized and transplanted into a cell. This effort was not only a conceptional milestone but also, and arguably even more so, a huge technical advancement for the field of synthetic biology. Techniques that arose from these efforts such as the 'Gibson assembly' have since become standard parts of the molecular toolkit.[26]

A follow-up effort deleted all the non-essential parts of the *Mycoplasma genitalium* genome in order to generate a truly minimal cell termed JCVIsyn3.0.[27] A striking phenotype of this minimal cell is its polymorphic appearance.[27] Given the drastic differences among cells within a population, single-cell sequencing would be predestinated to uncover the molecular mechanisms underlying the morphological heterogeneity of this artificial cell.

Initially, the *Mycoplasma genitalium* genome was chosen because of its simplicity. Naturally, the next step would be to 'minimize' more complex genomes. One interesting aspect to investigate would be communalities and differences across minimal genomes. For this purpose, single-cell sequencing would offer the highest resolution for transcriptomic comparisons which could help us gain fundamental understanding of the most minimal cells that currently exist.

2.2.2. Cellular Re-engineering

Beyond the basic understanding of the fundamental organization of a cell, top-down synthetic biology also aims to achieve applied goals. One application of synthetic biology in biotechnology is metabolic engineering. Here, the aim is to transform existing cells into molecular 'factories' which produce or metabolize a compound of interest. Exciting recent outcomes

from this efforts are the reengineering of *E.coli* to sequester and process CO_2[28] or the complete biosynthesis of the major cannabinoids.[29]

Similar to the biotechnological branch, the goal of the biomedical application of top-down synthetic biology is to steer preexisting cells towards artificial cells that have the potential to function as a 'living drug'.[30] Here, genetically engineered T-cells called 'Chimeric antigen receptor T cells' (CAR–T cells) have demonstrated great success in the treatment of hematological malignancies.[31] The ongoing clinical trials using CAR–T cells exemplifies the clinical success and the promise that synthetic biology holds for this and other therapeutic purposes.

Some applications of top-down engineered cells might also be useful for biotechnology as well as the biomedical field. Engineered cells could for example function as sensors or drug vehicles. Going one step further, sensor and vehicle function can also be combined by generating bacteria that were engineered to lyse synchronously at a threshold density of the cell population.[32]

Here, single-cell sequencing can be of instrumental help during the design process of the above described systems. In particular because gene circuit engineering builds the foundation for the above described cellular engineering examples.[33] Parallel testing of novel gene circuits in thousands of re-engineered cells in a single experiment could notably facilitate the design-build-test cycles. If the cost for single-cell sequencing continues to drop rapidly, single-cell sequencing could potentially replace simpler readouts such as fluorescence and thus provide a more information-rich readout.

3. Challenges

Despite the exciting prospects of using single-cell sequencing for synthetic biology a few technical challenges still need to be addressed. Single-cell sequencing still has room for improvement regarding detection sensitivity resulting from capturing only a subset of DNA/RNA molecules present in a cell. This is particularly relevant for exploring synthetic cells, for which the input amount of DNA/RNA could be very low depending on experimental system or scientific question and hence face challenges similar to sequencing single microorganisms.[34] Furthermore, the vast majority of single-cell sequencing is currently done using short-read sequencing approaches. This sequencing approach is inapplicable for long repetitive sequences and heavily relies on pre-amplifications of DNA/RNA. Current advances in single-molecule long-read sequencing technology might represent promising alternatives for synthetic cell sequencing experiments that might require to overcome these constrains.

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Conflict of Interest

The authors declare no conflict of interest.

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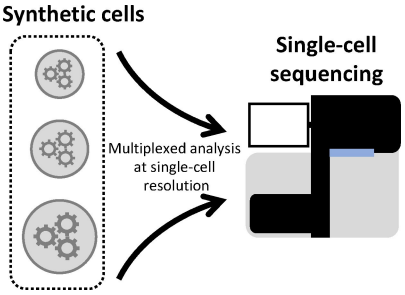
CONCEPTS

Single-cell sequencing technology aims to retrieve information from single cells by reading out their nucleotide content. By doing so, it has provided many novel insights into developmental- and cell- biology. Yet, other disciplines such as synthetic biology could also benefit from the advances brought about by this technology. This article highlights exciting opportunities for using single-cell sequencing in synthetic biology as well as other research fields at the interspace between chemistry and biology.



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1 – 6

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