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Sequencing enabling design and learning in synthetic biology

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Abstract

The ability to read and quantify nucleic acids such as DNA and RNA using sequencing technologies has revolutionized our understanding of life. With the emergence of synthetic biology, these tools are now being put to work in new ways – enabling *de novo* biological design. Here, we show how sequencing is supporting the creation of a new wave of biological parts and systems, as well as providing the vast data sets needed for the machine learning of design rules for predictive bioengineering. However, we believe this is only the tip of the iceberg and end by providing an outlook on recent advances that will likely broaden the role of sequencing in synthetic biology and its deployment in real-world environments.

Highlights

- Sequencing can capture detailed information about diverse biological processes.
- Synthetic biology is beginning to exploit sequencing to aid design.
- Large sequencing datasets are powering new machine learning approaches in biology.
- Emerging trends will see the application of sequencing in synthetic biology grow.

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Introduction

Sequencing technologies allow us to read DNA [1], RNA [2,3], and even some protein molecules [4], and monitor their changing abundance and composition over time. Such information provides a window into the inner workings of cells, helping us to better understand both their underlying genetic code and the ways it is interpreted to produce and regulate biological processes.

Synthetic biology aims to develop the means to modify existing biological systems or create new ones from scratch with our own desired behaviors [5]. This has resulted in engineered microbes that can sense and report the presence of cancer [6], efficiently self-regulate heterologous protein production to optimize yields [7] and allowed for the creation of programmable living materials that dynamically respond to their environment [8]. While these applications are impressive, they are the exception and not the norm as development of even simple bioengineered systems remains a challenge. Our limited ability to predict how biological parts or systems will function in new contexts [9,10] and the physical limitations and trade-offs when tapping into the limited resources of a host cell [11–13] means that generally numerous designs have to be built before a partially working version is found. Then, time-consuming “tinkering” is often required to optimize the performance further.

Sequencing offers solutions to some of these issues and emerging capabilities could open up more effective approaches for bioengineering [3,4,14,15]. In this review, we focus on recent developments in sequencing and how they are being applied to synthetic biology and the computational learning of biological design rules [10,16]. We summarize the wealth of sequencing methods available today and the diversity of information that can be captured (**Table 1**). Finally, we consider how new sequencing technologies might provide steps towards a more holistic spatiotemporal picture of engineered biological systems and support the more systematic design of synthetic biology across scales.

In search of new biological parts

In order to build novel living systems, synthetic biologists require parts that encapsulate simple functionalities. Examples include regulatory elements like promoters and terminators that control where transcription starts and stops, enzymes able to perform specific chemical conversions, and structural proteins that maintain the physical features of a cell. Existing organisms have a wealth of these parts encoded within their genomes and large DNA sequence databases like GenBank are a treasure trove of parts for synthetic biologists to choose from. The term “part mining” has even become commonplace when speaking about searching these repositories for sequences with a desired functionality. This revolution has been made possible by the rapidly falling costs of DNA sequencing (DNA-seq) over the past

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few decades. This has resulted in DNA-seq becoming the go to method for part discovery, allowing for genetic information to be extracted from virtually any environment and organism, including those not even culturable in the lab [17].

While DNA-seq is able to uncover sequences that encode biological parts, it does not capture any information about how they might perform. For parts controlling gene expression, such information is vital because precise levels of expression are often required for a device or system to function correctly. Computational models have been developed to try and bridge this gap [18,19], but their reliability is questionable when used outside of model organisms like *Escherichia coli*. For some key parts, such as transcriptional promoters and terminators, RNA sequencing (RNA-seq) can be used to measure part performance directly, providing a snapshot of RNA abundance at a point in time [2]. Furthermore, RNA-seq is able to characterize all promoters and terminators present in a cell simultaneously, if the transcripts produced are unique [15,20]. More recently, a system for DNA Regulatory Element Analysis by Cell-Free Transcription and Sequencing (DRAFTS) was developed to enable rapid high-throughput measurements of regulatory sequences controlling transcription [21]. This method brings together cell-free expression systems with multiplexed RNA-seq to allow for *in vitro* characterization of regulatory parts in a wide range of different organisms. This approach has been shown to display a good correlation with *in vivo* part performance and will be able to expand not only the number of parts available to bioengineers, but also provide crucial information about which non-model organisms they can be effectively used in.

Design and optimization of genetic and molecular parts

Biological parts taken directly from sequence databases rarely function exactly as desired. Optimization is therefore necessary to refine their behavior for specific applications. One of the most common biological parts used in synthetic biology are transcription factors (TFs), which allow for gene expression to be controlled in response to their abundance. TFs are proteins that generally bind DNA sequences near a promoter and either sterically block or recruit RNA polymerase (RNAP) to repress or activate downstream genes, respectively. While DNA-binding proteins can be inferred from acid sequence, the precise DNA sequence that is bound is more difficult to predict. Chromatin immunoprecipitation assays followed by sequencing (ChIP-seq) allow for the identification of the binding site of DNA-associated proteins and has been applied to asses genome-wide binding motifs and regulatory networks in cells (**Figure 1a**) [22]. ChIP-seq has also been used with cognate site identifier (CSI) arrays [23] to test the binding affinity of a TF to a library of possible DNA binding sequences *in vitro*. This allows for the DNA-binding motif to be accurately inferred and has been shown to have close correspondence to *in vivo* results. Such an approach has been used to exploit genome

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database mined repressors (TetR homologs) and to rapidly create synthetic regulated promoters based on CSI inferred binding motifs [24].

RNA-based parts and devices have become popular in recent years due to their portability across organisms and the potential for their *de novo* design using software able to predict RNA secondary structure [25,26]. Unfortunately, models struggle to capture the changes often necessary during the functioning of RNA-based devices making computational design a challenge. To support this effort experimentally, SHAPE-seq [27,28] applies selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) chemistry with multiplexed RNA sequencing to allow for the reactivity of each nucleotide to be calculated (**Figure 1b**). Low reactivities correspond to constrained nucleotides that are likely in secondary or tertiary structures. By performing SHAPE-seq for different conformations of a riboswitch or ribocomputing device, the experimentally measured structures for each state can be used to guide specific modifications for improved performance and enable a better understanding of structure-function relationships [29]. Recent developments have also extended this approach to capture nascent RNAs during transcription, allowing for cotranscriptional dynamics of a fluoride riboswitch to be observed at nucleotide resolution [30]. In addition to SHAPE-seq, standard RNA-seq has also been used to aid the design of riboswitches in mammalian cells where mRNA levels relate to switch activity [31].

Rather than optimizing a design through a sequential process of carefully chosen modifications, an alternative approach is to use high-throughput assays to build genotype-phenotype (GP) maps and extract design principles that can be readily exploited. Such approaches are supported by recent advances in massively parallel reporter assays (MPRAs) [32]. These combine chip synthesized oligos with combinatorial DNA assembly to generate a diversity of genetic constructs whose phenotype is linked to the expression of a fluorescent reporter protein. Due to the size of these libraries (normally >10,000s designs), each member could not be assayed in isolation. However, by pooling the designs and using fluorescence activated cell sorting (FACS) followed by DNA sequencing of the separate fluorescent sorted bins, a GP map for the entire library can be generated (**Figure 1c**). This methodology termed Flow-seq [32] has allowed MPRAs to unravel design principles for numerous biological systems, including: the inference of promoter regulatory logic in eukaryotes [32,33], assessing the composability of regulatory elements and the potential contextual effects that can arise [10], and dissecting the influence of RNA secondary structure, codon usage and other factors on translational efficiency in bacteria [16].

An alternative to screening increasingly complex combinatorial libraries of designs using MPRAs is to directly predict function from sequence by learning key relationships in the growing corpus of datasets. Statistical models ranging from linear regression [34] to deep learning architectures [35] have been used to realize this mapping. Compared to traditional

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machine learning methods, deep learning exploits vast datasets to derive informative representations in an unsupervised way [36]. While yielding substantial performance in many cases, these models are difficult to interpret. Opening up these “black box” models to better understand what they have learnt is still in its infancy and it is crucial that practitioners appreciate the fragility of their conclusions when looking at saliency maps [37] or applying other interpretive frameworks [38]. These predictive tools are currently able to guide research but are not reliable enough in most cases to prove causality alone.

Characterizing and debugging of large circuits and systems

The implementation of complex functions in living cells often requires the assembly of many biological parts/devices to create larger circuits and systems [9]. This is made possible by connecting parts whose inputs and outputs are based on a common signal [5,9,39,40]. For example, transcriptional devices often use RNAP flux as a signal and promoters to guide RNAP flux to specific points within a circuit [15,24]. These interconnections have to be carefully designed to ensure that regulatory signals have sufficient dynamic range to propagate correctly throughout an entire system. However, most of these internal signals and states are not directly observable, meaning that part failures are impossible to diagnose from the output alone.

To tackle this issue, RNA-seq has been applied to large genetic logic circuits to observe the steady-state concentration of mRNAs [20] and use these to infer the underlying RNAP flux present [15] (**Figure 2a**). This allows for transcriptional signals to be traced through the circuit and for each part/device to be characterized in the context of the larger system. Using this information, the root cause of failures can be quickly identified, and targeted modifications made to accelerate the construction of a working system [15]. More recently, this approach has been supplemented with ribosome profiling (Ribo-seq) [41] that allows for translational signals (i.e. ribosome flux) to be monitored concurrently (**Figure 2a**) [14,16]. Furthermore, quantification of these signals in absolute units has become possible through the introduction of external RNA standards (e.g. ERCC RNA spike-ins [42]) and measurements of key cellular properties (e.g. cell mass and growth rate) enabling the read counts from the sequencer to be converted into absolute RNAP/s and ribosome/s units for RNA-seq and Ribo-seq data, respectively [14]. Moving to more quantitative and calibrated measurements of biological parts and systems is of growing importance in helping to improve both the accuracy and reusability of data [14,42,43].

The 3D spatial organization of genetic constructs within a cell can also play a major role in gene expression and regulation [44,45]. Although exploiting such mechanisms in synthetic biology is currently rare [46], genome synthesis efforts such as the Sc2.0 project [47]

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have started to explore the effect of major structural rearrangements of genomes by using the Synthetic Chromosome Rearrangement and Modification by LoxPsym-mediated Evolution (SCRaMbLE) system [48]. SCRaMbLE causes massive changes in genome content and organization that can alter gene expression and enable the optimization of heterologous biosynthesis pathways by modification of both pathway and host genome simultaneously [49,50]. The Hi-C methodology [51,52] can be used to better understand how the physical organization of chromosomes might underlie these beneficial changes (**Figure 2b**). Hi-C works by crosslinking genomic loci that are close in space, performing a digestion and then ligation of the chromosomal DNA to connect these distant loci into a DNA fragment, and then reverse crosslinking them before deep sequencing [51–53]. From this data, a contact map can then be created to guide 3D models of the chromosomes and estimate their physical arrangement within the cell. Recent advances in Hi-C have used long-read sequencing to enable contacts between more than two loci to be measured simultaneously (IMN Ulahannan et al. bioRxiv doi: 10.1101/833590).

When applied to *Saccharomyces cerevisiae* cells containing original and SCRaMbLE'ed Sc2.0 chromosomes, Hi-C was able to show that the redesigned chromosomes in most cases maintained similar structures to the native versions and that removal of highly repetitive regions in the synthetic variants led to better resolved contact maps [54]. However, two major changes were observed: the loss of an interaction due to a gene deletion, and the relocation of an array of ribosomal RNA repeats to vicinity of the centromere cluster causing genome-wide conformational changes due to physical constraints within the nucleus [54]. As more comprehensive modifications are made to living cells, the importance of understanding their impact on the spatial organization of core cellular components and machinery will grow.

Looking to the future

Several new trends in sequencing have begun to emerge that further broaden the ways that sequencing can support biological design. From a technology perspective, the Oxford Nanopore Technologies (ONT) sequencing platform offers some interesting capabilities such as full-length reads of DNA and RNA molecules [3,55], as well as the ability to capture base modifications [56,57]. This opens up new avenues to support the development of regulatory mechanisms based on DNA/RNA modifying enzymes [58], and application of long-read DNA and RNA sequencing to monitor genome replication dynamics [59] and help dissect the influence of long-range interactions between genetic parts in a circuit. Another interesting development is the potential to use the same ONT hardware to perform protein sequencing and identification, through the use of disordered protein tags. These are designed to naturally

pass through a nanopore due to their charge and generate a unique disturbance in the measured electrical current (IMN Zhang et al. bioRxiv doi: 10.1101/837542) [4]. Capturing the variability between cells is a limitation of many current sequencing approaches that extract nucleic acids from large populations of cells and thus only provide data for population averages. While single cell sequencing is becoming more accessible and economical [60], the precision of the measurements made and inherent noise in these techniques at present makes their data challenging to use for engineering tasks. Longer term, as synthetic biology moves beyond single cells to multi-cellular consortia, tissues, and even to synthetic ecosystems, spatial sequencing approaches will also become valuable [61]. These will allow for cell/species abundance and transcriptional states to be linked to spatial positions/niches within complex structured environments – information that will be vital for the effective deployment of synthetic microbial consortia into real-world environments [62].

Conclusions

Sequencing has historically been seen as a tool to read genetic information and observe its regulation in natural contexts. However, advances in the breadth of data that can be collected and the ability for this to help inform biological design decisions, highlights it potential to underpin new bioengineering workflows. Established engineering fields rely on advanced monitoring and debugging tools to enable the rational construction and verification of complex systems before their deployment. As the field of synthetic biology matures, we expect the development of similar tools tailored to the unique features of living systems. Sequencing is well placed to take on this challenge, especially when combined with complementary analysis methods such as LC-MS [63]. Given these growing capabilities and falling costs, the application of sequencing to synthetic biology is likely to grow rapidly over the next decade, becoming a crucial tool to verify that engineered living systems work precisely as we expect before being used in real-world settings.

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Author Contributions

All authors helped to write the manuscript.

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Declaration of Interest

None.

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462 # Tables

463 **Table 1: Sequencing methods and the biological design applications they can support.**

Method	Type of Data	Applications	Refs.
DNA-seq	DNA sequence	• Verification of synthetic DNA constructs • Genome sequencing • Genetic part mining	[1,55]
	DNA structural variation	• Monitoring large DNA rearrangements (e.g. SCRaMbLE) • Characterization of recombinases and integrases • Monitoring of mobile genetic elements • Measurement of recombination rates during evolution	[49,63,64]
	DNA base modifications	• Engineering synthetic epigenetics • Novel DNA memory storage mechanisms	[56,57]
	Species identification	• Metagenomics • Microbiome engineering • Design of stable multi species consortia	[17]
RNA-seq	Transcript concentrations	• Monitoring steady-state mRNA concentrations • Calculating differential gene expression • Analysis of stress responses • Riboswitch design	[2,3,21,31]
	RNA polymerase flux	• Calculating initiation rate of promoters • Identifying transcription termination sites • Measuring termination efficiency of terminators • Calculating RNAP processivity	[15,20]
	Transcript isoforms	• Engineering controlled gene splicing • Discovery of protein variants • Refactoring gene sequences (intron removal)	[3]
	RNA base modifications	• Developing new mechanisms for gene regulation • Control of translation (tRNA modifications)	[3]
Ribo-seq	Ribosome occupancy	• Relative codon occupancy times • Identification of ribosome pausing sites • Optimization of protein translation efficiency	[14,41]
	Ribosome flux	• Measurement of stop codon termination efficiency • Measurement of translation initiation rates	[14]
ChIP-seq	Protein-DNA binding sites	• Identification of transcription start sites • Discovery of transcription factor binding motifs	[22–24]
SHAPE-seq	RNA secondary structure	• Design of RNA sensors and regulators • Monitoring of co-transcriptional RNA folding • Optimization of gene expression	[27–30]
Flow-seq	Gene expression (fluorescence)	• Construction of genotype-phenotype maps • Training machine learning models	[10,16,32–35]
Hi-C	Chromosome structure	• Control of chromosome structure • Engineering long-range genetic interactions	[44,45,51–54,65]

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Figures and captions

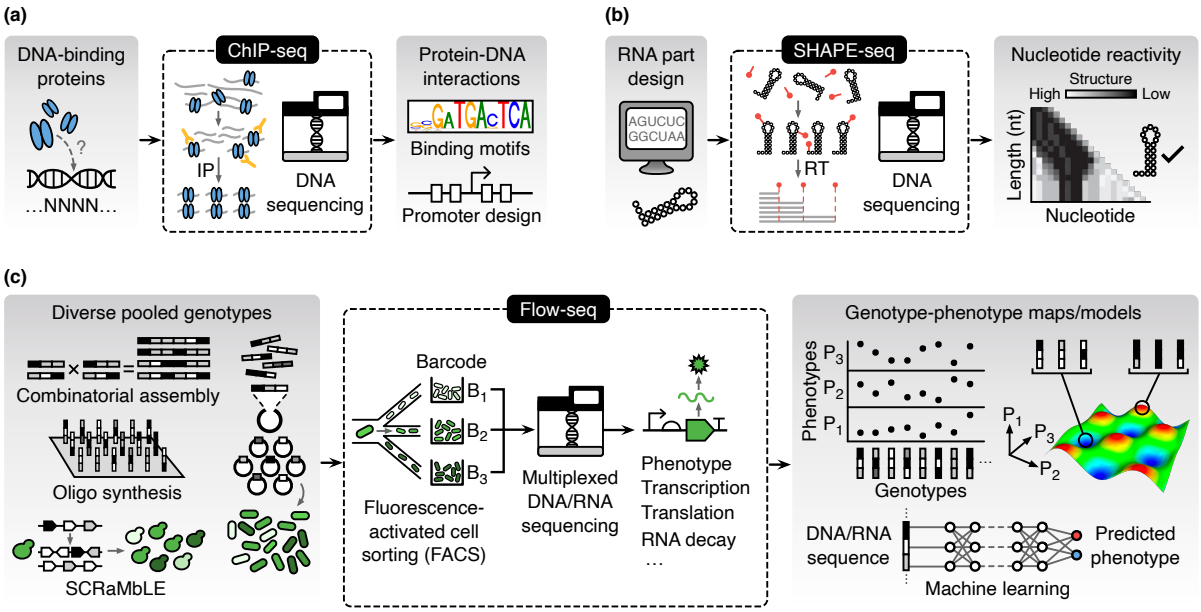

Figure 1: Sequencing methods to aid the design and optimization of genetic and molecular parts. (a) ChIP-seq can be used to study protein-DNA interactions allowing for the binding motifs of DNA-binding proteins to be measured [22]. These operator sequences can then be used to construct synthetic promoters [24]. **(b)** SHAPE-seq can probe the structural state of RNAs at a nucleotide resolution [27,28]. It relies on the use of SHAPE chemistry that selectively modifies unstructured nucleotides of RNA (red markers). These modified nucleotides can a reverse transcription (RT) to halt, providing the location of the unstructured nucleotide within the RNA. This data can then be used to generate the structure for the entire RNA. Nucleotide reactivity graph is adapted from data in [30] and is not related to the structure shown. **(c)** Flow-seq allows for a genotype-phenotype (GP) map to be generated for diverse pool of genetic constructs where phenotype is indicated by the expression of a fluorescent reporter [32]. This is made possible by fluorescence active cell sorting (FACS), barcoding of cells in each fluorescence label bin, and then multiplexed pooled DNA or RNA sequencing. Depending on how fluorescence is linked to the function of each genetic design, this approach can measure many types of phenotype related to transcriptional and translational processes.

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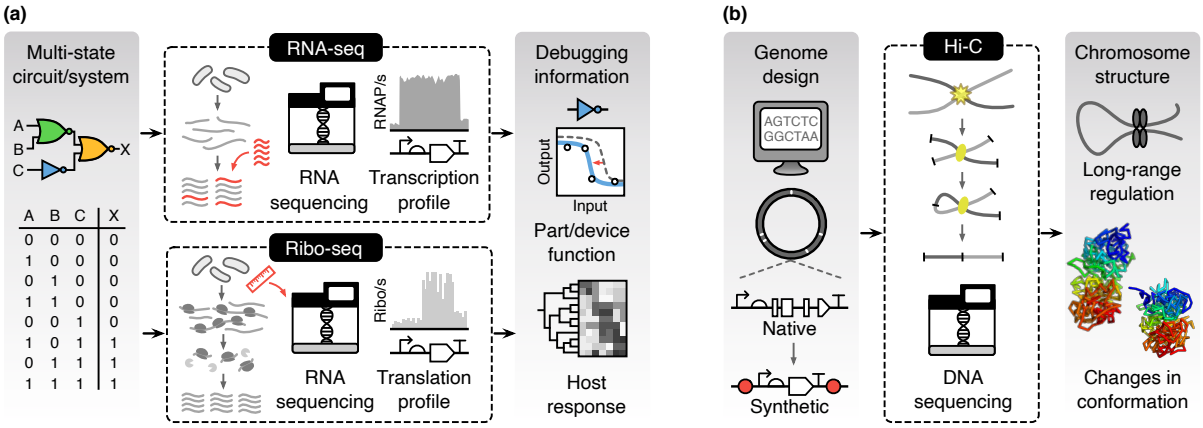

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483 **Figure 2: Applications of sequencing to large circuits and cellular systems. (a)** Large
484 multi-state genetic circuits can be debugged through a combination of RNA-seq and Ribo-Seq
485 [14,15] to provide detailed information about internal circuit states, the *in situ* function of parts
486 and devices, and the response of the host cell to the additional burden. RNA-seq can be
487 supplemented with synthetic RNA spike-ins and Ribo-seq with measurements of growth rate
488 and cellular mass (red features in dashed boxed) to convert read counts into absolute units
489 (i.e. RNAP/s and Ribosomes/s for transcriptional and translational signals, respectively) [14].
490 **(b)** Hi-C allows for chromosome structure to be measured [51,52]. This is valuable as synthetic
491 biology moves towards genome design [47] and begins to exploit long-range interactions to
492 regulate synthetic genetic circuits. Chromosome confirmations are adapted from [65].