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DNA synthesis, assembly and applications in synthetic biology

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The past couple of years saw exciting new developments in microchip-based gene synthesis technologies. Such technologies hold the potential for significantly increasing the throughput and decreasing the cost of gene synthesis. Together with more efficient enzymatic error correction and genome assembly methods, these new technologies are pushing the field of synthetic biology to a higher level.

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Gene synthesis

Conventional oligonucleotide synthesis takes place individually in small columns using microliter volumes of reagents. Chemicals and solvents are pumped through the columns, inducing stepwise addition of nucleotide monomers to the growing oligonucleotide chain. The reaction cycles proceed according to the standard phosphoramidite chemistry, which consists of four steps: (1) deprotection; (2) coupling; (3) capping and (4) oxidation [1,2]. The solid-phase phosphoramidite chemistry has been the method of choice for most commercial DNA synthesizers for the past decades. Because of limitations in chemical reaction efficiency, the length of synthetic oligonucleotides typically does not exceed 150–200 bases [3]. Beyond this length, side reactions and even modest inefficiencies within the stepwise chemical reactions would significantly compromise sequence integrity and product yields.

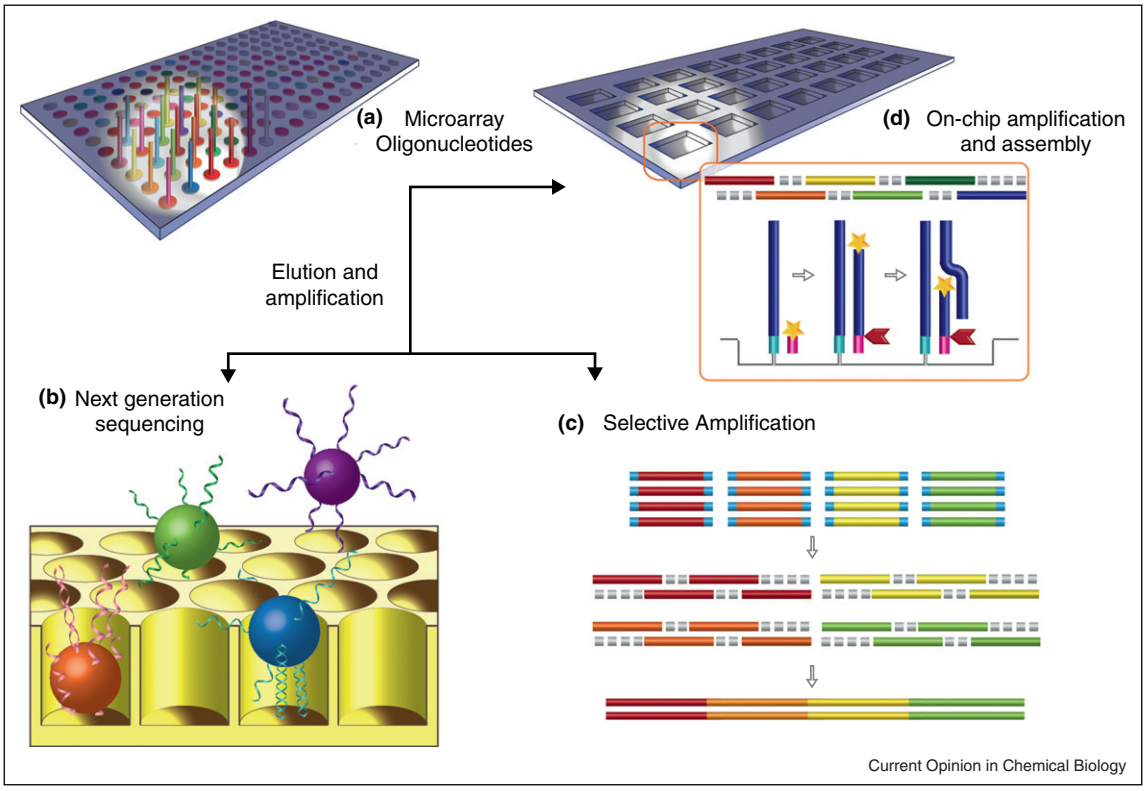
Traditionally, column-synthesized oligos are used as building blocks for gene construction using either DNA polymerase based [4] or DNA ligase based assembly methods [5]. More detailed evaluations of current gene assembly technologies can be found in recent reviews [6–9]. However, the high cost and limited throughput of column-based oligo synthesis has become a bottleneck for large-scale gene synthesis and genome assembly projects in the new synthetic biology era.

Microarrays have recently attracted attention as an inexpensive source of mixed pools of oligonucleotides. Synthesis on microarrays allows large numbers of unique oligo sequences to be generated in a miniaturized and parallel fashion and offers advantages in throughput, reagent consumption and cost (reviewed in [7]). Following the early demonstrations of gene assembly using microarray-derived oligo pools [10–12], the past couple of years witnessed exciting new developments in improving the quality, efficiency, and automation of microarray-based oligo synthesis and gene assembly. These new developments are summarized in Figure 1.

There exist a number of challenges in adapting microarray technology for gene synthesis. One significant challenge is the relatively low quality of microarray-generated sequences. Oligos synthesized on planar surfaces are more prone to errors and usually have higher error rates than column generated ones. One cause was attributed to 'depurination' from prolonged exposure to deprotecting/detritylation agents. By optimizing reagent flows and reaction conditions, Leproust's group at Agilent Technologies has developed procedures that allow high-fidelity synthesis of oligo pools up to 200 bases [3]. Another cause was due to the so-called 'edge effects' in microarray synthesis. Microarray synthesis typically relies on certain mechanisms to direct and spatially confine reactions on a silica chip. For example, Agilent uses inkjet-printing method to dispense picoliter reagents to specified locations on a chip. LC Sciences and Affymetrix control the deblocking step with light-activated photochemistry in a microfluidics system. CombiMatrix uses programmable microelectrode arrays to direct redox reactions at desired spots. In these cases, sequence integrity can be compromised by the 'edge effects' caused by droplets misalignment, light beam drifts or improper reagent sequestration. Such 'edge effects' can be minimized. In a study involving an inkjet chip synthesis platform, a plastic chip with patterned silica features was used to reduce the 'edge effects' and could effectively reduce the error rate of synthetic oligonucleotide pools from approximately 1 in 200 bases to 1 in 600 bases [13], which was comparable to column synthesized oligos.

Besides improving microarray quality, next-generation sequencing (NGS) has recently been used as a preparative tool to generate sequence-verified chip-made oligos for gene assembly (Figures 1b and 2) [14••]. Microarray-derived oligos are fed into a NGS instrument where

Figure 1



Gene assembly strategies from microarray-derived oligonucleotides. (a) Oligonucleotides synthesized on a microarray are less expensive, but the high heterogeneity and error rate requires appropriate retrieval and segregation technologies to assembly them into gene constructs. (b) Next generation sequencing was used to identify error-free oligo sequences for gene assembly. (c) Selective amplification of oligonucleotides from the pool. (d) Physically dividing a microarray into isolated subarrays. Oligonucleotides are amplified and assembled into gene fragments on chip within each reaction well.

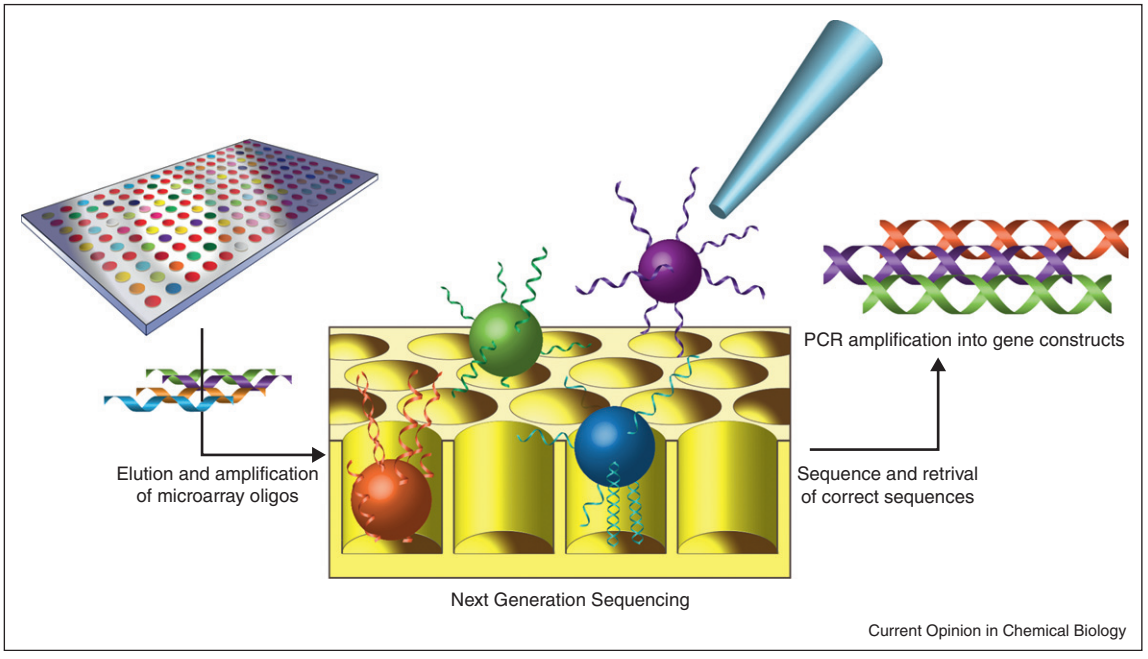
correct sequences were identified and retrieved. This strategy yielded an estimated 500-fold improvement in sequence accuracy [14••]. With further automation to reduce the number of human handling steps, millions of oligos can be sequenced and sorted in a single run, potentially enabling the construction of megabases of DNA sequences. Though translating this technology into routine practice may still require much optimization, the concept of integrating NGS and synthesis is attractive and promising.

Another challenge of utilizing microarray for gene assembly is increasing the efficiency and accuracy of gene assembly with chip-derived oligo pools. Typically, tens to hundreds of thousands of oligos can be generated from a single microarray. When such large numbers of oligos are amplified and collected from the surface, the resulting complex heterogonous pool makes subsequent gene assembly challenging and inefficient. A number of strategies have been developed recently to solve this problem. In one strategy, by applying oligo hybridization-selection principles [10], Borovkov et al. carefully optimized oligo design and gene assembly conditions to exclude erroneous oligos from incorporation into assemblies [15]. A block-based approach was developed to eliminate the expensive and labor-intensive oligo purification and amplification steps in preparation for gene assembly. Using this approach, genes could be directly assembled from unpurified pools of microarray-derived oligos which contained less than 5% perfect sequences.

Another approach to increase gene assembly efficiency is selective amplification of subpools of oligos from a chip. Kosuri et al. [16••] demonstrated this strategy by designing a quarter-million short primers that allow for the specific amplification of selected fragments for subsequent assembly (Figures 1c and 3). Error-free gene assembly of 40 single-chain antibody genes was demonstrated which had been shown previously to be difficult to synthesize due to high GC content and repetitive sequences [16••].

To reduce oligo pool size for efficient gene assembly, a third approach is to physically divide the microarray into subarrays. Our group tested this strategy by embossing up to 30 micro-wells on the surface of a single plastic chip

Figure 2



Schematic illustration of oligonucleotide error-correction by next generation sequencing. Microarray synthesized oligonucleotides are eluted and amplified before they were fed into a 454/Roche pyrosequencing instrument. The desired sequences are identified, sorted and retrieved with a microactuator-controlled micropipette, and PCR-amplified into full-length gene constructs [14••].

(Figures 1d and 4). A subarray was synthesized in each well and used to assemble a single gene construct.

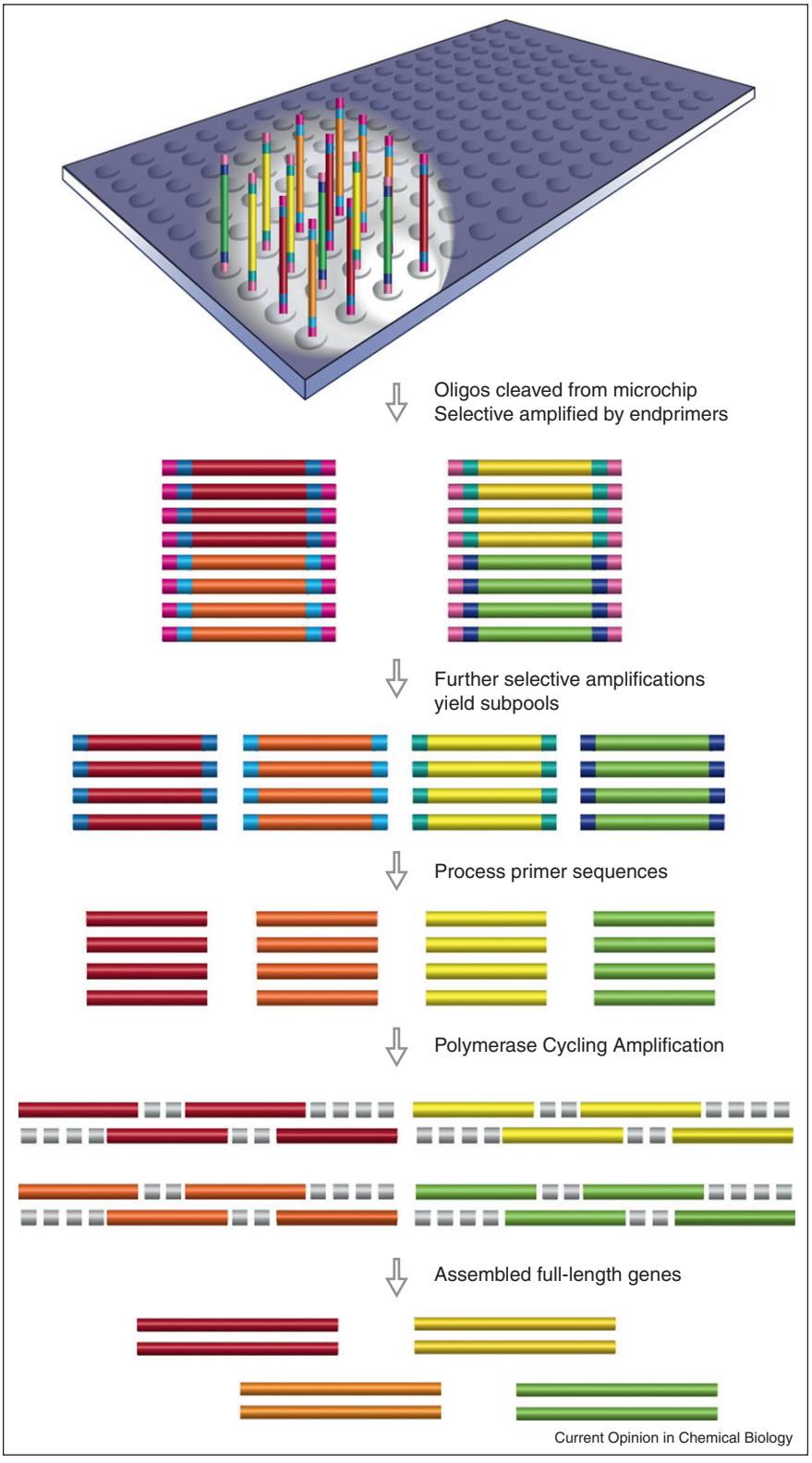
Oligo pools produced from microarray provide a cheap source for oligos but do not simplify or reduce the cost of downstream gene assembly process. In many cases, large complex oligo pools make gene assembly more challenging and prone to error. To address this issue, we integrated array oligo synthesis, amplification and gene assembly steps in individual wells on the same chip [17••,18]. First, instead of chemically cleaving oligonucleotides off the chip for gene assembly, we developed isothermal nicking and strand-displacement amplification reactions to amplify and release overlapping gene-construction oligonucleotides into the sealed wells. Then without switching buffer or hands-on manipulations, polymerase cycling assembly reaction takes place in the same cells to construct gene sequences, currently up to 1 kb each (Figure 4). The entire procedure is performed on-chip to increase throughput and minimize turn-around time and spurious hybridization. This platform can be coupled with downstream reactions to achieve miniaturized, automated high-throughput synthesis.

Long DNA synthesis

Constructing long DNA sequences beyond the lengths of individual genes need to address a different set of challenges. Besides traditional restriction enzyme digestion and ligation methods, genes can be linked together by the BioBrick™ [19] or BglBrick [20] based methods where standardized flanking sequences containing restriction sites allow for the pairwise joining of components to sequentially larger constructs. While efforts have been made to revise, standardize and automate the process [21,22], failure to deliver scar-free assemblies has restricted both BioBrick™ and BglBrick to generic pathways that can tolerate car sequences. More importantly, restriction enzyme digestion is often incompatible with longer constructs due to the limitation of 'inhibitory sequences' within the constructs. Several recent attempts using type-II restriction enzymes that cut around their recognition sequence have effectively mitigated the problem [23–26]; they are nevertheless laborious due to the sequential nature of the approaches.

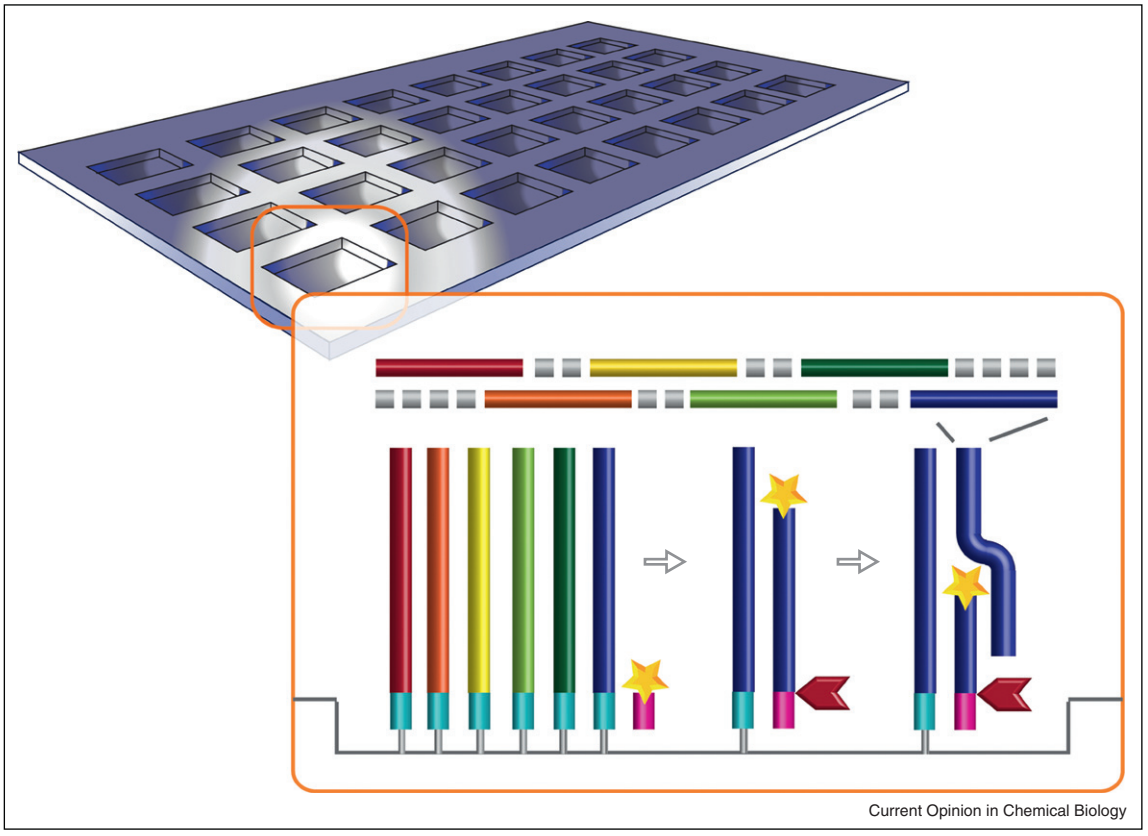
Alternatives to restriction/ligation methods include a number of overlapping extension techniques capable of scar-less and sequence-independent assembly. In a PCR reaction, homologous ends join adjacent DNA molecules together, and instantaneously prime their amplification for the next cycle. Circular polymerase extension cloning is a simple method that has been demonstrated successfully with both multi-way parallel assembly and combinatorial library construction [27,28]. Additional overlapping approaches include In-Fusion (commercial kit from Clontech®, CA) [29], Uracil-specific excision reagent (USER) [30] and sequence-independent and

Figure 3



Schematic illustration of gene assembly by selective amplification of microarray oligonucleotides. Oligonucleotides are eluted from the microchips to yield a heterogeneous pool. Specific short PCR primers are designed to selectively amplify oligonucleotides from the pool (hot and pale pink). Further selective amplification with end primers (shades of blue) generates assembly pools containing sequences for a single gene, which are then assembled in to gene constructs by PCR reactions [16••].

Figure 4



Schematic illustration of on-chip DNA amplification and gene assembly. The microchips are divided into physically isolated subarrays where oligonucleotides are amplified by on-chip strand displacement amplification. The released strands are assembled in to 0.5–1 kb gene fragments within the wells [40].

ligation-independent cloning (SLIC) [31]. These approaches are more applicable for plasmid or small pathway construction due to the drop in efficiency and rise in error rate of PCR reactions as product size scales up. Gibson isothermal assembly is exceptional in that it allows for assembly of genome-length constructs reaching hundreds of kilobases [32]. Similar approach has also been applied to the construction of a 16.3-kb mitochondrial genome directly from column-synthesized 60mers [33].

Despite numerous advancements, *in vitro* gene assembly methods seem to have reached a limit of how long a DNA sequence it can assemble. For more efficient assembly of longer constructs from multiple parts, attention has been turned to *in vivo* homologous recombination in yeast *Saccharomyces cerevisiae* thanks to its adaptability to very long constructs and accuracy as a result of sophisticated DNA repair mechanisms. Successful *in vivo* assembly examples include syntheses of 0.5–1 Mb bacteria genomes [34,35,36••], direct assembly from overlapping oligonucleotides [37] and various genetic pathway-engineering projects [38].

Error correction

Despite extensive attempts to remove errors in synthetic oligos through improved chemistries [3], stringent hybridization selection [10,15] or simply exhaustive purification, trace errors still carry over into the assembly process and accumulate in downstream gene constructs. Various enzymatic methods have therefore been developed to reduce errors at this stage, relying on either mismatch-binding or mismatch-cleavage mechanisms. A comprehensive elaboration on error-correction in synthetic gene technologies can be found in a recent review [39•].

Noticeably, two recent microarray mega synthesis studies both incorporated robust quality-control procedures using CEL-based mismatch specific endonucleases to significantly reduce error rate [16••,40]. In both cases, subpopulation of erroneous sequences are cleaved and eliminated while the remaining error-free populations are reassembled and enriched. In a recent study, two iterations of error-correction reduced the error rate by 16-fold to one in 8701 bp [18].

Applications in synthetic biology

The applications of synthetic DNA encompass a broad spectrum ranging from genetic circuits and metabolic pathways to synthetic genomes [33,41]. An expanding capacity to construct, manipulate and analyze DNA delivers the power to design, manipulate or even create artificial living systems. Scientists at the J. Craig Venter Institute performed *de novo* synthesis and assembly of a million base pair bacterial genome which, when placed inside a bacterial cell host devoid of its own genetic material, was able to successfully generate an artificial living cell [42]. This accomplishment marked a new milestone in synthetic biology and offered a potential framework for further developments.

Getting the genomic software to boot up is only the beginning of an extensive bioengineering process. The true design and engineering of any genome is still at its infancy. Even modest errors in genome construction could cause the genetic system to lose functionality. Very soon, the true limiting factor is not the ability to synthesize DNA, but the lack of a comprehensive understanding of the complex biological processes. In this sense, the synthesis of genomes will allow researchers to learn before they are ready to design.

A valuable domain of synthetic biology is metabolic engineering. By engineering parallel metabolic systems that interface with natural cellular metabolic machinery, researchers can program cells for practical applications including the synthesis of cost effective chemicals or drugs [43–45]. In one demonstration by Keasling and colleagues, the construction of a biosynthetic artemisinin pathway in yeast enabled the microbial production of this antimalarial compound at one-tenth of the cost of conventional production method of harvesting from the rare *Artemisia annua* plant [46].

The increasing fidelity of microchip-based synthesis gives researchers the opportunity to directly utilize complex libraries of oligonucleotides. Wang *et al.* developed a high-throughput genome prototyping system, called multiplex-automated genome engineering (MAGE) [47]. They achieved five times higher expression of a target protein by allowing the bacteria to receive a library of 'editing' DNA strands designed to target and manipulate multiple genome sites simultaneously. A more recent study adapted MAGE to perform site-specific synonymous codon substitutions at 314 sites in parallel across 32 *E. coli* strains. When combined with hierarchical conjugative assembly genome engineering (CAGE), codon modifications were applied across the entire genome [48]. This genome reengineering technology allows for simultaneous editing and evolution.

Another interesting study exploited the complexity and heterogeneity of microarray synthesized DNA. Quan *et al.* applied their on-chip gene synthesis method to codon optimize protein expression. A library of synonymous codon variants coding for the same gene was synthesized on-chip. Highly expressed codon variants were identified by expression screening. These constructed libraries were used to optimize expression of 74 *Drosophila melanogaster* transcription factors, which demonstrated the robustness of this technology [40].

Perhaps the most amenable target for whole genome synthesis with current technology is viral genomes. Viral genomes are relatively small and are good engineering targets for vaccine development. While others strive to optimize codon usage for higher gene expression in heterologous hosts, vaccine developers apply an opposite strategy, producing live attenuated forms of viruses by global codon de-optimization. Coleman and colleagues developed a process termed SAVE (synthetic attenuated virus engineering) which perturbs intrinsic viral genome bias through large-scale computer-aided viral genome redesign [49]. They recently demonstrated effective vaccine candidates with SAVE attenuated influenza viruses [50]. With rapid improvement in DNA fabrication technology, the redesign and synthesis of custom tailored whole virus genomes could become economically feasible.

Conclusions

Over the past four decades, *de novo* chemical DNA synthesis and enzymatic gene assembly technologies have continuously improved. The capacity to generate DNA assemblies increased from less than 100 bp to over 1 Mb [33,42]. Synthetic DNA is applied to a broad range of applications in metabolic engineering, gene circuit design and genome synthesis [33,45,46,51]. However, existing DNA fabrication technologies are still compromised by low throughput or high error rate, and are not mature enough for the convenient and economical engineering of large genomes. Continuing interdisciplinary efforts are being made to utilize new chemistries and strategies for DNA synthesis and gene assembly. Such efforts would facilitate the wide spread and economical construction of complex libraries and genomes, capabilities that could lead to significant impacts on research and society.

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- •• of outstanding interest

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