Published in final edited form as:

Wiley Interdiscip Rev Syst Biol Med. 2016 January ; 8(1): 5–21. doi:10.1002/wsbm.1319.

Accelerated Genome Engineering through Multiplexing

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Abstract

Throughout the biological sciences, the past fifteen years have seen a push towards the analysis and engineering of biological systems at the organism level. Given the complexity of even the simplest organisms, though, to elicit a phenotype of interest often requires genotypic manipulation of several loci. By traditional means, sequential editing of genomic targets requires a significant investment of time and labor, as the desired editing event typically occurs at a very low frequency against an overwhelming unedited background. In recent years, the development of a suite of new techniques has greatly increased editing efficiency, opening up the possibility for multiple editing events to occur in parallel. Termed as multiplexed genome engineering, this approach to genome editing has greatly expanded the scope of possible genome manipulations in diverse hosts, ranging from bacteria to human cells. The enabling technologies for multiplexed genome engineering include oligonucleotide-based and nuclease-based methodologies, and their application has led to the great breadth of successful examples described in this review. While many technical challenges remain, there also exists a multiplicity of opportunities in this rapidly expanding field.

Keywords

genome engineering; multiplexing; MAGE; ZFN; TALEN; CRISPR/Cas9

INTRODUCTION

Living organisms from bacteria to humans represent the most complex systems on earth, which despite centuries of research effort, still elude a complete understanding by scientists. Synthetic biologists harness the principles of the central dogma to engineer biological systems for improved and novel functions.^{1–4} Specifically, a desired phenotype can be generated by deleting or incorporating the corresponding genetic elements. Techniques enabling the precise alteration of genomic DNA sequences *in vivo*, termed as “genome

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In addition, we state that there is no conflict of interest.

engineering”, have been substantially developed in recent years and pushed the frontiers of cell biology, gene therapy, and industrial and agricultural biotechnology. Nonetheless, genome engineering in the early days was challenging, largely due to the inefficiency of introducing single-site mutations and inability to rapidly alter targeting sequences at will, which ultimately led to a high cost. As a result, conventional techniques usually target one locus on a genome in a single round, which severely limits the turnover of the design-build-test cycle.⁵ While this does not typically pose a problem for generating single modifications, such as correcting a point mutation associated with a monogenic disease,⁶ it does make combinatorial testing of multiple genome modifications unfeasible, which is usually required in strain and cell line development and complex disease modeling.^{7, 8}

Within this context, there exists an urgent need for tools enabling multiplexed and high-throughput genome engineering. To avoid possible confusion, we define “multiplex” as generating altered DNA sequences in two or more loci within a single genome in a single round of mutagenesis (Fig. 1a). As a result, population-based genome engineering with one modification per cell (Fig. 1b) will not be considered in this review. Besides, epigenome and transcriptome engineering (including RNA editing and regulation of gene expression levels) that do not introduce a change in DNA sequences will not be discussed. Readers who are interested in these topics are referred to other recent reviews.⁹⁻¹²

In this review, we will focus our discussion on recent technological advances in multiplexed genome engineering in various organisms, mostly from the past four years. We will first introduce oligonucleotide-mediated multiplexed genome engineering via homologous recombination in microbes. We will then move on to nuclease-mediated multiplexed genome engineering, with or without homologous recombination, in microbes as well as higher order organisms. Specifically, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered, regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) will be discussed. Finally, we will conclude by offering our perspectives on current challenges and future developments in the field of multiplexed genome engineering.

OLIGONUCLEOTIDE-MEDIATED MULTIPLEXED GENOME ENGINEERING

In bacteria, homologous recombination has been utilized as a robust mechanism for genome engineering.¹³ It was first demonstrated at the end of 20th century that phage-derived proteins can be used to replace DNA fragments in *Escherichia coli*, through the homologous recombination mechanism.¹⁴⁻¹⁶ Mechanistically, with the help from heterologous-expressed RecE/RecT or λ -*red* system, single-stranded DNA oligonucleotides harboring deletions, insertions or mismatches anneal to the lagging strand during DNA replication, resulting in the incorporation of customized sequence modifications in the newly synthesized genome (Fig. 2). This technique is termed as recombination-mediated genome engineering, or recombineering. Experimentally, multiple oligonucleotides can be transformed simultaneously into a single cell to allow editing of multiple loci at the same time.

The burst of multiplexed recombineering came in 2009 when the Church laboratory developed a high throughput multiplexed engineering methodology in *Escherichia coli*,

termed as Multiplex Automated Genome Engineering (MAGE).¹⁷ The relatively high efficiency of recombineering in MAGE makes it possible to omit selections for modified mutants, resulting in a highly diversified mutant pool where single cells can have multiple edits across the genome. The first example using MAGE successfully increased lycopene production by more than five-fold in *E. coli*, through optimizing the 1-deoxy-D-xylulose-5-phosphate (DXP) biosynthesis pathway.¹⁷ MAGE was also used to genetically recode the *E. coli* genome, replacing all the TAG stop codons with TAA codons.^{18, 19} The released TAG codon was further repurposed as a dedicated sense codon for synthetic amino acids and was introduced into 22 essential genes in the *E. coli* genome by MAGE, generating recoded organisms that are safe for use in the natural environment.²⁰ Of note, to enable multiple edits (typically more than 10) within a single cell, conjugative assembly genome engineering (CAGE) was used together with MAGE to merge different sets of edits through horizontal transfer. Besides random mutations and codon replacement, MAGE has also been used to introduce more focused functionalities such as promoter sequences. In one study, a MAGE variant called 'coselection' MAGE (CoS-MAGE) was used to combinatorially insert multiple T7 promoters simultaneously into 12 genomic operons, enabling the study of gain-of-function modifications and epistatic interactions within gene networks.²¹ Due to its popularity, other studies focusing on improving the efficiency²²⁻²⁴, *in silico* design²⁵ and reducing the cost²⁶ of MAGE have also been described.

Although groundbreaking, applications of MAGE are largely restricted to the model microorganism *E. coli*. Its success is not easily transferred to other microbial systems because of the inefficiency of homologous recombination using short oligonucleotides in other species. For instance, Yeast Oligo-Mediated Genome Engineering (YOGÉ) method used a similar strategy as MAGE, but in *Saccharomyces cerevisiae*.²⁷ After strain engineering, including knocking out mismatch repair proteins, overexpressing DNA recombinases, and optimization of transformation and oligonucleotide design, frequencies of recombination in yeast was significantly improved. YOGÉ was then applied to simultaneously modify two loci in the yeast genome in a cycled manner. Although successful, the efficiency of double modifications falls below 0.01% after three cycles, despite the use of the best performing oligonucleotides. Phenotypic screening or site-specific nuclease-mediated negative selection may be coupled with YOGÉ to further increase the efficiency of multiplexed yeast genome engineering.²⁸

Another study explored the possibility of multiplexed genome engineering in naturally competent microorganisms. Natural competence refers to the ability of diverse microbial species to take up DNA from the environment and integrate it into the genome by homologous recombination.²⁹ Taking advantage of natural competence, a multiplexed genome engineering method called Multiplex Genome Editing by Natural Transformation (MuGENT) was developed.³⁰ The effectiveness of this method was demonstrated by combinatorially editing the *Vibrio cholerae* genome via gene deletions, promoter replacements and tuning translation initiation of five genes to optimize the natural transformation trait. MuGENT was further used to simultaneously edit four redundant genes in another species, *Streptococcus pneumoniae*, lacking mismatch repair, demonstrating its broad utility as a multiplexed genome engineering tool.

TOOLS FOR NUCLEASE-MEDIATED MULTIPLEXED GENOME ENGINEERING

It is a well-known phenomenon that the introduction of a double-strand break (DSB) to the chromosomal DNA of a living cell significantly stimulates DNA repair functions,³¹ primarily through either non-homologous end-joining (NHEJ) or homologous recombination (HR). This phenomenon is unsurprising from an evolutionary perspective, as DSBs can be deleterious or lethal to the organism if unaddressed. From an engineering perspective, the stimulated repair response can be exploited as a means to prime specific loci of interest for genome editing events. In other words, by targeting a DSB to a precise chromosomal location, that location can become a “hotspot” for DNA sequence revision. Modification of the target site can then be achieved either via error-prone NHEJ or through HR by supplementing an editing template *in trans* that dictates the desired remodeled sequence.

While agents such as radiation or chemical mutagens can be used to introduce chromosomal DSBs *in vivo*, practical application of DSB-mediated genome editing necessitates precise control over the specific breakage point. This level of control can be afforded by endonucleases, enzymes that recognize specific DNA sequences and cleave within or near their recognition site. However, the most commonly used and well-studied restriction endonucleases (of the Type II class) recognize sequences that are too short to provide uniqueness in the total genomic context. As a result, additional cleavage events would occur at undesired loci bearing the same recognition sequence as the desired target, rendering these endonucleases impractical for all but the smallest genomes. Homing endonucleases (HEs), in contrast, recognize much longer DNA sequences, typically 14 – 40 nucleotides in length.³² This length typically satisfies the requirement for uniqueness, even in large metazoan and plant genomes. The key limitation, though, is that HEs have proven to be recalcitrant to extensive reprogramming of their DNA recognition sequences. HEs can be envisioned as bespoke enzymes, made to measure over evolutionary time for a particular recognition sequence. Consequently, DNA recognition and cleavage activity are difficult to decouple, as both are intricately interwoven in the overall protein fold. HE-mediated genome engineering is thus limited to cases where a wild type HE scaffold can be identified with sequence specificity that already closely matches the desired target^{32, 33}

For practical application of endonucleases in multiplexed genome engineering, what is needed is a set of “off-the-shelf” DNA recognition modules that can readily be assembled to generate custom endonucleases with designer specificity (Fig. 3). At the protein level, this modularity was first discovered in zinc finger (ZF) structural motifs, each of which can recognize a specific 3-nucleotide triplet (Fig. 3a).³⁴ Later on, the specificity code of the transcription activator-like effector (TALE) repeat domains, each of which recognizes a specific nucleotide on a 1:1 basis (Fig. 3b), was deciphered, further expanding the DNA recognition toolbox.^{35, 36} By fusing a set of DNA recognition domains with a nonspecific nuclease domain (such as that of the FokI endonuclease), these DNA binding proteins can be repurposed as site-specific endonucleases – namely ZFNs and TALENs. Most recently, the CRISPR-associated protein Cas9 has been demonstrated as perhaps the most versatile reprogrammable endonuclease. Its DNA sequence specificity is conferred by a short RNA

sequence with complementarity to its recognition site. Multiple short RNA sequences can be transcribed either separately or as a nuclease-cleavable polycistronic array (Fig. 3c).³⁷⁻³⁹ Thus, targeting Cas9 to multiple loci *in vivo* requires only the transcription of the corresponding guide RNA sequences, rather than construction of entirely new multi-domain proteins. As a result, though ZFNs and TALENs continue to see applications for multiplex genome engineering, it is Cas9 that has largely become the tool of choice for multiplexed genome editing.

By targeting multiple genomic loci simultaneously, three general classes of genome modifications are possible that would be difficult, tedious, or impossible to generate via single-locus targeting (Fig. 4). The first can be termed multi-allelic modification (Fig. 4a), in which a single engineered nuclease is employed to target multiple alleles in a multiploid organism. Though conceptually synonymous with single-locus editing (one nuclease/one target), the demands placed on editing efficiency make this a non-trivial extension of the single-locus platform. Further, the simultaneous generation of homozygous mutations provides clear advantages over alternative methods that would require screening of a population for the desired heterozygous mutation, followed by breeding and screening of progenies for the desired homozygous genotype. The second class involves deployment of multiple nucleases to enact simultaneous independent editing events (Fig. 4b). Again, this approach greatly facilitates the genome engineering process compared to single-locus methods that would require sequential selection or screening for each mutation of interest, often paired with intermediate counter-selection to remove selectable markers. Finally, the third class involves the concerted action of multiple nucleases to achieve outcomes inaccessible to single-locus targeting (Fig. 4c-4f). These outcomes include the manipulation of large fragments of chromosomal DNA, including excision, inversion, and translocation.

APPLICATIONS OF NUCLEASE-MEDIATED MULTIPLEXED GENOME ENGINEERING

Microbes

In bacterial systems, nuclease-mediated multiplexed genome engineering was only recently brought into play with the rapid development of the Cas9-based platform. This is perhaps due to the relative ease with which the most widely studied bacterial hosts can be sequentially manipulated by recombineering, diminishing the time- and effort-saving benefits of multiplexing. Nevertheless, Cas9-mediated bacterial genome editing has been demonstrated, starting with a landmark study in *Streptococcus pneumoniae*.⁴⁰ Here, the authors leveraged the native CRISPR/Cas system of the host by introducing an engineered CRISPR array containing two spacers and a selection marker, along with two editing templates dictating the desired mutations. Following co-transformation of all three elements, genotyping confirmed that 6/8 transformants were correctly editing at both loci, while the remaining two were edited at only one locus.

More recently, heterologous Cas9-mediated multiplexed genome editing has been demonstrated in *Streptomyces* spp.⁴¹ and *E. coli*.⁴² In *Streptomyces lividans*, two guide RNA (gRNA) cassettes and two editing templates were assembled into a shuttle plasmid

containing a codon-optimized *cas9* gene.⁴¹ Following conjugative transfer of the plasmid from an *E. coli* donor, four exconjugants were genotyped, and all were found to contain both desired short deletions. This approach was also demonstrated for the deletion of full gene clusters in *S. lividans* and *Streptomyces albus* by supplying gRNAs targeting both ends of the cluster along with a single editing template to bridge the gap between the two DSBs. In *E. coli*, a two-plasmid system was employed to simultaneously introduce double and triple deletions with efficiencies of 97% and 47%, respectively.³⁷ Additionally, the authors performed simultaneous deletion and insertion at separate loci with efficiency of 78%. Notably, their plasmid system included the λ -*red* *exo*, *beta*, and *gam* genes to provide the recombination rates necessary for efficient DSB repair, and longer homology arms (250 bp) were found to significantly enhance efficiency relative to short arms (40 bp).

In the model eukaryotic microbe *S. cerevisiae*, multiple groups have recently explored the multiplexing potential of Cas9. As an intrinsic benefit of this host, naturally high HR proficiency enables precision genome editing with relatively short editing templates (100 bp). For example, in the Homology-Integrated CRISPR/Cas (HI-CRISPR) system, editing templates are directly embedded with the spacer sequences in a CRISPR array, enabling an all-in-one approach in which Cas9, multiple spacers, and multiple editing templates are encoded on a single plasmid.⁴³ As a demonstration of this platform, two sets of three genes each were simultaneously disrupted with 8 bp deletions, with efficiencies ranging from 27% to 100%. In an alternative approach, gRNAs were fused to a self-cleaving ribozyme sequence to protect their 5' ends from exonuclease activity.^{44, 45} Using this so-called Multiplex CRISPR (CRISPRm) approach, highly efficient barcoding was achieved for up to three alleles in haploid and diploid laboratory strains, as well as for a single allele in a polyploid industrial strain. Most recently, up to five genes were simultaneously disrupted via co-transformation of a plasmid containing five gRNA cassettes with five separate 90 bp double-stranded oligonucleotide editing templates.⁴⁶ Disruption efficiency of 100% was confirmed for the quintuple knockout by genotyping a small subset of transformants. Besides merely gene inactivation, integration of metabolic pathway genes into up to three different loci of the yeast genome has been realized by different groups,⁴⁷⁻⁴⁹ which broadened the application scope of CRISPR/Cas and facilitated metabolic engineering in this important host.

Although TALENs have primarily only been expressed in *S. cerevisiae* as a prototyping platform for eventual application in other organisms,⁵⁰ a recent study demonstrated their application for multiplexed yeast genome editing as well.⁵¹ Here, four plasmids (each containing one TALEN of a heterodimer pair) were simultaneously introduced to the cell in order to disrupt two acyl-CoA synthetases, with the goal of improving intracellular free fatty acid accumulation. Of note, while separation of the TALENs on multiple plasmids aids plasmid stability both in construction and *in vivo*, it limits the number of TALENs that can be simultaneously introduced based on the number of available orthogonal selection markers.

Plants

As TALEs were originally identified in plant pathogens, it is logical that TALEN-mediated multiplexed genome editing has been attempted in plants. In *Oryza sativa*, TALENs were used to generate NHEJ-induced disruptions in the binding elements for pathogenic TALEs located in the promoter region of a blight susceptibility gene.⁵² Following introduction of TALEN pairs via *Agrobacterium*-mediated transformation, the progeny of primary transgenic plants were screened. In total, 18/53 genotyped plants contained biallelic mutations, of which 10 were homozygous. Expanding to greater numbers of targets has also led to success, but with predictably lower efficiency. For example, three pairs of TALENs were introduced via bombardment to *O. sativa* to disrupt three separate genes with the goal of improving rice fragrance and grain yield.⁵³ Screening of 207 plants revealed that only four were disrupted in all three positions, and none of them were homozygous at all three loci. In an allohexaploid wheat, three nearly identical copies of a mildew resistance locus were targeted with a single pair of TALENs.⁵⁴ Although only 27 plants were subsequently screened, one was identified to be heterozygous at all three loci. After rounds of self-pollination, triple homozygous progeny could be obtained, revealing the desired disease-resistant phenotype.

Cas9-mediated multiplexing has been evaluated in a variety of plants, including not only the model system *Arabidopsis thaliana* but also cereal crops such as corn and rice. In *A. thaliana*, when a single gRNA was employed to target two synonymous loci, a low mutation rate was observed at both sites, and presence of the desired double mutant was not confirmed.⁵⁵ Similarly, when two gRNAs were deployed to target the same gene, deletion of the region between the two loci was only observed in 5/91 genotyped transformants. However, in a later study, a significantly higher efficiency was observed when two gRNAs were used to target genes *CHL1* and *CHL2*, eliciting the anticipated albino phenotype of a double mutant in 24/36 plants.⁵⁶ Further, targeting three nearly synonymous loci with two gRNAs elicited the phenotype of a double or triple mutant in 26/33 members of the T1 generation; notably, though, the diversity of mutations found in a given transgenic plant suggested that editing had occurred after division of the fertilized egg. In *Zea mays*, chromosomal deletion was attempted by deploying two adjacent gRNAs, resulting in the expected deletion in 12/20 screened plants.

In *O. sativa*, multiplexed Cas9-mediated editing has proven to be very efficient. In one study, biallelic mutations were detected in 3/9 plants following bombardment of calli with plasmids containing a codon-optimized *cas9* and a gRNA.⁵⁷ In another study, biallelic editing with a codon-optimized *cas9* was observed with efficiencies 87%.⁵⁸ Notably, the efficiency dropped significantly when the gRNA was replaced with a tracrRNA/crRNA configuration. Introduction of two distant gRNAs was employed as a means to delete chromosomal fragments ranging from 115 – 245 kb in rice protoplasts. While the desired large deletions could be confirmed by PCR, sequencing of plants generated from transformed calli revealed deletion of the large fragment from one chromosome only, with mutation of the gRNA target sites on the homologous chromosome. Elsewhere, deployment of one gRNA targeting up to four homologous genes enabled six triple mutants to be identified from 13 regenerated rice plants, including five with triple mono-allelic mutations,

and one with two mono-allelic mutations and one bi-allelic mutation.⁵⁹ Note that the fourth potential target for this gRNA was unmodified in all plants screened, likely due to a single nucleotide difference close to the 3' end of the spacer that abrogated targeting. Most recently, an ingenious method to introduce many gRNAs in a single transcript was demonstrated in rice.³⁹ In this example, up to eight gRNAs were fused together with interstitial tRNA coding sequences as so-called polycistronic tRNA-gRNA (PTG) genes. This setup enables the plant's native tRNA processing machinery to splice out the individual gRNA coding sequences, which can then be used for multiplexed targeting. In one example, deployment of 8 gRNAs (four pairs targeting adjacent sites in four genes) in protoplasts enabled 4 – 20 % deletion efficiency between the two targeted sites in any single gene; individual clones, however, were not screened for the possible quadruple deletion. In intact plants, two-target PTGs enabled biallelic editing in up to 76% of screened plants, and an eight-target PTG created biallelic mutations in at least 5 loci in 7/14 plants, with mutations confirmed at all eight sites.

Metazoans

The importance of metazoans in diverse research areas, such as functional genomics and disease modeling, along with the time and labor intensity of their genetic manipulation, have motivated significant exploration of multiplexing technologies in these organisms. ZFNs, for example, have been introduced to CHO cells to create biallelic knockout lines. In a proof of concept study, three sites were targeted, and all three biallelic knockout events were observed at frequencies of >1 % without the use of selection.⁶⁰ The TALEN technology has been explored in a variety of metazoans, although largely as proofs of concept. In the silkworm *Bombyx mori*, two adjacent TALEN pairs were introduced to generate a deletion of 792 bp, and 14% efficiency was observed in the germline of the G1 brood.⁶¹ Taking this approach further, deletions, duplications, and inversions of an 8.9 Mb chromosomal fragment were observed when two distant TALEN pairs were introduced, although the specific efficiency of these events was not quantified.⁶² Small and large deletions have also been demonstrated by similar means in zebrafish. In one example, non-coding RNA clusters ranging from 1.2 kb to 79.8 kb were deleted with efficiencies ranging from 1.9% – 4.2%.^{63, 64} In another study, deletions of 200 bp – 4.2 kb could be introduced with 1 – 15% efficiency.⁶⁵ Note, however, that efficiency was not always directly proportional to deletion size. Deletions of 39 kb or 69 kb could be introduced with 3.2 and 4.9% efficiency, respectively, and a 5.5 Mb deletion (introduced by a combination of one ZFN and a TALEN pair) was introduced with 0.7% efficiency. Inversion events were also observed in several deletion experiments, but with lower efficiency as compared to the corresponding deletion events (<2%).

In the frogs *Xenopus laevis* and *Xenopus tropicalis*, eight single genes were targeted with TALENs, all of which exhibited 61.9 % efficiency.⁶⁶ Although not specifically measured, the high efficiencies observed at each site suggest that multiallelic editing has occurred in a subset of the transformed embryos. Of note, this study directly compared the TALENs to ZFNs, the latter of which exhibited greater toxicity at comparable doses and lower efficiency at lower doses. A subsequent study targeted three genes (two tyrosinase paralogs and EGFP) with two TALEN pairs or two genes (histone chaperone paralogs) with two

TALEN pairs, resulting in 40 – 90 % disruption efficiency at each locus.⁶⁷ Note that in this study, mutations that did not result in a frameshift (e.g., 6 bp deletion) were not counted as effective disruptions. The frequency of simultaneous editing at all targeted loci was not directly reported. In livestock, biallelic mutations have been introduced via injection of TALEN mRNA to bovine and porcine embryos.⁶⁸ With optimization of mRNA dosage, 2/4 bovine embryos and 6/56 porcine embryos (from 214 total, after an initial screen for EGFP fluorescence as an injection control) displayed biallelic mutation at the targeted locus. Further, five of six TALEN pairs tested in fibroblast culture showed biallelic modification in up to 17% of selected colonies. Deletion and inversion of a 6.5 kb region could be achieved with efficiencies of 10% and 4%, respectively, and modified fibroblasts could subsequently be used as nuclear donors for cloning.

Not surprisingly, the greatest attention for multiplexing in metazoan systems has been paid to Cas9. Though primarily in the proof-of-concept phase, results obtained so far have largely justified this interest. In the fruit fly *Drosophila melanogaster*, an optimized Cas9 toolkit was recently developed and shown to drive efficient biallelic editing in somatic and germline cells by either NHEJ or HR.⁶⁹ Supplying a donor sequence dictating insertion of *gfp* into the *wg* coding region, for example, led to GFP expression in 38% of the offspring of injected embryos. Targeting different loci in somatic cells enabled markerless biallelic editing suitable for phenotypic loss-of-function screening. Alternatively, by inserting the *cas9* sequence and a gRNA into the locus of interest, inheritance of the *cas9*-containing allele can be driven to near 100%, as recently demonstrated in a powerful gene drive system.⁷⁰ Such tools have clear applications in the engineering of entire insect populations, which could be a valuable means to prevent the spread of insect-borne diseases.

Cas9-mediated multiplexing has also been attempted in silkworms, with the goals of introducing deletions or simultaneously editing multiple loci. In one example, three gRNAs were employed to target three sites in the *BmKu70* gene.⁷¹ The efficiency, however, was quite low, with only 2/101 double-locus mutants and 1/101 triple-locus mutants observed. Two mutants were also observed with deletions between two of the targeted sites. In a second example, two gRNAs were shown to enable deletion or inversion of a 3 kb region, although the efficiency was not reported.⁷² To further test the limits of the system, ten gRNAs were deployed to target a total of six genes. While editing was observed at each locus, simultaneous targeting of each site in a single cell was not confirmed.

In zebrafish, while TALENs were largely explored for the introduction of small and large deletions, Cas9 based applications have focused more on the simultaneous introduction of small mutations in one or more targets. For example, using a codon-optimized *cas9* sequence, an efficiency of 75 – 99 % in single targeting of four endogenous loci and a reporter was observed, indicating a significant proportion of biallelic mutants.⁷³ Simultaneous targeting of all five loci also revealed high targeting efficiency at all five sites, and sequencing of a single embryo confirmed the presence of indels in each locus. Independent studies have also confirmed high single-locus editing efficiency,^{74, 75} demonstrated simultaneous targeting of five loci to edit three genes,⁷⁴ and shown deletion and inversion of a 7.1 kb fragment.⁷⁵ In *X. tropicalis*, high biallelic single-targeting efficiency with Cas9 was also confirmed, as was biallelic double-targeting modification.⁷⁶

Overall, the targeting efficiencies in *X. tropicalis* were comparable to those obtained with the TALEN technology.

In mammals and mammalian cell lines, numerous instances of Cas9-based multiplexing have been reported. In mice, a side-by-side comparison of TALENs and Cas9 showed that Cas9 was superior for the introduction of biallelic mutations.⁷⁷ In an early example in mouse embryonic stem cells, five genes were simultaneously targeted with five gRNAs, resulting in 10/96 clones modified by NHEJ at all eight possible alleles.⁷⁸ In mouse zygotes transformed with two gRNAs, 22/28 contained biallelic mutations. HR-mediated repair was also attempted, but with significantly lower efficiencies, suggesting NHEJ to be the dominant repair mechanism. In a separate study, three genes were targeted with three gRNAs in 60 embryos.⁷⁹ Of the nine pups obtained, all had mutations at all three loci, and 7/9 were mutated at all alleles. Here, a 95-base polyadenine tail was added to the *cas9* transcript to improve translation efficiency. Two-cut deletions could also be introduced using adjacent gRNAs; in mice, 3/12 co-injected embryos contained biallelic deletions between the two target sites.⁸⁰ An analogous experiment in rats yield only 1/15 rats separately disrupted at both targeted loci. In other studies, however, greater targeting efficiency in rats was achieved. For example, two gRNAs were designed to target the *Tet1* and *Tet2* genes, resulting in 15/24 rats mutated at both sites.⁸¹ A second set of two gRNAs targeting the same two genes yielded similarly high efficiency (15/20), with a significant proportion exhibiting biallelic mutations in both genes. Deploying two gRNAs each to target four genes simultaneously yielded 6/25 rats with mutations at all four loci. Finally, in CHO cells, Cas9 has been fused to GFP via a 2A linkage to enable an initial enrichment of Cas9-expressing transfected cells.⁸² In this experiment, 97 clones were deep sequenced after deployment of three gRNAs, resulting in 23/97 disrupted at two loci and 34/97 disrupted at all three loci.

Human cells

Compared to animal models, genome engineering in human cells provides a direct way to study human genetics and offers critical insights into the genesis of diseases. Abnormal genome rearrangements are hallmarks of complex diseases such as cancer. Thus, it is not surprising that the major application of multiplexed genome engineering in human cells is to treat or model complex diseases. Using nucleases for genomic deletion is a common proof-of-concept demonstration of multiplexed genome engineering. For example, ZFNs were used to generate deletions from several-hundred base pairs to 15 mega base pairs in human cancer cell lines, at frequencies from 10^{-3} to 10^{-1} .⁸³ ZFNs have also been engineered into nickases that only cleave one DNA strand in a DNA duplex, which is preferably repaired by the error-free homologous recombination pathway. Using two pairs of zinc finger nickases, which generated 4 chromosomal single strand breaks in homologous *CCR2* and *CCR5* loci, a 15 kb genomic deletion with precise junctions was created, though at low frequencies (0.01 % and 0.04 %).⁸⁴ Other than genomic deletion, ZFNs were also employed for generating chromosomal translocations, which are signatures of various cancers and lead to expression of oncogenic fusion genes. In one study, DSBs were induced at two loci on two chromosomes in both human embryonic stem cells (hES cells) and hES cell-derived mesenchymal precursor cells, leading to translocations at frequencies less than 10^{-6} .⁸⁵

Another study used ZFNs to model chromosomal translocations associated with Ewing sarcoma in hES cell-derived mesenchymal precursor cells.⁸⁶ Using an analogous strategy, TALENs were also used to model chromosomal translocations in anaplastic large cell lymphoma (ALCL) as well as to revert the ALCL translocations in a patient derived cell line. The overall reported efficiencies for chromosomal translocations using either ZFNs or TALENs in this particular study were between 10^{-4} and 10^{-2} . In castration-resistant prostate cancer cells, two TALEN pairs were introduced to generate intragenic androgen receptor gene rearrangements, including both deletion and inversion, with efficiencies not reported.⁸⁷

It is not surprising that CRISPR/Cas quickly became much more popular than ZFNs and TALENs in human cells, once again due to its ease in manipulation and avoidance of introducing multiple bulky proteins. The innate multiplexing nature of the crRNA expressing array was utilized for simultaneously editing *EMX1* and *PVALB* loci in human cells, by expressing two targeting spacers in a single crRNA expression cassette.⁸⁸ A 118 bp genomic deletion was also verified in 3 out of 182 amplicons after expressing two crRNAs targeting the *EMX1* locus. In another study, a 19 bp deletion was realized by expressing two gRNAs simultaneously, with efficiencies not quantified.⁸⁹ Due to its great promise in multiplexed genome engineering with more than just two edits, vector systems that allow simultaneous expression of multiple gRNAs on a single plasmid were also reported. For example, an all-in-one plasmid bearing two to seven gRNA expression cassettes can be sequentially assembled.⁹⁰ Editing efficiencies for an individual locus by a 7-gRNA plasmid varied from 4.3 % to 37.8 %, indicating the possibility of multi-locus editing. This vector system was also used to generate deletions, although the efficiencies were not reported. A second study assembled four gRNA expression cassettes through Golden Gate assembly into a single lentiviral vector, with each gRNA expressed under a different promoter.⁹¹ Using this vector, two loci in *IL1RN* and one locus each in *HBG1* and *AAVS1* were simultaneously edited. The individual editing efficiency of the four loci varied from 17.9 % to 33.3 % in HEK293T cells, and 4.8 % to 18.4 % in fibroblasts. Note that these efficiencies are comparable to those generated with single gRNA bearing vectors, again indicating the possibility of multi-locus editing. In dealing with diseases, CRISPR/Cas was utilized to generate a single large genomic deletion in Duchenne muscular dystrophy (DMD) patient myoblasts, which can correct up to 62% of DMD mutations.⁹² Although the efficiency of deletion was not quantified, this study showcased the potential of CRISPR/Cas in treating genetic diseases as well as cancers.

Factors affecting multiplexing capability

Thanks to the development of the aforementioned techniques, genome engineering has been greatly accelerated through multiplexing. Nonetheless, the level of multiplexing is still restricted, typically at only a few edits a time (Table 1). Increased multiplexing is highly desirable, yet technically challenging. What lies at the center of multiplexed genome engineering is a cost-effective way to generate mutagenizing reagents, be they oligonucleotides or nucleases. Ideally, it is the cheapest to use chemically synthesized short DNA oligos to directly introduce mutations into the genome, such as in the case of MAGE and YOG. These methods are also cloning free, which further reduces the cost. However, these methods can only be applied to microbial systems. ZFNs and TALENs, although have

been demonstrated for multiplexed genome engineering, suffer from high-cost, sophisticated assembly workflows. In contrast, Cas9 specificity solely relies on the gRNA sequence, which is very easy and cheap to synthesize. Thus, Cas9 seems a better choice over ZFNs and TALENs for multiplexed applications.

Another factor affecting multiplexing capability is the delivery of mutagenizing reagents. It is important to ensure that multiple oligonucleotides or nuclease-encoding plasmids/mRNAs can be efficiently introduced into a single cell. Taking CRISPR/Cas9 as an example, there are two general strategies to introduce multiple cr/gRNAs (Fig. 5). One strategy is to construct all the cr/gRNAs into one vector, by either sequential restriction digestion and ligation⁴⁵ or one-step assembly using customized protocols, such as Golden Gate^{39, 41, 43, 56}, USER⁴⁶ and BioBrick⁴² (Fig. 5a). Multiplexing capability in this strategy is restricted by the number of cr/gRNAs that can be cloned into one vector and functionally expressed. Another strategy is to construct each gRNA into one separate vector and introduce mixed plasmids or *in vitro* transcribed RNAs simultaneously into a cell (Fig. 5b). Multiplexing capability in this strategy is thus restricted by the number of constructs a cell can take at a time. Again, CRISPR/Cas9 has the advantage over ZFNs and TALENs, since multiple short cr/gRNA sequences can be readily made and easily delivered, as compared to the long gene fragments encoding multiple pairs of ZFNs and TALENs.

It is worth noting that each multiplexed genome engineering technique comes with advantages and limitations (Table 2). Thus, careful considerations must be taken when choosing from different tools. In microbial systems, though conceptually similar, oligonucleotide-mediated multiplexed genome engineering methods are customized for each species and are not easily transferable between different organisms. Among nuclease-mediated methods, ZFNs and TALENs have smaller protein sizes than Cas9 (from *Streptococcus pyogenes*), which is an advantage in single-locus genome engineering applications. However, in multiplexed genome engineering applications, introduction of multiple pairs of ZFNs or TALENs requires efficient delivery methods. In addition, mismatched ZFN or TALEN dimers could form, which aggravates off-target effects⁹³. In contrast, while off-target effects can still be a concern with Cas9, mismatching is not an issue, as Cas9 functions as a monomer and its targeting specificity resides solely in the cr/gRNA.

CONCLUSIONS AND FUTURE PROSPECTS

While true multiplexing has only recently been established as a viable genome engineering paradigm, a great number of very promising examples have already emerged. In diverse organisms ranging from bacteria to human cells, tools are being developed to enact complex genomic manipulations that would otherwise be tedious, difficult, or impossible to achieve by traditional means. Such tools have numerous applications in the fundamental and applied sciences, including multi-locus functional genomics studies in model organisms, the development of efficient industrial production strains, and the creation of models to study and treat complex diseases.

Moving forward, the development of these multiplexing tools beyond proof-of-concept studies will likely depend on two factors. First, continued optimization is necessary to increase the efficiency with which desired mutations can be introduced while reducing instances of off-target modification. This might include competency improvement to enhance the uptake of mutagenic oligonucleotides, promoter engineering to create optimal expression profiles for key elements (such as TALENs, Cas9, or gRNAs) in each organism of interest, or transcript optimization to improve the expression or stability of such elements. Protein engineering will also aid in the development of more efficient genome editing tools, as has been demonstrated with TALENs^{31, 94} and Cas9,⁴³ as will the continued development of tools and protocols to aid the selection of specific target sequences⁹⁵⁻⁹⁷ and increase the likelihood of desired editing events.^{98, 99}

Second, ongoing advances in high-throughput processing and laboratory automation will greatly influence future multiplexed genome engineering efforts. With increasing efficiency and a desire to target more and more loci simultaneously, the sizes of genome-wide libraries will increase exponentially. As a result, tools to rapidly generate targeting elements and screen such large libraries¹⁰⁰ will become a necessity. For the assembly of TALENs, generalized and scalable assembly schemes have already been developed.^{101, 102} Genome-wide application of Cas9 as well has benefitted from high-throughput generation of gRNAs¹⁰³ and the development of tools to screen the resulting libraries. Looking forward, automated platforms will likely become a staple to facilitate many aspects of multiplexed genome engineering, including DNA cloning, transformation, and cell culture.¹⁰⁴ Further, one can envision a future in which even decision-making processes are hardwired into laboratory robots, such that automated data analysis can close the loop of the design-build-test cycle.^{105, 106} Thus, with advances in both scale and efficiency, multiplexed genome engineering is poised to build upon its early successes and achieve further growth and applicability.

Acknowledgements

We thank the National Institutes of Health (GM077596), Roy J. Carver Charitable Trust (13-4257), and Carl R. Woese Institute for Genomic Biology at the University of Illinois at Urbana-Champaign for financial support in our development and application of genome engineering technologies. We thank all Zhao group members for giving critical comments during the preparation of this review.

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Figure 1.

Definition of multiplexed genome engineering in this review. **(a)** In multiplexed genome engineering, multiple mutations (colored ovals) are simultaneously introduced into a single genome (black line). **(b)** In genome-scale engineering, each genome receives one specific but different mutation.

Figure 2. Recombineering based multiplexed genome engineering. Mutagenic oligonucleotides recombine with the host genome to cause mutations during DNA replication. Multiple mutagenic oligonucleotides (black and grey lines) can be simultaneously introduced into the genome.

Figure 4.

Different types of nuclease-mediated multiplexing. **(a)** Multi-allelic modification. One nuclease or nuclease pair cleaves multiple copies of the same site. **(b)** Multi-gene disruption. Multiple nucleases/nuclease pairs target different loci across the genome. **(c)** Chromosomal excision. Two distant cleavages and splicing of the two break points cause the loss of a large chromosome fragment. **(d)** Chromosomal inversion. Two distant cleavages cause the inversion of a large chromosome fragment. **(e)** Chromosomal translocation. Two cleavages on two different chromosomes cause the translocation of chromosome arms. **(f)** Chromosomal duplication (a special case of chromosomal translocation). Two cleavages on two sites of two identical chromosomes result in the duplication of chromosome fragment in between the two cleavage sites. The brown star represents a duplicated gene.

Figure 5.

Common vector construction and delivery methods of multiplexed CRISPR/Cas systems. **(a)** Multiplexing at vector construction stage. Multiple gRNA or crRNA sequences can be constructed into one vector by either sequential digestion and ligation or one step assembly using customized methods. **(b)** Multiplexing at gRNA delivery stage. Each gRNA was constructed into one separate vector. Multiple vectors or *in vitro* transcribed gRNAs can be mixed and simultaneously delivered by microinjection or transfection.

Table 1

Reported efficiency values for nuclease-mediated multiplexed genome engineering

Organism	Endonuclease	Type of Modification	Targets	Efficiency (%)	Reference
<i>Streptococcus pneumoniae</i>	Cas9	multi-locus deletion	2	75	40
<i>Streptomyces</i> sp.	Cas9	multi-locus deletion	2	100	41
	Cas9	large deletion	2	100	41
<i>Escherichia coli</i>	Cas9	multi-locus deletion	2	97	42
	Cas9	multi-locus deletion/insertion	2	78	42
	Cas9	multi-locus deletion	3	47	42
<i>Saccharomyces cerevisiae</i>	TALEN	multi-locus deletion	2	~5 (phenotype)	51
	Cas9	multi-allelic insertion	1	70 - 100	44, 45
	Cas9	multi-locus deletion	3	27 - 100	43
	Cas9	multi-locus deletion	5	100	46
	Cas9	multi-locus insertion	2	43	45
	Cas9	multi-locus insertion	3	19	45
Rice (<i>Oryza sativa</i>)	TALEN	multi-allelic deletion	1	34	52
	TALEN	multi-locus deletion	3	1.9	53
	Cas9	multi-allelic deletion	1	33 - 100	39, 57 - 59
	Cas9	large deletion	2	4 - 20	39
Wheat (<i>Triticum aestivum</i> L.)	TALEN	multi-allelic deletion	1	3.7	54
<i>Arabidopsis thaliana</i>	Cas9	large deletion	2	5.5	55
	Cas9	multi-locus deletion	2	67 - 79	56
Corn (<i>Zea mays</i>)	Cas9	large deletion	2	60	56
Silkworm (<i>Bombyx mori</i>)	TALEN	large deletion	2	14	61
	Cas9	multi-locus deletion	3	1	71
Zebrafish (<i>Danio rerio</i>)	TALEN	large deletion	2	0.7 - 15	63 - 65
	TALEN	inversion	2	< 2	64
Fruitfly (<i>Drosophila melanogaster</i>)	Cas9	multi-allelic deletion	1	> 99	70
Livestock (cow and swine)	TALEN	multi-allelic deletion	1	0 - 17	68
	TALEN	large deletion	2	10	68
	TALEN	inversion	2	4	68
Mouse	Cas9	multi-locus deletion	2	79	78
	Cas9	multi-locus deletion	3	78	79
	Cas9	multi-locus deletion	5	10	78
	Cas9	large deletion	2	25	80
Rat	Cas9	multi-locus deletion	2	6.7, 63 - 75	80, 81
	Cas9	multi-locus deletion	4	24	81
Chinese hamster ovary	ZFN	multi-locus deletion	3	> 1	60
	Cas9	multi-locus deletion	3	35	82

Organism	Endonuclease	Type of Modification	Targets	Efficiency (%)	Reference
Human cell lines	ZFN	large deletion	2	0.01 - 10	83, 84
	ZFN	translocation	2	< 0.0001 - 0.01	85, 86
	TALEN	translocation	2	0.1 - 1	86
	Cas9	large deletion	2	1.6	88

Table 2

Advantages and limitations of various multiplexed genome engineering methods

Methods	Advantages	Limitations
<i>Oligonucleotide-mediated</i>		
MAGE	<ul style="list-style-type: none"> • Selection free • Low cost • No cloning required 	<ul style="list-style-type: none"> • Application restricted in <i>E. coli</i> • Only small (<20 bp) modifications can be efficiently introduced
YOGE	<ul style="list-style-type: none"> • Selection free • Low cost • No cloning required 	<ul style="list-style-type: none"> • Robust strain engineering required • Low multiplexing efficiency
MuGENT	<ul style="list-style-type: none"> • Selection free 	<ul style="list-style-type: none"> • Restricted to naturally competent microbial species
<i>Nuclease-mediated</i>		
ZFN	<ul style="list-style-type: none"> • Small protein size 	<ul style="list-style-type: none"> • Target sequence not easily customizable • Low or variable multiplexing efficiency • High off-target effects
TALEN	<ul style="list-style-type: none"> • High efficiency • Low off-target effects • Target sequence easily customizable • Effective in various organisms 	<ul style="list-style-type: none"> • Large protein size • Complex DNA assembly toolbox required
CRISPR/Cas9	<ul style="list-style-type: none"> • High efficiency • Low cost • Target sequence easily customizable • Effective in various organisms 	<ul style="list-style-type: none"> • Large protein size • Variable off-target effects