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Pooled screening with next-generation gene editing tools

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Abstract

Pooled screening creates a pool of cells with genetic variants, allowing for the simultaneous examination for changes in behavior or function. By selectively inducing mutations or perturbing expression, it enables scientists to systematically investigate the function of genes or genetic elements. Emerging gene editing tools, such as clustered regularly interspaced short palindromic repeats, coupled with advances in sequencing and computational capabilities, provide growing opportunities to understand biological processes in humans, animals, and plants as well as to identify potential targets for therapeutic interventions and agricultural research. In this review, we highlight the recent advances of pooled screens using next-generation gene editing tools along with relevant challenges and describe potential future directions of this technology.

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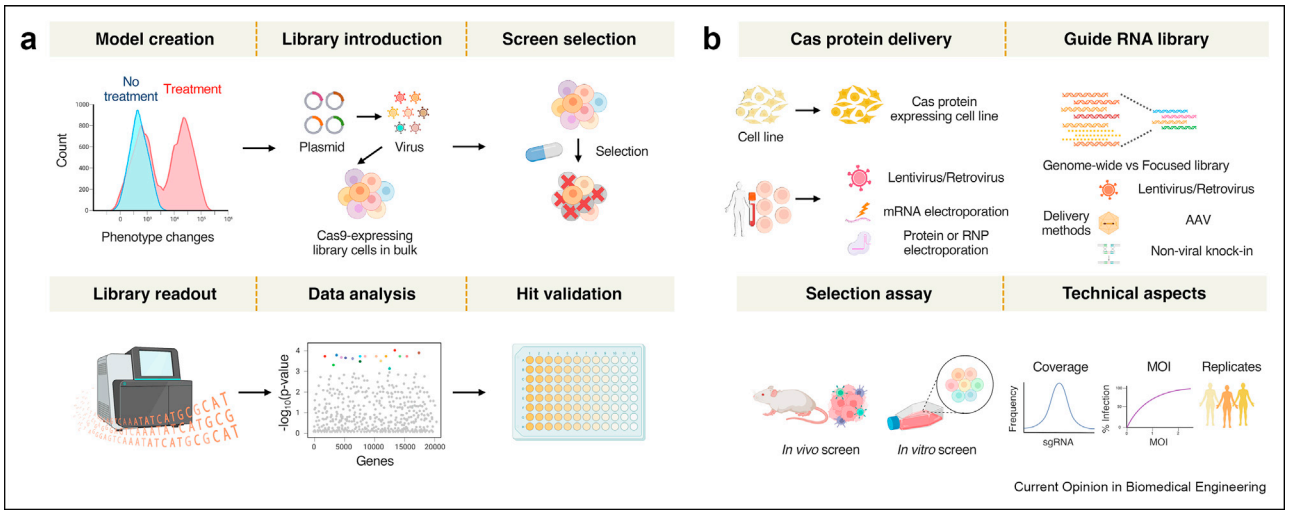
The workflow of modern pooled screening

Among the pooled-screening techniques, clustered regularly interspaced short palindromic repeats (CRISPR) screen, as a potent genetic tool, has enabled genome-scale genetic perturbations in the cells and revolutionized various fields in recent years [1]. The basic procedure of pooled CRISPR screens consists of six steps: (1) creating a biological model which has demonstrated substantial phenotype changes under different conditions, such as drug treatments; (2) introducing effector protein and the pooled CRISPR library into the cell populations; (3) performing screening experiments with selections of enrichment or depletion; (4) readout of the library; (5) screen analysis with computational algorithm(s); (6) (although not part of the screen per se) validation of top hits (Figure 1a).

Adapting the screening process to diverse cell types and more complex systems presents technical challenges, such as improving the efficiency, stability, and standardization of transduction cell pools while minimizing cellular toxicity. To achieve successful screening in different contexts, various strategies can be employed (Figure 1b). For instance, CRISPR transgenic animals have been developed to stably express Cas9 or other variants, allowing screening via straightforward introduction of sgRNA libraries [2–8]. However, it is not feasible to create stable Cas9 expression cell lines in human primary cells. To navigate this issue, strategies such as delivering sgRNA by lentiviral infection and Cas9 protein by electroporation or Cas9 ribonucleoprotein (RNP) electroporation have been developed. It enables genome-wide CRISPR screening in human primary T cells [9] or in hepatic stellate cells [10]. For Cas9 fusion proteins which the commercial protein and mRNA are not available, researchers have divided the delivery process into multiple steps of lentivirus infection steps [11].

Despite the popularity of lentivirus due to its high infection rate and relatively simple preparation, it can randomly integrate into the genome which may affect the experimental results. Additionally, false positive signal can occur in lentiviral transduction of primary cells, which can impact the accuracy of the multiplicity of infection (MOI). As an alternative, adeno-associated virus (AAV)-mediated screening is gaining traction.

Figure 1



Schematics of modern pooled screens.
(**a**) The pooled CRISPR screen process involves model creation, library introduction, screen selection, library readout, sequencing data analysis, and validation of hits.
(**b**) Specific strategies to be considered in a screen, including the proper ways to deliver effector proteins and guide RNAs, choosing the selection assay and testing important technical aspects. CRISPR, clustered regularly interspaced short palindromic repeats.

AAV does not integrate into the genome and has stable expression within a long time period. Researchers have leveraged AAV to conduct direct *in vivo* CRISPR screen to identify functional suppressors in glioblastoma [12] or mouse hepatocyte fitness [13], as well as developed a precise homology directed repair (HDR) knock-in method by AAV infection and Cpf1 mRNA electroporation to generate chimeric antigen receptor (CAR) T cells at specific genome locus [14]. This method enables to employ AAV-mediated pooled screening in human primary T cells and identify functional genes for anti-tumor immunity [15].

Modern gene editing tools for future pooled screens

Streptococcus pyogenes Cas9 (SpCas9), *Staphylococcus aureus* Cas9 (SaCas9), and *Acidaminococcus* sp. Cas12a (AsCas12a) are current popular CRISPR effector proteins for screening [16]. Emerging new tools have been developed to address challenges in pooled screening. The first obstacle to be overcome is the need for more efficient and accurate gene editing. Protein engineering methods, like iterative protein engineering and structural guided protein design, can be utilized to create more effective variants of common tools, such as enAsCas12a [17] and hyper Cas12a [18]. The editing accuracy depends on both gRNA sequence and the effector proteins. Efforts have been made to create Cas9 variants that have lower off-target effects, like evoCas9 [19] and HiFiCas9 [20], and to create better guide RNA optimization algorithms (e.g., CRISPick [21], sgDesigner [22]).

The size of the transgene inserted can significantly affect the delivery efficiency of the viral vector. For instance, AAV has a package capacity of approximately 4.7 kb. The sizes of conventional effector proteins, such as Cas9 and Cas12, range from 1000 to 1500 amino acids, while fusion proteins with other functional domains can easily exceed the AAV packaging limit. Recently discovered gene editing effector proteins have great potential to overcome this challenge. Several studies have reported and engineered Cas12f [23–26], TnpB, IscB, and IsrB [27,28] family proteins with gene editing ability in mammalian cells. Their sizes range from 350 to 550 amino acids, which make them promising potential tools for future pooled screening, especially in the situation where the guide, effector protein, and other transgene are packaged into 'all-in-one' constructs.

Other challenges include how to engineer a more efficient viral delivery system that can have tropism toward specific cell types. Structural engineering or directed evolution can modify AAV to infect specific cell types, such as CD4 targeting AAV [29], mouse T cell targeting AAV [30], and liver tropism AAV [31]. Chemical modifications could be applied to LNP/

mRNA-based delivery systems to enable them to target CD4+ T cells [32]. These modifications can increase the efficiency of *ex vivo* system delivery, which might enable direct *in situ* screens toward specific cell types.

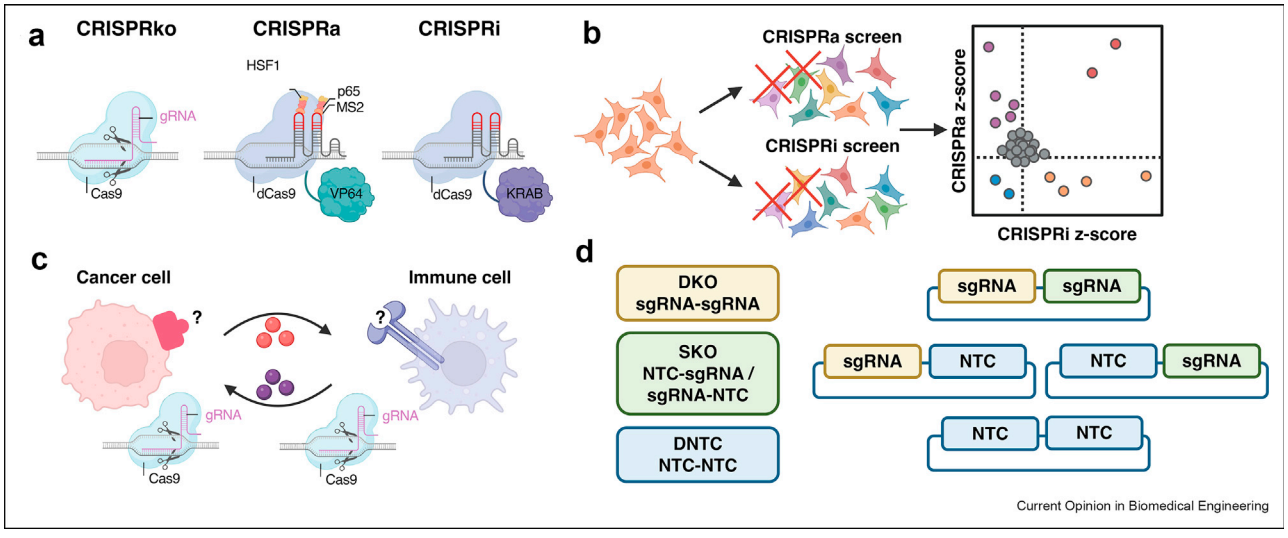
Different types of CRISPR-mediated perturbations

To date, the majority of CRISPR screens performed have utilized CRISPR knockout (CRISPRko), in which gRNAs direct Cas9 to cause DNA double-strand breaks (DSBs) and thereby knocking out the target genes, frequently as a result of frameshift mutations introduced by DNA repair machinery. However, introducing DNA DSBs can be toxic to cells. To overcome this limitation, endonuclease activity-dead Cas9 (dCas9) was developed and used for CRISPR interference (CRISPRi) and CRISPR activation (CRISPRa) [33] (Figure 2a). In CRISPRi, dCas9 is fused to transcriptional repressors, such as the Krüppel-assoiated box (KRAB) repressor to inhibit transcription of target genes without causing DNA damage [34]. Similar to this strategy, CRISPRa employs dCas9 fused with transcriptional activators, such as VP64, and co-delivered with MS2-p65-HSF1 (collectively referred to as synergistic activation mediator or SAM) to achieve robust gene activation at the target sites [35]. CRISPRi-based gene knockdown may result in weaker phenotypes than CRISPRko-based full gene knockout; however, it is less toxic to cells [36] and may produce a different phenotype as it is less likely to activate compensatory mechanisms [37]. Knockdown of housekeeping genes is also less likely to cause cell death compared to full knockout, making CRISPRi an appealing alternative to CRISPRko. However, compared to CRISPRko, CRISPRa and CRISPRi require continuous expression of dCas9 protein and gRNA to maintain transcriptional control and are thereby more challenging in certain primary cells due to poor lentiviral transduction.

While each approach has unique advantages and limitations, complementary gain-of-function and loss-of-function studies enable researchers to identify both the genes that play essential regulatory roles, and the genes that have complementary roles in regulating phenotypes of interest, thereby providing a more comprehensive view of functional regulators [11,38,39] (Figure 2b). For instance, Schmidt et al. performed paired CRISPRa and CRISPRi screens on drivers of T cell cytokine production. While CRISPRi identified required cytokine regulators, CRISPRa revealed key regulators that promote functions but are inactive in *ex vivo* cultured T cells. These two screens provide two different perspectives of the regulatory landscape of T cell cytokine production [11].

In addition to intracellular regulation, CRISPR screens also offer a versatile approach for investigating intercellular regulations and interactions, especially in the context of cancer cell-immune cell interaction systems (Figure 2c). For instance, Kamber et al. utilized complementary genome-wide CRISPRa and CRISPRko screens in cancer cells, and found that loss of APMAP in cancer cells can significantly boost macrophage phagocytosis. They then performed CRISPRko screens in macrophages and identified GPR84 as an essential gene for enhancing the uptake of APMAP-deficient cancer

Figure 2



Different types of CRISPR-mediated perturbations.
(**a**) CRISPR Knockout, CRISPR interference, and CRISPR activation.
(**b**) Complementary genome-wide gain-of-function and loss-of-function screens.
(**c**) CRISPR screens for investigation of intercellular regulations and interactions.
(**d**) Combinatorial double knockout screens to study genetic interactions. CRISPR, clustered regularly interspaced short palindromic repeats.

cells [40]. One advantage of this dual cell type screening approach is that it allows for the identification of target-specific immune receptors, which can be extended to other intracellular interaction studies. Overall, the use of multi-cell type CRISPR screens represents a powerful tool for studying both intracellular and intercellular functions, which is important for the study of the fundamental biology of complex diseases like cancer.

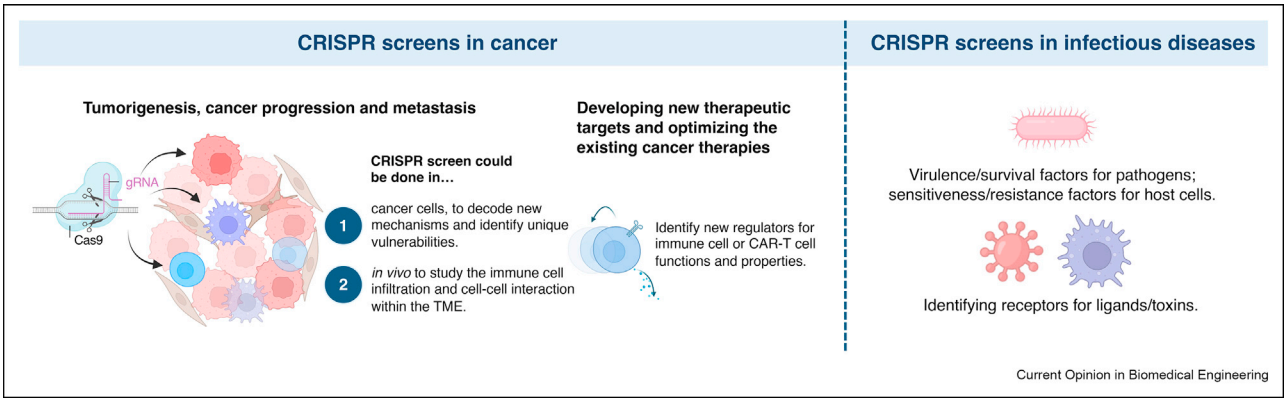
To date, most CRISPR screens have been employed to investigate the effect of individual gene on a desired phenotype. However, many intricate biological processes, such as the immune resistance of cancer cells, can be driven by interactions between multiple genes. Consequently, it is vital to identify genetic interactions by conducting dual or higher-dimensional perturbations in one cell for a more comprehensive understanding (Figure 2d). For instance, Park et al. performed a combinatorial double knockout screen on 1159 gene pairs in cancer cells to identify those where combined loss-of-function results in altered cellular response to T cell cytotoxicity [41]. While double knockout screens in immunology have just recently begun to emerge, they have the potential to be broadly applied in the future. In the meantime, the development of other mutational combinations, such as double activation screens and simultaneous activation-knockout screens, may enable the identification of novel genetic interactions. Additionally, the use of Cas12a in CRISPR screens enables higher-dimensional genetic screens by creating double, triple, or n-tuple perturbations simultaneously [42]. dCas12a-VPR based CRISPRa and dCas12a-KRAB based CRISPRi can add to the toolbox for future higher-dimensional genetic screens [43]. These higher dimensional perturbation screens open new opportunities to explore a more comprehensive view of genetic interactions involved in complex biological processes.

Applications of pooled CRISPR screening in disease models

Pooled CRISPR screenings have diverse applications in the study of human diseases (Figure 3). Cancer, for example, is associated with multiple hallmarks such as alteration in transcription and translation landscapes. While gene-coding mRNA has been extensively studied in cancer, the role of tRNA has often been overlooked. A recent study performed both genome-wide CRISPR screen and a focus screen targeting selected tRNA-biogenesis-related genes, revealing valine aminoacyl tRNA synthetase as a novel target to inhibit T cell acute lymphoblastic leukemia protein synthesis, opening the possibility of using dietary manipulation to treat cancer [44]. Unlike leukemia, the progression of solid tumor is heavily related to the tumor microenvironment (TME). To decode the complexity of cancer cell-TME interaction, one study developed Perturb-map, an approach for spatial functional genomics by engineering the cancer cell with a selected gRNA library and protein-barcodes (Pro-Code), which identified candidates such as TGFβ receptor 2 and Socs1 that can reprogram the TME [45]. Although innate immune pathway activation has been associated with a more inflamed TME and, thus, better outcomes, the antiviral signaling pathway central kinase TANK-binding kinase 1 (TBK1) has been identified by *in vitro* and *in vivo* screens as an inhibitor of cytotoxicity through the non-canonical TBK1-RIPK1-caspase axis [46].

CRISPR screens have also been applied to the study of various diseases other than cancer. For example, a genome-wide loss-of-function screen in the human pancreatic beta cell line EndoC-βH1 identified genes that regulate intracellular insulin content, which is an indicator of beta cell function [47]. While most CRISPR screens are performed in cell lines, large-scale screenings in primary cells pose several challenges, including low CRISPR machinery delivering efficiency, the

Figure 3



Diverse applications of pooled CRISPR screens in disease models
Depending on the gRNA targets and the species or cell types in which the screening is performed, CRISPR screen could be widely applied to study various types of biological questions in various disease settings. Some example scenarios for application are shown here. CRISPR, clustered regularly interspaced short palindromic repeats.

scarcity of certain populations, and difficulties in identifying and isolating specific cell types due to a lack of good surface markers. For primary cells other than blood cells, the acquisition of adequate samples is one of the principal obstacles to performing CRISPR screens in such cells. Alternatively, researchers have been using induced pluripotent stem cells (iPSCs)-derived cells that mimic human primary cells as substitutes. For example, iPSC-derived microglia were used to recapitulate Parkinson's disease (PD), which is characterized by microglial iron accumulation. A genome-wide CRISPR screen in such cells identified *SEC24B* as a novel target that regulates iron-dependent neurotoxicity in an *in vitro* co-culture system [48]. Similarly, another study which includes three screens using refined guide RNA libraries identified novel regulators for iPSC-derived microglia activities, which might link with neurodegenerative diseases [39]. Unlike the 2-dimensional *in vitro* culture system, the 3-dimensional (3D) organoids can better recapitulate the complexity of the microenvironment and cell-cell interaction *in vivo*. Therefore, when *in vivo* models are hard to access, the organoids combined with CRISPR screens can help identify key drivers in biological processes with higher authenticity. For example, a genome-wide CRISPR screen performed in kidney organoids identified not only key pathways in kidney development but also genes that account for congenital anomalies of the kidney and urinary tract [49].

Finally, CRISPR screens conducted on human pathogens provided insights for the discovery of novel targets that inhibit the host-pathogen interaction or the survival, proliferation, or virulence of the pathogens. For example, with the use of CRISPR screens, researchers have identified TFPI as a receptor for TcdB, a virulence factor in *Clostridioides difficile* that can induce gastroenteritis-associated death [50]. CRISPR screen can also be used to investigate viral pathogens, whose activity is entirely dependent on host cells. By engineering human fibroblasts with CRISPR machinery targeting the whole genome of the human cytomegalovirus (HCMV), followed by a subsequent low-MOI HCMV infection, factors for viral entry, replication, and killing of the host cells were identified [51]. CRISPR screens have also been applied to the discovery of host-pathogen interactions in SARS-CoV-2 [38,52,53].

Future directions and ethical considerations of CRISPR screens in complex biological systems

The advance in genetic modification and targeted delivery tools has enabled the application of CRISPR screen in systems with higher complexity. Additionally, the cost for CRISPR screen has decreased due to open access to well-developed gRNA libraries and the drop of next-generation sequencing price. As a result, CRISPR screen has become an unbiased and cost-efficient method for forward genetics studies. Here, we list several aspects of interests that future CRISPR screen could investigate. Many previous studies have been focused on cell lines. However, biological systems such as the immune system and the TME are highly complex. For example, the TME contains different types of cells from different sources. The fibroblasts are mostly local, while the endothelial cells and neurons are derived from nearby vasculature or nerve. The T cells are usually infiltrated at later stage from the capillaries. Given this fact, intravascular injection of gRNA library-transduced T cell could potentially recapitulate which T cell has gained better infiltration or killing capability. However, if the question is which endothelial cell is more permissive to T cell infiltration or cancer cell intravasation, an *in situ* CRISPR screen will be required. These kinds of questions could be either addressed by the development of targeted delivery system, or an inducible or spontaneous CRISPR machinery expression system. A recent paper has developed an inducible mosaic animal for perturbation [54] that could limit the CRISPR machinery expression to specific cell types, allowing for *in situ* generation of CRISPR knockouts for dozens of genes in a mosaic manner. In addition, the dynamic and cell-cell or cell-environment interaction has also been under-investigated in previous CRISPR screens. For example, secretory proteins or surface ligands could possibly influence the nearby cells during a pooled CRISPR screen, creating complexity in a cell non-autonomous manner. This is especially important in the context of developmental biology, in which the concentration of diffusible ligands and the presence of surface ligands determine the cell fate. CRISPR screen with spatial resolution will be beneficial to study these questions.

The rapid progress and widespread use of gene editing tools necessitate careful considerations of ethical concerns, including potential off-target effects, potential for increased social inequality due to access disparities, and misuse for non-therapeutic genetic enhancements [55]. In addition, the potential for germline editing, which could lead to inherited changes in future generations, adds another layer of ethical intricacy. Researchers' role in genome editing goes beyond the lab, and all scientists shall have high ethical standards to use these powerful tools. Researchers shall advocate for responsible use, urge for stringent testing, and support policies promoting equitable access. As we delve into the scientific development and applications of gene editing technology in this review, we underscore the importance of parallelly addressing these ethical issues, thereby promoting responsible advancement in gene editing research.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: SC is a (co)founder of EvolveImmune Tx, Cellinfinity Bio, Chen Consulting, NumericGlobal, and Chen Tech, all unrelated to this study.

Data availability

No data was used for the research described in the article.

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