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The future of scCRISPR-seq

Biomedical Sciences Master's Essay

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Abstract

This essay explores the combination of CRISPR technology and single-cell sequencing, offering a deep dive into understanding genetic changes. It discusses the background of CRISPR, which won the 2020 Nobel Prize in Chemistry. scCRISPR screens, a powerful application of CRISPR, are explained, emphasizing the integration of scRNA-seq for a more detailed analysis of cellular effects. Challenges and recent advancements in the scCRISPR field are explored, showcasing innovative studies in single-nucleotide variant analysis, spatial functional genomics, and genome-wide single-cell resolution screens. Applications across molecular and cell biology, cancer research, microbiology, medical genetics, and immunology are exemplified through noteworthy studies. The essay anticipates future possibilities, with a focus on *in vivo* methods, non-invasive single-cell transcriptomics, and single-cell metabolomics. The challenges and potential of these emerging areas are discussed, offering a glimpse into the future of genetics research. In conclusion, the essay highlights the crucial role of scCRISPR-seq in genetics research, covering the background, current capabilities, and future directions.

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Abbreviations

Cas = CRISPR-associated proteins
Cas9 = Cas9 protein from Streptococcus pyogenes
crRNA = CRISPR RNA
CRISPR = Clustered Regularly Interspaced Short Palindromic Repeats
DDR = DNA damage response
DSB = double-strand break
dCas9 = Cas9 dead endonuclease
gRNA = guide RNA
indels = insertions or deletions
KO = knock-out
NHEJ = non-homologous end-joining
RNP = ribonucleoprotein
scCRISPR = single-cell CRISPR
sci = single-cell combinatorial indexing
scRNA-seq = single-cell RNA-sequencing

Background: the possibilities of CRISPR

The CRISPR-Cas (Clustered Regularly Interspaced Short Palindromic Repeats - CRISPR-associated proteins) system is the natural bacterial and archaeal defence mechanism against bacteriophages and plasmid transfer (Barrangou et al., 2007; Koonin & Makarova, 2019). The CRISPR-Cas system works by inserting small pieces of the genome of the attacker into the host's genome, allowing it to produce small interfering crRNAs (CRISPR RNA) that complement the attacker and interfere with its function (Wang et al., 2022). It has been adapted to form a powerful tool, which has, since its discovery in 2012, revolutionized the field of genetics, even winning the 2020 Nobel Prize in Chemistry (Jinek et al., 2012; Nobel Prize Outreach AB, 2020). The interference system can be adapted to edit the genome of any target by combining a guide RNA (gRNA) with a Cas protein (Bock et al., 2022).

Cas9 beyond gene editing

There are different types of Cas proteins, both naturally occurring and engineered, that can perform various gene editing functions (Figure 1, Bock et al., 2022). The most studied and used Cas protein is spCas9 from the bacterium *Streptococcus pyogenes*, hereafter referred to as Cas9 (Gostimskaya, 2022). This is a protein of the second class of Cas proteins, which consists of single multi-domain proteins (Koonin & Makarova, 2019). This Cas9 protein, which has two nuclease domains, has been altered in different ways such that it affects the DNA differently (Makarova & Koonin, 2015). Cas9 itself will induce double-strand breaks (DSBs), which are then repaired by the DNA damage response (DDR) mechanisms present in the cell (Huang et al., 2022). Cas9 nickase has been mutated such that one of its nuclease domains has been inactivated (Eid et al., 2018). Therefore, it only induces a break in a single strand, rather than in both. dCas9, or Cas9 dead endonuclease, has been catalytically inactivated by mutating both nuclease domains and does not induce any DNA strand break.

Upon the occurrence of a DSB, wherein neither strand can function as a template for the repair of the other, the cell activates alternative DNA DDR pathways (Bock et al., 2022; Eid et al., 2018). To efficiently restore the break, the cell predominantly employs the non-homologous end-joining (NHEJ) mechanism. This repair process involves the direct ligation of the two fractured DNA ends without

the requirement for homologous sequence alignment. Notably, NHEJ is recognized for its swiftness; however, it is acknowledged to be error-prone and imprecise. Consequently, this may lead to the insertion or deletion (indels) of random nucleotides, potentially resulting in the generation of an early stop codon and subsequent gene knock-out (KO). Cas proteins have been engineered to work as CRISPR base editors, that induce targeted mutations, by integrating a base modification enzyme and a Cas nickase. The nickase induces a single-strand break in the non-edited DNA strand, promoting repair with the edited base. dCas9 is catalytically inactivated and thus does not damage the DNA in any way, rather it is used as a carrier for other proteins to the correct location in the DNA. dCas9 has also been engineered to be employed as an extra-gene editing tool, in particular with the purpose of gene expression regulation. Examples of this are CRISPR interference (CRISPRi), where dCas9 is attached to transcriptional repressors, and CRISPR activation (CRISPRa), where transcriptional activators are fused. Epigenetic marks can also be edited by adding epigenetic writer and eraser enzymes to dCas9. More variants of CRISPR-Cas editing are possible but are outside the scope of this essay.

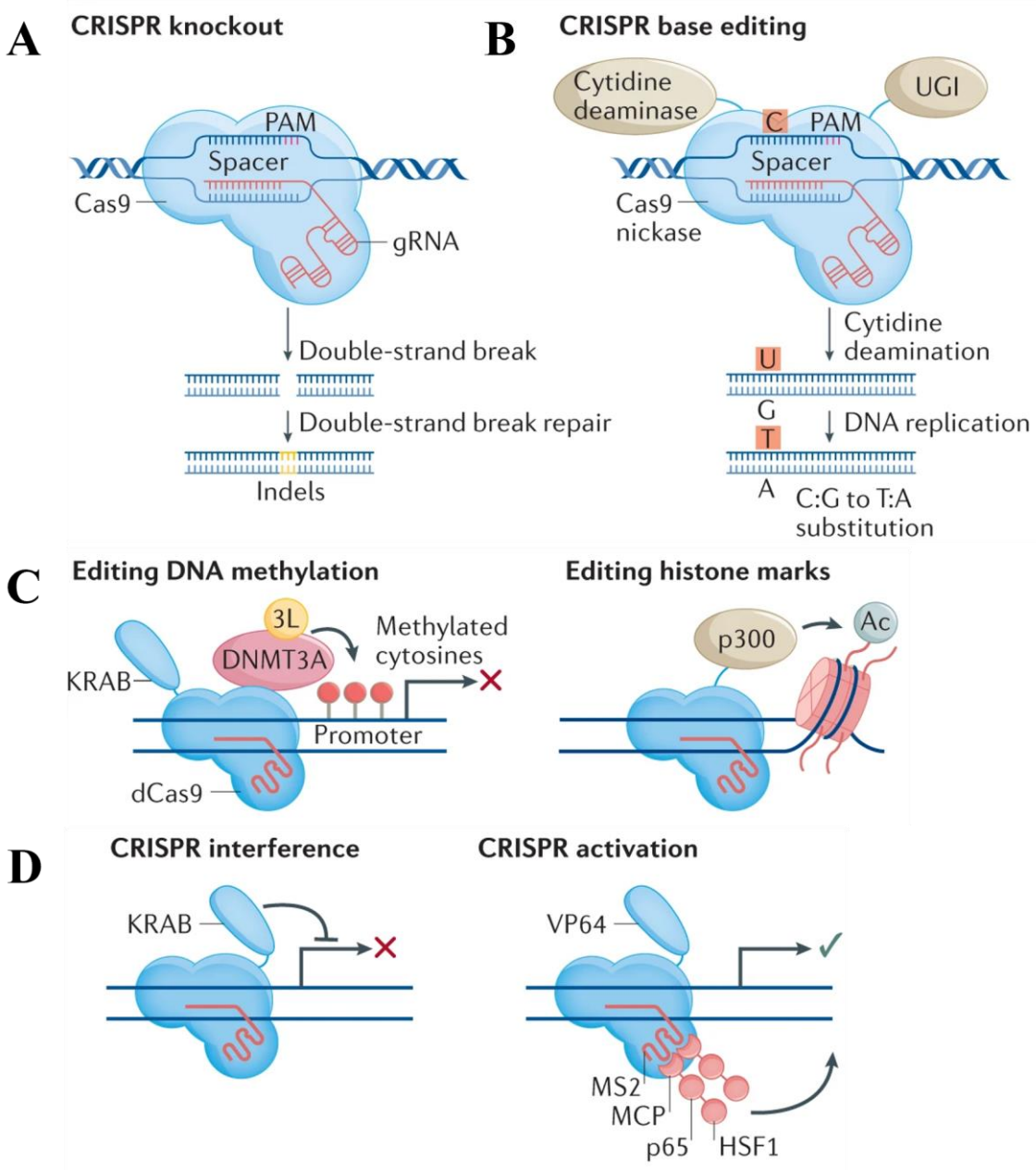

Figure 1: Diverse Applications of CRISPR Genetic Editing. *A) CRISPR KO: This approach involves the induction of a DSB by Cas9, guided by the gRNA. This results in the disruption of the targeted gene, as the DSB gets repaired by NHEJ and indels occur. B) Base editing: CRISPR base editors generate specific mutations by incorporating a base modification enzyme, an inhibitory domain impeding base excision repair, and a Cas nickase. The nickase initiates a single-strand break in the non-edited DNA strand, facilitating repair with the edited base. C) Epigenetic editing: Utilizing the dCas9 protein, this technique attracts enzymes for epigenome modification, including DNA methylation and histone mark editing. D) Gene expression: The dCas9 protein, coupled with the transcriptional activators and inhibitors, can interfere with or activate gene expression. dCas9 is only a carrier for the proteins. This mechanism demonstrates the utility of CRISPR for regulating gene activity at the transcriptional level. Figure adapted from Bock et al. (2022).*

CRISPR screen

CRISPR screens leverage the potency of CRISPR technology, employing a vast library of tens to hundreds of thousands of gRNAs. In a controlled process with low infection rates, cells incorporate a single gRNA, ensuring precision (Holcomb et al., 2022). For knockout (KO) or knock-in (KI) experiments, Cas protein and gRNA are commonly delivered using methods such as encoding in DNA, mRNA introduction via plasmid transfection, or as a protein/ribonucleoprotein (RNP) complex (Huang et al., 2022). In the context of screens, viral vectors are used more often, where Cas9 is inserted first, after which the library of guides is transduced.

Typically, a pooled in vitro CRISPR screen starts with the careful selection of a specific cell type that represents the desired biological condition (Bock et al., 2022). Following the delivery of the gRNA, a cell-based assay is employed. This assay, coupled with a selection system like drug administration, triggers a shift in the cellular phenotype, ultimately modifying the representation of sgRNAs in the cellular pool. In the analysis phase, the focus of most screens lies in assessing the depletion or enrichment of gRNAs, a process conducted through deep sequencing before and after the selection. Such changes in gRNA representation are indicative of cell death or proliferation, providing valuable insights into the impact of genetic perturbations on the cellular phenotype.

scRNA-seq and CRISPR

Another technology that has been transforming the field of genetics is the development of single-cell RNA sequencing (scRNA-seq) (Jovic et al., 2022). With this technique, each individual cell is sequenced, unlike bulk sequencing, where all cells are sequenced together. This technique allows scientists to look at the heterogeneity in the sequenced cell population rather than gathering an average of all cells. Most commonly, a microfluidics device is used that separates each cell into a nanodroplet. Each droplet contains a bead that captures the RNA and adds barcodes to the transcript that identify the unique RNA molecule and cell, which can then be used to recover the precise transcriptomic state of each cell. Another approach is combinatorial indexing, in which an extra round of barcoding is used to identify the cell rather than the droplet containing the cell-specific barcode (Vitak et al., 2017). Whilst scRNA-seq is the most used, similar single-cell approaches have allowed other sequencing methods to be adapted to a single-cell protocol such that one can also sequence the epigenome, the proteome, and the metabolome.

These two powerful techniques can be combined to create scCRISPR screening libraries. This method, where the cells in the CRISPR screen are read out on a single-cell level, provides a more detailed read-out of the effects of different gRNAs, going beyond just understanding cell survival and

general characteristics. However, it's important to note that practically, this process takes a lot of time. Growing or obtaining enough cells or samples, optimizing CRISPR, sequencing, and, most importantly, analyzing the data, all require a significant amount of time and effort.

In this essay, I will be describing the current state-of-the-art methods, some current successful applications of scCRISPR, and which techniques are in development. Lastly, I will discuss which of these techniques, in my opinion, show the most promise.

scCRISPR types and promising techniques

An overview of the development landscape of scCRISPR was published by Cheng et al. (Figure 2, 2023).

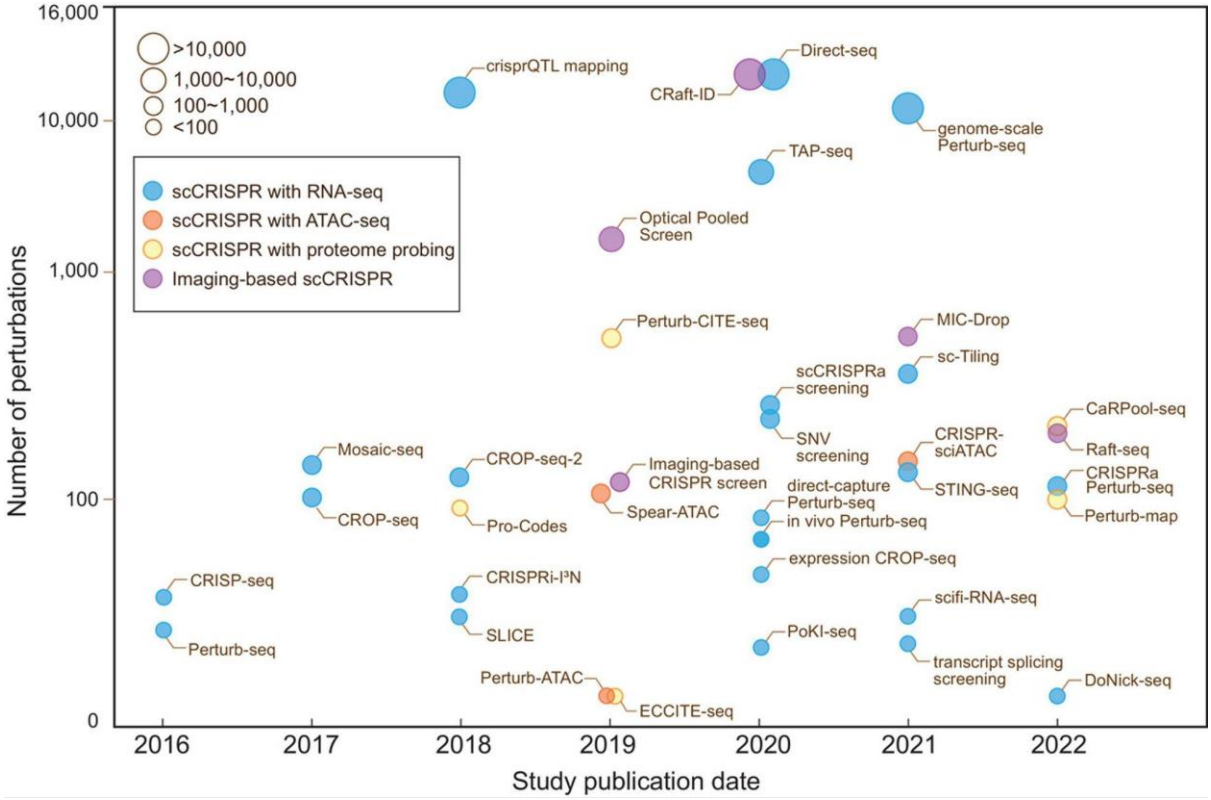

Figure 2: Development landscape of scCRISPR. *The study publication date is plotted against the number of perturbations, and the main type (RNA-seq, ATAC-seq, proteome probing or imaging-based scCRISPR) is visualized with colour. In the early years of scCRISPR, mainly RNA-seq techniques were developed, whilst in the following years, the focus shifted to the other types. Figure published in Cheng et al. (2023).*

As mentioned before, scRNA-seq and CRISPR screening libraries can be combined to inspect the transcriptome after CRISPR intervention and perturbation. The traditional scRNA-seq protocol cannot be directly used in a scCRISPR experiment as the gRNAs are not normally adenylated and are, therefore, not captured by the bead. There are three different approaches to solving this problem (Cheng et al., 2023). Firstly, a barcode can be added to the plasmid gRNA such that each mRNA read will also contain a unique gRNA barcode. Secondly, polyadenylating the gRNA by altering the RNA polymerase with which the transcript is made. Lastly, a capture sequence can be added to the gRNA such that the bead does capture it. The first method was employed by the pioneering techniques:

Perturb-seq, CRISP-seq and Mosaic-seq (Dixit et al., 2016; Jaitin et al., 2016; Xie et al., 2017). Since the development of scCRISPR with RNA-seq in 2016, many new techniques and improvements have been developed, thus allowing for larger studies with more gRNAs and more perturbations (Cheng et al., 2023). Recent developments have focused on improving the efficiency of the transduction of CRISPR-Cas9, increasing on-target efficacy, reducing off-target effects, and reducing costs by using the techniques and sequencing platforms more efficiently.

Besides RNA-seq, single-cell DNA methylation protocols have also been developed. The first method, Perturb-ATAC, was developed in 2019, which reads out the chromatin accessibility of the cells in the CRISPR screen (Rubin et al., 2019). However, this technique was far from perfect, as it had a limited throughput of 96 cells per run as well as other efficacy issues in the method. Thus, Spear-ATAC was developed, which overcame these limitations by reading out sgRNA spacer sequences directly from genomic DNA rather than off of RNA transcripts (Pierce et al., 2021). With these improvements, they increased throughput (35- to 100-fold) and reduced costs by 20-fold. A different approach, called CRISPR-sciATAC, which uses combinatorial indexing as its single-cell approach rather than the microfluidic approach, was developed by Liscovitch-Brauer et al. (2021). This is another approach to improving Perturb-ATAC without the use of commercial sc-platforms such as those from 10X. This method showed similar improvements in cost and throughput as Spear-ATAC and was published at a similar time as Spear-ATAC. However, since then, there has not been much development in the field, nor are the techniques used often.

Additionally, CRISPR-based proteome probing is also possible on a single-cell level, although it is much more limited. Two separate approaches exist, where one makes use of CITE-seq, which quantifies both the transcriptome and the epitopes present on the cells and modifies this to capture the gRNA as well. The other approach barcodes the cells before performing mass spectrometry to resolve the results on a single-cell level. The latter approach was developed first in a method called Pro-Codes in 2018 and is an easily scalable method in terms of the number of cells, but cannot detect many proteins at once (Wroblewska et al., 2018). Methods that use CITE-seq do not have this problem, but only epitopes can be detected (Cheng et al., 2023). The first method to do this was ECCITE-seq, in which both the transcriptome and the epitopes are sequenced (Mimitou et al., 2019). Several improved versions of these techniques, mainly coupling the protein approach with other modalities such as the transcriptome, have been published, some of which will be discussed later.

Lastly, imaging-based methods that inform on spatial context have been developed since 2019, as the aforementioned techniques cannot map all phenotypes to the genotypes. Also, here, there are two separate approaches, one in which fluorescence is used to map the gRNA to the cell, whilst the other approach utilizes the physical separation of the cells to identify the interesting cells to be genotyped (Cheng et al., 2023). Microscopy is very powerful on small numbers of cells but is harder to integrate with pooled screenings. The imaging-based methods are becoming more powerful, and big leaps are expected soon, by combining these methods with scRNA-seq and increasing scalability.

Whilst mainly the pioneering techniques in each type of single-cell readout have been discussed, I would now like to highlight some of the more recent and advanced methods, which apply an innovative approach to the scCRISPR field. The first study employed CRISPR RNA-guided deaminase and single-cell RNA sequencing to introduce and analyze substitution mutations in the exons of three genes associated with vemurafenib resistance (Jun et al., 2020). Using a library of 420 sgRNAs, the authors identified the E203K mutation in *MAP2K1* as enriched in melanoma cells with resistance to vemurafenib. The integration of scRNA-seq revealed transcriptomic changes, highlighting immune response activation with the E203K mutation and chemokine signalling in indel-

introduced cells. The method provides a single-nucleotide variant-based single-cell analysis, but careful validation is crucial to avoid misinterpretation. Challenges in the method lay in optimizing base editing efficiency and addressing off-target effects, emphasizing the need for precision in CRISPR-based mutation analysis.

The second study introduces Perturb-map, a spatial functional genomics approach combining CRISPR screens with imaging and spatial transcriptomics (Dhainaut et al., 2022). Applied in a lung cancer mouse model, Perturb-map allows simultaneous knockout of multiple genes while preserving tissue architecture. This enables the analysis of extracellular gene functions within the tissue context, providing insights into tumour growth, histopathology, and immune composition. Perturb-map broadens the scope of functional genomics beyond cell-intrinsic gene functions, facilitating the investigation of diverse processes through imaging analysis. The study demonstrates Perturb-map's utility in uncovering the nuanced effects of specific gene perturbations on tumour biology, including impacts on tumour immunity and morphology. While emphasizing its versatility, the study acknowledges limitations in the size of CRISPR libraries feasible for Perturb-map as the number of tumour lesions in a model animal is limited in a single model. Overall, Perturb-map is a valuable tool for spatial functional genomics, offering a comprehensive view of gene functions within the tissue environment.

In the last study, the authors utilized an impressively large-scale high-content phenotypic screen called Perturb-seq, combining CRISPR interference (CRISPRi) with single-cell RNA-sequencing readouts to perform genome-scale screens targeting all expressed genes in over 2.5 million human cells (Replogle et al., 2022). By use of this approach, the authors predicted the function of poorly characterized genes and revealed new regulators of ribosome biogenesis, transcription, and mitochondrial respiration. The single-cell transcriptional phenotypes provided in-depth insights into complex cellular phenomena, including RNA processing, differentiation, and the consequences of aneuploidy. While highlighting the potential of single-cell CRISPR screens for rich genotype-phenotype mapping, the study acknowledges limitations, such as cost constraints, technical aspects, and the need for careful experimental design. Overall, Perturb-seq emerges as a powerful tool for systematically exploring genetic and cellular function at a genome-wide single-cell resolution.

Applications

scCRISPR-seq is a powerful technique that can be applied in many different ways. Already, some papers have been discussed where the researchers utilized the method they developed in a proof-of-concept manner, but now some more general applications will be discussed.The main research areas where it is applied are molecular and cell biology, cancer research, microbiology, medical genetics, and immunology (Bock et al., 2022). I will be describing an example from each of these areas in order to showcase the versatility of scCRISPR-seq.

In a study by Saunders et al. (2023), the sci-RNA-seq3 protocol was employed to create a zebrafish single-cell atlas of perturbed embryos. This innovative approach falls within the realm of molecular and cell biology, providing a comprehensive dataset encompassing 23 genetic perturbations across 19 distinct time points in zebrafish embryonic development. By systematically applying CRISPR-based perturbations to genes associated with embryonic development, the researchers shed light on both well-studied and previously unexplored genes. This atlas, derived from 1,812 individually resolved zebrafish embryos and a total of 3.2 million cells, not only contributes to the understanding of gene functions in embryogenesis but also introduces the concept of whole-organism labelling for

systematic analysis of genetic perturbations at single-cell resolution. The study underscores the transformative impact of CRISPR technology in advancing molecular and cell biology research, particularly in unravelling the complexities of embryonic development and gene functions.

Li et al. (2023) employed a technique known as CRISPR–human organoids–single-cell RNA sequencing (CHOOSE) in the field of medical genetics. The study focused on investigating genes associated with developmental defects in autism spectrum disorder (ASD) using cerebral organoids. By utilizing verified guide RNAs and inducible CRISPR–Cas9-based genetic disruption, the researchers performed a pooled loss-of-function screening in mosaic organoids. The study revealed crucial insights into the impact of perturbing 36 high-risk ASD genes related to transcriptional regulation, uncovering vulnerabilities in specific cell types during early brain developmental stages. Notably, the perturbation of the BRG1/BRM-associated factor (BAF) chromatin remodelling complex was identified as influential, leading to the enrichment of ventral telencephalon progenitors. This study showcases the power of CHOOSE in systematically characterizing the phenotypic consequences of genetic perturbations in the context of cerebral organoids, providing a valuable tool for understanding the genetic basis of neurodevelopmental disorders.

In their research, Hein and Weissman (2022) applied the power of CRISPR technology, specifically employing Perturb-seq, to delve into the intricate dynamics of human cytomegalovirus (HCMV) infection within primary human fibroblasts. Operating within the field of microbiology, they strategically modified a core set of factors essential for virus replication, as identified in a previous pooled screen. Through Perturb-seq, the authors recorded the transcriptomes of tens of thousands of CRISPR-modified single cells, shedding light on the functional contribution of both viral and host factors during HCMV infection. Notably, their findings revealed that while perturbing essential host factors did not alter the typical transcriptional trajectory per se, it could significantly impact the timing and progression of infection, providing a valuable tool for pinpointing specific stages influenced by these factors. This innovative approach not only contributes to our understanding of host-pathogen interactions at a single-cell level but also holds promise for designing antiviral interventions and informing future studies across various virus-host systems.

In a study at the intersection of immunology and cancer biology, Belk et al. (2022) employed *in vivo* Perturb-seq to systematically identify key regulators of T-cell exhaustion. T-cell exhaustion represents a significant barrier to anti-tumour immunity, yet the molecular determinants of this process have been elusive. By conducting genome-wide CRISPR-Cas9 screens in chronically stimulated T cells, the researchers uncovered a notable enrichment of epigenetic factors, particularly the cBAF and INO80 chromatin remodelling complexes. The in vivo Perturb-seq experiments not only identified distinct transcriptional roles of these complexes but also highlighted the crucial role of the canonical BAF complex member, Arid1a, in maintaining an effector program and downregulating exhaustion-related genes in tumour-infiltrating T cells. Depletion of Arid1a not only limited the acquisition of exhaustion-associated chromatin accessibility but also demonstrated improved anti-tumour immunity. This research not only provides a comprehensive atlas of the genetic regulators of T cell exhaustion but also underscores the potential of modulating the epigenetic state to enhance T cell responses in cancer immunotherapy.

Future possibilities

The current state-of-the-art techniques have been discussed. Now I would like to highlight some of the most promising future possibilities that I think will greatly aid the field of scCRISPR. First of all, in vivo methods. The golden standard in any biomedical research is to work with immortalized cell lines. However, these cell lines have cancer-like mutations that allow the cells to reply indefinitely, which do not accurately reflect *in vivo* conditions. Therefore, research has slowly been moving towards researching *in vivo* models. There are still some limitations to overcome before this can be applied widely in CRISPR as well. Firstly, especially in large screens, there is a need for many replicates for the number of gRNAs (Kuhn et al., 2021). As *in vivo* models can be quite costly to set up, this could prove problematic as this is in addition to the increased cost of sequencing single cells rather than in bulk. Another problem, depending on the system, is the proper delivery of the gRNAs. Two methods exist: direct and indirect. In the direct method, a virus is used to distribute the gRNA, however, due to distribution issues, so far, this is only possible in organs such as the liver, lungs and the brain. The indirect method utilizes cell transplantation of cells already transduced with the gRNA. Already, some research is being done with *in vivo* models, such as Jin et al. (2020), who utilized Perturb-seq in mice embryos to study the effect of knocking out genes implicated in autism spectrum disorders. In a more recent example, Zhou et al. (2023) investigated a transcription factor library in Cas9-expressing CD8+ T cells, identifying potential regulators of intratumoral cytotoxic T lymphocyte accumulation in a mouse melanoma model. I expect that in the future, *in vivo* methods will become even more commonplace as costs go down and techniques improve as they are simply much more informative than traditional *in vitro* models.

Secondly, I would like to discuss non-invasive single-cell transcriptomics. Current methods require the user to lyse the cells, thus destroying any spatial or temporal context. If one wants to look at the changes over time, multiple cultures will be set up and sequenced at different time points. Chen et al. (2022) developed a method called Live-seq, which does not require lysing of the cells, they extract the RNA by using fluidic force microscopy, thus preserving the viability of the cells. The technique works by extracting RNA from a single cell by coupling force control with volume control with which they can recover up to 1 pg RNA per cell (Figure 3). Another method, by Lombard-Banek et al. (2021), in which capillary electrophoresis-electrospray ionization is used to aspirate 10 nL of the cytoplasm and then they performed mass spectrometry on it to characterize the metabolomic profile of Xenopus laevis embryos. Whilst these methods are not very scalable yet and take long to perform as they sample each cell individually, I expect that this technique is the start of a new revolution in non-invasive single-cell technology.

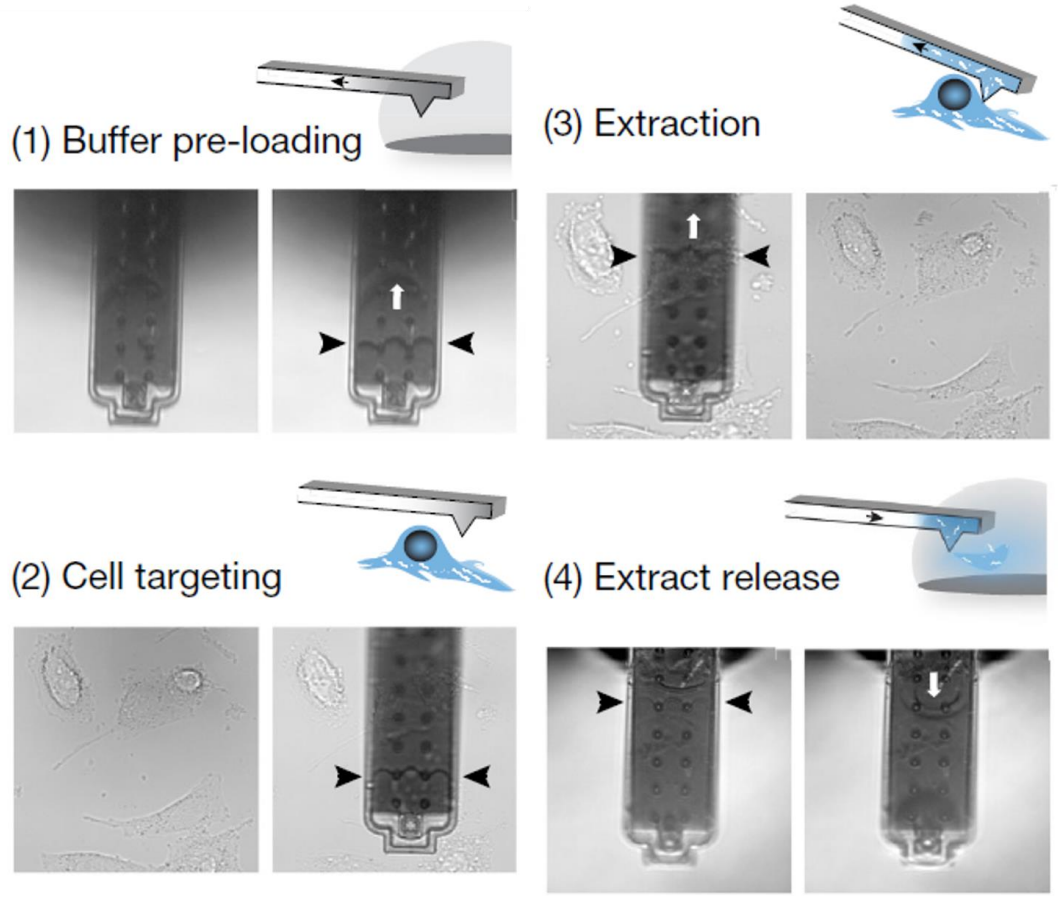

Figure 3: Live-seq method. *White arrows indicate the application of under/overpressure, whilst black arrows indicate the amount of buffer and extract in the probe. 1) The FluidFM probe was preloaded with mineral oil, followed by buffer loading and immersion in nuclease-free water. 2) The desired cell is targeted. 3) Using under pressure, on average 1.1 pl (ranging from 0.1 to 4.4 pl) cytoplasmic content was aspirated from a targeted cell into the probe, and mixed with sampling buffer. 4) The extract was then released into a lysis buffer drop for subsequent processing. The entire process, monitored in real-time, took approximately 15 minutes per sample, including loading, cell targeting, cytoplasmic extraction, and release into the lysis buffer. Figure published in Chen et al. (2022).*

Lastly, for now, mainly scRNA-seq has been discussed, although DNA methylation, proteomics and spatial sequencing have also been discussed. One area I feel is lacking in this overview is metabolomics. In metabolomics, the small molecules present in the cell are characterized by mass spectrometry. In general, this field is less developed than other omics techniques, but it has already been used in a single-cell CRISPR screening by Anglada-Girotto et al. (2022). The authors utilized CRISPR interference (CRISPRi) to analyze compound functionality in Escherichia coli, developing a high-throughput framework that linked genetic changes induced by CRISPRi with drug-induced alterations in metabolites. They established a reference map of metabolic changes through CRISPRi with 352 genes, allowing de novo predictions of compound functionality by comparing genetic and drug-induced metabolic changes. This approach identified antibacterials with unconventional modes of action, demonstrating the versatility of CRISPR technologies for high-throughput functional annotation of compound libraries and its potential impact on drug discovery across diverse biological systems. The future of metabolomics lies in seamlessly integrating metabolic information with protein and gene expression data at the single-cell level and in technical developments to increase the sensitivity and reliability of identification and quantification (Ali et al., 2022).

Discussion

The fusion of CRISPR technology with single-cell sequencing has transformed genetics research, offering an intricate understanding of genetic changes. Beyond the well-established scRNA-seq, scCRISPR-seq extends its potential to other modalities, encompassing DNA methylation, protein analysis, and imaging-based methodologies. Its utility spans across an array of biomedical research domains, ranging from cancer biology to microbiology.

Current endeavours predominantly focus on mitigating the limitations of previous techniques, with a focus on reducing costs, increasing scalability, and integrating different modalities. As technology rapidly evolves, new and innovative techniques arise. *In vivo* screenings emerge as a fusion of *in vivo* models with scCRISPR techniques, offering a dual advantage. However, the existing hurdles, particularly in the efficient delivery of guide RNAs and cost considerations, underscore the need for refinement. Non-invasive single-cell screenings, offering real-time insights into live cells, show promise but are so recent that scalability is still a major hurdle. Metabolomic screenings, though in their early stages, have enormous potential to reveal cellular responses to genetic changes.

Various techniques, each at different stages of development, have been explored, showcasing a spectrum of potential applications. While emerging approaches like non-invasive single-cell screening are in their early phases, more mature techniques, such as in vivo CRISPR screens, exhibit advanced development. In my perspective, the field with the most immediate and widespread utility in the coming years is *in vivo* CRISPR screens due to its relative advancement. As high-content CRISPR screenings gain further research attention, I anticipate a reduction in the cost per cell, opening avenues for larger-scale and more complex models. Additionally, the integration of modalities is a notable trend, where techniques combining transcriptomics with protein or imaging-based readouts are gaining traction and are likely to become more prevalent. The prospect of validating results through multi-modal approaches enhances the credibility of findings. In essence, this overview underscores the pivotal role of scCRISPR-seq in genetics research, spanning its historical roots, current capabilities, and future promise, expressing optimism for its continuous evolution and its impact on genetics.

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