Novel Approaches to Overcome CRISPR *In Vitro* Delivery Challenges

In order for CRISPR gene editing to reach its full potential, we must develop *in vitro* delivery approaches that will allow us to work with hard-to-transfect cells, large repair templates, multiplex editing projects, and minimise off-target effects

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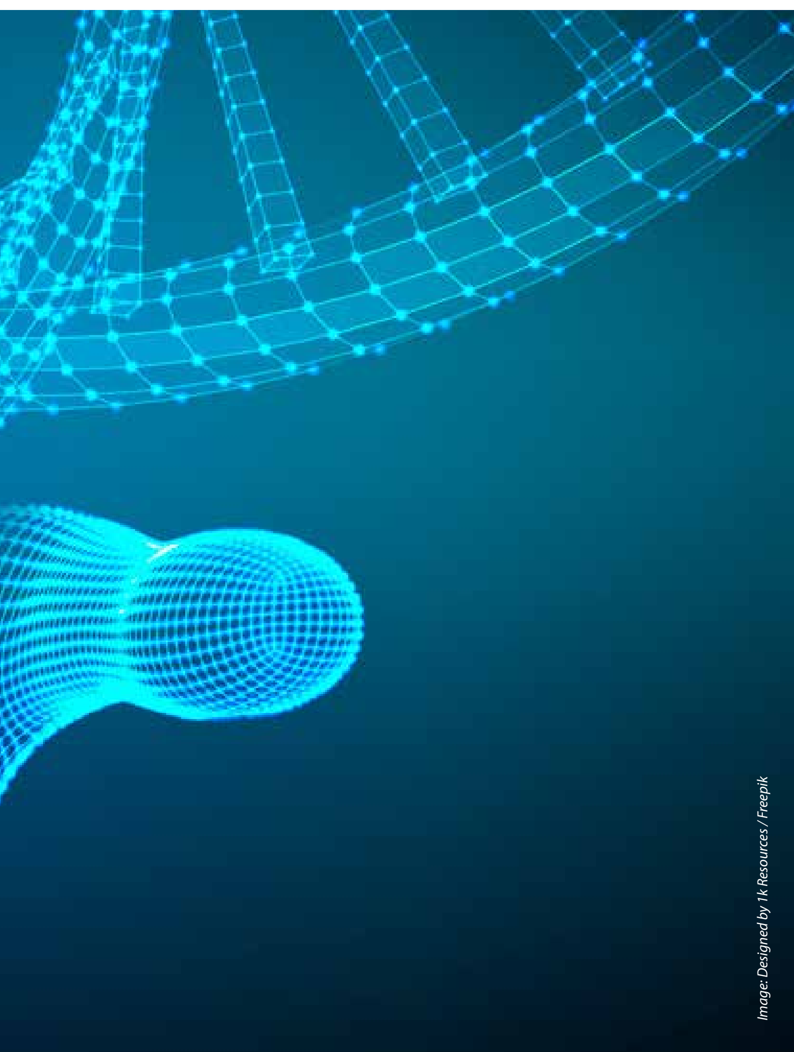
The discovery and development of the CRISPR system as a gene editing tool has been a major revolution in life science, from human health in gene and cell therapy, agricultural sciences, to fundamental biology where it is now used as a standard tool (1). Although CRISPR is widely used in research laboratories, many challenges regarding delivery and safety remain to be overcome, for both *in vivo* and *in vitro* applications, to fulfil its potential.

Safety is a key concern when considering gene therapy. Off-target mutations of the genome need to be minimised (2). This is also true when working in fundamental science, for example, when studying a signalling pathway where scientists need to ensure that the observed effect is caused by the targeted mutation and not off-target sites.

Another major challenge is the delivery of the CRISPR/Cas system into the nucleus of the targeted cells. *In vivo* is needed to pass through several barriers and be able to target a specific cell inside of a living organism. *In vitro*, the delivery of CRISPR/Cas system components into cell nuclei is often limited by cytotoxic and low efficiency methods. Batch transfection using lipofectamine or electroporation is well suited when working with immortalised cell lines and performing single knock out, which requires the delivery of a single guide RNA (gRNA) together with the Cas9 protein. The inherent cytotoxicity of these standard methods is overcome by the high number of cells that are available. In turn, these methods are much less appropriate when working with hard-to-transfect cells, large repair templates for homologous directed repair, or for multiplexing projects where many edits are required. Here, the use of different and novel transfection approaches becomes critical.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Challenge 1: Delivering CRISPR/Cas Systems to Hard-to-Transfect Cells

Conventional delivery methods like lipofection or electroporation can be:

- Very ineffective
- Highly toxic

This is the case for most primary cells, such as neurons and stem cells.

Viral transduction, especially using lentiviruses, can overcome these two main issues. The high efficiency and relative low toxicity have made viral transduction a method of choice, e.g., when working on CRISPR screening projects where millions of edited cells are needed (3).

Viral transduction, however, comes with its own drawbacks, which make it unsuitable for many applications, especially in therapeutics. The most important is undoubtedly the random insertion of the viral sequence into the host genome and the persistence of Cas9 expression. This is a vital issue in therapeutics where the safety of viral delivery approaches is a major concern, and has been shown to be responsible for severe pathologies (4-5). Random insertion is also problematic in fundamental research, where it might bring unwanted effects that can be difficult to distinguish from the effects induced by the CRISPR itself. Viral particles are also a biohazard and require higher level biosafety labs and apparatuses, which is a further limitation (6).

Researchers in immunology, haematology, CAR T cell therapy projects, and in biologics production often work with non-adherent cells. Some of these, such as B or T cells, are known to be very difficult to transfect (7). Therefore, finding a tool that can efficiently, gently, and safely deliver genetic material – or any kind of molecules – is particularly important for researchers in these fields.

Using novel injection approaches could be a solution for hard-to-transfect cells. Protocols of microinjection are well established for oocytes/zygotes. However, this approach fails to deliver into other mammalian cells efficiently and gently, mainly due to low viability and limited automation potential. Here, new technologies – like nano-injection with force feedback control – could overcome both viability and

automation limitation, thus offering a transfection solution for primary cells like neurons, T cells, and embryonic stem cells (8-9).

Cell Line Development with Hard-to-Transfect Cells
When creating a genome-engineered cell line, established top down approaches transfect millions of cells, and then go through an iterative and time-consuming selection process until they can identify, isolate, and expand the few clones of interest. This approach is well suited to immortalised cell lines; however, scientists working with rare or precious primary cells often cannot afford to lose thousands of cells as in these top down approaches. In contrast, bottom up approaches can start with a few cells and then keep track of each to isolate and expand only the selected cells of interest that have been transfected. Novel technologies that perform automated nano-injection and cell isolation are very promising in this regard (8, 10).

Challenge 2: Delivering Large Repair Templates for HDR

When precise editing of the genome is needed – for example, for protein tagging – the success of the CRISPR-induced mutation relies on the homologous-directed repair (HDR) pathway, an intrinsically low efficiency process. Many approaches have already been explored to increase this efficiency: for example, covalent binding of the repair template to the Cas9-gRNA RNP complex or synchronising the cell cycle of the targeted cells (11). Most of the current CRISPR gene editing projects, however, focus on small edits or insertions with small repair templates, as only these have a satisfactory HDR efficiency. Editing larger portions of the genome is of great interest, yet delivering large repair templates is a major challenge. Large insertions are, for example, needed when editing a cell to produce antibodies, or in immune-oncology projects involving the development of CAR T cells. Methods such as lipofection or electroporation usually fail to efficiently deliver large repair templates, together with the CRISPR/Cas system components, while viral transduction comes along with safety issues related to the use of viral particles.

Researchers who aim to achieve large edits have, therefore, been investigating the use of injection as a transfection method. Microinjection is well suited to deliver large repair templates, but is restricted to oocytes/zygotes as this approach has difficulty handling other mammalian cells. Technologies such as automated nano-injection with force feedback control of the nanosyringe overcome this issue and easily deliver large molecules (9).

Challenge 3: CRISPR Multiplexing

Targeting several loci in a multiplexing strategy approach is a growing trend in the life science field, whether for a multi-locus editing or for transcriptional regulation of many targets. This is particularly relevant for multi-genic disease studies or genome writing projects, such as de-extinction projects (12).

While editing a single locus with CRISPR/Cas system can be rather straightforward, editing several loci in the same cell is much more complex. Multiplexing faces two major challenges: the first is the toxicity brought by multiple double-strand breaks into the genome, which induces DNA damage response from the cell and can lead to apoptosis. The second is the simultaneous delivery of multiple gRNAs together with the Cas protein. Engineered Cas proteins have been developed to be able to read and process CRISPR arrays that encode multiple guide RNAs. However, creating these arrays is dependent on complex upstream molecular cloning steps, which prevent easy access and limit the number of

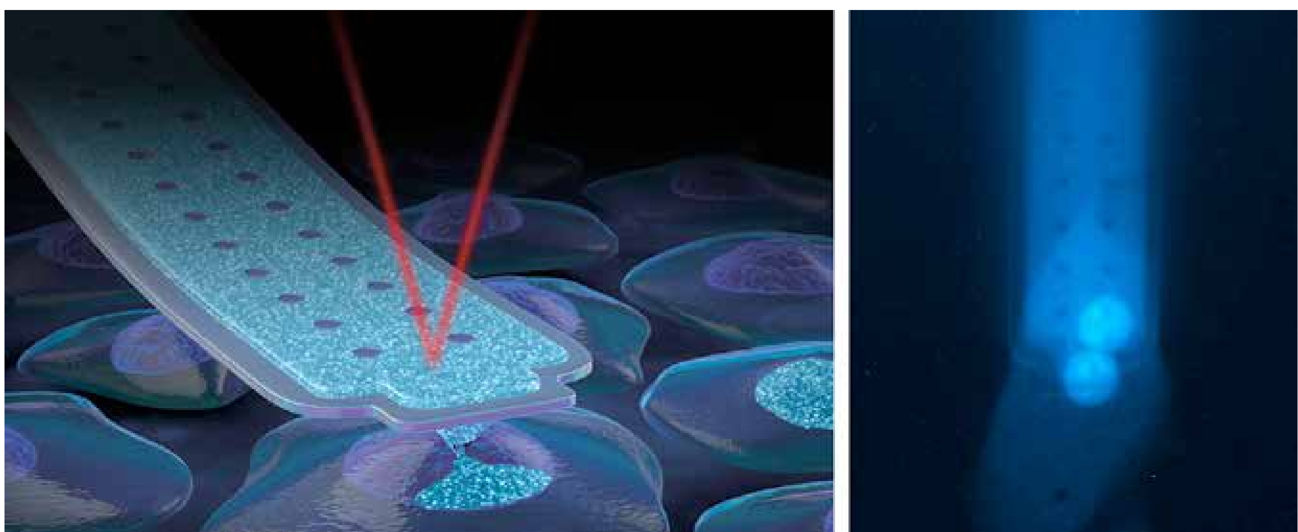

Figure 1: Delivering CRISPR/Cas systems into the nuclei of targeted cells. Newly developed methods, such as quantifiable force-controlled nano-injection, can help to overcome this challenge when working with hard-to-transfect cells, such as mouse primary hepatocytes, with large repair templates or in multiplex editing projects. Image courtesy of Cytosurge AG

gRNA that can be delivered. Today, the maximum number of genes that can be targeted in a multiplex editing experiment is limited to a few dozen – for example, 25 with the Cas12a or 30 targeted loci with Cas9 (13-14).

Emerging technologies could overcome this multiplex gRNAs delivery challenge. Nano-injection approaches can theoretically deliver hundreds of different gRNAs with a single injection into a specific cell (8). Toxicity might still limit the number of loci that can be edited per treatment when using Cas9. Injecting engineered Cas proteins, which do not induce double-strand breaks, might, therefore, help to push multiplex editing beyond this toxicity limitation. In that regard, base editors or dead Cas9 fused to an engineered reverse transcriptase enzyme for prime editing are particularly promising (15-16).

Challenge 4: Minimising Off-Target Mutations

Cas proteins can cut the genome at non-targeted loci, resulting in the apparition of off-target mutations (2). This creates concern over safety issues when using the CRISPR/Cas system for therapeutics. Several approaches have been developed to minimise the number of these off-target mutations, involving better engineering of new Cas proteins and gRNAs (17).

CRISPR off-target mutations are described to be a stochastic process that increases upon exposition time and Cas9 availability (18). However, traditional delivery methods, such as electroporation or lipofection, do not allow the precise control over the amounts of molecules that are delivered into the nucleus. Novel delivery technologies, such as quantifiable nano-injection methods, are very promising as they would allow the definition of precise dosage to optimise CRISPR efficiency while minimising off-target mutations (8).

Looking Ahead

It is essential to develop the ability to deliver measurable amounts of the CRISPR/Cas system components into the nuclei of cells, such as neurons, B cells, or embryonic stem cells, in a gentle process, which maintains high cell viability. It will open new doors for therapeutics development and fundamental research applications. It could also enable some of the more exotic research, such as de-extinction projects, which require high multiplexing capabilities.

New technologies tackling these delivery hurdles – e.g., automated force-controlled nano-injection and newly engineered Cas proteins – will help to increase CRISPR efficiency and unleash new applications in the gene editing field.

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Dr Paul Monnier obtained his PhD in Molecular Biology from University Pierre and Marie Curie in Paris, France, while working on non-coding RNAs involved in epigenetic growth control of the embryo. After a postdoctoral position at ETH Zurich, Switzerland, and working on CRISPR genetic screens in stem cells and X-chromosome inactivation, he joined **Cytosurge** to set up their new biology lab. Here, he develops applications and protocols related to FluidFM technology in the life science field.

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