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Review article

Single-cell transfection technologies for cell therapies and gene editing

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ABSTRACT

Advances in gene editing and cell therapies have recently led to outstanding clinical successes. However, the lack of a cost-effective manufacturing process prevents the democratization of these innovative medical tools. Due to the common use of viral vectors, the step of transfection in which cells are engineered to gain new functions, is a major bottleneck in making safe and affordable cell products. A promising opportunity lies in Single-Cell Transfection Technologies (SCTTs). SCTTs have demonstrated higher efficiency, safety and scalability than conventional transfection methods. They can also feature unique abilities such as substantial dosage control over the cargo delivery, single-cell addressability and integration in microdevices comprising multiple monitoring modalities. Unfortunately, the potential of SCTTs is not fully appreciated: they are most often restricted to research settings with little adoption in clinical settings. To encourage their adoption, we review and compare recent developments in SCTTs, and how they can enable selected clinical applications. To help bridge the gap between fundamental research and its translation to the clinic, we also describe how Good Manufacturing Practices (GMP) can be integrated in the design of SCTTs.

1. Introduction

Transfection, the intracellular delivery of nucleic acids and proteins, is a crucial part in the development of cell-based therapies. Cell-based therapies are innovative approaches making use of cells that have been genetically engineered to replace defective organ functions, treat diseases or model physiological and pathological behaviors in vitro. The most promising example so far is Chimeric Antigen Receptor (CAR) T cell immunotherapy: by introducing a tumor-targeting receptor in a cancer patient's T cells, they are turned into a "living cancer drug", essentially reprogramming the patient's immune system. CAR T cell therapies have shown tremendous success against B cell malignancies and two commercial products have recently been approved by the Food and Drug Administration for the treatment of lymphoma [1].

The clinical applications of genetically engineered cells are numerous. CAR T cells have also been harnessed to combat HIV. Other immune cells that have been reprogrammed to treat cancer include natural killer cells and macrophages [1]. Gene editing of stem cells offers the possibility to cure monogenetic diseases and generate a source of replacement tissues. Induced Pluripotent Stem Cells (iPSC) can generate more predictive, humanized disease models for patient stratification and drug discovery and testing, so-called "clinical trials in a dish". There is no doubt that modified human cells will take an important place in tomorrow's pharmaceutical toolbox [2–8].

Conventional transfection methods used to engineer cells have, however, significant limitations. The use of cuvette-based electroporation, cationic liposomes and viral vectors in scaled up manufacturing of cell products is hindered by low cell viability and delivery efficiency, virtually inexistent spatiotemporal resolution and labor-intensive, variability-prone manual operation. Manufacturing capabilities for clinical-grade viral vectors are inadequate, resulting in high prices and restricted availability [1,9].The use of viral vectors also introduces safety risks linked with insertional mutagenesis, uncontrolled expression of gene constructs and immunogenicity [10].

Single-Cell Transfection Technologies (SCTTs) are a promising alternative. We define them here as technologies capable of inducing

Abbreviation: Single-cell transfection technology, SCTT; Good Manufacturing Practices, GMP; Chimeric antigen receptor, CAR; Induced pluripotent stem cells, iPSC; Dendritic cell, DC; Antigen presenting cell, APC; Human leukocyte antigen, HLA; T cell receptor, TCR; Cas9-guide RNA ribonucleoprotein complex, RNP; Organ-on-Chips, OoC; Microelectrode arrays, MEA; Mouse Embryonic Fibroblast, MEF.

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membrane permeation through actuation at the single-cell level, allowing for a more efficient and less disruptive transfection. Moreover, they can feature substantial dosage control over the cargo delivery, single-cell addressability and integration in microdevices comprising multiple monitoring modalities.

This work presents a critical overview of the latest advances in SCTTs. We first list a selection of clinical applications and their requirements for more sophisticated transfection methods. Second, we address the most promising SCTTs published in the literature during the last five to ten years and explain how we expect their unique properties to drastically benefit, if not enable, the selected clinical applications. Finally, as we aim at fostering the further development of SCTTs and, most importantly, their translation toward clinical applications, we will discuss different aspects of the Good Manufacturing Practices (GMP) that govern the commercialization of safe and qualitative medical products.

2. Applications in cell-based medicine

2.1. From fundamental research to clinical applications

In this section, we highlight clinical applications whose key needs are not optimally met by conventional transfection methods. We argue that manufacturing of cell-based therapies, with an emphasis on immunotherapy, gene editing, iPSC technology, and personalized disease models, will be drastically improved by advanced transfection technologies, in particular SCTTs.

2.2. Immunotherapies

In the immunotherapy field, CAR T cell therapies currently receive the most attention in clinical trials [6]. Briefly, T lymphocytes are extracted from the patient's blood and are genetically modified to express an artificial receptor (the CAR) granting antitumoral activity. Up to a few hundred million modified cells are then reinfused back in the patient to fight the cancer [9,11,129]. Usually, the average manufacturing process takes about two weeks [12]. FDA-approved CAR T cell therapies have provided ample clinical evidence for the feasibility of an approach that requires ex vivo genetic engineering. However, the cost and complexity of the manufacturing process has been blamed for slowing down wide therapy uptake. Automation and miniaturization are expected to be key in solving these critical issues. Non-viral CAR delivery has been earmarked as a crucial step, and in addition, is promising improved safety over the use of viral vectors [13].

Electroporation-mediated delivery of mRNA has been shown as a promising alternative to viral vectors. Delivery of the CAR in mRNA form offers lower cytotoxicity than plasmid transfection and poses no risk from random genomic integration [14,15]. Cells engineered with mRNA show fast, transient expression of the CAR and require repeated infusions to sustain an antitumor effect. This dosage and temporal control over the CAR activity can reduce on-target, off-tumor effects and is thought to open CAR T therapies to solid tumors. Delivering CAR-encoding mRNA into freshly extracted peripheral blood mononuclear cells obviates the need for activation and expansion steps, which can reduce treatment manufacturing to a single day, thus reducing cost and the risk of disease complication while the treatment is produced [16]. Limiting the culture time ex vivo also enhances antitumoral activity by preventing T cell exhaustion [17]. This approach is currently being investigated in a Phase 1 clinical trial by the company MaxCyte as part of their CARMA (CAR mRNA) program, based on CAR mRNA delivery into non-activated lymphocytes for the treatment of solid tumors (NCT03608618). Finally, delivery of a purified mRNA construct, from which double strand RNA structures have been eliminated, drastically enhances the efficacy of the engineered CAR T cells [18].

Electroporation is the method of choice to deliver nucleic acids in primary immune cells [14]. Conventional bulk electroporation, however, perturbs T cell function [19]. The low throughput and manual operation of the method are additional issues. A suitable transfection method should robustly and reproducibly deliver large nucleic acids in around a billion suspended cells while preserving their immune function. In-flow transfection methods with high throughput are therefore best suited and will be discussed below. The company Cellectis, for instance, is currently developing universal CAR T cells therapies. It uses a proprietary bulk electroporation system to deliver mRNA-encoded gene editing enzymes targeting the TCR of the processed immune cells. Cellectis has multiple ongoing clinical trials (e.g. NCT04142619).

The immunotherapy field is also interested in the modification of Antigen Presenting Cells (APC) to act as cancer vaccines. APCs are scouts of the immune system that can organize an immune response against specific antigens. In the procedure, a patient's dendritic cells (DCs, which are potent APCs) are harvested and loaded with tumor-specific antigens ex vivo, which can be peptides, proteins or nucleic acids obtained from the tumor. They are then reinfused in the patient, where they will present the antigens to immune cells and prime an immune response against the tumor [20].

Electroporation is an effective way of delivering mRNA-encoded antigens or tumor lysate in DCs [21–23]. An advantage of the method is the possibility of targeting multiple mutant, tumor-specific antigens rather than a lineage-specific protein (such as CD19 in the case of CAR T therapy for lymphoma). It would enable the treatment of solid tumors, so far difficult for CAR T cells. While DC vaccines are safer than CAR T therapies, they have not yet demonstrated a similar efficacy [24]. Other cells more abundantly present in blood than DCs, including B cells and T cells, can also perform APC functions. Peptide antigen delivery in those cells is a simple, yet promising approach to improve cancer vaccines and modulate the immune system [14,25,26].

Delivery of gene editing molecules, such as the CRISPR-Cas9 enzyme and its guide RNA, is being investigated to improve the efficiency and availability of cell therapies. In immunotherapy, knocking out immune checkpoint receptors naturally present on T cells can prevent tumor cells relying on immunoinhibitory ligands as an evasion strategy. Gene editing is also key toward developing universal, off-the-shelf CAR T cells for allogeneic adoptive cell therapies. Indeed, deriving CAR T cells from a single donor to treat multiple patients would decrease the cost per treatment [1]. To reach this goal, knocking out endogenous T Cell Receptor (TCR) and Human Leukocyte Antigen (HLA) on allogeneic CAR T cells have been used as strategies to limit graft-versus-host disease and transplant rejection, respectively [27]. Gene editing is also applied to blood cells to treat other genetic diseases. For instance, transplantation of autologous hematopoietic stem cells, in which a single gene has been corrected, could cure inherited monogenic blood diseases such as sickle cell anemia [28].

The main obstacle against the clinical use of CRISPR-Cas9 technology lies in the off-target effects associated with high cellular concentrations of Cas9-guide RNA ribonucleoprotein complex (RNP) that arises from uncontrolled expression of gene constructs encoding the nuclease and its guide RNA. The transient, dosage-controlled intracellular delivery of the RNP, rather than nucleic acids, can minimize these effects [29]. Moreover, the avoidance of a DNA vector eliminates risks of genomic integration and the associated safety concerns. Several SCTTs offer such dosage control and are discussed below.

2.3. Induced pluripotent stem cells

Over a decade ago, researchers demonstrated that the introduction of four selected transcription factors in terminally differentiated somatic cells could reprogram them to gain embryonic stem cell-like pluripotency and proliferative potential [30]. Those induced Pluripotent Stem Cells (iPSC) have been lauded as a breakthrough in regenerative medicine and as a powerful tool for in vitro drug testing and development of personalized, more predictive disease-in-a-dish models [8]. Indeed, the collection and reprogramming of patient cells would allow for the in

vitro creation of a patient-specific tissue. Recent studies aim at using iPSC transplantations to treat spinal cord injury [31], macular degeneration and thalassemia [4]. Other promising results show, for instance, that transplantation of iPSC-derived dopaminergic neurons can alleviate symptoms in primate models of Parkinson's disease [32].

So far, iPSCs have been mostly adopted in disease modelling and drug screening [4,8,28,33], since In vitro study of patient-derived cells is a more accurate representation of processes happening in vivo in comparison to immortalized cell lines. Clinical trials in a dish are expected to give faster and more predictive pre-clinical studies of drug toxicity and efficacy [8]. Finally, iPSCs differentiated into distinct tissues could be used to create personalized disease models inside microdevices that integrate cell monitoring and stimulation modalities, so called Organ-on-Chips (OoC).

The limited understanding of the reprogramming barriers and lack of an appropriate transcription factor delivery method has resulted in low reprogramming efficiencies and prevented their adoption in the clinics [7,34]. A method that can deliver stoichiometric quantities of the different transcription factors has been identified as a key need to improve reprogramming efficiencies [35–37]. However, the different reprogramming factors are usually distributed across multiple plasmids and delivered in a series of transfection events that make dosage control difficult. Reliably delivering a controlled dose of a single, large plasmid containing each factor with optimal stoichiometry would be beneficial. Another approach, generating iPSCs through delivery of mRNAs [38] and proteins [39], is a DNA-free alternative leading to fast reprogramming. It eliminates any possibility of transgene integration in the cell's genome but may reduce control of delivered amount.

Transfection of iPSCs with lineage-specific transcription factors is a fast method to generate fully differentiated cells, compared to the weeks-long step-by-step protocols using a sequence of different culture conditions and growth factors. The shorter culture period will reduce cell heterogeneity within a cell line for more accurate analysis. Finally, an efficient way to generate a large and homogenous population of a given iPSC-derived cell type will benefit high-throughput screening of molecular compounds, which require large amounts of cells [40].

Among the most cited public health priorities, the study of neurodegenerative diseases will likely benefit most from OoC development. OoC are well suited for the investigation of neuronal networks, where pathological electrophysiological activity can be non-invasively recorded and analyzed through microelectrodes patterned on the device [41]. For instance, Wainger and colleagues have grown iPSC-derived motor neurons obtained from amyotrophic lateral sclerosis patients on a microelectrode array. Through electrophysiological monitoring, they could reveal the mechanisms underlying their hyperexcitability and identify a potential drug to treat this disease [42]. Such OoC will benefit from technologies that can "print tissues" with a physiologically relevant structure directly in the device. Through spatially resolved delivery of transcription factors in iPSCs, complex networks of different neuron types can be created, allowing for a tissue-level study of disease mechanisms.

2.4. Key needs for enabling clinical applications

From the above, it is clear that several key needs exist which are insufficiently met by current transfection technologies. Specifically, there is a demand for approaches that provide: non-viral delivery, especially for manufacturing cell therapies; delivery of non-DNA cargos such as mRNA, RNA, proteins and RNPs; robust and reproducible delivery, yielding a homogenous response from the target cells; high throughput; high efficiency and low toxicity; control of cargo dosage and stoichiometry; and spatiotemporal control of cargo delivery. Transfection technologies should also be amenable to automation, integration and miniaturization, preferably in a closed-loop configuration. Finally, they should facilitate GMP compliance of the entire manufacturing process that they are part of. In the next paragraph, we describe how SCTTs could resolve these outstanding challenges, stimulating adoption of state-of-the-art research results in the clinic.

3. SCTTs: A transition to next-generation transfection methods

Here, we present and discuss the most promising examples of SCTTs for clinical applications. SCTTs have the defining ability to reliably apply a membrane permeation stimulus through actuation at the single-cell level, rather than exposing an entire population of cells to a global stimulus. Those technologies offer higher yield, throughput, safety, versatility in cell and cargo types or scalability than conventional transfection methods. Among other features, some also display dosage control abilities and 2D spatial resolution that are unattainable with bulk transfection techniques. Moreover, they can display single-cell sensing modalities to monitor the membrane permeation process and the cell recovery. An overview of the different technologies discussed here is provided in Fig. 1 and Table 1. For conciseness, we limited our study to in vitro and ex vivo techniques having demonstrated delivery of nucleic acids and proteins, which is the main road toward cell engineering. Except for traditional microinjection, we have here selected the SCTTs having demonstrated almost perfect permeation efficiency and a transfection yield above 50%, which is defined as the fraction of surviving cells that is successfully transfected.

3.1. Microinjection

Microinjection was invented over a century ago [43] and is still widely used to introduce large cargos into cells and sample their content. Simply put, a sharp glass micropipette is punched through a cell's membrane, possibly in a targeted cellular compartment [44], and precise quantities [45] of its content can be delivered (Fig. 2A). Virtually any cargo type can be microinjected. The technique is ideally suited for processing samples of rare and precious cells and delivering very large cargo, such as during in vitro fertilization or nuclear transfer for cloning [14]. Apart from the difficulty of targeting small cells and cells in suspension, and the contamination-prone open dish procedure, microinjection suffers from low throughput and tedious operation: a skilled technician may process only tens of cells per hour.

Subsequent work has focused on automation: thanks to a microfluidic trap array with an open top, up to 250 suspended cells can be held at the same time under a microscope camera. Then, an image analysis routine detects the position of the cells and instructs a robotic arm-mounted pipette to target them for injection [46]. As such, this is an improvement for the processing of small pools of rare cells. However, the throughput is still limited by the holder size, limiting the utility of the technique for most applications. More recently, an innovative microfluidic approach consisting of a 2D array of single-cell traps enclosed in a microfluidic device has been developed. Etched in a silicon substrate, each trap contains a sharp, sub micrometer-sized spike and aspiration perforations [47]: Cells aspired in the traps get punctured by the spike. After their release and incubation with the cargo solution, the large, single pore created on the cell membrane allows for the uptake of large molecules (Fig. 2B). Remarkably low toxicity is shown while achieving more than 75% of plasmid transfection efficiency in primary human T cells, which makes it a promising tool for the delivery of CAR-encoding nucleic acids. However, the current design comprises 2500 capture sites per mm^2, and processing of millions of cells would require large devices possibly incompatible with microfluidics. This invention is currently being developed by a startup, Basilard BioTech, under the name SoloPore.

3.2. Electroporation

In electroporation, electric current pulses are delivered to a cell to transiently permeabilize the plasma membrane. Electroporation was popularized in the early 1980's [48] and is now widely used in

Table 1
Summary of SCTTs.

	Automated Microinjection	Microfluidic Microinjection	Cell Squeeze	Microfluidic Vortex Shedding	In-Flow Electroporation	Nanopore/ Nanostraw Electroporation	Electroporation on Microelectroe Array	Optical injection	Plasmonic Optical Injection
Delivery efficiency for small molecules*	Dextran: 58–88%	PI:93%	Dextran 3 kDa:50–90% 70 kDa:25–45% Antibodies:~35%	Not mentioned	Dextran 10 kDa: up to 95%	PI: >95% miRNA: Up to 100% Protein: Up to 80%	PI: 90%	Calcein efflux: up to 100%	Small Dye: 95% Dextran 10 kDa: 40%–90% 500 kDa: 25%–50% 2000 kDa: 45%
Survival rate	63–82%	~100%	50–95%	70%–95%	60%–90%	90–100%	Not mentioned	60%–90%	70–95%+
pDNA and mRNA transfection efficiency	pDNA: 18% (expression) mRNa: up to 80%	pDNA: 50–90%	(Electrophoresis aided) pDNA: up to 90%	mRNA: Up to 65%	pDNA: 70% - 90%	pDNA: 70%–90%	Not mentioned	pDNA: 55%–90%	pDNA: 45%–100%
Adherent cells	Yes	No	No	No	No	Yes	Yes	Yes	Yes
Suspended cells	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes
Cell amount / rate	~250 cells per chip ~2 min per chip	10^4 per chip, 2500 / mm^2 of chip surface	10^6–10^7 per chip (clogging) Up to 10^7 cells/s	2 × 10^6 cells / s	10^4–10^6 cells/s	4 × 10^4 per cm2	~100–10^4 per chip	5 cells/s, +10^6 per dish	20 cells/s in single cell mode, +10^6 per dish
Single cell addressability of transfection	Yes	No	No	No	Through IM-triggered EP	No	Yes	Yes	Yes
Built-in monitoring modalities	Microscopy	–	–	–	IM DEP sorting	Cytosol sampling (with nanostraws)	IM DEP	Microscopy	Microscopy
Chip integration	No	Yes	Yes	Yes	Yes	Yes	Yes	No	No
Main advantages	Delivery of large cargos, targeting subcellular compartments, Dosage control	Efficiently delivers plasmids in primary cells.	Simple to scale up, Little cell disruption, Fast processing rate	Simple to scale up, Little cell disruption, Fast processing rate	Efficient NA delivery, Fast processing rate, Can comprise monitoring modalities	Delivery of very large plasmid possible, Little cytotoxicity, Dosage control	Spatially-resolved delivery, comprises many monitoring modalities.	Spatially-resolved delivery, Virtually sterile.	Spatially-resolved delivery, Highest transfection efficiency.
Main disadvantages	Very slow processing rate. Open dish environment. Low plasmid expression if not injected in nucleus.	Challenging to scale up.	No unaided delivery of plasmids. Cell size variations may alter molecular uptake.	Lower efficiency compared to other in-flow techniques.	Possible disruption of cell function.	Challenging to scale up.	Limited processing rate and scalability.	Slow processing rate. Sensitive to laser misfocusing.	Use of nanoparticles or microfabrication intensive substrates.
Relevant applications	Nuclear transfer, Injection of particles	Car-T, NA delivery	Cancer vaccines, small molecule delivery	Car-T, mRNA delivery	Car-T, NA delivery	iPSC generation, Car-T	Tissue engineering, Clinical trials in a dish	Tissue engineering, Clinical trials in a dish	Tissue engineering, Clinical trials in a dish
Commercial applications / startups	–	Basilard Biotech	SQZ Biotech	Indee Labs	MaxCyte, Kytopen	–	–	–	TrinCE
References	[45,46]	[47]	[19,26,54,56,87–89]	[90]	[61–68,71]	[57,58,72–82]	[59,60,83–85]	[100–103,105]	[55,111,113–122,125,126]

Table 1) Summary of the SCTTs presented in this review. pDNA: Plasmid DNA; PI: Propidium Iodide; IM: Impedance Monitoring; NA: Nucleic Acids; DEP: Dielectrophoresis * The delivery efficiency for small fluorescent dyes is representative of the permeation efficiency. Dextrans are used as model cargo molecule to evaluate a transfection technology's ability to deliver proteins and small macromolecules in cells, compared to large nucleic acids.

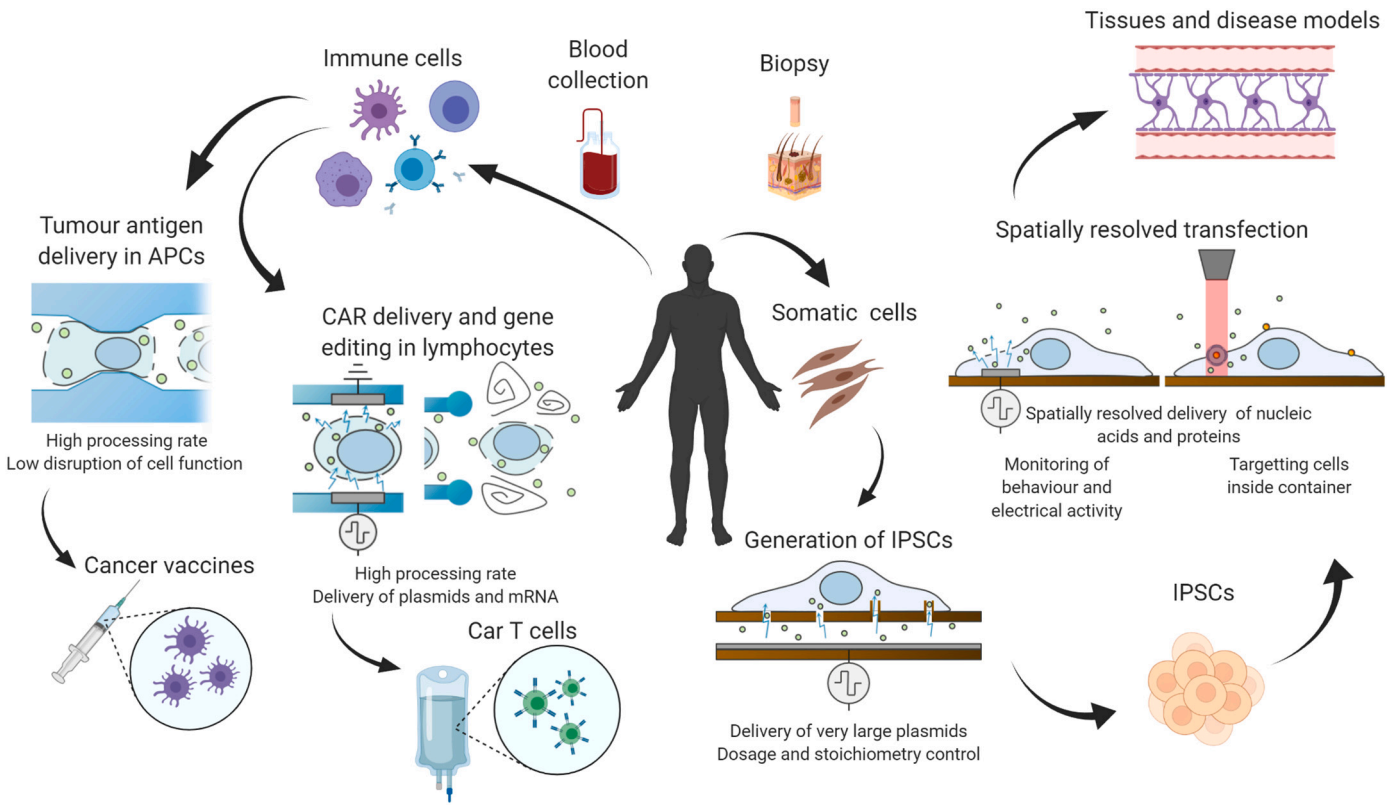

Fig. 1. Main applications of the most mature SCTTs presented in this work. On the left of the figure, immune cells are extracted from blood. In-flow transfection methods with high processing rates are used to deliver tumor antigens or nucleic acids in order to produce cancer immunotherapies. On the right, somatic cells such as skin fibroblast are taken from a tissue biopsy. Delivering large reprogramming plasmids in those cells with nanopore electroporation generates induced pluripotent stem cells. Then, the spatially-resolved delivery of differentiating factors in those iPSCs is used for the creation of more complex disease models and tissues with spatially designed architecture. Created with BioRender.com.

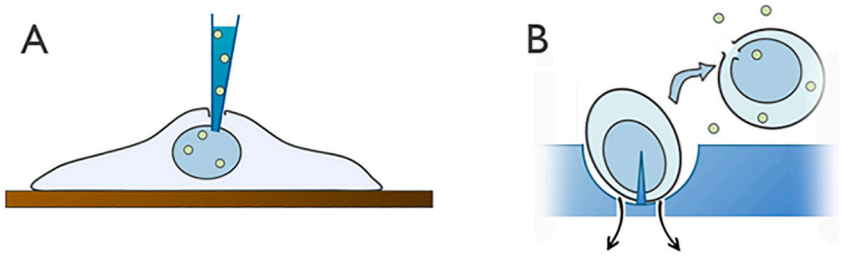

Fig. 2. Microinjection implemented in SCTTs. A) Traditional microinjection B) Microfluidic microinjection.

commercial transfection devices (for instance, the Nucleofector, commercialized by Lonza and the Neon Transfection System, marketed by Thermo Fisher Scientific). GMP-compliant platforms for non-viral transfection mainly rely on electroporation. They can make use of disposable cuvettes (Lonza's 4D-Nucleofector, Cellectis's Pulse Agile), enclosed fluidic systems with disposable elements (MaxCyte's devices), or fully integrated and automated cell manufacturing platform (CliniMACS Prodigy). They typically perform bulk electroporation: suspended cells are mixed with the cargo of interest and dispensed between two large electrode plates fitted in a cuvette. As a high voltage pulse is applied between the two plates, cytotoxicity is caused by over-exposure to high electric fields, pH changes, heating and release of toxic metal ions. The heterogenous distribution of the electric field causes variable exposure at the single-cell level, impacting individual cell survival and transfection efficiency [49].

Electroporation is a valuable technique to deliver nucleic acids: it triggers the uptake and intracellular trafficking of plasmids toward the nucleus, and electrophoresis during electroporation has been shown to drag nucleic acids into the target cells [50–52]. It is a major advantage over other physical transfection methods, which may struggle with the delivery of large charged molecules that are electrostatically repelled by the cell membrane or stall near the entry point in the cytosol [53–56].

Miniaturization of electroporation devices allows to perform controlled permeation at the single-cell level and offers other advantages: 1) Reduction of the distance between the electrodes allows to achieve similar electric fields, thus electroporation, at lower voltages, and smaller electrodes can apply highly localized currents to only electroporate a patch of the cell membrane for reduced cytotoxicity [57,58]. 2) In microdevices, reliable cell positioning with respect to the electrodes, as well as predictable electric fields further improve transfection efficiency [49]. 3) Miniaturization of the electroporation electrodes enables integration in devices possibly comprising thousands or millions of them, i.e. microelectrode arrays, that may also feature additional cell interfacing modalities [59,60]. Thus, single-cell electroporation devices can display higher efficiency, cell survival and control over transfection. Single-cell electroporation devices can be divided in

three main categories: In-flow electroporation, nanopore electroporation and electroporation on microelectrode arrays (Fig. 3).

3.2.1. In-flow electroporation

In-flow electroporation devices are simple microfluidic devices typically made of PDMS, glass or silicon fitted with electrodes. Suspended cells are flown in the microchannel, where an electric potential is applied between the electrodes, creating regions of high electric field strength. The duration and amplitude of the electric "pulse" applied to the cells can be adjusted by modulating the flow rate, channel geometry and number of electrodes. The ability to process cells suspensions grants those devices high throughput, from 10^4 to 10^8 cells per minute with close to 80% efficiency and cell viability [61,62].

Multiple approaches have been taken to increase transfection efficiency, e.g. fitting electrodes at both ends of the microchannel and alternating regions of high and low electric fields to permeate the cells [61,62], or hydrodynamically rotating the cells in the channel to improve efficiency by uniformly exposing the cell membrane to high electric fields [63–65]. A different approach is patterning microelectrodes in the channel. Wei et al. developed a laminar flow electroporation device that uses hydrodynamic flow focusing to align the cells between two longitudinal electrodes patterned on the entire length of the channel. The focusing greatly enhanced cell survival and transfection efficiency by reliably positioning the cells in the center of the channel and preventing exposure to the harsh conditions in the vicinity of the electrodes [66]. Addition of a dielectrophoretic sorter at the end of the electroporation channel to automatically remove cells that were lysed by the electroporation allowed to transfect fragile or hard-to-transfect cells, with viability varying from 20% to more than 80% in certain cases thanks to sorting [67]. Another device, featuring interdigitated comb electrodes that invert the electric field multiple times along the channel, boasts a very high delivery yield [68]. For most cell lines, in-flow electroporation often displays efficiencies in delivery and survival rates in the range of 70%–80%, sometimes up to 90%. Those numbers vary greatly with different cell types and can fall by half for some primary cells, showing that some cell types may be more amenable to electroporation than others.

Fitting electrodes in the microchannel opens the door to label-free electrical measurements [69]. Applied to electroporation, changes in membrane conductivity induced by electroporation can be quantified when a cell passes between a pair of electrodes by impedance measurements [70]. Impedance measurements have also been used to detect cells, trigger electroporation and monitor its outcome [71]. These built-in process monitoring strategies would be a step toward in-line quality control in cell therapy manufacturing.

Overall, in-flow electroporation devices show high processing rates, relatively high delivery efficiency for a variety of molecules and can easily integrate sorting and monitoring modalities based on electrical measurements. That is why they are a promising tool for manufacturing of CAR T therapies by delivering CAR-encoding plasmids or mRNA into T cells as well as gene editing RNP complexes. Consequently, in-flow electroporation emerges as a valuable tool for CAR T and APC engineering, thanks to its ability to deliver nucleic acids and proteins alike. The main commercial instance of in-flow electroporation is currently marketed by MaxCyte, which uses its proprietary, GMP-compliant, in-flow electroporation device to pursue clinical trials based on mRNA-engineered CAR T cells (NCT03608618). Finally, Kytopen, a recently founded biotech startup, is developing an in-flow electroporation technology aimed at gene-editing and research on cell therapy. The technology, called FlowFect, uses a microchannel with a narrow section. There, the electric fields are locally amplified, which allows for a controlled and efficient electroporation of cells flown in the device.

3.2.2. Nanopore electroporation

In nanopore electroporation devices, cells are funneled into nano-sized channels connected to a chamber filled with the cargo solution. Electric currents passing through the nanochannel are strongly condensed and electroporate a localized area of the cell membrane. Maintaining the electric field electrophoretically "injects" charged molecules in amounts controlled by the pulse duration. Nanopore electroporation offers virtually no cytotoxicity and fast expression of the delivered plasmids [72]. This efficient delivery platform was later applied to monitor the up- and downregulation of oncogene networks with delivery of miRNAs and RNA molecular beacons in AML cells [57]. In further nanopore electroporation designs, cells were grown on microfabricated planar nanoporous membranes with 40,000 pores per cm^2. Proof-of-concept studies have shown that the platform can transfect cardiomyocytes and also deliver very large reprogramming plasmids (13 kb) in Mouse Embryonic Fibroblasts (MEF) with 90% transfection efficiency, generating close to 1000 times more iPSC colonies than bulk electroporation [73]. Additionally, successful transfection of mice embryo primary neurons [74], reprogramming of MEF into iPSCs [75] and transdifferentiation of MEFs into neurons was demonstrated. Expression of neuronal phenotypic markers was faster than upon bulk electroporation and viral transfection [58].

Further improvements enabled the attraction of suspended immune cells to the pores by dielectrophoresis, extending the utility of the platform to non-adherent cells. After delivery of a CAR/GFP-reporter plasmid in natural killer cells, around 80% of the cells loaded on the chip showed GFP expression with little cell death [76]. As an alternative to dielectrophoresis, centrifugation of suspended cells deposited on the nanoporous membrane permitted to yield similarly high delivery efficiencies of proteins and nucleic acids and cell survival [77]. Such high transfection yield and low cell disruption combined with the possibility of using cell suspensions for higher throughput make it a very promising example of SCTT for both adoptive immunotherapies and iPSC generation. However, increasing the throughput will likely encounter the same problems as in the microfluidic microinjection device [47]. Prolonging the pores using nano-sized tubes [78–81], called nanostraws, additionally enables sampling of the cytosol thanks to a tight seal between the tube apertures and the cell membrane [82]. Finally, electroporation on nanostraws has been shown to minimally disturb cell division and gene expression [81].

The possibility to deliver controlled doses of very large plasmids with nanopore electroporation is an important step in iPSC research. Not only does packing of all reprogramming TFs in a non-viral vector limit the

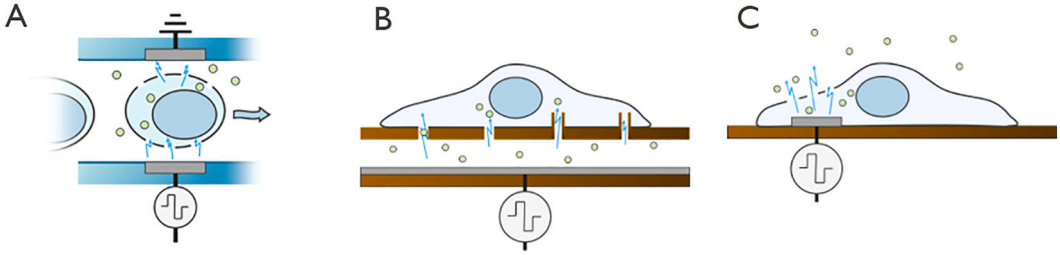

Fig. 3. Electroporation implemented in SCTTs. A) In-flow electroporation B) Nanopore / nanostraw electroporation C) Microelectrode array electroporation.

safety risks associated with viruses and possible uncontrolled expression of oncogenes [30], but tuning the ratios of the TFs including miRNA in a single plasmid to improve reprogramming efficiency will allow researchers to overcome the biological processes currently impeding cell reprogramming [7,58]. The co-delivery of a cocktail of mRNAs and miRNAs in such a device could also offer a higher-efficiency alternative to plasmids [7]. Combining a nanopore electroporation/Nanostraw device with a microfluidic system could in principle allow to formulate a solution composed of different cargos. Different cargo solutions could be sequentially or simultaneously pumped into the system for delivery, which would allow dosage and temporal control over transfection.

3.2.3. Electroporation on microelectrode arrays

The last type of electroporation devices consists of microelectrode arrays (MEA). In a MEA, micrometer-sized electrodes can electrically interface with individual cells for electrical stimulation and monitoring with an enormous versatility and at single-cell resolution. For instance, electrophysiological recordings and impedance monitoring offer a label-free real-time assessment of cell function and status [59]. Impedance monitoring can also be used to detect cells growing on top of the electrodes and to evaluate the quality of the cell-electrode coupling [83]. While there are examples of cell transfection on larger electrodes, instances of single-cell transfection on MEAs are scarce and only a few key studies that demonstrate proof of concept can be found.

By fitting a MEA in a microfluidic chamber comprising an array of electroporation/impedance monitoring electrodes and an array of positioning dielectrophoresis electrodes [60,84,85], suspended cells flown in the device can be "trapped" by dielectrophoresis to promote adherence on the electroporation/impedance monitoring electrodes. An electric pulse can deliver cargo such as plasmids into adherent cells while adhesion dynamics, resealing of the membrane and cell survival are assessed in real time through impedance monitoring.

MEAs are the ideal tool to evaluate drug efficacy and toxicity and study disease models of electrogenic cells. In our research, we are leveraging a state-of-the-art CMOS MEA for single-cell transfection and monitoring. The high density of individually addressable electrodes enables stimulation and electroporation, impedance monitoring and electrophysiological recording with single-cell addressability [59,86]. The chip has been used to successfully deliver plasmids in HeLa cells and human fibroblasts (our unpublished results). As for nanopore electroporation, the small size of the electrodes guarantees minimal cytotoxicity.

The main strength of MEA electroporation, compared to most other SCTTs, is plasmid delivery with single-cell addressability. By sequentially delivering different reprogramming or differentiation plasmids into adjacent cells, it is possible to generate complex cell patterns on the surface of the chip, to "print tissues". Combined with iPSC technology, MEA electroporation has profound implications in the creation of personalized disease models, in which the monitoring abilities can be used for label-free assessment of pathological cell functions and responses to drugs or gene editing treatments [42]. As such, it could greatly benefit the creation of brain-on-a-chip models from patients suffering from neurodegenerative diseases.

3.3. Mechanical stress in microchannels

In 2013, an intracellular delivery method based on rapid mechanical deformation of the cell's membrane rose to prominence. The technique, coined "cell squeezing", is outstandingly simple: as the cell flows at high speed through a narrow microchannel in a silicon chip, the transient pores created during the "squeezing" allow for molecules in the cell's surroundings to diffuse into its cytosol [54] (Fig. 4A). The technique can process up to millions of cells per minute and only requires a controllable pressure source to be operated. The channel constriction's geometry and number, as well as the target cell flow rate need to be adapted to specific cell types and sizes. Disadvantages of the technique may arise in the tedious optimization of the constriction geometry and flow rate for each cell type and the cell-to-cell variability in uptake of molecules due cell size distribution. Finally, it would benefit from the integration of monitoring modalities to track the delivery process.

Cell squeezing has been shown to deliver a large panel of small or uncharged molecules: dextrans, proteins, RNPs, nanoparticles and siRNA could be delivered in varied cell lines and primary cell types [19,26,54,87–89]. Multiple primary cell types were processed and kept above 75% of cell viability, while embryonic stem cells retained above 50% viability. They demonstrate the generation of iPSCs by sequential delivery of the four Yamanaka factors as recombinant proteins in human fibroblasts, resulting in ten times more colonies than when performed in a commercial electroporator [54]. Moreover, cell squeezing, compared to bulk electroporation, does not disrupt cell function: T cells edited via squeezing had higher tumor-killing properties than cells processed with bulk electroporation and displayed no release of cytokines, and squeezed hematopoietic stem cells had undisturbed proliferative and differentiation abilities (bulk electroporated hematopoietic stem cells were heavily disturbed) [19].

Cell squeezing has been developed by a company, SQZ Biotech, investigating the generation of APCs for cancer vaccines and immunomodulation (clinical trial NCT04084951). Protein antigens have been loaded in B cells and successfully presented at their surface. The loaded B cells induced activation and proliferation of CD8+ T cells [26]. In overall, cell squeezing appears as one of the most promising technologies for the development of APCs, with many key advantages. Thanks to a simple and efficient chip design, straight forward parallelization allows for high scalability. Its minimal disruptive effects on cell function are essential in the generation of therapeutically active immune cells.

The delivery of large, charged molecules such as nucleic acids has been rendered possible by the addition of electrodes after the narrow channels. As porated cells flow in between longitudinal electrodes, nucleic acids in solution are introduced by electrophoresis. Squeezing cells followed by electric field-enhanced DNA delivery allowed to lower the electric field strength necessary for transfection. It resulted in almost twice the transfection efficiency, with only a small decrease in survival, compared to cells treated with stronger electric fields without squeezing. This allowed to deliver a GFP plasmid in HeLa cells with efficiency close to 100% and around 90% viability. Plasmids reached the nucleus and was expressed under an hour, due to the disruption of the nuclear membrane by the high electric field generated between the electrodes

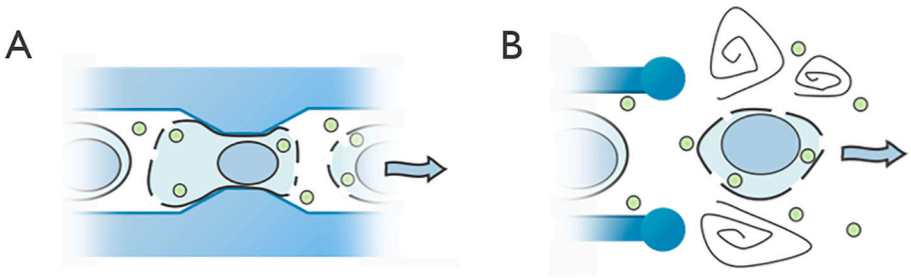

Fig. 4. SCTTs applying a mechanical stress on the cell membrane. A) Microfluidic cell squeezing B) Microfluidic vortex shedding (μVS).

[56]. This electric-field enhanced delivery of nucleic acids could allow for cell transduction with mRNAs for safer CAR delivery.

Other devices based on microfluidic mechanical membrane disruption have been developed. For instance, a technique based on shear stress-induced membrane poration uses vortices created at the end of a microfluidic channel at high flow rates (Fig. 4B). The invention, coined microfluidic vortex shedding (μVS), could deliver a 1 kb GFP mRNA in primary human T cells with more than 60% transfection efficiency and 80% cell viability while minimizing toxic side effects. Moreover, the permeation process does not rely on cell size and might be applicable to any cell type without discrimination. Finally, the device can process millions of cells per second, compatible with the output demanded for the generation of CAR T cell therapies [90]. The company behind μVS, Indee Labs, has built the μVS Delivery System™ around this concept. The device, that uses disposable cartridges and tubing, is aimed at offering an affordable way to deliver nucleic acids, proteins or gene editing constructs to T cells. Other examples of shear stress-induced membrane permeation have also demonstrated intracellular delivery of large molecules, such as DNA nanostructures [91,92]. For instance, in sonoporation, microbubbles exposed to ultrasounds rapidly expand and collapse, generating large shear stresses that permeate the nearby cells. While this technique has not yet reached high efficiency, it has shown close to 50% plasmid transfection efficiency in suspended cells [93], delivery of large dextran molecules in adherent cells [94] and single-cell addressability [95]. For further information, we direct the reader to other works [14,96,97].

3.4. Optical injection

In optical injection (also called photoporation or optoporation), a high-intensity laser pulse is condensed on a sub-micrometer dot, creating a nanosurgery tool able to dissect the plasma membrane and cellular compartments for molecular cargo delivery. The advantages of the technique are compatibility with most microscopy setups, processing of adherent and suspended cells, single-cell addressability and a virtually sterile procedure: cells can be porated without direct exposure to the environment [98]. The technique has been applied to deliver a wide range of molecules, including plasmid DNA, mRNA, siRNA and proteins [14].

The most effective light source for single-cell transfection are femtosecond lasers: ultrashort, high-energy pulses are focused on the membrane and generate a pore as big as a micrometer [99] for efficient plasmid delivery [100] (Fig. 5A). Combination with a microscopy setup and motorized sample stage creates a microinjection-like system, in which an operator manually searches for targets in a cell population and triggers the laser pulse. This system has shown consistently high efficiencies and cell viability in delivering plasmid molecules [98,101–103]. It has also been applied to stain selected retinal cells and study actin structures within whole explanted rat eyes [104]. In principle, the same method could be used for in vivo delivery of plasmids. Automation of the process increases throughput and reduces process variability, for instance through combination with image analysis software and cell selection routines [105]. Optical injection has also been adapted to deliver reprogramming minicircle vectors in human dermal fibroblasts as they flow in a microcapillary [106,107]. While formation of iPSC-like colonies has been shown [107], the 1% transfection efficiency of this method is marginalized by other cell suspension methods, like dielectrophoresis-assisted nanopore electroporation of plasmids or cell squeezing with reprogramming factors in purified protein form.

The main disadvantages of femtosecond-pulse transfection are the critical importance of vertical alignment between the laser and the cell membrane: micrometer-sized mismatches can drastically reduce the poration effect of the laser pulse. Moreover, the high price of the required light sources is an important access barrier [108]. As an alternative, researchers have explored optically absorbent materials (such as ITO and gold) that focus and amplify the energy of weaker lasers while solving the problem of vertical alignment. In those materials, the light is converted into heat, which induces the creation and rapid collapse of microbubbles near the cell membrane, which gets permeated by the fluid shear stress that is generated in the process [109]. Plasmonic materials are either used as a cell culture substrates and surfaces close to the cells for optical injection [110–116,119] (Fig. 5C) or as nanoparticles incubated with the cells for adsorption on their membrane [55,108,117,118,120,121] (Fig. 5B). Plasmonic nanoparticles extend the use of the technique to suspended cells and might have the advantage of simplicity over microfabrication-intensive substrates. Nanoparticle-enhanced optical injection has been shown to have low toxicity on primary T cells [122] and limited negative impact on cell behavior [123]. Antibody-functionalized nanoparticles allow targeting of a specific cell type within a mixed sample, and the clustering of nanoparticles on the targeted cells amplify the poration effect, reaching high plasmid transfection efficiency and cell viability in primary T cells [117]. The method has been applied to optical injection in a capillary. In other work, nuclear poration was achieved with functionalized nanoparticles delivered via electroporation and targeted to the nuclear envelope [124].

Finally, an automated nanoparticle-enhanced optical injection has been developed, based on a motorized sample stage and image recognition software. This Spatially-resolved Nanoparticle-enhanced Photoporation, coined SNAP, can process up to 200 cells per second. It can deliver large macromolecules in cells to either create predefined patterns of laser exposure or recognize individual cells based on certain characteristics, like a fluorescent label, and expose them to a porating laser pulse [55]. SNAP is the core technology behind LumiPore, a product currently developed by the startup TrinCE. TrinCE views tissue engineering and CAR-T cells as the main applications for their technology. For a convenient and reagent-free harvesting of cells transfected on a plasmonic surface, a surface coating with temperature-dependent cell adhesion properties has been developed [125]. Such strategies would allow for entirely contactless and automated cell transfection and harvesting. They also combined cationic polymers to enhance the delivery of plasmid DNA [126], which is an interesting combination of delivery methods to enhance transfection efficiency. It was developed as a platform to efficiently process hard-to-transfect cells, and boasts more than 80% of transfection efficiency in primary endothelial cells and MEF, with ~80% and ~ 100% of viability for those cell types, respectively. Interestingly, the different cationic polymers tested did not have the same toxic effects on both cell types.

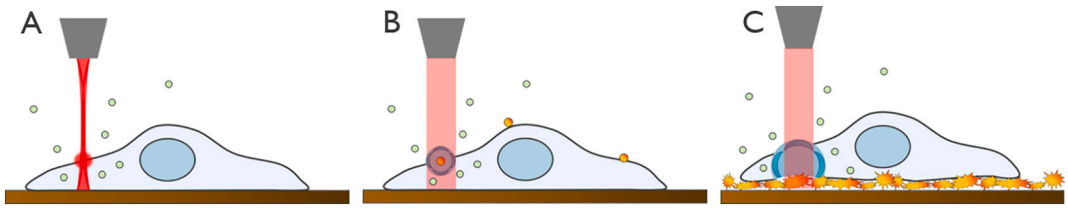

Fig. 5. Different implementations of Optical Injection A) "traditional" Optical Injection, such as fs-laser optical injection B) Plasmonic nanoparticle-based optical injection C) Plasmonic surface-based optical injection.

4. Toward clinically relevant, GMP-compliant SCTTs

Medicinal products that have been granted marketing authorization have to comply with GMP as early as from the clinical investigational phase. That is why GMP compliance should be considered as early as possible when developing a transfection technology with intended clinical applications. As our goal is to stimulate the translation of promising technologies to the clinics, we would like to put emphasis on how GMP compliance can be integrated in the design of SCTTs. Currently, there are only few GMP-compliant SCTTs commercially available. Electroporation platforms are the most predominant, such as in MaxCyte's in-flow electroporation devices.

GMP are a set of rules and guidelines overseeing the manufacturing of pharmaceutical products and ensure that clearly defined quality attributes are met. GMP focus on managing the risks involved in each manufacturing step by thoroughly documenting, validating and controlling processes. GMP are linked to an entire manufacturing process: they guide the manufacturing of medical products from starting material to stored, finished product in its final packaging, ready to be shipped to the patients. As such, GMP also cover topics like receiving goods, maintaining and cleaning the manufacturing premises, controlling suppliers, training employees and labelling of reagents and products. Box 1 describes the main GMP requirements for a generic process for manufacturing advanced therapy medicinal products [127]. When considering transfection as part of a cell product manufacturing, we can translate the points listed in Box 1 into features that must be implemented in a GMP-compliant SCTT.

1. The transfection must be reliable over time and generate a uniform cell population. Thus, an SCTT must display high transfection efficiency and cell survival. Uniformity is achievable with 1) precise dosage-controlled intracellular delivery; and 2) by limiting disruptive side effects. Dosage control has been demonstrated in microinjection and nanopore electroporation. On the other hand, nanoparticle-enhanced optical injection, where a variable number of nanoparticles may bind cells, or cell squeezing, which would not perform uniformly on cell samples with varying sizes, might struggle in delivering a uniform amount of cargo. Cell squeezing [19], nanopore electroporation [81] and some forms of optical injection [122,123] have been shown to minimize toxic side effects. The extensive approaches used by DiTommaso and colleagues [19] or Tay and Melosh [81], based-on functional and gene expression analysis are a solid foundation on which to build a standard analysis.
2. Real-time and in-process quality control strategies are necessary to track the transfection process efficiency and observe early on any deviation from quality requirements. Examples of built-in transfection monitoring are found in electroporation devices, where processed cells can be counted, and membrane permeation can be verified by impedance monitoring. Dielectrophoresis sorting of viable cell is also an elegant way to ensure product quality. In principle, these monitoring strategies could be, and should be, incorporated into other SCTTs that are not based on electroporation.
3. The transfection must be sterile and safe, and output a cell product free of contaminant like microorganisms or chemicals. SCTTs enclosed in microfluidic devices, and OP can conveniently maintain the sterility of the processed cells. Contamination risks generated by open dish techniques, like microinjection, need to be managed by performing the technique in an isolator or cleanroom, which pose additional challenges related to process and equipment validation. Moreover, contact between cells and non-disposable material should be minimized, thus techniques based on disposable devices, such as microfluidic chips and tubing, are best suited. For reusable devices on the other hand, such as MEAs too costly to dispose of, validated cleaning procedures must be put in place. Only GMP-grade reagents and biocompatible materials should be used, and their availability should be kept into account.
4. Automation of the process is necessary to reduce variability and possible mistakes leading to product contamination. Points to consider are the automation of the transfection itself, as well as the operation of the entire set up. Questions to keep in mind are: Are the fluidics control, the stimulus application and the cargo formulation automatable? In optoporation, is the cell selection and sample movement automated? As a general principle, SCTTs processing cell suspensions benefit from a simpler operation and automation than devices handling adherent cells, which need to be cultured on the device.
5. Commercially viable product requires a scalable manufacturing process. SCTTs with throughput high enough for their applications are necessary. In flow methods, such as cell squeezing and in-flow electroporation can process millions of cells in minutes and can be parallelized and combined with automated microfluidic handling to produce large quantities of cell product. Device cost is also to consider: MEA chips and powerful optoporation lasers might prevent their parallelization.
6. Process validation is the documented demonstration that a process performs reliably as intended and is key to GMP compliance. Validation shows that the process is adequate for its intended operation and that it meets all predefined requirements. Thus, each possible usage situation that could critically affect the product and process quality must be validated. While every SCTT can in principle be validated, the complexity of the method usually determines the complexity of the validation process, which should be taken into consideration during the design phase of the SCTT. electroporation for instance, uses a large pulse parameter space with complex interactions that are hardly predictable. A device that allowed complete freedom over the electroporation parameters could not realistically be validated, as each combination of the parameter space would have to be thoroughly tested. A viable approach is to limit the user's choice to a few sets of parameters, validated for certain cell types or cargos. It is the approach used by Lonza and their Nucleofector device. Moreover, any software control used during the process needs to perform in any situation encountered by the user: any automation must be proven to be reliable. In image-guided optoporation, the cell recognition routines need to be proven effective in every situation possibly encountered during use, such as different cell morphologies, surface densities, etc.

5. Conclusion/outlook

Cell therapies have demonstrated irrefutable success in clinical settings, and the next challenges are in scaling up the manufacturing of cell products. The SCTTs presented above offer such opportunity in multiple ways. First, these transfection methods can eliminate the tremendous costs, manufacturing complexity, regulatory burden and safety concerns linked with viral vectors. Second, their higher efficiency, propensity for automation enablement, versatility with cell types and cargos and built-in process monitoring modalities guarantee convenient integration in industrial setups, where they ensure the manufacturing of high-quality cell products. Finally, some unique features, such as dosage control, minimal cell perturbation and single-cell addressability among a population will uniquely enable applications previously unthinkable with bulk transfection methods.

Still, much remains to be done to mature the SCTTs presented in this review. Besides optimization of the membrane disruption stimulus, many aspects of transfection are relatively unexplored. Improving the efficacy of SCTTs by combining some of their features, or improving transfection efficiency by using chemical carriers, could be more common. For instance, combining sonoporation [128] or cell squeezing [56] with electrophoresis-mediated nucleic acid delivery is a great way to enable efficient transfection. The use of cationic polymers to facilitate nucleic acid entry in the cell, rather than naked nucleic acid, could benefit all SCTTs based on mechanical membrane disruption such as cell

Box 1
GMP and QBD.

GMP integrate the Quality By Design (QBD) principles. QBD is a validation strategy dictating that quality is built in the product by understanding the manufacturing process and the risks associated rather than solely screening it at the end. The QBD approach uses statistical, analytical and risk-management methodology (i.e. determination of critical quality attributes and critical process parameters) in the design, development and manufacturing of medical products. One of the goals of quality by design is to ensure that all sources of variability affecting a process are identified, explained and managed by appropriate measures. This enables the finished medicine to consistently meet its predefined characteristics from the start. Here are the key points to consider when developing an SCTT as part of a GMP-compliant cell manufacturing process.

Uniformity / Reproducibility

The process must be stable and reliable and have a predictable outcome. Process variation should be avoided and eliminated if possible.

- Can the outcome of the process be predicted with enough confidence?
- What are the expected transfection success rate or cell viability?
- Is the process stable over time? Are process variations limited with this technique?
- Are there Critical Process Parameters (CPP) that directly influence the Critical Quality Attributes (CQA)? How easy is it to control these with the technique? (i.e., flow rate in cell squeezing, voltage in electroporation, etc.)
- Can you determine the quantity of the starting materials and finished product?

Validation approach

The process needs to be validated. Process validation is the analysis of data gathered throughout the design and manufacturing of a product in order to confirm that the process can reliably output products of a determined standard.

- How complex is the validation of the process? Is there equipment involved and what is its complexity (e.g. pipette versus robot)? Does this equipment need validation too?
- What parameters are critical and in which playing field is the process or technique stable (see also uniformity / reproducibility)?
- Is there a software involved in the detection and automated systems? Does it require validation, or is it built on an approved software?

Use of GMP materials

The materials that are used during the process should not negatively interfere with the cell product. Biocompatibility and animal product-free reagents are key.

- Are disposables used and do they come in direct product contact?
- What about sterility and biocompatibility? Can the materials be used in combination with living cells without affecting their quality?
- What is their extractable / leachable profile? Extractables and leachable compounds (E/L) studies are critical to the identification and quantification of harmful leachable impurities which could migrate from container closure systems, process equipment and packaging to contaminate cell products.
- The used materials should themselves comply to "GMP-grade production". Is it possible to obtain these? Are their suppliers certified for this purpose?

In process controls / monitoring of the process

Built-in monitoring strategies are required to confirm that the process is running as validated and to assess the quality during the process itself.

- Does the technique offer possibilities for measurements during the process? Is it possible to integrate process controls? Are the CPP measurable?
- Is real time monitoring possible? Real time monitoring gives a quicker analysis of the process than microscope or lab testing.
- Can those methods / in-process controls be validated?

Quality control

Quality controls of the outcome of the process on the intermediate or final cell product (transfected cell) need to be performed.

- Is it possible to perform quality controls?
- Can those methods / in-process controls be validated?
- Which quality controls need to be performed?
- How is sampling performed?
- Can destructive sampling be avoided?

Cleanability

For non-disposable, reusable direct product contact equipment, it is necessary to prevent cross contamination or other contamination by cleaning / sterilizing the equipment.

- Is the used equipment cleanable?

Prevention of microbial or cross contamination / Sterility / Safety

Final products should be free from microbial contamination and endotoxins, absence of mycoplasma, in vitro adventitious agents and replication-competent retrovirus.

- Can direct product contact with the equipment be reduced?
- Is disposable / single use equipment involved?
- Is it possible to reduce the risk of contamination regarding equipment, materials, human intervention, and other agents?
- Is it possible to perform the process in a closed environment/under laminar flow/in an isolator?
- Is it possible to integrate the technique in a chip integration or closed dish environment?

Scalability in general

- Is the technique able to produce transfected cells on a large scale in a shorter timeframe?
- Are the transfection efficiency and cell viability high enough to generate the needed amount of cell product without extensive sorting?

Automation possible?

Can the process be automated? Automated detection, control systems, execution?

- Are manual interventions needed? Can the process be automated (in a cost-effective manner)?
- Are there detection methods or software that can assist in automation of the process?
- Is maintenance and calibration possible and easy for the automated systems?

Cell collection / harvesting

- Is the harvesting technique a time-consuming process?
- Is the cell viability not impacted?
- Are there additional contamination risks upon harvesting of the transfected cells?

squeezing and optical injection [126]. Another point to consider is the necessary distinction between the concepts of intracellular delivery and the actual biological effects generated by the delivered molecules. Optimization of the cargos' biological activity, such as in [18], and increasing our understanding of the triggered cellular processes, like in [58], is still required. Characterization of the disruptive effects that permeation has on cell function is another key to improve the efficacy of cell therapies and the relevance of disease models in a dish. As SCTTs are the most relevant to clinical applications when they have demonstrated efficacy on primary cells, each technology's effect on the viability and function of primary cells should be systematically studied.

Importantly, more applications should explore the use of SCTTs and drive their development. For instance, the field of tissue engineering and organs-on-a-chip would certainly benefit from techniques like optical injection, that could in principle transfect and differentiate cells to create complex cellular patterns. The fact that this has not been reported yet shows the effort that remains to be done in applying the unique abilities of SCTTs to applications that could benefit or be enabled by them. On the other hand, the very specific needs of certain applications should also be driving the development of dedicated, specialized SCTTs. It should be clear that no single SCTT can cater for all cell-based medicine applications discussed in this paper. Rather, developers of clinical applications should try to match their needs with the most suitable transfection technology. We hope our work will contribute to this goal.

Finally, a big attention point is compliance with regulatory and quality requirements. Researchers developing clinically relevant SCTTs should consider GMP compliance as early as possible in their designs. The optimization of specific aspects, such as implementing a sterile and reliable process may appear obvious. However, some non-trivial GMP aspects, such as the validation step and its complexity, or the availability of GMP compliant reagents to operate the SCTT, should not be overlooked. Addressing GMP compliance early in the development of SCTTs is key to improve novel technology uptake in clinical applications.

Declaration of Competing Interest

The authors Bastien Duckert, Dries Braeken and Maarten Fauvart declare no conflict of interest.

The author Steven Vinkx declares being employed as QA Project Manager at QbD, 2610 Antwerp, Belgium. QbD offers expertise and solutions in the field of Quality Management, Support and Regulatory Affairs for companies active in Pharma, Biotech, Healthcare and Medical Devices.

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