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In vitro and *ex vivo* strategies for intracellular delivery

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Intracellular delivery of materials has become a critical component of genome editing approaches, *ex vivo* cell-based therapies, and a diversity of fundamental research applications. Limitations of current technologies motivate development of next-generation systems that can deliver a broad variety of cargo to diverse cell types. Herein we review *in vitro* and *ex vivo* intracellular delivery approaches with a focus on mechanisms, challenges and opportunities. In particular, we emphasize membrane-disruption-based delivery methods and the transformative role of nanotechnology, microfluidics and laboratory-on-chip technology in advancing the field.

Despite its essential role in biological research and therapeutic applications, the efficient intracellular delivery of exogenous compounds and macromolecular cargo remains a long-standing challenge. The limitations of established delivery technologies have hampered progress in multiple areas as the potential of exciting new materials, insights into disease mechanism, and approaches to cell therapy are not fully realized due to their delivery hurdles. This challenge can be viewed through the lens of two broad parameters: cell type and target material. Existing technologies are mainly focused on addressing a subset of combinations, specifically nucleic acid delivery (that is, transfection) to immortalized cell lines and certain primary cells. Some of the most exciting target cell types, such as stem cells and immune cells, are also the most difficult to address. Thus, methods of delivering almost any cargo molecule to any cell type are much needed.

Although carrier-mediated delivery systems offer promise for nucleic acid transfection *in vivo*[1,2], membrane-disruption-based modalities are attractive candidates for universal delivery systems *in vitro* and *ex vivo*. In this review, we begin with motivations driving next-generation intracellular delivery strategies and suggest relevant requirements for future systems. Next, a broad overview of current delivery concepts covering salient strengths, challenges and opportunities is presented. Following that, our focus shifts to prevalent mechanisms of membrane disruption and recovery in the context of intracellular delivery. Finally,

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we highlight the potentially transformative role of nanotechnology, microfluidics and laboratory-on-chip approaches in shaping the field.

Next-generation intracellular delivery

Next-generation intracellular delivery solutions are required in diverse scenarios ranging from cell-based therapy and gene editing to regenerative medicine and fundamental biology (Fig. 1). *Ex vivo* cell-based gene therapies have demonstrated promise in clinical trials against human disease, with exciting examples including self-renewing haematopoietic stem cells and T cells for immunotherapy[3–6]. In haematopoietic stem cells, gene therapy to correct mutations in monogenic diseases such as severe combined immunodeficiency (SCID)-X1, Wiskott–Aldrich Syndrome, and β-thalassemia has been achieved[4]. For T cells, novel function against tumour targets can be instructed by induced expression of specific T cell receptors and chimaeric antigen receptors followed by adoptive cell transfer[5]. Non-essential proteins used by pathogenic processes can be deleted by delivery of genome-editing nucleases, such as was recently demonstrated with ablation of the HIV-dependent *CCR5* receptor necessary for infection in T cells[7] or the haematopoietic lineage[8]. Moreover, induced secretion of cytokines, or programmed drug resistance and safety switches, can be engineered into these cell types by *ex vivo* manipulation[4,6]. Recent breakthroughs in gene editing with programmable nucleases offer an unparalleled opportunity to reach many of these goals[9,10]. Further afield, in regenerative medicine the importance of not relying on potentially mutagenic viral vectors for induced pluripotent stem cell production has spawned reprogramming efforts by direct delivery of proteins[11], messenger (m)RNA transfection[12], and micro (mi)RNA delivery[13], among other possibilities. A common theme in these clinically relevant examples is the need to perform efficient and safe intracellular delivery.

In basic research, nucleic acids, proteins, peptides, metabolites, membrane impermeable drugs, cryoprotectants, exogenous organelles, molecular probes, nanodevices and nanoparticles are all potential target materials for intracellular delivery. Current limited delivery capabilities, largely centred around nucleic acid delivery to cell lines, have already yielded dramatic progress with plasmid (p)DNA and mRNA for gene expression and small interfering (si)RNA and miRNA for gene silencing. Meanwhile, systematic delivery of protein biologics into living cells, such as active inhibitory antibodies and stimulatory transcription factors, represent a powerful yet largely untapped tool for decoding and engineering cell function[14]. Measurement of intracellular chemical and physical properties with innovative devices, sensors and probes is another frontier[15]. Probes engineered from functional nanomaterials—including nanoplasmonic optical switches[16], carbon nanotubes[17] and quantum dots[18]—have generated excitement in research communities for decades but ineffective intracellular delivery, a poor understanding of their interaction with biological environments, and toxicity issues have retarded their deployment in the cellular context. These delivery challenges are particularly acute in the case of important patient-derived cell types such as immune cells, stem cells and neurons[19,20].

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Taken together, the preceding examples (Fig. 1) lay down compelling motivations and requirements for next-generation intracellular delivery systems. In Table 1 we propose a set of guidelines to be considered by inventors. Regarding cell-based therapies, for example, decades of clinical trials indicate that the risks of *ex vivo* culture include karyotype abnormalities, genotoxicity, and exhaustion of proliferative potential[3]. Hence rapid and safe delivery protocols that maximize efficiency and cell viability while minimizing time in culture are crucial. For gene editing, a key issue is that transient 'hit and run' exposure of nucleases is often more favourable than indirect expression from DNA or mRNA, because it yields more control over dosage concentration and efficacy, and reduces off-target effects[21]. For induced pluripotent stem cells, delivery challenges may impede the use of the ideal combination of proteins, nucleic acids, and small molecules to provide the optimal reprogramming outcome. Furthermore, low cost transfection remains a barrier in many sensitive cell types at the level of basic research, and is even more problematic when considering scale-up for clinical protocols or industrial processes. Thus, next-generation intracellular delivery strategies must strive for original solution to address the decades-old challenge of delivering diverse cargoes to the intracellular space of a wide range of cell types.

Many roads to one destination

Intracellular delivery can be achieved by a range of carrier-based or membrane-disruption-based techniques (Fig. 2). Membrane disruption modalities are primarily physical, involving the introduction of transient discontinuities in the plasma membrane via mechanical, electrical, thermal, optical or chemical means. These approaches can be thought of as permeabilization or direct penetration modalities. A cell becomes permeable to a substance when disruptions in the membrane are of sufficient size to allow passage through the membrane. Alternatively, direct penetration employs a solid conduit or vehicle to concurrently penetrate the membrane and introduce cargo. Carrier-based approaches comprise various biochemical assemblies, mostly of molecular to nanoscale dimensions. The purpose of carriers is threefold: (1) to package the cargo and protect it from degradation, (2) to gain access to the intended intracellular compartment, and (3) to release the payload with the appropriate spatiotemporal dynamics. Carriers can be bio-inspired, such as reconstituted viruses, vesicles, cell ghosts, and functional ligands and peptides. They may be based upon synthesis techniques from chemistry, materials science and nanotechnology, involving assembly of nanoparticles and macromolecular complexes from organic and inorganic origins. Most carriers enter through endocytosis but some may exhibit fusogenic potential, endowing them with the ability to merge directly with the target membrane.

In the case of nucleic acid delivery (that is, transfection), vectors are defined as constructs that contain foreign DNA for the purpose of expression or replication. Major vector types are plasmids, cosmids, episomes/artificial chromosomes, and viral vectors, of which only viral vectors are capable of unassisted entry. Viral vectors exploit the viral infection pathway to enter cells but avoid the subsequent expression of viral genes that leads to replication and pathogenicity[22]. This is done by deleting coding regions of the viral

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genome and replacing them with the DNA to be delivered, which either integrates into host chromosomal DNA or exists as an episomal vector. At present, viral vectors are the most clinically advanced nucleic acid delivery agents owing to their high efficiency and specificity. They have been implemented in clinical trials for decades, being viewed as a promising approach for gene therapy[22,23]. In 2015, more than half of the gene therapy submissions to the Federal Drug Agency (FDA) relied on viral vector platforms based on lentivirus, retrovirus, adenovirus, or adeno-associated virus. For *ex vivo* applications, lentiviral transduction of the haematopoietic lineage is a prominent example[3,4]. However, challenges such as immune response, safety, and complexity of preparation are concerns for viral vectors[22,23] and thus viral systems have struggled to gain FDA approval. To address these issues, researchers are developing new vectors and serotypes[4]. Table 2 presents a comparison of strengths, challenges and opportunities for viral vectors, non-viral carriers, and membrane disruption-based delivery.

Motivated by the limitations of their viral counterparts, hundreds of non-viral vectors and synthetic carriers have been designed, using vast combinations of lipid, polymer, and inorganic nanomaterials, sometimes featuring functionalization with ligands, cell-penetrating peptides and other targeting or stabilizing agents (see reviews[1,24–26]). Most carriers are designed for nucleic acid transfection, but recent efforts seek to expand their ability to co-deliver proteins or other biomolecules[27]. Nearly all carriers are taken up via specific endocytic pathways based on their cell surface interactions and physicochemical properties[28–30]. To reach the intended intracellular target, the cargo must escape endosomal progression, which otherwise leads to degradation in lysosomes or regurgitation back to the cell surface[31]. For lipid nanocarriers, which are considered the most advanced non-viral vectors for nucleic acid delivery, quantitative studies reveal approximately 1% of the nanocarriers escape from endosomes[32]. The exact mechanisms of escape remain elusive, however. Proposed explanations include endosome disruption, either by formation of transient lesions or vesicle lysis; active transport of dissociated products; or fusion of carriers or multi-vesicular bodies with the outer limiting membrane[30–32]. Apart from endosomal escape, another consideration is the kinetics of cargo release from the carrier, where delayed unpacking has been reported as a bottleneck to transfection efficiency[33]. Moreover, toxicity of carrier material and perturbation to membrane trafficking processes have been noted[32,34]. Manipulation of the host cell biology, using small molecules for example, represents an opportunity for boosting endosomal escape and delivery efficiency[35]. The design of stimuli-sensitive nanocarriers that respond to selective endosomal or intracellular conditions could also lead to improvements[26].

Carriers with fusion capabilities circumvent endocytosis by releasing their cargo directly into the cytoplasm. These systems were first inspired by viruses that deploy specialized surface proteins to induce fusion with target membranes[36,37]. Fusogenic carriers are bound by a phospholipid bilayer that hosts the fusion machinery. Examples include cell ghosts, dead cells that have their cytoplasm replaced with cargo[36,37], and virosomes, loaded vesicles reconstituted to present functional viral proteins[38]. More recently,

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cell-derived vesicles known as exosomes have been discovered to fuse with target cell membranes for the exchange of RNA and proteins between immune cells[39]. Although the exact fusion mechanisms are yet to be elucidated, such bio-inspired systems may represent a new generation of vehicles with which to overcome the poor efficiency and toxicity of synthetic carriers[40].

An inherent limitation of carrier systems is the restricted combination of feasible cargo materials and cell types. Target cells may not exhibit the appropriate receptors, surface interactions, endocytic activity, or endosomal escape pathways. Furthermore, potential cargo materials often display enormous variability in their properties, such as charge, hydrophobicity, size, mechanical properties, composition, and functional groups. They may not efficiently complex with the carrier, tolerate packaging, unpack properly, or be amenable to delivery in sufficient quantities for a given application. For example, cationic lipids readily form complexes with anionic nucleic acids to transfect most immortalized cell lines, but many blood and immune cells remain recalcitrant[20,41]. On the other hand, cell-type-specific uptake can be a characteristic deliberately employed to achieve controlled targeting of a cell population[42]. Furthermore, owing to their mechanisms of passive dispersion, carrier-based delivery is scalable and amenable to high throughput, with the ability to concentrate and protect limited amounts of cargo material for potent delivery. Related to these strengths, lipid nanoparticles and more minimalistic, compact conjugates are now in human clinical trials for *in vivo* delivery of therapeutic siRNAs[1,2].

Unlike carriers, membrane-disruption-based approaches are less dependent on cargo properties, being able to deliver almost any submicrometer material dispersed in solution. The ability to rapidly switch membrane-perturbing effects on and off enables temporal control and rapid, almost instantaneous delivery. A further strength of membrane-disruption techniques *in vitro* and *ex vivo* is the broad range of cell types and materials that can be addressed. Electroporation, for example, has a reputation for transfecting primary cell types that are otherwise recalcitrant to lipid nanoparticles and other non-viral transfection agents[43]. Membrane-disruption approaches may furthermore be combined with carriers to synergize the strengths of both, such as by delivering a nuclear-targeted DNA lipoplex to the cytoplasm[44]. They have also enabled several protein-delivery applications, including with antibodies, transcription factors and genome editing nucleases[14,21,45,46]. In primary human haematopoietic stem cells and T cells, for example, it was found that expression of Cas9 nuclease and guide RNA from plasmids was poorly tolerated, while direct delivery of Cas9–sgRNA complexes via electroporation improved efficiency, reduced off-target effects and normalized dosage control[21,46]. Such results highlight a trend towards direct delivery of macromolecules rather than their indirect expression from vectors.

Traditionally, key weaknesses of membrane-disruption strategies have been (1) the inconsistent level of cell-to-cell plasma membrane injury, with too little rendering insufficient delivery and too much causing excessive cell damage; (2) poor throughput and scalability (for example, microinjection); and (3) inadequate

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understanding of cell recovery, resulting in inefficient protocols[47,48]. Methods that employ severe thermal shock or electric fields may denature proteins or damage cell components[49]. Moreover, because of how membrane perturbation is administered in these techniques, they are often restricted to adherent or suspension cells. To overcome such challenges, new technologies are reinvigorating old approaches and concepts, suggesting that this may be a critical time in the development of *in vitro* and *ex vivo* intracellular delivery approaches (Box 1). Membrane disruption has seen promising advancement in recent years through nanotechnology, microfluidics, and laboratory-on-chip devices. Next, we cover the fundamentals of membrane disruption and repair before highlighting examples of technological progress in the field.

The make and break of a cell membrane

The plasma membrane can be perturbed by physical means, with mechanical force, thermal deviations, electromagnetic radiation and electric fields, or by appropriate biochemical agents, such as membrane-active peptides, detergents and pore-forming toxins[50] (Fig. 3a). A relatively straightforward mode is mechanical disruption, where in-plane tensile strains of 2%–3% rupture a lipid bilayer[51]. Such mechanical disruption can be administered through solid contact[52–54], fluid shear[55–57], or hydrostatic/osmotic pressure[58]. Depending on the contact area and strain rate the applied force may either disrupt the membrane immediately, or first deplete membrane reservoirs[59]. For example, sharp objects like microneedles concentrate the force to a small region and presumably penetrate rapidly, while the 'blunt' and relatively slower onset of an osmotic shock tends to deplete global reservoirs before producing disruptions[59]. Thermal deviations may promote membrane defects through several mechanisms. First, the higher kinetic energy associated with supraphysiological temperatures gives rise to more intense molecular fluctuations and subsequent dissociation of lipids[60]. In live cells it has been shown that leakage begins at 42° C while temperatures above 55° C promote rapid exchange of low-molecular-weight (<1 kDa) molecules[60]. Second, at the opposite end of the spectrum (<0° C**)**, formation of ice crystals can trigger mechanical expansion and cracking of the cell membrane, which may be reparable upon thawing[61]. Third, within the physiological range (0°–40° C), passing the membrane rapidly through thermal phase transitions may lead to the generation of holes, especially at phase domain boundaries[60,61]. In electroporation, the electric charging of non-conducting lipid bilayers and ever-present thermal fluctuations conspire to create and expand a heterogeneous population of pores[62]. As membrane potential rises beyond 0.2 V, hydrophobic defects of thermal origin readily transition over their energy barrier and expand into hydrophilic pores of >1 nm. Because small pores are poor conductors, their growth is energetically favourable while the field is maintained but slows down when they grow to a certain size[62]. Depending on the orientation of the electric field, these small pores may be unevenly distributed across the cell surface[63]. Alternatively, optoporation with lasers[64] produces a hole on a discrete point on the cell surface, and may involve a combination of mechanical, thermal and chemical effects, depending on pulse parameters[65]. Possibilities include thermal dissociation, thermoelastic stresses, effects beyond the focal region (such as shock-wave emission and shear stresses from induced cavitation

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bubbles) and generation of low-density free-electron plasma and reactive oxygen species. The latter leads to chemical degradation via peroxidation or reactive fragmentation of biomolecules[65,66]. Membrane defects from continuous-wave lasers are thought to arise from local heating, while nanosecond lasers produce a combination of heating, bubble formation and thermoelastic stresses. Femtosecond laser mechanisms are tunable on the basis of irradiance strength and repetition frequency, ranging from almost purely chemical effects to combinations of chemical and thermal degradation[66].

Various biochemical agents have been used to permeabilize cells, the most eminent of which are detergents and pore-forming toxins. Pore-forming toxins approach the cell membrane as soluble agents, bind to the cell surface, oligomerize, and insert as an assembled pore complexes[67]. The most often reported is Streptolysin O, owing to its ability to generate >30 nanometer large pores for the passage of proteins and large molecules[68]. Alternatively, detergents act by solubilizing membrane components. The amphiphilic plant glycosides digitonin and saponins are the most popular detergents for reversible permeabilization of cells[47,48]. Although the exact mechanisms are still a matter of study[69], it is known that digitonin and saponin interact with membrane cholesterol, making them specific for the cholesterol-rich plasma membrane. However, the reported pore sizes are inconsistent, varying from a few nanometres up to the micrometre scale[47,48,69].

Upon plasma membrane injury, the cell must either reseal or die. Along with the intended influx of cargo, there is the entry of Ca^{2+}, and the efflux of K^{+}, proteins, amino acids, metabolites, ATP, and other cytoplasmic contents to contend with[70]. Inundation with reactive oxygen species and other toxic molecules may cause further damage to endogenous proteins and biomolecules[49]. Thus, cells urgently deploy repair pathways to reseal membrane disruptions and recover from the damage imposed (see recent reviews[70–74]). This repair is an active process, primarily triggered by the influx of Ca^{2+} down its 10,000-fold concentration gradient[75]. There is a marked consistency in the properties of the repair pathways, regardless of whether the source of damage is electrical, mechanical, optical, or even chemical[70,74]. Instead, membrane recovery is thought to depend on disruption size, collateral damage, temperature, composition of the extracellular medium, and cell type. Up to six membrane repair pathways have been proposed, primarily involving membrane-trafficking processes around the defect[71]. As the exact mechanisms remain controversial[70,71,73,74], we illustrate three broad concepts here (Fig. 3b). First, Ca^{2+}-dependent exocytosis and fusion of membrane-proximal vesicles leads to resealing by patch formation[75], but may also serve to reduce tension around the wound, or release lysosomal signals for membrane remodelling[74]. Second and third, for smaller disruptions of several hundred nanometres or less, endocytosis or exocytosis, respectively, may extract the lesion into a disposable vesicle. The timescales of these repair processes are anywhere from a few seconds to several minutes.

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Taken together, to achieve effective membrane disruption-based delivery, the disruption must be sufficient to introduce the intended cargo, yet the cell must be capable of repairing itself without permanent damage. The membrane-disruption step usually occurs on a sub-second timescale; molecules then diffuse, or are driven, into the cell while repair and the associated contraction of holes takes place over seconds to minutes (Fig. 3c). This is followed by a longer phase involving restoration of cytoplasmic composition, stress response and possible alterations in transcription[70]. The influx and retention of molecules will depend on the size, lifetime and distribution of disruptions, as well as the properties of cargo molecules and their interaction with the cell[50]. Electroporation, for example, provides an electrophoretic force that may boost the influx of certain charged molecules[63,76].

Towards precision membrane disruption

For decades, a leading delivery technique has been electroporation, with its ability to introduce diverse biomolecules to millions of cells per run. In a conventional setup, a solution with suspended cells is dispersed between parallel plates that apply a series of electrical pulses of determinable voltage, duration, waveform and frequency[62,63,76,77]. Compared to bulk electroporation setups, microfluidic designs offer the ability to localize the electric field to the scale of the cell (Fig. 4a). They can reduce the required voltage (often by 100-fold), provide superior heat dissipation, and incorporate flexible design features, such as hydrodynamic focusing to distance cells from potentially damaging electrodes[78]. An elegant and uncomplicated design was reported by ref. 79, who combined constant voltage with cells flowing through constrictions. The pulse strength was dictated by the cross-sectional ratio between the main channel and the constrictions, while the pulse duration was determined by speed of passage, thus avoiding the need for a pulse generator. Electroporation with a constant direct-current voltage has also been demonstrated for cells in aqueous droplets[80]. Owing to the non-conductivity of oil, cells only experience a transient electric pulse when the conductive droplets pass the electrodes. At the nanoscale, ref. 81 described a nanochannel electroporation system that uses a ~90-nanometre aperture to localize the permeabilization to a single point on the cell surface. This innovation enables the generation of a single large hole rather than the numerous small pores characteristic of conventional electroporation, which is less amenable to free passage of large materials. For example, conventional electroporation appears to exploit the charge of nucleic acids, such as plasmids and mRNA, in order to partially embed them in pores, resulting in subsequent internalization through active membrane-trafficking pathways rather than direct delivery[81]. In contrast, nanochannel electroporation achieves improved dose control, enhanced electrophoretic delivery deeper into the cell, and the ability to deliver materials that bulk electroporation often struggles to deliver, such as quantum dots.

Classic options for membrane disruption via mechanical perturbation include scrape and bead loading of adherent cells[54] or syringe loading of cells in suspension by repeated aspiration and expulsion through small gauge needles[55]. These methods provide coarse, inconsistent damage over a target cell population while being high throughput and low cost. On the other hand, modern microfabrication

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technology is now enabling mechanical approaches with improved precision (Fig. 4b, c). Prominent examples include cell squeezing, nanoneedles, and exploding cavitation bubbles. Cell squeezing involves the rapid deformation of cells as they passage through microfluidic constrictions of around half to one-third of the cell's diameter[45]. Diffusive delivery of a variety of cargoes including proteins, nucleic acids, quantum dots, carbon nanotubes and other nanomaterials has been demonstrated[45]. A major strength of squeezing is simplicity of the device, with no moving parts or need for an external power supply. The energy for membrane disruption comes from the flow through a static structure. Although the technology has shown applicability across dozens of cell types at throughputs up to a million cells per second, a current limitation is the correlation between cell size and delivery efficiency. For an asynchronous population, cells that are too large may be lysed, such as multinuclear cells. Alternatively, cells that are too small do not experience sufficient deformation to disrupt the membrane properly. This has prompted the design of various constriction geometries to address different sizes of cell[45].

Nanoneedles are another key development over the last decade, involving the generation of nanometre-scaled features capable of penetrating the cell membrane and providing access to the cytosol[82]. In the reported modalities thus far, the target material is delivered through one of three modes: (1) dissociation from the penetrating structure upon cytosolic entry[82,83]; (2) direct injection through a hollow nanoneedle or "nanostraw"[84,85] or (3) the diffusion from the extracellular medium through holes after withdrawal of the needles[44]. For the first mode, the delivery of siRNA, peptides, DNA, proteins, and impermeable inhibitors to challenging cell types such as neurons and immune cells has been demonstrated[83,86]. Studies of the mechanism suggest that puncture does not occur upon initial cell contact, but requires active forces generated by cell spreading and formation of tension-promoting focal adhesions[87]. In the second mode, molecules have been successfully pumped into cells through nanostraws. A key benefit of this configuration is temporal control over delivery dynamics, volume, and dosage concentration, as well as possible gating with electric fields[84,85]. In the third mode, ref. 44 used a standard laboratory centrifuge to spin down a grid of diamond nanoneedles onto adherent cultured cells, followed by withdrawal and diffusive entry of cargo from solution. With this strategy it was possible to deliver a wide variety of cargo to primary neurons while maintaining >80% viability. For nanoneedles of diameter about 300 nm and height of about 4 μm, the required force for penetration was estimated to be 2 nN per needle. These mechanistic studies emphasize the need for active force to generate efficient membrane disruption. Thus far, the challenges of these systems have included the difficulty of fabricating high-precision structures and implementing the concept at scale.

Moving from solid to fluid (Fig. 4c)[55], cone-plate viscometers, devices for generating a determinable shear force over a surface, have also been shown to transiently permeabilize apical membranes in cell monolayers[56]. Such observations inspired the design of microfluidic devices to expose cells to shear forces proportional to flow velocity through 50-300 micron tapered microchannels[57]. Although an interesting concept, reproducibly controlling fluid shear forces at this scale has generally proven difficult. One way

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around this is to employ micrometre-sized cavitation bubbles[88]. Ultrasound phenomena, also known as sonoporation in the context of delivery, can produce cavitation bubbles in solution[89]and has been an intriguing permeabilization approach since its introduction in the 1980s[90]. However, a recent analysis across multiple published data sets indicates that ultrasound consistently struggles to deliver molecules with greater than 50% efficiency or 50% viability *in vitro*[89]. This was attributed to the operational mechanism of random and violent cavitation being heterogeneous, with some cells undergoing excessive damage while others remain unaffected. Targeted cavitation is a more promising idea, whereby a precisely positioned cavitation bubble is used to generate a local shear force at a given stand-off distance from a target cell[91,92]. Spatial control can be achieved by laser excitation of an absorbent particle or substrate. Recently, this concept was scaled up for deployment with cell monolayers[93]. Substrates arrayed with pores lined by metallic absorbers were irradiated underneath adherent cells. Exploding bubbles were synchronized with active pumping to successfully introduce large cargo, such as living bacteria greater than one micrometre, into the cytoplasm of several cell types.

Bulk thermal insults are capable of perturbing the membrane for delivery of small molecules but detrimental effects on cell function preclude their implementation[60,61]. Thermal disruption may be more feasible if confined to a localized area (Fig. 4d). Indeed, gene transfection has been demonstrated with cell solutions processed through thermal inkjet printers[94], although it is unclear whether membrane perturbation of passing cells arises from fluid shear effects at the nozzle, temperature spikes, or both. A more precise approach is the use of absorbent nanoparticles as nucleation sites for intense local heating[65]. Upon laser irradiation local perturbation effects may be due to thermal, chemical, or cavitation-mediated fluid shear phenomena. Recent efforts indicate that tuning the laser pulse parameters and properties of the absorbing particles can bias the mechanism towards a particular mode[95,96]. Although parameters are still being explored, a proof-of-concept study demonstrated conditions under which irradiation of gold nanoparticles was proposed to trigger localized thermal damage that permitted more than half of the treated cells to take up labelled antibodies[97].

As illustrated above, advances in micro- and nanotechnology are breathing new life into delivery modes that were once deemed impractical (Fig. 4). By concentrating precise membrane-perturbing effects to the cellular and subcellular scale, the potential exists to address applications that are underserved by current techniques. Early progress suggests that such techniques may be among the first real challengers to electroporation’s long-held dominance on membrane-disruption-based intracellular delivery. Nanoneedles and microfluidics, for example, have shown promising compatibility in stem cell reprogramming[45], functional interrogation of primary immune cells[86], and transfection of cultured neurons[44]. To achieve further advances, delivery efficacy and safety must be combined with scalability, tunable throughput, low cost and user-friendliness.

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Outlook

Every day in the research institutes and clinical centres around the world, scientists use kits and protocols based on viral vectors, lipid transfection agents, and electroporation, among other options. The complex mechanisms of established methods and their often unpredictable impact on cell behaviour have dramatically limited the scope of biological experiments and reduced efficacy of potentially promising cell therapy concepts. The biomedical research community would benefit greatly from a more mechanistic and transparent understanding of intracellular delivery, both to further the development of more robust techniques and to realize key medical and industrial applications.

Effective and safe intracellular delivery systems facilitate progress across multiple fields from cell-based therapies, gene editing and cutting-edge genomics to reprogramming cellular states and probing the intracellular environment (Fig. 1). Demand for effective solutions currently outstrips supply by a large margin, however. Nowhere is this aberration more greatly felt that in the treatment of primary and patient-derived cells, including various types of immune cells, neurons and stem cells. Deep interdisciplinary coordination will be required to transform imaginative engineering solutions into technological platforms with biological compatibility and relevance. Mechanistic studies must seek to gauge delivery performance quantitatively, and to assess influx mechanisms, cell damage, and off-target perturbations associated with treatment. More rigorous analysis of these factors will benefit the field, and counterbalance the temptation to hype up technologies that are yet to prove their usefulness in the clinic or laboratory[98,99]. Issues related to scale-up, cost and compatibility with current goods and manufacturing protocols must be considered at an earlier stage of development. To this end, we anticipate that our guidelines in Tables 1 and 2 will assist researchers in selecting appropriate methods and help aspiring inventors to understand key requirements and areas of opportunity.

As emerging intracellular delivery technologies take us beyond routine nucleic acid transfection and enable robust manipulation of previously recalcitrant cell types, we will be entering exciting new territory. The ability to deliver proteins, peptides, nanomaterials, molecular tags, and a variety of other compounds will enable unprecedented flexibility in our capacity to manipulate cell function and probe the intracellular environment. The systematic deployment of transcription factors to alter gene expression, or antibodies to label or block intracellular processes, could be revolutionary. With next-generation intracellular delivery, the development of molecular probes and nanomaterial sensors with which to analyse intracellular properties could facilitate both discovery and diagnostics with unprecedented accuracy. Future approaches could focus on targeting specific subcellular compartments—developing combinatorial strategies to direct materials to the endoplasmic reticulum, nucleus or mitochondria, for example[100].

Advances in nanotechnology and microfluidics will continue to expand the frontiers of membrane-disruption-based delivery. Already, platforms harnessing the power of exploding bubbles, microfluidic squeezing, and nanoneedles have been transformed into commercial ventures. On the other hand, carrier-

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based technologies continue to develop, with new generations of viral vectors, endosome disruption strategies, stimuli-sensitive functional materials, and biomimetic inspiration from pathogenic mechanisms and membrane-trafficking processes. Progress in these fields will lead to new challenges, such as off-target effects and innate cellular responses against carrier and cargo alike. Solving this next generation of problems may hinge on our ability to understand current delivery mechanisms and to implement the analytical approaches necessary to characterize cellular responses. Despite the barriers that remain, we anticipate that next-generation technologies will translate beyond academic endeavours into portable, personalized, cell-based diagnostics and the use of clinical intracellular delivery to engineer cell fate for therapeutic benefit.

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Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details are available in the online version of the paper. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to R.S.L. (rlanger@mit.edu) and K.F.J. (kfjensen@mit.edu).

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Figure 1 | Intracellular delivery is a key step in investigating and engineering cells. Examples of application areas and molecular tools that require intracellular delivery for their realization. The subsets are not mutually exclusive. For example, gene editing may be employed in cell-based therapies, regenerative medicine, and genetic perturbation. TCR/CAR, T cell receptor/chimaeric antigen receptor; iPSC, induced pluripotent stem cell

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Figure 2 | Map of the relationship between intracellular delivery approaches, basic mechanism and conventional physical and biochemical categorizations. Physical techniques produce membrane disruption either via permeabilization or direct penetration, while biochemical assemblies and viral vectors act as carriers to shuttle cargo through endocytosis. If a carrier has fusogenic potential, it may also enter through membrane fusion. Some biochemical approaches, such as detergents and pore-forming proteins, work via membrane permeabilization. For mechanical methods red arrows give an indication of active forces. Schematics in the centre show the four subcategories with molecular cargo (green), membrane (brown), and carrier material (purple).

Figure 3 | Membrane disruption and recovery in the context of intracellular delivery. a, Schematic of plasma membrane disruption by mechanical forces (solid contact and fluid shear), thermal effects, focused lasers, electric fields, lodging of pore-forming agents that assemble into complexes, and the action of detergents that solubilize membrane lipids. **b**, Selected mechanisms of plasma membrane repair in the literature. Small disruptions (<100 nm) can be removed via endocytosis (i) and exocytosis or shedding of blebs (ii). Repair of large disruptions involves patching with endomembrane from intracellular vesicles (contents in light blue) (iii). See recent reviews for further details[70–74] . **c**, Timescales of intracellular delivery via membrane disruption. Initially, membrane disruption permits the exchange of intracellular and extracellular contents, including cargo (green). Upon repair of membrane barrier function, delivered materials are retained while cellular recovery processes work to restore cytoplasmic homeostasis.

Figure 4 | Selected modes of bulk, microscale and nanoscale approaches for membrane-disruption-based intracellular delivery. Molecular cargo is shown in red.

Table 1 | Ideal features of next-generation intracellular delivery systems

Feature	Justification
Minimal cell perturbation	• The exogenous vectors, materials or physical forces required to facilitate delivery can lead to off-target effects and toxicity • Prolonged culture duration associated with delivery and verification can lead to unintended fate or phenotypic changes, loss of proliferative or homing potential, genotoxicity, and karyotyping abnormalities and accumulated mutations • By minimizing the physical or biochemical manipulation necessary to achieve delivery, one can reduce undesirable side effects and maximize efficacy of the delivery process
Scalability	• Effective delivery systems must be amenable to implementation at different scales of throughput • Studying a rare cell subset may require only 100 cells per sample whereas an adoptive transfer T cell therapy can involve transfer of over 10^8 cells per patient • Considering cGMP-compliant processes at an earlier stage may accelerate clinical translation
Universal across cell types	• An ideal delivery system should be able to accommodate the diversity of physical and biological properties of potential target cells to ensure efficacy in the applications of interest This could be accomplished through exploiting relatively universal mechanisms to facilitate delivery
Material independent	• Delivery materials of interest may have diverse chemical and physical properties • To facilitate robust delivery an ideal delivery system should rely on a delivery mechanism that is independent of material properties(for example, if a delivery technology relies on electrophoresis to facilitate delivery it may not be compatible with uncharged materials)

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Compatible with intracellular targeting	• Distinct materials have different target sites within the cell (for example, siRNA and mRNA should facilitate the desired gene knockdown or expression effects in the cytosol, whereas DNA transcription requires nuclear localization) • A preferred delivery system must be compatible with various intracellular targeting strategies; specifically, it should provide robust delivery of material to the cytosol and not interfere with targeting motifs (for example, a nuclear localization peptide sequence) on the material
Dosage control	• The ability to control the maximum and minimum intracellular concentration may be key in some applications • Strategies that rely on indirect delivery by expression from nucleic acids are subject to cell-to-cell variation and inherent signal amplification associated with transcription
Cost	• Cost and complexity of production/operation can limit the utility of a delivery technology • Effective delivery systems should use scalable, cost-effective designs that are amenable to clinical translation, compliance with cGMP standards, and large-scale manufacturing

Table 2 | Strengths, challenges and opportunities for intracellular delivery approaches

	Carriers		**Membrane disruption**
	Viral vectors	Non-viral	
Strengths	• Amenable to both *in vivo* and *in vitro* translation • Experience gained from advanced status in clinical trials • High efficiency of intracellular delivery due to viral exploitation of infection pathway	• Amenable to both *in vivo* and *in vitro* translation • Packaging of delivery material can protect payload from premature degradation and potentially enable more efficient use of valuable materials • Capable of cell-specific and intracellular targeting • Passive, potentially high throughput	• Delivery of diverse materials • Capable of addressing many cell types • Optionally vector-free (that is, non-immunogenic) • Transient, defined exposure to membrane disruption • Rapid, almost instantaneous delivery
Challenges	• Can trigger adverse host immune response • Limited to nucleic acid delivery (transduction) • Limited genome size • Many are cell-cycle dependent • Preparation may be expensive, time consuming, require extensive experience, and demand special safety measures (for example, BL2) • For integrating viruses, risk of genotoxicity • Limited tropism may restrict target cell types • Manufacturing challenges for scaling up, for example, quality control for vector potency	• Inefficient and slow delivery, especially for carriers that enter via endocytosis (about 1% endosomal escape) • Carrier materials may perturb cell function or cause toxicity in unpredictable ways • *In vivo* targeting outside the liver has been difficult • Often restricted to delivering particular types of cargo (such as nucleic acids) • Unpacking kinetics may be unfavourable • Complex, laborious and expensive biochemistry or materials synthesis may be required for carrier preparation and manufacturing	• Loss of cytoplasmic content • Some modalities (such as thermal and electric) can lead to excessive damage to organic molecules, protein denaturation, and internal membrane breakdown • Some approaches (such as microinjection) currently not amenable to high throughput • Some methods can be restricted to adherent-only or suspension-only cells • Less amenable to *in vivo* translation

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Opportunities	• Less-immunogenic vectors or evasion of immune response • Programmable tropism and specificity • Further development of hybrid viral serotypes • Potential of alternative viral species for new vector development • Improved production methods	• Novel carriers that can efficiently co-deliver diverse cargos (for example, proteins + nucleic acids) • Manipulation of target cell biology to regulate membrane trafficking and increase delivery efficiency • Stimuli-sensitive nanocarriers • Leveraging direct fusion to bypass endocytosis (for example, exosomes) • Implementation of biomimetic functionality inspired by viruses, bacterial machinery, and exosomes	• Microfabrication and nanotechnology enables fine control of physical phenomena and makes modalities that were previously intractable more feasible • A better understanding of cell recovery processes may allow mitigation of toxicity and functionality issues • Can be combined with carriers designed for subcellular targeting and controlled release • Engineered switchable valves in plasma membrane to dynamically control permeability • Versatile potential for *in vitro* and *ex vivo* applications

See text for further details and references.

Box 1 Snapshot of historical trends in intracellular delivery

The field of intracellular delivery arguably began with the advent of microinjection[52] in 1911. Since then a broad range of options have evolved, which can be categorized into carrier-mediated or membrane-disruption-mediated (Fig. 2). The above timeline highlights trends in the field.

For carrier-mediated approaches, early research noted that several cationic compounds readily complex with the negatively charged nucleic acids to facilitate uptake of DNA and RNA. Examples include precipitates formed with diethylaminoethyl(DEAE)-dextran and calcium phosphate. Inspired by these initial findings, chemical complexes and modified viruses were subsequently deployed as tools for DNA transfection. Since then hundreds of viral, lipid, polymer, and inorganic carriers have been developed, mostly for nucleic acid transfection[1,24,30]. Recent efforts have focused on multifunctional capabilities conferred through nanotechnology[26,29] and biomimetic strategies[25] such as exosomes[40], direct conjugates[2], and new generations of viral vectors[22,23].

Membrane-disruption-based approaches have evolved in parallel. Initially, available options included low-throughput microinjection[52] or membrane perturbation with hypotonic shock[58]. The demonstration of DNA transfection by electroporation in 1982[77] sparked an era of widespread experimentation with other membrane disruption modalities, including laser optoporation[64], scrape/bead loading[54], syringe loading[55], acoustic sonoporation[90], ballistic particles[53], and permeabilization with detergents and pore-forming agents[47,48]. Electroporation rapidly gained a foothold as commercial products were launched from the mid-1980s. Most other membrane disruption techniques were not broadly adopted, presumably owing to poor cell recoveries, limited throughput, need for specialized equipment, high cost, or skill-dependent operation[47,48]. In the past decade, nanotechnology, microfluidics, and laboratory-on-chip platforms are emerging as new possibilities to reinvigorate membrane-disruption-mediated approaches.