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Laser-assisted photoporation: fundamentals, technological advances and applications

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ABSTRACT

Laser-assisted photoporation is a promising technique that is receiving increasing attention for the delivery of membrane impermeable nanoscopic substances into living cells. Photoporation is based on the generation of localized transient pores in the cell membrane using continuous or pulsed laser light. Increased membrane permeability can be achieved directly by focused laser light or in combination with sensitizing nanoparticles for higher throughput. Here, we provide a detailed account on the history and current state-of-the-art of photoporation as a physical nanomaterial delivery technique. We first introduce with a detailed explanation of the mechanisms responsible for cell membrane pore formation, following an overview of experimental procedures for realizing direct laser photoporation. Next, we review the second and most recent method of photoporation that combines laser light with sensitizing NPs. The different mechanisms of pore formation are discussed and an overview is given of the various types of sensitizing nanomaterials. Typical experimental setups to achieve nanoparticle-mediated photoporation are discussed as well. Finally, we discuss the biological and therapeutic applications enabled by photoporation and give our current view on this expanding research field and the challenges and opportunities that remain for the near future.

ARTICLE HISTORY

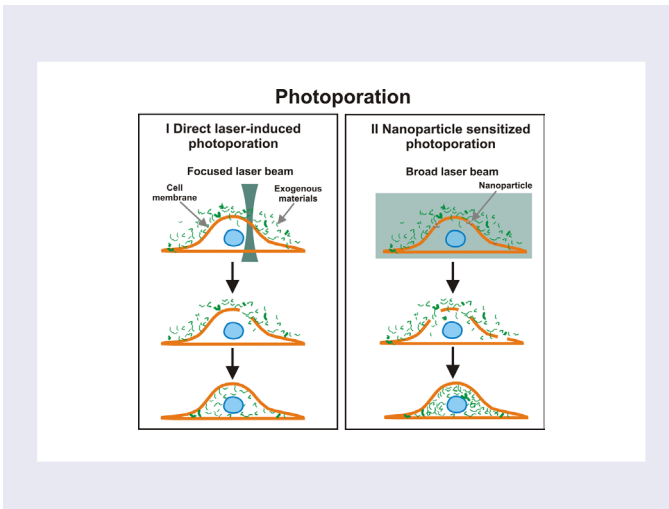
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87.15.mn Photoionization



1. Introduction

The incorporation of foreign materials into living cells is an important step not only for cell studies, but also for therapy and bio-imaging. DNA is, for instance, introduced into live cells for studying gene expression [1–5], mutation analysis [6], and gene therapy [7]. Similarly, mRNA incorporation into living cells helps to assess cell biological functions [8], while small interfering RNA (siRNA) is introduced for sequence-specific gene silencing [9–13]. Proteins also can be delivered into living cells for bio-imaging and therapies [14–16]. In recent years, the use of inorganic particles as intracellular labels is being investigated, such as superparamagnetic iron oxide nanoparticles (NPs) and Gadolinium complexes for magnetic resonance imaging [17–19], quantum dots (QDs) [20–22] and upconversion NPs [23] for fluorescence imaging and gold nanoparticles (AuNPs) for photoacoustic imaging [24,25]. In addition, QDs and AuNPs are also delivered into cells for *in vitro* microscopic imaging of subcellular structures and intracellular biosensing, respectively [26–28].

Delivering such nanomaterials into cells requires overcoming the cell membrane, which is a major biological barrier to charged, noncharged, and polar molecules as well as NPs. Numerous methodologies have been developed to allow these membrane-impermeable exogenous materials to cross the cell membrane, which can be broadly classified into biological, chemical and physical methods [29]. In the biological approach, foreign DNA is introduced into cells via a viral vector. Although virus-mediated transfection can provide highly efficient and sustainable transgene expression, the major disadvantages of this method are immunogenicity and toxicity [30,31]. To overcome these shortcomings of viral vectors, chemical vectors, often lipid or polymer based, are being explored as well [32–35]. These nanocarriers are generally internalized by cells through endocytosis, although the required subsequent escape from the endosomal compartments remains one of the major bottlenecks [36–38].

Physical approaches to permeate the cell membrane have attracted considerable interest as well. They typically offer generic applicability to a variety of cell types and enable direct delivery of the exogenous materials across the cell membrane into the cytoplasm [39–41]. Microinjection is a first example where the compounds of interest are injected into single cells [42–44]. Although it has demonstrated its usefulness for cell biological applications, it is technically challenging and only applicable to a limited number of cells. Electroporation is an alternative physical technique that can deliver molecules into a large batch of cells, but the high electric field often results in low cell viability [45–50]. The gene gun is another approach that uses micro- or nano-particles conjugated with e.g. nucleic acids which are shot into cells as a kind of bullets using pressurized gas [51,52]. More recently, sonoporation has been introduced as a method to permeabilize the plasma cell membrane by making use of ultrasound-responsive microbubbles. The acoustic response of the microbubbles can lead to the formation of micro-jets and shockwaves resulting in cell membrane poration [53–55]. However, the technique is rather developed for *in vivo* applications and less suited for *in vitro* work. It has also been reported that shear forces or elevated temperatures can lead to substantial cell damage and toxicity [39,56]. The use of a microfluidics device is also reported for the intracellular delivery of therapeutic molecules and labels with high throughput [57–60]. The cells are forced to flow through a narrow constriction in the channel so that pores are created in the cell membrane by friction forces. While it is tremendously fast at one million cells per second, it is limited to cells in suspension and the efficiency remains relatively low.

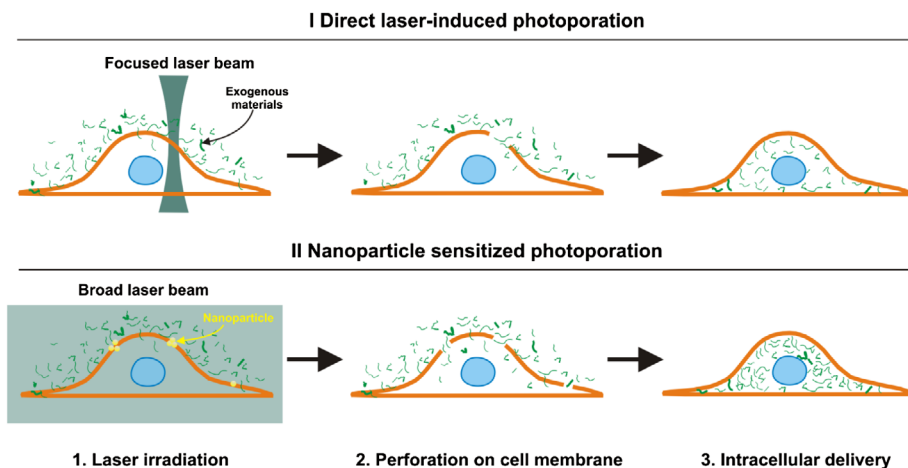


Figure 1. Direct laser-induced photoporation and NP-mediated photoporation. In direct photoporation (I), the laser beam is focused precisely on or slightly above the cell membrane to achieve a locally high photon density. For NP sensitized photoporation (II), the NPs are first incubated with the cells to allow them to adhere to the cell membrane.

Notes: A (low intensity) broad laser beam can then be used to photoporate many cells at the same time. Following laser irradiation, exogenous compounds in the cell medium can diffuse through the membrane pore(s) into the cell's cytoplasm.

Photoporation has been developed as a promising alternative technique offering distinct advantages over other physical delivery methods. In its standard form, pores are created in the cell membrane by high-intensity femtosecond (fs) laser pulses that are focused precisely with respect to the cell membrane (Figure 1) [61–66]. In the context of this review, the term ‘pore’ will be used to denote both water-filled holes in the membrane as well as local zones with increased membrane permeability, for instance, by localized reorganization of lipids. Just like in electroporation and sonoporation, exogenous nanomaterials that are present in the surrounding cell medium can then diffuse into the cell’s cytoplasm. Although it has proven to be useful for single cell studies, it requires the use of expensive fs lasers and remains relatively slow since the laser beam needs to be focused exactly in 3-D on the cell membrane and pores are created one at a time. Even by parallelization of laser beams, a limited throughput of only a few cells per second could be achieved [67]. However, using plasmonic NPs like AuNPs, it has been demonstrated in the last couple of years that photoporation throughput can be immensely enhanced (Figure 1) [68–70]. Most studies so far have used low intensity laser pulses or continuous wavelength (CW) laser light to excite AuNPs [71–74], causing thermal membrane permeabilization by a local phase transition of the lipid bilayer or by denaturation of integral glycoproteins [74]. Instead, the use of more intense laser pulses have shown to cause mechanical membrane poration by the generation of vapor nanobubbles (VNBs) [69,75]. In a comparative study of thermal vs. VNB mediated membrane poration, it has been found that VNB-mediated membrane poration actually allows more efficient uptake of exogenous nanomaterials with reduced cytotoxicity as compared to thermal poration at low laser intensity [76]. Although AuNPs are the most widely used for membrane poration, other nanomaterials like titanium nanostructures and carbon-based nanomaterials are also currently being evaluated as alternative sensitizing NPs [77–80].

In this review, we provide a detailed account on the history and current state-of-the-art of photoporation as a physical delivery technique. First, the historic form of photoporation is introduced with a detailed explanation of the mechanisms responsible for cell membrane pore formation. An overview is given of the most common experimental configurations for realizing direct laser photoporation. Next, we review the second and most recent method of photoporation that combines laser light with sensitizing NPs. The different mechanisms of pore formation will be discussed and an overview is given of the various types of sensitizing nanomaterials. Typical experimental setups to achieve NP-mediated photoporation will be discussed as well. Finally, the biological and therapeutic applications enabled by photoporation will be discussed including nucleic acid transfection, drug delivery, and cellular imaging. Taken together, it is clear that photoporation is acquiring immense interest and is expected to evolve substantially in the coming years, both in terms of technology and novel applications. Here, we give our current view on this expanding research field and the challenges and opportunities that remain for the near future.

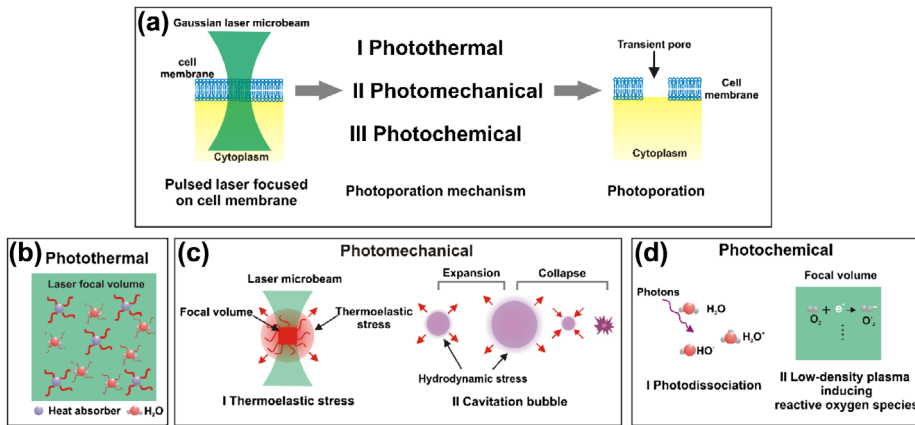


Figure 2. Main mechanisms contributing to direct laser-induced photoporation. (a) A pore (up to hundreds of nm) can be formed in the cell membrane by a focused laser beam through photothermal, photomechanical and photochemical effects. (b) The photothermal effect refers to a temperature increase following absorption of photons by molecules like water, but also proteins or DNA. (c) Under pulsed laser irradiation, thermoelastic stress or cavitation bubbles contribute to photomechanical poration of cell membranes. (d) With fs laser pulses or UV laser light, pores can be formed by photodissociation or the generation of ROS by actively free electrons.

2. Direct laser-induced photoporation

2.1. Mechanisms

In direct laser-induced photoporation, it is crucial to spatiotemporally confine the laser energy to the cell membrane to obtain sufficient laser energy density for the generation of membrane pores. To achieve this, a laser beam is focused to a very small spot size (typically $\sim 1\text{--}10\ \mu\text{m}$) through a microscope's objective lens [81]. Typically, a pulsed laser is used with a pulse duration from nanosecond (ns) down to fs to achieve a sufficiently high photon density. As schematically shown in Figure 2(a), a variety of possible mechanisms may contribute to pore formation, including photothermal, photomechanical, and photochemical processes [81–83]. The contribution of each of these processes depends on laser pulse duration, laser wavelength and intensity (which in turn is determined by the laser energy and beam size). In order to control pore formation, it is essential to understand these mechanisms of laser-induced photoporation.

2.1.1. Temporal laser energy confinement

Absorption of light can cause a transition between electronic and vibrational energy levels of the absorbing molecules so that heat is generated upon relaxation to the ground state (Figure 2(b)). In order to realize a substantial temperature increase in the focal spot, short intense laser pulses are preferred with a duration shorter than the characteristic thermal diffusion time t_d in the focal volume [84]:

$$t_d = \frac{0.124\lambda^2}{\kappa \cdot NA^2} \quad (1)$$

where λ is the laser wavelength, κ is the thermal diffusivity (in water $\kappa = 0.143 \text{ mm}^2/\text{s}$) and NA is the numerical aperture of the objective lens. For example, for a laser operating at $\lambda = 1064 \text{ nm}$ that is focused through a lens of $NA = 0.8$, the laser pulse duration should be less than 60 ns. Rapid localized heating may lead to a sudden thermal expansion of the medium, resulting in the generation of acoustic waves. In order to generate a substantial amount of this kind of thermoelastic stress, the time scale of laser energy deposition in the focal volume should not be longer than the characteristic time τ_s for acoustic wave propagation out of the focal volume, which is governed by the speed of sound in the medium [82]:

$$\tau_s = \frac{0.61\lambda}{c_w \cdot NA} \quad (2)$$

where c_w is the speed of sound in water (1484 m/s at 20 °C). For a typical NA in the range of 0.8 and a laser wavelength 1064 nm, the laser duration should be less than 550 ps for stress confinement.

Finally, multi-photon absorption can occur when using focused short laser pulses in the femto- to picosecond range. This may cause ionization of the absorbing molecules and the generation of a low density plasma [85]. Each of these three basic phenomena (heat production, acoustic cavitation, and plasma formation) can cause damage to cell membranes in a variety of ways, as will be explained in the following sections.

2.1.2. Photothermal pore formation

The photothermal effect refers to a temperature increase following single photon absorption by molecules like water, but also proteins or DNA (Figure 2(b)). Absorption in the UV and visible range corresponds to electronic transitions of molecules, while infrared is associated with vibrational transitions. Non-radiative relaxation to the ground state results in heat production. Localized heating can increase the permeability of the cell membrane by a local phase transition of the lipid bilayer or by thermal denaturation of integral proteins [74,86]. However, it was reported that photothermal heating by single photon absorption alone is not sufficient to effectively form pores in cell membranes [82,83,87]. This is mainly because water, lipids, and proteins have a relatively low absorption in the 350–1100 nm wavelength range. For that reason, dye molecules like phenol red are sometimes used to enhance light absorption and achieve a more efficient temperature increase [88–90].

2.1.3. Photomechanical pore formation

Pores can be created in cell membranes by mechanical stress, which can be induced by acoustic waves (cfr. Section 2.1.1) or by a phenomenon known as cavitation bubbles. Such bubbles are formed by localized vaporization of water, which can happen in two ways depending primarily on the laser pulse duration.

First, cavitation bubbles can be formed with fs laser pulses which cause plasma formation following a multi-photon absorption process. Free electrons in the plasma thermalize within tens of picoseconds. As this is much shorter than the characteristic time τ_s for acoustic wave propagation out of the focal volume (cfr. Equation (2)), which is in the order of 0.5 ns, the thermoelastic stress caused by the temperature rise will be confined to the focal volume. Subsequent propagation of the pressure wave causes substantial tensile stress in the center of the focal volume. If the tensile strength of the liquid (water) is exceeded, a cavitation bubble will be formed. A detailed description of the underlying physics can be found in the article by Vogel and colleagues [81]. The expanding bubble can lead to perforation of the cell membrane by hydrodynamic stress (Figure 2(c)). Furthermore, when the bubble has expanded to its maximum size, the bubble collapses by the surrounding hydrostatic pressure, inducing liquid jets or shockwaves that can form pores in the cell membrane. Based on SEM imaging, Sankin et al. visualized these pores and found that their size ranged from ~ 200 nm to ~ 2 μm [91]. A second way by which cavitation bubbles can be formed is using ns and picosecond (ps) laser pulses. Also here local plasma formation is involved, although the mechanism is somewhat different as for fs pulses. When using ns or ps laser pulses, only a small number of free ‘seed’ electrons can be generated following multiphoton absorption. These seed electrons can further absorb photons through a non-resonant process – known as Inverse Bremsstrahlung absorption (IBA) – until their kinetic energy is sufficiently high to produce other free electrons via impact ionization [92]. When the free electrons achieve a critical density, the irradiated material undergoes optical breakdown resulting in bubble formation as explained above. As avalanche ionization takes time to generate a sufficient amount of free electrons via IBA, the laser pulse duration should be typically more than 15 ps. For a pulse duration of tens of ps or even ns, the plasma will be further heated up to several thousands of degrees [93–95]. These high plasma temperatures will cause local evaporation of the medium (mostly water) and expansion of the plasma, which cause the formation of larger and more violent cavitation bubbles as compared to fs laser pulses. Rau et al. measured the bubble size as a function of time with laser fluences between $0.7\times$ and $3\times$ above the threshold (~ 250 J/cm²) for plasma formation ($t_p = 6$ ns, $\lambda = 532$ nm) and measured bubble sizes from ~ 200 to ~ 400 μm , which are more than 10 times bigger than those formed by fs laser pulses [85]. The entire process is visualized from plasma formation (bright luminescence in Figure 3(a) and (b)), over thermoelastic stress propagation (Figure 3(b) and (c)) and to bubble expansion and collapse (Figure 3(c)–(k)). The violent expansion and collapse of such bubbles could severely damage nearby cells, similar to fs induced cavitation bubbles (Figure 3(l)).

2.1.4. Photochemical pore formation

Photochemical reactions may also contribute to photoporation of cell membranes. When fs laser pulses are used below the threshold for optical breakdown or bubble

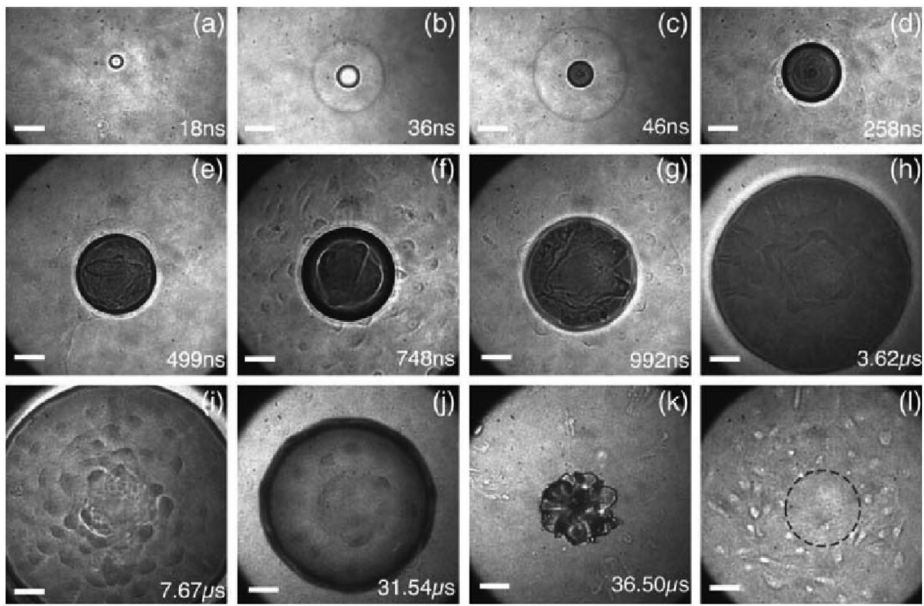


Figure 3. Time-resolved images show the process from plasma formation (a, b) over shock wave propagation (b, c) to cavitation bubble dynamics (c–k). Nearby cells are destroyed by the bubble shear forces (l).

Notes: A 6 ns laser pulse (at 532 nm) is used with a fluence three times above the plasma generation threshold ($\sim 250 \text{ J/cm}^2$). Scale bar is $50 \mu\text{m}$ and Potorous rat kidney epithelial (PtK2) cells were used (see Ref. [85]).

formation, reactive free electrons can be generated by multiphoton ionization of e.g. water molecules. The resulting highly reactive oxygen species (ROS) can locally induce cell membrane damage (Figure 3(d)) [96–98]. Alternatively, pulsed UV (typically ns or ps pulses) may damage the cell membrane by molecular fragmentation after absorption of highly energetic UV photons [83,99]. Indeed, UV photons have sufficient energy to cause dissociation of many organic molecular bonds [100,101]. This photo-induced molecular fragmentation plays a significant role in the localized ablation of the cell membrane with wavelengths shorter than 250 nm [83]. However, due to the toxicity of UV light, this process is not so commonly used.

2.2. Experimental procedures for direct laser-induced photoporation

Several approaches have been developed to achieve laser-induced photoporation. One of the widely used laser configurations makes use of a Gaussian laser beam that is focused on the cell membrane through a microscope objective lens (Figure 4(a)). In this procedure, pore formation occurs through photomechanical effects induced by thermoelastic stress or cavitation bubbles or photochemical reactions, depending on the laser pulse energy and duration. Here, it is crucial to position the focal volume exactly on the cell membrane, not only laterally, but also axially as a

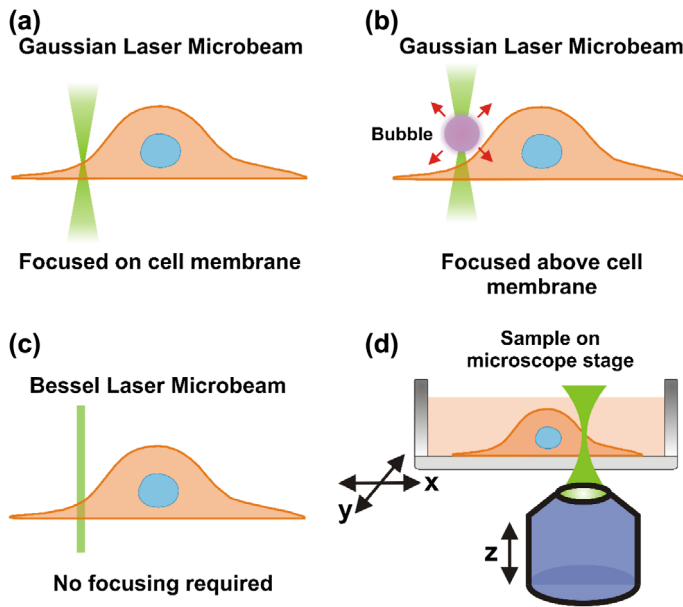


Figure 4. Experimental approaches for direct laser-induced photoporation. (a) A widely used approach in direct photoporation is to focus a Gaussian laser beam directly on the cell membrane. Pore formation in the cell membrane occurs through photomechanical or photochemical effects. (b) A ns pulsed laser can be used instead, which is focused a few micrometers above the cell membrane for the generation of micrometer sized vapor bubbles. (c) A non-diffracting Bessel laser beam can be used to enhance photoporation throughput as it does not require careful focusing on the cell membrane. (d) Cells can be photoporated in a sequential fashion by making use of a programmable x - y stage.

miss-focus of only 3 μm could reduce the transfection efficiency by as much as 50% [102]. As careful 3-D positioning of the focal volume is required, the photoporation throughput in this configuration is low at only a few cells/min [103,104]. It has been demonstrated that throughput can be increased by focusing a ns laser a few micrometers above the cell membrane to generate cavitation bubbles (Figure 4(b)). Tens of cells can be perforated by a single cavitation microbubble, so that a throughput of hundreds of cells/min can be reached [85,105]. Another way to increase throughput is the use of a Bessel laser beam that has a large depth of field (with a fluence of typically tens of mJ/cm^2 in the central part of the beam) and, therefore, does not need axial positioning [66,102]. With an array of multiple Bessel beams generated by a spatial light modulator, a throughput of ~ 100 cells/min could be achieved [67]. The downside of using a non-diffracting Bessel beam is that a high laser intensity is delivered throughout the cell, potentially causing membrane dysfunction and DNA strand breaks, thus leading to apoptosis-like cell death [81,106].

Cells can be photoporated in a sequential fashion by scanning the laser beam over the cells with a programmable x - y stage (Figure 4(d)). With image processing, the location of the individual cells can be determined. In combination with

an auto-focus system to control the axial position of the photoporation laser beam, this allows cells to be photoporated in an automated fashion [107,108]. In one example, it was demonstrated that ~ 300 cells/min can be photoporated that way [107]. Finally, a microfluidic approach has also developed in an attempt to increase photoporation throughput, with cells flowing one by one through the focused photoporation laser beam. In this case, a throughput of ~ 60 cells/min has been obtained [109].

3. NP sensitized photoporation

Nanomaterials can be used as sensitizers for the photoporation of cell membranes at lower laser energies, as was demonstrated first a little more than a decade ago [110,111]. While since then AuNPs have been used the most for this purpose, examples of other nanomaterials like titanium nanostructures and carbon-based nanomaterials are currently being explored as well. NP sensitized photoporation can offer high throughput since less laser energy density is needed so that the laser beam can be expanded to cover 10–100s of cells at the same time. Similar to direct photoporation, sensitizing NPs can lead to pore formation in the cell membrane through thermal, mechanical, and photochemical processes, as is discussed in detail in this section.

3.1. Plasmonic NP-sensitized photoporation

3.1.1. Laser interaction with plasmonic NP

For metallic NPs like AuNPs, the optical absorption is enhanced by Localized Surface Plasmon Resonance (LSPR). As indicated in Figure 5(a), LSPR corresponds to the interaction between a nanostructure and an electromagnetic field, typically laser light. Free electrons in the NP start to oscillate in synchrony with the incident laser electromagnetic field. These oscillating electrons are called localized surface plasmons. This oscillation generates an electric field opposite to the incident wave and forces the electrons back to their equilibrium position. When the incident laser frequency matches the resonant frequency of the localized surface plasmons, the electrons will oscillate with maximum amplitude, known as LSPR. The resonant plasmonic frequency depends on the size, shape, and composition of the metallic NP, as well as its environment [112–117]. Figure 5(b) shows the light scattering, absorption, and extinction cross-section for 50 nm AuNP in water, showing a LSPR peak at ~ 530 nm.

Due to oscillations of the localized surface plasmons, a series of sequential energy transfer processes occur (Figure 5(c)). First, the oscillating electrons become thermalized within a hundred of fs through electron–electron interactions [118,119]. After that, the thermalized electrons transfer their energy to the NP phonons (i.e. lattice vibrations) by electron–phonon coupling in ~ 1 –5 ps, resulting in an increase of the NP temperature [120–122]. Finally, thermal equilibration is

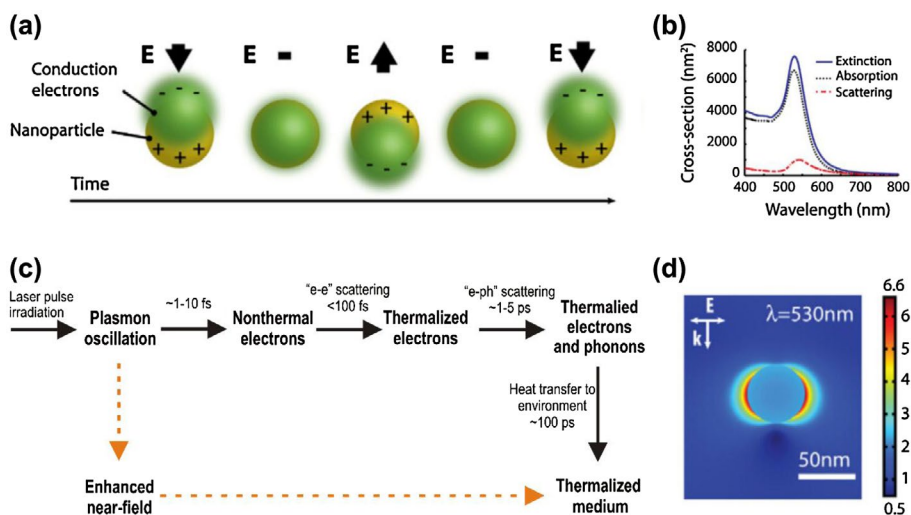


Figure 5. Principle of localized plasmon resonance and mechanisms of energy absorption and dissipation in laser-excited plasmonic NPs. (a) The incident electric field causes displacement of the NP's conduction electrons. Displacement of the electrons relative to the lattice ions creates its own restoration force. The interplay of the driving electric field and the dipole restoration force results in resonant behavior at certain light frequencies. (b) The scattering, absorption and extinction cross-section spectrum is shown for 50 nm NP in water. Localized plasmon resonance occurs around 530 nm. (c) Schematic overview of subsequent effects that happen in a NP upon absorption of incident light. (d) Electric field enhancement distribution around 20 nm AuNP at localized plasmon resonance of 520 nm.

Note: Figure 5(a), (b) and (d) adapted from Figure 2, Ref. [117].

achieved in ~ 100 ps inside the NPs, which is followed by heat transfer from the particle to the environment. Furthermore, the plasmon oscillation can also induce an enhanced electric field in the near-field (Figure 5(d)) [117,123]. In the intense nearfield, photothermal multi-photon absorption effects can happen similar as described for focused fs laser pulses in direct photoporation. These effects will be explained in more detail below.

3.1.2. Photoporation by heating of the plasma membrane

Cell membrane perforation can occur through direct heat transfer from the sensitizing NP that are adsorbed to the cell membrane [111]. This is typically achieved with CW laser irradiation or low intensity laser pulses (Figure 6(a)). A rise in local temperature has been reported ranging from tens to even a few hundreds of degrees [113,124]. Perforation of the cell membrane happens by a local phase transition of the lipid bilayer or thermal denaturation of integral glycoproteins which leads to opening of transient hydrophilic pores [72,74,125]. Heat-induced pore sizes have been reported ranging from tens to hundreds of nm depending on the NP size and laser intensity [71,74]. The main advantage of this approach is that CW lasers are relatively inexpensive and widely available. Throughput is,

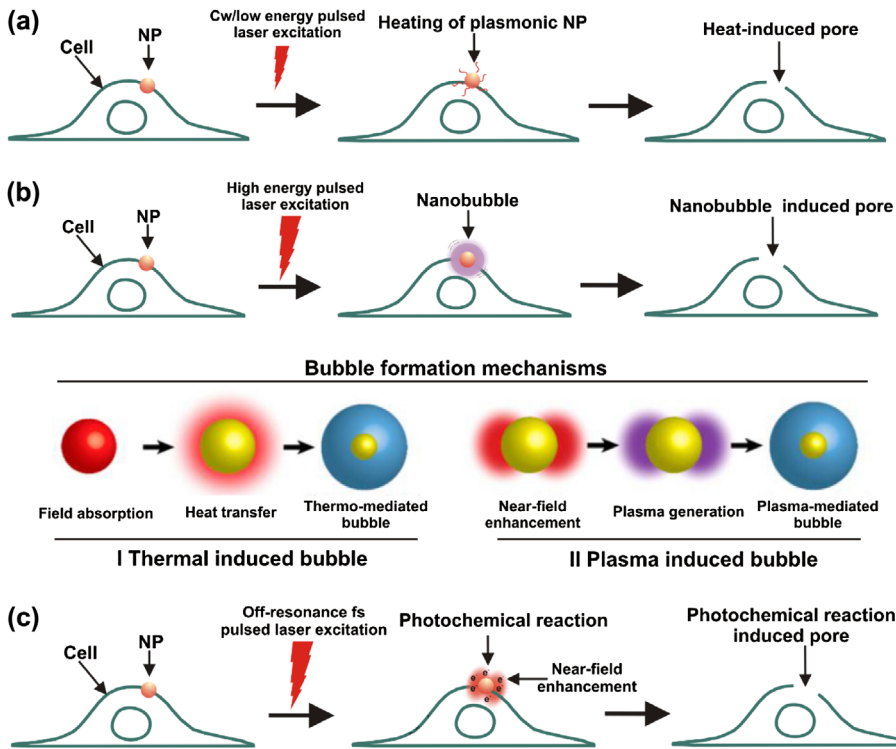


Figure 6. Mechanisms of plasmonic NP-sensitized photoporation. (a) When irradiated with CW laser light or low intensity pulsed laser light, plasmonic NPs become slightly heated and cause small pores in the cell membrane. (b) When short high-intensity laser pulses are used, water vapor nanobubbles can emerge around the NP whose mechanical force can form pores in the cell membrane.

Notes: Vapor nanobubbles may be thermally induced or plasma induced. In thermo-mediated nanobubble formation, strong absorption of the incident electric field leads to extreme heating of the NP, which in turn causes vaporization of the surrounding water and the formation of vapor nanobubbles. Alternatively, near-field enhancement around the NP can result in plasma formation via multiphoton ionization of the surrounding water molecules. The plasma relaxes and transfers its energy to the water, in turn leading to nanobubble formation (Figure 6 b down panel adapted from Figure 2, see Refs. [117] and [134]). (c) Photoporation of the cell membrane may also be caused by photochemical reactions that are triggered by multiphoton ionization of water and the generation of ROS.

however, limited since it can take up to tens of seconds or a few minutes to form heat-induced pores.

3.1.3. Nanobubble-induced photoporation

When using intense short laser pulses (<100 ps), the NP temperature can reach very high temperatures before heat can diffuse into the environment. In that way, the NP temperature can rapidly increase to several hundred or even thousand degrees, leading to evaporation of the water surrounding the NP. The resulting water vapor nanobubble is referred to as a thermo-mediated nanobubble [126–129]. The expansion and collapse of the VNBS can create pores in the cell membrane by high-pressure shockwaves or liquid jet formation (Figure 6(b)). A

particular characteristic of this phenomenon is that almost no heat is transferred to the environment due to the insulating effect of the vapor nanobubble [130]. This means that almost all incident laser energy is converted to mechanical energy of the expanding vapor nanobubble. The absence of heat transfer into cells may lead to better cell viability [76]. The size of VNBs can be tuned from tens to several hundreds of nm depending on the laser intensity and size of NPs. The application of thermo-mediated nanobubbles for photoporation was pioneered by the Lapotko group. They mostly used 10 ns, 500 or 70 ps pulsed laser light to irradiate gold nanospheres, gold nanorods, or gold nanoshells [131–133]. They found that the threshold for the formation of thermo-mediated nanobubbles significantly depends on the laser pulse duration and the types of NPs used. The laser intensity threshold for 10 ns pulsed laser light is more than 10fold higher than for 500 ps pulses. This is due to the fact that thermal equilibration and the onset of heat diffusion in the environment happen on the 100 ps time scale. A large part of ns laser pulses, therefore, do not contribute to the sudden rise in temperature that is needed for nanobubble formation. They also found that gold nanoshells often require much lower laser energy as compared to spherical AuNPs. Although diffusion is likely the predominant mechanism for exogenous compounds to enter cells through the membrane pores, Lukianova-Hleb et al. argued that an active flow of extracellular liquid might contribute as well [132]. The latter may be caused by transient nanojets which are formed by asymmetrical expansion and collapse of nanobubbles.

Apart from heat also plasma formation can lead to nanobubbles [123,134] (Figure 6(b), adapted figure in Ref. [134]). As mentioned before, SPR causes near field enhancement the NP dipole edges. In these regions, a plasma can be formed by multiphoton ionization of the medium. The plasma cools down by collision and recombination with water molecules, hence producing a quick temperature and pressure increase and leading to the generation of a water VNB around the irradiated NP. The use of plasma-mediated nanobubbles was first reported by the Meunier group, who used fs pulsed laser (45 fs) irradiation of 100 nm gold nanospheres [123,134,135]. Under these conditions, they found that the threshold of plasma-mediated nanobubble formation is ~ 100 mJ/cm².

3.1.4. Photochemical-induced photoporation

Photochemical processes could occur during laser interaction with NPs, especially in the region where the near field enhancement occurs (Figure 6(c)). Of special relevance is ionization of water molecules and the generation of ROS [73,136,137]. ROS and free radicals can initiate a damaging chain reaction of lipid peroxidation followed by decreased hydrophobicity of the lipid bilayer [74,86]. Baumgart et al. made use of this process by irradiating 100 nm AuNPs with a fs laser (45 fs pulse, $\lambda = 810$ nm) at a laser fluence of 60 mJ/cm² [135]. The authors argue that photochemical processes are the predominant photoporation mechanism at these settings since heating is limited using laser light at a non-resonant wavelength.

Similarly, VNBs were also thought not to be involved as the fluence was below the bubble threshold of $100 \text{ mJ}/\text{cm}^2$. Heisterkamp group performed photoporation experiments under similar conditions (120 fs pulse, $\lambda = 800 \text{ nm}$) using 200 nm AuNPs. Also Heinemann and Kalies claimed that multiphoton ionization of water was the main photoporation mechanism in their experiments, even though they used substantially longer 850 ps laser pulses (wavelength at 532 nm) in combination with 200 nm AuNPs [73,138].

3.2. Carbon nanostructure-sensitized photoporation

Recently, carbon nanomaterials were reported as alternative sensitizers for photoporation. Carbon black (CB) NP-sensitized photoporation was first reported by the group of Prausnitz [77,78]. They reported that thermal effects are not the main reason for membrane poration. Instead, they explain that a carbon–steam reaction $\text{C}(\text{s}) + \text{H}_2\text{O}(\text{l}) \rightarrow \text{CO}(\text{g}) + \text{H}_2(\text{g})$ induces cavitation shockwaves that can perforate the cell membrane [139–141]. Graphene Oxide (GO) is another carbon-based nanomaterial that was demonstrated to improve cell membrane permeability following laser irradiation [142–144]. In combination with CW laser light, the group of Liu reported that heating was the major photoporation mechanism. Also carbon nanotubes have been reported as sensitizers [79,145,146], as they are well

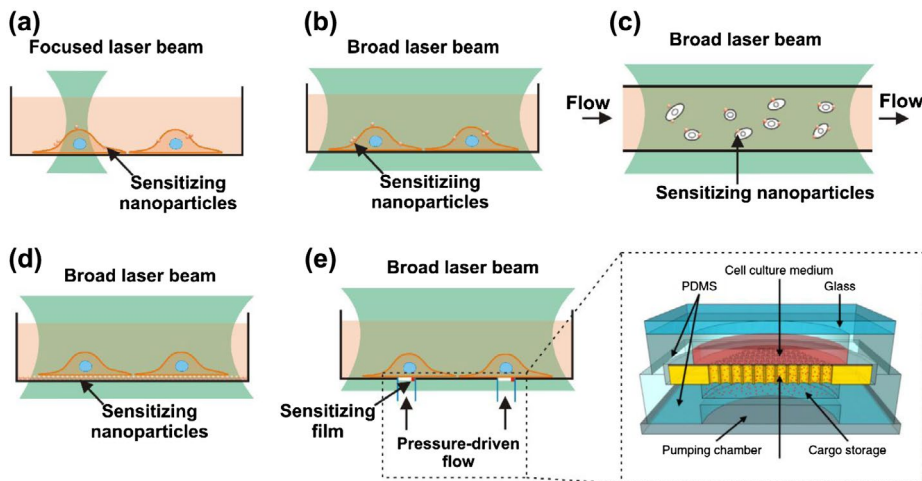


Figure 7. Approaches of NP-sensitized photoporation. (a) A focused laser beam can be applied to photoporate single cells. (b) Due to the sensitizing action of NPs, also a broad (low-energy) laser beam can be used to photoporate several cells at the same time so as to increase throughput. (c) A flow method was developed to photoporate cells in suspension. (d) Sensitizing NPs can be coated on the substrate onto which cells are grown. Pores are formed upon laser irradiation in the adherent part of the cell membrane. (e) Cells are grown on an array of micrometer-wide holes whose sides are asymmetrically coated with crescent-shaped titanium thin films.

Notes: The inset shows the microfluidic chip in more detail (Figure 7(e) Zoom-in figure adapted from Ref. [80]). Vapor bubbles are formed from the titanium structures upon laser irradiation, which form pores in the cell membrane. Cargo is then delivered into the cells by a pressurized fluid flow.

known to absorb laser energy and achieve high surface temperature upon laser irradiation [147,148]. The exact photoporation mechanism was not reported and needs further investigation.

3.3. Experimental procedures for NP-sensitized photoporation

The most frequently used approach to achieve NP membrane poration is by simple addition of the NPs to the cell medium so that they can adsorb to the cell membrane. There are even demonstrations that NPs can be actively attached to (or even injected through) the cell membrane by optical forces [149,150]. Laser irradiation can either be provided with a focused beam (Figure 7(a)) to photoporate single cells or with a broad beam that illuminates several cells at a time (Figure 7(b)). In the latter case, photoporation throughput is significantly enhanced with reported values of $>10^3$ cells/s. To photoporate cells in suspension, Lukianova-Hleb et al. used a broad laser beam illuminating cells flowing through an optically transparent cuvette. With this flow setup, a photoporation rate as high as 5×10^4 cells/s was achieved (Figure 7(c)) [132]. In another approach, to avoid direct adsorption of sensitizing NPs to the cell membrane, sensitizing nanostructures can be incorporated in the substrate onto which cells are grown [80,151] (Figure 7(d)). Using AuNPs-coated glass substrate, Wu et al. showed that gradually more compounds could be delivered into cells with repeated irradiation. The efficiency reached a plateau after six pulses probably due to release of the particles from the substrate. A downside of this approach is that the delivery efficiency was not as high as for NPs present on the cell membrane likely due to the fact that molecules have to diffuse through the narrow space underneath the adherent cells. Wu et al. tried to deliver calcein (0.6 kDa) and obtained ~58% positive HeLa cells. Instead, with membrane adsorbed AuNP, typically >80% positive cells are obtained, even when using larger molecules like 10 kDa FITC-dextran [76]. Substrate-mediated photoporation could be recently made more efficient by a 'laser-assisted surgery tool' was developed by Wu et al. to deliver large cargo in high throughput in cells [80]. The platform consists of a silicon chip providing an array of micrometer-wide holes whose sides are asymmetrically coated with crescent-shaped titanium thin films (Figure 7(e)). Underneath, the silicon chip was connected with vertical silicon channels providing pressure-driven fluid passage for cargo delivery. An array of bubbles is generated from the titanium nanostructures by providing pulsed laser irradiation onto the substrate, forming the pores in the adjacent cell membranes. The cargo is then gently driven into cells by pressurized flow. This platform has been used to deliver large cargos including bacteria, enzymes, antibodies and NPs into various cell types with high efficiency and viability. A similar platform was recently presented for spatially, temporally, and quantitatively controlled delivery of a broad range of molecules into selected cells via plasmonic nanotubes [152]. The delivery is achieved by a planar substrate with an array of protruding micro-fabricated gold nanotubes onto which cells are grown. By laser-irradiation of the

gold nanotubes, heating emerge from the tips that perforate the cell membrane. Compounds can then enter into the cells through the tubes that are directly connected to a microfluidic channel underneath.

4. Resealing of transient pores after photoporation

Following photoporation, cells reseal the membrane pores in a matter of tens of seconds to a few minutes, depending on the pore size. The repair mechanism is based on Ca^{2+} influx that induces exocytosis of lysosomes for ‘patching’ of the pores [153,154]. Based on inflow of fluorescent dextran after photoporation, the group of Kalies noticed that pores in the membrane (with a size of ~50 nm) were resealed in 15 s [136]. Daisuke Yamane reported a sealing time of 1 or 2 min after vapor nanobubble pore formation using an electrical impedance sensor [155]. Palankar found that transient nanopores in lipid membranes generated by photothermal heating of nanorods were resealed in tens of seconds. However, micrometer sized pores could not be repaired [156]. It is consistently observed that higher laser fluences (inducing large pores) or high concentration of NPs (inducing more pores) cause higher toxicity [73,132,135,138]. Therefore, careful optimization of photoporation parameters (like laser fluence, number of NPs) is needed in order to balance delivery efficiency with cell toxicity.

5. Applications of photoporation

5.1. Direct laser photoporation

Photoporation has been extensively explored for transfection of cells with nucleic acids (DNA, mRNA, or siRNA) as well as with proteins. Direct laser-induced photoporation (wavelength of 355 nm with 5 ns pulse duration) was already shown 30 years ago to enable efficient and contact-free delivery of DNA into cells [157]. Later on, fs-pulsed lasers were used to transfect cells with DNA with better efficiency and low cytotoxicity [62]. Spatially controlled introduction of mRNAs into specific regions (cell body or dendrites region) of primary rat neurons was reported by Barrett et al. using fs-pulsed direct laser photoporation [8]. Interestingly, they found that delivery of mRNA in dendrites produced cell death, whereas mRNA introduced in cell bodies did not cause cell death, shedding light on the importance of the dendritic environment on protein function. Recently, Dhakal et al. reported the use of a fs laser for the targeted transfection of single and multiple opsin-encoding genes into selected retinal cells *in vitro* [158]. They found that cells transfected with multiple opsins had a significantly higher white-light-induced photocurrent than cells expressing a single opsin, paving the way toward the restoration of lost vision in retinitis pigmentosa and age-related macular degeneration. Direct laser-induced photoporation was furthermore reported for transfecting ions, small molecules, siRNA, plasmid DNA, and semiconductor

nanocrystals into several cell types [105]. In recent years, cellular imaging by direct laser photoporation for delivery of dyes into live cells is being explored as a new and promising application. In particular, fs laser-assisted direct photoporation was used to deliver actin-staining fluorophores into rat cortical neurons for visualizing the cytoskeleton of dendrites [159].

5.2. NP-sensitized photoporation

NP-sensitized photoporation has been used for high-throughput transfection of cells with DNA, siRNA and proteins [110]. The group of Meunier has explored photoporation of naked DNA into human cancer cells by plasma-induced nanobubbles with a fs laser and found much higher transfection efficiency with very low toxicity compared to conventional lipofection [135]. Similarly, Lukianova-Hleb et al. used a pico-second laser for high-throughput transfection of 'hard-to-transfect' T-cells with GFP plasmids [132]. More studies are needed, however, to fully explore the potential of delivering large macromolecules like mRNA or pDNA in a variety of cell types by NP-sensitized photoporation. Xiong et al. compared the transfection efficiency of thermal-induced pores vs. pores created by thermo-induced VNBs and found that nanobubble-mediated poration allows more efficient entry of siRNA into cells [76]. Using CB sensitizing NPs, small molecules, proteins and DNA can be successfully delivered into living cells [77]. Titanium thin films were used as sensitizers to deliver large cargo like bacteria, enzymes, antibodies, and NPs into diverse cell types with high efficiency, cell viability and high-throughput [80]. Anti-cancer drugs have been delivered by photoporation into cancer cells for an enhanced chemotherapeutic effect [160]. The same group showed that direct delivery of the anti-cancer drug into the cell's cytoplasm by photoporation substantially enhanced the effect of chemoradiation *in vivo* [161]. Also GO NPs were used to deliver a photosensitizer into cells for enhanced photodynamic therapy efficacy against the cancer cells *in vitro* [142]. While photoporation is mostly used to deliver compounds into cells, it has also been shown that it can be used to release compounds from cells, which could be an interesting concept for light triggered drug release [74].

6. Perspectives and conclusion

Although photoporation has already been shown to be an exciting technology for efficient intracellular delivery of membrane-impermeable exogenous materials into living cells, there still lay plenty of challenges and opportunities ahead. On a fundamental level, it is of interest to get a better insight in the loading mechanism of molecules into the cytoplasm, especially for large and charged molecules like pDNA or mRNA. Translocation of molecules into the cytoplasm is mostly thought to happen by diffusion through the membrane pores. However, it has also been proposed that there might be an active 'nano-jet' mechanism involved as well

under certain conditions like the nanobubble-induced photoporation [132]. In order to be able to better control and predict the influx of molecules of various sizes and charges into the cytoplasm, the loading mechanisms need to be studied in more detail. Similarly, it is of fundamental interest to get a better view on the pore size, lifetime and repair mechanisms for the various photoporation approaches. While there have been a few studies along these lines, the reported values are quite different (e.g. pore lifetimes ranging from tens of seconds to tens of minutes), which is likely caused by differences in photoporation conditions, different cell types and different ways how the measurements are perforated. Therefore, more systematic studies are needed on this which in turn will be helpful to better understand and predict the loading process for different types of cells and molecules. Also systematic and in-depth cytotoxicity studies are needed, especially for NP-sensitized photoporation since there is the additional concern of NP-induced toxicity. Although simple cytotoxicity measurements are typically included in current research articles, in-depth understanding of the factors contributing to cytotoxicity is needed to expedite acceptance of photoporation as one of the standard transfection methods by the wider scientific community.

For NP-sensitized photoporation, there clearly lay opportunities on the material size as well. While AuNPs have clearly been used the most till now, it is of interest to look for NPs that are better resistant to the illumination conditions used in photoporation, especially when using pulsed laser light. Indeed, due to the substantial temperature increase the AuNP might melt and change shape (e.g. rods becoming spheres) which alters their wavelength-dependent absorption characteristics. Fragmentation of AuNP has been reported too, which renders them useless already after one laser pulse [69,76]. Carbon nanomaterial sensitized photoporation was recently explored as a promising alternative class of sensitizing NPs with superior thermal stability, facile synthesis, easily tunable surface functionalization and good biocompatibility. Further studies are needed to explore the potential functional benefits compared to AuNPs with cytotoxicity studies being performed in parallel.

Although photoporation has proven its value for delivering compounds into cells in culture, exploration of its *in vivo* potential has only just been started. Recently, the Lapotko group applied for the first time AuNP-sensitized photoporation of chemotherapeutics to *in vivo* cancer treatment. [161]. The same group recently showed *in vivo* elimination of residual head and neck cancer cells by tumor targeting gold colloids [162]. Undoubtedly more examples of *in vivo* applications of photoporation will follow in the near future. Here, it will be of interest to develop biocompatible sensitizing materials because of the toxicity concerns in relation to the *in vivo* usage of colloidal NPs [163]. In a recent report, it was, for instance, shown that VNBS can be formed by irradiating hemozoin crystals (malaria related organic crystals) in the blood circulation with laser light [164]. This shows that there might be opportunities to look for organic materials that could replace inorganic NPs.

Apart from certain *in vivo* applications, we do see a bright future for photoporation as a very flexible *in vitro* transfection technology, especially in combination with sensitizing NPs which enable high-throughput treatment. After about a decade of initial developments, we expect that photoporation will gradually become a more mature and widely available intracellular delivery technology. The portable photoporation device developed by the Heisterkamp group is a good example of this [73], as well as some first attempts to perform automated photoporation in a microfluidics device [109,165]. As cytotoxicity is typically limited under optimized conditions, we believe that photoporation may complement or in some cases even replace standard methods like electroporation which are well-known to induce substantial cell toxicity. Importantly, photoporation offers the unique possibility for spatially controlled delivery into cells as the laser beam can be easily tuned in size and position [152]. The possibility to transfect selected cells in a cell culture offers unprecedented opportunities not possible with any other of the current transfection technologies. One can imagine that this unique feature will be of benefit to study, for instance, cell bystander effects or to transfect one cell type in a co-culture of cells as is of interest for tissue engineering.

Taken together we can conclude that laser-assisted photoporation is a maturing promising technique for unprecedented flexible intracellular delivery of membrane impermeable substances that, thanks to its unique capabilities, will secure its place next to the more established intracellular delivery methods.

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