Supplementary Information

Photothermal nanofibers enable safe engineering of therapeutic cells

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Supplementary Table 1. Required biocompatibility testing of nanotechnological devices as set forward by the Scientific Committee on Health and Environmental Risks (SCENHIR), which is an independent scientific committee that provides advice to the European Commission with regard to emerging health and environmental risks. This includes health risks related to nanomaterials, for which guidelines are established in their 2015 report entitled “Guidance on the Determination of Potential Health Effects of Nanomaterials Used in Medical Devices”. Due to the risk of transferring essentially non-degradable NPs alongside reinfused modified cells in patients, NP-sensitized photoporation would be classified as a ‘long-term invasive medical device with medium exposure’, for which extensive biocompatibility testing needs to be performed as indicated in red. Instead, in case photoporation could be performed without direct exposure of cells to NPs, this approach would be classified as ‘short-term non-invasive with low exposure’, for which only minimal biocompatibility testing is required as indicated in green.

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1 [https://op.europa.eu/en/publication-detail/-/publication/e9899821-e4d4-4ceb-aada-0c62ce6fcfd3](https://op.europa.eu/en/publication-detail/-/publication/e9899821-e4d4-4ceb-aada-0c62ce6fcfd3)
| Name | Sequence                | Product name                  | Manufacturer    |
|------|-------------------------|-------------------------------|-----------------|
| Q1   | ACGGGCGTGACCTCCACATGA   | FlexiTube Genesolution        | Qiagen          |
| Q2   | CTGGTTGAGATGGCCCTGGA    | FlexiTube Genesolution        | Qiagen          |
| Q3   | CCCCTGGTTCTATTATA       | FlexiTube Genesolution        | Qiagen          |
| Q4   | ATCCGGAGAGCTCGTCTAAA    | FlexiTube Genesolution        | Qiagen          |
| S    | TTCCCTGTGGTCTATTATA     | On-Target PLUS specificity    | Dharmacon       |
|      |                         | Enhanced                      |                 |
| T1   | CGGAGAGCTTCGTGCUAAA     | Silencer                      | Thermofisher    |
| D2 (or siPD1) | TGGAGAGCTCGTCTAAA   | ON-TARGETplus                | Dharmacon       |
| siCTRL | TGGTTACATGTGACTAA         | ON-TARGETplus                | Dharmacon       |

**Supplementary Table 2.** siRNAs used for PD1 silencing in human donor-derived T cells.

| Antibody          | Dilution | Company (Cat no.)         | Remarks     |
|-------------------|----------|---------------------------|-------------|
| Mouse-anti-OCT4   | 1:700    | Santa Cruz (Sc-5279)      | Stem cells  |
| Rabbit-anti-SOX2  | 1:600    | Life Technologies (PA1-094) | Stem cells  |
| Rabbit-anti-NANOG | 1:600    | Life Technologies (PA1-097) | Stem cells  |
| Mouse monoclonal anti-cTnT | 1:250 | Life Technologies (MA5-12960) | Cardiomyocytes |
| Rabbit monoclonal anti-Nkx2.5 | 1:100 | Life Technologies (701622) | Cardiomyocytes |
| Goat-anti-Mouse IgG Dylight 488 | 1:1000 | Life Technologies (35503) | Secondary antibody |
| Goat-anti-Rabbit IgG Dylight 594 | 1:500 | Life Technologies (35561) | Secondary antibody |

**Supplementary Table 3.** Antibodies used to label proteins expressed on hESCs or cardiomyocytes
Supplementary Notes 1-3

Supplementary Note 1

Traditional NP-sensitized photoporation makes use of gold NPs which can be activated only once because they tend to fragment after already the first laser pulse, resulting in a loss of their photothermal functionality. We found that the same holds true for photoporation with free IONPs, in which case optimal delivery efficiency was obtained at a laser pulse fluence of 1.26 J/cm² (Supplementary Fig. 4a). Clearly the free IONPs lost their activity already after the first laser pulse (Supplementary Fig. 4b). At such high laser fluences vapor nanobubbles are formed (Supplementary Fig. 4d), resulting in particle fragmentation (Supplementary Fig. 4c). Considering that much lower laser fluences were sufficient to obtain optimal delivery with PEN webs in which IONPs are imbedded into PCL nanofibers, it is of interest to see if the PEN webs can perhaps be activated multiple times by repeated scanning of the laser beam. We hypothesized that this may be possible since the sensitizing particles are stabilized by the surrounding polymer material and are irradiated with less than 1/10th of the laser fluence that is typically used in traditional photoporation. We started by irradiating cells on a PEN substrate two times in a row. In the first round of PEN photoporation we delivered RD10 as before, after which the cells were washed and irradiated a second time on the same PEN substrate but now in the presence of 10 kDa green fluorescent FITC-dextran macromolecules (FD10). When examining the cells by confocal microscopy afterwards, it became clear that most cells had both red and green fluorescence (Supplementary Fig. 5a). It can be noticed that cells exhibiting strong green fluorescence do not necessarily have strong red fluorescence, and vice versa. This is likely due to the fact that there is about 10 min time between both experiments, which is the time needed to do the first photoporation treatment followed by washing of cells, adding the new dextrans and starting the second photoporation procedure. During that time cells might slightly move or reshape their cell surface so that they make contact with the fibers at different places, likely contributing to some variability in the delivery efficiency between both photoporation treatments. Nevertheless, quantitative analysis by flow cytometry confirmed that 90% of cells were positive for both RD10 as FD10 (Supplementary Fig. 5b). To provide further evidence of repeated photoporation with the same PEN webs, we continued photoporating HeLa cells up to 4 times with FD10. The FD10 concentration was doubled (from 0.2 mg/mL to 1.6 mg/mL) between each photoporation round to more easily see the increase in intracellular delivery (which is still diffusion driven). While the percentage of positive cells increased from ~70% to ~90% (Supplementary Fig. 5c), the increased delivery was most apparent from the relative mean fluorescence per cell (rMFI) which increased almost linearly with each round of photoporation (Supplementary Fig. 5d).
Having established that PEN webs can be repeatedly activated, we wanted to determine more precisely to which extent PENs web lose photoporation capacity with each additional scan. To this end we again performed repeated PEN photoporation, but now only added FD10 to the cells just before the last scan. For instance, in case of N=4 scans, the first three laser scans are performed with the cells still in normal culture medium (without FD10), while FD10 is added to cell medium prior to the fourth and last scan. When looking at the percentage of transfected cells, a small drop in PEN substrate functionality is observed when photoporing more than 2 times. However, the data clearly showed that the PEN web can be activated for at least 6 times, still transfecting 60-70% of the cells (Supplementary Fig. 5e). Interestingly, repeated photoporation of the cells did only have little effect on cell toxicity, with 75% viable cells even after 6 times PEN photoporation (Supplementary Fig. 5f).

The slight reduction in photoporation efficiency upon repeated activation may be due to either morphological changes happening to the IONPs (clusters) or possibly a release of (a part of) the IONPs from the fibers. Since the absence of IONPs release is a fundamental premise in our study, it will be investigated and discussed separately below. At this point we can already confirm that IONPs release does not happen, so that we here focus on potential morphological changes of the embedded IONPs after (repeated) laser irradiation which was investigated by SEM and TEM. As SEM and TEM images in Supplementary Fig. 6a show, IONPs remained unchanged after a single laser scan at the lowest fluence of 0.04 J/cm². At higher fluences the IONPs tended to melt and form larger spherical structures probably due to the high temperature that is reached. For instance, at a laser pulse fluence of 0.12 J/cm² a temperature of ~1800 °C is reached in the particles (see simulations further on), which is already above the iron oxide melting temperature (1565 °C). A similar phenomenon was observed when exposing the PEN webs to multiple laser scans (0.08 J/cm²), finding that gradually more and more IONPs clusters reshaped into larger spherical particles (Supplementary Fig. 6b). Since this reduces the effective photothermal area to some extent, this may explain why the photoporation efficiency dropped slightly upon repeated laser activation (Supplementary Fig. 6c).

Until now we evaluated intracellular delivery of a 10 kDa model marker, which is of a similar size as for instance antisense oligonucleotides or siRNA. In addition, it is of interest to evaluate to which extent larger macromolecules can be delivered, with a molecular weight closer to that of proteins or mRNA. To that end we used 40 kDa, 70 kDa, 150 kDa and 500 kDa FITC-dextran (FD40, FD70, FD150 & FD500) as model molecules, which were delivered in HeLa cells by 1x, 2x and 4x PEN photoporation. As shown in Supplementary Fig. 7a, b, delivery efficiency gradually decreased for increasing molecular weight, which is due to a combination of molecules becoming large compared to the pore size as well as slower molecular diffusion. Repeating the photoporation procedure generally resulted in slightly more positive cells, while it did not improve the amount delivered per cell. Still, we conclude that PEN
Photoporation is successful in transfecting cells with compounds up to at least 500 kDa, with a very substantial 65-90% transfected cells depending on the molecular size.

Supplementary Note 2
The mechanism of cell membrane permeabilization during PEN photoporation is transient local heating.

Given these positive results on adherent and suspension cells and having confirmed that the delivery process happens in a NPs-free manner, we continued investigating into greater details of the mechanism behind the cell membrane permeabilization by PEN photoporation. In traditional NPs-sensitized photoporation, membrane permeability can be induced by photothermal effects (heat or mechanical energy) or a photochemical process. Photochemical processes include the generation of reactive oxygen species (ROS) which primarily occurs when irradiating the sensitizing NPs with ultrafast femtosecond or picosecond pulsed lasers. Since in this work we used a much larger pulse width (7 ns), it seemed unlikely that permeabilization is caused by photochemical processes. Indeed, using 2',7'-Dichlorofluorescin (DCFH) as a fluorescent ROS indicator, we found no noticeable ROS after laser irradiation of PEN webs with IONPs content of up to 5% and laser fluence up to 0.16 J/cm² (Supplementary Fig. 9a). This means that a photothermal mechanism must be responsible for the cell membrane permeabilization, which can be either mechanical damage through the formation of vapor nanobubbles or thermal damage through direct heating of the cell membrane. We investigated the potential formation of VNBs with dark field microscopy by which VNBs can be easily visualized as reported before by us and others. Indeed, VNBs are visible in dark field images as brief localized bursts of light. When using a relatively low laser fluence of 0.14 J/cm², which is similar to the optimal condition for Hela’s (0.08 J/cm²) and Jurkats (0.16 J/cm²), no VNB could be observed (Supplementary Fig. 9b). Only at substantially higher laser fluences (>0.5 J/cm²) VNBs started to appear, similar to what we observed for free IONPs (Supplementary Fig. 4d). By counting the number of VNBs within the laser irradiation area for increasing laser fluence, one can determine the VNB threshold, defined as the laser pulse fluence at which 90% of the plateau of producing VNB. The VNB generation threshold was virtually identical for PEN webs with 0.02% and 2% IONPs with a value of 1.4-1.5 J/cm² (Supplementary Fig. 9c). This is about 10x higher than the highest laser fluence used for PEN photoporation, so that we can safely exclude VNBs formation as the dominant permeabilization mechanism.

This leaves a pure heating mechanism as the only left plausible mechanism for membrane permeabilization. It is of note that it cannot be simple bulk heating, since fibers with 5% IONPs irradiated by a single laser pulse of 0.16 J/cm² can only increase the bulk temperature by 0.005 K (see ‘Methods’ for details on the calculation). Therefore, what most likely happens is that the temperature
is rapidly and locally increased at the IONPs (cluster) sites within the fibers, which permeabilize locally
the cell membrane where it is in contact with the fiber hot spots. As it is virtually impossible to
investigate this experimentally due to the extremely short time and small spatial scales, we
investigated this more insight with theoretical calculations and numerical simulations of heat transfer
from IONPs to the surrounding following the absorption of a laser pulse, as schematically shown in
Supplementary Fig. 10a. From the Generalized Multiparticle Mie Theory simulations the optical
properties of the IONPs we calculated absorption cross-section which was further used to calculate
the initial IONPs temperature and numerically solved the heat transfer differential equations in 3D to
simulate heat transport from the IONPs into the fibers and surrounding cell medium. Note that
potential phase changes were neglected during heat diffusion, which seems a reasonable assumption
in the absence of VNBs generation. The calculated UV-VIS extinction spectrum as calculated from Mie
Theory matched well with the experimental spectrum of IONPs dispersed in DI water (Supplementary
Fig. 10b). Since we have already shown that IONPs tend to be present in clusters within the fibers, we
calculated the absorption cross section spectra for sets of IONPs in close proximity. As can be seen in
Supplementary Fig.11a, this does not have a great impact on the value of the absorption cross section
at 647 nm, probably due to only weak electromagnetic coupling between individual IONPs. We also
calculated the absorption cross section spectra for IONPs in different media (PCL polymer, water, or
an average of both), again finding that it does not appreciably change the value of the absorption cross
section at 647 nm (Supplementary Fig. 11b). As such we used the theoretical absorption cross section
at 647 nm of a single IONP to calculate its initial temperature \(T_0\) upon absorption of a single 7 ns laser
pulse (\(\lambda = 647 \text{ nm}\)) for various laser fluences. As shown in Supplementary Fig. 10c, initial
temperatures could easily exceed the iron oxide melting temperature of 1565 °C. For instance, already
at a laser fluence of 0.12 J/cm² a temperature of ~1600 °C is reached.

Next, a 3D model was built to simulate the heat transfer of a single 160 nm IONP to the surrounding
environment, consisting of a PCL fiber of 320 nm diameter and surrounded by water (Supplementary
Fig.11c). Simulations were performed for the absorption of a 0.08 J/cm² laser pulse by an IONP that is
separated by a distance \(h = 40 \text{ nm}\) from the fiber surface (cfr. Fig. 1m). Since the duration of a single
pulse is 7 ns, we started the simulations by setting the IONP temperature to 1069 °C for this duration.
The subsequent heat transfer is shown in Supplementary Fig. 10d and Supplementary Movie 2 where
temperatures >60 °C are color-coded in red, which is reportedly the temperature at which cell
membranes become completely permeable.\(^8\,9\,10\). As can be seen, a substantial area at the fiber surface
reaches >60°C, although within a very short time interval of tens of nanoseconds only. This area is
analyzed as a function of time for the upper side of the fiber. In Supplementary Fig. 10e, f the time
course of A is plotted, which represents the size of the areas >60°C, together with the average
temperatures $T$ over these areas. The average temperature remains $>60^\circ\text{C}$ for a time period of 137 ns, reaching a maximum of 110.1 °C after 27 ns and with a time average value $\overline{T} = 85.4 °\text{C}$. The area $>60^\circ\text{C}$ is on average $\overline{A} = 0.087 \mu\text{m}^2$, with a maximum size of 0.145 $\mu\text{m}^2$ which is reached after 57 ns.

Next, we investigated how $\overline{T}$ and $\overline{A}$ depend on the laser pulse fluence ($I=0.04-0.32 \text{ J/cm}^2$), the number of locally clustered IONPs ($N=1, 2, 4, 8$) and the distance $h$ (5, 40, 80 nm) from the fiber surface. Note that a maximum of $h=80 \text{ nm}$ was chosen since more than 90% IONPs had $h<80$ in this work. Similarly, a maximum of $N=8$ was considered since it covers the situation for 95% of the IONPs. For these calculations clusters of IONPs were modeled as linear arrangements of IONPs, which is a simplification of reality but nevertheless sufficient to capture the essence of the underlying phenomena. As can be expected, the closer the IONPs are to the fiber surface (smaller $h$) the higher the average temperature $\overline{T}$ will be (top panels in Supplementary Fig. 10g-j). Interestingly, however, it did not appreciably affect the area $\overline{A}$ (bottom panels in Supplementary Fig. 10g-j). The opposite is found when considering an increasing the cluster size (higher $N$), which did significantly affect the area $\overline{A}$ but not the average fiber surface temperature $\overline{T}$. Perhaps the most interesting is the observation that the laser fluence had a considerable effect on $\overline{A}$, but only up to a laser fluence of 0.08 to 0.16 $\text{J/cm}^2$ (depending on the IONPs cluster size). Indeed, when increasing further to 0.32 $\text{J/cm}^2$, the effect on $\overline{A}$ is very limited. If we consider $\overline{A}$ to be a measure for the effective pore size in the cell membrane, it predicts that increasing the laser fluence above 0.16 $\text{J/cm}^2$ will not much increase the delivery efficiency of large macromolecules, which is indeed what we have found for both HeLa and Jurkat cells. Seeking further confirmation for this, we delivered FDS00 in HeLa cells by PEN photoporation for increasing laser pulse fluence, as shown in Supplementary Fig. 12. Exactly as predicted by our simulations, the delivery efficiency in terms of both % positive cells and rMFI did not substantially improve further for a laser fluence above 0.16 $\text{J/cm}^2$. The simulations do show, however, that further improvements may be possible by increasing the cluster size (higher $N$), which points to an interesting avenue for future research and optimization of PEN photoporation.

Considering that the IONP temperature may reach up to 1600 °C, it is of interest to consider the possibility that vapor nanobubbles are formed when fibers are in contact with water. Vaporization of a liquid surrounding a nano-object happens when the liquid’s spinodal temperature is reached, which is 277 °C for water at ambient pressure$^{11, 12, 13}$. To see if a temperature $>277 °\text{C}$ is reached, we performed additional calculations for IONP at various depths below the fiber surface ($h=5, 10, 20$ and 40 nm). Simulations were performed for a laser pulse of 0.08 $\text{J/cm}^2$ or 0.16 $\text{J/cm}^2$, which are the optimal settings for adherent and suspension cells, respectively. In addition, we repeated these calculations for clusters of IONP consisting of 1 to 8 individual nanoparticles ($N=1, 2, 4$ and 8). From these simulations we calculated the ratio of the fiber surface area that reaches a temperature above 277 °C
(\(A_s\)) and above 60 °C (\(A_e\)). The ratio \(A_s/A_e\) indicates the relative importance of membrane pore formation by vapour nanobubbles (\(T > 277 °C\)) or by direct heating (\(T > 60 °C\)). **Supplementary Fig. 13** shows that for a laser fluence of 0.08 J/cm² \(A_s/A_e\) is less than 5% in all cases, and even is 0% when \(h \geq 20\) nm. For a laser fluence of 0.16 J/cm² \(A_s/A_e\) is less than 10% in all cases. Together these theoretical considerations show that, even though bubble formation cannot be fully excluded, under the conditions used in our work it is quite unlikely to take place, or at least is not a substantial contributor to the cell permeabilization process which is almost purely heat mediated. These calculations are further supported by dark field microscopy experiments in which vapor nanobubble generation was not observed under similar conditions (**Supplementary Fig. 9b, c**).

**Supplementary Note 3**

However, flow cytometry analysis tends to overestimate cell viability as it does not account for fragmented cells which are ‘lost’ in the debris background. Indeed, when we measured the viability of electroporated cells by flow cytometry after Calcein AM staining, an apparently high cell viability of up to 80% was found (**Supplementary Fig. 16a**). However, when the same cells were measured by the Cell Titre Glo assay, which measures the remaining metabolic activity of cells compared to the initial population, the viability was found to be much less, reaching 20-30% at best. To confirm that this apparent discrepancy is due to complete cell fragmentation, we determined absolute cell numbers by cell counting before and after electroporation of T cells labeled with Calcium AM (green) and Propidium Iodide (red) (**Supplementary Fig. 16b**). Quantification of microscopy images showed that it is in fact more than 30% of cells that are lost by fragmentation after EP (**Supplementary Fig. 16c**), both 1 h and 24 h after treatment. The fact that the difference in viabilities between flow cytometry and the Cell Titre Glo assay is even larger than that shows that many of the remaining ‘intact’ cells are actually not very healthy, with reduced metabolic activity even after 24 h of recovery.

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Supplementary Figure 1. Supplementary Figure 1. (a) Schematic representation of the electrospinning setup with a high voltage power supply, a digital syringe pump and a grounded rotating collector. (b) Photographs of microscope glass slides with a web of nanofibers on top containing different amounts of IONPs. (c) The size of individual IONP was quantified from TEM images and shown here as a size distribution (n=103). The mean diameter was 162 nm with a standard deviation of 41 nm. The inset shows an exemplary TEM image. (d) High resolution TEM images showing that IONP are embedded close to the PCL fiber surface but are still covered by a thin layer of PCL polymer. The outer surface of the IONP and nanofibers is demarcated by white and yellow dotted lines, respectively. (e) The parameters \( l_1, l_2 \) and \( h \) were quantified from TEM images of nanofibers prepared with 1% and 5% IONPs (n=53). (n=53, mean±SD, one-way ANOVA).
Supplementary Figure 2. (a) Schematic overview of the experimental procedure to deliver macromolecules into cells by PEN photoporation. (b) Photograph of microscope glass slides with nanofibers and adhesive stickers that are being sterilized by UV treatment in a laminar flow hood. (c) Schematic representation of how the homemade PEN culture wells are prepared. An eight well adhesive sticker (silicone) is applied onto a PEN web that is still attached to a glass slide. The top plastic layer is a removable protective layer on the top side of the silicone stickers. (d) After removal of the web and sticker from the glass slide, the sample is cut so as to obtain individual culture wells with PEN bottom. A photograph is shown of an individual PEN culture well held up in the air (top) or applied in a 6-well plate filled with water.
Supplementary Figure 3. (a) Schematic representation of the experimental procedure to modify the PCL fibers with collagen for better cell attachment. (b) Confocal images of HeLa cells grown in a PEN cell culture well (1% IONPs) without (left) and with collagen-coating applied to the fibers (right). Cells were labeled with the calcein-AM viability stain. (c) The cell density and cell area were quantified by image processing (n=7 from independent three samples, the thick horizontal lines in the box plot indicate the median value; the boxed area extends from the 25th to 75th percentile with whiskers from the 5th to the 95th). (d) The number of IONPs clusters available per cell was calculated by multiplying the IONPs density (see Supplementary Fig. 2i, n=10 images from three independent experiments,
mean ± SD) with the cell area for increasing percentages of embedded IONPs. (e) Red fluorescent dextran of 10 kDa (RD10) was delivered into HeLa cells by PEN (1% IONPs) photoporation. Confocal images show green fluorescence from the Calcein-AM viability staining and red fluorescence from RD10. Transfection efficiency increases with increasing laser pulse fluence (25.6% ± 13.0% RD10 positive cells for l=0.04 J/cm², 86.8% ± 6.9% RD10 positive cells for l=0.08 J/cm², and 92.2% ± 10.0% RD10 positive cells for l=0.12 J/cm²). At the highest laser fluence cell toxicity becomes apparent from a loss of green cells (88.6% ± 10.2% viability for l=0.04 J/cm², 87.8% ± 8.1% viability for l=0.08 J/cm², and 56.9% ± 19.6% viability for l=0.12 J/cm²).
Supplementary Figure 4. ‘Traditional’ photoporation with free IONP. (a) HeLa cells are incubated with free IONP, allowing the nanoparticles to attach to the cell membrane. Cells are subsequently washed and irradiated with pulsed laser light in the presence of FD10. The percentage of FD10 positive cells is quantified by flow cytometry, while cell viability is assessed in parallel by the Cell Titer Glo assay. IONP concentration was gradually increased from $2.25 \times 10^8$ to $9.0 \times 10^8$ NPs/ml in combination with a laser pulse fluence ranging from 0.32 to 1.60 J/cm$^2$. (n=3, independent experiments, mean ± SD) (b) Quantification of delivery efficiency after N laser irradiation steps (N=2, 3, 4 and 5). Cells treated with IONP ($4.5 \times 10^8$ NPs/ml) were first irradiated N-1 times, after which FD10 was added and delivered into the cells by the N$^{th}$ irradiation cycle. This shows the remaining photoporation efficiency upon the N$^{th}$ photoporation cycle. (n=3, independent experiments, mean ± SD) (c) IONP morphology after irradiation with N laser pulses of 1.26 J/cm$^2$. N=0 corresponds to unirradiated IONP. Scale bars are 500 nm. (d) The number of VNBs within the laser irradiation zone was counted for increasing laser fluence using free IONPs suspended in water. The solid line is a Boltzmann fit, from which the VNB threshold fluence can be derived ($I_{th} = 1.07$ J/cm$^2$), defined as the laser fluence at which 90% of the particles generate VNBs.
Supplementary Figure 5. (a) Repeated photoporation with PEN webs is demonstrated by sequentially delivering RD10 (red fluorescence) and FD10 (green fluorescence). The overlay shows that many cells have both green and red fluorescence. (b) Flow cytometry data showing red (RD10) fluorescence in the x-axis and green (FD10) fluorescence in the y-axis. Approximately 90% of the cells are positive for both red and green fluorescence after repeated photoporation. (c-d) Repeated photoporation of cells on PEN webs was demonstrated by FD10, the concentration of which was doubled between each scan from 0.2 to 1.6 mg/mL. The percentage of positive cells was quantified (c) along with the rMFI (relative mean fluorescence intensity) per cell (d). (e) The extent to which PEN webs loose transfection capacity upon each subsequent round of photoporation was investigated by photoporating cells N-1 times in the presence of normal cell medium (without marker) and the last time in the presence of FD10. The dashed line indicates the percentage of positive cell by one laser scan as a reference. (f) The corresponding cell viability was determined by Calcein AM. The dashed line indicates cell viability for one laser scan. (n=3 independent experiments, mean±SD)
Supplementary Figure 6. (a-b) SEM and TEM images of PEN webs after a single laser scan of increasing laser fluence (a), or after multiple laser scans at a fixed laser fluence of 0.08 J/cm² (b). The photoporation procedure was repeated with the same PEN substrates up to 4 times (N = 1, 2 and 4). (c) The effective photothermal area on the nanofiber surface was calculated for I=0.08 J/cm² as a function of the number of IONP either as a linear arrangement of individual neighboring IONP or as an equivalent larger spherical particle of the same total volume. The effective photothermal area is defined as the average area of the nanofiber surface that reaches a temperature above 60 °C.
Supplementary Figure 7. FITC-dextrans from 10 to 500 kDa were delivered into HeLa cells by PEN photoporation using fibers with 1% IONPs. Uptake was determined by flow cytometry and expressed as the percentage of positive cells (a) and rMFI (b). (n=3, independent experiments, mean±SD)
Supplementary Figure 8. (a) A z-projection confocal view of Jurkat cells collected on the PEN substrate is shown in the top panel. A sliced view is presented in the bottom panel showing a focal plane where both the fibers and the bottom of the cells are visible. The plasma membrane was labelled by CellMask (red) while the nanofibers were labeled with Coumarin 6 (green). (b) Quantification of the number of IONPs clusters per Jurkat cell for increasing percentages of IONPs embedded in the nanofibers (n=10 images from three independent experiments, mean±SD). (c) FD10 was photoporated into Jurkats with a PEN substrate containing 1% IONPs (n=3, independent experiments, mean±SD, one-way ANOVA). The nanofibers were either neutral (unmodified), positively charged (PAH treated) or collagen-coated. Photoporation was each time performed once with a laser pulse fluence of 0.08 J/cm². Differences between two datasets were assessed using one-way ANOVA. Statistical significance is indicated as follows: ns P≥0.05, **P<0.01, ***P<0.00.
Supplementary Figure 9. (a) ROS generation was determined using 2', 7'-Dichlorofluorescin (DCFH) as a fluorescent indicator. A 10 µM H$_2$O$_2$ solution was used as positive control. ROS generation was measured in DI water collected from PEN substrates containing different amounts of IONPs (0, 1, 5%), with and without laser irradiation at different fluences (0, 0.08, 0.16 J/cm$^2$) (n=3, independent experiments, mean±SD, one-way ANOVA). (b) Exemplary dark field images recorded just before, during and just after the arrival of a single laser pulse on a PEN web with 1.0% IONPs. At a laser pulse fluence of 0.56 J/cm$^2$, VNBs can be seen in the middle image within the green circles. At a lower laser fluence of 0.14 J/cm$^2$, on the other hand, VNBs could not be observed. Dashed circles indicate the laser irradiation region. (c) The number of VNBs within the laser irradiation zone was counted for increasing laser fluence using PEN substrates with 0.02% and 2% IONPs (n=15 images from three independent experiments, mean±SD). The solid line is a Boltzmann fit, from which the VNBs threshold fluence can be derived, defined as the laser fluence at which 90% of the particles generate VNBs.
Supplementary Figure 10. Local transient heating is the mechanism behind cell membrane permeabilization by PEN photoporation. (a) Schematic overview of theoretical calculations on laser-induced heat generation in IONPs followed by heat transport in the fiber and surrounding cell medium. (b) The extinction spectrum of 160 nm IONPs in dispersion was measured by UV-VIS spectrometry and calculated from Mie theory. (c) The temperature was calculated for a single IONP after absorption of a 7 ns laser pulse at 647 nm for increasing laser fluences. (d) Example of a simulation on the heat transfer from a heated IONP embedded in a PCL nanofiber and surrounded by water. The IONP initial temperature was calculated from the absorption of a 7 ns 0.08 J/cm² laser pulse. The IONPs were positioned at a distance \( h = 40 \) nm from the fiber surface. The orange dashed lines indicate the boundary of the nanofiber. (e-f) Time dependency of parameters \( T \) and \( A \) for \( N=1 \), \( h=40 \) nm and \( I=0.08 \) J/cm². Here, \( A \) is the total area of the fiber surface with a temperature >60 °C, while \( T \) is average temperature of this area. \( \bar{T} \) and \( \bar{A} \) are the time averages of \( T \) and \( A \). (g-j) Systematic evaluation of the effect of laser fluence (I), distance between IONPs and fiber surface (h) and number of clustered IONPs (N=1 (h),N=2 (i), N=3 (j), N=4 (k)) on \( \bar{T} \) and \( \bar{A} \) via numerical simulation.
Supplementary Figure 1. (a) The IONPs absorption cross-section spectrum was calculated from the generalized multiparticle Mie theory for different linear particle arrangements (N = 1, 2, 4 and 8 IONPs separated by 1 nm interparticle distance). The inserted graph shows the absorption cross-section at the wavelength of 647 nm for the various linear IONPs arrangements. (b) The absorption cross-section is shown, calculated from Mie theory, of a single IONPs in water, PCL polymer and a 50% water-50% polymer effective medium. (c) The overall 3D geometry of the model used for numerical simulations is shown (left) with a zoom-in of the central region that contains the IONPs embedded in the central fiber (right). (d) The simulation domain is discretized into a grid which is shown for the entire simulation space on the left, and for the central nanoparticle region on the right.
Supplementary Figure 12. FD500 delivery in HeLa cells by PEN photoporation with a 1% IONPs substrate for increasing laser fluences. The percentage of positive cells and the rMFI was determined by flow cytometry (n=3, independent experiments, mean±SD).
Supplementary Figure 13. The ratio of the fiber surface area that reaches a temperature above the spinodal temperature $A_s$ vs. the area that reaches a temperature above 60 °C $A_e$ is plotted as a function of depth $h$ of the IONP cluster below the fiber surface. Calculations were performed for clusters of $N=1, 2, 4$ and 8 IONP irradiated with a single laser pulse of (a) 0.08 J/cm$^2$, and (b) 0.16 J/cm$^2$. 
Supplementary Figure 14. (a) Singlet human T-cells were gated based on the forward scatter (FSC) and side scatter (SSC) signals. (b) The gated singlet cell population consisted of nearly 100% CD3+ T cells according to the PB450 CD3 antibody fluorescence. (c) The CD3+ T cell population included ~45% CD4+ cells labelled by the PC5.5 CD4 antibody. (d) The rest of the population primarily consisted of ~53% CD8+ cells labelled by the APC CD8 antibody.
Supplementary Figure 15. Intracellular delivery of FD10 in primary human T cells by PEN photoporation (neutral fibers) (a) and viability as measured by Cell Titre Glo after 2 h (b) and the delivery yield (c) were determined as a function of laser fluence and IONP content. The FD10 concentration was 2 mg/ml. (n=3, biological independent samples, mean±SD)
Supplementary Figure 16. T-cell viability after electroporation. (a) Human T cells were electroporated with Nucleofector programs EO-100, EO-115 and FI-115 according to the manufacture’s recommendations. Viability was measured by Calcein AM (live-death stain) and Cell Titer Glo (metabolic activity) after 2 h. (n=3, biological independent samples, mean±SD) (b) An exemplary large field of view confocal image of cells collected in a well of a 96-well titer plate. NTC = negative control cells, +EP = post electroporation with program EO-115. Viable cells were stained by Calcium AM (green) while dead cells were stained with Propidium Iodide (red). N is the total number of cells (live + dead) as counted by image processing. The insets show an enlarged image of part of the wells. (c) Quantification of the percentage of cells lost after EP, both 1h and 24h after electroporation with program EO-115. (n=3, biological independent samples, mean±SD)
**Supplementary Figure 17.** PD1 protein expression levels of human T cells 24 hours after transfection with 1 µM of various commercial PD1 siRNA constructs (cfr. Supplementary Table 3) (n=3, biological independent samples, mean ± SD).
Supplementary Figure 18. Representative flow cytometry histogram for CD70 tumor-antigen expression and PD-L1 expression on SKOV3 and H1650 cells. The values on the top left indicate the mean fluorescence intensity.
Supplementary Figure 19. (a) Tumor size are as a function of days post SKOV3 injection (n=4 for PBS, n=5 for CAR T cells, n=4 for PEN photoporated CAR T cells with siPD1, n=4 for CAR T cells with antibody administration biological independent animals, mean ± SD). They are shown for individual mice injected with (b) PBS (negative control, 4 mice), (c) CAR T cells (5 mice), (d) PEN photoporated CAR T cells with siPD1 (4 mice), and (e) CAR T cells with antibody administration (positive control, 4 mice).