Video Article

Optical Control of Living Cells Electrical Activity by Conjugated Polymers

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Abstract

Hybrid interfaces between organic semiconductors and living tissues represent a new tool for *in-vitro* and *in-vivo* applications. In particular, conjugated polymers display several optimal properties as substrates for biological systems, such as good biocompatibility, excellent mechanical properties, cheap and easy processing technology, and possibility of deposition on light, thin and flexible substrates. These materials have been employed for cellular interfaces like neural probes, transistors for excitation and recording of neural activity, biosensors and actuators for drug release. Recent experiments have also demonstrated the possibility to use conjugated polymers for all-optical modulation of the electrical activity of cells. Several *in-vitro* study cases have been reported, including primary neuronal networks, astrocytes and secondary line cells. Moreover, signal photo-transduction mediated by organic polymers has been shown to restore light sensitivity in degenerated retinas, suggesting that these devices may be used for artificial retinal prosthesis in the future. All in all, light sensitive conjugated polymers represent a new approach for optical modulation of cellular activity.

In this work, all the steps required to fabricate a bio-polymer interface for optical excitation of living cells are described. The function of the active interface is to transduce the light stimulus into a modulation of the cell membrane potential. As a study case, useful for *in-vitro* studies, a polythiophene thin film is used as the functional, light absorbing layer, and Human Embryonic Kidney (HEK-293) cells are employed as the biological component of the interface. Practical examples of successful control of the cell membrane potential upon stimulation with light pulses of different duration are provided. In particular, it is shown that both depolarizing and hyperpolarizing effects on the cell membrane can be achieved depending on the duration of the light stimulus. The reported protocol is of general validity and can be straightforwardly extended to other biological preparations.

Video Link

The video component of this article can be found at https://www.jove.com/video/53494/

Introduction

The possibility to manipulate the cellular activity with a precise spatial and temporal resolution represents a key strategy in neuroscientific research and in the treatment of neurological and psychiatric disorders.[1] Traditional methods are based on electrical stimulation of cells using electrodes positioned in proximity or in contact with the targeted system,[2] which can be of different complexity (single cell, cellular network, brain slices, *in-vivo* brain tissues). During the past century, the use of patch-clamp, metal and substrate-integrated electrodes have provided a detailed picture of the physiology and pathophysiology of single neurons and of the functioning mechanisms of neural networks. However, electrical stimulation suffers from important limitations. The first one is related to a generally poor spatial resolution due to the physical dimensions of the electrodes and their fixed geometry, which cannot be readily adapted to complex organized systems like biological tissues. Also, problems related to the electrodes impedance and of cross-talk between stimulation and recording systems may deteriorate the final signal-to-noise ratio of the measurements.[3] On the other hand, the use of light for stimulation may help to overcome many limitations of the electrical approach. First of all, it offers unprecedented spatial (< 1 μm) and temporal resolution (< 1 msec), making it possible to target specific cell types or even sub-cell compartments. In addition it is highly non-invasive since it avoids any physical contact with the tissue of interest and disentangles stimulation from recording. Moreover, both light intensity and wavelength can be precisely regulated and thus diverse stimulation protocols can be applied.[3,4]

However, the vast majority of animal cells do not present any specific sensitivity to light. Several strategies for optical stimulation have thus been proposed, either exploiting light-sensitive molecular mediators nearby or within the cells, or using photoactive device placed externally, close to the cell. The former category refers to endogenous mechanisms like the stimulation via visible or infrared (IR) light, as well as the use of either photoisomerizable/photocleavable compounds or the genetic expression of photosensitive molecular actuators (optogenetics). The latter class includes techniques for exogenous stimulation achieved with the use of inorganic nano/micro-particles or photoconductive silicon substrates.[5] Nevertheless, all these systems have bright sides and drawbacks. In particular, endogenous absorption of cells in the visible range is weak and not reliable, and the concomitant generation of reactive oxygen species may be harmful to the cell. In general, IR is used for inducing local thermal heating due to water absorption, but the extinction coefficient of water is small, thus requiring strong infrared light (from tens to hundreds of W/mm^2) that is difficult to deliver via standard microscope optics and may pose safety concerns for *in-vivo* applications. On the other hand,

photo-switchable caging compounds have a time limited action and often require UV light that is hard to deliver due to limited tissue penetration. In addition they suffer from diffusion problems of the activated compounds upon photolysis outside the illuminated area. Finally, optogenetic tools have allowed scientists to target specific cellular subpopulation and sub-compartments and are rapidly emerging as one of the key technologies in neuroscientific research. However, inserting an exogenous DNA segment via a viral vector raises important safety issues, especially in view of adoption on human patients.[5,6] For these reasons, research on new materials and devices capable of cell optical manipulation is an extremely hot topic.

Recently, a novel approach based on the use of light-sensitive conjugated polymers, able to efficiently transduce an optical stimulus into a modulation of cell electrical activity, has been proposed. The Cell Stimulation by Polymer Photoexcitation (CSPP) technique exploits many key-enabling features typical of organic semiconductors: they are intrinsically sensitive to light in the visible range;[7] they are biocompatible, soft and conformable and their mechanical flexibility allows an intimate interface with tissue both *in-vitro* and *in-vivo*[8-10]. Apart from that, they can be easily functionalized to better adapt to the interface with living cells, and to enable specific excitation, probing and sensing capabilities.[11,12] Moreover, they support electronic as well as ionic transport, making them ideal for the combination of electronics ad biology.[13,14] Interestingly, they can work in photovoltaic mode, avoiding the need to apply an external bias for efficient cell optical stimulation.[15]

The reliability of CSPP technique has been previously demonstrated in several systems, including primary neurons,[15,16] astrocytes,[17] secondary cell lines[18] and explanted retinal tissues.[16] In this work, all the steps necessary to fabricate a light-sensitive bio-polymer interface[19] for optical stimulation of *in-vitro* systems are described in detail. As a study case, a prototypical organic photovoltaic blend of region-regular poly(3-hexylthiophene) (rr-P3HT), functioning as the electron donor, and phenyl-C61-butyric-acid-methyl ester (PCBM), acting as the electron acceptor is employed. As the biological system, Human Embryonic Kidney (HEK-293) cells are used. An example of a photostimulation protocol with the relative recording of cell activity via electrophysiological measurements is provided.

The described platform is however of general validity, and it can be easily extended to the use of other conjugated polymers (by properly adjusting the solution preparation process and the deposition parameters), different cell types (by properly changing the cell culture protocol, plating procedure and time requested for cell seeding and proliferation) and different stimulation protocols (light wavelength, stimuli frequency and duration, photoexcitation density).

Protocol

1. Preparation of Photoactive Substrates

1. Prepare a P3HT:PCBM solution (1:1 w/w) in chlorobenzene at a P3HT concentration of 20 g/L. Mix the solution with a magnetic stirrer for at least 4 hr at 60 °C. Consider a volume of 150 µl of solution for each substrate to be prepared.
2. Clean ITO-coated glass slides (R_s = 10 Ω/sq, 18x18 mm^2, thickness 170 µm) with consecutive baths of deionized water, acetone and isopropanol in a sonicator (10 min for each step). Dry the coverslips with a nitrogen gun. Put the samples in a plasma cleaner at 100 W power for 10 min.
3. Deposit a thin film of the active layer on the cleaned substrates via spin-coating.
    1. With a diamond tip, cut a microscope glass slide, (thickness ≈ 1 mm) to the same lateral dimensions of the ITO-coated substrates. Remove the P3HT:PCBM solution from the hotplate and let it cool to ambient temperature.
    2. Set a two-steps spin-coater program: i) 30 sec at 800 rpm; ii) 30 sec at 1,600 rpm. Place the slide on the chuck of the spin-coater and start the vacuum to fix it. Put a drop of acetone on the slide and then the cleaned substrate, with the ITO-coated side facing upwards. Start the spin-coater rotation to get rid of the excess acetone.
    3. Put 150 µl of P3HT:PCBM solution on the substrates and start again the spin-coater (use the same protocol described in 1.3.2).
    4. After the spin-coater has finished, remove the sample and carefully detach the coated substrate from the glass slide. Clean the back of the substrate with a cleanroom swab dipped in acetone.
    Note: The photoactive substrates can be stored for few days in a dark box until used. For longer periods, keep them in a glovebox with inert gas.

2. Preparation of Cell Culture Medium and Electrophysiology Solutions

1. Prepare the complete growth medium (CGM) for HEK-293 cells: Dulbecco Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) not heat-inactivated, 100 U/ml penicillin and 100 µg/ml streptomycin. Sterilize the medium with a 0.2 µm filtration system and store at 4 °C.
2. Prepare the extracellular solution in ultrapure water (concentrations in mM): 135 NaCl, 5.4 KCl, 5 HEPES, 10 Glucose, 1.8 $CaCl_2$, 1 $MgCl_2$. Adjust pH to 7.4 with NaOH. Sterilize the solution with a 0.2 µm filtration system and store at 4 °C.
3. Prepare the intracellular solution in ultrapure water (concentrations in mM): 12 KCl, 125 K-Gluconate, 1 $MgCl_2$, 0.1 $CaCl_2$, 10 EGTA, 10 HEPES, 10 ATP-Na_2. Note that ATP-Na_2 is heat-sensitive and add it last. Adjust pH to 7.4 with NaOH. Sterilize the solution with a 0.2 µm filtration system, aliquot in 1 ml tubes and store at -20 °C.

3. Plating HEK-293 Cells on the Active Substrates

1. Place the photoactive substrate in a glass beaker covered with aluminum or a closed Petri dish and put in an oven at 120 °C for 2 hr for sterilization. From this moment onwards, perform all operations in sterile conditions. Put the samples in a sterile hood and let them cool down.
2. Coat the photoactive substrate with an adhesion layer to reduce the surface hydrophobicity and to promote cell adhesion.
    1. Place the active substrates in a P35 Petri dish or a 6 multiwell plate. Given the hydrophobicity of the conjugated polymer layer, make a polydimethylsiloxane (PDMS) well to fix the sample and confine the solutions.
        1. Mix PDMS and catalyst in a 10:1 proportion with a glass rod to obtain a viscous slurry.

2. Pour the slurry in a 6 multiwell plate, dispensing an amount sufficient to cover half of each well.
3. Wait for PDMS polymerization at RT.
4. Cut the cured PDMS in the middle for obtaining a square shape of the same dimension of the polymer sample.
5. Sterilize the PDMS wells by immersion in a 70% ethanol-water mixture for at least 30 min.

2. If using the PDMS well, scratch the photoactive layer along the entire border of the well with pointed tweezers, in order to avoid detachment of the entire layer when removing the PDMS well after cell culturing.
3. Cover the entire surface of each sample with a fibronectin solution (2 mg/L in PBS). A volume between 500-750 µl per sample is usually sufficient if a PDMS well is used (clear surface of the well: 15x15 mm^2). Without the PDMS well, about 2 ml of fibronectin solution are necessary to completely immerse the sample; pay attention that the substrate does not float while adding the solution. Incubate the samples at 37 °C for at least 1 hr.
4. Remove the fibronectin solution with a glass pipette and wash once with 2 ml of PBS.

3. Plate HEK-293 cells on the fibronectin-treated samples at a density of 15,000 cells/cm^2 in complete growth medium. Use a volume of 750-1,000 µl of CGM for each sample if a PDMS well is used, otherwise 2-3 ml of CGM are necessary. Incubate the cells at 37 °C and 5% CO_2 for 24-48 hr.

4. Optical Stimulation Protocol and Electrophysiological Measurements

1. Put the extracellular solution in a water bath at 37 °C to thermalize; thaw the intracellular solution, put it in a 1 ml syringe with a 34 gauge needle and keep it in contact with ice to avoid degradation of the ATP.
2. Take a cell-coated substrate from the incubator, carefully removing the PDMS well if present. Remove the growth medium with a glass pipette and rinse the sample with extracellular solution. Proceed slowly to avoid cell detachment.
3. Put the device into the sample-holder of the electrophysiology station with extracellular solution and the reference electrode. Prepare fresh glass micropipettes for patch-clamp with a puller (ideal resistance is in the range 2-4 MΩ). Fill half of the pipette with intracellular solution and mount it on the micromanipulator.
4. Look for a healthy cell to patch on the device. To avoid unwanted excitation of the photoactive substrate, use IR illumination for imaging, placing a long-wavelength pass filter (for example, a cut wavelength λ = 750 nm can be used for polymers absorbing in the visible) in front of the microscope illuminator.
5. Patch the selected cell and apply the desired protocol for optical stimulation by delivering light to the sample via the fluorescence excitation path of the microscope (**Figure 1**). A detailed description of the patch-clamp protocol can be found in reference 20.
    1. Position the patch pipette in close proximity to the cell membrane with a micrometric manipulator. During the positioning, apply overpressure to the pipette in order to avoid dirt sticking on the tip. The pipette should be few microns above the cell.
    2. Start lowering the pipette towards the cell membrane while controlling the pipette resistance on the patch control software. When the pipette resistance has increased about 1 MΩ, remove the overpressure from the pipette while applying a gentle suction, in order to form the gigaseal between the pipette and the cell membrane (*i.e.,* the measured resistance should increase at values greater than 1 GΩ).
    3. Apply a negative potential to the pipette close to the expected cell resting potential (for HEK293 cell a potential of -40 mV can be used) to help stabilize the seal. Compensate the capacitive transients due to the pipette capacitance with the relative commands on the patch amplifier and/or the patch control software.
    4. Break the membrane to allow electrical access to the cell cytoplasm by applying a brief and intense suction pulse to the pipette. Set the patch amplifier to current clamp mode (I = 0, *i.e.,* with no current injected into the cell) to track the cell membrane potential. Wait few minutes to see if the cell potential is stable.
    5. Apply the desired illumination protocol to the cell and record the effect on the membrane potential.
    Note: The illumination can be delivered to the sample either from the bottom or from the top, depending on the microscope architecture.
        1. Select an excitation wavelength that is well absorbed by the active material; for the P3HT:PCBM blend, a wavelength in the 450-600 nm range can be used. Suitable photoexcitation densities are in the range 10-100 mW/mm^2.
        Note: Short pulses of light (below 10-20 msec) result in a transient depolarization of the cell, while with prolonged illumination (hundreds of milliseconds or longer pulses) a sustained hyperpolarization is observed until the light is switched off.

Representative Results

Cells can be easily cultured on P3HT:PCBM substrates, provided that a suitable adhesion layer is deposited (like the fibronectin used in step 3.2 of the described protocol). P3HT:PCBM optical absorption peaks in the green part of the visible spectrum; however other light-sensitive conjugated polymers can be selected, according to the preferred photostimulation wavelengths range (**Figure 2**). Biocompatibility of these substrates has been demonstrated not only with cell lines[18,21] like HEK-293, but also with primary cultures of neurons[15] and astrocytes.[17] A typical micrograph of healthy HEK-293 cells cultured on a P3HT:PCBM film is shown in **Figure 3**. Optical stimulation of cells mediated by the photoactive substrates can result in different effects on the cell membrane, depending on the duration of the light stimulus.[18] Upon the onset of the light pulse, a fast spike (with an intensity of about 3 mV and a duration of about 1 msec) can be observed in the cell membrane potential recordings (**Figure 4**). This signal is due to a capacitive charging of the polymer/electrolyte interface upon charge generation in the active material.

After this initial fast spiking, a transient depolarization (with an intensity of about 1 mV and a duration on the order of tens of milliseconds) is observed in the cell (**Figure 4**). For short pulses of light, as the light is switched off, an opposite behavior is observed. These signals have been attributed to a variation in membrane capacitance due to local heating following light absorption.

For prolonged illumination, however, the initial depolarization during the light pulses turns into a cell hyperpolarization (**Figure 5**). This phenomenon has again a thermal origin, but in this case it is related to a variation of the membrane equilibrium potential due to a change in the ion channels conductivities induced by the increased temperature.

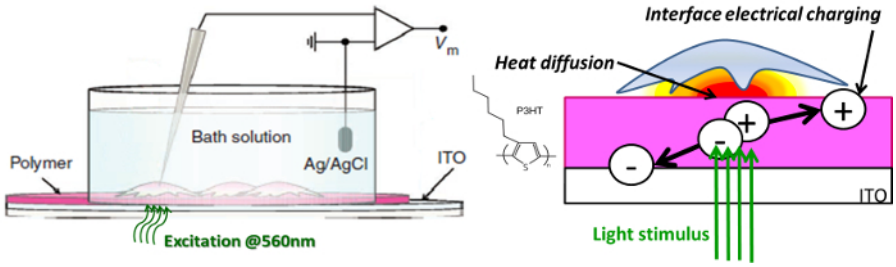

Figure 1. Sketch of the photoactive substrate. The photoactive interfaces used for cellular optical stimulation are made of a thin film of organic semiconductors deposited on a ITO-coated glass substrate. Cells can be grown directly on the device surfaces after it has been treated with a suitable adhesion layer (*e.g.,* fibronectin). Please click here to view a larger version of this figure.

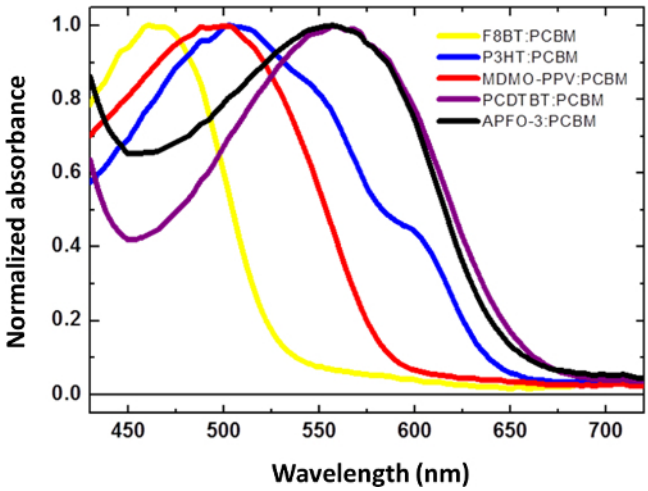

Figure 2. Absorption spectra of different conjugated polymers. The use of thin films of organic semiconductors may allow great tunability in excitation wavelength while retaining a partial transparence of the substrate. As an example, the absorption spectra of different conjugated polymers typically used in photovoltaics are reported. Please click here to view a larger version of this figure.

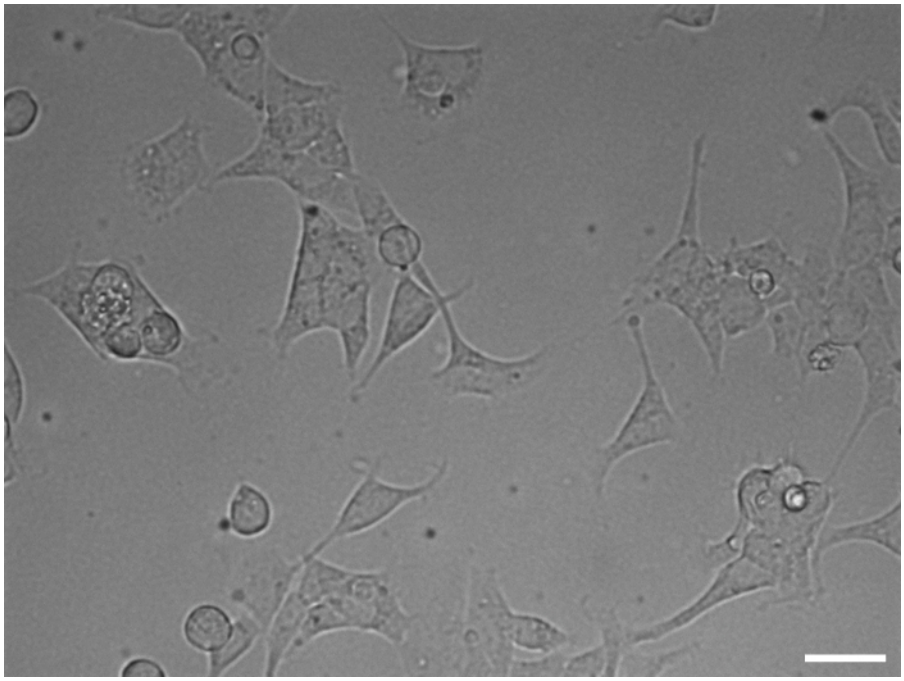

Figure 3. HEK-293 cells cultured on a photoactive substrate. Typical micrograph of HEK-293 cells cultured on the surface of a photoactive substrate after 24 hr of incubation. Scale bar, 20 µm. Please click here to view a larger version of this figure.

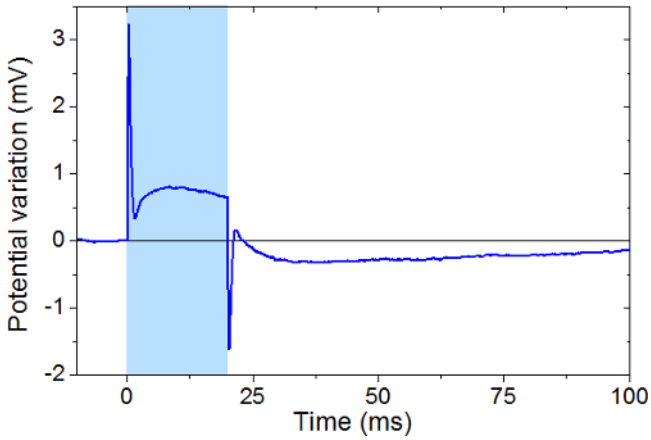

Figure 4. Optical stimulation of HEK-293 cells with 20 msec light pulses. Variation of the membrane potential of an HEK-293 cell elicited by a 20 msec pulse of light. An initial fast spike upon the onset of the light is followed by a depolarization of the cell during the pulse. As the light is switched off, an opposite behavior is observed. Please click here to view a larger version of this figure.

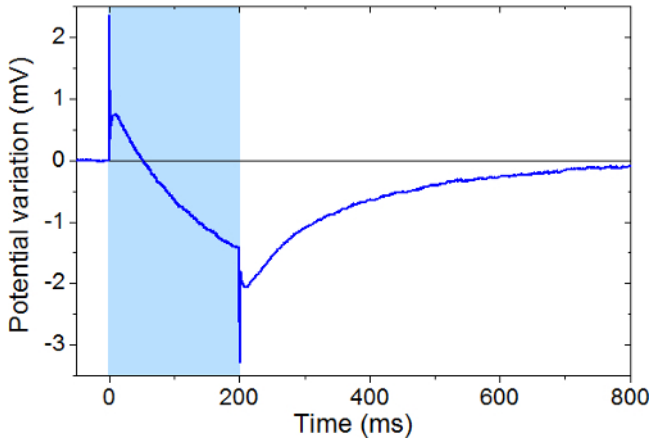

Figure 5. Optical stimulation of HEK-293 cells with 200 msec light pulses. Variation in the membrane potential of an HEK-293 cell elicited by a 200 msec pulse of light. The initial depolarization observed for short illumination is only a transient phenomenon, which after few tens of milliseconds of prolonged illumination turns into a hyperpolarization of the cell. Please click here to view a larger version of this figure.

Discussion

Critical steps of the reported protocol for *in-vitro* optical stimulation of cells mainly concern the choice of the light-sensitive polymer, the thermal sterilization parameters, the intensity and the duration of light stimuli. A P3HT:PCBM thin film was selected here, since it guarantees good temporal and electrochemical stability. However, one should notice that not all light-sensitive polymers can offer analogue performances,[22] more specifically upon illumination. In addition, in this case selected thermal sterilization parameters do not lead to sizable degradation of the polymer optoelectronic features; notice however that, in case the sterilization method or the thermal treatment parameters are changed, possible effects on the polymer properties should be carefully evaluated. It is known, for instance, that UV exposure rapidly leads to irreversible polymer bleaching and degradation, due to conjugation loss. Furthermore, different annealing temperatures and duration may lead to different morphological order of the polymer surface, with negative effects on the electrical charge transport properties. The parameters of the photoexcitation protocol should be carefully evaluated as well: excitation densities above 100 mW/cm^2, and/or prolonged stimuli, may easily lead to polymer degradation and failure of the photostimulation protocol. Another critical step in the protocol consists in the use of a protein adhesion layer, due to the high hydrophobicity degree of the P3HT:PCBM surface. If no adhesion layer is employed in the realization of the bio-polymer interface, cell seeding and proliferation might be seriously compromised.

Within the above mentioned critical steps and constraints, however, the reported protocol can be straightforwardly modified, according to the specific experimental needs and objectives. Other stable, light-sensitive polymers can be found,[7] from several commercial suppliers or by properly synthesizing new compounds. This allows the use of different excitation wavelengths, spanning from the blue to the red and NIR range. Moreover, the polymer deposition technique is not restricted to the spin coating use. Indeed, other methods ensuring good homogeneity and suitable film thickness can be envisaged, such as ink-jet printing, spray coating, or blading The effects of the prolonged contact with the cell growing media and of the sterilization method should be evaluated for each new material taken into consideration. Even though the use of protein adhesion layers is necessary to obtain good cell cultures on top of the polymer surfaces, the specific use of fibronectin, as reported here, is not mandatory. Different adhesion protein layers may be used, also in accordance with the cell line to grow;[21] for example, neuronal cells are usually cultured on a poly-L-lysine layer. Finally, illumination protocols can be changed and adapted to specific needs, in terms of wavelength, spot size, power excitation density, time stimuli duration and frequency, light incidence direction (from the substrate or from the electrolyte bath). As briefly stated above, however, the definition of the protocol parameters should take into account the polymer optical absorption range and possible degradation effects, which may vary a lot from case to case.

The limited temporal and electrochemical stability of some light sensitive polymers actually represents the main limitations of the technique. In addition, use of commercial polymers without any further purification may lead in some cases to limited repeatability of results among different batches of the material, due to different purity degree.

We also notice that the precise mechanisms at the base of the phototransduction effect in CSPP protocols still need to be further elucidated and characterized. Thermal, electrical and chemical phenomena are possibly involved, at different extents in different biological models. A complete understanding will be key to fully exploit the technique. In particular, the occurrence of heat-mediated phenomena should be reviewed as a limitation of the technique, since it can be hardly controlled, and can lead to local overheating effects, in particular in the case of *in vivo* applications. A specific implementation of the device, aimed at controlling the heat diffusion in the extracellular environment by proper engineering of heat-conductive paths, might reveal necessary in the next future for the full exploitation of the technique.

In literature, different techniques that use optical pulses for temperature-mediated stimulation of cells and tissues, both *in-vitro* and *in-vivo* can be found. In these studies, the absorption of IR laser beams by water is usually used as a transduction mechanism.[23-25] With respect to Infrared Neural Stimulation, the CSPP mechanism has distinct advantages, especially for *in-vitro* experimentation. CSPP is based on light in the visible range and of moderate intensity, so that stimulation can be provided by the excitation path of a standard fluorescence microscope. On the contrary, water absorption requires wavelengths in the IR (mainly around 1.45 µm and 1.93 µm), which have to be delivered to the sample via external sources like optical fibers micromanipulated in the proximity of the preparation, since the optical train of a standard microscope cannot be used at such wavelengths. In the case of CSPP, the light patterns obtained with laser scanning systems as well as the spatial light modulators

can be directly coupled to the optical train of most microscopes. In this way, the photostimulation of substrates based on organic semiconductors can make it possible to obtain stimulation of several cells in the field of view independently, with both high temporal and spatial resolution.

Another highly valuable alternative for optical stimulation is offered by genetic methods, based on targetable expression of genetically photoactivatable probes in cells. Optogenetics offers nowadays unprecedented temporal and spatial resolution, with typical illumination intensities in the range of few tens of mW/mm^2, can both excite and inhibit electrical activity, and can be genetically targeted to specific sub-populations of specific brain regions of interest. However, it still presents some issues that have to be solved. In particular, safety concerns regarding the use of viruses for gene expression, especially in humans, and the achievement of stable and controlled long-term heterologous protein expression still represent the major challenges. CSPP technique allows to circumvent the need for gene transfer, and all related safety concerns, which are particularly relevant for *in-vivo* applications.

All in all, the CSPP method offers several advantages with respect to endogenous photostimulation techniques. Since it is not invasive, it can be easily coupled to any existing electrophysiological working station and it does not require complex laser sources or genetic transfection, while maintaining high spatial and temporal selectivity. For these reasons, CSPP holds promise to become a complementary tool in neuroscience *in-vitro* investigations, and to open new perspectives in *in-vivo* applications. However, a big effort from the material science, physics and engineering communities is expected in the forthcoming years, needed to refine, optimize and fully exploit the technique.

Disclosures

The authors have nothing to disclose.

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