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Electrical stimulation of cells through photovoltaic microcell arrays

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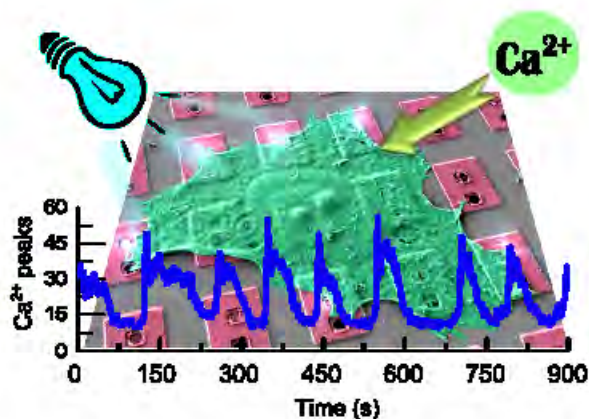
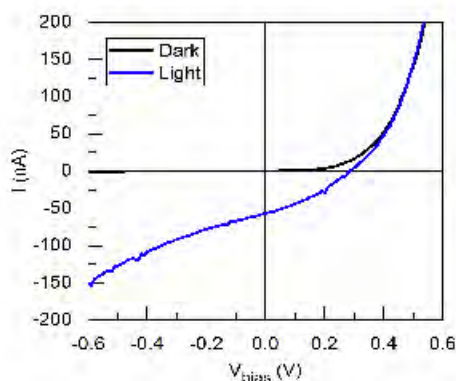
Abstract

Electrical stimulation may influence cell behavior associated with proliferation, differentiation, and migration, among others. The need for electrical tools to interact with living cells has pushed the technology to progressively develop less invasive devices. Technologies like energy harvesting have enabled wireless biological applications. Here we report the use of a photovoltaic microcell array (PVMA) based on silicon, as a wire-free interface to stimulate single cells with high spatial resolution. We demonstrate the effectivity of this microtool on osteoblast cells. The electrical stimulation triggered intracellular calcium transients as a response in 46% of the cells. The reduced dimension of the PVMA and its capacity to work in visible light show its potential for the wireless life science explorations.

Graphical abstract

¹ CV-E and AB contributed equally to this work.

From *Photoelectricity*



to *Biostimulation*

Keywords: photovoltaic array; silicon solar microcell; electrical cell stimulation.

1. Introduction

The possibility to modulate cell activity through electrical stimulation has become an interesting topic for cell research and the development of new therapeutic applications. There has been a wide exploration in this field, that goes from restoring or enhancing biological functions to preventing, for instance, loss of memory or muscular atrophy related to aging . In spite of this, many technological issues still need to be overcome in terms of spatial resolution, mechanical issues, and negative side effects such as the rejection of some electric probes. To address some of these problems, the tendency is to reduce the technology scale by enhancing spatial resolution and specificity, or even by developing highly flexible electrical probes. Furthermore, one alternative to overcome the limitations of the electrical stimulation is the energy harvesting technology . This technology obtains the energy directly from the environment and provides it in a wireless way . Energy harvesting devices make possible to explore the interactions of excitable cells with electric fields, as well as less invasive solutions to power autonomous biomedical devices. In terms of light harvesting, silicon-based photovoltaics have ruled the field due to their well-known efficiency, a favorable set of electrical and optical properties, their high natural abundance, their low cost and their biocompatibility . Furthermore, silicon-based devices rely on the existence of a mature and

established infrastructure which allows a continuous dimension reduction . The conductivity of bare silicon can be increased linearly with illumination thanks to the charge carrier generation produced by the photoelectric effect. Taking into account the aforementioned optical properties of silicon, some researchers have stimulated single living cells using electrically polarized substrates and a light source focused on the selected cell . Nevertheless, this system was still dependent on many external factors, such as the voltage polarization. With the introduction of pn-junctions, it was possible to create controlled photovoltaic microstimulators with high spatial selectivity for applications like the replacement of the retina neurons or spinal cord stimulation in animal models. The evolution of the technology is headed towards reducing the size of the devices to restore cell-to-cell communication in the less invasive way.

In the present article, we report the fabrication of a simple photovoltaic microcell array (PVMA) using a CMOS-compatible microfabrication technology. The PVMA has planar pn-junctions covered by a thin SiO₂ passivation layer (80 nm), with dimensions of 5 μm by 7 μm, and a separation of 3.5 μm horizontally and vertically in the array. The performance of the photovoltaic microcell was tested under dark and light conditions with different source lights (white led lamp, sun simulator and lasers). Additionally, we report the direct interaction of osteosarcoma cells with the PVMA and the generation of intracellular calcium transients in response to the light-induced electrical stimulation.

2. Materials and Methods

2.1. Fabrication of the PVMA

The fabrication process of the PVMA is described in Fig. 1. A thermal silicon oxide of 300 Å was grown on a 100 mm Ø p-type silicon wafer (Fig. 1A). Squares of 1 μm side were patterned in the silicon oxide via UV stepper photolithography (Nikon NSR-2205i12D i-Line Stepper) and anisotropic reactive ion etching (RIE; Alcatel 601E, France) to create the open areas for the p-type ionic implantation of boron (Fig. 1B). The implantation was performed with a dose of 1×10^{15} at/cm² and 50 keV of energy. Squares of 2 μm side were then patterned on the silicon oxide by

a photolithographic step to define the areas for the n-type ionic implantation of phosphorus (Fig. 1C). This implantation was performed with a dose of 4×10^{15} at/cm² and 50 keV of energy. After removing the photoresist, an annealing step was performed at 950 °C for 15 min to diffuse the implantations and repair the crystalline defects created. Then, a 50 nm-thick SiO₂ layer was deposited by plasma-enhanced chemical vapor deposition (PECVD). The silicon oxide layer was patterned in rectangles of 5 μm by 7 μm with two inner squared windows of 1 μm side by a photolithographic step and a RIE (Fig. 1D). Finally, a lift-off process was performed to create the electric contacts of the device. This process consisted in a photographic step (Fig. 1E) followed by the metallization of a 100 nm Tungsten layer by e-beam evaporation, in 1.5 μm squared areas on top of the oxide windows (Fig. 1F).

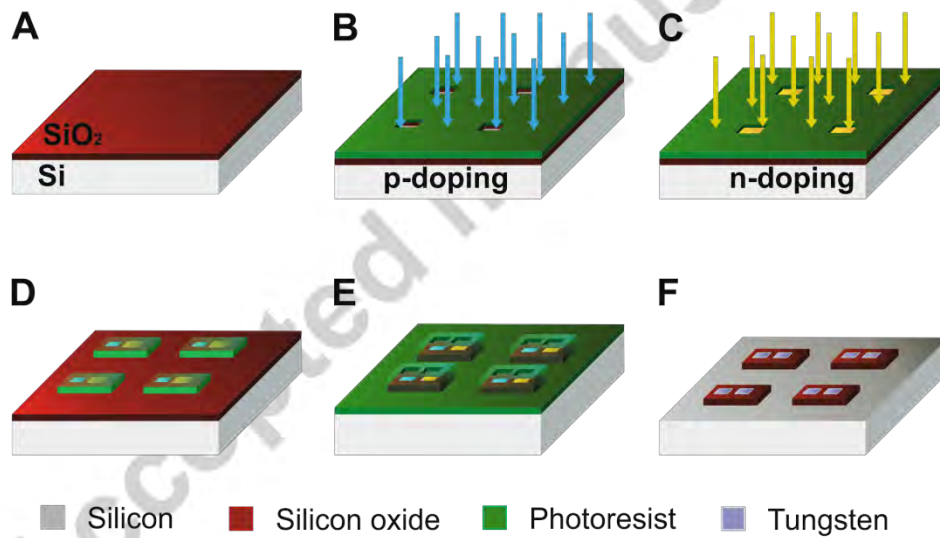


Fig.1. Fabrication process of the PVMA. (A) The fabrication started with a silicon wafer with 1 μm thick thermal oxide growth. Ionic implantation with areas defined by photolithography of (B) phosphorus (blue arrows) and (C) boron (yellow arrows). (D) A photolithography followed by a vertical etching of the initial thermal oxide defined the area of the pn-junctions. (E) The last photolithography defined the electrode area holes and (F) both doped regions were metallized with a layer of tungsten leaving the final array defined.

2.2. Characterization of the PVMA

Some test structures with pads of 150 μm² provided external electric contacts to validate the correct performance of the pn-junction. A test was carried out through electric microprobes (tip radius of 7 μm) in a probe station (Karl Süss PA200, Germany) by using a semiconductor characterization

system (Keithley 4200, USA) at room temperature (RT). The current–voltage characteristic was recorded polarizing the microdiode with a bias voltage ranging from -3 V to 3 V. Additionally, the photoresponse was also recorded using the lightbox station as source light (Karl Süss Lightbox150, Germany) focalized in a spot of 1 cm diameter with the help of the lenses of the optical microscope (Leyca MZ6, USA). The sample was totally enclosed in a chamber adapted to the system of the microscope that guaranteed a total darkness.

In a second approach, the wafer was diced in chips that were wire-bonded to the two external pads of a printed-circuit board (PCB). This allowed the optoelectronic characterization through current–voltage (I–V) dark and illuminated curves. The curves were obtained by applying an external bias ranging to from -3 V to 3 V and measuring the generated photocurrent with a Keithley2400 source meter. The illumination was established using a pre-calibrated Sun 3000 Class AAA solar simulator (Abet Technologies, USA) with an irradiance of 100 mW/cm^2 . The measurements were performed under standard test conditions (AM 1.5 illumination) at RT. Finally using the same packaged chip, the performance of the device was characterized under the measurement conditions used for the future biological test. The lasers of the confocal laser scanning microscope (CLSM, Leica TCS SP5, Germany) were used as source lights. The lasers were of different wavelengths that included blue (488 nm), green light (561 nm) and red light (633 nm). The scanned areas were observed with a $10\times$ objective, with a numerical aperture of 0.4 . The scanning spots were 1.5, 1.7 and 1.9 μm diameter for each laser respectively.

2.3. Osteosarcoma cell culture

Osteoblast-like Saos-2 cells, derived from primary human osteosarcoma, were purchased from ATCC (HTB-85) and maintained in Dulbecco's Modified Eagle medium (DMEM) with 10% foetal bovine serum (Gibco) under standard conditions (37°C and 5% CO_2).

2.4. Cell viability assay

The viability of cells growing directly on the PVMA was analysed using the Live/Dead Viability/Cytotoxicity kit for mammalian cells (Invitrogen), according to the manufacturer's

protocol. The PVMA were cleaned and sterilized with absolute ethanol and introduced into 4-multiwell plate. Then, 50,000 cells were seeded into each well and cultured at 37 °C and 5% CO₂. The cell viability assay was performed in three different conditions: after 24 h of culture, to test the potential cytotoxicity of the PVMA, and 24 h and 72h after light exposition, to analyse the effect of the electrical stimulation at short- and mid-term. In the latter case, the PVMA were exposed to blue laser light (488 nm) with an irradiance of 1.58 mW/cm² every second for 15 min. Then, cells were cultured for 24 h and the viability assay was performed. In addition, bare silicon substrates and glass coverslips were used as controls. All the experiments were held under controlled illumination conditions in a dark room.

Images from different regions of the samples were captured at 10× magnification using an Olympus IX71 inverted microscope equipped with epifluorescence. The experiments were performed in triplicate in independent culture preparations and a minimum of 300 cells per sample were analysed. The images were evaluated using the ImageJ software and the Cell Counter plugin.

2.5. Cell morphology analysis

After the viability assay of cells growing on PVMA without light activation, cultured cells were rinsed in phosphate buffered solution (PBS) and fixed in 4% paraformaldehyde (PFA; Sigma) in PBS for 15 min at RT. Cell dehydration was performed by 10 min washes in a series of ethanol (50%, 70%, 90% and twice 100%). Finally, samples were dried using hexamethyldisilazane reagent (Electron Microscopy Sciences) for 15 min, mounted on special stubs and analysed using scanning electron microscope (SEM; Merlin).

2.6. Cell adhesion and actin cytoskeleton distribution analysis

Cell adhesion onto the PVMA surface was analysed using an antibody against vinculin to determine the presence of focal contacts. At the same time, phalloidin was used to visualize the distribution of the actin filaments of the cytoskeleton. The same cell culture protocol described for viability studies was employed (section 2.4), but the cells were fixed in 4% PFA in PBS for 15 min at RT after 24 h of culture. Afterwards, the cells were permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 15

min and blocked with 1% bovine serum albumin (BSA; Sigma) in PBS for 25 min at RT. Samples were then incubated with 2 $\mu\text{g}/\text{ml}$ mouse anti-vinculin primary monoclonal antibody (Chemicon, MAB3574) for 60 min at RT and washed with 1% BSA-PBS. Subsequently, the samples were incubated with a mixture of 1.4 U/ml Alexa Fluor 594-conjugated phalloidin (Invitrogen), 6 $\mu\text{g}/\text{ml}$ Alexa Fluor 488 goat anti-mouse IgG1 and Hoechst 33258 (both from Sigma) for 60 min at RT. Finally, samples were washed in 1% BSA-PBS, air dried and mounted on bottom glass dishes (MatTek) using ProLong mounting solution (Life Technologies). Control analyses were performed on bare silicon substrates and glass coverslips. Sample evaluation was done with a confocal laser scanning microscope (CLSM, Olympus).

2.7. Intracellular calcium measurements

Saos-2 cells were cultured on PVMA for 24 h and then loaded with 2 μM Fluo-4 AM (emits fluorescence when binding to intracellular calcium) and 0.02% pluronic acid (both from Life Technologies) in serum free DMEM for 30 min at RT in the dark. Next, samples were washed and transferred to MatTek dishes with Minimum essential medium (MEM; Gibco) without phenol red. The dish was placed inside the incubator chamber of the CLSM (Leica TCS SP5, Germany) and illuminated for 15 min at 1000 Hz with the 488 nm laser, as previously described in section 2.4. Cell images were captured in the time-lapse mode, in the range of 500-515 nm, every second for 15 min. The same experiment was performed for cells growing on bare silicon substrates and glass coverslips, which were used as negative control of activation.

The time-lapse movies were analyzed by an ad-doc MATLAB algorithm programmed to automatically detect increases of intracellular fluorescence in cells. This code uses image enhancement and perimeter detection algorithms to detect the shape and position of the cultured cells. The average brightness of each cell was calculated. Finally, the algorithm uses a customized

peak detector to estimate the number of cells that were stimulated due to the illumination of the PVMA.

2.8. Statistical analysis

The number of live cells and the number of activated cells were analysed using the Fisher's exact test. Statistical significance was considered when $p < 0.05$. All statistical analyses were performed with the GraphPad PRISM software (6.01).

3. Results and Discussion

3.1. Electrical photoresponse of the PVMA

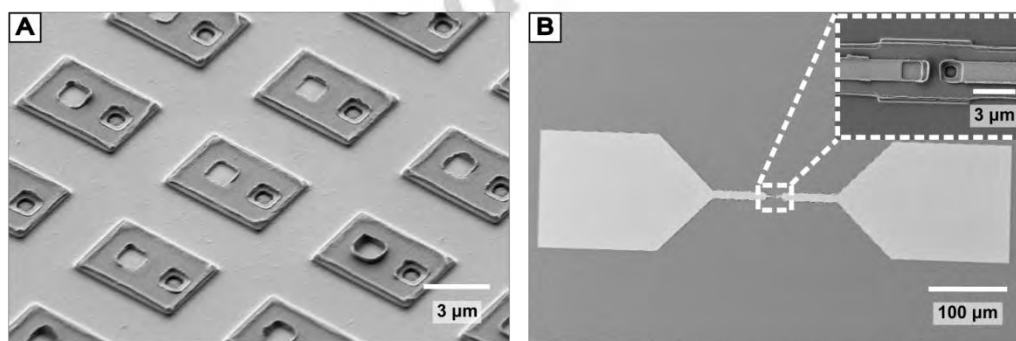


Fig.2. SEM image of (A) the PVMA fabricated and (B) the test structure made to reach the electrical contacts for the optoelectronic characterization (the inset shows in detail how the connection was made).

Fig. 2A shows the final devices observed using SEM (Carl Zeiss, Auriga Series, 3 KV, Germany). The test structures can be seen in Fig. 2B with the extensions of the electrical contacts that allowed the electrical connection to the microphotodiode. The performance of the device was characterized with an electric microprobe station with/without illumination using the optical microscope light and

with a polarization voltage ranged between -1 V and 1 V. The I-V data obtained from the test structures displayed the expected photodiode characteristic with an open-circuit voltage, V_{oc} , of 0.28 V and a short-circuit current, I_{sc} , of 56.53 nA under illumination (Fig. 3A).

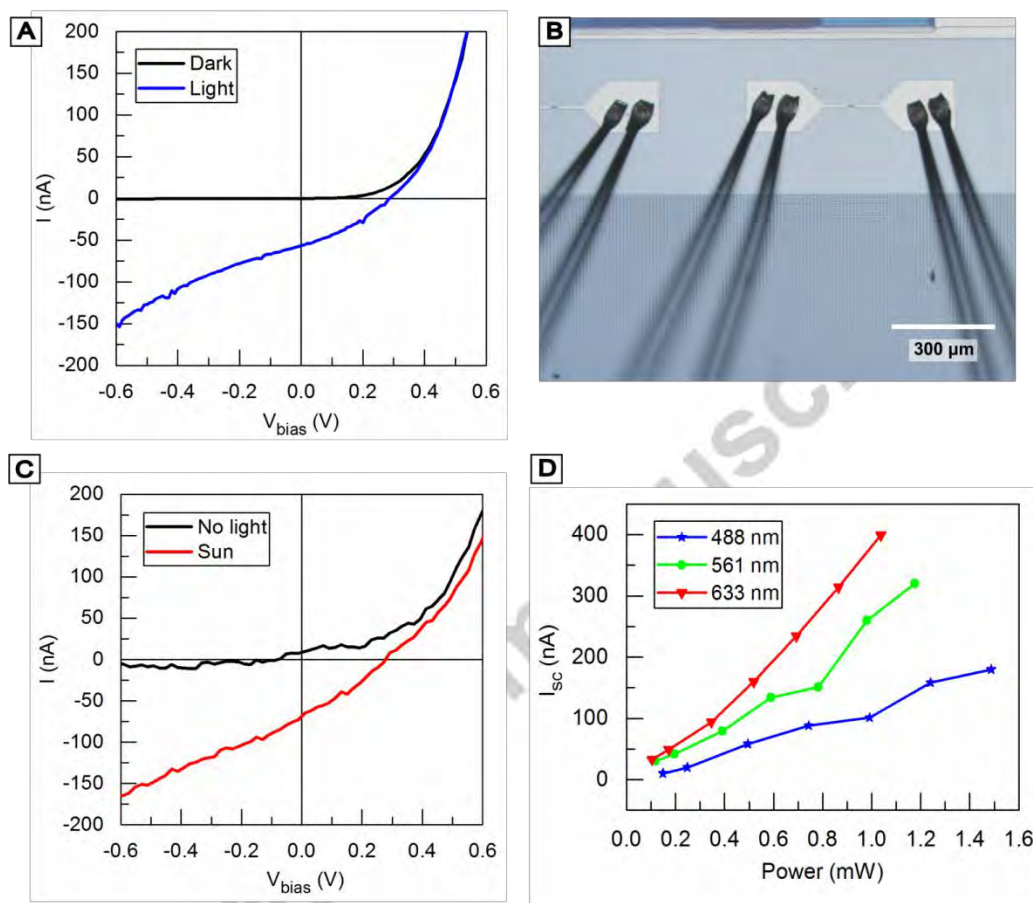


Fig.3. Electric characteristic of the PVMA. (A) I-V characteristic of a single microphotodiode under light/dark conditions measured with electric microprobes using test structures. (B) Optical microscope image of the microdiodes test structures with the four-point wire bonding. (C) Zoom in of the I-V characteristic of the wire-bonded microphotodiode under AM1.5 illumination and darkness. (D) Short-circuit current of the wire-bonded microphotodiode at different input luminous powers, for different wavelength laser, of the confocal microscope.

A second test was held with a more robust configuration, where the test structures were wire bonded with the traditional four-point probe (Fig. 3B). Using 1 sun illumination (under AM1.5 radiation at 25 °C), the following response shown in Fig. 3C was obtained. The V_{oc} was 0.27 V and the I_{sc} was 72.9 nA.

The response of the microdiodes was also observed under the CLSM using the microdiode test structure, as a reference of the microdiode response under some of the biological illumination conditions further tested. Fig. 3D shows the response of the device under various input power levels of the available lasers. It exhibits a relative linear I_{sc} dependence on the light intensity, in concordance with an increment of photogenerated carriers.

3.2. Osteoblast interaction with PVMA

The interaction of osteoblasts with the PVMA was first analysed without light stimulation to assess whether cells could adhere and grow on the photovoltaic microcells. After 24 h of culture on the PVMA, 90% of Saos-2 cells were alive and no significant differences in viability were observed when compared with control cells grown on a bare silicon chip (94%) and a glass coverslip (93%) (Fig. 4A and 4B). Silicon is widely used for the fabrication of devices, micro- and nanoparticles for multiple biological applications and it has been described as non-cytotoxic in numerous studies. The present results further indicate that neither the presence of tungsten contacts nor the array topography affect cell viability.

Analysis of cell morphology by SEM showed that, similar to control cells, osteoblasts grown on the PVMA presented a flat and spread morphology, and their nuclei contained several nucleoli, indicative of active transcription. As shown in Fig. 4C, each cell was spread over several microcells.

To confirm cell adhesion to the PVMA, immunofluorescence detection of vinculin and staining of actin fibres were also performed. Like in control cells, focal contacts could be clearly observed as an accumulation of vinculin at the end of the stress fibres (Fig. 4D). It is well-known that integrins and cytosolic proteins such as vinculin cluster to form the focal adhesion sites, through which cells interact with the substrate. Then, protein components of these focal adhesion sites activate actin polymerization to form stress fibres. Thus, the presence of both focal contacts and stress fibres in cells grown on the PVMA indicates that cells were fully adhered to the PVMA in agreement with

the SEM observations. The same focal contacts pattern and stress fibres distribution were observed in cells grown on bare silicon substrates and glass coverslips controls (Fig. 4E and 4F). Adhesion of the osteoblasts to the PVMA, which results in their flat morphology, together with the small size of the individual microcells in the PVMA, may enable the cells to simultaneously contact the two electrodes of several microcells in the array.

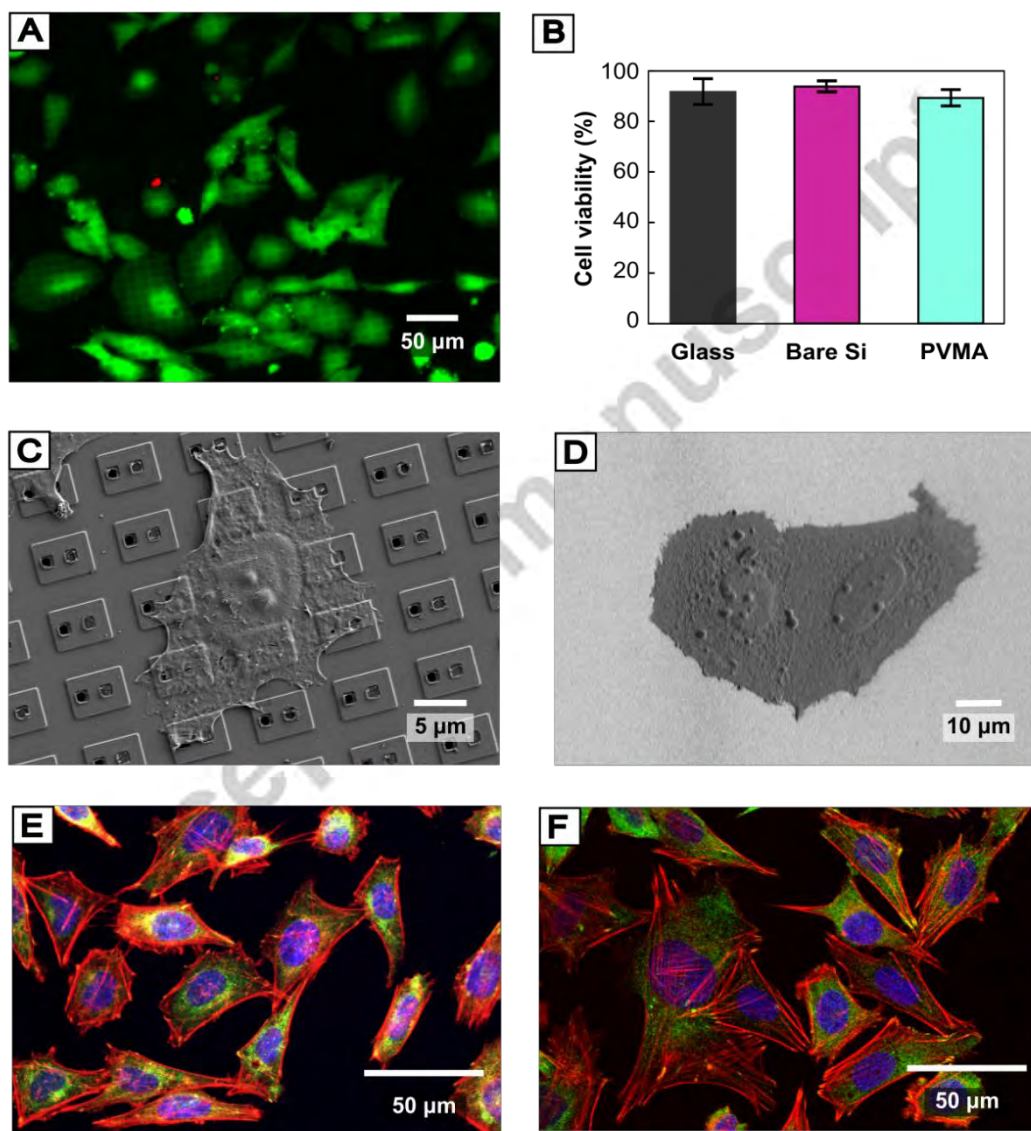


Fig.4 Saos-2 cells interaction with the PVMA. A) Fluorescence microscope image of cells growing on the PVMA, with living cells stained in green and dead cells stained in red. B) Percentage of live cells after 24 h in culture over PVMA and glass/bare silicon controls. C) Scanning electron microscope capture of Saos-2 cells growing on PVMA and D) on bare silicon. E) Confocal laser scanning microscope images of cells after 24 h of culture on the PVMA and F) on bare silicon. Stress fibers (red), focal contacts (green) and nuclei (blue) can be observed.

3.3. Osteoblast stimulation response

Ca^{2+} is a second messenger in multiple signalling pathways. It is present at very low levels in the cytosol of resting cells, but its concentration increases suddenly when a signal triggers the opening of specific Ca^{2+} channels in the endoplasmic reticulum or the plasma membrane. Electrically-responsive cells like osteoblasts, neurons and muscle cells can increase their cytosolic calcium levels when an electrical current or voltage is applied. In this regard, to analyse whether the activation of the PVMA could result in an electrical stimulation of Saos-2 cells, we measured the changes in the intracellular calcium concentration of cells grown on the PVMA when exposed to light.

The results showed that 46% of Saos-2 cells grown on the PVMA displayed intracellular calcium peaks in presence of light. By contrast, only 6% and 5% of Saos-2 cells presented calcium peaks under the same light conditions when grown on bare silicon substrates and glass coverslips controls, respectively (Fig. 5A and B, and in Supplementary information in Movie S1). Thus, the activation of PVMA by a specific laser wavelength was able to induce a cellular response, whereas no effect was observed on bare silicon in absence of the photovoltaic microcells. In order to study the effect of a reduced number of photovoltaic microcells, some areas of PVMA were covered by SU8 ink, generating windows where cells were able to contact a maximum of 4 photovoltaic microcells. The results obtained in terms of cell activation were similar to those obtained using the complete PVMA (Supplementary information).

Electrical stimulation of osteoblasts has been widely studied by several authors. In most cases, authors have explored the effect of electric fields or currents on cells by using a generator and two electrodes separated several millimetres, and with cells grown between the electrodes. In our study,

the two electrodes were only 1 μm apart and a single living cell could contact several pairs of electrodes simultaneously (Fig. 5C). Therefore, the effect of a PVMA on a single cell is different from the results published so far and it is interesting due to the local stimulation, paving the way for future bioelectronic applications at a single cell level. Furthermore, the possibility to work as a multielectrode device controlled by a selected light source allows applications where a large number of stimulation channels is required, such as visual or auditory prosthesis.

Finally, osteoblast viability 24 h and 72h after illumination and PVMA activation was analysed. As shown in Fig. 5C and 5D, no differences were observed between the cells grown on the PVMA and the ones grown on the glass control surface, after the same light exposition (observe additional results in Supplementary information in Fig. S1). Thus, neither the PVMA activation nor the light exposure had a cytotoxic effect at any of the two different time points. These results demonstrate the cytocompatibility of the PVMA after light stimulation at both short- and mid-term. Previous studies have shown that the use of electric fields can induce cell death when the voltage is too high or the exposure time is too long. Kumar et al. indicated that 1 V/cm applied for 10 min each day for 5 days induced apoptosis and that cell proliferation decreased. However, the use of alternative intervals and voltages did not cause cell death. In the present paper, the results showed that the wavelength used and short exposure time applied were able to induce a cellular response (increase of intracellular calcium concentration) without causing cell death, validating the potential use of the PVMA to induce intracellular changes controlled by light at single cell level without any external molecular addition as others have explored. This low-intensity electrical stimulation reduces side effects associated with power dissipation or even damages coming from the source that activates the device itself. In this regard, the use of energy harvesting devices such as PVMA as bioelectronic medicines has the advantage that they do not require any energy storage component. Actually, the use of nanogenerators for electrical stimulation has been growing in the past decade with applications that go from enhancers of healing to modulation of cell activity. In the same line,

previous studies by our group demonstrated that the interaction of Saos-2 cells with ZnO nanogenerators was able to generate local electric fields that induced a cellular response in the form of calcium transient. In that case, stimulation of the cells was mediated by cell adhesion forces, without the need for any other external stimulation, whereas in the PVMA the activation is controlled by light. In both cases, the local cell-scale stimulation with energy harvesting technologies opens the door to noninvasive therapeutic applications that go from low-intensity muscular activation to high special resolution retinal implants in a more accurate way.

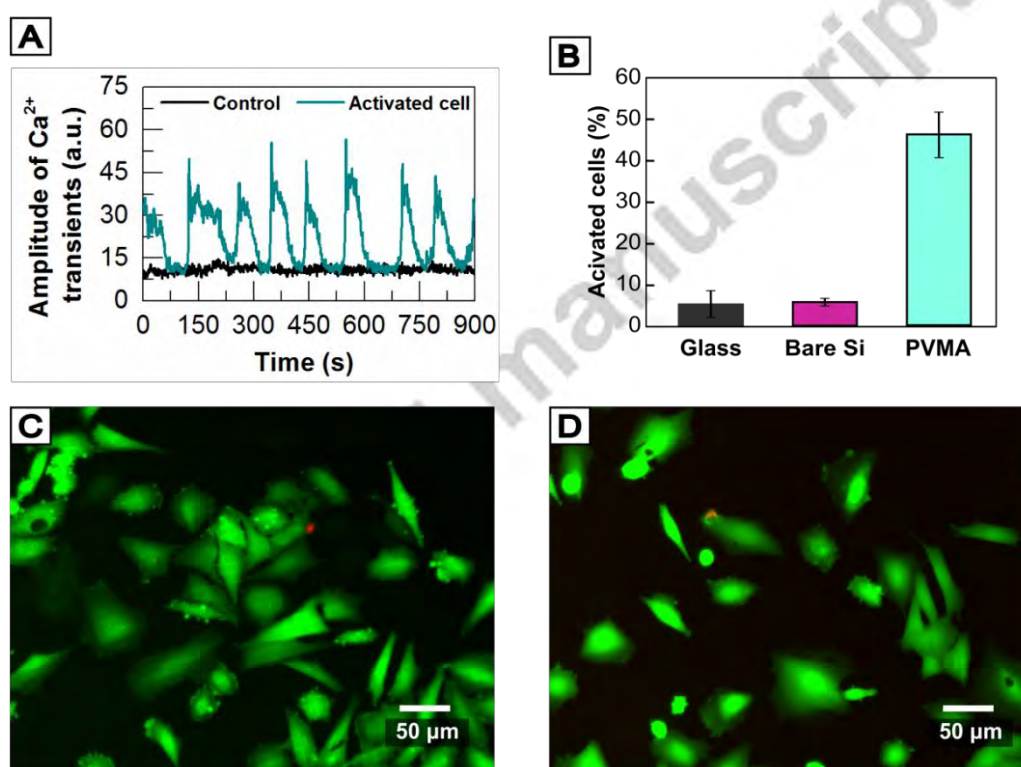


Fig.5. Saos-2 cells response to PVMA activation. A) Intracellular calcium transients pattern of a stimulated (activated) and an unstimulated (control) Saos-2 cell. B) Percentage of activated Saos-2 cells on PVMA and on glass coverslip/bare silicon controls for the same light stimulation. C) Fluorescence microscope images of cells growing on PVMA and D) control glass coverslip after 24 h of light stimulation. Live cells are stained in green and dead cells in red.

4. Conclusions

Silicon-based PVMA were successfully fabricated by means of microelectronic technology. Their reduced dimensions, planar morphology, simple fabrication process and their capability to harvest

energy from light and instantaneously convert it to an electric stimulus provides a stimulation tool of single cells without the limitation of external cables and electrodes. Furthermore, induction of cytosolic Ca^{2+} transients triggered by the electrical stimuli generated by the PVMA, on which osteoblasts cells were grown, shows the feasibility of this approach towards localized activation of excitable cells in a simple way. In summary, this technology affords new possibilities for the electric stimulation of single cells as a tool for life science explorations as well as for future biomedical applications based on controlling bioelectric signaling.

Acknowledgements

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Vitae



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Highlights

- ✓ A silicon photovoltaic microcell array (PVMA) capable to generate electrical current under visible light exposure is proposed.
- ✓ The electrical and photoelectrical performance is evaluated at the level of a single microdiode.
- ✓ The use of the PVMA as electrical micro-stimulators of living cells is evaluated.