Supporting Information

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Mirroring Action Potentials: Label-Free, Accurate, and Noninvasive Electrophysiological Recordings of Human-Derived Cardiomyocytes

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Figure S1. Device fabrication workflow. In the pictorial scheme, the developed fabrication workflow is reported. A brief description of each step is given below, while further technical details can be found in the Materials and Methods section.

a) The device was fabricated on a thin silicon nitride (Si$_3$N$_4$) membrane, obtained from a commercial wafer of silicon (Si) coated on both sides with 500 nm of Si$_3$N$_4$. By Reactive Ion Etching (RIE) and KOH wet-etching, Si$_3$N$_4$ and Si were removed from selected regions, obtaining square membranes with area 1.5x1.5 mm$^2$. The wafer was cleaved into samples with size 1x1 cm$^2$, each one endowed with a membrane. 

b) 20 nm of gold were deposited by e-beam evaporation on the planar side of the sample, with 5 nm of titanium as adhesion layer. Thereafter, arrays of nanopores were drilled by Ga$^+$ Focused Ion Beam (FIB) across the whole thickness of the membrane. Nanopores have a conical shape with diameter of 300 nm on the beam entry side and 200 nm on the exit side. 20 nm of gold were then sputtered on the planar side of the membrane in order to coat the border of the holes uncovered after FIB drilling. This step is fundamental in order to promote the successive galvanic growth inside the nanopores.

c) Nanopores were filled by gold through galvanic electrodeposition, creating an electrical connection between the two device compartments (the CIS and TRANS chambers, as described in the main text). As described in the work of A. Cerea at al., galvanic electrodeposition through nanopores culminates with the growth of 3D nanostructures, whose dimensions and roughness can be controlled by tuning process parameters, such as current and time duration. The obtained nanostructures have average diameter 1.3 μm and height 0.6 μm. During the process, the gold layer on the planar side of the membrane increased its thickness up to 150 nm.

d) By means of optical lithography methods, a resist mask was realized on the planar side of the membrane, formed by squares with lateral size 28 μm aligned with the nanostructures arrays. e) Nanostructures were covered with a drop of a common photoresist. Thereafter, wet gold etching was performed and electrically-disconnected electrodes pads were obtained. 

f) The photoresists on both sides of the membrane were removed by acetone immersion. g) A thin layer of silicon dioxide (SiO$_2$, 3 nm) was deposited by Atomic Layer Deposition (ALD) on the side of the membrane with the electrode pads. This layer aims at improving electrodes surface wettability and reducing fluorophore photobleaching from contact with the metallic electrodes.

h) A plexiglass ring was positioned on the top side of the membrane, to realize the chamber where to culture the cells.
Figure S2. Study of cell viability and device biocompatibility. LIVE/DEAD and Immunofluorescence staining of the cell culture grown on the device, imaged with an upright microscope (images above) and the inverted microscope used for APs recording (images below). For LIVE/DEAD images, the scale bars are 100 μm for the top view (30 μm for the zoomed image) and 30 μm for the bottom view. For Immunofluorescence images, the scale bars are 100 μm for the top view and 30 μm for the bottom view. The dark squares in the bottom view images are the floating electrodes pads responsible of APs signal transduction. LIVE/DEAD assay confirms that cells are healthy, due to the large presence of green signal for live cells (Calcein AM) and the small fraction of red signal for dead cells (BOBO™-3 Iodide). The fluorescence quantification has been conducted exploiting ImageJ tools, revealing a ratio of live cells of 87%, which is compatible with what expected in a confluent cell culture. In the top view zoomed image, electrodes nanostructures can be recognized. It is important to note that the cells that interface with the nanostructures express green fluorescence, confirming that nanostructures do not affect cells viability and the device has thus a good biocompatibility. Immunofluorescence study shows the presence of two key markers of the cardiac lineage: the cardiac troponin-T for cardiomyocytes stained in green (cTNT encoded by gene TNNT2), and the NKX2-5 marker for early cardiac mesoderm stained in red. In addition, the nuclear DNA is stained in blue. The wider expression of the green signal with respect to the red one highlights the correct maturation of cardiomyocytes in the cell culture. In the bottom view image, the green stained cardiac troponin-T appears as punctate fibrous striations across the cells, which is a hallmark of functional myocytes. A larger image of the immunofluorescence assay viewed from below is presented in the main text (see Figure 3a).
Figure S3. Measurement setup. a) The measurement setup is based on an inverted microscope for fluorescence microscopy, equipped with a TRITC filter cube set. A mercury lamp provides fluorescence excitation. Two neutral density (ND) filters allow for modulating the illuminating light intensity, in order to obtain a high fluorescence signal, while limiting fluorophore photobleaching. A halogen lamp above the microscope allows looking at the sample also in transmission mode. The device is assembled directly onto the microscope stage. First, the bottom electrode is fixed to the stage. Successively, a drop of the fluorophore solution is pipetted onto the electrode. Once the drop has spread over the electrode, the membrane with the cell culture is laid down over the fluorophore dispersion. Finally, a grounded Pt wire is immersed into the culture medium and the bottom electrode is connected to ground. b) Overlap between the absorption (green curve) and emission (red curve) spectra of rhodamine 6G (R6G) dissolved in ethylene glycol with the excitation (green) and emission (magenta) bands of the used TRITC filter. R6G dissolved in ethylene glycol shows an absorption maximum at $\lambda = 534$ nm and an emission maximum at $\lambda = 561$ nm. c) Schematics of image formation: fluorophore molecules are excited by the green light, and emit their fluorescence in all directions. Fluorescence light emitted by the fluorophores below the electrode pads is reflected by the metal towards the output readout. d) Image of the electrode pads as recorded by the camera: due to the reflection of fluorophore emitted light (see panel c), the electrodes appear brighter than the background (scale bar = 30 $\mu$m).
Figure S4. Study of fluorophore signal outside the virtual mirror cell. a) By turning on the microscope top halogen lamp, cells can be made visible. A region of the cell culture is chosen for APs recording. The scale bar is 30 μm. b) The microscope top halogen lamp is turned off and APs recording is performed by illuminating the sample only from below. The fluorescence signal is integrated in the region below the electrode pad (i.e. in the virtual mirror cell, blue area), and in the surrounding region (red area). The scale bar is 30 μm. c) The mean fluorescence intensity of the two regions is plotted versus time. Fluorescence of the electrode pad (blue curve) undergoes negative fluctuations, due to the motion of fluorophores pushed away from the electrode when an AP is fired. On the other hand, the region surrounding the electrode undergoes opposite fluctuations (red curve), caused by the accumulation of the fluorophores repelled from the electrode. The red curve has lower mean intensity and SNR than the blue one due to the less fluorescence light detected in the region outside the electrode pad (see Figure S3c,d). Moreover, the fluorophore motion away from the electrode, which in a first approximation can be considered as radial, gives rise to a decrease of molecule density, which contributes to the lower detected optical signal.
**Figure S5. Circuital simulation of the cell-device system.** a) Equivalent electrical circuit for the cell-device system. The cell-electrode interface was modeled following well-established works in literature.\[^2\] On the other hand, the mirror-cell was modeled according to the well-known model of an electrochemical cell.\[^3\]

The values for junctional (and non-junctional) resistance $R_j$ ($R_{nj}$) and capacitance $C_j$ ($C_{nj}$) were estimated from experimental values of membrane resistance and capacitance of hiPSC-derived cardiomyocytes.\[^4\] The junctional resistance and capacitance were normalized for the membrane area in contact with the array of nanoelectrodes of a gold pad.

The resistance $R_e$ and capacitance $C_e$ at the nanostructures surface interfacing with the cell were calculated from the corresponding values for gold reported in the literature,\[^5\] and normalized for the nanostructures surface area. The sealing resistance $R_{seal}$ was approximated equal to 100 MΩ, similarly to the case of Spira’s gold mushroom electrode-cell interface.\[^2\]

Considering the mirror-cell, the resistance $R_i$ and the capacitance $C_i$ at the electrodes-ethylene glycol interfaces were estimated from the corresponding average values of an electrochemical double layer,\[^3\] and normalized for the electrodes size. The resistance $R_s$ and capacitance $C_s$ of the ethylene glycol solution were calculated from ethylene glycol resistivity and dielectric constant, and the geometric dimensions of the mirror-cell underneath the electrode (lateral size = 28 µm; thickness = 3 µm). Therefore, the electrical components have the following values: $C_{nj} = 160$ pF; $R_{nj} = 650$ MΩ; $C_j = 1$ pF; $R_j = 1$ GΩ; $C_e = 25$ pF; $R_e = 500$ GΩ; $C_i = 150$ pF; $R_i = 500$ GΩ; $C_s = 0.08$ pF; $R_s = 700$ MΩ; $R_{seal} = 100$ MΩ.

b) Results of SPICE simulation. Input action potential (red curve) and voltage pulse measured at the electrode-fluorophore dispersion interface (blue curve). This latter pulse is the one generating the MAP signal. It reproduces reliably the shape of the input AP, featuring the same duration.
Figure S6. New device configuration with higher density of recording electrodes. a) Array of gold electrodes with smaller size and spacing. The electrodes have lateral size 8 µm and pitch 20 µm (scale bar 10 µm). b) 2 by 2 array of nanostructures on each gold electrode (scale bar 1 µm). c) The device is connected to a voltage generator as described in Figure 2d. When an external positive voltage pulse is applied, the fluorescence light intensity under each electrode pad decreases (voltage pulse amplitude = 500 mV, voltage pulse duration = 400 ms). To highlight the light intensity variation, a corresponding background image was subtracted.
Figure S7. Device testing with fast electrical pulses. Fluorescence signals measured upon application of external voltage pulses with time duration 0.7 ms (frequency = 1 Hz). Camera integration time = 0.633 ms. The device was connected to the voltage source as described in Figure 2d in the main text.
**Video S1. MAPs optical recording.** The video was recorded with both the bottom and the top lamps turned on, in order to visualize both the fluorescence fluctuations and the cardiomyocytes contractile motion, and thus correlate the two dynamics. In order to enhance signal visibility, a background image for the fluorescence fluctuations under the electrodes was subtracted. Fluorophore light was then colored in red, while cell culture in blue.

It is possible to see that the electrodes exhibit fluorescence fluctuations in the form of light intensity reductions, which are perfectly synchronous with cells contractile activity.

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