Electrical Impedance Spectroscopy for Microtissue Spheroid Analysis in Hanging-Drop Networks

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Supplementary Information

Fabrication of the hanging-drop network

The hanging-drop network has been fabricated by using well-established polydimethylsiloxane (PDMS) casting from an SU-8 mold (see Figure S-1A). The mold was fabricated on a 4-inch silicon wafer using three layers of SU-8 100 (MicroChem Corp., MA, USA; see also manufacturer's data sheet). The first two layers define the hanging drops and microchannels; the third layer adds a rectangular recess, in which the glass inlay containing the electrodes was later inserted during assembly. SU-8 100 was spin-coated at a rate of 1500 rpm for 30 s to achieve a layer thickness of 250 µm (first two layers) and at 800 rpm for obtaining a thickness of 500 µm for the third layer. After a soft-bake of 90 min at 95 °C, each layer was exposed to broad-band UV light by using a mask aligner and transparency masks, previously designed by using a 2D CAD software tool. After the final post-exposure bake, all SU-8 layers were developed for 1 h in mr-Dev600 (micro resist technology GmbH, Berlin, Germany).
Prior to PDMS casting, we deposited trichloro(1H,1H,2H,2H-perfluoro-octyl)silane (Sigma-Aldrich, Switzerland) in a vapor silanization process on the wafer to reduce adhesion of PDMS. PDMS (Sylgard 184, Dow Corning, USA) was mixed in a 10:1 ratio of elastomer and curing agent, degassed and poured onto the mold. Once cured at 80 °C after 2 h on a hotplate, the PDMS was peeled off and cut to the desired sizes. Holes were punched for the fluidic connections, and the PDMS was bonded to a microscopy glass slide for mechanical stability after a PDMS surface activation by using oxygen plasma. Prior to bonding, access holes were drilled into the glass slide.

Fabrication of the electrode inlay

The electrode inlays were fabricated on a 4-inch glass wafer by using lift-off technology (see Figure S-1B). First, lift-off resist (LOR 3B, MicroChem) and S1813 positive photoresist (Shipley Europe Ltd, Coventry, UK) were sequentially spin-coated onto a clean and dehydrated glass wafer and soft-baked. The electrode and wiring patterns were transferred from a transparency mask to the resist by means of a mask aligner and broad-band UV light. After development in MF319 (Shipley), 20 nm of TiW as adhesion layer and 200 nm of Pt were sputtered onto the wafer by using an IonFab300 (Oxford Instruments, UK). Then, the wafer was immersed into Remover1165 (Shipley) overnight for lift-off. A 500-nm silicon nitride passivation layer was deposited on the wafer using PECVD in a PlasmaLab80 (Oxford Instruments). The passivation was re-opened at the electrode and pad sites by using reactive-ion etching in a PlasmaLab100 (Oxford Instruments) and an S1813 photoresist mask. Two layers of SU-8 100 were processed using the same parameters as for the SU-8 master molds described above. Finally, the wafer was diced by using a precision saw yielding the small glass inlays, each of a size of 2.8 mm by 8.7 mm.

Cell culturing

Human colorectal carcinoma cells, transfected with eGFP (HCT-116 eGFP, Sirion Biotech, Germany) were cultured in RPMI 1640 growth medium (Chemie Brunschwig AG, Switzerland), supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 IU/ml penicillin, 100 µg/ml streptomycin (P/S, Chemie Brunschwig AG) and 0.3 µg/ml puromycin. The culture was maintained at 37° C in 5% CO_2 in a humidified incubator.
Subculturing was performed according to standard protocols. Briefly, upon reaching 80% confluence, the cells were trypsinized in 0.25% Trypsin/EDTA solution (Life Technologies,

Switzerland), washed and re-suspended in fresh medium. A new culture flask was seeded with a 10%-dilution of the cell suspension in medium. For experiments, the suspended cells were counted using a hemocytometer and diluted according to experimental requirements.
HCT-116 spheroids were produced using the GravityPLUS kit (InSphero AG, Schlieren, Switzerland). Suspended cells, prepared as described above, were diluted and introduced into the hanging-drop plate. We controlled the tissue size by varying the cell concentration from 250 to 2000 cells per drop (40 µl). The micro-tissues were grown at 37 °C and 5% CO_2 in a humidified incubator. Harvesting was possible after four or five days depending on micro-tissue size.
Human cardiac microtissue spheroids (hCdMT) and human liver microtissue spheroids (hLiMT) were provided by InSphero AG. Microtissues were maintained in the respective cardiac maintenance media (InSphero) at 37 °C and 5% CO_2 in a humidified incubator. Medium exchange was carried out every third day.

Supplementary Figures

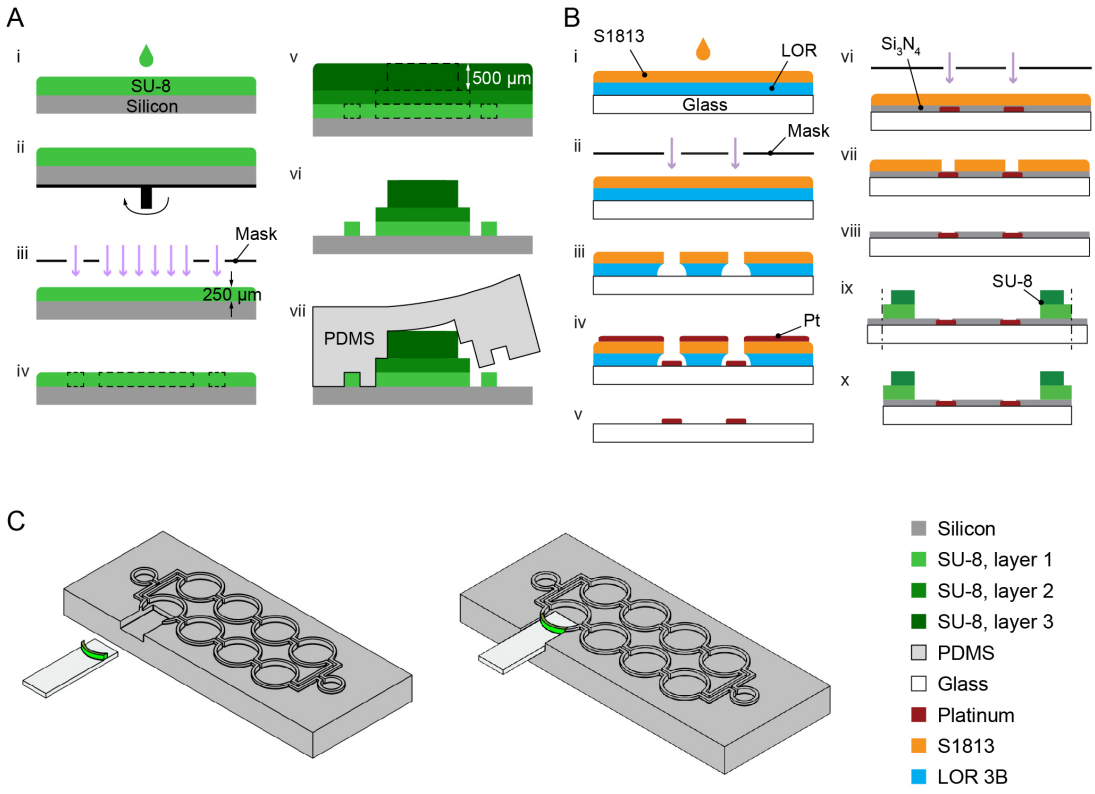

Figure S-1. Microfabrication steps. (A) Fabrication of the SU-8 master and subsequent PDMS molding of the hanging-drop network. SU-8 negative photoresist was poured (i) and spin-coated (ii) on a 4-inch silicon wafer. The resist was patterned by transferring the fluidic design from a transparency mask to the wafer by using a mask aligner (iii) and UV exposure. The wafer was baked to enhance the polymerization of the exposed resist (iv). Two additional layers were deposited sequentially onto the same wafer by using the respective masks (v). The wafer was developed (vi) and used as master to mold the fluidic structure of the hanging-drop network in PDMS (vii). (B) Fabrication of the glass inlays carrying the EIS electrodes. Lift-off resist (LOR 3B) and positive photoresist (S1813) were spin-coated on a 4-inch glass wafer (i). The positive resist was patterned by UV exposure through a transparency mask (ii). Subsequent baking and development of the resist resulted in a controlled undercut (iii), which was important to prevent the Pt from being sputtered onto the resist sidewalls (iv). The resist was removed (lift-off) leaving only the desired metal structures on the wafer (v). Silicon nitride (Si_3N_4) was deposited on the wafer in a PECVD process. This passivation was re-opened at the electrode and pad sites by reactive-ion etching using a photoresist (S1813) as etch mask (vi – viii). Two SU-8 layers were fabricated on the glass substrate by using the same photolithographic process as for the master mold (ix). Finally, the glass wafer was diced, yielding glass inlays that fitted into the recess of the PDMS device. (C) Schematic 3D view of device prior to assembly and once the glass inlay is inserted in the PDMS recess.

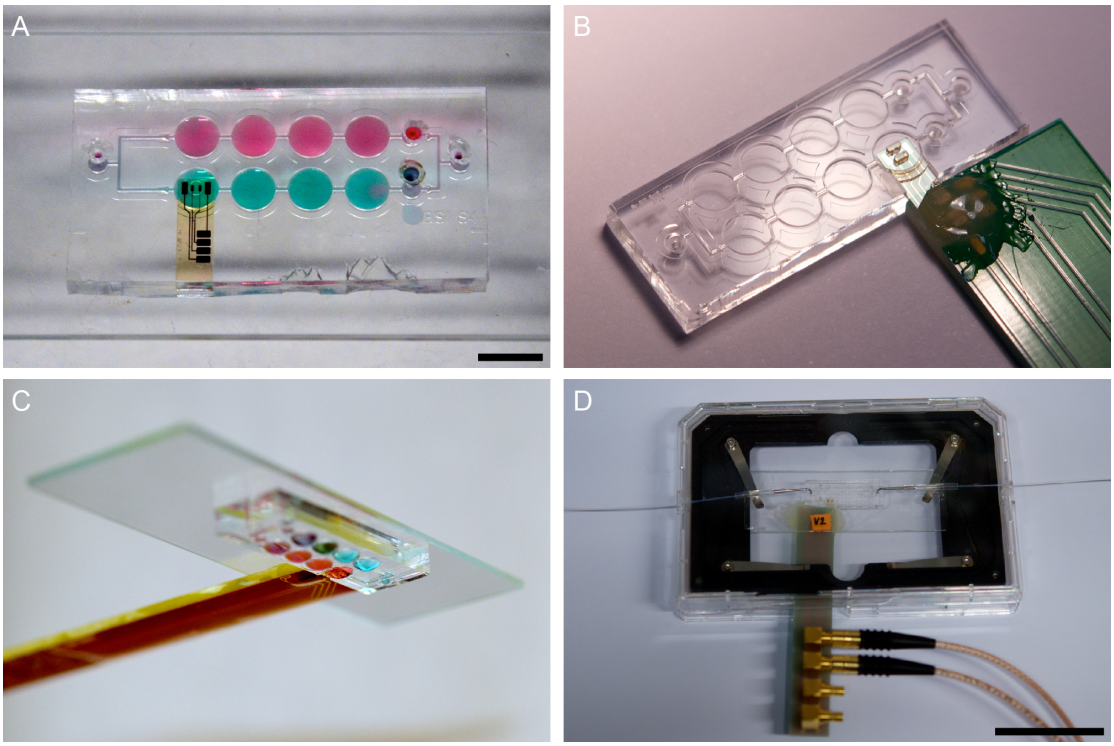

Figure S-2. Pictures of the fabricated device. (A) View from the top. Glass inlay placed in the PDMS hanging-drop chip. No PCB is present. Hanging-drop network was filled with colored water illustrating the two perfusion lanes. Scale bar is 5 mm. (B) View from the bottom. Glass inlay with PCB placed in the PDMS hanging-drop chip. Glass slide is not present. (C) View from the bottom. Assembled device was filled with colored liquid. Hanging drops are formed bellow the substrate. (D) View from the top. Assembled device placed in custom-made device holder inside a plastic box. Tubing is connected to the PDMS chip and cables connected to the PCB. Scale bar is 5 cm.

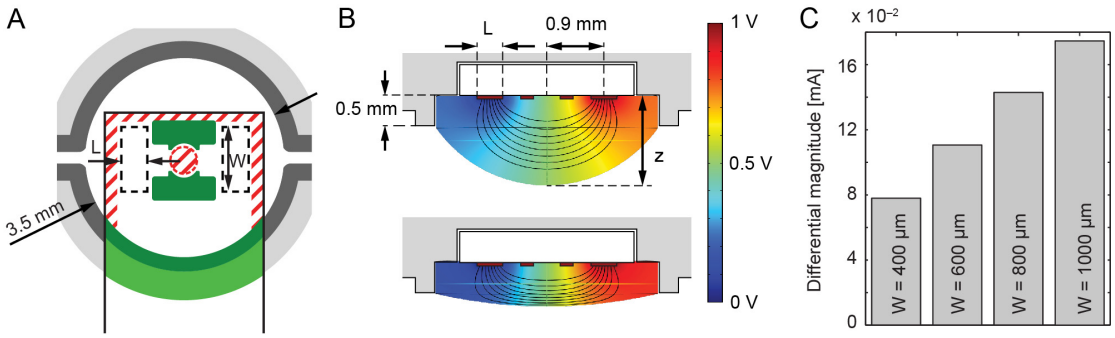

Figure S-3. 3D COMSOL simulation of drop size measurements by using impedance in hanging drops to find an optimal electrode design. (A) The model of the hanging drop consisted of a cylinder (diameter = 3.5 mm and height = 0.5 mm) merged with a spherical cap. Size and location of the electrodes have been limited by geometric constraints (red marked areas). The electrodes were parameterized with width, W, and length, L. (B) Simulated voltage drop and current field lines in a small (z = 700 µm) and a large (z = 1400 µm) hanging drop for an applied AC voltage of 1 V between the electrodes. (C) Differences (current in the large drop minus current in the small drop) in resulting currents of the hanging-drop model for four simulated electrode widths. The results are shown for an AC signal of 1 V at a frequency of 1 MHz.

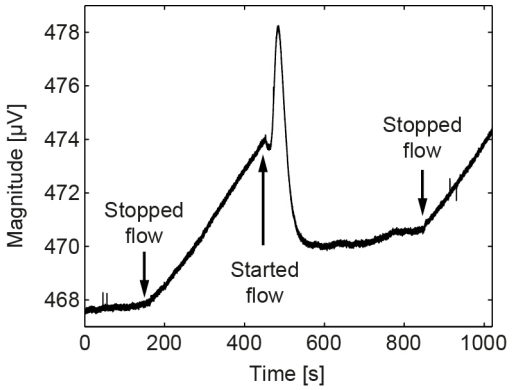

Figure S-4. Continuous signal over time (frequency 1 MHz) while switching on and off the flow at a flow rate of 5 µl/min. A stable baseline signal is achieve for flow rates >5 µl/min.

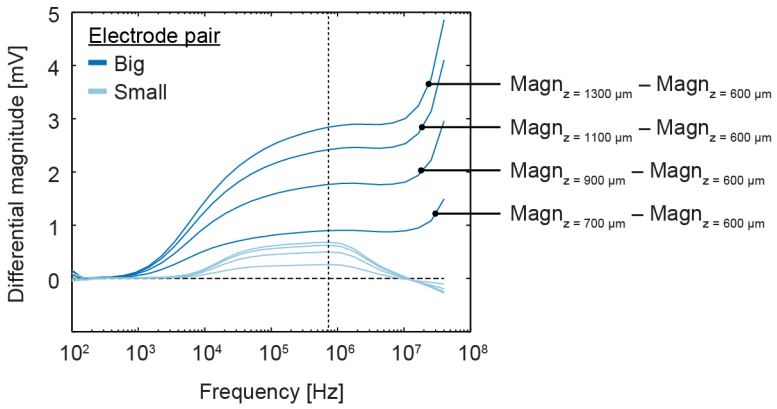

Figure S-5. Differential signal magnitude spectra with respect to the smallest drop (drop height of z = 600 µm). Differential magnitude spectra of the big electrodes (dark blue lines) were calculated as indicated. Differential magnitude spectra of the small electrodes are drawn in light blue and have the same order as indicated for the big electrodes. The dashed vertical line indicates the optimal frequency of 730 kHz maximizing the sensitivity for both electrode sizes.

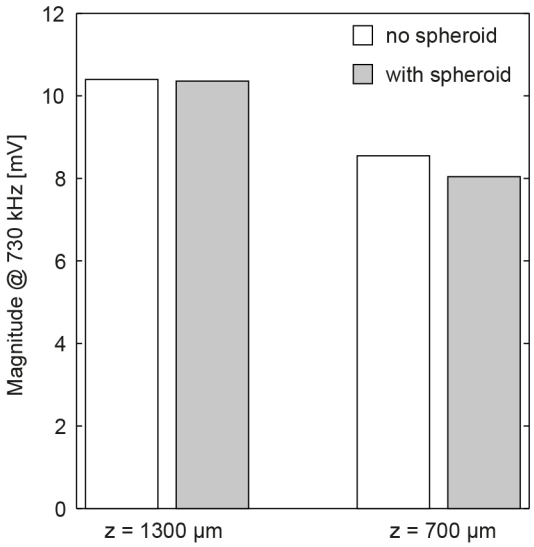

Figure S-6. Influence of the presence of a microtissue in the drop on drop-size measurement. Measurements were conducted at optimal frequency (730 kHz) for two different drop sizes (z = 700 µm and z = 1300 µm) by using the big electrode pair with and without the presence of a spheroid (600 µm diameter). For large drop heights (z = 1300 µm), the impedance signal was found to be largely independent of the presence of a spheroid in the respective drop.

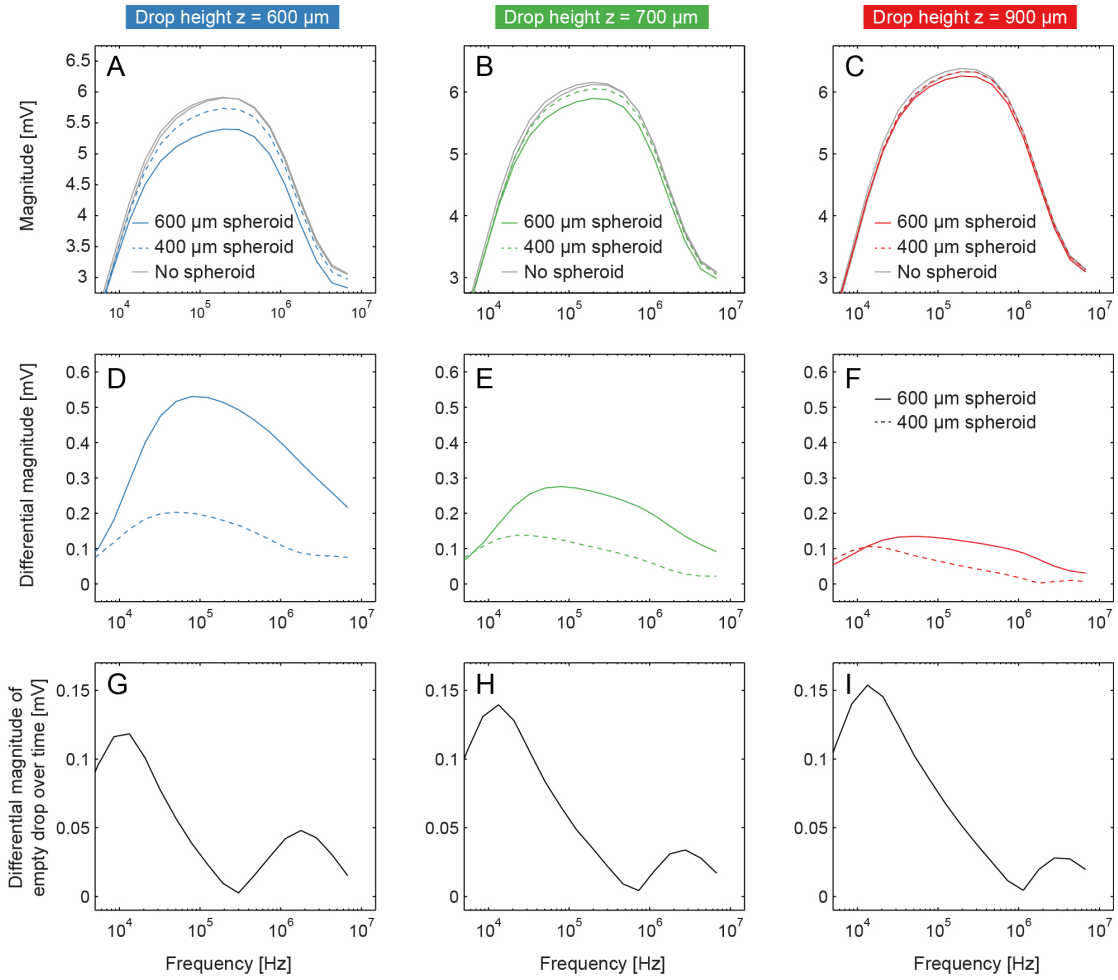

Figure S-7. (A-C) Measured signal magnitude spectra of differently sized spheroids (blue, green, red) for different drop heights and measured impedance signal magnitude spectra of the empty drops before and after the experiment (gray solid lines). (D-F) *Differential signal magnitudes* calculated as the signal of the drops containing a spheroid minus the signal of the empty drops. (G-I) *Differential signal magnitude spectra of the empty drops* measured before and after the experiments.

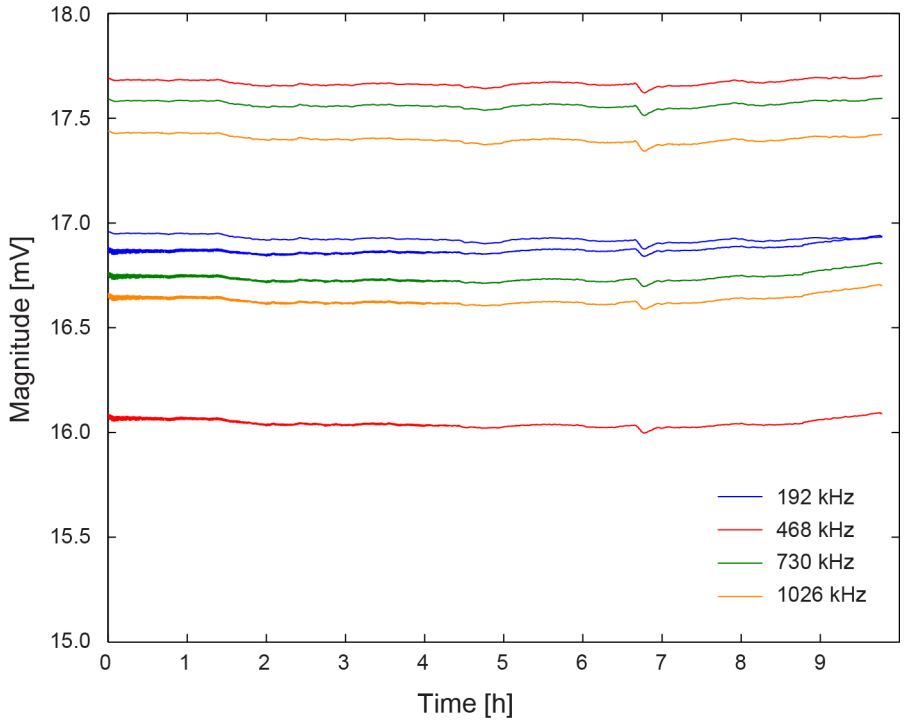

Figure S-8. Raw signal traces recorded continuously over 10 hours at four different frequencies (192, 468, 730 and 1026 kHz) in two empty drops in parallel by using the small electrode pair (z = 700 µm).