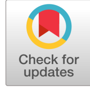

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## Research Paper

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# RF sensor dedicated to the dielectric characterization of spheroids between 500 MHz and 20 GHz

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**Abstract**

To achieve better accuracy in their investigations, biologists have recently been using three-dimensional models as intermediates between two-dimensional cell culture and the in vivo study of tissues. Some of these models are based on spheroids, which are cellular aggregates retaining many characteristics of in vivo behaviors while being still easy to use and implement in labs. To study such objects, multiple observation techniques can be used according to the objective of the study, including those using electromagnetic waves as nondestructive, noninvasive, and label-free analysis. Low-frequency (<1 MHz) ones are currently under investigation as electrochemical impedance spectroscopy. However, unlike microwaves (from 300 MHz to 60 GHz), they cannot penetrate the cell. Furthermore, most of the devices dedicated to microwave dielectric characterization are only focusing on cellular scales lower than spheroids (single cell, cell mats, or suspensions) or on tissues and organs. In this article, a microwave spectroscopy device using a coplanar waveguide adapted to the study of spheroids is presented with the dielectric characterization of fixed spheroids, with capacitance and conductance measurements from 0.5 to 20 GHz, aiming at filling the scale gap in the state of the art.

## Introduction

As soon as cancers were discovered, it became clear to biologists that the use of *in vitro* models independently of *in vivo* analyses would be essential, given the complexity of the mechanisms involved in these diseases. Two-dimensional (2D) cell culture has been used extensively for its ease of manufacture and low cost. However, this type of model is far from the reality of *in vivo* systems. Therefore, three-dimensional (3D) models of cell clusters in the form of spheres, also called spheroids, have been introduced as an intermediary between *in vivo* and 2D *in vitro* studies [1]. Indeed, spheroids are as easy and cheap to manufacture as 2D systems while having a complexity closer to physiological reality.

Numerous methods exist to analyze spheroids, the leading techniques being based on microscopy [2, 3]. In addition, electrical approaches are being studied, with a particular interest for microsensors using the application of low-frequency electromagnetic waves [4–7]. Indeed, they have the advantages of being noninvasive, nondestructive, label-free, and affordable. For example, impedancemetry, using waves with a maximum frequency of 1 MHz, makes it possible to study molecular exchanges with the extracellular medium and at the cell membrane surroundings [8].

Above a few hundred megahertz, microwave dielectric spectroscopy involves the penetration of electromagnetic waves into cells through the cell membrane, opening up the possibility of analyzing the intracellular contents. Various devices exist to analyze cells in the microwave range, whether at the scale of the single cell, the cell suspension, the tissue, or even the organ directly from the patient [9, 10]. However, for the intermediate spheroids scale, there is no device that allows their analysis in this frequency range. Thus, this article presents a radio frequency (RF) device designed for the analysis of spheroids of 300 μm in diameter in the frequency range of 500 MHz to 20 GHz.

First, the equipment and methods used will be detailed, including a description of the biological objects tested, the manufacture of the microwave sensor, and a reminder of the electrical model used for data analysis, since it is described in [11]. Then, the first dielectric characterization of a spheroid in the frequency range 0.5–20 GHz will be presented. Finally, a conclusion proposing future perspectives will be given.

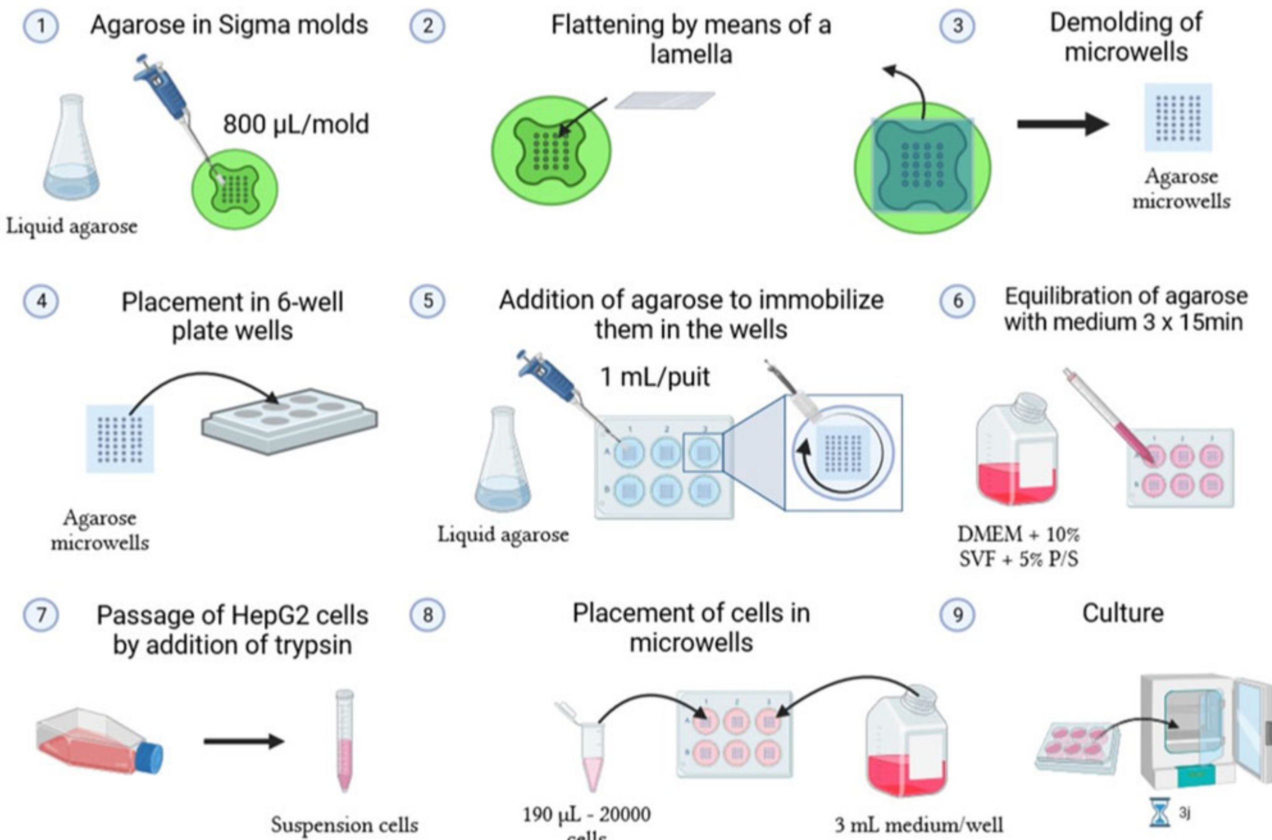


**Figure 1.** Experimental protocol for the manufacture of tumor spheroids.

## Materials and methods

### *Materials and reagents*

Formaldehyde (used with formalin solution, neutral buffered, 10%) was purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Gibco Dulbecco's Modified Eagle Medium (DMEM) (high glucose, GlutaMAX supplement and pyruvate), fetal bovine serum (FBS) (HyClone), pen-strep solution (100×) (Gibco), 0.05% Trypsin-EDTA (HyClone), Gibco Dubelcco's Phosphate Buffer Saline (DPBS) without Calcium, Magnesium (1×), Gibco Phosphate Buffered Saline 1× (PBS), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 1 M (Gibco) were purchased from Thermo Fisher Scientific (Illkirch-Graffenstaden, France). The HepG2 cell line (catalog number HB8065) was purchased from the American Type Cell Collection (LGC Standards, Molsheim, France).

### *Cell culture*

For our study, human hepatoma cells (HepG2, passages 15–35) were cultured at 37°C/5% $CO_2$ in DMEM medium complemented with 10% FBS and 1% pen-strep solution. Cells were grown up to 70–80% confluence.

### *HepG2 spheroids formation*

The spheroids formation protocol is illustrated in Fig. 1. The method we have chosen to make the spheroids is to use a mold made by Sigma, called Micro Tissue 3D Petri Dish size S, which is a 16 × 16 (256) cell array, sized to fit in plates containing 12 wells. Agarose (1%) is poured into these molds, left to cool, unmolded, and then placed in six-well plates. The immobilization of this network of 256 wells at the bottom of the wells is done with agarose. Balancing the pH of the agarose is then necessary to find conventional cell culture conditions before placing the cells. It is carried out by putting the culture medium for 45 min with a change of medium every 15 min. After this step, the micro-molds are ready to receive the cells.

The adherent cells cultured in flasks are passed through trypsin for 5 min, before undergoing centrifugation to resuspend them at the concentration of 20,000 cells/190 µL. The cell suspension is, then, deposited in the micro-molds. The whole is finally left in the culture for 3 days to form spheroids of approximately 300 µm in diameter (see Fig. 1 for a detailed diagram of the protocol).

The spheroids are retrieved with a P1000 micropipette after the 3 days of culture and deposited in an Eppendorf for fixation or for individualization in 96-well plates depending on the experiments planned. In both cases, the spheroids are, at this step, placed in DMEM culture medium containing no FBS but P/S and 25 mM HEPES (to buffer the pH of the medium at room temperature) to stop their growth. Individualized experiments can be done on them up to 3 days after recovery of the molds. The fixation of the spheroids is carried out after their recovery in pure formalin for 2.5 hours at room temperature.

### *The developed sensor*

#### *Architecture*

Figure 2 shows the developed biosensor. The biosensor consists of two distinct parts. A dielectric spectroscopy detection part with a

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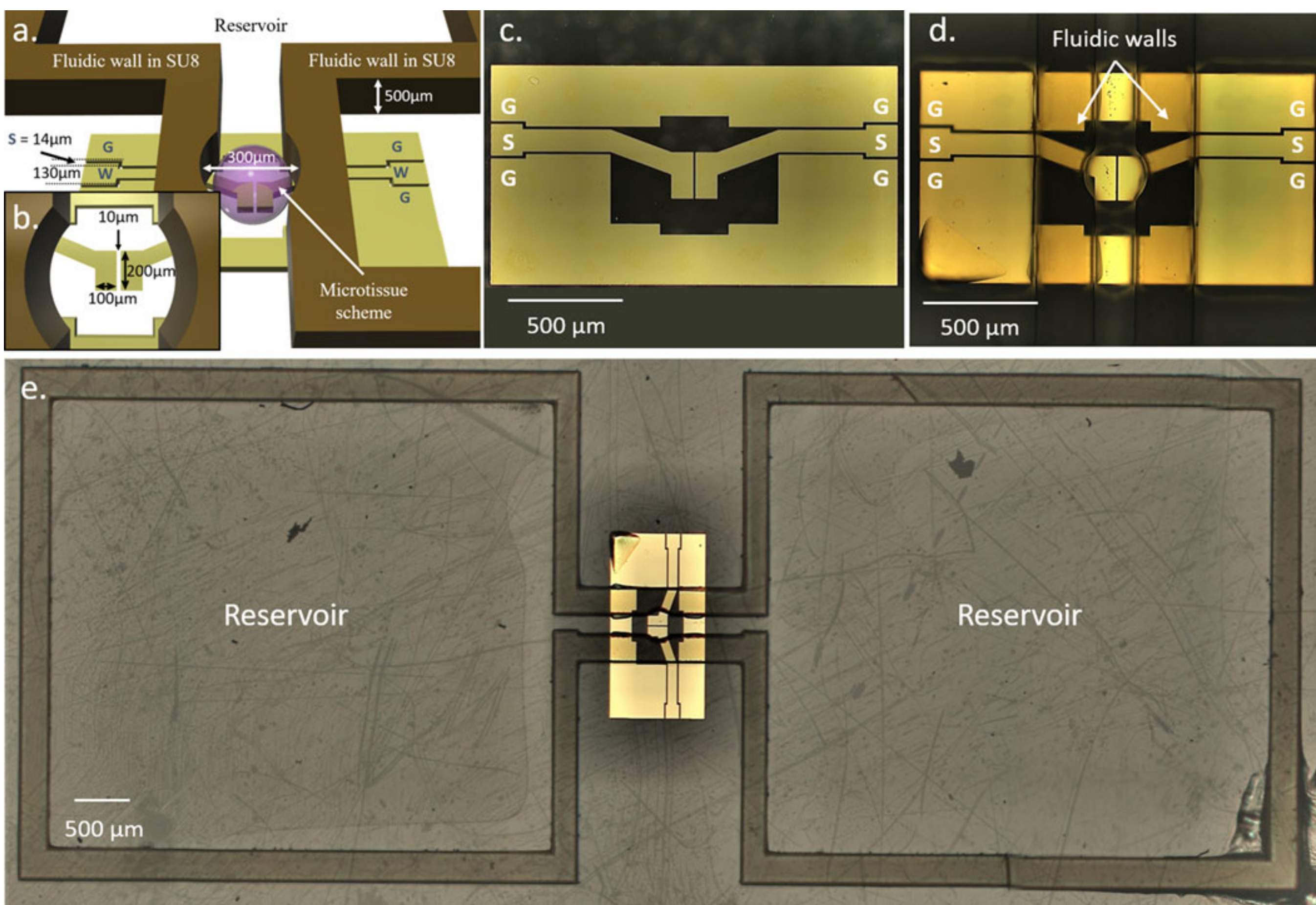


**Figure 2.** (a) and (b) Detailed diagram of the RF device with the schematic of a spheroid at its center in pink. (a) Global view; W = signal; S = slit; G = ground. (b) Zoom in on the central part of the sensor with the dimensions of the electrodes and the capacitive gap. (c), (d), and (e) Pictures taken with an HIROX microscope. (c) Picture of the device only with the first metallization. (d) Picture of the device after the second microfabrication step: the fluidic wall. (e) Global picture of the microdevice.

microwave circuit involving a coplanar waveguide of gold 300 nm thick on a quartz substrate, with a capacitive gap in the middle of the structure on the central conductor (cf. Fig. 2). Specifically, the RF accesses are implemented with a coplanar waveguide with a central conductor of width 130 µm, separated from the side grounds by a slot of 14 µm (cf. Fig. 2a). This coplanar configuration allows the placement of RF Ground-Signal-Ground (GSG) coplanar probes for characterization. The sensing area corresponds to the 10 µm wide capacitive gap in the center of the structure and the central conductor, as visible in Fig. 2b.

The fluidic part includes a fluidic channel and a micro-tank and two lateral reservoirs. The geometry of this channel allows it to hold 30 µL of the host medium (see Fig. 2d). This configuration is open, that is, without a cover over the channel and reservoirs. The spheroid is then manually positioned above the capacitive gap by means of a 500 µm high fluidic system placed perpendicular to the RF waveguide. The fluidic walls terminate in reservoirs to keep the fixed spheroid in 30 µL of its host medium, typically PBS, to later ensure the possibility to measure alive spheroids in future experiments.

### *Device microfabrication*

The fabrication of the biosensor is composed of two main steps based on classical microfabrication processes resumed in Fig. 3.

The electromagnetic sensor is fabricated on a quartz wafer (Corning Eagle XG series) previously cleaned for 2 min in a piranha mixture (50/50 v sulphuric acid $H_2SO_4$ hydrogen peroxide $H_2O_2$) followed by an oxygen plasma for 15 min. The metallization is carried out using the lift-off technique. Indeed, a first photolithography of negative AZ nLOF resin of 2.5 µm thickness is carried out to define the structure of the biosensor, then a metallization by evaporation of Titanium (Ti)/Gold (Au) is carried out. After metallization, a lift-off in acetone for about 1 hour allows to remove the gold adhering to the NLOF photoresist and to obtain only the gold forming the desired structure. The waveguide obtained is composed of a thin layer of Ti, helping the adhesion of a metal on the substrate, followed by a 300-nm-thick Au deposit. Figure 2c presents the obtained device. The second and final step corresponds to the elaboration of the fluidic walls and the two reservoirs placed on either side of the biosensor with a 500-µm-thick SU8-3050 layer deposited by gravity and then exposed and developed [11, 12]. The gravity method consists of manually depositing 6 g of SU8-3050 photoresist on the wafer, followed by baking with a slow temperature ramp up to 95°C (then leveling off for 11 h and naturally descending to room temperature) before carrying out the exposure at 365 nm with an energy of 3.6 J/cm$^2$, followed by the post-exposure bake (with temperature ramp up to 95°C and a 1 hour plateau) and finally the development for about 45 min. Figure 2d and e presents the obtained device.

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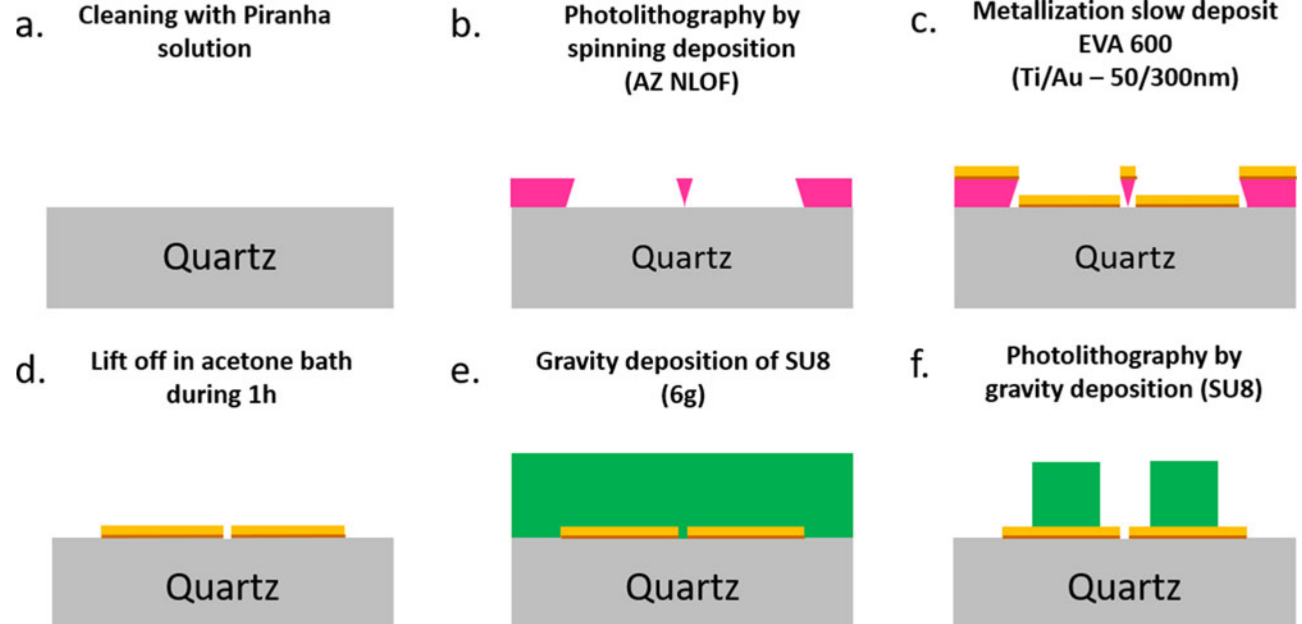


**Figure 3.** Diagram of the microfabrication of the biosensor. The representation of the photosensitive resin is shown in pink,, the gold conductive layer in yellow, and the titanium layer allowing the adhesion to the quartz substrate in brown. Finally, the thickness of the SU 8 polymer is shown in green. (a) First cleaning step of the wafer. (b) Result of the deposition and exposure of the 2.5 μm NLOF resin. (c) Ti/Au metal layer (50/300 nm). (d) Results obtained after a lift-off of about 1 hour in an acetone bath. (e) Gravitational deposition of a thick layer of SU8. (f) Fluid walls obtained after exposure and development of the SU8.

## Modelling and experimental protocol

The characterization of microwave components requires prior calibration of the two-port vector network analyzer (VNA). A Short, Open, Load, Thru (SOLT)-type calibration is performed with a Cascade Microtech calibration kit.

The test bench is presented in Fig. 4a. The probe station, shown on the right of Fig. 4a, is positioned on an anti-vibration table with the VNA at the rear. The positioning of the probes and the general control of the microdevice is done by means of an upright microscope, whose image is transcribed by a camera on a screen placed on the VNA.

After this calibration step, the device is connected to the VNA with two coplanar probes and cables. A first measurement of the *S* parameters is carried out while the sensor is unloaded. It allows to obtain the first measurement, which is necessary afterwards for data analysis, as developed in [11]. Next, the host medium alone is loaded into the device and measurements of the *S* parameters are performed for 25 min to check the measurement stability of the device before loading a biological object. The filling of the device is done by depositing 15 μL of the host medium, PBS in our case, in each of the reservoirs and checking that no bubble forms on the detection area. Finally, the tested object available in the culture medium is loaded into the middle tank. Just before manually positioning the object under study on the sensing zone using a micropipette, a measurement of the parameters *S* of the PBS host medium is performed. This measurement serves as a reference (giving $C_{ref}$ and $G_{ref}$). Measurements of the *S* parameters of the spheroid (or of the polystyrene beads) are then carried out for 20 min to extract its dielectric parameters.

In a few words, the data analysis is based on an electrical model, allowing us to extract a capacitance *C* and a conductance *G* of the device under test (DUT). With a contrast method using a reference measurement, $C_{ref}$ and $G_{ref}$, the specific influence of the studied object can be decorrelated from the culture medium in the form of $\Delta C$ and $\Delta G$ calculated with equations (1) and (2), respectively. The raw data, $C_{DUT}$, $G_{DUT}$, $C_{ref}$, and $G_{ref}$, and the standard deviations of the contrasts are given in [12] and [11], respectively.

$$\Delta C = C_{DUT} - C_{ref} \quad (1)$$

$$\Delta G = G_{DUT} - G_{ref} \quad (2)$$

## Results

In order to evaluate the stability of liquid measurements with our sensor, especially due to the open fluidic configuration, all PBS host medium measurements realized with different sensors and setup calibrations have been pooled and are presented in Fig. 4d and e. The blue curve corresponds to the average response of PBS measurements. It is defined by taking the average response of each independent PBS measurements into account. Those ones are previously obtained by averaging the 25 measurements of a PBS solution achieved every minute during 24 min, while their reference corresponds to the first PBS measurement realized after the stabilization procedure done within the sensor. The capacitive and conductive contrasts obtained with PBS shown in Fig. 4d and e are extremely close to the zero level. Maximum deviations of −1.39 fF and −0.02 mS in absolute value are obtained, which demonstrate the high robustness and repeatability of the microwave measurements of this sensor.

Next, to validate the microdevice and its proper functioning, two polystyrene beads are first measured and then three spheroids fixed with formalin are measured every minute for 24 min. Their capacitive and conductive contrasts in mean value over the measurement period are shown in Fig. 4d and e. The used reference is a PBS measurement realized just before the microwave characterization of each individual sample (bead or spheroid). The responses

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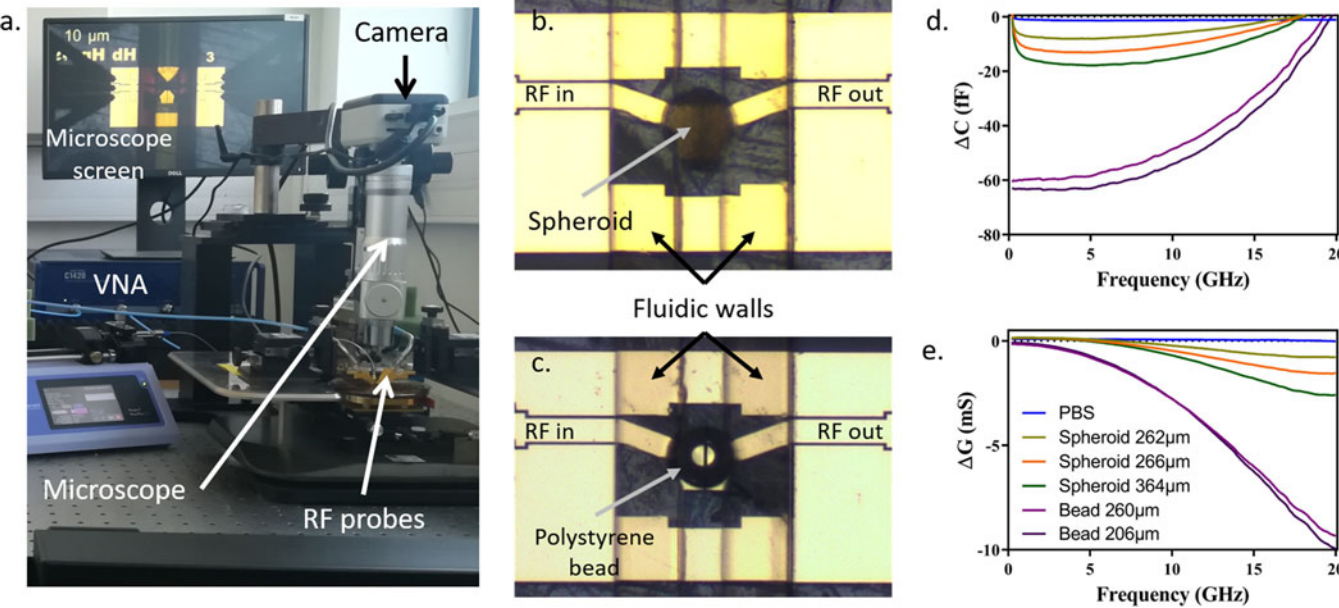


**Figure 4.** (a) Presentation photo of the microwave test bench with the different elements. (b) and (c) Pictures of the device loaded with a spheroid and a polystyrene bead, respectively. (d) and (e) Capacitive contrast $\Delta C$ and conductive contrast $\Delta G$ spectra obtained with the reference fluid in blue (PBS), polystyrene beads in green and fixed spheroid in red, purple, and beige, respectively. The curves represent the average of measurements made over 25 min.

obtained for the beads are represented by the curves in purple, while those of the spheroids are in green and orange, respectively.

A large variation between the dielectric responses acquired for the reference liquid (PBS) and the beads is obtained and may be easily explained by the fact that the beads present a low permittivity close to 3, whereas the PBS reference liquid exhibits a permittivity close to the one of water, i.e., frequency dependent and around 80 at 5 GHz. The extracted capacitive contrasts of polystyrene beads are located below −60 fF around 2 GHz, while the conductive contrasts at 20 GHz are close to −10 mS.

As far as the fixed spheroids are concerned, their capacitive and conductive contrasts are lower in absolute value compared to the polystyrene bead. The capacitive and the conductive contrasts of the fixed spheroids are indeed smaller than 20 fF around 2 GHz and 2.5 mS at 20 GHz in absolute value, respectively. These results are consistent since the permittivity of a spheroid is close to the reference liquid, PBS. This is all the more true that the spheroid has been fixed with formaldehyde, a treatment that causes permeabilization of the cell membrane. Thus, when stored in PBS, an equilibrium between the intra- and extracellular media happens, which decreases the dielectric contrasts in absolute value [13, 14]. Under these conditions, the measured contrasts are therefore lower in absolute value than for a living spheroid, allowing a proof of concept with a biological object that is easier to manipulate but more difficult to detect. Such fixed spheroids are indeed easier to prepare, may be stored for weeks, and used at convenience for experiments.

Finally, each spheroid presents a specific response, notably due to differences in shape and size. Indeed, despite a method that allows the creation of many spheroids in a short time, it is difficult to obtain perfectly round spheroids having exactly the same diameter.

## Conclusions

This article presents the first device allowing the dielectric characterization of spheroids and developed with its associated electrical model and characterized using first polystyrene beads and then formalin-fixed spheroids. The detailed protocol for the fabrication of the spheroids is also presented. The dielectric characterization results obtained are particularly promising for the use of microwave dielectric spectroscopy for the study of spheroids, since they allow the spheroids to be distinguished from each other despite the fact that they have undergone fixation, which induces very weak but nevertheless significantly observable capacitive and conductive contrasts with the host medium. These results show the feasibility of measuring spheroids in their culture medium by microwave dielectric spectroscopy, which had never been done before. The results of the measurement of polystyrene beads and fixed micro-tissues are consistent and open new analysis perspectives to this intermediate biological model as the microwave sensing technique is noninvasive, nondestructive, and label-free.

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**Competing interests.** None.

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