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Fast Bio-Impedance Analysis using Electrical Relaxation

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Abstract. The electrical characterization of biological material is a simple way for gaining information about process relevant measures like cell state, cell density or morphology. Although instrumentation for impedance and dielectric measurements is available in great variety, recent developments of applications require cheap, easy to use and miniaturized devices. Despite the availability of special IC, methods in frequency domain require more resources than methods in the time domain. Using electrical relaxation spectroscopy with the transformation of the result into frequency domain yields the impedance spectrum in a fraction of the time required in the frequency domain. Using non-equidistant sampling of the time signal reduces the amount of data by orders of magnitude while maintaining the accuracy of the measurement.

Single-use solutions often require disposable devices, especially in biomedical testing. Moreover, sensors incorporated in product packages are usually disposable. Fast monitoring of electrical properties is an emerging field in biotechnological and medical practice.

As an example of fast and efficient impedance detection with minimal hardware, monitoring electrical properties of multicellular spheroids passing a nozzle in order to assess the individual behavior is presented.

Keywords: relaxation spectroscopy, multicellular spheroids, bio-impedance,

1. Materials and Methods

The concept is based on relaxation spectroscopy in time domain where the hardware can be limited to a few components between a digital output and an analog-digital converter.

1.1 Electrical characterization based on relaxation

The generally employed electrical behavior for characterization of biological material is the impedance (Grimnes et al. 2014). For spectroscopic information in the frequency domain, a sweep through the interesting frequency range is performed with a measurement of magnitude and phase of the impedance. This takes time and is therefore not suitable for monitoring fast-changing objects. Moreover, sophisticated equipment is required which increases both power consumption and cost.

A much faster method is the application of a broad bandwidth signal and monitoring the system response (Min et al. 2015, Pliquett 2018). For LTI-systems (linear, time-invariant) Laplace transformation allows the transformation of the result into the frequency domain in order to obtain the impedance spectrum. This procedure yields a different, maybe more convenient presentation of the data but does not give any additional information. However, this approach is commonly favored by scientists because impedance spectroscopy is well established for monitoring biological objects while direct processing in the time domain does not really play a role.

Common broad bandwidth signals are the square wave, multi sine or maximum length sequence. All together, they are steady signals making processing simple by using fast Fourier transformation (FFT). A bottleneck is the requirement of equidistantly sampled signals, yielding huge data volume for broad bandwidth processing and even more pronounced, for continuous monitoring.

Despite more demanding processing of the response to transient signals, they exhibit great advantages with respect to data reduction and hardware requirements. Most popular transient signals are Dirac-, step-, and ramp function. The advantage of the step function is the robust generation but also comparatively simple data processing. The principle can be simply shown using an equivalent circuit for the material under test – here multicellular spheroids.

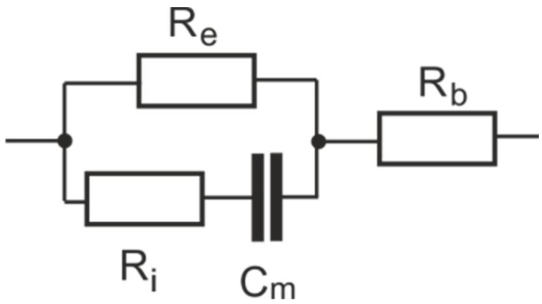


Figure 1: *Equivalent circuit mimicking spheroids in a nozzle*

Multicellular spheroids are spherical aggregates of cells that are extensively used in developmental biology and cancer research (Thielecke et al. 2001). In an equivalent circuit for spheroids (Fig. 1), R_e is the resistance of the extracellular medium, R_i mimics the cytosolic electrolytes and C_m depends on the capacitance of membrane structures. R_b is the resistance of the bulk electrolyte in the measuring path. In the ideal case of negligible R_b, the current response to a voltage step of U_0 is simply

$$I = \frac{U_0}{R_e} + \frac{U_0}{R_i} e^{-\frac{t}{R_i C_m}} = I_e + I_i e^{-\frac{t}{\tau}} \qquad (1)$$

where I_i, the current through the interior of the cells, crossing membrane structures at time $t = 0$, is the relaxation strength, i.e., the difference in elongation between beginning and end of the relaxation process. The relaxation process, where the charging of the membrane structures occurs, is further characterized by the time constant τ.

In the setup used in our study, however, R_b cannot be neglected. The current I_e with R_b taken into account is:

$$I_e = \frac{U_0}{R_e + R_b} \qquad (2)$$

For the current I_i through the cells we find:

$$I_i = \frac{U_0 {R_e}^2}{(R_e + R_b)(R_e R_b + R_i R_b + R_e R_i)} e^{-\frac{t}{\tau}} \qquad (3)$$

The time constant τ is

$$\tau = \frac{C_m (R_e R_b + R_i R_b + R_e R_i)}{R_e + R_b} \qquad (4)$$

The relation between frequency and time domain for LTI-systems is Laplace transformation or in the simpler case of steady state Fourier transformation. A relaxation process in the time domain, represented by a time constant and relaxation strength relates in the frequency domain to the characteristic frequency and circumference of the frequency dispersion.

The ideal case of a single time constant is seldom found in real measurements. Given the nature of the cell-based material, each individual cell exhibits electrical behavior which in summary is detected at the electrodes. Due to different cell size but also slight differences in the electrolytic composition of the cytoplasm, time constants are distributed. A good approach to this distribution is the Cole-model:

$$Z = R_\infty + \frac{R_0 - R_\infty}{1 + (j\omega\tau_0)^\alpha} \qquad (5)$$

R_0 is the resistance extrapolated to dc and corresponds to R_e in Fig. 1. R_∞ is the resistance at high frequencies (R_e parallel to R_i), and τ_0 is a frequency normalization factor corresponding to a medium time constant.

Because of the complex form of the analytical solution for transformation into the time domain, several well corresponding models are used. The most general approach is the distribution of time constants (DRT):

$$A(t) = A_0 + A_1 e^{-\left(\frac{t}{\tau}\right)^{\alpha}} \tag{6}$$

where A_0 accounts for a dc-offset and is mostly governed by R_e and R_b in Fig. 1.

In frequency as well as in time domain, α is a dimensionless parameter between 0 and 1. While 1 yields the ideal behavior of simple RC-combination, 0 yields a pure resistor.

For simple data processing, an equivalent circuit consisting of a serial combination of Cole- or RC-elements should be chosen for current excitation while parallel combinations of the corresponding admittances ($\underline{Y} = 1/\underline{Z}$) are preferred together with voltage excitation.

1.2 Sampling regime

A key feature of the step response of biological material in general – for current and voltage excitation – is the monotonically developing response with fast changes immediately after the step occurred and slowing down with time. It is not important when equidistant sampling is used. In this case, the sampling frequency should be twice as high as the highest frequency compound in the signal while the number of samples is the sampling frequency multiplied by the period time of the lowest frequency compound. For instance, if a frequency range between 10 Hz and 10 MHz is desired, the sampling frequency is minimally 20 MS/s while the number of samples is 20 MS/s * 0.1 s = 2 MS. Two million samples is feasible when one single measurement is performed but will exceed the capabilities of most equipment when continuous sampling with 10 measurements / s is required.

Using the features of the step response, like fast changes immediately after the step was applied while almost steady behavior after a multiple of the slowest time constant, the data volume can be greatly reduced by adapting the sampling regime. This means that fast sampling is necessary at the beginning of the response while the distance between sampling points can continuously increase with time (Fig. 2).

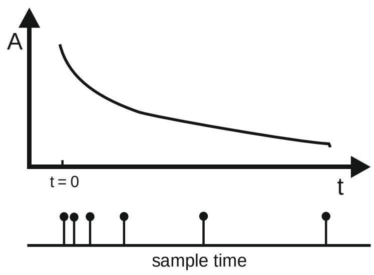


Figure 2: *Signal with sample times indicated*

Although it works well for signals without noise, serious problems arise in measurement practice. The reason is the violation of the Kotelnikov's sampling theorem which becomes increasingly pronounced with larger distances between sampling points.

Simply an anti-aliasing filter will comprise the high-frequency behavior. The way out is an adaptive anti-aliasing filter starting with high cutoff frequency at early times while it decreases with proceeding time. A simple hardware solution is to integrate the signal for instance by using an integration amplifier.

In practice, between 3 - 10 sample points per decade in time are required for good signal reconstruction (Fig. 3). This means, for a broad-bandwidth measurement over six decades (e.g., 10 Hz - 10 MHz) only 18 AD-conversions could be sufficient. In our case, we use ten sampling points for a range from 20 kHz to 4 MHz.

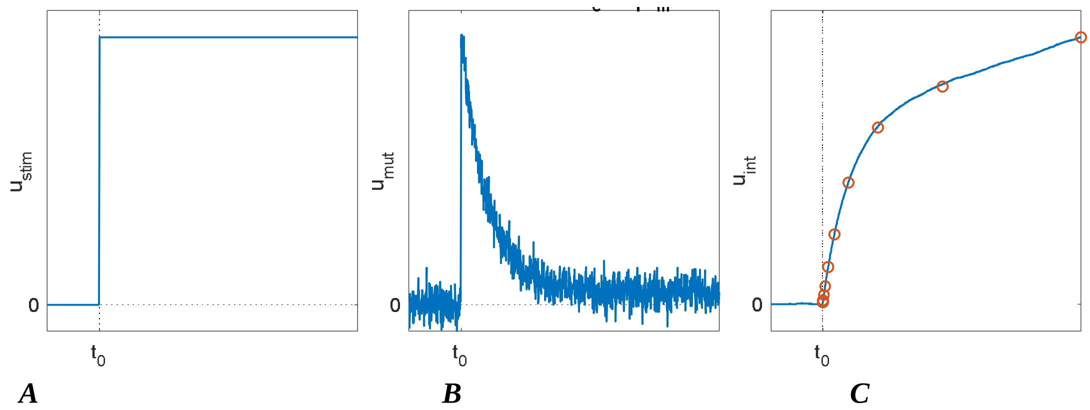


Figure 3. *The principle of electrical relaxation spectroscopy with integration and non-equidistant sampling. B) The current response to a voltage step (A) and an integrated signal (C) with indicated sample points (red circles)*

The practical solution involves a microcontroller, which contains a sample and hold (S&H) and an analog/digital converter (ADC). The data acquisition is carried out with a repetition rate of the stimulus of 1 kHz. The sampling frequency of microcontroller integrated sigma-delta ADCs is usually limited by almost 200 kHz. The reason for the low sample-rate is the time needed for the conversion by sigma-delta converters. But the S&H switch can be toggled as fast as one microcontroller cycle. To measure signals with an effective sampling frequency of several MHz, a sequential sampling method can be used. This can be done by applying one stimulus for each sampling point. In each sequence, the S&H-switch will switch at different accurate determined time points (Fig. 4). A critical requirement for using this sampling method is that the MUT should not change within the measuring time. Furthermore, it is important to know exactly, how long the time period from stimulus start until switching the S&H-switch is. The use of timer-interrupts is not recommended, because they will cause jitter of certain clock cycles. For determination of the acquisition time, a good way is to use assembler code and calculate the time based on the used instructions. Here a microcontroller with the main clock of 8 MHz is used. Therefore, the minimal effective sampling distance is 125 nanoseconds.

In practice, for the response arising from a relaxation process with only one time constant, 10 data points are used. Each data point is acquired after an individual step stimulus with a length of 50 µs with a varying time interval after the stimulus. This bears the advantage that the time interval between the two acquisitions is long enough for processing.

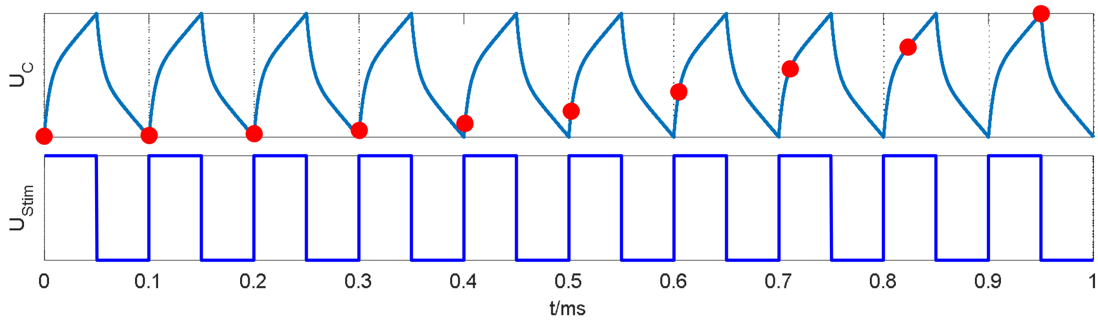


Figure 4. *The principle of sequential sampling. Measuring one signal with several step excitations.*

1.3 Hardware

The practical solution shown in Fig. 5 involves a microcontroller (MSP-EXP430FR6989 LaunchPad Development Kit, Texas Instruments, Dallas, TX, USA), which contains a sample and hold (S&H) and an analog/digital converter (ADC). For excitation, a digital output is used. In order to circumvent problems with electrochemical reactions but also field effects on biological material, a frontend ensuring low voltage (<100 mV) across the material under test (MUT) was used.

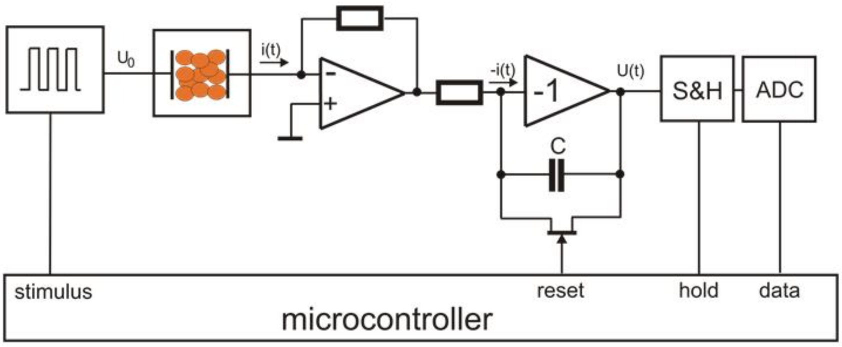
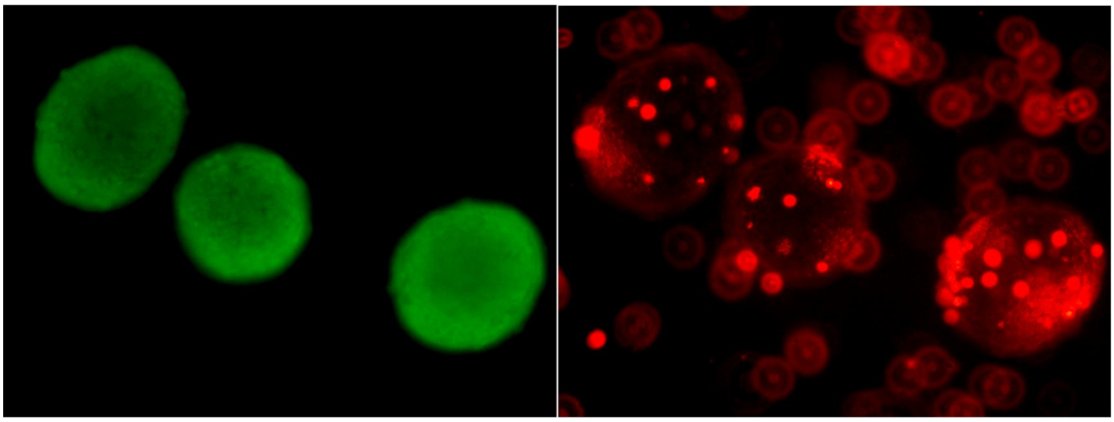


Figure 5. *Block diagram of the measuring system.*

The current through the MUT is transformed into an equivalent voltage by a transimpedance amplifier. The equivalent voltage is integrated by an operational amplifier (OPA) fed back by an integration capacitor with the reset switch. The integrated signal is sampled by the S&H circuitry and digitized by the ADC of the MCU. The time control was directly managed by the timers of the MCU which yield increasing times after the positive edge of a rectangular stimulus of 10 kHz. A full measurement cycle lasts 1 ms (10 periods) resulting in 10 different sampling times. The parts of the circuitry will be discussed in the following.

1.4 Characterization of multicellular spheroids

Multicellular spheroids are 3D-cell conglomerates commonly used as a model for the tissue. Embryonic kidney cells (HEK 293) were grown in culture flasks according to established protocol until a confluent monolayer was reached. DMEM (Dulbecco's Modified Eagle's medium) was used as culture medium throughout all experiments. After trypsinization, cells were washed and transferred to a spinning disc culture for three days. Within this time multicellular spheroids grow up to an average diameter of 100 µm and more (Fig. 6).



A **B**

Figure 6. *HEK293 spheroid stained with FDA for active metabolism (A) and Ethidium bromide (B) for labeling dead cells.*

In order to check the suitability of the MCS for our studies, an aliquot was taken and incubated for several minutes with fluorescein diacetate (FDA), a non-fluorescent, lipophilic molecule which easily penetrates the plasma membrane. The acetate groups are cut by esterase due to an active metabolism which yields fluorescing. In opposite to the FDA, fluorescein is not membrane permeant and highly green fluorescent (Fig.7A). Dead or inactive cells stay dark in fluorescence microscopy. In order to distinguish between inactive and dead cells, a co-staining with ethidium bromide (EB) was used, an almost non-fluorescent dye which does not penetrate intact membranes. However, dead cells loose their membrane integrity and EB enters the cell where it intercalates into the DNA. In DNA intercalated EB appears bright red fluorescent and is, therefore, a good marker for dead cells (Fig.6B).

Cultures of spheroids with more than 5 % dead cells or low metabolism were not used for experiments.

For electrical characterization, spheroids were harvested and pumped through a glass nozzle with a diameter of about 100 µm (Fig. 7) which was mounted inside a capillary of 900 µm diameter. At both sides of the nozzle, Ag/AgCl electrodes (Model E-205, Scientific Products GmbH, Hofheim, Germany) were mounted for electrical connection.

In order to validate the electrical measurements, a camera monitored the movement of spheroids through the nozzle. Impedance measurements using a voltage controlled square wave were conducted continuously with a repetition frequency of 10 kHz where ten periods were used for complete acquisition resulting in a repetition frequency of the measurement of 1 kHz. Only the positive steps were used for further processing. An additional sample was taken in the negative half wave for offset compensation.

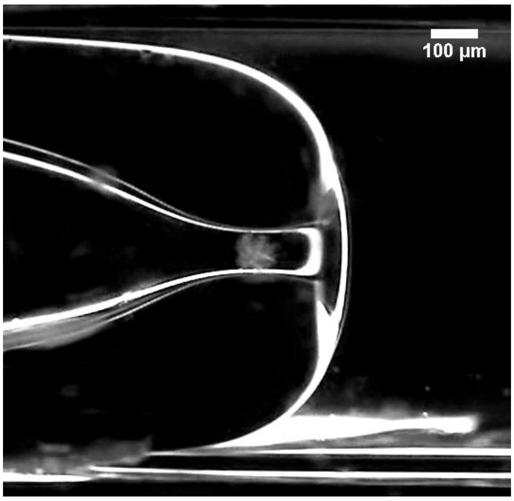


Figure 7: *Optical image of a spheroid in the glass nozzle. The spheroid is visible in the narrowest part of the nozzle.*

2. Results

The electrical behavior of multicellular spheroids passing through a nozzle was assessed by electrical means. For ensuring high repetition of the measurement, electrical relaxation spectroscopy (also termed time-domain spectroscopy) was used. This allows for multiple measurements during the passage of a single spheroid and therefore the development of electrically assessed parameters during the passage. For instance, shunting pathways around the cells decline when the pressure increases due to narrowing of the area inside the nozzle. Using the electrical parameters, biophysical properties like cell density or viability were deducted from the measured data.

The passage of a spheroid through the nozzle was detected by an overall decrease of the current between the electrodes (Fig.8). The step response during the passage (red circle in Fig.8B) was then fitted with the DRT model (Eq. 6) taking into account the serial bulk resistance R_b. The parameter α exhibited considerable variability when fitted together with the other parameters A_0, A_1, and τ. Because of this uncertainty influences all other parameters as well, it was set to a fixed value of 0.7 to ensure a stable fit throughout the experimental data. A typical plot of the fit parameters during the passage is shown in Figure 9.

Without the presence of a spheroid, the resistance of the bulk medium R_b was determined by the direct current resistance. With a spheroid present in the nozzle, all parameters were determined by a DRT fit. Only R_e is shown for the whole measurement, while R_i and C_m are shown only when a spheroid is present. R_b was set to the value of the dc resistance of the medium before the spheroid entered the nozzle.

The resistance of the extracellular medium R_e was increasing rapidly at the entrance of the spheroid to the nozzle. Spheroids with a diameter larger than the nozzle diameter were deformed and slowed down. During the passage, the intracellular resistance R_i and the membrane capacity C_m showed no clear trend.

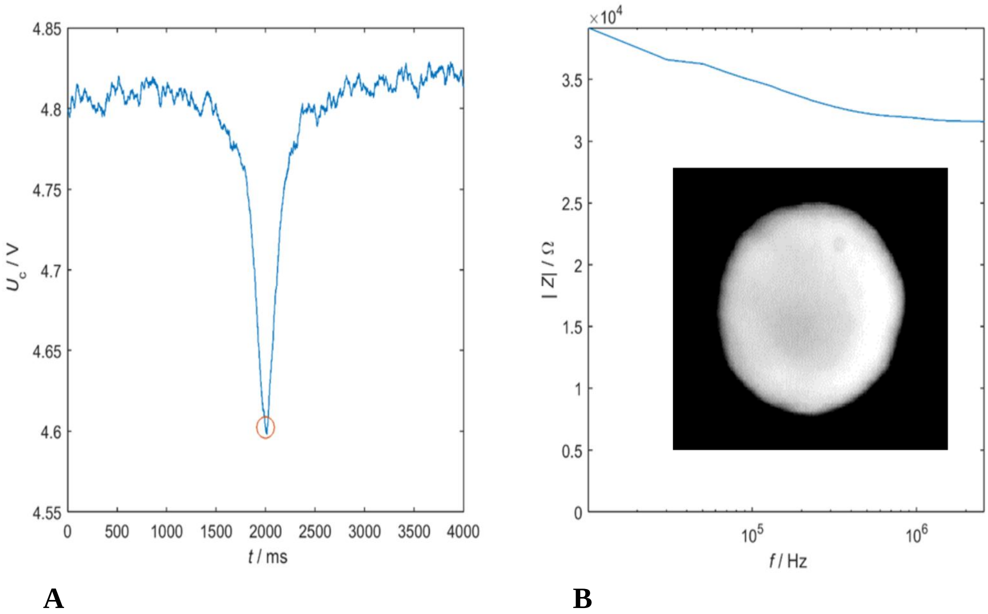


Figure 8: *(A) Time course of the 10th sample point during the passage of a spheroid. (B) The magnitude of the impedance as a function of frequency at the time point indicated by the red circle in (A). The image of the spheroid (B) was extracted from the video stream which was taken in parallel to the impedance measurements. For synchronization between the video stream and impedance measurements, the HSYNC-signal was traced in one audio channel while the step response was monitored using the other audio channel.*

3. Discussion

The fast acquisition of the electrical relaxation due to a voltage step makes it possible to analyze the properties of spheroids during the passage through the nozzle in detail.

The extracellular resistance R_e includes the resistance of the medium around the spheroid and the resistance of the medium between the cells. The tremendous increase of R_e during the passage can be attributed to changes in the resistance of the medium around the spheroid. This part was increasing because the spheroid covered the nozzle and blocked the current between the electrodes through the medium. After the spheroid covered the nozzle completely, R_e was further increased. This might be explained by the continuing adaptation of the spheroid's shape to the opening.

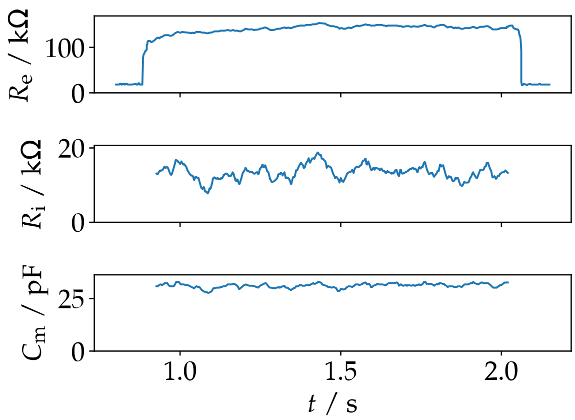


***Figure 9**: Passage of a single spheroid through the nozzle. R_e – Extracellular resistance, R_i – Intracellular resistance, C_m – Membrane capacity.*

The intracellular resistance R_i exhibits a relatively large variability due to the low absolute value of the parameter. The missing trend of the parameters R_i and C_m suggest that the internal structure of the cells in the spheroid is unchanged during the passage through the nozzle. Fluctuations in these values might arise from mechanical stressing during the nozzle. This makes the system ideal for measurements of spheroids with varying properties like diameter, incubation time, and the application of drugs. By adjusting the flow rate, a fast and continuous flow of spheroids can be obtained. The spheroids inflow can then be characterized at a very high velocity.

4. Conclusions

In this paper, we have shown that we can use an electrical relaxation to determine the bio-impedance of spheroids with a time resolution suitable for detecting fast events like the passage of spheroids or even single cells through a nozzle with the diameter of the spheroid or cell size. The setup is suitable for high-throughput measurements with continuous sampling.

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