REVIEW

Electrode-less Measurement of Cell Layers Impedance

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Summary

This paper is devoted to yet unpublished electrode-less methods (ELM) of cell layers impedance measurement based on transformer principle. The main advantage of ELM is elimination uncertainties caused by interface between electrodes and measured electrolyte. The method of avoiding distortion caused by non-ideal transformer transfer function ("deconvolution") and errors caused by residual voltage is described. The modification of original transformer based method allowing to measure an impedance of inserted object is proposed. Results of several calibration measurements confirming the proper function of ELM including example of transepithelial resistance of cells layer are presented. Crucial parts of measuring system and recommendation for their realization are included.

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Introduction

Most living cells in culture adhere to each other and to the suitable substrates (Loftus *et al.* 1980, Gailit and Ruoslahti 1988, Horton 1997). Confluent layers of cells could form relatively impermeable barrier. Namely some epithelial cells form layers with tight junctions between neighbouring cells with considerable electric parameters. Impedance of cell layers carries valuable information characterising the state of cells and can be used for observation and evaluation of many physiological phenomena as the quality of cell adhesion is a hallmark of their physiologic state. In this contribution the cell layer impedance is intended to be used for evaluation of toxic effects of tested material on living tissue. Basically the degree of toxicity of certain substance can be estimated by evaluation of impedance of cell exposed to tested substance. As general rule impedance of live and healthy cell layer is much higher than impedance of cell layer damaged by toxic substance. The quantification of toxic effects by impedance measurement is one of the alternatives to their evaluation from cell shape using image signal processing ("videometry") or from other biochemical and physiological parameters (Ďaďo 2009).

Measurement of electrical impedance of any object located between electrodes is well known and generally used approach. In case of cell impedance measurement electrodes are submersed to solution (electrolyte) in which cells are located. Several standard methods of measurement are used in electrophysiological laboratories, e.g. patch – clamp microelectrodes, TER method (Webster 2006), ECIS method (Giaever and Keese 1986), etc.

Measurement of transendothelial or transepithelial electrical resistances (TERs)

This straightforward *in situ* experimental approach is used for monitoring the modulation of barrier-forming cell-to-cell contacts (tight junctions) in

cultured cells grown on porous filters. As it is shown on Figure 1a the impedance of cell layer between two electrodes is formed by parallel combination of cell membrane capacitance C_{cl} (typical value 0.44 μF/cm^2) and membrane resistance TER (typically 380 Ω.cm^2) in series with resistance of electrolyte $R_{med1,2}$ (in order of 20 Ω) and Constant Phase Elements CPE$_1$, CPE$_2$

representing electrical behaviour of interface electrodes-electrolyte. The model of CPE was introduced to better describe the complicated processes on the double layer between metal electrode and liquid electrolyte. Instead of metal electrodes also Ag/AgCl electrodes in microfluidic version of TER method can be used.
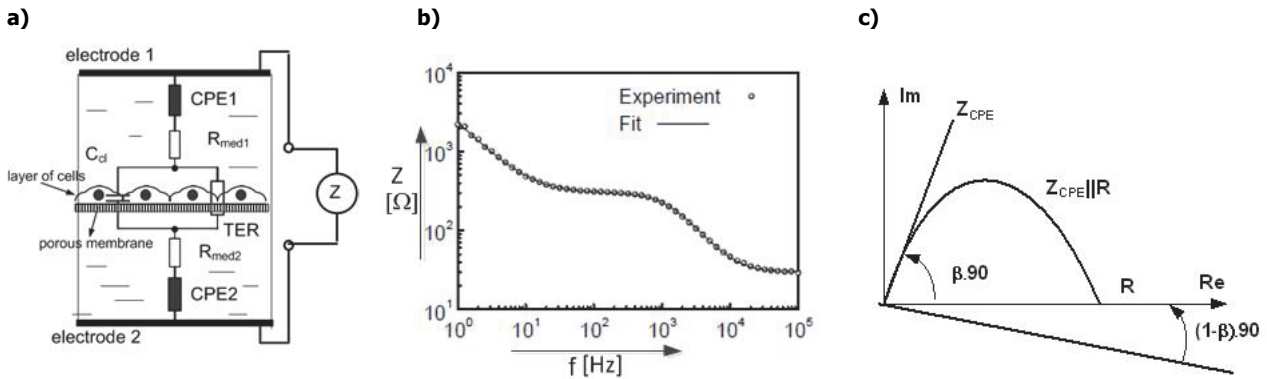

Fig. 1. a) Principle of TER method (Wegener *et al.* 2004). **b)** Typical graph of |Z(f)| (Wegener *et al.* 2004). **c)** Phasor diagram of Z$_{CPE}$ (straight line) and Z$_{CPE}$ in parallel with resistor R$_p$ (semicircle) (Webster 2006).

Electrode-less impedance measurement

In former model of interface composed from parallel connection of resistor and capacitor new circuit element CPE (empirical pseudo-capacitance) is used instead of normal capacitor. Impedance $\mathbf{Z}_{CPE}$ of CPE is approximated by expression (Webster 2006)

$$\mathbf{Z}_{CPE} = \frac{K}{(j\omega)^{\beta}} = \frac{K}{(j\omega)(j\omega)^{\beta-1}} = \frac{1}{j\omega \mathbf{C}_{eq}} \quad (1)$$

Equivalent pseudo-capacitance $\mathbf{C}_{eq}$ is inversely proportional to frequency

$$\mathbf{C}_{eq} = \frac{(j\omega)^{\beta-1}}{K} = \frac{1}{K(j\omega)^{1-\beta}} \quad (2)$$

Unit of equivalent capacitance magnitude **K** is Ωs^{-1}, exponent β is a constant, $0 < \beta < 1$. The phase angle φ of this empirical circuit element is constant $\varphi = \beta \pi/2$ and generally lies between 45° and 90°. Typically β has a value of 0.8 for many biomedical electrode systems (Fig. 1c).

Electrode-less impedance measurement

Characterization of disturbing phenomena on double layer interface metal electrode-electrolyte by CPE

is only approximation to real situation. In several cases contact of metal with electrolyte could change behavior of observed electrochemical or physiological process. Until now these effects were suppressed by implementation of the same type of electrodes in all laboratories, i.e. supposing that same "distortion effects" will occur in all experiments. Otherwise transferability of results could be jeopardized. Moreover electrodes from precious metals are expensive and mostly can be used only once (difficulties with cleaning and sterilization). Metal electrodes cannot be used in case when EM radiation absorbing in metal leads to increase of temperature. The methods of impedance measurement requiring no electrodes (electrode-less methods – ELM) could eliminate these drawbacks. One possible ELM of impedance measurement based on transformer principle is described below together with results of experiments confirming its feasibility and limitations.

Transformer method of electrode-less cells impedance measurement

Conversion of current through isolated tube filled by measured electrolytic solution to voltage by transformer (inductive method) is long known (Šalamon 1958) as one alternative in measurement of conductivity of electrolytes, but rarely used. The principle of the

method is depicted on Figure 2a. Isolated tube forms secondary winding of driving transformer (DT) and primary winding of current sensing transformer (CST). Normally voltage on secondary winding n_3 of CST is measured. Due to complicated equivalent circuit of this transformer, its transfer function is far from being ideal and it is very complicated to correct it in order to reach the same transfer factor for all frequencies. In

oscilloscope current probes, which might be also used for current I_2 measurement, this difficult task is accomplished by carefully chosen circuit correction components. Each probe must be corrected individually and this costly process increases enormously the price of probe and at the same time substantially decreases its sensitivity.
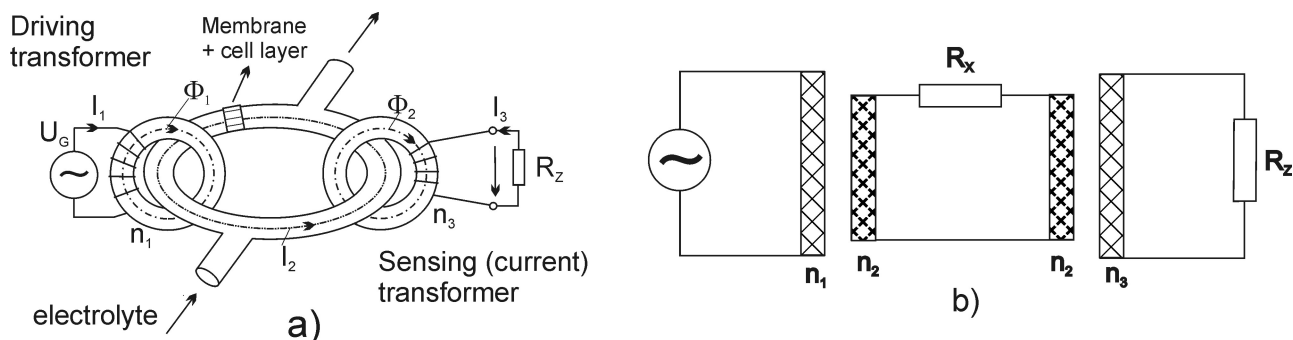

Fig. 2. a) Configuration of transformer based electrode-less method of impedance measurement. U_G – generator of measuring signal, $\Phi1$, $\Phi2$ – magnetic flux in toroid cores of transformers, I_1, I_3 – currents in windings of driving and sensing transformers, I_2 – current in measured electrolyte. For low values of I_2 (several mA) voltage or current on the winding n_3 of sensing transformer is measured by lock-in amplifier (LIA). **b)** Equivalent circuit, R_x – impedance of electrolyte in tube, R_z – input impedance of measuring instrument (Ďaďo and Kreidl 1996).

Fortunately in our case when dealing with harmonic signals the approach of inverse transfer function ("deconvolution", calibration) could be implemented. The response of output voltage on n_3 to current $\mathbf{I_2}$ through known impedance for different frequencies is measured (calibration process). The measured output values for unknown (measured) impedance for different frequencies are then corrected by means of data obtained by calibration process.

Another problem is nonzero output voltage of CST (initial voltage $\mathbf{U_{opz}}(f)$), when current $\mathbf{I_2}$ through tube (no electrolyte inside) is equal to zero. The penetration of DT voltage to CST *via* "illegal" way, (i.e. not through magnetic field) is caused mainly by capacitive coupling both transformers and limits the accuracy of the measured impedance. The influence of initial voltage can be partially decreased by shielding both transformers.

Further reduction of error due to initial voltage can be achieved by measuring and subtracting its amplitude and phase from phasor of total output voltage of CST.

In order to obtain information on both modulus (magnitude) and phase of measured impedance the principle of coherent demodulation realized by Lock In

Amplifier (LIA) should be used. Measuring the output CST by using current-to-voltage convertor (usually is included in LIA input channel) gives much better performance in terms of output signal value on high frequencies. Using current to voltage convertor has also advantage in elimination of influence of impedances (mainly capacitances) lying in parallel to secondary windings of CST.

Correction of transformer transfer function distortion by calibration

The Amplitude Frequency Characteristic (AFCH) of CST representing frequency dependence of ratio of voltage on secondary winding n_3 of CST to current $\mathbf{I_2}$ through electrolyte ($\mathbf{U_{op}}/\mathbf{I_2}$) is quite complicated due to non-ideal behavior of transformers. The influence of CST non-ideal, frequency dependent transfer function is eliminated by calibration. The calibration process consists in insertion of frequency independent element – resistor R_{cal} of known value in series with wire loop (calibration loop) located in place of tube ("secondary winding of driving transformer"). As voltage $U_2(f)$ on calibrating loop (Fig. 2b), and value of R_{cal} are known,

current through calibration loop is also known ($I_2 = U_2(f)/R_{cal}$).

Mostly amplitude of $U_2(f)$ does not depend on frequency, then $U_2(f) = U_2$. Changing the frequency of voltage $U_G(f)$ on primary windings n_1 of DT, the amplitude and phase of output voltage $U_{op}(f)$ on the winding n_3 of CST are measured by LIA.

In ideal case current-to-voltage transfer function $H_{cio}(f)$ [V/A] of CST is calculated from measured values $U_{op}(f)$ according definition

$$H_{cio} = \frac{U_{op}(f)}{U_2 \Big/ R_{cal}}$$

In reality it is necessary to avoid error due to initial voltage $U_{opz}(f)$. For finding the value of initial voltage the output voltage on winding n_3 of CST is measured for different frequencies f and open secondary of DT n_2, i.e. for empty tube, when $I_2 = 0$. Correct transfer function $H_{ci}(f)$ is then found from calibration equation

$$U_{opc}(f) = H_{ci}(f) \frac{U_2}{R_{cal}} + U_{opz}(f) \qquad (3)$$

in form

$$H_{ci}(f) = \frac{U_{opc}(f) - U_{opz}(f)}{U_2} R_{cal} \qquad (4)$$

During the measurement procedure, when measured impedance of electrolyte equals to $Z(f)$, the values of output voltage on CST $U_{opx}(f)$ are given by relation similar to (3).

Using (4), we obtain relation

$$U_{opx}(f) = \frac{U_2}{Z(f)} H_{ci}(f) + U_{opz}(f) =$$

$$\frac{U_2}{Z(f)} \frac{U_{opc}(f) - U_{opz}(f)}{U_2} R_{cal} + U_{opz}(f) \qquad (5)$$

On basis of (5) impedance $Z(f)$ is calculated using formula

$$Z(f) = \frac{U_{opx}(f) - U_{opz}(f)}{U_{opc}(f) - U_{opz}(f)} R_{cal} \qquad (6)$$

The variables $U_{opz}(f)$, $U_{opc}(f)$ and R_{cal} are measured once during calibration procedure for whole set of measured values $U_{opx}(f)$ corresponding to values of $Z(f)$ for given set of frequencies.

In case when impedance of the object (e.g. layer of cells) inserted to electrolyte is measured, the resistance of basic electrolyte R_{el} lies in series with object and forms part of total impedance $Z(f)$ and in order to obtain measured object impedance R_{el} should be subtracted. Generally it is necessary to measure impedance of electrolyte $Z_{el}(f)$ itself with measured object removed. Fortunately often the $Z_{el}(f)$ behaves as resistance and for obtaining important frequency dependent part of $Z(f)$ could be simply subtracted. Moreover in many cases R_{el} could be decreased to values much smaller then $Z(f)$ by increasing concentration of electrolyte or modification of geometry of measuring tube.

Experimental verification of deconvolution – correction of transformer transfer function

The magnitude and phase of the measured output voltage $U_{opc}(f)$ for frequency of driving voltage $U_G(f)$ changing in range from 0.2 to 100 kHz during calibration is depicted on Figure 3a. The resultant curves are in fact proportional to amplitude and phase of transfer function of CST. Proper function of deconvolution (correction of distortion due to transfer function) was verified for the case, when calibration resistor $R_{cal} = 490\ \Omega$ in wire loop was replaced by resistor $R = 1920\ \Omega$ playing role of unknown impedance $Z(f)$. Resulting value calculated by (6) was very closed to value of R. As it could be expected, the calculated value of resistance practically does not depend on frequency and its phase angle is close to 0° (Fig. 3b).

The similar verifying measurements were performed by connecting capacitor C=2.7 nF in series with wire loop. As expected, for capacitance frequency dependence of modulus has hyperbolic character, value of phase angle is close to 90° and values of modulus correspond to impedance of capacitor $1/\omega C$ for given range of frequencies (Fig. 3c).

In spite of non-ideal, frequency dependent transfer function of transformers (Fig. 3a) process of calibration and de-convolution eliminates the errors due to this distortion. For frequency independent components (resistors) de-convolution leads to correct results, i.e. modulus does not depend on frequency, and phase angle is close to zero (Fig. 3b). The same conclusion is valid also for frequency dependent component – capacitor (Fig. 3c).
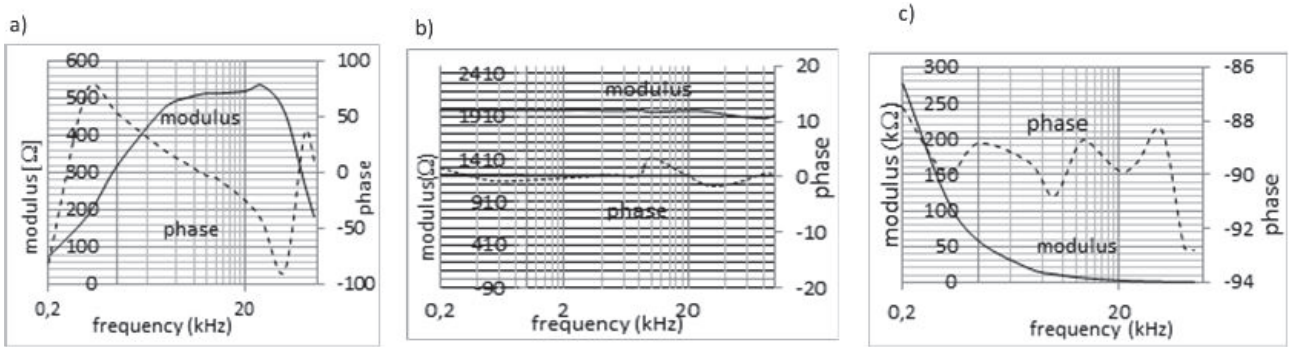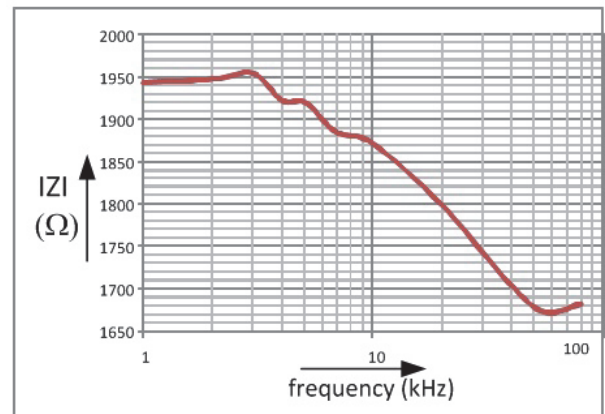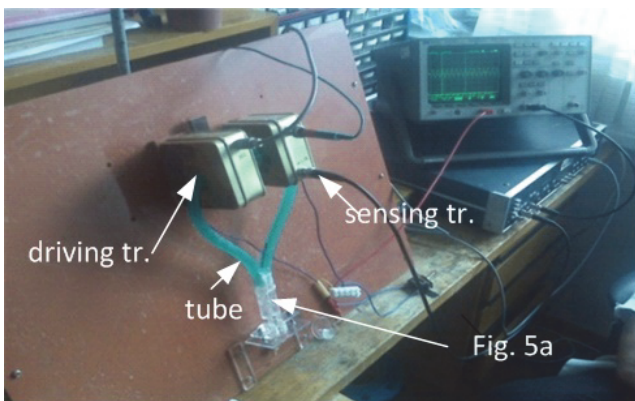
Fig. 3. a) Amplitude and phase of sensing transformer output voltage as function of frequency for current $I_2=(U_G/n_1)/R_{cal}$, (U_G/n_1=0.045 V, R_{cal}=490 Ω). **b)** Amplitude ("modulus") and phase of output voltage $U_{opx}(f)$ for $|Z(f)|$ =R=1920 Ω after correction using results of calibration procedure. **c)** Modulus and phase of capacitance.

Fig. 4. a) Experimental set-up. **b)** Modulus of impedance of cells layer $|Z(f)|$.
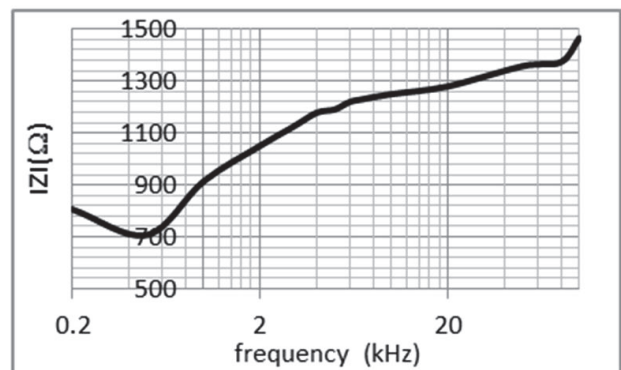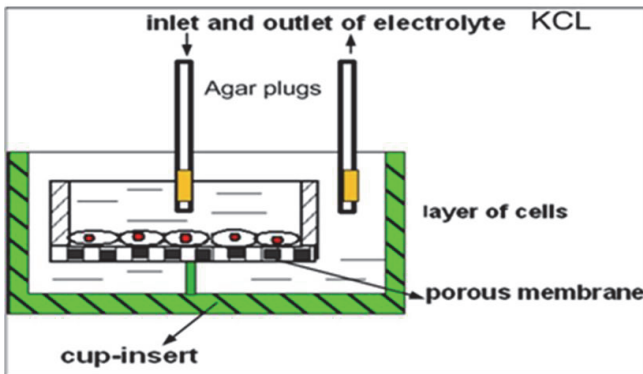

Fig. 5. a) The arrangement of most critical part of set-up: interface between cultivation solution and tube filled with KCl. The dish containing small cup in which porous membrane and cultivation solution can be seen on Figure 4a (the tied together ends of tube (hose). **b)** Anomalous course of cells impedance dependency on frequency ("peeling of cells"?).

Experimental set-up

The experimental set-up designed for measurement of cell impedance was derived from configuration analogical to TER measurement (Fig. 1). The layer of cells was located on polycarbonate filter inserts having several tens of millions of pores per cm^2 (Fig. 5a).

The Millicell Cell Culture Insert (diameter 12 mm) has shape of a dish with short legs on bottom side and is submersed to electrolyte in plastic measuring chamber. The bottom of Cell Culture Insert is formed by polycarbonate membrane with 0.4 μm pores which is covered with cell layer.

The top and bottom side of dish above and

below the cell layer is filled with the same cell bath electrolyte solution. The isolated tube filled with electrolyte passing through the windows of toroid transformers is by one end submersed to solution on upper part of cell layer and by other end to its down part.

Example of impedance of cell layer measurement

Human kidney epithelial HEK 293 cell line was cultivated in OPTIMEM (GIBCO) medium supplemented by 5 % of fetal calf serum. Cells were splitted 2 times weekly in polystyrene cultivating flasks. Cells for experiments were grown on polycarbonate membrane (pore size 0.4 μm) in 12 mm Millicell Cell Culture Inserts (Millipore) for one week to obtain the confluent layer.

The secondary winding of DT, i.e. hose with diameter 10 mm and length approximately 1 m is filled with saturated KCl electrolyte (Fig. 1). Layer of cells is seeded on the perforated polycarbonate membrane (product of Millipore) submersed in cell bath solution. Before the impedance measurement, cultivating solution (OPTIMEM) was replaced by the cell bath solution containing (in mM): NaCl 160, KCl 2.5, $CaCl_2$ 1, $MgCl_2$ 2, HEPES-NaOH 10, glucose 10, pH 7.3. As the cell-cell contacts and cell-substrate contacts are dependent on divalent cation concentration (Ca^{2+}, Mg^{2+}) we used divalent ion free solution to mimic the toxic conditions to release the cell-cell contacts (Loftus *et al.* 1990, Gailit and Ruoslahti 1988). The divalent ion free solution contained (in mM): NaCl 160, KCl 2.5, EGTA 5, HEPES-NaOH 10, glucose 10, pH 7.3. The lack of calcium and magnesium ions in the surrounding solution induced the loss of cell-cell and cell-substrate adhesion contacts in few minutes. In order to separate saturated KCl solution from the cell bath solution the ends of tube were narrowed and filled with agar plug (Fig. 5a). The agar plugs were prepared from cell bath solution with 2 % agar and were immobilized by nylon mesh attached to tube endings and were inserted to solution in above and bottom of membrane. The resistance of KCl electrolyte was approximately 400 Ω. Voltage on cells layer was chosen by setting voltage U_G on primary of DT to value fulfilling relation $U_G/n1=45$ mV. Then voltage on cells layers stays in recommended secure range below 45 mV (Giaever and Keese 1986). Using procedures of calibration and deconvolution (equations 3, 4, 5, 6), the module and phase of impedance was calculated by (6) from measured values of modulus and phase of output voltages $U_{opz}(f)$ and $U_{opx}(f)$ of CST. On Figure 4b is the

graph showing one example of dependence of cells impedance on frequency. The shape of graph reminds typical one displayed on Figure 1b and obtained by ordinary TER method (Wegener *et al.* 2004). In one experiment cell impedance after certain time since the start of measurement unexpectedly increased. On the contrary to expected decrease of impedance at high frequencies as well known from measurement with electrodes, the increase impedance with frequency demonstrates inductance behavior of electrolyte. This anomalous effect might be possibly caused by "peeling process", i.e. cell release from the substrate leading to opening of pores of membrane and consequent changes in electrical properties of electrolyte (Fig. 5b).

Limitations of transformer method of ELM

Although basic principle and circuitry of transformer based ELM of cells layer is simple, there are several difficulties accompanying it`s realization. Many of them stem from the low level of measured current due to low driving voltage required for avoiding electrocution of cells (several tens of mV). Relatively complicated instruments using coherent demodulation (Lock-in Amplifiers – LIA) are necessary for signal recovery from noise and for measuring amplitude and phase of signal harmonic components.

Transformer is obviously one of the most critical components of the measuring system. The high quality magnetic material should be used and distortion of signal due to hysteresis curve should be avoided. Distortion of signal waveform occurs when intensity of magnetic field shifts the working point to nonlinear part of hysteresis curve (close to saturation). Usually this effect occurs at low frequencies where impedance of coil is low and consequently current is high. The distortion of harmonic waveform of output voltage from sensing transformer should not apparently cause the problem when tracking band-pass filter is used in input channel of LIA. But even in this case the principle of superposition might not be valid and transfer characteristic of system could have nonlinear character.

The dimensions of transformers should be minimized in order to suppress penetration of signal by mutual capacitive coupling, to facilitate transformers shielding and to decrease the length of tube filled by measured medium.

Short tube with large diameter filled with basic electrolyte (KCl) has small resistance R_{el}, allowing to

measure weak current (high impedances) of electrolyte and limits influence of R_{el} on measured impedance $\mathbf{Z}(f)$. By minimizing the length of tube and increasing its diameter desirable small resistance of tube filled by electrolyte can be achieved even when culture medium is used also as basic electrolyte. Highly concentrated electrolytes are not compatible with cultivation solution of cells thus some kind of separating plugs (Fig. 5a) should be used on the interface tube-cultivation dish. Then agar plugs separating highly concentrated KCl from culture medium might not be necessary.

Fortunately modern high permeability magnetic materials facilitate realization of transformers with minimal dimensions.

Another difficulty arises from requirements for as low as possible concentration of air bubbles in electrolyte filling the tube. Also preparation of and manipulation with cell layer on perforated membrane could be complicated task requiring instrument construction capable of function in the milieu of cell culture CO_2 incubator.

Conclusion

The analysis and experiments performed in frame of this paper confirmed the feasibility of transformer based methods ELM of cell impedance measurement. The topic of the paper is in accordance with tendency for deeper understanding of electrochemical processes in electrolytes which are often "masked" by electrochemical phenomena on interface electrodes-electrolyte and does not allow to observe e.g. inductive character of electrolyte impedance (Bardos *et al.* 2005). Most of present limitations of ELM can be eliminated by proper design and optimization of transformers parameters and signal evaluating processes. Future work will be focused to the solution of above mentioned limitations and problems.

Conflict of Interest
There is no conflict of interest.

Acknowledgements

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