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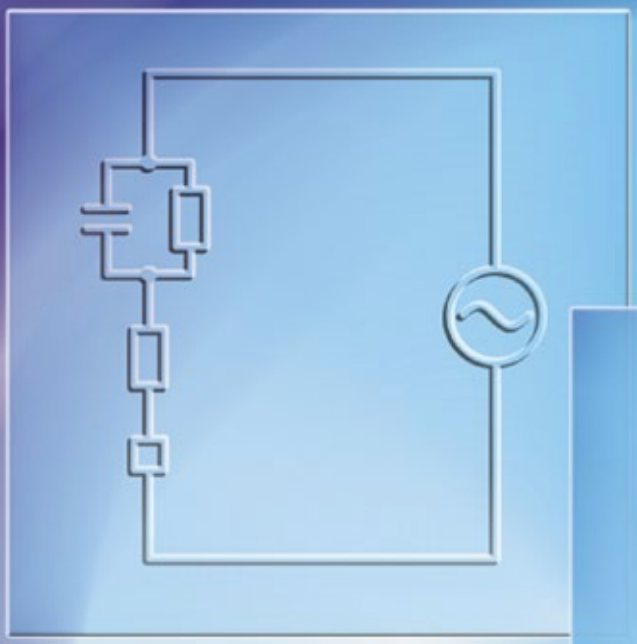

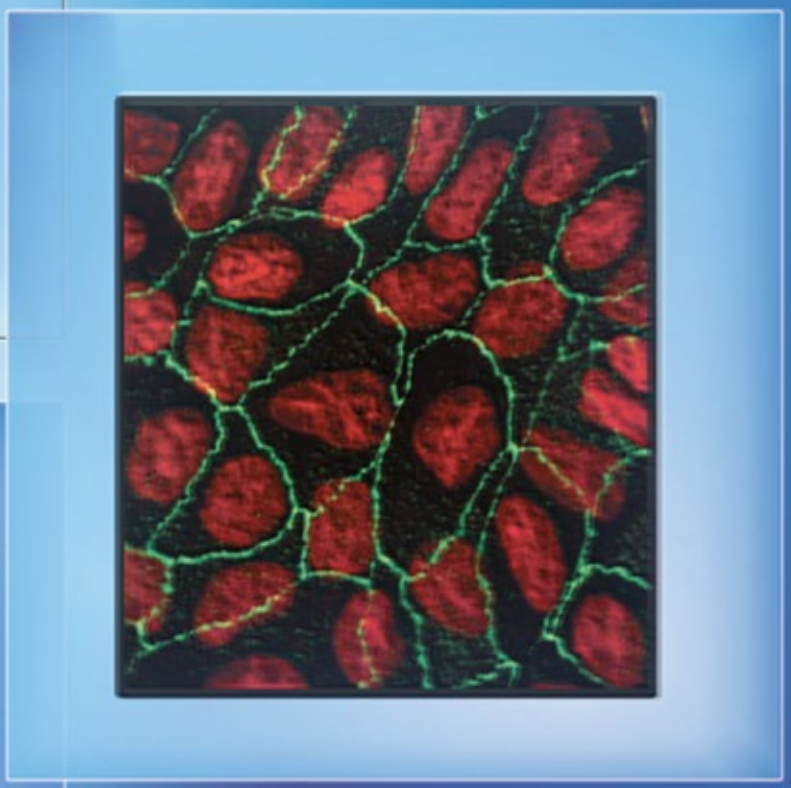

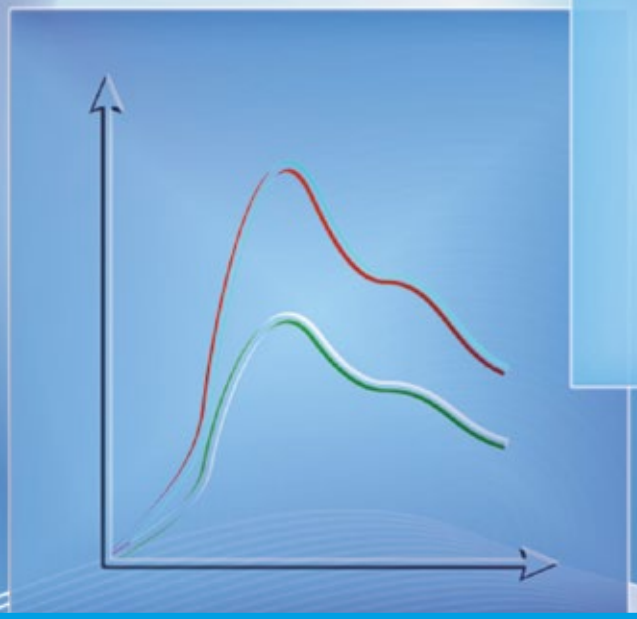

APPLICATION NOTE

Transepithelial Electrical Resistance and Impedance Measurements with ThinCert® Cell Culture Inserts and the cellZscope System

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1/ INTRODUCTION

Barrier-forming cells have become a popular *in vitro* model to study the paracellular transport of substances. Key to such experiments is the cultivation of endothelial or epithelial cells on permeable membrane supports (e.g. ThinCert® cell culture inserts). By culturing on porous membranes, these cells develop their specific features that are also found in intact tissues, such as the formation of dense layers with tight cell-cell junctions and established cellular polarity. An excellent tool to assess the barrier function of reconstructed epithelia and endothelia is the transepithelial or -endothelial resistance measurement (TEER). TEER measurements may be performed on vital cell cultures in a label-free manner. By exceeding certain TEER threshold, researchers can conclude that the cell layer is confluent and the formation of tight cell-cell junctions has occurred **(Fig. 1)**.

Figure 1: Formation of tight junctions between MDCK-II cells on a ThinCert® permeable membrane support.
MDCK-II cells have been cultivated on ThinCert® cell culture inserts. After three days *in vitro* the cells were fixed on the membrane, cross-sectioned and stained with an antibody against ZO1. Immunoreactivity against this protein (arrow heads) indicates the presence of tight cell-cell junctions (nuclei counterstained with DAPI).

The classical way of TEER measurement is the use of simple handheld devices with chopstick-type electrodes. This setup allows the approximate determination of the ohmic resistance of the barrier-forming cell layer **(Fig. 2A)**. Recently, impedance measurements have gained popularity for the characterisation of

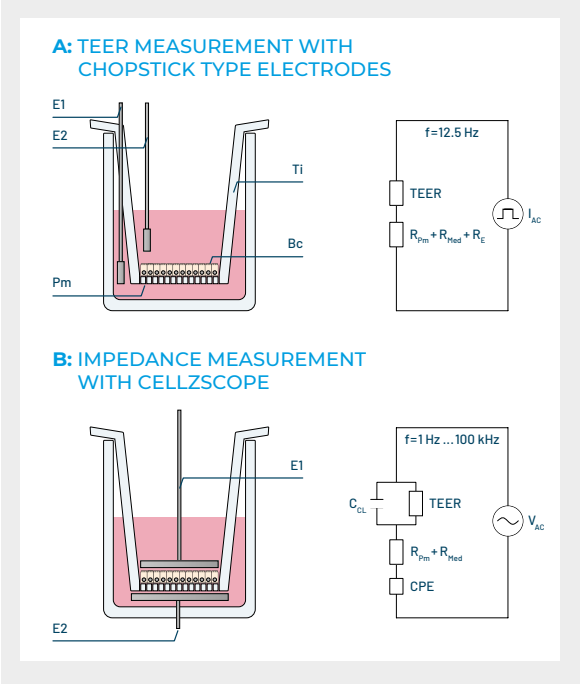

Figure 2: TEER and impedance measurements with chopstick-type electrodes and the cellZscope system.

A: Traditionally, chopstick-type electrodes (E1, E2) are used to measure the electric resistance of barrier-forming cell cultures (Bc) on ThinCert® (Ti) permeable membrane supports (Pm). The ohmic electric resistances of the barrier forming cell layer (transendothelial or transepithelial electric resistance, TEER), the cell culture medium in the upper and lower compartment (R_{Med}), the permeable membrane support (R_{Pm}), as well as the electrode-medium interface (R_E) contribute to the total electric resistance.

B: The cellZscope system records the frequency-dependent impedance of an AC circuit with two wide electrodes (E1, E2) exhibiting a homogeneous electric field across a barrier forming cell culture and a ThinCert® permeable membrane support. The mathematical model describing the impedance of this system includes the capacitance of the cell layer (C_{CL}), a constant phase element (CPE) representing the electrodes, the ohmic electric resistances of the cell layer (TEER), the permeable membrane support (R_{Pm}), and the cell culture media in the upper and lower compartments (R_{Med}). Applying a best fit algorithm the cell layer related parameters TEER and C_{CL} may be calculated from the impedance data set.

epithelial or endothelial cell layers *in vitro*. The electric impedance of a cell layer can be accurately measured by placing one electrode on each side of the membrane and applying a small AC voltage with varying frequency. The cell related parameters, resistance TEER and capacitance C_{CL}, can be deduced from the measured impedance spectra by applying a parametric function and a best fit algorithm **[1]**.

The capacitance C_{CL} may provide additional information about the cell layer's properties – in particular it is indicative of the formation of membrane protrusions, such as microvilli **[2]**.

This technical note illustrates the preeminent suitability of ThinCert® cell culture inserts for the reconstruction of barrier forming tissues *in vitro* and their characterisation by TEER or impedance measurements. Special emphasis is put on the newer cellZscope system from nanoAnalytics which allows real-time impedance recordings, thus providing TEER and C_{CL} of vital cell cultures on ThinCert® membranes.

In principal these electrical parameters have been shown to correlate with a multitude of cellular features, such as intercellular connectivity and cell morphology and may be used to draw conclusions upon cell migration **[3]**, probiotic activity **[4]** and changes in G-protein activity **[5]** and junctional tightness **[6]**.

2/ MATERIAL AND METHODS

2.1/ MATERIAL

Item	Manufacturer	Item No.
Alexa Fluor 488 goat anti-rabbit IgG	Invitrogen Corp.	A-11008
BSA	Sigma-Aldrich	A7979
DRAQ5™	Biostatus Limited	DR50050
EBSS	Invitrogen Corp.	14155
EGTA	Sigma-Aldrich	3777
EMEM	Sigma-Aldrich	M2779
FBS superior	Biochrom AG	S0615
L-Glutamine	Biochrom AG	K2083
MDCK-II	ECACC	621027
Paraformaldehyde	Sigma-Aldrich	P6148
PBS	Biochrom AG	L1825
Pen/Strep	Biochrom AG	A2212
Aqua-Poly/Mount	Polyscience	18606
ThinCert® cell culture inserts, different membrane qualities	Greiner Bio-One GmbH	665640, 665641, 665610, 662630, 662631, 662638
Rabbit anti-ZO-1	Invitrogen Corp.	61-7300
TritonX100	Sigma-Aldrich	T8787

Table 1: Material and suppliers.

2.2/ CELL CULTURE

MDCK-II cells were grown in EMEM medium with Earle's Salts supplemented with 5% FBS, 2 mM L-Glutamine and 1% Pen/Strep. Cells were fed twice a week and split once a week.

For the experiments cells were seeded on 12-well ThinCert® cell culture inserts at a density of 5×10^5 cells $\times$ cm^{-2}. For further cultivation and analysis inserts were placed in the cellZscope system from nanoAnalytics.

2.3/ IMPEDANCE (TEER AND C_{CL}) MEASUREMENTS

The impedance of the cell cultures were measured automatically with the cellZscope while the module holding the inserts remained in the incubator throughout the experiments (except for media exchange etc.) for maintaining physiological conditions.

In case of comparative TEER measurements with the EVOM hand-held device and STX2 chopstick-type electrodes (World Precision Instruments), the insert holding module or multiwell plates (Greiner Bio-One) had to be removed temporarily from the incubator and transferred to a flow bench. The chopstick-type electrodes were inserted in three different positions (120 deg turns) in each well and the readings averaged.

2.4/ IMMUNOCYTOCHEMICAL ANALYSIS

Cell layers were fixed at room temperature for 15 min in 4% Paraformaldehyde in PBS and then washed with PBS twice. For subsequent permeabilisation, cells were exposed to 0.5% TritonX100 in PBS for 10 min, followed by washing with PBS twice, exposure to 3% BSA in PBS for 20 min at room temperature and final washing with PBS three times. Fixed cell layers were incubated for 90 min at 37 °C with the primary antibody Rabbit anti-ZO-1 diluted 1:100 in PBS with 0.5% BSA, followed by washing in PBS three times, exposure to 3% BSA in PBS for 20 min at room temperature and final washing in PBS.

Thereafter, cell layers were stained for 60 min at 37 °C with the secondary antibody Alexa Fluor 488 goat anti-rabbit IgG diluted 1:1000 in PBS with 0.5% BSA, followed by washing in PBS three times. Finally, cell layers were stained for 10 min at room temperature with DRAQ5™ diluted 1:1000 in PBS, followed by washing in PBS three times. Samples were mounted in Aqua-Poly/Mount on cover slides for subsequent analysis with a SPE confocal laser scanning microscope (Leica).

2.5/ EGTA TREATMENT

One cell culture served as a reference and was fixed and stained prior to EGTA treatment. All the other cell culture inserts were washed two times with Ca^{2+}-free EBSS (Gibco) and further cultivated using serum free medium containing 0 (control), 1 or 4 mM EGTA. When TEER was reduced by more than 50%, cell cultures of each experimental condition were fixed and subjected to immunocytochemical analysis. The EGTA containing cell culture medium in the remaining inserts was replaced by normal, Ca^{2+}-containing medium and cell cultures were carried on.

3/ RESULTS

3.1/ REAL TIME MONITORING OF TIGHT JUNCTION FORMATION IN THE CELL LAYER

MDCK-II cells were seeded onto 12-well ThinCert® permeable membrane supports and TEER and capacitance values were monitored over 140 hours using the cellZscope system. The steep increase in TEER observed as early as 5 hours after cell seeding **(Fig. 3)** marks the early onset of tight junction formation in the cell layer.

After 10 hours, TEER reached a maximum, indicating fully established tight junctions. The subsequent decrease in TEER may be ascribed to an increasing number of cells and an increasing total perimeter of cell-cell contacts per surface area. Disturbances of the cell culture conditions such as the exchange of the cell culture medium and serum removal

after 70 hours are well reflected in TEER fluctuations **(dashed line in Fig. 3)**. After 100 hours in culture, TEER values reached a steady plateau between 70 and 120 Ω × cm^2. The onset of tight junction formation also correlated with decreasing C_{CL} values.

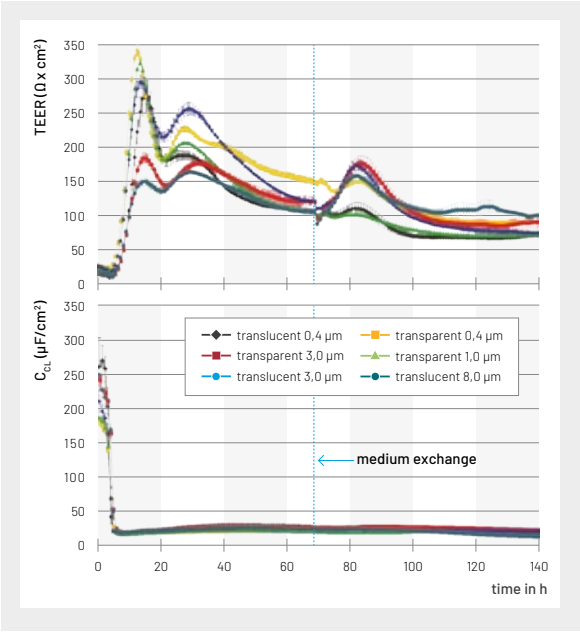

Figure 3: TEER and C_{CL} recordings on MDCK-II cells cultivated on ThinCert® cell culture inserts with different pore sizes.
Increasing transepithelial electric resistance values (TEER) correlate with a decreasing capacitance (C_{CL}) of the cell layer. After 60 hours the TEER values level out at 100 -160 Ω × cm^2, thus indicating confluence and formation of a tight cell layer. The dashed line indicates the time point of medium exchange.

In addition, the electric resistances of the ThinCert® membranes alone have been determined using the cellZscope system. Depending on the membrane characteristics, these values range from 25 to 75 Ω × cm^2 **(Table 2)**.

Item No.	Membrane quality	Pore size in µm	$R_{Pm} + R_{med}$ in Ω	
665640	translucent	0.4	26.7	± 0.8
665641	transparent	0.4	72.3	± 1.3
665610	transparent	1.0	33.3	± 0.5
665630	translucent	3.0	25.6	± 0.9
665631	transparent	3.0	28.4	± 0.2
665638	translucent	8.0	31.8	± 2.0

Table 2: Electric resistance (R_{Pm} + R_{Med}) of different ThinCert® membranes.

3.2/ REAL-TIME MONITORING OF TIGHT JUNCTION BREAK-UP

In another set of experiments, MDCK-II cells were cultivated on ThinCert® cell culture inserts to confluence in order to form tight cell-cell junctions **(Fig. 4B)**. TEER and C_{CL} were continuously recorded using the cellZscope system. After 169.8 hours EGTA was added to the cell cultures, thus leading to a depletion of extracellular Ca^{2+} and thereby to

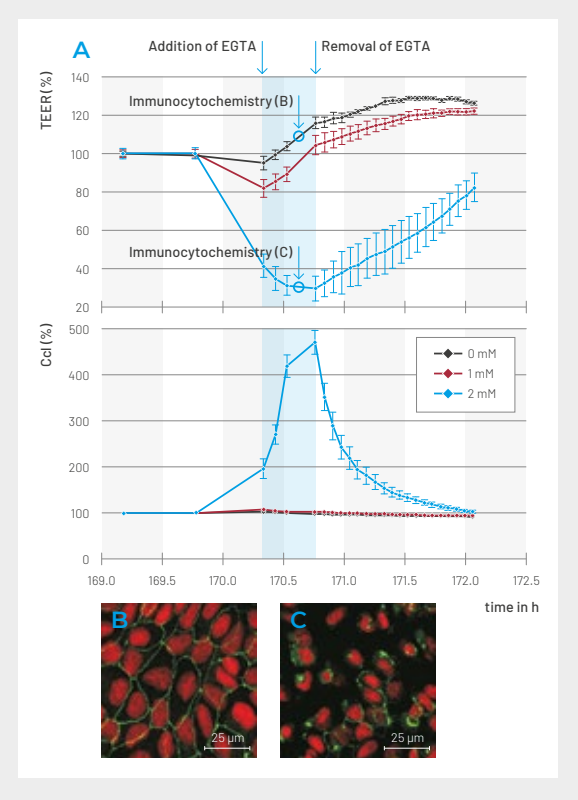

Figure 4: Break-up of tight junctions correlates with decreasing TEER and increasing C_{CL} values.

A: TEER and C_{CL} of a MDCK-II culture have been recorded continuously using the cellZscope system. After 169.8 hours *in vitro*, EGTA has been added to the cell culture medium at varying concentrations (0 mM, 1 mM, 2 mM) and tight junctions have been broken up. Tight junction break-up correlates with a severe drop of the transepithelial electric resistance (TEER) and an increase of the capacitance of the cell layer (C_{CL}).

B, C: Immunocytochemistry using an anti-ZO1 primary antibody confirms the presence of tight junctions in the cell layer (B) and their disappearance after treatment with 2 mM EGTA (C). Nuclei were counterstained with DRAQ5™. Images have been acquired with a confocal microscope. Note that the image in C represents the confocal plane with most remaining ZO1 immunogenicity, thus making nuclei appear smaller than in B.

disruption of the established tight junctions **[7] (Fig. 4C)**. This process was well reflected in decreasing TEER and increasing C_{CL}. After removal of EGTA, tight junctions could be restored – TEER and C_{CL} values changed accordingly **(Fig. 4A)**. The observed correlation of immunocytochemical findings and data deduced from impedance measurements strongly supports the concept of label-free analysis solely based on the electrical properties of the barrier-forming cell culture.

3.3/ COMPARISON OF TEER MEASUREMENTS USING CHOPSTICK-TYPE ELECTRODES AND THE CELLZSCOPE SYSTEM

Chopstick-type electrodes are still the most prevalent tool for TEER measurements on cells cultivated in inserts. For comparison of the two approaches for TEER measurement – the traditional chopstick-type electrodes and the newer cellZscope – an additional set of experiments was performed. MDCK-II cells were cultivated on ThinCert® membrane supports. One set of cell cultures was placed in a first cellZscope system, which remained in the incubator throughout the experiment and was employed to continuously record TEER values. A second cellZscope system was loaded with a similar set of cell cultures. Again, the cellZscope was used to automatically measure TEER. However, at certain time points, the module holding the inserts was temporarily removed from the incubator and comparative TEER measurements were performed with chopstick-type electrodes.

Data points taken with the chopstick-type electrodes give a rough indication of the typical time-dependent development of TEER during cell differentiation and tight junction formation **(Fig. 5)**. TEER values determined with the chopstick-type electrodes correlated with those continuously acquired with

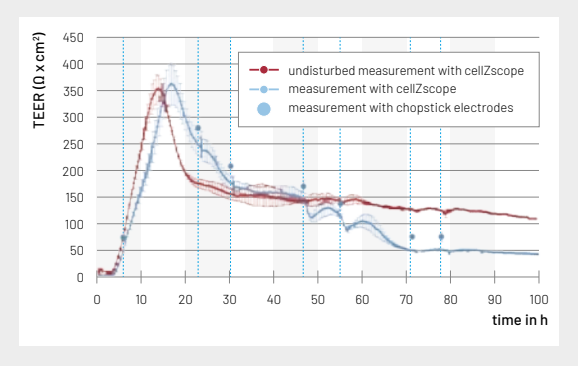

Figure 5: TEER measurements on MDCK-II cells cultivated on ThinCert® cell culture inserts using chopstick-type electrodes and the cellZscope system. MDCK-II cells cultivated on ThinCert® cell culture inserts develop higher TEER values when cultivated in the undisturbed cellZscope system as compared to cultivation in the cellZscope with repeated disturbance by manual measurements with chopstick-type electrodes. TEER values obtained with chopstick-type electrodes are slightly higher, but correlative with values acquired with the cellZscope system.

the cellZscope system, but were found to be slightly higher, which may be explained by inhomogeneous electrical fields between the chopstick-type electrodes, resulting in a systematic overestimation of TEER values. Interestingly, the TEER plateau reached after longterm cultivation was significantly higher in the experimental setup that was not disturbed by manual chopstick measurements outside the incubator. This finding is indicative of the relatively strong interference of this classical measurement approach with cell culture conditions. The more cells are cultivated in a continuous and undisturbed manner and culture conditions are kept constant, the more cellular parameters may reach a stable level. The cellZscope system supports this requirement with its non-invasive operating principle.

4/ CONCLUSION

ThinCert® cell culture inserts allow the restoration of *in vivo*-like growth conditions *in vitro* and hence the reconstruction of epithelia and endothelia from individual cells. The cultivated cell layers develop tissue-specific features, such as tight junctions, cellular polarity and barrier function, and may be used to study

tissue-specific phenomena, such as vectorial transport *in vitro* **[8]**. The transendothelial and -epithelial electric resistance provides a helpful tool to assess the properties of the cultivated cells in a non-invasive manner. Newer devices, such as the cellZscope system from nanoAnalytics, further extend the possibilities of cell-based research with ThinCert® cell culture inserts by enabling continuous real-time analyses, providing additional information about the capacitance of the cultivated cell layers and increasing assay throughput (for comparison of the classical and newer approach see **Table 3**). In the future, impedance-based analyses may be used to non-invasively analyse any cellular property that is correlated to altered cellular conductivity or cell shape. Such putative applications may include: studies on cell migration, alterations in membrane composition, changes of cell-cell contacts as well as the examination of signalling cascades leading to rearrangements of the cytoskeleton and changes of the overall cell shape and morphology.

Instrument	EVOM + STX2 electrodes	cellZscope
Suitability for ThinCert® cell culture inserts	yes	yes
Measured parameter of cell layer	TEER	TEER and capacity (impedance)
Related cellular features	/ conductivity of the cell layer / formation of tight cell-cell contacts	/ conductivity of the cell layer / formation of tight cell-cell contacts / formation of cellular protrusions / changes in cell shape and morphology
Application field	/ determination of barrier function of a cell culture / end point analysis / single or low throughput analysis	/ determination of barrier function of a cell culture / complex correlation of electrical and cell-biological properties of cell cultures / real-time analysis / medium throughput analysis
Acquisition cost	low	medium

Table 3: Comparison of classical and newer ways of analysis of cell cultures based on electrical properties.

5/ LITERATURE

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