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Direct assessment of individual skin barrier components by electrical impedance spectroscopy

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Measuring skin barrier components individually

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Abstract:

Background:

Expression of the tight junction proteins Cldn1 and 4 is altered in skin diseases such as atopic dermatitis, and Cldn1 deficiency affects skin barrier formation. Impedance spectroscopy (IS) has been proven to allow detection of alterations in the skin barrier but is currently unable to separate effects on viable epidermis (VE) and stratum corneum (SC).

Methods:

Effects of siRNA mediated Cldn1 and 4 knockdown in reconstructed human epidermis (RHE) on VE and SC barrier function were investigated with Ussing chamber-based IS. Barrier components were sequentially altered, employing iron oxide nanoparticles and EGTA, to identify their contribution to the impedance spectrum. Resistance changes due to apically applied hyperosmolar electrolyte were used to identify barrier defects non-invasively.

Results:

IS of RHE yielded two relaxation frequencies, representing the barrier properties of the SC (~1000 Hz) and VE (~100 Hz). As proof of concept it was shown, that the Cldn1 knockdown-induced resistance drop arises from the impairment of both SC and VE, indicated by a shift of both relaxation frequencies. Hyperosmolar electrolyte penetration allowed non-invasive detection of Cldn1 knockdown via time-dependent frequency shifts. The absence of Cldn4 knockdown-induced changes revealed the weaknesses of transepithelial electrical resistance analysis.

Conclusion:

In conclusion, the present technique allows to separately measure the barrier properties of SC and VE and further evaluate the Cldn1 and 4 knockdown impact on the skin barrier. As the measurement with agarose embedded electrolyte allowed non-invasive identification of the Cldn1 knockdown, this opens the way to detailed *in vivo* skin barrier assessment.

Keywords:

Impedance Spectroscopy, Reconstructed Human Epidermis, Skin Barrier, Tight Junctions, Transepithelial Electrical Resistance

1 Introduction

The healthy epidermis provides a tight barrier against the intrusion of pathogens, toxins and allergens, but also against the unregulated loss of water. Whereas the stratum corneum (SC) has long been identified as an important component of the skin barrier¹⁻⁴, it became clear during the past two decades that the tight junction also plays a role in skin barrier formation.^{5,6} Several skin diseases, such as atopic dermatitis, contact dermatitis, psoriasis, various forms of inflammation and different types of skin cancer are associated with impaired skin barrier function.⁷⁻⁹ Especially in allergy-associated diseases, this facilitates the uptake of allergens and perpetuates a vicious circle. Disease-related alterations of SC components (e.g. of Filaggrin or ceramides) are well established.^{7,10,11} However, many of these conditions are also accompanied by alterations in the expression of tight junction proteins in the viable epidermis (VE), especially the *stratum granulosum*. Particularly, a down-regulation of claudin-1 and -4 (Cldn1; Cldn4;^{6,12,13} for review see^{14,15}) was described. *In vitro*, mouse model and human skin studies demonstrated a correlation between the degree of Cldn1 down-regulation and the penetration of macromolecules of different sizes.^{13,16} On the other hand, temporal, controlled opening of skin barrier may be desirable for dermal drug delivery.¹⁷

The development of efficient treatments for barrier-linked skin diseases and drug delivery optimization requires full understanding of the contribution of each component towards skin barrier function. The overall skin barrier function is often characterized via trans-epidermal water loss¹⁸, transepithelial electrical resistance¹⁹, skin permeation of locally applied drugs^{20,21} and also impedance spectroscopy.^{22,23} However, these techniques lack the ability of differentiating between barrier properties of the SC and viable epidermis (VE) including tight junctions. Removing the SC by tape stripping²⁴ or affecting it via electroporation²⁵ can evaluate its contribution to the barrier but may also damage the VE.

Impedance spectroscopy is a common method for characterizing the barrier function of monolayered epithelia.²⁶ Measuring transepithelial impedance over a range of frequencies gives rise to a curve which contains relaxation frequency peaks. The peak position and height are determined by distinct resistance and capacitance values of the specific cell type. Impedance spectra can be interpreted by analysing an equivalent circuit model, containing electrical components that represent the sample's response to the applied alternating current. Six basic components can approximate a monolayered epithelium: resistance and capacitance of apical and basal side respectively, resistance of the tight junction and sub-epithelial resistance (Figure 1 A).²⁷

The present study aims to adapt the equivalent circuit model for use in stratified epithelia and thus allow to distinguish between SC and VE barrier components. As a proof of principle the technique is used to distinguish between the effects of Cldn1 and 4 knockdown. We used reconstructed human epidermis (RHE), to have an easily adjustable model of the skin, that allows to univocally identify the skin barrier components. Our results promise the prospect of future adaptation of the technology for *in vivo* applications.

2 Materials and Methods

2.1 Materials

Standard bath solution contained (113.6 mM NaCl, 21 mM NaHCO₃, 2.4 mM Na₂HPO₄, 0.6 mM NaH₂PO₄, 5.4 mM KCl, 1.2 mM CaCl₂, 1.2 mM MgCl₂, 10 mM Glucose, equilibrated with 5% CO₂/ 95% O₂ to obtain a pH value of 7.4).

Mouse ear control treatment was performed with phosphate buffered solution (PBS (+/+)) with calcium and magnesium (DPBS, Gibco™ – ThermoFisher Scientific). Acetone (JT Baker - Fisher Scientific GmbH) was used to disrupt the skin barrier of mouse ears.

To disrupt the tight junction strands, a 0.1 M EGTA stock solution was prepared (EGTA (Merck, Darmstadt, Germany) in ddH₂O titrated with 5 M NaOH to pH 7.0). To modulate SC capacitance, iron oxide nanoparticles (5 nm diameter, oleic acid functionalization, suspended in 10 mL toluene, Cytodiagnostics), Decamethylcyclopentasiloxane (TCI Chemicals, organic solvent commonly used in make-up removers) and a Neodymium magnet (10 mm diameter, 3 mm thickness, Webcraft GmbH) were used to coat the SC with the nanoparticles.

For cultivation of primary keratinocytes, human tissue was gained during surgical removal of foreskin anonymously from male donors (younger than 5 years; approved by the ethics committee of the Ärztekammer Hamburg, WF-61/12) gained during surgical removal of foreskin. All investigations were conducted according to the principles expressed in the Declaration of Helsinki.

siRNAs for human Cldn1 Hs_CLDN1_8: SI04279114, Cldn4 Hs CLDN4_7 (SI03064418) and AllStars negative control (siRNA ctrl; SI03650318) were purchased from QIAGEN (Hilden, Germany). Their effectiveness has been previously established.^{13,28}

2.2 Reconstructed Human Epidermis

Reconstructed human epidermis (RHE) was built essentially as described before.¹⁴ 2 x 10⁵ keratinocytes were seeded onto cell culture inserts (12 mm diameter, area 0.6 cm², 400 nm pore size, Merck Millipore, Tullagreen, Ireland) in 500 µl EpiLife Medium (Life Technologies, Carlsbad CA) including 1.5 mM CaCl₂. The basal compartment was filled with 2.5 ml EpiLife medium. Cells were lifted to the air-liquid interface after 30 h and medium in the basal part of the well was exchanged with 1.5 mL of EpiLife medium containing 1.5 mM CaCl₂, 92 µg/mL ascorbic acid (Merck, Darmstadt, Germany) and 10 ng/mL recombinant human keratinocyte growth factor (R&D Systems, Wiesbaden-Nordenstadt, Germany). Medium was changed every second day. Experiments were performed at day 4 and day 8 after air-liquid-interface (see also ^{13,14}). For characterization of the RHE-system concerning morphology, proliferation, differentiation and barrier function see ¹³. According to those findings, model batches were only used for evaluation if the SC surface was dry and TER of the siRNA untreated models was >600 Ohm*cm².

2.3 siRNA experiments

Confluent cells in passage 3 were trypsinized, resuspended in EpiLife medium with 1.5 mM CaCl_2 and subsequently transfected by using HiPerFect Transfection reagent (Qiagen, Hilden, Germany) at a cell density of 4×10^5 /mL with a final siRNA concentration of 80 nM. Transfected cells were directly used to build RHEs. For evaluation of knock-down efficiency see supporting information chapter 7.

2.4 Mice

All experiments were performed in accordance with the German law on animal protection and approved by the Landesamt für Gesundheit und Soziales (LAGeSo) Berlin (T0256/16 and TCH 0012/20). Adult male and female mice (22 – 34 weeks old, C57Bl/6NCrl background, see supporting information chapter 6) were sacrificed and organs were harvested for use in an unrelated experiment. The carcasses were then immediately used for application of impedance spectroscopy on ear skin as described below.

2.5 Manipulation of electrical parameters

To identify the VE barrier component of the RHEs, 300 μL of the 0.1 M EGTA stock solution was applied to the basal (orapical) side of the Ussing chamber (final concentration 3 mM) to disassemble TJ strands and thus reduce the VE resistance.²⁹

To coat the SC with iron oxide nanoparticles, the cell culture insert was removed from the Ussing chamber and placed in a cell culture incubator (37°C, 5% CO_2 , 85% humidity). Nanoparticles resuspended in Decamethylcyclopentasiloxane were added to the apical side (Figure S1) and a magnet was placed under the insert for 10 minutes to enrich the iron oxide nanoparticles at the SC. Subsequently, the inserts were submerged in standard bath solution, causing the iron oxide nanoparticles solution to rise, as the Decamethylcyclopentasiloxane density is lower than that of water. Thus, only nanoparticles that attached to the SC remained.

Mouse ear skin was manipulated by wiping both sides of the ear pinnae 10 times with a cotton swab, soaked with the treatment solution. As a control PBS(+ / +) was used while Acetone and hyperosmolar 0.5 M KCl solution were supposed to cause changes in the barrier properties. Acetone as an amphiphilic compound was expected to destabilize the structures in the VE if capable of penetrating through the SC.

2.6 Ussing chamber-based Impedance Spectroscopy

A standard Ussing chamber-based setup was used as described in Figure 2 A.²⁶ The electric setup consists of two current electrodes (Working Electrode, Counter Electrode) and two reference electrodes (RE1, RE2).

For the alternating current (AC) impedance measurement, a 25 μA sinusoidal current was applied through the current electrodes while the reference electrodes recorded the resulting transepithelial voltage drop and phase

angleusing a Newtons 4th Ltd PSM1700 phase sensitive multimeter. Each spectrum consisted of 48 data points ranging from 1.3 Hz to 65 kHz. The calculated impedance values, Z , for individual frequency data points are complex numbers that can be either expressed as real ($\text{Re}(Z)$) and imaginary ($\text{Im}(Z)$) part or magnitude ($|Z|$) and phase shift (ϕ).

RHE models mounted in the Ussing chamber divided the chamber into a basal and an apical compartment. Both compartments were filled with 10 mL standard bath solution. Continuous perfusion was achieved via bubble lift. Once the RHE models had reached stable transepithelial resistance values, impedance spectra recordings were started.

2.7 “static” Ussing chamber technique

The “static” Ussing chamber aimed to transfer electric characteristics of a standard Ussing chamber to a solid electrode as is necessary for *in vivo* implementation. Therefore standard bath solution was embedded in a 1% agarose gel in a 1.5 ml Eppendorf® tube. “Static” in this context refers to the fact, that there is no perfusion of the agarose-embedded electrolyte. Electrodes were attached as depicted in Figure 2 B and C. The bottom of the Eppendorf tube was cut so that the exposed agar could be directly placed onto the air-exposed SC, while the basal side of the RHE was placed in bath solution. Thus, the RHE models could be measured under air-liquid interface conditions without being damaged or removed from their culture well (Figure 2B).

In addition to the steady state measurements, time series of six impedance spectra (one spectrum/min) were recorded to investigate characteristic behaviour due to hydration of the SC.³⁰ To enhance the hydration induced resistance drop, hyperosmolar 0.5 M KCl solution embedded in a 1% agarose gel was used for the apical side of the chamber.

One-sided “static” Ussing chamber used the same principle as the “static” measurement, but both sides of the chamber were attached to the SC (Figure 2D and E). This mimics the measurement under *in vivo* conditions where the basal side of the skin is not accessible. For a detailed description of the “static” chambers see Figure S2.

As a proof of principle for *ex/in vivo* application, mouse ear skin was measured with a special aligned setup based on the static Ussing chamber as shown in the supplement (Figure S7 C,D). In the following this setup is denoted as “static mouse”. All impedance values were corrected by the measurement area of the corresponding chamber, which were 0.6 cm² for standard, 0.17 cm² for static and 0.07 cm² for one sided static and static mouse Ussing chamber measurement. In the case of one sided static RHE and static mouse skin measurements, the values were additionally corrected by a factor of 0.5 as the current passes the skin layers twice (Figure 2 E).

2.8 Impedance Spectra Evaluation

The general appearance and the interpretation principles of impedance spectra are explained in the supporting information chapter 1 (Figure S2). The spectra were fitted with appropriate equivalent circuit models (Figure 1 B, supporting information chapter 1, Figure S2) using the EIS Analyzer software.³¹

To assess the effect of substances manipulating the electrical properties of the tissue, pairwise analysis was performed. The value diff_R was calculated, describing the relative resistance change after treatment of the same sample:

$$\text{diff}_R = \frac{R_0 - R_{\text{treatment}}}{R_0}$$

Analogous calculations were carried out for capacitance changes. As the measured epithelia did not yield ideal capacitance behaviour the extraction of the capacitance value was performed with the help of the so called constant phase element as described in the supporting formation chapter 1 (Figure S2).

If equivalent circuit fitting was not sufficiently accurate, quantitative analysis of the spectra was performed by fitting the relaxation frequency peaks in the $-\text{Im}(Z)$ vs. $\log_{10}(\text{frequency})$ graph via Origin 2020 Peak Fitting tool (Figure S3). Here, only the position of the relaxation frequency f_i was taken into account for evaluation and not the peak amplitude, as the relaxation frequency is insensitive to changes in the electrode size and current pathway as long as the cells maintain their characteristics (supporting information chapter 1 and 4). The impact of 0.5 M KCl penetration on the spectrum was analysed by calculating the shift of the relaxation frequencies f_i before f_{i_0} and after $f_{i_{\text{KCl}}}$ KCl penetration:

$$\Delta f_i = \log_{10}(f_{i_{\text{KCl}}}) - \log_{10}(f_{i_0})$$

The transepithelial electrical resistance (TER) was evaluated by TER_{norm} , which is given by normalizing individual TER values by the maximum TER value of the RHE model batch.

2.9 Statistical Analysis

Statistical analysis was performed with GraphPad Prism 8.3.0 software. Data are expressed as a mean $\pm$ standard error of the mean (SEM). The sample size is annotated to each bar in the graphs. Unpaired student's t-test was performed to compare values of different conditions. When multiple testing was performed adjusted p-values were determined by Tukey's range test. The corresponding p-values are listed in the figure annotations.

3 Results

Many skin diseases comprise barrier defects. However, to which extent these defects concern the SC or the VE is often not clear. We used RHE as a model system for human skin which is easy to (genetically) manipulate and can also be prepared using cells from patients with skin diseases.³² Currently, there is no convincing equivalent circuit model for skin that describes all barrier components and allows to choose the optimum frequency for measuring the desired barrier component. To draft such an equivalent circuit model (Figure 1 B) potential barrier components were sequentially manipulated.

3.1 Standard Ussing chamber technique

Impedance spectra of RHE measured in standard Ussing chambers yielded two predominant relaxation frequencies, in the range of 10 Hz to 100 Hz (f_{low}) and in the range of 500 Hz to 10000 Hz (f_{high}) (Figure 3 A). The absolute values of these frequencies remained unchanged over time (Figure 3 B). Comparison of the fitting parameters resistance and capacitance over time (day 4 and day 8 after Ca^{2+} switch) showed an increase for the resistance value of f_{high} while its capacitance values dropped. There were no significant changes for f_{low} (Figure 3 C, D).

Figure 3 A also demonstrates, that basally applied EGTA mainly affected f_{low} while f_{high} remained unchanged. Apically applied EGTA impacted f_{low} only slightly and f_{high} not at all (Figure 3 A). Consistently, basal EGTA treatment caused the f_{low} resistance calculated by equivalent circuit fitting to drop significantly more than the f_{high} resistance, whereas there was no significant change for apical treatment (Figure 3E). For basal treatment no significant difference in capacitance change could be observed (Figure 3F). Furthermore, the relaxation frequency f_{low} was significantly higher after basal EGTA treatment.

Iron oxide nanoparticle deposition onto the SC affected predominantly f_{high} and in some cases f_{low} (Figure 4 A), causing a significantly higher decrease of the capacitance value and increase of the resistance and relaxation frequency value for f_{high} compared with f_{low} (Figure 4B, C), leading to an overall increase of the frequency value for f_{high} (Figure 4D). Basal EGTA post-treatment only showed significant decrease of the relative resistance $diff_R$ (Figure 4E).

The absence of a significant increase of the f_{low} frequency value upon EGTA post-treatment and details on the effect of nanoparticle deposition are discussed in the supporting information.

In summary, EGTA acts on f_{low} when applied from basal side and predominantly affected its resistance and frequency value. The frequency shift of f_{low} is predominantly caused by the resistance drop. Iron oxide nanoparticle deposition on the SC (from apical side) mainly affected f_{high} , while f_{low} was also affected in some cases. Here the frequency shift of f_{high} is dominated by the capacitance decrease. Throughout RHE cultivation the capacitance dropped for f_{high} while its resistance increases. Together, this indicates that f_{low} represents the VE, f_{high} the SC.

3.2 “static” Ussing chamber technique

For a future *in vivo* implementation, the “static” Ussing chamber technique was applied to RHE models and mouse ear pinnae.

The relaxation frequencies f_{low} and f_{high} were also detectable in static RHE, static one side RHE and static mouse skin (Figure 5A), with the latter yielding a lower relaxation frequency distance (exemplary spectra and fitting details see supporting information chapter 5). The peak heights $-Im(Z)$ of f_{low} and f_{high} were significantly lower for the standard Ussing chamber measurement, whereas the $-Im(Z(f_{high}))$ was much higher for the static mouse measurement (Figure 5B).

Time resolved measurement of SC hydration, used as an alternative to the nanoparticle based SC assignment in static RHE, showed a change in f_{high} , while f_{low} remained unchanged (Figure 5C). Alterations were mainly due to a decrease in R_{SC} (Figure 5D), with no change in the frequency. Subsequent basal application of EGTA was used to assure correct assignment of the relaxation frequencies for the static Ussing chamber setup (Figure 5C). Only f_{low} was affected by basal EGTA treatment with the resistance decreased (Figure 5D) and frequency value increased (Figure 5E). Apical penetration of 0.5 M KCl, used to enhance the effect of SC hydration, resulted in a resistance change for both relaxation frequencies (Figure 5D) while a significant increase in the frequency value was only observed for f_{high} (Figure 5E).

When mouse ear skin was measured with the static Ussing chamber, f_{low} was only affected by acetone treatment, with its frequency value being significantly increased. For f_{high} both acetone and 0.5 M KCl treatment increased the frequency value significantly, while PBS showed no effect (Figure 5F).

In summary, the frequencies found in the standard Ussing chamber could also be assigned with the static technique via SC hydration and basal EGTA treatment, i.e. f_{low} represents viable epidermis and f_{high} SC. *Ex vivo* testing with mouse ear skin showed, that the relaxation frequencies found in RHE are also present in mouse ear skin and can be affected with acetone and 0.5 M KCl.

3.3 Cldn knockdown evaluation

For standard Ussing chamber, spectra of RHE treated with different siRNAs and of untreated controls, normalized transepithelial resistance and relaxation frequency values were evaluated. Transepithelial resistance was significantly decreased for Cldn1 and Cldn4 knockdown compared to the control groups (Figure 6 A). For Cldn1 knockdown, significant increase of the frequency values was present for f_{low} (representing VE) and f_{high} (representing SC), whereas Cldn4 knockdown only shows slight changes in f_{low} (Figure 6 B).

When using the static Ussing chamber technique, both the impact of hydration on the RHE model and the absolute values of the relaxation frequencies were evaluated (Figure 6 A-D). In these experiments, hyperosmolar 0.5 M KCl solution was used from apical side, to enhance the impact of hydration on the resistance. Comparing the first and last impedance spectra of the time series, the impact of KCl penetration differed between Cldn1 knockdown and untreated controls (Figure 6 C, D). In untreated controls, only f_{high} was affected, whereas in Cldn1 knockdown f_{high} was only slightly affected and f_{low} highly. The ratio of the shift of f_{low} and f_{high} due to KCl penetration showed a significant difference for the Cldn1 knockdown (Figure 6 E). Furthermore, there was a highly significant difference for the absolute values of the relaxation frequencies for Cldn1 knockdown in comparison to the other siRNA treatments (Figure 6 F). This implicates that both barrier components have lower resistance and thus lower barrier integrity. In contrast to the transepithelial resistance evaluation in standard Ussing chamber, static Ussing chamber measurements showed no characteristic changes for the Cldn4 knockdown. This is further evaluated in supporting information chapter 4.

4 Discussion

The main goal of the present study was to draft an electrical model for the partial resistances of RHE and to evaluate the contribution of SC and VE to overall skin barrier function.

4.1 Standard Ussing chamber

Standard Ussing chamber impedance spectra of stratified epithelia revealed two relaxation frequencies, f_{low} and f_{high} .

Basally applied EGTA allowed to assign f_{low} to the VE, as it only acts in viable tissue via tight junction strand disassembly³³ and ion channel activity (e.g. TRP channel).^{34,35} The EGTA induced frequency increase was attributed to a resistance drop in the VE and was used as a measure for barrier impairment.

Apical nanoparticle deposition not only added an additional layer on top of the SC but also altered its electric properties (supporting information chapter 2) thereby predominantly decreasing the capacitance, increasing the resistance and solely affecting the relaxation frequency value of f_{high} . Thus, f_{high} could be assigned to the SC.

Finally, the development of capacitance and resistance values over time supports the assignment of the relaxation frequencies. Decrease in the SC capacitance, increase in resistance and unchanged relaxation frequency $1/(R_{sc} \cdot C_{sc})$ over time were due to the increasing thickness of the SC.¹³ Interestingly, no resistance increase or capacitance decrease could be observed for the VE over time. This is only possible if this relaxation frequency is predominantly represented by a barrier component with a fixed number of cells that do not change their properties over time and supports the finding that the main barrier forming tight junctions are found in a cell monolayer in the stratum granulosum.^{3,4,36,37}

The absolute values of the relaxation frequencies for SC and VE did not change over time and thus are characteristic for the two barrier components identified here. The equivalent circuit analysis explained the origin of the frequency shift due to EGTA and nanoparticle application. For further analysis the frequency shift could be used, as it is insensitive to changes in the electrode area.

4.2 "static" Ussing chamber

The standard Ussing chamber proved to be disadvantageous for applications on RHE (supporting information chapter 4, Figure S9). Yet, the assignment of the relaxation frequencies to the cellular barrier components VE and SC established with the standard Ussing chamber were validated for the static Ussing chamber via basal EGTA treatment and time resolved hydration observation of the SC. EGTA decreased f_{low} resistance and frequency values while hydration solely affected the f_{high} resistance. Both components could also be identified in *ex vivo* mouse skin (Figure 5F, Figure S10 E).

Using the static Ussing chamber technique in the one sided configuration in RHE and for *ex vivo* mouse ear skin measurement maintained relaxation frequencies discovered with the standard Ussing chamber, thereby demonstrating the suitability for *in/ex vivo* applications. Higher impedance values for the SC of mouse ear skin are

expected as *in/ex vivo* skin barrier is tighter than RHE barrier.³⁸ The differences in the frequency positions could be caused by an alternative current pathway such as sweat glands and hair follicles in the mouse ears acting as a shunt.

The ability to individually measure alterations in both SC and VE could be demonstrated under *ex vivo* conditions via acetone treatment which is a model for irritant dermatitis³⁹ and only SC via 0.5 M KCl treatment. The method thus allows to reassess previous *in vivo* approaches from the literature that were restricted to impedance measurements at 1 kHz.²³

In summary, the “static” technique has clear advantages over the commonly used techniques for transepithelial resistance measurement. Most importantly the “static” technique delivers separate information on the state of the VE and SC. The sample does not need to be submerged, so that the SC remains closer to its physiological state. The technique is independent of the insert used for RHE model cultivation as the area of current flow is limited by the electrode’s geometry. When the electrolyte is prepared under sterile conditions, the technique can be used for sterile measurements.

4.3 Cldn knockdown evaluation

Standard Ussing chamber measurements revealed significantly lower transepithelial resistance for Cldn1 and 4 knockdown, indicating a direct effect of these claudins on epidermal barrier function as has been shown previously *in vivo* and *in vitro* for Cldn1^{3,13,16} and in 2D-cultures for Cldn4.^{28,39} Furthermore, Cldn1 knockdown resulted in shifts of both relaxation frequencies, f_{low} (VE) and f_{high} (SC) to higher values while for Cldn4 knockdown only f_{low} (VE) is slightly altered.

The static Ussing chamber eliminated shunt contributions and verified characteristic frequency differences for the Cldn1 knockdown, but proved that Cldn4 knockdown did not result in any frequency changes (supporting information chapter 4 and Figure S11). Of note, no obvious skin barrier abnormalities were described in Cldn4 knock-out mice.^{40,41}

The shift of both relaxation frequencies to higher values and the lowered overall resistance indicates lower barrier integrity both in the SC and the VE caused by the absence of Cldn1, confirming previous studies on Cldn1 deficient mice.¹²

When considering implementing this technique for *in vivo* measurements, however, it will be problematic to rely on absolute resistance or relaxation frequency values, as these absolute values will vary for different individuals. This difficulty may be overcome by evaluating the time dependent development of the spectra during penetration of hyperosmolar electrolytes. Under these conditions we could identify Cldn1 knockdown induced barrier defects in RHE by analysing the relaxation frequency shift without the need of a reference resistance or frequency value.

5 Conclusion

The barrier properties of SC and VE of an epidermal stratified epithelium can be separately measured and individually characterized.

Cldn1 induced barrier defects can be identified by analysing the impact of hyperosmolar electrolyte penetration through the RHE model on the impedance spectrum over time. This demonstrates that tight junctions play an essential role in VE and thus in epidermal barrier function and opens up the possibility to exploit the present technique for a refinement of *in vivo* diagnostic tools for skin diseases with altered skin barrier such as allergic and irritant contact dermatitis.^{15,42}

This may help to understand the contribution of barrier defects in SC and VE to different skin diseases and their endotypes in patients but also in various mouse models of skin diseases.^{43, 44}

6 References

1. Proksch E, Brandner JM, Jensen J-M. The skin: an indispensable barrier. *Experimental Dermatology*. 2008;17(12):1063-1072.
2. Lee AY. Molecular Mechanism of Epidermal Barrier Dysfunction as Primary Abnormalities. *International journal of molecular sciences*. 2020;21(4).
3. Furuse M, Hata M, Furuse K, et al. Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. *J Cell Biol*. 2002;156(6):1099-1111.
4. Tunggal JA, Helfrich I, Schmitz A, et al. E-cadherin is essential for in vivo epidermal barrier function by regulating tight junctions. *EMBO J*. 2005;24(6):1146-1156.
5. Bäsler K, Bergmann S, Heisig M, Naegel A, Zorn-Kruppa M, Brandner JM. The role of tight junctions in skin barrier function and dermal absorption. *Journal of Controlled Release*. 2016;242:105-118.
6. Yokouchi M, Kubo A. Maintenance of tight junction barrier integrity in cell turnover and skin diseases. *Experimental Dermatology*. 2018;27(8):876-883.
7. Weidinger S, Novak N. Atopic dermatitis. *Lancet (London, England)*. 2016;387(10023):1109-1122.
8. Proksch E, Dähnhardt D, Dähnhardt-Pfeiffer S, Fölster-Holst R. [Epidermal barrier disorders in dermatoses]. *Der Hautarzt; Zeitschrift für Dermatologie, Venerologie, und verwandte Gebiete*. 2016;67(11):907-921.
9. Schmuth M, Blunder S, Dubrac S, Gruber R, Moosbrugger-Martinez V. Epidermal barrier in hereditary ichthyoses, atopic dermatitis, and psoriasis. *Journal der Deutschen Dermatologischen Gesellschaft = Journal of the German Society of Dermatology : JDDG*. 2015;13(11):1119-1123.
10. Kezic S, Novak N, Jakasa I, et al. Skin barrier in atopic dermatitis. *Frontiers in bioscience (Landmark edition)*. 2014;19:542-556.
11. Koppes SA, Engebretsen KA, Agner T, et al. Current knowledge on biomarkers for contact sensitization and allergic contact dermatitis. *Contact dermatitis*. 2017;77(1):1-16.
12. Sugawara T, Iwamoto N, Akashi M, et al. Tight junction dysfunction in the stratum granulosum leads to aberrant stratum corneum barrier function in claudin-1-deficient mice. *Journal of Dermatological Science*. 2013;70(1):12-18.
13. Bergmann S, von Buenau B, Vidal-y-Sy S, et al. Claudin-1 decrease impacts epidermal barrier function in atopic dermatitis lesions dose-dependently. *Scientific Reports*. 2020;10(1):2024.
14. Basler K, Brandner JM. Tight junctions in skin inflammation. *Pflugers Arch*. 2017;469(1):3-14.
15. Brandner JM, Zorn-Kruppa M, Yoshida T, Moll I, Beck LA, De Benedetto A. Epidermal tight junctions in health and disease. *Tissue barriers*. 2015;3(1-2):e974451.
16. Tokumasu R, Yamaga K, Yamazaki Y, et al. Dose-dependent role of claudin-1 in vivo in orchestrating features of atopic dermatitis. *Proceedings of the National Academy of Sciences of the United States of America*. 2016;113(28):E4061.
17. Benson HAE, Grice JE, Mohammed Y, Namjoshi S, Roberts MS. Topical and Transdermal Drug Delivery: From Simple Potions to Smart Technologies. *Current drug delivery*. 2019;16(5):444-460.
18. Alexander H, Brown S, Danby S, Flohr C. Research Techniques Made Simple: Transepidermal Water Loss Measurement as a Research Tool. *The Journal of investigative dermatology*. 2018;138(11):2295-2300.e2291.
19. Kiewewetter L, Littau L, Walles H, Boccaccini AR, Groeber-Becker F. Reepithelialization in focus: Non-invasive monitoring of epidermal wound healing in vitro. *Biosensors & bioelectronics*. 2019;142:111555.
20. Pradal J, Vallet CM, Frappin G, Bariguan F, Lombardi MS. Importance of the formulation in the skin delivery of topical diclofenac: not all topical diclofenac formulations are the same. *Journal of pain research*. 2019;12:1149-1154.

21. Shan J, Oshima T, Chen X, Fukui H, Watari J, Miwa H. Trypsin impaired epithelial barrier function and induced IL-8 secretion through basolateral PAR-2: a lesson from a stratified squamous epithelial model. *American Journal of Physiology*. 2012;303(10):G1105-G1112.
22. Ollmar S, Grant S. Nevisense: improving the accuracy of diagnosing melanoma. *Melanoma Manag*. 2016;3(2):93-96.
23. Rinaldi AO, Morita H, Wawrzyniak P, et al. Direct assessment of skin epithelial barrier by electrical impedance spectroscopy. *Allergy*. 2019;74(10):1934-1944.
24. Bashir SJ, Chew A-L, Anigbogu A, Dreher F, Maibach HI. Physical and physiological effects of stratum corneum tape stripping. *Skin Research and Technology*. 2001;7(1):40-48.
25. Lu F, Wang C, Zhao R, et al. Review of Stratum Corneum Impedance Measurement in Non-Invasive Penetration Application. *Biosensors*. 2018;8(2).
26. Gitter AH, Schulzke J-D, Sorgenfrei D, Fromm M. Ussing chamber for high-frequency transmural impedance analysis of epithelial tissues. *Journal of Biochemical and Biophysical Methods*. 1997;35(2):81-88.
27. Günzel D, Zakrzewski SS, Schmid T, et al. From TER to trans- and paracellular resistance: lessons from impedance spectroscopy. *Annals of the New York Academy of Sciences*. 2012;1257(1):142-151.
28. Kirschner N, Rosenthal R, Furuse M, Moll I, Fromm M, Brandner JM. Contribution of tight junction proteins to ion, macromolecule, and water barrier in keratinocytes. *The Journal of investigative dermatology*. 2013;133(5):1161-1169.
29. Tria S, Jimison LH, Hama A, Bongo M, Owens RM. Sensing of EGTA Mediated Barrier Tissue Disruption with an Organic Transistor. *Biosensors*. 2013;3(1):44-57.
30. Tagami H, Ohi M, Iwatsuki K, Kanamaru Y, Yamada M, Ichijo B. Evaluation of the skin surface hydration in vivo by electrical measurement. *The Journal of investigative dermatology*. 1980;75(6):500-507.
31. Johnson P. Progress in chemometrics research, A. L. Pomerantsev (ed.), Nova Science, New York, 2005, ISBN 1-59454-257-0, vii +325, pp. *Journal of Chemometrics*. 2005;19(4):266-267.
32. Niehues H, Bouwstra JA, El Ghalbzouri A, Brandner JM, Zeeuwen P, van den Bogaard EH. 3D skin models for 3R research: The potential of 3D reconstructed skin models to study skin barrier function. *Exp Dermatol*. 2018;27(5):501-511.
33. Rothen-Rutishauser B, Riesen FK, Braun A, Günthert M, Wunderli-Allenspach H. Dynamics of tight and adherens junctions under EGTA treatment. *The Journal of membrane biology*. 2002;188(2):151-162.
34. Toth BI, Olah A, Szollosi AG, Biro T. TRP channels in the skin. *Br J Pharmacol*. 2014;171(10):2568-2581.
35. Bodding M, Wissenbach U, Flockerzi V. The recombinant human TRPV6 channel functions as Ca²⁺ sensor in human embryonic kidney and rat basophilic leukemia cells. *J Biol Chem*. 2002;277(39):36656-36664.
36. Kirschner N, Houdek P, Fromm M, Moll I, Brandner JM. Tight junctions form a barrier in human epidermis. *European Journal of Cell Biology*. 2010;89(11):839-842.
37. Yoshida K, Yokouchi M, Nagao K, Ishii K, Amagai M, Kubo A. Functional tight junction barrier localizes in the second layer of the stratum granulosum of human epidermis. *J Dermatol Sci*. 2013;71(2):89-99.
38. Abd E, Yousef SA, Pastore MN, et al. Skin models for the testing of transdermal drugs. *Clinical pharmacology : advances and applications*. 2016;8:163-176.
39. Nakajima M, Nagase S, Iida M, et al. Claudin-1 Binder Enhances Epidermal Permeability in a Human Keratinocyte Model. *The Journal of pharmacology and experimental therapeutics*. 2015;354(3):440-447.
40. Kage H, Flodby P, Gao D, et al. Claudin 4 knockout mice: normal physiological phenotype with increased susceptibility to lung injury. *American journal of physiology Lung cellular and molecular physiology*. 2014;307(7):L524-536.

41. Fujita H, Hamazaki Y, Noda Y, Oshima M, Minato N. Claudin-4 deficiency results in urothelial hyperplasia and lethal hydronephrosis. *PLoS One*. 2012;7(12):e52272-e52272.
42. Zhai H, Leow YH, Maibach HI. Human barrier recovery after acute acetone perturbation: an irritant dermatitis model. *Clinical and experimental dermatology*. 1998;23(1):11-13.
43. Czarnecki T, He H, Krueger JG, Guttman-Yassky E. Atopic dermatitis endotypes and implications for targeted therapeutics. *The Journal of allergy and clinical immunology*. 2019;143(1):1-11.
44. Werfel T, Allam JP, Biedermann T, et al. Cellular and molecular immunologic mechanisms in patients with atopic dermatitis. *The Journal of allergy and clinical immunology*. 2016;138(2):336-349.

Figure 1 Equivalent circuit model of a cell monolayer **A:** Schematic illustration of a monolayered epithelium with its electric components. Ion passages are represented by resistors while lipid membranes act as capacitors. The tight junction is denoted as R_{para} , i.e. as the main resistor within the paracellular pathway. R_{ap} , C_{ap} , R_{bl} and C_{bl} are the resistances and capacitances of the apical and basolateral plasma membranes, respectively, R_{sub} is the resistance of the subepithelial tissues or filter supports. **B:** Schematic illustration of reconstructed human epidermis, with a conceivable model of its electric components.

Figure 2 Ussing chamber designs. **A:** Standard Ussing chamber setup. **1:** acrylic glass Ussing chamber. **2:** Cells on cell culture insert mounted vertically inside the chamber. **3:** Bath solution inlet. **4:** Bath solution outlet. **5:** heated water jacket as reservoir to keep the bath solution at 37°C. **6:** Bath solution, mixing and rapid perfusion of the Ussing chamber is achieved by using a bubble lift. **7:** Current injecting electrodes. **8:** Reference voltage electrodes. **9:** Differential amplifier calculating the potential difference. **10:** Phase sensitive multimeter. Image **(B)** and scheme **(C)** of the “static” Ussing chamber setup. Electrical current flow is narrowed down to the contact area at the SC. The second part of the chamber is placed in the bath solution. **D:** One-sided “static” setup and Scheme **(E)**. Both parts of the chamber are attached to the SC.

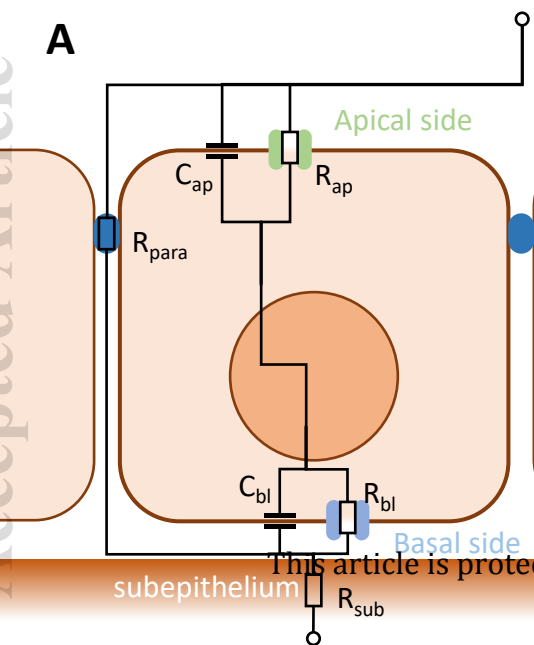
Figure 3 Effects of EGTA on RHE components. **A:** Exemplary plot of negative imaginary part of the impedance $-Im(Z)$ vs. frequency showing the impact of EGTA application from apical and basal side on the impedance spectrum. Only the low relaxation frequency in the region of 10 Hz to 100 Hz appears to be affected by EGTA treatment. The effect can already be seen after 5 minutes of treatment. After 10 minutes, the additional impact on f_{low} is low with respect to the 5 minute time point, thus indicating that the effect of EGTA is reaching an endpoint. **B:** Frequency values for relaxation frequencies f_{low} and f_{high} remain unchanged over time (d4/8: day 4/8 after calcium switch). **C, D :** Resistance and capacitance values for relaxation frequency f_{low} do not change over time. Resistance value for f_{high} increases significantly, while its capacitance value decreases. **D:** Secondary axis shows values for f_{high} . **E:** Impact of EGTA treatment from apical and basal side on resistance in RHE. Relative change of the resistance values before (R_0) and after 10 minutes (EGTA) treatment is shown. Only basal treatment showed significant difference in resistance change. **F:** No significant capacitance (C) change before and after 10 minutes EGTA treatment could be observed. **G:** The frequency value is significantly higher after basal EGTA treatment for relaxation frequency f_{low} . *: $P < 0.05$, **: $P < 0.01$, ****: $P < 0.0001$; numbers at bars, n

Figure4 Effects of iron oxide nanoparticles on RHE components. Iron oxide nanoparticles (FeNP) were applied apically for 10 minutes (supporting information chapter 2) on RHE models followed by basal EGTA treatment for 10 minutes. **A:** Exemplary graph showing the impact on the relaxation frequencies. FeNP causes major changes to f_{high} and minor to f_{low} , whereas EGTA acts only on f_{low} . **B, C:** Impact of FeNP treatment on resistance (R) and capacitance (C) values. Graphs show the relative change of the resistance and capacitance values before (0) and after (FeNP) nanoparticle treatment. Resistance (**B**) increase and capacitance (**C**) decrease is significantly higher for f_{high} . **D:** $\log_{10}$ of the frequency value shows no significant difference for f_{low} while for f_{high} it is significantly higher after apical nanoparticle (FeNP) treatment. **E:** Relative change of the resistance values of nanoparticle treated RHE models (FeNP) after basal EGTA application. Resistance significantly drops for f_{low} after EGTA treatment. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$; numbers at bars, n

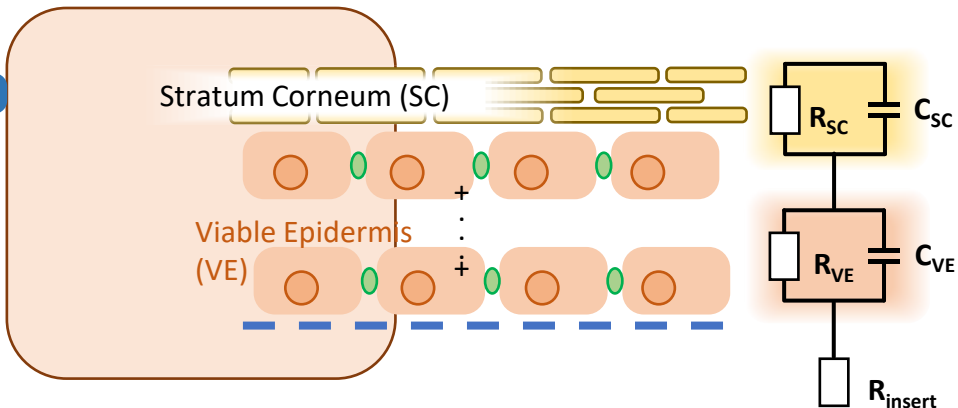
Figure 5 Comparison of standard and static Ussing chamber approaches. Different Ussing chamber setups (standard and static Ussing chamber) as well as different skin samples (RHE and mouse ear skin) were used. **A:** Evaluation of the distance of the relaxation frequencies by subtracting the $\log_{10}(\text{frequency})$ value of f_{low} from f_{high} . The distance is significantly lower for static mouse skin in comparison with the other measurements. **B:** Evaluation of the peak height $-Im(Z)$ for the corresponding relaxation frequencies. For f_{low} only standard RHE yields significantly lower values.

For f_{high} standard RHE values are also significantly lower while static mouse yields much higher values. All values were corrected for the chamber measurement area. Static one sided RHE and static mouse skin were additionally corrected by the factor 0.5, as the current passes two skin layers in these setups. **C:** Exemplary spectra demonstrating the hydration of the SC upon static Ussing chamber attachment within 2 minutes. Only f_{high} appears to be affected by the hydration. 10 minutes of subsequent basal EGTA treatment only affected f_{low} . **D, E:** Impact of Stratum Corneum (SC) hydration and basal EGTA treatment on RHE measured in static Ussing chamber. **D:** Graphs show relative resistance change before (R_0) and after ($R_{\text{treatment}}$) the treatment. SC hydrated with bath solution causes significant resistance decrease for f_{high} . Hyperosmolar 0.5 M KCl penetration of SC affects both f_{low} and f_{high} with no significant difference between them. Basal EGTA significantly decreases the resistance for f_{low} . **E, F:** Graphs show the $\log_{10}$ frequency value for the relaxation frequencies (RF) f_{low} and f_{high} . **E:** For f_{low} , the RF value after basal EGTA treatment is significantly higher than untreated control and bath solution hydrated RHE. 0.5 M KCl treatment shows no significant differences to the other conditions. For f_{high} , the RF value is significantly higher after 0.5 M KCl treatment. **F:** Impact of different apical treatments on mouse ear skin RF values measured with static Ussing chamber. For f_{low} the RF value is significantly increased after acetone treatment. For f_{high} both acetone and hyperosmolar 0.5 M KCl treatment increase the RF value significantly. PBS treatment shows no effect. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, ****: $P < 0.0001$; numbers at bars, n

Figure 6 Effects of Cldn1 and Cldn4 knockdown. A, B: Evaluation of RHE models measured with standard Ussing chamber **A:** Evaluation of impact of siRNA treatment on normalized transepithelial electrical resistance (TER_{norm}) shows significant differences for Cldn1 and Cldn4 compared to the control groups. 3 different batches of RHE models were measured on day 4 and day 8 after Ca^{2+} switch. **B:** The same samples from (**A**) were evaluated for their relaxation frequencies. Significant increase was present for Cldn1 knockdown for both f_{low} and f_{high} , whereas Cldn4 knockdown caused significant increase in f_{low} only. **C-F:** Evaluation of the siRNA treatment impact with static Ussing chamber techniques. One batch of RHE models at day 4 and 8 after Ca^{2+} switch was analysed. **C, D:** Exemplary spectra over time during 0.5 M KCl penetration through the RHE model. **C:** Only f_{high} is affected for the untreated control, while for Cldn1 knockdown (**D**) f_{low} is highly affected. **E:** The ratio of the shifts Δf_{low} and Δf_{high} due to 0.5 M KCl penetration in RHE shows significant difference for Cldn1 knockdown. **F:** Only Cldn1 knockdown relaxation frequencies significantly differ from the other treatments. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, ****: $P < 0.0001$

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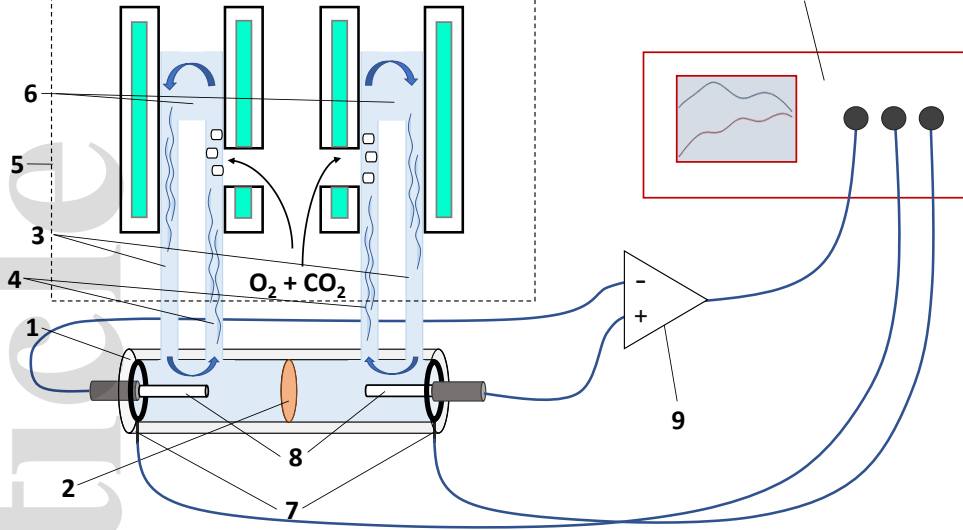
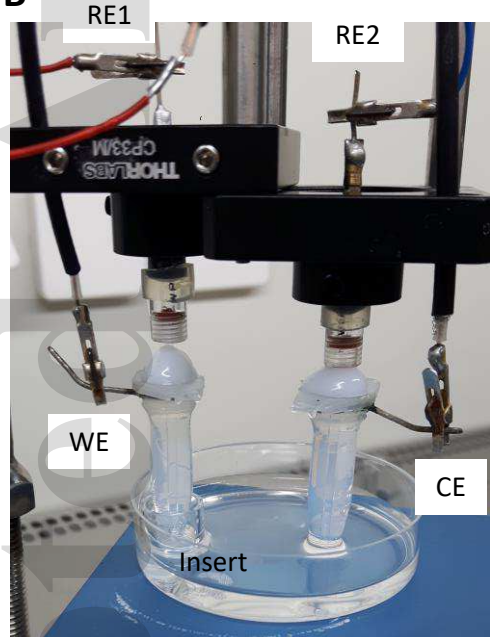
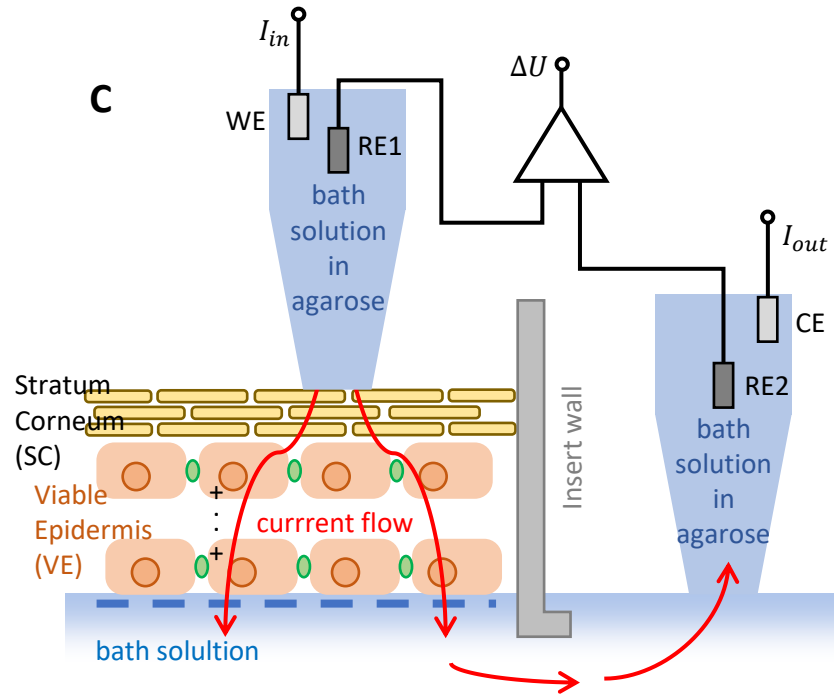
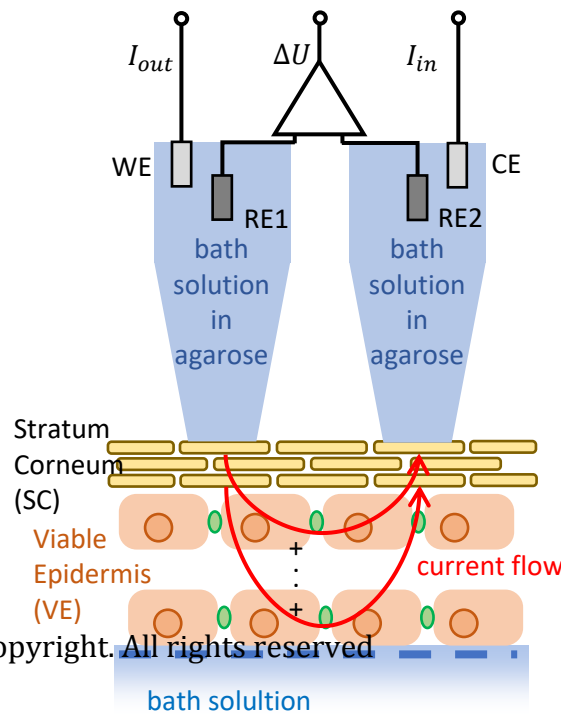
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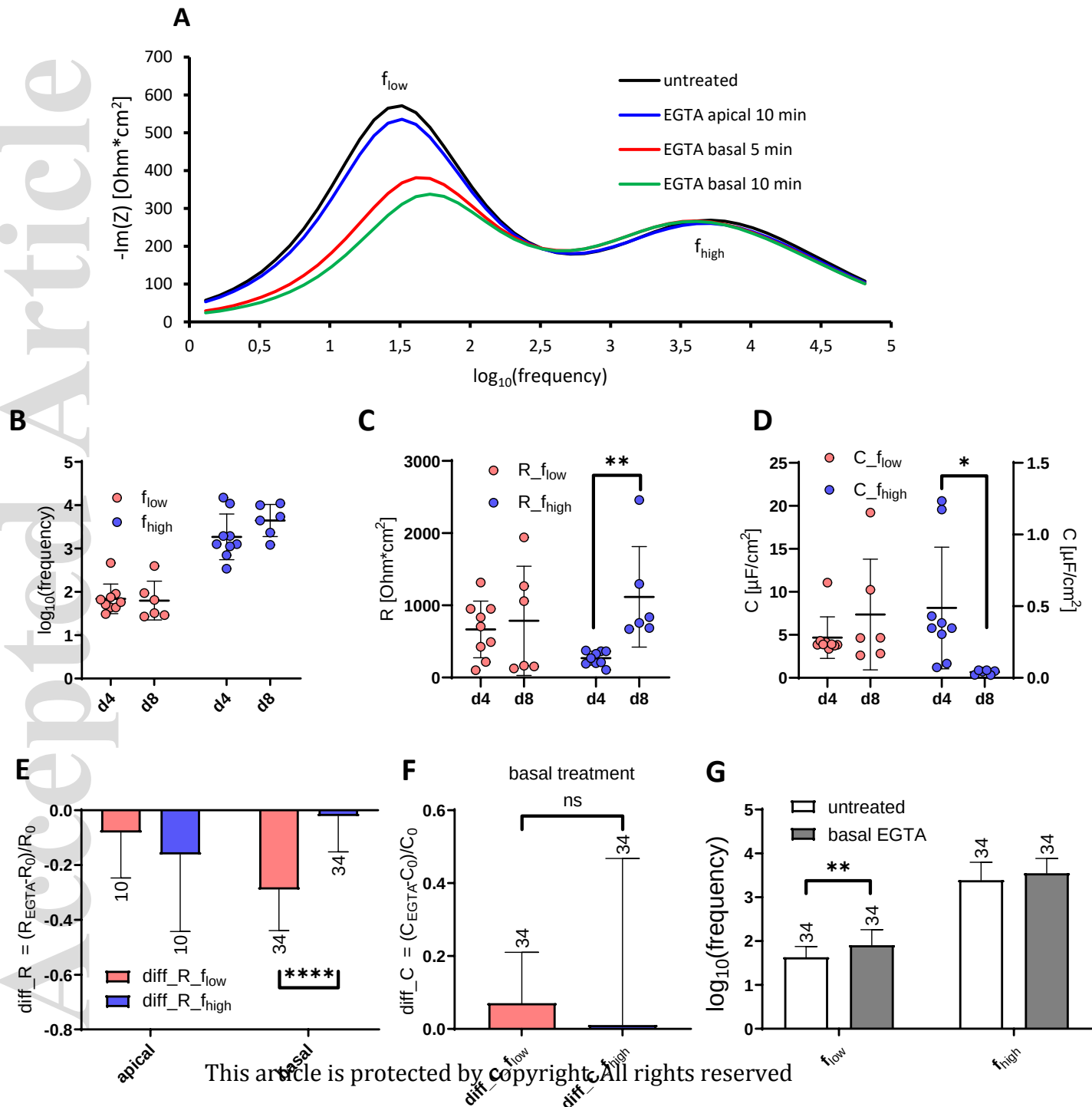
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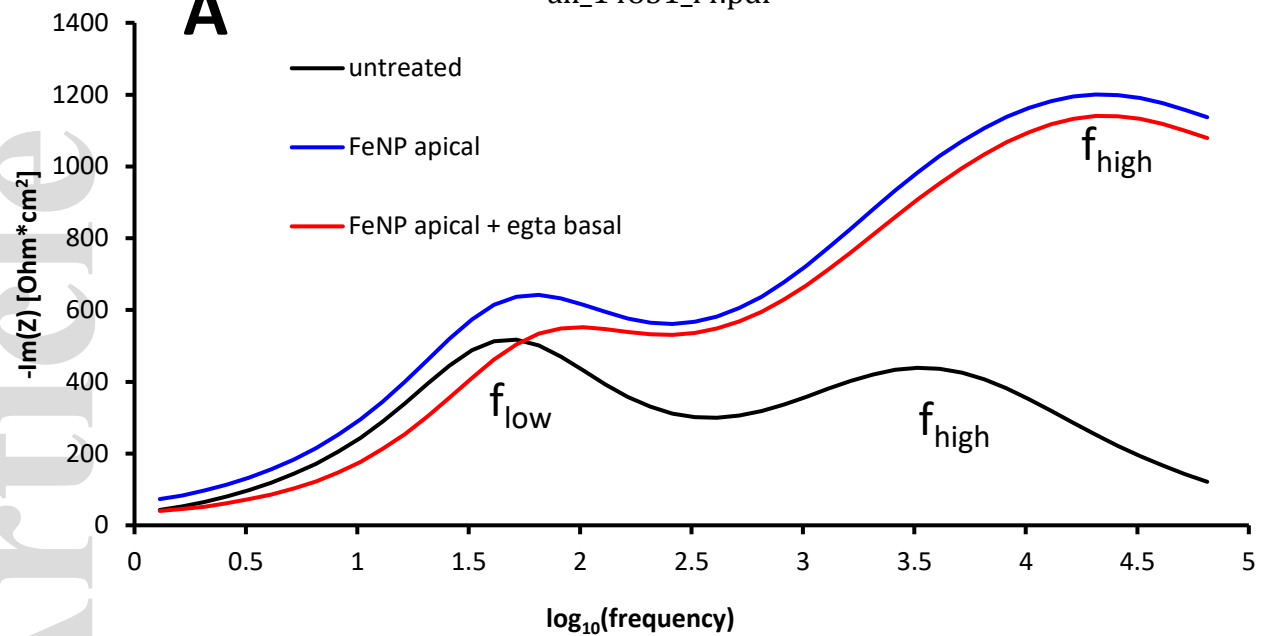
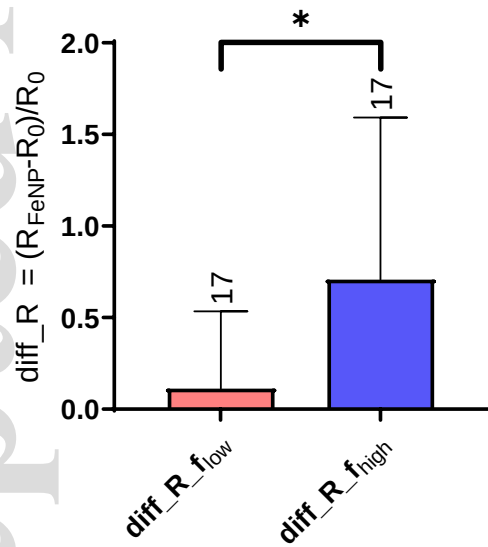
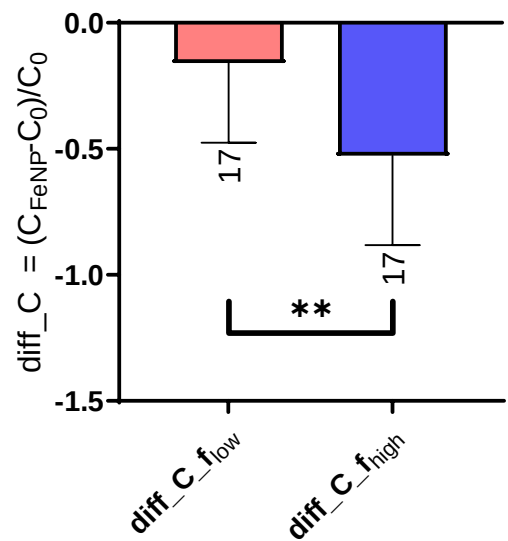
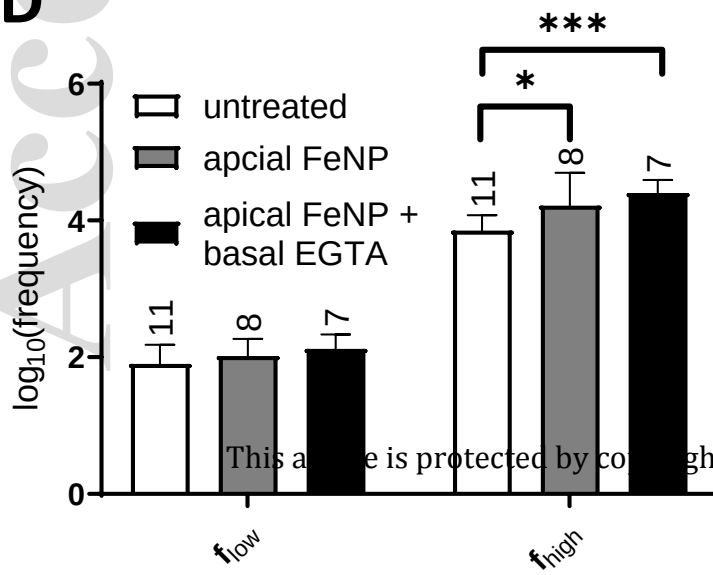
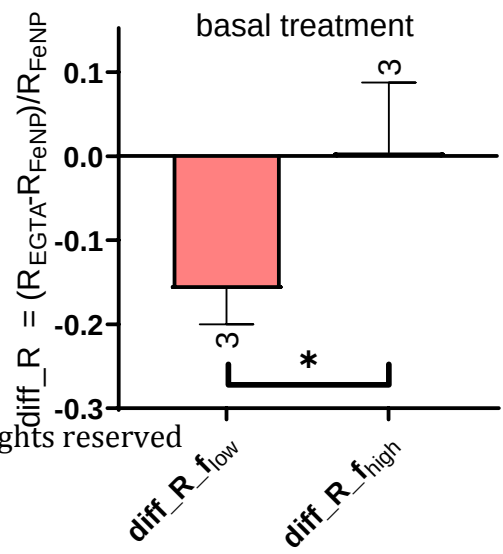
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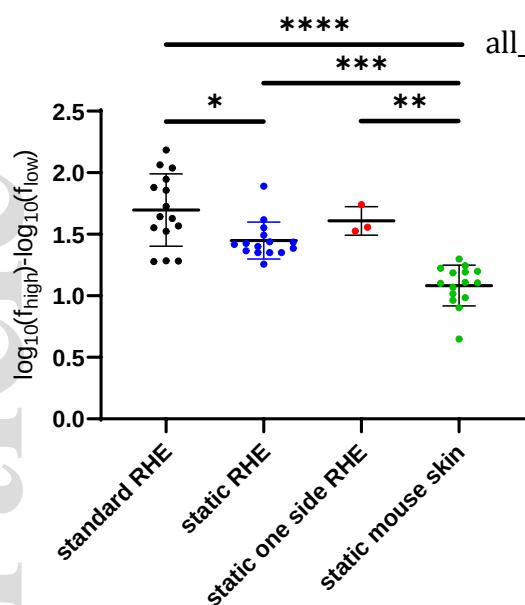
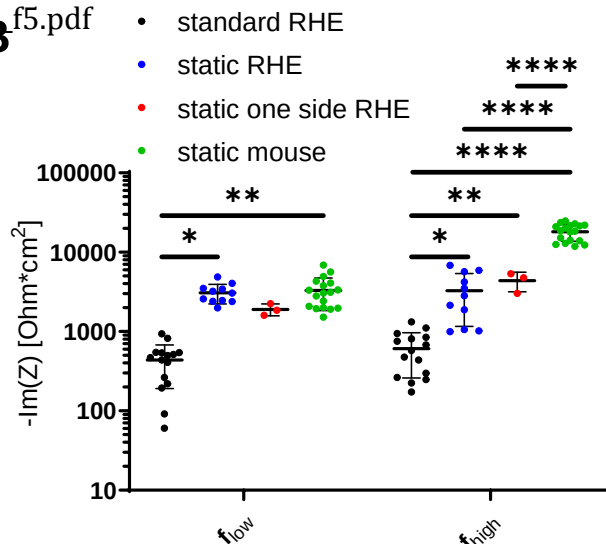
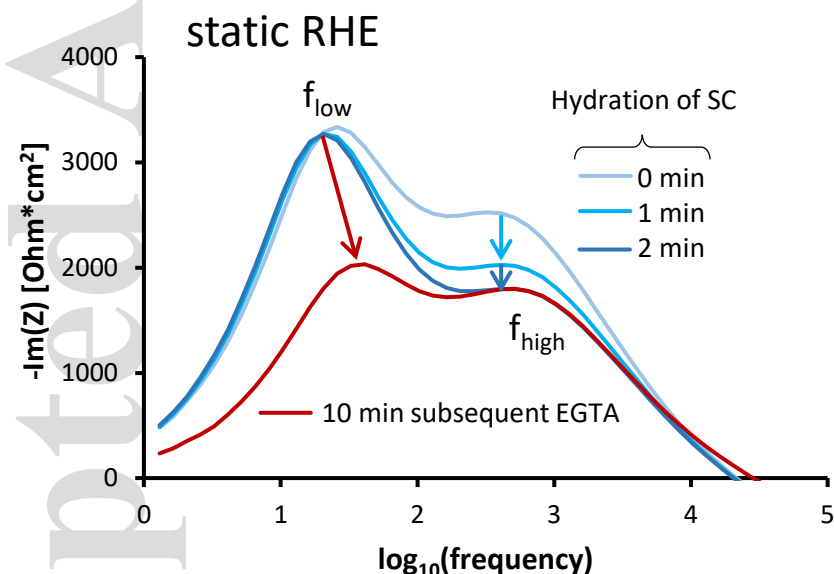
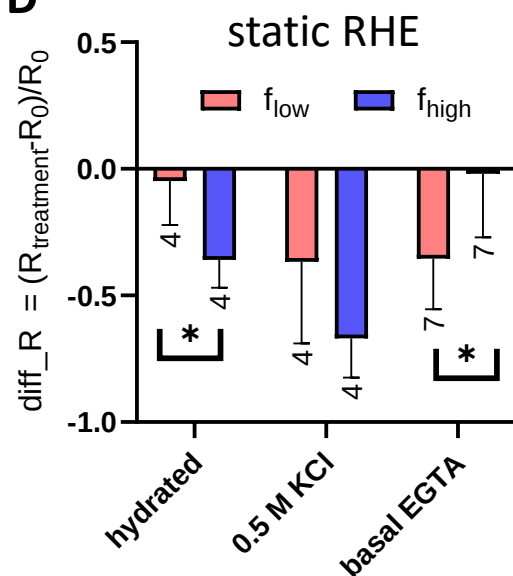
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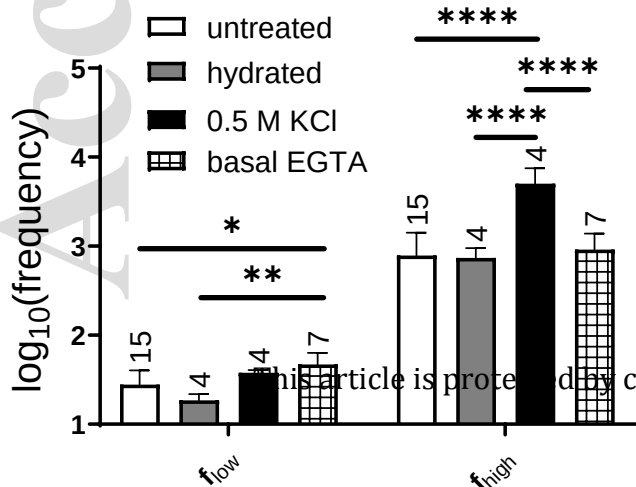
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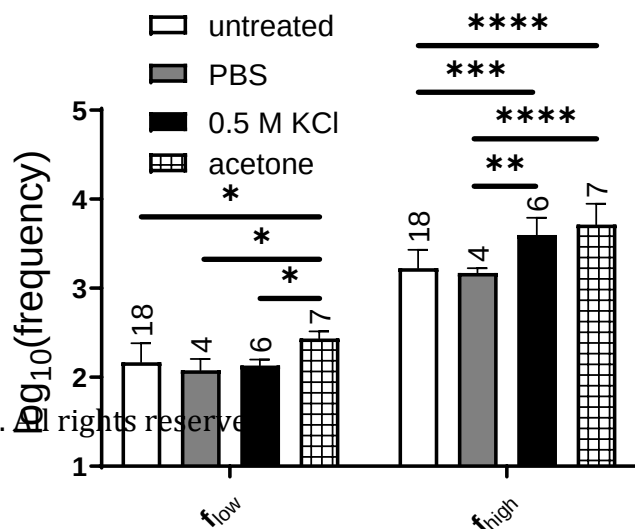
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A**B****C****D****E**

static RHE

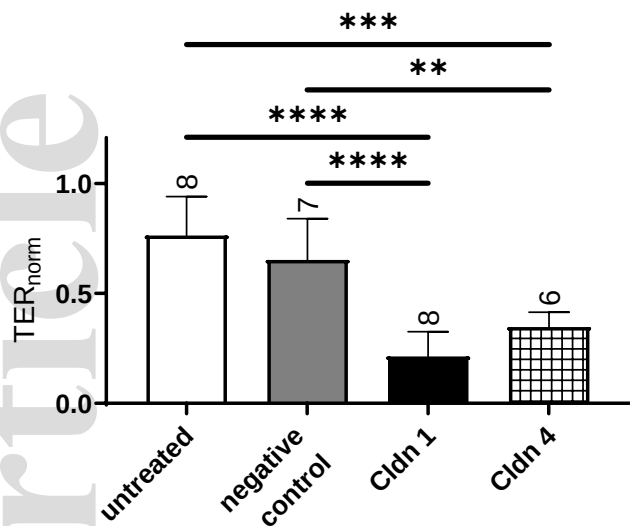
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static mouse skin



A

standard Ussing chamber

**B**

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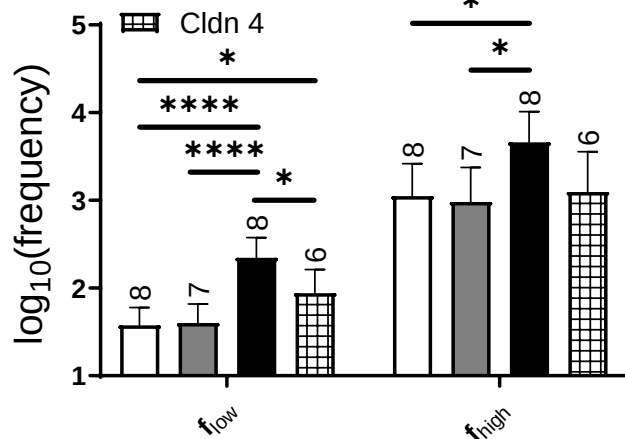
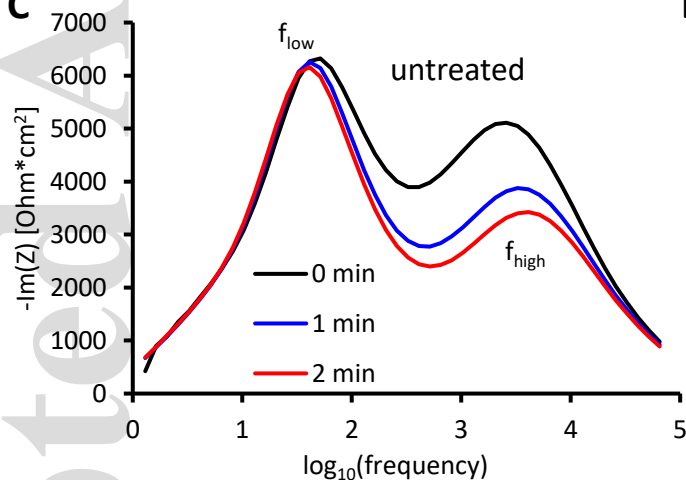
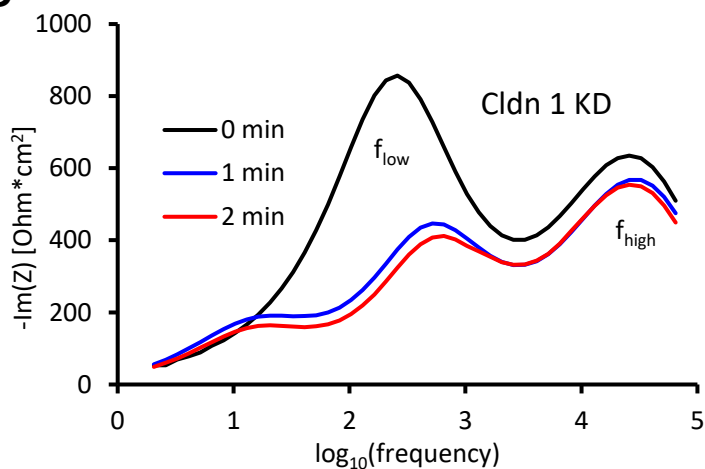
□ untreated

standard Ussing chamber

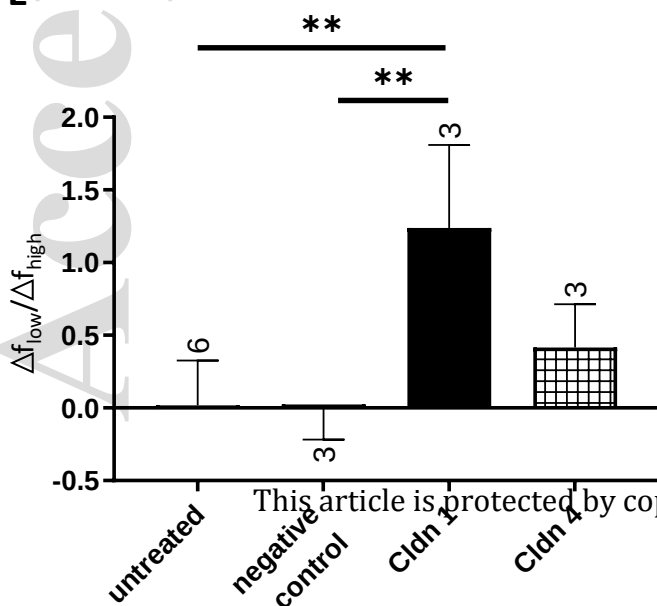
■ negative control

■ Cldn 1

▨ Cldn 4

**C****D****E**

Impact of 0.5 M KCl penetration

**F**

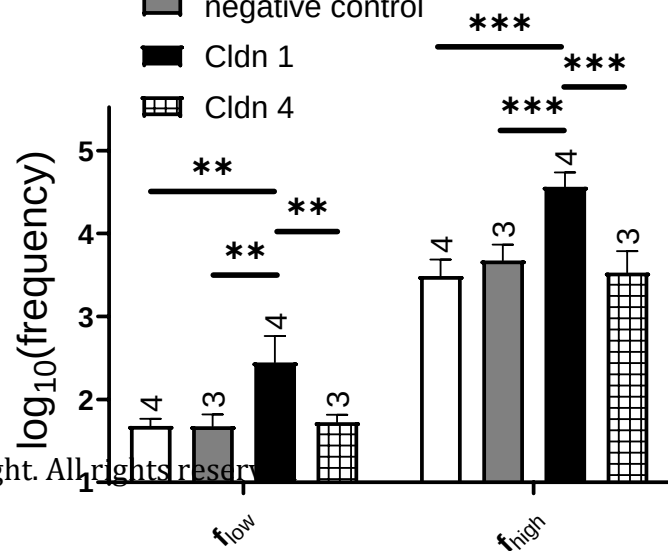
□ untreated

static Ussing chamber

■ negative control

■ Cldn 1

▨ Cldn 4



1 Impedance Spectra Fitting and Interpretation

The electric properties of the RHE are described using only resistors and capacitors, as the electrode resistance is not part of the impedance measurement chain and inductivities are not present in biological systems. The impedance of a resistor R is not dependent on the frequency f and the impedance of a capacitor with capacitance C is given by:

$$Z(\omega)_C = \frac{1}{i \cdot \omega \cdot C} \quad \omega = 2\pi \cdot f$$

The connection of a resistor and capacitor in parallel gives rise to a relaxation frequency, f_0 , at which the absolute value of the imaginary part of the impedance $\text{Im}(Z)$ reaches a local maximum (peak), which equals half of the resistance value R :

$$f_0 = \frac{1}{2\pi \cdot R \cdot C}$$

Electrochemical impedance spectra usually are presented as Nyquist plot, plotting the real part $\text{Re}(Z)$ of the complex impedance Z against the imaginary part $\text{Im}(Z)$ (see Figure 1 A). In the Nyquist plot the lowest frequency data point is located at the highest value of the $\text{Re}(Z)$ while the highest frequency data point can be found at the lowest values of $\text{Re}(Z)$. The relaxation frequencies appear as vertices of the semicircles in this representation. Alternatively, the negative imaginary part, $-\text{Im}(Z)$, was plotted against the $\log_{10}$ of the AC frequency to have both the frequency and resistance information in one graph (see Figure 1 B). The impact of the resistance R and ideal capacitance C are shown in Figure 1, with the resistance value affecting both the peak height and centre while C only affects the centre. High capacitance decreases the relaxation frequency value and the other way round (see Figure 1 F). The same applies to the resistance with the difference that it also affects the peak height (see Figure 1 E).

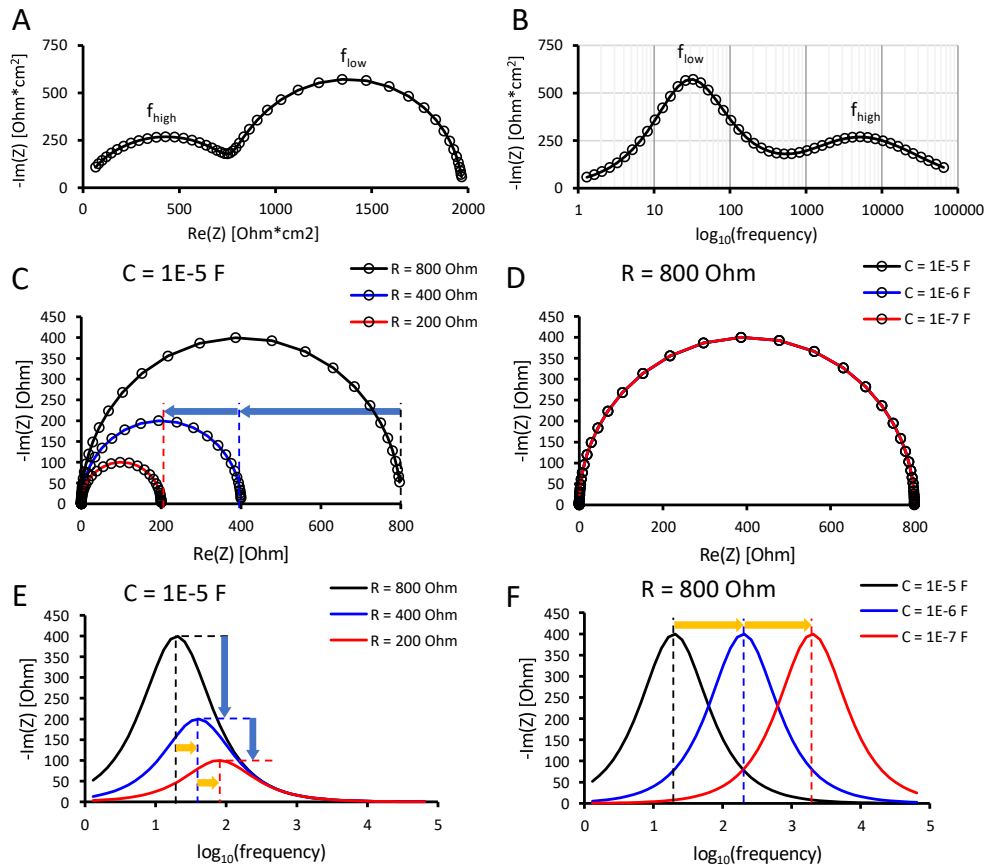


Figure 1 A, B: Exemplary impedance spectra of reconstructed human epidermis (RHE) yield two relaxation frequencies f_{low} and f_{high} . **A:** Nyquist plot – classical representation of electrochemical impedance spectra shows the real $\text{Re}(Z)$ and negative imaginary part $-\text{Im}(Z)$ of the complex impedance Z . Relaxation frequencies appear as semicircles. **B:** Alternative representation of impedance spectra – imaginary part of the impedance $\text{Im}(Z)$ vs. $\log_{10}$ of the AC frequency. Relaxation frequencies f_{low} and f_{high} appear as peaks. **C, D:** Nyquist diagrams. **C-E:** Simulated spectra showing the impact of resistance and capacitance **C:** Decreasing resistance leads to a decreasing semicircle in size. **D:** No effect on the size of the semicircle is visible if capacitance is changed. As the curves are overlapping, the change of the points along the semicircle curve is not visible. **C, D:** negative imaginary part of the impedance $-\text{Im}(Z)$ vs. $\log_{10}(\text{frequency})$. **E:** The resistance drop increases the relaxation frequency value (shown by yellow arrows) and decreases the peak height (blue arrows). **F:** The capacitance decrease does not affect the peak height but leads to an increase of the relaxation frequency value (yellow arrows).

An equivalent circuit model aims to extract resistance and capacitance values to obtain an overall measure for the properties of the tissue barrier components rather than attempting to present a detailed model that accounts for all the processes in the sample. For standard Ussing chamber measurements, an equivalent circuit model was used as presented in Figure 3 A. To account for imperfect capacitances¹ a so called constant phase element (CPE) was used instead of an ideal capacitor with the impedance Z depending on the frequency ω :

$$Z(\omega)_{CPE} = \frac{1}{Q(i\omega)^n}, n \in [0,1]$$

In this equation, Q represents the (adjusted) capacitance value, i is the imaginary unit and n is a correction factor. For $n = 1$ the CPE is equal to an ideal capacitor, while for $n = 0$ it acts as ohmic resistor. The relaxation frequency for this case is given by:

$$f_{0_CPE} = \frac{1}{2\pi \cdot \sqrt[n]{R \cdot Q}}$$

Figure 2 B shows an equivalent circuit model and corresponding values, used to demonstrate the impact of the parameter n of the constant phase element on the impedance spectrum. Decreasing values of n are flattening the curve in the Nyquist plot, leaving the DC resistance unchanged (Figure 2 C, x-intercept at 400 Ohm) and shifts the relaxation frequency to a higher value (Figure 2 D). Thus, the C -value is overestimated as the parameter n increases the relaxation frequency. So, to maintain the relaxation frequency position a higher C -value is needed that compensates for the shift that results from a parameter n lower than 1. Therefore the extracted capacitance values from the CPE fitting are overestimated and not used as a measure for the capacitance of the measured RHE models. On the other hand, fitting with an ideal RC-element would result in an underestimation of the resistance value (Figure 2 E, F). As a compromise the extracted values from R-CPE fitting were used to calculate the corrected capacitance C as follows:

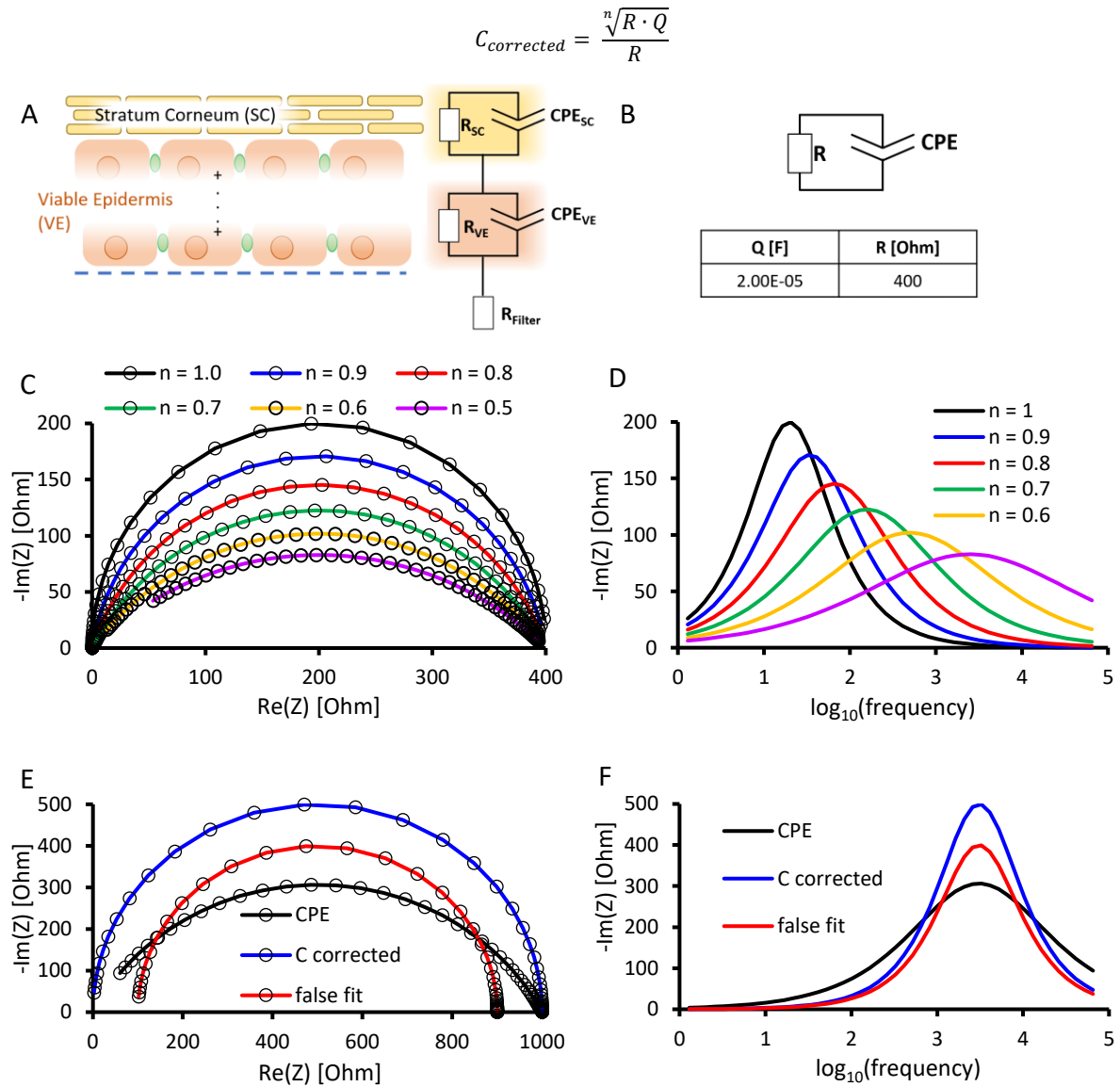


Figure 2 **A:** Equivalent circuit model of the RHE measured with standard Ussing chamber technique. **B:** Equivalent circuit model for demonstration of the impact of the parameter n of the constant phase element (CPE) on the impedance spectra. **C:** Sweep of parameter n (Nyquist Plot). **D:** Sweep of parameter n $-\text{Im}(Z)$ vs. $\log_{10}(\text{frequency})$. **E, F:** Different variants to extract R and C values from imperfect R - C -elements. CPE: ideal fit with R -CPE-Element, C -value is overestimated. C corrected: R - C -Element with the same R as the R -CPE-Element with adjusted C -value to match the RF position. False fit: Fitting result with R - C -element underestimates resistance value. **E:** Nyquist plot **F:** $-\text{Im}(Z)$ vs. $\log_{10}(\text{frequency})$

For the evaluation of static Ussing chamber measurements equivalent circuit fitting was not performed. Instead the $-\text{Im}(Z)$ vs. $\log_{10}(f)$ plot was used to extract the relaxation frequencies by gaussian peak fitting with Origin 2020 software (see Figure 3). For quantitative analysis of the spectra only the relaxation frequency position was taken into account as it is robust against changes in the electrode surface area and the current pathway.

A

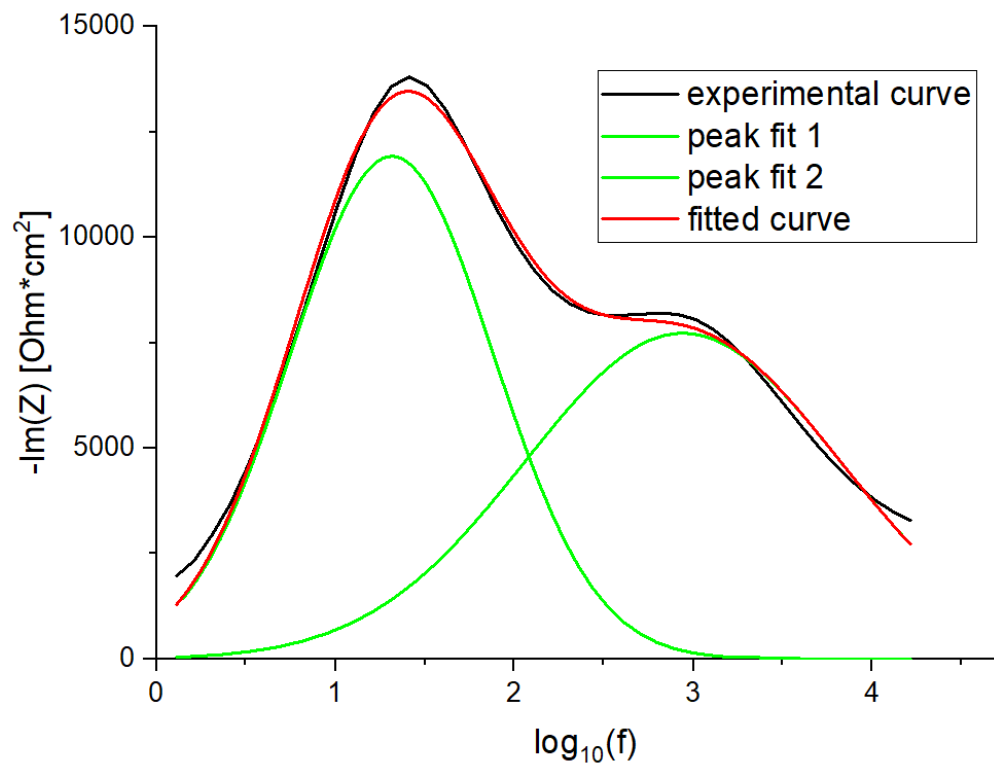


Figure 3 Schematic illustration of peak fit analysis of impedance spectra, that were not suitable for equivalent circuit fitting. Relaxation frequency peak positions are extracted by gaussian peak fitting. The green curves are the individual peaks while the red curve is the sum of the two individual peaks which is supposed to fit the experimental curve (black).

2 Stratum Corneum Coating with Iron oxide Nanoparticles

The toluene of the iron oxide nanoparticle suspension was evaporated at room temperature in a fume cupboard for 12 hours and the same amount of Decamethylcyclopentasiloxane (10 mL) was added. Afterwards, nanoparticles, suspended in Decamethylcyclopentasiloxane, were added to the apical side of the RHE model (Figure 4 A). The basal side of the RHE was immersed in cell culture medium in a petri dish. A magnet was placed underneath the petri dish for a duration of 10 minutes and attracted the nanoparticles which consequently attached to the surface of the SC. Afterwards the insert was submerged in bath solution, so that the siloxane lifted off, leaving only nanoparticles that attached to the SC (Figure 4 B).

However, variable nanoparticle layer thicknesses could reduce the effective measurement area, which would increase both the VE and SC resistance in the same way, leaving the relaxation frequency values unchanged. Therefore, only experiments with a change of diff_R for f_{low} lower than 1 were considered for evaluation. Furthermore, mechanical stress on the RHE during treatment could decrease VE resistance, making EGTA post treatment less effective. Therefore EGTA treatment yielded only significance in relative resistance change but not in the relaxation frequency value of f_{low} .

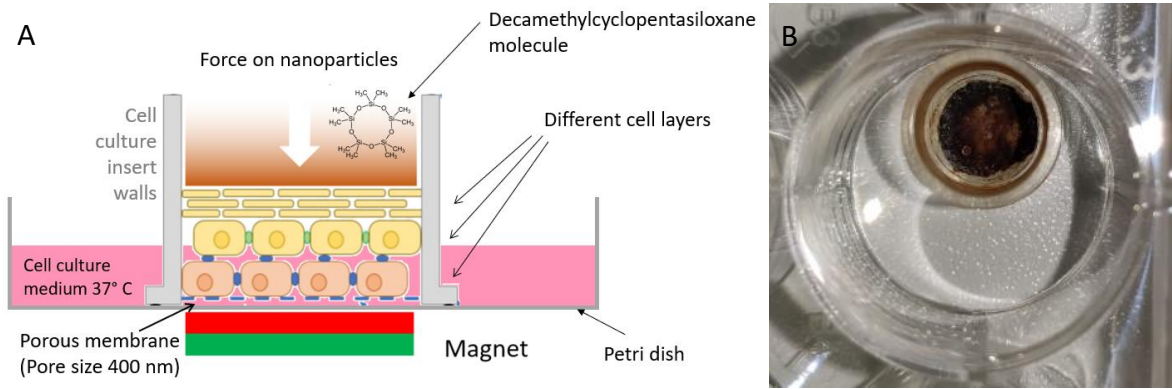


Figure 4 A: Schematic illustration of the iron oxide nanoparticle (FeNP, brown) deposition on the Stratum Corneum. **B:** Top view on RHE in cell culture insert, submerged in bath solution, after FeNP coating.

To further explain the impact of the nanoparticles on the SC (f_{high}), exemplary spectra are analysed with an advanced equivalent circuit model of RHE after apical iron oxide nanoparticle (FeNP) deposition as shown in Figure 5 A. This model assumes that there is an area fraction n_{FeNP} of the SC coated with the nanoparticles (SC_1), while the rest is uncoated (SC_2). This must be the case because otherwise the resistance would be infinite due to the Dodecanthiol functionalization of the particles, if the nanoparticles were densely packed and covering the whole SC area. Partial resistances and capacitances as shown in the equivalent circuit model (Figure 5 A) are calculated as follows:

$$R_{SC_1} = \frac{R_{SC}}{1 - n_{\text{FeNP}}}; R_{SC_2} = \frac{R_{SC}}{n_{\text{FeNP}}}$$

$$C_{SC_1} = C_{SC} \cdot (1 - n_{\text{FeNP}}); C_{SC_2} = C_{SC} \cdot n_{\text{FeNP}}$$

The initial parameters for the VE and SC were calculated from the experimental data of RHE before the nanoparticle treatment (see Figure 5 B, f_{VE} equals f_{low} , f_{SC} equals f_{high}). The simulation in Figure 5 C shows, that with increasing nanoparticle area fraction n_{FeNP} , the relaxation frequency f_{SC} decomposes into f_{SC} and f_{FeNP} . Fitting the experimental data under the assumption, that SC properties are not changed by FeNP deposition, such that only resistance R_{FeNP} , capacitance C_{FeNP} and area fraction n_{FeNP} are fitted, while all other parameters are fixed, does not deliver satisfying fitting results (see Figure 5 D). Assuming that the SC properties are changed by the nanoparticles leads to a better fit performance and therefore indicates that the nanoparticle coating does not only deposit a layer on top of the SC but also alters the properties of the SC itself. The parameter values from the fitting are displayed in Table 1.

Table 1: Impedance spectrum equivalent circuit fitting results of RHE after apical FeNP deposition, assuming that electric properties of the SC are changed or unchanged by the nanoparticles

Parameter	SC no change	SC change
n_{FeNP}	0.95	0.95
R_{FeNP} [Ohm*cm ²]	3200	2500
C_{FeNP} [μF/cm ²]	0.0001	0.0001
R_{SC} [Ohm*cm ²]	1009	1500
C_{SC} [μF/cm ²]	0.04	0.06
R_{VE} [Ohm*cm ²]	1100.1	1100.1
C_{VE} [μF/cm ²]	2.85	2.85

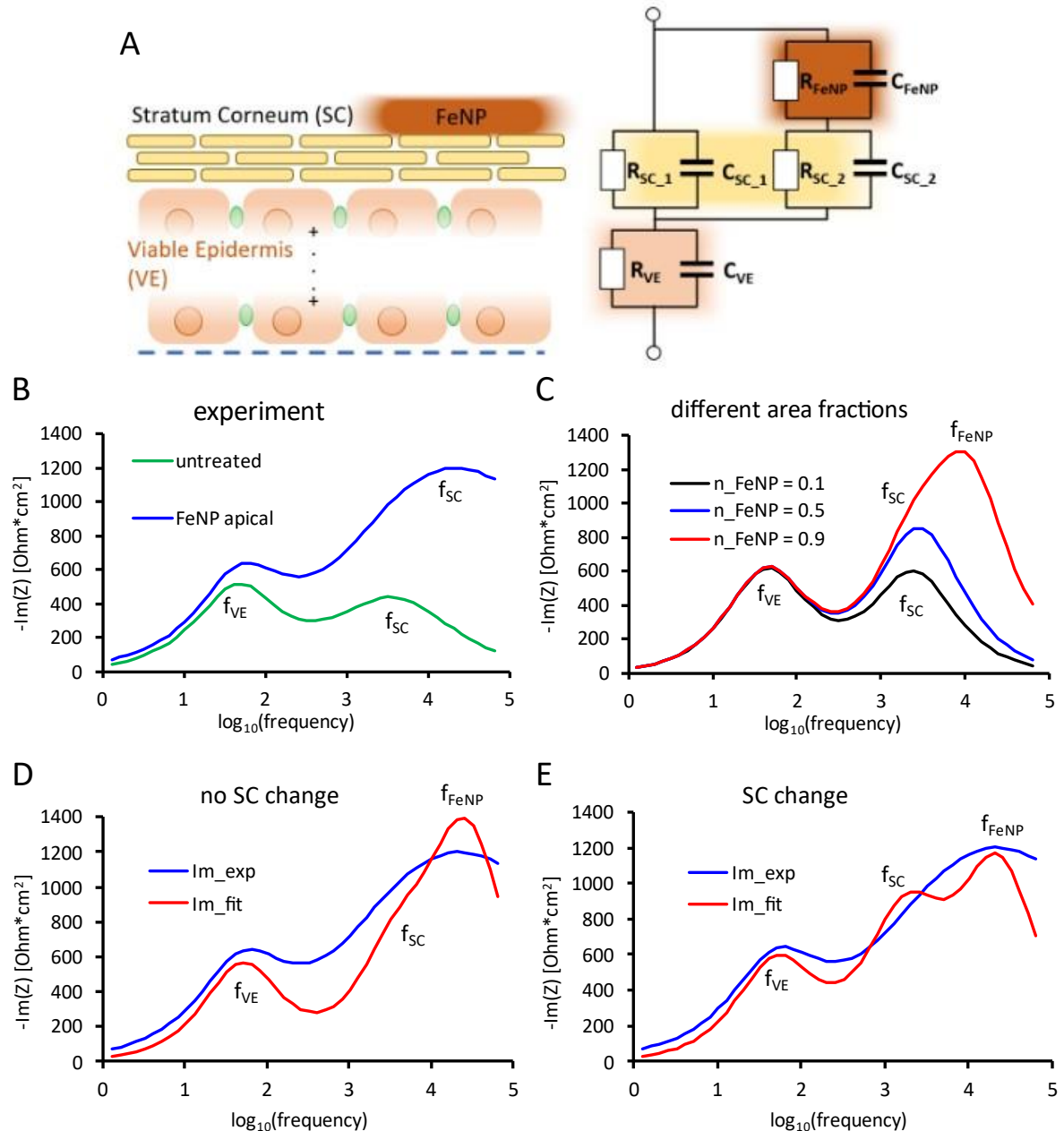


Figure 5 Iron Oxide Nanoparticle Impact **A:** Equivalent circuit model of RHE after apical iron oxide nanoparticle (FeNP) deposition. The Stratum Corneum is divided in the coated (SC₁) and uncoated (SC₂) area fraction. **B-E:** Experimental and simulated impedance spectra shown as negative imaginary impedance $-\text{Im}(Z)$ vs. $\log_{10}(\text{frequency})$. **B:** Experimental data – FeNP deposition affects f_{SC} , by increasing impedance and relaxation frequency value. **C:** Simulation of different area fractions of FeNP coating. With increasing FeNP area fraction, f_{SC} decomposes into f_{SC} and f_{FeNP} . **D:** Fitting experimental data assuming SC properties are not changed by FeNP deposition. **E:** Fitting experimental data assuming the SC properties are changed by the nanoparticles. Fit performs better if SC change is assumed.

3 Construction and implementation of static Ussing chamber

The body of the static Ussing chamber was based on a 1.5 mL Eppendorf tube. First, the lid was cut off and a hole of 1.5 mm in diameter was drilled into the centre of the lid. Another hole was drilled into the side wall of the lid to mount the current electrode wire into it (Figure 6 A). Next, a glass capillary with 1.5 mm outer diameter and 50 mm length was inserted into the hole in the centre of the lid and fixed with hot glue, such that 27 mm of the glass capillary extended into the Eppendorf tube (Figure 6 A).

Then the tip of the Eppendorf tube was cut to generate an opening of 4 mm in diameter (Figure 6 B). 0.5 M KCl solution and 1% (w/v) agarose were mixed and brought to the boil. The hot solution was filled into the glass capillary and left to cool until the agar had solidified. Subsequently, bath solution was boiled with 1% (w/v) agarose. The lid was clipped onto the body of the Eppendorf tube (Figure 6 C). If the one-sided static Ussing chamber was used, a flexible rubber tube was inserted into the tube's tip opening (Figure 6 D, E). In both cases (i.e. with or without the rubber tube extension), the hot bath solution - agarose mixture was poured into the tube. For the potential measurement the outer part of the glass capillary was inserted into a WPI microelectrode holder.

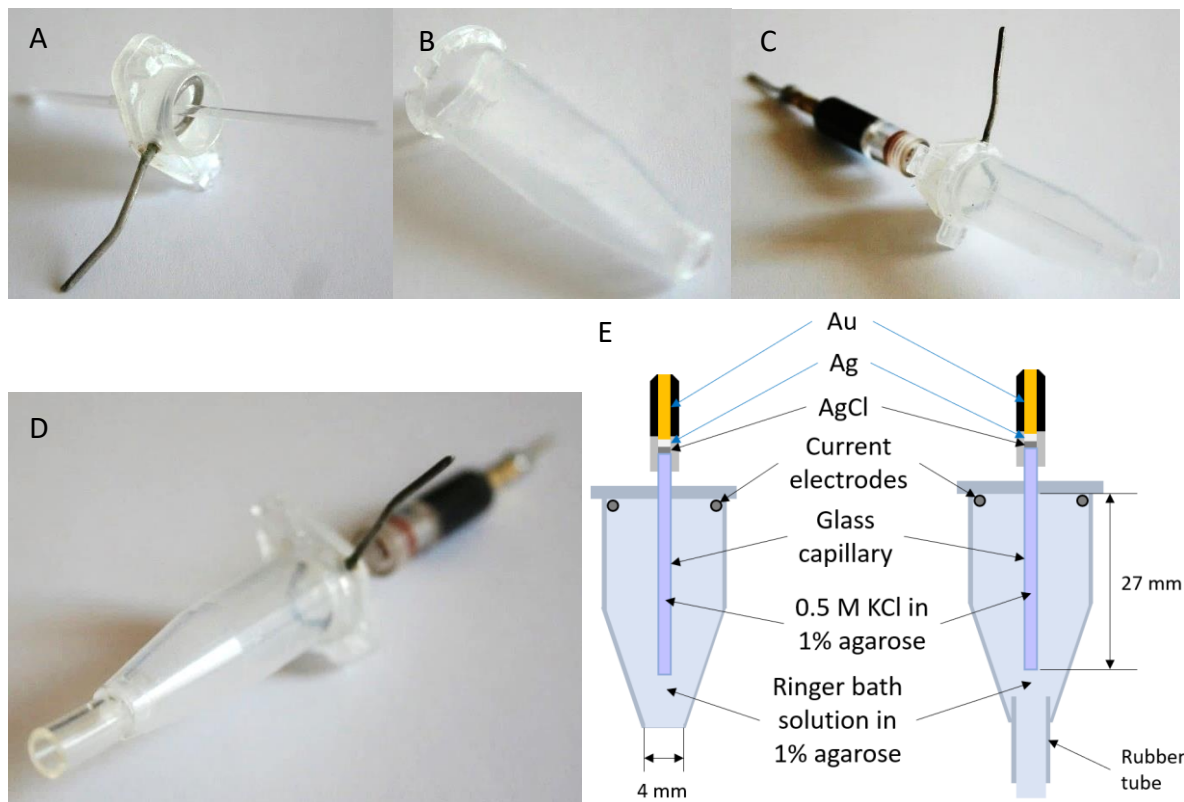


Figure 6 A: Eppendorf lid with glass capillary and current electrode. B: Eppendorf tube with truncated tip. C: Assembled static Ussing chamber (without agarose in tube). D: one sided static Ussing chamber with additional rubber tube mounted at the tip. E: schematic cross section views of the static and one sided static Ussing chamber.

When using the one-sided static Ussing chamber, the two parts of the chamber were mounted as shown in Figure 7 A. To check if no shunt is formed between the two parts of the chamber one can take a look at the bottom of the insert. Two separate dark spots mark the edges of the hydrated region indicating that no shunt is formed (Figure 7 B). For measuring mouse ear skin, the static Ussing chamber was mounted in a Thorlabs® cage system as shown in Figure 7 C, D. Here, ears from mouse cadavers were clamped between the two chambers in such a way that the outside of the ear was in contact with chamber X and inside of the ear was in contact with chamber Y. For evaluation, the impedance values were corrected by the corresponding chamber area. In the case of one sided static RHE and static mouse skin measurements, the values were additionally corrected by a factor of 0.5 as the current passes the skin layers twice.

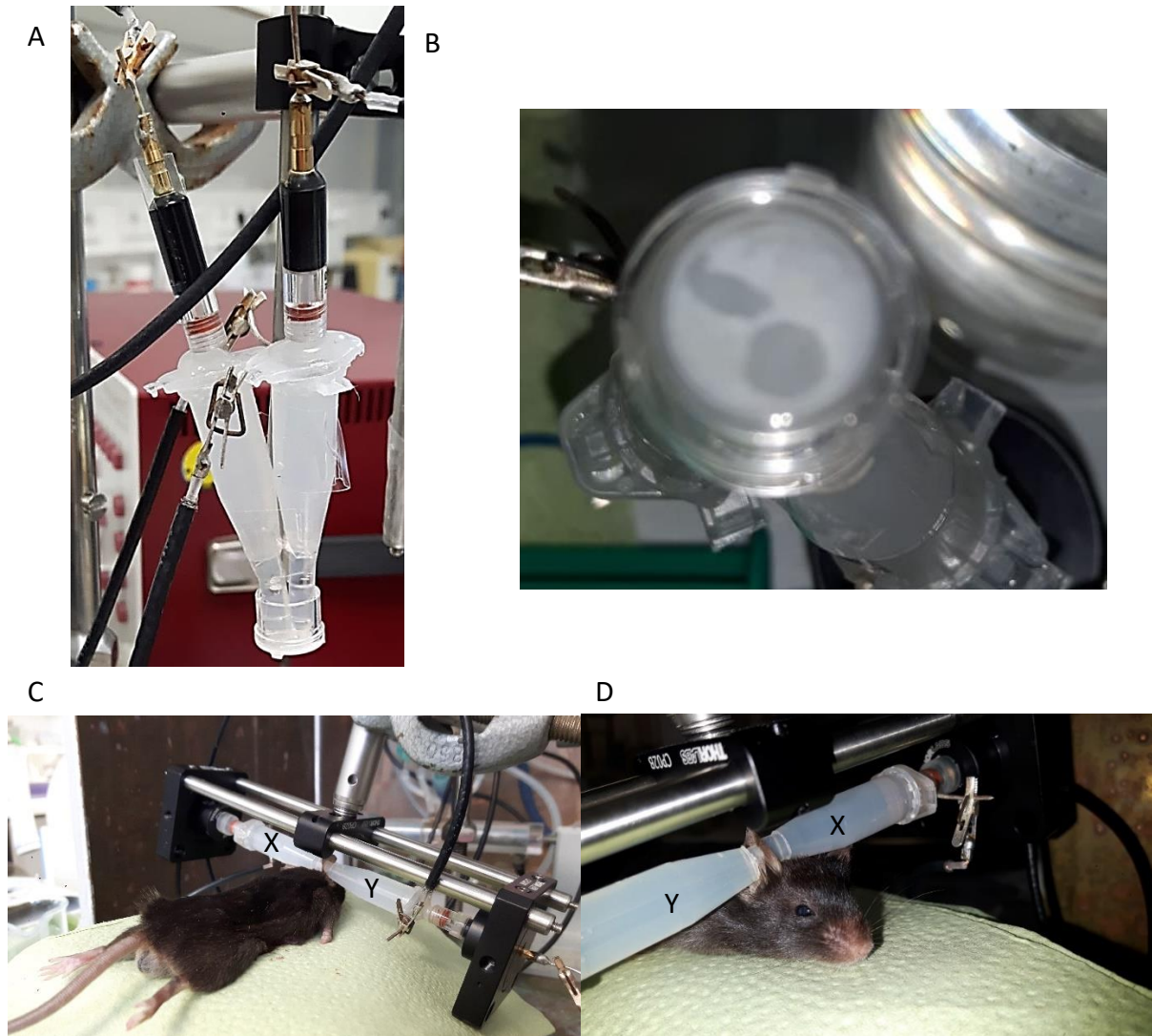


Figure 7 **A:** Experimental implementation of the one-sided static Ussing chamber. **B:** Bottom view of the cell culture insert during one sided measurement. Dark spots mark the region hydrated by the Agarose gel of the static Ussing chamber. **C, D:** Implementation of the static Ussing chamber for measurements on ears from mouse cadavers. Tubes are aligned via Thorlabs cage system mounting. Chamber tubes are marked by X and Y for referencing in text.

4 Investigation of the shunt

It could be observed, that curves recorded with the static Ussing chamber contained more spectral information, than the curves obtained with the standard Ussing chamber, such as an additional relaxation frequency f_x around 3 Hz (see Figure 8 A). Even though the relaxation frequencies f_{VE} and f_{SC} (denoted as f_{low} , f_{high} respectively in the main text) maintain their values for the same RHE models measured both in the standard and static Ussing chamber (Figure 8 B) on day 4 after the Calcium switch, the resistances are higher for Cldn4 knockdown RHE models measured in the static Ussing chamber (Figure 8 C).

This could be explained by a shunt (R_{shunt}), that may be present at the edge of the RHE, where the cells attach to the insert wall. In the equivalent circuit model this would be represented by a resistor (R_{shunt}) in parallel to all other electrical components representing the RHE (see Figure 8 D).

Figure 8 E demonstrates, that this model could indeed explain the decreased resistance in standard Ussing chamber measurements for Cldn4 knockdown models. The presence of a shunt decreases the contribution of f_x and f_{low} to the overall spectrum (Figure 8 E, blue curves). To some extent, such a shunt appeared to be present in all RHE models (regarding the loss of f_x component in the negative control in Figure 8 F compared to Figure 8 E), but was especially pronounced in Cldn4 KD models.

Direct comparison of the negative control and Cldn4 KD models from (Figure 8 A) measured in the standard Ussing chamber indicated a greater shunt conductance for Cldn4 KD in comparison to the negative control. The values, used for fitting the spectra are shown in Table 2 A. Table 2B shows that the simulated, shunt-induced frequency increase could explain the measured frequency shift in the experimental data. Together, this indicates that Cldn4 deficiency may increase the shunt conductance, e.g. by reducing cell adhesion to the plastic wall of the cell culture insert, but does not change barrier function. Furthermore, it was found that the TER does not correlate with the Cldn4 mRNA level (Figure 11), indicating that there could be a non-barrier related effect of the Cldn4 knockdown.

Thus, measuring with the static Ussing chamber indeed avoids the shunt but at the same time carries the risk of slightly varying electrode area, such that one should evaluate the relaxation frequency values instead of absolute resistance values, that are sensitive to changes in the electrode area.

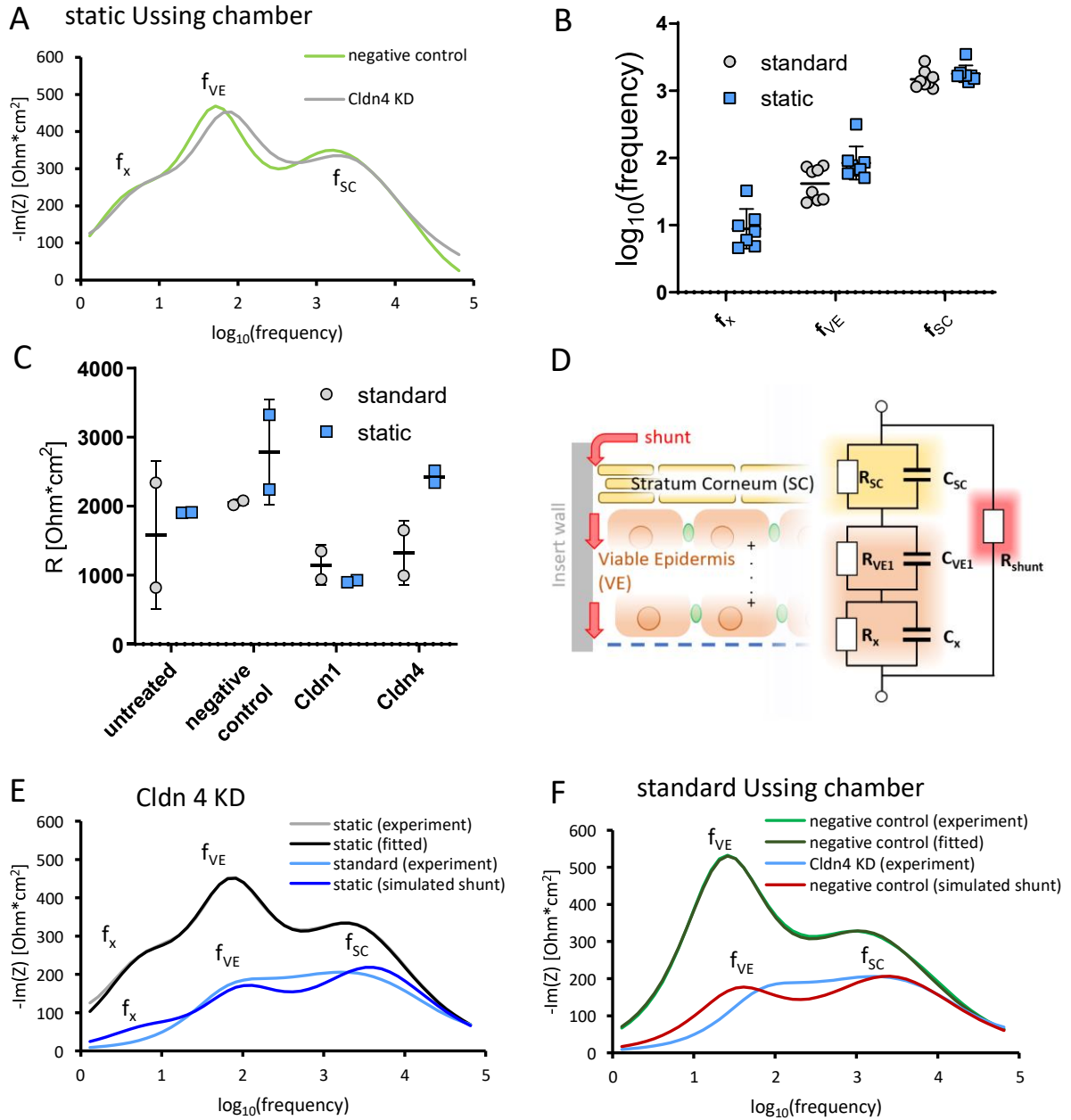


Figure 8 Effect of a shunt **A:** Exemplary impedance spectra of Cldn4 KD and negative control RHE measured in the static Ussing chamber, yielding spectra with 3 relaxation frequencies f_x , f_{VE} , f_{SC} and high similarity to one another. **B:** Relaxation frequencies f_{VE} and f_{SC} are in a similar range for static and standard Ussing chamber (all siRNA treatments merged). f_x is not detectable in standard Ussing chamber. **C:** Corresponding resistance values are similar for static and standard Ussing chamber for all treatments except Cldn4 KD. Here, resistance values for static measurement are higher. **D:** Schematic illustration of RHE and the corresponding equivalent circuit model used for simulation of the effect of a paracellular shunt. **E:** Direct comparison of the same Cldn4 KD RHE model measured on day 4 after Calcium switch in the standard and static Ussing chamber shows a behaviour that can be simulated by a shunt. Extracted parameters from fitting the static Ussing chamber spectrum (black curve) were used to simulate the shunt effect for the standard Ussing chamber measurement as shown in (D). **F:** Direct comparison of the negative control and Cldn4 KD models from (A) measured in the standard Ussing chamber indicates a greater shunt conductance for Cldn4 KD in comparison to the negative control. Here, only f_{VE} and f_{SC} were fitted for the negative control and used for simulation of the shunt. Parameters extracted from fitting are shown in Table 2.

Table 2 A: Parameters extracted from fitting the experimental curves in Figure 8 E and F. **B:** Comparison of the fitted and simulated frequency values with the experimental data. Calculated values lie within the range of $\mu \pm \sigma$. With μ : mean σ : standard deviation

A		static	standard
Fit Value	Cldn4 KD	neg. con.	
n_x	0.98		
n_{VE}	0.955	0.95	
n_{SC}	0.75	0.68	
C_x [$\mu F/cm^2$]	88		
C_{VE} [$\mu F/cm^2$]	3.7	8.2	
C_{SC} [$\mu F/cm^2$]	0.79	2	
R_x [$Ohm \cdot cm^2$]	360		
R_{VE} [$Ohm \cdot cm^2$]	790	1030	
R_{SC} [$Ohm \cdot cm^2$]	908	1030	
R_{shunt} [$Ohm \cdot cm^2$]	1947	1935	

B			
Treatment	Value	$\log_{10}(f_{VE})$	$\log_{10}(f_{SC})$
Cldn4 KD	$\mu - \sigma$	1.67	2.64
Cldn4 KD	$\mu + \sigma$	2.21	3.55
neg. con. shunt	simulation	1.71	3.31
neg. con.	$\mu - \sigma$	1.39	2.59
neg. con.	$\mu + \sigma$	1.82	3.37
neg. con.	fitting	1.41	3.01

The general presence of a shunt was further confirmed by evaluation of the permeability of the RHE to NHS-fluorescein (5- (and 6-)carboxyfluorescein, succinimidyl ester). To this end NHS-fluorescein at a final concentration of 100 μM in bath solution (stock solution: 100 mM in DMSO) was applied to the apical side of the RHE (see Figure 9 A1). NHS-fluorescein binds to primary amines and therefore preferentially to lysine residues in proteins, thus labelling the surface of the cell layer and marking all diffusion pathways across the cell layer.² After 45 min incubation, the inserts were washed with phosphate buffered saline (PBS), fixed with 4% PFA (paraformaldehyde) in PBS for 20 minutes and washed with PBS again. Subsequently the RHE was stained with DAPI at a final concentration of 10 $\mu g/ml$ in PBS for 20 minutes and washed again with PBS (see Figure 9 A2).

Finally, the RHE was placed on a glass coverslip and directly imaged with an inverted Zeiss LSM 780 (10x air objective) (see Figure 9 A3). Accumulation of fluorescein signal at the insert's edge could be observed indicating the presence of a shunt pathway for the electrical current (see Figure 9 B).

5 Comparison of different Ussing Chamber measurements

Comparison of the different Ussing chamber setups (standard and static Ussing chamber) as well as different skin samples (RHE and mouse ear skin) showed that all impedance curves yield the relaxation frequencies f_{VE} and f_{SC} (see Figure 10 A). Figure 10 B-E demonstrates where the relaxation frequencies have been located via peak fitting. Additional fitted peaks such as f_x for all static measurements and an additional relaxation frequency at 10^5 Hz for static one side RHE are not shown for reasons of clarity. For standard RHE, static RHE and static one side RHE the f_{VE} frequency values were located around 30 Hz whereas for f_{SC} they were around 1000 Hz (Figure 10 B-D). For static mouse skin both values are higher, f_{VE} around 100 Hz and f_{SC} around 3000 Hz (Figure 10 E).

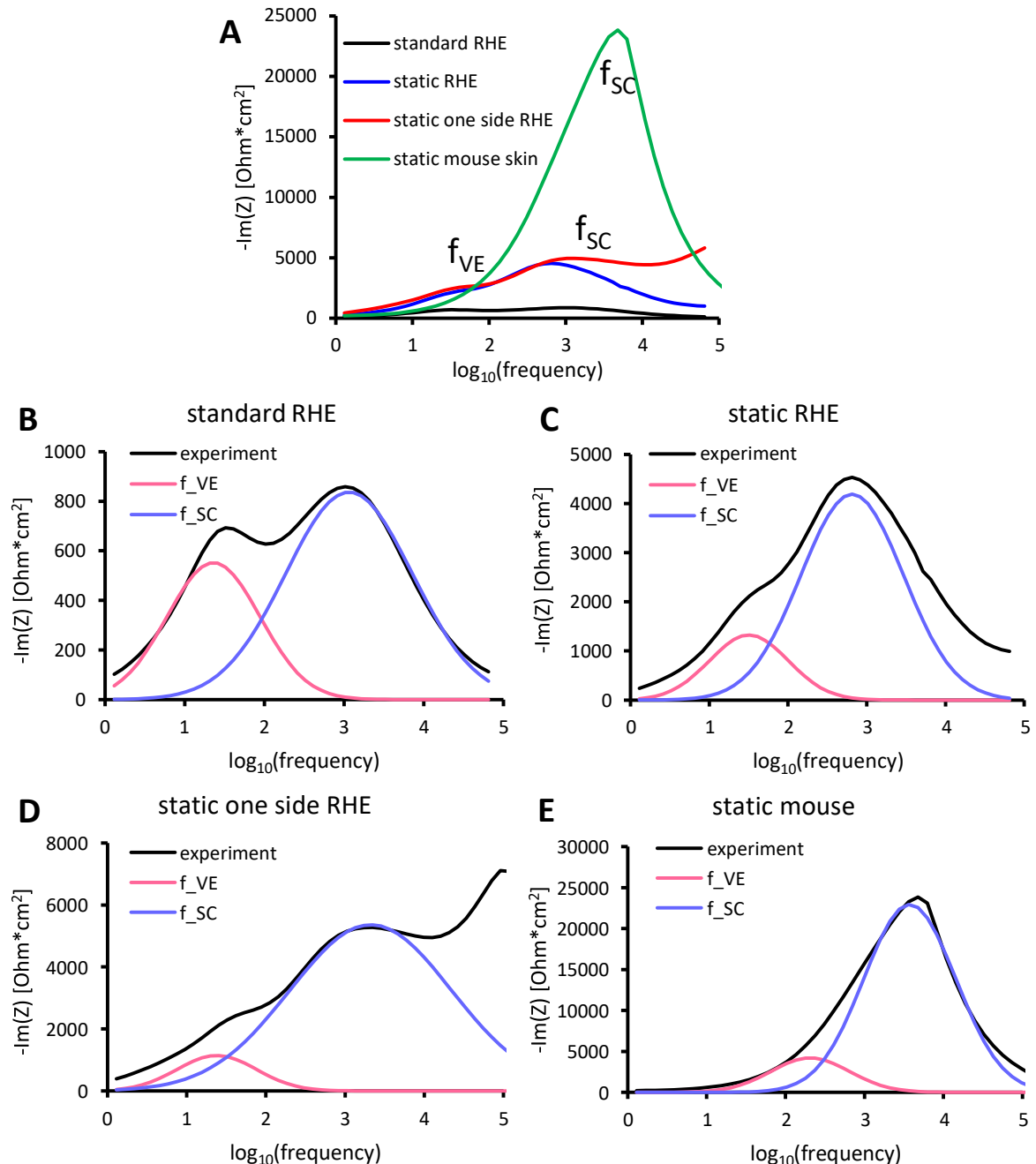


Figure 10: Comparison of different Ussing chamber measurements. Exemplary $-Im(Z)$ vs. $\log_{10}(\text{frequency})$ plots showing the differences and similarities between the different types of measurements. All curves are corrected for the chamber measurement area. Static one sided RHE and static mouse skin are additionally corrected by the factor 0.5, as the current passes two skin layers in these setups. **A:** Different Ussing chamber setups (standard and static Ussing chamber) as well as different skin samples (RHE and mouse ear skin) were used. f_{VE} and f_{SC} are present in all measurements. The total resistance of the standard RHE measurement is much lower compared to the other ones. Static and one sided static have equal curve shapes apart from a relaxation frequency arising at 10^5 Hz for static one side RHE. Static mouse skin has much higher peak height for f_{SC} , even considering the fact that it represents two SC layers on the two sides of the ear. For standard RHE (**B**), static RHE (**C**) and static one side RHE (**D**) f_{VE} the $\log_{10}(\text{frequency})$ value is located around 1.5 (~ 30 Hz) whereas for f_{SC} it is around 3 (~ 1000 Hz). For static mouse skin both values are higher, f_{VE} around 2 (~ 100 Hz) and f_{SC} around 3.5 (~ 3000 Hz).

6 Mice

Mice were housed under standardized conditions (12 hr light/dark cycle; 22–24°C temperature; 55%±15% humidity ad libitum access to standard diet and water). One animal was wildtype (male), 3 animals (all male) had an intestinal-specific knockdown of *Cldn10b* (C10bVilcKO VilCre+), 4 animals (2 male, 2 female) were deficient in *Cldn17* (C17KO -/-) and one animal (female) heterozygous for *Cldn17* (C17KO +/-). Neither protein is expressed in the epidermis, and none of the animals had any overt phenotype (Dr. Susanne M. Krug and Dr. Luca Meoli, Charité Universitätsmedizin Berlin, Germany, unpublished data). Mice were sacrificed by 200 mg/kg Ketamin + 30 mg/kg Xylazin (i.p) followed by cervical dislocation or by cervical dislocation alone.

7 siRNA Effectivity

After homogenisation of the RHEs in RLT-buffer including 1% Mercaptoethanol by using a Tissue Lyser (Qiagen, Hilden, Germany) for 3 min at 30 Hz the homogenate was applied onto a QIAshredder-column and processed as described by the manufacturer (Qiagen). Total RNA was isolated with the RNeasy Mini Kit (Qiagen) according to the manufacturers protocol. RNA concentration was analysed photometrically by using a NanoDrop (ThermoFisher Scientific, Waltham, MA, USA) at 260 nm, purity by evaluating the proportion of A260/A280 and A260/230. If purity was too low the samples were purified with RNease Mini spin columns (Qiagen). cDNA was synthesized by using the Maxima First Strand cDNA Synthesis for RT-qPCR kit (ThermoFischer Scientific) utilizing 1 µg RNA. For qPCR the following Taqman probes were used: Claudin-1: Hs00221623_m1, Claudin-4: Hs00533616_s1, GAPDH (housekeeping gene) (Applied Biosystems, Carlsbad, CA, USA). qPCR was performed with 4.5 µl of a 1:25 dilution of cDNA in a Light cycler 96 (Roche Diagnostics, Mannheim, Germany) with preincubation 120 sec, 50 °C and 600 sec 95 °C, amplification 10 sec 95 °C, 30 sec 60 °C and cooling 30 sec 37 °C. All samples were measured in triplicates. ΔC_t values were calculated by subtracting the C_t value of GAPDH from the C_t value of the gene of interest. $\Delta\Delta C_t$ values were calculated by subtracting the ΔC_t value of the sample (untreated, negative control (=Allstars), *Cldn1*-KD or *Cldn4*-KD) from the mean of ΔC_t values of untreated samples used in parallel in this experiment. Finally, $2^{-\Delta\Delta C_t}$ values were calculated to obtain data for the relative mRNA level. Figure 11 A, B demonstrates, that both the *Cldn1* and *Cldn4* targeting siRNA significantly decreases the corresponding mRNA levels. However, correlation analysis of the normalized TER vs. the mRNA level with Pearson's correlation coefficient shows only significant correlation for the *Cldn1* knockdown (Figure 11 C, D). The normalized TER is calculated by dividing TER value by the maximum TER value of the RHE model batch.

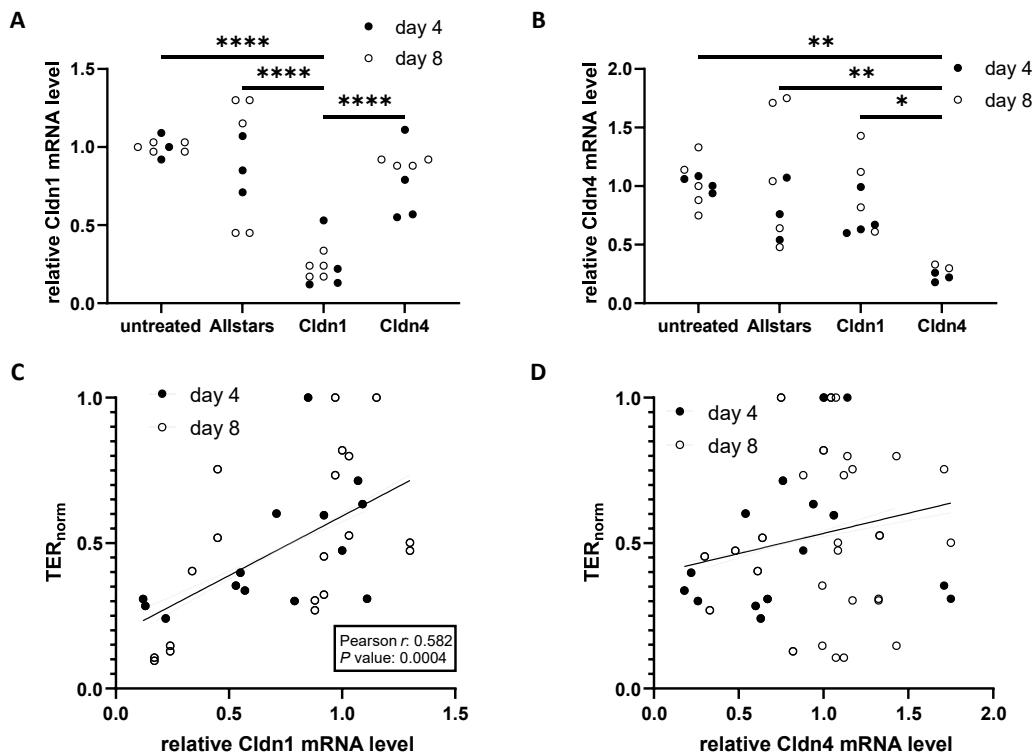


Figure 11: Cldn Knockdown efficiency A, B: relative *Cldn1/4* mRNA levels ($2^{-\Delta\Delta C_t}$) for the different treatments at day 4 and 8 after calcium switch. Both the *Cldn1* and *Cldn4* targeting siRNA yielded significantly lower corresponding mRNA levels. C, D: Correlation analysis of normalized TER (normalized with respect to the maximum TER value of each RHE model batch) vs. the mRNA levels. High correlation is present for *Cldn1* knockdown (C), whereas *Cldn4* KD is uncorrelated (D).

8 References

1. Kochowski S, Nitsch K. Description of the frequency behaviour of metal–SiO₂–GaAs structure characteristics by electrical equivalent circuit with constant phase element. *Thin Solid Films*. 2002;415(1):133-137.
2. Grabarek Z, Gergely J. Zero-length crosslinking procedure with the use of active esters. *Anal Biochem*. 1990;185(1):131-135.