Electrical impedance spectroscopy analysis to evaluate organotypic epidermis formation and barrier function *in vitro*

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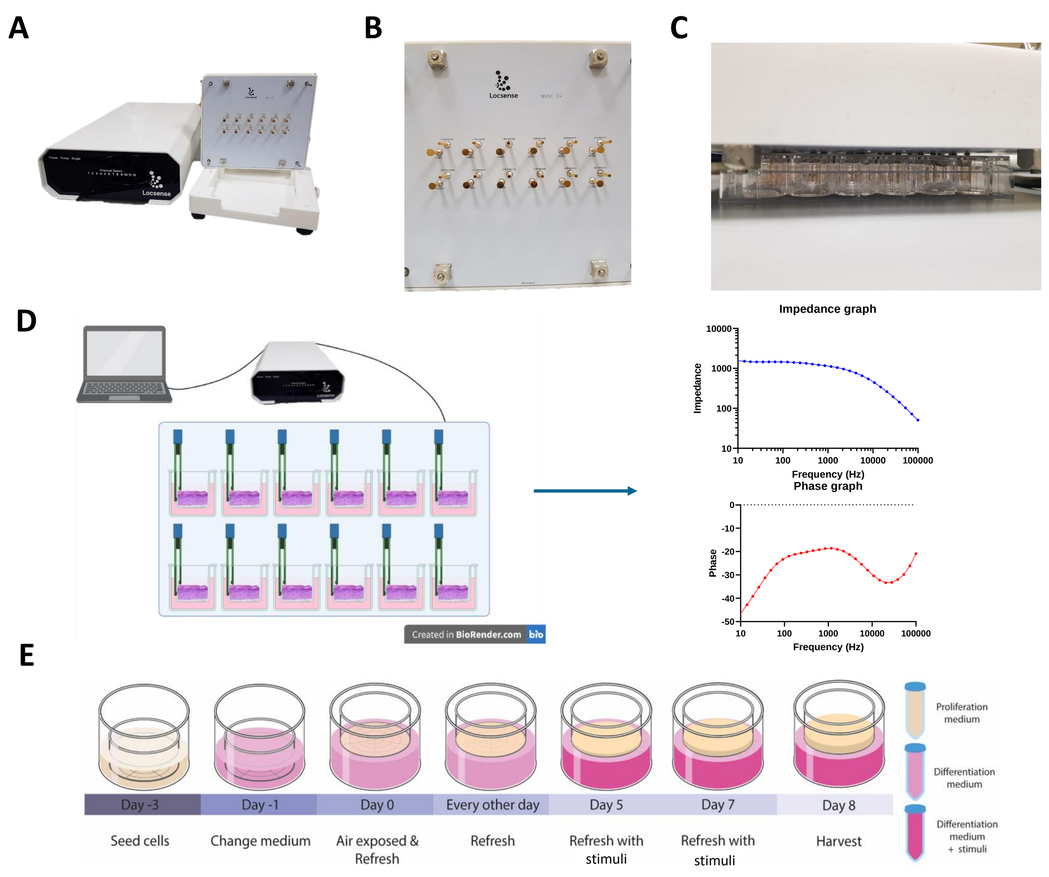
1 | Background

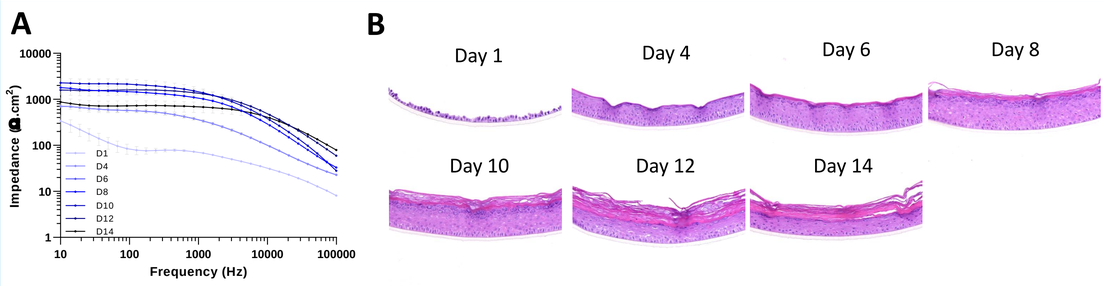
For research into skin biology, diseases and drug or chemical interactions, organotypic 3D human epidermal equivalents (HEEs) are frequently used. Studies heavily rely on end-points analysis for which HEEs are harvested to study cellular responses. Non-intrusive methods that enable longitudinal analysis by repetitive measurements can minimize batch effects, increase study reproducibility and maximize experimental throughput. Here we used a novel 12-well format electrical impedance spectroscopy (EIS) device, customized to fit with a 24-transwell cell culture system to replace conventional static and labor-intensive transepidermal electrical resistance (TEER) analysis using voltohmmeters.

2 | Measurement technique



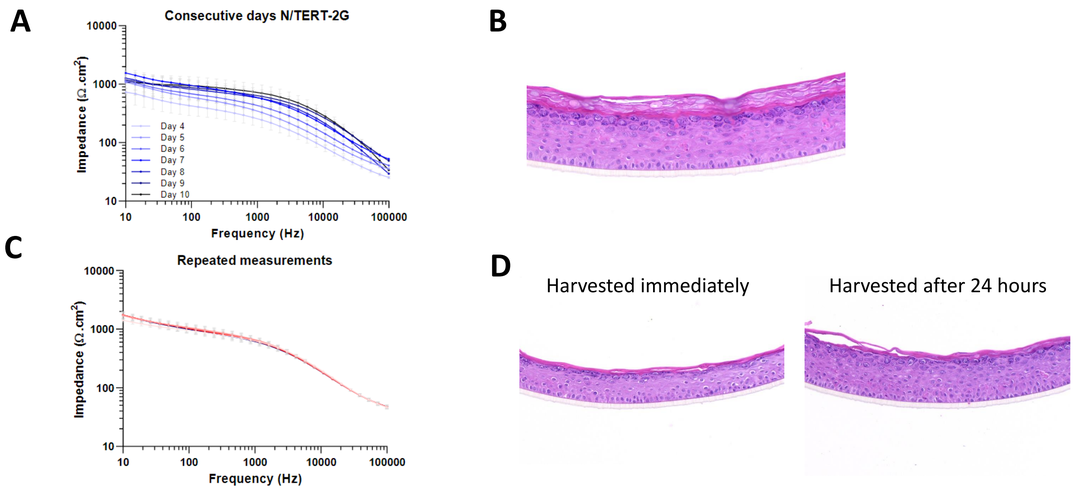
A) The Artemis, a novel EIS measuring setup developed by the company Locsense, provides a novel method of measuring EIS spectra optimized for simultaneous, multi-well measurements of 3D human epidermal equivalents in 24-wells plate setup. **B)** Close up of lid showing probes. **C)** Lid of the setup allows for easy cleaning of probes with ethanol. **D)** Schematic overview of measuring setup. These measurements provide both impedance data as well as phase data. **E)** Schematic overview of 3D human epidermal equivalent (HEE) culture. Stimulations were added from day 5 of air exposure onwards. EIS could be measured daily for HEEs from air exposure onwards.

3 |EIS increases over time of barrier formation



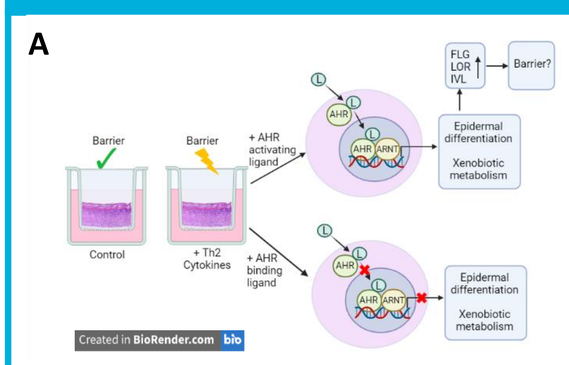
EIS was measured over time of HEE development to determine whether the formation of the barrier could be followed. After measurement, HEEs were immediately harvested. **A)** EIS impedance graphs showed an increase in barrier formation from day 1 until day 10 of HEE development, after which impedance is lowered in days 12-14. **B)** H&E stainings of HEEs show increasing epidermal stratification over time. However, at day 12 and 14 increase in stratum corneum and los of granular layer and epidermal thickness is observed, possibly explaining lowered EIS measurements.

4| Consecutive measurements are not harmful for epithelial development



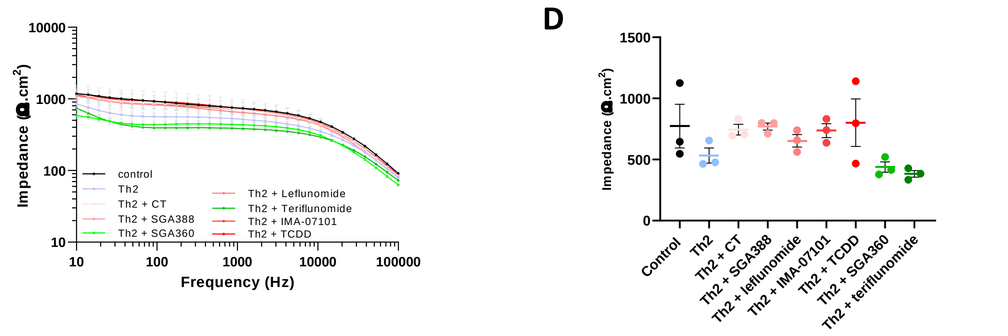
To determine whether EIS measurements are suitable for longitudinal analysis, whilst not being harmful for the constructs, 3 constructs were measured daily over development from day 4 until day 10 **A)** EIS impedance spectra show increase in resistance, indicating formation of barrier without damaging HEEs. **B)** Intactness of HEEs was also confirmed by H&E staining. Moreover, to determine harmfulness, constructs were measured six times within 1 hour. **C)** EIS impedance spectra remained consistent over measurements and **D)** constructs were not harmed by repeated measures

5 | Cytokine-induced reduction of EIS can be rescued by treatment with AHR agonists



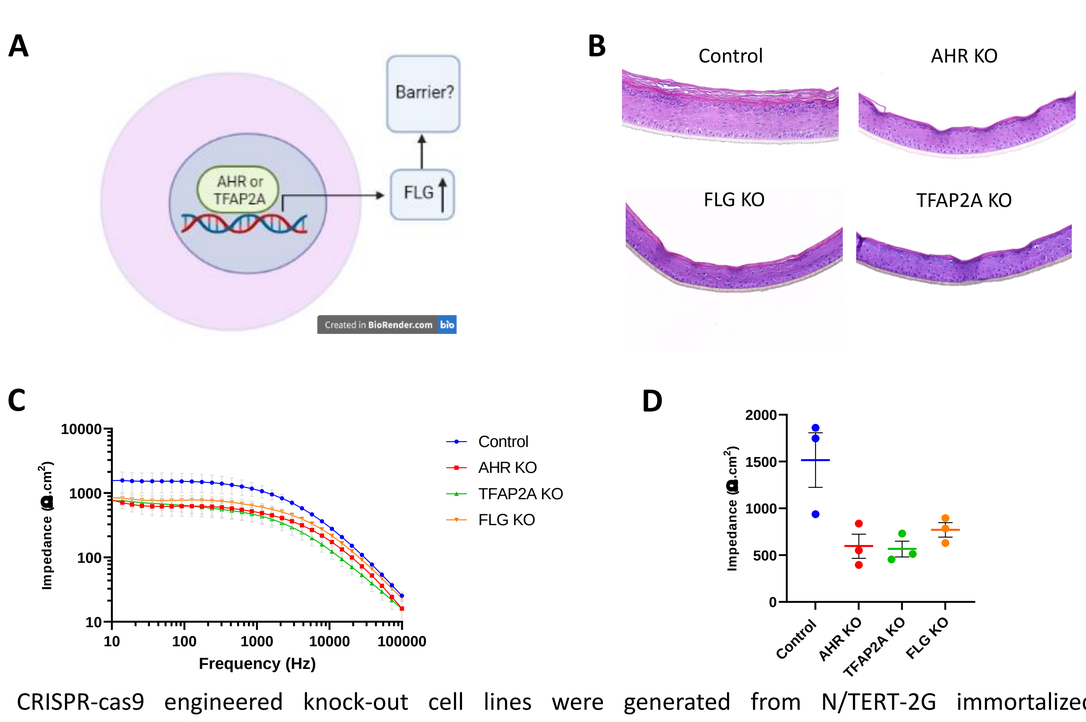
B

Ligand	AHR pathway activation?	Epidermal differentiation?
Coal tar (CT)	Yes	Yes
SGA388	Yes	Yes
Leflunomide	Yes	Yes
IMA-7101	Yes	Yes
TCDD	Yes	Yes
SGA360	No	No
Teriflunomide	No	No



A) To induce epithelial barrier defect, atopic dermatitis associated Th2 cytokines were added to the culture. Moreover, potentially therapeutic AHR agonists were added to determine whether barrier defects could be rescued. The AHR is a transcription factor, inducing epidermal differentiation upon activation. **B)** Some AHR ligands bind and activate the AHR pathway, whilst others only bind without activation. **C)** Addition of Th2 cytokines lowers EIS spectra and rescue is seen when treated with AHR activating agonists. No rescue is seen when treated with SGA360 or teriflunomide, however both of these ligands do not induce active AHR signaling. **D)** Similar effects are seen when impedance values are plotted where phase measurements are closest to zero.

6 |Barrier defects caused by knockout lower EIS measurements



CRISPR-cas9 engineered knock-out cell lines were generated from N/TERT-2G immortalized keratinocytes. **A)** AHR and TFAP2A were knocked out and both play a role as transcription factor in epidermal differentiation. Moreover, terminal differentiation gene filaggrin was knocked out, which is associated with maintaining the skin barrier. **B)** H&E stainings indicate varying terminal differentiation effects in knock out cell lines. **C)** All three knock-out cell lines show reduction in EIS spectra, indicating impaired barrier. **D)** Similar effects are seen when looking at values which are closest to a phase zero.

6 | Conclusion and perspectives

- This novel EIS measuring device proved ability to measure epidermal barrier formation in HEEs without damaging the construct or hampering development. Even after multiple measurements on consecutive days or within the same hour, constructs remained without damage.
- EIS measurement spectra showed decrease when HEEs were stimulated with Th2 cytokines. These EIS measurements proved that this barrier defect is also reproduced in Th2-stimulated HEEs and can be rescued upon treatment with known AHR activating compounds.
- Moreover, knock-outs of genes involved in the development and maintenance of the skin barrier showed an impaired EIS spectrum, indicating present barrier defects in the cell lines.
- For future research, this device provides a valuable tool for following development of cell culture models in a longitudinal fashion, without need for terminating the experiment by harvesting.
- Additionally, this provides a useful method to study effects of stimulating compounds, therapeutics or gene mutations on development and maintenance of the epidermal barrier.

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