

GPCR Mediated Signal Transduction in Endothelial Cells

Application Note

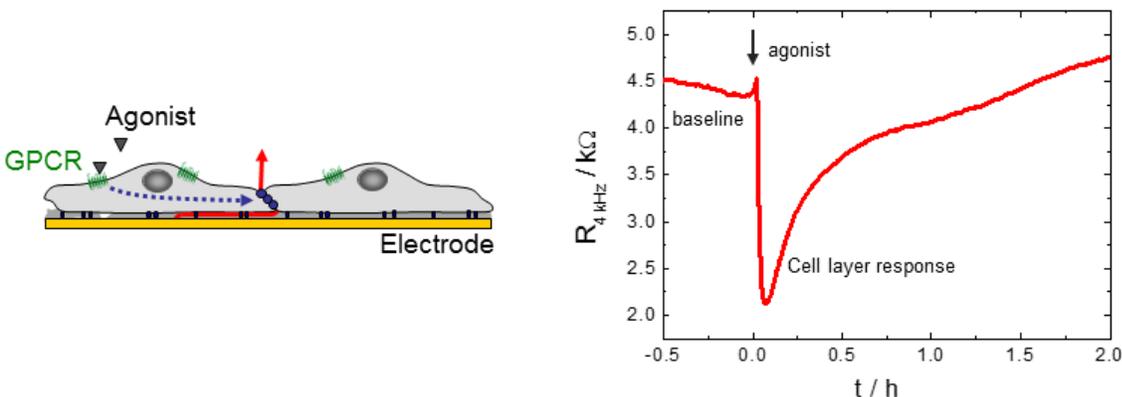


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Overview

	This AN's Model Experiment	Alternatives / Extended Use
Cell type	Endothelial cells HDMEC	Other endothelial cell types, Epithelial cells, other cell types
Array type	8W10E+	8W10E , 96W20idf / 8W1E, 96W1E
ECIS Machine	ECIS Z0, 16W array station	96W array station, ECIS Z
Specialized Materials	GPCR Agonists thrombin, histamine, S1P	Other receptor type agonists / receptor antagonists, combination with inhibitors, transfection for knock-down or overexpression
Data acquisition parameters	<u>Cell layer quality:</u> spectrum, MFT 48-96h <u>GPCR Assay:</u> SFT 4 kHz, 1h+	Monitoring time depends on cell type and agonist response kinetics. Optimum SFT Frequency is influenced by cell type, array type
Monitoring Parameter	<u>Cell layer quality:</u> Z , R, C <u>GPCR Assay:</u> R	Z can be used instead of R, when using the ECIS Z, the parameters R and C are not available.
Time including all preparations	4 – 6 days	depends on cell type and agonist response kinetics

Introduction

Cells communicate with their surroundings via cell surface receptors. Often, receptor stimulation triggers some kind of cell morphology change, typically through changes in the cytoskeleton or at cell-cell and cell-substrate binding proteins. G-protein coupled receptors belong to one of the most widely studied receptor type, as changes function or expression of members of the 7-transmembrane receptor group can be associated with numerous diseases.

The vascular endothelium is a prime example to illustrate the importance of GPCR activity studies. Various members of the GPCR family are associated with the regulation of endothelial barrier function, which is an essential means to control proper functionality of the vascular wall during infection and inflammation. Cell-cell junctions are temporarily opened to allow for fluid and immune cell extravasation to clear the affected tissue. However, chronic impairment of the endothelium induced by deregulation of GPCR signaling can lead to serious problems.

Therefore a proper understanding of signaling mechanisms and their implications belong to one of the central tasks of research. ECIS can be a useful tool to study the functional implications of receptor signaling. Naturally ECIS experiments are most successful when receptor stimulation results in changes in cell morphology, i.e. cell layer integrity and structure.

For example, endothelial barrier function is modulated by response to Histamine, Thrombin and Sphingosine-1-phosphate (S1P), which will be the show-examples in this application note.

Histamine and Thrombin lead to a transient breakdown of endothelial barrier integrity. However with different kinetics at different concentrations. S1P on the other hand is known to support endothelial barrier function. Histamine, Thrombin and S1P each represent a different class of agonists. Histamine is a small biogenic amine, binding to the H1-4 family of GPCR receptors. Thrombin acts as an enzyme and cleaves an N-terminal peptide from the extracellular portion of PAR receptor type. The newly formed N-terminus reacts with the GPCR binding site to activate signaling. S1P is a lipid binding to the S1P1-5 receptors family.

Equipment

Here we provide the equipment as it was used for the example experiments.

ECIS machine	ECIS Z0 + CO ₂ incubator
ECIS equipment	8W array station (96W array station)
Array types	8W10E+ (96W20idf)
Cells	Human Dermal Microvascular Endothelial Cells (HDMEC)
Medium	<u>Growth medium</u> EBM-2MV + Growth factor bullet kit (Lonza) + 10% FBS + Penicillin/Streptomycin + L-Glutamin <u>Serum-free medium</u> EBM-2MV +0.2% FBS + Penicillin/Streptomycin + L-Glutamin
Solutions	PBS Trypsin/EDTA HEPES
Agonists	Thrombin 5 nM Histamine 10 μM S1P 1 μM In serum-free medium or HEPES buffer

Materials

Reagents

- EBM2 basal medium (Lonza, cat. no. CC-3156)
- EGM2-MV bullet kit for microvascular endothelial cells (Lonza, cat. no. CC-4147)
- L-Glutamine 200 mM (100 x) (Thermo Fisher Scientific, HyClone™, cat. no. SH30034.01)
- Antibiotic-Antimycotic (Gibco®, Life Technologies, cat. no. 15240-062)
- sterile PBS (see REAGENT SETUP)

- sterile HBSS (see REAGENT SETUP)
- Sodium Chloride (NaCl) (Thermo Fisher Scientific, cat. no. L-11630)
- Potassium Chloride (KCl) (Thermo Fisher Scientific, cat. no. P217-500)
- Di-Sodium Hydrogenphosphate, anhydrous (Na₂HPO₄) (Thermo Fisher Scientific, cat. no. S374-500)
- Potassium-Dihydrogenphosphate (KH₂PO₄) (Thermo Fisher Scientific, cat. no. P285-500)
- Magnesium Chloride (MgCl₂·6H₂O) (Thermo Fisher Scientific, cat. no. M33-500)
- Calcium Chloride (CaCl₂·2H₂O) (Thermo Fisher Scientific, cat. no. C79-500)
- HEPES (Thermo Fisher Scientific, cat. no. BP310-500)
- Trypsin EDTA, 0.05% Trypsin / 0.53 mM EDTA in HBSS (Corning, cellgro[®], cat. no. 25-052-CI)
- Cysteine solution 10 mM in water (ECIS electrode-stabilizing solution) (Applied BioPhysics Inc, Troy)
- Gelatin (e.g. Acros organics, gelatin for analysis, granular, cat. no. AC410875000, cas 9000-70-8)
- Thrombin, from bovine plasma, (Sigma-Aldrich, cat. no. T4648)
- Histamine dihydrochloride (Sigma-Aldrich, cat. no. 53300)
- Sphingosine-1-phosphate (S1P) (Sigma-Aldrich, cat. no. 73914)
- Methanol (Sigma-Aldrich, cat. no. 494437)
- Fatty acid-free BSA from bovine plasma (Sigma-Aldrich, cat. no. A8806)

Equipment

- ECIS Z0 machine (Applied BioPhysics Inc. Troy, NY, USA) with 16W array station, humidified cell culture incubator and PC
- ECIS 8W10E+ 8-well Arrays
- Biosafety hood with vertical laminar flow equipped with UV light for decontamination
- CO₂ incubator
- Centrifuge (Eppendorf)
- Nitrogen stream supply
- Sonication bath
- Plasmalyzer (optional, for sterilization and cleaning ECIS electrodes)
- Vortexer
- Aspirator
- 15 ml and 50 ml conical tubes
- Sterile microtubes
- Micropipettes 1 – 10 µl, 2 – 20 µl, 20 – 200 µl, 0.1 – 1 ml
- Sterile serological pipettes 5 ml, 10 ml
- Cell flasks, T75
- Brueker cell counting chamber

Reagent Setup

1 x PBS

Dissolve 8.0 g NaCl, 0.2 g KCl, 1.42 g Na₂HPO₄ and 0.24 g KH₂PO₄ in 1 L H₂O and adjust pH to 7.4. Sterile filter or autoclave.

1 x HBSS

Dissolve 8.18 g NaCl, 0.35 g KCl, 0.23 g MgCl₂·6H₂O, 0.294 g CaCl₂·2H₂O and 2.38 g HEPES in 1 L H₂O and adjust pH to 7.4. Sterile filter or autoclave.

HDMEC Medium

Add 5 ml 100 × L-glutamine, 5 ml 100x Antibiotics/Antimycotics, the Serum and all supplements that are provided with the EGM-2V bullet kit to 500 ml EBM-2 basal medium. **Critical Step** Make aliquots of 50 – 100 ml and store at 4 – 8 °C and protect from light (e.g. wrap in aluminum foil). Prevent frequent and long-term re-heating to 37 °C.

Low (0.3 %) Serum Medium

Add 150 µl FBS, 500 µl 100 × Antibiotics-Antimycotics (see reagents) and 500 µl 100 × L-Glutamine solution (see reagents) to 50 ml EBM-2 basal medium.

0.2 % Gelatin solution for substrate coating

Make 5 % stock solution by mixing 5 g gelatin (see reagents) with 100 ml MilliQ in an autoclavable glass bottle. Note: Gelatin will not dissolve until heated. Sterilize by autoclaving at 121 °C for 20 min. Store stock solution at 4 – 8 °C and pre-warm to 37 °C to make 1:25 dilution in PBS. Make fresh 0.2 % gelatin solution about every 2 weeks and keep stored at 4 – 8 °C.

100 × Fatty Acid-Free BSA

Dissolve 3 mg fatty acid-free BSA (see Reagents) in 1 ml HEPES to give a 100 × stock solution. Sterile filter and store for up to 1 month at 4 – 8 °C.

Agonist Solutions

Note: For two 8-well arrays prepare minimum of 350 µl of agonist containing 20 × working solution. Instructions are given for final agonist concentrations of 5 nM Thrombin, 10 µM Histamine and 1 µM S1P.

Thrombin

! Caution Thrombin is a skin and respiratory sensitizer, avoid eye and skin contact (download MSDS from www.sigmaaldrich.com).

Reconstitute the thrombin lyophilisate by dissolving it in an appropriate amount of MilliQ water to give a 100 µM stock solution. Since the lyophilisate not only contains

pure thrombin, the amount of thrombin has to be calculated via the information on NIH units per protein and NIH units per solid in the product. Freeze aliquots a 1 – 10 μl at $-80\text{ }^{\circ}\text{C}$. Immediately before use in an experiment make a 100 nM working solution (for a final concentration of 5 nM, 20 μl per well) in pre-warmed low serum medium. Before adding to the cell layers briefly pre-warm to $37\text{ }^{\circ}\text{C}$ in a water bath.

Histamine

! Caution Histamine is toxic by ingestion, skin and eye irritant, respiratory sensitizer (download MSDS from www.sigmaaldrich.com).

Make 1M Histamine stock solution in MilliQ and store 1 μl aliquots at $-20\text{ }^{\circ}\text{C}$. Before use, make a 200 μM 20 \times working solution in serum-free medium (for a final concentration of 10 μM , 20 μl per well). Pre-warm to $37\text{ }^{\circ}\text{C}$ about 5 min before addition to the cells.

S1P

! Caution S1P is a skin and eye irritant and may cause respiratory irritation, toxic to target organs (download MSDS from www.sigmaaldrich.com).

Dissolve S1P in Methanol to give a stock solution of 1 mM. **! Caution** Methanol is flammable, toxic by inhalation, ingestion and skin absorption (download MSDS from www.sigmaaldrich.com).

Support reconstitution by vortexing followed by 5 min sonication at room temperature. Freeze aliquots of 10 μl at $-80\text{ }^{\circ}\text{C}$. To avoid changes in concentration by methanol evaporation over long-term storage and repeated opening of the vial evaporate methanol under gentle nitrogen stream and store the lipid films. Before use add 5 μl of the 100 \times fatty acid-free BSA directly onto the lipid film, let sit for 1 min and add 495 μl HBSS and sonicate for 5 min. Briefly pre-warm to $37\text{ }^{\circ}\text{C}$ and vortex before addition to the cells.

Limitations

Only GPCR responses that are associated with alteration in cell morphology and thus, cell layer structural aspects can be detected. And the sensitivity for catching cell morphology change events with ECIS depends on the initial impedance of the cell layer and on the activity of the signaling pathway under study.

The initial impedance of the cell layer is a function of cell type, cell density and state of cell-junctional maturation. Therefore, cell culture conditions should be controlled diligently.

For the GPCR assay applications presented here the cell layer under study should be in a confluent stage, ideally showing “steady state” behavior. When a GPCR signaling pathway acts on the opening of cell-cell junctions the cell layer under study needs to have junctions established in order to make the assay work. On the other hand, if an

agonist is supposed to enhance cell-cell junctions a cell layer with maximal matured junctions may not respond to the selected agonist, unless combined with barrier disrupting agents. We will discuss some of these examples in the protocol section Under sub-confluent conditions the effect of GPCR signaling on cell growth, attachment, spreading and maturation can be studied in principle, but is not covered by this application note.

Despite the strengths of ECIS for endothelial resistance monitoring various limiting aspects have to be considered. The temporal resolution of the measurement is limited by the degree of parallelization and information content, i.e. single frequency or multiple frequency data acquisition. The temporal resolution for two 8-well arrays (16 samples) measured at one frequency is 10 s. For very fast and transient responses to agonist stimulation or for a detailed analysis of multiphasic response profiles this resolution might be insufficient. Currently, maximal resolution of about 200 ms is obtained for single well recordings at one frequency. Parallelization can be increased when 96-well ECIS plates are used, even though limitations in temporal resolution have then to be considered.

As far as scientific conclusions based on ECIS measurements are concerned, the reader should still be aware that ECIS is an *in vitro* technique. There is no compartmentalization into a luminal and abluminal side and the substrate is rigid. Thus, changes in endothelial cell layer resistance measured with ECIS cannot reflect any tensional adjustments that would occur in soft tissue, i.e. contraction / dilatation of blood vessels.

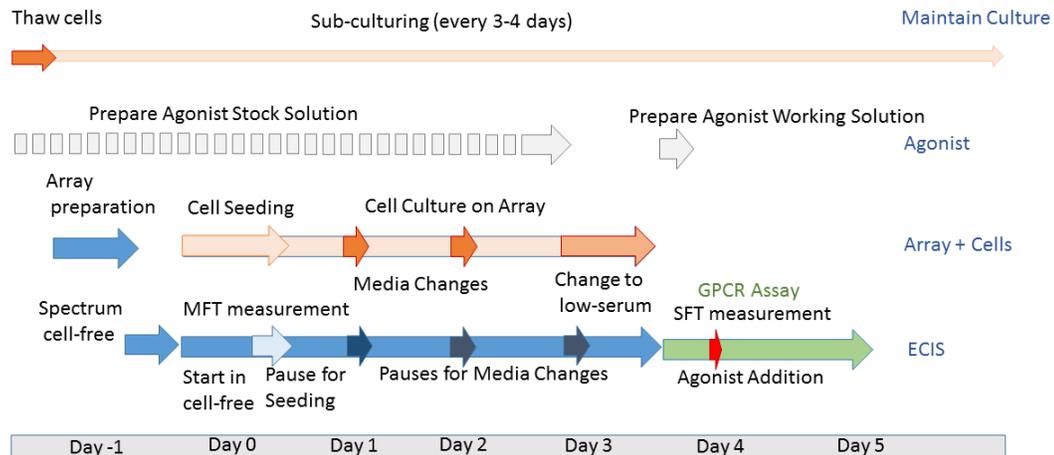
Experiment Plan

Assay Design

Necessary steps:

1. Array preparation
2. Cell seeding / culture on the array
3. Preparation of agonist / inhibitor solutions
4. ECIS measurement setup/ GPCR Assay procedure

Time Schedule



Array Type

The 8W10E+ format is very popular in endothelial barrier function studies, especially in a conventional laboratory setting.

The 2 x 20 electrodes, which are evenly distributed over the entire well area are great to obtain good statistical coverage over the cell layer. 8W10E+ is the second generation array type that followed the 8W10E electrodes with 10 electrodes in the center of the well. The 8W10E type can be used as an alternative to the 8W10E+ arrays, as the total impedance is in a similar range. 8W10E may be preferred over 8W10E+ when the cell layer is sensitive to shear stress by fluid exchange and tends to detach from the substrate at the well edges.

The decrease in sensitivity (smaller difference of impedance between cell-covered and cell-free area, and thus a smaller dynamic range for changes in between) is not problematic for endothelia (and epithelia) that form a good barrier.

For cell types with weaker cell-cell junctions and where the intensity of the response upon experimental manipulation is weak use of 8W1E may be of advantage. A possible disadvantage of increased sensitivity though is that weak response signals might be concealed by strong micromotion activity.

Negative control

Include one well without stimulation with agonist, by adding medium/carrier solution without agonist (same volume as in treated wells).

If the agonist is administered from a stock solution that contains residuals of solvent (DMSO, methanol, ethanol), make sure to include a solvent control.

Especially in the first experiment one should make sure that the solvent of the active substance has no effect on the cell layer, as they could interfere with the actual agonist activity.

- Medium/buffer (optional in later experiments, when any influence of the solvent can be excluded)
- Medium/buffer + solvent control (without agonist)
- Medium/buffer + agonist

Positive Control

Case1: Study of not yet established agonists

Use an established agonist for the respective receptor under study. E.g. if examining different potential H1R agonists use the natural agonist histamine as positive control

Case 2: Study of signaling cascade inhibitors/activators

In this type of experiments the agonist is added to induce the signaling cascade. Pre-incubation with an inhibitor – or simultaneous addition of agonist and inhibitor are performed to investigate the potential inhibitor's effect on the signaling event.

Use at least one well where no additional inhibitor is administered, but the agonist only is included. If the inhibitor solutions as used contain residuals of solvent (DMSO, ethanol), make sure to include a solvent control.

Specialized Materials

Agonist of choice (here: Thrombin, Histamine and S1P)

For S1P a sonication bath is needed for proper solubilization

If applicable some signaling pathway inhibitor

If applicable transfection reagents and DNA/siRNA of choice

Cell Cultivation

Please find a detailed protocol for cultivation of HDMEC on 8-well ECIS electrodes in the **Protocol** section.

Data Acquisition Parameters

First experiment with a given cell type/array type

A) MFT during attachment and maturation, including switch to low-serum medium ([Z], R, C)

B) Frequency Scan of cell-free and cell-covered electrode

C) SFT monitoring of response to GPCR agonist (R) at 4 kHz ($|Z|$ and C will be recorded automatically when using the Z θ)

Routine experiments

A) MFT Monitoring of attachment and spreading is optional, but not necessary when cell culture protocol is established

B) Frequency scan of the cell layer before starting the GPCR assay is recommended. It is a good way to check the quality of the cell layers

C) SFT monitoring of response to GPCR agonist (R) at 4 kHz

Protocol

Array Preparation

Timing ~ 30 min

ECIS culture ware comes sterile and clean when fresh from the shelf. For ECIS culture ware stored over a long time impurities might have adsorbed to the gold electrodes and 100 % sterility might not be guaranteed (> 6 month). In this case we recommend to follow steps 1 and 2. With fresh arrays steps 1 and 2 are optional (just maintain a standardized protocol).

1. If necessary (arrays have been on shelf for over about 3 months) clean and sterilize ECIS array by either 15 s oxygen plasma treatment or by 15 min UV exposure
2. Stabilize electrodes by 10 min incubation with 10 mM cysteine in distilled water (ECIS electrode-stabilizing solution). Rinse at least 3 times with MilliQ after cysteine treatment.
3. Add 300 μ l of 0.2 % gelatin in PBS (see REAGENT SETUP) to each 8-well chamber and incubate electrodes with gelatin for at least 10 min.
4. Remove gelatin and rinse with PBS or medium once.

Cell Preparation

HDMEC Cultivation

In this protocol ECIS studies on endothelial barrier function are performed with primary HDMEC, which are isolated from human foreskin as described elsewhere⁷⁵ and stored in vials of 10^6 cells in 1 ml FBS with 10% DMSO under liquid nitrogen.

Note that cultivation demands, growth behavior on ECIS electrodes, cell layer resistance and response profiles to agonists can differ from cell type to cell type.

Taking HDMEC in culture [Timing ~ 1 h](#)

1. Take cryovial (typically HDMEC of passage 3 – 4) from liquid nitrogen and place in -20°C for 30 min.
2. Prepare for the next steps: Pre-warm HDMEC medium, prepare aspirator and pipetor and place 50 ml conical tube under the hood.
3. Take vial from -20°C and rapidly thaw in 37°C water bath till only a small ice core is left.
4. Sterilize vial with 70% ethanol and transfer cells to 50 ml conical tube.
5. Immediately, but only drop-wise add 10 ml 37°C HDMEC medium.
7. Spin down cells for 10 min at 120 rpm at room temperature.
8. Aspirate medium and very gently re-suspend cells in 12 ml complete medium
9. Seed suspension on T75 flask.
10. After 1 day check cells under a microscope and change medium.

[Checkpoint: Trouble Shooting \(1\)](#)

11. Passage HDMEC at least once before using in ECIS experiments.

Critical Step Watch the passage number of your culture. HDMEC were not used beyond passage 9. This may vary for your cell type.

Plan enough time for thawing and passaging cells before barrier function studies are performed

HDMEC Subculturing [Timing ~ 1 h](#)

Passage HDMEC every 3 – 5 days, before reaching confluence (optimum $\sim 90\%$ confluence).

1. Gently rinse HDMEC on T75 flask with 12 ml 37°C PBS.
2. Aspirate PBS after first wash and add back fresh PBS for 5 – 10 min incubation in PBS at 37°C

Critical Step Pre-incubation with PBS loosens cell-cell junctions before trypsin treatment and prevents sheet-like detachment and aggregate-formation, which is important to receive single-cell suspensions. Time of pre-incubation in PBS depends on cell density and strength of cell-cell junctions within the cell layer on the flask. Check cell layer after 5 min, extend to up to 10 min for dense and tight cell layers.

3. Aspirate PBS, add 2.5 ml 37°C Trypsin EDTA and incubate for 5 – 8 min.

Critical Step Time of trypsin treatment depends on cell density and strength of cell-cell junctions. Check detachment of cells after 5 min, extend to 8 min if necessary. Tapping the flask with the long side edge to the work bench can support detachment.

4. Add 10 ml medium, suspend cells by pipetting up and down 3 – 4 times and spin cells down by centrifugation at 120 rpm for 10 min.

5. Re-suspend cell pellet in 10 ml HDMEC medium

Critical Step Re-suspend by pipetting up and down about 10 times to obtain single-cell-suspension.

6. Seed in a ratio of 1:3 to new T75 flask.

Cultivation of HDMEC cell layers on ECIS Electrodes Timing 2 – 4 d

1. Make single-cell suspension as described under steps **HDMEC Subculturing**. Cells harvested from one T75 flask at ~90 % confluence are usually sufficient for at least two 8-well ECIS arrays (or one 96-well plate).

Critical Step Use fresh HDMEC media for HDMEC cultivation.

2. Count cell density and adjust cell suspension to 200 000 cells/ml.

Check Point: Trouble Shooting (2)

3 Add 400 μ l of the 200 000 cells/ml suspension to each well of the 8-well ECIS array

4. Leave arrays with cells for ~ 10 min on the bench to allow the cells to settle to the array bottom uniformly.

5. Cultivate for 2 – 4 days (depending on the agonist study, see below) in a humidified incubator with 5 % CO₂ at 37 °C. Regularly inspect the cell layer under a microscope (about every 1 – 2 days).

Check Point: Trouble Shooting (3)

Critical Step The time of cultivation on the array critically influences maturation of cell-cell junctions and receptor expression. When barrier disrupting effects are to be monitored cells have to be cultivated on the arrays for at least 72 h, when cell layers have reached basal resistances of about 3 k Ω or above. Barrier enhancing effects on the other hand are best seen, when cell junctions are still in immature state after about 48 h.

6. Exchange half of the medium every 24 h by removing 200 μ l old medium and adding back 200 μ l fresh medium. **Critical Step** Without media changes the cell layers establish lower baseline resistances and higher variability in baseline values as well as response kinetics to agonists. The signal intensity was found to be negatively affected without regular media changes. Exchanging only 50 % of medium instead of a full exchange reduces mechanical stress on the cell layer.

7. 6 – 24 h before the experiment exchange complete medium for low serum medium.

Critical Step For some agonists serum starvation helps to increase the cell layer response, e.g. for histamine serum starvation for 6 – 12 h was found to pronounce the resistance drop at 4 kHz compared to histamine addition in complete medium or after serum starvation for 24 h. For S1P stimulation 6 h starvation gave strongest response, whereas intensity of thrombin response seem to be quite independent from time of serum starvation. However, other endothelial cell types might respond differently to serum starvation, i.e. reduce barrier function when incubated in low serum medium for too long.

Data Acquisition

Monitoring of HDMEC cell layer Maturation and Cell Layer Quality Control with ECIS (optional) **Timing ~5 d**

For initial experiments with HDMEC (or also other cell types) we recommend to monitor the entire attachment, growth and cell layer maturation process with ECIS at least once in order to select the right time frame for endothelial barrier studies for different agonists.

1. One day before cell seeding prepare ECIS arrays as described under **Cell Preparation** and include cysteine pre-treatment **Critical Step** cysteine treatment is critical for attachment and spreading measurements because the forming self-assembled cysteine monolayer on the gold establishes a clean, defined electrode surface, which is important for stable interface impedance. **Timing ~30 min**

2. Leave arrays in medium over night (at least 4 h)

Critical Step Equilibration in medium additionally helps to establish stable electrode-electrolyte interface impedance. If the electrodes are not well stabilized, a drift in baseline impedance of the cell-free electrodes will interfere with impedance signal from cell attachment and spreading).

3. Record a frequency scan of the cell-free electrodes. **Timing ~30 min**

Record impedance, resistance and capacitance for the frequency range between 10^2 and 10^5 Hz. Use the frequency default settings of the ECIS Z θ machine (31.25; 44.19417; 62.5; 88.38835; 125; 176.7767; 250; 353.55339; 500; 707.10678; 1000; 1414.2136; 2000; 2828.4271; 4000; 5656.8542; 8000; 11313.708; 16000; 22627.417; 32000; 45254.834; 64000; 90509.668 and 100000 Hz).

Check Point: Trouble Shooting (4)

4. Monitor HDMEC attachment, spreading and cell layer maturation. **Timing 4 d**

Use the multiple frequency time (MFT) collect mode to record the systems behavior for multiple frequencies, including the typical 4 kHz and 40 kHz, sensitive for cell-cell junctions or cell-coverage, respectively. The default settings do typically not include the 40 kHz, so either use 32 kHz from default settings instead or use custom frequencies and include 40 kHz (For setting the frequencies in the ECIS software: go to: Edit: sample frequencies: custom: type in default frequencies (62.5, 125, 250, 500, 1000, 2000, 4000, 8000, 16000, 32000, 64000 Hz) and include 40 000 Hz)

i) Record a baseline of the cell-free array with 400 μ l medium in each well over at least 1 h. The baseline should be flat and not drifting.

Check Point: Trouble Shooting (5)

ii) Prepare single cell suspension as described under **HDMEC Subculturing**.

iii) Pause ECIS data acquisition (klick pause button in ECIS software), remove array from ECIS holder, place under sterile hood to replace cell-free medium with 400 μ l cell suspension. Place the array back to the ECIS holder, check for electrical contact and resume the measurement (follow instructions within the ECIS software).

iv) After 24 h pause the measurement for media change. Under the hood remove 200 μ l used medium from each well and add back 200 μ l fresh pre-warmed medium and resume measurement.

v) After 48 h and 72 h repeat step 30iv. Optional: After 72 h or a later time point you may do a complete exchange of medium for low serum medium when you plan to use cell layers for an agonist stimulation experiment (e.g. histamine or thrombin).

[Check Point: Trouble Shooting \(6\)](#)

5. Record a frequency scan of the cell-covered electrodes. **Timing < 30 min**
Record impedance, resistance and capacitance for the frequency range between 10^2 and 10^5 Hz. Use the frequency default settings as described above (see step 29).

[Check Point: Trouble Shooting \(7\)](#)

Monitoring of HDMEC Response to Agonists

A) Monitoring of HDMEC Response to Thrombin

General Prerequisites

Use HDMEC after 4 days of cultivation on the ECIS electrodes as described under **Cultivation of HDMEC cell layers on ECIS Electrodes**. Note: Because of differences in cell source or endothelial cell type, the optimal time point for thrombin stimulation may vary differ as well as the sensitivity to serum starvation.

Change to serum-free medium 1 day (12 – 24 h) before the experiment (after ~ 72 h). Check the cell layer quality by phase contrast microscopy and by either recording an impedance spectrum or running a multiple frequency time collect measurement for 10 – 30 min. The resistance at 4 kHz should be in the range of 2 500 – 4 500 Ω for HDMEC grown on 8W10E+ or 8W10E electrodes. On 8W1E the resistance should be in the range of 20 – 35 k Ω .

Assay

1. To monitor agonist response use single frequency time (SFT) mode and select 4 kHz or other sensitive frequency (typically between 400 Hz and 4 kHz for barrier function studies).

Critical Step Be aware that the intensity of the signal depends on the selected monitoring frequency. The sensitivity of the monitoring frequency can vary with different cell types and electrode layouts. Sensitive frequency to use for monitoring of cell layer response can be determined as described above (divide resistance values of a cell-covered electrode by values of the cell-free electrode for each frequency of the frequency scan spectra. The maximum of the bell-shaped curve will indicating the frequency with highest dynamic range).

2. Take baseline data (minimum 10 min, or 5 data points)

[Check Point: Trouble Shooting \(8\)](#)

3. While recording the base line prepare 20 x thrombin solution in low serum medium (see REAGENT SETUP).

4. Add 20 x thrombin solution. While the measurement is running (do not pause) remove the lid from the ECIS 8-well chamber array, carefully remove 20 μ l old medium from the initially 400 μ l per well. Add 20 μ l of 20 x thrombin solution (100 nM) to the remaining 380 μ l to give a final concentration of 5 nM.

Critical Step The way of adding the agonist solution to the cell layer, essentially the volume of solution added is critical. Addition of agonist in too low volumes (e.g. only 2 μ l of a 200 x stock to a final volume of 400 μ l) may delay cell response in diffusion dependent manner and thereby influence reproducibility. Exchange of too high volumes (e.g. 200 μ l) will cause response to additional physical stimuli such as shear stress and temperature changes.

Add the 20 μ l into the middle of the well, by dipping the pipet tip just below the fluid meniscus and steadily releasing the agonist solution into the well (not too fast!).

Critical Step Reduce time of opening the system (open incubator, open ECIS wells) to a minimum, since temperature changes and changes in humidity influence the measurement.

5. Monitor cell response for at least 1 h after addition.

[Check Point: Trouble Shooting \(9\)](#)

B) Monitoring of HDMEC Response to Histamine

General Prerequisites

Use HDMEC after 3 – 4 days of cultivation on the ECIS electrodes as described under **Cultivation of HDMEC cell layers on ECIS Electrodes.**

Change to serum-free medium 6 – 12 h before the experiment (after ~ 72 h).

Check the cell layer quality by phase contrast microscopy and by either recording an impedance spectrum or running a multiple frequency time collect measurement for 10 – 30 min. The resistance at 4 kHz should be in the range of 2 500 – 4 500 Ω for HDMEC grown on 8W10E+ or 8W10E electrodes.

To monitor agonist response use single frequency time (SFT) mode and select 4 kHz or other sensitive frequency (typically between 400 Hz and 4 kHz for barrier function studies).

Assay

- 1.** Take baseline data (minimum 10 min, or 5 data points)
- 2.** While recording the base line prepare 200 μ M 20 x histamine solution in low serum medium (see REAGENT SETUP).
- 3.** Add 20 x histamine solution to the cell layer while the measurement is running to give a final concentration of 10 μ M (proceed as described for Thrombin, step 4).
- 4.** Monitor cell response for at least 1 h after addition.

Critical Step Consider critical steps described in protocol A

[Check Point: Trouble Shooting \(10\)](#)

C) Monitoring of HDMEC Response to S1P

General Prerequisites

Use HDMEC after 2 days of cultivation.

Change to serum-free medium ~ 6 h before the experiment.

Check the cell layer quality by phase contrast microscopy and by either recording an impedance spectrum or running a multiple frequency time collect measurement for 10 – 30 min. The resistance at 4 kHz should be in the range of 1 500 – 3 000 Ω for HDMEC grown. To monitor agonist response use single frequency time (SFT) mode and select 4 kHz.

Assay

1. Take baseline data (minimum 10 min, or 5 data points)

2. While recording the base line prepare 200 μM 20 x S1P solution in HBSS (see REAGENT SETUP).

Critical Step The following Steps are critical in preparing the S1P working solution, as otherwise signal stability and intensity are affected. Keep stock solution refrigerated until briefly before use.

Critical Step Evaporate methanol from 1 mM stock solution under gentle nitrogen stream. **Critical Step** Add 5 μl of the 100 x fatty acid-free BSA directly onto the lipid film, let sit for 1 min and add 495 μl HBSS and sonicate for 5 min. Briefly pre-warm to 37 $^{\circ}\text{C}$ and vortex before addition to the cells.

3. Add 20 x S1P solution to the cell layer while the measurement is running to give a final S1P concentration of 1 μM (proceed as described under step 38).

4. Monitor cell response for at least 1 hr after addition.

Critical Step Consider critical steps described in protocol A

Check Point: Trouble Shooting (11)

Post-ECIS procedures

Discard / clean arrays

Data Analysis

A) Attachment and cell layer maturation.

Ideally, plot R, $|Z|$ and C to obtain a full picture of your cell culture. R and $|Z|$ are by default (see below) often set to 4 kHz. C is typically monitored at 32 / 40 kHz (choose one, which one is available to you may depend on the default settings of your ECIS machine. These can be changed in the Z θ expert menu).

B) Spectra

Plot $|Z|$, R and C as a function of frequency for 1. your fully matured cell layer grown on the array and 2. The cell-free electrode from before cell seeding (if this was not recorded in advance, you can remove the cell layer by thorough trypsination in the end of the experiment and record cell-free values later).

This set of plots will provide a good overview of the electrical behavior of your system – and thus, your cell layer. Especially the $|Z|$ spectra provide an excellent reference for future experiments regarding the quality of your cell layers.

C) GPCR Assay

Plot absolute values of Resistance R and Impedance $|Z|$ as a function of time for the selected frequency (typically 4 kHz, out of historical reasons and therefore better comparability with existing literature).

Usually, presenting the resistance R is favored over showing the impedance $|Z|$ data. This also has practical and historical reasons 1. R describes the para-cellular current pathway and thus focusses on cell-cell- and cell-substrate junctions with less influence from electrode capacitance 2. R is more graspable for readers, 2.

However, R can lead into problems when the cell layer is very potent in forming an electrical barrier. The higher the resistance, the more the optimum frequency where this increase in R can be observed shifts to lower frequencies. At the same time R at higher frequencies will drop, even though the cell layer increases its barrier properties. At high cell-cell junctional barriers the current escapes through the membrane and is not picked up by R. This bears the risk of misinterpretations in a way that an actual impedance increase is interpreted as loss of barrier function.

Impedance $|Z|$ is not compromised by this effect, as it encompasses membrane resistance (which is mostly regarded as constant) as well.

Therefore, we recommend to plot both parameters, R and $|Z|$, at least for the first sets of experiments, to make sure that data are interpreted correctly with the choice of your monitoring frequency.

Looking at absolute values is essential to come to correct conclusions. Unforeseen events during cell culturing and shear stress by media exchanges can damage the cell layer. But also intended manipulations like transfections or a pre-incubation with a signaling pathway inhibitor can dramatically affect cell layer integrity.

Why is the absolute resistance so important for my assay?

The initial resistance of your cell layer determines the maximum dynamic range of possible cell layer response.

Case 1. GPCR activation leads to decrease of barrier function

Naturally, the signal drop from a high resistance level can be larger in magnitude than compared to a low resistance cell layer. The resistance of the cell-free electrode is the technical lower limit.

A cell layer with an exceptionally low baseline resistance will yield a significantly smaller signal drop than compared to the cell layers with high initial baseline resistance. When preceding manipulations are involved and differently treated populations have to be compared differences in starting resistance may lead to misinterpretations.

Normalizing values to the respective resistance value before manipulation brings all cell layers to the same level and can even more obscure variations in absolute barrier properties. Of course normalizing the data increases plot clarity, but should be supported with information on absolute values in the figure caption or text.

Case 2. GPCR activation leads to increase in barrier function

In cases where GPCR signaling increases barrier function one has to consider the natural limits of the cell layer to form cell-cell junctional structures. Thus, it may be beneficial to use immature barriers as a basis for the assay. A tight control of the absolute resistance of the cell layer can be essential. And it can help to interpret data in retrospect to the measurement, as missing cell responses could be explained by out-of-range baseline resistances.

Trouble Shooting

Step	Problem	Possible Reason (s)	Solution
(1)	only very few cells have attached, many cells are floating	cells did not survive freezing/thawing process	try other batch of cells, order new cell batch
(2)	not enough cells too low cell density	not enough cells on flask, too long incubation with trypsin EDTA low cell number, too high suspension volume	cancel experiment or give cells more time to establish a fully matured monolayer (monitor cell growth with ECIS) seed cells in higher volume (max. 500 μ l) or pellet cells and re-suspend in lower volume (for 2 \times 8-well minimum 6.4 ml)
(3)	cells did not adhere to the array surface	problems with cell suspension used for inoculation forgot coating of electrodes array fabrication errors (rare, but possible)	clean arrays by trypsination, rinse with sterile water, let dry and eventually re-use array after UV/plasma sterilization, contact abp or your local ECIS array supplier

(4)	frequency scan of cell-free electrode not as expected, e.g straight line, noisy	no contact to array defects in on electrode surface (scratches by aspirating or pipetting)	re-adjust array in holder perform contact check repeat frequency scan inspect electrodes under microscope, prevent mechanical destruction of array surface in wells
(5)	drifting baseline of cell-free electrode	electrodes not equilibrated properly	let arrays equilibrate in medium for additional ~ 6 h
(6)	software indicates no contact after media change	array misaligned in array holder; gold pads for contact with holder perforated by too tight pressure of holder pins	re-adjust array in holder perform contact check resume measurement
	resistance significantly higher than before media change	no contact	pause measurement, re-adjust array in holder perform contact check resume measurement
	resistance significantly lower (> 50%) than before media change	(partial) detachment of cells during media change In case of exchanges to low serum media (or other buffer solutions) cells might naturally exhibit lower resistance than in complete medium	let cells recover till initial resistance values are achieved let cells recover till stable resistance values are achieved
	resistance time course at 4 kHz shows gradual, constant decrease in resistance	contamination fluid loss (when medium gets in contact with upper rims of the chambers, capillary forces drag fluid out of the wells)	inspect array, discard if contaminated prevent contact of medium/other fluids with the upper rim and inner side of the lid pause measurement, remove fluid residues from outside the array chambers and inner lid side, refill with medium
(7)	frequency scan of cell-covered electrode not as expected, e.g. straight line, noisy, unexpected shape	no contact to array defects in on electrode surface (scratches by aspirating or pipetting)	re-adjust array in holder perform contact check repeat frequency scan inspect electrodes under microscope to identify defects prevent mechanical contact to the gold layer on bottom of wells
(8)	baseline resistance too low	cell density was too low cells (partially) removed from electrode during media changes cells are too old	inspect cell layer under microscope change for complete medium and monitor resistance for about 24h. If resistance

		isolation of poor quality	increases, use cells after reaching desired stable resistance values
	strong drift in resistance (up or down)	cell layer maturation still in progress natural oscillations in cell layer resistance (as may be dependent on media composition)	wait until stabilized
(9)	no response to thrombin	thrombin inactive (too old / stored at too high temperature) cell layer not confluent/fully matured (low baseline resistance) insufficient receptor expression (reasons: cell isolation/source, cultivation)	purchase fresh thrombin, follow suppliers instructions for reconstitution and storage control cell layer confluence
(10)	no response to histamine	histamine inactive (old, not stored properly) cell layer not confluent/fully matured (low baseline resistance) cells not equilibrated (upward drift in resistance) insufficient receptor expression (reasons: cell isolation/source, cultivation)	purchase fresh histamine, follow suppliers instructions for reconstitution and storage
(11)	no response to S1P	S1P inactive baseline resistance too high (physical maximum as depending on cell type, isolation and cultivation reached)	Purchase fresh S1P, follow suppliers instructions for reconstitution and storage. You can try to weaken an existing cell layer by incubation in Ca-free buffer before adding S1P Repeat experiment with new cell layer with shorter cultivation time on array.

Supplementary Information & References

Extension 1 – 96-well

8-well	96-well
200 000 / ml	150 000/ml
400 μ l	200 μ l
100 000 / cm^2	100 000 / cm^2
0.8 cm^2	0.3 cm^2
Normal pipets	Use 8-well channel pipet
Exchange 200 μ l	Exchange 100
Add 20 μ l agonist stock	Add 20
Agonist stock 20 \times	10 \times
Resolution SFT 1 Array < 1 min	Resolution SFT \sim 5min

Cultivation of HDMEC cell layers on ECIS 96 well plates Timing 2 – 4 d

1. Make single-cell suspension as described under steps **HDMEC Sub-culturing**. Cells harvested from one T75 flask at \sim 90 % confluence are usually sufficient for one 96-well plate.
2. Count cell density and adjust cell suspension to 150 000 cells/ml. You need about 20 ml of suspension.
[Check Point: Trouble Shooting \(2\)](#)
- 3 Add 200 μ l of the 150 000 cells/ml suspension to each well of the 96-well ECIS array. Use a 8-channel pipet. Repeatedly mix cell suspension to avoid well-to-well differences in cell density.
4. Leave arrays with cells for \sim 10 min on the bench to allow the cells to settle to the array bottom uniformly.
5. Cultivate for 2 – 4 days (depending on the agonist study, see below) in a humidified incubator with 5 % CO_2 at 37 $^\circ\text{C}$. Regularly inspect the cell layer under a microscope (about every 1 – 2 days).
[Check Point: Trouble Shooting \(3\)](#)
6. Exchange half of the medium every 24 h by removing 100 μ l old medium and adding back 100 μ l fresh medium. Use an 8-channel pipet to remove old medium and to add fresh medium.
7. 6 – 24 h before the experiment exchange complete medium for low serum medium.

A) Monitoring of HDMEC Response to Thrombin using 96 well plates

1. To monitor agonist response use single frequency time (SFT) mode and select 4 kHz or most sensitive frequency (was found to be 2 kHz for HDMEC on 96W20idf).
2. Take baseline data (minimum 10 min, or 5 data points)

[Check Point: Trouble Shooting \(8\)](#)

3. While recording the base line prepare 10 x thrombin solution in low serum medium (see REAGENT SETUP).

4. Add 10 x thrombin solution. While the measurement is running (do not pause) remove the lid from the ECIS 8-well chamber array, carefully remove 20 μ l old medium from the initially 200 μ l per well. Add 20 μ l of 10 x thrombin solution (50 nM) to the remaining 180 μ l to give a final concentration of 5 nM.

Add the 20 μ l into the middle of the well, by dipping the pipet tip just below the fluid meniscus and steadily releasing the agonist solution into the well (not too fast!).

5. Monitor cell response for at least 1 h after addition.

[Check Point: Trouble Shooting \(9\)](#)