EP4 receptor conformation sensor suited for ligand screening and imaging of extracellular prostaglandins

Michael Kurz¹, Michaela Ulrich¹, Alwina Bittner¹, Magdalena Martina Scharf², Jingchen Shao³, Imke Wallenstein¹, Horst Lemoine⁴, Nina Wetschureck³, Peter Kolb², Moritz Bünemann¹

¹ Institute for Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Philipps-University Marburg, Marburg, Germany

² Institute for Pharmaceutical Chemistry, Faculty of Pharmacy, Philipps-University Marburg, Marburg, Germany

³ Department of Pharmacology, Max Planck Institute for Heart and Lung Research, Bad Nauheim, Germany

⁴ Department of Laser Medicine, Heinrich Heine University, and LWL-Lab§, Himmelgeisterstr. 222, 40225 Düsseldorf, Germany

§Prof. Dr. H. Lemoine, info@lwl-lab.de
Running Title: EP4 receptor sensor to image prostaglandins

Corresponding Author:

Moritz Bünemann, Institute for Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Philipps-University Marburg, Karl-von-Frisch-Str. 2, 35043 Marburg, Germany. Tel.: +49 6421 2825702
E-mail: moritz.buenemann@staff.uni-marburg.de

The number of text pages: 32
Number of tables: 0
Numbers of figures: 7
Numbers of references: 42
Abstract: 250 words
Introduction: 491 words
Discussion: 1499 words

AA arachidonic acid
ASS acetyl salicylic acid
ANOVA analysis of variance
CFP cyan fluorescent protein
CI confidence interval
DHGLA dihomo-γ-linolenic acid
DMEM Dulbecco’s Modified Eagle’s Medium
EPA eicosapentaenoic acid
EP4 receptor prostaglandin E receptor subtype 4
eYFP enhanced yellow fluorescent protein

FRET Förster resonance energy transfer

eYFP/mTurq2 emission ratio

HEK human embryonic kidney

Ilo Iloprost

LPS Lipopolysaccharide

MDCK Madin-Darby canine kidney

Phenanthrene imidazole 3 MF63

mTurq2 mTurquoise fluorescent protein 2

PG Prostaglandin

YFP yellow fluorescent protein
Abstract

Prostaglandins are important lipid mediators with a wide range of functions in the human body. They act mainly via plasma membrane localized prostaglandin receptors, which belong to the G-protein coupled receptor class. Due to their localized formation and short lifetime, it is important to be able to measure the distribution and abundance of prostaglandins in time and/or space. In this study, we present a Foerster resonance energy transfer (FRET)-based conformation sensor of the human EP4 receptor, which was capable of detecting prostaglandin E₂ (PGE₂)-induced receptor activation in the low nanomolar range with a good signal to noise ratio. The sensor retained the typical selectivity for PGE₂ among arachidonic acid products. Human embryonic kidney (HEK293) cells stably expressing the sensor did not produce detectable amounts of prostaglandins making them suitable for a co-culture approach allowing us, over time, to detect prostaglandin formation in Madin-Darby canine kidney cells and primary mouse macrophages. Furthermore, the EP4 receptor sensor proved to be suited to detect experimentally generated PGE₂ gradients by means of FRET-microscopy, indicating the potential to measure gradients of PGE₂ within tissues. In addition to FRET-based imaging of prostanoid release, the sensor allowed not only for determination of PGE₂ concentrations, but also proved to be capable of measuring ligand binding kinetics. The good signal to noise ratio at a commercial plate reader and the ability to directly determine ligand efficacy shows the obvious potential of this sensor interest for screening and characterization of novel ligands of the pharmacologically important human EP4 receptor.

Significance Statement

We present a biosensor based on the prostaglandin EP4 receptor, which is well suited to measure extracellular prostaglandin E₂ (PGE₂) concentration with high temporal and spatial resolution. It can be used for the imaging of PGE₂ levels and gradients by means of Foerster resonance energy transfer
(FRET) microscopy, for determining PGE$_2$ release of primary cells as well as for screening purposes in a plate reader setting.

**Introduction**

The communication between cells is pivotal for the function of multicellular organisms. It is heavily dependent on secreted extracellular ligands such as neurotransmitters or local mediators, which are detected via receptors by recipient cells. Receptors activated by extracellular ligands induce cellular responses, which have been studied extensively by means of imaging spatiotemporal distribution of intracellular second messengers. However, the spatiotemporal distribution of the extracellular ligands is far more difficult to study due to the lack of suitable detectors. For the first time and only recently, technical advances have enabled the direct measurement of neurotransmitters such as norepinephrine, ATP, dopamine, endocannabinoids, acetylcholine and serotonin e.g. (Ravotto *et al.*, 2020; Wu *et al.*, 2022) with high spatial resolution. Instead of neurotransmitters, we focused on prostaglandins as local mediators and developed a Förster resonance energy transfer (FRET)-based assay suited to detect the concentration of an extracellular ligand by means of ratiometric imaging. Prostaglandins (PGs) are important lipid mediators with a variety of crucial functions and almost ubiquitous occurrence in the human body. They are formed in an enzymatic process inside the cell and can act by autocrine as well as paracrine signaling mainly via plasma membrane localized prostaglandin receptors, which belong to the G-protein coupled receptor (GPCRs) class (Woodward *et al.*, 2011). Prostaglandins are released from many different cell types and are short lived, likely leading to local unequal concentrations of PGs in tissues or organs. Currently, the state of the art method to measure prostaglandin concentrations is a discontinuous measurement for example by means of commercially available ELISA-based kits (Chen *et al.*, 2019; Ma *et al.*, 2019; Reis *et al.*, 2020). A recent study using mass spectrometry showed that the abundance of PGD$_2$ did not correspond to the abundance of prostaglandin synthases in uterine tissue emphasizing the need for a reliable in situ measurement method for prostaglandin distribution in tissue (Duncan *et al.*, 2021). In this study focusing on PGE$_2$ we generated a sensor based on the human EP4 receptor, which is currently the subject of extensive research including diverse EP4 receptor ligands in clinical trials (Das and Hong, 2021). Therefore, it
might also be interesting to use the sensor in the classical way, i.e. for screening purposes of this important receptor. This aspect is supported by our successful measurements with the EP4 receptor sensor in a commercial plate reader, laying the groundwork for future prostanoid screening. The sensor was sensitive to PGE₂ in the nanomolar range, and the activity could be blocked with the specific antagonist L-161,982 and provides the opportunity to study on and off kinetics of receptor activation with high precision. As PGE₂ is an important player in inflammatory processes, we set out to show the capability of the sensor by measuring the PGE₂ release of macrophages in real time in a coculture-based approach. Finally, we show that the EP4 receptor sensor can resolve PGE₂ gradients in space and time and thus may be suitable to image PGE₂ gradients in tissue.

Materials and Methods

Plasmids and agonist
cDNA encoding human EP4 receptor (Prostaglandin E4 receptor subtype 4 (PTGR4), AY429109, Catalog Number: #PER0400000) was obtained from the Missouri S&T cDNA Resource Center (http://www.cdna.org).

Cloning: All primers for vectors and fragments were designed to have a complementary overlap of at least 12 base pairs. The primers were designed using SnapGene Viewer (GSL Biotech LLC, United States). Generation of FRET-based EP4 receptor sensor: First, we generated EP4 receptor mTurq2 by deleting amino acids of the C-Terminus of EP4 after position S364 and fusing mTurquoise 2 (mTurq2). We flanked the mTurq2 with AgeI and EcoRV. For this EP4 receptor was amplified as a vector with fw: 5’- caagtaaGATATCCTCGAGTCTAGAGGGCCCG and rv: 5’-caccatACCGGTTGAGCAGTGCTGTCCGG and mTurq2 as a fragment was amplified with the following primers: fw: 5’- CTCAACCGGTatggtgagcaagggcgag and rv: 5’-ACTCGAGGATATCttacttgtacagctcgtccatgcc. Subsequently we inserted an eYFP in ICL3 of EP4 receptor mTurq2, flanked with NotI and SacII, between L223 and S259. The amino acids in between were removed. We amplified EP4 receptor mTurq2 as a vector with fw: 5’- TACAAGCCGCGGAGCTTCCGAGCTTCCGAGCGCATCG and rv: 5’-CATTGCGGCCGCCAGCGAGGTGCGGCG and eYFP as a fragment with fw: 5’-
GCGGCCGCAATGGTGAGCAAGGGCGAG and rv: 5' -
CTCCGCGGCTTGTACAGCTCGTCCATGCC. We used Q5® High-Fidelity DNA Polymerase and NEBuilder® HiFi DNA Assembly kit (New England Biolabs, Ipswich, United States).

In this study we used MF63 (13217), U-46619 (16450), arachidonic acid (90010), prostaglandin D2 (12010), prostaglandin E2 (14010), prostaglandin E3 (14990), 8-iso prostaglandin E2 (14350), prostaglandin E1 (13010), 8-iso prostaglandin E1 (13360), 8-iso prostaglandin F2α (16350), prostaglandin E2 ethanolamide (14012), 15-keto prostaglandin E2 (14720), L-161,982 (10011565) and Iloprost (18215) Manufacturer: Cayman Chemical, Ann Arbor, MI, USA (item number). The compounds were dissolved as stock solutions. The stock solutions were further diluted in buffer containing 0.1 % bovine serum albumin (Bovine serum albumin from heat shock fraction, protease free, fatty acid free, essentially globulin free, Sigma Aldrich, 9048-46-8). See Table S1 for the concentrations and the applied solvent of the prepared stock solutions. Acetyl salicylic acid (ASS) was a kind gift from Dr. Wibke Diederich (Institute for Pharmaceutical Chemistry, University of Marburg, Germany).

Cell culture and transfections

HEK293, HT22 and MDCK cell culture and transfections

In this study we carried out experiments with non-transfected, transiently, or stably transfected cells. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, 4.5 g L⁻¹ glucose) supplemented with 10% FBS, 2 mM L-glutamine, 100 U mL⁻¹ penicillin and 0.1 mg mL⁻¹ streptomycin at 37 °C in a humidified atmosphere with 5% CO₂. DMEM, FBS, L-glutamine, penicillin and streptomycin were from Capricorn Scientific GmbH (Ebsdorfergrund, Germany). To investigate the FRET-based EP4 receptor sensor, a stable cell line was generated by transfecting HEK293 with 1 µg of FRET-based EP4 receptor sensor plasmid cDNA using Effectene reagent (Qiagen, Hilden, Germany) according to the manufacturer’s instructions (in a dish with 6 cm Ø) and subsequently the cells were cultured under selection with G-418. G-418 sulfate was purchased from Capricorn Scientific GmbH (Ebsdorfergrund, Germany). HEK293T were transiently transfected in a manner analogous to the procedure described for the stable transfection of HEK293 cells with the EP4 receptor
sensor: Cells were transfected with 1 µg of either EP4 receptor sensor, EP4 receptor- mTurq2, EP4 wt-receptor- or empty pcDNA3 backbone vector. MDCK cells were transfected with 1 µg EP4 receptor sensor using METAFFECTENE® PRO (Biontex Laboratories GmbH, München, Germany) according to the manufacturer’s instructions. HT22 cells were transfected with 1 µg EP4 receptor sensor using Attractene (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Transfected cells were harvested on the subsequent day (except for cAMP measurements) and seeded for measurements on the following day either on sterile, poly-L-lysine-coated glass coverslips or on poly-L-lysine-coated or uncoated (cAMP measurements) microplates.

Single cell FRET measurements

FRET signals of EP4 receptor sensor were recorded from selected single cells using an inverted microscope (Axiovert 100) and a previously described measurement setup (Jelinek et al., 2021). In brief: mTurq2 was excited with short light flashes of 60 ms using a LED light source (pE-100; CoolLED) with excitation intensity set to 4% for 440 nm and the emitted donor (mTurquoise2) and acceptor fluorescence (eYFP) were recorded with a high-performance CCD camera with a sampling frequency of 0.5 - 1 Hz (see schematic representation in Fig. 1A). At the end of each measurement eYFP was excited using a LED light source (pE-100; CoolLED) with excitation intensity set to 10% for 500 nm and the eYFP fluorescence was recorded (referred to as “total eYFP”). Cells were continuously superfused with either external buffer (137 mM NaCl, 5.4 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, pH 7.32) or external buffer containing ligand in the respective concentration using a pressurized perfusion system (Fig. 1B,C, Fig. S1A,2,3 and 5). The measurements were performed at room temperature. Data was collected by the VisiView software (Visitron Systems). The collected data was corrected for background fluorescence, bleed-through and false excitation using Microsoft Excel. In detail: In each measurement, two regions of interest (ROI) were defined. One ROI included the cell(s), which was/were measured, while the other ROI was placed at a recorded region of the coverslip, without fluorescent cells. The eYFP fluorescence of the measured cell(s) was/were subtracted by the eYFP fluorescence of the background at any given time point. The mTurq2 fluorescence was corrected in an analogous manner. In a next step the background corrected eYFP
fluorescence was subtracted by the bleed through factor multiplied with the background corrected mTurq2 fluorescence and by the false excitation factor multiplied with the background corrected total eYFP fluorescence. The resulting eYFP fluorescence was then divided by the background corrected mTurq2 fluorescence. These data were referred to as emission ratio eYFP/mTurq2. Subsequently a correction for photobleaching (example for an uncorrected trace in Fig. S1A) was performed by an exponential baseline subtraction using Origin 2017. These data were referred to as Δ(YFP/CFP).

Determination of kinetics of receptor deactivation upon agonist withdrawal was performed by fitting the corrected FRET response of EP4 receptor-sensor (see Fig. S2) to monoexponential function, with constrained Y₀ (plateau before wash-out, which was set to 0). Only the first 30 s of wash-out were included for the fit. Curve-fitting and calculation of respective halftime values were performed using GraphPad Prism 8 (GraphPad Software).

FRET measurements of multiple cells in the plate reader

For FRET measurement of multiple cells a Spark 20M (Tecan) plate reader was used. Cells were counted and seeded in poly-L-lysine-coated Greiner Bio-One μClear™ bottom 96-well polystyrene microplates on the day before measurements. Mixtures of HEK293 and MDCK cells contained 100 000 cells each. If indicated cells were treated with 100 µM ASS or 5 µM MF63 or respective vehicle solution (0.1% DMSO final) starting on the day before measurement. The respective solutions were also present during the whole course of the measurements. For experiments with HEK293 stably expressing EP4 receptor sensor alone 120 000 cells were seeded per well. The culture medium was removed, and the cells were washed with external buffer. Subsequently 180 µl external buffer were placed in each well. At each application 20 µl external buffer or external buffer containing ligand solution were pipetted. Every protocol included the application of 20 µl external buffer at cycle 10 except for the Z-factor value measurements, which only were pipetted once with either external buffer or external buffer containing PGE2 at cycle 3.

Measurements were performed in the bottom reading mode using filters (bandwidth in brackets): mTurq2 was excited with 30 light flashes at 430 nm (35) and the emitted donor fluorescence (mTurq2) and acceptor fluorescence (eYFP) were recorded at 485 nm (20) and 535 nm (25). Intensities of eYFP
fluorescence was divided by that of mTurq2 fluorescence and the resulting ratio traces were corrected for photobleaching by subtraction of a monoexponential function using Origin 2017, excluding the Z-factor value measurements. In all measurements except for the Z-factor value measurements in each cycle first mTurq2 was excited and the mTurq2 emission was recorded for all measured wells, followed by the excitation of mTurq2 and the recording of eYFP emission for all measured wells (measurement “whole plate mTurq2 than whole plate eYFP”). In case of the Z-factor value measurements in each cycle every individual well was first illuminated with mTurq2 and mTurq2 was recorded and subsequently within the same well mTurq2 was excited and eYFP was recorded (measurement “well by well”).

As the measurements were performed using stable cell lines, every well was considered as an individual biological sample. Data points from at least two different days were included in every concentration response curve.

High resolution Multiple-Cell FRET measurements
Based on early development work in the Institute of Lasermedicine at the Heinrich Heine University of Düsseldorf, (Lemoine and Rood, 2006; Fischer et al., 2010), novel 12-channel readers (RasiDec-FRET) were developed in the LWL-Lab (info@LWL-Lab.de), equipped with 2 detectors with Si photodiodes (Hamamatsu, Japan) and high precision transpedance amplifiers (integrators, Texas Instruments, USA). A specialized injection technique with 12-channel injectors and thin stainless-steel needles (∅ 1.2 mm, length 50 mm) specially shaped to swirl the injected solutions was used to inject active substances while fluorescence measurement was in progress allowing to monitor the kinetics from the very beginning of drug action (Ilyaskina et al., 2018). The temperature of RasiDec-FRET readers was stabilized at 24 °C using refrigerated circulators (Julabo F25) to perfuse the aluminum housing of RasiDec-FRET readers.

HEK293 cells stably expressing EP4 receptor sensor were seeded for 18-24 h in black Greiner BioOne 384 well plates, 30 000 cells per well. Before starting the measurement DMEM medium was replaced by a buffer containing (mM) 130 NaCl, 5.0 KCl, 4.8 NaHCO3, 1.0 MgCl2, 2.0 CaCl2, 20
HEPES, pH 7.4 and 2% Brilliant Black at 24 °C with a final volume of 120 µl per well. Brilliant Black was purchased from Sigma-Aldrich, St. Louis, Missouri, USA. Injections of test compounds (PGE₂, antagonist) were made with a volume of 10.4 µl using the specialized injection technique described above.

For technical reasons, measurements were performed in duplicates. So, 4 runs of the 12 channels detector on wells organized in duplicates result in n=4 duplicate determinations for each concentration.

RasiDec-FRET readers are controlled by a software program (RasiNext RN1012, LWL-Lab; info@LWL-Lab.de) during the measurements, which is also used for the postprocessing of data stored in a SQLite database. Postprocessing routines were used for scaling, graphic export, the correction of the measurement curves on the basis of the control curves, calculation of mean value curves and mean values of equilibrium effects being exported to GraphPad Prism.

Optical/digital equipment used for multiple-cell FRET imaging: Fluorescence was excited with violet LED-light of 435 nm (additional band-pass of 435/20 nm) for 8 ms followed by darkness for 4 ms and the emitted light was split by a dichroic mirror (511 nm) and filtered by bandpass filters of 483/16 nm and 540/25 nm for the detection of blue (CFP) and yellow light (YFP), respectively. The emission of the split light was measured by a dual detector equipped with 2 x 12 channels for 2 emission wavelengths consisting of low noise silicone photodiodes (Hamanatsu Photonics, Japan) connected to so-called integrators (Texas Instruments, USA) for the smoothing of analog data, followed by digitizing with 96 kHz ADCs (analog-digital converters, Texas Instruments, USA) and further digital smoothing by sampling on chip before digitized data were sent (~ 100 Hz for 2x12 channels) to fast laptops (intel i5 processors with 8 GB Ram and 256 GB SSD hard drives).

Computer software (RasiNext RN1012, LWL-Lab) allows to control RasiDec-FRET readers (processing) during the experiment and provides a set of procedures for the evaluation of the data (postprocessing). RasiNext software allows a continuous recording of fluorescence, for the instantaneous display on the laptop screens, for the documentation of the injection marks, drug concentrations and any other descriptors of the experiment and for the storing of data in SQLite data files for a subsequent data analysis.
A subroutine of the RasiNext Programm 1.2.2.8 was programmed to fit exponential curves according to a single-term

Eq. 1: \[ F(t) = F_0 \cdot [1 - \exp(-k_{app}t)] \]

and a two-term exponential model

Eq. 2: \[ F(t) = F_0 \cdot [1 - f_1 * \exp(-k_{app1}t) - (1 - f_1) * \exp(-k_{app2}t)] \]

where \( F(t) \) and \( F_0 \) represent the fluorescence at time \( t \) and \( t = 0 \) sec, respectively,

\( k_{app} \) the apparent association constants for the single-term model,

and \( k_{app1} \) and \( k_{app2} \) the time constants for two classes of receptor states, a fast and a slow one, with the fractional occurrence of \( f_1 \), and \( (1 - f_1) \), respectively.

Nonlinear regression analyses were performed with R nls {stats} 3.6.2 integrated via an API (Application Programming Interface) in RasiNext Software package 1.2.2.8 using a Gauss-Newton algorithm. Parameter estimates are given as means ± S.E. (standard error) for the fit of individual curves (see Table S3). Means of parameter estimates are given ± SD (see Table S4). Residual standard errors varied between 0.7 and 3.8 on up to 45000 degrees of freedom.

Experimental design: HEK 293 cells were cultivated in black 384-well plates with a density of 20000 cells per well for 24 h to be assayed in the novel 12-channel readers (RasiDec-FRET) yielding full kinetics with high resolution. Experiments were performed at 37 °C PGE2. Effects of PGE2 were measured until equilibrium was reached using equilibrium effects to calculated concentration-response curves. PGE2 was concentrated up to 100 µM allowing the investigation of a wide concentration range of PGE2 of about 3 orders of magnitude.

The concentration ratio \( \log(CR) \) was calculated as the difference of the pEC50-values of the control curve and the curve in the presence of the antagonist B indexed with o and Bi, respectively.

\[ \text{Eq. 3: } \log(CR) = \text{pEC}_{50,0} - \text{pEC}_{50,Bi} \]
The Gaussian standard error of log (CR), SD_{CR}, was calculated according to the law of error propagation using the asymptotic standard error (ASD) of the nonlinear regression analysis of the respective PGE\textsubscript{2} concentration-response curve as follows

\textbf{Eq. 4}: \textit{SD}_{CR} = \sqrt{\left( (\text{SD}_0)^2 + (\text{SD}_B)^2 \right)}

From Eq. 3, the Schild-plot equation can easily be calculated as

\textbf{Eq. 5}: \textit{log}\ (CR - 1) = m \textit{log}\ ([B]) + pK_B

where the slope m close to 1 indicates a competitive mode of antagonism between agonist and antagonist B.

To take into account the dependence of the error size on the concentration-ratio log(CR-1) weighting factors were defined as

\textbf{Eq. 6}: \textit{SD}_{CR-1} = \frac{(CR)}{(CR-1)} \ast \textit{SD}_{CR}

which were included in the regression analysis of the Schild equation (Lemoine, 1992).

Cisbio cAMP accumulation assay

Downstream signaling was determined using the HTRF® cAMP Gs dynamic assay (Cisbio Bioassays, France), measuring cAMP accumulation. HEK293T cells were transfected with the respective receptor (EP4 wt-receptor, EP4 receptor – mTurq2, EP4 receptor sensor) or empty plasmid using effectene two days prior to measurement. To check for PGE\textsubscript{2} production by the cells, one dish of EP4 wt-receptor transfected cells was treated with 100 µM ASS on the day prior to the measurement. All other dishes were treated with the equivalent amount vehicle (0.1% DMSO final) for the same period of time. For the measurement, cells were harvested and resuspended in assay buffer (10 mM HEPES, 137 mM NaCl, 5.4 mM KCl, 2 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, pH 7.32; supplemented with 100 µM IBMX) at a cell concentration of 1000 cells/µL. Then 5 µL of the cell solution were transferred to each well of a white, low-volume 384-well plate and 5µL of the respective dilution of PGE\textsubscript{2} (2x final concentration in assay buffer) was added to the cells. The plate was then incubated for 1h at 37°C and 95% air, 5% CO\textsubscript{2}.

After incubation, 5 µL of each of the detection reagents cAMP-D2 and anti-cAMP-Cryptant (prepared according to the recommendations of the manufacturer in the provided lysis buffer) was added to each well and the plate was incubated for 1 h at room temperature. Subsequently, the plate was read on a Tecan Spark 20M plate reader using the settings recommended by the manufacturer.
As a control, cells were incubated with assay buffer instead of PGE2. For the negative control, cells were incubated with assay buffer and 5 µL of lysis buffer was added instead of the anti-cAMP-Cryptant. Each measurement was repeated four times in three technical replicates.

For analysis the HTRF ratio was calculated as described by the manufacturer. Data points were plotted and fitted using Graphpad Prism (log(inhibitor) vs. response):

\[
Y = Bottom + \frac{Top-\text{Bottom}}{1+10^{(X-\text{LogC50})}}
\]

FRET measurement of macrophages

Macrophages were induced and treated as described in Kuroda and Yamashita, 2003 (Kuroda and Yamashita, 2003). In brief, peritoneal cells were harvested 3 days after i.p. injection of thioglycolate into C57BL/6 mice. Isolated macrophages were subsequently cultured in RPMI (RPMI Medium 1640, Gibco, 21875-034) and treated for 18 h with either LPS (1 µg/ml, Lipopolysaccharides from Escherichia coli O111:B4, Sigma Aldrich, L4391) or vehicle solution. Stock solutions of LPS were prepared as 1 mg/ml LPS in Dulbecco’s Phosphate Buffered Saline (DPBS, Gibco, 14190-094). These cells were subsequently harvested by trypsinizing and the additional use of a cell scraper. The harvested macrophages were counted, and 50000 cells were seeded per 96 well plate together with 100000 HEK293 cells stably expressing EP4 receptor sensor. The subsequent measurement was performed at the previously described measurement setup (Jelinek et al., 2021) using a 40x objective (LD ACHROPLAN 40x/0.60 KORR Ph2 ∞ 0-2) with a sample frequency of 0.5 Hz. The traces were corrected for photobleaching by an exponential baseline subtraction using Origin 2017 and normalized to the response caused by PGE2 application. We measured macrophages from three different isolations. Each isolation contained the pooled cells of 5 animals.

Calculation of PGE2 per macrophage

To calculate the PGE2 mass per macrophage (Fig. 6D), we calculated in a first step the concentration [c] of PGE2 in the well. This was done by using the following term, which was derived from the standard Hill equation for receptor occupancy (Fischer et al., 2010) resulting after rearrangement in
Eq. 8: \[ \log [c] = \frac{\log \left( \frac{l}{o \cdot g} \right)}{\text{HillSlope}} - \log (EC_{50}) \]

where \( y \) is the basal sensor response normalized to \( \text{PGE}_2 \) max, \( EC_{50} = 27 \text{ nM} \) and \( \text{HillSlope} = 0.9 \).

\( EC_{50} \) and \( \text{HillSlope} \) values stem from the \( \text{PGE}_2 \) concentration response curve measured in the plate reader (Fig. 4C). The resulting \( \text{PGE}_2 \) concentration was then used to calculate the mass of \( \text{PGE}_2 \) per macrophage by the following formula: \( m(\text{PGE}_2) / \text{macrophage} = c \cdot V \cdot M(\text{PGE}_2) / \text{number of macrophages} \). The molecular mass of \( \text{PGE}_2 \) is 352.5 \( \mu \text{g/\mu mol} \) (data sheet Cayman Chemicals, item number 14010). As Volume (V), we used 200 \( \mu \text{l} \), which was the volume present in the well at the time point of the basal response. 50 000 macrophages were present per 96 well plate.

To compare our value with the result of Kuroda and Yamashita, 2003 (Kuroda and Yamashita, 2003) we read off the value for basal \( \text{PGE}_2 \) concentration of lipopolysaccharide (LPS) treated \( \text{C57BL/6} \) peritoneal mice macrophages from Fig. 1A based on the bar graph with a concentration of 8000 pg/ml. According to the methods section the cell number was given with \( 5 \times 10^5 \) cells/ml/well. Therefore, we calculated \( \text{PGE}_2 \) release per macrophage = \( (8000 \text{ pg/ml}) / (5 \times 10^5 \text{ cells/ml}) \) which resulted in 16 fg / cell which equals 0.045 fmoles/cell.

Measurements of artificial \( \text{PGE}_2 \) gradient (Fig. 7 and Fig. S6) and comparison of the expression of EP4 receptor- mTurq2 and EP4 receptor sensor

HEK293 cells stably expressing EP4 receptor sensor were seeded on glass coverslips. Measurements were performed on the following day. Micropipettes were pulled on each measuring day by using a P-87 micropipette puller. Micropipettes were filled with 10 \( \mu \text{M} \) \( \text{PGE}_2 \) solution. The pipette was placed under optical control using the microscope on the bottom right part of the visible area, as shown in the respective bright field images (Fig. 7 and Fig. S6). We applied pressure with a syringe that was combined with a three-way valve. FRET signals were recorded from selected regions of interest using an inverted microscope (Axiovert 135) and an oil-immersion objective (UPlanSApo 60x/1.35 Oil Olympus) with a sample frequency of 0.2 Hz. The CFP variant mTurq2 was excited with short light flashes of 50 ms using a LED light source (pE-100; CoolLED) with excitation intensity set to 7% for 440 nm and emission of mTurq2 and eYFP were split by an optosplit (Chroma) and detected with a
CCD camera (RETIGA-R1, Teledyne Photometrics) and stored with VisiView software (Visitron Systems). Data Shown in Fig. 7 and Fig. S6 were corrected for background fluorescence, bleed-through and false excitation using Microsoft Excel. Data shown in Fig. 7 was subsequently corrected for photobleaching using Origin 2017. Data Shown in Fig. 7 were normalized to the response to the pipetted PGE2. Traces shown in Fig. S6 were normalized to the initial emission ratio before local application of PGE2.

To determine the expression of EP4 receptor- mTurq2 and EP4 receptor sensor in HEK293 cells, HEK293T cells were transiently transfected with the respective construct and seeded on glass coverslips. The CFP variant mTurq2 was excited and the fluorescence was detected at the setup described above in this section. The YFP variant eYFP was excited at 500 nm with LED light source (precisExcite-100, 500 nm, CoolLED) with an excitation intensity of the light source set to 3%. In case of bleaching experiments, the bleaching was performed by illumination of the respective cells at 500 nm with LED light source (precisExcite-100, 500 nm, CoolLED) with an excitation intensity of the light source set to 50% for 1 min. After the bleaching procedure, mTurq2 and eYFP were again excited and the respective emissions were recorded. The first five values for each emission were averaged. The data are shown in Fig. S1C and Table S2. As the EP4 receptor sensor is a conformational sensor which shows FRET in the non-active state the mTurq2 fluorescence is consequently to low and therefore incorrect. To correct for this, eYFP was bleached as described above and the change in mTurq2 emission was calculated before and after bleaching. The median change of \((\text{mTurq2 (before bleaching)} / \text{mTurq2 (after bleaching)})-1\) was +29% with a median eYFP bleaching of 89%, see Table S2. We calculated the increase for mTurq2 with 33% in case of 100% eYFP bleaching and therefore multiplied all mTurq2 emission values of EP4 receptor sensor with the factor 1.33 to correct for initial FRET effect of the sensor. The individual corrected data are shown in Fig. S1C.

Data Analysis and Statistics
Data represent either an average of individual recordings or individual observations. Data is presented, if possible, as mean ± SD. The data were analyzed with Origin Pro 2017 or GraphPad Prism 8 (GraphPad Software) or Excel 2016. Statistical analysis was performed with one-way ANOVA with subsequent Tukey's multiple comparisons test as post hoc test. Differences were considered statistically significant for p≤0.05.

Analysis of concentration–response relationships

Concentration–response relationships were evaluated for PGE₂ (Fig. 1C) and for PGE₃ (Fig. S5A) at EP4 receptor sensor in single cell measurements. Single cells were superfused with concentrations tested, which was followed by a reference concentration. The responses of the tested concentrations were evaluated relative to the response of the reference concentration. Concentration–response relationships were evaluated for PGE₁ and PGE₂ (Fig. 4C), 8-iso PGE₂ (Fig. 4D) and arachidonic acid (Fig. 4D) in multiple cell measurements in the Tecan plate reader. Multiple cells were measured in a 96 well format. The procedure was done in a manner analogous to the PGE₂ concentration response curve at the EP4 receptor sensor measured in the high-resolution Multiple-Cell FRET system in 384 well format (Fig. 2). In each measurement one test concentration and a reference concentration of PGE₂ was applied. The responses of the tested concentration were evaluated relative to the response of the reference concentration of PGE₂. All concentration–response curves were fitted with Graphpad Prism 8 (log(agonist) vs. response -- Variable slope (four parameters)) with variable Hill slope and EC₅₀ while top and bottom were constrained, except for PGE₃ (Fig. S5A) with no constrain for top and for arachidonic acid (Fig. 5D) with no constrain for top and Hill slope set to 1. The following Eq. 9 was used for fitting:

Eq. 9: \[ Y = Bottom + \frac{Top-Bottom}{1+10^{\left((\log(\text{EC}50-X)}\right)Hill\text{Slope}} \]

Calculation of Z-factor values:
For comparison reasons Z-factor values were calculated as described in Schihada et al. using the term Eq. 10, which is based on the formula provided by Zhang et al. (Zhang et al., 1999):

Eq. 10:

\[
Z\text{-factor value} = 1 - \frac{3SD_c + 3SD_s}{\text{average } c - \text{average } s}
\]

where SD and average are the SDs and average ΔFRET [%] values of 1 µM PGE2 (sample, s) and external buffer control (control, c), respectively. Control wells are shown in black (buffer application) and sample wells in red (PGE2 application).

Results

Generation of a human EP4 receptor-based sensor to display prostaglandin concentration in real time

We constructed a human EP4 receptor conformation sensor in which mTurq2 was cloned to the truncated C-terminus and eYFP into intracellular loop three, to detect alterations in FRET upon receptor activation, analogue to the TP receptor sensor (Kurz et al., 2020). The subsequent characterization of the construct was initially carried out in single cell FRET experiments by means of dual emission fluorescence microscopy using a pressurized perfusion system (Fig. 1A). Upon stimulation with different concentrations of PGE2 the emission ratio (eYFP/mTurq2) decreased in a concentration dependent manner (Fig. 1B, top). The simultaneous eYFP-emission decrease together with an mTurq2-emission increase indicated the occurrence of FRET (Fig. 1B, bottom). The change in emission ratio was dependent on the concentration of PGE2 and reversible upon agonist withdrawal. The agonist induced decrease in emission ratio was in line with previously published analogously constructed GPCR-FRET-conformation sensors (Kauk and Hoffmann, 2018; Kurz et al., 2020). The traces shown in Fig. 1B were corrected for photobleaching by subtraction of a monoexponential function (for the respective uncorrected trace see Fig. S1A). Similar baseline corrections were done for all experiments in this study if not otherwise stated. To determine the sensitivity of the sensor we measured the dependence of the alterations in FRET by the concentration of PGE2. The curve gave rise to an EC50 value of 24 nM (95% CI: 22 nM to 27 nM) showing that this sensor allows the
detection of PGE$_2$ in the low nanomolar range (Fig. 1C). The observed EC$_{50}$ value is around one order of magnitude right shifted compared to the K$_i$ value reported in the literature to be about 1-2 nM for PGE$_2$ at the EP4 receptor (Boie et al., 1997; Kiriyama et al., 1997; Abramovitz et al., 2000; Davis and Sharif, 2000; Ross et al., 2002; Araki et al., 2017). This already indicates that the sensor is likely uncoupled from G-protein signalling. We supported this assumption by measurements using a commercial kit to quantify cAMP production. The EP4 receptor sensor was by a factor of about 75 less efficient to induce cAMP production compared to EP4 wt-receptors (Fig. S1B). Interestingly, we observed basal activity for EP4 wt-receptors and EP4 receptor labeled with mTurq2, presumably due to overexpression. Furthermore, even PGE$_2$ application to cells, which were only transfected with empty pcDNA3 vector, caused cAMP production in a concentration dependent manner with a right shifted EC$_{50}$ by a factor of about 565 compared to EP4 wt-receptors (Fig. S1B). HEK293 cells endogenously express EP4 receptor as well as other prostanoid receptors based on mRNA levels (Atwood et al., 2011), which presumably explains the response to PGE$_2$ of cells transfected with empty pcDNA3. The assay buffer was supplemented with 100 µM of the phosphodiesterase inhibitor IBMX (3-isobutyl-1-methylxanthine), leading to cAMP accumulation and therefore further enhances the sensitivity towards the PGE$_2$ induced increase in cAMP levels. We compared the expression level of EP4 receptor sensor to the expression of EP4 receptor labeled with mTurq2 and observed that the EP4 receptor sensor expression was 58% of the expression level measured for EP4 receptor labeled with mTurq2 (Fig. S1C), indicating, that a reduced expression of the EP4 receptor-sensor could account only to a very minor extent for the much-reduced cAMP production of the sensor compared to the EP4 receptor mTurq2.

We next measured EP4 receptor sensor stably expressed in HEK293 cells in 384 well format plates (30 000 cells/well) using the optimized high performance multicell reader system (RasiDec-FRET, LWL-Lab). These measurements indicated activation kinetics of PGE$_2$ at the EP4 receptor, which presumably directly reflect binding kinetics (Fig. 2) nicely showing the acceleration of drug onset with increasing PGE$_2$ concentrations and stable equilibrium-effects. We observed that the activation kinetics of concentrations higher than approx. 100 nM PGE$_2$ were biphasic and could be best fitted with a two-component exponential function. The time constant of the fast phase was inversely
dependent on the agonist concentration and its fractional size was positively correlated with the agonist concentration (Fig. 2 D, F) and, therefore, likely reflects the actual binding-induced conformational change of the receptor. Upon application of 1 µM PGE₂ the receptor activated with a half time of 4.4 s (Table S3, two-term model) indicating a speed of activation similar in range as observed for aminergic receptors at 1 µM agonist (Vilardaga et al., 2003; Hoffmann et al., 2005, 2012; Rochais et al., 2007). Additionally, we determined the half time of receptor deactivation upon agonist withdrawal from single cell FRET recordings using our pressurized perfusion system (Fig. S2). The monoexponential decay of the signal was fitted and gave rise to a half time of 21 s (median value; mean 28 s, SD: ± 21 s). The median of k_{off} (0.03325 s⁻¹) was then used together with the EC_{50} of 24 nM from Fig. 1C to calculate k_{on} with 8.3 x 10⁷ M⁻¹ Min⁻¹ similar in range as described for norepinephrine on the α₂A-AR (Rinne et al., 2013).

Next, we tested the PGE₂-activated EP4 receptor sensor for inhibition by L-161,982. Stable EP4 receptor sensor cells were pretreated with several concentrations of L-161,982 ranging from 3 nM to 1 µM assayed in the high resolution 12-channel readers at 37 °C. The exponential decline of the ratio signals by PGE₂ resulted in equilibrium effects, which were used to calculate concentration-response curves (Fig. 3A), the appropriate pEC_{50}-values and the concentration-ratios log (CR-1). The Schild-plot (Fig. 3B) exhibits a linear relationship of log (CR-1) on L-161,982 concentration with a slope close of unity (1.06±0.04) and the abzissa intercept (pK_{B}) of 8.47±0.08 (-log M) indicating a high affinity competitive antagonism by L-161,982.

Subsequently, EP4 receptor sensor signals were measured in a 96 well format (120 000 cells/well) commercially available plate reader (Fig. 4A, Fig. S3). The transition from single cell measurements to multiple cell FRET measurements in standard plate readers is often hampered by poor signal to noise ratios especially for classical YFP/CFP-based GPCR conformation sensors (Schihada et al., 2018). However, using the optimized sensor of the present study resulted in an improvement of signal to noise ratios measurable with a commercial plate reader and enabling determination of concentration response curves for PGE₂. We calculated the Z-factor value to be 0.302 ± 0.264 (Fig. S4, mean ± SD), leading to the recommendation for running a double assay for screening (Zhang et al., 1999).
The EC50 value of 27 nM (95% CI: 24 nM to 31 nM) PGE2 (Fig. 4B) at the EP4 receptor sensor at the plate reader and the EC50 value of 21 nM (95% CI: 16 to 30 nM) PGE2 (Fig. 2B) measured on the high performance RasiDec system were comparable to the EC50 value of the single cell experiments. The EC50 value of PGE1 with 65 nM (95% CI: 58 nM to 72 nM) was also in the same range (Fig. 4B). The EC50 value of PGE3 with 67 nM (95% CI: 52 nM to 89 nM) was also similar (Fig. S5A).

We chose the EP4 receptor as a base for our FRET sensor as this receptor possesses a high level of selectivity for PGE2 among arachidonic acid products already at the level of the binding affinity (>factor of 500) (Boie et al., 1997; Abramovitz et al., 2000). In line with the known wt-receptor binding properties, neither a high concentration of PGD2, Iloprost (a stable derivative of PGI2 with higher affinity towards EP4 receptor (Boie et al., 1997; Kiriyama et al., 1997; Abramovitz et al., 2000; Davis and Sharif, 2000)) nor U-46619 (a stable analogue of PGH2) were detected up to a concentration of 0.9 µM. A concentration of 9 µM of these compounds led to minor partial activation of the sensor (Fig. 4C). This selectivity also translated to other prostanoids such as 8-isoprostanes, the PGE2 metabolite 15-keto PGE2 and the prostamide PGE2 ethanolamide. 8-iso PGF2α did not lead to a detectable EP4 receptor sensor activation at all, while 8-iso PGE1 and 2 showed a pronounced right shift of between one or two orders of magnitude compared to PGE1 or PGE2, respectively (Fig. 4D). Neither the PGE2 metabolite 15-keto PGE2 with a binding affinity of between 3 and >15 µM at EP4 wt-receptor (Nishigaki et al., 1996; Endo et al., 2020) nor PGE2 ethanolamide, the main COX2 product of anandamide, with an affinity comparable to that of 15-keto PGE2 (Ross et al., 2002) were detected up to a concentration of 0.9 µM. A concentration of 9 µM these PGE2 analogs led to partial activation of our sensor, despite their high degree of structural similarity in comparison to PGE2 (Fig. 4E).

As a recently published paper on a P2Y1 receptor-based ATP sensor reported a difference in the affinity to ATP depending on the expression in HEK cells vs the expression in neurons of more than two orders of magnitude (Wu et al., 2022), we additionally tested the EP4 receptor sensor in Madin-Darby canine kidney (MDCK) and HT22 cells. A concentration of 30 nM PGE2, which is close to the
EC$_{50}$, caused a response in the same range in all three cell types indicating the robustness of our sensor independent of the applied cell type (Fig. S5B).

Real time recordings of PGE$_2$ release of different cell types

Many different cell types produce and release prostanoids. We expressed the EP4 receptor sensor stably in human embryonic kidney (HEK293) cells, which do not express detectable levels of cyclooxygenases and are therefore unable to produce prostaglandins themselves (Sood et al., 2014). Consequently, measurements of HEK293 cells stably expressing EP4 receptor sensor did not show a detectable PGE$_2$ production after arachidonic acid (AA) application (Fig. 5A). In contrast MDCK cells are well known to produce and secrete detectable amounts of PGs such as PGE$_2$ e.g.: (Levine and Ohuchi, 1978; Schaefers et al., 1996). In an experimental approach, we co-cultured HEK293 cells stably expressing EP4 receptor sensor together with non-transfected MDCK cells and performed FRET measurement of multiple cells in a 96 well format. Measurement of this co-culture showed PGE$_2$ production and release by MDCK cells. This could be specifically suppressed by either blocking the COX with acetyl salicylic acid or by using phenanthrene imidazole 3 (MF63), which has an inhibitory effect on certain prostaglandin E synthases (Fig. 5B). Taken together, this experimental approach enabled the recording of PGE$_2$ release in real time. The resulting PGE$_2$ formation was related to the concentration of added arachidonic acid (Fig. 5C,D).

Macrophages are well known to produce PGE$_2$ particularly upon activation (Kuroda and Yamashita, 2003). With a similar experimental approach as used on the MDCK cells, we measured the PGE$_2$ release by macrophages together with HEK293 cells stably expressing EP4 receptor sensor. These measurements showed that LPS treated macrophages caused activation of the EP4 receptor sensor which could be further enhanced by adding arachidonic acid (Fig. 6A,B) indicating the release of PGE$_2$. It cannot be completely excluded that PGE$_1$ or PGE$_3$ contributed to some extent to the basal response, since the EP4 receptor (sensor) is not fully selective. We were able to quantify the PGE$_2$ amount present in the wells via normalization. The experimental procedure included a maximum PGE$_2$ response using a concentration of more than 30 times EC$_{50}$ followed by a subsequent saturating
antagonist application (Fig. 6A-C). Our results for 2 out of the 3 isolations for the produced amount of PGE$_2$ per cell of LPS treated macrophages compared well to the literature values of 16 fg / cell calculated from (Kuroda and Yamashita, 2003), who used an antibody-based assay kit versus our results: isolation 1-3 (in fg / cell ± SD): 95 ± 96, 11 ± 3 and 14 ± 3 underlining the validity and precision of this approach (Fig. 6D).

Potential for spatial resolved PGE$_2$ measurements

Prostanoids are often formed and act locally (Woodward et al., 2011), therefore, in many cases their action is likely limited to the cells proximal to the prostanoid secreting cell. To see if our EP4 receptor sensor is principally capable of measuring spatial distribution of in situ produced prostaglandins, we tested, as a first step, if the spatial distribution of an artificially generated PGE$_2$ gradient can be measured. To do so, we filled a patch pipette with PGE$_2$, placed it above HEK293 cells stably expressing the EP4 receptor sensor, and applied pressure to distribute the ligand while generating a gradient (Fig. 7 top). Cells closer to the pipette reacted with a relatively stronger FRET change to PGE$_2$ than more distant cells, indicating that the sensor may be used to image the spatial distribution of PGE$_2$ (Fig. 7, Fig. S6).

Discussion

In the present study, we introduce an EP4 receptor-based conformation sensor, which selectively detects PGE$_2$ among arachidonic acid products in the nanomolar range with high sensitivity by means of FRET. It is well suited to measure the extracellular PGE$_2$ concentration and has the potential to resolve PGE$_2$ gradients in space and time and thus might be suited to image PGE$_2$ gradients in tissue. Due to its high signal to noise ratio the new sensor reports not only concentration dependent activation or inhibition of the EP4 receptor but also binding and dissociation kinetics of pharmacologically active ligands as well as their efficacy akin to those described in similar receptor sensors. Therefore, this novel sensor has the potential for being used for drug screening as it works on commercially available plate readers, however based on the Z-factor value of 0.302 ± 0.264 (Fig. S4, mean ± SD) it might not be applicable to HTS settings.
The selectivity of the EP4 receptor among arachidonic acid products for PGE2 is very high, which is in line with the results of our experiments using our EP4 receptor sensor (Fig. 4C). The observed selectivity of our EP4 receptor sensor against PGE2 ethanolamide (Fig. 4E) is especially striking as prostamides are often misidentified as prostaglandins by immunoassays, due to their structural similarity, which in consequence leads to the same set of antisera (Glass et al., 2005). Furthermore, it is a common feature of PGE2 that the first metabolization step eliminates much of the affinity for binding to EP4 receptors, while this affinity loss is much less pronounced at EP2 receptors. The selectivity factor for PGE2 over 15-keto PGE2 for EP2 receptor is about 50 in contrast to about 2140 for EP4 receptor, highlighting the biological differences of PGE2 and 15-keto PGE2 and emphasizing the need to detect both lipids separately (Nishigaki et al., 1996; Endo et al., 2020).

It could clearly be shown (Fig. 2, Table S3 and Table S4) that small PGE2 concentrations act monoexponentially, whereas higher PGE2 concentrations of 256 and 1024 nM act via a bi-exponential mechanism (half times differ more than 10-fold). We hypothesize, that the short half times (single-digit seconds) represent the true association to the receptor binding pocket, while the slow kinetics of the small concentrations are hampered by a slow lateral diffusion in the cell membranes. The calculated $k_{on}$ value of PGE2 at EP4 receptor sensor with $8.31 \times 10^7$ M$^{-1}$ Min$^{-1}$ was approximately in the range of reported antagonist $k_{on}$ values for the DP2 receptor ranging from $4.80 \times 10^7$ to $2.23 \times 10^8$ M$^{-1}$ Min$^{-1}$ (Sandham et al., 2017). Interestingly, the calculated $k_{on}$ for the binding of norepinephrine (NE) to $\alpha_{2A}$ adrenergic receptor was only about a 2 fold difference (our result: $k_{on}$ with $8.31 \times 10^7$ M$^{-1}$ Min$^{-1}$ VS $k_{on}$ 3.45 x $10^7$ M$^{-1}$ Min$^{-1}$ of Rinne et al. 2013, indicating that the completely different and far more complicated entry of PGE2 into the binding pocket of EP4 receptors from the plasma membrane compared to the more direct entry of NE into the binding pocket of the $\alpha_{2A}$-AR from the extracellular side does not make a major difference in the on-rate of binding (Rinne et al., 2013).

The Schild-plot analysis of the blockade by L-161,982 of PGE2 revealed a competitive binding mode of L-161,982 with an affinity of about 3 nM. The competitive binding mode is in line with the literature while calculated $K_i$ value is left shifted by about one order of magnitude (VS $K_i = 24$ nM (Machwate et al., 2001)). Possible reasons for this alteration are presumably differences in
experimental conditions such as incubation times or temperature, possibly leading to differences in the degree of equilibration.

In addition to the detection of the arachidonic acid product PGE₂, the EP4 receptor sensor also detects PGE₁ and PGE₃ in approximately the same concentration range (Fig. 4B, Fig. S5A). Among prostanooids, the 2-series (arachidonic acid products) are considered most abundant (Levin et al., 2002; Wada et al., 2007). Even an artificial increase of the ratio of dihomo-γ-linolenic acid/arachidonic acid to 2.8 in LL carcinoma cells only resulted in a ratio for PGE₁/PGE₂ of 0.41, emphasizing the low abundance of PGE₁ even if dihomo-γ-linolenic acid is increased (Levin et al., 2002). Therefore, it seems reasonable to assume that a major part of the receptor sensor signal will report PGE₂ rather than PGE₁ or PGE₃.

Furthermore there are applications in which the prostanoid series is predetermined by the experimental setting: e.g.; anandamide has recently been identified as a source for arachidonic acid in the lung, likely resulting in the formation of 2-series prostaglandins rather than those of the 1-series or 3-series (Simon et al., 2022).

While mass spectrometry allows for the simultaneous detection of different agonists, our sensor and ELISA are limited to one ligand at a time. Furthermore, also traditional ELISA showed a low specificity between series-1, -2, and -3 PGs (Kakutani et al., 2010). The sensitivity of our sensor is based on the binding properties of EP4 receptor. Based on Fig. 1B or Fig. 2A we would assume that it shows a sensitivity range for PGE₂ of about 4 nM up to 200 nM. This would translate to a range of 1.4 - 70 ng/ml. The reported range and sensitivity of mass spectrometry and antibody-based approaches is much higher compared to our sensor, due to the high affinity of antibodies and high sensitivity of modern mass spectrometry detectors. Dependent on the concentration range of prostaglandins in tissue which may range from picomoles to nanomoles per gram of tissue (Duncan et al., 2021), the best suited method to detect overall prostaglandin concentrations should be chosen. However, in contrast to other available methods (Kakutani et al., 2010) the FRET-based detection allows for real time measurement and spatiotemporal detection of local gradients.
Our experiments with MDCK cells and macrophages (Fig. 5B-D and Fig. 6) showed that our sensor was in the right sensitivity range to detect the prostaglandin concentrations of these types of experiments. Stimulated macrophages differ strongly in their rate of PGE$_2$ production. While the C57BL mice that we used result in prostaglandin concentrations in the range of our sensor, experiments with BALB mice with much higher PGE$_2$ levels after LPS stimulation would require a less sensitive sensor (Kuroda and Yamashita, 2003). In the future, it will be important to provide sensors with lower and higher affinity to cover all crucial physiological and pathological processes involving prostaglandins, which is laborious but necessary given the biological importance of prostanoids and specifically PGE$_2$.

We established a new co-culture-based approach for the measurement of prostanoid formation in real time using HEK cells, which do not produce prostanoids themselves due to their lack of detectable COX. This new assay not only allowed for the measurement of prostanoid formation in cells in cell culture such as MDCK cells but also for primary cells such as the macrophages.

The activation of our EP4 receptor sensor does not involve an amplification step, enabling a quantification of the conformation change-inducing agonist. In line with this, our results for 2 out of the 3 isolations for the basal produced amount of PGE$_2$ per LPS treated macrophage compared well to the literature value, which involved a commercial PGE$_2$ immunoassay kit: 16 fg / cell versus our result isolation 1-3 (in fg / cell): 95, 11 and 14. This underlines the validity and precision of this approach (Fig. 6D, Table S5, Kuroda et al. 2003).

A potential drawback of using intact cells for carrying the EP4 receptor sensor could be the possibility that these cells degrade PGE$_2$. The similar EC$_{50}$ values for the PGE$_2$-induced EP4 receptor sensor activation obtained in single cell assays with constant laminar superfusion of PGE$_2$ and in multicellular assays performed in the plate reader for which PGE$_2$ was pipetted in, suggest that a possible degradation of PGE$_2$ by HEK cells in the plate reader did not affect our results. Taken together, our co-culture approach offers new research possibilities and can be applied universally to almost all type of cells, as there is no limitation by poor transfection efficiency, which makes it a valuable new tool for real time measurements of prostanoids.
Lastly, we could show that the EP4 receptor sensor has the potential to resolve PGE$_2$ gradients with spatial and temporal resolution and thus might be suited to image PGE$_2$ gradients in cell culture or tissue (Fig. 7).

Our FRET-based receptor sensors in comparison to recently published GPCR-based sensors e.g. (Ravotto et al., 2020; Wu et al., 2022), is a ratiometric sensor, which has the potential to quantify prostanoid concentrations independent of the expression level of our sensor.

Besides the relevance for the investigation of the prostanoid pharmacology, our new approach to measure concentrations of extracellular ligands with a FRET sensor either in co-culture or in a knock-in model is of general interest. It could potentially be applied for different endogenous hormones and transmitters, such as opioids, lipid mediators or aminergic hormones and all other types of extracellular endogenous GPCR agonists.

**Acknowledgements**

We thank M.B. Münstermann and M. Nadskakula, precision mechanics workshop of the Department of Laser Medicine for help with the construction of the RasiDec fluorescence readers.

We thank Dr. Hannes Schihada for his help with the investigation of the EP4 receptor sensor Z-factor values.

**Data availability statement**

The data that support the findings of this study are available on request from the corresponding author.

**Authorship Contributions**

Participated in research design: Wetschureck, Kolb, Kurz and Bünemann.

Conducted experiments: Kurz, Wallenstein, Scharf, Shao, Lemoine, Bittner and Ulrich.

Performed data analysis: Kurz, Wallenstein, Scharf, Lemoine and Ulrich.
Wrote or contributed to the writing of the manuscript: Kurz, Lemoine and Bünemann.

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EP2 and EP4, differ in desensitization and sensitivity to the metabolic inactivation of the agonist.


Footnotes

This work was supported by Deutsche Forschungsgemeinschaft [Grant BU 1133/5-1].

H.L. is the owner of the commercial enterprise LWL-Lab (Düsseldorf) and performed experiments and data analysis using the Rasidec-FRET high performance plate reader and RasiNext analysis software from LWL-Labs in the present study. The other authors declare no competing interests.

Figure legends

Fig. 1 EP4 receptor sensor is sensitive to PGE₂ in the low nanomolar range.

(A) Scheme illustrating the principle of the FRET measurement with the EP4 receptor-based conformation sensor in single cells using a pressurized superfusion system as used for experiments shown in B and C. (B) Representative traces of the emission ratio (red, baseline corrected), mTurq2 (blue) and eYFP (yellow) emission (upon excitation at 440 nm, baseline-corrected and normalized to initial values) of the PGE₂ induced activation of the EP4 receptor sensor. (C) Concentration response curve based on experiments as shown in (B) n = 5 each, mean ± SD, EC₅₀ = 24 nM (95% CI: 22 nM to 27 nM); normalized to response at 5 µM PGE₂.

Fig. 2 Ligand binding kinetics recorded with the high resolution multiple-cell reader on EP4 receptor-sensor cells.

(A) Effects of increasing concentrations of PGE₂, n = 4 for each condition, were normalized to maximum effects induced with 10 µM PGE₂. A buffer injection without ligand was applied in control channels (red). Data were recorded with 41 Hz and plotted after gentle smoothing (rolling mean, k=4); the shaded areas show the S.E.M. at each point.

(B) Equilibrium effects were used to determine a concentration-response curve with an EC₅₀ of 21.3 ± 3.7 nM and a Hill slope of 1.01 ± 0.13 (data show mean and SD for n=4 duplicates). (C – F) Selected curves (N = 1) were taken from the data set of (A) and analysed according to a single-term
(dashed line) and, for higher PGE₂ concentrations, to a two-term exponential model (Eq. 3, solid line, D, F). Horizontal dashed lines at 0.35 (D) and 0.53 (F) indicate the proportion of fast components. Parameter estimates and standard errors (SE) are shown in Table S3. Kinetic data for the entire set of curves are given as means ± SD in Table S4.

**Fig. 3** Schild-plot analysis of the mode of antagonism of PGE₂ effects by L-161,982.

HEK293 cells stably transfected with the PGE₂ receptor sensor were pretreated with L-161,982 at 37°C for at least 15 min before a concentration-effect curve for PGE₂ was started. Experiments were carried out in the new 12-channel fluorescence measuring devices (RasiNext-FRET) which facilitate the measurement of equilibrium effects (compare Fig. 2) used to determine concentration-effect curves for PGE₂ (A, data are shown as n= 4 duplicates, ± SD). Curves were fitted by non-linear regression and pEC₅₀-values were used to calculated concentration-ratios (CR) for PGE₂ in the absence and presence of the antagonist. The double log plot of (CR-1)-values dependent on the concentration of L-161,982 (B) shows a linear relationship with a slope of 1.06±0.04 and an abzissa intercept of 8.47 ± 0.06 (-log M) characterizing L-161,982 as a competitive antagonist.

**Fig. 4** EP₄ receptor sensor is suited for measurements in a commercial plate reader and shows selectivity for PGE₂ among arachidonic acid products.

(A) Left: Scheme of FRET measurement in 96 well format at Spark 20M (Tecan) plate reader. Fluorescence of HEK293 stably expressing EP₄ receptor sensor (120k per well). Right: Example trace, representative recording out of n = 16. (B) – (E) Values were normalized to the response to 833 nM PGE₂. (B) PGE₂: EC₅₀ = 27 nM (95% CI: 24 nM to 31 nM), PGE₁: EC₅₀ = 65 nM (95% CI: 58 nM to 72 nM). (D) 8-iso PGE₂ EC₅₀ = 909 nM (95% CI: 855 nM to 967 nM). (C), (E) individual values are shown. (B), (D) Concentration response curves with the indicated agonist in 96 well format. Data were plotted as mean ± SD. n = 5-8 per data point.

**Fig. 5** Real-time measurement of prostaglandin formation in MDCK cells.
HEK293 cells stably expressing EP4 receptor sensor either alone or co-cultured with Madin-Darby canine kidney (MDCK) cells as indicated by the cartoons. The measurements were performed in 96 well format by means of a plate reader as described in Fig. 4. Cells were pretreated at least 16 hours before measurement with either 100 µM of the COX inhibitor acetyl salicylic acid (ASS), 5 µM of the microsomal PGE2 synthase-1 inhibitor MF63 or the respective vehicle solution. Data are shown as mean ± SD. FRET alterations were normalized to the response of 833 nM PGE2. The agonist induced response was blocked with 3800 nM L-161,982. (A) and (B) averaged data is shown of measurements in the plate reader performed side by side; n = 7 - 8 each, out of a total of (A) n = 15 - 16 and (B) n = 24 each. Compared are the mean values before max PGE2 application. (A) One-way ANOVA p = 0.0002 subsequent post hoc test: Tukey's multiple comparisons test w/o vs MF63 p = 0.0004***, w/o vs ASS p = 0.8426 and MF63 vs ASS p = 0.0017** (B) One-way ANOVA p < 0.0001 subsequent post hoc test: Tukey's multiple comparisons test w/o vs MF63 p<0.0001****, w/o vs ASS p<0.0001***** and MF63 vs ASS p = 0.7653 (C) n = 8 (D) Concentration response curve based on measurements as shown in (C) EC50 = 10 µM arachidonic acid (AA, 95% CI: 8 µM to 14 µM).

**Fig. 6 Measurement of prostaglandin release of primary macrophages.**

HEK293 cells stably expressing EP4 receptor sensor co-cultured with macrophages isolated from mice treated as described in Kuroda and Yamashita, 2003 with either LPS or vehicle; the measurements were performed in 96 well format at a fluorescent microscope. We measured macrophages from three different isolations. Each isolation contained the pooled cells of 5 animals. If indicated, final arachidonic acid concentration was at least 27 µM. The final concentration of PGE2 was 833-909 nM and of L-161,982 3846-4166 nM. All traces were normalized to the response to PGE2, while the response to the antagonist L-161,982 was set to 0. (A) Representative traces from isolation 3 (B) Overlay of traces measured as shown in (A) from isolation 3 during the course of arachidonic acid application, data shown as mean ± SD; n (LPS+) = 9, n (LPS-) = 2. (C) Representative traces of the short protocol from isolation 3 out of n = 4-6 each; (D) PGE2 quantity was calculated based on measurements as underlying figures (A) - (C), for details see methods and for the exact values for each
well of each isolation Table S3. The wells for each isolation with and without LPS treatment were averaged and plotted. The averaged data for each isolation with and without LPS treatment were considered as a group and connected with a line.

**Fig. 7 EP4 receptor sensor is potentially suited for PGE2 imaging.**

(A) Left: Representative brightfield microscopy image of HEK293 cells stably expressing EP4 receptor sensor, glass micropipette filled with 10 µM PGE2, right: respective fluorescent image, excited at 440 nM emission measured at 535 nM. (B), (C): the colors refer to the outline boxes in Fig. 7A. (B): Left FRET traces from top, the colored box indicates the region of interest of the respective color. All traces were normalized to the response to max PGE2 (final concentration: 2 µM).

Right: Magnified sequence of the FRET traces shown in (B) left, marked with the black box. (C) 4 more experiments were performed, similar to the experiment shown in (A) and (B), see Fig. S6. For comparison, the traces were normalized to the initial value before pressure application and not the maximum PGE2 response as done in (A) and (B), for details see methods section. Maximum values of each region of interest after local PGE2 application were compared and ranked. (C) shows the frequency distribution using the respective colors. 1 = maximum amplitude to 5 = smallest amplitude.
Fig. 1

A

agony buffer

EP4

B

$\Delta (eYFP/mTurq2)$

$\Delta F/F_0$

\[ \begin{array}{c}
20 \\
200 \\
5000 \\
\end{array} \]

PGE$_2$ [nM]

C

Norm. Response

PGE$_2$

log(M)

75 s
Fig. 2

A) Plot showing the effect of PGE$_2$ at different concentrations on eYFP/mTurq2 expression over time. The half-life ($t_{1/2}$) values are indicated for each concentration.

B) Graph illustrating the normalized response of PGE$_2$ at different concentrations.

C) Plot at 16 nM PGE$_2$, with $t_{1/2} = 197.6$ sec.

D) Plot at 256 nM PGE$_2$, with $t_{1/2} = 76.2$ sec.

E) Plot at 64 nM PGE$_2$, with $t_{1/2} = 138.6$ sec.

F) Plot at 1024 nM PGE$_2$, with $t_{1/2} = 88.5$ sec.