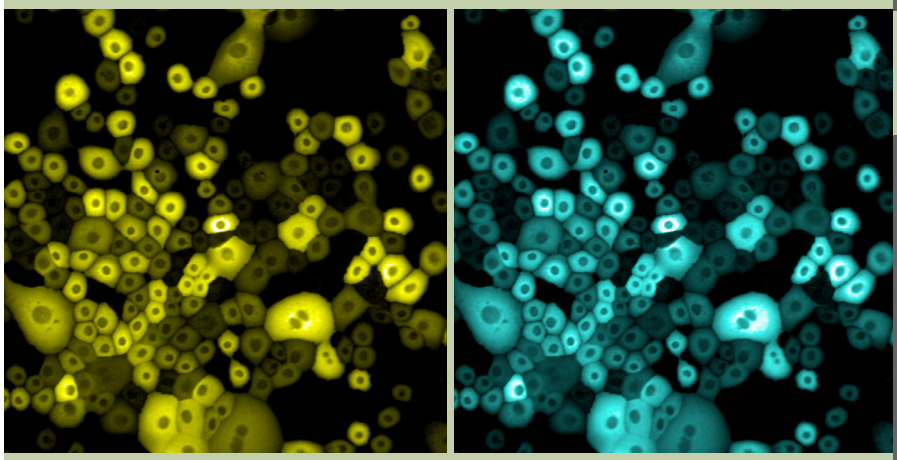

APPLICATION NOTE

Cellular Imaging and Analysis

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Analyzing ERK Signal Transduction in Live Cells Using a FRET-Based ERK Biosensor on the Operetta CLS High-Content Analysis System

Key Points:

- Ratiometric high-content FRET assay in living cells
- Quantify ERK signaling using an EKAREV biosensor
- Study signaling cascade-modulating compounds

Introduction

Extracellular signal-regulated kinase (ERK) is a key component in the regulation of embryogenesis, cell differentiation, cell proliferation and cell death.[1] The ERK pathway originates from an activated receptor in the plasma membrane and is propagated via Ras/Raf/MEK to ERK (Figure 1). The pathway is activated by different types of receptors, including receptor tyrosine kinases (e.g. EGF receptor) as well as G-protein coupled receptors.[2] As the final component of the signaling pathway, ERK phosphorylates different intracellular proteins including a large number of other kinases and transcription factors. The ERK signaling pathway is altered in various cancer types and hence is being investigated as a target for therapeutic intervention.[3]

Here, we describe how a live cell FRET (Förster Resonance Energy Transfer) assay, used to study ERK signaling, can be automated on the Operetta CLS™ High-Content Analysis system. This assay concept can support the drug discovery process for identifying signal-cascade modulating compounds.

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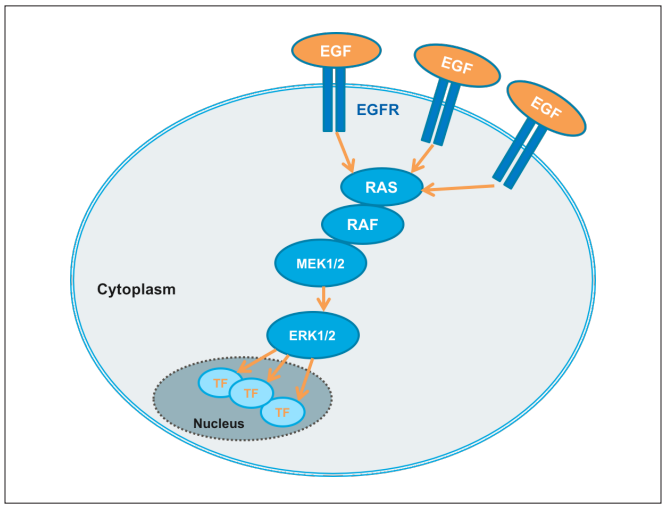

Figure 1. The Ras/Raf/MEK/ERK signaling cascade propagates signals from cell surface receptors such as the EGF receptor (EGFR) to intracellular proteins. ERK is the final component of this pathway and upon activation by growth factors, such as EGF (epidermal growth factor), downstream effects, for example, activation of kinases or transcription factors, are triggered.

FRET-based ERK Biosensor

FRET is the non-radiative transfer of energy from a donor molecule to an acceptor molecule.[4] The energy transfer requires the donor and acceptor to be less than 10 nm apart and thus provides a sensitive tool to study changes in molecular proximity, such as protein-protein interactions (intermolecular FRET) or conformational changes of proteins (intramolecular FRET).[5] More information on FRET and how it can be used for protein-protein interaction studies can be found in a previously published Application Note.[6] In this study, we focus on intramolecular FRET, using a CFP-YFP biosensor called EKAREV (ERK activity reporter with Evee backbone) (Figure 2). Cells stably expressing EKAREV were kindly provided by Dr. Somponnat Sampattavanich (Figure 3). In this biosensor, donor and acceptor fluorophores are encoded in a single fusion protein. The EKAREV biosensor was optimized to reduce randomly triggered basal FRET signals and to render it reliably distance-dependent - increasing gain and sensitivity.[7, 8] Phosphorylation of EKAREV by ERK triggers a conformational change moving CFP and YFP into close proximity to induce FRET.

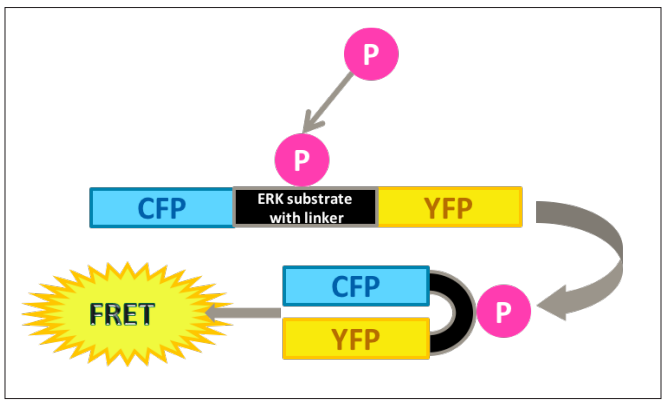

Figure 2. Schematic representation of the extracellular signal-regulated kinase activity reporter (EKAREV). In this biosensor, the two fluorescent proteins are separated by an ERK substrate domain, a linker, and a binding domain. Once the ERK substrate domain undergoes phosphorylation by ERK, a conformational change is triggered bringing CFP and YFP into close proximity and allowing FRET to occur.

The EKAREV biosensor is an example of intramolecular FRET where donor and acceptor are present at a fixed stoichiometry of 1:1. Therefore, it is sufficient to perform a two-channel ratiometric experiment, with channel 1 measuring acceptor emission ($I_{Acceptor}$) after exciting the donor and channel 2 recording the donor emission (I_{Donor}) excited by the donor. Subsequently, the two resulting fluorescence signal intensities are background-corrected and their ratio is calculated to give the relative FRET efficiency E_{FRET}:

$$E_{FRET} = FRET\ ratio = \frac{(I_{Acceptor} - I_{Background\ Acceptor})}{(I_{Donor} - I_{Background\ Donor})}$$

Application

Assay procedure: 1.2x10^4 EKAREV cells/well were plated in 150 µL growth medium (details in Table 1) into CellCarrier-96 Ultra microplates (PerkinElmer # 6055300). After 2 days of incubation (37 °C, 5% CO_2), cells were serum starved by washing them twice with 150 µL starving medium and incubating them for 5 hours in starving medium to decrease the basal ERK activity. Additionally, various concentrations of inhibitor or DMSO were added to the cells at the beginning of this starvation phase. After 4.5 hours, nuclei were stained with 4 µM DRAQ5 for 30 mins at 37 °C, 5% CO_2. Cells were then washed once with starving medium and 150 µL fresh starving medium, containing 8 µL of 20x concentrated inhibitors or DMSO controls, was added. As a reference, one time point was recorded followed by a break in the time series. During this break, 8 µL of 20x concentrated inducers (PMA or EGF) were added to the cells. To inhibit FRET signals, PD184352, SCH772984 and Ulixertinib were applied. Starving medium with or without the highest DMSO concentration of the tested compounds served as the control.

Table 1. List of assay reagents, compounds and media.

Growth Medium	Phenol-red-free DMEM/F12, Life Technologies, 11039-021 5% horse serum, Gibco, 16050-122 20 ng/mL EGF, Peprotech, AF-100-15 500 ng/mL hydrocortisone, Sigma, H0888 10 µg/mL insulin, Sigma, I2643 100 Units penicillin, 0.1 mg/mL streptomycin, Sigma, P4333 100 ng/mL choleratoxin, Sigma, C8052
Starving Medium	Phenol-red-free DMEM/F12, Life Technologies, 11039-021 500 ng/mL hydrocortisone, Sigma, H0888 100 Units penicillin, 0.1 mg/mL streptomycin, Sigma, P4333 100 ng/mL choleratoxin, Sigma, C8052
Nucleus Stain	DRAQ5, Biostatus, DR50200
Control	Phenol-red-free DMEM/F12, Life Technologies, 11039-021, containing highest present concentration of DMSO (dimethylsulfoxide), Sigma, D2650
Inhibitors	PD184352, Sigma, PZ0181 SCH772984, Cayman, 19166 Ulixertinib, Cayman, 18298
Inducers	EGF (epidermal growth factor), Peprotech, AF-100-15 PMA (phorbol 12-myristate 13-acetate), Sigma, P1585

Measurement: A time-lapse experiment was set up on the Operetta CLS system under climate controlled conditions (37 °C, 5% CO_2) using the 20x high NA objective lens (NA 0.8) in widefield mode. Images were acquired for a total of 97 minutes. After adding FRET-inducing compounds to the serum-starved cells, the time series was started with initially short measurement intervals - every 8 minutes – followed by one additional measurement after 25 minutes. Four channels were acquired in this setup: DRAQ5 (ex 615-645, em 655-760), CFP (ex 435-460, em 470-515), YFP (ex 490-515, em 525-580) and FRET (ex 435-460, em 515-580) (Figure 3).

Analysis Strategy: Automated image analysis was performed with Harmony® High-Content Imaging and Analysis Software. Briefly, images were segmented into cells and background. Donor and FRET intensities in the cytoplasm and background were calculated, followed by calculating the background corrected FRET ratio as final readout (Figure 4).

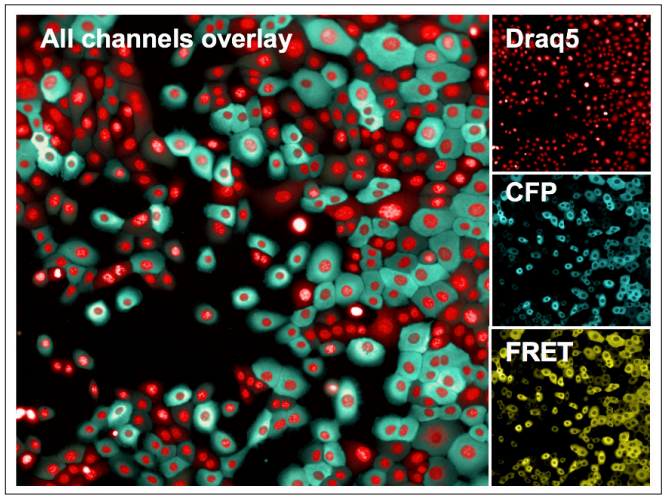

Figure 3. Human mammary epithelial cells stably expressing the EKAREV biosensor. Nuclei were stained with DRAQ5. Subsequently, cells were imaged using a 20x high NA objective in widefield mode on the Operetta CLS system. Although this is a stable cell line, note the relatively inhomogeneous expression levels of the biosensor.

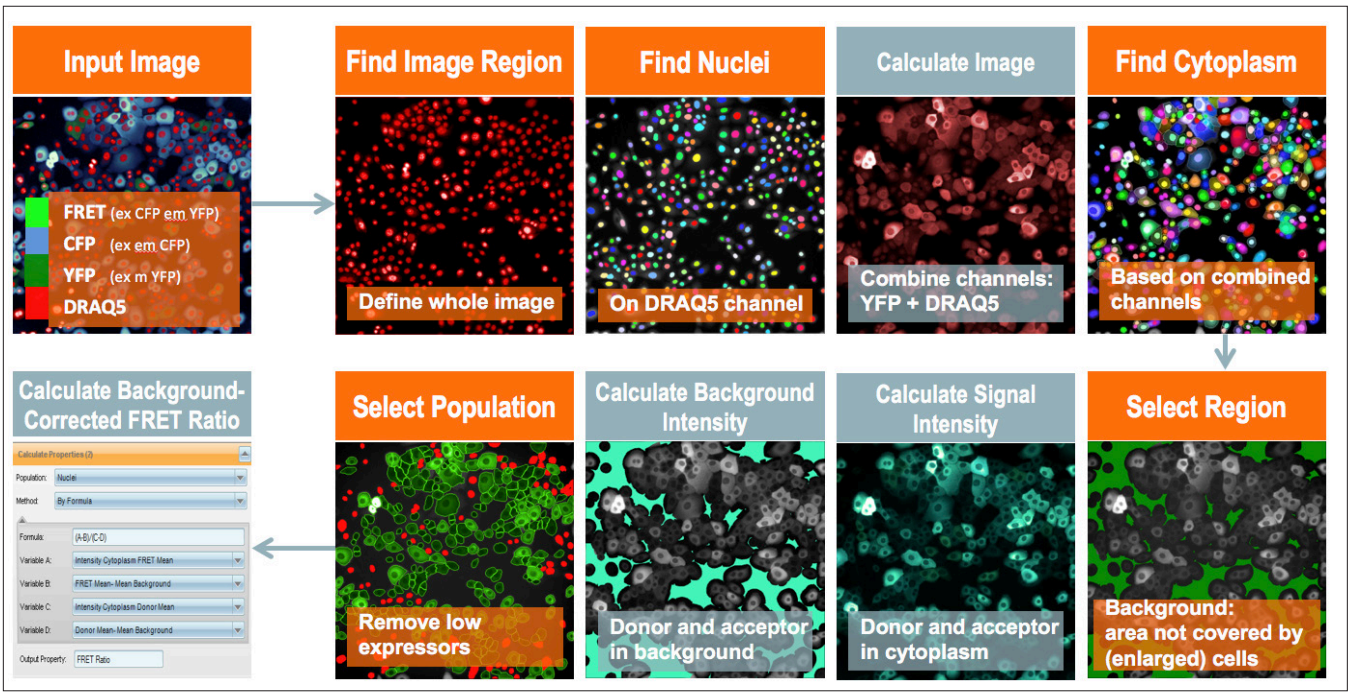

Figure 4. Image analysis workflow for ratiometric FRET quantification using Harmony software: cytoplasm of cells and background are segmented and low-expressing cells excluded by an intensity threshold. Intensity of donor and FRET channels and their appropriate backgrounds are quantified and the background-corrected FRET intensity ratio calculated. Subtracting background intensities is especially advantageous in live-cell applications where media with autofluorescent components often result in higher backgrounds and thus smaller assay windows.

Results

To explore whether modulations of ERK-signaling could be studied on the Operetta CLS using a FRET-based biosensor, EKAREV cells were treated with different ERK and MEK activators and inhibitors (Figure 5). PMA and EGF served as specific activators of the Ras/Raf/MEK/ERK signaling cascade. EGF specifically binds to the EGF-receptor on the cell surface, while PMA as a lipophilic, membrane-permeable molecule activates the pathway via direct activation of RAF. PD184352 can suppress the ERK pathway by selectively inhibiting MEK1/2, while both Ulixertinib and SCH772984 are potent and selective inhibitors of ERK1/2.

First, in order to learn more about the dynamic nature of FRET induction and inhibition, a 97 minute time-lapse experiment was recorded. As expected, treating the cells with either EGF or PMA alone leads to a strong increase in FRET ratio when compared to the untreated control (Figure 6). The signal plateaus on a high level after around 30 minutes. The control shows a lower level of ERK activation, and a steady increase over time is observed. Since ERK1/2 can be regulated via a variety of growth factors and mitogens, this can be caused by autocrine or paracrine signaling during live cell imaging. Co-treatment of the cells with various concentrations of an ERK inhibitor (SCH772984) leads to a dose-dependent decrease of the ERK response. At 5 µM SCH772984, ERK activation via EGF is almost negligible, suggesting ERK to be completely inhibited at this concentration. Please note that 0.5% DMSO, which was the highest concentration used in the experiment, does have an effect on the FRET ratio, and therefore this control needs to be included. Similar results were obtained with a second ERK1/2 specific inhibitor, Ulixertinib (data not shown).

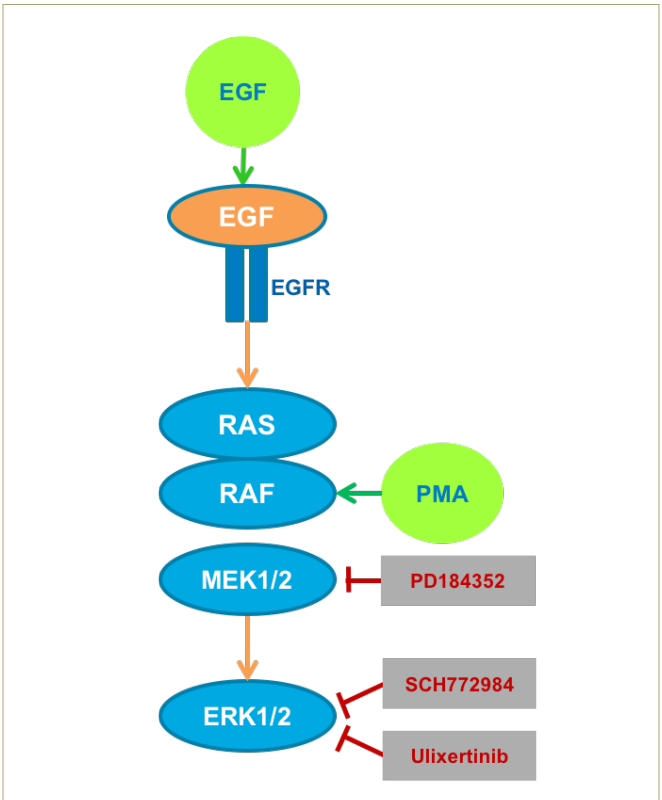

Figure 5. Schematic overview of exogenously added activators (green) and inhibitors (red) and their impact on the ERK signaling pathway. The EKAREV-expressing cells were treated with EGF or PMA to induce ERK activation, or, in addition, with one of three MEK and ERK-specific inhibitors (PD184352, SCH772984, Ulixertinib), interrupting signal transduction at different positions of the pathway.

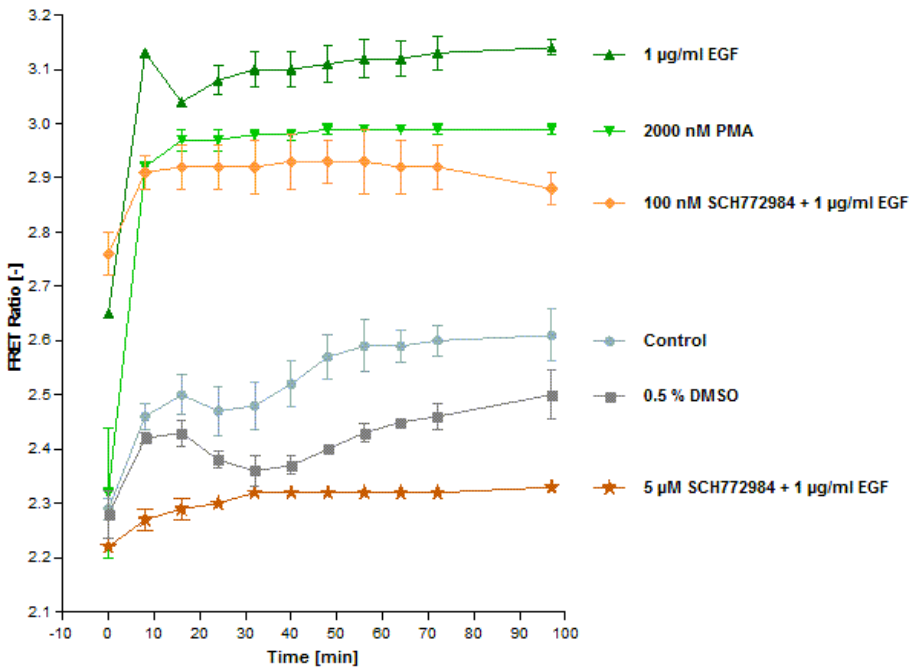

Figure 6. Time course of ERK signaling measured with the EKAREV FRET-based biosensor on the Operetta CLS system. Stimulation of ERK via EGF or PMA induces a rapid FRET signal increase that plateaus after about 30 min. High concentrations of SCH772984 (5 µM) lead to almost complete inhibition of ERK activation (in presence of 1 µg/mL EGF) as almost no FRET signal increase is measurable. Higher dilutions of SCH772984 only partially inhibit the EGF-induced ERK activation. The control curve shows an intermediate, slightly rising FRET signal without any treatment. FRET signals are slightly inhibited by 0.5 % DMSO, which was the highest concentration of DMSO used in the experiment. Assay statistics: Z`= 0.87 (as calculated at time point 32 min, DMSO being the negative and EGF the positive control).

As FRET signals reached a constant level after 32 min, this timepoint was selected to determine the IC_{50} value of SCH772984. The EKAREV cells were treated with 1 µg/mL EGF and a six point dilution series of SCH772984, ranging from 10 pM to 3 µM. The dose response curve with a calculated IC_{50} value of 272 nM is shown in Figure 7.

To explore whether the EKAREV FRET imaging assay can be used to study pathway modulations directly acting on MEK1/2, the effect of the MEK1/2 inhibitor PD184352 on PMA activated cells was tested (Figure 8). As shown, PD184352 inhibited the PMA-induced ERK activation.

Conclusions

As shown in this application note, the EKAREV FRET biosensor can be used in a live-cell imaging assay on the Operetta CLS system to study ERK activation and inhibition. Modulation of different targets within the cascade was easily measurable, hence this approach can contribute to the identification of new compounds interfering with the Ras/Raf/MEK/ERK signaling cascade. The assay is performed in living cells and therefore it can be used to analyze ERK signaling dynamics, while conventional biochemical techniques quantifying ERK phosphorylation are typically endpoint assays. Despite the relatively inhomogeneous biosensor expression levels (Figure 3) in the cell population, calculation of the FRET ratio provided exceptionally good assay data and statistics, with the Z` value being above 0.87. The optimized design of the EKAREV biosensor, the high quality imaging on the Operetta CLS system and the excellent tools for image analysis within Harmony all contributed to the robustness of the high-content FRET assay presented here. The building block concept of Harmony software allows the creation of image analysis sequences that are easy to set up and comprehend, and do not require expert image analysis knowledge. This assay also delivered comparable results and assay statistics on the Opera Phenix™ High-Content Screening system. As the Operetta CLS and Opera Phenix systems enable much higher throughput than conventional microscopes, high-content imaging of FRET based biosensors opens up new possibilities for drug discovery and basic research within cell signaling.

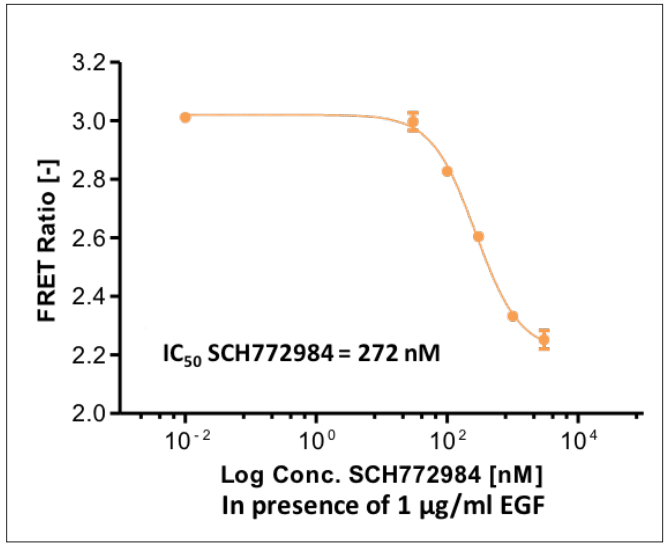

Figure 7. The ERK inhibitor SCH772984 leads to a dose-dependent decrease of FRET-based EKAREV signals. EKAREV cells were treated with increasing concentrations of SCH772984 in the presence of 1 µg/mL EGF. The FRET ratio was determined on the Operetta CLS system after 32 min of incubation, as signals stabilized at this time point. The high Z' value (Z' = 0.89) shows an excellent assay performance.

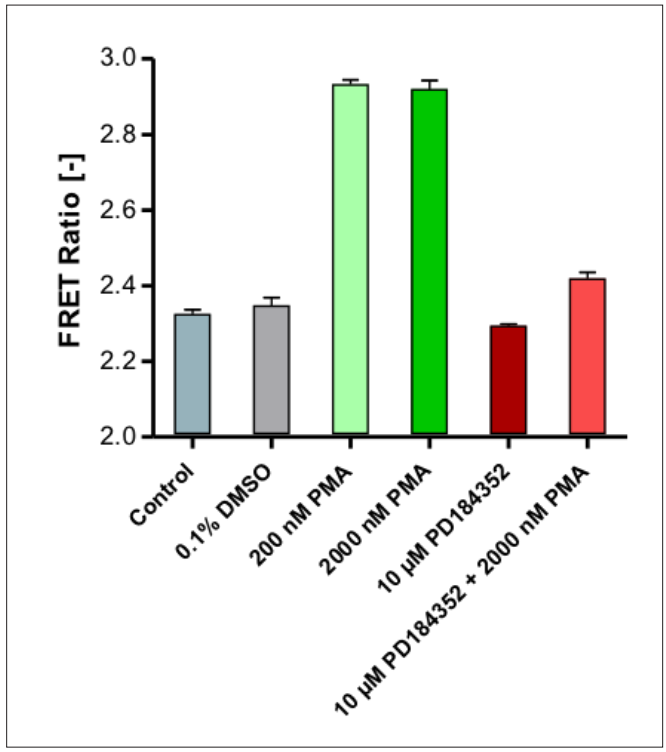

Figure 8. Inhibition of the PMA activated Ras/Raf/MEK/ERK signaling cascade by PD184352 measured on the Operetta CLS system. EKAREV cells were treated with an alternative set of activator and inhibitor (PMA + PD184352) acting further upstream on RAF/MEK (compare with Figure 5). EKAREV cells treated with either 200 or 2000 nM PMA show a high FRET response (32 min post induction). The activation is inhibited by co-incubating the cells with the MEK1/2 specific inhibitor PD184352 at a concentration of 10 µM.

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