1 A B C D

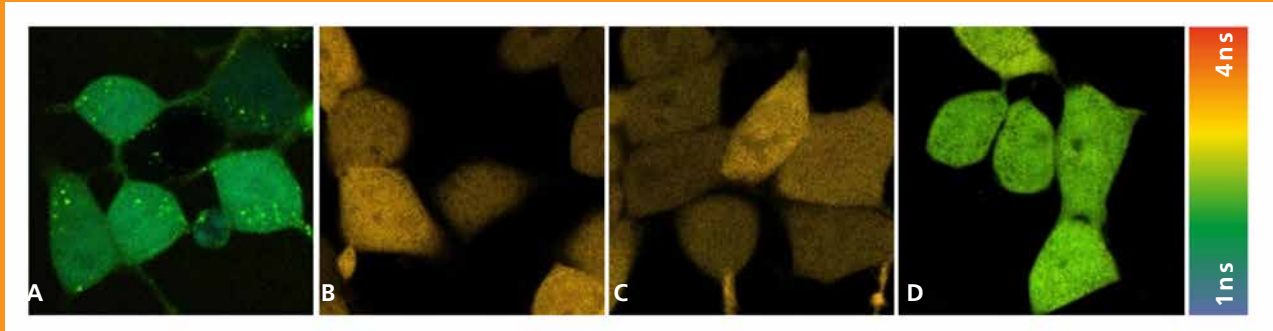**1** *FLIM images of HEK293T cells expressing FRET fluorophores alone (A, B), in combination (C) or as a fusion protein (D) © Goethe University Frankfurt and Fraunhofer IME / Verena Trümper, Andreas von Knethen, Tilo Knape.*

Fraunhofer Institute for Molecular Biology and Applied Ecology IME

Branch for Translational Medicine and Pharmacology of the Fraunhofer IME
Theodor-Stern-Kai 7
60596 Frankfurt/Main

Contact:
Dr. Tilo Knape
Phone: +49 69 8700-25072
tilo.knape@ime.fraunhofer.de

Prof. Dr. Andreas von Knethen
Phone: +49 69 6301-6989
andreas.von-knethen@ime.fraunhofer.de

Dr. Volker Laux
Phone +49 69 8700-25076
volker.laux@ime.fraunhofer.de

www.ime.fraunhofer.de/en/TMP

FLOW CYTOMETRY-BASED FRET ASSAY FOR ANALYSIS OF PROTEIN-PROTEIN INTERACTIONS
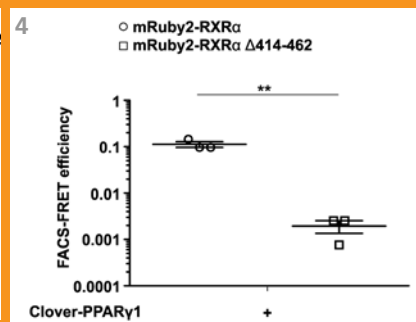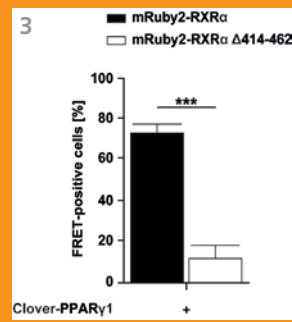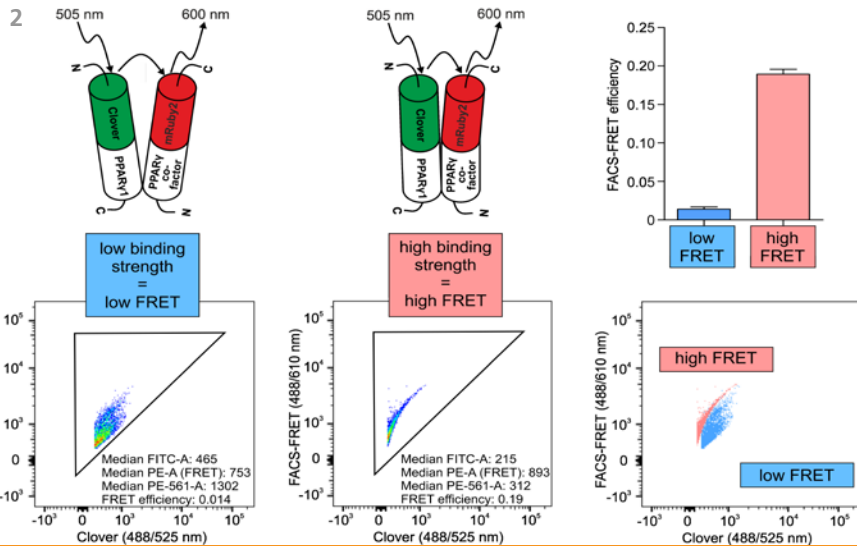
The detailed characterization of protein-protein interactions is highly important to study the development of diseases, to identify new molecular and pharmacological targets and to detect subtle changes in protein interactions upon drug treatment. To achieve this, Fraunhofer IME has developed a systematically improved, flow cytometry-based Förster`s resonance energy transfer (FRET) assay for molecular verification of specific protein-protein interactions. This for the first time, allows the close determination of protein-protein interactions with a special focus on binding intensity, allowing this system to characterize the role of protein domains as well as the effect of pharmacological agents on protein-protein interactions.

Field of application

- Basic signaling pathway research
- Testing novel therapeutics and drugs
- Detailed characterization of the binding profile of drugs
- Molecular and pharmacological target identification

Readout parameters

- Flow cytometry-based FRET of protein-protein interactions in living cells
- Determination of the cellular localization by fluorescence microscopy (Fig. 1)
- Verification of mechanistic functionality by reporter gene analyses
- Detection of protein expression by western blot

2 *Graphical abstract: Flow cytometry-based FRET combines the determination of FRET-positive cells as well as the FACS-FRET efficiency. This parallel approach allows to determine the binding strength of two interacting proteins in the living cell. © Goethe University Frankfurt and Fraunhofer IME / Verena Trümper, Andreas von Knethen, Tilo Knape.*

Vector and cell line generation

- Cloning of expression vector(s), encoding for the gene(s) of interest fused with donor (Clover) and acceptor (mRuby2) FRET fluorophores
- Generation of human and murine cell lines transiently and stably expressing gene(s) of interest

Quality management and validation

Flow cytometry-based FRET measurements are performed using a BD LSRFortessa™ flow cytometer with 488 nm and 561 nm lasers. To measure Clover and FRET, cells are excited with the 488 nm laser and fluorescence is detected in the green channel with a 525/50 nm filter, while the FRET signal is measured with a 610/20 nm filter in the channel. The mRuby2 cells are excited with the 561 nm laser, while emission is also detected with a 610/20 nm filter. For each sample, a minimum of 10,000 Clover- and/or mRuby2-positive cells are analyzed. Cells stably expressing Clover/mRuby2 alone or in combination and Clover fused (63bp) mRuby2 are used as positive and negative controls. The binding affinity is measured based on the determination of the FRET intensity via the measurement of the mean relative fluorescence value. Refining the system, Fraunhofer IME created a new readout (Fig. 2) to distinguish strong from weak interactions, focusing on nuclear hormone receptor (NHR)-binding to co-factors (CoF). Reporter gene analysis and co-localization studies by laser-scanning microscopy validated this system.

Application example changes in protein-protein interactions analyzed by flow cytometry-based FRET

Fraunhofer IME investigated the protein-protein interactions of the NHR, human peroxisome proliferator-activated receptor gamma 1 (PPARγ1), with a selected CoF, retinoid X receptor alpha (RXRα), and a deletion construct RXRα (RXRα Δ414–462) in living HEK293T cells to substantiate the functionality of the systematically improved flow cytometry-based FRET assay system. Consequently, due to the deletion (Δ414–462) of the C-terminus of RXRα, which is necessary for PPARγ1-binding, cells expressing RXRα Δ414–462 showed reduced binding to Clover-PPARγ1. In this case, only 11.5% of the double positive (Fig. 2) HEK293T cells expressing Clover-PPARγ1 and mRuby2-RXRα Δ414–462 showed a FRET signal whereas 72.9% of double positive HEK293T Clover-PPARγ1 + mRuby2-RXRα cells showed a FRET signal. Overall, the percentage of FRET-positive HEK293T Clover-PPARγ1 + mRuby2-RXRα cells was significantly higher compared to HEK293T cells expressing Clover-PPARγ1 and mRuby2-RXRα Δ414–462. Hence, flow cytometry-based FRET gives a robust readout for the interaction of this PPARγ1 with its CoF, RXRα, that is highly superior as compared to non-background corrected co-localization microscopy (Fig. 3). FRET efficiencies (Fig. 4) reflect FACS data.

Selected publications

- Trümper V, von Knethen A, Preuß A, Ermilov E, Hackbarth S, Kuchler L, et al. Flow cytometry-based FRET identifies binding intensities in PPARγ1 protein-protein interactions in living cells. Theranostics 2019; 9(19): 5444-5463.

3 *Comparison of the total percentages of FRET-positive living HEK293T Clover-PPARγ1 + mRuby2-RXRα and HEK293T Clover-PPARγ1 + mRuby2 RXRα Δ414-462 cells © Goethe University Frankfurt and Fraunhofer IME / Verena Trümper, Andreas von Knethen, Tilo Knape.*

4 *Flow cytometry-based FRET efficiencies of FRET-positive living HEK293T Clover-PPARγ1 + mRuby2-RXRα and HEK293T Clover-PPARγ1 + mRuby2 RXRα Δ414-462 cells © Goethe University Frankfurt and Fraunhofer IME / Verena Trümper, Andreas von Knethen, Tilo Knape.*