Introduction

FRET (fluorescence resonance energy transfer) is a physical process in which energy is transferred from one fluorophore (donor) to another (acceptor) by dipole-dipole interaction. A requisite for this process is a significant overlap of the donor emission spectrum and the acceptor absorption spectrum and the spatial proximity and orientation of two fluorophores (mostly within 10 nm) (Figure 1).

Applications utilizing FRET include the measurement of receptor interactions, transcription factors and binding proteins or caspase- and kinase- activities used for biochemical or immunological processes. Further, the excitation/emission properties of tandem conjugate fluorophore pairs used in flow cytometry (e.g., PE-Cy5, PE-Cy7 or APC-Cy7) are based on FRET.

This protocol describes how the interaction of the proteins interferon regulatory factor 1 (IRF-1) and myeloid differentiation factor 88 (MyD88) can be measured and visualized using the fluorescent protein moieties cyan fluorescent protein (CFP) as donor and yellow fluorescent protein (YFP) as acceptor.

Materials and Methods

Murine cDNA for MyD88, TRIF, IRF-1 was amplified by standard PCR technique from a RAW264.7 cDNA, sequenced and subcloned into expression plasmids for the generation of recombinant proteins.

HEK293T cells were transiently transfected with the respective plasmids and were stained 36h later with Ethidium monoazide (EMA) for dead cell exclusion (20 minutes in the dark, 4°C), followed by crosslinking of EMA (10 minutes on ice in light – 50W / 20 cm distance) and washing twice with PBS. Cells were fixed in PBS/1% paraformaldehyde.

To measure FRET from CFP to YFP on the CyAn ADP 9 Color, its original filter layout (Figure 2) is easily modified to a configuration (Figure 3) optimized to detect YFP (FL1 – excitation at 488 nm, dichroic filter 560LP, emission filter 550/30) and CFP (FL6 – excitation at 405nm, emission filter 467/26) and the FRET signal CFP -> YFP (FL7 – excitation at 405nm, emission dichroic filter 505LP, emission filter 550/30). Summit software v4.3 and FlowJo* software v8.2 were used for data analysis.

For single color controls (SCC) mock, single MyD88CFP, MyD88YFP, IRF1CFP, IRF1YFP transfected cells were used (data not shown). The CyAn ADP was set up in three steps:

1. Adjust PMT voltage for all parameters to set negative cells of the unstained controls gated on HEK293T cells in the first log decade and save a total of 25,000 events.
2. Run all stained SCC and additional controls and save a total of 25,000 events.
3. Run Auto Compensation function in Summit software using the SCC and the gate of live HEK293T cells.

After having set up the CyAn ADP, all SCC and further controls were acquired with a minimum of 100,000 total events to ensure validity of compensation and correct gates.
Technical Tips

- To reduce autofluorescence the medium should not contain phenol-red.
- Always use positive and negative controls to set up the flow cytometry analyzer.
- Set up an experimental condition to detect FRET in a positive control (CFP-YFP fusion protein transfected cells).
- Due to the need of spatial proximity and orientation of the FRET fluorophore pair, the optimal localization of the fluorophore (N- or C-terminal) determines the effectiveness of FRET.
- While CFP excitation characteristics may change upon fixation (Domin A. et al), living cells (with dead cells excluded by propidium iodide) may be used for analysis.
- Employ a fluorescence microscopy control to confirm FRET.

Results

Activation of interferon regulatory factor (IRF)-3 and/or IRF-7 drives the expression of antiviral genes and the production of α/β interferons (IFN), a hallmark of antiviral responses triggered by Toll-like receptors (TLRs) and certain cytoplasmic RNA helicase family members.

We recently described a novel antiviral signal pathway operating in myeloid (m) dendritic cells (DCs) and macrophages, which does not require IRF-3 and/or IRF-7 but is driven by IRF-1. IRF-1 together with myeloid differentiation factor 88 (MyD88) triggered IFN-β promoter activation upon physical interaction. The IRF-1 dependent IFN-β production by activated mDCs was paralleled by rapid transcriptional activation of IFN-stimulated genes. While the NF-κB dependent production of pro-inflammatory cytokines was independent of IRF-1, TLR-9 signaling through this novel pathway conferred cellular antiviral resistance while IRF-1 deficient mice displayed enhanced susceptibility to viral infection. These results demonstrate that TLR-9 activation of mDCs and macrophages contributes via IRF-1 to antiviral immunity.

Cells were measured and gated first on forward vs. side scatter (Figure 4A). After dead cell exclusion (EMA positive cells, Figure 4B) FRET was detected as double positive (CFP/YFP) cells. When gated on double transfected cells, the FRET positive population (MyD88-IRF-1 pair) is detected in FL7 (Figure 4C). Control transfected cells with non-interacting molecule pairs (TRIF-IRF-1 pair), on the other hand, show no FRET positive events (Figure 4E). To visualize this observation more intensely, FRET fluorescence normalized by donor fluorescence (FRETcalc.), is plotted against CFP (Figure 4D and 4F). Collectively, data from co-immunoprecipitation and FRET measurement confirm the interaction of MyD88 and IRF-1. Thus, FACS based measurement of FRET allows the easy and rapid detection of molecule interactions in living cells.

Discussion

The detection of molecular interactions is an integral part of cellular signal transduction research. The most widely used method of co-immunoprecipitation to detect such protein complexes often requires extensive optimization for the experimental conditions. The FRET based method and the corresponding experiment employed on both living and fixed cells can be easily established without the...
need of lysing the cells. In addition, a flow cytometry based approach allows a fast analysis providing statistically representative values. In contrast to FRET detection methods where the acceptor molecule is selectively bleached to increase donor emission intensity (acceptor photobleaching), the method described here is based on the excitation of the acceptor molecule (YFP) by the energy of an excited donor (CFP), while the acceptor itself is only sub-optimally excited by the violet laser light that is used for excitation of the donor.

Based on the results of this experiment, this powerful technique can be used for high throughput analysis of protein-protein interaction in living cells.

References


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Acknowledgements
We acknowledge the excellent technical assistance from Ingeborg Brosch and Monika Hammel. We thank Hermann Wagner and Dirk H. Busch for helpful discussions.

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