# Functional characterization of the human fractalkine receptor CX3CR1 using Photina™, a new highly sensitive photoprotein

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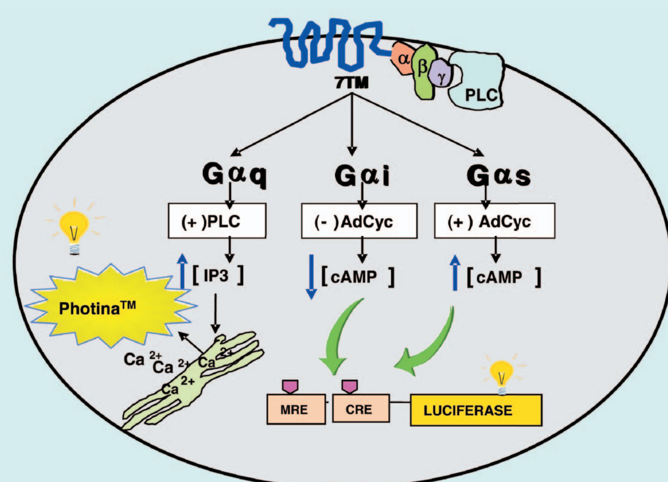
## Introduction

G-protein-coupled-receptors (GPCRs) are the largest family of cell surface receptors with an estimate total of 1000 members encoded in the human genome. These seven transmembrane domain receptors can be activated by a vast diversity of extracellular inputs such as amino acids, lipids, ions, proteases, nucleotides, peptides, large polypeptides, odorants, biogenic amines, and even photons. A wide variety of physiological functions are regulated by GPCRs: metabolism, secretion, neurotransmission, inflammatory and immune responses.
GPCRs currently represent the most important targets for drug development. High throughput screening (HTS) and ultra-HTS (uHTS) for human GPCRs are major goals for pharmaceutical companies.

CX3CR1 is a GPCR expressed in leukocytes and in myeloid cell lines. The receptor mediates a pathway for leukocyte trafficking upon activation by fractalkine (FKN) also called CX3CL1. FKN is a unique CX3C chemokine that functions not only as a chemoattractant but also as an adhesion molecule and it is expressed on endothelial cells. Soluble FKN causes migration of NK cells, cytotoxic T lymphocytes and macrophages; whereas the membrane-bound form captures and enhances the subsequent migration of these cells in response to secondary stimulation with other chemokines. Furthermore, stimulation through membrane-bound FKN activates NK cells, leading to increased cytotoxicity and interferon-γ production. Recently, accumulating evidence shows that FKN is involved in the pathogenesis of various diseases, such as atherosclerosis, glomerulonephritis, cardiac allograft rejection, and rheumatoid arthritis. In addition, polymorphisms in CX3CR1, which reduce its binding activity to FKN, have been reported to increase the risk of HIV disease and to reduce the risk of coronary artery disease.

CX3CR1 is a Gi-coupled and weakly Gq-coupled receptor able to inhibit adenylate cyclase and to stimulate intracellular $Ca^{2+}$ mobilization.
We have generated a cell line expressing the recombinant human CX3CR1 receptor in a stable way, in order to test its functionality following both the Gi- and Gq-coupled pathways.
Two different luminescence detection systems have been used: glow luminescence via a cAMP responsive-luciferase reporter vector and flash luminescence with a novel improved $Ca^{2+}$-sensitive photoprotein, Photina™. The scheme of this reporter cell line developed at Axxam is reported below.

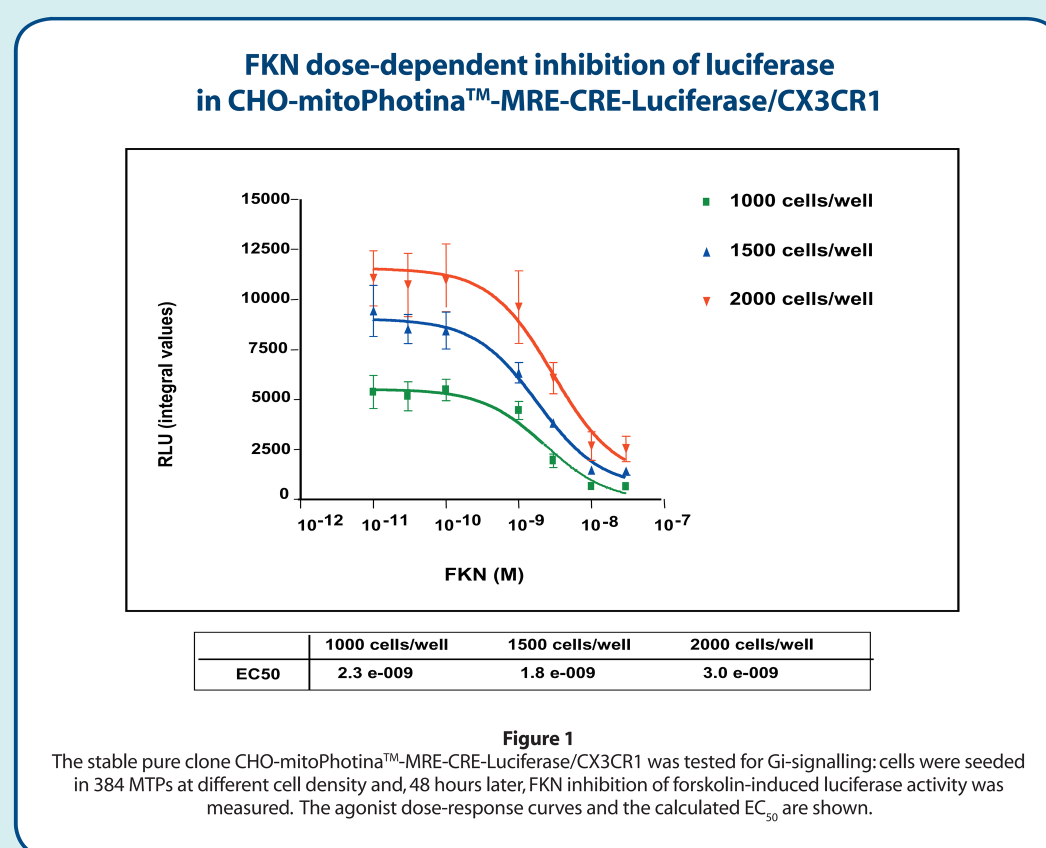
### GPCR reporter cell line




**CHO-mitoPhotina™-MRE-CRE-Luciferase cell line:**
GPCR sensitive reporter cell line developed in Axxam. The cell line allows compound screening for Gs- and Gi-coupled receptors via a cAMP responsive-luciferase reporter system and for Gq-coupled receptors via a novel improved $Ca^{2+}$-sensitive photoprotein, Photina™.

The CX3CR1-expressing cell line so far generated is a sensitive and reliable functional cell based assay suitable for HTS and uHTS of specific receptor antagonists. Furthermore, our data indicates that the newly developed photoprotein Photina™ represents a highly sensitive reporter for studying intracellular $Ca^{2+}$ mobilization mediated by the activation of GPCRs.

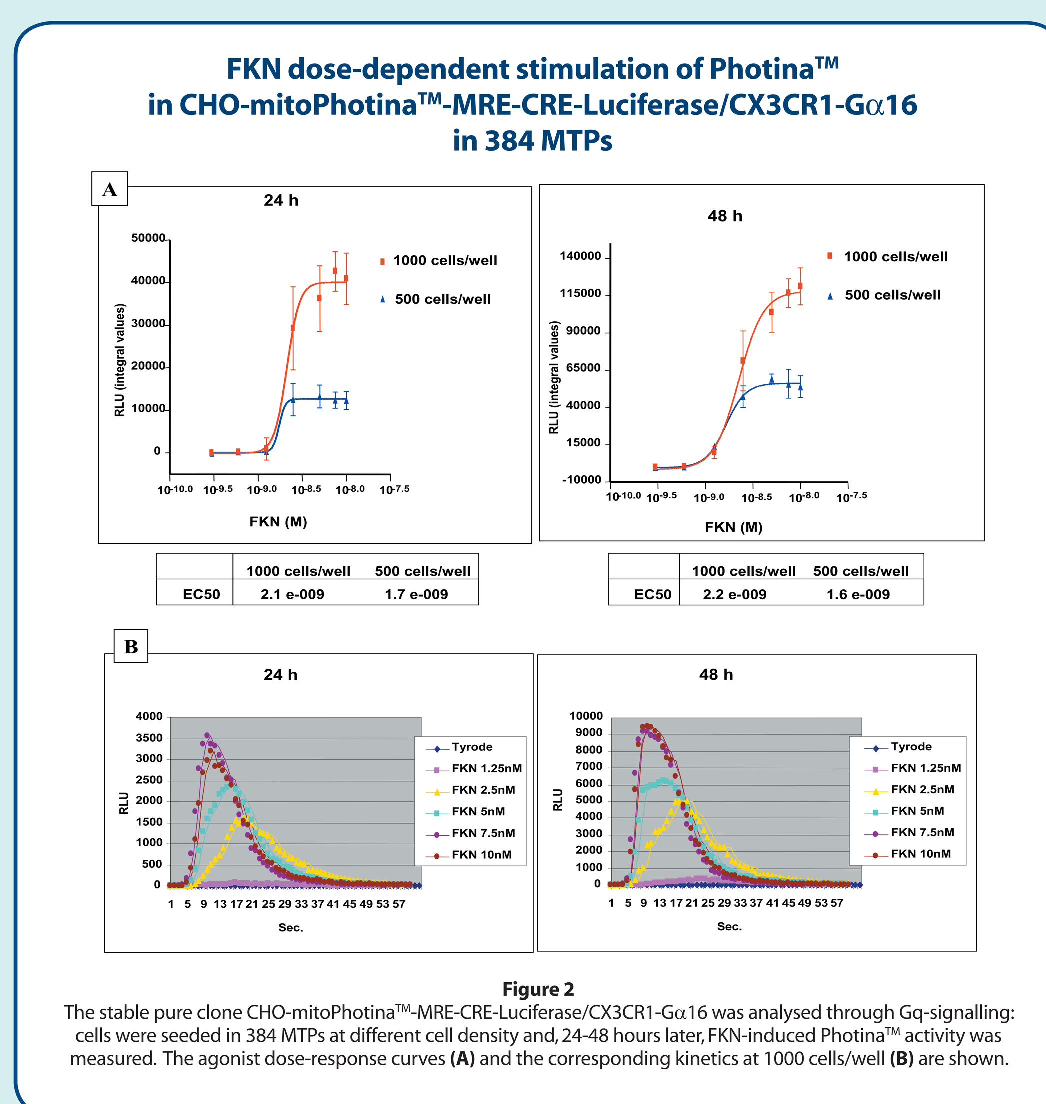
### FKN dose-dependent inhibition of luciferase in CHO-mitoPhotina™-MRE-CRE-Luciferase/CX3CR1




| | 1000 cells/well | 1500 cells/well | 2000 cells/well |
|---|---|---|---|
| EC50 | 2.3 e-009 | 1.8 e-009 | 3.0 e-009 |

**Figure 1**
The stable pure clone CHO-mitoPhotina™-MRE-CRE-Luciferase/CX3CR1 was tested for Gi-signalling: cells were seeded in 384 MTPs at different cell density and, 48 hours later, FKN inhibition of forskolin-induced luciferase activity was measured. The agonist dose-response curves and the calculated $EC_{50}$ are shown.

### FKN dose-dependent stimulation of Photina™ in CHO-mitoPhotina™-MRE-CRE-Luciferase/CX3CR1-Gα16 in 384 MTPs




24 h:

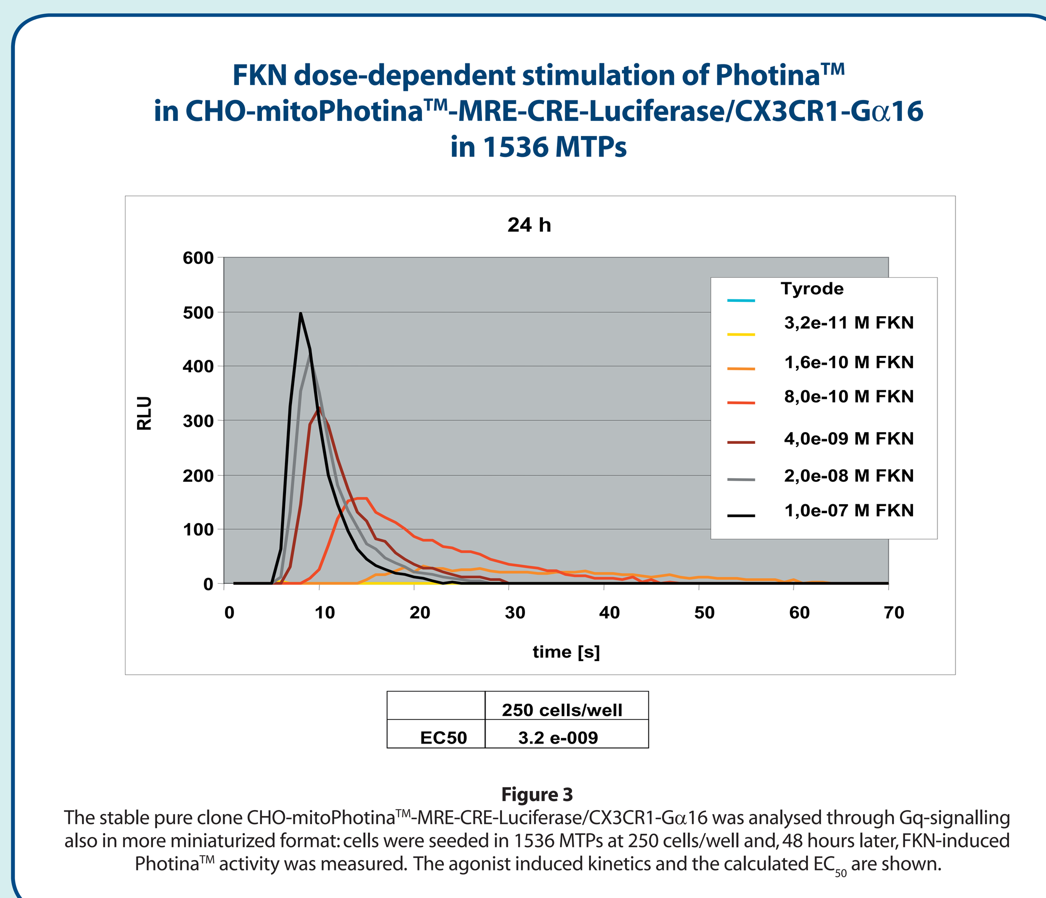
| | 1000 cells/well | 500 cells/well |
|---|---|---|
| EC50 | 2.1 e-009 | 1.7 e-009 |

48 h:

| | 1000 cells/well | 500 cells/well |
|---|---|---|
| EC50 | 2.2 e-009 | 1.6 e-009 |

**Figure 2**
The stable pure clone CHO-mitoPhotina™-MRE-CRE-Luciferase/CX3CR1-Gα16 was analysed through Gq-signalling: cells were seeded in 384 MTPs at different cell density and, 24-48 hours later, FKN-induced Photina™ activity was measured. The agonist dose-response curves **(A)** and the corresponding kinetics at 1000 cells/well **(B)** are shown.

### FKN dose-dependent stimulation of Photina™ in CHO-mitoPhotina™-MRE-CRE-Luciferase/CX3CR1-Gα16 in 1536 MTPs




| | 250 cells/well |
|---|---|
| EC50 | 3.2 e-009 |

**Figure 3**
The stable pure clone CHO-mitoPhotina™-MRE-CRE-Luciferase/CX3CR1-Gα16 was analysed through Gq-signalling also in more miniaturized format: cells were seeded in 1536 MTPs at 250 cells/well and, 48 hours later, FKN-induced Photina™ activity was measured. The agonist induced kinetics and the calculated $EC_{50}$ are shown.

### CHO-mitoPhotina™-MRE-CRE-Luciferase/CX3CR1-Gα16 analyzed at FLIPR

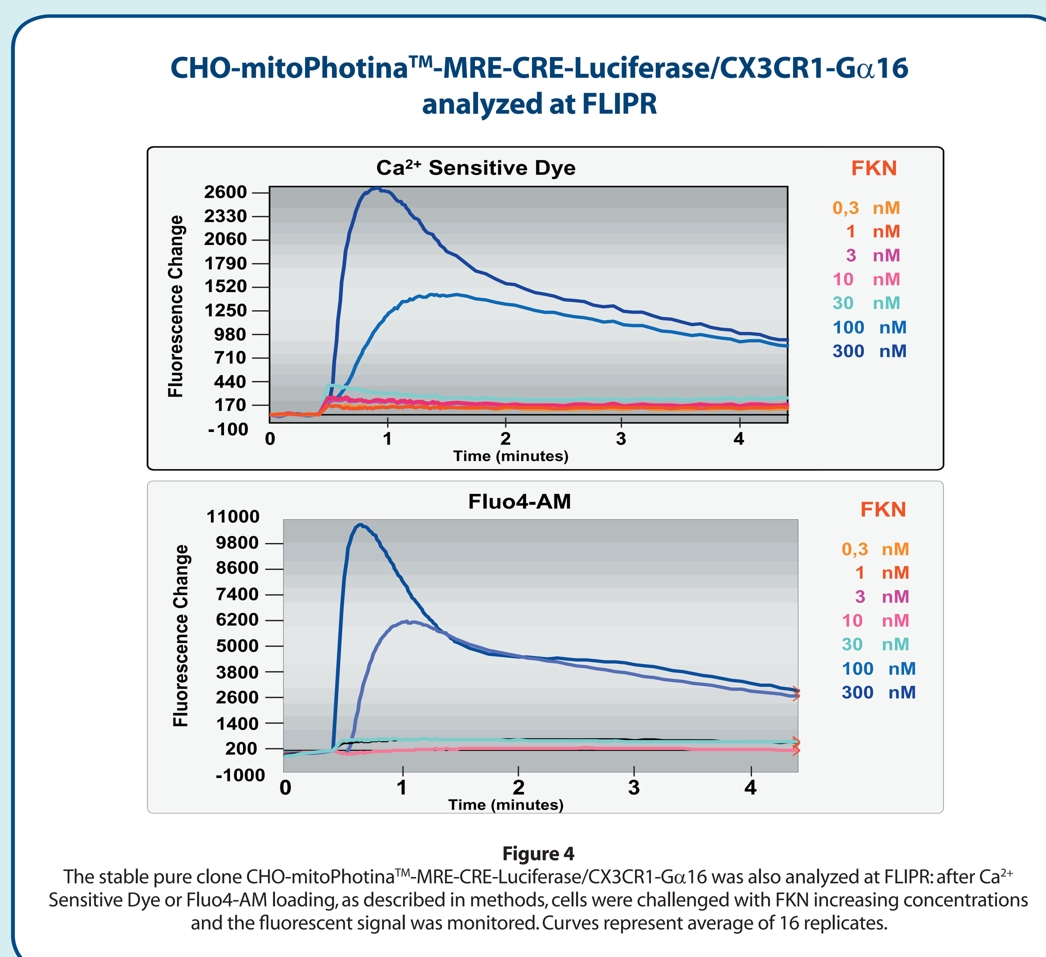



**Figure 4**
The stable pure clone CHO-mitoPhotina™-MRE-CRE-Luciferase/CX3CR1-Gα16 was also analyzed at FLIPR: after $Ca^{2+}$ Sensitive Dye or Fluo4-AM loading, as described in methods, cells were challenged with FKN increasing concentrations and the fluorescent signal was monitored. Curves represent average of 16 replicates.

## Methods

### Cloning

CX3CR1 (Acc. n° NM_001337) was amplified from human THP-1 cell RNA by RT-PCR and confirmed by sequencing. The PCR product was subcloned into pcDNA3 plasmid (Invitrogen) to generate CX3CR1-pcDNA3 and CX3CR1-Gα16-pcDNA3 expression vectors.

### Cell culture

CHO-mitoPhotina™-MRE-CRE-Luciferase cell line was cultured in DMEM/F12 with Glutamax (GIBCO) supplemented with 10% FBS, Hepes, $NaHCO_3$, NaPyruvate and 1% Penicillin/Streptomycin.
Cells were transfected with CX3CR1-pcDNA3 and CX3CR1-Gα16-pcDNA3 constructs by electroporation and stable pure clones were obtained through treatment with G418 (2 mg/ml) and limiting dilutions.

### Luminescence assays

24 or 48 hours before experiments cells were seeded in complete medium at 500-1000-1500-2000 cells/well in 384 MTPs or 48 hours before at 250 cells/well in 1536 MTPs.
Gi-signalling luminescence assay: 3 hours before analysis, medium was replaced with serum free medium containing the indicated concentrations of FKN plus 1 µM forskoline. Then triton-luciferine mix (1:1) was directly injected by the CCD Lumibox (Bayer) and luciferase activity was measured for 60 seconds. The inhibition of forskolin-induced luciferase activity was calculated.
Gq-signalling luminescence assay: 3 hours before analysis, medium was replaced with 2 mM $Ca^{2+}$ tyrode buffer, containing 5 µg/ml coelenterazine. Then the indicated concentrations of FKN were directly injected by the CCD Lumibox (Bayer) and 60 seconds measurement was performed.
The dose-response curves were obtained by fitting data to the Hill equation considering the total integral values as indicated in the figures.

### FLIPR analysis

Cells were seeded in black walled clear-based 384 MTPs at a density of 10000 cells/well in growth medium. 24 hours later cells were loaded with $Ca^{2+}$ Sensitive Dye (Molecular Devices) for 30 minutes or with 4 µM Fluo4-AM (Molecular Probes) for 1 hour at 37° C according to the protocols. Fluorescence was measured before and after agonist addition.

## Concluding remarks

- CX3CR1 has been stably transfected into CHO-mitoPhotina™-MRE-CRE-Luciferase cell line and assays suitable for HTS and uHTS have been developed.
- The Photina™ based detection system has displayed a much stronger ligand induced signal amplitude and sensitivity compared to the fluorescence based system.
- The high signal amplitude produced by Photina™ allows the use of a reduced number of cells/well and a lower concentration of the luminophore coelenterazine, decreasing the final cost/well.
- The overall data indicate that Photina™ is particularly useful for the development of cell-based systems suitable for the screening and identification of compounds able to modulate GPCRs.

## References

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