# Inducible protein-protein interactions in the cell membrane and the nucleus: Dynamics and function of interleukin receptors and nuclear receptors studied with single molecule sensitivity

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**Final report**

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Receptors in the plasma membrane and the nucleus transmit internal or external signals and regulate cellular processes by forming various complexes. The aim of the project was to investigate the parameters influencing the interactions and diffusion properties of membrane and nuclear receptors via using a combination of modern biophysical techniques, modeling and molecular biological tools. Bulk methods fail to resolve fine differences in the interaction patterns of molecular subpopulations involved in cellular signaling; to this end single cells studies and sometimes single molecule resolution are needed. We used modern biophysical techniques: microscopic and flow cytometric fluorescence resonance energy transfer to obtain a subcellular map as well as population level statistics of the studied interactions. Fluorescence correlation and crosscorrelation spectroscopy (FCS; FCCS) and single dye tracking was applied to measure the mobility and comobility (i.e. stability of associations) of proteins. A combination of these methods made it possible to tackle biological problems that could not be addressed with more conventional tools.

## I. Studying nuclear receptor function and interactions by modern biophysical and genomic tools

Nuclear receptors are transcription factors that regulate the expression of their target genes in a ligand dependent manner. According to the molecular switch model the receptors bind to DNA at the promoter/enhancer regions of the regulated genes as hetero- or homodimers. In the absence of ligand the receptors recruit a corepressor complex, which represses expression via its histone deacetylase activity. Upon ligand binding the corepressor dissociates and coactivator binds, inducing chromatin remodeling, histone deacetylation and eventually gene expression. Knowledge about receptor function has been mainly based on bulk biochemical and molecular biological techniques, which are unable to reveal the kinetics and single cell variability of these processes. In the present project we undertook to investigate receptor activation by live cell imaging techniques having single molecule sensitivity to measure kinetics, and complemented these with ChIP-Seq (chromatin immunoprecipitation followed by massive parallel sequencing) to reveal the genomic locations of the receptor binding sites (1, 2).

### I.1. Live-cell fluorescence correlation spectroscopy dissects the role of coregulator exchange and chromatin binding in retinoic acid receptor mobility (1)

The retinoic acid receptor (RAR) is a member of the nuclear receptor superfamily. This ligand-inducible transcription factor binds to DNA at the promoter/enhancer regions of its regulated genes as a heterodimer with the retinoid X receptor (RXR). The nucleus is a dynamic compartment and live-cell imaging techniques make it possible to investigate transcription factor action in real-time. We studied the diffusion of EGFP-RAR by

fluorescence correlation spectroscopy (FCS) to uncover the molecular interactions determining receptor mobility. Regarding the mechanism of receptor diffusion, we tested one- and two-component free and anomalous diffusion models, and found that the two-component free diffusion model was the minimal model accounting for the correlation curves with sufficient accuracy. In the absence of ligand, we identified two distinct species with different mobilities. The fast component had a diffusion coefficient of $D_1$=1.8-6.0 $\mu m^2$/second corresponding to small oligomeric forms, whereas the slow component with $D_2$=0.05-0.10 $\mu m^2$/second corresponds to interactions of RAR with the chromatin or other large structures. The RAR ligand-binding-domain fragment lacking direct DNA-binding capability also had a slow component, probably as a result of indirect DNA-binding through RXR, however, with lower affinity than the intact RAR-RXR complex. RAR-agonist treatment shifted the equilibrium towards the slow population of the wild-type receptor, but without significantly changing the mobility of either the fast or the slow population. By using a series of mutant forms of the receptor with altered DNA- or coregulator-binding capacity we proved that the slow component is probably related to chromatin binding, and that coregulator exchange, specifically the binding of the coactivator complex, is the main determinant contributing to the redistribution of RAR during ligand activation.

I.2. Ligand binding shifts highly mobile RXR to chromatin-bound state in a coactivator-dependent manner as revealed by single cell imaging (2)

Retinoid X Receptor (RXR) is the heterdimerizing partner of a series of nuclear receptors (RAR, vitamine D receptor, PPARg, LXR, etc.). We carried out the mapping of RXR binding sites by using ChIP-seq. With this method chromatin-bound receptors are fixed to DNA, chromatin is then fragmented by sonication and receptor-bound DNA segments are sequenced. We showed that the number of binding sites was ca. 6600 in the absence and 8300 in the presence of ligand, and binding sites including the consensus sequence of nuclear receptor response elements constituted 54% of all sites. Binding site occupancy increased significantly upon ligand binding, in accordance with results gained by single cell biophysical methods (see below). We characterized the nuclear dynamics of RXR during activation in single cells on the sub-second scale using live-cell imaging. We applied fluorescence techniques having different temporal resolution: fluorescence recovery after photobleaching (FRAP) to characterize receptor mobility at the time scale of seconds over the distance of a few microns and FCS to describe local mobility at the submicrometer scale in the the regime of milliseconds. Using these techniques, a highly dynamic behavior could be shown, which, similar to RAR, could be best described by a two-state model of receptor mobility. In the unliganded state most RXRs belonged to the fast population, leaving only ~15% for the slow, chromatin bound fraction. Upon agonist treatment, the ratio of the slow population increased to ~43% as a result of an immediate and reversible redistribution. Coactivator binding appeared to be indispensable for redistribution and had a major contribution to chromatin association. Cotransfection with a peptide inhibiting the binding of full length coactivator prevented the ligand induced transition to the chromatin bound state. Similarly to RAR, the transition also occurred for the mutant RXR lacking the whole DNA-binding domain, suggesting that the liganded, coactivator bound RXR could recruit a dimeric partner (such as PPARg) and this could induce DNA binding via the partner receptor. We applied a novel method, single plane illumination microscopy-FCS (SPIM-FCS) to map the nuclear mobility of GFP-RXR in the whole nucleus. SPIM-FCS showed that the ligand induced transition occurred throughout the nucleus, not just at a few sites (Fig. 1). These measurements were among the first applications of SPIM-FCS to measure molecular dynamics in live cells. Our results support a model in which RXR has a distinct, highly dynamic nuclear behavior and

follows hit-and-run kinetics upon activation. The high mobility of RXR (as compared to RAR) is in accordance with its role as a promiscuous heterodimerization partner of several nuclear receptors, allowing it two bind and unbind and switch partners easily. From these results a paper has been submitted (2) to the journal Molecular and Cellular Biology, which received a positive review; we are working on the revised version of the manuscript.

Taken together, the studied nuclear receptors are more mobile and interactions with the DNA have a more transient nature than anticipated, and they critically depend on the formation of coactivator complexes.

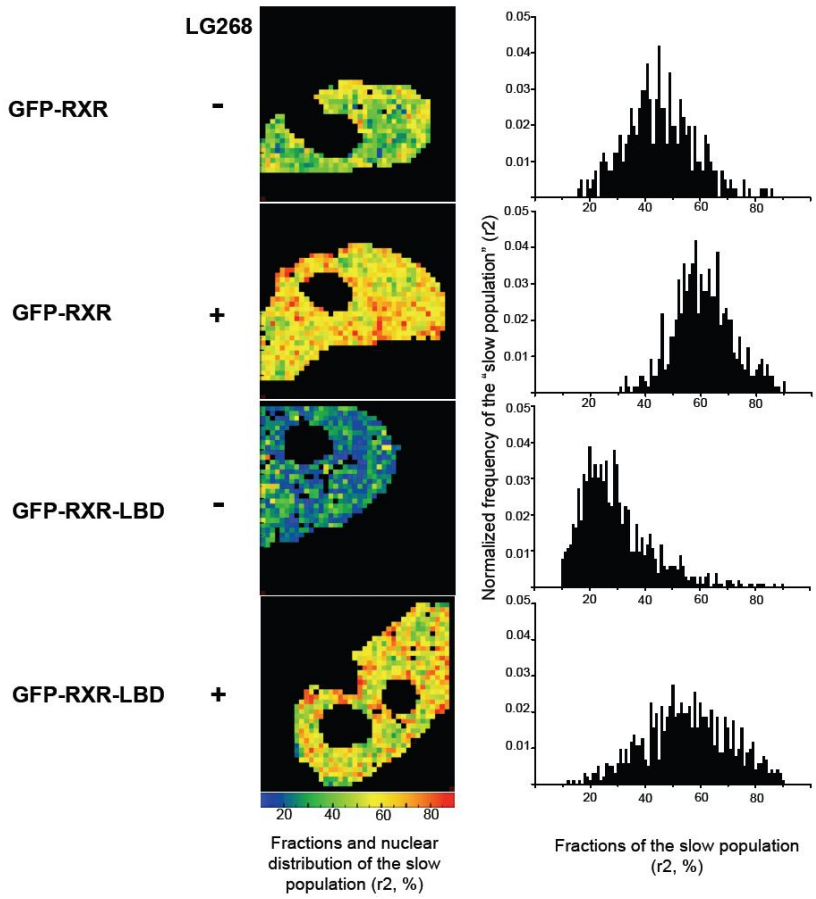


**Figure 1.** Results of mobility mapping by SPIM-FCS. The fraction of the slow population of GFP-RXR and GFP-RXR-LBD (ligand binding domain) increases upon binding agonist ligand (LG268).

I.3. Studying pairwise interactions between dimerizing receptors and cofactors

We also carried out fluorescence resonance energy transfer (FRET) experiments between RAR and RXR (as well as their different mutant forms), and detected significant FRET efficiencies (E~8%). This means that at the near-physiological concentrations used, RAR and RXR formed heterodimers in the absence of ligand, in line with their ability to recruit corepressor complex. Ligand binding induced a slight increase (by 1.5%) of the FRET efficiency indicating the formation of more dimers. We have also shown by FRET that binding of the activator peptide to RAR was enhanced in the presence of ligand. We used the two-color version of SPIM-FCS (SPIM-FCCS, fluorescence cross-correlation spectroscopy) to detect the co-diffusion of RAR and RXR in 2D, and showed that heterodimerization was

stable for at least a few tens of milliseconds. In the continuation of the project we are studying the factors governing receptor dimerization by FRET and SPIM-FCCS, as well as the mode of long range 3D diffusion and 1D motion along the chromatin by analyzing pixel-cross-correlation of SPIM-FCS data. These studies are expected to be completed next year.

**II. Dependence of interleukin receptor interactions and signaling on the cytoskeleton, the membrane potential and the expression of MHC glycoproteins**

Interleukin-2 and -15 receptors play a central role in the activation, survival and death of T lymphocytes. Previously we have shown by FRET and FCCS that they form supramolecular clusters with MHC I and II glycoproteins in lipid rafts of T cells. Receptor function can be modulated by several factors. We were interested, how the changes of the environment (membrane potential, associating partners, cytoskeleton, microdomain structure) influence receptor assembly, homo- and heteroclustering, mobility and function.

II.1. Membrane potential is a regulatory factor of receptor diffusion and signaling (3)

In damaged or inflamed tissues $K^+$ ions are released to the interstitial space, which can depolarize the membrane of T cells possessing $K^+$ channels. The common signaling beta and gamma chains of IL-2/15R are phosphorylated upon cytokine binding and gain a permanent dipole moment, thus their conformation, interactions, mobility and activity may be sensitive to the membrane potential. We induced depolarization on FT 7.10 T lymphoma cells by increasing the ec. $K^+$ level or by blocking Kv 1.3 voltage gated $K^+$ channels with margatoxin. FCS measuremens showed that the lateral mobility of Fab-labeled IL-2/15R and MHC I and II decreased upon depolarization. FRET efficiency measured between some elements of the IL-receptor/MHC cluster increased, which may reflect an increase of cluster size. IL-2-induced receptor activity monitored by STAT5-phosphorylation increased upon depolarization, whereas IL-15 induced phosphorylation did not change. Our results may reveal a novel regulatory mechanism of receptor function by the membrane potential. We have presented posters on these data at international conferences; a manuscript is in preparation.

II.2. Role of lipid microdomains and the cytoskeleton in the assembly, interactions and mobility of IL-2/15R and MHC glycoproteins

We were interested whether the microdomain organization of the membrane and the cytoskeleton were necessary to maintain protein-protein interactions of the IL-R/MHC clusters. Large apoptotic membrane blebs have no cytoskeletal connections, and display a homogenous distribution of proteins and lipids, in contrast to the patchy appearance characterizing lipid rafts of intact membranes. Thus, membrane blebs can be used as a control system when studying the influence of the cytoskeleton or the microdomain structure. We assessed molecular proximities within the clusters by confocal microscopic FRET imaging and measured mobility/comobility with FCS/FCCS. FRET efficiencies between the members of the cluster did not differ when measured in intact membranes or in blebs. Similarly, FCCS proved stable association of MHC I and II in both cases. This means that protein-protein interactions alone were sufficient to maintain the clusters. The mobility of all the studied

proteins was higher in blebs, indicating that the cytoskeleton probably interacts with the receptors in intact cells at least transiently. We confirmed this finding by measuring mobility with single dye tracking (SDT) in collaboration with Gerhard Schütz's group (TU Wien). SDT also revealed that diffusion in the membrane was anomalous, which can be due to molecular crowding, transient binding to immobile/slowly moving structures or confinement to membrane domains. Taken together, although the receptors probably interact with the cytoskeleton, interactions stabilizing the studied small-scale molecular clusters do not depend critically on the interaction with the cytoskeleton or higher order organization of the membrane. We presented these results as posters and conference talks; a manuscript is in preparation.

II.3. MHC I plays an organizing role in the maintenance of IL-2R/IL-15R/MHC clusters (5)

We applied RNAi to silence the expression of MHC I in order to study its possible role in receptor clustering. FRET measured on a cell-by-cell basis using flow cytometry indicated that the association of IL-2R and IL-15R with MHC I as well as between IL-2R and IL-15R weakened. Fluorescence correlation spectroscopy indicated an increase of receptor mobility also suggesting the partial disassembly of the clusters. The decrease of protein cluster size was confirmed by superresolution STED microscopy. Our results suggest that MHC I plays an organizing role in maintaining supramolecular receptor clusters. We presented these data at conferences, and a manuscript is close to completion.

**III. Development of fluorescence techniques to study protein-protein interactions**

Together with our collaborating partners at DKFZ, Heidelberg, we developed fluorescence correlation spectroscopy and fluorescence resonance energy transfer techniques to study protein-protein interactions. These have been described in four original publications (6-9), one protocol (10) and a book chapter (11).

III.1. Multiplexed multiple-tau auto- and cross-correlators on a single field programmable gate array (6)

Our earlier FCS setup allowed only the calculation of either autocorrelation (monitoring mobility) or cross-correlation (co-mobility) functions, thus, individual mobilities and co-diffusion of the interacting partners had to be determined in separate experiments. We introduced a new multiple-tau hardware correlator design that can detect two autocorrelation and two cross-correlation functions in real-time simultanously. This development makes it possible to detect the mobility and co-mobility (pair interaction) of two or more spectrally different fluorescent species. The correlator was implemented in LabVIEW on a National Instruments field programmable gate array (FPGA) card with a minimal sampling time of 400 ns. Use of hardware resources was minimized by scheduling the computation of different segments of the correlation functions on a single correlator block. Raw data are stored with a time resolution of 50 ns allowing detailed off-line analysis of photon statisctics (e.g. by counting histograms to determine oligomerization state from molecular brightness). The design can be adapted to other FPGA cards and extended to more input channels (more colors). We have provided the software for two German research groups upon their request.

III.2. Two-dimensional mobility mapping by SPIM-FCS: FPGA implementation of a 32x32 autocorrelator array for analysis of fast image series (7)

Molecular mobility can be heterogeneous in a cell because of selective interactions at different sites. Therefore, mapping pixelwise mobility in a whole optical section rather than just at a few selected points can add significant information about the subcellular distribution of molecular interactions. We participated in the software development and experimental testing of a single plane illumination microscope-FCS (SPIM-FCS), a tool to create 2D maps of the dynamics of fluorescent molecules inside living cells. The current implementation can calculate 1024 correlation functions at a resolution of 10 μs in real-time and therefore correlate real-time image streams from high speed single photon cameras with thousands of pixels. We applied this microscopic technique to study the diffusion of the RXR receptor (2), co-diffusion of RAR with RXR as well as homodimerization of c-Fos transcription factors (further manuscripts are in preparation).

III.3. High throughput FRET analysis of protein-protein interactions by slide-based imaging laser scanning cytometry (8)

Laser scanning cytometry (LSC) is a slide-based technique combining advantages of flow and image cytometry: automated, high-throughput detection of optical signals with subcellular resolution. Fluorescence resonance energy transfer (FRET) is a spectroscopic method often used for studying molecular interactions and molecular distances. We have developed a procedure for a commercial LSC instrument to measure FRET on a cell-by-cell or pixel-by-pixel basis on large cell populations, which adds a new modality to the use of LSC. As a reference sample for FRET, we used a fusion protein of a single donor and acceptor (ECFP-EYFP connected by a seven-amino acid linker) expressed in HeLa cells. The FRET efficiency of this sample was determined via acceptor photobleaching and used as a reference value for ratiometric FRET measurements. Using this standard allowed the precise determination of an important parameter (the alpha factor, characterizing the relative signal strengths from a single donor and acceptor molecule), which is indispensable for quantitative FRET calculations in real samples expressing donor and acceptor molecules at variable ratios. We worked out a protocol for the identification of adherent, healthy, double-positive cells based on light-loss and fluorescence parameters, and applied ratiometric FRET equations to calculate FRET efficiencies in a semi-automated fashion. To test our protocol, we measured the FRET efficiency between Fos-ECFP and Jun-EYFP transcription factors by LSC, as well as by confocal microscopy and flow cytometry, all yielding nearly identical results. Our procedure allows for accurate FRET measurements and can be applied to the fast screening of protein interactions. A pipeline exemplifying the gating and FRET analysis procedure using the CellProfiler software has been made accessible at our web site.

III.4. TripleFRET measurements in flow cytometry (9)

Protein complexes often consist of more than two components. FRET has traditionally been restricted to two fluorophores, allowing the study of pairwise interactions. Lately, several methods have been introduced to expand FRET methods to three species. We partipated in the development of a method that allows the determination of FRET efficiency in three-dye

systems on a flow cytometer. TripleFRET accurately reproduces energy transfer efficiency values measured in two-dye systems, and it can indicate the presence of trimeric complexes. We also discuss the interpretation of energy transfer values obtained with tripleFRET in relation to spatial distribution of labeled molecules and address the limitations of using total energy transfer to determine molecular distance.

III.5. FCS and FRET protocols

We published protocols with biological applications about the determination of molecular mobility with FCS (10) and FRET efficiencies by flow cytometry on a cell-by-cell basis on large cell populations (11).

## IV. Collaborations on related projects

IV.1. Plasticity of the asialoglycoprotein receptor deciphered by ensemble FRET imaging and single-molecule counting PALM imaging (12)

The stoichiometry and composition of membrane protein receptors are critical to their function. However, the inability to assess receptor subunit stoichiometry in situ has hampered efforts to relate receptor structures to functional states. We addressed this problem for the asialoglycoprotein receptor using ensemble FRET imaging, analytical modeling, and single-molecule counting with photoactivated localization microscopy (PALM). PALM is a superresolution microscopy technique having the ability to detect and localize single molecules with ~40 nm precision. We showed that the two subunits of asialoglycoprotein receptor (RHL1 and RHL2) can assemble into both homo- and hetero-oligomeric complexes, displaying three forms with distinct ligand specificities that coexist on the plasma membrane: higher-order homo-oligomers of RHL1, higher-order hetero-oligomers of RHL1 and RHL2 with two-to-one stoichiometry, and the homo-dimer RHL2 with little tendency to further homo-oligomerize. Levels of these complexes can be modulated in the plasma membrane by exogenous ligands. Thus, even a simple two-subunit receptor can exhibit remarkable plasticity in structure, and consequently function, underscoring the importance of deciphering oligomerization in single cells at the single-molecule level.

The PALM technique will also be used for studying the stoichimetry of interleukin receptor complexes.

IV.2. New cholesterol-specific antibodies remodel HIV-1 target cells' surface and inhibit their in vitro virus production (13)

We participated in a collaborative project with the Department of Immunology of the Loránd Eötvös University aimed at influencing HIV entry into target cells. Our role was to study the mobility of membrane proteins involved in HIV entry by using FCS. Our partners showed that treatment with anti-cholestrol antibodies rearranged membrane proteins and hindered HIV entry into T cells in vitro.

IV.3. Cancer cell targeting and imaging with biopolymer-based nanodevices (14)

We participated in a project aimed at synthesizing and testing nanoparticles capable of targeting MRI contrast agents (Gd-ions) via a folate moiety to tumor cells overexpressing folate receptors. Our role was to test the selective uptake of nanoparticles through the receptor with confocal microscopy and flow cytometry. Nanoparticles can also carry other materials such as DNA plasmids. Because T cells are difficult to transfect with plasmids, in the future we plan to try transfection using a similar nanoparticle based method by targeting nanoparticles to IL-2R with its ligand built into the nanoparticle.

IV.4. In vitro and in vivo activity of 4-thio-uridylate against JY cells, a model for human acute lymphoid leukemia (15)

We participated in a project to test the anti-proliferative effect of 4-thio-uridylate, a nucleotide derivative on JY B lymphoma cells. Our role was to perform fluorescence based apoptosis assays by confocal microscopy and flow cytometry. The treatment proved to be effective and tumor cell selective both in vitro and in vivo. Results suggest that s(4)UMP alone or in combination with other clinically approved anti-leukemic remedies should be further explored as a potential novel therapeutic agent. In the continuation of this collaboration we will test an improved version of this compound to a panel of different cancer cells including the IL-2R overexpressing T cells used in our studies described above.

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