THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Investigation of the interactions of nuclear receptors by quantitative fluorescence microscopic methods

by Dr. Rehó Bálint

Supervisor: György Vámosi PhD

UNIVERSITY OF DEBRECEN

DOCTORAL SCHOOL OF MOLECULAR MEDICINE

Debrecen, 2023

INVESTIGATION OF THE INTERACTIONS OF NUCLEAR RECEPTORS BY QUANTITATIVE FLUORESCENCE MICROSCOPIC METHODS

by Bálint Rehó, medical doctor

Supervisor: Vámosi György, PhD

Doctoral School of Molecular Medicine,
University of Debrecen

Head of the **Defense Committee**:	László Fésüs, PhD, DSc, MHAS
Reviewers:	Demény Máté Ágoston, PhD
	Tibor Pankotai, PhD
Members of the Defense Committee:	László Balkay, PhD
	Tamás Hegedűs, PhD

The PhD Defense takes place at the Lecture Hall of Bldg. „A", Department of Internal Medicine, Faculty of Medicine, University of Debrecen at 13:00, 19th October, 2023.

1. INTRODUCTION

Nuclear receptor superfamily

The nuclear receptor superfamily includes transcription factors that can influence the transcription of their target genes by a ligand-dependent manner. They play a crucial role in various cell functions, including the regulation of cell homeostasis, differentiation, metabolism, and death. Intensive research in this field has resulted in the identification of 48 nuclear receptors in humans. Their natural ligands are small lipid-soluble molecules that can diffuse freely across the cell membrane. These molecules are mainly fat-soluble vitamins, hormones, and steroids. The endogenous ligands of certain nuclear receptors are not known and are called orphan receptors.

Domain structure of nuclear receptors

The molecular weight of the nuclear receptors is between 50 and 100 kDa. Most of them contains 5 domains, and their domain structure shows a high degree of homology. The N-terminal end of the receptors contains the A/B domain. This is the least conserved sequence in the structure of nuclear receptors. The A/B domain is followed by the most conserved DNA-binding domain (DBD), which has two zinc fingers motifs, with 4-4 conserved cysteines each. The helix created by the two Zn-fingers can bind to the DNA major groove if it recognizes a specific response element. The D region forms a flexible bridge between the DBD and the ligand-binding domain (LBD). The ligand binding domain (LBD) is a true multifunctional domain. It plays a crucial role not only in ligand binding, but also in dimerization, ligand-dependent transcriptional activation (activation function 2, AF2), and coregulator binding as well. The F-domain shows a high degree of variability between different nuclear receptors. In some nuclear receptors, the F-domain is absent.

Classification of nuclear receptors according to their structure

The 48 types of nuclear receptors in humans can be divided into 7 major groups based on sequence homology. Among others, the NR1 group includes the retinoic acid receptors (RAR), the vitamin D receptor (VDR),. The natural ligands of retinoic acid are the active metabolites of vitamin A, the lipid-soluble trans-retinoic acid and 9-cis-retinoic acid. It is involved in many cellular processes, including cell growth and differentiation, but also has an immunomodulatory role. Abnormal retinoid signaling has been demonstrated in a number of malignancies, including leukemias, melanoma, lung, breast, ovarian, prostate, pancreatic and liver tumors, making RAR a potential chemotherapeutic target. RAR functions by dimerizing with the rexinoid receptor (RXR). The vitamin D receptor also forms a heterodimer with RXR, its endogenous ligand is the active metabolite of vitamin D, calcitriol. In contrast to RAR, VDR is found not only in the nucleus but also in the cytosol, but some research groups have also described its presence in the cell membrane. VDR is involved in the body's calcium transport, but several studies suggest that it also plays an important role in the immune response and tumorigenesis.

Molecular switch model

Nuclear receptor function is traditionally described by the molecular switch model. According to this model, in the absence of ligand, receptors bind to chromatin and associate with a corepressor complex, which inhibits the transcription of their target genes through histone deacetylase activity. In the presence of a ligand, the agonist binds to the ligand-binding pocket, triggering a conformational change: the H12 helix is locked onto the ligand-binding pocket. This conformational change decreases the affinity of the nuclear receptor for corepressors, but at the same time increases its affinity for

coactivators, so that coregulator exchange occurs. Co-activators bind additional proteins, and through their histone acetyltransferase activity, loosen the structure of chromatin, allowing the activation of the transcriptional machinery.

The dynamic model of nuclear receptor action

As a result of intensive research in the field, the above molecular switch model is beginning to be replaced by a more dynamic one. Transcription factors have been shown to scan the chromosome in three dimensions until they reach a response element, where they can spend longer periods of time under the right conditions and facilitate the initiation of transcription. In the case of the estrogen receptor, it has been shown that nuclear receptor complexes of different mobility are present within the nucleus. The fastest are nuclear receptor dimers that are not bound to chromatin, while the slowest are those that bind transiently to chromatin and associate with a coregulator complex. FRAP, ChIP and FCS studies with nuclear receptors have led to a new dynamic model, which has been termed the "hit-and-run" model. According to this model, receptors bind corepressors in the absence of ligand and then bind transiently to specific and non-specific response elements by scanning chromatin. In response to ligand treatment, coregulator exchange occurs, with the coreceptors binding to a high mass coactivator complex via the AF-2 domain. Upon reaching a specific response element, the nuclear receptors bind more stably to chromatin and activate the transcriptional machinery.

Confocal microscopy

The main disadvantage of epifluorescence microscopy is that the excitation light source excites fluorophores over the entire thickness of the sample, so that photons outside the focal plane are detected. If the thickness of the sample is negligible, this does not cause significant image degradation, but when

examining thicker samples, such as cells, it is difficult to distinguish the fluorescence signal from outside the focal plane. For these reasons, several new methods have been developed to exclude out-of-focus photons from detection. One such method is confocal laser scanning microscopy (CLSM), whereby the sample is illuminated with focused laser light and the emitted photons are then passed through a narrow slit, a pinhole. The pinhole ensures that only photons from the focal plane enter the detector. Since the excitation is done with a point laser beam, the microscopic image is obtained by scanning the sample, directing the laser light from point to point using mirrors, and then detecting the fluorescence intensity excited at each point.

Selective plane illumination microscopy (SPIM)

In contrast to traditional confocal microscopy, selective plane illumination microscopy illuminates a thin plane of the sample. During excitation, a cylindrical lens is used to form a plane of laser light perpendicular to the direction of detection. The excitation is made almost exclusively in the focal plane, so background excitation is minimal. This method allows optical slicing, significantly reducing photobleaching and phototoxicity to cells. Since excitation and detection are performed simultaneously in an entire plane, imaging is 100-1000x faster than CLSM.

Fluorescence correlation spectroscopy (FCS)

In fluorescence correlation spectroscopy, we use a confocal microscope to measure the variation in fluorescence intensity caused by fluorescence from small, femtolitre excited volumes entering and leaving the sample. In FCS studies, the excitation photons are provided by a focused laser beam projected onto the sample by an objective with a large numerical aperture (NA>0.9). The fluorescence signal emitted from the sample passes through the objective, a dichroic mirror, and an emission filter, and then enters the detector

through a pinhole. The sample is sampled over a longer period with sub-microsecond resolution, giving the time-dependent fluorescence intensity F(t) of the sample, which is proportional to the number of fluorophores in the detection volume at a given time. The length of the fluctuations depends on the velocity of the molecules, i.e., their diffusion constant. The autocorrelation function can be fitted with various model functions and the physical properties of the diffusion can be extracted from the fitting parameters. In the model selection process, a model is sought which describes the system as simply as possible but with sufficient accuracy.

Fluorescence cross-correlation spectroscopy (FCCS)

Fluorescence cross-correlation spectroscopy can be used to study the association of two co-diffusing molecules labelled with different fluorophores. In FCCS, the focal volume is excited by two overlapping laser beams and the incoming fluorescence signals are detected by two separate detectors. If the two fluorophore-labelled molecules associate with each other, the amplitude of the cross-correlation function will be high, and the extent of association can be inferred from the amplitude of the function.

Förster resonance energy transfer (FRET)

FRET is a sensitive method for determining the proximity of two molecules. FRET is achieved by a dipole-dipole interaction in the form of a non-radiative energy transfer between an excited state donor and an acceptor with appropriate spectroscopic parameters. The energy transfer requires a donor-acceptor distance of between about 1 and 10 nm, an overlap between the emission spectra of the donor and the absorption spectra of the acceptor, and the correct relative orientation of the molecules.

2. AIMS

In my thesis I focused on the dynamics of the RAR, VDR and RXR nuclear receptors in a live-cell environment using fluorescence microscopic methods. Our experiments were designed to answer the following questions:

- Is the nuclear receptor population homogeneous in the cell nucleus in terms of mobility?
- How does the mobility of nuclear receptors change in response to heterodimerizing partners or agonist ligands?
- Is the change in mobility a consequence of heterodimerization or of increased DNA binding?
- Does competition for RXR binding between VDR and RAR occur at the level of chromatin binding?

To answer the above questions, we performed fluorescence correlation studies using confocal microscopy, but we have also further developed a selective plane-illuminated microscopy (SPIM) based method to simultaneously study the association of molecules with each other and the mobility of interacting particles (SPIM-ALEX-FRET-FCCS).

The results of the present thesis may help in the future to identify therapeutic targets that affect the function of nuclear receptors and raise awareness of the importance of competition for therapies targeting nuclear receptors. Our improved microscopy method may also help to map molecular interactions and mobility for other proteins.

3. MATERIALS AND METHODS

Cell culture

Microscopic studies were performed using human embryonal renal carcinoma (HEK293), human cervical cancer (HeLa) and human colorectal adenocarcinoma (Caco-2) cells. Cells were grown in DMEM, containing phenol red dye supplemented with 10% FBS, 50 mg/l gentamycin and GlutaMax in a 37°C incubator in an atmosphere containing 5% CO2. Cells were passaged on Monday, Wednesday, and Friday. 48 hours prior to confocal microscopy measurements, cells were plated in 8-well ibidi chambers. The cells were then grown in 300 µl phenol red-free medium. Twenty-four hours before measurements, 65 ng of nuclear receptor plasmid and 0.3 ng of FuGene transfection reagent were added to the cells, along with 5 µl of serum-free DMEM solution according to the manufacturer's instructions.
For SPIM measurements, cells were plated on individually prepared coverslips previously treated with 0.01% poly-L-lysine solution. For this purpose, 120,000 cells were transferred to 3 35 mm petri dishes containing pre-poly-lysine-coated coverslips 48 hours before microscopic examinations. The cells were then transfected with a solution containing 400 ng of plasmid, 3 µl of FuGene and 50 µl of serum-free DMEM.
HEK293 cells expressing RXRα tagged with TagBFP, previously established by our group, were used for our competition assays and transfected with additional nuclear receptors as described above. In all cases, microscopic measurements were performed 24 hours after transfection.

Confocal microscopy

Our confocal microscopy measurements were performed on a Zeiss LSM880 - AiryScan confocal laser scanning microscope with a 40x magnification and a water immersion objective with a numerical aperture of 1.2. The images were

acquired from a field of view of 70.85 µm×70.85 µm at a resolution of 512×512 pixels with 16-bit depth of field. The relative expression levels of fluorophore-tagged nuclear receptors were determined using EGFP-mCherry and TagBFP-EGFP fusion proteins. To determine the relative expression levels of endogenous and transfected nuclear receptors in HEK293 cells, confocal microscopy combined with Western blotting was used.

Point FCS measurements

FCS measurements were performed by taking confocal images of the cell to be examined as described previously, and then performing FCS measurements at 2 freely chosen points in the nucleus with EGFP-labelled nuclear receptors. EGFP excitation was performed with a 488 nm Ar-ion laser. The laser intensity detectable at the objective was set to 1.7 µW at the beginning of each measurement day. The fluorescence intensity was then measured at the selected points for 10×8 s, from which the autocorrelation functions were calculated by the microscope's Zen software. All measurements were performed at room temperature (22.5°C). QuickFit3 software was used to evaluate the autocorrelation functions.

Selective plane illumination microscopy

For our SPIM microscopy measurements, we used a custom-built microscope. The cells were placed in a specially designed sample holder on slides described in the thesis. Plates were rotated about 45 degrees to the plane of the objective. The sample holder was filled with DMEM medium. Samples were excited with 488 nm and 561 nm solid-state lasers, and a plane excitation beam was created with cylindrical lenses and a single objective. The optical slices were 1.28 µm and 1.35 µm thick for 488 nm and 561 nm illumination, respectively. The measured laser intensities at the objective were 910 and 4750 µW. Fluorescent photons emitted by the fluorophores were collected perpendicular

to the excitation using a water-immersion objective at 60x magnification. The fluorescence photons were spectrally split (500-550 nm, >593 nm) using a DualView beam splitter, and the images of green and red photons were captured side by side with the same EMCCD camera on two fields of the photosensitive chip with a sampling rate of 530 μs at 51 μm × 8 μm (128 × 20 pixels). FRET and FCCS measurements were performed in the same plane, always evaluated pixel by pixel. For FRET measurements, we used preview images taken immediately before the FCCS measurement. Subsequently, FCCS images were acquired under continuous excitation with two lasers, during which green and red autocorrelation and cross-correlation curves were calculated for each pixel.

Alternating excitation (ALEX)

The disadvantage of the method described above is that the FRET efficiency calculated from the preview images and the FCCS amplitude are calculated at different times. Due to differences in intensity resulting from cell movement or photobleaching, the two measurements cannot be compared directly. In addition, the continuous excitation by two-lasers used for FCCS measurements must consider inter-channel transillumination and enhanced photo-bleaching. To avoid these artifacts, we have introduced alternating excitation (ALEX) in our SPIM microscopy measurements. During ALEX, we temporally separate the excitation with the two lasers, obtaining donor and transfer images (500-550 nm and >593 nm detection, respectively) during 488 nm excitation, and acceptor images (>593 nm detection) during 561 nm excitation, which we used for our FRET studies. Correlating the acquired donor and acceptor images allowed us to perform FCCS studies. With the above method, our FRET and FCCS studies are performed simultaneously using the same fluorescent photons, allowing direct comparison.

Ligand treatment

For ligand treatment, we used synthetic and natural ligands of nuclear receptors, AM580 for RAR, LG268 for RXR and calcitriol for VDR activation. For ligand treatment, ligands were added to cells at a concentration of 100 nM and measurements were performed after 20 min incubation. Cells were assayed for up to 1 h after ligand treatment, after which a new sample was subjected to a further ligand treatment.

4. RESULTS

Determination of the distribution and diffusion parameters of fluorescently labelled nuclear receptors in HEK293

Our microscopic studies have shown that in the absence of RAR ligand, it is mainly located in the nucleus. Nuclear receptors located in the nucleus can diffuse freely, transiently or stably bind to chromatin. The dynamics of these movements is expressed by the autocorrelation function (ACF) of fluorescence with respect to time. The autocorrelation function was fitted with the one- or two-component normal and anomalous diffusion models presented in the Methods section. In the case of EGFP-RAR, the one-component model function did not fit the measured ACF adequately. With two-component models, the function fitted well, but the 2-component anomalous diffusion model did not give a significantly better fit compared to the 2-component normal diffusion model. Based on our results, we further used the 2-component normal diffusion model for full-length coreceptors.

Agonist treatment or co-expressed heterodimerizing partner enhances chromatin binding of RAR

To investigate the diffusion parameters of RAR, EGFP-RAR and mCherry-RXR nuclear receptors were transfected into HEK293 cells and the cells were treated with AM580, a synthetic ligand for RAR. In the absence of an agonist ligand and heterodimerizing partner, the slow population (ρ_2) ofEGFP-RAR was approximately 22%, which was consistent with previous measurements by our group. When the cells were treated with AM580 ligand, the slow population fraction increased, while the diffusion constant of the slow population (D_2) decreased, which may be a consequence of stronger binding to chromatin, or a prolongation of the chromatin-bound state. When EGFP-RAR was transfected with mCherry-RXR, the slow component of RAR increased

even more, while the diffusion constant of the slow population decreased significantly, suggesting increased heterodimerization and enhanced chromatin binding. AM580 ligand treatment additively increased the ratio of the slow population. To understand the mechanism of RXR-induced enhanced chromatin binding, mCherry-tagged RXR ligand-binding domains (RXR-LBD) were transfected to EGFP-RAR. RXR-LBD can heterodimerize with RAR but is unable to bind to chromatin by itself in the absence of a DNA-binding domain. In contrast to full-length RXR, co-transfection of RXR-LBD did not increase the ratio of the slow population; addition of AM580 also induced only minimal changes. We concluded that dimerization of RAR with RXR only enhances chromatin binding of the complex if RXR also has DNA-binding ability.

VDR mobility is reduced only in the presence of a heterodimerizing partner and agonist ligand

In the absence of an agonist ligand - in contrast to EGFP-RAR - EGFP-VDR shows a homogeneous distribution within the cell. mCherry-RXR co-transfection and calcitriol treatment induce EGFP-VDR to migrate into the nucleus, as previously shown by our group and other research groups. In contrast to EGFP-RAR, the ratio of the slow population of EGFP-VDR was very low, so the affinity of VDR for chromatin was much lower than for RAR. Ligand treatment and mCherry-RXR co-transfection only slightly increased the fraction of slow population, while the diffusion constant of the slow population remained unchanged. Agonist ligand combined with mCherry-RXR co-transfection almost doubled the ratio of slow population, while the diffusion constant decreased, indicating an increased duration of chromatin binding

Ligand-mediated competition of RAR and VDR

In our group, we have previously shown by nuclear translocation studies that different nuclear receptors compete for RXR binding. Therefore, we were curious to see whether the competition described above also occurs at the level of DNA binding between RAR and VDR.

Our measurements showed that in the presence of all three receptors, the ratio of the slow population of RAR was slightly lower ($\rho_2 \sim 0.32$) than in the presence of RAR and RXR alone ($\rho_2 \sim 0.38$), suggesting that a portion of the RXR set binds to VDR, thereby reducing the proportion of RAR/RXR heterodimers.

In the case of VDR, no increased chromatin binding was observed when RXR was added, and the same result was obtained in the presence of all three receptors together.

No significant change in the fraction of slow population of RAR was observed upon addition of AM580 ligand, whereas calcitriol slightly decreased the slow population. No significant change was observed when AM580 and calcitriol were added together compared to the untreated sample.

The slow population of VDR was significantly increased with calcitriol and moderately decreased with AM580. When AM580 and calcitriol were used together, the fraction of the slow population fell between the values of the two treatments alone.

Based on our results, it can be concluded that when RAR and VDR are present simultaneously, RXR favours RAR over VDR and this is reflected in chromatin binding.

In the presence of all three nuclear receptors, LG268 increased the slow population of RAR in all combinations and decreased the slow population of VDR in all combinations.

For RAR, the chromatin-bound slow population was increased by LG268, in contrast to VDR, where a decrease was observed; this indicates an increased affinity of RXR for RAR. Co-administration of AM580 and LG268 further increased the proportion of the slow component of RAR compared to AM580 treatment, whereas for VDR, we observed a decrease with calcitriol and LG268 double treatment compared to calcitriol treatment.

The increased affinity of RXR for RAR was also evident when the ligand of RXR was co-administered with the ligand of the competent partner: the ratio of the slow component of RAR was higher in the presence of LG268 and calcitriol together than when calcitriol was present only; in contrast, the ratio of the slow component of VDR was not significantly changed with LG268 and AM580 treatment compared to AM580 treatment alone.

Taken together, our data suggest that ligand-driven competition between RAR and VDR occurs not only at the level of protein-protein interactions but also at the level of chromatin binding. Both in the absence of RAR ligand and in the presence of VDR and RAR ligand together, RAR bound to RXR with greater affinity than VDR, and the RAR-RXR complex bound more strongly to chromatin than the VDR-RXR complex.

Introduction of alternating excitation in Selective Plane Illumination Microscopy (SPIM-ALEX-FRET-FCCS)

In previous FCCS measurements, the simultaneous and continuous excitation with two lasers required additional corrections: among others, we had to consider the crosstalk of the donor into the red channel, the FRET and the excitation of the red dye with a 488 nm laser.

To overcome these problems, we adapted the SPIM microscope to use alternating excitation (ALEX). With this method, excitation at 488 nm results in donor and transfer signals, while excitation with a 561 nm laser produces an

acceptor image free of crosstalk. Donor and acceptor images are obtained with a difference of 0.53 ms and can be used for further FCCS analysis.

To validate the method, measurements were performed with continuous and alternating excitation on different control samples.

The standard deviation of the E values calculated using ALEX is higher than the E values calculated from the preview images. This is a consequence of the lower signal-to-noise ratio but can be corrected by summing several images from ALEX measurements.

With continuous excitation, even for the negative control, a relatively high relative cross-correlation amplitude (rCCF) is obtained, due to the crosstalk. If the crosstalk is corrected in software, the rCCF will be lower.

By using ALEX, higher values were obtained for positive controls compared to the corrected rCCF value, while lower values were obtained for negative controls, thus increasing the dynamic range of the measurement by about 45%, allowing better quantification of the degree of dimerization and increasing the sensitivity of the method.

As the excitation time is shorter, the use of ALEX also reduces photobleaching.

In alternating excitation, FRET and FCCS measurements are performed simultaneously, allowing the E and rCCF parameters to be plotted in a common coordinate system, thus allowing the molecular distance and co-diffusion rate to be quantified simultaneously.

Studying of nuclear receptor dimerization and chromatin binding simultaneously using SPIM-ALEX-FRET

The utility of the new method was first tested on EGFP and mCherry labelled nuclear receptors in HeLa cells.

To determine the FRET efficiency between the nuclear receptors, we used preview images taken before FCCS measurement due to the low E value

between the nuclear receptors. For full-length nuclear receptors in the absence of ligand, the FRET efficiency was found to be 1.6%, which was significantly increased by AM580 treatment. No significant increase was observed with LG268 and double ligand treatment.

In contrast to the value of E, the receptor comobility (rCCF) was already high in the absence of ligand. When AM580 was added, a marked increase in rCCF was observed, whereas LG268 showed a smaller increase. No significant change was detected upon double ligand treatment. LG268 may also dimerize RXR with endogenous receptors, which may explain the weaker effect than AM580.

To determine the diffusion parameters of nuclear receptors, autocorrelation function were fitted with a two-component normal diffusion model on each pixel of the cell. The diffusion constant of the fast population (D_1) was found to be 4-9 $\mu m^2/s$, while the slow population (D_2) was found to be 0.2 $\mu m^2/s$. These results are in agreement with our previous confocal FCS studies. The cross-correlation functions were fitted with a one-component normal diffusion model, and the diffusion constant of the cells was found to be around 0.3 $\mu m^2/s$, indicating chromatin binding of the dimer.

The determination of the fraction of the slow component (ρ_2) showed that the chromatin binding of RAR is less dependent on the addition of ligand: ρ_2 were already very high in the absence of ligand, as shown by our previous confocal FCS studies. However, for RXR, the fraction of slow component increased from 23% to 29% upon addition of AM580 and to 35% upon addition of LG268.

It can be said that AM580 and LG268 increase RAR-RXR dimerization, but to a lesser extent in the case of LG268, which is most likely because RXR dimerizes not only with EGFP-labeled RAR but also with other unlabeled endogenous nuclear receptors and thus binds to chromatin.

These results demonstrate the advantages of the SPIM-FRET-FCCS method: using FRET of FCCS alone: we can underestimate the extent of dimerization and would not detect differences in chromatin binding between RAR and RXR.

Analysis of ligand-binding domains of nuclear receptors by SPIM-FRET-FCCS

In order to study dimerization without the effect of chromatin binding, we also performed experiments with the ligand-binding domains of nuclear receptors. For this purpose, we co-transfected EGFP-tagged RAR-LBD and mCherry-tagged RXR-LBD. In the absence of ligand, the FRET efficiency measured between the two coreceptor LBDs was found to be higher than that for full-length coreceptors. This could be an indication of higher dimerization but could also be caused by a more preferred relative orientation of the two fluorophores or a smaller distance between them. The cross-correlation amplitude between the two nuclear receptor LBDs was lower compared to the value measured for full-length nuclear receptors, suggesting that chromatin binding or DNA-binding domain interactions may also contribute to dimerization.

Treatment with AM580 increased FRET efficiency by nearly two-fold, but did not significantly increase rCCF, suggesting that the increase in FRET efficiency was partly due to a decrease in the distance between the two fluorophores or their more favorable orientation rather than increased dimerization. LG268 slightly increased E, while rCCF remained unchanged. Double ligand treatment increased FRET efficiency almost two-fold, while rCCF remained unchanged.

In all cases, the fraction of the slow population was lower for LBDs than for full-length nuclear receptors. Since LBDs are unable to bind to chromatin, the slow population may consist exclusively of LBD dimers that bind a high

molecular weight coactivator complex or bind indirectly to chromatin through unlabeled endogenous partners.

5. SUMMARY

According to the intensive research on the field, the previously outlined molecular switch model being replaced by a more dynamic one.

Our FCS studies showed that there are two distinct populations of nuclear receptor with different diffusion properties are present in the nucleus: a fast population, in which nuclear receptors are bound to the chromatin with lower residence times, these receptors are scanning the DNA for specific response elements, but the stability of chromatin binding is low. Contrary, the receptors in the slow population are bound to the DNA (especially to response elements) with much longer residence times.

In the case of RAR, agonist treatment or RXR cotransfection increased the amount of the slow population which is due to the increased stability of chromatin-binding or increased residence time. In contrast to RAR, the slow population of the VDR only increased in the presence of both agonist and RXR, so the chromatin binding of the VDR stable only in liganded, RXR-bound form. By triple co-transfection of RAR, VDR and RXR, we showed that the competition between RAR and VDR for the binding of RXR is appeared on the level of chromatin-binding, which is at least partly responsible for the side effects of nuclear receptor targeted therapies. Without ligands, the RXR showed higher preference for RAR than VDR. In the presence of ligands, always the liganded receptor dominated. We also showed that RAR and VDR cannot heterodimerize with each other in living cells.

During the second part of my work, we further developed a microscopic method, which is capable of measuring the diffusion properties of molecules and their association at the same time in a plane of the cell. By using SPIM-FRET-FCCS we measured the dimerization a co-mobility of nuclear receptors

at the same time. The combination of the two methods, showed interactions between nuclear receptors, that couldn't be seen by just using FRET of FCCS separately. The application of ALEX made our method more precise by decreased photobleaching and increased dynamic range.

The results of this dissertation can help in the search for nuclear receptor-based therapies and raise attention to the importance of competition between nuclear receptors. The new microscopic method can be used in the investigation of other molecular systems as well.

6. PUBLICATIONS

UNIVERSITY of DEBRECEN

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

Registry number: DEENK/120/2023.PL
Subject: PhD Publication List

Candidate: Bálint Rehó
Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. **Rehó, B.**, Fadel, L., Brázda, P., Benziane, A., Hegedűs, É., Sen, P., Gadella, T. W., Tóth, K., Nagy, L., Vámosi, G.: Agonist-controlled competition of RAR and VDR nuclear receptors for heterodimerization with RXR is manifested in their DNA-binding.
   J. Biol. Chem. 299 (2), 1-16, 2023.
   DOI: http://dx.doi.org/10.1016/j.jbc.2023.102896
   IF: 5.486 (2021)
2. **Rehó, B.**, Lau, L., Mocsár, G., Müller, G., Fadel, L., Brázda, P., Nagy, L., Tóth, K., Vámosi, G.: Simultaneous Mapping of Molecular Proximity and Comobility Reveals Agonist-Enhanced Dimerization and DNA Binding of Nuclear Receptors.
   Anal. Chem. 92 (2), 2207-2215, 2020.
   DOI: http://dx.doi.org/10.1021/acs.analchem.9b04902
   IF: 6.986

List of other publications

3. Fadel, L., **Rehó, B.**, Volkó, J., Bojcsuk, D., Kolostyák, Z., Nagy, G., Müller, G., Simándi, Z., Hegedűs, É., Szabó, G., Tóth, K., Nagy, L., Vámosi, G.: Agonist binding directs dynamic competition among nuclear receptors for heterodimerization with retinoid X receptor.
   J. Biol. Chem. 295 (29), 10045-10061, 2020.
   DOI: http://dx.doi.org/10.1074/jbc.RA119.011614
   IF: 5.157

UNIVERSITY AND NATIONAL LIBRARY
UNIVERSITY OF DEBRECEN
H-4002 Egyetem tér 1, Debrecen
Phone: +3652/410-443, email: publikaciok@lib.unideb.hu

4. Simándi, Z., Pájer, K., Károlyi, K., Sieler, T., Jiang, L. L., Kolostyák, Z., Sári, Z., Fekecs, Z., Pap, A., Patsalos, A., Contreras, G. A., **Rehó, B.**, Papp, Z., Guo, X., Horváth, A., Kiss, G., Keresztessy, Z., Vámosi, G., Hickman, J., Xu, H., Dormann, D., Hortobágyi, T., Antal, M., Nógrádi, A., Nagy, L.: Arginine Methyltransferase PRMT8 Provides Cellular Stress Tolerance in Aging Motoneurons.
J. Neurosci. 38 (35), 7683-7700, 2018.
DOI: http://dx.doi.org/10.1523/JNEUROSCI.3389-17.2018
IF: 6.074

Total IF of journals (all publications): 23,703
Total IF of journals (publications related to the dissertation): 12,472

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

21 April, 2023

DEBRECENI EGYETEM * Egyetemi és Nemzeti Könyvtár *