Quantitative live cell imaging of glucocorticoid receptor dynamics in the nucleus

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1 INTRODUCTION

In this thesis, the focus lies on studying glucocorticoid receptor dynamics in living cells with the aim of understanding how this transcription factor finds its DNA target sites to regulate transcription. In addition, we have advanced the experimental techniques used to answer this question through development of novel analysis tools as well as investigations of alternative labeling methods.

1.1 THE PARADIGM

Proteins are essential for us to function properly. To make sure the right proteins are produced at the right time, the transcription of genes needs to be tightly regulated. Transcription factors do this job, by recognizing a specific DNA sequence and binding to it. This sequence can be located at the start of the gene, which is termed the promotor region, or at a more distant enhancer site. After the transcription factor has bound to the DNA, they will recruit other factors. These include, most importantly, RNA polymerase that will bind at the promotor and will initiate the transcription of a gene. This process includes partial unwinding of the double stranded DNA to form a complementary RNA strand. Not the whole sequence is translated into proteins. The introns are spliced out of the RNA transcript, such that only the coding regions, or exons, remain. This final mRNA product is then translated into protein. However, to regulate this whole process, the transcription factors must first find their specific target sites. With over a total of 6.5 billion basepairs of DNA within every human cell, and only a sequence of 5-10 nucleotides to recognize, this can be a daunting task.

1.2 NUCLEAR ORGANIZATION

In eukaryotic cells, the DNA is stored in the nucleus of the cell. The nucleus is separated from the rest of the cell by a double membrane, the nuclear envelope. To allow traffic of larger molecules, the nuclear membrane is dotted with nuclear pores through which molecules with a diameter up to 10 nm can diffuse passively (1). Proteins can also be transported actively when they carry a nuclear localization sequence (NLS) that is recognized by the nuclear pore complex. In this manner, proteins with a diameter of up to 40

nm can be translocated to the nucleus (2–4). Although the bare DNA only occupies around 0.65% of the nuclear volume (with one base pair has a volume of 1 nm^3 and a nuclear volume of 10^3 μm^3 (5,6)), there is still around 3 m of DNA that must be stored in an organized way in this compartment. Moreover, this must be done in a manner that still allows access of proteins, such as transcription factors, to the DNA. To package DNA, it is wrapped around histone proteins to form nucleosomes. Strings of nucleosomes form chromatin fibers of which the higher-order structure is a subject of active research. In turn, these fibers are folded in higher-order structures that in humans shape the 23 pairs of chromosomes. In spite of all of this compaction and organization, transcription factors can successfully bind to the DNA and initiate transcription.

1.3 MEET THE FAMILY: THE NUCLEAR RECEPTOR SUPERFAMILY

In this thesis, the glucocorticoid receptor (GR) is used as a model system to study transcription factor dynamics and distribution. This receptor is part of the nuclear receptor (NR) superfamily. Nuclear receptors are involved in the regulation of many essential processes such as reproduction, development, metabolism and the immune response. In contrast to most other receptor proteins that reside on the plasma membrane and bind molecules (ligands) that are outside the cell, nuclear receptors reside in the cytoplasm or nucleoplasm. Hence, their ligands must diffuse across the plasma membrane to activate their receptor inside the cell. Ligand binding induces a conformational change of the receptor that results in its activation. Once activated, nuclear receptors function as transcription factors that regulate gene transcription. Two modes of action exist: some nuclear receptors are bound to the DNA before the ligand is bound in a repressive capacity, and change their mode of action upon ligand binding. Others only bind to DNA upon ligand binding. About half of the NRs has been designated as orphan receptors, since no ligands have been identified for these receptors. In these receptors, the ligand binding pocket is either absent or constitutively occupied by a small lipid molecule or heme.

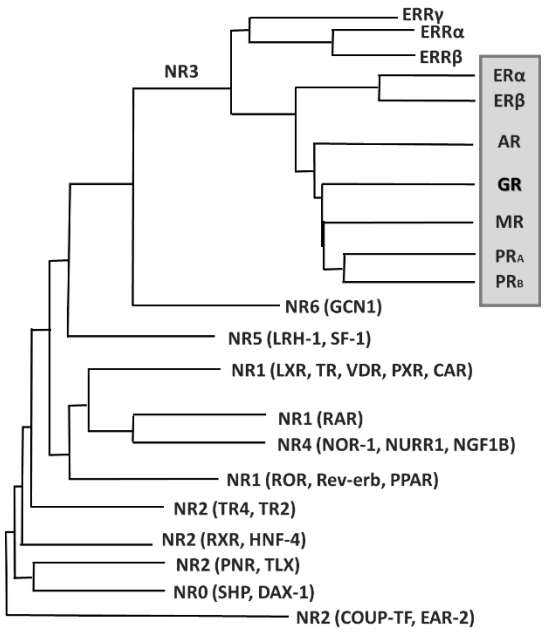

Figure 1. Nuclear receptor superfamily members. Steroid receptors (nuclear receptor subfamily 3; NR3) are boxed. Adapted from (7).

One class of nuclear receptors are the steroid receptors (figure 1). The ligands for these receptors are steroids, which are cholesterol-derived molecules that can easily traverse the plasma membrane. There are five classes of steroid hormones: androgens, estrogens, progestogens, mineralocorticoids and glucocorticoids. Secretion of these hormones by the endocrine organs in which they are produced (testis, ovary or adrenal gland) leads to circulation of these hormones in the bloodstream, so they can reach all cells in the body. Inside cells, these hormones are recognized by their respective receptors. This class contains the five steroid receptors: the androgen receptor (AR), estrogen receptor (ER), progesterone receptor (PR), mineralocorticoid receptor (MR) and glucocorticoid receptor (GR). Upon ligand binding, steroid receptors become activated, which enables them to bind specific target sites in the DNA, which are termed hormone response elements. These sites include repressive as well as activating sites. Subsequent recruitment of transcriptional coregulators can lead to either

transcription initiation or inhibition of associated genes. In addition to direct DNA-binding, steroid receptors can regulate gene expression through tethering to other transcription factors. These genomic effects of steroid receptors affect transcription and therefore the *de novo* synthesis of proteins, which are slow processes. Steroid receptors also perform more rapid non-genomic functions by playing on cytoplasmic signaling cascades, but much less is known about these latter pathways.

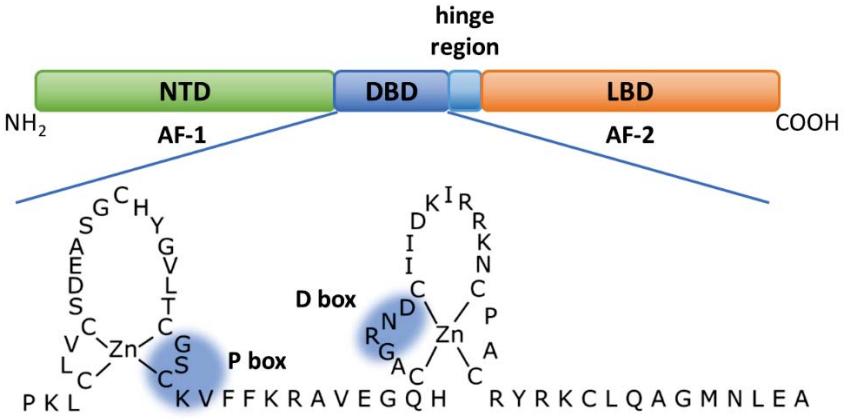

Figure 2. Modular structure of the GR. All steroid receptors share a common structure that includes an N-terminal-domain (NTD), a DNA-binding-domain (DBD) and a Ligand-binding-domain (LBD). Two transactivation domains (AF-1 & AF-2) allow for binding of coregulators to the GR. The amino acid sequence of the GR DBD is given. The DBD consists of two loops that are held together by Zinc residues. The proximal box (P box) contains the residues that are in direct contact with the DNA. The distal box (D box) contains residues that are involved in dimerization of the receptor.

All steroid receptors share a common structure encompassing three domains; an N-terminal domain (NTD), a DNA-binding-domain (DBD) and a ligand-binding-domain (LBD). In figure 2, the structure of the GR is given as an example. The N-terminal domain varies in length and is typically not well-conserved. It is thought to function as a docking site for transcriptional coregulators in the form of an activating function region (AF1) (8). The DBD is highly conserved and is involved in the recognition of specific DNA sequences. It contains two loops (so-called zinc fingers) that are shaped by

four cysteine residues that coordinate a zinc atom. The first loop contains the proximal box (P box), which includes residues that interact directly with the DNA. The second loop contains the distal box (D box) that is involved in dimerization of the receptor. The LBD is folded into multiple helices that together form the ligand binding pocket. Because different receptors bind slightly different ligands, the LBD is moderately conserved. The LBD also contains a transactivating domain (AF2) which interacts with different coregulators in a ligand-dependent way.

1.4 MEET THE MAIN ACTOR: THE GLUCOCORTICOID RECEPTOR (GR)

The main endogenous ligand of the glucocorticoid receptor in humans is cortisol. Cortisol is produced, in a diurnal rhythm and in response to stress, by the adrenal glands upon stimulation by ACTH from the pituitary, which in turn is under the control of the hypothalamic CRF secretion. Together these organs constitute the hypothalamic-pituitary (HPA) axis and are involved in maintaining homeostasis in a circadian and ultradian rhythm as well as controlling our response to stress. At the pituitary and the hypothalamus, cortisol functions as an inhibitor resulting in a negative feedback loop.

Cortisol affects a wide range of systems in our body. These effects are mediated by the GR and another steroid receptor, the MR. The MR has a higher binding affinity for cortisol and is activated under basal conditions, whereas the GR only becomes activated when cortisol levels are increased upon stress due to its lower affinity. The GR evokes different responses in different cell types. In general, it is tasked with maintaining metabolism and energy homeostasis. For example, during starvation or exercise the GR regulates gluconeogenesis and glycogenolysis in the liver. It is also involved in maintenance of blood pressure homeostasis through inhibition of vasodilators (9). Effects on brain function and behavior have been demonstrated as well. Elevated levels of cortisol have been associated with psychiatric disorders and studies indicate that functioning of the GR correlates with anxiety behavior (10). The GR has also been implicated in memory formation (11–13). Finally, the GR affects functioning of almost all cells of the immune system. In general activation of GR leads to suppression

of the immune system, although some stimulating effects have been observed as well (14).

The immunosuppressive effect of the cortisol is widely used clinically. Injections of cortisol were first used in 1940 by Philip Hench to treat patients with rheumathoid arthritis. Nowadays synthetic glucocorticoid drugs such as dexamethasone, prednisolone and fluticasone proprionate are commonly used in patients with immune-related diseases that include asthma, skin and ocular infections and patients undergoing organ transplants (14,15). Unfortunately, two major concerns impede the use of glucocorticoids in disease: serious side effects and glucocorticoid resistance. Up to one third of patients that receive chronic treatment with glucocorticoids show reduced glucocorticoid sensitivity (16). It is of note that not only exogenous supplementation of glucocorticoids, but also chronic stress leading to chronic glucocorticoid exposure is thought to lead to glucocorticoid resistance (16). Multiple mechanisms have been implicated in resistance, including an increase in the expression of the ß-isoform of the GR, changes in posttranslational modifications of the GR, reduced nuclear translocation of the GR, down-regulation of GR expression and repression by NF-κB (14,17). It has been suggested that the absence of the pulsatile changes that naturally occur under healthy conditions might also contribute. The side effects of glucocorticoid treatment include hyperglycemia, weight gain, osteoporosis, hypertension, depression, delayed wound healing, osteoporosis, reduced muscle regeneration and protein degradation (14,16). To reduce these side effects, selective GR agonists and modulators are being developed.

1.5 DNA BINDING UP CLOSE

In the absence of ligand, the GR is associated with heat shock proteins and immunophilins to form an inactive multiprotein complex. This conformation promotes high-affinity ligand binding, after which a conformational change most likely exposes as second nuclear localization signal of the GR, which facilitates active translocation of the GR to the nucleus, where it interacts with DNA. Several motifs have been associated with GR binding to DNA either directly or indirectly (figure 3). Classically, it can bind to specific target sites, so-called glucocorticoid-response elements (GREs). On these sites, the GR

homodimer binds to a half of the consensus sequence that is comprised of an inverted palindromic hexamer separated by three basepairs: AGAACAnnnTCTTGT. In the DBD of the GR, there are three amino acid residues that make base-specific contacts in the major groove of the DNA. Lysine 442 interacts directly with a thymine that is strongly conserved in the consensus GRE sequence. Valine 443 interacts with a guanine that is strongly conserved in all hormone response elements (18,19). The third residue to be involved in specific DNA binding is arginine 447. This residue has a direct interaction with a guanine that is present in canonical GREs. A stretch between the two zinc fingers of the DBD makes nonspecific contacts with the DNA helix backbone and the minor groove (18,20). Mutation of one of the residues involved in these interactions, arginine 477, leads to cortisol resistance in humans (21). The binding site as well as the sequence directly flanking the binding site influence the transcriptional effect of the GR, making the DNA an allosteric regulator of the GR (22–24). Recently it was shown that DNA binding can also trigger tetramerization of the GR, which might influence GR functioning (25).

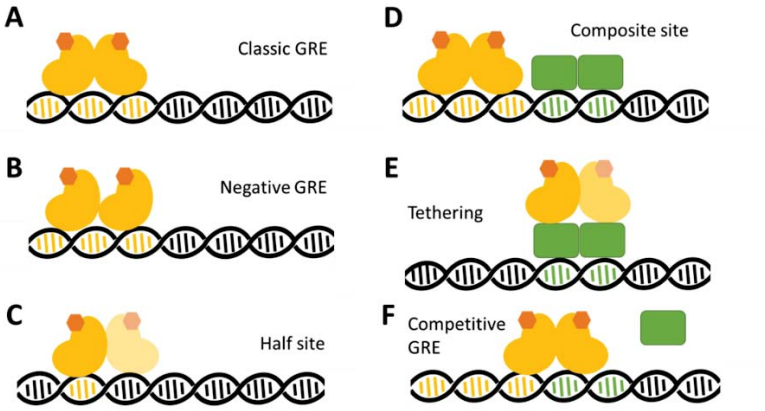

Figure 3. Types of GR binding in the nucleus. Four types of direct DNA binding and one type of indirect DNA binding have been identified. A. Binding of a homodimer to a GRE (yellow DNA). B. Binding of GR homodimer in a different conformation to negative GRE (nGRE). C. binding of GR to a GR half site (only one GR directly interacts with the DNA). D. Binding of GR that promotes interaction with another transcription factor. E. Indirect DNA binding through tethering to other transcription factors. F. Binding of GR to a GRE that prevents binding of transcription factors to neighboring DNA binding motifs.

Alternatively, the GR can bind to variations of the CTCC(n)$_{0-2}$GGAGA consensus sequence. A different GR dimer conformation has been correlated with these binding sites (26,27). These sequences were termed negative GREs because in general the activity of genes associated with them is downregulated by GR. In addition, it has been shown that the GR can bind to half sites as well as composite sites (14). A composite site is a functional unit of DNA with two or more different transcription factor binding sites. Through the interaction between the transcription factors gene expression is regulated (this can by synergistic or antagonistic). This term was originally coined for the composite site of a GR binding site that was found to be adjacent to an AP-1 binding site, which regulated the mouse proliferin promotor (28). Through binding of GR to other transcription factors (tethering), the GR has been shown to regulate their transcription. Tethering can occur through direct interactions between the transcription factors, but can also be mediated by a cofactor (29). Furthermore, the GR can bind to GREs and inhibit binding of transcription factors on a neighboring motif (30). Although recently it was suggested that the GR can also bind directly to other transcription factor motifs (31). Indeed, GR regulation of genes also involves many other transcription factors as was shown by analysis of gene regulatory networks (32). A number of genes is regulated by the GR without associated GR binding sites (32).

Between 6,000 to 50,000 target sequences may be bound by the GR, and this is highly context dependent (33–36). Of all GR binding sites only 4 to 11 percent overlaps between cell types (33). Moreover, genes associated with GR binding sites that overlap between cell types do not show the same magnitude of the response. Although the GR is able to recruit chromatin remodelers, 70-90% of GR binding sites is situated in pre-accessible chromatin (33). In line with these results, it was shown in an *in vitro* study that the GR has a higher affinity for DNA which is further away from a nucleosome and hence more accessible (24). Accessibility of DNA is regulated through epigenetic markers that locally compact or decompact chromatin and regulate nucleosome occupation. These variations in chromatin accessibility determine the cell type specificity of GR action. In addition, the cell type specific chromatin arrangement in the nucleus can bring binding

sites in enhancer regions and regulated genes together. It was shown that this arrangement is pre-established by other factors prior to binding of the essential transcription factor (37).

Further cell type specific regulation is conferred by posttranslational modifications brought about by different actors that are present in the cell (38). At least one posttranslational modification, phosphorylation, is dependent on the cell cycle, which suggests that there might be cell cycle dependent responses to glucocorticoids (39). Posttranslational modifications might affect the conformation and hence the function of the GR as different conformational changes lead to different coregulator binding patterns. Finally, although the GR is produced from one gene, different isoforms occur through alternative RNA splicing and variations in translation initiation. They have slightly different functions and abundancy, leading to fine tuning of GR signaling in a cell and tissue specific manner (40).

Gene expression is the result of a complex interplay between modification of GR through factors available in the cell, accessible DNA sequences and the ligand, which together induce conformational changes in the receptor, which enable different transcriptional coregulators to bind (figure 4). Once bound to DNA, the GR interacts with many different other proteins, the so-called transcriptional coregulators. The GR is known to recruit chromatin remodelers, e.g. the SWI/SNF complex, which moves or removes nucleosomes or replaces histone proteins in an ATP-dependent manner. This most likely further opens up the chromatin, resulting in increased DNA accessibility, as was suggested by FAIRE-seq experiments (41). Furthermore, histone methyl- and acetyltransferases, such as CARM1 and CBP/p300, interact with the GR to further make the chromatin accessible and mark the chromatin region by a specific pattern of acetylations and methylations on the tails of histone proteins (42). Interaction of the GR with mediating complexes, such as TRAP/DRIP, facilitate recruitment of the components of the basal transcription machinery like RNA polymerase II. Together, these factors enable the initiation of the transcription process. When different proteins, such as NcoR, are part of the complex, transcription can also be repressed.

Although the way the interactions are described here imply a successive buildup of factors that lead to transcription initiation, the process is not so linear. For example, recruitment of chromatin remodelers leads to dissociation of GR from DNA (43). In addition, p23 and the proteasome also play a role in the residence time of the GR on DNA (44). This residence time is relevant, because longer residence times are associated with greater transcriptional output (44). These results indicate that it is important to understand not only the interactions between factors, but also the dynamics of their interplay.

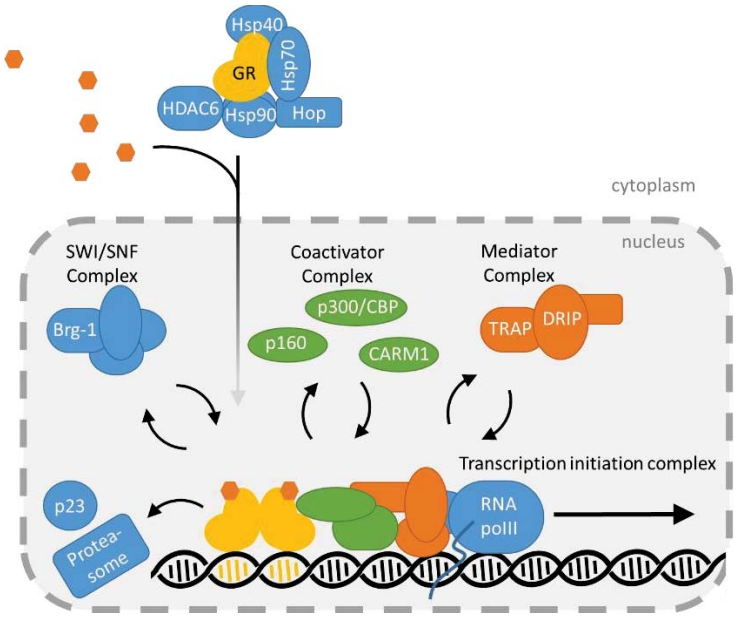

Figure 4. Coregulators of the GR. In the cytoplasm the GR is kept inactive through binding with heatshock proteins (Hsp), HDAC6 and Hop. Upon ligand binding the GR is shuttled to the nucleus and binds to DNA. At the DNA three main classes of coregulators interact with the GR in a dynamic manner, chromatin remodelers (SWI/SNF complex), coactivators (p160, p300, CRM1) and mediators (TRAP/DRIP). Subsequently, the transcription initiation complex is recruited, which includes RNA polymerase II (RNA polII). The proteasome and p23 play a role in the residence time of the GR on the DNA.

1.6 SEEING IS BELIEVING; CAPTURING THE INVISIBLE THROUGH MICROSCOPY

To understand the role of transcription factors in transcription regulation, we need to investigate both the distribution of molecules and their dynamics. In this thesis, advanced microscopy techniques are used to access this temporal and spatial information. In particular, single molecule microscopy and fluorescence recovery after photobleaching are employed.

In one of the earliest microscopes used for science in the late 17th century, Antoni van Leeuwenhoek used a single lens to magnify an object. Specimens were placed within the focal length of the lens and by placing the eye at a certain distance from the objective lens, a magnified image of the specimen would be projected in focus on the retina. This required a good eye and a lot of light. It took the advancement that Van Leeuwenhoek made in making the lenses to obtain the magnification important to life sciences. Even though larger magnifications were now possible, aberrations as a result of the different diffraction of different colors of light were limiting to the imaging of small objects. Compound microscopes, in which more than one lens were used to obtain a higher magnification, had already been discovered, but the aberrations limited the use of these microscopes.

Around 1850, the German instrument maker Carl Zeiss, joined by the chemist Otto Schott and the mathematician Ernst Abbe, made yet another improvement in microscopy. Otto Schott continued to optimize the glass formulation and optical properties of lenses, where Ernst Abbe aided the corrections for chromatic aberrations and developed a theoretical framework to understand the principles of microscopy. He created a formula that defines the resolution limit of a light microscope (equation 1), supporting it with experimental and theoretical work.

$$d = \frac{\lambda}{2\,N.A.} \qquad \text{Eq. 1}$$

with d the resolution and λ the wavelength of the light that is used. Abbe defined the numerical aperture (N.A.) of an objective lens as the sine of α (half the angle of the light cone that enters the objective), times the

refractive index of the medium between the objective and the cover glass. With this formula, we can calculate the resolution, which is defined as the smallest distance between two objects at which they can still be detected as individual objects. The highest quality objectives used in light microscopes today have an N.A. of 1.49, so for visible light (380-680 nm) the resolution *d* ranges between 128 and 2228 nanometer.

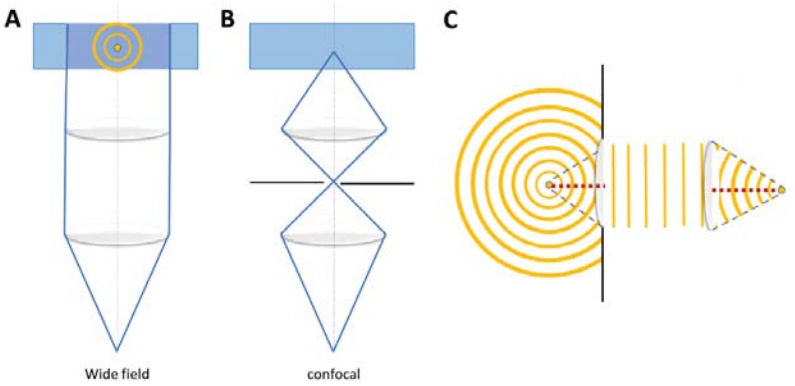

Figure 5. General principles of microscopy. A. Principle of wide field microscopy in which a large portion of the specimen (light blue bar) is illuminated (dark blue bar). The yellow point with concentric circles represents a single molecule that is illuminated. B. Principle of confocal microscopy. The captured light is restricted through a pinhole (black lines) and light from only a small volume of the sample is detected. C. A point source emits light in concentric circles. When a lens is placed at a distance that is equal to the focal length of the lens (red dashed lines), the wave propagates as a plane. When another lens is encountered, the image of the point source is in focus at the focal length of the lens. At this point, the image of the point source no longer looks like a point, but as an Airy disc (see text).

When the light source directly illuminates the whole sample, throughput its full width and depth, we speak of wide field microscopy (figure 5a). Although the whole depth of the sample is illuminated only a part of the sample is in focus, and other regions of the sample contribute to a high out-of-focus background signal. To reduce this noise, confocal microscopy was developed. By placing a pinhole in front of the detector, only the emitted light from a plane in the sample with a limited thickness reaches the detector, resulting in so-called optical sectioning (figure 5B). The pinhole also limits the portion

of the sample that is illuminated in the x,y-plane (figure 5B). To obtain a full image of the sample, the beam is scanned along the x,y plane of the image. To circumvent the time-consuming task of scanning, spinning disk microscopy was developed. In this microscope, through a series of thousand or more lenses combined with pinholes the sample is illuminated simultaneously. In this thesis both wide field microscopy as well as spinning disk microscopy have been used.

Light originating from a point source that is at the focal length from the objective lens, propagates as a spherical wave around the point source (figure 5C). The spherical wave is translated into a plane wave by the objective lens. Another lens, the imaging lens, can then convert the plane wave back to a spherical wave, which will focus into a point at the focal length of the imaging lens. If a camera is placed at the focal length of the imaging lens, we can capture an image of our object. However, when light passes through an object with a finite aperture, such as a lens, diffraction occurs. The resulting diffraction pattern due to interference of the diffracted waves causes the image that is obtained to be a series of concentric rings with decreasing intensities rather than a single point. This particular pattern is called an Airy disc. The diameter of this pattern is directly related to the wavelength and the size of the aperture. When two point emitters are close, their diffraction patterns overlap and the interference makes it impossible to distinguish the two objects. This caused Lord Rayleigh to adjust the resolution limit described by Abbe to be based on the smallest distance between two Airy discs where they can still be resolved separately (equation 2).

$$d = \frac{1.22\lambda}{2\ N.A.} \qquad \text{Eq. 2}$$

When the object is smaller than the diffraction limit, it appears larger through the interference pattern that is created. The point spread function is the description of the 3D image that the point object creates on the camera. As a general approximation, we can use a 2D-Gaussian to fit the central peak of the Airy disc and determine the center of the Gaussian with large accuracy (figure 6). This method is termed single molecule microscopy

and is used to observe single molecules in live cells. In this manner, the position of a molecule can be determined with precision of up to 30 nanometer, which is well beyond the resolution limit described by Abbe and Rayleigh. Through the use of modern high-speed cameras, their mobility can be captured with a time resolution of 5 milliseconds. Initially, experiments in live cells were focused on proteins inside membranes (45–48). In more recent years, the diffusion of proteins in 3D volumes, such as the nucleus, has also been explored. The first transcription factor study reported to use single molecule microscopy was the lac repressor in *Escherichia Coli* in 2007 (49). Soon after, many other transcription factors followed. In particular, the GR and other family members have been well characterized.

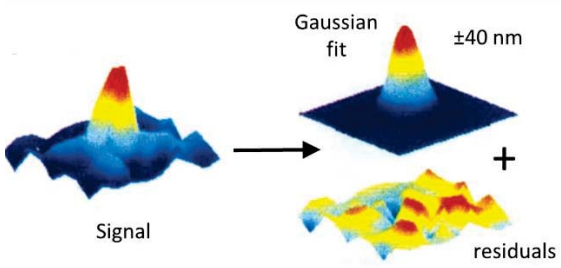

Figure 6. Single molecule identification. The signal obtained from the projection of a single molecule on the camera is fitted with a 2D Gaussian with an accuracy of ± 40 nm. The residuals compare to the background noise. Adapted from Schmidt et al. 1995 (50).

1.7 MAKING PROTEINS VISIBLE FOR MICROSCOPY

To observe proteins such as the GR using conventional microscopy the proteins are often conjugated to a fluorescent protein or tagged with a fluorescent dye. The principle of fluorescence relies on the excitation of an electron of the fluorescent molecule from the ground state to an excited state upon absorption of a photon. After a rapid relaxation to the lowest vibrational energy state, the electron falls back to the ground state and light is emitted. The emitted photon has a lower energy than the photon that excited the electron due to the first rapid relaxation (figure 7). Hence, the emission light has a longer wavelength than the excitation light and can be separated allowing for the detection of only the emitted photons.

Occasionally, the electron does not fall back to the ground state immediately, but is retained in a dark state. Once the photon decays to the ground state, it can be excited again. The detection of alternatively, direct decay to the ground state and retention in the dark state, is recorded on the camera as blinking behavior. Simultaneous absorption of two photons, with both photons carrying half the energy, can also result in excitation of the electron to the same excited state as mentioned before. This can be advantageous as the difference between the photons used to excite the electron and the photon emitted is larger, which results in lower background. Moreover, two photon excitation results in confocal excitation, and the lower energy photons incur less damage in live cells.

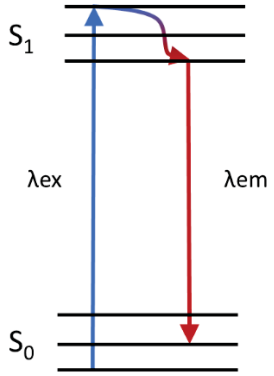

Figure 7. Jabloinski diagram of an electron. Excitation of an electron from the ground state (S_0) to the excited state (S_1) occurs due to absorbance of a photon. The first step consists of thermal decay. In the second step, the electron is returned to the ground state and a photon is emitted.

Photobleaching is the photochemical alteration of a molecule after excitation such that it can no longer be excited, and this process can be exploited to study the dynamics of proteins such as the GR in live cells. By sending a large number of photons to a well-defined area within the cell, the fluorescent molecules in that area are bleached, rendering these molecules invisible. By observing, over time, the dissipation of the bleached molecules to other areas in the cell as well as the movement of fluorescent molecules into the

bleached area we can infer the mobility pattern of the fluorescent molecules. This method is named fluorescent recovery after photobleaching (FRAP).

Because photobleaching and blinking of fluorescent proteins remains an issue in studying the diffusion of proteins over long time scales, efforts are being made in the search for alternative labels for the visualization of proteins inside living cells. Noble metals offer such an opportunity. In particular gold nanoparticles, spherical or rod-shaped have been used in live cells. Upon bombarding particles made of these materials with photons, rather than excitation and decay of a single electron, a collective oscillation of the free electrons on the surface of the particle is generated in a process termed plasmon resonance. Upon recombination of an electron with a hole in the lattice a photon is emitted that can be detected. For this type of interaction the optimal excitation wavelength is dependent on the ratio between the longitudinal and transverse axis of the particle. Interestingly, gold nanorods lend themselves well for anti-Stokes imaging. When after excitation (but before decay) additional energy is absorbed by the particle, for example through heat, there is a possibility that the emitted photon of a higher energy than the excitation wavelength, so light is emitted that has a shorter wavelength than the excitation light. As this process is rare for cellular structures, exclusive detection of photons that are produced via this anti-Stokes process results in near background-free imaging of gold nanorods in living cells (51).

1.8 From images to numbers; quantification.

Quantification of data obtained using microscopic imaging of biological samples is perhaps as difficult as getting the images in the first place, and therefore methods to analyze data are continually in development. In this thesis, in particular single molecule and FRAP experiments are quantitatively analyzed in detail.

Quantification of FRAP experiments by directly fitting the data is complicated by the large number of variables that need to be taken into account. This can be circumvented by using an approach in which the experimental data is compared to curves generated using Monte Carlo modeling. In this method,

simulations are made of diffusing molecules that can bind in an ellipsoid volume. A large set of curves is generated by varying the parameters that are involved within physiologically relevant ranges. The curves that best fit the experimentally obtained curves are then selected. The parameters used to construct these curves most likely reflect the true binding times of the transcription factors. Hence, this method for the quantification of FRAP experiments allows for the understanding of transcription factors in a quantitative manner.

To analyze single molecule experiments, different methods are required. As mentioned earlier we can determine the position of a single fluorescent protein with an accuracy of ~30 nm when we fit the distribution of light derived from a single molecule with a 2D-Gaussian. However, if we want to track the movement of this molecule over time we are limited by the bleaching and blinking properties of the fluorescent molecule. Since this results in short trajectories for individual molecules, most often the results of many molecules are pooled to obtain reliable data on their mobility. Furthermore, proper tracking of molecules requires some *a priori* knowledge on their mobility. In order to be able to use an unbiased approach, the Particle Image Correlation Spectroscopy (PICS) method was developed. By determining all possible correlations between the location of molecules in consecutive images, a cumulative distribution function (Cdf) of displacements *R* is generated (for details see chapter 2, 3 and (52)). From the cumulative distribution function we can determine, by curve fitting, whether there are multiple fractions with different mobility, the sizes of these fractions α and their respective diffusion coefficients *D*. Equation 3 can be used for curve fitting when three fractions of molecules occur.

$$Cdf(R^2) = 1 - \left(\alpha_1 \exp\left(\frac{R^2}{4D_1 t + 4\sigma^2}\right) - \alpha_2 \exp\left(\frac{R^2}{4D_2 t + 4\sigma^2}\right) - \alpha_3 \exp\left(\frac{R^2}{4\sigma^2}\right)\right)$$

Eq. 3

Using different intervals between images several cumulative distributions can be obtained for the same data. However, it is notable that as the interval increases a bias is created towards slower diffusing particles in settings where the depth of focus is limiting. Because particles that move slower

remain in the observable volume for a longer time than particles that diffuse faster, the α-coefficient is distorted. In chapter 3 of this thesis an analytical solution for is provided.

We can use the Cdf, obtained from the single-molecule data to extract additional dynamic parameters and time scales. Under the framework of a continuous-time Markov chain model, we can obtain the occupation probability distribution (for further explanation see (53). This allows us to get an insight into the probability of the GR to switch between binding and diffusion states as well as the time spent in these states.

1.9 And action! Dynamics of transcription factors

In this thesis, advanced microscopy studies on the dynamics and distribution of the GR in living cells are described. Early *in vitro* studies showed slow kinetics of transcription factors. Residence times on the DNA were found to be in the order of minutes to hours, which is in correspondence with the pace of transcription initiation. This static model was contradicted by a study in live cells (54). This study showed that after photobleaching of a small area of the nucleus containing an array of GREs, the recovery of fluorescently labeled GRs to this site and dissipation of photobleached GRs was in the order of seconds (54). A hit-and-run model was proposed, where the GR is only present at the GRE for a short period of time. These immobilizations of the receptor were associated with transcriptional activation (44). This dynamic behavior was confirmed by later studies and, amongst others, it was shown to be dependent on the type of ligand (55). There are indications that the contrast between *in vitro* and live cells experiments portrays an active disassembly of transcription factors from the chromatin in live cells (44,56).

In addition to its DNA binding, the diffusion of the GR in the nucleus has been studied extensively. Diffusion of transcription factors is necessary to find their binding sites as well as move between binding sites. Diffusion of the GR has been found to range around 3 $\mu m^2/s$ (57–60), which is comparable to other transcription factors (49,61–63). It has been proposed that effective search mechanisms are comprised of 3D as well as 1D diffusion (64,65), although 1D diffusion has only been demonstrated *in vitro* and in bacteria

(66–68). Different types of retarded diffusion have been proposed, such as 1D sliding along the DNA, hopping and intersegmental exchange (figure 8). Together, these studies have demonstrated the occurrence of multiple dynamic states for transcription factors like the GR. However, the temporal relations between these states is poorly understood because information on state-to-state transitions is not available. Furthermore, a consistent view describing all states including the biological processes underlying them is still lacking.

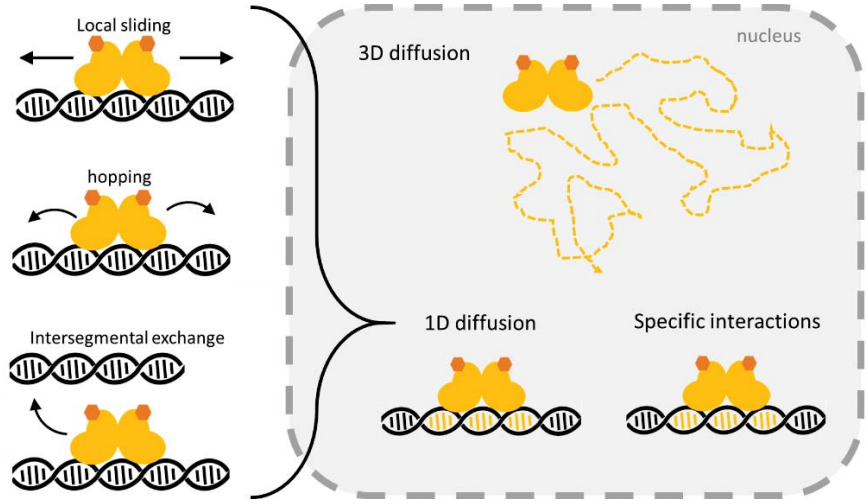

Figure 8. GR dynamics in the nucleus. Modes of 3D diffusion, 1D diffusion and specific binding have been identified. 1D diffusion might be based on non-specific interactions and can include local sliding, hopping and intersegmental exchange.

To salvage the apparent contradiction between the slow process of transcription with the fast binding kinetics of transcription factors, a probabilistic model has been suggested, which is based on stochastic interactions between transcription factors and DNA. In addition, most current models assume thermodynamic equilibrium. Although this makes modeling easier and provides meaningful results, there are certain underlying assumptions that may not always hold true. These include a balance of each reaction such that it takes place equally in both directions. These new directions highlight the need for a quantitative model of

transcription binding that dissects the behavior of individual transcription factors from that of the average population effect.

AIMS

The central aim of this thesis is quantification of the dynamics and distribution of the GR, a model transcription factor, in live cells. We have divided the work in the following objectives:

1. Generate the most complete description of GR dynamics to date
   a. Identification mobile and immobile states of GR using a combination of single molecule measurements and FRAP experiments
   b. Characterizing switching behavior of the GR between states
   c. Analyzing the biological mechanism underlying each states through the use of a series of six DNA-binding mutants
2. Development of novel analysis methods to better quantify GR diffusion
3. Characterize the distribution of GR in the nucleus of live cells
   a. Comparison of this distribution with ER, a closely related nuclear receptor
   b. Analyzing the biological mechanisms underlying GR distribution through the use of GR and ER mutants
4. Development of a novel method to track GR dynamics through the nucleus for arbitrarily long times

1.10 OUTLINE OF THIS THESIS

In this thesis, we have developed a framework to understand how the GR is spatially and temporally distributed in the nucleus of live cells, in order to better understand how it regulates transcription. Concomitantly, we have developed several experimental strategies to study these phenomena.

In **chapter 2** the GR is used to show novel mechanisms for DNA target site finding and binding by transcription factors. To this end a combination of single molecule microscopy and FRAP is used and a range of DNA-binding mutants is studied.

In **chapter 3** the analysis of single molecule data is extended to provide a solution for depth of focus loss of diffusive molecules. To this end the probability of losing molecules due to diffusion out of the depth of focus is taken into account in the analysis.

In **chapter 4** the spatial distribution of the GR and ER in the nucleus of live cells is analyzed. It is shown that the number of receptor hotspots and their size in the nucleus is dependent on the type of receptor. However, this is independent of the specific DNA binding properties of the receptor.

In **chapter 5** the potential of gold nanorods as a label for diffusive proteins is investigated. The diffusive behavior of gold nanorods is studied in two cellular compartments, the cytoplasm and nucleus. In addition, gold nanorods are functionalized with a nuclear localization signal resulting in successful translocation of these particles to the nucleus.

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