Nuclear Receptor Research
Vol. 1 (2014), Article ID 101090, 20 pages
doi:10.11131/2014/101090

AgiAl Publishing House
http://www.agialpress.com/

Review Article

Investigation of Interactions between DNA and Nuclear Receptors: A Review of the Most Used Methods

Juliana Fattori, Nathalia de Carvalho Indolfo, Jéssica Christina Lóis de Oliveira Campos, Natália Bernardi Videira, Aline Villanova Bridi, Tábata Renée Doratioto, Michelle Alexandrino de Assis, and Ana Carolina Migliorini Figueira

Brazilian Biosciences National Laboratory (LNBio), Brazilian Center for Research in Energy and Materials (CNPEM), P.O. Box 6192, Campinas-SP, Brazil

Corresponding Author: Ana Carolina Migliorini Figueira; email: ana.figueira@lnbio.cnpem.br

Recieved 29 April 2014; Accepted 5 August 2014

Editor: Mario Galigniana

Abstract. Nuclear receptors (NRs) comprise a superfamily of proteins modulated by ligands that regulate the expression of target genes. These proteins share a multidomain structure harboring an N-terminal domain, a highly conserved DNA binding domain, and a ligand binding domain, which has ligand dependent activation function. They play key roles in development, metabolism, and physiology being closely related to diseases. Most of the knowledge about this superfamily emerges from investigations on new ligands and are mostly centered in the ligand binding domain. However, more investigation focusing on interactions between DNA and DNA binding domain is necessary to shed light on important roles of NRs' participation in transcriptional mechanisms and in specific genes network. Here, our goal is to discuss some nuances of NRs-DNA interaction, describing details of the most used techniques in this sort of study, such as gel shift (EMSA), DNA footprinting, reporter gene assay, ChIP-Seq, 3C, and fluorescence anisotropy. Additionally, we aim to provide tools, presenting advantages and disadvantages of these common methods, when choosing the most suitable one to study NRs-DNA interactions to answer specific questions.

Keywords: nuclear receptors, DNA binding domain, protein-DNA interaction, ChiP-seq, DNA footprinting, EMSA, fluorescence anisotropy

1. Introduction

Nuclear receptors (NRs) are members of a superfamily of transcription factors modulated by ligands, which regulate the expression of target genes and, therefore, play key roles in development, metabolism, physiology, and disease [1–3]. The activity of the majority of NRs is induced by ligands or small lipophilic molecules such as steroids, hormones, vitamins, and fatty acids. However, this family also possesses the orphan nuclear receptors, for which once ligands remain unknown or could not exist, presenting unknown activity and suggesting that NRs' activity can also be regulated by other processes [2, 4, 5].

Traditionally, NRs act as homo- or heterodimers and share the same mechanism of action. In a classical view, unliganded NRs may be located in the cytoplasm, bound to

chaperones, or in the nucleus, bound to DNA, where they recruit corepressor complexes, which associate with HDAC (histone deacetyltransferases) to inhibit gene expression. Ligand addition induces translocation of cytoplasmatic NRs to the nucleus and promotes conformational changes in the NR structure, allowing corepressor dissociation and the recruitment of coactivator complexes that have HAT (histone acetyltransferase) activity to activate the transcription of target genes [1, 6].

However, alternative mechanisms of action have been described for this class of proteins, which includes both activation by posttranslational modifications and association with other transcription factors (TFs) [7]. For instance, the PPARγ sumoylation is a critical event that signals corepressor recruitment, even after PPARγ ligand binding, keeping inflammatory gene promoters in a transrepressed state [6, 8]. Moreover, the interaction between different TFs, resulting in cooperative enhancing or inhibition of gene expression, also named crosstalk, is an alternative NR action that explains, for example, the negative regulation of TSHβ gene by TR [7], or the blocked activity of NF-kβ by different NRs, such as GR, ER, PR, and AR [6].

Apart from sharing the general action mechanism, all NRs also present a similar modular structure including an aminoterminal domain, which contains the ligand independent activation function-1 (AF-1), a core DNA binding domain (DBD) that is responsible for recognizing and binding to DNA sequences, linked by a hinge to the ligand binding domain (LBD), which binds to ligands and recruits coregulators (Figure 1) [1].

Discovery of the NRs and most of the knowledge about them came from physiological investigations on hormones and ligands [9–12]. The identification of natural or synthetic ligands shed light over NRs roles in human physiology, where they are considered drug targets [13–17]. Meanwhile, there is still a lot to be understood about NRs in the context of specific gene networks' transcription [18]. In other words, there are many studies focusing on NRs binding to ligands, seeking molecules that can modulate them, and less studies focusing on how NRs bind to DNA.

In fact, the protein's ability to recognize DNA sequences, associated with the capacity of binding to specific sites across the genome, is a hallmark in gene regulation and maintenance [19, 20]. Initially, some codes for protein-DNA recognition were reported based on hydrogen bonding patterns between amino acids and nucleotides, in the DNA major groove. Also, arginine side chain was considered important to make these contacts [21]. Recently, the development of new technologies, such as computational simulations and high-throughput spectroscopic techniques, is uncovering new complexities of protein-DNA interactions, displaying that simple recognition codes for these interactions are much more complex. In this way, it is known that there are multiple modes of DNA binding to proteins, and variables such as spacers of DNA binding sites, multimeric protein binding, and alternative structural conformations should be considered together with cooperativity, allostery, and cofactor presence [19]. Particularly, to nuclear receptors, the main interactions between protein and DNA are made by the DNA binding domain. Albeit other interactions with N-terminal domain and LBD have been reported, they are specific to few receptors and still should not have been considered as functions [2, 3, 5].

1.1. A brief review of the DNA binding domain structure. For nuclear receptors, DNA recognition is controlled by the highly conserved DNA binding domain (or C domain). Structurally, the DBD is a very compact globular domain, generally composed by 100 amino acids which are organized in two main perpendicular helices, with conserved cysteines required for high-affinity DNA binding (Figure 2) [1]. The first DBD structure determination was in 1990 through nuclear magnetic resonance (NMR) [22]. However, since the first crystallographic structure of DBD on DNA was solved, in 1991 [23], X-ray crystallography has become a pivotal method in unraveling DBD-DNA interaction at atomic levels [24].

As shown in Figure 2, the DNA binding domain of all NRs presents two helices that pack together through their hydrophobic faces in a perpendicular way, forming the core domain with the two zinc fingers, contributing to the DBD integral fold [25]. The first α-helix, termed recognition helix, directly inserts itself to the DNA major groove, making contacts with the DNA phosphate backbone, forming a crucial substructure for the DNA-protein binding [25]. Amino acid residues at the base of the first zinc finger constitute the so-called P box that is involved in the discrimination of the response elements. Furthermore, residues in the second zinc finger form the D box and are involved in dimerization [1] and the C-terminal extension of DBD harbors T and A boxes, which contribute to contacts in the flanking DNA core recognition sequences [1, 26, 29].

Protein-DNA binding interfaces are also extended by ordered water molecules which make specific interactions between protein side chains and DNA functional groups. Apart from the specific interactions, there are several non-specific ones formed between basic side chains of the DBD and the DNA phosphodiester backbone [25–28].

Despite mediating DNA specificity, α-helix 1 contributes little to the discrimination of target-site symmetry. It has been shown that α-helix 2 is the main responsible for selecting symmetry for DNA recognition motifs, which also projects across the minor groove of the DNA, creating additional contacts that stabilize DBD-DNA binding. Additionally, some studies suggest that the specificity in DBD-DNA recognition is derived not only from the DNA sequence, but also from its geometry [25–28].

1.2. DBD-DNA binding and specificity. Although some NRs can bind to DNA as monomers, it was shown that the most common DNA binding happens when NRs are organized as

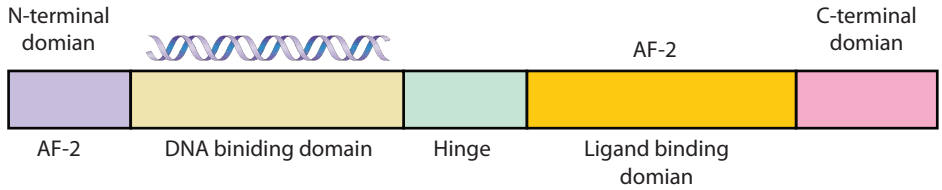

Figure 1: Typical domain organization of NRs, showing, in purple, the N-terminal domain, harboring the activation function ligand independent (AF-1); in light brown, the DNA binding domain, responsible for DNA binding; in light green, the hinge, which connects DBD and LBD; in yellow, the ligand binding domain (LBD) with the activation function ligand dependent (AF-2); and, in pink, the C-terminal portion.

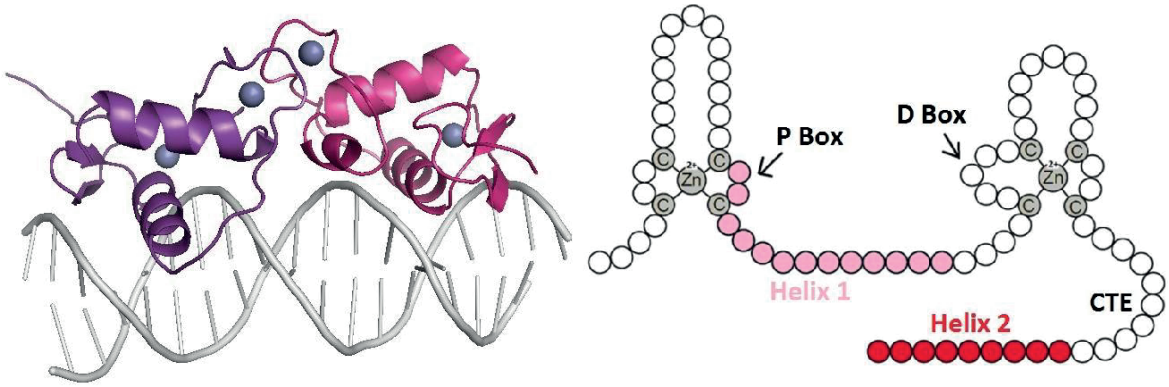

Figure 2: Illustration of the nuclear receptors' DBD. DBD is shown in dimeric form (in purple and pink) with two zinc ions (in gray) coordinated to each monomer bound to DNA (PDB: 1HCQ modified). On the right side, details of the two zinc fingers, each one being coordinated by four cysteines. Helix 1 or the recognition α-helix shown in light pink comprises the P box. The second zinc finger comprises residues forming the highlighted D box or dimerization interface. Finally, helix 2 is shown in red in the carboxiterminal extension (CTE). Adapted from 21.

homo- or heterodimers, with this being the best geometry array for recognition of binding sites [25]. Generally, binding to DNA occurs in specific DNA motifs, also termed as DNA response elements (REs). It has also been postulated that the formation of homo- and heterodimers between receptors DBDs occurs simultaneously to element response recognition [25].

In general, the REs are composed by hexameric sequences arranged in distinct configurations, including inverted, everted, and direct repeats. The specificities of one RE, to which each NR binds to, affects directly its function, and whether the receptor will work as an activator or repressor of the transcription [25]. The response elements may be located in regulatory sequences, generally found in the 5' region of the target gene, near the core promoter according to the NRs classical mechanism of action [1]. However, recent studies with new technologies posit that the majority of REs are found distally from promoters, as enhancers within transcription initiation site and, also, within intergenic and intronic regions [30, 31].

Typically, the consensus RE is composed by variations of two half-sites, which are in turn composed by hexameric sequences of 5'-PuAGGTCA-3'. These half-sites may be separated by different numbers of nucleotides [1], which can vary from 1 to hundreds of base pairs [32]. Idealized REs were divided into two generic groups: one is better recognized by steroid receptors, composed by 5'-AGAACA-3', and the other, composed by 5'-AGGTCA-3', is preferably bound by nonsteroidal and orphan receptors [1, 25, 28, 33]. Nevertheless, it has been shown that these sequences may be degenerated, since PuGGTCA and PuGGACA were identified as the best binders for ER and GR, respectively [27]. This variety of combinations may lead to increases in selectivity for each NR, as it was presented for TRs and RAR preferably binds to response elements comprised by AGGACA and AGTTCA, respectively [34]. Obviously, natural occurrence of these REs can present slight variations from the consensus sequence. Furthermore, structural studies have unraveled that the spacing between the half-sites is pivotal to the RE recognition and that the dimerization patterns of the NRs DBDs are reflected in their RE architectures [25].

However, to address questions about specificity and recognition, it is inevitable to mention the NR regarding chromatin context. While NRs act as sensors of external signals leading to a rapid regulatory response, chromatin accessibility plays as a way to specify the recruitment of NRs to regulatory elements [35]. In a classical mode, NRs bind to specific REs and recruit cofactors that modify the local chromatin structure and recruit RNA polymerases enhancing transcription (Figure 3). These structure modifications define

the chromatin state (active or silent) via histones modification.

In this context, the best characterized ones, mediated by NRs, are histone acetylation, deacetylation, and methylation [36]. While histone acetylation generally confers chromatin decondensation, via acetyltransferases recruitment (HAT), histone deacetylation, via deacetyltransferases (HDAC), counterparts by associating to chromatin condensation with the deacetylate histones complexes. The methylation process, on the other hand, is associated with both activation and repression, depending on the modified residue and on the number of incorporated methyl groups. For instance, methylation of lysine four (K4) in histone H3 activates a chromatin state, which stimulates transcriptional activation. Otherwise, methylation of K9 in histone H3 is associated with transcriptional suppression, inducing an inactivated chromosomal state [35, 36].

According to previous studies, the main functions of histone modification can be divided into two categories: (i) establishment, helping maintenance of the euchromatin accessibility for transcriptional machinery, and (ii) orchestration of DNA, giving support for DNA repair, replication, or chromosome condensation [40].

Besides the classical pathway of NRs action, the emergence of genome scale technologies has helped uncover new mechanisms that regulate NRs binding to DNA in the chromatin context. For instance, based on observations from the nucleosome crystal structure, it was proposed that these chromatin changes are, in fact, posttranslational modifications for histones. Moreover, it was suggested that histone N-terminal tails may be modified by processes such as acetylation, deacetylation, methylation, phosphorylation, ubiquitination, and sumoylation [35, 36, 41]. Another process involved in this new postulated mechanism is the ATP-dependent remodeling of nucleosomal arrays, which exposes new DNA naked sites, allowing for transcriptional machinery recruitment [36]. Essentially, according to this new hypothesis the chromatin, via its preexisting open sites, specifies NRs genomic localization and its interactions with regulatory elements. NR binding confers further changes to chromatin accessibility through remodeling of the underlying chromatin and associated nucleosomes by recruitment of cofactors and coregulators. Therefore, chromatin is though as an integral component in this mechanism, guiding NRs action in cell-type-specific and cell-state-dependent manners [35].

This brief discussion aims to show that the pathways that govern NRs selective activity in moderating cell- and signal-specific physiological programs are still a conundrum. However, new emerging genome scale technologies, along with new structural studies of full-length receptors, will shed light on the role of chromatin in selectively regulating NRs binding. Furthermore, these new approaches have also helped deciphering some new roles of NR cofactors in regulating DNA methylation, histone posttranslational modifications, and chromatin remodeling [35]. These events show that, from a global view, transcription selectively regulated by NRs through chromatin is a really well-orchestrated dance among chromatin histones, NRs, transcription factors, corepressors, coactivators, and modifying enzymes.

Moreover, the explanation of how NR-DNA specificity is achieved, considering that all NRs use a highly conserved DBD core region to specifically choose to bind to some DNA sequences is still obscure and need to be further investigated. Also, the chromatin context, conformational modifications, and further contacts between NRs and DNA should be considered in this discussion. However, it is important to highlight that this issue is crucial to the understanding of transcription regulation and studies with full-length NRs and chromatin are key points to answering this.

Regarding this issue, some reviews about DNA binding domain and hormone response elements have been reported recently [4, 18, 35]; herein, we focus on the description and applications of the most used analytical techniques to identify and study NR-DNA interaction. Our main goal in this review is to discuss advantages and disadvantages of the most applicable methodologies to NRs, providing a range of evidences that may assist in better methodological choice for the study of NRs.

1.3. Electrophoretic mobility shift assay (EMSA). Electrophoretic mobility shift assay (EMSA), also known as gel shift, is one method largely applied to study gene regulation and NR-DNA interactions. This method provides valuable information about sort of regulatory proteins involved in gene expression and may be adapted to a wide choice of cultured cell lines, DNA sequences, and transcription factors. In general, it is based on the propriety of retarded mobility in-gel, characteristics of protein-DNA complexes, when compared to free protein and DNA [42, 43].

Basically, gel shift assay consists of three key steps: binding reactions between DNA and protein, native electrophoresis, and probe detection. The DNA fragment used in this method is the target sequence, which is usually from restriction fragment, or a PCR product, or even, is obtained by DNA synthesis. The DNA labelling before is the most common approach, allowing for its specific detection after electrophoresis. Traditionally, DNA probes have been radiolabeled with ^{32}P, but, due to the concerns involving this kind of material, numerous nonradioactive labelling methods for performing EMSA have been developed, such as fluorescent probes that can be detected in-gel using an appropriate imaging system. Besides that, hapten-modified DNA probes can be visualized via secondary detection reagents, such as streptavidin or anti-DIG antibodies, in systems with enzymatic substrates similar to those used for Western blotting. Regarding the binding reaction components, there are many unique requirements for different nucleic acid binding proteins, so there is no universal set of reaction conditions for EMSA assays, which must be investigated in the literature for each specific case. Also important, the gels

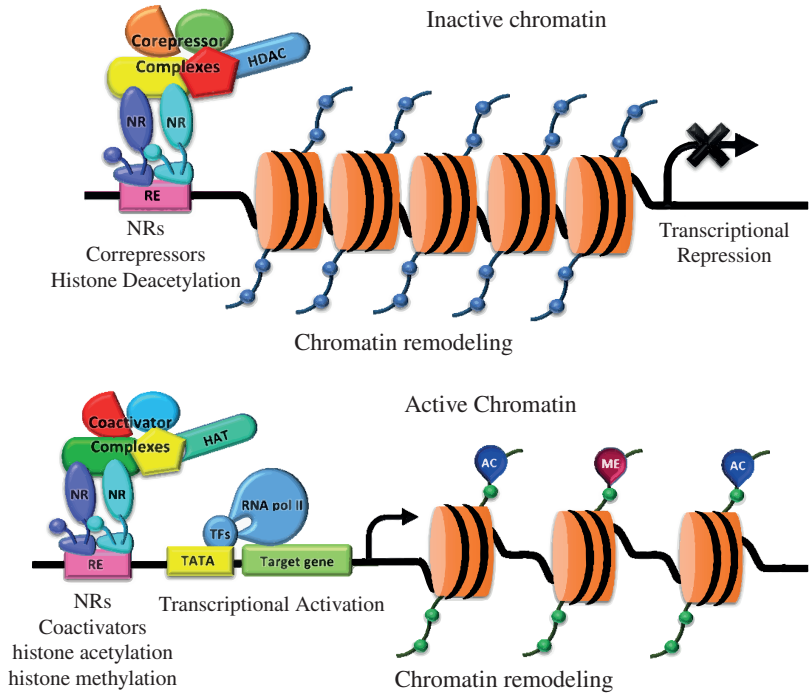

Figure 3: Scheme representing chromatin accessibility and NRs classical mechanism of action. Chromatin accessibility and histone modifications contribute to NR binding. The N-termini of histones have specific amino acids that are sensitive to posttranslational modifications, which contribute to chromatin status (active or silent). At the top of the scheme (inactive chromatin), there is an example of NRs bound to its RE and associated with transcription corepressor complexes. The presence of histone deacetylases (e.g., HDAC) leads to removal of any chromatin activating histone acetylation sites causing formation of transcriptionally repressed chromatin structure. At the bottom (active chromatin) there is an example of NRs heterodimers bound to the corresponding RE and associated with coactivator complexes. Formation of a coactivator complex induces histone modifications such as acetylation (Ac) by histone acetyltransferases (HAT) and methylation (Me) that in turn alter chromatin structure. This allows for the entry of the basal transcriptional machinery, including RNA pol II and transcription factors (TFs). The complete assembly then leads to the activation of target gene transcription (adapted from [37–39]).

used in this method are nondenaturing TBE-polyacrylamide gels or TAE-agarose gels [44].

EMSA is a reference technique that presents a great diversity of applications. In some cases, it may be applied in a simpler way to show DNA-NR binding events [45–50], or even to present that a heterologous expressed NR is able to binding to DNA [51]. But, in another way, more sophisticated questions have been answer using this sort of experiment. For instance, it was applied to show some particularities of TR isoforms in DNA binding, presenting that TRβ0 can bind as trimers to a subset of naturally occurring DNA elements and not just as homo- or heterodimers with Retinoid X Receptor (RXR), as it was thought [52]. Also, gel shift was employed to analyze the RXR/TR and RXR/PPAR heterodimerization and DNA binding [53]. In this study, the authors assessed the capability of these proteins to bind DNA in consensus target sites, with the heterodimers RXR/PPAR or RXR/TR efficiently bound to DR1 and DR4, respectively [53].

Other reports illustrate the use of EMSA in the study of nuclear receptors, identifying consensus DNA sequences that bind to different NR complexes. The interactions between coactivators and NRs on REs were studied by EMSA, searching for interactions among Vitamin D receptor (VDR), coactivators, and response elements. In this study, the effect of SRC-1 and TRAM-1 coactivators on VDR homodimer and VDR/RXR heterodimer was analyzed, showing that VDR forms stable homodimers after interaction with the coactivators on the VDRE DR3 (direct repeat spaced by 3 base pairs) and DR4 and DR5 REs may support these interactions, but in a weaker way [54].

In another example, a novel approach was developed to isolate large complexes of proteins associated with the DNA-bound estrogen receptor α (ERα) using an agarose-based EMSA, in order to understand how ERα regulates transcription of estrogen-responsive genes [55]. This method was adapted to other nuclear receptors and their responsive elements to provide better understanding of how they regulate gene transcription of certain genes.

Additionally, recent EMSAs were applied to study specificity in DNA–NR binding. It was reported that artificial DNA binding sites based on "AGGTCA" half-sites confer high affinity, but poor specificity, and that spacing alone does not account for the divergent DNA recognition properties of TRs and RARs, as it has been proposed [34]. In this case, the gel shift assay was used to explore the ability of TRα or RARα to bind to artificial DR4 and DR5 response elements

comprised of AGGTCA consensus half-sites and compare the binding profiles obtained from consensus REs to the two naturally occurring response elements, αMHC promoter (DR4), and the βRAR promoter (DR5). Although these elements were bound by their cognate receptors less strongly than were the artificial AGGTCA elements, they were bound with much greater specificity. Therefore, they conclude that half-site spacing contributes to DNA recognition by these receptors but is not the dominant discriminatory factor under these conditions [34]. All these results indicate the presence of particular abilities that different NRs have to discriminate between various promoter regions in similar arrays (as DRs), but with sequences that diverge slightly from the consensus.

All these examples, together with many others which apply EMSAs for NRs investigation [56–58], illustrate the wide application of this technique in the study of NR-DNA interaction. Though having some limitations such as samples that are not in the chemical equilibrium during the electrophoresis step or as that rapid dissociation during experiment may prevent detection of complexes or, even, the little direct information about the localization of the nucleic acid sequences; this assay is, in general, a rapid and sensitive method in the detection of protein-DNA interactions. The basic technique is simple to perform and highly sensitive, mainly with the use of radioisotope-labeled nucleic acids, allowing assays to be performed with small content of protein and nucleic acid.

Additionally, when this high sensitivity is not required, variants using fluorescence, chemiluminescence, and immunohistochemical detection are also available, avoiding the danger of radiation. Nucleic acids can be used in a wide range of sizes and structures and proteins can also include a range from small oligopeptides to transcription complexes. Another advantage is the possibility of using highly purified proteins and crude cell extracts, which accounts in large part for the continuing popularity of this assay [59]. In this way, the electrophoretic mobility shift assay is still a reference method generally used in the study of *in vitro* binding of nuclear receptors to DNA response elements.

1.4. DNA footprinting. Another important technique abundantly used to study interactions between NRs and DNA is DNA footprinting, which was one of the first's techniques applied in NRs field in this concern [60, 61]. This is an *in vitro* assay that investigates protein binding to specific DNA sites, studying protein-DNA interactions outside and inside the cell environment [62, 63]. It is based on the fact that when a transcription factor is bound to DNA, it is protected from degradation by nucleases, providing its "footprinting" on DNA sequence [62, 63]. Traditionally, this experiment was used in the studies on NRs to identify their binding sites in DNA. In the past, the DNA region of interest, harboring one or more transcription factors, was radioactively labeled, followed by DNAse I treatment, which digest unprotected or free DNA. After that, the digested DNA was separated by polyacrylamide gel and visualized on X-rays films [62, 63]. Nowadays instead, some other approaches have been associated with this method, such as the use of fluorescent probes over radioactive DNA labelling or the use of polymerase chain reaction (PCR) to amplify specific DNA regions over electrophoresis, in searching for the protected DNA sequences [64]. Additionally, application of synchrotron X-ray footprinting has been used to study time-resolved structural changes of nucleic acid conformation and protein-nucleic acid complexes. In this case, nucleases are substituted by X-ray to produce the cleavage patterns in DNA [65].

Remarkably, one of the first applications of this technique on NRs was the definition of specific REs, such as the elucidation of glucocorticoid response elements (GREs) in tyrosine aminotransferase gene promoter, which is regulated by GR in the presence of ligands [66]. Other applications include the regulation of tyrosine hydroxylase by Nurr1 orphan receptor [67] or, even, the binding of NGFI-B in the steroid 21-hydroxylase (21-OHase) gene promoter [68]. Some other regulatory regions in gene promoters were also found using footprinting, such as the regulation of growth hormones, GH1, and GC, by TR. In this case, authors found two regions that were selectively regulated by TR in strains of rat pituitary cells [69]. In the same way, other reports showed that the prostate antigen gene promoter was found to be regulated by AR [70], GR, and PR [71] and, also, by TR and ER [72, 73].

DNA footprinting also solved questions about how an NR recognizes specific promoters, considering that many receptors can bind to the same RE sequences. For example, the responsive elements composed by TGACC and TGTTCT sequences, known as glucocorticoid receptor REs, were found upstream several genes regulated by PR and AR. A more detailed investigation of these sequences, by using DNA footprinting, showed that one of these sequences was regulating rat probasin gene promoter by AR. This study also suggested that AR binds to another sequence in the same promoter with a different affinity, but the highest androgen induction was reached when both sites are filled with AR in a cooperative and mutually dependent manner [74].

Later, other studies using DNA footprinting revealed conformational changes in DNA upon NR binding, as it was observed for estrogen related receptor α 1 (ERRα-1) binding to a silencer element (S1), downregulating the action of human aromatase gene promoter. In this case, DNA footprinting was used to confirm the previous data obtained by gel shift mobility assay and presented the exact binding site for ERRα-1. However, it showed some intriguing conformational changes of DNA upon binding of ERRα-1[75], another important feature obtained by EMSA.

It is undoubted that DNA footprinting is a fairly standardized technique and has numerous applications in NRs studies as showed above. However, the development of these experiments has been presented as quite laborious and it is a

costly expensive method, generating hard data to be analyzed. To overcome these problems some improvements have been made, such as the development of softwares for quantitative analysis of gel images, to reduce the time of data analysis [76]. Also, digital approaches to evaluating regulatory protein occupancy on genomic DNA, using massively parallel DNA sequencing, have changed the paradigm of the absence of informatics tools specifically designed for footprinting analysis, which makes it a less tedious, time consumable and overwhelmed experiment [77].

These improvements and updates in DNA footprinting methods allowed for the identification of protein-binding footprints with high resolution on a genome scale. Digital DNase I Analysis is one of these advances, providing a perspective of the genome mapping and quantifying the accessibility of chromatin and the NRs occupancy [78, 79]. The approach is based on techniques of chromatin analysis that have been developed and widely used to detect regulatory regions, which uses data generated from chromatin by DNase I digestion with parallel massive sequencing [78, 80]. In the context of nuclear receptors, the Digital DNase I was applied to map GR accessibility in chromatin regarding genome wide scale [81]. According to this qualitative analysis [82], GR was qualified as an NR capable of autonomous binding to genomic DNA target sites, resulting in local chromatin remodeling, and 95% of these sites were recognized as preexisting accessible sites in chromatin [81].

However, even with the advances in technology, to infer accurately the location of these footprints remains a challenging computational task. To avoid this, the development of dynamic Bayesian network had improved the identification and statistical calculation of protein binding sites from the genome digital footprinting data in a probabilistic framework [83].

Parallel to this, the development of new DNA labelling methodologies, avoiding the radiolabelling, combined with new bioinformatics tools for data processing and, moreover, the development of *in silico* footprinting [84] have greatly aided the use of DNA footprinting, which continues to be a powerful tool to answer many questions, especially about the NR-DNA interaction. On the other hand, it is important to mention that the identification of REs depends on different factors, like cell type and treatment conditions, which makes this process more complicated. Nowadays, the advent of chromatin immunoprecipitation assays made it possible to identify large genomic fragments to which NRs bind directly and indirectly, evaluating cellular contexts [85]. The insertion of this technology on genome wide scale after the advent of Digital DNase I analysis updated this assay, which still may be associated with different methods in the elucidation of more complex questions concerning NR-DNA interactions.

1.5. Transactivation assays—transfection and reporter gene assays. In addition to the techniques mentioned above, a widely used *in vivo* method to measure NR activity is the reporter gene assay. In general, this method is based on inserting a DNA construction inside the cell, which owns an RE followed by a reporter gene. This is a fundamental tool to monitor cellular events associated with gene expression, regulation, and signal transduction. After the development of this assay, researchers have acquired one sensitive, reliable and convenient assay, providing one efficient report of the activation of particular NRs and their effects on gene expression [86, 87].

Aiming to understand the biological role of NRs in gene expression by using this assay, primarily it is important to monitor the protein in the cellular context. In order to achieve this, the first step would be the introduction of the target gene inside the cell; otherwise, it is still possible to consider endogenous levels of NR, which is one of many advantages of this technique [88]. However, when the introduction of interest gene is required, transfection is a widely used procedure and a powerful analytical tool for study gene and protein function and regulation [88]. There are two major transfection types: stable, where gene is integrated to genome, and transient, where a plasmid containing the gene supports expression for short periods of time [89, 90].

In the first one, transgene becomes as dependent as other genes of transcription regulation machinery, which could be considered a disadvantage if compared with the second one, but it is more similar to the cell real conditions. Within the first scenario, in a natural *in vivo* system, transgene may be associated or not with histones in a dense chromatin waiting to be transcripted and translated [35, 36]. In contrast, in the second method, plasmids probably are in a supercoiled shape, but the regulation sequence access is still easier because of the absence of histones and chromatinized DNA [90, 91], with this approach being widely applied for fast transactivation assays. On the other hand, it is an artificial system for cell metabolism. Overall, both transfections types have advantages and disadvantages, and the best choice between them should be done after some questioning about cell toxicity, transfection efficiency, effects on normal physiology, and also reproducibility [88].

The second step for reporter assays is the choice of reporter gene since there are several types of systems available today. Remarkably, some important features have to be considered in the reporter's choice: effectiveness and sensitivity, level of expression, stability of expressed protein, and, background, due to endogenous protein [86, 92].

Historically, chloramphenicol acetyltransferase (CAT) was the first reporter gene to be used. However, it has become obsolete nowadays mostly because of the fast decay of the enzymatic activity [87, 92]. To overcome this, luciferase (LUC), which is more stable than CAT, is widely used. LUC assay is highly sensitive, it requires fewer cells than CAT assay, and its response can be measured within 25 hours after transfection. Nevertheless, it remains a simple, rapid, and sensitive method for NR activity on promoters [93]. Overall, *Renilla* luciferase together with firefly luciferase

reporters is considered the most efficient ones, emitting the highest bioluminescence signal, allowing for the detection of subattomoles amounts of enzyme. Both are excellent markers for gene expression, as they lack posttranslational modifications, have absent endogenous proteins or enzymes, and exhibit fast enzymatic interactions.

Moreover, luciferase reporter gene system can be used for monitoring gene expression *in vivo*. One example was the generation of the ERE-LUC transgenic mice [94], which had the luciferase reporter gene under the control of an estrogen-responsive element (ERE-LUC). In this report the luciferase activity in estrogen cycles indicated that the highest transcriptional activity of ER occurred during proestrus in reproductive tissue [94]. These ERE-LUC model mice also facilitated kinetics, monitoring, and quantitative analysis of ER activity in specific tissues [87].

Later, a novel reporter assay system was developed as an improvement of luciferase reporter gene system. This assay, termed as the tricolor reporter in vitro assay system, consists of the use of green- and red-emitting *Phrixothrix* luciferases, as dual reporters, and of blue-emitting *Renilla* luciferase, as internal control. This system was developed firstly to study the RAR-related orphan receptor alpha (RORα) and was successfully employed to verify the clock effects of gene products on the enhancer elements of Bmal1 and Per1 promoters [95].

As already mentioned, the reporter gene technique is widely applied and may provide data to solve distinct questions. One example was the study highlighting the need to investigate the (anti-) androgenic activity of compounds depending on cellular and promoter context [96]. In this study, the reporter activity of plasmids containing AR response elements derived from the human secretory component (rat probasin gene), as well as, the GREs, was evaluated together with mouse mammary tumor virus promoter [96].

Interestingly, another application of reporter gene method encompasses its use as an adjuvant to corroborate protein structural hypothesis. For example, to understand TR LBD conformation and its conformational changes after ligand binding, experiments of H/D exchange MS were performed and suggested a new regulation step in coactivator recruitment [97]. This hypothesis was tested by site direct mutagenesis and TR transactivation assays, showing that the detected changes in TR conformation are important, influencing the activation of this receptor [97].

The development of a new reporter gene assay to test NR-coregulators interactions inside the cells using a chimeric system was also described as another application of this assay for NR, combining reporter gene assay with two-hybrid mammalian system [98–102]. Additionally, reporter gene assay technique is also capable of searching and understanding novel NRs' interactions with DNA itself, investigating gene regulation by NRs inside promoter regions. Moreover, it is common to use reporter gene along with EMSA and ChIP-chip to discover and validate where transcription factors are binding to [103–105]. As an example, a recent report shows that TM (thrombomodulin) expression and activity were upregulated by FXR activation in vascular endothelial cells [106]. FXR activation significantly enhanced the transcriptional activity of human TM gene promoter, as seen in reporter gene assays, and EMSA and Chip-chip indicated that FXR induced TM expression by binding to a novel FXR-responsive element.

Remarkably, there are a considerable number of studies in the literature searching for selective ER modulators with balanced high affinity for ERα and ERβ, which could act as therapeutics for the treatment of hormone-response breast cancer, osteoporosis, and many other diseases using these reporter gene assays [107–112]. One of them is aimed to discover novel selective ligands for ERβ, through development and characterization of a cell-based Gal4-ERβ β-lactamase reporter gene assay (GERTA) for ligand-induced activation of the human ERβ. This assay was optimized for high-throughput screening, using 3,456-well nanoplate format, and it was successfully used to screen a large compound collection for ERβ agonists [111]. Alternatively, one study focused on the development of second- and third-generation selective ER modulators, with the goal of reducing toxicity and improving tissue-selective efficacy, developing a new cell-based ERα-transactivation assay, where ERα-specific antagonists were screened after only 4h of incubation time, using a fully automated ultrahigh throughput screen, and a number of valuable leads were identified [112].

So far, there are some different methods to understand the activity of NRs in DNA response elements, and novel ones are under development right now. Among all, the most widely used and easiest way to standardize in mammal cells continues to be transactivation using luciferase enzyme system. One of the reasons for this preference is its reproducibility and easy way to perform measurements. However, it is important to mention that LUC activity does not always imply a direct interaction of NR-DNA, and this activity may be a response to an indirect interaction via different partners through crosstalk, for example, which may be confirmed by EMSA or DNA footprinting assay.

Nevertheless, LUC assays are excellent markers for gene expression and, altogether, the discussed characteristics turn luciferase reporter gene assay into the most sophisticated and robust method used nowadays to study nuclear receptor interactions with DNA *in vivo*. Here we show that this assay may be applied to answer a large spectrum of questions, as the analysis of NR activity in different cell contexts, or new promoters; or as the evaluation of interactions with coregulator and new ligands; and, even, to confirm structural hypothesis. Also important, this assay allows for the study of NRs in naked DNA reporters or in chromatinized DNA, according to the chosen transfection method, which,

depending on the objective of the study, may present more realistic results, closer to cellular environment.

1.6. Chromatin immunoprecipitation sequencing (ChIP-Seq). While EMSA and DNA footprinting are mostly used for *in vitro* analyses of NR and DNA interactions, another technique used to investigate this interaction in cellular context, as reporter gene assay, is the chromatin immunoprecipitation-sequencing, or ChIP-S Nowadays, as far as we know, this is one of the most used assays for investigation of NRs-DNA binding.

ChIP-Seq is applied to map NR binding sites in genomic-wide scale throughout the DNA, without significant cellular modification [113], and to map chromatin modifications and nucleosome positions [114]. The method is an evolution of ChIP [115] and ChIP-chip procedures, which initially were based on applications of specific primers and DNA microarrays, towards next generation DNA sequencing (NGS), allowing for the study through the whole genome [116]. The first ChIP-Seq application mapped histone modifications in the entire genome, identifying DNA binding sites, and the location of different histone methylation patterns in lysine and arginine residues, in human CD4CT cells [117].

ChIP-Seq and ChIP-chip are currently the two main competing technologies for the genome-wide identification of chromatin immunoprecipitated material [118]. The general principle of ChIP is immunoprecipitation of specific proteins cross-linked together with their associated DNA. In classical protocols DNA-proteins complexes from cells extracts are cross-linked and the chromatin is fragmented by sonication. During basic ChIP procedure, after DNA purification, a PCR with specific primers for known DNA sequences is performed [115]. In the ChIP-chip procedure, purified DNA is applied in a microarray plate, which allows for the positive recognition of several, but limited, known DNA sequences. Differently, in ChIP-Seq procedures, the purified DNA is submitted to the NGS and most of the binding sites are identified.

When compared with the other two ChIP methodologies, ChIP-Seq is the one that provides a large amount of results. In a comparison between ChIP-chip and ChIP-Seq, the latter has higher signal-to-noise ratios, is less expensive, and requires lower amounts of DNA for genome analysis [113]. Also, the sequencing step did not limit ChIP-Seq to the few binding sites, allowing for analysis of the whole genome [119]. Therefore, based on ChIP-Seq advantages, as it is largely used nowadays in NRs field, our review will focus on this ChIP method.

The importance of ChIP-Seq assay is totally related to understanding how a huge number of transcriptional factors in living cells can interact with some specific DNA sequences, improving our knowledge about the functional sites. Several studies show ChIP-Seq experiments applied in the search of NRs binding sites in DNA, using distinct cell types. For example, ChIP-Seq together with ChIP found 8,848 GR binding sites in mouse adipocytes treated with synthetic glucocorticoid dexamethasone [84]. Also in adipocytes, more than 5,000 PPARγ binding sites were isolated [120]. In human lung adenocarcinoma cells, more than 4,000 GR binding sites were identified [121], while in human breast cancer cell lines more than 10,000 ER binding sites [122] and 20,000,000 PPARβ/δ binding sites were found [123].

Importantly, we observe in the abovementioned examples that the diversity of cell types and experimental conditions results in data variability in ChIP-Seq, being impossible to define regular guidelines appropriate to a more general rule [124]. Although, taking together the ChIP-Seq results from distinct NRs and cell lines, the identification of overlapping or unique DNA binding sites can lead to new therapeutic strategies [125].

ChIP-Seq also has been extensively used to map the *in vivo* genome-wide binding (cistrome) of NRs in both normal and cancer cells due to evidences that NRs play a differential role in cancer cells [126]. ChIP-seq assays have confirmed these evidences as presented in studies of AR in prostate cancer cell [127, 128] and of ER [129–131], GR [132], and PPARβ [133] in breast cancer cells. In these investigations authors found new insights into the DNA sequences, in which ones NRs can bind and identify cooperating transcription factors. Also, they identified potential NRs regulated genes that are not seen in normal cells, providing enlightenment into the biological processes regulated by them. This kind of application may elucidate meta-analysis data of the same cancer cell line and generate consensus cistrome and expression profiles, which can be used to understand the pathologies and guide new therapeutics developments for cancer treatment [125].

As therapeutics targeting for NRs are mainly ligands, ChIP-Seq may also be applied to identify and characterize NRs behavior in presence and absence of them. As an example, it was observed that triiodothyronine hormone (T3) treatment altered TRβ1 binding at distinct genomic sites and also change expression patterns, suggesting a new mechanism of regulation of target genes by TRβ1 [134]. Another recent development of ChIP-Seq allowed performing this assay with a low input of cells, as it was showed in ERα ChIP-Seq that was successfully performed with an input of only 5,000 cells by using single tube linear amplification (LinDA) [135].

Besides the large amount of examples of ChIP-Seq, it is important to highlight the difficulty in finding specific antibodies for NRs. Essentially, the success of the experiment depends on validations and use of highly specific antibodies [124]. An example showing the efforts in validation of specific antibodies could be seen in the case of LXR genome-wide mapping of binding sites studies [136]. One alternative to solve this issue may be outlined with a tag-based approach, by using transgenesis to express NRs tagged with an epitope or a tag as EGFP. By this way, the only required antibody would be the one specific to the tag. This approach allows for

the use of just one antibody to study several proteins [137], as it was shown in ChIP-Seq study of 24 NRs expressed in breast cancer cell lines [125].

Another important point of ChIP-Seq application for NRs is the huge amount of data generated by NGS and the requirement of both bioinformatics and statistics tools to process the data and turn them into understandable results. Moreover, big amounts of data generated by ChIP-Seq studies are available on databanks and can be analyzed in parallel. The development of novel bioinformatics analyses can compare some patterns of REs, distance distributions of transcription start sites of known genes, evolutionary conservations, and collaborating partners of several NRs [126], also generating consensus cistromes and expression profiles among NRs.

However, albeit the binding sites can be predicted by *in silico* studies, this kind of prediction still did not achieve sufficient success or accuracy, qualifying ChIP-Seq as one technique capable of performing an effective detection of NRs genome-wide *in vivo* binding sites or cistromes [126]. As an example, although some computational analyses determined a potential of 105 – 106 binding sites to VDR responsive elements- (VDRE-) like sequence motifs, ChIP-Seq showed that less than 1000 sites were generally occupied by the VDR in the absence of ligand and between 2000 and 8000 sites were occupied following vitamin D treatment [116].

In another recent example, the comparison between ChIP and ChIP-Seq assays of human and mice had demonstrated a common feature of NRs in recognizing relatively short AT-rich motifs [124]. In addition, it was verified that NRs bind to introns and distal intergenetic regions far away from transcription start sites [126] and counterpoint the classical statement which suggests REs may be located in the 5' region of the target gene, closely to the core promoter.

Other evident and specific limitations of ChIP-seq are that it only provides information about the NRs binding sites and regions nearby, which may harden the identification of genes that are under particular regulation. Moreover, it has been shown that many NRs binding sites land in distal intergenic regions or introns, according to recent data in the literature. The AR ChIP-seq in prostate cancer cells suggested AR binding in nonpromoter regions and action through chromatin loopings [138]. Despite the difficulties to use ChIP-seq to predict target genes that are not straightforward [126] and as some binding sites are distal from gene promoter; it is difficult to predict what distal NRs binding sites are nonfunctional fortuitous binding sites and what are involved in transcriptional activity through a remote control mechanism [139]. This sort of difficult could be overcome by the application of recently developed methods, such as chromosome conformation capture (3C), which have been performed to observe long-range chromatin interactions between DNA elements engaged in transcriptional regulation [140].

Overall, taking together, the advantages of ChIP-seq include the capacity of whole genome analysis of responsive elements both *in vivo* and *in vitro*, the low input of cells requiring the reliability of results compared with *in silico* predictions, the higher signal to noise ratio, the large amount of results, and the capacity of understand network regulations that will generate new therapeutic approaches. With the development of high-throughput sequencing platforms, like Illumina, Genome Analyzer, and SOLiD, and, with the availability of ChIP-grade antibodies, ChIP-Seq has become one of the most widely used methods for determining *de novo* functional elements in the sequenced genome [113].

1.7. Chromosome conformation capture (3C). The 3C is a technique that investigates chromosomes' organization in a cell's natural state. It was originally developed in 2002 aiming at the identification, location, and mapping physical interactions between genetic elements located throughout the human genome [140]. Basically, it is based on binding proximity to investigate the interaction between any two genomic loci, in the same or different chromosomes, revealing their relative spatial disposition [140]. Knowledge about structural properties and spatial organization of chromosomes is important for the understanding of the regulation of gene expression. A chromosomal region that folds in order to bring an enhancer and associated transcription factors within close proximity of a gene is an example of how chromosomal interactions can influence gene expression, as it was shown firstly in the beta-globin locus [141]. Also, the development of 3C technique enables researchers to investigate this kind of interaction/regulation.

The general 3C procedure comprises the isolation of intact nuclei and fixation to cross-link proteins to other proteins and DNA. The interacting segments will be physically bound via cross-linking and digested with restriction enzymes. Following this step, these bound fragments are subjected to ligation at very low DNA concentration, which favors the ligation of relevant DNA fragments over the random ones. Finally, cross-linking is reversed and individual ligation products are detected and quantified by the polymerase chain reaction (PCR), using locus-specific primers [140, 142].

One of the first applications of 3C in NRs studies investigated ER in breast cancer cells [143]. In this study, it was observed that carbonic anhydrase XII gene, which is widely related to breast cancer, is regulated by estrogen via ERα. Also, applying 3C authors that observed this regulation involves a distal region giving new insights into CA12 regulation mechanism and its strong relationship with ER in breast cancer [143]. In another example, 3C method was used to identify distal chromosomal regions which interact with GR-induced Lipocalin2 (Lcn2) gene [144]. Through these studies, GR activation in the Ciz1-Lcn2 locus by long range interactions was observed, suggesting a relationship between a chromatin looping and gene regulation tissue specific [144].

Moreover, recent studies have found that the distal-binding AR transcription complex, including AR, associated transcription factors, and coactivators, regulates the expression of several AR target genes involved in prostate cancer growth, through chromatin looping. By using a global 3C assay, future studies should address whether such a long-range combinatorial regulation can be generalized to include other AR-dependent genes in the genome [138].

At last, a recent study used 3C to investigate whether PPARγ locus position is changed during cell differentiation over other adipogenic genes. These results allowed the observation that the genome organization is remodeled in response to adipogenic signaling [145].

Besides 3C advantages, such as detecting remote chromatin interactions between DNA elements engaged in transcriptional regulation, to overcome the ChIP-seq limitation and to understand a specific protein-DNA interaction that has a role in gene expression; this method also presents limitations. Some of them are the requirement of prior knowledge of different complexes to choose the best DNA primers [146], which may be limited to detection of one-point or partial sites in genome. In addition, it is incapable of *de novo* detecting genome-wide chromatin interactions, also presenting low signal-to-noise ratios [139].

Due to these limitations, improvements of 3C resulted in new technologies, such as 4C, 5C, and 6C. Circular chromosome conformation capture (4C) was developed to overcome the requests of previous knowledge of the different complexes identity by applying the maternally inherited H19 imprinting control region primer present near to the target sequence, during ligation [146]. Chromosome conformation capture-Carbon Copy (5C) is a high-throughput 3C approach, which employs microarrays or quantitative DNA sequencing using 454-technology as detection methods [147]. Another improvement is the combined 3C-ChIP-Cloning assay (6C) that combines the standard looping approaches previously defined with an immunoprecipitation step to investigate involvement of a specific protein that may mediate long-range chromatin interactions [148]. This merge of ChIP and 3C aims to reduce noisy and increase specificity for chromatin interaction detection; however, new approaches to separate chromatin complexes from nonspecific chromatin fragments are necessary to overcome high levels of false positives [139].

Overall, a common problem found in 3C and its derivatives methods is the frequent random collisions of chromosomal regions to one another, which means that the detection of a product does not always indicate a specific interaction between two regions. Therefore, a specific interaction between two regions is only confirmed when the interaction occurs at a higher frequency than with neighboring DNA. Another disadvantage of these techniques (3C, 4C, 5C, and 6C) is the requirement of a large number of cells, especially in the high-throughput methodologies. Experiments using the 4C technique, for example, routinely process ten million cells for analysis on a single microarray. However, in contrast with 3C and 5C, the 4C method does not require the prior knowledge of both interacting chromosomal regions [142].

Finally, 3C technology now becomes a standard method for studying the relationship between nuclear organization and transcription in the native cellular state. It allows researchers to analyze the folding of chromatin in the native cellular state at a resolution beyond that provided by current microscopy techniques. Moreover, considering the shape of the genome is thought to play an important part in the coordination of transcription and, more specifically that NRs mechanism of action involves REs distant from target genes, 3C emerges as a remarkably method in NRs field. Furthermore, together with ChIP-Seq, 3C technologies are indicated assays to discover new relationship between NRs and DNA or to monitor previously described interactions and, also, to study particularities on genome.

1.8. Fluorescence anisotropy. Apart from the techniques described above, there is also another *in vitro* method largely used to identify and characterize protein-DNA interactions, termed fluorescence polarization (FP), or fluorescence anisotropy (FA). Classically, this sort of experiment is able to measure interactions between molecules in solution, in a quantitative way, informing whether they physically are capable of interacting. More detailed, it is a biophysical measurement, based on the timescale of rotational mobility of biological macromolecules and excited-state lifetime. Essentially, when a molecule is excited by polarized light, the fluorescence emission will be depolarized in relation to the incident light, mainly due to rotation mobility of molecules in solution [149]. For example, fluorescence polarization increases as rotational mobility decreases or, indirectly, as the size of the molecule increases. Therefore, FP is affected by molecular size, viscosity of the medium, and temperature. The FP experimental setup involves a vertical polarizing filter for the exciting monochromatic light, which makes that only the properly oriented molecules in the vertically polarized plane to absorb light and become excited. The emitted light from these excited molecules is then measured in both the horizontal and vertical planes. In this sort of measurements the calculated parameters are polarization (P) and anisotropy (r), obtained from the equations below:

$$\begin{aligned} P &= \frac{I_{VV}-I_{VH}}{I_{VV}+I_{VH}} \\ r &= \frac{I_{VV}-I_{VH}}{I_{VV}+2I_{VH}}, \end{aligned} \tag{1}$$

where I_{VV} is the fluorescence emission intensity measured in the plane parallel to the plane of vertically polarized excitation and I_{VH} is the fluorescence emission intensity, measured in the plane perpendicular to the plane of vertically polarized excitation [149].

Furthermore, applying Hill approach to fit the anisotropy data, as showed in the following equation, one can determine

dissociation constant (K_d) and Hill cooperativity coefficient (n) [150, 151]:

$$r_{obs} = ri + \left(r_f - r_i\right)^* \left\{ \frac{\left[\frac{NR+n}{k^n}\right]}{\left[1 + \left(\frac{NR^n}{k^n}\right)\right]} \right\}, \qquad (2)$$

where r_{obs} is the observed anisotropy at total protein concentration NR, r_i and r_f are the lower and upper anisotropy values, k is the K_d value, and n is the Hill cooperativity coefficient. There are other plots which could be applied to fit anisotropy data; however, it was observed [151–154] for the majority of NR-DNA interactions that the occupancy of some binding sites may affect the affinity for the unfilled ones. Therefore the Hill approach, which accounts for the possibility that not all receptor sites are independent (cooperativity), may be properly applied in these systems.

Many examples of fluorescence anisotropy application in studies of DNA-NRs interactions can be found since the beginning of these studies until nowadays [155–158]. One of them applied fluorescence anisotropy assays to verify the interaction of RAR: RXR heterodimer with DNA in the presence of ligands was also investigated by FP [159]. In this study presence or absence of both agonists (retinoic acid and 9-*cis* retinoic acid) did not influence the heterodimer affinity to DNA. However, when they tested a number of other antagonists, it was noticed that the DNA binding was destabilized directly or by destabilizing the heterodimer [159]. Later, the TR DBD homodimerization was also investigated with the same assay, leading to the conclusion that the DBD is also responsible for TR dimerization [160].

Specificity is another variable investigated by FP, as it was reported in an affinity study of ER by different ERE. Firstly, estradiol (E2) roles and salt dependence in ER-ERE binding were studied, presenting almost the same binding affinity in presence or absence of E2, suggesting ER-DNA binding is E2 independent. Moreover, the more complete is protein constructs, it is more able to distinguish between the two different DNA sequences, allowing for the conclusion that other regions of the protein, besides DBD, are important in ERE binding and specificity [161].

Following the same line, other detailed report aiming at deciphering whether minimal TR domains are capable to distinguish among different DNA sequences was performed [154]. In this study, FP assays investigated the affinity of three different constructs of TR (containing just Helix 1, or just DBD, or both DBD and LBD) to four different arrays of AGGTCA (PAL0, IP-6, DR-4, and DR-1) [154]. Based on this study, we found firstly that TR binds to DNA as dimer. We verify that only a small peptide derived from the DBD (Helix 1) is sufficient for recognition of the DNA, the entire DBD is sufficient to bind with high affinity to F2, PAL, and DR-4, but the highest specificity was achieved when LBD is present in the protein, defining differences in Kds, in low nM range [154]. These results confirm that more complete NRs better distinguish among different REs, indicating that other domains may be important for selectivity of NRs in DNA binding, as it was shown for ER [161].

Apart from this, some interactions of NR-protein and NR-Protein-DNA also were defined by fluorescence anisotropy, as it was shown in a study involving ER, transcription intermediary factor 1-alpha (hTIF1α), and ERE binding [162]. A trial of anisotropy of ER interacting with the ERE sequence was performed, and the interaction of this complex with ER-DNA-hTIF1α was verified. It was found that ER interacts with the hTIF1α bound to DNA in hormone dependent manner and that, especially in the absence of E2, the hTIF1α interacts better with ERβ than with ERα, with DNA being requested in ER-hTIF1α binding.

With similar purposes, in 2010, we reported a fluorescence anisotropy study of binding affinities of TR and GATA2 on TSHβ promoter, to postulate a model of interaction where in absence of ligand (T3) thyroid hormone receptor binds to its TRE, while GATA2 binds to GATA-RE. However, in presence of T3, TR-TRE bond is weakened, facilitating the interaction of TR with GATA2 zinc finger domain, which, in turns, binds to GATA-RE [153].

Interestingly, FP studies have shown particularities in the measured systems, like the addition of ligands and ions, which may change affinities of some NRs to specific DNA arrays, helping to elucidate mechanisms of actions of NRs in DNA [153, 154, 161]. As a memorable technique, fluorescence anisotropy has been widely applied for testing interactions between different molecules. Among several advantages presented by FP, this assay is also very well employed in high-throughput screening assays [163]. Similar technologies for large-scale identification of pharmaceutical compounds and environmental have been currently presented [164, 165], whose compounds that alter the ability of ER to bind to DNA are searched, since this interaction is known as a good target for cancer treatment [165].

There are no doubts that the application of fluorescence anisotropy in studies of DNA-protein interactions may present several advantages over other standard methods of assessing these interactions. On the other hand, it is clear that this sort of experiment may present disadvantages, like the need for purified protein and labeled samples, which is quite laborious, but it is feasible for different sorts of proteins, such as NRs. In addition this is an artificial *in vitro* assay that does not consider physiological conditions, like the cell environment. Apart from this, FP is a quantitative technique that provides definition of thermodynamic parameters for biological systems and allows for inferring about physical conditions for biomolecules interaction systems. The measurements are performed in solution and in the binding reaction equilibrium, opposed to methods such as EMSA and DNA footprinting, which involves separation of free and bound ligand, disturbing this equilibrium. Also, in contrast with DNA footprinting and EMSA, no hazardous radioactive waste is generated. FP has

Table 1: Advantages and disadvantages of techniques on studies of NR-DNA interaction.

Technique	Application	Advantages	Disadvantages
EMSA	Investigation of interaction between NR and specific DNA sequences.	• Simple technique • Rapid and Sensitive	• Radioactive labeling • Rapid dissociation of complexes
DNA footprinting	Identify interactions with DNA.	• Whole genome analysis • *in vitro*	• Radioactive labelling • X-rays films
Reporter gene	Quantitative measure of NR activation/repression.	• *In vivo* • Easy to standardize	• Expensive • NR could act indirectly
Chip-seq	Identification of DNA binding sites.	• Whole genome analysis • *in vitro* and *in vivo*	• Require specific antibody • Huge amount of data
3C	Identification and mapping NR-DNA physical interactions	• Detection of remote chromatin interactions in native cellular state	• Require prior knowledge • Low signal-to-noise ratios
Fluorescence anisotropy	Quantitative measurement of affinity of interaction of NR-DNA.	• Quantitative fast	• Require pure protein • Fluorescent labeling

a lower limit of detection, in subnanomolar range; therefore, low quantity of sample is needed. Also, all components are in solution, requiring relatively simple instrumentation, which makes this method more applicable than calorimetry. Moreover, this technique is generally applicable and does not need molecules with dissimilar size to measure equilibrium binding, allowing for real-time measurements for kinetic assays. Several conditions of buffer, ionic strength, and others can be tested rapidly [166] and it is feasible to test more physiological like systems, such as the NRs binding to reconstituted chromatin.

1.9. Overview of discussed methods to detect NR-DNA interactions. This minireview aimed to discuss some of the most common techniques used to study DNA-protein interactions, as summarized in Table 1. Although other methods, such calorimetry and surface plasmon resonance, may also be applied to study these interactions [167–173], the techniques discussed above have been used to elucidate details of NRs-DNA interactions since they were first characterized. Here we present applicability, advantages, and disadvantages of such methods, discussing some details and providing evidences found in literature, aiming to help choosing the best one to perform NR experiments.

Among all the discussed assays, DNA footprinting and EMSA were largely used in the beginning of the investigations regarding interactions between NR and DNA. However, the development of new technologies and instruments allowed the outbreak of modern techniques, and their applications have been decreasing. This is also due to the use of radioactive reagents, which is an inconvenient of these two techniques, and due to the fact that these experiments have been considered quite laborious. Apart from these inconveniences, both techniques are very informative and provide specific information of DNA binding, especially for NR. While EMSA is applied to verify NR binding to specific DNA sequences *in vitro*, DNA footprinting provides information on which sequences of NRs are bound to, taking into account cellular context. As already discussed, the development of fluorescent and chemiluminescent probes makes easier the manipulation of samples for both assays, avoiding radioactivity. Specifically to DNA footprinting, the advent of Digital DNase I Analysis increased its usability, facilitating analysis of huge amount of data.

Parallel to this, ChIP-seq and transactivation assays are applied to cell systems, abundantly investigating NR-DNA interactions. Transfection/reporter gene assays are used since the beginning of this sort of investigation, suffering constantly improvements. In other words, it seems that this technique will continue to be applied for many years as one way to measure activity of NRs. Despite this assay not even being applied in most physiological conditions, due to the fact that many times the RE and the reporter gene are artificially inserted in the cell environment, this is still the best way to verify NRs behavior. Remarkable, ChIP-seq starts to be applied to NR-DNA interaction investigation more recently, being developed from ChIP and ChIP-chip assays. Nowadays, it is one of the most used techniques to investigate the binding sites of NRs in chromatin context, allowing for the identification of roles in gene regulation by NRs. However, as discussed before, this assay also presents some limitations, as many reports have shown that NRs bind to distal sites from gene promoters, making it difficult to predict NR transcriptional activity through a remote control mechanism. In this context, the development of 3C technique increased the possibilities of observation of long-range chromatin interactions between DNA elements engaged in transcriptional regulation by NRs. Also, combining both methodologies applied to NRs (6C) could result in a huge advance to a better understanding of transcriptional regulation mediated by NRs.

Finally, fluorescence polarization might be considered an outside point in this review, as it is a more biophysical and *in vitro* technique, which may be judged as artificial. But it is important to consider that this has been used in NRs

research for many years, providing valuable information as some thermodynamic parameters, quantifying affinities for different DNA sequences, and also providing some clues about selectivity. Regarding all its advantages discussed above and its application to HTS systems, the FP makes investigation faster and may be combined with other cell assays like ChIP-seq and transactivation assays. Together with the abovementioned techniques, FP added to other structural methods (such as NMR and X-ray crystallography), which are very important and useful in studying NR-DNA interaction, may broaden horizons in the best understanding of RN-DNA interactions, answering important questions about gene regulation and transcriptional networks regulated by NRs.

2. Conclusion

This review aimed to discuss different methodologies that may be applied to the study of nuclear receptors and their interactions with DNA. Apart from all the particularities of this superfamily of proteins, we presented different methodological aspects of each technique that can be applied to NRs, aiming at better understanding different aspects of its interaction with DNA, which could improve overall knowledge of some of its roles in transcriptional regulation.

Acknowledgments

The authors thank FAPESP for financial support (Grant no. 2013/08743-2). They also acknowledge T. M .A. Moreira for reviewing this manuscript.

References

[1] A. Aranda and A. Pascual, Nuclear hormone receptors and gene expression, *Physiological Reviews*, **81**, no. 3, 1269–1304, (2001).

[2] P. Huang, V. Chandra, and F. Rastinejad, Structural overview of the nuclear receptor superfamily: insights into physiology and therapeutics, *Annual Review of Physiology*, **72**, 247–272, (2009).

[3] J. C. Nwachukwu and K. W. Nettles, The nuclear receptor signalling scaffold: insights from full-length structures, *The EMBO Journal*, **31**, no. 2, 251–253, (2012).

[4] C. Helsen, S. Kerkhofs, L. Clinckemalie, L. Spans, M. Laurent, S. Boonen, D. Vanderschueren, and F. Claessens, Structural basis for nuclear hormone receptor DNA binding, *Molecular and Cellular Endocrinology*, **348**, no. 2, 411–417, (2012).

[5] D. J. Mangelsdorf, C. Thummel, M. Beato, P. Herrlich, G. Schütz, K. Umesono, B. Blumberg, P. Kastner, M. Mark, P. Chambon, and R. M. Evans, The nuclear receptor super-family: the second decade, *Cell*, **83**, no. 6, 835–839, (1995).

[6] K. De Bosscher, W. V. Berghe, and G. Haegeman, Cross-talk between nuclear receptors and nuclear factor κB, *Oncogene*, **25**, no. 51, 6868–6886, (2006).

[7] G. M. Santos, L. Fairall, and J. W. R. Schwabe, Negative regulation by nuclear receptors: a plethora of mechanisms, *Trends in Endocrinology and Metabolism*, **22**, no. 3, 87–93, (2011).

[8] G. Pascual, A. L. Fong, S. Ogawa, A. Gamliel, A. C. Li, V. Perissi, D. W. Rose, T. M. Willson, M. G. Rosenfeld, and C. K. Glass, A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-γ, *Nature*, **437**, no. 7059, 759–763, (2005).

[9] E. V. Jensen, On the mechanism of estrogen action, *Perspectives in Biology and Medicine*, **6**, 47–59, (1962).

[10] R. M. Evans, The steroid and thyroid hormone receptor superfamily, *Science*, **240**, no. 4854, 889–895, (1988).

[11] W. B. O'Malley, Mechanisms of action of steroid hormones, *The New England Journal of Medicine*, **284**, 370–377, (1971).

[12] K. R. Yamamoto, Steroid receptor regulated transcription of specific genes and gene networks, *Annual Review of Genetics*, **19**, 209–252, (1985).

[13] J. P. Overington, B. Al-Lazikani, and A. L. Hopkins, How many drug targets are there? *Nature Reviews Drug Discovery*, **5**, no. 12, 993–996, (2006).

[14] A. L. Hopkins and C. R. Groom, The druggable genome, *Nature Reviews Drug Discovery*, **1**, no. 9, 727–730, (2002).

[15] S. Mandrekar-Colucci and G. E. Landreth, Nuclear receptors as therapeutic targets for Alzheimer's disease, *Expert Opinion on Therapeutic Targets*, **15**, no. 9, 1085–1097, (2011).

[16] G. Landreth, Q. Jiang, S. Mandrekar, and M. Heneka, PPARγ agonists as therapeutics for the treatment of Alzheimer's disease, *Neurotherapeutics*, **5**, no. 3, 481–489, (2008).

[17] M. Makishima, Nuclear receptors as targets for drug development: regulation of cholesterol and bile acid metabolism by nuclear receptors, *Journal of Pharmacological Sciences*, **97**, 177–183, (2005).

[18] D. Cotnoir-White, D. Laperrière, and S. Mader, Evolution of the repertoire of nuclear receptor binding sites in genomes, *Molecular and Cellular Endocrinology*, **334**, no. 1-2, 76–82, (2011).

[19] T. Siggers and R. Gordan, Protein. DNA binding: complexities and multi-protein codes, *Nucleic Acids Research*, 1–13, (2013).

[20] R. Rohs, S. M. West, A. Sosinsky, P. Liu, R. S. Mann, and B. Honig, The role of DNA shape in protein-DNA recognition, *Nature*, **461**, no. 7268, 1248–1253, (2009).

[21] N. C. Seeman, J. M. Rosenberg, and A. Rich, Sequence specific recognition of double helical nucleic acids by proteins, *Proceedings of the National Academy of Sciences of the United States of America*, **73**, no. 3, 804–808, (1976).

[22] J. W. R. Schwabe, D. Neuhaus, and D. Rhodes, Solution structure of the DNA-binding domain of the oestrogen receptor, *Nature*, **348**, no. 6300, 458–461, (1990).

[23] B. F. Luisi, W. X. Xu, Z. Otwinowski, L. P. Freedman, K. R. Yamamoto, and P. B. Sigler, Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA, *Nature*, **352**, no. 6335, 497–505, (1991).

[24] Y. Brélivet, N. Rochel, and D. Moras, Structural analysis of nuclear receptors: from isolated domains to integral proteins, *Molecular and Cellular Endocrinology*, **348**, no. 2, 466–473, (2012).

[25] S. A. Chasse and F. Rastinejad, Physical structure of nuclear receptor-DNA complexes, *Methods in Molecular Biology*, **176**, 91–103, (2001).

[26] F. Rastinejad, P. Huang, V. Chandra, and S. Khorasanizadeh, Understanding nuclear receptor form and function using structural biology, *Journal of Molecular Endocrinology*, **51**, T1–T21, (2013).

[27] P. Germain, L. Altucci, W. Bourguet, C. Rochette-Egly, and H. Gronemeyer, Nuclear receptor superfamily: principles of signaling, *Pure and Applied Chemistry*, **75**, no. 11-12, 1619–1664, (2003).

[28] S. Khorasanizadeh and F. Rastinejad, Nuclear-receptor interactions on DNA-response elements, *Trends in Biochemical Sciences*, **26**, no. 6, 384–390, (2001).

[29] M. Pawlak, P. Lefebvre, and B. Staels, General molecular biology and architecture of nuclear receptors, *Current Topics in Medicinal Chemistry*, **12**, no. 6, 486–504, (2012).

[30] B. O'Hara, D. A. de la Rosa, and V. M. Rajendran, Multiple mineralocorticoid response elements localized in different introns regulate intermediate conductance K+ (Kcnn4) channel expression in the rat distal colon, *PLoS ONE*, **9**, Article ID e98695, (2014).

[31] J. S. Carroll, C. A. Meyer, J. Song, W. Li, T. R. Geistlinger, J. Eeckhoute, A. S. Brodsky, E. K. Keeton, K. C. Fertuck, G. F. Hall, Q. Wang, S. Bekiranov, V. Sementchenko, E. A. Fox, P. A. Silver, T. R. Gingeras, X. S. Liu, and M. Brown, Genome-wide analysis of estrogen receptor binding sites, *Nature Genetics*, **38**, no. 11, 1289–1297, (2006).

[32] S. Kato, H. Sasaki, M. Suzawa, S. Masushige, L. Tora, P. Chambon, and H. Gronemeyer, Widely spaced, directly repeated PuGGTCA elements act as promiscuous enhancers for different classes of nuclear receptors, *Molecular and Cellular Biology*, **15**, no. 11, 5858–5867, (1995).

[33] F. Claessens and D. T. Gewirth, DNA recognition by nuclear receptors, *Essays in Biochemistry*, **40**, 59–72, (2004).

[34] T. Q. Phan, M. M. Jow, and M. L. Privalsky, DNA recognition by thyroid hormone and retinoic acid receptors: 3,4,5 rule modified, *Molecular and Cellular Endocrinology*, **319**, no. 1-2, 88–98, (2010).

[35] S. C. Biddie and S. John, Conversing with chromatin: the language of nuclear receptors, *Molecular Endocrinology*, **28**, 3–15, (2014).

[36] M. Kishimoto, R. Fujiki, S. Takezawa, Y. Sasaki, T. Nakamura, K. Yamaoka, H. Kitagawa, and S. Kato, Nuclear receptor mediated gene regulation through chromatin remodeling and histone modifications, *Endocrine Journal*, **53**, no. 2, 157–172, (2006).

[37] X. Zhang and S.-M. Ho, Epigenetics meets endocrinology, *Journal of Molecular Endocrinology*, **46**, no. 1, R11–R32, (2011).

[38] R. M. Gadaleta and L. Magnani, Nuclear receptors and chromatin: an inducible couple, *Journal of Molecular Endocrinology*, **52**, R137–R149, (2014).

[39] M. W. King, The medical biochemistry, http://www.themedicalbiochemistrypage.org.

[40] T. Kouzarides, Chromatin modifications and their function, *Cell*, **128**, no. 4, 693–705, (2007).

[41] K. Luger, A. W. Mäder, R. K. Richmond, D. F. Sargent, and T. J. Richmond, Crystal structure of the nucleosome core particle at 2.8 Å resolution, *Nature*, **389**, no. 6648, 251–260, (1997).

[42] W. Hendrickson, Protein-DNA interactions studied by the gel electrophoresis-DNA binding assay, *BioTechniques*, **3**, no. 3, 198–207, (1985).

[43] A. Revzin, Gel electrophoresis assays for DNA-protein interactions, *BioTechniques*, **7**, no. 4, 346–355, (1989).

[44] I. P. Chernov, S. B. Akopov, L. G. Nikolaev, and E. D. Sverdlov, Identification and mapping of DNA binding proteins target sequences in long genomic regions by two-dimensional EMSA, *BioTechniques*, **41**, no. 1, 91–96, (2006).

[45] M. A. Lazar, T. J. Berrodin, and H. P. Harding, Differential DNA binding by monomeric, homodimeric, and potentially heteromeric forms of the thyroid hormone receptor, *Molecular and Cellular Biology*, **11**, no. 10, 5005–5015, (1991).

[46] R. C. J. Ribeiro, P. J. Kushner, J. W. Apriletti, B. L. West, and J. D. Baxter, Thyroid hormone alters in vitro DNA binding of monomers and dimers of thyroid hormone receptors, *Molecular Endocrinology*, **6**, no. 7, 1142–1152, (1992).

[47] K. Shulemovich, D. D. Dimaculangan, D. Katz, and M. A. Lazar, DNA bending by thyroid hormone receptor: influence of half-site spacing and RXR, *Nucleic Acids Research*, **23**, no. 5, 811–818, (1995).

[48] Y. Wu, Y.-Z. Yang, and R. J. Koenig, Protein-protein interaction domains and the heterodimerization of thyroid hormone receptor variant α2 with retinoid X receptors, *Molecular Endocrinology*, **12**, no. 10, 1542–1550, (1998).

[49] L. F. R. Velasco, M. Togashi, P. G. Walfish, R. P. Pessanha, F. N. Moura, G. B. Barra, P. Nguyen, R. Rebong, C. Yuan, L. A. Simeoni, R. C. J. Ribeiro, J. D. Baxter, P. Webb, and F. A. R. Neves, Thyroid hormone response element organization dictates the composition of active receptor, *Journal of Biological Chemistry*, **282**, no. 17, 12458–12466, (2007).

[50] Y. Chen and M. A. Young, Structure of a thyroid hormone receptor DNA-binding domain homodimer bound to an inverted palindrome DNA response element, *Molecular Endocrinology*, **24**, no. 8, 1650–1664, (2010).

[51] A. C. M. Figueira, S. M. G. Dias, M. A. M. Santos, J. W. Apriletti, J. D. Baxter, P. Webb, F. A. R. Neves, L. A. Simeoni, R. C. J. Ribeiro, and I. Polikarpov, Human thyroid receptor forms tetramers in solution, which dissociate into dimers upon ligand binding, *Cell Biochemistry and Biophysics*, **44**, no. 3, 453–462, (2006).

[52] B. J. Mengeling, S. Lee, and M. L. Privalsky, Coactivator recruitment is enhanced by thyroid hormone receptor trimers, *Molecular and Cellular Endocrinology*, **280**, no. 1-2, 47–62, (2008).

[53] M. Mulero, J. Perroy, C. Federici, G. Cabello, and V. Ollendorff, Analysis of RXR/THR and RXR/PPARG2 heterodimerization by bioluminescence resonance energy transfer (BRET), *PLoS ONE*, **8**, Article ID e84569, (2013).

[54] A. Takeshita, Y. Ozawa, and W. W. Chin, Nuclear receptor coactivators facilitate vitamin D receptor homodimer action on direct repeat hormone response elements, *Endocrinology*, **141**, no. 3, 1281–1284, (2000).

[55] J. R. Schultz-Norton, Y. S. Ziegler, V. S. Likhite, and A. M. Nardulli, Isolation of proteins associated with the DNA-bound estrogen receptor alpha, *Methods in Molecular Biology*, **590**, 209–221, (2009).

[56] J. T. Read, H. Cheng, S. C. Hendy, C. C. Nelson, and P. S. Rennie, Receptor-DNA interactions: EMSA and footprinting, *Methods in Molecular Biology*, **505**, 97–122, (2009).

[57] E. F. Greiner, J. Kirfel, H. Greschik, D. Huang, P. Becker, J. P. Kapfhammer, and R. Schüle, Differential ligand-dependent protein-protein interactions between nuclear receptors and a neuronal-specific cofactor, *Proceedings of the National Academy of Sciences of the United States of America*, **97**, no. 13, 7160–7165, (2000).

[58] J. J. Eloranta, C. Hiller, S. Häusler, B. Stieger, and G. A. Kullak-Ublick, Vitamin D3 and its nuclear receptor increase the expression and activity of the human proton-coupled folate transporter, *Molecular Pharmacology*, **76**, no. 5, 1062–1071, (2009).

[59] L. M. Hellman and M. G. Fried, Electrophoretic mobility shift assay (EMSA) for detecting protein-nucleic acid interactions, *Nature Protocols*, **2**, no. 8, 1849–1861, (2007).

[60] T. E. Wilson, A. R. Mouw, C. A. Weaver, J. Milbrandt, and K. L. Parker, The orphan nuclear receptor NGFI-B regulates expression of the gene encoding steroid 21-hydroxylase, *Molecular and Cellular Biology*, **13**, no. 2, 861–868, (1993).

[61] O. Chumsakul, K. Nakamura, T. Kurata, T. Sakamoto, N. Ogasawara, T. Oshima, and S. Shikawa, High-resolution mapping of in vivo genomic transcription factor binding sites using in situ DNase I footprinting and ChIP-seq, *DNA Research*, **20**, 325–337, (2013).

[62] D. J. Galas and A. Schmitz, DNAase footprinting: a simple method for the detection of protein-DNA binding specificity, *Nucleic Acids Research*, **5**, no. 9, 3157–3170, (1978).

[63] A. Ralston, Do transcription factors actually bind DNA? DNA footprinting and gel shift assays, *Nature Education*, **1**, p. 121, (2008).

[64] A. J. Hampshire, D. A. Rusling, V. J. Broughton-Head, and K. R. Fox, Footprinting: a method for determining the sequence selectivity, affinity and kinetics of DNA-binding ligands, *Methods*, **42**, no. 2, 128–140, (2007).

[65] B. Sclavi, S. Woodson, M. Sullivan, M. R. Chance, and M. Brenowitz, Time-resolved synchrotron X-ray "footprinting", a new approach to the study of nucleic acid structure and function: application to Protein-DNA interactions and RNA folding, *Journal of Molecular Biology*, **266**, no. 1, 144–159, (1997).

[66] P. B. Becker, B. Gloss, W. Schmid, U. Strähle, and G. Schütz, In vivo protein-DNA interactions in a glucocorticoid response element require the presence of the hormone, *Nature*, **324**, no. 6098, 686–688, (1986).

[67] K. Sakurada, M. Ohshima-Sakurada, T. D. Palmer, and F. H. Gage, Nurr1, an orphan nuclear receptor, is a transcriptional activator of endogenous tyrosine hydroxylase in neural progenitor cells derived from the adult brain, *Development*, **126**, no. 18, 4017–4026, (1999).

[68] T. E. Wilson, K. A. Padgett, M. Johnston, and J. Milbrandt, A genetic method for defining DNA-binding domains: application to the nuclear receptor NGFI-B, *Proceedings of the National Academy of Sciences of the United States of America*, **90**, no. 19, 9186–9190, (1993).

[69] Z. S. Ye and H. H. Samuels, Cell- and sequence-specific binding of nuclear proteins to 5′-flanking DNA of the rat growth hormone gene, *Journal of Biological Chemistry*, **262**, no. 13, 6313–6317, (1987).

[70] P. H. J. Riegman, R. J. Vlietstra, J. A. G. M. Van der Korput, A. O. Brinkmann, and J. Trapman, The promoter of the prostate-specific antigen gene contains a functional androgen responsive element, *Molecular Endocrinology*, **5**, no. 12, 1921–1930, (1991).

[71] M. Truss, G. Chalepakis, and M. Beato, Contacts between steroid hormone receptors and thymines in DNA: an interference method, *Proceedings of the National Academy of Sciences of the United States of America*, **87**, no. 18, 7180–7184, (1990).

[72] M. D. Driscoll, C. M. Klinge, R. Hilf, and R. A. Bambara, Footprint analysis of estrogen receptor binding to adjacent estrogen response elements, *Journal of Steroid Biochemistry and Molecular Biology*, **58**, no. 1, 45–61, (1996).

[73] C. M. Klinge, D. L. Bodenner, D. Desai, R. M. Niles, and A. M. Traish, Binding of type II nuclear receptors and estrogen receptor to full and half-site estrogen response elements in vitro, *Nucleic Acids Research*, **25**, no. 10, 1903–1912, (1997).

[74] S. Kasper, P. S. Rennie, N. Bruchovsky, P. C. Sheppard, H. Cheng, L. Lin, R. P. C. Shiu, R. Snoek, and R. J. Matusik, Cooperative binding of androgen receptors to two DNA sequences is required for androgen induction of the probasin gene, *Journal of Biological Chemistry*, **269**, no. 50, 31763–31769, (1994).

[75] C. Yang, D. Zhou, and S. Chen, Modulation of aromatase expression in the breast tissue by ERRα-1 orphan receptor, *Cancer Research*, **58**, no. 24, 5695–5700, (1998).

[76] R. Das, A. Laederach, S. M. Pearlman, D. Herschlag, and R. B. Altman, SAFA: semi-automated footprinting analysis software for high-throughput quantification of nucleic acid footprinting experiments, *RNA*, **11**, no. 3, 344–354, (2005).

[77] J. R. Hesselberth, X. Chen, Z. Zhang, P. J. Sabo, R. Sandstrom, A. P. Reynolds, R. E. Thurman, S. Neph, M. S. Kuehn, W. S. Noble, S. Fields, and J. A. Stamatoyannopoulos, Global mapping of protein-DNA interactions in vivo by digital genomic footprinting, *Nature Methods*, **6**, no. 4, 283–289, (2009).

[78] J. R. Hesselberth, X. Chen, Z. Zhang, P. J. Sabo, R. Sandstrom, A. P. Reynolds, R. E. Thurman, S. Neph, M. S. Kuehn, W. S. Noble, S. Fields, and J. A. Stamatoyannopoulos, Global mapping of protein-DNA interactions in vivo by digital genomic footprinting, *Nature Methods*, **6**, no. 4, 283–289, (2009).

[79] M. Sekimata, M. Pérez-Melgosa, S. A. Miller, A. S. Weinmann, P. J. Sabo, R. Sandstrom, M. O. Dorschner, J. A. Stamatoyannopoulos, and C. B. Wilson, CCCTC-binding factor and the transcription factor T-bet orchestrate T helper 1 cell-specific structure and function at the interferon-γ locus, *Immunity*, **31**, no. 4, 551–564, (2009).

[80] M. O. Dorschner, M. Hawrylycz, R. Humbert, J. C. Wallace, A. Shafer, J. Kawamoto, J. Mack, R. Hall, J. Goldy, P. J. Sabo, A. Kohli, Q. Li, M. McArthur, and J. A. Stamatoyannopoulos, High-throughput localization of functional elements by quantitative chromatin profiling, *Nature Methods*, **1**, no. 3, 219–225, (2004).

[81] S. John, P. J. Sabo, R. E. Thurman, M.-H. Sung, S. C. Biddie, T. A. Johnson, G. L. Hager, and J. A. Stamatoyannopoulos, Chromatin accessibility pre-determines glucocorticoid receptor binding patterns, *Nature Genetics*, **43**, no. 3, 264–268, (2011).

[82] P. Becker, R. Renkawitz, and G. Schütz, Tissue-specific DNaseI hypersensitive sites in the 5′-flanking sequences of the tryptophan oxygenase and the tyrosine aminotransferase genes, *The EMBO Journal*, **3**, no. 9, 2015–2020, (1984).

[83] X. Chen, M. M. Hoffman, J. A. Bilmes, J. R. Hesselberth, and W. S. Noble, A dynamic Bayesian network for identifying protein-binding footprints from single molecule-based sequencing data, *Bioinformatics*, **26**, no. 12, Article ID btq175, i334–i342, (2010).

[84] J. D. Turner, L. P. L. Pelascini, J. A. MacEdo, and C. P. Muller, Highly individual methylation patterns of alternative glucocorticoid receptor promoters suggest individualized epigenetic regulatory mechanisms, *Nucleic Acids Research*, **36**, no. 22, 7207–7218, (2008).

[85] R. De Bruyn, R. Bollen, and F. Claessens, Identification and characterization of androgen response elements, *Methods in Molecular Biology*, **776**, 81–93, (2011).

[86] L. H. Naylor, Reporter gene technology: the future looks bright, *Biochemical Pharmacology*, **58**, no. 5, 749–757, (1999).

[87] T. Jiang, B. Xing, and J. Rao, Recent developments of biological reporter technology for detecting gene expression,

Biotechnology and Genetic Engineering Reviews, **25**, 41–76, (2008).

[88] T. K. Kim and J. H. Eberwine, Mammalian cell transfection: the present and the future, *Analytical and Bioanalytical Chemistry*, **397**, no. 8, 3173–3178, (2010).

[89] F. Recillas-Targa, Multiple strategies for gene transfer, expression, knockdown, and chromatin influence in mammalian cell lines and transgenic animals, *Molecular Biotechnology*, **34**, no. 3, 337–354, (2006).

[90] D. J. Glover, H. J. Lipps, and D. A. Jans, Towards safe, non-viral therapeutic gene expression in humans, *Nature Reviews Genetics*, **6**, no. 4, 299–310, (2005).

[91] D. L. Nelson, A. L. Lehninger, and M. M. Cox, *Lehninger Principles of Biochemistry*, W.H. Freeman, New York, NY, USA, 4th edition, 2005.

[92] I. Bronstein, J. Fortin, P. E. Stanley, G. S. A. B. Stewart, and L. J. Kricka, Chemiluminescent and bioluminescent reporter gene assays, *Analytical Biochemistry*, **219**, no. 2, 169–181, (1994).

[93] T. M. Williams, J. E. Burlein, S. Ogden, L. J. Kricka, and J. A. Kant, Advantages of firefly luciferase as a reporter gene: application to the interleukin-2 gene promoter, *Analytical Biochemistry*, **176**, no. 1, 28–32, (1989).

[94] P. Ciana, M. Raviscioni, P. Mussi, E. Vegeto, I. Que, M. G. Parker, C. Lowik, and A. Maggi, In vivo imaging of transcriptionally active estrogen receptors, *Nature Medicine*, **9**, no. 1, 82–86, (2003).

[95] Y. Nakajima, M. Ikeda, T. Kimura, S. Honma, Y. Ohmiya, and K.-I. Honma, Bidirectional role of orphan nuclear receptor RORα in clock gene transcriptions demonstrated by a novel reporter assay system, *FEBS Letters*, **565**, no. 1–3, 122–126, (2004).

[96] S. Simon and S. O. Mueller, Human reporter gene assays: transcriptional activity of the androgen receptor is modulated by the cellular environment and promoter context, *Toxicology*, **220**, no. 2-3, 90–103, (2006).

[97] A. C. M. Figueira, D. M. Saidemberg, P. C. T. Souza, L. Martínez, T. S. Scanlan, J. D. Baxter, M. S. Skaf, M. S. Palma, P. Webb, and I. Polikarpov, Analysis of agonist and antagonist effects on thyroid hormone receptor conformation by hydrogen/deuterium exchange, *Molecular Endocrinology*, **25**, no. 1, 15–31, (2011).

[98] C. M. Tyree and K. Klausing, The mammalian two-hybrid assay for detection of coactivator-nuclear receptor interactions, *Methods in Molecular Medicine*, **85**, 175–183, (2003).

[99] C. Pan, Y.-P. Liu, Y.-F. Li, J.-X. Hu, J.-P. Zhang, H.-M. Wang, J. Li, and L.-C. Xu, Effects of cypermethrin on the ligand-independent interaction between androgen receptor and steroid receptor coactivator-1, *Toxicology*, **299**, no. 2-3, 160–164, (2012).

[100] Z.-G. Sheng, Y. Tang, Y.-X. Liu, Y. Yuan, B.-Q. Zhao, X.-J. Chao, and B.-Z. Zhu, Low concentrations of bisphenol a suppress thyroid hormone receptor transcription through a nongenomic mechanism, *Toxicology and Applied Pharmacology*, **259**, no. 1, 133–142, (2012).

[101] M. Nakka, I. U. Agoulnik, and N. L. Weigel, Targeted disruption of the p160 coactivator interface of androgen receptor (AR) selectively inhibits AR activity in both androgen-dependent and castration-resistant AR-expressing prostate cancer cells, *International Journal of Biochemistry and Cell Biology*, **45**, no. 4, 763–772, (2013).

[102] L. Nagy, H.-Y. Kao, D. Chakravarti, R. J. Lin, C. A. Hassig, D. E. Ayer, S. L. Schreiber, and R. M. Evans, Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase, *Cell*, **89**, no. 3, 373–380, (1997).

[103] J. Ang, J. Sheng, K. Lai, S. Wei, and X. Gao, Identification of estrogen receptor-related receptor gamma as a direct transcriptional target of angiogenin, *PLoS ONE*, **8**, no. 8, Article ID e71487, (2013).

[104] S. Swami, A. V. Krishnan, L. Peng, J. Lundqvist, and D. Feldman, Transrepression of the estrogen receptor promoter by calcitriol in human breast cancer cells via two negative vitamin D response elements, *Endocrine-Related Cancer*, **20**, no. 4, 565–577, (2013).

[105] F. Fang, Q. Zheng, J. Zhang, B. Dong, S. Zhu, X. Huang, Y. Wang, B. Zhao, S. Li, H. Xiong, J. Chen, N. Wu, S. W. Song, C. Chang, and Y. Yang, Testicular orphan nuclear receptor 4-associated protein 16 promotes non-small cell lung carcinoma by activating estrogen receptor β and blocking testicular orphan nuclear receptor 2, *Oncology Reports*, **29**, no. 1, 297–305, (2013).

[106] X. He, Z. Xu, B. Wang, Y. Zheng, W. Gong, G. Huang, L. Zhang, Y. Li, and F. He, Upregulation of thrombomodulin expression by activation of farnesoid X receptor in vascular endothelial cells, *European Journal of Pharmacology*, **718**, 283–289, (2013).

[107] B. S. Katzenellenbogen and J. A. Katzenellenbogen, Estrogen receptor alpha and estrogen receptor beta: regulation by selective estrogen receptor modulators and importance in breast cancer, *Breast Cancer Research*, **2**, no. 5, 335–344, (2000).

[108] B. R. Henke, T. G. Consler, N. Go, R. L. Hale, D. R. Hohman, S. A. Jones, A. T. Lu, L. B. Moore, J. T. Moore, L. A. Orband-Miller, R. G. Robinett, J. Shearin, P. K. Spearing, E. L. Stewart, P. S. Turnbull, S. L. Weaver, S. P. Williams, G. B. Wisely, and M. H. Lambert, A new series of estrogen receptor modulators that display selectivity for estrogen receptor β, *Journal of Medicinal Chemistry*, **45**, no. 25, 5492–5505, (2002).

[109] M. J. Meyers, J. Sun, K. E. Carlson, G. A. Marriner, B. S. Katzenellenbogen, and J. A. Katzenellenbogen, Estrogen receptor-β potency-selective ligands: structure-activity relationship studies of diarylpropionitriles and their acetylene and polar analogues, *Journal of Medicinal Chemistry*, **44**, no. 24, 4230–4251, (2001).

[110] H. A. Harris, L. M. Albert, Y. Leathurby, M. S. Malamas, R. E. Mewshaw, C. P. Miller, Y. P. Kharode, J. Marzolf, B. S. Komm, R. C. Winneker, D. E. Frail, R. A. Henderson, Y. Zhu, and J. C. Keith Jr., Evaluation of an estrogen receptor-β agonist in animal models of human disease, *Endocrinology*, **144**, 4241–4249, (2003).

[111] N. T. Peekhaus, M. Ferrer, T. Chang, O. Kornienko, J. E. Schneeweis, T. S. Smith, I. Hoffman, L. J. Mitnaul, J. Chin, P. A. Fischer, T. A. Blizzard, E. T. Birzin, W. Chan, J. Inglese, B. Strulovici, S. P. Rohrer, and J. M. Schaeffer, A beta-lactamase-dependent Gal4-estrogen receptor beta transactivation assay for the ultra-high throughput screening of estrogen receptor beta agonists in a 3456-well format, *ASSAY and Drug Development Technologies*, **1**, no. 6, 789–800, (2003).

[112] X. Shi, W. Zheng, J. E. Schneeweis, P. A. Fischer, B. Strulovici, and N. T. Peekhaus, A short-incubation reporter-gene assay for high-throughput screening of estrogen receptor-α antagonists, *ASSAY and Drug Development Technologies*, **3**, no. 4, 393–400, (2005).

[113] D. Raha, M. Hong, and M. Snyder, ChIP-Seq: a method for global identification of regulatory elements in the genome, *Current Protocols in Molecular Biology*, no. 91, 1–14, (2010).

[114] W. Sikora-Wohlfeld, M. Ackermann, E. Christodoulou, K. Singaravelu, and A. Beyer, Assessing computational methods for transcription factor target gene identification based on ChIP-seq data, *PLOS Computational Biology*, **9**, Article ID e1003342, (2013).

[115] M.-H. Kuo and C. D. Allis, In vivo cross-linking and immunoprecipitation for studying dynamic protein:DNA associations in a chromatin environment, *Methods*, **19**, no. 3, 425–433, (1999).

[116] J. Pike and M. Meyer, Fundamentals of vitamin D hormone-regulated gene expression, *The Journal of Steroid Biochemistry and Molecular Biology*, **144, part A**, 5–11, (2014).

[117] A. Barski, S. Cuddapah, K. Cui, T.-Y. Roh, D. E. Schones, Z. Wang, G. Wei, I. Chepelev, and K. Zhao, High-resolution profiling of histone methylations in the human genome, *Cell*, **129**, no. 4, 823–837, (2007).

[118] J. Wu, L. T. Smith, C. Plass, and T. H.-M. Huang, ChIP-chip comes of age for genome-wide functional analysis, *Cancer Research*, **66**, no. 14, 6899–6902, (2006).

[119] B. G. Hoffman and S. J. M. Jones, Genome-wide identification of DNA-protein interactions using chromatin immunoprecipitation coupled with flow cell sequencing, *Journal of Endocrinology*, **201**, no. 1, 1–13, (2009).

[120] R. Nielsen, T. Å. Pedersen, D. Hagenbeek, P. Moulos, R. Siersbæk, E. Megens, S. Denissov, M. Børgesen, K.-J. Francoijs, S. Mandrup, and H. G. Stunnenberg, Genome-wide profiling of PPARγ:RXR and RNA polymerase II occupancy reveals temporal activation of distinct metabolic pathways and changes in RXR dimer composition during adipogenesis, *Genes and Development*, **22**, no. 21, 2953–2967, (2008).

[121] T. E. Reddy, F. Pauli, R. O. Sprouse, N. F. Neff, K. M. Newberry, M. J. Garabedian, and R. M. Myers, Genomic determination of the glucocorticoid response reveals unexpected mechanisms of gene regulation, *Genome Research*, **19**, no. 12, 2163–2171, (2009).

[122] W.-J. Welboren, M. A. Van Driel, E. M. Janssen-Megens, S. J. Van Heeringen, F. C. Sweep, P. N. Span, and H. G. Stunnenberg, ChIP-Seq of ERα and RNA polymerase II defines genes differentially responding to ligands, *The EMBO Journal*, **28**, no. 10, 1418–1428, (2009).

[123] T. Adhikary, D. T. Brandt, K. Kaddatz, J. Stockert, S. Naruhn, W. Meissner, F. Finkernagel, J. Obert, S. Lieber, M. Scharfe, M. Jarek, P. M. Toth, F. Scheer, W. E. Diederich, S. Reinartz, R. Grosse, S. Müller-Brüsselbach, and R. Müller, Inverse PPARβ/δ agonists suppress oncogenic signaling to the ANGPTL4 gene and inhibit cancer cell invasion, *Oncogene*, **32**, 5241–5252, (2013).

[124] T. S. Furey, ChIP-seq and beyond: new and improved methodologies to detect and characterize protein-DNA interactions, *Nature Reviews Genetics*, **13**, no. 12, 840–852, (2012).

[125] R. Kittler, J. Zhou, S. Hua, L. Ma, Y. Liu, E. Pendleton, C. Cheng, M. Gerstein, and K. P. White, A comprehensive nuclear receptor network for breast cancer cells, *Cell Reports*, **3**, no. 2, 538–551, (2013).

[126] Q. Tang, Y. Chen, C. Meyer, T. Geistlinger, M. Lupien, Q. Wang, T. Liu, Y. Zhang, M. Brown, and X. S. Liu, A comprehensive view of nuclear receptor cancer cistromes, *Cancer Research*, **71**, no. 22, 6940–6947, (2011).

[127] C. E. Massie and I. G. Mills, Global identification of androgen response elements, *Methods in Molecular Biology*, **776**, 255–273, (2011).

[128] K. R. Chng and E. Cheung, Sequencing the transcriptional network of androgen receptor in prostate cancer, *Cancer Letters*, **340**, 254–360, (2012).

[129] Q. Li, H. Wang, L. Yu, J. Zhou, J. Chen, X. Zhang, L. Chen, Y. Gao, and Q. Li, ChIP-seq predicted estrogen receptor biding sites in human breast cancer cell line MCF7, *Tumor Biology*, **35**, 4779–4784, (2014).

[130] W. Zwart, R. Koornstra, J. Wesseling, E. Rutgers, S. Linn, and J. S. Carroll, A carrier-assisted ChIP-seq method for estrogen receptor-chromatin interactions from breast cancer core needle biopsy samples, *BMC Genomics*, **14**, no. 1, article no. 232, (2013).

[131] M. Ding, H. Wang, J. Chen, B. Shen, and Z. Xu, Identification and functional annotation of genome-wide ER-regulated genes in breast cancer based on ChIP-Seq data, *Computational and Mathematical Methods in Medicine*, **2012**, Article ID 568950, 10 pages, (2012).

[132] D. Pan, M. Kocherginsky, and S. D. Conzen, Activation of the glucocorticoid receptor is associated with poor prognosis in estrogen receptor-negative breast cancer, *Cancer Research*, **71**, no. 20, 6360–6370, (2011).

[133] T. Adhikary, D. T. Brandt, K. Kaddatz, J. Stockert, S. Naruhn, W. Meissner, F. Finkernagel, J. Obert, S. Lieber, M. Scharfe, M. Jarek, P. M. Toth, F. Scheer, W. E. Diederich, S. Reinartz, R. Grosse, S. Müller-Brüsselbach, and R. Müller, Inverse PPARβ/δ agonists suppress oncogenic signaling to the ANGPTL4 gene and inhibit cancer cell invasion, *Oncogene*, **32**, 5241–5252, (2013).

[134] P. Ramadoss, B. Abraham, L. Tsai, Y. Zhou, R. Costa-E-Sousa, F. Ye, M. Bilban, K. Zhao, and A. Hollenberg, Novel mechanism of positive versus negative regulation by thyroid hormone receptor β1 (TRβ1) Identified by genome-wide profiling of binding sites in mouse liver, *Journal of Biological Chemistry*, **289**, 1313–1328, (2014).

[135] P. Shankaranarayanan, M.-A. Mendoza-Parra, W. Van Gool, L. M. Trindade, and H. Gronemeyer, Single-tube linear DNA amplification for genome-wide studies using a few thousand cells, *Nature Protocols*, **7**, no. 2, 328–339, (2012).

[136] M. Boergesen, T. Å. Pedersen, B. Gross, S. J. van Heeringen, D. Hagenbeek, C. Bindesbøll, S. Caron, F. Lalloyer, K. R. Steffensen, H. I. Nebb, J.-A. Gustafsson, H. G. Stunnenberg, B. Staels, and S. Mandrup, Genome-wide profiling of liver X receptor, retinoid X receptor, and peroxisome proliferator-activated receptor α in mouse liver reveals extensive sharing of binding sites, *Molecular and Cellular Biology*, **32**, no. 4, 852–867, (2012).

[137] I. Poser, M. Sarov, J. R. A. Hutchins, J.-K. Hériché, Y. Toyoda, A. Pozniakovsky, D. Weigl, A. Nitzsche, B. Hegemann, A. W. Bird, L. Pelletier, R. Kittler, S. Hua, R. Naumann, M. Augsburg, M. M. Sykora, H. Hofemeister, Y. Zhang, K. Nasmyth, K. P. White, S. Dietzel, K. Mechtler, R. Durbin, A. F. Stewart, J.-M. Peters, F. Buchholz, and A. A. Hyman, BAC TransgeneOmics: a high-throughput method for exploration of protein function in mammals, *Nature Methods*, **5**, 409–415, (2008).

[138] D. Wu, C. Zhang, Y. Shen, K. P. Nephew, and Q. Wang, Androgen receptor-driven chromatin looping in prostate cancer, *Trends in Endocrinology and Metabolism*, **22**, no. 12, 474–480, (2011).

[139] M. J. Fullwood and Y. Ruan, ChIP-based methods for the identification of long-range chromatin interactions, *Journal of Cellular Biochemistry*, **107**, no. 1, 30–39, (2009).

[140] J. Dekker, K. Rippe, M. Dekker, and N. Kleckner, Capturing chromosome conformation, *Science*, **295**, no. 5558, 1306–1311, (2002).

[141] B. Tolhuis, R.-J. Palstra, E. Splinter, F. Grosveld, and W. De Laat, Looping and interaction between hypersensitive sites in the active β-globin locus, *Molecular Cell*, **10**, no. 6, 1453–1465, (2002).

[142] M. Simonis, J. Kooren, and W. de Laat, An evaluation of 3C-based methods to capture DNA interactions, *Nature Methods*, **4**, no. 11, 895–901, (2007).

[143] D. H. Barnett, S. Sheng, T. H. Charn, A. Waheed, W. S. Sly, C.-Y. Lin, E. T. Liu, and B. S. Katzenellenbogen, Estrogen receptor regulation of carbonic anhydrase XII through a distal enhancer in breast cancer, *Cancer Research*, **68**, no. 9, 3505–3515, (2008).

[144] O. Hakim, S. John, J. Q. Ling, S. C. Biddie, A. R. Hoffman, and G. L. Hager, Glucocorticoid receptor activation of the Ciz1-Lcn2 locus by long range interactions, *Journal of Biological Chemistry*, **284**, no. 10, 6048–6052, (2009).

[145] S. E. LeBlanc, Q. Wu, A. R. Barutcu, H. Xiao, Y. Ohkawa, and A. N. Imbalzano, The PPARγ locus makes long-range chromatin interactions with selected tissue-specific gene loci during adipocyte differentiation in a protein kinase A dependent manner, *PLoS ONE*, **9**, 1–12, (2014).

[146] Z. Zhao, G. Tavoosidana, M. Sjölinder, A. Göndör, P. Mariano, S. Wang, C. Kanduri, M. Lezcano, K. S. Sandhu, U. Singh, V. Pant, V. Tiwari, S. Kurukuti, and R. Ohlsson, Circular chromosome conformation capture (4C) uncovers extensive networks of epigenetically regulated intra- and interchromosomal interactions, *Nature Genetics*, **38**, no. 11, 1341–1347, (2006).

[147] J. Dostie, T. A. Richmond, R. A. Arnaout, R. R. Selzer, W. L. Lee, T. A. Honan, E. D. Rubio, A. Krumm, J. Lamb, C. Nusbaum, R. D. Green, and J. Dekker, Chromosome Conformation Capture Carbon Copy (5C): a massively parallel solution for mapping interactions between genomic elements, *Genome Research*, **16**, no. 10, 1299–1309, (2006).

[148] V. K. Tiwari, L. Cope, K. M. McGarvey, J. E. Ohm, and S. B. Baylin, A novel 6C assay uncovers Polycomb-mediated higher order chromatin conformations, *Genome Research*, **18**, no. 7, 1171–1179, (2008).

[149] E. M. Goldys, *Fluorescence Applications in Biotechnology and the Life Sciences*, Wiley-Blackwell, Hoboken, NJ, USA, 2009.

[150] A. V. Hill, The possible effects of the aggregation of the molecules of haemoglobin on its oxygen dissociation curve, *The Journal of Physiology*, **40**, 4–7, (1910).

[151] L. M. T. R. Lima and J. L. Silva, Positive contribution of hydration on DNA binding by E2c protein from papillomavirus, *Journal of Biological Chemistry*, **279**, no. 46, 47968–47974, (2004).

[152] *Fluorescence Polarization Technical Resource Guide*, Invitrogen Co., Madison, Wis, USA, 4th edition, 2006.

[153] A. C. M. Figueira, I. Polikarpov, D. Veprintsev, and G. M. Santos, Dissecting the relation between a nuclear receptor and GATA: binding affinity studies of thyroid hormone receptor and GATA2 on TSHβ promoter, *PloS ONE*, **5**, no. 9, Article ID e12628, (2010).

[154] A. C. M. Figueira, L. M. T. R. Lima, L. H. F. Lima, A. T. Ranzani, G. D. S. Mule, and I. Polikarpov, Recognition by the thyroid hormone receptor of canonical DNA response elements, *Biochemistry*, **49**, no. 5, 893–904, (2010).

[155] S. Wang, C. Zhang, S. K. Nordeen, and D. J. Shapiro, In vitro fluorescence anisotropy analysis of the interaction of full-length SRC1a with estrogen receptors α and β supports an active displacement model for coregulator utilization, *Journal of Biological Chemistry*, **282**, no. 5, 2765–2775, (2007).

[156] C. Zhang, S. K. Nordeen, and D. J. Shapiro, Fluorescence anisotropy microplate assay to investigate the interaction of full-length steroid receptor coactivator-1a with steroid receptors, *Methods in Molecular Biology*, **977**, 339–351, (2013).

[157] V. Pogenberg, J.-F. Guichoul, V. Vivat-Hannah, S. Kammerer, E. Pérez, P. Germain, A. R. De Lera, H. Gronemeyer, C. A. Royer, and W. Bourguet, Characterization of the interaction between retinoic acid receptor/retinoid X receptor (RAR/RXR) heterodimers and transcriptional coactivators through structural and fluorescence anisotropy studies, *Journal of Biological Chemistry*, **280**, no. 2, 1625–1633, (2005).

[158] M. Boyer, N. Poujol, E. Margeat, and C. A. Royer, Quantitative characterization of the interaction between purified human estrogen receptor α and DNA using fluorescence anisotropy, *Nucleic Acids Research*, **28**, no. 13, 2494–2502, (2000).

[159] N. Poujol, E. Margeat, S. Baud, and C. A. Royer, RAR antagonists diminish the level of DNA binding by the RAR/RXR heterodimer, *Biochemistry*, **42**, no. 17, 4918–4925, (2003).

[160] Y. Chen and M. A. Young, Structure of a thyroid hormone receptor DNA-binding domain homodimer bound to an inverted palindrome DNA response element, *Molecular Endocrinology*, **24**, no. 8, 1650–1664, (2010).

[161] M. S. Ozers, J. J. Hill, K. Ervin, J. R. Wood, A. M. Nardulli, C. A. Royer, and J. Gorski, Equilibrium binding of estrogen receptor with DNA using fluorescence anisotropy, *Journal of Biological Chemistry*, **272**, no. 48, 30405–30411, (1997).

[162] S. Thénot, S. Bonnet, A. Boulahtouf, E. Margeat, C. A. Royer, J.-L. Borgna, and V. Cavailles, Effect of ligand and DNA binding on the interaction between human transcription intermediary factor 1α and estrogen receptors, *Molecular Endocrinology*, **13**, no. 12, 2137–2150, (1999).

[163] S. Y. Wang, B. S. Ahn, R. Harris, S. K. Nordeen, and D. J. Shapiro, Fluorescence anisotropy microplate assay for analysis of steroid receptor-DNA interactions, *BioTechniques*, **37**, no. 5, 807–817, (2004).

[164] R. Bolger, T. E. Wiese, K. Ervin, S. Nestich, and W. Checovich, Rapid screening of environmental chemicals for estrogen receptor binding capacity, *Environmental Health Perspectives*, **106**, no. 9, 551–557, (1998).

[165] C. Mao, N. M. Patterson, M. T. Cherian, I. O. Aninye, C. Zhang, J. B. Montoya, J. Cheng, K. S. Putt, P. J. Hergenrother, E. M. Wilson, A. M. Nardulli, S. K. Nordeen, and D. J. Shapiro, A new small molecule inhibitor of estrogen receptor α binding to estrogen response elements blocks estrogen-dependent growth of cancer cells, *Journal of Biological Chemistry*, **283**, no. 19, 12819–12830, (2008).

[166] J. R. Lundblad, M. Laurance, and R. H. Goodman, Fluorescence polarization analysis of protein-DNA and protein-protein interactions, *Molecular Endocrinology*, **10**, no. 6, 607–612, (1996).

[167] B. Cheskis and L. P. Freedman, Modulation of nuclear receptor interactions by ligands: kinetic analysis using surface plasmon resonance, *Biochemistry*, **35**, no. 10, 3309–3318, (1996).

[168] B. J. Cheskis, S. Karathanasis, and C. R. Lyttle, Estrogen receptor ligands modulate its interaction with DNA, *Journal of Biological Chemistry*, **272**, no. 17, 11384–11391, (1997).

[169] J. N. Siew, X. Su, and J. S. Thomsen, Surface plasmon resonance study of cooperative interactions of estrogen receptor α and transcriptional factor Sp1 with composite DNA elements, *Analytical Chemistry*, **81**, no. 9, 3344–3349, (2009).

[170] B. J. Deegan, K. L. Seldeen, C. B. McDonald, V. Bhat, and A. Farooq, Binding of the ERα nuclear receptor to DNA is coupled to proton uptake, *Biochemistry*, **49**, no. 29, 5978–5988, (2010).

[171] L. Y. Low, H. Hernández, C. V. Robinson, R. O'Brien, J. G. Grossmann, J. E. Ladbury, and B. Luisi, Metal-dependent folding and stability of nuclear hormone receptor DNA-binding domains, *Journal of Molecular Biology*, **319**, no. 1, 87–106, (2002).

[172] M. L. Cutress, H. C. Whitaker, I. G. Mills, M. Stewart, and D. E. Neal, Structural basis for the nuclear import of the human androgen receptor, *Journal of Cell Science*, **121**, no. 7, 957–968, (2008).

[173] B.-D. K. Putcha and E. J. Fernandez, Direct interdomain interactions can mediate allosterism in the thyroid receptor, *Journal of Biological Chemistry*, **284**, no. 34, 22517–22524, (2009).