Androgen receptor complexes probe DNA for recognition sequences by short random interactions

Running title: Single-molecule analysis of AR mobility


*equal contribution

1Department of Pathology, Erasmus MC, The Netherlands
2Erasmus Optical Imaging Center, Erasmus MC, The Netherlands
3Physics of Life Processes, Institute of Physics, Leiden University, The Netherlands
4Molecular Cell Biology, Institute of Biology, Leiden University, The Netherlands

Correspondence to:
M. J. M. Schaaf
Institute of Biology, Gorlaeus Laboratory
Einsteinweg 55, 2333CC Leiden
The Netherlands
Tel.: (+31)071-527 4975
E-mail: m.j.m.schaaf@biology.leidenuniv.nl

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Summary

Due to the tremendous progress in microscopic imaging of fluorescently labeled proteins in living cells, the insight into the highly dynamic behavior of transcription factors has rapidly increased over the past decade. However, a consistent quantitative scheme of their action is still lacking. Using the androgen receptor as a model system, we combined three different fluorescence microscopy assays: single-molecule microscopy, photobleaching and correlation spectroscopy, to provide a quantitative model of the action of this transcription factor. This approach enabled us to distinguish two types of AR-DNA binding: very brief interactions, in the order of a few hundred milliseconds, and hormone-induced longer-lasting interactions, with a characteristic binding time of several seconds. In addition, freely mobile ARs were slowed down in the presence of hormone, suggesting the formation of large AR-coregulator complexes in the nucleoplasm upon hormone activation. Our data suggest a model in which mobile, hormone-induced transcription factor-coregulator complexes probe DNA by briefly binding at random sites, and only forming relatively stable transcription initiation complexes when bound to specific recognition sequences.
Introduction

The androgen receptor (AR) is a ligand-activated transcription factor that specifically regulates genes involved in the development and maintenance of the male phenotype, and it also plays a role in the growth of prostate cancer. Like all steroid receptors (SRs), the AR has a modular structure composed of an N-terminal domain, a DNA-binding domain (DBD) and a C-terminal ligand-binding domain. Upon activation by agonistic ligand binding SRs translocate from the cytoplasm to the nucleus where they bind hormone response elements in promoter and enhancer regions of target genes. When bound to the target sequences SRs initiate the recruitment of specific transcriptional coregulators which alter local chromatin structure in order to enhance transcription initiation. Subsequently, the basal transcription machinery is recruited, inducing transcription of target genes (McKenna and O'Malley, 2002).

In the past decade, fluorescent labeling of proteins in living cells and advances in quantitative live microscopy has greatly influenced our view on the organization of nuclear processes. Approaches like fluorescence recovery after photobleaching (FRAP) (Van Royen et al., 2009) and fluorescence correlation spectroscopy (FCS) (Weidtkamp-Peters et al., 2009) have provided novel insight into their mechanism of action. Initial FRAP studies revealed unexpectedly high mobilities and the occurrence of only brief immobilization events for proteins involved in many nuclear processes, including DNA replication (Leonhardt et al., 2000), DNA damage repair (Essers et al., 2002; Houtsmuller et al., 1999), gene transcription (Dundr et al., 2002; Kimura et al., 2002; McNally et al., 2000; Schaaf et al., 2006), and RNA processing (Kruhlak et al., 2000; Phair and Misteli, 2000). A multitude of FRAP studies have shown that SRs share this common behavior. Importantly, the observed transient immobilizations appear to be dependent on ligand activation and DNA binding ability of receptors, suggesting that activated SRs move freely through the nucleus and are chromatin bound for only short time periods (Farla et al., 2004; Farla et al., 2005; Klokk et al., 2007; Marcelli et al., 2006; McNally et al., 2000; Meijsing et al., 2007; Mueller et al., 2008; Rayasam et al., 2005; Schaaf and Cidlowski, 2003; Schaaf et al., 2005; Stenoien et al., 2001; Van Royen et al., 2007).

Using kinetic modeling, a quantitative analysis of FRAP has been performed in several studies (Farla et al., 2005; Hinow et al., 2006; Mueller et al., 2008; Phair et al., 2004; Sprague et al., 2004). However, due to the large number of variables (e.g. number of binding sites, on- and off-rates, relative sizes of free and bound fractions), the variety of analytical
approaches and inaccuracy of FRAP at short time intervals, results of these quantifications have not yet provided a consistent view on transcription factor mobility, and the nature and timing of their interactions with DNA (Van Royen et al., 2011).

To some extent, this problem can be addressed by a complementary approach. FCS has already been applied to SRs in several studies (Jankevics et al., 2005; Mikuni et al., 2007a; Mikuni et al., 2007b), and recently, the group of McNally (Stasevich et al., 2010) cross-validated FRAP and FCS measurements on GR dynamics. Although the findings obtained with the two approaches were relatively consistent, uncertainties due to a number of approximations in the FRAP and FCS analyses call for additional approaches to obtain conclusive knowledge on the nature and dynamics of DNA interaction by nuclear proteins.

The most powerful approach to complement the limitations of both FRAP and FCS is to study protein behavior by single-molecule microscopy (SMM) in living cells. Using a laser-based fluorescence microscopy setup equipped with a high-sensitivity and high-speed CCD camera (Schmidt et al., 1996a), SMM has successfully been applied to proteins fused to autofluorescent proteins like GFP, providing insight into the mobility patterns of several proteins at a time resolution of ~5 ms and a positional accuracy of ~40 nm (de Keijzer et al., 2008; Harms et al., 1999; Iino et al., 2001; Lommerse et al., 2005). Initially, these studies mostly focused on membrane proteins, but in recent years data on three-dimensional mobility of fluorescently labeled proteins in the nuclei of living cells have been extracted using this approach. The intra-nuclear mobility was determined of fluorescently labeled inert proteins like streptavidin (Grunwald et al., 2008) and ovalbumin (Speil and Kubitscheck, 2010), showing that these proteins appear to be immobilized transiently inside the nucleoplasm for ~10-20 ms. The first SMM study on a transcription factor in a living cell was performed on the lac repressor in E. coli cells, in which brief immobilizations (<5 ms) were observed as well (Elf et al., 2007). Recently, the transcription factor STAT1 has been studied by SMM revealing that activated STAT1 diffuses freely through the nucleus and is transiently immobilized, showing residence times of up to 5 s (Speil and Kubitscheck, 2010).

In the present paper, we have combined SMM with FRAP and FCS in order to study the intra-nuclear dynamics of the AR in detail. This combination of techniques provides consistent quantitative data on the mobility pattern of AR in the nucleus. Our results show the occurrence of a freely diffusing fraction and two different binding events, representing sequence-specific and non-specific DNA binding. The combination of these three techniques enables the determination of the relative size of the different fractions, the diffusion coefficient of freely moving molecules and binding residence times.
Results

Analysis of single YFP dynamics in 3D

In order to validate our methods of detection of molecules and analysis of the dynamic behavior in a 3D environment, we first studied the free diffusion of YFP in a 50% glycerol solution. Images of this solution captured using an SMM setup (Schmidt et al., 1996b) showed individual fluorescence intensity peaks (Fig.1A) representing single molecules, since they fitted well to a Gaussian distribution with an intensity and width similar to single YFP fluorescence intensity peaks previously observed using an identical setup (Harms et al., 2001; Lommerse et al., 2004; Schaaf et al., 2009). The observed signal to noise ratio defined as the fluorescence intensity of an individual fluorophore divided by the standard deviation of the background signal was ~17, resulting in a positional accuracy of the localization of these individual molecules of ~33 nm (Schmidt et al., 1996a).

Image sequences were acquired using time intervals of 6.25, 12.5, and 25 ms, and protein mobility was analyzed using the Particle Image Correlation Spectroscopy (PICS) analysis method described previously (Fig.1B-D) (Semrau and Schmidt, 2007). The obtained cumulative distribution function of squared displacements \( P_{cum} (l) \) fitted well to a one-population model, and for each time lag used the mean squared displacement (MSD) was calculated using this fit model (Fig.1D). These values were plotted as a function of the time lag, and the resulting curve showed a straight line reflecting free diffusion of YFP molecules in the 50% glycerol solution (Fig.1E) with a diffusion coefficient \( D \) of 7.35 ± 0.99 \( \mu \text{m}^2/\text{s} \).

The determined \( D \) for YFP in 50% glycerol is in the range of the expected value (9.4 \( \mu \text{m}^2/\text{s} \)), which was determined based on the estimated hydrodynamic radius of YFP, using Eq.4 (see Materials and Methods section). Deviations in temperature or glycerol concentration or inhomogeneity of the solution may underlie the difference between the expected and determined value. Subsequently, we studied the dynamics of YFP-labeled histone protein H2B in nuclei of living (Hep3B) cells. Histone proteins are known to be stably bound to DNA and therefore predominantly immobile. Similarly to the data on free YFP, the data on H2B-YFP fitted to a one-population model. The MSD plot showed a straight line with a diffusion coefficient approximately 200-fold lower than that of YFP in 50% glycerol was determined \( (D = 0.040 \pm 0.0023 \ \mu \text{m}^2/\text{s}) \), which probably reflects the slow movement of chromatin in these live nuclei (Fig.1F).
In silico validation of analysis of 3D protein dynamics

Since we image two-dimensional projections of molecules moving in three dimensions, and the thickness of the ‘optical slice’ from which this projection is made is limited, one can argue that molecules ‘escaping’ in the z-direction create a bias in our analysis. To determine the potential limitations of our analysis, we generated data in a series of Monte Carlo simulations and studied whether the optical slice thickness affected the analysis of molecular dynamics (see Figs.S1 and S2 in supplementary material). The results of these in silico experiments showed that our approach is well suited for analysis of 3D dynamics of a single fraction of freely diffusing molecules ($D = 0 - 10 \mu m^2/s$) (Fig.S1). In addition, when an immobile fraction was introduced, the simulations demonstrated that molecular dynamics can still accurately be determined using our approach, when the immobile fraction was determined at the shortest time lag used (6.25 ms) (Fig.S2).

Quantitative analysis of individual AR dynamics

To obtain a detailed description of the dynamic nuclear behavior of ARs of living cells we applied SMM on Hep3B cells stably expressing YFP-labeled ARs treated with synthetic AR agonist metribolone (R1881) or antagonist OH-flutamide (OHF) (Fig.2 and Fig. 3). Unlike the data of YFP and H2B-YFP, the cumulative distribution function $P_{cum}(l)$ of the ARs best fitted a two-population model (Fig.3A). From this the relative fraction size ($\alpha$) and their mean squared displacements (MSD$_1$ and MSD$_2$) were determined and plotted as a function of the time lag.

For wild type AR in presence of R1881 the size of the fast fraction was found to be 46.0 ± 2.9 % (Fig.3B, Table 1). The MSDs of the fast and slow fraction plotted against the time lag fitted to a straight line, indicating free diffusion of both fractions through the nucleus at this time scale (Figs.3C and 3D). The diffusion coefficient of the fast fraction ($D_1$) was calculated to be 1.13 ± 0.09 $\mu m^2/s$ (Fig.3G, Table 1), whereas the diffusion coefficient for the slow fraction ($D_2$) was 0.056 ± 0.003 $\mu m^2/s$ (Fig.3H, Table 1). The latter diffusion coefficient is in the same range as that found for H2B-YFP, strongly suggesting that this fraction of ARs is bound to chromatin (see also Figs.1A and 1C).

To further study the role of AR activity on its mobility, we performed SMM on OHF bound AR. The size of the fast fraction increased dramatically to 88.5 ± 2.8 % of ARs (Fig.3B, Table 1). The diffusion coefficient of this fast fraction was higher than in the presence of R1881 (2.31 ± 0.10 $\mu m^2/s$, Figs.3C and 3G, Table 1), whereas the slow fraction
diffusion coefficient was unchanged and in the same range as chromatin bound H2B (0.063 ± 0.008 μm²/s, Figs.3D and 3H).

To verify that the slow fraction of ARs is a result of DNA binding, an AR was used with a point mutation in the DBD (R585K). This mutant, in which an important amino acid is mutated that is involved in base-specific interaction with AR target sequences in DNA (Shaffer et al., 2004), has been found in a patient with complete androgen insensitivity syndrome (Sultan et al., 1993). In line with these findings, it was shown that this AR mutant is transcriptionally inactive (Lobaccaro et al., 1999) and lacks stable DNA binding (Van Royen et al., 2012). Together, these data suggest that the R585K mutation disrupts specific interaction of ARs with its target sites in the genome. Like wild type AR, the AR R585K mutant showed two fractions of molecules. In the presence of R1881, the fast fraction size of mutant ARs (61.1 ± 6.7 %, Fig.3B) was increased as compared to wild type AR. In addition, both the diffusion coefficient of the fast (1.42 ± 0.08 μm²/s, Figs.3E and 3G, Table 1) and the slow fraction (0.077 ± 0.005 μm²/s, Figs.3F and 3H, Table 1) were only slightly increased. Thus, the results show that the AR R585K mutant dynamics are only slightly changed as compared to the wild type receptor, indicating that the mutant is still able to bind to chromatin for periods within the time scale of the experiment (<50ms).

The presence of the antagonist OHF slightly increased the size of the fast fraction of the mutant AR (79.7 ± 4.0 Fig.3B) in comparison to R1881, but the difference between R1881 and OHF is remarkably smaller than for the wild type receptor (Fig.3B, Table 1). The diffusion coefficient of the slow fraction (0.090 ± 0.013 μm²/s, Figs.3F and 3H) was unaltered, whereas the diffusion coefficient of the fast fraction (2.24 ± 0.19 μm²/s, Figs.3E and 3G, Table 1) was increased. Apparently, the difference between the sizes of the fast fractions of R1881-bound and OHF-bound wild type ARs depends on the ability to bind DNA, whereas the difference in diffusion rate of the fast fraction is not dependent on the DNA-binding capacity of AR.

In summary, the results of our SMM experiments indicated the presence of two AR fractions, that both show free diffusion through the nucleus at the time scale of our experiments. The diffusion coefficient of the slow fraction is approximately 20-fold lower than that of the fast fraction. Antagonist treatment dramatically decreased the size of the slow fraction, and increased the diffusion rate of the fast fraction. Interestingly, a DBD mutation decreased the difference in the size of the fast fraction between agonist- and antagonist-bound ARs, but left the difference in diffusion rate of the fast fraction intact.
Combining SMM analysis of ARs with FCS and FRAP

To verify the parameters obtained by SMM and expand the time scale of measurements on the dynamic behavior of the AR, the cell lines stably expressing YFP-AR and its R585K mutant used in the single-molecule analysis were subjected to both FCS and FRAP (Fig. 4 and 5, respectively). For a good comparison it must be realized that the FCS approach used in this study, in which intensity fluctuations are measured for 20 seconds, is essentially blind for molecules that are immobile for periods in the range of seconds and longer due to photobleaching and the small number of long events in this time frame, and that inaccuracy in FRAP at short time intervals limits the ability to extract diffusion parameters, especially for highly mobile molecules.

Therefore in FCS, only diffusion rates were extracted from the retention times of the YFP-tagged molecules in the diffraction-limited spot, using a two-population free-diffusion triplet-state model (Fig. 4A and Fig. S3 in supplementary material). The FCS data showed for wild type AR a lower diffusion coefficient \( D \) in the presence of R1881 than in the presence of OHF (1.61 ± 0.26 and 2.42 ± 0.37 \( \mu \)m\(^2\)/s respectively; Fig. 4B). Although the absolute diffusion rates are slightly lower, they are in the same range as those found with SMM (1.13 ± 0.09 \( \mu \)m\(^2\)/s and 2.31 ± 0.10 \( \mu \)m\(^2\)/s; Fig. 3G, Table 1). This trend was also observed for the R585K mutant AR, for which the diffusion rates \( D \) determined with FCS are 1.78 ± 0.19 \( \mu \)m\(^2\)/s and 2.64 ± 0.39 \( \mu \)m\(^2\)/s in the presence of R1881 and OHF respectively (Fig. 4B and Fig. S3 in supplementary material), and 1.42 ± 0.08 \( \mu \)m\(^2\)/s and 2.24 ± 0.19 \( \mu \)m\(^2\)/s in SMM (Fig. 3G, Table 1). Thus, the diffusion rates determined by FCS were consistent with the findings from our single-molecule experiments (Table 1).

Subsequently, FRAP experiments were performed, and the resulting FRAP data were fit to curves obtained using computer modeling described previously (e.g. Farla et al., 2005; Van Royen et al., 2009). The large immobile fractions for agonist bound wild type AR found in SMM could not fully be attributed to long immobilization events, but required the inclusion of short immobilizations in the model. Note that in previous reports (Farla et al., 2005) these short immobilizations were explained by slower diffusion, but in combination with the SMM experiments presented here this model is no longer sufficient.

The diffusion coefficients obtained using SMM and FCS were averaged and used as fixed parameters in the FRAP analysis and the curves were fitted to a reaction diffusion model with two immobile fractions, one previously found long interacting fraction (Farla et al., 2005) and one additional fraction of ARs with short interactions with the DNA (Fig. 5 and...
Fig.S4). As SMM does not discriminate between these long and short immobilizations because of the temporal resolution of this technique (<50ms), the sum of the two fractions in the FRAP data corroborates the results from the SMM experiments (Table 1).

FRAP on agonist-bound wild type AR showed a fraction of ARs (28 ± 3 %) with binding time of 8 ± 2 s (Fig.5C and D). FRAP data of antagonist-bound AR and in the AR R585K mutant did not fit well to models that included these immobilization. Thus, only the agonist-bound wild type AR displays stable interactions with chromatin. In contrast, for all agonist and antagonist bound wild type AR and AR R585K a significant fraction of ~30% with sub-second binding times (0.5 - 0.8 s) was found (Fig.5D).

In summary, FCS confirmed that antagonist-bound ARs show a faster diffusion rate in comparison with agonist bound (wild type and mutant) ARs. FRAP showed that only a significant long immobilization was found for agonist bound wild type AR, whereas an additional briefly immobilized fraction is found for all agonist or antagonist bound wild type AR or AR R585K mutant (Table 1).
Discussion

The view on how proteins find their way through the nucleus to identify and bind their target sites in the vast amount of DNA has been subject to intensive discussion (e.g. Erdel et al., 2011; Halford and Marko, 2004; Mueller et al., 2010; Mueller et al., 2008; Phair et al., 2004; Sprague and McNally, 2005; Sprague et al., 2004; Van Royen et al., 2011). In a simple model proteins diffuse freely though the nucleoplasm and find their targets by random collision resulting in binding to specific and non-specific binding sites (Gorski et al., 2006; Halford and Marko, 2004; Hoogstraten et al., 2008; Houtsmuller et al., 1999; McNally et al., 2000; Mueller et al., 2008). However, more sophisticated models based on in vitro experiments using isolated DNA, suggest sliding along the DNA strand (one-dimensional (1D) diffusion) or over the chromatin surface enabling proteins to bypass obstacles (2D diffusion) (Blainey et al., 2009; Gorman et al., 2007; Kampmann, 2004).

Although live cell imaging methods like FRAP and FCS have revealed high mobility of nuclear proteins and the very dynamic nature of their interactions with chromatin, a large variation in quantitative estimates of diffusion rates and DNA-binding kinetics still exists (Van Royen et al., 2011 and references therein). This variation is mostly caused by differences in the choice of analytical methods and by different assessment of experimental parameters regarding microscopic properties such as laser intensity distribution or photophysical properties of fluorescent labels, like blinking or photobleaching (e.g. Houtsmuller, 2005; Mueller et al., 2012). In addition, differences in shape and size of the cell nucleus are often not taken into account in spite of the fact that these may have considerable influence on FRAP recovery curves (as discussed in Houtsmuller, 2005; Mueller et al., 2010; Van Royen et al., 2011). In the present paper we argue that errors caused by such methodological and analytical limitations can be largely eliminated by applying and using the strengths of several complementary approaches.

Therefore, we combined FRAP, FCS and SMM in order to provide a consistent quantitative model of the mobility and DNA interactions of a ligand-dependent transcription factor, the AR. We obtained mobility and interaction data at different time-scales, from milliseconds in FCS, up to tens of milliseconds in single molecule tracking assays, and hundreds of milliseconds to seconds in FRAP. From this we determined diffusion rates using FCS and SMM, which gave consistent results (Table 1). In addition we determined the fraction of immobile molecules using SMM and FRAP also yielding similar results. Further
analysis of the FRAP data allowed us to dissect the fraction of immobile molecules into two fractions with distinct kinetics (Table 1).

The results are consistent with a model of activated ARs diffusing freely in the nucleoplasm with frequent, stochastically driven, short binding events probably representing immobilizations by non-specific DNA interactions in the sub-second range as well as less frequent but more stable interactions typically in the order of tens of seconds (Table 1, Fig.6). The latter immobilization events most likely represent associations of transcriptionally active ARs with their cognate recognition sequence in promoter/enhancer regions of androgen-regulated genes, since it is absent in an AR mutant (R585K) that is unable to identify its cognate recognition sequences in promoter/enhancer regions in androgen-regulated genes (Shaffer et al., 2004). The increased binding stability of wild type AR might well result from the association of stabilizing (coregulating) factors during formation of transcription complexes, or changes in chromatin structure due to remodeling. This explains the absence of this fraction in the presence of the antagonist OHF, which does not result in binding of these factors (Fig.6).

Next to the long-binding fraction (~25%), we also observed short immobilization events in agonist-bound wild type AR, but also in mutant (R585K) and antagonist-bound ARs (Table 1). These short immobilizations may well reflect a general non-specific DNA-binding capacity which is independent of sequence and agonist binding. This behavior could well reflect a very general mechanism by which nuclear proteins find their target sequences: free 3D diffusion through the nucleus, combined with frequent random collisions with chromatin leading to short interactions. It was previously hypothesized that nuclear proteins bind repeatedly in the same region interspersed with only short 3D diffusion events to enhance their chances of finding their target sites (sometimes referred to as ‘hopping’ and ‘jumping’), (Gorski et al., 2006; Halford and Marko, 2004; Loverdo et al., 2009; and as previously discussed in Van Royen et al., 2011). Although hopping and jumping indeed are often described as distinct models, molecules display essentially the same behavior in a model of 3D diffusion with random collisions which is supported by our data. Importantly, since our data fit well to a model in which the presented diffusion and binding parameters explain the data, it does not suggest the occurrence of one-dimensional diffusion along the DNA helix (‘sliding’). However, the existence of very short one-dimensional sliding behavior over a small distance cannot be ruled out, since this may be undetectable by any of the used technologies. Furthermore, AR dimerization does, in theory, allow binding to a distant site that is brought into proximity by looping of DNA before dissociating from the initial site.
(‘facilitated diffusion’), but these slowly moving molecules have not been detected by SMM in the present study. Moreover, the crowded nature of regulating proteins bound to DNA will limit the ability of these proteins to scan the DNA for target sites, which by itself makes it an unlikely scenario.

Very recently, a live cell study combining data from SMM, FCS and FRAP experiments also indicated sequence-specific and –nonspecific DNA binding by the transcription factor p53 (Mazza et al., 2012). In this study a continuum in chromatin residence times that included both sequence-specific and non-specific binding was observed. However, in the present study two distinct populations of residence times were found for AR, and only the short immobilization was found for the AR mutant R585K as well (Table 1). Although this seems to present a discrepancy, both studies present a very similar model of sequence-specific and –nonspecific DNA binding. This is a similar model as was suggested for the lac repressor in Escherichia coli (Elf et al., 2007). Numerical differences between p53 and AR could well be explained by differences in binding affinity or formation of complexes after DNA binding.

Interestingly, SMM and FCS consistently indicated a significant agonist-induced approximately two-fold decrease in diffusion rate of the freely mobile pool of wild-type and R585K mutant ARs (Table 1). This surprising decrease in diffusion rate could be due the formation of large hormone-induced AR complexes that diffuse at lower diffusion coefficients (Fig. 6). Since the diffusion coefficient is linearly related to molecule radius and therefore to the cube root of the molecular weight, the observed decrease of a factor of approximately 1.6 would require four-fold increase of the molecular weight of such complexes. It has been shown that ARs dimerize upon agonist binding (Van Royen et al., 2012). It is very conceivable that these activated AR dimers associate with a number of coregulator proteins forming a complex with a molecular weight four-fold higher than that of the AR monomer, thereby decreasing the diffusion coefficient of this complex 1.6-fold as compared to the AR monomer (Fig. 6). In addition, the data does not exclude the contribution of very brief (≤1 ms) binding events (on top of the previous described short interactions in the 0.5-0.8 ms range) to the diffusion rate decrease. These very short immobilizing interactions, representing an additional scanning behavior would be enhanced by agonist binding resulting in a lower effective diffusion rate.

In conclusion, combining SMM, FCS and FRAP appears to be a powerful approach to obtain a detailed quantitative description of the dynamic behavior of nuclear proteins in living cells. The results presented here point to a model of free diffusion, random collision with
DNA and two classes of DNA-binding events, relatively long DNA binding, most likely in transcription complexes and short interactions that may represent search mechanisms.
Materials and Methods

Expression constructs and cell culture

The constructs expressing N-terminally YFP-tagged AR (mutants) were generated as described before (Van Royen et al., 2012). In all constructs expressing AR fusion proteins the AR was separated from the fluorescent tag by a flexible (GlyAla)6 spacer (Farla et al., 2004). All new constructs were verified by sequencing and sizes of expressed ARs were verified by western blotting. The YFP-H2B expression plasmid was a generous gift from Dr. H. Kimura (Kyoto University, Kyoto, Japan).

Cell lines stably expressing YFP-labeled proteins at very low levels were generated as described before (Van Royen et al., 2009). Stably expressing cell lines were maintained in α-MEM (Cambrex) supplemented with 5% FBS (HyClone), 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/ml streptomycin and 600 μg/mL G418 (active concentration).

Single molecule microscopy

Cultured Hep3B cells were studied by SMM at 37ºC using a previously described wide-field fluorescence microscopy setup (Harms et al., 2001; Lommerse et al., 2004; Schmidt et al., 1996a). The microscope (Axiovert 100TV, Zeiss) was equipped with 100x oil-immersion objective (NA=1.4, Zeiss). A region-of-interest was set to 50x50 pixel at a pixel size of 220 nm. Excitation was done using a 514 nm argon laser line (Spectra Physics, Mountain View, CA) combined with an acousto-optic tunable filter (AOTF), illuminating the region of interest for 3 ms with a power of ~2 kW/cm². The time lag between subsequent illuminations was varied between 6.25 and 25 ms and camera frame rate was synchronized with the AOTF. Fluorescent light was filtered by a combination of filters (DCLP530, HQ570/80 (Chroma Technology, Brattleboro, VT) and OG530-3 (Schott, Mainz, Germany)) and detected by a liquid-nitrogen-cooled slow-scan charge-coupled device (CCD) camera (Princeton Instruments, Trenton, NJ).

At least seven cells were studied by taking 10 sequences of 120 images in each individual experiment. Positional data from three experiments were pooled for the (PICS) analysis of the mobility patterns. This way, positional information derived from approximately 50,000-100,000 fluorescence intensity spots attributed to individual molecules were analyzed together.

Three different time lags were used (6.25, 12.5, and 25 ms). For each time lag, data were obtained for different step sizes in the image sequence (e.g. using a time lag of 6.25 ms,
data was obtained for 6.25, 12.5, 18.75, 25, 31.25, 37.5 and 43.75 ms). The generated series of data points ranged from 6.25 to 43.75 ms, and for some time point more than one data point was generated (e.g. the 12.5 ms data point was generated twice, using the 6.25 and 12.5 ms time lag).

Analysis of YFP-AR mobility patterns

Analysis of individual molecules was done as described previously (Lommerse et al., 2004; Schutz et al., 1997). The signals from fluorescence intensity spots attributed to individual molecules were fitted to a two-dimensional Gaussian surface, permitting the localization of the molecule with a positional accuracy that is determined by the quotient of the full-width-at-half-maximum of the Gaussian fit and square root of the number of photons detected (Bobroff, 1986).

The two-dimensional mobility patterns were analyzed using the Particle Image Correlation Spectroscopy (PICS) analysis method (Semrau and Schmidt, 2007). Briefly, the cross-correlation between single-molecule positions at two subsequent time points is calculated (see Fig.1B). To correct for the effect of random proximity, the contribution from uncorrelated molecules in close proximity (which is described by a linear function) is subtracted (Fig.1C). This results in the cumulative distribution function $P_{\text{cum}}(l, \Delta t)$ for length $l$ of diffusion steps during time lag $\Delta t$ (Fig.1D).

For each time lag $P$ is fitted to one of the following two models (Fig.1D). The first model is described by the following function:

\[
(1) \quad P_{\text{cum}}(l, \Delta t) = 1 - \exp\left(-\frac{l^2}{\text{MSD}_0(\Delta t)}\right).
\]

It describes the probability that the Brownian particle starting at the origin will be found within a circle of radius $l$ at time lag $\Delta t$. It is described by the mean squared displacement $\text{MSD}_0(\Delta t) = 4 \cdot D \cdot \Delta t$. If the population of molecules segregates into two fractions of molecules, one with a fast and one with a slow mobility, Eq.1 becomes

\[
(2) \quad P_{\text{cum}}(l, \Delta t) = 1 - \left[ \alpha \cdot \exp\left(-\frac{l^2}{\text{MSD}_1(\Delta t)}\right) + (1 - \alpha) \cdot \exp\left(-\frac{l^2}{\text{MSD}_2(\Delta t)}\right) \right].
\]

This equation describes the second model, characterized by mean squared displacements $\text{MSD}_1$ and $\text{MSD}_2$, and relative fraction $\alpha$ and $(1-\alpha)$, respectively (Schutz et al., 1997). Subsequently, $\text{MSD}_1$ and $\text{MSD}_2$ are plotted against $\Delta t$. These plots (Figs. 3C-F) reveal the diffusional behavior of individual fractions.
The plots were best fitted using a free diffusion model:

\[ \text{MSD}(\Delta t) = 4 \cdot D_i \cdot \Delta t \]

in which \( D_i \) is the diffusion coefficient. The diffusion coefficient and fraction sizes (± s.e.m.) are given.

The expected diffusion coefficient for YFP in 50% glycerol was determined using the equation

\[ D = \frac{k_B \cdot T}{6 \cdot \pi \cdot \eta \cdot r^2}, \]

in which \( k_B \) is the Boltzmann constant, \( T \) the temperature (25°C), \( r \) the hydrodynamic radius of YFP (3 nm), and \( \eta \) the viscosity (\( \eta \) \( \text{H}_2\text{O}:\text{glycerol} 1:1 \text{v:v} = 7.7 \text{cPoise} \) (Cheng, 2008)).

**Fluorescence recovery after photobleaching**

At least one day prior to the experiment cells stably expressing YFP labeled proteins were plated. The medium was replaced at least 12 h before the experiment by medium with 5% charcoal-stripped FBS, supplemented with the appropriate hormone (100 nM R1881 or 1 \( \mu \)M OHF). The quantitative FRAP procedure was performed on a Zeiss LSM510 META Confocal Laser Scanning Microscope equipped with a 40x/1.3A NA oil immersion objective, a Lasos LGK 7812ML-4 Laser Class 3B Argon laser (30mW) and AOTF (Carl Zeiss MicroImaging, Jena, Germany). Temperature was controlled by a heatable stage and lens-heating device (37°C). For FRAP analysis a narrow strip spanning the nucleus was scanned at 514 nm excitation with 100 ms intervals at low laser power (Van Royen et al., 2009). Fluorescence intensity of YFP was recorded using a 560-nm longpass filter. After 40 scans, a high-intensity, 100-ms bleach pulse at 514 nm was applied photobleach YFP inside the strip. Subsequently, scanning of the bleached strip was continued at 514 nm at low laser intensity. Because of the previously shown absence of a permanently immobile fraction (Farla et al., 2004; Van Royen et al., 2009), the curves are normalized using equation:

\[ I_{\text{norm},t} = \frac{I_t - I_0}{I_{\text{final}} - I_0} \]

in which \( I_0 \), and \( I_{\text{final}} \) are the fluorescent intensities immediately after the bleach and after complete recovery, respectively.

The FRAP data was quantitatively analyzed by comparing the experimental data to curves generated using Monte Carlo modeling (Van Royen et al., 2009). Computer simulations used to generate FRAP curves for the fit were based on a model that simulates...
diffusion of molecules and binding to immobile elements in an ellipsoidal volume based on the average size of measured nuclei. The in silico generated curve fitting best to an experimental curve under evaluation (by ordinary least squares) was picked from a large set of computer simulated FRAP curves in which two or four parameters representing mobility properties in one or two immobile fractions were varied: fraction sizes (ranging from 0-40 %), and time spent in immobile state for each fraction (ranging from 0.1-15 s). The diffusion coefficients obtained using SMM and FCS were averaged and used as fixed parameters in this analysis. The laser bleach pulse was simulated based on experimentally derived three-dimensional laser intensity profile, which was used to determine the probability for each molecule to get bleached considering their 3D-position. The simulation of FRAP curve was run using discrete time steps (\(\Delta t\)) corresponding to the 21 ms experimental scan interval. The number of molecules in the simulations was \(10^6\), which was empirically determined by producing curves that closely approximate the data with comparable fluctuations. Diffusion was simulated at each new time step \(t + \Delta t\) by deriving the new positions \((x_{t+\Delta t}, y_{t+\Delta t}, z_{t+\Delta t})\) of all mobile molecules from their current positions \((x_t, y_t, z_t)\) by \(x_{t+\Delta t} = x_t + G(r_1), y_{t+\Delta t} = y_t + G(r_2),\) and \(z_{t+\Delta t} = z_t + G(r_3)\), where \(r_i\) is a random number \((0 \leq r_i \leq 1)\) chosen from a uniform distribution, and \(G(r_i)\) is the inverse of a cumulative Gaussian function with \(\mu = 0\) and \(\sigma^2 = 2D\Delta t\), where \(D\) is the diffusion coefficient. Immobilization was derived from simple binding kinetics described by:

\[
\frac{k_{on}}{k_{off}} = \frac{F_{imm}}{F_{mob}},
\]

where \(F_{imm}\) is the relative number of immobile molecules and \(F_{mob} = 1 - F_{imm}\). The probability for each particle to become immobilized (representing chromatin-binding) is defined as:

\[
P_{immobilize} = k_{on} = \frac{F_{imm}}{(T_{imm} \cdot F_{mob})},
\]

where \(T_{imm}\) is the characteristic time spent in the immobile state. The probability to be released is given by:

\[
P_{mobilize} = k_{off} = \frac{1}{T_{imm}}.
\]

The parameters of the top 7-10 best fitting (least square fitting) were averaged to represent the properties of the molecules in the experimental data \((\pm 2 \times s.e.m.)\) and to generate the fit curves in Fig.5A and Fig.S4 in supplementary material. For more details see (Farla et al., 2004; Van Royen et al., 2009).
Fluorescence correlation spectroscopy

For FCS the cells were prepared as described for FRAP. FCS experiments were performed on a Zeiss LSM510 confocal laser-scanning microscope equipped with a Confocor-2 FCS unit (Carl Zeiss MicroImaging, Jena, Germany). Temperature was controlled by a heatable stage and a lens-heating device (37°C). After an initial bleaching period of 3 s, the fluorescence intensity at a randomly chosen location in the nucleus was measured five times for 20 s (excitation 514 nm emission longpass filter 560-nm). Data were analyzed with the SSTC data processor (Scientific Software Technologies Center, Minsk, Belarus.). The raw data were auto-correlated (Eq.9), and the autocorrelation curves were analyzed as a two-component free diffusion triplet state model to determine the different retention times in the diffraction limited spot (Eq.10). In this model, the first component reflects YFP blinking (50%, 100 ms, obtained from direct comparison of YFP and GFP, data not shown) and the second component represents free diffusion. From this the appropriate diffusion time $\tau$ and diffusion coefficient $D$ were determined ($\pm$ s.d.).

\[
G(\tau) = \frac{\langle \delta I(t) \cdot \delta I(t+\tau) \rangle}{\langle I(t) \rangle^2}, \text{where } \delta I(t) = I(t) - \langle I(t) \rangle \text{ is the deviation from the mean intensity}
\]

\[
G(\tau) = 1 + \frac{1}{N} \cdot \frac{1 - T + T e^{-\tau/\tau}}{(1 - T)} \sum_{i} \left[ 1 + \frac{\tau}{\tau_{diff,i}} \right] \cdot \left[ 1 + \frac{\omega_{xy,i}^2}{\omega_z^2} \right] \cdot \frac{\tau}{\tau_{diff,i}},
\]

where $\omega_{xy}$ and $\omega_z$ describe the distance in lateral and axial direction at which the intensity $I$ decays to $I = I_0 \cdot e^{-2}$ (Rigler et al., 1993).

Statistical analysis

Fraction sizes determined using SMM in three individual experiments (shown in Fig.3B) were analyzed by two-way ANOVA, in order to determine the effect of the ligand and the mutation. Average MSDs determined using SMM (shown in Figs.3C-F) were analyzed using three-way ANOVA, so the effect of the ligand, the mutation, and the time point was determined. Individual FCS and FRAP curves (of which averages are shown in Figs.4 and 5 respectively) were analyzed by three-way ANOVA, in order to determine the effect of the ligand, the mutation and the time point. In all analyses, interactions between variables were determined as well. Statistical significance was accepted at $p<0.05$. 

\[
(9)
\]

\[
(10)
\]
Funding

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Author contributions

Designed research: MEvR, TS, ABH, MJMS
Performed research: MEvR, MJMS
Contributed analysis tools / analyzed data: MEvR, WAvC, BG, TS, ABH, MJMS
Wrote the paper: MEvR, MJMS
References


Figure Legends

Figure 1. Single-molecule microscopy and analysis of YFP-AR mobility patterns by Particle Image Correlation Spectroscopy (PICS). A. Representative image of YFP molecules in a 50% glycerol solution captured using the single-molecule microscopy (SMM) setup. Scale bar represents 2 μm. The 3-D representation of the fluorescence intensities shows two fluorescence intensity peaks that can be attributed to single YFP molecules. B. Schematic representation of the PICS algorithm (see also (Semrau and Schmidt, 2007)). For each molecule in image $I_a$ (blue circles) the number of molecules in image $I_b$ (red circles) closer than $l$ is counted. In this example, for three molecules in image $I_a$, one molecule in image $I_b$ closer than $l$ is counted, and for two molecules in image $I_a$ two molecules are counted. Thus, a total of seven molecules are counted: five counts are due to diffusion, whereas two counts are a result of random proximity of the molecules. C. Cumulative correlation function $C_{\text{cum}}(l)$. $C_{\text{cum}}(l)$ was obtained for individual molecules with a time lag $\Delta t$ of 6.25 ms (gray curve). Subtraction of a correction term derived from a linear fit of the long distance data (red line), representing the contribution of random proximity, yields the cumulative distribution function $P_{\text{cum}}(l)$ for the length $l$ of diffusion steps during this time lag (black curve). D. Curve fitting of $P_{\text{cum}}(l)$. Fitting was performed using a mono-exponential probability function (red line, Eq.1), reflecting a one-population model. E. Mean squared displacement (MSD) of YFP molecules in a 50% glycerol solution, plotted against time. The line represents the curve fit using a free-diffusion model (Eq.3). The value of the diffusion coefficient $D$ determined this way is indicated. F. Mean squared displacement (MSD) of YFP-H2B in Hep3B cells plotted against time. The line represents the curve fit using a free-diffusion model (Eq.3). The value of the determined diffusion coefficient $D$ is indicated.

Figure 2. Confocal images of agonist- and antagonist-bound wild type and mutant AR. Hep3B cells transiently transfect with YFP labeled ARs. Agonist (R1881)-bound AR shows a typical speckled distribution whereas the mutant AR R585K, which has a point mutation in the DNA-binding domain disabling interactions with the cognate AR target sequence, is homogeneously distributed in the presence of R1881 (see also (Van Royen et al., 2012)). When bound with antagonist (OHF), both wild type and AR R585K show a more homogeneous distribution. Scale bar represents 5 μm.
Figure 3. Quantitative analysis of the dynamic behavior of individual ARs in Hep3B cells by single molecule microscopy. A. Cumulative distribution function for individual YFP-AR molecules in the nuclei of Hep3B cells with a time lag $\Delta t$ of 25 ms (black diamonds). Curves are based on data from three experiments (at least seven cells per experiment), yielding positional data from approximately 50,000-100,000 fluorescence intensity spots attributed to individual molecules that were analyzed together. Curve fitting was performed using a mono-exponential (green line, Eq.1) and a bi-exponential (red line, Eq.2) probability function, reflecting a one- and two-population model respectively. The results of these fits indicated the occurrence of two fractions of molecules. B. Relative size of the fast fractions ($\alpha$) of wild type YFP-AR and YFP-R585K molecules, in the presence of R1881 and OHF, determined at time lag 6.25 ms. Data show that in the presence of OHF the fast fraction is larger than in the presence of R1881. The mutation in the DNA binding domain results in a slightly increased size of the fast fraction and abolishes the difference between R1881 and OHF. Two-way ANOVA revealed a significant interaction between the effects of the ligand and the occurrence of the mutation ($F(1,8)=10.7, p=0.01$). C – F. Mean squared displacements (MSDs) of the fast (C and E) and slow (D and F) fraction of YFP-AR (C and D) and YFP-R585K (E and F) molecules plotted against time. Black diamonds show data for molecules in presence of R1881, gray diamonds show data for molecules in presence of OHF. Lines represent curve fits using a free diffusion model (Eq.3, black line for R1881, gray line for OHF). Three-way ANOVA of the data of the fast fraction revealed a significant interaction between the effects of the ligand and the time point ($F(6,16)=2.90, p=0.04$). No significant interaction involving the mutation or main effect of the mutation was detected. A similar analysis of the data of the slow fraction revealed a significant interaction between the effects of the ligand and the mutation ($F(1,16)=27.2, p<0.0005$). G. Diffusion coefficient $D$ of fast fractions obtained using the curve fits shown in C and E. Both for the wild type and the R585K mutant receptor the diffusion coefficients obtained in the presence of OHF are higher than those obtained in the presence of R1881. H. Diffusion coefficient $D$ of slow fractions obtained using the curve fits shown in D and F. Diffusion coefficients obtained for the R585K mutant receptor are increased compared to those obtained for the wild type receptor. The determined positional accuracy ($dx$) of 33 nm led to a constant offset ($r^2(0)$) in MSD of 0.0044 $\mu m^2 (= 4 \cdot (dx)^2)$.
**Figure 4.** FCS analysis of YFP-AR dynamics in Hep3B cells. A. Correlation curves (left panel) and fit curves (right panel) of wild type and R585K mutant YFP-AR, in the presence of R1881 and OHF. The presented data are averages of at least 100 curves, obtained in three independent experiments in which at least 20 cells were used. Residuals of the fit are shown in Fig.S3. The main determinant in these curves appears to be the ligand. The curves for the R585K mutant and the wild type receptor in the presence of R1881 are similar, as are the two curves generated in the presence of OHF. However, statistical analysis of these data by ANOVA revealed a significant main effect not only of the ligand but also of the mutation, although the latter effect is small (F(1,100860)=280.95, p<0.0005, and F(1,100860)=38.84, p<0.0005 respectively). In addition, a significant interaction between the effects of the ligand and the mutation was detected (F(1,100860)=24.06, p<0.0005). B. Diffusion coefficients of the freely diffusing fraction, determined by fitting of the curves shown in A. Both for the wild type and the R585K mutant receptor the diffusion coefficients obtained in the presence of OHF are higher than those obtained in the presence of R1881. These data can be compared to those shown in Fig.3G, which shows the diffusion coefficients of the same fractions, determined by SMM.

**Figure 5.** FRAP analysis of YFP-AR dynamics in Hep3B cells. A. FRAP curves (left panel) and fit curves (right panel) of wild type and R585K mutant YFP-AR, in the presence of R1881 and OHF. Residuals of the fit are shown in Fig.S4. The FRAP curves represent the average data of at least 25 curves obtained from individual cells in at least two independent experiments. The wild type receptor in the presence of R1881 shows a slower recovery compared to the other three curves which display similar recovery rates (left panel). Statistical analysis by ANOVA revealed a significant interaction between the effect of the ligand and the mutation (F(1,54135)=2675.59, p<0.0005). These curves were subsequently analyzed quantitatively using Monte Carlo simulations. A large set of simulated FRAP curves were generated and compared to the experimentally generated curves. This way, the size of a freely diffusing fraction, and one or two transiently immobile fraction(s) and their residence times were determined. Curves representing the average parameters of the top 7-10 best fits are presented in the right panel. B. The relative size of the freely diffusing fraction. The wild type receptor in the presence of R1881 shows a low fraction size compared to those determined for the other three groups, which display similar fraction sizes. These data can be compared to those shown in Fig.3B, in which the sizes of the same fractions determined by SMM are shown. Error bars represent 2*SEM. C. Fraction size and residence time of the transiently
immobile fraction(s). Data labels represent residence times of the respective fraction. The size of the fraction showing a short immobilization time (0.5-0.8 s) is similar between all groups (~30%), but the wild type receptor shows an additional fraction with a longer (8 s) residence time.

Figure 6. Kinetic model of nuclear AR. Both ARs bound by antagonist (inactive) or agonist (active) move freely though the nucleoplasm frequently interspersed by short non-sequence specific interactions with DNA. Only activated ARs show a longer, more stable binding, most likely representing promoter binding. A mutation in the DNA binding domain disables the longer specific binding events but does not interfere with short interactions with DNA. Activated ARs diffuse slower than inactive ARs, indicating that agonist-bound AR dimerize (Van Royen et al., 2012) and are assembled into complexes with cofactors prior to DNA-binding whereas inactive antagonist-bound ARs remain in monomeric state.
<table>
<thead>
<tr>
<th></th>
<th>Fraction size (%)</th>
<th>( D ) (( \mu \text{m}^2/s ))</th>
<th>( D ) (( \mu \text{m}^2/s ))</th>
<th>Fraction size (%)</th>
<th>Imm. time (s)</th>
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<tbody>
<tr>
<td>AR wt</td>
<td>R1881</td>
<td></td>
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<tr>
<td></td>
<td>Free diff.</td>
<td>46.0 ± 2.9 &amp; 1.13 ± 0.09 &amp; 1.61 ± 0.26</td>
<td>38 ± 4 &amp; −</td>
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<td></td>
<td>Short imm.</td>
<td>54.0 ± 2.9 &amp; 0.056 ± 0.003 &amp; 28 ± 3 &amp; 8 ± 2</td>
<td>34 ± 4 &amp; 0.8 ± 0.2</td>
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<tr>
<td></td>
<td>Long imm.</td>
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<tr>
<td>OHF</td>
<td>Free diff.</td>
<td>88.5 ± 2.8 &amp; 2.31 ± 0.10 &amp; 2.42 ± 0.37</td>
<td>68 ± 5 &amp; −</td>
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<tr>
<td></td>
<td>Short imm.</td>
<td>11.5 ± 2.8 &amp; 0.063 ± 0.008 &amp; 32 ± 5 &amp; 0.5 ± 0.2</td>
<td>32 ± 5 &amp; 0.5 ± 0.2</td>
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<tr>
<td></td>
<td>Long imm.</td>
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<tr>
<td>AR R585K</td>
<td>R1881</td>
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<td></td>
<td>Free diff.</td>
<td>61.1 ± 6.7 &amp; 1.42 ± 0.08 &amp; 1.78 ± 0.19</td>
<td>66 ± 6 &amp; −</td>
<td></td>
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<tr>
<td></td>
<td>Short imm.</td>
<td>38.9 ± 6.7 &amp; 0.077 ± 0.005 &amp; 34 ± 6 &amp; 0.6 ± 0.3</td>
<td>34 ± 6 &amp; 0.6 ± 0.3</td>
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<td></td>
<td>Long imm.</td>
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<tr>
<td>OHF</td>
<td>Free diff.</td>
<td>79.7 ± 4.0 &amp; 2.24 ± 0.19 &amp; 2.64 ± 0.39</td>
<td>71 ± 6 &amp; −</td>
<td></td>
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<tr>
<td></td>
<td>Short imm.</td>
<td>20.3 ± 4.0 &amp; 0.090 ± 0.013 &amp; 29 ± 6 &amp; 0.5 ± 0.2</td>
<td>29 ± 6 &amp; 0.5 ± 0.2</td>
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<tr>
<td></td>
<td>Long imm.</td>
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Figure 1

A. An image showing a 3D visualization of a cellular structure.

B. A diagram illustrating the positioning of images $I_a$ and $I_b$.

C. A plot showing the cumulative distribution function $C_{cum}(t)$.

D. A plot showing the cumulative population $P_{cum}(t)$.

E. A plot showing the mean squared displacement (MSD) for YFP, with a slope indicating diffusion coefficient $D = 7.35 \pm 0.99 \mu m^2/s$.

F. A plot showing the MSD for H2B, with a slope indicating diffusion coefficient $D = 0.040 \pm 0.0023 \mu m^2/s$.
Figure 2
Figure 3

A

\[ P_{\text{cum}}(l) \]

\[ \ell (\mu m^2) \]

B

Size of fast faction (%)

C

Fast fraction

D

Slow fraction

E

AR R585K

F

AR R585K

G

D (\mu m^2/s)

H

D (\mu m^2/s)
Figure 4

A

Autocorrelation function G(t)

Log time t (ms)

B

D (µm²/s)

AR wt R1881

AR wt OHF

AR R585K R1881

AR R585K OHF

AR wt

AR R585K
Figure 5

A

B

C
Figure 6

Inactive AR

- 68% of DBD mutation R585K
- $D = 2.4 \, \mu m/s^2$
- 0.5 s
- 32%

Activated AR

- 38% of DBD mutation R585K
- $D = 1.4 \, \mu m/s^2$
- 0.8 s
- 34%
- 8 s
- 28%