

A high-throughput platform for single-molecule tracking identifies drug interaction and cellular mechanisms

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Abstract

The regulation of cell physiology depends largely upon interactions of functionally distinct proteins and cellular components. These interactions may be transient or long-lived, but often affect protein motion. Measurement of protein dynamics within a cellular environment, particularly while perturbing protein function with small molecules, may enable dissection of key interactions and facilitate drug discovery; however, current approaches are limited by throughput with respect to data acquisition and analysis. As a result, studies using super-resolution imaging are typically drawing conclusions from tens of cells and a few experimental conditions tested. We addressed these limitations by developing a high-throughput single-molecule tracking (htSMT) platform for pharmacologic dissection of protein dynamics in living cells at an unprecedented scale (capable of imaging $> 10^6$ cells/day and screening $> 10^4$ compounds). We applied htSMT to measure the cellular dynamics of fluorescently tagged estrogen receptor (ER) and screened a diverse library to identify small molecules that perturbed ER function in real time. With this one experimental modality, we determined the potency, pathway selectivity, target engagement, and mechanism action for identified hits. Kinetic htSMT experiments were capable of distinguishing between on-target and on-pathway modulators of ER signaling. Integrated pathway analysis recapitulated the network of known ER interaction partners and suggested potentially novel, kinase-mediated regulatory mechanisms. The sensitivity of htSMT revealed a new correlation between ER dynamics and the ability of ER antagonists to suppress cancer cell growth. Therefore, measuring protein motion at scale is a powerful method to investigate dynamic interactions among proteins and may facilitate the identification and characterization of novel therapeutics.

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This work presents an **important** technological advance, in the form of a high throughput platform for Single Particle Tracking allowing us to measure millions of cells and thousands of compounds per day. Analysis of the diffusional behaviour of fluorescently-tagged targets permits the identification of, and differentiation between, small molecules that bind directly or affect the target indirectly. The evidence provided is **compelling**, although some methodological information is undisclosed.

Introduction

Both the activity and mobility of proteins within the crowded cellular environment are profoundly influenced by interactions with their surroundings^{1,2,3}. Under these conditions, where diffusion is no longer well described by the Stokes radius of the protein monomer⁴, changes in protein motion might be expected to correlate closely with changes in the activity of these proteins. The development of increasingly sophisticated live-cell microscopy techniques, including early ensemble methods like fluorescence recovery after photobleaching (FRAP) and fluctuation correlation spectroscopy (FCS), have informed our understanding of protein dynamics in cellular biology⁵. A myriad of technical improvements, such as enhanced labeling methods^{6,7}, better live-cell compatible fluorophores⁸, new forms of light microscopy⁹, dramatic increases in computational power¹⁰, and the addition of machine learning approaches to data analysis^{10,11} have together enabled a new era of imaging-based studies across biological contexts^{3,5,12}.

Applying any microscopy technique at scale presents challenges, but recent advances have shown the power of high content imaging techniques to address both mechanistic biological questions as well as to generate leads for new chemical matter in drug discovery¹³. Even these conceptually simple experiments involving fixed cells stained with well-characterized commercial reagents take careful experiment design and sophisticated computational approaches to execute¹⁴. It is no wonder, then, that attempts to combine high content imaging workflows with more advanced super-resolution microscopy methods have thus far been limited. Such advances have enabled the development of systems for fixed-cell STORM imaging at an impressive 10,000 cells per day¹⁵, though the appropriate application of this increase in scale remains an open question.

In single molecule tracking (SMT), a fluorescently labeled protein of interest is imaged at high spatiotemporal resolution to track its motion in a live cell¹⁰. The information embedded in these trajectories has been used to investigate diverse cellular phenomena including protein oligomerization state and function^{15–17}, inter-organelle communication¹⁸, nuclear organization¹⁹, and transcription regulation^{20–23}. Of particular utility are “fast-SMT” approaches which use high frame rates and stroboscopic illumination to minimize motion-induced blurring, and hence can measure diffusive states over a large dynamic range^{24,25}. Specifically, proteins that diffuse rapidly throughout the cell are often missed in alternative tracking approaches, biasing the resulting data. In spite of the potential biological discoveries that depend on the application of SMT on a large scale, SMT in general (and fast-SMT in particular) has not been adapted to a high-throughput setting that would enable the analysis of complex, multi-component systems, or the identification of compounds that affect protein motion.

Steroid hormone receptors (SHRs) are a class of transcription factors that play crucial roles in normal human development and in disease pathogenesis. SHRs like the estrogen receptor (genes ESR1 and ESR2), androgen receptor (AR), and progesterone receptor (PR), as examples, contribute decisively to the acquisition of secondary sex characteristics, while the glucocorticoid receptor (GR) helps to orchestrate both metabolism and inflammation²⁶. In their ligand-free state, SHRs are kept sequestered in multiprotein complexes by the chaperone HSP90²⁷. Canonically, in the presence of hormone they dimerize and bind their cognate genomic response elements, recruiting epigenetic modifiers and transcription machinery^{26–28}. At the same time, steroid hormone receptor-derived signals impose a large disease burden by promoting the growth of breast cancers (ER)^{26,29} or prostate cancers (AR)²⁶; or by imposing immune and metabolic dysfunction (GR)^{26,30}. SHRs therefore provide an excellent proof-of-concept system to study the relationship between protein dynamics and protein function due to the wealth of information and reagents²⁹ already available for these systems as well as previous reports characterizing some aspects of their cellular dynamics^{20,22,31–33}.

Here, we present the first industrial-scale, high-throughput, fast-SMT (htSMT) platform capable of measuring protein motion from more than 13,000 individual assay wells (>1,000,000 individual cells) per day. Using ER as a test system, we demonstrate that chemical screening using htSMT is specific, robust, and reproducible. The increase in throughput enables classical drug discovery activities, including compound library screening and the elucidation of structure-activity relationships (SAR), yielding accurate and reproducible results that are inaccessible or unmeasurable with other techniques or using SMT on a smaller scale. Importantly, we demonstrate that htSMT can be used to characterize both known and novel pathway contributions to the ER protein interaction network. More than a proof-of-concept for the htSMT platform, these data confirm that analysis of protein motion itself on a large scale reveals detailed information about pathway interactions and signaling.

Results

Creation and validation of a high throughput SMT platform

We developed a robotic system capable of handling reagents, collecting high-quality fast SMT image series, processing time-ordered raw images to yield molecular trajectories, and extracting features of biological interest within defined cellular compartments. To examine htSMT system performance across a broad spectrum of diffusion coefficients, we generated three U2OS cell lines ectopically expressing HaloTag⁶ fused proteins with well-established behaviors in the cell. These HaloTag fusions allow the subsequent addition of bright and photostable organic fluorophores like JF₅₄₉⁸ which produce high signal spots to detect and track. Histone H2B-Halo, which is predominantly incorporated into chromatin and therefore effectively immobile over short timescales²⁵, was employed to estimate localization error. A prenylation motif (Halo-CaaX) embedded in the plasma membrane exhibits moderate diffusion³⁴. Unfused HaloTag was chosen to represent the upper limit of cellular “free” diffusion. Single-molecule trajectories measured in these cell lines yielded the expected diffusion coefficients (**Figure 1A**). Using the immobile H2B-Halo trajectories, we found the localization error of the htSMT system to be 39 nm (**Extended data 1A**), comparable to other benchmark stroboscopic illumination datasets^{25,35}.

We then tested whether our htSMT platform can extract accurate molecular trajectories at scale. We employed 384-well plates where free Halo, Halo-CaaX, and H2B-Halo cell lines were mixed in equal proportions in each well. Imaging with a 94 μm by 94 μm field-of-view (FOV), we achieved an average of 10 nuclei simultaneously (**Figure 1B**, **Extended data 1B**, Supplemental video 1), enough that most FOVs contained cells from each cell line. To limit ambiguity in cell assignment, we considered only the trajectories that fell within nuclear segmented regions. The

probability distribution of diffusion states cleanly distinguishes between the three cell types (**Figure 1C**). More importantly, by looking at the single-cell state distribution profiles of 103,757 cells from five separate 384-well plates, grouped by their distribution profile, we recovered highly consistent estimates of protein dynamics at the single cell level (**Figure 1D**).

While single-cell measurements are powerful, the number of trajectories in one cell is limited, and so estimates of diffusive states can be broad. Combining trajectories from multiple cells, however, provides the expected distribution of diffusive states (**Figure 1E**). Moreover, combining trajectories derived from many cells makes model-based²⁵ or model-agnostic³⁵ state analysis possible, where as few as 10^3 trajectories permit satisfactory inference of the underlying diffusion states. We determined that imaging six fields of view (FOV), for 1.5 seconds each, yielded enough trajectories ($> 10,000$) to accurately estimate protein dynamics, bringing the overall throughput of the platform to more than 13,000 individual wells ($> 90,000$ FOVs; $> 1,000,000$ cells/day), a rate of data acquisition that enables compound screening on a feasible timescale (**Extended Data 1C**).

Using htSMT to measure protein dynamics of SHRs

Equipped with an htSMT system capable of measuring protein dynamics broadly, we sought to understand how measuring protein motion can be used to characterize protein activity. SHRs transition between inactive and active states via ligand binding (**Extended Data 2A**), a phenomenon that has been previously observed at the single molecule level^{20,22}, and we hypothesized that the large dynamic range and orders-of-magnitude increase in throughput of our platform could capture these differences in the context of compound screening. We generated HaloTag fusions to ER, AR, and PR through ectopic expression, and GR through endogenous knock-in. Similar to previous approaches^{20,36}, we used clonal cell lines in a U2OS cell background to minimize effects of comparing dynamics in different cell types. Clones were carefully selected such that the HaloTag fusion SHRs were comparable to each other in transcript abundance, and not higher than transcript levels in tissue-specific cell lines like MCF7 and T47d, which are both ER and PR positive (**Extended Data 2B**).

In the absence of hormone, all four SHR proteins exhibit similar dynamic profiles: a small immobile fraction and a large freely diffusing fraction with a $3.4 - 4.3 \mu\text{m}^2/\text{sec}$ average diffusion coefficient (**Figure 2A**, **Extended Data 2C**). No correlation between diffusion and monomeric protein size was observed, highlighting the differences between cellular protein dynamics versus purified systems. Upon addition of agonist, a dramatic increase in immobile trajectories is observed, which we attribute to chromatin binding. Using a conservative upper-bound of chromatin mobility in the nucleus and chromatin-associated transcription factors³⁵, we define the bound fraction (f_{bound}) for each SHR as the fraction of trajectories diffusing less than $0.1 \mu\text{m}^2/\text{sec}$ (**Figure 2A**). Using this threshold, f_{bound} of histone H2B is 0.92 on average, consistent with previous reports²⁵. D_{free} we defined as the occupation-weighted average diffusion coefficient of the non-bound states (**Extended data 2C**). Some SHRs had a higher proportion of bound molecules than others; The ligand-induced effect is most pronounced for ER, with 34% bound in basal conditions and 87% bound after estradiol treatment (**Figure 2A**, Supplemental video 2 and 3).

SHRs are highly selective for their cognate agonists in biochemical binding assays, which we confirmed by measuring the dose-dependent change in dynamics as a function of agonist concentration. The maximal change in f_{bound} (**Figure 2B**) and decrease in D_{free} (**Extended Data 2D**) differed between SHRs. The dose titration curves also showed variable potencies (EC_{50}) for each SHR/hormone pair, with ER-estradiol being both the most potent and most selective pair. RNA-seq after estradiol stimulation showed a marked induction of hallmark ER-dependent gene sets³⁷, confirming that the increase in chromatin binding we observed by SMT has a functional effect in promoting ER-responsive gene programs, even in the ectopic expression setting

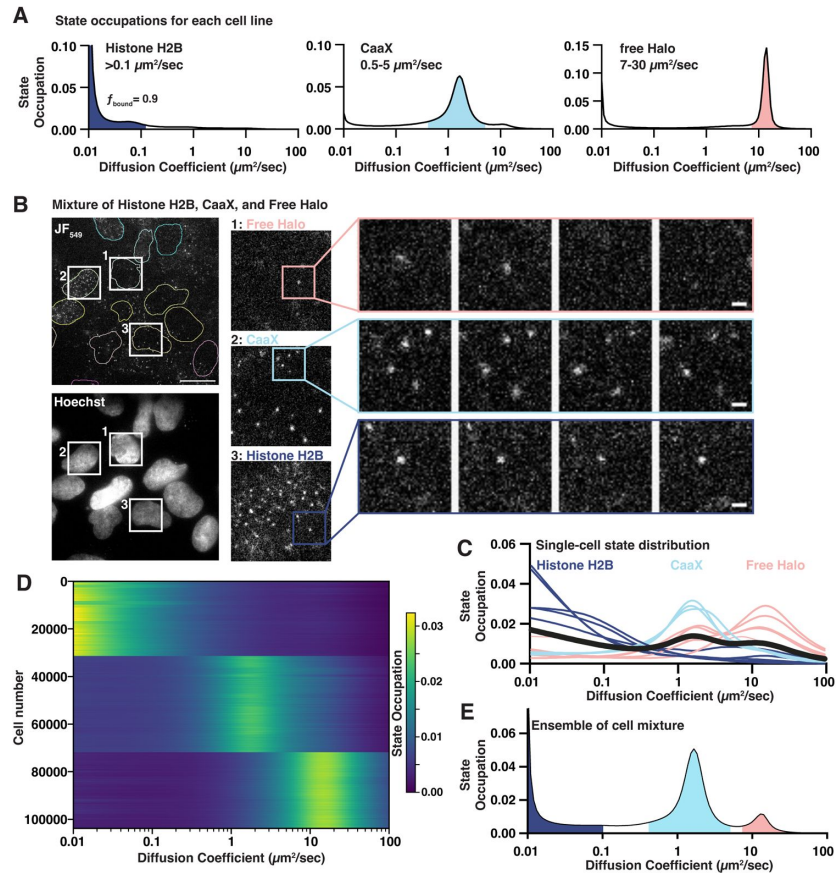


Figure 1.

Benchmarking a high throughput single molecule tracking platform

- Diffusion state probability distributions from three cell lines expressing Histone H2B-Halo, Halo-CaaX, or Halo alone. Shaded bins represent the diffusive states characteristic of each cell line.
- Example field of view. Equal mixture of H2B-Halo, Halo-CaaX and free Halo cell line single molecule images (top) and reference Hoechst image (bottom). Insets show zoom-ins to individual cells and to sequential frames of individual molecules. Image intensities are equivalently scaled across panels.
- Single-cell diffusive states extracted from (c), colored based on similarity to the H2B-, CaaX-, or free-Halo dynamics reported in (b).
- Heatmap representation of 103757 cell nuclei measured from a mixture of Halo-H2B, Halo-CaaX, or Free Halo mixed within each well over 1540 unique wells in five 384-well plates. Each horizontal line represents a nucleus. Cells were clustered using k-means clustering and labels assigned based on the diffusive profiles determined in (b).
- Ensemble state occupation of all trajectories recovered from a mixture of Halo-H2B, Halo-CaaX, free Halo cells.

(**Extended Data 2E, F** [↗](#)). Thus, SMT can detect functionally-relevant changes in transcription factor dynamics and accurately differentiate the ligand/target specificity directly within the cellular environment.

Screening a diverse bioactive chemical set identifies known and novel modulators of ER dynamics

Our characterization efforts of ligand selectivity for AR, ER, GR, and PR collectively suggested that we could use SMT to interrogate the effects of compounds on protein dynamics at a throughput conducive to high throughput screening. We first identified a structurally diverse set of 5,067 molecules with heterogeneous biological activities as a useful screening set. Having determined that the same data acquisition parameters (6 FOVs imaged for 1.5 sec each) were sufficient to recover more than 10,000 trajectories per well, we could achieve a throughput of 15,000 wells per day which would permit us to interrogate the whole library in a single day. We chose to screen this bioactive compound set against ER, assessing change in f_{bound} at 1 μM compound versus the vehicle DMSO (**Figure 3** [↗](#)). The screen was run twice to assess reproducibility, showing a high degree of agreement between replicates for ER-active molecules (**Extended Data 3B** [↗](#)). This screen illustrates some important advantages of our htSMT platform over more manual lower-throughput approaches.

From plate to plate, the assay window for the screen was robust³⁸ [↗](#) (average Z'-factor = 0.79 over 72 plates), the measured potency of the control estradiol in each instance remained within three-fold of the mean (**Extended Data 3C-E** [↗](#)), and the distribution of negative control wells centered tightly on zero (**Extended Data 3F** [↗](#)). Each compound measurement was averaged from SMT trajectories of between 94 and 161 cells (25th to 75th percentiles; **Extended Data 3G** [↗](#)). Of the 30 compounds we identified from the bioactive set that we expected to modulate ER, either as agonists or antagonists^{29,39} [↗](#), all significantly increased f_{bound} measured by SMT. This includes agonistic molecules like estradiol, but also notable examples such as 4-hydroxytamoxifen, fulvestrant, and bazedoxifene (**Figure 2C** [↗](#), **Extended Data 3B, H** [↗](#)).

The somewhat counter-intuitive finding that either strong agonism or antagonism can lead to an increase in chromatin binding has been reported for ER³¹ [↗](#), but this appears not to be a general feature of SHRs. While the PR antagonist mifepristone⁴⁰ [↗](#) behaves similarly to ER (**Extended Data 4A** [↗](#)), antagonists of AR like Enzalutamide and Darolutamide⁴¹ [↗](#), and antagonists of GR like AL082D06⁴² [↗](#) cause a decrease in chromatin binding. This decrease occurs when administered singly or when co-administered in competition with the cognate agonist (**Extended Data 4B and C** [↗](#)). These results show how the cellular context and interaction partners are critical to understand the effect of a compound on its intended target. To underscore this point, in addition to binders of the ER ligand-binding domain, we also identified a number of active compounds targeting diverse nodes in the ER interaction network, including modulators of the proteasome, chaperones, kinases, and others (**Figure 2C** [↗](#)).

Cellular ER dynamics elucidate structure-activity relationships (SAR) of ER modulators

Our screen revealed that, surprisingly, all the known ER modulators—both agonists like estradiol and potent antagonists like fulvestrant—caused an increase in f_{bound} (**Figure 4A** [↗](#)). We therefore characterized a subset of selective ER modulators (SERMs) and selective ER degraders (SERDs) in more detail. These molecules all bind competitively to the ER ligand binding domain²⁹ [↗](#). As in the bioactive screen, both SERDs and SERMs increased f_{bound} (**Figure 4A** [↗](#)) and slightly decreased measured D_{free} (**Extended Data 5A** [↗](#)), with potencies ranging from 9 pM for GDC-0927 to 4.8 nM for GDC-0810 (**Figure 4B** [↗](#)). Despite different physical-chemical properties, all five increased f_{bound} within minutes of compound addition (**Figure 4C** [↗](#), **Extended Data 5B** [↗](#)), with no

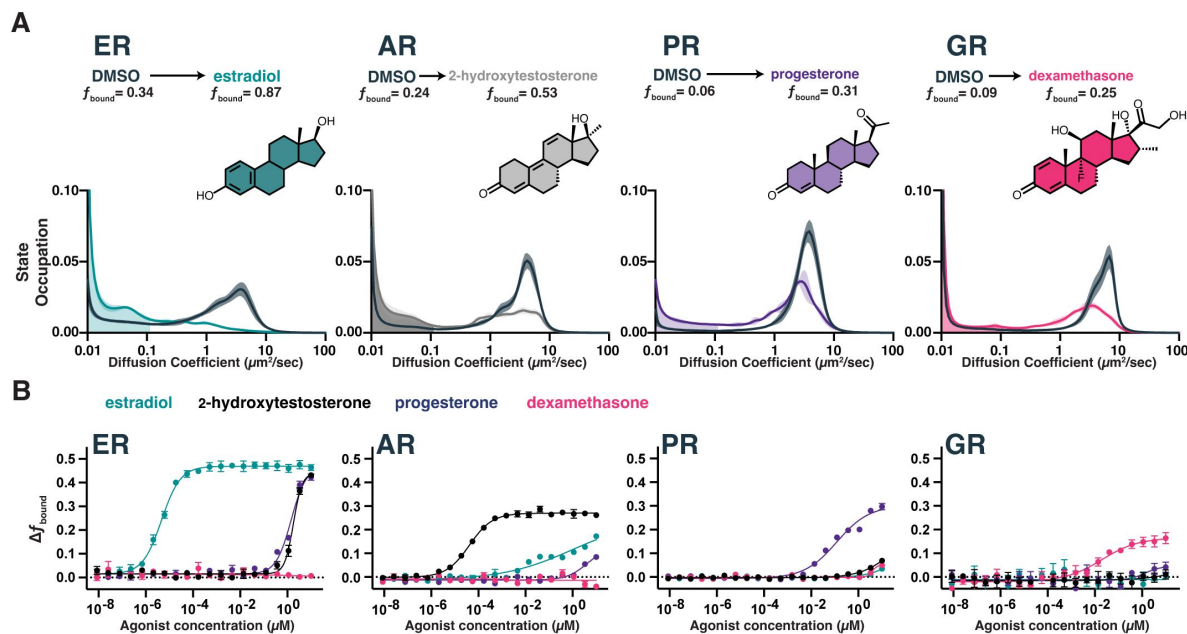


Figure 2.

Chromatin binding by steroid hormone receptors is affected by compound treatment

a) Distribution of diffusive states for Halo-AR, Halo-ER, Halo-GR, and Halo-PR in U2OS cells before and after stimulation with an activating ligand. The area in the shaded region is f_{bound} . Shaded error bands represent S.D.

b) Selectivity of individual SHRs to their cognate ligand compared with other steroids, as determined by f_{bound} . Error bars represent SEM.

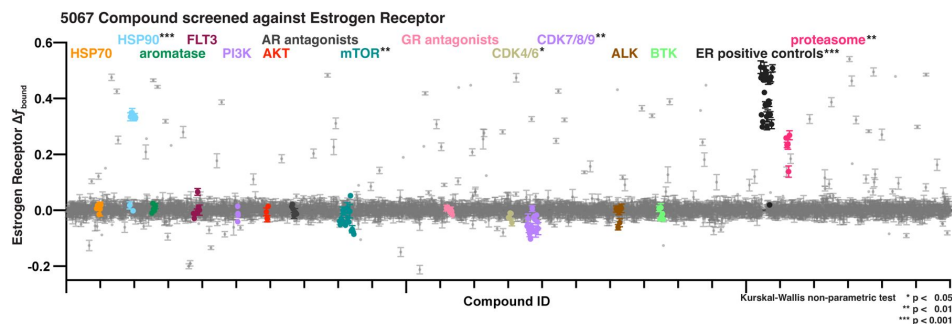


Figure 3.

Bioactive screen results of change in estrogen receptor f_{bound} for two biological replicates of 5067 compounds.

Select inhibitors are grouped and uniquely colored by pathway or target. Error bars are SEM.

evidence of transient diffusive states (**Extended Data 5C** [↗](#)). Presumably, ER dissociation from the chaperone complex, dimerization, and chromatin binding occur on rapid and seemingly comparable timescales. Since we cannot distinguish individual steps in these transitions, we consider the on rate of the entire process to have the effective rate constant k_{on}^* . Importantly, selective antagonists of AR and GR did not induce significant modulation of ER dynamics, further highlighting the utility of htSMT in characterizing the specificity of interactions between small molecule modulators of protein function and their cognate targets (**Extended data 5D** [↗](#)).

Interestingly, SERMs 4-hydroxytamoxifen (4OHT) and GDC-0810 show lower maximal increases in f_{bound} compared with the SERDs fulvestrant and GDC-0927 (**Figure 4D** [↗](#)). Similar effects have been described previously using fluorescence recovery after photobleaching³¹ (FRAP), which we confirmed using our Halo-ER cell line (**Figure 4E** [↗](#)). The delay in ER signal recovery after two minutes in FRAP was consistent with the changes in f_{bound} measured by SMT (**Figure 4F** [↗](#)). Although FRAP was used to measure these f_{bound} differences, the technique suffers from challenges in scalability and depends heavily on prior assumption of the underlying dynamical model in the sample. SMT, alternatively, enables detailed characterization of the potency of 4OHT and GDC-0810 relative to other ER ligands in their ability to increase ER chromatin binding on a tractable timescale.

Neither FRAP nor htSMT can discriminate between recovery driven by an increase in residence time (decreasing k_{off}^*) or increasing the rate of chromatin binding (increasing k_{on}^*), either of which would result in increasing f_{bound} . By changing SMT acquisition conditions to reduce the illumination intensity and collect long, 250 msec continuous frame exposures, only immobile proteins form spots. Under these imaging conditions, the distribution of track lengths provides a measure of relative residence times^{20,21,43,44}. Both agonist and antagonist treatment led to longer binding times compared to DMSO, suggesting that ligand binding decreases k_{off}^* (**Figure 4G** [↗](#), **Extended Data 5E** [↗](#)). Consistent with FRAP, estradiol, GDC-0927, and fulvestrant show longer binding times compared with other ER modulators. Using f_{bound} and k_{off}^* measurements, one can infer the k_{on}^* . In all cases the changes in dissociation rate are not proportional to the increase in f_{bound} , and so ligand-imposed increases in k_{on}^* likely contribute to the observed change in the chromatin associated ER fraction (**Extended Data 5F** [↗](#)). These data are consistent with a model wherein ER rapidly binds to chromatin irrespective of which molecule occupies the ligand binding domain, but some ligands induce a conformation that can be further stabilized on chromatin by cofactors. Consequently, these data support the hypothesis that ER may engage chromatin in mechanistically different ways³¹. An efficacious ER inhibitor may promote rapid and transient chromatin binding that fails to effectively recruit necessary cofactors to drive transcription.³³

htSMT reveals a relationship between ER dynamics and efficacy in ER-dependent cell toxicity

As the name implies, next-generation ER degraders like GDC-0927, AZD9833, and GDC9545 were optimized to enhance degradation of ER^{31,45}. We indeed observed compound-induced ER degradation via immunofluorescence, both in established breast cancer model lines and our U2OS ectopic expression system (**Extended Data 6A-B** [↗](#)). Structural analogs of GDC0927 have been reported and optimized for ER degradation, however the correlation between ER degradation and cell proliferation is poor^{29,46} (**Figure 5A** [↗](#), **Extended Data 6C-E** [↗](#)). We hypothesized that by measuring protein dynamics we might obtain more precise measurements of inhibitory activity than can be achieved by assessing protein degradation. We therefore determined the potency and maximal effect of structural analogues of GDC-0927 using htSMT.

Overall, these analogues exhibited a potency range of 15 pM to 12 nM and increased ER f_{bound} by 0.4 to 0.56 (**Figure 5B** [↗](#)). Small changes in the chemical structure produced measurable changes in both compound potency and maximal efficacy as determined using SMT, a critical feature if an

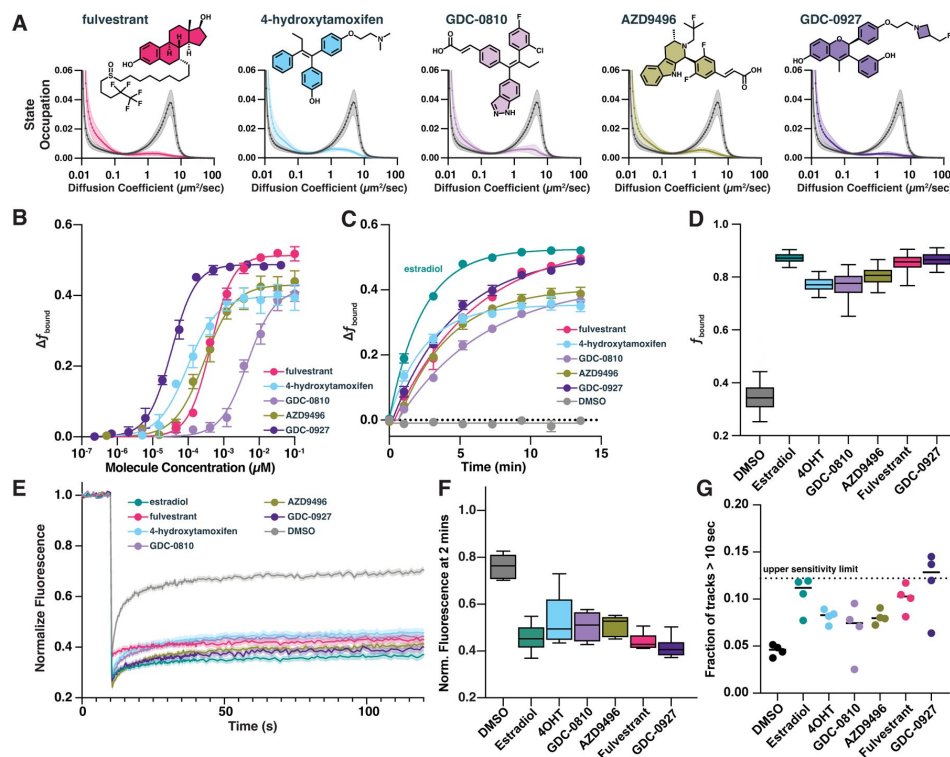


Figure 4.

Selective ER modulators and degraders induce DNA binding measurable through htSMT

- Diffusion state probability distribution for ER treated with 100 nM of exemplified SERMs and SERDs. Shaded regions represent the S.D.
- Change in f_{bound} as a function of a 12-pt dose titration of fulvestrant (5), 4-OHT (6), GDC-0810 (8), AZD9496 (9), or GDC-0927 (10) with fitted curve. Compounds colored as in (a). Error bars represent the SEM of three replicates.
- Change in f_{bound} as a function of time after agonist or antagonist addition, fitted with a single exponential. Compounds colored as in (a). Estradiol (1, green) and DMSO added for comparison. Error bars represent SEM.
- Maximum effect of SERMs and SERDs on f_{bound} . Each box represents quartiles while whiskers denote the 5-95th percentiles of single well measurements, measured over a minimum of four days with at least 8 wells per compound per day.
- Fluorescence Recovery After Photobleaching of ER-Halo cells, treated either with DMSO alone or with 100 nM SERM/D. Curves are the mean $\pm$ SEM for 18-24 cells, colored as in (a). Error bands represent SEM.
- Quantification of FRAP recovery curves to measure recovery 2 minutes after photobleaching. Whiskers denote the 5-95th percentiles of single cell measurements.
- Quantification long-lived tracks, each point representing the fraction of trajectories greater than 10 seconds for a single biological replicate consisting of 3-10 wells per condition. Dashed line represents the median fraction of trajectories lasting longer than 10 sec for Histone H2B-Halo, which is the upper limit of measurement sensitivity.

assay is to be used to iteratively optimize a compound for potency or efficacy.

We compared the potencies of GDC-0927 and analogues determined either via ER degradation or SMT, with the ability of each of these compounds to block estrogen-induced breast cancer cell proliferation. Potency assessed by ER degradation was not a good predictor of potency in the cell proliferation assay (**Figure 5A**). By contrast, SMT measurements of f_{bound} strongly correlate with cell viability (**Figure 5C**; R^2 of 0.83 for T47d and 0.84 for MCF7).

Intriguingly, SMT EC_{50} values were on average 10-fold lower than those observed in the cell growth assay, suggesting that SMT may be sensitive enough to identify chemical series that would not show effects in other cellular assays, enabling the identification of starting points for medicinal chemistry that could not be obtained by other methods. This correlation between effects on protein dynamics (f_{bound}) and protein function (suppression of cell proliferation) coupled with the throughput of the SMT system make this an attractive approach for the identification of protein modulators with novel properties.

Screening of a diverse chemical library using htSMT enables unbiased pathway interaction analysis by monitoring protein dynamics

In addition to known ER active modulators, many other compounds in our bioactive library provoked easily measurable changes in f_{bound} . To define a threshold for calling a molecule from the screen “active,” we selected 92 compounds with different magnitudes of change in f_{bound} to retest in a dose titration (**Extended Data 7A, B**). We found that a 5% change in f_{bound} was sufficient to reproducibly distinguish active compounds. Using this approach, we identified 239 compounds in the bioactive library that affected the ER mobility (**Extended Data 3B**). Among these compounds, the correlation between the two screen replicates was high ($R^2 = 0.92$) and the level of activity was reproducible (the slope for active molecules was 0.94). Some active compounds could be clustered based on scaffold homology, but most clusters consisted of one or only a few members (**Extended Data 7C-D**). Structural clustering was employed to identify known ER modulators where the vendor-provided annotation was poorly defined (**Extended data 7C**). Our results demonstrate that htSMT is reproducible and robust when screening large collections of molecules.

Most active molecules from the screen were not structurally related to steroids (**Extended Data 7C, D**). On the other hand, many compounds could be grouped based on their reported biological targets or pathways (**Figure 3**, **Extended Data 8A-B**). For example, heat shock protein (HSP) and proteasome inhibitors consistently increased f_{bound} , whereas cyclin dependent kinase (CDK) and mTOR inhibitors decreased f_{bound} . Though many CDK inhibitors lack within-family specificity⁴⁷ (**Extended Data 8A**, pan-CDK), we found that CDK9-specific inhibitors more strongly affected ER dynamics than did CDK4/6-specific inhibitors. Furthermore, as with selective AR and GR antagonists, inhibitors targeting ALK, BTK, and FLT3 kinases that have not been shown to interact with ER have no impact on ER dynamics when assessed using SMT (**Figure 3**).

For the inhibitors of cellular pathways we identified, we used a dose titration to better characterize the effect of each on ER dynamics. Potencies ranged from the sub-nanomolar to low micromolar (**Figure 6B**), similar to the reported potencies of these compounds against their cellular targets^{48–59}. Additionally, we tested these molecules against AR (**Extended Data 8C**) and PR (**Extended Data 8D**). Each SHR differed meaningfully from the others in terms of the response to compounds identified through an ER-focused screening effort. Again, the magnitude of ER SMT effect was largely consistent within a target class (**Figure 6A**, **Extended Data 8A-D**). The finding that structurally distinct compounds exhibited similar effects based on their biological

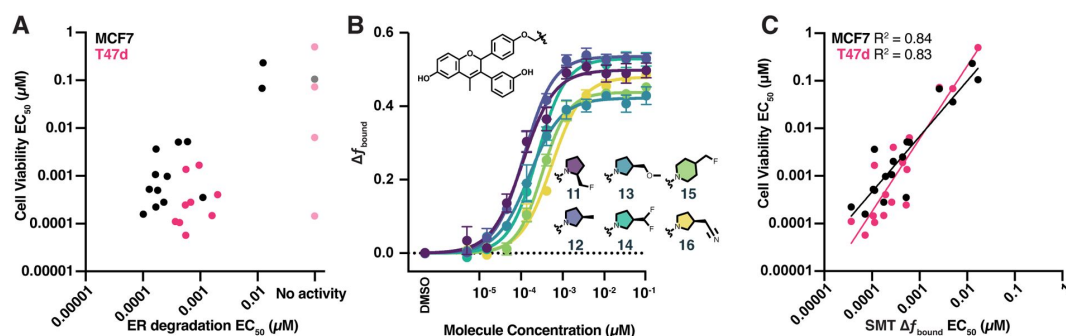


Figure 5.

htSMT can be used to determine chemical structure-activity relationships

- Correlation of potency measured by ER degradation and cell proliferation in MCF7 cells (black) and T47d cells (magenta) for compounds in the GDC-0927 structural series.
- Change in f_{bound} across a 12-pt dose titration of compounds 11 through 16, colored by structure. Points are the mean $\pm$ SEM across three biological replicates.
- Correlation of potency measured by change in f_{bound} and cell proliferation in MCF7 cells (black) and T47d cells (magenta) for compounds in the GDC-0927 structural series.

targets favors the view that these biological targets must themselves interact with ER, and that the compounds therefore affect ER dynamics indirectly. HSP90 is a chaperone for many proteins, including SHRs. In the canonical model, hormone binding releases the SHR-HSP90 complex. Indeed, HSP90 inhibitors increased f_{bound} for ER, AR, and PR, consistent with the hypothesis that one function of the chaperone may be to adjust the equilibrium of SHR binding to chromatin (Extended data 8B-D). Proteasome inhibition also leads to ER immobilization on chromatin⁶⁰, which aligns with the results that we obtained in our htSMT screen of bioactive compounds. ER has been shown to be phosphorylated by CDK⁶¹, Src⁶², or GSK-3 through MAPK and PI3K/AKT signaling pathways⁶³, and therefore inhibition of these pathways could reasonably be expected to affect ER dynamics measured using SMT. While CDK inhibition led to an increase in ER mobility, inhibition of PI3K, AKT, or other upstream kinases showed no effect (**Figure 3**).

Interestingly, SMT dynamics of an ER triple point mutant engineered to lack previously defined phosphorylation sites important for transactivation⁶³ (S104A/S106A/S118A) were affected by CDK and mTOR pathway inhibitors (Extended Data 9), suggesting that additional phosphorylation sites can mediate the effects of CDK9 and PI3K/AKT signaling, or that other molecular targets of CDK and PI3K/AKT can act indirectly to alter the motion of ER. The change in ER protein dynamics for characterized pathway inhibitors such as those targeting CDK and mTOR is subtle but consistent across compounds, suggesting biological meaning in these observations and highlight the need for accurate and precise SMT measurements. Hence htSMT screening offers the promise of providing comprehensive pathway interaction information or revealing novel interaction mechanisms.

Kinetic htSMT Facilitates Evaluation of Small Molecule Mechanism of Action

Since SMT can identify compounds that act either directly on a fluorescent target, or through some intermediary process, we sought to distinguish between these alternative modes of action. We hypothesized that by investigating the rate at which changes in protein motion emerge, SMT could be used to distinguish direct versus indirect effects on ER activity. Given the live-cell setting of SMT, we configured a data collection mode that allows for measurement of protein dynamics in set intervals after compound addition (kinetic SMT or kSMT). Both ER agonists and antagonists rapidly induce ER immobilization on chromatin when measured in kSMT ($t_{1/2} = 1.6$ minutes for estradiol; **Figure 4C**). On the other hand, HSP90 inhibitors like ganetespib and HSP990 exhibit a delay of 5 to 7 minutes before alterations in ER dynamics appear, after which we observed an increase in f_{bound} with a $t_{1/2}$ of 19.3 and 17.5 minutes, respectively. The overall effect of these compounds reached a plateau after an hour (**Figure 6B**). Proteasome inhibitors, e.g. bortezomib and carfilzomib, acted even more slowly, with changes in ER dynamics emerging only after 40 minutes, and slowly increasing over the four-hour measurement window (**Figure 6C**). Hence this exploration of SMT kinetics represents an important tool that can facilitate differentiation between on-target and on-pathway modulators. We believe that this approach will permit, for example, rapid mechanistic characterization of active compounds in a drug discovery setting.

To further differentiate the effect of pathway inhibitors on ER protein dynamics, we sought to characterize relative ER residence times for each such molecule. In contrast to the SERDs and SERNs, although HSP90 inhibition by HSP990 and ganetespib resulted in an increase in f_{bound} , we observed a decrease in the total number long binding events by two- and four-fold, respectively, while the binding times were similar to that observed with DMSO alone (**Figure 6D-E**). These results suggest HSP90 inhibition primarily increases k^*_{on} while leaving k^*_{off} largely unaffected. On the other hand, inhibition of the proteasome led to an increase in both the number and duration of long binding events. These results demonstrate that ER-chromatin binding can be modulated by changing the rate of association or disassociation, and that the inhibition of specific

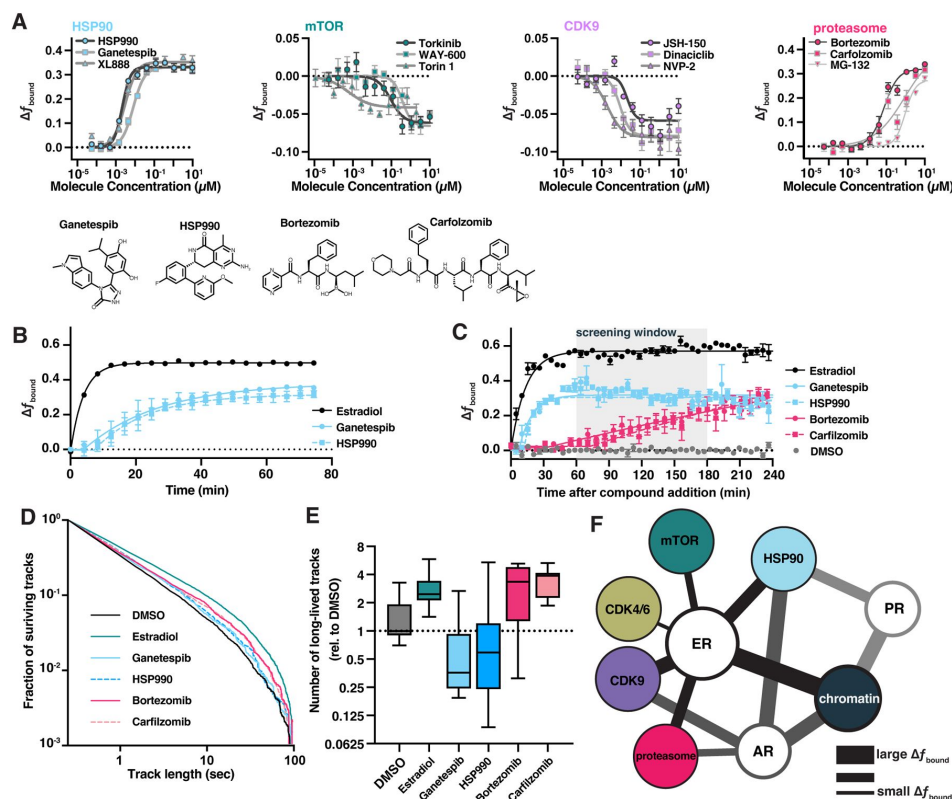


Figure 6.

Bioactive molecules targeting pathways associated with ER affect its dynamics

- Bioactive screen results with select inhibitors grouped and uniquely colored by pathway. Change in f_{bound} across a 12-pt dose titration of three representative compounds targeting each of HSP90, mTOR, CDK9 and the proteasome. Individual molecules are denoted by specific shapes. Error bars represent SEM.
- Change in f_{bound} as a function of time after compound addition. Estradiol treatment (black) is compared to ganetespib (blue circles) and HSP990 (blue squares). Points are bins of 4 minutes. Error bars represent SEM.
- Change in f_{bound} as a function of time after compound addition. Estradiol treatment (black) is compared to HSP90 inhibitors ganetespib (blue circles) and HSP990 (blue squares); proteasome inhibitors bortezomib (red circles) and carfilzomib (red squares). Points are bins of 7.5 minutes of htSMT data, marking the mean $\pm$ SEM. The shaded region denotes the window of time used during htSMT screening.
- Track length survival curve of ER-Halo cells treated either with DMSO alone, with estradiol stimulation, or with 100 nM HSP90 or proteasome inhibition. Track survival is plotted as the 1-CDF of the track length distribution; faster decay means shorter binding times.
- Quantification of the number of long-lived trajectories as a function of treatment condition. All conditions were normalized to the median number of trajectories in DMSO.
- Diagram summarizing pathway interactions based on htSMT results for ER, AR, and PR.

cellular partners can affect these rates differentially. Taken together with the different kinetics for direct ER, HSP90, and proteasome modulators, our data suggest that each class of molecule alters ER dynamics through distinct mechanisms.

Discussion

Many pathways that regulate the fundamental biochemistry of cells depend upon the interaction of protein “sensors” with distinct protein “effectors” that engage transiently to trigger a change in cell physiology. Although the fundamentals of this process have long been appreciated, biochemical investigation of these protein interactions has typically required *in vitro* reconstitution or has been interrogated through pull-down assays after cell permeabilization. Here we report the combination of SMT, a type of super-resolution microscopy, with high content microscopy as a means of visualizing individual protein motion in millions of live cells, and under circumstances where the effect of small molecule inhibitors can be assessed quantitatively. We demonstrate the capabilities of the htSMT platform by analyzing the behavior of SHRs, a class of sensors that mediate hormone-induced modulation of gene expression, and in particular the dynamics of the ER.

To validate our htSMT platform, our analysis initially focused on the very rapid immobilization of SHRs on chromatin observed in cells exposed to their established, cognate steroid ligands. The technique proved to be highly quantitative, effectively evaluating ligands whose potency differ by more than four orders of magnitude, and readily characterizing differences in both the sensitivity and the selectivity of the steroid receptor family. These observations prompted us to apply htSMT to screen thousands of bioactive compounds, which we hypothesized would reveal new chemical matter as well as enable comprehensive pharmacologic dissection of ER pathway interactions. As further validation of the technique, automated screening of ER dynamics using htSMT identified all 30 known steroid ligands from a library of 5,067 bioactive compounds. The potency of these steroid ligands with respect to alterations in ER dynamics varied across a thousand-fold range, demonstrating the dynamic range of this single experimental setup. Among molecules known to behave as ER signaling inhibitors, the change in ER dynamics correlated closely with the ability of these compounds to block estrogen-induced proliferation of estrogen dependent breast cancer cells, demonstrating the ability of htSMT to document structure-activity relationships in a chemical series across disparate and biologically relevant readouts. Since our analysis relies only on detection of changes in protein dynamics, this unbiased readout will prove broadly useful in screening libraries of compounds to identify starting points for the development of new therapeutics. In fact, our analysis identified 209 non-steroidal molecules that affect ER dynamics, which are likely to act elsewhere in the network of ER-interacting proteins.

The observation that by screening large libraries of bioactive compounds for an effect on protein motion, htSMT can define networks of biochemical signaling pathways is a critical outcome of this high-throughput platform. Protein dynamics in the cell are not governed by singular interactions between any two proteins but by biochemical networks within the cell that intersect with the protein under observation. Our work enables construction of a putative interaction network connecting nodes involving different proteins, the identities of which were deduced based on the impact of known inhibitors on the dynamics of steroid receptors (**Figure 6F** [↗](#)). The interaction map derived from unbiased htSMT screening recapitulates many known biological interaction partners of the ER, in a single experimental setup using protein motion as a sole readout. More experiments are necessary to determine which nodes represent direct physical interactions and which occur through intermediaries. We believe that identifying additional cellular interaction networks through htSMT will provide an important foundation for the broader understanding of biochemical regulatory mechanisms. For example, CDK4/6 inhibitors are co-administered with SERDs and SERMs to improve therapeutic outcomes^{64,65} [↗](#), CDK4/6 inhibition only minimally

affects ER dynamics in SMT (**Figure 3**), supporting the view that the combination of CDK4/6 inhibition with ER antagonists functions through a non-redundant ER-independent mechanism.⁶⁶

Most cell-based assays cannot easily be configured for kinetic analysis of treatment effects, as these typically involve fixed-endpoint, aggregate readouts. With such endpoint assays, biochemical feedback and compensatory mechanisms often confound interpretation of the direct effect of a change in treatment conditions. SMT, however, when implemented in a high-throughput system that exhibits consistency over long time intervals, permits kinetic analysis of treatment effects on protein dynamics in real time with high reliability. Such kinetic analyses help to define pathway cascades; in a first instance, they can be used to rapidly identify compounds that likely engage directly with a therapeutic target.

Mechanistically, an increase in f_{bound} provoked by a change in cell conditions suggests that molecular interactions with the protein being analyzed have been stabilized (either made more probable or longer-lasting), while a decrease in f_{bound} suggests the opposite: molecular interactions that have been made less robust. Unexpectedly, we observed both SERDs and SERMs, which antagonize ER, cause an increase in nuclear bound fraction, likely via chromatin binding, in a way that mimics what is seen with traditional ER agonists. We showed that this mechanism is not a commonality among all SHRs, and indeed inhibitors of AR and GR behaved more like the prototypical competitive antagonist. Inhibition of HSP90 also produces a marked increase in ER f_{bound} , though these binding events are more transient (**Figure 6E**). Transcription factors are thought to find binding sites through free 3D diffusion, 1D sliding, and transient, non-specific DNA binding.^{5,12,20,21,24} ER antagonists may function by promoting ER binding to non-specific decoy chromatin sites, thus reducing the amount of ER able to activate transcription at ER-responsive genes.^{31–33} Previous work has also shown that SERDs, and to a lesser extent SERMs, induce an alternate conformation of ER, thereby inhibiting co-factor recruitment.³¹ If co-factors like CBP and p300 stabilize ER-chromatin binding, then efficacious inhibitors might exhibit shorter binding times compared with agonist stimulation.³¹ The SERMs 4OHT and GDC-0810 dramatically increase ER-chromatin binding frequency and only modestly increase binding times; fulvestrant and GDC-0927 strongly increase both ER-chromatin binding frequency and residence time. Therefore, htSMT suggests that agonists and antagonists of SHR signaling operate through a previously unappreciated, unified mechanism of chromatin immobilization, meriting further investigation.

Lastly, we note that fast-SMT, as implemented here, can define relationships between the structures of chemical inhibitors and their effects on a fundamental property of protein regulatory elements: their dynamics in living cells. In our hands, these measurements proved far more reliable in predicting the ability of an ER antagonist to block cell proliferation than an ER degradation assay, a conclusion that was only able to be drawn due to the scale of htSMT screening capabilities. Notably, saturable dose responses were observed for ER antagonists at much lower concentrations using htSMT than with ER degradation assays, suggesting that this method will be more sensitive for identifying novel therapeutic agents than with corresponding traditional assay formats. Thus, SMT can identify promising compounds the activity of which might not otherwise be measurable, provide insight into their mechanism(s) of action, and in the native environment of the cell. While ER is a transcription factor, the same principles may apply broadly to regulatory mechanisms of proteins with diverse function. In this context, we conclude that the application of technologies for measuring protein dynamics at scale will prove broadly applicable to the elucidation of biological mechanisms.

Author Contributions

D. A., H. P. B., D. T. M., & Y. T. conceived the research project; D. A., H. P. B., X. D., E. G., A. He., J. H., H. L., D. T. M., S. E. P., R. T., & Y. T. contributed to study design; H. C., K. F., E. G., R. Kr., H. L., D. T. M., S. E. P., A. S., & R. T. performed experiments; H. C., E. G., H. L., D. T. M., A. S., & R. T. generated reagents; L. A., D. A., A. Ha, A. He., H. L., D. T. M., C. S. & R. T. analyzed data; S. A., R. B., M. B., R. G., A. Ha, A. He, S. L. J., R. Ke., A. K., J. L., & L. M. designed and wrote software; R. B., X. D., R. Ke., K. L., B. M., & D. T. M. designed and built hardware; D. A., H. P. B., R. B., J. H., A. K., & R. Ke. supervised the work; L. A., D. A., A. He., H. L., D. T. M., S. E. P., & R. T. wrote the original draft; L. A., D. A., H. P. B., R. B., X. D., A. He., J. H., H. L., D. T. M., R. T., & Y. T. reviewed and revised of the original draft.

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Declaration of interests

The authors are employees and/or shareholders of Eikon Therapeutics.

Extended Data

Methods

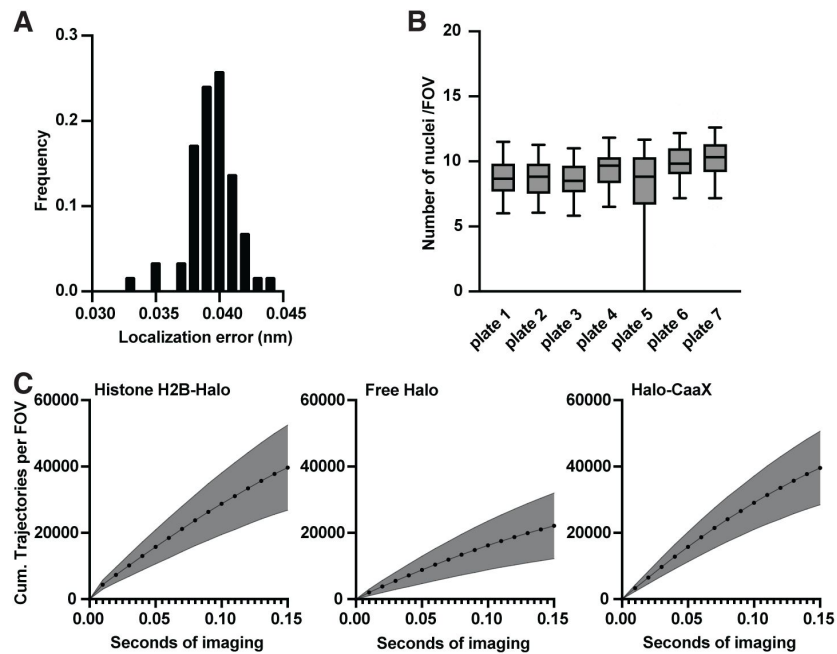
Cell Lines

U2OS (ATCC Cat. No. HTB-96), MCF7 (ATCC Cat. No. HTB-22), T47d (ATCC Cat. No. HTB-133) and SK-BR-3 (ATCC Cat. No. HTB-30) were grown in DMEM (Cat. No. 1056601,

Gibco DMEM, high glucose, GlutaMAX Supplement, ThermoFisher) supplemented with 10% Fetal Bovine Serum (Cat. No. 16000044, ThermoFisher) and 1% pen-strep (Cat. No 15140122, Thermo Fisher) and maintained in a humidified 37 °C incubator at 5% CO₂ and subcultivated approximately every two to three days.

HaloTag-expressing cell lines

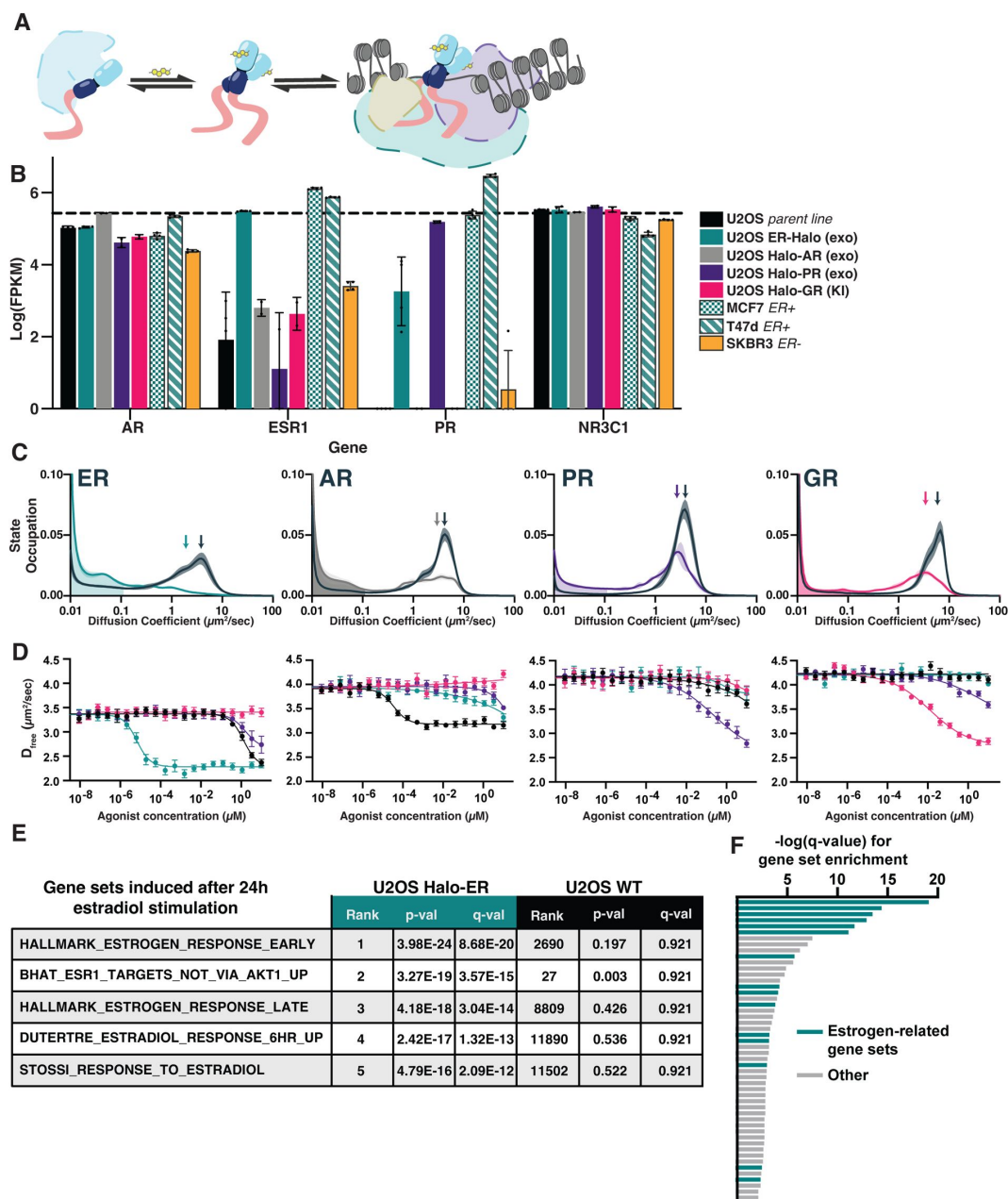
For ER, AR, and PR-HaloTag fusions, mammalian expression vectors containing the fusion gene under the control of a weak L30 promoter and containing a Neomycin resistance marker were transfected into U2OS cells at 70% confluence using FuGENE 6 (Cat. No. E2691, Promega). Transfected cells were selected with G418 (Cat. No. 10131027, Thermo Fisher) at 500 µg/mL, then clonally isolated. Clones expressing the desired fusion gene were determined first by staining with 100 nM JF₅₄₉-HTL (Cat. No. GA1110, Promega) and 50 nM Hoechst 33342 and identifying clones with the expected distribution of JF₅₄₉ signal. Between three and six clones were subsequently tested using SMT conditions for response to a control compound, and the most homogenous clones were subsequently expanded for further testing. Unless otherwise specified, all experiments are with a single, clonally isolated cell line. Because U2OS cells express GR endogenously, HaloTag was



Extended Data figure 1.

Characterization of htSMT system performance.

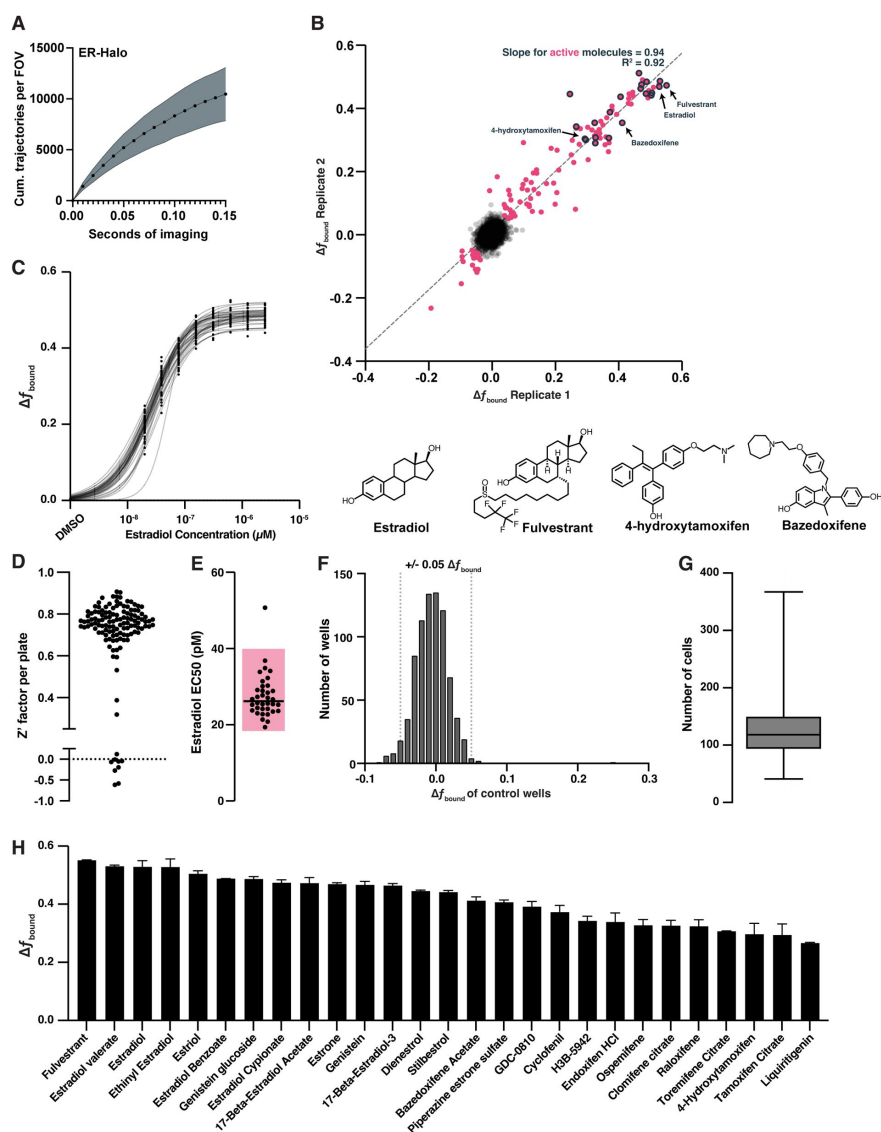
- Distribution of localization error measured across multiple independent wells using Histone H2B-Halo cells. Median localization error of 39 nm.
- Number of measurable nuclei in a 94 by 94 μm FOV. Each box plot is the distribution over wells in one 384-well plate.
- Cumulative number of trajectories as a function of imaging time for each individual cell type for cells labeled with 10 pM JF₅₄₉. Shaded error bars are 1 S.D.



Extended Data figure 2.

Activation of steroid hormone receptors changes free diffusion and impacts downstream gene expression.

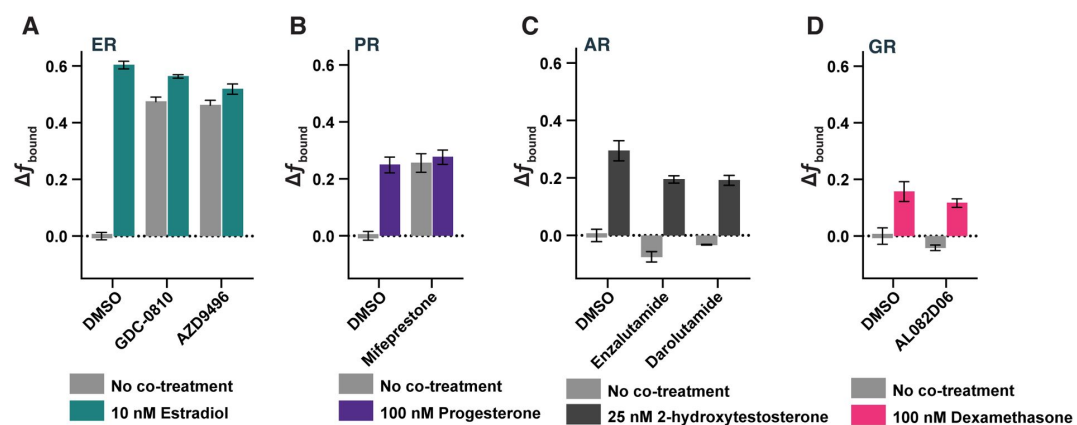
- Cartoon illustration of SHR function. Under basal conditions, SHRs are sequestered in complex with HSP90 and other cofactors. Upon ligand binding, the receptor dissociates from the inactive complex, dimerizes, and binds to DNA.
- mRNA transcript levels in log(FPKM) of AR, ESR1, PR, and NR3C1 for the engineered U2OS cell lines compared to the parental U2OS line as well as three reference breast cancer cell lines.
- Distribution of diffusive states for Halo-AR, Halo-ER, Halo-GR, and Halo-PR in U2OS cells before and after stimulation with an activating ligand. Arrows indicate the mean D_{free} . Shaded error bands represent 1 S.D.
- Selectivity of individual SHRs to their cognate ligand compared with other steroids, as determined by D_{free} . Error bars represent SEM.
- Reference gene sets induced after 24 hrs of stimulation with 25 nM estradiol. The top five induced gene sets for Halo-ER ectopic expression increases were not significantly induced in the parental U2OS line.
- Bar plot showing the $-\log(q\text{ value})$ from the top 50 most significantly induced gene sets after estradiol stimulation. Gene sets characteristic of ESR1 or the estrogen response are colored green.



Extended Data figure 3.

Screen of bioactive molecules produces robust data with good assay performance.

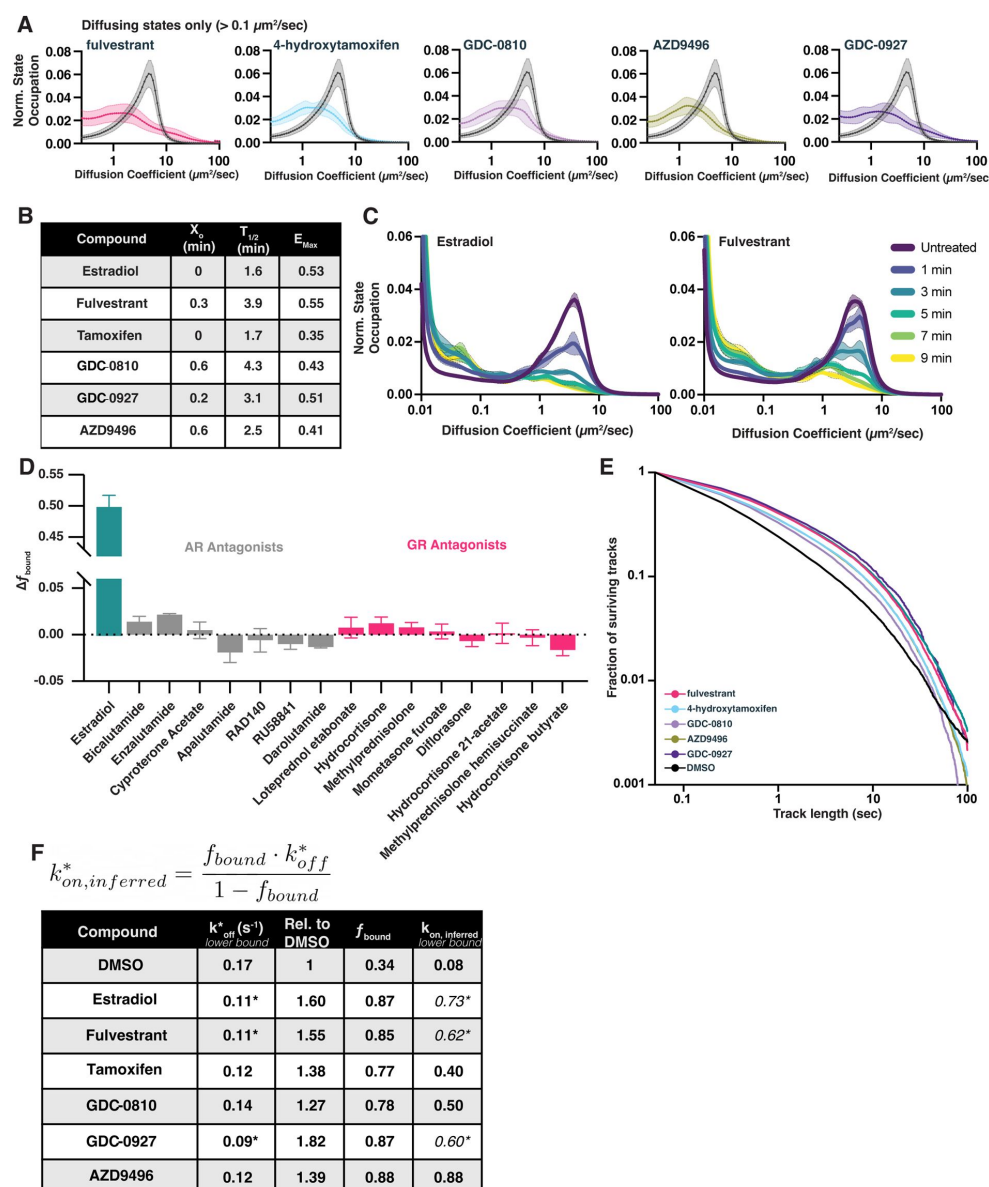
- Cumulative number of trajectories as a function of imaging time for DMSO-treated cells labeled with 20 pM JF₅₄₉. Shaded error bars are 1 S.D.
- Change in f_{bound} repeatability of screening results assessed over two biological replicates. Compounds in magenta were identified as active if their magnitude of change in f_{bound} when averaged across both replicates was greater than 0.05. Of the expected 30 positive control compounds, 26 significantly increased f_{bound} in both replicates (gray outline). Three compounds increased f_{bound} but were only present in one replicate after filtering. Exemplified positive controls including the agonist estradiol (1) and the antagonists fulvestrant (5), 4-OHT (6) and bazedoxifene (7) are exemplified. A linear regression was fit to the magenta compounds to determine slope and correlation.
- Z'-factor analysis for the bioactive screen. Each point represents the Z'-factor of a single 384-well plate, measuring the difference between DMSO and 25 nM estradiol treatment. Plates with very low Z'-factors were removed from further analysis.
- Dose titration of estradiol for each plate in the bioactive molecules screen fit with a logistic regression to determine potency.
- EC₅₀ values extracted from each curve fit in (c). Shaded region is a three-fold range in potency.
- Distribution of change in f_{bound} for control DMSO wells.
- Boxplot showing the number of cell nuclei measured for each compound tested. Whiskers are the 1st and 99th percentiles.
- Change in f_{bound} for 30 known ER-interaction molecules circled in (b), ordered by the magnitude of effect. Error bars are the SEM.



Extended Data figure 4.

Antagonists of ER (a), PR (b), AR (c) and GR(d) show distinct effects on target protein dynamics.

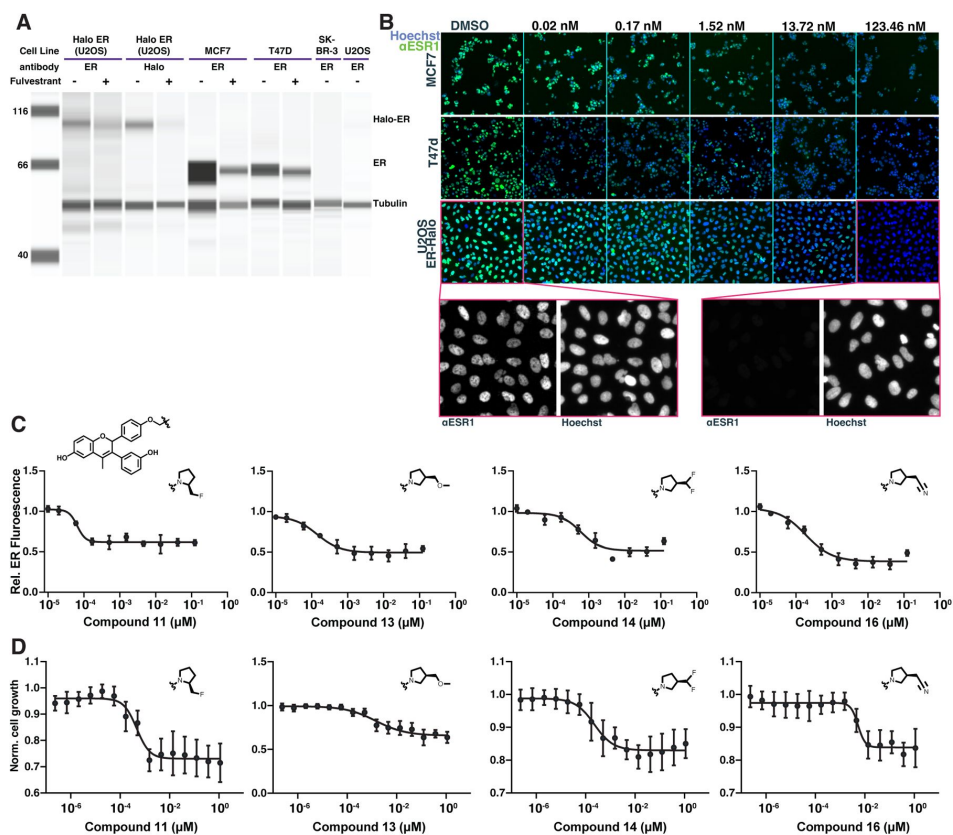
Change in f_{bound} after addition of 1 μM antagonist either in the absence or presence of the cognate agonist.



Extended Data figure 5.

SERMs and SERDs decrease free diffusion and increase f_{bound} rapidly after addition.

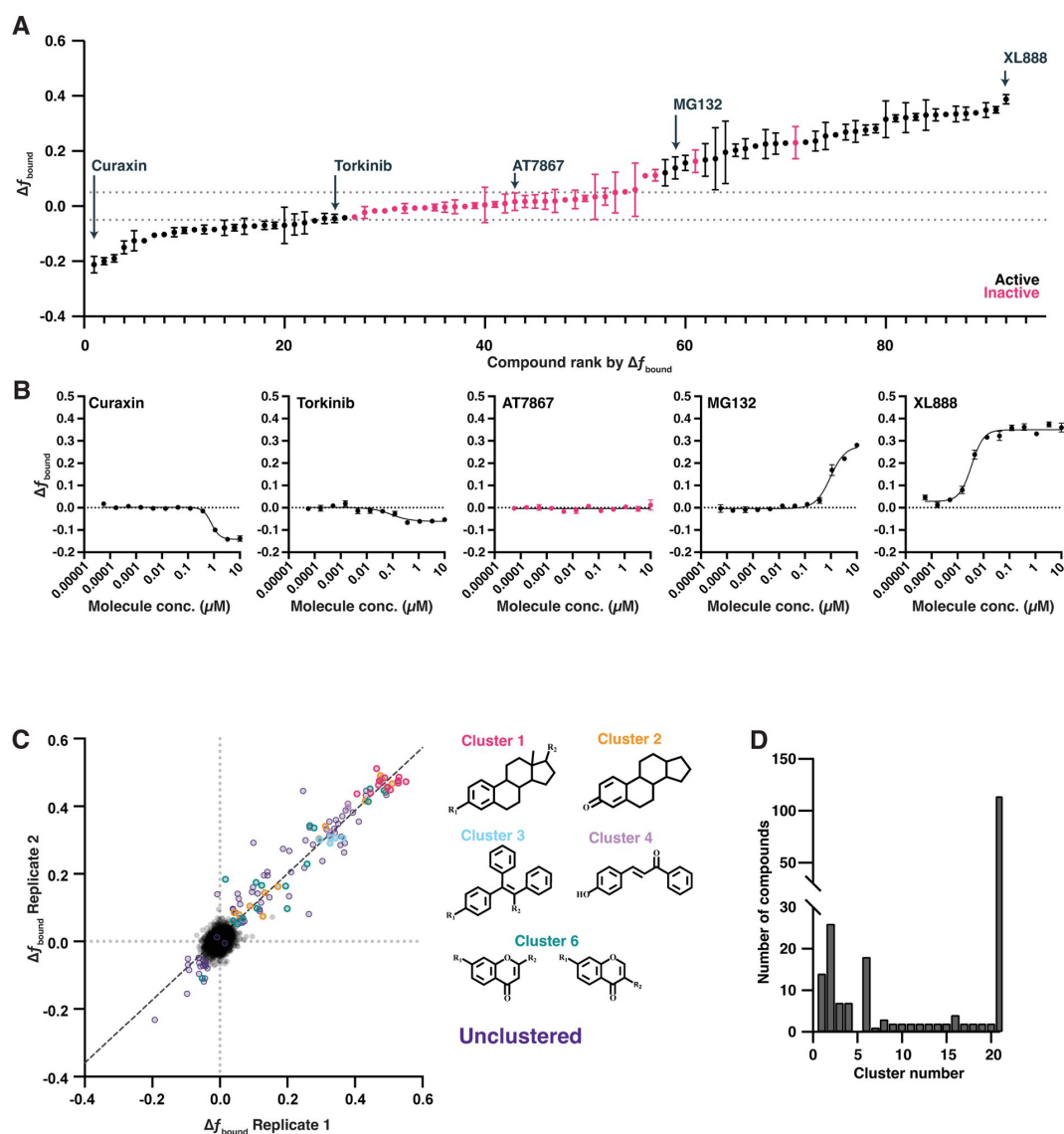
- Normalized occupation of diffusive states (diffusion coefficient $0.2 - 100 \mu\text{m}^2/\text{sec}$) for 100 nM SERD or SERM-treated samples compared with DMSO. Histograms are normalized to integrate to 1. Shaded regions represent bin-wise standard deviations. Curves are the average of 3-4 biological replicates, with 8 well replicates per condition (>1200 cells total)
- Results from a single-exponential association fit to the data in **Figure 4C**.
- Change in diffusive state distribution as a function of time after compound addition for estradiol and fulvestrant. Each curve represents the mean of three biological replicates. Shaded regions are the bin-wise standard deviation.
- Change in f_{bound} of selective AR or GR antagonists from the bioactive screening set.
- Track length survival curve of ER-Halo cells treated either with DMSO alone or with SERM/D. Track survival is plotted as the 1-CDF of the trajectory length distribution; faster decay means shorter binding times.
- Table of results of the slow decay rate constant (k_{slow}) from residence time SMT curve fits. Fits were performed on the aggregate of three biological replicates. Taken together with the f_{bound} determined in 4D, an upper limit of the inferred k_{on} can be calculated from the equation assuming all bound molecules will have $k^* = k_{\text{slow}}$. Cells marked with an asterisk are ones in which k_{slow} could not reliably be distinguished from photobleaching.



Extended Data figure 6.

GDC-0927 structural variants characterized by ER degradation or cell proliferation assays.

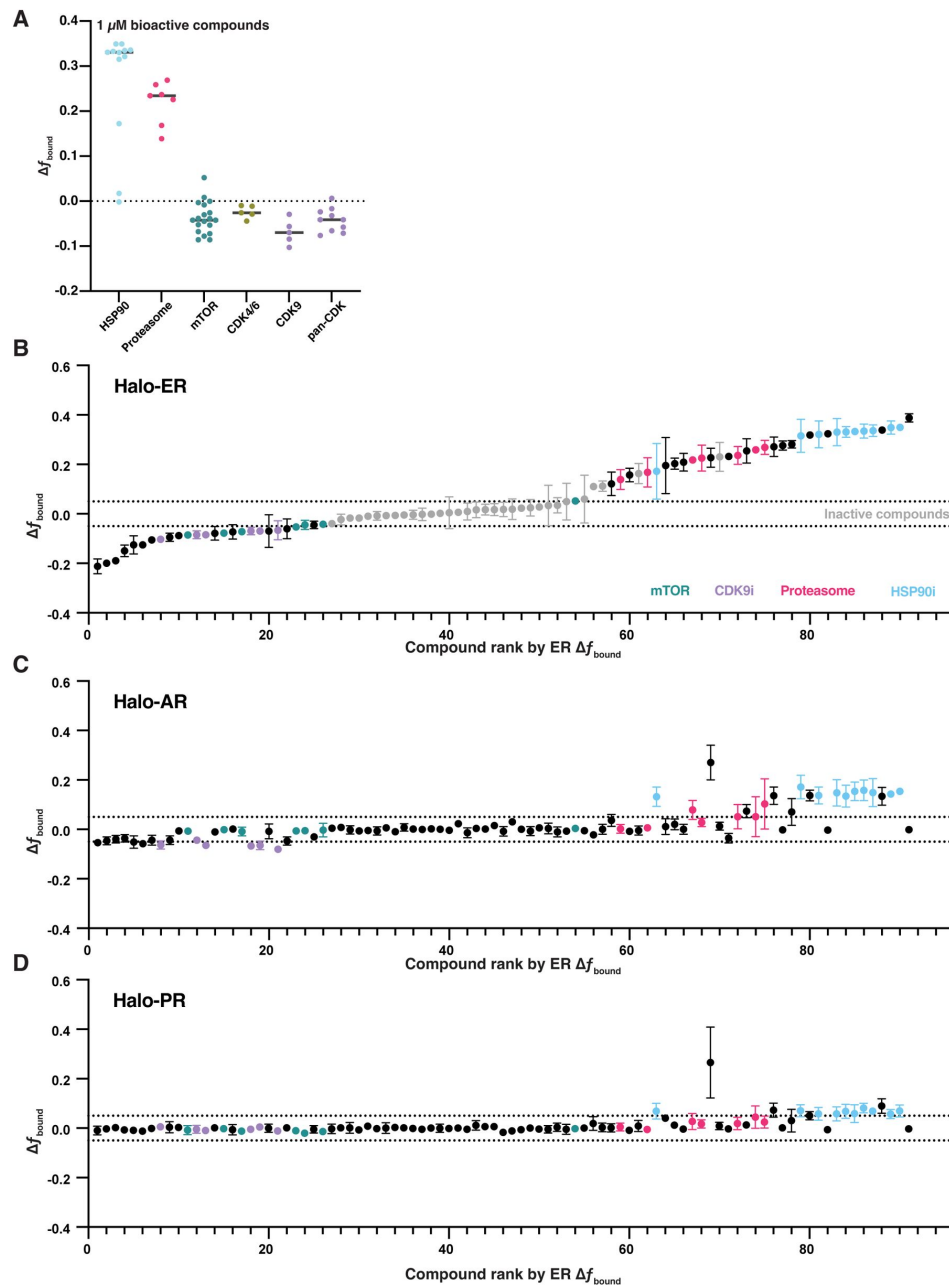
- Western blot of the ER-expressing breast cancer cell lines MCF7 and T47d compared to ER expression in ER-null lines SK-BR-3 or U2OS. Samples were treated with fulvestrant for 24 hours prior to lysis. Fulvestrant treatment leads to the degradation of ER, even when fused to HaloTag.
- Example of GDC-0927 induced degradation of ER in different cell lines measured by immunofluorescence against ER. Cells were exposed to compound for 24 hours prior to fixation. Image pixel intensities are equivalently scaled.
- Example compound dose titrations showing change in mean nuclear intensity as a function of compound concentration.
- Example compound dose titrations measuring cell proliferation, normalized to DMSO-treated cells, of cells treated with analogs of GDC-0927.



Extended Data figure 7.

Deeper investigation of bioactive screening data identifies cutoff for active molecules.

- Examples of compound effects on $\text{ER } f_{\text{bound}}$ from a dose titration experiment. 92 compounds from the primary screen are ranked based on the magnitude of their effect. Compounds colored in black repeatably showed dose-dependent changes; magenta compounds were inactive in a dose titration.
- Example dose titration of compounds of increasing overall change in $\text{ER } f_{\text{bound}}$.
- Two replicates of the bioactive screen as in [Figure 3](#). Active compounds are colored based on structural scaffold.
- A quantification of the number of compounds associated with any given cluster. Singletons (cluster 21) represent the majority of active compounds.



Extended Data figure 8.

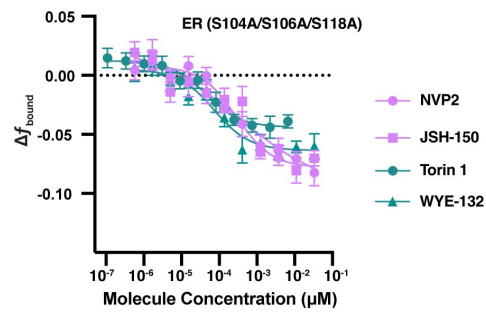
Some pathway inhibitors modulating ER dynamics are specific to ER.

a) Change in f_{bound} of ER following treatment with inhibitors of HSP90, the proteasome, mTOR, and CDK from the initial bioactive screen. CDK inhibitors were further stratified into CDK4/6 inhibitors, CDK9 inhibitors, or inhibitors without strong selectivity for a particular family (pan-CDK). Line represents the median for each target.

b) Change in f_{bound} of ER for 97 bioactive molecules, colored by their pathway annotation. Compounds are plotted in their rank order based on their effect in ER. Error bars are the SEM of three biological replicates.

c) Change in f_{bound} of AR for the same molecules from (b). Compounds are plotted in their rank order based on their effect in ER. Error bars are the SEM of three biological replicates.

d) Change in f_{bound} of PR for the same molecules from (b). Compounds are plotted in their rank order based on their effect in ER. Error bars are the SEM of three biological replicates.



Extended Data figure 9.

Dose titration plots of ER(S104A/S106A/S118A) with mTOR and CDK9 compounds.

Each point represents the mean and SEM of three biological replicates.

inserted right before the stop codon of endogenous NR3C1 via homology-directed repair using CRISPR/Cas9. The HaloTag knock-in was validated by imaging using HTL-JF₆₄₆ staining and through DNA sequencing⁶⁷.

Western Blot

Cells were grown in the same conditions as described previously. 1.5×10^6 cells were seeded per well in a 6-well plate in DMEM overnight, followed by compound treatment (DMSO or 100nM fulvestrant) the following day for 24 hours. Cells are lysed in 200 μ L 1X Cell Lysis Buffer (catalogue number 9803, Cell Signaling). Protein lysate concentration is then determined using BCA protein assay kit (Catalog number 23225, Pierce™ BCA Protein Assay Kit) following manufacturer instructions. Capillary Western Immunoassay were performed using Jess Protein Simple following manufacturer's instruction (protein simple, USA). Levels of α ER (1:100, RM-9101) were normalized to loading control β -tubulin (1:100, NC0244815 LI-COR 92642213, Thermo Fisher). The peaks were analyzed with the Compass software (Protein Simple, USA).

RNA-seq

Cells were seeded into 12-well tissue-culture treated plates at densities of 250,000 cells (U2OS-WT), 200,000 cells (U2OS-ER), or 300,000 cells (MCF7, SKBR3, T47d) per well. 24 hours later, cells were treated with estradiol at a final concentration of 25nM for 24 hours. To process cells for total RNA, cells were washed twice with ice-cold PBS, lysed with 350 μ L Buffer RLT (Qiagen 79216), scraped off the plate (Fisher 08100241), frozen on dry ice and stored at -20 degrees C. Cell lysates were then thawed, homogenized using QIAshredder columns (Qiagen 79656), and processed through the Qiagen RNeasy Micro kit (Qiagen 74004) using the standard protocol and including the optional on-column DNase digestion step (Qiagen 79254). All samples had a RIN score of 10 by TapeStation (Agilent 5067-5576). RNA sequencing libraries were prepared from total RNA by Novogene (CA). In brief, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads and fragmented. First-strand synthesis was performed using random hexamer primers, second-strand synthesis was performed using dTTP, and libraries were prepared after end repair, A-tailing, adapter ligation, amplification, and purification. Libraries were sequenced on an Illumina NovaSeq with paired 150 cycle reads. For data analysis, paired-end reads were aligned to the hg38 reference genome using Hisat2 v2.0.5, featureCounts v1.5.0-p3 was used to count the number of reads mapped to each gene, and differential expression analysis was performed using DESeq2 (1.20.0).

Single Molecule Tracking Sample preparation

Cells were seed on tissue culture treated 384-well glass-bottom plates at 6000 cells per well. Seeded cells were then incubated at 37 °C and 5% CO₂ to allow adhesion overnight. For all SMT experiments, cells were incubated with 5-100 pM of JF₅₄₉-HTL (Cat. No. GA1110, Promega) and 50 nM Hoechst 33342 for an hour in complete medium. Cells were then washed three times in DPBS and twice in imaging media, which is fluoroBrite DMEM media (Cat. No. A1896701, Thermo Fisher) supplemented with GlutaMAX (Cat. No. 35050079, Thermo Fisher) and the same serum and antibiotics as growth media. Where appropriate, compounds were serially diluted in an Echo Qualified 384-Well Low Dead Volume Source Microplate (0018544, Beckman Coulter) to generate dose-titration source material. Compounds were administered at a final 1:1000 dilution in cell culture medium. Each dose of a compound has at least 2 replicates per plate and 3 plate replicates, 20 DMSO control wells and 2 no dye control wells were randomized across each plate. Unless otherwise specified, compounds were allowed to incubate for an hour at 37 °C prior to image acquisition.

Image Acquisition

Unless otherwise stated, all image acquisition using SMT was performed on a custom-built HILO microscope based on a Nikon Ti2, motorized stage, stage top environmental chamber (OKO labs), quad-band filter cube (Chroma), custom laser launch with 405 nm, and 561 nm wavelengths, delivering >10 mW and >150 mW of power to the back focal plane of the objective, respectively. Fluorescence emission was passed through a high-speed filter wheel (Finger Lakes Instruments) and collected with a backlit CMOS camera (Prime 95b, Teledyne). Images were acquired with a 60X 1.27 NA water immersion objective (Nikon). Environmental chamber was set to 37° Celsius, 95% humidity, and 5% CO₂. For each field of view, 200 SMT frames were collected at a frame rate of 100 Hz, with a 2 msec stroboscopic laser pulse. 10 frames of the Hoechst channel were collected at the same frame rate for downstream registration of trajectories to nuclei.

Image Analysis

Image acquisition produced one JF₅₄₉ movie and one Hoechst per field of view. The JF₅₄₉ movie was used to track the motion of individual JF₅₄₉ molecules, while the Hoechst movie was used for nuclear segmentation. Tracking was accomplished in three sequential steps – detection, subpixel localization, and linking – using a combination of existing methods. Briefly, spots were detected using a generalized log likelihood ratio detector⁶⁸. After detection, the estimated position of each emitter was refined to subpixel resolution using Levenberg-Marquardt fitting^{69–71} with an integrated 2D Gaussian spot model⁷² starting from an initial guess afforded by the radial symmetry method⁷³. Detected spots were linked into trajectories using a custom modification of a hill-climbing algorithm^{10,74}. The same detection, subpixel localization, and linking settings were used for all movies used in this manuscript.

For nuclear segmentation, all frames of the Hoechst movie were averaged to generate a mean projection. This mean projection was then segmented with a neural network trained on human-labeled nuclei⁷⁵. Each spot was assigned to at most one nucleus using its subpixel coordinates.

To recover dynamical information from trajectories, we used state arrays³⁵, a Bayesian inference approach, with the “RBME” likelihood function and a grid of 100 diffusion coefficients from 0.01 to 100.0 $\mu\text{m}^2 \text{s}^{-1}$ and 31 localization error magnitudes from 0.02 to 0.08 μm . After inference, localization error was marginalized out to yield a one-dimensional distribution over the diffusion coefficient for each field of view. For single-cell analysis, we performed SMT and nuclear segmentation on a mixture of U2OS cells bearing H2B-HaloTag, HaloTag-CaaX, or free HaloTag. We then evaluated the marginal likelihood of each of a set of 100 diffusion coefficients on the set of trajectories within each segmented nucleus³⁰. These marginal likelihood functions were clustered with k-means (3 clusters), and the marginal likelihood functions for each cell were ordered by their cluster index to produce the heat map. To estimate the fraction bound (f_{bound}), we integrated the state array posterior distribution below 0.1 $\mu\text{m}^2 \text{s}^{-1}$. To estimate the free diffusion coefficient (D_{free}), we computed the mean of the posterior distribution above 0.1 $\mu\text{m}^2 \text{s}^{-1}$.

Data analysis

Tracking results from the automated processing pipeline were analyzed using KNIME or Spotfire (TIBCO). Individual f_{bound} or D_{free} measurements were associated with experimental metadata and aggregated by condition. Change in f_{bound} was calculated as the difference between the f_{bound} of each well and the median f_{bound} of DMSO in the same plate. Wells that had no cells in the field of view or in which the field of view was out of focus were omitted from further analysis. Compounds were assessed for assay interference using the median fluorescence intensity of the tracking channel and omitted if it was more than 3 standard deviations higher than the median intensity of the DMSO wells. Similarly, plates where the active and negative controls could not be clearly resolved or where the significantly deviated from the performance of the rest of the screen were removed from further analysis. Finally, compound with a variance more than three standard

deviations higher than the average compound variance (41 compounds; 0.08%) were removed from downstream analysis. Z'-factor between the active controls on a plate and DMSO was calculated as previously described³⁸. EC₅₀ values were calculated in Prism (GraphPad) by first log-transforming the molecule concentrations and then fitting to a four parameter logistic curve.

Clustering active molecules

Chemical structure-based clustering was performed on molecules identified as active (239 in total). Molecular frameworks were computed as described by Murcko et al and as implemented in Pipeline Pilot⁷⁶. Molecular frameworks were clustered using functional class fingerprints (FCFP₄) with a similarity threshold cut-off of 0.3 Tanimoto distance. A total of 21 clusters were obtained with singletons being the major class (124 molecules). The next largest group was the flavone class represented by 27 members, followed by a couple of diverse classes within the steroidal class with 14 and 20 members respectively. The other category is the stilbene class with 7 members representing tamoxifen as one of the members. The remaining actives (47 molecules) were grouped into one 3-membered cluster and all the others with 2 members per cluster.

Kinetic Experiments

Cells were seeded into a 384-well plate the day before, dyed, and washed as described above. 1 well with 25 FOVs per well were taken as a baseline reading. Then, while imaging, compound was manually added to each well to a final concentration of 100 nM. Data was then collected for 20 wells. A pause was included between each FOV such that the entire imaging regime covers the assay window. Change in f_{bound} was determined per-well relative to $t=0$.

For assays extending to 4 hours, the plate was imaged twice with 8 FOVs per well with different FOV locations per readthrough to prevent photobleaching from impacting data. All data presented represents was performed in three different biological replicates.

Residence Time Imaging

Sample preparation and execution of residence time imaging experiments were conducted in a similar manner to the single molecule tracking assay described above with a few exceptions. Samples were dyed with 1 – 10 pM JF₅₄₉ (Promega) and 50 nM Hoechst 33342 for an hour. 400 frames per field of view were collected with a camera integration time was set to 250 msec, and laser sources reduced to 5 mW at the objective. During image acquisition, lasers were on continuously. Compound incubation ranged from 1 to 4 hours. At least 8 well replicates were collected per condition.

Residence Time analysis

Quantifying transcription factor binding times on DNA is an open problem with multiple proposed solutions^{21,77,78}. Here we adopted an approach similar to Hansen et. al.¹⁹. Image processing, including spot detection, localization, and track reconnection were performed using the same methods described above. Because residence time imaging selectively tracks slow-diffusing molecules, individual localizations were limited to a 300 nm maximum displacement for individual jump reconnections. Sets of trajectories for each field of view were binned into 1-CDF distributions as previously described and fit to a two exponent decay model:

$$CDF(t) = A(Fe^{-k_{\text{fast}}t} + (1 - F)e^{-k_{\text{slow}}t}).$$

The k_{slow} term comprises both the rate of molecule unbinding (k_{off}) as well as photobleaching (k_{bleach}) and diffusion of chromatin out of the focal volume. Often approaches attempt to derive the unbinding rate by applying a correction such as subtracting a bleaching rate measured either directly in the sample or by using a separate control sample^{19,21}, where $T_{\text{corrected}} = (k_{\text{off}} - k_{\text{bleach}})^{-1}$. The inverse relationship between $T_{\text{corrected}}$ and k_{bleach} makes it highly sensitive and

nonlinear to noise in k_{bleach} . Because the 1-CDF distributions of compound-treated ER samples are so close in decay rate to the control Histone H2B, background subtraction yields unfeasible k_{off} values after correction. Instead we report only the uncorrected k_{slow} values with the understanding that these represent a lower-bound of the actual k_{off} , but that within-experiment comparisons can be made between conditions.

Fluorescence Recovery After Photobleaching

Images were acquired on a custom-built HiLo microscope as described above with a Spectra Light Engine RS-232. Stimulation was directed using a miniscanner coupled with a Coherent OBIS 561nm 100 mW laser. All imaging was performed using a 60X 1.27 NA water immersion objective (Nikon). All experiments were performed at 37° Celsius. For FRAP experiments, Cells were seeded into a 384-well plate the day before, labeled with 50 nM HTL-JF₅₄₉, and washed as described above. Compound was added to 100 nM final an hour before imaging. Then, a prebleach image was acquired by averaging 10 consecutive images. Then 8-10 regions were bleached (2 background, 6-8 cells) and 2 regions in cells were unbleached. Regions that were bleached were bleached at 10% power without scanning. For the next 30 seconds, an image was acquired every 200 ms, then every 1 second for 2 minutes. The background-subtracted average intensity was measured in the region of interest over time and normalized to the average of the fluorescence in the baseline images, then normalized to the unbleached regions to account for readout-induced photobleaching of fluorophores. Data from 18-24 cells were pooled per experiment for 3 biological experiments.

Immunofluorescence

Cells were grown in conditions as described previously. Cells were seeded in glass bottom 384-well plates coated with 0.05mg/ml PDL (Cat. No. A3890401, Thermofisher) at 6000 cells per well for Halo-ER U2OS cells and 8000 for MCF7 and T47d cells. Cells were grown overnight followed by compound treatment on the second day for 24 hours at 37 °C and 5% CO₂. Compounds were serially diluted in an Echo® Qualified 384-Well Low Dead Volume Source Microplate (0018544, Beckman Coulter) to generate a 21-point dose response at 1:3 dilution starting from a concentration of 10mM. Compounds were administered at a final 1:1000 dilution in cell culture medium. An 8 to 12-point dose response was selected based on the potency of each compound. Each concentration was replicated at least once per plate and has at least 2 plate replicates. Cells were fixed by addition of paraformaldehyde (Cat. No. 15710-S; Electron Microscopy Sciences), with a final concentration of 4% for 20 minutes. Cells were then permeabilized using blocking buffer containing 1% bovine serum albumin and 0.3% TritonX100 in 1x PBS for an hour at room temperature. Immunofluorescent staining of ER was carried out using α ER antibody (1:500, RM-9101) diluted in the same blocking buffer for 1 hour at room temperature. Extensive washing with PBS was performed prior to secondary antibody staining. Secondary antibody staining was carried out using Alexa fluor 488 conjugate anti-rabbit IgG (1:1000, Cat. No. A32731, thermos Fisher) for an hour. Nuclear staining was carried out using Hoechst 33342 solution at 1mg/ml. Imaging of immunofluorescence was done using the ImageXpress Micro (Molecular Devices) at 10x magnification and 4 field-of-view per well. Fluorescence intensity within the nucleus were quantified using CellProfiler⁷⁹. All analysis and curve fitting were carried out using Prism with DMSO as a baseline.

Cell proliferation

Cells were grown and seeded in conditions as described above. Cells were seeded in 384well plates (Cat. No. 353963, Corning) at 1000 cells per well for Halo-ER U2OS, 1200 cells for SKBR3 and 1800 cells for MCF7 and T47d. Cells were grown overnight, then treated with compounds the following day. Compound concentration and administration are the same as described previously for the immunofluorescence assay. Plates are scanned in the IncuCyte live cell analysis system (Sartorius)

at 24-hour intervals for a total of 5 days using phase contrast. Cell proliferation quantification was carried out by the built-in analysis function using whole well confluency mask. All analysis and curve fitting were carried out using Prism with DMSO as a baseline.

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Reviewer #1 (Public Review):

Summary:

The authors set up a pipeline for automated high-throughput single-molecule fluorescence imaging (htSMT) in living cells and analysis of molecular dynamics.

Strengths:

htSMT reveals information on the diffusion and bound fraction of molecules, dose-response curves, relative estimates of binding rates, and temporal changes of parameters. It enables the screening of thousands of compounds in a reasonable time and proves to be more sensitive and faster than classical cell-growth assays. If the function of a compound is coupled to the mobility of the protein of interest, or affects an interaction partner, which modulates the mobility of the protein of interest, htSMT allows identifying the modulator and getting the first indication of the mechanism of action or interaction networks, which can be a starting point for more in-depth analysis.

Weaknesses:

While elegantly showcasing the power of high-throughput measurements, the authors disclose little information on their microscope setup and analysis procedures. Thus, reproduction by other scientists is limited. Moreover, a critical discussion about the limits of the approach in determining dynamic parameters, the mechanism of action of compounds, and network reconstruction for the protein of interest is missing. In addition, automated imaging and analysis procedures require implementing sensitive measures to assure data and analysis quality, but a description of such measures is missing.

- <https://doi.org/10.7554/eLife.93183.1.sa2>

Reviewer #2 (Public Review):**Summary:**

McSwiggen et al present a high throughput platform for SPT that allows them to identify pharmaceuticals interactions with the diffusional behavior of receptors and in turn to identify potent new ligands and cellular mechanisms. The manuscript is well written, it provides a solid new mentor and a proper experimental foundation

Strengths:

The method capitalizes and extends to existing high throughput toolboxes and is directly applied to multiple receptors and ligands. The outcomes are important and relevant for society. 10^6 cells and >400 ligands per is a significant achievement.

The method can detect functionally relevant changes in transcription factor dynamics and accurately differentiate the ligand/target specificity directly within the cellular environment. This will be instrumental in screening libraries of compounds to identify starting points for the development of new therapeutics. Identifying hitherto unknown networks of biochemical signaling pathways will propel the field of single-particle live cell and quantitative microscopy in the area of diagnostics. The manuscript is well-written and clearly conveys its message.

Weaknesses:

There are a few elements, that if rectified would improve the claims of the manuscript.

The authors claim that they measure receptor dynamics. In essence, their readout is a variation in diffusional behavior that correlates to ligand binding. While ligand binding can result in altered dynamics or /and shift in conformational equilibrium, SPT is not recording directly protein structural dynamics, but their effect on diffusion. They should correct and elaborate on this.

L 148 What do the authors mean 'No correlation between diffusion and monomeric protein size was observed, highlighting the differences between cellular protein dynamics versus purified systems'. This is not justified by data here or literature reference. How do the authors know these are individual molecules? Intensity distributions or single bleaching steps should be presented.

Along the same lines, the data in Figs 2 and 4 show that not only the immobile fraction is increased but also that the diffusion coefficient of the fast-moving (attributed to free) is reduced. The authors mention this and show an extended Fig 5 but do not provide an explanation. How do potential transient ligand binding and the time-dependent heterogeneity in motion (see comment above) contribute to this? Also, in line 216 the authors write "with no evidence" of transient diffusive states. How do they define transient diffusive states? While there are toolboxes to directly extract the existence and abundance of these either by HMM analysis or temporal segmentation, the authors do not discuss or use them.

The authors discuss the methods for extracting kinetic information of ligand binding by diffusion. They should consider the temporal segmentation of heterogeneous diffusion. There are numerous methods published in journals or BioRxiv based on analytical or deep learning tools to perform temporal segmentation. This could elevate their analysis of Kon and Koff.

- <https://doi.org/10.7554/eLife.93183.1.sa1>

Reviewer #3 (Public Review):

Summary:

The authors aim to demonstrate the effectiveness of their developed methodology, which utilizes super-resolution microscopy and single-molecule tracking in live cells on a high-throughput scale. Their study focuses on measuring the diffusion state of a molecule target, the estrogen receptor, in both ligand-bound and unbound forms in live cells. By showcasing the ability to screen 5067 compounds and measure the diffusive state of the estrogen receptor for each compound in live cells, they illustrate the capability and power of their methodology.

Strengths:

Readers are well introduced to the principles in the initial stages of the manuscript with highly convincing video examples. The methods and metrics used (fbound) are robust. The authors demonstrate high reproducibility of their screening method ($R^2=0.92$). They also showcase the great sensitivity of their method in predicting the proliferation/viability state of cells ($R^2=0.84$). The outcome of the screen is sound, with multiple compounds clustering identified in line with known estrogen receptor biology.

Weaknesses:

- Potential overstatement on the relationship of low diffusion state of ER bound to compound and chromatin state without any work on chromatin level.
- Could the authors clarify if the identified lead compound effects are novel at any level?
- More video example cases on the final lead compounds identified would be a good addition to the current data package.

- <https://doi.org/10.7554/eLife.93183.1.sa0>