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Off-DNA DNA-Encoded Library Affinity Screening

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Abstract

DNA-encoded library (DEL) technology is emerging as a key element of the small molecule discovery toolbox. Conventional DEL screens (i.e., on-DNA screening) interrogate large combinatorial libraries via affinity selection of DNA-tagged library members that are ligands of a purified and immobilized protein target. In these selections, the DNA tags can materially and undesirably influence target binding, and therefore the experiment outcome. Here, we use a solid-phase DEL and droplet-based microfluidic screening to separate the DEL member from its DNA tag (i.e. off-DNA screening), for subsequent in-droplet laser-induced fluorescence polarization (FP) detection of target binding, obviating DNA tag interference. Using the receptor tyrosine kinase (RTK) discoidin domain receptor 1 (DDR1) as a proof-of-concept target in a droplet-scale competition binding assay, we screened a 67,100-member solid-phase DEL of drug-like small molecules for competitive ligands of DDR1 and identified several known RTK inhibitor pharmacophores, including azaindole- and quinazolinone-containing monomers. Off-DNA DEL affinity screening with FP detection is potentially amenable to a wide array of target classes, including nucleic acid binding proteins, proteins that are difficult to overexpress and purify, or targets with no known activity assay.

Graphical Abstract

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The Supporting Information is available free of charge on the ACS Publications website. Experimental details and additional results

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Keywords

DNA-encoded library; fluorescence anisotropy; microfluidics; one-bead-one-compound; miniaturization; high-throughput screening

Introduction

DNA-encoded library (DEL) screening¹ has rapidly emerged as an efficient and scalable strategy for furnishing novel bioactive compounds, among the key first steps in prosecuting a target for drug discovery. DELs are large, ~million-member collections of almost never-before-seen chemical matter^{2–8} that are efficiently produced in highly parallel split-and-pool encoded combinatorial synthesis⁹ and screened in affinity selections against immobilized protein targets. DEL screens have raised ligands for numerous target classes,^{10–16} and at least two DEL hits are clinical candidates.^{17,18} Meanwhile, DEL technology has continued to evolve, with many recently reported DNA-compatible reactions expanding access to exciting new chemical space.^{19,20}

Until very recently, screens of these novel diversity sets were limited to affinity selections of the DNA-linked, or “on-DNA”, library members. Affinity selection, while highly parallel, only discovers ligands; functional ligand discovery is serendipitous. Further, the DNA tag attached to each library member can participate in or abolish binding, contributing to false positive and negative rates.^{21,22} These observations lead to the development of bead-based solid-phase DEL synthesis protocols²³ and accompanying microfluidic instrumentation for activity-based measurements in microscopic droplets of enzyme activity assay.^{24,25} This hybrid of high-throughput screening (HTS) and DEL leverages the bead to deliver many copies of a single library member ($\sim 10^{10}$) to a droplet for subsequent photocleavage of the library member from the host bead and into the droplet,²⁶ eliminating the aforementioned DNA tag interference. However, this new screening mode requires a fluorescence-based activity assay, which may not be available or applicable for every target, prompting our technology development efforts to widen the target scope.

Fluorescence polarization (FP) is an alternative biophysical measurement that finds routine use in HTS assay development for its generality and simplicity in identifying protein target

ligand candidates.^{27–29} FP measurements rely on the fact that excitation of a fluorophore elicits fluorescence emission polarized parallel to its dipole. The dipole of a freely rotating fluorophore is likely to be displaced from its original orientation at excitation, with emission polarization component intensities, $I_{\parallel}$ and $I_{\perp}$ ($I_{\parallel}$, parallel emission to excitation; $I_{\perp}$, perpendicular emission to excitation) reflecting a randomized excited state fluorophore dipole orientation ($I_{\parallel} @ I_{\perp}$). However, a fluorophore bound to a macromolecular target exhibits restricted rotation, limiting dipole displacement from its original orientation at excitation. Emitted light therefore retains a higher degree of polarization compared to the freely rotating fluorophore ($I_{\parallel} > I_{\perp}$). Making such FP-based measurements of macromolecular target engagement in droplets is possible,^{30–33} and would significantly expand the target scope for solid-phase DEL screening. However, FP is not a demonstrated droplet sorting criterion,^{34,35} and the feasibility of implementing FP-based binding assays within the microfluidic solid-phase DEL screening workflow is completely unknown.

In this study, we demonstrate that laser-induced FP is sufficiently sensitive to detect off-bead, off-DNA macromolecular binding in microfluidic droplets and sort droplets based on that signal. We establish that FP-based detection of macromolecular binding is statistically robust in microfluidic droplets by detecting probe binding to the model target streptavidin, and two clinically relevant drug targets: the phosphodiesterase autotaxin (ATX) and the receptor tyrosine kinase (RTK) discoidin domain receptor I (DDR1).³⁶ We demonstrate polarization-activated droplet sorting in a competition binding screen for ligands of DDR1, which lacks a suitable fluorescence-based activity assay. A screen of a 67,100-member solid-phase DEL²⁵ produced ligands that validated as competitive DDR1 ligands. This droplet-scale competition binding screen enables interrogation of library members for affinity to a target without attachment of a linker or DNA tag while screening.

Results & Discussion

FP-based binding assays were developed for several macromolecular targets and then miniaturized to the droplet scale for microfluidic off-DNA DEL screening. FP probes, known ligands coupled to a fluorescein (FAM) reporter, were synthesized for DDR1 and ATX (Figure 1A) in addition to the control target, streptavidin. The streptavidin FP probe was a commercially available FAM-labeled biotin (Figure S1). DDR1 and ATX ligands were discovered during previous DEL screens (Figure S2 and S3).^{25,37} Probe performance was evaluated in binding assays to determine conditions for saturation of emission polarization (Figure 1B). Saturation emission polarization for DDR1 and ATX FP probes reached $mP = 157 \pm 1$ and 300 ± 2 , respectively. Flow injection analysis in microfluidic droplets³⁸ (Figure S4) was used to make initial laser-induced FP-based measurements of macromolecular binding (Figure S5). Each droplet's profile in the $I_{\parallel}$ and $I_{\perp}$ channels was reduced to a single FP value. Droplets containing FP probe alone exhibited significantly lower FP signal compared to droplets containing FP probe and saturating target (Figure 1C). Droplet FP populations from the analyses of both DDR1 and ATX (Figure 1D) were used to extract mean (μ) and standard deviation (σ) FP values for each droplet population via Gaussian fit. Using these values, the statistical assay quality score, Z' ,^{26,39} was 0.56 and 0.67 for the DDR1 and ATX droplet-scale binding assays, respectively.

These results substantiate the feasibility of detecting macromolecular binding via laser-induced FP in microfluidic droplets. The signal separation between droplet populations containing unbound and bound FP probe was statistically robust for both DDR1 and ATX, demonstrating the generality of the approach to address multiple target classes. Achieving sufficient signal-to-noise ratio for both targets required using the entire raw droplet profile to calculate each droplet's FP value. Using the modified data acquisition code, there was still a ~15% reduction in saturation emission polarization when measuring the same binding interaction in droplets versus plates. The larger number of elements in a confocal LIF optical train are likely to blame, and further engineering of the detection system may be fruitful. Nevertheless, the high Z' of the DDR1 and ATX binding assays prompted further in-depth studies.

The newly developed FP detection system and associated binding assays were next used to evaluate potential droplet-scale library screening strategies. To detect target site engagement by a ligand other than the FP probe, one can measure either probe displacement or competition binding. Displacement and competition binding assay formats were tested using positive control competitor beads that displayed the DDR1 ligand on a photocleavable linker (Figure 2A, S6). The competition binding assay resulted in >80% depolarization compared to control versus ~20% for the displacement binding assay (Figure S7). Adapting the competition binding assay to flow, the DDR1 FP probe (20 nM) and positive control photocleavable DDR1 ligand competitor beads (25 beads/ μ L) were introduced through one aqueous input and DDR1 (1 μ M) was introduced through the second aqueous input at identical flow rates (400 nL/min). Unlabeled control competitor was released into droplets by UV irradiation and concomitant linker photolysis in flow.²⁶ After UV dosing, droplets were incubated (12 min)³⁸, then interrogated for FP. Bead-occupied droplets exhibited statistically significantly depolarized emission compared to unoccupied droplets (Figure 2B). Histogram analysis showed two separate droplet populations with the FP signal of bead-occupied droplets being 6σ below the mean of the unoccupied droplet FP signal (Figure 2C). Competition binding signal from positive control beads required UV dosing. Probe emission remained highly polarized when the UV source was turned off (Figure S8).

Droplet FP assay results using the photocleavable positive control ligand beads confirmed that competition binding is robustly detectable in droplets. The difference in droplet FP signal between droplets containing positive control beads and unoccupied droplets placed occupied droplets' FP signal below the dynamic FP signal hit threshold. This threshold dynamically adjusts during the course of the droplet screen. A droplet is classified as a hit if its FP signal is $<4\sigma$ below the mean FP value of the previous 1000 droplets that did not classify as hits.²⁵ Importantly, this signal was only detectable in droplets that were irradiated with UV, indicating that binding of the target to the ligand-displaying bead did not influence assay performance. Based on the bead diameter (17 μ m swollen), approximate DDR1 radius (20 Å), and DDR1 load (100 amol), we estimate that the beads' maximal surface packing is equal to the droplet load of DDR1. Previous work⁴⁰ has shown that protein loading onto TentaGel beads is 0.001%, far lower than the expected stoichiometry, in agreement with our observations. Thus, although bead interactions with the protein target in the droplet likely occur, neither calculations nor empirical observations suggests that they would confound off-bead binding assay outcomes and therefore library screening.

The validated off-bead DDR1 competition binding assay and FP detection system were deployed in a solid-phase DEL screen for novel ligands of DDR1. The DEL was prepared by acylating 110 amino acids with 610 carboxylic acids. The two-cycle combinatorial DNA-encoded solid-phase synthesis²³ yielded a 67,100-member library.²⁵ DDR1 FP probe (20 nM) and DEL beads (~2500 beads/ μ L) were introduced through aqueous input LIB, and DDR1 (1 μ M) was introduced through the separate input TAR. After DEL bead encapsulation in DDR1 binding assay droplets and UV dosing to cleave the library member from the bead, the droplets flow through the incubator (~12 min), and pass through the laser focus for detection (6000 Hz) of FP signal. Droplets exhibiting FP $<4\sigma$ below the mean FP of unoccupied droplets were sorted as hits (Figure 3A). Most droplets did not contain a competitor ligand. The FP of these droplets remained high and relatively consistent over the course of the screen (Figure 3B). Multiple library equivalents (~15 eq, 10^6 beads) were screened.^{41,42} The observed hit rate was ~0.1%. Hit droplets tended to exhibit a spread of polarization values below the 4σ sort threshold.

System performance during the DEL screen was in agreement with previous off-bead screening and soluble library selection experiments. We had already screened this library against ATX using a homogeneous fluorescence-based enzyme activity assay and observed a similar spread of hit bead signals below the sort threshold. Hit droplet FP signal variance could indicate a range of competitor binding affinities, heterogeneity in photocleavage efficiencies, or synthesis yields.⁴³ The hit rate of the ATX screen was much higher (0.7%) and occurred at a fraction of the UV dose (0.2 V). Selection experiments against ATX using an analogous DEL were ~10-fold more productive against ATX than DDR1. Our observations in droplets agree with those findings.⁴⁴ Low hit rate notwithstanding, the DEL signal observed $<4\sigma$ (1000 droplets) was significantly more than the predicted false discovery rate (FDR) based on a normal distribution of droplet FP signals ($p = 6 \times 10^{-5}$, FDR = 0.006%, 84 occupied droplets), suggesting that the off-bead, off-DNA competition binding screen output should be sequenced.

The hit collections from the DDR1 competition binding library screens were processed to reveal the hit structures. Briefly, DNA tags on hit beads were prepared for deep sequencing. Reads were pattern matched, trimmed, and filtered by a unique molecular identifier (UMI) threshold (>10) to reduce sequencing noise.^{24,25} Encoding sequences were marked as replicates if more than one bead-specific barcode was associated with that encoding sequence.¹⁴ Bead encoding sequences represented only once in the hit collection were culled. The k class > 2 hit collection contained 304 unique compounds (Figure S9). The k class > 3 hit collection contained 23 unique compounds (Figure 4A). Of the k class > 3 hits, 13/23 shared monomer usage with other k class > 3 hits. In an analysis of the k class > 2 hit collection monomer usage, the four most observed monomers are present in compounds in the k class > 3 hit collection (Figure 4B). Among k class > 2 hits, 7-hydroxytetrahydroisoquinoline and quinazolinone monomers were most frequently observed in cycle 1, with the latter appearing in four $k > 3$ hits. A tetrahydrocarbazole and an elaborated azaindole were most frequently observed in cycle 2. Both the azaindole and the tetrahydroisoquinoline appear in k class = 4 hits.

Newly discovered DDR1 competitor hit structures and monomer usage in the hit collection generally reflected known kinase-binding properties.^{45,46} However, the *k* class representation was lower than expected. High *k* class hits — those structures observed as hits on multiple, distinct beads — are far more likely to validate and are therefore higher priority candidates for synthesis.⁴² The highest *k* class hits (*k* class > 3) contained azaindoles and quinazolinones, both known RTK pharmacophores.⁴⁷ Furthermore, the monomer usage in the hit collection is biased toward these structures, and highly used monomers tend to be seen in *k* class > 3 hits, further substantiating their importance. However, the highest observed *k* class = 4, was much lower than the expected *k* class based on the number of library equivalents screened (15).⁴² The reduced separation of signal between the photocleavable DDR1 positive control bead-occupied droplets and unoccupied droplets in conjunction with the lower mean *k* class of the hit collection highlights hit signal variance as a previously unappreciated factor influencing the representation of the hit collection.

Hit selection criteria included *k* class, commercial monomer availability, and structural novelty. Thirteen hits (Table S1) were prepared via low-scale (~ 300 nmol) solid-phase synthesis on a photocleavable linker. The hits were photocleaved, and the photocleavage reaction supernatants were tested in a DDR1 competition binding assay without purification. All 13 hits reduced the probe's FP signal compared to a negative control compound (Figure 5). Droplet-scale competition binding assays were repeated for six of the hits (**2**, **4**, **5**, **8**, **9**, **12**), the negative control, and the positive control. DDR1 ligand positive control competitor beads produced a population of droplets with statistically significantly depolarized emission compared to unoccupied droplets, whereas negative control competitor beads did not. Droplet-scale analysis of the six hits yielded a range of emission depolarization falling between that observed for the negative and positive control bead-occupied droplet FP populations (Figure S10). Quantitative competition binding analysis yielded equilibrium binding constants for **4** and **5**, ($K_d = 35 \mu\text{M}$ and $7.6 \mu\text{M}$, respectively). Hits **9** and **12** reached the limits of compound solubility. These data are consistent with initial single-concentration and droplet-based validation data, where **4** and **5** show strong competition.

Most of the hits were competitive in the initial, single-concentration competition binding assays, implying that the screen accurately sorted hit droplets. The results of droplet-based competition binding assays for the six selected hits (**2**, **4**, **5**, **8**, **9**, **12**) generally agreed with the single-concentration, competition binding assays conducted in microtiter well plates, indicating that the two validation techniques are consistent. Finally, four of the thirteen hits (**4**, **5**, **9**, **12**) were synthesized at milligram scale for quantitative competition binding-based measurements of their equilibrium binding constants. The four hits chosen for quantitative competition binding experiments represent a range of competition for binding to DDR1 based on initial single-point competition binding assay results. Hits **9** and **12** showed some incongruity between droplet-based competition binding assays and single-concentration binding assays in plates.

Finally, the hit collection output from the off-DNA DEL competition binding screen was compared to the hit collection from an on-DNA DEL affinity screen.³⁷ The solid-phase DEL (67,100 members) used in the off-DNA DEL competition binding screen was much smaller

than the on-DNA DEL (866,000 members) used in the affinity selection of DDR1 ligands. However, 13,260 on-DNA DEL members were present in the off-DNA DEL screen. To compare the two hit pools, we used Tanimoto chemical similarity scoring to cluster the 304 off-DNA DEL screening hit compounds into 36 of 47 hit clusters found during the on-DNA DEL affinity screen. Overlap of chemical space between the two hit collections is represented by overlaying each on-DNA hit cluster with the cumulative k class value for all off-DNA hits mapped to that cluster (Figure 6). The majority (7/8) of off-DNA hits with high cumulative k class (> 9) are found in the top 50% of on-DNA hit clusters. The highest enrichment score on-DNA hit cluster also contains the highest cumulative k class of off-DNA competition binding screen clustered hits. However, off-DNA hit **8** of this cluster did not validate in the single concentration competition binding screen and produced very weak depolarization of droplet FP signal. Based on monomer conservation, further exploration of this cluster would be warranted.

Here we describe a new approach to solid-phase DEL analysis that permits affinity screening free of the library bead. Cleaving the library member from the bead dodges avidity effects that tend to drive up false discovery rates of one-bead-one compound library screening^{48–50} and inspired technology development efforts in automated bead handling and analysis.⁵¹ Our approach integrates the significant advances of droplet-scale microfluidic automation and miniaturization with the high-throughput hit deconvolution capabilities of DEL technology. This strategy resulted in the discovery of several DDR1 ligand families from a 2-cycle solid-phase DEL. The ligand families contain both fragments known to bind kinases⁴⁷ and recently disclosed novel chemical matter.³⁷ Parallel assay development efforts suggest that the approach is extensible to other diverse target classes, such as ATX.

Freeing the library member from the bead in droplets also frees it from the DNA encoding tag, enabling off-DNA affinity binding DEL screens. Analysis of multiple exemplar molecules demonstrates that FP-based observation of DDR1 binding required both a bona fide DDR1 ligand and photocleavage of the ligand from the bead. Binding was undetectable absent photocleavage, implying that the bead-bound species do not detectably interact with assay reagents in the droplet. The library member is free to engage the target, which need only be stable in the droplet. These aspects of the analysis are advantageous for targets that natively bind nucleic acids or that are recalcitrant to overexpression and purification. Such targets (e.g. transcription factors, polymerases) are not ideal candidates for conventional on-DNA affinity-based screening,^{3,10} but may be tractable in droplets with solid-phase DELs.

These studies further establish the intriguing possibility of measuring library productivity in real time. During the first library screens for DDR1 ligands, it was immediately apparent that the library contained far fewer DDR1 hits than ATX hits, recapitulating observations from previous affinity selections against DDR1 and ATX.^{25,37} Importantly, reaching this conclusion using the microfluidic DEL screening platform did not require DNA sequencing. Furthermore, determining an approximate hit rate required screening < 1 library equivalent.^{41,42} In fact, it is possible that DNA encoding is unnecessary for such expeditions into chemical space. It would be interesting to compare library productivity in on-DNA, off-DNA, and activity-based screening results using the same target to understand how the

various approaches are complementary or orthogonal to one another. These studies are underway using ATX as the target.

In conclusion, the development of new detection methods is critically enabling for microfluidic chemical space analysis. This work demonstrates that FP is a feasible droplet sorting criterion for DEL screening and the assay development advantages of FP are still applicable to the droplet scale. This new detection capability dramatically widens the scope of accessible targets for off-DNA DEL - particularly those targets with unknown function.⁵² Other detection methods have also been explored in flow, such as resonance Raman scattering⁵³ and mass spectrometry,^{54,55} and could profoundly advance DEL analysis by offering label-free detection. Time-resolved fluorescence, another recently disclosed droplet sorting criterion,³⁵ raises the prospect of accessing the established TR-FRET assay paradigm and its attendant advantages of high sensitivity and low background in complex matrices (e.g., lysate) for DEL screening. Exploring these diverse detection modes is therefore likely to be fruitful both for expanding the scope of combinatorial library analysis and in the discovery of novel bioactive compounds to address difficult, emerging targets.

Experimental Procedures

Microfluidic device operation.

Aqueous solutions were loaded into syringes (1 mL, BD Medical, Franklin Lakes, NJ). Oils (OIL1, OIL2, OIL3) were also loaded into syringes (1 mL, 10 mL, 3 mL, BD Medical). Droplet generation oil (OIL1) was squalene (TCI America, Portland, OR) containing KF-6038 (4% w/w, ShinEtsu, Tokyo, Japan). The droplet generation oil was prepared by combining squalene and KF-6038 and mixing with rotation (16 h, 500 rpm). The spacing (OIL2) and flow focusing oil (OIL3) were neat squalene. All syringes were fitted with blunt-tip Luer-Lok needles and connected to fluidic inputs via microbore Tygon tubing (0.01" × 0.03" IC × OD, Saint Gobain, Valley Forge, PA). Displacement syringe pumps (Legato 100, KD Scientific, Holliston, MA) drove fluids from syringes through the circuit. Oil2 and OIL3 were flowed (8 and 3 μ L/min, respectively), while hit and waste tubing were clamped to backfill the circuit. The hit and waste outlets were unclamped once the incubator was primed with oil. The aqueous inputs, library (LIB) and Target (TAR) and OIL1, OIL2, OIL3 flows were held constant (0.4, 12.7, 1.3 μ L/min, respectively). Flow was equilibrated (25 min), then data acquisition and screening began.

Confocal laser-induced fluorescence detection system data acquisition.

Polarized droplet fluorescence emission was detected using a custom confocal laser-induced fluorescence (LIF) microscope that was built using 30-mm cage system components (ThorLabs, Inc., Newton, NJ). The emission of a diode laser ($\lambda = 488$ nm, OBIS-488 20 LS, Coherent Inc., Santa Clara, CA) was attenuated using a neutral density filter (0.3 OD, ThorLabs) and filter using a linear polarizer (480–550 nm, ThorLabs) before coupling in the main optical train using a long-pass dichroic mirror (505 DRLP, Omega Optical Inc., Brattleboro, VT). The laser excitation was focused on the microfluidic channel through a microscope objective (20 $\times$, 0.40 NA, 3.9 mm WD, Nikon, Tokyo, Japan). Polarized emission was collected through the same objective. The collimated emission was transmitted

through the long-pass dichroic mirror, reflected off a broadband dielectric coated elliptical mirror (ThorLabs), and filtered through a short-pass dichroic mirror (600 nm Dichroic Shortpass Filter, Edmund Optics Inc., Barrington, NJ) and bandpass filter (520BP20 RAPIDBAND, Omega Optical). The spectrally filtered emission is split into p - and s -polarization (polarizing beamsplitter cube, 420 — 680 nm, ThorLabs). p -polarized emission ($I_{\parallel}$) is transmitted by the polarizing beamsplitter, focused by a plano-convex lens ($f/D = 30$ mm/25.4 mm), and spatially filtered with a pinhole (50 μ m) prior to detection with a photon counting head PMT (H7828, Hamamatsu, Middlesex, NJ). s -polarized emission ($I_{\perp}$) is reflected by the polarizing beamsplitter and follows an identical optical train. The PMT signals are digitized by a data acquisition board (NI-USB-6341, National Instruments, Austin, TX). LabView code written in-house controls acquisition. A 620 — 630 nm LED illuminator (Littlite, Hamburg, MI) is used to image the microfluidic device with a high-speed camera (I-SPEED 210, Dynamic Imaging, Brentwood, TN). Droplet data acquisition, signal processing, and all electronic equipment control was accomplished in LabVIEW (6 kHz). The PMT signals were digitized (DAQ, NI USB-6341, National Instruments). Droplets were detected based on passing above and then falling below a pre-set fluorescence threshold in the $I_{\parallel}$ channel (threshold = 400 RFU). Median smoothing (window width = 3) was applied to the signal in real-time. Once a droplet was detected, the average of digitized PMT signals for each direction of polarization, after smoothing, was used as the droplet's $I_{\parallel}$ and $I_{\perp}$ values for calculating FP.

Droplet-scale flow injection analysis of DDR1 binding assay quality FP assays.

For probe only experiments, LIB contained biotin FP probe (20 nM, 0.5% BSA, 0.01% Tween-20 PBS) or DDR1 FP probe (20 nM, DDR1 binding assay buffer). For probe only analysis, TAR contained only buffer (0.5% BSA, 0.01% Tween-20 PBS for streptavidin assay; DDR1 binding assay buffer for DDR1 assay). For probe and target experiments, LIB contained the biotin FP probe (20 nM, 0.5% BSA, 0.01% Tween-20 PBS) or DDR1 FP probe (20 nM, DDR1 binding assay buffer). TAR contained streptavidin (400 nM, 0.5% BSA, 0.01% Tween-20 PBS) or DDR1 (1 μ M, DDR1 binding assay buffer). Droplet data were acquired for 3 minutes (10,000 droplets) for all flow injection analysis conditions.

Microfluidic droplet-based FP competition binding screen.

Luer-Lok needle-fitted syringes (1 mL, BD Medical) connected to microbore Tygon tubing filled were filled with liquid metal galinstan (Gallium Indium Tin eutectic, Alfa Aesar) and pumped into the microfabricated electrode channels (V_{AC} and ground).⁵⁶ Droplet-scale FP assays were conducted as described with minor modifications. LIB contained the DDR1 FP probe (20 nM) in buffer (DDR1 binding assay buffer, 19% w/v sucrose). A library aliquot was washed and quantitated and added to LIB input (2500 beads/ μ L). TAR input contained DDR1 (1 μ M) in buffer (DDR1 binding assay buffer, 19% sucrose). Circuit priming and equilibration was performed as described. A dynamic sorting threshold was calculated in real-time to identify droplets with statistically significantly reduced FP as hits. The dynamic threshold was calculated as $\mu - 4\sigma$, where μ and σ were the mean and standard deviation of the last 1,000 droplets' FP values, respectively. Hit droplets were excluded from the fluorescence values used to calculate population mean and standard deviation. When a hit droplet was detected (droplet FP < $\mu - 4\sigma$ counts), LabVIEW output a TTL pulse from the

DAQ board to a waveform generator (Agilent 33210A, Agilent Technologies, Santa Clara, CA), triggering a square wave pulse output (0–6.50 V, 10.0 kHz, 350 cycles) that was amplified (gain = 100 V/V, TREK Model 2210 high-voltage power amplifier, TREK Inc., Lockport, NY) and conducted through liquid metal microfabricated electrode channels (V_{AC} and ground). The electric field generated between the active electrode and ground electrode deflected the hit droplet to the hit output for collection.^{24,34,57}

Bulk resin photocleavage.

TentaGel amino-functionalized resin (10 μ m dia., Rapp-Polymere) was filtered from mixed-scaled hit synthesis resin as described. Hit beads were washed (0.4% w/v Tween-80 DDR1 binding assay buffer without DTT, 3 $\times$ 1 mL) and resuspended (0.4% w/v Tween-80 DDR1 assay buffer without DTT, 1 mL) for counting with a hemocytometer. Hit beads (0.3 μ moles) were transferred to a PCR tube (200 μ L) and centrifuged (30 s, 6,000 rpm). Supernatant was removed and beads were resuspended (DDR1 binding assay buffer, 100 μ L). PCR tubes containing hits were affixed to a microplate shaker, placed into a 365-nm UV-crosslinker (CL-1000L, Analytik Jena, Jena, Germany), exposed (16 h, 1,000 rpm), and centrifuged (30 s, 6,000 rpm). The supernatant was removed and assayed for competition in the DDR1 FP assay. Expected photocleavage product was confirmed via MALDI-TOF MS. A dilution of the photocleavage supernatant (1:10 in 50:50 ACN: 0.1% TFA in H_2O , 100 μ L) was spotted (1 μ L) on a MALDI-TOF MS plate, dried, covered with HCCA matrix solution (1.5 mg/mL HCCA in 1:2 ACN:0.1% TFA in H_2O) and analyzed with MALDI-TOF MS (Microflex, Bruker Daltonics, Inc., Billerica MA).

Qualitative hit validation assay.

Hit validation resin photocleavage supernatant (3.3 μ L) was added to FP probe (15 nM, 3.3 μ L in DDR1 binding assay buffer) and DDR1 (1.5 μ M, 3.3 μ L, in DDR1 binding assay buffer) in a 384-well black microplate (Greiner, low-volume). After incubation (15 min), FP was measured. The mP for probe binding was calculated by subtracting the FP of probe-only wells from the FP of target-containing wells.

Quantitative competition binding assay.

Increasing concentrations of compound **4**, **5**, **9**, or **12** (3.3 μ L in DDR1 binding assay buffer, < 9% DMSO) were added to FP probe (15 nM, 3.3 μ L in DDR1 binding assay buffer) and DDR1 (1.5 μ M, 3.3 μ L, in DDR1 binding assay buffer) in a 384-well black microplate (Greiner, low-volume). After incubation (15 min, RT) fluorescence anisotropy (r) was measured. The r for probe binding was calculated by subtracting the anisotropy of probe-only wells from the FP of target-containing wells. The competition binding curves were fit to the variable slope model dose response regression model (Eq. 1) to find I_{50} , the total concentration of competing ligand causing displacement of 50% of the FP probe, where x = [competitor], h = hill slope coefficient.

$$r = \min r + \frac{(\max r - \min r)}{1 + \frac{x^h}{I_{50}^h}} \quad (1)$$

To determine K_d of the DDR1 ligand FP probe, increasing concentrations of DDR1 (5 μ L in DDR1 binding assay buffer) were added to FP probe (10 nM, 5 μ L in DDR1 binding assay buffer) in a 384-well black microplate (Greiner, low-volume). After incubation (15 min, RT), r was measured. The r for probe binding was calculated by subtracting the anisotropy of probe-only wells from the FP of target-containing wells. The FP probe binding curve was fit to a one-site specific binding model (Eq. 2) for a calculated K_d of DDR1 FP probe binding to DDR1 of 32 +4 nM, where $x = [\text{DDR1}]$.

$$r = \frac{(\max r * x)}{(K_d + x)} \quad (2)$$

K_d values for compound **4** (35 μ M) and **5** (7.6 μ M) were calculated using the competitor ligand I 50 values (Compound **4**, 1600 + 200 μ M); Compound **5**, 350 + 60 μ M), K_d of DDR1 ligand FP probe (32 nM), concentration of DDR1 ligand FP probe (5 nM), and total concentration of DDR1 (500 nM).⁵⁸

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

NGS	next-generation sequencing
HTS	high-throughput screening
DEL	DNA-encoded library
ATX	autotaxin
AA	amino acid
CA	carboxylic acid
QC	quality control
UMI	unique molecular identifier
FDR	false discovery rate
HDNA	headpiece DNA
OP	oligonucleotide paired stock
DESPS	DNA-encoded solid-phase synthesis

TR-FRET	time-resolved fluorescence resonance energy transfer
FP	fluorescence polarization

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Figure 1. FP-based detection of macromolecular binding in microfluidic droplets.

(A) Droplet-scale FP binding assays contained a macromolecular target and an FP probe. The FP probes contain a fluorescein (FAM) label coupled to known ligands of DDR1 and ATX. (B) DDR1 FP probe and ATX FP probe (5 nM) emission polarization increases upon addition of DDR1 and ATX, respectively. (C) Example raw transient droplet fluorescence data illustrate four droplet detection events ($I_{||}$, black trace; $I_{\perp}$, cyan trace) and their associated FP values (purple bars). FP probe droplets (*top*) contained DDR1 FP probe (5 nM) or ATX FP probe (10 nM) and no target. FP probe + target droplets (*bottom*) contained DDR1 FP probe (5 nM) and DDR1 (500 nM) or ATX FP probe (10 nM) and ATX (300 nM). (D) Droplet FP populations for either probe-only droplets (gray histograms) or probe + target droplets (green histograms) were used to calculate Z' . Droplet assay quality score was

$Z' = 0.56$ for DDR1 FP probe binding to DDR1 and $Z' = 0.67$ for ATX FP probe binding to ATX.

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Figure 2. Off-bead droplet FP assay using a positive control competitor ligand.

(A) The DDR1 ligand is synthesized on TentaGel resin (10 μ m) displaying a photocleavable linker to yield positive control PC-DDR1 ligand beads. (B) Example raw transient droplet fluorescence data (I_{||}, black; I_⊥, cyan; FP purple) illustrate unoccupied and PC DDR1 ligand bead-occupied ("hit") droplets. (C) In a population analysis of unoccupied droplets (green histogram) or bead-occupied droplets (blue histogram), bead release of an unlabeled competitor significantly depolarizes probe emission (Δ mP = 110 $\pm$ 20) when the UV LED is on (+ UV) but not when the UV LED is off (- UV). Bead-occupied droplet population data are magnified 50-fold.

Figure 3. Microfluidic competition binding screen of a 67,100-member DEL.

(A) Raw transient droplet fluorescence data ($I_{\parallel}$ raw data, black points; $I_{\parallel}$ median smoothed, black trace; $I_{\perp}$ raw data, cyan points; $I_{\perp}$ median smoothed, cyan trace) and each droplet's associated FP value (purple bars) illustrate FP-based detection of hit-containing droplets. Median smoothing effectively removes signal spikes that occur when a bead crosses the detection volume. A droplet was sorted if the droplet's FP value was less than the dynamically calculated sort threshold (green, 4σ). (B) Transient histogram visualization of the DEL screen reveals a distribution of droplets exhibiting competition binding to DDR1, resulting in varying degrees of depolarized emission. Droplet FP values were binned (1 mP per bin) in 30-s windows.

Figure 4. DDR1 Hit Collection Monomer Representation.

(A) Hit bead sequences (k class > 3) were decoded to determine the synthesis cycle 1 and 2 monomer identities of each hit. Dashed lines indicate monomer use conservation in synthesis cycles 1 (vertical) and 2 (horizontal). Numbered compounds were synthesized for validation in competition binding assays. (B) The most represented monomers for synthesis cycles 1 and 2 were identified from an analysis of the k class > 2 hits. Monomers observed many times (purple bars) were statistically significantly represented (4σ) compared to the expected number of observations in a random sample.

Figure 5. Hit validation.

Thirteen compounds were synthesized and analyzed in DDR1 competition binding assays. Two million beads of each compound were photochemically cleaved from the bead into assay buffer. The photocleavage reaction supernatants were used without purification in FP competition binding assays (black bars). A negative control (inactive compound, -), positive control (DDR1 ligand, +), and 100% FP probe binding signal (gray line) are indicated. Error bars and the 100% FP probe binding signal line width reflect the SEM. Six compounds, the negative control, and the positive control were analyzed in droplet-based FP competition binding assays. Validation for droplet-based competition binding proceeded via population analysis of FP signal for bead-occupied droplets versus unoccupied droplets (blue points). Error bars reflect the SEM. The average sort threshold across the five droplet-based FP competition binding assays (green line) is represented with the width of the line reflecting the SEM of the average sort threshold.

Figure 6. Comparison of hits from a droplet FP screen of DDR1 to hits from a solution DEL screen of DDR1.

Hits (k class = 2) from the droplet-based binding screen were clustered by chemical similarity with on-DNA DEL screening hits. Each point on the plot represents a cluster of hits found in the on-DNA DEL screen, shaded by the sum of the k class values of the off-DNA DEL screening hits within that cluster. The clusters are ordered according to cluster enrichment during the on-DNA DEL affinity screen. Points highlighted in orange represent hit clusters with the highest similarity between on-DNA and off-DNA screening hits (Tanimoto similarity score ≥ 0.6).