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Communication

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Selection of DNA-encoded Libraries to Protein Targets Within and On Living Cells

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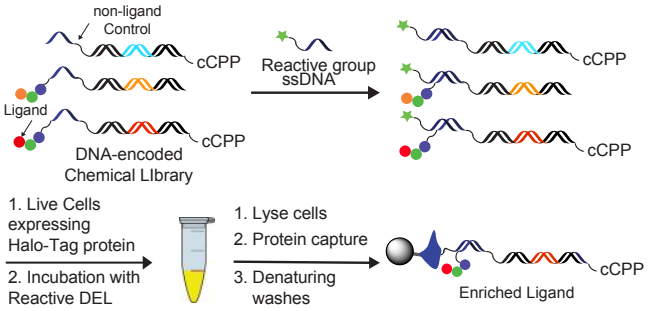
Supporting Information Placeholder

ABSTRACT: We report the selection of DNA-encoded small molecule libraries against protein targets within the cytosol and on the surface of live cells. The approach relies on generation of a covalent linkage of the DNA to protein targets by affinity labeling. This crosslinking event enables subsequent co-purification by a tag on the recombinant protein. To access targets within cells, a cyclic cell-penetrating peptide is appended to DNA-encoded libraries for delivery across the cell membrane. As this approach assesses binding of DELs to targets in live cells, it provides a strategy for selection of DELs against challenging targets that cannot be expressed and purified as active.

The discovery of small molecule ligands to protein targets is of fundamental importance for biological research and therapeutic development. DNA-encoded chemical libraries (DELs) have emerged as an important tool in molecular discovery.[1] The high sensitivity and low cost of DELs allow exploration of large portions of chemical space. These benefits arise because DNA-encoding allows collective, rather than individual, assessment of molecular function via an *in vitro* selection assay.[2] Unlike high throughput screening, the cost and difficulty of selection assays do not scale appreciably with the number of compounds. The traditional DEL selection using purified, immobilized protein targets cannot be applied to many important drug target classes.[3] This includes proteins that cannot be expressed recombinantly and purified in their fully active form. An approach to enable selection of DELs to targets within live cells would not only remove the onerous requirement for a pure, active protein but also assess proteins in a more functionally relevant state, where critical binding partners or posttranslational modifications can be maintained.

Prior work by our lab and others has applied covalent crosslinking by affinity labeling to expand the capabilities of DEL selections.[4,5] By trapping the transient interaction of DEL ligands to the target, crosslinking can give improved enrichment of ligands over non-ligands, particularly for low affinity binders or with protein targets at low concentration. Crosslinking also allows assessment of binding interactions in solution and can be conducted in complex environments such as cell lysates. Extension of this approach into live cells would have further advantages in limiting protein dilution, which is typically 10-fold or greater in cell lysates.[6]



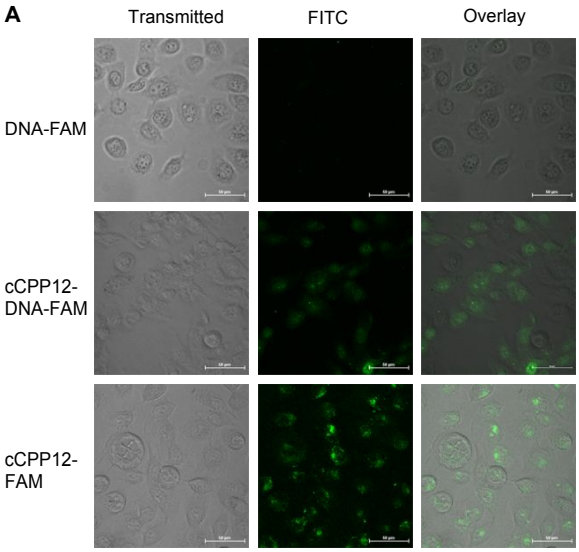
Scheme 1. Crosslinking approach to live cell DEL selections.

While there are reports of cell-based selections for aptamers,[7] this has been relatively unexplored with DELs. Israel and coworkers applied a cell-based selection with DELs to a cell surface protein, the NK3 Tachykinin GPCR.[8] This approach required high expression of the protein target. Thus, limitations arise for proteins that are toxic when expressed at high levels (ion channels, e.g.) or for discovery of ligands with low or modest affinity. Here, we apply covalent crosslinking to enable DEL selections against targets both within and on living cells.

For selection of targets within live cells, delivery of DELs into the cytosol presents a challenge. To achieve this, we conjugated DNA-linked molecules to a cyclic, cell-penetrating peptide developed by Pei and coworkers,[9] which has shown high cell entry and endosomal escape efficiency. Our approach is outlined in Scheme 1. A protein target is expressed in cells containing a fusion tag to enable later covalent capture. Upon cytosolic delivery of a DEL, the binding event between a protein and a DNA-linked ligand enables a covalent bond to be formed between the protein and DNA by affinity labeling. Cells are then lysed, and all target proteins are captured on beads. After stringent,

protein-denaturing washes, enriched ligands are identified by DNA sequencing.

To evaluate the cellular uptake, we appended cCPP12 to a 20-base oligonucleotide via a disulfide bond (Schemes S1 and S2), such that constructs might accumulate in the cytosol under reducing conditions. This cCPP oligo was hybridized to a 6-carboxyfluorescein amide (FAM)-linked oligo to yield a 20-base pair (bp), double-stranded DNA (cCPP12–DNA–FAM). Cell entry was assessed by fluorescence microscopy (Figure 1A). We observed comparable cell entry of cCPP12–DNA–FAM to cCPP12-fluorescein, while no fluorescence was observed with DNA–FAM.



B

DNA length	140 bp	60 bp
DNA only	0.093%	0.064%
cCPP12-DNA	1.2%	11%
Fold Difference	13	170

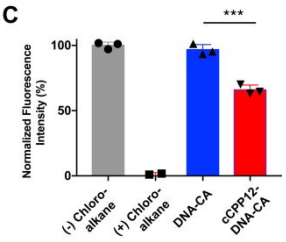


Figure 1. Cell penetration of DNA is enabled by cCPP12. (A) Live cell entry of a dsDNA-cCPP12 conjugate. 20-base dsDNAs containing FAM labels and a cCPP12-FAM conjugate were incubated with HMLE cells at 2 μM for 4.5 hours and imaged. (B) qPCR analysis of DNA cell entry. (C) CAPA performed with 5 μM 20-base dsDNA. P-values were calculated using unpaired Student's two-tailed t-test: ***P < 0.001.

While the cCPP12 allows uptake of DNA, the amount of amplifiable DNA remaining in cells is another key consideration. Nuclease degradation of DNA constructs may be problematic. DNase II is the predominant nuclease within endosomes and lysosomes that degrades exogenous DNA.[10,11] To determine the amount of amplifiable DNA remaining in cells, we performed qPCR analysis of cell lysates prepared after treatment of cCPP12-DNA. We also investigated the effect of DNA length on cellular uptake. When delivering a 60 bp DNA, ~11% cCPP12–DNA was recovered, 170-fold higher than the recovery of DNA lacking the cCPP12. In contrast, the recovery of a 140 bp DNA by cCPP12 was ~1% (Figure 1B). These qPCR tests indicated that significant amount of DNA was amplifiable after incubation and that cell entry is length-dependent. Treatment of cells with cationic lipid transfection reagents (Lipofectamine, e.g.) showed slightly improved DNA uptake compared to cCPP12 (Table S3); however, subsequent affinity labeling and selection experiments were unsuccessful, suggesting that DNA in these cases may be trapped within endosomes.

In addition to overall uptake, the actual cytosolic delivery of DNA to protein targets is critical. To evaluate the cytosolic penetration of cCPP12-DNA, we performed the HaloTag-based[12] chloroalkane penetration assay (CAPA).[13] When cells were treated with 5 μM cCPP12-linked chloroalkane (CA)-DNA (20-base dsDNA), the fluorescence intensity was reduced by ~30% (Figure 1C), indicating cCPP12 allows cytosolic delivery of DNA. Results were similar, but less pronounced (~20% reduction), using 2.5 μM CA-DNA (Figure S1).

The cGAS–STING pathway detects the presence of cytosolic DNA and triggers an immune response.[14] While prior experiments showed no concerns with cell viability, we sought to avoid this and investigated cell lines that lack cGAS or STING expression: HEK293T, FreeStyle™ 293-F, U937 cGAS$^{-/-}$ and U937 STING$^{-/-}$.[14] We found the FreeStyle™ 293-F cells, which are adapted to suspension culture, gave the best results. These cells showed high transfection efficiency and were easy to handle at high cell density, which gave improved cell entry of DNA by qPCR (Table S4).

We evaluated interactions between DNA-linked ligands and cytosolic proteins by affinity labeling and gel analysis. Within 293-F cells, we expressed two HaloTag fusion proteins: the Chromobox Protein Homolog 7 Chromodomain (CBX7-ChD) and *E. coli*-dihydrofolate reductase (eDHFR). Affinity labeling within live cells used DNA constructs containing a ligand, FAM, and a sulfonyl fluoride as a reactive crosslinker (Figure S2). For CBX7-ChD, we used a previously reported modified peptide ligand (4-BrBA) (Figure 2A, K_d ~ 270 nM off-DNA, 26 nM on-DNA).[15] For eDHFR, a modified trimethoprim (TMP) was used (Figure 2B, K_d ~1 nM off-DNA).[16] After incubation, cells were washed and lysed directly with SDS-PAGE buffer and excess free ligand to ensure that labeling occurred within the cell rather than the lysate. Labeling was observed and was dependent on the presence of the cCPP12, the ligand, and the sulfonyl fluoride on the DNA. With Halotag-eDHFR tests, addition of excess TMP ligand eliminated labeling (Figure 2B, lane 5), additionally verifying that labeling occurred within the cell. Bands occurred at expected molecular weights for the proteins linked to 40 bp DNA. Although Lipofectamine gave higher cellular DNA delivery as

assessed by qPCR, the absence of conjugated band in this case (Figure 2A, lane 4) suggests poor cytosolic entry.

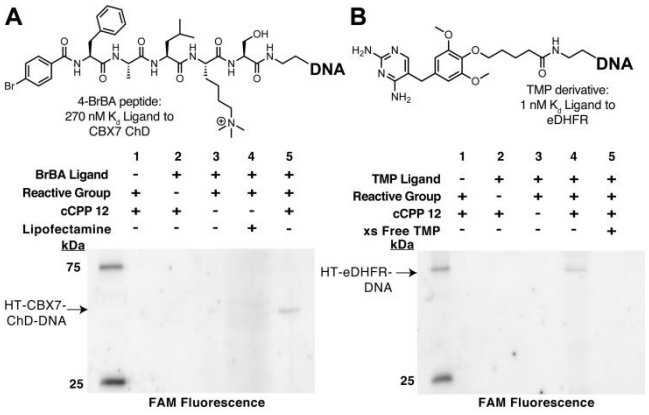


Figure 2. Assessing DNA-linked ligand-target interactions inside live cells. Affinity labeling of CBX7-ChD (A) and eDHFR (B) with DNA-linked ligands analyzed by SDS-PAGE.

Encouraged by successful labeling experiments, we performed a test selection assay against Halotag-CBX7-ChD within live cells using three model constructs (Figure 3A and S3). An encoding DNA of 60 bp was used, which is the approximate size within many DELs.[1] In an initial test, the recovery of the positive control ligand P1 (BrBA-60 bp DNA-cCPP12, Figure 3A) was 0.012%, 20-fold higher than that of N1 (non-ligand control, Figure 3A) and 22-fold higher than that of N2 (non-cCPP12 control, Figure 3A). Enrichments were significantly greater (~600-fold, Table S5) in analogous experiments in cell lysates, suggesting that much of the DNA recovered at cell lysis was unable to access the cytosol. Enrichment and recovery values are critical parameters that govern the amount and complexity of DELs used for a selection to ensure against under sampling of the library.[17]

We constructed a 96-membered DEL by varying 24 building blocks at each of four monomer positions within a known CBX7 ChD ligand (4-BrBA) to create a positional scanning library (PSL). Constructs from the 3-member test selection were included as controls. Selections were additionally performed against Halotag-CBX7-ChD in lysates and purified Halotag-CBX7-ChD. Consistent with the qPCR selection, recovery of the positive control ligand was ~20 fold higher than the non-ligand control (Figure 3C) when assessed by DNA sequencing (Table S5). An additional control, N2, which contained the BrBA ligand but lacked the cCPP, showed no enrichment in the live cell selection (Figure 3C), but showed comparable enrichment to the positive control in cell lysate selections (Table S5 and S11). Sequencing results showed differential enrichment of molecules (Figure S6 and S7) consistent with the reported SAR to the CBX7 ChD.[15] Enrichment of each library member in the live cell selection correlated well with the cell lysate selection (Figure 3B). To validate selection hits, we synthesized 10 molecules off-DNA and assessed their IC_{50} values in a ligand displacement assay.[15] Values roughly correlated to observed enrichments (Figure 3D and Table S13).

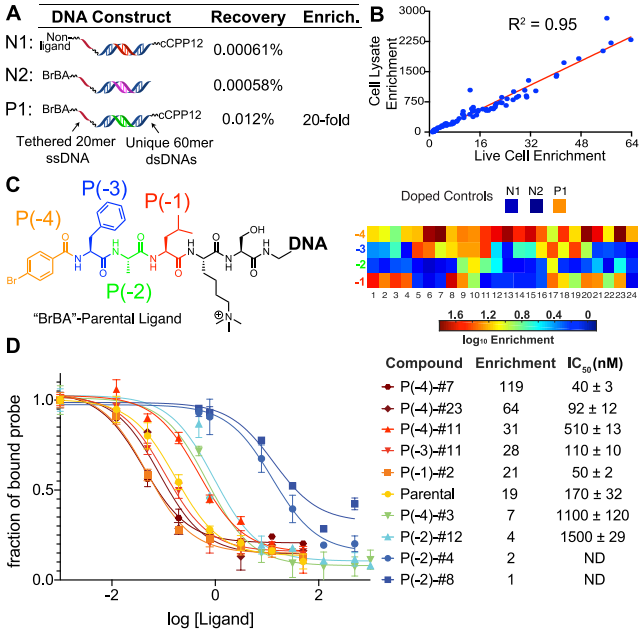


Figure 3. DEL selection against Halotag-CBX7-ChD within living cells. (A) Recovery and enrichment of tested DNA constructs in a mock selection at 1000-fold dilution. (molar ratios P1:N1:N2 = 1:500:500; [P1] = 1 nM). (B) Correlation plot of live cell and cell lysate selections. (C) The parental peptide for a DNA-encoded PSL and a heat map of live cell enrichments. The color scale represents the log enrichment relative to the non-ligand control. See Figures S6 and S7 for structures. (D) IC_{50} values of off-DNA hits in binding assay to Halotag-CBX7-ChD. Line colors correspond to live cell enrichment values from the selection, as indicated on the color scale.

In addition, we sought to test this approach with an integral membrane protein target, the δ opioid receptor (DOR), as an example of a target that is not amenable to purification and immobilization. First, we prepared an on-DNA control ligand for this target, Dmt-Tic-Lys (DTK, 2,6-dimethyl-L-tyrosine (Dmt), 1,2,3,4-tetrahydroisoquinolone-3-carboxylic acid (Tic)), which binds with high affinity (K_i ~ 150 pM, Figure S4).[18] Imaging of HEK-293T cells expressing SNAPtag-DOR after treatment with fluorescently-labeled DNA constructs showed that retention of the fluorescent signal was dependent on both the ligand and the crosslinker (Figure S5). Application of the selection approach with 293F cells expressing DOR (Figure 4A) gave a 180-fold enrichment of the DTK ligand over a non-ligand control at 100-fold dilution (Figure S4). Similar enrichment and recovery were observed when reducing the DTK ligand concentration 10-fold (from 1 nM to 0.1 nM).

We constructed a 96 compound DEL using Dmt-Pro-Phe-Phe (K_i ~ 28 nM)[19] and Tyr-Tic (K_i ~ 240 nM)[20] as

parental peptides and applied the selection. Compounds from the higher affinity parental peptide were more greatly enriched (Figures 4B, S8 and S9). The DTK ligand doped into the DEL gave 300-fold enrichment. Similarly, the Dmt-Tic pair was the highest enriching ligand among the low affinity set (LA-P1-#1, 13-fold). We synthesized 8 off-DNA ligands and evaluated their K_i by a radiolabeled ligand displacement assay.[21] K_i values roughly correlated to the enrichments with the exception of (HA-P1-#6), which did not show binding (Figure 4C and Table S14). Compounds HA-P(2)-#10 and HA-P(2)-#11 (both containing non-alpha amino acids) are novel DOR ligands of high affinity.

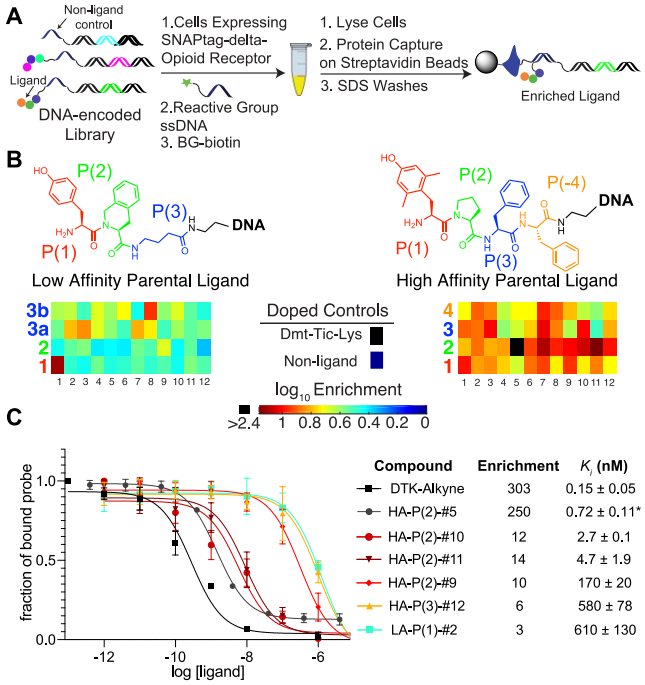


Figure 4. DEL selection against SNAPtag-DOR on live cell surfaces. (A) Crosslinking approach to DEL selections on live cell surfaces. BG-biotin = O-(6)-benzylguanine-biotin. (B) Parental peptides for DNA-encoded PSLs and heat maps of live cell enrichments. The color scale represents the log enrichment relative to the non-ligand control. See Figures S8 and S9 for structures. (C) K_i values of off-DNA hits binding to SNAPtag-DOR. Line colors correspond to live cell enrichment values from the selection, as indicated on the color scale. *K_i of HA-P(2)-#5 was determined on Prolink-tagged DOR (DiscoverX).

In summary, the covalent crosslinking approach described here enables use of DELs within and on living cells. Because protein concentration drives the binding equilibrium to DEL ligands in typical selections, high specific activity of protein targets is critical, which makes assessment of binding in live cells desirable. To our knowledge, this work represents the first demonstration of targeting DELs to proteins within living cells and will expand the target scope of DELs beyond purified proteins. While still reliant on recombinant protein expression, further work may enable targeting endogenous proteins by using immunopurification or incorporation of affinity tags on targets using CRISPR/Cas. In addition to the PSLs demonstrated here, we expect this approach to find application in *de novo* ligand discovery with combinatorial DELs, on-DNA hit validation, and DNA-based protein activity assays.[22]

ASSOCIATED CONTENT

Supporting Information. Synthetic procedures, evaluation of DNA cell entry, protein expression, cell culture, selection details, off-DNA hit validation, library information, and characterization data is available free of charge on the ACS Publications website.

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Notes

DK is the founder and CEO of Dencoda, LLC, a company that uses DNA-linked molecules as sensors for diagnostic and drug discovery purposes.

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