A suspension cell-based interaction platform for interrogation of membrane proteins.

Patrick Krohl[1], Kook Bum Kim[1], Lance Lew[1], Derek VanDyke[1], Seth Ludwig[1], and Jamie Spangler[1]

[1]Johns Hopkins University

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Abstract

The majority of clinically approved therapeutics target membrane proteins (MPs), highlighting the need for tools to study this important category of proteins. To overcome limitations with recombinant MP expression, whole cell screening techniques have been developed that present MPs in their native conformations. Whereas many such platforms utilize adherent cells, here we introduce a novel suspension cell-based platform termed "biofloating" that enables quantitative analysis of interactions between proteins displayed on yeast and MPs expressed on mammalian cells, without need for genetic fusions. We characterize and optimize biofloating and illustrate its sensitivity advantage compared to an adherent cell-based platform (biopanning). We further demonstrate the utility of suspension cell-based approaches by iterating rounds of magnetic-activated cell sorting (MACS) selections against MP-expressing mammalian cells to enrich for a specific binder within a yeast-displayed antibody library. Overall, biofloating represents a promising new technology that can be readily integrated into protein discovery and development workflows.

Topical Heading

Biomolecular Engineering, Bioengineering, Biochemicals, Biofuels, and Food

Key Words

Membrane proteins; Biomolecular engineering; *In vitro* evolution; Cell panning; Yeast surface display

1 Introduction

Membrane proteins (MPs) are important therapeutic targets as they are responsible for facilitating a wide range of biological functions critical to maintaining homeostasis. MPs transduce signals into cells, catalyze biochemical reactions, adhere to surfaces, and transfer ions across the cell membrane. The ability to manipulate cellular processes, particularly when these processes are dysregulated in the context of disease, therefore rests on our capacity to specifically engage MPs. Indeed, over 60% of all clinically approved drugs target MPs.[1] Due to their high affinity, specificity, and favorable pharmacokinetic properties, antibodies represent a particularly desirable and growing class of drugs against MPs.[2,3] Antibodies are generally discovered either through immunization of animals or using *in vitro* display technologies such as phage or yeast display.[4–8] *In vitro* display methods enable the screening of large combinatorial protein libraries by linking genetic information to phenotypic responses. Antibodies are typically screened in display platforms through presentation of their minimal binding moieties, known as single-chain variable fragments (scFvs). Discovered scFvs can later be formatted into full-length antibodies as potential therapeutics.[9] *In vitro* display technologies allow for fine tuning of the biophysical properties of antibodies such as target affinity and specificity.[10–13] Although the phage display platform allows for greater library diversity compared to yeast display, the latter has several important advantages, including: (i) more accurate recapitulation of native post-translational

modifications in mammalian proteins through use of a eukaryotic system; (ii) more sophisticated secretory apparatus to allow for expression of disulfide-bonded proteins; and (iii) capacity to perform dual-color sorts using fluorescently activated cell-sorting (FACS) to enhance selection of high affinity clones.[10]

In order to select for translationally relevant antibodies against MPs, the antigen must be displayed in a conformation that closely represents its native state in the cell membrane. Although there has been great success in generating antibodies against relatively straightforward MP targets such as single-pass membrane proteins, generation of antibodies targeting complex multipass transmembrane proteins such as G protein-coupled receptors (GPCRs) or ion channels has been more challenging. This is due to poor expression or solubility of multipass transmembrane proteins when produced recombinantly, which complicates their use as selection targets for immunization or display strategies. Addition of detergents to stabilize multipass MPs or selections against truncated peptide sequences found within MPs have been implemented; however, these approaches can enrich for antibodies against intracellular rather than extracellular epitopes or antibodies that bind non-native conformations of the protein.[14–16]Alternative approaches such as nanodisk technology have facilitated display of MPs, although such methods require significant optimization.[17]

Another approach to achieve native antigen presentation for multipass transmembrane proteins involves the use of whole cells expressing the MP of interest. Whole cell screening approaches allow for the target to be displayed in its native conformation, with appropriate post-translational modifications, and in an orientation that is directly relevant for antibody targeting. Tillotson et al established a technique known as yeast biopanning, which involves incubating a yeast-displayed library of proteins with monolayers of mammalian cells expressing the target MP for discovery of specific binders.[18] In addition, the authors established a solubilized whole cell lysate-based approach to interface yeast-displayed protein libraries with detergent-treated MP-expressing mammalian cells. Cell lysate-based evolution has proven to be an effective engineering approach, although it requires significant optimization of detergent conditions.[18] A recently reported yeast/mammalian cell interaction platform allowed for live cell incubation in suspension.[19] This platform enriched for specific clones within a yeast-displayed library using FACS, through detection of fluorescent proteins genetically incorporated into both the yeast and mammalian cells. In this study, we establish a platform denoted "biofloating," which enables quantitative analysis of the interaction between proteins on the surface of yeast and MPs on the surface of mammalian cells. This approach detects yeast/mammalian cell interactions via fluorescently-labeled antibody staining of an epitope tag commonly incorporated into the yeast display platform and intracellular staining of MP-presenting mammalian cells. In contrast with previous approaches, biofloating enables interrogation of yeast/mammalian cell interactions in a fully suspension-based system without the need for genetic incorporation of fluorescent proteins. We characterize and optimize the biofloating platform by studying the interaction between scFvs on the surface of yeast and the MP programmed death-ligand 1 (PD-L1) on mammalian cells, comparing the performance of our platform to traditional biopanning techniques using a range of affinity and avidity conditions.

To extend our suspension cell-based approach to high-throughput library screening, we used magnetic-activated cell sorting (MACS) to enrich for specifically interacting scFvs against a target MP. MACS was recently shown to be a useful technique for yeast/mammalian cell screening,[20] given its superior library sampling capacity compared to FACS.[19] Here, we demonstrate the use of MACS-based yeast/mammalian cell selections to enrich for a specific clone spiked into a naïve yeast-displayed scFv library (10^9 diversity). The biofloating technique complements this suspension cell-based MACS selection approach, providing a high-throughput flow cytometry-enabled screening approach that can be integrated into the protein engineering workflow.

2 Materials and Methods

2.1 Tissue culture

Human embryonic kidney (HEK) 293F cells (Thermo Life Technologies) were cultured in FreeStyle 293 Expression Medium (Thermo Fisher Scientific) containing penicillin-streptomycin (10 μg/mL) (Thermo Fisher Scientific). CHO-K1 cells were cultured in F-12K medium containing 10% fetal bovine serum (FBS), L-

glutamine (2 mM), and penicillin-streptomycin (500 μg/mL). CHO-K1 cells (ATCC) stably transduced with PD-L1 were kindly provided to us by Dr. Sridhar Nimmagadda (Department of Oncology at Johns Hopkins Medicine) and cultured in F-12K medium (Thermo Life Technologies) containing 10% FBS, L-glutamine (2 mM), G418 Sulfate (2 mg/mL) (Thermo Fisher Scientific), and penicillin-streptomycin (500 μg/mL). H2444 cells were cultured in RPMI-1640 medium with 10% FBS, L-glutamine (2 mM), and penicillin-streptomycin (500 μg/mL). MDA-MB-231 cells were cultured in DMEM medium with 10% FBS, L-glutamine (2 mM), and penicillin-streptomycin (500 μg/mL). All cells were grown at 37°C with a humidified atmosphere and 5% CO_2.

2.2 Protein expression and purification

The extracellular domain of human PD-L1 (amino acids 19-228) and the anti-PD-L1 scFvs atezolizumab, D12, and A1 (comprised of the variable heavy [V_H] chain followed by the variable light (V_L) chain of the respective antibodies) were expressed via transient transfection of HEK 293F cells. The encoding DNA was cloned into the gWiz mammalian expression vector (Genlantis) containing a 6x His-tag for purification. The PD-L1 construct also contained a biotin acceptor peptide (BAP) GLNDIFEAQKIEWHE sequence immediately prior to the His-tag. HEK 293F cells were grown to 1.2×10^6cells/mL and diluted to 1.0×10^6 cells/mL on the day of transfection. Midiprepped DNA and polyethyleneimine (PEI, Polysciences) were independently diluted to 0.05 and 0.1 mg/mL in OptiPro medium (Thermo Life Technologies), respectively, and incubated at room temperature for 15 min. Equal volumes of DNA and polyethyleneimine were mixed and incubated at room temperature for an additional 15 min. Subsequently, the diluted HEK 293F cells and the DNA/PEI (40 mL/L) mixture were added to a shaking flask and incubated at 37@C and 5% CO_2 with rotation at 125 rpm for 72 hr. Secreted protein was harvested from HEK 293F cell supernatants after 72 hr by Ni-NTA (Expedeon) affinity chromatography. PD-L1 was biotinylated overnight at 4ºC using BirA ligase enzyme in 0.5 mM Bicine pH 8.3, 100 mM ATP, 100 mM magnesium acetate, and 500 mM biotin (Avidity). scFvs and biotinylated PD-L1 were further purified using a Superdex 200 sizing column on a fast protein liquid chromatography (FPLC) instrument (GE Healthcare), equilibrated in HEPES-buffered saline (HBS). Purity (>98%) was confirmed by SDS-PAGE analysis.

2.3 Yeast display and PD-L1 affinity characterization

General yeast display methodologies were carried out with EBY-100 yeast cells, as previously described.[7,21] The V_H chain followed by the V_L chain of the anti-PD-L1 antibody atezolizumab and the anti-programmed cell death protein (PD-1) antibody nivolumab were cloned into the yeast display vector pCT3CBN, separated by a $(G_4S)_3$linker (scFv format). The PD-L1-binding scFvs D12 and A1 were selected from a previously reported naïve yeast-displayed scFv library.[22] These clones were displayed on yeast using the pCTCON2 expression vector. Yeast clones were grown in SD-CAA medium at 30ºC while shaking at 200 rpm. Yeast were induced in SG-CAA at an initial optical density (OD) of 1.0 (1×10^7 cells/mL).

To titrate PD-L1 affinity, atezolizumab, D12, and A1 scFv-displaying yeast were grown overnight, induced for 24 hr, and then aliquoted into 96-well plates at 10^5 cells/well. Cells were then pelleted and resuspended in PBE buffer (phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (BSA) and 1 mM ethylenediaminetetraacetic acid (EDTA)) containing various concentrations of biotinylated PD-L1 and incubated for 2 hr at room temperature. Cells were then pelleted and washed with PBE and incubated for 15 min at 4°C with 50 nM Alexa647-conjugated streptavidin (SA-647) (Thermo Fisher Scientific) diluted in PBE. A final wash was conducted and binding was analyzed via flow cytometry using a CytoFLEX analyzer (Beckman Coulter). Data was analyzed in Prism software (GraphPad) and fitted using a single logistic model. Equilibrium dissociation constant (K_D) values were determined for each clone. The experiment was performed in triplicate and repeated twice to ensure reproducibility.

2.4 Bio-layer interferometry measurements of PD-L1 binding kinetics

Binding kinetics measurements were obtained using bio-layer interferometry (BLI) on an OctetRED96 instrument (Molecular Devices). Biotinylated PD-L1 was immobilized to SA-coated biosensors (Molecular Devices) in 0.45 μm filtered PBSA (phosphate-buffered saline pH 7.2 containing 0.1% BSA). Once baseline

measurements were collected in PBSA, binding kinetics were measured by submerging the biosensors in wells containing 3-fold serial dilutions of each anti-PD-L1 scFv for 300 seconds (association) followed by submerging the biosensors in wells containing only PBSA for 600 seconds (dissociation). Tips were regenerated in 0.1 M glycine pH 2.7. Curves were fitted using the Octet Data Analysis HT Software version 7.1 (Molecular Devices) assuming a 1:1 binding model to determine the association rates and dissociation rates.

2.5 Quantification of target expression levels on cells

PD-L1-transduced CHO K1 cells, H2444 cells, and MDA-MB-231 cells were all quantified for PD-L1 expression using Quantum Simply Cellular anti-mouse IgG beads (Bangs Laboratories), according to the manufacturer's protocol. Standard beads were incubated with an APC-conjugated mouse anti-human PD-L1 antibody (BioLegend, clone MIH2) (1:50 dilution) in PBSA for 30 min at room temperature. The beads were then pelleted, washed, resuspended in PBSA, and analyzed via flow cytometry to generate a standard curve. Cells were grown to 60-90% confluency and detached with trypsin-EDTA (Thermo Fisher Scientific) and quenched via addition of culture medium. 2×10^5 cells were then aliquoted into microcentrifuge tubes, washed, and resuspended in PBSA containing APC-conjugated mouse anti-human PD-L1 antibody (BioLegend, clone MIH2) (1:50 dilution) and incubated for 30 min at 4°C with rotation. Finally, the cells were washed, resuspended in PBSA, and analyzed via flow cytometry. Mean fluorescence intensity (MFI) values were compared to the generated calibration curve to determine PD-L1 expression levels.

2.6 Biofloating assays

Mammalian cells were grown to 60-90% confluency, detached with trypsin-EDTA, and quenched via addition of culture medium. Dissociated cells were washed and centrifuged at 400×g for 5 min twice with PBS and stained with CellTrace^{TM} Violet dye (Thermo Fisher Scientific). The cells were incubated with 2.5 μM dye in PBS at 1×10^6 cells/mL for 20 min at room temperature. Following incubation, the mammalian cells were washed three times with PBSA and then aliquoted into a 96-well plate at 5×10^4cells/well in a volume of 10 μL. Induced yeast cells were washed and centrifuged at 3,500×g for 3 min and resuspended in PBSA containing anti-cmyc Alexa647 antibody (Cell Signaling Technology, clone 9B11) (1:50 dilution). The cmyc-labeled yeast samples were aliquoted into the 96-well plate containing the CellTrace^{TM}Violet-labeled mammalian cells at 10 μL/well (total volume of 20 μL/well) at a final ratio of 10:1 yeast:mammalian cells (5×10^5 yeast cells/well). Incubation proceeded at 4°C for 2 hr with rotation. The cells were then pelleted, washed, and resuspended in PBSA for analysis on a CytoFLEX flow cytometer. Percent bound to yeast was quantified based on the cmyc-positive fraction of the CellTrace^{TM} Violet-labeled mammalian cells. No forward/side scatter gating was implemented.

Kinetic assays varied the incubation timepoints, wherein the 0 min timepoint sample consisted of adding the yeast to the mammalian cells and immediately pelleting and washing the sample. The yeast:mammalian cell ratio was held constant at 10:1 for kinetic assays. Titration studies were carried out by varying the yeast:mammalian cell ratios while keeping the incubation period constant at 2 hr. The percent of mammalian cells bound to scFv-expressing yeast was plotted for each ratio and fitted to a single logistic model using Prism software.

2.7 Biopanning assays

The biopanning assays were carried out using established protocols[18,23]. Tissue culture treated 6-well plates (Corning) were coated with [?]300,000 g/mol Poly-D-Lysine (PDL) (Sigma-Aldrich) by following the procedure described by the manufacturer. Sterile water was used to dissolve the PDL powder to 0.1 mg/mL. Surfaces were coated with PDL solution at 1 mL/25 cm^2, and allowed to incubate for 5 min. The solution was then aspirated and plates were washed twice with sterile water and then left to dry overnight. Mammalian cell monolayers were grown in the PDL-coated 6-well plates to 70-100% confluency and washed three times with PBSCMA (PBS containing 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$, and 0.1% BSA). Induced yeast samples were washed twice with PBSCMA and centrifuged at 3,500xg for 3 min. Yeast samples were resuspended in PBSCMA at 5x10^7 yeast cells/mL. 1 mL of the yeast sample was added to each well (5x10^7yeast cells/well – an approximate yeast:mammalian cell ratio of 40:1 assuming that a confluent 6-well plate contains [?]1.2x10^6

cells (Thermo Fisher specifications)). Co-incubation was carried out for 2 hr, after which five washes with PBSCMA were conducted. Washes consisted of adding 1 mL of buffer to each well and gently rocking the plate back and forth 25 times followed by five rotations and then aspirating. Binding was detected via phase contrast microscopy using an EVOS XL (Thermo Fisher Scientific) 40x objective and quantified through manual counting of images in ImageJ. Kinetic assays were conducted by varying the yeast/mammalian cell incubation times at a fixed ratio of 40:1. Titrations were carried out by varying the yeast:mammalian cell ratios while keeping the incubation period constant at 2 hr.

2.8 Construction of mock library and enrichment tracking

The pCT3CBN yeast expression vector containing the atezolizumab scFv construct was modified to include a FLAG-tag (DYKDDDDK), replacing the original C-terminal myc tag. On day 1, a naive synthetic scFv library[22] with a diversity of approximately 1x10^9 was thawed and grown overnight in SD-CAA. Concurrently, competent yeast were electroporated with FLAG-tagged atezolizumab scFv and grown in SD-CAA for 2 days. On day 2, the naive scFv library was passaged in SD-CAA to an O.D. of 1, and on day 3 the library was induced for 2 days in SG-CAA. Also on day 3, atezolizumab scFv-expressing yeast were induced in SG-CAA and incubated for 2 days. On day 5, a mock library was constructed by adding atezolizumab scFv-expressing yeast into the naive library at a dilution of 1:1000. Rounds of selections were carried out by both biofloating and biopanning enrichment protocols, as described below. Atezolizumab scFv-expressing yeast enrichment was analyzed by staining the yeast population after each round of selection using anti-cmyc Alexa647 antibody (Cell Signaling Technology, clone 9B11) (1:50 dilution) and anti-FLAG DyLight 488 (20 μg/mL) (Thermo Fisher Scientific). FLAG detection was used to quantify the atezolizumab scFv-displaying yeast population and cmyc detection was used to identify expressing yeast from the naïve scFv library.

2.9 Suspension cell-based enrichment via MACS

Non-transduced CHO-K1 (PD-L1$^-$ CHO-K1) cells and PD-L1-transduced CHO-K1 (PD-L1$^+$ CHO-K1) cells were grown to 70-90% confluency, detached with trypsin-EDTA, and quenched via addition of culture medium. Cells were pelleted at 400×g for 5 min, washed three times with PBS pH 8, and then biotinylated using EZ-Link Sulfo-NHS-SS-Biotin (Thermo Fisher Scientific). Per manufacturer's protocol, PD-L1$^-$ CHO-K1 and PD-L1$^+$CHO-K1 cells were each resuspended at 25×10^6 cells/mL in PBS pH 8 containing 2 mM of EZ-Link Sulfo-NHS-SS-Biotin reagent and incubated at room temperature for 30 min with rotation. Three washes were then conducted using PBSA (pH 7.3) to quench the reaction and remove excess byproducts. Mock library yeast induced in SG-CAA were pelleted at 3,500×g for 5 min, washed twice with PBSA, and resuspended in 5 mL of PBSA containing biotinylated PD-L1$^-$ CHO-K1 cells. In round 1, 10^7 PD-L1$^-$ CHO-K1 cells were incubated with 10-fold diversity of the yeast mock library (10^{10} yeast) for a final yeast:mammalian cell ratio of 1000:1. The cell mixture was then incubated for 1 hr at 4°C with rotation (negative selection). After 1 hr, 250 μL of magnetic beads coated with SA (Miltenyi Biotec) were added to the cell mixture and incubation proceeded for 20 min at 4°C with rotation. The cell mixture was then washed once with PBSA and centrifuged at 400×g for 5 min. The pellet was gently resuspended in 20 mL of PBSA and cells were separated over four magnetic columns (Miltenyi Biotec) ([?]5 mL/column), according to the manufacturer's protocol. The flow-through solutions from each column were pooled and pelleted at 3,500xg for 5 min. The pellet was then resuspended in 5 mL of PBSA containing biotinylated PD-L1$^+$ CHO-K1 cells (10^7PD-L1$^+$ CHO-K1 cells were used in round 1) and allowed to incubate for 2 hr at 4degC with rotation (positive selection). After 2 hr, 250 μL of magnetic beads were added to the cell mixture and incubation proceeded for 20 min at 4°C with rotation. The cell mixture was then washed once with PBSA and centrifuged at 400×g for 5 min. The pellet was gently resuspended in 20 mL of PBSA and separated over four magnetic columns ([?]5 mL/column), according to the manufacturer's protocol. Eluted cells from each column were pooled and centrifuged at 3,500xg for 10 min. The pellet was then resuspended in SD-CAA and grown overnight. The following day, the yeast were induced with SG-CAA and incubated for 2 days.

Subsequent rounds were carried out identically to round 1 with the following exceptions: (i) 10^8 yeast cells and 10^6 mammalian cells (100:1 ratio) were co-incubated; (ii) 50 μL of magnetic beads were incubated with the cell mixture; and (iii) 1 magnetic column was used for separation of the yeast/mammalian cell mixture.

2.10 Adherent cell-based enrichment

PD-L1$^-$ CHO-K1 and PD-L1$^+$ CHO-K1 cells were grown to 90-100% confluency in tissue culture-treated 150 mm petri dishes (Corning) that were PDL-coated. The adhered mammalian cells were washed three times with PBSCMA and the induced yeast mock library was washed twice. For the first round of selections, 10^{10} yeast cells were resuspended in 15 mL of PBSCMA and added to PD-L1$^-$ CHO-K1 cells at a ratio of 550:1 yeast/mammalian cells for 30 min at 4°C (negative selection). The supernatant was slowly collected from the plate and cells were washed five times with PBSCMA, while collecting the supernatant for each wash. The pooled supernatant was then spun down and resuspended in 15 mL of PBSCMA and incubated with PD-L1$^+$ CHO-K1 cells for 1 hr at 4°C (positive selection). Five washes were then conducted, discarding the supernatant from each wash. Cells were then scraped from the plate, and washed twice with 15 mL of PBSCMA to collect the mammalian and yeast cells. The cells were then spun down at 3,500×g for 10 min, resuspended in SD-CAA, and grown overnight. The following day, yeast were induced in SG-CAA and incubated for 2 days.

Subsequent rounds were carried out identically to round 1 with the following exceptions: (i) selections were carried out in a single well of a 6-well plate; (ii) 1 mL of PBSCMA was used for each wash; and (iii) 10^8 yeast cells and 1.2×10^6mammalian cells (83:1 ratio) were co-incubated.

3 Results

3.1 Development and characterization of the biofloating platform

We designed a new platform known as biofloating for quantitative analysis of the interaction between yeast displayed scFvs and target MPs on mammalian cells (Figure 1a). Biofloating entails the co-incubation of yeast and mammalian cells in suspension to enable ready compatibility with flow cytometry analysis (Figure 1b). A system was previously reported that detects interactions between scFv-expressing yeast and MP-expressing mammalian cells by incorporating fluorescent proteins into both cell types.[19] In contrast, our approach detects yeast/mammalian cell interactions via staining of yeast with fluorescently-labeled anti-cmyc antibody and intracellular staining of mammalian cells.

We compare our new platform to biopanning, an established technique for analysis of yeast/mammalian cell interactions in which yeast are incubated with mammalian cells adhered to a plate and binding is observed using phase contrast microscopy (Figure 1c). Here, we interrogate differences between the biofloating and biopanning platforms under varying affinity and avidity conditions. We use PD-L1 as our target MP for comparison of these platforms. To evaluate the effects of affinity on specific yeast/mammalian cell interactions, we use 3 scFvs with varying affinities to PD-L1: the scFv format of the FDA-approved anti-PD-L1 antibody drug atezolizumab and 2 anti-PD-L1 scFvs our lab discovered from a previously reported synthetic library,[22] denoted D12 and A1. We conducted yeast surface binding titrations with these clones against soluble PD-L1. Atezolizumab, D12, and A1 scFvs showed yeast surface $K_D$values of 1.1 nM (high), 60 nM (medium), and 650 nM (low), respectively (Table 1). We also interrogated the kinetic profiles of the soluble anti-PD-L1 scFvs binding to immobilized PD-L1 using bio-layer interferometry (BLI). Association rate constants (k_{on}) and dissociation rate constants (k_{off}) are reported in Table 2. The effects of avidity on binding were investigated by using 3 cell lines with varying PD-L1 expression. The PD-L1$^+$CHO-K1, H2444, and MDA-MB-231 cell lines were found to express PD-L1 at 2.0×10^6 molecules/cell (dense), 1.3×10^6 molecules/cell (medium), and 3×10^5 molecules/cell (sparse), respectively (Table 3). In each PD-L1$^+$ cell line, cells uniformly expressed the antigen (Figure S1).

3.2 Effects of antibody affinity on yeast/mammalian cell interactions

We sought to compare the impact of antibody binding affinity on yeast/mammalian cell interactions in the biofloating and biopanning platforms. For biofloating characterization, yeast cells expressing the atezolizumab, D12, A1, and nivolumab (an anti-PD-1 antibody serving as a negative control) scFvs were co-incubated with PD-L1$^+$CHO-K1 cells in suspension with a yeast:mammalian cell ratio of 10:1. The co-incubation period was varied from 0 to 180 min to gauge the kinetics of this system under different affinity conditions. We

found that yeast displaying the high affinity atezolizumab and medium affinity D12 scFvs were fully bound to PD-L1$^+$ CHO-K1 cells virtually instantaneously (Figures 2a and 2c). However, the low affinity clone A1 showed a time-dependent increase in mammalian cell binding, and did not achieve the same degree of binding as the high affinity or medium affinity scFvs, even after 180 min. As anticipated, no binding of atezolizumab, D12, or A1 scFvs was detected on PD-L1$^-$CHO-K1 cells via biofloating (Figure S2a). For biopanning characterization, scFvs were co-incubated with the PD-L1$^+$ CHO-K1 monolayers. We found that yeast displaying the high and medium affinity clones showed detectable binding to PD-L1$^+$ CHO-K1 cells within 60 min, whereas yeast displaying the low affinity clone did not show detectable binding to mammalian cells within 180 min (Figures 2b and 2d). As expected, no binding of atezolizumab, D12, or A1 scFvs was detected on PD-L1$^-$ CHO-K1 cells via biopanning (Figure S2b). In contrast with their instantaneous saturation using the biofloating platform, interaction between the high and medium affinity clones and the mammalian cells increased over time, demonstrating the stronger kinetic dependence of yeast/mammalian cell binding for the biopanning versus the biofloating platform. Moreover, detection of interaction between yeast displaying the low affinity clone and mammalian cells in the biofloating but not the biopanning setup indicates the superior sensitivity of the former.

We next sought to compare the impact of antibody binding affinity on the optimal yeast:mammalian cell co-incubation ratio in both the biofloating and biopanning platforms. To this end, yeast displaying atezolizumab, D12, A1, and nivolumab (negative control) scFvs were incubated with PD-L1$^+$ CHO-K1 cells at various yeast:mammalian cell ratios while keeping the incubation time constant. For biofloating experiments, binding was detectable for ratios as low as 0.57 for the high, medium, and low affinity clones (Figure 3a). The potency of binding corresponded with scFv affinity, with the high affinity clone requiring the lowest yeast:mammalian cell ratio to achieve saturation (Figure 3c). Based on these findings, a 10:1 yeast:mammalian cell ratio was determined to be sufficient for biofloating studies. Analogous biopanning studies demonstrated that >10-fold higher yeast:mammalian cell ratios were required to achieve saturation, as compared to biofloating (Figures 3b and 3d). Binding of yeast displaying the low affinity scFv to mammalian cells was barely detectable, even at the highest yeast:mammalian cell ratio, again supporting the enhanced sensitivity of binding detection for the biofloating platform. Our results also confirm that the 40:1 yeast:mammalian cell ratio previously implemented for biopanning studies[18] is sufficient to reach saturation for high affinity scFv clones.

3.3 Effects of avidity on yeast/mammalian cell interactions

We next sought to compare the effects of avidity on yeast/mammalian cell interactions in the biofloating and biopanning platforms through studies using mammalian cell lines with varying PD-L1 expression levels: PD-L1$^+$ CHO-K1 (dense), H2444 (medium), and MDA-MB-231 (sparse). For biofloating characterization, yeast cells expressing the atezolizumab and nivolumab (negative control) scFvs were co-incubated with the 3 PD-L1-expressing cell lines in suspension at a yeast:mammalian cell ratio of 10:1. We found that yeast displaying the atezolizumab scFv were fully bound to dense and medium PD-L1-expressing cell lines almost immediately (Figures 4a and 4c). Minimal specific binding of atezolizumab scFv-expressing yeast to the sparse PD-L1-expressing cell line was observed compared to the nivolumab scFv-expressing yeast control. Also, the percent of PD-L1-expressing mammalian cells bound to scFv-expressing yeast declined with decreasing antigen density. For biopanning characterization, yeast cells expressing the atezolizumab and nivolumab scFvs were incubated with monolayers of the 3 PD-L1-expressing mammalian cell lines. We found that atezolizumab scFv-expressing yeast bound to the dense PD-L1-expressing cells in a time-dependent manner, whereas almost no specific binding was detected to the medium and sparse cell lines for all timepoints when compared to the nivolumab scFv-expressing yeast control (Figures 4b and 4d). Collectively, these experiments demonstrate the superior sensitivity for the biofloating versus the biopanning platform in detecting yeast/mammalian cell interactions at lower levels of antigen expression.

We next studied the effects of avidity on the optimal yeast:mammalian cell co-incubation ratio in both the biofloating and biopanning systems. Yeast displaying the atezolizumab or nivolumab (negative control) scFv were incubated with the dense, medium, and sparse PD-L1-expressing cell lines at various yeast:mammalian cell ratios while fixing the incubation time at 2 hr. For biofloating experiments, atezolizumab scFv-expressing

yeast binding to the dense cell line was detected at ratios as low as 0.06:1, whereas the medium cell line required yeast:mammalian cell ratios of >2:1 for detection (Figures 5a and 5c). Specific binding to the sparse cell line was barely detectable by biofloating. Notably, the maximum percentage of PD-L1-expressing mammalian cells bound to scFv-expressing yeast declined with decreasing avidity conditions. These results reinforce that the 10:1 yeast:mammalian cell ratio determined from affinity titrations is optimal for biofloating analysis. Similar to the affinity titration studies, the analogous biopanning characterization for avidity effects demonstrate that >10-fold higher yeast:mammalian cell ratios were required to achieve saturation, as compared to biofloating (Figures 5b and 5d). Almost no specific binding could be detected to the medium and sparse cell lines using biopanning, again reiterating the higher sensitivity of the biofloating platform.

3.4 Enrichment of atezolizumab scFv-expressing yeast using suspension versus adherent methods

In addition to characterizing yeast/mammalian cell binding interactions on the biofloating platform, we explored whether a soluble yeast/mammalian cell interaction system could be exploited for enrichment of a specific clone spiked into a naïve scFv library. Enrichment using a soluble yeast/mammalian cell interaction platform has been conducted previously via FACS to enrich specific yeast clones from a library of diversity 2.5×10^7.[19] To increase the throughput of enrichment and allow for selection of higher diversity libraries, we combined MACS with a suspension cell-based yeast/mammalian cell interaction platform. Lown et al previously used magnetic bead-immobilized target-expressing mammalian cells to enrich yeast displaying a target-specific fibronectin spiked into a pool of non-displaying yeast.[20] Resonating with this work, we created a mock library consisting of a naïve yeast-displayed scFv library[22] of diversity 10^9 with atezolizumab scFv-expressing yeast spiked in at 1:1000. Enrichment of FLAG-tagged atezolizumab scFv-expressing yeast was tracked over 3 rounds of selection using the suspension cell-based platform compared to an adherent cell-based platforms.

For MACS-enabled suspension cell-based selections, mammalian cells were biotinylated and immobilized on SA-coated magnetic beads. Yeast and biotinylated mammalian cells were first co-incubated in suspension, and SA-coated magnetic beads were then added prior to separation over a magnetic column (Figure 6a). The first round of selections used 10^{10} yeast cells from the mock library (10-fold oversampling of scFv diversity) and 10^7 mammalian cells, for a 1000:1 yeast:mammalian cell ratio. In each round, a negative selection was carried out using PD-L1$^-$ CHO-K1 cells to deplete the yeast library of scFvs binding to irrelevant molecules on the mammalian cell surface. A positive selection was then conducted against PD-L1$^+$ CHO-K1 cells to enrich for PD-L1-binding yeast-displayed scFvs. Flow cytometry analysis was performed following each round to track specific scFv enrichment (Figure 6b). Rapid enrichment of atezolizumab scFv-expressing yeast was observed over the first 2 rounds, with the first round enriching to 10% specific yeast compared to 0.1% in the unsorted library. The library was almost fully converged post-round 2 and plateaued post-round 3 (63% and 66% atezolizumab scFv-expressing yeast, respectively).

For adherent cell-based selections, monolayers of mammalian cells were co-incubated with the yeast-displayed mock library for directed evolution following traditional screening methods (Figure 6c). The first round of selections used 10^{10} yeast cells from the mock library (10-fold oversampling of scFv diversity) and 1.8×10^7 mammalian cells for a 550:1 yeast:mammalian cell ratio. For each round, negative selections were performed by clearing binders to PD-L1$^-$ CHO-K1 cells prior to positive selections that enriched for binders to PD-L1$^+$ CHO-K1 cells. Flow cytometry analysis was conducted after each round to track scFv enrichment (Figure 6d). Minimal enrichment of atezolizumab scFv-expressing yeast was detected after round 1 (1.4% atezolizumab scFv-expressing yeast), whereas dramatic enrichment was observed post-round 2 (52% atezolizumab scFv-expressing yeast). Further library enrichment was observed after round 3, reaching a final atezolizumab scFv-expressing yeast percentage of 71%. Although comparable library enrichment was observed post-round 3 for the suspension and adherent cell-based platforms, suspension cell-based evolution led to faster enrichment compared to adherent cell-based evolution.

4 Discussion

In vitro display platforms have been employed extensively over the past 3 decades for protein discovery and characterization.[7] Within the past 10 years, whole cell selection technologies have empowered selection approaches against MP targets by simulating the natural context of the protein relevant for ligand recognition.[18] In this study, we build upon these whole cell selection technologies by presenting and characterizing a novel platform that we call biofloating to interrogate protein interactions within the context of a yeast/mammalian cell system. In contrast with previous yeast/mammalian cell interaction systems, our approach enables incubation of yeast with mammalian cells in suspension and utilizes quantitative flow cytometry analysis, without requirement for the incorporation of genetic fusions into either the yeast or mammalian cells. Hence, this versatile interaction system allows for compatibility with existing yeast display infrastructure. Moreover, we found that the biofloating platform demonstrated superior sensitivity compared to biopanning, both in terms of kinetics and equilibrium interactions investigated in this study. In particular, dramatic differences in binding behavior were evident in the context of low target binding affinity or reduced target expression (avidity). Of note, the biofloating approach detected high, medium, and low affinity interactions between yeast-displayed scFvs and target antigen-expressing mammalian cells, whereas biopanning detected only high and medium affinity interactions, and with significantly weaker potency (Figure 3). In addition, the biofloating method detected scFv-displaying yeast interactions with dense and medium density antigen-expressing mammalian cells (Figure 5), whereas biopanning was only able to detect interactions with antigen-dense cells, and with significantly weaker sensitivity.

Interestingly, the biofloating platform achieved maximum yeast/mammalian cell binding virtually instantaneously in the case of high and medium affinity interactions, whereas the biopanning platform exhibited a more gradual rise in binding (Figure 2). The biofloating approach also led to immediate saturation of dense and medium density target-expressing cell lines, whereas the biopanning strategy effected a time-dependent rise in binding for only the dense cell line (Figure 4). The significant differences in on-rate kinetics between the two platforms likely results from distinct spatial arrangements and mixing capacities between the 2-dimensional biopanning and 3-dimensional biofloating systems. In the biopanning platform, yeast gradually settle on top of the mammalian cell monolayer, thus manifesting a diffusive limitation in achieving cell-cell binding. In contrast, in the biofloating platform, yeast cells are well mixed in suspension with mammalian cells, thereby eliminating any diffusive barriers.

A major advantage of the biofloating platform is integration with flow cytometry, which enables precise quantification of binding dynamics. This gives us access to important molecular parameters that define yeast/mammalian cell interactions, such as the number of yeast bound per mammalian cell and the percentage of mammalian cells that interact with yeast. In addition, the biofloating platform interrogates protein interactions fully in suspension, which can be exploited for *in vitro* evolutionary schemes. To this end, we demonstrated enrichment of yeast displaying a specific scFv from a library of scFv-displaying yeast using a fully suspension cell-based MACS selection approach. This work builds upon a recently reported MACS-based selection platform[20] to achieve specific enrichment of a clone spiked into a naïve scFv library and to elucidate the dynamics of enrichment over of multiple rounds of evolution. We showed that this suspension cell-based approach led to robust enrichment of yeast specific for the mammalian cell-expressed target antigen, and this enrichment was more rapid than for an analogous adherent cell-based selection approach (Figure 6). Importantly, substantial enrichment occurred in the first round using the suspension cell-based approach. This observation is significant since MACS is most useful in early rounds to debulk large libraries, compared to alternative approaches such as FACS, which take prohibitively lengthy amounts of time to screen highly diverse libraries. Indeed, the throughput of our approach enabled rapid screening of a 10^9 member scFv library incorporating a 10-fold excess of yeast cells. We ultimately envision integration our methodologies into a hybrid MACS/FACS selection workflow. Initial rounds of MACS could be implemented to accommodate large library sizes, followed by subsequent rounds using FACS for superior control over the affinities of the enriched proteins. The 96-well plate biofloating format we have developed could then be implemented for high-throughput screening of individual clones from the evolved library to monitor selection progress and identify lead clones. This post-selection screening would be logistically challenging and significantly less sensitive if implemented using the biopanning format, underscoring the advantage for our novel biofloating

platform. Furthermore, although FACS was not used in this study, our characterization of yeast/mammalian cell interactions via flow cytometry offers useful insight that could inform the design of FACS-based selections in future implementations. An important limitation to keep in mind is that specific scFv-expressing yeast were spiked into the naïve yeast scFv library at a ratio of 1:1000. Representation of a binding clone within the library would likely be much lower; thus, additional rounds of selection could be required to achieve adequate enrichment. In addition, these selections were carried out using a high affinity anti-PD-L1 scFv, which may not be present in a naïve library. Indeed, Lown et al found that enrichment of yeast displaying a target-specific fibronectin using MACS-based selections against target-expressing mammalian cells was more challenging for fibronectin clones with lower affinity.[20] Thus, the selection process may require optimization to enable enrichment of lower affinity clones.

Antibody discovery against MPs has immense potential to advance scientific research and therapeutic development. The ability to study protein interactions and perform selections against MPs in their native conformations in the context of the cell membrane empowers the characterization and manipulation of complex MPs such as GPCRs and ion channels, for which the native antigen display has been historically difficult. Our novel biofloating platform enables quantitative investigation of MP interactions in a fully suspension cell-based system that is compatible with existing yeast display infrastructure. This work also paves the way for development and optimization of new selection technologies that will advance MP-focused therapeutic design.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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TABLE 1 Anti-PD-L1 scFv equilibrium binding affinities measured on yeast

Yeast-Displayed scFv	**K_D (nM)**
Atezolizumab	1.1 ± 0.28
D12	60 ± 6.7
A1	650 ± 130

TABLE 2 Anti-PD-L1 scFv binding kinetics measured by bio-layer interferometry (BLI)

scFv	**k_{on} ($M^{-1}s^{-1}$)**	**k_{off} (s^{-1})**
Atezolizumab	$6.4 \pm 0.09\times10^{6}$	$7.7 \pm 0.48\times10^{-5}$
D12	$6.4 \pm 0.20\times10^{5}$	$4.1 \pm 0.040\times10^{-3}$

scFv	k_{on} ($M^{-1}s^{-1}$)	k_{off} (s^{-1})
A1	$4.4 \pm 0.33\times10^{4}$	$5.6 \pm 0.060\times10^{-3}$

TABLE 3 Cellular PD-L1 expression levels

Cell line	PD-L1 molecules/cell
PD-L1^{+} CHO-K1	$2.0 \pm 0.37\times10^{6}$
H2444	$1.3 \pm 0.25\times10^{6}$
MDA-MB-231	$3.0 \pm 0.89\times10^{5}$

Figure Legends

Figure 1: Yeast/mammalian cell-cell selection platform layouts. (a) Schematic of yeast surface display of an scFv (left) and mammalian cell expression of a target MP (right). (b) In the biofloating platform, yeast and mammalian cells are co-incubated in suspension. (c) In the biopanning platform, yeast are incubated on top of mammalian cell monolayers.

Figure 2: Effects of scFv affinity on kinetics of yeast/mammalian cell interactions for biofloating versus biopanning platforms. Yeast expressing the anti-PD-L1 atezolizumab (High), D12 (Medium), or A1 (Low) scFvs or the anti-PD-1 (control) scFv were co-incubated with PD-L1^{+} CHO-K1 cells for varying lengths of time. (a) Flow cytometry histogram overlays depicting yeast binding (as measured by cmyc detection) within the mammalian cell population via biofloating for various incubation timepoints. (b) Phase contrast microscopy images of yeast binding to mammalian cells via biopanning for various incubation timepoints. (c) Quantification of biofloating results shown in (a), displaying the percentage of mammalian cells that are bound to yeast cells at various timepoints. (d) Quantification of biopanning results shown in (b), presenting the number of bound yeast cells per image at various timepoints.

Figure 3: Effects of scFv affinity on equilibrium yeast/mammalian cell interactions for biofloating versus biopanning platforms. Yeast expressing the anti-PD-L1 atezolizumab (High), D12 (Medium), or A1 (Low) scFvs or the anti-PD-1 (control) scFv were co-incubated with PD-L1^{+} CHO-K1 cells over a range of yeast:mammalian cell ratios. (a) Flow cytometry histogram overlays showing yeast binding (as measured by cmyc detection) within the mammalian cell population via biofloating for various yeast:mammalian cell ratios. (b) Phase contrast microscopy images of yeast binding to mammalian cells via biopanning for various yeast:mammalian cell ratios. (c) Quantification of the data shown in (a), displaying the percentage of mammalian cells that are bound to yeast cells at various yeast:mammalian cell ratios. (d) Quantification of the results shown (b), displaying the number of bound yeast cells per image at various yeast:mammalian cell ratios.

Figure 4: Effects of avidity on kinetics of yeast/mammalian cell interactions for biofloating versus biopanning platforms. Atezolizumab (PD-L1-specific) or nivolumab (control) scFv-expressing yeast were co-incubated with PD-L1^{+} CHO-K1 (Dense), H2444 (Medium), or MDA-MB-231 (Sparse) cells for varying lengths of time. (a) Flow cytometry histogram overlays depicting yeast binding (as measured by cmyc detection) within the mammalian cell population via biofloating for various incubation timepoints. (b) Phase contrast microscopy images of yeast binding to mammalian cells via biopanning for various incubation timepoints. (c) Quantification of biofloating results shown in (a), displaying the percentage of mammalian cells that are bound to yeast cells at various timepoints. (d) Quantification of biopanning results shown in (b), presenting the number of bound yeast cells per image at various timepoints.

Figure 5: Effects of avidity on equilibrium yeast/mammalian cell interactions for biofloating versus biopanning platforms. Atezolizumab (PD-L1-specific) or nivolumab (control) scFv-expressing yeast were co-incubated with PD-L1^{+} CHO-K1 (Dense), H2444 (Medium), or MDA-MB-231 (Sparse) cells over a range

of yeast:mammalian cell ratios. (a) Flow cytometry histogram overlays showing yeast binding (as measured by cmyc detection) within the mammalian cell population via biofloating for various yeast:mammalian cell ratios. (b) Phase contrast microscopy images of yeast binding to mammalian cells via biopanning for various yeast:mammalian cell ratios. (c) Quantification of the data shown in (a), displaying the percentage of mammalian cells that are bound to yeast cells at various yeast:mammalian cell ratios. (d) Quantification of the results shown (b), displaying the number of bound yeast cells per image at various yeast:mammalian cell ratios.

Figure 6: Enrichment of a specific clone spiked into a naïve yeast-displayed scFv library using suspension cell-based versus adherent cell-based selections. (a) Suspension cell-based selection scheme using MACS wherein biotinylated target antigen-expressing mammalian cells are incubated with a yeast-displayed scFv library and immobilized within a magnetic column via streptavidin-coated magnetic beads to allow for enrichment of target antigen-specific yeast. (b) Analysis of the yeast population following each round of evolution performed using the suspension cell-based selection scheme shown in (a). A naïve yeast-displayed scFv library doped with 1:1000 PD-L1-specific atezolizumab scFv-displaying yeast was evolved against PD-L1$^+$ CHO-K1 cells. Enrichment of atezolizumab scFv-expressing yeast (red) and depletion of other scFvs (blue) was monitored by flow cytometry. (c) Adherent cell-based selection scheme wherein a yeast library is incubated over mammalian cell monolayers and washed to allow for enrichment of target antigen-specific yeast. (d) Analysis of the yeast population following each round of evolution performed using the adherent cell-based selection paradigm shown in (c). A naïve yeast-displayed scFv library doped with 1:1000 PD-L1-specific atezolizumab scFv-displaying yeast was evolved against PD-L1$^+$ CHO-K1 cells. Enrichment of atezolizumab scFv-expressing yeast (red) and depletion of other scFvs (blue) were monitored by flow cytometry.

Figure S1: Expression of PD-L1 on various mammalian cell lines. Flow cytometry histograms depicting PD-L1 levels on the PD-L1$^+$ CHO-K1 (left), H2444 (middle), and MDA-MB-231 (right) cell lines. Staining with fluorescently labeled isotype control antibody (red) and anti-PD-L1 antibody (blue) are presented. Note that antigen expression is uniform on each cell line.

Figure S2: Selective binding of anti-PD-L1 scFv-expressing yeast clones to PD-L1$^+$ but not PD-L1$^-$ CHO-K1 cells on biofloating and biopanning platforms. Yeast expressing the anti-PD-L1 atezolizumab (High), D12 (Medium), or A1 (Low) scFvs were co-incubated with PD-L1$^-$ CHO-K1 or PD-L1$^+$ CHO-K1 cells for 180 min. (a) Flow cytometry histogram overlays depicting yeast binding (as measured by cmyc detection) within the mammalian cell population via biofloating. (b) Phase contrast microscopy images of yeast binding to mammalian cells via biopanning.

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