A cell–cell interaction format for selection of high-affinity antibodies to membrane proteins

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Contributed by Richard A. Lerner, June 3, 2019 (sent for review May 22, 2019; reviewed by Raymond Allen Dwek and Sanford M. Simon)

Generating and improving antibodies and peptides that bind specifically to membrane protein targets such as ion channels and G protein-coupled receptors (GPCRs) can be challenging using established selection methods. Current strategies are often limited by difficulties in the presentation of the antigen or the efficiency of the selection process. Here, we report a method for obtaining antibodies specific for whole cell membrane-associated antigens which combines a cell–cell interaction format based on yeast display technology with fluorescence-activated cell sorting of dual fluorescent complexes. Using this method, we were able to direct the affinity maturation of an antagonist antibody specific for the proton-gated ion channel ASIC1a and showed that both the affinity and potency were improved. We were also able to use this method to do kinetic selections to generate clones with better dissociation profiles. In addition, this method was employed successfully to handle the difficult problem of selecting antibodies specific to a GPCR target, the mu-opioid receptor.

antibody drug discovery | yeast display | membrane proteins | ion channels | GPCRs

Membrane proteins are essential for cell fate decisions, proliferation, metabolism, and other important aspects of maintaining homeostasis. This group includes ion channels, G protein-coupled receptors (GPCRs), enzymes, transporters, and receptors—all of which are major pharmaceutical targets. As membrane proteins they are more accessible than intracellular targets to biomolecules such as antibodies, making them ideal pharmaceutical targets. In addition, previous studies have shown that targeting membrane proteins, which are mutated or expressed at an abnormal level, can result in therapeutic drugs with higher specificity. Nowadays, in recognition of these advantages, more than 60% of drug targets are membrane proteins (1).

The usual procedure of selecting or optimizing binding antibodies against membrane protein targets often involves multistep processes such as preparation of antigens in different forms, presenting those antigens to either in vivo immune systems or artificial antibody libraries using surface display technologies, and finally selecting the resulting binding antibodies. Although it is difficult to obtain antibodies with desired properties using animal immunization, this strategy is still widely used to generate specific antibodies for both therapeutic and research purposes (2, 3). In addition, if the goal is to identify drug candidates, antibodies obtained via animal immunization must be derived from transgenic humanized mice or humanized at a later stage in their development.

An alternative approach is to use surface display technology, best exemplified by phage and yeast display, to isolate binding antibodies from large human antibody libraries (4, 5). Each display technology has its own characteristic advantages and disadvantages. For example, phage display systems offer the advantage of large library size, but the selection process can suffer from nonspecific binding and insufficient modifications in prokaryotic expression systems. However, yeast display systems benefit from the power of employing fluorescence-activated cell sorting (FACS) and posttranslational modification machinery, while having the limitation of small library size (6). A parallel study comparing yeast and phage display using the same antibody library and target antigen revealed that selection with yeast display yielded more novel binders (7). Furthermore, the eukaryotic protein expression also enables the yeast system to display peptides and proteins having complex structures, such as toxin peptides (8).

A major bottleneck in screening or improving the binding affinity of antibodies against membrane proteins using the surface display technologies is the preparation of the antigens. This has proved especially challenging for targets such as ion channels and GPCRs (9, 10). Some of these membrane proteins may be poorly expressed and/or poorly soluble, requiring extensive efforts to optimize the purification conditions. It can also be difficult to maintain their native conformation following purification. Additional techniques such as nanodisc and styrene–maleic acid lipid particles are often needed to help these proteins fold properly (11, 12). Alternative strategies such as using extracellular peptide segments or truncated peptides as antigens have also been explored (13, 14). However, antibodies enriched in this way may lose or show reduced binding ability to their natural conformation or have less chances of being functional. Previously, researchers have tried to avoid this problem by selecting antibodies against targets

Significance

Antibodies are an important class of drugs. We developed a simple, easy-to-use method for antibody drug discovery targeting membrane proteins including gated ion channels and G protein-coupled receptors. While phage or yeast display provides a convenient tool for antibody screening, the development of therapeutic antibodies against membrane proteins still encounters bottlenecks, because it is usually difficult to purify membrane proteins in native conformation for selecting functional antibodies. To address this challenge, we designed a whole-cell screening platform based on yeast–mammalian cell interaction, which bypasses the step of membrane protein purification and enables the direct selection of antibodies against "difficult-to-purify membrane proteins" in native state.

Author contributions: Z.Y., Y.W., T.Z., and R.A.L. designed research; Z.Y., Y.W., P.T., M.Q., X.D., C.-W.L., and T.Z. performed research; Z.Y., Y.W., P.T., M.Q., X.D., C.-W.L., G.Y., T.Z., and R.A.L. analyzed data; and Z.Y., Y.W., T.Z., and R.A.L. wrote the paper.

Reviewers: R.A.D., University of Oxford; and S.M.S., Rockefeller University.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1908571116/-/DCSupplemental.

Published online July 8, 2019.

expressed on whole cells using panning with phage or yeast display libraries (15, 16). In these experiments, phage- or yeast-expressing antibodies are added to cultured cells displaying the target protein. Specific binding antibodies are enriched by washing away the nonbinding phage or yeast (17). These experiments are challenging with nonadherent or poorly adherent cell types and can be plagued by nonspecific binding of the phage or yeast itself.

In the research reported here, we describe a different strategy where the yeast and the antigen-bearing target cell were labeled with two different fluorophores and sorted using FACS to identify the yeast–target cell complexes (Fig. 1). Each yeast cell displays a unique member of the antibody repertoire, and mammalian cells express a membrane protein of interest. When a library of yeast cells was mixed with mammalian cells, yeast may interact with and attach to mammalian cells if antibodies on the yeast surface bind to the membrane proteins overexpressed in mammalian cells, leading to the formation of yeast–mammalian cell complexes. Using this cell–cell interaction format in combination with FACS sorting of complexes, we were able to direct the affinity maturation of an antagonist antibody specific for human acid-sensing ion channel 1a (*h*ASIC1a) and showed that both the affinity and potency were improved. We also demonstrated that this method allowed us to do kinetic selections to generate clones with better dissociation profiles. In addition, this method was employed successfully to handle the difficult problem of selecting antibodies specific to a GPCR target, the mu-opioid receptor.

Results

FACS Sorting of the Cellular Complexes. To determine whether yeast–target cell complexes would be sufficiently stable for FACS sorting, we chose to pair *h*ASIC1a with its binding venom peptide

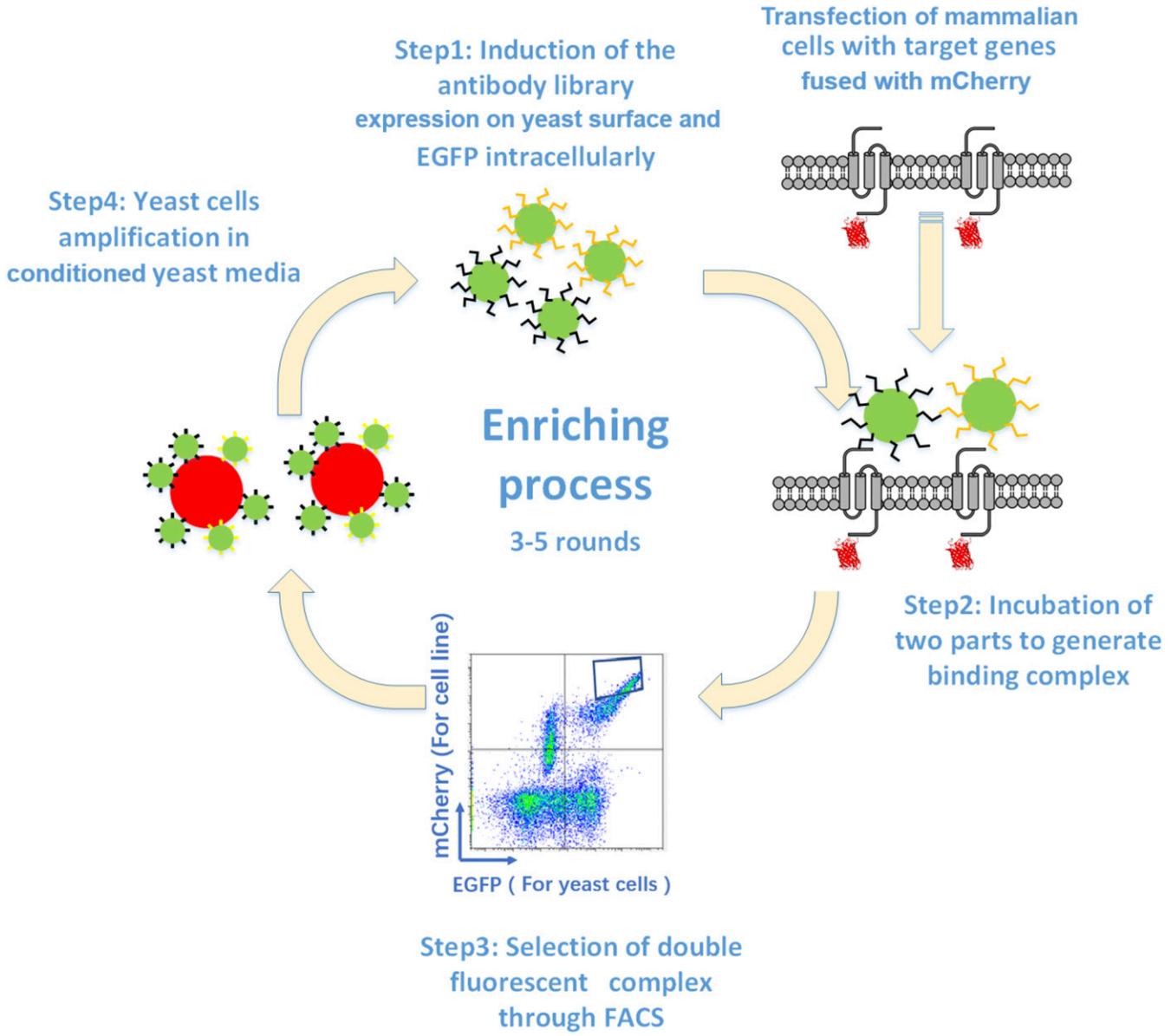

Fig. 1. Cartoon of the antibody or peptide selection process using cell–cell interaction between yeast and mammalian cells: 1) Yeast cell-surface display of peptide or antibody library with intracellular expression of EGFP and overexpression of the membrane protein target fused with mCherry in mammalian cells; 2) incubation of the yeast cells together with mammalian cells; 3) sorting of the dual fluorescent yeast–mammalian cell complexes using FACS; and 4) recovery and amplification of yeast cells from selected dual-fluorescent cell complexes in conditioned yeast culture medium.

PcTx1 for initial analysis (18). A stable CHO-K1 cell line overexpressing *h*ASIC1a was tagged with C-terminal mCherry fusion as described. The PcTx1 venom peptide was displayed on the yeast surface using a human IgG Fc fusion, and the resulting PcTx1-expressing yeast cells were tagged with anti-c-Myc-FITC antibody. Incubating the two cell constructs yielded a large population of dual fluorescent complexes sufficiently stable to be analyzed using FACS. Controls pairing the *h*ASIC1a-expressing CHO-K1 cells with yeast cells expressing an irrelevant antibody were negative (Fig. 2*A*). It is worth noting that varying incubation conditions, such as temperature and time, as well as using different yeast tagging strategies, significantly influenced binding efficiency between the mammalian and yeast cells (*SI Appendix*, Fig. S1). To avoid both possible steric hindrance by secondary fluorescence antibody, and the time-consuming and expensive tagging process of yeast expression, we decided to use an alternative yeast display construct in which a T2A-EGFP sequence fused to the C terminus of the displayed protein allows intracellular EGFP expression to be correlated with display level of the surface proteins (Fig. 2*B*) (19). We observed that the EGFP expression level indicates a similar expression profile for PcTx1. Compared with the previous construct in which PcTx1-displaying yeast cells were tagged with anti-c-Myc-FITC antibody, this construct showed similar display signals and similar binding efficiency with the *h*ASIC1a-overexpressing CHO-K1 cell line (*SI Appendix*, Fig. S2). To investigate whether high-affinity binding peptides can be enriched through FACS gating of dual fluorescent populations, the PcTx1 displaying yeast cells were added to a toxin peptide library at ratio of 1:4,000, and the library was screened against *h*ASIC1a-overexpressing cells. After just two rounds of selection, the percentage of double-positive counts in the FACS sorting results increased from 0.06 to 3.54%, and sanger sequencing of 50 clones in the final output consistently showed PcTx1 identity (Fig. 2*C*). An imaging flow cytometer was used to visualize the yeast–mammalian binding complexes during the FACS analysis. Multiple yeast cells binding one mammalian cell was a commonly observed pattern, including clusters of yeast cells bridging two or more mammalian cells (Fig. 2*D*).

Affinity Maturation of an Antibody to a Membrane Protein. Having shown that the system outlined in Fig. 1 can be used to select for dual fluorescent yeast–mammalian cell complexes indicative of protein-target cell binding, we then used an antibody specific for the membrane protein *h*ASIC1a (ASC06) to study directed affinity maturation and to determine whether the intensity of the dual fluorescent signals is positively correlated with affinity. This ASC06 antibody was previously reported to be a potent and specific antagonist that blocks ASIC1a (20). A mutagenic library was generated in the heavy chain CDR3 region of ASC06 by using a pool of oligonucleotide primers in which each single oligonucleotide in the pool encodes a CDR3 variant carrying no more than four mutated positions (21). Five rounds of screening were done to enrich high-affinity binding antibodies. The positive dual fluorescent signals increased from 0.075% of the initial library to 3.16% after round 4 of selection, indicating enrichment of the high-affinity binding antibodies (Fig. 3*A*). One hundred clones were isolated and sequenced after the five rounds of selection, and the results were analyzed to identify affinity-matured variants (Fig. 3*B*).

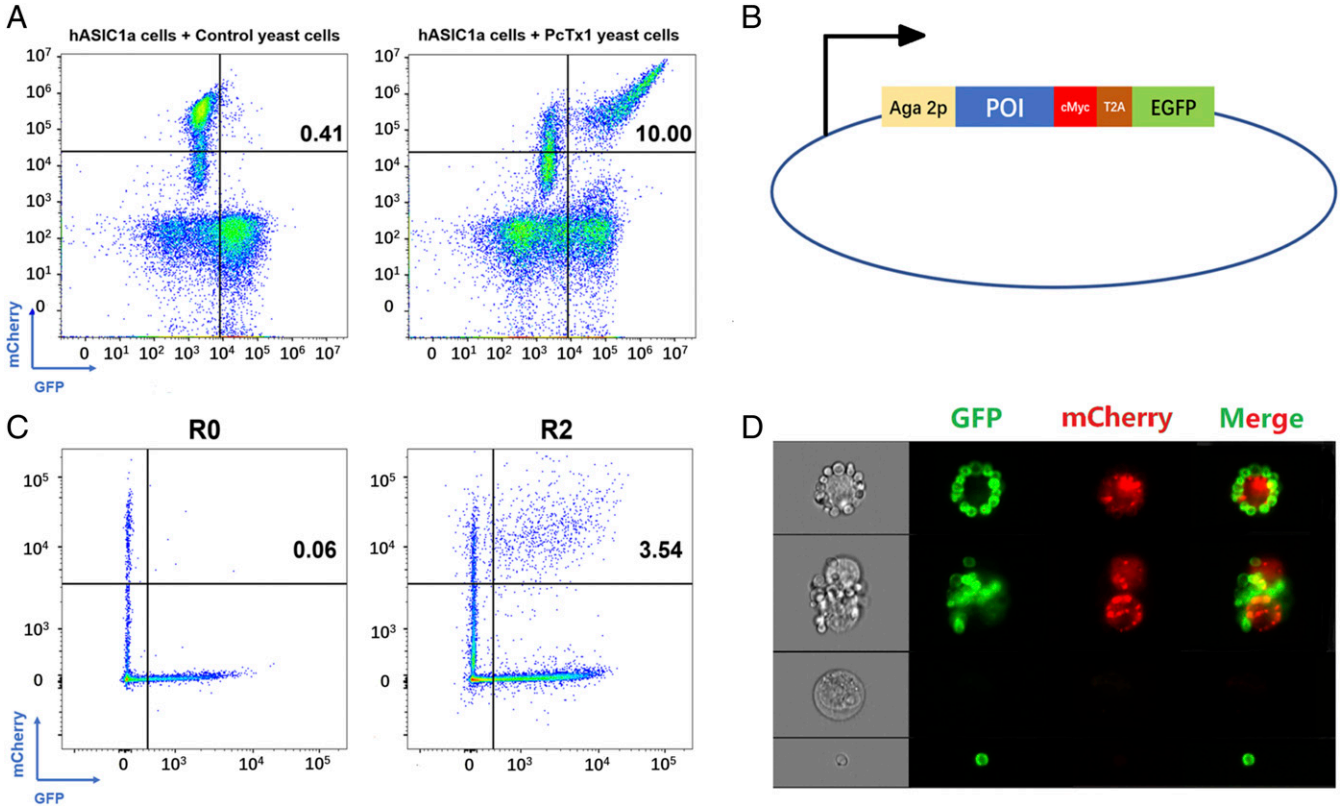

Fig. 2. Characterization of the complexes formed by incubating yeast cells and mammalian cells overexpressed with binding partners. (*A*) FACS analysis of PcTx1 displayed yeast cells or control yeast cells incubated with *h*ASIC1a-mCherry overexpressed CHO cells. (*B*) The yeast display construct expressing both protein of interests (POI) and intracellular EGFP via a T2A sequence. (*C*) FACS analysis of the initial library (R0) with PcTx1-expressed yeast cells added at the ratio of 1:4,000, and the second-round screening result (R2) of the library against *h*ASIC1a-mCherry overexpressed CHO cells. (*D*) Analysis of the mixture of CHO/*h*ASIC1a-mCherry cells and PcTx1 yeast cells using the imaging flow cytometers. Images of yeast cells, mammalian cells, or their complexes in bright field, GFP channel, and mCherry channel were taken during the analysis.

Of the 100 clones sequenced, two major types of mutations were identified: YF replacing FY in the original DSFYGYSKGD sequence (41 clones) and RA replacing YS (19 clones). The wild-type sequence was present in 12 clones. Two mutants, ASC03 (YF mutation) and ASC28 (RA mutation), plus the wild type were purified in whole IgG form, and the affinity of these antibodies was measured by surface plasmon resonance (SPR) using the purified intact extracellular domain of *h*ASIC1a trimer proteins (Fig. 3*C*). The SPR results showed that the affinity of the two mutants increased two- to threefold compared with wild type (from 0.28 nM wild type to 0.10 nM ASC03 and 0.12 nM ASC28) (Table 1). This improvement in affinity was also evidenced by an increase in dual fluorescent populations compared with wild type when yeast cells expressing either ASC03 or ASC28 were incubated with mammalian cells overexpressing *h*ASIC1a (*SI Appendix*, Fig. S3). The SPR data revealed that the increased affinity of ASC03 (YF mutation) is mainly due to an improved association profile compared with the wild-type ASC06, while with ASC28 (RA mutation), it is due to slower dissociation. We also determined the IC_{50} of these three antibodies by measuring the membrane potential using voltage-sensitive dye through fluorometric imaging plate reader (FLIPR) in the presence of different concentrations of antibody. The two mutant antibodies showed better IC_{50} compared with wild-type antibody, indicating improved potency in terms of their ability to inhibit acid-induced *h*ASIC1a currents (Fig. 3*D* and *SI Appendix*, Fig. S4).

During the traditional affinity maturation process using a yeast display platform, competitive kinetic selection is frequently introduced to screen for antibodies with better dissociation profiles (22). This is often achieved by adding a saturating amount of untagged target antigens to compete with their fluorescent-stained counterparts. Similarly, we conducted kinetic selection using our cell–cell interaction system by adding *h*ASIC1a-YFP overexpressed cells to the mixture of round 4 yeast cells and *h*ASIC1a-mCherry overexpressed cells. The ratio of dual fluorescent populations decreased with increasing competition time. After 3 h of competition, the remaining positive complexes were sorted by FACS and the results were analyzed by sequencing (Fig. 4*A*). Compared with previous output without the kinetic selection, the results revealed that the "RA" mutation frequency increased significantly from 19 to 37% in the output of kinetic selection, consistent with our previous observation that those two mutation residues may be important for improving dissociation profiles (Fig. 4 *B*, *Left*). We also did parallel affinity maturation of ASC06 using the soluble extracellular domain of *h*ASIC1a as antigens and four rounds of equilibrium selection followed by one round of kinetic selection. In terms of the mutation frequency distribution, the 100 clones sequenced here showed results

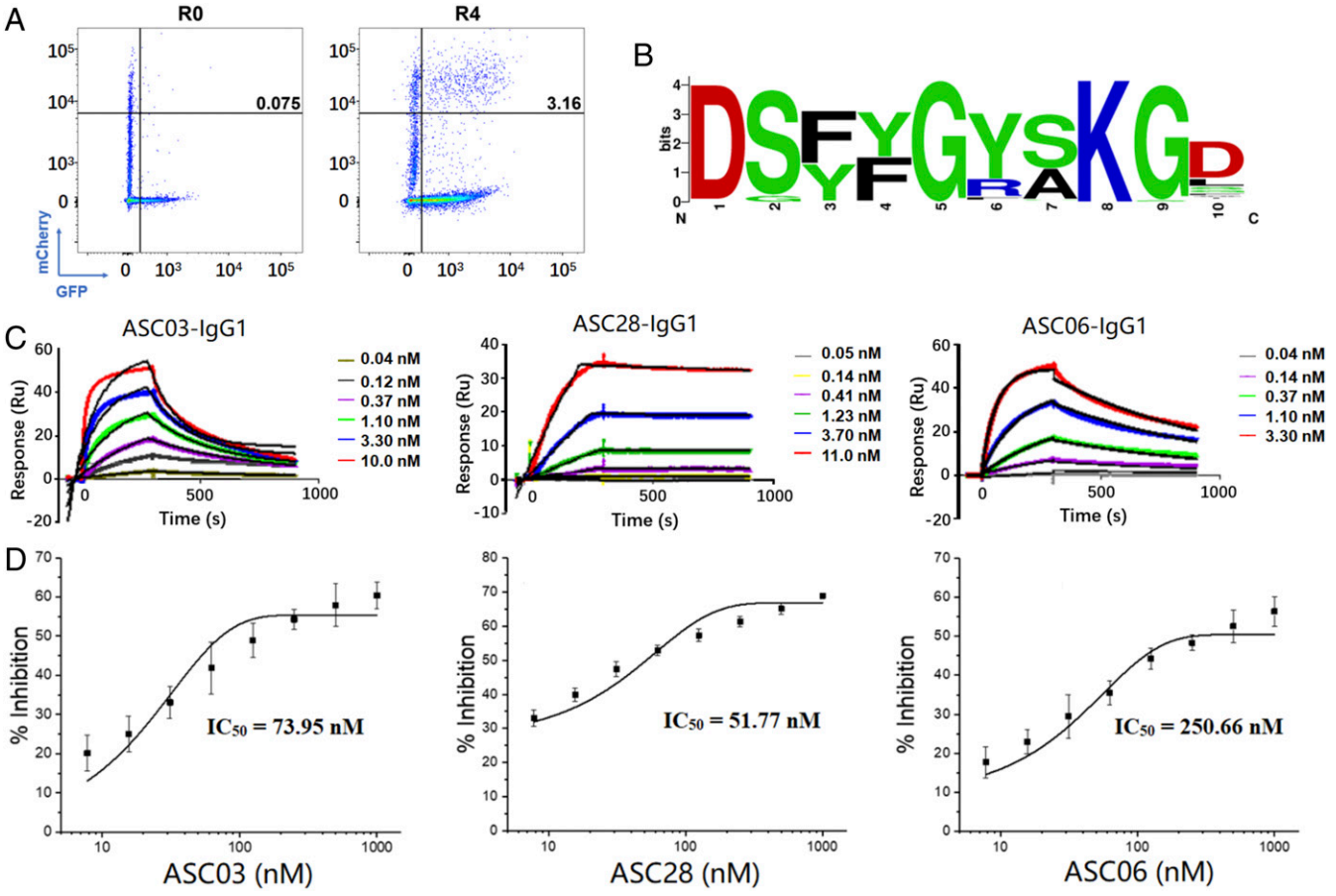

Fig. 3. The affinity maturation of the *h*ASIC1a targeting antibody ASC06 and the characterization of the enriched clones. (*A*) FACS analysis of the mixture of CHO/*h*ASIC1a-mCherry cells with the initial yeast library (R0) or the round 4 screening results (R4). (*B*) A WebLogo plot of the H-CDR3 of 100 clones sequenced after five rounds of selection was generated to show the amino acid distribution at each position within H-CDR3. (*C*) The affinity profiles of the purified antibody ASC03, ASC06, and ASC28 were measured by SPR. Antibodies were analyzed at multiple concentrations for the fitting of the affinity. The raw data were represented by the colorful lines while the black lines are the fitted curves. (*D*) IC_{50} of the antibody ASC03-IgG1, ASC06-IgG1, and ASC28-IgG1 in terms of their ability to inhibit the acid-induced *h*ASIC1a currents. The inhibitory effect of these three antibodies on the acid-induced *h*ASIC1a currents was measured using voltage-sensitive dye through FLIPR in the presence of different concentrations of the antibody (n = 6 for each concentration).

Table 1. The kinetic and activity profiles of the affinity improved antibodies and the parental ASC06

Clone no.	H chain CDR3	k_{on}, 1/Ms	k_{off}, 1/s	K_D, M	IC_{50}, nM
ASC06	DSFYGYSKGD	4.7e+06	1.3e−03	2.8e−10	250.66
ASC03	DSYFGYSKGD	9.7e+06	1.0e−03	1.0e−10	73.95
ASC28	DSFYGRAKGS	6.8e+05	8.2e−05	1.2e−10	51.77

Results were simulated and calculated by BIA evaluation software. The IC_{50} was measured by FLIPR membrane potential assay and fitted by maximal fluorescent intensity.

similar to the 100 clones obtained by cell–cell interaction kinetic selection (Fig. 4 *B*, *Right* and *SI Appendix*, Fig. S5).

Antibodies to the Human mu Opioid Receptor. GPCRs are important drug targets, but it has been challenging to raise antibodies specific for these membrane-spanning proteins using established methods, largely because of the difficulty in presenting antigen in the correct conformation both in vivo and in vitro (23). In an effort to determine if our system would overcome this problem, we paired yeast cells expressing a large human naïve library with mammalian cells overexpressing the human GPCR mu opioid receptor (*h*MOR). A naïve human scFv library with 2.5×10^7 members was expressed in yeast by subcloning scFv fragments from a nonimmunized combinatorial human scFv phage library into the yeast display vector. The human mu opioid receptor is one of four major types of opioid receptors that are targets for opioids such as morphine and has recently taken center stage because of the opioid epidemic (24, 25). Since it is such an important drug target, extensive efforts have been made to understand the biology of this GPCR. We overexpressed the *h*MOR protein with mCherry fused to the C terminus using Lenti virus infection. These cells were then used to select anti-*h*MOR binding antibodies from the yeast display library. Four rounds of selection were done to enrich the antibody against the *h*MOR-mCherry–overexpressing cells, and 30 clones were sequenced to analyze the enrichment (Fig. 5*A*). Three clones with repetition in their sequences were picked for deeper examination while two of them showed specific binding in staining the *h*MOR-overexpressed cells without the mCherry fusion compared with that of wild-type CHO cells (Fig. 5*B*). Immunofluorescence staining using a series of different antibody concentrations revealed a binding affinity of 76 nM and 280 nM for Ab3 and Ab17, respectively (Fig. 5*C*).

Discussion

Recently, combining large antibody libraries with yeast display technologies has been shown to greatly facilitate selection of specific antibodies for both research and therapeutic purposes. Despite these successes, much work to improve the affinity and specificity of selected antibodies is still ongoing. Many phage and yeast display systems have been tailored to manipulate desired antibody properties or to access difficult targets. In this study, we proposed a way of identifying and isolating antibodies specific for difficult membrane protein targets by direct selection of dual fluorescent cellular complexes. Thus, combining the power of large antibody libraries with enhanced presentation of antigens and the efficiency of FACS sorting allows enrichment of high-affinity binding antibodies specific for difficult targets such as GPCR proteins and ion channels.

In whole-cell screening systems, the antigen density on the presenting cell surface can be an important factor in terms of both the efficiency and the specificity of the final outcome. In the system reported here, we used a strong promoter to initiate overexpression of target proteins, so that the density of these proteins on the presenting cell surface should be significantly higher than the density of other endogenously expressed membrane proteins on the same cell. While some membrane proteins have a low expression level even when driven by strong promoters like CMV, this was not observed for two targets in our studies when the expression level of two membrane proteins studied is good enough for recovering of high-affinity binders. Nonspecific binding has been a significant issue when panning whole cells with either phage or yeast display systems. Such strategies often require

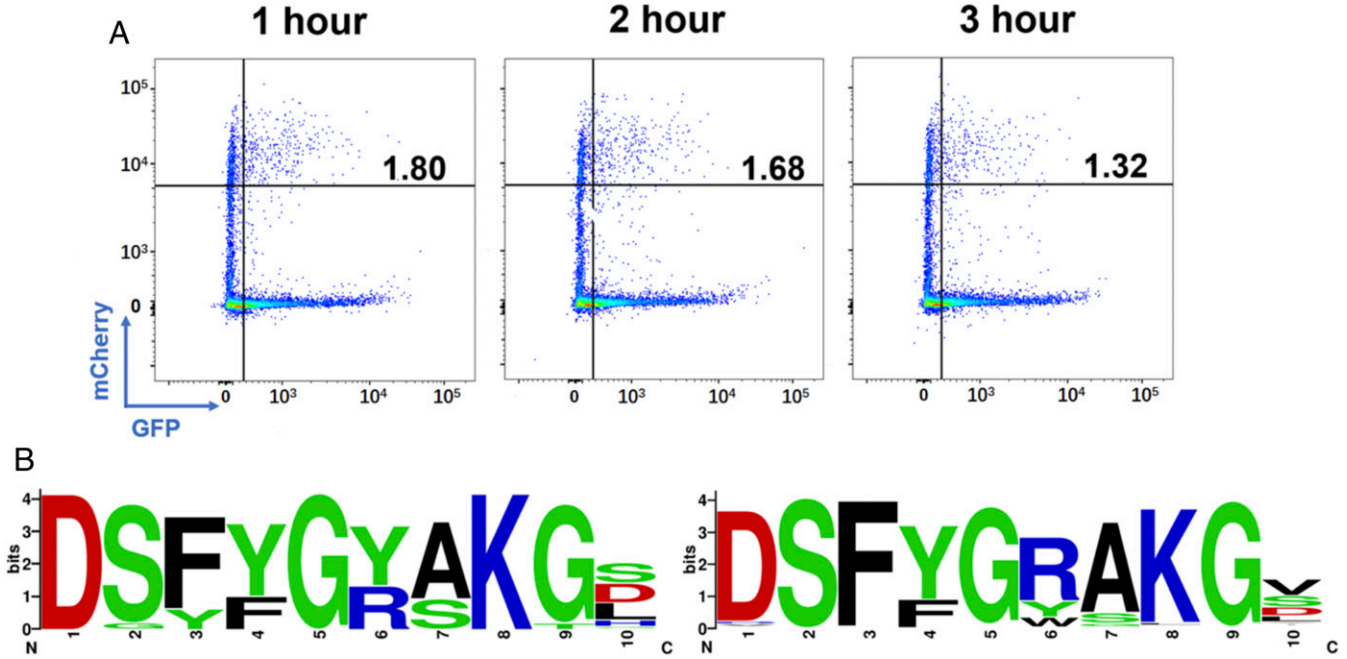

Fig. 4. Kinetic selection after four rounds of equilibrium selection. (*A*) FACS analysis of the mixture of the round 4 yeast cells and CHO-K1/*h*ASIC1a-mCherry cells after 1 h, 2 h, or 3 h of competition. (*B*) The WebLogo presentation of mutation distribution within the H-CDR3 region of sequenced clones after kinetic selection using whole-cell antigens (*Left*) and soluble antigens (*Right*).

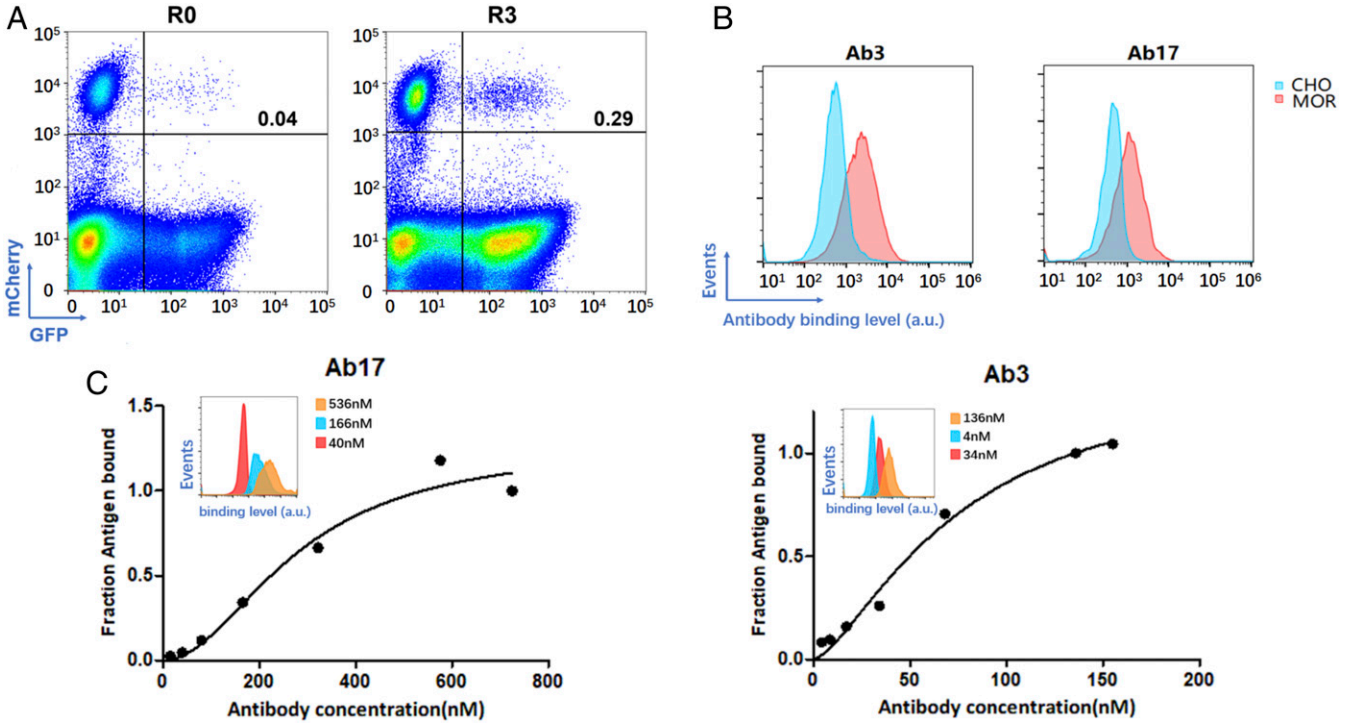

Fig. 5. Antibody selection against *h*MOR-mCherry overexpressed cells and the characterization of identified antibodies. (*A*) FACS analysis of the initial library (R0) and the library after three rounds of selection (R3) incubated with the *h*MOR-mCherry overexpressed CHO cells. 500,000 events were displayed in the plot. (*B*) FACS analysis of wild-type CHO cells and *h*MOR-overexpressed CHO cells stained with two of the selected *h*MOR targeting antibodies: Ab3 and Ab17. (*C*) The binding affinity of Ab3 and Ab17 was measured by staining the *h*MOR overexpressed cells with the antibodies at multiple concentrations.

subtractive screening and extensive washing processes to remove nonspecific binding antibodies. The unique nature of our method is that it is based on assessing antibody binding through visualization of resulting cellular complexes in solution. Since the formation of these cellular complexes is based on antigen-antibody union, their stability may require a higher-affinity antibody present in sufficient numbers to result in a greater avidity than is necessary in traditional screening methods. Hence, the background of nonspecific binding can be minor when expression levels of other endogenous membrane proteins are significantly lower than the overexpressed targets. During the screening against *h*MOR, we did identify one enriched antibody specific for an unknown, and therefore unmanipulated, membrane protein on the CHO cell (*SI Appendix*, Fig. S6). This suggests that our method is also sufficiently sensitive to detect antibodies specific for membrane protein targets routinely expressed on tissue or tumor cells (26).

Due to limitations in the speed of most cell sorters, analysis of large cell populations can require a long time. For instance, analysis of a population numbering 10^9 can require a full day. Hence, when dealing with libraries of large size, extra rounds of magnetic-assisted cell sorting (MACS) selection are often used to enrich binding antibodies (27). In our system, the mammalian cells overexpressing target antigen take the place of the antigen-coated magnetic beads such that the FACS sorting speed is no longer an impediment to efficient sorting of large libraries. Moreover, as the imaging flow cytometer results suggested, a single mammalian cell often binds multiple yeast cells, so the absolute number of complexes per library is reduced. Therefore, the whole selection process requires less time. Thus, this system can take advantage of the powerful FACS equipment while eliminating the time restriction on the size of libraries that can be studied.

One advantage of using in vitro display technologies is that antibodies with desired properties can be effectively engineered by emphasizing certain antibody properties during the selection process. Such engineering can be difficult to achieve by immunization. Here, we showed that using our method, the affinity maturation of an *h*ASIC1a binding antibody (ASC06) was engineered to improve both affinity and function. After five rounds of selection followed by sequencing of 100 selected clones, we found that several clones dominated. Two of the dominant mutants tested showed improvement in both affinity and function (inhibition of channel opening at low pH). In this case, the increase in affinity relative to wild type is modest compared with other reports on affinity maturation. This result is not surprising because the original sequence still comprised about 10% of the final sequence. In addition, the result may have been influenced by the limited number of residues mutated and/or the high affinity of the wild-type antibody. We also conducted kinetic selection to optimize the dissociation profile of the antibody, and the results showed significant increase of percentage of clones with slow dissociation rates. The wild-type clones were also gone in the sequencing results, indicating that the competition process of kinetic selection decreased the clones with fast dissociation. Overall, the results showed that our method is capable of detecting small differences in affinity and can be used to select antibodies with higher affinity.

Developing antibodies that can recognize native, fully folded membrane protein targets is essential for both research and therapeutic purposes. However, antibodies isolated using a soluble or truncated extracellular domain may not bind to the target when it is presented in its native, fully folded conformation. In a study that selected broadly neutralizing antibodies against HIV-1 envelope protein displayed on the surface of mammalian cells, a number of antibodies capable of recognizing the envelope proteins in a cell-based bait selection system but not in their soluble form were obtained (28). Since our system presents membrane proteins in their native form, it is possible that this increases the likelihood

of selecting antibodies capable of both binding these proteins in their native state and triggering function. To summarize, in this study, we report a whole cell-based antibody selection system and engineering strategy that can be applied to selecting or improving antibodies specific for membrane protein targets in an efficient way. In addition, this method can be used to accelerate the process of generating or improving potential drug biomolecules against important yet challenging targets such as GPCRs and ion channels.

Materials and Methods

Yeast Cells Culture, Induction, and Library Constructions. Yeast strain EBY100 was used for the yeast display and cultured in YPD medium. EBY100 cells transfected with vectors containing protein or library of interests were maintained in the SD-CAA medium, and the expression of the displayed proteins was induced in SGR-CAA medium at 20 °C for 24 h with shaking. The yeast display vector used in this study is based on pCTcon2 (Addgene) modified with human IgG Fc, T2A, and EGFP sequences. For the mutagenic library, a pool of mutant oligonucleotide primers spanning the H-CDR3 region of ASC06 were synthesized by GENEWIZ, Inc., and the yeast display vector was linearized with homologous region shared by the oligonucleotide primer pool. The library was generated by cotransfecting the primer pool and the linearized vector into the yeast competent cell. For the yeast naïve human antibody library, the scFv fragments were amplified from a nonimmunized human scFv phage display library by PCR and those scFv DNA fragments were cotransfected with linearized yeast display vector into the yeast competent cells.

Cell Culture and Stable Cell Line Construction. The CHO-K1/*h*ASIC1a-mCherry stable cell line was generated as described previously (20) and cultured in F-12K medium supplemented with 10% FBS and 200 μg/mL geneticin. The CHO-K1/*h*MOR-mCherry cells was generated using lentivirus transfection. Briefly, cDNA of *h*MOR fused with mCherry was cloned into a lentiviral vector, and the plasmid was transfected into 293T cells along with packaging plasmids for the production of lentivirus. CHO/K1 cells were infected with the lentivirus to integrate *h*MOR-mCherry DNA into the genome. Infected CHO-K1 cells with high mCherry expression were sorted by FACS.

Incubation, Analysis, and Sorting of the Yeast/Mammalian Cell Mixture. For the analysis of PcTx-displayed yeast cells and CHO/*h*ASIC1a cells, yeast and CHO/*h*ASIC1a cells were coincubated at a ratio of 1:5 in FACS buffer (phosphate-buffered saline [PBS] with 0.5% bovine serum albumin [BSA]) for 1 h at room temperature. The mixture was washed with FACS buffer and then resuspended for FACS analysis. The mixture was also analyzed by Amnis imaging flow cytometers to acquire photos of the complexes, the yeast cells, and mammalian cells alone. For the library screening, the CHO-K1 cells overexpressing target proteins and the induced yeast library were coincubated at a ratio of 1:10. The mixture was incubated for an hour at room temperature and then washed with FACS buffer and sorted using a BD FACSAria III flow cytometer. The yeast/CHO cell complexes with dual fluorescent signals were selected for the recovery of yeast cells in the SDCAA medium overnight. For the kinetic selection against CHO/*h*AISC1a-mCherry, yeast cells were first incubated with CHO/*h*AISC1a-mCherry at room temperature for an hour. Then, *h*AISC1a-YFP overexpressed CHO cells were added into the mixture and incubated at 37 °C with rotation for 3 h. The CHO/*h*AISC1a-YFP cells are three times more than CHO/*h*AISC1a-mCherry cells in the mixture. The mixture was analyzed and sorted with a BD FACSAria III flow cytometer, and the yeast/CHO cell complexes with both GFP and mCherry fluorescent signals were selected.

Antibody Expression and Purification. For the scFv-Fc fusion proteins, the scFv/yeast display vectors were digested with SfiI enzyme and the scFv cDNA was subcloned into pFUSE expression vector (pfusehg1fc2; Invivogen), which contains the Fc domain of human IgG1. For the full-length IgG protein, the variable heavy chain and light chain sequence were cloned into modified pFUSE expression vectors, which contain corresponding constant regions of IgG1 protein. The expression vectors were transfected into Expi293F cells by ExpiFectamine 293 reagent, and the supernatant was collected 5 d after the transfection. A HiTrap Protein A HP column (no. 17-0403-03; GE Healthcare) was used to capture antibodies in the ÄKTA purifier 100 (GE Healthcare), and those antibodies were eluted with glycine buffer (pH 2.7). The eluted antibody was neutralized with Tris buffer and stored in PBS after buffer exchange using Ultracel 30 kDa (Merck Millipore). The concentration of antibodies was determined by Qubit Protein Assay Kit (Thermo Fisher Scientific).

Cell Immunofluorescence Staining. For the cell immunofluorescence staining, wild-type cells and cells that overexpressed *h*ASIC1a or human MOR were detached with trypsin and resuspended in FACS buffer (PBS, 0.1% BSA). Cells (100,000 cells) were incubated in FACS buffer containing antibodies with different concentrations at room temperature for 30 min, washed twice, and then resuspended in FACS buffer containing FITC-conjugated anti-human IgG Fc (1:800 [vol/vol] dilution; Life Technologies). After 30 min at room temperature, cells were washed twice and then analyzed by FACS.

The Measurement of Antibody Affinity. For the SPR analysis of ASC06 and its mutants, the *h*ASIC1a ectodomain was produced as the antigen. Briefly, *h*ASIC1a ectodomain fused with a FLAG tag was expressed in HEK293 cells and purified with anti-FLAG M2 magnetic beads. The protein was further purified with gel filtration, and the *h*ASIC1a trimer was collected. The *h*ASIC1a trimer protein was biotinylated and loaded onto the SA chip surface (GE Healthcare). Antibodies (ASC06 and its mutants) with different concentrations were then loaded in running buffer (25 mM Hepes, pH 7.4, 500 mM NaCl and 0.05% EDTA) and analyzed on the Biacore T200 (GE Healthcare).

FLIPR Membrane Potential Assay. The experiment was performed using the FLIPR Membrane Potential Assay Kit (Molecular Devices). Briefly, the *h*ASIC1a-YFP cells were seeded in 96-well plates (Greiner, catalog no. 655090) at a density of 15,000 cells per well 1 d before the experiment. Antibody solution was prepared in a serial dilution using the buffer (20 mM Hepes in Hanks' balanced salt solution [HBSS], pH 7.4). The same buffer is used in the preparation of the control antibody H8, calcium channel blocker Amiloride, and FMP dye (FMP kit, MD, catalog no. R8126). Cell media was removed and changed to the buffer (20 mM Hepes in HBSS, pH 7.4) with or without the antibodies. After 30 min at 37 °C, the freshly prepared indicator dye was added into each well and the plate was incubated at 37 °C for 30 min. The plate was then analyzed using the FDSS/μCELL plate reader (Hamamatsu). The compound plate was filled with either pH 7.4 or pH 6.0 stimulation buffer. The fluorescence intensity was recorded after the stimulation, and the data was analyzed and fitted to calculate the IC_{50}.

ACKNOWLEDGMENTS. We thank Chunchun Liu, Lishuang Zhang, and Pengwei Zhang from Shanghaitech FACS core facility for the help with FACS sorting. This work was supported by the JPB Foundation.

1. M. L. Jones *et al*., Targeting membrane proteins for antibody discovery using phage display. *Sci. Rep.* **6**, 26240 (2016).
2. G. Köhler, C. Milstein, Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**, 495–497 (1975).
3. A. R. Bradbury, S. Sidhu, S. Dübel, J. McCafferty, Beyond natural antibodies: The power of in vitro display technologies. *Nat. Biotechnol.* **29**, 245–254 (2011).
4. J. McCafferty, A. D. Griffiths, G. Winter, D. J. Chiswell, Phage antibodies: Filamentous phage displaying antibody variable domains. *Nature* **348**, 552–554 (1990).
5. E. T. Boder, K. D. Wittrup, Yeast surface display for screening combinatorial polypeptide libraries. *Nat. Biotechnol.* **15**, 553–557 (1997).
6. G. M. Cherf, J. R. Cochran, Applications of yeast surface display for protein engineering. *Methods Mol. Biol.* **1319**, 155–175 (2015).
7. D. R. Bowley, A. F. Labrijn, M. B. Zwick, D. R. Burton, Antigen selection from an HIV-1 immune antibody library displayed on yeast yields many novel antibodies compared to selection from the same library displayed on phage. *Protein Eng. Des. Sel.* **20**, 81–90 (2007).
8. L. R. Pepper, Y. K. Cho, E. T. Boder, E. V. Shusta, A decade of yeast surface display technology: Where are we now? *Comb. Chem. High Throughput Screen.* **11**, 127–134 (2008).
9. C. J. Hutchings, P. Colussi, T. G. Clark, Ion channels as therapeutic antibody targets. *MAbs* **11**, 265–296 (2019).
10. C. J. Hutchings, M. Koglin, F. H. Marshall, Therapeutic antibodies directed at G protein-coupled receptors. *MAbs* **2**, 594–606 (2010).
11. I. G. Denisov, S. G. Sligar, Nanodiscs for structural and functional studies of membrane proteins. *Nat. Struct. Mol. Biol.* **23**, 481–486 (2016).
12. T. J. Knowles *et al*., Membrane proteins solubilized intact in lipid containing nanoparticles bounded by styrene maleic acid copolymer. *J. Am. Chem. Soc.* **131**, 7484–7485 (2009).
13. J. Naylor, D. J. Beech, Extracellular ion channel inhibitor antibodies. *Open Drug Discov. J.* **1**, 36–42 (2009).
14. A. Gupta *et al*., Conformation state-sensitive antibodies to G-protein-coupled receptors. *J. Biol. Chem.* **282**, 5116–5124 (2007).

15. H. R. Hoogenboom *et al*., Selection-dominant and nonaccessible epitopes on cell-surface receptors revealed by cell-panning with a large phage antibody library. *Eur. J. Biochem.* **260**, 774–784 (1999).
16. X. X. Wang, E. V. Shusta, The use of scFv-displaying yeast in mammalian cell surface selections. *J. Immunol. Methods* **304**, 30–42 (2005).
17. B. J. Tillotson, Y. K. Cho, E. V. Shusta, Cells and cell lysates: A direct approach for engineering antibodies against membrane proteins using yeast surface display. *Methods* **60**, 27–37 (2013).
18. P. Escoubas, C. Bernard, G. Lambeau, M. Lazdunski, H. Darbon, Recombinant production and solution structure of PcTx1, the specific peptide inhibitor of ASIC1a proton-gated cation channels. *Protein Sci.* **12**, 1332–1343 (2003).
19. J. Grzeschik *et al*., A simplified procedure for antibody engineering by yeast surface display: Coupling display levels and target binding by ribosomal skipping. *Biotechnol. J.* **12**, 1600454 (2017).
20. M. Qiang *et al*., Selection of an ASIC1a-blocking combinatorial antibody that protects cells from ischemic death. *Proc. Natl. Acad. Sci. U.S.A.* **115**, E7469–E7477 (2018).
21. Z. Yang *et al*., Affinity maturation of an TpoR targeting antibody in full-length IgG form for enhanced agonist activity. *Protein Eng. Des. Sel.* **31**, 233–241 (2018).
22. M. J. Feldhaus, R. W. Siegel, Yeast display of antibody fragments: A discovery and characterization platform. *J. Immunol. Methods* **290**, 69–80 (2004).
23. A. S. Hauser *et al*., Pharmacogenomics of GPCR drug targets. *Cell* **172**, 41–54.e19 (2018).
24. C. Stein, Opioid receptors. *Annu. Rev. Med.* **67**, 433–451 (2016).
25. C. Contet, B. L. Kieffer, K. Befort, Mu opioid receptor: A gateway to drug addiction. *Curr. Opin. Neurobiol.* **14**, 370–378 (2004).
26. X. X. Wang, Y. K. Cho, E. V. Shusta, Mining a yeast library for brain endothelial cell-binding antibodies. *Nat. Methods* **4**, 143–145 (2007).
27. M. J. Feldhaus *et al*., Flow-cytometric isolation of human antibodies from a non-immune Saccharomyces cerevisiae surface display library. *Nat. Biotechnol.* **21**, 163–170 (2003).
28. F. Klein *et al*., Broad neutralization by a combination of antibodies recognizing the CD4 binding site and a new conformational epitope on the HIV-1 envelope protein. *J. Exp. Med.* **209**, 1469–1479 (2012).