Published in final edited form as:

Methods Mol Biol. 2015 ; 1319: 65–78. doi:10.1007/978-1-4939-2748-7_4.

Yeast display-based antibody affinity maturation using detergent-solubilized cell lysates

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Summary

It is often desired to identify or engineer antibodies that target membrane proteins (MPs). However, due to their inherent insolubility in aqueous solutions, MPs are often incompatible with *in vitro* antibody discovery and optimization platforms. Recently, we adapted yeast display technology to accommodate detergent-solubilized cell lysates as sources of MP antigens. The following protocol details the incorporation of cell lysates into a kinetic screen designed to obtain antibodies with improved affinity via slowed dissociation from an MP antigen.

Keywords

Membrane protein; single-chain antibody (scFv); yeast surface display; cell lysates; affinity maturation

1. Introduction

Membrane proteins (MPs) comprise a large and growing class of therapeutic antibody targets because of their physical accessibility and involvement in the regulation of many disease states (1). Because of limited solubility and relatively low natural or recombinant abundance, MPs can be troublesome antigens for antibody discovery and lead optimization using various platforms including animal immunization (2, 3) or *in vitro* antibody display techniques (4, 5). In order to overcome these limitations, MP antigens must frequently be produced as soluble recombinant proteins. This often involves deletion of transmembrane domains followed by transformation into a heterologous host, expression, and purification (6, 7). This process is laborious and can result in a peptide or protein that, while antigenic, may not be physiologically relevant (8).

Yeast surface display (YSD) is well developed for the engineering and optimization of antibody affinity, stability, and specificity (4, 9); but, its use for MP antibody engineering has been limited to a few special cases (10, 11). Therefore, to facilitate YSD-based affinity maturation of antibodies against MPs, we developed a technique wherein the MP antigen is presented in the form of a detergent-solubilized cell lysate. By lysing cells in buffers

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containing non-denaturing detergents, the MPs are extracted from the membrane, and solubilized via detergent interactions with their membrane-spanning hydrophobic domains (12). This allows the solution phase presentation of the MP of interest in a near-native state without the need for truncation and heterologous production. In this way, MPs in detergent-solubilized cell lysates can be used directly in YSD-based screens (13–15). Antibody-MP binding on the yeast surface can be detected through selective biotinylation of surface-accessible MP epitopes prior to cell lysis. Alternatively, by lysing cells without biotinylation, solubilized, but unlabeled MP antigens can compete for antibody binding in a kinetic (dissociation rate) screen (See Fig 1) (15). Altogether, these features and the detailed protocol discussed below, extend the YSD platform to handle antibody engineering against MP antigens.

As an illustrative example, we affinity matured a single-chain antibody (scFv), H7, recognizing the human transferrin receptor (TfR) that was previously identified in a phage display screen for internalizing scFv by Poul and colleagues (16). Two- to four-fold improvements in the dissociation rate constants were obtained by kinetic screening with HEK293 lysates containing solubilized TfR (See Fig 1). Dissociation rate constants and apparent affinity improvements were quantitatively assayed with scFvs displayed on the yeast surface. These yeast surface binding parameters translated to an up to 7-fold improvement in equilibrium binding affinity when soluble scFv were titrated against cell surface TfR. (15). Importantly, although the screen was performed under detergent-based conditions, the improvements translated to the physiological situation.

2. Materials

2.1. Mammalian cells and cell culture components

1. HEK293 cells (CRL-1473), or cell line expressing MP of interest (see Note 1)
2. HEK293 Growth medium: Minimum Essential Medium (Alpha Modification) supplemented with 1X PSA (Penicillin, Streptomycin, Amphotericin B), 10% Fetal bovine serum, 2mM L-glutamine, 20mM HEPES buffer pH 7.3
3. Phosphate buffered saline (PBS) pH 7.4: 10mM Na₂HPO₄, 2mM KH₂PO₄, 137mM NaCl, 2.7mM KCl.
4. PBSCM: supplement PBS with 0.9mM CaCl₂ and 0.49mM MgCl₂
5. Tissue culture-treated 75cm² polystyrene flasks (T75 flasks)
6. 50 µg/mL Poly-D-Lysine in sterile ddH₂O

2.2. Lysate generation

1. EZ-Link™ Sulfo-NHS-LC Biotin (Thermo/Fisher) (see Note 2)

¹HEK293 cells were chosen as they express the human transferrin receptor (hTfR), and were immunoreactive to the wild-type anti-human TfR scFv, H7. In theory, any cell line expressing the antigen of interest can be used as the source for the creation of *B*- and *U*-lysates.

2. PBSCM with 100mM glycine
3. Cell lysis buffer: 1 mL PBS, 1% (v/v) Triton X-100 or alternative MP compatible detergent (see Note 3), 1× Protease inhibitor cocktail (PIC), 2 mM Sodium EDTA (see Note 4)
4. Sterile cell scrapers

2.3. Yeast surface display (see Note 5)

1. *S. cerevisiae* strain EBY100 (17)
2. Wash buffer (PBSCMA): Supplement PBSCM with 1g/L protease-free bovine serum albumin (See Note 4), store at 4°C
3. Detergent wash buffer (PBSD): PBS supplemented with the same concentration and type of detergent selected for creation of cell lysates (see Note 3)
4. SD-CAA: 20.0 g/L dextrose, 6.7 g/L yeast nitrogen base, 5.0 g/L casamino acids, 10.19 g/L Na₂HPO₄•7H₂O, 8.56 g/L NaH₂PO₄•H₂O, add kanamycin (50 µg/mL) when indicated below
5. SG-CAA: SD-CAA replacing dextrose with 20 g/L galactose
6. Detection antibodies (see Note 6)
7. Surface display plasmid harboring the scFv gene of interest, e.g. pCT-ESO-scFv (15, 18)

²There are numerous commercially available biotinylation reagents that vary in their reaction chemistry, cell permeability, linker length, and linker cleavability. When engineering scFvs against plasma MPs it is most often desirable to use a cell impermeable reagent to ensure selective biotinylation of the cell surface and help minimize background lysate binding signal on the yeast. We chose Sulfo-NHS-LC-biotin as the negatively charged sulfate group renders the reagent water soluble and as a result impermeable to cell membranes. In addition, the N-Hydroxysuccinimide (NHS) group reacts with primary amines thereby appending a single biotin to lysine residues and the amino-terminus, and the long linker chain renders the biotin more accessible to detection reagents by limiting steric hindrance from surrounding amino acid residues. Alternatively, if the MP target is intracellular or if there is a large intracellular pool, cell permeable biotinylation reagents, such as NHS-LC-Biotin, can be employed in the detergent-solubilized lysate platform (14).

³Triton X-100 was the chosen detergent in the H7 affinity maturation study as it was found to effectively solubilize TfR (21). However, not all membrane proteins will be efficiently solubilized by any one detergent so care should be taken to choose the detergent that best suits the target protein (12). Non-denaturing detergents are favored so that scFv properties engineered in the cell lysate environment maintain their improved affinity in a physiological (non-detergent) environment (13–15). In our hands N-Octyl-β-D-glucoside (OG), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and Radio-Immunoprecipitation Assay (RIPA) buffer are also suitable for MP solubilization while maintaining the affinity of scFv-antigen binding (13). The working concentration of detergent in lysate and PBSD should be above the critical micelle concentration (CMC) for efficient MP solubilization (22). Based on this heuristic, 1% v/v or w/v of detergent is usually sufficient. MP expressing cell lines and other solubilizing detergents can be rapidly assessed by yeast surface display as described in this protocol, giving the investigator immediate feedback on the suitability of cell lysates as a screening tool, and a straightforward means of adapting the lysate-based techniques.

⁴Adding protease inhibitor cocktail (PIC) to the cell lysis buffer is critical to prevent degradation of target proteins and scFvs as many proteases will be liberated during cell lysis. For mammalian cell lysates we find Roche cOmplete mini EDTA free PIC to be the most reliable choice, however PIC for mammalian cells from Sigma-Aldrich has also been used. Additionally, EDTA should be added to the lysis buffer to act as a chelating agent thereby inhibiting the activity of metalloproteases. Care should also be taken to use protease-free BSA to eliminate any additional source of proteases.

⁵The yeast surface display method for screening of combinatorial libraries has been developed and described previously (17, 23). These references provide additional details on the key components and general techniques used.

⁶For flow cytometric analysis and sorting, we typically label for full length scFv expression using mouse-anti-*c-myc* antibody (9E10) (Covance) at 1:200 dilution followed by anti-mouse-Alexa488 conjugate at 1:500 dilution and detect biotinylated antigen binding using streptavidin-phycoerythrin conjugate (SA-PE) at 1:500 dilution. Alternatively, We have also successfully used mouse-anti-biotin (BTN.4) (Pierce) instead of SA-PE and rabbit-anti-*c-myc* (Pierce) instead of mouse-anti-*c-myc* in cell lysate-based yeast display protocols.

3. Methods

3.1. Cell culture and generation of detergent-solubilized cell lysates

The procedures described in this section have been optimized for adherent cell culture. However, biotinylation and cell lysis are easily adaptable to suspension culture. Lysate created from biotinylated cells is termed *B*-lysate while lysate created from unlabeled cells is termed *U*-lysate.

1. Coat T75 flasks to improve HEK293 cell adherence by incubating flasks with 10mL of poly-D-lysine solution for 30 min at 37°C. Aspirate coating. Rinse twice with 10 mL sterile PBSCM.
2. Culture HEK293 cells at 37°C / 5% CO₂ in growth medium so that they are 80–90% confluent on the day of lysate creation (See Note 7).
3. Prepare 1 mL cell lysis buffer per T75 flask of HEK293 cells. Store on ice.
4. Immediately prior to use, prepare 10mL biotinylation reagent per T75 flask by diluting Sulfo-NHS-LC-Biotin to 1 mg/mL in PBSCM (see Note 2).
5. Wash HEK293 cells twice with 10 mL sterile PBSCM at room temperature.
6. Incubate cells with biotinylation reagent for 30 min at 37°C (see Note 8).
7. Alternatively, cells not undergoing biotinylation are washed once with 10 mL PBSCM prior to lysis.
8. All subsequent steps should be performed at 4°C. Aspirate the biotinylation solution and quench the reaction by washing twice with 10 mL ice cold PBSCM+100mM glycine and once with 10 mL ice cold PBS.
9. Aspirate the washing solution, and add 1mL ice-cold lysis buffer to each flask. Scrape the cells from the growth surface with the cell scraper, and collect the solution by micropipette into a 1.5 mL microfuge tube.
10. Vortex briefly, then rotate for 15 min at 4°C.
11. Centrifuge the lysates at 4°C, 18,000×g for 30 min to remove insoluble material.

⁷Biotinylation and cell lysis were carried out at 80–90% confluence as these conditions were determined to maximize the levels of biotinylated TfR in the cell lysate (i.e. optimizing the number of cells and amount of MPs available for solubilization versus artifacts from biotinylation of dead cells and cell debris that occur at higher cell densities). Typical cell counts for the HEK293 line growing in a T75 at 80% confluence were 2×10^7 viable cells at >90% viability. At higher confluence cell viability decreased.

⁸The time and temperature of the biotinylation reaction may be varied based on the nature of the target MP. For example, many MPs undergo endocytosis with subsequent degradation or recycling of the receptor back to the cell surface (24). Given the dynamic nature of membrane protein biogenesis, recycling, and degradation, there may be a large intracellular pool of receptors not accessible to biotinylation at a given time (25). For our purposes 37 °C and 30 minutes was chosen to take advantage of cell surface resident TfR turnover via endocytosis and recycling which occurs on the order of 10–15 minutes (26). Alternatively, incubation can be carried out at 4 °C for 2 hours to suppress endocytosis.

12. Transfer the supernatant that contains detergent solubilized MPs to a new 1.5 mL microfuge tube. These ~1 mL aliquots comprise the *B*- and *U*-lysates employed in the affinity maturation (See Note 9).

3.2. Yeast culture and induction of surface display

1. Inoculate yeast into sterile SD-CAA media and grow overnight at 30°C and 260rpm. For clonal yeast, a single colony from an agar plate is sufficient for 3 mL overnight culture. For combinatorial libraries, use liquid culture inoculating between 10 to 100 times the library size to a final cell density of $<1 \times 10^7$ yeast/mL (1×10^6 yeast/mL is preferable if the required culture volume is feasible). It is important to inoculate between 10 to 100 times the library size to limit loss of rare clones (see section 3.5 for more details regarding library creation strategies).
2. After 12–18 hours of culture growth, measure the optical density at 600 nm (OD_{600}) (dilute samples appropriately to stay within the instrument's linear range). A reading of 1.0 OD_{600} corresponds to approximately 1×10^7 yeast cells per mL. After 12–18 hours of growth, the initial yeast culture should have expanded to 5–10 OD_{600} . Passage the yeast by diluting into fresh SD-CAA media to a density of 0.3 OD_{600} .
3. Grow at 30°C and 260rpm until the culture reaches 1.0 OD_{600} . Typically, 1.0 OD_{600} is reached within 4 hrs.
4. Pellet the yeast by centrifuging at 2,500×g for 5min. Discard the spent SD-CAA media.
5. Induce scFv expression by re-suspending the yeast in an identical volume of SG-CAA media and incubate cultures at 20°C and 260rpm for 16–20hrs.

3.3. Determination of scFv dissociation rate constant (k_{off}) by yeast surface display

In order to select the ideal competition time for the dissociation rate screen, it is necessary to first determine the dissociation rate constant of the wild-type scFv-MP antigen interaction. Dissociation rate is measured by loading surface-displayed scFv with biotinylated antigen (in the form of *B*-lysate), removing the solution, then adding an excess of soluble competitor (in the form of *U*-lysate) and incubating for a range of competition times (see Note 10).

1. Prepare *B*-lysate and *U*-lysate in sufficient quantities as described above (see Note 11).

⁹Once created, cell lysates are perishable and should be used immediately, or stored at 4°C for a maximum of 24 hr prior to use.

¹⁰Provided there is a high enough MP concentration in the undiluted *B*-lysate such that the yeast surface bound, biotinylated MP is detected at reasonable signal to background levels, it will be possible to measure the dissociation rate constant as described in section 3.3 and Note 12. This applies whether or not the scFv is saturated by the MP ligand and opens up affinity maturation by kinetic screening to any lysate resident MP whose abundance can yield a detectable signal on the yeast surface.

¹¹Ratios of 50 μ L *B*-lysate and 100 μ L *U*-lysate per 2×10^6 yeast were used for k_{off} determination and kinetic screening of H7 mutants. Large libraries will necessarily require a large volume of detergent solubilized lysate for screening. As a result, cell culture should be scaled up accordingly. In lieu of multiple T75 flasks, 500 cm² culture plates can be used. The volumes of *B*- and *U*-lysate were chosen to maintain an excess of unlabeled MP during the competition step. When labeled MP antigen dissociates, it will in essence be

2. Grow and induce yeast displaying a clonal scFv as described above. Collect 2×10^6 yeast per desired competition time point. In order to accurately quantify the dissociation rate constant, prepare enough samples to cover a range of competition times from 0 minutes (no competition) up to at least 10 times the expected dissociation half-time.
3. Wash yeast twice with 500 μ L PBSCMA. Washes are performed as follows: Pellet yeast by centrifugation at $18,000 \times g$ for 1 min, aspirate the supernatant, add wash buffer, and vortex to re-suspend the cells.
4. Incubate yeast as one large batch with 50 μ L undiluted *B*-lysate per 2×10^6 induced yeast, rotating, at room temperature for 2 h.
5. Wash the yeast once with PBS-D using 100 μ L per 2×10^6 yeast cells (see Note 12).
6. Incubate yeast with 100 μ L undiluted *U*-lysate per 2×10^6 , rotating, at room temperature for the desired competition time.
7. All subsequent steps are performed at 4°C , with reagents on ice to avoid further antigen dissociation. At each competition time, withdraw 2×10^6 yeast from the *U*-lysate incubation. Pellet the yeast, aspirate *U*-lysate, then wash the yeast once with 100 μ L ice cold PBS-D and twice with 100 μ L ice cold PBSCMA, storing the washed yeast pellet on ice until all competition samples have been collected.
8. Label yeast for flow cytometry and analyze on a flow cytometer (see 3.4). Cells should be labeled for full-length scFv expression with anti-*c-myc* antibody (9E10) and for biotinylated antigen binding with streptavidin-phycoerythrin (SA-PE) or equivalent alternatives (See Note 6).
9. Quantify antigen binding at each time point by determining the geometric mean fluorescence intensity (MFI) of the antigen binding population from each sample using FlowJo or a similar software package. To remove background fluorescence from the measurement, the MFI for the non-displaying yeast population should be subtracted from these values.
10. MFI values at each time point can be fit to a mono-exponential decay model to determine the dissociation rate constant (see Note 13) (See Fig. 2a for example dissociation curve for the H7 scFv).

eliminated from re-binding the scFv as the excess unlabeled MP in *U*-lysate will compete favorably for scFv binding. This ensures accurate measurement of a pseudo-irreversible first-order dissociation rate constant.

¹²The effects of detergents on yeast cells should be considered throughout the screening procedure. Yeast can be permeabilized by non-ionic detergents at concentrations $<0.1\%$ (v/v) (27). By our observations, yeast cells did not lyse under these conditions, the surface display of scFv was not disrupted, and scFv-antigen binding was maintained (see Note 2). However, after incubation with detergent solutions and subsequent washes with non-detergent containing buffers, the yeast cells do not pellet well and there is an increased risk of aspirating the pellet. Care should be taken to limit loss of yeast cells.

¹³The dissociation rate constant, k_{off} , can be determined from background-subtracted MFI data at each competition time point by fitting the data to the following mono-exponential dissociation model:

$$\text{Fraction Bound} = (\text{MFI}(t) - \text{MFI}_{\text{inf}}) / (\text{MFI}_0 - \text{MFI}_{\text{inf}}) = \exp(-k_{\text{off}} \times t)$$

3.4. Labeling yeast for flow cytometry

ScFv-MP antigen binding and competition steps are carried out at room temperature unless otherwise specified, however, after kinetic competition, care must be taken to keep all reagents on ice and to perform all steps at 4°C to prevent unwanted antigen dissociation. The volumes of labeling reagents and buffers quoted below are calculated based on 2×10^6 yeast per sample and should be adjusted proportionally based on the number of yeast actually used.

1. Pellet the yeast by centrifugation at $18,000 \times g$ for 1 min and aspirate the supernatant.
2. Re-suspend yeast by vortexing in 50 μ L primary antibody (e.g. 9E10) and incubate on ice for 1 h (See Note 6).
3. Wash yeast twice with 100 μ L PBSCMA.
4. Re-suspend yeast by vortexing in 50 μ L secondary antibody solution (e.g. anti-mouse-Alexa488 and SA-PE) and incubate on ice for 30 min.
5. Wash yeast twice with 100 μ L PBSCMA. For sorting, proceed to Section **3.5, Step 9**. For analysis only, re-suspend in 500 μ L PBSCMA and analyze on a flow cytometer (see Notes 14 and 15).

3.5. Affinity maturation of scFvs

Prior to implementing the kinetic screening strategy described below, the competition time of the screen should be chosen based on wild-type dissociation rate data (see **3.3**). The “optimal” competition time is calculated using straightforward mathematical models that are designed to maximize the difference in biotinylated antigen binding between wild-type and putative improved antibody clones in the library (19). In order to enable affinity maturation, a combinatorial library of mutant scFv should be created from the parental antibody via the method that best suits the goals of the screen. For example, in the H7 affinity maturation case study presented here, error-prone PCR (20) was used to introduce random point mutations into the H7 scFv gene yielding a library of 5×10^7 clones (15, 16). Since mutagenesis can ablate antigen binding in a portion of the initial scFv library, one should first screen the starting library without competition, aiming to enrich the library pool for clones that retain MP antigen binding in undiluted lysate. In the H7 case study, this strategy resulted in recovery of approximately 1.5×10^7 TfR-binding clones. Subsequently, the kinetic screen can be carried out for a number of rounds until the desired enrichment of

Where MFI_0 is the MFI at zero competition time, MFI_{inf} is the MFI at complete antigen dissociation, and t is the competition time. (19)

¹⁴In order to correctly analyze antigen binding on the yeast surface by flow cytometry and establish proper gating strategies, certain controls must be prepared in parallel with experimental samples. The following controls are typically used: (A) Double negative control- Unlabeled yeast (negative for both scFv expression and antigen binding) , (B) Negative binding control- Irrelevant scFv (e.g. anti-fluorescein scFv 4-4-20 (17)) incubated with lysate (or wild-type incubated in the absence of lysate) and labeled for flow cytometry, (C) Wild-type control- wild-type scFv incubated with lysate and labeled for flow cytometry (e.g. wild-type anti-hTfR scFv H7). The double negative control is used to establish background signal intensity for the double negative population (non-binding, non-expressing), while the negative binding control provides a benchmark for signal intensities from a population of surface-expressed but non-binding scFvs. The wild-type control is used for comparison to mutants and as a benchmark for gating.

¹⁵Data from the relevant flow cytometer fluorescence channels should be recorded for at least 10,000 events per sample. Quantification of antigen binding to scFv via flow cytometry has been described previously (23, 28).

putative improved clones is reached. We describe below the protocol for a kinetic screen as performed for the H7 scFv.

1. Prepare *B*-lysate and *U*-lysate in sufficient quantities as described above (see Note 11).
2. Grow and induce the yeast-displayed scFv library as described above. Collect a 10-fold excess of the library size. The clonal wild-type scFv and appropriate negative controls should always be included (i.e. prepared and labeled in tandem at the exact screening conditions) when isolating improved clones via FACS in order to establish proper gating strategies (see Note 14).
3. Wash the yeast twice with 500 μ L PBSCMA.
4. Incubate yeast with an appropriate volume of undiluted *B*-lysate (50 μ L lysate per 2×10^6 yeast) for 2 hours at room temperature.
5. Wash the yeast once with ice cold PBS-D using $2 \times$ the lysate volume used in **Step 4**.
6. Incubate yeast with undiluted *U*-lysate using $2 \times$ the lysate volume used in **Step 4**. Rotate at room temperature for the optimal competition time arrived at using data from section **3.3**.
7. At the end of the competition period, pellet the yeast by centrifugation for 1 min at $18,000 \times g$, aspirate *U*-lysate, and wash the yeast once in ice cold PBS-D and twice in ice cold PBSCMA using an equivalent volume to the lysate volume used in **Step 6**.
8. Label yeast for flow cytometry (see **3.4**). Cells should be labeled for full-length scFv expression with an antibody to the *c-myc* epitope and for biotinylated antigen binding with SA-PE or equivalent alternatives (See Note 6).
9. Re-suspend the labeled yeast at 1×10^7 cells/mL in PBSCMA.
10. Analyze and sort on a flow cytometer (e.g. BD FACSAria) (see Note 16). First, collect data for 10,000 events for negative control, wild-type, and library yeast. Next, set a logical sorting gate to recover improved clones based on the wild-type binding population. The bottom of the gate (in the binding direction) should lie roughly above the maximum wild-type binding signal. Also, ensure that the gate is set to recover only scFv expressing clones. Finally, sort the entire library sample based on this gating strategy (See Figure 2b for example gating). The stringency of gating will vary from round to round. In the first round of the H7 affinity maturation, with a large starting library, 5% of the library was recovered. In subsequent rounds the stringency was increased in order to recover

¹⁶Yeast cells are extremely durable and can be sorted at very high pressure, through a small orifice to achieve event rates around 5×10^7 /hr. Therefore, libraries with upwards of 5×10^7 clones can be sorted in a day (assuming 10 times the library size is sorted).

those clones with the greatest improvement in dissociation rate. Percent recovered in sorting rounds 2 through 4 were 1%, 0.3%, and 0.1%.

11. Collect the sorted yeast in sterile SD-CAA supplemented with kanamycin.
12. Culture recovered yeast overnight at 30°C and 260 rpm in SD-CAA with kanamycin until an OD₆₀₀ of 1.0 is reached.
13. Repeat the procedure for additional rounds of kinetic screening and flow cytometric sorting until the desired enrichment of improved clones is achieved (see Notes 17 and 18).

Acknowledgments

The work described here was supported by National Institutes of Health grants NS071513, NS056249 and AI072435.

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¹⁷Four rounds of kinetic screening and flow cytometric enrichment were carried out for the single round of mutagenic affinity maturation of H7 (15). The number of screening rounds will vary depending on the size and diversity of the enriched populations as well as the desired dissociation rate (affinity) improvement. In general, as the number of screening rounds increases the library diversity decreases. Once a sufficiently enriched population is achieved individual clones should be selected and their dissociation rate quantified via the methods described above. If necessary, additional rounds of mutagenesis and screening can be performed to further improve the dissociation rate.

¹⁸After four rounds of kinetic screening, mutant clones (e.g. TKe308) were identified that had 2–4 fold improved dissociation rates on the surface of yeast (see Fig 2c). These improved dissociation rates translated to 7-fold improved equilibrium dissociation constants for soluble mutant scFvs binding to cell surface TfR (15), indicating that scFv-MP interactions engineered in a detergent-solubilized lysate environment translated to the physiological situation (no detergent, soluble scFv, cell surface TfR).

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Fig. 1. Schematic representation of dissociation rate screening of a mutagenic, yeast display scFv library with MP antigen presented in a detergent-solubilized cell lysate. Specific example shown is for anti-TfR scFv. **(a)** Lysate creation consists of plasma membrane-selective biotinylation and subsequent lysis in a buffered detergent solution. A cell-impermeable biotinylation reagent is used to tag plasma membrane proteins, including the desired antigen (TfR), yielding biotin-tagged lysate (*B*-lysate). If the antigen-expressing cells were not biotinylated prior to detergent lysis, unlabeled cell lysate (*U*-lysate) results and acts as the soluble competitor in a kinetic screen. **(b)** A mutagenic scFv yeast display library is allowed to bind antigen present in the *B*-lysate. Antigen binding is detected using antibodies or streptavidin conjugates against the biotin tag, or antibodies against the MP of interest. Full-length scFv expression is detected by an antibody directed against the C-terminal *c-myc* tag. **(c)** After washing, an excess of *U*-lysate is applied in step (c) for a predetermined competition time. **(d)** ScFvs that retain *B*-lysate binding after competition, as distinguished by both binding (biotin) and *c-myc* signals, are isolated by flow cytometry to recover mutant scFvs that have an improved dissociation rate. **Figure reproduced from Ref (15) by permission of Oxford University Press.**

Fig. 2.

Measurement of the dissociation rate of wild-type H7-TfR binding on the yeast surface was used to determine optimal competition time for dissociation rate engineering. **(a)** Dissociation kinetics of the H7-TfR binding interaction were assayed using detergent-solubilized lysates as described in section 3.3. The half time of dissociation ($\tau_{1/2}$) of TfR from H7 on the yeast surface was 45 minutes. Data from 10 independent experiments along with a line representing the fitted solution of a mono-exponential dissociation curve and a theoretical dissociation curve for a H7 mutant with slowed dissociation rate are shown. A

competition time for the kinetic screen of 180 minutes was calculated based on this data using mathematical models described previously(19). **(b)** Binding populations after 180 minutes of competition time for (i) Wild-type H7, (ii) Mutant library sorted during Round 1 of kinetic screening, and (iii) Final library recovered from four rounds of kinetic screening. Sample sort gates are shown to give an example of the type of gating described in section 3.5 as well as to illustrate the enrichment of clones that exhibit slowed dissociation during the H7 kinetic screen. **(c)** Dissociation kinetics of affinity matured clones TKe308 and TKe218 were assayed using detergent-solubilized lysates as described in section 3.3. The resultant dissociation half times as measured on the yeast surface were improved by 4 and 2 fold, respectively compared to wild-type H7. Data from two independent experiments are plotted along with fitted mono-exponential dissociation curves. **Figure modified from Ref (15) by permission of Oxford University Press.**