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Cell-Binding Assays for Determining the Affinity of Protein–Protein Interactions: Technologies and Considerations

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Abstract

Determining the equilibrium-binding affinity (K_d) of two interacting proteins is essential not only for the biochemical study of protein signaling and function but also for the engineering of improved protein and enzyme variants. One common technique for measuring protein-binding affinities uses flow cytometry to analyze ligand binding to proteins presented on the surface of a cell. However, cell-binding assays require specific considerations to accurately quantify the binding affinity of a protein–protein interaction. Here we will cover the basic assumptions in designing a cell-based binding assay, including the relevant equations and theory behind determining binding affinities. Further, two major considerations in measuring binding affinities—time to equilibrium and ligand depletion—will be discussed. As these conditions have the potential to greatly alter the K_d , methods through which to avoid or minimize them will be provided. We then outline detailed protocols for performing direct- and competitive-binding assays against proteins displayed on the surface of yeast or mammalian cells that can be used to derive accurate K_d values. Finally, a comparison of cell-based binding assays to other types of binding assays will be presented.

1. INTRODUCTION

Proteins, as ligands, signaling partners, or messengers, form vast networks that require specific interactions to carry out their functions. Generally, these binding events can be thought of as the propensity of two protein-binding partners to either associate, or conversely dissociate at different concentrations (Kuriyan, Boyana, & Wemmer, 2013). Protein partners that come together rapidly in solution at low relative concentrations, and stay bound together for an extended period of time are thought to have a low propensity for dissociation, and are thus thought of as stronger binders (Kuriyan et al., 2013).

While protein interactions are easy to conceptualize in theory, in practice they can be challenging to measure accurately. Protein interactions are measured using binding assays, each of which follows a general pattern. Methods require a soluble or cell surface displayed protein (eg, receptor) of interest, varying concentrations of soluble ligand, and a mechanism through which bound ligand can be measured (de Jong, Uges, Franke, & Bischoff, 2005; Hulme & Trevethick, 2010; Kuriyan et al., 2013). Bound ligand is measured over a variety of starting concentrations, and the resulting dose response curve can be fit to determine

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specific binding values (Berson & Yalow, 1959; Hulme & Trevethick, 2010; Kuriyan et al., 2013). Historically, ligand/receptor-binding assays were performed by using radioactively labeled ligands, allowing for quantification of high-affinity binding interactions with great specificity (Crevat-Pisano, Hariton, Rolland, & Cano, 1986; Maguire, Kuc, & Davenport, 2012; McKinney & Raddatz, 2006). Safety concerns and limitations in radioligand labeling, along with recent advances in imaging technology have given way to fluorescent labeling and detection of ligands, which is commonly used in cell-binding assays today (de Jong et al., 2005). A number of label-free methods have also been developed to measure protein-protein binding interactions in solution.

Here we present a practical guide for measuring 1:1 binding events between soluble ligands and binding partners expressed on the surface of yeast and mammalian cells. Further, we describe several important considerations: (1) how to properly set up a cellular-binding assay, (2) how to avoid experimental errors that will lead to inaccurate values, and (3) how cell-based binding assays compare to other assays used in the field.

2. GENERAL BINDING THEORY AND RELEVANCE OF K_d

Analysis of the binding of proteins and small molecules was initially proposed by Scatchard (1949), and later refined for protein-protein interactions by others (Berson & Yalow, 1959; Feldman, 1972; Rosenthal, 1967; Stephenson, 1956). The collective model states that the binding interaction of two proteins (here a ligand and receptor) can be described by the following equation, where L represents free ligand, R represents unbound receptor, and LR represents the bound ligand-receptor complex (Berson & Yalow, 1959; Kuriyan et al., 2013):

A binding reaction consists of a dynamic exchange between bound and unbound states, described by two rate constants of the reaction, the k_{on} and k_{off} (units of $M^{-1}s^{-1}$ and s^{-1} , respectively). Over time, reactions proceed to equilibrium as more and more ligand binds to receptor, until the concentrations of [L], [R], and [LR] are held in steady state.

Under these equilibrium conditions, binding can then be modeled further, using the law of mass action (Guldberg & Waage, 1864; Hulme & Trevethick, 2010; Kuriyan et al., 2013; Pollard, 2010). At equilibrium, the rates of the forward ($k_{on}[L][R]$) and reverse ($k_{off}[LR]$) reactions are equal. This relationship, shown in Eq. (2), can be rearranged to derive a ratio known as the equilibrium-binding association constant, K_a with units of inverse molarity (M^{-1}) (Berson & Yalow, 1959; Hulme & Trevethick, 2010; Pollard, 2010). This relationship can be modeled by the following equations:

$$k_{on}[L][R] = k_{off}[LR] \quad (2)$$

$$\frac{[LR]}{[L][R]} = \frac{k_{on}}{k_{off}} = K_a \quad (3)$$

However, binding is usually not thought of in terms of the association reaction, but instead the dissociation reaction. This is given by the inverse of the association constant and is known as K_d and has the units of molarity (M) (Berson & Yalow, 1959; Hulme & Trevethick, 2010; Kuriyan et al., 2013; Pollard, 2010). The equation for K_d can be written as follows:

$$\frac{1}{K_a} = \frac{[L][R]}{[LR]} = \frac{k_{off}}{k_{on}} = K_d \quad (4)$$

It can be useful to think of the K_d in terms of the total fraction of ligand bound (Kenakin, 2016; Klotz, 1982). To determine this relationship, an equation for the total receptor concentration must first be established. This equation is based on the assumption that the total concentration of receptor $[R]_T$ present is simply the sum of the concentration of free receptor $[R]$ and that of bound receptor $[LR]$. Thus:

$$[R]_T = [R] + [LR] \quad (5)$$

Rearrangement of Eq. (4) gives:

$$[R] = \frac{[LR]K_d}{[L]} \quad (6)$$

Substituting into Eq. (5) for $[R]$, the following equation is derived:

$$[R]_T = \frac{[LR]K_d}{[L]} + [LR] \quad (7)$$

The fraction of receptor bound (f) can be thought of as the ratio of the concentration of bound receptor $[LR]$ to the total receptor $[R]_T$: $[LR]/[R]_T$. Thus, by rearranging Eq. (7), the following equation is derived, which gives the fraction of receptor bound in terms of the concentration of free ligand and K_d :

$$\frac{[LR]}{[R]_T} = f = \frac{[L]}{K_d + [L]} \quad (8)$$

Eq. (8) can be fitted to either a hyperbolic shape (on a normal axis) or a sigmoidal shape (on a logarithmic axis) (Hulme & Trevethick, 2010; Klotz, 1982; Pollard, 2010). Most typically,

this equation is depicted as a sigmoidal curve on a semilog plot, with the ligand concentration as the abscissa (on a log-scale axis) and the fraction bound as the ordinate (Fig. 1) (Klotz, 1982). As K_d is thought of in units of molarity, it is easiest to derive the K_d from this equation when the value is equal to that of the ligand concentration. In other words: $K_d = [L]$.

Substituting $[L]$ for K_d under these circumstances in Eq. (8), the following is derived for the fraction bound (f):

$$f = \frac{K_d}{K_d + K_d} = \frac{K_d}{2K_d} = \frac{1}{2}$$

Thus, when 50% of the receptor is bound by ligand, the K_d will be equal to the ligand concentration, meaning that its value can be extrapolated from the fit sigmoidal curve (Fig. 1).

For any given binding reaction, the lower the concentration of ligand required for half of the maximal binding events possible to occur, the more tightly the two binding partners must interact. Moreover, as the K_d is defined as k_{off}/k_{on} , there are two mechanisms through which a K_d can take on a low value, a slow off-rate (k_{off}), or a fast on-rate (k_{on}). Thus, when altering a binding interaction to have a lower K_d , both of these parameters may be modulated to achieve a tighter binding interaction (Chen, de Picciotto, Hackel, & Wittrup, 2013).

3. GENERAL PITFALLS IN CELL-BASED BINDING ASSAYS

The K_d derivation equation (Eq. 8) relies on a number of assumptions that, if unaccounted for, may introduce significant error into the assay. Two major considerations are the time to equilibrium of the binding reaction and ligand depletion that occurs during the binding assay.

3.1 Time to Equilibrium

The K_d equation is based on a system at equilibrium, which requires that a binding reaction must come to equilibrium for measured K_d values to be accurate (Berson & Yalow, 1959; Bylund & Toews, 1993; Hulme & Trevethick, 2010; Kuriyan et al., 2013; Pollard, 2010). Allowing binding reactions to come to equilibrium is highly dependent on the concentration of ligand used and the strength of the binding interaction, and thus may require the reaction to be incubated for several hours or days. Mathematically, equilibrium time can be calculated from the k_{off} , $[L]$, and K_d (Chen et al., 2013; Hulme & Trevethick, 2010). A useful parameter is the half-time of the equilibrium equation ($t_{1/2}$):

$$t_{1/2} = \frac{\ln(2)}{k_{off} \left(1 + \frac{[L]}{K_d}\right)} \quad (9)$$

The reaction will reach one half of the remaining concentration ratio toward equilibrium after each half-time. Thus, it takes $5 \times t_{1/2}$ to achieve 97% of the final equilibrium value. As additional time after this point will only lead to minimal increases towards complete equilibrium, $5 \times t_{1/2}$ can be thought of as the time required to reach equilibrium in a binding reaction (Chen et al., 2013; Hulme & Trevethick, 2010).

Establishing the time to equilibrium is critical for assuring that a binding assay has been performed correctly, and requires knowledge of k_{off} , $[L]$, and K_d (Eq. 9). However, for a novel binding interaction, the k_{off} and K_d will be unknown, making determination of the half-time parameter at the outset difficult. The k_{off} can be determined from an off-rate assay (Boder & Wittrup, 1998). This value, in conjunction with an estimated K_d (derived from literature or pilot experiments) can then be used to determine the time to equilibrium at the lowest concentration of ligand tested to determine the appropriate incubation time. In addition, it is often useful to perform the binding assay using a longer incubation time to determine if a similar K_d is obtained. If so, this provides additional confidence that a binding reaction has come to equilibrium.

Failure to allow binding reactions to come to equilibrium results in a right-ward shift in the binding curve (Fig. 2), meaning that the K_d is determined as a higher value than it actually is and the tightness of binding is underestimated (Hulme & Trevethick, 2010; Pollard, 2010). When the reaction has not come to equilibrium, the ratio of free ligand and receptor to bound receptor ($[L][R]/[LR]$) is higher, as not all of the free ligand has yet bound receptor in contrast to when the system is at equilibrium.

3.2 Ligand Depletion

It is essential to be able to determine or assume an accurate value for the concentration of free ligand $[L]$ when performing a binding assay (Hulme & Trevethick, 2010; Kuriyan et al., 2013). While it is theoretically possible to measure the concentration of ligand that remains free at equilibrium, it is much easier to assume that the initial concentration of ligand used is the same as the free ligand concentration at equilibrium. This assumption is only possible if the initial concentration of ligand introduced to the system is far greater than the concentration of receptor present (Kuriyan et al., 2013; Limbird, 1995). Ligand depletion can be modeled by the fraction of ligand bound by receptor, described using the following equation, with δ as ligand depletion, $[LR]$ as the concentration of bound receptor, and $[L]_T$ as the total concentration of ligand in the system (Hulme & Trevethick, 2010):

$$\delta = \frac{[LR]}{[L]_T} \quad (10)$$

In this case, it is assumed that all of the receptor present is bound by ligand; therefore $[LR]$ becomes equivalent to $[R]_T$, or the total concentration of receptor. The ratio for ligand depletion then becomes:

$$\delta = \frac{[R]_T}{[L]_T} \quad (11)$$

If the total concentration of receptor $[R]_T$ is 10% of the concentration of total ligand $[L]_T$ ($\delta = 0.1$), then the initial concentration of ligand introduced will be roughly the same as the final concentration of free ligand $[L]$ when the system reaches equilibrium (Colby et al., 2004). Thus, the $[L]_T$ added at the beginning of the assay can be used in place of $[L]$ when calculating K_d . A more in depth description of the mathematical rationale for ligand depletion is discussed in review elsewhere (Colby et al., 2004; Hulme & Trevethick, 2010).

Based on Eq. (11), ligand depletion can be modulated by controlling two factors of the binding assay: the total concentration of receptor present $[R]_T$ or the total concentration of ligand present $[L]_T$. To decrease the value of δ to 0.1 or below, $[R]_T$ can be reduced or $[L]_T$ can be increased. The main technique used to control for $[R]_T$ in cell-binding assays is to control the number of cells present. However, in most experiments there is a minimum number of cells that must be used to measure a significant binding signal to derive reproducible data ($\sim 10^5$ cells) (Colby et al., 2004). Alternatively, to increase $[L]_T$, the total moles of ligand added to the reaction and the total volume of the reaction can be increased. As a result of increasing the volume of the reaction, the overall concentration of receptor $[R]_T$ will decrease, as the total number of receptors (based on cell number) is held constant in each condition. Thus, volumetric increase is critical to avoid ligand depletion at low ligand concentrations.

Table 1 shows the minimum volume necessary for varying concentrations of ligand, given a receptor number of 10^{10} (10^5 cells $\times 10^5$ receptors/cell), a standard estimate when testing proteins expressed on the yeast cell surface (Boder & Wittrup, 2000) or mammalian cell surface (discussed in Sections 4 and 5, respectively). As is clearly evident, the effects of ligand depletion become the most apparent when starting concentrations of ligand become very low (<1 nM). For this reason, it is often challenging to quantify high-affinity binders using cell-based assays because of the difficulty in recovering small numbers of cells from large (>15 mL) assay volumes.

If compensation for ligand depletion is not taken into account, the effect will create a rightward shift of the binding curve and an overestimation of the K_d (Hulme & Trevethick, 2010; Limbird, 1995; Moore & Cochran, 2012). This is due to the mechanism of analysis of K_d from binding curves, reliant on the assumption that the free ligand concentration $[L]$ used in fitting the curve is equal to the total starting ligand concentration $[L]_T$ (Kuriyan et al., 2013). If ligand depletion exists, then a significant portion of the free ligand will be bound by receptor, and as the assay progresses, the effective free ligand concentration $[L]$ will no longer be equal to the initial $[L]_T$. The binding signal measured will thus be reflective of a concentration of free ligand that is significantly lower than the initial ligand concentration. This phenomenon is demonstrated in Fig. 3 and Table 2.

Given the number of considerations required in order to set up an accurate binding assay, it is perhaps unsurprising that there are often a variety of K_d values (often over-estimations) provided in the literature for the same protein–protein interaction (Kastritis & Bonvin, 2013; Kastritis et al., 2011). This is an especially important consideration in engineering proteins for tighter binding, as “improved” variants are benchmarked against wild-type counterparts, often based on literature K_d values.

4. MEASURING BINDING ON THE SURFACE OF YEAST

A powerful method for analyzing and engineering novel protein variants and interactions is yeast surface display (YSD), pioneered by Boder and Wittrup. YSD relies on the fusion of a protein of interest to a yeast surface protein (Aga2p), along with epitope tags (c-myc or hemagglutinin (HA)) to monitor protein expression after induction (Fig. 4) (Boder & Wittrup, 1997). YSD as a technology to engineer proteins has been reviewed extensively (Chen et al., 2013; Cherf & Cochran, 2015; Colby et al., 2004; Moore & Cochran, 2012). YSD is also a useful platform for performing characterization of binding interactions with a soluble target, obviating the need for large-scale expression and purification of a protein of interest (Gai & Wittrup, 2007). Importantly, affinities of proteins that are displayed on the surface of yeast have been demonstrated to be comparable to the same proteins in a soluble form, as measured by other techniques (Gai & Wittrup, 2007). However, binding values can be confounded if the binding interaction being measured on yeast is more complicated in the endogenous setting, for example, due to binding interactions that are not 1:1, or if the displayed protein adopts an unnatural conformation.

Here we will describe the general procedures for binding assays using yeast that have been transformed with the appropriate vector to display the protein of interest (Boder & Wittrup, 1998; Chen et al., 2013; Colby et al., 2004; Moore & Cochran, 2012).

4.1 Materials

4.1.1 Yeast Cells—*Saccharomyces cerevisiae* yeast strain EBY100 transformed with the pCTCON2 or pTMY vector containing a protein of interest. pCTCON2 displays the protein of interest as an N-terminal fusion to Aga2p (with a C-terminal c-myc expression tag) (Boder & Wittrup, 1998), while pTMY displays the proteins as a C-terminal fusion to Aga2p (with an N-terminal HA expression tag) (Jones et al., 2011) (Fig. 4).

4.1.2 Solutions and Media—*SD-CAA Media* (Growth media): 20 g/L dextrose, 6.7 g/L yeast nitrogen base (Becton Dickinson, catalog no. 291940), 5 g/L casamino acids (Becton Dickinson, catalog no. BP1424), 5.4 g/L Na_2HPO_4 (anhydrous), 8.6 g/L $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ in deionized H_2O , filter sterilize with a 0.2- μm filter and store at 4°C.

SG-CAA Media (Induction media): identical to SD-CAA, except replace dextrose with galactose (20 g/L).

0.1% BPBS (also known as PBS-BSA, PBSA—“Binding Assay Buffer”): Dissolve 1 g of BSA in 1 L of 1 × PBS. Filter sterilize using a 0.2 μm filter and store at 4°C. In cases of specialized binding buffers, a 1:1 mixture of the selective binding buffer and 0.1% BPBS can

be used. Inclusion of a low concentration of BSA in the binding assay buffer helps to prevent nonspecific binding.

4.1.3 Proteins/Antibodies—*Binding protein of interest* (ligand): Should be tagged for binding recognition (biotinylation, His₆-tag, fluorescent dye, etc.).

Primary expression antibody (to be used against expression tag)—Example: Chicken-Anti *c-myc* (Life Technologies A21281, 1:500 dilution, for pCTCON2).

Fluorophore-conjugated secondary expression antibody (to be used against Primary Expression Antibody, typically R-phycoerythrin [PE] conjugated)—Example: Goat-Anti Chicken-R-phycoerythrin (Santa Cruz Biotechnologies D1715, 1:100 dilution, for pCTCON2).

Fluorophore-conjugated secondary binding antibody (to be used against tag on ligand-binding partner, typically FITC conjugated)—Example: Rabbit-Anti His₆-FITC (Bethyl A190-114F, 1:100 dilution).

4.2 Method

Day 1: Inoculation

1. Over flame, inoculate a single pCTCON2- or pTMY-transformed yeast colony into 5 mL SD-CAA. Place at 30°C for 24 h, or until the OD_{600nm} = 3–5.

Day 2: Induction

1. Determine the OD_{600nm} of all samples using a spectrophotometer.
2. Seed induction samples at an OD_{600nm} of 1. Transfer the appropriate volume to a 1.5-mL Eppendorf tube. Spin down all samples at 3600 rpm for 4 min. Discard the supernatant.
3. Resuspend in 500 µL of SG-CAA, add to 5 mL SG-CAA in new culture tube.
4. Place at the optimal induction temperature for 24 h.
 - a. *Note:* Yeast are typically induced to express protein at either 20°C or 30°C. The optimal expression condition for the protein of interest should be tested and determined prior to starting a binding assay (Fig. 5).

Day 3

1. Determine the range of ligand concentrations (two orders of magnitude above and below the K_d) (Hulme & Trevethick, 2010; Moore & Cochran, 2012) and volumes needed to avoid ligand depletion. Prepare dilutions of ligand. Include expression, secondary antibody, and cells only controls (Table 3).
2. Measure the OD_{600nm} of each induced yeast culture.

3. Add 10^5 yeast to labeled Eppendorf tubes (OD_{600nm} of 1 is equal to 1×10^7 cells/mL). Wash with 50 μ L binding assay buffer. Spin at $14,000 \times g$ for 30 s and discard supernatant.
4. Resuspend in binding assay buffer for the final binding incubation volume (step 1). Transfer yeast to larger tubes for larger volume incubations, if needed.
5. Add the ligand to each sample. Do not add ligand to the control tubes for expression, nonspecific secondary binding, and cells only. Incubate tubes on a rotator at room temperature or 4°C until reactions reach equilibrium (Section 3).

Postincubation

1. Keep samples at 4°C . Spin down samples at $14,000 \times g$ for 30 s at 4°C , and remove the supernatant. For larger tubes (>2 mL), spin at 3600 rpm for 4 min.
2. Resuspend each tube with 50 μ L of cold binding assay buffer. Add primary expression antibody at the appropriate dilution (Anti-HA for pTMY or Anti-*c-myc* for pCTCON2). Do not add primary antibody to the nonspecific binding secondary only and cells only control tubes. Incubate on a rotator at 4°C for 30 min.
3. Wash each sample with 0.5 mL of cold binding assay buffer. Spin at $14,000 \times g$ for 30 s at 4°C . Remove supernatant, leaving samples on ice.
4. Resuspend each sample in 50 μ L of secondary antibody containing binding assay buffer (1:100 dilution). Keep tubes with secondary antibody in the dark. Incubate at 4°C on a rotator for 15 min.
5. Wash as in step 3.
6. Analyze the samples on a flow cytometer. Binding values can be determined from the average FITC value of each sample corrected for autofluorescence (expression only). Plot fraction bound vs ligand concentration (log scale). Fit a sigmoidal curve using nonlinear regression analysis. The K_d value can be derived from the ligand concentration at half the fraction bound (Fig. 1).

5. MEASURING BINDING ON THE SURFACE OF MAMMALIAN CELLS

Measuring binding of receptors or membrane-bound proteins on the surface of mammalian cells offers a unique ability to assay for binding values in endogenous settings, instead of using displayed or soluble protein (Bylund & Toews, 1993). Mammalian systems also give the advantage of being able to assay against receptor complexes and can present proteins that may not fold or be displayed properly on the surface of yeast. There are two major types of mammalian cell-binding assays: direct and competition (Moore & Cochran, 2012). Both protocols follow similar steps to the yeast cell-binding assay, however, mammalian cells are much more fragile than yeast and should be treated with care. The potential pitfalls and logic behind the experimental design are the same for both types of cell-based assays.

5.1 Direct Binding

1. Aliquot 5×10^4 mammalian cells expressing the target protein of interest to labeled 1.5 mL Eppendorf tubes. Make sure to include tubes for cells only and cells with antibodies only controls, if needed.
2. Spin down cells at $800 \times g$ for 5 min, ensuring cells are still viable by try-pan blue staining. Remove supernatant, and resuspend in binding assay buffer, using appropriate volumes to avoid ligand depletion (Section 3).
 - a. *Note:* Binding assay buffer can be anything from media to BPBS. All components necessary for the binding interaction of interest should be included (salts, cofactors, etc.).
3. Add soluble ligand to each tube at varying concentrations, spanning two orders of magnitude above and below the anticipated K_d .
4. Allow the reaction to incubate at 4°C until it has come to equilibrium, generally a number of hours.
5. If the ligand is fluorescently labeled, proceed to step 6. If the ligand has an epitope tag, first wash the tubes with 0.5 mL of cold BPBS, and spin them down at $800 \times g$ for 5 min at 4°C . Remove the supernatant and then resuspend the cells in 50 μL with a 1:100 dilution of the appropriate fluorescently labeled antibody (eg, Rabbit Anti-His₆-FITC). Allow the antibody to incubate for 20–30 min at 4°C .
6. Wash cells with 0.5 mL of cold BPBS. Spin down at $800 \times g$ for 5 min at 4°C . Remove the supernatant.
7. Analyze the cells using flow cytometry. Analyze data as described in the yeast binding assay (Postincubation, step 6).

5.2 Competition Binding

The competition assay follows very similar steps to those listed in the direct-binding assay. Differences are noted below.

1. Follow steps 1 and 2 as in the direct-binding assay.
2. For step 3, incubate with the same range of ligand concentrations as described but also include a constant concentration of competitor in each tube. Competitor should bind to the target receptor and should be fluorescently labeled or contain an epitope tag for detection. Competitor should be used at a value lower than its K_d , such that it can still be detected but is able to be competed off. Allow to incubate at 4°C for a number of hours.
3. If competitor is fluorescently labeled, follow steps 6–7 of the direct-binding assay. If not, wash and incubate with the appropriate fluorescently labeled antibody, as described in step 5 of the direct-binding assay. Then follow steps 6–7.

4. When the curve is analyzed it will give the IC_{50} of the binding reaction, not the K_d . The K_d can be calculated from the Cheng–Prusoff equation (Cheng & Prusoff, 1973):

$$K_d = \frac{IC_{50}}{1 + [competitor]/K_d \text{ competitor}} \quad (12)$$

The assumptions of Eq. (12) are very similar to those of the general-binding Eq. (8) described earlier (Krohn & Link, 2003). Notably, the reaction must be at equilibrium, represent a 1:1 binding interaction, and not contain depleted ligand (Ehlert, Roeske, & Yamamura, 1981; McKinney & Raddatz, 2006). Further, the K_d of the competitor must be known under the binding conditions being used. With these parameters in place, the K_d can be calculated using the competition assay (Fig. 6).

6. OTHER METHODS OF MEASURING BINDING: KINETIC EXCLUSION ASSAY AND SURFACE PLASMON RESONANCE

While cell-binding assays are advantageous in the speed and ease with which they can be carried out, they are limited in their ability to measure high-affinity binding interactions and kinetic parameters (Bylund & Toews, 1993). In these cases, the kinetic exclusion assay (KinExA) or surface plasmon resonance (SPR) can be used (Blake, Pavlov, & Blake, 1999; Darling & Brault, 2004; Myszk, 2000; Patching, 2014). Here we will give a brief introduction to each and list the major advantages and disadvantages of each technology. Note that the designations of ligand and receptor in the examples below are arbitrary and can be reversed.

6.1 Kinetic Exclusion Assay

Binding interactions measured by KinExA (Blake et al., 1999) make use of a column of packed beads to which an immobilized ligand is affixed (Darling & Brault, 2004). Binding reactions are set up with varying concentrations of ligand and constant concentrations of receptor, allowed to come to equilibrium, and are then run over the column to capture unbound receptor by the affixed ligand (Darling & Brault, 2004). The amount of bead-bound receptor is then detected and quantified using a fluorescent antibody (Fig. 7A–C). Thus, KinExA allows direct measurement of free receptor remaining at equilibrium in each binding reaction.

Kinetic exclusion refers to the rapid speed at which the binding reaction is flowed over the column (<0.5 s), such that the bound ligand–receptor complex does not have time to dissociate and increase the free receptor concentration in solution (Darling & Brault, 2004). Thus LR dissociation is “kinetically excluded” from occurring. This critical feature means that the amount of receptor captured by the immobilized ligand is proportional to $[R]_{\text{free}}$, providing a method through which K_d can be determined without having to consider factors

underlying cell-binding assays, such as ligand depletion (Blake et al., 1999; Darling & Brault, 2004; Drake, Myszk, & Klakamp, 2004).

Since binding is measured directly and extrapolated from a small amount of measured $[R]_{\text{free}}$, KinExA allows for the determination of the K_d of ultra high affinity binding interactions, and can also measure kinetic binding parameters (Darling & Brault, 2004). This is done by mixing predetermined concentrations of $[L]_0$ and $[R]_0$ together, and then measuring $[R]_{\text{free}}$ over time (Darling & Brault, 2004; Drake et al., 2004). These data can be plotted and fit to the standard biomolecular rate equation and extrapolated to determine the k_{on} (Darling & Brault, 2004). For more details, KinExA as a technology for determining binding affinities has been reviewed extensively elsewhere (Darling & Brault, 2004; Drake et al., 2004).

6.2 Surface Plasmon Resonance

SPR relies on the measurement of refractive index change from receptor attached to a gold surface after soluble ligand, or analyte, is flowed over the chip (Fig. 7D) (Jönsson et al., 1991, 1993; Myszk, 2000; Patching, 2014). These measurements are carried out in real time, and the magnitude of refractive index change is directly proportional to the molecular weight of the analyte (Myszk, 2000; Patching, 2014).

To measure kinetic parameters, the SPR protocol makes use of varying concentrations of analyte flowed over chips of immobilized receptor. Real-time measurements are made of the association phase, as analyte is flowed, and of the dissociation phase, as buffer is flowed (Myszk, 2000; Patching, 2014). These data generate a set of binding curves from which the k_{on} (association), k_{off} (dissociation), and K_d can be extrapolated (Myszk, 2000; Patching, 2014).

Unlike KinExA and cell-based binding assays, SPR does not require reactions to come to equilibrium, advantageous for binding reactions that have very long equilibrium times. However, because it requires measuring k_{on} and k_{off} , if either of these values is too fast or too slow, respectively, SPR can become no longer reliable in measuring these parameters and another type of binding assay should be used. For more details, the use of SPR to determine binding affinities has been extensively reviewed elsewhere (Myszk, 2000; Patching, 2014).

6.3 Comparison

Each binding assay covered in this review has a specific set of circumstances under which its use is optimal. The advantages and disadvantages of each type of binding assay are summarized in Table 4. All four methods of measuring binding interactions can be used to characterize wild-type and engineered proteins. For example, YSD can be used to rapidly express multiple protein variants and test them for equilibrium binding or kinetic off-rate of binding to a protein of interest, without having to carry out large scale purification and expression of individual proteins. Ligands can also be tested using direct or competition mammalian cell-binding assays to probe for receptor or membrane-bound protein interactions in an endogenous setting. Further characterization, including in-depth kinetic analysis and a more rigorous testing of the K_d , especially if the engineered affinity is very

tight, can be measured using either KinExA or SPR, depending on the properties of the binding interaction (see Table 4). These four methods can be also used in concert to obtain confidence in the accuracy of binding measurements.

7. SUMMARY

In general, whenever they are possible cell-based binding assays provide facile and robust methods to measure affinities of protein–protein interactions. In this review we cover basic principles underlying the design of cell-based binding assays, discuss potential pitfalls that can occur in determining the K_d of protein–protein interactions, and provide protocols to determine the binding affinities of protein interactions using cell-based assays. We discuss steps to establish the proper time to equilibrium and incubation volumes to avoid or minimize ligand depletion, and demonstrate how these factors can lead to errors in determining K_d values. Additionally, we compare cell-based binding assays to KinExA and SPR, and offer rationale for when each assay might best be used.

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Fig. 1. Sigmoidal binding curve of varying concentrations of ligand bound to cell surface receptor. *Dashed lines* demarcate the K_d (1 nM) as the ligand concentration at 0.5 fraction bound.

Fig. 2.

The effects of equilibrium time on ligand binding to displayed receptor. *Dashed lines* represent the K_d of each binding curve. The equilibrium-binding curve (represented by *black circles*) shows a binding reaction that was allowed to go to equilibrium and reveals the true K_d of 150 pM. The nonequilibrium-binding curve (represented by *red (gray in the print version) triangles*) shows the results of a binding reaction that was not allowed enough time to come to equilibrium. The measured K_d of this curve is now 2 nM, a roughly tenfold difference above the actual K_d .

Fig. 3. The effects of ligand depletion on cell-binding assay measurements. *Dashed lines* represent the K_d of each binding curve. The equilibrium-binding reaction curve (represented by *black circles*) was performed using appropriate volumes at each concentration to avoid ligand depletion, while the ligand depletion reaction curve (represented by *blue (dark gray in the print version) diamonds*) did not. The measured K_d of this curve is 2 nM, a 10-fold difference above the actual K_d of 150 pM. These data are tabulated in Table 2.

Fig. 4.

A schematic of yeast surface display. In this case, the binding partner protein is detected using a fluorescently labeled antibody against a His₆-tag, although other epitope tags or a fluorescently labeled binding partner can be used. The pCTCON2 vector layout and display system is shown. The N-terminus of the protein of interest is fused to the C-terminus of Aga2p; a C-terminal c-myc tag allows the expression of full-length protein to be measured with a fluorescent secondary antibody binding to an anti-c-myc antibody. Alternatively, the pTMY vector layout and display system (not shown) results in a C-terminal fusion of the protein of interest to N-terminus of Aga2p, with an exposed HA expression tag. Expression is then detected with an anti-HA antibody.

Fig. 5.

Expression histograms of example protein displayed on the surface of yeast. The y -axis is the number of cells and the x -axis is the PE signal. (A) Expression at 20°C, with a tightly defined expressing population (*arrow*). (B) Expression at 30°C, with a poorly defined expressing population (*arrow*). Differences between A and B are due to yeast growth and overall protein folding and expression levels.

Fig. 6. Mammalian cell competition assay. Competitor was fluorescently labeled with Alexa Fluor 488 prior to assay. Reactions were carried out with varying concentrations of ligand. *Dotted lines* mark the IC_{50} (4 nM).

Fig. 7.

(A–C) KinExA. (A) Close up of the KinExA bead-based detection system. Ligand affixed to beads in a column bind soluble, free receptor, which is detected via fluorescent antibody binding to receptor. (B and C) Schematic of the KinExA assay. (B) Soluble ligand and receptor are incubated until equilibrium is reached. This reaction is flowed over the column, allowing free receptor at equilibrium to bind bead-affixed ligand. (C) After washing, bound receptor is detected using specific fluorescent antibodies and quantified. (D) Surface plasmon resonance. Receptor is affixed to a gold film surface. Ligand, or “analyte,” is flowed over the surface. Alterations in resonance due to binding are measured via a detector from light shone through a prism and analyzed. Note that for KinExA and SPR, the designation of ligand and receptor is arbitrary and can be reversed.

Table 1

Minimum Volumes Needed to Avoid Ligand Depletion in a Cell-Binding Assay with 10^{10} Receptors Present

Ligand Conc. $[L]_T$ (nM)	Ligand Depletion Volume (μ L)	Ligand Conc. $[L]_T$ (pM)	Ligand Depletion Volume (mL)
500	0.33	500	0.33
100	1.66	100	1.66
50	3.32	50	3.32
10	16.6	10	16.6
5	33.2	5	33.2
1	166	1	166
0.5	330	0.5	330

Large volumes are required to avoid ligand depletion for low ligand concentrations.

Table 2

Binding Assay Conditions Used in Nonligand Depleting and Ligand Depleting Experiments

Perceived [Ligand] (nM)	Equilibrium-Binding Conditions		Ligand-Depleting Conditions	
	Volume (μL)	Fraction Bound	Volume (μL)	Fraction Bound
30.00	20	1.00	20	1.00
10.00	20	0.95	20	1.28
3.00	50	0.98	20	<i>*0.52</i>
1.00	200	0.90	20	<i>*0.12</i>
0.30	500	0.54	20	<i>*0.08</i>
0.10	2000	0.34	60	<i>*0.03</i>
0.03	5500	0.24	200	<i>*0.04</i>
0.01	20,000	0.14	600	<i>*0.05</i>
0.003	55,000	0.07	1500	<i>*0.07</i>

The perceived ligand concentration is the concentration of ligand that was used as a starting point at each listed volume. The resultant fraction bound values are derived from analyzing the final reactions. Values that are under ligand depletion conditions ($\delta > 0.1$) are italicized and asterisked. Data are plotted in Fig. 3. While this table provides useful guidelines, ligand depletion (or lack thereof) should be determined empirically by measuring binding affinities under different volumes to confirm similar K_d values are achieved.

Table 3

Preparation for a Binding Assay Set Up

Sample No.	Purpose	Primary Condition	Secondary Condition
1	Binding/expression (100 nM ligand)	0.4 μ L of 5 μ M ligand stock+primary expression Ab (20 μ L total volume)	Secondary binding Ab+ secondary expression Ab
2	Expression only	Primary expression Ab	Secondary expression Ab
3	Secondary only	N/A	Secondary binding Ab+ secondary expression Ab
4	Cells only	N/A	N/A

An example is provided for binding measured at 100 nM of ligand, stored at a stock concentration of 5 μ M (Sample 1). The appropriate detection antibodies to be used are listed. Controls are also shown (Samples 2–4).

Table 4**Advantages and Disadvantages of Cell-Binding Assays, KinExA, and SPR**

Assay	Advantages	Disadvantages
Yeast surface display	<ul style="list-style-type: none"> No need for large-scale expression and purification of protein of interest Easy to measure binding of multiple protein variants Relatively high throughput Low technical and time requirements Protein expression can be quantified and normalized with binding Compatible with flow cytometry 	<ul style="list-style-type: none"> Unable to accurately measure high-affinity interactions (<10 pM) Protein immobilization on the surface of yeast might not mimic natural environment Measurement of high-affinity binders requires cognizance of ligand depletion and time to equilibrium Requires separate assays to measure equilibrium and kinetic parameters
Mammalian cell	<ul style="list-style-type: none"> Replicates natural binding conditions (for membrane-bound proteins) No need to express or purify the receptor of interest Compatible with flow cytometry Relatively high throughput Can perform direct- or competition-binding assays 	<ul style="list-style-type: none"> Unable to accurately measure high-affinity interactions (<10 pM) Mammalian cells are fragile, dead cells can nonspecifically bind to ligand or reagents Measurement of high-affinity binders requires cognizance of ligand depletion and time to equilibrium Requires separate assays to measure equilibrium and kinetic parameters
KinExA	<ul style="list-style-type: none"> True solution-based measurements of unmodified proteins Accurately measures high-affinity interactions (<10 pM), can measure fM Avoids nonspecific cell binding Measures amount of $[R]_{\text{free}}$ at equilibrium removing concerns of ligand depletion Measurements can be made with unpurified mixtures 	<ul style="list-style-type: none"> Relatively low throughput, although autosampler is available Nonspecific bead binding may occur Weaker binders can be measured but may require extra care Solution binding might not mimic natural environment Requires separate assays to measure equilibrium and kinetic parameters
Surface plasmon resonance	<ul style="list-style-type: none"> Advanced automation Low quantities of samples needed Temperature control Able to measure k_{on} and k_{off} simultaneously Reactions do not need to reach equilibrium to be evaluated 	<ul style="list-style-type: none"> Binding partner is immobilized to a surface Requires purified proteins Limited by mass transport effects in measuring very fast association rates or very slow dissociation rates Can be relatively low throughput, depending on the instrument