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Titration of apparent *in-cellula* affinities of protein-protein interactions

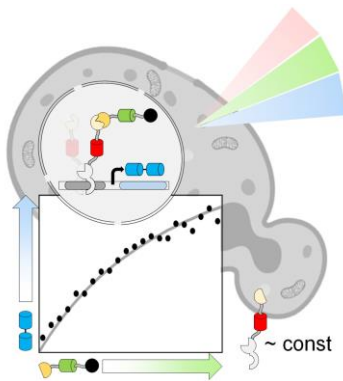
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Graphical abstract:



Using fluorescent probes, we estimate at the cellular level the fraction of Bait molecules bound by Prey molecules in a quantitative yeast-two hybrid assay. A titration-like analysis of flow cytometer data permits the extraction of the apparent *in-cellula* dissociation constant.

KEYWORDS: protein-protein interactions, quantitative yeast-two hybrid, flow cytometry, single-cell analysis.

Abstract: A genetic assay permits simultaneous quantification of two interacting proteins and their bound fraction at the single-cell level using flow cytometry. Apparent *in-cellula* affinities of protein-protein interactions can be extracted from the acquired data through a titration-like analysis. The applicability of this approach is demonstrated on a diverse set of interactions with proteins from different families and organisms and with *in-vitro* dissociation constants ranging from picomolar to micromolar.

(Introduction)

The quest for methods that permit rapid and reliable determination of the affinity of protein-protein interactions (PPI) is unbroken. In contrast to biochemical *in-vitro* methods such as Isothermal Titration Calorimetry (ITC) and Surface Plasmon Resonance (SPR) that require purified proteins, quantitative genetic assays rely on the expression of the proteins of interest in cells. Many of these assays^[1–6] are inspired by the yeast two-hybrid (Y2H) technique^[7–9] which is based on the *in-cellula* expression of two proteins, usually named Bait and Prey, fused to an DNA-binding domain (BD) and an activation domain (AD), respectively. Upon physical interaction of the BD-Bait and AD-Prey proteins, a functional transcription factor is reconstituted that drives the expression of a reporter gene. The stronger the interaction, the higher should be the expression level of the reporter.^[1] However, the expression level of the BD-Bait and AD-Prey play an important role, too.^[10]

We recently introduced a quantitative yeast-two hybrid system (qY2H) that permits for the first time simultaneous quantification of BD-Bait, AD-Prey and the reporter at the single-cell level without the need of any antibodies or purified proteins.^[10] Instead, we take advantage of fluorescent fusion proteins that can be detected by standard flow cytometers. Here we show how this qY2H method can be exploited to perform a titration-like analysis to extract the apparent *in-cellula* affinity of PPIs by applying the following two important improvements:

1) Cellular contents of fluorescent proteins are determined in units of Molecules of Equivalent Soluble Fluorochrome (MESF), so that measured quantities become independent of the applied apparatus setup. It facilitates the future transferability of the qY2H measurements to other flow cytometers and allows researchers to consistently compare their results.

2) The cultured populations of yeast cells can be considered as collections of microvessels^[11] with distinctly different content of BD-Bait, AD-Prey and reporter. The fluorescence data of these collections can be analyzed by a titration-like procedure. This allows the straightforward extraction of apparent *in-cellula* dissociation constants for BD-Bait:AD-Prey interactions.

In a proof of concept, we apply this *in-cellula* titration approach to a diverse set of PPIs with dissociation constants ranging from 117 pM to 17 μ M (Table 1). We consider the set of Table 1 as an ideal choice for mainly three reasons: a) the set covers a wide range of affinities, protein families and protein sizes; b) when fused to BD-or AD-, the proteins do not display any detectable auto-activation under the applied experimental conditions;^[10] c) all PPIs have been extensively studied *in-vitro* (see footnotes of Table 1). As in *in-vitro* SPR experiments, each PPI can be measured by Y2H in two different orientations (by exchanging the proteins between BD-Bait and AD-Prey). Here we study only the orientation that produced the higher reporter level.^[10] This orientation is considered as the molecular configuration with the higher accessibility of the PPI binding interface.^[1]

(Methodology)

In our qYH2 experiments, diploid yeast cells with constitutive expression of BD-Bait and induced expression of AD-Prey are cultured for two hours. Then, their fluorescence intensity is measured by flow cytometry in the three channels TagRFP-H (corresponding to BD-Bait), EGFP-H (AD-Prey), and TagBFP-H (reporter). Due to phenotypic variations, BD-Bait and AD-Prey are expressed at different levels among these cells which can be exploited to

“prepare samples” for a titration-like analysis. By gating, we can split the global heterogeneous ensemble of cells into several homogenous subensembles (bins). Each bin contains only cells within two specific, narrow intervals of red and green fluorescence intensity centered at values R and G , respectively. Assuming a linear relationship between fluorescence intensity and molecule numbers, R and G can be considered as measures for the mean cellular content of BD-Bait and AD-Prey in the corresponding bin. Cellular contents of fluorescent proteins are determined in units of Molecules of Equivalent Soluble Fluorochrome (MESF), so that measured quantities become independent of the applied apparatus setup. It facilitates the future transferability of the qY2H measurements to other flow cytometers and allows researchers to consistently compare their results.

With the mean value of the blue fluorescence intensity, we can calculate for each bin the normalized reporter level φ . It is obtained by forming the ratio of the expression level for the interaction of interest, $E_{\text{interaction}}$ (Fig. 1A) and the level for a covalent BD-AD fusion, E_{covalent} (Fig. 1B). This normalization renders φ dimensionless and independent of the acquisition apparatus (assuming again a linear relationship between molecule number and fluorescence intensity). Most importantly, we consider that φ reflects the time-averaged fraction of BD-Bait bound by AD-Prey during the reaction (as explained in the caption of Fig. 1). Thus, titration curves can be obtained when φ is plotted as a function of G while keeping R fixed (Fig. 1C). The curves can be fitted with the following Langmuir-type equation:

$$\varphi(G) \cong \beta \frac{G/\alpha}{K'_d + R + G/\alpha}$$

Eq. 1

where K'_d is the apparent *in-cellula* dissociation constant (in units of MESF of EGFP) and α and β are dimensionless parameters that empirically account for the fact that φ is a time-integrated property. The parameter α reduces the final cellular content of AD-Prey (measured

at the end of the reaction, G) to the time-averaged content (over the entire reaction course, $\langle G \rangle$). Since the induction kinetics under the GAL1-promoter in yeast^[12] displays a quadratic-like time dependence (for short induction times), a reasonable choice for α is 3 [$\langle G \rangle = \int_0^t G \, dt = G/3$]. The prefactor β , on the other hand, integrates differences in the expression kinetics of the reporter for $E_{\text{interaction}}$ (induced expression) and E_{covalent} (constitutive expression). It can be determined experimentally by monitoring φ for $G \rightarrow \infty$ using a high-affinity couple (such as BD-Barstar29F/AD-BarnaseH102A).

We recommend that the titration-like analysis is carried out with the lowest possible value of $R=R_{\text{min}}$ (as defined by the detection limit of TagRFP by flow cytometry, see “Methods”). It limits overexpression and associated protein burden effects.^[13] Furthermore, the auto-activation potential of the BD-Bait fusion is kept at a minimum, too.^[14] Most importantly, it mimics the condition of *in-vitro* affinity titration experiments^[15] where the concentration of the titrated species (here BD-Bait) is kept fixed and as low as possible to avoid saturation effects. For the titrations with $R=R_{\text{min}}$ the parameters $\alpha=3$ and $\beta=1.35$ were used to extract the K_d -values.

Our *in-cellula* assay differs significantly from *in-vitro* titration experiments (as previously discussed^[10] in detail). The *in-vitro* experiment measures the affinity under well-defined buffer-controlled equilibrium conditions. In contrast, our *in-cellula* experiments take place in a non-equilibrium biological environment^[11] where the interaction partners BA-Bait and AD-Prey can interact with the endogenous complex solution of biomolecules. This may lead, for example, to effectively smaller concentrations of the reaction partners. The measured *in-cellula* dissociation constant should therefore be perceived as an apparent dissociation constant. Also, only bimolecular interactions can be investigated with the *in-cellula* approach.

(Results and Discussion)

Despite the substantial differences between our *in-cellula* system and *in-vitro* setups,

the apparent *in-cellula* affinities strongly correlate with those from *in-vitro* measurements ($R^2=0.91$, Fig. 1D). The slope of the regression line in the log-log plot of the dissociation constants is 0.84. Other *in-cellula* assays usually find lower Pearson correlation coefficients (< 0.9) and significantly lower values for the slope of the regression line (0.2-0.6).^[4,5,16–18] This is even more remarkable if one considers that the tested set of PPIs in this work is significantly more diverse. The regression slope in the log-log plot indicates the power law between the *in-cellula* quantity and the *in-vitro* quantity. If the apparent *in-cellula* affinity was linearly correlated with *in-vitro* affinity, we would observe a value of 1 for the regression slope. A decrease of the regression slope in the log-log plot (*in-cellula* vs *in-vitro*) potentially points to a decreased sensitivity of the cellular assay: the extent of the BD-Bait:AD-Prey affinity has little impact on the read-out of the cellular assay, *i.e.*, the read-out is largely dominated by cellular processes that do not depend on the affinity between BD-Bait and AD-Prey. Thus, the higher regression slope in the case of our qY2H titration approach (with respect to other *in-cellula* assays) may indicate a higher sensitivity; more testing will be necessary to confirm this surmise.

From the regression analysis (intercept) it is possible to derive a rough estimate for the conversion factor that relates units of MESF of $K'_{d, \text{in-cellula}}$ to units of molarity. With the relation $150 \text{ MESF} \approx 1 \text{ pM}$; appropriate values in units of molarity are obtained (Table S1, Supporting Information). This conversion relies on the assumption that the *in-cellula* affinities are on the same order of magnitude as the *in-vitro* affinities. This is obviously not correct (see above) and converted values should therefore be used with caution.

We have optimized the experimental conditions in order to cover an affinity range between 100 pM and 10 μM . Thus, the couples BarstarY29F:BarnaseH102A and Grb2:Vav1 represent the minimal and maximal interaction strength that can be studied with these experimental conditions. Stronger interactions lead to saturation and weaker interactions cannot

be distinguished from noise. Not surprisingly the estimate of K'_d for couples BarstarY29F:BarnaseH102A and Grb2:Vav1 is connected with the largest (relative) error (ca. 1; see Table S1, Supporting Information); most other couples feature relative errors between 0.15 and 0.3. To enable the study of interactions with apparent affinities in the lower picomolar range, the experimental conditions need to be changed. The concentration of the AD-Prey inducer (galactose), for example, could be decreased or the AD-Prey could be induced for less than 2h. Similarly, the study of PPIs with weaker affinities than Grb2:Vav1 would require a higher concentration of inducer or a longer induction period of Prey expression.

(Conclusion)

The presented protocol is robust as witnessed by the small error bars in the titration curves (Fig. 1c) and the generally low relative error of K'_d . All steps of the protocol have been optimized in liquid phase that can be easily automated for the use of microplates and integrated within robotic pipelines. It sets the stage for high-throughput affinity screenings of PPIs using cross-mating approaches^[19,20] with libraries of yeast clones. As an outlook, affinity-based networks^[21] can be created by attributing weights to the PPI edges according to their apparent affinities. It contrasts standard Y2H screens that yield networks with only binary information (YES or NO). The topology of edge-weighted spring-embedded networks^[22] may help identifying key pathways within the network, and how they change as a function of environmental conditions (stress, metabolism, *etc*). Thus, we anticipate that high-throughput qY2H affinity data would boost the modelling of interactomes and thereby advance significantly systems biology.

Methods

Construction of the plasmids pSB_1Bait, pSB_1Prey and pBFP2 for the expression of BD-Bait, AD-Prey and the reported, respectively, has been described in detail elsewhere.^[10]

Chemo-competent EGY42 (MATa; trp1, his3, ura3, leu2) and TB50 (MATα; trp1, his3, ura3, leu2, rme1) yeasts were generated as previously described.^[23] The liquid-phase qY2H experiments, acquisition by flow cytometry, conversion to units of MESF^[24] and statistical analyses are described in the Supporting Information.

For the titration-like analysis we selected only yeast cells with a red fluorescence intensity of 800 ± 100 in TagRFP-H channel, “H” indicates signal height. This red fluorescence bin is located just above the 95% threshold of the non-fluorescent cells,^[10] and therefore defines R_{\min} . To ensure that these cells have been indeed transfected with all three vectors, we selected for the analysis only large (=growing) cells with a forward scatter range $75\,000 < \text{FSC-H} < 125\,000$.

The mean Tag BFP-H value was then calculated for bins of varying G values from -500 to 25500 yEGFP-H (bin size 1000). For each bin we calculated:

$$\varphi(G) = \frac{\langle F_{\text{BFP,interaction}} \rangle_G - \langle F_{\text{BFP,CTRL}} \rangle_G}{\langle F_{\text{BFP,covalent}} \rangle_G - \langle F_{\text{BFP,CTRL}} \rangle_G}$$

Eq. 2

where $\langle F_{\text{BFP,X}} \rangle_G$ is the mean blue fluorescence intensity. The subscripted X refers to the physical interaction, covalent fusion or control couple. The control couple BD-Empty / AD-Empty^[10] permits to remove the background of the reporter system.

Finally, G values were converted into MESF of EGFP using calibration beads (Takara, reference 632594) following the manufacturer’s protocol (see Supporting Information for more details). For the conversion of R_{\min} , we performed independent calibration measurements with diploid yeast cells expressing the fluorescent tandem fusion protein BD-TagRFP-yEGFP (under the same condition as the qY2H experiments). Cells with a red fluorescence intensity of $R_{\min} = 800 \pm 100$ TagRFP-H displayed a mean green fluorescence intensity of 370 000 MESF of EGFP.

Experiments and analyses were performed at least three times for each interaction and

213 averaged titration curves were least-square fitted with Eq. 1.

214

215 **Acknowledgments**

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Graphical elements

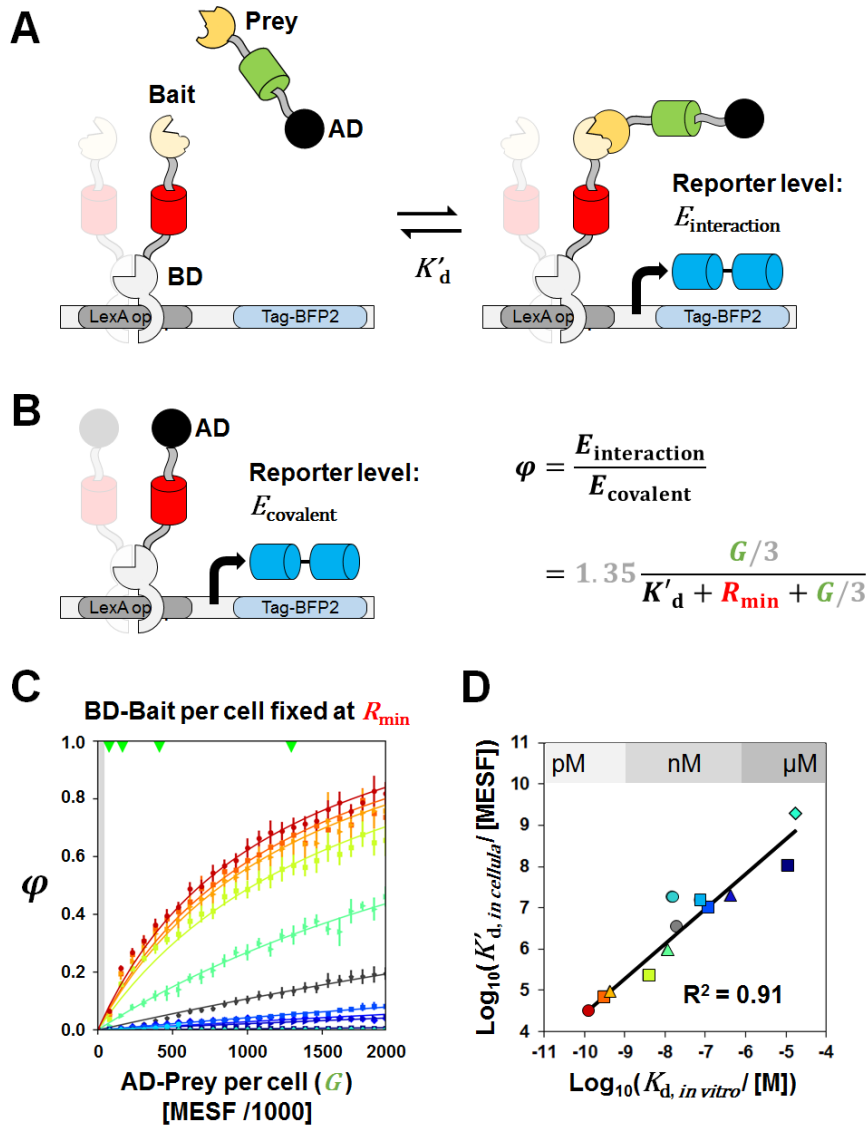


Figure 1: The qY2H system and its application to a titration-like analysis.

(A) In our qY2H system, red-fluorescent BD-Bait interacts with green-fluorescent AD-Prey to reconstitute a transcription factor that drives the expression of a blue-fluorescent reporter. Our hypothesis is that the expression level of the reporter, $E_{interacting}$, reflects the number of BD-molecules bound to the promoter corrected by the fraction of BD-Bait bound to AD-Prey. This fraction is influenced by the affinity between BD-Bait and AD-Prey, but also by their expression levels. (B) When the activation domain is covalently linked to the DNA-binding domain, the expression level $E_{covalent}$ depends only on the number of BD-Bait molecules bound the promoter. Thus, when forming the quantity φ by dividing $E_{interacting}$ with $E_{covalent}$, we obtain a measure for the fraction of BD-molecules bound by an AD-Prey molecule. To determine $E_{covalent}$, we constructed a BD-AD fusion protein. Unfortunately, the activation domain B42 (as used in **a**) turned out to be toxic for our yeast strains when used in the BD-AD construct. Instead, we used the activation domain B112. Difference in the activation potential between B42 and B112 are integrated in the parameter β of Eq. 1. (C) The quantity φ can be monitored as a function of different levels of EGFP Molecules of Equivalent Soluble Fluorochrome (MESF) corresponding to different cellular levels of AD-Prey. In this titration-like analysis, the level of BD-Bait is kept fixed at the lowest possible value (see “Methods”). For the interaction TEM/BLIP1 (cyan line) the titration-like analysis can be performed only up to one third of the titrant quantity due to expression problems of AD-BLIP1.^[10] Green triangles at the top vertical axis indicate the position of used calibration beads. (D) When the titration curves are fitted with Eq. 1, we can extract the apparent dissociation constant in units of MESF (K'_d). The estimated K'_d -values show a remarkable correlation with the dissociation constants measured from alternative *in-vitro* experiments (with matching symbols of Table 1).

Table 1: Investigated protein-protein interactions and their *in-vitro* affinities (K_d).

Bait proteins						Prey proteins					
Organism	Family	Name	Mutant	MW	K_d (pM)	Symbol	Organism	Family	Name	Mutant	MW
<i>B. amylo-liquefaciens</i>	RNase inhibitor	Barstar	WT	10	320 ^a	■	<i>B. amylo-liquefaciens</i>	RNase	Barnase	H102A	12
			Y29A	10	420 ^a	▲					
			Y29F	10	117 ^a	●					
			W38F	10	4 000 ^a	■					
			D35A	10	25 000 ^h	●					
			D39A	10	420 000 ^b	▲					
<i>H. sapiens</i>	GTPase	HRas	G12V & C186A	21	122 000 ^c 11 000 ^d	■ ▲	<i>H. sapiens</i>	Kinase	CRaf RBD	WT A85K	9 9
<i>H. sapiens</i>	Kinase regulatory subunit	CksHs1	WT	10	77 000 ^e	■	<i>H. sapiens</i>	Kinase	CDK2	WT	34
<i>E.coli</i>	β-Lactamase	TEM	WT	31	15 000 ^f	●	<i>S. clavuligerus</i>	β-Lactamase inhibitor	BLIP1	WT	21
<i>HIV1</i>	Virulence factor	Nef	LAI	23	11 400 000 ^g	■	<i>H. sapiens</i>	Kinase	SRC SH3	WT	7
<i>H. sapiens</i>	Adapter	Grb2 SH3	WT	7	17 000 000 ⁱ	◆	<i>M. musculus</i>	Nucleotide exchange factor	Vav1 SH3	WT	8

^a ITC, 50mM Tris/HCl, pH 8 at 25°C.^[25]

^b Mean values from two studies^[25,26] with ITC, 24mM Hepes, pH 8, 1 mM DTT at 25°C.

^c Mean values from four studies of Ras G12V (without the membrane anchor): SPR, 50 mM Tris/HCl, pH 7.4, 100mM NaCl, 5 mM MgCl₂;^[27] SPR, 50 mM Tris/HCl, pH 7.4, 100mM NaCl, 5 mM MgCl₂;^[28] SPR, 10 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM MgCl₂, and 0.01% Nonidet P-40 25°C;^[29] ITC, 50 mM Hepes, pH 7.4, 125 mM NaCl, 5 mM MgCl₂, 25°C.^[30]

^d ITC, 50 mM Hepes, pH 7.4, 125 mM NaCl, 5 mM MgCl₂, 25°C.^[30] The dissociation constant of the CRaf RBD A85K mutant was measured with HRas WT loaded with a GTP-analogue. The mutant HRas G12V is known to decrease the dissociation constant for the interaction with CRaf RBD WT by a factor of 11.^[31] The given value applies the same correction factor.

^e SPR, 10 mM Hepes, 3.4 mM EDTA, 150 mM NaCl, 0.001% surfactant P20, pH 7.4.^[32]

^f SPR, 10 mM Hepes, 3.4 mM EDTA, 150 mM NaCl, 0.05% surfactant P20, pH 7.4.^[33]

^g ITC, 20 mM phosphate buffer, pH 7.5, 150 mM NaCl, 2 mM EGTA, and 5 mM DTT, 25°C.^[34]

^h SPR, 10 mM Hepes-Na, pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.005% (v/v) Tween 20, 25 °C.^[35]

ⁱ SPR, 25°C.^[36]

^h Free-energy calculations.^[10]

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316