CHAPTER FOURTEEN

A Toolkit of Protein-Fragment Complementation Assays for Studying and Dissecting Large-Scale and Dynamic Protein–Protein Interactions in Living Cells

Stephen W. Michnick,* Po Hien Ear,* Christian Landry,† Mohan K. Malleshaiah,* *and* Vincent Messier*

Contents

* Département de Biochimie, Université de Montréal, C.P. 6128, Succursale Centre-ville, Montréal, Québec, Canada
† Département de Biologie, Institut de Biologie Intégrative et des Systèmes, PROTEO-Québec Research Network on Protein Function, Structure and Engineering, Pavillon Charles-Eugène-Marchand, Université Laval, Québec, Canada

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Abstract

Protein-fragment complementation assays (PCAs) are a family of assays for detecting protein–protein interactions (PPIs) that have been developed to provide simple and direct ways to study PPIs in any living cell, multicellular organism or *in vitro*. PCAs can be used to detect PPI between proteins of any molecular weight and expressed at their endogenous levels. Proteins are expressed in their appropriate cellular compartments and can undergo any posttranslational modification or degradation that, barring effects of the PCA fragment fusion, they would normally undergo. Applications of PCAs in yeast have been limited until recently, simply because appropriate expression plasmids or cassettes had not been developed. However, we have now developed and reported on several PCAs in *Saccharomyces cerevisiae* that cover the gamut of applications one could envision for studying any aspect of PPIs. Here, we present detailed protocols for large-scale analysis of PPIs with the survival-selection dihydrofolate reductase (DHFR) reporter PCA and a new PCA based on a yeast cytosine deaminase reporter that allows for both survival and death selection. This PCA should prove a powerful way to dissect PPIs. We then present a method to study spatial localization and dynamics of PPIs based on fluorescent protein reporter PCAs and finally, two luciferase reporter PCAs that have proved useful for studies of dynamics of PPIs.

1. Introduction

In the protein-fragment complementation assay (PCA) strategy, protein–protein interactions (PPIs) are measured by fusing each of the proteins of interest to complementary N- or C-terminal peptides of a reporter protein that has been *rationally* dissected using protein engineering strategies (Michnick *et al.*, 2000; Pelletier and Michnick, 1997; Pelletier *et al.*, 1998). The reporter protein fragments are brought into proximity by interaction of the two interacting proteins, allowing them to fold together into the three-dimensional structure of the reporter protein, thus reconstituting the activity of the reporter (Fig. 14.1). PCAs have been created with many different reporter proteins and thus provide for different types of readouts, depending on the desired application. This generality means that PCA is not a single reporter assay, but rather a toolkit. PCAs have also been developed to study spatial and temporal changes in PPIs under different conditions and also survival-selection assays that provide a simple readout for large-scale systematic analyses of protein interaction networks or directed evolution experiments (reviewed in Michnick *et al.*, 2007). Finally, there are two unique features of PCAs we must note: first, by nature of the fact that interactions between two proteins must occur in such a way that the reporter protein can fold, PCAs can provide structural and topological

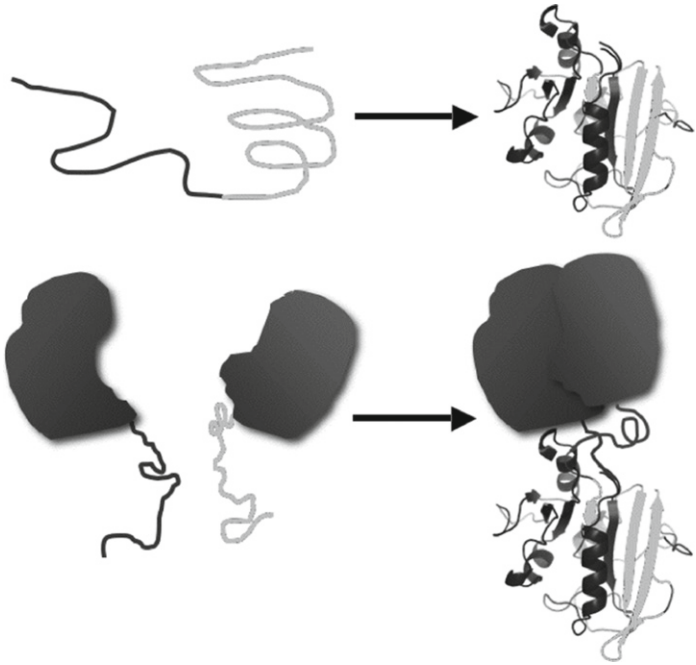

Figure 14.1 Conceptual basis of protein-fragment complementation. The spontaneous unimolecular folding of a protein from its nascent polypeptide (upper) can be made a protein–protein interaction-dependent bimolecular process by fusing two interacting proteins to one or the other complementary N- or C-terminal peptides into which a protein has been dissected (lower). PPI-mediated folding of a reporter protein from its complementary fragments results in reconstitution of reporter protein activity.

details of how a PPI is formed or if such complexes undergo conformation changes under specific conditions (Remy *et al.*, 1999; Tarassov *et al.*, 2008). Second, contrary to intuition, most PCAs are fully reversible, allowing for direct studies of the dynamics of both formation and disruption of PPIs.

2. General Considerations in Using PCA

Measuring PPI in living cells by any method entails that one reconsider any suppositions that we may have about the nature of a PPI, particularly if it has only been studied with *in vitro* methods and most importantly by indirect methods such as affinity or immunopurification. PCAs detect direct binary or indirect proximal interactions between proteins and thus, if it is assumed that there is such an interaction based on experiments that only suggest association of proteins in a complex, it is possible that no interaction

will be detected. Our advice is: ''life is short, experiment.'' However, we can make some general statements about what to consider when setting up any PCA experiment in order to maximize the probability of a successful outcome.

First, we consider the sensitivity of PCAs. Like any analytical technique, the sensitivity of the assay depends on the sensitivity of the detection method and background signal that may arise from cells. Regardless of the properties of the reporters, the range of signal detectable will depend in all cases on the quantity of complexes formed, which in turn is determined by the abundances of the proteins studied and their affinity for each other. We have only explored these parameters in great detail for the dihydrofolate reductase (DHFR) PCA (Section 3). We have demonstrated that for this simple survival-selection assay, the number of complexes needed to support survival under the selection conditions was as low as approximately 25 per cell (Remy and Michnick, 1999) for a complex for which the dissociation constant was in the range of 1 n*M*. We recently showed that we could generalize this result across a proteome, demonstrating that the distribution of detected interactions covered the range of protein abundances down to less than 100 molecules per cell (Tarassov *et al.*, 2008). We have also shown that an upper limit of the dissociation constant for detection of PPI is likely in the range of 10 μM–100 μM for the DHFR (Campbell-Valois *et al.*, 2005) and OyCD (Section 4) PCAs (Ear and Michnick, 2009). These observations suggest that PPI can be detected by PCA within ranges of protein abundances and complex affinities that are commonly observed. However, PPI may or may not bedetected depending on the PCA reporter used. For instance, a PPI studied with a fluorescent protein-PCA reporters (Section 5) might not be detected if the abundance of complexes is lower than necessary to reconstitute enough fluorescent proteins. In this case, signal will not be high enough to overcome background fluorescence of cells in the range of wavelengths over which the fluorophore emits. On the other hand, there are no background issues for luciferase-based PCAs (Section 6) and thus detection is limited only by the sensitivity of the detector used. Finally, an issue of particular importance to studies in yeast where the complementary PCA fragments are fused to gene open reading frames (ORFs) by homologous recombination is whether the genes are hetero- or homozygous for the fusions in diploid cells. In this case, the untagged proteins (A and B) will compete for binding with those that are tagged (A′ and B′), resulting in a reduced number of reconstituted PCA reporter proteins and thus, reporter signal. Only the A′B′ complex (out of the four possible AB, AB′, A′B, and A′B′) results in a reconstituted PCA reporter protein, leading to a fourfold reduction in signal. The number of reconstituted complexes necessary for signal detection in assays performed in diploid cells (Tarassov *et al.*, 2008) is, therefore, much lower than what is expected for the abundances of the interacting partners alone.

A second set of considerations in using PCA is how the fusion of complementary PCA reporter fragments could affect the proteins of interest and the ability to detect PPI. First, as with any fusion construct, it is critical to test the fusions in established functional assays in order to assure that the tags themselves do not impair the function of the protein or lead to gain of function. One should also not assume that a functional fusion protein with a particular tag ensures that other PCA tags will lead to functional fusions. Different tags may have different effects. Second, we can ask if the orientation of fusion (N- or C-terminus) or identity of the fragment may affect the outcome of a PCA experiment. This can only be determined empirically. We have tested all possible combinations and permutations of tagging individual test proteins that are known to interact (8 total per protein pair) and found that in some cases it made no difference how the proteins were tagged while for others, only an individual arrangement worked (unpublished results).

As we described above, PCAs are sensitive to whether the complementary N- and C-terminal fragments can find each other in space and this depends on the distances between the termini of the interacting proteins to which the fragments are fused. To assure that PCA can occur, we typically insert a 10–15 amino acid flexible polypeptide linker consisting of the sequences $(Gly.Gly.Gly.Gly.Ser)_n$ between the proteins of interest and the PCA reporter protein fragments. We chose the $(Gly.Gly.Gly.Gly.Ser)_n$ linker because it is the most flexible possible and we have empirically observed that linkers of these lengths are sufficiently long to allow for fragments to find each other and fold, regardless of the sizes of the interacting proteins to which the fragments are fused (Remy and Michnick, 2001).

3. DHFR PCA Survival-Selection for Large-Scale Analysis of PPIs

The DHFR PCA was previously developed for *Escherichia coli*, plant protoplasts and mammalian cell lines (Pelletier *et al.*, 1998, 1999; Remy and Michnick, 1999; Subramaniam *et al.*, 2001) and has recently been adapted for large-scale screening of PPIs in yeast (Tarassov *et al.*, 2008). The principle of the DHFR PCA survival-selection assay is that cells lacking endogenous DHFR activity, here achieved by inhibiting the *Saccharomyces cerevisiae* scDHFR with methotrexate, are enabled to proliferate by simultaneously expressing PCA fragments of a methotrexate-resistant DHFR mutant that are fused to interacting proteins or peptides. If the proteins interact and thus allow refolding of the DHFR reporter, cells that are grown in the presence of methotrexate can proliferate (Fig. 14.2) (Remy and Michnick, 1999). To adapt the DHFR PCA for high-throughput screening

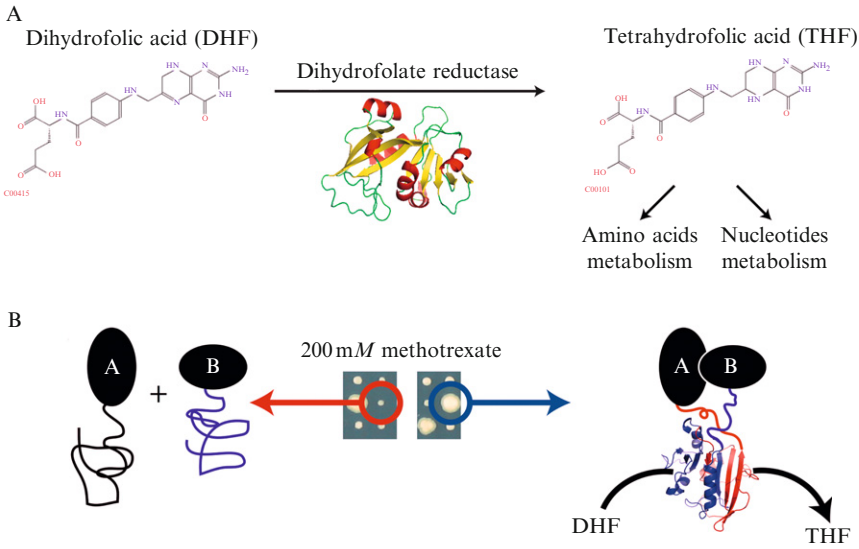

Figure 14.2 (A) DHFR catalyzes the reduction of dihydrofolate to tetrahydrofolate, which is required for nucleotide and in some organisms, amino acid synthesis. This reaction can be inhibited by an antifolate, methotrexate. (B) In the DHFR PCA strategy, the two proteins of interest are fused to complementary fragments of a mutant DHFR that is insensitive to methotrexate. The PCA fragments are inactive in the absence of an interaction. If the proteins interact, the DHFR fragments are brought together in space and fold into the native structure, thus reconstituting the activity of the mutant DHFR and allowing cells to proliferate in the presence of methotrexate.

in *S. cerevisiae,* we created a double mutant (L22F and F31S) that is 10,000 times less sensitive to methotrexate than wild-type scDHFR, while retaining full catalytic activity (Ercikan-Abali *et al.*, 1996). The assay can be used with strains harboring yeast expression vectors of the target protein ORF fused to PCA fragment coding sequence. It is also sensitive enough to be used with genomic recombinant strains, expressing proteins fused PCA fragment under the control of their endogenous promoters. We created two universal oligonucleotide cassettes encoding each complementary DHFR PCA fragment and two unique antibiotic resistance enzymes to allow for selection of haploid strains that have been successfully transformed and recombined with one or the other homologous recombination cassettes (Tarassov *et al.*, 2008). The resulting universal templates were used to create homologous recombination cassettes for most budding yeast genes by PCR using 5′ and 3′ oligonucleotides consisting of 40-nucleotide sequences homologous to the 3′ end of each ORF (prior to the stop codon) and a region approximately 20 nucleotides from the stop codon. Below are protocols to perform DHFR PCA at a large scale with recombinant strains or with yeast transformed with expression plasmids.

3.1. Materials

3.1.1. Reagents

- Glycerol stocks of *MAT***a** recombinant strains in which ORFs are fused to the complementary DHFR PCA F[1,2] fragment (Open Biosystems).
- Glycerol stocks of *MAT*α recombinant strains in which ORFs are fused to the complementary DHFR F[3] PCA fragment (Open Biosystems).
- 3% agar solidified YPD medium in Nunc omniplates.
- 3% agar solidified YPD medium with 100 μg/ml nourseothricin for *MAT***a** recombinant strains (WERNER BioAgents, Jena, Germany) or 250 μg/ml hygromycin B for *MAT*α recombinant strains (Wisent Corporation, Quebec, Canada) in omniplate.
- 3% agar solidified YPD medium with both 100 μg/ml nourseothricin (WERNER BioAgents) and 250 μg/ml hygromycin B (Wisent Corporation) in omniplate.
- 4% noble agar (purified Agar, Bioshop) solidified synthetic complete (SC) medium with 200 μg/ml methotrexate (prepared from a 10-mg/ml methotrexate in DMSO stock solution) in omniplate.

3.1.2. Facultative

Antibodies against DHFR fragments: anti-DHFR polyclonal antibody that specifically recognizes an epitope in the N-terminal F[1,2] fragment (Sigma D1067, 1:6000; Sigma-Aldrich, St. Louis, MO) and an anti-DHFR polyclonal antibody that specifically recognizes an epitope in the C-terminal F[3] fragment (Sigma D0942, 1:5000; Sigma-Aldrich).

3.1.3. Equipment

- *Pintool*: Robotically manipulated (96 pintool) (0.910 mm flat round-shaped pins, AFIX96FP4, V&P Scientific Inc., San Diego, CA), 384 pintool (0.356 mm flat round-shaped pins, custom AFIX384FP8 BMP Multimek FP8N, V&P Scientific Inc.) and a 1536-pintool (0.229 mm flat round-shaped pins, custom AFIX1536FP9 BMP Multimek FP9N, V&P Scientific Inc.) or manually manipulated (96 pintool) (1.58 mm, 1 μl slot pins, 45 mm, VP 408Sa, V&P Scientific Inc.).
- *Plate imaging*: At least a 4.0-mega pixel camera (Powershot A520, Canon), a stationary arm (70 cm mini repro, Industria Fototecnica Firenze, Italy) and a plate-shooting platform.

3.2. Procedure

The general strategy for performing a screen is to generate an array of "prey" strains as indexed colonies grown in a regular grid on agar and then mate them with individual "bait" strains of the opposite mating type to

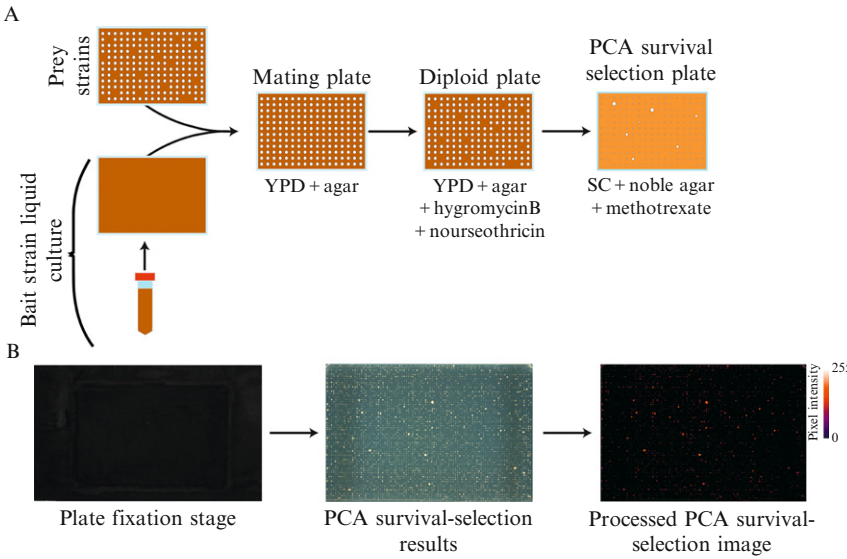

Figure 14.3 (A) The DHFR PCA screen is performed as show in this schematic. The bait reporter strain is incubated in liquid culture. The prey reporter strains are printed on solid medium and incubated to be used on multiple assay plates. The mating plate is produced by sequentially printing the bait strain and the prey strains on sold agar containing rich medium, allowing strains to mate and grow. Resulting haploids and diploid mixture strains are transferred to solid agar plates containing diploid selective medium. The resulting diploid strains can be transferred onto plates containing PCA survival-selection medium (containing methotrexate). (B) The resulting PCA survival selection plate, here a 6144-density plate grown for 2 weeks, can be imaged using a black velvet covered plate fixation platform and a basic digital camera. The image can be processed to remove plate sides, allowing image analysis to be performed only on the region containing colonies and images can be corrected for nonuniform illumination as described in (http://www.mathworks.com/products/image/demos.html?file=/products/demos/shipping/images/ipexrice.html) and small objects, correspond to bubble, gel background and other anomalies can removed using the imopen function. Finally, the integrated pixel density is computed using pixel intensity, represented here as a color- or gray-coded scale, integrated on the area of each colony.

select for diploids and then transfer these to a methotrexate-containing plate for survival selection (Fig. 14.3). The choice of whether to use the *MAT***a** or *MAT*α strains as bait or prey is arbitrary. Here we describe a procedure in which the *MAT***a** strains are bait and *MAT*α are the prey strains. Baits can also be expressed as fusions to DHFR PCA fragments from expression plasmids available in our lab and transformed into appropriate strains.

3.2.1. Experimental procedure

(1) Incubate individual bait strains picked from glycerol stocks into 45 ml liquid culture of strain selective media (YPD with 100 μg/ml nourseothricin for *MAT***a** recombinant strains or 250 μg/ml hygromycin

B for *MAT*α recombinant strains) and allow culture to reach saturation at 30 °C.

(2) Print prey strains picked from glycerol stocks onto a 35-ml agar solidified omniplate of strain selective media (3% agar + YPD with 100 μg/ml nourseothricin for *MAT***a** recombinant strains or 250 μg/ml hygromycin B for *MAT*α recombinant strains) using four 96 manual or robotic pintool prints for a total of 384 prints per plates and incubate 16 h at 30 °C.

Note: For the prey strain, step 2 can be repeated from the 384 prints to be transferred to a maximum of four other 1536 pintool prints per omniplate to achieve a density of up to 6144 colonies.

Critical steps:

- Centrifuge a saturated culture of bait strain at 500 × *g* for 5 min and resuspend in 15 ml of YPD.
- Bait culture must be saturated to print enough cells for efficient mating on solid phase.
- Pintool must be cleaned between each cell transfer. We soak the pins twice in a solution of 10% bleach containing glass beads followed by a 10% bleach wash and two sterile water bath washes.

(3) Transfer bait strain suspension in to an empty omniplate.

(4) Print the bait strain suspension from the empty omniplate to a 35-ml agar solidified rich medium omniplate (YPD + 3% agar) at the same density as the prey strains using a pintool appropriate for the desired colony array density.

(5) Transfer prey strains onto the bait strains on an omniplate containing 35 ml-solid agar containing rich medium (YPD + 3% agar) using the appropriate pintool. Allow mating to occur and incubate for 16 h at 30 °C.

(6) Transfer the mixed haploid and diploid colonies from Step 5 onto an omniplate containing solid agar containing diploid selective medium (3% agar + YPD with both 100 μg/ml nourseothricin and 250 μg/ml hygromycin B) using the appropriate pintool. Incubate for 16 h at 30 °C.

(7) Transfer diploid selected strains onto a solid noble agar solidified synthetic minimal media omniplate with methotrexate (4% noble agar + SC + 2% glucose + 200 μg/ml methotrexate) using an appropriate pintool. Incubate at 30 °C and acquire pictures of the colony array every 96 h for approximately 2 weeks.

3.2.1.2. Timeline

Endogenous recombinant strain screen setup (steps 1–2): 8 h to 3 days (depending on the screen density achieved).

Transferring haploids for mating (steps 3–6): 6 min or more per bait strain (depending on screen density and robotic routine efficiency) + 16 h incubation.

Diploid cell selection (step 7): 5 min or more per bait (depending on screen density and robotic routine efficiency) + 16 h incubation.
DHFR PCA survival selection (step 8): 5 min or more per bait (depending on screen density and robotic routine efficiency) + 2 weeks incubation (maximum).

3.2.1.3. Anticipated results and controls To evaluate a DHFR PCA screen, both positive controls (known PPIs) and negative controls (fragments alone or non-interacting protein partners) should be tested on every plate. These non-interacting protein partner strain colonies exhibit background growth that should stop after a few days of incubation on methotrexate-containing plates. Colonies containing interacting baits and preys will continue to grow. The PCA fragment fusions expressed alone should not result in cell proliferation because the individual PCA fragments have no activity, thus if individual strain colonies do grow for unknown reasons, they should not be considered for further analysis. The most critical controls to do are those for spontaneous PCA; cases where a protein-PCA fragment fusion interacts with the complementary fragment alone. We found in our own screen that about 5% of bait or prey protein-expressing strains would grow in the presence of methotrexate when mated to a strain harboring an expression vector encoding the complementary fragment alone (Tarassov *et al.*, 2008). These complementary DHFR PCA fragment expression vectors are available upon request form our lab. Other controls can be included to test how the PCA screen performs. For instance, we have used the engineered heteromeric $SspB_{YGMF}$: $SspB_{LSLA}$ interaction as a positive control to validate DHFR PCA activity as suggested in the troubleshooting section (Table 14.1). Another elegant control to examine the range of dissociation constants for which the DHFR PCA is sensitive is to use a complex for which single-point mutations are known by other methods disrupt the interaction to different degrees. To this end, we have used in our own work, mutants of the Ras binding domain of Raf (Campbell-Valois *et al.*, 2005). A potential source of false positives in a PCA screen could be through trapping of nonspecific complexes due to irreversible folding of the DHFR fragments. However, we have used the adenosine 3′,5′-monophosphate dependent dissociation of the yeast protein kinase A complex as a control (Stefan *et al.*, 2007) to show that the DHFR PCA is fully reversible, and thus the trapping of complexes is unlikely (Tarassov *et al.*, 2008). Another control one could use is a condition-dependent PPI. We have used in our own work, the FK506-binding protein that binds to rapamycin and this complex then binds the target of rapamycin (TOR) (Pelletier *et al.*, 1998). All of these reagents are available upon request.

Table 14.1 Trouble shooting large-scale DHFR PCA screen

Step	Problem	Possible reason	Solution
1–2	Strains are not growing or incomplete prey array growth	Erroneous haploid selection	Verify protocol for appropriate culture conditions
		Low glycerol viability	Strains can be streaked on solid agar-selective medium Petri dishes prior to inoculation to increase viability
		Technical problem	Verify that all pins of the pintool touch glycerol stocks and the recipient omniplate
7	Low number or no colonies on diploid selective plates	Erroneous haploid strains type	Verify mating type of haploid strains
		Technical problem	Pintool alignment might have changed. No modifications to the pintool positioning should be done between transfers
8	No colony growth on DHFR PCA survival-selective medium	Erroneous selective conditions	Use heteromeric complex $SspB_{YGMF}$:$SspB_{LSLA}$ as a positive control to validate DHFR PCA activity
		Erroneous DHFR PCA	Verify by a strain diagnostic PCR the complementarity of PCA fragments
			Verify DHFR PCA fragment recombinant insertion by genomic sequencing
		DHFR PCA fragment expression	Verify DHFR PCA fragment expression by western blot
	All colonies grow at the same rate on DHFR PCA survival-selective medium	Erroneous selective conditions	Use DHFR PCA fragment controls alone as negative control
		Methotrexate solubility	Verify methotrexate solubility under conditions used. Stock solution should not exceed 10 mg/ml in DMSO and final concentration in solid agar plates should not exceed 200 mg/ml

3.2.2. Analysis of large-scale DHFR PCA screens

The goal of this section is to turn the size of the colonies on the selection plate into binary data that will represent PPIs. First, the digital images have to be transformed into tables containing colony intensities. Second, these colony intensities have to be turned into PPI confidence scores.

3.2.2.1. Image analysis Several bioinformatics tools are available to perform colony size measurements from digital images of high colony density plates (Carpenter *et al.*, 2006; Collins *et al.*, 2006; Memarian *et al.*, 2007). Alternatively, tools developed for analysis of spotted DNA microarrays can be modified to estimate the sizes of the colonies spaced on regular grids (Dudley *et al.*, 2005). Globally, the analysis consists of measuring the number of pixels per colony position. In cases where high density plates are used (above 1536 position grid), more involved analyses methods have to be utilized to separate adjacent colonies that may touch each other (Tarassov *et al.*, 2008). However, because PPIs are rare, most colonies will have a very slow growth rate and this problem is mostly negligible at lower densities. Thus, when lower densities are used for the screens, simple macros can be implemented in publicly accessible image analysis software such as ImageJ (http://rsb.info.nih.gov/ij/). In this case, digital images of plates are first converted to 8-bit grayscale format and colonies are measured by positioning the measurement tool on a colony center and estimating the integrated pixel intensity in an area that corresponds to the maximal colony size allowed. The process is iterated over all the grid positions and then all the plates, and the grid positions and intensity values are exported to text files for further processing in your favorite spreadsheet or statistical analysis software (Example ImageJ scripts that we use are available at our web site: http://michnick.bcm.umontreal.ca). It is important to note that colonies should always have the same positions on the images. If this is not the case, some of the tools cited above include a step that positions the analysis grid onto the colony positions prior to colony size measurements.

3.2.2.2. Statistical analysis of raw colony data: From continuous to binary data A PCA screen based on survival assay will only be useful if there is a confidence score attached to each of the putative interactions. Raw colony intensity data are continuously distributed, that is, they cover a wide range of values and cannot be directly turned into ''yes'' or ''no'' binary scores. Further, not all the colonies that can grow due to protein-fragment complementation will do so at exactly the same rate. As described above, every PCA experiment should include a set of positive controls consisting of pairs of baits and preys that interact with each other, and negative controls, consisting of pairs or baits and preys that do not interact with each other. These will be used for quality control in order to detect mis-positioning of

the grid and batch effects (variation in media, incubation, drug concentration) that affect global growth rate of the different plates. Finally, the positive controls can provide a first, visual analysis of the data, whereby the growth rate of the positive controls indicate roughly the intensity threshold above which we expect strains with interacting bait–prey pairs to grow. Beyond these ''qualitative'' controls, a statistical analysis should be used to separate the interacting pairs from the noninteracting pairs.

The statistical analysis globally includes two steps. First, it has to be determined whether there is a significant difference in growth rates among the plates before applying a global analysis to the data. If there is significant variation, the data should be normalized such that all the plates have the same average colony size. Alternatively, data could be transformed into relative scores, such as Z-scores, whereby each data point is transformed to become the number of standard deviations that data point is from the average of the plate. We found that combining the Z-score and the raw intensity worked best for our large-scale screen (Tarassov *et al.*, 2008). Then continuous values must be turned into binary values by setting a threshold of intensity above which proteins are inferred to interact, and establishing a confidence score for this particular threshold. One way to assign confidence values to PCA interactions is to benchmark the intensity values against a set of data containing interactions that should be detected in the screen (a set of real positives) and others that should not (a set of real negatives). The real positives set can be derived from a set of known and well-supported interactions. The real negative set has however to be approximated because it is impossible to show that two proteins never interact. Sets of proteins that are most likely not interacting can be used for this purpose, for instance proteins that are not localized in the same cell compartments and that have negatively correlated expression profiles (Collins *et al.*, 2007). One can then predict, for a given intensity threshold, what should be the proportion of true positive interactions and false positive interactions. In order to decide on the threshold, the ratio of true positive interactions divided by to the total number of inferred positives (true positives + predicted false positives)—known as the positive predictive value (PPV)—is calculated as a function of threshold of intensities. For instance, at a PPV of 95% percent, one expects 5% of positives to be false. Lower and higher thresholds can be used depending on how stringent one wants the analysis to be. It is important to note that the estimated PPV is only accurate if the relative occurrence of positives and negatives in the reference sets is similar to that of the real positives and negatives (Jansen and Gerstein, 2004). In the case of a genome-wide, comprehensive screen, this fraction corresponds to a very low prior probability of finding interactions among all pairwise possibilities. On the other hand, a small-scale screen of a specific biological process will contain a greater proportion of real positives than a random screen. The reference set therefore needs to be tailored for the actual screen being

performed, that is, the space of the interactome that is covered. For a formal treatment of these issues, refer to Jansen and Gerstein (2004). Beyond these statistical considerations, analysis such as Gene Ontology enrichment and visualization of interaction clusters should be used to further assess the confidence in the data set being produced. For instance, the matrix of binary interactions can be clustered to identify groups or complexes of interacting proteins. Finally, sets of true positives and negatives are not a panacea and the functional and evolutionary characterization of PPIs is the only way to provide a definitive answer as to whether an interaction is functionally relevant or not for the cell (Levy *et al.*, 2009).

4. A Life and Death Selection PCA Based on the Prodrug-Converting Cytosine Deaminase for Dissection of PPIs

In this section, we present a PCA based on an optimized mutant form of the reporter enzyme yeast cytosine deaminase (OyCD). The choice of yCD as a reporter was based on its role in a pyrimidine salvage pathway and the availability of a prodrug 5-fluorocytosine (5-FC), which is converted to 5-fluorouracil (5-FU) by yCD. Bacteria and yeast can convert cytosine to uracil and use it for the synthesis of UTP and TTP, which are required for cell survival (Kurtz *et al.*, 1999). In *S. cerevisiae*, yCD is encoded by the *FCY1* gene and is the enzyme that catalyzes this reaction. In addition to deaminating cytosine, yCD can also deaminates 5-FC to 5-FU. 5-FU will be further processed by enzymes of the pyrimidine salvage pathway to 5-FUTP, a toxic compound that causes cell death. These particular properties of yCD make it an ideal reporter for a life and death selection PCA (Fig. 14.4A) (Ear and Michnick, 2009).

The OyCD PCA allows death and survival assay to be performed without changing the reporter system. In a two-step selection process, we can engineer mutant forms of a protein in order to dissect its different functions; disrupting interactions with one partner, while retaining interaction with others (Fig. 14.4B). For example, protein A interacts with both protein B and protein C. First, we can screen for mutant forms of protein A that disrupt interaction with protein B. Second, we select for protein A mutants that still interact with protein C. Using OyCD PCA, neither of these selection steps requires replica plating. In addition, no expensive reagent or equipment is required. Specific mutants can be obtained in about 4 weeks.

Both the survival and death selection assays are performed in *fcy1* deletion strains. For the survival selection assay, uracil must be removed from the selection medium. Only cells that have OyCD PCA activity will be able to synthesize uracil and survive. For the death selection assay, cells

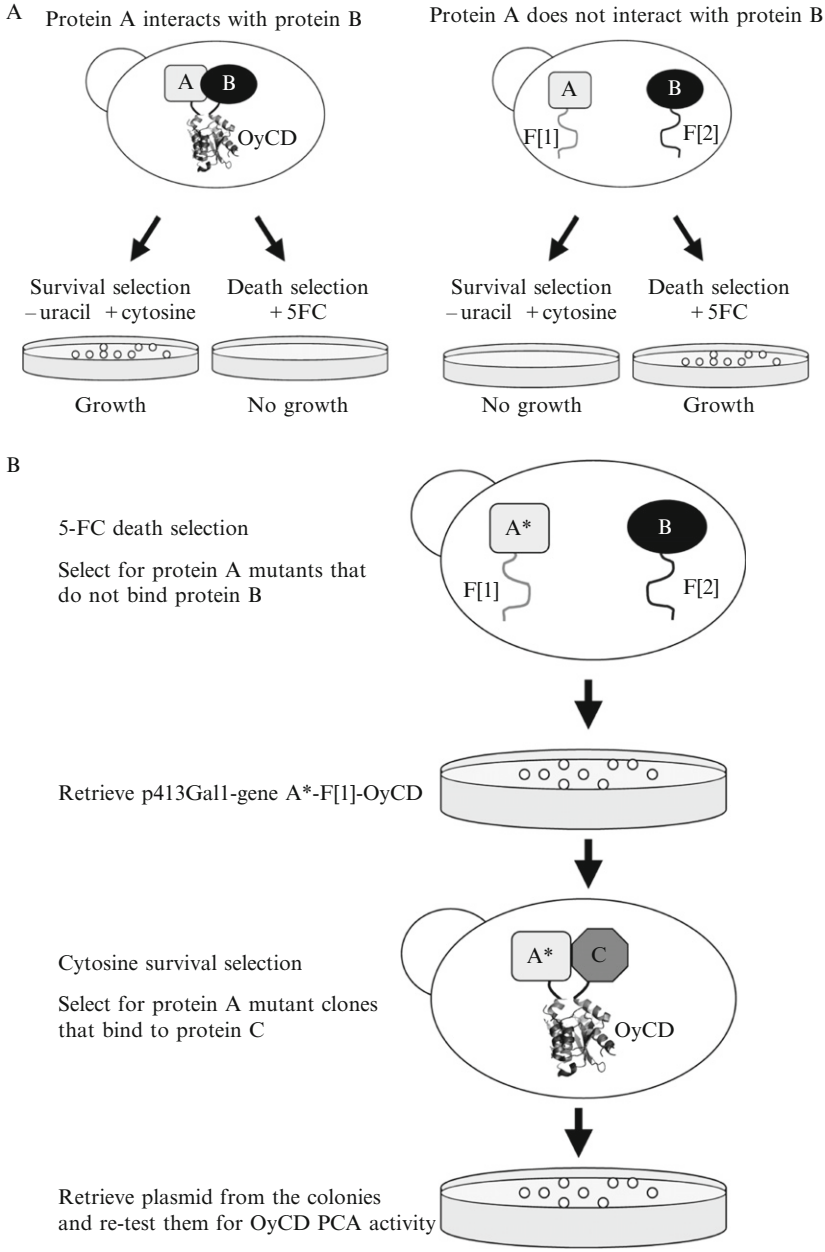

Figure 14.4 (A) A dual selection PCA. The OyCD PCA can serve as a reporter for formation of a protein–protein interaction provided that the reconstituted reporter enzyme supports growth under one condition (survival assay) or no growth under another condition (death assay). In the case where the two test proteins do not interact,

are grown in a selection medium in the presence of 5-FC. In this death assay, cells that have OyCD PCA activity will be sensitive to 5-FC.

4.1. Preparation for a two-step OyCD PCA screen

The proteins of interest are fused to the N-terminal of OyCD fragment 1 or fragment 2 (protein A-OyCD-F[1], protein B-OyCD-F[2] and protein C-OyCD-F[2]). For some proteins, PPIs can only be detected when they are fused at the C-terminal of OyCD fragment 1 (e.g., OyCD-F[1]-protein A). OyCD-F[1] corresponds to amino acid residues 1–77 of yCD with an A23L point mutation. OyCD-F[2] corresponds to amino acid residues 57–158 of yCD with the following point mutations: V108I, I140L, T95S, and K117E (Ear and Michnick, 2009). The proteins of interest and OyCD fragments are separated by a 15-amino acid flexible polypeptide linker (Gly.Gly.Gly.Ser)$_3$. The nucleotide sequences encoding these fusion proteins are cloned into yeast expression vectors. We used the p413Gal1 and p415Gal1 expression plasmids (Mumberg *et al.*, 1995). Before proceeding with a screen, verify that interactions of your proteins of interest are detected by OyCD PCA. Titrate the amount of cytosine and 5-FC required for detecting your interactions. We normally try a range between 50 and 1000 μg/ml of cytosine or 5-FC. For many proteins, 100 μg/ml of either substrate is sufficient for detecting OyCD PCA activity. Use well known interacting and non-interacting proteins as controls. For the two-step OyCD PCA screen, a library of your gene of interest can be generated by methods such as error-prone PCR. For example, if the goal is to engineer mutant forms of protein A that can specifically disrupt interaction with protein B while preserving interaction with protein C, an ideal library of protein A carrying 1–3 mutations is desired.

4.2. Materials

4.2.1. Reagents

- BY4741, BY4742, or BY4743 strains with a deletion in the *FCY1* gene (*fcy1*Δ) (Giaever *et al.*, 2002) that are resistant to G418.

the reverse scenarios are observed. (B) Screen for mutants of protein A that do not bind to protein B but retain binding to protein C using sequential death followed by survival selection OyCD PCA. The first death selection screen consists of screening the library of protein A mutants fused to OyCD-F[1] (A*-F[1]) with protein B fused to OyCD-F[2] (B-F[2]) and screen for clones that show loss of OyCD PCA activity (growth in the presence of 5-FC). The second survival selection step consists of screening A*-F[1] clones harvested from the first death selection screen against protein C fused to OyCD-F[2] (C-F[2]) for clones that show OyCD PCA activity using the life assay (growth in presence of cytosine).

- SC medium with the appropriate amino acid drop out according to the chosen expression plasmids.
- Your genes of interest fused to the OyCD fragments in yeast expression vectors.
- Sorbitol buffer (1 *M* sorbitol, 1 m*M* EDTA, 10 m*M* Tris (pH 8.0), 100 m*M* lithium acetate)
- PLATE solution (40% PEG 3350, 100 m*M* lithium acetate, 10 m*M* Tris (pH 7.5), and 0.4 m*M* EDTA)
- Dimethylsulfoxide (Fisher)
- Sterile distilled water
- G418 (Wisent)
- Cytosine (Sigma)
- 5-Fluorocytosine (Sigma)
- Agar (Bioshop)
- Noble agar (Bioshop)
- DH5α or MC1061 *E. coli* electrocompetent cells
- LB medium
- DNeasy Tissue Kit (Qiagen)

4.2.2. Facultative

- Antibodies against yCD fragments: anti-yCD polyclonal (Biogenesis).

4.2.3. Equipment

- Genepulser II electroporator system (Bio-Rad) or Electroporator 2510 (Eppendorf)
- Electroporation cuvette with 1 mm wide slot (Sigma)
- Glass spreader
- 100 mm Petri dishes
- Shaking incubators, preset to 30 and 37 °C
- Incubator, preset to 30 and 37 °C

4.2.4. Experiment preparation

10 mg/ml stock solution of cytosine: Dissolve 100 mg of cytosine in 10 ml of distilled water. Vortex the solution and incubate at 37 °C to make it dissolve. Filter the solution and store at room temperature. It is better to make this solution fresh and use it within a week.

10 mg/ml stock solution of 5-FC: Dissolve 100 mg of 5-FC in 10 ml of distilled water. Vortex the solution and incubate at 37 °C to make it dissolve. Filter the solution and use it right away or aliquot in sterile tubes and store at −20 °C.

Control plates: Make SC plates for selection of clones harboring the expression plasmids. We used the p413Gal1 and p415Gal1 expression vectors, therefore, our control plates contain SC medium without histidine and leucine, with 2% agar, 2% raffinose, and 2% galactose.

Cytosine survival selection plate: Make SC plates without uracil and selection for the expression plasmids. We used the p413Gal1 and p415Gal1 expression vectors, therefore our selection plates contain SC medium without uracil, histidine, and leucine, with 3% noble agar, 2% raffinose, 2% galactose, and cytosine (we use 100 μg/ml of 5-FC for our proteins of interest).

5-FC death selection plates: Make SC plates with 5-FC and selection for the expression plasmids. We used the p413Gal1 and p415Gal1 expression vectors, therefore our selection plates contain SC medium without histidine and leucine, with 2% noble agar, 2% raffinose, 2% galactose, and 5-FC (we use 100 μg/ml of 5-FC for our proteins of interest).

4.3. Procedure

4.3.1. Death selection screen

(1) Transform (Knop *et al.*, 1999) 1 μg of the library encoding mutant forms of protein A (protein A*) in BY4741 *fcy1*Δ strain that already carry a plasmid expressing protein B (Fig. 14.4B).
Critical step: Make sure that the efficiency of the transformation gives enough colonies to cover six times the size of the library in order to have good coverage of potential mutants. For example, if the size of the library of protein A* is 5000 clones, make sure to obtain more than 30,000 clones.

(2) Plate half of the transformation on the control plates to select for the presence of both expression plasmids (p413Gal1-gene A*-OyCD-F[1] + p415Gal1-gene B-OyCD-F[2]). These plates serve as controls for reporting the efficiency of the transformation. Plate the other half of the transformation on 5-FC death selection plates.
Critical step: Test the efficiency of your competent yeast cells to determine how many cells to plate per 100 mm Petri dish. Do not plate more than 5000 cells per 100 mm Petri dish.
Pause point: Make glycerol stock of the pooled yeast colonies obtained on the control plates as a backup source or for future screens if required.

(3) Incubate plates at 30 °C for 2–3 days. Compare the number of colonies obtained on the 5-FC death selection plates to the control plates.
Critical step: We should expect 10–50% less colonies on the 5-FC death selection plates in comparison to the control plates. This variability depends on the pair of interaction chosen and the number of mutations per clone in the library.

(4) Colonies that grow on 5-FC selection plates are pooled and harvested for DNA extraction (Qiagen DNeasy Tissue Kit or a genomic DNA purification protocol using phenol-choroform) in order to recover the plasmids that express protein A★.
Pause point: Yeast cell pellet can be store at −20 °C for months.

(5) Digest the extracted DNA with enzyme(s) that cut in the plasmids expressing protein B-OyCD-F[2] but not the plasmids expressing protein A★-OyCD-F[1] library. We use AflII, BspmI, HpaI, MunI, NarI, or XcmI since they cut in p415Gal1 and not in p413Gal1 plasmid or the gene of interest. This step is not required if the two expression plasmids do not have the same antibiotic resistance gene.

(6) Use 2 μl of extracted DNA for electroporation into electrocompetent *E. coli* cells. We use the MC1061 *E. coli* strain since it has higher transformation efficiency than the DH5α strain. Plate the *E. coli* on LB plates with appropriate antibiotic selection. We use LB with ampicillin for the p41XGal1 plasmids.

(7) Pool *E. coli* colonies and extract the plasmid DNA using your miniprep kit of choice.
Pause point: *E. coli* cell pellet can be store at −20 °C for months.

4.3.2. Survival selection screen

(8) Transform according to Knop *et al.* (1999) the library encoding for mutant forms of protein A (protein A★) retrieved after the death selection screen in BY4741 *fcy1*Δ strain that already carry a plasmid expressing protein C (Fig. 14.4B).
Critical step: Make sure that the efficiency of the transformation gives enough colonies to cover six times the size of the library in order to have a good coverage of potential mutant clones.

(9) Plate half of the transformation on the control plates to select for the presence of both expression plasmids (p413Gal1-gene A★-OyCD-F[1] + p415Gal1-gene C-OyCD-F[2]). These plates serve as control for reporting the efficiency of the transformation. Plate the other half of the transformation on cytosine survival-selection plates.
Critical step: Test the efficiency of your competent yeast cells to have an idea how much cells to plate per 100 mm Petri dish. Do not plate more than 2000 cells per 100 mm Petri dish.
Pause point: Make glycerol stock of the pooled yeast colonies obtained on the control plates as a backup source or for future screens if required.

(10) Incubate plates at 30 °C for 3–7 days.
Critical step: We can expect to obtain from a few to hundreds of colonies at this step. This variability depends mostly on the pair of interaction that was chosen and the complexity of the library.

(11) If the screen resulted in less than 50 colonies, inoculate each yeast colony separately in 5 ml of selection medium and harvest cells for DNA extraction (Qiagen DNeasy Tissue Kit or a genomic DNA purification protocol using phenol-choroform). If over 50 colonies were obtained, pooled all the colonies and extract DNA from the pooled cells.
Pause point: Make glycerol stock of the single or pooled yeast colonies as a backup source.

(12) Digest the extracted DNA with enzyme(s) that cut in the plasmid expressing protein C-OyCD-F[2] but not the protein A*-OyCD-F[1] library. We use AflII, BspmI, HpaI, MunI, NarI, or XcmI since they cut in p415Gal1 and not in p413Gal1 plasmid or the gene of interest. This step is not required if the two expression plasmids do not have the same antibiotic resistance gene.

(13) Use 2 μl of extracted DNA for electroporation into electrocompetent MC1061 *E. coli* cells. Plate the *E. coli* on LB plates with the appropriate antibiotic selection.

(14) For samples obtained from a single yeast colony in step 11, inoculate one or two *E. coli* colonies for plasmid DNA extraction. For samples obtained from pooled yeast colonies in step 11, inoculate over 90 *E. coli* colonies for plasmid DNA extraction.
Pause point: Make glycerol stock of the single or pooled bacterial colonies as a backup source.

(15) Digest the isolated plasmids with appropriate restriction enzymes or perform diagnostic PCR to confirm the presence of gene A*-OyCD-F[1].

(16) Retransform individually the purified plasmids expressing protein A mutants in BY4741 *fcy1*Δ strain carrying a plasmid expressing protein B and C, respectively, and test for OyCD PCA activity.

(17) Send the purified plasmids expressing protein A mutants for sequencing in order to identify the mutation(s).

4.3.3. Timeline

5-FC death selection (steps 1–3): 2–3 days.
Cytosine survival selection (steps 8–10): 3–7 days.
Isolation of DNA from yeast (steps 4 and 11): almost 1 day.
Further characterization of the individual clones (steps 14–17): several days depending on the number of clones obtained.
Troubleshooting advice can be found in Table 14.2.

4.3.4. Additional information

We have also generated destination vectors carrying the OyCD fragments that are compatible with the Gateway cloning system. With these plasmids, we can take advantage of the existing Gateway expression clones

Table 14.2 Troubleshooting an OyCD PCA screen

Step	Problem	Possible reason	Solution
3	Less than 10% of colonies died on the 5-FC selection plates	Too many yeast plated on the selection plate	Plate less than 1000 cells per 100 mm Petri dish Increase 5-FC concentration
6 and 13	No *E. coli* colonies or very few colonies	Electrocompetent *E. coli* cells not very competent	Use freshly prepare electrocompetent MC1061 *E. coli* cells
10	Several hundreds of colonies grew on the cytosine selection plates Small colonies form around the initial large colony after 4 days of incubation	Too many yeast plated on the selection plate Uracil can diffuse out of cells that have OyCD PCA activity and allow for cells that do not have OyCD PCA activity to grow	Plate less than 1000 cells per 100 mm Petri dish Decrease cytosine concentration Pick only the large colony at the center

(distributed by Open Biosystems) to facilitate the process of generating the fusion between the genes of interest and OyCD fragments.

5. Visualizing the Localization of PPIs with GFP Family Fluorescent Protein PCAs

The first fluorescent protein PCA was described by Lynne Regan's group for GFP (Ghosh *et al.*, 2000; Magliery *et al.*, 2005; Wilson *et al.*, 2004) and we and others have described different color and behavioral variants (Cabantous *et al.*, 2005; Hu *et al.*, 2002; Macdonald *et al.*, 2006; Nyfeler *et al.*, 2005; Remy and Michnick, 2004; Remy *et al.*, 2004). PCAs based on fluorescent proteins have both unique features, but also the most caveats to their application. Notably, and unlike other PCAs, those based on these fluorescent proteins are irreversible, which can be both useful (trapping and visualizing rare and transient complexes) but also require care in interpretation of turnover or localization of interacting proteins (Hu *et al.*, 2002; Magliery *et al.*, 2005). It is important that the kinetics of relocalization of protein interactions observed with fluorescence PCAs be confirmed by immunofluorescence or by monitoring the localization of the same proteins fused to full-length fluorescent proteins. Fluorescent protein PCAs are also limited to the temporal range of dynamics that can be studied. Because different variants of these proteins take minutes to hours to fold and mature, they are obviously not appropriate for studying most dynamic processes in a quantitative way, though many important slower processes can be studied. PCAs based on luciferase enzyme reporters are, like the DHFR PCA, fully reversible and can be used to capture kinetics on the second time scale (see Section 6) (Remy and Michnick, 2006; Stefan *et al.*, 2007). As we previously demonstrated, PPIs that occur within a specific biochemical pathway can be modulated in predicted ways by conditions or molecules that activate or inhibit the pathway. We and others have shown that at least changes in the formation of complexes can be detected with the GFP and YFP PCAs (Remy and Michnick, 2004). Further, the subcellular location of stable complexes and changes in their locations following perturbation can also be detected in intact living cells with the YFP PCA (Macdonald *et al.*, 2006; Remy and Michnick, 2004; Remy *et al.*, 2004). It is this ability to detect the location and intracellular movements of protein complexes that make fluorescent protein-based PCAs unique. Because GFP/YFP-based PCAs do not require additional substrates or cofactors for emission of fluorescence, they are particularly simple to implement. We have shown that PPIs can be monitored by fluorescence microscopy, flow cytometry, and spectroscopy using GFP- and YFP-based PCAs (Macdonald *et al.*, 2006; Remy and Michnick, 2004; Remy *et al.*, 2004). We have applied these assays to the

detection and quantification of protein interactions, localization of complexes in living cells, and cDNA library screening in mammalian cells (Benton *et al.*, 2006; Ding *et al.*, 2006; Macdonald *et al.*, 2006; Nyfeler *et al.*, 2008; Remy and Michnick, 2004; Remy *et al.*, 2004). In addition, we have used the YFP-based PCA to detect protein interactions in specific subcellular compartments of *S. cerevisiae*, such as cytoplasm, nucleus, plasma membrane and the bud neck (Fig. 14.5) (Manderson *et al.*, 2008). In the following protocol we describe methods for studying PPI with the "Venus" mutant of YFP (Nagai *et al.*, 2002).

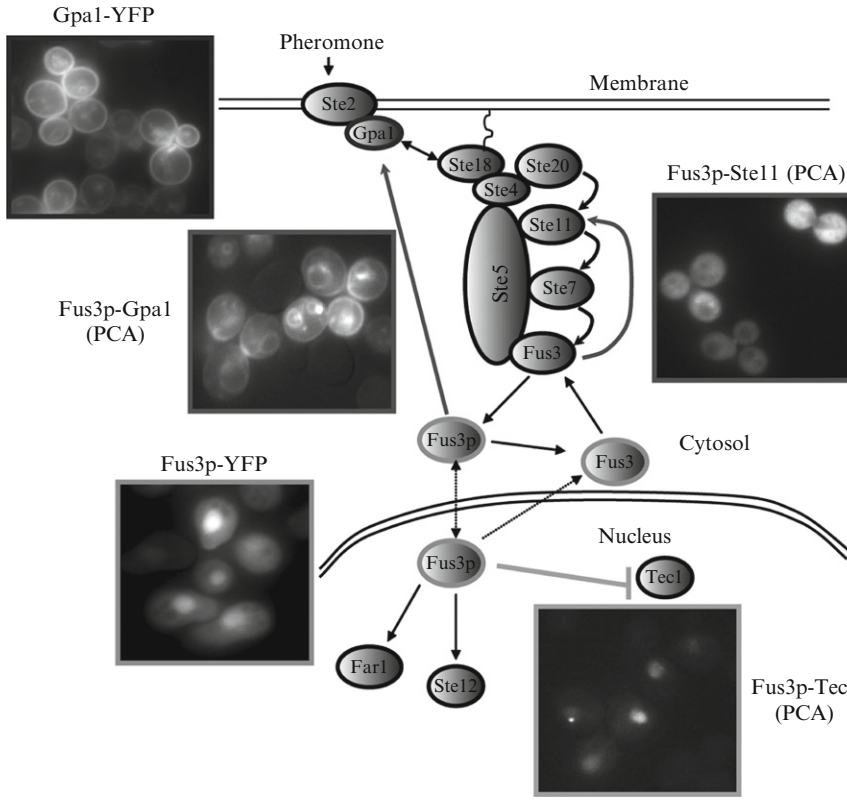

Figure 14.5 Venus YFP PCA allows for detection of precise location of protein complexes within living cells. Illustration for visualization of protein complexes in different regions within cells using yeast pheromone response mitogen activated protein kinase pathway as an example. Images show the location of interactions of Fus3p with Gpa1 (Metodiev *et al.*, 2002) to the membrane, with Ste11 (Choi *et al.*, 1994) to the cytoplasm and with Tec1 (Chou *et al.*, 2004) to the nucleus. As controls for different localizations, Gpa1 fused to full-length Venus YFP protein is shown to be at the membrane while Fus3-Venus YFP is found in both cytoplasm and the nucleus. Cells containing Fus3-venus YFP were treated with 1 μ*M* alpha-factor pheromone for 2–3 h to induce its translocation to membrane and nucleus.

5.1. Materials

5.1.1. Reagents

- Competent *MAT**a*** or diploid yeast prepared according to Knop *et al.* (1999).
- SD medium (6.7 g/l yeast nitrogen base, without amino acids)
- SD agar (SD medium with 2% agar)
- 10× amino acid mix -his, -leu, -lys:

Adenine sulfate	0.4 g/ml
Uracil	0.2 g/ml
L-Tryptophan	0.4 g/ml
L-Arginine HCl	0.2 g/ml
L-Tyrosine	0.3 g/ml
L-Phenylalanine	0.5 g/ml
L-Glutamic acid	1.0 g/ml
L-Asparagine	1.0 g/ml
L-Valine	1.5 g/ml
L-Threonine	2.0 g/ml
L-Serine	3.75 g/ml
Methionine	0.2 g/ml (do not include when growing diploid yeast)

- 10× low fluorescence yeast nitrogen base without riboflavin and folic acid (Sheff and Thorn, 2004)
- 20% glucose solution
- PLATE solution (40% polyethylene glycol 3350, 100 m*M* LiOAc, 10 m*M* Tris (pH 7.5), 0.4 m*M* EDTA)
- DMSO
- poly-L-lysine mol. wt. 30,000–70,000 (Sigma P2636) or concanavalin A (Sigma L7647)

5.1.2. Equipment

- Fluorescence microscope (Nikon Eclipse TE2000U inverted microscope with a CoolSnap HQ Monochrome CCD camera (Photometrics))
- 96-well black, glass-bottom plate (Molecular Machines)
- 6-well culture plate or Petri dish
- Appropriate sterile tubes to grow yeast
- Spectrophotometer spectra MAX GEMINI XS (Molecular Devices)

5.1.3. Plasmids

The proteins to test for interaction are fused to the N- and C-terminal fragments of an enhanced YFP (Venus YFP; Nagai *et al.*, 2002), in 5′ or 3′ of the fragments (protein A-vYFP-F[1], vYFP-F[1]-protein A, protein B-vYFP-F[1], vYFP-F[1]-protein B). vYFP-F[1] (N-terminal) corresponds to amino acids 1–158, and vYFP-F[2] (C-terminal) corresponds to amino acids 159–239 of Venus YFP. The fusions are subcloned into yeast expression vectors p413ADH for the vYFP-F[1] fusion and p415ADH for the vYFP-F [2] fusion (Mumberg *et al.*, 1995). We typically insert a 10-amino acid flexible polypeptide linker consisting of $(Gly.Gly.Gly.Gly.Ser)_2$ between the protein of interest and the vYFP fragments (Table 14.3). In yeast fragments can also be fused to the genes of interest at their chromosomal loci using a homologous recombination method (Ghaemmaghami *et al.*, 2003). For this purpose the PCA fragments are cloned into nonexpression vectors that provide a selection marker (e.g., antibiotic resistance).

5.2. Procedure

5.2.1. Cotransformation of competent yeast

(1) Thaw competent yeast cells on ice.
(2) Mix 10 μl of cells with 1 μl (~250 ng) of each yeast expression plasmid (e.g., p413ADH and p415ADH, Mumberg *et al.*, 1995) encoding the Venus YFP PCA fusion partners (protein A fused to vYFP-F[1] and protein B fused to vYFP-F[2]), 60 μl of PLATE solution and 8 μl DMSO.
(3) Heat shock yeast at 42 °C for 20 min.

Table 14.3 Troubleshooting vYFP PCA experiments

Step	Problem	Possible reason	Solution
5.2.1. (6)	No colonies after transformation	DNA or cells used is less	Increase quantity of cells and DNA. Increase the volume of cells plated on the Petri dish or six-well plate
	Too many colonies after transformation	Plated lot of cells	Dilute cells before plating on the Petri dish or six-well plate
5.2.2. (3)	Fusion protein is not functioning correctly	Fragment fusion interferes with protein expression/ function/stability	Fuse the PCA fragment to the other end of the protein

Critical step: Shorter or longer incubation times at higher or lower temperatures can result in decreased efficiency of transformation.

(4) Centrifuge at 2000 rpm for 3 min. Remove supernatant and resuspend cells in 500 μl SD medium without amino acids or glucose.

(5) Plate 20 μl of cell suspension per well on SC agar (SD agar + 2% glucose + 1× amino acids (-his, -leu, -lys for *MAT**a***; -his, -leu, -lys, -met for diploids)) in a 6-well plate.

(6) Incubate at 30 °C for 48–72h.

5.2.2. Preparation of cells for fluorescence microscopy

(1) Inoculate a fresh colony for each sample into 3 ml of SC medium (SD medium + 2% glucose + 1× amino acids (–his, -leu, -lys for *MAT**a***; -his, -leu, -lys, -met for diploids)) and grow overnight at 30 °C with shaking.

(2) The following day, measure the OD_{600} of the overnight culture and inoculate a fresh culture of LFM (1× low fluorescence yeast nitrogen base + 2% glucose + 1× amino acids (-his, -leu, -lys for Mat A; -his, -leu, -lys, -met for diploids)) with enough cells to obtain an OD_{600} of approximately 0.1–0.3 at the time of analysis.

Critical step: It is particularly important for the cells to be in the log phase of growth in order to avoid including dead and unhealthy cells. These cells are highly autofluorescent and thus would confound quantitative analysis. Cells in the lag phase can be used if they are appropriate to study a particular interaction(s) as long as the condition of the cells is verified by bright field microscopy.

(3) Coat the wells of a glass bottom 96-well plate (Molecular Machines) with a solution of 1 mg/ml poly-L-lysine, or 50 μg/ml concanavalin A for 10 min, rinse with distilled water and allow to dry. Transfer 70 μl of cell suspension to each well. Wait 10 min to allow the cells to settle in the wells. Acquire images with a fluorescence microscope equipped with a CCD camera, using a YFP filter cube and ~750 ms of exposure time.

Critical step: It is best to use a 60× or 100× objective to discriminate subcellular structures. Bright field or phase contrast images can be acquired for each field of view to compare the morphology of the yeast with fluorescent PCA signal. Specific functional assays to further characterize a PPI might be performed here.

5.2.3. Timeline

Cotransformation of competent yeast (steps 1–5): 30–45 min (depending on the number of samples) plus 48–72 h for cell growth (step 6).

Preparation of cells for fluorometric analysis (steps 1 and 2): 24 h.

Fluorescence microscopy (step 3): 30 min to hours, depending on the number of samples.
Microplate reader analysis (step 3): a few minutes or more, depending on the number of samples.

5.2.4. Anticipated results

The fluorescence intensity of the reassembled Venus YFP PCA varies with the expression levels and the interaction dissociation constants for the protein pairs attached to the PCA fragments. In the case of our simplest positive control (GCN4 leucine zipper pair fused to the PCA fragments: Zip-vYFP-F[1] + Zip-vYFP-F[2]), the reconstituted PCAs represent approximately 10–20% of the activity of the full-length Venus YFP. The PCA fusions expressed alone should not result in detectable fluorescence (compared to nontransformed cells) because the individual PCA fragments have no activity. For each study, positive (known interaction) and particularly negative (noninteracting proteins) controls should always be performed in parallel. A PCA response should not be observed if non-interacting proteins are used as PCA partners.

6. Studying Dynamics of PPIs with Luciferase Reporter PCAs

It has been a major challenge to measure and quantify the dynamics of protein complexes in their native state within living cells. Here, we describe protocols for implementing two luciferase enzyme based PCAs; *Renilla* luciferase (*R*luc) and *Gaussia* luciferase (Gluc) that are designed specifically to investigate the dynamics of assembly and disassembly of protein complexes. We have applied these assays to the detection and quantification of protein interactions in mammalian cells as well as yeast. These assays are sensitive enough to detect interactions among proteins expressed at endogenous levels *in vivo* and to study dynamic changes in both the formation and disruption of PPIs over seconds without altering the kinetics of binding (Remy and Michnick, 2006; Stefan *et al.*, 2007). Both of these luciferases catalyze the oxidation of substrate coelenterate luciferins (coelenterazines) in a reaction that emits blue light (at a peak of 480 nm) and requires no cofactors (Tannous *et al.*, 2005). The substrates readily diffuse through cell membranes and into all cellular compartments, enabling quantitative analysis in live cells. *R*luc and Gluc are monomeric proteins of 312 (36 kDa) and 185 amino acids (19.9 kDa). Gluc PCA has some advantage in that the reporter protein is smaller and has 10 times higher activity to native coelanterizine than Rluc. However, at present, Rluc has the advantage that stable substrates (e.g., benzyl-coelenterizine) can be used with this

reporter allowing for easier handling and integration of signal over longer times. In contrast to fluorescent protein-based PCAs, both Rluc and Gluc are fully reversible; a prerequisite to study signaling events by the dynamics of protein complex assembly and disassembly (Remy and Michnick, 2006; Stefan *et al.*, 2007). Both Rluc and Gluc PCAs provide for extremely high signal-to-background ratio due to lack of any cellular luminescence and can easily be measured spectroscopically on whole cell populations or by imaging single cells. Finally, the luciferase PCAs allow for accurate measurements of time- (for time constants greater than 10 s) and dose-dependence of pharmacologically induced alterations of protein complexes.

6.1. Materials

6.1.1. Reagents

- cDNAs encoding the Rluc and Gluc PCA fusion partners in suitable expression vectors
- Coelenterazine and benzyl-coelenterazine (Nanolight)
- Competent *MAT**a*** or diploid yeast prepared according to Knop *et al.* (1999).
- SD medium (6.7 g/l yeast nitrogen base, without amino acids)
- SD agar (SD medium with 2% agar)
- 10× amino acid mix -his, -leu, -lys:

Adenine sulfate	0.4 g/ml
Uracil	0.2 g/ml
L-Tryptophan	0.4 g/ml
L-Arginine HCl	0.2 g/ml
L-Tyrosine	0.3 g/ml
L-Phenylalanine	0.5 g/ml
L-Glutamic acid	1.0 g/ml
L-Asparagine	1.0 g/ml
L-Valine	1.5 g/ml
L-Threonine	2.0 g/ml
L-Serine	3.75 g/ml
Methionine	0.2 g/ml (do not include when growing diploid yeast)

- 10× low fluorescence yeast nitrogen base without riboflavin and folic acid (Sheff and Thorn, 2004)
- 20% glucose solution
- PLATE solution (40% polyethylene glycol 3350, 100 m*M* LiOAc, 10 m*M* Tris (pH 7.5), 0.4 m*M* EDTA)

- DMSO
- poly-L-lysine mol. wt. 30,000–70,000 (Sigma P2636) or concanavalin A (Sigma L7647)

6.1.2. Equipment

- Luminescence microplate reader (LMax II^{384} Luminometer, Molecular Devices)
- Luminescence microscope (Nikon Eclipse TE2000U inverted microscope with a CoolSnap HQ Monochrome CCD camera (Photometrics))
- 96-well white plates (Molecular Machines)
- 6-well culture plate or Petri dish
- Appropriate sterile tubes to grow yeast
- Spectrophotometer

6.1.3. Plasmids

The proteins to test for interaction are fused to the coding sequences for N- and C-terminal fragments of Rluc or Gluc, in 5′ or 3′ of the fragments (e.g., protein A-Rluc-F[1], Rluc-F[1]-protein A, protein B-Rluc-F[2], Rluc-F[2]-protein B). Rluc-F[1] (N-terminal) corresponds to amino acids 1–110, and Rluc-F[2] (C-terminal) corresponds to amino acids 111–312 of Rluc (Stefan *et al.*, 2007). Similarly Gluc-F[1] corresponds to amino acids 1–63 and Gluc-F[1] to amino acids 64–185 of Gluc (Remy and Michnick, 2006). The fusions are subcloned into yeast expression vectors, for example, p413ADH for the Rluc-F[1] or Gluc-F[1] fusion and p415ADH for the Rluc-F[2] or Gluc-F[2] fusion (Mumberg *et al.*, 1995). In yeast fragments can also be fused to the genes of interest at their chromosomal loci using a homologous recombination method (Ghaemmaghami *et al.*, 2003). For this purpose the PCA fragments are cloned into nonexpression vectors that provide a selection marker (e.g., antibiotic resistance). For example, pAG25-Rluc-F[1] and pAG32-Rluc-F[2] plasmids are used for the Rluc PCA fragment fusions.

6.2. Procedure

6.2.1. Cotransformation of competent yeast

(1) Thaw competent yeast cells on ice.
(2) Mix 10 μl of cells with 1 μl ($\sim$250 ng) of each yeast expression plasmid (e.g., p413ADH and p415ADH, Mumberg *et al.*, 1995) encoding the Rluc or Gluc PCA fusion partners (protein A fused to Rluc-F[1] or Gluc-F[1] and protein B fused to Rluc-F[2] or Gluc-F[2], 60 μl of PLATE solution and 8 μl DMSO).
(3) Heat shock yeast at 42 °C for 20 min.

Critical step: Shorter or longer incubation times at higher or lower temperatures can result in decreased efficiency of transformation.

(4) Centrifuge at 2000 rpm for 3 min. Remove supernatant and resuspend cells in 500 μl SD medium without amino acids or glucose.
(5) Plate 20 μl of cell suspension per well on SC agar (SD agar + 2% glucose + 1× amino acids (-his, -leu, -lys for *MAT**a***; -his, -leu, -lys, -met for diploids)) in a 6-well plate.
(6) Incubate at 30 °C for 48–72 h.

6.2.2. Fusion of PCA fragments at the chromosomal loci

(1) PCR amplify the Rluc or Gluc PCA fragment cassettes (containing the PCA fragment followed by a terminator that is followed by an antibiotic selection marker; available upon request)
(2) Transform the PCR product in to suitable competent cells: mix 10 μl of thawed competent cells with 10 μl (∼1–2 μg) of each PCR amplified cassette DNA encoding the Rluc or Gluc PCA fragments along with a resistance marker, add 85 μl of PLATE solution; incubate for 30 min at room temperature; add 9.5 μl DMSO followed by heat shock at 42 °C for 20 min; centrifuge at 2000 rpm for 3 min, remove supernatant and resuspend cells in 500 μl YPD medium and incubate at 30 °C with shaking for 4 h; centrifuge the cells, remove supernatant and resuspend cells in 200 μl of YPD; plate 60 μl per well in 6-well plate or entire 200 μl on Petri dish that contain the suitable antibiotic; incubate the plates at 30 °C for 48–72h; the colonies can be further verified by colony PCR methods.

6.2.3. Preparation of cells for bioluminescence assay

(1) Inoculate a fresh colony for each sample with plasmids into 3 ml of SC medium (SD medium + 2% glucose + 1× amino acids (-his, -leu, -lys for *MAT***a**; -his, -leu, -lys, -met for diploids)) and grow overnight at 30 °C with shaking. For cells with fragments fused at chromosomes, grow them in SC medium with suitable antibiotic.
(2) The following day, measure the OD_{600} of the overnight culture and inoculate a fresh culture of LFM (1× low fluorescence yeast nitrogen base + 2% glucose + 1× amino acids (-his, -leu, -lys for Mat A; -his, -leu, -lys, -met for diploids)) or LFM complete with suitable antibiotics with enough cells to obtain an OD_{600} of approximately 0.1–0.3 at the time of analysis.

Critical step: It is particularly important for the cells to be in the log phase of growth in order to avoid including dead and unhealthy cells.

(3) Transfer 160–180 μl of cell suspension (cells equivalent to 0.1–0.3 OD_{600}) to each well. Manually add or inject 20–40 μl of suitable substrate using the Luminometer injector and initiate the

Table 14.4 Troubleshooting Rluc or Gluc luciferase PCAs

Step	Problem	Possible reason	Solution
6.2.1. (6)	No colonies after transformation	Not enough DNA or cells	Increase quantity of cells and DNA. Increase the number of cells plated on the Petri dish or six-well plate
	Too many colonies after transformation	Too many cells plated	Dilute cells before plating on the Petri dish or six-well plate
6.2.2. (2)	Fusion protein is not functioning correctly	Fragment fusion interferes with protein expression/function/stability	Fuse the PCA fragment to the other end of the protein
6.2.3. (3)	Poor Luminescence signal of Luminescence assay	Signal integration time is too short	Optimize the signal Integration times
		Not enough substrate	Increase the substrate concentration
		Not enough cells used	Increase the number of cells used per assay
	No or low signal modulation after Stimulus or Inhibitor treatment	Number of cells and signal integration times are not optimal	Optimize the number of cells and signal integration times
		Stimulus or Inhibitor concentration are too low or duration of treatment is not optimal	Try different stimulus or inhibitor treatment times and or concentrations
		Signal detection time is not optimal	Peak signal occurs immediately after addition of colelantrezines. Try optimizing the beginning of signal integration after substrate addition
		Signal-to-background ratio is low	If the signal is very low, find an optimal way to extract the meaningful signal from background. Test appropriate positive and negative controls for the interaction you are studying

bioluminescence analysis. Optimize the signal integration times depending on the bioluminescence signal strength. For real time kinetics experiments, add or inject the substrate, immediately initiate the bioluminescence readings with the optimized signal integration time continuously for the desired period. Then, background correct the bioluminescence signals to obtain meaningful signal. Afterwards, normalize the data to total protein concentration in cell lysates if desired (Bio-Rad protein assay).

Critical step: Specific functional assays to further characterize a PPI might be performed here. For example, incubation of cells with agents, such as specific enzyme or transport inhibitors, can be performed for various amount of time, prior to the Luminometric analysis. Troubleshooting advice can be found in Table 14.4.

6.2.4. Timeline

Cotransformation of competent yeast (steps 1–5): 30–45 min (depending on the number of samples) plus 48–72 h for cell growth (step 6).

Fusion of PCA fragments at the chromosomal loci: 5 h (depending on the number of samples) plus 48–72 h for cell growth.

Preparation of cells for Luminometric analysis (steps 1–3): 24 h.

Microplate reader analysis: a few minutes to hours, depending on the signal integration time and the number of samples.

6.2.5. Anticipated results

The luminescence intensity of the reassembled Rluc and Gluc PCAs vary with the strength of interaction between the protein pairs attached to the PCA fragments. In the case of our simplest positive control (GCN4 leucine zipper pair fused to the PCA fragments: e.g., Zip-Rluc-F[1] + Zip-Rluc-F[2]), the reconstituted PCAs represent approximately 10–30% of the activity of the full-length Rluc or Gluc enzymes. The PCA fusions expressed alone should not result in detectable luminescence (compared to nontransfected cells) because the individual PCA fragments have no activity. For each study, positive (known interaction) and particularly negative (non-interacting proteins) controls should always be performed in parallel. A PCA response should not be observed if noninteracting proteins are used as PCA partners.

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