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Analyzing P-bodies in *Saccharomyces cerevisiae*

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Abstract

Cytoplasmic processing bodies, or P-bodies, are RNA-protein granules found in eukaryotic cells. P-bodies contain untranslating mRNAs and proteins involved in mRNA degradation and translation repression. P-bodies, and the mRNPs within them, have been implicated in mRNA storage, mRNA degradation and translational repression. The analysis of mRNA turnover often involves the analysis of P-bodies. In this chapter, we describe methods to analyze P-bodies in the budding yeast, *Saccharomyces cerevisiae* including procedures to determine if a protein or mRNA can accumulate in P-bodies, if an environmental perturbation or mutation affects P-body size and number, and methods to quantify P-bodies.

1. Introduction

The control of translation and mRNA degradation can involve conserved cytoplasmic RNA granules. One class of such RNA granules is the cytoplasmic processing body, or P-body. P-bodies are dynamic aggregates of untranslating mRNAs in conjunction with translational repressors and proteins involved in deadenylation, decapping and 5' to 3' exonucleolytic decay (Parker and Sheth, 2007). P-bodies and the mRNPs assembled within them are of interest since they have been implicated in translational repression (Holmes *et al.*, 2004; Collier and Parker, 2005), normal mRNA decay (Sheth and Parker, 2003; Cougot *et al.*, 2004), nonsense-mediated decay (Sheth and Parker, 2006; Unterholzner and Izaurralde, 2004), miRNA-mediated repression (Liu *et al.*, 2005; Pillai *et al.*, 2005), and mRNA storage (Brenques *et al.*, 2005; Bhattacharyya *et al.*, 2006). At a minimum, P-bodies serve as markers proportional to the concentration of mRNPs complexed with the mRNA decay machinery and may have additional biochemical properties that affect the control of mRNA translation and/or degradation.

Given the concentration of mRNAs and mRNA decay factors in P-bodies, the analysis of mRNA turnover can involve examining aspects of P-body composition and function. In this chapter we describe methods to analyze P-bodies in the budding yeast, *Saccharomyces cerevisiae*, to determine: a) Does a given protein or mRNA accumulate in P-bodies? b) does a specific perturbation (e.g. mutation, over-expression, or environmental cue) change P-body size or number? and c) is there a quantifiable change in the number and size of P-bodies in a population of cells?

2. Determining if a specific protein can accumulate in P-bodies

2.1 Markers of P-bodies

A common experimental goal is determining if a given protein accumulates in P-bodies. Previous work has identified many proteins enriched in yeast P-bodies (summarized in Table I). These include a conserved core of proteins found in P-bodies from yeast to mammals that

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consists of the mRNA decapping machinery. These core P-body components include the decapping enzyme, Dcp1p/Dcp2p, the activators of decapping Dhh1p, Pat1p, Scd6p, Edc3p, the Lsm1p-7p complex, and the 5' to 3' exonuclease, Xrn1p. Some proteins observed in yeast P-bodies, such as proteins involved in nonsense-mediated decay (Sheth and Parker, 2006), are only observed in P-bodies under certain mutant, cell-type, stress, or over-expression conditions (Table I). These proteins may normally rapidly transit through P-bodies, but under some conditions accumulates to detectable levels.

To determine if a given protein can accumulate in P-bodies one simply needs to examine the subcellular distribution of the protein relative to other P-body components. In the absence of good antisera, the simplest way to do this is to use the relevant protein tagged with GFP, or some other fluorescent protein, and then examine the potential co-localization of a component with one or more of the core P-body components. For this type of experiment, many of the core P-body components are available as fusions to fluorescent proteins on yeast plasmids (Table II). Thus to examine if a protein accumulates in yeast P-bodies one should take the following steps:

- a. Obtain a fluorescent tagged version of the protein of interest. Note that most of the yeast ORFs fused to GFP are available from a genomic collection (Huh *et al.*, 2003) and can be purchased from Invitrogen.
- b. Determine if the fusion protein is functional by some criteria.
- c. Compare the localization of the tagged protein of interest with a core P-body marker tagged with a different fluorescent protein as described below. In our experience, the most reliable and brightest components of yeast P-bodies are Dcp2p and Edc3p.

2.2 Preparation of samples

To determine if a protein can accumulate in P-bodies we recommend examining its subcellular distribution in mid log phase and also in a condition where P-bodies are enhanced such as glucose deprivation, hyperosmotic stress, high OD, or when decapping is inhibited (Teixeira *et al.*, 2005; Sheth and Parker, 2003; Figure 1). Note that it is important to be careful when preparing cells for examining P-bodies microscopically as P-bodies are dynamic and can change rapidly in response to a variety of stresses (Teixeira *et al.*, 2005). Therefore, proper aeration and if required, a carbon source are essential. In all cases, care should be taken to reduce centrifugation and handling times as variations can alter P-bodies. A final caveat is that growth conditions can alter P-body composition, for example different results can be obtained from growth in rich versus minimal media for non-core proteins. Protocols for examining P-bodies in mid-log cultures or with glucose deprivation or osmotic stress are described below.

2.21 Examination of P-bodies in mid-log growth

1. Grow a 5–10 ml yeast culture in rich or minimal media as appropriate to mid log phase, with an absorbance between 0.3 and 0.6 at 600 nm.
2. Pellet 5 ml of the cells in a clinical centrifuge at room temperature for 1 minute.
3. Resuspend in 1 ml minimal media supplemented with amino acids and the same carbon source that the cells were originally grown in, then transfer the sample to a microfuge tube.
4. Pellet 1 ml of cells in a table top microfuge for 20 seconds, aspirate off the supernatant and resuspend them in 50–100 ul of minimal media supplemented as above. Keep in a microfuge tube with constant flicking by hand to maintain aeration.

5. Add the suspension to the slide at the microscope, then place a coverslip on the sample and examine immediately. This is important as cells under a coverslip lack aeration, which can eventually induce a stress response and artifactually increase the presence of P-bodies.
6. Take the pictures rapidly to allow accurate assessment of the P-body state of the cells. As two channels need to be taken to co-localize the P-body markers with the protein of interest, it is optimal to use a microscope that splits the beam to record the two channels simultaneously.
7. If cells are moving, which can make colocalization difficult, then it may be necessary to immobilize the cells by coating the coverslips with the lectin concanavalin A, which binds to the yeast cell wall. The protocol we use to coat coverslips is to wash coverslips overnight in sterile filtered 1 M NaOH. After washing well with distilled water, add concanavalin A solution (0.5 g/L Sigma #L7647, 10mM phosphate buffer pH 6, 1 mM CaCl₂, 0.02% azide) for 20' with gentle shaking. After removal of the solution, rise once in distilled water, pour off the liquid and let dry overnight. The coverslips can be stored at room temperature after coating.
8. If longer exposures or time-lapse data are required, we use an inverted Deltavision microscope with concanavalin A coated glass bottom microwell dishes (MatTek Corporation #P35G-1.5-14-C) with the coverslip immersed in enough minimal media (supplemented as appropriate for the experiment) to fully cover it. This avoids both the problem of cell movement as well as lack of aeration.

2.22 Examination of P-bodies under glucose deprivation or osmotic stress

1. Grow a 5–10 ml yeast culture in rich or minimal media as appropriate to mid log phase, with an absorbance between 0.3 and 0.6 at 600 nm.
2. Pellet 5 ml of the cells in a clinical centrifuge at room temperature for 1 minute.
3. After pelleting the cells, aspirate off the supernatant and wash with 1 ml minimal media to be used for the induction. For example, in a glucose deprivation experiment, the media should be identical to that used for the initial growth but lacking glucose. For hyperosmotic stress, the media should be the starting media but with KCl added to a final concentration of 1 M.
4. Pellet the cells and resuspend them in 1 ml induction media and transfer into 4 ml of pre-warmed induction media in a small conical flask with shaking to maintain aeration. Grow for the appropriate induction time with shaking at the temperature that cells were grown prior to induction. The induction time should be determined experimentally, for example during glucose depletion or osmotic stress P-bodies are substantially increased after 10 minutes of stress (Teixeira *et al.*, 2005).
5. Pellet 1.5 ml of cells in a table top microfuge for 20 seconds, aspirate off the supernatant and resuspend them in 50–100 ul of minimal media supplemented as above. Keep in a microfuge tube with constant flicking by hand to maintain aeration.
6. Add the suspension to the slide at the microscope, then place a coverslip on the sample and examine immediately. This is important as yeast cells under a coverslip lack aeration, which can influence the induction or presence of P-bodies.
7. Take the pictures rapidly to allow accurate assessment of the P-body state of the cells. As two channels need to be taken to colocalize the P-body markers with the protein of interest, it is optimal to use a microscope that splits the beam to record the two channels simultaneously, although not necessary. If need be cells can be immobilized and time-lapse images can be taken as described above.

If a protein shows substantial overlap in its subcellular distribution with core P-body proteins, it can be inferred to accumulate in, or near, P-bodies. We recommend confirming and extending such possible interactions by using standard approaches to perform a co-immunoprecipitation of the P-body component and probing for the target protein.

3. Monitoring messenger RNA in P-bodies

In some cases it is useful or important to determine whether bulk mRNA or a specific transcript is accumulating in P-bodies. There are two general approaches to determine if specific mRNAs are accumulating in P-bodies. First, one can use in situ hybridization techniques to monitor the presence of bulk mRNA using an oligo(dT) probe, or specific mRNAs using sequence specific probes. Although such approaches have worked well in mammalian cells (Pillai *et al.*, 2005; Franks and Lykke-Andersen, 2007), robust techniques for in situ hybridization of yeast P-bodies have not yet been described.

In an alternative method, one can use "GFP-tagged" RNAs to follow the localization of specific transcripts in yeast and determine if they accumulate in P-bodies. To visualize specific mRNAs, binding sites for a RNA binding protein fused to a fluorescent protein are inserted into the 3' UTR of the mRNA of interest allowing its subcellular distribution to be examined by following the location of the RNA binding protein fused to the fluorescent protein. Most commonly, the well-characterized U1A or the MS2 binding sites are inserted into the 3' UTR of the mRNA of interest (Brodsky and Silver, 2000; Bertrand *et al.*, 1998). These mRNA constructs are then co-expressed with either the U1A-GFP (Brodsky and Silver, 2000) or the MS2 coat protein fused to GFP (Bertrand *et al.*, 1998). Both of these have nano to pico molar affinity for their respective binding sites allowing detection of the mRNA.

Several of these types of engineered mRNAs have been constructed on plasmids and used to demonstrate the accumulation of specific yeast mRNAs in P-bodies. Available plasmids expressing "tagged" version of the stable PGK1 and the unstable MFA2 mRNA are described in Table III. In addition, variants of the tagged PGK1 mRNAs are available with premature nonsense codons in specific positions, which can be used for examining the accumulation of mRNA in P-bodies due to the action of NMD (Sheth and Parker, 2006). A variety of plasmids expressing the MS2 or U1A proteins fused to GFP are also available (Table III).

4. Determining if a mutation or perturbation affects P-body size and number

4.1 Conditions to observe increases or decreases in P-bodies

A common experimental issue is determining if a mutation, protein over-expression, or an environmental cue affects the size and number of P-bodies. To examine if P-bodies are altered under a certain condition we make three suggestions. First, one should use multiple markers of P-bodies to ensure that any differences seen are not unique to a single protein. Second, since a specific mutant may affect P-bodies only under certain conditions, we recommend that P-bodies be examined under multiple conditions (e.g. mid-log growth, glucose deprivation, osmotic stress, high cell density). Finally, we recommend quantification of the amount and number of P-bodies by computational methods to allow unbiased calculation of the size and number of P-bodies present in a given situation.

In practice, there are different methods for examining if a perturbation reduces or increases P-body size or number. To determine if a perturbation reduces P-bodies it is most convincing to examine conditions where P-bodies are large and easily detectable. For example, P-bodies are large under stress conditions such as glucose deprivation, high cell density, and when decapping or 5' to 3' degradation are inhibited (Teixera *et al.*, 2005). Therefore, these are ideal conditions to examine if P-bodies are reduced by a mutation or condition (for examples see

Teixera and Parker, 2007; Decker *et al.*, 2007). Conversely, P-bodies are small when cells are undergoing mid log growth (Teixeira *et al.*, 2005), which makes this condition an ideal situation to see if P-bodies increase (Teixeira and Parker, 2007).

4.2 Interpreting alterations in P-body size and number

Any alteration in P-body size and number observed due to a specific mutation or alteration in growth can be due to a variety of effects and should be interpreted with care. This is because a variety of changes in cell physiology will affect P-body size and number due to the flux of mRNP in and out of these structures (Table IV). For example, P-body size and number can be increased by defects in mRNA decapping or 5' to 3' degradation, which increase the pool of mRNPs in P-bodies by decreasing the destruction of mRNAs in this compartment, or by defects in translation initiation, which increase the pool of untranslating mRNPs associated with P-bodies. Alternatively, P-bodies can be reduced in size and number by inhibiting translation repression, by removing interactions that promote aggregation of the individual mRNPs into larger structures, or by reductions in the level of the P-body marker being examined. Note that in order to be confident of the underlying mechanism affecting P-body size and number additional experiments should be performed to identify the true cause of the defect (Table IV).

5. Quantification of P-body size and number

A common and important issue is determining whether there are differences in P-body size and number between different conditions or mutants. When comparing two conditions or mutants to determine if there is a difference in P-body size and number it is important to use unbiased approaches to determine the effects. One method is to score P-bodies in the two conditions by comparing a large number of cells (e.g. 100) by visual inspection, recording the number and approximate size distribution in each cell. However, given that such an approach can introduce experimental bias, we recommend that if P-bodies are scored by visual means, it be done in a blind manner with one experimenter preparing the images and a second scoring them without knowing the identity of any image. In addition, we recommend that manual scoring only be used for dramatic differences in P-body size and number.

An unbiased and more quantitative approach is to use semi-automated software programs for image analysis based on computational algorithms. This can be accomplished using ImageJ, a free downloadable software from the NIH (Abramoff *et al.*, 2004). On the ImageJ website, there are instructions and downloadable plugins, which allow direct loading of raw images from the microscope. The quantification of P-bodies is accomplished by setting a threshold mask, which allows regions of the image above a certain intensity to be scored as “on” and the rest of the image “off”. The P-bodies are counted by the number of “on” regions and their area and number can be computed. To further reduce bias, the masking is performed automatically using the Otsu Thresholding Filter (Otsu, 1979). Once the images, ImageJ and the Otsu plugin are obtained, the image can be quantified. A protocol for quantifying such images is detailed below:

1. Open the image in ImageJ to quantify. Normally, an entire Z-stack is used that captures the entire thickness of the cell. Once the stack is collapsed, the P-bodies can be analyzed.
2. In the ImageJ program, go to Process, and select (“Smooth”). Then go to Process, select Math and then select Subtract to remove the background. This is the most important step in the process, and will probably need to be modified for different microscopes as well as marker proteins or RNA. It should be noted that because of threshold subtractions, such quantifications are not necessarily absolute numbers or areas of P-bodies but provide a systematic and unbiased measure of relative P-body number and area within experiments.

3. Go to Plugins, select Filters and then select "Otsu Thresholding". This option will only be available if you have downloaded the plugin and placed it in the correct directory on your computer.
4. Go to Image, select Lookup Tables and then select "Invert LUT". This will reverse the image, allowing the P-bodies to be considered "on".
5. Go to analyze and select "Analyze Particles..." to set the pixel size range to be counted. In our experience it should be between 7 and 500, but it will vary depending on samples. Optimization is required so random speckles do not count as P-bodies and large fluorescent regions unrelated to P-bodies are not counted (for example outside those the cells).
6. Select show Masks, which will graphically demonstrate the calculated P-bodies as compared to the P-bodies' thresholded image. This procedure generates a table that lists the number of foci, average area and total area.
7. The number of cells in each image must be calculated manually, however all steps except cell counting can be automated by writing a macro to perform them to obtain more high throughput analyses.

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Figure 1. P-bodies under different growth and induction conditions

The P-body marker used is the genomically integrated DCP2-GFP (yRP2162). The cells were grown in YEPD to (A) mid log growth phase, then treated with (B) glucose deprivation for 10' in complete media or (C) hyperosmotic (1 M KCl) stress for 15' in complete minimal media with glucose as described in the text.

TABLE I

Protein Components of P Bodies

Core Components	Function
Dcp1p	Decapping enzyme subunit ^a
Dcp2p	Catalytic subunit of decapping enzyme ^a
Dhh1p	DEAD box helicase required for translational repression; decapping activator ^a
Edc3p	Decapping activator ^b
Lsm1p-7p	Sm-like proteins involved in decapping ^a
Pat1p	Decapping activator and translational repressor ^a
Scd6p	Protein containing Sm-like and FDF domain; Involved in translation repression ^c
Xrn1p	5' to 3' exonuclease ^a
Ccr4p/Pop2p/ Not1-5p	Major cytoplasmic deadenylase ^{ad}
Proteins involved in NMD	Function
Upf1p	ATP-dependent helicase required for NMD, accumulates in yeast P bodies in dcp1Δ, xrn1Δ, dcp2Δ, upf2Δ, and upf3Δ strains ^e
Upf2p	Component required for NMD, accumulates in P bodies in dcp1Δ, dcp2Δ, and xrn1Δ mutants ^e
Upf3p	Component required for NMD, accumulates in P bodies in dcp1Δ, dcp2Δ, and xrn1Δ mutants ^e
Ebs1p	Putative ortholog of human Smg7, accumulates in glucose deprivation ^f
Translation and Translational Repression	Function
Cdc33p	eIF4E: mRNA m7G cap binding protein ^{g, h}
Pab1p	Predominant poly A binding protein ^{g, h}
Rbp1p	RNA-binding protein, localizes to P bodies during stress ⁱ
Sbp1p	Facilitates mRNA decapping ^j
Tif4631p	eIF4G1: Component of eIF4F initiation factor ^{g, h}
Tif4632p	eIF4G2: Component of eIF4F, induced at stationary phase ^{g, h}
Additional Components	Function
Dsc2p	Nutrient stress-dependent regulator of the scavenger enzyme Dcs1 ^k
Pby1p	Putative tubulin tyrosine ligase ^l
Rpb4p	Subunit of RNA polymerase II ^m
Rpm2p	Protein component of the mitochondrial RNaseP ⁿ

^a (Sheth and Parker, 2003)

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TABLE II

Core P-body components available on plasmids as fluorescent protein fusions

Protein	Fluorescent Tag	Plasmid Marker	Lab Plasmid Number
Dcp2p full length	GFP	LEU2	pRP1175 ^a
	GFP	TRP1	pRP1316 ^b
	GFP	URA3	pRP1315 ^c
Dcp2p full length	RFP	LEU2	pRP1155 ^d
	RFP	TRP1	pRP1156 ^e
	RFP	URA3	pRP1186 ^f
Dcp2p truncated (1–300)	RFP	LEU2	pRP1167 ^g
	RFP	TRP1	pRP1165 ^h
	RFP	URA3	pRP1152 ^h
Dhh1p	GFP	LEU2	pRP1151 ^g
Edc3p	mCherry	URA3	pRP1574 ⁱ
	mCherry	TRP1	pRP1575 ⁱ
Lsm1p	GFP	LEU2	pRP1313 ^a
	GFP	URA3	pRP1314 ^j
Lsm1p	mCherry	LEU2	pRP1400 ^k
Lsm1p	RFP	URA3	pRP1084 ^l
	RFP	LEU2	pRP1085 ^l
Pat1p	GFP	URA3	pRP1501 ^m

^a(Coller and Parker, 2005)^bSegal, S.P., Dunckley, T., and Parker R. (2006). Sbp1p affects translational repression and decapping in *Saccharomyces cerevisiae*. *Mol Cell Biol.* **26**,5120–5130.^cUnpublished, Parker Lab.^d(Teixeira *et al.*, 2005)^e(Sheth and Parker, 2006)^f(Teixeira *et al.*, 2005)^gSweet, T.J., Boyer, B., Hu, W., Baker, K.E., and Coller J. (2007). Microtubule disruption stimulates P-body formation. *RNA* **13**,493–502.^hMuhlrad, D., and Parker, R. (2005). The yeast EDC1 mRNA undergoes deadenylation-independent decapping stimulated by Not2p, Not4p, and Not5p. *EMBO J.* **24**,1033–1045.ⁱUnpublished, Buchan, R., and Parker, R.^jTharun, S., Muhlrad, D., Chowdhury, A., and Parker R. (2005). Mutations in the *Saccharomyces cerevisiae* LSM1 gene that affect mRNA decapping and 3' end protection. *Genetics* **170**,33–46.^kBeckham, C.J., Light, H.R., Nissan, T.A., Ahlquist, P., and Parker, R., Noueiry A. (2007). Interactions between brome mosaic virus RNAs and cytoplasmic processing bodies. *J Virol.* **81**,9759–9768.^l(Sheth and Parker, 2003)

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TABLE III

Plasmids for localizing mRNA in yeast cells: GFP fusion proteins that bind to specific binding sites in mRNA engineered in their 3' UTR

Protein + Tag	Plasmid Marker	Promoter		Lab Plasmid Number
MS2 CP-GFP	HIS3	Met25		pRP1094 ^a
U1A-GFP	TRP1	GPD		pRP1187 ^b
U1A-GFP	LEU2	GPD		pRP1194 ^c
RNA + Binding Seq.	Plasmid Marker	Promoter	Description^h	Lab Plasmid Number
MFA2 pG MS2	URA3	GPD	two MS2 sites 3' to poly(G) tract in 3' UTR	pRP1081 ^d
MFA2 MS2	URA3	GPD	two MS2 sites in 3' UTR	pRP1083 ^e
MFA2 U1A	URA3	GPD	PGK1 3' UTR with sixteen U1A binding sites	pRP1193 ^c
MFA2 U1A	URA3	Tet-Off	PGK1 3' UTR with sixteen U1A binding sites	pRP1291 ^c
PGK1 pG MS2	URA3	PGK1	two MS2 sites 3' to poly(G) tract in 3' UTR	pRP1086 ^c
PGK1 U1A	URA3	PGK1	sixteen U1A sites in 3' UTR	pPP1354 ^b
PGK1 early PTC U1A	URA3	PGK1	PGK1 U1A with nonsense mutation at position 22	pRP1295 ^f
PGK1 late PTC U1A	URA3	PGK1	PGK1 U1A with nonsense mutation at position 225	pRP1296 ^f
PGK1 U1A	URA3	GAL	sixteen U1A sites in 3' UTR	pRP1303 ^g

^aBeach, D.L. and Bloom, K. (2001). ASH1 mRNA Localization in Three Acts. *Mol. Biol. Cell.* **12** 2567–2577.

^bBrodsky and Silver, 2000).

^cBrengues *et al.*, 2005)

^dM. Valencia-Sanchez and R. Parker, unpublished

^eSheth and Parker, 2003), has short polyG tract in 3' UTR that does not inhibit exonucleolytic decay.

^f(Sheth and Parker, 2005).

^gU. Sheth and R. Parker, unpublished.

^hAll mRNA constructs have their native 5' and 3' UTR except where noted

TABLE IV

Dissection of effects on P-body size and number

Observation	Possible Cause	Follow-up Experiments
Increase in P-Body size and number	1) Inhibition of decapping or 5' to 3' degradation 2) Defects in translation initiation	1) Examine mRNA degradation rate and whether mRNAs are degraded accurately 2) Measure the rate of protein synthesis by polysomes or ³⁵ S incorporation.
Decrease in P-Body size and number	1) Decreases in translation elongation 2) Enhanced translation 3) Reduced expression of marker proteins	1) Polysome analysis to determine if their size is increased 2) Measure the rates of protein synthesis by polysomes or ³⁵ S incorporation. 3) Western blot to ensure P-Body expression levels