Pil1 co-tethering assay to detect protein-protein interactions in the fission yeast *Schizosaccharomyces pombe*

Zhao-Qian Pan, Yu-Sheng Yang, and Li-Lin Du

Abstract

Protein-protein interactions play critical roles in biological processes. We have previously developed the Pil1 co-tethering assay, an imaging-based method to detect protein-protein interactions in living *Schizosaccharomyces pombe* cells. This assay leverages the distinct localization pattern of the Pil1 protein by fusing a bait protein to Pil1 and examining whether a prey protein co-localize with the Pil1-fused bait. Here, we present an improved protocol of the Pil1 co-tethering assay. In this protocol, modified stable integration vectors (SIVs) with a NotI site as the linearization site are used to express bait and prey proteins. We expect that this protocol will enhance the application of the Pil1 co-tethering assay for studying protein-protein interactions.

Keywords: Protein-protein interactions, Live cell imaging, Pil1 co-tethering assay, Fission yeast, *Schizosaccharomyces pombe*

1. Introduction

Protein-protein interactions are the basis of numerous cellular processes [1, 2]. A variety of *in vitro* and *in vivo* methods have been developed to detect protein-protein interactions [3–5]. We have recently developed an imaging-based method called the Pil1 co-tethering assay to detect protein-protein interactions in the fission yeast *Schizosaccharomyces pombe* [6]. In this method, the *S. pombe* Pil1 protein, which forms filamentary structures associated with the plasma membrane [7], is fused with mCherry and a bait protein, and co-expressed with a prey protein fused with mECitrine. If the bait and prey proteins interact, co-localization of mCherry and mECitrine signals will be observed in living fission yeast cells (Fig. 1).

In our published method [6], bait and prey proteins are expressed using pDUAL vector-based integrating plasmids, which integrate into the genome through ends-in

recombination [8, 9]. We have observed that transformants of such plasmids frequently exhibit failed or incorrect integration. To address this, we have revised the method by adopting stable integration vectors (SIVs) developed by Sophie Martin's laboratory for expressing bait and prey proteins [10]. We have modified the SIVs by introducing a NotI site as the linearization site, making the SIVs more universally applicable (*see* **Note 1**).

2. Materials

2.1 Media

1. Rich medium: yeast extract medium with supplements (YES). For 1 L liquid YES medium, mix 5 g yeast extract, 30 g glucose, 40 mL 25 × supplements (5 g leucine, 5 g histidine, 5 g adenine, and 5 g uracil in 1 L water), add water to 1 L and autoclave. For solid medium, add 2% (w/v) agar prior to autoclaving.
2. Minimal medium: Edinburgh minimal medium (EMM). For 1 L liquid EMM medium, mix 3 g potassium hydrogen phthalate, 5 g ammonium chloride, 2.2 g sodium phosphate dibasic, 20 g glucose, 20 mL 50 × salt stock (52.5 g magnesium chloride hexahydrate, 0.735 g calcium chloride dihydrate, 50 g potassium chloride, and 2 g sodium sulfate in 1 L water), 1 mL 1000 × vitamin stock (1 g pantothenic acid, 10 g nicotinic acid, 10 g inositol, and 10 mg biotin in 1 L water, stored at 4 °C), 0.1 mL 10000 × mineral stock (5 g boric acid, 4.5 g manganese sulfate monohydrate, 4 g zinc sulfate heptahydrate, 2 g iron(III) chloride hexahydrate, 0.4 g molybdic acid, 1 g potassium iodide, 0.4 g copper sulfate pentahydrate, and 10 g citric acid monohydrate in 1 L water, stored at 4 °C), add water to 1 L and autoclave. For solid medium, add 2% (w/v) agar prior to autoclaving.

2.2 Plasmid digestion

1. The restriction enzyme NotI.
2. A 10 × restriction enzyme buffer appropriate for NotI.

2.3 Transformation

1. 1.0 M Lithium acetate: Dissolve 51 g of lithium acetate dihydrate in 450 mL of water. Adjust volume to 500 mL with water. Autoclave and store at room temperature.

2. 50 % w/v PEG MW 3350: Dissolve 100 g of PEG 3350 in 120 mL of water. Adjust volume to 200 mL with water. Autoclave and store at room temperature (*see* **Note 2**).

3. 2.0 mg/mL carrier DNA: Dissolve 200 mg of salmon sperm DNA in 100 mL of TE buffer (10 mM Tris-HCl, 1 mM Na2-EDTA, pH 8.0) using a stir plate at 4 °C. Aliquot and store at -20 °C (*see* **Note 3**).

2.4 Modified SIV plasmids for expressing bait and prey proteins

We have constructed SIV plasmids that contain the *P41nmt1* promoter, the *ScADH1* terminator, and the coding sequence of Pil1-mCherry, mECitrine, or mTurquoise2 (Tables 1, 2, and 3). These plasmids are available from Addgene (https://www.addgene.org/) and the Yeast Genetic Resource Center of Japan (YGRC/NBRP, https://yeast.nig.ac.jp/). Table 1 includes four plasmids (pDB5225-pDB5228) that contain the coding sequence of Pil1-mCherry and can be used for expressing bait proteins. They differ in the selection marker and the locus of integration. Table 1 also includes a control bait plasmid (pDB5230) expressing Pil1-mCherry fused with SpAtg8(1-115). Table 2 lists four plasmids (pDB4875-pDB4884) for expressing prey proteins fused with mECitrine and a control prey plasmid (pDB5253) expressing CtNbr1 fused with mECitrine. Table 3 lists four plasmids (pDB4887-pDB4896) for expressing prey proteins fused with mTurquoise2.

3. Methods

3.1 Plasmid construction

To generate a plasmid expressing a bait protein, the coding sequence of the bait

protein can be cloned into the BglII site of the SIV plasmids containing the coding sequence of Pil1-mCherry (Fig. 2). To generate a plasmid expressing a prey protein, the coding sequence of the prey protein can be cloned into either the NheI or BglII site of the SIV plasmids containing the coding sequence of mECitrine or mTurquoise2 (Fig. 2). The availability of plasmids containing these two different fluorescent proteins facilitates the detection of ternary protein interactions (*see* **Note 4**).

3.2 Yeast transformation

1. Inoculate a yeast strain in 5 mL of liquid YES medium and incubate overnight at 30 °C with rotary shaking. Determine the cell concentration by measuring the optical density at 600 nm (OD_{600}). Dilute the culture into a flask containing fresh liquid YES medium to a OD_{600} of 0.1-0.2. Incubate the flask at 30 °C with rotary shaking (*see* **Note 5**).

2. When the OD_{600} of the culture reaches 0.4-0.8, harvest the cells by centrifuging at 3000 g for 2 min, and wash the cells with sterilized water once. 5 OD_{600} units of cells are used for each transformation.

3. Perform plasmid digestion by mixing 3 μL 10 × restriction enzyme buffer, 1 μL NotI, 1 μg plasmid, adding water to a total volume of 30 μL, and incubating at 37 °C for 1 h.

4. Boil carrier DNA for 5 min and then chill in an ice-water bath immediately.

5. Prepare the transformation mix (240 μL 50% PEG 3500, 36 μL 1 M LiAc, and 50 μL carrier DNA for one transformation) and add the mix into a 1.5 mL tube containing the pellet of 5 OD_{600} units of cells (*see* **Note 6**).

6. Add the 30 μL NotI digestion mix into the tube containing the cells and the transformation mix. Add sterilized water to a final volume of 360 μL. Resuspend the cells by vortexing. A bait plasmid and a prey plasmid can be co-transformed together (*see* **Note 7**).

7. Incubate the tube in a 42 °C water bath for 40 min.

8. Centrifugation at the top speed of a microcentrifuge for 1 min and remove the

supernatant.

9. Resuspend the cells with 100 μL of sterilized water and spread the cells on a YES plate. Incubate the plate at 30 °C for 24 h.

10. Replica plate onto an antibiotic-containing YES plate. Incubate the plate at 30 °C for 2-3 days (*see* **Note 8**).

3.3 Transformant clone verification

1. Pick colonies grown on the selection plate, and re-streak to single colonies on a YES plate.

2. When the single colonies are grown up, replica plate onto a selection plate. If a transformant clone contains stably integrated plasmid(s), all single colonies derived from it should grow on the selection plate.

3. Further verify the clones by examining the fluorescence signals with microscopy and by performing colony PCR (*see* **Note 9**).

3.4 Microscopy analysis

1. Inoculate a yeast strain in 2 mL of liquid EMM medium and incubate overnight at 30 °C with rotary shaking (*see* **Note 10**).

2. Dilute and grow the culture at 30 °C for 24 h, diluting at least twice during this period to ensure the culture remains in continuous log-phase growth prior to microscopy.

3. Collect 1 mL of culture in a 1.5-mL tube and centrifuge at 3000 g for 30 s (*see* **Note 11**).

4. Using a micropipette, collect 1-1.5 μL of cell pellet from the bottom of the tube and place it onto a coverslip. Gently place a glass slide on top to create a sandwich with the cells between the slide and coverslip (*see* **Note 12**). If the cells do not spread into a single layer, make a new sandwich.

5. Acquire micrographs of the cells using a fluorescence microscope (*see* **Note 13**).

3.5 Quantification of the co-localization

To quantify the degree of co-localization between bait and prey proteins in the Pil1 co-tethering assay, we routinely use the Pearson correlation coefficient (PCC) calculated by the Coloc 2 plugin of the Fiji distribution of the ImageJ software [6].

1. Open a multi-channel image file in Fiji and use the "Image > Color > Split Channels" command to obtain separate files for different wavelength channels.

2. Use the "Freehand selections" tool (the fourth tool in the Fiji toolbar) to outline individual cells in one channel (*see* **Note 14**). The outlined cells are called regions of interest (ROIs) in Fiji.

3. Open the Coloc 2 plugin in Fiji (Analyze > Colocalization > Coloc 2). In the "ROI or mask" pull-down menu, choose the channel in which ROIs have been selected in the last step.

4. After running the Coloc 2 plugin, the plugin-reported "Pearson's R value (no threshold)" for a cell is recorded as the PCC value for that cell. We usually analyze a total of 10 cells and present the PCC values as mean±s.d. (*see* **Note 15**).

Here we use the positive control plasmids pDB5230 and pDB5253 (Tables 1 and 2; available from Addgene and YGRC/NBRP) to demonstrate PCC-based co-localization analysis. pDB5230 expresses the bait protein Pil1-mCherry-fused SpAtg8(1-115), a C-terminally truncated version of *S. pombe* Atg8. pDB5253 expresses the prey protein mECitrine-fused *Chaetomium thermophilum* Nbr1 (CtNbr1). We have previously shown that these two proteins interact in the Pil1 co-tethering assay [11]. Fluorescent signals from these two proteins exhibit strong visual co-localization in both top/bottom and mid-plane Z-sections (Fig. 3). As a negative control, Pil1-mCherry alone does not co-localize with mECitrine-CtNbr1 (Fig. 3). PCC values obtained using the Coloc 2 plugin in Fiji align with the visual observation (Fig. 3).

4. Notes

1. The original SIV plasmids developed by the Martin laboratory use the restriction site of AfeI, PmeI, BstZ17I, or StuI as the linearization site [10]. AfeI, BstZ17I, and

StuI are 6-base cutters and their restriction sites frequently exist in intended inserts, thus rendering a SIV plasmid incompatible with an intended insert. We modified the SIVs so that all of them use the restriction site of the 8-base cutter NotI as the linearization site.

2. The bottle containing the PEG 3350 solution should be tightly capped to prevent evaporation, as an increased concentration of PEG in the transformation reaction can negatively affect the yield of transformants.

3. Carrier DNA should be denatured by boiling in a heat block for 5 min and chilled immediately in an ice-water bath before use. Denatured carrier DNA can be used three or four times without loss of activity, and it should be boiled again afterwards or a new aliquot should be used.

4. To detect a ternary interaction, one bait protein fused with Pil1-mCherry and two prey proteins fused respectively with mECitrine and mTurquoise2 can be co-expressed, as has been done in our published study [6].

5. It is important to allow the cells to complete at least two doublings before collecting the cells for transformation.

6. When preparing multiple transformation reactions, make a transformation mix with a volume for n+1 reactions (where n is the number of planned transformations), then aliquot the mix into the individual tubes. This ensures sufficient volume is prepared to account for any pipetting losses during the aliquoting process.

7. The transformation efficiency when co-transforming more than one plasmid is lower compared to transforming a single plasmid. To account for this, at least 1 μg of DNA should be used for each plasmid when performing co-transformations.

8. For co-transformation of two plasmids, replica plate onto a selection plate containing two different antibiotics (e.g. YES + hygromycin + G418).

9. In our experience, the rate of correct SIV plasmid integration is quite high. The pUra4^{NotI}, pAde6^{NotI}, and pHis5^{NotI} plasmids show rates of correct integration of 90% or above, while the pLys3^{NotI} plasmids exhibit a lower rate that is still above 50%. PCR primers for assessing whether correct integration has happened are those reported by the Martin laboratory [10].

10. The *P41nmt1* promoter is a thiamine-repressible promoter. Since the commonly used rich medium for fission yeast (the YES medium) contains thiamine, minimal media lacking thiamine, such as the EMM medium, should be used for expression of proteins under the control of the *P41nmt1* promoter.

11. Gently pouring off some, but not all, of the supernatant can make the following pipetting step easier.

12. Cleanness of the coverslip and slide is critical, as dust particles can impede proper cell spreading. Gently placing the heavier glass slide on top aids in spreading cells into a single layer without applying additional pressure that may cause cell rupture.

13. We typically use images focused on the top or bottom plasma membrane for visualizing co-localization. At least 5 optical Z-sections with a 0.2 μm Z-interval should be acquired to ensure that at least one Z-section has the plasma membrane-associated Pil1 filaments in focus. Performing deconvolution can enhance image quality.

14. Choose a channel in which the cell boundary is more visually discernable for outlining.

15. PCC values range from −1 to 1, with higher values indicating stronger co-localization between the two channels.

References

1. Alberts B (1998) The Cell as a Collection of Protein Machines: Preparing the Next Generation of Molecular Biologists. Cell 92:291–294

2. Gavin A-C, Superti-Furga G (2003) Protein complexes and proteome organization from yeast to man. Current Opinion in Chemical Biology 7:21–27

3. Wiens MD, Campbell RE (2018) Surveying the landscape of optogenetic methods for detection of protein-protein interactions. Wiley Interdiscip Rev Syst Biol Med 10:e1415

4. Zhou M, Li Q, Wang R (2016) Current Experimental Methods for Characterizing Protein-Protein Interactions. ChemMedChem 11:738–756

5. Xing S, Wallmeroth N, Berendzen KW et al (2016) Techniques for the Analysis of Protein-Protein Interactions in Vivo. Plant Physiol 171:727–758

6. Yu Z-Q, Liu X-M, Zhao D et al (2021) Visual detection of binary, ternary and quaternary protein interactions in fission yeast using a Pil1 co-tethering assay. J Cell Sci 134:jcs258774

7. Kabeche R, Baldissard S, Hammond J et al (2011) The filament-forming protein Pil1 assembles linear eisosomes in fission yeast. Mol Biol Cell 22:4059–4067

8. Matsuyama A, Shirai A, Yashiroda Y et al (2004) pDUAL, a multipurpose, multicopy vector capable of chromosomal integration in fission yeast. Yeast 21:1289–1305

9. Wei Y, Wang H-T, Zhai Y et al (2014) Mdb1, a Fission Yeast Homolog of Human MDC1, Modulates DNA Damage Response and Mitotic Spindle Function.

PLoS ONE 9:e97028

10. Vještica A, Marek M, Nkosi PJ et al (2020) A toolbox of stable integration vectors in the fission yeast Schizosaccharomyces pombe. J Cell Sci. https://doi.org/10.1242/jcs.240754

11. Zhang J, Wang Y-Y, Pan Z-Q et al (2022) Structural mechanism of protein recognition by the FW domain of autophagy receptor Nbr1. Nat Commun 13:1–11

Tables

Table 1. SIV plasmids for expressing a bait protein fused with Pil1 and mCherry

Plasmid ID	Addgene ID	NBRP ID	Descriptive name	Locus of integration in the *S. pombe* genome	Antibiotic-resistance marker
pDB5225	215492	FYP6282	pUra4^{NotI}-P41nmt1-Pil1-mCherry-terminator^{ScADH1}-natMX	*ura4*	*natMX*
pDB5226	215493	FYP6283	pAde6^{NotI}-P41nmt1-Pil1-mCherry-terminator^{ScADH1}-hphMX	*ade6*	*hphMX*
pDB5227	215494	FYP6284	pLys3^{NotI}-P41nmt1-Pil1-mCherry-terminator^{ScADH1}-	*lys3*	*bsdMX*

			bsdMX		
pDB5228	215495	FYP6285	pHis5^{NotI}-P41nmt1-Pil1-mCherry-terminator^{ScADH1}-kanMX	*his5*	*kanMX*
pDB5230	215496	FYP6299	pAde6^{NotI}-P41nmt1-Pil1-mCherry-SpAtg8(1-115) terminator^{ScADH1}-hphMX	*ade6*	*hphMX*

Table 2. SIV plasmids for expressing a prey protein fused with mECitrine

Plasmid ID	Addgene ID	NBRP ID	Descriptive name	Locus of integration in the *S. pombe* genome	Antibiotic-resistance marker
pDB4875	215497	FYP6300	pUra4^{NotI}-P41nmt1-mECitrine-terminator^{ScADH1} natMX	*ura4*	*natMX*
pDB4878	215498	FYP6301	pAde6^{NotI}-P41nmt1-mECitrine-terminator^{ScADH1} hphMX	*ade6*	*hphMX*
pDB4881	215499	FYP6302	pLys3^{NotI}-	*lys3*	*bsdMX*

			P41nmt1-mECitrine-terminator^{ScADH1}-bsdMX		
pDB4884	215500	FYP6303	pHis5^{NotI}-P41nmt1-mECitrine-terminator^{ScADH1}-kanMX	*his5*	*kanMX*
pDB5253	215501	FYP6304	pUra4^{NotI}-P41nmt1-mECitrine-CtNbr1 terminator^{ScADH1}-natMX	*ura4*	*natMX*

Table 3. SIV plasmids for expressing a prey protein fused with mTurquoise2

Plasmid ID	Addgene ID	NBRP ID	Descriptive name	Locus of integration in the *S. pombe* genome	Antibiotic-resistance marker
pDB4887	215502	FYP6305	pUra4^{NotI}-P41nmt1-mTurquoise2-terminator^{ScADH1} natMX	*ura4*	*natMX*
pDB4890	215503	FYP6306	pAde6^{NotI}-P41nmt1-mTurquoise2-	*ade6*	*hphMX*

			terminator^{ScADH1} hphMX		
pDB4893	215504	FYP6307	pLys3^{NotI}-P41nmt1-mTurquoise2-terminator^{ScADH1}-bsdMX	*lys3*	*bsdMX*
pDB4896	215505	FYP6308	pHis5^{NotI}-P41nmt1-mTurquoise2-terminator^{ScADH1}-kanMX	*his5*	*kanMX*

Figure legends

Fig. 1 A schematic of the Pil1 co-tethering assay. The bait protein is fused with Pil1-mCherry and is localized on filamentary structures. If the mECitrine-fused prey protein can interact with the bait protein, it will exhibit co-localization with the bait protein on the same filamentary structures.

Fig. 2 Schematics of SIV plasmids for expressing bait or prey proteins (pUra4^{NotI} backbone as example). SIV plasmid backbone is shown in grey and the NotI linearization site is indicated. The coding sequence of a bait protein can be cloned into the BglII site of the plasmid shown on top, resulting in a plasmid expressing a Pil1-mCherry-bait fusion protein. The coding sequence of a prey proteins can be cloned into either the NheI or BglII site of a plasmid shown at the bottom, resulting in a plasmid expressing the prey protein either C-terminally or N-terminally fused with mECitrine or mTurquoise2.

Fig. 3 Detection of the interaction between SpAtg8(1-115) and CtNbr1. SpAtg8(1-115),

lacking the glycine 116 residue required for lipidation, was fused to Pil1-mCherry as bait and co-expressed with mECitrine-fused CtNbr1 in *S. pombe*. Pil1-mCherry served as a negative control bait. Deconvolved images focused on the top/bottom or mid-plane sections of the cells are shown. PCC values for the cells indicated by arrows are displayed. Scale bar, 2 μm.

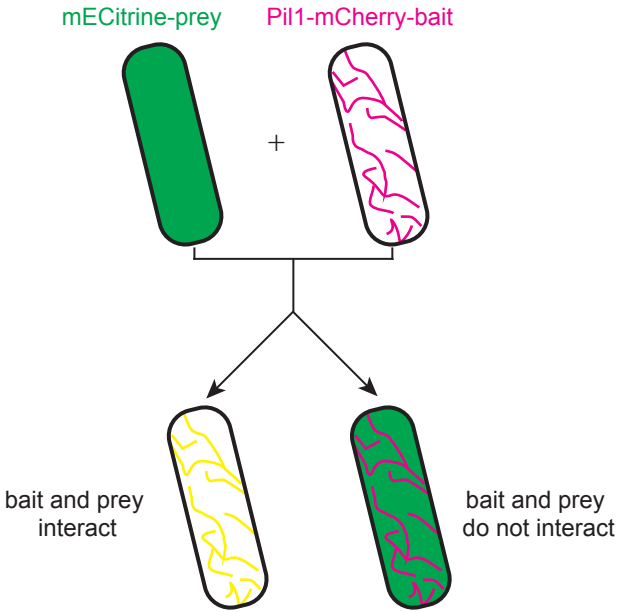
mECitrine-prey
Pil1-mCherry-bait
+
bait and prey
interact
bait and prey
do not interact

Figure 1

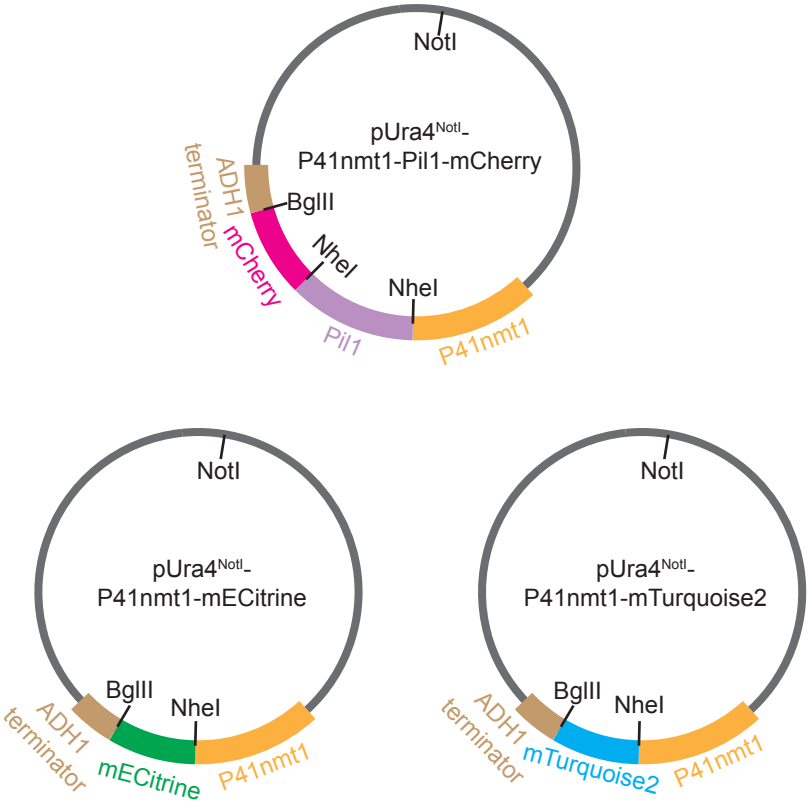
NotI
pUra4NotI-
P41nmt1-Pil1-mCherry
ADH1
terminator
BglII
mCherry
NheI
NheI
Pil1
P41nmt1
NotI
pUra4NotI-
P41nmt1-mECitrine
BglII
NheI
ADH1
terminator
mECitrine
P41nmt1
NotI
pUra4NotI-
P41nmt1-mTurquoise2
BglII
NheI
ADH1
terminator
mTurquoise2
P41nmt1

Figure 2

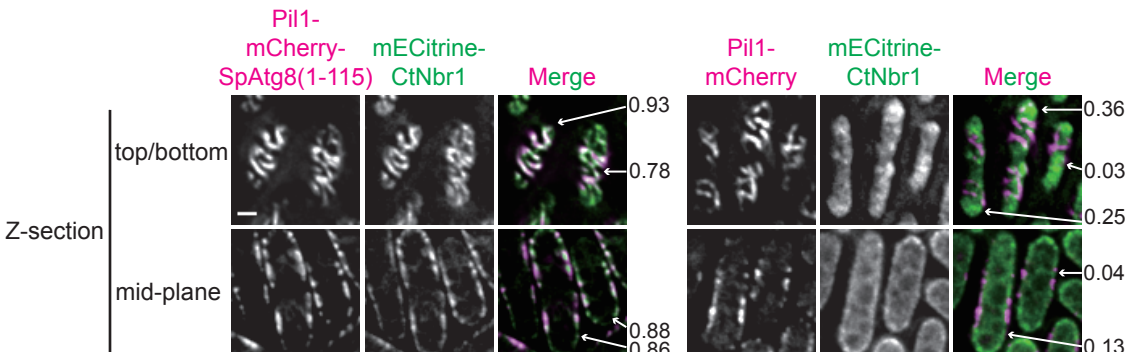
Pil1-
mCherry-
SpAtg8(1-115)
mECitrine-
CtNbr1
Merge
Pil1-
mCherry
mECitrine-
CtNbr1
Merge
Z-section
top/bottom
mid-plane
0.93
0.78
0.88
0.86
0.36
0.03
0.25
0.04
0.13

Figure 3