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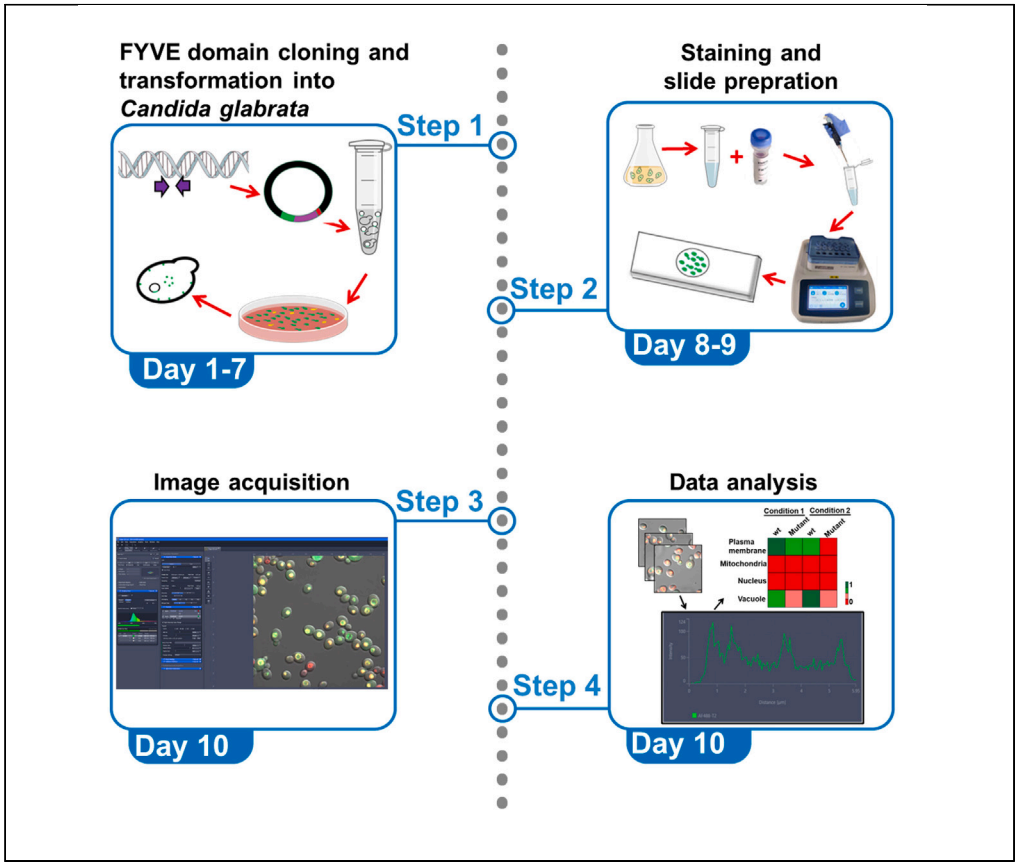
Protocol for determination of phosphatidylinositol 3-phosphate levels and localization in *Candida glabrata* by confocal microscopy



Phosphatidylinositol 3-phosphate (PI3P) levels govern membrane trafficking in *Candida glabrata*. Here, we present a confocal imaging-based protocol for PI3P localization analysis using the GFP-FYVE (found in Fab1, YOTB, Vac1, and EEA1) fusion protein. We describe steps for cloning the FYVE domain into the GFP-containing vector backbone, transforming FYVE-GFP into *C. glabrata*, and preparing slides with FYVE-GFP-expressing *C. glabrata* cells. We then detail procedures for acquiring and analyzing images and quantifying signal data. This protocol is adaptable to subcellular localization analysis of other low-abundant lipid and protein molecules.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

FYVE domain fusion with GFP fluorophore for expression in *Candida glabrata*

Step-by-step guide for sample and slide preparation

Details on setting up imaging acquisition configurations for enhanced signal quality

Interpretation and analysis of captured images

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Protocol for determination of phosphatidylinositol 3-phosphate levels and localization in *Candida glabrata* by confocal microscopy

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SUMMARY

Phosphatidylinositol 3-phosphate (PI3P) levels govern membrane trafficking in *Candida glabrata*. Here, we present a confocal imaging-based protocol for PI3P localization analysis using the GFP-FYVE (found in Fab1, YOTB, Vac1, and EEA1) fusion protein. We describe steps for cloning the FYVE domain into the GFP-containing vector backbone, transforming FYVE-GFP into *C. glabrata*, and preparing slides with FYVE-GFP-expressing *C. glabrata* cells. We then detail procedures for acquiring and analyzing images and quantifying signal data. This protocol is adaptable to subcellular localization analysis of other low-abundant lipid and protein molecules.

For complete details on the use and execution of this protocol, please refer to Askari et al. (2023).[1]

BEFORE YOU BEGIN

Candida (*Nakaseomyces*) *glabrata*, an opportunistic human fungal pathogen, causes mucosal and invasive infections, with the latter being associated with a mortality rate of 27%–58%.[2–6] The current protocol employs the phosphatidylinositol 3-phosphate (PI3P)-binding FYVE (found in Fab1, YOTB, Vac1, and EEA1) domain containing a double zinc-finger motif, and serves as a guide for examining the subcellular distribution and amounts of PI3P in *C. glabrata*. Notably, the phosphatidylinositol 3-kinase (CgVps34) converts phosphatidylinositol to PI3P, regulates retrograde trafficking of the high-affinity iron transporter from the plasma membrane, and is essential for virulence of *C. glabrata*.[1,7] To delineate how the PI3P lipid molecule, which is mostly confined to membrane microdomains,[8] controls trafficking of the plasma membrane proteins, we have cloned the FYVE domain-encoding sequence upstream of the GFP (Green Fluorescent Protein)-encoding sequence in the replicative pCU-PDC1-GFP plasmid (Addgene #45324).

We transformed and expressed the FYVE-GFP-containing plasmid in the uracil auxotroph, *wild-type* (*wt*) strain of *C. glabrata*. To maintain the pCU-PDC1-FYVE-GFP plasmid, *C. glabrata* strains were cultured in the Casamino Acid (CAA) medium that lacks uracil. To learn about the available vector backbones for cloning and expression of the FYVE domain, any other domain or a protein of interest (PoI) in *C. glabrata*, the articles by Zordan et al., 2012[9] and Carrillo et al., 2015[10] may be referred to. Further, we have used the counterstaining dyes for the plasma membrane (TMA-DPH) and the vacuole (FM 4–64) to study subcellular distribution of PI3P. Some other organelle-specific dyes, which have previously been used in *C. glabrata*, are listed in Table 1. Therefore, as per the experimental requirement, the FYVE domain may be cloned in an appropriate vector backbone, followed by staining with the organelle-specific dyes for subcellular localization analysis.

Table 1. List of organelle-specific dyes used in *C. glabrata*

Organelle	Dye	Reference
Cell wall	Calcofluor white	Dagher et al.[11]
Plasma membrane	TMA-DPH	Sharma et al.[12]
Vacuolar membrane	FM 4–64	Sharma et al.[12]
Vacuolar lumen	CMAC	Askari et al.[1]
Endoplasmic reticulum	ER-Tracker Blue-White DPX	Askari et al.[1]
Mitochondria	MitoTracker Green FM	Sharma et al.[12]
Nucleus	DAPI	Roetzer et al.[13]
	Hoechst	Bhakt et al.[14]

Important vector backbone details are described below.

Type of vector. Replicative and integrative plasmids, viz., pGRB2.3 (addgene #45343) and pCU-PDC1-GFP (addgene #45324), and pYC54 (addgene #63909) and pYC56 (Addgene #63910), can be used to express the PoI ectopically and from the genomic locus, respectively.

Choice of the fluorophore. GFP, YFP (Yellow Fluorescent Protein), CFP (Cyan Fluorescent Protein) and mCherry (Monomeric red fluorescent protein) are some fluorophores that can be used.

⚠ CRITICAL: It is important to ensure that the excitation and the emission wavelength spectrum of the chosen fluorophore does not overlap with that of the requisite organelle-specific staining dyes.

Terminus for tagging. The fluorophore can be tagged at the C-or the N-terminus of the domain or protein.

Promoter strength. The endogenous promoter for the gene of interest, or promoters with varying strengths, *CgEGD2*pr < *CgHHT2*pr < *CgPDC1*pr, can be used.

Type of promoter. The constitutive promoters, *CgEGD2*pr, *CgHHT2*pr and *CgPDC1*pr, or the regulatable promoters, *CgACO2*pr, *CgLYS21*pr and *CgMET3*pr, can be used.

Selection marker for the *C. glabrata* plasmid. The uracil biosynthesis (*CgURA3*) or the nourseothricin resistance-conferring (*nat1*) gene can be used.

C. glabrata strain background. For nat^R-marked vector, *wt* (BG2) or any other *C. glabrata* strain can be used, while the uracil auxotroph strain (BG14) of *C. glabrata* should be used for the plasmid carrying *CgURA3* as a selection marker.

Institutional permissions

Candida glabrata is a human opportunistic fungal pathogen. *C. glabrata* work described in this study was approved by the Institutional Biosafety Committee at the Centre for DNA Fingerprinting and Diagnostics. Researchers, who intend to adapt this protocol for pathogenic microbes, should obtain appropriate approvals from their institution.

FYVE domain cloning

Timing: 2–3 days

1. Design and order primers for amplification of the FYVE sequence using *C. glabrata* genomic DNA as template.

a. The corresponding nucleotide sequence for the FYVE domain, identified using the smart tool (http://smart.embl.de/), in any of these three PI3P-binding proteins, CgFab1 (CAGL0K10384p; 273–341 a.a), CgVac1 (CAGL0M01452p; 74–148 and 217–325 a.a) and CgVps27 (CAGL0I05830p; 165–231 a.a), can be retrieved from the *C. glabrata* genome database (http://www.candidagenome.org/).

Note: We have designed primers to amplify the FYVE domain-encoding sequence from the *CgFAB1* gene. The Forward primer sequence is CATATCTAGAATGCTATCGAAGGAA TATTG, and the Reverse primer sequence is CATACCCGGGGTTATCGGCGTGTTTGTAGC. The underlined sequences mark the restriction enzyme sites for cloning. The digestion sites of chosen restriction enzymes should fall within the multiple cloning site region of the vector backbone, and should be absent from the FYVE-encoding sequence

⚠ CRITICAL: For FYVE domain tagging at the C-terminus, add the start codon 'ATG' in the Forward primer. For the N-terminus tagging, add both the start codon 'ATG' in the Forward primer and the Stop codon 'TGA, TAA or TAG' in the Reverse primer.

2. Clone the FYVE domain-encoding sequence in the pCU-PDC1-GFP plasmid.
   a. Transform the ligation mixture into the *Escherichia coli* DH5-α strain.
   b. Select two independent *E. coli* transformants carrying pCU-PDC1-FYVE-GFP plasmid.
   c. Extract plasmid DNA using QIAGEN Miniprep kit, and store at −20°C for further use.

Transformation of the *C. glabrata* strain with the pCU-PDC1-FYVE-GFP plasmid

Timing: 3–5 days

3. To revive the *C. glabrata* uracil auxotroph strain from a frozen vial stored at −80°C, scrape the vial content with a tip, and use the tip to streak cells on a YPD plate.

Note: Based on the experimental requirements, multiple mutant and *wild-type* strains of *C. glabrata* can be transformed with the pCU-PDC1-FYVE-GFP plasmid.

4. Incubate the plate at 30°C for 16 h.
5. Pick a single colony from the YPD plate, and inoculate it into a tube containing 10 mL YPD medium.
6. Grow cells at 30°C, with continuous shaking at 10 × *g* for 12 h.
7. Measure culture absorbance at 600 nm in a spectrophotometer.
8. Use the 12 h grown cultures to set up a secondary culture at an initial OD_{600} of 0.1 in 10 mL YPD medium.
9. Grow cultures for ∼ 4 h, till OD_{600} reaches 0.5–0.6.
10. Harvest cells, and transform them with 150–300 ng of pCU-PDC1-FYVE-GFP plasmid using the lithium acetate method.[15]
11. Select *C. glabrata* transformants on CAA plate.
12. Purify uracil prototroph transformants by streaking for single colony on CAA plate.
13. Check FYVE-GFP expression in transformants either by Western blot analysis using anti-GFP antibody, or by microscopy.
14. Store FYVE-GFP-expressing *C. glabrata* strains at −80°C for further use.

Note: Store a minimum of three FYVE-GFP-expressing transformants for each strain.

⚠ CRITICAL: The FYVE-GFP-expressing *C. glabrata* strains should be stored at −80°C within 3–5 days of expression confirmation.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
FM 4–64 dye	Invitrogen	T3166
TMA-DPH (N,N,N-trimethyl-4-(6-phenyl-1,3,5-hexatrien-1-yl) phenylammonium p-toluenesulfonate)	Sigma	T0775
Tris (hydroxymethyl)aminomethane	MP Biomedicals	2103133
EDTA (ethylenediaminetetraacetic acid)	Sigma	E6635
Glucose	BD Difco	215530
Miniprep Kit	QIAGEN	27014
PBS (phosphate-buffered saline)	HiMedia	TL1031
YPD (yeast peptone dextrose)	BD Difco	242810
YNB (yeast nitrogen base without amino acid)	BD Difco	291940
CAA (casamino acid)	BD Difco	223120
LB (Luria broth)	BD Difco	244620
Glass slide	HiMedia	CG098
Coverslip	Blue Star	Blue Star micro cover glass
pCU-PDC1-GFP plasmid	Addgene	45324
Experimental models: Organisms/strains		
Escherichia coli DH5-α strain [*Δ(argF-lac)U169 supE44 hsdR17 recA1 endA1gyrA96 thi-1 relA1(φ80lacZΔM15)*]	Laboratory collection	D Hanahan[16]
Candida glabrata strain (BG14; *ura3Δ::Tn903 G418^R*, YRK19)	Laboratory collection	Cormack and Falkow[17]
BG2	Clinical isolate	Fidel et al.[18]
Oligonucleotides		
CATATCTAGAATGCTATCGAAGGAATATTG (OgRK2835; *FYVE* domain_forward primer, Xma*I* restriction site)	Eurofins Genomics India Pvt. Ltd.	N/A
CATACCCGGGGTTATCGGCGTGTTTGTAGC (OgRK2836; *FYVE* domain_reverse primer, Xba*I* restriction site)	Eurofins Genomics India Pvt. Ltd.	N/A
Software and algorithms		
Adobe Photoshop CS3	Adobe	http://www.adobe.com
Adobe Illustrator CS3	Adobe	http://www.adobe.com
Zen Black	Zeiss	https://www.zeiss.com/microscopy/en/products/software/zeiss-zen.html
Zen Blue	Zeiss	https://www.zeiss.com/microscopy/en/products/software/zeiss-zen.html
Other		
Zen LSM microscope	Zeiss	N/A

STEP-BY-STEP METHOD DETAILS

Preparing cells for imaging

Timing: 2–3 days

This step outlines the necessary procedures for sample preparation before proceeding for the imaging process.

1. Revive FYVE-GFP-expressing *C. glabrata* strain on a CAA plate 1–2 days, prior to the experiment.

Note: The revived strain can be stored at 4°C for a maximum of 15 days.

2. Prepare cells for imaging.
   a. Inoculate and grow the strain in 10 mL CAA medium for 16 h.

   Note: *C. glabrata* culturing conditions depend on the type of the experiment being performed, and can be changed accordingly.

   b. Use these cultures to set up a secondary culture at an initial OD_{600} of 0.1 in 10 mL CAA medium.
   c. Grow the culture at 30°C for 4 h to obtain logarithmic-phase cells.
   d. Next, stain the vacuole with FM 4–64.
      i. Harvest 4–5 OD_{600} logarithmic-phase cells at 2000 × *g* at 25°C.

   ⚠ CRITICAL: Use cells of same OD_{600} for all samples.

      ii. Suspend cells in 100 μL CAA medium containing 16 μM FM 4–64 dye.

   Alternatives: For vacuole staining, the CMAC dye, which stains the vacuolar lumen, can also be used.

      iii. Incubate at 30°C with continuous agitation at 10 × *g* for 2 h.
      iv. Wash cells three times with 1 mL CAA medium at 25°C. Collect cells via centrifugation at 2000 × *g* for 5 min.

   ⚠ CRITICAL: Cells need to be washed properly, before proceeding for the FM 4–64 chasing step

   e. Grow FM 4-64-labeled cells in 10 mL CAA medium for 2 h at 30°C.

   Note: This step of FM 4–64 chasing is to ensure that FM 4–64 reaches and stains the vacuolar membrane.

   Note: For FM 4–64 staining in the YPD medium, labeling time should be reduced to 30 min.

   f. Wash the cells thrice with 1 mL PBS at 2000 × *g* for 5 min at 25°C.
   g. Stain the plasma membrane with TMA-DPH dye.
      i. Incubate 4–5 OD_{600} FM 4-64-labeled cells in 100 μL Tris-HCl buffer [1 M Tris (pH 7.5), 0.5 M EDTA] containing 0.5 μM TMA-DPH dye at 25°C for 10 min with continuous agitation at 10 × *g*.

   ⚠ CRITICAL: For plasma membrane staining with TMA-DPH dye, always prepare Tris buffer fresh.

      ii. Wash cells three times with chilled 1 mL PBS at 4°C and place on ice.
      iii. Suspend cells in 200 μL of chilled PBS and store on ice.

   ⚠ CRITICAL: Since *C. glabrata* cells are not fixed in this protocol, images should be acquired on the same day.

Confocal imaging of FYVE-GFP-expressing *C. glabrata* cells

Timing: 3–4 h

This step highlights important parameters for image acquisition, and gives directives for image analysis.

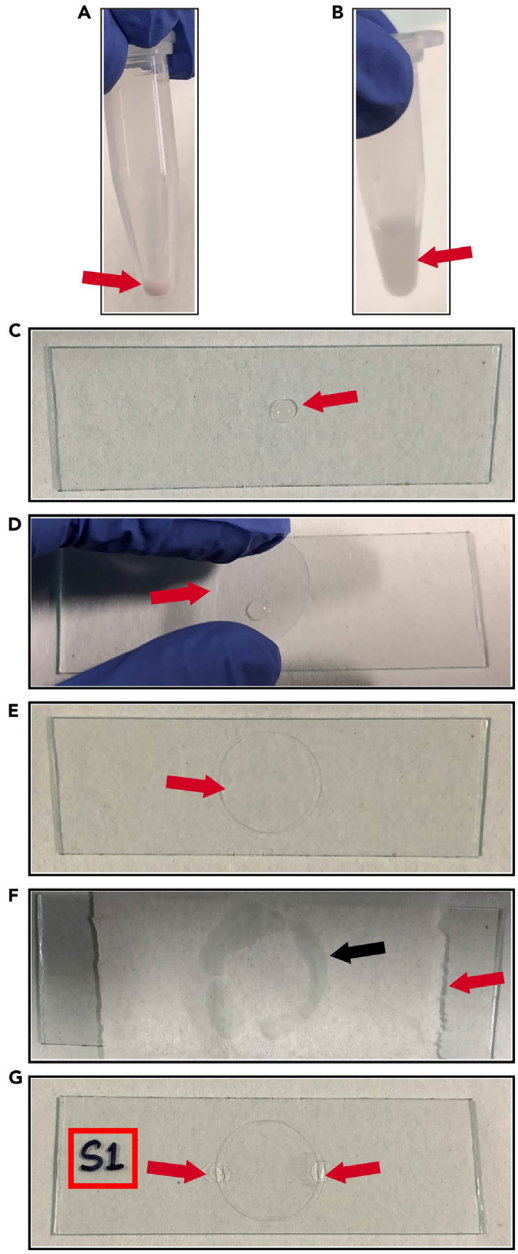

Figure 1. Steps for preparation of slides for imaging

(A) A microcentrifuge tube displaying pelleted-*C. glabrata* cells.

(B) A microcentrifuge tube showing PBS-cell suspension.

(C) A 6 μL sample drop in the middle of the slide.

(D) The gentle placement of an inclined coverslip over the sample drop.

(E) A properly-positioned coverslip.

(F) A tissue paper kept gently on the top of the coverslip (indicated by the red arrow) to remove excess cells, as indicated by the black arrow.

(G) A coverslip sealed with the nail paint, and the slide is labeled as S1 (Sample 1) on the left-hand side with a marker.

3. Clean glass slides and coverslips with Kimwipes dipped in 70% ethanol, and air dry, before slide preparation. Protect glass slides and coverslips from dust. The slide preparation steps, mentioned below, are illustrated in Figure 1.

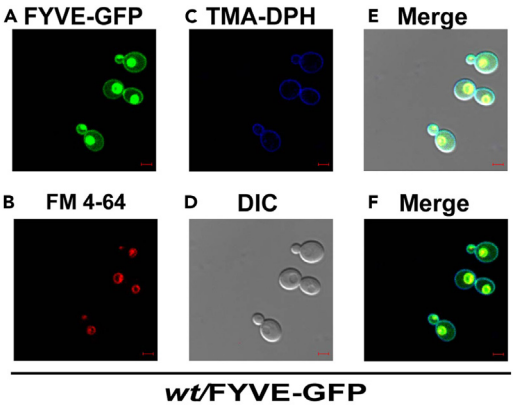

Figure 2. Representative confocal images of FYVE-GFP-expressing, logarithmic-phase *wild-type* (*wt*) *C. glabrata* cells

Scale bar = 2 μm.

(A) PI3P cellular localization, as depicted by FYVE-GFP fluorescence.

(B) Vacuolar membrane stained with FM 4–64 dye.

(C) Plasma membrane stained with TMA-DPH dye.

(D) Differential Interference Contrast (DIC) image.

(E) Overlay of Panels, A, B and C, with the DIC image (Panel D).

(F) Overlay of A, B and C Panels.

a. Place 6 μL PBS cell suspension in the middle of the slide.
b. Keep the coverslip gently on the top of the cell suspension, avoiding the entry of air bubbles. Remove any excess cells by placing a tissue paper over the coverslip.

⚠ CRITICAL: The slides and coverslips should be clean, and totally dust- and smudge-free.

c. Put the nail paint on two edges of the cover slip to seal the coverslip.

Note: In case of multiple samples, slides are freshly prepared for each sample.

d. Wait for 30–60 s to let the cells settle down, before proceeding for image acquisition.

Note: The slides may get dry while imaging. Thus, to acquire images for more than 30 min, prepare a fresh slide with the same sample.

Alternatives: Slides can be coated with poly-L-lysine or concanavalin A to immobilize cells.

4. Image cells using the confocal microscope. One representative acquired image is shown in Figure 2.
   a. Switch on the microscope system, along with other components viz., laser and detector. It ensures that the hardware necessary for image acquisition is ready.
   b. Switch on the Zen Black software.

Note: We have used the Zeiss confocal microscope. Its inbuilt software, Zen Black and Zen Blue, were used for image acquisition and analysis, respectively.

Alternatives: Any other confocal microscope such as Leica, Nikon etc. can be used.

   c. Select the objective lens, laser lines, detectors and scanning parameters (Figures 3A–3D).
      i. Select the objective lens in the Zen Black software under the 'Locate Menu' command, which is encircled in yellow in Figure 3A.

Note: We have used the Plan-Apochromat 63x/1.40 Oil objective lens. This lens magnifies the image 63 times of the actual size, and has 1.4 Numerical aperture (NA). NA determines the lateral and axial resolution in the sample. The higher the NA of the objective lens, the better will be the resolution. The Plan-Apochromat lens aids in accurate color reproduction and yields better quality images. The immersion oil increases the resolution and improves the image quality. Overall, based on the magnification and the resolution requirement of the experimental set-up, the appropriate lens can be chosen.

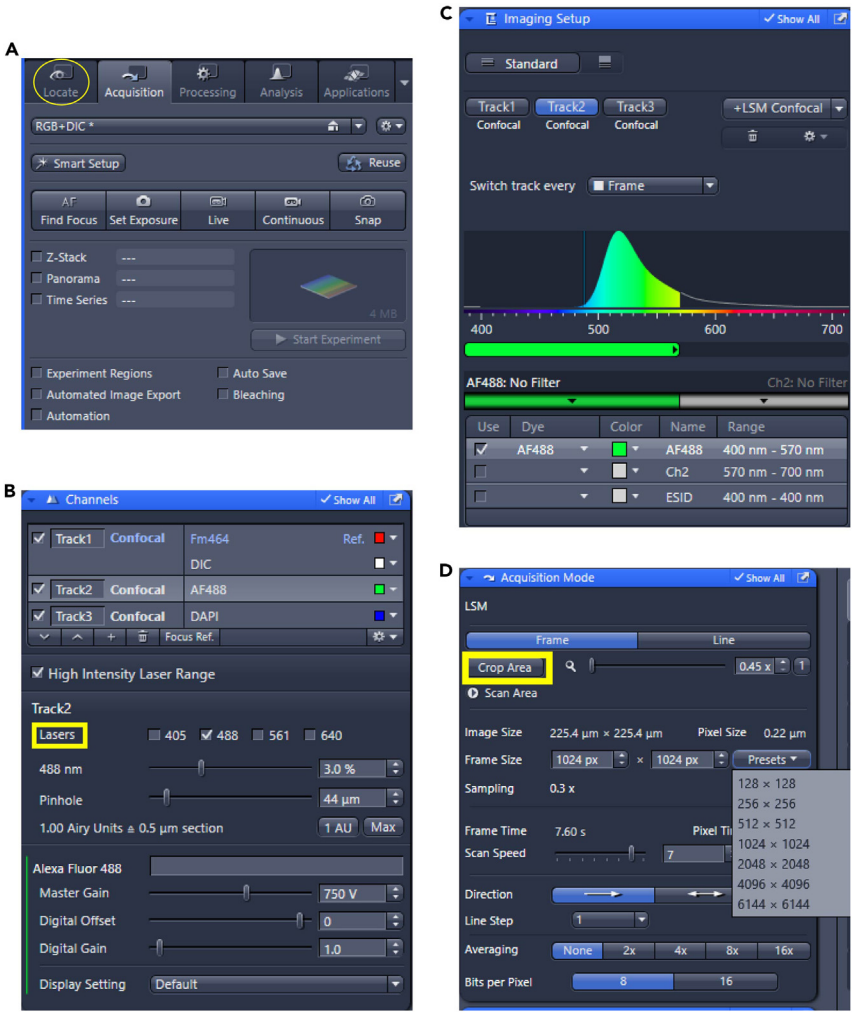

Figure 3. Image acquisition parameter settings in the Zen Black software

(A) The 'Locate' option is used to select the objective lens, and is marked by the yellow circle. Adjacent to it, is the 'Acquisition' option, which is used to open the settings for image acquisition parameters.

(B) The 'Channels' option allows laser selection, and the laser power and gain can be adjusted using further settings, under this option.

(C) The 'Imaging Setup' is used to adjust detector settings.

(D) The 'Acquisition mode' is used to set-up scanning parameters, and to select the region of interest to acquire image from the whole field, using the 'Crop Area' option.

ii. Adjust the laser line and its settings, as shown under the 'Channels' option in Figure 3B.

Note: We have used three different laser lines, 405, 488 and 561, for FM4-64, GFP and TMA-DPH, respectively. The laser line choice depends on the excitation wavelength of the fluorophores and dyes being used in the experiment.

iii. Select the detector settings, as shown under the 'Imaging setup' option in Figure 3C.

Note: The detector is selected based on the emission spectrum of the fluorophores and dyes being used in the experiment.

⚠ CRITICAL: The accurate selection of laser line and detector minimizes the issues of photobleaching, autofluorescence and bleed-through in the samples.

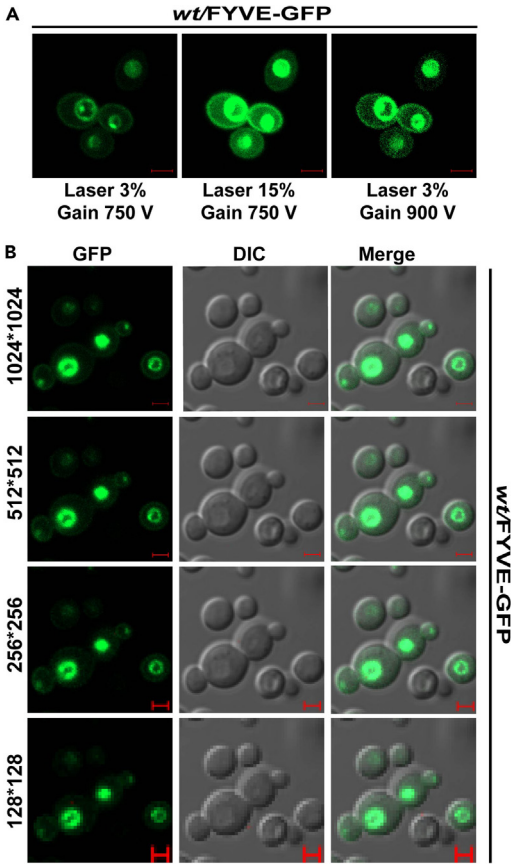

Figure 4. Image acquisition parameter comparison to improve the image quality

Scale bar = 2 μm.

(A) Images illustrating signal saturation and signal haziness under settings of increased laser power (percentage) and laser gain (Voltage; V), respectively.

(B) Images displaying resolution variations in images acquired using different pixel sizes.

d. Set the laser power and detector gain. These settings can be adjusted, as shown under the 'Channels' option in Figure 3B.

Note: The laser power should be selected to excite fluorophores and dyes optimally. While the higher laser power can cause photobleaching, phototoxicity or oversaturated signal, the low laser power may yield weak or no fluorescence signal.

Note: The optimal detector gain is important to minimize the signal to noise ratio.

e. Confirm the accuracy of adjusted settings.

Note: Figure 4A shows both the impact on signal saturation, when samples are illuminated with laser power higher than required, and on image quality when using higher than required gain.

f. Set up the scanning parameters including scanning mode, scan speed and pixel size (Figure 3D).

Note: These features help in balancing the time required for image capturing and acquiring best image resolution. Figure 4B shows the images captured at four different pixel sizes, 1204*1204, 512*512, 256*256 and 128*128.

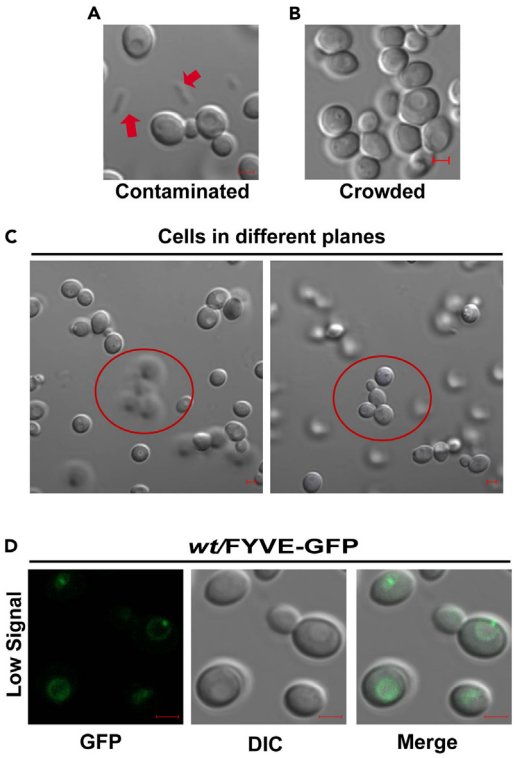

Figure 5. Some problems appearing during image acquisition

Scale bar = 2 μm.

(A) *C. glabrata* cells contaminated with rod-shaped bacteria (marked by the red arrow).

(B) Dense cell suspension.

(C) Cells present in distinct focal planes. One plane is marked by the red circle.

(D) Cells displaying low GFP signal intensity.

Note: The Pixel size correlates with image resolution, the fewer the pixels are, the less-resolved the image will be.

g. Define the region of interest (ROI). It can be selected from the 'Acquisition mode' option (Crop Area), as shown in Figure 3D.
h. Acquire images. Figure 5 marks the cells that can impact image acquisition and quality. These issues should be resolved prior to image capturing.
i. Save images and proceed for analysis.

Pause point: Saved images can be processed for signal quantification either on the same day or at a later time.

Quantification and analysis of GFP signal intensity

Timing: 2–3 h

These steps provide instructions for measurement and quantification of the GFP signal intensity from various acquired images.

5. Analyze images to extract the quantitative information.
   a. Open images in the Zen Blue software.

Alternatives: Images can be analyzed by various other software including LAS X, NIS-Elements, and ImageJ/Fiji etc.

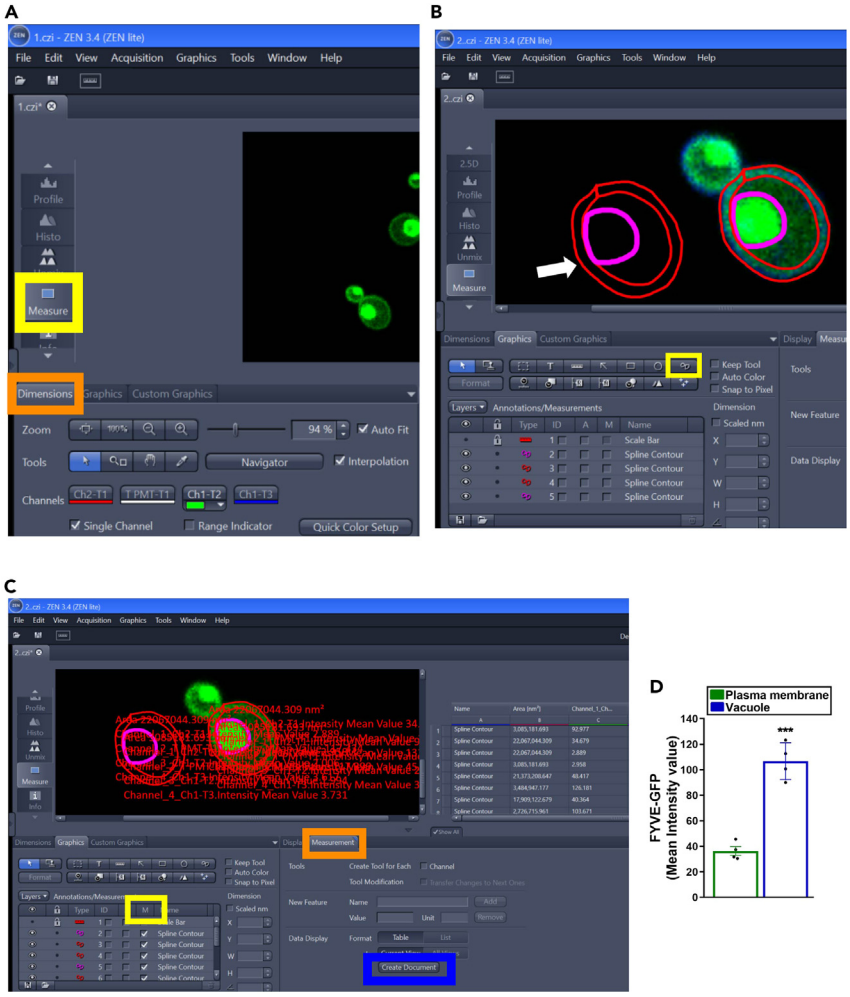

Figure 6. Steps for signal quantification in the Zen Blue software

(A) Image illustrating 'Measure' and 'Dimensions' options, marked by yellow and orange boxes, respectively, for FYVE-GFP signal intensity quantification.

(B) Steps for drawing the spline contour. Yellow box marks the 'Spline contour' option. The red and pink spline contours mark the plasma membrane and vacuole, respectively. The blank space is marked by a white arrow.

(C) Image illustrating FYVE-GFP signal intensity data generated in a tabular format. The 'M' option marked by the yellow box under the 'Graphics' menu enables the table display on the right-hand side. The "Create Document" under 'Measurement' option, marked by the blue and orange box, respectively facilitates export of the table.

(D) Bar plot showing higher FYVE-GFP levels in the vacuole, compared to the plasma membrane. Four cells were used to quantify signal, and sample mean intensity values are plotted arbitrary units as. Data represent mean ± SEM (n = 4). $p \leq 0.001$; Unpaired, two-tailed Student's t-test was applied. (Signal should ideally be quantified in 100–150 cells).

b. Quantify FYVE-GFP signal intensity on the plasma membrane and the vacuole by selecting the 'Measure' option from the left side, and the GFP Channel from the 'Dimensions' menu, as marked by yellow and orange boxes, respectively, in Figure 6A.

c. From the 'Graphics' menu, select the 'spline contour' option, as marked by the yellow box in Figure 6B.

d. Draw one spline contour close to the cell boundary and another one enclosing the vacuole for measuring FYVE-GFP signal intensity at the plasma membrane and in the vacuole, respectively.

   i. Copy the same spline contour to the blank space where cells are not present.

   ii. The intensity values obtained from the blank space are considered as background signal.

e. To access information on the area and mean intensity values for the selected GFP channel under the spline contour, tick off the 'M' option within the 'Graphics' menu (indicated by the yellow box in Figure 6C).

Note: This step will create a 'table containing desired information' on the right-hand side of the image (Figure 6C).

f. Select the 'Measurement' option (marked by the orange box in Figure 6C), and then select the 'Create document' (marked by the blue box in Figure 6C) to export the table as a comma-separated values (CSV) file.
g. Subtract the background intensity values from sample mean intensity values, and plot data as arbitrary units in the GraphPad prism (Figure 6D).

Note: Statistical significance of fluorescence intensity differences between two organelles (plasma membrane and vacuole; Figure 6D) can be determined by employing an appropriate statistical test, such as Student's t-test.

h. Next, choose the image that accurately reflects the findings of all acquired images.
i. Select the 'Profile plot' option from the left side, as marked by the white box (Figure 7A) or from the 'Graphics' menu, as shown in Figure 7A.
j. Click and drag the profile plot arrow (highlighted by the yellow box in Figure 7A), along with the selected path marked by the yellow circle in Figure 7A.
k. Generate the graph plot displaying pixel intensity differences for all fluorophores, covered within the drawn line.

Note: Figure 7B shows the colocalization of all three fluorophores, along with the line drawn. Depending on the experimental requirement, fluorophore intensity plot combinations can be selected. We observed FYYE-GFP distribution on the plasma membrane as well as in the vacuole with varying degrees of intensity (Figure 7B).

Note: By choosing the 'Channels' option, marked by the orange box in Figure 7A, fluorophore localization either solely at the plasma membrane or in the vacuole, or at both cellular organelles can be illustrated.

l. Modify the intensity plot data against the distance generated for the fluorophore by selecting and changing, respectively, the 'Profile view' and 'X and Y-axis settings' options, marked by the yellow box and the orange box in Figure 7C.

Note: The profile plot can also be exported as a CSV file, as highlighted by the yellow circle in Figure 7C. These data can further be used in the GraphPad prism or Microsoft Excel programs to visualize and illustrate results in multiple ways.

m. Generate the profile plot that will provide the spatial distribution and the intensity of fluorescent signals across the line drawn.

EXPECTED OUTCOMES

We observed a distinct subcellular localization of FYVE-GFP in *C. glabrata*, with the signal being present at the plasma membrane and the vacuole. Notably, GFP signal intensity was higher in the vacuole, compared to the plasma membrane (Figure 6D). Since the FYVE domain was used as a read-out of PI3P, we infer that PI3P is localized at both the plasma membrane and the vacuole, *albeit* PI3P levels are higher in the vacuole. Since PI3P amounts may vary in response to environmental conditions, gene mutations or deletions, this detection method can be used to investigate intracellular

Figure 7. Steps for generation of profile plot in the Zen Blue software

(A) Image illustrating the process of drawing the profile plot. 'Graphics' and 'Profile' options are marked by white boxes. The yellow circle denotes a cell with the line drawn across the cell. Yellow and orange boxes mark, "Arrow" and "Channel" respectively.

(B) Profile plots obtained using the pixel intensity combinations of different channels (Red, Green and Blue).

(C) Images illustrating options for profile plot editing (yellow and orange boxes), and signal intensity data export (yellow circle).

PI3P dynamics in different strain backgrounds, and under different growth and host-mimicking conditions. For this analysis, FYVE-GFP construct can be transformed into appropriate strains/clinical isolates, followed by confocal imaging of transformants.

QUANTIFICATION AND STATISTICAL ANALYSIS

A total of 100–150 cells should be counted in each experiment, and the experiments should be repeated at least thrice. Data, the percentage of cells expressing fluorophore signal in a specific organelle, can be plotted as a scatter plot, with error bars representing standard error of the mean. Based on the experimental set-up, data may also be normalized against the *wild-type* strain or untreated condition. Significance of the observed differences among samples can be determined using the t-test or ANOVA test.

LIMITATIONS

Since FYVE is a PI3P-binding domain, the obtained FYVE-GFP signal data can be used for determining relative intracellular PI3P levels across different samples/conditions, but not for measuring absolute intracellular PI3P levels. Further, the intracellular FYVE-GFP distribution may not precisely match with the actual PI3P location inside the cell, as GFP signal arising from the high-abundant PI3P

cellular sites may mask the signal of organelles containing low amounts of PI3P. Lastly, rapidly-occurring changes in PI3P turnover may be missed due to the kinetics of FYVE-PI3P binding.

TROUBLESHOOTING

Problem 1

Unable to detect PI3P localization or intensity differences between control and experimental samples (Step3: before you begin).

Potential solution

- Choose another control sample.
- Change the concentration and/or duration of the stressor treatment.

Problem 2

Fluorescent signal of the FYVE-GFP construct is weak. (Step 13: before you begin).

Potential solution

- Express FYVE-GFP from a strong promoter.
- Express FYVE-GFP from a multicopy plasmid.
- Insert a small linker region between FYVE domain and GFP.
- Choose another fluorophore, instead of GFP.

Problem 3

The FYVE-GFP signal partially colocalizes with the organelle-specific dye (Step 4c: step-by-step method details).

Potential solution

- Use Z-stack images. It will capture the entire three-dimensional structure of the cell. This will help to determine if the FYVE-GFP signal truly coexists with the organelle-specific stain in specific cellular compartments.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Rupinder Kaur (rkaur@cdfd.org.in).

Materials availability

All the reagents used are listed in the key resources table. The FYVE-GFP construct is available on request.

Data and code availability

This protocol has not generated any new dataset. The confocal images acquired are available from the lead contact, upon request.

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AUTHOR CONTRIBUTIONS

F.A. and R.K. conceived the idea, established the protocol, prepared figures, and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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