

Submission ID #: 71709

Scriptwriter Name: Ruchira Mukherji

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Title: Contactless Co-Culture Assays for Morphometric Studies During Inter-Species Interactions in Fungi

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Author Questionnaire

1. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **Yes**

If **Yes**, can you record movies/images using your own microscope camera? **Yes**

Nikon Eclipse Ti2 laser free confocal microscope.

2. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes**

3. Filming location: Will the filming need to take place in multiple locations? **No; it will be within the lab.**

4. Testimonials (optional): Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 16

Number of Shots: 44

Interviews

Videographer: Obtain headshots for all authors available at the filming location.

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Rajesh Patkar:** We study molecular communication in host–pathogen and fungal interspecies interactions, asking how secreted signals shape microbial morphology and behavior.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Rajesh Patkar:** Existing methods can be laborious, costly, or confusing; this protocol offers a simple, adaptable contactless assay to study novel microbial inter-species interactions.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the research contexts or systems in which this protocol can be applied.~~

- 1.3. **Rajesh Patkar:** This protocol supports fundamental and translational research on interspecies and cross-kingdom interactions across diverse biological and clinical systems.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.4. **Parth Trivedi, PhD Student (Fungal Patho-biology lab, IITB):** The protocol enables researchers to investigate multispecies coexistence by measuring morphological responses to interspecies signaling within microbial communities.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.5. **Parth Trivedi, PhD Student (Fungal Patho-biology lab, IITB):** The key challenge here is ensuring molecular exchange across the membrane, which is addressed by plate agitation and maintaining sufficient hydrostatic pressure.

1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.6. **Parth Trivedi, PhD Student (Fungal Patho-biology lab, IITB):** Live-cell imaging of organellar dynamics could reveal how molecular communication mechanistically shapes interspecies interactions.

1.6.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

2. Preparing Fungal Strains for Co-Culture

Demonstrator: Parth Trivedi

2.1. Begin by placing a Glucose Minimal Medium agar plate, with colonies of *Candida glabrata* (*can-dee-duh gluh-brah-tah*) CBS138 (*see-bee-es one-three-eight*), a sterile loop, and a tube containing sterile Yeast-extract Peptone Dextrose or YPD (*Y-P-D*) broth inside the biosafety cabinet [1]. Pick a single isolated colony from the plate and inoculate it into 10 milliliters of YPD broth [2]. Incubate at 37 degrees Celsius with continuous agitation, at 200 rpm for 18 hours [3].

2.1.1. WIDE: Talent arranging the GMM agar plate, sterile inoculation loop, and a culture tube of YPD broth inside the biosafety cabinet.

2.1.2. CU: Talent picking a single *Candida glabrata* CBS138 colony and inoculating it into 10 milliliters of YPD broth.

2.1.3. Talent placing the culture tube in the orbital shaking incubator and setting it to 37 degrees Celsius and 200 rpm.

***Videographer:** Please obtain multiple reusable takes of 2.1.3, as these will be used later in the protocol.*

2.2. Take the overnight culture, verify yeast morphology, and the absence of contamination by light microscopy [1]. Transfer the culture to a 15-milliliter conical tube, centrifuge at 5,000 g for 5 minutes at 25 degrees Celsius [2]. Gently discard the supernatant and save the cell pellet for the membrane insert assay [3].

2.2.1. SCOPE: 71709_2.2.1.v2.tif

2.2.2. Talent transferring the culture to a 15-milliliter conical tube and placing the tube in the centrifuge.

2.2.3. CU: Talent removing the supernatant without disturbing the *Candida glabrata* cell pellet and keeping the tube aside.

2.3. For the target *Candida albicans* (*can-dee-duh al-bee-cans*) strain, inoculate a single colony into 5 milliliters of GMM (*gee-em-em*) broth for a diploid strain, or YPD broth

for a haploid strain [1]. Incubate the inoculated culture at 37 degrees Celsius, with agitation at 200 rpm for 12 hours [2]. Check the optical density of the overnight culture at 600 nanometers [3]. Next, dilute the culture with pre-warmed GMM to a final volume of 1 milliliter and a final OD at 600 nanometers of 0.1 [4].

2.3.1. CU: Talent inoculating a single *Candida albicans* colony into 5 milliliters of GMM or YPD broth, according to the strain ploidy.

2.3.2. *Please reuse 2.1.3. NOTE: A similar shot has been added here, because the medium used in 2.1.3 is different from the one used in 2.3. The videographer has tagged it as 2.3.2.*

2.3.3. Shot of talent placing a cuvette, with an overnight culture *Candida albicans* into the spectrophotometer, to measure the optical density.

2.3.4. Shot of talent adjusting the optical density of the culture with pre-warmed GMM broth.

Videographer: Please obtain multiple reusable takes of 2.3.4, as these will be used later in the protocol.

3. Membrane Insert Well Plate Co-Culture Assay

3.1. Take a sterile 24-well tissue culture plate [1], add 1 milliliter of *Candida albicans* suspension with an OD at 600 nanometers of 0.1 into each selected lower, or the abluminal chamber of a dual-compartment cell culture plate [2].

3.1.1. WIDE: Talent placing a sterile 24-well tissue culture plate inside the biosafety cabinet.

3.1.2. CU: Talent dispensing 1 milliliter of the normalized *Candida albicans* suspension into each required lower chamber.

3.2. Next, retrieve the *Candida glabrata* pellet and resuspend it in 1 milliliter of GMM broth [1]. Centrifuge at 5,000 *g* for 3 minutes and discard the supernatant [2]. Repeat the wash twice [3]. Resuspend the final pellet in fresh GMM broth [4] and adjust the OD at 600 nanometers of the cell suspension to 5 [5].

3.2.1. CU: Talent resuspending the retained *Candida glabrata* pellet in 1 milliliter of sterile GMM broth.

Videographer: Please obtain multiple reusable takes of 3.2.1, as these will be used later in the protocol.

3.2.2. Shot of talent placing the tube with the cell suspension in the centrifuge and discarding the supernatant afterwards.

Videographer: Please obtain multiple reusable takes of 3.2.2, as these will be used later in the protocol.

3.2.3. *Please reuse 3.2.1 and 3.2.2.*

3.2.4. CU: Talent resuspending the washed pellet in fresh GMM broth.

3.2.5. *Please reuse 2.3.4.*

3.3. Next, using sterile forceps, place a sterile membrane insert with a 0.4-micrometer-pore polycarbonate membrane into each working well [1]. Secure the insert firmly in the wells [2] and dispense 750 microliters of the prepared *Candida glabrata* suspension into each upper, or luminal, chamber [3].

3.3.1. CU: Talent holding a sterile membrane insert, with a track-etched 0.4-micrometer-pore polycarbonate membrane, with forceps over a well.

3.3.2. CU: Talent lowering the membrane insert into a working well and securing it in place with the flanges on the middle notches.

3.3.3. CU: Talent adding 750 microliters of *Candida glabrata* suspension to the upper insert chamber.

3.4. Cover the assembled plate and incubate it at 37 degrees Celsius, static, for 3 hours [1]. After incubation, use sterile forceps to remove each upper insert and discard it safely [3]. Position the 24-well plate on an inverted microscope, equipped with DIC (*dee-i-see*) optics and 10X (*ten-ex*), 20X (*twenty-ex*), and extra-long-working-distance 40X (*forty-ex*) objectives for morphological evaluation [4].

3.4.1. WIDE: Talent placing the covered membrane insert plate, static, in a 37 degrees Celsius incubator.

3.4.2. CU: Talent removing each upper insert from the plate wells and discarding them into the biohazard waste.

3.4.3. WIDE: Talent positioning the 24-well plate on the inverted microscope for monitoring morphological changes in the culture during growth.

4. High-Resolution Microscopy and Digital Image Analysis

4.1. After the required incubation period [1], scan the well floors with the 10X objective to verify uniform cell distribution and identify tracking errors or anomalous clustering

[2]. Capture at least 10 separate fields of view with the calibrated 20X objective for the core filament-count and population-distribution dataset [3]. For fine phenotypic analysis and cell-boundary measurements, capture micrographs with the 40X extra-long-working-distance objective [4]. Then, save all raw image files [5].

4.1.1. WIDE: Talent sitting at the computer attached to the inverted microscope to begin morphological evaluation.

4.1.2. SCREEN: 71709_4.1.mp4: 00:16-00:30

4.1.3. SCREEN: 71709_4.1.mp4: 00:49-00:53, 01:02-01:02, 02:00-02:05, 02:14-02:15

4.1.4. SCREEN: 71709_4.1.mp4: 03:23-03:27, 03:44-03:50

4.1.5. SCREEN: 71709_4.1.mp4: 03:55-04:05.

4.2. Launch the latest version of Fiji (*FEE-jee*) software [1]. Open the target micrograph by selecting **File**, followed by **Open** [2].

4.2.1. SCREEN: 71709_4.2.mp4: 00:00-00:10

4.2.2. SCREEN: 71709_4.2.mp4: 00:11-00:20

4.3. To measure hyphal length, right-click the **Line Selection** icon and select the **Segmented Line Tool** [1]. Place the cursor at the junction where the filament emerges from the mother yeast cell wall [2]. Trace the central longitudinal axis of the hypha, adding anchor points to follow curves and bends, and ending at the apical tip [3]. Run the **Measure** command using **Control-M** on Windows or **Command-M** on macOS to log the total length, **L**, in micrometers in the **Results** window [4].

4.3.1. SCREEN: 71709_4.3.mp4: 00:00-00:06

4.3.2. SCREEN: 71709_4.3.mp4: 00:07-00:10

4.3.3. SCREEN: 71709_4.3.mp4: 00:10-00:22

4.3.4. SCREEN: 71709_4.3.mp4: 00:22-en

4.4. Use the **Brush Tool** to mark the measured cell and prevent duplicate analysis [1]. Locate its attached mother yeast cell [2]. With the **Segmented Line Tool**, draw and measure a straight line across the maximum long axis of the cell to record the major axis, **a** [3]. Then draw and measure a line perpendicular to the major axis across the widest transverse span to record the minor axis, **b** [4]. Calculate the mother yeast cell volume, **CV** (**C-V**), in cubic micrometers using the prolate-spheroid model, included in the software [5-TXT].

4.4.1. SCREEN: 71709_4.4.mp4: 00:00-00:02

4.4.2. SCREEN: 71709_4.4.mp4: 00:02-00:04

4.4.3. SCREEN: 71709_4.4.mp4: 00:04-00:09

4.4.4. SCREEN: 71709_4.4.mp4: 00:09-00:15

4.4.5. SCREEN: 71709_4.5.mp4: 00:32-00:33 **TXT- Cell Volume = $\frac{4}{3} \pi ab^2$**
Video Editor: 4/3 is a fraction **NOTE: Highlight D1 and D2 cells**

4.5. To measure hyphal thickness, use the **Segmented Line Tool** to draw an outer-wall-to-outer-wall line perpendicular to the hyphal length axis **[1]**. Record the diameter at three equidistant positions along the filament **[2]**. Calculate the arithmetic mean of the three diameters to obtain the average thickness, **d**, and divide it by 2 to obtain the radius, **r**. Calculate the total hyphal volume, **HV**, in cubic micrometers by treating the unbranched filament as a cylinder **[4 - TXT]**.

4.5.1. SCREEN: 71709_4.5.mp4: 00:00-00:05.

4.5.2. SCREEN: 71709_4.5.mp4: 00:05-00:21

4.5.3. SCREEN: 71709_4.5.mp4: 00:21-00:38

4.5.4. SCREEN: 71709_4.5.mp4: 00:21-00:38 **TXT - Hyphal Volume = πr^2**

5. Statistical Design and Analysis

5.1. Calculate the mean or median cell measurement for each biological replicate and use these replicate-level values for downstream statistical comparisons **[1-TXT]**.

5.1.1. Shot of talent recording multiple values for cell measurements from all biological and technical replicates and calculating the mean. **TXT- Refer to the text protocol for replication and cell measurement details**

5.2. Before applying parametric methods, assess the biological replicate dataset for normality using the Shapiro-Wilk or D'Agostino-Pearson test **[1]**. For a two-group comparison, use an unpaired, two-tailed Student's T-test when the dataset follows a normal distribution **[2]**. After performing the statistical analyses and preparing the graphical representations, apply consistent statistical notations across all figures **[3]**.

5.2.1. SCREEN: 71709_5.2..mp4: 00:12-00:35

5.2.2. SCREEN: 71709_5.2..mp4: 00:41-00:56, 01:09-01:25

5.2.3. Shot of talent at the computer preparing graphs and applying statistical notations to figures.

Results

6. Results

6.1. *Candida albicans* cells developed true hyphae in two contactless assay systems, either when exposed to membrane-separated *Candida glabrata* cells [1] or filtered *Candida glabrata* cell-free supernatant [2]. The control cells, on the other hand, predominantly retained yeast and pseudo-hyphal morphologies [3].

6.1.1. LAB MEDIA: Figure 2 **Video Editor:** Please highlight the image labeled '*C. glabrata in luminal well*' in Figure 2A, where a white arrowhead is pointing to - true hyphae.

6.1.2. LAB MEDIA: Figure 2 **Video Editor:** Please highlight the image labeled '*C. glabrata CFS*' in Figure 2A, where a white arrowhead is pointing to - true hyphae.

6.1.3. LAB MEDIA: Figure 2 **Video Editor:** Please highlight the image labeled '*YPD Control*' in Figure 2A, where a pink arrowhead points to yeast cells and black arrowheads to pseudo-hyphae.

6.2. No significant differences were observed between the membrane-separated coculture and filtered CFS assays, both of which produced comparable hyphal lengths [1], hyphal volumes [2], and thicknesses [3].

6.2.1. LAB MEDIA: Figure 2 **Video Editor:** Please highlight the two rightmost data groups, corresponding to '*C. glabrata in luminal well*' and '*C. glabrata CFS*'- along with the '*ns*' value in Figure 2B.

6.2.2. LAB MEDIA: Figure 2 **Video Editor:** Please highlight the two rightmost data groups, corresponding to '*C. glabrata in luminal well*' and '*C. glabrata CFS*'- along with the '*ns*' value in Figure 2C.

6.2.3. LAB MEDIA: Figure 2 **Video Editor:** Please highlight the two rightmost data groups, corresponding to '*C. glabrata in luminal well*' and '*C. glabrata CFS*'- along with the '*ns*' value in Figure 2D.

1. Glucose

Pronunciation link: <https://www.merriam-webster.com/dictionary/glucose>

- IPA: /'gluːˌkoʊs/
Phonetic Spelling: GLOO·kohs
2. Candida glabrata
Pronunciation link: No confirmed link found
IPA: /'kændɪdə gləˈbrɑːtə/
Phonetic Spelling: KAN·dih·duh gluh·BRAH·tuh
 3. Yeast-extract Peptone Dextrose
Pronunciation link: No confirmed link found
IPA: /jiːst ˈɛkˌstrækt ˈpepˌtoʊn ˈdɛkˌstroʊs/
Phonetic Spelling: YEEST EK·strakt PEP·tohn DEK·strohs
 4. Peptone
Pronunciation link: <https://www.merriam-webster.com/dictionary/peptone>
IPA: /ˈpepˌtoʊn/
Phonetic Spelling: PEP·tohn
 5. Dextrose
Pronunciation link: <https://www.merriam-webster.com/dictionary/dextrose>
IPA: /ˈdɛkˌstroʊs/
Phonetic Spelling: DEK·strohs
 6. Biosafety
Pronunciation link: No confirmed link found
IPA: /ˌbaɪoʊˈseɪfti/
Phonetic Spelling: bye·oh·SAYF·tee
 7. Inoculate
Pronunciation link: <https://www.merriam-webster.com/dictionary/inoculate>
IPA: /ɪˈnoːkjəˌleɪt/
Phonetic Spelling: ih·NOK·yuh·layt
 8. Agitation
Pronunciation link: <https://www.merriam-webster.com/dictionary/agitation>
IPA: /ˌædʒəˈteɪʃən/
Phonetic Spelling: aj·uh·TAY·shuhn
 9. Morphology
Pronunciation link: <https://www.merriam-webster.com/dictionary/morphology>
IPA: /mɔːrˈfɑːlədʒi/
Phonetic Spelling: mor·FOL·uh·jee
 10. Microscopy
Pronunciation link: <https://www.merriam-webster.com/dictionary/microscopy>
IPA: /maɪˈkrɑːskəpi/
Phonetic Spelling: my·KROS·kuh·pee
 11. Centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /ˈsentrəˌfjuːdʒ/
Phonetic Spelling: SEN·truh·fyooj
 12. Supernatant
Pronunciation link: <https://www.merriam-webster.com/dictionary/supernatant>

- IPA: /ˌsuːpərˈnɛntənt/
Phonetic Spelling: soo·per·NAY·tuht
13. Candida albicans
Pronunciation link: No confirmed link found
IPA: /ˈkændɪdə ˈælbɪˌkænz/
Phonetic Spelling: KAN·dih·duh AL·bih·kanz
14. Diploid
Pronunciation link: <https://www.merriam-webster.com/dictionary/diploid>
IPA: /ˈdɪˌplɔɪd/
Phonetic Spelling: DIP·loyd
15. Haploid
Pronunciation link: <https://www.merriam-webster.com/dictionary/haploid>
IPA: /ˈhæˌplɔɪd/
Phonetic Spelling: HAP·loyd
16. Ploidy
Pronunciation link: No confirmed link found
IPA: /ˈplɔɪdi/
Phonetic Spelling: PLOY·dee
17. Cuvette
Pronunciation link: <https://www.merriam-webster.com/dictionary/cuvette>
IPA: /kjuːˈvɛt/
Phonetic Spelling: kyoo·VET
18. Spectrophotometer
Pronunciation link: <https://www.merriam-webster.com/dictionary/spectrophotometer>
IPA: /ˌspektroʊfoʊˈtɑːmətər/
Phonetic Spelling: spek·troh·foh·TOM·uh·ter
19. Optical density
Pronunciation link: No confirmed link found
IPA: /ˈɑːptɪkəl ˈdensəti/
Phonetic Spelling: OP·tih·kuhl DEN·suh·tee
20. Abluminal
Pronunciation link: <https://www.merriam-webster.com/medical/abluminal>
IPA: /ˌæbˈluːmənəl/
Phonetic Spelling: ab·LOO·muh·nuhl
21. Polycarbonate
Pronunciation link: <https://www.merriam-webster.com/dictionary/polycarbonate>
IPA: /ˌpɑːliˈkɑːrbənət/
Phonetic Spelling: pol·ee·KAR·buh·nuht
22. Luminal
Pronunciation link: <https://www.merriam-webster.com/medical/luminal>
IPA: /ˈluːmənəl/
Phonetic Spelling: LOO·muh·nuhl
23. DIC
Pronunciation link: No confirmed link found

- IPA: /ˌdiː aɪ ˈsiː/
Phonetic Spelling: dee·eye·SEE
24. Phenotypic
Pronunciation link: <https://www.merriam-webster.com/dictionary/phenotypic>
IPA: /ˌfiːnəˈtɪpɪk/
Phonetic Spelling: fee·nuh·TIP·ik
25. Micrograph
Pronunciation link: <https://www.merriam-webster.com/dictionary/micrograph>
IPA: /ˈmaɪkrəˌɡræf/
Phonetic Spelling: MY·kruh·graf
26. Hyphal
Pronunciation link: <https://www.merriam-webster.com/dictionary/hyphal>
IPA: /ˈhaɪfəl/
Phonetic Spelling: HY·fuhl
27. Hypha
Pronunciation link: <https://www.merriam-webster.com/dictionary/hypha>
IPA: /ˈhaɪfə/
Phonetic Spelling: HY·fuh
28. Longitudinal
Pronunciation link: <https://www.merriam-webster.com/dictionary/longitudinal>
IPA: /ˌlɒːndʒəˈtuːdənəl/
Phonetic Spelling: lon·juh·TOO·duh·nuhl
29. Apical
Pronunciation link: <https://www.merriam-webster.com/dictionary/apical>
IPA: /ˈeɪpɪkəl/
Phonetic Spelling: AY·pih·kuhl
30. Transverse
Pronunciation link: <https://www.merriam-webster.com/dictionary/transverse>
IPA: /ˈtrænzˌvɜːs/
Phonetic Spelling: TRANZ·vurs
31. Prolate spheroid
Pronunciation link: No confirmed link found
IPA: /ˈproʊˌlert ˈsfɪrɔɪd/
Phonetic Spelling: PROH·layt SFEER·oyd
32. Filament
Pronunciation link: <https://www.merriam-webster.com/dictionary/filament>
IPA: /ˈfɪləmənt/
Phonetic Spelling: FIL·uh·muhnt
33. Biological replicate
Pronunciation link: No confirmed link found
IPA: /ˌbaɪəˈlɒːdʒɪkəl ˈreplɪkət/
Phonetic Spelling: bye·uh·LOJ·ih·kuhl REP·lih·kuht
34. Parametric
Pronunciation link: <https://www.merriam-webster.com/dictionary/parametric>

- IPA: /ˌpærəˈmɛtrɪk/
Phonetic Spelling: pair·uh·MET·rik
35. Shapiro-Wilk
Pronunciation link: No confirmed link found
IPA: /ʃəˈpɪroʊ wɪlk/
Phonetic Spelling: shuh·PEER·oh WILK
36. D'Agostino-Pearson
Pronunciation link: No confirmed link found
IPA: /ˌdægəˈstiːnoʊ ˈpɪrsən/
Phonetic Spelling: dag·uh·STEE·noh PEER·suhn
37. Pseudo-hyphal
Pronunciation link: No confirmed link found
IPA: /ˌsuːdoʊ ˈhaɪfəl/
Phonetic Spelling: soo·doh·HY·fuhl
38. Coculture
Pronunciation link: No confirmed link found
IPA: /ˌkoʊ ˈkʌltʃər/
Phonetic Spelling: koh·KUL·cher
39. Cell-free supernatant
Pronunciation link: No confirmed link found
IPA: /ˈsɛl ˌfriː ˌsuːpərˈneɪtənt/
Phonetic Spelling: SEL·free soo·per·NAY·tuhnt