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Cryptococcus neoformans: mating and genetic crosses

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Abstract

The *Cryptococcus* pathogenic species complex is a group of opportunistic human fungal pathogens that cause cryptococcal meningoencephalitis, an infection associated with unacceptably high mortality rates. The public health relevance of these pathogens has galvanized extensive research over the past several decades and led to characterization of their sexual cycles. This research has allowed several *Cryptococcus* species to develop into model fungal organisms for both pathogenesis and basic science studies. Many of these studies require observation of the meiotic process and its associated mating structures, as well as the generation of meiotic progeny with novel phenotypes and genotypes. Herein, we describe how to set up genetic crosses between *Cryptococcus* strains and observe their mating phenotypes, as well as how to recover progeny from these crosses for further analysis.

Keywords

Cryptococcus; Genetic cross; Mating progeny

INTRODUCTION

The human pathogenic *Cryptococcus* species complex belongs to the phylum Basidiomycota and is currently composed of seven species, defined based on genetic divergence and reproductive isolation (Sun and Xu 2007; Sun and Xu 2009; Hagen *et al.* 2015; Kwon-Chung *et al.* 2017). Most of the species in this complex are haploid opportunistic human pathogens, staying dormant in healthy hosts and only causing disease when the host immune system becomes compromised, such as in AIDS or organ transplant patients. It has been estimated that *Cryptococcus* was responsible for 223,100 cases of meningitis in 2014, with an associated 20–70% mortality rate for patients in care (Rajasingham *et al.* 2017). The global impact of this pathogen is magnified by the limited number of antifungal drug treatment options, as well as the recent outbreak of *C. gattii* infections in the Pacific Northwest of the USA and Canada, which suggests an expansion of the geographic niches of some of these human pathogens (Byrnes *et al.* 2010).

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Pathogenic *Cryptococcus* species have a bipolar mating system, with two mating types (*MAT***a** and *MAT*α) defined by which of the two alternative alleles is present at the single *MAT* locus (Lengeler *et al.* 2000; Hull *et al.* 2002). Close contact between cells of opposite mating types results in the formation of zygotes that are able to undergo filamentous growth and eventually produce basidia and basidiospores. Basidiospores are the products of meiosis followed by multiple rounds of mitosis and are therefore the results of exchange of genetic material between the two parental cells.

Numerous tools and resources are available for *Cryptococcus* sexual reproduction and genetic studies. Genetic manipulation in *Cryptococcus* can be achieved by biolistic transformation, *Agrobacterium*-mediated random insertional mutagenesis, and a novel CRISPR-mediated electroporation method (Toffaletti *et al.* 1993; Toffaletti and Perfect 1994; Davidson *et al.* 2000; Esher *et al.* 2015; Fan and Lin 2018; Jung *et al.* 2018). Congenic strain pairs (i.e. strains that are genetically identical with the exception of the *MAT* locus) have been constructed for *C. neoformans*, *C. deneoformans*, and *C. deuterogattii* (Heitman *et al.* 1999; Nielsen *et al.* 2003; Zhai *et al.* 2013). High quality, end-to-end chromosomal level genome assemblies are becoming available for all of the major lineages in the species complex (Loftus *et al.* 2005; Janbon *et al.* 2014; Farrer *et al.* 2015), as well as closely related non-pathogenic sister species (Sun *et al.* 2017). Additionally, collections of deletion strains of lacking all non-essential genes, transcription factors, and kinases in the *C. neoformans* genome have been or are being constructed (Liu *et al.* 2008; Jung *et al.* 2015; Lee *et al.* 2016). All of these advances that facilitate genetic manipulation and analyses of specific genes in *Cryptococcus* have allowed this opportunistic pathogen to become a fungal model organism (Idnurm *et al.* 2005; Heitman *et al.* 2011). These resources have aided fungal pathogenesis studies to elucidate fungal virulence factors and host-pathogen interactions, which have allowed significant advancements in our knowledge and treatment of these human pathogens. Furthermore, studies on basic fungal biology in *Cryptococcus* have contributed to our understanding of sexual reproduction, genome evolution, and evolution of the fungal mating type locus and centromeres (Heitman *et al.* 2011; Sun *et al.* 2012; Sun *et al.* 2017; Yadav *et al.* 2018).

Sexual reproduction plays important roles in *Cryptococcus* pathogenesis, as well as in the population dynamics and evolution of natural isolates. Cryptococcal infection begins when desiccated yeast cells or spores, the products of *Cryptococcus* sexual reproduction, are inhaled into the lungs. Genomic analyses suggest that sexual reproduction readily occurs in populations of *Cryptococcus* environmental and clinical isolates, providing a route to generate genetic diversity and the potential for increased pathogenicity (Hiremath *et al.* 2008; Sun *et al.* 2014; Roth *et al.* 2018). Therefore, it is crucial to understand the genetic and molecular basis of sexual reproduction in this species. In the following sections, we will illustrate how standard mating assays of *Cryptococcus* strains are carried out and what one can expect to observe in these assays. Additionally, we will demonstrate how to recover mating products via either spore dissection or screening for phenotypic markers.

STRATEGIC PLANNING

There is a wide range of mating phenotypes and efficiencies among *C. neoformans*, *C. deneoformans*, and *C. gattii* strains. Therefore, consideration should be taken for each genetic cross to determine the appropriate mating medium and to approximate the incubation period that will be required for sufficient mating structure and basidiospore chain production for either imaging or dissection. Murashige and Skoog (MS) agar medium is commonly used for *C. neoformans* and *C. deneoformans* dissections, but V8 (pH=5) agar medium can also be used for *C. deneoformans* dissections. Filamentation and mating structures typically appear within the first 1–4 days of incubation, making this an ideal time for determining mating responses. Figures 1A and 1B show typical mating structure production in a *C. neoformans* cross on MS and a *C. deneoformans* cross on V8 (pH=5), respectively. Figures 1C and 1D depict mating structures, including hyphal filaments, fused clamp cells, basidia, and protruding spore chains, from a *C. neoformans* cross on MS after approximately two weeks of incubation. Dissection from *C. neoformans* crosses should be done approximately 2 to 3 weeks post inoculation on the mating medium, while basidiospores from *C. deneoformans* crosses should be dissected later, around 3 to 4 weeks after inoculation.

BASIC PROTOCOL 1: Mating Assays

Genetic crosses can be performed between *Cryptococcus* strains to observe various mating phenotypes and their associated sexual structures, to generate progeny of a desired genotype or phenotype, and to probe species boundaries. Crosses typically consist of two strains of opposite mating types (*MAT***a** and *MAT*α), although unisexual reproduction between *C. deneoformans* parents of the same mating type is also possible. See Figure 2A for appropriate plate setup.

Materials

Cryptococcus strains (overnight culture or cells on agar medium)

Sterile deionized water

1.5 ml microcentrifuge tubes (Eppendorf)

Inoculation picks

Spectrophotometer

Vortex

Mating medium of choice (approximately 1 agar plate for every two unique genetic crosses)

Protocol steps

1. Combine *Cryptococcus* cells for desired cross
    a. If using cells from agar plates, prepare a microcentrifuge tube with 200 μL of dH_2O. Using inoculation picks, add cells of two chosen strains

for the cross (an inoculum approximately the size of a matchstick head for each strain). Vortex the cells from the two strains together in the water.

b. If using cells from an overnight culture, spin the culture down in a tabletop centrifuge at 3,000 rpm for 5 minutes and wash twice with 5 ml sterile dH_2O. Resuspend cells in sterile dH_2O to an absorbance of OD_{600} = 1.0. Combine 100 μL of the two strains to be crossed in a single microcentrifuge tube and vortex until thoroughly mixed.

2. Spot 20 μL of the mixed strains onto the mating medium of choice.

a. Ensure that each mating spot is approximately 2 cm apart from any other mating spot to minimize interference from other spots on mating. Each agar plate can accommodate up to 9 total spots, including positive and negative controls (Figures 2A and 2D).

b. On each mating plate, a positive control involving common laboratory strains should be used to ensure there is no deficiency in the mating medium. For *C. neoformans* (serotype A) strains, the positive control may be a cross between KN99**a** x KN99α (Nielsen *et al.* 2003). For *C. deneoformans* (serotype D) strains, the positive control may be a cross between JEC20 x JEC21 (Kwon-Chung *et al.* 1992; Heitman *et al.* 1999). Negative controls, where each parental strain involved in the experimental cross are spotted alone, should also be included on the mating plate to determine if contamination or self-filamentation may confound results (Figure 2A).

3. After mating spots have dried (approximately 20 minutes), place mating plates face up in a completely dark area (such as a drawer) at room temperature. Do not place mating plates upside down, parafilm plates, or seal them in any way as this can inhibit mating.

4. Incubate mating spots for up to six weeks, imaging and dissecting spores as necessary.

Note: To observe filamentation, check plates every day; for basidia and basidiospore observation, check every day after day 4. For spore dissection, begin checking mating structures after approximately two weeks of incubation.

ALTERNATE PROTOCOL 1: Liquid-free Mating Assays

High humidity environments are known to decrease the mating efficiency of *Cryptococcus* strains. If the genetic cross set up following ***BASIC PROTOCOL 1*** fails to produce sufficient mating structures, setting up a cross involving no additional liquid may help improve spore production. This method also produces more dispersed mating structures around the mating patch than the method described in ***BASIC PROTOCOL 1***.

Materials

Cryptococcus strains (on agar medium)

Inoculation picks

Mating medium of choice (approximately 1 agar plate for every two unique genetic crosses)

Protocol steps

1. Using a sterile inoculation pick, make a small circle of cells of one parental strain on the mating medium of choice.
2. With a second sterile inoculation pick, make a small circle of the same size with the other parental strain directly on the same spot from step 1.
3. Include the same positive and negative controls that were described in ***BASIC PROTOCOL 1***.
4. Place plates in dark, room temperature incubation area as in ***BASIC PROTOCOL 1*** and incubate accordingly.

SUPPORT PROTOCOL 1: Confrontation Assays

Unlike mating assays, confrontation assays are utilized to determine if a strain of interest will produce mating structures in response to pheromone or small molecule production by another strain. This can be determined by placing the two strains in close proximity to each other and observing the production of hyphae in the space separating the two isolates over time. See Figure 2B for appropriate plate setup.

Materials

Cryptococcus strains (on agar medium)

Inoculation picks

V8 (pH=5) or V8 (pH=7) medium plates

Protocol steps

1. Using a sterile inoculation pick, streak a thin line of one of the two strains on the V8 medium plate.
2. Using another sterile inoculation pick, streak a thin line of the other strain in parallel with the first strain, approximately 1 to 2 mm apart, beginning approximately 1 cm below where the first line was struck and extending an additional centimeter beyond where the first line ended to establish zones of non-interaction. Similar to *BASIC PROTOCOL 1,* a positive control should be included on each plate in which two strains known to undergo robust, successful mating are confronted. See plate layout in Figure 2B.
3. Incubate the plates as in ***BASIC PROTOCOL 1***. Hyphal production should be visible after 1 to 3 days of incubation for mating partners with vigorous

interaction. Extended incubation may be needed to observe hyphal production between strains with low-efficiency mating responses.

BASIC PROTOCOL 2: Recover basidiospores by random spore dissection

Isolating *C. neoformans* basidiospores via microdissection is an important technique for generating progeny with novel genotypes and phenotypes and for measuring germination frequencies to determine species boundaries or meiotic proficiency. To dissect random basidiospores, they must first be isolated from the periphery of a mating patch and moved to nutrient-rich Yeast Extract Peptone Dextrose (YPD) agar medium. Following this isolation, the spores can be individually micromanipulated and spread across the agar plate to ensure each resulting colony is the product of a single progeny. See Figure 2C for how to set up the YPD agar plate that spores will be dissected onto and Figures 2F and 2G for microscopy images of what the experimenter may see during the dissection process.

Materials

Mating spot with sufficient basidiospore chains from ***BASIC PROTOCOL 1***

9” glass Pasteur pipette (VWR cat. no. 14672–380)

Unfolded paperclip

YPD agar plates

Dissection microscope (Zeiss AxioScope.A1 HAL 50, specifically configured for tetrad dissection of yeast)

Dissection needle (Optical Fiber Dissection Kit, 25-micron, Cora Styles Lab Supplies, cat. no. 1025)

Parafilm

Protocol steps

Isolate spores from mating plate and transfer to YPD agar plate

1. Observe the periphery of a mating spot under a light microscope and identify an area with robust spore chain production that is as far as possible from the dense mating patch. On the bottom of the mating plate, mark this area for easy identification in step 5.
2. Break off the end of a long tip (9 inch) glass Pasteur pipette to obtain a small-diameter piece approximately 2 inches in length.

   Note: you will want a paperclip that can fit into the broken Pasteur pipette piece as tightly as possible for the full 1 to 2-inch length. You will also want both ends of the broken pipette piece to be as flat as possible.
3. Insert the straightened paperclip fully into the broken pipette piece and sterilize them together over an open flame.

4. After cooling, remove the pipette piece from the paper clip and flame the more uneven end of the pipette piece.

5. Insert the more uneven end of the pipette piece into the agar at the marked spot from step 1.

   Note: Take care to avoid getting any of the dense mating spot itself in the pipette piece, as this area contains many yeast cells.

6. Carefully remove the pipette piece with the agar plug remaining inside of the pipette (twist and slowly lift up; angle pipette piece if necessary).

7. Briefly flame the other end of the pipette piece (the flatter end) being careful not to let the agar plug with spore chains come too near the flame.

8. After cooling, slide the paperclip into the end of the pipette piece where the agar plug is and push the plug to the opposite end of the pipette piece so that the agar plug surface with the spore chains protrudes very slightly.

9. Drag the agar plug surface with the spore chains lightly along the surface of a YPD agar plate in a single straight line to deposit spore chains while keeping the agar plug within the pipette piece and avoiding gouging the YPD agar. See Figure 2C for dissection plate layout.

Spore dissection

10. Center the dissection needle within the field of view on the dissection microscope and clean the dissection needle by inserting and removing the needle several times in a new, sterile YPD agar plate.

11. Place the YPD agar plate from step 9 upside down (so that the agar surface is facing the dissection needle) on the stage of the dissection microscope.

12. Move the dissection needle to an area of the plate that does not contain yeast cells and will not be used for dissection later (normally the top most region of the plate, see Figure 2C). Bring the dissection needle into focus so that it is just touching the surface of the agar.

    Note: Needle will appear to “jump” slightly as it comes into contact with the agar.

13. Move needle away from agar, keeping agar surface within focus.

14. Move field to where spores were distributed onto plate and identify spores for dissection.

    Note: spores are smaller and slightly elongated in shape (Figure 2G), while yeast cells are typically larger, almost perfectly round, and have a distinct black border around the cell (Figure 2F).

15. Slowly bring dissection needle to the agar surface so that the perimeter of the needle comes just into contact with the spore.

    Note: Make sure that the needle does not come into contact with any yeast cells.

16. After the needle has contacted the agar surface and the needle edge has come into contact with the spore, slowly withdraw the needle and ensure that the spore has remained attached to the needle (it should no longer be in the field of view).

    Note: gently tapping the microscope base can help to ensure the spore is stuck to the dissection needle before withdrawing the needle.

17. Move the stage to a specific coordinate on the lower region of the plate (see Figure 2C), bring the needle with the spore to the agar surface, lightly tap microscope base to dislodge spore from needle. Continue bringing the needle to the surface (without puncturing agar), lightly tapping, and withdrawing needle as needed until spore has been visibly deposited on agar surface.

18. Record coordinates at which the spore was placed.

19. Return to area where spores were distributed from mating plate in step 9, identify additional spores for dissection, and repeat steps 14–18.

20. After dissecting the appropriate number of spores, remove the YPD agar plate from the dissection scope, parafilm the YPD plate, and leave it to incubate upside down at room temperature.

21. Purify germinated spores from the YPD agar dissection plate by streaking them onto the appropriate medium.

    Note: spores typically take 3 to 5 days to germinate, but spores from genetically distant crosses may take up to two weeks to germinate. Additionally, if contamination is observed on YPD plate that spores were dissected onto, the progeny from this dissection cannot be used to estimate germination frequencies as contaminating organisms and their secreted compounds could affect germination efficiency.

ALTERNATE PROTOCOL 2: Recover basidiospores by single basidium dissection

The progeny recovered through random spore dissection are, as indicated by the name of the technique, random. That is, there is not information on the “genealogy” of the spores dissected, and they are considered to represent a population sampled from a large number of meiotic events, with the four meiotic products from each meiosis not necessarily all being represented in the progeny dissected. Random dissection is advantageous in cases where a broad sampling of the progeny population is the objective, such as when analyzing genome-wide meiotic recombination, or when isolating a progeny that has a certain combination of the genotypes of the two parents at multiple loci. However, sometimes it is necessary to know which spores were produced from the same meiotic event and in these cases, dissecting spores from a single basidium is required. Because it is known that only one meiotic event occurred within each basidium of *Cryptococcus* (Idnurm 2010), single basidium dissection in *Cryptococcus*, to some extent, resembles tetrad dissection in *Saccharomyces cerevisiae*.

Materials

Mating spot with sufficient basidiospore chains from ***BASIC PROTOCOL 1***

Dissection microscope equipped with dissection needle (see ***BASIC PROTOCOL 2***)

YPD agar plates

Protocol steps

1. Collect basidiospores from a single basidium on the mating plate from Basic Protocol 1.
    - **a.** Place the mating plate upside down (so that the mating spot is facing the dissection needle) on the stage of the dissection microscope.
    - **b.** Make sure that the needle is away from and not touching the plate surface and mating spot.
    - **c.** Focus the objective onto the agar surface of the YPD medium plate.
    - **d.** Locate an area where the basidia and basidiospores are abundant.
    - **e.** Find one basidium with chains of basidiospores that is well isolated from the other basidia and spores chains.
    - **f.** Move this basidium into the center of the field.
    - **g.** Bring the needle closer to the plate surface (this should be done very slowly using the turning knob) until the needle's shape first appears in the field. (Figure 2H panel i)
    - **h.** Using the handle, slightly move the needle closer to the targeted basidium and spore chains (now the needle will come into focus).
    - **i.** Spore chains will be "pulled" onto the needle surface (likely due to electrostatic attraction). (Figure 2H panel ii)
    - **j.** Using the handle, slightly move the needle away from the plate surface, and in the process, breaking the spore chains, which will result in a portion of the spores from that basidium remaining on the tip of the needle. (Figure 2H panel iii)
    - **k.** Using the turning knob, move the needle as far as possible from the plate surface.
    - **l.** Remove the mating plate from the stage of the dissection microscope.
2. Transfer the basidiospores onto a fresh YPD plate.
    - **a.** Place a fresh YPD plate on the stage of the dissection microscope.
    - **b.** Move the stage, so that the needle is underneath a spot on the plate that is located on the vertical axis of the plate, and about 5–10 cm from the top (upper region of plate in Figure 2C).

c. Move the objective to focus on the agar surface of the plate.

d. Using the turning knob, very slowly move the needle towards the plate surface until the shape of the needle appears in the field.

e. Using the handle, move the needle even closer to the surface until it comes into focus.

f. Using the handle, move the needle slightly up and down so that it makes repeated contacts with the agar plate surface. With each contact, a portion of the spores on the tip of the needle will be placed onto the plate surface.

g. Repeat until no additional spores are placed onto the agar plate surface with additional needle-plate contacts.

Note: it is helpful to move the stage very slightly following each contact to make sure the spores are relatively well separated from each other while placing the spores onto the plate surface, which will facilitate the following spore separation steps.

3. Separate the basidiospores

a. Move the field to where spores were distributed onto plate.

b. Follow step 15 to step 21 in ***BASIC PROTOCOL 2***.

SUPPORT PROTOCOL 2: Recover mating progeny using dominant drug markers

When sexual reproduction is between two strains that are genetically modified to express different dominant drug resistance markers (e.g. *NAT*, *NEO*, or *HYG*), meiotic progeny can be recovered by simply screening for double-resistant colonies using YPD medium plates supplemented with both drugs.

Materials

Mating spot with sufficient basidiospore chains from ***BASIC PROTOCOL 1***

Sterile scalpel or razor blade

1.5 ml microcentrifuge tubes (Eppendorf)

Sterile deionized water

Vortex

YPD agar plate supplemented with drugs

Parafilm

Protocol steps

1. Recover and resuspend mating products.
   a. Locate areas on the mating plate that contain abundant basidiospores.
   b. Using sterile scalpel (or razor blade), cut out the regions with spores and transfer them into a microcentrifuge tube.
   c. Add 1 ml sterile dH_2O into the microcentrifuge tube.
   d. Vortex for 30 seconds.
2. Plate 100 µl of the cell suspension onto YPD solid medium supplemented with the two drugs to which the parental strains are resistant.
3. Parafilm the plate and incubate the plate upside down at 30 °C for 3 days.

   Note: it is recommended that 10x, 100x, and 1000x dilutions of the cell suspension from step 1 should also each be plated onto YPD solid medium supplemented with drugs, so that a plate with a desirable number of colonies (100 – 200) will be obtained.

REAGENTS AND SOLUTIONS

V8 agar medium

- 50 ml V8 juice
- 0.5 g KH_2PO4
- 950 ml dH_2O
- 40 g Bacto agar (VWR cat. no. 90000–762)

  (Note: The pH of the media should be at 5 inherently and can be adjusted to 7 with KCl if necessary)
- Autoclave for 40 minutes

Murashige Skoog (MS) agar medium

- 4.328 g MS mix (entire contents of one foil packet) (Sigma, M5524)
- 16 g Bacto agar (VWR cat. no. 90000–762)
- 1000 ml dH_2O
- Adjust pH to 5.8
- Autoclave for 40 minutes
- Add 1ml 1000X vitamin mix after autoclaving (Sigma, M7150)

Yeast Extract Peptone Dextrose (YPD) agar medium

- 65 g YPD agar (VWR cat. no. 90003–278)
- 5 g Bacto agar (VWR cat. no. 90000–762)

- Autoclave 40 minutes
- Potential antibiotic drug modifications:
  - If adding antibiotic drugs, allow agar to cool to 50–55°C before addition.
  - Add Nourseothricin to a final concentration of 100 μg/ml.
  - Add G418 to a final concentration of 200 μg/ml.
  - Add Hygromycin to a final concentration of 200 μg/ml.

Synthetic Drop-out (SD) medium

- 6.7 g YNB without amino acids
- 20 g Bacto agar
- 800 ml dH_2O
- Autoclave for 40 minutes
- After autoclaving, add:
  - 100 ml 20% glucose
  - 100 ml 10X amino acid stock mix lacking appropriate amino acid

10X amino acid stock mix

- Do not add the amino acid for whichever auxotrophy you will be selecting for.
- 850 ml dH_2O
- 300 mg L-isoleucine
- 1.5 g L-valine
- 200 mg adenine sulfate
- 200 mg L-histidine-HCl
- 1 g L-leucine
- 300 mg L-lysine
- 200 mg L-methionine
- 500 mg L-phenylalanine
- 200 mg L-tryptophan
- 300 mg L-tyrosine
- 200 mg uracil
- 4 g L-serine
- 1 g L-glutamate
- Autoclave 40 minutes, cool to room temperature, then add:

- 50 ml 4% (w/v) L-threonine
- 100 ml 1% (w/v) L-aspartate

YNB + 5-fluoroorotic acid (5-FOA)

- 20 g Bacto agar
- 6.7 g YNB without amino acids
- 50 mg uracil
- 900 ml dH_2O
- Autoclave 40 minutes
- While autoclaving, combine:
  - 100 ml 10X amino acid stock without uracil
  - 1 g 5-FOA
  - 20 g glucose
  - Heat while stirring to dissolve solutes, making sure not to boil
  - Filter sterilize
- Add filter-sterilized solution to autoclaved medium (no need to cool)

COMMENTARY

Background Information.

It is thought that the cryptococcal bipolar mating system evolved from a tetrapolar system that is ancestral to Basidiomycetes (Metin *et al.* 2010; Findley *et al.* 2012; Sun *et al.* 2017). The *Cryptococcus* sexual cycle is well-characterized and was first identified and described in *C. neoformans* over 40 years ago (Kwon-Chung 1975; Kwon-Chung 1976a; Kwon-Chung 1976b). When mating compatible *MAT***a** and *MAT*α cells come into close contact, conjugation between the two cells is initiated, resulting in a dikaryotic zygote. The zygote undergoes a morphological transition and grows as a filament. Fused clamp cells form along the hyphae, bridging over the septa that divide the hyphae into compartments and ensuring accurate nuclear distribution during hyphal development. Eventually, the hyphal tip develops into a basidium, within which the two nuclei fuse and undergo karyogamy and one round of meiosis, producing four meiotic products. These four meiotic products then undergo repeated rounds of mitosis to produce additional nuclei that are packaged into basidiospores, which are located on the surface of the basidium and form four long chains of basidiospores. Thus, *Cryptococcus* mating bears all of the hallmarks of sexual development in Basidiomycetes, making it an excellent model organism to study fungal sexual reproduction. In addition to mating between cells of opposite mating types, unisexual reproduction can also occur between *MAT*α strains in *C. deneoformans* (Lin *et al.* 2005; Feretzaki and Heitman 2013; Sun *et al.* 2014; Sun and Heitman 2015; Roth *et al.* 2018)

Controls.

As in all biological experiments, proper controls (positive and negative) should be included in the analysis of sexual reproduction in *Cryptococcus* for both confrontation and mating assays. For mating assays, a positive control should be a mixture of two strains known to undergo efficient mating. For confrontation assays, a positive control should be a confrontation between two strains with opposite mating types that are known to interact and produce mating structures. The common strain pairs used for positive controls in mating assays are congenic *C. neoformans* strains KN99**a** and KN99α or *C. deneoformans* strains JEC20 (*MAT***a**) and JEC21 (*MAT*α). *C. neoformans* H99 (*MAT*α) *crg1*Δ and KN99**a** *crg1*Δ deletion strains are frequently used in confrontation assays due to their hyper-sensitivity to pheromones (see comment below). Negative controls are typically the solo culture of the strains being tested for mating or mating interactions. Positive and negative controls should be on the same plate as the mating pairs being tested. Figures 2A and 2B depict common layouts of the plates for both mating as well as confrontation assays and include all of the proper controls.

Super-maters.

The mating ability, such as hyphal production, of *Cryptococcus* isolates has been shown to be a quantitative trait (Lin *et al.* 2006). Therefore, the absence of signs of successful mating does not necessarily indicate that the strains being tested are sterile. There are several genetically modified *Cryptococcus* isolates that have been shown to exhibit enhanced mating abilities, such as a *crg1*Δ strain in which the *CRG1* gene that is involved in attenuating the pheromone response has been disrupted (Fraser *et al.* 2003) and a $CPR2^{OE}$ strain where a constitutively active pheromone receptor-like gene, *CPR2*, has been overexpressed (Hsueh *et al.* 2009). These genetically engineered super-maters can be used in cases where mating interaction is absent when standard tester strains are used.

Mating medium.

The most commonly used medium for *Cryptococcus* mating assay are MS, V8 (pH=5), and V8 (pH=7). It should be noted that if an MS medium plate is used for crosses that involve strains that are auxotrophic for certain amino acids (e.g. adenine or uracil), these amino acids need to be supplemented in the MS medium to ensure proper growth of the auxotrophic strains. Mating agar plates should also be prepared with at least 25 ml of medium to ensure that they do not dry out for up to six weeks of incubation at room temperature. Studies have shown that certain modifications of these standard mating medium plates could enhance mating or induce mating structures, and thus, increase the chance of observing successful sexual reproduction. For example, supplementing media with inositol has been shown to stimulate mating. It has also been indicated that the presence of copper in media has a positive effect on mating. Additionally, glucosamine as the sole carbon source in nutrient-rich media as well as the presence of quorum sensing peptides have also been shown to induce filamentation in *Cryptococcus* in recent studies (Xue *et al.* 2007; Kent *et al.* 2008; Xu *et al.* 2017; Tian *et al.* 2018).

Progeny recovery.

When mating is between two strains, each with either a dominant drug resistant marker or an auxotrophic marker, isolation of the mating products based on these phenotypic markers (see ***SUPPORT PROTOCOL 2***) needs to be carried out in two steps because the drugs are ineffective in the minimal medium that is typically used to screen against auxotrophic markers (e.g. SD medium). For example, if a cross is between a nourseothricin-resistant strain and a uracil auxotrophic strain, cells from the mating mixture should first be screened for drug resistant colonies on YPD+nourseothricin medium plates, and then the colonies from the YPD+nourseothricin plates should be replica-plated onto agar plates containing 5-fluoroorotic acid (5-FOA) to screen for uracil auxotrophic isolates. The resulting colonies from the 5-FOA plates will be nourseothricin resistant and uracil auxotrophic, a combination of the phenotypes of the two parental strains. Alternatively, if the nourseothricin resistance marker and the uracil auxotrophic marker are in the same strain and this strain is crossed with an isolate that is wild type regarding these markers, the cells should first be screened on YPD+nourseothricin medium plates and then replica-plated onto SD-uracil solid medium to screen for uracil prototrophic colonies, which represent mating progeny in which the drug marker and uracil auxotrophic marker have segregated. Another method of isolating spores from mating mixtures based on gradient purification using centrifugation was also recently developed, which generates a progeny population resembling that from the random spore dissection method described in ***BASIC PROTOCOL 2*** (Botts *et al.* 2009).

Troubleshooting.

One of the most common issues that might occur in studies of sexual reproduction in *Cryptococcus* is that no successful mating and spore production is observed. There are several possible explanations if this happens. First, make sure the medium and controls worked properly (see comments above). If not (e.g. no mating is observed in the positive control), prepare fresh medium plates and repeat. Second, as mentioned in previous comments, mating ability is strain-specific and quantitative, and successful mating could also depend on environmental factors. It might be that the strains being tested have low mating efficiency. If possible, retest the strains with super-maters, or modify the mating media according to previous comments. Third, the absence of successful mating and spore production is a real biological outcome, especially after attempts to induce mating by changing testing strains and modifying mating environments have all failed. Another issue could be low germination rates among the recovered mating progeny. In this case, possible explanations could be that the cross is between strains with highly diverged genomes. This has been observed among the progeny recovered from inter-species crosses in Cryptococcus (Sun and Xu 2007). Genotyping of the strains using MLST markers could help shed light on possible causes of this problem.

Time considerations.

Cryptococcal matings vary greatly in the time they take to produce various mating structures and sufficient basidiospore chains for dissection depending on the species involved in the cross, the mating medium used, and the genetic divergence between the two parental strains. Hyphae protruding from mating mixtures can be visible as early as 1 to 3 days after the

mating is set up in efficient crosses and can take up to one week (or even longer) to be visible in crosses where one or both of the strains are less fertile or have mating deficiencies. The most efficient matings will also begin producing basidia and spore chains after approximately 5 to 7 days of incubation, and will continue to produce these structures as incubation is continued. Ideally, *C. neoformans* spore dissection takes place around two weeks after inoculating the mating medium and *C. deneoformans* dissections take place 3 to 4 weeks post-inoculation. Spores may be dissected up to 6 weeks after a cross is set up, although germination frequencies tend to decline over time.

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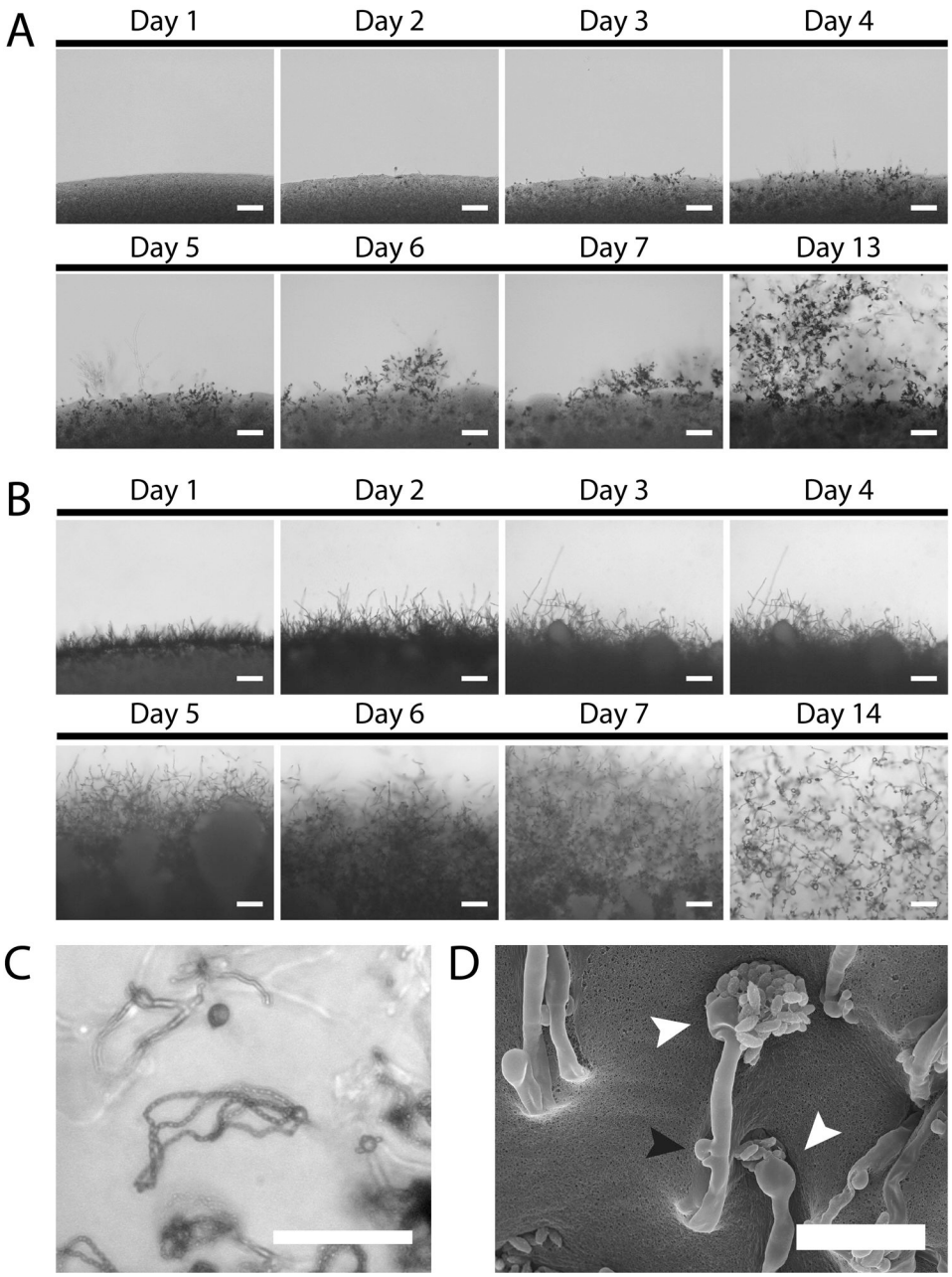

Figure 1. Mating progression of *Cryptococcus* reference strains.

A. Filamentation and mating structures produced by a cross between *C. neoformans* strains H99α and KN99**a** on MS medium. Hyphal filaments begin appearing after two days of incubation on the MS medium, while basidia appear on day 3. Spore chains are visible by the fifth day, but are robust and far enough from the central mating patch for dissection by day 13. Scale bars represent 100 μM. B.Filamentation and mating structures produced by a *C. deneoformans* cross between JEC20**a** and JEC21α on V8 (pH=5) medium. Robust filamentation occurs within the first day and continues with prolonged incubation. After two weeks, basidia with individual spores protruding are visible, although it typically takes 3 to 4 weeks of incubation before enough spore chains will be available for dissection. Scale bars

represent 100 μM. C. Spore chains emerging from a basidium produced by a cross between KN99α and KN99**a**. Basidia producing individual spores and hyphal filaments can be observed in the periphery. Scale bar represents 50 μM. D. Scanning electron microscopy of mating structures produced by a cross between KN99α and KN99**a** after approximately two weeks of incubation on MS medium. White arrowheads indicate basidia producing spore chains. The black arrowhead indicates a fused clamp cell along a hyphal filament. Scale bar represents 15 μM.

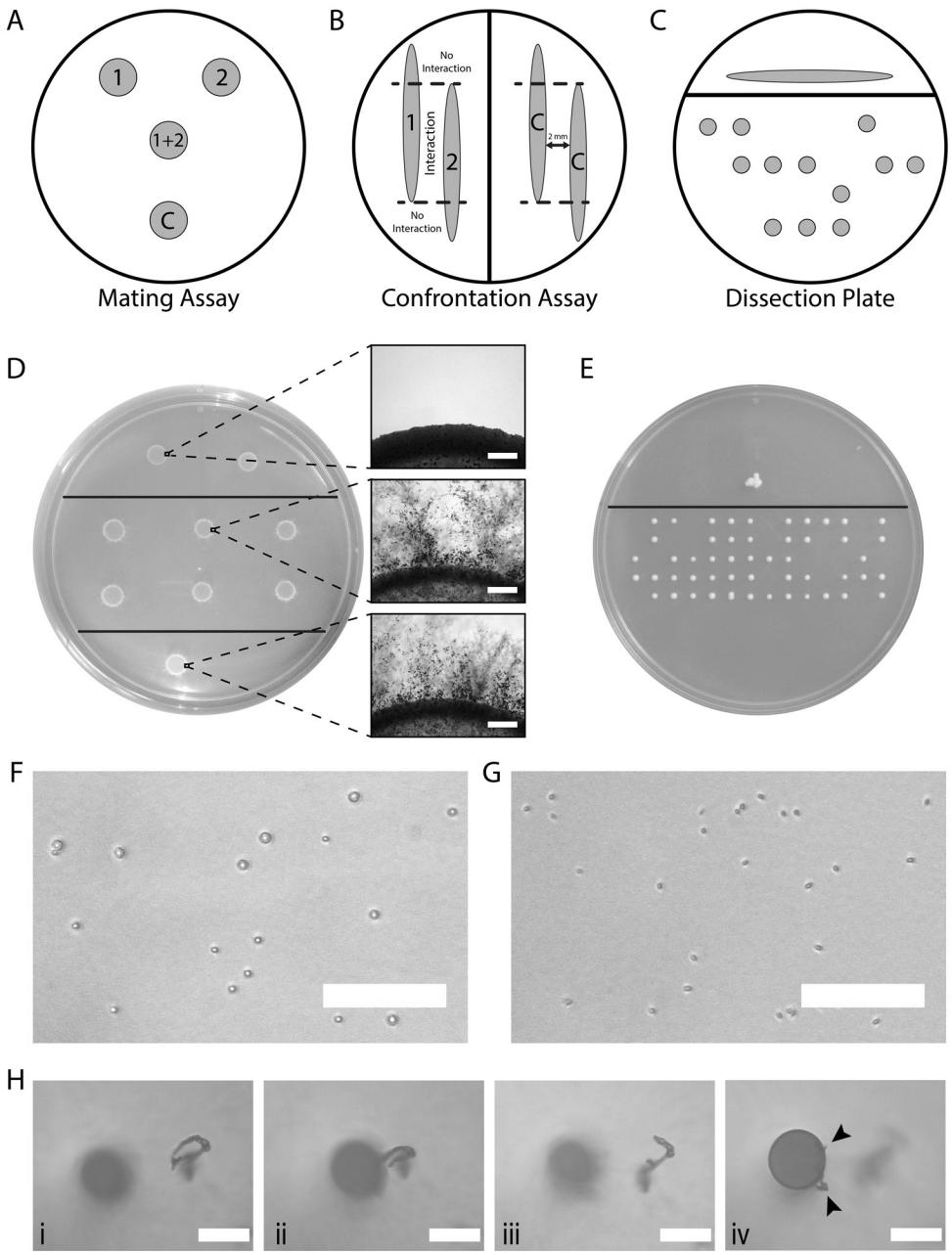

Figure 2. Plate layouts for mating assays, confrontation assays, and spore dissections as well as a comparison of yeast cells and spores.

A. The schematic depicts a basic plate layout that can be used for mating assays as described in Basic Protocol 1. It includes spots of both parental strains alone (1 and 2), to display any possible self-filamentation or confirm that either parent is not contaminated. Below the parents is the combination of parents (1+2), which is the mating patch that will be used for imaging and/or dissection. Finally, the bottom patch (C) should be a control mating with strains known to robustly undergo sexual reproduction together to ensure that the medium supports mating. With appropriate spacing, additional replicates of the mating patch (1+2) can also be included on the plate. B. An example of how to set up confrontation assays to

determine if a strain (1) will produce mating structures in response to another strain (2). The approximately 2 mm region between the two strains (area labeled “Interaction”), will be where potential interactions and mating structures can be observed. Outside of the dashed lines (area labeled “No Interaction”), the researcher can determine if either strain is self-filamentous or is responding to the medium alone. The right half of the plate depicts a positive control, which should be between two strains known to strongly interact. C. A representative example of how a dissection plate will appear after incubation. Spores from the mating plate should be placed above the solid black line. Following incubation, this will become a large patch including a mixture of yeast cells and basidiospores. Below the solid black line is where spores from above will be micromanipulated and arranged into a grid. D. A representative mating plate depicting a cross between two KN99α x KN99**a** mutant strains and appropriate controls on MS medium after a two-week incubation. The top portion of the plate contains either parental strain alone, the middle portion has replicates of the KN99α and KN99**a** mutant cross, and the bottom portion has a control mating between KN99α and KN99**a**. Insets show filamentation along the border of each corresponding spot. Scale bars represent 400 μM.E. An example of a dissection plate after four days of incubation with progeny from a KN99α and KN99**a** cross. Panels F and G are representative images of what will be observed when performing random basidiospore dissections. F. Yeast cells distributed on YPD agar. Note the large size, round shape, and distinct black outline. Scale bar represents 50 μM. G. Spores on YPD agar. Note that spores are smaller and more elongated than the yeast cells. Scale bar represents 50 μM. H. Sequential images of what will be observed during dissection of basidiospores from a single basidium, as the needle i) comes into focus, ii) makes physical contact with a chain of basidiospores, and iii) is retracted, separating spores from the basidium. The final panel (iv) shifts focus to the end of the needle and arrowheads indicate where spores can be seen affixed to the sides of the needle. Scale bars represent 50 μM.