How to separate the components of a heterokaryon.

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Background

Methods are needed in a variety of situations for resolving a heterokaryon into its components.

- Heterokaryons may have been created purposely to shelter lethal, disadvantaged, unstable, or infertile strains (see *How to use helper strains*). For subsequent characterization and determination of recoverability, the sheltered component must be obtained as a monokaryon, free of the *helper* component.
- Stocks in culture may need purification because they have accumulated unwanted recessive mutations (see, for example, Kafer 1982).
- During mutant hunts, newly induced mutations may fuse with nonmutants to become heterokaryotic.
- The question may be asked, whether a null mutation (knockout) is viable. To determine this, the sheltered component must be recovered as a monokaryon or shown to be unrecoverable.
- The question may be asked whether strains from nature are heterokaryotic (Pandit *et al.* 1994, Navaraj *et al.* 2000).

Extraction of the desired component as a monokaryon can be accomplished in several ways. Streaking or plating conidia may serve to achieve the separation, using sorbose and selective media if appropriate, and confirming purity by repeated streaking. The task is easier if phenotypic differences can be distinguished visually. Although most macroconidia are multinucleate and may be heterokaryotic, a minority are uninucleate. Also, some of the multinucleate conidia may be monokaryotic.

Use of microconidia, which are nearly 100% uninucleate, makes separation of components straightforward. Macroconidiating strains can be induced to produce nearly pure microconidia in large numbers by following the procedure of Pandit and Maheshwari (1993). Alternatively, microconidia can be separated from macroconidia by filtration (Ebbole and Sachs 1990). Large numbers of microconidia can also be obtained using macroconidiating strains that carry the *mcm* mutation (Maheshwari 1991). See *How to obtain microconidia*.

Purity of the extracted component is desired and may be essential. Forced heterokaryons that appear normal may nevertheless be unbalanced, with only a few percent of the complementing minority component present (Davis 1966). By introducing a thymidine kinase gene tk^+ into *N. crassa*, Metzenberg and Sachs (2002), Sachs *et al.* (1997) made it possible to be completely certain that the *tk*-negative component of a *tk* + tk^+ heterokaryon is pure. This depends on the lethality of FUDR for any cell that contains the tk^+ gene. The following is from Metzenberg and Sachs 2002):

"Many of the most interesting and useful strains encountered in research readily die in storage and/or are semi-sterile. If such strains are Oak Ridge-compatible, they can be carried and even crossed as heterokaryons with the sterile but vigorous strain from the Griffiths lab, a^{m1} *ad-3B cyh-1* (FGSC 4564). We have used this strain ["*Helper-1*"] extensively for improving the fertility of crosses. [See *How to use helper strains.*] However, it has one

shortcoming. When one wants to retrieve a homokaryotic culture of the fragile component for purposes other than crossing, it is at best a nuisance and often even a challenge to isolate it from the heterokaryon."

We have deposited at the FGSC three new strains, *Helpers* 2, 4, and 5, that have a deletion of the *mat* locus and, in addition, contain an insertion of the herpes simplex thymidine kinase gene, tk^+. The encoded enzyme causes 5-fluorouracil-2'-deoxyriboside (FUDR) to be phosphorylated, turning a minimally toxic pro-drug into a powerful inhibitor. Thus tk^+ can be regarded as a dominant sensitivity gene (Sachs *et al.* 1997), and this can be used to select strongly against the outgrowth of heterokaryotic conidia or germlings."

Procedures

- *Macroconidia*. Where the desired component can be recognized or selected, streaking to sorbose-glucose-fructose agar medium and picking three times will often suffice to obtain pure colonies originating from monoiaryotic macroconidia.. Growth at 34°C on 0.5 ml medium in small tubes for 1-1/2 days will produce conidia for the next round of suspending and streaking.

- Microconidia. Microconidia can be recovered from macroconidiating strains by filtration (Ebbole and Sachs 1990) or by the cellophane overlay technique of Pandit and Maheshwari (1993). See *How to obtain microconidia*, which also describes use of the *mcm* mutant and of *pe fl, fl;dn,*and *fl* mutant strains, which produce only microconidia.

  The following is excerpted from Ebbole and Sachs (1990):

  "Sterile 16 x 150 mm glass culture tubes containing 6 ml of a sterile, molten solution consisting of 2% agar/0.5% sucrose/0.1 x SC and plugged with pre-autoclaved foam plugs, were adjusted to 50-60°C in a water bath. Then 60 µl of 0.1 M sodium IAA (Sigma Catalog # I2512, freshly prepared, filter-sterilized in water) was mixed with the contents of each tube (final concentration 1.0 mM). Tubes were slanted at room temperature until solidified, and stored refrigerated for up to one week.

  Slants were inoculated using an agar plug of mycelia (obtained by coring a colony with a sterile pasteur pipet) or using a drop of macroconidia suspended in water. Cultures were incubated at 25°C with a 12 hour light/dark cycle for 7-10 days. The production of microconidia was checked by lightly scraping an area of the sparse surface growth (away from tufts of macroconidia) with a sterile, wet inoculating loop. The loopful of culture was transferred to 20 µl of water and examined microscopically.

  Microconidia were harvested from cultures by adding 2.5 ml of sterile water to tubes followed by rigorous Vortex mixing for 30-60 sec (more microconidia were obtainable by repeating this harvesting step). The conidial suspensions were passed through 5 µm Millex Durapore filter units (Millipore catalog number SLSV025LS) using sterile conditions. Typically 0.1-1% of the microconidia were recovered. The yield as determined by counting with a hemacytometer varied from 10^3-10^6 microconidia per slant. Filtrates with low numbers of microconidia were concentrated by pelleting the microconidia in a clinical centrifuge (Beckman RT6000, 2000 × g, 5 min). Most of each supernatant was removed by aspiration and the pellets resuspended in the remaining liquid.

  Microconidia were germinated at 34°C after spreading on freshly prepared Vogel's/sorbose agar plates. Microconidial viability varied from 1-20%, as determined by comparison of the number of colony forming centers/ml after seven days growth to the number of microconidia/ml as determined by direct counting. We typically plated 2000 microconidia/plate to obtain homokaryotic cultures, and picked colonies after 2-3 days. Vegetative homokaryotic stocks were obtained by transferring individual colonies to slants of Vogel's sucrose medium."

  The following is excerpted from Pandit and Maheshwari (1993):

"The cultures were initiated by placing a trace quantity of macroconidia at the center of a cellophane circle which covered the surface of 2% water agar in a Petri dish. The cellophane (a transparent cellulose sheet made from viscose) was pierced at the point of inoculation. The circles were cut from cellophane sheets purchased from market, boiled for 5 min in 1% KOH to remove soluble impurities, washed by swirling in distilled water, spread between wet Whatman filter paper, and autoclaved before laying over the surface of solidified agar. The Petri dishes were inverted and kept for 7-12 days at 22-25 C in a room which received intermittent illumination. The stacked Petri dishes along with a beaker containing water were covered by a bell jar to provide a humid atmosphere.

A thin mycelial growth developed over cellophane and produced patches of aerial microconidiophores in 7-12 days. However, some macroconidiophores were also produced. If the cellophane was peeled off after approximately 10 days of mycelial growth on cellophane, then microconidiophores developed selectively within 3-5 h from the thin mycelium which had grown in the agar under the cellophane. The young microconidiophores generally were associated with a liquid droplet.

The microconidia were harvested 24 h after removing the cellophane by adding 1-2 ml sterile water, tilting the Petri dish, and removing the microconidial suspension with a pipette. A second and third crop of pure microconidia could be obtained after harvest of the previous crop.

The production of microconidiophores both on cellophane and subsequently on agar was increased substantially if water agar was replaced by Westergaard and Mitchell synthetic crossing (SC) medium supplemented with iodoacetate (Rossier *et al.* 1973) as modified by Ebbole and Sachs (1990). This medium contained 0.1 x SC, 0.5% sucrose, 1 mM iodoacetate (IAA) and 2% agar. Since the development of macroconidiophores precedes that of microconidiophores (Springer and Yanofsky 1989), the initiation of mycelial growth on cellophane followed by its removal was expedient for the production of virtually pure microconidiophores. All strains tested produced microconidiophores by this method. Microconidia were counted by a haemocytometer and 150-200 were spread on sorbose plating medium (Davis and de Serres 1970). The number of colonies formed was used to estimate their viability."

- *Thymidine kinase helpers*. (Recommended when assurance is needed that a minority component is no longer present.) The following is excerpted from Metzenberg and Sachs (2002):

"The new helpers are: *mat*^{D} *tk*$^{+}$ *cyh-1; Bml pan-2; inl* ("*Helper 2*", FGSC 8745), *mat*^{D} *his-2 tk*$^{+}$ *cyh-1; Bml pan-2; inl* ("*Helper 4*", FGSC 8746), and *mat*^{D} *his-3; hyg*^{R} *tk*$^{+}$ *Bml pan-2* ("*Helper 5*", FGSC 8747). [*mat*^{D} signifies *mating type* locus deleted.]

In our hands, optimal conditions for isolating strains not bearing *tk*$^{+}$ from heterokaryons with a strain that is *tk*$^{+}$ (sensitive) are as follows: FUDR, 2 micromolar, and uracil, 1 millimolar. [A rationale for adding uracil is given.] Streaking or plating a water-suspension of conidia from such a heterokaryon to appropriately supplemented sorbose-glucose-fructose agar medium results in colonies which are homokaryons of the component that does not carry *tk*$^{+}$. In our hands, 1-2 micromolar FUDR is the minimum concentration which gives only homokaryons, but at least 10 micromolar FUDR works equally well and may be preferred for the most exacting work. The uracil concentration is 1 millimolar. The method works about equally well at 23°C or 34°C with either the salts base of Vogel or of Westergaard-Mitchell."

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