How to analyze and quantify conidial anastomosis tube (CAT)-mediated cell fusion

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Background

Conidial anastomosis tubes (CATs) are specialized cell protrusions formed from asexual conidia or germ tubes (GTs) of *Neurospora crassa* and other filamentous fungi. They function to establish cell fusion connections between conidial germlings during colony initiation (Figs. 1A-B). CATs of the wild type are morphologically distinguishable from GTs by being thinner (2-3 µm in width), unbranched, and exhibiting determinate growth (typically with a maximum length < 10 µm). GTs are usually thicker (4-5 µm in width), often branch, show indeterminate growth, and differentiate into vegetative hyphae, which branch profusely, and ultimately form the mature fungal colony. Another key distinguishing feature of CATs is that they exhibit positive chemotropism towards each other whereas, in contrast, GTs tend to avoid each other, and thus display negative chemotropisms (Roca *et al.*, 2005; Read *et al.*, 2009, 2010). Another useful way to distinguish CATs from GTs is based on their resistance to the microtubule polymerizing inhibitor, benomyl; CAT formation, homing, and fusion occur in the absence of microtubules while the formation of elongated GTs is microtubule-dependent (Roca *et al.*, 2010).

The study and analysis of CAT-mediated cell fusion in *N. crassa* has proven to be useful in a number of different ways, including:

1. As a simple, experimentally amenable and genetically tractable eukaryotic model system for studies on self-signalling and self-fusion (Read *et al.*, 2009, 2010). Dependent on temperature and nutrient availability, the whole process from conidial inoculation to CAT formation and cell fusion occurs within 4-6 h, which is much faster than in other filamentous fungi, such as *Colletotrichum lindemuthianum* (Ishikawa *et al.*, 2010). Quantification of CAT-mediated cell fusion is straightforward and generates highly reproducible results.
2. Screening for mutants compromised in CAT formation, homing, or fusion (e.g. Roca *et al.*, 2005, 2010; Fleissner *et al.* 2005, 2009).
3. Analyses of the inhibitory effects of pharmacological agents or other chemical treatments on CAT chemotropism and cell fusion (e.g. Roca *et al.*, 2010).
4. Generation of genetically compatible heterokaryons stably expressing multiple fluorescently labelled proteins by means of vegetative cell fusion (Roca *et al.*, 2010).
5. Reduction of over-expression artefacts of ectopically introduced protein constructs through vegetative heterokaryon formation with non-expressing strains (Roca *et al.*, 2010).

Procedures

Protocol for the quantification of CAT-mediated cell fusion

1. Grow the desired *Neurospora crassa* strain on solid Vogel's agar medium containing 2% (w/v) sucrose for 4-5 days at 35°C or until sufficient conidia have been developed. Conidiation can be promoted by placing the cultures for 1-2 days in daylight before harvesting the conidia.
2. Prepare a cell suspension by harvesting the conidia from the plate cultures in liquid medium. Start with 1 ml liquid and gently rinse the conidia repeatedly off the culture surface without damaging the mycelium. If the liquid is soaked up by the culture, use an additional ml and repeat rinsing, until a sufficient amount of run-off (~ 1 ml) becomes cloudy with conidia. Finally, collect the conidial suspension in an Eppendorf tube.
3. Note the time. In the presence of nutrients the conidia will start the germination process; thus, this is the first time point of your experiment.
4. Vigorously vortex the harvest suspension for at least 1 min to break up conidial chains and produce a homogenous conidial suspension.
5. Determine the number of conidia per ml of harvested cell suspension, using a Fuchs-Rosenthal cell counting chamber or hemocytometer. For convenient counting, prepare a 1:100 dilution of your original harvested conidial suspension, or a stronger dilution if appropriate.
6. Prepare a working conidial suspension by adjusting your harvest suspension with Vogel's medium to a concentration of 1×10^6 conidia per ml, which is the optimum concentration for CAT formation and

germling network formation. Ensure that you have at least 800 µl of adjusted conidial working suspension for the experiment.

7. Place 200 µl of the working suspension into four separate compartments of a slide culture chamber (we use an eight-well slide culture chamber from Nalge Nunc International, Rochester, NY). You will therefore have 4 x 200 µl samples to analyse. You may want to prepare duplicates, i.e. 2 x 200 µl of each condition on two separate slides.
8. Incubate your samples in the dark at 35°C until the desired time point to record cellular development. Lower incubation temperatures can alternatively be used to slow down CAT formation and fusion. Both occur more rapidly at 35°C than at 25°C (e.g. the amount of CAT formation and cell fusion after 5 h at 35°C is roughly equivalent to that after 6 h at 25°C).
9. Examine the samples at room temperature by using brightfield or differential interference contrast optics with a 60x or 100x objective, and record 10-15 images per sample condition for subsequent analysis. We routinely quantify CAT formation and cell fusion in the wt after 4 and 6 h (for two time points) or after 5 h (for one time point only) of incubation at 35°C. This will need to be adapted for slower or faster developing mutant strains, or when drugs that influence cellular development are applied (Fig 1A-B).
10. Quantify CAT-mediated cell fusion as the percentage of conidia or conidial germlings involved in cell fusion. For each condition and time point analyse 100 conidia in the 4 independent samples (n = 400) and calculate the mean. We routinely also determine the percentage of total germination from the same samples. For this, germination is defined as the event of cell symmetry breaking, i.e. we quantify the number of conidia that form protrusions that develop into either GTs or CATs.
11. Incubate the samples at 35°C between recordings. Be very careful not to shake samples when transferring them between the incubator and the microscope as this may mechanically perturb polarized growth and displace interacting cells.
12. Finally, produce a histogram/graph showing percentage of conidial germination and CAT-mediated cell fusion at the various time points (Fig. 1D).

Alternative harvest procedure for short-term spore storage

Harvesting and maintaining a concentrated cell suspension in sterile water prevents conidial germination and allows storage of this batch of conidia for a few days in the fridge. This stored cell suspension can be used for subsequent or repeated experiments, thus saving the time and avoiding the error potentially associated with preparing conidial suspensions on different occasions. Furthermore, using a concentrated stock suspension allows easier preparation of samples with defined cell densities.

1. Grow the desired strains as described in point 1 above.
2. Harvest conidia as described in point 2 above, but instead of using Vogel's medium, use sterile (autoclaved) dH_2O.
3. Determine the cell concentration per ml of harvested conidial suspension, as described in point 5 above.
4. Adjust the number of conidia in suspension with water to a concentration of 1×10^7 cells/ml. The high conidial density (probably resulting in an increased concentration of a germination self-inhibitor) and the absence of nutrients prevents germination and allows storage of the cell suspension for at least up to a week at 4°C.
5. Place 180 µl of medium in each chamber of a culture slide and add 20 µl of your stored conidial suspension (at a concentration of 1×10^7 cells/ml) to yield the final, optimal spore concentration of 1×10^6 cells/ml. This will be the 0 h time point of your experiment. The dilution of the Vogel's medium can be neglected, as no statistically significant differences in cell development or the amount of CAT-mediated cell fusion between undiluted (100%) down to half-diluted (50%) Vogel's medium have been observed.
6. Continue from point 8 in the protocol above.

C. References

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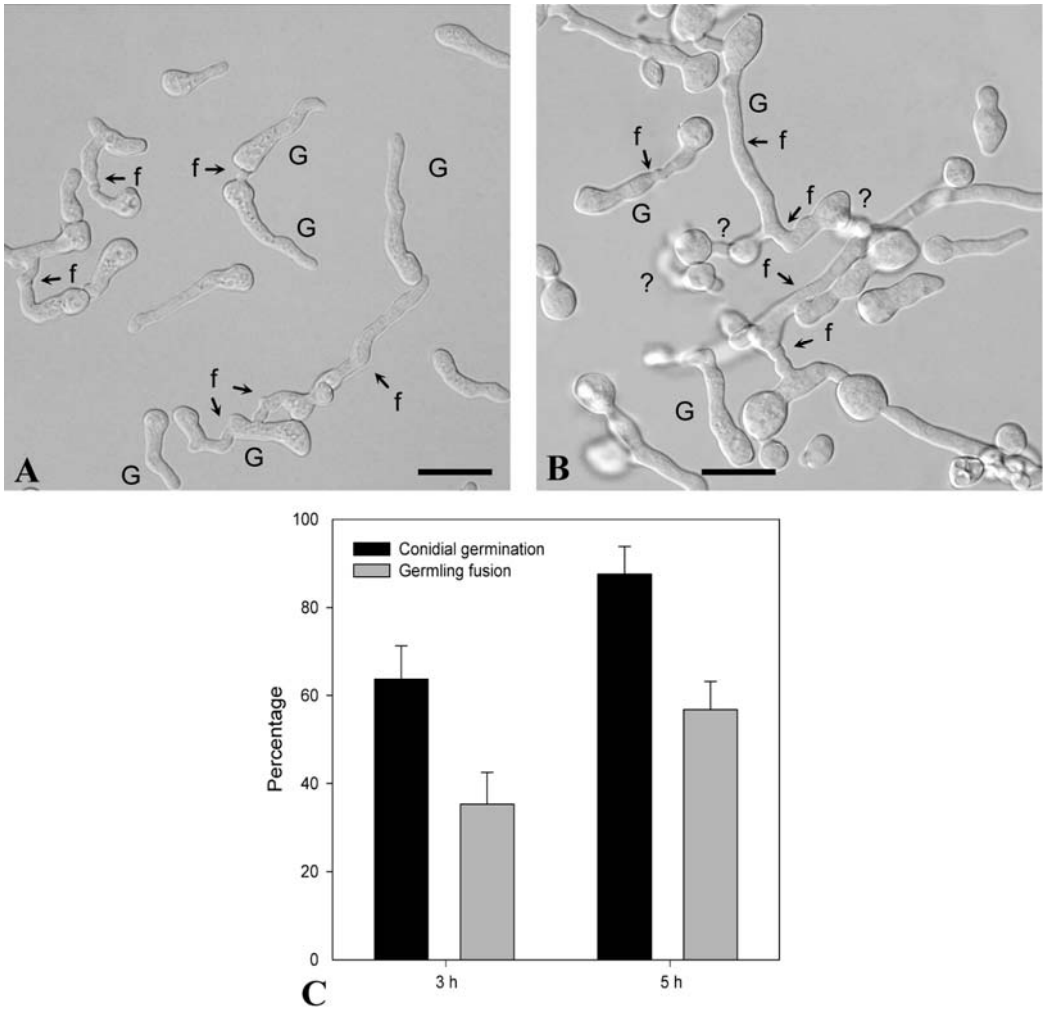

Figure 1. CAT-mediated cell fusion in *Neurospora crassa*. **(A)** fused germlings after 5 h of incubation in liquid Vogel's medium; **(B)** fused germlings after 5 h incubation in a more dense field of view but otherwise identical conditions; **(C)** Histogram showing quantification of CAT-mediated cell fusion and conidial germination in liquid media. Bars: 10 µm. f, fusion connections; G, germ tube; ?, ambiguous fusion connection not counted.