How to distinguish conidial anastomosis tubes (CATs) from germ tubes, and to discriminate between cell fusion mutants blocked in CAT formation and CAT homing

Alexander Lichius, M. Gabriela Roca and Nick D. Read
Fungal Cell Biology Group, Institute of Cell Biology, University of Edinburgh, Edinburgh EH9 3JH, UK

Background

Fusion between conidial germlings of filamentous fungi involves the formation and chemotropic interaction of specialized cell protrusions, which are different from germ tubes (GTs), and have been termed *conidial anastomosis tubes* (CATs) [6-8] .

The initial characterization of CATs was made in the plant pathogen *Colletotrichum lindemuthianum* [8] and the saprotrophic species *Neurospora crassa* [6]. CATs were shown to have the following combination of characteristics which distinguishes them from GTs:

1. CATs are thinner and shorter than GTs and exhibit determinate growth. GTs differentiate into vegetative hyphae of the mature colony, whereas the sole function of CATs is to form a germling network through the establishment of cell fusion connections between individual asexual spores (conidia) [1, 4-6].
2. CATs are unbranched and aseptate, while GTs undergo branching and form septa [1, 6].
3. CATs in *N. crassa* can arise directly from conidia, from GT tips or as branches of GTs, often subapically. A single spore can form multiple CATs, whereas under normal conditions only one GT is being formed [6, 7, 9].
4. CAT induction is dependent on conidial density. In *N. crassa*, the optimum cell number to achieve maximum CAT formation and CAT-mediated cell fusion is 1×10^6 cells/ml [6]. Germ tube formation in *N. crassa* is not dependent on conidial density over the range of 1×10^2 to 1×10^6 conidia/ml (M.G. Roca and N.D.Read, unpublished data).
5. CATs show positive chemotropism towards each other, whilst GTs exhibit negative chemotropism (avoidance) away from other GT tips or conidia. CAT chemoattraction (homing) has been most unambiguously demonstrated using optical tweezer micromanipulation. When the relative position of two cells with homing CATs was changed using laser tweezers, both cells readjust the growth direction of their CATs in order to make contact and fuse at their tips [3, 6, 7, 10].
6. CATs are under separate genetic control to that of GTs. A growing number of mutants have been identified that are inhibited at specific stages of CAT-mediated cell fusion, but undergo normal GT formation [4, 5].
7. CAT-mediated cell fusion is independent of microtubules. Addition of microtubule disrupting drugs, such as benomyl, blocks the formation of elongated GTs, but does not interfere with CAT-mediated cell fusion [9]. CAT formation, homing and fusion relies entirely on the F-actin cytoskeleton [2, 9]. This remarkable functional separation of both cytoskeletal elements can be exploited to distinguish CATs from GTs, and to discriminate between cell fusion mutants blocked in CAT formation and CAT chemoattraction.

Assay to distinguish CATs from GTs

1. Grow the desired *Neurospora crassa* strains on solid Vogel's medium (VM) containing 2% (w/v) sucrose for 3-4 days at 35 °C, or until sufficient conidia have been formed, which in developmentally delayed mutant strains might take significantly longer compared to the wt. Conidiation can usually be promoted by placing the cultures for 1-2 days in daylight before harvesting the cells.
2. Prepare a conidial cell suspension by collecting conidia from plate cultures in sterile ddH_2O. Start with 1 ml of sterile ddH_2O and gently rinse the conidia repeatedly off the culture surface without damaging the mycelium. If the water is soaked up by the culture, use one or more additional mls of sterile ddH_2O and repeat rinsing, until ~ 1 ml of ddH_2O 'run-off' becomes cloudy with a slight orange colour. Finally collect the conidial suspension in an Eppendorf tube.
3. Vigorously vortex the harvested conidial suspension for about 1 min to break up conidial chains and produce a homogenous conidial suspension.
4. Determine the cell number per ml in the harvest suspension using a Fuchs-Rosenthal cell counting chamber or a hemocytometer. For convenient counting, prepare a 1:100 dilution of your original harvested suspension, or a stronger dilution if appropriate.
5. Prepare a stock conidial suspension by adjusting with sterile ddH_2O to a concentration of 1 x 10^7 cells/ml. The high conidial density (probably resulting in an increased concentration of a germination self-inhibitor) and the absence of nutrients are believed to be responsible for preventing germination. Storage of the conidial suspension at 4 °C allows it to be re-used for < 1 week.
6. Prepare a 1000x benomyl stock solution by diluting 3.5 mg benomyl (e.g. cat no. 45339, Fluka) in 1 ml DMSO (e.g. cat no. D2650, Sigma; ideally from a septum-sealed bottle). The maximum solubility of benomyl in an aqueous solution is 3.6 µg/ml at pH 5 and 25 °C
7. Place 180 µl of liquid 60% VM (i.e. VM diluted with sterile ddH_2O in order to prevent precipitation of benomyl) in each chamber of an 8-well chambered culture slide (#155411, Nalge Nunc) and add 20 µl of your adjusted conidial suspension (1 x 10^7 cells/ml) to yield the final, optimal spore concentration of 1 x 10^6 cells/ml. Addition of the conidia to the liquid growth medium marks the 0 h time point of your experiment. Diluting the VM by < 50% with ddH_2O has been found to not significantly affect GT formation, GT elongation, CAT formation or CAT-mediated cell fusion. Greater VM dilution can lead to changes in the GT/CAT ratio (A. Lichius and N.D. Read, unpublished data).
8. Add 0.2 µl of the 1000x benomyl stock to 200 µl of conidial suspension in each well. The final concentration of DMSO will be 0.1%. To prevent cytotoxic damage it is generally recommended not to exceed a final DMSO concentration of 0.5 %.
9. Prepare duplicate samples in separate 8-well chamber slides (slides A and B). For a larger number of different experimental conditions per slide (> 4), separate slides A and B by the time it takes you to record all the necessary images from slide A. 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 cell fusion. In the presence of benomyl, i.e. in the absence of microtubules, conidia still induce the formation of germ tubes and occasionally protrude GT buds. These, however, remain swollen and do not elongate (compare Figs. 1A and 1E). Therefore, any polarized cell protrusion with the typical CAT dimensions (2-3 µm in width and < 10 µm in length) emerging from the spore body is most likely to be a CAT. Furthermore, in cell fusion competent strains, CAT-mediated cell fusion

occurs unhindered and leads to the formation of interconnected conidia (Fig. 1E). Note that due to extended isotropic growth in the presence of benomyl, the average spore diameter will increase compared to the untreated controls (Fig. 1A-D).

10. Examine the samples at room temperature by using brightfield or differential interference contrast optics with a 60x objective, and record 10-16 images per sample condition for subsequent analysis. To correct for continued cell development during image acquisition do the following: in case you have 8 samples take 5-8 images in each sample proceeding from sample 1 to 8. Then reverse the order and take a second set of 5-8 images per sample proceeding from sample 8 to 1. Changes in morphology and the GT/CAT ratio will be averaged out in the subsequent quantification. We routinely quantify cell development after 4 and 6 h of incubation at 35 °C. This however might need to be adapted for slower or faster developing mutant strains, or when drugs that influence cellular development are being used.
11. Incubate the samples at 35 °C between recordings. Avoid shaking the samples during transfer between incubator and microscope as this will mechanically perturb polarized growth and potentially displace interacting cells.
12. In the untreated control samples (Fig. 1A-D), quantify the percentage of conidia that break cell symmetry and germinate by the formation of a GT or CAT, form elongated GTs (longer than the spore diameter; on average > 10 µm in length), and form CAT or CAT-like structures that are unfused or have undergone cell fusion. A cell symmetry breaking event (germination) is scored when any protrusion emerges from the conidium.
13. Similarly quantify cell symmetry breaking, elongated GTs, and CATs and CAT-like protrusions in the benomyl treated samples.
14. Finally, produce a histogram displaying the percentage of conidia that have undergone cell symmetry breaking, formed elongated GTs, and formed CATs and CAT-like protrusions (Fig. 2).

References

1. **Araujo-Palomares, C. L., E. Castro-Longoria, and M. Riquelme.** 2007. Ontogeny of the Spitzenkörper in germlings of *Neurospora crassa*. Fungal Genetics and Biology **44:**492.
2. **Berepiki, A., A. Lichius, J.-y. Shoji, J. Tilsner, and N. D. Read.** 2010. F-actin dynamics in *Neurospora crassa*. Eukaryotic Cell **9:**547-57.
3. **Fleißner, A., S. Sarkar, D. J. Jacobson, G. M. Roca, N. D. Read, and N. L. Glass.** 2005. The *so* locus is required for vegetative cell fusion and postfertilization events in *Neurospora crassa*. Eukaryotic Cell **4:**920-930.
4. **Read, N. D., A. Fleißner, M. G. Roca, and N. L. Glass.** 2010. Hyphal fusion. *In* K. A. Borkovich and D. Ebbole (ed.), Cellular and Molecular Biology of Filamentous Fungi. American Society of Microbiology, Washington DC.
5. **Read, N. D., A. Lichius, J. Shoji, and A. B. Goryachev.** 2009. Self-signalling and self-fusion in filamentous fungi. Current Opinion in Microbiology **12:**608-615.
6. **Roca, G. M., J. Arlt, C. E. Jeffree, and N. D. Read.** 2005. Cell biology of conidial anastomosis tubes in *Neurospora crassa*. Eukaryotic Cell **4:**911-919.
7. **Roca, G. M., N. D. Read, and A. E. Wheals.** 2005. Conidial anastomosis tubes in filamentous fungi. FEMS Microbiological Letters **249:**191-198.
8. **Roca, M. G., L. C. Davide, M. C. Mendes-Costa, and A. Wheals.** 2003. Conidial anastomosis tubes in Colletotrichum. Fungal Genetics and Biology **40:**138-145.
9. **Roca, M. G., H.-C. Kuo, A. Lichius, M. Freitag, and N. D. Read.** 2010. Nuclear dynamics, mitosis and the cytoskeleton during the early stages of colony initiation in *Neurospora crassa*. Eukaryotic Cell **9:**1171-1183.
10. **Wright, G. D., J. Arlt, W. C. K. Poon, and N. D. Read.** 2007. Optical tweezer micromanipulation of filamentous fungi. Fungal Genetics and Biology **44:**1-13.

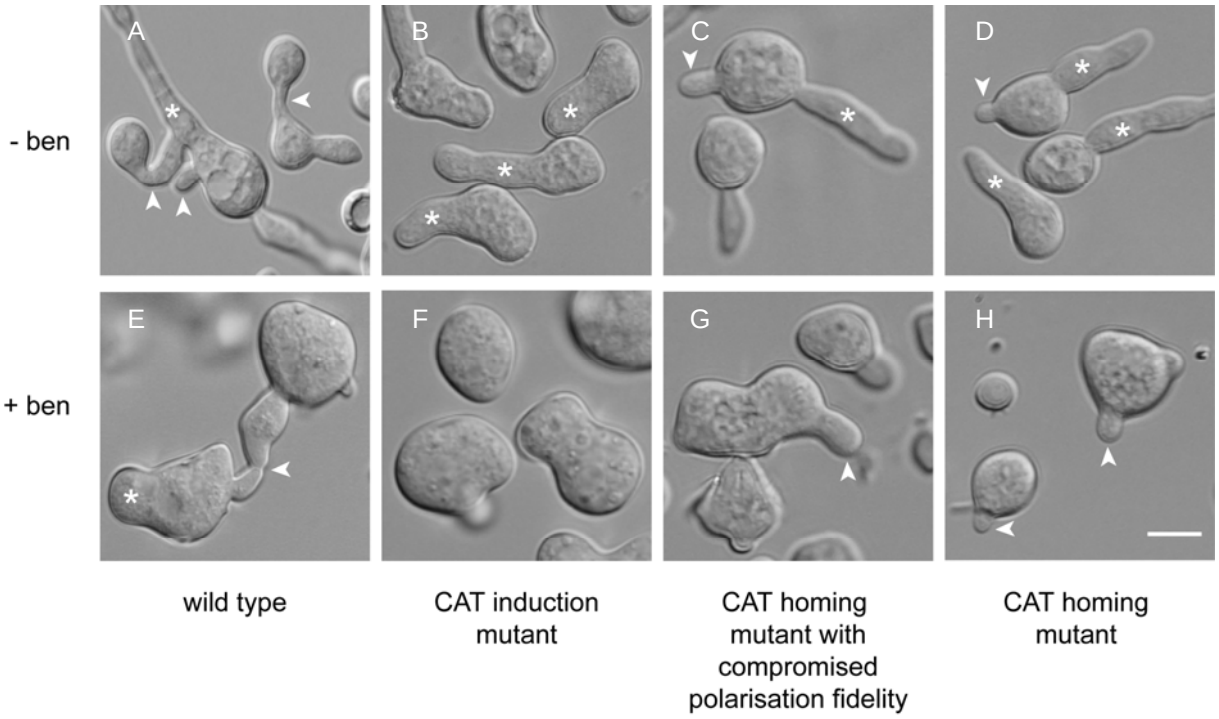

Figure 1. CAT formation and CAT-mediated cell fusion is revealed in the presence of benomyl. (A-D) In the absence of the microtubule polymerizing inhibitor benomyl (ben), elongated GTs (asterisks) were formed in the wild type and cell fusion mutants of *N. crassa*. **(A)** In the wild type, CAT formation and CAT-mediated cell fusion occurred normally (both indicated with arrowheads). **(B)** In gene deletion mutants which are unable to form CATs, only elongated GTs were formed. **(C and D)** In CAT homing mutants, CAT-like protrusions were formed in addition to elongated GTs. On the cellular level, morphological differences between CAT homing mutants which are compromised in polarization fidelity (C) and those that are not (D), are only very subtle, but can lead to an increased spore diameter before cell symmetry is broken. Differences, however become more apparent upon quantitative analysis of the cell population (see Fig. 3). **(E-H)** In the presence of ~ 3 µg/ml benomyl development of elongated GTs was efficiently supressed. **(E)** Cell symmetry breaking through the initiation and protrusion of GT buds could be observed (asterisk), as here indicated for the wt. **(F)** CAT induction defective strains tend to show increased istropic extension and were unable to break cell symmetry through the protrusion of CATs. Due to the lack of clearly identifiable morphological features, formation of GT buds is generally difficult to assess, but does occur. However, neither elongated GTs nor CATs were being formed. **(G-H)** Conidia of CAT homing mutants still formed CAT-like protrusions in the presence of benomyl, but were unable to chemotropically interact and fuse. Scale bar, 5 µm. All cells imaged after 4 h of incubation at 35 °C in the presence or absence of benomyl, respectively.

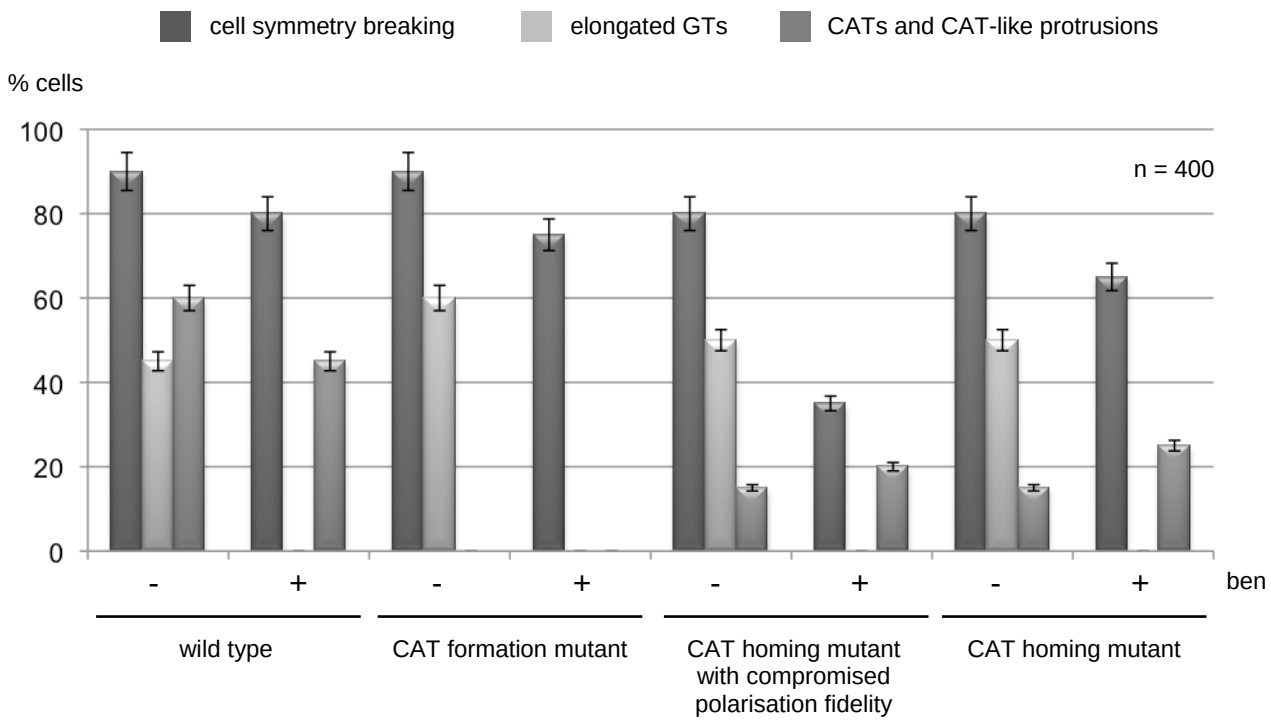

Figure 2. Quantification of cellular development of strains shown in Fig. 1 after 4 h of incubation in the presence or absence of ~ 3 µg/ml benomyl at 35 °C. In the presence of benomyl, conidial germination can be slightly delayed in the wt, leading to a reduced number of cells that broke cell symmetry after 4 h of incubation. Nevertheless, CAT formation and CAT-mediated cell fusion occurred normally, and after 6 h usually reached levels as high as in the untreated control (not shown). Slightly delayed germination in the presence of benomyl was usually also observed in mutants unable to form CATs. Importantly, in the presence of benomyl, no elongated GTs nor CAT-like protrusions were formed, confirming the inability of these strains to induce CATs. All CAT homing mutants identified to date, generally showed a much reduced level of CAT-like protrusions compared to the wild type, which was usually < 20 % of the cell population. Blocking GT development with benomyl can lead to a slight increase in the CAT formation in these strains, a feature not observed in the wt. Fusion mutants compromised in cell polarization fidelity, were more sensitive to benomyl treatment and displayed much reduced germination in the presence of the drug compared to fusion mutants which broke cell symmetry normally. Most importantly, however, both types of mutants do form CAT-like protrusions in the absence of microtubules, justifying their classification as CAT homing mutants. Error bars represent standard deviations.