Inverted Race Tube Assay for Circadian Clock Studies of the Neurospora Accessions.

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Although the *Neurospora crassa* circadian clock has been studied for forty years, population studies of natural accessions have been limited by technical difficulties associated with the conventional race tube assay (CRTA) that is used to measure asexual development (conidiation). Due to the buildup of CO2 in the CRTA that represses banding, a mutant strain band (bd) has been utilized for increased visualization of the banding phenotype. In order to study the circadian clock in natural accessions of Neurospora multiple techniques have been explored. One such technique, the rubidium chloride-supplemented race tube assay (RRTA) has been used successfully. Here we present a new technique, the Inverted Race Tube Assay (IRTA) that is a simple modification of the CRTA. We analyzed 5 natural accessions of Neurospora using CRTA, IRTA and RRTA and discuss the advantages of the IRTA in natural variation studies in Neurospora.

N. crassa has proved to be a successful model for studying the molecular bases of circadian clocks. Significant insights into the mechanism of the clock have been obtained in N. crassa including a) transcriptional/translational feedback loops in the clock that opened up the conceptual foundation for our understanding of the biological clock (Aronson et al.1994); b) the resetting mechanisms by light and temperature (Liu *et al.* 1998; Crosthwaite et al. 1995); c) circadian gating of cellular activities (Heintzen *et al.* 2001); and d) the involvement of anti-sense RNA in the circadian regulatory circuit (Kramer *et al.* 2003).

A critical step in studying the circadian clock is the ability to easily observe and measure circadian regulated behavior. The rhythmic behavior that has been most extensively studied in *N. crassa* is asexual spore development using the CRTA. A race tube is a long glass tube (28 inch long) that is bent up at both ends to hold an agar medium. Mycelia or conidia are inoculated at one end of the race tube and the fungus grows toward the other end. To synchronize the cells to the same time of day, the cultures are germinated in constant light for a day and then transferred to constant darkness. Under these conditions, Neurospora initiates asexual development about every 22 hrs (Loros and Dunlap 2001). As a result of asexual development, Neurospora produces orange colored spores creating the “banding” phenotype. This easily tractable clock phenotype has been integral in dissecting the genetic structure of the Neurospora circadian oscillator.

All laboratory Neurospora strains used in clock studies contain the mutation band (*bd*) (Sargent and Woodward 1969). In the absence of the *bd* mutation, the rhythmic asexual development of Neurospora in a race tube is suppressed by the accumulated CO_2, a byproduct of respiration (Sargent and Kaltenborn 1972). Before the discovery of the *bd* mutant, researchers relied on a device to blow fresh air into the race tube to prevent the accumulation of CO_2. However, blown air easily disperses the highly hydrophobic Neurospora spores inside the race tube, ruining the experiment. In the *bd* mutant background, the rhythmic pattern of asexual development can be visualized without the air circulation device. It was proposed that the *bd* mutation desensitizes the repressive role of CO_2 in rhythmic asexual development in Neurospora, although its specific role is unknown (Sargent and Kaltenborn 1972). This repressive role of CO_2 has been a major obstacle in studying natural variation of the circadian clock in wild-collected Neurospora accessions (Morgan and Feldman 1998). In addition to the adoption of blowing fresh air into the race tube, crossing natural accessions with the *bd* mutant, or the rubidium chloride-supplemented race tube assay (RRTA) (Morgan and Feldman 1998) have been suggested as methods to monitor banding in Neurospora accessions. However, each of these solutions created additional issues. First, crossing the accessions with the *bd* strain is not desirable because it does not maintain the heterogeneity of the genomic structures between accessions. Second, the RRTA introduces another variable; the mode of rubidium chloride action is not characterized, adding an unknown environmental factor that further complicates quantitative studies (Morgan and Feldman 1998).

With all these technical problems in mind, we developed a modified race tube assay, the Inverted Race Tube Assay (IRTA) (Fig. 1). We reasoned that if it is truly only the CO_2 trapped in the bottom of the race tube that prevents the rhythmic asexual development [8], one could remove the heavy CO_2 by simply inverting the race tube. 25 ml of media (1X Vogel, L-arginine 0.17%, D-glucose 0.1%, 1.5% agar) is poured into the race tube and autoclaved (20 min. 121 ˚C). As the media cools down, the media solidifies; due to the water condensation on the inside surface of the race tube, the solid media can settle on the bottom (what used to be the top) of the race tube as the race tube is turned upside down. The race tube is then placed on a flat surface overnight until the excess water inside the race tube is dried.

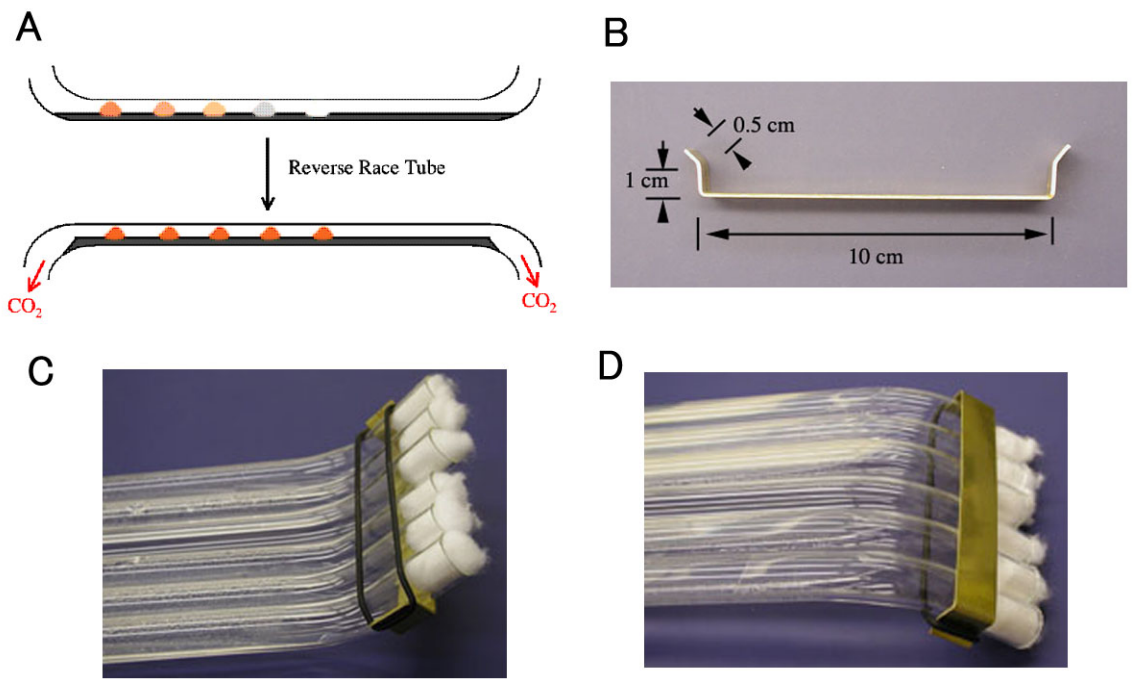

Figure 1. Inverted race tube assay. A) A cartoon of the inverted race tube. B) design of the race tube holder. The holder is made of stainless steel (Incodema Inc. Ithaca, NY). C) and D) shows different sides of the race tubes. For convenience of cleaning and maintenance, we used o-rings (o-ring 2-143, Web Seal Inc. Rochester, NY) to hold the six race tubes as shown in C and D.

Illustrated in Figure 2, the banding phenotypes in five representative accessions were compared in the three different race tube assay methods: IRTA, RRTA, and CRTA. Banding was evident in all three assays; however, the banding pattern was clearer and more robust in the IRTA compared to both the CTRA and RRTA (Fig.2). In general, the accessions grew more slowly in RRTA than in CTRA and RRTA. There were only marginal differences between growth rates between CTRA and ITRA. In the RRTA, sporadic and non-circadian asexual development obscured accurate scoring of circadian parameters, as compared to the IRTA. There is natural variation in terms of the Neurospora accessions' ability to cope with the adverse effects of CO_2 in asexual development.

Here we present data comparing the CRTA, IRTA and RRTA in assessing the circadian clock of Neurospora accessions. The IRTA increases both the percentage of banding phenotype in accessions as well as the clarity of banding as compared to either RRTA or CRTA. Furthermore, the IRTA does not require additional equipment or media modifications from the CRTA, making the results from the IRTA parallel with current and past CRTA studies. The optimized IRTA will significantly contribute to the study of circadian clock natural variation, as well as population and ecological studies in Neurospora.

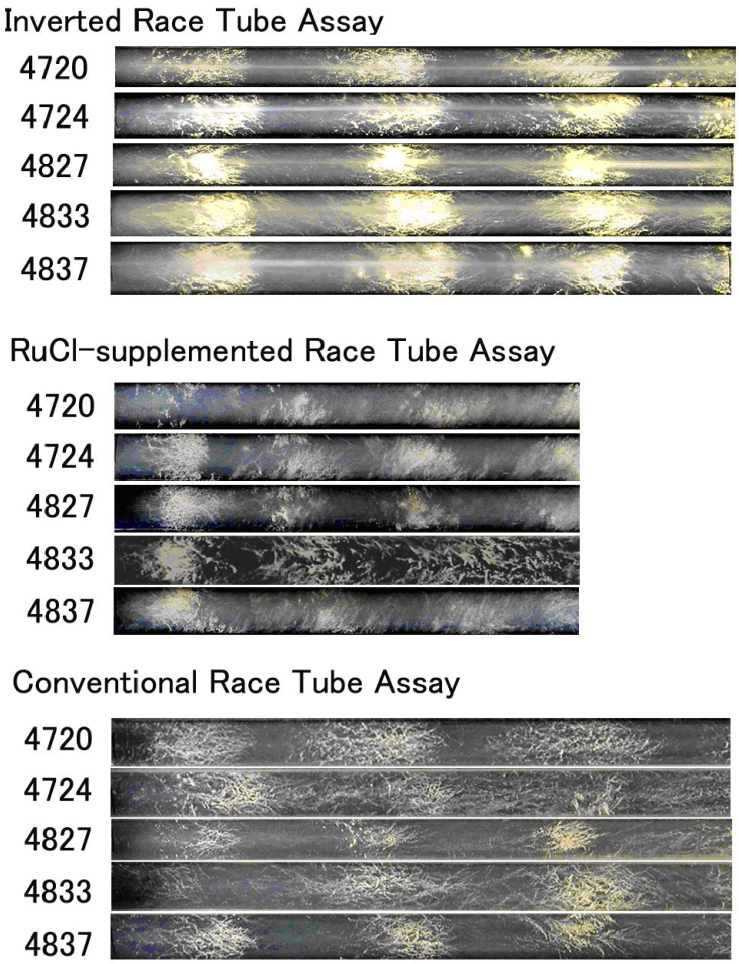

Figure 2. Comparison of the three race tube assay methods.
The numbers in the left column represent the Fungal Genetics Stock Center numbers. After inoculation, the races were incubated in constant light (500 ?moles/m2/sec) and 25 ˚C for 12 hr until there was an even growing front. Then the race tubes were incubated in constant darkness for several days. We observed that the growth rate of the accessions in the RRTA was about three-fourth of that in the inverted race tube. The images of IRTA and RRTA were taken from the bottom of race

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