Mycofluidics:
The Fluid Mechanics of Fungal Chimerism

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Abstract

The resilience of fungi is a major contribution to their pronounced effect on human lives. To protect our health and economic transactions, billions of dollars have been spent to control certain fungal species. Along with a general lack of understanding on how fungi operate, the chimeric aspect of fungi, or their ability to support multiple genetically different nuclei within a single spore,is thought to increase their adaptibility in the face of eradication [1]. It has previously been shown that nuclear mixing within the fungal mycelium keeps genetically different nuclei well-mixed and in stable proportions throughout a fungus [2]. Our paper is an attempt to see whether this genetic diversity can be maintined over time and distance. Our experiment consisted of growing *Neurospora crassa* in race tubes, and then looking at how p_r, or the probability that an individual nucleus was red, fluctuated over time. We found that over the length of the race tube, p_r tended to oscillate around the intial p_r, suggesting that in the case of a fungus with genetically different nuclei, a mixture could be maintained over time and space.

1 Introduction

Genetic diversity within an organism contributes to the resilience of the species as a whole. In syncytial organisms, such as filamentous fungi, genetically diverse nuclei are free to move within the same cytoplasm. This leads to the idea of chimerism, in which a single fungal spore may be multinucleate. This capability offers wide internal genetic diversity and is often thought to contribute to the virulence of fungi [1]. It has been shown that fungi employ nuclear mixing within the fungal mycelium to keep these genetically different nuclei well-mixed and in stable proportions thoughout the fungus. This mixing is further augmented by hyphae, which are the branching filamentous structures of a fungus that grow at their tips. In most filamentous fungi, hyphae fuse with each other, forming an interconnected network within the mycelium. This enables nuclei to take different paths to reach the same hyphal tip. The use of different hyphal paths varies the time and velocity that the nuclei arrive at the hyphal tip. Since these times are often longer than the time it takes the tip to grow, the nuclei may be delivered to the colony edge far enough apart that they end up in different hyphal tips. This variation augments nuclear

mixing already occurring thoughout the syncytium [2].

Although it is known that nuclear mixing with the aid of hyphal fusions keeps the multinucleate population well-mixed and stable [2], it is still unkown whether fungi are able to maintain this internal genetic diversity over a period of time and distance away from the center of the colony. The ability to upkeep a stable and mixed proportion throughout the entire mycelium would greatly aid to a species' evolutionary adaptability. To examine this, we innoculated colonies of *Neurospora crassa*, a filamentous fungi and common bread mold, along a series of race tubes. The *N. crassa* were genetically modified to possess dsRed and gfp-labeled proteins such that when samples were underneath a flourescence, nuclei possessing the dsRed protein emitted a red flourescence, and those that possessed the gfp protein flouresced green [2]. This allowed for easier identification of genetically-varied nuclei. The innoculations were performed at one end of each tube, which enabled us to monitor the change in nuclear mixing over time and length.

Image analysis and statiscal methods were used to evaluate the change in samples along the tube. Using an automated particle analysis through MATLAB, the number of spores with only red nuclei, spores with only green nuclei, and mixed spores were recorded. Also recorded was the frequency of nuclei per spore. With this data, p_r, or the probability that an individual nucleus was red, could be fitted appropiately by maximization of the log-likelihood that a certain sampling of spores were to occur. The fluctuations in p_r were used to evaluate the fluctuations in nuclear mixing over the entire tube.

The Moran process is a stochastic process that models processes affecting genetic diversity, such as mutation and natural selection. The dynamics of two different populations of nuclei within a fungus can be modeled as a two species Moran process [2]. At each step in this process, randomly chosen individuals are chosen for reproduction and death. The offspring of the reproduction then replaces the eliminated individual, keeping the population constant. The primary characteristic of this process is that over time only two equilibriums are possible–the entire population is either one type or the other. The p_r fluctuations we fitted could determine whether a Moran process is indeed an appropriate model for a filmentous fungi such as *N. crassa*.

2 Methods

We started with two strains of *N. crassa*: one that contained dsRed protein, the other which contained gfp protein. This way we were able to distinguish between the two strains under fluorescent light, since nuclei that contain dsRed fluoresce red and nuclei that contian gfp fluoresce green. We made approximately 20 test tubes each of the dsRed strain, the gfp strain, and a control strain that contained neither dsRed or gfp. These samples were grown on agar and kept in a Low Temperature Diurnal Illumination Incubator (Model: LI15) at 20° C.

To culture the spores, we created a 50/50 dsRed/gfp mixture. Samples were created in a LabConco Purifier Class II Biosafety Cabinet. Once the colonies had sporalated, we used an applicator to collect spore samples and placed them in an Eppendorf with 1000 μm of autoclaved water. The solution was then vortexed using a Southwest Science SBV1000 Vortex Mixer. We used the hemocytometer to check the concentration of the spores (ideally we wanted about 30 spores in 5 randomly chosen squares). The spore solution was then diluted or concentrated appropriately. A 50/50 solution was made in a new Eppendorf and vortexed. We took approximately 20 μL of the mixture using an Eppendorf Research Plus 10-1000 μL micropippetter and placed it on one end of a prepared plastic racetube. The racetubes were filled halfway with agar on which the fungus could grow. Holes had been previously drilled into the racetube approximately 2 cm apart and spanned the entire race tube (about 2 feet). The holes were positioned such that they were aligned slightly above the surface of the agar for easy collection of the spores. Once the tubes were inoculated, the ends were plugged using autoclaved cotton balls and the holes were covered with Parafilm.

We prepared a total of nine tubes. The first three tubes were stored horizontally to simulate unassisted growth. The next three tubes were stored vertically with the inoculation point on the bottom. This way gravity would not carry spores to other positions on the agar and thus also acted as a control. The last three tubes were positioned horizontally with the inoculation point at the top. The vertical tubes were lightly shaken for 20 seconds 1-2 times per day so simulate natural disturbances to the fungus.

To count the spores, we filled an Eppendorf with 1000 μL of autoclaved water. We then used an applicator to get a sample of spores out of the race tube through the holes. The spores were placed in the Eppendorf. The solution was vortexed for about 10 seconds, to thoroughly mix the spores in the water. To get rid of any extra debris, we filtered the solution using a filter tip, then vortexed the solution again. To keep spores from moving while we imaged them, agar slides were prepared. We then placed about 10 μL of the filtered solution on the agar slide.

To image the samples, we used a Zeiss Imager M2 microscope with Zeiss 2011 software. We attempted to collect images of about 200 spores per sample at 20$\times$ magnification. At every position on the slide, we took three images: one in DIC, one in dsRed, and one in gfp. We manually set the exposure to minimize the amount of over-exposure while still getting as many spores as possible. For this experiment, the exposure for dsRed was 40 ms and the exposure for gfp was 120 ms. The pictures were exported in CZI format. These had to be manually converted into TIF files. We then ran our image processing code on the images to count the number of spores in each image.

2.1 Image Processing

Our image processing code, titled "thresh.m", was written in Matlab 2011. The program is a modified version of Otsu's method for segmentation. Because spores do not take up much space on the image, most of the image is black, so the intensity histograms of the image were heavily skewed toward the lower end of the histogram. To fix this, we first ran Otsu's method on the image to give us a threshold in order to subtract out the black pixels. We then ran Otsu's method again on the resulting histogram, which now represents the higher end of the histogram, containing information about the spores. This process was run on both the dsRed image and the gfp image. We assumed that the histogram would have a bimodal distribution, where one of the modes represents the presence of a spore, while the other mode represents the presence of a particular kind of nucleus. Once the new thresholds were calculated, the image was then segmented and converted into a labeled image. The program could then count how many spores contained red nuclei and how many contained green nuclei. We then used the principle of inclusion exclusion to get the total number of spores that contained both kinds of nuclei.

Once we had the spore counts, we wanted to predict the number of red nuclei present in the sample. We assumed that the probability that a given spore was purely red followed the equation

$$p_R = \sum \mathbb{P}(N_{nucl} = n) \times p_r^n,$$

where p_R is the probability that a spore is purely red, n is the number of nuclei in a spore and p_r is the probability that a particular nucleus is red. Similarly, the probability that a spore is purely green is given by

$$p_G = \sum \mathbb{P}(N_{nucl} = n) \times (1 - p_r)^n.$$

The coefficients for these polynomials were obtained by collecting samples from three positions on a tube: one at the beginning, one in the middle, and one towards the end. We then took Z-stack images of the samples at $40\times$ magnification and hand counted the number of nuclei in the spores. We used the resulting histogram of nuclei counts as the coefficients in the polynomial. The value of the coefficients used was dependent on where in the racetube the sample was taken from.

We assumed that the distribution of spores followed a multinomial distribution. Thus, the likelihood function was represented by

$$L(N_R, N_G, N_Y) = \frac{N!}{N_R! N_G! N_Y!} p_R^{N_R} p_G^{N_G} p_Y^{N_Y},$$

where N is the total number of spores and N_x is the total number of spores of a particular color. To calculate p_r, we found the maximum of the log of the likelihood function. Thus, we solved

$$\frac{N_R}{p_R}\frac{dp_R}{dp_r} + \frac{N_G}{p_G}\frac{dp_G}{dp_r} - \frac{N_Y}{1 - p_R - p_G}\left(\frac{dp_R}{dp_r} + \frac{dp_G}{dp_r}\right) = 0$$

for p_r. A function was written in Matlab to perform this calculation. In addition, we noticed that the images were very sensitive to the threshold value. Thus, for each position, we created a vector of thresholds containing the threshold values for each image taken of the sample from that position and calculated the mean (μ) and standard deviation (σ) of these thresholds. We could then calculated p_r using three different threshold values: μ, $\mu + \sigma$, and $\mu - \sigma$.

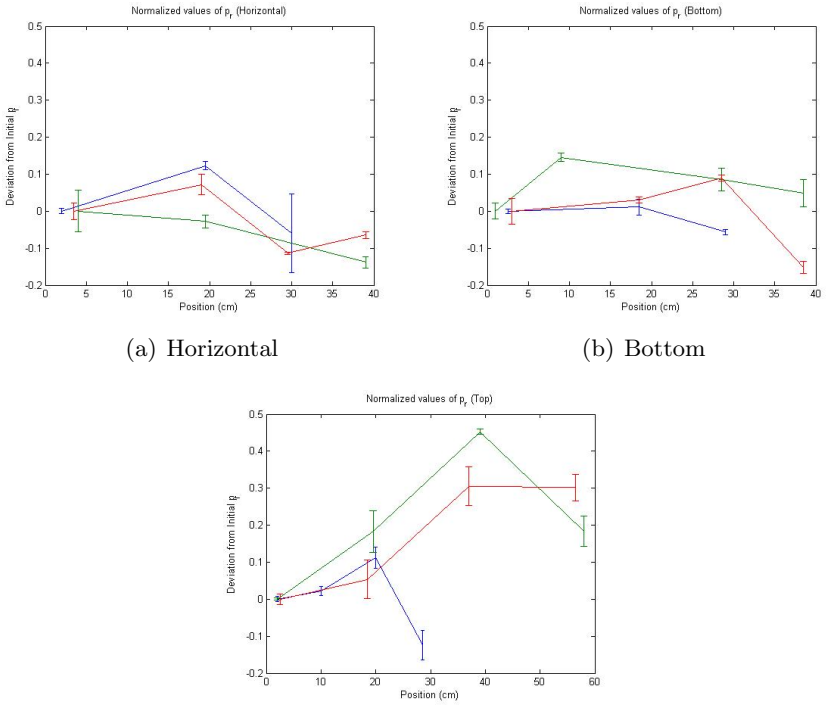
3 Results

Using the iterative Otsu scheme for image thresholding and a multinomial maximum likelihood function for parameter fitting, we found values for p_r at three to four points along each race tube. The horizontally oriented tubes served as a control group, as well as the vertically oriented tubes in which spores grew from the bottom. In contrast, the vertically oriented tubes in which spores grew from the top experienced accelerated growth due to the additional mechanism of spore dispersal, which was induced by shaking the tubes once or twice a day.

One common feature throughout all of the data was that the mixing dynamics were not indicative of a Moran-like process, in which stochastic fluctuations allow for one type of nucleus to dominate over time. Instead, the value of p_r at various points along the tubes would simply deviate around the initial values. As the spores reach farther distances along the race tubes, the frequency histogram of these deviations resembles a Gaussian distribution, suggesting that the values for p_r along the tubes are normally distrubted about the initial proportion. Thus, the proportions of red and green nuclei seem to stay relatively well-mixed throughout the tube, though the deviations become longer further along the tube due to aggregated stochastic effects.

For the two types of control tubes, the data looked very similar - there were small deviations from the initial value of p_r in both directions. However, the deviations were much larger in the vertical tubes in which the spore colonies progressed downward. One possible explanation for this is that the additional downward flux of spores induced by periodically shaking the tubes separated subpopulations of spores from the mycelial network growing from the top of the tube.

As noted in [1] and [2], recent research suggests that genetic diversity is maintained by complex mixing flows of nuclei throughout the hyphal network, driven by the action of pressure gradients between the center of the mycelium and the growing peripheral hyphae. This is due to controlled hyphal branching and interconnectedness. This nuclear mixing process is now thought to be largely responsible for maintaining genetic diversity, keeping stable and well-mixed proportions of nuclei despite the natural tendency to segregrate during growth. By seperating some of the spores from the interconnected mycelial network via induced dispersal, new mycelial sectors are created, which may cause less well-mixed proportions to develop away from the primary sector growing from the top of the tube.



(a) Horizontal

(b) Bottom

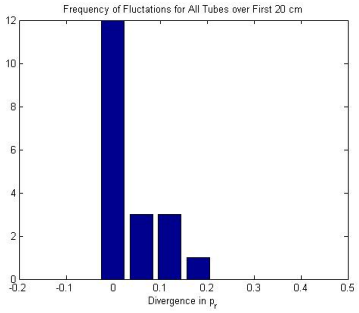
(c) Top

Figure 1: Normalized deviations of p_r from the initial proportions throughout the race tubes. The top tubes have larger deviations horizontal and bottom tubes due to the effects of induced spore dispersal.

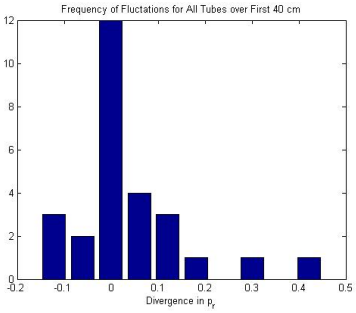
4 Prospectives

We seem to have verified the recent hypothesis that fungi are able to maintain genetic diversity through mixing flows within the hyphal network. The values of p_r along the race tubes did not reflect dynamics dictated by a Moran process, and instead seemed to deviate from the initial proportions according to a normal distribtuion. However, more work should be conducted on the effects of spore dispersal. Our preliminary results suggest that dispersal disrupts the interconnected mycelial network and causes p_r to deviate further away from the initial proportions. A more in-depth analysis is needed, perhaps elucidating the underlying fluid mechanics involved by using a controlled airflow to induce the dispersal. It may be interesting to study how well the fungi can maintain genetic diversity in other stressed conditions, such as by using more hostile media for growth or altering physical parameters.

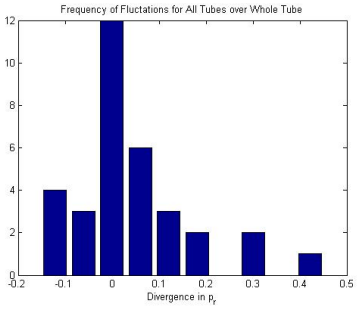
One feature of our model which could be investigated further is the fitting procedure for p_r. We took a simple approach by assuming that trials were independent, but it may be that red nuclei are more likely to be found near other red nuclei. Even if the overall populations remain well-mixed, different sectors of the myeclium may



(a) 0 to 20 cm



(b) 0 to 40 cm



(c) 0 to 60 cm

Figure 2: Frequency histograms of the normalized deviations of p_r from 0 to 20 cm, 0 to 40 cm, and 0 to 60 cm. Note that further along the tubes, the cumulative frequency histogram begins to resemble a normal distribution.

be more homogeneous, especially toward the tips. Hence, we may wish to investigate the effects of correlation by using a multinomial distribution with dependency parameters. Another way to approach this is to see what role fitness plays in dictating local and overall mixing.

In addition, there are some changes that can be made to the image segmentation code in order to improve accuracy. There is currently a trade-off between being able to properly segment clumps of spores and making sure to include each spore in the initial segmentation. If the initial threshold is too low, the spores clump together, causing groups of individual spores to be treated as one large mass. However, if the threshold is too high, then some spores will not pass the treshold, allowing for stray nuclei that will not be counted by our algorithm, as they do not appear to reside in any spores. One way to go about this is to use a low threshold (i.e. scaling the first threshold computed by Otsu's method by a factor of 0.5 or 0.25), and then segmenting clumps of spores by using a watershed algorithm. Another issue arising in the segmentation process was inconsistency due to uneven illumination and saturation. We chose an ad hoc value for the saturation parameter which seemed to work well with most of our images, but subtle changes in this parameter have a

large impact on the intensity histograms, causing the images to be tresholded differently. A more consistent approach that would work for arbitrary sets of images would likely require more sophisticated filtering and other pre-processing techniques.

Despite some of the problems with thresholding the images, this work presents a step in the right direction, as it is accurate enough to give us insight about the nuclear mixing process and the effect of spore dispersal. There are several directions for this research in both the computational and experimental realms, which will provide exciting discoveries in future work.

5 Acknowledgements

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6 References

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