Cell Expression in Congenital Heart Disease

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Introduction

What to study?

- The difference of cell expression in the cells from healthy babies (controls) and the cells from diseased babies (CHDs).

- Cell expression analysis with quantification of cell shape (aspect ratio, orientation, size), notch distribution in each cell, and the fraction of abnormal adherens junctions in each cell image.

Experiments

- What cells do we analyze? Endothelial cells from umbilical cords of newborns.
- Experimental Design: (Experiments are conducted by the Arispe Lab from the MCDB department)

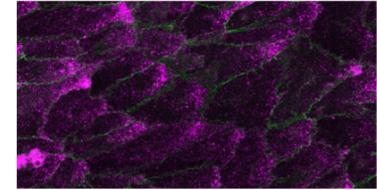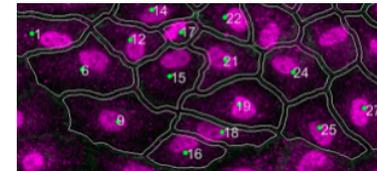| Control group	Experimental group
Cells from healthy babies	Cells from babies with congenital heart disease (CHD)
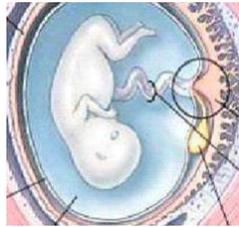
Flow

Seed Cells

Live Imaging

Imaging Processing

Data Extraction

Data Clustering

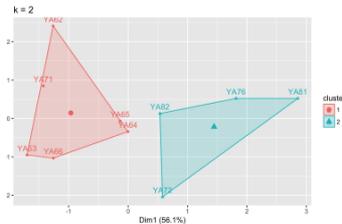| AspectRatio	theta	Area	TotalNotch
1.0578041	1.2791249	9859	20293859
1.2374456	0.9233659	2633	7820372
2.436259	0.090187	7359	22314890
1.4531769	0.9577022	5447	13658022
1.9570792	0.063368	9170	19028681
1.2125212	0.8784018	8110	16271354

Image Processing

1. Cell Segmentation

```
Input Image
     ↓
Image
Enhancement
     ↓
Canny Edge
Detector
     ↓
Edge
Enhancement  ──┐
```

1. Morphological Operation
2. Gradient Method
3. Holes Closure
4. Edge Thinning
5. Skeleton Clean Up

2. Nuclei Segmentation

```
Input Image
     ↓
Image
Enhancement
     ↓
Extended-minima
transform
     ↓
Watershed
Method
```
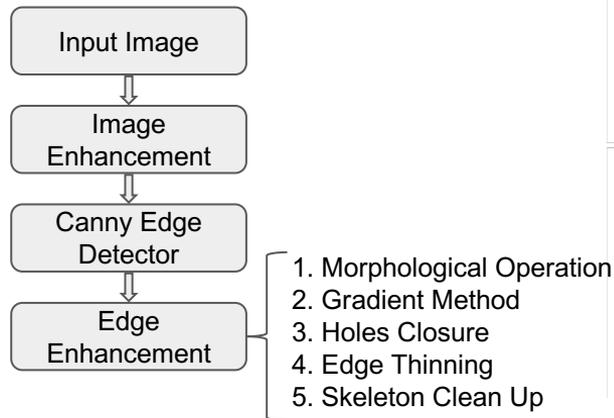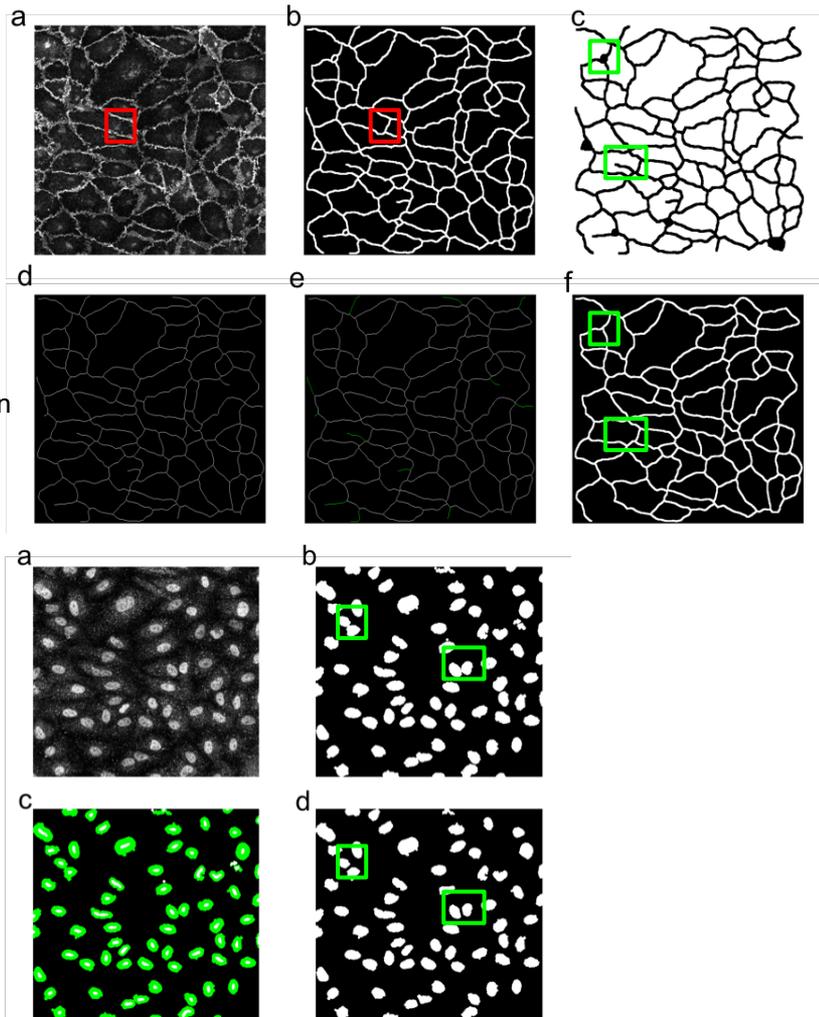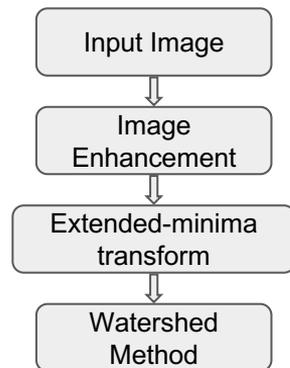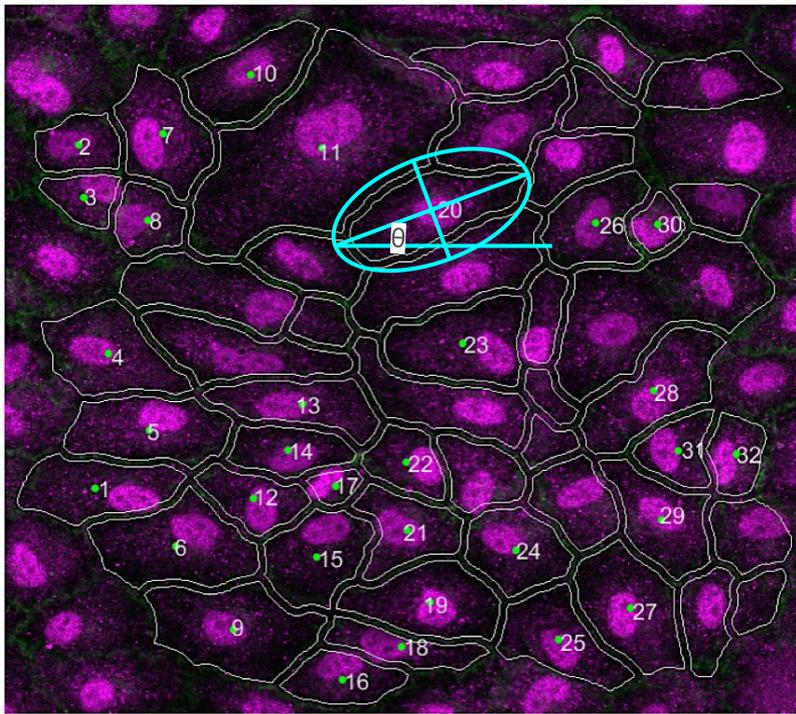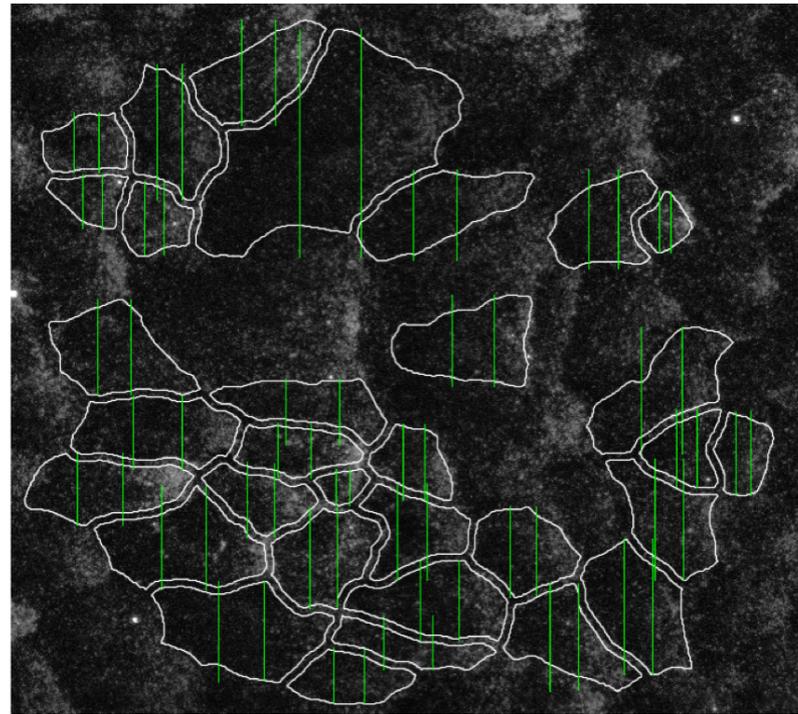
Cell expression analysis with quantification of cell shape (size, aspect ratio, theta) and notch distribution.
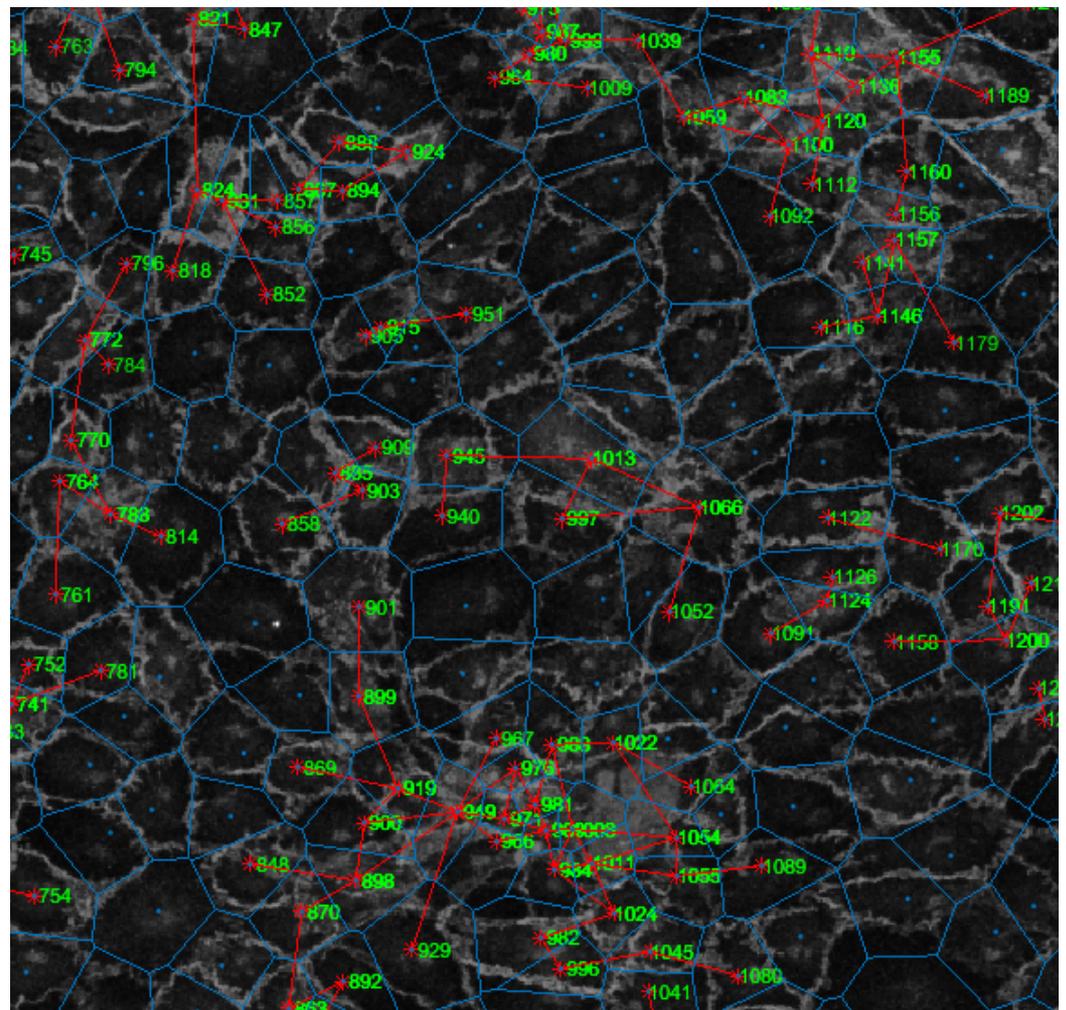
Cells Shape Analysis Demo

Notch Distribution Analysis Demo

Cell expression analysis with quantification of abnormal adherens junctions.
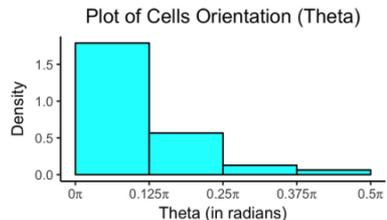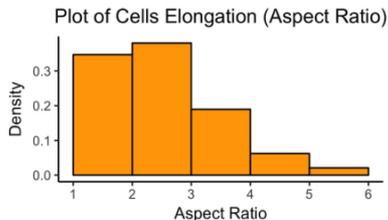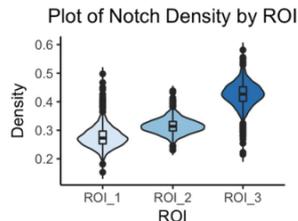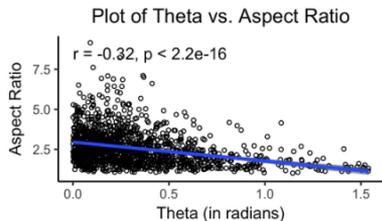
Threshold based on:
(1) the integrated intensity along the line connecting each pair of cell;
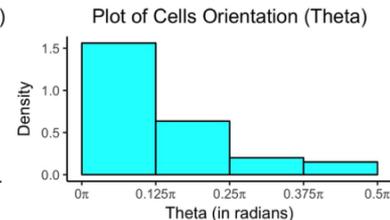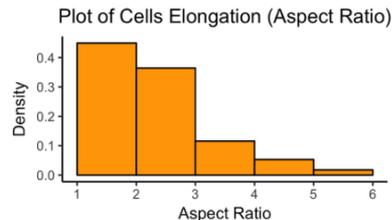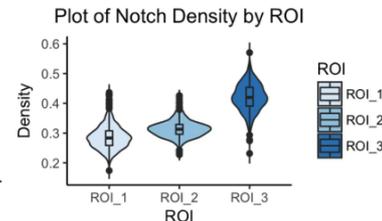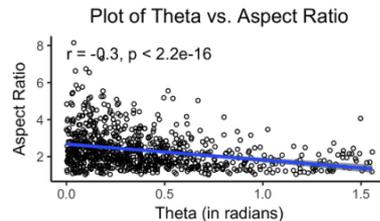(2) the sum of widths of intensity peaks at half maximum along each line.

Data Visualization
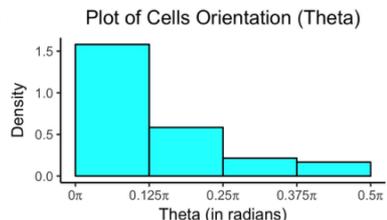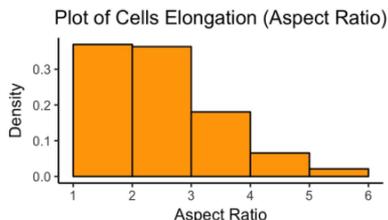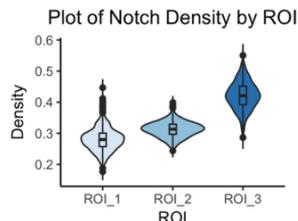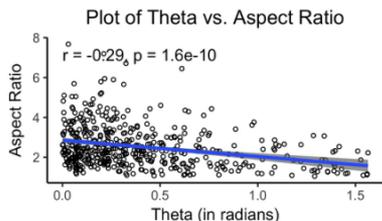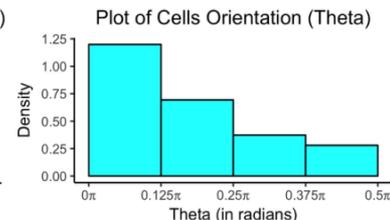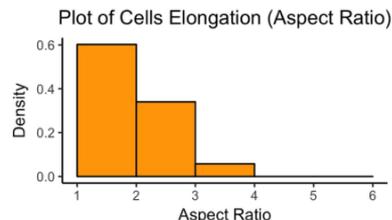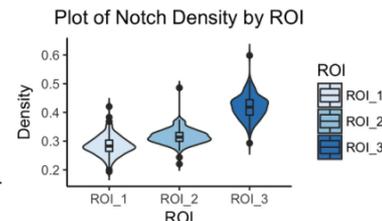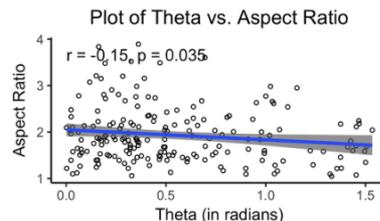
Fusion Between Genetically Different *Neurospora Crassa* Spores (Project 2)

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Abstract

The ability to obtain resources is vital for an organism to survive and reproduce. Especially in hostile environments, the organism that has better skills to take advantage of available resources is considered more fit, and is thus more likely to survive. This project investigates the fitness of a filamentous fungus *Neurospora Crassa*. *Neurospora* spores that are genetically different will not fuse in natural environments. We are interested in whether they will overcome this genetic difference when there is a need for the spores to obtain resources from each other. We tested the hypothesis that genetically different Neurospora Crassa spores fuse and cooperate under stressful environments to become more fit.

1. Introduction

Neurospora Crassa is a type of fungal species that can fuse with each other during growth. It is filamentous and multinucleated. Fusion between spores is a form of cooperation. Through fusion, spores can share their resources with each other, which may or may not improve the chance of their survival. The downside to fusion is that one party may get exploited by the other.

The motivation for spores to fuse may be to become more fit. An important concept in evolution is selection. Selection is a process in which different types of individuals compete for resources (1). During this process, the more advantageous and strong individuals tend to survive and reproduce. Over time, the genes for advantageous traits will be passed to new generations. This long-term process results in the presence of more advantageous traits in the species.

Genetic difference that prevents two strains of N. Crassa spores from fusion can be viewed as a barrier to avoid exploitation of resources by one from the other. The genetic difference in the strains that we are using in the experiment is in the seg-9 locus. The two strains each lack the ability to make an important amino acid/vitamin that is vital to their survival. The first strain, T 2.7.3, lacks the ability to make pantothenate. The second strain, Karine Dementhon 14, lacks the ability to make histidine and pyridine. When we put them together in liquid minimal media, the only way for them to obtain their missing vitamin/amino acids is through fusing with the other strain, even though their seg-9 gene is different and should prevent them from fusing.

The purpose of this experiment is to explore whether T 2.7.3 and the Karine Dementhon 14 strains will overcome their genetic incompatibility and fuse to obtain the vitamin they lack from the other strain. To effectively do so, we are using PDMS as our

microfluidic device. PDMS is a biocompatible silicone (2). It is clear and thus makes biological contents on it visible and imaginable (2).

2. Experimental Methods

The experiment is completed in three main steps: preparing spore suspension, loading PDMS chip, and imaging using microscope. The experiment set up consists of a PDMS chip loaded with spores. The PDMS chip is made and plasma cleaned in Dino Di Carlo's lab. Before each experiment, the chip must be plasma cleaned and immediately put in 1.5% liquid minimal media. This allows the liquid to adhere to the PDMS wells so that spores can grow in them. The PDMS chip consists of approximately 10,000 wells, which are circular pockets for spores to settle in. The loaded PDMS chip will be sealed with a cover glass, which will be separated from the chip surface by a layer of dielectric oil placed around the imaging region. The PDMS chip will be placed in a large petri dish, and wet paper towels will also be placed in the dish to surround the chip. The large petri dish will be closed during the experiment. This setup minimizes condensation on the cover glass and ensures the clarity of the image will not be affected by condensation.

In the first step, spores are mixed into sterile water to form a low concentration spore suspension. The concentration is not measured to an exact number, but rather an approximate one to ensure that every well on the PDMS chip will contain anywhere between zero to fifteen spores. After mixing the spores, the suspension is vortexed to make sure the spores are evenly distributed in the solution, and then filtered to ensure unwanted particles are excluded. If an experiment involving two strains of spore is being conducted, then the process will be repeated for the other strain, and the two solutions will be mixed together to form a suspension with spores of both strains.

In the second step, the PDMS chip is taken out from the liquid minimal media, and dried using a Kim wipe. Around 150-200 µl of liquid minimal media will be pipetted onto the chip surface. Then, a 3*3 array of 1 µl spore suspension will be pipetted onto the well region. The chip will be left for 20 minutes for the spores to settle into the wells. The liquid media left on the surface of the chip will then be scraped off using a cover glass. This ensures that there is no media between wells. The chip will then be sealed by putting four strips of dielectric oil around the well region and then stacking a cover glass on top. The separation of the cover glass and the chip surface allows the spores to obtain oxygen. The chip is placed on a microscope slide and put into a large petri dish. The large petri dish will contain wet paper towels around the chip to minimize condensation. After closing the large petri dish with a lid, the sample is ready for imaging.

In the third step, the sample is placed under the microscope. A portion of the well region is selected as the region of interest. The region of interest will be divided into 256 tiles. The microscope will move in the XY-plane to capture all tiles, and stitch the tiles together afterwards. The microscope will also move in the Z direction (up and down) to capture different depths. The z-stack consists of 37 z-positions. The range will allow us to capture the very top of the wells to the very bottom of the wells. Having a thick z-stack

ensures that the best focused image is included. The microscope is programmed to take a full image of the region of interest every 6 hours for 24 hours in total.

3. Image Analysis

To detect growth and fusion of the spores, we first conduct several experiments with genetically compatible strains to know what fusion looks like in our experiment. When the images are exported, each tile and each z-position is exported into a separate file. We first find the optimal z-position for each tile at which the hyphal structure is the clearest, and then we stitch the best focused image of all tiles together into a large image. This image is what we use to detect growth.

3.1. Creating Hyphae/Well Mask by Thresholding Intensities

When the spores grow out and fuse, they appear in hyphal structures that can span more than one well (Figure.1). To quantify growth, our approach is to quantify the hyphal networks that appear on the image.
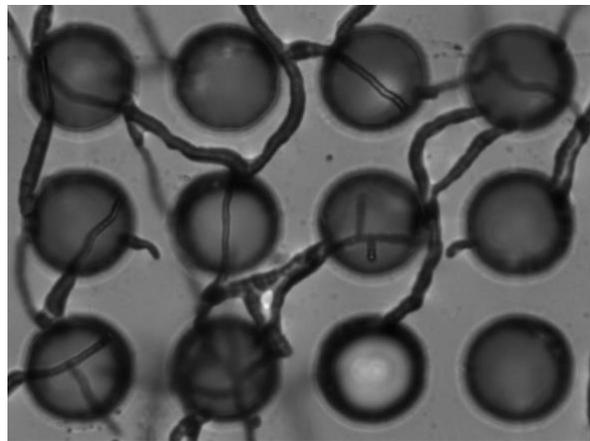

Figure.1 - hyphal structure of compatible strains of *N. Crassa* spores

After stitching the tiles, our first step is to create a mask of the wells and hyphal structures. In the best focused z-position, the well and the hyphal structures have a lower intensity (Figure.2). The black strips in the stitched image are due to microscope light box failure.
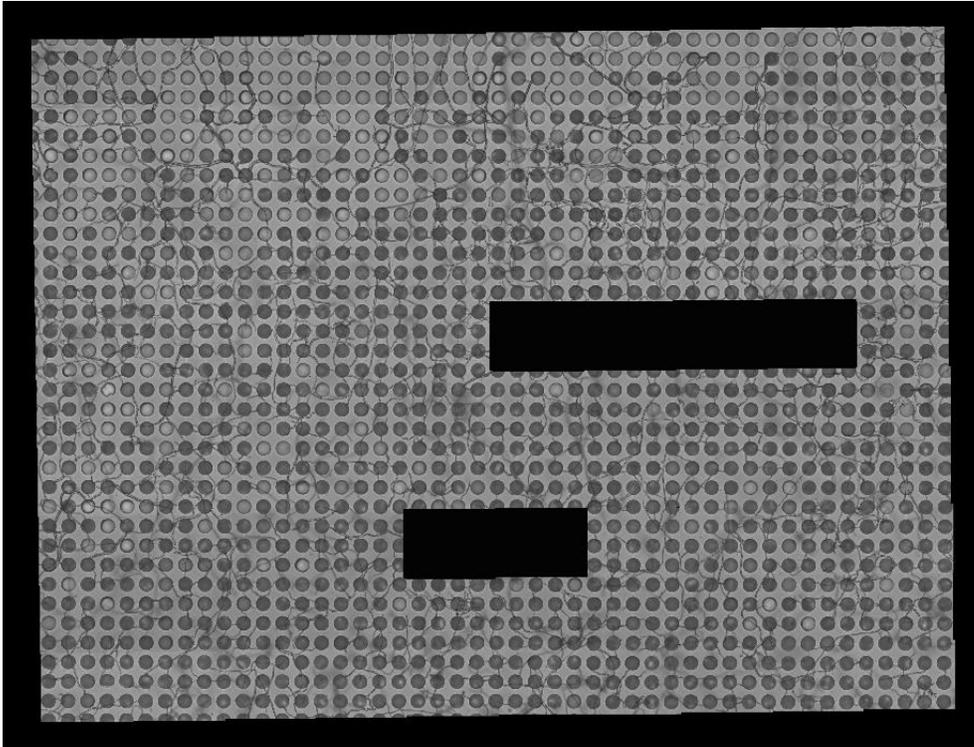
Figure.2 - stitched image from experiment

To create the mask, we take a histogram of intensities in the stitched image. The hyphae and the wells appear much darker than the surrounding well surface on the image, and so we find the threshold in between the intensity of the hyphae/wells and the intensity of the surroundings. To create the mask, we keep only the intensities that are lower than the threshold, and this will give us a binary image that shows the hyphae and the wells.

To find the threshold, we find the two maximum peaks in the histogram. One peak will correspond to the bin in which the intensities of the wells and hyphae pixels will fall into, and the other peak will correspond to that of the surrounding well surface. We take an average of the two peaks, and create a mask that contains only the pixels that have intensities lower than the threshold.

The next step is to clean up the mask obtained from thresholding intensities. The first step is to fill the holes inside the wells. Sometimes thresholding can give us holes inside wells because the center of the wells tends to appear lighter than the outer circle. We do so by constructing a well mask that only shows the circular wells by using a distance transform, and create the mask by creating a binary image showing all pixels that are within 110 pixels from the nearest well center. By using the and operator on this well mask and the previous hyphae/well mask, we obtain a new well mask that excludes the hyphae structures and only leaves the wells, but sometimes with holes inside them. We then fill these holes on this new well mask. We exclude the hyphae before doing so

because filling the holes will also cause small gaps and hollow areas in hyphae networks to be filled if we don't exclude them.

After thresholding, the hyphal structures can sometimes appear unclear, so we do a closing on the mask after filling the holes. This will thicken the hyphal structures and make them appear more solid. This step will help us later in the process to detect them.

The last step to post-processing the mask is removing any dirt and unwanted particles on the well surface. These particles usually appear darker and are between the wells and on the well surface. Because of their low intensity, they get segmented along with the hyphae and the wells when we threshold the image. To get rid of them, we simply do a reconstruction using the well mask created from distance transform as the marker, and the hyphae/well mask as the mask. This will only keep the connected components that are connected to the wells. After this step, we obtain a desired hyphae/well mask (Figure.3).
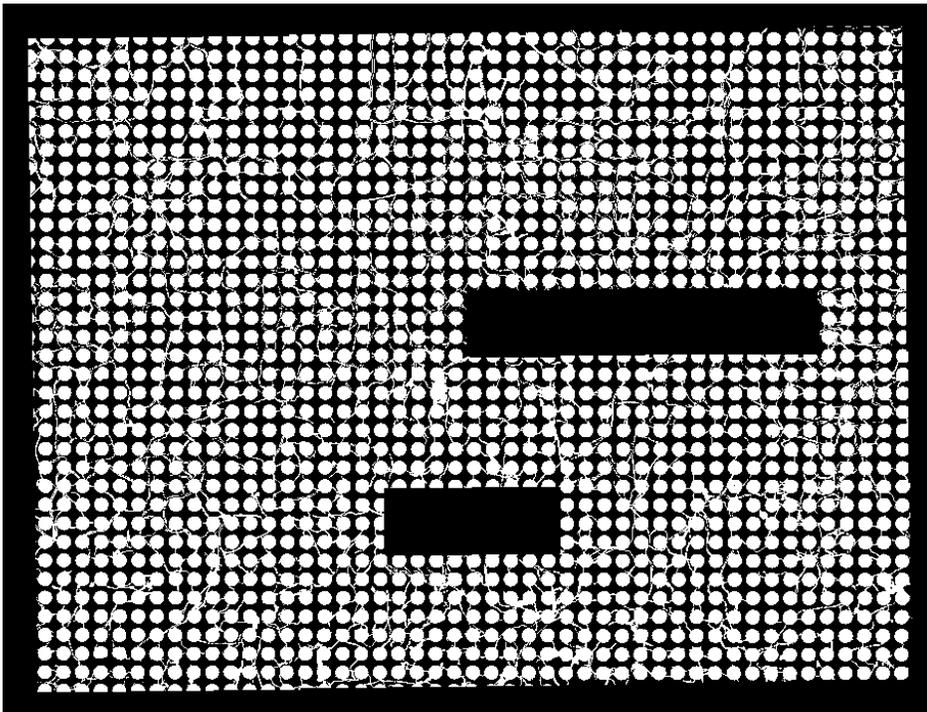
Figure.3 - hyphae/well mask obtained after thresholding, hole filling, closing, and reconstruction

3.2. Labeling Connected Components

To quantify growth, our approach is to count the number of connected components in the hyphae/well mask that either includes more than one well or includes only one well but has an area larger than a certain area threshold. The first case indicates the hyphae has grown out of the well and has fused with another hypha that grew out from the other well. This is a hyphae network. In images from compatible strain experiments, a hyphae network can span up to eighty wells. The results for incompatible strain experiments

will be discussed later. The second case indicates there is hyphae growing out of the well, but it does not fuse with hyphae from other wells. This is also counted as growth.

To implement this growth quantification, we used bwlabel function in MATLAB to label our hyphae/well mask. This function gives each connected component a color, which will be represented by an integer. For example, if the connected components have three pixels, all three pixels will have a certain number after labeling. The pixels with the same label/color are in one connected component.

Before we start labeling, we create a mask containing only wells with a slightly larger radius, and we call this the wells-filled mask. We use the mask obtained right after filling the holes during the process of creating the hyphae/well mask. This mask contains circular wells with no hyphae, but the outer edge of each well is not a perfect circle. The edge will be the same as the actual edge on the wells from the image, which can sometimes be slightly deformed and not perfect. We then dilate this mask by 20 pixels, which will give us an enlarged radius. We will refer to this mask as wells-filled.

To allow steps later to be easier, we create three labeled images: labeled image, labeled wells-filled, labeled hyphae/well mask. Labeled image contains both pixels from wells-filled and the hyphae/well mask. We do so because wells-filled contains the wells that have centers outside of our imaging region, but intersects with our imaging region for some range. For example, we may see a little less than half of a circle on the edge of the image. When we find the well centers in the very beginning, we will also find centers of these wells, so when we do the distance transform and create this well mask, we also take into consideration the wells with centers outside of the image. To create the labeled image, we first use the or operator on the wells-filled mask and the hyphae/well mask, and we use the imlabel function on it (Figure.4).
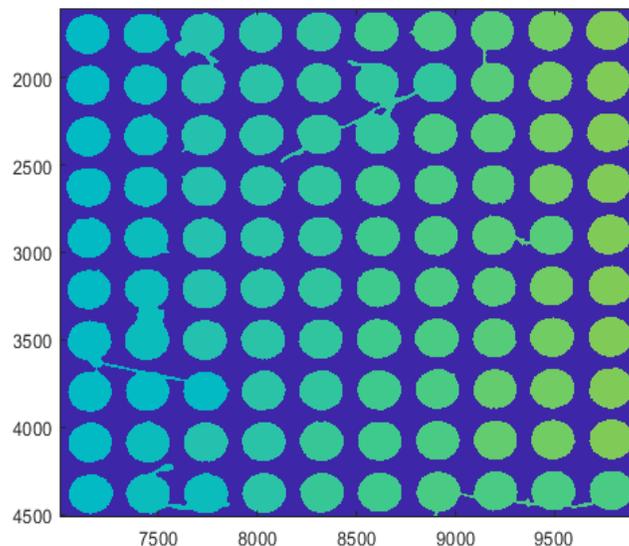

Figure.4 - labeled image (each connected component will have a distinct color)

14

After obtaining labeled image, we also label the wells-filled mask the hyphae/well mask separately. The difference between the two is that labeled wells-filled will only contain wells, including the ones on the boundary; labeled hyphae/well mask will contain both wells and hyphae, but only partial wells on the boundary.

3.3. Detecting and Quantifying Growth

To quantify growth, we first keep an array of well labels. The index of this array are well numbers, and each entry will be the label of the corresponding well. For example, if well 1 and well 2 are in the same connected component, then the first and second entry of this array will be the same number. We will call this array well-labels.

The next thing we want to do is to keep track of how many wells there are in each connected component. We do this by using the unique function on well-labels. This will give us a cell array that has length of the number of connected components. We then loop through each entry of this cell array and record the number of wells there are by using the find function in well-labels. The find function will return the indices of the well-labels array in which the entry is same as the value we want to find. For example, if well 1 and well 2 are both in connected component 1. Then the first entry of this connected component array will consist of 1 and 2, which represents the well numbers.

This array gives us information about all the connected components with more than one well. For the wells that have hyphae growing out but do not connect to other wells, we use an area-based approach to determine growth. We create another cell array that records growth areas. The length of this array will also be the number of connected components. To calculate the area of the hyphae connected to each well, we first update the labeled well mask to a new version that contains only hyphae and no wells. Although the wells are no longer present, we are still able to know which hyphae belongs to which connected components because the mask is labeled already. We then loop through every item in the growth area array, and calculate how many pixels there are in the updated labeled hyphae mask, and this number will represent the area.

Apart from numerical data, our algorithm also outputs a labeled image that gives us a visual sense of the rate of fusion (Figure.5).
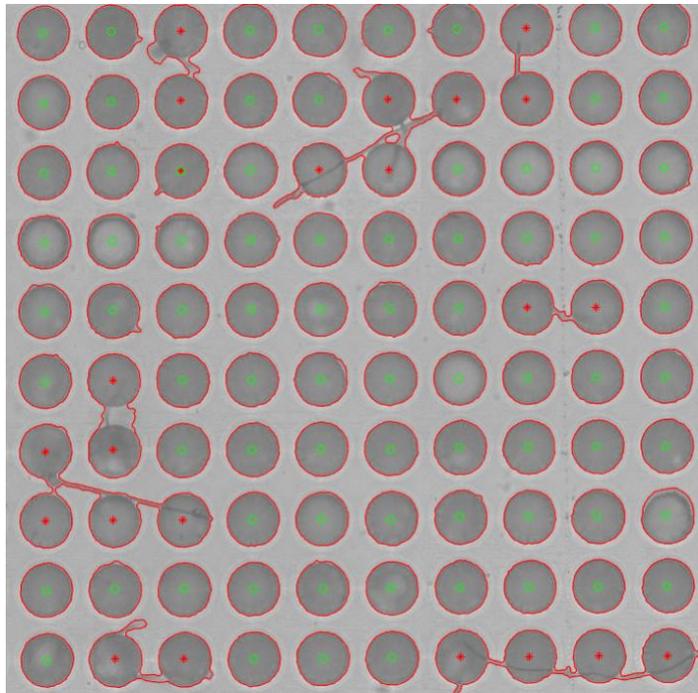
Figure.5 - labeled experimental image (red contours represent hyphae/well mask; red stars are wells that are in a connected component with other wells; green stars are wells that are disconnected to other wells)

4. Results

Analyzing experimental images with our algorithm shows that genetically different spores do fuse when they need an essential vitamin that they cannot produce themselves. In fact, they fuse at a much higher rate than we imagined. The results are significantly different from the control experiments, in which we put the two strains of spores separately in the same liquid media and there are absolutely no hyphae that grew outside of the wells.

From the contoured image that our algorithm outputs, we can see that there are 39 connected components that include more than one well. We call these hyphae networks (marked in red stars in Figure.6).
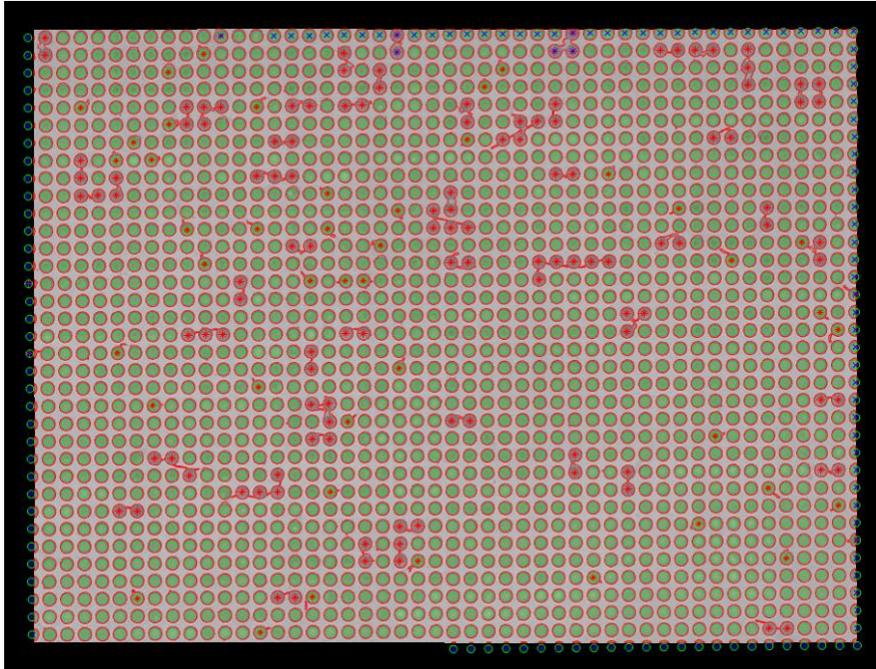
Figure.6 - contoured image of experiment with incompatible strains of *N. Crassa* at time point 5 (24 hours into experiment)

Compared to an experimental image with a pair of compatible strains of *N. Crassa* spores (Figure.7), the density of hyphae networks is much lower. This indicates that the fusion rate of incompatible strains with lacking abilities to make vitamins is much lower than that of compatible strains, but it still occurs.
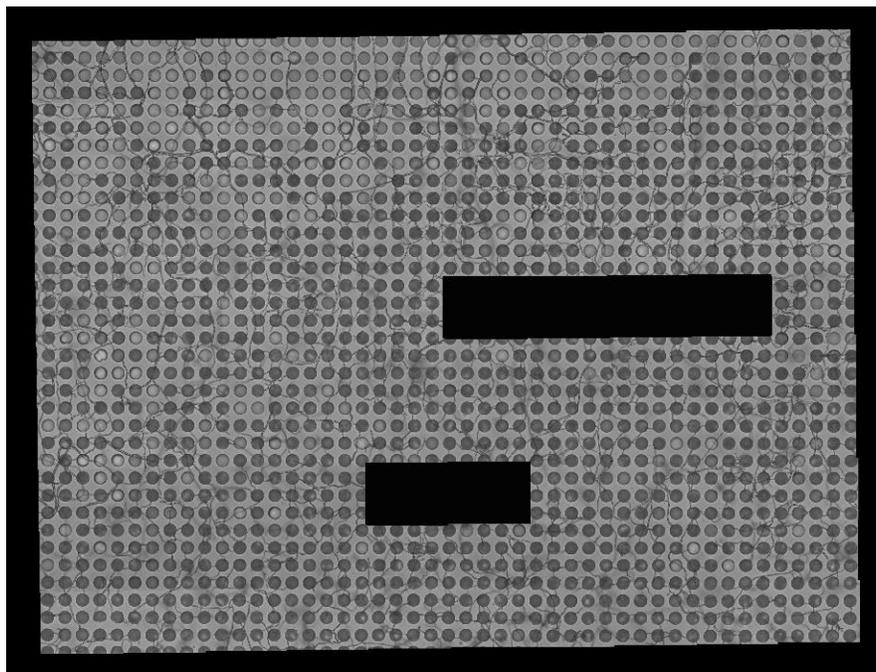

Figure.7 - experimental image with compatible strains of *N. Crassa* spores (black strips are due to microscope failure in some tiles)

Our hypothesis is not refuted so far, yet more accurate results and quantification need more repetitions of experiments to achieve. Experiments in the future will help quantify the growth rate of incompatible *N. Crassa* strains in a more accurate way. Furthermore, we are also interested seeing how different environmental pressures will affect the growth and fusion of *N. Crassa* spores.

References

1. Nowak, M. A. Evolutionary dynamics: exploring the equations of life. (Belknap Press of Harvard University Press, 2006).

2. Marshall, J. et al. Microfluidic device enabled quantitative time-lapse microscopic-photography for phenotyping vegetative and reproductive phases in Fusarium virguliforme, which is pathogenic to soybean. *Scientific Reports* 7, (2017).