HEMOCYTOMETRY COLLABORATION

2017

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1) Introduction

Greetings from the IISc iGEM Team of 2017! It's great to have you on board for this collaboration. This year, our team is working with two species of yeast: *Saccharomyces cerevisiae* and *Pichia pastoris*. Both of these are important players in eukaryotic recombinant protein synthesis. But taking Optical Density (OD) measurements to monitor their growth become unreliable- due to their large size their OD increases to the point where it does not linearly correlate to biomass.

This means **hemocytometry** is used in most yeast labs to monitor the growth-rate and biomass of yeast cultures. But this process involves tedious labour in counting cells for each measurement taken (in triplicates), and thus is much more time consuming than using a spectrophotometer. Some workarounds to this include:
   a) Automatic Cell counters, which are standalone machines and also expensive.
   b) Cell counting softwares, which are paid software and/or not robust enough to measure cell count accurately.

2) Objective of the Collaboration

This calls for a better, precise and time effective way to measure the cell count of yeast cultures. With this collaboration, we aim to collect enough data to train a **machine learning algorithm** we have designed for counting these cells! An introduction to machine learning can be found here: https://www.sas.com/en_us/insights/analytics/machine-learning.html

Within this collaboration, each team is expected to generate images for the algorithm to analyze by monitoring one growth curve each for *Saccharomyces cerevisiae* and *Pichia pastoris*. In case one of these organisms is not available, the team can perform **two** growth curves with the organism available. We have explained all important details in this document, but for any further queries or corrections, please refer to contact details!

3) Contact Details

For queries on **culturing yeasts** and **imaging**, contact:
> Sai
> 9148045314
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For queries on **hemocytometry** and **staining**, contact:
> Kunal
> 9980584211

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4) Important Notes

GENERAL

a) Read through this document carefully before starting the experiments.
b) Doing one growth curve of each organism is recommended. If one of the organisms is not available, please perform two independent growth curves of the same organism.
c) Each growth curve takes **16 hours** to complete.
d) Sample clarity is better with an inverted objective microscope, but using an upright microscope is also fine.
e) Adjust exposure settings on your phone/microscope camera such that good contrast is obtained between cells and the background. If your microscope camera does not give good contrast, use a cell phone to take pictures!
f) Do not vortex cells harshly or centrifuge cells after staining to avoid disintegration of dead cells (this will ensure reliable estimation of the health of yeast culture).

Saccharomyces cerevisiae

a) GENERATION TIME: ~80 Minutes
b) SAMPLING INTERVAL: 1 hour
c) Recommended media: **YPD** Medium / **YPAD** Medium (if yeast strain used is Ade-)

RECIPE (1L):

Peptone	20 g
Yeast Extract	10 g
(Adenine Sulphate	40 mg)*
Water	950 mL
Autoclave	
40%(w/v) Glucose solution (filter sterilized)	50 mL
* Add for YPAD	

NOTE: Add filter sterilized glucose solution **after** autoclaving medium to avoid Maillard reactions.

Pichia pastoris

d) GENERATION TIME: ~80 Minutes
e) SAMPLING INTERVAL: 1 hour

f) Recommended media: **YPD** Medium (see above for recipe)

5) Materials Required

a) **5 mL** (for primary inoculum) + **250 mL** (for secondary inoculum) <u>YPD/YPAD</u> medium
b) PBS (1x)
c) 1L conical flask
d) *Pichia pastoris* colony
e) *Saccharomyces cerevisiae* colony
f) **4% (w/v) Safranin soln** (if unavailable, use any of the other two dyes instead)
g) **4% (w/v) Methylene Blue soln** (if unavailable, use any of the other two dyes instead)
h) **1% (w/v) Trypan Blue soln** or **1% (w/v) Erythrosine B soln** (if unavailable, use any of the other two dyes instead)
i) Centrifuge
j) Hemocytometer
k) Microscope

6) Method

DAY 1: Primary Inoculum

a) In a sterile hood, inoculate a colony of *S.cerevisiae* in **5 mL** of **YPD/YPAD**.
   <u>NOTE</u>: Use an autoclaved 50 mL falcon tube and a 200 uL pipette tip to inoculate the yeast for optimal agitation and aeration of inoculum.
b) Similarly, inoculate a colony of *P.pastoris* in **5 mL** of **YPD**.
c) Incubate both at **30°C, overnight**, at **180 RPM**.

DAY 2: The Growth Curves

a) MAKING THE SECONDARY INOCULUM
   i) Add 2.5 mL of primary inoculum to 247.5 mL of YPD/YPAD in a 1L flask in sterile environment.
   ii) Swirl flask gently.
   iii) Take **'0'th timepoint** reading for both organisms with hemocytometer after swirling.
   iv) Seal mouth of flask with a cotton plug, incubate at **30°C** and **180 RPM**.
   v) Take measurements **every one hour** for the growth curve for **16 hours**.

b) TAKING MEASUREMENTS

i) Resuspending Culture

1) For each time point, pipette **1 mL** of culture into an eppendorf in a **sterile environment** .
2) Centrifuge aliquot at **1500 RPM (or 2000g)** for **2 mins** to pellet cells.
3) In sterile environment, remove the supernatant and resuspend cells in 1 mL PBS

ii) Staining

1) Make triplicates of 200 uL PBS + 200 uL culture
2) Add 10 uL of safranin to one, 10 uL of methylene blue to one and 10 uL of trypan blue to the other.
3) Schematic:

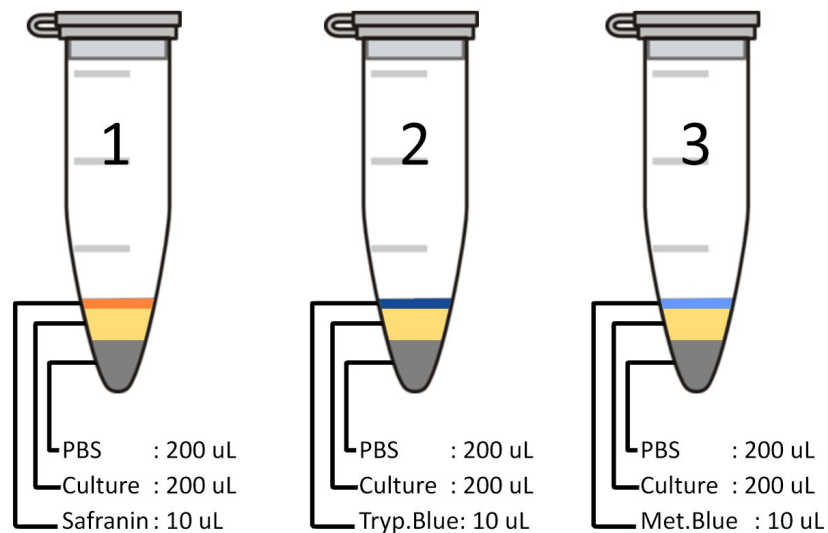### FOR EACH TIMEPOINT, MAKE THREE DILUTIONS FROM RESUSPENDED CULTURE AS SUCH:

Fig. 1

PBS   : 200 uL
Culture : 200 uL
Safranin : 10 uL

PBS   : 200 uL
Culture : 200 uL
Tryp.Blue: 10 uL

PBS   : 200 uL
Culture : 200 uL
Met.Blue : 10 uL

AFTER WHICH, YOU MUST VORTEX GENTLY TO STAIN THE CELLS

4) Vortex gently and let solution sit for 3 - 5 minutes (**max**) at room temperature
5) Load and visualize all of the above samples.

iii) Hemocytometer: Loading

1) Clean hemocytometer surface and coverslip with 70% ethanol.

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2) **Place coverslip on hemocytometer.**
3) Then, take 10 uL of sample and load it onto the hemocytometer carefully as such:
   (a) Invert the test tube several times to resuspend the cells
   (b) Pipette 10 uL of sample at once with the smallest micropipette tip available
   (c) Add the 10 uL of sample to the V-shaped groove in the hemocytometer.
   (d) If your hemocytometer does not have a V-shaped groove, place the micropipette tip on the edge of the coverslip and release the sample between the coverslip and hemocytometer gently, letting capillary action do most of the work.
   (e) Reference video for handling hemocytometer: **http://tinyurl.com/hemocytometer**

iv) Hemocytometer: Visualizing
1) Let the sample rest on the hemocytometer for **2 mins** before viewing under microscope. If cells are on a different focal plane from the hemocytometer, **let the cells rest longer** or **load lesser volume** of sample.
2) View under 10x magnification, center central grid of hemocytometer under objective. **Capture image of central grid.**
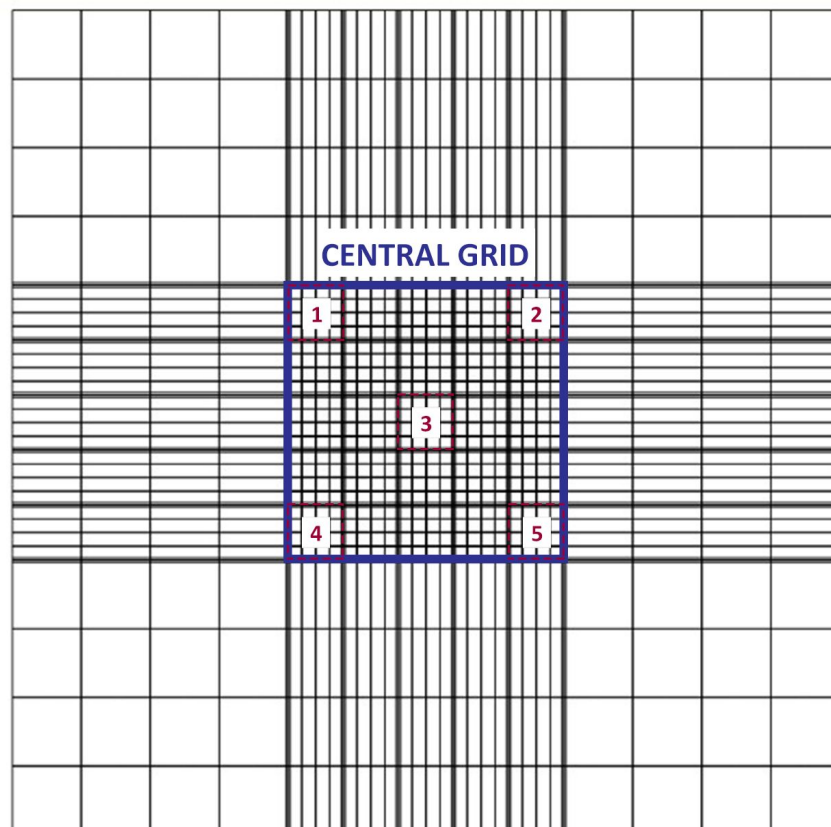3) View under 40x magnification, **take images of grid locations 1,2,3,4 and 5.**

CENTRAL GRID

Fig. 2

c) COUNTING CELLS
  i)   Count cells **only from images taken**, and not directly from the hemocytometer.
  ii) Instead of conventional hemocytometry counting, count all cells **fully** or **more than halfway** residing inside required area.
  iii) Enter counting data into the <u>spreadsheet</u> which you can download from:
       http://tinyurl.com/hemodatalog

7) Sending Data

a) There are two main categories of data to be sent: **Image Files** and **Counted Data**.

b) IMAGE FILES:
  i)   Store the images in an organized manner in folders to avoid confusion.
  ii) Rename image files as "TeamAbbreviation_OrganismNumber_TimePoint_DyeNumber_GridLocationNumber"
  iii) Do not have any spaces in the file name! (This makes it easier for us to parse the data)
  iv) To know more about the above point, check the **excel sheet to be downloaded** for recording data from the "<u>Counting Cells</u>" section.
  v)   Rename the images as soon as possible from when they were taken. Best done in between consecutive readings.

c) COUNTED DATA:
  i)   Count cells from the images as specified in the "<u>Counting Cells</u>" section.
  ii) Log data in excel sheet as per instructions in the "<u>Counting Cells</u>" section.

d) SENDING BOTH:
  i)   These data are to be stored in a folder named "TeamName_HemocytometryCollab", compressed and sent as **ZIP** or **RAR** files to **igem.2017.iisc@gmail.com**
  ii) Alternatively, the "TeamName_HemocytometryCollab" folder can be uploaded onto your team's **Google Drive** or other **file sharing platforms** and shared with **igem.2017.iisc@gmail.com**

8) Future Directions

Thank you for patiently going through the protocol, and once again for participating in this collaboration! Though it is intense, it can be immensely useful to yeast researchers and breweries if successful. We are also currently exploring whether **MTT Assays** are a viable alternative to hemocytometry for measuring the cultured biomass of yeasts. This can also be an extension of this collaboration, and if we can get it to work, will be much less time consuming than hemocytometry. For any further queries, ideas or corrections, please do not hesitate to contact us!

With regards,
The IISc Bangalore iGEM Team