

Pombe microscopy sample preparation

by Lucia Martin Caballero as indicated by Ramon Ramos Barrales as of 22/08/16

Day 0: Set 3 ml precultures of the strains to be analyzed on YES and put them to grow at 30 °C.

Day 1: In the morning dilute the precultures to an OD=0.2 in 5-10 ml. In the evening set 10 ml cultures with an OD according to their growth (lem mutants should be set with ODs 0.05-0.08, for wt or wt-like growing cultures ODs 0.01-0.03 should do).

Day2: Measure ODs of the cultures. Take cultures with OD 0.4-0.5. From those culture, take some 200-300 µL into an eppendorf. If cultures are to be re-imaged, bring them back to grow at 30 °C.

1. Unfreeze in water a tube of lectin 2x. Once melted, keep it on ice.
2. Dilute the lectin 2x to 1x with the medium in which your yeast is diluted (in our case YES).
3. Place one of the special coverslips for yeast imaging on a tissue. Always keep on a tissue to avoid scratches in the imaging surface and also avoid touching it.
4. Add 100 µL of the 1x lectin into the glass (opening of the plastic), spread over the crystal and incubate 5 min at RT.
5. Remove and discard the lectin from the crystal.
6. Resuspend the culture in the eppendorf up and down to ensure cells are not clumped for imaging.
7. Add 100 µL of the resuspended and mixed culture into the crystal and incubate 5 min at RT.
8. Remove and discard the culture from the crystal.
9. Wash the crystal with 1 ml of medium (YES) by pipetting up and down, not harsh directly into the crystal but rather from the sides (not to fully detach all yeast from the crystal. Remove and discard the 1ml of the wash.
10. Add 3 ml medium (YES) into the slide and bring to microscope.

Materials:

Lectin – Dissolve lectin (Sigma L1395) in autoclaved water in a concentration of 1mg/mL.

Glass Bottom Culture Dishes – MatTek Corporation (35mm Dishes with 10mm glass (1.5 thickness))(P35G-1.5-10-C)

Pombe microscopy

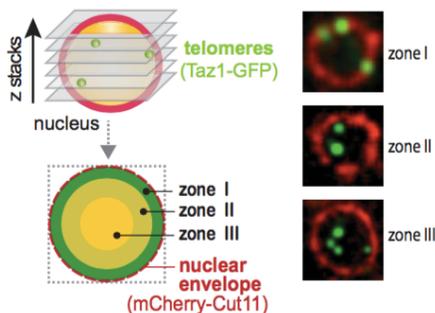
1. Turn on the equipment
2. First the computer and enter username/pw
3. Turn on the three power strips
4. Turn on lasers, for us 488 and 532/561
5. Set up the temperature to 30 °C without CO2 (clear alarm msg)
6. Pull shuttle to the outside and turn on upper camera
7. Open the ZEN software
8. In locate, light path, choose the 100x oil objective
9. Turn on the light from inside the microscope temperature chamber and add carefully into the side of the lense, without touching it, a drop of the immersion oil.
10. Put and adjust the coverslip on top of the lense.
11. Still with the light on, start going up with the focus knob (towards you) until you touch the immersion oil, then turn off the lights.
12. In locate, choose BF and vis and go into the eyepiece and get cells into focus. Once focused, turn off BF by pressing shutter
13. Go to acquisition, use the profile for the imaging (in this case GFP mCherry for sme2 cut11)
14. In case you know the settings are good just proceed into taking images. Otherwise, optimize the lasers (in general not more than 45% laser intensity):
 - a. In acquisition, channels, click on each of the lasers separately and go into live
 - b. If image too bright, decrease laser intensity, exposure and gain until signal/noise is optimal
 - c. If red signal is too few, might be that the green (which was normally acquired first) bleaches a bit in the red. To avoid this, exchange the channels (bring up the red)
 - d. If more light is needed for the BF, in locate, light path settings, click on the bulb and change the volts
15. To select cells to image, in acquisition, channels, select BF and click live
16. Wait for 5-10 min for the oil to temperate. In this time focus might change
17. Once the cells are selected, and to acquire z-stacks go into Z stack
 - a. focus the cells and press 'set last'
 - b. go down in the Z until you take 11 stacks and press 'set first'
 - c. go back up in Z until you have another 11 stacks after the middle point and press 'set last'. In total should be 22 stacks, adjust to this number.
18. Click 'start experiment'. Acquire 15 images per condition/strain
19. Rename the images by right clicking into their names and save them in format date_strain
20. To save the images, go into processing and drag all the images into the list
21. Click->batch->OME tiff. Check that in the naming section there is %N and apply. Transfer takes some time
22. Go into pictures->temp and there are all the photos. Copy the photos from temp into a new folder in the pictures and from there into big data->users->...
23. Close the program
24. Turn off the equipment
 - a. Turn off computer
 - b. Turn off the three power strips
 - c. Turn off lasers, for us 488 and 532/561
 - d. Turn off the temperature setting and leave the little window open for the microscope to reach RT for next user



Live

Pombe Microscopy analysis

1. Download Fiji in your computer
2. Have your files randomized to not be biased towards analysis (ask Sigurd to do it)
3. Open the image from the server (ask Gyula to get access to the images)
 - a. Open the .tiff file
 - b. Select open as hyperstack and split the channels
4. Image->adjust->brightness and contrast (leave this menu open on the side the whole time).
5. In the brightfield channel, add B&C until you see the cells, if in 1 layer, then go on.
6. Close the brightfield image
7. Image->color->merge channels
 - a. C0= red (normally it is green, but in this case we put the red to avoid the bleaching with the strong green signal)
 - b. C1=green
 - c. (C2=brightfield) We already closed it, we don't really need it!
8. Save the image as a .tiff file, that will have now the green and red channels merged, in case I want to go back to counting in the future, without having to do again the open with split channels and merging.
9. Play with the B&C and enhance the signal until it is as good as possible.
10. For the analysis itself, do per "coded" image:
11. Measure the diameter with the  tool, dragging a line between the 2 points of interest and then pressing M. A table will be generated with all the measurements. Once having counted all diameters, copy this table to excel and use it to calculate the average diameter of the cells (per condition)
12. Measure the distance from the middle of the point/s to the middle of the nuclear envelope with the  tool, dragging a line between the 2 points of interest and then pressing M. It is easiest to observe the envelope when removing the green channel. For the sme2 locus or red1, count when there is only 1 point. In general, count only points when the nuclei are elongated. Also do not count points that are very up or down the envelope. For this, focus the z where the spot is brightest and then move a couple of z's up and down. Count only when also the envelope is in focus/bright. A table will be generated with all the measurements. Once having counted all distances to the envelope, copy this table to excel.
13. Once all photos have been counted and data has been copied on excel, decode the photos, figuring to what strain/condition they belong to and regroup them according to this.
14. Per strain/condition, calculate:
 - a. Average diameter of the cells
 - b. Spots per zone



1	$0,184*r$	$\cong 0,197$
2	$0,422*r$	$\cong 0,452$
3	$0,578*r$	
NOTE!! $r = \text{diameter}/2$		