Protocol for ploidy measurement of snowflake yeast using fluorescence microscopy

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Overview

Procedure: collect exponential-phase cells, ethanol fixation, RNase treatment, PI staining, imaging, image analysis for nuclei segmentation and PI fluorescence quantification, R data analysis

- Propidium iodide (PI) for DNA staining: bind to DNA/RNA (thus RNase digestion), enter only permeabilized cells but not cells with intact cell membranes (thus ethanol fixation), PI is a charged molecule thus keep cells in PI solution when imaging to prevent PI from leaking out of the cells, keep PI and PI-stained samples in dark to prevent photobleaching (PI does not photobleach very fast under ambient light, but it does not hurt to be extra cautious)
- Why exponential-phase cells: contain mostly actively dividing cells including G1 and G2 cells

Keys:

- Fluorescence quantification requires careful design and execution of experiments and paying attention to many technical details that may affect fluorescence intensity
- For successful fluorescence quantification, snowflake yeast clusters must be crushed into a single cell layer and the focal plane must be selected accurately and consistently
- Keep constant across experiments for reproducibility: cell density, DNA stain concentration, microscope settings
- Include ploidy control strains in every experiment for (1) providing reference fluorescent values which experimental samples are compared to for ploidy estimation (2) between-experiment comparisons to check if experimental setup are consistent (3) checking if microscope (e.g., exposure/gain) is working as usual

Time consideration:

- Day1 afternoon: inoculate cultures
- Day2 morning: transfer cultures and grow 4h
- Day2 afternoon: sample prep (including 2h ethanol fixation, 2h RNase digestion and overnight PI/RNase incubation)
- Day3 morning or afternoon: imaging (5-10min per sample)
- Note: PI-stained samples (Day3 morning) might be stored at 4 degrees for a few days before imaging, which does not seem to affect fluorescent intensity significantly (confirmed in a quick test). This allows for more flexible experimental scheduling. However, I would still recommend using freshly prepared samples (Day3 morning) for imaging, and if samples previously stored at 4 degrees are used, ideally the ploidy control samples processed in the same way (e.g., stored at 4 degrees for the same amount of time) should be used

References:

- Todd2018 (yeast ploidy measurement using flow cytometry)
- Roukos2015 (mammalian cell ploidy measurement using fluorescence microscopy)

Materials

- 70% ethanol
- 50mM sodium citrate: 7.35g sodium citrate dihydrate + 0.5L H2O, autoclave, store RT (prepared by Vivian in early 2022)

- 20mg/ml RNase A in 40% glycerol: 100mg RNase A (MP Biomedicals, #101076) + 5ml 40% glycerol, dissolve at 37C for 1-2h, aliquot 1ml per 1.5ml tube, store -20C (liquid does not freeze) (prepared on 2022/9/19)
- 1mg/ml propidium iodide in H2O (Thermo Fisher, #P1304MP): 100mg + 100ml H2O (final 1mg/ml, 1.5mM), aliquot 1ml per 1.5ml tube, store dark at 4C (for short-term storage, stable >6 months, minimize frequent freeze-thaw) or -20C (for long-term storage) (prepared on 2022/9/19)
- Yeast control strains: 2N multi and 4N multi (Y55 background, grande, isogenic with ho::hphNT1 and ace2::kanMX, 2N is a/alpha and 4N is a/a/alpha/alpha)

Sample prep

1. **Grow culture**: for yeast strains of interest and ploidy control strains (2N, 4N, optionally 1N), inoculate into 10ml YPD and grow overnight, transfer 500ul overnight culture into 10ml YPD and grow 4h to exponential phase
2. **Collect cells**: transfer 250ul culture into 1.5ml tube, spin 5000g 1min, remove sup
3. **Wash**: resuspend in 1ml H2O, spin 5000g 1min, remove sup
    - For macroscopic strains: resuspend in 100ul H2O, use 100ul regular-bore tip to pipette break clusters into small pieces (as small as possible, crucial for fixation/RNase/staining and crushing into single cell layer), then add another 900ul H2O, spin 5000g 1min, remove sup
        - Pipette break clusters: first insert tip to close to tube bottom and pipette several times to break big clusters into small pieces, then push tip onto tube bottom and pipette slowly up and down several times (be careful to not accidentally suck liquid up into the pipettor), which forces clusters to pass through the tiny space between tip and tube bottom and breaks them into even smaller pieces
    - Note: pellet volume should be ~10-20ul (allow easier fixation/digestion/staining and crushing below), if more then you can pipette resuspend cells (before removing H2O sup) and remove some cell suspension and spin again
4. **Ethanol fixation**:
    a. Resuspend in 1ml 70% ethanol, immediately vortex at max level to mix, then immediately put on mini-rotator and rotate at max speed
        - Recommend: complete this step one sample at a time, otherwise bigger clumps may form during ethanol fixation
        - Note: more input cells may cause more clumping
    b. Incubate at room temperature on mini-rotator with max speed for 2h
        - Recommend: after 1h of ethanol fixation with rotation, vortex samples at max level to dissociate clumps, then continue fixing with rotation for another 1h
    c. Spin 5000g 1min, remove sup
        - Note: if you get a loose pellet or a smear on the side of tube, rotate the tube by 180 degrees and re-spin for 30sec to get a tighter pellet
5. **Wash and rehydration**: [resuspend in 1ml 50mM sodium citrate, vortex mix, spin 5000g 1min, remove sup] x twice
    - During spinning: set heat block to 37C, prepare sodium citrate with RNase as below
    - Note: if you get a loose pellet or a smear on the side of tube, rotate the tube by 180 degrees and re-spin for 30sec to get a tighter pellet
6. **RNase treatment**:

a. Resuspend in 200ul 50mM sodium citrate containing 0.5mg/ml RNase A, pipette to mix (no vortex after adding RNase)
    - Prepare solution: for each sample, mix 5ul 20mg/ml RNase (in 40% glycerol) and 200ul 50mM sodium citrate, then use 200ul
b. Incubate at 37C on heat block for 2h, with manual inversion every 30min
    - Manual inversion: invert the tube and shake it to let liquid drop from bottom, then quickly invert the tube and let liquid go to bottom, do this 3-4 times

7. **PI staining**:
    a. Add 5ul 1mg/ml PI solution into a new 1.5ml tube (wrapped with aluminum foil to prevent photobleaching) per sample
    b. Transfer 195ul cells into this new 1.5ml tube (final 25ug/ml PI), immediately pipette to mix, then immediately put on mini-rotator and rotate at min speed
        - Recommend: complete this step one sample at a time (you can bring samples, pipettor, tips and waste beaker to 30C incubator), otherwise clusters can settle quickly to the tube bottom within minutes while processing other samples, which may hamper PI staining
        - Why transfer to a new tube: (1) pipetting cells onto 5ul PI and mixing should allow more even staining than adding 5ul PI into ~200ul cells then mixing (2) tube wall of the previous tube is wetted thus liquid may rotate (3) the remaining ~15ul cells can serve as unstained negative control
    c. Incubate 30C dark (wrap tube with aluminum foil) on mini-rotator with min speed overnight
        - Note: 200ul liquid does not do end-to-end rotation under this setting but just stays at the tube bottom, but this rotation keeps clusters from settling and should help with staining and continued RNase digestion
        - Note: 37C incubation may be better but we do not have a free 37C incubator that can fit in the mini-rotator, and 30C appears to be working fine so far
8. **Prepare slides for imaging** (after setting up the microscope below): pipette mix clusters, drop 5ul on microscope slide, apply coverslip quickly and clusters should spread out and air bubbles should be minimized, use two fingers of left hand to hold two left corners of coverslip to prevent its movement, use one finger of right hand to press on the coverslip to crush clusters into a single cell layer
    - Important: crushing clusters into a single cell layer (can be examined under BF channel) is crucial for accurate quantification of nuclei intensity, if not single cell layer then prepare the slide again
    - Key: to crush clusters into a single cell layer, clusters should be small enough (thus pipette break clusters for macroscopic strains above), and clusters should spread out rather than clump together on the slide, to achieve this (1) clusters should be diluted enough (thus input 250ul mid-log-phase culture above and finally suspended in 200ul PI/RNase solution) (2) apply coverslip in a way that spreads out clusters
    - Recommend: for each sample, prepare the slide right before imaging, to prevent evaporation

Imaging

1. DO NOT put the slide on the microscope stage before "For each sample" step below, to prevent accidental photobleaching of PI under Red channel
2. Turn on the microscope and let laser warm up for 10-15min

3. Make sure there is enough space on local disk or external hard drive
4. Make sure that microscope hardware are at their default settings (esp., all filters are kept to the right, zoom knob is at 1.00x)
5. Turn room light OFF and close the curtain
6. Ti Pad window: **20x** objective, Light Path L100, (turn on BF channel and Live) Lamps **5.0V**, Zoom 1.00x
    - Lamps: turn on channel [BF] Remote Control green / Switch Lamp on [Red] Remote Control green / Switch Lamp off
        - Note: these should be default, otherwise set them like this and set this as default by "right clicking the channel button on toolbar -> Assign current microscope setting"
    - Why 20x magnification: compared to 40x, get more nuclei per field of view for smoother nuclei intensity histogram, easier to scan and find clusters, better integration of multiple focal planes
7. DS-Qi2 Settings window: Resolution **14-bit 4908x3264**, set exposure and gain [BF] **100ms** (or auto exposure if does not look right) **4.1x** [Red] **600ms 2.2x** (after setting exposure/gain on one channel, switch to another channel and switch back to check if the settings are correctly saved)
    - Choice of exposure and gain for BF channel: minimize gain (reduce noise but not as strict as Red channel, okay to be above 1.0x), use short exposure to allow setting focus easily and quickly at 20x magnification
    - Choice of exposure and gain for Red channel: minimize gain (reduce noise for fluorescence quantification), while keeping exposure as long as possible (hundreds of ms) but not too long to potentially cause photobleaching especially when imaging multiple z slices
        - Important: the brightest objects (here G2 nuclei of 4N cells) should be within the range of detection (ideally ~80% of max) rather than seeing saturation, check by "View -> Visualization controls -> Histogram"
    - Note: if these gain settings are not default, set them by "right click the channel button on toolbar -> Assign current camera setting"
8. Open XYZ Navigation window: "View menu -> Acquisition Controls -> XYZ Navigation"
9. Open ND Acquisition window: "View menu -> Acquisition Controls -> ND Acquisition"
    a. Load XML file: "Load -> Load from XML file …. -> Desktop/Kai/ploidy_measurement_v1.xml"
    b. Save to File: specify Path to a folder
    c. Deselect XY tab
10. For each sample:
    - Image 4N multi and 2N multi control strains first to check if all settings look good (esp. intensity histogram is not saturating and has expected intensity i.e. 4N multi max ~12k, 2N multi max ~8k, non-cell background ~400)
        - Optional: check images of ploidy control strains by image analysis and confirm good before proceeding to other samples

    a. Make sure Red channel is NOT on, then place the slide on microscope stage with coverslip facing down
    b. Specify Filename in ND Acquisition window
        - Recommend: use <sample-name>_001.nd2, because after taking the first image, the file name for the next image will be automatically updated to <sample-name>_002.nd2 ,etc.

c. Image **5-10 fields of view (FOVs)** (recommend 10 FOVs for ploidy control strains), and for each FOV:
    1) Turn on BF channel and Live
    2) Move to a good XY position (a bit away from previously imaged positions to avoid photobleaching and accidentally imaging the same clusters again)
        - Good XY position: many clusters crushed into single cell layer, avoid big debris > 500um^2 (small debris is fine but affects max intensity when viewing histogram), (for applications that involve cluster-level measurements, e.g. some method of population-level ploidy measurement) avoid touching clusters
    3) Adjust z position using slowest mode to get cells in focus, where cells are gray rather than darker or brighter (key for fluorescence quantification)
        - Why adjust to gray then move down by 1um: setting accurate and consistent focus is critical for fluorescence quantification, and I find setting cells in focus (in the middle plane where cells look gray, while moving down causes cells to look brighter and moving up causes cells to look darker) would be the easiest to do and have the lowest person-to-person subjective bias, and then move down from there by 1um to get to the nuclei focal plane
    4) Use XYZ Navigation window to move z position down by **1um** (this is where nuclei are in focus), confirm that cells look slightly brighter
    5) Click "Run Now" in ND Acquisition window
    6) Check output image that is displayed on the screen (you may check the first 2-3 FOVs for each sample, and then skip this for the subsequent FOVs to save time):
        - Red channel: check intensity histogram (1) max pixel intensity is expected for the sample and (2) three z slices have similar histogram (the first one usually has slightly higher intensity than the other two)
            - Note: if debris is present in the image (debris is often very bright), then max intensity may go beyond what is expected for the sample
        - BF channel: should be slightly brighter than gray

XML file ("ploidy_measurement_v1.xml") used for imaging:

- XY tab: one selected point, include Z, uncheck "Split multipoints"
- λ tab: **Red offset 0um, Red offset -0.3um, Red offset +0.3um, BF offset 0um**
    - Why three z slices (later used for max intensity projection): tolerate (1) slight human error/bias in setting focus (2) potential focal plane difference across nuclei in a crushed cluster (3) potential focal plane change across one field of view
    - Why BF channel in the end: return to BF channel at the end of image acquisition, instead of letting Red channel be on in the end to continue unwanted photobleaching…

Image analysis

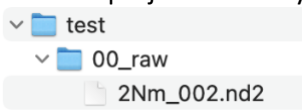
Overview:

1. Perform maximum intensity projection of three z slices of PI channel for nuclei segmentation and fluorescence quantification (note that I retain an option to use one z slice for nuclei segmentation and fluorescence quantification by modifying the “nucleus_channels” parameter at the beginning of the script)

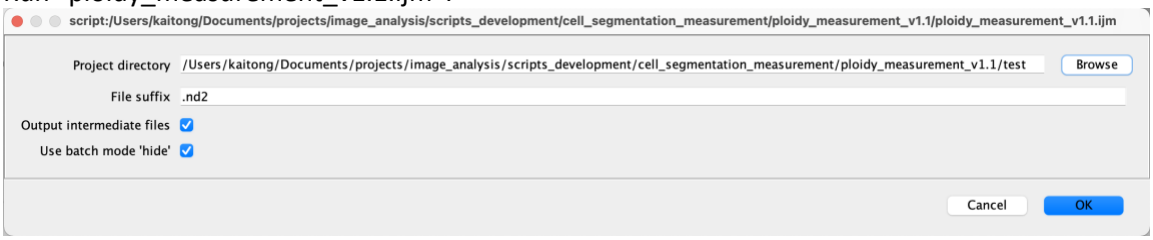
2. Segment clusters, single nuclei (remove touching nuclei using min circularity threshold) and cytoplasm
3. Measure total PI fluorescence intensity of each nucleus with background subtraction using median intensity of cytoplasm region in each cluster (method 1, work better than method 2) or using median intensity of non-cluster background region of the whole image (method 2)
4. Also measure other nucleus metrics (e.g., area, circularity) and cluster metrics (i.e., area, roundness, if touching image edges)

Requirement: ImageJ/Fiji, install bioVoxxel plugin

1. Create a project directory, store all raw image files in a subdirectory named “00_raw”

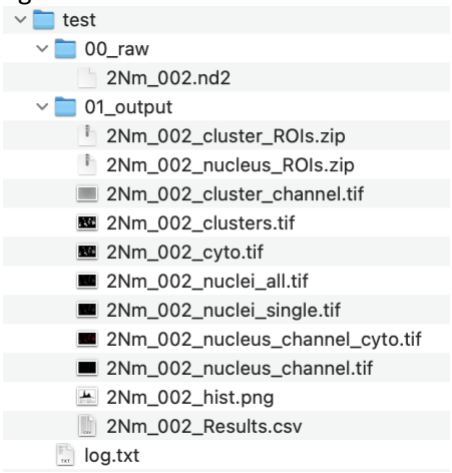


2. Run “ploidy_measurement_v1.1.ijm”:



   a. Specify project directory and input raw image file suffix (default .nd2)
   b. If you want to see all intermediate files (which are all the TIFF files below) for troubleshooting or checking, select "Output intermediate files"
   c. If you want to run the script in the background, select "Use batch mode 'hide'"

3. Check output: a new "01_output" subdirectory is created and stores all output files, as well as a log.txt file



4. Check log.txt file: “Non-cell bgInt” is method 2 background intensity (calculated per image) and should be ~400, “cyto bgInt” is method 1 background intensity (calculated per cluster) and should be ~1000 and generally similar across all clusters in the same image

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Running ploidy_measurement_v1.1 ...
ImageJ version: 2.3.0/1.53q
OS: Mac OS X
Project directory: /Users/kaitong/Documents/projects/image_analysis/scripts_development/cell_segmentation_measurement/ploidy_measurement_v1.1/test2
Input directory: /Users/kaitong/Documents/projects/image_analysis/scripts_development/cell_segmentation_measurement/ploidy_measurement_v1.1/test2/00_raw
Output directory: /Users/kaitong/Documents/projects/image_analysis/scripts_development/cell_segmentation_measurement/ploidy_measurement_v1.1/test2/01_output
File suffix: .nd2
Output intermediate files: Yes
Use batch mode 'hide': Yes

Processing sample 2Nm_002 ...
Detect 12 clusters
Non-cell bgInt: 442
Cluster 1: cyto bgInt 978 | 69 nuclei
Cluster 2: cyto bgInt 966 | 98 nuclei
Cluster 3: cyto bgInt 1109 | 251 nuclei
Cluster 4: cyto bgInt 1044 | 86 nuclei
Cluster 5: cyto bgInt 933 | 62 nuclei
Cluster 6: cyto bgInt 1077 | 166 nuclei
Cluster 7: cyto bgInt 1115 | 667 nuclei
Cluster 8: cyto bgInt 955 | 87 nuclei
Cluster 9: cyto bgInt 991 | 124 nuclei
Cluster 10: cyto bgInt 995 | 354 nuclei
Cluster 11: cyto bgInt 1004 | 267 nuclei
Cluster 12: cyto bgInt 972 | 160 nuclei
Detect 2391 nuclei in total
Complete processing.

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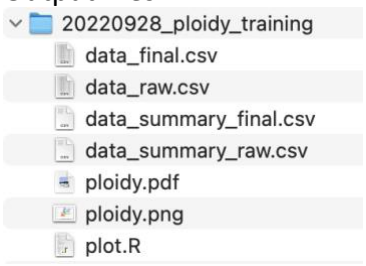
R data analysis

Overview: remove small objects (segmentation artifacts) and outlier images, plot density plot of nuclei intensity for each strain

Procedure: run “plog.R”, modify code where needed

Output files:



Output plot (example):