Microcolony growth assay – Protocol and details

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If you have any questions or encounter any problems, feel free to contact me at nz375@nyu.edu

- This document contains a general protocol for doing microcolony growth experiments. It contains important details specific to the assay, as it is set-up in the Berman lab at Tel-Aviv University.
- For more information about the different experiment types, assumptions and usage of scripts and functions, read MicrocolonyExperiments.docx
- For more information about the underlying code, read MicrocolonyScripts.docx
- For information about how to set-up the assay in a new lab, read MicrocolonyAssay.docx

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Pre experiment considerations

Pre experiment growth conditions should be considered carefully. My usual routine in the Berman lab was: Day 1 – streak from -80 to single colonies, Day 2 – inoculate small colony into 3ml media in tube, Day 3 (+24hr) – dilute culture into 3ml new media (e.g., 1:100) with/without Fluconazole, grow for 4 hours, dilute (e.g., 1:2000) into new media with/without Fluconazole. All media used for the microcolony assay should be filter-sterilized.

Experiment day

Coating the plate with ConA

Take an aliquot from the -80 freezer containing 5X Con-A solution (1mg/ml), these contain 5 ml or 2.5 ml. Add appropriate volume of water (20 or 10 ml) to bring solution to 1X (200 ug/ml), water should be autoclaved DDW. After the solution thaws, filter-sterilize using a syringe into a new tube. Add the appropriate volume (see table) to each well.

This is a good time to make sure the microscope incubator is turned on, the water receptacle within the incubator is filled with DDW and the plate adaptor is set-up (see below).

After a few hours (1.5 hr was typical in NYU and 3-4 in TAU), remove the con-A, wash with water twice and put your cells in the plate. Removing the Con-A/water with a vacuum is helpful, especially for 96 or 384 well plates. Do not let the Con-A dry on the plate, if your cells are not ready, leave the second wash in the wells and remove when the cells are ready to be put in the plate

Setting up the plate and placing it on the microscope

Before setting up your plate, change the plate adaptor on the microscope. There are little screws meant to help push the adaptor up. These should be flush against the bottom of the plate adaptor. Put the adaptor on and make sure it is sitting flat. There are three large white screws that hold the adaptor, start to screw each one of these, do each one half way and than do the other half.

Sonicate (to ensure cells are separated) and dilute your cells. Cells should be at 2X10^4 cells/ml. Place appropriate volume (see table) of cells in well.

Before placing the plate on the microscope, spray bottom with anti-duster. Open the microscope software NIS-elements, under devices tab, click initiate stage. When the stage is done moving, place the plate on the microscope (make sure the objective is down.

Wait 30 minutes to allow the plate to reach incubator temperature, this is a good time to ensure your plateID and experiment ID files are updated and ready to use.

Volumes used during set-up

Plate	Volume of Con-A	Volume of cells
24-well	1000 ul	2000 ul
96-well – TAU	100 ul	200 ul
384-well	50 ul	75 ul

Microscope settings

There are a number of microscope settings that help ensure images look right. The additional glass that breaks light above the condenser should be out. The density filter (filter on top left with 'd') should be out. The aperture controlling the spread of light (small black knob on condenser) should be quite closed.

Focusing routine

Starting with the objective down, raise the objective until the light indicating the readiness of the perfect focus system (PFS) has turned on (FOCUS), turn on the PFS by pushing ON button, the focus is now adjusted using the PFS wheel (next to the keyboard). Focus on cells and adjust lighting conditions and exposure (e.g., ~20ms exposure when lamp is set to ~35). Cells should be white with black rims, the background should be grey, and the cells should not be too fuzzy. Add a xy-point (press + in xy tab in ND Acquisition section) and note the PFS value.

Open Cygwin and run NewMultipoints.sh, answer questions, ask for 1 field per well, and give the PFS value you just saw. Load multipoints.xml newly created in f/Multipoints. Turn the PFS off/on to see values. Manually adjust the PFS value of each point (press little arrow next to point after changing focus), ensuring optimal focus for each well. Save the adjusted xy points as template.xml also in f/Multipoints.

Run NewMultipoints.sh again, this time choose the number of fields per well (I used 100, 16 and 4 for 24, 96 and 384 well plates), see table for maximum values based on size, an additional consideration is the time interval between timepoints. Also write NA when asked for a focus offset. Load the new file created at (f/Multipoints/multipoints.xml). Turn the PFS off/on to see values. Look at a few fields to make sure things look good.

Set up the Time tab in the ND Acquisition section. Typical values are 1 hr interval and 19 hour duration (resulting in 20 timepoints). Only the Time and xy tabs should be checked.

Maximum number of fields per well based on size

Plate	Objective	Magnifier	Maximum number of fields
24-well	10	1	42
24-well	10	1.5	110
24-well	40	1	810
24-well	40	1.5	1886
96-well	10	1	6
96-well	10	1.5	16
96-well	40	1	132
96-well	40	1.5	288
384-well	10	1	2
384-well	10	1.5	6
384-well	40	1	56
384-well	40	1.5	132

Starting analysis

In the ND Acquisition section, the box 'save as TIFF series' should be checked, the path should be f/GrowthAssay and the subfolder should be defined as intials_date_id_GR (e.g., NZ_150908_1_GR), matching your experiment ID. In the xy tab, 'Leave PFS offset ON between points' should be checked. Press 'run now' to start the experiment. Copy the multipoints.xml file to the experiment folder. Open Cygwin and run Intialize.sh, choose your ID files. Calculate how much space the experiment will need (each image is 11.1 MB) and make sure there is enough room on the microscope and processing computers.

ID files

Avoid using spaces, commas and special characters in the ID files.

plateID

The plate ID file specifies which conditions (strain and media) are in each well of your plate. This should be saved as csv file in ~X/plateIDs on the processing computer (contains examples). You can rewrite these files (in this location) frequently as a copy is always present in the resulting experiment output folder.

experimentID

The experiment ID file specifies parameter values controlling all aspects of the automatic analysis flow, there is a short explanation of each parameter in template file. This should be saved as csv file in ~X/experimentIDs on the processing computer (contains examples). You can rewrite these files (in this location) frequently as a copy is always present in the resulting experiment output folder. There is a short explanation of each parameter

Output folder

The output folder is created when you run Intialize.sh in ~/X/out/GR/. At first it contains empty folders that will be filled as the analysis progresses.
When analysis is complete it contains:

1. **ID files –** the folder will immediately contain two copies of your plate and experiment ID files, with original and generic names
2. **multipoints.xml –** a copy of the multipoints file you used will appear at the second phase of analysis (after all image analysis)
3. **jpgGRimages folder –** all experiment images, saved as jpegs, created in first (image analysis) phase of analysis
4. **overlays folder –** black and white images defining cells/microcolonies, created in first (image analysis) phase of analysis
5. **rprops folder –** matlab files containing the information (area, centroid position…) for all white blobs in the corresponding overlay, created in first (image analysis) phase of analysis
6. **colors folder –** matlab files containing information about the 'color' of microcolonies in each image, only relevant for FC type experiments, created in the second phase of analysis (after all image analysis)
7. **matout folder –** matlab files containing information (area, centroid position…) about all microcolonies in a given field, also contains information about how each microcolony was aligned in different time-points, is created in the second phase of analysis (after all image analysis)
8. **datafiles folder –** text files containing information (area, centroid position…) about all microcolonies in the experiment, also files with information about the plate, is created in the second phase of analysis (after all image analysis)
9. **logs folder –** contains log files, GRp_out.txt can be useful for identifying analysis problems
10. **movies folder –** contains movies, both movies that are automatically generated or manually generated using the 'Movies' matlab function
11. **Rplots folder –** contains a series of plots for assessing the experiment
12. **R files –** these files can be loaded into R and contain certain variables (variable names match file names, e.g., d for d.Rfile)
    a. **d.Rfile –** a list containing the following elements:
        i. d$well.vector - a 0 or 1 vector of if a well has been used
        ii. d$well.list - a list of the colony identifiers in each well
        iii. d$wells.used - a vector of only the wells used
        iv. d$areas - a matrix of colony areas (rows are colonies, columns are timepoints, row number is colony identifier)
        v. d$imnumber - a vector of the image number (field identifier) for each colony
        vi. d$plate - a matrix of information about the plate and field replicated for each microcolony (plate row, plate column, imnumber, microscope X, microscope Y, microscope Z, PFS offset)

vii. d$cX - the X position of the colony centroid in the field (rows are colonies, columns are timepoints, row number is colony identifier)
viii. d$cY - the Y position of the colony centroid in the field (rows are colonies, columns are timepoints, row number is colony identifier)
ix. d$color - the 'color' of the colony (rows are colonies, columns are timepoints, row number is colony identifier), only relevant for FC type experiments
x. d$times - matrix of image times for each colony (rows are colonies, columns are timepoints, row number is colony identifier)
xi. d$fint - a matrix of sum of the fluorescent intensity for each colony (rows are colonies, columns are timepoints, row number is colony identifier), only relevant for fluor-type experiments
xii. d$perimeter - a matrix of the perimeter for each colony (rows are colonies, columns are timepoints, row number is colony identifier)
xiii. d$pID - a copy of the plateID
xiv. d$eID - a copy of the experimentID

b. **gr.Rfile –** a list containing the following elements:
   i. gr$areas.raw – identical to d$areas
   ii. gr$colparam.raw – matrix with information about growth calculation for microcolony (rate, r^2, foldX, lag, t0 and t-last (or points – depends on analysis method))
   iii. gr$areas – processed areas matrix (NA for microcolonies without growth rates)
   iv. gr$colparam – processed colparam matrix (additional filtering that depends on analysis method)
c. **md.Rfile –** a list containing the following elements:
   i. md$cxm – mean microcolony centroid X position for timepoints used for growth rate calculation
   ii. md#cym – mean microcolony centroid Y position for timepoints used for growth rate calculation
   iii. md$mindist – minimum distance (between centroids) to other tracked objects in field

Additional data analysis

Creating movies

You can create additional assay movies after analysis is complete by directly using the matlab Movies function. The movies are created in the movies folder of the experiment output folder.

The function has the following arguments:

1. input_folder – path to folder containing images (can be jpgGRimages)
2. output_folder – path to experiment output folder (must have experimentID, overlays folder, rprops folder, datafiles folder (with platerow.txt, platepos.txt, plate.txt), matout folder and movies folder)
3. xypos – vector of field numbers to create movies for (can be one number or more)
4. resize – parameter that controls the resizing of the movie, during automatic analysis, it is set to 0.4, can be set higher to create movies with higher resolution

Usage (examples of commands to be run in Matlab):

```
>>Movies('/Volumes/IN/GR/NZ_150811_1_GR',
'~/X/out/GR/NZ_150811_1_GR', 1810, 0.4)
```

For multiple fields:

```
>>Movies('/Volumes/IN/GR/NZ_150811_1_GR',
'~/X/out/GR/NZ_150811_1_GR', [1810 1811 1812], 0.4)
```

For higher resolution:

```
>>Movies('/Volumes/IN/GR/NZ_150811_1_GR',
'~/X/out/GR/NZ_150811_1_GR', 1810, 1)
```

For jpegs:

```
>>Movies('~/X/out/GR/NZ_150811_1_GR/jpgGRimages',
'~/X/out/GR/NZ_150811_1_GR', 1810, 1)
```

Using get.info.R

get.info is a useful R function for organizing data from multiple experiments (or only one). It basically loads Rfiles, extracts information and organizes it in a dataframe. It has built in options allowing you to specify what type of information to curate from the experiments.

It has the following arguments:

1. folders – single or multiple experimental directory names (e.g., 'NZ_150908_1_GR' or c('NZ_150907_1_GR','NZ_150908_1_GR')), no default

2. pre.data – data from previous use of the get.info function, either a data frame or list with 2 or 3 elements, must match all current options, (default is NULL)
3. get.size – option for obtaining additional info about microcolony fold change, time tracked and centroid position, (default is F)
4. get.plate – option for obtaining additional info about field position in plate, (default is F)
5. get.areas – option for getting matrix of areas, (default is F)
6. get.fint – option for getting matrix of fint (fluorescent intensity), (default is F)
7. cam – used to filter microcolonies based on position within a field, coordinate limits in X and Y, (default is c(0,Inf,0,Inf))
8. path – used to specify the path to directories given as 'folders', (default is '~/X/out/GR/')

It creates the following output:

1. If get.areas and get.fint is F, the function returns a single dataframe with the following columns:
    a. GR – microcolony growth rate
    b. Lag – microcolony lag duration
    c. Isize – microcolony size at first timepoint
    d. mindist – minimum distance (between centroids) to other tracked objects in field
    e. color – most frequent 'color' of microcolony
    f. Genotype – microcolony genotype as appears in plateID
    g. Environment - microcolony environment as appears in plateID
    h. colony – unique colony identifier in experiment
    i. field – microcolony image number (field)
    j. well – microcolony well name, nested in plate
    k. plate – experiment identifier
2. If get.areas is set to T and get.fint is F, the function returns a list with two elements, the first is the dataframe described above and the second is a corresponding matrix of microcolony areas, all experiments will be trimmed to have the same number of timepoints
3. If get.areas and get.fint is T, the function returns a list with three elements, the first is the dataframe described above, the second is the matrix of areas described above and the third is a corresponding matrix of fint (summed fluorescent intensity), all experiments will be trimmed to have the same number of timepoints.
4. If get.size is T, additional columns will be added to the dataframe described above
    a. foldX – fold increase in microcolony area while tracked
    b. t0 – first timepoint colony area tracked or used for GR calculation (depends of analysis method)
    c. len.track – number of points colony tracked or used for GR calculation (depends of analysis method)
    d. cX – mean microcolony X coordinate position within field
    e. cY– mean microcolony Y coordinate position within field

5. If get.plate is T, additional columns will be added to the dataframe described above.
    a. plate.row – row number in plate
    b. plate.col – column number in plate
    c. fieldX – field X coordinate position on microscope
    d. fieldY– field Y coordinate position on microscope
    e. pfs – field PFS values

Usage (examples of commands to be run in R):

First, get access to function
> source('~/X/code/R/get.info.R')

One experiment
> mydata<-get.info('NZ_150901_1_GR')

Multiple experiments
> mydata<-get.info(c('NZ_150901_1_GR','NZ_150908_1_GR'))

Using pre.data and path arguments (example of multiple experiments saved in different locations)
> mydata<-get.info(c('NZ_150901_1_GR','NZ_150908_1_GR'))
> mydata<-get.info('NZ_150903_1_GR', pre.data=mydata, path='/Volumes/IN/out/GR/')

Using options
> mydata<-get.info(c('NZ_150901_1_GR', 'NZ_150908_1_GR'), get.plate=T, get.areas=T)

Data clearance and back-up

Data from experiments include original images and output folders. Images are stored temporarily (typical experiment size: 200-800 GB), output folders are saved and backed-up (typical experiment size: 5-50 GB). Folders are automatically removed or backed-up. Clearance and backing up occurs each morning at 7AM.

The following locations exist in the Berman lab: the microscope computer has a 4T internal drive (f), the processing computer has a 250 GB internal drive (/) and a 4T external drive (IN), and there is also a 12T NAS server.

Image folders are automatically removed from the microscope computer 3 weeks after the last modification (this relies on location and correct naming of folders). Image folders are automatically removed from the processing computer (/Volumes/IN) 3 weeks after the last modification (this relies on location and correct naming of folders). Output folders are created on the processing computer (~/X/out/GR) and backed up to both (/Volumes/IN/out/GR) and the

NAS server (Shared/MicrocolonyGrowth/out/GR). Folders are removed from the internal drive 2 weeks after last modification and from the external drive 4 weeks after last modification (this relies on location and correct naming of folders). Folders are not removed from the NAS.

Details for different experiment types

Experiments with fluorescence

To set up experiments that include fluorescence images at every timepoint, you should check the lambda tab (in addition to the Time and xy) in the ND acquisition section of NIS elements. Make sure the first channel is brightfield and that all channels have appropriate exposure settings. Choose the number of fields and time interval carefully, as switching between channels takes time (usually faster if all channels have the same gain settings).

Don't forget to change the experiment type in your experiment ID. The d variable produced from these experiments will contain 'fint', a matrix of summed fluorescent intensity values for each microcolony at each time-point (the microcolony is defined by the brightfield image). You almost always want to divide the fint value by the area value to obtain average fluorescence per pixel. If the experiment had multiple fluorescent channels, the fint matrix for each channel is horizontally concatenated (e.g., for two fluorescence channels and 10 timepoints, the fint matrix will have 20 columns, the first 10 representing the first channel and the following 10 representing the second channel).

Experiments with mixed genotypes in well (FC)

These experiments depend on the different genotypes being differentiated by fluorescence images taken at the end of the experiment. When setting up the experiment, choose the appropriate fluorescence channels in the lambda tab of the ND acquisition section of NIS elements, do not include brightfield. After setting up the tab, uncheck it (leaving only the Time and xy tabs checked). Instead of hitting 'run now', click 'Run Macro' under the 'Macro' tab (make sure the active macro is FC.mac). The macro works by first starting the experiment as it is defined, when it is finished, a new experiment will start that only includes the xy and lambda tabs of the ND acquisition section. There is currently a bug that does not allow specifying the name of the new experiment (maybe you can fix that), hence the second folder ends up as 001 (or similar). You will have to manually fix that by changing the name of the folder after the experiment finishes (no more images are taken). The name should match the first phase, with an FC ending instead of GR (NZ_150911_1_FC).

Choose the duration of the experiment thoughtfully; microcolonies should not be very overgrown when the fluorescent images are taken. The 'color' element of the d variable defines the different genotypes. Don't forget to change the experiment type in your experiment ID.

Experiments with two phases (AT)

When setting up these experiments, cells should be more dilute (5X10^3 cells/ml). The folder for the first experiment phase should have the regular GR ending; the second should have AT (e.g., NZ_150911_1_GR and NZ_150911_1_AT). The experiment should contain at least 166 fields in the whole plate. Be careful when removing and returning the plate. Make sure the treatment does not change the lighting conditions on the plate (like creating condensation), to ensure the microcolonies continue being tracked. The utility of the get.info R function for AT experiments is limited. Don't forget to change the experiment type in your experiment ID.