AutoCalibrationCurve: Determining the ratio between fluorescence intensity and number of molecules

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Epstein, A.E., S.V. Espinoza Sanchez, and T.D. Pollard. 2018. Phosphorylation of Arp2 is not essential for Arp2/3 complex activity in fission yeast. *bioRxiv*.

Li, T., H. Mary, M. Grosjean, J. Fouchard, S. Cabello, C. Reyes, Y. Gachet, and S. Tournier. 2017. MAARS: A novel high content acquisition software for the analysis of mitotic defects in fission yeast. *Mol Biol Cell*. 28.

This software was designed to automate the calibration curve, used to determine the ratio between a number of fluorescent molecules and their total fluorescence intensity under given imaging conditions. This ratio is determined by plotting the total intracellular fluorescence intensity for seven strains of *Schizosaccharomyces pombe* expressing GFP-tagged proteins against the number of molecules of each protein per cell, as previously determined by quantitative immunoblotting (Wu and Pollard, 2005). Note that once this ratio has been obtained, it can be used to quantify the number of GFP-tagged molecules in any region for any organism, as long as it has been imaged under the same conditions.

This software determines the Relevant *S. pombe* strains must be imaged according to a specific protocol. The cells are segmented using the Mitotic Analysis And Recording System (MAARS) (Li et al., 2017)

Installation: Drag the following files into the Fiji /plugins folder: Adjustable_Watershed.java, maars_bfSeg_1.0-SNAPSHOT.jar, and maars_lib 1.0-SNAPSHOT.jar.

In Fiji, go to Plugins→Macros→Install, and select "Epstein_CalibrationCurveMacro.ijm".

Sample data can be obtained from Google Drive:
Unprocessed: https://drive.google.com/open?id=1_cCgzw1Vcq55VcAWNwc4dTRjCs-3M31X
Processed: https://drive.google.com/open?id=1nsI8HmWnRydpAKkcgO8p0_UY1kePy-nU

Preparing cells and image acquisition

Grow the seven calibration curve *S. pombe* strains (Ain1, Myo2, Acp2, ArpC5, Arp2, Arp3, Fim1) along with untagged *S. pombe*. For more information, see the attached growth protocol (credit to Dr. Samantha E.R. Dundon). Image all strains, following the instructions below. Use of a confocal microscope is recommended.

AutoCalibrationCurve was designed for use with the µManager plugin (Stuurman et al., 2010), but can also be used with other imaging software.

When taking images, make sure that the file name of each image *starts* with the name of the relevant calibration curve protein (Ain1, Myo2, Acp2, ArpC5, Arp2, Arp3, Fim1, and the name of your untagged strain; in the sample data, FY528). These names may be case sensitive. The ending of the file name is not important.

For best results, it is recommended to obtain 50-60 cells per strain. This can require imaging multiple fields. For ease of naming these images, the ending of the file names is not important (i.e. it is fine to have Ain1_1, Ain1_2, Ain1_3, etc.)

In addition to the below images, you will need to take an uneven illumination correction image, which should be normalized so that the brightest pixel has a value of 1. You may want to average several of these together for best results.

3-channel imaging: If your microscope software is capable of turning all light sources off to take camera noise images without manual intervention, use this method. Image cells with the settings below. The order of the channels is important! A sample data set of this form has been provided for reference.

All channels: 21 Z-sections, 0.6 µm spacing. This is very important—segmentation may not work otherwise!

- Channel 1: Camera Noise. Light and laser should be off. Make sure the exposure time is the same as the exposure time you want to use for your GFP channel (Channel 3)!
- Channel 2: Bright field. Adjust the exposure time to maximize image quality; it does not need to match the exposure times of Channels 1 and 3.
- Channel 3: GFP. Make sure the exposure time matches that from Channel 1.
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2-channel imaging: If your microscope software cannot turn off all light sources at once (e.g. it is necessary to block the laser physically in order to take camera noise images), take a two-channel image stack using the same settings mentioned above, but with only channels 2 and 3. Separately, take a camera noise image (you may want to average several of these together).

Single-channel imaging: If your microscope software cannot take multi-channel image stacks, you will need to take fluorescence and bright-field image stacks separately. Make sure that your sample does not move in-between taking these images. In this case, your fluorescence images can have however many Z-slices you like; however, make sure to use 21 Z-slices and 0.6 µm for the bright field image stack. Fluorescence images should be named as described above. However, the filenames for bright-field image stacks should be of this format: "MAARS_imagename". For instance, the fluorescence images for Ain1 might be called "Ain1_1.tif", "Ain1_2.tif" and "Ain1_blahblahblah.tif"; in this case, the bright-field image stacks

should be called “MAARS_Ain1_1.tif”, “MAARS_Ain1_2.tif” and “MAARS_Ain1_blahblahblah.tif”.

Preparing to run the software: All of your image stacks, along with the camera noise correction (if necessary) and uneven illumination images, should be located either in a single parent folder, or within folders inside the parent folder (as in the sample data). The organization of these images should not be important, as long as you do not place them in folders called “Curve Images” or “MAARS Images”, as those names are reserved for use by the software. In addition, you cannot have more than two layers of folders (the parent folder and up to one subfolder), as the software will not look deeper than that. Also remember to check that your image file names correspond to the guidelines mentioned in the previous section.

The uneven illumination correction image should be called “UnevenIllumination.tif”. If performing 2-channel imaging, there should also be a camera noise image called “CameraNoise.tif”.

Single-channel imaging only (separate bright-field and fluorescence image stacks): Within the parent folder, create a subfolder called “MAARS Images”. Place all bright-field image stacks directly into this folder, with no subfolders in between. Fluorescence images can remain in the parent folder or within their own individual folders; it is not important.

Before running the software, make a backup of your data! The program may make changes to your files which are not reversible.

Running the software:

In Fiji, go to Plugins→Macros→Epstein_CalibrationCurveMacro (if it does not appear in the dropdown list, then click “Run” and then find the “Epstein_CalibrationCurveMacro.ijm” file).

Settings: When you run the software, a “Calibration curve settings” dialog box will appear. The first four checkboxes tell the software which of these four basic steps to run:

1. Split stacks, correct background noise and uneven illumination: Separates image stacks into camera noise, fluorescence and curve images and organizes them into subfolders. Corrects fluorescence image stacks for camera noise and uneven illumination and makes sum projections.
2. Segment images with MAARS: Uses the MAARS plugin (Li et al., 2017) to segment *S. pombe* cells. Creates one ROI for each cell and overlays the ROIs onto the sum projections of the fluorescence image stacks.
3. Sum fluorescence and plot curve: Determines the total fluorescence in each *S. pombe* cell. Finds the mean value for each strain (mean total intracellular fluorescence). Subtracts the mean total intracellular fluorescence of untagged *S. pombe* from the other strains. Plots the mean total intracellular fluorescence of each strain against the number of fluorescent molecules per cell, as previously determined by quantitative

immunoblotting (Wu and Pollard, 2005) (*Note: the x-values and horizontal error bars are stored in the code)*. Obtains and prints the slope.

4. Find total fluorescence for additional images (optional): This section can be used to find the mean total intracellular fluorescence for another strain, or for any sample/intracellular structure (not necessarily limited to *S. pombe*). Make sure that the "Curve Images" folder contains sum projections of the desired images and that all filenames for the same sample start with the same few characters, and that cells/regions of interest have been outlined in ImageJ and the resulting ROIs have been overlaid onto the image.

These functions are all somewhat independent of each other. For example, you can run Step 1 without running Steps 2 and 3, or run steps 1 and 2 without running step 3. In addition, if one step goes wrong (most often the segmentation in Step 2), you can try again without having to re-run step 1.

Other settings:

- Number of Z-slices to sum: 21 Z-slices was chosen for optimal segmentation, but normally fewer than 21 slices is sufficient to capture the entire cell—and using more will simply add out-of-focus light. This setting determines how many Z-slices of the fluorescence images are actually used. 15 or 17 slices is suggested. Note that Z-slices are taken from the center of the stack. For instance, if you input "15", the 15 central Z-slices of the fluorescence image stacks will be summed, and the three on the top and three on the bottom will be ignored.
- Filename path separating character: This may depend on your operating system. On Macs, it is a forward slash (/); on PCs, in theory it should be a backslash (\) but in practice the forward slash sometimes works while the backslash does not.
- Wild type name: Enter the name of your untagged strain (in my case FY528). This should be the same name that is at the beginning of the filenames of your untagged strain.
- Error type: switches between using standard deviation (recommended) and standard error for the vertical error bars of the calibration curve. Horizontal error bars will always use standard deviation.
- Calibration/target image type: Used if your calibration images and your target images (i.e. the images in which you want to measure number of molecules) have different bit depths. Note that this assumes 16-bit images are stored as integer arrays, and 32-bit images are stored as floating points with four decimal places (and that either both are unsigned or both are signed). If your calibration and target images have the same bit depth, then just make sure both settings are the same (e.g. if your calibration and target images are both 8-bit, you can make both the calibration and target image type 32 bit).
- Calibration/target exposure time: Use if your calibration images and target images were taken with different exposure times. The slope of the calibration curve will be divided by the calibration curve exposure time and multiplied by the target exposure time.

- Use batch mode? In batch mode, the software (during steps 1 and 3) will not open images and will run substantially faster. Occasionally batch mode can cause the program to crash; if this occurs, try selecting "No".

Section 1: Split stacks, correct background noise and uneven illumination: This section should run on its own, with no outside intervention. When it is finished, there should be two folders called "MAARS Images" and "Curve Images". Sum projection fluorescence images should be in "Curve Images" and bright-field stacks in "MAARS Images".

Section 2: Segment images with MAARS: If you have run this section before and segmented some images, a box will appear asking "Clear and redo existing segmentations"? If you click "Yes", then those images will be re-segmented; if you click "no", then only images that have not been segmented before will be segmented.

A bright-field image stack will appear, along with a dialog box that says "MAARS: bright-field segmentation". Click "Load" and then "Run". Wait a brief moment until a dialog box appears that says "Optimize (or not) segmentation of…". The box may be behind all other Fiji windows. Click OK.

Wait a moment; several images should rapidly appear and disappear. Then a new "MAARS: bright-field segmentation" box will pop up. Proceed as before: click "Load" and then "Run", wait for the "Optimize (or not) segmentation of…" box to appear, and click "OK". Repeat for every additional image.

Checking and correcting segmentation: Generally this is not necessary, but if you want to be extra careful you can curate the segmentations and eliminate any that are wrong (occasionally two cells will be circled as one, for example. If you want to check/edit segmentation for an image, then after running Step 2, go into the "Curve Images" folder and open it in Fiji. Go to Image→Overlay→To ROI Manager. Make sure the macro is not running while you do this. Click on any ROI in the manager you want and edit or delete it if needed. Then, select all ROIs in the ROI manager by clicking on the top one and shift-clicking on the bottom one, and go to Image→Overlay→From ROI Manager. Save and close the image.

For an example segmentation, see Epstein *et al* (2018), Fig. S4A.

Step 3: Sum fluorescence and plot curve: This step should, like Step 1, run on its own. At the end, it will generate a calibration curve, which it will save as "CalibrationCurveFinal.tif". It will also save the calibration intensities, standard deviations and number of cells per strain in the file "Calibration_intensities.txt". For an example curve, see Epstein *et al* (2018), Fig. S4B.

Step 4: Find total fluorescence for additional images (optional):

- In the first dialog box that appears, insert the prefixes for up to four additional samples/strains (i.e. the text that appears at the beginning of their movie files). If you have fewer, leave the extras as "N/A".

- In the second dialog box that appears, enter the slope of the calibration curve (see CalibrationCurveFinal.tif).
- A text file should appear with the results ("Calibration_intensities_all_movies.txt"). Strains you entered will be at the top of the table; other movies that you did not mention will be at the bottom.

Troubleshooting/FAQ

- *The program crashed in Step 2, possibly with a popup saying "There are no images open".* This is a tough one, it happens sometimes. Your best bet is to close all open images and try running Step 2 again. Sometimes you can only get through 2-3 images at a time. Click "No" when it asks if you want to "clear and redo existing segmentations".
- *The top of the Settings box does not appear!* I'm not sure why this happens, but it can be resolved by clicking within the missing area of the box.
- *I can't find the "Adjustable Watershed" box with the "OK" button!* On some computers, this box will appear behind the other ImageJ windows. Drag them aside and find it.
- *The segmentation didn't work well!* Make sure you are using the recommended spacing settings when acquiring your images. Also, make sure your images really are bright-field. They should look like the example image provided in the "Image Acquisition" section.
- *My microscope doesn't have a "bright-field" setting!* Sometimes an improperly adjusted differential-interference contrast (DIC) setup will function like bright-field imaging. You can try taking "DIC" images; if they look like the sample images provided in the "Image Acquisition" section, they may work.
- *I already took some images using a different acquisition protocol. How can I process them with this software?* Unfortunately, it is not currently possible to segment *S. pombe* cells reliably without the bright-field image stacks that the MAARS program requires. Therefore, you will have to segment these images manually as follows:
    - Perform step 1 to correct the images for uneven illumination and background noise. Make sure that the "UnevenIllumination.tif" and "CameraNoise.tif" files are present.
    - Segment each .tif file in "Curve Images" manually by using the Polygon tool in ImageJ to circle cells. Make each cell into an ROI.
    - Select all ROIs in the ROI manager and go to Image→Overlay→From ROI manager. Then save the .tif file.
    - Perform step 3 to obtain the calibration curve.
- *Does the software correct for photobleaching?* Currently, no; this may make the reported ratio slightly inaccurate. It is recommended to minimize photobleaching by shortening exposure time. You can also try decreasing the number of Z-sections, however this may worsen segmentation.
- *The software crashes with an error code during Step 1 or Step 3, or behaves weirdly in other ways*. Sometimes using batch mode causes the software to run too fast. Try turning off batch mode and running the software again.

References:

Li, T., H. Mary, M. Grosjean, J. Fouchard, S. Cabello, C. Reyes, Y. Gachet, and S. Tournier. 2017. MAARS: A novel high content acquisition software for the analysis of mitotic defects in fission yeast. *Mol Biol Cell*. 28.

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