ImageJ/FIJI Particle Analyser

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[1]In-house protocol

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1 Works for me This protocol is published without a DOI.

Eadewunm

ABSTRACT

ImageJ/FIJI Particle Analyzer

PROTOCOL CITATION

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46088

GUIDELINES

An example macro is included below:

```
run("Fit Circle to Image", "threshold=-195705");
run("Enlarge...", "enlarge=-0.05");
setBackgroundColor(4, 4, 4);
run("Clear Outside");
run("8-bit");
run("Subtract Background...", "rolling=50");
run("Auto Threshold", "method=MaxEntropy");
setOption("BlackBackground", false);
run("Make Binary");
run("Fill Holes");
run("Watershed");
run("Analyze Particles...", "size=0.0005-Infinity circularity=0.50-1.00 show=Outlines
display exclude clear summarize");
```

MATERIALS TEXT

Image plates
Photo doc machine

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DISCLAIMER: THIS WORK IS IN PROGRESS. IT IS FOR INFORMATIONAL PURPOSES ONLY; USE AT YOUR OWN

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BEFORE STARTING

Macros

- To create a macro, open FIJI and open any image of the type the macro is intended to quickly process and analyse.
- *Plugins > Macros > Record...*- This will begin recording all your actions into a script format that can later be run automatically.
- Process the image as you normally would (Steps 4-12).
- Name your macro and click *Create*. A new window will open, from here select *File > Save As* and choose a file location you will remember for this macro.
- To run an individual macro, simply open it via *Plugins > Macros... > Run...*and then select *Run > Run*. Alternatively, you can use *Run > Run Selected* to only execute the lines of the script you have selected - this can be useful for troubleshooting.

Batching

Batching allows you to process all files in a particle folder in the same way via a macro and then consolidates all analysis data in a summary table.

- Open FIJI, then select *Process > Batch > Macro* to open the batching window. Select the input and output folders. FIJI will not destroy the original images.
- Open your macro via *Plugins > Macros > Edit...* and copy the commands into the Batch Process window. Select *Process*.
- The summary window will contain the averaged data for each image. You can add or remove metrics via *Results > Set Measurements...*
- If any images return an error, try running them manually. It's likely the auto threshold failed and you should try a different threshold method for that particular image

Caution:

Batching can result in inaccurate data if images are not sufficiently similar. Please examine the final data carefully for outliers (especially particle *area* and *circularity*), and run these images manually if necessary.

steps

1 Image plates with photo doc machine. Choose plates where colonies are well spread, and not clumped - especially against the sides, where the image will be cropped.

Overcrowded plates have inherently smaller colonies. Ensure that the images are all taken at the same distance/zoom.

2 Download FIJI from "https://imagej.net/Fiji" if you don't already have the program. This is an open-source distribution of ImageJ with many tools/plugins built-in.

3 Open FIJI, and open your image (*File > Open... > [Select yourFIle] > Open*)

4 First, we must select your image and crop out most of the non-particle background (in this case, the walls of the plate). You have two options here:

4.1 **Recommended:** *Edit > Selection > Fit Circle to Image*. This will create a circular selection area that contains the majority of your colonies. It may not be perfectly centered on the plate, but generally, it's good enough to get a representative selection of colonies. The default threshold is usually fine, but you may test other values. Optionally, you can also use *Edit > Selection > Enlarge* and input a value to grow or shrink the selection (a negative value to shrink) if you think this better represents the colonies.

4.2 Use the oval select tool to manually draw out the region of the plate containing the colonies, cropping out the sides of the plate. This option is not suited for batching but may help resolve anomalous data from batching.

5 *Edit > Clear Outside* to remove most of the background.

We do not want to use the crop tool here, because this changes the resolution of the image, which influences the particle analyzer - by using a set of images all the same resolution, we can properly compare colony size between images.

6 *Image > Type > 8-Bit* - This allows the remaining commands to work properly.

7 *Process > Subtract Background* - This seems to make the thresholding step more reliable but may be optional. Try with and without, and see what works better.

8 The thresholding step has a large impact on the final analysis, and again you have two options here:

8.1 Manually: *Image > Adjust > Threshold* - From here you must play with the settings until you feel you have filtered out any non-colony particles but not caused any colonies to shrink/change shape. The ideal setting will not be consistent between plates due to differences in image brightness. This option is not suited for batch processing.

8.2 **Recommended:** *Image > Adjust > Auto Threshold* - You can test it with Try All method first to see the difference between the options (the names will be below each image in a tiny font) - MaxEntropy seems to be a decent middle ground that removes erroneous particles while preserving colony shape.

9 *Process > Binary > Make Binary* - Required for steps 10 and 11

10 *Process > Binary > Fill Holes* - This will fill in holes in the particles, which may appear if colonies are particularly shiny.

11 *Process > Binary > Watershed* - This draws a one-pixel wide line where the software detects two separate particles/colonies are merging. If you aren't confident in the software or zero colonies are touching, you may exclude

this step.

12 *Analyze > Analyze Particles* - There are a few settings potentially worth modifying from their default options :

12.1 Size - This setting is not intuitive, as the units (squared inches/pixels) don't easily correspond to the real world colony size. I recommend running this first default and using the “Area” analysis output to get a reasonable idea of how small your smallest colonies are - from here you can set more exact size boundaries.

12.2 Circularity - This essential setting defines how circular our particles must be to get included in the analysis. Typically 0.60 - 1.00 is a safe range to exclude all remaining background.

12.3 Checkboxes: Display Results, Exclude on edges, Clear results, Summarize

13 Check the resulting image to ensure only colonies were analyzed. Particles will be numbered on the new image, so you may reference them against the tabulated data. A summary window will also be generated containing the averaged data of each particle analysis you run. The tables can be copied directly to Excel.