

SAMA

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What is SAMA?

Software for Automated Morphological Analysis or SAMA is a plugin that is operated via the imaging platform, FIJI. Although the analysis is done using R, the user-friendly interface of SAMA integrates imaging and analysis in FIJI.

SAMA aims to describe morphological aspects of many objects observed in 3d, in particular but not exclusively epithelial structures in 3d culture.

SAMA is released under the [Gnu Public License](#).

[Download SAMA](#) (compatible with windows, mac OSX and Linux)

[Download example dataset](#)

[Source code](#) for the Rbind plugin

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Website: <http://montevil.theobio.org/en/content/sama>

Table des matières

SAMA.....	1
What is SAMA?.....	1
Installation of SAMA.....	2
Dependencies.....	2
Programs required:.....	2
FIJI Plugins required:.....	2
Optional FIJI Plugins:.....	2
Installation of Sama.....	2
How to use SAMA?.....	3
Overview.....	3
Example dataset.....	3
Folders.....	3
Type of images to be analyzed.....	4
SAMA-images.....	4
Tier 1:.....	4
Tier 2:.....	5
Tier 3:.....	5
SAMA-analyze.....	5
Preliminary: setConditions.....	5

SAMA-analyze.....	6
Graph descriptions.....	7
Batch analyze treatment effect:.....	7
Analyze reproducibility:.....	8
Quantities.....	8
Basic morphometrics.....	8
Thickness measures (from bonej).....	9
Lumen analysis.....	10
Branching analysis.....	10
PCA analysis.....	10
Troubleshooting.....	10
Check list.....	10

Installation of SAMA

Dependencies

Programs required:

If they are not already installed, install FIJI and R.

- FIJI - <http://fiji.sc/Installation>
- R - <http://cran.r-project.org/>

WARNING for Microsoft Windows users: it is better to install R and Fiji outside Program Files to avoid problems of permissions.

FIJI Plugins required:

- 3D-ROI Manager http://imagejdocu.tudor.lu/doku.php?id=plugin:stacks:3d_ij_suite:start

As of now, Sama is compatible with version 3.5 and later.

This plugin can be installed by going to *help> update fiji*. And in the new window going to *Manage update sites* and activating the 3D ImageJ Suite.

Optional FIJI Plugins:

- BoneJ <http://bonej.org/#install>
- Puredenoise <http://bigwww.epfl.ch/algorithms/denoise/>

Installation of Sama

- Download SAMA:
<http://montevil.theobio.org/sites/montevil.theobio.org/files/sama/Sama.zip>
- Extract Sama.zip and copy the SAMA folder in the plugins folder of FIJI.
- (Re)start FIJI.

- In FIJI, run *Plugin>Sama>install or update R component*. Then select install in the dialog and hit ok. This process will install the required R packages, including the R SAMA components.
- Windows only: SAMA will ask you for the path to the R executable. It usually is in /root folder/R/R-x.x.x/bin/. In case of a mistake, it is possible to change this path in SAMA > updateR. Let us recall that R should not be installed in Program files because of issues with permissions.

How to use SAMA?

Overview

In imageJ, go to Plugin > Sama. There are 2 main components available – **SAMA-images** and **SAMA-analyze**.

Sama-images performs the image analysis part, and produce quantitative data for every image stack. The processing is modular, for example, the user may want to do only Tier 1 with a few options, and compute other options or tiers later.

Sama-analyze gathers the output of sama-images and enables to represent, analyze and export it. The analysis includes both treatment effects and the comparison between the different replicates to check the robustness of the results.

Example dataset

The simplest way to first use SAMA and check your installation is to download the sample set of images available [here](#) and follow the steps below.

- Unzip Example_SAMA.zip.
- In Plugin > Sama, run SAMA-images and uncheck the lumen options in tiers 1, 2 and 3. Only Basic morphometrics and Branching measurement should be checked.
- In the "choose source directory" dialog select the *denoised* folder in the *example_SAMA* folder.
- Wait for the processing to finish.
- In Plugin > Sama, run SAMA-Analyze. Uncheck Lumen and hit ok.
- Enjoy the pdfs produced in the folder SAMA-analyze output. The main results are in the coarseplot pdfs (the different pdfs correspond to different filters on the structures, see below).

Folders

Important: create a folder where the analysis of the dataset will take place, and put all the source image files in a subfolder of this main folder.

SAMA will create other subfolders as follows:

- **Sama-object map** (image stacks where all structure have a color corresponding to its label),
- **Sama-prelumen images** (the images preprocessed for lumen analysis)
- **Sama-tree analysis** (the skeletonized images)
- **Sama-Image data** (the various raw output sheets)
- **Sama-analyze output** (the pdf and reported sheets).

The folders Sama-object map and Sama-tree analysis can be used to check the validity of the image analysis.

Type of images to be analyzed

SAMA supports any file format supported directly by Fiji.

There is no intrinsic limit to the size of the original image but the memory available to Fiji. Note that you can [change the later](#). Also in case of tiling, the stitched image should be used.

SAMA takes into account the scales that can be set in Image >properties. In particular:

- Relevant results will follow these units.
- Thickness analysis requires that the scale is the same in the three direction to provide relevant results.

SAMA is designed to analyze a population of structures and not a few structures at high magnification. In the later case, the patterns generated by cells may disturb the result.

SAMA-images

Important:

- *Computation may take a long time depending on the number of images, their size and the computer used. SAMA-images is designed to separate automatic parts (tier 1 and 3) which require a substancial amount of computing time from the interactive part which is fast to avoid waiting time.*

Tier 1:

- Basic morphometrics: 3D structures are counted and parameters that define shape, volume and position are measured. These data are saved in an excel spreadsheet in SAMA-image data. The threshold is set by default to 30.
- Additional filtering: 3 choices: "none", "Enhance boundaries" which consolidates the luminosity boundaries in order to have more stable outcomes, less dependant on the threshold and "Split neighbors" which tries to separate structure that are touching yet distinct (might generate artifacts).

- The filters by size are in unit³ (for example μm^3 , pixel³=voxel, ...). The default of 500 is reasonable in voxels.
- Check threshold for basic morphometrics enables the user to choose an image. The image will be preprocessed and the user will be asked to choose a valid threshold.
- Compute convex hull: This is an option for the basic morphometrics. By default this has been unchecked. While this option gives complementary parameters about the shape of the structures, we rely on a function of 3D suite which is not completely stable and may abort the process.
- Lumen measurement: In this step, the images are processed and stored in SAMA-prelumen analysis for further analysis in Tiers 2 and 3.
- Branching measurement: In this step, the images are processed and branching is analyzed by the 3d Skeleton plugin.
- Thickness measurement: By default this has been unchecked because it requires BoneJ. Similar to Convex hull option, this option gives useful parameters about the branching of the structures but is memory-consuming. See: Hildebrand T, Rüeggsegger P (1997) A new method for the model-independent assessment of thickness in three-dimensional images. J. Microsc. 185: 67-75. doi:[10.1046/j.1365-2818.1997.1340694.x](https://doi.org/10.1046/j.1365-2818.1997.1340694.x).

Tier 2:

The user manually sets the threshold for lumen analysis after the lumen analysis part of tier 1 has been performed. In this step, the user determines a threshold value for lumen in the images. If the user checks this option, the program will first run through Tier 1 and then pause at Tier 2. The first image in SAMA-Prelumen analysis will be opened and a user interface asks the user to choose a threshold. Once the user selects the optimum threshold, the program will continue to open the other images in the folder one at a time for the same action.

The user should aim for a threshold where the boundary of the lumen are above the threshold (in red) and the lumen is below (i.e. dark).

Tier 3:

The final lumen measurement is done. Once the user has defined all the threshold of the images in Tier 2, this step automatically carries out the final analysis of the lumen parameters.

SAMA-analyze

This plugin analyzes all the data generated from images processed in SAMA-images.

Preliminary: setConditions

Important: Before starting SAMA-analyze, it is **MANDATORY** to save a file “conditions.csv” in “SAMA-image data” folder describing every image stack. Users should either start from the template (conditions.csv) provided [here](#) or use the macro **setconditions** which will create automatically the id of every images and guide the user to input the other required informations.

To edit conditions.csv, Excel or Open Office can be used and the file should be saved in the .csv format (coma separated value). The different columns have to be separated by **commas**. It is also possible to do the changes with a text editor (wordpad).

The conditions.csv file has 4 columns or more:

1. **name**: corresponds to the filename of the image (which has to be a unique code).
2. **Plate**: This is the experiment # and is used to analyze reproducibility of experiments.
3. **Well**: This is the replicate # within each experiment to analyze reproducibility within each experiment.
4. All the remaining columns will be considered a **treatment** column and at least one is needed. Those are the name/condition of the experimental treatments and will be used in the data graphs. Several columns can be used If needed (for example one column for the cell type and one for the hormones).

SAMA-analyze

- **Were these parameters analyzed?**

Choose the parameters that you have processed with Sama-images. Note that basic morphometrics is always performed and does not require any of these options.

- **Data handling**

- Put data together: **Important**: this part has to be run once after any changes in sama-images for them to be taken into account.
- Export sheet: This option produces a sheet with the data of all structure in “Sama-analyze output”, called all-export.csv. This sheet is intended for use with your favorite statistical analysis / plotting software.

- **Data analysis**

- Batch analyze treatment effect: produces pdfs named to summarize the data. Several pdfs are produced: by filtering the acceptable structures with:

no filter (with the prefix size0) / size above size threshold (see below) / above 2*size threshold / above 4*size threshold / quality above quality threshold and size above size threshold / structures with lumen and size above size threshold.

Corresponding sheets are also produced if the user wants to produce alternative plots with another software.

- Analyze reproducibility: produces pdf to check the consistency of the data between experiments and between replicates. Note that if there is only one experiment performed, this part will have limited results.

See below for a description of the graphs.

- **Choose a subset of the conditions?**

This option will lead to another dialog in order to select the condition to be shown on graphs. The user has two ways to achieve this:

The user can use a filtering system on the treatments: for example choosing Only Treatments WITH criterium: E2, will show only the data which have “E2” in their description.

Alternatively, there is a manual choice option which enables to pick the conditions to be plotted manually, and add a custom string to the plots obtained for convenience.

- **Options for analysis**

- Quality threshold: see Batch analyze treatment effect.
- Size threshold: see Batch analyze treatment effect. The threshold is for the volume and is in the same unit than the scale of the image (typically in μm which leads then to μm^3).

- **Options for graphs**

- Choose labels in PCA. The possible option are: *none*, *treatment*, *treatment and plate*, *plate*, *filename*. This option aim to workaround the label overcrowding when there are too many points by choosing the information displayed.
- Color graphs: this option produces color graph, when unchecked the graphs are black and white.

Graph descriptions

Batch analyze treatment effect:

Let us recall that several pdfs are produced: by filtering the acceptable structures with:

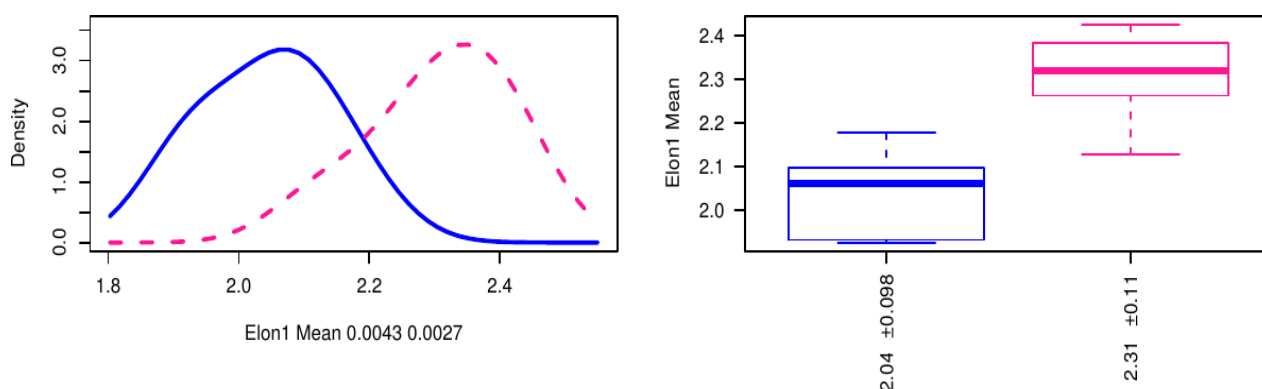
*no filter (with the prefix size0) / size above size threshold (see below) / above 2*size threshold / above 4*size threshold / quality above quality threshold and size above size threshold / structures with lumen and size above size threshold.*

The size threshold is for the volume and is in the same unit than the scale of the image (typically in μm which leads then to μm^3). It is set in SAMA analyze with a default value of 500.

For every image, there are several structures which can be summarized by taking either the mean

value for each quantity, the coefficient of variation (standard deviation/ mean), standard deviation or the median. Every pdf uses all these methods successively.

For every quantity, the population of replicate is plotted as an estimation of a distribution and as a boxplot. Under the first graph, the quantity plotted is given, here *Elon1*, then the function in order to go from all the structures in one replicate to one point (here the mean), then the p-value is given for an ANOVA and for the Wilcoxon rank test (when there is only two treatments). Under the boxplot, the average and standard deviation of every Treatment is given.



The graph include also a PCA analysis, based on the *factominR* package for R. This include a plot of the similarity between the two first dimension of the PCA, and aof the original quantity, a scatterplot of the replicates on these dimensions and an automatic clustering based on the 4 first dimensions of the PCA.

Also, the 5 first dimensions are displayed like the original quantities as *dim.1* to *dim.5*.

Analyze reproducibility:

The reproducibility analysis produces similar plot than above but where the experiment number takes the place of the treatment in the analysis and instead of the mean for each replicate, all structures are taken into account..

Moreover, a pdf called *distribution* plots the distribution of the structures for every replicate, in order to check their shape and their stability. Comparisons are performed between replicates in the same conditions. P-values are obtained by ANOVA, and check the significance of the plate (experiment) number and of the well (replicate) number. Note that unlike above the comparison is performed at the level of the whole population of structures, not at the level of the replicates, which means that small differences will be significant.

Quantities

Summary of the relevant quantities, by order of appearance in the pdf produced.

Basic morphometrics

Note: axis refers to the axis when fitting the structure to an [ellipsoid](#).

- **"Quality"** Elon1-Elon2 without lumen analysis. $\text{Elon1-Elon2} + 5 \times \text{lumenosity}$ with lumen analysis.

Proportions of the structure:

- **"Elon1"** elongation: length of the main axis of the structure/length of the second axis of the structure
- **"logElon1"** Logarithm of $\text{Elon1} - 1$.
- **"Elon2"** flatness: length of the second axis of the structure/length of the third axis of the structure
- **"logElon2"** Logarithm of $\text{Elon2} - 1$.

Sizes

- **"logvol"** Logarithm of the volume of the structure, in units (ex: μm^3).
- **"logEll_MajRad"** logarithm of the length of the largest axis of the structure.
- **"logFerret"** logarithm of the largest distance between two points of the structure.

Comparison with other shapes

- **"Sphericity"** ratio of volume to area set to 1 for spheres (and smaller for any other shape). That is $S^3 = 36 \pi V^2 / A^3$.
- **"RatioVolEllipsoid"** volume of the structure / volume of the embedding ellipsoid. Describes how convoluted the structure is that is to say how different from an ellipsoid it is.
- **"logRatioVolEllipsoid"** Logarithm of $1 - \text{RatioVolEllipsoid}$.
- **"RatioVolbox"** Volume of the structure/ Volume of its bounding box.
- **"logRatioVolbox"** Logarithm $1 - \text{RatioVolbox}$.
- **"logMoment1"** **"logMoment2"** **"logMoment3"** logarithms of the three moments invariant by rotation and translation discussed in Sadjadi, Firooz A.; Hall, Ernest L., "Three-Dimensional Moment Invariants," in *Pattern Analysis and Machine Intelligence, IEEE Transactions on*, vol.PAMI-2, no.2, pp.127-136, March 1980 doi: 10.1109/TPAMI.1980.4766990
<http://ieeexplore.ieee.org/stamp/stamp.jsp?tp=&arnumber=4766990&isnumber=4766983>
- **"logMoment4"**, **"logMoment5"** Moments also invariant by scaling.

Thickness measures (from bonej)

Thickness is a quantity that can be computed for every point inside a structure: it is the radius of the largest ball which contains this point and that fit inside the structure.

- **"Thickness_Mean"** Each structure is represented by its mean thickness.
- **"Thickness_SD"** Each structure is represented by the standard deviation of its thickness.

- **"Thickness_Max"** Each structure is represented by the thickness of its thickest point.
- **"logThickness_Mean", "logThickness_SD", "logThickness_Max"** Logarithm of the above.
- **"rThickness_Mean"** reduced thickness: Mean Thickness / volume of the structure (power 1/3, to compare a length with a length)
- **"rThickness_SD"** same for standard deviation.
- **"rThickness_Max"** same for maximum.
- **"rbThickness_SD"** standard deviation / mean
- **"rbThickness_Max"** max/ mean

Lumen analysis

- **"lumenosity"** volume of lumen / volume of the structure
- **"lumen"** 1 if there is a lumen 0 otherwise. (when the mean is applied, it is the proportion of structures with lumena).

Branching analysis

- **"complexity"** logarithm of the number of branches of the skeleton.
- **"complexity2"** 0 if there is one branch and 1 if there is 2 branches or more.
- **"complexity3"** logarithm of the cumulative length of branches.
- **"complexity4"** "entropy" of the branches (sum -p log(p) with p=length of the branch/ cumulative length of branches).

PCA analysis

- **"Dim.1" "Dim.2" "Dim.3" "Dim.4" "Dim.5"** The first 5 dimension from pca analysis. The variable taken into account depend on the analysis options (with or without lumen analysis, branching analysis, etc.).

Troubleshooting

Check list

- The folder with original images contains only the original images.
- Conditions.csv is in the *SAMA-images data* folder, and the columns are separated by commas.
- RoiManager3D 3.52 is not currently compatible with Java versions newer than version 6 on mac. While a fix is planned, currently, this incompatibility will stop SAMA from running. To work around this issue, delete JavaAppletPlugin.plugin from your applications folder and then install the legacy Java 6 runtime for OS X (https://support.apple.com/kb/DL1572?locale=en_US) and install.