User manual

MSSegregation

Macro package for Fiji-ImageJ

ver 1.48

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Software and hardware requirements:

All MSSegregation macros run in Fiji-ImageJ application. Installation of Fiji-Image application before using MSSegregation macros is required (for details see the step-by-step guide of individual macros). The macros have been tested in Windows7/10, 64-bit, Fiji-ImageJ 1.53c

The MSSegregation package was run on two different notebook computers and desktop computers (8Gb RAM, 64-bit Intel/AMD processors). We believe typical modern desktop computer can successfully run our macros. However, handling large image sets requires more powerful computer.

Availability

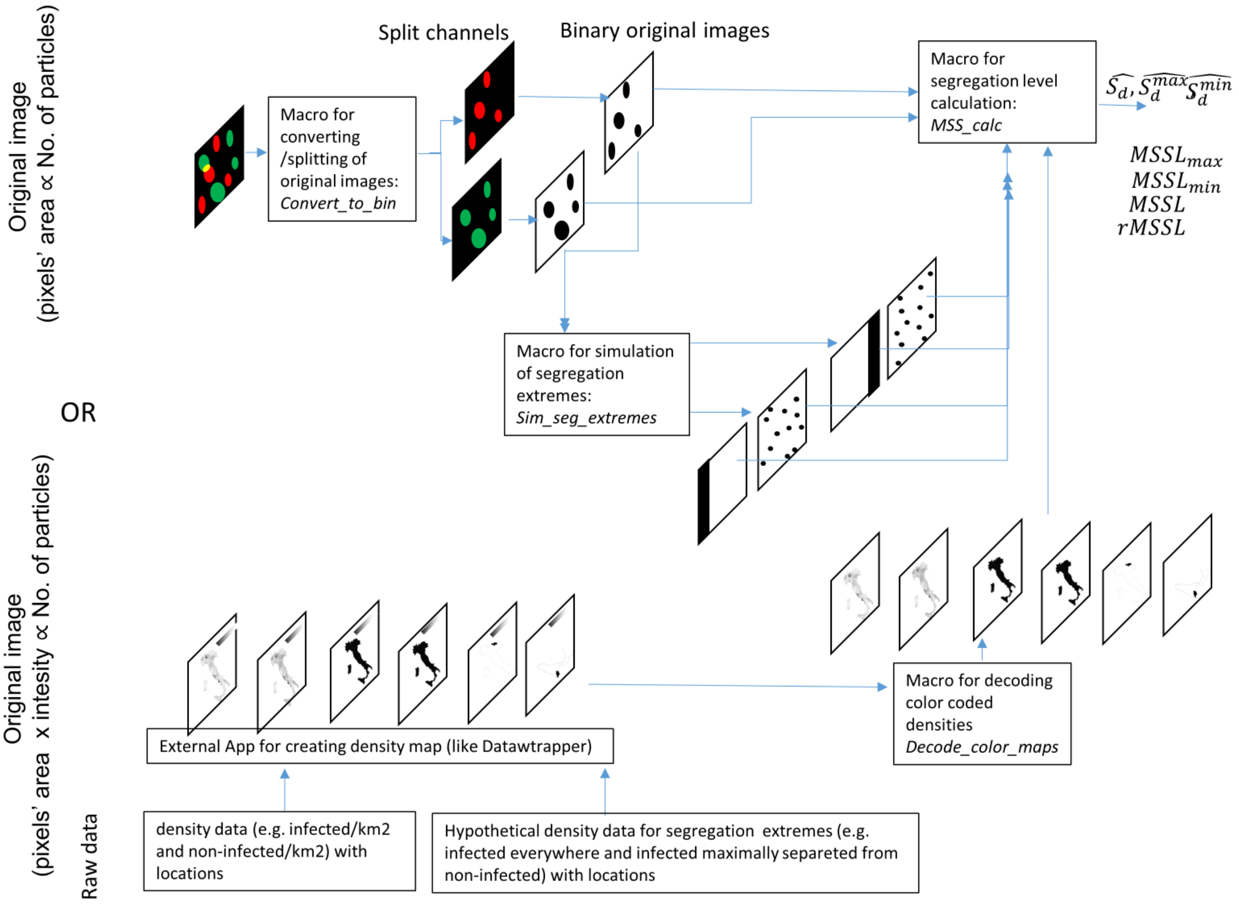
The ImageJ distribution package FIJI is freely available online: https://imagej.net/Fiji

The MSSegregation package is freely available at https://github.com/IztokD/MSSegregation-package

Package: MSSegregation

A brief overview

The purpose of MSSegregation package is to perform segregation analysis in digital images of two component mixtures (mixtures of two types of particles, particles and background…). Two types of digital images can be analysed, images, where particle quantity is proportional to the number of pixels that they occupy in an image (like the number of individual bacteria in microscopy image), or images, where particle quantity is proportional to the number of pixels and to the pixel intensity (like in density maps). MSSegregation package consists of four scripts that can be run as macros in Fiji (ImageJ) environment. The macro *Convert_to_bin_ver1.3.ijm* is used to split input images depicting two components into two separate images, where each image represent only one component. The macro *Sim_seg_extremes_ver1.3.ijm* simulates and depicts maximal and minimal segregation of two components system depicted in input digital images. Based on digital images of individual components, their maximal segregation and minimal segregation images, the Macro *MSS_calc _ver1.3.ijm* can perform spatial scale segregation analysis. Macro *Decode_color_maps_ver1.3.ijm* can be used to decode colour coded densities in density maps (e.g. in choropleth maps) to obtain images where pixel intensity value is proportional to the number of particles at particular pixel location. These images can then be analysed by *MSS_calc _ver1.3.ijm.* The workflow of multiscale spatial segregation analysis of digital images is shown in the next page. For more details about usage, please refer to the individual descriptions of macros.


Original image
(pixels' area ∝ No. of particles)
Split channels
Binary original images
Macro for converting /splitting of original images: *Convert_to_bin*
Macro for segregation level calculation: *MSS_calc*
$\widehat{S_d}, \widehat{S_d^{max}} \widehat{S_d^{min}}$
$MSSL_{max}$
$MSSL_{min}$
$MSSL$
$rMSSL$
Macro for simulation of segregation extremes: *Sim_seg_extremes*
OR
Original image
(pixels' area x intesity ∝ No. of particles)
External App for creating density map (like Datawtrapper)
Macro for decoding color coded densities *Decode_color_maps*
Raw data
density data (e.g. infected/km2 and non-infected/km2) with locations
Hypothetical density data for segregation extremes (e.g. infected everywhere and infected maximally separeted from non-infected) with locations

Workflow of multiscale spatial segregation analysis of digital images. The original image containing two types of particles (for example, microscopy image displaying red and green bacteria) is split into single type particle images, following conversion into binary images (i.e. black & white). Binary original images serve as input for macro that generates maximum and minimum segregation images for each type of the particle. In such images the number of displayed particles is typically proportional to the area occupied by the pixels representing them. From binary original images the segregation level (*Sd^*) as a function of dimension (*d*) of field of view (FOV) is calculated; maximum and minimum segregation images are used for calculation of maximum and minimum segregation level (*Sdmax^, Sdmin^*). Finally, multiscale spatial segregation levels (*MSSL*), relative multiscale spatial segregation level (*rMSSL*) are calculated from segregation levels. The number of particles can be represented in an image also by the colour (pixel) intensity, as is typical for density maps that can be created from raw data by external app (e.g. https://www.datawrapper.de/). The original values of density in such images must be decoded from colours before spatial segregation analysis can be performed.

Macro 1: *Convert_to_bin_ver1.3.ijm*

Description

This script runs as a macro in Fiji (ImageJ) environment. Its purpose is to split and convert a digital image that depicts a mixture of two components, (two types of particles, particles and background…, called *ch1*, *ch2* particles) into two images in a 8-bit binary format (black and white), where each image represent one type of the particle. The separation is done via user assisted thresholding of pixel values. These images can serve as input for Multiscale spatial segregation analysis implemented in script *MSS_calc_ver1.3* and in *Sim_seg_extremes_ver1.3* for more details refer to part of this manual corresponding to these macros). The stacks of images are supported by the macro.

Step by step guide

1. If you do not already have Fiji, download and install it from: https://imagej.net/software/fiji/downloads
2. Run Fiji (ImageJ-win64.exe file, if you are running 64-bit version).
   If asked to make an update, you can choose »Remind me later«, the update is usually not needed to successfully run *Convert_to_bin_ver1.3.ijm*

A. Installation

3. In Windows explorer (or equivalent File browser) go to the folder where you have installed Fiji and find plugins folder within Fiji folder. In our case:
   C:\Users\iztokd\Downloads\fiji-win64\Fiji.app\plugins
   Create folder \MSSegregation within plugins folder

   In running Fiji app press Ctrl+shift+M (=Install Macro) and choose a file *Convert_to_bin_ver1.3.ijm* .

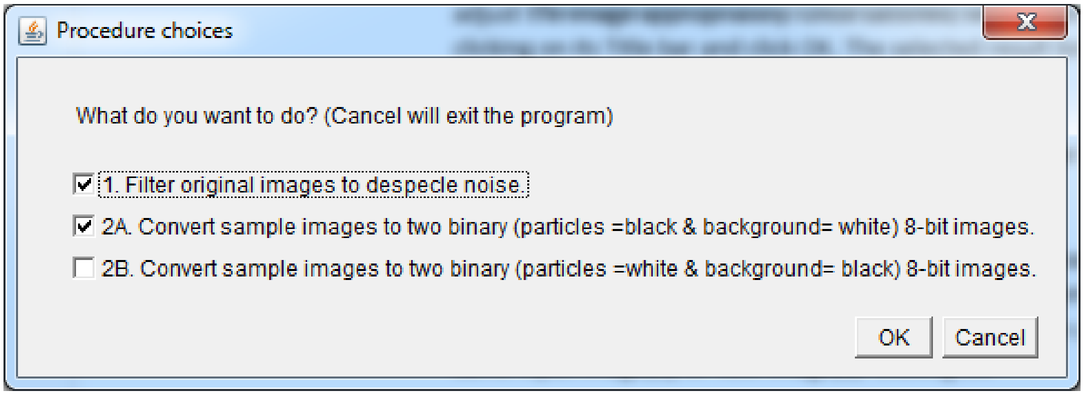
   Save it to directory \MSSegregation, created above.
   Close and restart Fiji app. If asked to save LUTMenuTool.ijm, just say "No".

4. Now you can run the macro from the Menu Bar: Plugins> MSSegregation>Convert_to_bin_ver1.3.

B. Direct run of the Macro

3. Open Windows explorer and "drag and drop" the file *Convert_to_bin_ver1.3.ijm* to the running Fiji application.
4. New window with displaying the code of *Convert_to_bin_ver1.ijm* will open (script editor) and you can run it by pressing Ctrl+R or by choosing Run>Run from menu of the script editor.

5. First you will be asked, What do you want to do?



What do you want to do? (Cancel will exit the program)

☑ 1. Filter original images to despecle noise.

☑ 2A. Convert sample images to two binary (particles =black & background= white) 8-bit images.

□ 2B. Convert sample images to two binary (particles =white & background= black) 8-bit images.

Option 1:
By default the filtering of original (input images) of pixel noise is selected. Usually this improves the output.

Option 2:
Also, because the main purpose of this macro is to obtain two binary images (8-bit), where on first image the particles of one type are shown (with bit value of 255) and on the second image the particles of other type are shown (with bit value of 255) the option 2 is selected by default. The background or anything which is not a particle of the particular type shown in the image will have bit value of 0. By (ImageJ) default the particles in these 8-bit images are displayed in black colour (option 2A), but you can also choose to display them in white (option 2B).

6. After you decided what you want to do, press "OK". Now you will be asked to choose a directory to save the output binary images. Select the directory and press "Select".

7. Now the menu "Image type choice" should appear:

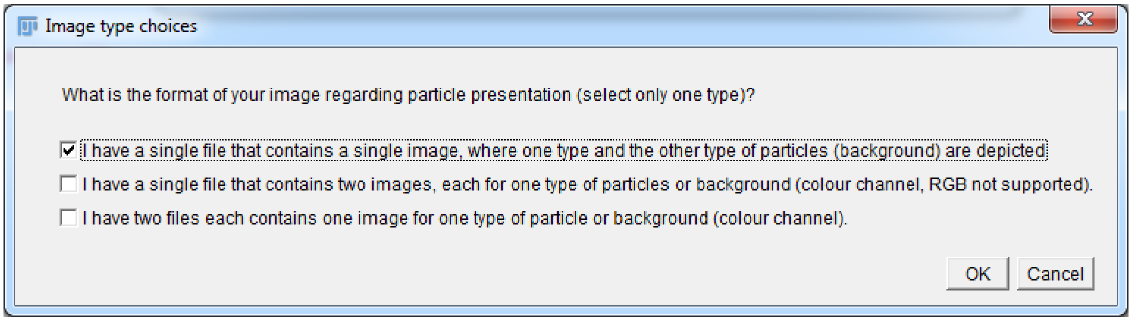


Image type choices

What is the format of your image regarding particle presentation (select only one type)?

☑ I have a single file that contains a single image, where one type and the other type of particles (background) are depicted

☐ I have a single file that contains two images, each for one type of particles or background (colour channel, RGB not supported).

☐ I have two files each contains one image for one type of particle or background (colour channel).

Your input image(s) representing the mixture of two components (particles) can be of various formats:

Option 1:

user can have one file in which one digital image is decoded where both type of the particles are depicted (i.e. singe file, single image). As the result of the opening procedure, two identical images will be displayed (duplicates), if the image is not RGB. In case of an RGB image, the original image will be split to three grayscale images (red, green, blue).

Option 2:

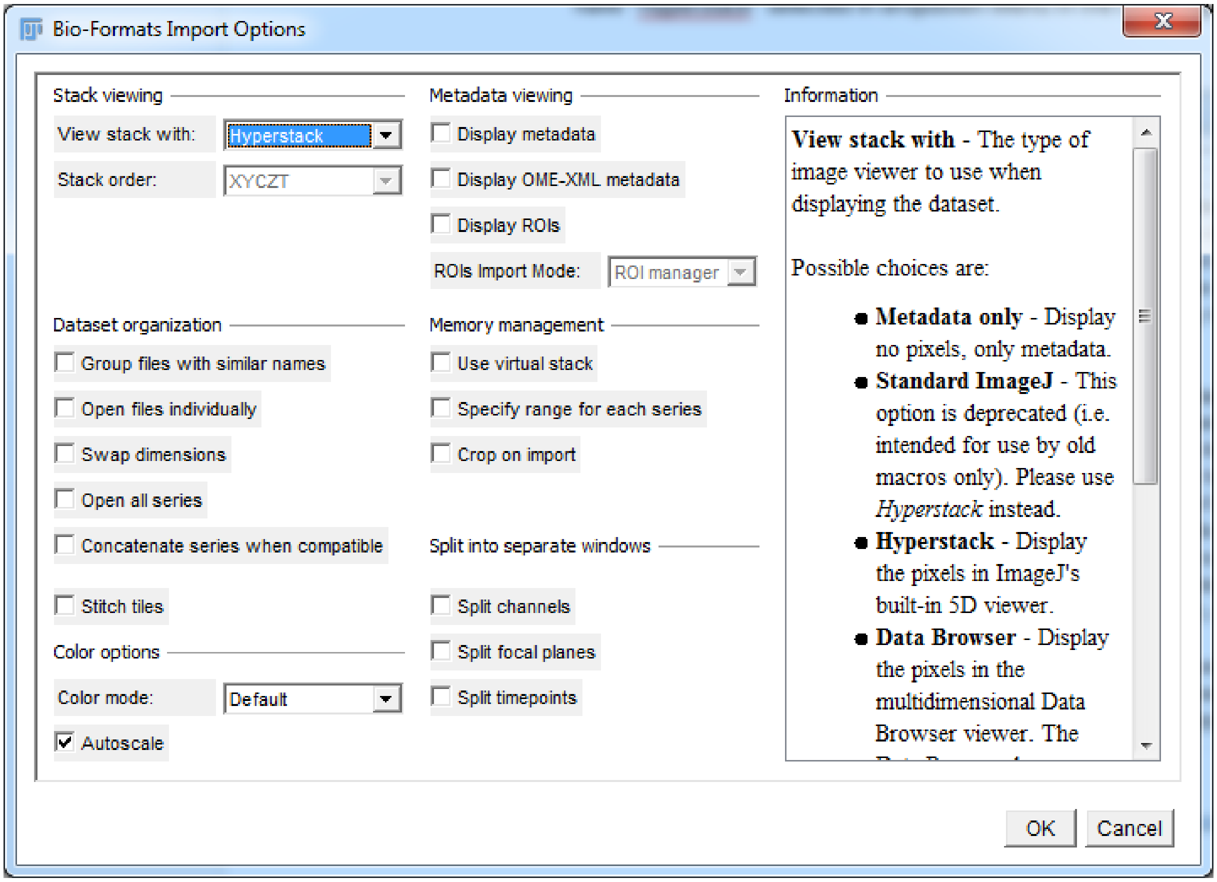
user can have one file in which two digital images are decoded, each for one type of the particles (i.e. singe file, two images). As the result of the opening procedure, two images will be displayed.

Option 3:

as last, you can have two files that each decode one image representing one type of the particle. As the result of the opening procedure, two images will be displayed.

In short, the user needs to know, which of the above holds for his input image and make the corresponding choice in the displayed menu and then click “OK”.

8. In next step select the corresponding image file and click “Open”. Note that varies file formats are supported by ImageJ (tif, jpg…) and many more in Fiji through the plug-in Bio-formats that automatically starts, when file of particular format is to be opened (like in the case of Zeiss .czi format). If the Bio-formats menu pops up, you should not thick anything (you can leave Autoscale), but you need to have “Hyperstack” selected in dropdown Menu in the upper left corner. Once you click “OK” the next Bio-Formats window may appear, offering you a selection of the size of the images that are going to be opened. By default, choose ONLY the biggest resolution series.



Bio-Formats Import Options

Stack viewing

View stack with: Hyperstack

Stack order: XYCZT

Dataset organization

□ Group files with similar names

□ Open files individually

□ Swap dimensions

□ Open all series

□ Concatenate series when compatible

□ Stitch tiles

Color options

Color mode: Default

☑ Autoscale

Metadata viewing

□ Display metadata

□ Display OME-XML metadata

□ Display ROIs

ROIs Import Mode: ROI manager

Memory management

□ Use virtual stack

□ Specify range for each series

□ Crop on import

Split into separate windows

□ Split channels

□ Split focal planes

□ Split timepoints

Information

View stack with - The type of image viewer to use when displaying the dataset.

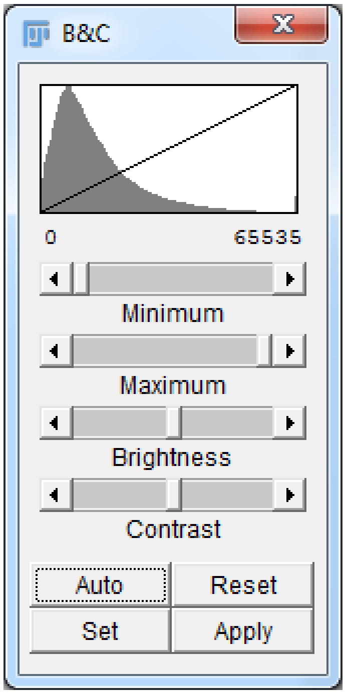
Possible choices are:

- **Metadata only** - Display no pixels, only metadata.
- **Standard ImageJ** - This option is deprecated (i.e. intended for use by old macros only). Please use *Hyperstack* instead.
- **Hyperstack** - Display the pixels in ImageJ's built-in 5D viewer.
- **Data Browser** - Display the pixels in the multidimensional Data Browser viewer. The

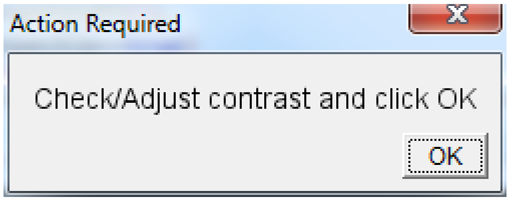
9. In the case the user has opened an RGB image (*Option 1 in step 7*) three images will be displayed, representing red, green, blue channels. (S)he will be asked to close the image with the worst contrast of the particles of one type against the other type. So, the remaining two images, should show, each separately, as good as possible one type of the particles.

   There are now two images opened (we will call them *image_ch1* & *image_ch2*), each representing one type of the particle and to observe them in best possible contrast the auto-contrast option is automatically applied by the macro. Each of the two images will be now sequentially converted to two binary images.

10. Let us start with the *image_ch1*. The user can manually improve the contrast through B&C menu that appears simultaneously on the right hand side next to the image in focus, if this is necessary.



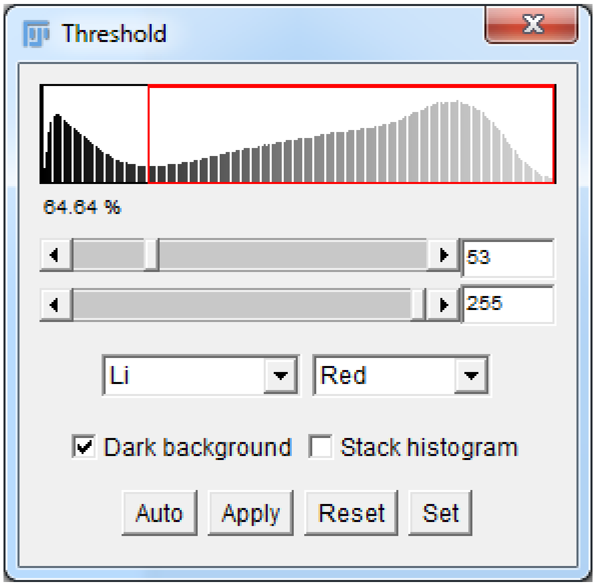
Unless the user is an expert, we suggest, only the sliders are adjusted in this menu if this is necessary at all. Once satisfied, the user should click “OK” in the simultanously displayed window:



Check/Adjust contrast and click OK

Note that contrasting is applied on selected window (*image_ch1*), which can be accidentally changed by clicking the other window (*image_ch2*). In the case you did accidentally click on the *image_ch2*, just click again on the *image_ch1* and then adjust contrast. Also, in the case you have two stacks of images (like in the case of CLSM images of biofilms composed from two bacterial strains, labelled with different fluorochromes), you can slide through the stack prior clicking “OK”, just to make sure you adjusted the contrast satisfactorily through all slices in stack.

11. After clicking OK in previous step, the Threshold menu will open. The red area in the *image_ch1* denotes the particles that are supposed to be extracted from the *image_ch1*. By default it is assumed that the background in *image_ch1* is darker than particles. If this is not the case the user can uncheck the “Dark background” choice. By using the sliders in the Threshold menu the user can adjust the lower and upper boundary of pixel values to be included in the selection to select the particles of interest in the best possible way.



Threshold

64.64 %

53

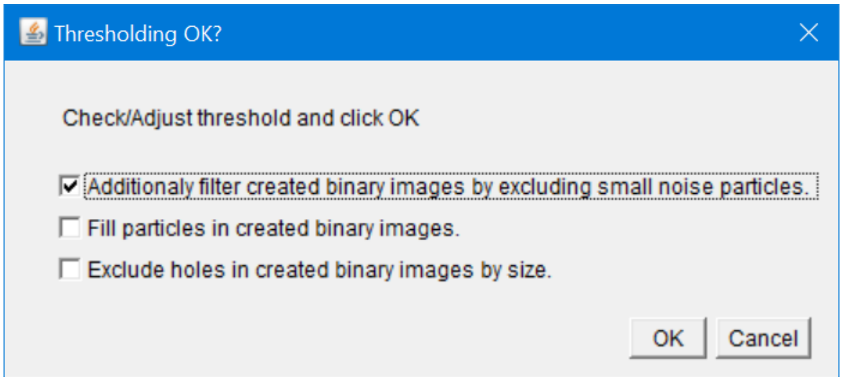
255

Li | Red

☑ Dark background ☐ Stack histogram

Unless the user is an expert, we suggest, only the sliders (and choice of Dark background) are adjusted in this menu if this is necessary.

Sometimes the thresholding cannot be set ideally- there are some parts of the particles that are not coloured red and some pixels or group if pixels that are not part of the particles of interest, but are coloured red. In this case it is a good idea to select Option 1 and 2 in the simultaneously displayed window:



Thresholding OK?

Check/Adjust threshold and click OK

☑ Additionaly filter created binary images by excluding small noise particles.

☐ Fill particles in created binary images.

☐ Exclude holes in created binary images by size.

Option 1:
The obtained binary image may display particles (noise, like some shadows…) that are not part of the “true” particles that you want to obtain. Often they are small in size, and can be filtered out. Choose this option if needed.

Option 2:
The obtained binary image may display particles that are the “true” particles that you want to display. However, some pixels that are part of them are missing (displayed as background, due to some

shadows etc.). If the extend of missing pixels is not too high, you can fill the missing parts by choosing this option. However, all holes in the particles will be filled regardless of their size.

Option 3:

This option differs from the above option. Here, you can specify the maximum size of the hole that will be filled.

Once satisfied with set thresholds, you can click “OK” to continue.

12. If you checked *Option 1* in previous step you will be asked to encircle the biggest particle (that are not of your interest) you want to remove. You can use the Oval or Rectangle tool, which you can select from ImageJ-Fiji toolbar. All particles of the encircled and smaller size will be removed after clicking “OK”.

13. Now the result binary image is displayed. You will be asked if you are satisfied. If Yes, select the result binary image by clicking on its Title bar (the top of the image with title) and click OK. If Not, you can manually use additional built-in tools in ImageJ-Fiji to adjust the image appropriately. Once satisfied, select the result binary image by clicking on its Title bar and click OK. The selected result binary image will be saved to the save directory you have set in step 6 and closed.

14. The steps from 10 to 13 are now repeated, but this time the *image_ch2* is in the focus.

15. Once *image_ch1* and *image_ch2* had been converted to two binary images (*ch1_bin* and *ch2_bin*), where each image separately presents one type of the particle (or component, background) the user can choose either to process next image set (user can select again the same input image(s), or new image(s). Clicking OK brings the user again to step 5, while No or Cancel terminates the macro.

    The saved output images from step 13 can serve as input images in macro *sim_seg_extremes* and *MSS_calc.*

Macro 2: *Sim_seg_extremes_ver1.3.ijm*

Description

This script runs as a macro in Fiji (ImageJ) environment. Its purpose is to simulate and depict maximal and minimal segregation of two components system (or particles of two types, called *ch1* and *ch2* particles) depicted in input digital images. Two input images (8-bit binary format, black and white) are required, each representing one type of the particle (*ch1_bin_, ch2_bin_*) in the same space. The two images can be obtained by the macro *Convert_to_bin_ver1.3.ijm*, but the user can obtain them also in other ways (e.g. manually by splitting via ImageJ tools). The output of the simulation are images (8-bit) representing minimal and maximal segregation case for each particle type (component type), together four images (*segmin_ch1_bin_, segmax_ch1_bin_* and *segmin_ch2_bin_, segmax_ch2_bin_*) stored to the disk as corresponding files. The particles are simulated as circles of user defined diameter; their number is obtained by dividing particle area in an input image by the sphere area. In addition, the macro can also simulate segregation for only part of the image (ROI) i.e. by creating the user defined mask. The masked original images are also outputted (*M_orig_ch1_bin_, M_orig_ch2_bin_*), together with the image representing mask itself (*Mask_*) and stored to the disk as corresponding files. Also, the stacks of images are supported by the macro. *segmin* and *segmax* files together *ch1_bin, ch2_bin* serve as input for *MSS_calc_ver1.3*, where Multiscale spatial segregation analysis is performed.

Step by step guide

1. If you do not already have Fiji, download and install it from: https://imagej.net/software/fiji/downloads
2. Run Fiji (ImageJ-win64.exe file, if you are running 64-bit version).
   If asked to make an update, you can choose »Remind me later«, the update is usually not needed to successfully run *Sim_seg_extremes_ver1.3*, unless you are running really old version of Fiji-ImageJ.

A. Installation

3. In Windows explorer (or equivalent File browser) go to the folder where you have installed Fiji and find \plugins folder within Fiji folder. In our case:
   C:\Users\iztokd\Downloads\fiji-win64\Fiji.app\plugins
   Create folder (if it is not there already) \MSSegregation within plugins folder

   In running Fiji app press Ctrl+shift+M (=Install Macro) and choose a file *Sim_seg_extremes_ver1.3.ijm*

   Save it to directory \MSSegregation, created above.
   Close and restart Fiji app. If asked to save LUTMenuTool.ijm, just say “No”.

4. Now you can run the macro from the Menu Bar: Plugins>MSSegregation> *Sim_seg_extremes_ver1.3*

B. Direct run of the Macro

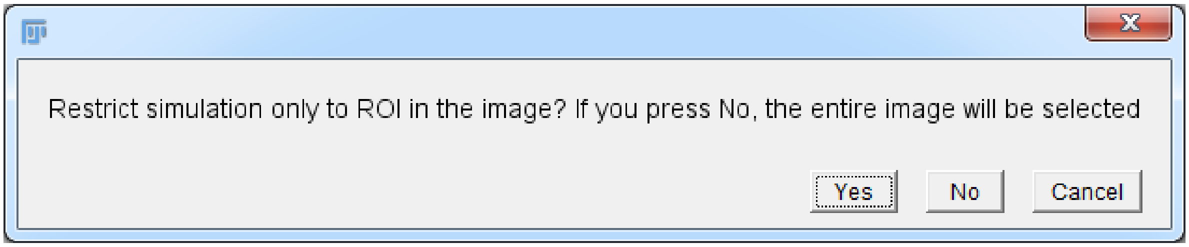
3. Windows explorer (or equivalent File browser) and "drag and drop" the *Sim_seg_extremes_ver1.3.ijm* to the running Fiji application.

4. New window with displaying the code of *Sim_seg_extremes_ver1.3.ijm* will open (script editor) and you can run it by pressing Ctrl+R or by choosing Run>Run from menu of the script editor.

5. Now you will be asked to choose a directory to save the output binary images, which are the result of the simulation. Select directory and press "Select".

6. Next you will be asked to select an image file that represent *ch1* particles. The image needs to be in 8-bit format. The particles must have pixel value of 255 the rest of pixels in the image must be 0. If you had used the macro *Convert_to_bin_ver1.3* than this is the file that ends with *_ch1_bin.tif* Click "OK" and select the file. Repeat the same for the image file that represent *ch2* particles.

7. Next the following question appears:



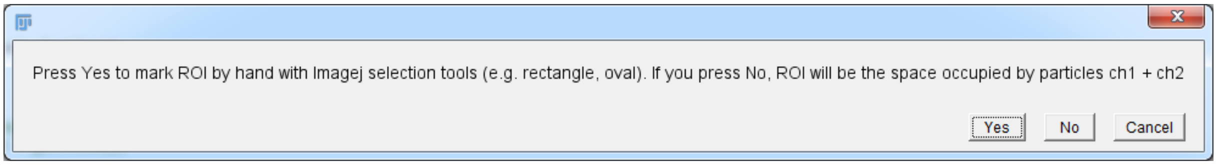
Restrict simulation only to ROI in the image? If you press No, the entire image will be selected

If you press "Yes" the macro will simulate segregation extremes only on the specified region (ROI = region of interest) of the input images from step 6. This can be handy, if the image has some background that you do not want to include into analysis and/or particles can occupy only one particular region in the image.

If you press "No" the macro will simulate segregation extremes on the entire area of input images from step 6. This is the most common situation. In this case you can go directly to step 9.

If you press "Cancel" the macro will terminate.

8. If you pressed “Yes” in previous step the following will appear on your screen:



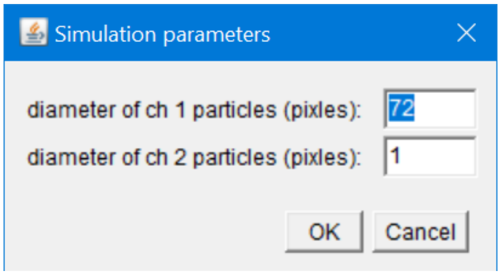
Press Yes to mark ROI by hand with Imagej selection tools (e.g. rectangle, oval). If you press No, ROI will be the space occupied by particles ch1 + ch2

If you press “Yes” you will be asked to mark ROI on ch1 image (*_ch1_bin*). Use ImageJ-Fiji selection tools from the toolbar (e.g. Oval, Rectangle…) to encircle the ROI. The same ROI will be automatically copied and applied to ch2 image (*_ch2_bin*).

If you press “No”, the ROI will become the space that is occupied by the particle *ch1* plus the space occupied by the particle *ch2*. This is useful, when two component or particles are restricted to certain area in the image. For example, oats and raisins can be restricted by the plate that occupies only part of the entire image. Oats or raisins cannot “move” out of the plate. Further example: you want to preserve the shape of two species biofilm in microscopy image and just simulate segregation within biofilm. You will be also asked to fill the holes in the common space. If they are not any holes present or the displayed holes are true empty space, just press No, otherwise press OK. If you are satisfied press OK, when asked about the result, No will undo fill.

If you press “Cancel” the macro will terminate.

9. In this step you need to specify the diameter of the particles (component).



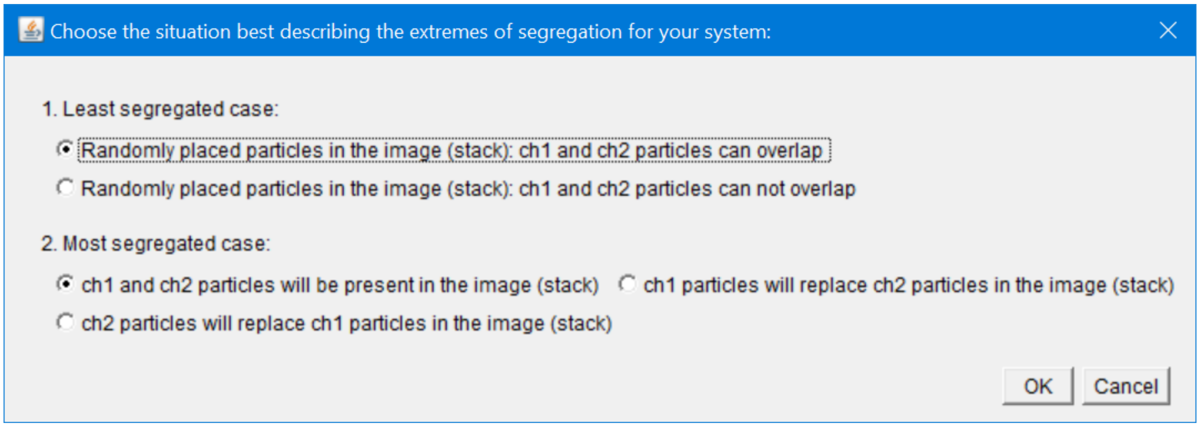
Simulation parameters

diameter of ch 1 particles (pixles): 72

diameter of ch 2 particles (pixles): 1

The particles can be only 1 pix in size, but can be also bigger. Only circular particles are supported at present time. It is advisable that user opens the image of particular particles in a separate instance of Fiji app and measures the particle size by, for example, by Straight tool from ImageJ-Fiji toolbar. However, if particles can be regarded as a continuous matrix than their size is the image resolution, i.e. 1 pixel. For example, in the case of mixture of oats and raisins, the oats can be considered as continuous matrix (diameter 1 pix) and raisins as circular particles(diameter 72 pix).

10. After you entered the diameter of respective particles and have pressed “OK” the following menu will appear:



Choose the situation best describing the extremes of segregation for your system:

1. Least segregated case:

● Randomly placed particles in the image (stack): ch1 and ch2 particles can overlap

○ Randomly placed particles in the image (stack): ch1 and ch2 particles can not overlap

2. Most segregated case:

● ch1 and ch2 particles will be present in the image (stack) ○ ch1 particles will replace ch2 particles in the image (stack)

○ ch2 particles will replace ch1 particles in the image (stack)

1. *Least segregated case:*

Here you decide, if the randomly placed particles of one type (*ch1*) can overlap (i.e. share the same location in an image) with particles of other type (*ch2*) in the image. By default particles of the same type cannot overlap.

2. *Most segregated case:*

Here you decide about the type of maximal segregation:

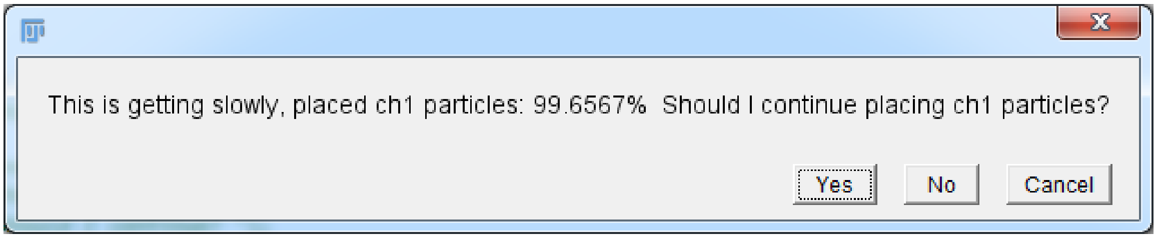
The two type of particles (*ch1* and *ch2*) are restricted to the space of image (or ROI, step 8) and cannot “escape” from it. However, they can segregate to the sides of the image in order to maximize the distance from not alike particles. On the other hand, the alike particles will be placed maximally to each other, their individual shape will be ignored (the area of the particles is held constant).

If the most segregated case is such that particles *ch1* can completely exclude particles *ch2* then choose the second option.

If the most segregated case is such that particles *ch2* can completely exclude particles *ch1* then choose the third option.

11. After clicking OK in the previous step the simulation of placing the particles in the image will begin, without displaying anything, except in Log window “start of the simulation:” together with time will be printed.
    However, you may get warnings that “Total area of simulated spherical particles outlined by square exceeds the available image space”. This can happen in the case you did not allow particle overlap in step 10 and the area of square outlined spherical particles is higher than total area of image or ROI. Outlining spheres by squares is very conservative approach in calculating total area, so there is a good chance that the simulation will be able to place all the particles, especially if at least one of the

particles is very small. Therefore, you should try and click “OK” and soon you will likely encounter a message like this:



This is getting slowly, placed ch1 particles: 99.6567% Should I continue placing ch1 particles?

This occurred because the space available to particles become very scarce during simulation and not all of the particles have been placed. If the total area of simulated particles exceeds the available image space than the placing all of the particles without overlapping will never happen. If the percentage is small, you should press Yes and see in the next message if the percentage improved significantly. If it did, than you should press Yes again and continue pressing Yes as long as you see significant improvements. Once you have reached high enough number of the % of placed (typically >95 %) you can press No to finish simulation and check the result images. On the other hand, if you did not observe significant steps in increase in % of placed particles after clicking Yes, you can either abort the macro by clicking Cancel and rerun the simulation with different parameters (allowing overlap, or different size of particle) or press No to finish simulation and check the result images.

12. Once all the particles *(ch1* and *ch2*) have been placed or user agrees not to continue placing the particles (read step 11) the simulation finishes, prints to Log window “end of simulation:” together with time and displays the result images (three dots in the name means the name of the original image):

    1. Two Images showing minimally segregated cases (best mixed):
    segmin_ch1_bin_…, segmin_ch2_bin_…

    2. Two Images showing maximally segregated cases (worst mixed):
    segmax_ch1_bin_…, segmax_ch2_bin_…

    3. One Image showing the mask (i.e. the image that shows the area, where particles were allowed to be placed. The pixel value of 1 (black) indicates this area. If no restriction (ROI) of simulation space was selected (see step 7) then this image will be black:
    *Mask_*

    4. Two Images showing the original input images through the mask (if the mask was uniform black (no restrictions) than these images are the same as original input images):
    M_orig_ch1_bin_…, M_orig _ch2_bin_…

    5. One image showing overlap of *segmin_ch1_bin_…* and *segmin_ch2_bin_…* images (useful to see how severe overlap is, if it was permitted- black colour (pixel value 512) indicates overlapping of *ch1* and *ch2*, grey (pixel value 255) means particle is there, and white (pixel value 0) means empty space):

overlap of ch1 min seg and ch2 min seg

6. In addition, Log window is displayed, where most important data of simulation is displayed.

   All of these images are stored as .tif files beginning with the above names in the save directory selected in step 5, together with Log window, stored as: *Log_Sim_seg_extremes_ver1.3.txt* **.** The image files from 1. and 2., together with *ch1_bin_, ch2_bin_* image files serve as input files for macro *MSS_calc_ver1.3*, where Multiscale spatial segregation analysis is performed.

Macro 3: *MSS_calc _ver1.3.ijm*

Description

This script runs as a macro in Fiji (ImageJ) environment. Its purpose is to calculate the measures of segregation in images of two components system (mixture of two particles, particle and background….). Two components are called *ch1* and *ch2* (particles). The main quantity to be calculated is spatial size scale dependent segregation level (*Sd^*), i.e. *Sd^* as a function of dimension, *d*, of field of view (FOV). This is obtained by scanning the image by randomly placing the square of size *d* x *d* (= FOV) and calculating the dominance of one particle over the other in each placed FOV. This is done *n* times and then averaged to calculate *Sd^* (for the details, please check the reference Dogsa et al., 2021, once it is published). To do this the two input images (8-bit binary format, black and white) are required, each representing one type of the particle (*ch1_bin_, ch2_bin_*) in the same space (like an image of oats-*ch1* and raisins-*ch2* in the same plate). The two images can be obtained by the help of macro *Convert_to_bin_ver1.3.ijm*, but the user can obtain them also in other ways (e.g. manually doing the splitting and converting via ImageJ tools). To compare *Sd^* of your sample to the positive and negative controls, i.e. to extremely segregated and extremely non-segregated cases in the same space by the same particles the images displaying these situations are required for *ch1* and *ch2* particles. These images (*segmin_ch1_bin_, segmax_ch1_bin_* and *segmin_ch2_bin_, segmax_ch2_bin_*) can be obtained by the macro *Sim_seg_extremes_ver1.3.ijm.* The stacks of images are supported by the macro. Besides calculating the segregation level (*Sd^*) as a function of dimension of field of view (FOV), the macro can also calculate:

1. multiscale spatial segregation level (*MSSL*), which is *Sd^* averaged over all *d* (from *dmin* to *dmax*) of FOV to give distance averaged segregation level for users (sample) image and for min and max segregation images.
2. multiscale spatial segregation distance (*MSSD*), which is *Sd^* integrated over all *d* of FOV
   It can be interpreted as the distance (dimension) over which segregation level is 1 (i.e. maximal segregation level), if we theoretically compact all segregation levels, *Sd^* , from *dmin* to *dmax* to the distances from *dmin* to *MSSD*. This distance roughly corresponds to dimension of particles in the hypothetical image, where these particles form homogenous aggregates of *MSSD* and contain either one or the other type of the particle.
3. Relative multiscale spatial segregation level (*rMSSL*). This segregation measure takes into account *MSSL* of users (sample) image and *MSSL* of min and max segregation images to calculate relative FOV independent segregation measure.

Step by step guide

1. If you do not already have Fiji, download and install it from: https://imagej.net/software/fiji/downloads

2. Run Fiji (ImageJ-win64.exe file, if you are running 64-bit version).
If asked to make an update, you can choose »Remind me later«, the update is usually not needed to successfully run *MSS_calc _ver1.3*, unless you are running really old version of Fiji_ImageJ.

A. Installation

3. In Windows explorer (or equivalent File browser) go to the folder where you have installed Fiji and find \plugins folder within Fiji folder. In our case:
C:\Users\iztokd\Downloads\fiji-win64\Fiji.app\plugins
Create folder (if it is not there already there) \MSSegregation within plugins folder

   In running Fiji app press Ctrl+shift+M (=Install Macro) and choose a file *MSS_calc _ver1.3.ijm*

   Save it to directory \MSSegregation, created above.
Close and restart Fiji app. If asked to save LUTMenuTool.ijm, just say “No”.

4. Now you can run the macro from the Menu Bar: Plugins> MSSegregation> *MSS_calc _ver1.3*

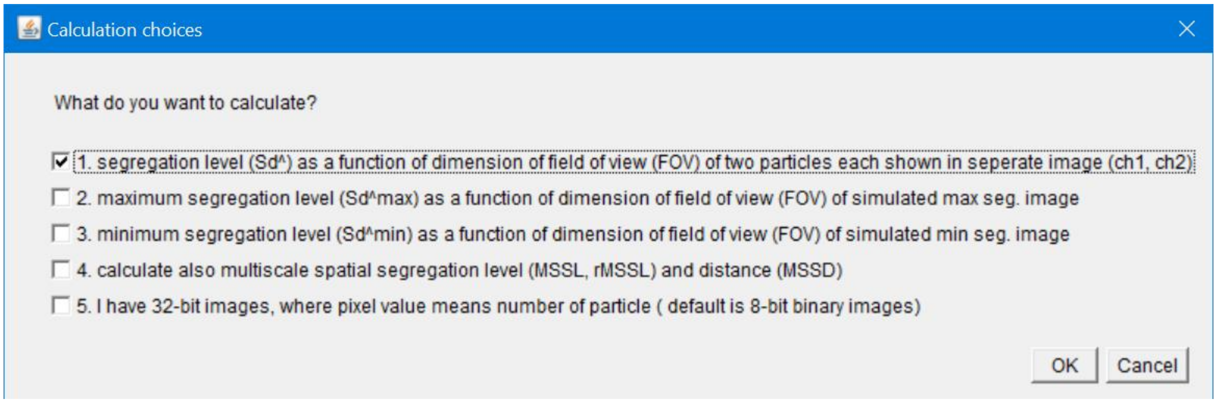
B. Direct run of the Macro

3. Open Windows explorer (or equivalent File browser) and “drag and drop” the *MSS_calc _ver1.3.ijm* to the running Fiji application.

4. New window with displaying the code of *MSS_calc _ver1.3.ijm* will open (script editor) and you can run it by pressing Ctrl+R or by choosing Run>Run from menu of the script editor.

5. Now you will be asked to choose a directory to save the output calculations, which are the result of the simulation. Select directory and press “Select”.

6. Then you will be asked what to calculate.



Calculation choices

What do you want to calculate?

☑ 1. segregation level (Sd^) as a function of dimension of field of view (FOV) of two particles each shown in seperate image (ch1, ch2)

□ 2. maximum segregation level (Sd^max) as a function of dimension of field of view (FOV) of simulated max seg. image

□ 3. minimum segregation level (Sd^min) as a function of dimension of field of view (FOV) of simulated min seg. image

□ 4. calculate also multiscale spatial segregation level (MSSL, rMSSL) and distance (MSSD)

□ 5. I have 32-bit images, where pixel value means number of particle (default is 8-bit binary images)

Option 1:
This option is by default checked, as this is the most fundamental purpose of this macro. To calculate segregation level (*Sd^*), you will need two binary images, each representing one type of the particle or component in the common mixture, which can be obtained by splitting and converting the original sample image by *Convert_bin_ver1.3.ijm* macro.

Option 2:
This option is used to compare calculated segregation level of sample image to the positive control, i.e. to the segregation level of hypothetically maximally segregated sample image. This image can be obtained by *Sim_seg_extremes_ver1.3.ijm* macro.

Option 3:
This option is used to compare calculated segregation level of sample image to the negative control, i.e. to the segregation level of hypothetically minimally segregated sample image. This image can be obtained by *Sim_seg_extremes_ver1.3.ijm* macro.

Option 4:
If the Option 1 to 3 is checked the macro will calculate the segregation measures derived from segregation level, *Sd^.*

Option 5:
By default the input images need to be 8-bit with pixel value of 255 indicating the presence of the particle and 0 of its absence. If this option is checked, the user can also input 32-bit images, where pixel value represents the number of particles in the location of the pixel. This is useful, for example, in the case of choropleth maps.

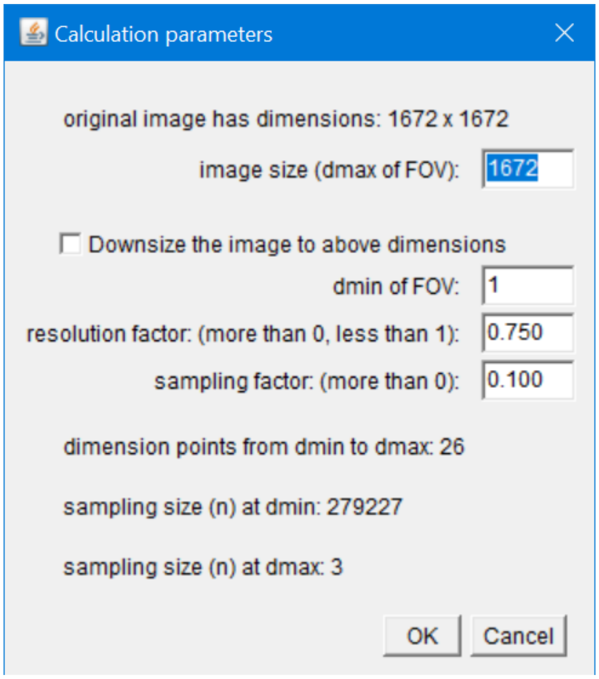
Pressing “OK” will get you to the next step, while pressing “Cancel” will exit the macro.

7. Now you will be asked to select a directory to save the calculation results. Click on the chosen directory and press “Select”.

8. Next you will be asked to select an image file that represent *ch1* particles. The image needs to be in 8-bit format (unless you have checked Option 5 in step 6). If you had used the macro *Convert_to_bin.ijm* than this is the file that ends with *_ch1_bin.tif.* Click “OK” and select the file. Repeat the same for the image file that represents *ch2* particles.

9. If you have checked Option 3 and 4 in step 6, you will be asked to choose corresponding 8-bit (32-bit if you have checked option 5 in step 6) images, representing maximal and minimal segregation cases. If these images were obtained by the macro *Sim_seg_extremes_ver1.3.ijm.*, then the corresponding files are named as: *segmax_ch1_bin_*....tif, *segmax_ch2_bin_....tif* and *segmin_ch1_bin_....tif, segmin_ch2_bin_....tif*). For each image click “OK” and select the file.

10. In this step you can adjust calculation parameters.



original image has dimensions: 1672 x 1672

image size (dmax of FOV): 1672

□ Downsize the image to above dimensions

dmin of FOV: 1

resolution factor: (more than 0, less than 1): 0.750

sampling factor: (more than 0): 0.100

dimension points from dmin to dmax: 26

sampling size (n) at dmin: 279227

sampling size (n) at dmax: 3

Image size (dmax of FOV) [pixels]:

By default, image size in pixels is displayed corresponding to the maximum dimension (*dmax*) of FOV that will be applied to an image to calculate FOV dimension dependant segregation level, *Sd^*. The user can decrease the *dmax* of FOV for segregation level calculation, but in this case (s)he will not obtain the segregation level, *Sd^* of entire area of image.

Downsize the image to above dimensions:

If the time taken for the calculation is very long and the image has unnecessary high pixel resolution, the user can downsize the input images to new *dmax* [pixels]. In this case the *Sd^* of entire area of image can be obtained.

dmin of FOV [pixels]:

The user can set the smallest dimension (*dmin*) of FOV. This can improve the calculation speed significantly, if *Sd^* at *dmin* of 1 pix is not necessary to be calculated, than this number should be increased. For example, in the mixture of oats & raisins that are in size about 50 pix, there the *Sd^* at *dmin* of 1 pix will for sure be 1 as the two constituents cannot interpenetrate each other.

resolution factor:

This factor determines the number of different dimensions, *d* of FOV at which *Sd^* will be calculated and by which factor the values of *d* will be spaced, in the direction from *dmax* to *dmin*. For example factor of 0.99, means that starting from *dmax*, every next *d* will be smaller by factor of 0.99 in the direction to *dmin*. Unless the range from *dmax* to *dmin* is very low, this means a lot of *d* points.

sampling factor:

This factor determines the number, *n*, of local segregation levels will be calculated from randomly placed FOVs of dimension *d* in the image , which get then averaged to obtain *Sd^* at *d*. The more the better the statistics, but of course more time is required for the calculation. Maximal sampling size per *d* is 1 000 000, but often a few thousands will be enough. This factor impacts the duration of the calculation significantly, so try first with *n* about few thousands and check the estimated experimental error of the calculated quantities in the end of simulation, maybe they are small enough and you will not need to increase sampling factor.

Note that the total time of calculation depends on the number of points to be calculated (distance points from *dmax* to *dmin*), the number of sampling at each point (sampling size, *n*, and dmin and dmax) and the resolution of image.

If the changes were made to whatever parameters default values in this menu, the parameters (*distance points from dmax to dmin*), (*sampling size* at *dmin* and *dmax*) will get refreshed after clicking “OK”. Once no more changes to the parameters values (and parameter values are within their ranges) had been made, clicking “OK” will start the calculations and calculation start time will appear in the Log window.

During the calculations, the user can follow the progress in Log window. For each calculation point the data about the dimension, *d*, and number, *n*, of FOV (sampling size) will be displayed in Log window. Once the *Sd^* at certain *d* is calculated, the macro proceeds to calculate next *Sd^* at next *d*, until all *Sd^* from *dmax* to *dmin* are calculated. When this happens the calculation end time will appear in the Log window and the calculation results will be displayed in Results window, including all the segregation measures selected in step 6 together with their standard errors. The Result table and Log window are saved to the saving directory specified in step 6, having the names *Sd_MSSDL_ver1.3.txt* and *Log_Sd_ver1.3.txt*. In addition, the file *statistics_of_segregation_Sd_ver1.3.txt* containing additional segregation indices that can help very advanced users to further analyse their segregation cases is also stored to the saving directory.

Step 11: As a last step, the user can choose that the segregation level as a function of *d* of FOV gets plotted, which will be than displayed.

Macro 4: *Decode_choropleth ver1.3.ijm*

Description

This script runs as a macro in Fiji (ImageJ) environment. Its purpose is to convert digital images, where value of the pixel intensity carries information about number of particles (particles can be cells, people, molecules…) at the pixel location into a format that can be used by *MSS_calc_ver1.3* for multiscale spatial segregation analysis. The original values that are encoded into the pixel colour get decoded and the value of pixels intensity becomes original value. Two types of colour decoding are supported: (i) the original values are linear in RGB space or (ii) the continuous colour scale is depicted in the image that needs to be decoded. The original digital images can be of any type supported by Fiji (ImageJ), but macro has been optimized for 8-bit grayscale images. The higher bit original images will get first converted to 8-bit grayscale images and then converted to 32-bit grayscale images with pixel intensities of original values. The typical application of this macro are density maps (e.g. choropleth maps). Ideally, they are created from raw data, by external application (like https://www.datawrapper.de/maps/choropleth-map), but they can be also obtained by simply copying images from other sources, as long as they are of sufficient quality.

Besides providing original image for conversion, the user needs also to input an image (mask) that clearly marks the region where particles can be positioned. For details see, Step by step guide.

Step by step guide

1. If you do not already have Fiji, download and install it from: https://imagej.net/software/fiji/downloads
2. Run Fiji (ImageJ-win64.exe file, if you are running 64-bit version).

If asked to make an update, you can choose »Remind me later«, the update is usually not needed to successfully run *Decode_choropleth ver1.3.ijm*

A. Installation

3. In Windows explorer (or equivalent File browser) go to the folder where you have installed Fiji and find plugins folder within Fiji folder. In our case:

C:\Users\iztokd\Downloads\fiji-win64\Fiji.app\plugins
Create folder \MSSegregation within plugins folder

In running Fiji app press Ctrl+shift+M (=Install Macro) and choose a file *Decode_choropleth ver1.3.ijm*.

Save it to directory \MSSegregation, created above.
Close and restart Fiji app. If asked to save LUTMenuTool.ijm, just say “No”.

4. Now you can run the macro from the Menu Bar: Plugins> MSSegregation> *Decode_choropleth ver1.3.ijm*

B. Direct run of the Macro

5. Open Windows explorer and “drag and drop” the file *Decode_choropleth ver1.3.ijm* to the running Fiji application.
6. New window with displaying the code of *Decode_choropleth ver1.3.ijm* will open (script editor) and you can run it by pressing Ctrl+R or by choosing Run>Run from menu of the script editor.

7. After pressing Run, you will be asked to choose a directory to save the converted images, which are the result of the simulation. Select directory and press “Select”.

8. Next you will be asked to select an image file that you want to convert. This image, called sample image, can be of any type supported by Fiji, but to avoid possible precision loss, the 8-bit format grayscale is preferred as macro is optimised for these images. Click “OK” and select the file. In general, you could input already published images, even printed ones, by taking photos of them, but note that bad quality images, like uneven colour intensity representing the same density, or low resolution images, will produce unprecise results. Also boundaries encircling regions with the same colour (density), reduce the accuracy of the final segregation analysis in the *MSS_calc-ver1.3.ijm*.

9. After the selected image is opened, you will be asked to mark the region within background by, for example, rectangular tool of ImageJ. If your background pixels are white and have 0 value than you can just say “No”, but most of the images that have white background have pixel values of 255. This step is important to distinguish background pixels from coding pixels. Pressing “Cancel” will exit the macro. If you press “Yes” you have to make a selection of some background pixels, so the program will know what background is. Make a selection and press “OK”.

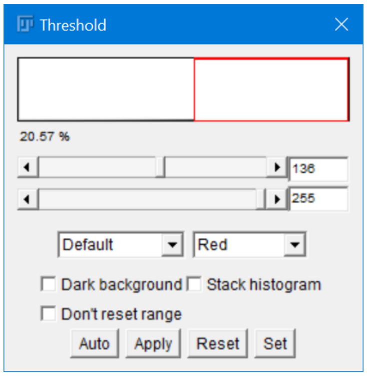
10. Next you will be asked to select an image file that is masking the region of interest. This means that on this image the space where your “particles” could be present is clearly distinguished from the background, where “particles” cannot be present. For example, the density map of SARS-CoV2 of Italy that is dark on white background (the “particles” can be only present in Italy). Also the mask image, must be of the same size as sample image. If you already made a masking image in some previous round of analysis you can keep the checkbox "The mask image is already created" checked and you will only select it in the step 11 and skip step 12.

    If you did not created it, you should uncheck this checkbox. In principle you can use the sample image (step 8) for masking image, as long as the space where your “particles” could be present is clearly distinguished from the background, where “particles” cannot be present. Alternatively, you can create masking image by other means, as for example by https://www.datawrapper.de/maps/choropleth-map (see Example 3), where you input the same data for all regions on the map.

11. After the selected masking image is opened, you will be asked to mark the region within background by, for example, rectangular tool of ImageJ. If your background pixels are white and have 0 value than you can just say “No”, but most of the images that have white background have pixel values of 255. This step is important to distinguish background pixels from pixels marking the “particle” space. Pressing “Cancel” will exit the macro. If you press “Yes” you have to make a selection of some background pixels, so the program will know what background is. Make a selection and press “OK”.

12. If the proposed masking image contains whatever additional objects (like colour scale in the legend, some titles…) that are not part of the space, where “particles” can be present, you can remove them in this step. When asked, press “Yes” and mark the objects by ImageJ tools (e.g. Recetangular tool) and they will be removed. If no such objects are present, than you can press “No” and macro will continue. Pressing “Cancel” will terminate the macro. After removing the unwanted objects, the macro automatically converts the masking image to binary image (black and white image displayed in the background). This binary masking image is than applied on sample image in order to remove everything except the region of interest as indicated in the masking image.

13. To further refine distinction of background from “particle” space you will be asked via Action Required window to Check/Adjust threshold and a threshold window will appear:



Threshold

20.57 %

136

255

Default

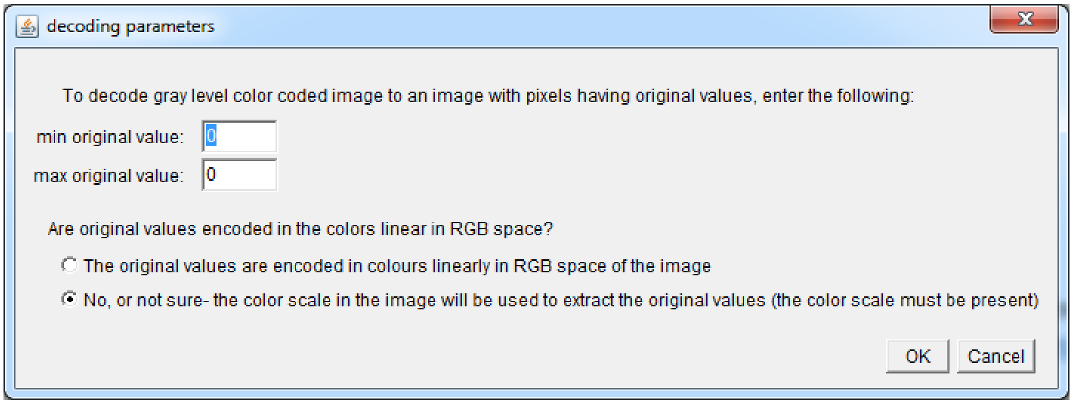
Red

□ Dark background □ Stack histogram

□ Don't reset range

Adjust the two sliders so, that you will exclude all the space in the image that should not be considered as “particle” space. For most accurate results you should exclude also the boundaries between regions that are typically white or black in the choropleth maps. Once done click “OK” on Action Required window.

14. Next, a dialog box, where you can set decoding parameters for sample image appears:



To decode gray level color coded image to an image with pixels having original values, enter the following:

min original value: 0

max original value: 0

Are original values encoded in the colors linear in RGB space?

○ The original values are encoded in colours linearly in RGB space of the image

● No, or not sure- the color scale in the image will be used to extract the original values (the color scale must be present)

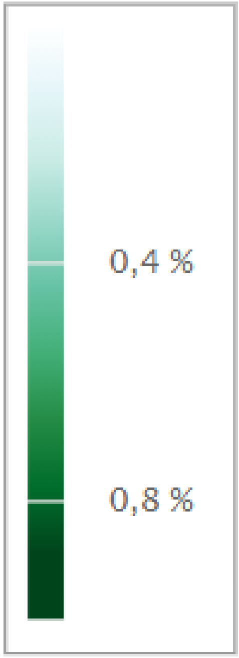
min original value, max original value:

To properly decode colours to original values the program needs to now what are the minimal and maximal original values. In many cases you can read these values out of the colour scales depicted in the legend of sample image or if you created sample image via external software (e.g. https://www.datawrapper.de/maps/choropleth-map) than you know exactly these values. Enter these values without units.

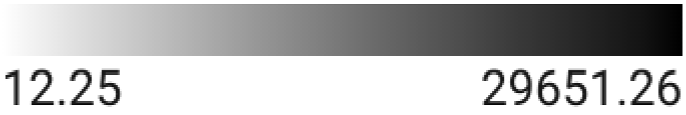
Are original values encoded in the colors linear in RGB space:

Use this option, if colors in the image are linear with the original values. For example, in this case black would mean max original value, white min original value and gray (50% black, 50% white) = min [original value + max original value / 2].

Choose second option if you do not know and your sample image includes a legend with color scale. The supported color scales must be linear, meaning that the original values increase (or decrease) linearly with the physical length of the color scale. For example, this means that if the start of the color scale has min original value and the end of the scale has max color value than the middle of the scale has middle original value (= [min original value + max original value]/ 2). The color scales can be depicted in the sample image either horizontally or vertically. Best if they are not interrupted or just Minimally. The two examples of valid color scales are given below:



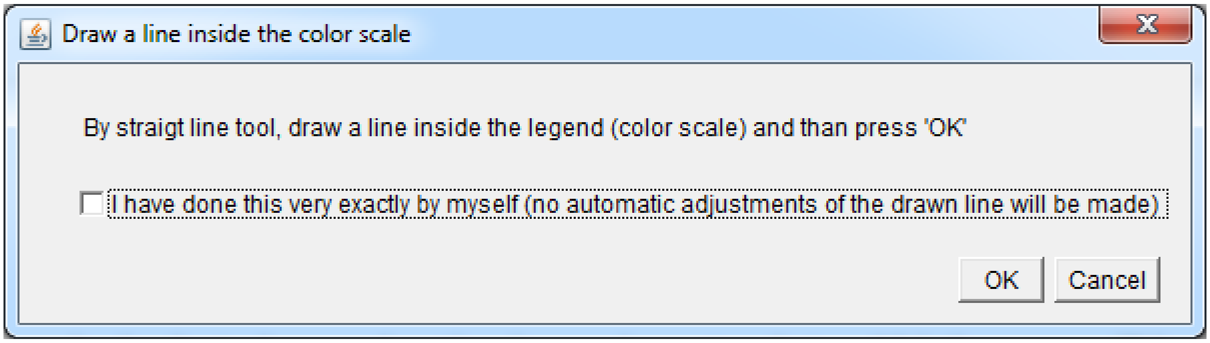
Example of linear vertical color scale that is minimally (two times) interrupted by white lines. As it is linear the min and max values must be 0,0 % and 1,0 %, repectively.



Example of linear continuous horizontal color scale. Min and max values are indicated

After entering min and max original values and selecting decoding mode you can click “OK” to continue with decoding.

15. If you had selected first option in previous step (step 14) than you should go to step 19 of this manual. If you had selected second option then you will be asked to mark the scale by drawing a straight line onto the color scale itself:

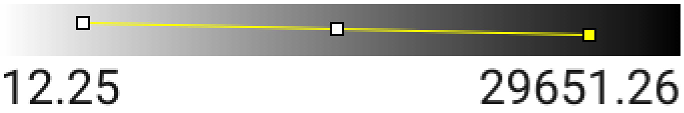


Draw a line inside the color scale

By straigt line tool, draw a line inside the legend (color scale) and than press 'OK'

□ I have done this very exactly by myself (no automatic adjustments of the drawn line will be made)

Do not go over the scale. Use Magnifying glass tool, if necessary. A good example is shown below:



A line drawn onto the color scale by Straight tool

The program will, after clicking “OK”, adjust the line automatically, but if you prefer to omit the automatic adjustment than you should place the line very exactly and select “I have done this very exactly by myself (no automatic adjustments of the drawn line will be made) and then click “OK”.

16. After line is automatically positioned you will be asked to confirm that the positioning is OK. If the beginning and end of the color scale are connected by straight line that runs straight over the color scale, than you can click “Yes”, if not, you should say “No” and you go back to step 15.

17. A plot depicting Distance vs Gray level will appear. This plot shows how the Gray level scales with physical distance of the depicted color scale. In general, the line should be monotonous. If there are some peaks that correspond to the interruptions in the color scale, and there are just a few, the program will ignore them by decoding. Note that the more of the peaks the less precise will be the conversion of gray levels to original values.

18. After confirming by clicking “OK”, new plot appears that shows how the Gray levels in the sample image will be decoded. Click “OK” to continue.

19. The sample image gets now converted to 32-bit image and each pixel has now original value. Once the decoding is done, the “decoding done” appears in Log window. This decoded image is displayed and saved to the disk to the directory specified in step 7. Such image can then serve as an input image for *MSS_calc _ver1.3.ijm,* where segregation analysis is performed. In addition, also the mask image is saved, so it can be also used to decode maximal and minimal segregation images necessary for *MSS_calc _ver1.3.ijm* . The window asking to continue to next image is displayed. If you press “Yes” you can proceed with new image (step 8). No, or Cancel will terminate macro.

Examples

This section comprises three examples of MSSegregation package application. For each macro applied the input images entered at certain step along with input parameters are given. Also, the output images and most important results at certain steps of the macros are shown. The order of appearance of macros within the particular examples is the same as the run order of the macros. To understand how to run and use certain macros, refer to the previous sections of this manual. The input/output files for all three examples are given on github: https://github.com/IztokD/MSSegregation-package. You can also watch online movies, where we have shown how to remake this available on FigShare (the links are given in the text below). In Example 3 we have used free online external application to plot choropleth maps (https://www.datawrapper.de), but in general the user can obtain the choropleth maps also in other ways (e.g. by other software, drawing by hands, copying from digital sources…).

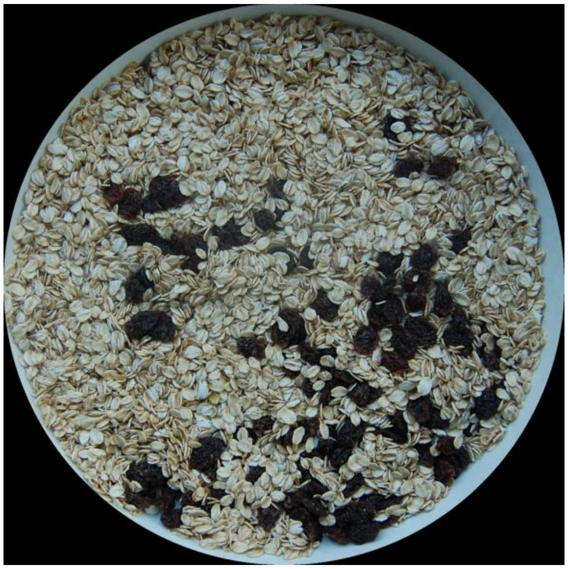
Example 1: Oats &Raisins

Convert_to_bin_ver1.3.ijm

Parameters used: step 5 (*Option 1 and 2*), step 7 (*Option 1*), step 9: (closed image = blue), step 11 for image (red) = ch1: *Option 1, 2*, lower-threshold [upper slider] =1, upper-threshold [lower slider] = 61; for image (green) = ch2: *Option 1, 3*, lower-threshold =67, upper-threshold = 255

Movie showing the procedure:
Convert_bin_oats_rasins.wmv at https://figshare.com/s/5a08f7b84a76586e1601

Input images (step 8):



t3.tif (RGB image)

Output of images (step 13):

t3.tif (red)0_ch1_bin.tif

t3.tif (green)0_ch2_bin.tif

Sim_seg_extremes_ver1.3.ijm

Parameters used: step 7 (*Yes*)*,* step 8 (*No*), step 9: ch1 = *72* pix, ch2 = *1* pix, step 10: *Randomly placed particles in the image (stack): ch1 and ch2 particles can not overlap*, *ch1 and ch2 particles will be present in the image (stack)*, step 11, (*No,* after >99% ch2 partciles placed).

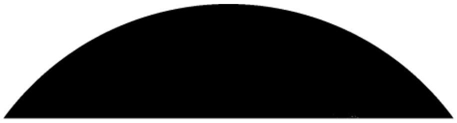
Movie showing the procedure:
segregation_extremes_oats_raisins.wmv at https://figshare.com/s/5a08f7b84a76586e1601

Input images (step 6):

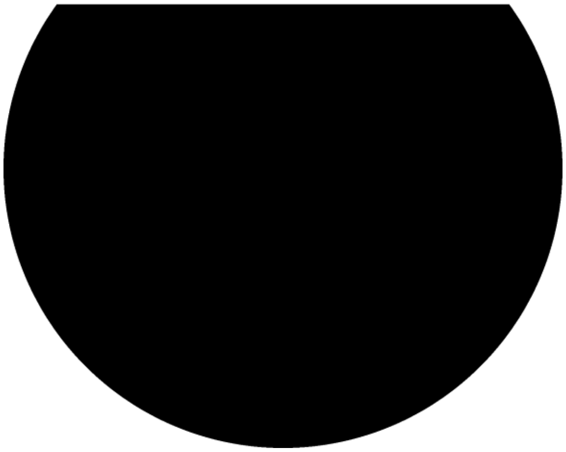
Output of *Convert_to_bin_ver1.3.ijm :*

t3.tif (red)0_ch1_bin.tif t3.tif (green)0_ch2_bin.tif

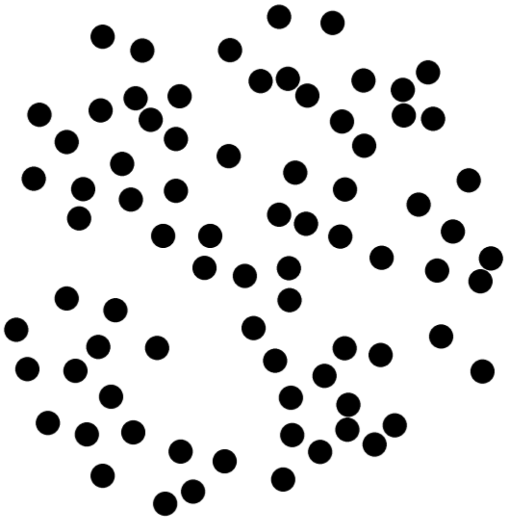
Output images (step 15):



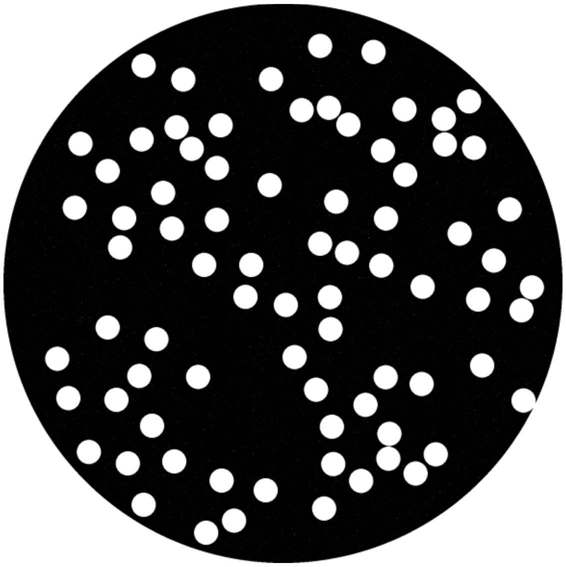
segmax_ch1_bin_t3.tif (red)0_ch1_bin



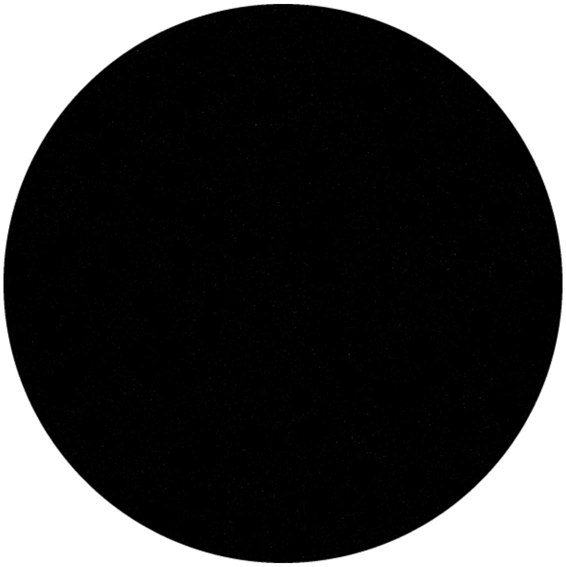
segmax_ch2_bin_t3.tif (green)0_ch2_bin



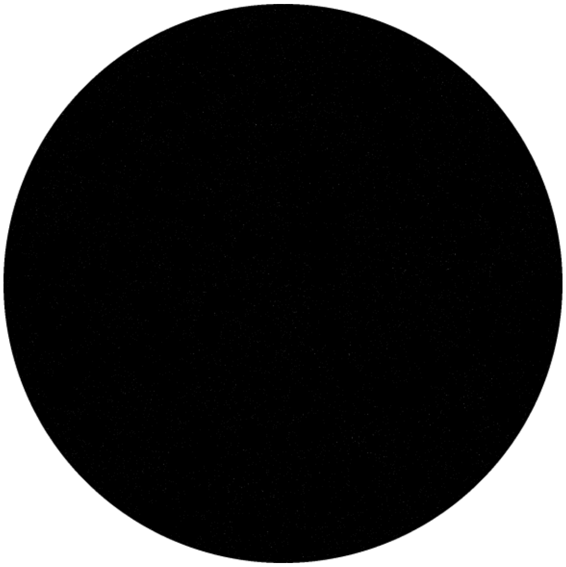
segmin_ch1_bin_t3.tif (red)0_ch1_bin.tif



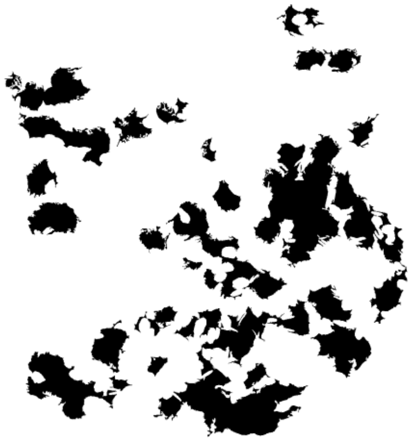
segmin_ch2_bin_t3.tif (green)0_ch2_bin.tif



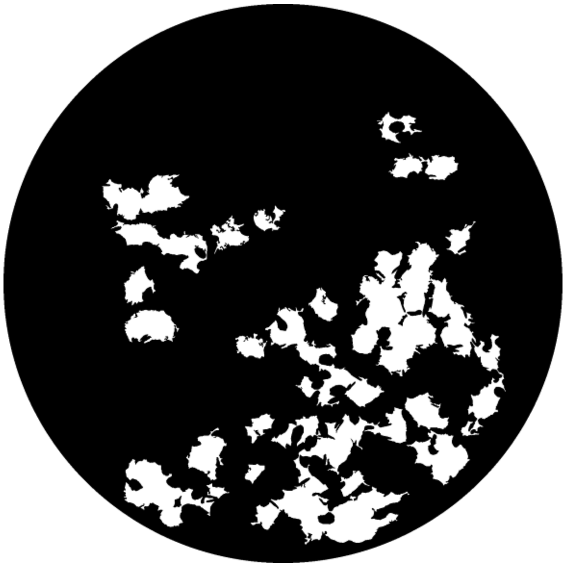
overlap of ch1 min seg and ch2 min seg.tif



Mask_t3.tif (red)0_ch1_bin t3.tif (green)0_ch2_bin.tif



M_orig_ch1_bin_t3.tif (red)0_ch1_bin.tif



M_orig_ch2_bin_t3.tif (green)0_ch2_bin.tif

MSS_calc _ver1.3.ijm

Parameters used: step 6 (*Option 1 to 4*), *step 10 (dmax =1663, dmin =5, res_factor =0.75, sampling_factor =0.01), step 11 (Yes)*

Movie showing the procedure:
MSS_analysis_oats_raisins.wmv at https://figshare.com/s/5a08f7b84a76586e1601

Input images (step 8 and 9):

1. Output of *Convert_to_bin_ver1.3.ijm :*

t3.tif (red)0_ch1_bin.tif
t3.tif (green)0_ch2_bin.tif

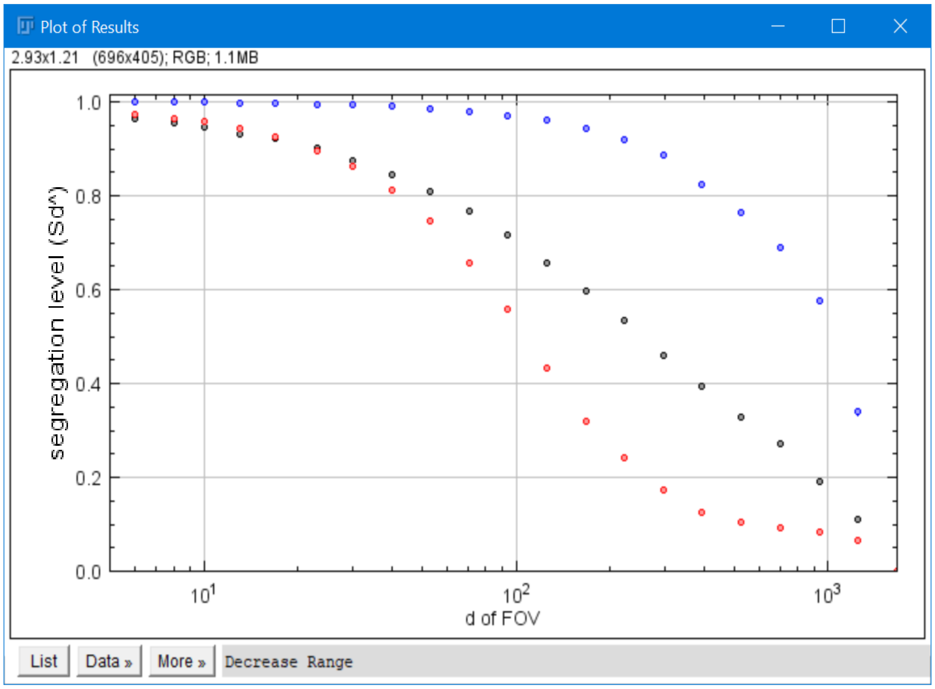
2. Output of *Sim_seg_extremes_ver1.3.ijm:*

segmax_ch1_bin_t3.tif (red)0_ch1_bin.tif
segmin_ch1_bin_t3.tif (red)0_ch1_bin.tif

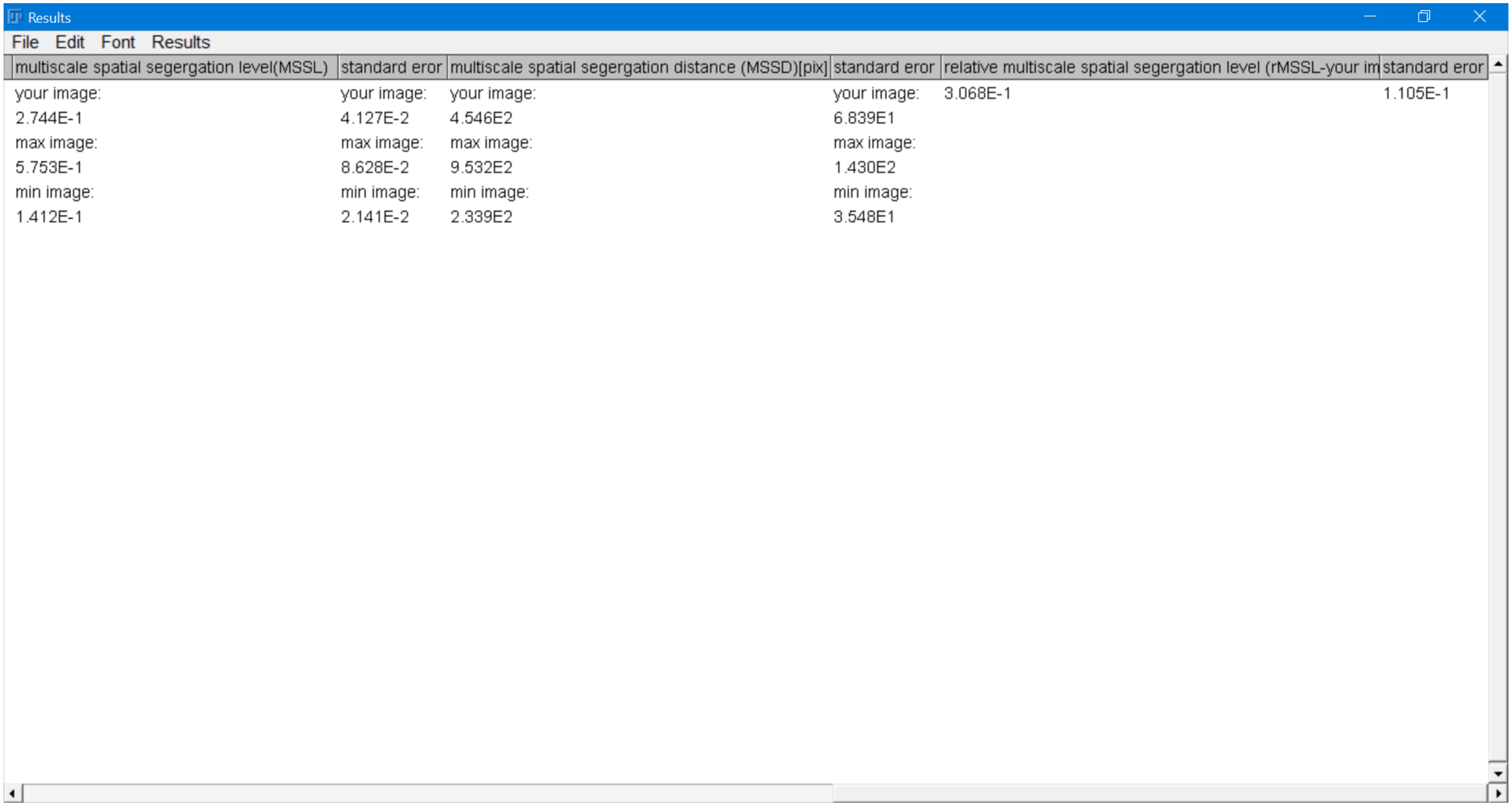
segmax_ch2_bin_t3.tif (green)0_ch2_bin.tif
segmin_ch2_bin_t3.tif (green)0_ch2_bin.tif

Output result (most important):

-the graph of segregation level vs d of FOV for user's sample image (= Your image, black), max seg image (blue) and min seg image (red), (step 11 , *Yes*) :



● Sd^min, ● Sd^ (user's sample image) and ● Sd^max
Part of the Results table showing average segregation measures:



multiscale spatial segergation level(MSSL)	standard eror	multiscale spatial segergation distance (MSSD)[pix]	standard eror	relative multiscale spatial segergation level (rMSSL-your im	standard eror
your image:	your image:	your image:	your image:	3.068E-1	1.105E-1
2.744E-1	4.127E-2	4.546E2	6.839E1		
max image:	max image:	max image:	max image:		
5.753E-1	8.628E-2	9.532E2	1.430E2		
min image:	min image:	min image:	min image:		
1.412E-1	2.141E-2	2.339E2	3.548E1		

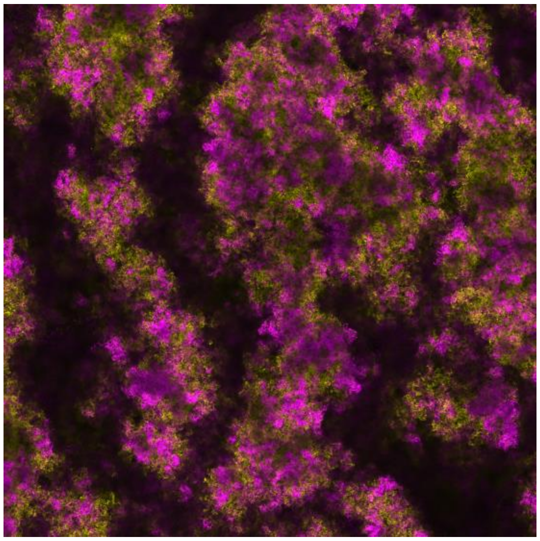
Example 2: bacterial biofilm composed of two strains (microscopy)

Convert_to_bin_ver1.3.ijm

Parameters used: step 5 (*Option 1 and 3*), step 7 (*Option 2*), step 11 (images ch1 and ch2: default threshold of Li Method applied on middle slice) (No other *Options*).

Movie showing the procedure:
Convert_bin_biofilms .wmv at https://figshare.com/s/5a08f7b84a76586e1601

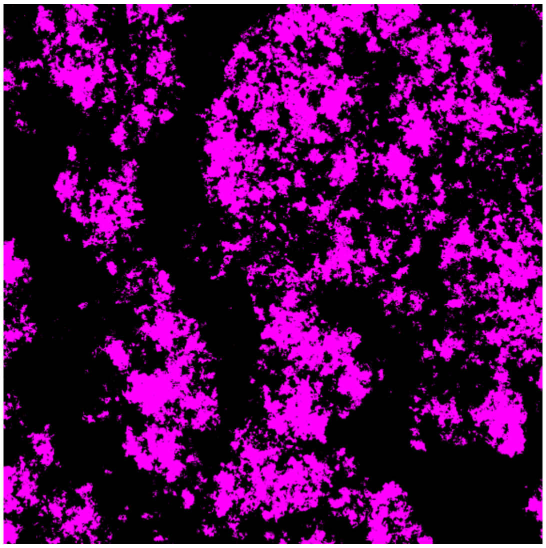
Input images (step 8):



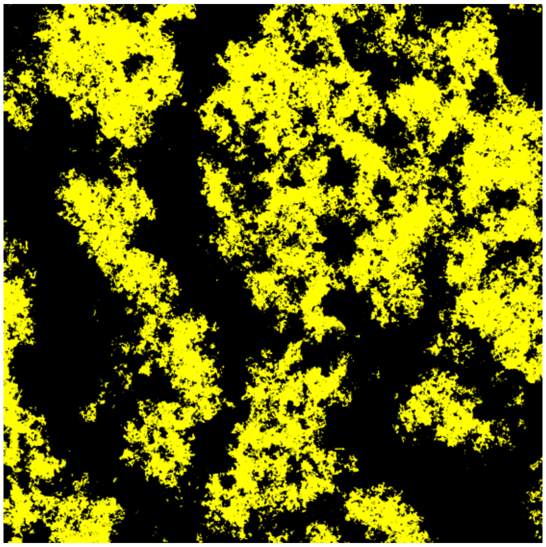
mk + yfp.czi (Zeiss. czi image stack of 17 slices, only middle slice (=9) is shown)

Note that images in this example where pseudolocored in ImegeJ by ch1 = pink snd ch2 = yellow. This is not necessary in order to correctly perform MSS segregation analysis.

Output of images (step 13):



C1-mk + yfp.czi - mk + yfp.czi #10_ch1_bin.tif



C2-mk + yfp.czi - mk + yfp.czi #10_ch2_bin.tif

Sim_seg_extremes_ver1.3.ijm

Parameters used: step 7 (*Yes*), step 8 (*No*), step 9: ch1 = *3* pix, ch2 = *3* pix, step 10: *Randomly placed particles in the image (stack): ch1 and ch2 particles can overlap*, *ch1 particles will replace ch2 particles in the image (stack)*, step 11, (*Yes,* after cca. 75% ch1 particles placed, the rest can be placed by partial overlap; the same for ch2).

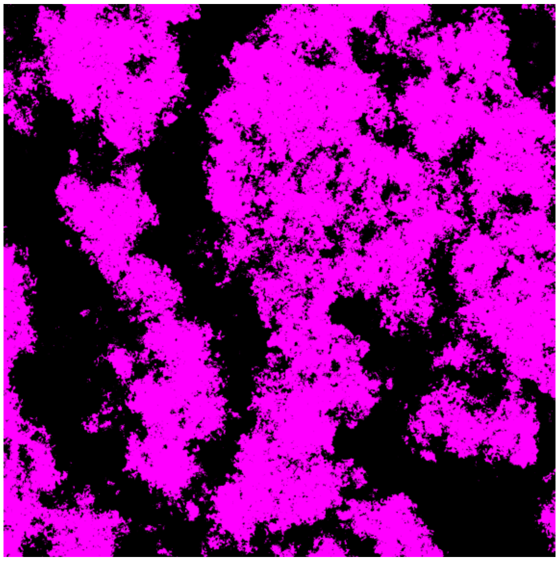
Movie showing the procedure:
segregation_extremes_biofilms.wmv at https://figshare.com/s/5a08f7b84a76586e1601

Input images (step 6):

Output of *Convert_to_bin_ver1.3.ijm :*

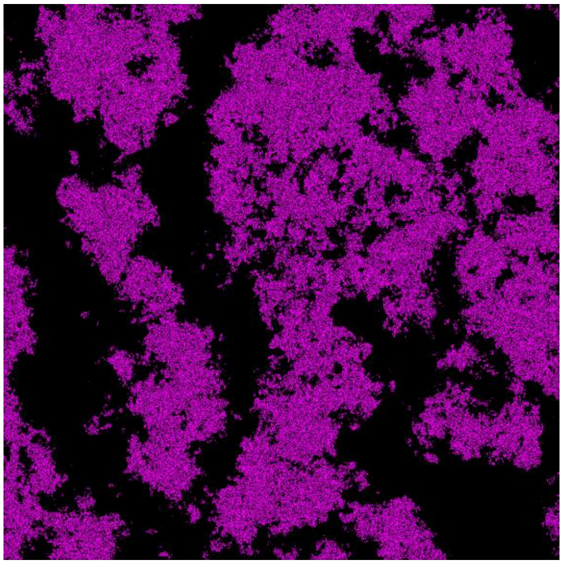
C1-mk + yfp.czi - mk + yfp.czi #10_ch1_bin.tif   C2-mk + yfp.czi - mk + yfp.czi #10_ch2_bin.tif

Output images (step 15):

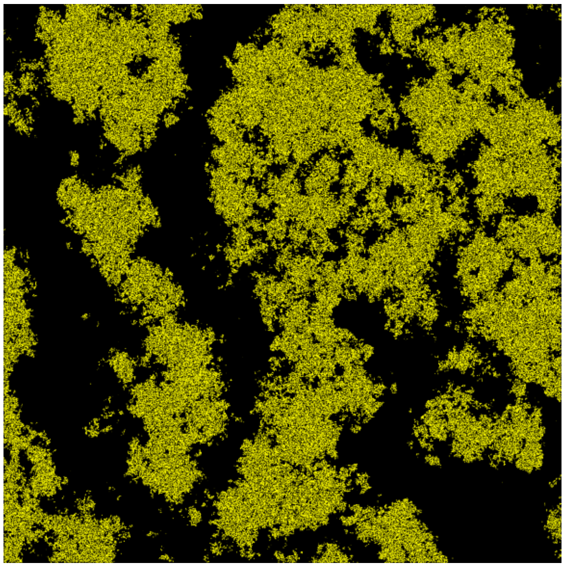


segmax_ch1_bin_C1-mk +yfp.czi -… _ch1_bin.tif

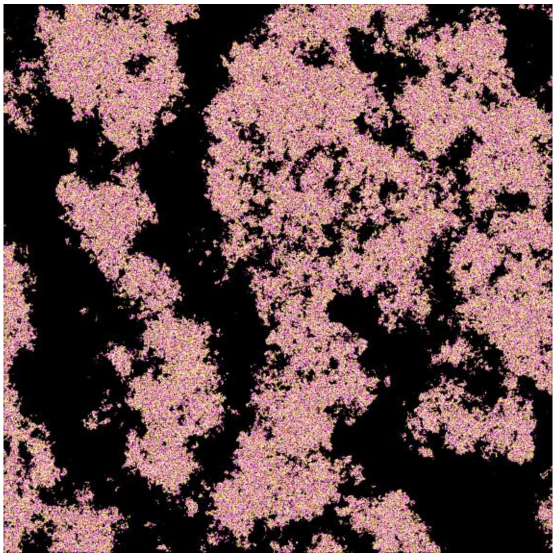
segmax_ch2_bin_C2-mk + yfp.czi - …_ch2_bin.tif



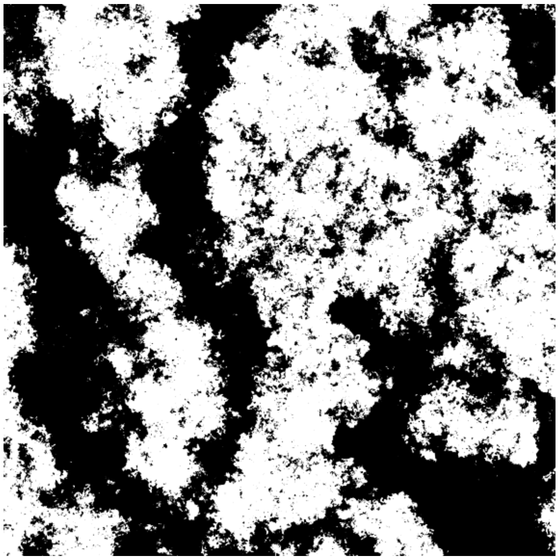
segmin_ch1_bin_C1-mk +yfp.czi -… _ch1_bin.tif



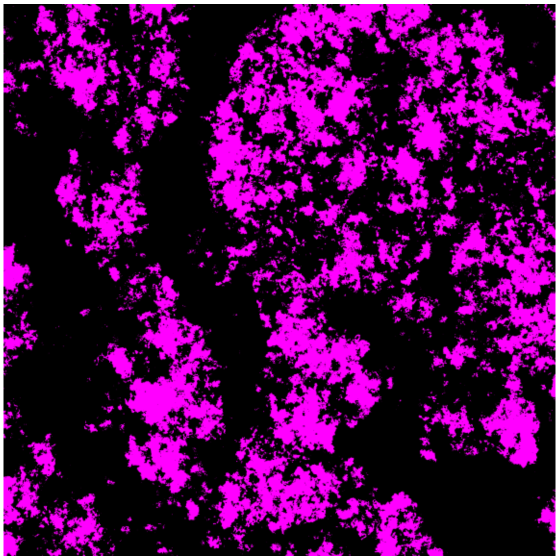
segmax_ch2_bin_C2-mk + yfp.czi - …_ch2_bin.tif



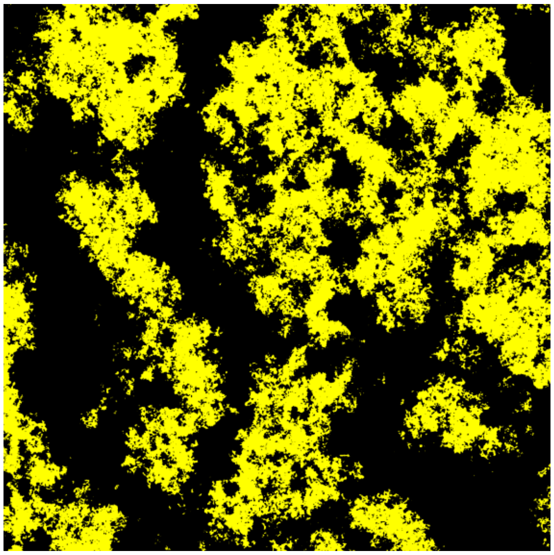
overlap of ch1 min seg and ch2 min seg.tif



Mask_C1-mk + yfp.czi -mk + yfp... _ch2_bin.tif



M_orig_ch1_bin_C1- mk + yfp.czi-..._ch1_bin.tif



M_orig_ch2_bin_C2- mk + yfp.czi -..._ch2_bin.tif

MSS_calc _ver1.3.ijm

Parameters used: step 6 (*Option 1 to 4*), *step 10 (dmax =1964, dmin =7, res_factor =0.75, sampling_factor =0.001), step 11 (Yes)*

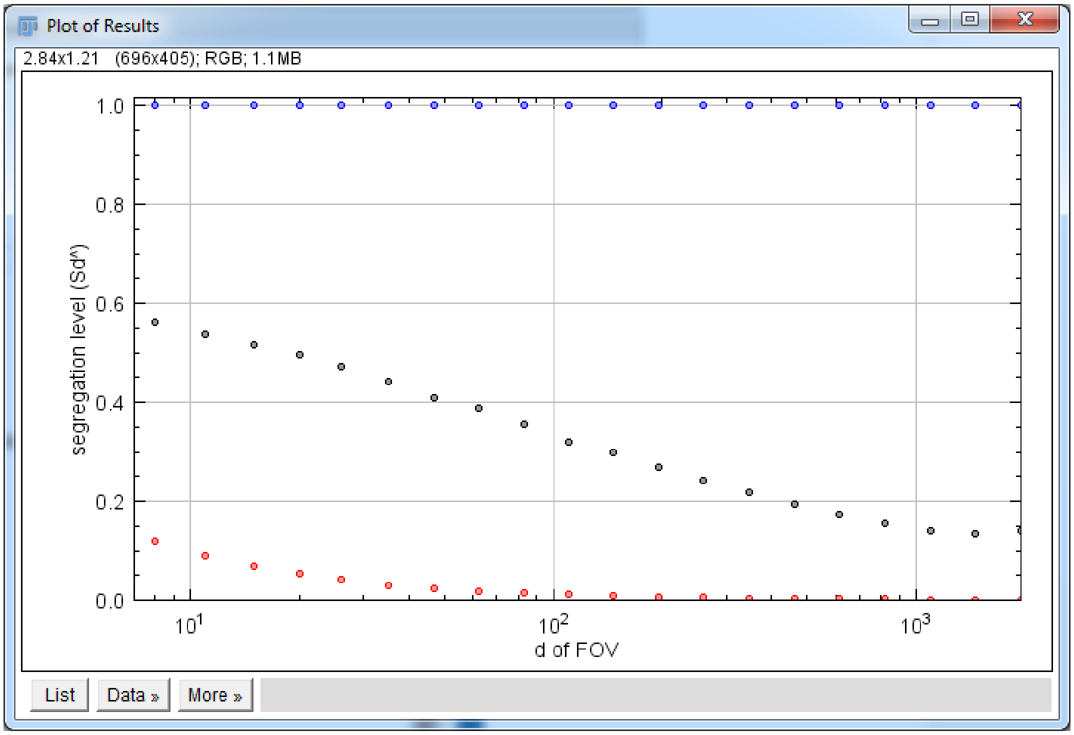
Movie showing the procedure:
MSS_analysis_biofilms.wmv at https://figshare.com/s/5a08f7b84a76586e1601

Input images (step 8 and 9):

1. Output of *Convert_to_bin_ver1.3.ijm :*
   C1-mk + yfp.czi - mk + yfp.czi #10_ch1_bin.tif
   C2-mk + yfp.czi - mk + yfp.czi #10_ch2_bin.tif

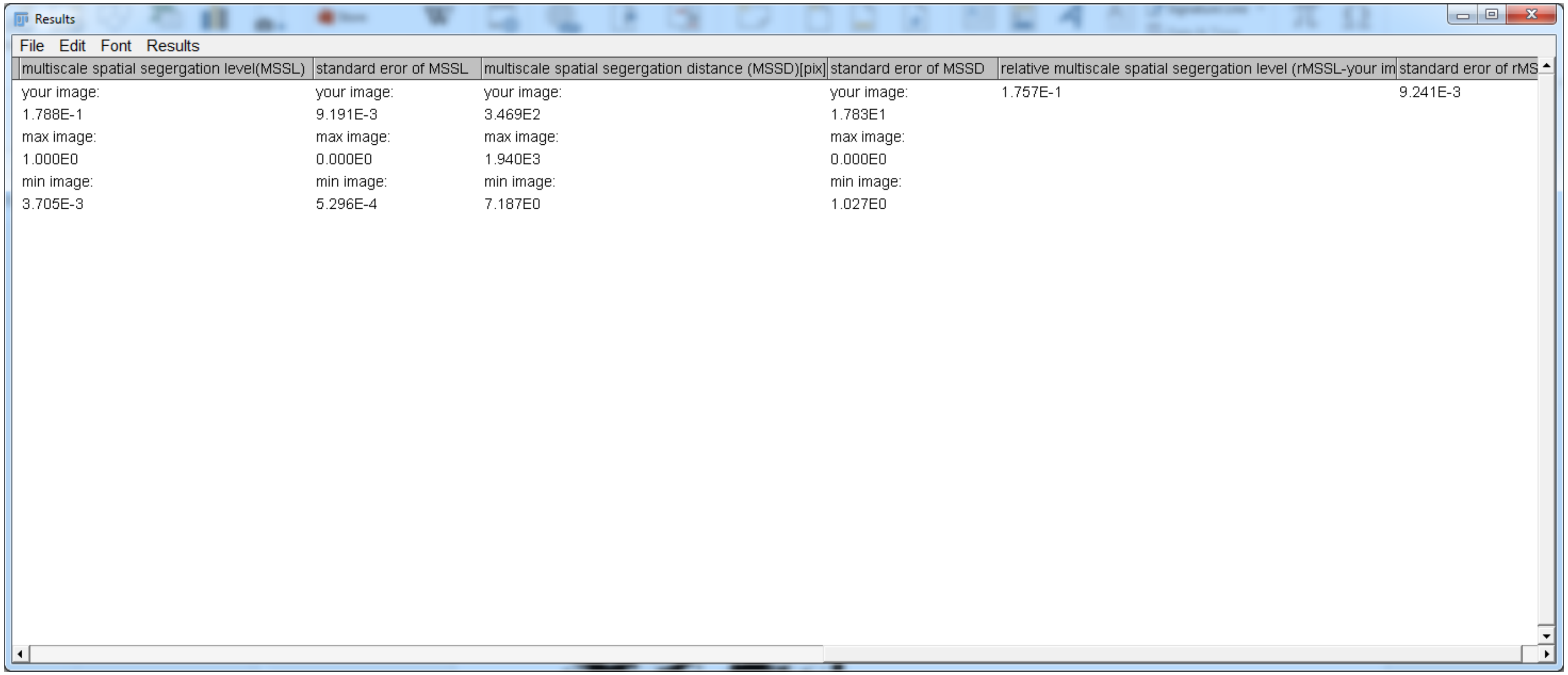
2. Output of *Sim_seg_extremes_ver1.3.ijm:*
   segmax_ch1_bin_C1-mk +yfp.czi -… _ch1_bin.tif
   segmax_ch2_bin_C2-mk + yfp.czi - …_ch2_bin.tif
   segmin_ch1_bin_C1-mk +yfp.czi -… _ch1_bin.tif
   segmin_ch2_bin_C2-mk + yfp.czi - …_ch2_bin.tif

Output result (most important):
-the graph of segregation level vs d of FOV for user's sample image (= Your image, black), max seg image (blue) and min seg image (red), (step 11 , *Yes*) :



● Sd^min, ● Sd^ (user's sample image) and ● Sd^max

Part of the Results table showing average segregation measures:

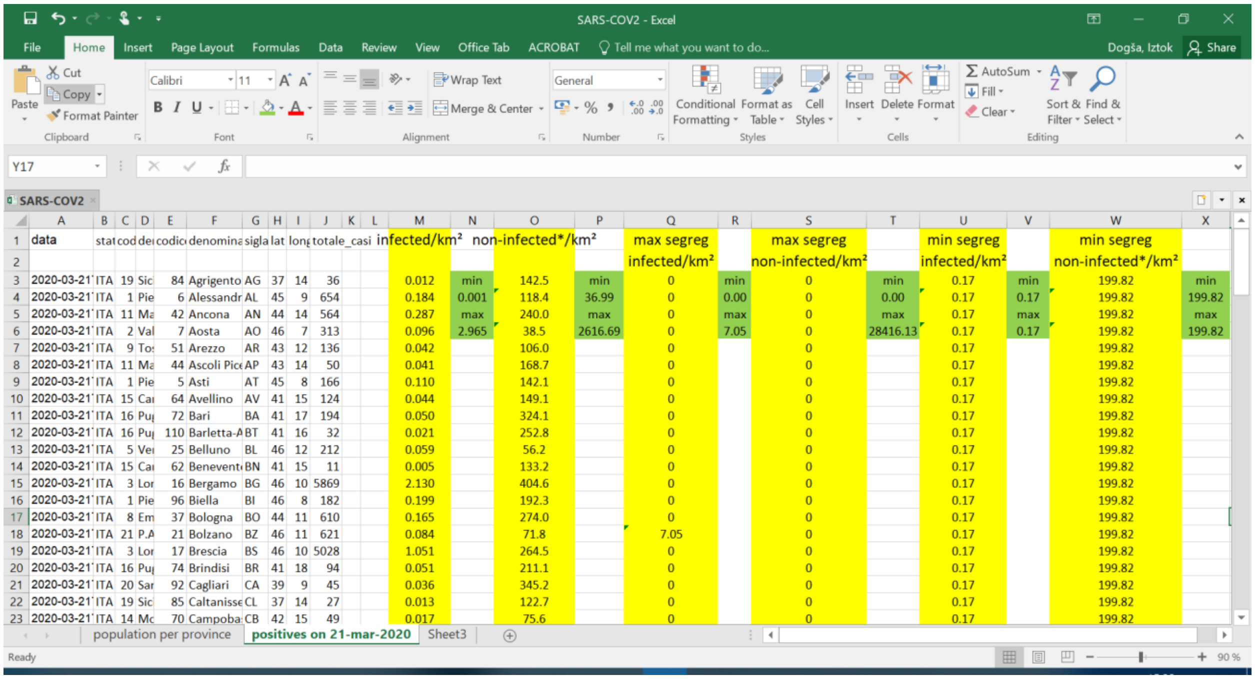


multiscale spatial segergation level(MSSL)	standard eror of MSSL	multiscale spatial segergation distance (MSSD)[pix]	standard eror of MSSD	relative multiscale spatial segergation level (rMSSL-your im	standard eror of rMS
your image:	your image:	your image:	your image:	1.757E-1	9.241E-3
1.788E-1	9.191E-3	3.469E2	1.783E1		
max image:	max image:	max image:	max image:		
1.000E0	0.000E0	1.940E3	0.000E0		
min image:	min image:	min image:	min image:		
3.705E-3	5.296E-4	7.187E0	1.027E0		

Example 3: Mixing of SARS-CoV-2 infected population with non-infected

Obtaining the raw data

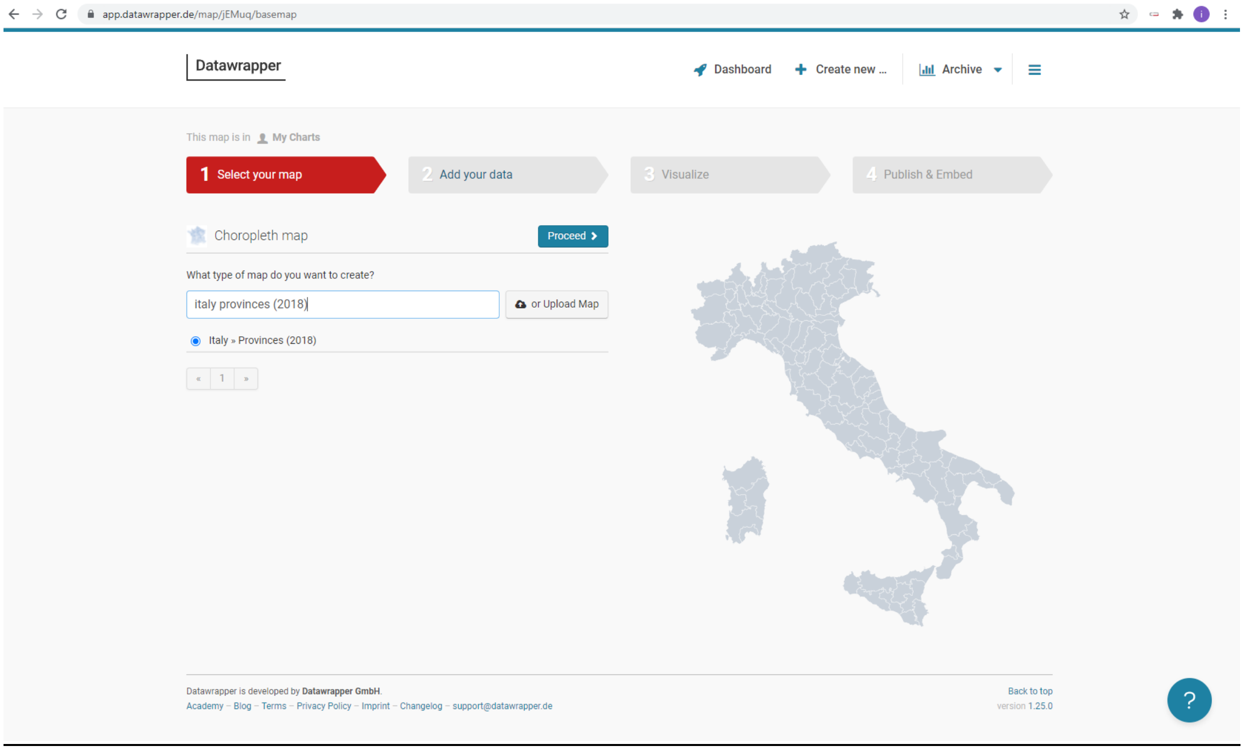
This example is based on epidemiologic data from Italy (https://github.com/pcm-dpc/COVID-19/blob/master/dati-province/dpc-covid19-ita-province-20200321.csv) provided by Italian Civil Protection Department et al. (2020). This data shows how the number of SARS-CoV-2 positives is distributed in all of 107 Italian provinces. The data was imported into excel spreadsheet SARS-COV.xlsx (Sheet name: “positives on 21-mar-2020”) and complemented by data of total population per provinces (Sheet name: “population per province”) obtained by https://www.tuttitalia.it/province/popolazione/. The data was sorted alphabetically by name of the province, excluding the unsigned cases (“in fase de definizione/aggiornamento”). The data in five columns (yellow colored) represents population densities calculated from epidemiologic data and population and density data given in both sheets. The data in green corresponds to min and max values of each column. The data in yellow columns served as input data for choropleth map construction. The data in green serves as input parameters for *Decode_choropleth ver1.3.ijm* . Each column corresponds to one choropleth map of Italy that will be constructed in the next step. The excel spreadsheet (SARS-COV.xlsx) can be obtained on github in folder \Example 3



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
1	data	stat	cod	de	codic	denomina	sigla	lat	long	totale_casi			infected/km^2		non-infected*/km^2		max segreg		max segreg		min segreg		min segreg	
2																	infected/km^2		non-infected/km^2		infected/km^2		non-infected*/km^2	
3	2020-03-21	ITA	19	Sic	84	Agrigento	AG	37	14	36			0.012	min	142.5	min	0	min	0	min	0.17	min	199.82	min
4	2020-03-21	ITA	1	Pie	6	Alessandr	AL	45	9	654			0.184	0.001	118.4	36.99	0	0.00	0	0.00	0.17	0.17	199.82	199.82
5	2020-03-21	ITA	11	Ma	42	Ancona	AN	44	14	564			0.287	max	240.0	max	0	max	0	max	0.17	max	199.82	max
6	2020-03-21	ITA	2	Val	7	Aosta	AO	46	7	313			0.096	2.965	38.5	2616.69	0	7.05	0	28416.13	0.17	0.17	199.82	199.82
7	2020-03-21	ITA	9	To	51	Arezzo	AR	43	12	136			0.042		106.0		0		0		0.17		199.82	
8	2020-03-21	ITA	11	Ma	44	Ascoli Pice	AP	43	14	50			0.041		168.7		0		0		0.17		199.82	
9	2020-03-21	ITA	1	Pie	5	Asti	AT	45	8	166			0.110		142.1		0		0		0.17		199.82	
10	2020-03-21	ITA	15	Ca	64	Avellino	AV	41	15	124			0.044		149.1		0		0		0.17		199.82	
11	2020-03-21	ITA	16	Pu	72	Bari	BA	41	17	194			0.050		324.1		0		0		0.17		199.82	
12	2020-03-21	ITA	16	Pu	110	Barletta-A	BT	41	16	32			0.021		252.8		0		0		0.17		199.82	
13	2020-03-21	ITA	5	Ve	25	Belluno	BL	46	12	212			0.059		56.2		0		0		0.17		199.82	
14	2020-03-21	ITA	15	Ca	62	Benevent	BN	41	15	11			0.005		133.2		0		0		0.17		199.82	
15	2020-03-21	ITA	3	Lor	16	Bergamo	BG	46	10	5869			2.130		404.6		0		0		0.17		199.82	
16	2020-03-21	ITA	1	Pie	96	Biella	BI	46	8	182			0.199		192.3		0		0		0.17		199.82	
17	2020-03-21	ITA	8	Em	37	Bologna	BO	44	11	610			0.165		274.0		0		0		0.17		199.82	
18	2020-03-21	ITA	21	P.A	21	Bolzano	BZ	46	11	621			0.084		71.8		7.05		0		0.17		199.82	
19	2020-03-21	ITA	3	Lor	17	Brescia	BS	46	10	5028			1.051		264.5		0		0		0.17		199.82	
20	2020-03-21	ITA	16	Pu	74	Brindisi	BR	41	18	94			0.051		211.1		0		0		0.17		199.82	
21	2020-03-21	ITA	20	Sar	92	Cagliari	CA	39	9	45			0.036		345.2		0		0		0.17		199.82	
22	2020-03-21	ITA	19	Sic	85	Caltanisse	CL	37	14	27			0.013		122.7		0		0		0.17		199.82	
23	2020-03-21	ITA	14	Mc	70	Campoba	CB	42	15	49			0.017		75.6		0		0		0.17		199.82	

Obtaining choropleth maps

The choropleth maps that served as input images for *Decode_choropleth ver1.3.ijm* were constructed by online app available at https://www.datawrapper.de/maps/choropleth-map .The basic version of app used for this example is freely available. To construct the choropleth maps visit https://app.datawrapper.de/select/map and select "Choropleth map"



app.datawrapper.de/map/jEMuq/basemap

This map is in My Charts

What type of map do you want to create?

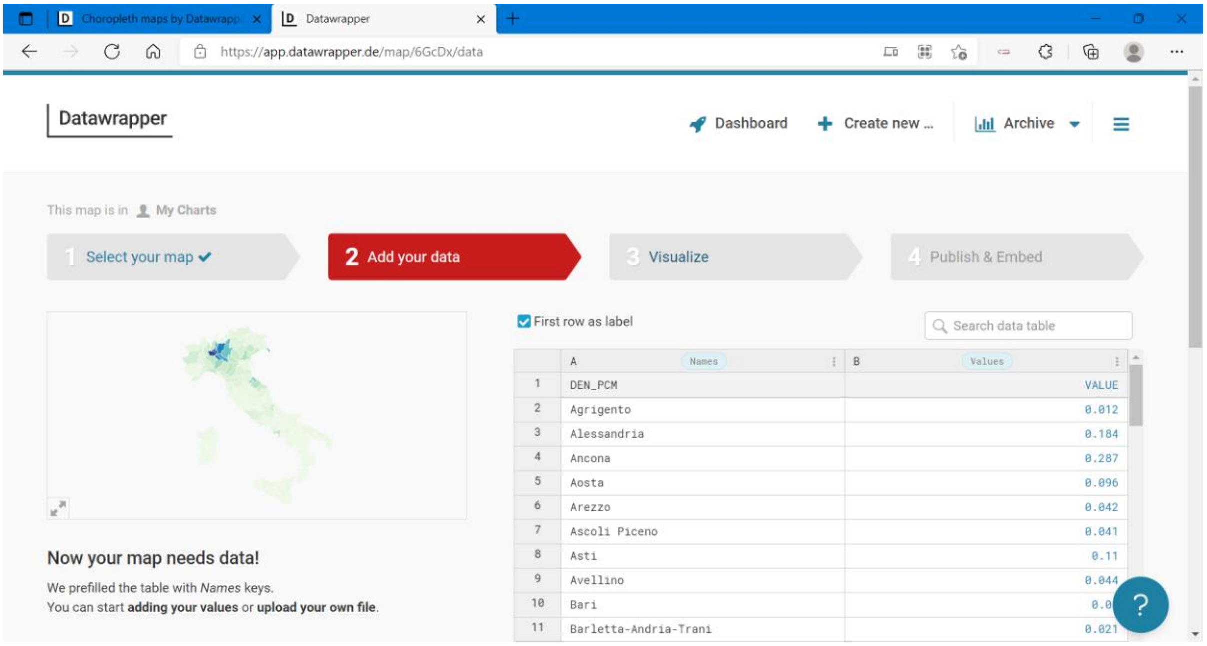
italy provinces (2018)

● Italy » Provinces (2018)

Datawrapper is developed by Datawrapper GmbH.
Academy – Blog – Terms – Privacy Policy – Imprint – Changelog – support@datawrapper.de

version 1.25.0

In step 1 select your map by typing "Italy provinces (2018)" and then go to step 2, where you should copy/paste the data in yellow columns of SARS-COV.xlsx spreadsheet. Each column correponds to one map data needs to be created seperately.


Choropleth maps by Datawrapp
Datawrapper
https://app.datawrapper.de/map/6GcDx/data
Datawrapper
Dashboard
Create new ...
Archive

This map is in My Charts

1 Select your map ✓ | 2 Add your data | 3 Visualize | 4 Publish & Embed

☑ First row as label

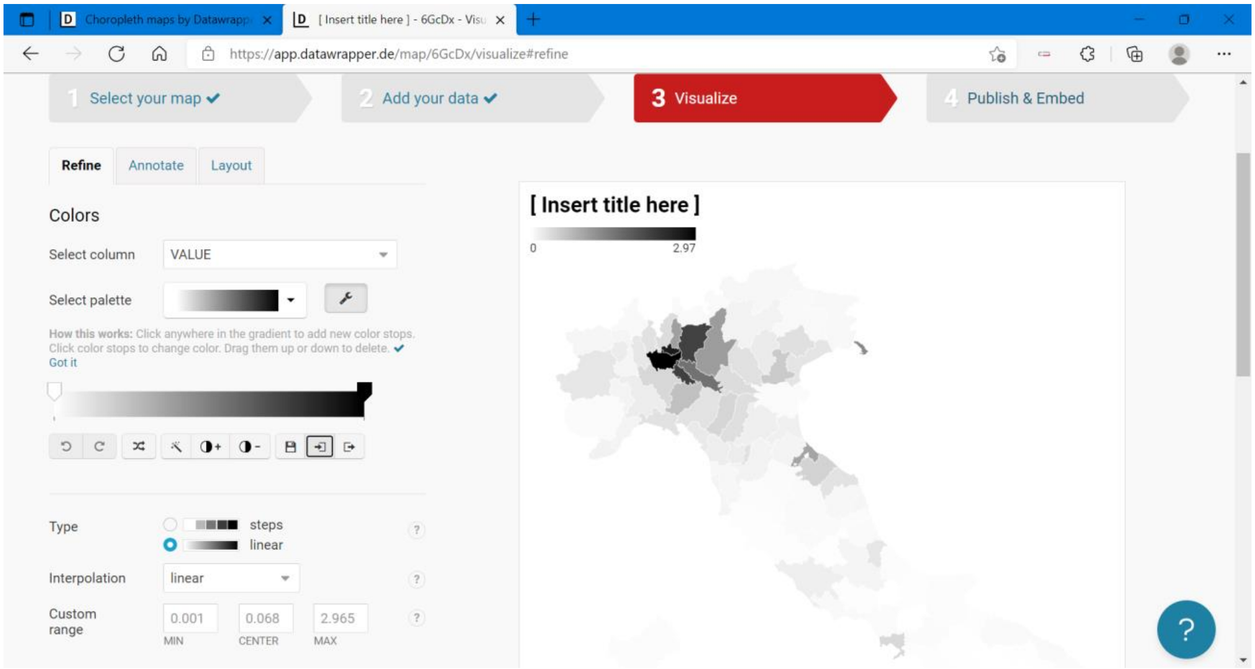
Search data table

	A (Names)	B (Values)
1	DEN_PCM	VALUE
2	Agrigento	0.012
3	Alessandria	0.184
4	Ancona	0.287
5	Aosta	0.096
6	Arezzo	0.042
7	Ascoli Piceno	0.041
8	Asti	0.11
9	Avellino	0.044
10	Bari	0.0
11	Barletta-Andria-Trani	0.021

Now your map needs data!

We prefilled the table with *Names* keys.
You can start **adding your values** or **upload your own file**.

In step 3 you should visualize the data by creating best colour palate suitable for *Decode_choropleth ver1.3.ijm*. This is black and white linear color palate, which however is not avaialable in the “Select palate” in the current version of the datawrapper app. However, one can easily create it by clicking the wrench tool icon 🔧 and then “Import colors” icon .In the pop up window type #FFFFFF,#000000 and click OK. After this you should see this:


Choropleth maps by Datawrapp
[Insert title here] - 6GcDx - Visu
https://app.datawrapper.de/map/6GcDx/visualize#refine

1 Select your map ✓ | 2 Add your data ✓ | 3 Visualize | 4 Publish & Embed

Refine | Annotate | Layout

Colors

Select column: VALUE

Select palette

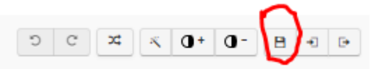
How this works: Click anywhere in the gradient to add new color stops. Click color stops to change color. Drag them up or down to delete. ✓ Got it

Type: ○ steps ● linear

Interpolation: linear

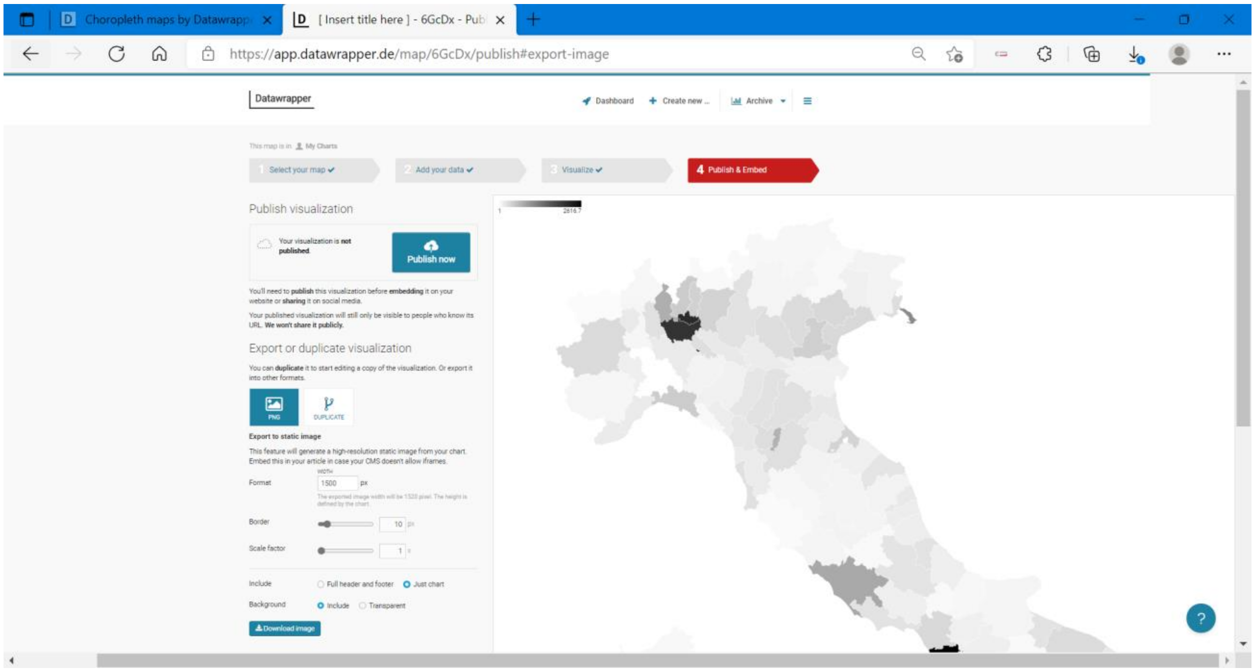
Custom range: 0.001 (MIN) 0.068 (CENTER) 2.965 (MAX)

[Insert title here]

Press the diskette icon  to keep the new color palette for later.

In the bottom of the page (not seen above) select maximal Max. height to 1500 px (if you need higher/lower resolution you can adjust this value).

In step 4 "Publish & Embed" select PNG, format width 1500 px, Border 10 px and Scale factor 1x. Include Just chart and Include Background.



Publish visualization

Your visualization is **not published**.

Publish now

You'll need to **publish** this visualization before **embedding** it on your website or **sharing** it on social media.

Your published visualization will still only be visible to people who know its URL. **We won't share it publicly.**

Export or duplicate visualization

You can **duplicate** it to start editing a copy of the visualization. Or export it into other formats.

PNG DUPLICATE

Export to static image

This feature will generate a high-resolution static image from your chart. Embed this in your article in case your CMS doesn't allow iframes.

Format WIDTH 1500 px

The exported image width will be 1520 pixel. The height is defined by the chart.

Border 10 px

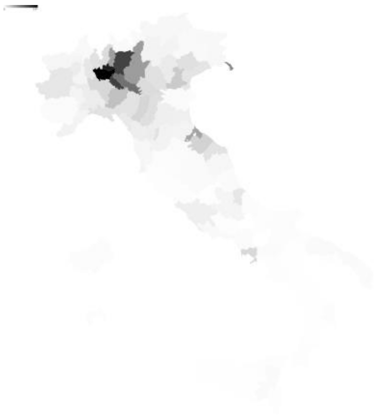
Scale factor 1 x

Include ○ Full header and footer ● Just chart

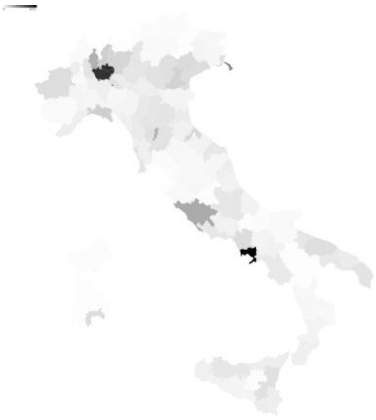
Background ● Include ○ Transparent

Download image

Finally, press Download image and you will obtain choropleth map that serves as an input image for *Decode_choropleth ver1.3.ijm*. Going back by clicking on step 2 "Add your data" enables you to replace the current data by the data from next yellow column in SARS-COV.xlsx spreadsheet. By keeping the rest of the paramteres settings the same (in all steps) and going through step 3 and 4, you will finally obtain all six choropleth maps that are neccesary to perform seggregation analysis. In this way obtained choropleth maps can be accessed on github in folder \Example 3 and serve as input for *Decode_choropleth ver1.3.ijm*



21-03-2020_infected.png



21-03-2020_non-infected.png

21-03-2020_infected_max_seg.png

21-03-2020_non-infected_max_seg.png

21-03-2020_infected_min_seg.png

21-03-2020_non-infected_min_seg.png

Decode_choropleth ver1.3.ijm

Input images: step 8: (21-03-2020_infected.png), step 10 (21-03-2020_non-infected_min_seg.png)

Parameters used: step 9 (Yes), step 10 (uncheck “the mask image is already created, OK), step 11 (Yes), step 12 (Yes, by Rectangular tool grey scale in upper left corner was selected), step 13 (lower-threshold [upper slider]: 136, upper-threshold [lower slider]: 255), step 14 (*min original value: 0.001, max original value: 2.965, option: "No, or not sure- the color scale in the image will be used to extract the original values (the color scale must be present)",* step 15 (OK), step 16 (Yes), step 17 (OK, OK).

For rest of the choropleth input images rerun the *Decode_choropleth ver1.3.ijm* and input them in step 8; step 10: mask_21-03-2020_infected_min_seg.tif

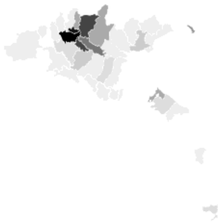
Parameters used: step 9 (Yes), step 10 (check “the mask image is already created, OK), step 14 (*min original value: XX, max original value: YY, option: "No, or not sure- the color scale in the image will be used* *to extract the original values (the color scale must be present)",* step 15 (OK), step 16 (Yes), step 17 (OK, OK).

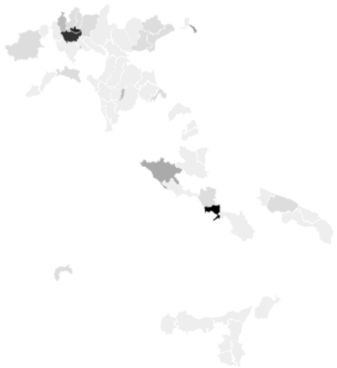
Input values XX and YY in step 14 are obtained from SARS-COV.xlsx for corresponding input images

	XX	*YY*
21-03-2020_non-infected.png	*36.99*	*2616.69*
21-03-2020_ infected_max_seg.png	*0.00*	*7.05*
21-03-2020_ non-infected_max_seg.png	*0.00*	*28416.13*
21-03-2020_infected_min_seg.png	*0.17*	*0.17*
21-03-2020_non-infected_min_seg.png	*199.82*	*199.82*

Movie showing the procedure:
decoding_choropleths.wmv at https://figshare.com/s/5a08f7b84a76586e1601

Output images (step 17, after decoding of all choropleths):1





decod_21-03-2020_infected.tif   decod_21-03-2020_non-infected.tif   decod_21-03-2020_infected_max_seg.tif

decod_21-03-2020_non-infected_max_seg.tif decod_21-03-2020_infected_min_seg.tif decod_21-03-2020_non-infected_min_seg.tif

mask_21-03-2020_infected_min_seg.tif

MSS_calc _ver1.3.ijm

Parameters used: step 6 (*Option 1 to 5*), *step 10 (dmax =1520, dmin =1, res_factor =0.75, sampling_factor =0.003), step 11 (Yes)*

Movie showing the procedure:
MSS_analysis_decoding_choropleths_2.wmv at https://figshare.com/s/5a08f7b84a76586e1601

Input images (step 8 and 9): Output of *Decode_choropleth ver1.3.ijm*

Step 8:

decod_21-03-2020_infected.tif

decod_21-03-2020_non-infected.tif

step 9:

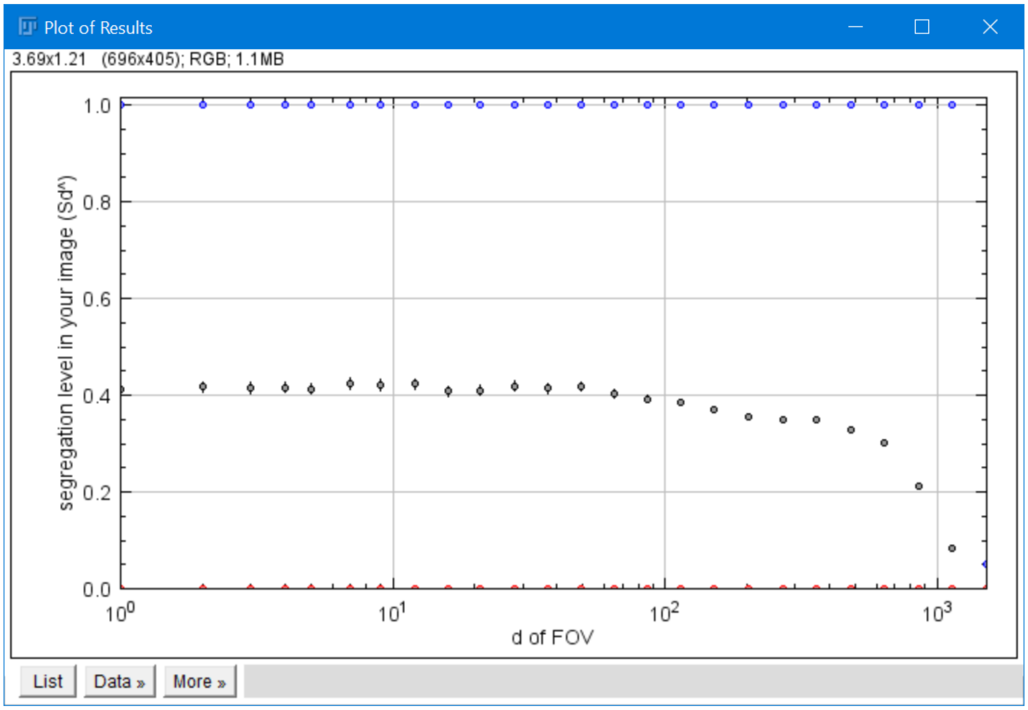
decod_21-03-2020_infected_max_seg.tif

decod_21-03-2020_non-infected_max_seg.tif

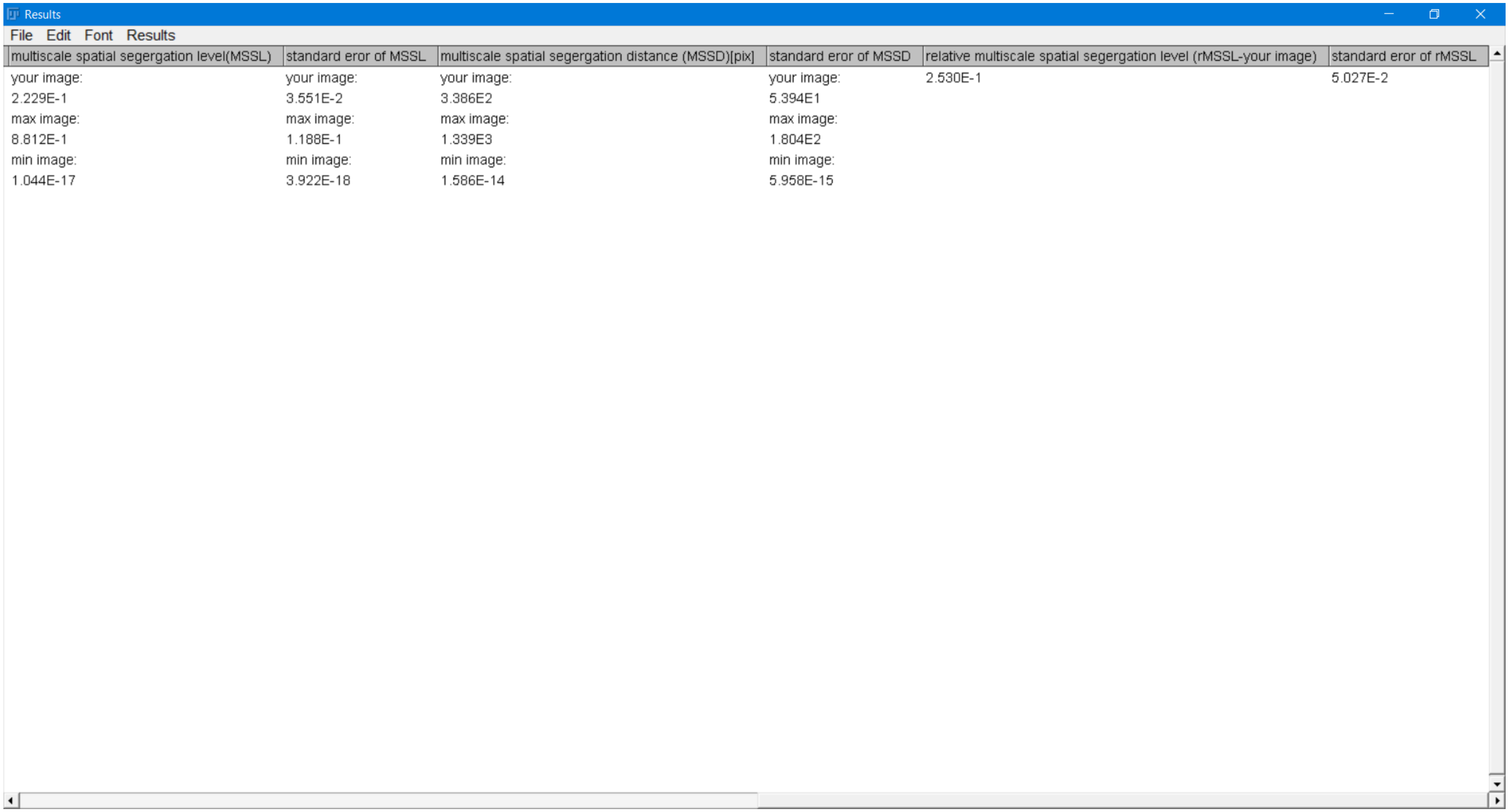
decod_21-03-2020_infected_min_seg.tif

decod_21-03-2020_non-infected_min_seg.tif

Output result (most important):
-the graph of segregation level vs d of FOV for user's sample image (= Your image, black), max seg image (blue) and min seg image (red), (step 11 , *Yes*) :



● Sd^min, ● Sd^ (user's sample image) and ● Sd^max



multiscale spatial segergation level(MSSL)	standard eror of MSSL	multiscale spatial segergation distance (MSSD)[pix]	standard eror of MSSD	relative multiscale spatial segergation level (rMSSL-your image)	standard eror of rMSSL
your image:	your image:	your image:	your image:	2.530E-1	5.027E-2
2.229E-1	3.551E-2	3.386E2	5.394E1		
max image:	max image:	max image:	max image:		
8.812E-1	1.188E-1	1.339E3	1.804E2		
min image:	min image:	min image:	min image:		
1.044E-17	3.922E-18	1.586E-14	5.958E-15		

References

Italian Civil Protection Department, Morettini, M., Sbrollini, A., Marcantoni, I., Burattini, L. COVID-19 in Italy: Dataset of the Italian Civil Protection Department. Data in Brief 30,105526–33 (2020).