Microscopy Image Analysis Software for Medical Applications

MIASMA

Particulate Matter Analysis

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Overview of *MIASMA*

MIASMA is Microscopy Image Analysis Software for Medical Applications, the collective name for a number of projects involving image analysis in which I am collaborating with medics. I am the author of software for image analysis of scanning microscopy images, principally for applications in nanoscience and related disciplines. The software that I have written, and continue to develop and expand, is *Image SXM*. Although written for scanning microscopy applications, I have found that *Image SXM* is an excellent platform on which to develop specialist image analysis solutions for the specific needs of users, including those who obtain images from light microscopes. *MIASMA* is the result of a number of these specialist applications having some common ground and so benefiting from being considered as part of a larger, overarching project.

Particulate Matter Analysis

Image SXM contains routines that have been written to identify and quantify the amount of particulate matter in macrophage cells. In the following pages the process by which the particulate matter is quantified is outlined and the effects of user input are explained. These notes are not intended to be comprehensive documentation, but should be enough to give the user an idea of how the processing is carried out and allow the user to use reasonable judgement in selecting input options.

For more information on MIASMA see the web page http://www.liv.ac.uk/~sdb/MIASMA
For help using the other functions of *Image SXM* see http://www.ImageSXM.org.uk
If you have any problems using *Image SXM*, email me S.D.Barrett@liv.ac.uk

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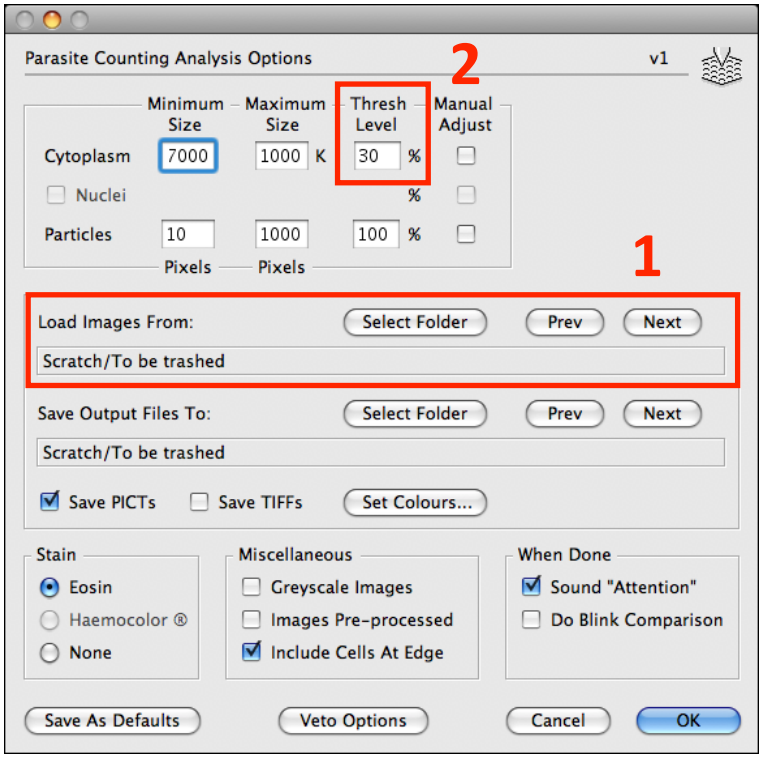
The Analysis Process

The raw (TIFF) images are loaded from a folder selected by the user [1], or a set of folders within the folder selected by the user. The images are processed folder by folder.

Firstly, images within the selected folder (the top folder) are processed. Secondly, images within any sub-folders are processed.

A maximum of 256 images in each of 32 folders can be processed at a time.



For each image, the following generic processing is carried out...

Cells
- create an image that makes cells look dark (*cf* background)
- check histogram of pixel values and note lowest and highest values
- threshold at value specified in dialog box [2] — see p3
  [threshold at low values (~25%) to include most of cytoplasm]
  [threshold at high values (~75%) to include only denser region]
- optionally allow user to adjust threshold manually

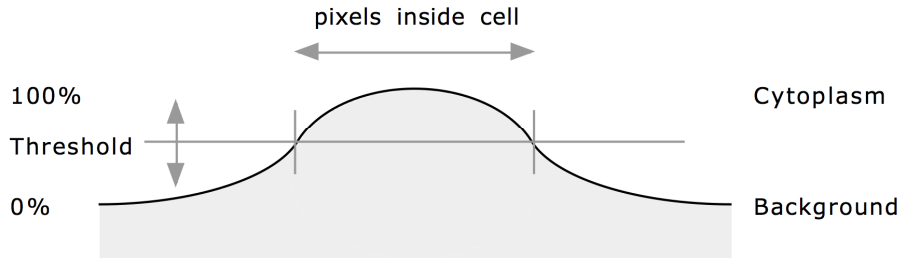
Nuclei
- create an image that makes nuclei look dark (*cf* particulate matter)
- threshold at fixed level (adjusted by user–specified value in dialog box)
- optionally allow user to adjust threshold manually

Particles
- create an image that makes particulate matter look dark (*cf* nuclei)
- threshold at fixed level (adjusted by user–specified value in dialog box)
- optionally allow user to adjust threshold manually
- invert the image and threshold again to find interior 'holes' in pm

The algorithms that create the images and generate the threshold levels that discriminate between these objects depend on the stain applied and are described in Appendix 1.

Setting Thresholds

For cytoplasm the threshold level specified in the dialog box relates to the range between the background intensity and the mean cytoplasm intensity.



The default value of 50% should be appropriate for many analyses. Set the threshold to a lower value (~ 25%) to include more of the cytoplasm edges or to a higher value (~ 75%) to include only the denser regions.

For nuclei and particulate matter the threshold levels specified in the dialog box are expressed as a percentage of the corresponding threshold levels determined by the analysis algorithms. These should be changed from the default values of 100% only if there is a consistent under/ overestimate of the calculated areas (as determined by, for instance, the Blink Comparator).

Calculation of Particulate Matter / Cytoplasm Ratio

- identify pixels inside cell outline = C
- identify pixels inside nuclei outline = N (optional)
- identify pixels inside particle outline = oP
- identify pixels inside pm interior holes = iP

- P = oP – iP (subtract interior holes from pm)

- reject pixels inside N but outside C
- reject pixels inside P but outside C

- N = N + iP (count interior holes as nuclei)
- Cy = C – N (cytoplasm = non-nuclear cell)

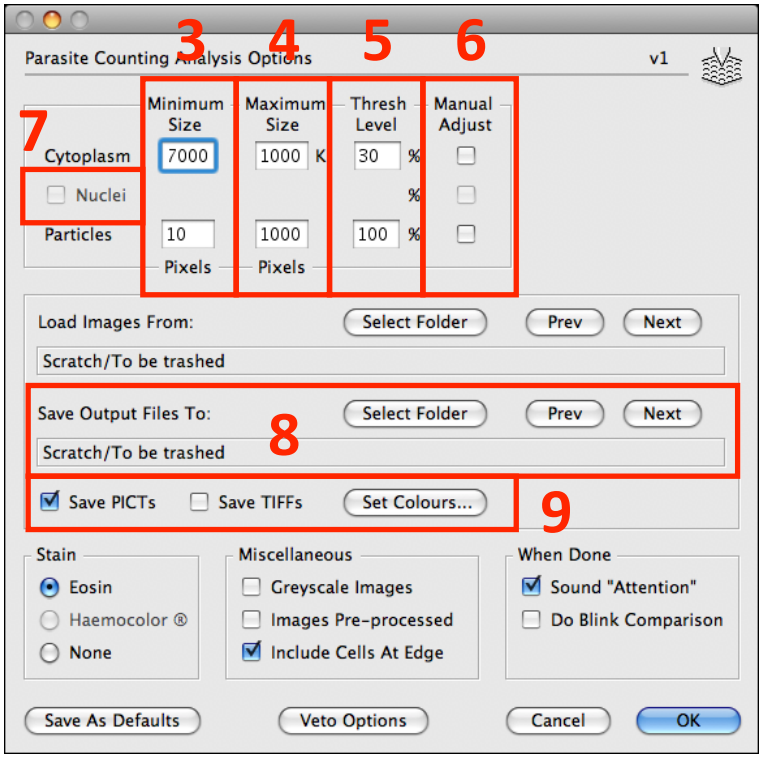
- count pixels in P
- count pixels in N
- count pixels in Cy

- particulate matter = P/Cy

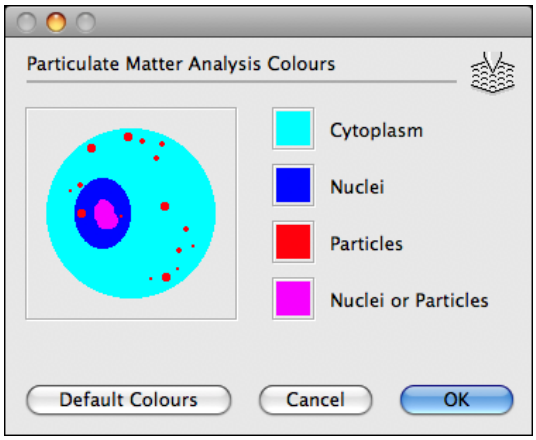
Selecting Options

Lower [3] and upper [4] limits to the sizes of objects that are included in the analysis can be set. The sizes are specified in pixels for the minimum size and thousands of pixels for the maximum size.

Thresholds for each object are set here [5]. The value for cytoplasm affects the measured areas of cells that have spread out (see p2). The value for nuclei and particles are percentages of the values calculated by the program, and should only be changed from 100% if the values calculated by the program are consistently wrong.



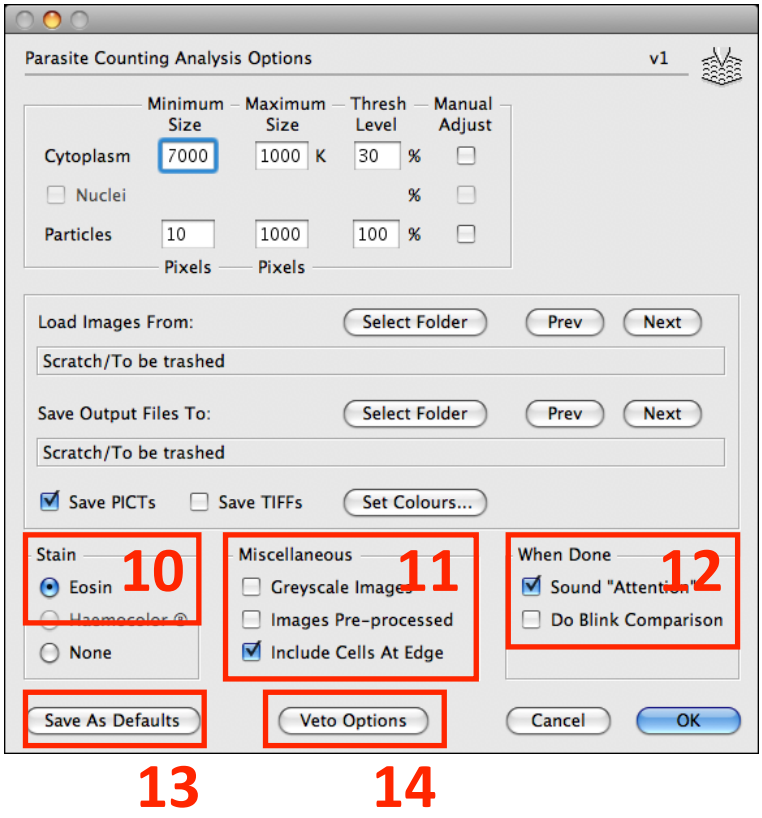
Thresholds calculated by the program can be manually adjusted by the user on an image-by-image basis [6]. The threshold level is displayed in the Info window and the values before and after user adjustment are recorded in the log file. If you are unhappy with an image even after making these manual adjustments, press the 'V' key to veto that image from the calculation of the mean value of the relative area of particulate matter. Nuclei can be ignored completely in the analysis by unchecking this item [7]. When the eosin stain is selected, this item is disabled.



Output files (see p6) are saved to a folder selected by the user [8]. Optionally, colour 'maps' showing which pixels were identified as cytoplasm, nuclei or particulate matter can be saved as PICT or TIFF images [9]. The colours used can be specified in a separate dialog box [9] (left).

If the cells have been stained using eosin then select this item [10]. These analysis routines may not work with any other stain unless the code is modified — email Steve Barrett <S.D.Barrett @ liv.ac.uk> if you use Haemocolor ® or a different stain.

Select the appropriate items [11] if the images are greyscale (rather than 24-bit or 36-bit colour TIFFs) or have been pre-processed to remove the background and cell nuclei. Cells touching the edge of an image can be excluded from the analysis if you think that this will effect the calculation of the relative area of particulate matter.



The Blink Comparator [12] can be selected to launch when the analysis is complete so that you can carry out a visual check of the results.

The Save As Defaults button [13] saves the Preferences file with the current settings of the dialog box so that these will be the default values next time *Image SXM* is launched.

Some images may be vetoed from the calculation of the average value of P/Cy [14]. See p6 for details.

Output Files

Three text files are generated for each analysis run

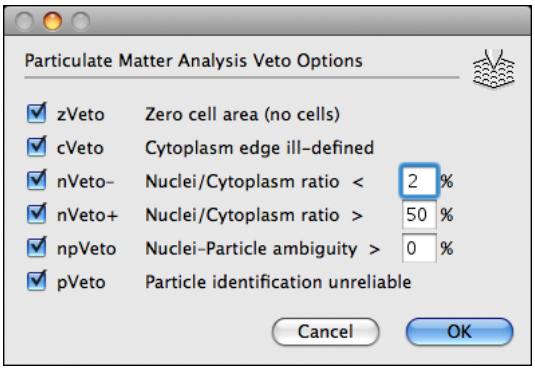
Log-yymmddhhmm.txt
PM-A-yymmddhhmm.txt
PM-G-yymmddhhmm.txt

where yymmdd are the date and hhmm the time at which the analysis ended. The log file contains full information of the analysis parameters and the areas (in kPixels) of cytoplasm, nuclei and particulate matter in each image. The PM-A and PM-G files contains just the (arithmetic and geometric) mean P/Cy values and their standard deviations for each folder of images analysed. If the analysis fails to complete due to an error, the file 'Log-Crash.txt' records the results for all folders analysed up to the time at which the error occurred.

Some images may be vetoed from the calculation of the average value of P/Cy. These are indicated in the log file with the following flags:

Flag	Reason for veto
zVeto	zero cytoplasm area (no cells)
cVeto	cytoplasm edge ill–defined
nVeto–	nuclei too small as fraction of cytoplasm
nVeto+	nuclei too large as fraction of cytoplasm
npVeto	discrimination between nuclei and PM ambiguous
pVeto	identification of particulate matter ambiguous
uVeto	veto by user when manually inspecting images

Each veto can be enabled/disabled individually and the criteria fine–tuned in the Veto Options dialog box.



If the pVeto is disabled but the analysis determines that an image is underexposed (too many dark pixels) or overexposed (too many bright pixels) then this will be indicated in the log as *UnEx* or *OvEx*. The image will be counted even though the value of P/Cy may be unreliable.

Using the Analysis Routines

I would recommend that you start by analysing just a few typical images to establish whether or not the default settings are appropriate. Either copy a few images into a test folder, or analyse one image at a time by pressing the shift key before selecting the Particulate Matter Analysis item from the Cells menu (the menu item changes to 'PM Analysis for Single Image'). You can then analyse the rest of your images in one of two ways:

i) Run the analysis on one or more folders containing your images with all the settings at the default values. On automatic, the analysis will take a few seconds per image. The time remaining to complete the analysis is indicated in the Info window in the bottom left corner of the screen. Go and have a cup of tea.

   When the analysis is complete, use the Blink Comparator to check each image with the output 'map' showing where pixels were identified as cytoplasm, nuclei and and particulate matter. The Blink Comparator toggles between the original image and its corresponding map so that you can judge whether or not the identification was satisfactory.

   Note whether or not there is any consistent under/overestimate of the areas of any of the objects. If there is, adjust the threshold levels (see p4) and run again. Lowering (raising) a threshold level will count more (less) pixels and hence increase (decrease) the measured area of an object.

   If there is no consistent under/overestimate of the areas but some objects in some images have been identified incorrectly, either:

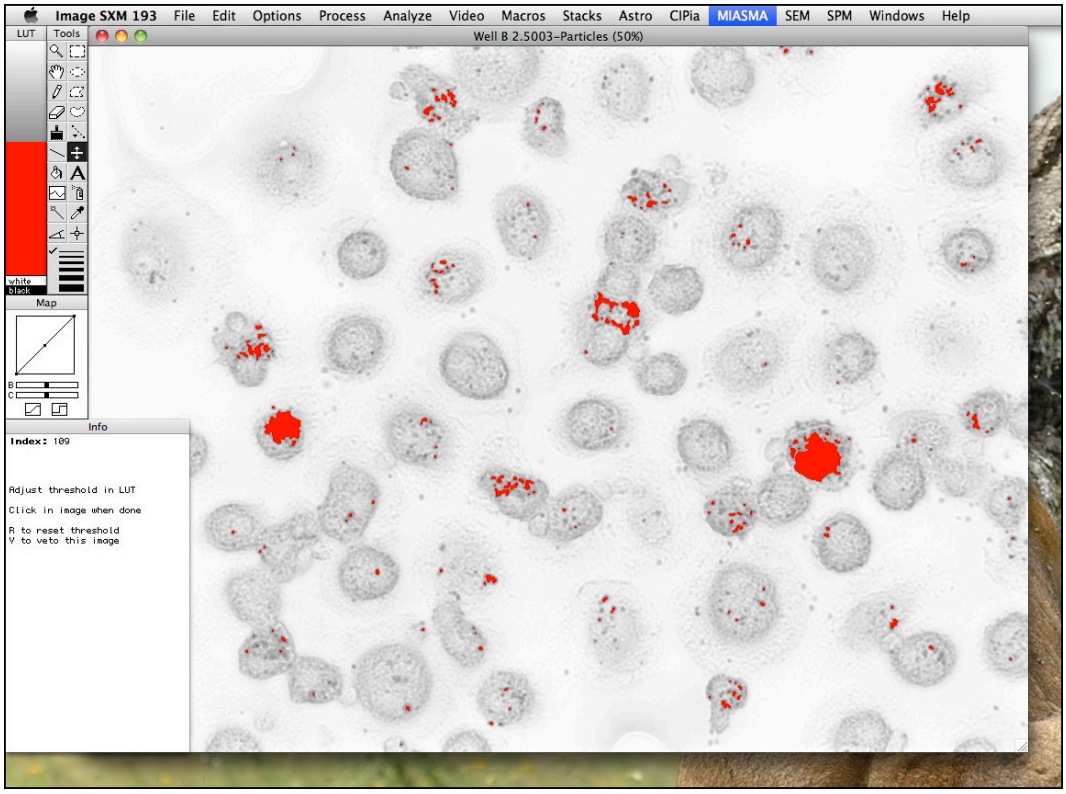
   (a) remove the files from the folder and re-run the analysis, or

   (b) re-run the analysis with the Manual Adjust option selected and either adjust the threshold until you are happy with the area measured or veto the image.

ii) Run the analysis with the Manual Adjust options selected. For each image, the analysis will pause and allow you to adjust the thresholds that determine the areas of cytoplasm, nuclei and particulate matter. Adjust the threshold until you are happy with the area measured or veto the image. Both the automatic and the manually adjusted threshold values are recorded in the log file.

Manual adjustment of a threshold is carried out by dragging the red/grey boundary line in the look-up table (LUT) window whilst looking at the effect this has on the pixels in the image.

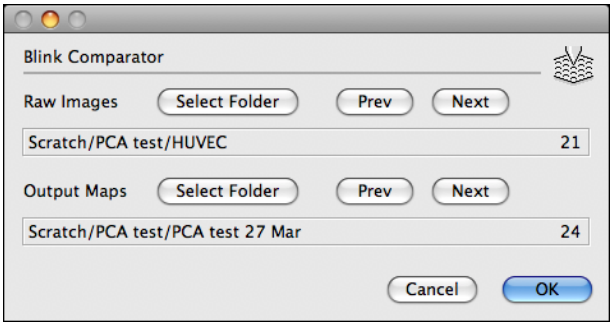
> >



When you are happy with the threshold, click in the image. An image can be vetoed by pressing 'V' at any time during manual threshold adjustment.

Blink Comparator

For a quick way to check whether or not the PMA routines have done their job correctly use the Blink Comparator to jump back and forth (blink) between the original images and the corresponding colour maps of cytoplasm/nuclei/particulate matter.



Select the folders containing the original images and the output maps using the 'Select Folder' buttons, or the Prev/Next (or, with the option key, Up/Down) buttons to jump quickly between folders. The number at the end of the folder path name shows how many images are in the folder — a quick check that you have selected the correct folder(s). When blinking, use the Tab or Space keys to blink between images, or the Caps Lock key to blink continuously every half second. Use the arrow keys to move to the next image in a folder. Instructions are summarised in the Info window.

Menu Location

By default the 'Particulate Matter Analysis' menu item appears in the menu structure of *Image SXM* in a series of sub-menus:

Analyze > Specialist Analysis > MIASMA > Particulate Matter Analysis

Most users of *Image SXM* will not use this menu item and so it is tucked away where it will not get in anybody's way. Those of you who intend to use PMA extensively will probably prefer to have it available directly from the menu bar. If you press the option and control keys and select the PMA sub-menu you will find an extra item 'Move This Menu To Menu Bar'. This creates a new 'MIASMA' menu in the menu bar, which will appear every time you run *Image SXM* (on that Mac). If you want to move it back, repeat the process.

Appendix 2

History of changes to Particulate Matter Analysis routines in *Image SXM*

v1	First public release of PMA code (*Image SXM* v184) Algorithms written for Haemocolor stain	28 Jun 2007
v2	Added display of threshold % when doing manual adjustment Added option to mute 'Attention' sound Added option to set colours of PICT or TIFF maps	8 Jul 2007
v3	Changed file prefix to include decimal points Added separate contrast enhancement for each image colour	4 Oct 2007
v4	Fixed bug that overestimated cytoplasm area	21 Nov 2007
	Public release of *Image SXM* v185	7 Jan 2008
v5	Algorithms rewritten for Eosin stain	17 Feb 2008
v6	Fixed bug in manual adjustment display of threshold % Algorithms adjusted to give more reliable PM areas	5 Mar 2008
	Public release of *Image SXM* v186	20 Apr 2008
	Public release of *Image SXM* v187	4 Oct 2008
v7	Added background subtraction to compensate for vignetting	14 Feb 2009
	Public release of *Image SXM* v188	14 Feb 2009
	Public release of *Image SXM* v189	23 Aug 2009
	Public release of *Image SXM* v190	14 Apr 2010
	Public release of *Image SXM* v191	23 Dec 2010
	Public release of *Image SXM* v192	18 Apr 2011
	Public release of *Image SXM* v193	28 Apr 2012

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v7 May 2012