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| **Step** | **Action** | **Parameters** | **Snapshot** |
| 0. | **Load the dataset**  *Where in MIB:*  ***a)*** *Select files in the “Directory Contents Panel” using the left mouse button*  ***b)*** *Press the right mouse button to call a context menu*  ***c)*** *Choose the “Combine selected datasets” option* | NA |  |
| 1. | **Perona-Malik**  **anisotropic diffusion filtering**  to eliminate local noise while preserving edges of organelles  *Where in MIB:*  ***a)*** *Image Filters Panel→Perona Malik anisotropic diffusion→Filter*  ***b)*** *Save the filtered dataset to the hard drive for future use under a different filename, Menu→File→Save As* | Type: Regions  Number of iterations (Iter): 10  Edge stopping parameter (K): 4  Diffusion step (lambda): 0.15  All: on |  |
| 2. | **Morphological bottom-hat filtering**  to temporally remove all large organelles such as chromosomes and mitochondria, while keeping ER  *Where in MIB:*  *Menu→Image→*  *Morphological operations→Bottom-hat filtering* | Mode: 2D, full dataset  Strel element, shape: rectangle  Strel element, Size: 5 |  |

**Download link to the dataset:** [**http://mib.helsinki.fi/tutorials/mitoticcell\_segmentation/MitoticCell\_Segmentation\_workflow.zip**](http://mib.helsinki.fi/tutorials/mitoticcell_segmentation/MitoticCell_Segmentation_workflow.zip)

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| 3a. | **Hessian based Frangi Vesselness filter in the XY plane**  to segment ER tubules and sheet remnants that are perpendicular to the XY plane  *Where in MIB:*  *Mask Generators Panel (turned on using a popup menu in the Directory Contents panel)→Frangi filter→Do It* | Mode: 2D all  Range: 1-2  Ratio: 1  beta 1: .55  beta 2: 13  B/W thresholding: 0.15  Object size limit: 12  Black on while: unchecked | The segmented ER plotted against the original dataset |
| 3b. | **Hessian based Frangi Vesselness filter in the ZX plane**  to segment ER tubules and sheet remnants that are perpendicular to the ZX plane  *Where in MIB:*  ***a)*** *Toolbar→ZX button to change orientation*  ***b)*** *Mask Generators Panel→Frangi filter→the right mouse click above the Do It button and select “Generate a new mask and add it to the existing mask”*  *Assign the generated mask to the ER material of the model* | Range: 1-2  Ratio: 1  beta 1: .55  beta 2: 13  B/W thresholding: 0.15  Object size limit: 12  Black on while: unchecked |
| 3c. | **Add a new material “ER” to the model and assign results of the Frangi filter**  *Where in MIB:*  ***a)*** *Segmentation panel→”+”→”ER” →OK*  ***b)*** *Press the Shift+A shortcut to add selection to ER* | Segmentation Panel*→*  *Add to: 1* |
| 3d. | **Filter the results and save the model**  ***a)*** *Segmentation Panel→ER→*  *Right mouse click→Get Statistics…*  ***b)*** *Save model to the hard drive, Menu→Models→Save model as…* | Slices: Whole volume  Detect Objects: 3D objects  Press the Run button  - Highlight Range: 1 – 200  - Press the Do button to highlight the objects  - Click on the main window of MIB and press Shift+S to subtract highlighted objects from the ER material | C:\Users\ibelev\AppData\Local\Temp\SNAGHTML4273cbf2.PNG |
| 4. | **Subtract the model of ER from the anisotropically filtered dataset**  to simplify the data  *Where in MIB:*  ***a)*** *Load anisotropically filtered dataset, stored to the hard drive in step 1.*  ***b)*** *Load the model stored in the step 3b Menu→Models→Load model*  ***c)*** *Select the “ER” material and replace with the background the corresponding areas in the dataset.* | Segmentation Panel→Select from→1→right mouse click→NEW selection (ALL)  *Menu→Selection→Replace selected area in the image*  New intensity: 190  Slice number: 0  Color channels: 1 |  |
| 5. | **Black and white thresholding**  to segment dark, heavily stained organelles, such as lipid droplets and lysosomes  *Where in MIB:*  *Segmentation Panel→Selection type→BW Theresholding* | Low Lim: 0  High Lim: 131  all: checked  Select from: Ext  Fix selection to material: checked  *Remember uncheck the Fix selection to material checkbox after thresholding* |  |
| 6. | **Erode and dilate in 3D**  to remove small irrelevant objects  *Where in MIB:*  ***a)*** *Selection Panel→Er*  ***b)*** *Selection Panel→Di*  ***c)*** *Assign results to the new material (LD) of the model* | Color channel: Ch 1  3D: checked  Strel: 3;2  *similar to 3c, but*  Segmentation Panel*→*  *Add to: 2* | The segmented lipid droplets and lysosomes plotted against the original dataset |

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| 7. | **Subtract the model of LD from the dataset**  to further simplify the data  *Where in MIB:*  ***a)*** *Select the “LD” material and replace with the background the corresponding areas in the dataset.* | Segmentation Panel→Select from→2→right mouse click→NEW selection (ALL)  *Menu→Selection→Replace selected area in the image*  New intensity: 190  Slice number: 0  Color channels: 1 | The masked area is indicated with magenta contour |
| 8a. | **Perona-Malik**  **anisotropic diffusion filtering**  additional filtering  *Where in MIB:*  ***a)*** *Image Filters Panel→Perona Malik anisotropic diffusion→Filter* | Type: Regions  Number of iterations (Iter): 20  Edge stopping parameter (K): 6  Diffusion step (lambda): 0.25  All: checked |
| 8b. | **The Brush tool and the shape interpolation**  to mask the central part of the cell containing chromosomes  *Where in MIB:*  *a) Segmentation Panel→Selection type→Brush*  *b) Menu→Selection→*  *Interpolate as Shapes* | NA |
| 8c. | **Local Black and White thresholding within the masked area**  to segment chromosomes  *Where in MIB:*  *Segmentation Panel→Selection type→BW Theresholding* | Low Lim: 0  High Lim: 173  all: checked  Masked area: checked |  |

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| 9a. | **Erode and dilate in 3D**  to remove small irrelevant objects  *Where in MIB:*  ***a)*** *Selection Panel→Er*  ***b)*** *Selection Panel→Di*  ***c)*** *Assign results to the new material (Chromosomes) of the model* | Color channel: Ch 1  3D: checked  Strel: 4;2  *similar to 3c, but*  Segmentation Panel*→*  *Add to: 3* | The segmented chromosomes plotted against the original dataset |
| 9b. | **Filtering results based on size and intensity properties of the segmented objects**  to eliminate too small or too dark objects  *Where in MIB:*  *Segmentation Panel→Chromosomes→*  *Right mouse click→Get Statistics…* | Slices: Whole volume  **a)** Detect objects: 2D objects  Mode: Intensity  Parameter: MeanIntensity  Remove objects darker than 165  **b)** Detect objects: 2D objects  Mode: Object  Parameter: Area  Remove objects smaller than 150  **c)** Detect objects: 3D objects  Mode: Object  Parameter: Area  Remove all small objects |
| 10. | **Subtract the model of Chromosomes from the dataset**  to further simplify the data  *Where in MIB:*  ***a)*** *Select the “Chromosomes” material and replace with the background the corresponding areas in the dataset.* | Segmentation Panel→Select from→3→right mouse click→NEW selection (ALL)  *Menu→Selection→Replace selected area in the image*  New intensity: 179  Slice number: 0  Color channels: 1 |  |
| 11a. | **Perona-Malik**  **anisotropic diffusion filtering**  additional filtering  *Where in MIB:*  ***a)*** *Image Filters Panel→Perona Malik anisotropic diffusion→Filter* | Type: Regions  Number of iterations (Iter): 20  Edge stopping parameter (K): 15  Diffusion step (lambda): 0.25 |  |
| 11b. | **Strel mask generator**  to segment mitochondria  (*the* *slowest step*)  *Where in MIB:*  *Mask Generators Panel→Strel filter* | Strel: 25  B/W threshold: 0.07  Size limit: 50  Mode: 3D  Black on white: checked  Press the “Do it” button |
| 12a. | **Select Mask that does not belong to any other material and assign to a new material of the model**  *Where in MIB:*  ***a)*** *Highlight “Ext” in the Segmentation Panel using the left mouse button*  ***b)*** *Menu→Mask→to Selection…→All Frames→Replace*  ***c)*** *Add a new material: “Mitochondria”*  ***d)*** *Press the Shift+A shortcut to assign selection to the new material* | Segmentation Panel→Fix selection to material: checked  Segmentation Panel→Fix selection to material: unchecked | The segmented mitochondria plotted against the original dataset |
| 12b. | **Filtering results based on size properties of the segmented objects**  to eliminate too small objects  *Where in MIB:*  *Segmentation Panel→Mitochondria→*  *Right mouse click→Get Statistics…* | Slices: Whole volume  Mode: Object  Parameter: Area  **a)** Detect objects: 2D objects  Remove objects smaller than 50 pixels  **b)** Detect objects: 3D objects  Remove objects smaller than 1200 pixels |
| 12c. | **Erode and dilate in 3D**  to remove small irrelevant objects  *Where in MIB:*  ***a)*** *Select the “Mitochondia” material*  ***a)*** *Selection Panel→Er x2 times*  ***b)*** *Selection Panel→Di x2 times* | Segmentation Panel→Select from→4→right mouse click→NEW selection (ALL)  *Selection Panel:*  Color channel: Ch 1  3D: checked  Strel: 1;1 |
| 12d. | **Smooth selection**  *Where in MIB:*  *Menu→Selection→Smooth selection* | Mode: 3d  XY Kernel size: 5  Z Kernel size: 5  Sigma: 5 |
| 12e. | **Replace the “Mitochondria” material with the current selection**  ***a)*** *Segmentation Panel→Add to: 4*  ***b)*** *Press the Shift+R shortcut to replace the material* |  |
| 12f. | **Filtering results based on size properties of the segmented objects**  to eliminate too small objects  *Due to the low contrast of mitochondria, an additional manual poling using the Brush tool is required after this step* | As in step 12b. |
| 13. | **Visualization of the model**  *Where in MIB:*  *Menu→Models→Save model as…* | Format: AmiraMesh binary |  |