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DOI https://doi.org/10.1007/978-1-0716-2071-7_4

CITATION Anwar T., Eskelinen EL. (2022) Modified LC3 Dot Quantification Method. In: Norberg H., Norberg E. (eds) Autophagy and Cancer. Methods in Molecular Biology, vol 2445. Humana, New York, NY.
https://doi.org/10.1007/978-1-0716-2071-7_4

Modified LC3 dot quantification method

Running Title: Modified LC3 dot quantification

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Abstract

Since the discovery of autophagy genes and proteins in the beginning 1990ies, numerous previously unknown physiological and pathological functions have been discovered for autophagy. At the same time, precise monitoring of autophagy has become important, and western blotting and fluorescence microscopy of the marker protein LC3 are widely used for this purpose. Here we describe a modification of the widely used method, number of LC3 dots per cell. This protocol provides the proportion of vesicular LC3 staining over the total LC3 staining in the same cell. The approach is well suitable for quantification of endogenous LC3.

Key words Autophagy, LC3, Immunofluorescence, Fluorescence microscopy, Image analysis, Cell Profiler

1 Introduction

Microtubule-associated protein 1 light chain 3 (LC3) is a widely used autophagy marker protein [1]. Endogenous LC3 can be monitored by western blotting, which separates the non-lipidated, cytosolic LC3-I, and the lipidated, autophagosome-associated LC3-II [2]. In addition, endogenous or transfected, fluorescent-tagged LC3 is widely used for fluorescence microscopy based quantification of autophagy. In both approaches, one has to keep in mind that an increase in LC3-II or the number of LC3-positive dots can be due to two reasons, which are not mutually exclusive: increased formation of autophagosomes, and decreased clearance of autophagosomes, amphisomes or autolysosomes. These two can be separated by inhibiting lysosomal degradation and autophagosome-lysosome fusion by adding drugs such as bafilomycin A [2].

The number of LC3 dots per cell is a widely used measure to monitor autophagy. However, it is not always easy to define the number of dots, e.g. if the size of the dots is widely variable between cells or between different samples, or if the dots are very close to each other. Therefore, some researchers have opted to use total intensity or total area of LC3 dots per cell instead of number of dots per cell. In this Chapter, we describe a protocol that monitors the proportion of LC3 in dots over total LC3 staining, the latter including both diffuse cytoplasmic LC3, and LC3 in dots (Figure

1) [3]. Since the diffuse cytoplasmic LC3 is likely to represent non-lipidated LC3-I, and the LC3 in dots is likely to represent lipidated, membrane-bound LC3-II, this approach is in principle monitoring the same entities as western blotting. This approach is well suited for microscopic autophagy assay of endogenous LC3 on cells transiently transfected with a protein of interest, since it allows using the non-expressing cells in the same samples as an internal control. The protocol is well suited for immunofluorescence of endogenous LC3, but a similar approach can be used to ectopically expressed LC3 as well, provided the level of ectopic expression is close to endogenous level of LC3. The protocol utilizes the open-source software CellProfiler [4,5] and an ImageJ plugin with Otsu thresholding [6]. The protocol described here gives repeatable results on autophagy activation by amino acid starvation, and on the effect of beclin 1 overexpression on autophagy, in mouse embryonic fibroblasts (Figure 1D).

2 Materials

1. Glass cover slips, suitable for fluorescence microscopy such as #1.5
2. In case you wish to use transient transfection of a protein of interest, you need the suitable expression plasmid, tagged with e.g. green fluorescent protein (GFP).
3. Transfection reagent suitable for the cell line of your choice. For example, Xfect™ (BD Biosciences Clontech 631318) can be tested.
4. Cell culture medium suitable for your cell line
5. Cell line of your choice
6. Starvation medium. Earl's Balanced Salt Solution (EBSS, Gibco 2401043) can be used.
7. Bafilomycin A (e.g. Sigma B1793).
8. Phosphate-buffered saline (PBS, 140 mM NaCl, 2.7 mM KCl, 4.6 mM Na₂HPO₄ 2H₂O, 1.5 mM KH₂PO₄, pH 7.4)
9. Fixative: 4% paraformaldehyde in PBS
10. 0.2% saponin (e.g. Amresco 0163) in PBS
11. 3% bovine serum albumin (e.g. Biowest P6154) in PBS/saponin
12. Mouse anti-LC3 (Cosmo Bio CTB-LC3-2-IC)
13. Alexa Fluor 594 conjugated goat anti mouse IgG (Invitrogen A-11032)
14. Microscope slides
15. Mounting medium suitable for fluorescence microscopy. Mowiol (Calbiochem, 475904), containing the antifading agent 1,4-Diazabicyclo [2.2.2] octane (DABCO, Sigma D-2522) and the nuclear stain 4',6-diamidino-2-phenylindole (DAPI, Pierce 62247) can be used. Mowiol mounting medium is prepared as follows:
 - Weigh 6g glycerol in a beaker and check the pH using a pH paper. The pH should not be acidic.
 - Slowly add 2.4g Mowiol while mixing. Add Mowiol over the course of 1 h.
 - Add 6 ml MilliQ water, mix for 1 min, and let stand at room temperature overnight, covered.
 - Heat 12 ml of 0.2 M Tris, pH 8.5, to +50°C in a water bath.

- Add the prewarmed 0.2M Tris to the Mowiol and keep at +50°C for 10 min with occasional mixing. The mixture can be kept at +50°C for 1 h with agitation every 10 min. Undissolved Mowiol may be left in the beaker.
- Pellet undissolved Mowiol by centrifugation at 5000g at +4°C, 15 min.
- Separate clear supernatant and let it stand at +4°C for 15-30 min to allow air bubbles to rise to the top.
- Aliquot in Eppendorf tubes, and store at -20°C.
- Before use, add DABCO to 2.5% (10X DABCO stock in Tris buffer, aliquot in 0.1-ml aliquots, and store frozen).
- To stain nuclei, add 1 µl DAPI stock (1 mg/ml in DMSO, store at -20°C protected from light) per 1 ml Mowiol.
- Make sure DABCO and DAPI are properly mixed before you use the Mowiol. The ready mixture can be frozen and thawed several times.

16. Open-source software CellProfiler_2.1.1 [4,5] and an ImageJ plugin with Otsu thresholding [6]. The ImageJ plugin (macro) should be added to CellProfiler Analysis module RunImageJ/Macro. The plugin reads as follows:

```
rename("input");
run("LoG 3D...", "sigmax=2 sigmay=2 sigmaz=2 displaykernel=0 volume=1");
selectWindow("LoG of input");
rename("output");
selectWindow("input");
close();
selectWindow("output");
run("Invert");
run("8-bit");
run("Auto Threshold", "method=Otsu white");
```

17. Two additional .jar files, Auto_Threshold-1.16.jar and LoG_3D.jar [6], should be added to CellProfiler (File – Preferences – ImageJ Plugins directory). Auto_Threshold is a plugin of Fiji, available on github.com: https://github.com/fiji/Auto_Threshold/blob/Auto_Threshold-1.16.2/src/main/java/fiji/threshold/Auto_Local_Threshold.java. LoG_3D can be found on this website: <http://bigwww.epfl.ch/sage/soft/LoG3D/>.

3 Methods

3.1 Seeding the cells, transfection and immunofluorescence staining of LC3

1. Plate cells on glass coverslips (see Note 1).
2. 24 h after seeding, transfect cells with GFP-tagged construct of interest using Xfect™ transfection reagent according to the manufacturer's instructions (see Note 2).

3. 24 h after transfection, perform the following treatments: 1) starve the cells in EBSS for 1 h to induce autophagy, 2) starve the cells in EBSS with 100 nM bafilomycin A for 1h, and 3) keep the cells in full culture medium as a control.
4. Fix the cells in 4% paraformaldehyde in PBS at room temperature for 30 min.
5. Permeabilize the cells with 0.2% saponin in PBS for 10 min.
6. Block with 3% bovine serum albumin in PBS/saponin for 30 min.
7. Incubate with primary antibody at room temperature for 1 h: mouse anti-LC3 (1:100) in 3% bovine serum albumin in PBS/saponin (see Note 3).
8. Wash three times with PBS/saponin.
9. Incubate with secondary antibody conjugated to Alexa Fluor 594 (goat anti mouse, 1:500) in 3% bovine serum albumin in PBS/saponin, in the dark for 1 h.
10. Wash three times with PBS/saponin and once with MilliQ water.
11. Mount coverslips on microscope slides with Mowiol containing DABCO and DAPI.
12. Let Mowiol settle at room temperature, in the dark, overnight. Usually there is no need to use any further actions in order to fix the cover slips to the microscope slides.
13. Store the slides at +4 °C in the dark.

3.2 Imaging and quantification

1. Image at least 50 cells per sample with an upright fluorescence wide field microscope using a 63x objective suitable for fluorescence microscopy. The images shown in this article were taken using a Leica DM6000B microscope, 63x/1.40-0.60 HCX PL APO Lbd.bl. oil objective wd = 0.10 (Figure 2). Image Alexa Fluor 594 and DAPI, as well as GFP in case you used transfection.
2. Build a pipeline in CellProfiler as explained in Materials, and analyze the images as detailed in the following steps.
3. Drop DAPI, GFP and LC3 images from the cell of interest under the images tab in CellProfiler (Figure 3A).
4. Assign a name to each image using the NamesAndTypes tab (Figure 3B). It is important to carefully choose the names (e.g. to match the channels DAPI, GFP and LC3) as they will be used by the software in all the following modules.
5. Smooth DAPI (nuclei) images with a Gaussian filter and then identify them with IdentifyPrimaryObjects module (Figure 4A). Use default automatic threshold settings.
6. Identify cell borders with IdentifySecondaryObjects module (Figure 4B). Use adaptive thresholding.
7. Detect LC3 dots (Alexa Fluor 594) in the image of interest by running an ImageJ plugin with Otsu thresholding [6] (Figure 5A) and IdentifyPrimaryObjects module. Set a manual correction factor to precisely identify the dots apart from background noise and diffuse LC3 staining.
8. Recognize GFP expressing cells using MeasureObjectIntensity and module. Filter GFP expressing cells using mean intensity of the GFP image in FilterObjects module (Figure 5B).

9. Mask objects (LC3 dots) using MaskObjects module (Figure 6A).
10. Use the GrayToColor and OverlayOutlines modules to create outlines of the image of interest (Figure 6B), that is, cells and dots (Figure 1C).
11. Use MaskObjects module to mask GFP expressing cells and non-expressing cells (Figure 7).
12. Use DisplayDataOnImage to display measurement of the object of interest in GFP expressing cells (CellsGFPMinusSpots). This will allow you to label the cell of interest in the analyzed image.
13. Use the MeasureObjectIntensity and MeasureObjectSizeShape modules to measure LC3 dots in the cell of interest.
14. Use the RelateObjects to relate the analyzed dots to the parent cell.
15. Use ExportToSpreadsheet and ExportToDatabase modules to save the collected data.
16. Use the SaveImages module to save the overlay images created during the analysis.

3.3 Calculating the proportion of LC3 intensity in dots

The final results are calculated as proportion of LC3 staining located in dots, calculated as follows:

1. In order to get the total amount of LC3 staining within the cell of interest, sum together values for LC3 dots staining (Sum_Intensity_IntegratedIntensity_LC3), and diffuse cytoplasmic LC3 staining [Intensity_IntegratedIntensity_LC3 (cellsGFPminusSpots)] obtained from the CellProfiler analysis.
2. To obtain the proportion of vesicular LC3 staining for the cell of interest, divide the intensity of LC3 dots by the total amount of LC3 (see Note 4).
3. Follow this protocol for both cells expressing the GFP-tagged construct, and for non-expressing cells (see Note 5).

4 Notes

1. Optimal cell density for the analysis is subconfluent. It becomes difficult to separate the cells from each other if the culture is too dense. In addition, cells tend to be more flat in a subconfluent culture.
2. You may need to optimize the transfection reagent that is best suitable for your cell line. XfectTM is suitable for mouse embryonic fibroblasts. In addition, some transfection reagents may be fluorescent and thus not suitable for use in fluorescence microscopy.
3. Please check the suitability of the anti-LC3 to your cell line. Cosmo Bio CTB-LC3-2-IC is a mouse monoclonal IgG1 with reactivity against human and mouse LC3. It is well suitable for mouse embryonic fibroblasts.
4. Diffuse LC3 signal in the cells may change due to different reasons (for example, if protein synthesis is increased), and this may affect the LC3 dots/total LC3 intensity ratio. In case you are analyzing ectopically expressed LC3, make sure the expression level is close to the endogenous level.

- Analyze at least 40-50 cells for each sample.

Acknowledgements

This work was supported by The Academy of Finland and Magnus Ehrnrooth Foundation. We thank the Light Microscopy Unit, Institute of Biotechnology, University of Helsinki, especially Harri Jääliñoja, for technical help and availability of instruments.

Figure legends

Fig. 1 Schematic presentation of the work flow in quantification of proportion of vesicular LC3 intensity over total LC3 intensity, in cells stained for endogenous LC3 using immunofluorescence.

Fig. 2 Quantification of proportion of LC3 dots over total LC3 staining in mouse embryonic fibroblasts. The quantification was done for cells expressing GFP-tagged beclin 1 and for non-expressing cells on the same cover slip. (A) Black-and-white image of endogenous LC3 staining. (B) Overlay of LC3 (magenta) and DAPI (blue). (C) The same image as in panels A and B, saved after CellProfiler analysis steps, showing the cell borders and the LC3 dots. The two cells lined with green express GFP-tagged beclin 1. (D) Results of LC3 quantification from GFP-tagged beclin 1 expressing cells (black columns) and non-expressing cells (grey columns), incubated in full culture medium (Full medium), EBSS (AA starvation), or EBSS with bafilomycin A (AA starvation + Baf), for 1 h. The asterisks indicate a statistically significant difference.

Fig. 3 Screenshots of the CellProfiler analysis, 3.2 steps 3 (A) and 4 (B).

Fig. 4 Screenshots of the CellProfiler analysis, 3.2 steps 5 (A) and 6 (B).

Fig. 5 Screenshots of the CellProfiler analysis, 3.2 steps 7 (A) and 8 (B).

Fig. 6 Screenshots of the CellProfiler analysis, 3.2 steps 9 (A) and 10 (B).

Fig. 3 Screenshot of the CellProfiler analysis, 3.2 step 11.

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Figure 1

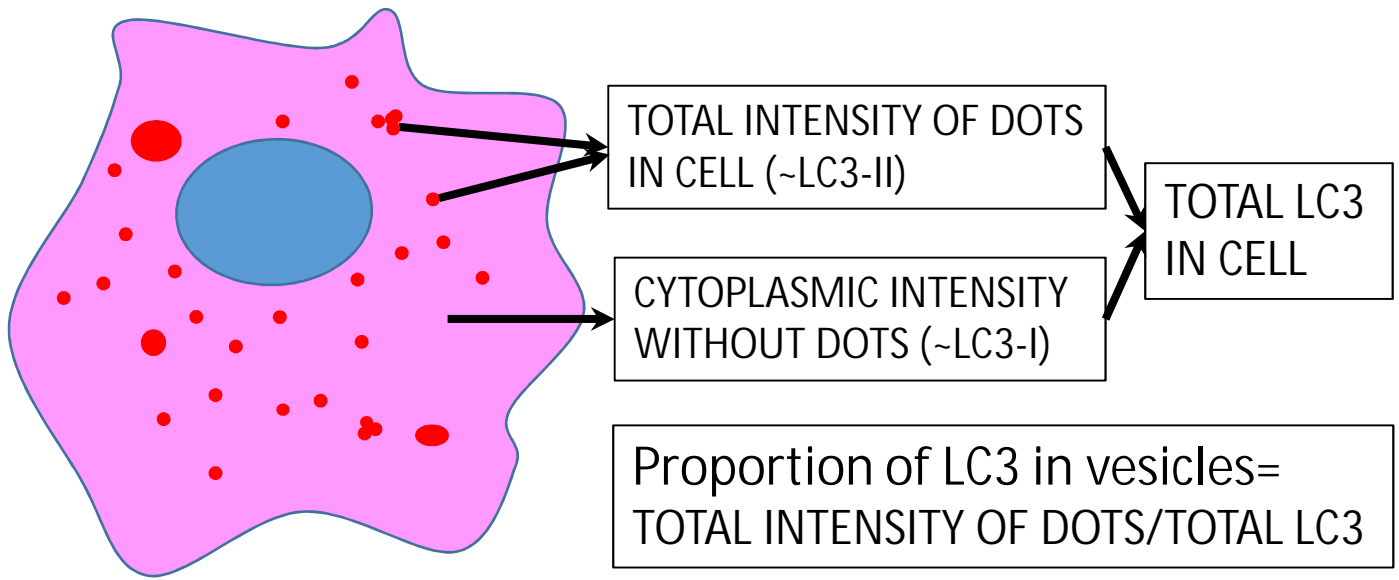


Figure 2

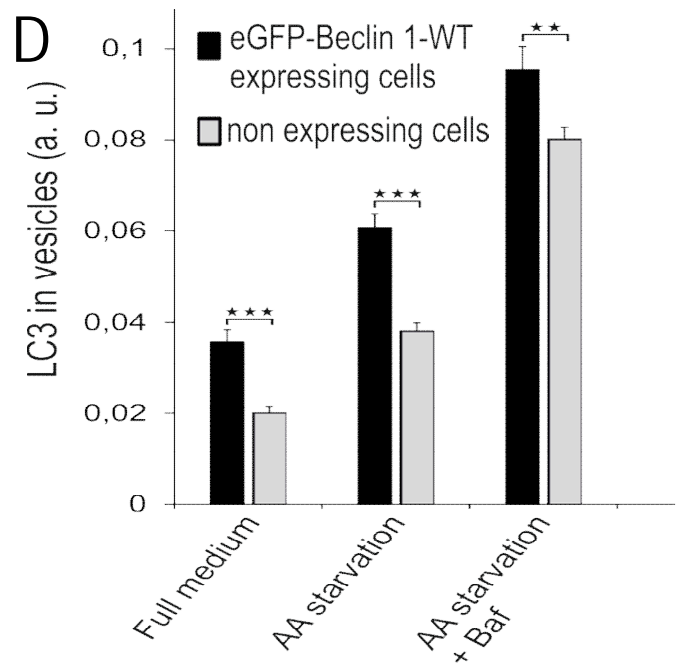
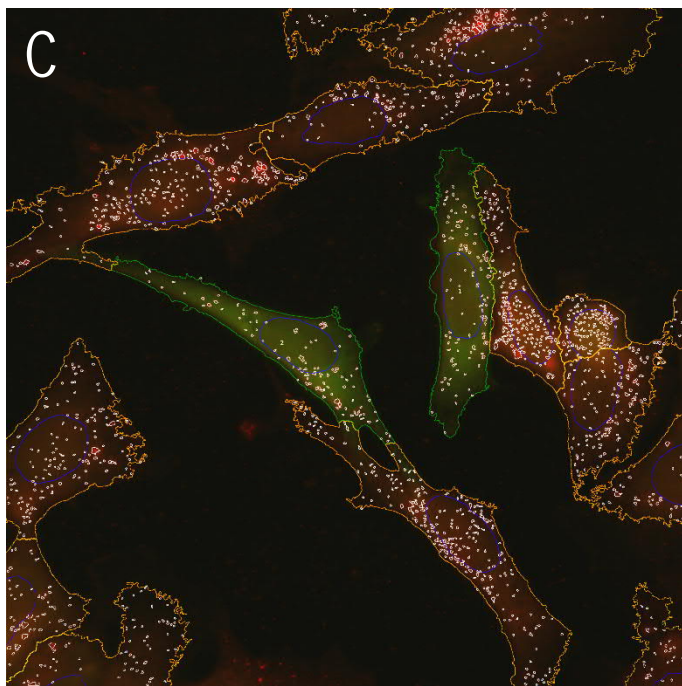
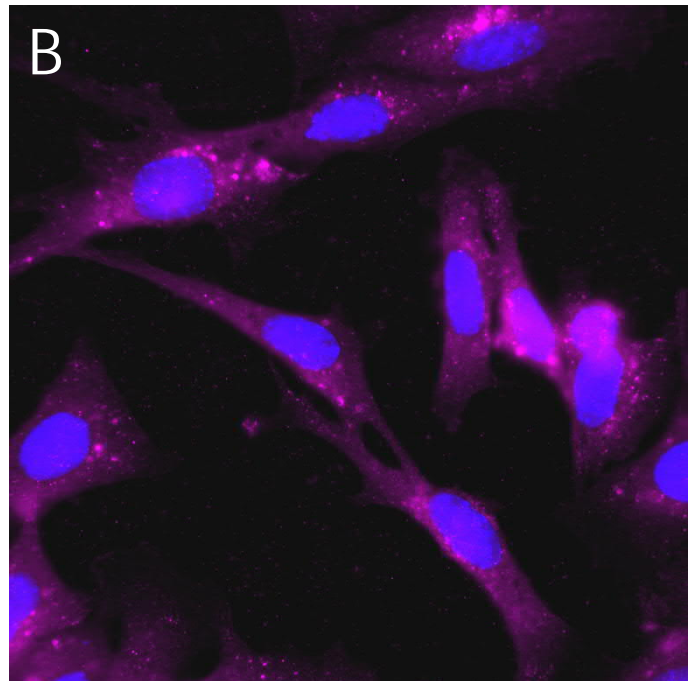
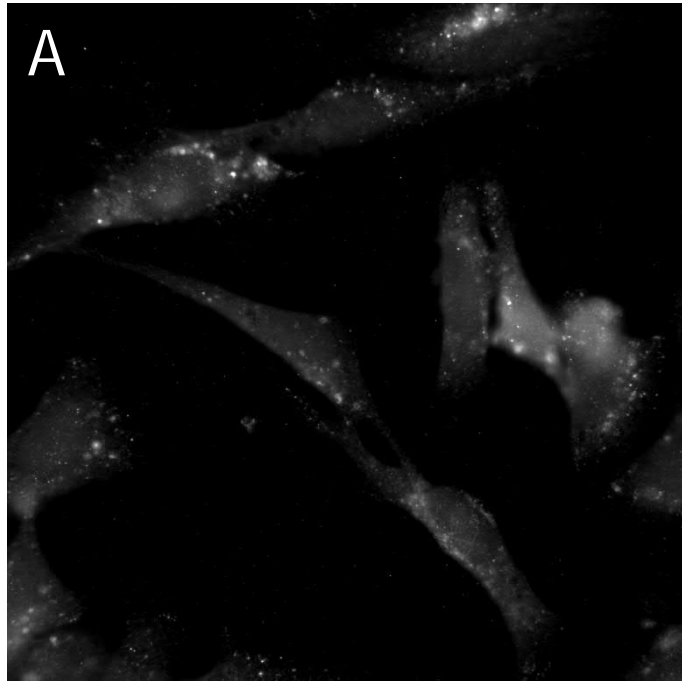


Figure 3

File Edit Test Data Tools Window Help

A

Pipeline

Input modules

- Images
- Metadata
- NamesAndTypes
- Groups

Analysis modules

- Smooth
- IdentifyPrimaryObjects
- IdentifySecondaryObjects
- RunImageJ
- IdentifyPrimaryObjects
- MeasureObjectIntensity
- FilterObjects
- FilterObjects
- MaskObjects
- MaskObjects
- GrayToColor
- OverlayOutlines
- DisplayDataOnImage
- MaskObjects
- MaskObjects
- DisplayDataOnImage
- DisplayDataOnImage
- MeasureObjectIntensity
- MeasureObjectSizeShape

Module notes

To begin creating your project, use the Images module to compile a list of files and/or folders that you

File list

Drop files and folders here

Show files excluded by filters

Module settings

Filter images? Custom

Select the rule criteria Match All of the following rules

Extension Is the extension of an image file

Apply filters to the file list

File Edit Test Data Tools Window Help

B

Pipeline

Input modules

- Images
- Metadata
- NamesAndTypes
- Groups

Analysis modules

- Smooth
- IdentifyPrimaryObjects
- IdentifySecondaryObjects
- RunImageJ
- IdentifyPrimaryObjects
- MeasureObjectIntensity
- FilterObjects
- FilterObjects
- MaskObjects
- MaskObjects
- GrayToColor
- OverlayOutlines
- DisplayDataOnImage
- MaskObjects
- MaskObjects
- DisplayDataOnImage
- DisplayDataOnImage
- MeasureObjectIntensity
- MeasureObjectSizeShape
- RelateObjects
- RelateObjects
- ExportToSpreadsheet
- ExportToDatabase
- SaveImages

Module notes

The NamesAndTypes module allows you to assign a meaningful name to each image by which other modules will refer to it.

Module settings

Assign a name to Images matching rules

Select the rule criteria Match All of the following rules

File Does Contain ch00

Name to assign these images DAPI

Select the image type Grayscale image

Set intensity range from Image metadata

Select the rule criteria Match All of the following rules

File Does Contain ch02

Name to assign these images GFP

Select the image type Grayscale image

Set intensity range from Image metadata

Remove this image

Select the rule criteria Match All of the following rules

File Does Contain ch01

Name to assign these images LC3

Select the image type Grayscale image

Set intensity range from Image metadata

Remove this image

Figure 4

File Edit Test Data Tools Window Help

Pipeline

Input modules

- Images
- Metadata
- NamesAndTypes
- Groups

Analysis modules

- Smooth
- IdentifyPrimaryObjects
- IdentifySecondaryObjects
- RunImageJ
- IdentifyPrimaryObjects
- MeasureObjectIntensity
- FilterObjects
- FilterObjects
- MaskObjects
- MaskObjects
- GrayToColor
- OverlayOutlines
- DisplayDataOnImage
- MaskObjects
- MaskObjects
- DisplayDataOnImage
- DisplayDataOnImage
- MeasureObjectIntensity
- MeasureObjectSizeShape
- RelateObjects
- RelateObjects
- ExportToSpreadsheet
- ExportToDatabase
- SaveImages

Module notes

Module settings

Select the input image: SmoothDAPI (from Smooth #05)

Name the primary objects to be identified: Nuclei

Typical diameter of objects, in pixel units (Min,Max): 50 500

Discard objects outside the diameter range? Yes No

Discard objects touching the border of the image? Yes No

Threshold strategy: Automatic

Method to distinguish dumped objects: Shape

Method to draw dividing lines between dumped objects: Shape

Automatically calculate size of smoothing filter for dedumping? Yes No

Automatically calculate minimum allowed distance between local maxima? Yes No

Speed up by using lower-resolution image to find local maxima? Yes No

Retain outlines of the identified objects? Yes No

Name the outline image: NucleiOutlines

Fill holes in identified objects? After both thresholding and dedumping

Handling of objects if excessive number of objects identified: Continue

File Edit Test Data Tools Window Help

Pipeline

Input modules

- Images
- Metadata
- NamesAndTypes
- Groups

Analysis modules

- Smooth
- IdentifyPrimaryObjects
- IdentifySecondaryObjects
- RunImageJ
- IdentifyPrimaryObjects
- MeasureObjectIntensity
- FilterObjects
- FilterObjects
- MaskObjects
- MaskObjects
- GrayToColor
- OverlayOutlines
- DisplayDataOnImage
- MaskObjects
- MaskObjects
- DisplayDataOnImage
- DisplayDataOnImage
- MeasureObjectIntensity
- MeasureObjectSizeShape
- RelateObjects
- RelateObjects
- ExportToSpreadsheet
- ExportToDatabase
- SaveImages

Module notes

Module settings

Select the input image: GFP (from NamesAndTypes)

Select the input objects: Nuclei (from IdentifyPrimaryObjects #06)

Name the objects to be identified: Cells

Select the method to identify the secondary objects: Watershed - Image

Threshold strategy: Adaptive

Thresholding method: Otsu

Two-class or three-class thresholding? Two classes

Minimize the weighted variance or the entropy? Weighted variance

Select the smoothing method for thresholding: No smoothing

Threshold correction factor: 1.2

Lower and upper bounds on threshold: 0.0 1.0

Method to calculate adaptive window size: Image size

Fill holes in identified objects? Yes No

Discard secondary objects touching the border of the image? Yes No

Retain outlines of the identified secondary objects? Yes No

Name the outline image: CellOutlines

Figure 5

File Edit Test Data Tools Window Help

A

Input modules

- Images
- Metadata
- NamesAndTypes
- Groups

Analysis modules

- Smooth
- IdentifyPrimaryObjects
- IdentifySecondaryObjects
- RunImageJ
- IdentifyPrimaryObjects
- MeasureObjectIntensity
- FilterObjects
- FilterObjects
- MaskObjects
- MaskObjects
- GrayToColor
- OverlayOutlines
- DisplayDataOnImage
- MaskObjects
- MaskObjects
- DisplayDataOnImage
- DisplayDataOnImage
- MeasureObjectIntensity
- MeasureObjectSizeShape
- RelateObjects
- RelateObjects
- ExportToSpreadsheet
- ExportToDatabase
- SaveImages

Module notes

Module settings

Run an ImageJ command or macro? Macro

Macro

```

rename("input");
run("LoG 3D...", "sigmax=2 sigmay=2 sigmaz=2 displaykernel=0 volume=1");
selectWindow("LoG of input");
rename("output");
selectWindow("input");
close();
selectWindow("output");
run("Invert");
run("8-bit");
run("Auto Threshold", "method=Otsu white");
    
```

Input the currently active image in ImageJ? Yes No

Select the input image LC3 (from NamesAndTypes)

Retrieve the currently active image from ImageJ? Yes No

Name the current output image LoG_LC3

Function to run before each group of images? Nothing

Function to run after each group of images? Nothing

Wait for ImageJ before continuing? Yes No

Show ImageJ Show

File Edit Test Data Tools Window Help

B

Input modules

- Images
- Metadata
- NamesAndTypes
- Groups

Analysis modules

- Smooth
- IdentifyPrimaryObjects
- IdentifySecondaryObjects
- RunImageJ
- IdentifyPrimaryObjects
- MeasureObjectIntensity
- FilterObjects
- FilterObjects
- MaskObjects
- MaskObjects
- GrayToColor
- OverlayOutlines
- DisplayDataOnImage
- MaskObjects
- MaskObjects
- DisplayDataOnImage
- DisplayDataOnImage
- MeasureObjectIntensity
- MeasureObjectSizeShape
- RelateObjects
- RelateObjects
- ExportToSpreadsheet
- ExportToDatabase
- SaveImages

Module notes

Module settings

Name the output objects CellsGFP

Select the object to filter Cells (from IdentifySecondaryObjects #07)

Select the filtering mode Measurements

Select the filtering method Limits

Select the measurement to filter by Category: Intensity

Measurement: MeanIntensity

Image: GFP

Filter using a minimum measurement value? Yes No

Minimum value 0.08

Filter using a maximum measurement value? Yes No

Add another measurement Add

Retain outlines of the identified objects? Yes No

Relabel additional objects to match the filtered object? Add an additional object

Figure 6

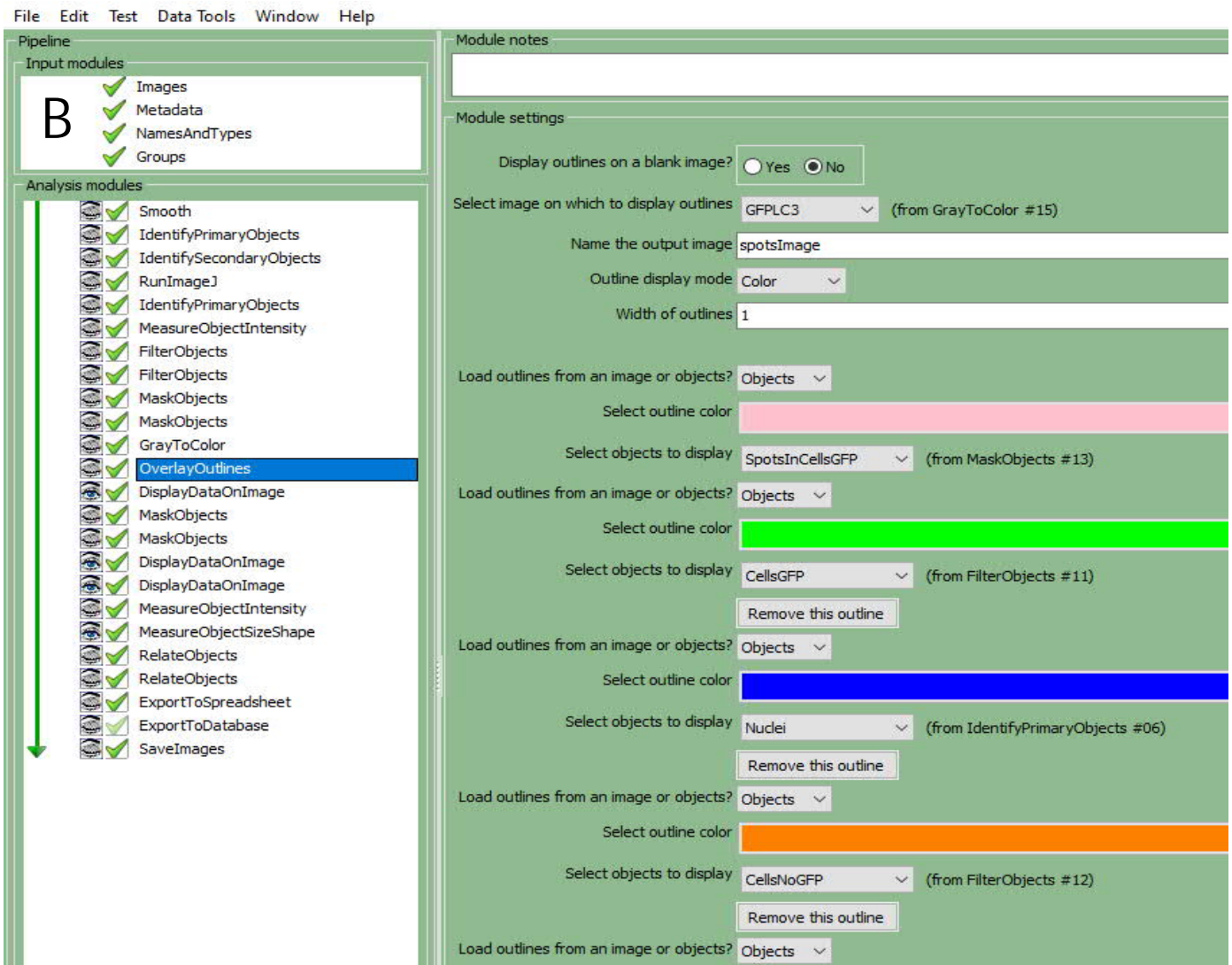
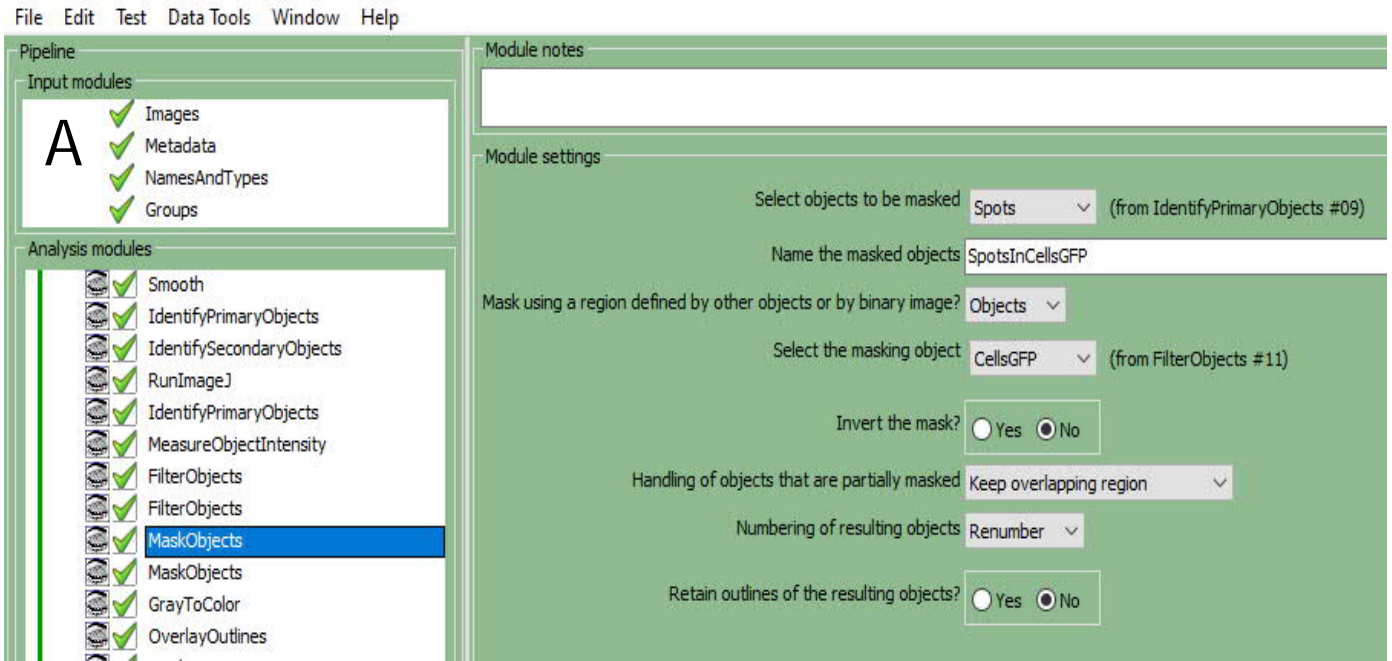


Figure 7

File Edit Test Data Tools Window Help

The screenshot displays a software interface for configuring a pipeline. On the left, a 'Pipeline' panel is divided into 'Input modules' and 'Analysis modules'. The 'Input modules' list includes Images, Metadata, NamesAndTypes, and Groups. The 'Analysis modules' list includes Smooth, IdentifyPrimaryObjects, IdentifySecondaryObjects, RunImageJ, MeasureObjectIntensity, FilterObjects, MaskObjects, GrayToColor, OverlayOutlines, DisplayDataOnImage, MaskObjects, and MaskObiects. The 'MaskObjects' module is highlighted in blue. On the right, the 'Module settings' panel for 'MaskObjects' is shown. It includes a 'Module notes' section, a 'Select objects to be masked' dropdown set to 'CellsGFP', a 'Name the masked objects' text field containing 'CellsGFPMinusSpots', a 'Mask using a region defined by other objects or by binary image?' dropdown set to 'Objects', a 'Select the masking object' dropdown set to 'SpotsInCellsGFP', an 'Invert the mask?' section with 'Yes' selected, a 'Handling of objects that are partially masked' dropdown set to 'Keep overlapping region', a 'Numbering of resulting objects' dropdown set to 'Renumber', and a 'Retain outlines of the resulting objects?' section with 'No' selected.

Module notes

Module settings

Select objects to be masked CellsGFP (from FilterObjects #11)

Name the masked objects CellsGFPMinusSpots

Mask using a region defined by other objects or by binary image? Objects

Select the masking object SpotsInCellsGFP (from MaskObjects #13)

Invert the mask? Yes No

Handling of objects that are partially masked Keep overlapping region

Numbering of resulting objects Renumber

Retain outlines of the resulting objects? Yes No