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**Protocol status:** Working  
 We use this protocol and it's working

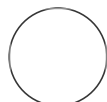
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## A computational pipeline to quantify perinuclear lysosomes in fibroblasts using CellProfiler

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### ABSTRACT

Here we present a CellProfiler software pipeline to quantify the distribution of lysosomes in MEF cells. The lysosomes were stained using anti-LAMP1 antibody, and nuclei were labeled using DAPI. The images were acquired using a Zeiss laser scanning confocal microscope and were maximum intensity projected in FIJI.

### IMAGE ATTRIBUTION

We thank Dr. Wondwossen Yeshaw for providing images for this analysis.

### MATERIALS

Latest edition CellProfiler 4.04 (or later)  
 Maximum intensity projected raw .TIF files

## Import files into CellProfiler and extract metadata

- 1 A. Select the **Images** module, drag and drop the maximum intensity projected .TIF files as indicated

B. Select the **Metadata** module

In the Metadata module:

Extract metadata? Yes.

Metadata extraction method: Extract from file/folder names

Metadata source: File name

Regular expression to extract from file name :

“Regex” will be as follows:

`^(?P<celltype>[a-z]{2})MEFs_(?P<treatment>[A-Z]{4})-(?P<position>[0-9]{2})`

for an example file name “wtMEFs\_DMSO-01.czi\_max.tif”. This step helps to extract the cell type, treatment, and position from each file name.

In Regex,

^ indicates the beginning of the file name

`(?P<celltype>[a-z]{2})` tells the program to name the captured field “celltype” and recognize two letters that follow

`(?P<treatment>[A-Z]{4})` tells the program to name the captured field “treatment” and recognize four letters that follow

`(?P<position>[0-9]{2})` tells the program to name the captured field “position” and recognize two digits that follow

Extract metadata from: All images

Add another extraction method

Metadata extraction method: Extract from image file headers

Extract metadata from: All images

Hit "Extract metadata"

Metadata data type: Text

Hit "update" to populate the metadata field

## Group individual channels and create image subsets

### 2 A. Go to **NamesAndTypes** module

Assign a name to : "Images matching rules"

Process as 3D : No

Select the rule criteria

Match "All" of the following rules

"Metadata/Does/Have C matching 0"

Name to assign these images: LAMP1

Select the image type: Grayscale image

Set intensity range from : Image metadata

Add another image

Match "All" of the following rules

"Metadata/Does/Have C matching 1"

Name to assign these images: DAPI

Select the image type: Grayscale image

Set intensity range from : Image metadata

Hit "update" to populate the names and types field

B. Select **Groups** module

Do you want to group your images? *Yes*

Metadata category: celltype

Add another metadata item

Metadata category: treatment

Add another metadata item

Metadata category: position

This groups images based on cell type, treatment, and position as identified in the metadata module.

## Identify lysosomes as objects

- 3 Click on the "+" sign at the bottom next to Adjust Modules. One can choose different modules by double-clicking from the list or by typing in the search box. Under the module category, object processing, Add **Identifyprimaryobjects** module

Use advanced settings? *Yes*

Select the input image: LAMP1

Name the primary objects to be identified: LAMP1objects

Typical diameter of objects, in pixel units: 3 - 100

Discard objects outside the diameter range? *Yes*

Discard objects touching the border of the image? *Yes*

Threshold strategy? *Global*

Thresholding method? *Otsu*

Two-class or three-class thresholding? *Two classes*

Threshold smoothing scale 1.3488

Threshold correction factor 1.0

Lower and upper bounds on threshold 0.25 and 1.0

Log transform before thresholding? Yes

Method to distinguish clumped objects? Intensity

Method to draw dividing lines between clumped objects? Intensity

Automatically calculate size of smoothing filter for declumping? Yes

Automatically calculate size for smoothing filter for declumping? Yes

Automatically calculate minimum allowed distance between local maxima? Yes

Speed up by using lower-resolution image to find local maxima? Yes

Display accepted local maxima? No

Fill holes in identified objects? After both thresholding and declumping

Handling of objects if excessive number of objects identified? Continue

#### Note

Note : These parameters will need to be optimized for each image set. Check by clicking "Start Test Mode" and hitting the green triangle next to the **IdentifyPrimaryObjects** module each time a parameter is changed to find the best parameters for each image set. Green outlines represent valid objects whereas yellow and red represent invalid objects, as they are either touching the border or outside the diameter range set.

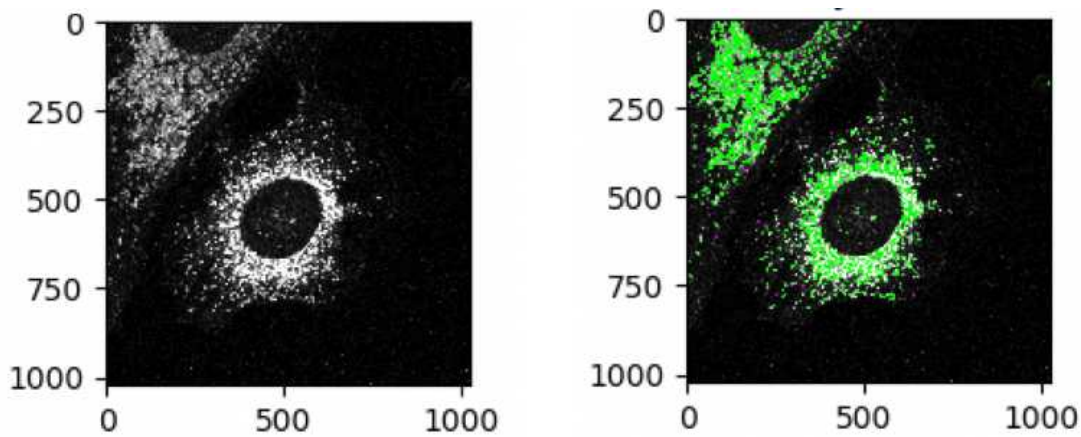


Figure 1: Lysosomes in MEF cells stained using anti-LAMP1 (left) and the LAMP1 objects identified by CellProfiler (right). Green regions represent valid objects.

## Segment nuclei

### 4 Add **Identifyprimaryobjects** module

Use advanced settings? Yes

Select the input image: DAPI

Name the primary objects to be identified: Nuclei

Typical diameter of objects, in pixel units: 110 - 500

Discard objects outside the diameter range? Yes

Discard objects touching the border of the image? Yes

Threshold strategy? Global

Thresholding method? Minimum Cross-Entropy

Threshold smoothing scale 1.3488

Threshold correction factor 1.0

Lower and upper bounds on threshold 0.1 and 1.0

Log transform before thresholding? No

Method to distinguish clumped objects? Intensity

Method to draw dividing lines between clumped objects? Intensity

Automatically calculate size of smoothing filter for declumping? Yes

Automatically calculate size for smoothing filter for declumping? Yes

Automatically calculate minimum allowed distance between local maxima? Yes

Speed up by using lower-resolution image to find local maxima? Yes

Display accepted local maxima? No

Fill holes in identified objects? After both thresholding and declumping

Handling of objects if excessive number of objects identified? Continue

#### Note

Note: Check by clicking “Start Test Mode” and hitting the green triangle next to the **IdentifyPrimaryObjects** module each time a parameter is changed to find the best parameters for each image set.

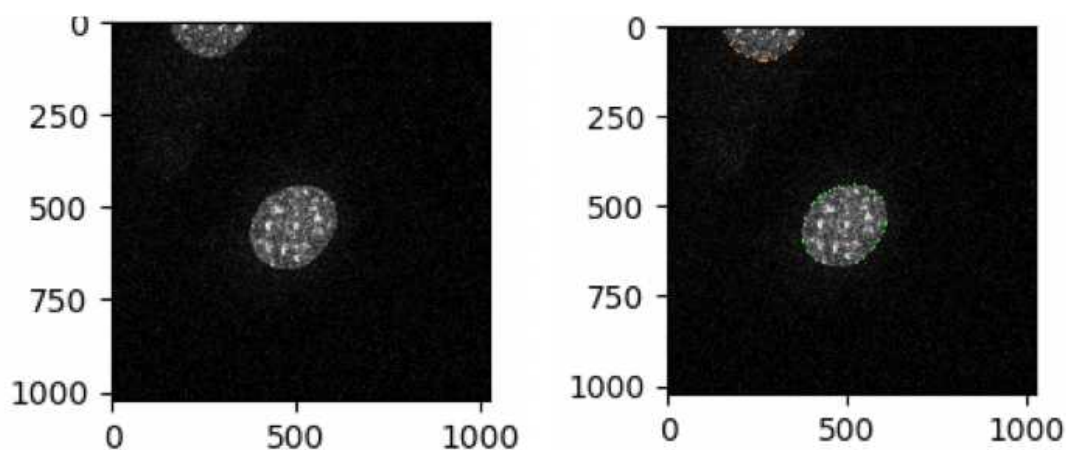


Figure 2: Nuclei in MEF cells labeled using DAPI (left) and the nuclei objects identified by CellProfiler (right). Green outlines represent valid objects.

## Define the perinuclear region

### 5 A. Add **ExpandOrShrinkObjects** module from the “+” sign at the bottom

Select the input objects : Nuclei

Name the output objects: Expandednuclei

Select the operation: Expand objects by a specified number of pixels

Number of pixels by which to expand or shrink : 20

This expands the nuclei by 20 pixels and this ring can be used to define perinuclear region.

#### Note

In this case, we used 20 pixels. However, larger cells may require a larger number of pixels to capture the perinuclear region adequately.

### B. Add **OverlayOutlines** module

Display outlines on a blank image: No

Select image on which to display outlines: LAMP1

Name the output image: outline20pixels

Outline display mode: Color

How to outline: Thick

Select objects to display: Expandednuclei

Select outline color: Maraschino

### C. Add **SaveImages** module

Select type of image to save: Image

Select the image to save: outline20pixels



Constructing file names: From image filename

Image name for file prefix: LAMP1

Append a suffix to the image file name? Yes

Text to append to the image name: outline20pixels

Saved file format: tiff

Image bit depth: 8-bit integer

Save with lossless compression? Yes

Output file location: choose a folder where images should be saved

Overwrite existing files without warning? No

When to save? Every cycle

Record the file and path information to the saved image? No

Create subfolders in the output folder? No

#### Note

Steps B and C allow you to save and verify CellProfiler's interpretations for each image.

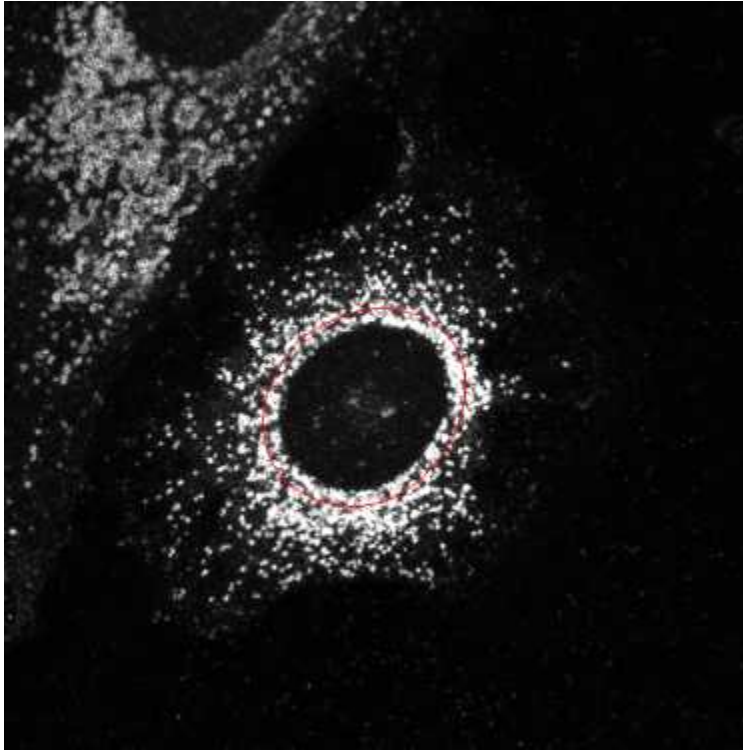


Figure 3: The red outline indicates the region within 20 pixels of the nucleus.

## Segment lysosome objects in the perinuclear region

- 6 A. Add **RelateObjects** module to relate parent objects (“nuclei expanded by 20 pixels”) with child objects (“LAMP1 objects”). Parent objects are the objects that encompass the child objects and child objects will be contained within the parent object.

Parent objects: Expandednuclei

Child objects: LAMP1objects

Calculate per-parent means for all child measurements? *No*

Calculate child-parent distances? *None*

Do you want to save the children with parents as a new object set? *Yes*

Name the output object : Relate objects\_perinuclearLAMP1

- B. Add **OverlayOutlines** module

Display outlines on a blank image: No

Select image on which to display outlines: LAMP1

Name the output image: PerinuclearLAMP1

Outline display mode: Color

How to outline: Outer

Select objects to display: Relate objects\_perinuclearLAMP1

Select outline color: Maraschino

C. Add **SaveImages** module

Select type of image to save: image

Select the image to save: PerinuclearLAMP1

Constructing file names: From image filename

Image name for file prefix: LAMP1

Append a suffix to the image file name? Yes

Text to append to the image name: PerinuclearLAMP1

Saved file format: tiff

Image bit depth: 8-bit integer

Save with lossless compression? Yes

Output file location: choose a folder where images should be saved

Overwrite existing files without warning? No

When to save? Every cycle

Record the file and path information to the saved image? No

Create subfolders in the output folder? No

#### Note

Steps B and C allow you to save and verify CellProfiler's interpretations for each image.

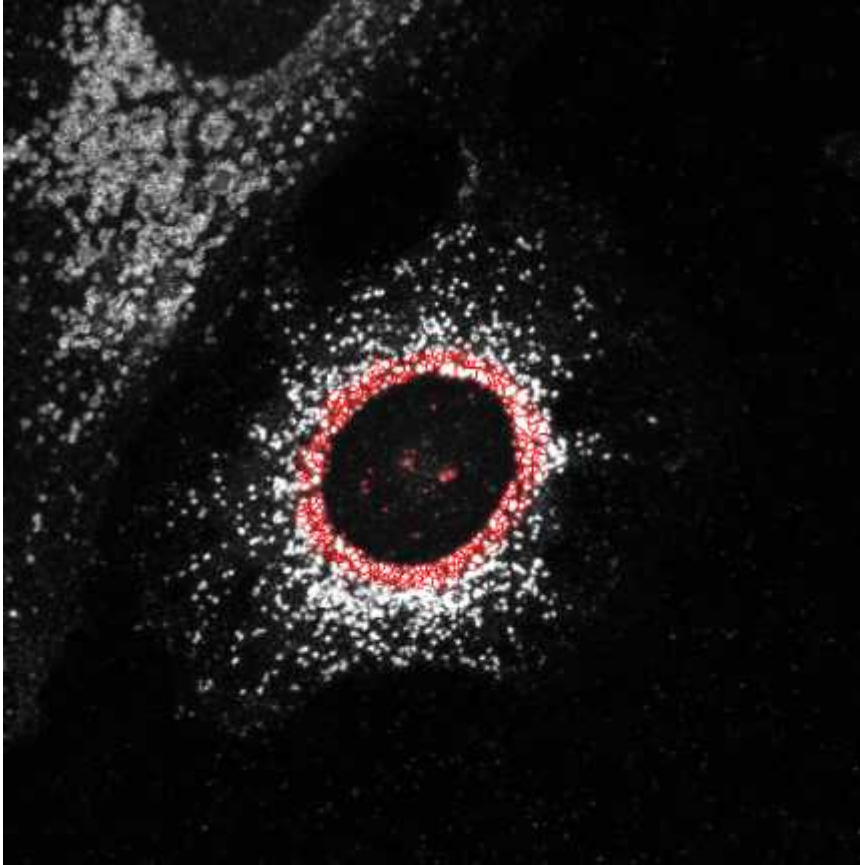


Figure 4: The objects outlined in red indicate the LAMP1 objects in the perinuclear region.

## Identify cell boundaries

- 7 A. Add **IdentifySecondaryObjects** module to identify the whole cell using the nucleus as the primary object.

Select the input image: LAMP1

Select the input objects: Nuclei

Name the objects to be identified: wholecells

Select the method to identify the secondary objects: Propagation

This module finds the dividing lines between secondary objects that are touching.

Threshold strategy: Global.

Thresholding method: *Minimum Cross-Entropy*

Threshold smoothing scale: 1.3488

Threshold correction factor: 1.0

Lower and upper bounds of threshold: 0.048-1.0

These numbers may need to be changed per experiment depending on the quality of staining, background noise etc.

Log transform before thresholding? No

Regularization factor: 0.01

Fill holes in identified objects? *Yes*

Discard secondary objects touching the border of the image? No

B. Add **OverlayOutlines** module

Display outlines on a blank image: No

Select image on which to display outlines: LAMP1

Name the output image: Cell outline

Outline display mode: Color

How to outline: Thick

Select objects to display: *wholecells*

Select outline color: Maraschino

C. Add **SaveImages** module

Select type of image to save: image

Select the image to save: Celloutline

Constructing file names: From image filename

Image name for file prefix: LAMP1

Append a suffix to the image file name? Yes

Text to append to the image name: Celloutline

Saved file format: tiff

Image bit depth: 8-bit integer

Save with lossless compression? Yes

Output file location: choose a folder where images should be saved

Overwrite existing files without warning? No

When to save? Every cycle

Record the file and path information to the saved image? No

Create subfolders in the output folder? No

## Segment lysosome objects in the non-perinuclear region

### 8 A. Add **MaskObjects** module

Select objects to be masked: LAMP1 objects

Name the masked objects: NonperinuclearLAMP1

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

Select the masking object: RelateObjects\_perinuclear LAMP1

Invert the mask: Yes

Handling of objects that are partially masked: Keep overlapping region

Numbering of resulting objects: Renumber

This step masks the LAMP1 objects in the perinuclear region.

B. Add **RelateObjects** module

Parent objects: wholecells

Child objects: NonperinuclearLAMP1

Calculate per-parent means for all child measurements? *No*

Calculate child-parent distances? *None*

Do you want to save the children with parents as a new object set? *Yes*

Name the output object : RelateObjects\_nonperinuclearLAMP1

This step segments the LAMP1 objects in the non-perinuclear region.

C. Add **OverlayOutlines** module

Display outlines on a blank image: No

Select image on which to display outlines: LAMP1

Name the output image: NonperinuclearLAMP1

Outline display mode: Color

How to outline: Outer

Select objects to display: RelateObjects\_nonperinuclear LAMP1

Select outline color: Maraschino

D. Add **SaveImages** module:

Select type of image to save: Image

Select the image to save: NonperinuclearLAMP1

Constructing file names: From image filename

Image name for file prefix: LAMP1

Append a suffix to the image file name? Yes

Text to append to the image name: NonperinuclearLAMP1

Saved file format: tiff

Image bit depth: 8-bit integer

Save with lossless compression? Yes

Output file location: choose a folder where images should be saved

Overwrite existing files without warning? No

When to save? Every cycle

Record the file and path information to the saved image? No

Create subfolders in the output folder? No

#### Note

Steps C and D allow you to save and verify CellProfiler's interpretations for each image.



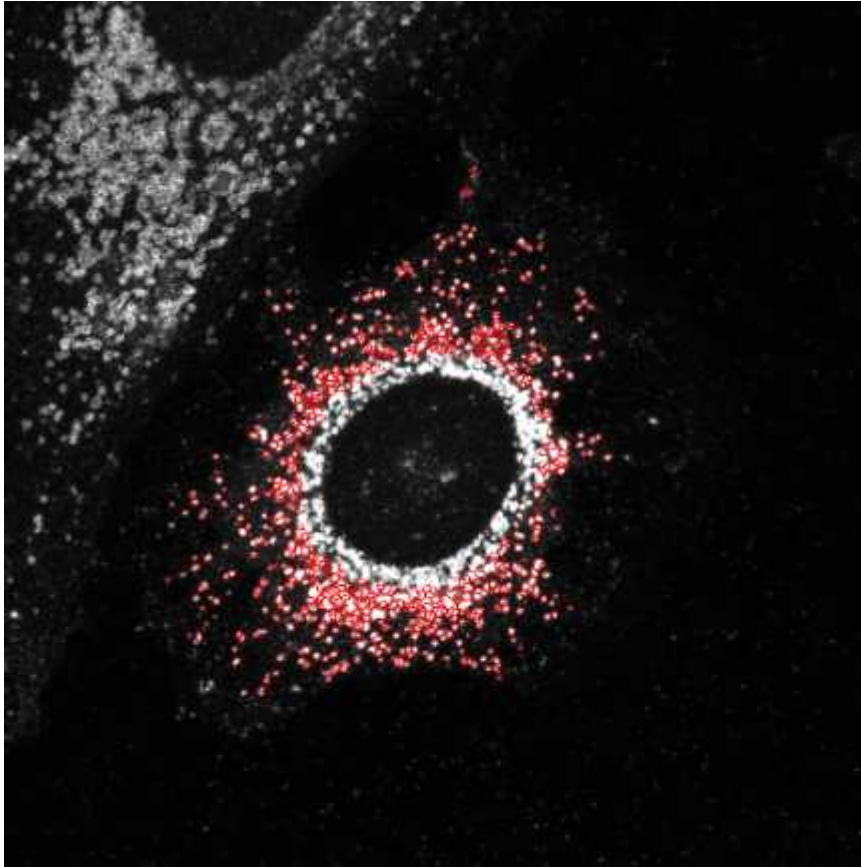


Figure 6. The objects outlined in red color show the non-perinuclear LAMP1 objects identified by CellProfiler.

## Measure the integrated intensity of LAMP1

### 9 Add **MeasureObjectIntensity** module

Select images to measure: LAMP1

Select objects to measure: RelateObjects\_PerinuclearLAMP1, RelateObjects\_nonperinuclear LAMP1

## Export the data

### 10 Add **ExportToSpreadsheet** module from the + at the bottom

Select the column delimiter: Tab

Output file location: choose a folder where you want the images to be saved.

Add a prefix to file names? Yes.

File name prefix: Add experiment identifier.

Overwrite existing files without warning? No

Note: While the pipeline is run for optimizing the parameters, choose Yes to avoid being asked to rewrite each file.

Add image metadata columns to your object data file? Yes

Add image file and folder names to your object data file? Yes

Representation of Nan/Inf: NaN

Select measurements to export? Yes

Press button to select measurements:

Under "Expandednuclei" select: Number

Under "RelateObjects\_PerinuclearLAMP1" choose Intensity and then integrated intensity.

Under "RelateObjects\_nonperinuclearLAMP1" choose Intensity and then Integrated intensity

Calculate the per-image mean values for object measurements? No

Calculate the per-image median values for object measurements? No


Calculate the per-image standard deviation values for object measurements? No

Create GenePattern GCT file? No

Export all measurement types? No

Export all measurement types? No

Save the pipeline from File-Save Project and hit **Analyze Images** on bottom left.



The pipeline will run and export the data to the folder previously specified.

The output file can be opened in Excel software. Distinct columns indicate the number of nuclei in each image and the integrated intensity of individual objects – perinuclear and non-perinuclear.