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🌐 Immunostaining and quantification of intracellular accessible cholesterol using ALOD4-mNeon in Human Fibroblasts

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

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Abstract

Here we present a protocol for quantitative immunostaining of intracellular accessible cholesterol using ALOD4-mNeon in human fibroblasts.

Guidelines

Be sure to use freshly prepared ALOD4-mNeon (within 14 days of preparation). Store at 4°C and do not freeze.

Materials

1. Paraformaldehyde 16% Solution (Electron Microscopy Sciences # 15710) diluted fresh in 1X PBS
2. Glass coverslips (Fisher Scientific #12-545-81)
3. Rat tail collagen (Gibco by Life Technologies # A10483-01)
4. Saponin (Sigma # S7900)
5. Bovine serum albumin (Equitech-Bio, Inc. #BAH65)
6. Human fibroblasts (Coriell Institute of Medical Research)
7. ALOD4-mNeon (https://dx.doi.org/10.17504/protocols.io.rm7vzx2qxgx1/v1) freshly prepared and never frozen
8. Mowiol mounting solution
9. FIJI/ImageJ
10. Latest edition CellProfiler software 4.04 (or later)

ALOD4 Staining and image acquisition

1 This section describes plating and staining of cells and image acquisition.

2 Seed 0.2 X 10^6 cells onto 3-4 collagen-coated 12 mm coverslips, in each well of a six-well plate

3 16-18 hr after plating, wash the cells once with 1X PBS

4 Transfer each coverslip to a well of a 24-well plate containing 500µl [M] 4 % (v/v) PFA per well; fix the cells for ⏱ 00:15:00 at 🌡 Room temperature   `15m`

5 Wash cells 3X with 1X PBS

6 Permeabilize the cells by addition of 0.1% Saponin in 1X PBS for ⏱ 00:05:00 at 🌡 Room temperature   `5m`

7 Wash cells 3X with 1X PBS

8 Block fixative by addition of 1% BSA in 1X PBS for ⏱ 00:30:00 at 🌡 Room temperature   `30m`

9 Dilute ALOD4-mNeon to 4µM in 1% BSA/1X PBS

10 Add 50 µl, 4µM ALOD4 per coverslip

11 Incubate cells for ⏱ 01:00:00 at 🌡 Room temperature   `1h`

12 Wash cells 3X in 1X PBS and mount coverslips onto clean glass slides with 4 µl Mowiol. Air-dry coverslips overnight or at least 4-5 hours; store in dark at 🌡 Room temperature

13   Images below were acquired using a Zeiss laser scanning microscope.

Batch process images for maximum intensity projection and background subtraction

14   Images need to be batch processed for maximum intensity projection and uniform background subtraction. Process the images for CellProfiler analysis as described in
dx.doi.org/10.17504/protocols.io.3byl4bpo8vo5/v1
FOOTNOTE: For Zeiss images, the user needs to specify the folder to save the processed images and run lines 26, 27, and 29. Processing includes maximum intensity projection and background subtraction. Choose a value for background subtraction based on the images analyzed.

Import files and Segment Cells

15   In CellProfiler, select the Images module, drag and drop the background subtracted and maximum intensity projected, .TIF files created in Step 14 above. Here we subtracted a value of 50 to remove the background noise.

16   Select the Metadata module

In the Metadata module:
Extract metadata? Yes.

Metadata extraction method: Extract from image file headers

Extract metadata from: All images

Hit "Extract metadata"

Click Add another extraction method

Metadata extraction method: Extract from file/folder names

Metadata source: File name

Regular expression to extract from file name: "Regex" will be as follows: ^GM(?P[0-9]{4}) .*#(?P[0-9]{2}) for an example file name "GM01607.czi #01_max.tif".
This step helps to extract the cell line, and position from each file name. In Regex, ^ indicates the beginning of the file name (?P[0-9]{4}) tells the program to name the captured field "Cell line" and recognize five digits that follow .* indicates any character (?P[0-9]{2}) tells the program to name the captured field "position" and recognize two digits that follow.

Metadata data type: Text

Hit "update" to populate the metadata field

17   Go to NamesAndTypes module

In the 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: ALOD4

Select the image type: Grayscale image

Set intensity range from: Image metadata

Hit "update" to populate the names and types field

18   Go to Groups module

In the Groups module:
Do you want to group your images? Yes

Metadata category: Celltype

Metadata category: position

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

19   Segmentation of cells:
Click on the "+" sign at the bottom next to Adjust Modules. Under the module category, Image processing, Add RescaleIntensitymodule.

Select the input image: ALOD4

Name the output image: RescaleIntensity_ALOD4

Rescaling method: Divide each image by the same value

Divisor value: 0.001

Rescaling the intensity of images makes it easier to segment cells in the step below.

20   Add Identifyprimaryobjects module

In the Identifyprimaryobjects module:
Use advanced settings? Yes

Select the input image: RescaleIntensity_ALOD4

Name the primary objects to be identified: cells

Typical diameter of objects, in pixel units: 95 - 800

Discard objects outside the diameter range: Yes

Discard objects touching the border of the image: No

Threshold strategy: Global Thresholding method: Otsu

Two-class or three-class thresholding? Two classes

Threshold smoothing scale: 1.6

Threshold correction factor 1.0

Lower and upper bounds on threshold 0 and 1.0

Log transform before thresholding? Yes

Method to distinguish clumped objects? None

Fill holes in identified objects? After both thresholding and declumping

Handling of objects if excessive number of objects identified? Continue

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.

21   Add OverlayOutlines module

In the OverlayOutlines module:
Display outlines on a blank image: No

Select image on which to display outlines: ALOD4

Name the output image: celloutline

Outline display mode: Color

How to outline: Thick

Select objects to display: cells
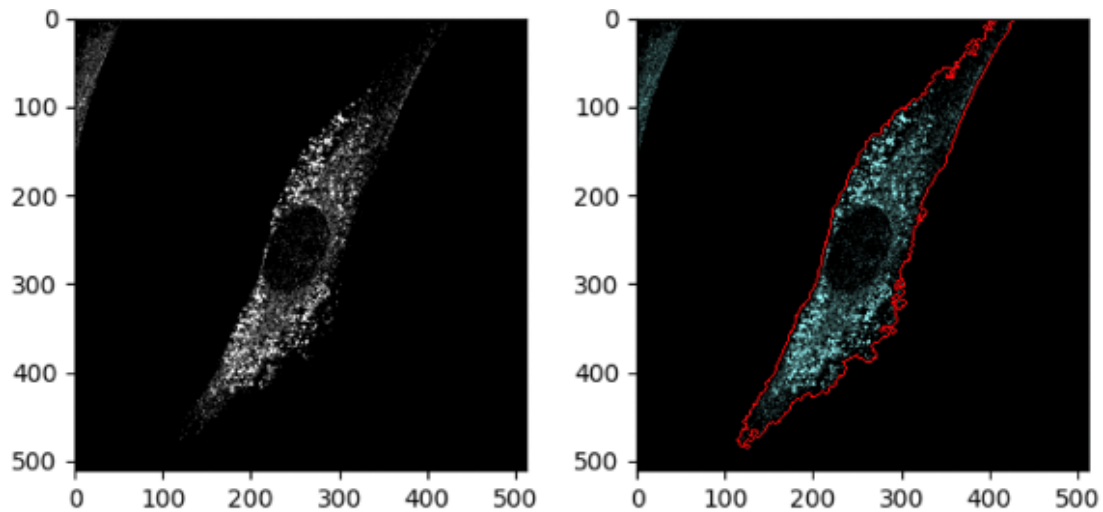
Select outline color: Maraschino

Figure 1. Example of CellProfiler Segmentation.

Measure ALOD4 intensity

22   Add MeasureObjectIntensity module
In the MeasureObjectIntensity module:
Select images to measure: ALOD4

Select objects to measure: cells

This module measures ALOD4 intensity in segmented cells.

23 Add MeasureObjectSizeShape module

In the MeasureObjectSizeShape module:
Select object sets to measure: cells

Calculate Zernike feature? No

Calculate the advanced features? No

24 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 "cells" choose AreaShape -> Area, and Intensity ->
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

Data to export: cells Use the object name for the file name? Yes

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 using Excel software. Columns indicate Cell line, image number, area of each cell, and integrated intensity of ALOD4 in each cell.
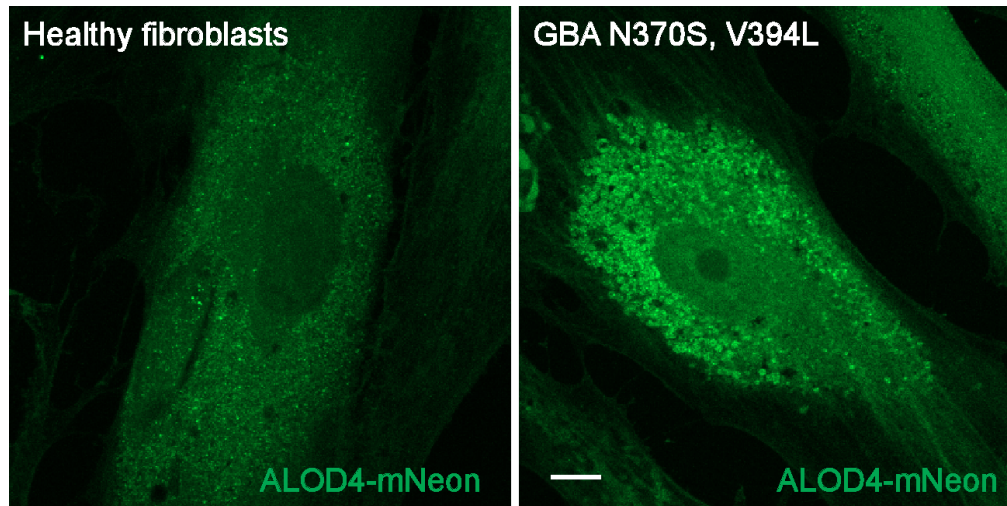

Figure 2. Example of Intracellular accessible cholesterol staining. Human fibroblasts from healthy control and an individual with GBA N370S/V394L mutations stained with ALOD4-mNeon according to this protocol. Scale bar, 10 μm.

Protocol references

Stirling DR, Swain-Bowden MJ, Lucas AM, Carpenter AE, Cimini BA, Goodman A (2021). CellProfiler 4: improvements in speed, utility and usability. BMC Bioinformatics, 22 (1), 433. PMID: 34507520 PMCID: PMC8431850.