Oct 30, 2020

Semi-automated quantitation of macroautophagy with the auto-QC counter

Francois Singh[1], Graeme Ball[2], Ian G Ganley[3]

[1]MRC Protein Phosphorylation and Ubiquitylation Unit, University of Dundee, Dundee DD1 5EH, UK; [2]Dundee Imaging Facility, School of Life Sciences, University of Dundee, Dundee DD1 5EH, UK; [3]University of Dundee

1 Works for me dx.doi.org/10.17504/protocols.io.bnzamf2e

IG Lab

Francois Singh
MRC Protein Phosphorylation and Ubiquitylation Unit, Univers...

ABSTRACT

General macroautophagy is a naturally occurring phenomenon. Based on our *auto-QC* reporter, which consists of a tandem mCherry-GFP tag associated with the N terminus of MAP1LC3B/LC3B, we developed the *auto*-QC counter macro for FIJI. This macro enables the semi-automated detection and quantitation of autophagosomes and autolysosomes with the auto-QC reporter. Here we describe how this macro works, and provide a step-by-step guide on how to use it.

The “auto-QC counter” for Fiji/ImageJ (Schindelin et al., 2012; Schneider, Rasband, & Eliceiri, 2012) macro allows semi-automated batch analysis of autophagy in a set of images. Autophagy is identified using differences in fluorescence intensity between the red mCherry and green GFP proteins of an auto-QC reporter delivered to autophagosomes. Similar to the mito-QC reporter (Allen, Toth, James, & Ganley, 2013; McWilliams et al., 2016; Montava-Garriga, Singh, Ball, & Ganley, 2020), the auto-QC reporter displays similar red and green channel intensities outside the lysosome using typical imaging parameters giving a red/green intensity ratio close to 1. In autophagosomes, quenching of the GFP leads to a relatively higher red/green intensity ratio. In contrast to our previously published “mito-QC counter” macro, which first identifies punctae in a red/green ratio channel and then filters according to red intensity, the autophagy counter macro we describe here first identifies punctae in the red channel and then interrogates the red/green ratio. The advantage of this approach is that “yellow” (red+green) punctae are identified for quantification as well as the red-only punctae (the caveat is that the red channel must be relatively “clean” and free from background fluorescence, which is less of a limitation when using the mito-QC counter approach of identifying punctae in the red/green ratio image first).

In terms of usage, the autophagy counter macro takes as input a folder of images in which an outline for each cell of interest has been created and added to the image overlay in Fiji/ImageJ before saving as tiff. Upon running the macro the user is first asked to select a folder containing images to analyse. A Dialog then prompts the user to check/adjust the following parameters: 1) and 2) green and red channel index, 3) spot size (in microns), 4) a peak finding threshold to identify punctae, 5) a minimum red intensity for punctae of interest, and 6) a red/green intensity ratio threshold to classify punctae as “red” rather than “yellow”. These parameters are discussed in more detail in the next paragraph. Batch analysis of the images in the input folder then begins, and a new output folder is created with a date and timestamp in the name. A new Results table is created for each run, with one row of results per cell region-of-interest, and this is saved to the output folder with a “spots” image corresponding to each input image that shows the location and classification of the punctae identified.

To help use the macro effectively we describe here in words the procedure it follows, as well as taking a more detailed look at the input parameters and results. After choosing the input images and updating parameters the macro first applies a median filter to all channels to reduce the impact of noise on the results. The scale of the *median filter* is determined by the third macro parameter “spotSize” (in calibrated units, typically microns) – this should be around the same size as the smallest autophagosomes, since making it larger will degrade the resolution to a point where individual autophagosomes are not detected. Next, the macro uses ImageJ’s built-in “*Find Maxima*” function to identify peaks in a filtered version of the red channel image: the macro implements a “Difference of Gaussian” (DoG) filter to enhance spots at the scale of interest and suppress local background. In layman’s terms this is achieved by subtracting a very blurred version of the original image from a less blurred

Citation: Francois Singh, Graeme Ball, Ian G Ganley (10/30/2020). Semi-automated quantitation of macroautophagy with the auto-QC counter. https://dx.doi.org/10.17504/protocols.io.bnzamf2e

version of it. The scale of the less blurred version is set according to the spotSize parameter, and the more blurred version is several times this scale (the non-user-adjustable parameter DoGmultiplier = 5 by default). The *Find Maxima* function used for peak detection then takes an input parameter called *Prominence*, which determines how much above local background an intensity peak must be to be detected. The *Prominence* value used for peak detection is determined by the fourth macro parameter "peakNstd": we apply an autothresholding strategy where we calculate standard deviation (std) of the DoG-filtered red channel intensities and use a threshold *Prominence* = std x peakNstd. The peakNstd value should be more than zero and less than 5, and we suggest a default value of 2. Having detected candidate intensity peaks in the DoG-filtered red channel, the macro then looks at the original (median-filtered) red channel intensity of each peak. Again, an auto-thresholding strategy is used: peak red intensity values must be greater than or equal to the average (mean) red channel intensity plus a number of standard deviations to be counted as a red spot. The number of standard deviations used to calculate this threshold is set according to the fifth macro parameter, "redMinimumSD" (default = 1). Spots that fail to meet this criterion are classified as "black" to signify insufficient red intensity. Finally, spots are classified as "red" only or "yellow" (i.e. red+green) by looking at the ratio of red channel intensity to green channel intensity: the minimum red/green intensity ratio to be counted as "red" only is determined by the sixth macro parameter "rThresh". The appropriate value for rThresh will obviously depend on the imaging parameters used for the red and green channels (i.e. exposure time, laser power, dwell time etc.) but should not vary between images if these parameters are kept fixed. The Results table produced by the macro displays: nBlack, the number of spots below the red minimum intensity; nRed, the number of red spots with a high red/green ratio; nYellow, the number of red spots that also coincide with significant green intensity; and then the four main input parameters spotSize, peakNstd, rThresh and redMinimumSD are recorded for future reference (redMinimum is the actual threshold red intensity value calculated using the redMinimumSD parameter).

Allen GF, Toth R, James J, Ganley IG (2013). Loss of iron triggers PINK1/Parkin-independent mitophagy.. EMBO reports.
https://doi.org/10.1038/embor.2013.168

McWilliams TG, Prescott AR, Allen GF, Tamjar J, Munson MJ, Thomson C, Muqit MM, Ganley IG (2016). mito-QC illuminates mitophagy and mitochondrial architecture in vivo.. The Journal of cell biology.
https://doi.org/10.1083/jcb.201603039

Montava-Garriga L, Singh F, Ball G, Ganley IG (2020). Semi-automated quantitation of mitophagy in cells and tissues.. Mechanisms of ageing and development.
https://doi.org/10.1016/j.mad.2019.111196

Citation: Francois Singh, Graeme Ball, Ian G Ganley (10/30/2020). Semi-automated quantitation of macroautophagy with the auto-QC counter. https://dx.doi.org/10.17504/protocols.io.bnzamf2e

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A (2012). Fiji: an open-source platform for biological-image analysis.. Nature methods.
https://doi.org/10.1038/nmeth.2019

Schneider CA, Rasband WS, Eliceiri KW (2012). NIH Image to ImageJ: 25 years of image analysis.. Nature methods.

DOI

dx.doi.org/10.17504/protocols.io.bnzamf2e

PROTOCOL CITATION

Francois Singh, Graeme Ball, Ian G Ganley 2020. Semi-automated quantitation of macroautophagy with the auto-QC counter. **protocols.io**
https://protocols.io/view/semi-automated-quantitation-of-macroautophagy-with-bnzamf2e

KEYWORDS

Autophagy, auto-QC, FIJI, Autophagosome, Autolysosome

CREATED

Oct 26, 2020

LAST MODIFIED

Oct 30, 2020

PROTOCOL INTEGER ID

43778

GUIDELINES

This section should help you adjust the parameters of the auto-QC counter to detect autophagosomes and autolysosomes in an optimal manner.

Minimum red intensity for spots (stdDevs above mean)
That parameter adjusts the threshold above which red spots are detected.
- Increase that parameter to detect less spots
- Decrease that value to detect more spots

Number of stDevs above backgroung for peaks
That parameter adjusts the sensitivity of peak detecting in the ratio channel.
- Increase that value to detect less peaks of intensity (less yellow (autophagosomes) and red (autolysosomes)

spots, more black (unspecific) spots)
- Decrease that value to detect more peaks of intensity (more yellow (autophagosomes) and red (autolysosomes), less black (unspecific) spots)

Minimum red/green intensity ratio for red dots
That parameter defines the ratio threshold to distinguish yellow dots from red only dots
- Increase that parameter to have a larger proportion of autophagosomes (yellow) and less autolysosomes (red only)
- Decrease that parameter to have a smaller proportion of autophagosomes (yellow) and more autolysosomes (red only)

ABSTRACT

General macroautophagy is a naturally occurring phenomenon. Based on our *auto-QC* reporter, which consists of a tandem mCherry-GFP tag associated with the N terminus of MAP1LC3B/LC3B, we developed the *auto*-QC counter macro for FIJI. This macro enables the semi-automated detection and quantitation of autophagosomes and autolysosomes with the auto-QC reporter. Here we describe how this macro works, and provide a step-by-step guide on how to use it.

The “auto-QC counter” for Fiji/ImageJ (Schindelin et al., 2012; Schneider, Rasband, & Eliceiri, 2012) macro allows semi-automated batch analysis of autophagy in a set of images. Autophagy is identified using differences in fluorescence intensity between the red mCherry and green GFP proteins of an auto-QC reporter delivered to autophagosomes. Similar to the mito-QC reporter (Allen, Toth, James, & Ganley, 2013; McWilliams et al., 2016; Montava-Garriga, Singh, Ball, & Ganley, 2020), the auto-QC reporter displays similar red and green channel intensities outside the lysosome using typical imaging parameters giving a red/green intensity ratio close to 1. In autophagosomes, quenching of the GFP leads to a relatively higher red/green intensity ratio. In contrast to our previously published “mito-QC counter” macro, which first identifies punctae in a red/green ratio channel and then filters according to red intensity, the autophagy counter macro we describe here first identifies punctae in the red channel and then interrogates the red/green ratio. The advantage of this approach is that “yellow” (red+green) punctae are identified for quantification as well as the red-only punctae (the caveat is that the red channel must be relatively “clean” and free from background fluorescence, which is less of a limitation when using the mito-QC counter approach of identifying punctae in the red/green ratio image first).

In terms of usage, the autophagy counter macro takes as input a folder of images in which an outline for each cell of interest has been created and added to the image overlay in Fiji/ImageJ before saving as tiff. Upon running the macro the user is first asked to select a folder containing images to analyse. A Dialog then prompts the user to check/adjust the following parameters: 1) and 2) green and red channel index, 3) spot size (in microns), 4) a peak finding threshold to identify punctae, 5) a minimum red intensity for punctae of interest, and 6) a red/green intensity ratio threshold to classify punctae as “red” rather than “yellow”. These parameters are discussed in more detail in the next paragraph. Batch analysis of the images in the input folder then begins, and a new output folder is created with a date and timestamp in the name. A new Results table is created for each run, with one row of results per cell region-of-interest, and this is saved to the output folder with a “spots” image corresponding to each input image that shows the location and classification of the punctae identified.

To help use the macro effectively we describe here in words the procedure it follows, as well as taking a more detailed look at the input parameters and results. After choosing the input images and updating parameters the macro first applies a median filter to all channels to reduce the impact of noise on the results. The scale of the *median filter* is determined by the third macro parameter “spotSize” (in calibrated units, typically microns) – this should be around the same size as the smallest autophagosomes, since making it larger will degrade the resolution to a point where individual autophagosomes are not detected. Next, the macro uses ImageJ’s built-in “*Find Maxima*” function to identify peaks in a filtered version of the red channel image: the macro implements a “Difference of Gaussian” (DoG) filter to enhance spots at the scale of interest and suppress local background. In layman’s terms this is achieved by subtracting a very blurred version of the original image from a less blurred version of it. The scale of the less blurred version is set according to the spotSize parameter, and the more blurred version is several times this scale (the non-user-adjustable parameter DoGmultiplier = 5 by default). The *Find Maxima* function used for peak detection then takes an input parameter called *Prominence*, which

Citation: Francois Singh, Graeme Ball, Ian G Ganley (10/30/2020). Semi-automated quantitation of macroautophagy with the auto-QC counter. https://dx.doi.org/10.17504/protocols.io.bnzamf2e

determines how much above local background an intensity peak must be to be detected. The *Prominence* value used for peak detection is determined by the fourth macro parameter “peakNstd”: we apply an autothresholding strategy where we calculate standard deviation (std) of the DoG-filtered red channel intensities and use a threshold *Prominence* = std x peakNstd. The peakNstd value should be more than zero and less than 5, and we suggest a default value of 2. Having detected candidate intensity peaks in the DoG-filtered red channel, the macro then looks at the original (median-filtered) red channel intensity of each peak. Again, an auto-thresholding strategy is used: peak red intensity values must be greater than or equal to the average (mean) red channel intensity plus a number of standard deviations to be counted as a red spot. The number of standard deviations used to calculate this threshold is set according to the fifth macro parameter, “redMinimumSD” (default = 1). Spots that fail to meet this criterion are classified as “black” to signify insufficient red intensity. Finally, spots are classified as “red” only or “yellow” (i.e. red+green) by looking at the ratio of red channel intensity to green channel intensity: the minimum red/green intensity ratio to be counted as “red” only is determined by the sixth macro parameter “rThresh”. The appropriate value for rThresh will obviously depend on the imaging parameters used for the red and green channels (i.e. exposure time, laser power, dwell time etc.) but should not vary between images if these parameters are kept fixed. The Results table produced by the macro displays: nBlack, the number of spots below the red minimum intensity; nRed, the number of red spots with a high red/green ratio; nYellow, the number of red spots that also coincide with significant green intensity; and then the four main input parameters spotSize, peakNstd, rThresh and redMinimumSD are recorded for future reference (redMinimum is the actual threshold red intensity value calculated using the redMinimumSD parameter).

Allen GF, Toth R, James J, Ganley IG (2013). Loss of iron triggers PINK1/Parkin-independent mitophagy.. EMBO reports.
https://doi.org/10.1038/embor.2013.168

McWilliams TG, Prescott AR, Allen GF, Tamjar J, Munson MJ, Thomson C, Muqit MM, Ganley IG (2016). mito-QC illuminates mitophagy and mitochondrial architecture in vivo.. The Journal of cell biology.
https://doi.org/10.1083/jcb.201603039

Montava-Garriga L, Singh F, Ball G, Ganley IG (2020). Semi-automated quantitation of mitophagy in cells and tissues.. Mechanisms of ageing and development.
https://doi.org/10.1016/j.mad.2019.111196

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A (2012). Fiji: an open-source platform for biological-image analysis.. Nature methods.
https://doi.org/10.1038/nmeth.2019

Citation: Francois Singh, Graeme Ball, Ian G Ganley (10/30/2020). Semi-automated quantitation of macroautophagy with the auto-QC counter. https://dx.doi.org/10.17504/protocols.io.bnzamf2e

Schneider CA, Rasband WS, Eliceiri KW (2012). NIH Image to ImageJ: 25 years of image analysis.. Nature methods.

Download and install the auto-QC counter

1 This section explains where to find and how to install the auto-QC counter

1.1 The file "*autoQC_counter.ijm*" ("version 1.0", DOI: 10.5281/zenodo.4158361) is available at the following address: https://github.com/graemeball/autoQC_counter

1.2 Paste this file in the following folder:
Windows: (the folder where you saved FIJI)\fiji-win64\Fiji.app\plugins\Scripts\Plugins
Mac: Right click on the FIJI program and select "show package contents".
Select plugins→Scripts > Plugins and paste the file in this subfolder.

1.3 Start FIJI and install the plugin:
→Plugins→Macros→Install…
Restart FIJI

1.4 We advise creating a shortcut for the plugin:
Plugins→Shortcut→Add Shortcut…
(Example: F7)
Restart FIJI

Find the appropriate settings

2 This section explains how to adjust the settings on a single picture to detect autophagosomes and autolysosomes

2.1 Open a picture with high autophagy (*e.g.* cells stimulated with EBSS):
Circle cells *as* described in

Montava-Garriga L, Singh F, Ball G, Ganley IG (2020). Semi-automated quantitation of mitophagy in cells and tissues.. Mechanisms of ageing and development.
https://doi.org/10.1016/j.mad.2019.111196

2.2 Start the auto-QC counter plugin (F7)
A new dialog widow will appear.
Leave the "Batch mode?" box unticked for now.
Make sure your green and red channels correspond to the values shown
The spot size in calibratedd units of 0.100 microns is in general appropriate.

Citation: Francois Singh, Graeme Ball, Ian G Ganley (10/30/2020). Semi-automated quantitation of macroautophagy with the auto-QC counter. https://dx.doi.org/10.17504/protocols.io.bnzamf2e

Start with the default settings and adjust if necessary.
See guidelines for more information on how to adjust it.

Use the batch mode

3 This section explains how to use the batch mode of the auto-QC counter

3.1 Open the first picture and outline the first cell.
Press "CTRL" + "B" add the outline to the image overlay.
Circle all the other cells in the picture in the same manner.
Save the picture as a Tiff in a new folder (create a new folder for each experimental condition).
For the first image: File --> Save as... --> Tiff...

3.2 Repeat for all the images.
Images can now be saved by pressing "CTRL" + "S"

3.3 Start the auto-QC counter plugin (F7).
Tick the "Batch mode?" box.
Adjust the parameters as previously defined in the single-image mode.
Press OK.
The plugin automaticaly saves:
- a table of your results (.csv file)
- a "spots" file diaplaying the autophagosomes (Yellow) and autolysosomes (Red) detected when opened with FIJI.
This image also shows the spots below threshold in black.

Citation: Francois Singh, Graeme Ball, Ian G Ganley (10/30/2020). Semi-automated quantitation of macroautophagy with the auto-QC counter. https://dx.doi.org/10.17504/protocols.io.bnzamf2e