

Specifications for a successful analysis Standard Tool: WimAutophagy

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1 Description of WimAutophagy

WimAutophagy is specifically designed to detect autophagosomes in the cytoplasm of cells for Autophagy determination and other similar assays. It has been developed to detect autophagosomes using immunofluorescence assays with LC3 antibodies or cells transfected with GFP-LC3 or other LC3 reporters.

This tool is designed to work with many different types of image acquisition such as epifluorescence and confocal microscopy in adherent cell lines. Besides autophagosome number, size and intensity WimAutophagy also determines other parameters such as number of cells and the area of the nuclei.

WimAutophagy has been optimized to distinguish between isolated and clustered autophagosomes, in order to be able to estimate the number autophagosomes per cell or the number of puncta per cell.

The nuclei are detected at the beginning of the analysis, then the input image is filtered and standardized to enhance autophagosomes. After that, autophagosomes are detected and assigned to the nearest nuclei. The different parts of analysis can be seen in more detail in appendix A

2 Specifications for a successful analysis

2.1 Upload requirements

WimAutophagy only analyzes images. Images can be organized and uploaded in different folders. The maximum upload size is 1 GB per order

In order to assure the objectivity of the evaluation; all images of the same order should be acquired using the same microscopy parameters (image size, magnification, illumination conditions, etc). We also recommend including all images of the assay you want to evaluate in the same order.

2.2 Valid Formats

WimAutophagy accepts the following file formats. If you want to use another format, please contact us.

Images

- jpg/jpeg
- jp2
- tif/tiff
- png
- gif
- bmp
- mpeg/mpg

Videos and Stack

- tif/tiff
- mov
- avi
- wmv
- zvi

2.3 Characteristics of the input

- Input image should be a RGB image (3 layers).
- In the case of confocal microscopy the image to be analyzed can be the projection of all z-stacks.
- The client has to specify the color layer for autophagosomes (green, red or blue) and the nucleus (green, red or blue).
- Low or medium density of cells and no clustering
- Background darker than the objects
- Autophagosomes brighter than the cytoplasm

- Images should not contain other objects such as scale bar. Magnification can be specified in a input parameter to be taken into account.
- No artifacts, noise or debris

Figure 1 and Figure 2 display valid images for wimAutophagy analysis.

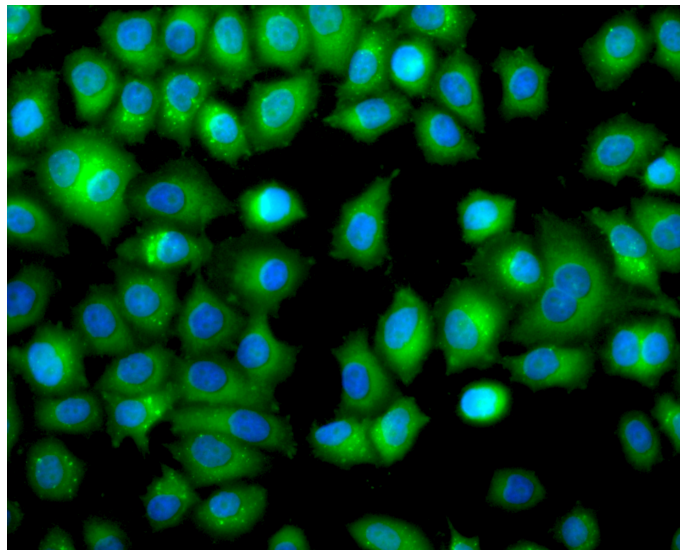


Figure 1: Example of valid image for epifluorescence in stable HeLa GFP-LC3 cells with the autophagosomes in the green layer and nuclei in the blue layer.

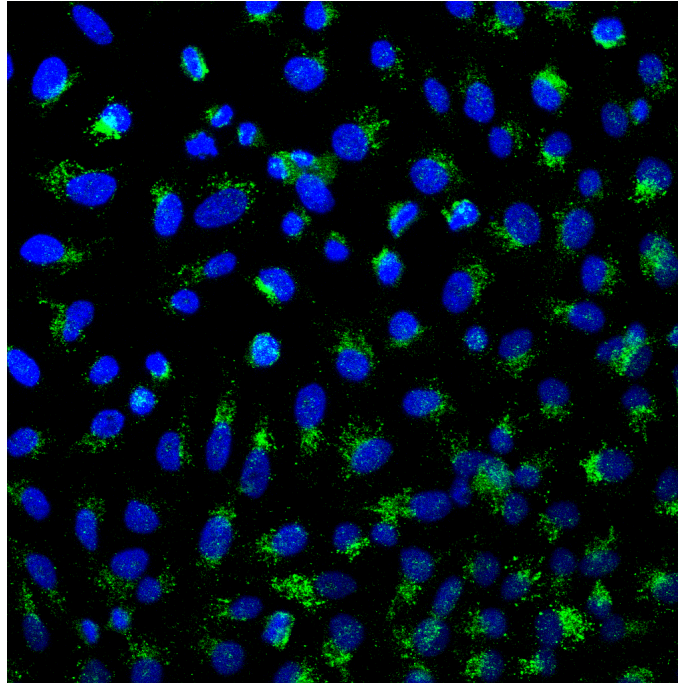


Figure 2: Example of valid image for confocal microscopy using immunofluorescence staining for LC3 with the autophagosomes in the green layer and nuclei in the blue layer.

2.4 Mandatory parameters of the analysis

Users have to specify three the parameters of their assay:

1. Type image acquisition: this parameter is used to choose the type of image acquisition.
 - Epifluorescence
 - Confocal (default)
2. Layer Autophagosome: this parameter is used to indicate the color of the autophagosomes layer
 - Red
 - Green (default)
 - Blue

3. Layer nuclei: this parameter is used to indicate the color of nuclear layer

- Red
- Green
- Blue (default)

2.5 Optional parameters of the analysis

- Scale [$\mu\text{m}/\text{px}$]. If the user enters the $\mu\text{m}/\text{px}$ conversion factor indicated by the microscope, the data readouts will be given in μm instead of px.

The following two options must only be changed if you are familiar with image analysis and are an experienced WimAutophagy user. Otherwise the estimation algorithm may not work properly.

- Area Autophagosome: With this parameter the user can indicate the mean autophagosome area in pixels. This area will be applied in all images of the same order.
 - Automatic (default and strongly recommended)
 - User defined
- Minimum Area Autophagosome: This parameter specifies the minimum area of autophagosome. The dots smaller than this value will not be taken into account (they are probably noise or artifact)
 - Automatic (default and strongly recommended)
 - User defined

3 Results

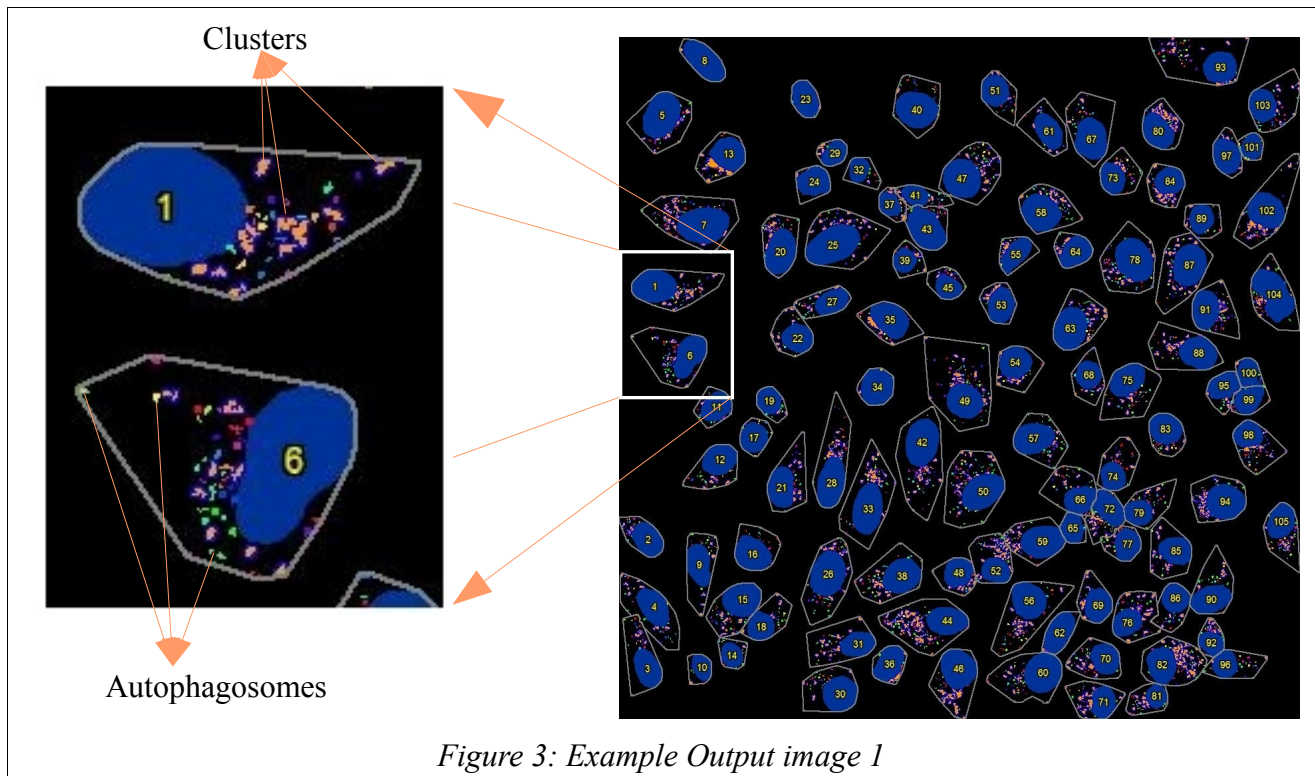
Results are given in a zip file. Files included in the zip are:

- Output image 1 (one per uploaded image).
- Csv file (one per uploaded image).
- Order Summary (one per order).

3.1 Output Image1

Output Image1 is named as imageName_Ext01.jpg. One file is provided per uploaded image. This is an artificial image where the following objects are highlighted:

- Nuclei, labeled with the cell ID number
- Perimeter of the cell marked in gray.
- Individual autophagosomes, labeled in different colors.
- Clusters, labeled in orange with a red perimeter



3.2 Description of CSV file

The CSV file is a document for each image with the values of the parameters quantified by WimAutophagy in the Autophagy assay. It is a csv file, whose name is composed as follows imageName_Stats01.csv. One file is provided per uploaded image.

The information in the Csv file is organized in two parts (Figure 4).

Lines 1,2 and 3: This first part is the information about overall data of the analysis for that image that later will appear in the summary file.

Line 5 onwards: This part displays the Individual Cell Metrics (all the parameters per cell organized in one row per each cell)

- **Column A, ID:** ID to identify the cell in the control image.
- **Column B, Cytoplasm Area [px]:** Area of the cytoplasm for this cell.
- **Column C, Nucleus Area [px]:** Nucleus Area in px.
- **Column D, Number Isolated APs (A):** Number of isolated autophagosomes. Number of Autophagosomes which area is less than twice the estimated autophagosome area. (See Appendix 4 for clarification)
- **Column E, Number Clusters (B):** Number of Autophagosomes which area is bigger than twice the estimated autophagosome area.
- **Column F, Number Estimated APs in Cluster (C):** This value is calculated dividing the area of each cluster by the estimated area of autophagosome. These values are rounded
- **Column G, Puncta per cell (A+B):** Total number of Autophagosomes in the cell without estimating the autophagosomes in cluster.

$$\text{Puncta per cell} = \text{Number Isolated APs} + \text{Number Cluster}$$

- **Column H, Total Number Estimated APs (A+C):** Total number of Autophagosomes in the cell estimating the number of Autophagosomes in each cluster.

$$\text{Total Number Estimated APs} = \text{Number Isolated APs} + \text{Number Estimated}$$

- **Column I, Total AP Area [px]:** Sum of the area of all Autophagosomes in the cell.

$$\text{Total AP Area} = \sum Area_{APi}$$

- **Column J, Mean Intensity of Total AP Area**
- **Column K, Total Area Cluster:** Sum of the area of all Cluster in the cell.

$$Total\ Cluster\ Area = \sum Area_{Cluster_i}$$

	A	B	C	D	E	F	G	H	I	J	K
1	Autophagy	Assay Key Metrics									
2	No. Cells	Estimated Area AP [px]	Mean AP per cell	Mean Puncta per cell	Mean AP Intensity	Mean Nucleus Area [px]	Std AP per cell	Std Puncta per cell	Std Nucleus Area [px]	Total Area AP [px]	
3	95	6	35.37	29.55	0.32	1819	25.1	19.42	534	19000	
4	Individual Cell Metrics										
5	ID	Cytoplasm Area [px]	Nucleus Area [px]	Number Isolated APs (A)	Number Clusters (B)	Number Estimated APs in Cluster (C)	Puncta per cell (A+B)	Total Number Estimated APs	Total AP Area [px]	Mean Intensity of	Total Area Cluster [px]
6	1	5935	3144	45	4	11	49	56	324	0.28	90
7	2	3658	1408	24	1	2	25	26	130	0.23	23
8	3	5105	1737	31	3	9	34	40	247	0.21	61
9	4	2564	1726	26	0	0	26	26	146	0.29	0
10	5	2098	1818	18	0	0	18	18	81	0.34	0
11	6	1870	1794	26	1	2	27	28	141	0.4	19
12	7	8907	2423	65	12	28	77	93	636	0.49	224
13	8	2234	1607	26	6	20	32	46	251	0.42	103
14	9	1884	1522	31	3	6	34	37	205	0.35	53
15	10	8601	2223	37	5	10	42	47	270	0.32	79
16	11	1174	1756	13	2	4	15	17	101	0.27	33
17	12	5250	2258	41	4	9	45	50	268	0.28	66
18	13	5383	1534	23	2	2	24	25	115	0.23	13
19	14	1578	1310	27	6	12	33	39	249	0.31	106
20	15	2936	1856	29	1	2	30	31	166	0.32	16
21	16	1938	1939	19	4	11	23	30	185	0.45	95
22	17	763	1421	12	1	3	13	15	65	0.21	13
23	18	1195	1725	14	3	7	17	21	126	0.29	40
24	19	652	1483	5	0	0	5	5	23	0.24	0
25	20	1509	1330	14	4	9	18	23	128	0.27	66
26	21	2521	2231	25	4	9	29	34	185	0.41	73
27	22	4722	2020	51	11	36	62	87	605	0.5	241
28	23	1044	1839	10	1	3	11	13	56	0.23	12
29	24	1072	1651	14	2	4	16	18	85	0.23	26
30	25	8970	3775	74	6	16	80	90	502	0.37	169
31	26	3407	860	29	3	6	32	35	215	0.35	65
32	27	4681	1814	44	8	27	52	71	392	0.39	175
33	28	1069	1445	14	0	0	14	14	75	0.26	0
34	29	3698	2199	31	0	31	31	31	148	0.22	0
35	30	1708	1374	22	1	2	23	24	144	0.27	16
36	31	1556	1983	18	2	2	20	22	126	0.28	24
37	32	1551	1741	13	0	0	13	13	71	0.33	0
38	33	1293	1895	14	1	3	17	17	68	0.19	12
39	34	941	1368	19	0	0	19	19	90	0.24	0
40	35	5139	2315	45	10	24	55	69	413	0.31	168
41	36	1036	1780	12	1	2	13	14	78	0.24	15
42	37	3695	1558	25	2	4	27	29	179	0.23	40
43	38	1543	1820	19	1	2	20	21	101	0.31	12
44	39	1607	2006	10	0	0	10	10	41	0.25	0
45	40	3565	1927	43	9	22	52	65	369	0.27	150
46	41	2042	1520	21	5	34	26	55	392	0.76	272
47	42	1094	1444	9	3	7	12	16	103	0.26	61
48	43	7572	3485	48	4	9	52	57	294	0.24	63
49	44	893	1450	19	3	7	22	26	176	0.4	52
50	45	9116	2632	69	13	38	82	107	606	0.48	273
51	46	3403	1438	10	0	0	10	10	268	0.40	188

Figure 4: CSV File

3.3 Order Summary.csv

This is a document with relevant important information of the order. It is a csv file, whose name is formed as follows orderNum_Summary.csv. One Order Summary file per order is provided. The Order Summary file has a line for each image and specifies the following data for each image:

- **Column A**, Name of the image
- **Column C**, **No. Cells:** Total numbers of cells in the image
- **Column D**, **Estimated Area AP [px]:** Estimated Area of Autophagosomes in px.
- **Column E**, **Mean AP per cell:** Mean of total number estimated APs per cell.

$$\text{Mean AP per Cell} = \frac{1}{N} \sum \text{NumberEstimatedAPs}_i$$

- **Column F, Mean Puncta per cell:** Mean of Puncta per cell

$$\text{Mean Puncta per cell} = \frac{1}{N} \sum \text{PunctaPerCell}_i$$

- **Column G, Mean AP Intensity:** Mean Autophagosomes Intensity. We have considered only the cells that have some autophagosomes to calculate this value

$$\text{Mean AP Intensity} = \frac{1}{N} \sum \text{IntensityAP}_i$$

- **Column H, Mean Nucleus Area [px]:** We have considered all cells to calculate this value

$$\text{Mean Nucleus Area} = \frac{1}{N} \sum \text{AreaNucleus}_i$$

- **Column I, Std AP per cell:** Standard deviation of total number estimated APs

$$\text{Std AP per cell} = \sqrt{\frac{1}{N} \sum (\text{APPerCell}_i - \text{MeanAPPerCell})^2}$$

- **Column I, Std Puncta per cell:** Standard deviation of Puncta per cell (A+B)

$$\text{Std Puncta per cell} = \sqrt{\frac{1}{N} \sum (\text{PunctaPerCell}_i - \text{MeanPunctaPerCell})^2}$$

- **Column J, Std Nucleus Area:** Standard deviation of Nucleus Area [px]

$$\text{Std Nucleus Area} = \sqrt{\frac{1}{N} \sum (\text{NucleusArea}_i - \text{MeanNucleusArea})^2}$$

- **Column L, Total Area AP:** Sum of the area of all Autophagosomes in the image

$$\text{Total Area AP Image} = \sum \text{Total Area AP Cell}_i$$



	A	B	C	D	E	F	G	H	I	J	K	L
1	File Name	Autophagy Assay	Key Metrics									
2		No. Cells	Estimated Area AP*	Mean AP per cell	Mean Puncta per cell	Mean AP Intensity	Mean Nucleus Area [px]	Std AP per cell	Std Puncta per cell	Std Nucleus Area [px]	Total Area AP [px]	
3	Atg7/PN5_At7_-_suero_NL_*	31	8	42.03	26.29	0.31	2079	32.82	20.22	981	8917	
4	Atg7/PN5_At7_-_suero_NL_*	22	8	2	0.45	0.1	2050	8.14	1.74	584	356	
5	Atg7/PN5_At7_-_suero_NL_*	21	8	45	30.52	0.25	2202	33.36	21.28	1078	6173	
6	Atg7/PN5_At7_-_suero_NL_*	25	8	34.36	23.36	0.23	2316	24.5	14.78	957	5692	
7	Atg7/PN5_At7_-_suero_NL_*	22	8	22.91	16	0.2	2317	29.95	18.99	745	3207	
8	Atg7/PN5_At7_-_suero_NL_*	43	8	25.53	17	0.22	2396	28.65	16.97	958	7217	
9	Atg7/PN5_At7_-_suero_a_L*	38	8	15.29	12.68	0.2	3161	15.05	11.82	1311	3769	
10	Atg7/PN5_At7_-_suero_b_L*	30	8	15.97	12.73	0.19	2928	13.48	10.36	711	3030	
11	Atg7/PN5_At7_-_suero_c_L*	20	8	49.45	31.85	0.33	2519	33.58	13.94	472	6566	
12	Atg7/PN5_At7_-_suero_d_L*	49	8	45.92	30.55	0.35	2623	29.12	18.91	1084	14824	
13	Atg7/PN5_At7_-_suero_e_L*	76	8	28.67	21.58	0.28	2351	27.01	19.41	925	14270	
14	Atg7/PN5_At7_-_suero_f_L*	54	8	36.48	27.48	0.31	2397	27.4	21.19	979	12915	
15	Atg7/PN5_At7_control_NL_*	32	8	8.56	7.66	0.19	1315	8.63	7.46	592	1652	
16	Atg7/PN5_At7_control_NL_*	20	8	22.65	18.2	0.21	2708	17.3	12.38	929	2906	
17	Atg7/PN5_At7_control_NL_*	37	8	7.89	5.46	0.21	2310	10.61	3.86	882	1947	
18	Atg7/PN5_At7_control_NL_*	20	8	6.3	5.35	0.21	3225	11.63	8.19	1142	769	
19	Atg7/PN5_At7_control_NL_*	25	8	6.84	3.24	0.19	2667	20.23	6.49	1002	1247	
20	Atg7/PN5_At7_control_a_L*	51	8	6.12	3.69	0.22	1860	15.83	7.06	589	2079	
21	Atg7/PN5_At7_control_c_L*	39	8	4.26	3.28	0.17	2213	5.41	2.93	656	1056	
22	Atg7/PN5_At7_control_d_L*	17	8	8.29	5.18	0.2	2748	12.31	6.01	1231	962	
23	Atg7/PN5_At7_control_e_L*	59	8	5.14	2.95	0.23	1889	14.71	5.97	629	2101	
24	Atg7/PN5_At7_control_f_L*	24	8	6.46	5.08	0.18	2305	5.71	3.69	857	1011	
25	L2a/PN5_L2a_-_suero_NL_a*	44	8	2.25	2.23	0.23	2134	1.63	1.63	656	614	
26	L2a/PN5_L2a_-_suero_NL_b*	32	8	5.97	4.53	0.26	2435	10.22	6.71	878	1185	
27	L2a/PN5_L2a_-_suero_NL_c*	27	8	3.26	3.07	0.22	3053	3.61	3.32	1007	564	
28	L2a/PN5_L2a_-_suero_NL_d*	43	8	1.51	1.44	0.21	1895	1.72	1.64	912	428	
29	L2a/PN5_L2a_-_suero_NL_e*	43	8	3.44	2.98	0.25	2380	4.81	3.17	786	903	

Figure 5: Order Summary

4 Appendix A: Analysis Phases Autophagy

The main phases of the analysis are three, preprocessing, processing and data merge.

4.1 Preprocessing

This phase detects the area occupied by the autophagosomes by statistical calculations. The steps to calculate the area of autophagosomes are:

- Detection of the all puncta for each image and estimating its area in pixels
- Estimate a probability density function of these areas
- Find the estimated area in this function. If we can distinguish the two populations in the probability density function as shown in Figure 6, the maximum area of the autophagosome will be between both curves. Puncta bigger than that will be considered as clusters. If only one population is detected as in Figure 7 the probability density function will be a Gaussian probability density function and the maximum area will be set where the area is almost 1 indicating that in these images there are a very small number of clusters.

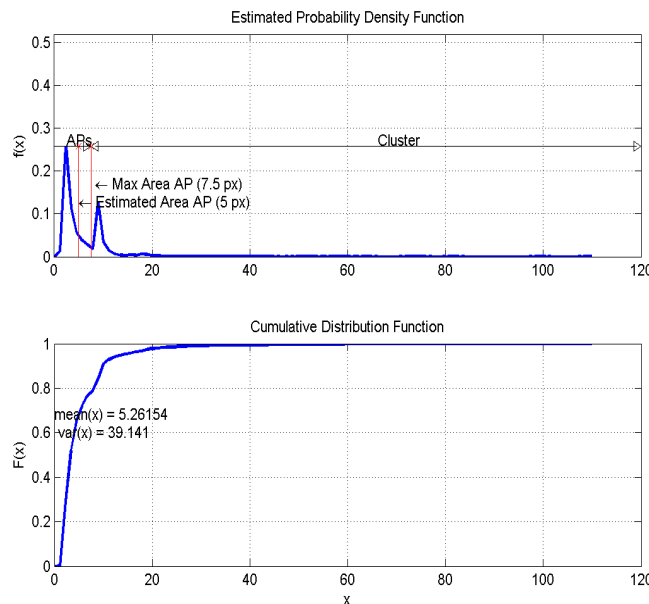


Figure 6: Probability density function of image with two kind of puncta populations

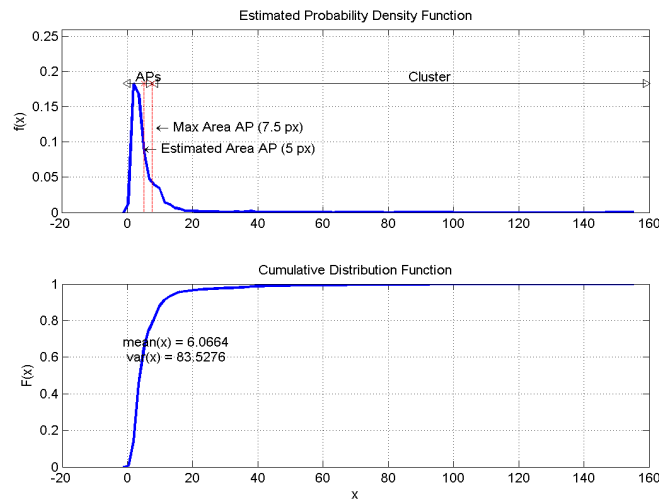


Figure 7: Probability density function of image with one population

See types of dots (isolated autophagosomes and clusters) in image Figure 3

4.2 Processing

The different steps of processing are:

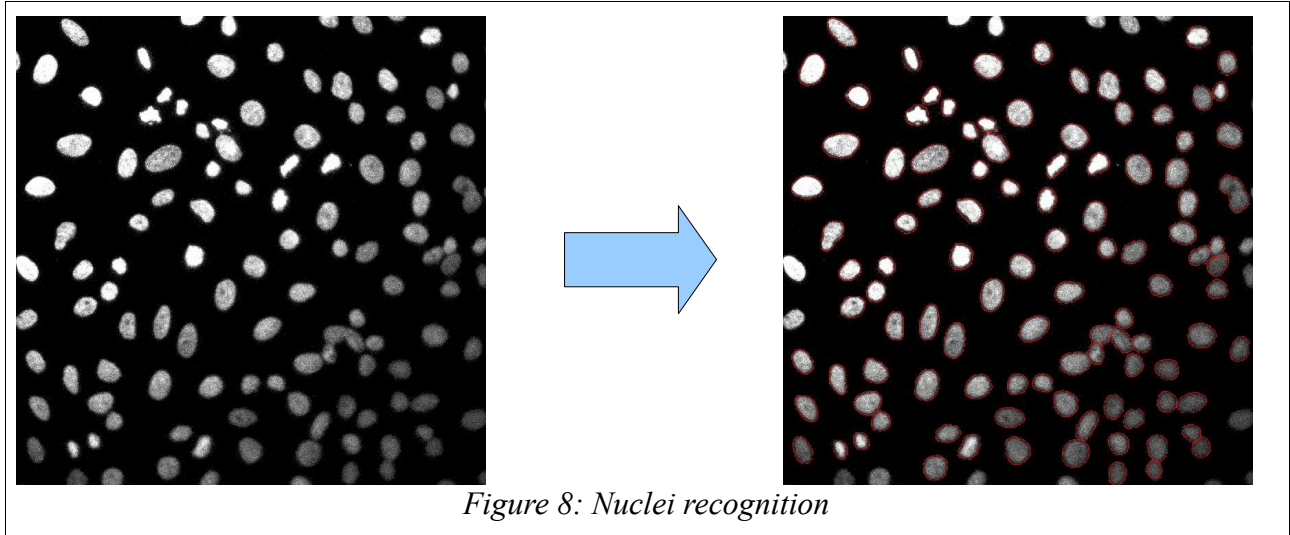
4.2.1. Filtering of input Images (Standardization)

The input image is a RGB image, in this phase of the analysis the algorithm will:

- Split the input image in the different layers.
- Standardize image size.
- Increase contrast.
- Correct not uniform illuminations.

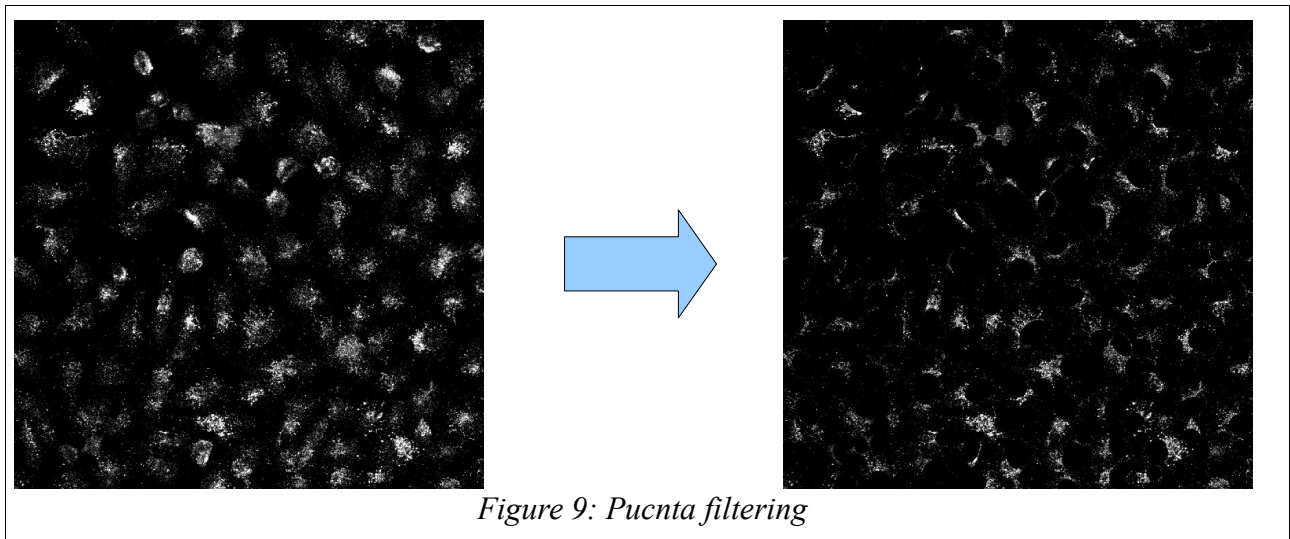
4.2.2. Nuclei recognition

In this phase of the analysis the nuclei are identified by a red perimeter and numbered. Cells whose nuclei are not completely inside the image will not be used for the analysis and will not be labeled in output image 1.



4.2.3. Filtering of LC3 images.

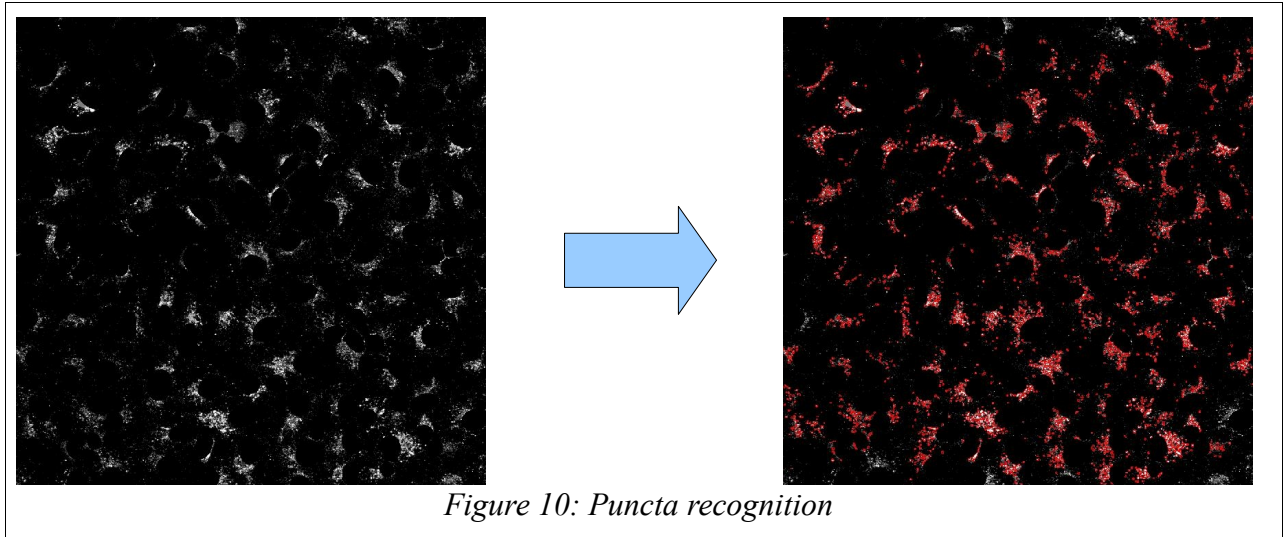
Different types of filters are applied to enhance puncta.



4.2.4. Puncta Recognition

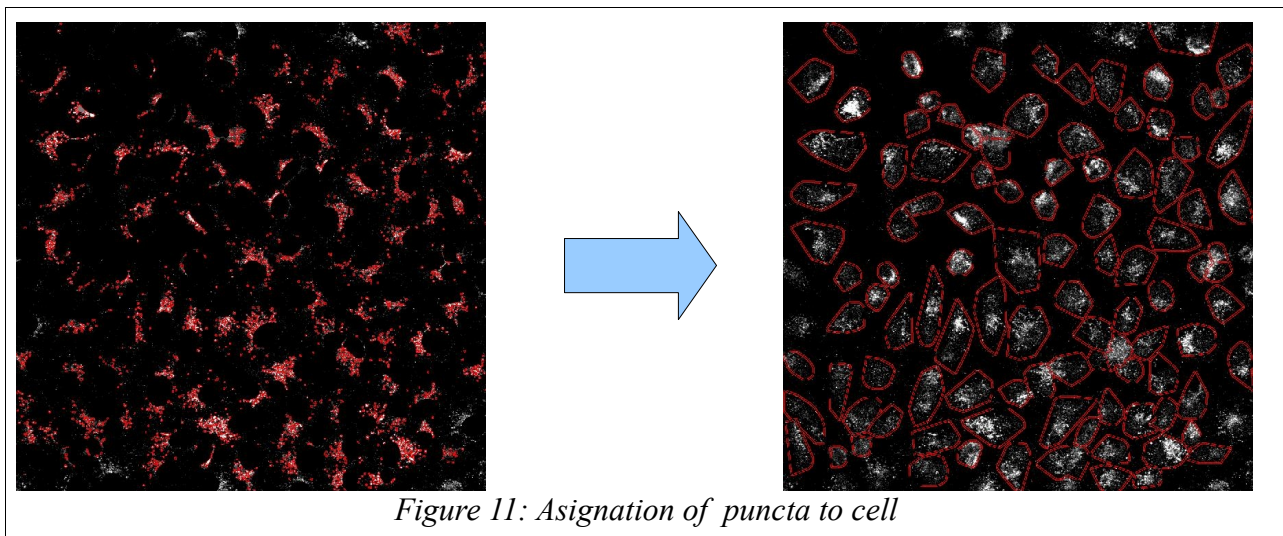
The resulting image, after filtering and processing techniques, will have only the puncta (bright dots within the cytoplasm). Puncta are detected by means of iterative techniques of segmentation of

intensity. The brightest puncta are detected in the first iterations and the autophagosomes whose intensity is lower in the last iteration.



4.2.5. Relate puncta to Cell and Cell Indexing

Detected puncta are assigned to the nucleus through morphology and distance techniques.



4.2.6. Data Processing

With all the obtained parameters the algorithm will calculate all data to be exported. The the number of

AP will be calculated by adding the number of isolated APs plus the APs present in clusters.

4.3 Data Merge

Summary.csv file will be created in this part of analysis by collecting all the data obtained in the previous steps.