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An improved Immunohistochemistry image post-processing method for the semi-automatic quantification of astrocytes number and activation

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Co-Chaired by PROF. DR. MARIA EMÍLIA SOUSA

pharmaceuticals MDPI

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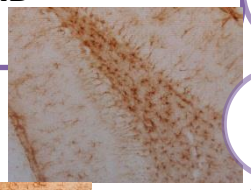
CINTESIS Health. Research.

An Improved IHC image post-processing method for the semi-automatic quantification of astrocyte number and activation

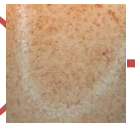
Conventional analysis of photomicrographs from IHC staining with DAB

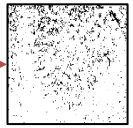
- Manual quantification
- User subjectivity
- Innapropriate for large sampling processing
- Complex photomicrographs
- Dependent of image quality



Improved semi-automatic method

- Open-source software
- Appropriate to any sampling size
- Rapid
- Applicable to low magnification images
- Determination of cell and branch number and size





Abstract

Immunohistochemical staining of cell and molecular targets in brain samples is a powerful tool that can provide valuable information on neurological mechanisms. However, post-processing of photomicrographs acquired after 3, 3'-Diaminobenzidine (DAB) staining can be particularly challenging due to the complexity, size and number of the samples, the targets being analyzed, image quality, and even the subjectivity related with image analysis and morphological appreciation by different users. Conventional analysis of these data usually relies on the manual quantification of distinct parameters in a large set of images like, for example, the number and size of cells or, in more complex analysis, the number and size of cell branching (as in Sholl analysis, e.g.). These prove to be extremely time-consuming and complex tasks, inappropriate for the processing of high amounts of information.

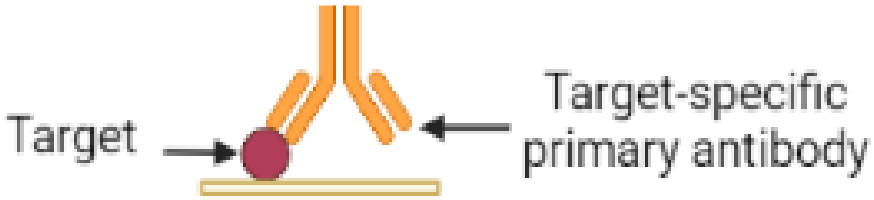
Here we describe an improved semi-automatic method to quantify glial fibrillary acidic protein (GFAP)-labelled astrocytes in immunohistochemistry images of rat brain, at a magnification as low as 20x. This method is a straightforward adaptation of the Young & Morrison method, using the ImageJ plugin Skeletonize, coupled to an intuitive data processing in datasheet-based software.

This method allows a faster and more efficient post-processing of brain tissue samples for the quantification of astrocytes size and number, area occupied, as well as astrocyte branching and branch length (indicative of astrocyte activation). Thus, contributing to better understand the possible inflammatory response developed by astrocytes.

Keywords: astrocytes, GFAP, ImageJ; quantification; semi-automatic method; skeletonize.

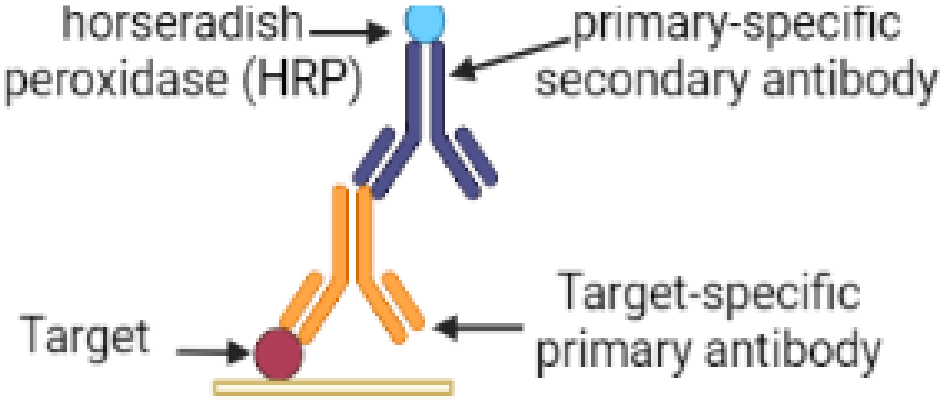
Young, K., Morrison, H. Quantifying Microglia Morphology from Photomicrographs of Immunohistochemistry Prepared Tissue Using ImageJ. J. Vis. Exp. (136), e57648, doi:10.3791/57648 (2018)

IMMUNOHISTOCHEMISTRY - a powerful tool to provide information on cells and molecular targets



Exploits the **specific relation** between a **target** and its respective **antibody**;

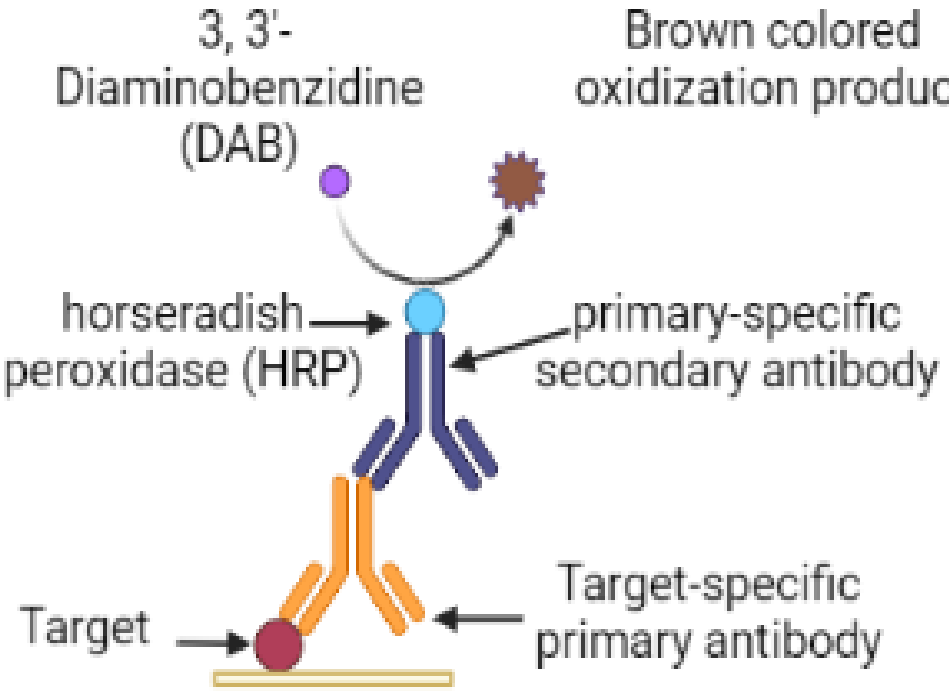
IMMUNOHISTOCHEMISTRY - a powerful tool to provide information on cells and molecular targets



Secondary antibody is associated with **horseradish peroxidase (HRP)**;

Exploits the **specific relation** between a **target** and its respective **antibody**;

IMMUNOHISTOCHEMISTRY - a powerful tool to provide information on cells and molecular targets

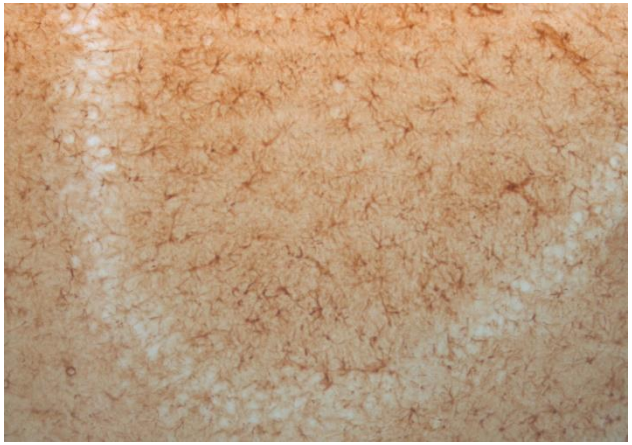


HRP that oxidizes **DAB**, forming a **brown precipitate.**

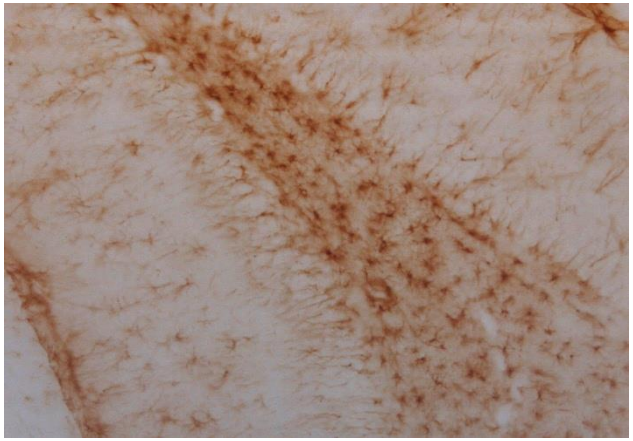
Secondary antibody is associated with **horseradish peroxidase (HRP)**.

Exploits the **specific relation** between a **target** and its respective **antibody.**

Typical GFAP IHC-stained photomicrographs

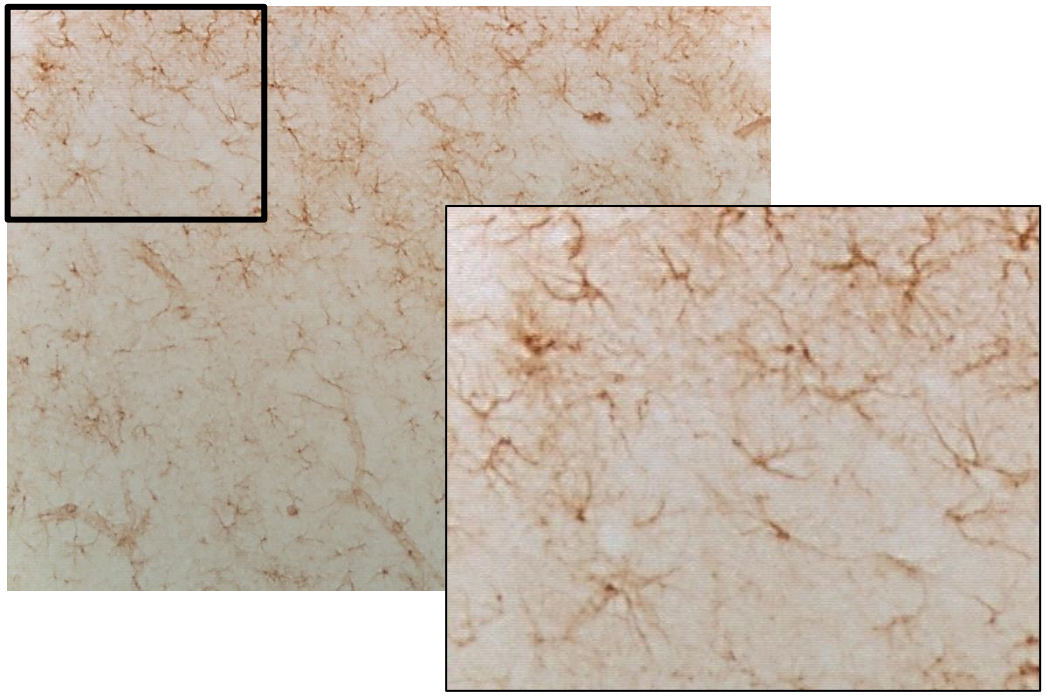


CA3 area of the hippocampal formation



Dentate Gyrus and Hilus areas of the hippocampal formation

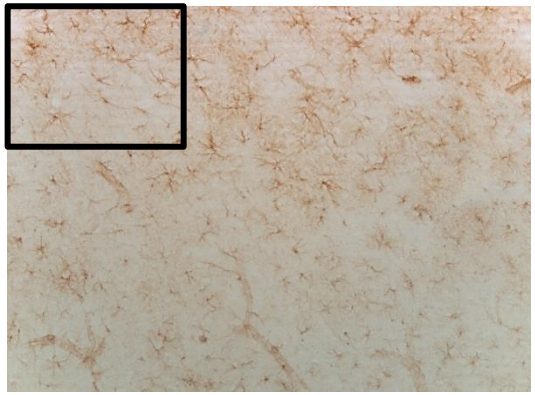
GFAP-immunostained astrocytes



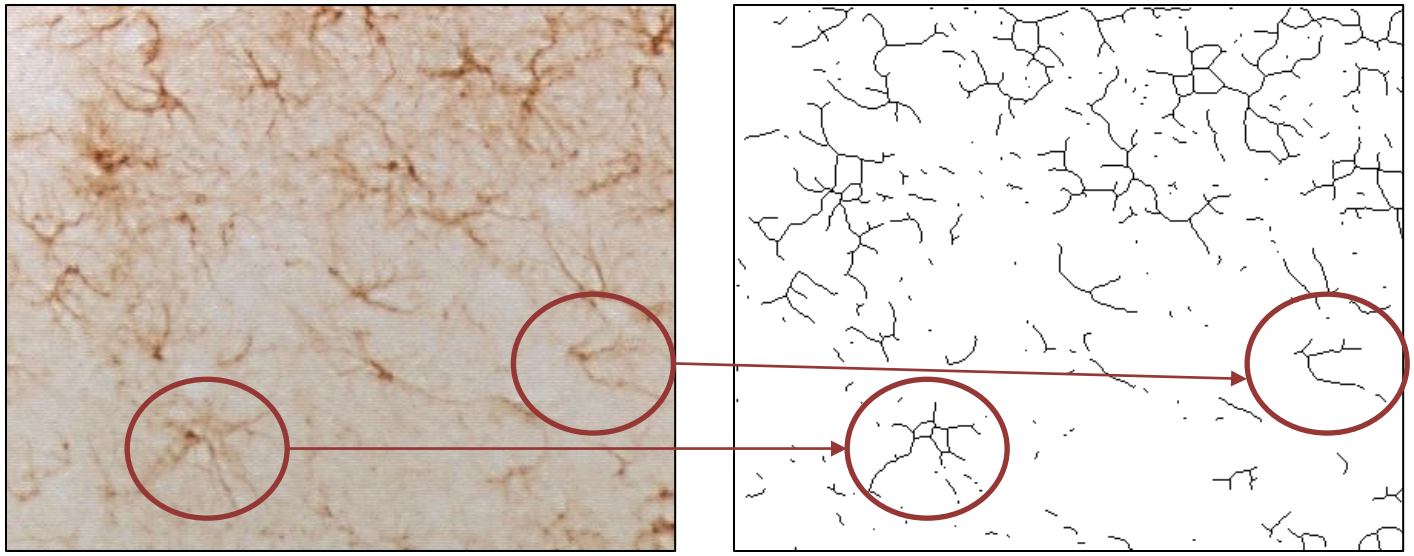
Prefrontal cortex area of rat brain sample

Quantification of cell number and branche size and number is a dauting task.

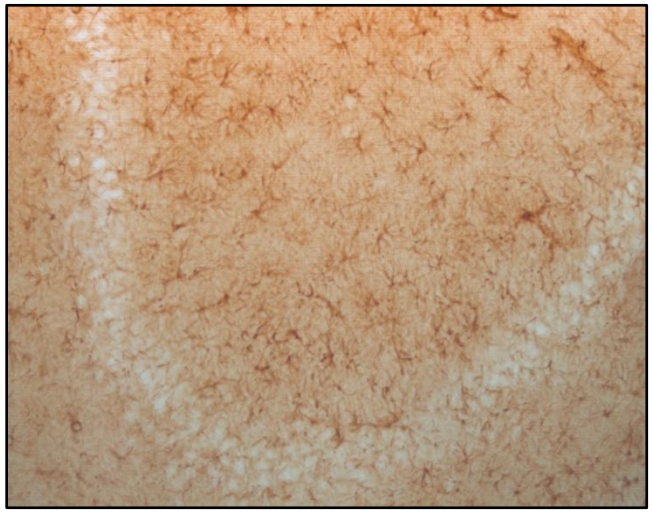
Typical GFAP IHC-stained photomicrographs

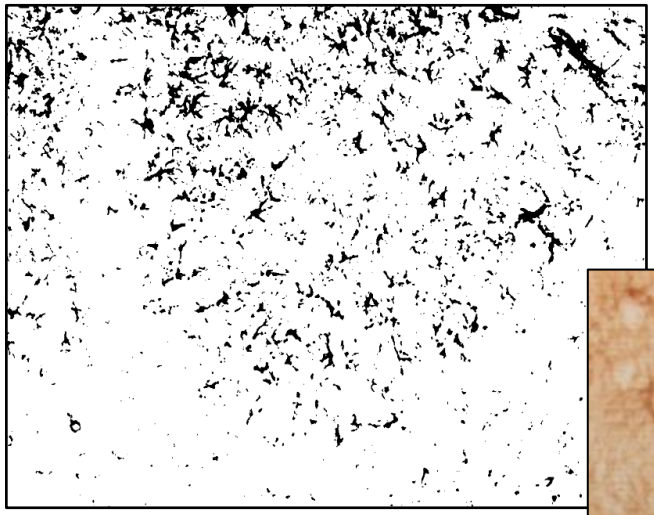


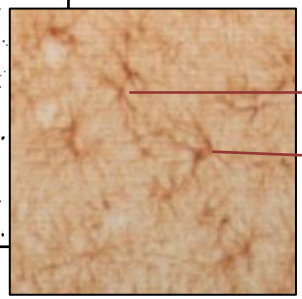
Prefrontal cortex area of rat brain sample

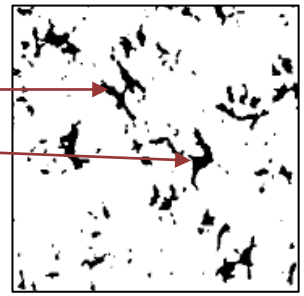


OUR METHOD: from microphotograph to analisable mask - ImageJ









Scaling

1. Set scale;
2. Preselect of area and measure;

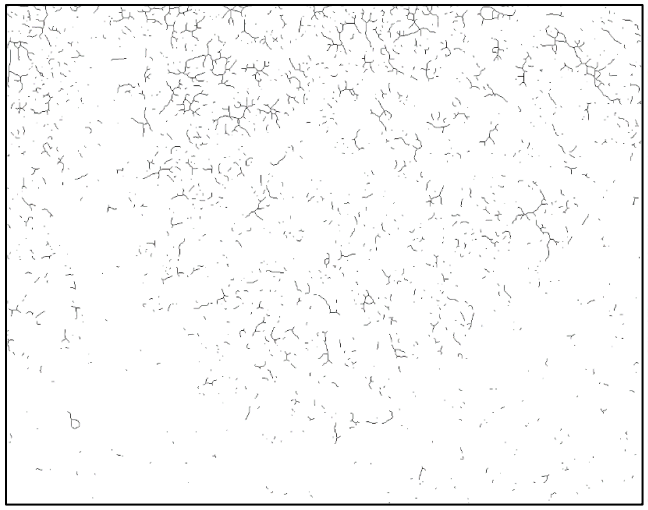
Image Processing

3. Convert to grey scale;
4. Unsharp mask;
5. Clear noise with Despeckle,

Threshold

6. Using the Threshold tool:
   - Set algorithm to MaxEntropy;
7. Define accordingly to area occupancy.

Skeletonize and Analysis- ImageJ



Branch information

File Edit Font

	Skeleton ID	Branch length	V1 x	V1 y	V1 z	V2 x	V2 y	V2 z	Euclide
1	14	17.728	14	141	0	26	152	0	16.279
2	20	24.314	25	586	0	27	597	0	11.180
3	20	11.828	25	586	0	27	597	0	11.180
4	23	4.828	33	187	0	35	183	0	4.472
5	36	37.314	66	96	0	96	96	0	30.000
6	36	21.828	63	116	0	66	96	0	20.224
7	36	21.071	55	66	0	60	85	0	19.647
8	36	10.657	63	120	0	70	126	0	9.220
9	36	10.485	58	90	0	66	96	0	10.000
10	36	9.414	70	126	0	79	125	0	9.055
11	36	7.243	50	119	0	56	116	0	6.708
12	36	7.000	56	116	0	63	116	0	7.000
13	36	5.828	58	90	0	60	85	0	5.385
14	36	4.828	63	116	0	63	120	0	4.000
15	37	18.899	57	15	0	72	7	0	17.000

Mask Processing

8. Despeckle clearing;
9. Removal of outliers

Skeletonize

10. Skeletonize maks;
11. Analyze Skeleton

Data acquisition

ImageJ supplies a data table with skeleton identification and Branch length.

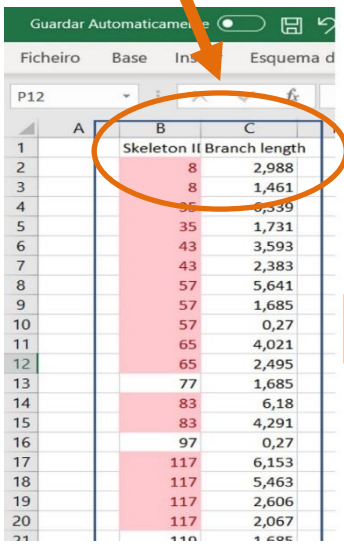
- Save the table with the respective spreadsheet file extension.

Spreadsheet analysis

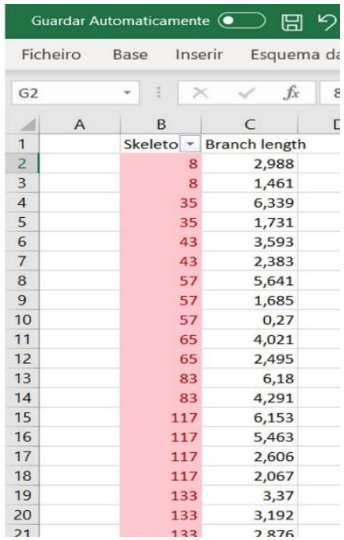
From the data acquired from ImageJ, **skeleton ID** and **Branch length** is selected

Single Skeleton IDs entries are **removed**

Data is sorted acording to the **number of branches per cell** and **total branch length**.



	A	B	C
1		Skeleton II	Branch length
2		8	2,988
3		8	1,461
4		35	6,339
5		35	1,731
6		43	3,593
7		43	2,383
8		57	5,641
9		57	1,685
10		57	0,27
11		65	4,021
12		65	2,495
13		77	1,685
14		83	6,18
15		83	4,291
16		97	0,27
17		117	6,153
18		117	5,463
19		117	2,606
20		117	2,067



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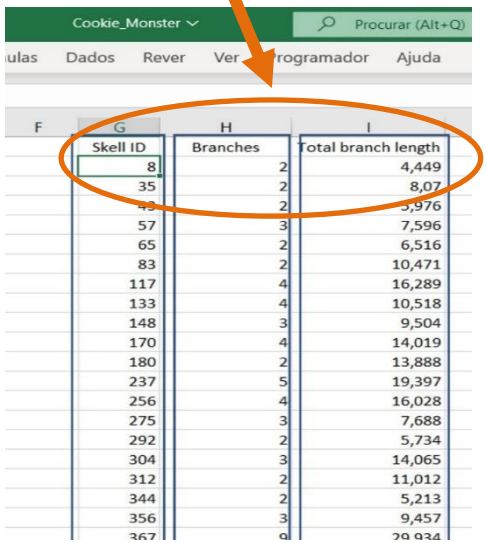
12		65	2,495
13		83	6,18
14		83	4,291
15		117	6,153
16		117	5,463
17		117	2,606
18		117	2,067
19		133	3,37
20		133	3,192
21		133	2,876



	F	G	H	I
		Skell ID	Branches	Total branch length
		8	2	4,449
		35	2	8,07
		43	2	5,976
		57	3	7,596
		65	2	6,516
		83	2	10,471
		117	4	16,289
		133	4	10,518
		148	3	9,504
		170	4	14,019
		180	2	13,888
		237	5	19,397
		256	4	16,028
		275	3	7,688
		292	2	5,734
		304	3	14,065
		312	2	11,012
		344	2	5,213
		356	3	9,457
		367	9	29,934

Data trimming allows the quantification of the number of cells, branch/ cell and branch length

Spreadsheet analysis

Data can be organized by the **number of cells = Skeleton IDs**;

Mode of the **branch length**;

Total branch length

Area, measured initially.

Sample group analysis further includes **Branch number/cell; cell/ área and length/cell**.

	A	B	C	D	E	F	G	H	I	J	K	L	M
1						Mode							
2			Area	Astrocyte number	branch number	[0,1; 2,5[	[2,5; 4,5[	[4,5; 5000,5[	[5000,5; 8,5[	[8,5; 10,5[	>=10,5	Total Branch length	
3	g			48	162	1,38	1,00	1,00	0,17	0,06	0,04	586,93	
4													
5													
6													
7													
8													

Treatment group	sample identification	Area	Astrocyte number	Total number of branches	Mode [0,1; 2,5[	[2,5; 4,5[	[4,5; 6,5[	[6,5; 8,5[	[8,5; 10,5[	>=10,5	total branch length	branch number/ astrocyte	astrocyte /area (x10000)	length/a strocyte
1	259	1580297,639	1785	14311	212,7	139,3	106,7	29,2	13,1	10,3	55083,91	8,02	11,30	30,86
	264	1310245,94	1012,00	5439,00	96,5	99,4	69,2	26,4	9,5	6,7	21548,02	5,37	7,72	21,29
	310	1072722,168	1432	8785	115,5	80,9	55,8	14,3	6,9	5,1	30776,29	6,13	13,35	21,49
	315	943224,431	630	3128	85,6	90,8	67,1	19,7	5,7	7,7	12309,01	4,97	6,68	19,54
2	260	1311943,206	1616	10539	169,9	115,8	89,8	24,6	11,0	9,0	39375,02	6,52	12,32	24,37
	265	973140,613	805	4836	88,7	107,3	76,8	23,7	9,9	6,5	19661,05	6,01	8,27	24,42
	262	1197367,99	1396	8184	130,3	98,6	74,1	20,6	8,0	7,4	29166,31	5,86	11,66	20,89
	311	1404340,004	1929	12746	145,1	122,5	91,6	26,9	9,9	9,8	47453,4	6,61	13,74	24,60
	317	800829,058	513	3023	48,6	52,8	43,1	11,4	4,1	3,4	11826,18	5,89	6,41	23,05
3	263	543071,44	384,00	1873,00	23,0	26,0	23,5	5,3	2,5	1,7	7382,43	4,88	7,07	19,23
	266	901991,89	777	4370	59,9	56,7	47,8	12,7	4,2	3,3	16332,34	5,62	8,61	21,02
	261	663283,428	861	5156	61,8	49,6	35,5	9,9	3,9	2,8	18895,35	5,99	12,98	21,95
	312	1388273,59	1931,00	14710,00	127,1	99,9	71,3	21,0	8,9	5,7	55225,48	7,62	13,91	28,60
	314	1445793,496	1141	5965	75,2	77,0	66,6	17,9	7,0	4,9	23708,3	5,23	7,89	20,78

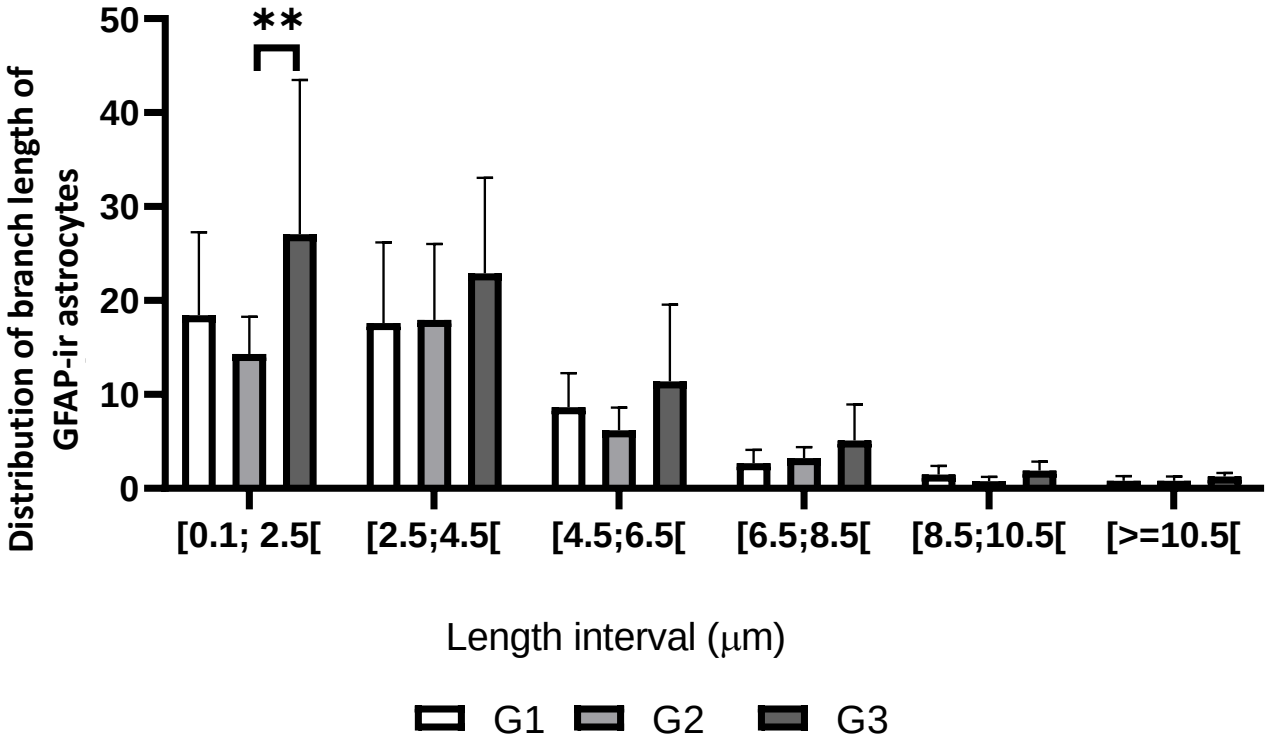
Single sample analysis presents the number of cells, total banch length and a branch length mode

Graphical Representation – mode, histogram and scatterplot

Mode of the branches' size

The organization of the **branches size**, into a **mode**, allows the **comparison** of this particular characteristic **between treatment groups**.

This mode allows the **perception of the increased size in branches of the sampled cells**. Potentially indicating a **change of homeostasis** status.



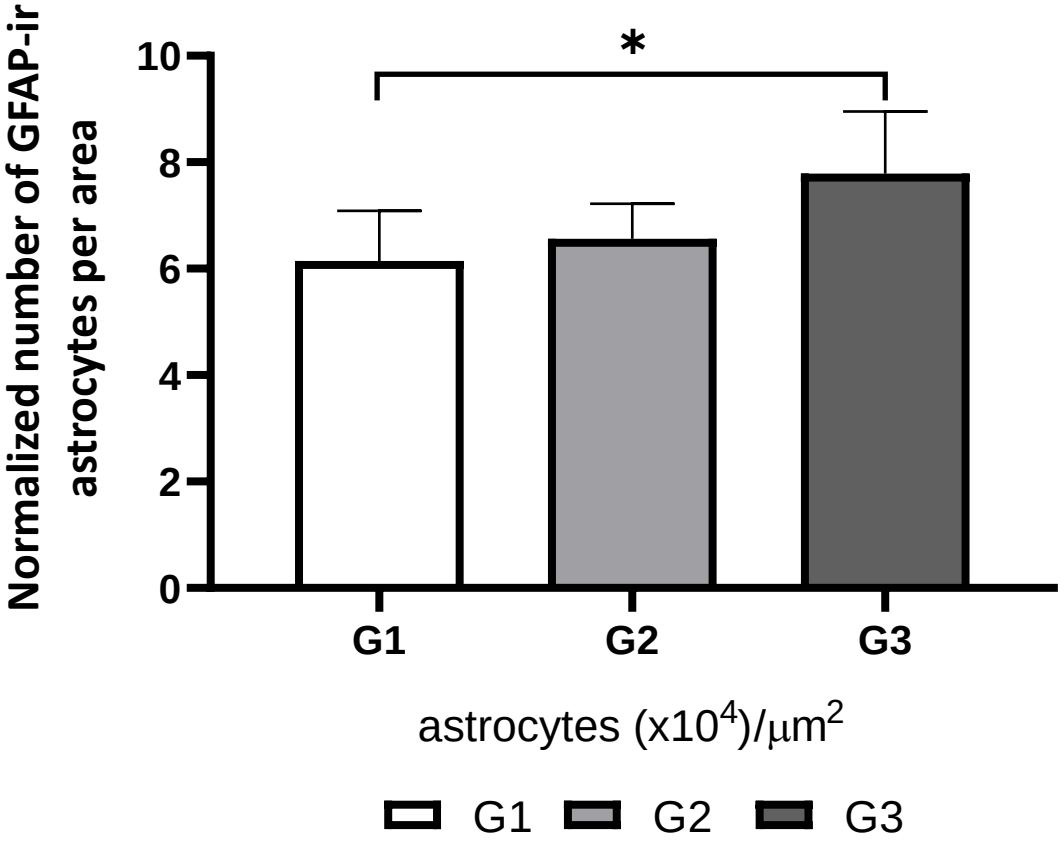
Exemplary graphical representation

Graphical Representation – mode, histogram and scatterplot

Cell/area

This method quantifies the **area and number** of cells in each microphotograph.

In astrocytes, the presence of a higher number of cells in an area may indicate a **change in tissue homeostasis**.



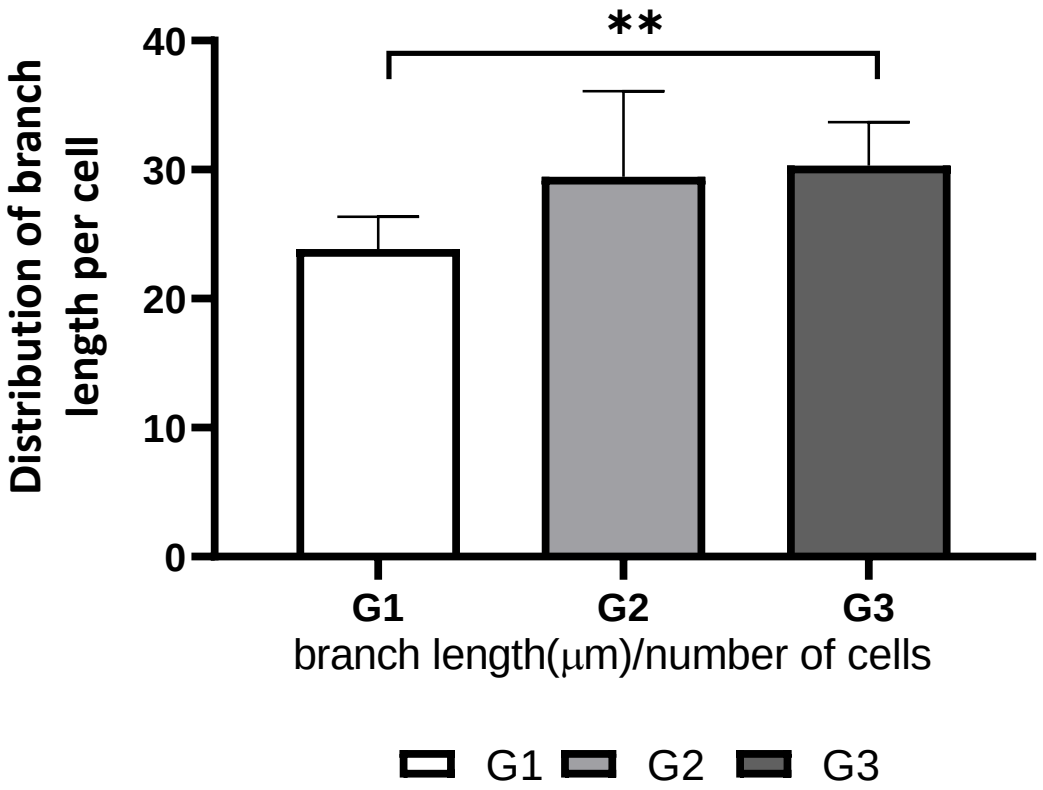
Representative graphical representation

Graphical Representation – mode, histogram and scatterplot

Branch length/cell

Branch length per cell directly indicates the mean of the branch length each cells presents.

In astrocytes, the **activated stated** is characterized by a **higher branch size** and **number**.



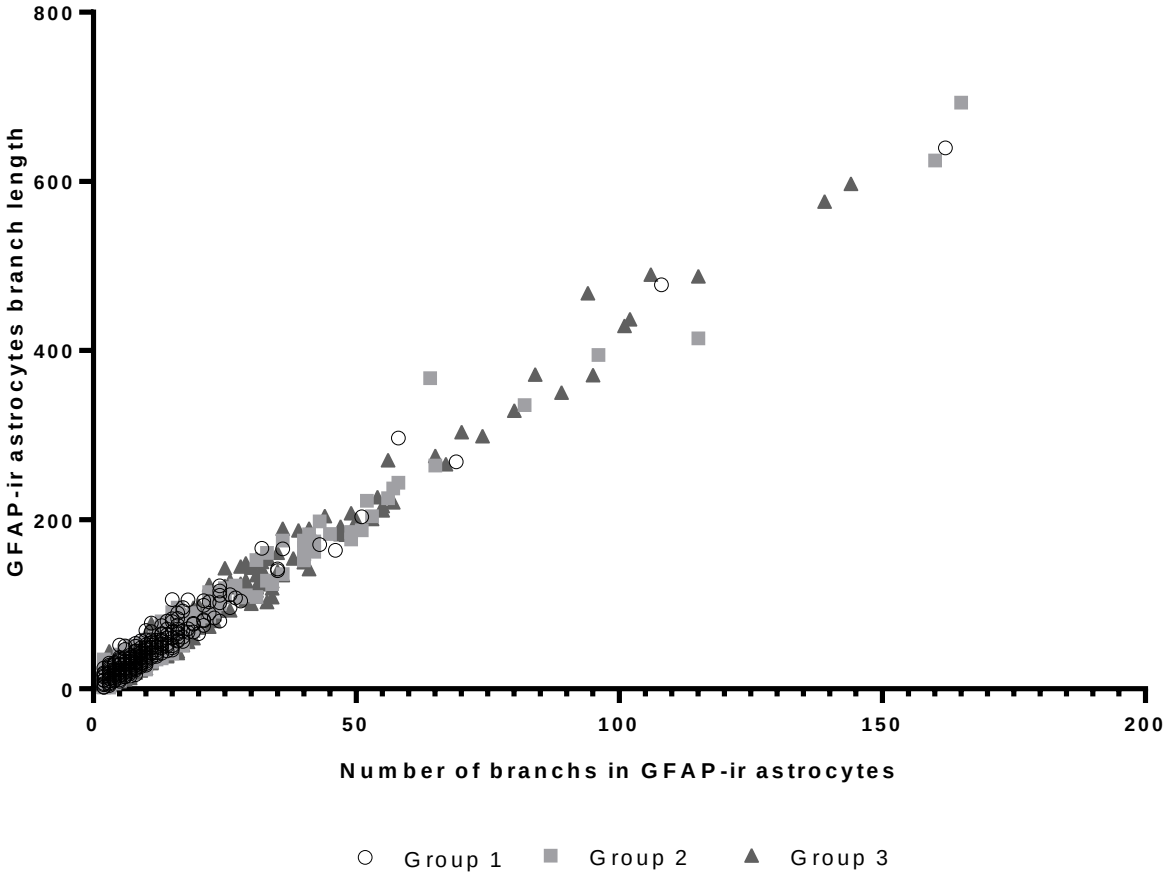
Representative graphical representation

Graphical Representation – mode, histogram and scatterplot

Cell Virtual Size

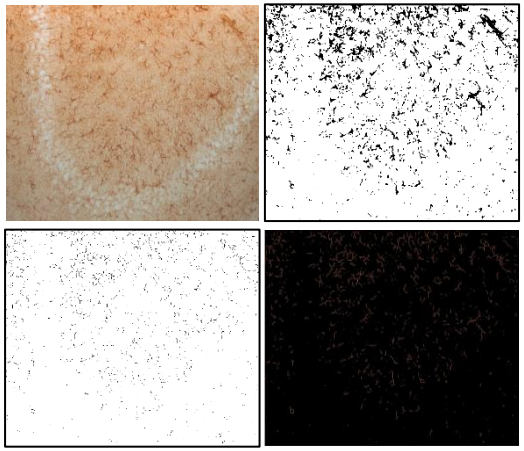
With the **number of branchs per cell** and the **total branch length per cell**, it is possible to infer a **virtual size to each cell**.

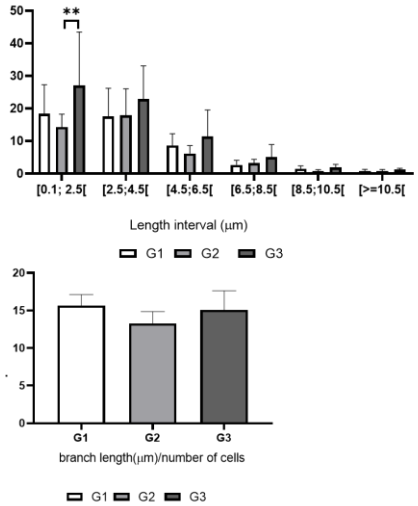
This scatter-plot allows the **perception of the increased size of the sampled cells**. Potentially indicating a **change of homeostasis** status.

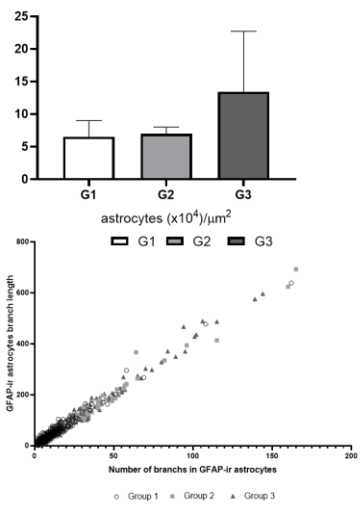


Representative graphical representation

Our Method is FASTER, RELIABLE, SIMPLE and FREE







SEMI AUTOMATIC METHOD

- **SUITABLE FOR BIG SAMPLING SIZES;**
- **Standardizable,** and **precise** data aquisition;
- **Accurate** for photomicrographs with **low amplification**, such as **20x**;
- Use of **open-source software ImageJ**, **skeletonize** plugin and common **spreadsheet** program;
- **Intuitive** and **straightfoward** data representation.

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Prof. Helena Carmo

Prof. Félix Carvalho

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Department of Anatomy @ Faculty of Medicine (UPorto)

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