Validation process of the plugin NeuronZigzagJ

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In order to validate the plugin NeuronZigzagJ, we have undertaken two experimental studies. In the first experience, the aim was to investigate the role of the maximal projection ($\sqcup$) and the union ($\cup$) strategies (with the corresponding filter and thresholding methods). In the second experiment, a more systematic study was carried out to estimate the accuracy of our program with respect to the observations of some human experts. The files containing the images we have worked with are available at `http://www.unirioja.es/cu/anromero/repository-of-images.html`, in order to allow other researchers to reproduce our programming experiments.

1 First experiment

First of all, we have considered a series of actual neurophysiology images and we have applied the plugin using both the *maximal projection* and the *union* strategies (with the corresponding filter and thresholding methods). Concretely, we have applied our plugin starting from 1146 slices (coming from 12 GFP-Actin stacks of images, and 11 DiI stacks).

The results of the experimental study are collected in the table of Figure 1. The description of the columns in the table is as follows. Column 1 contains an identification number, and column 2 is the name of the file containing each stack. Column 3 indicates the number of slices in each stack. Data of column 4 are provided by a human, who inspected the relevant number of connected components (i.e., significant fragments of dendrites) in each stack; it is the column of quality control of the table. Then, each row is divided into two subrows, depending on the kind of operation used to compute the zigzag persistence: the union $\cup$ or the maximal projection $\sqcup$ (let us stress that each operation comes with its own filtering and thresholding methods, as explained before). Finally, the last column indicates the slices from which it is necessary start from to get some of the components (in brackets the number of connected components reached from each slice).

The conclusions that we can extract from these experimental data are the following.

1. An examination of table in Figure 1 shows the "right" slice to start from is not always located at the same place in the stack (in principle, our conjecture was that the intermediate slice would contain most of the relevant

information); this justifies our decision of letting the user choose the initial slice.

2. In DiI images, in 40% of cases there is not a unique starting slice allowing recovering all the information (because dendrites can sprout at distant layers in the stack).

3. Our decision of treating differently GFP-Actin and DiI images is supported by the experiments, since in the former case in a 33% of cases no information is obtained with the union $\cup$ operation, while in the latter case both operations behave similarly, and $\cup$ is preferred because it is cheaper to be computed.

4. To reinforce the previous conclusion, it was checked (but we are not able to reflect it in the table), that the chosen combination filtering-operation-thresholding produced a more accurate segmentation (other combinations were tested, but getting always worst results; in any case, let us recall that all these parameters can be fixed in the user interface, easing the reproduction of our computer experiments).

5. Information in the graphical and barcode outputs is complementary, without obtaining one as a by-product of the other one.

The last observation is specially interesting, because our preconception was that the barcode information would be simply a visual aid to a better understanding of the graphical output. This impression was biased because we underestimate the importance of the choosing of the starting slice. These experiments suggest a way of working with our plugin:

1. First, focus on the barcode diagram, looking for long bars and also considering when two longs bars are not intersecting in the same column (in this second case, it implies that no starting slice could produce all the relevant homological information).

2. Second, produce the corresponding graphical outputs starting from one or from several slices, determined by the barcode examination.

2 Second experiment

In a second experiment, we have selected 60 images (30 GFP images and 30 Dil images). They has been randomly divided into three blocks of 30 images, and each block has been assigned to a researcher to make a manual analysis. In that way, we got that some images were analyzed by more than one human, trying to measure the influence of subjective behaviour in the study. Two of the observers were biologists, and the third one a computer scientist (to also control in this way possible biases with respect to the initial training of the testers).

Actin-GFP	Title Image	No of Slices	Main Components	Operation	Slices which show components
1	Actin01	4	1	$\cup$	None
				$\sqcup$	all(1)
2	Actin02	6	1	$\cup$	None
				$\sqcup$	5,6(1)
3	Actin03	4	2	$\cup$	3,4(2)
				$\sqcup$	3,4(2)
4	Actin04	5	1	$\cup$	4,5(1)
				$\sqcup$	all(1)
5	Actin05	8	1	$\cup$	all(1)
				$\sqcup$	all(1)
6	Actin06	4	2	$\cup$	all(2)
				$\sqcup$	all(2)
7	Actin07	4	1	$\cup$	2(1)
				$\sqcup$	all(1)
8	Actin08	5	1	$\cup$	all(1)
				$\sqcup$	all(1)
9	Actin09	3	1	$\cup$	all(1)
				$\sqcup$	all(1)
10	Actin10	5	1	$\cup$	None
				$\sqcup$	1(1)
11	Actin11	5	1	$\cup$	all(1)
				$\sqcup$	all(1)
12	Actin12	5	2	$\cup$	4,5(2)
				$\sqcup$	4,5(2)
DiI	Title Image	No of Slices	Main Components	Operation	Slices which show components
1	R3N2S2C3	26	2	$\cup$	7-12(1)/14-23(1)
				$\sqcup$	7-12(1)/14-23(1)
2	R3N2S2C5	26	2	$\cup$	4-14(1)/17-26(1)
				$\sqcup$	4-14(1)/17-26(1)
3	R3PbR1N1C3	40	3	$\cup$	1-9(2)/14(1)
				$\sqcup$	1-8(2)/None
4	R3PbR1N1C4	59	3	$\cup$	4-9(2)/11-23(1)/24-31(1)/35-59(1)
				$\sqcup$	4-11(2)/18-23(1)/24-31(1)/35-59(1)
5	R3PbR1N1C5	59	3	$\cup$	11,12(3)
				$\sqcup$	11,12,13(3)
6	R3PbR1N1C6	59	1	$\cup$	18-43(1)
				$\sqcup$	18-43(1)
7	R3PbR1N1C7	59	2	$\cup$	36-44(2)
				$\sqcup$	39-47(2)
8	R3PbR1N1C8	59	4	$\cup$	17-18(3)/ 30-33(1)
				$\sqcup$	16-17(3)/27-32(1)
9	R1S8C5	44	2	$\cup$	36(2)
				$\sqcup$	35,36(2)
10	R1S8C2	40	1	$\cup$	11-34(1)
				$\sqcup$	11-34(1)
11	R1S8C4	44	2	$\cup$	28-38(2)
				$\sqcup$	27(2)

Figure 1: Results of first experiment.

For each image, the human observer had to annotate the number of crossings, the number of connected components, and also the exact location of each crossing and of each dendrite. In addition, a free text box was included where the observer could write some comments about his/her interpretation (quality of the image, ambiguities and so on). Figures 2 and 3 include a summary of the results of the three observers. We also include in Figures 4, 5 and 6 the individual studies.

The result of the crossover study was clear: there wasn't any relevant discrepancy among the interpretations of the three observers, when looking at a same image. Even the free comments were quite similar in each case. This increases the reliability of the aggregated results obtained in the final table.

The success of the plugin was remarkable with respect to GFP images (90% of hits) and reasonable with respect to Dil images (77.6% of hits). Here *hit* means that the plugin found the same results than the human observers, up to some small ambiguity present in the image. If we consider *full hits*, that is to say, exact equality with respect to the four measured features (number of crossing, number of dendrites, and location of both), the figures are 86% for GFP images, and 66% for Dil images. The poorer performance for Dil images is explained because each image has around 45 slices; then the human eye didn't perceive all the intricacies contained in the image.

Title Image	Tester 1	Tester 2	Tester 3	Conclusion
DiI01	OK	OK	OK	1
DiI02	OK		OK	1
DiI03			OK	1
DiI04	It doesn't fit all			0
DiI05	It doesn't fit all	There are more dendrites in the PI because they are in some slice with low intensity		0
DiI06	OK			1
DiI07	It counts one dendrite as 2 but is just a ramification	OK		1
DiI08	There are too many dendrites superpossed, it is very difficult even by eye	There are more dendrites in the PI because they are in some slice with low intensity		0
DiI09	There is one piece that corresponds to a dendrite after the crossing but it is recognized as part of the other one			0
DiI10	OK	OK	OK	1
DiI11			The plugin finds one dendrite that the human doesn't	1
DiI12	OK		The plugin finds one dendrite that the human doesn't	1
DiI13	OK	OK		1
DiI14	Very difficult to say as there are a lot and they all cross (not even sure that there are 18) The program counts a lot of them as the same in white			0
DiI15			OK	1
DiI16	OK			1
DiI17	OK	OK		1
DiI18		It doesn't recognize one dendrite		0
DiI19		OK		1
DiI20		OK		1
DiI21		OK		1
DiI22			OK	1
DiI23		There are more dendrites in the PI because they are in some slice with low intensity		0
DiI24			OK	1
DiI25			OK	1
DiI26			The plugin detects a crossing that seems to be real although it is not seen in the maximal projection	1
DiI27			OK	1
DiI28			The plugin detects a crossing that seems to be real although it is not seen in the maximal projection	1
DiI29			OK	1
DiI30			OK, it is an axon	1
			Total	23

Figure 2: Summary of second experiment - DiI images.

Title Image	Tester 1	Tester 2	Tester 3	Conclusion
GFP01	OK, but a piece of dendrite from one of them is missing in the plugging result	Loses half dendrite of one of them		1
GFP02			OK	1
GFP03	OK			1
GFP04	OK	OK		1
GFP05	They all came from the same soma, so it finds them persistent from there but they are 3 diferent dendrites		OK	1
GFP06	OK	OK	OK	1
GFP07	OK		OK	1
GFP08		OK		1
GFP09	It has desconnected one dendrite	Loses half dendrite (it has a disconection point)	The plugin sees a dendrite which doesn't exist	0
GFP10	It loses the smallest dendrite		OK	1
GFP11		OK		1
GFP12	OK, one of them is noise			1
GFP13		OK (this image has a soma, and it's recognized too)		1
GFP14	OK	OK	OK	1
GFP15		OK		1
GFP16	OK, but they are probably a bit connected but they are 2 I would say	OK	OK, there are three of them but they are in the same cell, perfect	1
GFP17	It misses some pices		It loses one dendrite	0
GFP18	Join both dendrites			1
GFP19	OK	OK (a few noise but it's ok)	OK	1
GFP20	OK			1
GFP21		OK		1
GFP22			OK	1
GFP23		OK		1
GFP24			OK	1
GFP25			OK	1
GFP26		There are more dendrites in the PI because they are in some slice with low intensity		0
GFP27			OK	1
GFP28		OK		1
GFP29		OK	OK	1
GFP30		OK		1
			Total	27

Figure 3: Summary of second experiment - GFP images.

	Kind of stained		Original image(OI)		Processed image(PI)		Comparative OI vs. PI	
Title Image	DiI	GFP	Dendrites	Crossings	Dendrites	Crossings	Dendrites which coincide	Crossings which coincide
DiI01	1		2	1	2	1	2	1
DiI02	1		2	0	2	0	2	0
DiI04	1		5	3	3	3	2	3
DiI05	1		4	4	3	2	3	2
DiI06	1		3	0	3	0	3	0
DiI07	1		4	1	5	3	4	2
DiI08	1		10	3	8	2	8	2
DiI09	1		2	1	2	1	2	1
DiI10	1		2	1	2	1	2	1
DiI12	1		1	0	1	0	1	0
DiI13	1		2	0	2	0	2	0
DiI14	1		18	5	10	2	10	2
DiI16	1		2	0	2	0	2	0
DiI17	1		1	0	1	0	1	0
GFP01		1	2	0	2	0	2	0
GFP03		1	1	0	1	0	1	0
GFP04		1	2	0	2	0	2	0
GFP05		1	3	1	1	0	1	0
GFP06		1	1	0	1	0	1	0
GFP07		1	1	0	1	0	1	0
GFP09		1	1	0	2	0	1	0
GFP10		1	2	0	2	0	1	0
GFP12		1	1	0	2	0	1	0
GFP14		1	2	0	2	0	2	0
GFP16		1	2	0	1	0	1	0
GFP17		1	1	0	1	0	1	0
GFP18		1	2	1	1	0	1	0
GFP19		1	2	0	2	0	2	0
GFP20		1	1	0	1	0	1	0

Figure 4: Results of second experiment - first observer.

	Kind of stained		Original image(OI)		Processed image(PI)		Comparative OI vs. PI	
Title Image	DiI	GFP	Dendrites	Crossings	Dendrites	Crossings	Dendrites which coincide	Crossings which coincide
DiI01	1		2	1	2	1	2	1
DiI05	1		2	1	4	2	2	1
DiI07	1		3	0	3	0	3	0
DiI08	1		4	0	6	0	6	0
DiI10	1		2	1	2	1	2	1
DiI13	1		2	0	2	0	2	0
DiI17	1		2	0	2	0	2	0
DiI18	1		3	0	2	0	2	0
DiI19	1		1	0	1	0	1	0
DiI20	1		2	0	2	0	2	0
DiI21	1		2	0	2	0	2	0
DiI23	1		1	0	2	0	1	0
GFP01		1	2	0	2	0	2	0
GFP04		1	2	0	2	0	2	0
GFP06		1	1	0	1	0	1	0
GFP08		1	1	0	1	0	1	0
GFP09		1	1	0	1	0	1	0
GFP11		1	1	0	1	0	1	0
GFP13		1	2	0	2	0	2	0
GFP14		1	2	0	2	0	2	0
GFP15		1	2	0	2	0	2	0
GFP16		1	2	0	2	0	2	0
GFP19		1	1	0	1	0	1	0
GFP21		1	1	0	1	0	1	0
GFP23		1	2	0	2	0	2	0
GFP26		1	1	0	2	0	1	0
GFP28		1	2	0	2	0	2	0
GFP29		1	3	0	3	0	3	0
GFP30		1	1	0	1	0	1	0

Figure 5: Results of second experiment - second observer.

	Kind of stained		Original image(OI)		Processed image(PI)		Comparative OI vs. PI	
Title Image	DiI	GFP	Dendrites	Crossings	Dendrites	Crossings	Dendrites which coincide	Crossings which coincide
DiI01	1		3	1	3	1	3	1
DiI02	1		2	0	2	0	2	0
DiI03	1		3	1	3	1	3	1
DiI10	1		2	1	2	1	2	1
DiI11	1		1	0	1	0	1	0
DiI12	1		1	0	2	1	1	0
DiI15	1		1	0	1	0	1	0
DiI22	1		2	0	2	0	2	0
DiI24	1		2	0	2	0	2	0
DiI25	1		1	0	1	0	1	0
DiI26	1		4	0	5	1	4	0
DiI27	1		2	0	2	0	2	0
DiI28	1		2	0	3	0	2	0
DiI29	1		2	0	2	0	2	0
DiI30	1		2	1	3	1	2	1
GFP02		1	3	0	3	0	3	0
GFP05		1	3	0	3	0	3	0
GFP06		1	1	0	1	0	1	0
GFP07		1	3	1	3	1	3	1
GFP09		1	1	0	2	0	1	0
GFP10		1	2	0	2	0	2	0
GFP14		1	2	0	2	0	2	0
GFP16		1	3	0	3	0	3	0
GFP17		1	2	0	1	0	1	0
GFP19		1	1	0	1	0	1	0
GFP22		1	1	0	1	0	1	0
GFP24		1	2	0	2	0	2	0
GFP25		1	1	0	1	0	1	0
GFP27		1	1	0	1	0	1	0
GFP29		1	3	0	3	0	3	0

Figure 6: Results of second experiment - third observer.