Quantifying the morphologial changes induced on neurons by chemical stimulation.

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Abstract: This report focuses on different morphometric tools providing estimates of shape changes in cell cultures stimulated by environmental factors. To test our methods we used the fluorescence microscopy observations on cultures of rat hippocampal neurons treated with Cytotoxic Necrotizing Factor 1 (CNF1). The 'global' data concerning all the cells in a microscopic image, reported in a recent work on the toxin-induced changes in the cell soma, as well as in the dendrites number, length and diameter, were: i) fully confirmed by our estimates, and ii) reinforced by morphometric analyses carried out on single cells. Such results underline the importance of high-resolution studies of cell morphometry to dissect-out the structure-function relationships, and justify the efforts: i) to investigate the time-dependent changes in shape, and ii) to exploit the euristic power of modeling and simulating the functional modifications associated to given morphological changes.

1 INTRODUCTION

Morphometry has a widely recognized utility in biological research, and a critical importance in Paleontology, Evolutionary and Developmental Biology etc [1]. Even at the microscopic, cellular level, however, it is difficult to overestimate the contribution that quantitative assessment of shapes and time-dependent shape-changes may provide. In particular, the recent, fast increase in the number of sophisticated microscopic imaging techniques and of powerful and manageable software tools for static and dynamic image analysis [1–4], caused the emergence of a deep interest concerning the possible understanding of cellular functions at the space-resolution of cell organelles and at the time scale of minutes/seconds. Thus, it becomes worth of attention any experimental strategy aiming to exploit the synergic combination of the above mentioned tools.

The architectural intricacies typical of neuron networks are directly reflecting (and conditioning) their highly sophisticated functions. Thus, the complex molecular and cellular phenomena associated to their *in vivo* dynamic behaviour always require a careful design of the *in vitro* experiments in order to find an appropriate balance between biologically sound and easy-to-control conditions or, in other words, between significant and measurable quantities. Having achieved that, an appropriate coupling of morphological and functional information showed essential for a reliable mechanistic account of the underlying subcellular and molecular events in a countless number of examples [5, 6]. In such a context, we decided to apply our morphometric approach to an interesting phenomenon characterized in the lab of one of us [7], namely the drastic suppression of dendritic arborization induced by Cytotoxic Necrotizing Factor 1 (CNF1), a protein produced by certain pathogenic strains of Escherichia coli, on cultures of pure neurons.

It has been reported that CNF1 permanently activates the regulatory Rho, Rac, and Cdc42 GTPases in eukaryotic cells, by deamidation of a glutamine residue. This modification promotes new cellular activities, such as gene transcription, cell proliferation and survival [8]. Quite recently, it has been also reported that CNF1 *in vitro* exerts a direct action on neuronal cells morphology during differentiation in pure primary cultures [7]. Although fully reversible upon removal of CNF1 from cultures, the CNF1 action has been described by the appearence of philopodia-like, actin-positive projections from the cell soma and, at the level of neurites, by thickened, poorly branched dendrites also less rich in synapses.

Morphological modifications of neurons are traditionally characterized by fluorescence and confocal microscopy analyses and quantified by morphometric analysis [9]. Thus, we classified the cells according to the size of the cell body and to the dendritic arborization by means of parameters such as soma area (SA), and checked the significant differences between treated and control cells by tests carried out on the whole set of cells in a microscopic image as well as on a selection of individual cells.

2 MATERIALS AND METHODS

2.1 Cell cultures.

Pure neuronal cultures obtained from the hippocampus of Wistar rat (Charles River) embryos at gestational day 18 and grown at 35 ^oC and 5% CO_2 as described in [7], were treated with CNF1 for 14 days and fixed on day 14 of growth. After fixation, mixed hippocampal cultures were permeabilized in Triton X-100 (0.2%) and incubated with monoclonal antibodies anti Microtubule Associated Protein 2 (MAP-2). After washing in PBS, Alexafluor 546-conjugated goat anti-mouse IgG were used as secondary antibodies (Molecular Probes). The cultures were observed by a fluorescence microscope (Eclipse 80i Nikon; Nikon), equipped with a Video Confocal system1 [10].

2.2 2D Images.

The images of the MAP-2 marked cells were imported into the Image J software, and analyzed as 8-bit images. Background artifacts (if present), were digitally removed and images appropriately thresholded to put in evidence the binary images of neurons. The images were then quantitatively analyzed focusing on the size of the soma and the branching complexity of the dendritic arbor. The bottom-left histogram in Figure 1 refers to the means of the Total Area (TA) marked by MAP-2, namely TA = SA + DA, where SA, bottom-right histogram in Figure 1, is

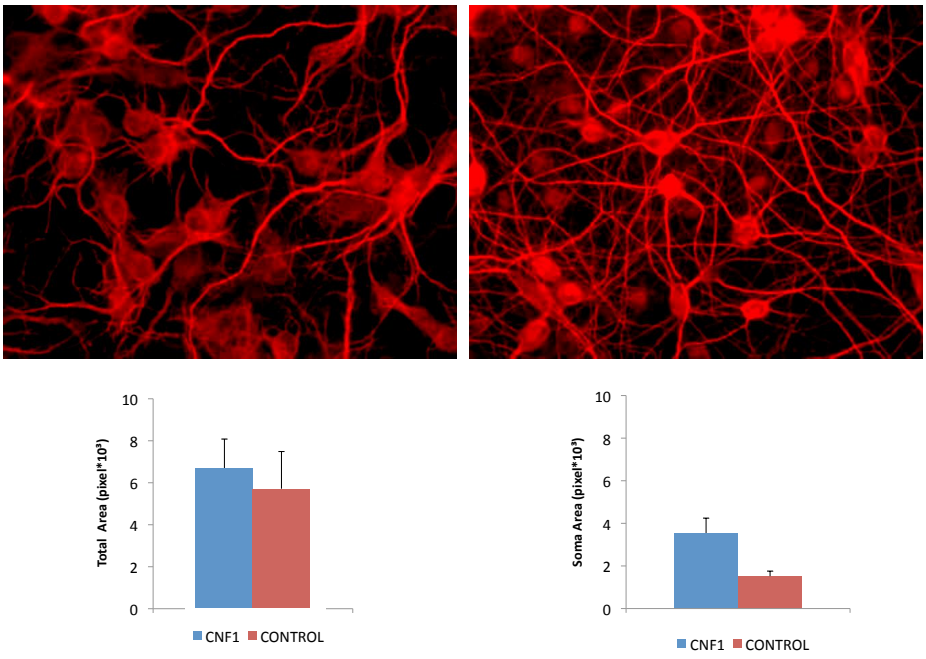


Figure 1: *Shape changes induced by CNF1 in primary cultures of hippocampal neurons.* Upper left panel: hippocampal cells subjected to the action of CNF1 for 14 days; upper right panel: control. Cell cultures (labeled with MAP-2) and micrographs were obtained as described in [7]. Bottom panels: The histograms represent the mean value $\pm 1SEM$ of the total area (TA) positive to MAP-2 (*left*), and the soma area (SA) (*right*). 20 images of comparable technical quality were included in the analysis and, in each image, a minimum of 30 cells were considered in order to estimate SA.

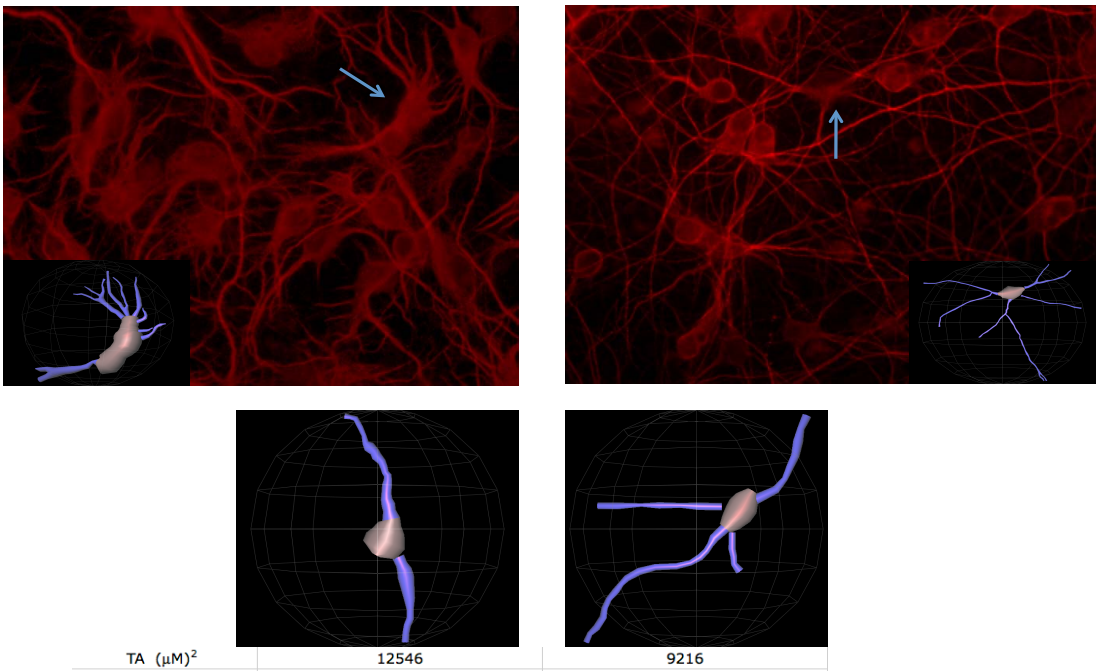
a global estimate of the soma area of all the cells included in the picture. Under the same condition, a parallel, independent estimate of DA, the (total) Dendrites Area, is difficult; however, such an estimate can be obviously obtained by subtracting from TA the area of the cell soma (SA).

2.3 Volume reconstruction.

The 3D pictures of single cells reported in Figure 2 have been obtained by Neuromantic [11], a software tool assembling a number of sophisticated algorithms for 2D image quantification and 3D volume rendering of neuron cells. At difference with the better known algorithms of 3D rendering based on 2D 'slices' as obtained by any tomographic technique, in our case the much less demanding requirements were: i) the assumption of a roughly cylindrical shape of the various sections of the object to be rendered, and ii) the (even manual) definition of a number of points on the 2D image to be used as references in the volume reconstruction.

3 RESULTS

The top panels of Figure 1 contain some typical images of CNF1 treated (left) and control (right) cells from pure rat hippocampal cultures. In the bottom panels of the same figure the results of the related morphometric analysis of both CNF1 treated and control cells, including 20 images and at least 30 cells in each image, are reported. Under these conditions we determined, in each of the 20 images, the estimate of the following parameters: total area positive to the MAP-2 (TA), Soma Area (SA), and Dendrites Area (DA). The averaged estimates



TA $(\mu M)^2$	12546	9216
DA $(\mu M)^2$	7610	6772
SA $(\mu M)^2$	4936	2543

Figure 2: *Volume analysis of CNF1 treated hippocampal neurons.* Upper panels: CNF1 treated and control hippocampal cells are shown in the right and left panel, respectively. Each panel contains, in the inset, the 3D reconstruction of a single cell whose location in the image is marked by an arrow. The culturing and picture-recording conditions are identical to those described in Figure 1. Bottom Panels: 3D reconstruction of typical single cells from the CNF1-treated (left) and control (right) groups, together with the corresponding TA, SA and DA shape descriptors. Such descriptors, as well as the volumes, were calculated by Neuromantic [11] which includes a number of algorithms particularly suited for 3D rendering of neuron cells, as described in the text.

and the variability of TA and SA over the 20 images under consideration $\pm 1 SEM$ are reported in the histograms of Figure 1.

Treatment with the toxin produced significant changes in the immunoreactivity of MAP-2 through an increase of SA and of the TA (total area positive to the MAP-2) in the analyzed regions. The non parametric test of Mann Whitney assessed a significant difference between control and treated cells not for TA ($p=0.11$) but solely for SA ($p=0.03$). Some independent information from Immunofluorescence data [10] showed that CNF1 treated neurons were poorer in dendrites, which became somehow thicker as compared to controls. Thus, some shape changes of different size induced by CNF1 on the soma and on the neurites can be invoked to explain such results: to an effect of large size exerted on the soma, corresponds a much smaller one exerted on the neurites.

This conclusion is in line with the analysis reported in Figure 2. The lower left and right panels in the figure refer to cells included in the image of the corresponding upper panels. Besides the added value of visualizing in 3D the CNF1 induced structural changes, from a purely statistical viewpoint the comparison of two cell populations through the study of single elements in each population is an obvious improvement as compared to the analysis of a global parameter, like the MAP-2 immunoreaction distributed over the whole population.

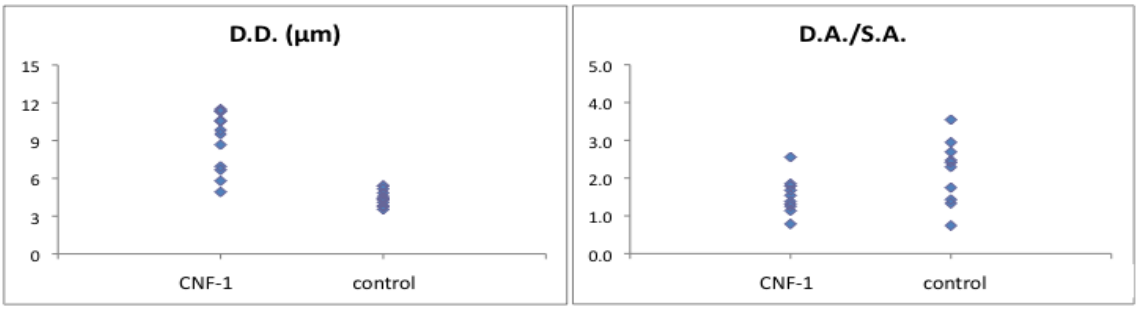


Figure 3: *CNF1 induced changes in the dendrites of hippocampal neurons.* Left and middle panels: dendrites diameter and soma area of CNF1 treated neurons; right panel: form factor of CNF1 treated neurons as defined by the DA/SA ratio (see the text for details).

4 Discussion

The present work should be considered as a preliminary report of a research program aiming to a systematic correlation of structural (morphometric) features and functional properties of cultures of excitable cells. The focus will be on the study of the response to a number of chemical and physical stimuli, and the adopted methodology will benefit of some specific computational tools for quantitative image-analysis and simulation of complex, electrophysiological signals.

In this frame, the experimental system of election has been identified in a neuron culture endowed with a set of well characterized morphofunctional properties *in vitro*, comparable to those observed in the *in vivo* environment. Thus, we decided to tackle first an intriguing problem connected to the apparently opposite effects of CNF1 on an animal model *in vivo* or on pure neuron cultures. In the former and latter case, in fact, CNF1 seems to enhance or inhibit, respectively, the dendritic tree development and the synapse formation. Although these phenomena have been rationalized [7] invoking a mediator role played *in vivo* by astrocytes, the block of differentiation and the remodeled cytoscheleton architecture caused by CNF1 on pure neuron cultures, remains *per se* an interesting phenomenon.

In the analysis of CNF1 treated neuron cultures we focused on the size of the soma area (SA) and on the branching complexity of the dendritic arbor (DA) by careful and systematic use of the plug-ins included in the ImageJ software [12] and of Neuromantic, a more specific tool for neurons morphometric analysis [11]. We could confirm the significant difference in the soma area and in dendrites area between CNF1 treated cells and not, observed by Malchiodi et al. [7] by following essentially the same strategy of those authors, based on the consideration of whole images of the type shown in Figure 1. However, the powerful algorithms included in the ImageJ software allowed us to analyze such a difference also at the level of single cells, as shown in Figure 2. The results of the two types of analysis, centering on whole images or on single cells, are in good agreement, as summarized in Table 1.

As a final comment it is worth stressing that the ability to pick up from high resolution images any quantitative morphometric details from single excitable cells, is of great help to understand the associated functional features. To such an approach, initiated several years ago by Anderson and collaborators [13] a new dimension has been recently added by realistic simulations of time dependent electrical activities [5, 14]. We plan to contribute to such an endeavour by adding to standard morphometric descriptors the parameters stemming from the fractal analysis of cell shapes and volumes [15]. This should substantially refine the available structural information, in order to facilitate any mechanistic modeling and make reliable any functional simulation.

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	Total A.	**Soma A.**	**Dendrite A.**	**Dendrite Diameter**	**Dendrite A. / Soma A.**
Whole Image (Fig.1)	0.11	**0.03**	0.88	/	/
Single Cells (Fig. 2)	/	**0.02**	/	$<<$ **0.01**	0.06

Table 1: *CNF-1 induced changes on neurons morphological parameters.*
The significance levels of differences between CNF-1 treated and control neurons are reported as p-values provided by a 2-tails t-test. Values lower than the critical level of 0.05 are in bold.

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