Assessment and comparison of neural morphology through metrical feature extraction and analysis in neuron and neuron–glia cultures

L. Billeci · G. Pioggia · F. Vaglini · A. Ahluwalia

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Abstract The morphology of dissociated single cerebellar Purkinje cells obtained from wild-type P1 CD1 mice was assessed in the absence and in the presence of glia. A dedicated noninvasive technique based on optical microscopy was developed. Image processing algorithms were implemented to extract metrical features characterizing cell structure and dendritic arborization. The morphological features were analyzed in order to identify quantitative differences in Purkinje cell morphology due to interactions with astrocytes.

Keywords Neuron branching organization · Morphological analysis · Purkinje cells · Glia

1 Introduction

Recent discoveries indicate that astrocytes are an active in neuronal signaling, suggesting that information processing is based on multiple cells rather than only neurons [1–4]. In fact, one of the roles of astrocytes is to sense and modulate synaptic activity. So, the network of information processing is not made up only by neurons but it is the result of a dialogue between two parallel networks: one consisting of neurons and one consisting in astrocytes.

Moreover, there is wide ranging evidence that a bilateral relation between activity and topological or geometrical constraints may affect network activity and vice versa: networks dynamics due to plasticity of neurons/synapses modifies this topology [5]. Astrocytes in particular can modify neuronal morphology in order to influence the functional processing of neural networks. For example, it has been shown that astrocytes change the number

L. Billeci (✉) · A. Ahluwalia
Interdepartmental Research Center “E. Piaggio”, University of Pisa, via Diotisalvi 2, Pisa, Italy
e-mail: lucia.billeci@ing.unipi.it

G. Pioggia
Institute of Clinical Physiology, CNR, via G. Moruzzi 1, Pisa, Italy

F. Vaglini
Department of Neuroscience, University of Pisa, via Roma 67, Pisa, Italy

Fig. 1 Effects of astrocytes on neuronal morphology

of synaptic connections of neurons. This change can occur in two ways (Fig. 1). First, astrocytes change the number of synaptic connections directly via synaptogenic signals: they release factors that enhance or inhibit synaptic transmission. Zhang et al. demonstrate that ATP released by glial cells act as activity-dependent signaling molecules in neuron–glia communication, resulting in astrocyte Ca^{2+} wave and synaptic modulation in hippocampal slices [6].

Astrocytes also act indirectly by modifying the morphology of axons and dendrites and thus the potential for synapse formation. These modifications are due to the secretion of factors such as laminins [7], neurotrophins [8], S100B [9, 10] and activity-dependent neurotrophic factors [11] that act on neuronal morphology.

From this prospective, investigations on “neuron–glia” interactions as the basis of human brain information processing are currently taking off. So far, there are no extensive mathematical studies on the effect of neuron–glia interactions on neuronal morphology. Morphometric analyses are relevant for the study of alterations in the dendritic/axonal field of neurons or neuronal morphological correlates of diseases that can be due to inappropriate functionality of glial cells [12].

In this work, a specific technique for an accurate quantitative analysis of the evolution of microscale neurostructural features during development is established. This technique allow us to assess how astrocytes alter the morphological development of neurons in an in vitro culture.

Our study focuses on Purkinje cells, a population of neurons of the cerebellar cortex. The Purkinje cell is the only efferent neuron of the cerebellar cortex and develops before the other neuron populations in the cerebellum. Therefore, the analysis of Purkinje structural and morphological development could have important consequences for subsequent connections and information processing for cells such as granular and Golgi neurons.

Experiments on organotypic cultures show a crucial role of astrocytes in the structural maturation of cerebellar cells [13] and in their process of migration [14]. It can be supposed that if the function of the glia is compromised, the maturation and migration of Purkinje cells is abnormal. Indeed, there is wide-ranging evidence to show that loss and abnormalities in both Purkinje cells and their associated glia compromise cerebellar development and outputs in autism spectrum disorder [15–17]. For example, the absence of gliosis (the proliferation of neuroglial tissue that can follow neural damage) has been observed in post-autopsy tissue from autistic subjects [18]. The evaluation of the quantitative changes of Purkinje cell morphology due to their interaction with astrocytes can be useful to understand in what way alterations or the absence of glia can affect Purkinje cell functionality.

In this work, a quantitative microstructural analysis of branching organization of Purkinje cells isolated from mice in the absence and in the presence of glia was performed. A time-dependent analysis was made on cells cultured in a medium without glial cells and then

the results of this analysis were compared to that obtained analyzing cells cultured in the presence of glia at their maturation stage.

The most common methods employed for the analysis of microscale neuronal structure in vitro are based on optical imaging [19]. In fact, computational image analyses can be used to quantitatively measure and assess morphological characteristics of neuronal branching patterns [12].

A powerful imaging technique is that of fluorescence microscopy and several reports describe neural growth dynamics and network organization using fluorophores [20–22]. In order to discriminate Purkinje cells from the other cells of the culture, it is necessary to use specific dyes. However, most of these specific dyes are cytotoxic, which makes it impossible to evaluate the dynamics of a network. In order to achieve this objective, we used a noninvasive technique. The position of each cell was labeled using a grid and the evolution of its dendritic tree was recorded using time-lapse optical microscopy. Dedicated algorithms were then implemented in order to extract relevant features, such as morphometric variables, fractal dimension, and directionality, from the acquired images of the cultures. Sholl and fractal analyses were performed to unravel correlations between morphometric variables in wild-type mice and allow classification of cells according to their morphology.

The originality of this study lies in the analysis technique which allows automatic processing of a large amount of quantitative information on cell morphology. In our study, data are obtained using a purpose-written software that allows extraction of all the morphological features of interest. Images of a single cell are opened automatically after choosing the cell to be analyzed and all the data are collected in a single window. The technique developed in this work for the dynamic analysis of neurons is fully described for cells in absence of glia and preliminary results obtained from a comparison between the final morphology of these cells and of those cultured in the presence of glia are presented.

2 Materials and methods

2.1 Culture preparations

Unless stated, all the culture reagents were from Sigma (Sigma, Italy) and materials from Falcon (Falcon, Italy). Purkinje cells were prepared from P1 CD1 mice pups and maintained according to previously described procedures [23, 24]. Timed pregnant CD1 mice were purchased from Harlan, Italy. P1 mice cerebella were dissected in Ca^{2+} / Mg^{2+} free phosphate buffered saline (PBS) containing gentamicin (10 $\mu\text{g}/\text{ml}$) and placed in a 15-ml plastic tube (Falcon 2096). The tube was centrifuged at 1,000 rpm, 4°C for 1–3 min. After the supernatant was aspirated, fresh PBS was poured into the tube. Tissue dissociation was realized both enzymatically and mechanically. The enzymatic dissociation was obtained by the addition of trypsin (0.1% *w/v* in PBS), maintained at 33° for 13–15 min, and, after two rinses in PBS, DNase (5 U/ml in PBS) was added. The mechanical dissociation was obtained using a borosilicate-coated fire-polished Pasteur pipette, after which the cell suspension was centrifuged at 1,200 rpm, 4°C for 5 min. After removing the supernatant, cells were counted and the density was adjusted to 5×10^6 cells/ml using the seeding medium consisting of 100 ml of Dulbecco's modified Eagle medium (DMEM)/F-12 in a 1:1 mixture containing 10% fetal calf serum. A 100- μl drop of the medium containing the cells was placed on 15- $\mu\text{g}/\text{ml}$ polylysine-coated culture plates (radius 360 mm). After 3 h incubation, 1 ml of culture medium was added to each plate. Culture medium consisted

of DMEM/F-12 in a 1:1 mixture containing 20 $\mu\text{g/ml}$ of transferrin, 40 nM of progesterone, 20 $\mu\text{g/ml}$ of insulin, and 0.5 ng/ml of tri-iodothyronine. Cells were maintained in a humidified cell culture incubator (37°C, 5% CO₂). In the culture without glia, every 3 days, half of the medium was replaced with a fresh one enriched with 100 $\mu\text{g/ml}$ of bovine serum albumin (BSA) and 4 mM of Ara-C (cytosine arabinoside—Upjohn, USA), a glial proliferation inhibitor. In the culture with glia, after 2 h of incubation with the medium with serum, it was replaced with a serum-free medium without Ara-C. Every 3 days, half of the medium was replaced with fresh medium. Purkinje cells survived in culture for about 20 to 25 days.

2.2 Optical imaging

Cells were observed daily and photographed using a microscope (Olympus PR54, Italy) with a $\times 20$ objective interfaced to a digital camera (Alkeria, Pisa). In order to observe the same area of the culture dish, a labeled transparent two-dimensional square grid realized with CorelDraw® (Corel Corporation, Inc.) was glued to the bottom of each well. The unit length of the grid was 100 μm . A total of 25 cells in the presence of Ara-C were analyzed and a total of ten cells with glia were analyzed.

2.3 Image processing and data collection

The acquired images were preprocessed using MIPAV [25] and Image J software [26] in order to remove noise and enhance contrast. After these preprocessing steps, dedicated algorithms were implemented in Matlab® code (The MathWorks™, Inc., USA) in order to perform the image processing. The Matlab GUIDE tool was used to create a graphical user interface (GUI) environment. The GUI was provided with morphological data extraction capabilities. Data extracted were organized in a three-dimensional matrix structure and were organized into cells, morphometric variables, and time.

2.4 Morphological data analysis

The morphological analysis allows the contribution of metrical features and their evolution to be studied quantitatively. Using the GUI, the metrical features relevant to the cell's structure and morphology were directly extracted. The morphological variables were chosen from those generally reported in the literature [27, 28] and divided into two groups, local and global variables. The former variables are referred to the dendritic tree, while the latter variables, such as radial extension, soma area, cone angle, and fractal dimension, relate to the whole cell structure. Following the Sholl method [29], each cell skeleton circumscribed a coordinate system consisting of a series of concentric circles centered on the soma. Local variables were extracted by counting the number of intersections between each circle and the cell's dendrites. A list of variables assessed is given in Table 1. In order to generalize the data processing algorithms and GUI, all the lateral dimensions are represented in terms of pixels. All the values in the figures and tables are therefore expressed in pixels. At the magnification used here, one pixel corresponds to 0.182 μm .

2.4.1 Sholl analysis

The Sholl analysis is a method for the quantitative study of the radial distribution of the neuronal dendritic arborization pattern around the cell's soma [29]. This analysis

Table 1 List of variables and variables selected for 3-way PCA analysis

Variables
Intersections
Critical radius (R_c)
Maximum number of intersections (N_m)
Schoenen ramification index
Regression coefficient for log–log method (k_{LL})
Regression coefficient for semilog method (k_{SL})
Correlation coefficient for log–log method (R_{LL})
Correlation coefficient for semilog method (R_{SL})
Determination ratio (Δ)
Minimum length vectors
Angles between minimum length vectors
Minimum pathway (P_m)
Radial extension (E)
Cone angle (C_a)
Soma area (S_a)
Fractal dimension (F_d)

allows dendritic geometry, ramification density, and dendritic branching patterns to be evaluated and is applied to binary images representing the cell skeleton. The Sholl analysis can be applied in three different graphical modalities as demonstrated by Milošević and Ristanović [30]:

1. Linear method: the plot of the number of dendritic intersections calculated per intersecting circle area versus the circle radius
2. Semilog method: the plot of the logarithm of the number of dendritic intersections per intersecting circle area versus the circle radius
3. Log–log method: the plot of the logarithm of the number of dendritic intersections per intersecting circle area versus the logarithm of the circle radius

As demonstrated by Sholl [29], at least in the semilog or log–log methods, a linear behavior is apparent. The method which better approximates a straight line provides relevant cues about the dendritic tree structure.

The linear method is characterized by:

- R_c (critical radius): the radius of the circle with the maximum number of intersections
- N_m : maximum number of intersections
- Schoenen ramification index: quotient of the maximum number of intersections and the number of primary dendrites of the cell (the branches directly arising from the soma)

The semilog and the log–log methods are characterized by:

- k_{SL} (Sholl regression coefficient): slope of the fitted straight line in the semilog plot. It measures the decay rate of intersection numbers versus the distance from the soma
- k_{LL} (Sholl regression coefficient): slope of the fitted straight line in the log–log plot. It measures the decay rate of intersection numbers versus the distance from the soma
- R_{SL} (correlation coefficient): degree of data correlation in semilog method
- R_{LL} (correlation coefficient): degree of data correlation in log–log method

- Δ (determination ratio): $\Delta = \frac{R_{SL}^2}{R_{LL}^2}$. It allows the more appropriate graphical method to be determined. If $\Delta < 1$, the log–log method is preferred, while if $\Delta > 1$, the semilog method is better

2.4.2 Morphometric parameters

Besides the Sholl analysis, other morphometric variables relative to the cells were calculated. If the cell is not uniformly extended in all directions, it is also possible to extract the principal axis of each cell, which corresponds to the thickest dendrites which determines the direction of extension of the cell. From the intersections obtained from the Sholl analysis and the principal axis, other variables such as the minimum length vectors, the angles between minimum length vectors, and the minimum pathway (P_m) can be evaluated. The minimum length vector between two adjacent circles can be extracted by evaluating the minimum Euclidean distance between the cell intersections on the two circles and the corresponding angle with the radial axis. The minimum pathway is the sum of the magnitudes of the minimum length vectors.

Other parameters were:

- Radial extension (E): in order to evaluate the cell dimensions, the radial extension of the cells can be assessed as the Euclidean distance from the cell's center to the furthest pixel of the cell skeleton
- Cone angle (C_a): in order to characterize the cell's orientation, the angle of the cone in which the cell is contained can be extracted. Once the direction of extension is assessed, cones can be drawn on the cell image using a semiautomated procedure, and the cone angle subtended by the cell is computed using Carnot's theorem. If the cell is uniformly extended in all directions, the cone angle is fixed at 360°
- Soma area (S_a): the soma area can be calculated by approximating each soma to an ellipse. After manually marking the major and minor axes of the ellipse, the algorithm determines their lengths and the area of the ellipse
- Fractal dimension (F_d): as argued in several studies [31–36], neurons are fractal objects and the fractal dimension represents an important parameter for the characterization of their dendritic structure. Fractal dimension is a measure of a feature common to several biological systems, their fractality. An object is fractal if it expresses the property of self-similarity: it is made up of copies of itself at different scales. There are several methods to evaluate the fractal dimension; in our study, we used the box-counting method [37] implemented in a specific Matlab Library (<http://www.fast.u-psud.fr/~moisy/ml/boxcount/html/demo.html>). In this method, cell image is inscribed in a squared grid of sides r . The size r of each square or box in the grid is initially equal to the image size and is progressively decreased, while the number of nonempty boxes, $N(r)$, is counted. After this calculation, a plot of $\log(N(r))$ versus $\log(r)$ is obtained. This plot can be fitted with a straight line, of slope S , where S is an estimation of the fractal dimension (F_d)

$$F_d = S.$$

While a mathematical fractal requires infinite orders of magnitude of power-law scaling and therefore is fractal over all scales, physical, biological, and other structures in nature are fractal over a relatively small scaling range [38]. Therefore, the fractal dimension can be computed using the box-counting-slope method in which we plot the local slope versus r . In this way, we can appreciate if the object has some fractal properties only over a limited

range of box size r (where the slope, S , maintains a constant value) and evaluate the fractal dimension in this range.

3 Results

3.1 Morphological analysis

Purkinje cells with glia were imaged and analyzed at the mature stage of their growth, about the 18th day. The presence of glial cells within the cultures alter the development of dendrites and the directionality of cells to favor their connection with other neurons (Fig. 2a). The survival and the dendrogenesis of the Purkinje cells are favored by supplying cell culture medium and by the presence of other cells able to provide signals necessary for their growth.

Fig. 2 **a** Purkinje cell cultured in the presence of glia. **c** Growth of a Purkinje cell cultured in the absence of glia. **b**, **d** Skeletons of Purkinje cells

Purkinje cells without glia were imaged starting from the first day of dendritic development, generally the 7th–12th day (Fig. 2c). During the growth period, we observed three major changes:

- Increase of size and dimensions
- Development of dendrites
- Orientation of the cells in a specific direction

During the last few days of culture, we observed a decrease in the cell size and in the number of dendrites, indicative of suffering and cell initiation of death. After the image preprocessing to obtain the cell skeleton (Fig. 2b, d), all the images obtained were submitted to the GUI. In the GUI morphological data analysis, we implemented the linear, semilog, and log–log methods of the Sholl techniques. We considered two coordinate systems: one with a constant number of circles and the other with a constant intercircle distance. The results obtained with the two systems were similar, so we choose to use the constant number of circles for its graphical simplicity. Figure 3a shows that the variation of number of intersections with radius for the cells in the presence of glia has a peak near the soma of the cell (Fig. 3a). The distribution of the value of number of intersections can be approximated

Fig. 3 **a** Interpolation of the number of intersections as a function of radius (given in units of pixels) and logarithmic fit of the number of intersections per circle area as a function of radius for a typical cell cultured in the presence of glia. **b** Unimodal distribution of the number of intersections as a function of radius and logarithmic fit of the number of intersections per circle area as a function of radius for a typical cell cultured in the absence of glia

to a Poisson distribution. The number of intersection versus radius for the cells without glia follows a unimodal distribution, i.e., low near the soma, high in the middle, and low far from the soma as shown in Fig. 3b. In both cases, the number of intersections/area decreases logarithmically with the distance from the soma (Fig. 3a, b).

The semilog and log-log methods are reported in Fig. 4. The results obtained for the cells with glia are not general; in fact, for some cells the semilog method is better (Fig. 4a) while for some other cells, the log-log fits the data better (Fig. 4c). In agreement with this result, for some cells, Δ is higher than 1, and for some others is lower, although Δ

Fig. 4 **a** Semilog method for a cell in the presence of glia. **b** Semilog method for a cell in the absence of glia. **c** Log-log method for a cell in the presence of glia. **d** Log-log method for a cell in the absence of glia. **e** Value of Δ for all the cells cultured in the presence of glia. **f** Value of Δ for all the cells cultured in the absence of glia. The radius r is given in units of pixels

remains close to 1 in both cases (Fig. 4e). For all the cells without glia, the semilog method (Fig. 4b) approximates a straight line while the regression error is worse in the log–log method (Fig. 4d) indicating the former one as the most suitable for modelling dendritic organization in the cells. This is also confirmed in Fig. 4f, where the determination ratio Δ for all the cells is higher than 1. The values of Δ are consistently higher than the values obtained for the cells with glia (Fig. 4e, f).

Dynamic behavior of the cells without glia was assessed through plotting the number of intersections and the maximum number of intersections versus time. Figure 5a shows a typical plot of intersections versus time for a sample cell. For all cells, peaks coinciding with the days after the culture medium was changed were observed (days 11, 13, 16, and 18). This indicates that the cells reacted to nutrient supply by developing new dendrites and can be seen clearly in the plot of the maximum number of intersections versus time (Fig. 5b). Toward the end of culture period, the cells began to suffer and did not react to the nutrient supply, as shown by the low rate of dendrite growth (Fig. 5a) and the downward trend of Fig. 5b.

Fig. 5 **a** Number of intersections for all the *circles* as a function of time for a typical cell cultured in the absence of glia. **b** Maximum number of intersections as a function of time for all the cells

Figure 6a, b shows the cell vectors of minimum length and angles, respectively, for a typical cell in the presence of glia. The plot of the minimum length vectors is quite linear (Fig. 6a) while the plot of angles has a high number of peaks (Fig. 6b). This means that the axis is tortuous but with a regular structure. On the other hand, the analysis of the evolution of the principal axis during the development of cells without glia shows that on the first few days, the minimum length vectors do not change in magnitude (Fig. 6c), while the angles are small and maintain a narrow range (Fig. 6d). This implies that the principal axis starts developing perpendicular to the cell soma. During active cell growth (days 15–20), there is an increased number of peaks in both plots (Fig. 6c, d) and as time goes on and the cell begins to die, the behavior is once again linear and the range of angles is small.

In the cells of the culture with glia, the principal axis always corresponds to the radial extension. The mean radial extension of these cells is $92\text{ }\mu\text{m}$ (Fig. 7a). In cells without glia, the principal axis is not always the longest dendrite. Figure 7b shows that the mean minimum pathway varies logarithmically with time. The minimum value is around $40\text{ }\mu\text{m}$, while the maximum $90\text{ }\mu\text{m}$ and the principal axis grows at $4.5\text{ }\mu\text{m}$ per day. In agreement with the dynamic cellular growth theory [39], the mean radial extension for all the cells shows a logarithmic increase as reported in Fig. 7c. The minimum extension is around $45\text{ }\mu\text{m}$ and the maximum extension around $100\text{ }\mu\text{m}$, so the cells grow at an average of

Fig. 6 **a** Vectors of minimum length as a function of circles for a typical cell cultured in the presence of glia. **b** Angles between vectors of minimum length as a function of circles for a typical cell cultured in the presence of glia. **c** Vectors of minimum length as a function of circles on three different days of growth for a typical cell cultured in the absence of glia. **d** Angles between vectors of minimum length as a function of circles in three different days of growth for a typical cell cultured in the absence of glia

Fig. 7 **a** Radial extension (given in units of pixels) for all the cells cultured in the presence of glia and mean value (*dashed line*). **b** Mean minimum pathway as a function of time and logarithmic fit. **c** Mean radial extension as a function of time and logarithmic fit

4.5 μm per day. Both groups of cells, with and without glia, have similar mean values of radial extension.

A plot of the cone angle for all the cells with glia, shown in Fig. 8a, shows that these cells are very oriented. The cone angle is always narrow and is about 50° . The dynamic analysis of cells without glia shows that, for many cells, the angle subtended decreased to about $100\text{--}150^\circ$, indicating a preferential direction of extension, although they are less oriented than cells with glia (Fig. 8c). Moreover, a few cells maintain an isotropic radial extension throughout the culture period, since the cone angle is always 360° throughout the culture period (Fig. 8c).

The soma size of the cells cultured with glia is quite small (Fig. 8b). The variation in soma size fell into two main categories (Fig. 8d). Cells with isotropic extension increased their soma area, while the soma of cells with a preferential direction of extension increased during the first few days of culture and then suddenly decreased. This is probably because the cells extended along a particular direction, and the soma becomes thinner in comparison with the cells having isotropic extension, in which somas maintain a more rounded shape.

It can be noticed that the soma size of cells with glia was similar to that obtained for the group of cells cultured in Ara-C with a preferential direction of extension. This is a confirmation of the fact that cells with glia are very oriented.

For the fractal analysis, we applied both the box-counting and the box-counting-slope method, comparing our results with ones obtained with a space-filling 2D image of the

Fig. 8 **a** Cone angle for all the cells cultured in the presence of glia and mean value (*dashed line*). **b** Soma size for all the cells cultured in the presence of glia and mean value (*dashed line*). **c** Cone angle as a function of time for an isotropic cell and for a directional one, both cultured in the absence of glia. **d** Soma size as a function of time for an isotropic cell and for a directional one, both cultured in the absence of glia

same dimensions (Fig. 9a, c). The $n(r)$ versus r plot for Purkinje cells in both groups follows a power law. The discrepancy between the two curves indicates that the structures are not completely space-filling. Plotting the local slope of the curve versus r , we confirmed the presence of fractal properties in a particular range of r and the fractal dimension was evaluated in this range. The range of r in which the slope of curve is constant is smaller for cells in presence of glia (Fig. 9b, d). The fractal dimension of both cells with and without glia is about 1.97 (Fig. 9e, f). This value is very close to 2; our results indicate that Purkinje cells are neither fractal nor pure space-filling objects but fall somewhere in between. The fractal properties of biological systems and, in particular, of neurons are widely debated particularly because most measurements of fractal dimension cover only relatively few decades of radii, so that some authors have suggested that most biological patterns are not fractals but space-filling objects [40] while others demonstrates that they are fractal [41].

In order to analyze the evolution of fractality over time, we plotted the mean fractal dimension versus time (Fig. 9f). The figure shows that the fractal dimension is almost constant, indicating how cells grow regularly while maintaining the initial fractal dimension.

4 Discussion

The glia has recently come into the limelight for its role in regulating and coordinating synaptic transmission. That astrocytes and glial cells, in general, are involved in

Fig. 9 **a** Box-count method for a typical cell cultured in the presence of glia compared with a space-filling image. **b** Box-count slope method for a typical cell cultured in the presence of glia. **c** Box-count method for a typical cell cultured in the absence of glia compared with a space-filled image. **d** Box-count-slope method for a typical cell cultured in the absence of glia. **e** Fractal dimension for all the cells cultured in the presence of glia and mean value (dashed line). **f** Mean fractal dimension as a function of time for all the cells cultured in the absence of glia

regeneration or rather the failure of neurons to generate following injury is well established. Furthermore, several publications show that glial contact provides guidance to axonal and dendritic growth both in vivo and in vitro [42]. This interaction is two-way, since glial development is also influenced by the axonal microenvironment [43]. Despite the great interest in glial-neuronal communication, there is surprisingly little quantitative information on the structural and morphological interactions between the two.

This work therefore represents an important step in the establishment of a systematic and automated method for assessing quantitative differences in neuron morphology in a variety of experimental conditions. Using this method, the role of the glia and the way in which its composition, density, and characterization modulate neuronal morphology can be easily identified and quantified.

Our method was used to analyze primary Purkinje cells dissociated from the cerebellum of P1 mice *in vitro* in the absence and presence of glia, respectively, in order to quantify morphological differences between the two cultures. This population of neurons was selected because their abnormal structure and development have been widely implicated in autism and many studies suggest that this malfunctioning of glial cells plays a role in the evolution of these abnormalities. Furthermore, the morphological evolution of a Purkinje cell culture in the absence of glia was studied over a period of 23 days using the optical microscopy. A labeled culture plate enabled the same neuron to be located and photographed at regular intervals.

It should be noticed that cells *in vivo* are organized into a three-dimensional architecture while the morphometric analysis presented here have been conducted on dissociated cells in two dimensional cultures. However, as in vascular and other branching networks, the basic topology of the three-dimensional system should remain the same when projected on a two-dimensional framework. This is because all natural networks have been shown to obey the same laws of energy minimization [44, 45].

An important aspect of this study is the automation of the analysis method: through a set of computation algorithms in Matlab implemented in a GUI framework, it was easy to access the program and have a global view of the results. The algorithms permitted the extraction of metrical variables which characterized the cell morphology.

Table 2 summarizes the differences found between the two cell populations. As is evident from the table, as well as the results, there is a clear distinction between the two cultures. In the presence of glia, the Purkinje cells are more oriented, and smaller dendritic branching is concentrated close to the soma and close to the principal axis. In this sense, the glia provide the Purkinje cells with a framework which maintains compactness and orientation, as well as a greater level of structural organization and consequently a less random branching. On the other hand, cells cultured with Ara-C are larger and have a more casual organization:

Table 2 Comparison of features extracted from image, Sholl, and fractal analysis for Purkinje cells with and without glia

Variable	Purkinje with glia	Purkinje without glia	Comments
Plot of the number of intersections versus r	Maximum at r small and smaller at r large	Gaussian	Dendrites localized near the soma in the presence of glia
Plot of log–log and semilog method	Both, depends on cell	Semilog is better	Without glia all neurons are clearly more branched and disorganized
Value of Δ	≈ 1	> 1	Dendrites are longer and less branched in cells supported by glia
Plot of minimum length vectors	Same	Same	
Plot of angles between minimum length vectors	Many large-amplitude peaks	Small peaks	The principal axis is more tortuous in cells with glia
Radial extension	Same	Same	
Cone angle	About 50°	Variable	Cells with glia are highly oriented
Soma size	Small	Variable	Cells with glia are smaller
Fractality	High only at small r	High for a wide range of r	Linearity in a small range of r

for example, some are oriented and others have an isotropic structure. In fact, the most interesting structural difference due to the presence of glia was the increase of directionality of the cells. The cone angle was very narrow and the soma was small and oriented in the direction of cellular extension. This increase in directionality was probably due to the chemical stimuli secreted by the glial cells that orient the cells in order to favour the formation of synapses with other cells.

The quantitative morphometric data provided by the automated image and structural analysis algorithms also show how morphological features can be analyzed in time, to observe morphological changes during development. This type of analysis forms a starting point for comparative dynamic morphological studies between Purkinje cells cultured in the absence of glia and cells cultured with glial cells. From this comparison, it is possible to identify and quantify the differences in cell morphological development due to their interactions with astrocytes and other components of the glia. Future studies could then be geared toward modulating the morphology of abnormal neurons by chemical or other means, in order to correct and cure developmental abnormalities in neuron structure.

5 Conclusions

The purpose of this work was to quantify differences in morphology between Purkinje cells *in vitro* in the presence and in the absence of glia using an image and data processing framework designed for the extraction and analysis of relevant morphological features of neurons. Using nondestructive imaging processing methods, we developed a methodology for the morphometric and statistical analysis of Purkinje cells or other neurons in the presence and absence of glia. The method is shown to be highly efficient for the classification and analysis of cells and cell variables pertaining to neural morphology and to their evolution in time. The information obtained in this study provides a starting point for a comparative dynamic study between Purkinje cells isolated from mice in different cultures and microenvironmental conditions in order to better understand how glial cells alter Purkinje cell morphology during development.

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