Networks of Neuroblastoma Cells on Porous Silicon Substrates Reveal a Small World Topology

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Supporting Information #1. Ultra high resolution SEM images for determining the average pore size and pore size distribution of the macro porous (MaP) silicon substrates. For estimating the pore size distribution in the porous substrates, we used ultra-high resolution SEM images. These images have a sufficient level of detail to allow a precise evaluation of the pore size distribution in the low macro-porous regime, that is, for pores larger than 50 nm, even without the need of adsorption/desorption isotherms. From these images, using image analysis algorithms used and described in^{1,2}, we derived the average pore size as S=75 nm. Notice that the pore size distribution may have tails that reach and surpass the 90 nm limit and, in some cases, may be lower than 50 nm. Here, we include additional SEM images of the sole pore substrates to further validate the pore size distribution.



Supporting Information Figure S1.1 SEM image of a MaP substrate revealing the pore size distribution. Magnification factor $10^4 \times$.



Supporting Information Figure S1.2 SEM image of a MaP substrate revealing the pore size distribution. Magnification factor $3 \ 10^5 \times$.



Supporting Information Figure S1.3 SEM image of a MaP substrate revealing the pore size distribution. Magnification factor $3 \ 10^4 \times$.



Supporting Information Figure S1.4 SEM image of a MaP substrate revealing the pore size distribution. Magnification factor $3 \ 10^4 \times$.



Supporting Information Figure S1.5 SEM image of a MaP substrate revealing the pore size distribution. Magnification factor $3 \ 10^4 \times$.



Supporting Information Figure S1.6 SEM image of a MaP substrate revealing the pore size distribution. Magnification factor $3 \ 10^4 \times$.

Supporting Information #2. N2A cells wiring diagrams obtained on meso porous (MeP) silicon substrates. In the Supporting Information Figure S2.1, we report DAPI confocal images of N2A cells 36 h after incubation on (a) a SP MeP silicon surface and (d) a LP MeP silicon substrate. The topology of those cells in a plane is revealed by the corresponding graphs (b-e) that are a simplified pictorial representation of the nuclei of the cells (which are, the nodes) and the signal connections between those cells (which are, the edges), that were calculated using the Waxman model described in the Materials and Methods. The distribution of the number of edges that you may find at different nodal distances is reported in the histograms in (c-f). The described distributions double peaked for either the SP and LP MeP configuration, similarly to the case of macro porous silicon reported in the main text. This confirms that a nano-scale porous structure boost the assembly of N2A cells into clusters.



Supporting Information Figure S2.1

Supporting Information #3. N2A cells wiring diagrams obtained on flat silicon substrates with increased initial cell density (for direct comparison with porous substrates at constant effective cell density). On observing the networks of N2A cells reported, for flat silicon, MaP and MeP silicon in the Figure 5 in the main text, and in the Supporting Information Figure S2.1, one may speculate that the networking differences between flat and porous surfaces are influenced by different cell densities. Even if we seeded the same amount of cells on both surfaces at the initial time (see the Materials and Methods), maybe because flat silicon presents less anchorage points with respect to modified porous silicon, cell density on flat silicon after 24 hours is largely reduced. To surpass this limitation, we performed additional experiments, herein reported, in which the initial cell density on flat silicon is artificially augmented from 3 to 5 times the initial value (from $\sim 10^5$ cells per substrate up to $\sim 5 \times 10^5$ cells per substrate). In doing so, we could compare the behavior of N2A cells on different substrates (that are, flat and porous) at the same *effective* cell density (that is about 200 to 400 cells/mm², see also Figure 4 in the main text). The corresponding N2A cells wiring diagrams, and the distributions of the number of edges of these diagrams (that are single peaked) are reported in the Supporting Information Figures S3.1,2 and confirm the behavior of neural cell on the surface is not influenced by cell density. The propensity of cells to form complex patterns is thus regulated by the sole substrate topography, that is a notable result.



Supporting Information Figure S3.1 (cell density ~3x10⁵ cells per substrate)



Supporting Information Figure S3.2 (cell density ~5x10⁵ cells per substrate)

Supporting Information #4. Determining the metrics of neuronal networks starting from confocal images – the image analysis algorithm. Confocal images of neuronal cells on porous silicon surfaces were taken according to the biological protocol and the nanofabrication process reported in the methods in the main text. Here, we describe with some details the image analysis algorithm used to analyze the confocal DAPI stained images of N2A cell on the porous substrates and to extract, from those, the topological properties of the networks of N2A cells. All the operations below were performed in MatlabTM upon correctly importing the original image files. Each file is a composite Irgb image that contains 3 matrices (512x512 pixels), corresponding to the red R, green G and blue B color channels. Those channels were combined to obtain an individual image I in which the R, G and B components were considered with the following weights R=1.1956, G=2.348, B=0.4560. The resulting image was therefore converted into a binary image, in which cells are clearly distinguished from the background, and the noise (Supporting Information Figure S4.1) is disregarded. The k-means algorithm was used to segment the image color intensity. k is the number of different groups in which the color range is divided (a priori). It works as an optimization problem: after setting k centroids (clusters) (sometimes randomly), it computes the distance from each centroids (cluster) to the pixels. Therefore, it assigns each point (pixel) to the centroid (cluster) it is nearest to. Then, it iterates over the above two steps till the sum of squared within group errors cannot be lowered any more.

Supporting Information Figure S4.1. Region of interest (640x640 mm²) of Neuro-2A nuclei (a). Pixels details showing noisy image (b).

At this point, the image is segmented into k levels and upon assigning a reference value to the nuclei, a binary image in which the pixels are either zero-valued (that are, cells) and one-valued (background) is derived as in the **Supporting Information Figure S4.2**.

Supporting Information Figure S4.2 Segmented image using k-means algorithm (k=20) (a). Detailed color cluster of a group of nuclei (b).

Still, the resulting image comprises clusters of multiple pixels which correspond to individual cells. That is, a single cell is composed by an average number n of pixels (we say that the size of a cell is n) with n strictly larger than 1. The aim now is to *reduce* the number of pixels in the image without loss of information. In other terms, we want to assign a single pixel to each cell: in doing so, each cell in the ROI may be described by one couple of coordinates, and the network of cells and the operations thereof may be easily simplified.

Consider the example in the **Supporting Information Figure S4.2a**. The image is sub-divided into regions where the size n of each region is strictly related to the size of a nucleus (**Supporting Information Figure S4.2**). Each of this region has an intensity value that is the average of intensities of the pixels that fall in that region. In mathematical terms, the following *average* function is operated:

$$I_{2}(i,j) = \frac{\sum_{i=1}^{l/n} \sum_{j=1}^{l/n} I(s_{1},s_{2})}{N-1}, i=1, 2..., l/n; j=1, 2..., l/n; N=n^{2}$$
S4.1

In which, I₂ is the target image, I is the original image of size lxl. The indices i, j, run from 1 to l/n. s₁, s₂ are two arrays taking into account the indices of a generic sub-region: s₁=[(i-1)n+1, i n]; s₂=[(j-1)n+1, j n]; S4.2 i=1, 2...l/n; j=1, 2..., l/n; N=n²

Notice that, upon application of Eq.(S4.1), the target image I_2 shall have a size that is 1/n times the size of the original image I. I_2 is therefore *rescaled* to have the original size 1 (Supporting Information Figure S4.3)

$$I_3(in/l, jn/l) = I_2(i, j)$$

S4.3

3) Reallocation in a new image

Supporting Information Figure S4.3. Rescaling operation of the binary image with the cells to a the original size.

In I_3 , the cells in the image are replaced by their nuclei, which are the coordinates of single points approximately at the center of the original cells. These nuclei, alternatively named nodes, serve to describe the neuronal network over an area of 512×512 pixels and compute steadily the adjacency matrix, the clustering coefficient and characteristic path length of a distribution of cells in the plane, as described in the Methods of the main text.

Supporting Information Figure S4.4 Cell diagram of a generic distribution of N2A cells on a MaP substrate. The diagram was derived upon application of the algorithms described in the present Supporting Information file, and in the methods of the main text. The lines defining the links were generated by the Bresenham' line algorithm where each pair of points were provided by the adjacency matrix of N2A.

Supporting Information #5. Determining the Small World properties of N2A cells on porous substrates. In the main text, we argued that N2A cells cultured on porous silicon substrates reveal a small world topology. Small world networks lies between the extremes of order and randomness^{3,4}: they exhibit short paths and high clustering differently from regular or random geometries. Formally, we recognize that a graph Λ with a specific Cc and Cpl shares the properties of a small world network if $Cc \square C_r$ and $Cpl_r Cpl_r$, where Cc_r and Cpl_r are the clustering coefficient and characteristic path length of a random network with the same number of nodes and edges of Λ^{5} . In the panels in the Supporting Information Figures S5.1 to S5.4, we show the connectivity graphs of N2A cells on the MaP porous substrates for all the considered times. that are, 24 h, 36 h, 48 h, 60 h. In the panel in the Supporting Information Figures S5.5 to S5.8, we report randomly generated networks with the same number of nodes as in the experimental MaP case. Similarly, in the panels in the Supporting Information Figures \$5.9 to **S5.14**, we show the connectivity graphs of N2A cells on the SP and LP MeP porous substrates for all the considered times (the randomly networks with the same number of nodes as in the experimental MeP cases were likewise generated and are not shown here). The Cc and Cpl values of the described networks are reported in a separate Supporting Information Table S5.1. From a direct comparison of these values, we may observe that $Cc \Box C_r$ and $Cpl \sim Cpl_r$, for the considered range of values, this proves mathematically the initial hypothesis that cells on porous surfaces reveal a small world topology.

Supporting Information Figure S5.1 connectivity graphs of N2A cells on MaP substrates at the time point t=24 h.

Supporting Information Figure S5.2 connectivity graphs of N2A cells on MaP substrates at the time point t=36 h.

Supporting Information Figure S5.3 connectivity graphs of N2A cells on MaP substrates at the time point t=48 h.

Supporting Information Figure S5.4 connectivity graphs of N2A cells on MaP substrates at the time point t=60 h.

Supporting Information Figure S5.5 randomly generated networks with the same number of nodes as in the experimental MaP case at time point t=24 h.

Supporting Information Figure S5.6 randomly generated networks with the same number of nodes as in the experimental MaP case at time point t=36 h.

Supporting Information Figure S5.7 randomly generated networks with the same number of nodes as in the experimental MaP case at time point t=48 h.

Supporting Information Figure S5.8 randomly generated networks with the same number of nodes as in the experimental MaP case at time point t=60 h.

Supporting Information Figure S5.9 connectivity graphs of N2A cells on SP MeP substrates at the time point t=24 h.

Supporting Information Figure S5.10 connectivity graphs of N2A cells on SP MeP substrates at the time point t=36 h.

Supporting Information Figure S5.11 connectivity graphs of N2A cells on SP MeP substrates at the time point t=48 h.

Supporting Information Figure S5.12 connectivity graphs of N2A cells on SP MeP substrates at the time point t=60 h.

Supporting Information Figure S5.13 connectivity graphs of N2A cells on LP MeP substrates at the time point t=24 h.

Supporting Information Figure S5.14 connectivity graphs of N2A cells on LP MeP substrates at the time point t=36 h.

Supporting Information Figure S5.15 connectivity graphs of N2A cells on LP MeP substrates at the time point t=48 h.

Supporting Information Figure S5.16 connectivity graphs of N2A cells on LP MeP substrates at the time point t=60 h.

	24 h	36 h	48 h	60 h
N2A on Macro Porous silicon	0.383±0.060	0.6653±0.047	0.6836±0.050	0.640±0.043
Substrates				
Random Networks (MaP)	0.1310±0.014	0.1670±0.020	0.1501±0.0121	0.111±0.0313
N2A on SP MeP Si	0.450±0.760	0.600±0.051	0.6510±0.0557	0.6510±0.076
Random Networks (SP MeP Si)			•	
	0.213±0.023	0.134±0.037	0.1901±0.0481	0.140±0.023
	0.213±0.023	0.134±0.037	0.1901±0.0481	0.140±0.023
N2A on LP MeP Si	0.213±0.023 0.492±0.045	0.134±0.037 0.382±0.0397	0.1901±0.0481 0.372±0.045	0.140±0.023 0.320±0.065
N2A on LP MeP Si Random Networks (LP MeP Si)	0.213±0.023 0.492±0.045 0.301±0.072	0.134±0.037 0.382±0.0397 0.184±0.084	0.1901±0.0481 0.372±0.045 0.201±0.05	0.140±0.023 0.320±0.065 0.114±0.039

Clustering Coefficient (Cc)

Characteristic Path length (CPL)

	24 h	36 h	48 h	60 h
N2A on Macro Porous silicon	1.775±0.409	2.449±0.311	2.791±0.702	2.7156±0.304
Substrates				
Random Networks (MaP)	1.3030±0.253	1.8492±0.284	2 . 9460±0 . 552	2.1397±0.401
N2A on SP MeP Si	2.23±0.477	1.39±0.259	1.62±0.562	1.76±0.364
Random Networks (SP MeP Si)	1.63±0.653	0.954±0.353	1.773±0.662	1.536±0.432
N2A on LP MeP Si	2.68±0.614	2.16±0.363	2.40±0.632	3.09±0.274
Random Networks (LP MeP Si)	2.24±0.428	2.420±0.784	2.128±0.692	2.602±0.543

Supporting Information Table S5.1 In the table, we report the values of Clustering Coefficient Cc and Characteristic Path Length CPL derived for the (i) networks of N2A cells cultured on porous substrates at different time steps 24 h, 36 h, 48 h, 60 h, and for (ii) randomly generated networks with the same number of nodes as in the experimental porous cases. The Cc and Cpl values of for all the considered cases are such that $Cc\Box C_r$ and $Cpl~Cpl_r$, that is equivalent to say that cells on porous surfaces reveal a *small world* topology.

Supporting Information #6. Connectivity graphs of N2A cells on flat silicon substrates. In the panels in the **Supporting Information Figures S6.1 to S6.4**, we show the connectivity graphs of N2A cells on silicon substrates for all the considered times of the analysis, that are, 24 h, 36 h, 48 h, 60 h. In the panel in the **Supporting Information Figures S6.5 to S6.8**, we report randomly generated networks with the same number of nodes as in the experimental silicon case. The silicon substrates were used as a control for comparison with the porous nano-structured surfaces.

Supporting Information Figure S6.1 connectivity graphs of N2A cells on Si substrates at the time point t=24 h.

Supporting Information Figure S6.2 connectivity graphs of N2A cells on Si substrates at the time point t=36 h.

Supporting Information Figure S6.3 connectivity graphs of N2A cells on Si substrates at the time point t=48 h.

Supporting Information Figure S6.4 connectivity graphs of N2A cells on Si substrates at the time point t=60 h.

Supporting Information Figure S6.5 randomly generated networks with the same number of nodes as in the experimental Si case at time point t=24 h.

Supporting Information Figure S6.6 randomly generated networks with the same number of nodes as in the experimental Si case at time point t=36 h.

Supporting Information Figure S6.7 randomly generated networks with the same number of nodes as in the experimental Si case at time point t=48 h.

Supporting Information Figure S6.8 randomly generated networks with the same number of nodes as in the experimental Si case at time point t=60 h.

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