Supplementary Information

Asymmetric confinement for defining outgrowth directionality

Paul M. Holloway[1,2]*, Grace Hallinan[3]*, Manjunath Hegde[1], Simon I.R. Lane[3], Katrin Deinhardt[3] and Jonathan West[1†]

[1]Cancer Sciences, Faculty of Medicine, University of Southampton, UK.

[2]Investigative Medicine Division, Radcliffe Department of Medicine, University of Oxford, UK.

[3]Biological Sciences, Faculty of Environmental and Life Sciences, University of Southampton, UK.

* equal contribution

† corresponding author

Materials and Methods

Design, Fabrication, Replication and Assembly

Different devices were designed for the different asymmetric outgrowth structures (arch, heart, pretzel and arrowhead). The devices had two culture compartments interconnected over a length of 800 μm via multiple 7.5-μm-wide outgrowth channels including zero to five microstructured motifs. The different structures were replicated five-fold in each device to enable statistical rigour. The asymmetric structures introduce additional edge length that was matched using symmetrical control motifs (see SI CAD). Double layer SU-8 photolithography requiring alignment features was used to develop a first layer with culture compartments and outgrowth channels to a height of 2.5 μm, and a second layer was used to extend the culture compartment height to 60 μm.

Standard PDMS moulding was used for device replication, and a 5-mm-diameter biopsy punch was used to prepare the inlet and outlet wells. Glass coverslip substrates (22x55 mm, Smith Scientific) were used to enclose the PDMS channels. These were first treated with 1M NaOH for 1 hour, thoroughly rinsed in water and then treated with 1N HCl for 1 hour and again thoroughly rinsed with water. Overnight 70% (v/v) ethanol incubation was used to sterilise the glass, dried and incubated with poly-*D*-lysine (PDL, 100 μg/mL, Sigma-Aldrich) at 37°C overnight, followed by a triple sterile water rinse. The PDMS devices were then mounted on the glass substrate, flushed with 10 μL ethanol and then incubated with PDL (100 μg/mL, Sigma-Aldrich) at 37°C overnight, after which the PDL was exchanged with Neurobasal media supplemented with 2% B27 and 0.5 mM GlutaMAX (Gibco) that was replaced every 2 hours for 6 hours and incubated overnight to passivate surfaces and prevent the loss of growth factors during neuronal culture.

Neuron Isolation and Culture

All experiments were carried out in accordance with the Animals Act 1986 (Scientific Procedures) set out by the UK Home Office. Primary cultures were prepared as described previously[1]. Briefly, hippocampal neurons were isolated from embryonic day 15–18 C57BL/6 mouse brains. Dissociated neurons were suspended in Neurobasal medium supplemented with 2% B27 and 0.5 mM GlutaMAX

(Gibco) at a density of 7000 cells/µL for culture in the microfluidic devices with partial media exchanges every 2–3 days.

Immunocytochemistry and Imaging

Once compartmentalised cultures reached 14DIV, media was removed from the devices and cells were washed with PBS. Cells were fixed *in situ* with ice cold 100% methanol for 2 min, the PDMS device was carefully removed from the glass substrate and methanol allowed to cover the axonal region for a further 1 min to ensure complete fixation of both somal and axonal compartments. Cell cultures were then washed 3 times with PBS with 0.1% Tween 20 before blocking with PBS with 0.1% Tween 20, 1% BSA and 10% goat serum in PBS for 30 min. A 1h incubation with E7 mouse anti-βIII tubulin (Developmental Studies Hybridoma Bank) diluted 1 in 500 in blocking buffer was used to stain the neurons followed by 1h incubation with Alexa 555 conjugated anti-mouse diluted 1 in 1000. Following washing a 5 min incubation with Hoechst® 33342 at 5 µg/mL was used to stain nuclei. Slides were then mounted with ProLong™ Diamond Antifade Mounting media (ThermoFisher) Fluorescent and differential interference contrast (DIC) images of compartmentalised neuron cultures were obtained using a 60x/1.42NA Oil Plan APO objective on a DeltaVision Elite system (GE Life Sciences) with SSI 7-band LED for illumination and a monochrome sCMOS camera, using SoftWoRks software (version 6).

Image Analysis

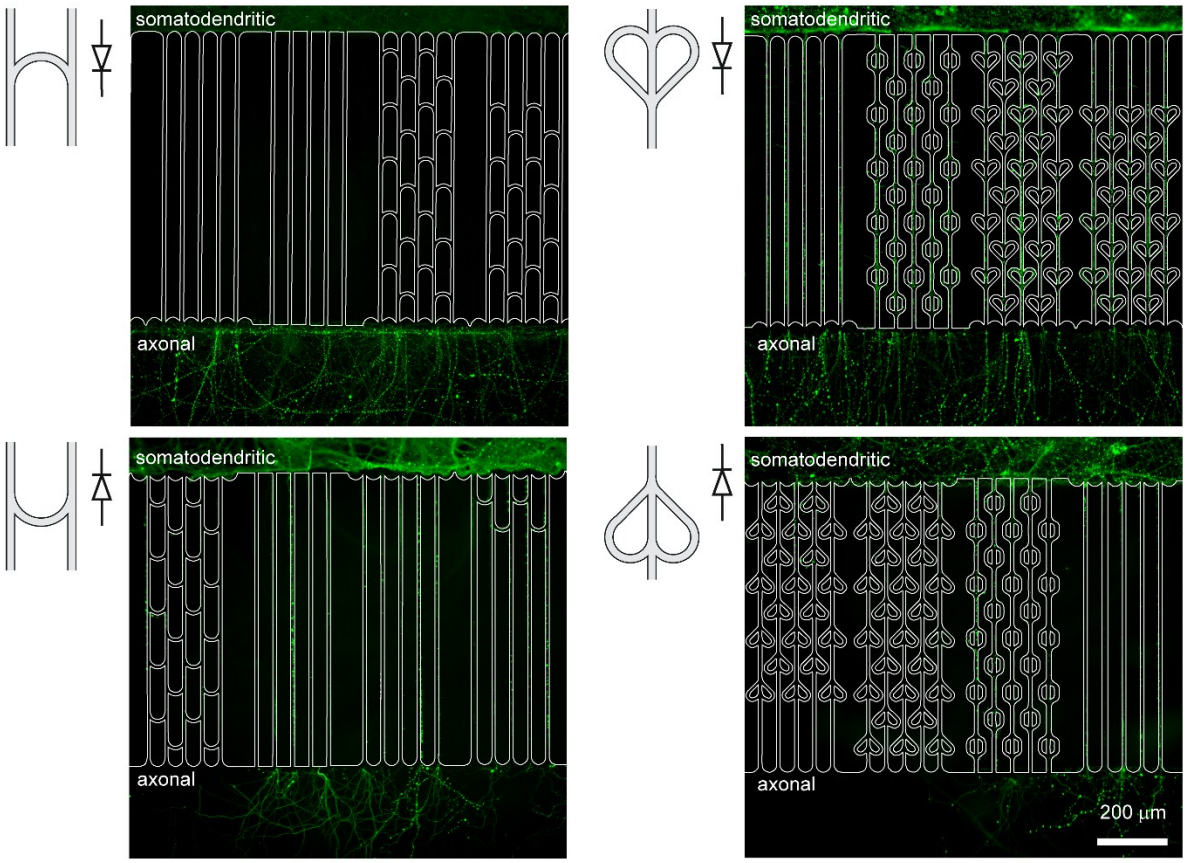
Axonal projection between compartments was calculated for each channel using the following:

$$axonal\ projection = {PI_o}/{PI_i}$$

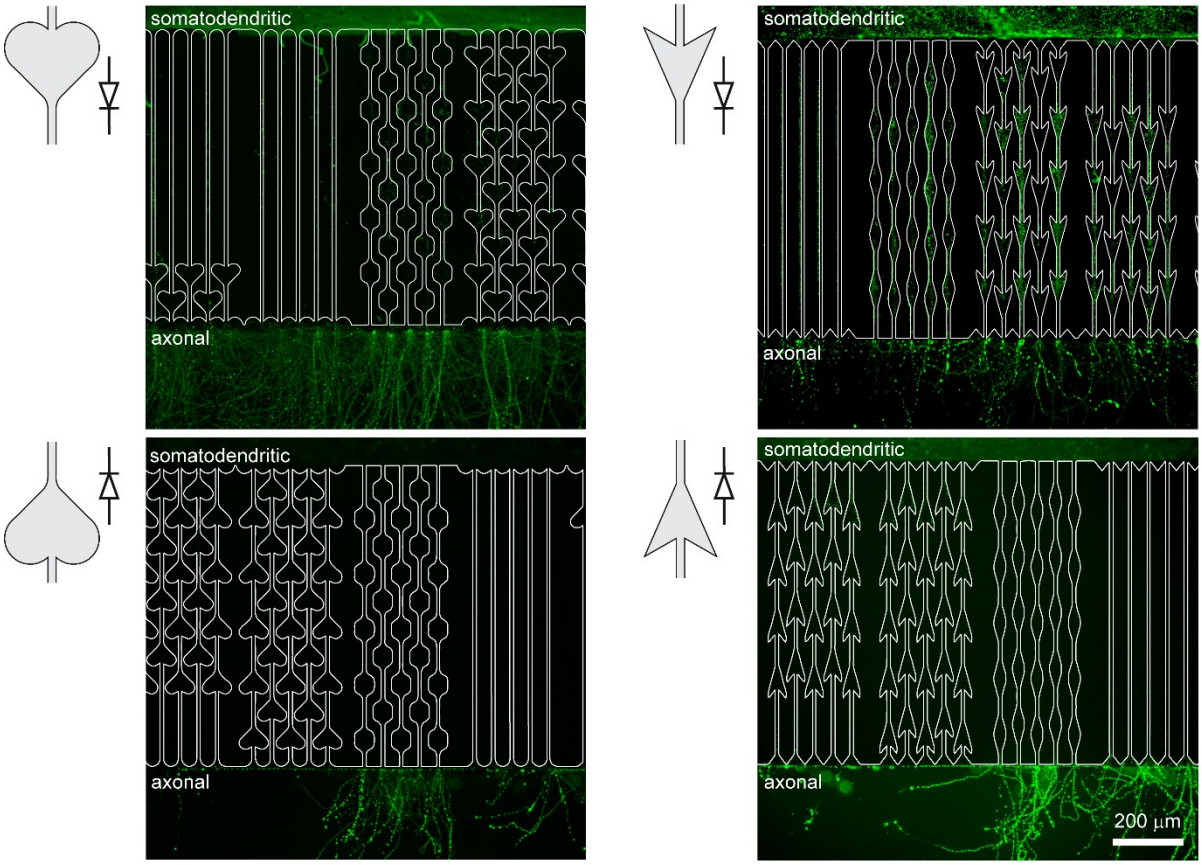
where PI_o is the integrated pixel intensity of the βIII tubulin signal at the outgrowth channel outlet and PI_i is the integrated pixel intensity at the corresponding channel inlet. In order to obtain values for the inlet and exit integrated pixel intensity, 8 bit plot profiles of average pixel intensity were created from a 5 µm wide and 250 µm long region of interest. Each ROI spanned 5 repeats of each type of outgrowth channel and was positioned 20 µm into channel inlets and outlets. Pixel intensity plot values were analysed using a custom batch processing macro in Origin 2015 version 92E (OriginLab Corporation, Massachusetts, USA). Briefly the peak analyser function was used to integrate pixel intensity peaks across the 7.5 µm wide outgrowth channel. To correct for background signal variations a floating baseline was subtracted, with anchor points determined using a 2nd derivative function and connected by interpolation. Axonal projection values for each type of axonal guidance channel design were averaged across 3 sets of 5 channels to give a single value per channel design for each device. Mean and standard deviation results were obtained from 3 independent experiments using different neuronal preparations.

Dynamic data was extracted from image sequences using Image J (NIH, United states): Image stacks were duplicated and one stack was shifted by one frame. This frame shifted image stack was subtracted from the original, creating a new image series that eliminated common features and revealed changes between subsequent frames. These changes were colour coded using a bespoke ImageJ macro to reveal change over time (progressing from blue to red from first to last frame

respectively) and then maximally z-projected. Alternatively a maximal z-projection of the specified period was made to show motion with the ImageJ 'red hot' lookup table applied. Unprocessed original time-lapse movies are shown in supplementary movies 1–3.



SI Figure 1. Large field of view comparison of 14DIV outgrowth levels during confinement in arch and pretzel microstructures in the permissive and prohibitive directions. Outgrowths are stained for βIII tubulin.



SI Figure 2. Large field of view comparison of 14DIV outgrowth levels during confinement in heart and arrowhead microstructures in the permissive and prohibitive directions. Outgrowths are stained for βIII tubulin.

Movie SI1. Branching and re-routing of an axon growing in the prohibitive direction in the pretzel motif. An image was taken every 5 min in the 195–865 min period. Image size, 79 x 73 µm.

Movie SI2. Retraction of an axon growing in the prohibitive direction in the pretzel motif. An image was taken every 5 min in the 1750–2295 min period. Image size, 79 x 73 µm.

Movie SI3. Axon edge-avoidance in the prohibitive direction in the arrow motif. An image was taken every 15 min in the 0–930 min period. Image size, 48 x 103 µm.

Movie SI4. Acute axon re-routing in the prohibitive direction in the arrow motif. An image was taken every 15 min in the 0–1185 min period. Image size, 48 x 99 µm.

References

1. K. Deinhardt, T. Kim, D. S. Spellman, R. E. Mains, B. A. Eipper, T. A. Neubert, M. V. Chao and B. L. Hempstead, *Science Signaling*, 2011, **4**, ra82-ra82.