Published in final edited form as:

J Neurosci Methods. 2011 June 15; 198(2): 230–235. doi:10.1016/j.jneumeth.2011.04.012.

A high-throughput method for generating uniform microislands for autaptic neuronal cultures

Allyson E. Sgro¹, Amy L. Nowak², Naola S. Austin², Kenneth L. Custer³, Peter B. Allen¹, Daniel T. Chiu^{1,*}, and Sandra M. Bajjalieh^{2,*}

¹Department of Chemistry, University of Washington, Seattle, WA 98195-1700

²Department of Pharmacology, University of Washington, Seattle, WA 98195-7750

³Graduate Program in Neurobiology and Behavior, University of Washington, Seattle, WA 98195-7270

Abstract

Generating microislands of culture substrate on coverslips by spray application of poly-D lysine is a commonly used method for culturing isolated neurons that form self (autaptic) synapses. This preparation has multiple advantages for studying synaptic transmission in isolation; however, generating microislands by spraying produces islands of non-uniform size and thus cultures vary widely in the number of islands containing single neurons. To address these problems, we developed a high-throughput method for reliably generating uniformly-shaped microislands of culture substrate. Stamp molds formed of poly(dimethylsiloxane) (PDMS) were fabricated with arrays of circles and used to generate stamps made of 9.2% agarose. The agarose stamps were capable of loading sufficient poly D-lysine and collagen dissolved in acetic acid to rapidly generate coverslips containing at least 64 microislands per coverslip. When hippocampal neurons were cultured on these coverslips, there were significantly more single-neuron islands per coverslip. We noted that single neurons tended to form one of three distinct neurite-arbor morphologies, which varied with island size and the location of the cell body on the island. To our surprise, the number of synapses per autaptic neuron did not correlate with arbor shape or island size, suggesting that other factors regulate the number of synapses formed by isolated neurons. The stamping method we report can be used to increase the number of single-neuron islands per culture and aid in the rapid visualization of microislands.

Keywords

autapses; neuron patterning; electrophysiology; cell culture; cell patterning; dendritic arbors

INTRODUCTION

Patterned neuronal cell culture has been a valuable technique in the modern neuroscience toolbox, especially for the generation of autapses in primary neuronal culture (Allen, 2006; Bekkers, 2003; Bekkers and Stevens, 1991; Branch et al., 1998; Chang et al., 2003; Corey and Feldman, 2003; Furshpan et al., 1976; Martinoia et al., 1999; Scholl et al., 2000; Segal and Furshpan, 1990; Takano et al., 2002; Vogt et al., 2003). Autaptic neurons, which are neurons that form synapses onto themselves, contain both pre- and post-synaptic processes in a single cell. This preparation allows the study of neurotransmission in isolation, and thus has been widely used by synaptic physiologists aiming to understand the molecular basis of

* Authors to whom correspondence should be addressed. bajjalie@u.washington.edu; chiu@chem.washington.edu.

synaptic events (Allen, 2006; Bekkers, 2003; Bekkers and Stevens, 1991; Segal and Furshpan, 1990).

Autaptic neuronal cultures are generated by culturing dissociated neurons on microislands of permissive substrate. By restricting microisland size, one can increase the number of synaptic contacts per neuron. This in turn increases the magnitude of synaptic events observed with whole-cell patch-clamp methods, making them easier to detect and quantify. However, current stamping techniques for producing microislands on coverslips suffer from one of two major drawbacks. Techniques that require repeated loading can be time consuming because the stamps usually have to be loaded with permissive substrate for extended periods of time prior to stamping (Chang et al., 2003; Furshpan et al., 1976; Mayer et al., 2004; Scholl et al., 2000). An alternative method with a short stamp loading time has a more laborious permissive-substrate application process (Vogt et al., 2003). Thus, these methods are not suitable for laboratories that aim to record from many autaptic neurons per study. Spraying culture substrate is very rapid, but produces few usable microislands per coverslip because sprayed droplets vary in size and often merge into single large islands (Allen, 2006; Banker and Goslin, 1998). As quantitative electrophysiology experiments require recordings from dozens of neurons to draw conclusions, a technique that allows for efficient generation of large numbers of microislands capable of supporting autaptic neurons, and which is also accessible to electrophysiology labs, would be a useful addition to the biological toolbox.

This paper describes a high-throughput method for generating patterned arrays of uniformly-shaped microislands that are suitable for use in autaptic neuronal cultures. This technique results in more single microislands per coverslip than the spraying approach. Additionally, unlike the spraying method that forms random spots, this technique generates microislands in an array format that facilitates rapid identification of single-neuron microislands. We also report that, surprisingly, the number of synapses formed per neuron does not correlate with island size. Finally, we observed that the neuritic arbors formed by isolated neurons had three distinct shapes and that these were also independent of island size.

METHODS

Fabrication of agarose stamps

Silicon wafers with features composed of SU-8 negative photoresist were used as masters for poly(dimethylsiloxane) (PDMS) molds for the agarose stamps, and they were fabricated using established photolithography and replica molding techniques (McDonald et al., 2000). To make the positive silicon master, a 300 μm -thick layer of SU-8-2150 negative photoresist (Microchem, Newton, MA, USA) was spun on top of a 3" diameter silicon wafer (WRS Materials, San Jose, CA, USA) using a spin coater and exposed to UV light through an exposure mask with the desired stamping pattern (generated in AutoCAD, printed by FineLine Imaging, Colorado Springs, CO, USA). After development of the features by removing any unexposed photoresist with propylene glycol methyl ether acetate for 30 minutes, followed by a 30 minute hard bake at 150 °C, the master was exposed overnight to tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane vapor to aid in removing the negative PDMS molds from the master.

Negative-relief stamp molds were formed out of PDMS (SYLGARD 184, Dow Corning, Midland, MI, USA) through replica molding. A 10:1 ratio of pre-polymer to catalyst was degassed under vacuum in a desiccator, poured in a 4–6 mm thick layer on top of the master, and baked at 65 °C for 4 hours before being cut out and removed from the master. These negative-relief PDMS molds can be used repeatedly, but should be boiled in MilliQ H₂O (deionized and purified water produced using a MilliQ Advantage A10 Ultrapure Water

Purification System, Millipore, Billerica, MA, USA) prior to each use to remove dust and residual agarose.

Agarose stamps are composed of 9.2% agarose (GenePure LE Agarose, ISC BioExpress, Kaysville, UT, USA) in MilliQ H₂O. For making up to six agarose stamps, 250 mL of 9.2% agarose was heated in a 1 L Erlenmeyer vacuum flask in the microwave until completely dissolved and boiling. The agarose was then aspirated for 30–60 seconds to remove residual dissolved air, and poured on top of the PDMS mold to form a 2 mm-thick layer of agarose. Any bubbles formed between the PDMS and agarose layer were aspirated, after which glass tubes (0.5 inch in diameter) were centered over each pattern repeat on the mold. The glass tubes were then filled with the remaining agarose to better help stabilize the agarose stamp within the glass tube holder. Prior to use, agarose stamps were allowed to cool and harden completely, and then they were gently removed with a scalpel. Stamps could be stored indefinitely in MilliQ H₂O to prevent dehydration.

Generation of microislands

Prior to stamping, 24-well cell culture plates were filled with glass coverslips and coated with 0.15% agarose to discourage cell adhesion outside the patterned areas (Bekkers and Stevens, 1991). Permissive substrate, consisting of 1350 μ L of 17 mM acetic acid, 600 μ L Type I collagen (3.52 mg/mL), and 150 μ L poly-D-lysine hydrobromide (1 mg/mL in MilliQ H₂O) was made fresh prior to each use. Each stamp was coated with 200 μ L of permissive substrate and then blown with clean, dry air to encourage it to fill the areas between posts and to form an even layer. Stamps were then blotted on a test coverslip to remove any excess substrate before stamping on the coverslips, after which the stamps were pressed on each coverslip to pattern microislands of permissive substrate. After stamping, plates were allowed to age for at least two weeks prior to use.

Neuronal culture

Primary cultures of mouse hippocampal neurons were prepared using slight modifications on a previously established procedure (Bekkers and Stevens, 1991). Astrocyte cultures were prepared from the cortices P0–P1 wild-type mice and grown to confluence for 10 days in T-25 flasks before plating on the stamped microisland plates. The night before plating, the flasks containing the astrocytes were wrapped in aluminium foil and were shaken for 12–16 hours at 250 rpm at room temperature. The following morning, the astrocyte's media was changed and they were placed in the incubator to recover for 4–6 hours before they were removed from the flask surface using trypsin-EDTA, washed, dissociated by trituration with a glass pipette, and plated on the microislands at a density of 6250 cells/well (12,500 cells/mL). The plated astrocytes were allowed to grow for at least 2–3 days on the microislands before neurons were added. Hippocampi were isolated from P0–P1 mice and then incubated for 30 minutes in papain (Worthington Biochemical Corporation, Lakewood, NJ, USA), washed, dissociated via pipetting, filtered with a 70 μ m cell filter (BD Biosciences, Bedford, MA, USA), and diluted to a density of 5000 cells/mL before adding 0.5–0.75 mL to each well of the stamped 24-well plate with astrocytes. Astrocyte medium contained Dulbecco's modified eagle medium supplemented with 9% fetal bovine serum, 50 U/ml penicillin, and 50 μ g/ml streptomycin. Hippocampal culture medium contained minimum essential medium supplemented with 10% fetal horse serum, 25 mM HEPES, 20 mM glucose, 50 U/ml penicillin, 50 μ g/ml streptomycin, 1:200 N2 supplement, 1:100 B27 supplement, and 0.1 mM sodium pyruvate. Mitotic inhibitors were not used in our cultures.

Immunocytochemistry

Coverslips of 10–14 d.i.v. neurons on stamped microislands were washed with 1 $\times$ PBS, fixed in 4% paraformaldehyde for 20 minutes, and then blocked in blocking solution (2%

Normal Goat Serum, 0.4% saponin, and 1.0% BSA in 1X PBS) for 1 hour. Blocked coverslips were then incubated with anti-SV2A (1:1000, (Bajjalieh et al., 1994)) and anti-MAP2 (1:400, Sigma-Aldrich, St. Louis, MO, USA) antibodies in blocking solution for 1 hour, rinsed, and incubated for 1 hour with Alexa Fluor 488 and Alexa Fluor 568 secondary antibodies (Invitrogen, Carlsbad, CA, USA) at a ratio of 1:1000 in blocking solution. Following a 10 minute incubation in Hoechst stain (1:10,000, Invitrogen, Carlsbad, CA, USA) or a 5 minute incubation in TO-PRO-3 (1:1000, Invitrogen) coverslips were mounted on slides using Fluoromount-G (Electron Microscopy Sciences, Hatfield, PA, USA) for fluorescence imaging.

Imaging

Brightfield images were taken on a Nikon TE300 inverted microscope (Tokyo, Japan) using a Nikon D40 digital SLR camera. Fluorescence images for dendritic-arbor shape analysis were taken on a Nikon TE300 using a mercury lamp for illumination. Fluorescence images for synapse quantification were taken with Leica TCS SP/NT confocal microscope (Leica Microsystems CMS GmbH, Mannheim, Germany) used in conjunction with 488 nm Argon laser, a 561 nm DPSS laser, and a 633 nm HeNe laser.

Image analysis

Synapses were identified and quantified using NIH's ImageJ software in conjunction with the Multiple Thresholds available on their website (Poburko, 2009). Puncta were required to have a fluorescence intensity at least one standard deviation above background and be larger than four pixels but less than forty pixels. Minimum and maximum thresholds were auto-calculated by the macro and the background region of the sample was determined visually for each image. Images for figures were processed using Adobe Photoshop CS3 (Adobe Systems, San Jose, CA, USA).

RESULTS AND DISCUSSION

High-throughput stamping of multiple coverslips

Agarose was chosen as the stamp material because of its known biocompatibility and previous use in stamping techniques (Mayer et al., 2004; Stevens et al., 2005). To generate agarose stamps, we formulated a three-step process that included a master mold, a stamp mold, and an agarose stamp. Master molds were formed of silicon patterned with photoresist using photolithography. The master is then used to generate reusable molds using of poly(dimethylsiloxane) (PDMS) using standard replica molding techniques (McDonald et al., 2000). Finally, the PDMS molds were used to form the agarose stamps, which were made by pouring hot 9.2% agarose into glass tubes placed over the PDMS mold. We found this concentration of agarose to both be stiff enough to produce posts that did not deform significantly during stamping and yet have a low enough viscosity to flow into the PDMS mold when hot. Figure 1 shows the procedure for generating stamps for microisland patterning. After testing multiple stamp patterns, we chose the pattern shown in the inset in Figure 1, a thickly-lined box surrounded by an array of circles with a diameter 150 μm spaced 500 μm apart. This pattern offers two major advantages. First, the thick lines provide stabilization to the agarose stamp and offer support during stamping, thus preventing deformation of the more delicate posts in the center of the stamp. Second, as part of the pattern of permissive substrate on the coverslip, the large bars also provide area for significant numbers of glia and neurons to grow and to supply sufficient neurotrophic factors to the culture, thus circumventing a major problem of sparse cultures (Banker and Goslin, 1998; Ullian et al., 2004). Each silicon wafer master mold contained a six-by-six array of these boxes to allow for multiple agarose stamps to be created from the same PDMS mold.

Culture substrate is applied directly to the stamp with a micropipette, briefly dried with a stream of air, and then applied to a glass coverslip with a gentle rolling motion. Each application to the stamp provides enough culture substrate to produce 4–7 coverslips. Varying the pressure on the stamp as it is rolled across the coverslip allows a range of island sizes to be generated. We found that islands smaller than 150 μm usually had no neurons, whereas those above 500 μm were likely to have multiple neurons per island. Thus we recommend limiting islands to this size range.

Using the agarose stamp and the above procedure, we were able to generate hundreds of stamped coverslips per hour. Because culturing is most successful with stamped coverslips that have been aged a minimum of two weeks, and coverslips can be stored for up to a year, a large stock of plates can be rapidly generated in a single session for use over a long period of time. Although agarose stamps can be used indefinitely, because pressure tends to deform the posts on the stamp, fresh stamps should be fabricated for each stamping session to avoid pattern degradation.

Figure 2a shows neurons growing on stamped microislands, and for comparison, Figure 2b shows neurons growing on sprayed microislands. While the stamped microislands were uniformly shaped and were laid out in an array format that made them easy to locate, the sprayed microislands were both erratically shaped and randomly located. Additionally, many sprayed islands merged to form larger islands that had multiple neurons per island. While these larger areas of sprayed permissive substrate provided areas for the large numbers of glia and neurons which produce neurotrophic factors, they were randomly distributed and occurred with a much greater frequency than the small microislands suitable for autapse formation. Stamping permissive substrate produced, in a similar amount of time as spraying, large numbers of microislands surrounded by big regions of supporting cells. As a result, we think this method is useful for patterning substrates where large numbers of single neuron islands are required.

Hippocampal neurons grown on uniform-sized islands displayed three patterns of neurites

Somewhat to our surprise, inspection of immunolabeled neurons revealed that isolated neurons had neuritic arbors that fell into one of three morphological categories, which we refer to as “well-defined”, “circular”, and “perimeter”. Well-defined neurons were characterized by a classic neuritic arbor that spread away from the cell body. Circular neurons had arbors that looped one or more times around the cell body, often forming a tightly packed circle of neurites. Perimeter neurons had neurites that encircled the edge of the microisland and were most often seen in neurons whose cell body was on the edge of the microisland. Figure 3a–c shows examples of each shape class.

In a sample of 27 randomly-chosen autaptic neurons 18 (66.7%) had well-defined arbors, 6 (22.2%) were circular-shaped, and 3 (11.1%) were perimeter-shaped. In general, we found that the shape of neuritic arbor was not predicted by island size (Figure 4). Small islands, between 150 and 300 μm in diameter, had neurons of all shapes, and neurons with circular-shaped arbors were found exclusively in this size-range. Among single neurons on islands larger than 300 μm , the well-defined arbor shape was most prevalent (14/19 islands). Consistent with this neurons with well-defined arbors were observed largely on islands larger than 300 μm (14 of the 18 well-defined neurons). Perimeter-shaped arbors were observed when the neuron attached near the edge of the island, however, these occurred so infrequently it isn't possible to draw conclusions about what causes this growth pattern.

Uniform microisland size does not produce uniform numbers of synapses per neuron

One of our motivations for generating uniform microisland cultures was based on two assumptions 1) that the number of synapses formed per autaptic neuron was a function of island size, and 2) that this produced the large variability in the magnitude of neuronal response observed in these cultures. Having generated coverslips with uniformly-sized islands, we next determined if island size correlated with the number of synapses formed per neuron.

Hippocampal neurons cultured from wild-type mice were grown on stamped microislands, fixed and immunolabeled with antibodies against the synaptic vesicle protein SV2A to mark synapses. To visualize dendrites, we also labelled cultures with antibodies against the dendritic protein MAP2 (data not shown). We collected confocal microscopy images and then analyzed them for the number of SV2A-labeled puncta (Figure 3). We found that within the 200–300 μm size that as most likely to result in single neuron islands, the number of synapses a neuron formed was largely independent of the size of the microislands. The number of synapses varied widely across the range of island sizes we tested. For example, counts ranged from 383 to 2449 synapses on $\sim 200 \mu\text{m}$ islands and from 198 synapses to 1208 synapses on $\sim 275 \mu\text{m}$ islands (Figure 5). Indeed, the largest island we analyzed ($\sim 450 \mu\text{m}$ in diameter) with a single neuron had a low number of synapses (311). Furthermore, a small number of whole-cell path-clamp recordings from neurons grown on uniformly sized microislands indicated that evoked excitatory postsynaptic current (EPSC) amplitudes varied widely among neurons (data not shown). Therefore, island size surprisingly does not appear to influence the number of synapses formed, or the sizes of the electrical responses from neurons grown in isolation. We also considered whether or not the shape of the dendritic arbor correlated with the variability in the number of synapses (Figure 5). We found that neurons with circular-shaped arbors varied the most in synapse number, ranging from 383 to 2449 synapses/cell. Additionally, the majority of neurons with more than 1000 synapses exhibited circular-shaped arbors (7 of the 10 were circular-shaped). Additionally, the majority of neurons with more than 1000 synapses exhibited circular-shaped arbors (7 of the 10 were circular-shaped). Thus selecting well-defined or perimeter-shaped neurons may provide more consistency when comparing responses between neurons.

CONCLUSIONS

In conclusion, we described a new protocol for stamping microislands that encourage the formation of healthy autaptic neuronal cultures. This technique easily produces dozens of coverslips with a high probability of having multiple single-neuron islands arranged in an array format. Using this technique to generate single-neuron islands, we showed that both island size and neuritic-arbor shape do not directly correlate with the number of self-synapses that an autaptic neuron forms. What uniform microislands did provide was a higher number of single-neuron islands when compared to coverslips onto which substrate had been sprayed. Thus the stamping method provides a way to decrease the number of coverslips needed per study. Additionally, because this approach provides a uniform array of islands, which facilitate finding usable autapses, we anticipate this method will be of great interest to electrophysiologists who study synaptic function.

Acknowledgments

The authors thank Lisa Baldwin for animal husbandry. We gratefully acknowledge support of this work by the National Institute of Mental Health (R01MH059842 to SMB) and National Institute of Neurological Disease and Stroke (NS052637 to D.T.C. and S.M.B.).

References

- Allen TGJ. Preparation and maintenance of single-cell micro-island cultures of basal forebrain neurons. *Nat Protocols*. 2006; 1:2543–50.
- Bajjalieh S, Frantz G, Weimann J, McConnell S, Scheller R. Differential expression of synaptic vesicle protein 2 (SV2) isoforms. *J Neurosci*. 1994; 14:5223–35. [PubMed: 8083732]
- Banker, G.; Goslin, K., editors. *Culturing Nerve Cells*. 2. MIT Press; Cambridge, MA: 1998.
- Bekkers JM. Synaptic Transmission: Functional Autapses in the Cortex. *Curr Biol*. 2003; 13:R433–R5. [PubMed: 12781151]
- Bekkers JM, Stevens CF. Excitatory and inhibitory autaptic currents in isolated hippocampal neurons maintained in cell culture. *PNAS*. 1991; 88:7834–8. [PubMed: 1679238]
- Branch D, Corey J, Weyhenmeyer J, Brewer G, Wheeler B. Microstamp patterns of biomolecules for high-resolution neuronal networks. *Med Biol Eng Comput*. 1998; 36:135–41. [PubMed: 9614762]
- Chang JC, Brewer GJ, Wheeler BC. A modified microstamping technique enhances polylysine transfer and neuronal cell patterning. *Biomaterials*. 2003; 24:2863–70. [PubMed: 12742724]
- Corey JM, Feldman EL. Substrate patterning: an emerging technology for the study of neuronal behavior. *Exp Neurol*. 2003; 184:89–96.
- Furshpan EJ, MacLeish PR, O’Lague PH, Potter DD. Chemical transmission between rat sympathetic neurons and cardiac myocytes developing in microcultures: evidence for cholinergic, adrenergic, and dual-function neurons. *PNAS*. 1976; 73:4225–9. [PubMed: 186792]
- Martinoia S, Bove M, Tedesco M, Margesin B, Grattarola M. A simple microfluidic system for patterning populations of neurons on silicon micromachined substrates. *J Neurosci Meth*. 1999; 87:35–44.
- Mayer M, Yang J, Gitlin I, Gracias DH, Whitesides GM. Micropatterned agarose gels for stamping arrays of proteins and gradients of proteins. *Proteomics*. 2004; 4:2366–76. [PubMed: 15274132]
- McDonald JC, Duffy DC, Anderson JR, Chiu DT, Wu H, Schueller OJA, Whitesides GM. Fabrication of microfluidic systems in poly(dimethylsiloxane). *Electrophoresis*. 2000; 21:27–40. [PubMed: 10634468]
- Poburko, D. ImageJ Multiple Thresholds Macro. 2009.
- Scholl M, Sprössler C, Denyer M, Krause M, Nakajima K, Maelicke A, Knoll W, Offenhäusser A. Ordered networks of rat hippocampal neurons attached to silicon oxide surfaces. *J Neurosci Meth*. 2000; 104:65–75.
- Segal MM, Furshpan EJ. Epileptiform activity in microcultures containing small numbers of hippocampal neurons. *J Neurophysiol*. 1990; 64:1390–9. [PubMed: 2283535]
- Stevens MM, Mayer M, Anderson DG, Weibel DB, Whitesides GM, Langer R. Direct patterning of mammalian cells onto porous tissue engineering substrates using agarose stamps. *Biomaterials*. 2005; 26:7636–41. [PubMed: 15979701]
- Takano H, Sul J-Y, Mazzanti ML, Doyle RT, Haydon PG, Porter MD. Micropatterned Substrates: Approach to Probing Intercellular Communication Pathways. *Anal Chem*. 2002; 74:4640–6. [PubMed: 12349965]
- Ullian EM, Christopherson KS, Barres BA. Role for glia in synaptogenesis. *Glia*. 2004; 47:209–16. [PubMed: 15252809]
- Vogt AK, Lauer L, Knoll W, Offenhäusser A. Micropatterned Substrates for the Growth of Functional Neuronal Networks of Defined Geometry. *Biotechnol Progr*. 2003; 19:1562–8.

Figure 1. Schematic showing the formation of stamps

A patterned master made of SU-8 photoresist features on a silicon wafer was used to cast a negative PDMS template for the stamp. This PDMS template was then covered in hot agarose and while still hot, a glass tube was placed over the stamp. The glass tube was then filled with hot agarose and allowed to set. Once set, the tube and patterned area were fused and could be separated from the PDMS mold to form an agarose stamp surrounded by the glass tube, which made it easy to handle during stamping. Large posts are shown as example features for clarity in the schematic. A sample pattern repeat that would appear on one stamp surface is shown in the inset. Each wafer had a six-by-six array of these pattern repeats. The wide barrier lines are 2 mm thick and each stamp pattern is separated from each other by 6 mm. Each island on the stamp is 150 μm in diameter and the islands are spaced 600 μm apart.

Figure 2. Example of a stamp pattern

a) Hippocampal neurons cultured on the stamped microislands. b) Hippocampal neurons cultured on sprayed microislands. Scale bars are 200 μm . Neuronal cell bodies are indicated by white arrowheads.

Figure 3. Dendritic arbor classes and quantifying synapse number

Examples of SV2A labeling we used for counting synapses from arbors that were a) well-defined, b) circular, and c) perimeter. d–f) show the corresponding masks we used to identify and count the synapses. All scale bars are 100 μm .

Figure 4. Frequency of occurrence of dendritic arbors of different shapes
Histogram plotting the number of different shaped arbors on islands of different sizes.
Black: well-defined arbors; white: circular-shaped arbors; grey: perimeter-shaped arbors.

Figure 5. Quantifying synapse density

A plot showing the number of synapses is not correlated with the size of the island on which the neurons reside. Grey squares: neurons with perimeter arbors; white circles: neurons with circular arbors; black triangles: neurons with well-defined arbors. Linear regression analyses of the combined data as well as for each arbor class returned poor fits ($R^2 < 0.0816$ for all fits), consistent with no correlation between island size and synapse number.