Supporting Information

Aligning Synthetic Hippocampal Neural Circuits via Self-Rolled-Up Silicon Nitride Microtube Arrays

Olivia V. Cangellaris[1,2,3,4], Elise A. Corbin[1,3,5], Paul Froeter[3,6], Julian A. Michaels[3,6], Xiuling Li[3,6], and Martha U. Gillette[1,3,4,7,8]*

[1]Department of Bioengineering, University of Illinois at Urbana-Champaign, Urbana, IL 61801

[2]Medical Scholars Program, University of Illinois College of Medicine at Urbana-Champaign, Urbana, IL 61801

[3]Micro and Nanotechnology Laboratory, University of Illinois at Urbana-Champaign, Urbana, IL 61801

[4]Beckman Institute for Advanced Science and Technology, University of Illinois at Urbana-Champaign, Urbana, IL 61801

[5]Cardiovascular Institute, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104

[6]Department of Electrical and Computer Engineering, University of Illinois at Urbana-Champaign, Urbana, IL 61801

[7]Department of Cell and Developmental Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801

[8]Neuroscience Program, University of Illinois at Urbana-Champaign, Urbana, IL 61801

Corresponding Author
*Email: mgillett@illinois.edu

Local Alignment Thresholding

Evidence of the trend of global alignment on the μ-Tubes is apparent in the distribution of the angles of neurites on three substrates and the differences in the relative spectral energy on the SPμTb substrate compared to the two controls. These observations caused us to develop a consistent measure of alignment. The alignment parameter and neurite angle for pooled data-sets over time (**Figure S2A**), and at 4, 7, 10, and 14 DIV (**Figures S2B-E**) were compiled as 2D histograms in order to establish appropriate threshold values for alignment. The two properties of each neurite were considered together, and an angle of $\leq 30°$ was determined as the angle cutoff, while an alignment parameter of ≥ 0.75 was chosen as the alignment parameter cutoff. **Figures S2B-E** depict the distribution of only the neurites that fall within these cutoff bounds. For data sets sufficiently large ($n \geq 90$), the chosen cutoff bounds successfully capture the training data at a rate of 97% or better. These results validate the decision to choose a universal cut-off of an angle $\leq 30°$ and alignment parameter ≥ 0.75 as an indicator for alignment for all DIV.

Orientation of Neurites on Control Substrates

Neurons cultured on glass or planar SiN_x control substrates demonstrated a lack of alignment of neurites compared to that seen on the μ-Tube substrates. Neurites extended in random directions on both control substrates. Representative fluorescent images are shown (**Figure S3**).

Minimum Distance between Gradient-Length μ-Tube and Aligned Neurites

The minimum distance analysis performed for the gradient length μ-Tube array revealed no significant difference between alignment and distance between soma-originating neurites and the nearest μ-Tube for any tube length. Additionally, no μ-Tube length conferred any better relationship between minimum distance and aligned processes (**Figure S4A**).

For the GLμTb substrate, neurites with 90%-95% of their length classified as aligned and interacting with 70 - 80 μm-long μ-Tubes were significantly closer to the microtube than for similarly aligned neurites interacting with 40 μm-long μ-Tubes (**Figure S4B**). These data suggest that the longer μ-Tubes provide a more robust cue for alignment than shorter (40 μm) μ-Tubes.

Incidence of Aligned Neurites Across Substrates

Two additional measures of neurite alignment were explored: frequency of alignment of neurites inside the microtube compared to outside the microtube, and fraction of aligned neurites compared to all neurites on the substrate.

To calculate the frequency of neurite alignment inside or outside the microtubes, we randomly selected 3 images/experimental condition, and aligned neurites in contact with the μ-Tubes were classified (**Table S1**). The sampled-set reveals that, generally, alignment occurs more frequently along the outside of the microtube (low-frequency SiNx). However, relative to the other standard-pitch substrate, more instances of alignment are observed along the inside of the microtubes (high-frequency SiNx) in the gradient-pitch substrate (26.04%). On the gradient-length substrate, the greatest incidence of neurites aligned inside the μ-Tubes is found in those with a length of 40 μm (36.84%). We suspect that the increased frequency of neurites aligned inside the μ-Tubes on the gradient-pitch substrate compared to the standard-pitch substrate is due to the increased frequency of topographical cues offered by the more closely spaced μ-Tubes. Additionally, the neurons are more likely to be situated close to a μ-Tube after seeding, further increasing the instances of neurite extension through the microtube.

When comparing the total fraction of aligned neurites to all traced neurites in our complete data set, we found ~36-43% of the neurites are aligned to the μ-Tube pattern (**Table**

S2). In a previous study, alignment of axons of cultured cortical neurons was found to be 83% in the direction of the microtube array.[1] There are several differences between the previous study and what we report here: 1) Differences in neurite studied; we examined both dendrites and axons. 2) Differences in cellular populations studied; our work utilized hippocampal neuron cultures that contain predominately pyramidal neurons *vs.* the mixed population of neurons in a cortical culture. 3) The range of μ-Tube orientations studied (standard-pitch, gradient-pitch, and gradient-length) 4) The location sampled on the culture plate.

Table S1: Fraction of Neurite Alignment Outside or Inside Microtube

Substrate	Neurites Aligned Outside µTb	Neurites Aligned Inside µTb
Standard-Pitch	86/92 = 93.48%	6/92 = 6.53%
Gradient-Pitch	125/169 = 73.96%	44/169 = 26.04%
Gradient-Length	118/136 = 86.76%	18/136 = 13.24%
30 µm	*12/17 = 70.59%*	*5/17 = 29.41%*
40 µm	*12/19 = 63.16%*	*7/19 = 36.84%*
50 µm	*10/11 = 90.91%*	*1/11 = 9.09%*
60 µm	*15/15 = 100%*	*0/15 = 0%*
70 µm	*14/16 = 87.5%*	*2/16 = 12.5%*
80 µm	*51/55 = 92.73%*	*4/55 = 7.27%*

Table S2: Fraction of Total Aligned Neurites

Substrate	Total # Neurites (pooled across DIV)	# Neurite Aligned (pooled across DIV)	% Alignment
Standard-Pitch	5,074	1,811	35.69%
Gradient-Pitch	4,253	1,561	36.70%
Gradient-Length	1,341	573	42.73%
30 µm	*119*	*34*	*28.57%*
40 µm	*156*	*75*	*48.08%*
50 µm	*231*	*103*	*44.59%*
60 µm	*282*	*121*	*42.91%*
70 µm	*275*	*108*	*39.27%*
80 µm	*277*	*131*	*47.29%*

Table S3: Compilation of p-values for gradient-length relative energy data.

A p-value	4 DIV 30 µm	4 DIV 40 µm	4 DIV 50 µm	4 DIV 60 µm	4 DIV 70 µm	4 DIV 80 µm
7 DIV 30 µm	1.40e-06	0.079	0.999	1	2.67e-03	0.930
7 DIV 40 µm	2.24e-07	2.41e-07	0.199	0.066	2.24e-07	2.39e-04
7 DIV 50 µm	2.24e-07	2.32e-07	0.274	0.090	2.24e-07	2.66e-04
7 DIV 60 µm	2.24e-07	2.24e-07	4.38e-03	4.08e-04	2.24e-07	4.11e-07
7 DIV 70 µm	2.24e-07	2.24e-07	2.24e-07	2.24e-07	2.24e-07	2.24e-07
7 DIV 80 µm	2.24e-07	2.24e-07	2.26e-07	2.24e-07	2.24e-07	2.24e-07

B p-value	4 DIV 30 µm	4 DIV 40 µm	4 DIV 50 µm	4 DIV 60 µm	4 DIV 70 µm	4 DIV 80 µm
4 DIV 30 µm	--	2.88e-02	2.24e-07	2.24e-07	0.294	1.39e-05
4 DIV 40 µm	--	--	1.65e-04	1.15e-04	0.991	0.704
4 DIV 50 µm	--	--	--	0.999	2.52e-07	0.332
4 DIV 60 µm	--	--	--	--	2.28e-07	0.427
4 DIV 70 µm	--	--	--	--	--	5.48e-02

C p-value	7 DIV 30 µm	7 DIV 40 µm	7 DIV 50 µm	7 DIV 60 µm	7 DIV 70 µm	7 DIV 80 µm
7 DIV 30 µm	--	0.290	0.390	2.40e-02	2.29e-07	5.88e-07
7 DIV 40 µm	--	--	0.999	0.999	3.67e-03	4.19e-02
7 DIV 50 µm	--	--	--	0.994	4.87e-04	9.25e-03
7 DIV 60 µm	--	--	--	--	2.66e-02	0.202
7 DIV 70 µm	--	--	--	--	--	0.999

Shaded cells indicate significance; dashed boxes highlight significance between similar tube lengths. Comparison of p-values (A) between 4 DIV and 7 DIV time points (Two-way ANOVA, Tukey's post-hoc), and for (B) 4 DIV and (C) 7 DIV (one -way ANOVA, Tukey's post-hoc).

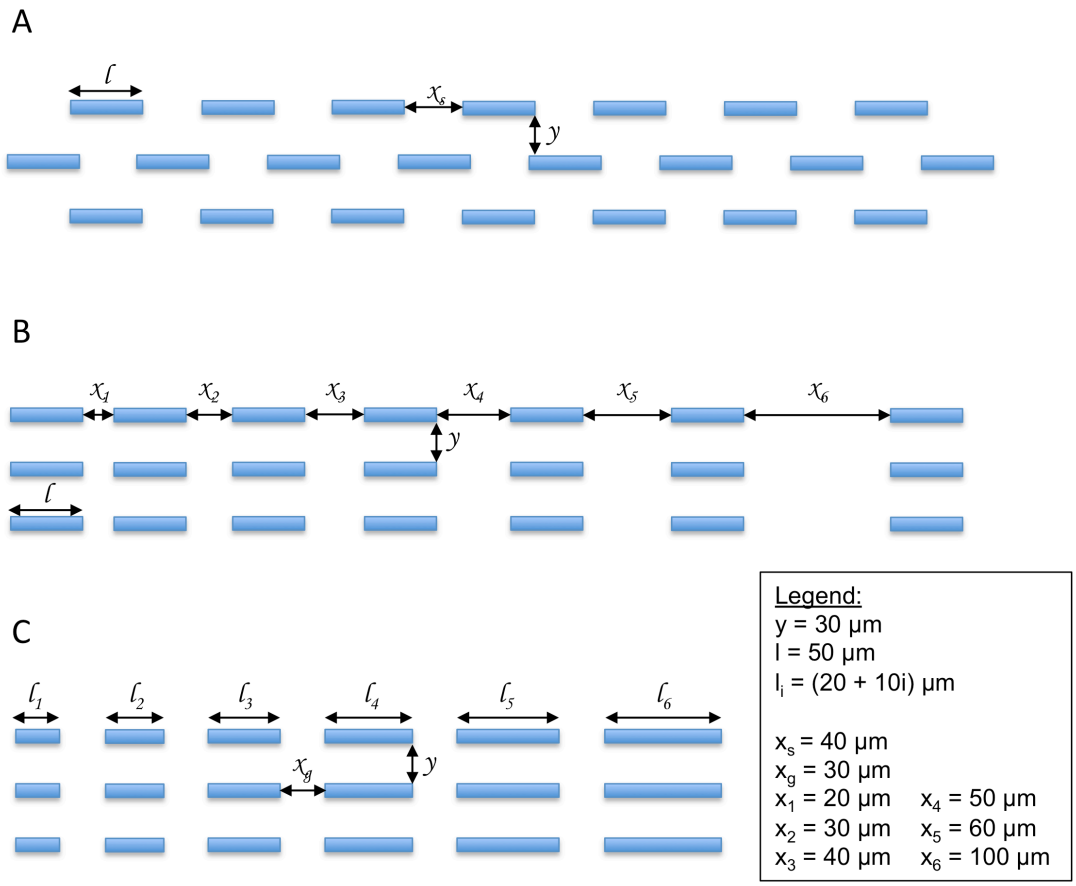

Figure S1. Schematic illustrating parameters of experimental µ-Tube substrates. (A) The standard-pitch µ-Tube array has a constant longitudinal pitch, x_s, a constant lateral pitch, y, and a constant µ-Tube length, l. **(B)** The gradient-pitch µ-Tubes array has a longitudinal pitch that varies between 20 µm – 60 µm and 100 µm, x_i, a constant lateral pitch, y, and a constant µ-Tube length, l. **(C)** The gradient-length microtube array has a constant longitudinal pitch, x_g, a constant lateral pitch, y, and the length of the microtubes varies between 30 µm and 80 µm long, l_i.

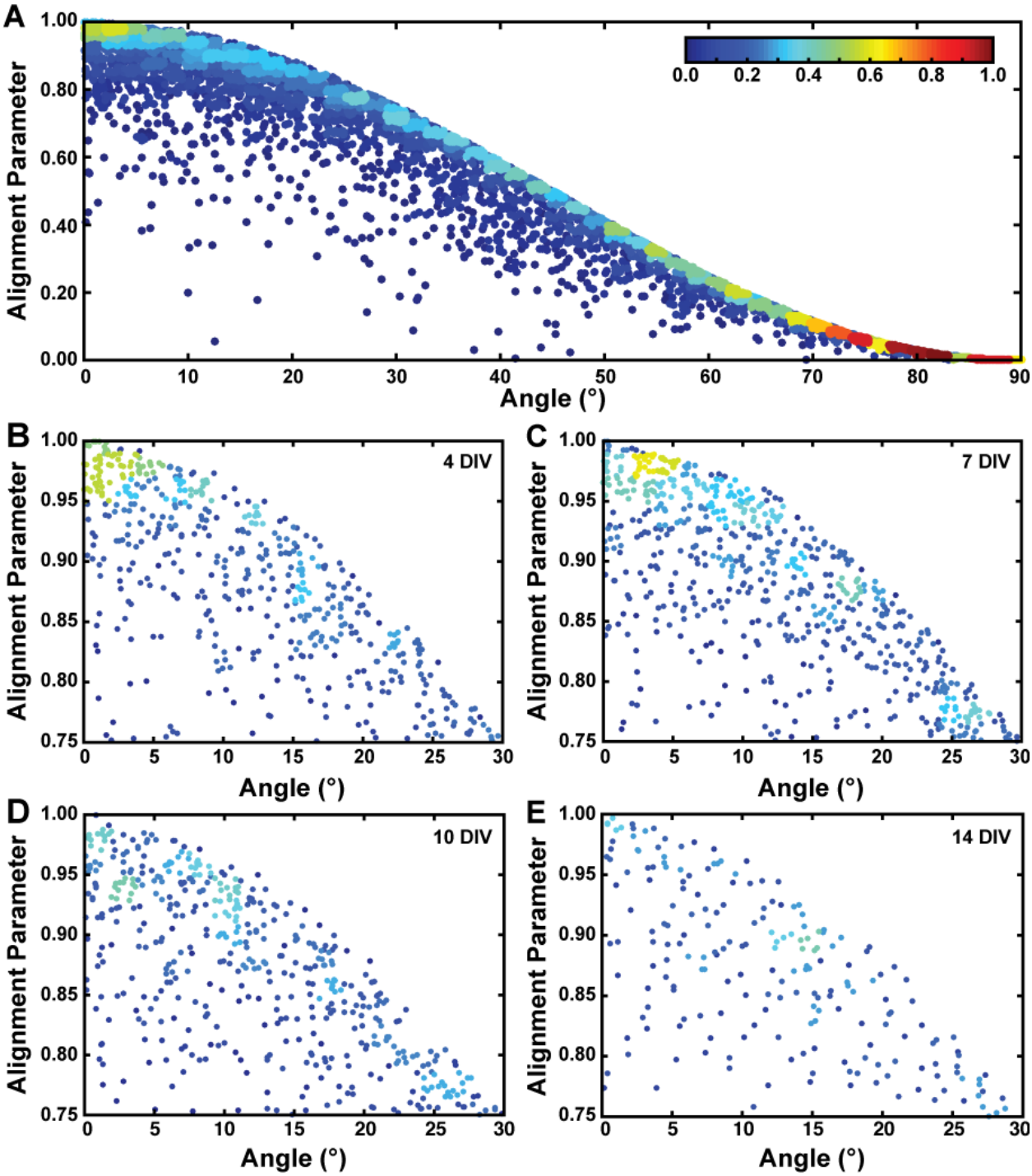

Figure S2. 2D histograms (normalized) of alignment parameter and process angle for (A) pooled data sets over time, and (B) 4, (C) 7, (D) 10, and (E) 14 DIV within the cutoff bounds. The color indicates the number of processes binned at each point. The cutoff for alignment is set as any process with an alignment parameter of ≥ 0.75, and an angle of $\leq 30°$.

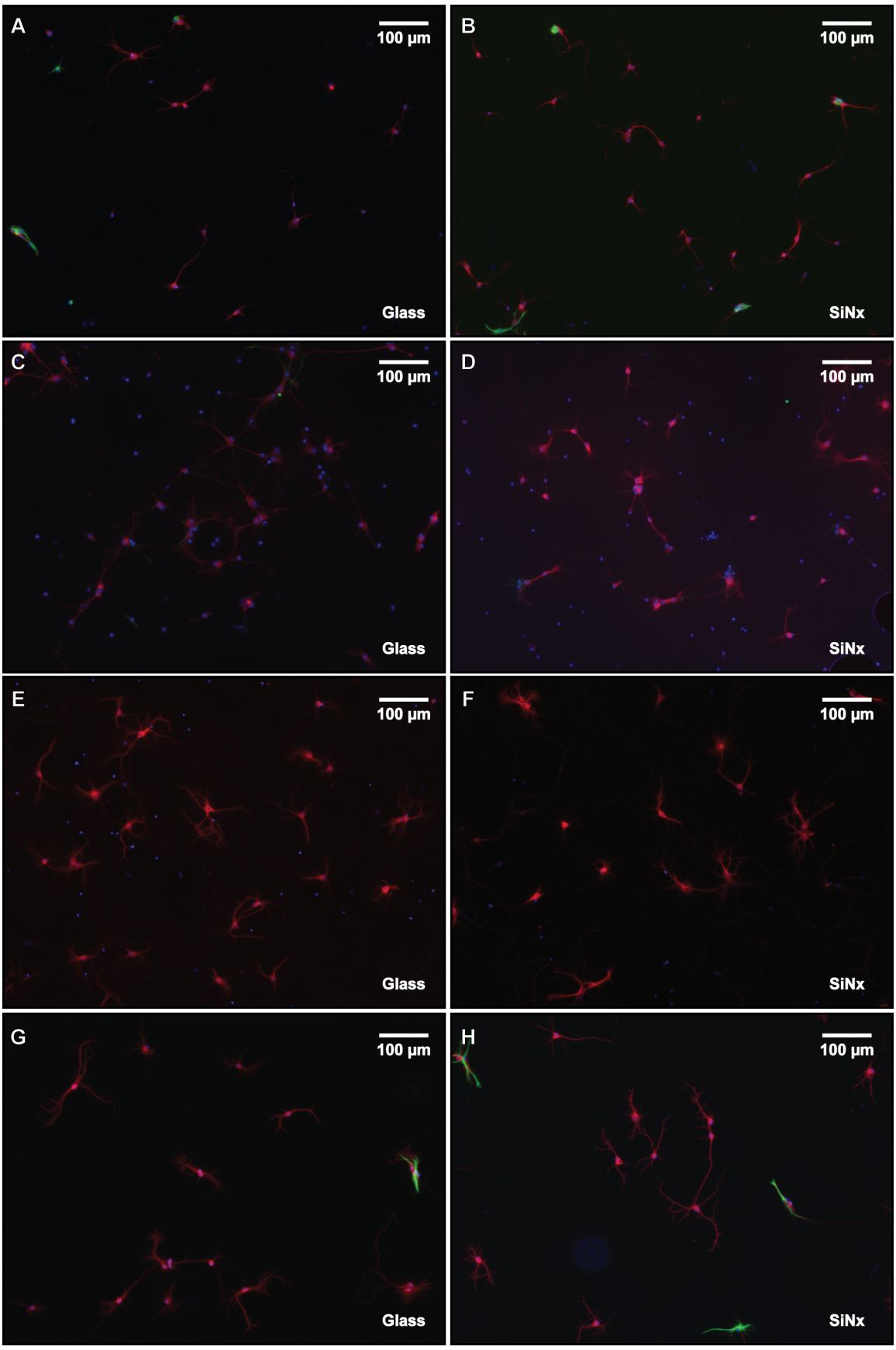
A
100 µm
Glass
B
100 µm
SiNx
C
100 µm
Glass
D
100 µm
SiNx
E
100 µm
Glass
F
100 µm
SiNx
G
100 µm
Glass
H
100 µm
SiNx

Figure S3. Fluorescent images of rat hippocampal neurons on glass and planar SiN_x control substrates. Immunocytochemistry shows MAP2 (red, neuronal marker), GFAP (green, glial marker), and DAPI (blue, nuclear marker). Neurons fixed at 4 (A-B), 7 (C-D), 10 (E-F), and 14 (G-H) days *in vitro* (DIV).

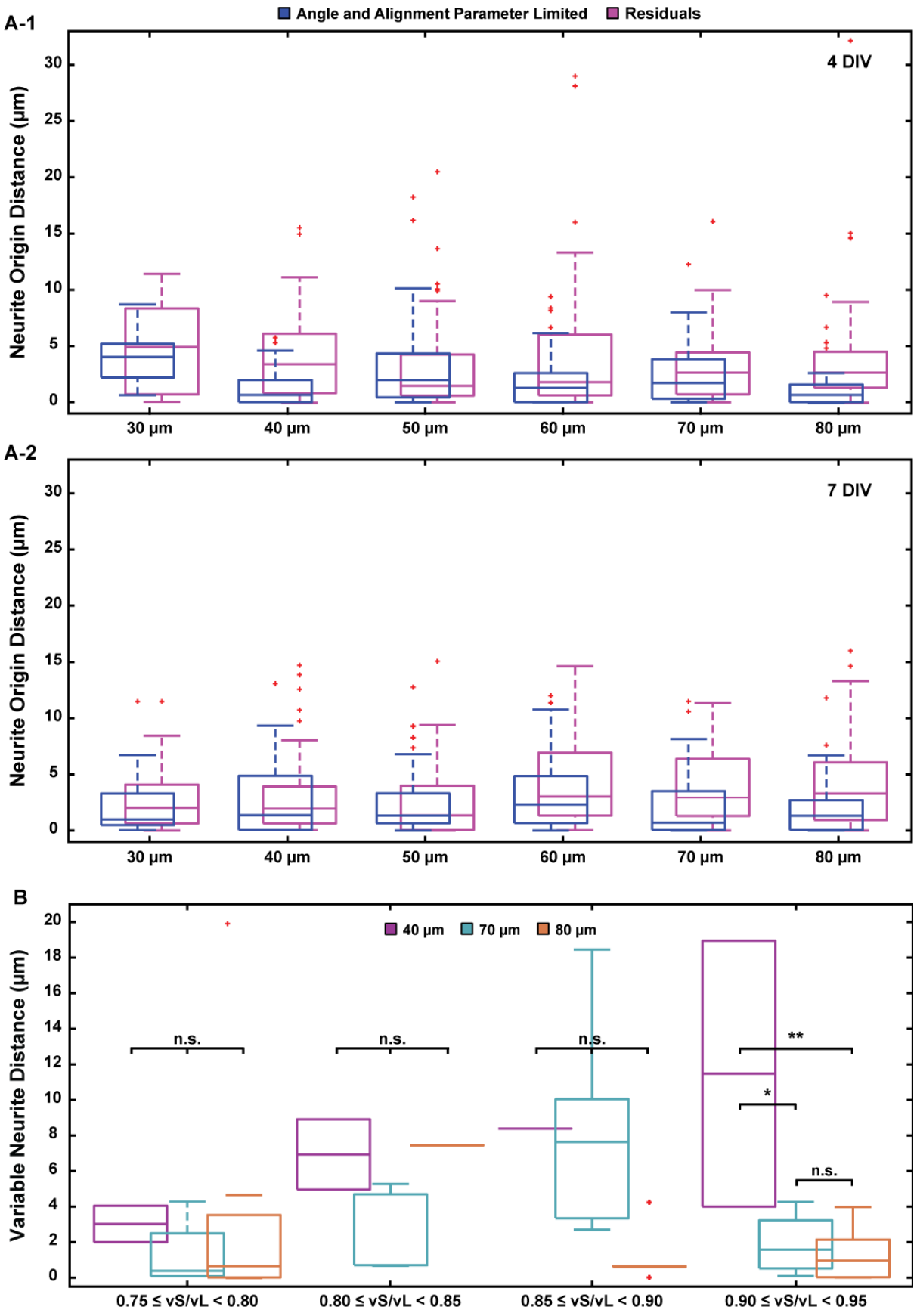
A-1
Angle and Alignment Parameter Limited
Residuals
4 DIV
Neurite Origin Distance (μm)
30 μm
40 μm
50 μm
60 μm
70 μm
80 μm
A-2
7 DIV
Neurite Origin Distance (μm)
B
40 μm
70 μm
80 μm
Variable Neurite Distance (μm)
n.s.
n.s.
n.s.
**
*
n.s.
0.75 ≤ vS/vL < 0.80
0.80 ≤ vS/vL < 0.85
0.85 ≤ vS/vL < 0.90
0.90 ≤ vS/vL < 0.95

Figure S4. Analysis of distance and neurite alignment on gradient-length μ-Tube substrate. (A) The relationship between neurite origin distance *vs.* alignment is presented as angle and alignment parameter limited (blue), and residuals (pink) for each μ-Tube length. Data are plotted for (A-1) 4 DIV and (A-2) 7 DIV. No significant difference was found between alignment and distance between the origin of soma-originating neurites and the closest μ-Tube for any tube lengths (one-way ANOVA, $p > 0.05$), nor was there significance between minimum distance of aligned neurites and μ-Tube length (one-way ANOVA, $p > 0.05$). (B) The relationship between the minimum distance at the variable length of the neurite *vs.* ratio of remaining projected length (vS) to remaining total length (vL) for neurites associated with μ-Tubes of lengths 40, 70, and 80 μm. Significance was found only between 40 μm and 70 μm ($p = 0.042$) and 40 μm and 80 μm ($p = 0.009$) for the vS/vL ratio in the range of 0.90 to 0.95 (one-way ANOVA, Tukey's post-hoc, * $p < 0.05$, ** $p < 0.01$). Red crosses indicate outliers from the boxplots.

References

1. Froeter, P., Huang, Y., Cangellaris, O. V., Huang, W., Dent, E. W., Gillette, M. U., Williams, J. C., and Li, X. Toward Intelligent Synthetic Neural Circuits: Directing and Accelerating Neuron Cell Growth by Self-Rolled-Up Silicon Nitride Microtube Array. *ACS Nano* **8**, 11108–11117 (2014).