Electronic Supplementary Material (ESI) for Lab on a Chip.

Supplementary Information

Electric Field Temporal Interference Stimulation of Neurons *in Vitro*

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- Figures S1-S9
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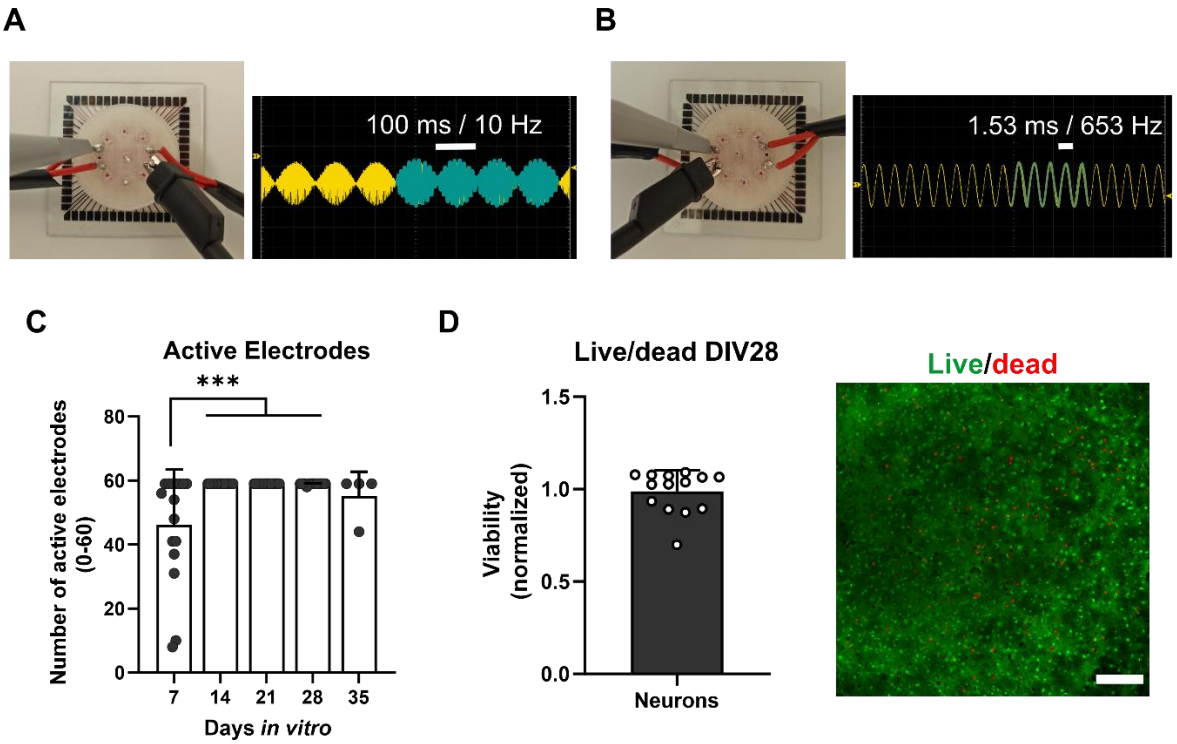
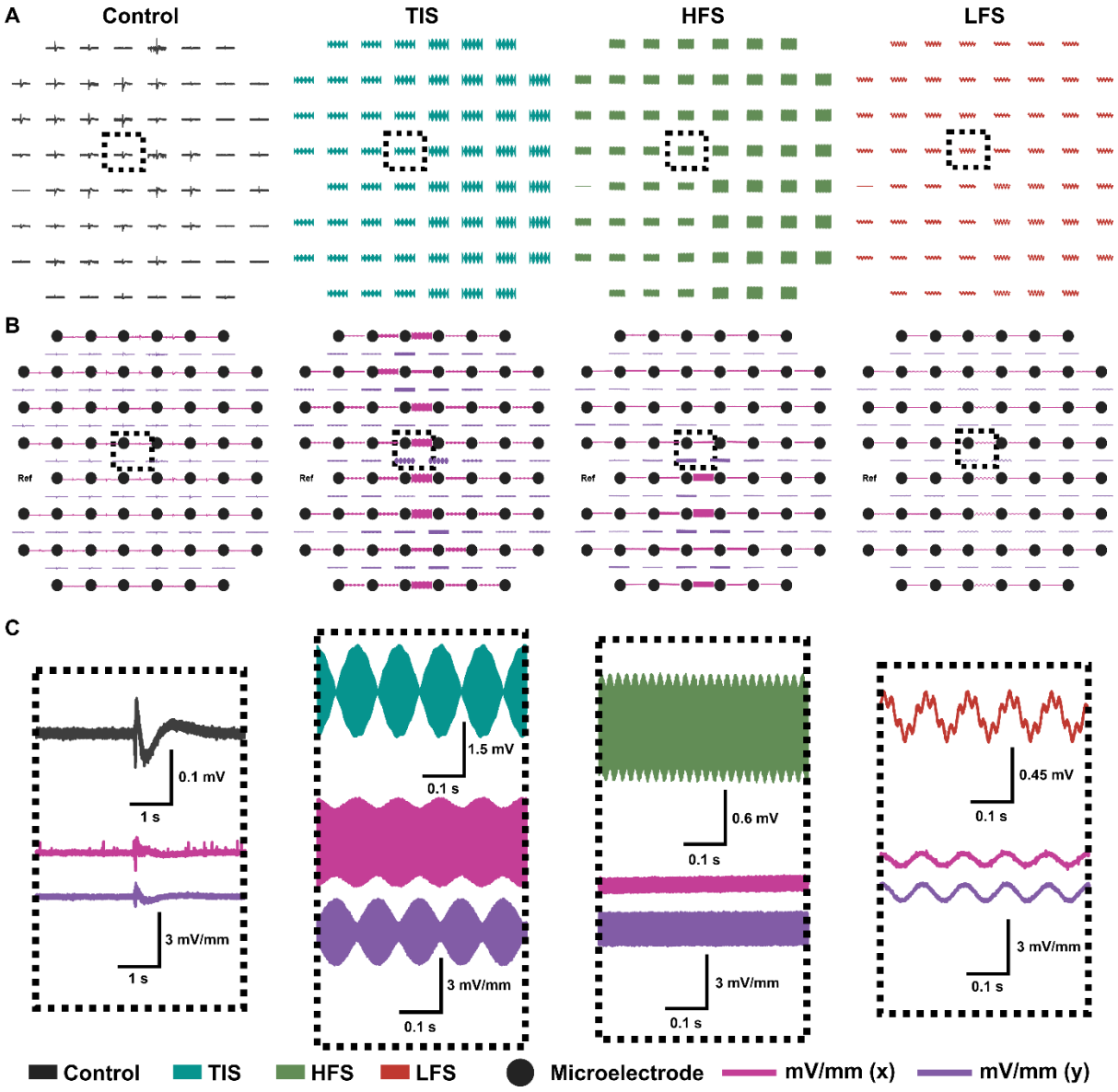
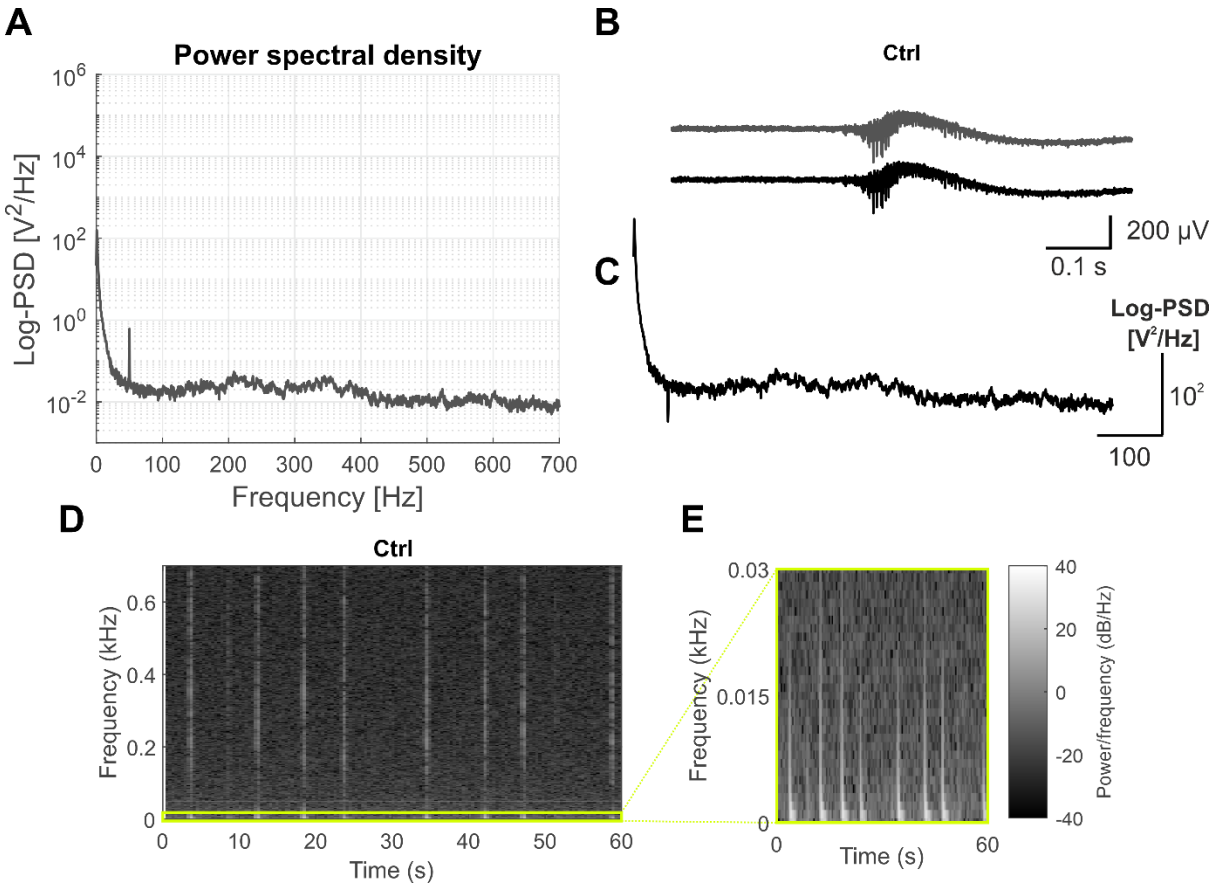


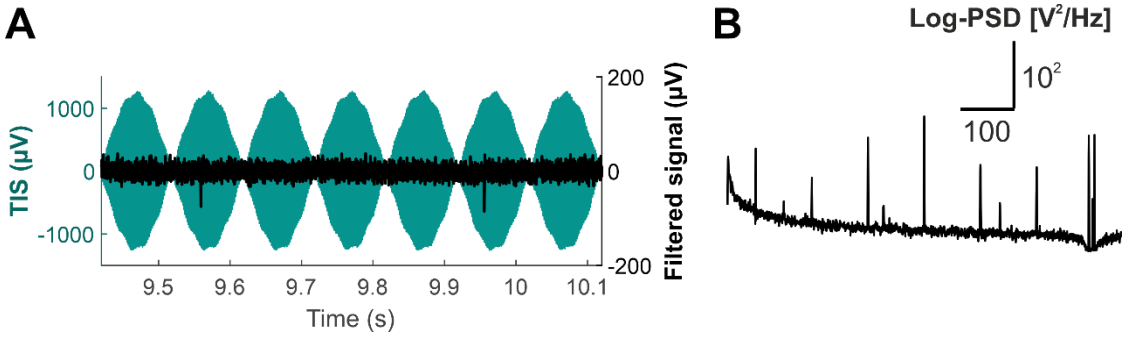
Supplementary Figure S1. *(**A**) Oscilloscope measurements of the TIS signal at 500 µA. TIS envelopes are indicated in blue. (**B**) Oscilloscope measurements of high-frequency signals at 500 µA. High-frequency signals are indicated in green. (**C**) The number of electrically active electrodes during maturation. After DIV7, all electrodes were active through the whole culture period, except for one MEA at DIV35 (One-way ANOVA followed by Tukey's multiple comparisons test. Adjusted p-values are indicated as ***p<0.001 (n=16 MEAs, except for DIV35 n=4 MEAs). (**D**) Live/dead assay of neuronal cultures at DIV28. Bar graph shows means±SD with data points (n=14 regions of interest [ROIs] in total).*



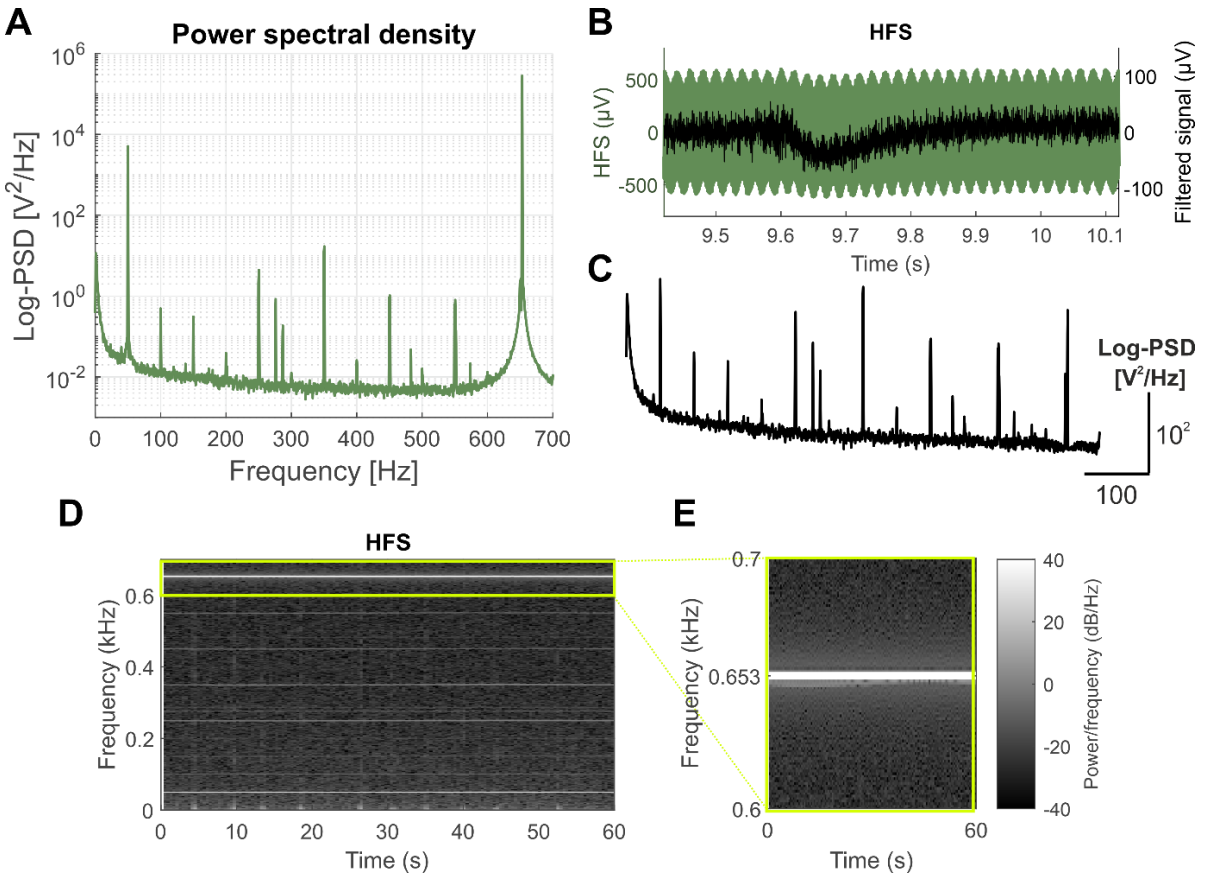
Supplementary Figure S2. *Electrical stimulation system and its validation. (**A**) Signal traces of TI, HF, and LF-stimulated cultures (during stimulation) and sham. (**B**) The electric field potentials (indicated with pink and purple traces) between two adjacent microelectrodes in both horizontal and vertical directions (E_x [mV/mm] and E_y [mV/mm], respectively). For TIS, both electric field potentials were lower at the sides of the electrode area and highest in the middle of it, suggesting the steerability of our TIS system. The microelectrodes are depicted as dark gray circles. (**C**) Close-ups of the signals depicted in A and B from microelectrode ID 44.*



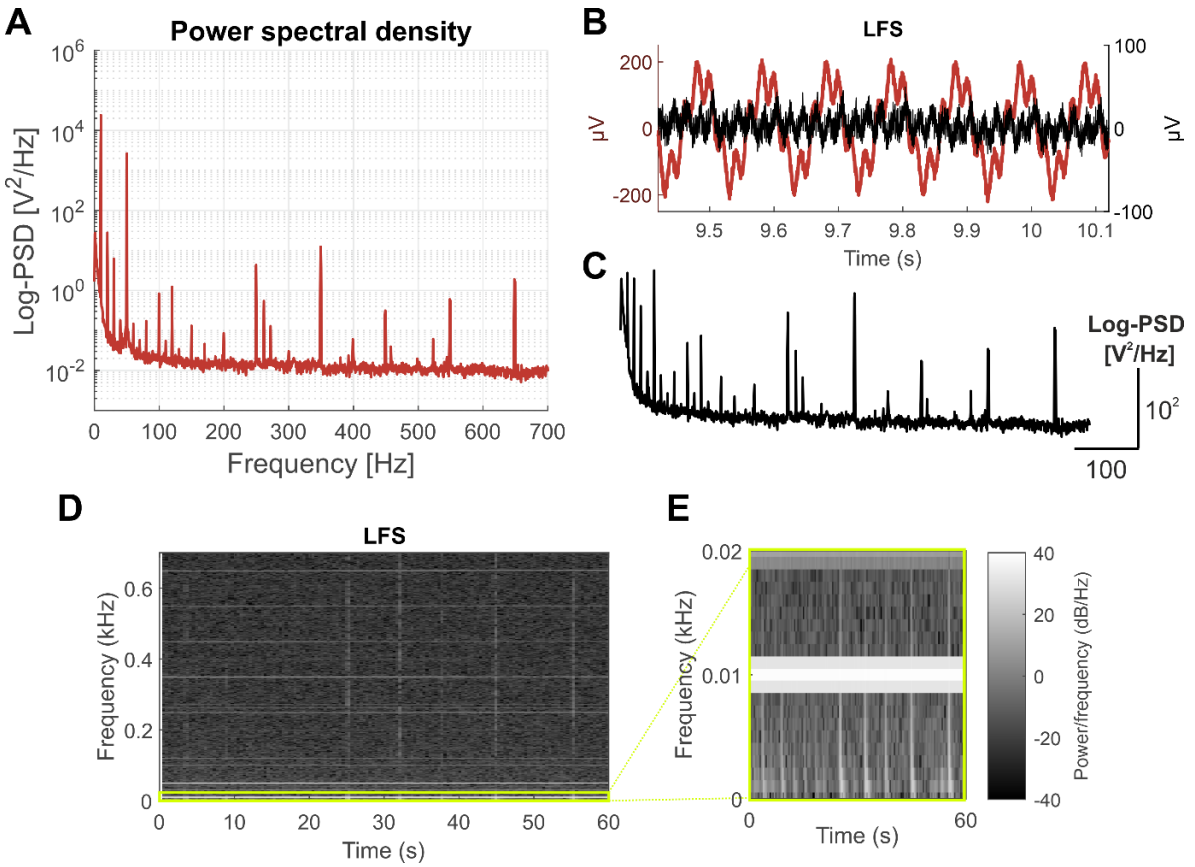
Supplementary Figure S3. *(**A**) PSD from unfiltered control data during sham. (**B**) Unfiltered signals (gray) and filtered signals (black) during sham. Neurons had spiking activity. (**C**) PSD of filtered signal during sham. (**D**) Spectrogram of sham signal during 'stimulation'. (**E**) Close-up of (**D**). In all cases, the data is derived from one respective electrode that was used to generate all different images.*



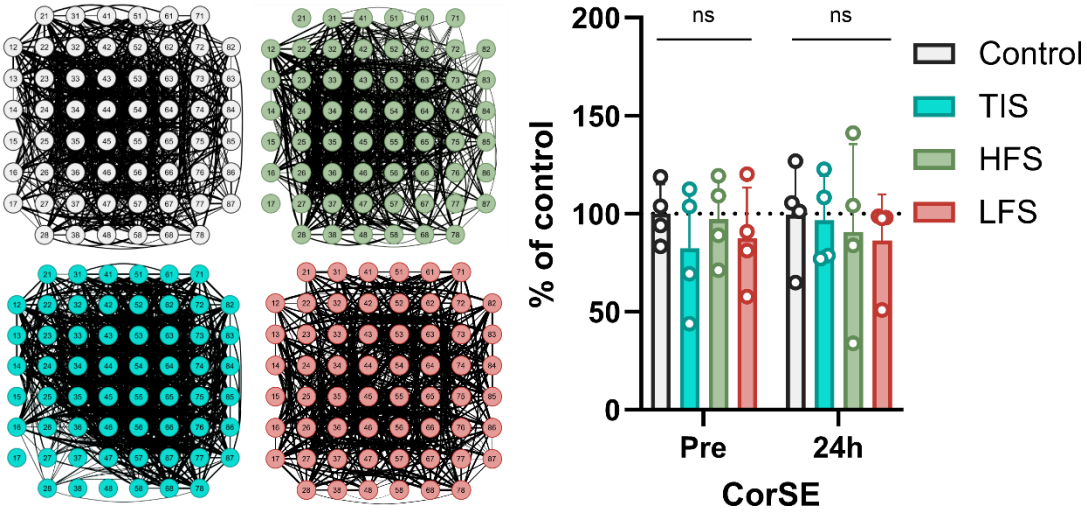
Supplementary Figure S4. *(**A**) Unfiltered signals (blue) and filtered signals (black) during TIS. Neurons had some spiking activity also during stimulation. Left y-axis represents the unfiltered signal during stimulation and the right y-axis the filtered signal of the same electrode during stimulation. (**B**) PSD of the filtered signal during TIS.*



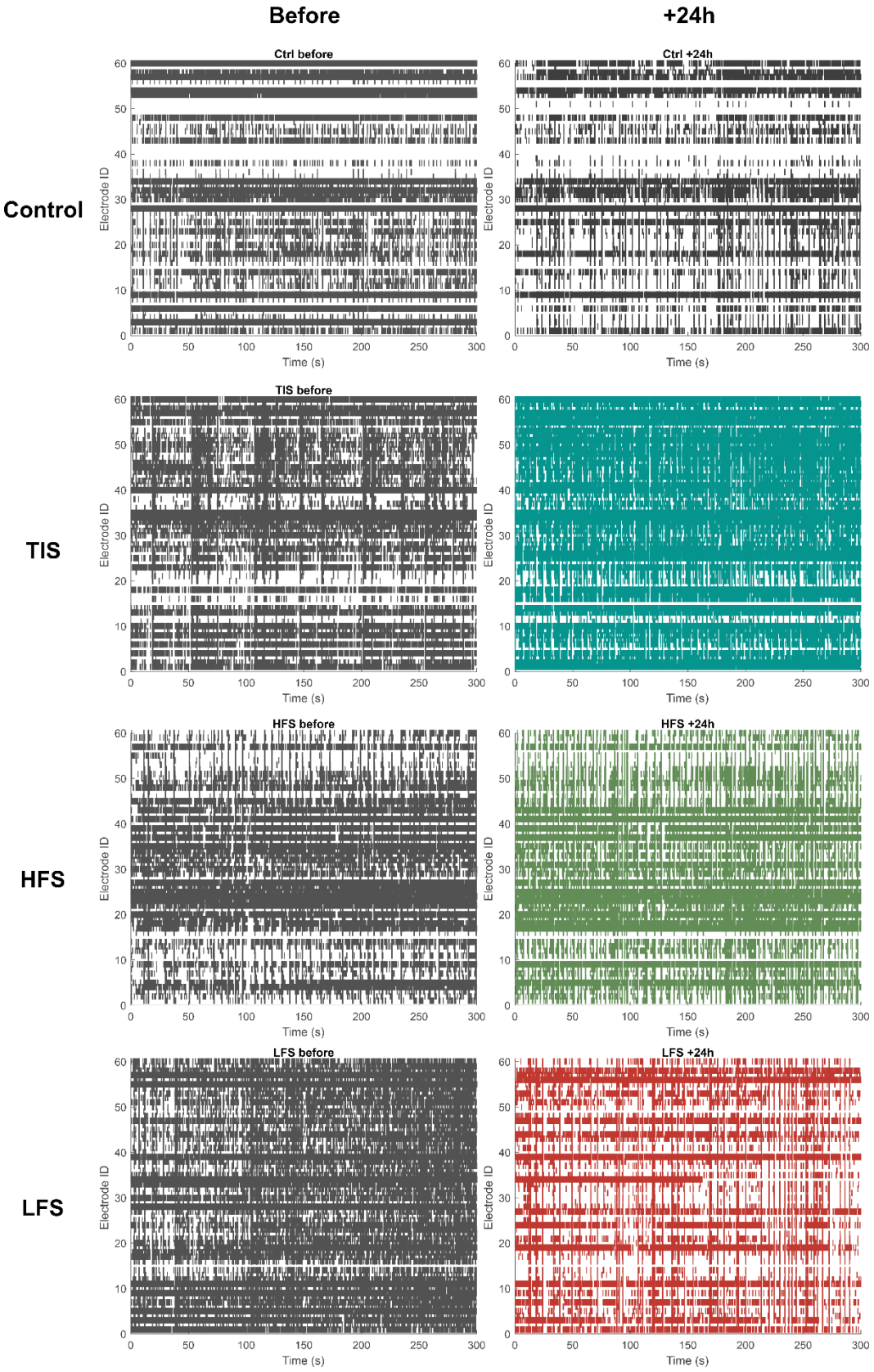
Supplementary Figure S5. *(**A**) PSD from unfiltered HFS data during stimulation. (**B**) Unfiltered signals (green) and filtered signals (black) during HFS. Neurons had spiking activity also during stimulation. Left y-axis represents the unfiltered signal during stimulation and the right y-axis the filtered signal of the same electrode during stimulation. (**C**) PSD of filtered signal during HFS. (**D**) Spectrogram of HFS signal during stimulation (**E**) Close-up of (**D**). In all cases, the data is derived from one respective electrode that was used to generate all different images.*



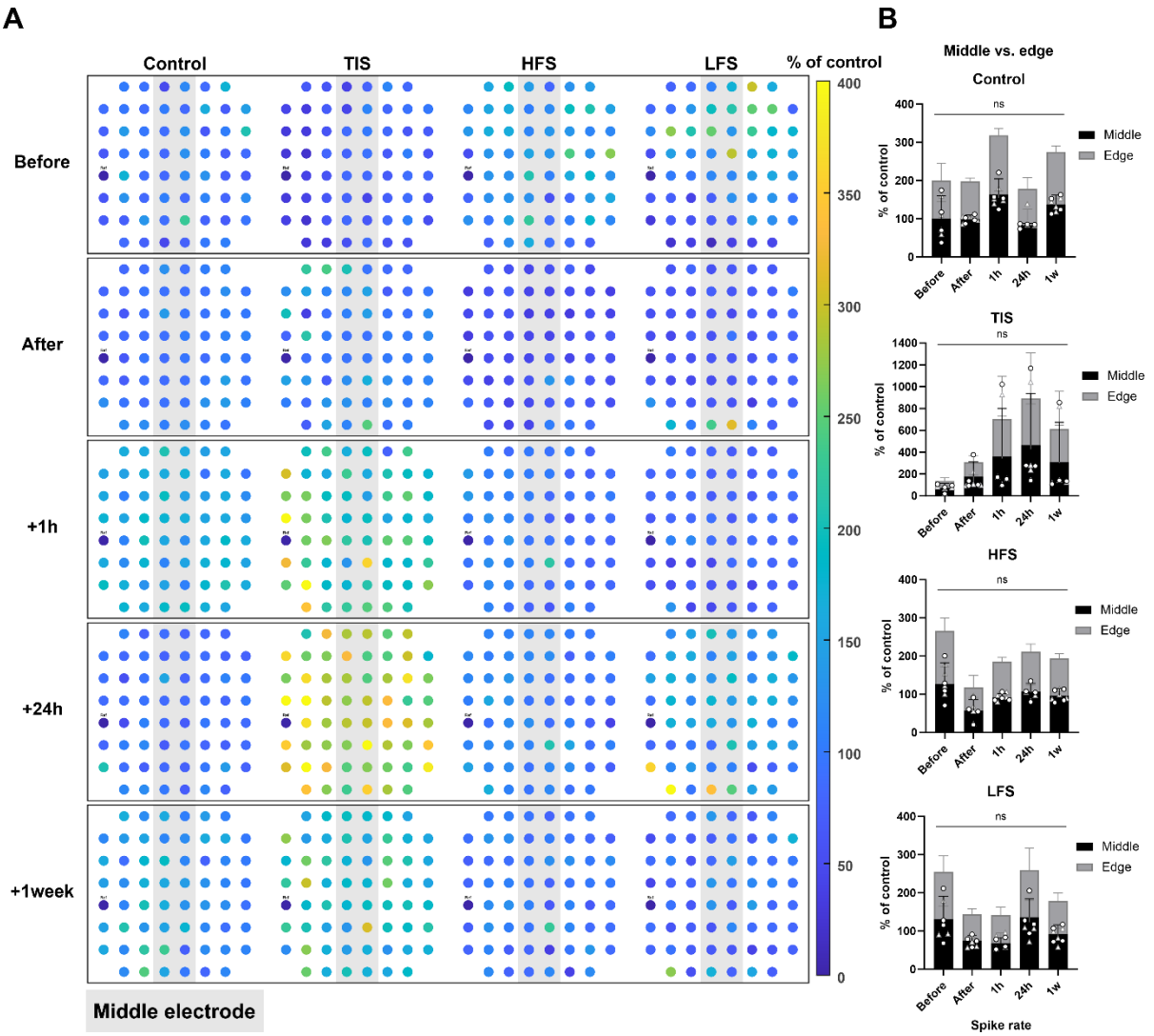
Supplementary Figure S6. *(**A**) PSD from unfiltered LFS data during stimulation. (**B**) Unfiltered signals (orange) and filtered signals (black) during LFS. Neurons had spiking activity also during stimulation. Left y-axis represents the unfiltered signal during stimulation and the right y-axis the filtered signal of the same electrode during stimulation. (**C**) PSD of filtered signal during LFS. (**D**) Spectrogram of LFS signal during stimulation (**E**) Close-up of (**D**). In all cases, the data is derived from one respective electrode that was used to generate all different images.*



Supplementary Figure S7. *CorSE analysis 24 hours after the stimulation. There were no differences in the connectivity between the groups. ns=not significant (n=4 MEAs/condition. Mann-Whitney U test was used for the CorSE analysis).*



Supplementary Figure S8. *Representative raster plots of neuron cultures before (left) and 24 hours after stimulation/sham (right).*



Supplementary Figure S9. *Percentual changes (compared to control) of the electrodes in the middle and edge of the microelectrode array area. (**A**) The TI-stimulation increased the activity of neurons in the electrodes residing in the middle of the MEA area (indicated with a gray background) as well as on the edge of the electrode area. Reference electrode is indicated with dark blue (0% change). (n=16 electrodes from 4 MEAs for each condition [middle]; n=43 electrodes from 4 MEAs for each condition [edge]). (**B**) There were no significant differences between the activated electrode areas for any of the culture conditions (middle: averages of 16 electrodes; edge: averages of 43 electrodes [from n=4 MEAs]; two-way ANOVA followed by Šídák's multiple comparisons test).*

Supplementary Table S1. *Maturation of the cultures. Mean±SD shown below, p-values for the maturation above.*

Electrophysiology – maturation

Original one-way ANOVA followed by Tukey's multiple comparisons test for corrections (α=0.05)

Significance ; adjusted *p*-values

ns=not significant; * *p*<0.05; ** *p*<0.01; * *p*<0.001**

Neurons

	Spike rate	Burst rate	Burst duration	Spikes In Bursts	Burst Spike Ratio	ISI in bursts	Active electrodes
F(df), p	F(4, 63) = 25.90, p<0.0001	F(4, 63) = 12.37, p<0.0001	F(4, 63) = 1.809, p=0.1383	F(4, 63) = 4.757, p=0.0020	F(4, 63) = 61.79, p<0.0001	F(4, 63) = 17.78, p<0.0001	F (4, 63) = 6.703, p=0.0001
DIV7 vs. DIV14	**0.0020****	**0.0441***	0.9126 ns	0.0559 ns	**p<0.0001*****	**p<0.0001*****	**0.0007*****
DIV7 vs. DIV21	**p<0.0001*****	**0.0023****	0.2444 ns	**0.0008*****	**p<0.0001*****	**p<0.0001*****	**0.0007*****
DIV7 vs. DIV28	**p<0.0001*****	**p<0.0001*****	0.2592 ns	0.0547 ns	**p<0.0001*****	**p<0.0001*****	**0.0008*****
DIV7 vs. DIV35	**p<0.0001*****	**p<0.0001*****	0.3878 ns	0.9021 ns	**p<0.0001*****	**0.0007*****	0.3312 ns
DIV14 vs. DIV21	**0.0164***	0.8450 ns	0.7397 ns	0.6078 ns	**0.0004*****	0.6082 ns	>0.9999 ns
DIV14 vs. DIV28	**0.0004*****	0.0536 ns	0.7588 ns	>0.9999 ns	0.3297 ns	0.8866 ns	>0.9999 ns
DIV14 vs. DIV35	**0.0001*****	**0.0026****	0.7214 ns	0.9087 ns	0.9763 ns	0.9904 ns	0.9344 ns
DIV21 vs. DIV28	0.7637 ns	0.4058 ns	>0.9999 ns	0.6132 ns	0.1074 ns	0.9861 ns	>0.9999 ns
DIV21 vs. DIV35	0.0662 ns	**0.0179***	0.9889 ns	0.3987 ns	0.2000 ns	0.9917 ns	0.9344 ns
DIV28 vs. DIV35	0.3031 ns	0.2401 ns	0.9870 ns	0.9068 ns	0.9728 ns	>0.9999 ns	0.9381 ns

Electrophysiology - maturation

Mean ± SD

Neurons

	Spike rate (spikes/min)	Burst rate (bursts/min)	Burst duration (ms)	Spikes In Bursts	Burst Spike Ratio (0-1)	ISI in bursts (ms)	Active electrodes (0-60 (icl.ref))
DIV7	92±75	3±1	184±119	20±22	0.3±0.1	25±16	46±17
DIV14	453±209	12±9	164±43	34±11	0.7±0.1	8±4	59±0
DIV21	751±273	16±6	136±23	41±9	0.8±0.1	4±2	59±0
DIV28	860±371	21±15	137±31	34±11	0.8±0.1	6±2	59±0
DIV35	1144±350	32±13	119±46	27±11	0.7±0.0	6±2	55±8

Supplementary Table S2. *Stimulation results. Mann-Whitney U test, comparisons done by comparing the stimulation results to control change at each time point in question.*

Electrophysiology – stimulation (%)

Mann-Whitney-U-test

Significance (median1, median2, U), *p*)

*** *p*<0.05**

Spike rate

Neurons	Pre	After	1h	1d	1w
ctrl vs. TIS	(86.48, 77.69, 5), 0.4857	(96.67, 111.8, 4), 0.3429	(145.6, 168.8, 6), 0.6857	(81.75, 263.8, 0), **0.0286 ***	(141.3, 128.3, 7), 0.8857
ctrl vs. HFS	(86.48, 131.3, 4), 0.3429	(96.67, 61.57, 2), 0.1143	(145.6, 95.46, 0), **0.0286 ***	(81.75, 103.0, 3), 0.200	(141.3, 91.16, 0), **0.0286 ***
ctrl vs. LFS	(86.48, 114.3, 4), 0.3429	(96.67, 71.37, 0), **0.0286 ***	(145.6, 71.90 6), **0.0286 ***	(81.75, 112.3, 6), 0.6857	(141.3, 86.94, 0), **0.0286 ***
TIS vs. HFS	(77.69, 131.3, 0), **0.0286 ***	(111.8, 61.57, 1), 0.0571	(168.8, 95.46, 1), 0.0571	(263.8, 103.0, 0), **0.0286 ***	(128.3, 91.16, 0), **0.0286 ***
TIS vs. LFS	(77.69, 114.3, 2), 0.1143	(111.8, 71.37, 0), **0.0286 ***	(168.8, 71.90, 0), **0.0286 ***	(263.8, 112.3, 1), 0.0571	(128.3, 86.94, 0), **0.0286 ***
HFS vs. LFS	(131.3, 114.3, 7), 0.8857	(61.57, 71.37, 6), 0.6857	(95.46, 71.90, 3), 0.200	(103.0, 112.3, 6), 0.6857	(91.16, 86.94, 6), 0.6857
Burst rate					
Neurons					
ctrl vs. TIS	(100.5, 73.48, 6), 0.6857	(117.8, 101.9, 5), 0.4857	(293.9, 118.5, 5), 0.4857	(98.49, 326.8, 0), **0.0286 ***	(226.1, 132.4, 5), 0.4857
ctrl vs. HFS	(100.5, 127.7, 4), 0.3429	(117.8, 48.25, 0), **0.0286 ***	(293.9, 74.06, 0), **0.0286 ***	(98.49, 92.85, 6), 0.6857	(226.1, 90.87, 0), **0.0286 ***
ctrl vs. LFS	(100.5, 141.1, 3), 0.2000	(117.8, 66.45, 0), **0.0286 ***	(293.9, 52.42, 0), **0.0286 ***	(98.49, 104.6, 8), >0.9999	(226.1, 86.79, 0), **0.0286 ***
TIS vs. HFS	(73.48, 127.7, 3), 0.2000	(101.9, 48.25, 1), 0.0571	(118.5, 74.06, 2), 0.1143	(326.8, 92.85 0), **0.0286 ***	(132.4, 90.87, 1), 0.0571
TIS vs. LFS	(73.48, 141.1, 3), 0.2000	(101.9, 66.45, 1), 0.0571	(118.5, 52.42, 0), **0.0286 ***	(326.8, 104.6 0), **0.0286 ***	(132.4, 86.79, 0), **0.0286 ***
HFS vs. LFS	(127.7, 141.1, 6), 0.6857	(48.25, 66.45, 7), 0.8857	(74.06, 52.42, 0), **0.0286 ***	(92.85, 104.6, 6), 0.6857	(90.87, 86.79, 7), 0.8857
Burst duration					
Neurons					
ctrl vs. TIS	(104.2, 95.54, 4), 0.3429	(81.93, 101.0, 5), 0.4857	(54.09, 191.6, 1), 0.0571	(88.93, 83.21, 7), 0.8857	(91.05, 94.74, 5), 0.4857
ctrl vs. HFS	(104.2, 99.34, 7), 0.8857	(81.93, 102.7, 5), 0.4857	(54.09, 168.6, 0), **0.0286 ***	(88.93, 103.3, 5), 0.4857	(91.05, 114.8, 3), 0.2000
ctrl vs. LFS	(104.2, 81.31, 3), 0.2000	(81.93, 151.3, 0), **0.0286 ***	(54.09, 165.9, 0), **0.0286 ***	(88.93, 146.9, 2), 0.1143	(91.05, 146.2, 0), **0.0286 ***
TIS vs. HFS	(95.54, 99.34, 7), 0.8857	(101.0, 102.7, 8), >0.9999	(191.6, 168.6 6), 0.6857	(83.21, 103.3, 5), 0.4857	(94.74, 114.8, 6), 0.6857
TIS vs. LFS	(95.54, 81.31, 5), 0.4857	(101.0, 151.3, 2), 0.1143	(191.6, 165.9 8), >0.9999	(83.21, 146.9, 2), 0.1143	(94.74, 146.2, 3), 0.2000
HFS vs. LFS	(99.34, 81.31, 3), 0.2000	(102.7, 151.3, 2), 0.1143	(168.6, 165.9 6), 0.6857	(103.3, 146.9, 2), 0.1143	(114.8, 146.2, 6), 0.6857
Spikes in Burst					
Neurons					
ctrl vs. TIS	(94.57, 96.83, 7), 0.8857	(82.07, 90.65, 5), 0.4857	(48.97, 144.3, 0), **0.0286 ***	(89.00, 84.21, 6), 0.6857	(70.37, 91.48, 0), **0.0286 ***
ctrl vs. HFS	(94.57, 85.90, 6), 0.6857	(82.07, 96.25, 6), 0.6857	(48.97, 131.2, 1), 0.0571	(89.00, 101.8, 5), 0.4857	(70.37, 90.97, 1), 0.0571
ctrl vs. LFS	(94.57, 64.10, 3), 0.2000	(82.07, 116.0, 0), **0.0286 ***	(48.97, 144.9, 0), **0.0286 ***	(89.00, 113.2, 1), 0.0571	(70.37, 114.7, 0), **0.0286 ***
TIS vs. HFS	(96.83, 85.90, 8), >0.9999	(90.65, 96.25, 7), 0.8857	(144.3, 131.2, 6), 0.6857	(84.21, 101.8, 4), 0.3429	(91.48, 90.97, 7), 0.8857
TIS vs. LFS	(96.83, 64.10, 3), 0.2000	(90.65, 116.0, 3), 0.2000	(144.3, 144.9, 7), 0.8857	(84.21, 113.2, 1), 0.0571	(91.48, 114.7, 3), 0.2000
HFS vs. LFS	(85.90, 64.10, 4), 0.3429	(90.65, 116.0, 4), 0.3429	(144.9, 131.2 5), 0.4857	(101.8, 113.2, 4), 0.3429	(90.97, 114.7, 5), 0.4857

Burst Spike Ratio					
Neurons					
ctrl vs. TIS	(99.30, 85.89, 4), 0.3429	(100.7, 94.29, 6), 0.6857	(90.77, 112.3, 0), **0.0286 ***	(97.26, 108.6, 2), 0.1143	(89.23, 147.2, 0), **0.0286 ***
ctrl vs. HFS	(99.30, 91.94, 0), **0.0286 ***	(100.7, 91.20, 0), **0.0286 ***	(90.77, 109.0, 0), **0.0286 ***	(97.26, 99.78, 6), 0.6857	(89.23, 121.3, 0), **0.0286 ***
ctrl vs. LFS	(99.30, 90.29, 4), 0.3429	(100.7, 94.92, 4), 0.3429	(90.77, 103.9, 0), **0.0286 ***	(97.26, 99.48, 6), 0.6857	(89.23, 132.3, 0), **0.0286 ***
TIS vs. HFS	(85.89, 91.94 4), 0.3429	(94.29, 91.20, 6), 0.6857	(112.3, 109.0, 6), 0.6857	(108.6, 99.78, 4), 0.3429	(147.2, 121.3, 2), 0.1143
TIS vs. LFS	(85.89, 90.29, 7), 0.8857	(94.29, 94.92, 6), 0.6857	(112.3, 103.9, 7), 0.8857	(108.6, 99.48, 5), 0.4857	(147.2, 132.3, 5), 0.4857
HFS vs. LFS	(91.94, 90.29, 6), 0.6857	(91.20, 94.92, 4), 0.3429	(109.0, 103.9, 7), 0.8857	(99.78, 99.48, 8), >0.9999	(121.3, 132.3, 4), 0.3429
ISI in bursts					
Neurons					
ctrl vs. TIS	(97.50, 115.9, 4), 0.3429	(94.10, 116.1, 2), 0.1143	(123.5, 124.1, 8), >0.9999	(97.95, 113.6, 6), 0.6857	(142.4, 101.6, 4), 0.3429
ctrl vs. HFS	(97.50, 130.1, 4), 0.3429	(94.10, 109.1, 3), 0.2000	(123.5, 133.3, 6), 0.6857	(97.95, 99.20, 7), 0.8857	(142.4, 127.8, 5), 0.4857
ctrl vs. LFS	(97.50, 109.4, 7), 0.8857	(94.10, 121.7, 0), **0.0286 ***	(123.5, 118.6, 8), >0.9999	(97.95, 127.8, 2), 0.1143	(142.4, 126.6, 7), 0.8857
TIS vs. HFS	(115.9, 130.1, 5), 0.4857	(116.1, 109.1, 8), >0.9999	(124.1, 133.3, 6), 0.6857	(113.6, 99.20, 8), >0.9999	(101.6, 127.8, 3), 0.2000
TIS vs. LFS	(115.9, 109.4, 7), 0.8857	(116.1, 121.7, 4), 0.3429	(124.1, 118.6, 8), >0.9999	(113.6, 127.8, 5), 0.4857	(101.6, 126.6, 4), 0.3429
HFS vs. LFS	(130.1, 109.4, 7), 0.8857	(109.1, 121.7, 5), 0.4857	(133.3, 118.6, 7), 0.8857	(99.20, 127.8, 2), 0.1143	(127.8, 126.6, 8), >0.9999
CorSE					
Neurons					
ctrl vs. TIS	(98.90, 86.49, 5), 0.4857			(103.3, 93.53, 8), >0.9999	
ctrl vs. HFS	(98.90, 99.13, 8), >0.9999			(103.3, 94.04, 7), 0.8857	
ctrl vs. LFS	(98.90, 86.04, 5), 0.4857			(103.3, 97.79, 3), 0.2000	
TIS vs. HFS	(86.49, 99.13 5), 0.4857			(93.53, 94.04, 8), >0.9999	
TIS vs. LFS	(86.49, 86.04, 7), 0.8857			(93.53, 97.79, 6), 0.6857	
HFS vs. LFS	(99.13, 86.04, 7), 0.8857			(94.04, 97.79, 7), 0.8857	

Supplementary Table S3. *Absolute values (mean±SD, standard error of mean [SEM]) of the electrical stimulation results.*

Electrophysiology - stimulation

Mean ±SD, SEM

Spike rate (spikes/min)

	Pre	After	1h	1d	1w
Control	809.2±376.2, 188.1	777.5±351.2, 175.6	1215±476.6, 238.3	713.6±290.7, 145.3	1144±349.9, 174.9
TIS	531.6±249.8, 124.9	608.9±169.1, 84.53	1682±579.2, 289.6	1353±330.9, 165.5	1295±361.2, 180.6
HFS	1086±320.2, 160.1	588.3±234.8, 117.4	1568±590, 295	1016±327.9, 164	1418±427.3, 213.7
LFS	1012±370, 185	675.6±190.8, 95.41	1183±698.5, 349.3	1255±1021, 510.7	1258±714.4, 357.2

Burst rate (bursts/min)

Control	15.41±5.075, 2.537	19.31±6.595, 3.298	41.57±16.74, 8.369	15.58±3.517, 1.759	32.47±12.91, 6.456

TIS	14.42±12.21, 6.105	19.27±7.762, 3.881	51.92±21.46, 10.73	44.65±17.55, 8.774	47.59±19.84, 9.919
HFS	25.70±15.76, 7.880	14.08±5.515, 2.758	54.36±29.38, 14.69	26.92±12.35, 6.176	45.33±18.52, 9.260
LFS	30.21±21.03, 10.51	18.88±4.970, 2.485	46.73±40.24, 20.12	43.42±49.41, 24.71	53.68±41.30, 20.65

Burst duration (ms)

Control	150.2±30.21, 15.10	108.7±34.10, 17.05	89.43±23.85, 11.92	123.2±42.90, 21.45	118.7±45.68, 22.84
TIS	134.1±30.55, 15.28	95.15±29.53, 14.77	134.2±45.43, 22.72	96.75±24.97, 12.49	110.7±10.46, 5.230
HFS	149.2±21.90, 10.95	109.7±27.05, 13.52	139.8±18.22, 9.112	125.9±25.19, 12.59	147.9±40.38, 20.19
LFS	114.7±36.42, 18.21	121.9±43.48, 21.74	123.2±19.95, 9.973	138.3±53.95, 26.98	130.3±47.69, 23.85

Spikes in Burst

Control	40.06±12.33, 6.163	31.12±7.804, 3.902	21.61±4.831, 2.415	34.59±11.95, 5.976	27.39±10.95, 5.474
TIS	35.05±10.96, 5.482	24.61±6.907, 3.454	25.88±7.607, 3.803	24.23±7.066, 3.533	22.52±8.025, 4.012
HFS	35.48±12.75, 6.377	24.57±8.971, 4.485	21.99±2.633, 1.317	29.08±7.983, 3.992	21.68±3.667, 1.833
LFS	26.27±7.814, 3.907	24.92±5.921, 2.961	20.63±3.961, 1.980	25.59±5.090, 2.545	19.27±5.111, 2.556

Burst Spike Ratio (0-1)

Control	0.8171±0.0303, 0.0151	0.8254±0.0357, 0.0179	0.7431±0.0251, 0.0126	0.7957±0.0236, 0.0118	0.7248±0.0332, 0.0169
TIS	0.7339±0.0979, 0.0489	0.7043±0.0710, 0.0355	0.7304±0.0322, 0.0161	0.7687±0.0541, 0.0271	0.7290±0.1115, 0.0557
HFS	0.7519±0.0194, 0.0097	0.6129±0.1737, 0.0868	0.7279±0.0570, 0.0285	0.7374±0.0678, 0.0339	0.6299±0.0487, 0.0243
LFS	0.7314±0.117, 0.0585	0.7532±0.0858, 0.0429	0.7252±0.0484, 0.0242	0.7573±0.0985, 0.0492	0.6937±0.1121, 0.0561

ISI in bursts (ms)

Control	4.741±0.4361, 0.218	4.333±1.218, 0.6088	5.778±1.091, 0.5454	4.626±0.8505, 0.4253	6.323±1.713, 0.8565
TIS	5.689±1.448, 0.7239	5.888±1.805, 0.9026	8.592±3.219, 1.610	6.371±3.553, 1.777	8.307±4.258, 2.129
HFS	6.399±2.037, 1.018	6.905±2.175, 1.087	9.996±1.549, 0.7743	6.409±1.603, 0.8016	10.73±3.064, 1.532
LFS	6.284±2.990, 1.495	7.097±2.610, 1.305	9.351±3.276, 1.638	8.224±5.239, 2.619	10.30±4.370, 2.185

CorSE (0-1)

Control	0.68±0.10, 0.05	0.67±0.18, 0.09
TIS	0.56±0.22, 0.11	0.52±0.19, 0.10
HFS	0.66±0.15, 0.07	0.62±0.31, 0.16
LFS	0.60±0.18, 0.09	0.54±0.25, 0.12

Supplementary Table S4. *Other results (viability and CorSE).*

Other results **Mann-Whitney-U-test**		
	Mean±SD	
	Viability DIV28	**CorSE DIV28**
Neurons	83±10	0.62±0.16
Impact electrodes ('middle' vs. 'edge')		
Two-way ANOVA followed by Šídák's multiple comparisons test for corrections (α=0.05) **Significance ; adjusted *p*-values** **ns=not significant; * *p*<0.05; ** *p*<0.01; *** *p*<0.001**		
	F(df), *p* (column factor)	**Middle vs. edge (*p*) (before, after, 1h, 24h, 1w)**	Significance
Control	F (1, 30) = 0.001216, *p*=0.9724	>0.9999, >0.9999, 0.9963, 0.9795, >0.9999	ns
TIS	F (1, 30) = 0.05899, *p*=0.8098	>0.9999, >0.9999, >0.9999, >0.9999, >0.9999,	ns
HFS	F (1, 30) = 0.1368, *p*=0.7141	0.9867, 0.9998, >0.9999, >0.9999, >0.9999	ns
LFS	F (1, 30) = 0.2480, *p*=0.6221	0.9994, 0.9996, 0.9999, 0.9907, 0.9997	ns