# Multichannel Silicon Probes for Laminar Cortical and Hippocampal Recordings in Large Animals

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**Abstract:** 1) Laminar information across different deep brain structures and cortical regions need to be decoded in order to understand the spatiotemporal ensembles that represent cognition and memory. Pig models are widely used in translational research, and may provide an excellent bridge between rodents and non-human primates for CNS disease models due to their gyrencephalic neuroanatomy and significant white matter composition. One of the major obstacles to addressing the research questions and approaches currently accessible to smaller-brained animals, are technical limitations in microelectrodes. 2) We tested various designs of multichannel silicon probes developed for large animal electrophysiology acutely in Yucatan pigs by recording neurophysiological signals from laminar structures under anesthesia. 3) Electrophysiological parameters of single units and local field potentials were analyzed to evaluate performance of given silicon probes acutely. 4) Cross-sectional area of multichannel silicon probes was found to be a crucial determinant of silicon probes' performance. EDGE style probes have the highest yields during cortical and intra-hippocampal recordings in pigs, with a potential for use in chronic implantations and awake behavior. The new CAMB 64-channel probes with EDGE style and a poly-2 site arrangement have an even better single unit separation and a denser sampling of the laminar structure, identifying them as potential candidates for chronic implantations. This study provides a comprehensive analysis of acute silicon probes designed for large animal laminar electrophysiology.

**Keywords:** large animals, pigs, laminar cortical and hippocampal electrophysiology, multichannel silicon probes.

## 1. Introduction

Oscillatory activity and corresponding power bands in the brain are different within layers of laminar structures as different inputs are driven at different frequencies depending on their area of origin [1-5]. Laminar analyses of oscillatory activity have proven valuable for analyzing circuit dynamics as well as changes between states in both cortex and hippocampus [5, 6]. Traditionally, single neurons have been analyzed independently on the basis of their tuning to sensory stimuli or movement. Although tuning curve approaches are unaffected by growing numbers of simultaneously recorded neurons, newly developed techniques that analyze interactions between neurons become more accurate and more complex as the number of recorded neurons increases [7]. For spike - field entrainment analyses, it is also important to know where inputs that drive the cells are arriving from and how they interact locally. Cell types, dendritic arborization, local and long-range axonal projections all distribute unevenly across layers, shaping the integration and segregation of neural signals. Ultimately, we must decode laminar information

across different structures and cortical regions to understand the spatiotemporal ensembles that represent cognition and memory [6].

The hippocampus is an example of a deep laminar structure, and is highly involved in encoding episodic memory formation presumably via spatiotemporal ensembles of neurons. Once multichannel silicon probes were developed, they quickly became a standard tool for hippocampal neurophysiological recordings in rodents. Increasing the density of laminar contacts in deep structures allows researchers to gain more information about these structures and more recently, study the potential effects of neurological disease states on single unit activity [8-10]. Although rodent cortical and hippocampal laminar structures were studied in great detail, the transition to large animal models has been slower. One of the major obstacles to addressing the research questions and approaches currently accessible to smaller-brained animals, are technical limitations in laminar silicon microelectrodes.

Much of the large animal brain is still inaccessible due to insufficient length of available silicon probes. In large animals and humans, the hippocampus is located deep inside the brain and is difficult to study electrophysiologically. Even many cortical targets cannot be reached with the current technology such as those typically utilized for brain machine interface (BMIs) such at the Utah electrode. In addition, laminar structure cannot be detected with a single wire or even tetrode recordings. Existing non-laminar solutions can scale to high density, but have single contacts at the tip, limiting the ability to differentiate single units as well as localize them within the laminar circuitry. These are therefore not suitable for understanding the relationships of neurons within laminar structures, as no information can be gathered about the relationships between neurons and their laminar inputs.

In order to reach deep regions of cortex and subcortical structures, silicon probes need to be long enough while still remaining narrow and balance flexibility to better match brain tissue properties to reduce gliosis and neuronal death. Technology necessary to produce finer features at longer length has only recently became available, including stitching across the die in the photomask process to utilize smaller features. New multichannel silicon probes designed for large animal electrophysiology allow for simultaneous recordings of fields and spikes from multiple layers of laminar structures such as cortex and hippocampus. In this study, we tested laminar silicon probes of various designs suitable for large animals and compared variables such as electrode size and coating material, probe thickness and width, and site arrangement and their effects on insertion ability, neurophysiological characteristics and biomechanical compatibility across probe designs.

## 2. Materials and Methods

Animals

Male Yucatan miniature pigs, which were chosen for their docile temperament, ease of handling, and slow growth rate for future chronic studies were used in this study. Pigs were purchased from Sinclair and underwent the current studies at the approximate age of 5 - 6 months at a mean weight of 38 ± 3 kg (n = 17, mean ± SEM). We chose this age as the pigs are post-adolescent with near-fully developed brains, but young enough to be of a manageable weight for procedures and behavior [11-13]. All pigs were pair housed when possible, and were always in a shared room with other pigs. All animal procedures were performed in accordance with the University of Pennsylvania animal care committee's regulations, an AALAC accredited institution.

Surgical Procedure

Yucatan miniature pigs were fasted for 16 hours then induced with 20 mg/kg of ketamine and 0.5 mg/kg of midazolam. Animals were intubated with an endotracheal tube and anesthesia was maintained

with 2-2.5% isoflurane per 2 liters $O_2$. Each animal was placed on a ventilator and supplied oxygen at a tidal volume of 10 mL/kg. A catheter was placed in an auricular vein to deliver 0.9% normal saline at 200 mL per hour. Additionally, heart rate, respiratory rate, arterial oxygen saturation, end tidal $CO_2$ and rectal temperature were continuously monitored, while pain response to pinch was periodically assessed. All of these measures were used to titrate ventilation settings and isoflurane percentage to maintain an adequate level of anesthesia. A forced air warming system was used to maintain normothermia throughout the procedure.

All animals in this study underwent electrophysiological recordings and analysis as described previously [14]. Briefly, pigs were placed in a stereotactic frame, with the surgical field prepped and draped, and a linear incision was made along the midline. A 13-mm diameter burr hole was made centered at 7 mm lateral to the midline and 4.5 mm posterior to bregma, and the bony opening was subsequently expanded using Kerrison punches. Skull screws were placed over the contralateral cortex as a ground and non-probe reference signal. The dura was opened in a cruciate manner and the dorsal hippocampus was first mapped with high-impedance Tungsten electrode (∅ = 125 μm, length = 60 mm, impedance = 0.5 MΩ; FHC, Cat# UEWSEGSEBNNM) in the sagittal plane, utilizing observed spiking activity to generate a two-dimensional map. We then chose coordinates for insertion of multichannel silicon probes based on the map such that the spread of electrode contacts would span the laminar structure of the dorsal hippocampus at its maximal thickness in the dorsal-ventral plane, perpendicular to the individual hippocampal layers. The cortical recordings were done in the same track on the way to dorsal hippocampus. At the end of the procedure the anesthetic level was deepened (to 5% isoflurane) and pigs were sacrificed for histological analysis to locate tracks generated by probe insertion.

Multichannel Silicon Probes

32-channel silicon probes were purchased from NeuroNexus (P-trode probes: catalog # P1x32-70mm-100-314-HP32, P1x32-70mm-100-314r-HP32, and P1x32-70mm-300-707-HP32; custom design Vector probe: catalog # V1x32-80mm-275-tet-177-HP32; and EDGE probe: catalog # V1x32-Edge-10mm-200-312-Ref) and ATLAS Neuroengineering (catalog # E32-35-S01T-L20.0). In addition, two novel 64-channel research probes (developed in a collaboration with SBM (Scientific & Biomedical Microsystems) and Cambridge NeuroTech) have either a linear or poly-2 style arrangement of sites for comparative purposes. The arrangement of the channels for all probes is shown in Scheme 1. Some silicon probes (P-trode w/ref, Vector, EDGE, and CAMB probes) were designed with one low-impedance channel placed 1-2 mm above the next most-proximal channel, which was used as a reference signal. For dorsal hippocampal targeting, this design usually provides 31 (or 63) channels for intra-hippocampal recordings and results in the reference channel being positioned within the temporal horn of the lateral ventricle dorsal to the hippocampus.

Neural Data Collection and Analysis

All electrophysiological recordings were made under isoflurane anesthesia (2 - 2.5 %). Neural signals were amplified and acquired continuously at 32 kHz on a 64-channel Digital Lynx 4SX acquisition system with Cheetah recording and acquisition software (Neuralynx, Inc.).

Spike Detection and Analysis: Signals acquired from the tungsten monopolar electrode during the mapping procedure were bandpass filtered (600 Hz to 6 kHz) and thresholded for spike detection in real time. Thresholds were chosen based on observed signal to noise ratios during the session. Recorded spike trains and waveforms then underwent off-line automated spike sorting using KlustaKwik software (http://klusta-team.github.io/klustakwik/, RRID: SCR_014480) and further manual refinements using SpikeSort3D (Neuralynx, Inc.). Neural signals acquired from the 31 channels on the silicon probe were bandpass filtered (0.1 Hz to 9 kHz) in real time prior to sampling. Off-line spike detection and sorting was performed on the wideband signals using the Klusta package (http://klusta-team.github.io/klustakwik/, RRID: SCR_014480), which was developed for higher density electrodes, and manually refined with

KlustaViewa or Phy (https://github.com/klusta-team/klustaviewa). The Klusta packages are designed to construct putative clusters from all probe channels simultaneously by taking advantage of spoke timing and the special arrangement of channels [15]. After manual refinement, resulting single-unit clusters were then imported into Matlab software, version R2017a for visualization and further analysis using custom and built-in routines (https://www.mathworks.com/products/matlab.html, RRID: SCR_001622). In order to minimize waveform shape distortion for visualization, the wideband signal was high pass filtered using a wavelet multi-level decomposition and reconstruction filter (level 6, Daubechies 4 wavelet) [16]. Waveforms were then extracted from this filtered signal and averaged for display. Spike trains were imported into NeuroExplorer software, version 6 for further analysis and to generate autocorrelograms and interspike intervals histograms (http://www.neuroexplorer.com/, RRID:SCR_001818).

Analysis of Local Field Potentials (LFP): Acquired wideband LFP recorded from all channels of the silicon probe were down-sampled to 3 kHz for further analysis. Signals were imported into Matlab software, version R2017a (https://www.mathworks.com/products/matlab.html, RRID: SCR_001622) and processed using a combination of custom and modified scripts from the freely available Matlab packages FMAToolbox (http://fmatoolbox.sourceforge.net, RRID:SCR_015533), Chronux (http://chronux.org, RRID: SCR_005547), and EEGLAB (http://sccn.ucsd.edu/eeglab/index.html, RRID:SCR_007292) [17, 18].

Tissue Handling and Histological Examinations

Histological analyses were performed on brain tissue from male Yucatan miniature pigs in order to locate tracks generated by electrodes' insertion. While under 5 % isoflurane anesthesia, all animals underwent transcardial perfusion with 0.9% heparinized saline followed by 10% neutral buffered formalin (NBF). After post-fixation for 7 days in 10% NBF at 4°C, each brain was dissected into 5 mm blocks in the coronal plane and processed to paraffin using standard techniques. 8 μm sections were obtained at the level of the hippocampus in either the coronal or sagittal planes. Standard hematoxylin & eosin (H&E) staining was performed on all animals to visualize electrode tracks. For Luxol Fast Blue / Cresyl Violet (LFB/CV) Staining, tissue sections were dewaxed in xylenes and rehydrated to water via graded ethanols before being immersed in 1% LFB solution (Sigma, S3382) at 60°C for 4 hours. Excess stain was then removed by immersion of sections in 95% ethanol. Differentiation was performed via immersion in 0.035% lithium carbonate for 10 seconds followed by multiple immersions in 70% ethanol until the gray and white matter could be clearly distinguished. Slides were rinsed and counterstained via immersion in preheated 0.1% CV solution (Sigma, C5042) for 5 minutes at 60°C. After further rinsing, slides were differentiated in 95% ethanol with 0.001% acetic acid, followed by dehydration, clearing in xylenes and cover slipping using cytoseal-60.

Statistical Analysis

The data was analyzed using Graphpad Prism software, version 7 (http://www.graphpad.com/, PRID: SCR_002798). Single unit waveforms recorded with silicon laminar probes are presented as mean ± SD. Impedance of silicon probes are shown as mean ± SEM.

## 3. Results

Performance of multichannel silicon probes was tested acutely in Yucatan pigs during cortical and hippocampal recordings under anesthesia as described previously [14]. We have acutely tested silicon probes from various manufacturers and evaluated these probes based on their ability to record laminar oscillatory and single unit activities in cortical and hippocampal deep structures. Silicon probes were also evaluated based on their ability to cleanly separate distinct single-units.

### *3.1. Silicon Probes for Laminar Recordings in Swine*

#### 3.1.1. Silicon Probe Design

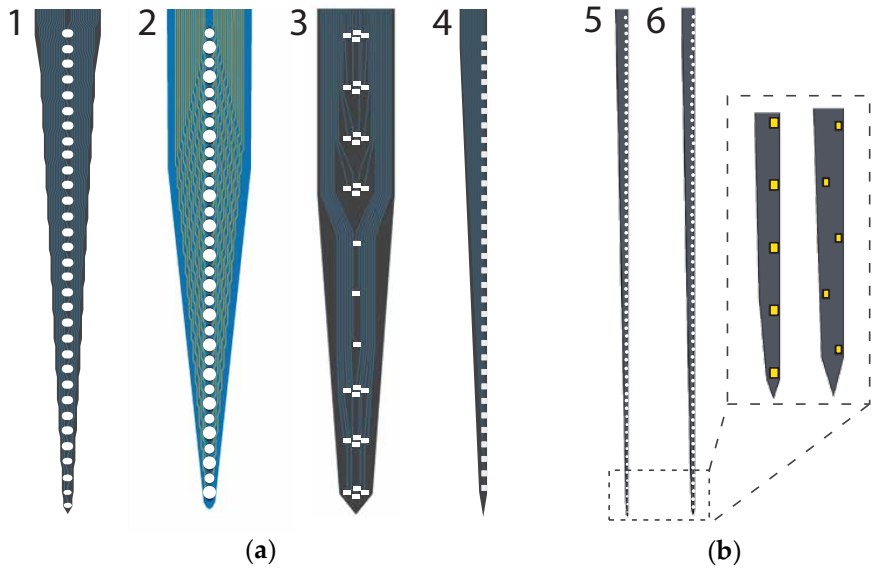
Specific parameters of multichannel probes tested in this study are summarized in Table 1. Later version of silicon probes used in the study (P-trode w/ref, Vector, EDGE, and CAMB probes) were designed to have one channel substituted for a low-impedance reference site, which is placed 1-2 mm above the most-proximal probe site (depending on the probe design). The individual probe sites consisted of iridium oxide (IrOx) on NeuroNexus probes, platinum (Pt) on ATLAS probes, and gold (Au) on CAMB probes.

**Table 1.** 32- and 64-channel silicon probes for acute cortical and hippocampal recordings in pigs.

| Silicon Probe | Layout | Spacing (μm) | Area (μm²) | Imp (MΩ) | Electrode Material | Thickness (μm) | Width (μm) |
|---|---|---|---|---|---|---|---|
| P-trode | linear | 100/300 | 707 | 1.61 | IrOx | 100 | 198 |
| P-trode (w/ref [1]) | linear | 100 | 314 | 1.61 | IrOx | 100 | 198 |
| ATLAS | linear | 35 | 962/491 [2] | 0.75 | Pt | 100 | 250 |
| Vector | tetrode + linear [3] | 275 [4] | 312 | 1.68 | IrOx | 50 | 225 |
| EDGE | edge [5] | 200 | 312 | 1.81 | IrOx | 50 | 80 |
| CAMB | linear | 100 | 165 | 0.06 | Au | 18 | 148 |
| CAMB | poly-2 [6] | 100 | 165 | 0.06 | Au | 37 | 154 |

[1] the most proximal site was substituted for a reference site with surface area of 4200 μm²; [2] sites with alternating diameters, Ø = 35 μm and Ø = 25 μm; [3] an arrangement of four electrode sites placed close together; [4] spacing between individual tetrodes (at first site) as well as between linear sites; [5] similar to the linear layout, but electrode sites are strategically positioned at the edge of the substrate; [6] similar to the linear layout, but electrode sites are off-set by 21 μm relative to each other.

Multichannel silicon probes used in this study had a total of either 32 or 64 electrode sites and can be divided into sub-categories based on the site arrangement (Scheme 1). Most of the multichannel silicon probes used in pig hippocampal recordings were of linear design to resolve laminar structure (P-trode, ATLAS, EDGE and CAMB probes). For dorsal hippocampal targeting, this design provides 31 (or 63) channels for laminar recordings and results in the reference channel being positioned within the temporal horn of the lateral ventricle sitting just above the hippocampus (not shown).




**Scheme 1.** Design of multichannel silicon probes used for hippocampal recordings in pigs under anesthesia. **(a)** The individual site arrangement for 32-channel silicon probes is shown for (1) P-trode, (2) ATLAS, (3) Vector, and (4) EDGE type probes. Some probes (P-trode w/ref, Vector, and Edge) are designed to include a reference site placed 1-2 mm above the most-proximal electrode (not shown); **(b)** The individual site arrangement for CAMB 64-channel silicon probes is shown for (5) linear and (6) poly-2 type probes. The poly-2 design is similar to the linear design but electrode sites are off-set by 21 μm relative to each other (see insert). Both 64-channel probe types have a reference site placed 1.75 mm above the most-proximal electrode site (not shown). Drawings are not to scale, and are utilized only to depict site locations/arrangement (i.e. 4, 5, and 6 are twice as long as 3.)

Detailed description of multichannel silicon probes used acutely in pig cortical and hippocampal recordings are listed below:

1. P-trode style 32-channel probes have linear site arrangement, with site spacing of either 100 μm or 300 μm (Scheme 1a, panel 1). A later version of this silicon probe (P-trode w/ref) has an internal reference (surface area = 4000 $\mu m^2$) placed 1 mm above the most-proximal channel of the probe (not shown). The total coverage of P-trode recording sites are: 3200 μm (100 μm site spacing), 9600 μm (300 μm site spacing), and 3100 μm (100 μm site spacing with reference). The individual sites are iridium oxide (IrOx) composition and have an impedance of 1.61 ± 0.15 MΩ (mean ± SEM, $n_{probes}$ = 14).
2. ATLAS style 32-channel probes have linear site arrangement, with 35 μm site spacing (Scheme 1a, panel 2; $n_{probes}$ = 1). The total coverage of ATLAS recording sites is 1120 μm. The individual sites have an alternating diameter of either 35 μm or 25 μm and are platinum [19]. The impedances measured on these probes are: 0.75 ± 0.02 MΩ (mean ± SEM, $n_{sites}$ =16) for a big site (Ø = 35 μm) and 1.04 ± 0.04 MΩ (mean ± SEM, $n_{sites}$ =16) for a small site (Ø = 25 μm).
3. Vector style 32-channel probes a have custom designed site arrangement with most of the individual sites arranged in groups of four closely spaced sites or tetrodes (Scheme 1a, panel 3). Vector style probes have an internal reference (surface area = 4200 $\mu m^2$) placed 2 mm above the most-proximal site of the probe (not shown). During hippocampal recordings, the top four tetrodes are placed in pyramidal CA1 layer, while the bottom three tetrodes are placed in granular cell layer. Three linear sites in between groups of tetrodes are added to cover pig hippocampal layers of strata radiatum, lacunosum-moleculare and moleculare. The top site on each tetrode along with linear sites are placed

275 μm to form 10 equally spaced sites for laminar hippocampal recordings. The total coverage of Vector recording sites is 2750 μm. Individual sites are iridium oxide (IrOx) and have an impedance of 1.68 ± 0.20 MΩ (mean ± SEM, $n_{probes}$ = 13).

4. EDGE style 32-channel probes have a linear site arrangement, with 200 μm site spacing. The individual sites were strategically positioned at the edge of the substrate (Scheme 1a, panel 4). The total coverage of EDGE recording sites is 6200 μm. Edge type probes have an internal reference (surface area = 1400 $\mu m^2$) placed 1.5 mm above the most-proximal site of the probe (not shown). Edge probe sites are iridium oxide (IrOx) and have an impedance of 1.68 ± 0.20 MΩ (mean ± SEM, $n_{probes}$ = 13).
5. CAMB style 64-channel probes with a linear site arrangement have 100 μm site spacing (Scheme 1b, panel 5; $n_{probes}$ = 1). MTEC-64 linear probes have an internal reference (surface area = 1404 $\mu m^2$) placed 1.75 mm above the most-proximal site of the probe (not shown). The total coverage of MTEC recording sites is 6300 μm. Probe sites are gold (Au) and have an impedance of 0.063 ± 0.001 MΩ (mean ± SEM, $n_{sites}$ = 63).
6. CAMB style 64-channel probes with a poly-2 site arrangement have 100 μm site spacing and 21 μm off-set (Scheme 1b, panel 6; $n_{probes}$ = 1). MTEC-64 poly-2 probes have an internal reference (surface area = 1404 $\mu m^2$) placed 1.75 mm above the most-proximal site of the probe (not shown). The total coverage of MTEC recording sites is 6300 μm. Probe sites are coated with gold (Au) and have an impedance of 0.064 ± 0.001 MΩ (mean ± SEM, $n_{sites}$ = 63). Smaller feature of CAMB silicon probes are achieved with a process of high-resolution projection (stepper) lithography of 0.5μm resolution.

### 3.1.2 Laminar Structure is Revealed by Site Spacing

The laminar structure of pig dorsal hippocampsus was examined electrophysiologically with various designs of silicon probes (Table 1). Laminar structure of the dorsal hippocampus shown in Figure 1 was recorded with Edge type silicon probe (Scheme 1a, panel 4). The corresponding depths for each layer are: alveus (A) = 173.8 ± 11.55 μm, oriens (O) = 178.3 ± 15.01 μm, pyramidale (P) = 213.2 ±15.9 μm, radiatum (R) = 394.1 ± 22.36 μm, lacunosum-moleculare (L-M) = 223.1 ± 11.7 μm, moleculare (M) = 335.1 ± 17.85 μm, granulosum (G) = 81.3 ± 4.5 μm (see [14]). Since the hippocampal region of interest (at our standard recording coordinates in medial-lateral (ML) and anterior-posterior (AP) planes) is about 1600 μm, a silicon probe with 100 - 200 μm site spacing will provide good coverage for the laminar structure with enough resolution to identify every layer (Figure 1). While the P-trode type silicon probe with 300 μm could be potentially used to examine the whole dorsal hippocampus (total length = 9600 μm), some of the layers could be easily missed (such as L-M). Similarly, while the ATLAS type silicon probe with 35 μm site spacing could be potentially used to analyze some hippocampal layers in more detail, it does not provide enough coverage to cover all hippocampal layers (total length = 1120 μm).

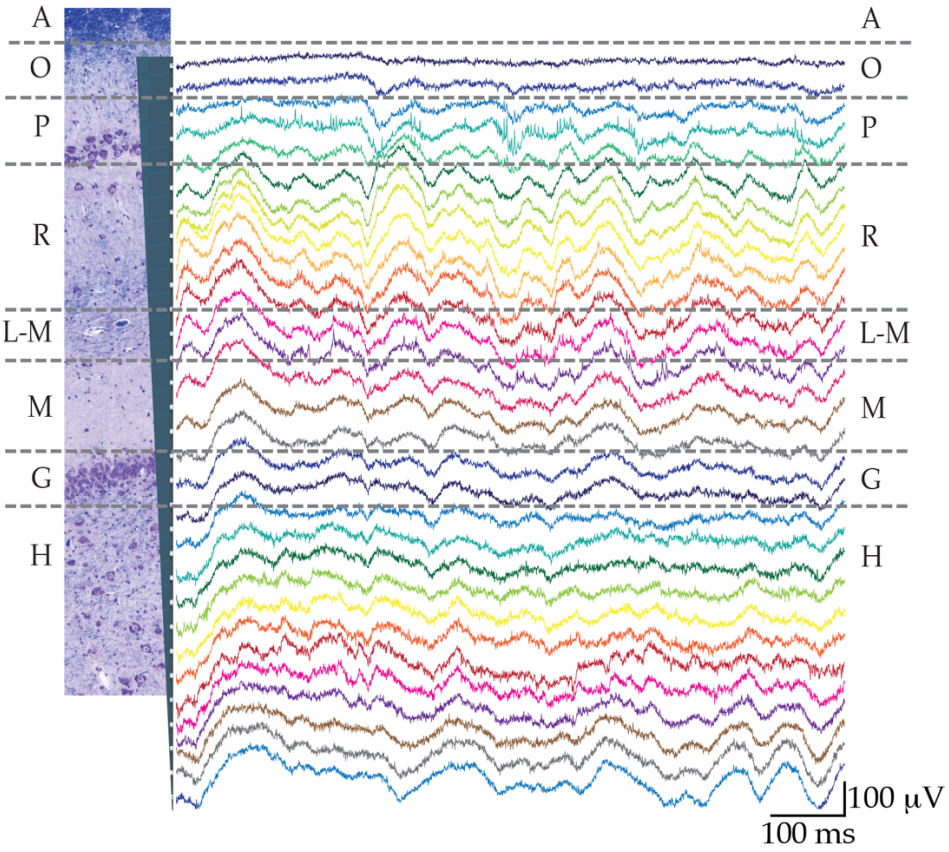



**Figure 1.** Laminar structure of pig hippocampus. Recording with a linear EDGE style probe (200 μm site spacing) was done under isoflurane anesthesia. Silicon probe placement within hippocampal layers of stratum oriens (O), pyramidale (P), radiatum (R), lacunosum-moleculare (L-M), moleculare (M), granulosum (G), and hilus (H) are first identified electrophysiologically and then later confirmed histopathologically. Representative histopathological section is overlaid on laminar oscillatory activity to match representative layers as described previously [14].

### 3.1.3. Internal Reference Reduces Noise During Laminar Fields Recordings

Designing the probes with an internal reference for hippocampal recordings may help to reduce noise conferred from the electrical environment by using an electrically inactive but conductive structure such as the CSF of the ventricle in contrast to ECoG style skull screws. To estimate how an introduction of the internal reference affects the noise during hippocampal recordings, local field potetnial (LFPs) were recorded with EDGE style probe similar to the experiment displayed in Figure 1. Power specrum density (PSD) was then calculated at the lavel of pyramidal CA1 layer (Figure 2). During the same recording, the signals were first referenced to the skull screw similar to standard referencing protocol used in rodent and human recordings (Figure 2, red line). Afterwards, the internal reference on Edge probe was used, which eliminated most of the slow "drift" oscillations as well as 60 Hz frequencies presumably from AC noise (Figure 2, blue line). Frequencies in 120-300 Hz band (Figure 2) and above were not affected. Since single

unit activity usually lies within 600 – 6,000 Hz frequency band, the choice of skull vs. internal reference will not affect the single unit recordings.

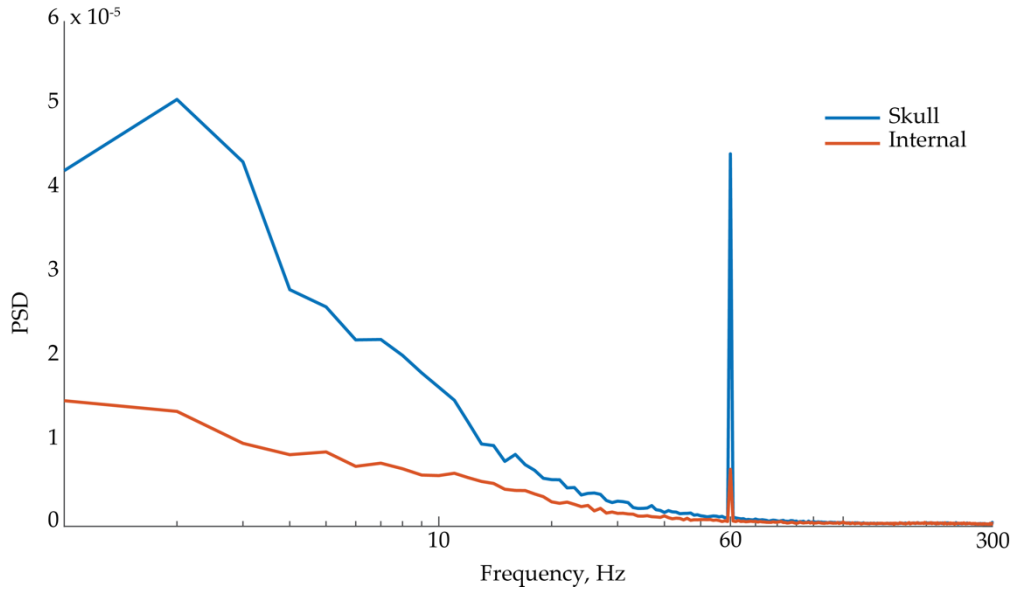


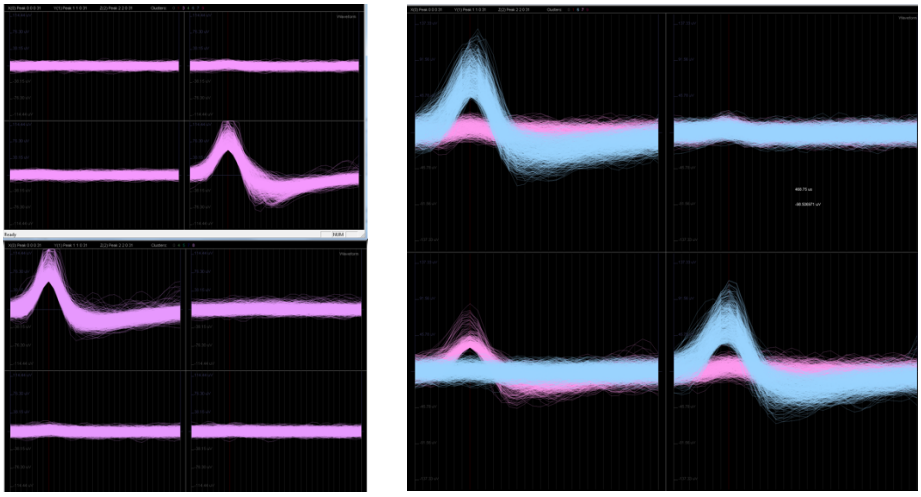

**Figure 2.** Noise reduction with the internal reference on silicon probe. During the same recording, the signals were first referenced to the skull screw similar to standard referencing protocol used in rodent and human recordings. Power Spectral Density (PSD) of the channel presenting stratum radiatum was calculated in 2-300 Hz frequency band and then normalized by the its peak value and displayed in logarithmic scale (Figure 2, blue line). Afterwards, the reference on EDGE style probe was switched to the internal reference. PSD was again calculated and normalized by the its peak value (Figure 2, red line). The internal reference eliminated most of the slow "drift" oscillations as well as most of the noise produced in 60 Hz-cycle frequencies (the peak at 60 Hz).

### 3.1.3. Sites Arrangement for Single Unit Detection and Sorting

In planar silicon probes, the individual electrode sites are placed on a face of the designs (P-trode, ATLAS, and Vector probes). In an attempt to decrease the damage near electrode sites and increase the exposure of the electrode sites to the parenchyma, we implemented a probe with EDGE design (Scheme 1a, panel 4). In contrast to P-trode and Vector style probes, the individual electrode sites on Edge probe are strategically positioned on the edge of the substrate, potentially reducing the interference of the insulator with the surrounding signals.

To characterize the laminar structure of the pig hippocampus, silicon multichannel probes with a linear design were used throughout the study. While the linear design is fit for a wide range of applications, isolation of single unit activity may be difficult with only a linear site arrangement. To utilize commercial spike sorting software such as SpikeSort 3D (Neuralynx), individual sites on silicone probes with a linear design were artificially grouped into sets of four (tetrodes) and then sorted. Single units recorded with 32-channel probes of the earlier designs (P-trode, Scheme 1a, panels 1; and ATLAS, Scheme 1a, panels 2) could not be properly isolated with spike sorting software available at the time (Figure 3a), partially due to potential overlap between putative units between the artificial "tetrodes" (Figure 3a). A single unit (pink) was recorded on two adjacent sites, which got placed into separate tetrodes (the last site of top tetrode and

the first site of bottom tetrode). As a result, this single unit was counted twice, creating a duplicate unit. To address this issue, we designed the Vector probe with custom arrangement of electrode sites (Scheme 1, panel 3). Most of the individual sites were arranged in four sites placed close together (tetrodes), allowing for high-quality cell discrimination in hippocampal recordings, with extra sites in between groups of tetrodes in order to maintain the laminar analyses. Four tetrodes were placed in pyramidal CA1 hippocampal layer (top part of the probe), while three tetrodes were placed in granular cell layer (bottom part of the probe). Single units recorded with Vector style probes and sorted with Spike Sort 3D software are shown in Figure 3b. The same unit could be registered on multiple sites of the same tetrode (blue color, first and last site) to facilitate spike sorting. As Vector style probes were created with the individual sites arranged closely to form tetrodes, more single units were isolated but cross-over of the units onto neighboring tetrodes were still observed occasionally in the CA1 layer due to the large size and dendritic arbor of these neurons (not shown). Since the advent of laminar spike-sorting software [15] the necessity no longer exists for specific tetrodes to be recreated in the silicon probes, however probe geometry is still an important part of the spike-sorting process (see section 3.2.3 below).



(**a**) (**b**)

**Figure 3.** Sites Arrangement Defines Single Unit Separation. Single units were sorted with Spike Sort 3D software available from Neuralynx at the time. (**a**) Single units recorded with linear P-trode type probe (300 mm site spacing). A single unit (pink) was recorded on two adjacent sites, which got placed into separate tetrodes (the last site of top tetrode and the first site of bottom tetrode). In result, this single unit was counted twice, creating a duplicate unit. (**b**) Single units recorded with tetrode type Vector probe. The same unit is registered on multiple sites of the same tetrode (blue color, first and last site) which facilitates spike sorting by increasing the probability of isolating clusters.

### 3.1.4. Electrode Material Affects Spike Shape

To compare signal-to-noise quality as well as single unit separation, we compared standard electrode material irridium oxide (IrOx) used in rodent silicon probes with platinum (Pt) electrode material used in ATLAS style probes. Vector and ATLAS probes were tested acutely in the same animal by consequitive insertion in the cortical column at the same distance from the brain surface (dorsal-ventral (DV) coordinates). Single units were recorded and then sorted with Spike Sort 3D software for either IrOx Vector

probe (Figure 4a) or Pt ATLAS probe (Figure 4b). While single units of various distinct shapes were sorted for Vector probe (Figure 4), all single units sorted for ATLAS probe did not have the standard action potential shape, suggesting a filtering effect for higher frequencies (Figure 3b). There was also a lower number of units picked up during recording with ATLAS probe, potentially due to the lower signal to noise ratio. The total of 45 single units were recorded and sorted in cortex and hippocampus (in the same one animal), with 18 single units on big electrode sites (Ø = 35 μm) and 27 small electrode sites (Ø = 25 μm) picking up unit activity. The size of an individual site doesn't appear to affect recordings of single unit activity as the ratio of units registered with both big and small sites in the same recording was about the same (40% and 60 % respectively).

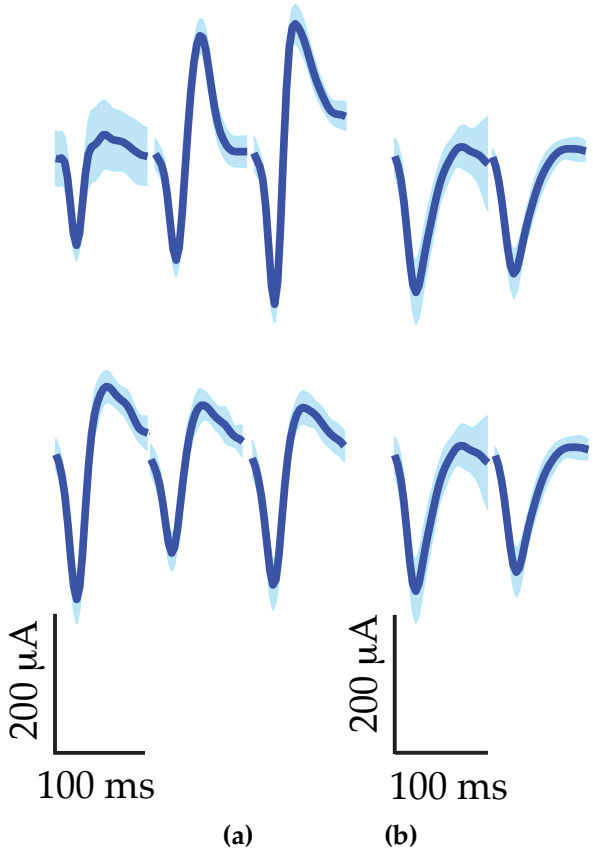


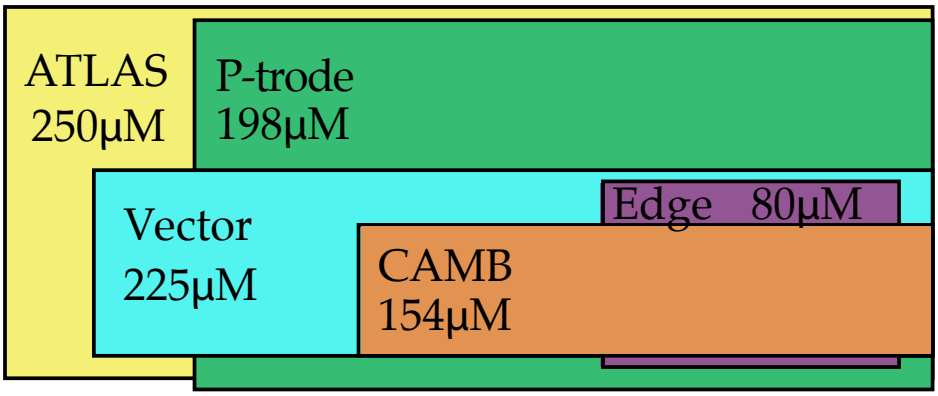

**Figure 4.** Site coating affects a shape of single units (displayed as mean ± SD). *S*ingle units were recorded and sorted with Spike Sort 3D for: (**a**) Vector probe coated with standard IrOx composition; and (**b**) ATLAS probe coated with Pt composition.

### *3.2. Silicon Probes Dimensions and Technical Advances*

#### 3.2.1 Width and Thickness of Silicon Probes Defined by Production Methodology

The available silicon probe technology for large animals suffers from the limitations of low-resolution lithography, which determines the size and spacing of the lines (approx. 4μm features) and thereby limits the number of sites and increases the size of the probes (typical thicknesses of 50-100 μm and widths of 225-250 μm; Scheme 2). NeuroNexus large animal depth probes are 50μM thick, stiff and brittle, thus increasing insertion force but making them prone to fracturing during handling and decreasing yields beyond 10-15mm. This necessitated a novel interface cable solution whereby a cable is threaded through a 250μm stainless steel tube and bonded to the contacts at the end of the probe, allowing the probes to be

produced in lengths great enough to reach any part of the large animal brain. In addition, 32 individual sites are the current upper channel limit with these probes using contact lithography while maintaining reasonable widths. In order to produce longer silicon probes without the need for the extension tube, novel CAMB 64-channel flexible silicon probes are produced with newer lithography techniques with 1-4μm resolution features, and utilizing a new process that allows for stitching across the stepper lithography dies. This technology allows for a doubling of the number of sites on the probe, leading to the first 64-channel probes for large animals that are made exclusively from silicon wafer (Scheme 2, orange panel). Additional testing revealed that mock CAMB style probes with 35 μm thickness and 80 μm width (not shown) could be inserted into large animal brain with an 80μm width, giving a minimum dimension for the probes as stiffness increases linearly with width.




**Scheme 2.** Schematic comparison of cross-sectional area of silicon probes. The corresponding widths are 250 μm (ATLAS probe, yellow), 225 μm (Vector probe, blue), 198 μm (P-trode probe, green), 168 μm (CAMB probe, orange), and 80 μm (Edge probe, purple). The thickness of silicon probes is 100 μm (for ATLAS and P-trode probes), 50 μm (for Vector and Edge probes), and 35 μm (for CAMB probe).

Increasing a probe's width imparts greater mechanical stiffness of the silicon probe, which benefits insertion, but causes a further compliance mismatch between probe and brain tissue, potentially contributing to gliosis and local cell death for chronic implementations. By reducing the tissue impact during surgery, smaller, more flexible silicon probes may increase the survival rate of neurons in close proximity to the travel path.

### 3.2.3 CAMB 64-channel Silicon Probes

We also compared single-unit separation between a linear 64-channel CAMB probe and a 64-channel CAMB probe with a poly-2 design, which is similar to the linear layout, but electrode sites are off-set by 21 μm relative to each other (Scheme 1b). For the single units on the laminar probe, modern laminar spike sorting methods that classify units based on waveforms across all channels on the probe simultaneously (i.e. Klusta) lead to clusters with poorly defined classic separation measurement thresholds (i.e. L-ratio, Mahalanobis distance) for higher dimensions than tetrodes [15, 20] To answer an open question whether non-linear probe designs are necessary for true resolution of single units using modern software such as Klusta/PHY, CAMB silicon 64-channel type probes were inserted in the pig cortex. Single units were recorded with CAMB 64-channel probes of either linear (Scheme 1b, panel 5) or poly-2 (Scheme 1b, panel 6) design, 30mm in length. Single units sorted with PHY software for both designs are shown in Figure 5.

For single unit separation, the geometry of the poly-2 design (Figure 5a) appears to better separate single cells compared to the linear design (Figure 5b) and is quantified below.

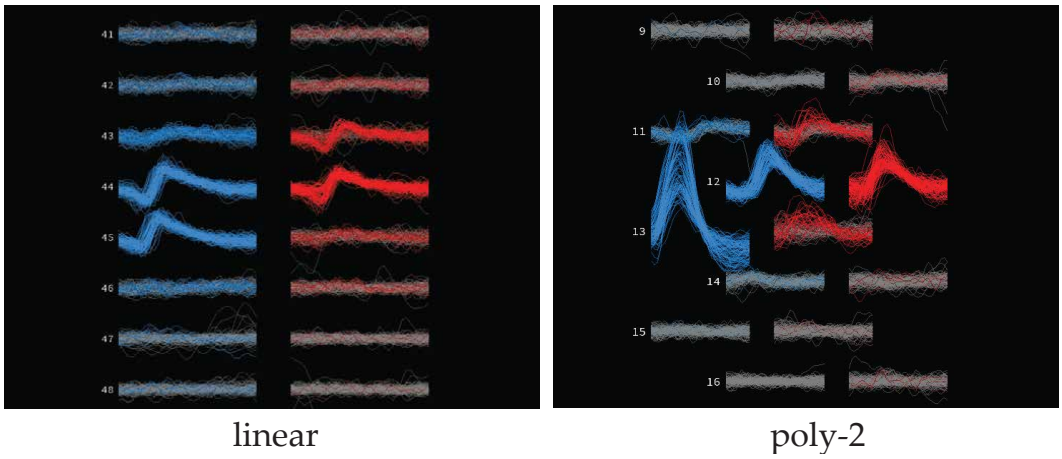



**Figure 5.** Single units were isolated for a linear and a poly-2 designs CAMB 64-channel silicon probes. A 21 μm off-set on a poly-2 design CAMB probes (right panel) helps to better separate single units (red and blue) than a linear design CAMB probe (left panel). Note that the blue unit on the poly-2 style probe could have easily been classified with the red unit had the offset geometry not revealed the higher amplitude action potentials at the same time stamps.

Many separated clusters from the CAMB linear 64-channel probe recording contained multiple units, which were not well isolated from each other with current spike sorting methods. While some clusters from CAMB 64-channel poly-2 design probe contained multiple units, the proportion of multi-unit clusters was less when compared to the linear design (33% and 54% respectively) as shown in Figure 6.

**Figure 6.** Cortical units were isolated for recordings with CAMB 64-channel style probes of linear and poly-2 design. (**a**) Number of single units (green, n = 17) vs. multi-units (red, n = 20) sorted for the linear design (37 units total); (**b**) While some clusters the poly-2 design probe contained multiple units (red, n = 24), two

times more single units (green, n = 48) were identified. Also, the proportion of multi-unit clusters for poly-2 design was less when compared to the linear design (33% and 54% respectively).

## 4. Discussion

In this study, we implemented acute *in vivo* electrophysiology to characterize multichannel silicon probes from various manufacturers, and compared designs of a new higher density research probe. Silicon multichannel probes designed and used for large animal cortical and hippocampal electrophysiology allow for a longer area of coverage in contrast to single site recordings with tungsten electrodes, and also allowing for a slight drift in electrode placement in case of chronic implantations. They may also replace the need for multiple-insertion experiments if the animal is chronically implanted and/or a drive is utilized. Most of our silicon multichannel probes were designed with an internal reference to improve signal-to-noise ratio while leaving physiological slow oscillation like alpha and theta rhythms intact. In addition to having an extra reference signal to utilize or switch to in case of reference failure, it can also be used to compare the signals to the signals recorded with standard skull reference in rodent and human studies.

We tested various designs of silicon probes available from different manufacturers for a quality of single unit isolation. Linear, tetrode plus linear and poly-2 designs arrangement design were evaluated for single unit vs. multi-unit cluster isolation. Although most of the probes with a linear design site arrangement (P-trode, ATLAS, and EDGE style probes) were able to record neuronal activity of individual neurons, silicon probes with a tetrode and a poly-2 design site arrangement had better cluster separation with currently available sorting algorithms, as the proportion of single units to multi-units recorded with a given probe increased. While electrode material should be carefully considered as some materials affect shape of single units suggesting a filtering effect for higher frequencies, the site size did not appear to influence the number of single units recorded. Previous acute examinations in large animals have also noted the usefulness of the parallel geometry in isolating units, even prior to the advent of new sorting techniques [21].

We have also identified the advantages of minimizing probe dimensions such as shank width and thickness while increasing channel counts. Since the current commercially available probe technology employs contact lithography, it sets the resolution of patterned features to 4 μm and limits the number of electrode sites on the probe without increasing widths to those that will create impediments to long-term chronic recordings. Research probes described here (CAMB 64-channel) have been designed with maximal taper at the active electrode sites as well as placing at least one row at the edge of the probe is has been accomplished with the NeuroNexus EDGE probe. Application of newer photo-lithography techniques decreases the width/electrode ratio, minimizes local tissue damage, and increases the length that silicon probes can be produced for large animal work. Neuropathological examination of CAMB mock probes demonstrated that thinner probes produced less neuropathology, which is important point to consider for chronic probe implantations. Although a key benefit of long thin shanks is that they offer mechanical flexibility, thereby minimizing the mechanical compliance mismatch with the brain, long probes must remain stiff enough to penetrate the brain. By testing CAMB 25mm-long probes, we showed that 35μm shank thickness for 80μm wide probes strikes an ideal compromise between ease of tissue penetration and appropriate targeting vs. the need to maintain small device dimensions. This allowed for production and testing of the 30mm long 64-channel probes described above, which yielded substantially more units in the poly-2 style probe.

## 5. Conclusions

This study is a comprehensive survey of multichannel silicon probes designed for large animal electrophysiology available for use, as well as comparing them to a new design and process. NeuroNexus EDGE style probes were determined to be ideal commercially available probes for acute recordings from laminar structures due a linear site arrangement, 6mm of coverage, and the highest single-unit yields, with a potential for use in chronic implantations and awake behavior. In addition, cross-sectional area was found to be a crucial determinant of silicon probes' performance. Novel CAMB 64-channel probes with a poly-2 design were found to have an even better single unit separation and a denser sampling of the laminar structure than linear probes. By increasing channel density, we were able to better visualize laminar structure and to reduce tissue damage from probe insertion, identifying them as potential candidates for chronic implantations. Channel density, site arrangement, and physical profile of the silicon probe are all important factors to consider when designing probes for acute and chronic implantations to study laminar structures over time in future awake behaving animals. These results should be confirmed and expanded in chronic preparations, as it is likely that acute unit visibility immediately (<12 hours) post insertion is not predictive of chronic neural interface response and recovery post implantation. We hope these results will lower the threshold for adoption in acute and future chronic implantations, by demonstrating consistent yields in laminar electrophysiological recordings in large animals using commercially available probes, leading to new discoveries in both hippocampal and neocortical neurophysiology. In addition, better detection and understanding of laminar circuitry and changes in human disease (i.e. epilepsy, traumatic brain injury) are needed, and therefore viable electrodes need to be tested first in translational large animal models.

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