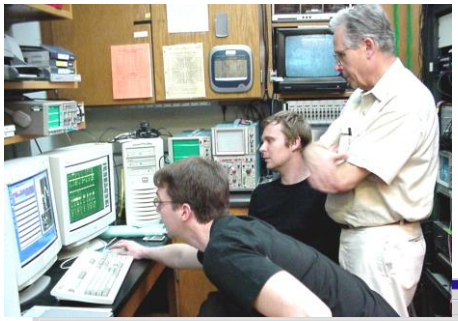
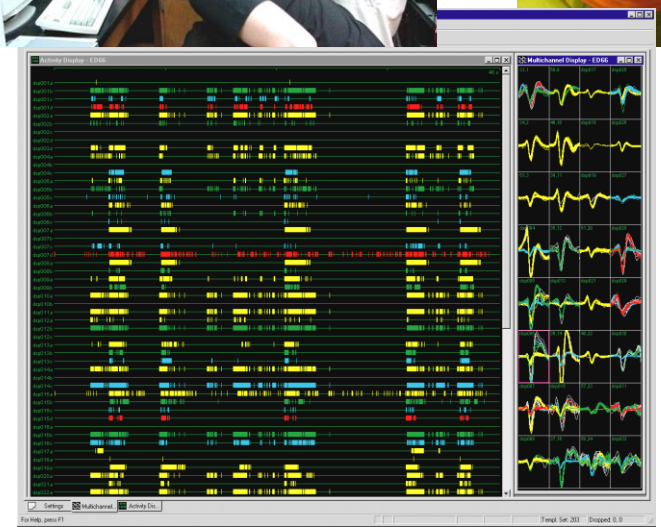
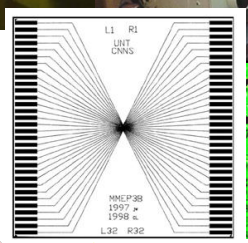
CNNS

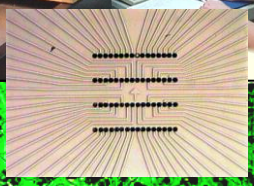
Center for Network Neuroscience

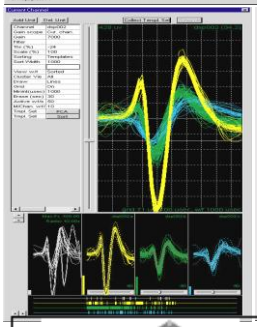
UNIVERSITY of NORTH TEXAS

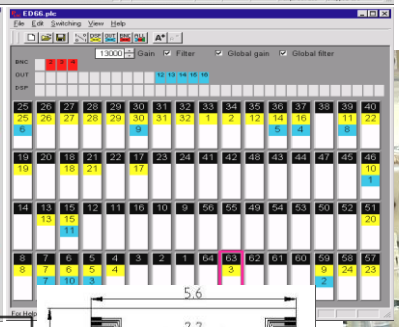


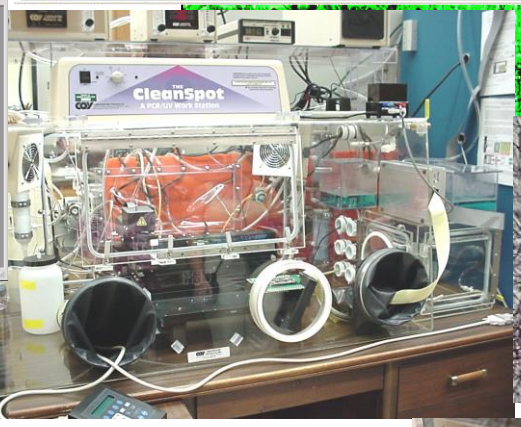


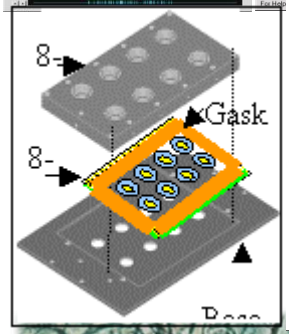


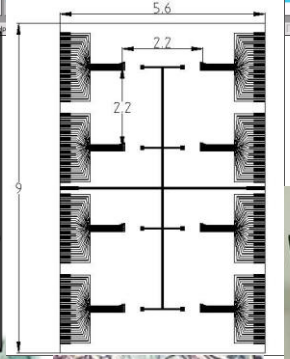


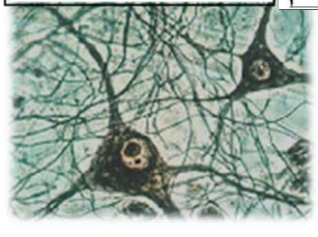


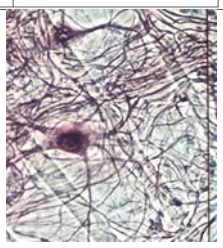


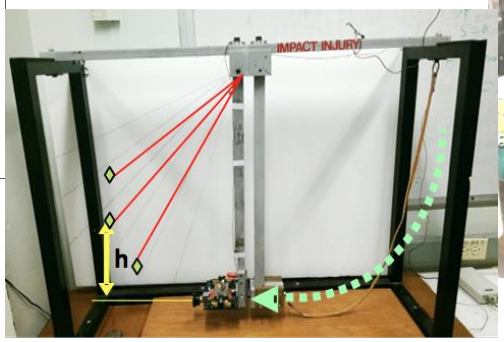


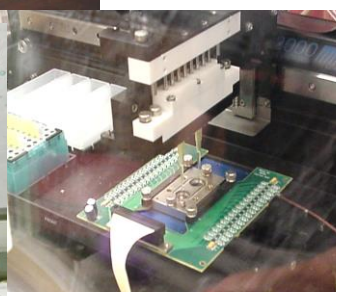












Version 2.23.17

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Perspective from the CNNS Director

Recent advances in growing electrically active cells on substrate-integrated, thin-film microelectrode arrays in cell culture have led to experimental systems in which spontaneous activity can be monitored by 64 or more electrodes for periods of time extending to six months. These systems are accessible pharmacologically and have shown high sensitivity to metabolically active, neuroactive, and neurotoxic compounds. For primary neuronal cultures, tissue-specific responses are now routinely observed, suggesting that cultured networks share the pharmacological sensitivity profiles of the parent tissue. Although the number of cells, the ratios of different cell types, and detailed circuit structure may vary among cultures, pharmacological responses are robust and highly reproducible. Such networks offer new assay and sensing systems that lie between biochemical and whole animal experiments and provide rapid, quantitative information on neurophysiological responses to chemicals and toxins.

MEA designs have matured and now provide highly reliable support for routine experiments using nerve cells in culture. The large numbers of electrodes and stable cell-electrode coupling that can be attained provide massive data on the internal dynamics of such networks. Given the experimental progress in pharmacology, the road to meaningful applications in drug development, industrial and environmental toxicology, and the domain of tissue-based biosensors is now open. Future challenges are shifting to multinetwork platforms for parallel (high throughput) recording, as well as to more sophisticated multichannel data analysis, display, and interpretation. The same methodology can also be applied to other electrically active tissues derived from glands (e.g. pancreas), heart, and muscle.

The first step in the organization of central nervous system function occurs at the small nerve cell ensemble level, yet we know little about origins of pattern generation and rules governing pattern processing dynamics. Perhaps the greatest contributions MEA technology can make will be in the area of information processing strategies and mechanisms. Given the substantial progress in recent years, nerve cells *in vitro* can no longer be considered “different” from nerve cells *in vivo*, at least pharmacologically. With further progress, networks *in vitro* will also be accepted as functional, dynamic entities that can provide unique and highly useful information on a whole spectrum of unknown mechanisms. Here, the simultaneity of information and long-term monitoring that is offered by MEA technology and methodology will prove to be crucial. *To me, it is self-evident: We will not understand information processing in the brain until we understand pattern processing in small nerve cell networks.*

Finally, applications to neurological diseases and trauma have been initiated. In these research domains, methods that provide functional electrophysiological signatures in parallel with morphological observations will provide unique insights into network activity changes linked to cellular and subcellular damage.

Guenter W. Gross, Ph.D.; Director, CNNS

CNNS Advisory Board

(as of September 2013)

LOCAL

Jeff Hamer	President, *Asset Direction Incorporated*, *jmhamer@gmail.com*
Dr. Ernest Moore	Chair, Dept. of Speech and Hearing Sciences, UNT *ejmoore@unt.edu*
Tahir Rana (PhD/MD)	Medical Director, Cancer Care of North Texas *cancercareofnorthtexas@yahoo.com; trana16@yahoo.com*
Dr. Fritz Schwalm	Chair, Biology Department, TWU (Emeritus) *fritzeschwalm@verizon.net*
Dr. Joseph Pancrazio,	Vice Provost & Professor, Department of Biomedical Engineering, UTD *Joseph.pancrazio@utdallas.edu*

INTERNATIONAL

Dr. Enric Calverol-Tinture	Director and CEO, Afferent Technologies 2016 - UAB Research Park, Eureka Building, 08193 Bellaterra, Barcelona *eclaverol@eclaverol.com*

Short Resumes

Enric Claverol-Tinturé,

Dr. Claverol-Tinturé has industrial and academic experience in the area of biomedical technology, as CEO of a bioinstrumentation startup and head of Neuroengineering at the Catalonia Institute for Bioengineering (IBEC). He was scientific coordinator of two international projects funded by the European Commission in the area of neural engineering and frequent evaluator of EU R&D programs. He is presently the CEO of Afferent Technologies 2016 - UAB Research Park, Eureka Building, 08193 Bellaterra, Barcelona. His Business Administration and Venture Capital training took place at ESADE and Berkeley Haas Business Schools, having previously carried out research at Caltech and Los Alamos National Laboratory. Dr. Claverol-Tinturé holds a PhD by the University of Southampton, United Kingdom.

Jeff Hamer, President of Asset Direction Incorporated.
Asset Direction Inc. is asset management consultancy and General Partner of a small private market-neutral equity hedge fund. Previously he co-founded and was CEO of *CADG*, venture-backed producer of enterprise software marketed worldwide including *IBM*-logo, used by a majority of the largest *Fortune 500* firms. Mr. Hamer was Assistant Professor in the Graduate School of Architecture and Urban Planning at *UCLA*, as well as teaching invited courses worldwide including Harvard and MIT. He is author of *Facility*

Management Systems (Van Nostrand Reinhold, New York) and winner of the *International Facility Management Association's "Distinguished Author Award"* as well as hundreds of published technical and managerial papers and invited keynotes.

Dr. Ernest J. Moore, Professor & Chair, Dept. of Speech and Hearing Sciences, UNT.
Dr. Moore earned a B.S. in Speech-Language Pathology and Audiology at Tennessee A. & I. State University (Nashville), a M.A. in Audiology from Northern Illinois University (DeKalb), and a Ph.D. in Communicative Disorders, with a specialization in Auditory Electrophysiology from The University of Wisconsin (Madison). Dr. Moore has received numerous awards, fellowships, and grants, including those from the National Institutes of Health, Deafness Research Foundation, the National Science Foundation, the Montel Williams MS Foundation, and the Once Upon a Time Foundation. Dr. Moore came to UNT from the Medical School at Northwestern University in Chicago, where he held an appointment of Research Professor and Knowles Scholar in the Department of Molecular Pharmacology and Biological Chemistry/Center for Drug Discovery and Chemical Biology. Dr. Moore's current research interest lies in the investigation of molecular ion channel activity that might underlie tinnitus.

Joseph J. Pancrazio*, *Professor, Department of Biomedical Engineering and Vice Provost University of Texas at Dallas
Before coming to UTD in 2015, Dr. Pancrazio served as the chair and founder of the Biomedical Engineering program at George Mason University. Prior to this appointment, Dr. Pancrazio was director for neural engineering and the neural prosthesis program at the National Institutes of Health, and served as laboratory head and principal investigator at the US Naval Research Laboratory where he developed and demonstrated cellular and neuronal based biosensor platforms. His undergraduate degree was in electrical engineering from the University of Illinois, followed by his masters of science and PhD degrees in Biomedical Engineering from the University of Virginia. Dr. Pancrazio has authored over 90 peer reviewed publications and holds two patents. His research interests include advanced materials for neural interfaces, neural stem cell electrophysiology, and neuropharmacological assays.

Tahir M. Rana, M.D., Ph.D., D.A.B.R. - Radiation Oncologist
Dr. Rana is the medical director of Cancer Care of North Texas. Receiving his medical degree from Bahaauddin Zykryia in Multan, Pakistan, Dr. Rana was awarded a Medical Research Council Fellowship and received his Ph.D. in tumor immunology from the University of Cambridge, Darwin College in the United Kingdom. He completed a bone marrow fellowship at the University of Nebraska and a residency in oncology at Wayne State University, Michigan. Having served as a guest speaker at many institutions, Dr. Rana has a special interest in the areas of prostate, breast and lung cancer treatment. He specializes in IMRT techniques for the delivery of radiation therapy in the treatment of cancer. He is board certified in radiation oncology by the American Board of Radiology.

Dr. Fritz E Schwalm, Prof. emeritus, Texas Woman's University
Dr. Schwalm graduated Dr. rer. nat. from the Philipps University in Marburg, Germany. His research interests focused on morphogenesis in embryonic development of insects and the molecular determinants deposited in the egg during oogenesis. After research appointments at the University of the Witwatersrand (Johannesburg, South Africa) and at the University of Notre Dame, he worked as faculty member at the University of Illinois with graduate teaching assignments in Developmental Biology and Developmental Neurobiology. He was Chair of the Department of Biological Sciences at Illinois State University (1978-1980) and at the Texas Woman's University from 1982 – 2001.

Introduction to the CNNS

The CNNS at the University of North Texas is a unique research organization dedicated to the study of fundamental mechanisms of the nervous system with a primary focus on the self-organization and function of small nerve cell networks grown in cell culture on microelectrode arrays. Recently, it has been established that such networks are pharmacologically very similar to the parent tissue, allowing lucrative applications to the fields of toxicology, drug development, and artificial intelligence through efforts in computational neuroscience.

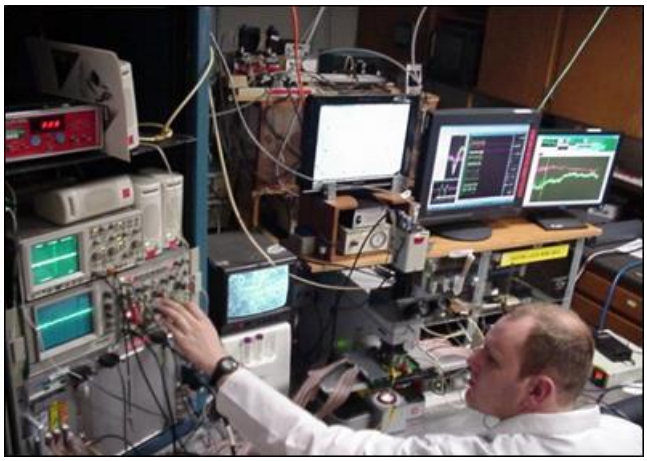
The CNNS is seeking support from private sources for ongoing research and for expansion of research to key disease states, physical trauma, and development of high throughput pharma-tox platforms.

We have extensive experience in neuronal cell culture, fabrication of microelectrode arrays, and novel multichannel recording techniques. As a pioneer in microelectrode array recording, the CNNS has international visibility. Since 1990, we have trained over 35 scientists, technicians and graduate students from the US and 5 different foreign countries. This includes senior personnel at NeuroProof, a successful new company in Rostock, Germany, which is applying nerve cell network analyses to drug development.

The CNNS presently sells microelectrode array plates, recording chambers, miniature microscope incubators for cell culture life support, but cannot expand sales or focal research areas with current personnel and facility limitations.

The establishment of a Service and R&D Company is needed.

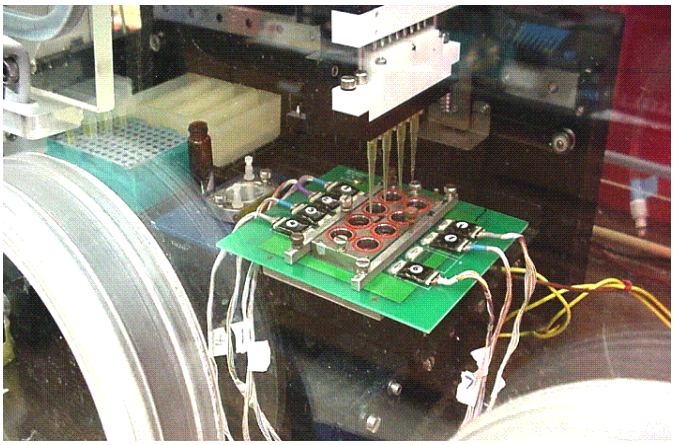
Mouse spinal cord neurons growing on microelectrode arrays featuring transparent indium-tinoxide (ITO) conductors. Such neurons form networks that are always spontaneously active. Changes in that electrical activity are used to evaluate new chemical compounds that are toxic or pharmacologically active. Continuous optical and electrophysiological monitoring is possible.



One of 4 CNNS workstations showing recording and monitoring equipment. At the controls is Matthias NIssen, visiting Ph.D. student from the University of Rostock, Germany. This particular station is coupled to a laser microbeam system (pulsed nitrogen laser) that allows surgery on the cellular level as well as laser stimulation of neurons.

Why the CNNS needs your help

- Continual federal and state research support is becoming increasing difficult to maintain. This leads to loss of experienced personnel, project disruptions, and substantial inefficiencies.
- New insights and promising ideas can often not be pursued because of lack of immediate funding for pilot experiments (new grants require 8-10 months for funding and presently have success rates below 10%).
- Highly interested and very talented students are often turned away because specific scholarships and other student support are not available.
- Technical modifications that would lead to enhanced research efficiencies and increased CNNS sales are frequently not possible.
- Advertising and website functionality are also impeded under the present financial constraints.
- **Many functions and products such as sales of MEAs, custom recording chambers, training support, and (especially) sales of networks grown on MEAs and shipped in the living state to end-users should eventually transition to a small R&D company in the local area. Such a development requires private investors.**



8-Network recording platform in life support chamber with robotic maintenance.

These developments are essential to achieve the high throughput required by industry.

Technical advances that allow recording from 16 to 48 networks simultaneously must be supported to assure that the key technologies emerge in the US.

Routine and widespread application of high throughput assay platforms using nerve cell networks on microelectrode arrays will result in a drastic reduction of experimental animals presently used by industry and government agencies.

Donation Categories

Category 1. General Support
Donations are used for general lab supplies, student assistant hourly wages, and electronics or fine mechanics repair or construction. This category gives the lab maximum flexibility.

Category 2. Targeted Donations
Donations support specific research of interest to the donor. This includes investigations on neurotoxicity, pharmacology, toxicology, and pancreatic tissue. Focus on construction of novel research instruments, specific neurological diseases, impact trauma with associated recovery methods, and pain suppression with pulsed magnetic fields is also feasible.

Category 3. New Research Directions
The CNNS lab can be diverse in its research and many new research directions lie within the capabilities of the Center. Category 3 donations allow a sponsor to focus on new research areas of interest to the sponsor. (Requires approval from Advisory Committee.)

Category 4. Scholarships
Sponsors may generate scholarships in their name, or the name of their company, for general undergraduate student support or for specific graduate research projects.

Sponsor Benefits

- Participation in an emerging company.
- Participation in patents.
- Honorable mention in publications, press interviews, and at conferences.
- Lab visits and participation in research, if desired.
- University recognition.
- Optional Advisory Committee membership.
- Quarterly updates on research progress.

For more information please call or e-mail

Professor Guenter W, Gross (Director, CNNS)
Office: 972.565.3615
Cell: 972.300.3525
e-mail: gwgross@unt.edu

Or visit the CNNS labs on appointment.

CNNS History

The small neuronal network is one of the least explored, yet profoundly important organizational unit in neuroscience. It holds the secrets of pattern generation, recognition, and storage (the mechanism underlying behavior), of neurobiological fault tolerance, and of pharmacologic and toxic response mechanisms.

The CNNS at the University of North Texas has pioneered research methods that allow exploration of these network phenomena. Dr. Gross was the first researcher to record neuronal signals with substrate integrated microelectrode arrays[1] and held the first patent in this area[7]. The CNNS was stablished in 1987 with large grants from the Texas Advanced Technology Program and the Communities Foundation of Texas, made possible through Dallas developer W. W. Caruth, Jr. with a focus on facilitating transdisciplinary studies of the self-organization and electrophysiological dynamics of mammalian networks in cell culture. In 1996, Matsushita Electric (Panasonic, Japan) purchased world-wide, non-exclusive rights to this patent.

The mission of the CNNS is pursued with a unique experimental strategy: the monitoring of networks growing on arrays of substrate integrated, photoetched microelectrode arrays (MEAs), a method largely pioneered at UNT. The CNNS has international visibility because its pioneering efforts[1, 2], the introduction of transparent metal conductors to electrophysiology[3], and early as well as ongoing efforts to use MEA systems as reliable biosensors for pharmacology, toxicology, and drug development [4,5]. MEA technology is now used world-wide and an international conference is held every two years.

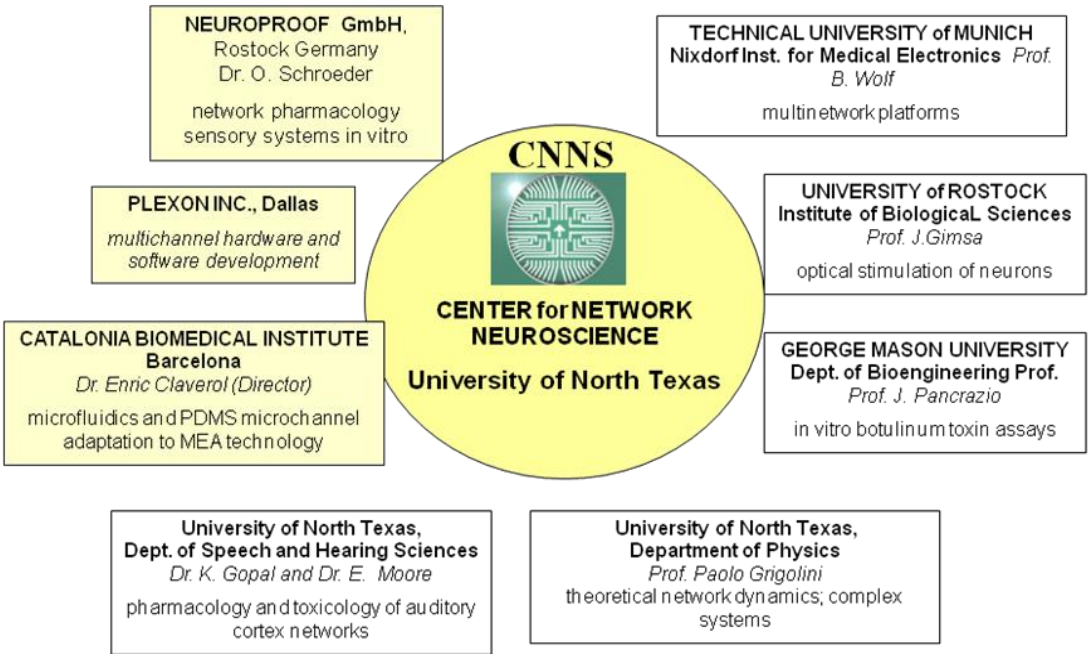
Versatility of MEA Applications

The MEA methodology is not limited to the nervous system but can be expanded to studies of cardiac tissue and glandular tissue (which are also electrically active). Powerful in vitro investigations can be initiated in the areas of Alzheimer's and Parkinson's disease states, as well as functional and cellular toxicity of gland cells (such as pancreatic tissue). The neurotoxicity of the cancer drug Cisplatin and protection by D-Methionine has recently been investigated and published.[6]

The combination of optical and electrophysiological observations in an isolated and chemically defined environment (not possible in animals), represents a unique and presently not yet fully implemented research tool of considerable promise.

(1) Gross, G.W., Rieske, E., Kreutzberg, G.W. and Meyer, A. (l977). A new fixed-array multimicroelectrode system designed for long-term monitoring of extracellular single unit neuronal activity in vitro. Neurosci. Lett. 6: 101-105.
(2) Gross, G.W. (l979). Simultaneous single unit recording in vitro with a photoetched laser deinsulated gold multi-microelectrode surface. IEEEE Trans. Biomed. Eng. BME-26: 273-279.
(3) Gross, G.W., Wen, W. and Lin, J. (l985). Transparent indium-tin oxide patterns for extracellular, multisite recording in neuronal cultures. J. Neurosci. Meth. 15: 243-252.
(4) Gross, G.W. and Pancrazio, J.P.P. (2007) Neuronal Network Biosensors. In: Smart Biosensor Technology (G.K. Knopf and A.S. Bassi, eds), Taylor and Francis Publishers, CRC Press.pp 177-201.
(5) Gross GW (2011) Multielectrode Arrays. Scholarpedia, 6(3): 5749
(6) Gopal KV, Wu C, Shresta B, Campbell KCM, Moore EJ, Gross GW (2012) D-methionine protects against cisplatin-induced neurotoxicity in cortical networks. Neurotoxicology and Teratology, Volume 34, Issue 5, 495-504.
(7) P 27 41 638.2 ; (U.S patent (7) 4,231,660) Nov. 1979. Microscope slide with electrode arrangements for cell study and method for its construction. G..W. Gross, A. Meyer, E. Remy, E. Rieske ,

2013 COLLABORATIONS



Dormant Collaborations Awaiting Funding

Southern Methodist University, Departments of Electrical and Mechanical Engineering
Prof. Marc Christensen, Prof. Volkan Otugen

University of Texas at Dallas, Department of Material Science: *Prof. Duncan Macfarlane*

Santa Fe Institute , New Mexico *Dr. Luis Bettencourt;*

Los Alamos National Laboratory: *Dr. Michael Ham*

Research Support since 1987: $6,757,759

A. State of Texas ($1,644,671)
Advanced Research and Technology Grant

B. Federal Grants ($4,728,636)
NSF, NIH, DARPA, Los Alamos National Laboratory

C. Foundation Support (1,237,326)
Communities & Hillcrest Foundations of Dallas

D. Industrial Support: ($284,452)
Sandoz Inc. (Basel), Plexon Inc. (Dallas)

E. Endowments: ($100,000)
Charles and Josephine Bowen Memorial Endowment for Neuroscience Research.

Facilities

1. Photolithography/MEA Fabrication	**SRB 248**
2. Cell Culture	**SRB 246**
3. Multichannel Recording	**SRB 247**
4. Laser cell Surgery & Photonic Stimulation	**SRB 248**
5. High Throughput Platforms	**SRB 248**

MEA Fabrication

Science Research Building (SRB) Room 248

The Center houses a modest photolithography facility that fabricates microelectrode arrays (MEAs) with line widths of 6 - 8 um. Patterns are etched into sputtered indium-tin oxide (ITO) on barrier glass (Applied Films Corp., Boulder, CO). The following MEA designs are being produced:

1. M- 3: 64 microelectrodes arranged in 4 rows and 16 columns with lateral spacing of 40 um and vertical spacing (between rows) of 200 um.

2. M- 4: 64 microelectrodes arranged on an 8 x 8 matrix with equal spacing of 150 um.

3. M- 5: 64 microelectrodes forming two separate recording areas (32 electrodes each) with 150 um equidistant spacing.

4. M- 6: 64 microelectrodes arranged in 3 recording areas served by 16 electrodes in each area with linear interconnecting paths covered by 8 electrodes each.

5. M- 8: 256 microelectrodes arranged as 8 separate recording areas served by 32 microelectrodes each.

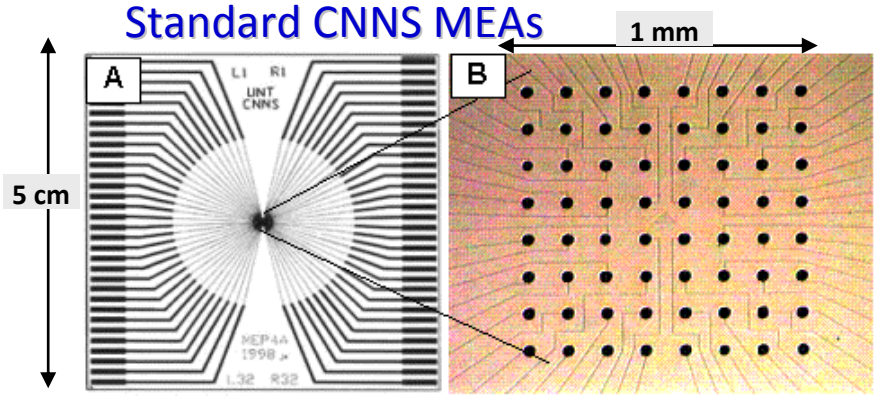
6. M-8.256 Single network configuration with 256 recording electrodes using the same plate dimensions and amplifier contacts as MMEP-8

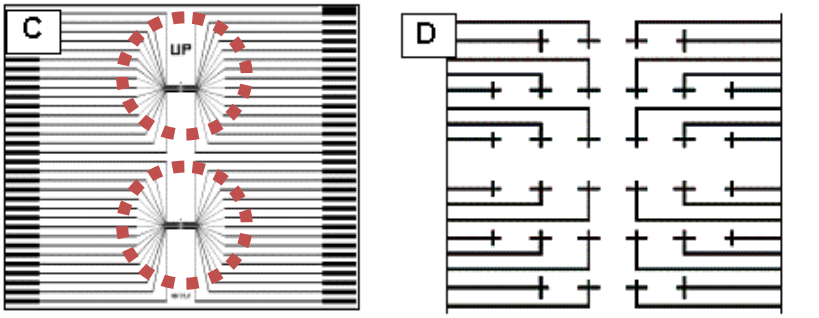
M- 3 to 6 consist of 5x5 cm glass wafers, 1 mm thick with identical amplifier contact finger layout. MMEP 8 is an 8 x 10.5 cm glass plate with a very dense contact finger design (300 um width and 300 um pitch).

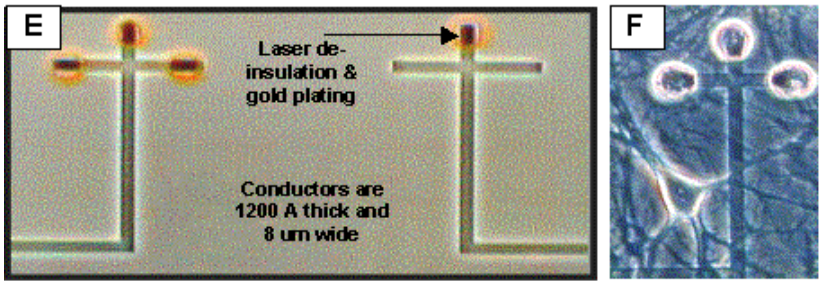
Ahmet Ors. MEA Fabrication Technician

We are using optically flat glass plates, together with transparent conductors (indium-tin oxide), to assure high magnification phase contrast optical access during recording. This allows monitoring of cell stress, observation of circuitry, and discovery of bacterial contamination that can influence results.

Standard CNNS MEAs





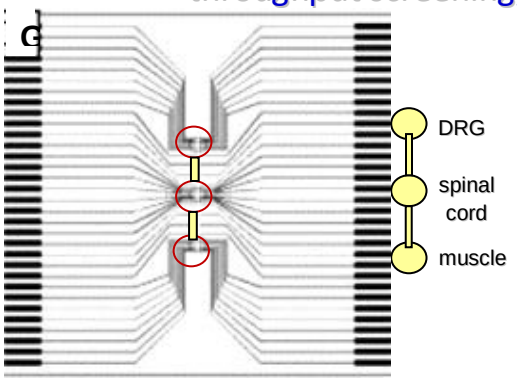


MICROELECTRODE ARRAYS FABRICATED at UNT

Plates A to D measure 50 x 50 x 1.1 mm. Amplifier edge contacts are the same for all these arrays (32, each side).
(A, B) M - 4 with 64 conductors terminating in a 1 mm^2 recording area in an 8 x 8 matrix . Electrodes are spaced equidistant at 150 μm. Impedance @1kHz: 0.8 - 1 megohms.
(C) M-5. Electrode array plate featuring two separate recording islands with 32 microelectrodes each. Center-to-center distance: 2.24 cm; electrode spacing: 200 μm. Dashed circles indicate location of O-ring after chamber assembly. Cruciform electrodes increase the recording probability in low-density cultures (D, E, F).

Below: 8-network array plate (90 x 56 x 1.1 mm) served by 32 microelectrodes per recording area (256 total). Amplifier contact fingers: 300 μm; pitch of 300 μm. Designed for high throughput, parallel multi-network assays.

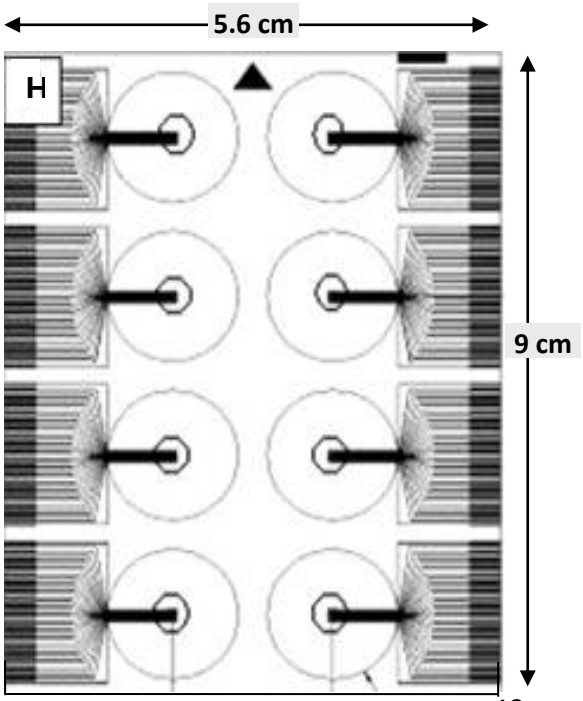
New designs for regeneration and HTS (high throughput screening)



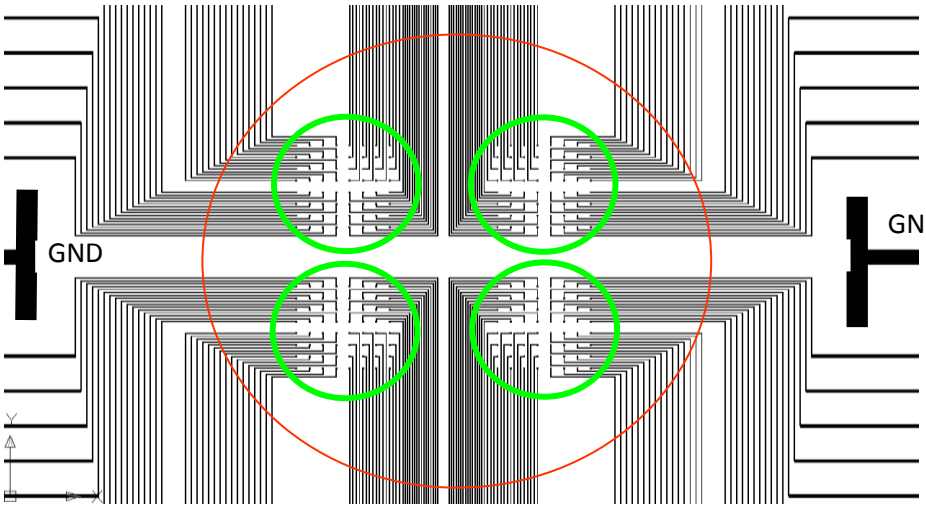
Above: M-6 with three recording areas connected by two conduits of 8 electrodes each. For co-cultures of different tissues, regeneration studies, and studies of system dynamics.

8 network
256 electrode array
(32 E each)

Contact finger width: 300 μm
Pitch 300 μm



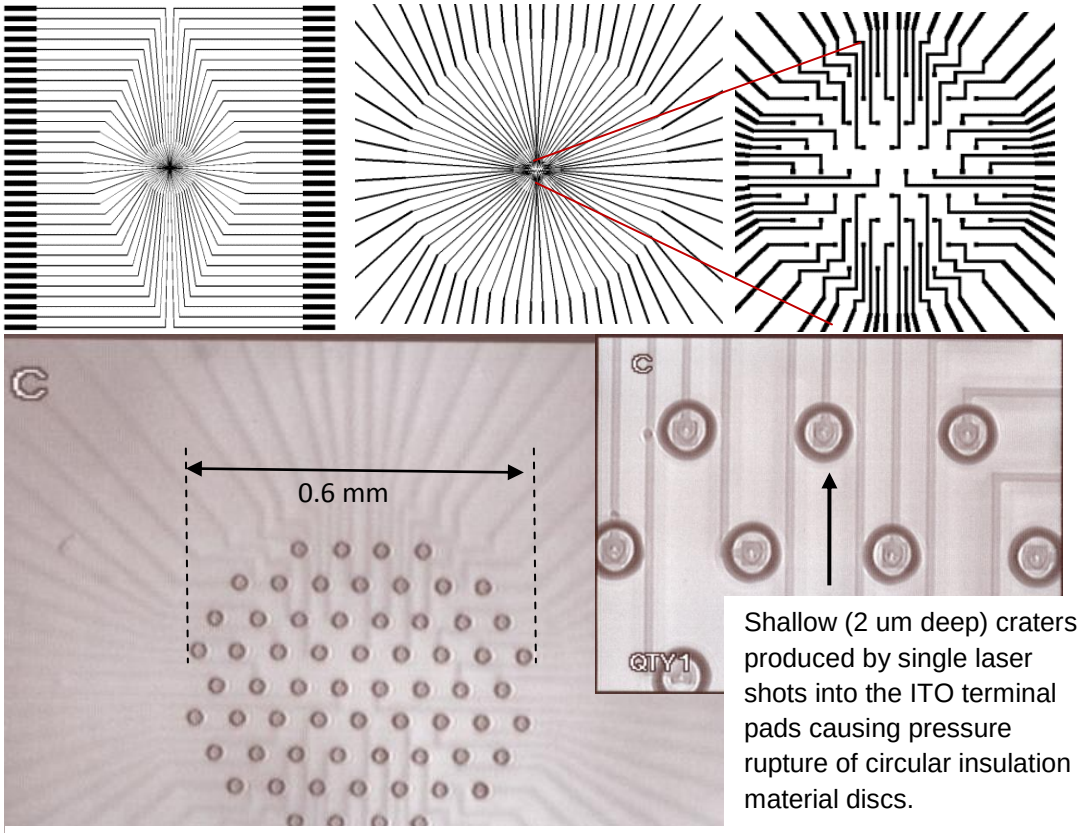
256-electrode (4 x 64) MEA for theoretical studies of large networks



Electrode spacing: 200 micrometer (um)
Field separation (circles):

Edge contacts: 300 um;
Pitch 300 um

M-4R: 64-electrode array designed for Cornell University Med. Ctr., New York (Dr. Sheila Nirenberg; studies of vertebrate retina)

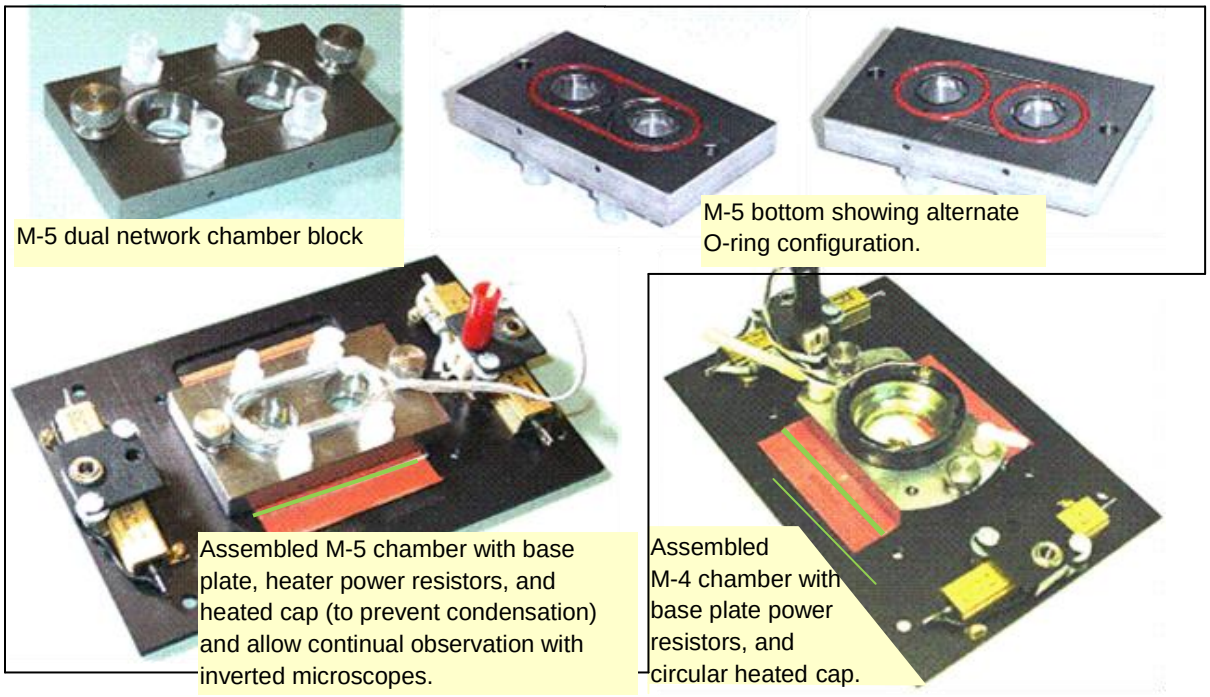


Shallow (2 um deep) craters produced by single laser shots into the ITO terminal pads causing pressure rupture of circular insulation material discs.

Description: Transparent ITO conductors: thickness: 1200 A; width: 6 micrometer (um)
terminal pads: 18 um x 18 um; de-insulation (recording) crater: 25 um diam;
insulation material: methyltrimethoxysilane; insulation thickness: 2 um
impedance after gold plating * : 1 megOhm * *picture shows MEA before gold plating*
Electrode terminal spacing: 70um (lateral and vertical)

Recording Chamber Development

With the support of a State of Texas "Advanced Technology" grant, the CNNS developed a variety of recording chambers that allow continual optical monitoring during electrophysiological recording and greatly facilitate test compound additions medium changes via Luer ports in the stainless steel chamber block. In all cases, 1 mm diameter medium entrance and exit ports are located inside the O-ring proper.



M-5 dual network chamber block

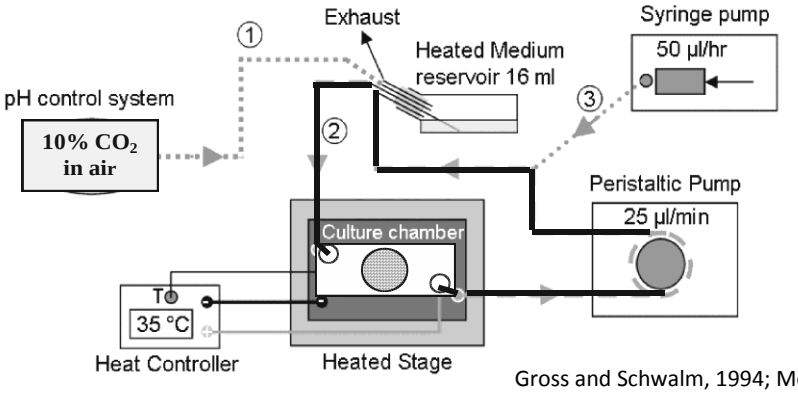
M-5 bottom showing alternate O-ring configuration.

Assembled M-5 chamber with base plate, heater power resistors, and heated cap (to prevent condensation) and allow continual observation with inverted microscopes.

Assembled M-4 chamber with base plate power resistors, and circular heated cap.

These chambers, if combined with a gas flow (10% CO_2 in air, 10 ml/min) and gentle water injection to compensate for evaporation (infusion pump, ~50 ul/hr) form **highly cost-effective mini-incubators on the microscope stage**. Luer connections provide access to the medium inside the O-ring for test compound additions, medium changes, and water injection (for maintaining constant medium osmolarities).

Closed Circulation Chambers: Addition of glass or quartz cover glass to a recessed area at the bottom of the chamber block converts the open chamber into a closed chamber.



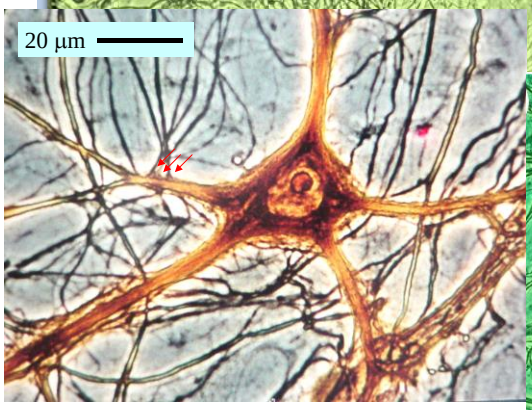
Life-support system for cell culture chambers (bottom middle). Line 1: 10% CO_2 + 90% air supply for pH stability in the medium reservoir. Line 2: medium supply. Line 3: sterile water supply for osmolarity control in the medium reservoir. CO_2-impermeable Pharmed® tubing is used. The supply medium is maintained between 39 and 40 °C to eliminate gas bubble formation in the culture chamber maintained at 35°C.

Gross and Schwalm, 1994; Meyer, Wolf, and Gross (2009)

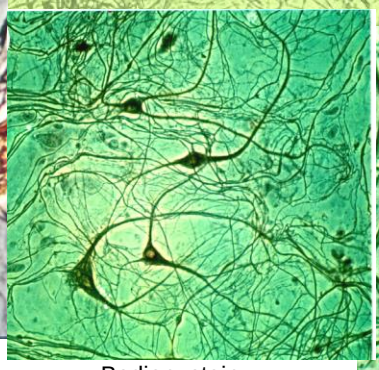
Cell Culture

Growing nerve cells in vitro (outside the animal in cell culture) is difficult. Growing them on microelectrode arrays, assure spontaneous activity, assure pharmacological responsiveness, and maintain such networks functionally active for several months is daunting.

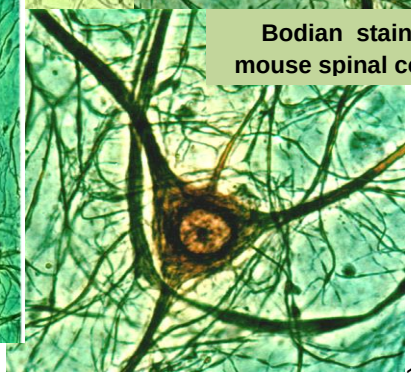
The CNNS has 33 years of experience in neuronal cell culture and can be counted as one of the top laboratories world-wide in this area.

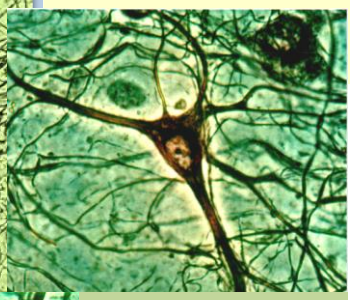


Neurofilament antibody stain, mouse cortex



Bodian stain



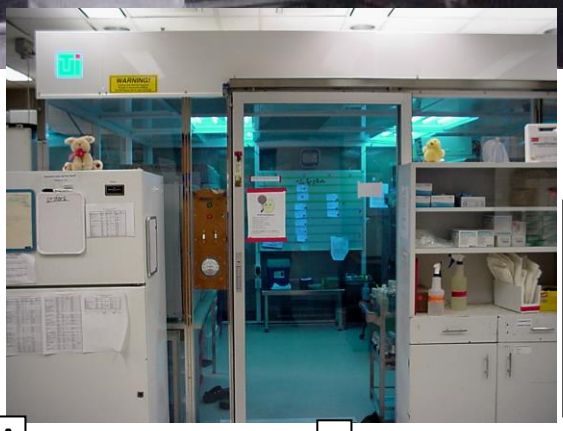


Bodian stain, mouse spinal cord

The CNNS has a well-equipped cell culture facility (600 ft^2) with 6 incubators, one biohazard hood, two laminar flow hoods, one inverted microscope with camera and monitor, 2 pH meters, and 2 osmometers.

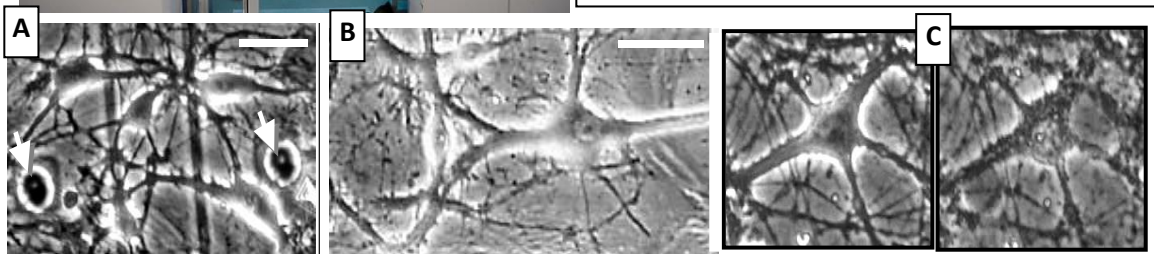
Left: Cell dissociation and seeding onto MEAs.

Graduate students Jason Gibson and Nicole Calderon at laminar flow hood with undergraduate HHMI* stipend recipient, Son Le, standing in support.
* HHMI: Howard Hughes Medical Institute



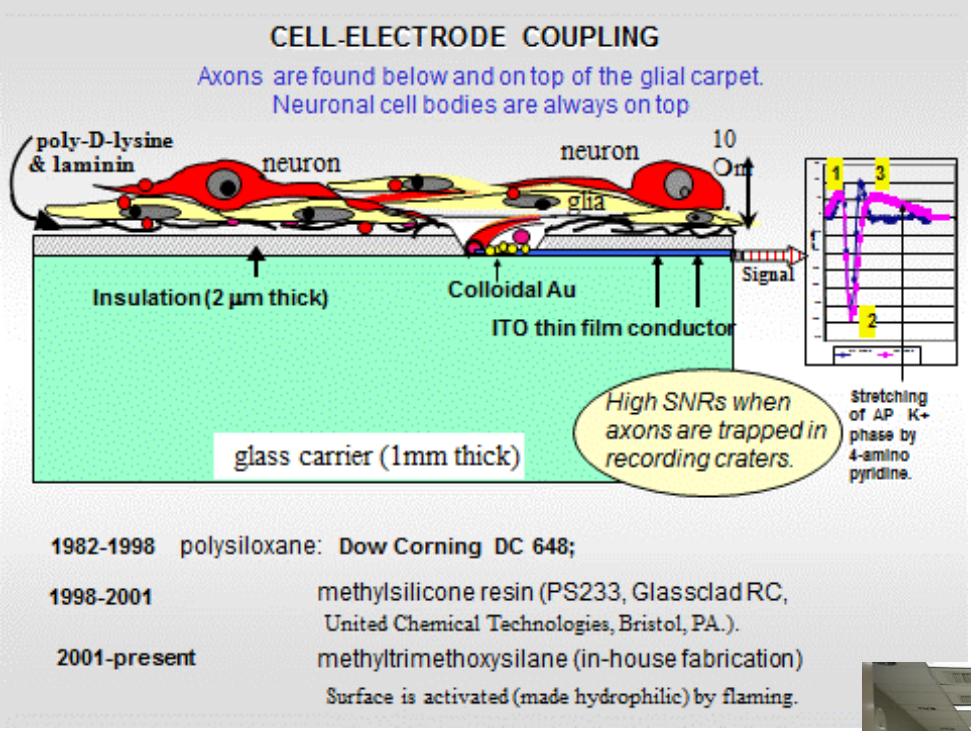
Above: Cell culture technician Jennifer Mcanally performing biweekly feedings of cultures (1/2 medium changes).

Left: Sterile culture room during UV exposure.



Above: Living neurons (embryonic mouse frontal cortex) after 30-40 days in culture, growing on MEA surfaces. Arrows in (A) point to recording sites. Panel (C) shows a multipolar neuron before and after 100uM exposure to zinc acetate. The right panel reveals necrosis and cell death. Phase contrast microscopy of low density cultures shows remarkable detail of neurons and circuitry in the living state.

Although a low electrode impedance is desirable, the most important factor determining high signal-to-noise ratios is cell-electrode coupling. The neuronal compartment that is generating action potentials (mostly axons, but cell bodies and dendrites also participate) must be in close proximity to the metal-electrolyte interface that responds to the extremely minute ionic currents produced by action potentials. The interface forms what is called a "Helmholtz Double Layer" where ions are found in a less random state than in the bulk medium. Together with electrons in the metal, this area forms a capacitor. A slight disturbance of the Helmholtz layer by ionic currents resulting from action potentials produced by local nerve cells, now causes a transient movement of electrons in the metal conductors leading away from the recording site to the amplifier. Those signals are in the microvolt range, but can be detected and amplified with appropriate equipment.

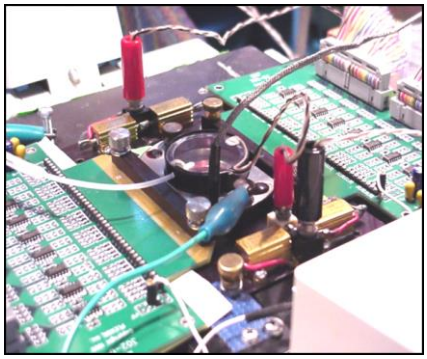


The electrode surface must be decorated with adhesion molecules such as poly-D-lysine and laminin. On this surface glia cells (non-neuronal components of neural tissue) form a "carpet" on which nerve cells (neurons) reside. Once established, such networks can survive in vitro (i.e. in the incubator for over 6 months. But it takes constant care: biweekly feeding (medium removal and additions), maintenance of osmolarities (proper water content of the medium that can be lost by evaporation), and maintenance of strict sterility.

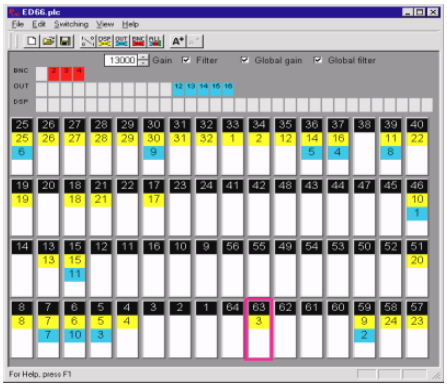
Multichannel Recording

Network research and application require the capture of simultaneous information from many nerve cells in an ensemble.

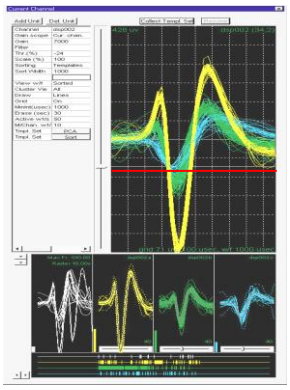
Single Network Assembly and Real Time Data Acquisition



Chamber on microscope stage with preamplifier hookup



Geometrically true display of electrode pattern and selection of electrodes for display.



Automatic or manual thresholding and waveshape template selection. Neurons are identified and followed based on waveshapes. Up to 4 active units can be separated on each electrode in real time. 4- kHz scan rates gives digitized signals with 25 us resolution (Plexon Inc.)

Left: Time stamp display (raster) for all discriminated units. Colors represent different units on the same electrode (4 max). The right panel shows discriminated waveshapes

All amplifiers and digitizing as well as display software is obtained from Plexon Inc., Dallas, TX.

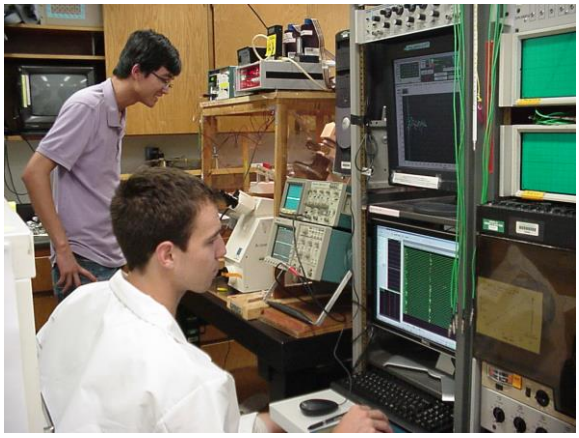
Recording with electrodes outside the neurons (i.e extracellular recording) is non-invasive and non-destructive. However, the electrical signals are minute: usually only a few hundred microvolts in amplitude.

Multichannel Recording Workstation

The CNNS has 4 operations workstations. Each station consists of:

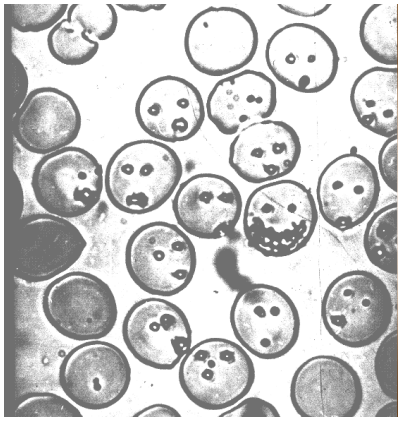
1. An inverted microscope with analog and digital cameras.
2. A Plexon Inc. MNAP system for 64 channel parallel recording, real time raw and processed data displays, digitized data storage.
3. Network life support micro-incubator system (CNNS development) that features: (a) 10 % or 5% CO2 in air for pH maintenance (10 ml/min into heated chamber cap), (b) water evaporation compensation (infusion pump, ~50 ul/hr) to maintain medium osmolarities; (c) feedback heater control to maintain temperatures to +/- 1 deg C. Osmolarities and pH must be checked manually via 10 ul and 100 ul samples, respectively.

Graduate students Edward Keefer and Alexandra Voss (Univ, of Rostock, Germany) at workstation 1 in 2003. Both are now established researchers in industry (Plexon Inc., Dallas and Neuroproof, Rostock). Dr. Keefer received a postdoctoral position at the prestigious Neuroscience Institute in La Jolla, CA, before returning to Texas.

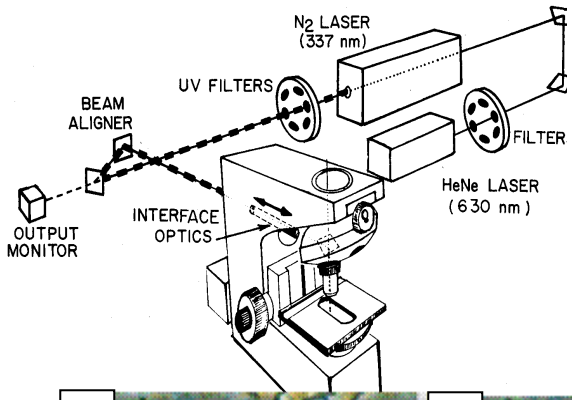


Undergraduate students David Smith and Son Le monitoring multichannel recording and culture life support equipment during a quinolinic acid toxicity experiment.

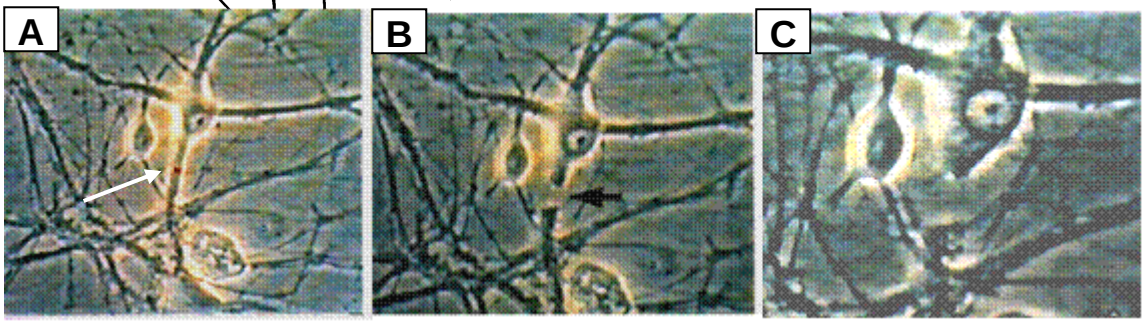
Laser Surgery, Trauma, and Stimulation

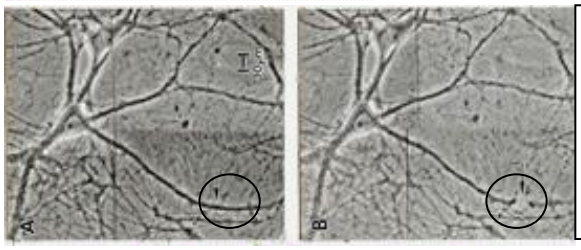


If you want to write your name into a red blood cell, that is possible (if your name is short). The human red blood cells to the left are only 6 micrometer in diameter and show laser perforations of about 1 micrometer. Such a precision is achieved by firing a pulsed near-UV nitrogen laser through a microscope equipped with quartz optics. The CNNS has one of these rare systems. It was built by BTG, GmbH of Munich Germany, sold to the Sandoz Corporation in Basel Switzerland, and finally donated to the CNNS. The system is used for cell surgery to study trauma, simplify networks to explore fault tolerance by selective elimination of cells and/or their connections, and explore photonic stimulation of nerve cells.



Laser microbeam system operational at the CNNS. The near UV nitrogen work laser (337 nm) and the HeNe positioning laser are aligned collinearly, reflected into a Leitz Orthoplan microscope, and focused onto the specimen in special recording chambers via quartz objectives. A x32 objective can produce a minimum focus of 2.3 μm at an energy density of approximately 3 $\mu J/\mu m^2$. Neurite transections can be achieved via substrate vaporization shock waves, or more gently via multiple shots that probably cause mitochondrial disruption due to specific absorption by NADH and NADPH, Ca++ release, disruption of the cytoskeleton and gradual process transection.





CELLULAR TRAUMA. Above: Laser transection of large dendrite close to the cell body. (A) Before transection with target laser at arrow. (B & C) 5 & 10 min after transection. The target neuron is displaying necrotic cell death. A smaller satellite neuron appears normal. Left: Dendritic pruning 100 um from cell body (circle).

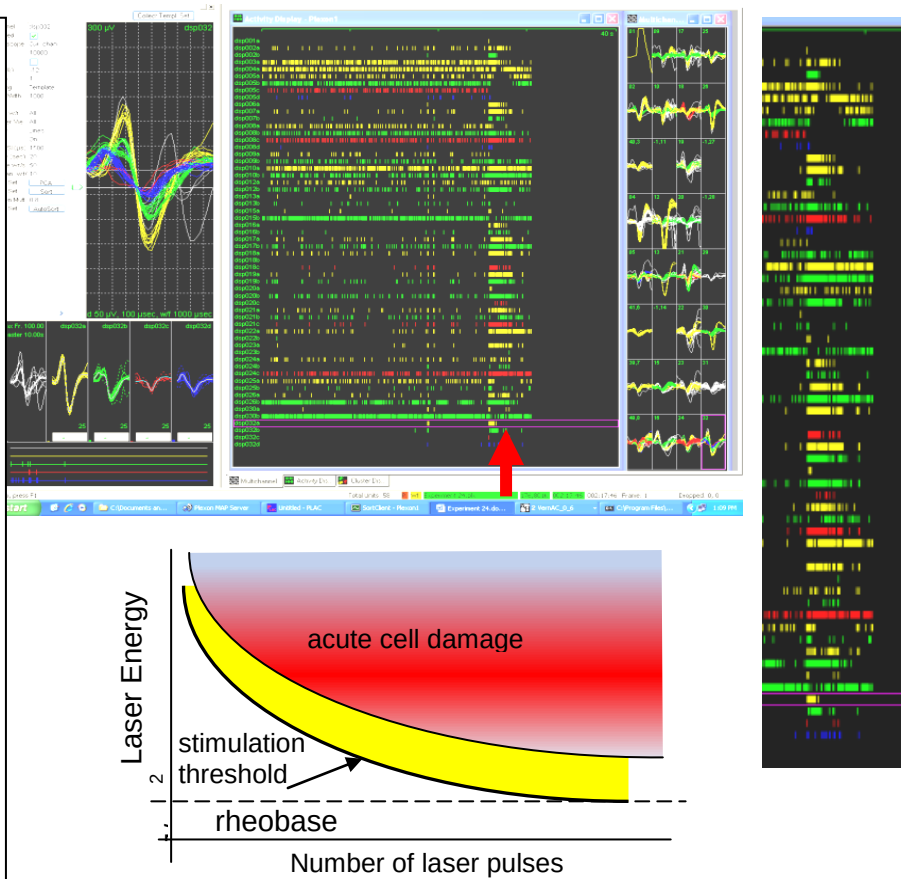
Such targeted, controlled injuries allow quantitative studies of cell survival and exploration of new pharmacological interventions. Laser surgery also has theoretical significance as it can be combined with multichannel recording from networks on microelectrode arrays (MEAs).

Direct Photonic Stimulation

Electrical stimulation of neural tissue has many drawbacks in that it is not highly localizable, is affected by electromagnetic interference (EMI), and produces undesirable biochemical effects at the metal/electrolyte interfaces. Both the military via DARPA and the NSF have shown increased interest in "biophotonics". Photonic stimulation will add new dimensions to present stimulation methods and may, in the future, dominate the neurostimulation domain. The reasons are: artifact-free stimulation, immunity to electromagnetic interference (EMI), high spatial specificity, and the potential for rapid multiplexing of stimulation signals at different wavelengths. Compared to electrical stimulation, chemical reactions may also be minimized.

Photonic stimulation phenomena have not yet been explored systematically across wavelengths ranging from the infrared to the UV. The combination of array recording from networks in vitro with laser stimulation provides a powerful tool for the systematic, quantitative exploration of stimulation efficacy and stimulation limits at all wavelengths.

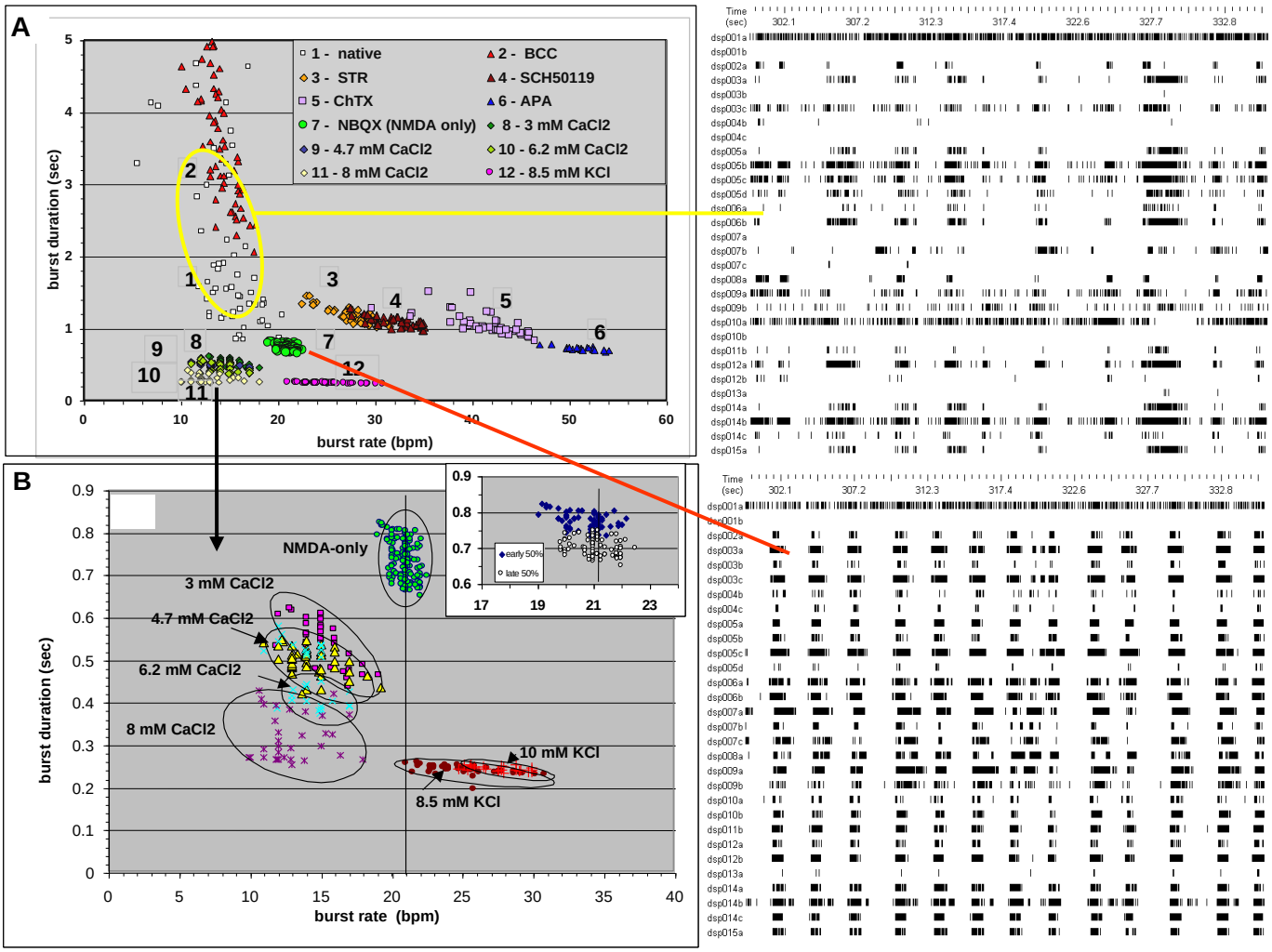
Network population burst (arrow) in response to a single laser pulse. Both inhibition and extensive excitation are seen (see enlarged panel at the far right). Up to 4 different waveshapes, representing separate neurons can be discriminated in real time on one electrode resulting in the different colors in the raster display. The raster display consists of time stamps (25 microsec resolution) that are converted to tick marks on the computer screen (Plexon Inc., Dallas). The challenge will be to establish damage thresholds and develop methods allowing routine, repetitive stimulation without crossing the damage threshold.



The techniques described allow transitions from reversible, repetitive stimulation to cell process transection and surgical elimination of specific neurons *simply by increasing the laser output power.* UNPUBLISHED DATA

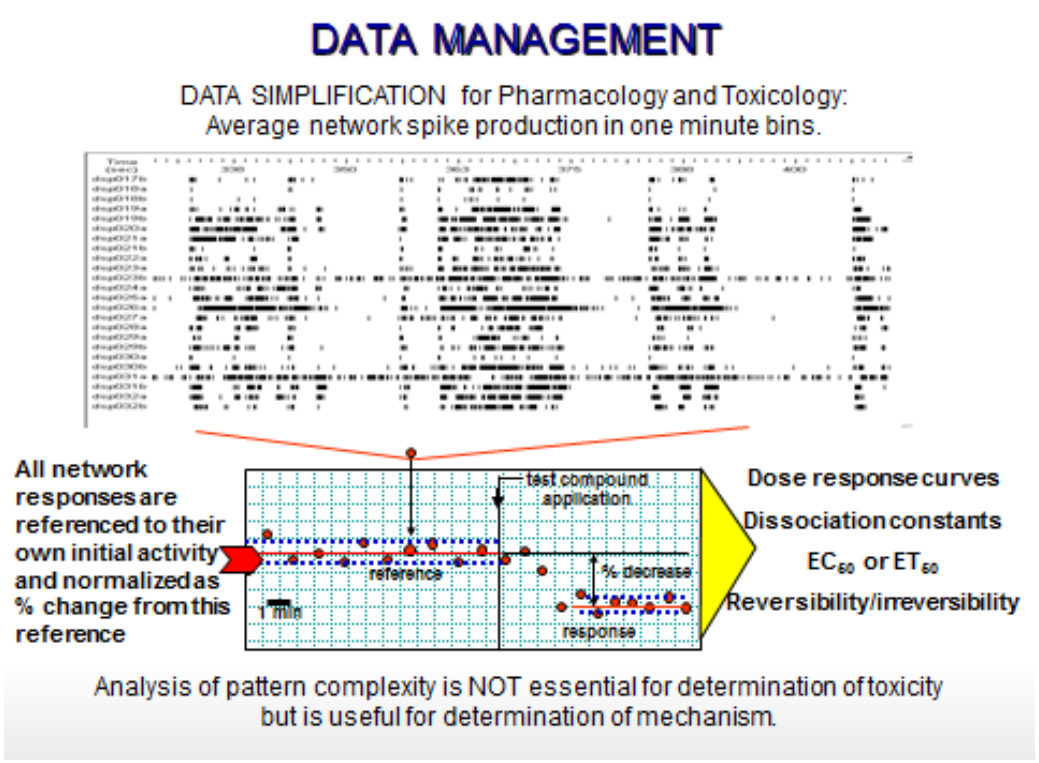
Applications to Pharmacology, Toxicology, and Drug Development

A remarkably reliable and specific sensitivity to compound additions to the medium!!



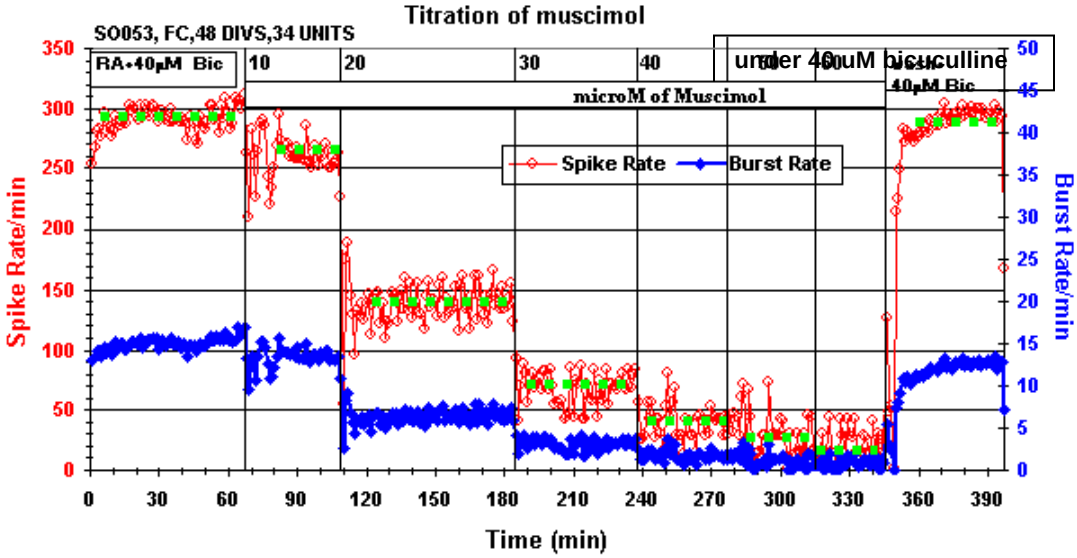
The biochemical sensitivity of spontaneously active nerve cell networks on MEAs is shown above with 11 different pharmacological substances. Each application result in a reliable pattern change expressed here as clusters of burst rate plotted against burst duration. The native raster plot and that for a much more organized burst oscillation state (induced by blocking all synapses except NMDA synapses) are shown in the panels to the right (see also tight cluster, State 7 in A). State 11 at different Ca++ concentrations is expanded in B.

Keefer, E.W., Gramowski, A., and Gross, G.W. (2001c) NMDA receptor dependent periodic oscillations in cultured spinal cord networks. J. Neurophysiol. 86: 3030-3042.

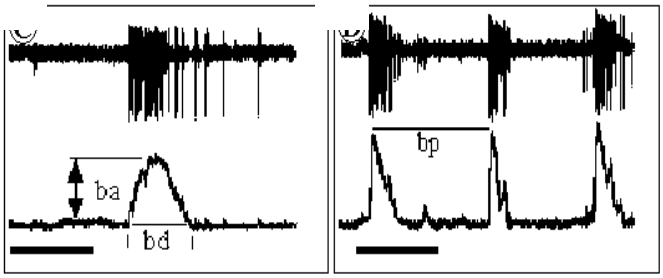


One of the most difficult problems in neuroscience is the quantification of the myriad of action potential (AP) patterns. The panel to the left shows a one minute segment of network activity from only 24 discriminated neurons. Such a pattern cannot be expressed mathematically without simplification. Fortunately applications to pharmacology and toxicology allow the condensing of a complex on-minute pattern into one data point.

The Primary Experimental Display:
Network spike and burst production

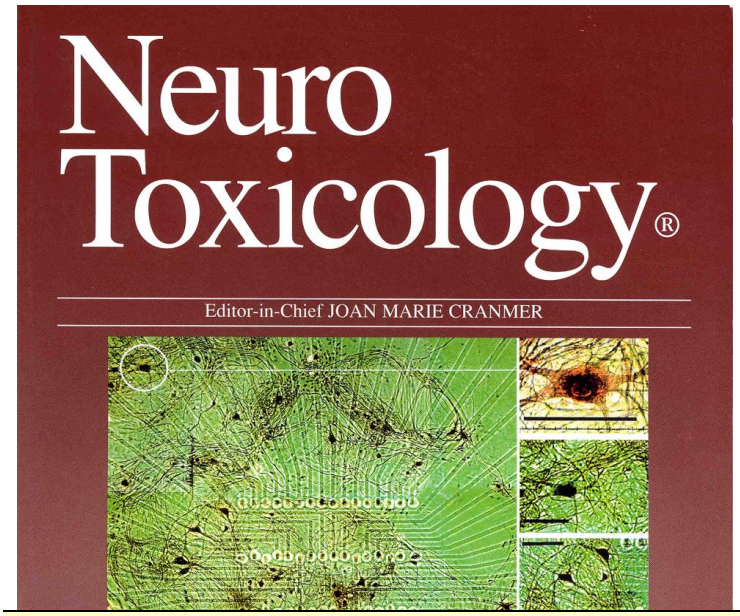


Muscimol is a deadly but reversible toxin derived from the toadstool mushroom. It mimics the action of the inhibitory transmitter GABA and reduces activity in a concentration-dependent manner (in animals and in cultured networks).



Bursts are clusters of high frequency action potentials that play a major role in brain function ranging from memory formation to precise muscle movement. For quantification, we integrate bursts, which provides measurable properties such as burst duration (bd), burst period (bp), and integrated burst

An Example of a Practical Application in Pharmacology and Toxicology



7 weak AChE blockers designed for alleviation of Alzheimer's syndrome were newly synthesized at the University of Perugia, Italy (Prof. Vincenzo Talesa) and tested at the CNNS.

The biochemical data confirmed binding to the AChE enzyme (acetyl cholinesterase)

However, such binding does not reveal other binding sites for compounds that had never existed before on this planet!

Functional Testing is necessary. **Screening with networks on MEAs saves time, money, and animals.**

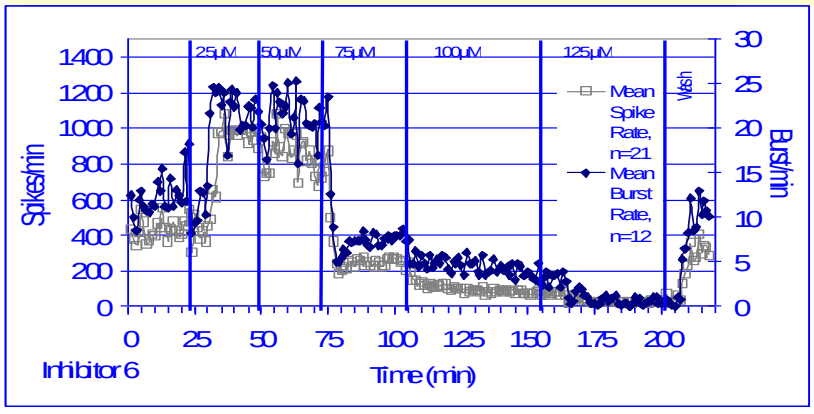
NOVEL ACHE Blockers

	AChE Inhibition. Const Ki (M)	Inhibit. Type	Excitat.	Inhibit.	Reversi-bility	No of experiments
$Ch^+O\text{-}CO\text{-}S(CH_2)_2S\text{-}CO\text{-}OCh^+$	$1.0x10^{-6}$	Mixed	10		R	2
$Ch^+O\text{-}CO\text{-}S(CH_2)_3S\text{-}CO\text{-}OCh^+$	$1.4x10^{-6}$	Mixed	50		R	3
$Ch^+O\text{-}CO\text{-}S(CH_2)_4S\text{-}CO\text{-}OCh^+$	$1.0x10^{-6}$	Mixed	10		R	2
$Ch^+O\text{-}CO\text{-}S(CH_2)_5S\text{-}CO\text{-}OCh^+$	$1.4x10^{-6}$	Mixed	NE	NE	--	2
$Ch^+O\text{-}CO\text{-}S(CH_2)_6S\text{-}CO\text{-}OCh^+$	$1.4x10^{-6}$	Mixed		350	I	2
$DMEA^+O\text{-}CO\text{-}S(CH_2)_4S\text{-}CO\text{-}DMEA^+$	$3.6x10^{-7}$	Mixed	25	125	R	3
$DMEA^+O\text{-}CO\text{-}S(CH_2)_6S\text{-}CO\text{-}DMEA^+$	$5.0x10^{-7}$	Mixed		200	I	2

NE: no effect; R: reversible inhibition (2 medium changes); I: irreversible inhibition (3 medium changes and 2 hr wait)

Ch^+O- represents choline residues, $DMEA^+$- represents N,N-dimethylethanolamine residues.

Two of the 7 substances were toxic and killed the networks. One was inhibitory, but reversible, and 3 were excitatory. Of those three, one showed the expected response. (see below)



Network spike and burst production as a function of time. Each dot represents a one minute average. Spontaneous activity between 0 and 25 min (Reference) is followed by a near doubling of the activity in response to 25 µM of test substance. No further increase occurs at 50 uM. At 75 µM, the activity drops 30% below reference and at 125 µM all activity is lost. A single medium changes restores the original activity.

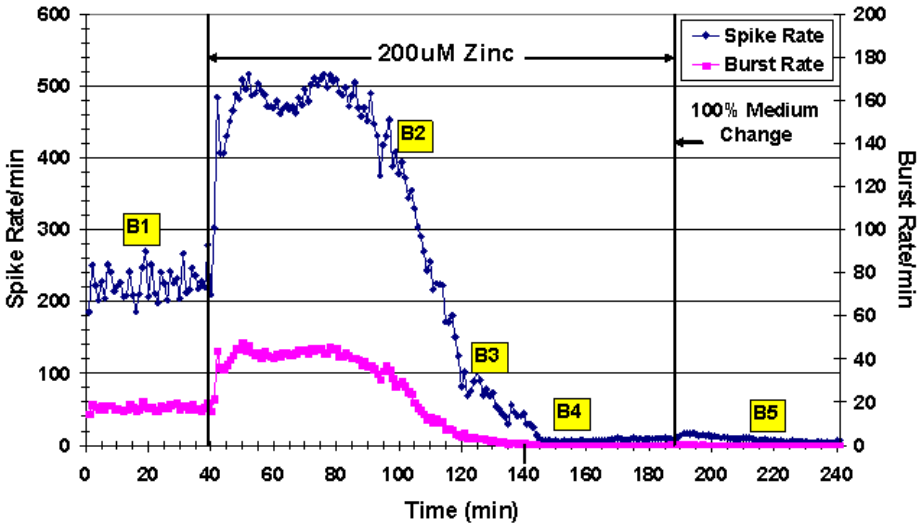
One experiment with one network on an MEA provided rapid screening data on the new compound in 220 min: (1) desired efficacy at 25 and 50 uM, (2) inhibition at 75 uM, and (3) dangerous activity loss between 100 and 125 uM, (4) reversibility. Such information can make drug development rapid and cost effective with a minim al use of experimental animals. Keefer, E.W., Norton, S.J., Boyle, N.A.J., Talesa, V., and Gross, G.W. (2001) NeuroToxicology 22: 3-12.

Environmental and Industrial Toxicology

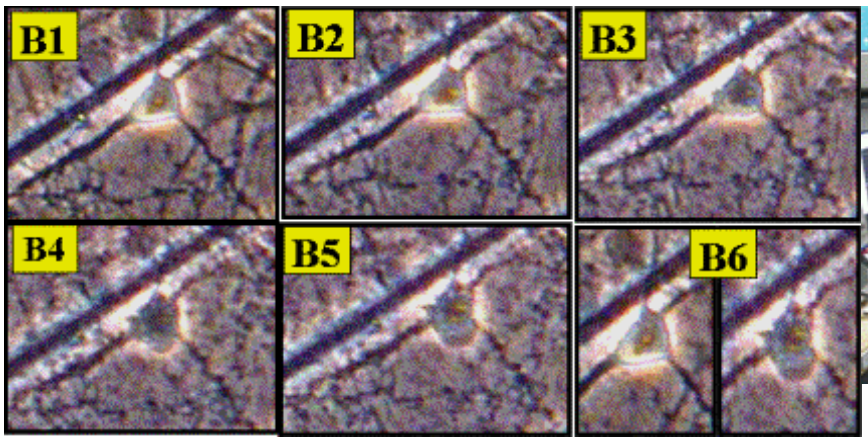
ZINC

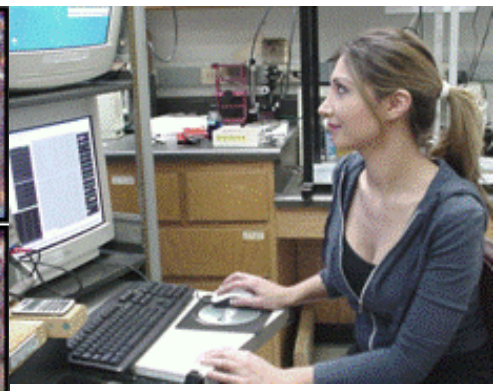
The zinc ion is a necessary dietary component and is required by numerous biochemical reactions in the nanomolar range (10^{-9} M).

HOWEVER, if the concentration is increased by a factor of 1,000 zinc becomes toxic in the micromolar range. Zinc is found everywhere in our environment: dental adhesives, vitamin supplements, lipstick, baby powder, rubber tires, waste water, and sunscreen (a large amount if applied to 80% of the body).



200 uM zinc acetate causes excitation followed by irreversible activity loss
.
Morphological observations during recording show cell body swelling and eventual necrosis. (compare B1 and B5 in the figure below).



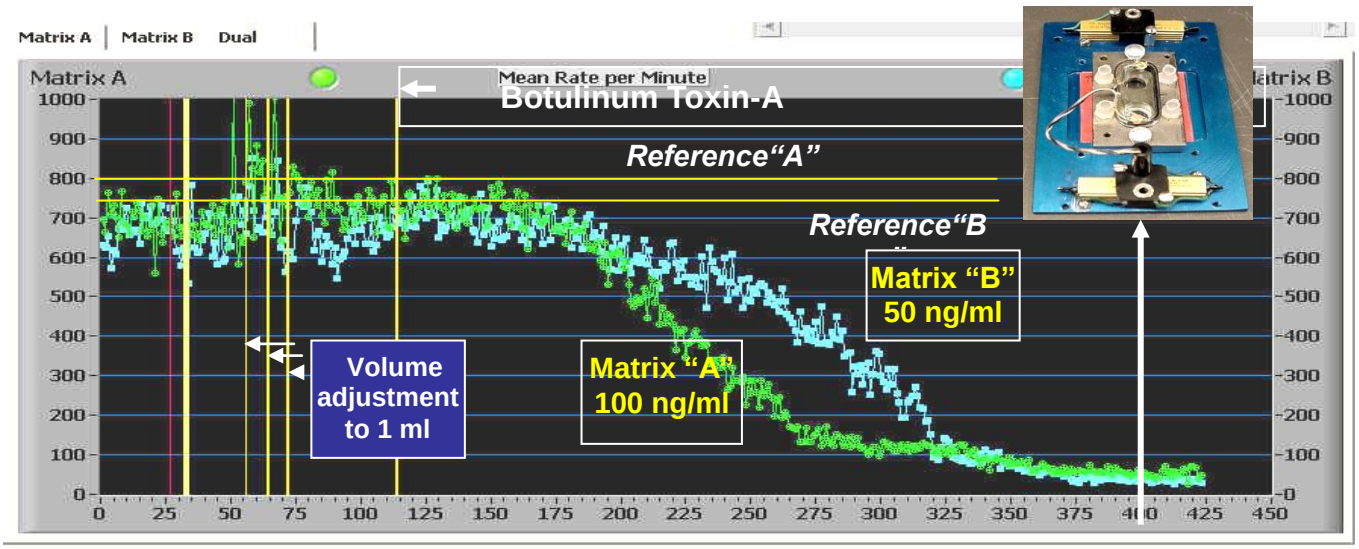


Maryam Parviz, PhD research

Parviz, M. and Gross, G.W. (2007) Quantification of zinc toxicity using neuronal networks on microelectrode arrays. NeuroToxicology 28: 520-531.

Botulinum Neurotoxin (BoNT)

Present methods for testing botulinium toxins (A - E) are time-consuming, expensive, and require a large number of aimals. Because BoNT does not cross the blood-brain barrier, the primary focus has been on using peripheral nervous system cholinergic synapses. However, all synapses are affected by BoNT because they all use identical proteins (snap 25) for exocytosis. It is this molecule that is affected by BoNT after it enters synapses. Networks derived from cortical tissue can be effectively used for BoNT research, with higher accuracy, less time, and at a much reduced cost.



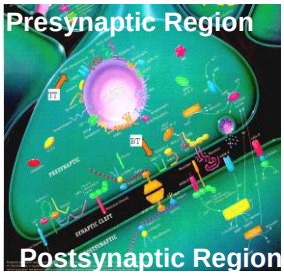


Diagram of a chemical synaptic terminal. It is the most complex area of a nerve cell.

Responses to botulinum toxin A

Dual network array experiment

Matrix A: 100 ng/ml (660 pM)
Matrix B: 50 ng/ml (320 pM)

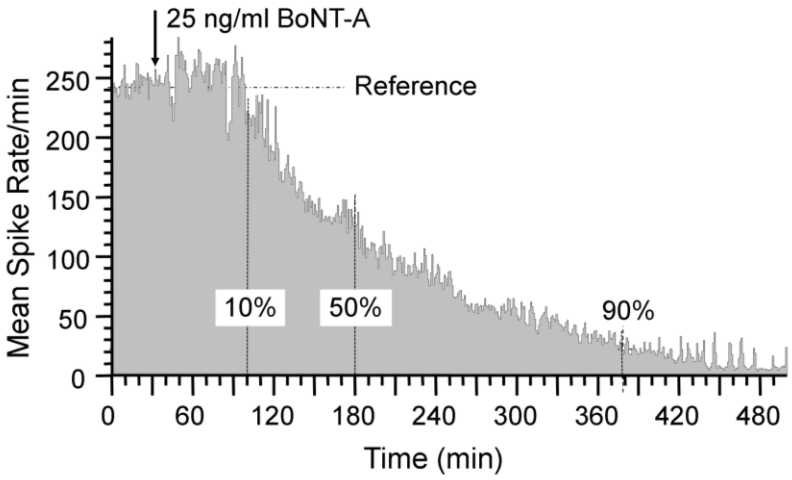
Time to 50% activity decay:

100 ng/ml: 1.9 h
50 ng/ml: 2.9 h

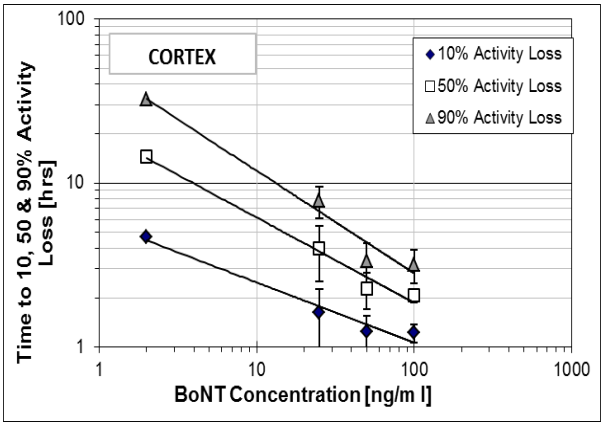
Real-time computer display (CNNS NACTAN program).

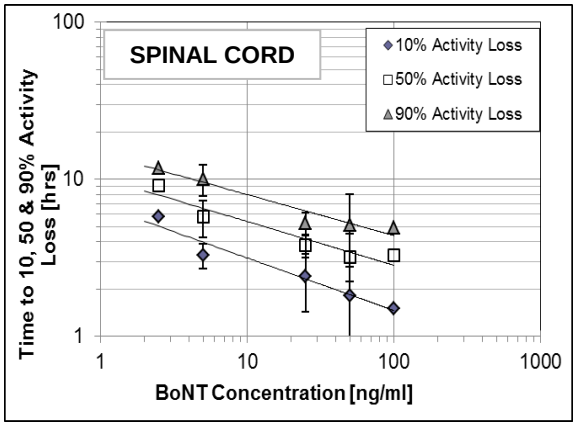
Recording chamber assembly for the dual network array. Shown with heated cap and power resistor plate heaters.

Botulinum toxin stops neve cell function by entering synapses and interfering with the fusion of vesicles (containing neurotransmitters) with the membrane. This blocks the process of "excocytosis" and prevents most cell-cell communication. The toxin does not kill cells, but without cell-cell communication all vital functions stop and the organism dies. This toxin is one of the most lethal substances found in nature.

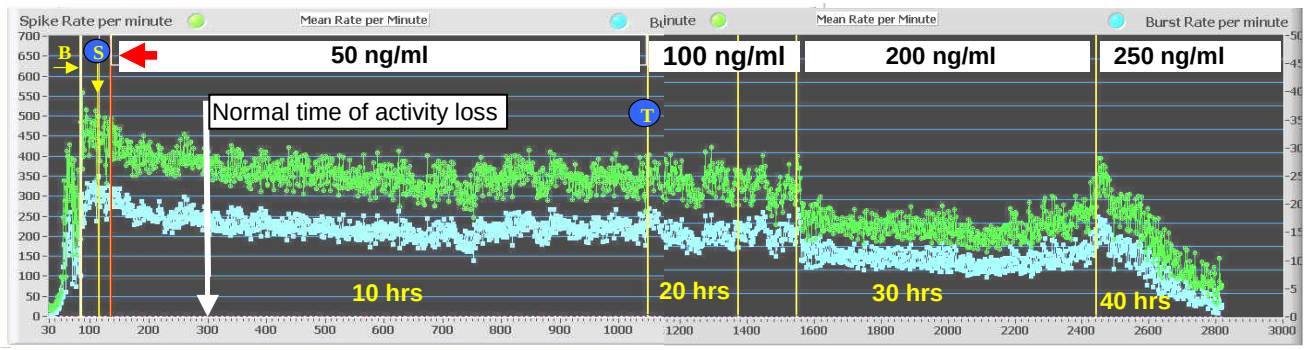


Network activity suppression after addition of 25 ng/ml botulinum toxin. The graph shows the average network spike production per minute as a function of time derived from 37 discriminated units. Activity changes are determined as percent decreases relative to the native activity (Reference). **Graphs (below**) allow prediction of when activity will have decayed by 10, 50, or 90% from reference as a function of BoNT concentration.





A direct and simple test for protection of nerve cells by antisera to botulinum toxin*



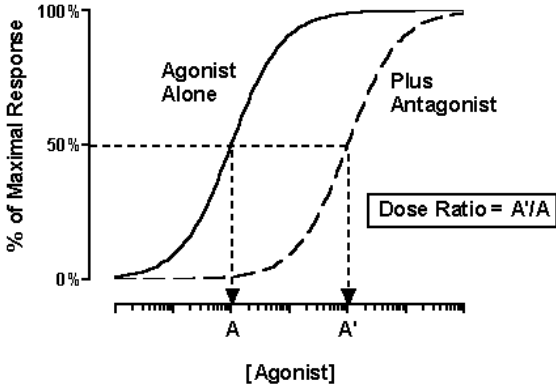
Protection of network activity with antisera pretreatment. The network maintained spontaneous activity for 40 hrs despite increases in BoNT concentrations from 100 to 200 ng/ml. Activity was finally stopped by 250 ng/ml. Without the serum, 90% of the activity would have been lost at 300 min. (white arrow). Codes: B: 40 uM bicuculline; S: application of antiserum at 112 min; T: time base switch from 1 min to 2 min. 50 ng/ml BoNT was added 20 min after application of antiserum.

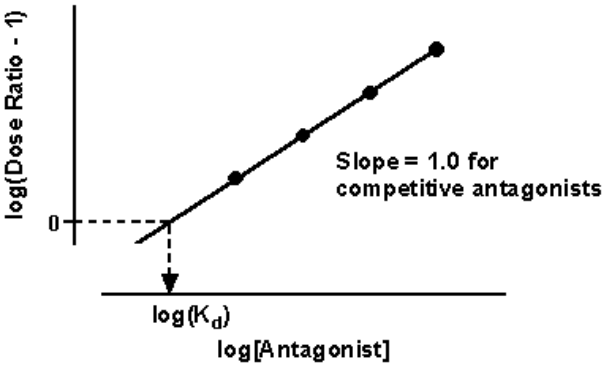
~100,000 mice are being used per year just for antitoxin research (Fort Detrick, MD)

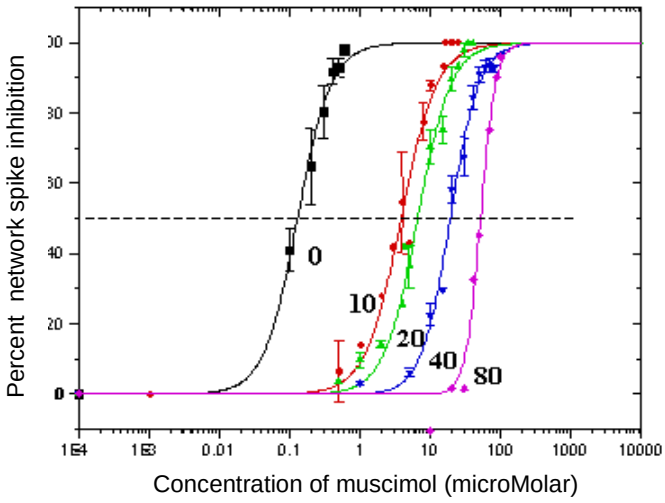
Pancrazio JP, Keefer E, Gopal K, Gross GW (2014) Botulinum Toxin Suppression of CNS Network Activity In Vitro. Journal of Toxicology, vol. 2014, Article ID 732913, 10 pages. doi:10.1155/2014/732913.

Quantitative Pharmacology: Determination of Dissociation Constants

The culture, life support, and multichannel recording methods have evolved to a level that allows the determination of dissociation constants (i.e. a measurement of molecular binding strength to a particular receptor). The approach uses the shifting of concentration response curves (shown below) to higher concentrations by the action of antagonists

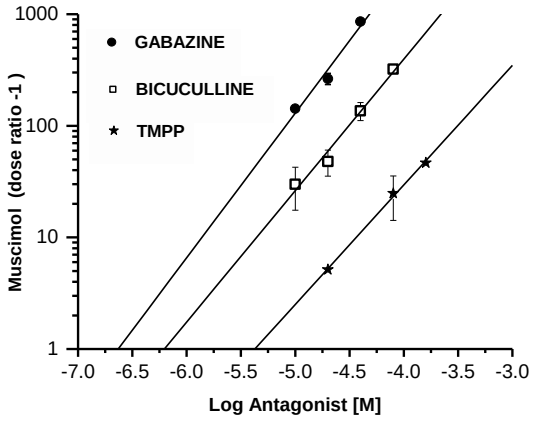






If the agonist and antagonist are competitive, the Schild plot will have a slope of 1.0 and the X intercept will equal the logarithm of the K_b of the antagonist.

Sabnam Rijal-Oli in her MS research shifted the dose responses of muscimol to the right with the antagonists bicuculline, gabazine, and trimethylolpropane phosphate. She was able to calculate the dissociation constants for all three antagonists.



Shild plot (left) and numerical results (table below). A comparison with other methods in the literature is given on the next page.

	Gabazine	Bicuculline	TMPP
pA2 (-log K_B)	6.64	6.21	5.40
K_B (X- intercept)	0.23 μM	0.61 μM	3.98 μM
R^2 (linear fit)	0.98	0.99	0.99

Comparison with data from established preparations

Compound	K_B (µM)	pA2	Animal	Tissue	References
Bicuculline	**0.62**	6.21	Mouse networks	FC	UNT/CNNS study
Bicuculline	**1.02**	5.9	Xenopus laevis	oocytes	Smart et al., J. Neuropharmacol, 1996
Bicuculline	**1.0**	6.0	Rat	Medial septal slice	Schneggenburger et al., J. Physiol, 1992
Bicuculline methoidodide	**1.2**	5.92	Rat	Cerebellar slices	Hussain et al., Gen Pharmacol, 1990
Bicuuclline methochloride	**0.79**	6.10	Rat	Hippocamplal slices	Kemp et al., Br.J Pharmacol, 1986
Bicuculline	**1.12**	5.98	Rat	cuneate nucleus slices	Simmonds et al., Eur J Pharmacol, 1982
Bicuculline	**1.12**	5.98	Rat	Dorsal funiculus fibres	Simmonds, MA.Eur. J.Pharmacol, 1982
Bicuculline methochloride	**1.3**	5.88	Rat	Dorsal funiculus fibers	Simmonds, MA.Eur. J.Pharmacol, 1982
Bicuculline	**4.4**	5.35	Rat	rat cuneate nucleus slices	Simmonds MA, Br.J. Pharmacol, 1978
Bicuculline	**1.7**	5.76	Rat		Ueno et al., J. Neurosci, 1997

Of the many methods used for the determination of binding constants, which will be considered most accurate and reliable?

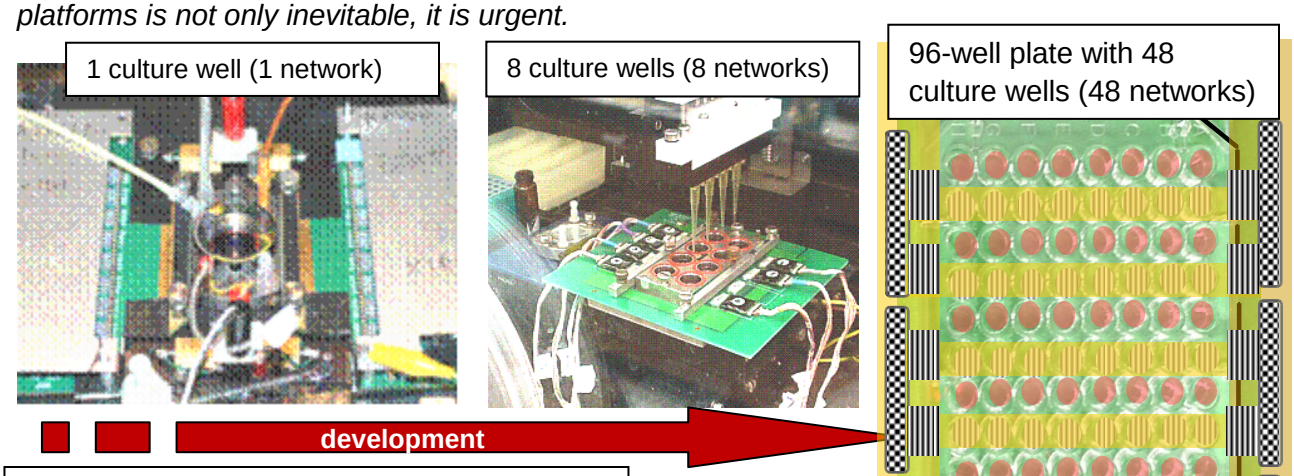
A strong advantage of this technique is that it determines binding constants in the receptors' native environment, in the presence of all the normal cell types, neuromodulators, transmitters, and while the system is in a functional, spontaneously active state.

Rijal-Oli, S. and Gross, G.W. (2008) Determination of dissociation constants using spontaneous neuronal network activity recorded with microelectrode arrays *in vitro.* Journal of Neuroscience Methods 173 (2008) 183–192.

Methods for High Throughput Toxicity and Neuroactivity Screening

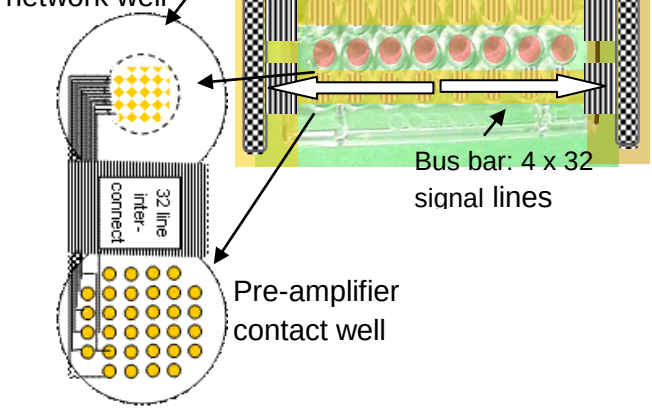
Rationale for High Throughput Screening

Of the thousands of chemicals distributed in international commerce, 93% are missing one (or more) set of internationally agreed upon tests, and 43% are missing all of these tests[1]. A similar problem exists with pharmaceuticals and new materials, such as nanotubes and C-60 fullerenes. The commercial ability to produce new compounds has far exceeded the ability to test them for safety. *The development and commercialization of new high throughput physiological screening platforms is not only inevitable, it is urgent.*



The 96-well plate has alternate rows of 8 wells each with arrays of 32 microelectrodes. Adjacent rows of wells have 32 contact pads for VLSI pre-amplifiers that will be inserted in each well. A bus bar carries signals to output connectors. In this manner, a standard 96 well plate can be used in a robotic system with 48 networks providing information in parallel.

Issued US Patent "MULTINETWORK NERVE CELL ASSAY PLATFORM WITH PARALLEL RECORDING CAPABILITY" UNT Ref: 10GG-2005-09 - ATTY Ref: UNTD-0010WO/US (122302.119). June 2013



**(EPA; Chemical Information Collection; Chemical Hazard Data Availability Study, June 23, 2004)*

A workshop (ICCVAM/NICEATM/ECVAM, Scientific Workshop on Alternative Methods to Refine, Reduce, and Replace the Mouse LD_{50} Assay for Botulinum Toxin Testing, Silver Springs, MD., 13-14 Nov 2006) emphasized the need for replacing the mouse LD_{50} assay with a more efficient safety testing technology. The conference reviewed the existing state-of-the-science

and current knowledge of alternative methods targeted to reduce, replace, and refine (less pain and distress) the use of mice for botulinum toxin testing and identify priorities for research, development, and validation efforts needed to advance the use of alternative methods. Neuronal networks on microelectrode arrays provide such an alternative method. One pregnant mouse with 12 embryos can provide neuronal cell pools that allow the seeding of up to 1,000 networks (if most regions of the central nervous system are used). This translates to1,000 assays; a tissue yield that is unprecedented.

Based on 30 years of research activities with neuronal networks on microelectrode arrays, faculty and staff that make up the Center for Network Neuroscience (CNNS) have the experience, know-how, core organization, international visibility, and technical contacts to address the challenges of working with a large number of parallel networks in the development of advanced multipurpose platforms for basic and applied neuroscience research, as well as for routine industrial compound screening.

SUMMARY of Pharmacological Applications

No single test system can provide all the answers.
However, some systems provide more simultaneous information than others.

NEURONAL NETWORKS on MICROELECTRODE ARRAYS report

cytotoxic, neurotoxic, and functional effects.

This platform provides high content data. Combined with high throughput technology it may become the leading screening platform in toxicology, pharmacology, and drug development.

DEFINITIONS

CYTOTOXICITY:	death of all types of cells
NEUROTOXICITY:	death only of neurons or some subpopulations of neurons *(eg. Parkinson syndrome)*
FUNCTIONAL TOXICITY:	loss of electrical functions in the absence of neurotoxicity *(e.g. tetrodo-toxin; botulinum-toxin)*
CHANGES in NEUROACTIVITY:	the influences of ALL medicines and drugs are reflected in the activity changes of networks
RECEPTOR REGULATION:	reflected in changes in dose response curves

NOTE: In addition, electric, magnetic, and other physical exposures reach “toxic” levels at some dose. Such exposures can be quantified *in vitro* before animal experiments commence.

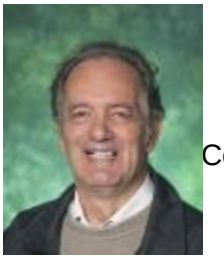
Theory, Modeling, & Computational Neuroscience

We will not understand information processing in the brain until we understand pattern processing in small networks.

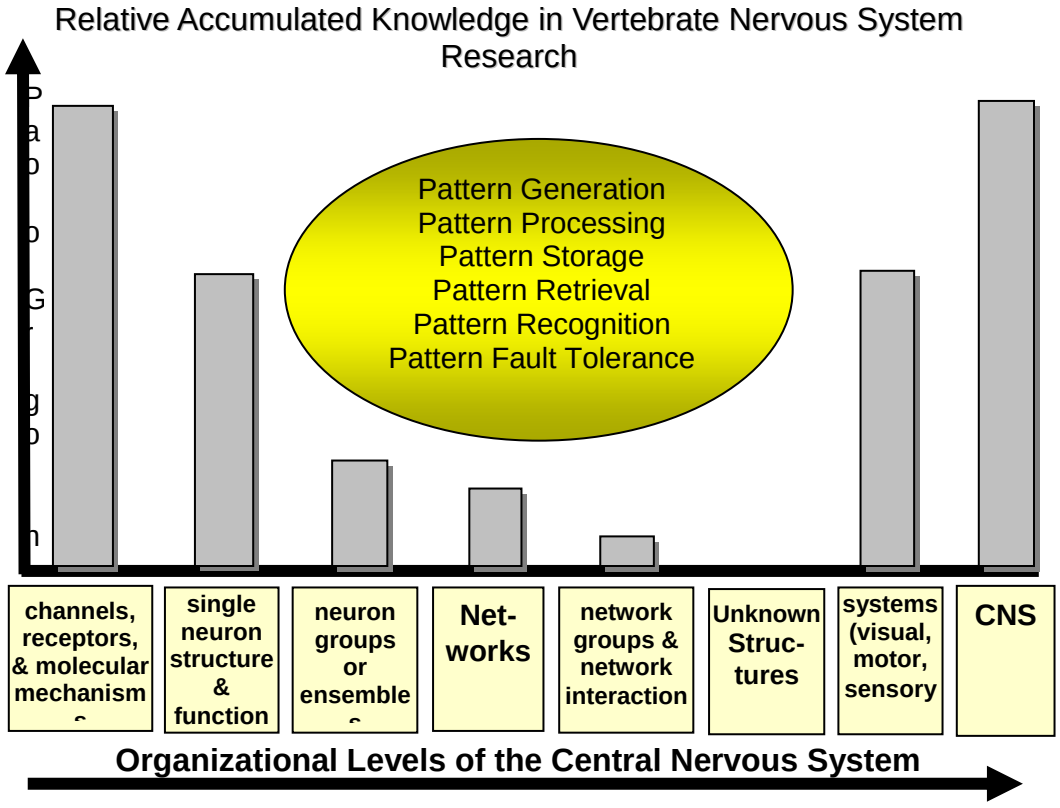
Michael Ham, Los Alamos

Jacek Kowalski
UNT

Luis Bettencourt, Los Alamos N
Greg Stephens, Princeton G.W. Gross, UNT



Paolo GrigolinI, UNT
Center for Complex Systems



A focus on networks and complex systems

Of all the organizational levels of the brain, the network level is least understood.

Theoretical constructs of brain function based only on cellular properties face almost insurmountable complexities due to the vast number of synaptic constellations and the extremely variable morphological influences on temporal and spatial summation of membrane voltage fluctuations. *It is reasonable to suspect that the small network provides an effective study system of population dynamics that does not require detailed knowledge of all cellular and synaptic* components. It is now possible to extract vital new information from small network growing on microelectrode arrays (MEAs).

The MEA technology and methodology described in this brochure focuses on such small networks of nerve cells. In vertebrates, neuronal populations and not single neurons may be considered the "fundamental processing unit" of the brain. Basic pattern generation, processing, and fault tolerance emerge on the small network level and information processing in neural tissue cannot be effectively explored without including such fundamental emerging network properties.

The CNNS has made substantial contributions to Theoretical Neuroscience. However, to cover this area adequately will require another brochure.

Below are some key publications that reflect our efforts in this important subdomain of neuroscience.

Kowalski, J.M., Ansari, A., Prueitt, P., Dawes, R., and Gross, G.W. (1988) On synchronization and phase locking in strongly coupled systems of planar rotators. Complex Systems 2: 441-462.

Kowalski, J.M. and Gross, G.W. (1990) Coupled relaxational oscillators as a model of spontaneous activity in neuronal networks. Proceedings of the International Joint Conf. on Neural Networks, Paris, Vol 1, pp 488-491.

Kowalski, J.M., Albert, G.L., and Gross, G.W. (1990) On the asymptotically synchronous chaotic orbits in systems of excitable elements. Physical Review A, 42: 6260-6263.

Gross, G.W. and Kowalski, J.M. (1991) Experimental and theoretical analyses of random network dynamics. In: Neural Networks, Concepts, Application and Implementation, Vol. 4 (Antognetti and Milutinovic, eds), Prentice Hall, N.J., pp 47-110.

Kowalski, J.M., Albert, G.L., Rhoades, B.K., and Gross, G.W. (1992) Neuronal networks with spontaneous, correlated bursting activity: theory and simulations. Neural Networks 5: 805-822.

Tam, D.C. and Gross, G.W. (1994) Extraction of dynamical changes in neuronal network circuitries using multi-unit spike train analysis. In: Enabling Technologies for Cultured Neural Networks (D.A. Stenger and T.M. McKenna, eds), Academic Press, N.Y.

Gross, G.W. and Kowalski, J.M. (1998) Emergent dynamical properties of biological neuronal ensembles and their theoretical interpretation and significance. AIP Conf. Proc. 437: 577-594.

Gross, G.W. (1998) Emergent Properties of Self-Organizing Nerve Cell Ensembles in Culture. Proc. of the 4th Internat. Conf. on Intelligent Materials, Tokyo, Japan. pp 326-329.

Gross, G.W., Kowalski, J.M. and Rhoades, B.K. (1999) Spontaneous and evoked oscillations in cultured neuronal networks. In: Oscillations in Neural Systems (D. Levine, V. Brown, T. Shirey, eds) Erlbaum Assoc., N.Y.pp 3-29.

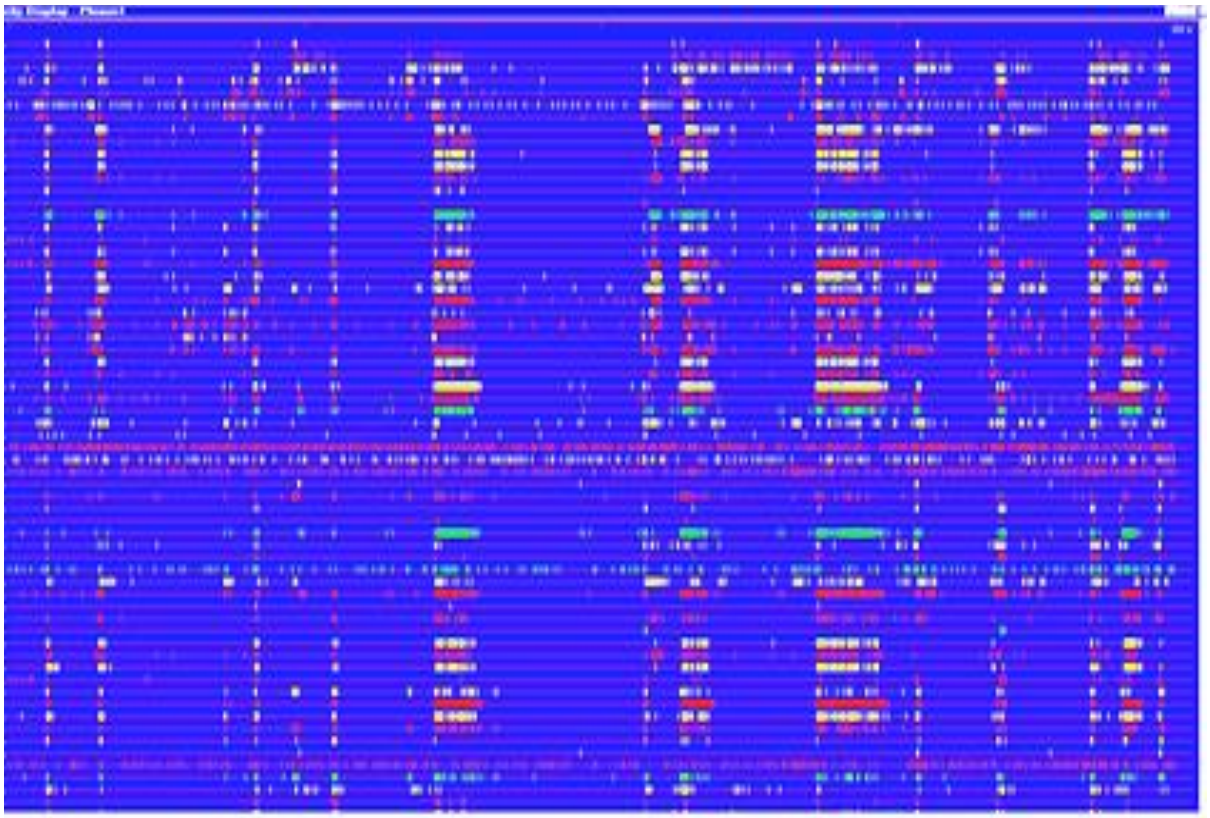
Gross, G.W. and Kowalski, J.M. (1999) Origins of activity patterns in self-organizing neuronal networks in vitro. Journal of Intelligent Material Systems and Structures 10: 558-563.

Turnbull, L. and Gross, G.W. (2005) The string method of burst identification in neuronal spike trains. J. Neurosci. Meth. 145: 23-35.

Bettencourt L.M, Stephens, G.J., Ham, M., and Gross, G.W. (2007). Functional structure of cortical neuronal networks grown *in vitro.* Physical Reviews E **75**, 021915:1-10.

Ham, M., Bettencourt, L. M., McDaniel, D, and Gross, G.W. (2008). Spontaneous coordinated activity in cultured networks: Analysis of multiple ignition sites, primary circuits, and burst phase delay distributions. J. Computational Neuroscience 24: 346-357.

Ham, M.I., Gintautas, V., and Gross, G.W. (2008) Spontaneous coordinated activity in cultured networks: Analysis of multiple ignition sites, primary circuits, burst phase delay distributions, and functional structures. Proceedings of the 6th International Meeting on Substrate-Integrated Micro Electrode Arrays. pp. 29-32.



The mystery of intelligence, behavior, and control of organs - such as the heart and muscles- lies in the generation of spatio-temporal patterns of a myriad of electrical pulses called "action potentials". Nerve cell networks, whether inside the organism as part of the intact nervous system or outside the animal growing as isolated systems on microelectrode arrays (MEAs), are always spontaneously active and form such patterns. We can now monitor such patterns but know little about the basic mechanisms that generate, shape, store, and trigger such patterns.

Whether driven by interest in basic neuroscience research or by lucrative applications in electronics or computer design, this research area is of fundamental importance.

Applications to Traumatic Brain Injury (TBI)

Quantification of Damage Thresholds *in vitro* and Methods of Recovery Enhancement

TRAUMATIC BRAIN INJURY is the leading cause of death in America between 1-44 years of age [1]. This includes military personnel exposed to blast concussions and other head injuries. 5.3 million US citizens are living with TBI-related disabilities

The severity of consequences and the enormous cost associated with medical care and social disruption make accelerated research efforts essential and urgent.

Despite extensive research efforts on TBI the
> complexity,
> high number of variables out of the control of investigators,
> non-standardized data,
> and limited quantification,
have made this research area difficult and frustrating.

This complex pathology requires research on all levels:
from holistic brain injury to cellular and even synaptic disruption.

(Reviews: Chen et al 2009 ; Bruns and Hauser, 2003; Dixon et al.,1987),

MILD BRAIN INJURY

(1) Physical evidence usually does not exist (standard CTI or MRI scans are not sensitive enough).

(2) The damage is microscopic and causes no bleeding.

(3) Diffuse axonal damage after minor head injury has been demonstrated (Povlishock et al., 1983; Greer et al., 2012).

(4) Diffuse brain injury now also includes:
inflammatory responses (Kelley et al. 2007) &
calpain-mediated cytoskeletal changes (McGinn et al.,2009).

In the animal (or patient), it is extremely difficult to identify mild TBI and link it to functional deficits.

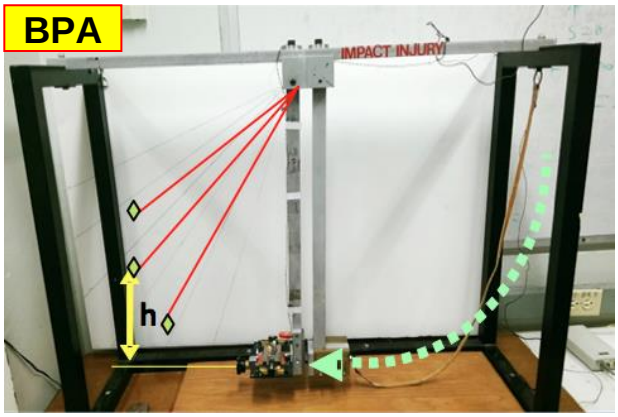
Such investigations are possible *in vitro* where cellular damage can be linked to functional damage and quantified.

Most important is the ability to study recovery strategies through biochemical and pharmacological interventions.

The highly controlled physical and biochemical environment allows long term studies of interventions to enhance recovery.

The exact impact forces causing specific responses may differ between in vitro and in situ models, **but the recovery from morphological and cellular damage as well as the efficacy of interventions can be very effectively determined in vitro and should be scalable to the animal model.**

Methods and Preliminary Data

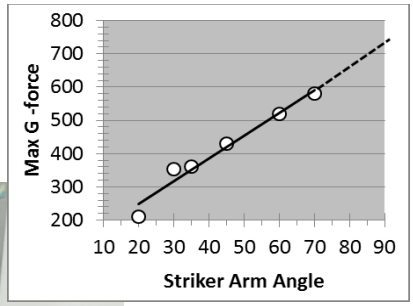


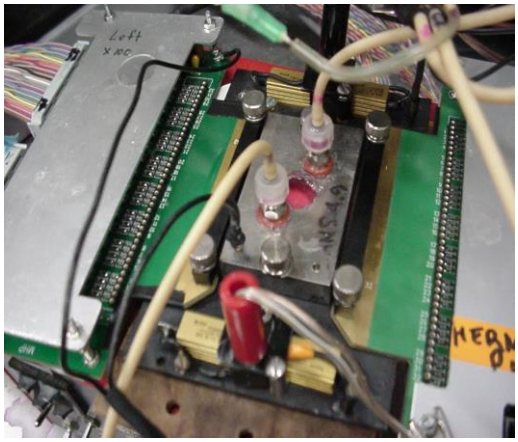
Prototype ballistic pendulum apparatus (BPA). Left arm: target with network in recording chamber; right arm: striker. g-forces can be estimated form the height reached by the target arm (h) caused by the peak kinetic energy upon impact: ½ mv^2 = mgh, where v is the maximum velocity reached immediately after impact.

$V_{max} = [2gh]^{1/2}$

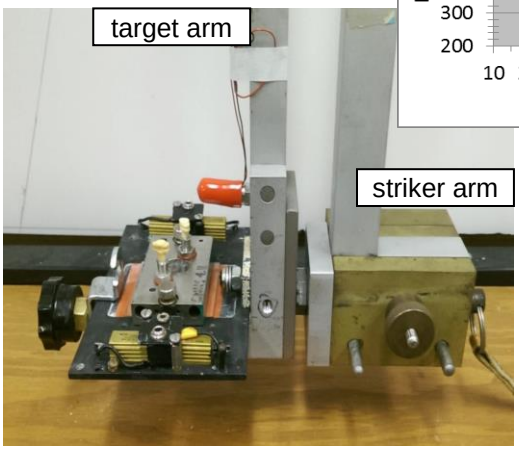
$a = (V_{max} - Vo) / t_{max} - t_o$

$a = (2gh)^{1/2} / t$.

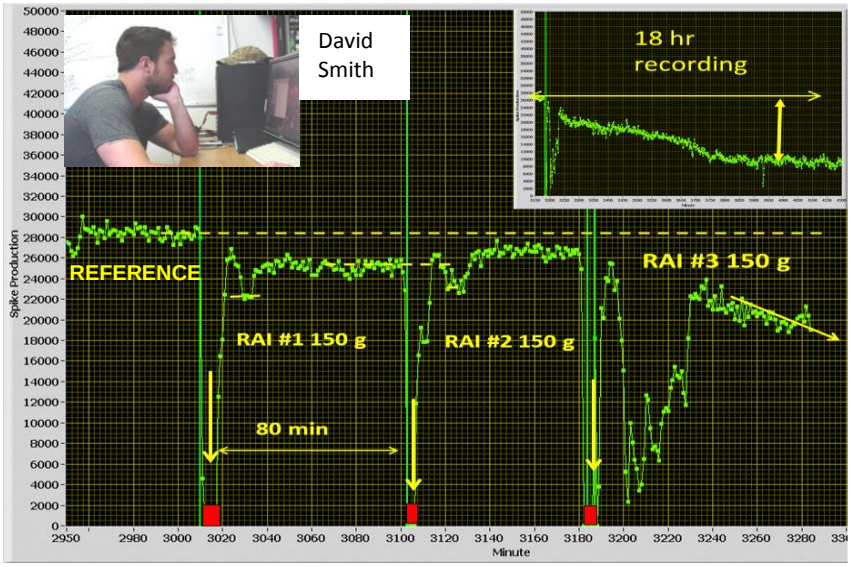




Assembled MEA in flow chamber and on microscope stage.



Chamber and base plate disconnected from amplifiers and attached to target arm.



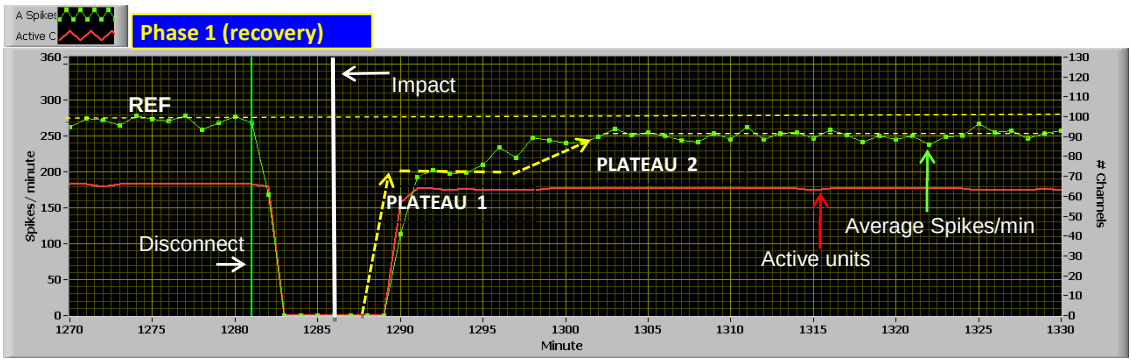
Network responses to MULTIPLE IMPACTS. The figure to the left shows total network activity per minute (green trace). Activity recovers temporarily to within 10% of reference, forms a 6 min depression at 20% of baseline, and produces a new baseline 12% below reference. NOTE: This represents activity reduction and not a loss of channels. RAI 3 (~150 g): A short recovery peak followed by a 26 minute major activity suppression (max. 90%). Thereafter, activity recovered to 80% of Ref., followed by a slow decay to 27% of reference over 18 hours (see Insert). Data: David Smith, 2014.
*RAI: rapid acceleration injury.

In the fall of 2014, David Smith joined "Smith and Nephew, Ltd", and the project was transferred to **MS student Edmond A. Rogers**.

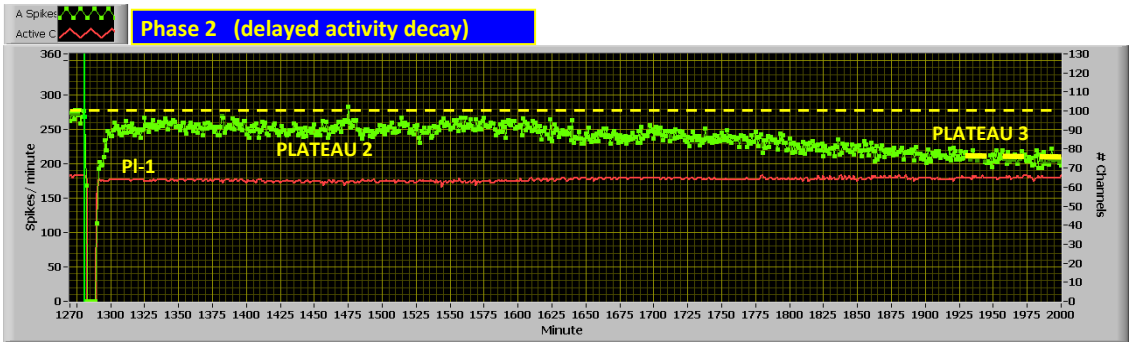
Edmond Rogers

Subsequent extensive experimentation showed rapid progress and a welcome consistency in response profiles. The dominant profile is a two phase, three-plateau activity response shown in below**.** Phase 1 consist of two activity plateaus: Pl-1 is characterized by a rapid return of activity (1 to 2 minutes) that remains stable for 5 minutes at 25% below reference; Pl-2 stabilizes at 10 % below reference for five hours, preceded by a gradual increase over a 7 min period. Phase 2 describes a subsequent slow activity loss to approximately 40% of reference over 24 hours, stabilizing as Plateau 3. This long-term damage profile appears stable and has not yet shown activity recovery to Reference in any experiment.

A



B



Dominant response profile **(**example: ER52). **(A)** Phase 1. A brief (5 min) partial activity recovery to 28% of reference (Plateau 1), is followed by a 4 minute climb (slope= +12.5 spikes per min) to within 10% of reference, where activity remains stable for ~300 min (Plateau 2). Dashed yellow lines represent established activity plateaus. REF is native culture spontaneous activity. **(B)** Phase 2. Expanded time scale of Phase 1 showing ~300 min Plateau 2 stabilization followed by a subsequent decay at 0.06 spikes per minute to Plateau 3 at 30% below reference activity with 98.5% of active unit retention. Note: Dead time includes temperature increase to 37°C before amplifier reconnect. The number of active units stays constant (red line), implying no cell death or major disruption of cell-electrode coupling.

The profile data to date has been summarized in Table 1 below. The Table shows results from 12 two-phase experiments, which includes one preliminary experiment by David Smith (2014). In all cases, plateau 1 represent a decrease, plateau 2 a partial recovery, and plateau 3 a subsequent delayed activity decay. These are major response features that are consistent. Although standard deviations are still relatively high, technical problems such as measuring acceleration or measuring response angles play a role in the data fluctuations.

			PLATEAU 1			PLATEAU 2			PLATEAU 3	
EXP #	**FORCE Angle/G**	**Units Lost**	**Time to P1 (min)**	**% Dec from REF**	**Time Durat. (min)**	**Time to P2 (min)**	**% Dec (Ref)**	**Time Durat (min)**	**Activity Decr.**	**start AT hrs post Impact**
DS 028	80/ 640	2/40	2	-63 %	18	23	- 19%	90		
ER 09	**35/ 350**	**0/49**	2	**-11%**	4	2	**-8%**	10	stopped	
ER 10	**60/ 520**	**18/48**	2	**-34%**	14	6	**-20%**	60	-50%	17 h
ER 12	**60/ 520**	**0/100**	3	**-28%**	8	11	**-17%**	>40	stopped	
ER 18	**60/ 520**	**0/44**	2	**-15%**	6	5	**- 5%**	35	no decay	P3
ER 21-1	**60/ 520**	**0/11**	2	**-17%**	6	9	**-5%**	60	stopped	
ER 24-1	**30/ 350**	**0/45**	2	**-40%**	5	2	**29%**	30	-69%	6.3 h
ER 28	**30/ 350**	**0/29**	1	**-21 %**	4	2	**-6%**	450	-12%	10.9 h
ER 30	**30/ 350**	**4/15**	2	**-28%**	5	4	**-14%**	100	-37%	10 h
ER 39	**30/ 350**	**1/20**	2	**-19%**	5	13	**- 4%**	10	-38%	16 h
ER 50	**60/ 520**	**0/28**	2	**-29 %**	6	4	**- 9%**	270	-15%	11 h
ER 52	**60/ 520**	**1/65**	2	**-28 %**	5	8	**- 10%**	300	-40%	17 h
				24.5±8.7%SD			**12.4±9.1%SD**			**37.2±19.6%SD**

Table 1. A current table of impact experiments, including one experiment from a former CNNS member (DS 028, Smith and Gross, 2014). Red numbers reference the relatively large number of units lost in one experiment. The 'Units lost' column shows the units that could not be recovered after impact with the total number of selected discriminated units during the reference period. "Angle" refers to the initial angle before release of the striker arm. Acceleration g's were measured but the accelerometer has not been calibrated. All changes are compared to the reference activity and all are negative. Positive changes (increased activity) have not yet been seen.

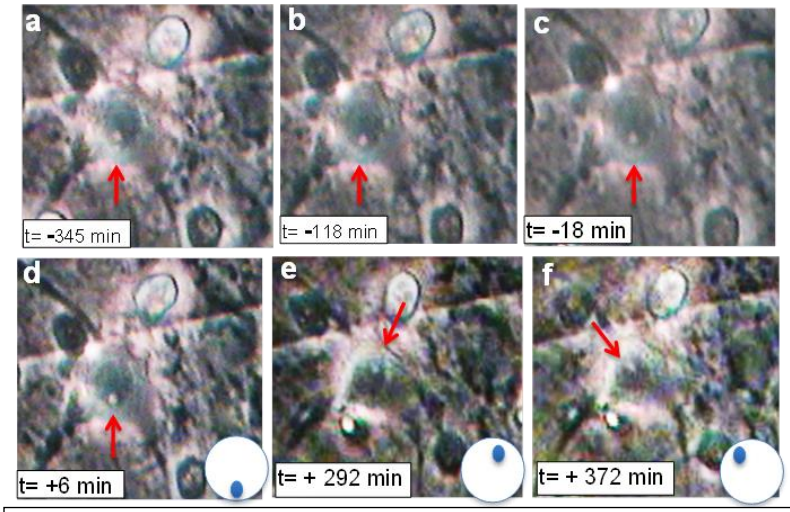
Phase 2 and the associated Plateau 3 were often truncated (stopped) to prepare for a second impact or due to failure of the life support system. The last column refers to the time post-impact when a level plateau is re-established, albeit always below the reference. ER18 showed a gradual activity decay to that never formed a plateau 3.

Movement of Nucleus after Impact

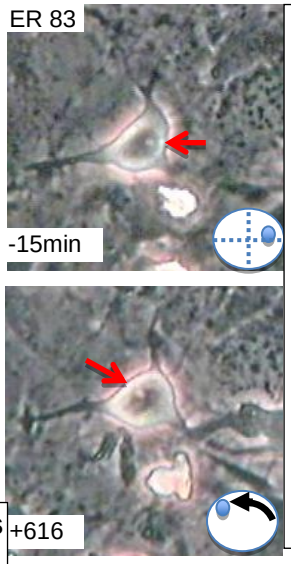
The investigations by Edmond Rogers revealed an unexpected morphological response of major significance. **Nuclei in nerve cells began to rotate after impact with a delay of 30 to 60 min**.

In highly polarized cells, such as nerve cells, the nucleus must be stable to ensure specific protein trafficking into axons or dendrites. Inappropriate transport of proteins to the wrong target can cause loss of function in the absence of cell death. Synaptic dysfunction, for example, results in memory loss and performance or behavioral changes. Whereas we expected immediate nuclear displacement after impact and cytoskeletal stress, such a response was not seen frequently. Instead, the delayed nuclear rotation suggests release of calcium ions from internal stores and gradual enzymatic weakening of the cytoskeleton and, especially, the anchoring of the nuclear membrane to the cytoskeleton. Such a delay would explain the observed Phase 2 deficits in activity.

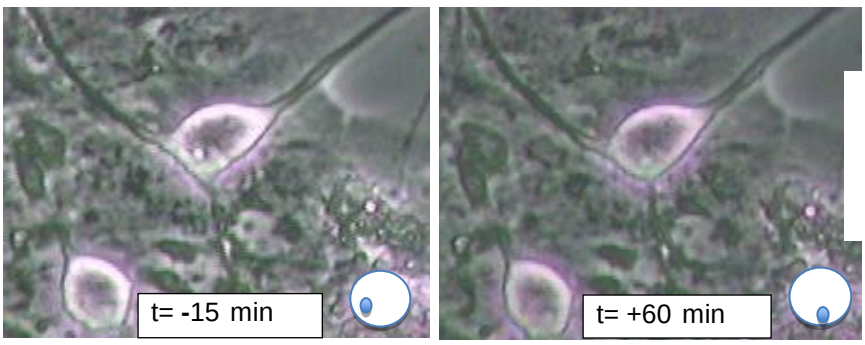
It is not known how the cell would repair such a transport misalignment but the in vitro method described in this section makes it feasible to systematically explore pharmacological interventions that may speed up recovery or prevent further damage after impact.



EXP ER48. Rotation of nucleus after impact at t=0. **Nucleus is stable for 345 min before impact.** Rotation did not start until 100 min after impact. Rate: 1.4 deg per min.



EXP ER83: 150 º Rotation CCW.



EXP. ER 100: 40 º CCW rotation in 60 min

TOTAL MORPH. EXPERIMENTS	***number 28***	***Nuclear rotation***
Time lapse	*5*	*80%*
control (no I)	*3*	*0%*
Micrographs	*14*	*93%**
Controls (no Impact)	*1*	*0%*
MEA breakage	*5*	*N/A*

Table 2. Summary of morphological observations listing time lapse experiments and manual micrographs. In both cases nuclear rotation was observed in 83% and 93% of the observations, respectively.

*Multiple cells per experiment

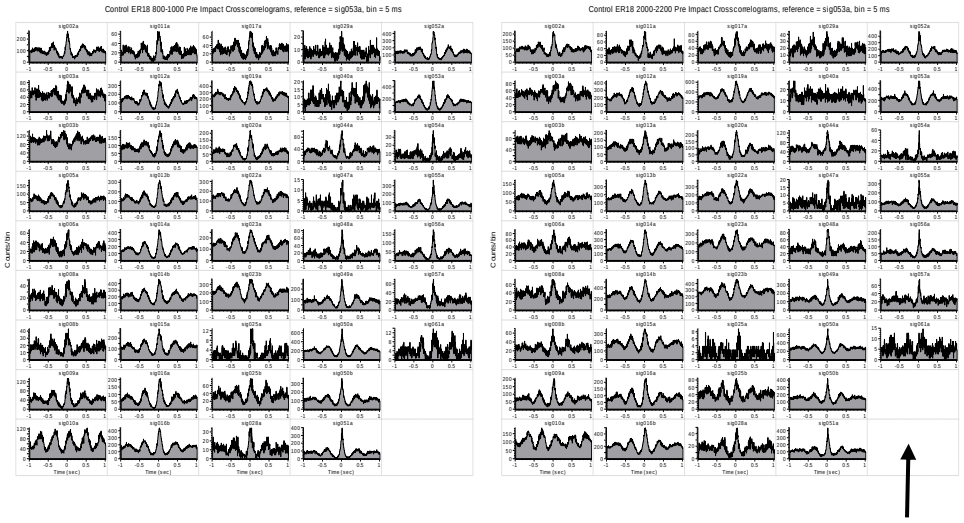
SUMMARY

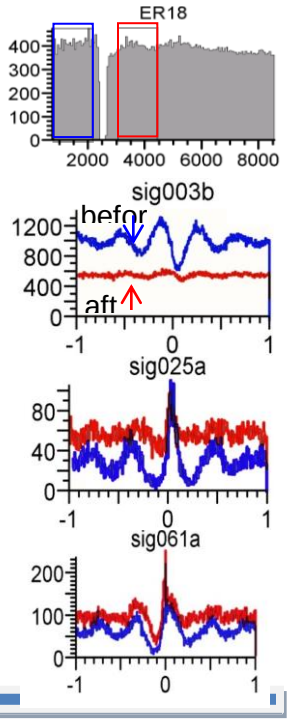
(1) No significant loss of adhesion has so far been observed (300g max.). Activity loss is indicative of cellular damage, possibly due to:
(A) Membrane Microporation;
(B) Synaptic Damage:
Preliminary data from cross-correlograms *suggests synaptic damage.*

(2) Network spike production and spike patterns are affected *before* cell death occurs.

(3) Changes in network action potential production and types of patterns expressed are *cell culture correlates* of changes in animal performance and behaviour.
This method of injury reveals subtle, repeatable, electrophysiology alterations, some of which may not be reversible.

CONTROL: **stable cross correlation patterns during reference period**

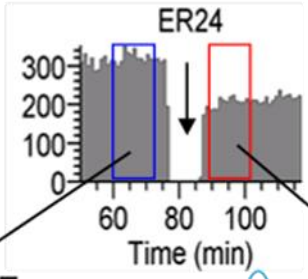




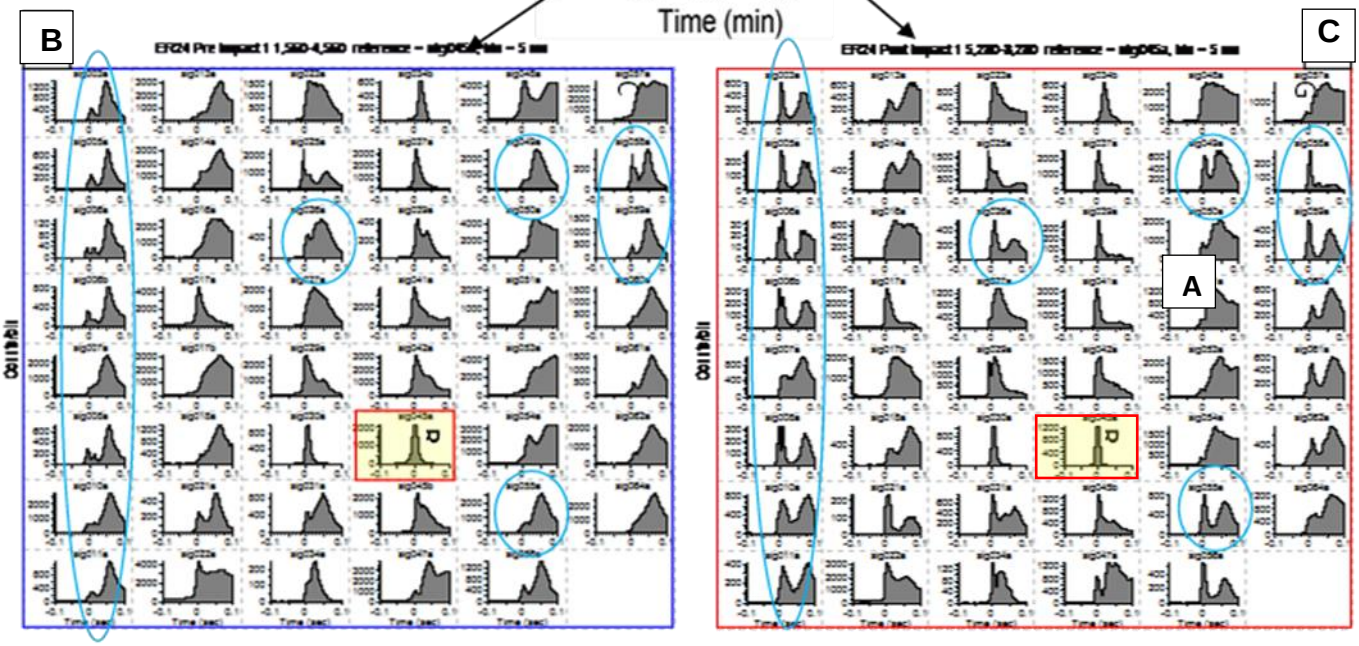
ER18 43 channel correlations 20 min apart BEFORE Impact: 200 sec data files.

Strongly damped oscillations after 400 G impact (red trace) ⟶

Experiment ER24
Cross correlation profiles from 15 minute data segments before and after impact (identified by rectangles in the population vector: A).



Major changes in the CCP's are evident before (B) and after impact (C)



Detailed features cannot be quantified due to the complexity of the profiles. The major qualitative changes are an enhanced spiking close to the zero point (see first column) and a general development of a biphasic response profile. NOTE: culture was under 40 microMolar bicuculline.

Objectives

Strategic Goals for the next 5 Years

1. Develop high throughput 8, 16, and 48 network platforms for parallel analysis, complete with automated life support, data analysis and display, and a sophisticated, user-friendly interface.
   Application Areas: industrial and environmental toxicology, quantitative pharmacology, drug development.

2. Expand the current platform technology for use as broad-band biosensors. The systems proposed are not olfactory systems but rather physiological neuronal sensors. They show parallel alteration in activity at concentrations that also affect mammals. As living tissues, they respond to unknown or uncharacterized compounds and agents. Current technology sensors require a molecular signature and *a priori* knowledge of what agents may be used. They cannot respond to unknown agents.
   Application Area: Homeland Security

3. Expand platforms for use with non-neural tissues that are electrically active (pancreatic cells, cardiac cells).
   Application Area: Medicine & Disease States

4. Provide single network recording platforms with 256 (or more) electrodes for high density interrogation of network dynamics. These systems will be designed for basic research in network physiology, network pattern processing, theoretical models, and computational neuroscience.

5. Modify the proposed platforms to allow long-term testing. Neuronal networks can live for over 9 months in culture and chronic testing is possible if the appropriate long-term life support conditions are met.

6. Develop an impedance spectroscopy capability to measure cell layer impedances with primary application to CANCER CELL proliferation and pharmacological arrest or destruction.
   Application Area: Patient- and cancer tissue- specific drug efficacy evaluation.

7. **Validate the in vitro Traumatic Brain Injury method and initiate studies of pharmacological and physical interventions that enhance recovery.**

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Sponsors are recognized in publications for their supporting donations.

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