Braincubator: an incubation system to extend brain slice lifespan for use in neurophysiology

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Abstract— **In vitro brain slice preparations are instrumental in developing our understanding of the nervous system. However, the current lifespan of an acute brain slice is limited to approximately 6-12 hours. This reduces potential experimentation time and leads to considerable waste of neural tissue. We have designed, developed and tested a novel incubation system capable of extending the lifespan of these brain slices. This is done by controlling the temperature and pH of the artificial cerebral spinal fluid in which the slices are incubated while continuously passing the fluid through a UVC filtration system. This system is capable of maintaining extremely low bacterial levels and significantly extending the brain slice lifespan to at least 24 hours. Brain slice viability was validated through electrophysiological recordings as well as live/dead cell assays.**

I. Introduction

Much of our understanding of neural function is derived from in vitro brain slice preparations. This experimental technique has been employed since the early 1950's [1]–[3] and has become a fundamental and powerful method, instrumental in answering a huge variety of neuroscience questions from brain anatomy to neural electrophysiology [4]–[7].

In vitro brain slice preparations offer a rapid means of examining metabolic and electrophysiological properties without contamination from anesthetics, relaxants or intrinsic regulatory substances. Unlike cultures or cell homogenates, brain slices maintain the brain structure allowing the study of neural circuits and networks in isolation [4], [8], for example, the thalamocortical pathway [9], [10]. In vitro electrophysiological recordings are not affected by heartbeat or respiration, allowing longer periods of cellular recording compared to in vivo methods. As cells may be directly visualized, researchers can locate, identify and access particular cells of interest [11].

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The use of in vitro brain slice preparations has considerably advanced our understanding of the central nervous system and will continue to do so for many years to come. However, this experimental method is not without its limitations. One of the major constrains in the use of brain slices is their relatively short lifespan. A typical brain slice, e.g. from a rat or mouse, may be expected to be in a healthy usable state for a period of only 6-12 hours. If hippocampal or neocortical thin slices are used, this period may be as short as 4 hours [12].

Extending the lifespan of brain slices would have several benefits for researchers. As experiments can take extended periods of time to complete, brain tissue is often wasted, as cells are dead by the time the slice can be used. By removing to some degree the time critical nature of this work more slices may be used. This would increase the quantity of experiments that may be conducted with a single animal. It would also reduce both the number of animals required and researcher time spent preparing slices over the course of the entire research study.

Several factors affect the longevity of a brain slice. They are environmentally defenceless and susceptible to changes in pH, temperature, oxygen, glucose levels and bacteria [13]–[17]. Bacteria numbers display a characteristic four-phase pattern of growth [18]. Initially growth is slow (Lag Phase) during a period in which the bacteria adapt to their environment. This is followed by a period of exponential growth (Log Phase) during which bacteria numbers double with every replication cycle. As numbers increase the supply of nutrients becomes a limiting factor and the rate of multiplication equals the rate of death (Stationary Phase) before nutrient supply begins to run out and bacterial death occurs at an accelerating rate (Logarithmic Decline Phase).

Currently, researchers use a variety of ad hoc and largely uncontrolled vessels to store brain slices prior to use. We hypothesised that by controlling the brain slice environment (temperature, pH, oxygen and glucose) and maintaining low bacterial levels, we could considerably extend the lifespan of the slices. To test this hypothesis we examined the viability, electrophysiological response and bacterial load of brain slices treated normally and with environmental control via a newly developed incubation device, which we call a Braincubator.

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II. METHODS

A. Animals

We used 2-5 week old Wister rats in this study. All animals were healthy and handled with standard conditions of temperature, humidity, twelve hours light/dark cycle, free access to food and water, and without any intended stress stimuli. All experiments were approved by the University of Western Sydney committee for animal use and care (Animal Research Authority #A9452).

B. Slice Preparation

Animals were anesthetized by inhalation of isoflurane (5%), decapitated, their brains quickly removed and placed into ice-cold physiological solution (artificial cerebral spinal fluid, aCSF) containing (in mM): 125 NaCl, 2.5 KCl, 1 $MgCl_2$, 1.25 NaH_2PO_4, 2 $CaCl_2$, 25 $NaHCO_3$, 25 dextrose and saturated with carbogen (95% O_2 -5% CO_2 mixture; pH 7.4). Parasagittal brain slices (300 µm thick) were cut with a vibrating microtome (Camden Instruments, UK) and transferred to a holding chamber containing carbogenated aCSF for 30 min at 35 °C.

Sequentially, slices were either allowed to cool to room temperature (~22 °C) in the same recovery chamber (Control, built as reported by [11]) or transferred to the Braincubator. Slices were kept in the incubation system (either control or in the Braincubator) for at least 30 min before any measurement.

C. The Braincubator

This device allowed continuous and close control of the brain slice environment. A photograph of the device is shown in Fig. 1 and a block diagram in Fig. 2. Brain slices are held suspended on a mesh platform in a chamber containing aCSF (same as that used in slice preparation). This chamber also contained pH and temperature probes and was bubbled with carbogen to maintain a pH of 7.2-7.4.

A peristaltic pump continuously circulated the aCSF through a UVC filter (12ml/min) where it was exposed to 1.1W UVC light (254 nm, 5W/2 Philips Ultra Violet sterilizer lamp) to eradicate bacteria. The UVC light was programmed to turn ON for times varying between 15 and 26 minutes every 15 to 30 minutes. This was done to prevent excessive heating of the aCSF and prevent the increase of bacterial resistance to UVC.

A Peltier thermoelectric cold plate cooler (TE technology, Traverse city, MI) in series with the other elements enabled the solution to be maintained at 16 °C.

D. Bacteria Culturing

Brain slice samples were collected from either the Braincubator or control recovery chamber at set time points (1, 6, 12, 16, 20 and 24 hrs) after slicing. The brain slice and 1 mL of aCSF solution was aspirated from each group by sterile syringe, smashed into a thick suspension and centrifuged (1000 rpm for 5 min). Supernatant was collected, diluted and cultured on agar plates for 24 hrs at 37 °C. Following incubation, colony-forming units (CFU) were counted and inspected qualitatively for common colonies.

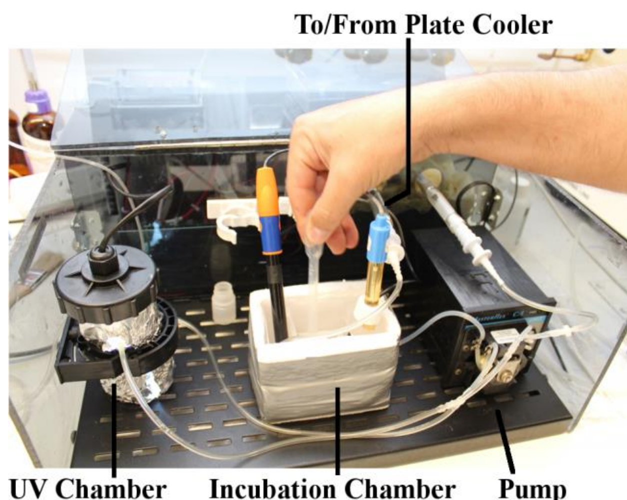

Figure 1. Braincubator.

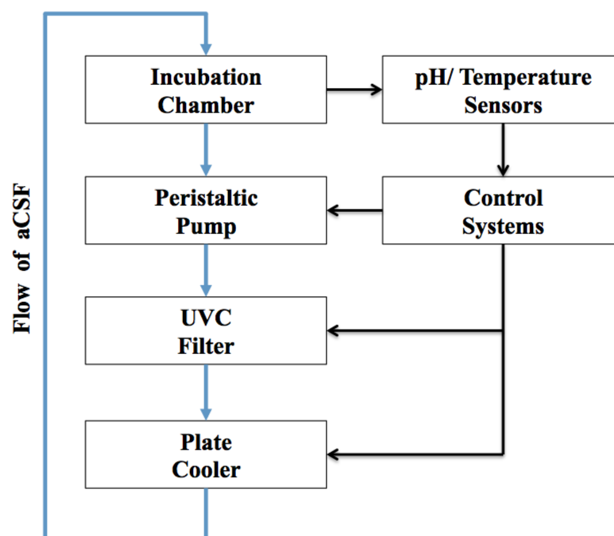

Figure 2. Block diagram of aCSF and control flow in the Braincubator.

E. Brain Slice Viability

Brain slices were incubated for 15 min with the selective dead cell fluorescent marker propidium iodide (PI, 1 µg/ml) [19] and co-incubated with the nuclear marker DAPI (1µg/ml) for total cell counts. Slices were subsequently washed with fresh aCSF for 10 min. Images were acquired using a Zeiss LSM-510 Meta confocal microscope (Carl Zeiss, Oberkochen, Germany) using a 40×-oil immersion objective in the inverted configuration. Z plane optical sections (4 µm) were taken at -20 to -70 µm depth from the surface of the cerebral cortex to produce an image stack. The DAPI signal was obtained using Argon laser excitation at 488 nm; PI was excited with 543-nm HeNe laser. Images were visualized using ZEN software and processed using ImageJ. Slice viability was assessed as the ratio between dead/total cells in the visual field.

F. Brain Slice Viability

The recording chamber was mounted on an Olympus BX-51 microscope equipped with IR/DIC optics. During

recordings the slices were kept at room temperature, ~22°C, and constantly perfused (2–3 ml/min) with oxygenated solution as reported previously [7]. Whole cell recordings were performed from the soma of layer 5 pyramidal neurons in the somatosensory cortex with patch pipettes (5–7 MΩ) containing (in mM) 130 K-Methansulfate, 10 HEPES, 0.05 EGTA, 7 KCl, 0.5 Na_2GTP, 2 Na_2ATP, 2 MgATP, 7 phosphocreatine, 0.1 Alexa Fluor-488 (Molecular Probes) and titrated with KOH to pH 7.2 (~285 mOsm).

Stimulation protocols were designed using the pClamp 10 software suit (Molecular devices, Sunnyvale, CA) and stimulation currents were injected through the recording electrodes. Voltages were recorded in current clamp mode using a multiclamp 700B dual patch-clamp amplifier (Axon instruments, Foster city, CA), digitally sampled at 30-50 kHz, filtered at 10 kHz, and analysed off-line using pClamp software. Access resistance was corrected on-line and recordings included if <30MΩ. Cells were considered suitable for analysis if the access resistance, input resistance and resting membrane potential did not change by > 20% during recording. At the end of each experiment, the location and morphology of neurons were examined by fluorescence microscopy and digitally recorded (ROLERA-XR, Q-Imaging).

III. RESULTS

Bacterial growth in control conditions demonstrated a typical lag phase of approximately 12 hours followed by exponential multiplication or log phase (Fig. 3). In the Braincubator, bacterial numbers were maintained in the lag phase for the duration of the experiment. Significant differences in bacterial count were found from the 12-hour point onwards ($p<0.01$). Two types of gram-negative bacteria were identified: Pseudomonas species and stenotrophomonas maltophilia

In terms of cell viability, brain slice deterioration was significantly reduced when incubated with the Braincubator compared to slices incubated in the control condition. Confocal microscopic serial images (Fig. 4) of brain slices following different incubation times are shown in Fig. 4. All sections were sliced at the same time. The combination of DAPI (blue, all cells) and PI (red, dead cells) allows the simultaneous visualization of all cells in the slice. Expansion of the marked areas after 24 hours under control conditions demonstrates changes occurring in morphology of some of the dead cells. This is probably due to membrane deterioration.

Following incubation of just six hours, a significant difference of the live/total cell ratio was detected (68±5% vs 56.4±4%; $p \leq 0.007$), reaching a maximal difference of 28% after 24 hours (45±4% vs 17±4%, $p<0.0001$). These changes over time can be seen in figure (Fig. 5).

All cells had the morphology of pyramidal neurons and respond to a depolarizing current stimulus with tonic, adapting patterns of action potentials, which categorize them as regular spiking (RS) neurons [20], [21]. Basic

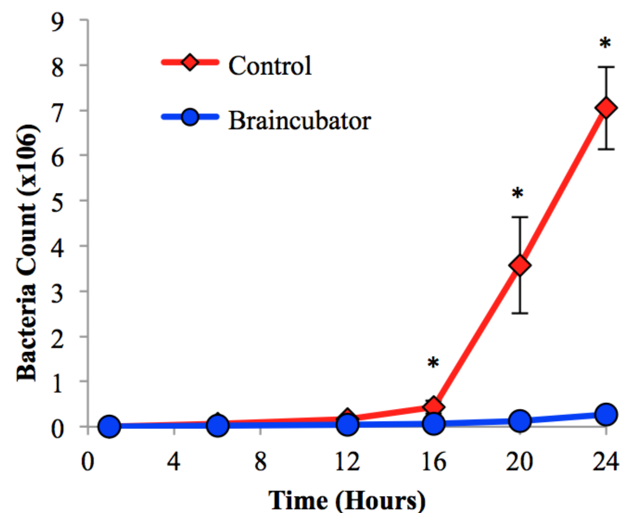

Figure 3. Brain slice bacterial growth over time using typical laboratory incubation (Control) and the Braincubator. Error bars are ±S.E. * denotes significant difference between control and Braincubator ($p < 0.05$).

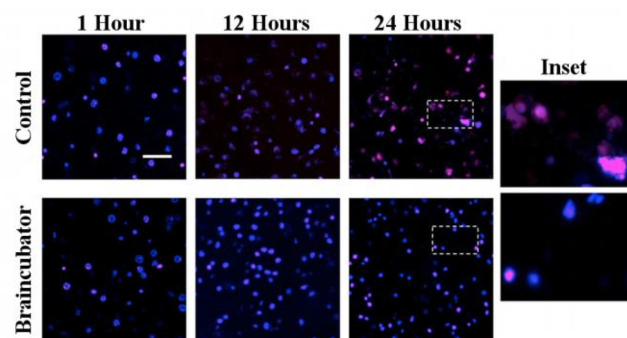

Figure 4. Confocal microscopic serial images (x40) of acute brain slices following different incubation time in control and Braincubator conditions. DAPI (blue, all cells), PI (red, dead cells), Scale bar =50 µm. Select marked areas of 24 hour images are expanded in inset.

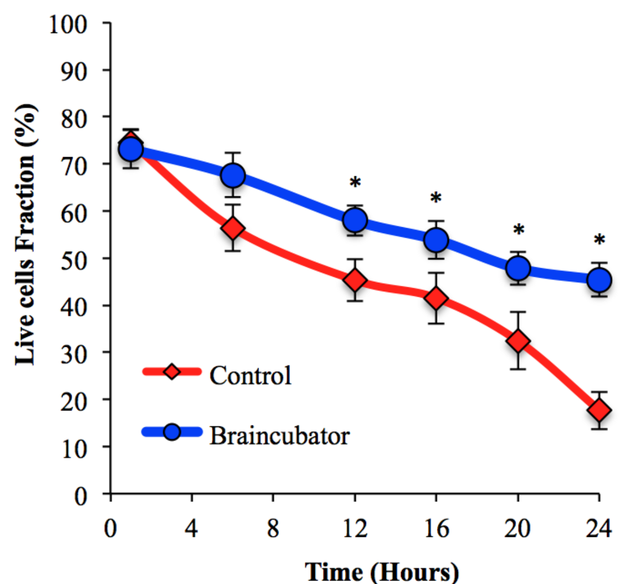

Figure 5. Change in live cell fraction over time using typical laboratory incubation (Control) and the Braincubator. Error bars are ±S.E. * denotes significant difference between control and Braincubator ($p < 0.05$).

electrophysiological parameters were monitored after 1 hour and 24 hours in the Braincubator. No significant differences were detected in terms of resting membrane potential, input resistance, membrane time constant, resonance frequency or first spike amplitude ($p > 0.05$ in all cases). Moreover, No significant differences were detected between neurons from slices that were incubated in the Braincubator for less than 3 hours and neurons incubated under control conditions for the same period of time.

IV. Discussion And Conclusion

In this work we investigated the use of environmental controls system, the Braincubator, to increase the lifespan of in vitro brain slices. We found that this use of this system had a highly significant effect on bacterial growth, maintaining low bacteria numbers and growth in the lag phase. The combination of low temperature (16°C) and UVC irradiation of the aCSF solution in the Braincubator will have led to this massive reduction in bacterial growth UVC irradiation leads to bonding of adjacent thymine bases, thus creating a dimmer that prevents DNA replication [22]. We found two species of mesophilic bacteria in our slices. These bacterial species have an optimum growth temperature of ~37°C and a minimum temperature of 10-15°C [23]. The lowered aCSF temperature will certainly have impeded bacterial growth.

Typically, a brain slice is useable up to a maximum of 12 hours post brain extraction. This point coincides with a live cell fraction of ~45% in our study. Using the Braincubator, the live cell fraction was maintained above this level for a further 12 hours. Indeed at this 24 hour time point we saw substantial morphological changes in the control cells but not in the Braincubator samples. Indeed, electrophysiological properties of pyramidal neurons were unchanged after 24 hours incubation in the Braincubator.

Based on the results of this experimental work we believe the useable lifespan of brain slices may be extended to a full 24-hour period, twice that currently achievable. Clearly, this will have an impact on the number of slices that may be used in experimental work before cell death. As a result the number of animals required for a given set of experiments may potentially be reduced or the number of results from a given number of animals increased substantially. Furthermore, preparation of brain slices can take a considerable period of time (up to 3 hours). As fewer animals may be required, the researcher may spend less time preparing slices and more time performing experiments. Currently no standardized method of incubating brain slices exists, a device such as the Braincubator may provide this capability and lead to greater repeatability in individual laboratories and between laboratories.

Several variables will have had an impact on the longevity of brain slices in our experiments. Intensity and duration of UVC exposure, aCSF flow rate, temperature and pH can all be expected to have a marked effect. Future work is required to investigate the impact of these individual parameters with the aim of finding an optimal setup for greatest longevity.

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