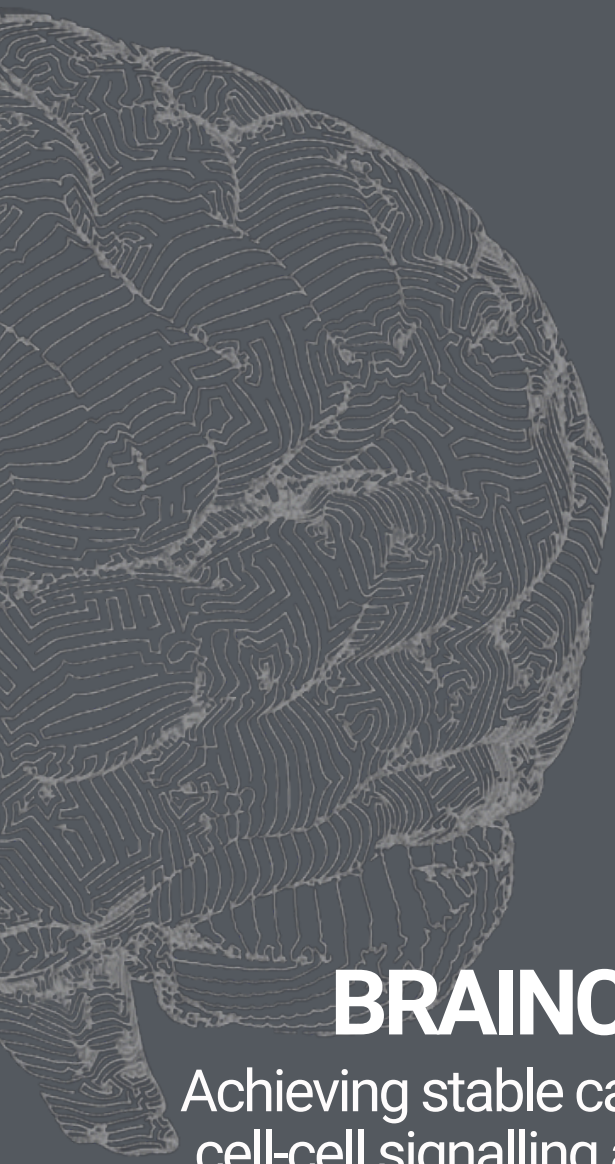
APPLICATION NOTE



BRAINCUBATOR™

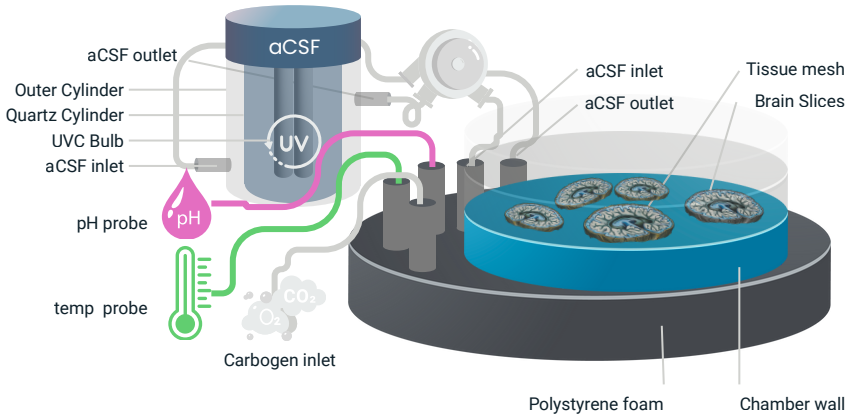
Achieving stable calcium events and cell-cell signalling across slices over 24h as assessed via Ca2+ imaging

Morven Cameron , Orsolya Kékesi , John W. Morley, Jonathan Tapson, Paul P. Breen, André van Schaik, Yossi Buskila

This study examined the **activity of acute brain slices and excised retinae** loaded with calcium indicators **up to 24 hours post tissue dissection**. Collected slices were incubated for varying periods using the **Braincubator,** which **regulates pH, temperature, carbogen flow, and continuous fluid decontamination to precisely mimic the physiological levels exhibited in the brain**. Calcium dynamics were analyzed using Fura-2 AM and Fluo-4 AM dyes.

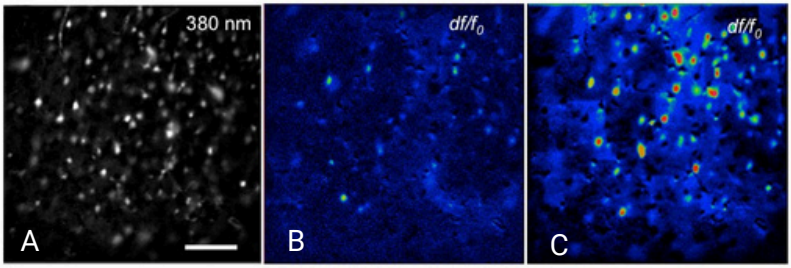
In order to compare between groups, cells were classified into three categories based on calcium signaling: Loaded Cells (LC), Spontaneously Active Cells, and Evoked Cells. The LC portion remained stable across all slices over 24 hours, indicating dye and membrane integrity. Furthermore, there were **no significant differences in the percentage of spontaneously active cells or their calcium event frequency between slices imaged at <4 hours and >24 hours post-slicing**. Similarly, evoked calcium responses following KCl or glutamate stimulation were comparable between short- and long-term incubated slices, confirming the presence of functional voltage-gated calcium channels within tissue sections.

In retinal wholemounts, papain digestion was used to remove the inner limiting membrane for better dye loading. Retinal tissue from rd/rd mice demonstrated stable spontaneous and evoked calcium activity over 24 hours, with comparable fluorescence responses and calcium recovery dynamics. These **results confirm that calcium signaling and second messenger systems remain intact in both brain slices and retinae for at least 24 hours post-slicing**.



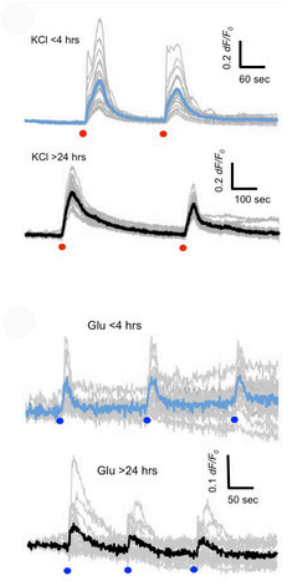
BRAINCUBATOR™ system - Schematic Diagram

STABILITY OF CALCIUM DYNAMICS IN ACUTE BRAIN SLICES

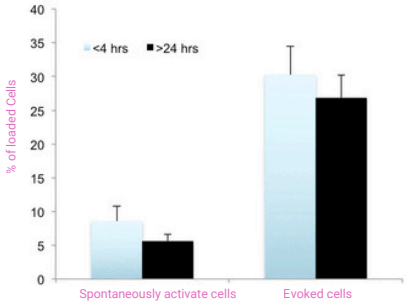


Fluorescence images of a neocortical slice show ubiquitous staining of cortical neurons and glia with Fura-2-AM.

Fluorescence imaging of a neocortical slice stained with Fura-2-AM reveals widespread labeling of cortical neurons and glial cells. A) Representative image of Fura-2-AM-loaded cells, captured using 380 nm excitation and a 20× objective (scale bar = 100 µm). B) Spontaneous calcium activity observed prior to stimulation. C) Evoked calcium response following application of 30 mM KCl, showing a marked increase in the 340/380 nm fluorescence ratio (ΔF/F) relative to baseline (F_0). Red indicates regions of high intracellular calcium concentration, while blue indicates low calcium levels.

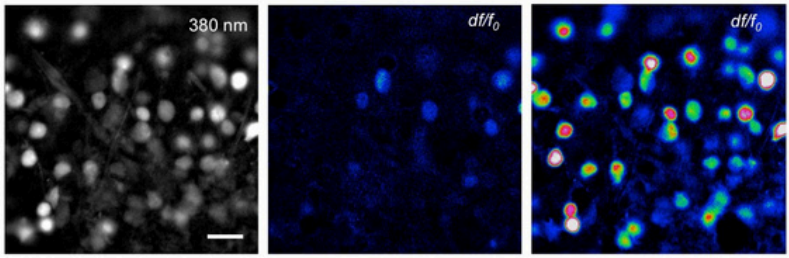


Intracellular calcium signals the time points of local KCl & Glutamate application



In response to depolarization with 30 mM KCl, the proportion of evoked cells remained constant between slices imaged <4 hours and >24 hours post slicing (27±3% vs. 24±3%, p>0.4). Additionally, the amplitude of calcium transients following KCl application was not significantly different (0.15±0.03 vs. 0.19±0.03 dF/F, p>0.5). Importantly, calcium recovery mechanisms were intact, as indicated by the comparable percentages of evoked cells restoring calcium levels post-stimulation (85±9% vs. 98±1% for KCl; 90±5% vs. 85±13% for glutamate, p>0.7).

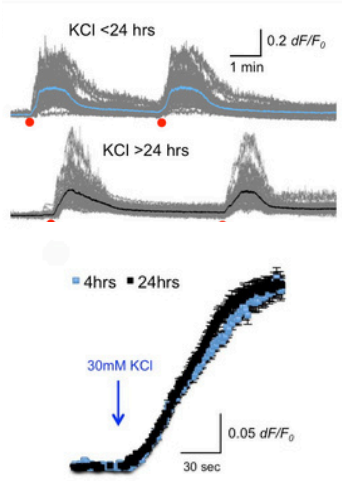
RETINAL CALCIUM SIGNALING INTEGRITY



Fluorescence image of the ganglion cell layer of a wholemount retina shows ubiquitous staining with Fura-2-AM

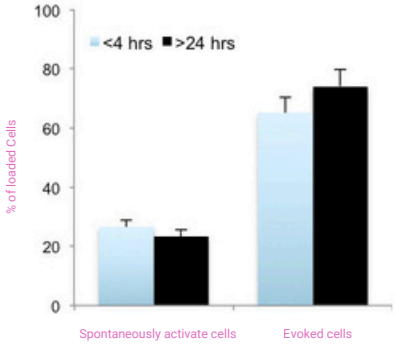
Retinal calcium signaling integrity was assessed by examining retinal wholemounts, where the integrity of calcium dynamics was preserved over 24 hours following dissection. Enzymatic removal of the internal limiting membrane (ILM) allowed for efficient dye loading in the ganglion cell layer, which facilitated reliable calcium imaging of retinal cells.

The proportion of spontaneously active cells in the retina did not significantly differ between retinae imaged within 4 hours of dissection and those imaged after more than 24 hours (23±3% vs. 27±3%, p>0.4). Similarly, the proportion of evoked cells also remained similar between the two time points (74±5% vs. 65±9%, p>0.4), suggesting that retinal neurons retain their functional properties over extended incubation periods.



The percentage of cells restoring baseline calcium levels remained stable.

The evoked **calcium transients**, measured following the application of KCl, **remained similar between the 4hr and 24hr time points** (0.28±0.01 vs. 0.26±0.01 dF/F, p>0.5) respectively.

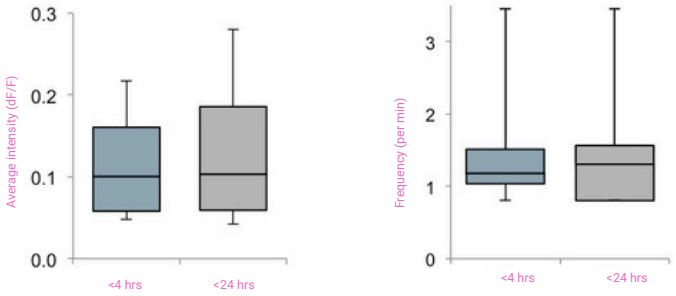


Proportion spontaneously active of cells

This indicates that the ability of retinal neurons to respond to depolarization stimuli remained stable over time. Moreover, the percentage of cells restoring baseline calcium levels post-stimulation was also consistent (94±3% vs. 95±1%, p>0.7).

These findings provide strong evidence that retinal calcium signaling dynamics remain intact for at least 24 hours after dissection, demonstrating the stability and reliability of retinal preparations for long-term activity studies.

IMPLICATIONS FOR LONG-TERM EX VIVO STUDIES



Spontaneous calcium signals in brain slices are a key indicator of neuronal activity and viability.

Experimental results demonstrate that the percentage of spontaneously active cells remained consistent over 24 hours post slicing, with no significant difference between slices imaged less than 4 hours and those imaged more than 24 hours post slicing (8.5±2.2% vs. 5.7±3.5%, p>0.4). These **spontaneous calcium transients reflect the ongoing basal activity of neurons** and suggest that the **cells maintain their functional properties even under prolonged incubation**. The persistence of spontaneous calcium signals over time underscores the viability of neuronal tissue and its maintained capacity for intrinsic activity. This stability suggests that spontaneous calcium transients serve as a reliable indicator of tissue health, supporting their utility as a key metric for evaluating the physiological integrity of brain slices in long-term ex vivo studies

Precisely regulates pH, temperature, and media

Tissue viability up to 36hrs

Halve the number of animals sacrificed

The stability of calcium signaling in both brain slices and retinae maintained in the Braincubator suggests that acute preparations can be used for extended periods without compromising data quality. This is particularly relevant for studies requiring prolonged observation of neuronal function, pharmacological testing, or dynamic assessments of synaptic plasticity. The observed consistency in spontaneous and evoked calcium responses supports the use of prolonged incubation as a viable approach for maintaining tissue viability, reducing variability, and increasing the reproducibility of experiments.

Overall, our findings provide strong evidence that acute neuronal tissue preparations can remain physiologically viable for at least 24 hours under optimized incubation conditions. This extended viability enhances the feasibility of long-term ex vivo studies and contributes to more reliable interpretations of neuronal and glial function over time.

METHOD – STANDARD CONTROL VS BRAINCUBATOR

The study evaluated calcium signaling in brain slices and retinal wholemounts using Fura-2 AM and Fluo-4 AM calcium dyes. The tissue was prepared from healthy animals, incubated in a controlled environment, and imaged for calcium dynamics, with statistical analysis performed to compare cell populations and slice groups. A custom-built incubation system maintained tissue viability, and fluorescence changes were analyzed using ImageJ/FIJI software.

Animals:

We used 3–30-week-old Wistar rats, C57BL/6, and C3H/HeJArc (rd/rd) mice, all born in the Western Sydney University animal facility. Animals were maintained under standard conditions with free access to food and water, a 12-hour light/dark cycle, and no stress stimuli. All experiments were approved by the University of Western Sydney Animal Care and Ethics Committee.

Brain Slice and Retinal Wholemount Preparation:

Animals were anesthetized with isoflurane (5%), decapitated, and their brains were removed and placed into ice-cold artificial cerebrospinal fluid (aCSF). Parasagittal brain slices (300 µm) were cut using a Leica microtome and incubated in a custom system, the Braincubator, which controls pH, temperature, and carbogen flow. Retinal wholemounts were prepared by enucleating eyes, cutting along the ora serrata, and placing them in aCSF at room temperature. The inner limiting membrane was removed with papain digestion, and retinal tissue was loaded with calcium dyes before being transferred to the Braincubator.

The Braincubator™ – Recovery Incubation System:

A custom incubation system was designed to regulate pH, temperature, and bacterial contamination. The system consisted of a main chamber housing brain slices and a separate UVC chamber for bacterial sterilization. The main chamber was insulated with polystyrene foam and monitored for pH and temperature. The UVC chamber contained a 5W Philips UVC lamp (254 nm), which operated intermittently (15–26 min every 15–30 min) to minimize heat buildup and prevent bacterial resistance. Solution was circulated between chambers via a peristaltic pump, and temperature was controlled using a Peltier thermoelectric cooler (TE Technology, MI).

Braincubator™ System

Calcium Imaging:

Calcium dyes (Fura-2 AM or Fluo-4 AM) were loaded into the tissue by bath application at concentrations of 10 µM (brain slices) or 20 µM (retina). Dye loading was performed at 37°C for brain slices and at room temperature for retinae. After washing, tissue was placed in the Braincubator. For imaging, tissue was positioned under an Olympus BX51W microscope and perfused with oxygenated aCSF. Fura-2 was imaged using a ratiometric method (340/380 nm excitation), while Fluo-4 was imaged at a single excitation wavelength (460–490 nm). Image sequences were analyzed using ImageJ/FIJI software, with fluorescence intensity changes measured and plotted for analysis.

Statistical Analysis:

Data are presented as mean ± S.E.M. Statistical comparisons were made using an unpaired two-tailed Student's t-test, with significance set at $p < 0.05$.

Cameron M, et al. (2016) Calcium Imaging of AM Dyes Following Prolonged Incubation in Acute Neuronal Tissue. PLoS ONE 11(5): e0155468. doi:10.1371/journal.pone.0155468

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Sengupta, A. et al (2017): **Basolateral amygdala neurons maintain aversive emotional salience.** Journal of Neuroscience, 38:2460–17. doi: 10.1523/JNEUROSCI.2460-17.2017

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Buskila, Y. et al (2019): **Dynamic interplay between H-current and M-current controls motoneuron hyperexcitability in amyotrophic lateral sclerosis.** Cell Death & Disease, 10, Article number: 310 (2019)

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BRAINCUBATOR™

prolongs tissue slice lifespan, reduces animal usage, supports extended experimental time, and ensures more reliable experimental conditions

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