Video Article

Prolonged Incubation of Acute Neuronal Tissue for Electrophysiology and Calcium-imaging

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Abstract

Acute neuronal tissue preparations, brain slices and retinal wholemount, can usually only be maintained for 6 - 8 h following dissection. This limits the experimental time, and increases the number of animals that are utilized per study. This limitation specifically impacts protocols such as calcium imaging that require prolonged pre-incubation with bath-applied dyes. Exponential bacterial growth within 3 - 4 h after slicing is tightly correlated with a decrease in tissue health. This study describes a method for limiting the proliferation of bacteria in acute preparations to maintain viable neuronal tissue for prolonged periods of time (>24 h) without the need for antibiotics, sterile procedures, or tissue culture media containing growth factors. By cycling the extracellular fluid through UV irradiation and keeping the tissue in a custom holding chamber at 15 - 16 °C, the tissue shows no difference in electrophysiological properties, or calcium signaling through intracellular calcium dyes at >24 h postdissection. These methods will not only extend experimental time for those using acute neuronal tissue, but will reduce the number of animals required to complete experimental goals, and will set a gold standard for acute neuronal tissue incubation.

Video Link

The video component of this article can be found at https://www.jove.com/video/55396/

Introduction

Electrophysiology and functional imaging (calcium, voltage sensitive dyes) are two of the most commonly used experimental techniques in neuroscience. Brain slice preparations and retinal wholemount, which will be examined here, provide a means of examining electrophysiological properties and synaptic connectivity without contamination from anesthetics or muscle relaxants. Brain slices and retinal wholemount maintain their structural integrity, unlike cultures or cell homogenates, allowing the study of specific circuits and brain networks[1]. Recordings from isolated tissue have advantages over *in vivo* recordings as movements associated with the heartbeat and respiration are eliminated. Moreover, direct visualization allows specific classes of cells to be targeted, and local application of pharmacological tools [2,3].

Patch-clamp recordings and calcium dye-loading in retinal wholemount is complicated by the existence of the Inner Limiting Membrane (ILM), which covers the Retinal Ganglion Cell (RGC) layer and prevents direct access to the cells. Typically, this membrane is scraped away with a glass pipette to allow direct application of a patch pipette and formation of a gigaohm seal on a single cell. In addition, bath-applied calcium dyes do not cross the ILM and must either be injected beneath this membrane[4], retrogradely transported following injection at the optic nerve[5] or electroporated through the tissue[6]. Furthermore, when utilizing a rodent model of retinitis pigmentosa, the *rd/rd* mouse, the ILM is thicker and more impenetrable. Here, we use a technique to remove the ILM with enzymatic digestion[7], to allow both ubiquitous calcium dye-loading, and direct access to retinal ganglion cells for patch-clamp recordings[8].

Successful recordings from either brain slices or retinal wholemount depend on dissection and incubation of viable neuronal tissue. Typically, tissue is extracted on the morning of the experiment and incubated in artificial cerebrospinal fluid (aCSF) until it is used for recordings. Usually, tissue remains viable for 6 - 8 h, with significant degradation following this time window. However, both brain slices and wholemount retinae preparations usually produce more tissue than can be recorded from within this short time period. Consequently, tissue is often discarded at the end of the day and the dissection is completed again on subsequent days. This means another animal is utilized and ~2 h of setup and dissection/staining repeated. The following protocol describes a method for extending the life of neuronal tissue for more than 24 h, meaning fewer animals are utilized, and more experimental time is available. Tissue viability was assessed through recording electrophysiological properties and calcium dynamics, and these properties were indistinguishable between <4 h and >24 h postdissection.

These results indicate that not only are single cell properties intact and functional after prolonged incubation, but network activity, as assessed by calcium-imaging and electrophysiological recordings, is unchanged >24 h postdissection. Moreover, we show that calcium dyes can remain in cells for prolonged periods without causing any detrimental effects. Application of this protocol demonstrates that the functional activity of neurons in acute neuronal tissue can be maintained for long periods, once the external environment is highly regulated. Moreover, as

tissue viability varies greatly between laboratories due to different incubation protocols, this method establishes a gold standard for the ideal parameters that should be applied to reduce variability in the health of acute neuronal tissue.

Protocol

The protocol below describes the preparation of C57BL/6 and C3H/He (retinally degenerate) mouse neuronal tissue, but similar techniques can be applied to other species. All animals were healthy and handled with standard conditions of temperature, humidity, 12 h light/dark cycle, free access to food and water, and without any intended stress stimuli. All experiments were approved and performed in accordance with Western Sydney University Animal Care and Ethics committee and according to the animal use and care guidelines (Animal Research Authority #A9452, #A10396 and #A8967).

1. Brain Slice Preparation

1. Anesthetize the animal by inhalation of isoflurane (5%) and decapitate using a rodent guillotine. Remove the brain quickly, as described previously[9] and place it into ice-cold physiological solution (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; 310 mOsm; pH 7.4).
2. Cut brain slices, in the region of interest, 300 µm thick with a vibrating microtome.
3. Transfer slices to a custom built incubation system that closely monitors and controls pH levels (pH 7.2 - 7.4), carbogen flow, and temperature as previously described[10,11].
4. Set initial chamber temperature to 35 °C for 15 - 30 min, and then slowly reduce to 15 - 16 °C as shown in **Figure 1C**. Then incubate slices in the incubation system until needed, either for electrophysiology or imaging.
   NOTE: If slices are to be used for calcium-imaging, follow steps below before cooling tissue below RT.

2. Retinal Wholemount Preparation and Inner Limiting Membrane Removal

1. Prepare retinal wholemounts under either normal laboratory lighting conditions or dim red/infra-red light.
2. Euthanize animal by cervical dislocation and immediately enucleate eyes. Make a small cut along the ora serrata, and place in either Ames media, or aCSF containing (mM): 125 NaCl, 25 $NaHCO_3$, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 0.5 L-glutamine, and saturate with carbogen (95% O_2/5% CO_2 mixture; ~300 mOsm; pH 7.4), at RT.
3. Immediately remove the cornea, lens and vitreous by cutting along the ora serrata with small scissors and removing the lens and vitreous with forceps. Place tissue in the incubation system at RT.
   NOTE: Retinal tissue can be maintained in the eye-cup, after slow temperature reduction to 15 - 16 °C, for >24 h in the incubation system until needed.
4. To remove the ILM, transfer the eye-cup containing the retina to a small glass jar containing 30 U/mL papain, 1 mM L-cystine, 0.5 mM EDTA and 0.005% DNase in Earl's-Balanced Salt Solution (BSS) at 37 °C for 20 min. Apply 95% O_2/5% CO_2 to the solution through the lid but do not bubble. If using tissue from young animals (<6 weeks), dilute the solution to half-strength.
   1. Stop enzymatic digestion, by placing the tissue in an ovomucoid (10 mg/mL) and BSA (10 mg/mL) solution for 10 min in Earl's BSS. Wash tissue thoroughly with aCSF before transfer to the incubation system and reduce temperature to 15 - 16 °C.
5. Maintain retinal tissue in the eye-cup until needed. For transfer to the microscope, isolate the retina from the eye-cup and cut into 4 pieces with a razorblade. If the entire retina is required, make four small cuts in the periphery of the retina to allow it to lie flat.
   NOTE: For imaging experiments, load with calcium-dyes before transfer to the incubation system, see below.

3. Maintaining Tissue in the Incubator

1. Place tissue in the main chamber containing probes for pH and temperature measurements (**Figure 1A, B**).
2. Circulate aCSF through a second chamber (UVC chamber, built as previously described[12]) isolated from the main chamber and exposed to 1.1 W UVC light (254 nm, 5W/2P sterilizer UV lamp) in order to eradiate bacteria floating in the solution (**Figure 1A**). Control UVC light timing via a programmable timer using a random feature that turns ON at times varying between 15 and 26 min every 15 - 30 min to avoid excessive heating of the aCSF.
3. Set the flow rate in the UVC chamber to 12 mL/min as previously reported[12]. Cover the UVC chamber with aluminum foil to prevent UVC illumination outside the chamber, which can damage neuronal tissue.
   NOTE: For dark-adapted retinal tissue, apply a custom-made cover to the main chamber to exclude light.
4. Use a peristaltic pump to circulate the solution (aCSF) through the two chambers and a Peltier thermoelectric cold plate cooler to either cool or heat the main chamber to the desired temperatures in the range of 0 - 50 °C. For optimal tissue incubation, use 15 - 16 °C for long-term viability.

4. Calcium Dye Loading

1. Select calcium dyes based on the preference of the individual researcher. Here we use Fura-2AM, Fluo-8AM or Fluo-4AM. However, this method can be applied to other dyes as well: dissolve calcium dyes in DMSO to a 1 mM solution and 1% block copolymers (*e.g.*, pluronic acid-127) to a final volume of 50 µL and sonicate for 10 min.
2. Add solution to aCSF to a final concentration of 10 µM, 0.01% block copolymers for brain slices, and 20 µM (retina), 0.02% block copolymers for the retina. In retinae (papain treated) and brain slices from young animals, load to the bath for 45 min at 37 °C (Fura-2 AM) or at RT (Fluo-4 AM; Fluo-8 AM).
   1. For adult animals, (>12 weeks) pipette the dyes (50 µL) directly onto the brain slices, and maintain for 75 min to allow better penetration of the dye into the deep layers.

2. To ensure adequate oxygenation of the submerged slice during dye incubation, prepare a glass loading chamber with a closed lid (circular jar of diameter 2.5 cm, 3.5 cm height) with calcium dyes diluted in 2.5 mL of aCSF. Oxygenate continuously with 95% O_2/5% CO_2. Do not bubble.

3. Following dye loading, wash the tissue with aCSF and transfer to the incubation system, slowly reduce the temperature to 15 - 16 °C until experimental use.

5. Electrophysiological Recordings and Imaging

1. Place tissue in a submerged recording chamber under a microscope, and perfuse with oxygenated aCSF at a flow rate of 4 - 5 mL/min either at RT (~22 °C) or physiological temperature (~35 °C). Hold the tissue in place by using a custom made "harp", made of nylon or gold threads stretched and glued across a U-shaped piece of gold or platinum wire.
2. For electrophysiology:
    1. Prepare recording pipettes from 1.5 mm (1.19 mm ID) borosilicate glass using a micropipette puller to achieve a final resistance of 5-6 MΩ.
    2. Fill the pipette with 3 - 4 µL of internal solution as previously described[13] and visualize cells under infrared Differential Interference Contrast (IR-DIC) using a CCD camera. The intracellular solution should be carefully tailored to each experiment to achieve experimental outcomes.
    3. Position pipette, while maintaining positive pressure applied through a suction port on the pipette holder, on the membrane of a cell using a micromanipulator. Since the ILM has been removed from the retina, no prior membrane scraping is required. Once the pipette is on the cell, apply gentle negative pressure to the pipette to achieve a gigaohm seal. Then rupture the cell membrane with a brief amount of negative pressure.
    4. Make whole-cell current- or voltage-clamp recordings using standard techniques as appropriate.
3. Calcium-imaging:
    1. For ratiometric imaging of Fura-2, use an ultra-high speed wavelength switcher to provide excitation wavelengths of 340 nm and 380 nm. Pass emitted light from individual cells through an emission filter of 510 ±20 nm, and capture with a high sensitivity, high-speed digital camera.
    2. For a single excitation wavelength of Fluo-4, filter excitation light through a 460 - 490 nm band pass filter and emitted light through a 515 - 550 nm band pass filter. For the fastest and most sensitive recording use a high speed digital camera such. Acquire images as required.
    3. Measure alterations in fluorescence as a function of time either at a single wavelength (Fluo-4) or using the wavelength ratio method (Fura-2), as previously described[8,14].

Representative Results

Tight regulation of the bacterial load and temperature of the aCSF during incubation is essential to maintain neuronal tissue viability. This can be optimized through irradiation with UVC light and maintaining the temperature of the aCSF at 15 - 16 °C (**Figure 1**). Furthermore, the aCSF parameters stamp (APS; **Figure 1C**) provides the experimenter with a record of the environmental conditions (pH and temp.), thus offering a gold standard for parameters when incubating neuronal tissue, which, if followed, will reduce variability between experiments.

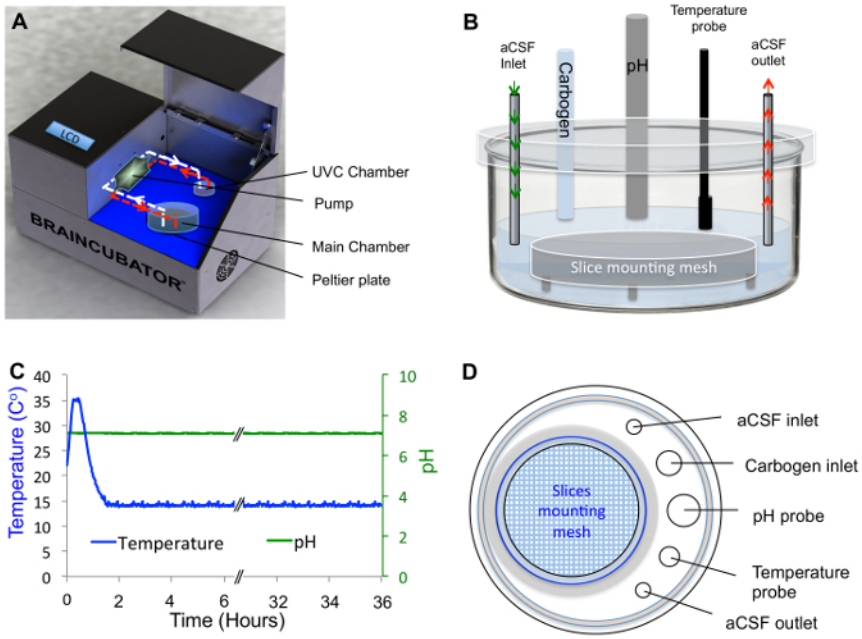

Figure 1. An Incubation System That Enables Tight Regulation of Tissue Environment. A, Schematic diagram of the incubation system composed of a cooling Peltier plate, main chamber, peristaltic pump and UVC chamber. **B**, Side view of the main chamber showing the inlet and outlet points of the aCSF, as well as the carbogen inlet, temperature and pH probes. Tissue is placed on the mesh as indicated. All measuring probes are attached to the lid to allow structural stability. **C**, aCSF Parameters Stamp describing the temperature and pH of the aCSF during incubation. **D**, Top view of the main chamber showing the mounting holes for the measuring probes. Please click here to view a larger version of this figure.

Tissue viability can be measured by means of network activity (**Figure 2**), as well as various imaging methods, including calcium responses (**Figure 2B, C**). To monitor the network activity, we applied aCSF containing a high concentration of potassium chloride (KCl; 30 mM) locally, which led to depolarization of neurons and glia within the vicinity of the applied K^+. This depolarization can be observed by the increase in calcium transients (**Figure 2B, C**) and spiking activity (**Figure 2D**), which also serves as a physiological indicator for the cell viability. Our results show that spiking activity in slices that were incubated for >24 h in the incubation system was similar to "fresh" slices incubated for shorter periods (>4 h; **Figure 2D**). Furthermore, individual neuron viability, which was monitored through intracellular electrophysiological recordings of both passive and active membrane properties, including the resting membrane potential, input resistance, time constant, and action potential was comparable to parameters reported in the literature[15] (**Figure 2E, F**).

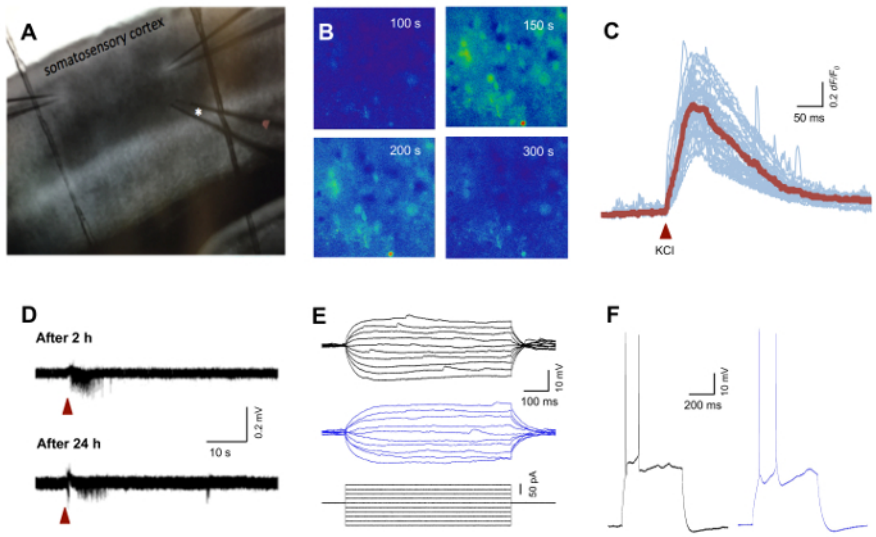

Figure 2. Electrophysiological and Calcium Properties of Brain Slices after Prolonged Incubation. A, Image of a brain slice taken 28 h after slicing with two extracellular recording electrodes used for measuring the network activity. A third electrode (marked with asterisk) is used to puff 30 mM of KCl. **B**, Time lapse images of slices loaded with Fluo-4AM for >24 h, depicting an increase in intracellular calcium transients following bath application of KCl. **C**, Intracellular calcium transients following brief application of KCl (30 mM; 1 s). Red - average signal, Grey - calcium traces in single cells as shown in **B**. **D**, Extracellular physiological recording of the network activity before and after the application of KCl (red arrows) were largely unchanged between "fresh" slices (<4 h postdissection) and those that were incubated for >24 h in the incubator. Note the increase in spiking activity immediately after KCl application indicating viable neurons that respond to increase in $[K^+]$ with depolarization. **E&F**, Passive (**E**), and active (**F)** membrane properties of pyramidal neuron following 2 h (blue traces) or 26 h (black traces) of incubation in the incubation system. Please click here to view a larger version of this figure.

To provide direct access to the cells of the ganglion cell layer of the wholemount retina of retinally degenerate *rd/rd* mice, the ILM was digested by the enzyme papain[7,8]. After digestion, RGCs and displaced amacrine cells can be clearly visualized under DIC illumination (**Figure 3A**), and cells can be targeted for patch-clamp recordings without any prior scraping of the ILM. Representative current-clamp recordings from a RGC are shown in **Figure 3B**. Depolarization of the cell via the patch pipette caused dose-dependent action potential generation, indicating the viability of the cells following papain treatment.

Removal of the ILM also allowed ubiquitous staining of the ganglion cell layer (GCL) with Fura-2AM (**Figure 3D**) and cells responded to 30 mM KCl application with a large increase in 340/380 nm ratio relative to F_0, signifying a large increase in intracellular calcium concentration (**Figure 3E**). Calcium levels returned to baseline following stimulation and cells could be subsequently stimulated to produce a similar amplitude response. Moreover, these responses were indistinguishable between retinae recorded at <4 h and >24 h postdissection (**Figure 3F, G**).

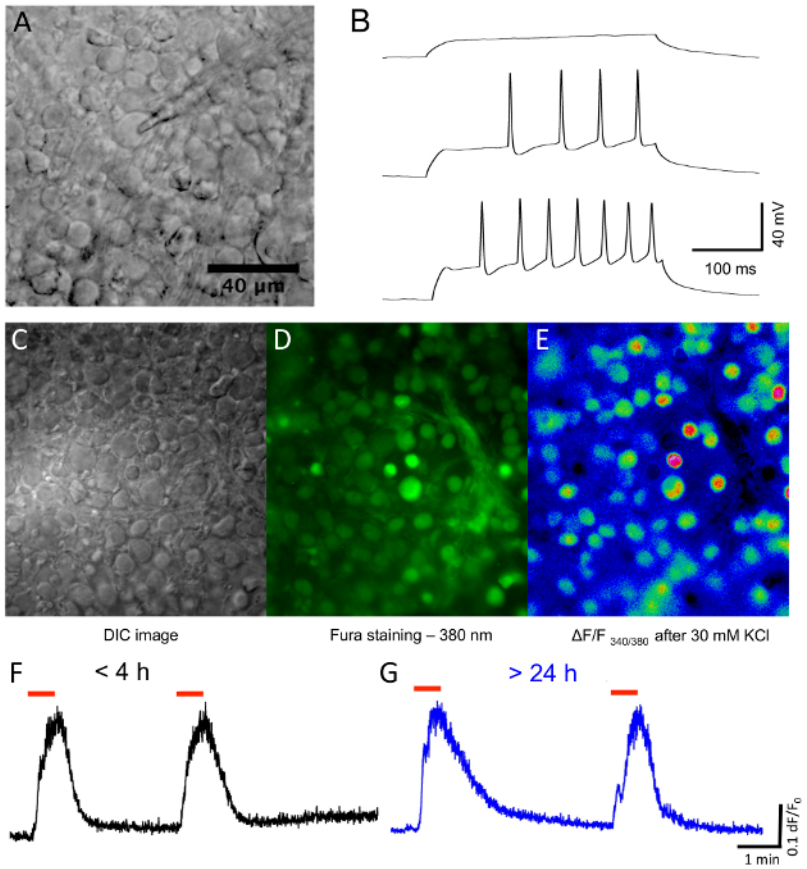

Figure 3: Electrophysiological and Calcium Recordings in Retinal Wholemount. A, Differential interference contrast image of a patch-clamp recording from a retinal ganglion cell in the GCL after papain digestion. **B**, Cells retain dose dependent spiking responses when depolarized with 50, 100 and 150 pA respectively. **C&D**, Removal of the inner limiting membrane before bath application of Fura-2AM allows ubiquitous loading of retinal ganglion cells, image taken with 380 nm excitation. **E**, When 30 mM KCl ASCF is applied, 85% of stained cells responded with an increase in 340/380 ratio. **F&G**, The increase in 340/380 ratio returns to baseline after 30 mM KCl application (red bars), and cells respond to subsequent stimulation both <4 h and >24 h following dissection. Please click here to view a larger version of this figure.

Discussion

This article describes an incubation method to extend the viability of acute neuronal tissue for imaging and electrophysiological experiments, thereby reducing the animal numbers needed to complete experimental goals. Two main processes govern the deterioration of neuronal tissue over time: i) increased bacteria levels, and the accompanying increase in bacterial endotoxin released, and ii) excitotoxicity which follows the traumatic slicing procedure[10]. As acute neuronal tissue is environmentally defenseless, tight regulation of the tissue environment through close monitoring of pH, temperature and bacteria levels is essential for maintaining the cellular activity as well as network integrity. Dissection, and tissue treatment (papain digestion, calcium dye loading) can take over 2 h to complete, causing a reduction in experimental time. However, since the tissue can be kept in the incubation system for >24 h without a measurable loss in viability[7,8], this preparation only needs to be completed once to obtain >24 h of results. As part of the 3Rs strategy *(Replacement, Reduction, Refinement)* aimed at providing guiding principles for ethical use of animals[16], this method will have a large impact on reducing the number of animals used in these types of experiments.

Digestion of the ILM of retinal wholemount tissue allows easy targeting of cells in the GCL for patch-clamp recordings and ubiquitous calcium dye loading by bath application. These techniques are particularly useful for degenerate retinae in which the ILM is much thicker and harder to breach by scraping with a glass pipette. Furthermore, the papain preparation provides a way to perform paired-cell recordings using two electrodes to record from gap-junction coupled cells in the GCL, something that has not been previously possible. However, papain digestion likely has some effects on normal retinal physiology as K^+ receptor expression has been shown to be altered in photoreceptors following dissociation with papain[6]. It is also possible that retinal architecture is affected. Velte and Masland showed that although some RGC somas and dendrites were damaged by papain treatment to remove the ILM, they could successfully record from RGC dendrites distal to the soma in most neurons, indicating the structural integrity of the cells and processes[5]. Importantly, leaving the retina attached to the retinal pigment epithelium (RPE) and sclera, as described above, isolates the outer retina from papain diffusion and may reduce any adverse effects on photoreceptor outer segments when this protocol is applied to WT retinae. In both instances, papain treatment, or scraping of the ILM, some cell damage will occur, the experimenter must choose the method appropriate to the experimental question.

Disclosures

Yossi Buskila and Paul Breen declare that they are co-owners of PAYO Scientific Pty Ltd, a company specialized in building incubation systems for acute neural tissue. All other authors report no financial interests or potential conflicts of interest related to the current study.

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