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Thin slice CNS explants maintained on collagen-coated culture dishes

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Abstract

We have developed a simple and inexpensive procedure for explant culture termed 'thin slice culture' that relies on the use of thin sections of CNS tissue (≤ 150 μm) which adhere directly to the bottom of collagen-coated culture dishes (or glass coverslips within culture dishes). Microscopic visualization and tissue oxygenation are enhanced due to the reduced slice thickness, and the reduced volumes of incubation media required lessen the amount of expensive agents used (e.g. growth factors). We show that thin slice cultures of spinal cord, brainstem and hippocampus remain viable for at least several weeks and are suitable for many experimental approaches including time-dependent studies, immunocytochemistry and electrophysiology.

Keywords: Organotypic; Organ culture; Spinal cord; Brainstem; Hippocampus; Development; Regeneration; Co-culture

1. Introduction

Explant culture combines the strengths of the CNS tissue slice preparation (in situ network organization and cell identity) with cell culture (long-term study and chemical control of incubating media). Historically, CNS explant cultures of 1 mm^3 were developed to examine in situ properties of functionally interconnected networks of cells in vitro (Crain and Peterson, 1963; Crain, 1965). In an attempt to optimize experimental conditions, explants were reduced in thickness, usually between 250 and 500 μm, the contemporary approach to organotypic culture (Gähwiler, 1981; Stoppini et al., 1991). Since different regions can re-establish functionally appropriate interconnections when cultured together (e.g. Crain and Peterson, 1963; Gähwiler and Brown, 1985; Boltz et al., 1990; Streit et al., 1996) slice co-culture is also a powerful approach for the study of cellular interactions between adjacent or distant CNS regions in vitro.

Although powerful, investigations on cell and network properties using explant culture have only recently been extensively exploited and the dissociated cell culture approach for neurophysiological investigations in culture still predominates. Part of the preference for dissociated cell culture may be due to the technological difficulties involved in explant culture or to limitations in cell visualization. One common method, the roller tube method (Gähwiler, 1981) facilitates cell visualization by producing a virtual monolayer of cells and permits imaging and patch clamp electrophysiological recordings (Gähwiler, 1981; Knöpfel et al., 1990). However, the roller tube method is technically intricate (Gähwiler, 1988) and cannot be used for studies of long-term, time-dependent phenomena within a given slice (e.g. axonal migration) because periodic microscopic examination cannot be undertaken in the test tube (Gähwiler, 1981). A technically less demanding approach, the transwell or interface method, optimized for widespread use by Stoppini et al. (1991), involves the culturing of explants on expensive porous membranes which act as an interface between nutrient supply (media) and oxygenation. Using this

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method, 400–500 μm explants may flatten to a final thickness of 150 μm (Stoppini et al., 1991). While in situ tissue organization remains intact, the explants remain comparatively thick, and their placement on non-transparent membranes makes visual identification of cells more difficult, but clearly amenable to 'blind' electrophysiological approaches (Stoppini et al., 1991; Xie and Ziskind-Conhaim, 1995). Furthermore, microscopic examination of the tissue following histological procedures may be burdensome due to slice attachment to the membrane insert.

We have developed a simple and inexpensive alternative method for explant culture that relies on the use of thin sections which adhere directly to the bottom of a culture dish or glass coverslip. Tissue oxygenation is maintained due to the reduced slice thickness, while the small volumes of culture media required minimize the use of expensive agents (e.g. growth factors). The explants flatten from a maximum initial thickness of 150 μm or less to allow for cell visualization. Our thin slice culture technique is extremely useful for histochemical studies, and more importantly, allows for powerful experimental approaches which require visualization (e.g. patch clamp electrophysiology and imaging) and an assessment of time-dependent changes (Parsley et al., 1996; Song et al., 1996).

2. Materials and methods

2.1. Culturing procedure

Although rat tail collagen is available commercially, we prefer the quality of that prepared in our laboratory. Collagen solution is prepared monthly from adult rat tails according to the procedure of Elsdale and Bard (1972), with slight modifications. For coating the plastic dishes and glass coverslips, rat tail collagen is used at a concentration of 50 μg/ml. Twenty-four hours prior to a culture experiment and under sterile conditions, 35 mm culture dishes (Corning) are coated with 0.5 ml of rat tail collagen and allowed to dry in a culture hood for 4–6 h. The dishes are rinsed twice with 1 ml of sterile deionized water, and then soaked in 1 ml of Neurobasal or DMEM–F12 media (GIBCO) overnight in an incubator at 37°C in an atmosphere of 5% CO_2, 95% air (Narco). On the day of the experiment the media is replaced with 0.5 ml of new media and B27 supplement (GIBCO) (Brewer et al., 1993). Culture dishes are then returned to the incubator until the tissue slices are ready to be placed in them. For electrophysiological studies, collagen-coated glass coverslips are prepared 48 h prior to the experiment. The desired number of round 25 mm glass coverslips are cleaned according to the method of Fitzgerald (1989). Coverslips are sterilized by autoclaving, coated with 0.3 ml of rat tail collagen, placed on the bottom of 35 mm culture dishes and treated the same as collagen-coated dishes.

Timed-pregnant rats are anesthetized, and the ensuing procedures are performed under sterile conditions. E15–E18 fetuses are removed, the brainstem and spinal cord from each fetus is dissected out (see Xie and Ziskind-Conhaim, 1995), embedded in 2.5% agarose and cut into transverse sections of 80–150 μm thickness using a Leica VT1000 vibrating blade microtome. Slices are transferred into sterile dishes containing dissecting media (in mM: NaCl, 150; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 4; *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 4.2; and D-glucose, 11; at pH 7.3), where they are cleaned of agarose and meninges and placed into collagen-coated culture dishes. Co-culture slices are placed approximately 1 mm apart in a dish, while single cultures are placed in the center of an individual dish. Five or six individual culture dishes are then placed into sterile 150 mm culture dishes (VWR) in the incubator. Cultures are fed every 4–5 days by removing 0.2 ml, then adding 0.2 ml of fresh Neurobasal and B27 media. For several experiments, antimitotics (fluorodeoxyuridine 10 μM and uridine 10 μM) are added at day 3 for 48 h to reduce proliferation of non-neuronal cells (Banker and Goslin, 1991).

Photographs of the cultures are taken as often as every 3–4 days on a Nikon Diaphot 300 inverted microscope equipped with Hoffman modulation optics. As a comparison to cultures using thicker explants, we also cultured slices at a thickness of 300 μm. Postnatal hippocampal (P3–6) and spinal cord cultures (P8–16) are undertaken using an oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl, 125; KCl, 2.5; $NaHCO_3$, 26; NaH_2PO_4, 1.25; D-glucose, 25; $MgCl_2$, 1; and $CaCl_2$, 2.

2.2. Histochemistry

2.2.1. Live/dead cell assay

Using room temperature solutions, culture media are removed and cultures are washed three times for 5 min each in oxygenated ACSF. Then, 1.6 μl/ml of a 1% stock solution of propidium iodide and 2 μl/ml of a 10% stock solution of fluorescein diacetate is added. After a 10-min incubation at room temperature cultures are then washed three times for 5 min each in oxygenated ACSF. The cultures are immediately photographed using standard fluorescein and rhodamine filter sets on either a Nikon Labophot-2 light/epifluorescence microscope, or Nikon Diaphot 300 inverted light/epifluorescence microscope.

2.2.2. Immunohistochemistry

Cultures are fixed in phosphate-buffered 4% paraformaldehyde at room temperature or at 4°C for 3 days. The cultures are then washed six times for 20 min

each in phosphate buffered saline – 0.3% Triton (PBS-T). The following primary antibody series are then added: mouse microtubule associated protein (MAP-2) at 1:1000 μl (Sternberger), mouse Panaxonal at 1:1000 μl (Sternberger), and rabbit glial fibrillary acidic protein (GFAP) at 1:1000 μl (Chemicon). Cultures are placed in a sealed container at room temperature or at 4°C for another 3 days, then washed three times for 20 min each in PBS-T. The secondary antibody series of anti-mouse Cy3 at 1:100 μl (Sigma) and anti-rabbit FITC at 1:100 μl (Sigma) are incubated at room temperature for 1.5 h, then washed twice for 20 min each in PBS-T, and 20 min in 50 mM Tris–HCl (pH 7.4). Cultures are desiccated overnight and then photographed under epifluorescence illumination as described above or coverslipped with a glycerol-based anti-fade media (Valnes and Brantzaeg, 1985) and stored in the freezer for future morphological studies.

2.3. Electrophysiology

A glass coverslip containing the explant culture is removed from an individual culture dish. The coverslip is then adhered to the bottom of a round Plexiglas ring (25 mm outer diameter) with high vacuum grease (Dow Corning), becoming the experimental chamber. A perfusion system is attached to the chamber and ACSF is perfused at approximately 2 ml/min. Whole-cell patch clamp recordings were obtained (Hamill et al., 1981) with an Axopatch 1D amplifier (Axon Instruments) and data were acquired using pCLAMP software (v 6.0, Axon Instruments). Microelectrodes were pulled on a two-stage upright puller (Narishige PP-83) with resulting resistance values between 3 and 6 MΩ in a recording solution containing (in mM): K-gluconate or CsF, 140; ethylene glycol-bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 11; KOH, 35; HEPES, 10; $CaCl_2$, 1. pH was adjusted to 7.3.

3. Results

3.1. Explant viability and topography (spinal cord)

CNS slices were cultured for periods of up to 5 weeks with a contamination rate under 1%. Sample results of spinal cell viability assessed with live and dead cell indicators for the different section thicknesses of 300, 150 and 80 μm at culture day 14 are presented in Fig. 1. Using standard fluorescein and rhodamine filter sets, live cells fluoresce green while the nuclei of dead cells fluoresce red. A slice culture prepared at 300 μm thickness is presented in Fig. 1A. There was a considerable number of living cells (Fig. $1A_1$), but at this thickness it was difficult to visualize individually labeled cells within a focal plane in the slice region. Cell viability assays confirmed widespread cell death (Fig. $1A_2$) and slices usually appeared mottled upon examination using interference optics. Nonetheless, electrophysiological recordings could be obtained from neurons using a 'blind' approach. Overall, $50 \pm 7\%$ of cells were viable at this section thickness after 14 days in culture ($n = 4$).

A representative example of results on assessing cell viability from the 150 μm slices is shown in Fig. 1B. In comparison to slices prepared at 300 μm thickness, the live cells in the 150 μm slices are well labeled and easy to visualize within the slice. Only the live cell assay is presented since a barely detectable fluorescent signal for dead cells was obtained. Overall, $95 \pm 3\%$ of cells were viable at 150 μm section thickness after 14 days in culture ($n = 4$).

Preparation of slice cultures at 80 μm thickness is presented in Fig. 1C. Because of the reduced slice thickness following culture for 14 days, cell visualization was superior and the tissue integrity appeared to be generally well preserved. However, an increased number of dead cells were observed within the slice (Fig. $1C_2$), possibly due to the mechanical trauma of microtome slicing at this thickness. Overall cell viability after 14 days in culture was $78 \pm 7\%$ ($n = 4$). A Nissl stain of a 80 μm thick slice is shown in Fig. 1D demonstrating the topography of cell labeling. Note that general spinal cord topography appears to be retained and there was a dense labeling of cell bodies which were rather uniformly distributed throughout the slice.

3.2. Immunohistochemistry (spinal cord and brainstem)

Immunohistochemical staining of spinal cord slices with Panaxonal and MAP-2 antibodies demonstrates extensive interconnection of the neuronal population (Fig. $2A_1$). Many neurites can be observed radiating from the slice explant proper (Fig. $2A_2$). Additionally, MAP-2 positive neuronal cell bodies (indicated by arrows) can be observed migrating out from the slice explants (Fig. $2B_1$). There is a large number of GFAP-positive stained glia both within and outside the slices (Fig. $2B_2$). Fig. $2C_1$ shows an example explant co-culture of spinal cord and brainstem containing axonal interconnections traveling as bundles (Fig. $2C_2$) or individually (Fig. $2C_3$). Neuronal cell bodies can be easily visualized within the slice proper, as exemplified in a brainstem slice (Fig. $2C_4$).

3.3. Hippocampus

Since organotypic slice topography and cytoarchitecture is difficult to examine in spinal cord or brainstem, we performed several experiments using postnatal hippocampal slices. Overall topography of the hippocampus was well maintained (Fig. $3A_1$ and Fig.

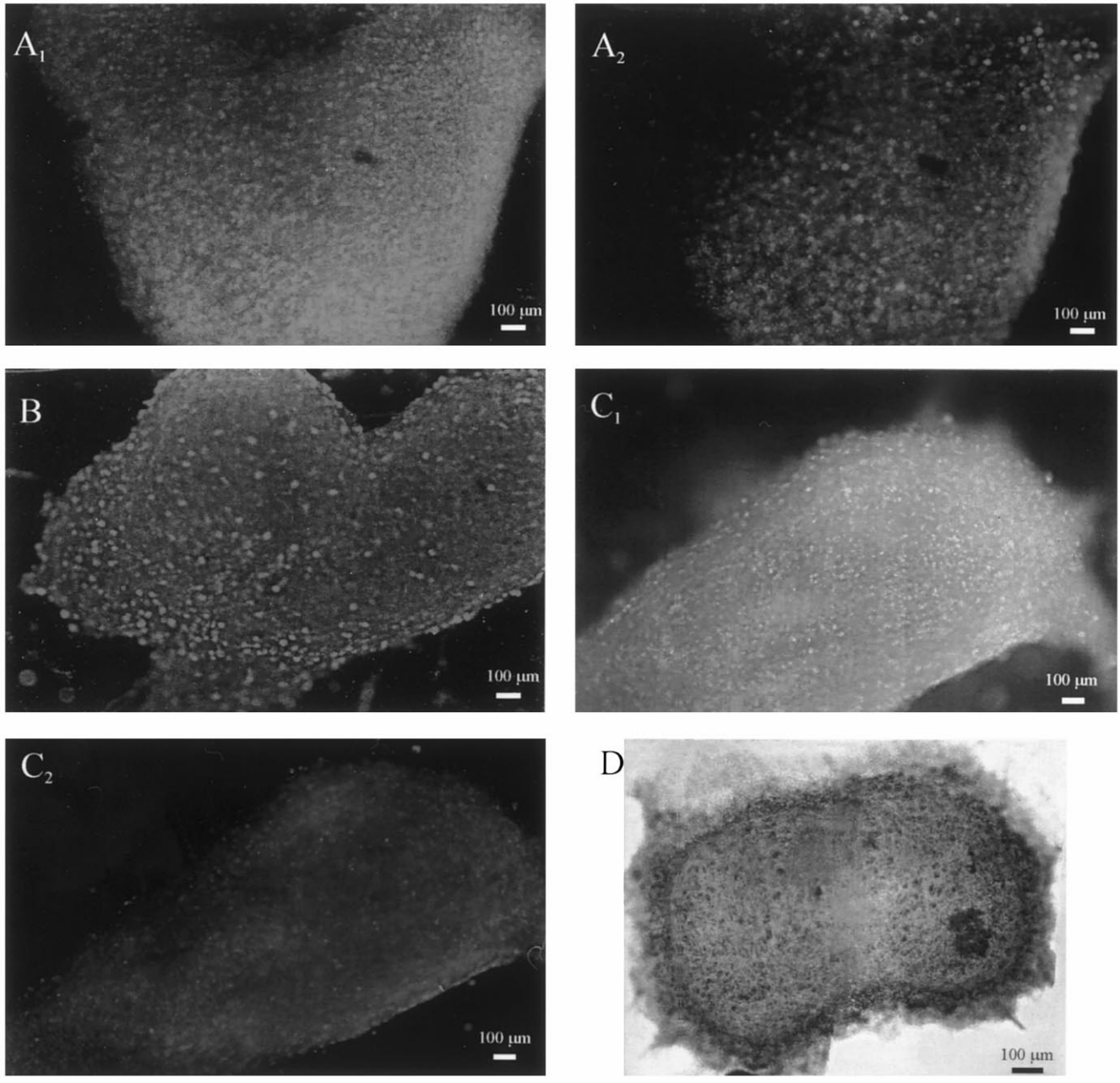

Fig. 1. Live/dead cell assays after 14 days in culture demonstrate an abundance of living cells within spinal cord slices. The live cell indicator, fluorescein diacetate, shows living cells in slice cultures at 300 (A_1) 150 (B) and 80 μm (C_1) thicknesses. The dead cell indicator, propidium iodide, identifies dead cell nuclei at 300 and 80 μm thicknesses (A_2 and C_2, respectively). D Nissl stain of an 80 μm slice culture to demonstrate cellular topography.

4B). MAP-2 positive cell bodies and dendrites, and Panaxonal labeled axons were well visualized at both CA1 pyramidal (Fig. $3A_2$) and dentate granule cell regions (Fig. $3A_3$). GFAP staining of the CA1 region is also provided to illustrate glia within the slice (Fig. $3A_4$). Few MAP-2 positive staining neurons were found in the CA3 region in this culture (not illustrated). An example of MAP-2 and Panaxonal labeling in a 150 μm thick hippocampal slice (including subicular region) is presented to show the generally maintained slice topography and abundant interconnections (Fig. $3B_1$). There also appeared to be an increase of neurons observed in the CA3 region in 150 μm slice cultures over the 100 μm slice cultures. Neurons which migrated out from the subicular region of the slice culture are also shown (Fig. $3B_2$). Hippocampal cultures have been successfully maintained for periods of at least 4 weeks.

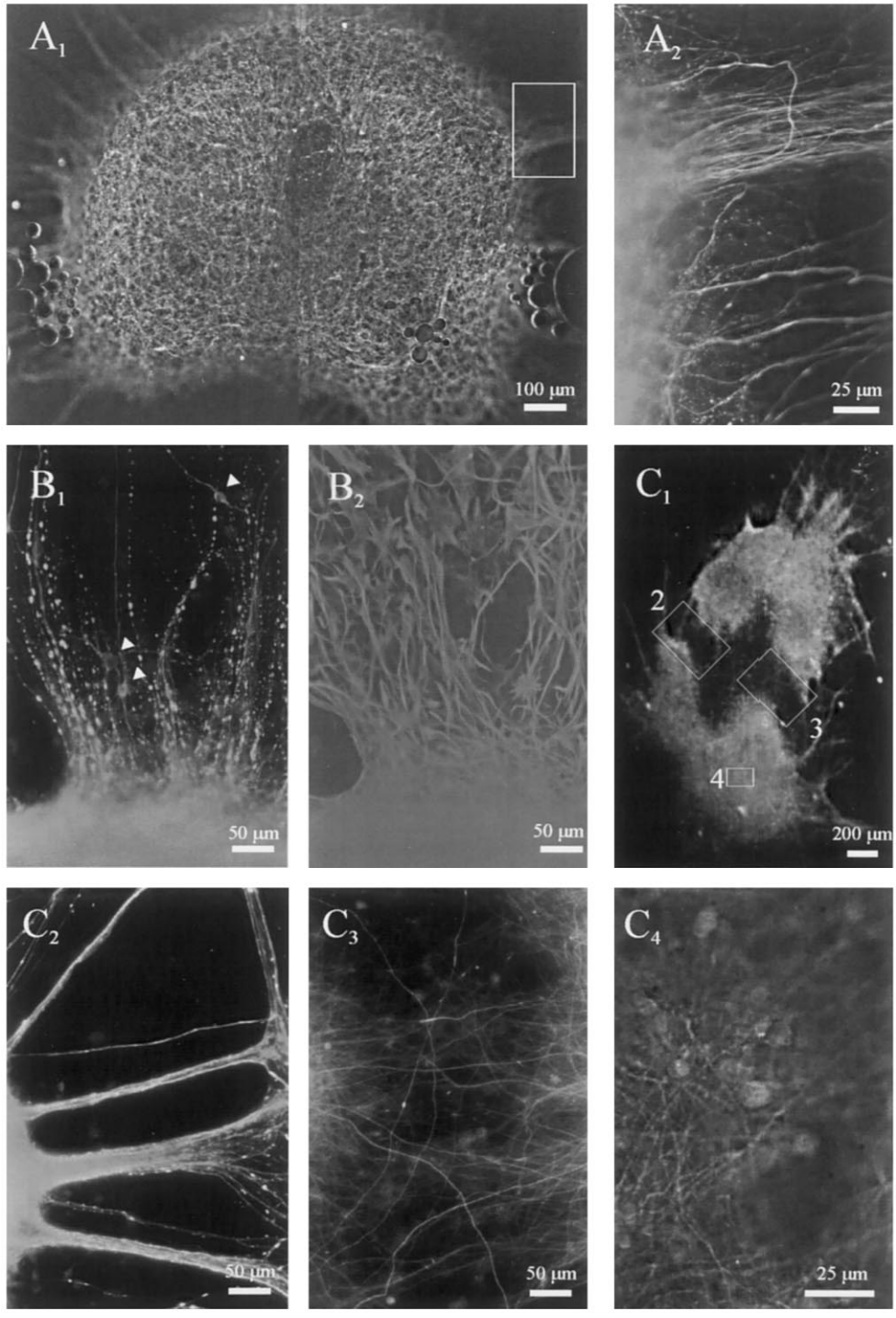

Fig. 2. Immunocytochemical procedures to assay slice organization and composition. All slices were sectioned at 150 μm thickness and processed at culture day 14. A_1 Immunostaining for Panaxonal and MAP-2 to demonstrate extensive arborizations within the spinal cord slice. A_2 Enlargement of boxed area in A_1 to show projections extending out from the slice. B_1 Axons and neurons labeled with Panaxonal and MAP-2. Note the labeled cell bodies which have migrated out from the slice (arrowheads). B_2 A spinal cord slice stained with GFAP to illustrate presence of glia (same region as B_1). C_1 Brainstem-spinal cord co-culture. C_{2-4} Expanded regions as indicated with boxes in C_1 (but not in same focal plane). C_{2-3} Distinct patterns of axonal projections interconnecting slices are visualized using Panaxonal and MAP-2 immunostaining. C_2 Axons travel in bundles between the slices. C_3 Single axons projecting between slices. C_4 Nuclei within brainstem slice.

3.4. Electrophysiology

We have obtained several blind (from 300 μm slices) and visual (from 150 and 80 μm slices) patch clamp recordings from cultured neurons and glia in our spinal cord and brainstem slice cultures. An example of the cellular resolution provided for visual patch recordings using an 80 μm spinal slice culture is presented in Fig.

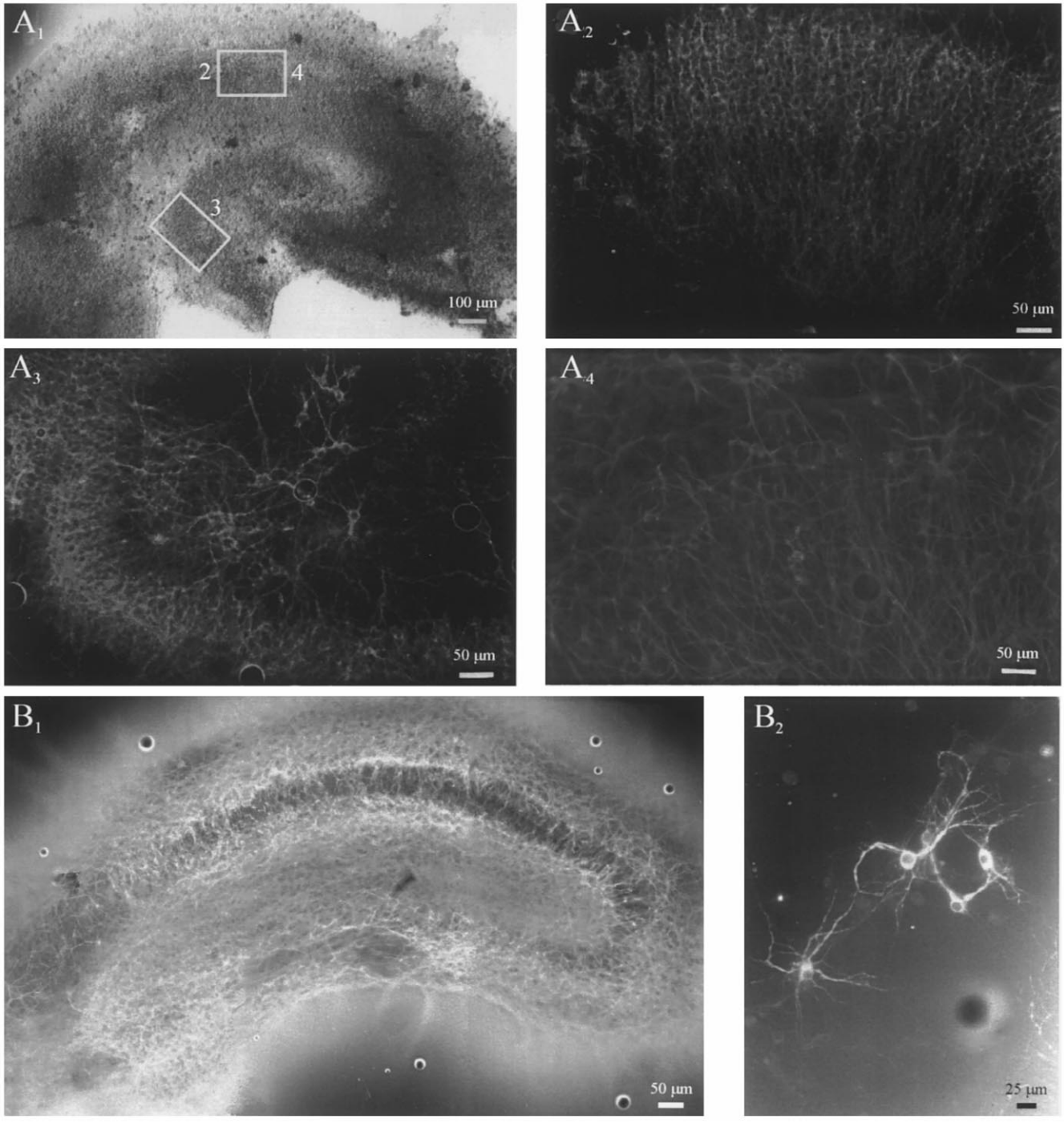

Fig. 3. Hippocampal slice culture. A_1 Hippocampal slice (100 μm) after 6 days in culture (P4 animal). A_2 Immunostaining of CA1 neurons with Panaxonal and MAP-2. A_3 Immunostaining of dentate granule cells with Panaxonal and MAP-2. A_4 Immunostaining of CA1 region for glia using GFAP. B_1 Panaxonal and MAP-2 staining to demonstrate overall neuronal organization of a 150 μm hippocampal slice culture maintained for 7 days (P3 animal). B_2 Neurons from subicular region have migrated out of the slice proper.

4A and for hippocampus in Fig. 4B. Notice the ease of cellular visualization at both low and high magnification using Hoffman modulation optics. Sample recordings are presented in Fig. 5 from two neurons in the same spinal cord slice under both voltage and current clamp configurations (Fig. 5A and B). After 14 days in culture both single and repetitive firing can be observed in different neurons and further, spontaneous synaptic events are also seen (Fig. 5C).

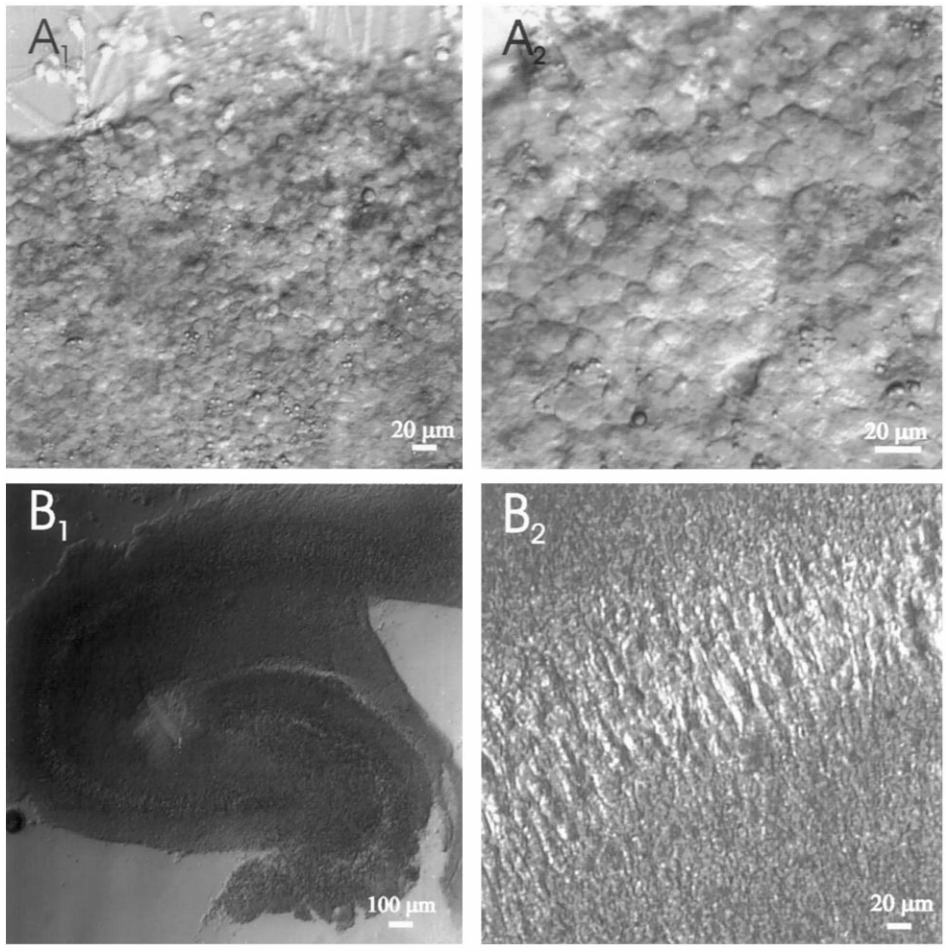

Fig. 4. Hoffman modulation optics images of spinal cord (A) and hippocampal slice cultures (B) to show the ease of cellular visualization for electrophysiological targeting. A. An 80 μm thick spinal slice after 14 days in culture at two magnifications. B. An 80 μm thick hippocampal slice culture (P8) after 27 days displays a well preserved overall topography (B_1) including neurons in the CA3 region (B_2).

3.5. Time-dependent observation (spinal cord and brainstem)

The use of our culture method to study time dependent changes is represented in Fig. 6. Photographs of cultures were taken to show the extension of processes at 2, 8 and 14 days in culture for spinal cord (A_1–A_3) and brainstem (B_1–B_3). At day 2 for both spinal cord (A_1) and brainstem (B_1) there is little process extension. By day 8, some processes can be observed to extend for over 1 mm (A_2, B_2). By day 14, continued process extension is observed to have distances >1.3 mm (A_3) or 2.1 mm (B_3), as well as having become much more extensively arborized. Panels C_1–C_3 demonstrates the development of a presumed axon bundle interconnecting a spinal cord-brainstem co-culture by culture day 14. Although general size and morphology may change slightly over time (A_1 vs. A_3, B_1 vs. B_3, or C_1 vs. C_2), Nissl stains demonstrate the preservation of the overall morphology (e.g. Fig. 1D).

4. Discussion

We have demonstrated the importance of slice thickness by culturing explants of 300 μm, and then compared them to explants of 150 and 80 μm thicknesses. Although many cells were viable in the 300 μm spinal cord slice cultures after 14 days (Fig. 1), visualization of these individually labeled cells was difficult. The 300 μm spinal cord slice cultures also contained large areas of pronounced cell death, which coincided with the darker areas of the slice observed with interference optics. These darker areas occurred preferentially in the center of the slices, presumably where the exchange of nutrients and/or gases is most compromised. Slice cultures produced at 80 μm thicknesses were optimal for cell visualization, and therefore for visual electrophysiological recording techniques. Although thin slice cultures for both spinal cord and hippocampus (80 and 100 μm, respectively) are optimal for studies requiring cell visualization, there was a slight increase in cell

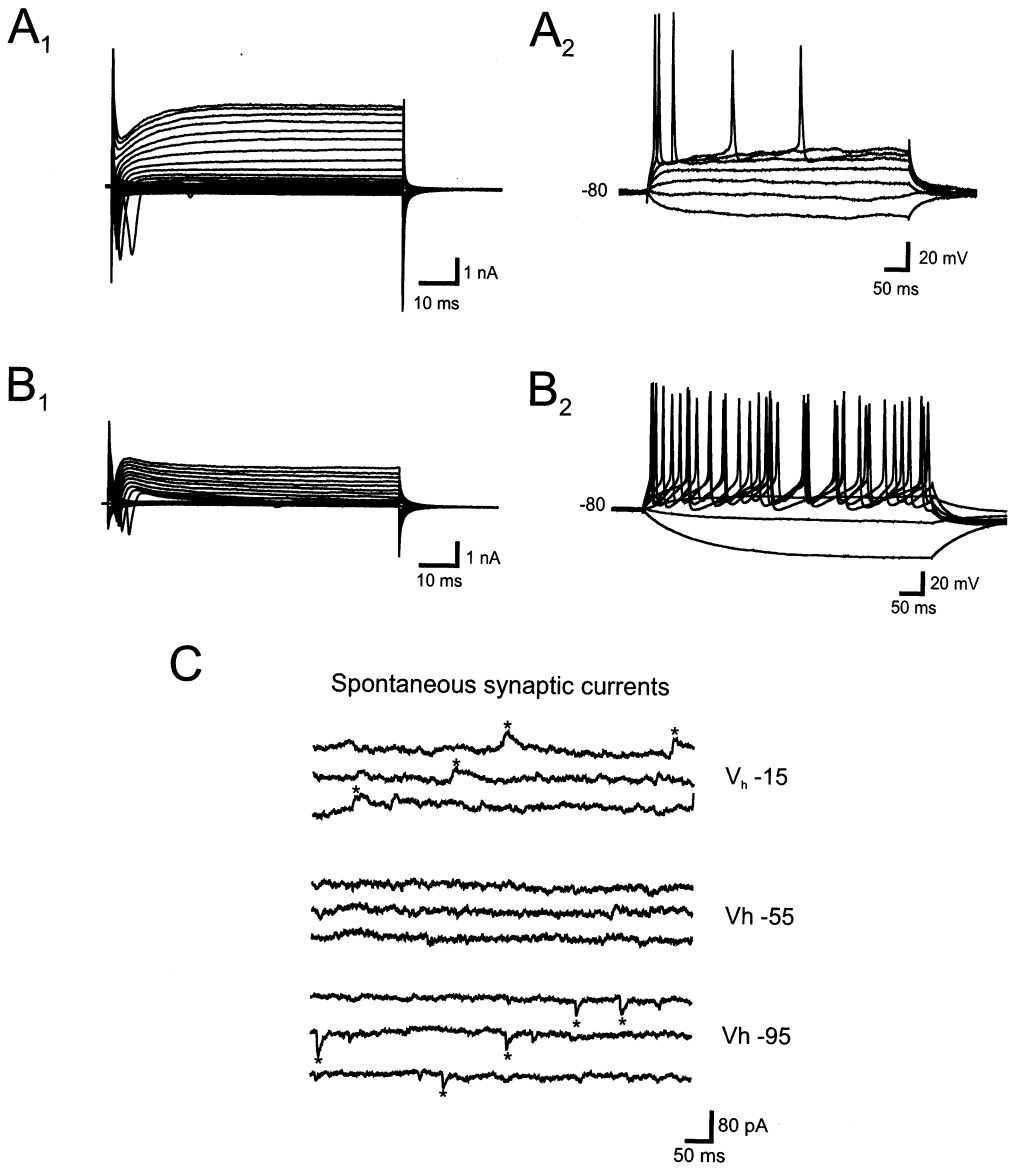

Fig. 5. Response properties of two neurons (A and B) in the same spinal slice after 14 days in culture. A_1 and B_1 Voltage clamp recordings showing currents evoked from a voltage step protocol. A_2 and B_2 Current clamp recordings showing action potentials generated from current steps. C. Spontaneous synaptic activity is observed in slice culture. Spontaneous inhibitory synaptic currents reverse at ~ -55 mV.

death within the slice probably due to trauma suffered from slicing at such a reduced thickness. The incidence of cell death seen in 150 μm slice cultures was less in both spinal cord and hippocampus with cell viability in 150 μm spinal cord sections being 95%. While not quantified, it appears that an increased number of viable cells remained in 150 μm hippocampus slices as well. Therefore, future studies employing our method should use 150 μm slices if visualization at a near monolayer is not required, while 80 μm slices are suggested for studies requiring more optimal cellular visualization.

Thin slice culture permits visual patch recording from neurons located at or near the slice surface while using an inverted microscope. This contrasts the requirement for an upright microscope with water immersion objectives and interference optics to visualize neurons with the interface method. Furthermore, an inverted scope permits greater access to the preparation for the positioning of electrodes and other devices.

After 14 days in culture, heterogeneity of neuronal firing properties were observed (Fig. 5A and B) and the additional observation of spontaneous synaptic events in neurons (Fig. 5C) suggests that the physiological properties of the neuronal population are maturing in vitro (Xie and Ziskind-Conhaim, 1995). Support for continued developmental maturation or plasticity in vitro is further provided by the time-dependent changes

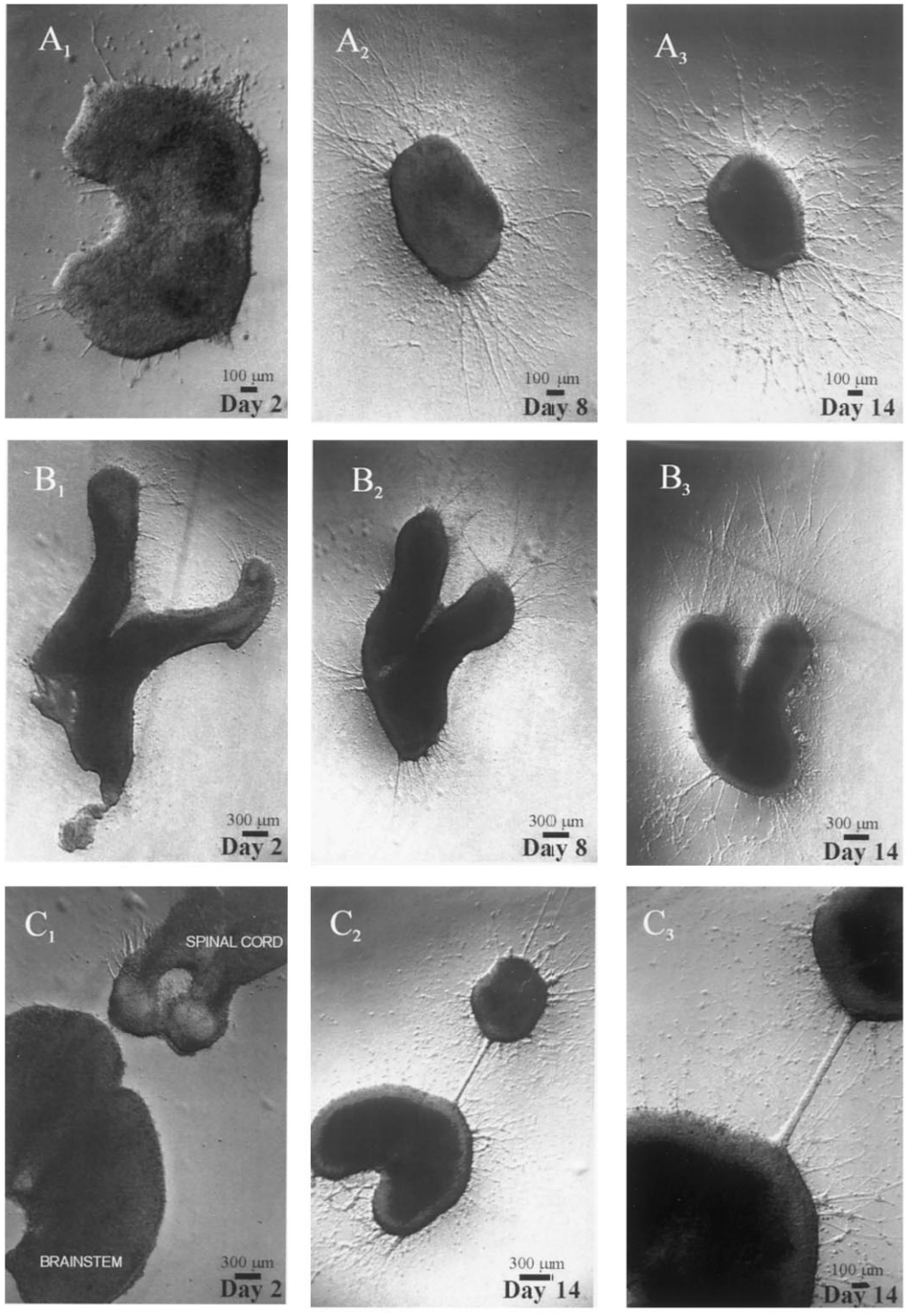

Fig. 6. Slice culture allows assessment of time-dependent changes. All slices were sectioned at 150 μm thickness. A_{1-3} Spinal cord. B_{1-3} Brainstem. C_{1-3} Brainstem – spinal cord co-culture. In all note the time-dependent extension of processes which are seen forming a bridge in co-culture ($C_{2,3}$).

in slice morphology (Fig. 6). As single slices remain in culture over time (spinal cord and brainstem), the elongation and continued arborization of processes can be visualized. In co-cultures, maturation is also suggested by the development of bundles (presumably axonal) that interconnect the slices.

In summary, we have developed a procedurally simple and inexpensive explant culture technique that allows CNS slices to be maintained in organotypic culture. The technique relies on the use of comparatively thin tissue sections (80 or 150 μm) as compared to those used in other culture methods (250 to 500 μm;

see Gähwiler, 1981; Stoppini et al., 1991). Tissue viability was verified both by live/dead cell assay and electrophysiological recordings. These explants appear to maintain in situ tissue organization, contain neurons that demonstrate maturing neuronal properties and flatten to a final thickness that allows visualization of individual cells for visual patch clamp recordings on an inverted microscope. While this method offers a visual resolution already available with the roller tube method, it may offer additional advantages. Firstly, because thinner tissue sections are used, reduced topographical distortion due to slice flattening would be expected. Secondly, immunostaining procedures are performed easily, and are not hampered by difficulties associated with attachment to a porous membrane (Stoppini et al., 1991). Thirdly, unlike the roller tube method, our method allows for continuous microscopic evaluation of cultures throughout the culturing period while maintaining sterility. This is particularly useful for experiments which assess time-dependent changes. Since explants can be studied daily as either single cultures or co-cultures, this method is useful for studies on development or regeneration. Finally, due to the use of serum-free media, our culture method provides the controlled environment necessary to study the effects of specific molecules and/or neurotrophic factors on development and functionality of CNS neurons. We are currently capitalizing on these advantages to study the effects of trophic factors on regeneration between brainstem and spinal cord regions.

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