Applications Brief

Near-monolayer Sectioning of Live CNS Tissue with the Leica VT1000 S Vibrating Blade Microtome

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Introduction

The in situ study of sections of living tissue maintained in vitro is a powerful method to elucidate many aspects of cellular and network function in the CNS, both in acute slice and long-term culture (organotypic) preparations. Recently, there has been an increased demand for technologies that enhance optical resolution at the synaptic/cellular level in order to better identify particular cell types or examine the properties of distinct spatial regions within individual cells.

Since the ability to resolve detailed cellular features is generally limited to 40-50 µm from the slice surface, one strategy for enhanced cell visualization is to section CNS tissue at a reduced thickness which would also improve image quality by minimizing light scatter. This can be achieved with the LEICA VT1000S vibrating blade microtome as described below.

Experimental Procedures

Materials and methods: Isolated cerebellum, hippocampus or spinal cord from neonatal rats (P1- P21) were embedded in AGAR (2.5 % w/v), affixed to the specimen holder with cyanoacrylate (Krazy glue), then fastened to the buffer tray bottom which contained ice-cold artificial cerebrospinal fluid (ACSF). The buffer tray temperature was maintained by applying ice cubes to the outer holding chamber of themicrotome.

Sectioning was performed with the LEICA VT1000 S vibrating blade microtome using stainless steel razor blades (Personna - Personna Medical, Staunton, VA). The microtome was operated at the following adjustable settings: knife angle, 5-7°; sectioning speed, 0.05 - 0.2 mm/sec; oscillation frequency, 80-100 Hz; and oscillation amplitude, 0.6 mm.

The original ACSF used was (in mM): NaCl 125, KCl 2.5, $MgCl_2$ 1, NaH_2PO_4 1.25, $NaHCO_3$ 26, D-glucose 25, and $CaCl_2$ 2. The ACSF was maintained at ~4 °C and oxygenated with 95 %O_2 and 5 %CO_2. Several modifications to the original ACSF were tested and compared in an attempt to enhance preservation of cell viability during slicing. This included substituting equiosmolar sucrose for NaCl, incubation with antioxidants, using a nominally Ca^{2+}-free solution and/or inclusion of glutamate receptor antagonists to minimize excitotoxicity, and use of a high osmolarity solution by including an additional 200 mM sucrose.

Acute slices: Slices were transferred to the recording chamber with Pasteur pipettes. The conventional use of a platinum frame with attached nylon fibres to submerge slices in the recording chamber was too gross and indelicate for these wafer-thin slices. Hence 3 alternate mounting procedures were tested.

(1) The sucrose-ACSF solution was drained from the recording chamber until only a small drop remained covering the slice. Filter paper pieces cut into long wicks were used to remove additional solution so that only a meniscus was left covering the slice. A short period of time (~ 1 minute) was allowed for the perimeter of the solution to evaporate creating a syrupy base for slice adherence. ACSF was then reintrodced into the chamber. This was achieved by gently applying drops of ACSF using a Pasteur pipette immediately over the tissue (to prevent tissue lifting from hydrostatic forces) until the section was completely submerged in solution.

(2) The coverslip bottom of the recording chamber was first coated in hexamethyldisilazane (HMDS; Sigma) then airdried. HMDS acts as a biocompatible interface material for both hydrophobic and hydrophilic surfaces. Slice adherence following contact to the HMDS-coated coverslip bottom of the chamber was greatly enhanced.

(3) Appropriately sized U-frames or O-rings of silver wire were constructed then pressed flat (~2000 psi) to cover the slice perimeter.

Slice culture: Slices of hippocampus and spinal cord (50-150 µm) were maintained in culture using collagen-coated 35 mm culture dishes or coverslips (within culture dishes) with 0.5 ml of Neurobasal and B27 media (GIBCO). Media replacement consists of removing 0.2 ml, then adding 0.2 ml of fresh media every 4-5 days.

Results

Acute slices: Sectioning tissue with equiosmolar sucrose replacing NaCl in the ACSF was the single most important variable for increased cell viability. While all methods for slice fixation following transfer to the recording chamber worked, HMDS proved to be the easiest and most reliable method.

Slice viability was assessed using fluorescein diacetate and propidium iodide as markers for live and dead cells respectively.

Figure 1 presents examples of live cells in hippocampus (A) and spinal cord (B) sectioned at 50 µm. Generally, a large fraction of cells remain viable following sectioning, but there is considerable variability between slices. In the example of Figure 1A, CA1 pyramidal and dentate granule cells were preferentially spared as compared to neurons from the CA3 region.

Figure 2 presents acute slice sections of hippocampus observed with Hoffman optics. Enlargements of 50 µm sections of CA1, CA3 and dentate granule regions of the hippocampus are presented in Figure $2A_{2\text{-}4}$. For the CA1 region, primary apical dendrites and pyramidal-shaped cell somas are easily visualized. While neurons from the CA3 region also display a relative ease of visualization of primary apical dendrites (e.g. at top left of Fig. $2A_3$) cell somas are clearly spindle-shaped. Cell somas of dentate granule cells (Fig. $2A_4$) appear more rounded without an obvious parallel array of process extensions. Hippocampal slice integrity was not maintained at 20 µm as the shearing stress of hydrostatic forces probably exceeded the adhesiveness of extracellular matrix to maintain neuropil integrity (Fig. 2B). However single neurons and neuropil were fortuitously separated introducing the possibility of isolation of single neurons with long processes with proximal and distal regions clearly visible (Fig. $2B_2$).

Figure 3 presents a 50 µm sagittal section of cerebellar cortex. Gross architecture of the cerebellar folia are maintained (Fig 3_1) and distinct molecular, purkinje and granule cell layers are clearly visible (Fig. $3_{2,3}$). At higher magnification, neuronal processes are apparent in the sub-granule interneuronal regions (Fig. 3_4).

Figure 4 presents a 20 µm section from neonatal rat sacral spinal cord. Enlargements of dorsal central canal region (Fig. $4_{2,4}$ lamina X) and dorsal horn (Fig. 4_3) are also presented. Individual cell bodies can be easily identified even at low magnifications and evidence of a retained complex morphology is seen at high magnification (Fig. 4_4).

Slice culture: Figure 5 presents an example of hippocampal slice culture at 100 µm. Overall topography of the hippocampus was well maintained (Fig. $5A_1$). Immunostained neurons were well visualized at both CA1 pyramidal (Fig. $5A_2$) and dentate granule cell regions (Fig. $5A_3$). Few positive staining neurons were found in the CA3 region at this thickness (not illustrated) possibly due to slicing-induced cellular trauma or mishandling. Neurons which migrated out from the subicular region of another hippocampal slice culture are also shown (Fig. 5B).

General Discussion

The enhanced transparency due to reduced slice thickness clearly permits superior optical resolution of cell bodies and their processes throughout the slice thickness. Live/dead cell staining revealed that many cells remained viable in all CNS regions examined. Though not illustrated, whole-cell patch clamp experiments were obtained in normal ACSF, however replacement of sucrose ACSF with control ACSF had to be executed gradually and delicately or slice viability deteriorated rapidly.

We conclude that "monolayer" slices retain viable populations of cells for a multitude of experimental applications including: (i) Neuropil visualization. This is due to enhanced optical resolution. Tissue sectioned at 20-50 µm provides a near-monolayer of living cells with in situ organization and use of an inverted microscope permits imaging experiments to be performed with high numerical aperture immersion objectives without concern for working distance. (ii) Electrophysiology. The reduced section thickness permits visual patch clamp recordings using an inverted microscope and hence a greater ease and flexibility in the placement of experimental equipment (electrodes and local perfusion systems). Further, the reduced slice thickness would hasten drug equilibration and washout times. (iii) Organotypic culture. Since sections are at near monolayer thickness, limted tissue topographical distortion would be expected to occur due to slice flattening.

Light Microscopical Analysis

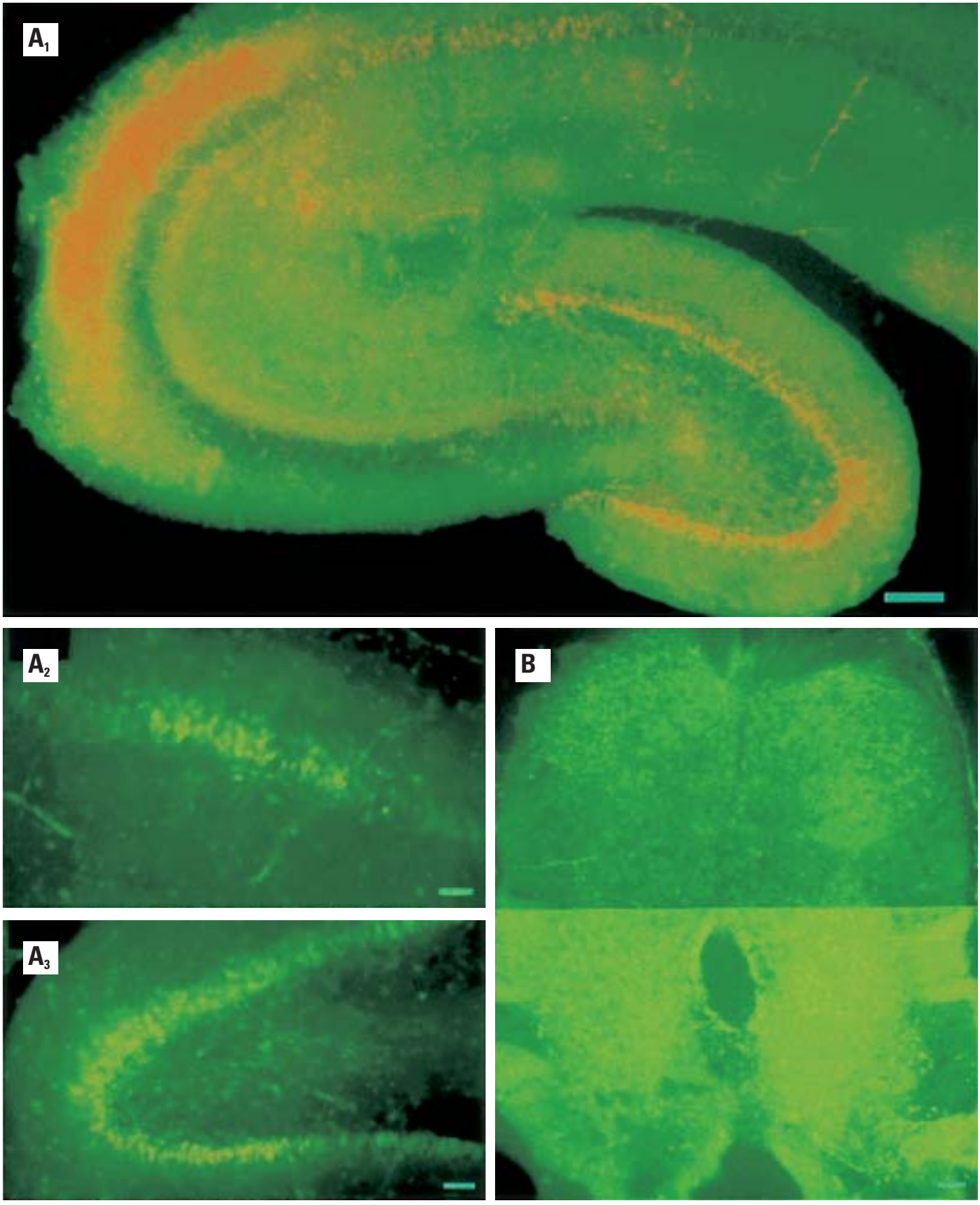
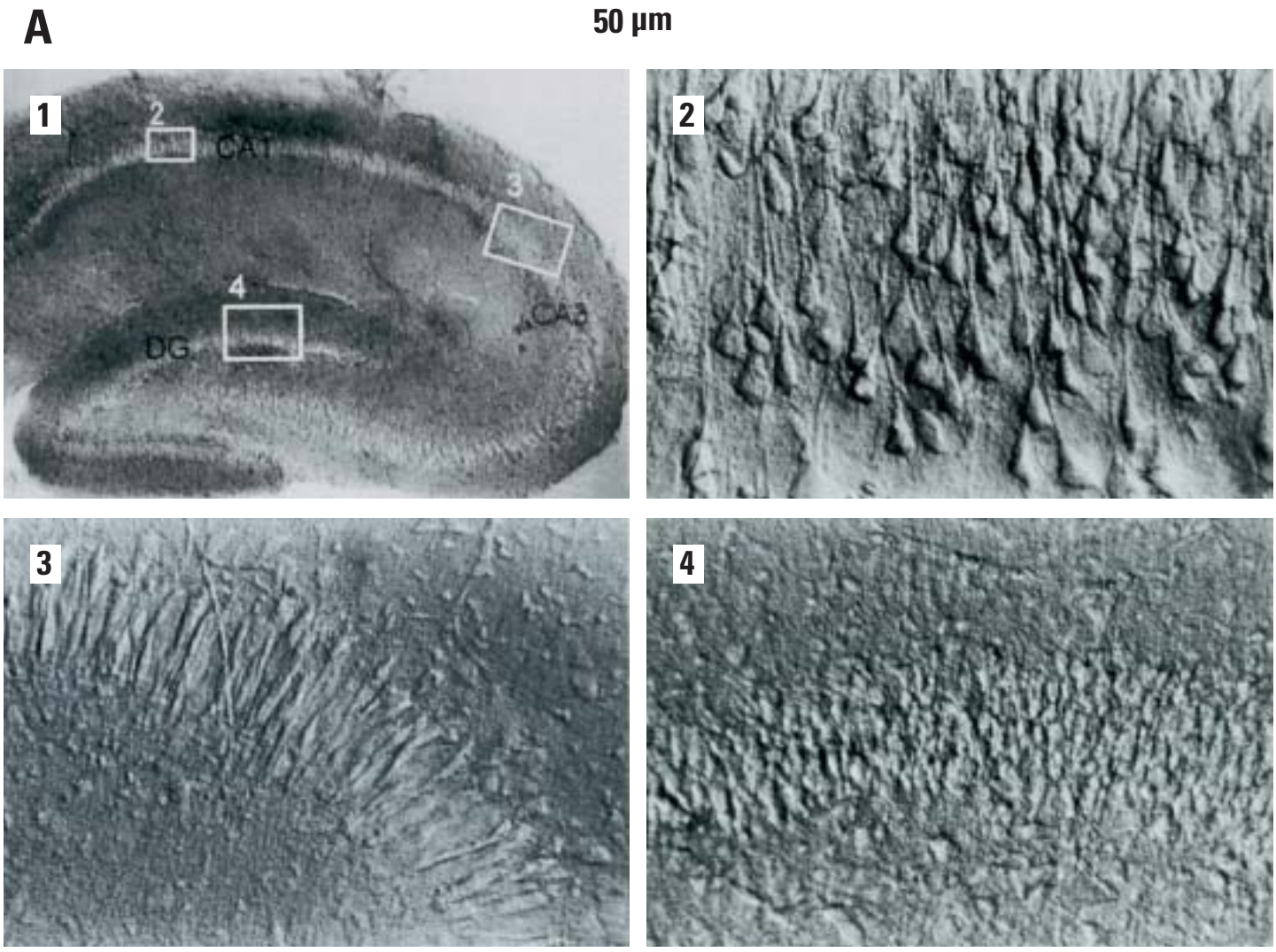


Figure 1. Live/dead cell assay reveals viable neurons (fluorescing green) in 50 µm sections of hippocampus (**A**) and spinal cord (**B**). A_2 and A_3. Enlargement of CA1 and dentate granule regions respectively. Scale bar = 100 µm in A_1 and 50 µm in A_2, A_3, and **B**).

Light Microscopical Analysis



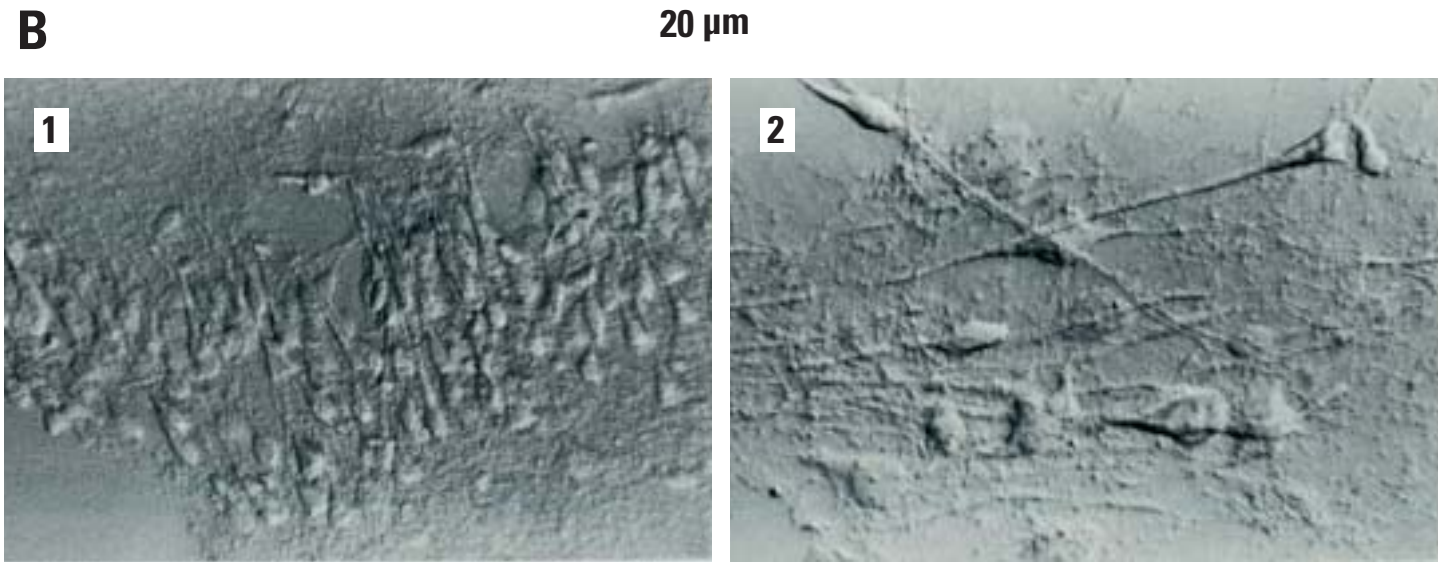


Figure 2. Hippocampal slice at 50 (**A)** and 20 µm (**B**) section thickness. **A1**. Coronal slice from a postnatal day 8 (P8) rat (scale bar = 100 µm). **A2.** Enlargement of CA1 region showing pyramidal neurons with apical dendritic processes (scale bar = 10 µm) **A3**. CA3 region (scale bar = 20 µm). **A4**. Dentate gyrus (DG) (scale bar = 20 µm). **B1.** CA1 region (P8) (scale bar = 20 µm) **B2.** Higher power magnification of CA1 region (scale bar = 10 µm). Photomicrographs for Figures 1-3 were taken in sucrose ACSF.

Light Microscopical Analysis

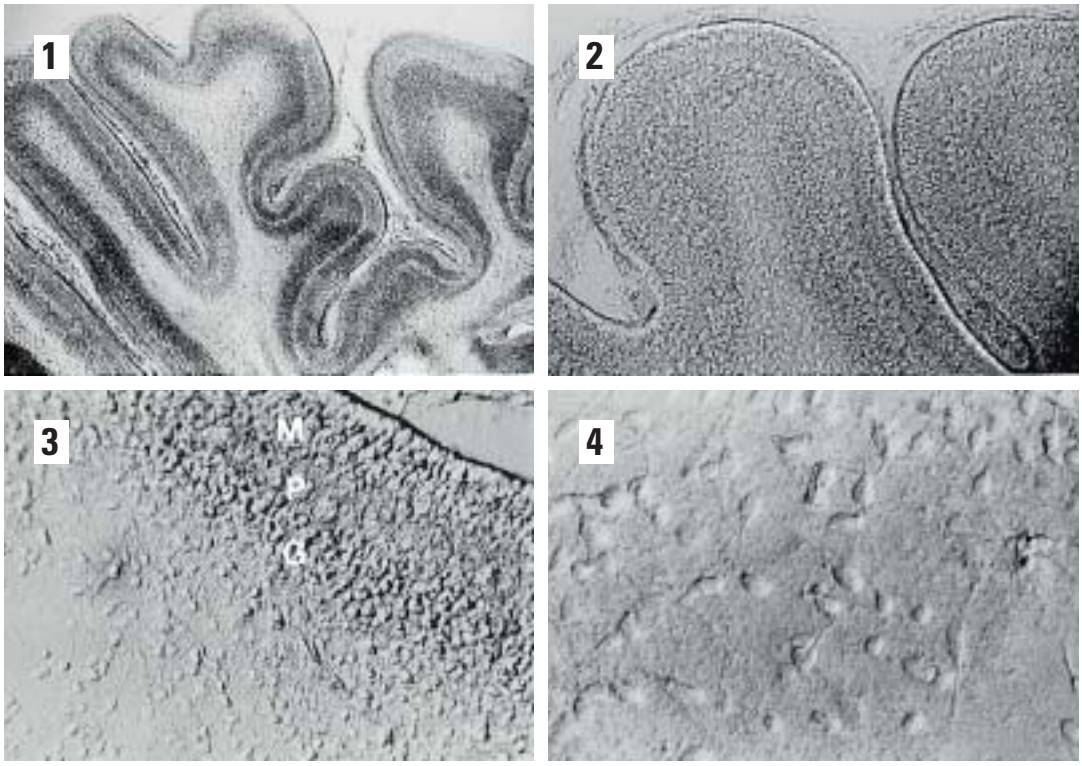


Figure 3. Cerebellar slice (50 µm). **1.** Sagittal section showing cerebellar folia (scale bar = 100 µm); **2.** Enlargement showing Cerebellar cortex and secondary fissure (scale bar = 50 µm); **3.** Enlargement showing cortical cell layers (scale bar = 20 µm); **4.** High magnification of cells in granular region (scale bar = 10 µm). (**M**: Molecular layer; **P**: Purkinje cell layer; **G**: Granular layer)

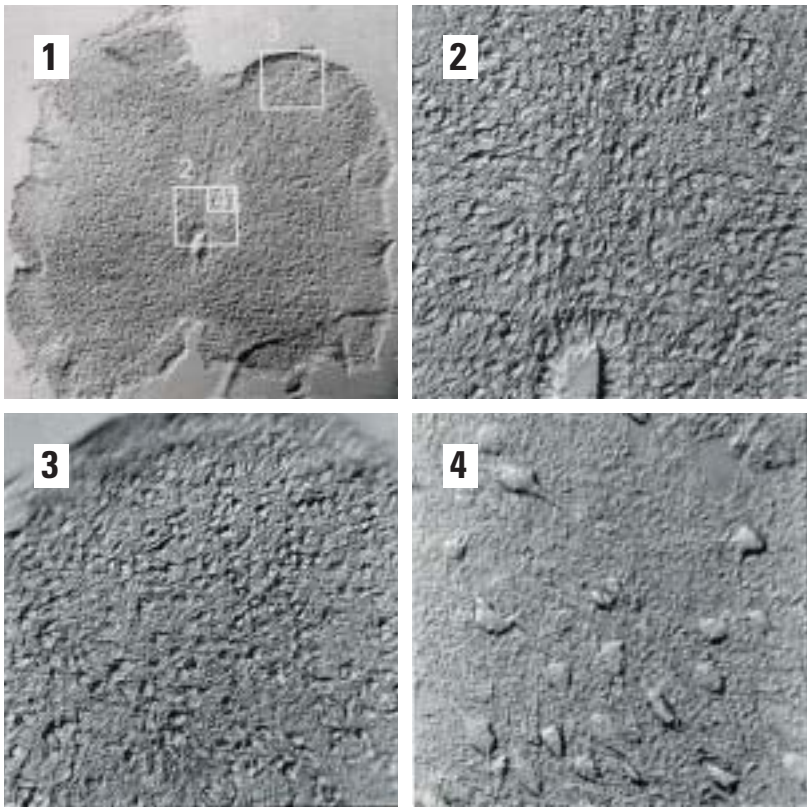


Figure 4. Spinal cord slice at 20 µm thickness (P4).
1. Transverse slice (scale bar = 50 µm).
2. Dorsomedial region above central canal (scale bar = 20 µm).
3. Superficial dorsal horn (scale bar = 20 µm).
4. Enlargement showing spinal neurons with processes in deep dorsal horn (scale bar = 10 µm).

Light Microscopical Analysis

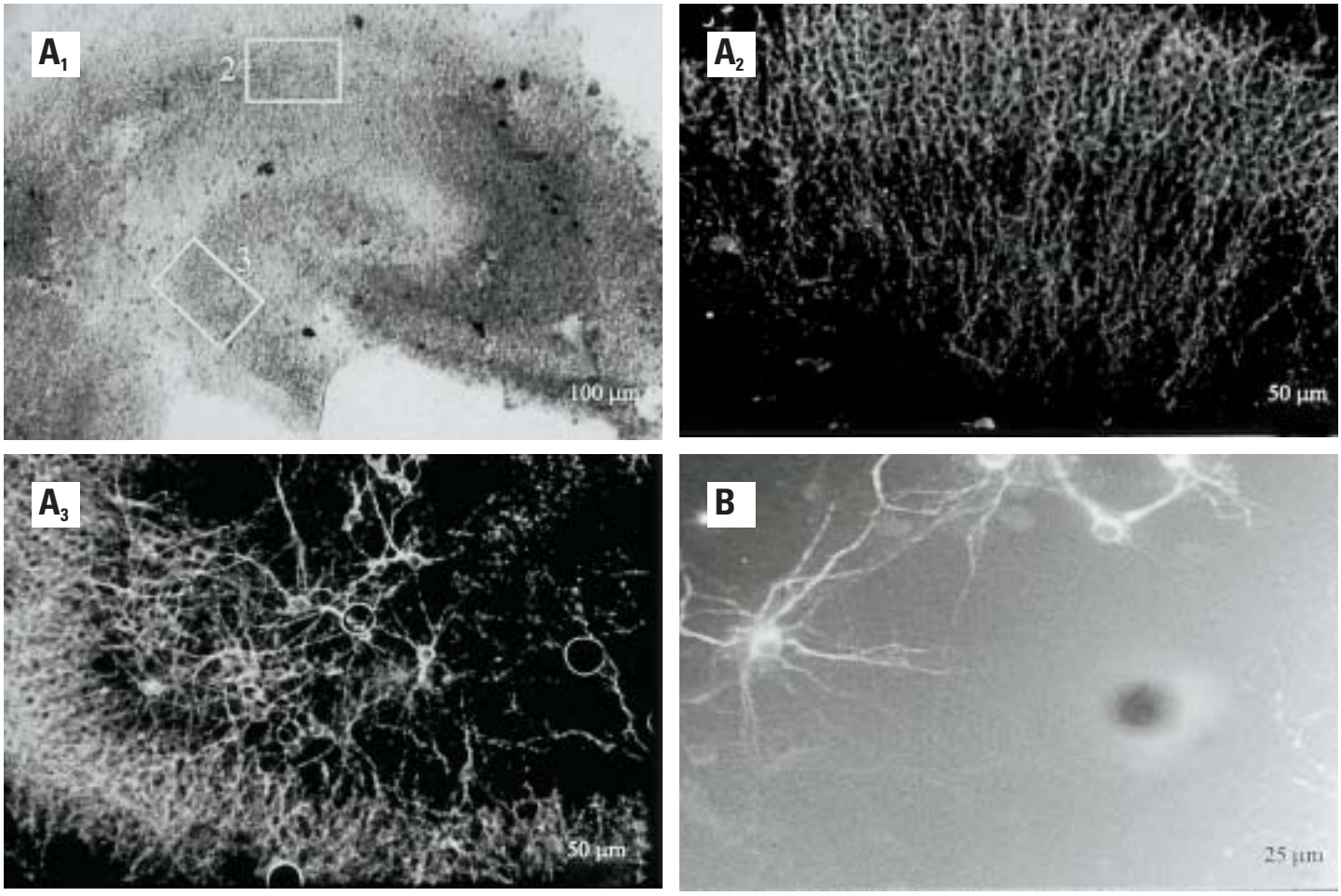


Figure 5. Hippocampal slice culture. $\mathbf{A_1}$ Hippocampal slice (100 µm) after 6 days in culture (P4 animal). $\mathbf{A_2}$ and $\mathbf{A_3}$ Immunostaining of CA1 and dentate granule neurons with Panaxonal and MAP-2.
B. Neurons from subicular region have migrated out of the slice proper.

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Leica VT1000 S

The vibrating blade microtome for advanced applications

The VT1000 S vibrating blade microtome can produce sections of living CNS tissue as thin as 20 µm that permit enhanced optical resolution with aspects of in situ organization retained. Additionally, use of an inverted microscope permits electrophysiological and imaging experiments to be performed with high numerical aperture immersion objectives without concern for working distance. The VT1000 S can also produce thin slice explants for organotypic culture (50-150 µm). At this section thickness, slices can be incubated using standard culture procedures in 35 mm culture dishes with minimal topographical distortion. Hence the VT1000 S should facilitate and economize experimental studies where cellular visualization is critical.

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