CHAPTER I

INTRODUCTION

1. General Introduction

Brain slice preparation is an in vitro technique utilized in study of mammalian central nervous system (CNS) by employing thin (100-700 um thick) fresh brain tissue. The first electrophysiological investigation was introduced when successful recording of resting membrane potential was obtained from neocortical slices (Li and McIlwain, 1957; Hillman and McIlwain, 1961; Gibson and McIlwain, 1965). However, at that time, there was no report of electrical signals evoked as a response to any given stimulus. Thereafter, by employing olfactory cortical slice synaptic field potentials could be observed extracellulary following stimulation applied of the incoming lateral olfactory tract (Yamamoto and McIlwain, 1966). Responses of the nature have also been found in the laminate structure of hippocampus (Yamamoto, 1972). These findings have substantiated a rapid gain in the value of the brain slice preparation.

A decade later, this method has invaded into various mammalian CNS areas where exact neuronal

circuitry are known; these are : cerebellum (Gardner-Medwin, 1972), lateral geniculate body (Yamamoto, 1974), medulla (Fukuda and Loescheke, 1977), caudate nucleus (Miller and Rutherford, 1978), cuneate nucleus (Simmonds and Pickles, 1978), spinal cord (Takahashi, 1978), interpeduncular nucleus (Brown and Halliwell, 1979; Ogata, 1979), hypothalamus (Hatton, Doran, Tweedle, and Salm, 1980), and neostriatum (Bak, Misgeld, Weiler and Morgan, 1980; Kitai and Kita, 1984).

In the cerebellum, evoked responses related to parallel fiber stimulation were observed in guinea-pig cerebellar slices (Gardner-Medwin, 1972). Major advantage in studying the cerebellum can be attributed to a great deal of knowledge about the morphology, anatomical, as well as physiological connections of the cerebellar cortical neurons (Eccles, Ito and Szentagothai, 1967). Therefore, the cerebellar slice preparation has been extensively employed in many fields of study, e.g. physiology, pharmacology, biochemistry and morphology (Yamamoto, 1973; Okamoto and Quastel, 1973, 1976; Okamoto, Quastel and Quastel, 1976; Garthwaite, et al., 1979, 1980; Llinas and Sugimori, 1980a,b; Crepel and Dhanjal, 1981a,b; Crepel, Dhanjal and Garthwaite, 1981; Okamoto and Sakai, 1981; Crepel, Dhanjal and Sears, 1982; Basile, Hoffer and

Dunwiddie, 1983; Hounsgaard and Nicholson, 1983; Basile and Dunwiddie, 1984; Crepel, Dupont and Gardette, 1984; Gardette, Debono, Dupont and Crepel, 1985a,b; Okamoto, Kimura and Sakai, 1984; Garthwaite and Garthwaite, 1985; Flint and McBride, 1986; Sekiguchi, Okamoto and Sakai, 1986; Sakurai, 1987).

In our laboratory, previous works deal with electrophysiology of the cerebellum in in vivo condition have been presented (Saguanrungsirikul, 1983; Tongroach, Saguanrungsirikul, Tantisira and Kunluan, 1984; and Sutthisunk, 1987). However, due to the problems in maintaining extracellulary recording over a prolonged periods as frequently required in order to complete processing of spike data following various manipulation and as in application of drugs or substances, a stable preparation such as brain slice seems to promise a reasonable alternative to the existing in vivo techniques.

2. Cerebellar Cortex; An Overview.

The cerebellum possesses a well-defined cortical structure (Eccles, Ito and Szentagothai, 1967; Eccles, 1973; and Ito, 1984) characterized by a folded sheet of gray matter in which the white matter is covered. Structurally, it consists of three different cortical layers according to the composition of

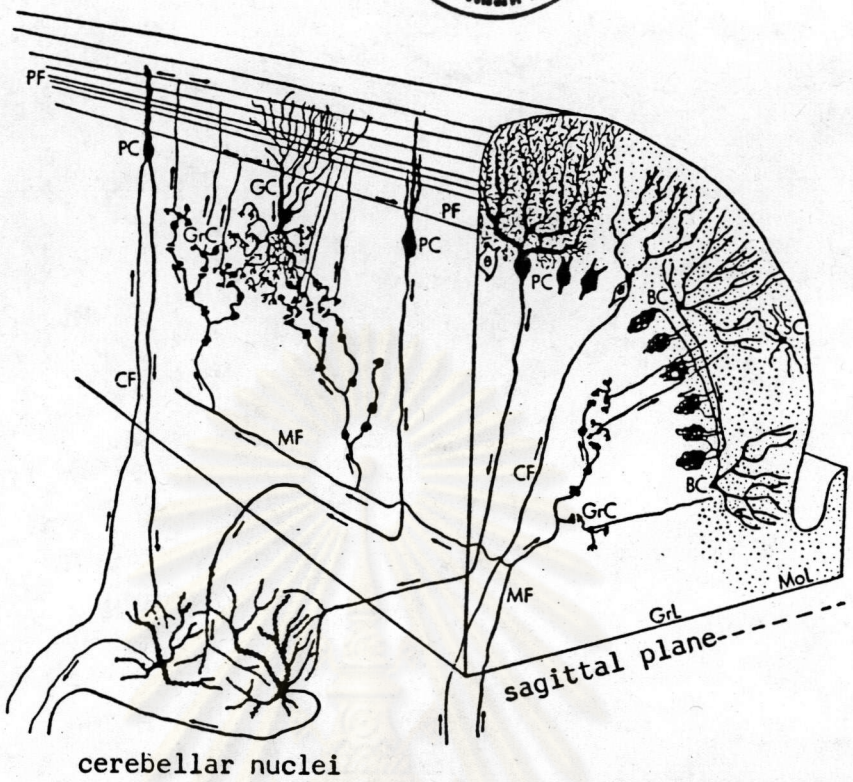

Fig. 1. Schematic picture of a segment of a cerebellar folium. There are two main afferent fibers projected to the cerebellar cortex. The climbing fibers (CF) that twin around the Purkinje cells dendrites and the mossy fibers (MF) that branch enormously and synapse on the little granule cells (GrC) in the granular layer (GrL) whose axons pass up to the molecular layer (MoL) to bifurcate and form the parallel fibers (PF) that run along the folium. Thus they synapse to the dendritic trees of the Purkinje (PC), basket (BC), and stellate cells (SC). The Golgi cells (GC) also receive synapses from the parallel fibers and have profusely branched axons that end on the granule cell dendrites. (adapted from Eccles, 1973).

neuronal elements, as shown in Fig. 1.

The outermost molecular layer has the thickness of about 200 um in adult rat (Garthwaite et al., 1980). It composes of two types of interneurons, the basket and the outer stellate cells, which are local elements of the cortex. The dendrite of Purkinje cell, or dendritic arborization, extend outward in the direction perpendicular to numerous small axons running along the long axis of the folia, the so-called parallel fibers.

Purkinje cell bodies with thickness of about 40 um (Garthwaite, et al., 1980), according to the long axis of the soma, lies along the middle layer of the cortex. Under light microscopic observation, this layer could be localized as a thin line at the junction between the molecular and the granular layers.

The innermost granular layer is a dense pack of granule cells mixed with Golgi cells. Thickness of the granular layer is uncertain and seems to vary with degree of innervation of Purkinje cells present in that area.

3. Electrical Activity of the Cortical Neurons.

In intact animals, Purkinje cells as well as other interneurons usually generate spontaneous

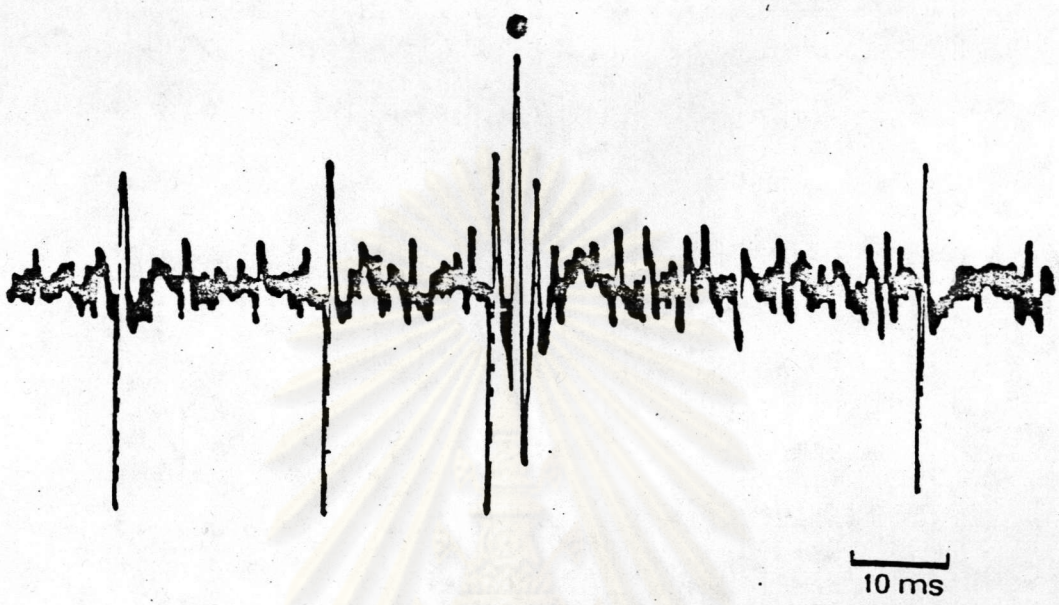

Fig. 2. Back ground continuous discharge of a Purkinje cell extracellulary recorded from an anesthetized rat showing two different action potentials of a simple and a complex (black dot) spikes. (adapted from Saguanrungsirikul, 1983)

activities which can be recorded electrophysiologically. The detail description of the spontaneous activities have been presented by Eccles et al. (1967) and Bell and Grimm (1969) as the following summary. The background discharge of Purkinje cells is usually found in combination of a simple spike and a complex spike at the depth between 250 and 300 um from the pia surface (Fig. 2). The discharge of this cell has a large amplitude of spike and occurs at relatively high frequency of 20-50 impulses/sec or, sometimes, up to 200 impulses/sec. The activity often starts with discharges of single impulses in a more or less regular rhythmic series, and follows with the complex spike. Next, inhibitory interneurons discharge spontaneously at a low and rather irregular frequency which usually is between 7 and 30 /sec. The activity of the basket cells can be recorded at the depth about 350 um, whereas that of the Golgi cells about 500 um. Finally, the smallest fine discharge of the granule cells, usually can be recorded from several cells simultaneously, is observed at the depth of about 500 um.

4. Reviews of Cutting Procedures.

There are two main mechanisms used in preparing the brain slices. The first is a chopping action of

the thin knife over the tissue block against a moving stage. The early model of the chopper has been designed by McIlwain, therefore called McIlwain mechanical tissue chopper (McIlwain and Buddle, 1953; McIlwain, 1962). It is successfully used for cutting pre-piriform area (Yamamoto and McIlwain, 1966), hippocampus (Schiff and Somjen, 1985), and cerebellum (Flint and McBride, 1986). Another model of tissue chopper is a Sorvall TC-2 which is a fully automatic machine. The Sorvall provides user with ability to adjust position of the blade as well as and orientation of the tissue block. It is convenient for preparing hippocampus (Spencer et al., 1976; Schwartzkroin, 1981) and cerebellum (Basile et al., 1983; Basile and Dunwiddie, 1984). The last model is a simple tissue chopper which is developed by Duffy and Teyler (1975). This model features a simple mechanical construction, however, there are only a few use such as in hippocampus (Teyler, 1980; Hatton, Doran, Salm and Tweedle, 1980). Nevertheless, the chopping mechanism has been claimed not to be suitable in case of cerebellar slice (Garthwaite et al., 1979; Alger et al., 1980; Dingledine, Dodd and Kelly, 1980).

The second technique, on the contrary, requires slicing, or sawing, action of the blade that slowly cuts through the brain block mounted on a tissue stage.

The development of this technique can be described as two different procedures concerning the apparatus used.

Hand-Slicing Technique. This is the oldest technique which is still popular in metabolic studies as well as in electrophysiology. The early apparatus used in this method was developed by Stadie and Riggs in 1944 (cf. Elliott, 1969) so-called the Stadie-Riggs' cutter. This instrument keeps the blade at a fixed distance from the tissue while the motion of the blade is guided in a fixed direction. It is no longer in used due to excessive damage of the lower tissue stump produced by rubbing and shearing with surface of the blade (McIlwain, 1962). The latter model designed by Majno and Bunker (1957) consists of a microtome knife mounted along the edge of a plastic plate. Slicing is performed by moving the tissue manually across and along the cutting edge. This apparatus, like the former model, is limited only to a few use since it is so elabolated and cumbersome. Finally, the most wildly used is developed by McIlwain (1962) and still extensively used in preparing of various brain areas for electrophysiological studies in particular : e.g. olfactory cortex (Richards and Sercombe, 1968), interpeduncular nucleus (Brown and Halliwell, 1979; Ogata, 1979), and cerebellum (Okamoto and Quastel, 1973; Crepel et al., 1981a,b; Crepel, Dupont and

Dupont and Gardette, 1984). The apparatus required is a bow cutter, a guide, a strip of razor blade, and a cutting table (McIlwain, 1962; Alger et al., 1984).

The Slicer Machine. A variety of the brain slicer machines have been developed as an alternative to hand-slice techniques. The early model was constructed by Franck (1972), which is called a cutting table. The mechanism used is sawing motion of a running stretched nylon thread that trims across the tissue.

A novel vibrating blade Tissue Slicer. This model acting with a transverse vibration of the knife for cutting the tissue block. At present, there are at least two types of machine available commercially such as (1) the Oxford Vibratome, and (2) the Vibroslice.

(1) Oxford Vibratome. This instrument has been designed for slicing freshy or lightly fixed tissue (Smith, 1970; Clouser, 1977; Cuello and Carson, 1983). It provides an automatic movement of the solenoid driven vibrating blade that trims into the brain block mounted on the fixed stage in a sectioning bath. Thickness of the slice can be set at each beginning of the section by means of a vertical setting of the blade. The sectioning is performed in a 500 ml bath filled with cold medium (2^{o}-4^{o}C). The bathing solution

has a four-fold function ; to lubricate the knife as sections are cut; to prevent heating or drying of the specimen; to float the sections for transfer; and to preserve or enhance specimen characteristics. The best cutting angles are found with the blade depressed between 15^{o} and 25^{o} from the horizontal (Clouser, 1977).

(2) Vibroslice. Developed by Jefferys (1982), the machine has particularly an economical design and has more convenient to use. In common with the Vibratome, the Vibroslice cuts horizontally under cold medium using a razor blade. It differs in other respects. Slice thickness is set by lowering the blade holder between successive cuts. The blade vibration frequency is controlled by a variable speed electric motor (less than 3000 rev/min) which oscillates the blade horizontally. The block of tissue is glued to a stage and immerged in a small capacity bath (200 ml), stage and bath are removable separately for the ease of cleaning. The tissue block is cut by advancing it onto the vibrating blade using either a hand-operated lead screw or a motorized advancing mechanism. Since this machine is of lower cost than the original, it is now becoming commonly used in many brain research laboratories (Gardette et al., 1985a,b; Garthwaite and Garthwaite, 1985; Hubbard, 1988).

The vibrating blade type microtome has been suggested to be most suitable for slicing the cerebellum (Richards, 1981; Hatton, 1983; Alger et al., 1984; and Hubbard, 1988). Therefore, this type was chosen as a typical model for construction of the brain slicer machine in this study.

5. Slice Thickness.

Since the cutting procedure destroys predominantly superficial neuronal elements (Garthwaite et al., 1979; Bak et al., 1980), it becomes more probable to find vital neurons if thicker slices are prepared. The thickness, however, is restricted because the total slice has to be supplied with oxygen by diffusion from its surface.

Practically, most laboratories employ 350-450 um slices, although some have reported using preparation of up to 700 um. At 400 um a compromise is achieved between having a useful core of healthy tissue and the diffusion barriers posed by increasing in tissue thickness (Teyler, 1980).

In preparation of cerebellar slices, it is found that 380 um slices prepared by hand showed better morphology of Purkinje cells as compared to those prepared by the tissue chopper at the same thickness

(Garthwaite et al., 1979). Moreover, comparative study of diffusion time between 300 and 700 um cerebellar slices predict the rate of penetration of substances into a slice, indicated that the faster diffused time was found in the thin slice (Nicholson and Hounsgaard, 1983). Recent study of pO_2-profile and morphology in a 1000 um thick hippocampal slice revealed that a minimum pO_2 value related to a large number of swollen neurons was found at a depth below 150 um.

6. Slice Bath.

A tissue bath serves as physiological support with artificial cerebrospinal fluid (ACSF) whose composition is controllable to provide suitable metabolic substrates, oxygenation and appropriate temperature. The bath used in brain slice can be classified into 2 types with regards to the manifestation of bathing media : (1) a circulating bath, or a superfusion bath, and (2) a static bath, or a stagnant pool.

The circulating bath features a continuous flow of the bathing media. There are two different techniques of perfusion which have been designed for the different purposes. The first is a sub-perfusion bath which is designed for keeping the slices suspended on the mesh. Hence, the tissue are incubated between

the covering warm humidified carbogen (95% O_2 and 5% CO_2) and the subperfusing solution. This bath is sometime known as a interface chamber and was originally designed by Li and McIlwain (1957), latter modified by Gibson and McIlwain (1965), and some other authors (Dore and Richards (1974), Schwartzkroin (1975), Richards and Tegg (1977), Hatton et al. (1980), and Teyler (1980)). In addition, a simplified model of this bath was designed by Haas, Schaerer and Vosmansky (1979) and later modified by Kitai and Kita (1984), and Schurr et al. (1985). The advantages of this chamber is that it provides a good stability and ease of electrode placement. The second type, on the other hand, keeps the slices fully submerged in the perfused media. This type was firstly developed by White, Nadler and Cotman (1978) and subsequently by Koerner and Cotman (1983), and Nicoll and Alger (1981). The submersion chamber has been designed for facilitating the use in pharmacology which requires a rapid changing of perfusing media. The summary of advantages and disadvantages associated with various types of bath are presented in table 1.

The static bath, on the other hand, gives some advantages for stability in intra- or extracellular recording of bioelectrical activities since there is no or a little mechanical disturbance and preferably with

Table 1. Advantages and disadvantages of three types of tissue bath used in brain slice preparation.

Type of bath	Advantage	Disadvantage
A static bath	1. vary good stability. 2. easy to place electrodes.	1. difficult to control medium temperature and osmolarity. 2. difficult to apply drug.
An interface bath	1. high mechanical stability 2. easy to place the recording and stimulating electrodes. 3. techniques of local drug application can use : - iontophoresis - nanodrop	1. limitation of perfusing rate (0.5 to 1 ml/min) 2. drying of slice surface 3. difficult to control oxygen tension, temperature, and fluid level. 4. continuous pump or suction should be used.
A submersion bath	1. easy to construct. 2. simple perfusion system can be used. 3. no problem with O_2 supply. 4. good temperature control. 5. facilitate diffusion at both side of tissue slice. 6. fast exchange of the tested solution.	1. poor mechanical stability. 2. difficult to see electrodes.

small dead space if drugs are to be applied. The static chamber was developed initially by Spencer et al. (1976) and later by Teyler (1980). The former design features as a true stagnant pool which is suitable for electrophysiological investigations while the latter design allows convenient changing of the new medium that is favorable for pharmacological investigations. There are, however, some disadvantages that should be considered such as difficulties to maintain O_2, temperature, and osmolarity of the incubating media. Additionally, theoretical calculation of diffusion capability of substances into the slice showed that this static pool design takes the longest period of penetration through the center portion of a 300 um thick slice (Nicholson and Hounsgaard, 1983).

7. Bathing Medium.

The bathing medium pays an important role as a life supporting element which provides the tissue not only an appropriate level of oxygen and glucose, but also a suitable environment such as an optimized pH, osmolarity and temperature. Since it is now clear that the extracellular environment of nerve cells corresponds not to an ultrafiltrate of plasma but to a cerebrospinal fluid or CSF (cf. Richards, 1981). So, the media that is commonly used in brain slice is a

bicarbonate buffered medium, so-called an artificial CSF (ACSF), whose compositions are comparable to the mammalian CSF (McIlwain, 1975).

The list of ionic compositions found in rat and guinea-pig is presented in table 2 and the chemical compositions used in preparing ACSF in table 3. Table 2 show marked difference of Ca^{2+} concentrations which are lower in CSF as compared with those in serum or plasma. However, approximately 50 % of the calcium in body fluid is bound (Harvey and McIlwain, 1969), and recent studies with ion selective microelectrodes indicate that extracellular calcium concentrations in cortex and cerebellar slices are between 1.0 and 1.5 mM (Nicholson et al., 1978). Moreover, recent study on the use of lower calcium concentration (0.75 mM) in the hypothalamic slice revealed that the neurons exhibited spontaneous activity during perfusing with 0.75 mM Ca but ceased firing when the Ca concentration was raised to 1.26-2.0 mM (Pittman, Hatton and Bloom, 1981; Sibbald, Sirett and Hubbard, 1987). Hence, these reports have strongly indicated that a lower concentration of calcium should be used in ACSF.

Table 2. List of ionic compositions (in mM) in mammal CSF.

Species	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	HCO_3^-	Glu	$H_2PO_4^-$	mOsm	References
Rat	134	5.1	1.3	3	102	22.0	6.1	2.4	-	McIlwain, 1975
Rat	157	3-3.5	-	1.3	143	-	-	-	-	Dingledine, et al., 1980
Guinea-pig	151	3	-	0.86	126	-	-	-	-	cf. Harvey & McIlwain, 1979
Cat	158	2.69-3.28	1.33-1.47	1.5-1.67	138-144	18.3-25.6	3.68	-	314.8	
Rabbit	149	2.9	0.87	1.24	130	22.0	5.35	-	305.2	cf. Alger et. al., 1984.
Human	139-147	2.82-2.84	0.97-1.12	1.14-1.32	113-123	23.3	3.3	-	289.0	

Table 3. Variety of ACSF used in cerebellar slice preparation.

	NaCl	KCl	KH_2PO_4	$MgSO_4$	$CaCl_2$	$NaHCO_3$	Glu	NaH_2PO_4	$MgCl_2$	Species	Cutting Technique	Thickness
Llinas & Sugimori, 1980	124	5	1.2	1.3	2.4	26	10	-	-	G	Vibratome	200
Gardette et al., 1985	124	5	1.15	1.15	2.5	25	10	-	-	R(SD)	Vibroslice	500
Kimura et al., 1985	125	5	-	1.0	2.0	24	11	1.0	1	G	Vibratome	-
Okamoto et al., 1981, 1984	125	5	-	1.0	2.0	24	11	1.0	1	G	Vibratome	150, 160-170
Crepel et al., 1981, 1984	124	5	1.15	1.15	2.0	25	10	-	-	R(P)	Bow cutter	400, 500
Yamamoto, 1973	124	5	1.24	1.3	2.4	26	10	-	-	G	Vibratome	250
Sekiguchi et al., 1986	125	5	-	-	2.0	24	5	1.0	1	G	Vibratome	160-170
Garthwaite & Garthwaite, 1985	118	4.7	1.18	1.19	2.5	25	11	-	-	M(A)	Vibroslice	350
Basile and Dunwiddie, 1984	124	4	1.2	1.2	2.0	25	10	-	-	-	Sorvall TC-2	300, 350
Hounsgaard & Nicholson, 1983	124	5	1.0	-	2.0	25	10	1.0	1	G	Vibratome	200
Okamoto & Quastel, 1973, 1976	125	5	-	1.0	2.0	24	10	1.0	1	G	Stadie-Riggs	500
Crepel et al., 1982	124	5	1.15	1.15	2.5	25	10	-	-	R(W)	Vibroslice	400
Sakurai, 1987	124	5	-	1.3	2.4	26	10	1.24	1.24	G	Microslicer	300-330
Basile et al., 1983	124	2.8	1.2	2.4	2.5	25.7	10	-	-	M(HS)	Sorvall TC-2	-
Hubbard, 1988	124	5	1.25	1.3	0.75	26	10	-	-	R(W)	Vibroslice	300

NOTE : G = Guinea-pig, M(A) = Mouse (Albino), M(HS) = Mouse (Heterogeneous Stock),

R(P) = Rat (Portal), R(SD) = Rat (Sprague-Dawley), R(W) = Rat (Wistar)

8. The Present Study : Rationale.

The aims of this study is to develop an experimental set-up of brain slice which has been divided into three main elements, i.e., slicing procedure, tissue chamber, and perfusion system. The attempt focuses on the cerebellum which is a well defined cortical structure. In addition, the preparation has also been prepared base on the previous works. Validity of the set-up is indicated later by testing viability of the slice as well as by general observation of tissue survival using electro-physiological methods as well as visual inspection. Response of neurons to environmental change (e.g. temperature) and drug application (e.g. glutamate) serves as supporting criteria for tissue survival.