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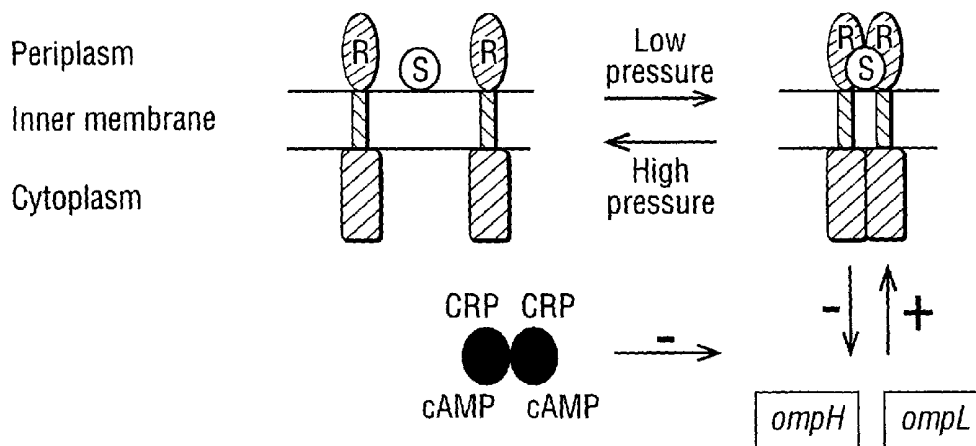
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(54) Title: BIOLOGICAL SENSOR



(57) Abstract: A biological sensor, especially a barosensor, which can be operated using a neuronal network, is described. Neuronal cultures suitable for use in such a sensor are also described.

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BIOLOGICAL SENSOR

- 5 This invention relates to biological sensors. More particularly the present invention relates to biological sensors, which use micro-organisms or living cells as the biological component of the sensor.

10 In the present invention, the sensor may be electrical, magnetic, optical, thermal, chemical or acoustic. In the description that follows, the invention will be described with particular reference to its preferred application in the sensing of pressure in the field of underwater acoustics. However, it is not intended that the present invention be strictly limited to this field since it finds equal utility in other areas.

15 It is known that biological materials exist in nature that employ micro-systems capable of performing physical sensing functions. The benefit of employing such materials is that the materials are likely to be sensitive, efficient, abundant and adaptable. More importantly, when using live materials it is likely that they will work better since the systems would, in nature, be of little use to a dead organism.

20

Problems exist in the identification and isolation of the appropriate organisms, and with the harnessing of their outputs in a repeatable and coherent manner.

25 It is already known, for example, that neurons exist which can perform many of these basic sensing functions. It is also known, from early work in Japan (FUKADA E, YASUDA I. On the piezoelectric effect of bone. J. Phys. Soc. of Japan. 12, 1158-1162. FUKADA E (1968). Piezoelectricity in polymers and biological materials. Ultrasonics. 68, 229-234. FUKADA E, ANDO Y. (1988). Bending piezoelectricity in a microbially produced poly-b-hydroxybutyrate. Biorheology 297-302) that dried bone possesses piezo-electric properties.

30

Further work has been performed more recently at the "Centre Technique des Systèmes Navals" (CTSN) Toulon in conjunction with the University of Marseilles under funding from the French government. In the more recent work the investigations have included vegetable protein structures (cellulose), fungal material, algae and yeasts, all of which have been found to possess interesting sensory properties.

The present inventors have found that the use of micro-organisms, particularly bacteria, in a biological sensor has many distinct advantages. For example, bacteria are generally easy to culture, are robust and are more readily amenable to manipulation. One objective of present inventors was to identify, isolate, and then to manipulate pressure-sensing bacteria that could then be exploited in a biological acoustic sensor.

The pressure sensing system of the deep-sea bacterium *Photobacterium* SS9 has been identified as a potential candidate for biological acoustic sensor development. This system has the advantage that *Photobacterium* can be routinely cultured in the laboratory. The inventors wished to increase the sensitivity of the system to enable response to microPascal changes of pressure.

Work was performed to manipulate the mechanism involved in pressure sensing by the ToxR protein of *Photobacterium* SS9. Mutations of the pressure-sensing region of this protein were made by the present inventors. These mutants have been screened at high and low pressure to isolate any mutants with altered ToxR activity.

ToxR protein has been purified as a maltose binding protein to enable X-ray crystallography studies to be performed. Structural analysis will provide information about important sites in the ToxR protein.

Bacteria adapt to fluctuations in their environment by altering their gene expression. In order to do this they need to be able to sense their environment

and changes in it. The moderately barophilic marine bacterium *Photobacterium* SS9 is sensitive to changes in pressure. It possesses an environmental sensing system whereby small changes in pressure can be detected resulting in a change in gene expression [WELCH T J, BARTLETT D H (1998)

- 5 Identification of a regulatory protein required for pressure-responsive gene expression in the deep-sea bacterium *Photobacterium* species strain SS9. *Molecular microbiology* 27, 977-985.]. These changes in gene expression ultimately result in changes in the molecular make-up of the bacterium that allows the bacterium to survive and grow at low or high pressures. Two of the
- 10 proteins that are regulated in this way are Outer Membrane Protein H (OmpH) which increases in abundance as pressure increases and OmpL, which is produced maximally at low pressures. Whilst the level of production of these proteins could be used to measure pressure changes, it is likely that there is a lag in the time between the pressure change and changes in the amount of
- 15 protein. Therefore, it is more appropriate to work with the "membrane sensor" of the bacterium.

- Two cytoplasmic membrane proteins have been identified as having an involvement in pressure sensing/adaptation in *Photobacterium* SS9, ToxS and
- 20 ToxR [WELCH T J, BARTLETT D H (1998) *supra*]. ToxR is a transmembrane DNA binding protein which spans both the cytoplasm and the periplasm whilst ToxS, although also membrane associated, is located exclusively in the periplasm [MILLER V L, TAYLOR R K, MEKALANOS J J (1987) Cholera toxin transcriptional activator toxR is a transmembrane DNA binding protein. *Cell*.
- 25 48, 271-279. DIRITA V J, MEKALANOS J J (1991) Periplasmic interaction between 2 membrane regulatory proteins, toxR and toxS, results in signal transduction and transcriptional activation. *Cell* 64, 29-37.]. Gene expression is modulated by dimerisation of the ToxR protein. ToxS plays an influential role in this dimerisation event by facilitating association of ToxR monomer [Miller,
- 30 *supra*]. Based on the activity of ToxR/ToxS homologues in *Vibrio cholerae* [Miller *supra*; Dirita *supra*] a model has been proposed for ToxR/ToxS pressure sensing in *Photobacterium* SS9 [Welch *supra*]. This model is illustrated in Figure 1 of the accompanying drawings.

At low pressure ToxR exists as a dimer. In this form ToxR has a regulatory effect on the expression of two outer-membrane proteins, OmpH and OmpL, with a negative regulatory effect on transcription of ompH and a positive effect on ompL transcription [Miller *supra*. WELCH T J, BARTLETT D H (1996) Isolation and characterisation of the structural gene for OmpL, a pressure-regulated porin-like protein from the deep-sea bacterium Photobacterium species strain SS9. J. of Bacteriology. 178, 5027-5031. BARTLETT D H, WELCH T J (1995) OmpH gene-expression is regulated by multiple environmental cues in addition to high-pressure in the deep-sea bacterium Photobacterium species strain SS9. J. of Bacteriology. 177, 1008-1016.].

However when pressure is raised ToxR monomerises, relieving repression of ompH and preventing expression of ompL [Miller *supra*]. At a molecular level the ability of the ToxR protein to dimerise and monomerise must be mediated by specific interactions between amino acid side chains which are exposed on the surface of the molecule. However, at this time the nature of the amino acids involved in these interactions is not known.

Pressure changes of a few MPa have been observed to elicit changes in ToxR/S mediated omp expression [Miller *supra*]. Photobacterium SS9 thus possesses a highly effective environmental sensor of pressure changes. It is, therefore, proposed that these pressure-sensing systems may be exploited to provide the basis for biological sensors, and that modulation of the ToxR dimer/monomerisation may allow the development of systems with increased pressure sensitivity. Such increases in the sensitivity of the sensing system can be achieved by modifying the ToxR protein, so that the interactions between the ToxR molecules occur in response to smaller pressure changes.

The present inventors sought to construct hypersensitive ToxR mutants since construction of site-directed or random mutants of the ToxR-like protein may allow identification of amino acids that are critical for pressure sensing. Such mutants may be derived in two ways, either by modifying amino acids by site-

- directed mutagenesis using an overlap polymerase chain reaction (PCR) method, or by using chemical mutagenesis. Using these processes mutant forms of ToxR may be identified with reduced or enhanced pressure responses. Mutants that show enhanced responses to pressure will be especially useful in this project. The derivative of *Photobacterium* that is needed as a host for these studies will be a *toxR* deletion mutant containing a gene encoding an *ompH::lacZ* fusion protein. This system will allow pressure-induced gene expression to be monitored.
- 10 Sufficient quantities of the ToxR protein were produced to allow determination of the structure of the ToxR protein by X-ray crystallography. This allowed the identification of surface exposed amino acids, and the identification of amino acid side chains that are involved in the dimerisation process. This allowed protein engineering of the ToxR protein to alter its pressure sensing properties.
- 15 The ToxR protein is known to be composed of two domains, one is embedded in the outer membrane of the bacterium, with the other domain exposed on the surface of the bacterium (the sensor domain).

- Construction of an *ompH::lux* fusion allowed the determination of the kinetics of response of mutant strains to small changes in pressure. This construct responds to pressure changes by producing light (bioluminescence) which will then be measured.
- 20

- The ToxR pressure sensing system of *Photobacterium* SS9 is capable of responding to pressure changes of several MPa. In order to function as an acoustic sensor an increase in sensitivity is required. Subtle changes to the pressure-sensing region of the ToxR protein (C-terminus) may result in changes to the sensitivity of the pressure sensing system. With no *a priori* knowledge of which areas are influential a random mutation approach was decided upon. Results from X-ray crystallography and defining the mutations most influential on pressure sensing will enable a more site-directed approach to be adopted in the future.
- 25
- 30

Generation of hypersensitive ToxR protein requires the completion of a sequence of experimental steps. Firstly, a strain of host Photobacterium that has a suitable genetic background and contains a reporter gene construct is required. A bank of ToxR protein with randomly introduced mutations in the pressure sensing region is inserted into this strain. Through screening, the ability of the mutated ToxR protein to respond to pressure is assessed. Those mutations that confer an increased sensitivity to pressure are processed through additional rounds of mutation and screening until a hypersensitive variant is produced. Each successive round yields more information concerning the site(s) that confer the pressure sensing capability of the ToxR protein. This information coupled with data from the X-ray crystallisation studies, enables more precise, site-directed changes to the ToxR protein to be employed.

Accordingly, in one aspect the present invention provides a recombinant or genetically modified Photobacterium S99 able to detect microPascal pressure changes.

Preferably the recombinant or genetically modified Photobacterium S99 is produced by performing a toxR deletion mutant in Photobacterium SS9 containing an ompH::lacZ reporter construct.

Accordingly, the present invention also provides a toxR-deletion-mutant Photobacterium SS9 containing an ompH::lacZ reporter construct.

In order to study the effect of mutations on the barosensing activity of ToxR it was necessary to construct a toxR deletion mutant, in which toxR is disabled in the DNA sequence, in a strain harbouring an ompH::lacZ reporter system.

Using this strain the barosensing properties of introduced ToxR protein can be assessed by measuring the corresponding ompH activity.

Alternatively, the sensors of the present invention may use neurological cell cultures. The advantage of using neuronal cells in a biological acoustic sensor is that they are likely to be already endowed with very sensitive pressure sensing systems and have an inherent reporter system. The problem is in the
5 detection and interpretation of a suitable response from a population of such cells.

In vivo measurements of auditory evoked potentials have long been routine (e.g. Urbani and Lucertini (1994), Hearing Res. 76: 73) and studies on
10 individual isolated neurones, such as dorsal root ganglion cells, were made possible by voltage- or current-clamping techniques (Christenson *et al* (1993) Brain Res. 608: 58). Measurement of some physiological responses to, for instance, oxygen, carbon dioxide and ammonia levels, had been possible since at least the 1960s by means of microelectrode sensing from single neurones
15 (Negishi and Svaetichin (1966) Pflueg Arch 292: 177).

The present inventors sought to develop suitable electrophysiology equipment for the monitoring of neuronal activity and to identify and culture potential pressure sensitive neurons.
20

Accordingly, in another aspect of the invention a barosensory neuronal cell culture is provided.

The present inventors have prepared useful neuronal cells and cell lines
25 together with specialised electrophysiological equipment. Initial investigations of the spontaneous output from neuronal cells have been made.

Advantageously, the growth conditions of spontaneously active neuronal networks on the specialised equipment have been optimised. Modifications
30 were made to the experimental set up to enable pressure stimuli to be applied.

The present inventors have found that it is in fact possible to produce electrically active dorsal root ganglia (DRG) cultures. Previously, it has been

shown that active rat DRG cultures may be produced from excised tissue but electrical activity has only been analysed by intracellular techniques. Such studies have shown oscillation of the membrane potential and periodic firing [AMIR R, DEVOR M Spike-evoked suppression and burst patterning in dorsal
5 root ganglion neurons of the rat. J. Physiol. (London) 501, 183-196.] The results of the present inventors constitute the first documented case of extracellular recording of electrical activity oscillations from DRG cultures. Such findings have important implications as the DRG cells are beginning to be analysed in terms of pain and nerve injury.

10

Accordingly, one aspect of the invention is a pressure sensing apparatus comprising living neuronal cells contacting a substrate, wherein said substrate comprises a plurality of electrodes capable of detecting the electrical activity of said cells.

15

Preferably, said neuronal cells are primary dorsal root ganglion cells.

Alternatively, said neuronal cells are selected from the group comprising ND7/23 cells, neuromast cells, P-cells, stretch receptor cells, nodose cells,
20 Merckel cells, phaeochromocytoma cells, Immortomouse cells and hair cells.

In a preferred embodiment, the pressure sensing apparatus of the present invention comprises a diaphragm capable of transmitting an external acoustic signal to the fluid medium contacting said neuronal cells.

25

A related aspect of the invention is the method of using such a pressure sensing apparatus to detect acoustic signals.

The system of the present invention may be used in a drug screening method.
30 Surprisingly, the present inventors have found that the addition of antibiotics to the cell culture medium has an acute and chronic effect on neuronal activity. This effect has not previously been documented in the literature.

In another aspect of the invention, an antibiotic sensing apparatus comprising living neuronal cells contacting a substrate, wherein said substrate comprises a plurality of electrodes capable of detecting the electrical activity of said cells is provided.

5

Embodiments of the invention will now be described in more detail with reference to the following drawings of which;

10

Figure 1 is a model of ToxR/S regulation of omp gene expression in *Photobacterium* S99 showing the associated ToxR/S protein as both a repressor and an activator of gene expression and has been described earlier;

15

Figure 2 is a schematic representation of the FPLC trace and SDS PAGE analysis of protein peaks eluted from the gel filtration column during purification of the ToxR C-terminus MBP fusion;

Figure 3 shows a mature culture of foetal rat hippocampal neurons;

20

Figure 4 shows an example of a trace recording of spontaneous activity from a 4-week old culture of hippocampal neurons;

25

Figure 5 shows three waveforms characteristic of spontaneous activity in hippocampal neuronal cultures (a) is classical action potential profile, (b) is a sodium spike and (c) is a rare chloride spike;

Figure 6 shows spontaneous activity recorded from mature cultures of foetal rat spinal cord neurons;

30

Figure 7 shows a culture of primary dorsal root ganglia cells from foetal rats;

Figure 8 is a representative trace of spontaneous activity recorded from mature cultures of primary foetal rat dorsal root ganglia cells;

Figure 9 shows a characteristic waveform of spontaneous events recorded from a 4-week old culture of dorsal root ganglia cells;

Figure 10 shows the effect of the acute addition of antibiotics to the spontaneous activity of hippocampal neuronal cell cultures, (a) Penicillin G, (b) Streptomycin, (c) Penicillin G/ Streptomycin, (d) Kanamycin (arrow shows point of addition);

Figure 11 shows the modifications to the MMEP system to enable application of static pressures to neuronal cells;

Figure 12 shows the modification of the burst intervals in DRG spontaneous activity in response to an applied static pressure, (a) is a representative trace with pressure applied at the arrow and (b) is a graph indicating burst interval timings between bursts;

Figure 13 is a trace showing the stimulation of DRG burst activity in response to a drop of medium falling 10 cm onto the culture;

Figure 14 shows the bioacoustic calibration tube;

Figure 15 shows the MMEP base plate and culture chamber modifications to allow pressure application to neuronal cultures present in the bioacoustics calibration tube;

Figure 16 is a trace showing spontaneous activity from foetal rat spinal cord neurons present inside the bioacoustics calibration tube, and

Figure 17 is a recording obtained from a 2 week old differentiated culture of the ND7/23 (dorsal root ganglia) cell line where a pressure of 1 atm was applied transiently at point P1 and the trace was switched off at point O for approximately 3 minutes before the pressure was re-applied at point P2.

Example 1.

- Sufficient quantities of the ToxR protein were produced to allow determination of the structure of the ToxR protein by X-ray crystallography. This allowed the
5 identification of surface exposed amino acids, and the identification of amino acid side chains that are involved in the dimerisation process. In turn, this allowed protein engineering of the ToxR protein in order to alter its pressure sensing properties.
- 10 The ToxR protein is known to be composed of two domains, one is embedded in the outer membrane of the bacterium, with the other domain exposed on the surface of the bacterium (the sensor domain). A number of options for the production of ToxR protein were investigated.
- 15 Kinetics of response to changes in pressure: construction of an ompH::lux fusion allowed the determination of the kinetics of response of mutant strains to small changes in pressure. This construct responds to pressure changes by producing light (bioluminescence) which can then be measured.
- 20 Progress generating hypersensitive bacterial pressure sensing protein: the ToxR pressure sensing system of Photobacterium SS9 is capable of responding to pressure changes of several MPa. In order to function as an acoustic sensor an increase in sensitivity is required. Subtle changes to the pressure-sensing region of the ToxR protein (C-terminus) resulted in changes
25 to the sensitivity of the pressure sensing system. With no *a priori* knowledge of which areas are influential a random mutation approach was decided upon. Results from X-ray crystallography and defining the mutations most influential on pressure sensing enabled a more site-directed approach to be adopted.
- 30 Generation of hypersensitive ToxR protein required the completion of a sequence of experimental steps. Firstly, a strain of host Photobacterium that has a suitable genetic background and contains a reporter gene construct was required. A bank of ToxR protein with randomly introduced mutations in the

pressure sensing region was inserted into this strain. Through screening, the ability of the mutated ToxR protein to respond to pressure was assessed.

Those mutations that conferred an increased sensitivity to pressure were
5 processed through additional rounds of mutation and screening until a hypersensitive variant was produced. Each successive round yielded more information concerning the site(s) that conferred the pressure sensing capability of the ToxR protein. This information coupled with data from the X-ray crystallisation studies, enabled more precise, site-directed changes to the ToxR
10 protein to be employed.

Production of a *toxR* deletion mutant in *Photobacterium* SS9 containing an *ompH::lacZ* reporter construct: in order to study the effect of mutations on the barosensing activity of ToxR it was necessary to construct a *toxR* deletion
15 mutant, in which *toxR* is disabled in the DNA sequence, in a strain harbouring an *ompH::lacZ* reporter system. Using this strain the barosensing properties of introduced ToxR protein were assessed by measuring the corresponding *ompH* activity. Changes in *ompH* activity result in a coupled change in the activity of the reporter gene *lacZ*. The reporter gene *lacZ* encodes the enzyme β -
20 galactosidase which, when produced in the presence of the chromogenic substrate X-GAL, turns bacterial colonies from white to blue. Thus increases or decreases in ToxR activity can be measured directly by alterations in the colour of bacterial colonies.

25 Construction of randomly mutated ToxR mutant bank: two approaches were used to generate random mutants of the pressure-sensing region of the ToxR protein. Initial attempts used a known "spiked" PCR protocol to perform oligonucleotide directed mutagenesis, in which mutations are introduced into the DNA of interest by amplification of the DNA under conditions in which one
30 of the nucleotide bases is in limited supply. The result is that mistakes are introduced into the DNA and by varying the level of spiking the number of mutations introduced can be controlled. The level of spiking used was such

that there would be 2.5 random mistakes per DNA region produced, equivalent to approximately one amino acid alteration.

5 Using the mutated oligonucleotides as primers with the M13 vector a functional toxR gene was regenerated. A single-stranded copy of the toxR gene, with the mutated pressure region incorporated, was produced. From this construct a double stranded plasmid harbouring the toxR mutation was generated.

10 The mutagenised genes were PCR amplified and cloned into a broad host range vector. Two such vectors are available for use in Photobacteria, pKT231 and pRL10. The mutant bank, present in the broad host range vector, is then transferred to the toxR deletion strain of Photobacterium T41 by the process of conjugation.

15 The toxR mutant bank was conjugated into Photobacterium T41 using the pRL10 vector. Unfortunately the efficiency of conjugation between Photobacterium T41 and Escherichia coli XL2 blue was surprising low, even in the presence of a helper strain. It is thought that this was due to a plasmid-mediated problem preventing efficient inter-species conjugation.

20 Due to insufficient numbers of exconjugants being produced using pRL10, it was decided to adopt a similar approach using the pKT231 vector.

25 Transformation of the pKT231 cloned toxR mutant bank into E. coli XL2 blue gave a low number of transformants, possibly due to the large size of the vector (13 kb). However, the efficiency of conjugation of this mutant bank with Photobacterium T41 gave a high number of exconjugants that were forwarded for screening.

The toxR mutant bank was subjected to screening at high and low pressure.

30 Since cyclic AMP (cAMP) acts as a repressor of ompH activity it was important to ensure that the Photobacterium remain in glucose rich media throughout the

screening process. Optimal concentrations of the chromogenic substrate X-Gal and glucose were determined experimentally.

Low pressure screening the pKT231 toxR mutant bank at 1 atmosphere
5 revealed no distinction between the mutant bank and the toxR deletion negative control (Photobacterium T41 with ompH::lacZ construct). Both populations were blue in colour. The fact that the positive control, consisting of a wild type toxR gene borne on the pKT231 plasmid, was also blue suggests that toxR expression was not occurring using this plasmid. It is likely that the kanamycin
10 promoter on pKT231, used to drive the toxR gene expression, was unable to do so effectively.

Work on the high pressure screening of the toxR mutant bank concentrated on developing protocols for screening at elevated pressures in the temperature
15 controlled pressure chamber at the Scripps Institute of Oceanography. Experiments were performed to optimise the growth in plastic bags of discrete colonies of Photobacterium in a semi-solid medium. An intermediate pressure of 140 atmospheres was chosen and the corresponding optimal levels of X-GAL, glucose and low-melting point agarose determined experimentally. The
20 pKT231 toxR mutant bank was incubated in sealed bags at a pressure of 140 atm. for 5 days at a temperature of 19° C. Positive and negative controls were included to provide a direct comparison of colony colour. The expected colour changes are associated with growth at 140 atm (atmospheres). At this intermediate pressure, strains with normal ToxR activity will only have toxR
25 partially expressed and a pale blue phenotype exhibited; full expression is only exhibited at 280 atms. Thus any hypersensitive ToxR strains can be distinguished by a dark blue phenotype.

The result of the high pressure revealed no distinction between the mutant
30 bank and the toxR deletion strain T41. This confirmed the observations made during the low-pressure screen, reflecting the inability of the pKT231 plasmid to drive toxR expression.

In order to rectify the problem of driving expression of *toxR* in *Photobacterium* it was decided to construct the mutant bank with the inclusion of the wild type *toxR* promoter as well as the *toxR*-coding region. The *toxR* coding and promoter regions were amplified by PCR, cloned into the M13 vector and single-stranded template DNA generated. Unfortunately, attempts to generate single stranded DNA from the mutant oligonucleotide primers were unsuccessful. Problems were encountered with annealing the oligonucleotide primers to the *toxR* template DNA. Previous experiments using the *toxR* template without the promoter region did not encounter this problem. It was therefore concluded that the addition of the *toxR* promoter significantly altered the annealing conditions required. Despite investigating a variety of annealing conditions, production of mutant DNA could not be achieved. This approach was abandoned and the use of mutator strains investigated.

The *Escherichia coli* XL-1 Red mutator strain (Stratagene) can be used to perform random mutagenesis of target genes. This strain is deficient in three of the primary DNA repair pathways in *E. coli*, *mutS*, *mutD* and *mutT*, making its mutation rate approximately 5,000-fold higher than the wild type. For mutagenesis the mutator strain is transformed with a plasmid carrying the target DNA and grown overnight. During this growth period the plasmid DNA is reproduced but, due to the deficiencies in the repair pathways, any mistakes that are produced are not corrected. Using one round of this approach the target DNA will contain approximately one mutation in every 2000 bases. Additional rounds of propagation achieve increased mutation rates.

The entire *toxRS* operon was cloned into the high copy number plasmid, pUC18. The resulting plasmid, pUC*toxR*, was transformed in the mutator strain and grown overnight to introduce mutations. A further four rounds of propagation were performed producing five separate mutant banks, pUC*toxR*1 – pUC*toxR*5, with increasing levels of mutation in the *toxRS* operon region.

In order to localise the mutations to the pressure sensing region of the ToxR protein it was necessary to perform overlap PCR. Using overlap PCR the

pressure-sensing region from the mutant banks pUCtoxR1 – pUCtoxR5 was exchanged with the corresponding region from a wild type toxR gene. The resultant, reconstituted toxRS operon, harbouring mutations in the pressure region, was cloned into the broad host range vector pKT231 and conjugated
5 into the toxR deletion Photobacterium T41 containing the ompH::lacZ reporter system. The five random mutant banks were designated pKTtoxR1 – pKTtoxR5.

All five mutant banks were screened at 1 atmosphere using 96 well microtitre
10 plates. Assay conditions were optimised using the X-GAL and glucose concentrations determined previously. It became apparent early on that the screening process had an absolute dependency on the cell numbers in each well. False positives were produced in which a simple increase in the number of bacteria present was sufficient to produce confusing results. An alternative
15 method for screening was therefore required.

One alternative method for measuring β -galactosidase activity uses the breakdown of the chromogenic substrate o-nitrophenol galactopyranoside (ONPG). The result is the production of a yellow colour change that is
20 quantifiable by spectrographic measurement at 450 nm. Moreover, using this assay system the relative cell numbers can be determined through a simple protein assay.

Test strains T41 ompH::lacZ (toxR-) and DB110 ompH::lacZ (toxR+) were
25 cultured in microtitre wells. A 10 μ l aliquot was processed for β -galactosidase activity using the ONPG assay for detailed protocol). Colour development was stopped after ten minutes and clear distinction was present between the test strains. Using this assay the toxR- strain appeared bright yellow, whereas the toxR+ strain was pale yellow. These are expected results for toxR activity at 1
30 atmosphere.

All five mutant banks, pKTtoxR1 – pKTtoxR5, were screened at 1 atmosphere using the ONPG assay described above. In total 420 mutants from each

mutant bank were processed. No mutants with increased toxR activity were identified. This is not unexpected as it is unlikely that mutants exhibiting increased pressure sensitivity will be identified through the low-pressure (1 atm.) screen. Screening at high pressure would better detect such mutants.

5

Colonies were identified from each mutant bank that appeared to exhibit decreased toxR activity. Plasmid DNA was extracted from these mutants and sequenced for base changes in the pressure-sensing region. Sequencing of these mutants did not reveal any mutations in the pressure-sensing region.

10

Characterisation of pressure sensing regions of the ToxR protein: by determining the structure of the ToxR protein through crystallographic studies the sites that are important for pressure sensing can be identified. This information would eventually allow protein engineering of the ToxR protein to alter its pressure sensing abilities.

15

In order to undertake these studies high yields of pure ToxR protein are required. A number of options for the production of ToxR protein were investigated.

20

Attempts were made at producing purified, full length ToxR protein. The complete ToxR coding region was produced as a GST fusion protein in *Escherichia coli*. Unfortunately, expression of this full length ToxR fusion protein was very low. Similar results were obtained when the full-length ToxR protein was expressed as either a poly-histidine or maltose binding protein fusion protein. The ToxR protein is a membrane protein and therefore very hydrophobic in nature. This hydrophobicity is likely to prevent high levels of expression occurring in the bacterium. Consequently, very small amounts of the protein are produced. It was decided, therefore, to concentrate on the production of a ToxR C-terminal fusion protein. The C-terminal region of ToxR contains the pressure-sensing region of interest and, as it is present in the cell cytoplasm, is likely to be hydrophilic in nature.

25

30

Production of ToxR C-terminal fusion protein: a truncated form of ToxR, consisting of the 36 kDa C-terminal portion of the protein containing the pressure sensing region, was generated as a GST fusion protein. The fusion protein was expressed at high levels in *E. coli* JM109 but was present

5 predominantly in an insoluble form, unsuitable for affinity chromatography purification. Large quantities of the fusion protein were detected in the insoluble pellet after the sonication step. Several factors may contribute to this insolubility including strain variation, growth temperature and the detergent used. These factors were investigated in an attempt to increase solubility.

10 Neither production of the fusion protein in other host strains, lowering the growth temperature, or heat-shocking the cultures resulted in any increase in protein solubility. The use of different detergents, however, during fusion protein recovery was able to improve protein solubility slightly.

15 Scaling up of the purification process presented many problems. Initial attempts were made applying the cell lysate to a glutathione sepharose column. The eluate was further purified by subjecting it to fast protein liquid chromatography (FPLC) gel filtration, on a Superdex 200 (RTM) column. The

20 main problem encountered was achieving good separation of the fusion protein from contaminating breakdown products. Ion exchange chromatography was unable to resolve this problem. Alternative fusion protein production systems were examined.

25 The expression of the truncated ToxR protein as a poly-histidine fusion protein was examined. A variety of conditions were used but no significant increase in fusion protein yield could be obtained.

30 Truncated ToxR protein was also expressed as a maltose binding protein (MBP) fusion protein. The advantage of using the MBP system is that the fusion protein can be expressed either in the periplasm (pMAL-P2) or cytoplasm (pMAL-C2) depending on the vector used. Periplasmic and cytoplasmic expression of the truncated ToxR MBP fusion protein was examined. As expected periplasmic preparations gave low yields of expressed

protein in comparison to cytoplasmic preparations. Large quantities of soluble ToxR C-terminus MBP fusion protein was detected from cytoplasmic preparations.

5 Large-scale purification of the MBP fusion protein was performed. Purification consisted of affinity chromatography through an amylose resin. Relevant fractions were pooled and subjected to FPLC using a Sephadex (RTM) 200 gel filtration column for further resolution. Analysis of the collected fractions by SDS-PAGE revealed the presence of two protein bands, a contaminating band
10 at 95 kDa and the fusion protein at 55 kDa. The 95 kDa-contaminating band co-eluted with the majority of the fusion protein in the first protein peak eluting from the column, designated Peak 1. This may be the result of some association between the contaminant and the fusion protein. Some free fusion protein could, however, be distinguished from this contaminant-fusion protein
15 peak eluting in the second protein peak, designated Peak 2. A schematic representation of the peaks eluted from the gel purification column and their appearance by SDS PAGE analysis is shown in Figure 2.

Both the Peak 1, containing the MBP fusion and the contaminating protein, and
20 Peak 2 contaminant free fusion protein were concentrated and sent to the Department of Crystallography at Birkbeck College.

Crystallisation studies of the pressure sensing region of ToxR. Conditions for the crystallisation of the fusion protein have been carried out at the Department
25 of Crystallography, Birkbeck College. Preliminary experiments yielded small crystals of ToxR C-terminus MBP. Unfortunately these crystals were unsuitable for crystallography analysis. A variety of techniques were explored to enable the production of larger crystals.

30 Example 2 : Neuronal Biological Sensor

Experiments have been performed investigating the effects of a wide range of antibiotic compounds on neuronal network activity in cultured hippocampal cells.

- 5 Preliminary pressure response experiments were performed on active networks of dissociated primary dorsal root ganglia cells. The results showed an alteration of neuronal activity in response to an applied pressure stimulus.

- Experiments have also been performed attempting to generate neuronal
10 networks from cell lines. Two cell lines have been tested and, the cells readily differentiate into a neuronal-like morphology.

- Nerve cell culture: typically nerve cells are cultured by two methods, tissue slices and dissociated cell culture. Nerve tissue slices represent the most
15 highly organised neuronal cultures and are no more than thin slices of the nerve tissue of interest. Neuronal tissue slices are typically maintained for study for up to 6 hours. Dissociated neuronal cultures, on the other hand, are prepared by total dispersion of the neural tissue into single cells by mechanical and enzymatic methods. The cells are then seeded at an appropriate density in
20 tissue culture-treated plasticware containing nutrient medium and incubated at 37°C. Neuronal cells usually require additional coating of the plastic surface to improve attachment, prevent clumping, and stimulate the growth of processes. Over a period of a few days functional connections (synapses) are established between cells and within approximately seven days the cells reach full maturity.
25 Mature neuronal cultures have been reported to survive for up to several months [BANKER G, GOSLIN K (1991) Culturing nerve cells. MIT Press].

- Dissociated cell preparations are referred to as 'primary' cultures because they are derived directly from living tissue. Cell lines, however, usually originate
30 from a tumour extracted from an animal or human and can be allowed to multiply up to potentially unlimited quantities in appropriate conditions. A neuronal example is the Phaeochromocytoma (PC12) cell line originally derived from a tumour of a rat adrenal gland. The PC12 line multiplies under standard

tissue culture conditions as clumps of small, round, non-adherent cells.

Additional coating of the tissue culture plasticware, and the inclusion of Nerve Growth Factor (NGF) in the culture medium produces cell cultures with true neuronal morphology [GREENE L A, TISCHLER A S (1976) Establishment of
5 a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proc.Natl.Acad. Sci. USA 73, 2424-2428.].

Cell lines can also be produced artificially by chemical or viral 'immortalisation' or fusion of cells with an already immortal cell line. A relatively recent

10 innovation involves the development of the 'Immortomouse' [HOLLEY M C, LAWLOR P W (1997) Production of conditionally immortalised cell lines from a transgenic mouse. Audiol. Neurotol. 2, 25-35.]. Dissociated cells from tissue from the 'Immortomouse' remains immortalised at 33°C. Transfer of these cells to the normal body temperature of the mouse (39°C) stops cell division and
15 they develop their normal characteristics.

Electrophysiological techniques: the classical method for measuring electrical phenomena associated with neuronal cells involves the insertion of ultrafine glass microelectrodes, filled with electrolyte solution, into isolated or cultured
20 mammalian nerve cells. Recordings of this type indicate that neuronal cells have a resting membrane potential of (-70mV (the inside being negatively charged). An important advantage of a system of this type, that is, a fixed-array, multi microelectrode system, is that it is non-invasive.

25 Changes in electrical potential with different characteristics and magnitude are recorded as a nerve impulse travels along an axon, depending on the positioning of the electrodes. In the case of a single electrode, in contact with the surface of a nerve cell, and another at a remote location, i.e. in the bathing medium, there is an initial rise in potential followed by a return to normality, a
30 decrease in potential and a final return to resting conditions. The changes are representative of an initial alteration in the permeability of the membrane to Na⁺ and K⁺ ions and subsequent cross-membrane fluxes.

The main disadvantage of the electrodes mentioned above is the need for them to be manipulated into close contact with the neurons of interest. This results in an inherently unstable and short-lived preparation. Recent advances have been made in the use of fixed-array, multi-microelectrode systems. A number of systems have been developed and designed for long-term monitoring of extracellular single unit neuronal activity in vitro. Such systems have been used to monitor spontaneous and electrically or drug induced activity in monolayer cultures of neuronal cells [GROSS G W, WEN W Y, LIN J W (1985).

Transparent indium-tin oxide electrode patterns for extracellular, multisite recording in neuronal cultures. J. Neurosci. Meths. 15, 243-252. GROSS G W, SCHWALM F U (1994). A closed flow chamber for long-term multichannel recording and optical monitoring. J. Neurosci. Meths. 52, 73-85. GROSS G W (1994). Internal dynamics of randomised mammalian neuronal networks in culture. In Enabling Technologies for Cultured Neural Networks 13, pp 277-317. Academic Press. GROSS G W, RHOADES B K, AZZAZY H M E, WU M-C (1995) The use of neuronal networks on multielectrode arrays as biosensors. Biosensors & Bio-electronics 10, 553-567.]. Sophisticated electronics have been developed in parallel for the acquisition and analysis of large-scale multichannel activity data [ABUZAID M A, VITHALANI P V, GOSNEY W M, HOWARD L L, GROSS G W (1991). A VLSI peripheral system for monitoring and stimulating action potentials of cultured neurons. Proc. of the 1st Great Lakes Symp. on VLSI. Kalamazoo, MI, pp. 170-175.].

Mechanoreceptor cells: an exhaustive search was performed to identify potential cells that could be screened, in conjunction with the acquired electrophysiological equipment, for production of signals in response to mechanical stimulation. A report of cultured cells having been used in this manner was not identified. It was, however, demonstrated that a single leech mechanosensory neuron (P cell) did not exhibit spontaneous activity when placed on a linear electrode array, although responses could be evoked by electrical or mechanical stimulation.

Potentially useful cell types identified are listed below.

- Neuromast cells: Isolated from the 'lateral line' of fish and 'stitches' distributed over the body of amphibia. Neuromast cells are a complex conglomeration of different phenotypes, with the 'hair' cell as the basic sensory unit. There are no reports of successful long-term culture of these cells.
- 5 - 'P' Cells: Isolated from the leech ventral nerve cord. Although they have been examined for responses to mechanical and electrical stimulation on microelectrode arrays they are not conducive to large scale or long term culture conditions.
- Stretch receptor cells: Isolated from crayfish, they have been used in
10 patch-clamp electrophysiological experiments to study the characteristics of single-channel currents. There are no reports of these cells in tissue culture studies.
- Nodose sensory neurons: Isolated from 'nodose' ganglia of mammals that lie just exterior to the cranial cavity in the upper neck region. The 'nodose'
15 ganglia contain the cell bodies of nodose sensory neurons that innervated the major organs of the body. Nodose sensory neurons are bipolar neurons that are stimulated by distension of the cell membrane. Standard procedures exist for the culture of these neurons and they have been successfully maintained for up to 12 days in vitro [SHARMA RV, CHAPLEAU M W, HAJDUCZOK G,
20 WACHTEL R E, WAITE L J, BHALLA R C, ABBOUD F M (1995). Mechanical stimulation increases intracellular calcium concentration in nodose sensory neurons. Neurosci. 66, 433-441.] by which time well-developed neurites are formed.
- Merckel cells: These are mammalian mechanoreceptor cells that
25 transmit pressure signals from the skin to the primary sensory nerve fibres. A pure monolayer culture of Merckel cells has been reported, and a tumour cell line of human origin is available, although no electrophysiological studies have yet been performed.
- 30 'Hair' cells: Two different types of cells are involved in the transduction of mechanical stimuli in the cochlea of the mammalian ear. The inner hair cells (IHCs) and the outer hair cells (OHCs). Isolated hair cells have been cultured but only very small populations of sensory neurons are obtained. Several lines

from the inner ear of the 'Immortomouse' have been isolated and are currently in the process of characterisation.

Neuronal cell lines: A number of cell lines of neuronal origin are commercially available. Although most are not from source tissue that is mechanoreceptive
5 by nature, the possibility exists that certain surface receptors may be deformed and hence activated by mechanical stress. One potential mechanoreceptive cell line is the ND7/23 cell line. The progenitor tissue of this cell line is the dorsal root ganglion of the mouse.

10 Example 3

Electrophysiological Equipment

In order to produce a neuronal biological sensor of acoustic pressure a robust
15 system for measuring neuronal outputs is required. A system that enables neuronal activity to be measured using extracellular substratum electrodes was identified. This system had only recently become feasible due to advances in neuronal cell culture and sophisticated amplification technologies. It has the advantages of being capable of measuring electrical responses from a
20 population of living cells growing on the substratum, in a non-invasive way, over a protracted period of time.

Miniature microelectrode plate (MMEP) system. This system that has been developed over the last 20 years by Guenter Gross, CNNS, Denton, Texas,
25 USA (Gross *et al* (1997) Biosensors and Bioelectronics 12: 373–393) comprises of three basic components:

a metal base plate fitted with a central recess to accept the microelectrode plates and an optical port allowing microscopic examination of neuronal cells. The plate is fitted with power resistors to enable heating of the cultures to
30 occur;

a microelectrode plate consisting of a 2 inch square indium tin oxide (ITO) - sputtered glass slide which has 64 central terminals (four rows, 16 columns with 200 and 40µm spacing respectively) in an area of 0.8mm². The plates are

coated with a 2µm thick layer of insulating polysiloxane resin that is removed over the central terminals by laser pulses. The ITO thus exposed is plated with a thin layer of gold to complete the electrode;
a stainless steel culture chamber comprising a central circular aperture and
5 sealing ring that is clamped over the microelectrode plate.

Electrophysiology amplification equipment. Studies presented herein been performed using the 'Neurolog' system supplied by Digitimer Ltd., Welwyn Garden City, Hertfordshire. A single channel A.C. recording arrangement
10 consists of an impedance buffer headstage unit connected to a preamplifier unit capable of up to 20k gain. The signal is further processed through a filter unit and a gated amplitude discriminator. The signal is displayed visually on an oscilloscope or as sound through an audio amplifier unit. Optimal settings of 10K gain and a filter range of 500Hz to 6kHz are used routinely (as
15 recommended by Gross et al. *supra*).

Multichannel Neuronal Amplifier. The major limitation of using the Neurolog system is that only one channel can be monitored at any one time. Need of multichannel equipment was revealed.

20 The neuronal amplifier system consists of two 32-channel pre-amplifier boards that are connected to a 64-channel amplifier card. Initial trials using MMEP plates and culture media indicate that the system is capable of providing a gain of 100,000 and has low noise characteristics (background noise 17 nanovolts /
25 (Hz). Using the system neuronal outputs from cultures present inside the BioAcoustic Tube have been recorded.

Real time data acquisition software. Software has been developed to enable the real time analysis and recording of multiple channels of neuronal output
30 data. All 64 channels are interfaced to a dedicated PC with 16, user defined, channels displayed in real time. These channels can be flagged to indicate the presence of activity and the data logged to disk. Saved data can be replayed for analysis or transferred to other software packages for presentation.

Cell Culture Methodology

5 Detailed methods for the preparation and maintenance of primary cell cultures and cell lines have been described comprehensively in the literature and are well known to the person skilled in the art.

10 Primary cell culture. The tissue of interest is dissected from a freshly terminated animal in a laminar airflow hood under sterile conditions. For neuronal tissue from the Central Nervous System (CNS) younger animals, in particular embryonic, will yield the best cultures. Tissue is minced, subjected to enzymatic digestion, and titrated to produce a single cell suspension. Cell density is determined and cells are dispersed (seeded) into tissue culture plasticware containing an appropriate volume of medium.

15 The cultures are held at 37°C in an incubator with an atmosphere of 5% CO₂ in air which interacts with the bicarbonate buffer of the medium to maintain pH at a neutral 7.3.

20 Hippocampal / spinal cord / dorsal root ganglia cells. Hippocampal cultures are produced according to well-documented methods. The dissection of both spinal cord and Dorsal Root Ganglia (DRG) can be performed from the same E15 day fetuses. The head is removed and skin peeled away from the back of the fetus. The spinal cord is then loosened from the spinal canal by teasing
25 with fine watchmaker's forceps, and finally stripped out from the head end down. DRG can be carefully plucked from the cord, the meninges can then be peeled away and the cord isolated. Enzymatic digestion and cell suspension methods are essentially the same as for hippocampal cultures, except DNase I at .05% is usually included in spinal cord digests. A range of seeding densities
30 has been tested between 50,000 and 200,000 per cm². In this manner, electrically active DRG cultures can be prepared.

Cell line maintenance. 'Immortal' cells can be cultured over long periods of time and expanded up to copious quantities. Routine maintenance involves removing the cell layer by mechanical or enzymatic means and sub-culturing them into fresh medium at a lower cell density. Some cell lines require coating factors to be present on the tissue cultureware (e.g. PC-12) and many require the presence of specific factors to be present to enable a morphological change to occur. This change is referred to as differentiation and is typified by a stop on multiplication and a change in the characteristics of the cell.

Tissue culture ware. All tissue culture plasticware used was as previous [1]. The use of Heraeus FlexipermTM tissue culture wells has enabled the production of good quality neuronal cell cultures. They are particularly useful for seeding very small areas of the MMEP plates. This is especially useful for producing dorsal root ganglia cultures as only small numbers of these cells are generated in any one dissection run.

Culture medium. Basic medium formulations are available from a variety of commercial sources. The basic formulation is generally supplemented with glutamine, antibiotics and serum to ensure healthy cultures. Media formulations used in this project are:

Hippocampal; spinal cord; DRG cell medium:

Seeding out: Neurobasal medium (Gibco) + B27 supplement + L-Glutamine (2mM) + 10% Foetal bovine serum (FBS)

Culture media: Neurobasal medium (Gibco) + B27 supplement + L-Glutamine (2mM)

ND7/23 media formulations:

Routine culture: DMEM + 10% FBS + L-Glutamine (2mM)

Differentiation: DMEM + 0.5% FBS + L-Glutamine (2mM) + dibutyl cAMP (1mM) + 2(g/ml Nerve growth factor (NGF)

P19 cell line:

Routine culture: MEM + 10% FBS + L-Glutamine (2mM) + Non-essential Amino acids (1x)

Differentiation: MEM + 0.1% FBS + L-Glutamine (2mM) + Non-essential

5 Amino acids (1x) + 30uM retinoic acid

Neurobasal + B27 + Non-essential Amino acids (1x) + L-Glutamine (2mM) + 30uM retinoic acid

10 PC12 culture medium:

Routine culture: RPMI 1640 basal medium + 5% FBS + 5% Horse serum + L-glutamine (2mM) + Penicillin (100IU/ml) + Streptomycin (0.1mg/ml)

Differentiation: As routine medium with addition of NGF (100ng/ml)

15

Merckel cell line medium:

Routine culture: MEM with Earles salts + 10% FBS + L-Glutamine (2mM) + Non-essential Amino acids (1x) + Penicillin (100IU/ml) + Streptomycin

20 (0.1mg/ml)

Fish/amphibia medium:

Routine culture: Leibovitz L-15 + D-glucose (10mM) + Gentamycin 50(g/ml) + 5% FBS

25

Primary cells and cell lines investigated and acquired

Rat Hippocampal neurons: Cultures of these cells from the learning/memory centre of the brain (embryonic rat) are prepared routinely and some have been
30 utilised in initial calibration of the MMEP system and antibiotic experiments.

Rat Spinal cord cells: Rat foetal spinal cord cells are prepared from E15 day foetus by standard procedures and have been used in initial calibration experiments.

- 5 Rat Dorsal Root Ganglia cells. Rat dorsal root ganglia cells are prepared from dissected ganglia by standard techniques. Neuronal cells from the DRG innervate regions of the skin and are, therefore, potentially mechanosensory. ND7/23 cell line: The ND7/23 dorsal root ganglia cell line was acquired from the European Collection of Animal Cell Cultures, Salisbury, UK. This cell line is
10 cultured routinely using standard techniques and has the potential of being mechanosensory.

- P19 cell line: The P19 cell line was acquired from the European Collection of Animal Cell Cultures, Salisbury, UK. This cell line is cultured routinely using
15 standard techniques.

PC12 cell line: This cell line is cultured routinely and has been the main source of neuronal cultures for the initial calibration of the MMEP system.

- 20 Nodose ganglia sensory neurons: These concentrations of cell bodies (ganglia) have been identified in the necks of young rats and processed by standard procedures.

- Merckel cell line: This cell line has been acquired from Dr Tapas Das Gupta,
25 Specialised Cancer Centre, University of Illinois, Chicago, USA.

- Neuromast cells: Lateral line cells of fish and amphibia are potential sources for mechanosensory cells. Amphibians, such as the African Clawed Toad (*Xenopus laevis*), possess lateral line "stitches" arranged as ring around each
30 eye. A dissection procedure was developed to remove the ring of stitches intact, complete with underlying nerve supply. The tissue was further processed by standard procedures in attempts to produce dissociated cell

cultures. Preliminary investigations have also been conducted to assess the possibility of producing dissociated cell cultures from the lateral line of trout.

Long term maintenance of active neuronal cultures

- 5 General tissue culture. Contamination of cultures by bacteria, yeast and/or fungi represents a considerable problem for the long-term maintenance of cells. This is exacerbated by the need to omit antibiotics from the culture medium (see following sections).
- 10 The introduction of the use of Flexiperm tissue culture rings has resulted in a large reduction in the volume of medium required for culture maintenance. This is particularly important when expensive medium supplements are required (e.g. nerve growth factor, NGF). It has also produced an improvement in culture quality and longevity due to reduced dilution of soluble trophic factors,
- 15 secreted by glial cells or the neurons themselves.

- Effects of substrate pre-treatment: many cultures of spinal cord and hippocampal neurons successfully achieved 4-week maturity under a variety of different substrate and media combinations. Neuronal attachment was best
- 20 facilitated by the pre-treatment of MMEP'S with PEI, whether the surface has been flamed or not. Although P-D-L and P-L-L coating alone does not result in long-term attachment of hippocampal and spinal cord cells to the polysiloxane resin the use of these factors following flaming of the MMEP plate produces good cell adhesion. In conclusion, methods have been developed to ensure
 - 25 good attachment of neuronal cells and processes using PEI, P-D-L or P-L-L as the attachment factor.

- Cell culture medium effects: the use of a serum free medium formulation used by Gross (SSM-A) generally resulted in poor cultures. Other formulations were
- 30 investigated including the use of glial cell conditioned medium and top-grade foetal bovine serum. Although a few instances of electrophysiological activity were recorded the cell cultures produced were generally of a very poor quality.

Refinements made to the production of active neuronal cell cultures as follows: the critical factor that had prevented neuronal activity was identified as the use of antibiotics in the culture medium. This result has fundamental implications. In addition, a commercial source of a specially formulated serum-free medium
5 for neuronal cells was identified. This is now routinely used.

Investigations have determined the exact requirements for the production of an active network of primary neuronal cells. These are as follows:

Good Quality MMEP plates, effective coating of plates, good primary cultures,
10 neurobasal serum free medium, maturation period of at least 3 weeks and no antibiotics in the culture medium.

Routine recordings of spontaneous activity were achieved from cultures of hippocampal neurons at 3 – 6 weeks' maturity. A typical hippocampal neuronal
15 network culture is shown in Figure 3. Typically, activity from these cultures can be observed on 75% or more of the 64 channels, with activity ranging from ca. 100(V – 1,500(V (peak to peak). In general, it appears that the activity becomes stronger and more copious as the cultures become increasingly
20 mature.

An example trace showing spontaneous activity from a single electrode site in a hippocampal neuronal network is presented in Figure 4. Upon digital analysis, the waveforms appear to present in 3 forms. The first, Figure 5(a), represents a typical, externally recorded action-potential event with an initial positive spike,
25 followed by a larger negative spike and a period of after-hyperpolarisation. The second, Figure 5(b) is the most common waveform, lacks the initial positive spike, and is therefore a pure sodium channel event. The third category of waveform, Figure 5(c), is quite rare, consisting of a positive spike only, probably representative of chloride channel activity.

30 Primary spinal cord cultures. Neuronal network activity has been routinely observed from rat spinal cord cultures using the MMEP system. A typical trace, highlighting the bursting pattern, is shown in Figure 6. Analysis of the

spontaneous activity from spinal cord cultures reveals that they have a similar profile to activity recorded from hippocampal neurons. Typically, activity is present as a series of bursts and individual events can be classified into three categories.

5

Primary dorsal root ganglia cultures: the dissection of dorsal root ganglia from embryonic rat foetuses and the subsequent culture of dispersed cells were perfected. A representative culture of DRG cells is shown in Figure 7. Analysis of spontaneous activity recorded from mature cultures shows a marked

10 difference from that seen with hippocampal and spinal cord cells.

Typical DRG spontaneous activity consists of short bursts of a few spikes, followed by an interval of silence lasting on average 40 seconds (see Figure 8). Occasionally there are intermittent single spikes, or single regular spikes with

15 no bursting. The amplitude of the individual events are generally smaller than that for the hippocampal and spinal cord cultures with the largest event observed to date consisting of a 300V peak to peak waveform.

Furthermore, the overall activity appears to be less than that for hippocampal

20 and spinal cord cultures. Typically only a very few channels are detected, although on one occasion 50% of the 64 channels showed activity. When analysed, the predominant waveform present consists of a negative spike with some after-hyperpolarisation (Figure 9) similar to the most common type seen with hippocampal and spinal cord cultures (compare Figure 5(a)).

25

Effects of antibiotics on neuronal network activity: chronic administration of antibiotics to neuronal cell cultures. The chronic effect of antibiotics on neuronal network activity was investigated. Experiments were performed on hippocampal cultures grown in parallel, from the same cell preparation, in the

30 presence or absence of penicillin-streptomycin or gentamycin at the recommended doses. Cell cultures were grown over a 4-week period and analysis of corresponding activity revealed that the addition of antibiotics caused an almost complete cessation of activity. Those few channels that

exhibited any activity were very weak (<100 (V peak-to-peak) and often short lived. In contrast, those cultures grown in the absence of antibiotics regularly exhibited activity in excess of 75% of all channels, with some peak-to-peak amplitudes in excess of 1mV, although 300-500 (V was more usual).

5

In conclusion, it became apparent to the inventors that the production of active neuronal networks required an absence of antibiotics in the culture medium. All techniques were adjusted accordingly. It is important to note that although antibiotics are used routinely in tissue culture applications, there is very little documented evidence that these compounds have any detrimental effect on cell growth. It was decided, therefore, to investigate further the effects antibiotic compounds have on neuronal network activity.

Acute administration of antibiotic compounds to neuronal cell cultures: the majority of experiments involved the investigation of acute addition of a range of antibiotics to hippocampal cultures grown in antibiotic-free Neurobasal medium for 4 weeks. In each case MMEP's were screened for activity and a strong, reliable channel was selected for experimentation. Each antibiotic was tested on at least 3 individual cultures and the antibiotic addition was started at the concentration used routinely in tissue culture studies.

As controls, the anti-fungal agents Nystatin and Amphotericin B (Fungizone), and the anti-mycoplasma (PPLO) agent Tylosin were also tested. In addition, the agent in which the antibiotic compound was dissolved (usually deionised water or 0.9% sodium chloride), as well as normal medium, were also tested for any effects on neuronal activity.

A list of the all the agents tested for the effects of acute addition to hippocampal cultures is presented in Table 1. Particular interest was paid to the effects of penicillin G, streptomycin and penicillin/streptomycin mixtures. These are routinely used in tissue culture medium and therefore any possible toxic effects are particularly relevant.

Penicillin G. The addition of 10 μ l penicillin G caused an immediate increase in the firing rate of the channel being monitored, with a small associated decrease in the amplitude (see Figure 10 (a)). Subsequent further 10 μ l additions up to 100 μ l at approximate 10 second intervals induced a further decrease in the amplitude with eventual slowing of the firing rate.

Streptomycin. Addition of 10 μ l of streptomycin to an active culture of neuronal cells caused an immediate cessation of firing. If a particularly strong (>500 V) channel was under investigation, the activity would usually begin to recover, without a change of medium, after a silence of approximately 30 seconds. The normal activity was, thereafter, resumed within a few minutes. Further sequential additions of 10 μ l aliquots of Streptomycin once again caused immediate interruption of activity, which recovered spontaneously after longer time intervals (see Figure 10 (b)).

If a channel with weak activity ($< 500 \mu$ V) was under study, a single 10 μ l addition of Streptomycin was usually sufficient to inhibit activity for several minutes, or until a medium change was performed.

In all cases changing the culture to the antibiotic-free formulation reversed the effect. Furthermore, equivalent additions of medium or 0.9% sodium chloride produced no effect in these cultures.

Penicillin G / Streptomycin mix. Further experiments, using standard mixtures of penicillin and streptomycin, were performed to establish a threshold value for inhibition of activity. The concentration for the "all-or-nothing" response lies very close to the normal concentration used in tissue culture medium, a single 10 μ l addition to a final concentration of 100U/0.1mg/ml being sufficient to inhibit activity (see Figure 10(c)). A final concentration 80-90% of the normal concentration was usually ineffective at inhibiting activity. Very strong channels of activity were again more resistant to inhibition and could recover activity spontaneously.

Gentamicin. The antibiotic gentamicin is also commonly used in tissue culture applications. Addition of this antibiotic, to levels recommended by the supplier, results in a cessation of neuronal activity. This inhibition is reversed by a
5 complete medium change within the MMEP culture apparatus.

Other aminoglycoside antibiotics. Streptomycin belongs to the aminoglycoside group of antibiotics and it was therefore interesting to investigate whether other structurally similar compounds elicit a similar response. A number of
10 aminoglycoside antibiotics were tested and the pattern of inhibition was very similar to that seen for streptomycin addition. Kanamycin was the only exception, with sequential additions of 10µl aliquots, up to 10 times the normal concentration, generally insufficient to inhibit spontaneous activity (see Figure 10(d)). Some minor modification in the firing pattern was, however,
15 occasionally observed.

Other compounds tested. Polymyxin 'B' also caused inhibition of activity, whereas chlortetracycline induced a modification somewhat similar to penicillin G.
20

No inhibition of activity was seen with the anti-fungal agents amphotericin B and Nystatin, the anti-PPLO (mycoplasma) agent Tylosin, or any of the control additions up to 10 times the usual volume.

25 The poor frequency of activity detection seen when cultures were grown for several weeks in the presence of Penicillin-Streptomycin or Gentamicin indicates a toxic effect of these compounds after long-term presence. Noughnejad & Salehian [NOUGHNEJAD P, SALEHIAN P (1989) Toxicity and mechanism of action of aminoglycoside antibiotics (gentamycin and amikacin)
30 at the level of neural membranes. Asia Pacific Journal of Pharmacology. 4, 227-231.] It has previously been shown that high doses of gentamicin can cause major structural changes in neuronal cytoarchitecture when administered chronically to guinea pigs.

The fact that some channels (especially those with strong activity) recommenced firing in the acute presence of Streptomycin, without the need to change the chamber medium, suggests that short-term channel blocking effects can be overcome in certain circumstances. Thus also suggests an alternative mechanism for long-term toxic effects.

It is interesting that Penicillin on its own appeared to induce an initial acceleration of the firing rate, with an accompanied reduction in spike amplitude, at up to 10 times the recommended concentration. This is most likely due to the difference in chemical structure of Penicillin related to its mode of action in interfering with the final stage of the synthesis of the bacterial cell wall. Aminoglycoside antibiotics, on the other hand, interfere with bacterial protein synthesis at the level of the ribosomes.

Also of interest is the reduced effectiveness of Kanamycin when compared with other antibiotics of aminoglycoside structure, and similar traditional mode of action. The concentration tested is equivalent to that of the other aminoglycosides used, so the explanation most likely lies in small structural differences making Kanamycin less effective as a channel blocker.

The mode of action of Polymixin 'B' is to bind to the bacterial cytoplasmic membrane and interfere with the permeability of the cell, thus suggesting its' apparent channel blocking activity. Tetracycline functions in a similar manner to the aminoglycosides, by blocking binding of RNA to the 30S subunit of ribosomes, thus inhibiting protein synthesis. It also appears to have a modulatory role on neural activity, without a blocking effect, although the concentration recommended and tested was 0.1-1 times that of the aminoglycosides.

Neuronal network response to pressure stimuli.

Modifications to the MMEP apparatus. Minor modifications were made to the culture apparatus to enable preliminary pressure response experiments to be performed. In brief, this modification consisted of a 50 ml syringe barrel

connected and sealed to the stainless-steel culture chamber (see Figure 11). A further modification has been developed in which a membrane is introduced above the cells to allow the transmission of a physical effect without a damping effect. A lightly weighted syringe barrel is released from various heights above the membrane.

Response of hippocampal cells to static pressure. Using the syringe barrel modification of the MMEP system, the effect of a 2 atmospheres increase in static pressure on hippocampal cell activity was investigated. It was observed that the application of pressure did not result in any modification of spontaneous activity. This result is not unexpected, as it is unlikely that hippocampal cells possess an inherent pressure sensing system.

Response of Dorsal Root Ganglia cells to static pressure. The pattern of spontaneous activity in DRG neurons was markedly different to that observed in hippocampal or spinal cord cultures. Activity occurs in a series of bursts separated by a reasonably constant time delay. Timing of individual burst intervals was made over a 40 min period and a total of 60 bursts occurred with a mean burst interval of 39.8 seconds.

When a pressure of +2 atmospheres was applied from a gas cylinder, via a 50ml syringe barrel clamped to the MMEP chamber, the normal pattern of burst activity was interrupted but resumed upon release of pressure, unless there has been loss of medium from the chamber. This is demonstrated in Figure 12 where no burst occurred after the pressure was applied for a further 4 minutes. At this point the pressure was released and a normal pattern of bursting was resumed. The normal bursting pattern continued for a further 10 minutes (13 bursts).

The demonstration of an inhibitory effect on regular DRG cell bursting with the application of pressure is highly intriguing and suggests a disruption of the "Tensegrity" model, suggested by Ingber [INGBER D E (1997) The

architectural basis of cellular mechanotransduction. Annual Rev. Physiol. 59, 575-599.], associated with normal mechanoreceptor function.

5 Response of DRG cells to DC pressure. If drops of medium are allowed to fall from the top of the syringe barrel (a distance of 10cm), an audible and visible burst of spikes is stimulated (see Figure 13). These appear to have the same waveform profile as spontaneous activity. There is no stimulus artefact if drops are allowed to fall into an MMEP chamber containing medium, but no cells growing on the plate.

10

Further analysis of one burst event induced by a falling drop, shows it to be composed of 9 single, well spaced, events occurring over a 160 millisecond time period. Each individual event has a classical waveform pattern thus confirming that they are not artefacts.

15

Response to pressure induced by a falling weight approach. Using the modification described above the response of a DRG culture to a light weight falling on a membrane lying just above the cells was examined. Initial studies were very promising showing an effect following impact of the weight. Further studies, however, showed that these large artefacts were induced in the presence or absence of cell cultures. Thus, the effect observed was not real.

20

BioAcoustics Calibration Tube. Subjecting neuronal cell cultures, grown on the MMEP system, to defined acoustic and/or pressure fields requires a unique piece of equipment. The BioAcoustics Calibration (BAC) tube was designed and procured (see Figure 14). The Tube allows the culture of neuronal and bacterial cells under static or acoustic pressure and allows electrical contact to be made at 60 sites within the culture chamber. Additional equipment has been developed to enable the BAC tube to be pressurised. All necessary health and safety checks have been performed on the BAC tube and all ancillary equipment.

25

30

Additional developments have been made to enable transmission of acoustic and/or static pressure to neuronal cultures grown on the MMEP system. A smaller base plate has been built that can fit into the chamber of the BAC tube. Furthermore, a water-tight sealed system has been produced ensuring no leakage of culture medium upon experimentation. The system consists of a rubber diaphragm and O-ring that is placed into the stainless steel MMEP culture chamber (see Figure 15). A purpose built steel top plate is bolted onto the culture chamber using the screw holes for the medium re-circulating taps. Neuronal cell cultures on MMEP plates are flooded with medium and the diaphragm put in place, avoiding trapping of air. The O-ring and top plate are bolted down producing a water-tight seal. Using this system spontaneous activity from a foetal rat spinal cord culture present inside the BioAcoustics tube has been recorded (see Figure 16).

The use of the elastomere zebra strip results in a decrease in signal strength. For this reason a Pin-jig arrangement is currently being developed that will enable contact with the 32 edge connectors through spring-loaded gold pins. A miniaturised pre-amplifier board will be added to this arrangement that will fit into the BAC tube. Once the capability of measuring neuronal responses from 64 channels of a MMEP plate has been established, cultures of dorsal root ganglia cells will be subjected to defined acoustic fields and associated pressure responses characterised.

Differentiation studies on cell lines: cell lines provide an almost unlimited supply of material and, therefore, are of enormous benefit to the development of a biological acoustic sensor.

Neuronal cell lines exist in an immature, immortal state to enable their routine culture. The development of a neuronal-like morphology is achieved through the process of differentiation. In this process, cues are given to the cell to instruct it to undergo a pre-determined course of gene expression. These cues are typically, signalling molecules such as polypeptides (e.g. NGF) or chemical compounds (e.g. retinoic acid), that can be added to the cell culture medium.

Dorsal root ganglia cell line ND7/23: the ND7/23 cell line is of particular interest because it was developed from the dorsal root ganglia and, therefore, is potentially pressure responsive. Neurons are produced from the cell line by
5 reducing the serum content (down to 0.5%) and adding dibutyl cAMP and NGF to the cell culture medium as described by Wood *et al.* [WOOD J N, BEVAN S J, COOTE P R, DUNN P M, HARMAR A, HOGAN P, LATCHMAN D S, MORRISON C, ROUGON G, THEVENIAU M, WHEATLEY S. (1990) Novel cell lines display properties of nociceptive sensory neurons. Proc. R. Soc.
10 Lond. B 241, 187-194.].

Transfer of the ND7/23 cells to differentiation medium results in the cessation of multiplication and the development of a classical sensory neurone-like bi-polar morphology within a week in many cells (see Figure 16). A dense
15 network of processes is, however, never achieved and the cells appear morphologically unhealthy within about 2 weeks. This is undoubtedly due to the very low serum content of the differentiation medium. Experiments with a higher serum content (1%) have shown that this is not low enough to prevent the continued vigorous multiplication of the cells.

20 A culture of differentiated ND7/23 cells, set up on an MMEP plate, was screened for spontaneous activity. Using the syringe barrel arrangement a static pressure equivalent to 2 atmospheres was applied briefly to the culture. On the fifth electrode chosen a 26 second period of activity was recorded
25 coincidental with the pressure application (see Figure 17). The pressure pulse was repeated within a few minutes and resulted in a similar period of neuronal activity, although of greater amplitude and lesser duration. A digitised sample was taken and showed the activity present as a series of negative spikes of – 60µV followed by a short period of hyper-polarisation.

30 Further pressure application failed to elicit a response and microscopic analysis of the culture revealed that the cells had become detached from the MMEP plate.

P19 cell line: studies on the P19 cell line have indicated that neuronal cells produced are electrically active [MACPHERSON P A, JONES S, PAWSON P A, MARSHALL K C, MCBURNEY M W. (1997) P19 cells differentiate into glutamatergic and glutamate-responsive neurons *in vitro*. Neuroscience. 80, 487-499.]. The P19 cell line is a mouse teratocarcinoma cell and the neuronal cells are believed to originate from the neocortex. It is unlikely that such cells will possess inherent pressure sensing capabilities, however, the availability of an electrically active cell line would be of enormous benefit to the development of a neuronal acoustic sensor.

P19 cells are embryonic in nature and, therefore, have the potential to differentiate into a number of different phenotypes under the influence of specific factors. Addition of DMSO into the cell culture medium produces muscle cells, whereas retinoic acid supplementation results in the production of neuronal-like cells [JONES-VILLENEUVE E M, MCBURNEY M W, ROGERS K A, KALNINS, V I. (1982) Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells. J. Cell Biol. 94, 253-262. MCBURNEY M W, JONES-VILLENEUVE E M, EDWARDS M K, ANDERSON P J. (1982) Control of muscle and neuronal differentiation in a cultured embryonal carcinoma cell line. Nature 299, 165-167.]. Moreover, maintenance of P19 cells in retinoic acid supplemented medium for 28 days produces a dense network of neural processes.

Differentiation experiments have been performed on the P19 cell line using retinoic acid. In initial experiments it was observed that the P19 cells respond to retinoic acid and adopt neuronal-like morphology. However, only 60-70% of the cells responded to the stimulus, such that over an extended period the non-neuronal cells proliferate vigorously and kill the neuronal cells. The use of serum-free medium has overcome this problem but cultures appear unhealthy after a period of 2 weeks.

Rat phaeochromocytoma (PC-12) cells: although networks of fully differentiated, apparently healthy, PC-12 cultures have been produced on

MMEP plates no electrical activity has been detected. It has yet to be documented in the literature whether PC-12 cells are electrically active or not. In the light of experiments on the effects of antibiotics on neuronal activity this matter has yet to be resolved as these experiments were performed with a
5 penicillin-streptomycin antibiotic mixture present in the cell culture medium.

	<u>Antimicrobial</u>	<u>Class of Agent</u>	<u>Company</u>	<u>Cat No</u>	<u>Concentration/ml</u>
	Penicillin G	HYDRABAMINE	SIGMA	P-3032	100U
5	Streptomycin	AMINOGLYCOSIDE	SIGMA	S-0890	0.1mg
	Pen-Strep	MIXED	SIGMA	P-4458	50U/0.05mg
	Pen-Strep	MIXED	SIGMA	P-4333	100U/0.1mg
	Gentamicin	AMINOGLYCOSIDE	SIGMA	G-1397	0.05mg
	Geneticin	AMINOGLYCOSIDE	SIGMA	G-7034	0.05mg
10	Kanamycin	AMINOGLYCOSIDE	GIBCO	15160/021	0.1mg
	Neomycin	AMINOGLYCOSIDE	GIBCO	15310/022	0.1mg
	Lincomycin	AMINOGLYCOSIDE	GIBCO	25600/016	0.05mg
	Tetracycline	CYCLIC AMINE	GIBCO	15280/019	0.01mg
	Polymixin 'B'	DAB-AMINE	GIBCO	15350/010	100U
15	Nystatin	POLYENE	GIBCO	15340/029	100U
	Amphotericin B	POLYENE	SIGMA	A-2942	0.25µg
	Tylosin	MACROLIDE	GIBCO	15220/023	0.01mg

20 **Table 1: List of antimicrobial agents tested for effects of acute addition to hippocampal cultures**

CLAIMS

1. Pressure sensing apparatus comprising living neuronal cells contacting a substrate, wherein said substrate comprises a plurality of electrodes
5 capable of detecting the electrical activity of said cells.
2. Pressure sensing apparatus according to Claim 1 wherein said neuronal cells are primary dorsal root ganglion cells.
- 10 3. Pressure sensing apparatus according to Claim 1, wherein said neuronal cells are selected from the group comprising ND7/23 cells, neuromast cells, P-cells, stretch receptor cells, nodose cells, Merckel cells, phaeochromocytoma cells, Immortomouse cells and hair cells.
- 15 4. Pressure sensing apparatus according to any of Claims 1 to 3, wherein external acoustic signals are transmitted, via a diaphragm, to a fluid medium contacting said neuronal cells.
- 20 5. A method of detecting acoustic signals by means of a sensing apparatus according to any of Claims 1 to 4.
- 25 6. Antibiotic sensing apparatus comprising living neuronal cells contacting a substrate, wherein said substrate comprises a plurality of electrodes capable of detecting the electrical activity of said cells.
7. Antibiotic sensing apparatus according to Claim 4, wherein said neuronal cells are hippocampal cells.
8. A drug screening method using the apparatus of either of Claims 6 or 7.
- 30 9. Sensor apparatus comprising living neuronal cells contacting a substrate, wherein said substrate comprises a plurality of electrodes capable of detecting the electrical activity of said cells.

FIG. 1

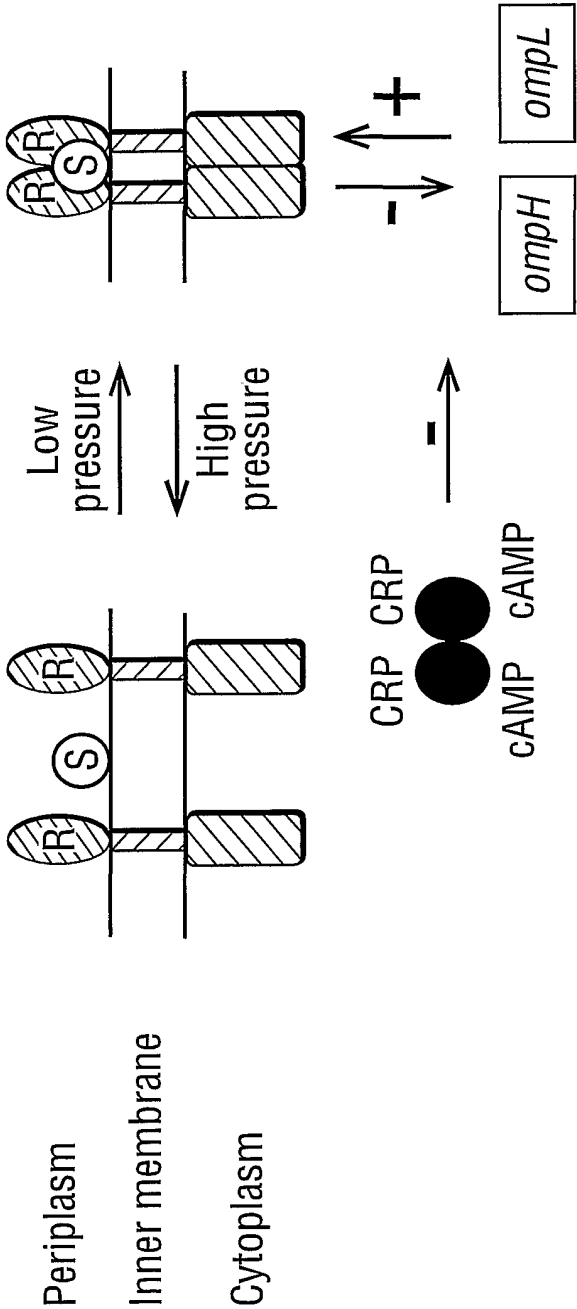
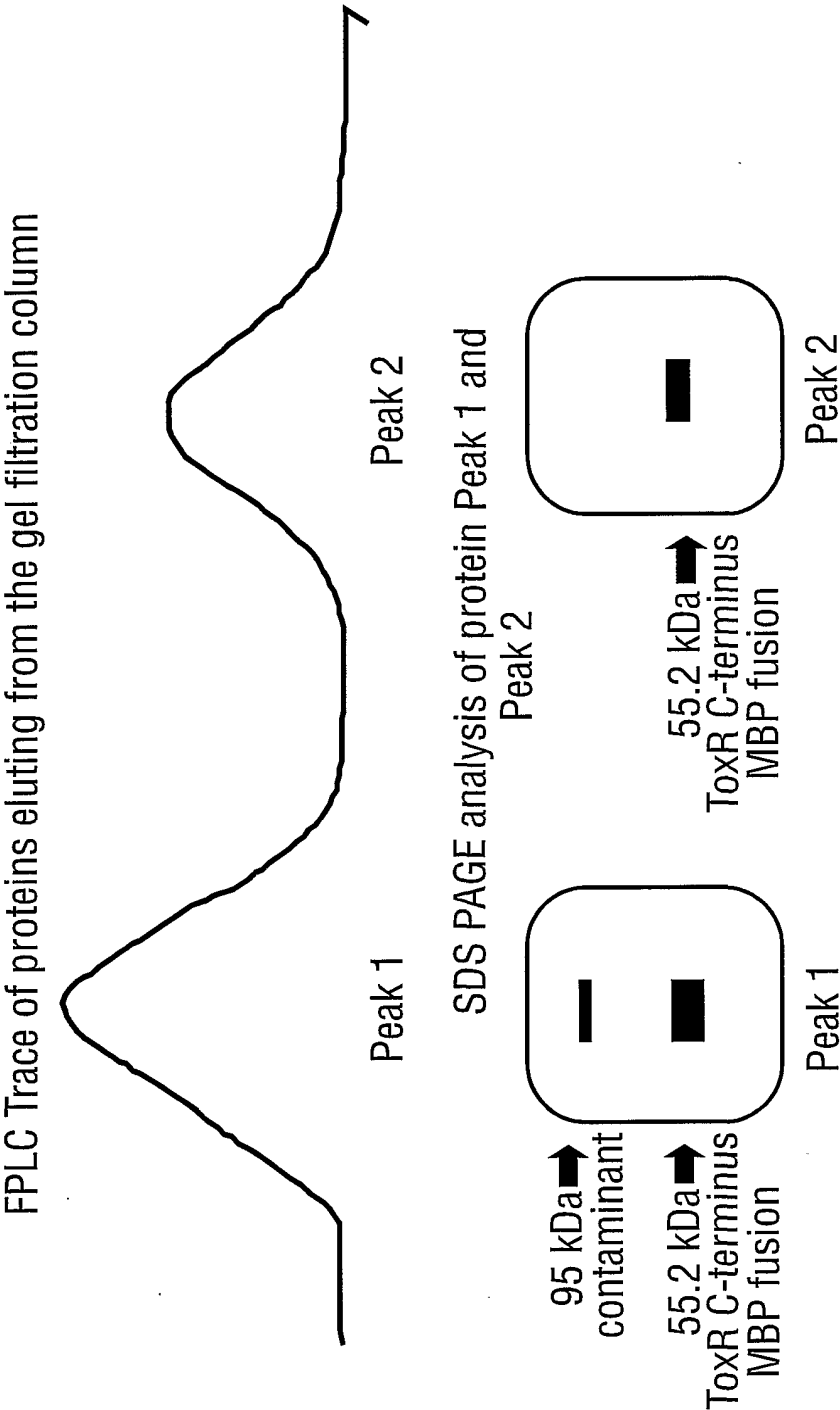
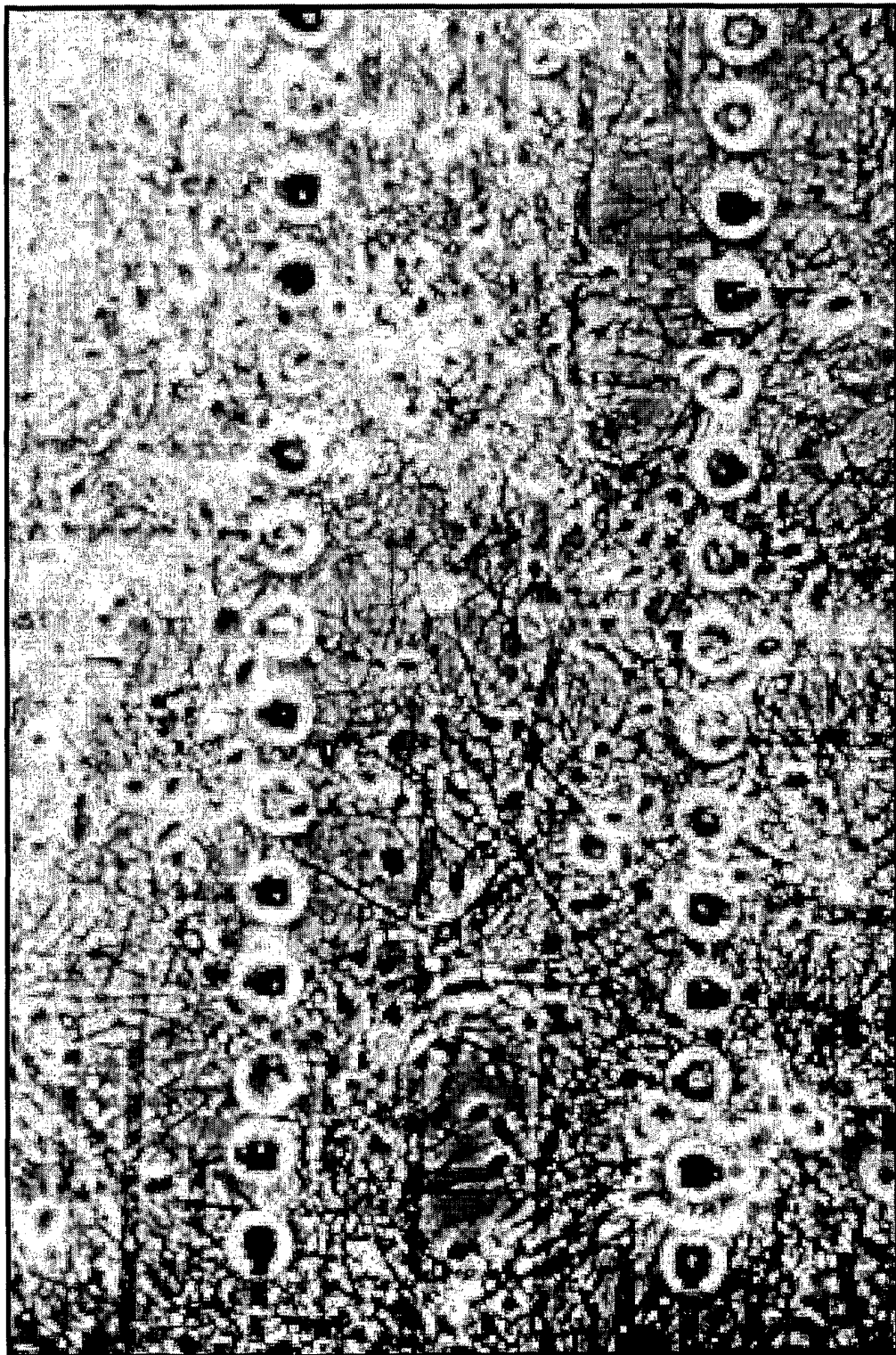


FIG. 2



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FIG. 3



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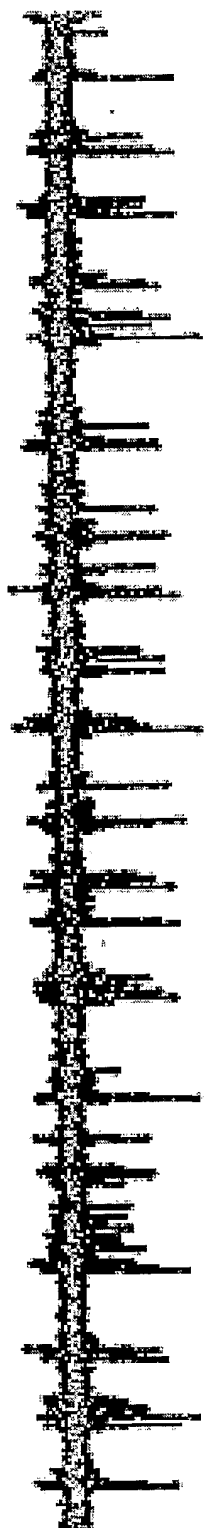


FIG. 4

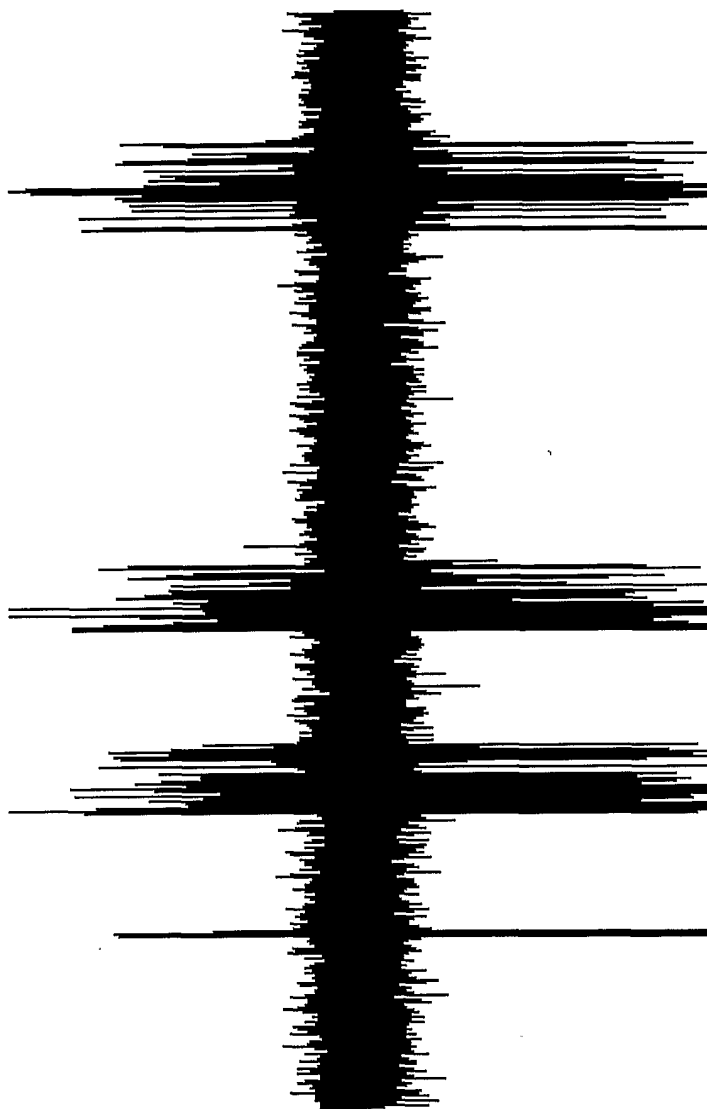
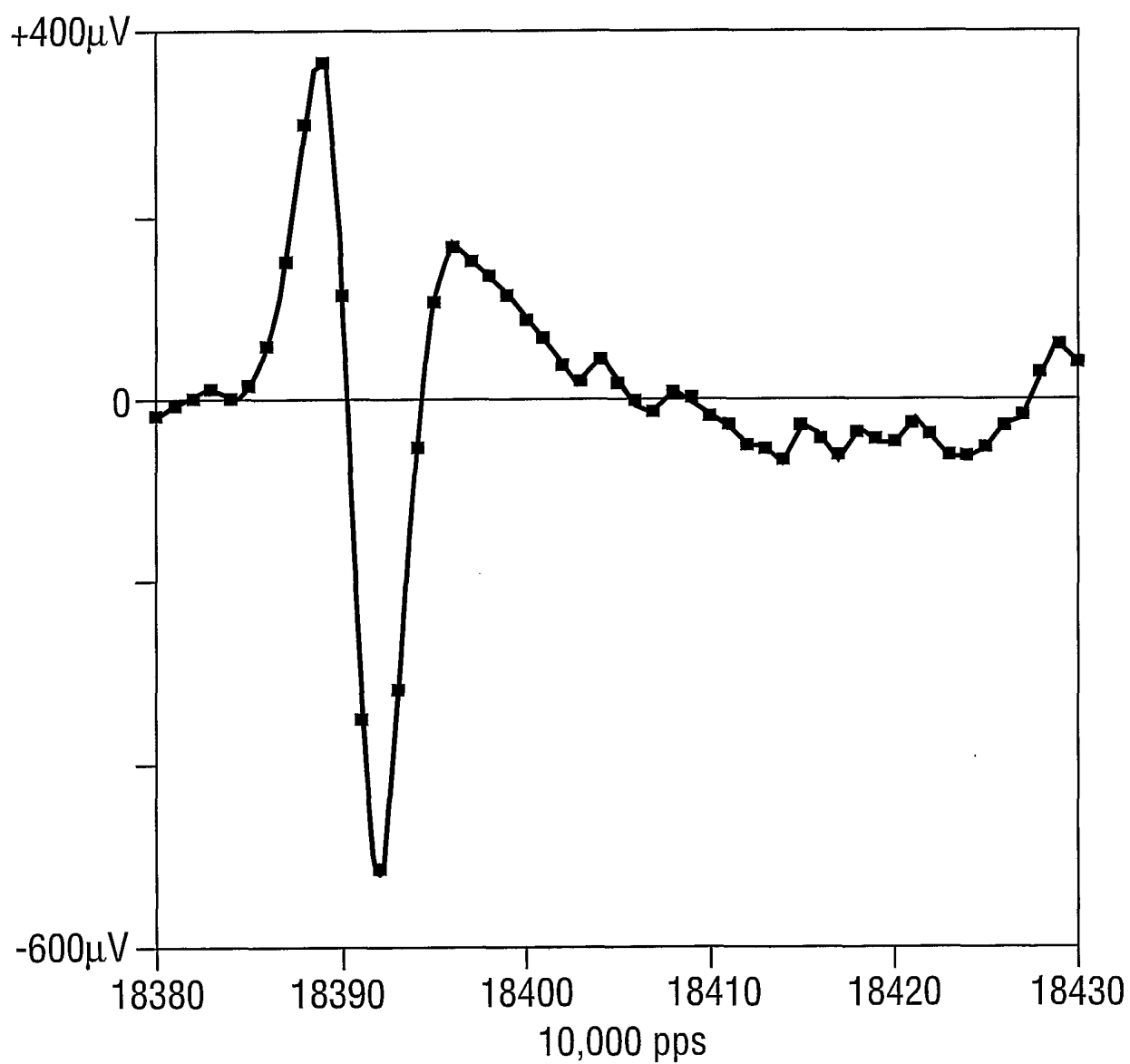


FIG. 6

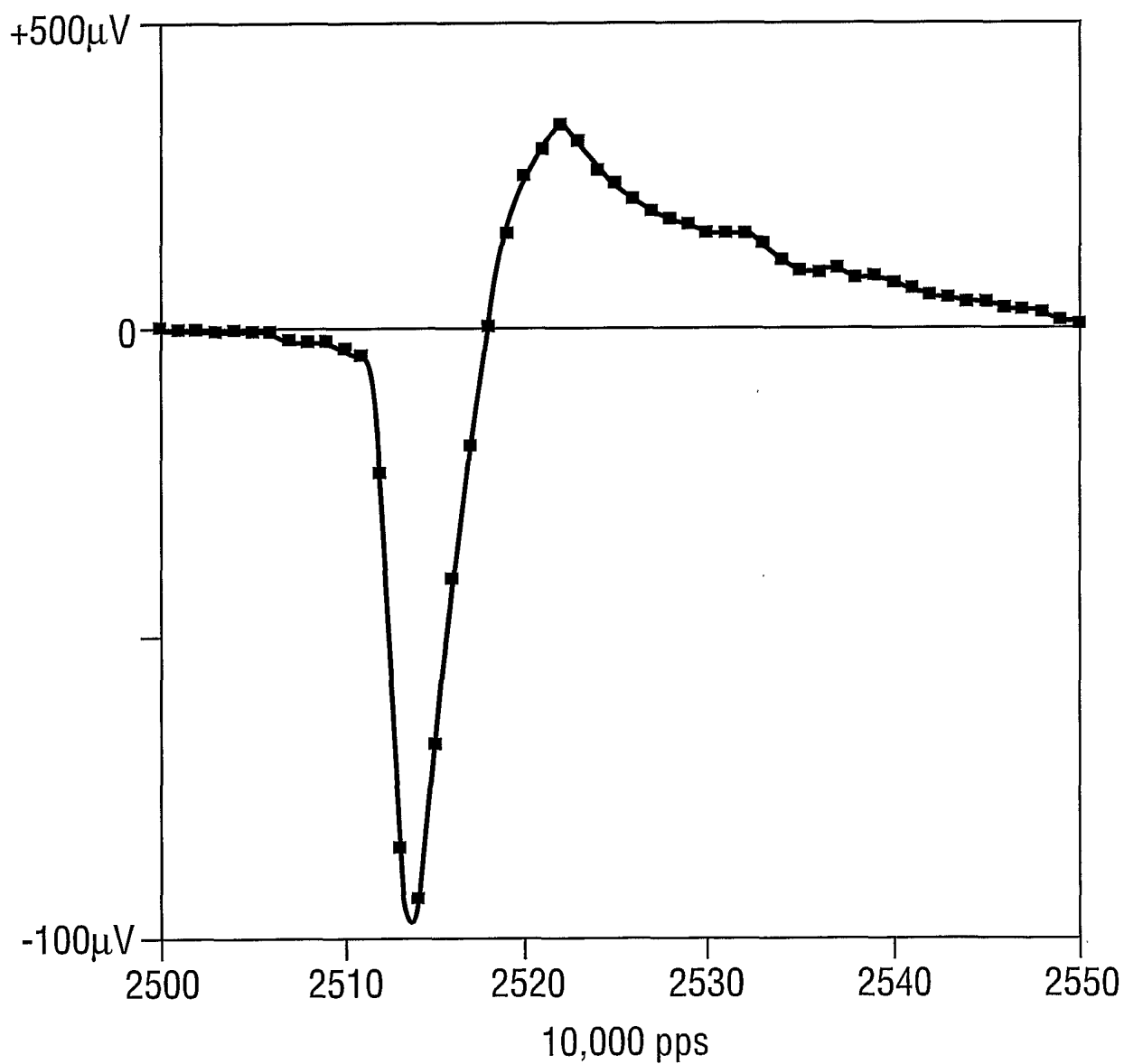
5/13

FIG. 5a



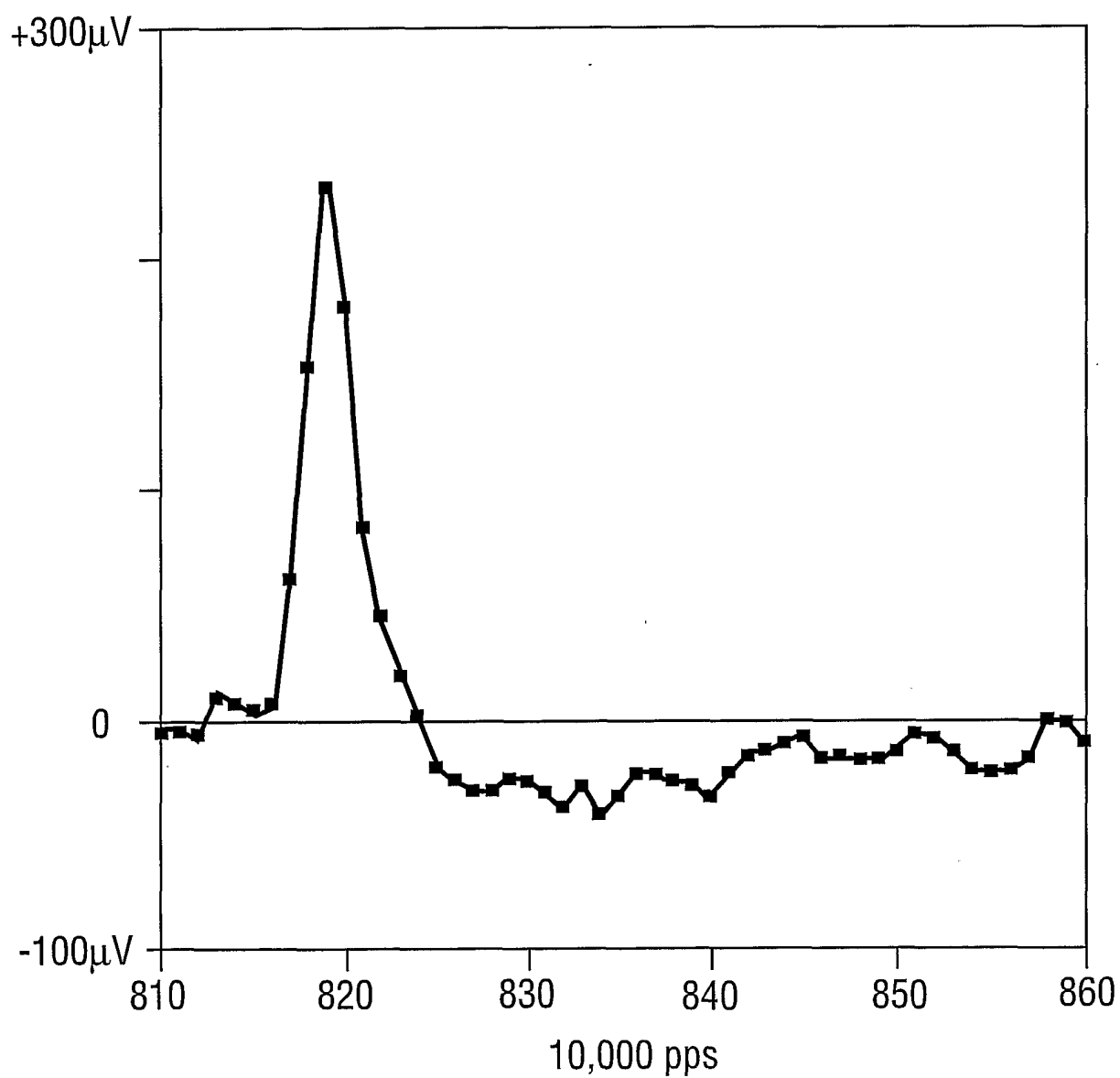
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FIG. 5b



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FIG. 5c



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FIG. 7

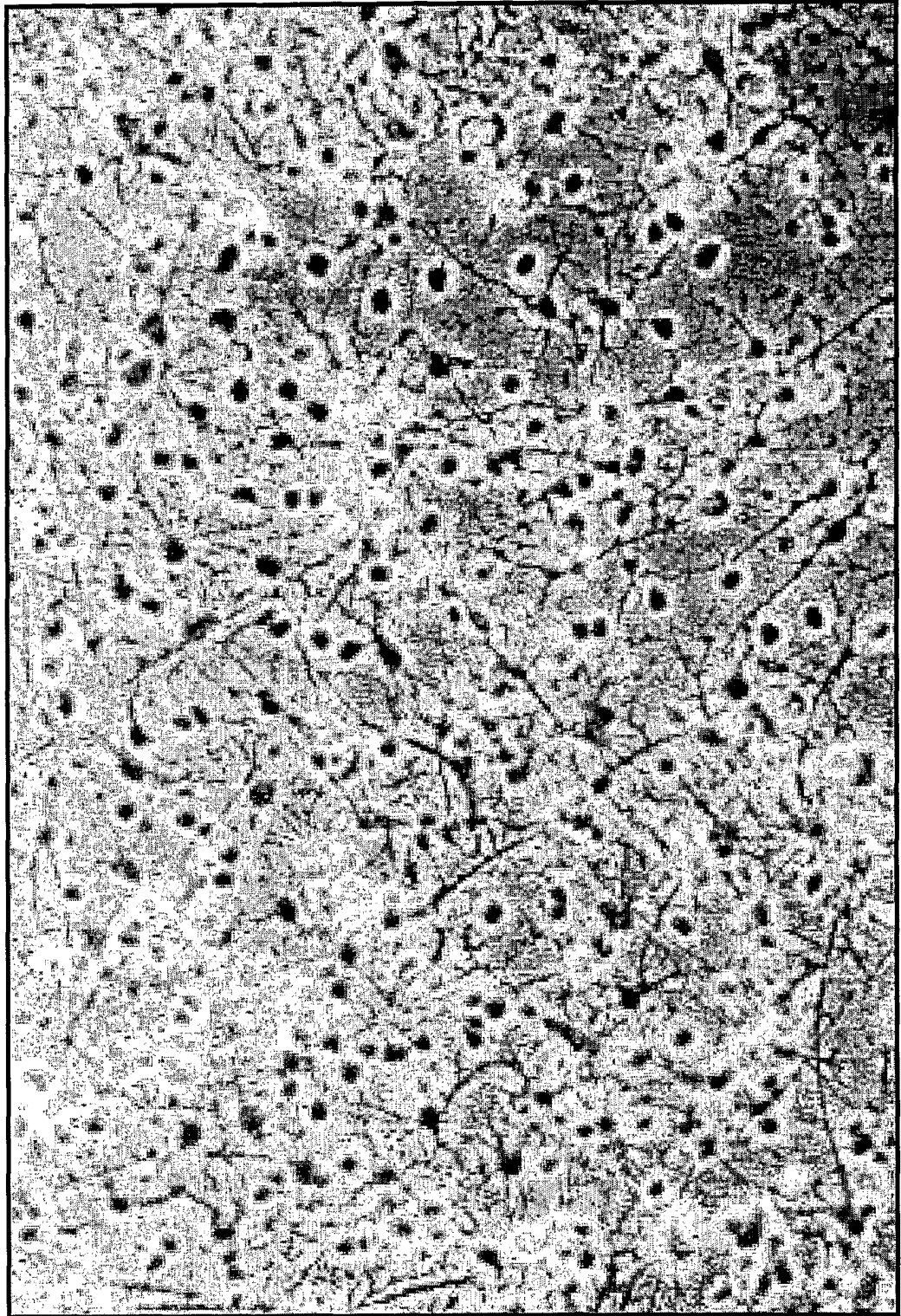




FIG. 8

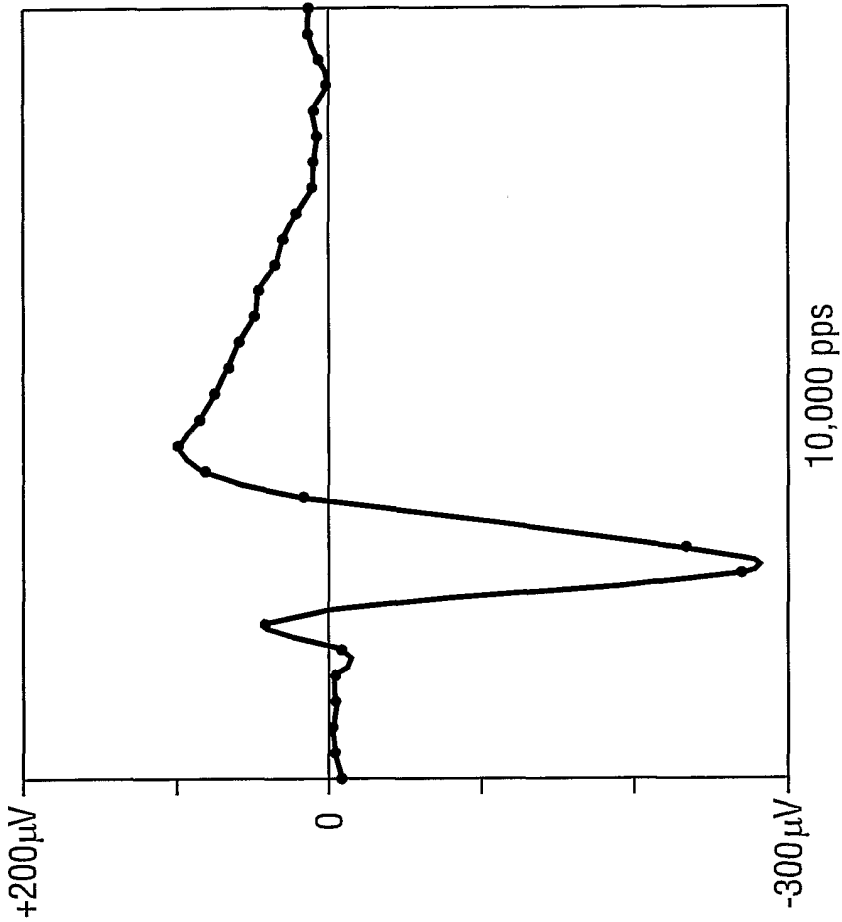


FIG. 9

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FIG. 10a

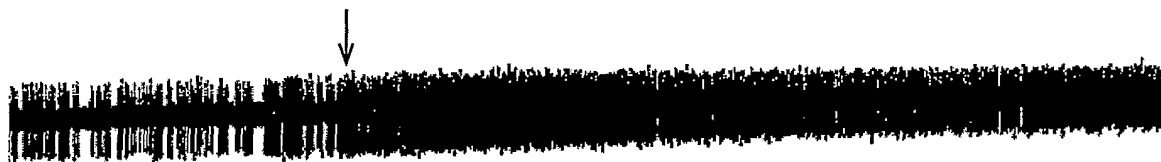


FIG. 10b



FIG. 10c

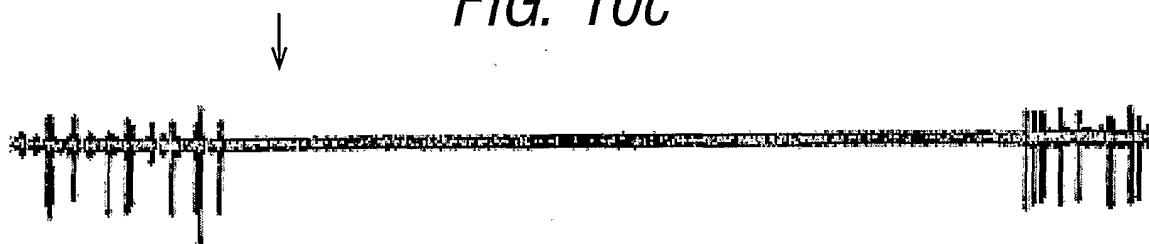
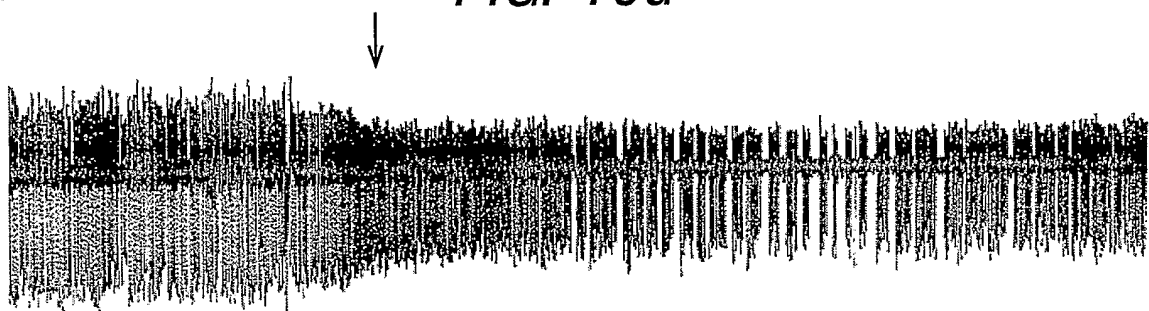


FIG. 10d



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FIG. 11

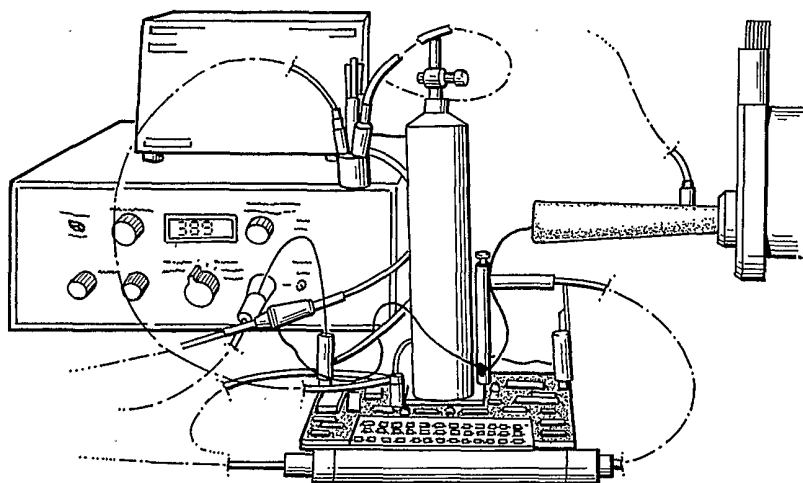
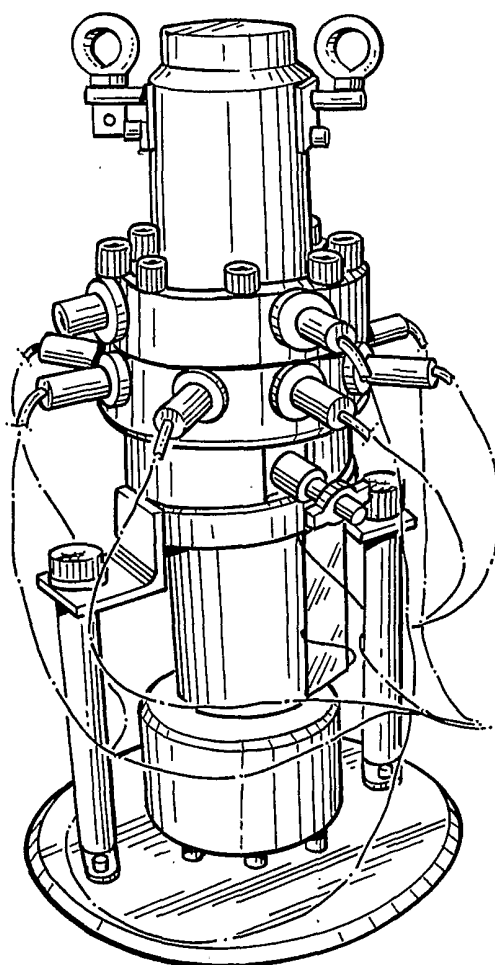


FIG. 14



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FIG. 12a

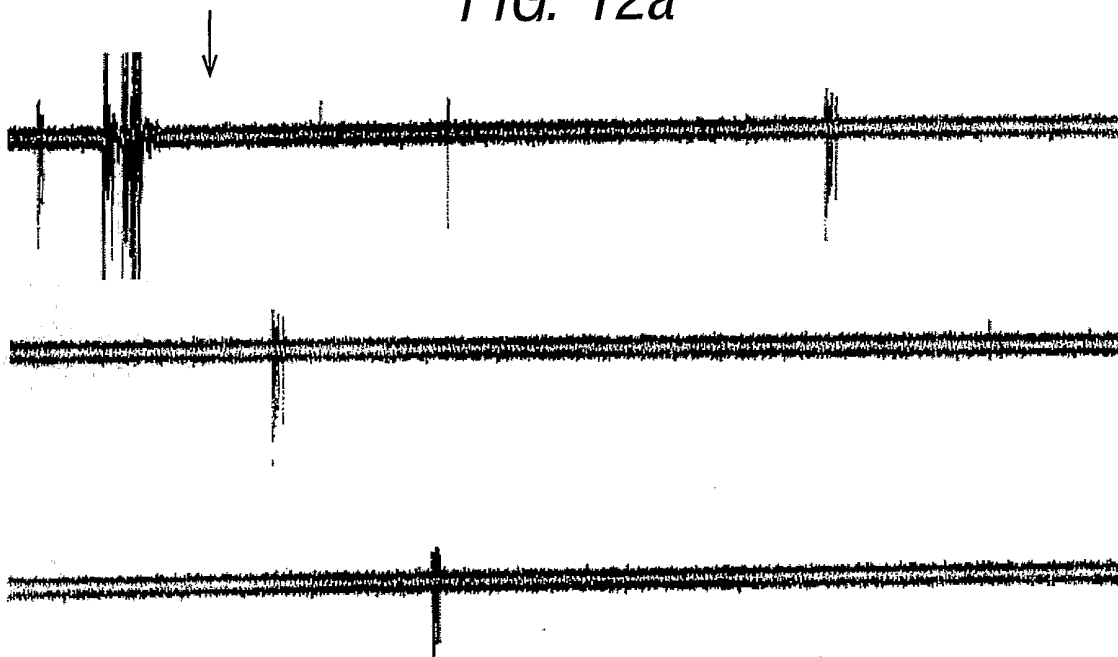
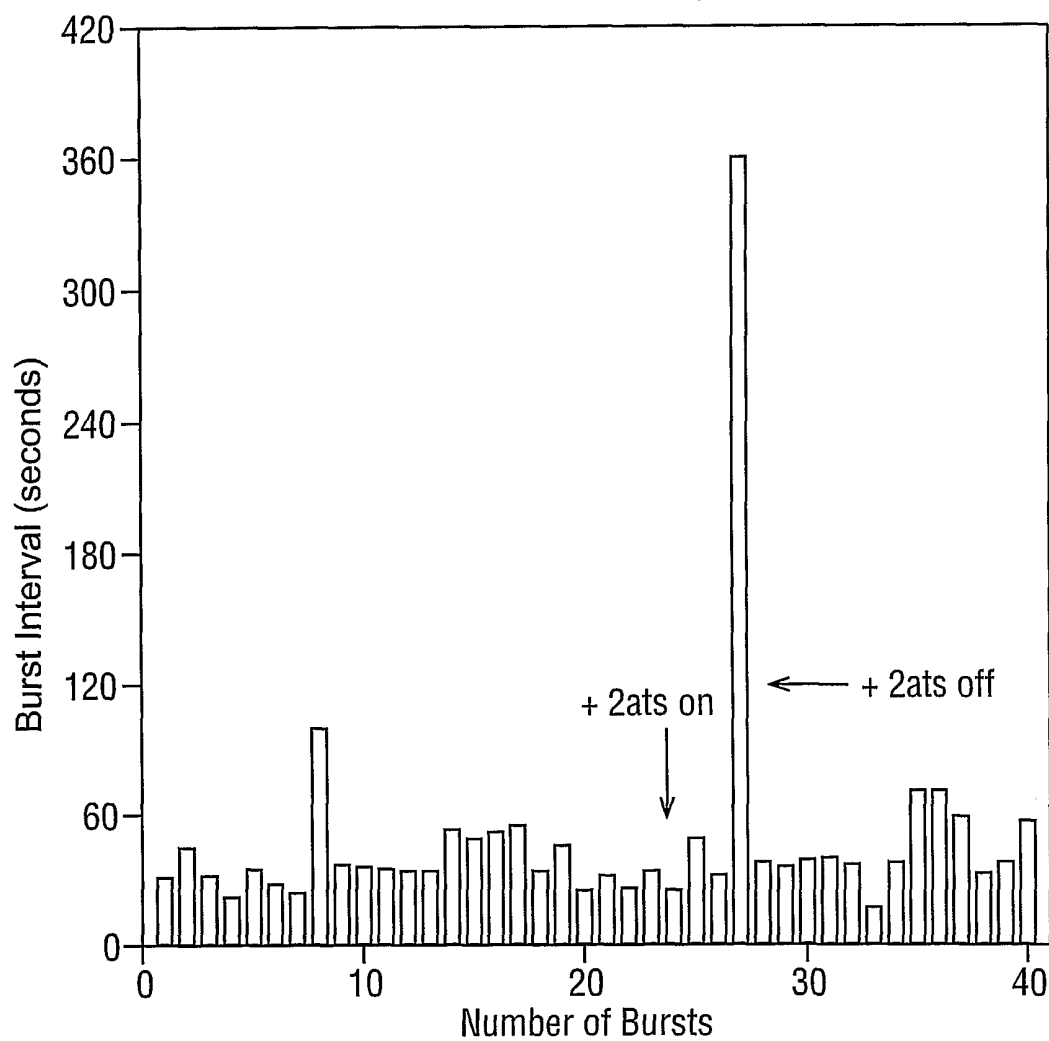


FIG. 12b

DRG culture in Nb 4wks⁺ (July 23, Ch 4)



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FIG. 13



FIG. 15

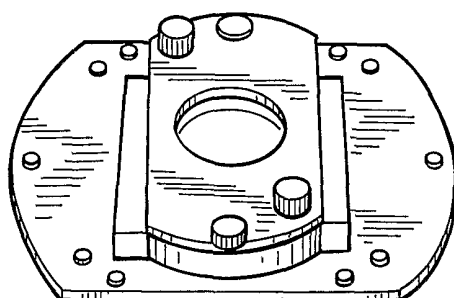


FIG. 16



FIG. 17

