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BIOLOGICAL ASSAY OF FRESH WATERS BY A NEW METHOD, DIELECTROPHORESIS

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TABLE OF CONTENTS

LIST OF FIGURES

BIOLOGICAL ASSAY OF FRESH WATERS BY A NEW METHOD, DIELECTROPHORESIS

I. RESEARCH OBJECTIVES

Rapid and selective methods for assaying cellular organisms in fresh water are being sought, through the application of nonuniform electric field effects (NUFE).

The original objectives, it will be recalled, were twofold. First, a direct attempt was to be made to develop a rapid but selective assay using NUFE, and Chlorella, sp. as the model organism. Secondly, the development of a continuous rather than a batch type of dielectrophoretic separator using NUFE was to be done. The latter would have wide application in studying fresh water biota.

II. INTRODUCTORY COMMENTS ON NUFE

Since the NUFE techniques are relatively new, the next few paragraphs will present a brief introduction to the basic principles of its operation before going on into a discussion of the research procedures.

1. Nonuniform Field Effects (NUFE)

A gentle force is exerted upon all matter by a nonuniform electric field. Recently it has been shown to be usefully applicable in the study of single cell organisms. The nonuniform field effect, dielectrophoresis, is the translational motion produced by the action of a nonuniform electric field upon neutral polarizable bodies. It is especially useful in studies of suspensions of cells. Normal and abnormal cells respond differently, as do cells of different species, and may be separated on the basis of their differing polarizational response

Because the phenomenon depends upon the polarization and not the charge of the particles, subtle new differences are available for exploitation by dielectrophoresis. Operations can be carried out in a high frequency electric field. The technique is rapid and simple. The frequency "spectrum" of cellular response to dielectrophoresis varies with the species, and with the physiological state of the individual cell. Cells may be studied individually or in large numbers at a time. The technique works best in media of low conductivity, such as in fresh water.

It is a familiar fact that in a uniform electric field, a force is exerted upon a charged object. If the field is made nonuniform, that type of force persists, and a new type of force arises, due to polarization effects. In the latter case of nonuniform fields, even neutral objects become subject to a force, one proportional to their polarizability. The two phenomena are distinguished by the terms electrophoresis for that on charged objects; and dielectrophoresis for that of neutral objects being pulled along by a nonuniform field.

We shall be most concerned with the latter, dielectrophoresis and polarization effects in the present work. With it one can obtain information as to the electrical polarizabilities and related biological mechanisms. Moreover it is easily possible to use the nonuniform field forces to produce useful physical separations to particulate materials such as mixtures of cells or other particles.

Nonuniform electric fields are easily produced in a controlled fashion. Since the polarizability of material bodies, especially living cells, varies strongly with the applied frequency, it is a relatively straightforward matter to obtain a "spectrum" for the dielectrophoretic responses of cellular suspensions. It is observed that different species show distinct "spectra" with up to three characteristic peaks or "relaxation times." As we shall see, Chlorella vulgaris appears to be unique in having four characteristic peaks or relaxation times.

In general, before dielectrophoresis is applied to a particular test system, several decisions concerning the experimental approach must be made. The important considerations are the configuration of the electrodes which will produce the nonuniform field, the selection of a critical parameter which reflects changes in the dielectrophoresis and thus measure the effect of the field, and the determination of the varible quantities upon which the chosen critical parameter depends.

A nonuniform field will be produced by an electrode design other than parallel plates. The number of possible configurations can be greatly reduced by requiring that the electric field be easily calculable. This desire for mathematical simplicity leads to the standard geometries of concentric spheres and concentric cylinders. Other configurations[3] have been devised which can be approximated as cylinders or spheres but which are easier to construct and offer experimental advantages. These include a wire perpendicular to a flat plate (pin-plate), a wire parallel to a flat plate (wire-plate), two coaxial wires end-to-end, (pin-pin) and two parallel wires side-by-side (wire-wire). One other calculable shape is that which gives a constant dielectrophoretic force[3], the "isomotive field". The choice between these five designs for a particular experiment depends upon the experimental characteristics desired. Probably the most popular type is the pin-pin arrangement due to its ease of construction and observation.

Once a field shape has been chosen, the next problem is to select a dependent variable which will give an indication of the effect of the field. Several such critical parameters exist, including the dielectrophoretic force, the velocity of a test particle, and the rate at which materials is built up at one of the electrodes. The force has been used as the critical parameter on several occasions and is convenient when dealing with a single, large particle. The force can also be used for single microorganisms in a variation of the Millikan oil drop experiment where the dielectrophoresis force is balanced by gravity. The velocity of a particle has not been used as the dependent variable, but there are no obvious reasons why it would not be useful in certain systems. The most widely used critical parameter is the rate of collection of particles at an electrode. It is most often expressed as a "yield", which is the amount collected within a specified time, and is best suited to systems which are suspensions of large numbers of particles. For use with very dilute suspensions of cells; a counting procedure of considerable precision is available. It consists of collecting cells for a short period (one minute, say) at a "driving" voltage (20 volts, say) then cutting the applied voltage to a "holding" value (2 volts, say) then counting the number of cells touching the electrode wire along a prechosen length. Using a mechanical stage, the count density of collected (single) cells can be checked along several regions of the wire-wire electrode and the results averaged. Cell suspensions containing only about 100 to 1000 cells/mm3 can be conveniently studied in this manner.

After the dependent variable has been selected, the various independent variables need to be ascertained so that the necessary equipment and procedures can be provided with the yield as the critical parameter, these independent variables are; the size and shape of the particles, the conductivities and permittivities of both the particles and the suspending medium, the concentration of particles, the frequency and strength of the applied field, and the elapsed time.

In a typical experiment involving suspensions, the ranges of the parameters are: particle diameter, 1-50 microns; frequency of applied field, 10^2 - 10^9 Hz; applied voltage 5-30 volts for closely spaced (3mm) electrodes; suspension conductivity, less than 10-1 mho/m. Low frequencies and high conductivities lead to excessive heating and other disruptive effects. An important point is the strong dependence of the results on frequency and conductivity. The previous simple theoretical treatment, which works well for insulating materials in insulating media, attributes motion to a difference in permittivities only and has no provision for the inclusion of these other two variables. A more general treatment has been given which includes these parameters. The predicted results using a spherical four-medium model agree with yeast cell behavior. However, the approach has not been checked against other systems and so at present the applicability of the special model to them requires further study and, doubtless, modification.

To summarize, dielectrophoresis is a new method for handling living cells in fresh water. It has been shown capable of distinguishing not only living and dead cells, but also cells which are different in kind or even in their physiological state.

III. SPECIFIC OBJECTIVES

The original objectives of this study were to develop an assay method specifically for a model organism, Chlorella vulgaris suitable for application to fresh water samples; and to develop a continuous broadly applicable NUFE separation procedure.

To date progress has been made towards both objectives. Because of the usually low concentration of planktonic biota, it was early realized that "large" volumes of fresh water (at least 100 ml) would have to be sampled in order to obtain fair and representative analyses. Earlier NUFE studies worked with sample volumes of 0.01 ml to 0.5 ml. Accordingly, it proved desirable to develop the NUFE method for the handling of rather larger volumes. This meant the development of the continuous type of dielectrophoresis to practicable levels before the first objective could be satisfactorily handled. The major emphasis during this past year has been on the development of techniques which could apply NUFE to large volumes. Concurrently, the specific organism, Chlorella vulgaris was studied and shown to collect readily using the NUFE. The collection rate in nonuniform electric fields was found to depend strongly upon the applied frequency of the ac electric field. A "yield spectrum" of of this response has been measured to help guide the separation operations.

IV. EXPERIMENTAL SECTION

A. Biological Materials

The microorganisms chosen for this study were a yeast (Saccharomyces cerevisiae) and an alga (Chlorella vulgaris). The yeast was grown in

Sabouraud broth. The culture was shaken gently during growth to minimize clumping of the cells. In all cases a 48 hour culture was used for experiments. Yeast was used in most experiments since it has been studied in this laboratory extensively and its dielectric characteristic and responses are well documented.[1,2,3]

One of the areas of study of this project was to make a similar study of algae. The alga (Chlorella vulgaris) was grown on a Wood's Hole formula[7] in 4 l. aspirator bottles under fluorescent light with stiring to minimize clumping of the cells.

B. The Dielectrophoretic Collection Spectrum of an Alga

The algal response curve was studied using a batch method and a wire-wire configuration for the electrodes. Figure 1 shows the dielectrophoretic collection rate (DCR) vs. frequency curve for both normal Chlorella vulgaris and for C. vulgaris stained with crystal violet. This data allows us to select appropriate frequencies and voltage in our later separation experiments.

C. The Development of Continuous Separators

The main area of this research has been the development of an effective and dependable apparatus for continuous dielectrophoresis. A large number of different flow geometries and electrode designs were investigated.

Originally, a cylindrical electrode design originated by Pohl[4] and later used by Mason and Townsley[5] in their studies was used. This design incorporates a cylindrical electrode with a central wire (Figure 3). This design concentrated a flowing suspension of cells about the central wire where they could be collected. Another design consisted of a flow chamber through which a pair of wire electrodes ran diagonally (Figure 4A). Yet a third chamber after Verschure and Ijlst[6] was designed consisting of one flat plate electrode and a wire mesh electrode to provide the nonuniform field (Figure 4B).

All of the above chambers were designed with one inlet port and two outlet ports. A flowing suspension of cells would be concentrated by the applied non-uniform electric field toward one of the outlet ports. Thus one outlet port should have a greater concentration of cells than the other. The opical density of the two cell suspensions was measured by a photometer to determine the effect of the field. (Figure 2)

Another cell design consisted of multiple pairs of wire electrodes (Figure 4C). The amount of cells collected on the wires is a measure of its biomass.

The designs discussed above were all subject to some problems. A cell at different positions in the non-uniform field experienced different dielectrophoretic forces. The cells also flowed at different speeds according to position, those in mid-stream flowing faster and being subjected to the field for a shorter period of time than those near the wall. These problems were eliminated by a new method of great promise: Stream-centered Continuous Dielectrophoresis (SCD). Accordingly the previous designs were then discarded in favor of the SCD method.

D. The Development of Stream-centered Continuous Dielectrophoresis

In this type of chamber (Figure 5), a central stream of very concentrated cell suspension is introduced into a surrounding support stream. The stream

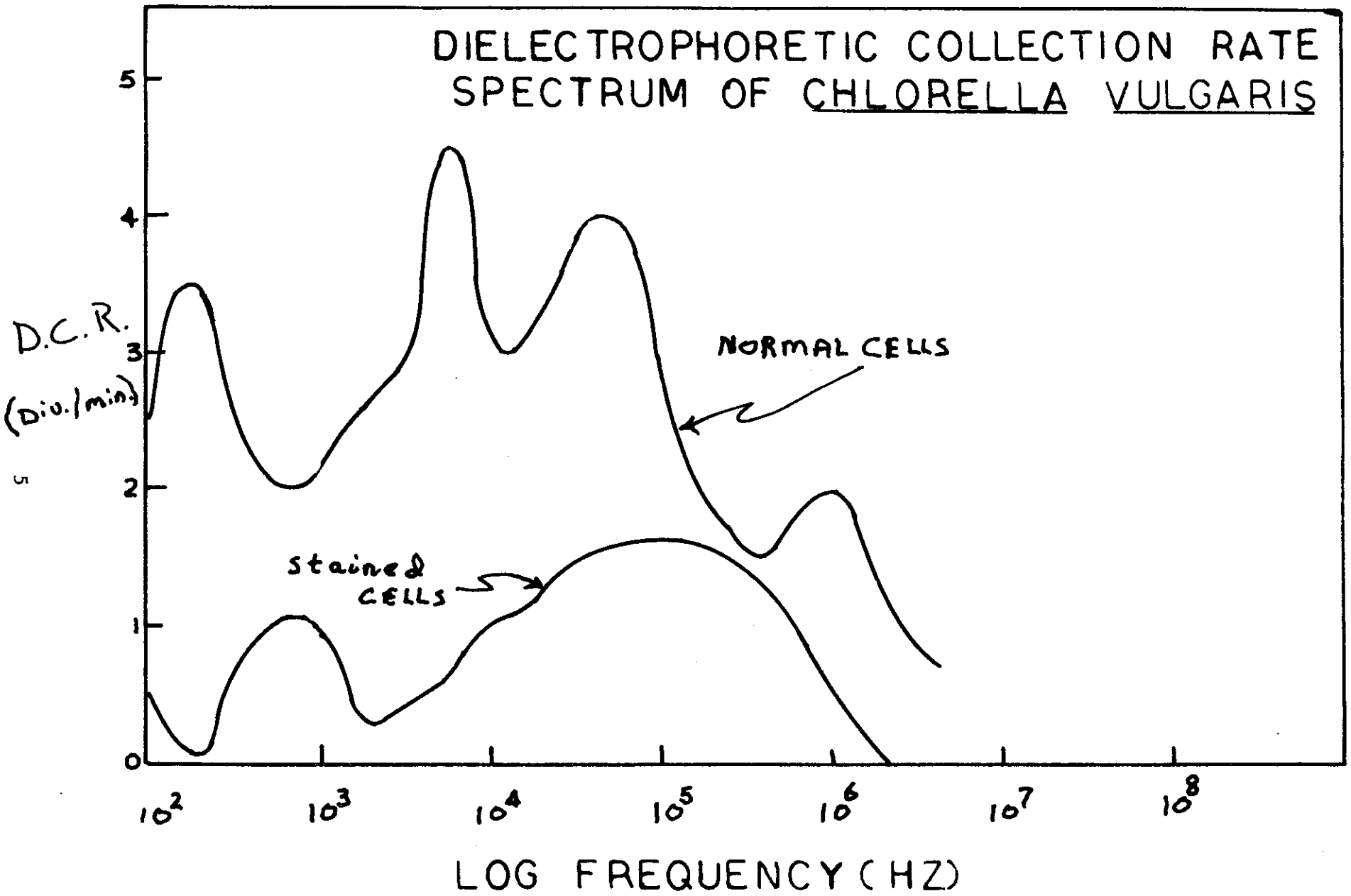

DIELECTROPHORETIC COLLECTION RATE SPECTRUM OF CHLORELLA VULGARIS
D.C.R. (Div./min)
NORMAL CELLS
stained CELLS
0
1
2
3
4
5
10^2
10^3
10^4
10^5
10^6
10^7
10^8
LOG FREQUENCY (HZ)

FIG. 1

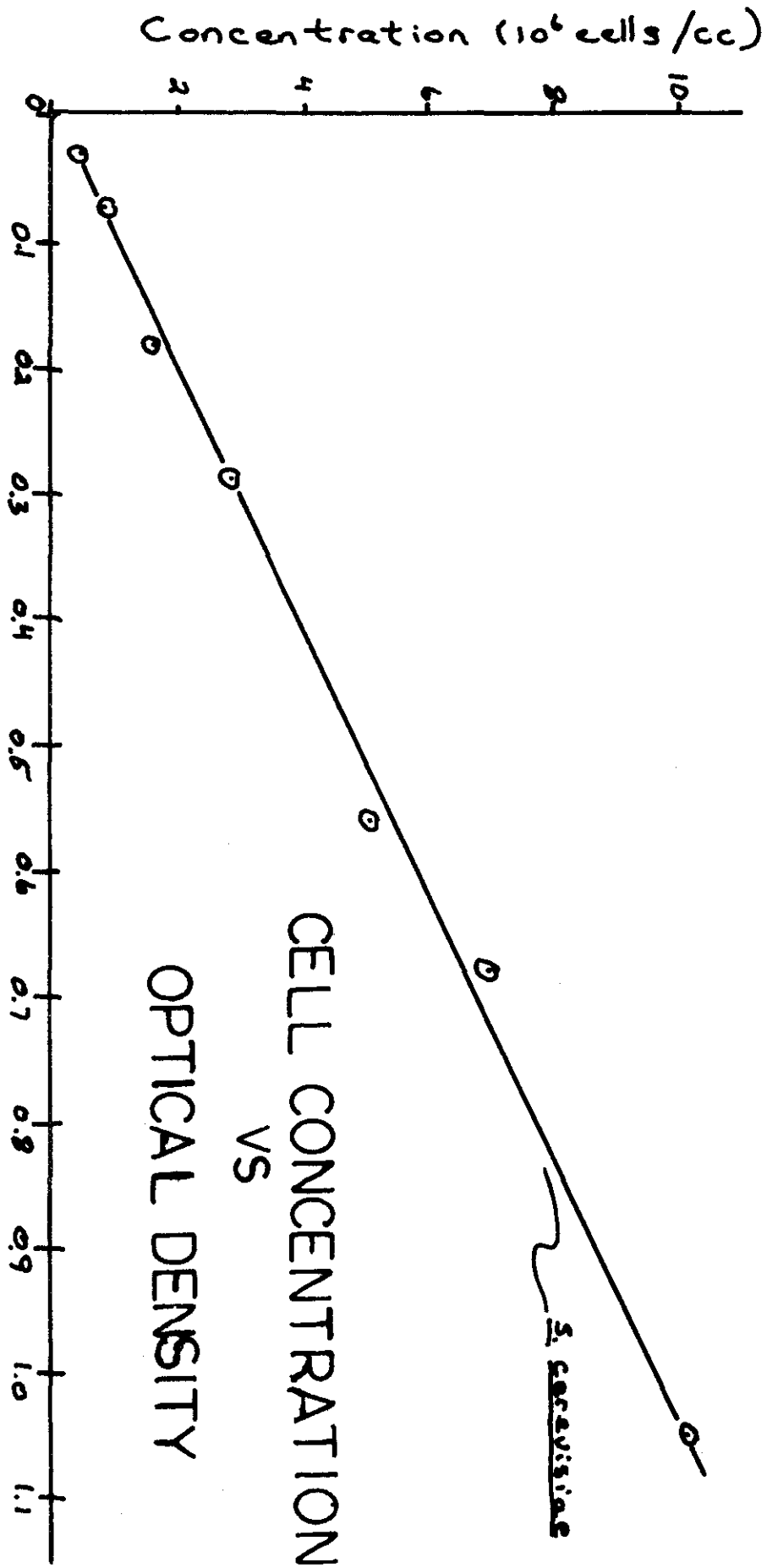
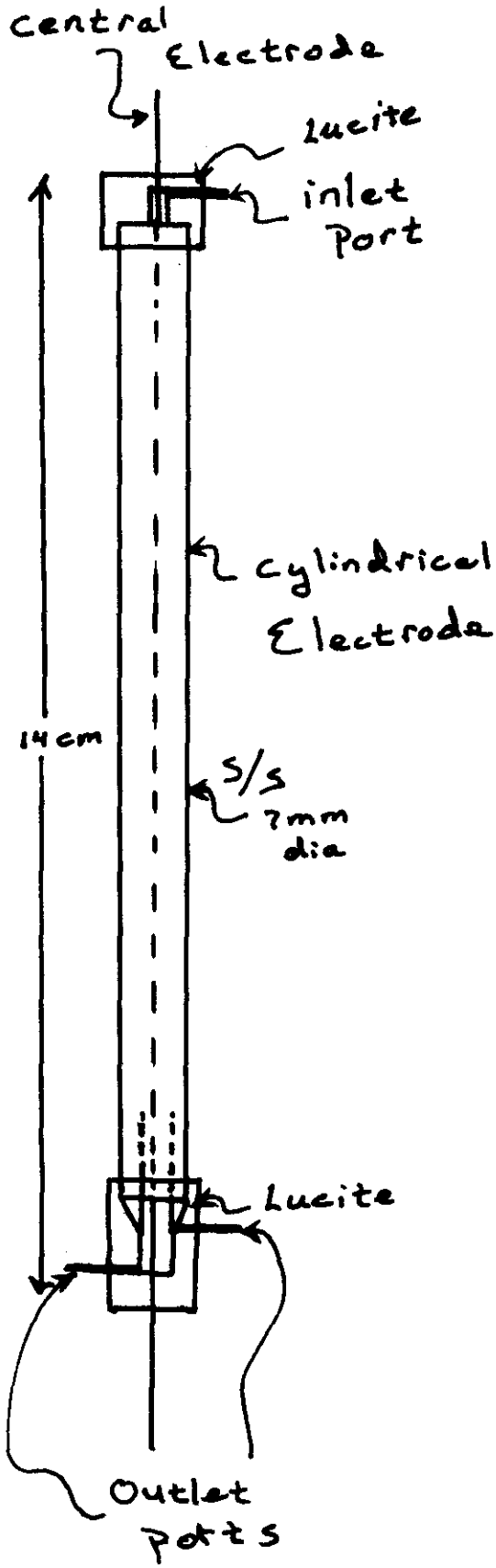
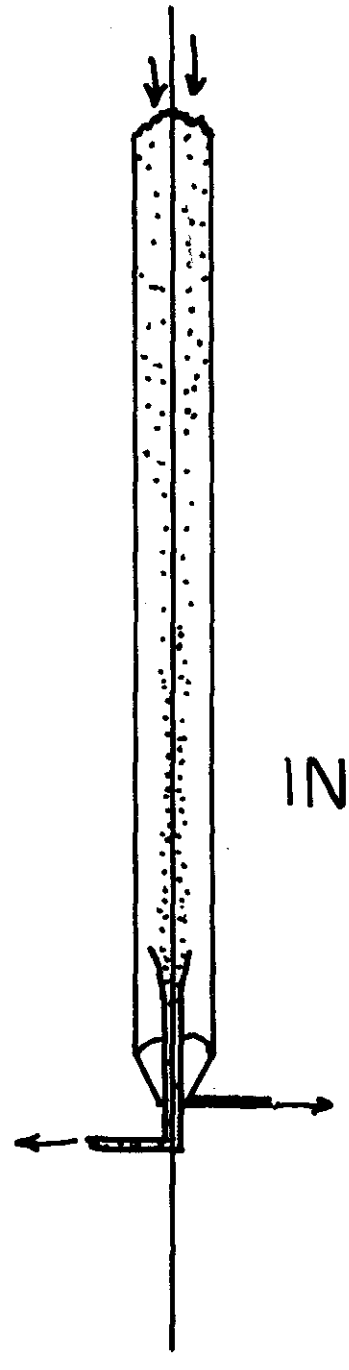

Concentration (10^6 cells/cc)
0
2
4
6
8
10
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
O.D.
CELL CONCENTRATION
VS
OPTICAL DENSITY

FIG. 2





INTERIOR
VIEW

FIG. 3

CYLINDRICAL
ELECTRODE CHAMBER

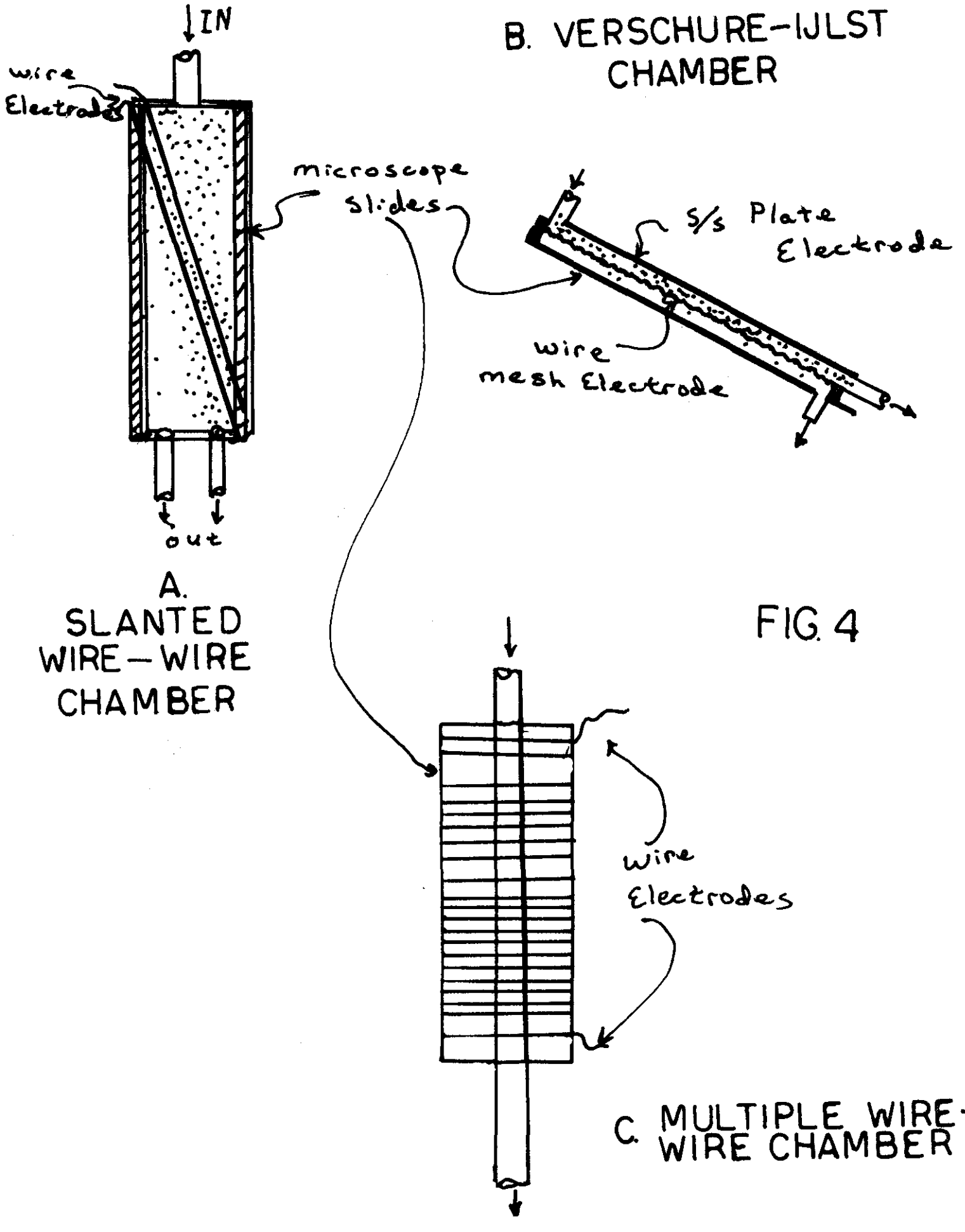

IN
wire Electrodes
microscope slides
out
A. SLANTED WIRE-WIRE CHAMBER
B. VERSCHURE-IJLST CHAMBER
S/S Plate Electrode
wire mesh Electrode
wire Electrodes
C. MULTIPLE WIRE-WIRE CHAMBER

FIG. 4

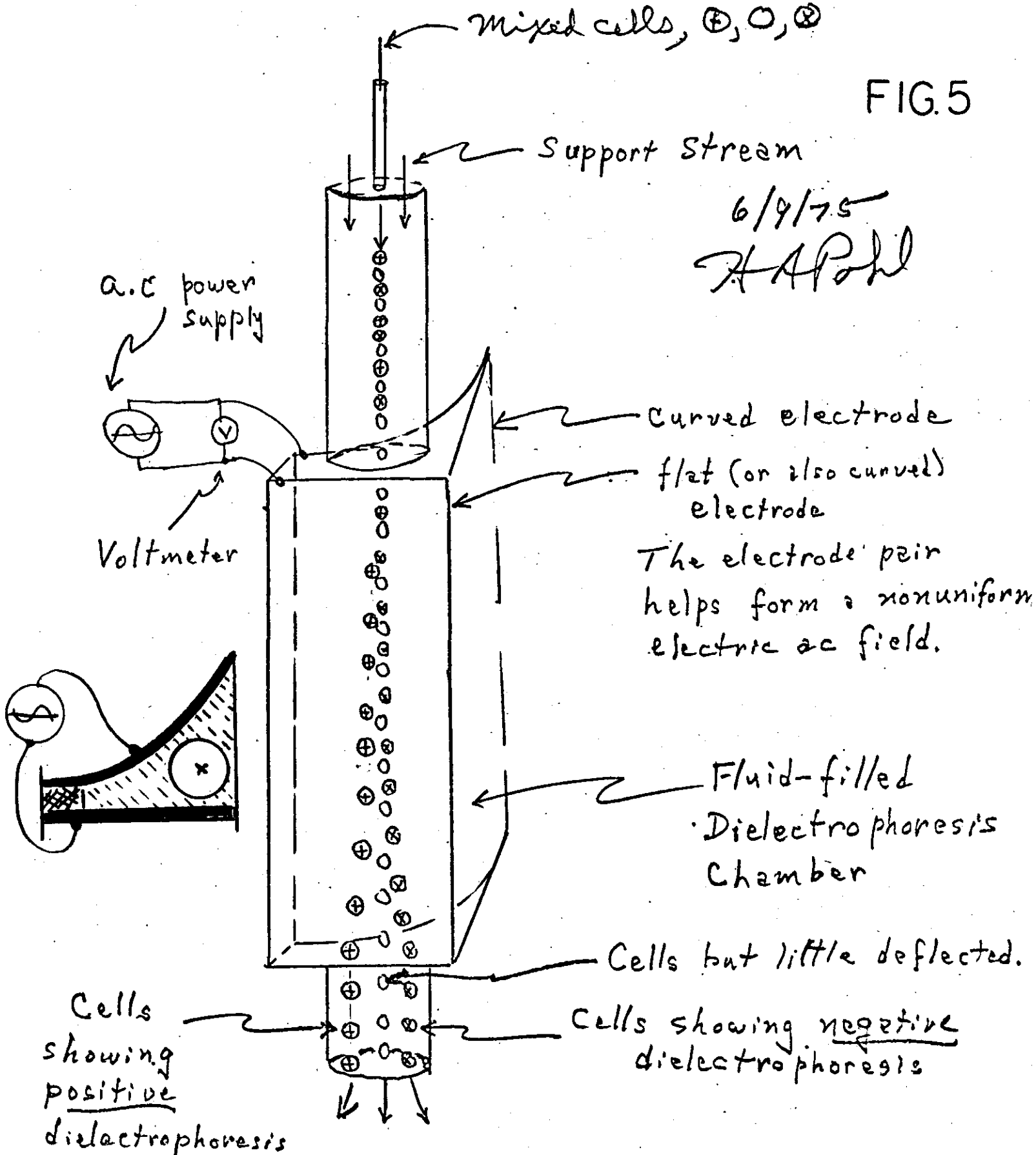


FIG. 5

6/9/75
H. A. Pohl

The electrode pair helps form a nonuniform electric ac field.

STREAM-CENTERED CONTINUOUS DIELECTROPHORESIS

of cells experiences a lateral force due to the applied electric field. Different particles will experience a different force and therefore deflect a different distance. This method has the potential to separate many different species of organism and allow their collection and enumeration from large volumes of liquid (e.g. one liter).

Most of the past year was applied to the development of the SCD method. The first chambers made were from 7 to 10 cm long and used various arrangements of electrodes to produce the non-uniform field. The most effective design was an isomotive[3] configuration. Unlike other field configurations, the isomotive force does not diminish with distance. The isomotive shape is relatively easy to approximate and construct (Figure 3).

The first small, successful chamber of this type produced a noticeable deflection (approximately 2mm) of the central stream of yeast with only 3 to 5 volts applied while running along a path of 5 cm. A larger chamber with multiple outlets yielded very encouraging results with a maximum deflection of 7 mm using an applied field of 8 volts (Figure 6). These results, obtained with small voltages (10 Vrms) suggested that longer chambers with correspondingly longer times in the field for the cells should yield excellent results with the same voltages.

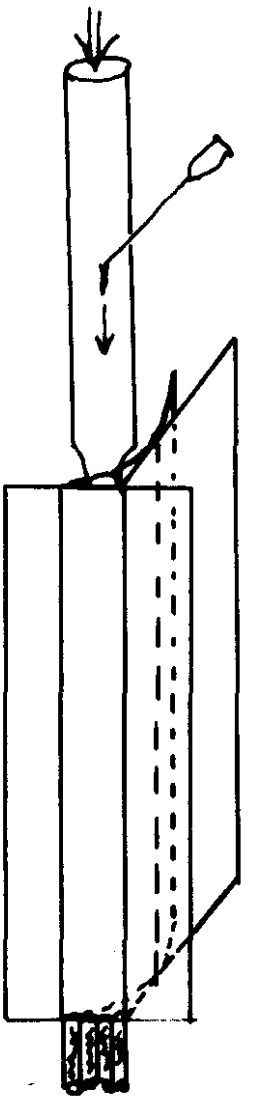
The larger chambers, however, exhibit greater problems with turbulence which destroys the flow pattern and masks effects of the electric field. Turbulences arise through the geometry differences of the flow chambers, electrode charge injection, the flow rate of the liquid, and temperature differences (due, in part, to dielectric heating) causing convection currents. Two larger chambers have been built, one 15 cm long and one 45 cm long. We are currently investigating the use of an agar gel filling in these chambers to achieve a simple cylindrical geometry throughout the system and to reduce electrode charge injection. Preliminary results with the use of de-ionized 2% agar gel to partially fill the electrode area (Figure 7) show excellent results to date. Despite some limited turbulence, noticeable deflection (3 to 5 mm) has been observed in the larger chamber at 5 to 7 volts using S. cerevisiae. At present, a method of thermostatting throughout the system is being studied to help reduce the remaining turbulence.

V. SUMMARY AND CONCLUSIONS

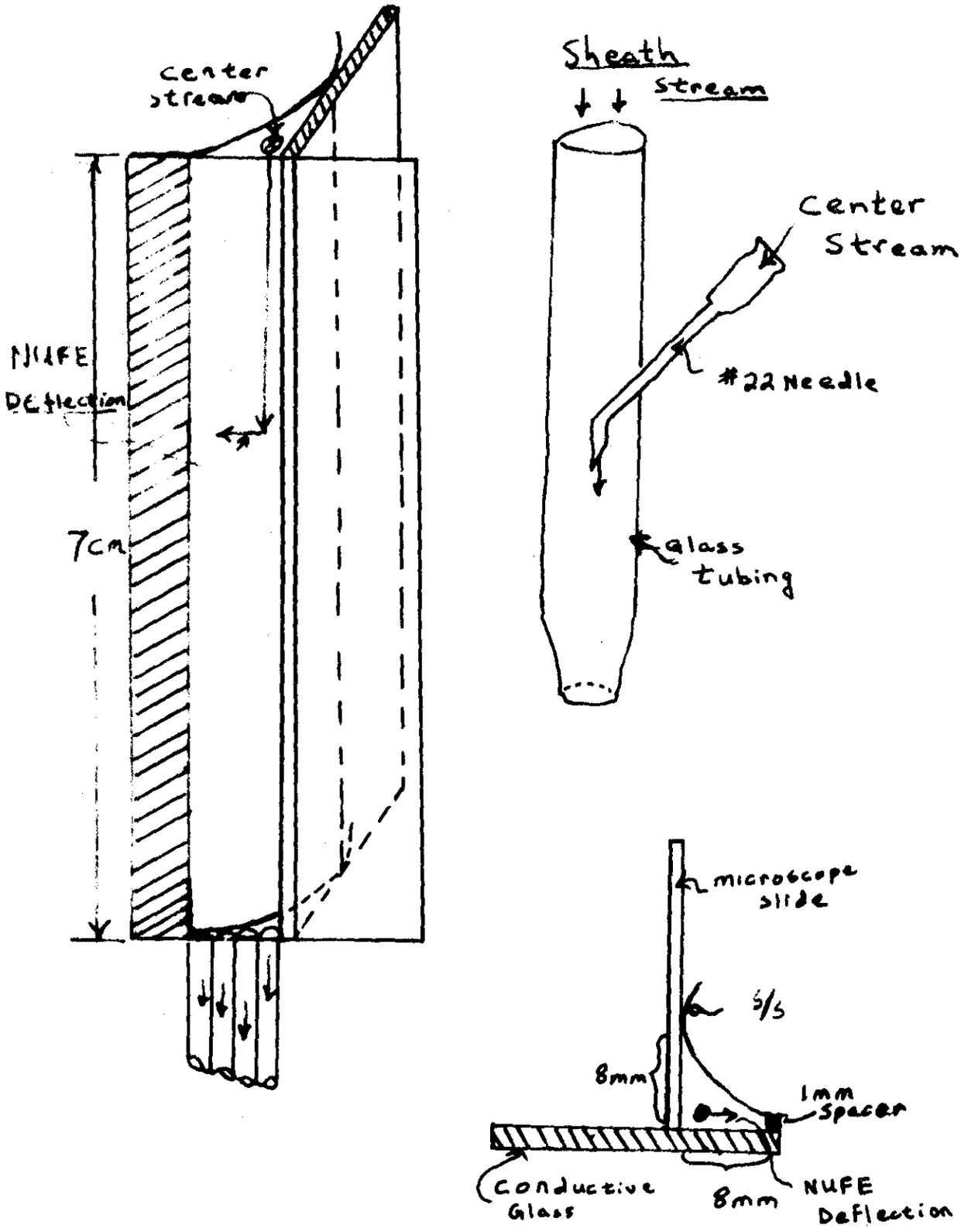
We have shown that the fresh water alga, Chlorella vulgaris exhibits positive dielectrophoresis over a wide frequency range, 100 Hz to 4 MHz. This means that the cells are pulled toward the region of highest field strength in an ac electric field. The collection is observed to be rapid and to vary strongly with the applied frequency. The later results provides a "yield" spectrum showing the varied polorization responses as the applied frequency changes and is a "diagnostic" of the organism. Most cells have yield spectra with three or fewer peaks. C. vulgaris is unusual in that its yield spectrum has at least four peaks. A comparison between the yield spectra of normal and dye-stained algal cells showed strong differences.

In order to develop an assay method using NUFE as applied to fresh water samples, we have needed to modify the conventional NUFE techniques. Previously these all dealt with only very small volumes (about 0.01 to 0.5 ml). For dealing with fresh water assays of biota, rather larger volumes (circa 100 ml or larger) are needed. To this end, our main thrust has been to develop the NUFE technique so as to handle these larger volumes. This meant the development of continuous methods whereby the larger volumes could be made to pass through the electrode treatment area.

FIG. 6A



STREAM-CENTERED
CONTINUOUS DIELECTROPHORESIS
CHAMBER



SCD CHAMBER

FIG 6B

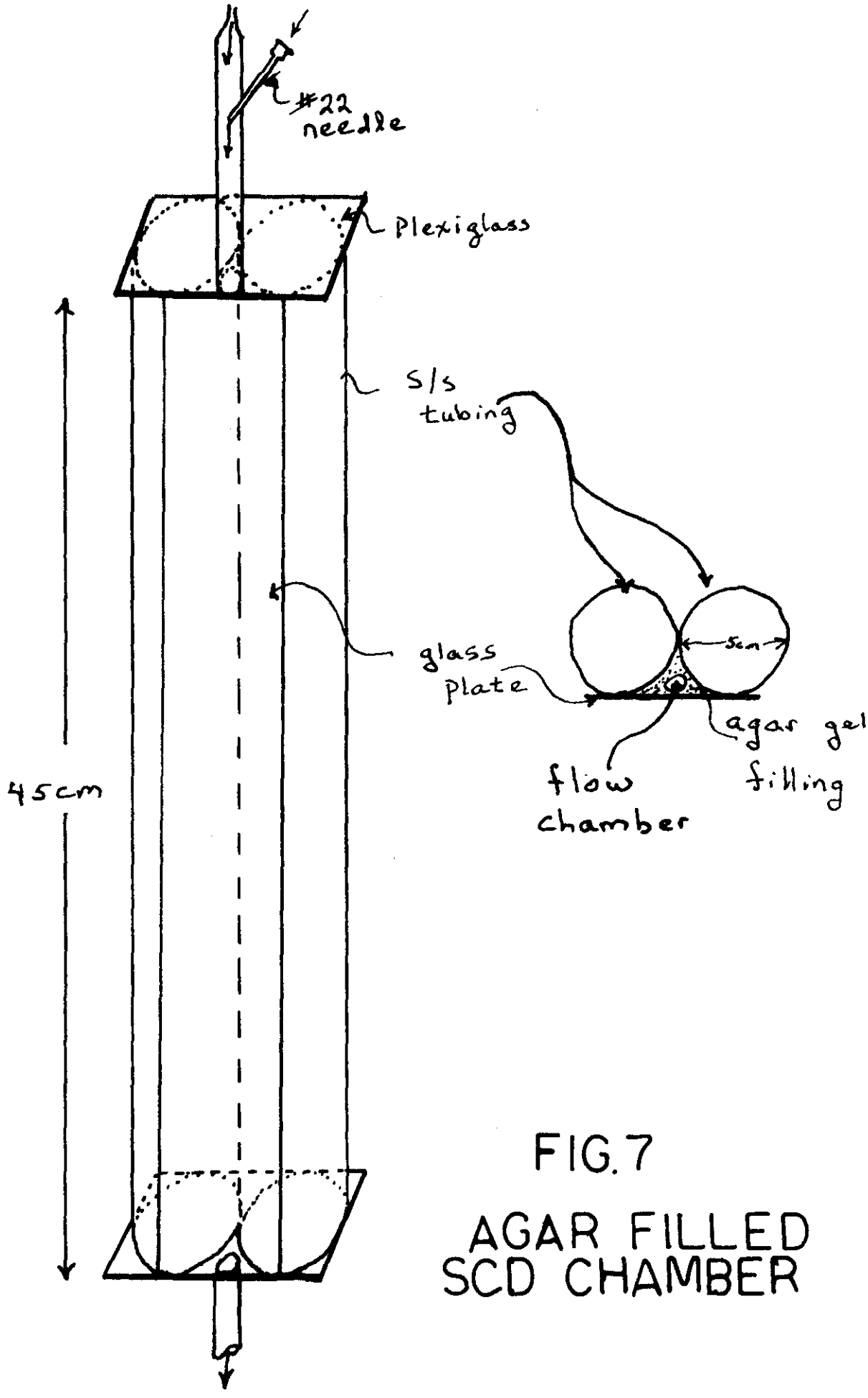


FIG. 7

AGAR FILLED SCD CHAMBER

The development of a continuous NUFE process has come, after many trial equipments were built and tested, to one which we believe has much promise. It subjects the desired water stream to a nonuniform electric field after first surrounding it with a protective thick layer of the suspending medium. The technique is referred to as "stream-centered continuous dielectrophoresis", (SCD). Preliminary tests with suspended cells show it to operate reasonably well. The results are so encouraging as to deserve strong further efforts to perfect and standardize the technique.

The stream-centered continuous dielectrophoresis method appears to have enormous potential. Not only does it seem well suited for bio-assay problems of fresh water, but it should also be applicable to subtle investigations in health problems. It should be ideally suited to handling suspensions of many components such as whole blood, and could presumably even distinguish slight differences among cells in a single species. Active development of this powerful technique appears to warrant intense study.

VI. GOALS FOR FURTHER STUDY

1. Perfect and standardize the stream-centered continuous dielectrophoresis technique for the analysis and separation of living cells.
2. Application of the SCD technique to the problem of bio-assay.
3. Exploration of the capabilities of this SCD method. For example, possible application to the determination of the population type and density in a body of water. Or, a possible application to human health problems such as leukemia or sickle cell anemia.

REFERENCES

1. Pohl, H. A. and I. Hawk, "Separation of Living and Dead Cells by Dielectrophoresis", Science, 152, 647 (1966).

2. Pohl, H. A. and J. S. Crane, "Dielectrophoresis of Cells", Biophysical J., 11, 711 (1971).

3. Pohl, H. A. and J. S. Crane, "Biological Aspects of Dielectrophoresis", Chapter 15 in A. D. Moore's editing of Electrostatics and Its Applications, Wiley and Sons.

4. Pohl, H. A., J. Appl. Phys., 29, 1182 (1958).

5. Mason, B. D. and P. M. Townsley, "Dielectrophoretic Separation of Living Cells", Can. J. Microbiol., 17, 879 (1971).

6. Verschure, R. H. and L. Ijlst, "Apparatus for Continuous Dielectric-medium Separation of Mineral Grains", Nature, 211, 619 (1966).

7. Nichols, H. W., "Growth Media-Freshwater", pp 7-24 (1973). In: Handbook of Phycological Methods, (Ed) J. Stein, Cambridge University Press, Cambridge.