Biological, Physical, and Electrical Parameters for In Vitro Studies With ELF Magnetic and Electric Fields: A Primer

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This paper presents material which is intended to assist researchers in identifying and controlling a range of biological, electrical, and other physical parameters that can affect the outcome of in vitro studies with extremely low frequency (ELF) magnetic and electric fields. Brief descriptions of power line magnetic and electric fields are provided and methods for the generation of 60 Hz as well as other ELF fields in the laboratory are surveyed. Methods for calculating and measuring exposure parameters in culture medium are also described. Relating in vitro and internal in vivo exposure conditions across different animal species is discussed to aid researchers in selecting levels of field exposure. The text is purposely elementary, and sometimes brief, with references provided to aid the interested reader in obtaining a fuller understanding of the many topics. Because the range of experimental parameters that can influence the outcome of in vitro studies with ELF fields is so broad, a multidisciplinary approach is normally required to carry out the research. © 1993 Wiley-Liss, Inc.*

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CONTENTS

- **1. INTRODUCTION**
- 2. POWER LINE ELECTRIC AND MAGNETIC FIELDS AND EXPOSURE PARAMETERS
- 3. EXPOSURE SYSTEMS FOR IN VITRO STUDIES WITH MAGNETIC FIELDS
 - 3.1 Apparatus for Generating Magnetic Fields
 - 3.1.1 Magnetic field of rectangular loops of many turns
 - 3.1.2 Magnetic field of circular coils
 - 3.1.3 Magnetic field of solenoid
 - 3.1.4 Current sources for magnetic field coils
 - 3.1.5 Stray magnetic fields and shielding
 - 3.1.6 Parallel plates, rectangular solenoids, and stray fields
 - 3.1.7 Rotating magnetic fields
 - 3.1.8 Systems with AC and DC magnetic fields
 - 3.2 Chamber Design
 - 3.2.1 Experimental geometry and exposure parameters
 - 3.2.2 Biological considerations in designing exposure chambers
 - 3.2.3 Some notes on exposure chambers and their fabrication

3.3 Other Biological and Physical Considerations

- 3.3.1 CO₂/O₂ tension, buffering, and pH
- 3.3.2 Temperature
- 3.3.3 Osmolality
- 3.3.4 Humidity
- 3.3.5 Viscosity
- **3.3.6 Surface tension**
- 3.3.7 Normal growth and sampling
- 3.3.8 Initial cell density
- 3.3.9 Light
- **3.3.10 Incubators**
- 3.3.11 Incidental magnetic field exposure
- **3.4 Experimental Results**
- 4. MEASUREMENT OF MAGNETIC FIELDS
 - 4.1 Instrumentation
 - 4.2 Calibration of Magnetic Field Meters
 - 4.3 Harmonic Content Measurements
- 5. EXPOSURE SYSTEMS FOR IN VITRO STUDIES WITH ELECTRIC FIELDS
 - 5.1 Methods for Generation of Electric Fields
 - 5.2 Generation of Electric Fields With Current-Passing Electrodes
 - 5.2.1 Surface current density and electrode resistance
 - 5.2.2 Uniformity of the electric field and exposure conditions
 - 5.2.3 Toxicity problems with in vitro electric field systems
 - **5.2.4 Constant current sources**
 - 5.2.5 Compatibility of in vitro electric field systems with electrophysiological recordings
 - 5.2.6 Resistive heating in culture medium
 - 5.2.7 Magnetic fields from currents in culture medium

- 5.2.8 Preparation of silver-silver chloride and platinum black electrodes by electrolysis
- 5.3 Generation of Electric Fields Between Capacitor Plates
- 6. MEASUREMENTS OF ELECTRIC FIELDS OF IN VITRO SYSTEMS
- 6.1 Measurements of Electric Field Uniformity and Strength in Conducting Media Using Dipole Probes
 - 6.1.1 Calibration of electric field dipole probes
- 6.2 Measurement of Electric Field Using Single Electrode Probe
- 6.3 Determining Electric Field From Resistivity and Current Density Measurements
- 6.4 Measurement of Electric Fields Induced by Magnetic Fields 6.5 Reporting Exposure Conditions
- 6.5 Reporting Exposure Conditions

7. RELATING IN VITRO AND IN VIVO EXPOSURE CONDITIONS

8. CONCLUSIONS

9. ACKNOWLEDGMENTS

1. INTRODUCTION

Since the mid 1970s, many laboratory studies have been conducted to examine biological effects in cells exposed in vitro to extremely low frequency (ELF) magnetic and electric fields. Concurrent with these studies have been investigations of ELF electric and magnetic field effects on small animals. While there exists some guidance for establishing and characterizing exposure conditions for the small animal studies [Misakian, 1984], there is limited guidance for the in vitro research. The purpose of this paper is to fill part of the information void by identifying electrical and biological parameters that are encountered during in vitro studies with ELF magnetic and electric fields, and by describing how the fields can be produced for exposure purposes and how the fields can be measured and, in some situations, calculated. The influence of the geometry of the culture medium-magnetic field combination on the exposure conditions for cells during in vitro studies with magnetic fields will also be examined. The text will provide discussions of the many biological and physical (other than electrical) parameters that introduce experimental constraints and must be recognized when an exposure system is designed and operated. For example, biological factors that must be considered include cell type and assay endpoints. Cell type affects a number of culture conditions that vary from cell to cell, including method of culture (e.g., monolayer or suspension), choice of substratum for growth, medium constituents, pH, CO₂/O₂ tension, etc. Assay endpoints also affect the criteria for selecting an exposure chamber and exposure conditions.

This paper is intended to serve as a primer for biologists engaged in in vitro bioeffect studies with ELF magnetic and electric fields and for physicists or engineers that design the exposure system (in collaboration with the biologists) and provide equipment and measurement support. To achieve valid results when exposing cells to magnetic and electric fields, dosimetric and biological considerations must be carefully balanced. Good biological data at the expense of dosimetry is of little value, as is good dosimetric data from cells that are either contaminated with microorganisms or exposed under conditions that are not physiological. Conditions in the chambers (sham or control) should not alter the health and/or viability of the cells. It will become apparent in the following sections that developing an exposure system in

which the exposure conditions are well defined and in which the biological requirements are satisfied requires a multidisciplinary effort. The research effort will be most productive when investigators with different expertise work in close collaboration and exchange information pertaining to the biological aspects of the experiment and the characteristics of the exposure parameters.

Experiments with sinusoidal ELF fields, i.e., 3 Hz to 3 kHz [Jay, 1984], are the primary focus of this primer. Experiments with pulsed fields that have ELF repetition rates are not considered because of the possibility that the fields contain frequency components outside the ELF range due to the fast risetime of the pulse waveform. These higher frequency components or harmonics (see 4.3, Harmonic Content Measurements) can reach megahertz values [Barker and Lunt, 1983], and the generation and measurement of fields with such frequencies require more guidance than is provided by this primer. Non-sinusoidal magnetic fields with frequency components only in the ELF range, e.g., the "sawtooth" ELF magnetic field produced by televisions [IEEE, 1991], could be generated and measured using the techniques that are described.

It should be noted that throughout this primer the sinusoidal variation of the magnetic and electric fields and associated currents will be suppressed and only root-mean-square (rms) values will be used unless noted otherwise. The rms or "effective value" of an alternating current (ac) is equal to the steady direct current (dc) that is required to develop the same amount of heat as the ac current passing through the same resistance in the same time. The rms value of a pure sinusoidal quantity (current, magnetic flux density, electric field strength, etc.) is equal to the peak value divided by $\sqrt{2}$.

2. POWER LINE ELECTRIC AND MAGNETIC FIELDS AND EXPOSURE PARAMETERS

Because research that is addressed by this primer is often stimulated by interest in the effects of power line fields, we briefly describe the electric and magnetic fields near these lines. However, laboratory exposure systems for in vitro research do not resemble small scale models of power lines. Rather, laboratory systems take a number of more practical forms as discussed in the following sections.

The electric and magnetic field lines of a single current-carrying conductor oriented perpendicularly to the page, energized with 60 Hz voltage, and located above a ground plane are shown schematically at some instant in Figure 1. The magnetic flux density, B, at a point can be represented by a vector that oscillates in magnitude at a frequency of 60 Hz along a line tangent to a circular magnetic field line. Similarly, the electric field strength, E, can be represented as an oscillating vector tangent to an electric field line. These fields are sometimes referred to as single-phase ac fields [IEEE, 1978] or as being linearly polarized because the vectors oscillate along a fixed direction.

Transmission line circuits normally have three or more conductors (or conductor bundles) with multiple voltage phases. The electric and magnetic fields of a three-phase transmission line have been calculated and illustrated by Deno [1976] and a somewhat simplified sketch of the electric field in the vicinity of a three-phase transmission line is given in Figure 2. Three conductors are shown above the ground plane and the phase of the 60 Hz voltage applied to each conductor differs by 120°

In Vitro Studies With ELF Fields: A Primer



Fig. 1. Electric- and magnetic-field lines of a single conductor oriented perpendicular to the page above a ground plane. The \vec{E} and \vec{B} represent the respective fields at the points indicated.



Fig. 2. Electric-field ellipses at representative points in vicinity of 3-phase transmission line (after Deno [1976]) with conductor-length oriented perpendicularly to the page. During each period, the electric field vector at a point in space rotates and traces an ellipse in a plane perpendicular to the conductors. The period of rotation coincides with the period of the ac voltages applied to the conductors. At distant points and on the ground plane, the ellipses become lines and the field resembles a single-phase AC field.

with respect to the adjacent conductors, e.g., sinusoidal voltages $V\sin(\omega t)$, $V\sin(\omega t + 120^{\circ})$, and $V\sin(\omega t + 240^{\circ})$ applied to adjacent conductors, where V is the peak voltage, ω is the angular frequency (equal to $2\pi f$, where f is the frequency), and t is the time.

In general, the electric field strength, E, at a point in space can be represented as a rotating vector that traces an ellipse in a plane perpendicular to the conductors as shown in Figure 2. Near ground level, the field ellipse degenerates to a nearly vertical line. In the absence of nearby objects or irregular terrain, the field strength changes slowly from ground level to a height of about 1 or 2 m, i.e., the field is approximately uniform. At ground level, the field vector oscillates along a line perpendicular to the ground plane like a single phase field. Thus, laboratory simulations of three-phase power line electric fields for in vitro studies can be closely approximated by using single phase electric fields in the culture medium (see 5, Exposure Systems For In Vitro Studies With Electric Fields).

Although not shown in Figure 2, the magnetic field at a point in space near a three-phase transmission line can also be represented by a rotating vector in space.

However, in contrast to the electric field, the vector B is a rotating vector even at ground level [Deno, 1976]. Therefore, laboratory simulations of three-phase power line magnetic fields for in vitro studies would require an apparatus which can generate an approximately uniform, rotating (i.e., elliptically polarized) field (see 3.1.7, Rotating magnetic fields). Nevertheless, linearly polarized magnetic fields are routinely used in bioeffect studies and can provide important information.

Because of the time variation of the magnetic field, an externally applied ac magnetic field will also induce an electric field and associated current in the body and in a culture medium. Consequently, during in vitro studies with ac magnetic fields, cells are exposed to at least three candidate exposure parameters (see note below regarding dc magnetic fields): the magnetic field, the induced electric field, and an electrical current. The dependence of these induced exposure parameters on the geometry of the exposure chamber and ac magnetic field direction is discussed further in 3.2.1, Experimental geometry and exposure parameters.

In closing this section, it is noted that for environments away from power lines, the electric and magnetic fields can be more complex [IEEE, 1991]. For example, in residential, occupational, and transportation environments, the fields can be highly nonuniform and contain harmonics of the power frequency (see 4.3, Harmonic Content Measurements) as well as other frequencies in the ELF range. In addition, there can be transient, intermittent, as well as more gradual temporal variations. While the possible biological effects of these fields are of current interest, it is also noteworthy that research unrelated to the fields in the above environments, e.g., experiments with potential medical applications, employ ELF fields. The usual practice during laboratory studies with ELF fields has been to use exposure systems that produce approximately uniform fields at a single frequency to insure that all cells are exposed to the same applied field. Experiments have also been conducted during which biological endpoints have been examined as a function of frequency [Blackman et al., 1985a] which can be of interest because of the possibility of harmonics in ambient fields. In addition, speculation that the earth's (dc) magnetic field may be a factor in the outcome of biological studies with ac magnetic fields has led to investigations with combined ac and dc magnetic fields [Liboff, 1985; Blackman et al., 1985b]. The studies with combined ac and dc magnetic fields suggest that even if the ambient dc magnetic field is not being considered as an exposure parameter, its magnitude and direction in the exposure apparatus should be recorded for possible future reference.

3. EXPOSURE SYSTEMS FOR IN VITRO STUDIES WITH MAGNETIC FIELDS

3.1 Apparatus for Generating Magnetic Fields

Several types of apparatus for producing approximately uniform ELF magnetic fields for in vitro studies are described in the technical literature. The most commonly used system is one that employs circular or rectangular loops of wire of many turns. However, Crawford cells [Spiegel et al., 1987] and flat plates [Gundersen et al., 1986; Miller et al., 1989] have also been used successfully. The latter approaches are similar in that the magnetic field is produced by sheets of current. When three parallel plates are used in a vertical stacked configuration, and appropriately energized [Miller et al., 1989], the stray fields decrease more rapidly than the more commonly used coil systems described below. This feature can be an important consideration for place-



Fig. 3. Coordinate system for Eq. (2) and geometry of rectangular loops for generating magnetic fields. The current, I, in each loop is in the same direction.

ment of sham-exposed cells and is discussed further in 3.1.5, Stray magnetic fields and shielding.

3.1.1 Magnetic field of rectangular loops of many turns. Figure 3 shows two rectangular loops consisting of many turns of wire with dimensions $2a \times 2b$ separated by a distance *s*. The z-component of the magnetic flux density at a point P(x,y,z) due to a current in the lower loop is given by the expression [Weber, 1965; Kotter and Misakian, 1977]

$$B_{21} = \frac{\mu_o}{4\pi} IN \sum_{\alpha=1}^{4} \left[\frac{(-1)^{\alpha} d_{\alpha}}{r_{\alpha} [r_{\alpha} + (-1)^{\alpha+1} C_{\alpha}]} - \frac{C_{\alpha}}{r_{\alpha} (r_{\alpha} + d_{\alpha})} \right], \tag{1}$$

where

$$C_{1} = -C_{4} = a + x \text{ and } r_{1} = \sqrt{(a + x)^{2} + (b + y)^{2} + z^{2}},$$

$$C_{2} = -C_{3} = a - x \quad r_{2} = \sqrt{(a - x)^{2} + (b + y)^{2} + z^{2}},$$

$$d_{1} = d_{2} = y + b \quad r_{3} = \sqrt{(a - x)^{2} + (b - y)^{2} + z^{2}},$$

$$d_{3} = d_{4} = y - b \quad r_{4} = \sqrt{(a + x)^{2} + (b - y)^{2} + z^{2}},$$

N is the number of turns, I is the current in amperes, μ_o is the magnetic permeability of free space $(4\pi \times 10^{-7} \text{ in mks units})$, the dimensions are in units of meters, and B_{z1} is in units of tesla (10^4 gauss = 1 tesla). The conductors in the loop are assumed to be of negligible cross section. By superposition of fields, the flux density at P(x,y,z) due to a current I in both loops, assuming the same number of turns and current direction in each loop, is

ł

$$B_{z} = B_{z1} + \frac{\mu_{o}}{4\pi} IN \sum_{\alpha=1}^{4} \left[\frac{(-1)^{\alpha} d_{\alpha}}{r_{\alpha}' [r_{\alpha}' + (-1)^{\alpha+1} C_{\alpha}]} - \frac{C_{\alpha}}{r_{\alpha}' (r_{\alpha}' + d_{\alpha})} \right], \quad (2)$$

where

$$r_1' = \sqrt{(a + x)^2 + (b + y)^2 + (s - z)^2},$$

$$r_2' = \sqrt{(a - x)^2 + (b + y)^2 + (s - z)^2},$$

$$r_3' = \sqrt{(a - x)^2 + (b - y)^2 + (s - z)^2},$$

and

$$r'_4 = \sqrt{(a + x)^2 + (b - y)^2 + (s - z)^2}.$$

It can also be shown, following steps outlined by Weber [1965], that the x- and y-components of the flux density for the two loops are given by

$$B_{x} = \frac{\mu_{o}}{4\pi} IN \sum_{\alpha=1}^{4} \left[\frac{(-1)^{\alpha+1}z}{r_{\alpha}(r_{\alpha} + d_{\alpha})} + \frac{(-1)^{\alpha}(s-z)}{r_{\alpha}^{'}(r_{\alpha}^{'} + d_{\alpha})} \right],$$
(3)

and

$$B_{y} = \frac{\mu_{o}}{4\pi} IN \sum_{\alpha=1}^{4} \left[\frac{(-1)^{\alpha+1}z}{r_{\alpha}[r_{\alpha} + (-1)^{\alpha+1}C_{\alpha}]} + \frac{(-1)^{\alpha}(s-z)}{r_{\alpha}'[r_{\alpha}' + (-1)^{\alpha+1}C_{\alpha}]} \right].$$
(4)

Figure 4 shows the variation along the z-axis of the normalized flux density, B/B(0,0,0.1), for square coils of 0.4 m side dimension and 0.2 m spacing. Because of symmetry considerations, B_x and B_y vanish along the z-axis and B_z is equal to the total flux density B. Also shown are the variations of the normalized x-, y-, and z-components of the flux density along a vertical line passing through the point (0.1,0.1,0). The corresponding numerical values are given in Table 1. For this sample calculation, B_z , which is normally regarded as the exposure field, is within 4% of B(0,0,0.1) in a central volume of 20 cm \times 20 cm \times 8 cm. The x and y components of the field in this region are each less than 3.6% of B_z (Table 1). Generally, a field nonuniformity of $\pm 5\%$ is considered very good. In some cases, it is necessary to accept variations as large as 15%.

Beginning with Eq. (1), the principle of superposition can be used to develop expressions for the magnetic flux density due to two or more rectangular loops of many turns with different values of a, b, N, I, current phase, and spacing provided that their z-axes coincide. The magnetic field produced by sets of loops with different axes can be determined by calculating the field components from each set of loops and using the principle of superposition. Orthogonally oriented sets of coils have been used to produce approximately circularly polarized magnetic fields (see 3.1.7, Rotating magnetic fields).

The magnitude and time dependence of the magnetic flux density at a point is determined by the sinusoidal current I once the dimensions and the number of turns of the loops have been fixed.



Fig. 4. Normalized magnetic flux-density profiles along z-axis and along vertical line through point x = 0.1 m, y = 0.1 m. The magnetic field is produced with two square coils of 40 cm side dimension and separated by a distance of 20 cm (see Fig. 3 for geometry and Table 1 for numerical values). The dominant magnetic field component is in the z-direction.

TABLE 1. Normalized	Flux Density	Profiles for	Two Square	Loops of	Wire With	Dimensions
40 cm × 40 cm × 20 c	m Spacing					

z(cm)	$B_z(0,0,z)/B(0,0,0.1)$	$B_z(0.1,0.1,z)/B(0,0,0.1)$	$B_{x,y}(0.1,0.1,z)/B(0,0,0.1)^{a}$
0	0.9335	1.1476	-0.0807
2	0.9637	1.1471	-0.0247
4	0.9830	1.0994	0.0128
6	0.9937	1.0377	0.0254
8	0.9986	0.9903	0.0179
10	1.0000	0.9730	0.0000
12	0.9886	0.9903	-0.0179
14	0.9937	1.0377	-0.0254
16	0.9830	1.0994	-0.0128
18	0.9637	1.1471	0.0247
20	0.9335	1.1476	0.0807

^aNegative sign indicates reversal of field direction.

3.1.2 Magnetic field of circular coils. The z-component of the magnetic flux density at a point $P(r, \theta)$, between two coils of radius b with N turns of wire separated by a distance of 2d as shown in Figure 5 is given by [Ramo and Whinnery, 1944]



Fig. 5. Geometry of coils for generating magnetic field and coordinate system for Eq. (5). The current, I, in each loop is in the same direction.

$$B_{z}(r,\theta) = \frac{\mu_{0} l b^{2} N}{2(b^{2} + d^{2})^{1.5}} \left[2 + A_{2} P_{2}(\cos\theta) r^{2} + A_{4} P_{4}(\cos\theta) r^{4} + \ldots\right], \quad (5)$$

where

$$A_2 = \frac{15d^2 - 3(b^2 + d^2)}{(b^2 + d^2)^2}$$

and

$$A_4 = \frac{15(b^2 + d^2)^2 - 210d^2(b^2 + d^2) + 315d^4}{4(b^2 + d^2)^4}$$

and μ_o is the permeability, *I* is the current, *r* and θ are the distance and angle in spherical coordinates, and $P_m(\cos\theta)$ are Legendre polynomials of order *m*. Again, the conductor cross sections are assumed to be negligible.

For the condition 2d = b, A_2 vanishes and the field becomes highly uniform in a small volume near the origin. For example, in a region |z| < (b/10) along the z-axis (i.e., $\theta = 0$), $B_z(z)$ varies from the central value by less than 0.012%. Coils that satisfy the condition 2d = b are known as Helmholtz coils. The horizontal field component can be calculated but only with some difficulty [Stratton, 1941; Nagaoka, 1921] and is not considered here. As for the case with square loops (see 3.1.1, Magnetic field of rectangular loops of many turns), the horizontal field component near the center of the coil system (r = 0) is expected to be small.

3.1.3 Magnetic field of solenoid. A solenoid can be described as N turns of



Fig. 6. Angles formed between the axis of a solenoid and lines joining a point, P, along the axis to the outer edges of the solenoid.

wire closely wound on a cylindrical form of length L and radius a as shown in Figure 6. Assuming the cross-sectional area of the wire is small compared with the area of the cylinder, the magnetic field along the axis of the solenoid is given by [Reitz and Milford, 1960]

$$B_{z}(z) = (\mu_{\alpha} N I/2L) (\cos \theta_{1} + \cos \theta_{2}), \qquad (6)$$

where θ_1 and θ_2 are both positive angles as shown in Figure 6, μ_o and *I* are as previously defined, and the *z*-axis coincides with the cylinder axis. At the center of the solenoid, Eq. (6) becomes

$$B_{z}(z = L/2) = \mu_{0} N I / \sqrt{4a^{2} + L^{2}}.$$
(7)

For a long cylinder, where L >> a, the field within the solenoid is nearly uniform except near the ends and close to the turns of wire.

3.1.4 Current sources for magnetic field coils. Power frequency current for the magnetic field coils that have been described in the above sections can be provided with a variable auto-transformer and current limiting resistor. Use of a line conditioner is desirable to reduce fluctuations of the line voltage to the autotransformer and to minimize harmonics that may be present on the line voltage. A function generator-power-amplifier combination may also be used to provide the current. The function generator allows for the production of current with ELF frequencies other than 60 Hz and normally provides isolation from fluctuations in the line voltage. Knowledge of the current-limiting resistance and measurement of the voltage across the resistor allows one to determine the current, *I*, with the use of Ohm's law. The power rating of the current limiting resistor must be large enough to prevent significant change in its resistance due to heating.

It should be recognized that as the number of turns, N, increases, there will also be an increase in stray capacitance between the windings. The equivalent circuit can be roughly modelled as an inductor, L, and a capacitor, C, in parallel which has a resonance frequency, f_r , given by $1/2\pi\sqrt{LC}$. The magnetic field coils should be operated at frequencies well removed from f_r because near resonance, some current will be shunted through the stray capacitance and not contribute to production of the magnetic field. At power line frequencies and with N equal to tens-of-turns, the resonance phenomenon is not expected to be a significant factor because of the small capacitance and inductance. However, if the coil system is operated at higher frequencies (e.g., at 3 kHz, the upper limit of the ELF range), some attempt should be

made to estimate the resonance frequency. One approach is to measure the voltage across the coil system as a function of frequency, keeping the current to the coils constant. At frequencies, f, well removed from f_r , the voltage will increase linearly with frequency, i.e., the voltage is given by $2\pi f L$ in our rough model. As f approaches f_r , the voltage increase will become more rapid because the parallel impedance of L and C will become significant. In any event, the magnetic fields produced for exposure purposes should be characterized with measurements (see 4.1, Instrumentation).

As a final note, when magnetic field coils are energized, provision should be made for the current to be turned on (and later off) in a gradual fashion to avoid producing short-lived transients in the magnetic field, i.e., the exposure field should not be turned on or off suddenly with a switch.

3.1.5 Stray magnetic fields and shielding. Equations 1 to 4 show that the magnetic field decreases as a function of distance from the field-generating loops. For example, the magnetic flux density along the z-axis for a set of square loops 0.4 m \times 0.4 m and 0.2 m spacing decreases to 0.44% of the central value (between the loops) at a distance of 1.5 m from the center of the loop. For a central flux density of 2×10^{-4} tesla (2 gauss), the stray field 1.5 m away is 8.8×10^{-7} tesla (8.8 milligauss), a value which is more than eight times the average ambient magnetic field levels reportedly found in buildings [Silva et al., 1989].

In general, it is difficult and costly to shield sham-exposed cells from stray ELF magnetic fields. Metal sheets with large permeability or high conductivity can be used for attenuating the stray fields to an "acceptable" (to be set by the researchers) level if the exposed and sham-exposed cells cannot be separated with sufficient distance. Metals with large permeabilities concentrate and confine the magnetic field lines to a degree that depends on the angle of incidence of the flux lines and the magnitude of the permeability [Corson and Lorraine, 1962]. Limited attenuation also occurs in metals of low permeability, but good conductivity, such as copper, because the varying magnetic field induces eddy currents in the metal that reduce the incident flux density. The distance, δ , into the metal at which the magnetic field is attenuated to e^{-1} of its value at the metal surface is known as the skin depth and is given for copper by the expression [Plonus, 1978]

$$\delta = 0.066/f^{0.5},\tag{8}$$

where f is the frequency of the field in Hz and δ is in meters. For 60 Hz magnetic fields, the skin depth in copper is nearly 1 cm.

Metal sheets also attenuate ac magnetic fields by a "reflection" mechanism [Bannister, 1968]. For many practical situations, this attenuation can exceed that predicted by Eq. (8). A convenient expression for predicting shielding effectiveness for simple geometries, which takes into account skin depth and "reflection" losses, has been derived by Bannister [1968].

If shielding is required, a trial and error approach using alternating metal sheets of high permeability and good conductivity followed by measurements of the residual field may be the most expeditious way to develop adequate shielding for the shamexposed cells. Theoretical treatments of magnetic shielding effectiveness with multiple sheets can also be found in the technical literature [Wadey, 1956; Thomas, 1968].

3.1.6 Parallel plates, rectangular solenoids, and stray fields. Because of

In Vitro Studies With ELF Fields: A Primer



Fig. 7. Current and magnetic field direction at some instant for parallel plate apparatus.

stray magnetic fields from the exposure apparatus and difficulty in shielding these fields from the sham-exposed cells, this section notes an experimental design that produces stray fields that decrease more rapidly with distance than the coil arrangements described above. Figure 7 shows a parallel plate magnetic field apparatus for bioeffect studies similar in principle to one reported by Miller et al. [1989]. The sheet of current in the central plate divides equally between the upper and lower plates and produces a magnetic field parallel to the plates in each half of the apparatus as shown schematically in Figure 7 at some instant. Because the fields in the two sections are in opposite directions, stray fields from the apparatus decrease more rapidly than the field from either section alone. Approximate values of the field between the plates as well as the stray fields can be calculated using the law of Biot-Savart. An accurate theoretical treatment of the problem is made difficult because skin effects make the current density in the plates nonuniform, i.e., an ac current is more concentrated along the side edges compared to a dc current. However, nearly uniform fields between the plates can readily be obtained with systems of similar design [Miller et al., 1989].

Figure 8 shows an equivalent apparatus consisting of two rectangular solenoids. The fields in each section as well as the stray fields can be calculated using the equations for rectangular loops given in 3.1.1, Magnetic field of rectangular loops of many turns, and the principle of superposition. The design shown in Figure 8 eliminates significant skin effects and requires less current than the plates to produce the same field because of the many turns of wire. As for the parallel plate version, the stray field from the "coupled" rectangular solenoids will decrease significantly faster than either solenoid alone. Stray fields have also been reduced from sets of rectangular loops of wire that have been similarly coupled by their close proximity in a quadrupole configuration [Harvey, 1987; Stuchly et al., 1991].

3.1.7 Rotating magnetic fields. As noted in Section 2, Power Line Electric and Magnetic Fields and Exposure Parameters, the magnetic field from a three-phase transmission line is a rotating field in a vertical plane at and near ground level. Circularly polarized magnetic fields have been used to simulate transmission line fields in bioeffects studies sponsored by the New York State Power Lines Project [Baum et al., 1991] and the production of such fields is considered here briefly.

Circularly polarized magnetic fields can be produced at a point by two sets of orthogonally oriented magnetic-field loops or coils provided that the field components at the point are equal in magnitude and frequency and differ in phase by 90°. If the magnetic field components can be made nearly uniform in an exposure system where the culture medium and cells are located, the cells will experience a nearly circularly



Fig. 8. "Coupled" rectangular solenoids which can reduce stray magnetic fields.

polarized magnetic field. Because the time dependence of the field components is a function of the sinusoidal currents to the coils, the 90° phase difference between the field components is obtained by phase-shifting the current to one set of coils by 90° with respect to the other set.

Figure 9 is a diagram of an apparatus which can generate a rotating magnetic field. The arrangement shown in Figure 9 is for illustrative purposes. Other arrangements such as employing more than two loops or coils along a given axis (with unequal turns) or energizing the coils with three-phase line voltage can also be used [Baum et al., 1991].

3.1.8 Systems with AC and DC magnetic fields. Recent in vitro experiments with brain tissue and diatoms [Blackman et al., 1985b; Smith et al., 1987] indicate that dc magnetic fields can affect the outcome of a bioeffects study. The production of combined ac and dc magnetic fields is straight-forward in that the field generating apparatus described earlier can be used for generating dc fields if ac currents are replaced with dc currents. The equations for predicting the fields remain the same with I(dc) replacing I(rms). Because some commercially available function generators (which drive the power amplifier for the coils) can provide sinusoidal and dc voltages simultaneously, the same coils can be used for producing both fields. Alternatively, separate coils with different sources of energization can be used.

3.2 Chamber Design

In vitro studies with ELF magnetic fields have often been conducted using circular petri dishes and rectangularly shaped vessels as chambers for cells. When the chamber containing the culture medium and cells is introduced into the magnetic field, an electric field and an associated current are induced in the liquid by the time-varying magnetic field. Thus, the cells are exposed to three candidate exposure parameters associated with the ELF field that may individually or in combination cause a biological effect, i.e., the magnetic field or flux density (B), the induced electric field strength (E), and the induced current density (J). As noted earlier, the ambient dc magnetic field may or may not be considered an exposure parameter, but in any event its magnitude and direction in the exposure apparatus should be characterized. While all the cells will experience essentially the same magnetic field, McLeod et al. [1983] have shown that the exposure to E and J will depend on the size and shape of the liquid volume, the direction of the ELF magnetic field with respect to the liquid volume, and the location of the cell in the liquid volume. Consequently, the design of the chamber for the culture medium and cells must be considered an important part of the exposure system. However, as noted earlier, to obtain valid results when exposing cells to ELF fields, dosimetric and biological considerations

In Vitro Studies With ELF Fields: A Primer



Fig. 9. Diagram of an apparatus for generating rotating magnetic fields.

must be carefully balanced. Therefore, following a discussion of exposure conditions for several experimental arrangements that have been used for in vitro studies, a careful examination is made of the biological parameters that introduce constraints in the design of the chamber.

3.2.1 Experimental geometry and exposure parameters. We consider the three experimental configurations shown in Figure 10: a cylindrical liquid volume with the ELF magnetic field parallel and perpendicular to the cylinder axis (Fig. 10a,c) and a rectangular liquid volume with the magnetic field perpendicular to one of the faces (Fig. 10b). It should be noted that the direction and magnitude of any dc magnetic field may be of significance if tests of a resonance model are being conducted, but the impact of ambient level dc magnetic fields on the induced electric field and current will not be significant. As indicated in Figure 10, the induced electric field lines and current paths are circular when the magnetic field is parallel to the cylinder axis. In general, the electric field lines and current paths form noncircular loops for the remaining two cases. The equations for the induced current density and electric field are simplest when the magnetic field is parallel to the cylinder axis. Then the current density is given by [McLeod et al., 1983]

$$J = \sigma \pi f B r, \tag{9}$$

where σ is the conductivity in units of siemens/meter, f is the frequency, B is the flux density in units of tesla, and r is the radial distance in meters from the cylinder axis. Because the current density is equal to the product σE , the induced electric field is just

$$E = J/\sigma = \pi f B r, \tag{10}$$

which we note does not depend on conductivity.

The expressions for the induced current density in the rectangular volume are more complicated and are given by [McLeod et al., 1983]

$$J_{y} \simeq i \frac{\omega \sigma B2a}{\pi^{2}} \sum_{n_{odd}} \frac{4}{n^{2}} \left[\pm \cos\left(\frac{n\pi}{2a}\right) y \right] \left(\frac{\sinh \frac{n\pi}{2a}z}{\cosh \frac{n\pi}{2a}h} \right)$$
(11)

an et al.





and

$$J_z \simeq -i \frac{\omega \sigma B 2h}{\pi^2} \sum_{m_{odd}} \frac{4}{m^2} \left(\pm \cos \frac{m\pi}{2h} z \right) \left(\frac{\sinh \frac{m\pi}{2h} y}{\cosh \frac{m\pi}{2h} a} \right)$$
(12)

where 2a is the width of the liquid volume, 2h is the depth, ω is 2π times the frequency, f, and i is $\sqrt{-1}$. The \pm sign indicates that the terms in the summations are alternatively added and subtracted. As above, the corresponding expressions for the electric field are found by dividing Eqs. (11) and (12) by the conductivity σ . As shown by McLeod et al. [1983], the case of the cylindrical volume with the magnetic field perpendicular to the cylinder axis can be treated by cutting the cylinder into

approximately rectangular volumes as shown in Figure 10c and using Eqs. (11) and (12) for each rectangular volume. The rectangular geometry is examined first in more detail.

Rectangular geometry. With the rectangular configuration indicated in Figure 10b, it can be shown, using Eqs. (11) and (12), that the induced current and electric field vanish along the x-axis as well as along the four edges parallel to the x-axis. The maximum values of the induced electric field and current density in the y and z directions occur at points along the liquid boundary. The cells will all experience the same magnetic field because the external magnetic field, which is nearly uniform, is not significantly perturbed by the chamber, the liquid, or the magnetic field associated with the induced current. However, the electric field and current density experienced by the cells can vary from zero to the maximum value depending on the location of the cell. If the region the cells occupied during exposure to the magnetic field is known, their exposure to *E* and *J* can be determined using Eqs. (11) and (12). Such information is in general not known when experiments are conducted with cell suspensions.

Our ability to describe the exposure conditions improves if the cells can be cultured on the bottom surface of the chamber. Then the vertical components of E and J vanish and approximately uniform values of E and J in the horizontal direction can be produced along the bottom surface of the liquid if the liquid depth is small compared to the width, i.e., $2h \ll 2a$. Figure 11a shows normalized values of E or J calculated at the bottom surface of the liquid volume for 2a = 6 cm and 2h = 0.2cm. It can be seen for this example that the region of approximately uniform E or Jextends almost to the side edges (i.e., within ~ 2.5 mm), where E and J rapidly decrease to zero. Increasing the depth of the liquid by 1 mm decreases the uniformity, but because the liquid depth, 2h, is still much smaller than the side dimension, 2a, the uniformity is still very good as shown in Figure 11b. While the 1 mm difference in depth has had a small effect on the uniformity of E and J in this example, the increase in magnitude of E and J is not insignificant, amounting to 50% over most of the area. This sensitivity of E and J to liquid depth for configurations with B parallel to the bottom of the chamber may be an important consideration when attempts are made to replicate the results of an earlier in vitro study. The biological significance of liquid depth is noted in 3.3.1, CO₂/O₂ tension, buffering, and pH. Researchers, when reporting their experimental conditions, should indicate the experimental configuration as well as the values of induced E and J.

It should also be noted that cells cultured on the bottom surface of the enclosure will experience an "uneven" exposure to the approximately uniform E and J values described above because a portion of the underside of the cell, which is in contact with the plastic or glass chamber, will not be exposed to the full current density or electric field in the liquid. In addition, it is expected that as the number of the cells increases to the point where cells are close to one another, there will be perturbations of E and J due to proximity effects. The first problem can be reduced if the cells can be cultured in agar that has the same conductivity as the liquid. However, the use of agar can introduce undesirable biological effects (see 3.2.2, Biological considerations in designing exposure chambers) and cause significant changes in the exposure conditions. For example, if liquid 2 mm deep covers a layer of agar 2 mm thick on the bottom surface of a rectangular chamber 3 cm wide, and if the cells are cultured near the upper surface of the agar, they will experience little or no exposure to E or J





because they are now located at the center of the conducting volume where the current density is zero.

Summarizing the above discussion, use of a rectangular chamber allows for the exposure of large numbers of cells to uniform values of B, E, and J if they are cultured on the bottom surface of the chamber. The values of E and J are sensitive to liquid depth and can be calculated using Eqs. (11) and (12). The equations for determining E and J are not elementary but can be readily evaluated with a small computer. The use of an agar substratum can significantly influence the exposure parameters and also has biological consequences.

Cylindrical geometry: Magnetic field perpendicular to axis. As noted earlier, the case of a cylindrical liquid volume with the magnetic field perpendicular to the cylindrical axis can be treated by imagining the cylinder being cut into rectangular sections and using the theory for rectangular geometry. Thus, the above remarks regarding the characterization of exposure conditions for cells in suspension and in agar, and cells cultured on the bottom of the chamber again apply for each section. It is readily shown that very good uniformity in the values of E and J can again be achieved at the bottom of the chamber when $2h \ll 2a$. As for the rectangular chamber, the magnitude of E or J is sensitive to the liquid depth. A comparison of E, J, and the



Fig. 12. Normalized values of E or J in cylindrical volume of culture medium with B parallel to cylinder axis.

rate of energy deposition when B is either linearly or circularly polarized are given in Misakian [1991].

Cylindrical geometry: Magnetic field parallel to axis. When the magnetic field is parallel to the cylinder axis, the problem of describing the exposure conditions for the cells becomes more simple. The equations describing E and J [Eqs. (9) and (10)] are elementary and indicate that both E and J increase linearly with radial distance from the cylinder axis. As noted earlier, the electric field lines and current density form concentric circular loops. Significantly, Eqs. (9) and (10) indicate that E and J are independent of liquid depth.

Figure 12 shows normalized E and J values for a cylindrical volume 6 cm in diameter. While the lack of uniformity of these exposure parameters may at first seem to be a disadvantage when characterizing cell exposure, it is possible to make use of the nonuniformity to obtain several ranges of exposure to E and J during the same experimental run [Misakian and Kaune, 1990; Bassen et al., 1992]. For example, cells can be segregated into annular sections with circular walls; the range of E and J values experienced by the cells in each section then can be determined from Eqs. (9) and (10) and the radii that define the section. The exposure geometry and design of a chamber that would be suitable for short term studies for cells in suspension are shown in Figure 13. While the cylindrical volume in Figure 13 has been partitioned into three sections, more or fewer sections could be used. For more extended periods of exposure, lasting as long as 6 days, the enclosure can be fitted with a cap and "sealed" with commercially available laboratory film which prevents significant evaporation (see 3.3.4, Humidity) but allows adequate air exchange. Vertical stacking of the enclosures is one technique for increasing the number of cells that are exposed during a single experimental run. Introducing a layer of agar at the bottom of the enclosure, as shown in Figure 13, provides for a more uniform exposure of the cells (top and underside) to E and J. Unlike the case with B parallel to the bottom of the chamber, the agar will not affect the values of E and J because E and J are independent of liquid or agar depth assuming the agar conductivity matches that of the culture medium. However, use of agar can have significant biological effects as noted below (see 3.2.2, Biological considerations in designing exposure chambers).

If the circular walls shown in Figure 13 introduce an impediment for cells plated





Fig. 13. Experimental design for short term in vitro studies with cells in suspension (a) top view. Directions of E and J are schematically indicated (b) cross sectional view of enclosure containing agar and liquid medium. The use of agar is discussed in the text. Circular walls partition the enclosure into regions of different exposure conditions.

directly on the chamber floor, it may be possible to replace the walls with concentric circles marked on the underside of the chamber. Other techniques for characterizing the radial positions of the cells include growing the cells on glass cover slips placed in the chamber or indicating the annular regions on a flat surface upon which the chamber is placed.

Because of the spatial uniformity of B and the readily determined variation of E and J with position in the chamber, the arrangement described here can be used to investigate directly questions regarding the relative significance of interaction mechanisms involving the magnetic field and/or induced electric field and current density. For example, the observation of a biological effect at the same level in all sections of the chamber would suggest a mechanism involving direct magnetic field interaction, whereas an effect that differed between sections could be indicative of a mechanism involving the induced electric field or electric field-magnetic field combination (see

3.4, Experimental Results). For the latter case, a single experimental run could provide data for a range of E or J levels.

Summarizing the above results, the cylindrical configuration with the magnetic field parallel to the cylinder axis results in induced electric fields and current densities that are easily visualized, and elementary equations for calculating E and J that are independent of liquid depth. In addition, the amount of information pertaining to dose-response during a single experimental run can be maximized, and it may be possible to distinguish between a purely magnetic field bioeffect and a bioeffect that involves the electric field or current density-magnetic field combination. The configuration just discussed does not permit exposure of large numbers of cells to the same E and J values as does the rectangular case considered earlier. The use of agar does not have significant effect on E and J values, but can have biological effects.

3.2.2 Biological considerations in designing exposure chambers. In the following discussion, we have attempted to examine some of the biological requirements for cell cultures that must be considered in addition to exposure parameters when developing and using suitable exposure chambers. As noted earlier, the biological factors that must be considered during the design of an exposure chamber include cell type and assay endpoints. Cell type affects a number of culture conditions that vary from cell to cell, including method of culture (e.g., monolayer or suspension), substratum for growth, medium constituents, pH, CO_2/O_2 tension, etc. Culture conditions are reviewed thoroughly in a tissue culture manual such as that by Freshney [1987]. For more specific applications see Jakoby and Pastan [1979] or Pollard and Walker [1990].

Assay endpoints can affect the criteria for selecting an exposure chamber and exposure conditions. For example, if cell attachment and morphology are endpoints, the optical quality of the chamber surface must be sufficiently good that the cells can be monitored by microscopy. Other important endpoints that are often monitored are cell growth rates, cloning efficiencies, metabolic activities (e.g., RNA, DNA, or protein synthesis; enzyme activities, calcium uptake, etc.), cell-cell interactions, mutation frequencies, and transformation frequencies (for a review see Cleary [1987]). Each of these endpoints places certain requirements and constraints on the exposure chamber. Some metabolic studies require direct extraction using organic solvents; this can affect substratum selection. Other endpoints require chambers with large surface areas or large numbers of cells (e.g., cloning efficiency studies, mutation frequency determinations, or transformation assays; a full account of these methods is given in Freshney [1987]). Finally, avoiding microbial contamination is critical to the outcome of all these assays.

Substrata. Several features should be considered when choosing a culture surface or substratum for use in an exposure system. These include cell yield, whether the cells will be grown as suspensions or monolayers, whether the cultures will be vented to the atmosphere or sealed, the types and form of sampling required, and cost.

Growth in suspension is usually done only with blood cell lines, ascites tumors, and cell lines especially adapted or selected for growth in suspension. Therefore, the majority of cells cultured in vitro are grown as monolayers on an artificial substratum [Freshney, 1987].

An exposure chamber for monolayer culture should have a reasonably flat surface for cell growth, and a cap that can provide a good seal (if necessary). The

parts (e.g., cap liners) should be constructed of nontoxic components. It should also be shaped to facilitate harvesting of monolayer cells by trypsinization and allow efficient washing if the chambers are to be reused. For monolayer cultures, cell yield is proportional to the available surface of the chamber. The absolute cell numbers that can be obtained are influenced by the size of the cells being grown and their sensitivity to contact inhibition. Therefore, if the chambers are small, and large numbers of cells are required for assay, the size and number of chambers required may be limiting. For this reason, suspension cultures have a significant advantage: production and harvesting of large quantities of cells may be achieved without increasing the surface area of the substratum because the yield of cells from a suspension culture can be increased simply by increasing the volume, maintaining adequate agitation, and assuring that the gas phase is mixed with 5% CO₂ in air.

Cells appear to require two types of interaction with the substratum: 1) adhesion, which allows the attachment and spreading necessary for proliferation [Folkman and Moscona, 1978; Fisher and Solursh, 1979], and 2) specific interactions between cells and a basement membrane, with extracellular matrix constituents, or with adjacent tissue cells [Auerbach and Grobstein, 1958; Finbow and Pitts, 1981]. The attachment of cells to each other and to the culture substratum is mediated by cell surface glycoproteins, Ca^{++} , and Mg^{++} ions. Other proteins, derived from the cells and serum, become associated with the cell surface [Gospodarowicz et al., 1980; Vlodavsky et al., 1980]. The surface of the substratum also serves to facilitate cell adhesion [Folkman and Moscona, 1978]. It is important to realize that the interaction of one cell with another is different from the interaction of cells with synthetic substrata. Cell-cell interactions can cause changes in morphology and reduce proliferative potential, while interactions with substrata usually do not. In fact, interaction between cell and substratum is usually necessary for cell proliferation [Folkman and Moscona, 1978].

Glass has been used as a substratum for cell culture because it has reasonably good optical properties and a negative surface charge which allows cell attachment. Glass is also inexpensive, easily washed, and can be sterilized. Cell attachment and growth can be improved by pretreating the substratum in a variety of ways [Barnes et al., 1984]. For example, used glassware supports growth better than clean glassware, probably because of surface etching or residues released by the cells [Reid and Rojkind, 1979; Hauschka and Konigsberg, 1966]. Inexpensive soda glass bottles are often more suitable than Pyrex [NIST, 1992] or other high-grade glass that contains lead. For complete details on preparation and handling of glass bottles for tissue culture purposes see Freshney [1987].

Cells can also be grown on a wide variety of other artificial surfaces, including polyvinylcholoride (PVC), polycarbonate (PC), polytetrafluoroethylene (PTFE), thermanox (TPX), and a number of other plastics of which polystyrene (PS) is the most common [Freshney, 1987]. Plastics such as polystyrene make excellent substrata for cell culture. They have good optical quality and flat surfaces, and generally give results that are uniform and reproducible. However, manufactured polystyrene is hydrophobic and does not provide a suitable surface for cell attachment. Therefore, in order to support cell attachment, these plastics must be treated with gamma radiation, UV irradiation, chemicals, or an electric arc to produce a charged surface which is wettable. Since the resulting products may vary in quality among the different manufacturers, it is important to test and compare growth rate and plating efficiencies under low serum growth conditions as described by Freshney [1987].

Poor adhesion or overcrowding of cells inhibits cell proliferation. Cells that require attachment for growth are called anchorage dependent. Cells that have been transformed may become anchorage independent and may grow when stirred or held in suspension with a semisolid medium containing agar or methylcellulose.

If measurement of changes in the cell proliferation rate is not the objective of the study, cells can be grown attached to each other, either as spheroids in suspension or as a secondary layer on top of a confluent monolayer. While these cells may proliferate more slowly than under standard conditions, their metabolic processes may reflect more accurately their behavior in vivo. Specifically, they may exhibit normal differentiated cell functions.

During exposure to magnetic fields, it is important to recognize that attachment of the cell sometimes may be undesirable because of the difficulty that cell attachment can introduce in determining exposure to current. For example, the uneven exposure of attached cells to the current density was briefly noted in 3.2.1, Experimental geometry and exposure parameters. The principles involved are prevention of attachment on the floor of the chamber, minimization of cell spreading, and immobilization of cells. Because the cells are approximately spherical and essentially in suspension, dose calculations are simplified. The agents most often used are agar, agarose, or methylcellulose. The first two are gels and the methylcellulose is a high viscosity sol. The cells will slowly pass through the sol, so it should be used with an underlay of agar.

It is well known that the physiological condition of cells contained in an agar gel affects their colony-forming ability and inhibits cell growth. In most agars, this selectivity of agar is due to the inhibitory effects of acidic and sulfated polysaccharides on normal cell multiplication [Neugut and Weinstein, 1979]. In addition to attachment, normal cells also require space to extend cellular projections on a solid substratum in order to undergo cell division. Agar medium in which the polyanions have been complexed with DEAE-dextran can support cell extension and allow at least limited multiplication of cell lines that cannot grow in untreated agar. Agarose, which is agar with most of the large charged molecules removed, also supports the growth of some cells. Conversely, an increase in the concentration of polyanions by the addition of dextran sulfate reinforces the inhibitory effect of the agar gel [Montagnier, 1968]. Tissue culture agar may also lead to an increased number of chromosome breaks and sister-chromatid exchanges in the cultured cells [Cohen et al., 1988].

Washing agar can eliminate many of these effects. In addition to removing soluble toxins, the washing process causes the agar to swell properly which results in the formation of a uniform gel after autoclaving. Both washed and unwashed agar should be evaluated before use, by testing their effects on the growth rate and plating efficiency of cells in semisolid agar at various dilutions.

Different cells respond quite differently to different substrata. For example, polyacrylamide layers allow the cloning of tumor cells but not normal fibroblasts [Jones and Haskill, 1973, 1976], and transformed cells proliferate on PTFE while most others will not; macrophages will also attach to PTFE but they do not proliferate [Parenjpe et al., 1975]; and collagen gels favor the proliferation of epithelial cells [Lillie et al., 1980]. Transformed cell cultures demonstrate reduced anchorage de-

pendence for cell proliferation. The selective effect of substrata (e.g., glass, plastics, agar, etc.) on growth may depend on differential rates of both attachment and growth, although in practice the two are indistinguishable.

Selection of a substratum for cell growth also depends on the methods used for sampling and analysis. If processing of the sample involves extraction in certain organic solvents, then polystyrene cannot be used.

Preparation and sterilization. All apparatus in direct association with the culture medium must be thoroughly cleaned. New materials (e.g., silicone tubing, stoppers, etc.) should be soaked in detergent overnight, thoroughly rinsed, dried, and sterilized. Used items should be rinsed in tap water and immersed in detergent immediately after use (not allowing protein to dry on the surfaces) and then processed as if they were new material. It is particularly important that silicone be completely removed using an appropriate solvent before the standard cleaning process is started. A tissue culture manual such as Freshney [1987] should be consulted for complete cleaning, washing, and sterilizing protocols.

Preparation and sterilization of glassware for cell growth requires not only a clean surface for growth, but the surface must also carry the charge that will allow cell attachment. Caustic alkaline detergents render the surface unsuitable for cell attachment. The necessary charge can be restored by an acid treatment [Freshney, 1987]. There are several products available for cleaning glassware without destroying the charge on the surfaces.

Used glassware must not be allowed to dry out! Immediately after use, the soiled glassware should be put in a sterilizing agent such as 0.1% sodium hypochlorite to prevent microbial contamination. After removing the glassware from the bleach, it should be rinsed five times with tap water or deionized water. It should then be washed in a nonionic detergent, rinsed thoroughly five times in deionized water, five times in distilled water, inverted, dried, and sterilized using dry heat or an autoclave that uses distilled or deionized water. If the autoclave is connected to a common steam line, toxic residues can be deposited on the surface of the glassware [Freshney, 1987].

Since microbial contamination invalidates any results from the affected cultures, care must be taken in sterilization of all materials that come into contact with the cells. Sterilization methods depend on the type of material being processed. Tissue culture handbooks that provide guidance for sterilizing most items are available [Freshney, 1987]. Plastic tissue-culture dishes can also be washed, sterilized, and reused for certain culture applications. Cell morphology, attachment, and proliferation kinetics in the rewashed dishes are similar to those of new dishes. However, the lag phase (see 3.3.7, Normal growth and sampling) can be two times longer and the cloning efficiency is decreased by about 50%. Most plastics cannot survive the temperatures required for autoclaving or dry heat sterilization. Therefore, alternative procedures must be employed. There are four methods for sterilizing these items: 1) immersion in 70% alcohol for 30 min, followed by drying under sterile conditions (e.g., uv light in a laminar flow cabinet); 2) gamma-irradiation at 100,000 rad; 3) exposure to ethylene oxide; items so exposed cannot be used for 2 to 3 weeks following treatment because of residual ethylene oxide on the plastic surfaces; and 4) microwave sterilization, involving irradiating the plastic three times with the power selector on high. Each heat cycle involves 10 s with the power on followed by a 10 min interval without irradiation, in order to avoid excessive heating and damage to the plastic dishes. A beaker of water in the microwave is sometimes used as a load on the microwave generator.

Detection of toxicity. There are three main types of tests that can be used to determine if there are cytotoxic effects due to the chamber: 1) plating efficiency, 2) growth curve at regular passage densities and up to saturation densities, and 3) expression of special function (e.g., differentiation in the presence of an inducer, expression of a specific antigen). The values obtained with the exposure chamber should be comparable to those obtained under normal cell-culture conditions. The plating efficiency should be examined for a range of serum concentrations since serum may mask toxic factors in the medium.

Cloning efficiency tests are excellent for detecting cytotoxic or inhibitory factors associated with exposure chambers. Isolated cells require a greater range of nutrients at low densities since cell-derived diffusible signals or conditioning factors that are present in high-density cultures are absent or too dilute at low densities. In other words, while the intracellular metabolic pool of a leaky cell in a dense population will soon reach equilibrium with the surrounding medium, that of an isolated cell will not.

Morphology can also be used to determine whether the cells are being subjected to stress. Signs of morphological changes include rounding and detachment of cells, cytoplasmic vacuoles, granularity around the nuclei, etc. However, these changes can also result from an inadequate or toxic medium, contamination, or aging of the cell line. All of these cytotoxicity tests are thoroughly described and explained in Freshney [1987].

3.2.3 Some notes on exposure chambers and their fabrication. A discussion of the relations between some commonly used experimental geometries and their impact on exposure conditions was given in 3.2.1, Experimental geometry and exposure parameters. An important geometrical factor was the shape and size of the culture medium. Because these parameters are determined in large part by the chamber, some comments regarding the commercial availability of chambers and their fabrication are given here. Commercially available flasks with a range of sizes are available as chambers for the rectangular geometry shown in Figure 10b. Large rectangularly shaped flasks are useful for studies requiring large numbers of cells or large cell-growing surface areas (e.g., cloning efficiencies, mutation assays, and transformation assays). If such vessels are used, the depth of the culture medium should be carefully noted in order to determine the values of the induced current and electric field as given by Eqs. (11) and (12) [McLeod et al., 1983; Bassen et al., 1991].

Commercially available plastic culture dishes with cylindrical geometries are also available. Use of such vessels has some dosimetric advantages as noted in 3.2.1, Experimental geometry and exposure parameters, but there are limits on the duration of the experiments that can be performed. Annular sections, as shown in Figure 13, can be formed by gluing small cylindrical vessels within larger ones. However, the biological effects of the adhesive must be carefully evaluated. The chamber should also be able to withstand repeated cleaning if it is to be reused.

Since the chamber is an important part of the exposure system, a case can be made for the added expense of fabricating large cylindrical chambers with several annular sections in place of the commercial "off the shelf" vessels. If this approach

is taken, the biological considerations discussed in the previous section should be carefully examined before their use.

3.3 Other Biological and Physical Considerations

3.3.1 CO_2/O_2 tension, buffering, and pH. The significant gas-phase constituents for cell culture are oxygen and carbon dioxide. Cells vary in their oxygen requirements. Most cell cultures prefer atmospheric or lower oxygen tension [Cooper et al., 1958; Balin et al., 1976]. In serum-free medium, the oxygen tension should be carefully controlled during incubation.

In addition to possibly influencing the exposure parameters (see 3.2.1, Experimental geometry and exposure parameters), the depth of the culture medium can affect the rate of oxygen diffusion to the cells. It is advisable to keep the depth within the range of 2 to 5 mm ($0.2-0.5 \text{ ml/cm}^2$) in static cultures. If the depth of the medium is more than 5 mm, then gaseous diffusion can become growth limiting. With monolayer cultures in exposure systems, this is difficult to overcome; suspension cultures merely need mixing.

The role of carbon dioxide is complex because of the interactions of dissolved CO_2 and HCO_3^- concentrations and pH level. The atmospheric CO_2 tension regulates the concentration of dissolved CO_2 directly as a function of temperature. This, in turn, produces H_2CO_3 , which dissociates to H^+ and HCO_3^- . Since HCO_3^- has a fairly low dissociation constant with most available cations, it tends to reassociate, leaving the medium acidic. As a result of increasing atmospheric CO_2 , the pH is depressed. To counter the effect of elevated CO_2 tension, the bicarbonate concentration is increased, causing the equilibrium to shift so that less HCO_3^- is in solution, resulting in an appropriate pH [Freshney, 1987].

When HEPES buffer was first introduced, there was some speculation that CO_2 would no longer be required for cell growth, since it was no longer required to stabilize pH. This has been proven untrue, at least for a large number of cell types. The need for CO_2 is most obvious at low cell concentrations. Carbon dioxide is essential to obtain maximum cloning efficiency for most cells. While eliminating dissolved CO_2 from the medium appears to limit cell growth, it is not clear whether cells require dissolved CO_2 , HCO_3^- , or both [Itagaki and Kimura, 1974].

In summary, cell cultures at low concentrations in an open vessel need to be incubated in an atmosphere of air and CO_2 . The concentration of CO_2 selected should be that which equilibrates with the sodium bicarbonate in the medium. For most cultures at very low cell concentrations it is necessary to add CO_2 to the gas phase of sealed chambers. At high cell concentrations, that will not be necessary. In open dishes, on the other hand, CO_2 may be required. In cultures that produce large quantities of acid, and high endogenous production of CO_2 , it may be necessary to loosen the cap of a culture flask or chamber to allow the excess CO_2 to escape. Culture flasks usually have loose-fitting lids to allow the excess CO_2 to escape in excessive acid-producing cell lines.

Where CO_2 is a problem, it is advisable to incorporate HEPES in the medium to stabilize the pH. HEPES is a much stronger buffer in the pH 7.2 to 7.6 range than bicarbonate and is now used extensively. When HEPES is used with exogenous O_2 , the HEPES concentration must be more than double that of the bicarbonate for adequate buffering. HEPES is the most effective buffer in the pH 7.2 to 7.8 range, and TRICINE is most effective in the pH 7.4 to 8.0 range. However, caution must be exercised when HEPES concentrations greater than 20 mM are used because of possible toxicity effects [Spierenburg et al., 1984]. Most cells grow well at pH 7.4, although the optimum pH for cell growth can vary with different cells. Fibroblasts prefer a pH between 7.4 and 7.7, transformed cells grow best between 7.0 to 7.4, and some epidermal cells will grow at a pH level as low as 5.5. For an extensive discussion on pH, O_2/CO_2 , and buffering of cultures see Chapter 7 of Freshney [1987].

Small differences in environmental pH have pronounced effects on the in vitro propagation of mammalian cells as well as on various experiments employing cells maintained in vitro. These effects include changes in the production of cell-directed proteins. Thus, the reproducibility of experimental observations of altered gene expression may reside with the ability to monitor and adjust the pH of the medium under conditions which do not alter the physiological state of the cells. This poses a technical problem when cultures are incubated with small volumes of medium. Furthermore, cultures at high cell concentrations use up medium faster and change the pH more rapidly than cultures containing cells at low concentrations.

3.3.2 Temperature. Cultured cells can tolerate considerable drops in temperature. They can survive several days at 4 °C, but they cannot tolerate temperatures more than 2 °C above their normal growth temperature for more than a few hours. Most mammalian cells die rapidly at temperatures of 40 °C and above. Regulation of temperature in experiments should be kept within ± 0.2 °C, but it is even more important to be consistent. Cells will grow quite well between 33 °C and 39 °C, but they will vary in growth rate and metabolism. The incubation temperature should be constant both in time and at various locations in the incubator. Air should be circulated by a fan to give a more even temperature distribution in the incubator. If magnetic field shielding (see 3.1.5, Stray magnetic fields and shielding) is introduced in the incubator, it should be positioned to minimize perturbation of the circulating air currents. Continuous monitoring of the temperature at several locations in the vicinity of the chamber(s) as well as mapping the temperature profile in the incubator is strongly urged. See Freshney [1987] for a full discussion of incubation temperature. Continuous monitoring of the temperature permits the detection of temperature changes due to unexpected events, e.g., short term interruption of power to incubator.

It must be recognized that the systems described earlier for producing magnetic fields also can act as a heat source, the magnitude of which depends on the current to the coils or plates. The source of heat will therefore depend on the applied magnetic field. As an engineering note, the current carrying capacity of insulated wire in the magnetic field coils will have to be reduced from the value specified for use in air by the manufacturer because of the "bundled" configuration [Jordon, 1986].

A check should also be made for vibrations from the coils when the coils are operated at the maximum magnetic field level and whether the vibrations can couple to the exposure chamber(s). Vibration measurements can be made with commercially available accelerometers provided that the transducer can be isolated from the magnetic field (transducer function will be affected by ac magnetic fields [Stuchly et al., 1991]). What can be regarded as an acceptable level of vibration for the exposure chamber is unknown at this time. It is not uncommon for researchers to report that vibrations of the exposure chamber were not detectable by touch.

In addition to its direct effect on cell growth, temperature also influences pH due to the increased solubility of CO_2 at lower temperatures and, possibly, because

of changes in ionization and the pK_a of the buffer. When preparing culture medium, the pH should be adjusted to 0.2 pH units lower than desired for culture because the medium is prepared at room temperature and the cultures are grown at 36.5 °C. To evaluate the pH at cell culture temperatures when making up a medium for the first time, it is advisable to make up the medium complete with serum and incubate for a sample overnight at 36.5 °C under correct gas tension. Measurements of pH should be made before and after the incubation period to insure that the desired level is maintained.

During experiments with electric and magnetic fields, temperature can be extremely important, especially when sham-exposed cultures are in different incubators. Significant effects can result from 0.5 to 1.0 °C differences in incubation temperature (see also 3.3.10, Incubators). The importance of temperature as well as temperature history on field induced alteration of calcium ion release from in vitro brain tissue was recently reported by Blackman et al., [1991].

It is readily shown that heating effects in the culture medium due to induced currents (see 3.2.1, Experimental geometry and exposure parameters) will be negligible. In addition, magnetic fields associated with the induced currents are negligibly small [Polk, 1990].

3.3.3 Osmolality. Most cells have a fairly wide tolerance for osmotic pressure. Osmolalities between 260 mOsm/kg and 320 mOsm/kg are usually acceptable. Therefore, if evaporation will occur during culture (e.g., in Petri dish culture), it is better to use slightly hypotonic medium. It is important to monitor osmolality if alterations are made in the constituents of the medium (e.g., addition of HEPES, drugs, or other components that require neutralization) [Waymouth, 1970].

3.3.4 Humidity. The incubator should be well humidified (95 to 100%) if cultures are to be maintained for more than a few hours. Evaporation of the medium from loosely capped exposure chambers can significantly affect conductivity and induced current densities [see Eq. (9)]. It will also alter osmolality of the solution and severely impair cell proliferation. This evaporation effect is readily observed as a positional effect on growth rates or cloning efficiency of cells cultured in multiwelled plates. The outermost chambers yield lower cloning efficiencies and slower growth rates than the interior wells. To avoid this problem, high humidities must be maintained. Another method for addressing this problem is to fill the outer wells only with sterile medium or distilled water. This provides protection for the cells grown in the inner wells. The type of artifact described here could result from using the chamber shown in Figure 13 for long-term exposure studies. Comparing the results from the inner and outer wells of the sham exposed cultures would be one method for detecting the artifact.

3.3.5 Viscosity. Viscosity becomes important only after trypsinization or other manipulations which may damage cell membranes thus making them more sensitive to changes in viscosity. The viscosity of cell culture medium is influenced mainly by the serum content and, in most cases, has little effect on cell growth. However, viscosity should be monitored closely at low serum concentrations or in the absence of serum. Medium viscosity can be increased by the addition of carboxy methylcellulose or polyvinyl pyrolidone [Birch and Pirt, 1971; Freshney, 1987].

3.3.6 Surface tension. Although the physiological effects of foaming are not clear, foaming may affect protein denaturation; it also increases the risk of contamination [Freshney, 1987].

29



Fig. 14. Diagram of growth curve for cells showing the lag, log, and stationary or plateau phases.

3.3.7 Normal growth and sampling. The phase of cell growth also may be an important consideration when cells are exposed to magnetic fields [Aaron and Ciombor, 1991; McLeod et al., 1992]. The cell growth cycle is divided into the lag, log, and stationary (or plateau) phases as shown schematically in Figure 14. The lag phase is a period of adaptation, during which the cell synthesizes the components necessary to repair any damage caused during transfer (trypsinization, etc.), attachment to the substratum, and spreading outward. During this spreading, the cytoskeleton reappears, and its reappearance is probably an important part of the spreading process. Enzymes being synthesized include DNA and RNA polymerases, which are rapidly followed by synthesis of new DNA, RNA, and structural proteins. Once this process begins, specialized cell products may disappear and not reappear until cessation of cell proliferation at high cell density.

The log phase is a period of exponential increase in cell numbers, which continues until one or two divisions after confluence is reached. This is the most reproducible aspect of the culture, and it is the optimal time for sampling the population, because it is quite uniform and the viability is high. Toward the end of the log phase the culture becomes confluent, the growth rate of the cells decreases and often cell proliferation nearly ceases. Normal cells stop dividing at high cell concentrations due to depletion of growth factors and other key metabolites. They remain blocked at the G1 phase of the cell cycle, but they can be maintained for 2 to 3 weeks without much loss of viability. Transformed cells, continuous cells, and some other cell types deteriorate rapidly at these same cell densities unless the medium is changed daily or unless they are subcultured. During the stationary phase, there is usually an increase in the synthesis of specialized versus structural proteins, and the constitution and charge of the cell surface can change [Freshney, 1987].

3.3.8 Initial cell density. Cells should be cultured at initial cell densities that will allow the culture to be in log phase (see 3.3.7, Normal growth and sampling) at the time of sampling unless some specialized differentiated function is being studied.

3.3.9 Light. Cells should be cultured in the dark and should be exposed to room lighting as little as possible since visible light has been shown to cause the deterioration of culture medium [Wang, 1976]. Incandescent lighting is preferable to fluorescent lighting as it causes less degradation of medium components. Exposure to fluorescent lights also can increase mutation frequency by increasing the concentration of hydrogen peroxide and related free radicals [Sanford et al., 1986].

3.3.10 Incubators. Incubators have been discussed in 3.3.1 to 3.3.4 (CO_2/O_2 tension, buffering, and pH; Temperature; Osmolality; and Humidity) in connection with CO_2/O_2 tension, pH, temperature, and humidity. It should also be noted that probably all commercially available CO_2 incubators have background gradients of ac magnetic fields ranging from a few tenths of microtesla (a few milligauss) to approximately 1×10^{-4} tesla (1 gauss), and which can be time dependent. These variations depend on location in the incubator and the distance to the sources (e.g., motors, solenoids, and heating elements). Incubators should be modified for bioeffect studies with ac magnetic fields so that these background fields are reduced to what would be considered negligible levels. This can be accomplished by removing to external locations such things as motors for fans, adding shielding (see "Temperature"), redesigning electrical components such as solenoids or heating elements or substituting low field components.

Because ferromagnetic materials are typically used in the construction of incubators, the incubator will likely affect the ambient dc magnetic field. Thus, dc magnetic field values measured outside an incubator probably will not be representative of values inside the incubator. It should also be recognized that the use of ferromagnetic materials in building construction can significantly perturb the local geomagnetic field. In addition, residual magnetism in building materials after welding [Suzuki et al., 1978], forming, or machining [Kihara et al., 1984] could make measurable contributions to the dc magnetic field in a room. Permanent magnets which can influence the ambient dc magnetic field also can be found in such common laboratory objects as magnetized tools, magnetic door latches, universal motors, and magnetic field should be measured in the exposure system at the location of the dc magnetic field should be measured in the exposure system at the location of the chamber (measurements in air will be equal to those made in the culture medium because of the nonmagnetic nature of culture medium) and reported.

3.3.11 Incidental magnetic field exposure. Because research laboratories typically have a variety of electrically driven appliances or equipment that produce stray magnetic fields, it is necessary to characterize and report ambient magnetic field levels that cells and tissue experience during preparation. In addition to apparent magnetic field sources such as magnetic stirrers, freezers, centrifuges, and incubators, researchers should be aware of unseen sources such as transformers in adjacent rooms or large current-carrying conductors in walls, floors, or ceilings. Obviously, exposure of cells and tissue to significant levels of ambient magnetic fields will compromise the results of any exposure study with ELF magnetic fields.

3.4 Experimental Results

In this section, some of the earlier topics that were discussed are brought together by examining briefly an in vitro study with ELF magnetic fields. As noted in "Experimental geometry and exposure parameters," by using a cylindrical exposure vessel with annular rings, it is possible to expose cells to one magnetic field level



Fig. 15. (a) Exposure system used by Greene et al. [1991] consists of solenoid surrounded by mu-metal shield that is placed inside a CO_2 equilibrated water-jacket incubator. Cell cultures are in ten 60 mm diameter dishes. (b) Profiles of measured magnetic flux density and induced electric field strength as function of distance from center of culture dish. The electric field was induced by a 1 millitesla (10 gauss) magnetic field. Redrawn with permission from Academic Press.

while simultaneously exposing them to a range of electric field and current density values. It is recalled that such an arrangement allows the investigation of the relative importance of B or the induced exposure parameters, E or J, on the outcome of the experiment. Using an annular ring approach, Greene et al., [1991] have examined the effects of the electric and magnetic fields on transcription in human HL-60 cells. The exposure system as well as examples of the magnetic field and induced electric field profiles used by Greene et al. are shown in Figure 15.

Exposure of the cells in an outer annular ring to a 60 Hz sinusoidal magnetic field of 1.0 millitesla caused a transient increase in the rate of total RNA synthesis, as measured by the incorporation of ³H-uridine into RNA during a 15 min exposure. Transcription rates increased to a maximum of 50–60% above control levels at 20–120 min of exposure and declined to control levels by 18 h. This enhancement was determined to be due largely to the induced electric field because simultaneous exposure of cells in an inner annular ring showed less enhancement in the total rate of RNA synthesis. Figure 16 shows the percent of enhancement in ³H-uridine incorporation over that for corresponding control cultures, for cells in the outer (open circles) and inner (closed circles) annular rings. The reader should consult Greene et al. [1991] for further details of the experimental conditions and results.

It should be noted that under similar exposure conditions, Krause et al. [1991] observed no differences in the expression of the specific messages for c-myc, betaactin, and histone H2B when total cellular RNA was extracted from exposed cells, gel



Fig. 16. Comparison of ³H-uridine incorporation for cells in outer (open circles) and inner (closed circles) annular rings following exposure to a 60 Hz 1 millitesla magnetic field. The cells in the outer annular ring experienced induced electric fields near 3.4 mV/m and cells in the inner annular ring experienced induced electric fields near 0.34 mV/m. The data are presented as percent enhancement over corresponding control cultures [Greene et al., 1991]. Reprinted with permission from Academic Press.

electrophoresed, northern blotted to nylon membranes, and probed using ³²P-labeled cDNAs. Dot-blot analysis also did not detect any change in the expression of these mRNAs.

4. MEASUREMENT OF MAGNETIC FIELDS

A brief discussion is provided here on measuring ELF and dc magnetic fields and calibration procedures for the instrumentation. Techniques for measuring the induced electric field are more complicated and are discussed in Section 6, Measurement of Electric Fields Of In Vitro Systems. If the experimental geometry matches one of the configurations discussed in 3.2.1, Experimental geometry and exposure parameters, the induced electric field can be calculated [e.g., Eq. (10)] once the magnetic field is determined. Similarly, if the conductivity of the culture medium can be measured, the induced currents can also be calculated. As noted previously, measurement of the magnetic field in air at the location where the chamber(s) will be positioned in the exposure system will also be indicative of the field in the culture medium because of the nonmagnetic nature of the liquid.

4.1 Instrumentation

Sinusoidal magnetic fields can be measured with a field meter consisting of an electrically shielded coil of wire (probe) and portable voltmeter-detector shown schematically in Figure 17. The principle of operation is based on Faraday's Law which predicts that a voltage is produced at the ends of an open loop of wire placed in a

In Vitro Studies With ELF Fields: A Primer



Fig. 17. Magnetic field probe and voltmeter detector.

changing magnetic field. Specifically, the instantaneous voltage, V_s , is equal to the negative of the time rate-of-change of the magnetic flux, B_sA , through the loop

$$V_s = -d[B_s A]/dt, \tag{13a}$$

where B_s is the time-varying magnetic flux density (assumed to be uniform) perpendicular to the area, A, of the loop. For a sinusoidal magnetic field, B_s , given by B_o sin ωt , and a loop of N turns of wire, the rms voltage, V, is

$$V = 2\pi f NAB, \tag{13b}$$

where ω is equal to $2\pi f$, and f is the frequency of the magnetic field. The minus sign is dropped because rms values are assumed for V and B. It is also assumed that the magnetic field due to the induced current in the probe (after the probe is connected to a voltmeter) and the stray capacitance associated with the probe are negligible. Stray capacitance combined with the probe's inductance and resistance can lead to, when connected to the detector, output voltages that are a function of frequency but differ significantly from that predicted by Eq. (13b) [Beiersdorfer and Clothiaux, 1983]. Equation (13b) predicts that the value of V produced by a coil of 500 turns and with an area of 10^{-2} m² when placed in a field of 1×10^{-4} tesla (1 gauss) is 0.188 V. The coil probe and detector should be well shielded to prevent electrical pick-up.

If measurements of exposure fields of different frequencies are to be performed, the simple coil probe-voltmeter detector shown in Figure 17 should be replaced with a magnetic field meter which has a nearly constant response (rms indication) as the frequency of the magnetic field (with fixed magnitude) is varied over the frequency range of interest. This "flat" frequency response is obtained by incorporating a stage of integration in the detector circuit and using a probe for which the ratio of induced voltage [Eq. (13b)] to output voltage remains constant as a function of frequency over the same frequency range. The stage of integration is required in order to accomplish the inverse mathematical operation performed by the probe, i.e., differentiation [Eq. (13a)]. The response of the probe-detector combination then becomes independent of magnetic field frequency. Further details of magnetic field meters with coil probes that have a flat frequency response are given in Fulcomer [1985].

Coil-type magnetic field meters with different frequency responses can be fabricated and are also commercially available. Some Hall-effect magnetic field meters which are commercially available can also be used for ELF magnetic field measurements. However, Hall-effect meters tend to have insufficient sensitivity for measure-

ment of ambient $(10^{-7}$ tesla) flux densities. Measurements of small ac magnetic fields ($<0.5 \times 10^{-4}$ tesla) with Hall-effect field meters may also be in error because the probe responds to the total magnetic field, including the earth's dc field.

Hall-effect meters can be used for measuring dc magnetic fields, but care should be exercised if ac magnetic fields of comparable magnitudes are also present because, as noted above, the probe will respond to the total magnetic field and possibly cause a measurement error.

In recent years fluxgate magnetometers which previously had been used for only dc magnetic field measurements have been adapted for measuring both ac and dc fields.

4.2 Calibration of Magnetic Field Meters

Calibration of a magnetic field meter is typically performed by introducing the probe into a nearly uniform magnetic field of known magnitude and direction. Helmholtz coils (see 3.1.2, Magnetic field of circular coils) have frequently been employed to generate such fields, but the more simply constructed single loop consisting of many turns with rectangular geometry can also be used. The simplicity in construction is at the expense of reduced uniformity, but sufficient accuracy is readily obtained. The z-component of the magnetic flux density of a rectangular loop is given by Eq. (1). It is noted for purposes of reference that the magnetic field at the center of a square loop of side dimension 2a is $\mu_a IN \sqrt{2}/\pi a$.

Equation (1) has been used to calculate the field values at and near the center of a square loop of dimensions $1 \text{ m} \times 1 \text{ m}$. Figure 18 shows the percentage of departure from the central magnetic field value at nearby points in the plane of the loop and 3 cm above and below the plane of the loop (indicated in parentheses). Also shown in Figure 18 is an outline (scale drawing) of a magnetic field probe 10 cm in diameter. For this probe, the calibration field is within 1% of the central value. Uncertainty in the values of I and a will combine with the 1% uncertainty associated with the nonuniformity. Increasing the size of the loop increases the region of uniformity and decreases the amount of uncertainty associated with the calibration. A 1% change in loop dimension changes the value at the probe by about 1%. Calibrations should be performed with sinusoidal current at the same frequency planned for the exposure studies.

4.3 Harmonic Content Measurements

The harmonic of a sinusoidal magnetic field of frequency f_o , sometimes referred to as the fundamental frequency, is itself a sinusoidal field having a frequency equal to an integral multiple of f_o . For example, if the harmonic has a frequency of three times f_o , it is referred to as the third harmonic. Harmonics may occur because of distortions in the current waveform produced by the power supply for the magnetic field coils. The harmonic content in the magnetic field is characterized by determining the magnitude and order (harmonic number) of the harmonics that are present (see below) [Jay, 1984]. The expression "total harmonic distortion" which is also used to describe the harmonic content is defined as the ratio of the rms value of the harmonic content in the field to the rms value of the total field [IEC, 1983].

To reduce the possibility of attributing biological effects that may occur at frequencies other than that intended for use in the experiment, the harmonic content

In Vitro Studies With ELF Fields: A Primer

35



Fig. 18. Enlarged central region of square loop $1 \text{ m} \times 1$ m showing scale drawing of magnetic field probe 10 cm in diameter. The percentage departure of B_z from $B_z(0,0,0)$ is indicated at representative points in the plane of the square loop and at points 3 cm above and below the plane of the loop (parentheses). The departure, in percent, from the central value $B_z(0,0,0)$ at each point is determined using Eq. (1).

in the magnetic field should be checked. When determining an acceptable level of harmonic content, it should be noted that the current induced in the culture medium by a particular harmonic field component will be weighted by its harmonic number. The weighting occurs because the induced current will be proportional to the time derivative of the magnetic field. This result is also implied by Eqs. (9) to (13), i.e., the induced current, electric field, and voltage are proportional to frequency. If, for example, the magnetic field, B_t , contains 3% third harmonic, i.e., $B_t = B_o \sin \omega t + 0.03 B_o \sin 3\omega t$, the induced current (and associated electric field) in the culture medium due to the third harmonic field component will be 9% of the induced current due to the fundamental frequency.

This result is shown by considering the expression for induced current density, Eq. (9), with the presence of the third harmonic in the magnetic field, before the rms value is considered. For the above example, there will be a second term on the right hand side of Eq. (9), due to the 3rd harmonic field component, $0.03 \times 3\sigma\pi fB_o r \cos 3\omega t$, i.e.,

 $J = \sigma \pi f B_o r \cos \omega t + 0.03 \times 3 \sigma \pi f B_o r \cos 3 \omega t,$

where the factor 3 and the cosines occur because a time derivative of the magnetic field must be taken to obtain the induced current or current density. The second term is 9% of the current density due only to the fundamental frequency, as indicated above. It is interesting, however, that there is very little difference between the rms

value of the total current density with and without the presence of the third harmonic in the magnetic field, i.e., it can be shown that the difference amounts to only 0.4%.

If the harmonic distortion in the magnetic field is of the order of 2 to 3% or greater, the magnetic field probe described in 4.1, Instrumentation, very often can be used with an oscilloscope to verify the presence of the harmonics in a semiquantitative fashion. This verification is possible because the distortion of a sinusoidal waveform is discernible visually by the presence of 6% of the third harmonic and lesser amounts of the higher order harmonics when simultaneously compared with a pure sinusoidal waveform. Because the portion of the signal from the magnetic field probe due to harmonics will be enhanced [i.e., V is proportional to frequency, Eq. (13)], it will be possible to detect 2 to 3% third harmonic *in the field* when the signal is viewed on an oscilloscope.

The time derivative waveforms of magnetic fields of order 10^{-4} tesla (1 gauss) can usually be observed by connecting the coil probe directly to an oscilloscope, because of the relatively large voltages induced by the magnetic field [Eq. (13)]. If a quantitative measure of the harmonic content in the magnetic field is desired, the waveform can be decomposed into its harmonic components by replacing the oscilloscope with a spectrum or waveform analyzer. However, proper accounting should be made of the weighting of each harmonic component.

5. EXPOSURE SYSTEMS FOR IN VITRO STUDIES WITH ELECTRIC FIELDS

The design of systems for exposure of cells and tissues to electric fields must address several vexing problems that can not always be solved to total satisfaction. The most frequent problems arise in achieving field uniformity over the volume occupied by cells or tissues, in maintaining a constant field strength throughout the duration of an experiment, and in monitoring and measuring fields. Most electric field exposure systems involve an electric current between electrodes in a conducting saline medium. As a result, one common problem involves potentially toxic electrolytic products created at the electrodes. Most experiments are free from problems that arise from heat produced by resistive losses in the medium. However, at very high field strengths, the heat generated may be a factor as well. As is true for the magnetic field systems already discussed, the maintenance of well-controlled temperature, humidity, and gas tension are essential for keeping cells and tissues in a normal, unstressed condition.

In vitro studies may involve acute exposures or long-term exposures and have many possible biological endpoints. Acute studies, for example electrophysiological studies or studies of the biochemical elements of cell signaling that last for only minutes or hours, make less stringent demands on the field generating system when compared to chronic studies that require cells be kept in culture for days or weeks. In addition, some acute studies permit continuous perfusion of the physiological medium at rates that flush the chamber with fresh medium every minute or so, thereby greatly diluting any toxic products that might be introduced. In contrast, chronic experiments may involve only weekly changes of, for example, 10 ml of medium, and have a greater risk of contamination by electrode by-products if the electrodes are in contact with the solution.

Cell growth can be affected by metallic ions liberated from silver, platinum, or stainless electrodes at micromolar and, in one report, at submicromolar concentrations [Baldi et al., 1988; Davis et al., 1991; Jackson and Duling, 1983; Moutin et al., 1989; Schnetkamp and Szerencsei, 1989; Sharma and Jande, 1989; Semeykina and Skulachev, 1990]. Mild effects may be detected with plating efficiency tests, but more severe effects will be self-evident by a large drop in the numbers of viable cells. The toxic effects of metal ions provide an example of the manner in which an apparent "field effect" may really be an artifact of the exposure system. Agar is often used as a barrier to the diffusion of toxic electrode by-products [Krauthamer et al., 1991].

5.1 Methods for Generation of Electric Fields

In Section 3, Exposure Systems For In Vitro Studies With Magnetic Fields, we discussed in vitro magnetic field exposures that are typically performed by placing dishes, flasks, etc. into a coil system that produces the magnetic field in the space within the coils. In principle, in vitro electric field exposures can be performed in much the same way: an electric field could be generated between parallel plates with the dishes or flasks placed between the plates. In fact, although this method is normally used for exposure of laboratory animals, it is used infrequently for in vitro studies. This is because very high electric fields in air are required to achieve the electric fields within the medium which are relevant to environmental exposures of humans, such as exposures to power transmission line fields. For in vitro electric field exposure, it is more practical and much more efficient to generate the field within the culture medium itself. In this way the voltage drop associated with generation of an electric field occurs entirely across the culture medium rather than primarily across an air space or chamber wall thereby allowing generation of relatively high fields with low voltages.

In the first and most common of the three schemes for generation of electric fields, an electric current is passed through the saline medium surrounding the cell or tissue specimen. Only two elements are needed: electrodes in contact with the medium (referred to below as current-passing electrodes) and a source of electric current. However, whenever electric currents are carried by metals in contact with ionically dissociated solutions, the electrical energy required to create the current also does chemical work that can affect the electrical properties of the electrode by altering its surface and may also liberate metal ions or other toxic products. A constant current source is usually needed because it is desirable to maintain a constant field in the medium despite variations in electrical properties of electrodes.

In the second scheme, considered further in 5.3, Generation of Electric Fields Between Capacitor Plates, an electric field is generated in a conducting medium without electric contact between the electrodes and the medium. This scheme, which is only possible with alternating currents, requires relatively high voltages on electrodes that form a parallel plate capacitor that contains the conducting medium and biological sample [Lymangrover et al., 1983; Tobey et al., 1981]. A variant of capacitive coupling is achieved by using ceramic electrodes that are in contact with the medium but interact with the electrolyte solution through a displacement current rather than a conduction current [Carstensen and Miller, 1984].

The third and final scheme employs magnetic fields to induce electric currents in the medium, as has already been discussed in Section 3, Exposure Systems For In Vitro Studies With Magnetic Fields.

Throughout the discussion that follows, it is useful to write interchangeably

about the electric field (*E*) and the associated current density (*J*). These quantities are related by Ohm's Law, $E = \rho J$, where *E* is usually given in units of V/m, *J* in A/m² (and often μ A/m² or mA/m²), and ρ in ohm-m. Assuming, as is usually the case for liquid or agar media, that the resistivity is uniform, a uniform current density is associated with a uniform electric field in the medium.

5.2 Generation of Electric Fields With Current-Passing Electrodes

For the purpose of creating electric fields, the ideal current-passing electrode would have zero impedance, the geometrical shape needed to create a uniform field in the particular chamber, and electrochemical stability both over time and over the required range of current densities. While one would wish that electrode functions also might not be influenced by the electrolyte solution and temperature, these factors are immutable aspects of the electrochemical reactions. In addition, an ideal electrode would not be the source of toxic contaminants, allow easy attachment of a metal wire, and have attractive mechanical properties of strength and flexibility.

It is easiest to achieve uniform current densities in a rectangular chamber using a pair of electrodes at either end of the chamber. To achieve a totally uniform current density, the depth of the medium should be constant throughout and the electrodes should completely span the side of the chamber. In addition, the electrode surfaces should behave uniformly so that each region produces an identical current density, that is, the electrode should have a uniform surface resistance.

For tissue confined to only a part of the chamber, as is usual in electrophysiology, good results are obtained in practice so long as the uniform depth requirement is satisfied. This is because the high conductivity of the culture medium usually assures a uniform current density over the volume containing biological tissue. This situation is common because the distance between electrodes is usually large compared with the scale of any inhomogeneities attributable to an electrode too short to span the chamber's width or to current variations along the electrode. In effect, the medium "shorts out" any inhomogeneities that arise near the electrode surface (see 5.2.2, Uniformity of electric field and exposure conditions). Nonetheless, the experimenter should assure that electrode spacing is sufficient, that the electrodes are in good condition by visual inspection, that they are not extensively poisoned by proteins, nor their surfaces damaged mechanically. Electrodes poisoned by adherent proteins will be unstable and have increased resistance. Mechanical damage is a problem for the relatively fragile coatings on silver/silver chloride and platinum black electrodes, but, especially for current-passing purposes in biological studies, perfect surfaces are not a necessity.

Common among materials used for current-passing electrodes are silver/silver chloride, stainless steel, platinum, and carbon (graphite). Ceramics can also be used like metal electrodes for ac circuits. For best electrochemical properties, silver electrodes should be chloridized before use, and platinum platinized (see 5.2.8, Preparation of silver-silver chloride and platinum black). When passing alternating currents, over time bare silver will spontaneously acquire a silver chloride surface as a result of the chloride ions normally present in the culture medium and the reversible chemical reaction between silver chloride and metallic silver.

5.2.1 Surface current density and electrode resistance. The interface between a metal electrode and the culture medium, an electrolyte, is the site of a transfer of the current carried by electrons in a metal to an ionic current in the electrolyte. This

In Vitro Studies With ELF Fields: A Primer



Fig. 19. Resistance and capacitance in an electrode-electrolyte system. (a) Simplified schematic illustration of a layer of adsorbed anions at the surface of a current-carrying metal electrode immersed in an electrolyte with hydrated anions and cations. Within the metal, conduction is by electrons and in the electrolyte by ions; a chemical reaction on the electrode surface accomplishes the transition from ionic to electronic conduction. (b) The equivalent circuit for an electrode through which a current generator drives a current *I*. The electrochemical interface generates a potential difference V, direct current flows through a resistive path of resistance R_o , and alternating currents flow through a parallel branch with capacitance C in series with resistance R_s . For ac measurements, R_o can be ignored. R_s and the reactance $X_c = 1/(2\pi fC)$, are often roughly equal in magnitude; for example, for silver R_s is about one-third X_c . As a result, X_c/R_s , the tangent of the phase angle between the voltage and current in the electrode circuit varies from 45° for copper to 71° for silver and 80° for platinum when operated at low current density [Ragheb and Geddes, 1991].

is made possible by a chemical reaction in which the electrode and electrolyte exchange ions. For example, at the silver/silver chloride anode electrode, silver metal gains a chloride ion while the silver chloride salt dissolved in the electrolyte loses a chloride ion. The opposite reaction occurs at the cathode where silver ions are created on the electrode surface while silver chloride is formed in the electrolyte. The chemical reactions on the metal surface involve charges distributed over a very thin region. It is this physical arrangement of the charges that is responsible for the unusual and unstable properties of the electrode-electrolyte interface.

With the passage of direct current between the electrode and electrolyte, work is done to rearrange the charges at the electrode-electrolyte interface so that charge distributions of opposite polarity exist in the electrode and electrolyte as shown in Figure 19a. Because a direct current is maintained in the presence of the polarized

charge distributions, an equivalent circuit of a resistor in series with a battery, representing the potential difference between the charge distributions, can be used to approximately model the interface region. An electrode-electrolyte interface for which there is such a potential difference is said to be "polarized."

For alternating currents, the electrode interface can be modelled as a battery, resistor, and capacitor in series with the external current source. The combined behavior of an electrode for both direct and alternating currents is illustrated by the model shown in Figure 19b. The resistance and capacitance of electrodes depend strongly on frequency and current density, and the properties for different metalelectrolyte combinations vary considerably. At low current densities, the changes in resistance and capacitance vary inversely with frequency; electrode polarization is greatest for direct currents and decreases rapidly with increasing frequency.

Ragheb and Geddes [1991] examined the frequency dependence of eight metal electrodes in 0.9% saline solution to determine the frequency dependence of electrode impedances over the range 100 to 20,000 Hz. In summary, they found,

$$R = (0.005/S)Af^{-\alpha}$$
(14)

$$X_c = (0.005/S)Bf^{-\beta}, \tag{15}$$

and

$$Z = \sqrt{(R^2 + X_c^2)},$$
 (16)

where S is the electrode area in cm², and A, B, α , and β are experimentally determined parameters independent of the frequency, *f*. For silver at 0.025 mA/cm², Ragheb and Geddes [1991] reported that A = 104 k Ω , α = 0.674, B = 307 k Ω , and β = 0.741 for 100 Hz to 20 kHz. The seemingly large values of A and B in reality are only reflections of the very small surface area, 0.005 cm², to which the formula is normalized. For example, at 100 Hz a silver/silver chloride electrode 0.5 cm² in area would have a resistance of about 47 Ω . The lowest value of A, 10.8 k Ω , was found for rhodium, followed by copper (44.3 k Ω), silver (104 k Ω), platinum (253 k Ω), and stainless steel (989 k Ω). Since copper, rhodium, and silver also had low values of B, and since polarization capacitance is inversely related to B, copper, rhodium, and silver had the highest polarization capacitances.

The key parameter in the use of metal electrodes is the current density at the metal surface. As surface current density rises, so does the potential difference between the bulk metal and the liquid medium. The resulting increase in electrode polarization is in proportion to the degree of chemical activity at the metal surface. Increased electrode polarization has the undesirable effect of further increasing surface resistance and, in a vicious circle, causes additional changes at the metal-electrolyte interface. These physical-chemical changes at the electrode surface are responsible for the non-linearity of electrodes with increasing surface current density. The properties of electrode surfaces have been studied extensively with the greatest emphasis on the physical and electrochemical properties that affect the use of electrodes for potential measurements [e.g., Janz, 1961].

Because of progressive increases in electrode polarization or electrode poisoning, the voltage needed to maintain a given current from an electrode may grow with time to a much greater level than that needed at the outset. For this reason, it is necessary to use constant current sources (see 5.2.4, Constant current sources) to energize electrode circuits.

For silver-silver chloride electrodes, a current density of 50 A/m^2 (5 mA/cm²) measured at the electrode surface is a reasonable upper limit, since at much higher current densities the electrode resistance rises significantly and unstably. The corresponding upper limit on the voltage applied to the electrode is about 1 V. For example, in the case of the 0.5 cm², 47 ohm silver/silver chloride electrode considered above, 0.12 V is required at 100 Hz to produce a 5 mA/cm² current density. It should be noted that this current density is orders of magnitude larger than that induced in biological organisms by electric or magnetic fields comparable in magnitude to those found in the vicinity of power lines (see Section 7, Relating In Vitro And In Vivo Exposure Conditions). Therefore, surface current density need not be a factor in achieving fields suitable for most experiments.

In order to reduce surface current density, the electrode surface area can be increased by using a coiled electrode or rectangular foil instead of a short straight wire. Sintered silver-silver chloride electrodes are also useful since they have large effective surface areas that result from compaction of small grains of silver chloride onto a silver surface. However, sintered wire is usually provided in relatively short lengths of about 1 cm and cannot be bent into coils without damage.

Because of the reversibility of the silver and silver chloride chemical reactions at each electrode, the silver-silver chloride system is particularly well-suited for use with direct currents and at very low frequencies, such as in the ELF region. At frequencies above 10 Hz and extending to 250 MHz, platinum black electrodes are widely used in measurements of tissue and cell impedance [Schwan, 1963; Schanne and P.-Cerretti, 1978].

The surfaces of silver-silver chloride and platinum black electrodes require the extra steps needed for their fabrication and they are delicate, requiring care to avoid mechanical injury. In many cases, bare silver metal electrodes and bare platinum electrodes may be used, although their initial properties will change with use. Unlike silver and platinum, stainless steel electrodes require no surface preparation and are especially durable, but have a larger surface resistance.

As noted earlier, precautions must be taken to prevent potentially toxic electrolytic products produced at the electrodes from reaching cells in the culture medium. Ceramic "electrodes" are free of the electrochemical concerns that apply to metals. However, while wires can be soldered directly to some metallic electrodes, electrical contact with others, e.g., ceramic and graphite, may require clips or a pre-chamber with a metal electrode and liquid medium (see 5.2.3, Toxicity problems with in vitro electric field exposure systems).

5.2.2 Uniformity of the electric field and exposure conditions. Assuming a homogeneous medium for which the resistivity is everywhere the same, the requirements for a uniform electric field can, by Ohm's Law, just as well be discussed in terms of the current density. Because the description in terms of the flow of current leads to a more natural picture of the problems, electric field uniformity is usually evaluated in terms of the factors affecting current density. As discussed in this section, the principal factors affecting current density are the shape of the chamber and the shape of the electrodes.

In order that the current density, \vec{J} , be equal at all vertical cross sections through the chamber, the areas of the vertical cross sections must be made the same, as shown

in Figure 20a. If this cannot be done, then it is usually satisfactory to require only that the cross sectional areas in the region surrounding the biological specimen be kept constant. The need for constant areas follows simply from the fact that \vec{J} is defined as the quotient of a current, I, and cross-sectional area, $A: \vec{J} = \hat{e}_k I/A$, where \hat{e}_k is a unit vector in the direction of current flow. Since I is fixed by the current source, and \hat{e}_k by the electrode and chamber geometry (assuming a homogeneous medium), the only quantity that could affect uniformity is the area A. Hence, to have \vec{J}_n , the current density in the n-th section remain constant for all cross-sectional areas, it is necessary that the medium in the chamber have top and bottom surfaces that are parallel (and therefore level), that the surfaces be flat (that is, without significant irregularities in the surface of the chamber floor), and that there be no objects located close to the specimen that could obstruct the current flow. Figure 20b,c shows the effects of a surface that is not level and a surface irregularity on the chamber floor on the current density in the chamber.

In small chambers, the top surface of the medium may be significantly curved by the effects of surface tension acting at the perimeter of the chamber and near objects such as perfusion lines, drain lines, recording electrodes, and temperature measuring devices that are typically found in electrophysiologic chambers but are not a problem in cell culture dishes. A practical guideline is simply to design the experimental chamber with a flat bottom surface and with all objects as remote as possible from the region occupied by the tissue specimen. In a well thought-out design the perturbations produced by surface tension and objects have only local effects on the current density and there is no deviation in current density at the location of the biological specimen. Unlike electrophysiology chambers, rectangularly shaped cellculture dishes are devoid of problematic objects so that only placement on a level surface is required in order to assure uniform cross sectional area.

The second requirement for a uniform current density involves the shape and size of the current-passing electrodes relative to the shape and size of the chamber. In order to have a uniform current density at a cross section close to the electrode surface, the shape of the electrode must match the cross section of the chamber. Hence, for the geometry shown in Figure 20a, rectangular electrodes would be ideal and electrodes in the shape of a point or line would be least desirable (Fig. 20d). As one moves to points far from the electrode, its shape has less influence on the

Fig. 20. Effects of the geometry of the chamber and electrode on the uniformity of the current density and electric field. Equipotential lines are indicated approximately with dashed lines. (a) Equal vertical cross sectional areas through the chamber, e.g., A_1 , A_2 , result in a uniform current density in the culture medium. (b) The effect of non-level surfaces is illustrated. The cross sectional area of the culture medium decreases going from right to left in the chamber. As a result, the current density and electric field increase from right to left. (c) The presence of an object on the floor of the chamber leads to smaller cross sectional areas and larger current densities in the vicinity of the object. (d) Approximate current density and electric field lines near "line" (rod) and "point" (sphere) electrodes. The uniformity of the current density and electric field improves at points distant from the electrodes. A number of equipotential lines in vertical and horizontal planes that bisect the electrodes are approximately indicated. (e) For a circular chamber with parallel electrodes, the current density can be approximately uniform in the central region, but nearly zero in the regions labelled 'z' behind the electrodes. (f) Effects of a defect in a portion of the rectangular electrode shown in (a). The current density in the chamber is altered by a defect in a portion of the rectangular electrode which sharply increases the current density over the electrode's active length. At points distant from the defective electrode, the current density becomes uniform.



Legend amount on th



Fig. 20d-f. Legend appears on p. 42.

uniformity of the current density since the highly conducting medium will not support large differences in current density across any cross section unless there is a nearby electrode surface to create that difference. One can say that the medium "shorts out" inhomogeneities in current density and tends to make the current density uniform.

In practice, the problem of mating electrode shape to chamber shape is most difficult for circular dishes because the cross section varies continuously as one moves along a diameter of the dish. The problems are of more concern for cell culture studies than for electrophysiological studies. Figure 20e shows a map of isopotentials in a circular dish used for electrophysiology studies in which the region of satisfactory uniformity includes the area occupied by tissue. However, Figure 20e illustrates the difficulties associated with electric field studies of cells grown in a circular dish. Although the current density near the center of the volume is quite uniform. cells located in regions near the electrodes (inner faces) are in a higher current density with a substantial spatial gradient, and cells located in the regions behind each electrode (i.e., outer faces) are unexposed since there is essentially no current behind the electrodes. Because the region of uniform current density can be small for circular chambers, the design shown in Figure 20e is not normally used for cell culture research. Figure 20 indicates that reasonably uniform current densities can be obtained over almost all of the chamber if well-shaped electrodes are used, and over a limited region even if poorly suited electrodes are used. The use of agar near the electrodes to provide isolation of cells and tissue from toxic electrode byproducts (see 5.2.3, Toxicity problems with in vitro electric field exposure systems) may also serve to position the cells and tissue away from the nonuniformities.

Thus far, a homogeneous conducting medium has been assumed. However, the biological specimen and any objects in the chamber create regions of inhomogeneous conductivity. In studies with cells at usual densities, their volume is too small to have any significant effect on the resistivity of the medium, but pieces of tissue can perturb the resistivity in the experimental chamber and thereby cause an inhomogeneous current density and inhomogeneous field in the region immediately surrounding the tissue. It is customary to describe exposures in terms of the unperturbed current density and to ignore the perturbations produced by the tissue, but a detailed analysis of exposure within the tissue would be required to treat perturbations in the tissue. The degree to which the tissue mass affects current density in the exposure chamber depends on the conductivity of the tissue as compared with the conductivity of the medium. Tissues without sheets of connective tissue or fibrous networks and in which the cells are not very densely packed will have a considerable volume of interstitial space that is filled with physiological solution and as a result have a conductivity similar to that of the bulk medium. On the other hand, a considerable part of the volume within muscle, brain, and bone tissues is occupied by materials with poor conductivity and as a result the tissue resistivity is much higher than that of the surrounding medium. The measured resistivities at low frequency of various tissues are several times higher than that of standard physiological media. See Table 2 for a selected listing of some tissue conductivities at 100 Hz, which range over nearly two orders of magnitude.

As noted earlier in 3.2.1, Experimental geometry and exposure parameters, at a microscopic level cells adherent to a non-conducting plastic surface will be exposed to anisotropic fields. This occurs because the current density at the adherent membrane is nearly zero, depending on the degree to which ion flow is impeded by the

	σ(S/m)	k	Source ^a
Brain	0.33		2
Bone	0.013	3.8×10^{3}	1
Blood	0.60	en lo man <u>o</u> ri ser .so	1
Blood plasma	1.0	sension endails microit	3
Cytoplasm (Purkinje cell)	1.0	r diamoter - the dish. T	4
Fat	and and the state	1.5×10^{5}	1
Liver in situ	0.13	8.5×10^{5}	1
Lung in situ	0.09	4.5×10^{5}	1
Skeletal muscle oriented			
parallel to E-field	0.52	1.0×10^{6}	1
Skeletal Muscle oriented			
perpendicular to E-field	0.076	3.2×10^{5}	1

TABLE 2. Va	rious Tissue	Conductivities (r) and	Relative	Permittivities	(k)	at	100	Hz
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^aSources: (1) Foster and Schwan, 1986, Table 6. See therein for primary references. (2) Adey, 1975. (3) Schwan and Kay, 1956. (4) Biology Data Handbook, 1973, p. 1234.

network of proteins that cause cell adhesion. As a result, on a cell-by-cell basis, less of the surface of adherent cells would be exposed than for cells maintained in suspension, and cells packed closely in a monolayer would be even less exposed. Moreover, in tissues, the microscopic pathways for currents are significantly affected by the volume of fluid-filled intercellular space and the volume of poorly conducting biological materials (e.g., myelin, bone, sheets of connective tissue, and fat). Similarly, the close, extended intercellular contacts that occur with support cells alter current densities at the level of the single cell. For example, the glial cells of the central nervous system may form extended close contacts with nerve cells. Likewise, Schwan cells greatly affect the extracellular environment of the axons in myelinated nerves. For these reasons, even in the case of an ideal exposure system that produces perfectly uniform fields in a homogeneous medium, there will be variations in the fields for cells in different parts of the tissue and in different subcellular regions of a single cell within the tissue. There is, of course, no practical way to take into account these variabilities at the cellular and sub-cellular levels and as a result the unperturbed current density or electric field universally is used to specify exposure. Polk [1987] and Polk and Song [1990] provide detailed examinations of the effects of timevarying electric and magnetic fields at cell surfaces and interiors.

The growth of cells in agar medium presents no new concerns regarding field uniformity since the agar gel is an electrically homogeneous conductor that acts similarly to liquid media.

5.2.3 Toxicity problems with in vitro electric field systems. The major biocompatibility issues for electrode systems concern the toxicity of metal ions, particularly, silver, platinum, and copper ions. In general, no electrodes can be safely used for chronic exposures in contact with a medium that is not changed frequently (or changed by continuous perfusion). Instead, a plug or layer of agar is interposed between the electrodes and the solution containing cells or tissue. Agar conducts electricity because it is porous to ions, but agar greatly attenuates the transport of metal ions since bulk movement of ions is inhibited by the extensive semi-solid matrix of sugars. Diffusion down the concentration gradient still occurs, but this is a much slower process. For long-term studies, the barrier should be long enough to

In Vitro Studies With ELF Fields: A Primer



Fig. 21. Agar bridge. The agar bridge was made by cutting glass tubing of two different diameters along their lengths. The half-cylinders were then cut to lengths that matched the width of the rectangular exposure dish and a pair of half-cylinders of different diameter were joined by melting the glass at the ends to form a seal. This left a hollow space between the half-cylinders with two slot-like openings to the outside. A sterile agar prepared with physiological medium was then poured into the opening and allowed to set before use. Because of the low resistance of this system, a series arrangement of bridges from one dish to the next (only one dish shown) allows one amplifier/monitoring system to be connected to a string of several dishes containing biological cells or tissue. Carbon rod electrodes connected to the current source were placed into the end dishes which did not contain biological matter.

limit diffusional transport. Agar or media bridges [McLeod et al., 1987] are often used to couple current from a pre-chamber to the test chamber. A stainless steel or silver-silver chloride electrode typically is placed in the pre-chamber and carries the current from the current source.

The use of an agar bridge can present a problem if the agar is held in a tube of cylindrical cross section because most experimental chambers do not have a cylindrical cross section. As a result, this type of bridge would not give a uniform exposure in most chambers or in cell culture flasks. An ingenious solution to this problem has been devised by a number of researchers [Davidson and Basu, 1987; Cain et al., 1987]. The apparatus used by Cain and coworkers is shown in Figure 21. They chose a rectangularly shaped dish and fabricated an agar bridge with a slot-like opening so that it coupled current to nearly the entire width of the dish, thus achieving a highly uniform current density in the medium and avoiding the problems of isolation for chronic studies was devised by McLeod et al. [1987] who incorporated a media bridge between platinum electrodes located in wells at the ends of a rectangular trough and the central well in which the tissue was placed. They used a cross-linked collagen membrane to confine the media and limit convective mixing between the outer and central wells (Fig. 22).

For long-term growth of cells in culture, the concentration of ions of silver, platinum, and copper must be kept below toxic levels by use of a barrier to ion transport, usually a bridge of the type already described. For example, silver ions have been reported to be toxic at levels as low as 10^{-8} mol/L (M) [Semeykina and Skulachev, 1990]. More commonly, silver ions have been found to have physiological or toxic effects at concentrations of 1 to 50×10^{-6} mol/L [Baldi et al., 1988; Moutin et al., 1989; Schnetkamp and Szerencsei, 1989]. Physiological effects of copper also have been reported [Sharma and Jande, 1989]. In addition current-



Fig. 22. Exposure system used by McLeod et al. [1987] to minimize contaminants from the electrodes reaching the biological specimen. A constant current source provides current to the platinum electrodes and culture medium and the cross-linked collagen membranes isolate the electrode contaminants from the tissue sample. The lumen of the tissue holder defines a cross sectional area which is used in the calculation of the current density and electric field strength [see 6.3, Determining Electric Field From Conductivity and Current Density Measurements]. Redrawn with permission from AAAS. Copyright 1987 by the AAAS.

carrying electrodes have well-known bactericidal effects that vary with electrode material [Davis et al., 1991; Jackson and Duling, 1983; Uezono, 1990]. The potential toxicity of the medium can be assessed directly by a control experiment using medium previously conditioned by passing current through the chamber without any tissue present.

For the purposes of chronic cell culture studies, even non-metallic electrodes are not trouble-free [Lyle et al., 1988]. Carbon electrodes are attractive because the carbon itself is biologically innocuous and the electrode can pass large currents. However, unless very pure grades (such as spectroscopically pure graphite) are used, the passing of current through the graphite will liberate machine oil, binders or other undesirable materials trapped in the porous surface of the material. Even the ultrapure materials may have significant surface contamination and the electrode materials need to be thoroughly cleaned and sterilized, for example, by prolonged soaking in ethanol, acetone and water.

5.2.4 Constant current sources. Because of large variations in electrode characteristics (for example, 2-fold or even 10-fold changes in resistance), it is usually necessary to use a constant current arrangement for the current source. The two types of constant current source are either a passive circuit or an active, amplifier-based circuit. The passive circuit is simplest and often adequate. In this case a large resistance is placed in series with the electrodes such that most of the voltage drop occurs across the resistor and little (for example, <5%) across the electrodes. The total current through the circuit is set largely by the fixed resistance so that a doubling of



Fig. 23. Constant current arrangement using a series resistance. Current from the generator passes through series resistor R_s and load R_e . Since $R_s \ge R_e$ the magnitude of the current is determined mainly by the voltage, V, at the generator and R_s , but is nearly independent of R_e .

the electrode resistance would represent an increase of, for example, only 5% in the total resistance. The fixed resistor is often called the "current limiting resistor" or "series resistor." In principle, current regulation can be improved by using a higher series resistance. However, to do so also requires higher voltages from the source since, for a given current, a greater and greater voltage drop occurs across the series resistor as its value increases.

A practical example of a constant current arrangement is shown in Figure 23. From Ohm's law, under steady-state conditions, the current through the circuit, *I*, is

$$V/(R_e + R_s), \tag{17}$$

where V is the applied voltage, R_e is the variable electrode resistance, and R_s is a fixed resistor. For $R_s \ge R_e$, to first order, a change in R_e to $R'_e = R_e + \Delta R_e$ changes the current from I to

$$I' \approx I[1 - (\Delta R_e/R_s)]. \tag{18}$$

Hence, for current-limiting circuits, the percentage regulation is given approximately by

1

Current Regulation (percent)
$$\approx 100(\Delta R_e/R_s)$$
. (19)

Taking typical values of $R_e = 10 \ \Omega$ and $R_s = 10,000 \ \Omega$, even an upwards shift in R_e by $\Delta R_e = 50 \ \Omega$ (500%) results in a change of just 0.5% in the applied current, while a 5-fold reduction to 2 Ω , for which $\Delta R_e = 8 \ \Omega$, results in a shift in the current of just 0.08%.

Though simple, this form of voltage regulation can be effective so long as the current required is small, as in the foregoing example where, if $I = 10^{-4}$ A, the required voltage, V is 1 V. However, to use the same circuit for a current of 10^{-2} A would require a 100 V source. As an alternative to high voltages, a lower degree of current regulation might be accepted by choosing a smaller value for R_s in order to reduce the required voltage, V. As an alternative to the passive circuit described in Figure 23, relatively simple circuits with operational amplifiers can be used to generate nearly constant currents. The Howland Pump and another operational amplifier-based circuit for generation of constant currents are shown in Figure 24.

The current can be measured and monitored either by placing a sensitive electronic ammeter ("picoammeter") in series with the circuit or by measuring the voltage across a precision resistor (e.g., 300 Ω) placed in series with the electrode circuit. In either case, for electrophysiologic studies, it will be necessary to take precautions that these additions to the circuit do not introduce unwanted stray signals.



Fig. 24. Constant current operational amplifier circuits. The operational amplifiers (U) may be such common devices as types 741 or 356. (a) A bipolar constant current amplifier (Howland Pump) in which the load (the current-passing electrodes in a bath) is connected to circuit ground [Frederiksen, 1984, p. 193; Jung, 1974, p. 182]. The input resistors R_1 and R_2 are equal in value (typically, 150 k Ω). R_{se} is the sense resistor across which is developed the potential that the amplifier senses to maintain a constant current. R_{se} is chosen so that there is a voltage drop of 1 or more volts at a current level near the desired level. Typically R_{se} is about 1–2% of R_f . The feedback resistance, R_f , is chosen according to the approximate magnitude of the current so that there is a suitable current in the feedback loop (typically, R_f is 50 k Ω to 1 M Ω). (b) This simple circuit also acts as a constant current amplifier with unity gain (and opposite phase) between the input current and the current in the feedback loop into which the load is placed [Jung, 1974, p. 172]. An attractive feature of this circuit is that it is immune to dc offset potentials at the input or to those generated in the load. Note that the load circuit does not reference the circuit ground. The current through the load is constant as long as V/ R_1 remains constant.

In the case of an electronic picoammeter, especially when used at levels near and below 1 μ A, it is necessary to use a battery-powered unit and to remove the plug from the ac outlet. This is necessary even in instruments with carefully designed power supplies that are intended to provide good isolation of the measuring circuit from the mains ground because of stray capacitance in even the best-designed transformers. Moreover, the entire ammeter should be placed in a shield (for example, copper screening) to isolate the instrument from stray 60 Hz electric fields in the laboratory, and it should be located away from strong 60 Hz magnetic fields. In order to maintain a high degree of isolation from ground, all wires should have high grade insulation such as polytetrafluoroethylene (PTFE) and contacts with grounded objects should be avoided wherever possible.

The use of a direct-coupled constant current device makes possible the generation of dc potentials ("dc offsets"). These may not be easily noticed, particularly when using instruments set up to measure alternating current signals. The offset potential may begin small and grow over a period of hours or days to a very high value. The source of the offset currents may be an unbalanced amplifier or, more

In Vitro Studies With ELF Fields: A Primer 51

commonly, battery-like chemical reactions at the various electrodes in the circuit. The electrochemical potential associated with a single electrode is called the half-cell potential. In the case of series-connected exposure chambers, the potentials found in practice can be considerable, for example, reaching levels of 1.5 V or more. Because these incidental electrochemical cells generally have high internal resistance, they can not maintain significant potentials when connected to a low resistance load; that is, they can not drive a large current. However, the high impedance amplifier circuits associated with constant current devices may amplify the offset potentials with serious consequences. In the worst case, the amplified potential can drive a current of many tens or hundreds of microamperes through the exposure chambers. In addition, offset potentials may cause the amplifier to greatly distort the waveform if the offset voltage causes the amplifier to clip the output signal.

Blocking offset-produced direct currents by coupling to the electrodes through a capacitor requires an impracticably large non-polarizing capacitor at extremely low frequencies. Also, blocking capacitors cannot be used with constant current amplifier circuits that require a direct current pathway. Therefore, this problem is best solved by careful design of the constant current circuit to assure that the circuit appears as a low impedance load to the electrochemical elements and by checking for dc offset potentials at various times throughout a prolonged experiment.

The design of the constant current source must also take into account the largest electrode resistance that might occur in order to have sufficient voltage to drive the current. For example, to obtain a 10 mA peak current through an electrode circuit of 1,000 Ω resistance requires 10 V peak; if the constant current source uses only a 5 V power supply, Ohm's Law makes it certain that the current waveform would be clipped at one-half the intended amplitude. In such cases where the current source can not supply the needed voltage, the result is significant distortion of the applied signal and a reduced average field amplitude. For this reason, when using electrodes at high surface current densities (i.e., with chemically significant potentials of about a volt or more) it is always necessary to monitor the current source output waveform using an oscilloscope (Fig. 21).

5.2.5 Compatibility of in vitro electric field systems with electrophysiological recordings. For electrophysiological setups where it is desired to avoid artifactual potentials at the microelectrode, the driving circuit (whether an active or passive constant current circuit) should be operated from batteries that are isolated from the recording circuit. This isolation assures that there is only one ground point in the bath, thereby avoiding distortions of the current density. Isolation also allows electrophysiological recording without significant interference from the electric current flowing in the chamber.

Electrophysiological recordings of cells require high resistance microelectrodes and very high resistance input circuits because of the high impedance of cells. As a result, the recording systems are sensitive to stray electric and magnetic fields in the laboratory. However, the major problem in making recordings during electric field exposures is the fact that the recording microelectrode is a sensitive voltmeter that is designed to sense the potential difference of 50 to 80 mV across a cell membrane or the much smaller voltages (e.g., 100 μ V) that are observed extracellularly. This voltmeter will also detect the potential differences that result when current passes through the resistive medium (Fig. 25a). This means that there will be an artifact of the applied field imposed on the physiological potentials. The magnitude of the



Fig. 25. Sources of artifact potentials in electrophysiological recordings. (a) A rectangular chamber with current-passing electrodes EL1 and EL2 is shown with a microelectrode M impaled into a cell C lying along equipotential "2." An indifferent (reference) electrode, I_R, lies along equipotential "1." The recording microelectrode and indifferent electrode are attached to a high impedance voltmeter, usually an electrophysiological pre-amplifier with an input impedance of $10^{11} \Omega$ or more. The potential difference between "1" and "2" is detected by the electrodes and appears superimposed on the physiological signal. By movement of I_R with respect to M, this potential difference can be reduced nearly to zero. R_I shows a possible leakage path between the current-passing circuit and the recording circuit that can affect the signal seen at the voltmeter. (b) This circuit identifies equivalent circuit elements R_{FL1} and R_{FL2} . representing surface resistances at the electrodes, R_{B1} and R_{B2} , showing the bath resistance between the electrode site and locations "1" and "2." R_{B3} is the series resistance between the indifferent electrode at "1" and the recording electrode site at "2." The microelectrode, M, introduces additional circuit elements (resistance, capacitance, and half-cell potentials) as indicated by the open box and likewise for the indifferent electrode and cell, C. The ground loop created by the leakage resistance R1 permits current flow through EL_1 which will appear at the voltmeter as a potential that is in addition to that developed across the small bath resistance, R_{B3} . The alternating current through EL₁ would be significant only if R_1 were comparable to $R_{EL2} + R_{B2}$. Although not indicated explicitly on the schematic, the current through electrodes EL_1 , EL_2 , and I_R is subject to phase shifts as a result of the capacitive nature of the electrodes. Consequently, instabilities at the electrodes may change both the potentials and phase of the signal derived from the bath current as measured at the voltmeter, V.

artifact is dependent only on the potential difference between the recording and reference (indifferent) electrodes so long as the recording and current passing circuits have no common electrical path. To reduce the artifact potential difference that arises from the electric field in the medium, it is possible to place the recording electrode and its reference electrode so that they lie on the same equipotential surface in the culture medium. This potential is "ground" for the recording circuit.

Although the current-passing electrodes create potential differences at each electrode interface and throughout the chamber, there is (ideally) an infinite resistance between the two circuits and therefore no interaction between them. However, total elimination of the artifact from the current in the chamber is not possible for a number of reasons. There are imperfections in isolation of the circuits and capacitive coupling between them that leads to an artifact signal. In addition, the finite size of the reference electrode and potential variations on its surface mean that it will not define a single equipotential line in the chamber. Finally, because there is generally some source of an artifact signal input to the recording circuit, the adjustment of the two probes of the recording circuit to reduce the observed artifact signal will actually put the two probes along different equipotential lines, that is, at the potentials that give the best cancellation of the artifact. Under some circumstances, all these effects may be too small to be important, but with high-resistance micropipettes and high-gain voltage measurement systems, complete and stable cancellation of the artifact is not possible. Because of the capacitive reactance of electrodes, small differences in the phase of the potential differences at each electrode become a major obstacle when trying to fully cancel the artifactual voltages from two electrodes. If phase and amplitude of the voltages were stable, better cancellation would not be so problematic, but time-dependent variations in microelectrode and metal electrode polarization result in continual small shifts in phase and magnitude of the measured potential differences.

The potential difference between the recording electrode and its reference in the bath is not the only potential difference seen by the microelectrode. There is a potential drop across each current-passing electrode that may amount to many tens or hundreds of millivolts, depending on electrode polarization and the magnitude of the current (Fig. 25b). If there is also a leakage path to the recording circuit ground, the recording electrode will respond to the current-passing electrode's potential. However, the presence of the reference electrode in the bath places the leakage path in parallel with the low resistance path between the recording and reference electrodes, and unless the leakage resistance is comparably low (for example, as low as hundreds or thousands of ohms), there will be no observable artifact as a result of the leakage path.

Figure 26 shows an electrophysiological recording made during electric field exposures to illustrate the degree to which artifact-free recordings can be made by isolation of recording and current passing circuits, by careful placement of the reference electrode, and with stable, well-prepared electrodes.

5.2.6 Resistive heating in culture medium. The flow of current through culture medium results in ohmic losses that produce heat in the medium. The average power density, W (W/cm³) is given by $\frac{1}{2} (\rho J^2)$ for a sinusoidal current density with amplitude or peak value J (not rms, which is usually measured by most instruments; see Section 1, Introduction). For experiments at low and moderate current densities, this is a small and unimportant factor in the overall flow of heat from the environ-



Fig. 26. Electrophysiological recording during electric field exposure. Spontaneous action potentials from a pacemaker nerve cell of the marine mollusk Aplysia were recorded intracellularly before, during, and after 10 min exposures to 60 Hz electric fields carried by silver/silver chloride electrodes in the recording chamber. The data, which show slowing of the neuron firing rate during exposure, were unaffected by artifacts from the 60 Hz current in the chamber. Had there been interference, it would be visible as additional noise in the baseline and as variable alterations in the height of the action potentials according to the phase of the interfering signal at the moment of the action potential.

ment. However, for the somewhat extreme peak current density of $J = 10^3 \text{ A/m}^2$ (100 mA/cm²) in a medium of 0.5 Ω -m (50 Ω -cm) resistivity, the heat produced is 2.5×10^5 W/m³ (0.25 W/cm³). This is a significant heat flux that, in an insulated volume, would raise the temperature of water by 3.6 °C per min. Since the heat input and therefore the temperature increase are proportional to J^2 , reducing the current density by a factor of 10 (to 10^2 A/m^2) reduces the calculated heat input and temperature rise by a factor of 100, that is, to 2.5×10^3 W/m³ (0.0025 W/cm³) and 0.036 °C per minute, respectively. At these and still lower current densities which are used in the majority of experiments (see Section 7, Relating In Vitro And In Vitro Exposure Conditions), heat input from resistive losses in the bath are too small to be significant. Since most chambers are not insulated and are in contact with a substantial thermal reservoir, thermal problems are further lessened.

5.2.7 Magnetic fields from currents in culture medium. We briefly noted in 3.3.2, Temperature, that magnetic fields associated with the typically small currents that are induced in the culture medium by an external magnetic field are negligible. We estimate here the approximate magnitude of magnetic fields produced by the potentially larger currents introduced in the culture medium by current-passing electrodes. The current through a rectangular chamber is approximately modelled as a sheet of current infinitely long and of finite width. It can be shown that the magnetic field at the surface of the current sheet, midway between the edges, is equal to $\mu I/2W$ [Halliday and Resnick, 1966], where μ is the permeability of the culture medium (nearly equal to that of vacuum, $4\pi \times 10^{-7}$ H/m), W is the width of the current sheet, and I is the current through the chamber. Assuming a current of 0.05 A (e.g., a current density of 100 A/m² through a cross sectional area of 0.5 cm by 10 cm), the

magnetic field at the surface of the current sheet is just 0.3 µtesla (3 milligauss) which is somewhat greater than background fields (i.e., away from sources) reported in buildings [Silva et al., 1989].

5.2.8 Preparation of silver-silver chloride and platinum black electrodes by electrolysis. Although many variations exist, the following technique makes electrodes suitable for the purposes of passing currents. A bare silver wire or the bare part of a PTFE coated silver wire [typically 0.025 cm (0.010 inch) diameter, ≥99% pure] or silver foil [typically 0.025 cm (0.010 inch) thick] is cleaned of any contamination by fingerprints or other oils using alcohol and/or immersion in 0.1 N H₂NO₃ for 2 min. Thereafter, the metal is handled with forceps. When desired, the metal can be formed into coils or other shapes. Coils are easily formed by winding the silver wire around a mandrel made of stiff wire.

Before depositing the layer of silver-silver chloride, the chemically clean silver surface should also be cleaned electrolytically. This is done by reversing the direction of current flow one or two times after periods of a few minutes of electrolytic action. The clean surface will appear bright and shiny. Finally, to deposit the silver-silver chloride, the anode (at which the electrode will be made) is left attached to the positive terminal of a dc power supply or a battery circuit set to deliver a constant current.

The electrolytic deposition of silver-chloride onto silver is done in a dilute solution of a chloride salt, for example, 0.9% NaCl, or a dilute acid such as 0.1 N HCl. A direct current is passed between a cathode and the silver anode where AgCl is formed. The result is a fine layer of silver chloride deposited onto the silver surface. The desired current density during the electrolysis is in the range 10 µA/cm² to 1 mA/cm^2 (0.1 to 10 A/m²) [Bures et al., 1967] or 5 mA/cm² [Geddes and Baker, 1975]. At current densities near the lowest level of 10 µA/cm², the chloridization process may require several hours, or conveniently, be allowed to run overnight in accord with a current density-time product of about 100 to 400 mA-s/cm². In contrast, Geddes and Baker [1975] suggest a current density of 5 mA/cm² for 100 to 400 s, corresponding to a current density-time product of 500 to 2,000 mA-s/cm². A typical coil of 24 turns of 0.025 cm diameter wire will contain about a 5 cm length of wire and a total area of about 0.4 cm². When this coil carries 4 μ A into the medium, the surface current density is 10 µA/cm².

A fine layer of silver chloride will have a light brown fine-grained matte appearance. By using current densities at the low end of the suggested range, the process can be allowed to proceed for several hours or overnight. It is desirable to avoid depositing too thick a layer of silver chloride because an overly thick layer is more easily damaged by slight contact and has an undesirably high resistance. Electrochemical theory and studies [Janz, 1961] show that the physical and electrochemical reactions at electrodes occur on the metal surface and in an immediately adjacent region that is less than a nanometer in thickness. Since silver chloride is photosensitive, it is often stated that the electrolysis best be done in relative darkness, although the consequences of photoreactions on recording electrodes are in question [Janz, 1961, p. 218 ff] and the relevance to electrodes used to pass current is unknown.

The low frequency resistance of platinum electrodes is reduced by forming a porous coat of platinum black on the surface (platinization). Platinum metal is platinized electrolytically at the cathode of a cell in which the electrolyte is a solution of 3% chloroplatinic acid (H_2 PtCl₆) and 0.025% lead acetate and the anode is pure

platinum [Cote and Gill, 1987]. These authors recommend using a current density of 0.45 mA/mm² and a current density-time product of 550 mA-s/mm² for optimal quality in the electrical and mechanical coatings. The electrode so fabricated had a lower resistance than silver/silver chloride for frequencies > 0.25 Hz.

5.3 Generation of Electric Fields Between Capacitor Plates

When a steady voltage is applied to parallel metal plates (a capacitor) in a dielectric (e.g., air), a current flows for a moment while charge builds up on the plates. Of course, in the region between the electrodes, no conduction current can flow through the insulating (dielectric) material, but whenever the applied plate voltage changes, there is a rearrangement of charges within the dielectric material between the plates. This movement of charges constitutes a portion of a displacement current that lasts as long as the voltage changes. The resulting rearranged charge distribution in the dielectric combined with the charge distribution on the metal plates establishes an electric field in the dielectric.

Since displacement currents last for only an instant when charging a capacitor with direct current, there is no lasting displacement current for steady (dc) capacitor voltages. Thus, displacement currents useful for experiments require a time-varying voltage on the capacitor plates. Sinusoidal voltages are used often, but repetitive pulsed voltages, although not considered in this primer, also produce displacement currents.

If a sinusoidal voltage is applied to the capacitor plates, it will produce a displacement current that is 90° out of phase with the plate voltage. Although within a capacitor filled with a dielectric the charges are bound and not free to move very far, the displacement current is nonetheless a true current insofar as it originates from the movement of charge. As a result, if a conductor is placed inside the dielectric (where it cannot contact the capacitor plates), there will be an alternating current in the conductor. The arrangement of a capacitor with a conductor within it represents the situation that exists when metal electrodes are placed on the outside faces of a plastic flask containing a conducting fluid medium. One capacitor is formed by the metal electrode, plastic dielectric, and interface with the conducting medium. The second capacitor is formed by the other metal electrode, plastic dielectric, and interface with the conducting medium. (Often, there is the added factor of an air gap between the top plastic face and the liquid.) In terms of an equivalent circuit, the conducting medium is in series with two capacitors. The current in the medium, like current everywhere in the circuit, is in phase with the displacement current in the dielectric. Of course the current in the conducting medium involves the movement of free charges, yet in this case the current in the medium is often called a "displacement current" just as for the displacement current in the dielectric.

For a sinusoidal applied voltage, the displacement current in a parallel plate capacitor of area A, filled with a dielectric material of relative dielectric constant k, and subjected to a sinusoidal electric field, E at an angular frequency $\omega = 2\pi f$ is

$$I = \omega k \epsilon_o E A, \tag{20}$$

where ϵ_o is the dielectric constant of free space, 8.854 $\times 10^{-12}$ F/m and f the frequency. Thus, the displacement current density, J, is $\omega k \epsilon_o E$.

To apply these ideas to electric field studies, the conducting liquid medium is the physiological buffer. Electrically, it is in series with the dielectric materials of the air and the glass or plastic walls that enclose the experimental chamber. The metal plates are placed adjacent to the glass or plastic walls of the chamber which serve as the dielectrics for the capacitors. For example, aluminum foil or metal sheet electrodes can be placed on the top and bottom of a standard tissue culture flask and connected to a voltage source. Because of the relatively large impedances of the capacitors, most of the voltage applied to the metal plates appears across the capacitors and very little across the culture medium. Since the electric field in the culture medium is equal approximately to the voltage across the liquid divided by the liquid depth (for simplicity, we assume the flask is completely filled with culture medium), the electric field in the culture medium will be, in general, very small.

As Eq. (20) shows, the size of the displacement current in the medium is determined by the dimensions and dielectric properties of the system as well as by the frequency, but these properties are stable and therefore stable exposures require only a stable voltage source. Since no electrodes are in contact with the medium, there can be no problems with the toxic electrode products that occur when exposures are made with electrodes in the medium (see 5.2.3, Toxicity problems with in vitro electric field exposure systems). In addition, it is easier to maintain cells without contamination by fungi or bacteria. The major drawback of this system is that a modest current density of, for example, 1 mA/m², requires several thousand volts at 60 Hz for a typical configuration. Because such voltages are unwieldy and dangerous, this form of electric field generation is not used often. In addition, high voltages may produce corona with attendant ultraviolet light and ozone. Since both of these products are toxic to cells, it may be necessary to take precautions to eliminate the corona so that neither interacts with the cells.

Capacitive systems have been used in studies at moderately high field strengths within the medium by several researchers. Lymangrover et al. [1983] used voltages of up to 10 kV (60 Hz) to achieve an electric field in the medium of 17 mV/m (0.17 mV/cm), corresponding to current densities of 23 mA/m² (2.3 µA/cm²). Tobey et al. [1981] used a chamber in which applied voltages of up to 10 kV produced peak fields in the medium of up to 0.3 V/m (3 mV/cm) and a current density of up to 0.55 A/m^2 (55 $\mu A/cm^2$). The "effects" observed during this study were later thought to be an artifact due to corona because they could not be replicated with a currentpassing electrode system. In contrast to these high voltage systems, Fitzsimmons et al. [1986] used a capacitive system energized directly by the low voltages at the output terminals of an electronic oscillator (≈10 V) in studies for which the electric field strength in the culture medium was estimated to be about 10^{-5} V/m.

6. MEASUREMENTS OF ELECTRIC FIELDS OF IN VITRO SYSTEMS

By definition, the electric field strength at a point in the culture medium is the potential gradient at that point. Although calculations can usually indicate the average field strength in the medium to reasonable accuracy, a detailed characterization of the field is especially important in small chambers. Unfortunately, when working in small chambers with small probes there are considerable technical difficulties in making direct measurements of the electric field strength.

Because voltmeters can only measure potential differences, electric fields cannot be measured directly, but must be obtained from measurements of the change in electric potential from one point to another. When the distance between the two points

is small compared with the desired degree of spatial resolution, the potential difference provides a useful measure of the electric field on the scale of the distance between the points of measurement.

If the only number desired is an average electric field strength within the chamber, all that is necessary is to measure the potentials V_1 and V_2 at two widely separated points within the region of current flow and the separation of these points, $x_2 - x_1$. The quotient $(V_2 - V_1)/(x_2 - x_1)$ gives the average electric field over the distance $x_2 - x_1$.

However, if field uniformity is at issue, the field must be evaluated at a number of points within the chamber. For example, to evaluate for electric field non-uniformities of 10% or more over distances of 1 mm or less, it is necessary to measure pairs of potentials and the associated positions every millimeter over the area of the chamber. The uncertainty of each measurement must be significantly less than 10% so that the resulting quantity, $(V_2 - V_1)/(x_2 - x_1)$, is uncertain to less than 10%. By making measurements with a fixed pair of wires (a dipole), the quantity $(x_2 - x_1)$ is kept constant and subject only to a single measurement error, thereby improving the absolute accuracy and eliminating variability from one observation to the next. For a dipole pair, the numerator $(V_2 - V_1)$ is also determined immediately as a single differential quantity without the additional errors inherent in separate measurements of V_1 and V_2 . For these reasons, dipole probes are preferred for measurements of electric field strength in conducting media, although single wire probes can be used to measure the potentials at individual sites to obtain the potential gradient. A third method for determining the electric field strength involves the measurement of current and the cross sectional area of the culture medium. All three approaches are discussed in the following sections. Comparisons of measurements using the three approaches are discussed by Gundersen and Greenebaum [1985].

6.1 Measurements of Electric Field Uniformity and Strength in Conducting Media Using Dipole Probes

The determination of the electric field uniformity in an in vitro system is best made by using a small dipole probe in a chamber filled with a very dilute electrolyte solution. The separation between the dipole electrodes sets a lower limit to the spatial resolution of the map. The diluted solution is used because, for a given current density, the magnitude of the measured potential difference is enhanced by the higher resistivity of the dilute solution. Without this "trick" there would be an impractically small potential difference (<1 mV) for a 1 mm dipole at a typical current density. A 1:100 dilution of the normal electrolyte solution (i.e., that intended for the exposure study) into water and a current density of 100 μ A/cm² often yield satisfactory conditions. The goal of the measurements is to develop a "map" showing spatial variations in the field over the region used for exposure of the biological specimen.

The principles set out below describe problems encountered in using small dipole probes. These principles can be easily modified for a dipole probe with large separation between the electrodes. The large dipole probe would be useful for accurate determination of the average electric field strength in the physiological medium actually used for the exposure study. In contrast, the small dipole probes used for mapping are inherently less accurate for absolute measurements of the electric field strength because of difficulty in accurately measuring the smaller separations of the

electrodes and the smaller potential differences in the higher conductivity physiological medium used for exposure purposes.

Because the metal electrodes of the dipole probe extend from the surface to some depth, distortions in the liquid surface by the probe wires can affect measurements. The probe wires perturb the field they are measuring because the probe wires displace medium and provide a lower resistance path than the medium. There is also a local disturbance of the surface tension at the point where the wires penetrate the surface. If the ratio of wire diameter to separation is too large, these perturbations may be significant. In practice a ratio of at least 5:1, as in the use of 0.020 cm diameter wires separated by > 1.0 mm, is sufficient. For optimum operation of the dipole probe, it is necessary to minimize the potential difference across the wire probe/culture medium interface. This, it is recalled (see 5.2.1, Surface current density and electrode resistance), is achieved by minimizing the current density across the interface. Immersion of the probe wires to sufficient depth (i.e., voltage readings are independent of depth) and use of high input impedance voltage measurement systems are both methods for reducing the current density [Gundersen and Greenebaum, 1985]. Before use, the probe should be tested for the degree to which readings are independent of depth of immersion and to assure that the electrodes are noise-free and stable enough for potentiometric use. This immersion test should be performed in a region of the culture medium where the electric field is nearly uniform, i.e., independent of depth.

A dipole probe with a separation of about 1 mm can be made from a pair of metal wires (silver or silver-silver chloride for stable performance) cemented to the outside of a glass capillary tube support about 1 mm in diameter (Fig. 27a). To minimize noise signals from stray background electric fields, the insulated portion of the probe wires can be electrically shielded. The tubes used for microelectrodes in electrophysiology make good supports. The ends of the wires should extend several millimeters beyond the edge of the supporting glass tube so that the liquid surface is not disturbed by the glass tube. Wetting of the glass support may also contribute to the shorting of the electrodes outside the medium and should be avoided. To reduce the chance of breakage, all but the last few millimeters of the glass tube may be reinforced by placing it inside a plastic tube made of a semi-rigid material like polyethylene or a heavy gauge heat-shrinkable tubing. A slightly different construction of a dipole probe is described in the paper by Gundersen and Greenebaum [1985].

Before use, the separation of the wires is measured using a microscope with a graduated reticle. Considerable care needs to be taken so that the probe wires are not struck during use, thereby requiring re-measurement and re-calibration of the probe.

In order to make simultaneous measurements of the two orthogonal horizontal components of the field, it is useful to make a three wire probe such that the three parallel wires lie in two orthogonal planes. The central wire lies in the intersection of the two planes and is electrically common to the other two wires. It is best to have a pair of voltmeters with high input impedances to allow simultaneous readings of the voltages from the two orthogonal directions. Figure 27b shows an arrangement in which the common wire lies in the lumen of a micropipette and two other wires are fixed at orthogonal positions on the outside of the tube. An alternative probe construction for simultaneous measurements of two field components and the connections to two voltmeters is shown in Figure 27c.

foil) at opposite ends of a rectangular chamber (Fig. 20a) create uniform, accurately determined fields. The magnitude of the field in the calibration chamber is obtained from precise measurements of the total current, chamber dimensions, electrolyte resistivity, and by computing E using Ohm's Law, $E = \rho J$. Precautions should be taken to control for evaporation during the calibration process; use of an electrolyte having conductivity similar to that in the exposure chamber is recommended.

A check for electrical pickup by the dipole probe should be made during calibrations by observing whether the voltage reading from the dipole probe is zero when the current through the calibration chamber is removed. If a substantial amount of stray pickup occurs and is not eliminated, the electric field readings will be in error. During calibrations, the waveform of the current through the calibration chamber should also be monitored with an oscilloscope for evidence of distortion. Distortion of the waveform is more likely to occur during calibrations in a low conductivity solution since a much larger driving voltage must be applied to the electrodes and in such cases the voltage capabilities of the amplifier may be exceeded.

6.2 Measurement of Electric Field Using Single Electrode Probe

It is also possible to map the electric field using a single electrode which then shows the potential difference between a location in the chamber and the potential in the metal phase of the reference electrode. As a result, the measured potential differences also reflect the reference electrode's polarization resistance, which may be unstable, especially if the current-passing electrode is chosen to serve as the reference electrode as well. Since this technique does not measure the potential difference in the liquid phase using equivalent electrodes, it is subject to errors arising from changing electrode polarization and is inherently less desirable. The reference potential in this system may be common for both the voltmeter and the current source, that is, both circuits may be grounded in common for the purposes of mapping the field. In this case, when the test probe is close to the electrode energized by the current source, the measured values will be very large, while measurements made close to the other electrode-which must be at the same potential as the voltmeter ground-will yield very small voltages and greater inaccuracies. These inaccuracies may not be significant, however, if the electric field region of interest is at points well removed from the electrodes. Finally, this technique does not permit simultaneous measurements of the two orthogonal components. Thus, the single probe technique, and especially use of a current-passing electrode as the reference electrode, present some disadvantages when compared with the dipole method for characterizing electric fields in an experimental chamber. With care, however, useful data can be obtained with a single-axis probe [Gundersen and Greenebaum, 1985].

6.3 Determining Electric Field From Resistivity and Current Density Measurements

We briefly note here that electric fields of known value can be established in the culture medium by simple calculations from a few measured quantities. The electric field in an exposure chamber with rectangular geometry and with electrodes that match the shape of the culture medium (Fig. 20a) is readily determined from measurements of the total current and cross sectional area (to obtain current density) and resistivity, and the use of Ohm's Law, $E = \rho J$.

6.4 Measurement of Electric Fields Induced by Magnetic Fields

When a magnetic field is used to induce the electric field in culture medium (see Section 3, Exposure Systems For In Vitro Studies With Magnetic Fields), measurement of the electric field is complicated by the presence of the magnetic field. Despite the added difficulties, the induced electric field can be measured (and should be measured if any deviation from simple patterns, such as those for a circular cylinder with *B* vertical, occur). Small probes with two or more electrodes have successfully been employed by several research groups for measuring induced electric fields associated with in vitro studies, including McLeod et al. [1983], Miller [1991a,b], and Bassen, et al. [1992].

For induced electric fields, the voltages measured are typically small. For example, from Eq. (10) for a cylindrical dish 10 cm in diameter with a 60 Hz, 1 millitesla magnetic field parallel to its axis, the maximum induced electric field at the edge of the dish is 94 μ V/cm. If a differential probe with a 0.4 cm electrode spacing were used to measure this field, the voltage would be only 37.7 μ V. Such small voltages can be difficult to measure without specialized laboratory equipment. In addition, the "trick" mentioned earlier (see 6.1, Measurement of Electric Field Uniformity and Strength in Conducting Media Using Dipole Probes), which involves lowering the conductivity of a medium in a constant-current system in order to increase the voltage across the electrodes, does not work for magnetically induced electric fields because such fields are independent of conductivity [see Eq. (10)]. The use of relatively high conductivities such as that of isotonic saline (1.3 S/m) actually helps with low voltage measurements because the medium reduces the impedance of the probe and thus the noise voltages due to magnetic pickup (see below).

Measurement of the relatively low voltages noted above is further complicated by magnetic induction of voltages in the wires used to measure the induced electric fields. For example, Eq. (13) gives 37.7μ V for the voltage induced in a loop of wire 1 cm² in area in a 60 Hz, 1 millitesla field perpendicular to the plane of the loop. Note that this background noise (''pickup'') is of the same magnitude as the expected maximum signal from the 10 cm dish described above. The problem is so severe for the single electrode method described above (see 6.2, Measurement of Electric Field Using Single Electrode Probe) that the single electrode method cannot be used for magnetically induced electric field systems. To reduce the pickup problem which arises even with dipole probes, care must be taken to twist the leads and use a probe geometry which minimizes the area of any loop (of any shape) which is crossed by the magnetic fields. In addition, exceptional care must be taken to avoid forming loops in the ground wires (i.e., ''ground loops''). These loops are often relatively large in area so that even stray magnetic fields can induce artifactual voltages that obscure the desired signal from the electric field induced in the liquid medium.

6.5 Reporting Exposure Conditions

The favored graphic presentations of the results are either as electric field vectors at selected points in the chamber or as contour lines showing the isopotentials. An example where equipotential contours are indicated is shown in Figure 28. For cell culture work it may be useful to determine the percentage of total volume (for suspended cells) or of total area (for adherent cells) within which the field has a given value. Such percentile scores are a good way to characterize the uniformity of ex-



Fig. 28. Equipotential lines in an in vitro electrophysiology chamber 26 mm diameter, with two silver/silver chloride electrodes, EL. The equipotentials, shown at 20 mV increments were obtained using a saline solution diluted 1:100. A tissue sample (dotted line) was located in the nearly uniform central region. The current flow is slightly influenced by the inlet line, IN, and a mound of insulating adhesive sealant in which a thermistor, T, was buried. End effects at the electrodes affected current flow close to the electrodes, but the location of the drain, D, behind the electrode caused no discernible effects on the current flow.

posure. The extremes of field strength and the fraction of the area or volume in which they occur can also be reported.

7. RELATING IN VITRO AND IN VIVO EXPOSURE CONDITIONS

The goal of this section is to outline general aspects of the ELF field dosimetry and to compare the biophysical parameters for human beings and animals as they pertain to in vitro experiments. Such information is critical for rational development of exposure systems and selection of appropriate exposure parameters. Since one objective of in vitro experiments can be to simulate internal in vivo exposure conditions, dosimetric information is needed for extrapolation of laboratory in vitro experimental results to environmental exposures of humans. In addition, this information is needed for the consideration of potential mechanisms of biological effects of ELF fields.

Exposures of human beings and animals to ELF electric or magnetic fields in air are typically described in terms of the external field direction and rms magnitude in the absence of the body. This description of external exposure is unfortunately of little value for communicating the internal exposure parameters critical for bioeffects stud-

ies. An external electric field is greatly attenuated inside the body and enhanced at the surface, owing to the high electrical conductivity of the body relative to air. In contrast, external magnetic fields penetrate the body virtually unperturbed and induce small electric fields and currents inside the body. These small electric fields and currents induced by an externally applied ELF electric or magnetic field constitute the candidate internal exposure parameters of cells and tissue. In the latter case, the magnetic field must also be considered a candidate exposure parameter. The induced internal dosimetric parameters are proportional to the external field strength but are also strongly dependent on the size, shape, and conductivity of the body. During in vitro studies, similar considerations apply to the size and shape of cell suspensions. However, a greater degree of control is possible in the range of parameters, e.g., in the relative magnitudes of the induced current and electric field, and the magnetic field in the medium (see 3.2.1, Experimental geometry and exposure parameters).

External electric field exposure produces an internal electric field E and current density J. A surface charge is also generated on an animal, but this aspect of an in vivo exposure is not usually simulated in vitro. Magnetic field exposure produces an internal electric field and current density, and in addition involves the presence of the magnetic field B itself. Which of these three internal dosimetric quantities (E, J, or B) is of interest in a particular experiment depends on the situation under consideration and the mechanisms of interest. For example, for consideration of the proposed ion cyclotron resonance mechanism [Liboff, 1985], the induced electric field and current might be unimportant, and other parameters like the local geomagnetic field might be important. Dosimetric determination, in vivo or in vitro, of the value of B itself is trivial, since it is unperturbed even inside very large animals, and so we will concentrate on the dosimetry of the induced electric field and currents.

The electric field and current density are related by Ohm's law, $J = \sigma E$, in which σ is the conductivity of the medium. Typical values of σ are 0.013 S/m for bone, 0.13 S/m for liver, and 0.6 S/m for blood (Table 2). An overall in vivo average value is 0.2 S/m [Tenforde and Kaune, 1987]. For comparison, the conductivity of isotonic saline, and culture media such as F-12 with 10% fetal bovine serum added, are about 1.4 S/m.

The currents and fields induced in vivo by ELF electric and magnetic fields are difficult to calculate or measure in realistic detail, and therefore these internal parameters have normally been estimated from calculations and measurements made on homogeneous models. Induced currents have been measured for 60 Hz electric field exposure [Kaune and Forsythe, 1985]. For electric field exposure, the induced current is not dependent on the size or conductivity of the body but is strongly dependent on its shape. Using a model filled with saline solution, the average total induced current in the torso of a standing human with grounded feet (the worst-case situation), in a vertical field of 10 kV/m, was found to be about 2.5 mA/m² [Kaune and Forsythe, 1985]. This current density yields an electric field of 12.5 mV/m in a 0.2 S/m medium. The induced current is independent of conductivity, and exists throughout the body, as illustrated in Figure 29. There is considerable variation in the magnitude of the current in different positions, with higher values in the neck and especially the ankles for grounded feet (about six times higher than in the torso). As indicated by the arrows, the direction is primarily vertical, except near the arms (of course, the directions reverse each half cycle of the 60 Hz alternating field). For a similar exposure of a rat, the induced current was 0.2 mA/m² in the torso of a saline model

Field type	Human exposure	Rat	Induced internal electric field	
Industrial:				
Electric	10 kV/m	125 kV/m	12 mV/m	
Magnetic	0.2 mT	1.0 mT	12 mV/m	
Residential:				
Electric	100 V/m	1.2 kV/m	120 µV/m	
Magnetic	0.2 μT	1.0 µT	12 μV/m	

TABLE 3. Comparison of Typical 60 Hz Electric and Magnetic Field Exposures to Human Beings, Exposure Levels for Equivalent Dosimetric Treatment of Rats, and Representative Induced Electric Fields Inside the Human Body Estimated From Homogeneous Models

tric and magnetic fields. Of course, testing at higher fields might be of interest in simulating conditions in which currents are applied directly to the body (e.g., in electrostimulation) or for basic research where an understanding of mechanisms rather than exact simulation of exposure is the goal. The general considerations presented here provide guidance for selecting relevant levels of exposure parameters during in vitro studies; however, specific systems and parameters must be devised for addressing the unique problems of each specific scientific question under study.

8. CONCLUSIONS

The conclusions drawn from this primer can be expressed very briefly. There is a multitude of parameters that can affect the outcome of biological experiments with ELF magnetic and electric fields, and it requires researchers from the disciplines of biology and physics/electrical engineering, working collaboratively, to adequately characterize and report them. For example, we have seen that details of the culture medium geometry and magnetic field direction significantly influence the magnitude and direction of the induced electric field and current density in the culture medium, that such details as electrode contamination, humidity (by its effects on evaporation rate and pH), cell type (e.g., anchorage dependence), glassware or plasticware preparation, and incubator conditions (CO₂, airflow, and magnetic fields) can be significant, and that exposure of cells and tissue to significant levels of ambient magnetic fields during preparation should be avoided. We recognize that as long as the mechanisms for the reported biological effects remain unknown, determining which parameters are the relevant ones sometimes will be difficult and that it may be necessary to "overreport" the experimental conditions for now. The question that researchers must ask when reporting the results of their study is has enough information been provided to permit a valid replication of the study elsewhere.

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