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Experimental Requirements for *in vitro* Studies Aimed to Evaluate the Biological Effects of Radiofrequency Radiation

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1. Introduction

In the last years human exposure to electromagnetic fields (EMF) in the radiofrequency (RF) range has increased rapidly becoming unavoidable. As a matter of fact, many sources of RF fields are present at home, at work, and in the environment. In addition, other sources of occupational RF field exposure include equipments such as medical devices, dielectric heaters, induction heaters, diathermy machines, plasma discharge equipment and radars. Moreover, the rapidly increased use of mobile telecommunication has lead to exposure of a large amount of the population to RF fields. It has been estimated that 4.9 billions of mobile phone subscriptions will be active by the end of 2012. Although these technologies have highly improved the quality of life, at the same time they have given rise to great concern about possible health effects of such non-ionizing radiation at low exposure levels, with particular attention to cancer risk. As a matter of fact, heating is the most widely accepted mechanism of RF radiation with biological systems, and the current guidelines of human exposure are based on thermal effects, but subtle effects due to chronic exposures cannot be excluded. The latter, non thermal effects, are hypothesized to occur in the absence of local or whole body increases in temperature, although there are no generally accepted biophysical mechanisms that could explain such effects.

Three main approaches, epidemiological studies, in vivo studies and in vitro studies, providing different and complementary information, can be followed in addressing the evaluation of biological effects induced by exposure to RF fields. The epidemiological studies aim to test, on a statistical basis, whether a causal nexus between exposure to an environmental agent and its putative health effects on the health status of the exposed subjects could exist. They use specially designed studies that try to determine statistical

associations between independent (level of exposure) and dependent (health status, disease occurrence, etc.) variables by collecting data from population samples. In addition, in relation to RF-based wireless communications, there are two different exposure situations: to RF in the far field, emitted by base stations, WiFi access points, etc. and to RF in the near field, emitted by handheld devices (e.g. mobile phones). According to the World Health Organization publication on Electromagnetic Fields, Environmental Health Criteria series [1, 2], to proper address human health risk assessment, epidemiological research should allow for sufficient latency, sufficient range of exposure, including high exposure, and ability to accurately classify individuals into several exposure groups.

In vivo studies are carried out on human volunteers or animals and provide information concerning the interaction of RF radiation with living systems displaying the whole body functions, such as immune response, cardiovascular changes, and behaviour. There are obvious limitations in the exposure conditions to be tested on humans due to ethical issues, therefore most of the in vivo studies are carried out on laboratory animals (mainly rodents). However, extrapolation to humans to provide an estimation of health risk is not straightforward due to the differences in physiology and metabolism between species as well as differences in life expectancy and many other variables.

In vitro studies, carried out mainly on cell cultures or isolated tissue samples, are used extensively in toxicological investigations. This is because they can provide essential information about the potential effects of chemicals and other agents such as radiation on specific cell properties, and provide a more rapid and cost-effective approach to molecular and mechanistic studies than conventional laboratory animal models.

In the last 20 years, scientific investigations on whether RF radiation used in these technologies could have short-, medium- or long-term biological effects, and whether they could represent a health hazard to human population have largely increased, since any detectable detrimental effect of RF radiation, even small, could be very important, due to its widespread use, the large numbers of people exposed on a daily basis, and the social, economical and health impact this could have.

Recently, on May 2011, the International Agency for Research on Cancer (IARC) classified RF electromagnetic fields as possibly carcinogenic to humans (Group 2.B) [3, 4]. The IARC evaluated available literature about the carcinogenicity of RF electromagnetic fields and found the evidence to be "limited for carcinogenicity of RF-EMF, based on positive associations between glioma and acoustic neurinoma and long term exposure". The conclusion of the IARC was mainly based on the INTERPHONE epidemiological study, which found an increased risk for glioma in the highest category of heavy users (30 minutes per day over a 10 year period), although no increased risk was found at lower exposure. The evidence for other types of cancer was found to be "inadequate" [5]. In vivo and in vitro studies, carried out so far, have provided only limited support for the above mentioned classification [6], mainly due to the difficulty in comparing the results of the available studies to draw general conclusion. The difficulty often arises from the quality of the studies, in terms of study design, specific methodologies and analysis of the results.

In this chapter the focus is on in vitro studies, which are the studies providing insight into the basic mechanisms by which effects might be induced in more complex animal or human organisms. As a matter of fact, in vitro studies, carried out on tissue or cell cultures of animal or human origin, transformed or not transformed, are relatively simple and represent well described models, where the control of exposure and experimental conditions is significantly greater than in live animals or human volunteers.

In vitro studies, the most common in the evaluation of biological effects of RF radiation, are mainly aimed to investigate cellular endpoints related to cancer occurrence. Carcinogenesis is a multi-step process in which direct (genotoxic) and indirect (non-genotoxic) DNA damage is involved, as schematically depicted in figure 1. Therefore, cancer related studies can be classified as genotoxic and non-genotoxic. Genotoxic effects include DNA strand breaks, micronucleus formation, mutation and chromosomal aberration. Non-genotoxic effects refer to changes in cellular function, and include cell proliferation, oxidative metabolism, apoptosis or programmed cell death, cellular signal transduction, and gene expression (RNA and protein).
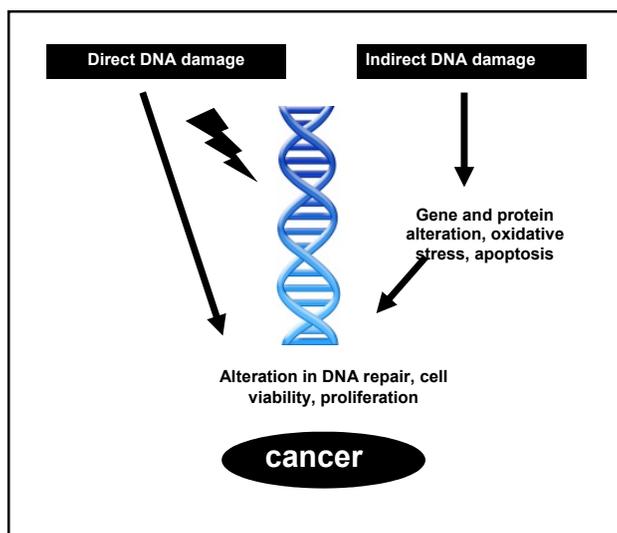

Figure 1. Schematic representation for genotoxic and non-genotoxic carcinogenesis

On the basis of the conclusions reported in the above mentioned reviews [1-3, 6], also confirmed by more recent scientific literature, the following considerations can be drawn. Results on genetic effects and other cellular endpoints, like cell proliferation and differentiation, apoptosis and cell transformation, are mainly negative, and some of the few positive findings may be attributable to a thermal insult rather than to the RF-exposure as such. As a matter of fact, some studies following replication, under more controlled conditions, failed to be confirmed by independent research groups [7]. The same is for expression of cancer-related genes (e.g., proto-oncogenes and tumor suppressor genes), and studies, carried out using powerful high-throughput screening techniques capable of examining changes in the expression of very large numbers of genes and proteins. It should be pointed out that the results achieved by high-throughput techniques need to be confirmed by quantitative methods since methodologies are not sufficiently standardized.

In order to improve the quality of in vitro studies and of the future research, high methodological quality is needed to address uncertainties in technical and biological aspects, and to determine whether the achieved results reflect "true" biological response or they are related to some unknown uncontrolled variable.

The aim of this chapter is to describe the main requirements for a well conducted in vitro study to overcome methodological limitations. Due to the inter-disciplinary nature of the bioelectromagnetic research, basically, biological and electromagnetic requirements can be identified.

2. Biological requirements

Cell cultures are mainly used as an inexhaustible source of experimental material. The use of cell cultures offers the advantage of investigating the specific interactions at cell or molecular level, and the ability to perform large series of experiments under the same conditions. Cell cultures are achieved through enzymatic or mechanical disaggregation of a tissue sample. Human and animal cell cultures are derived either from a primary explant or a cell line. A fresh isolate of cells which is cultured in vitro is called a 'primary culture' until cells are subcultured or passaged. Primary cell cultures are generally heterogeneous, with a low fraction of growing cells, but they contain a variety of cell types which are representative of the tissue. The subculture allows the propagation of the culture, which is now called a 'cell line': it appears to be more uniform, but specialized cells and functions can be lost. The greatest advantage of a cell line is the availability of a large amount of homogeneous material to be used for long periods of time due to their ability to propagate and divide into replicates. After several passages a cell line may die (finite cell line) or 'transform' to become an established or continuous cell line. Most of the continuous cell lines originate from neoplastic tissues, but several continuous cell lines are derived from normal embryonic tissue. They can be characterized and stored by freezing.

According to the Good Cell Culture Practice [8], the maintenance of high standards is fundamental to all good scientific practice, and is essential for ensuring the reproducibility, reliability, credibility, acceptance and proper application of any produced results. The standardization of cell culture is required, and is achieved by controlling the materials, such as cells and culture medium, that interact and determine the properties of the total system. However, the potential for variation can also be considered for each separate component. It is recommended that authenticated stocks of continuous cell lines are purchased from recognized national and international cell banks. Cell culture medium is a defined base solution including salts, aminoacids and sugars supplemented with cell type specific components. The more complex component is the serum that origins from a pool of donations taken from a large number of animals, thus expressing a large variability among different manufactures. Primary cell cultures require complex nutrient media, supplemented with animal serum and other non-defined components, thus primary cell culture systems are difficult to standardize. Immortalized cell lines, being able to multiply for extended periods, can be expanded and cryopreserved as cell bank deposits. They represent a more stable and reproducible system than primary cells.

A wide variety of cell types, ranging from stem cells to highly differentiated tissue specific cells, can be used. The appropriate cell model has to be used for specific experimental approaches for the proper identification of biological effect. It has to be chosen on the base of the cellular target investigated. For instance, human lymphocytes are largely employed, since they are of human origin and easy to obtain by venipuncture. They represent one of the best suited cell model for the investigation of genotoxic effects of chemical and physical agents, including EMF.

More than one endpoint have to be investigated, for each cellular target, also to balance mechanistic vs. toxicity studies. Thus, a combination of techniques, confirming and/or complementing each other, is recommended for the reliable detection of effects. For instance, to study direct DNA damage (genotoxicity), mainly cytogenetic techniques are employed, which allow to investigate the frequencies of chromosomal aberrations, sister chromatid exchanges and micronuclei whose increased level in human lymphocytes are predictive for cancer risk [9-12]. However, such assays essentially reveal severe genetic damage and could not be the optimal choice to detect most of the subtle indirect effects that may be induced by RF radiation. More sensitive techniques, like single cell gel electrophoresis (comet assay) or the detection of γ-H2AX phosphorylated histone, will help in this case. The alkaline version of the comet assay, first introduced in 1988 [13], allow to detect the combination of DNA single-strand breaks (SSBs), double-strand breaks (DSBs) and alkali-labile sites in the DNA, although the results and the interpretation of the biological relevance of the damage can be misleading if not compared with other measures of DNA damage. For the assay, cells are embedded in agarose on a microscope slide, lysed with detergent, subjected to electrophoresis and stained with a fluorescent DNA-binding dye and subsequently observed by fluorescence microscopy. Negatively charged loops/fragments of DNA migrate out of the nuclei forming a tail in the direction of the anode, giving the nuclei the appearance of a comet [14]. In Figure 2 human lymphocytes processed for comet assay are shown.

The phosphorylation of the histone variant H2AX to γ-H2AX–containing nucleosomes [15] is one of the earliest marks of a DNA double-strand break in eukaryotes. γ-H2AX is essential for the efficient recognition and/or repair of DNA double-strand breaks and many molecules, often thousands, of H2AX become rapidly phosphorylated at the site of each nascent double-strand break. It was shown that this simple method was suitable to monitor response to radiation or other DNA-damaging agents [16].

As for cytogenetic techniques, there is no single parameter that defines other cellular functions related to carcinogenesis, like apoptosis, cell viability and oxidative stress. Therefore a combination of techniques is recommended for their reliable detection to ensure valid conclusions.

Apoptosis is a process of programmed cell death that is essential in the shaping of organs during embryonal development and in the maintenance of tissue homeostasis in adult life [17]. It also occurs as a response to an insult, and is implicated in diseases such as cancer [18]. The main feature of apoptotic cell death is the fast and efficient removal of dying cells

by macrophages. This process ensures the uptake of death-destined cells before their membrane lysis, thus preventing inflammation and homeostasis disturbance [19].

Figure 2. Ethidium bromide stained human lymphocytes processed for comet assay, as appear under fluorescence microscope after treatment with 0 (top left), 5 (top right), 10 (bottom left) and 25 (bottom right) μM methylmethanesulphonate (MMS), a well known DNA damaging agent. Cells experiencing increasing doses of MMS show increasing DNA migration (tails)

Apoptotic cells have to be first recognized by the characteristic change. Then, using timed inductions, and comparing relationships between cell populations expressing multiple endpoints aimed to evaluate biochemical changes, it is possible to estimate the relative order in which the different aspects of an apoptotic process become evident within a given cell model.

In the case of cell viability, assays that measure membrane integrity and metabolic activity are needed [20]. Modification of the oxidative status of cells should be monitored by measuring Reactive Oxygen Species (ROS) formation together with the activity of antioxidant enzymes and concentration of antioxidant molecules [21].

A general requirement for the biological assay in a well designed in vitro experiment is the high sensitivity, and particular care must be devoted to set up accurate experimental control samples. Negative and positive controls provide evidence for controlled experimental conditions, as for generic toxicological investigations, moreover in bioelectromagnetic experiments it is preferable to use also sham exposure as a control condition.

As a matter of fact, negative control samples (cell cultures placed in standard cell culture CO_2 incubator) provide information on the background level of the endpoint under examination; positive control samples (cell cultures treated with a well known agent inducing the effect under investigation) provide evidence that the cells respond to the damaging agent, and the biological technique is carried out in the proper way, able to show effect when induced. The sham control samples (cell cultures placed in a RF exposure device identical to the one employed for the exposure but with zero field) represent the true

control, taking into account the microenvironment in the exposure device that could affect the cellular endpoint under examination.

Furthermore, it is mandatory to perform experiments in a blind manner to minimize experimenter bias: experiments have to be carried out with samples coded so that their treatment group is unknown until the data are analyzed. This is of crucial importance in any comparison between laboratories and especially when a slight variation is expected, as for RF exposures.

Analysis of the results also represents a critical aspect of an in vitro study. Although, in some cases, the results may be so clear-cut that it is obvious that any statistical analysis would not alter the interpretation, the results of most experiments should be assessed by an appropriate statistical analysis. The aim is to extract all the information present in the data, in such a way that it can be interpreted, taking account of biological variability and measurement error. Appropriate statistical tools must be used when designing a study, in order to evaluate the properties (power, bias, variance) of the statistical test. Sample sizes are of crucial importance and should be based on the expected variation. Both the number of parallel samples during the experiment, and the number of independent replicates of an experiment have to be considered.

The method of statistical analysis depends on the purpose of the study, the design of the experiment, and the nature of the resulting data. Quantitative data are usually summarised in terms of the mean, n (the number of samples), and the standard deviation as a measure of variation. The median, and the inter-quartile range may be preferable for data which are clearly skewed. The statistical analysis is usually used to assess whether the means, medians or distributions of the different treatment groups differ. Quantitative data can be analysed by using parametric methods, such as the t test or the analysis of variance, or by using non-parametric methods, such as the Mann-Whitney test. Parametric tests are usually more versatile and more powerful, so are preferred, but depend on the assumptions that the variances are approximately the same in each group, that the residuals (i.e. deviation of each observation from its group mean) have a normal distribution, and that the observations are independent of each other. Non parametric tests are usually employed for data not normally distributed [22].

The magnitude of any significant effects should always be quoted, with a confidence interval, standard deviation or standard error to indicate its precision, and exact p-values should normally be given. It should be considered that it is possible for an effect to be statistically significant, but of little or no biological importance. Lack of statistical significance should not be used to claim that an effect does not exist, because this may be due to the experiment being too small or the experimental material being too variable. Where an effect is not statistically significant, a power analysis can sometimes be used to show the size of biological effect that the experiment was probably capable of detecting.

On the whole, a balance between statistical and biological significance of the detected effect has to be taken into consideration to draw valid conclusions.

3. Electromagnetic requirements

The design and realization of the RF exposure set up is another critical aspect to take into account, since well defined and characterized exposure conditions are needed for reproducible and scientifically valuable results, and represent the bases for health risk assessment [23]. Moreover, though the World Health Organization (WHO) in the EM Field Project has emphasized the importance of accurate dosimetry in the study of biological effects of RF radiation [24], this aspect has been underestimated by research groups for a long time, thus preventing possible comparison among results gained under different conditions.

Exposure systems employed in bioelectromagnetic research have not been standardized, due to the different biological tests and protocols to be conducted. However, general guidelines and minimal requirements have been defined and published in the literature [25-28] suggesting specific procedures and methods to be followed in the realization of RF in vitro exposure setups in order to pursue reliability and reproducibility of the results.

In general, the design and realization of an RF exposure system for in vitro bioelectromagnetic experiments is driven by the electromagnetic conditions to be reproduced (frequency, modulation scheme, required SAR level inside the sample, polarization of the EM field with respect to the sample, duration of the exposure), which are generally defined on the basis of specific "real life" conditions (exposure to EM fields employed for communication systems or for therapeutic applications), and by the biological protocols and assays to be carried out (number of sample to be exposed at the same time, biological test to be conducted off-line or in real-time with the exposure). All these conditions are relevant towards the selection of the hardware and software solutions to be implemented in the experimental set up.

An RF exposure set up is usually made up with the following basic elements:

- an RF source, which allows to set the main characteristics of the signal (frequency, amplitude, modulation scheme);
- active or passive components for the signal conditioning (amplifiers or attenuators, couplers, splitters, etc…);
- components for monitoring and adjusting the signal according to pre-defined requirements (power meters, PC for remote control, etc…);
- RF applicator, i.e. the structure that allows the propagation of EM field and the sample exposure (waveguide, TEM cells, wire patch cells, etc…);
- components for monitoring the relevant biological and environmental parameters (temperature, CO_2, humidity, etc…).

For a proper choice of the components listed above both biological and electromagnetic aspects must be taken into account, and this requires a strict cooperation between biologists and engineers. As a matter of facts, RF exposure setups for in vitro studies must comply with the basic requirements for maintaining cell cultures (temperature, pH, CO_2 concentration and humidity). These can be gained by placing the exposure unit inside an

ordinary cell culture incubator or by providing the unit with equipment to maintain the environment required for cell culture. Generally, cell cultures are placed in Petri dishes or flasks, and different technical solutions are chosen as RF applicator taking into account the volume efficiency e.g. the ratio between the sample area (for adherent cells) or volume (for floating cells) versus the space requirements for the entire exposure unit. The volume is critical since if the volume efficiency is scanty, the setup cannot be placed inside an incubator, which would increase the effort for environmental control [27]. On the other hand, any RF exposure system must assure uniform and well defined exposure conditions for the entire cell population, in order to allow adequate interpretation and reproducibility of the results. This can be achieved by means of accurate dosimetric analyses of the experimental conditions.

Dosimetry is the evaluation of the magnitude (dose) and distribution of electromagnetic energy absorbed by the exposed biological sample, when the characteristics of the incident electromagnetic field (frequency, modulation, polarization), the physical and electromagnetic properties (mass density, dielectric permittivity and conductivity) of the materials and the environmental conditions in which the exposure takes place are known.

The specific absorption rate or SAR (expressed in W/kg or mW/g) represents the basic dosimetric quantity, and is formally defined as the time derivative of the incremental energy absorbed by an incremental mass contained in a volume of a given density [25]. The SAR is related to the electric field (E) induced in the sample as well as to the heating rate, as described by the following equations:

$$SAR = \frac{\sigma}{\rho}E^2$$

$$SAR = c\frac{dT}{dt}$$

where σ, ρ and c are the electric conductivity (S/m), the mass density (kg/m^3) and the specific heat capacity (kcal/kg·°C) of the sample material, respectively and E is the root-mean-square value of the induced electric field.

Moreover, SAR is the reference parameter for the international regulations regarding the protection against electromagnetic fields, and it is suitable to compare the biological effects observed under different exposure conditions. Thus, a well defined characterization of the power deposition pattern inside the sample is mandatory towards the accomplishment of reliable results. Moreover, the performance of the exposure system can be assessed by considering:

• the uniformity of SAR distribution inside the sample, which must be as high as possible, although an overall standard deviation from homogeneity of less than 30% is considered acceptable [26];
• the SAR efficiency, which is defined as the ratio between the average SAR and the input power at the feeding end of the RF applicator, and can be increased by optimizing the coupling condition between the induced electromagnetic field and the sample;

- the thermal increase in the biological sample, which, in the framework of the evaluation of non-thermal effects of EM fields, should be insignificant (< 0.1 °C). This means that either the SAR level throughout the exposure must be low enough to avoid sample heating, or the exposure system must be provided with specific thermoregulation tools that counteract the undesired thermal increases.

Various procedures, both numerical and experimental, are available for dosimetric analyses.

With the development of 3D simulation tools for EM fields, numerical dosimetry has become essential for the design of RF applicators. Different numerical approaches can be employed, either in the time domain (Finite Integration Technique, FIT; finite difference time domain, FDTD) or in the frequency domain (finite element method, FEM). In all cases, Maxwell's equations are solved by partitioning the space into subdomains where solutions can be found more easily and more efficiently. The use of computational codes and modern computers has greatly improved the performance of this type of analysis, allowing on the one hand, the representation of increasingly accurate and realistic models of exposed systems, and on the other hand, fast and efficient resolution of complex electromagnetic problems. This numerical analysis requires: 1) the creation of a geometrical model of the RF applicator and of the biological sample, 2) the knowledge of the electromagnetic (dielectric permittivity, electric conductivity, magnetic permeability) and physical (mass density) properties of the materials, 3) the imposition of boundary conditions (electric, magnetic, absorbing, periodic, etc.) describing the operative space of the simulation and 4) the discretization of the structure to be simulated in mesh cells that define the computational domain. All these aspects must be precisely defined and critically chosen for gaining accurate results while keeping down the time required for the analysis. The final results of the numerical dosimetry is the field level and distribution inside the sample with a certain level of accuracy. An example is reported in figure 3, showing the electric field distribution, calculated by means of the FIT technique, in the cross-sectional area of a cell culture exposed to an RF, 1950 MHz EM field inside a short-circuited waveguide.

Numerical results have to be validated by means of experimental procedures measuring the dosimetric quantities directly in the exposed sample (experimental dosimetry). Local SAR measurements in in vitro exposure systems are usually carried out by means of thermal sensors: fiber optic thermometers and thermocouples are employed for local temperature measurements, which are performed in a number of points throughout the sample for mapping the spatial distribution of SAR; infrared cameras can be used to detect the heating pattern on the upper surface of the sample. Moreover, evaluations of the average SAR are also performed by measuring the S parameters of the applicator when loaded with the sample. Examples of experimental dosimetry have been reported in previous papers [29, 30].

The choice of the RF applicator is particularly critical. Different solutions can be devised depending on several factors, such as the number of samples to be contemporaneously exposed, the possibility of hosting the structure inside an incubator or of providing it with tools for maintaining environmental conditions, the polarization of the EM field with respect to the sample, the required SAR efficiency and homogeneity.
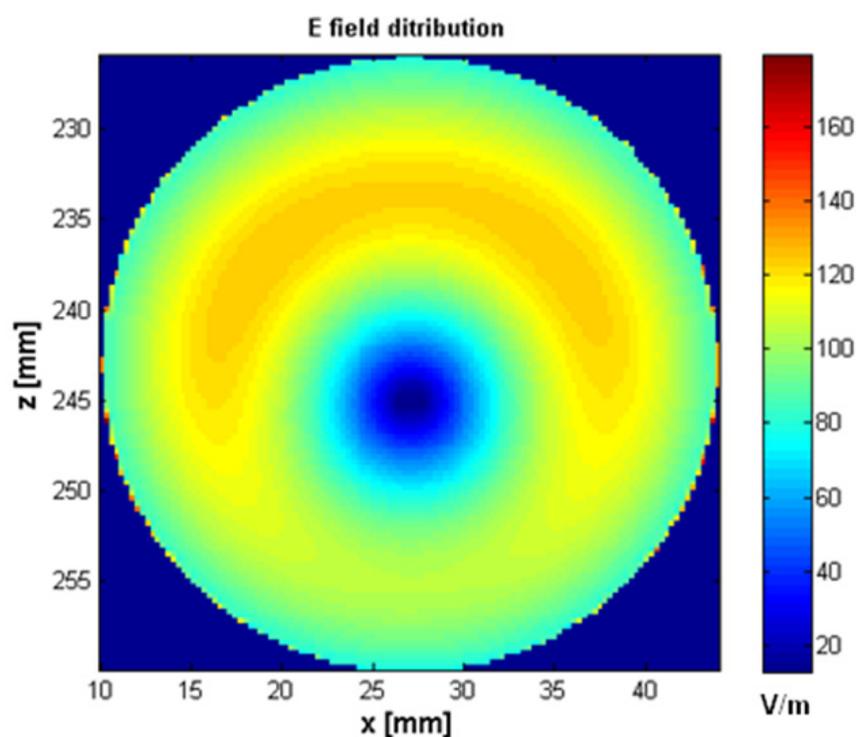
Figure 3. Electric field distribution in a simulated biological sample under 1950 MHz radiofrequency field in a high efficiency and uniformity applicator (FIT method).

Different kinds of RF applicators are used in bioelectromagnetics, that can be generally classified in radiating, propagating or resonant systems, as suggested by [28]. Among them, transverse electromagnetic (TEM) cells, waveguides (mainly rectangular, but also circular, radial and coplanar), radial transmission lines and wire patch cells are the most used for their versatility and good performance. Both TEM cells and rectangular waveguides can be placed in commercial incubators and host the most common cell culture containers (Petri dishes, flasks, multiwells). TEM cells (figure 4) provide exposure conditions similar to the free-space and are very versatile. They provide high performance in terms of homogeneity of SAR distribution especially when used with low number of samples [31].

Waveguide-based set up are also widely used in bioelectromagnetic studies: cell culture holders can be oriented in either E, H or k polarization, and the structure operates over wide frequency ranges.

Beyond working as propagating structure, rectangular waveguides are also widely used as resonant structures, allowing standing wave exposures. This is achieved by terminating one end of the waveguide with a short circuiting plate and allows to increase the efficiency of the applicator. Since they are based on resonance, the operative frequency band is quite narrow, and the performance is strongly affected by the position and size of the biological sample, but, in spite of this, SAR homogeneity is increased. Optimized systems have been described (27, 28, 29, 32, 33). In figure 5a, waveguides allowing simultaneous exposure of four cell culture dishes, currently employed in our laboratory, are shown. Petri dishes are

Figure 4. TEM cell placed vertically inside a cell culture incubator. TEM cell hosts, on both right and left side of the septum, two pyrex flasks over a 1.5 mm thickness plexiglas shelf.

placed on a four-layer Plexiglass stand (figure 5b), and two different SAR values are available at the same time by exploiting the symmetries of the waveguide and the unperturbed TE10 fundamental mode, as well as those of the cell container.

a)   b)

Figure 5. Short-circuited waveguides hosted in a cell culture incubator (panel a) and sample holder hosting cell cultures to be placed in the waveguide (panel b)

The Radial Transmission Line (RTL) consists of a circular parallel plate applicator, driven at its center by a conical antenna and terminated radially by microwave absorbers or a

matching load [34]. It can be used for a wide frequency band, and several samples can be exposed at the same time.

The wire patch cell (WPC) is made up with parallel plates fed in the centre and short-circuited by special props at the corns, resulting in large E fields between the plates [35]. In comparison with a TEM cell, this device generates energy of high levels. Furthermore, WPCs can easily be built and used inside incubators because of their small size and the simplicity of its structure. As a matter of fact, it can be placed into an incubator, leading to better ventilation for cell samples and avoiding a possible temperature increase inherent to closed systems. WPC also allows simultaneous exposure of several biological samples to the same energy level, thus enhancing the statistical power of biological studies. Figure 6 shows the WPC that our research group employed as RF applicator in the four-channel exposure setup, realized in the replication study performed in the framework of a Cooperative Research and Development Agreement (CRADA) between the Cellular Telecommunications & Internet Association (CTIA) and the U.S. Food and Drug Administration [30].
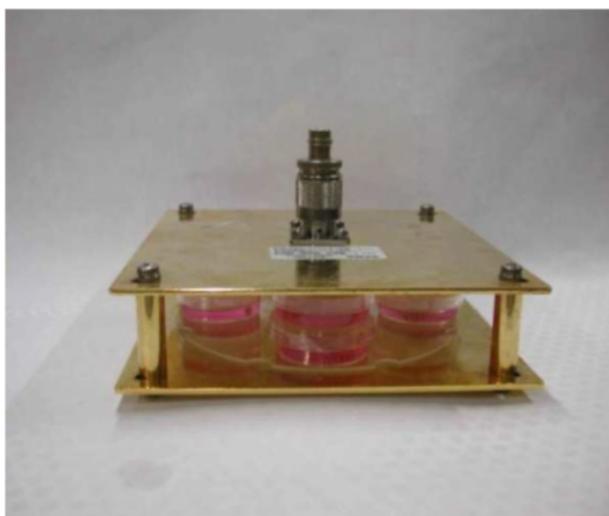

Figure 6.   Wire patch cell able to host 4 Petri dishes.

An important feature of an RF exposure setup is the stability of electromagnetic exposure, which depends on a number of details, not always strictly controllable, e.g., location of the flasks with respect to the exposure unit, amount of cell culture medium, changes of the dielectric properties of the medium, amplifier and frequency drift, and others. Therefore, well-defined mechanical properties of the exposure chamber and continuous monitoring of exposure conditions are a prerequisite for high-quality experiments.

Special attention must be paid to temperature control. For plastic flasks surrounded mainly by air, the thermal coupling between the medium and the temperature controlled environment is poor, and even SAR values much below 2 W/kg may result in an unacceptable temperature rises [32, 34]. In some cases, RF exposure chamber are equipped with circulating water jacket to counteract undesired temperature increase inside the cell cultures, and temperature is monitored in dummy cultures through the RF exposure period, by means of fiber-optic thermometers, not perturbing the EMF. Additionally, identical

environmental parameters for exposure and sham must be ensured, e.g., temperature differences between exposure and sham should be less than 0.1° C.

The main steps in designing and realizing an experimental set up for in vitro studies, in which the connection between biological and electromagnetic requirements are emphasized, are schematically summarized in figure 7.
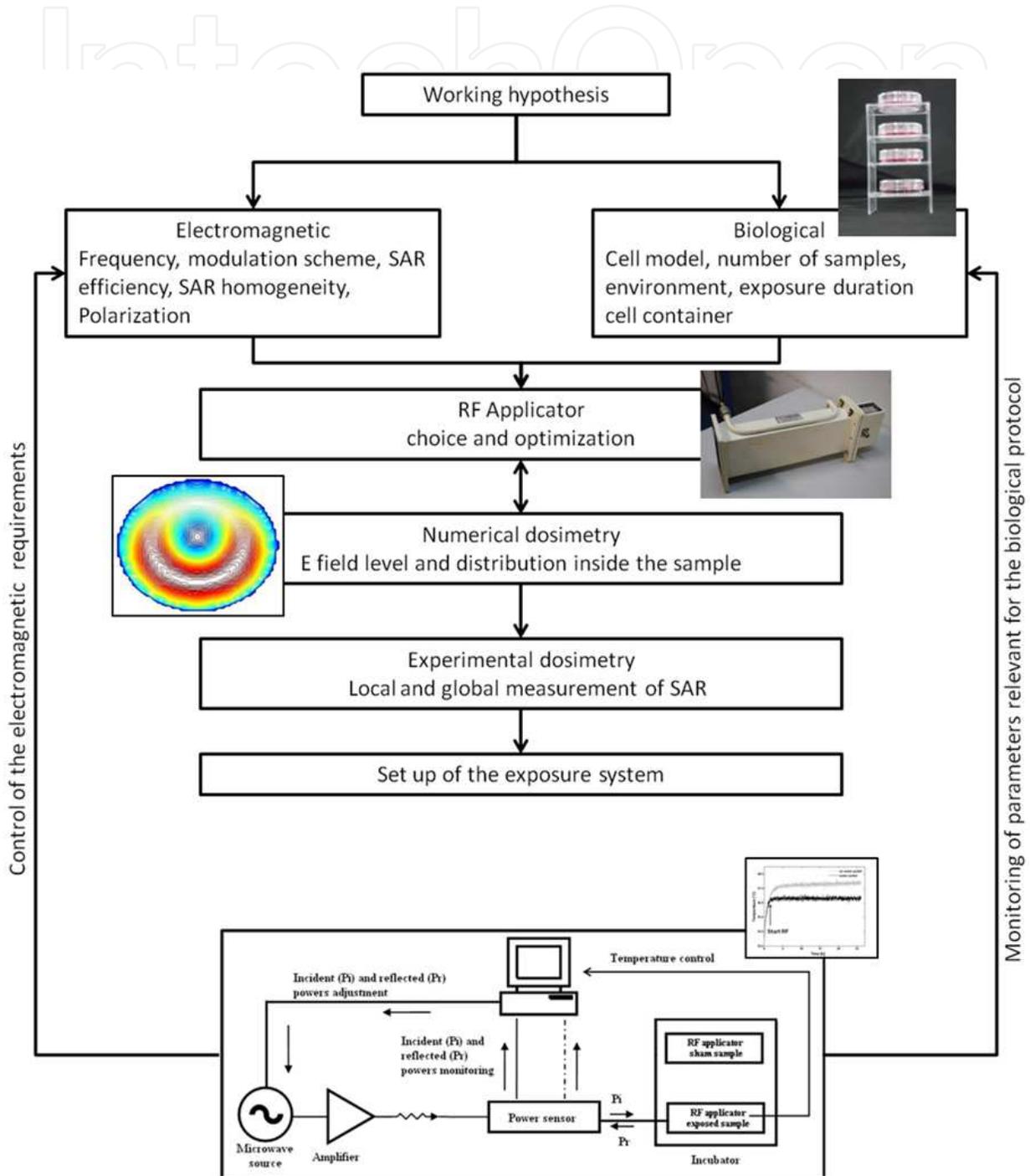

Figure 7. Schematic representation of the main steps in designing and realizing an experimental set up for in vitro studies

4. Conclusion

Because of the ubiquity of RF exposure and the remaining uncertainties regarding possible low level effects, it is crucial to perform good quality in vitro investigations in order to yield information on plausible interaction mechanisms. To achieve their full potential, in vitro experiments have to be well designed taking care of both biological and electromagnetic aspects. To this end, a strict cooperation between biologists and engineers is required, and the final procedures established in preliminary experiments, have to be preserved in writing and strictly followed throughout the experiments in a Good Laboratory Practices (GLP) like approach, and have to allow understanding of what was done, why and how, to assess the biological relevance of the study and the reliability and validity of the findings. There should be also enough information to allow the experiments to be repeated in independent laboratories.

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