NextGEM

Next Generation Integrated Sensing and Analytical System for Monitoring and Assessing Radiofrequency Electromagnetic Field Exposure and Health

D4.8: *In vitro* and *in vivo* investigations – Final report

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Glossary of terms and abbreviations used

Abbreviation / Term	Description
ANOVA	Analysis of Variance
ATP	Adenosine triphosphate
BE(2)C	Human neuroblastoma cells
CAT	Catalase
CBPI	Cytokinesis-block Proliferation Index
CIMNE	Center Internacional De Mèetodes Núumerics en Enginyeria
CNR	National Research Council of Italy
CpG	Cytosine-phosphate-Guanine
CSIC	Agencia Estatal Consejo Superior de Investigaciones Científicas
DAPI	4',6-Diamidino-2-Phenylindole
DC	Direct Current
DCF	Dichlorodihydrofluorescein
DDI2	DNA Damage Inducible homolog 2
DECT	Digital Enhanced Cordless Telecommunications
DEG	Differentially Expressed Genes
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DO	Detector Oligos
EMF	Electromagnetic Fields
EM-seq	Enzymatic methyl-seq
EMS	Ethyl Methanesulfonate
FBS	Foetal Bovine Serum
FCS	Foetal Calf Serum
FITC	Fluorescein-5-isothiocyanate

FR1/FR2	Frequency Range 1/2
GA	Grant Agreement
Gen	Generation of *C.elegans*
GFP	Green Fluorescence Protein
GHz	Giga Hertz
GLP	Good Laboratory Practice
GO	Gene Ontology
GPx	Glutathione Peroxidase
GSM	Global System for Mobile Communication
GUI	Graphic User Interface
H2DCF-DA	Dichloro-dihydro-fluorescein diacetate
HaCaT	Human epidermal keratinocyte line
HDF	Human dermal fibroblasts
HEKa	Human Epidermal Keratinocytes, adult
HeLa	Henrietta Lacks cervical cancer cells
hLEC	Human Lens Epithelial Cells
HTR-8/SV	Human Trophoblast HTR-8/SV neo cells
HUJI	The Hebrew University of Jerusalem
IARC	International Agency for Research on Cancer
ICNIRP	International Commission for Non-Ionizing Radiation Protection
ICT	Information and Communication Technologies
IEEE	Institution of Electrical and Electronic Engineers
IT'IS	The Foundation for Research on Information Technologies in Society
L1-L4	Developmental stages 1-4 in *C. elegans*
LMP	Low Melting Point

LTE	Long Term Evolution
MD	Menadione
MMC	Mitomycin-C
MMS	Methyl methanesulfonate
NGM	Nematode Growth Medium
NGS	Next Generation Sequencing
NR	New Radio
NRF-1	Nuclear respiratory factor 1
NRF-3	Nuclear respiratory factor 3
OECD	Guidelines for the testing of chemicals
PBS	Phosphate Buffered Saline
PI	Propidium Iodide
PS	Phosphatidylserine
Q1	Quadrant 1
Q2	Quadrant 2
Q3	Quadrant 3
Q4	Quadrant 4
RA	Risk assessment
RC	Reverberation Chamber
RF	Radiofrequency
RFU	Relative Fluorescence Unit
ROS	Reactive Oxygen Species
RT-qPCR	Reverse Transcript-quantitative Polymerase Chain Reaction
SAR	Specific Absorption Rate
SC	Sciensano

Sh	Sham
SH-SY5Y	Human neuroblastoma cell line
SOD	Superoxide dismutase
SOP	Standard Operating Procedure
TAC	Total Antioxidant Capacity
TempO-Seq	Templated Oligo-Sequencing
UCAS	University of Cassino e del Lazio Meridionale
UV	Ultraviolet
WG	Waveguide
WHO	World Health Organization
WiFi	Wireless-Fidelity
WP4	Work Package 4

Executive Summary

The deliverable D4.8 "*In vitro and in vivo investigations –Final report*" is part of Work Package 4 (WP4) and provides a framework for the experimental activities planned in Task 4.3 aimed at investigating the effects of 4G or 5G RF-EMF exposure on cancer-related endpoints in mammalian cell cultures (*in vitro*) or *C. elegans* samples (*in vivo*) in the absence and presence of other physical or chemical agents (combined exposure). In particular, a brief overview of the biological effects of RF exposure/co-exposure on cancer-related endpoints is presented to lay the foundation for the experimental activities planned and performed in WP4. The deliverable describes the experimental procedures carried out so far at different partner sites. Since highly controlled experiments are crucial for generating robust and scientifically valuable data, the detailed experimental protocols following a GLP-like approach are described in the "Standard Operating Procedures (SOPs)", which are included as Annexes. The preparatory work and the results obtained so far on human neuroblastoma (SH-SY5Y) cells exposed to a 4G LTE signal alone, in combination with a chemical treatment or with a WiFi signal, and on human (HaCaT) keratinocytes and *C. elegans* samples exposed to a 5G signal are presented and discussed. No statistically significant effects were detected between RF-exposed and Sham-exposed samples in the experimental conditions analysed so far in the above mentioned biological models.

1 Introduction

Over the past three decades, human exposure to RF-EMF emitted by wireless communication systems has exponentially increased and become crucial for the modern lifestyle.

Currently, humans are exposed to RF-EMF used for wireless communications utilizing multiple standards from the 2nd generation (2G) Global System for Mobile Communication (GSM) to the 3rd generation (3G) systems based on technologies such as the Wideband Code-Division Multiple Access (WCDMA)/Universal Mobile Telecommunication System (WCDMA/3G UMTS), the 4th generation (4G) mobile service and the 5th generation (5G) networks.

Studies of the possible adverse health effects of RF exposure and combined exposure to RF and other physical or chemical agents mainly cover 2G and 3G signals. In comparison, limited information is available on the potential hazards associated with exposure to the 4G and 5G frequency ranges of EMF. Many expert groups within international organizations have analyzed data available in the literature, and the overall results often conflict within the different lines of evidence of epidemiological, *in vivo,* and *in vitro* studies. Some studies did not report effects on the analyzed outcomes/endpoints, while others reported effects that were not confirmed when replicated by independent research groups. Among the possible reasons, the quality of the investigations on both electromagnetic and biological aspects is emerging as a critical issue that affects the results. It prevents any reasonable conclusion on the possible risk to human health [1] [2].

In order to generate robust and reliable data for health risk evaluation, NextGEM planned and is performing *in vitro* and *in vivo* studies under strictly controlled electromagnetic and biological conditions to evaluate the effects of RF exposure on cancer-related endpoints in relevant *in vitro* and *in vivo* models. The cooperation with the partners involved in Task 4.2 allowed the optimization of the 4G and 5G RF exposure systems in terms of numerical and experimental dosimetry, and the definition of the procedures for RF exposures of the *in vitro* and *ex vivo* human cell cultures and of *C. elegans* samples. Moreover, the optimization of the biological procedures for evaluating cancer-related endpoints in the biological models under examination was set in preliminary experiments and used for the RF experiments.

1.1 Mapping NextGEM Outputs

The purpose of this section is to map NextGEM's Grant Agreement (GA) commitments, both within the formal Task description and Deliverable, against the project's respective outputs and work performed.

Table 1: Adherence to NextGEM's GA Tasks and Deliverables Descriptions

TASKS	
Task Number & Title	**Respective extract from formal Task Description**
Task 4.3- *In vitro* and *in vivo* studies	This task aims to evaluate the effects of EMF exposure to 4G and 5G signals *in vitro* on human neuroblastoma and keratinocyte cells , *ex vivo* on human lymphocytes and *in vivo* on *C. elegans* , as a small organism model. *In vitro* and *ex vivo* exposure to EMF, given alone, as multiple frequencies/signals and in combination with chemical (Menadione) or physical (UV-B) agents, will be considered. We will investigate cancer-related endpoints such as oxidative stress, apoptosis, cell cycle progression, DNA damage and exploration of the epigenetic effects. Concerning *in vitro* experiments, selected exposure conditions will be tested in two independent laboratories (CNR and SC) to perform the same experiments to carry out a repetition study, useful to validate the results. Based on the obtained results, selected exposure conditions will be defined for (epi)genetic analysis . This will allow identifying the genes to be explored in human studies (CNR and SC). Reproduction-related endpoints (number of progenies, development of second generation) will be analysed in *C. elegans* (wild-type and sensitive mutant strains). For both *in vitro* and *in vivo* investigations, dose-response studies will be carried out by varying SAR values and exposure duration.
DELIVERABLE	

Deliverable: D4.8: *In vitro* and *in vivo* investigations - Final report (M35)

This deliverable will present the final results of 4G and 5G exposures and multiple combined exposures cytogenetic and epigenetic tests, and gene expression analysis.

1.2 Deliverable overview and report structure

Based on the objectives and work carried out under Task 4.3, the document starts with the Executive Summary, followed by the document's introduction in Section 1.

Section 2 presents an overview of the biological effects of RF fields.

Section 3 describes the quality criteria in experimental studies.

Section 4 includes the setup of the experimental procedures.

Section 5 presents the experimental results.

Section 6 concludes the deliverable.

Section 7 includes a reference list.

Finally, the developed Standard Operating Procedures (SOPs) are included in Annexes.

1.3 Update from previous deliverable D4.3: *in vitro* and *in vivo* investigation – Initial report

This deliverable, D4.8: "*In vitro* and *in vivo* investigation – Final report", provides a follow-up on the deliverable D4.3. Apart from the updated content in all sections, based on the new experimental activities carried out since the submission of the initial report, some additions were required, which can be summarized as follows:

- New paragraphs have been added under Section 4 – Setup of experimental procedures, while the paragraph on UVB exposure setup has been replaced with a new one employing a new UV source.
- New paragraphs have been added under Section 5 – Results.
- New Standard Operating Procedures (SOPs) have been developed, which add to the previous ones. All the SOPs have been enclosed as Annexes and the list has been updated accordingly.

2 Overview of the biological effects of radiofrequency fields (RF)

The impact of exposure to RF-EMF (300 kHz < f ≤ 300 GHz) on human health has been the subject of intense debate for many decades, due mainly to mobile phone use and the development of new wireless technologies [1][2].

When health risks of exposure to potentially harmful compounds or conditions are discussed, the cancer-related effects are of the greatest public concern. Numerous studies have been carried out to explore the possible association between exposure to mobile phones and carcinogenesis. The studies cover the evaluation of the lines of evidence resulting from mechanistic, *in vitro*, *in vivo,* and epidemiological investigations. This chapter provides an overview of the currently available literature, focusing specifically on the studies essential for Task 4.3. The effects of RF exposure alone are considered along with the effects of combined exposure to RF and other physical or chemical agents (cooperative effects). The study of cooperative effects mimics human exposure in a real-life scenario where humans are exposed to different environmental stressors. Moreover, these studies provide hints into the mechanisms of interaction of RF with biological material when chemical/physical agents, acting via well-known mechanisms, are used.

2.1 Possible biophysical and biochemical mechanisms

RF-EMF interactions with biological systems depend on the EMF characteristics (frequency, waveform, and strength of the induced fields), as well as the physical properties and dimensions of the body, leading to different patterns of EMF distribution within the system and complex biological responses. Understanding biophysical and biochemical mechanisms of interaction between RF-EMF and biological systems is of pivotal importance for health risk assessment (RA).

Under ordinary circumstances, the energy associated with RF-EMF (non-ionizing radiation) is too weak to induce the ionization of biological molecules such as Deoxyribonucleic Acid (DNA). Instead, RF-EMF induces relaxation phenomena in the biological systems by acting on the polar molecules, including water and other charged or polarized micro- and macromolecules [3].

The biological effects of RF-EMF can be attributed to thermal or non-thermal causes. Thermal effects are mainly associated with the absorption of RF energy resulting from the electrical conductivity and dielectric losses of most biological media. Most biochemical processes are sensitive to changes in temperature. International guidelines limiting human exposure to RF-EMF are largely based on the well-known thermal effects of these fields [4].

Molecular mechanisms of non-thermal effects remain a matter of debate. They might be associated with other processes induced by the RF electric or magnetic field at different levels of the biological scale of complexity.

Apollonio and co-workers attempted to draft a hierarchical classification of the possible interaction mechanisms, starting from atomic and molecular scales and moving up to the levels of macromolecules, cells, and cell aggregates [5]. Depending on the size of the target and the nature of the processes triggered by the EMF, different thresholds for frequencies and energies are required to cause the effects. The type of EMF and the modulation may also affect the threshold.

At the subatomic level, the magnetic field interferes with biochemical reactions involving radical pairs. Although this mechanism is usually associated with the action of static or low-frequency magnetic fields, low-intensity RF radiation has been reported to shift the equilibrium and potential, which has an important role in several diseases, including cancer [6].

At the level of macromolecules, one of the most studied hypotheses of interaction with RF fields is the possibility of inducing conformational changes in proteins, such as enzymes, ion channels and pumps [7].

2.2 Effects on cancer-related endpoints in mammalian cell cultures

The interest in studying RF-EMF and cancer stems from historical investigations into the health effects of EMF in general. Early concerns were raised in the 1970s and 1980s about the potential association between field exposure from power lines (50/60 Hz) and the incidence of childhood leukaemia [8]. Although conclusive evidence is missing, these studies laid the groundwork for broader investigations into the health impacts of EMF.

Epidemiological studies have been conducted to examine potential associations between RF-EMF exposure and various types of cancer, especially certain types of brain tumours. The head is assumed to be one of the target tissues

for RF exposure in general since the phone is typically held close to the ear during a call. These epidemiological studies analyze data from large populations to assess whether there is an increased risk of cancer among individuals exposed to RF-EMF from mobile phones or other sources.

As a result of these studies, international organizations such as the International Agency for Research on Cancer (IARC), a part of the World Health Organization (WHO), have assessed the carcinogenicity of RF-EMF. In 2011, IARC classified RF-EMF as "possibly carcinogenic to humans" (Group 2B) based on limited evidence from epidemiological studies suggesting a possible association of RF-EMF exposure with glioma, a type of brain tumour. It is important to note that a classification in category 2B does not mean that RF-EMF definitively causes cancer. Instead, it reflects a level of uncertainty where further research is needed to clarify the potential risks. The evidence supporting this classification is considered weaker or less consistent compared to agents classified in Group 1 (known carcinogens) or Group 2A (probable carcinogens). Recently, two large WHO-funded systematic reviews with meta-analyses were published in order to establish the current status of cancer research and RF-EMF exposure in human observational studies, where they both looked at frequently studied and less studied outcomes.

For the most researched outcomes, for mobile phones (near-field exposure to the head), there is moderate certainty that exposure does not increase the risk of glioma, meningioma, acoustic neuroma, pituitary tumours, salivary gland tumours in adults, or paediatric brain tumours. For cordless phones (near-field), there is low certainty that they may not increase the risk of glioma, meningioma, or acoustic neuroma. For fixed-site transmitters (far-field, whole-body exposure), there is moderate certainty that it likely does not increase the risk of childhood leukaemia. There is low certainty that it may not increase the risk of paediatric brain tumours. There are no eligible studies for assessing cancer risk in adults as a result of exposure to cordless phones. For occupational RF-EMF exposure, there is low certainty that it does not increase the risk of brain cancer/glioma, and no studies were found on occupational RF-EMF on leukaemia [9].

For less-researched outcomes, there is low to very low certainty that exposure to radiofrequency electromagnetic fields (RF-EMF) does not increase cancer risk. For mobile phones (near-field exposure to the head), there is low certainty that there is no increased risk of leukaemia, non-Hodgkin's lymphoma, or thyroid cancer. For occupational exposure, there is very low certainty that there is no increased risk of lymphohematopoietic cancers or oral/pharyngeal cancers. For Fixed-site transmitters (far-field, whole-body exposure) there is also insufficient evidence to assess cancer risk. For other cancers and sources of RF-EMF, there is a lack of sufficient evidence for conclusions [10].

In addition to epidemiological studies, laboratory research using animal models and cell cultures has been employed to investigate the biological mechanisms by which RF-EMF could potentially promote cancer development. This includes studies examining genotoxic effects, oxidative stress, alterations in the apoptotic process and cell cycle, and cellular signalling pathways following RF-EMF exposure.

These investigations cover mostly 2G and 3G signals, while a limited number of investigations addressed 4G and 5G. The available literature has been analyzed by many expert groups within national and international organizations, and the need for additional studies fulfilling both electromagnetic and biological quality criteria is always highlighted [1][2][4][11].

Another but less investigated aspect is the possible establishment of cooperative effects resulting from combined exposure to RF-EMF and other physical or chemical agents.

2.2.1 RF exposure alone

2.2.1.1 Oxidative stress

Oxidative stress occurs when there is an imbalance between reactive oxygen species (ROS) production and antioxidant defence mechanisms in cells. In addition to the superoxide anion ($O_2^{\cdot-}$) and hydroxyl ($^{\cdot}OH$) radicals, ROS include hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), as well as organic compounds. Although these reactive molecules can potentially cause damage to biological material and impede functionality, their presence and production should not generally be considered harmful. For example, H_2O_2 is required for wound-healing processes or the correct formation of protein structures. The organism needs to keep temporal and spatial control of ROS production and clearance. This control is achieved through precise control over the ROS-generating system and through the orchestrated action of antioxidants and enzymatic protection mechanisms. Indeed, (patho)physiological levels of ROS interfere with many vital cellular processes and functions, such as inflammation, cell proliferation and differentiation, wound healing, neuronal activity, reproduction, and behaviour by altering biochemical and signalling processes or even

resulting in oxidative damage to DNA, RNA, and proteins or the peroxidation of fatty acids. If this condition persists over a long period, it can lead to alterations of the physiological state of cells, as well as to changes to the genetic and epigenetic information, and, ultimately, to health-related malfunctions.

Different methods for the detection of ROS generation have been developed. In most cases, dyes that turn fluorescent upon contact with ROS are used, even though they have limited specificity and sensitivity. The activity or amount of superoxide dismutases (SODs), catalases (CATs), or peroxidases can also be used as an indicator of oxidative stress, as well as the ratio of reduced to oxidized glutathione (GSH/GSSG). In addition to direct measurements of ROS production and scavenging by the antioxidative defense systems, damage to biomolecules or their degradation products can serve as indicators for sustained oxidative stress [12].

Several studies have reported an increase in ROS levels and oxidative damage markers following RF-EMF exposure, indicating a potential role of RF-EMF in generating oxidative stress.

Different hypotheses have been put forward to explain the induction of oxidative stress. One is that RF-EMF might lead to an increase in free radical production in various cell types and biological systems. Another possibility is that RF-EMF may somehow affect the antioxidant defence mechanisms, for example by altering antioxidant enzyme activity (such as SOD, CAT, GPx).

However, it is important to note that there is currently no scientific consensus on RF-EMF-induced oxidative stress. Some research has reported conflicting results or failed to observe significant oxidative effects following RF-EMF exposure, which might be attributed to variability in exposure parameters (frequency, modulation, SAR level, and duration) or tissue/cell type-specific responses. The latter might influence the biological response, making it difficult to establish consistent outcomes across studies. In addition, cellular response variability (cell type, tissue specificity) and methodological limitations (variations in experimental protocols, assay techniques, and data interpretation) can impact the reliability and reproducibility of oxidative stress studies. Several studies are burdened with methodological uncertainties and weaknesses, or are not very comprehensive regarding exposure time, dose, number, and quantitative analysis of the biomarkers used.

In a recent review paper, Schuermann and Mevissen summarized findings from animal and cell studies on oxidative stress and EMF exposure by organ systems and related cell types and assessed their relevance for human health. The authors highlighted that most of the animal and more than half of the cell studies evidenced an increase of oxidative stress caused by RF-EMF exposure, although the quality of the single studies was not considered [12].

In a WHO-requested systematic review with meta-analysis investigating the effects of RF-EMF on oxidative stress biomarkers, considering both *in vitro* and *in vivo* studies, the results show that, overall, there are inconsistent and variable effects on oxidative stress biomarkers across tissues and species. Some studies report large increases, others show decreases or no effect, indicating uncertainty and variability in biological responses. In animal studies, several outcomes were examined: brain, liver, blood, gonads, thymus, and other organs. In the brain, the results showed highly inconsistent effects on biomarkers for oxidized DNA, proteins, amino acids, and lipids, with results showing both large decreases and large increases across different studies in rodents and rabbits. Regarding the liver, there were mixed results for oxidized DNA bases; both increases and decreases were reported. A moderate increase was found for protein and amino acid modifications in rodents. For the blood, inconsistent effects were found on oxidized DNA bases in rodent blood, no significant effect on protein/amino acid biomarkers, and a significant increase in oxidized DNA biomarkers in rodent plasma. In the gonads, increased oxidative DNA damage in the testis was observed, along with inconsistent effects in ovaries, ranging from small to large. In the thymus, a strong increase in biomarkers for modified proteins/amino acids has been documented. Finally, a significant increase in oxidized DNA in rodent cells was found, along with a non-significant moderate increase in lipid oxidation. *In vitro* studies examined the effects on rodent and human cells. In rodent cells, the literature observed a large but not statistically significant increase in oxidized DNA bases and a moderate, non-significant effect on protein/amino acid biomarkers [13].

More extensive studies under standardized conditions are necessary to clarify inconsistencies in study outcomes, elucidate the underlying mechanisms, and assess the potential health implications of RF-EMF-induced oxidative damage.

2.2.1.2 Apoptosis

Apoptosis is a process of controlled cell death, that is highly conserved within multicellular organisms and genetically controlled. It serves to remove damaged, dysfunctional, or no longer necessary cells to promote homeostasis and

survival of organisms [14][15][16]. Due to its pivotal role in tissue homeostasis, the regulation of the apoptotic program is decisive to avoid cancer occurrence. Moreover, abnormalities in cell death regulation, whether they feature insufficient or excessive apoptosis, can be a significant component of diseases such as autoimmune lymphoproliferative syndrome, AIDS, ischemia, and neurodegenerative diseases such as Parkinson's, Alzheimer's, Huntington's diseases, and Amyotrophic Lateral Sclerosis. Two pathways are involved in apoptosis that work synergistically to ensure the removal of defective cells. The intrinsic, or mitochondrial, cell death pathway, is activated by the cell itself upon detection of cell damage *via* several intracellular sensors. The extrinsic cell death pathway is activated by the interaction between a cell of the immune system and a damaged cell [16].

Different methods for the detection of cell damage and death have been developed over time, concurrently with the growing knowledge of the apoptosis phenomena. The methods mainly rely on morphological and biochemical analysis aimed at identifying features of apoptotic cells such as shrinkage, membrane blebbing and chromatin condensation, DNA fragmentation, detection of caspases, cleaved substrates, regulators and inhibitors, externalization of phosphatidylserine, alteration of mitochondrial membrane potential, the release of cytochrome-c, analysis of apoptotic or anti-apoptotic regulator proteins such as Bcl-2-associated X protein (Bax), as well as BH3-interacting domain death agonist (Bid), and BCL2 apoptosis regulator (Bcl 2) [16].

Several *in vitro* and *in vivo* experimental studies have addressed the effects of RF-EMF exposure, at frequencies and signals typical of ICT, on the apoptotic process. These studies have been carried out under different conditions and experimental regimens with conflicting results.

Manna and Gosh reviewed the effects of RF-EMF exposure in cultured mammalian cells on several biological outcomes, including apoptosis. The authors concluded that RF-EMF exposure might affect the apoptotic process *in vitro*, with results depending on the type of modulation, exposure modality, and cell model [17]. An extensive meta-analysis of data from *in vitro* studies on the effects of weak RF-EMF from mobile phones, published between 1990 and 2015, revealed a lack of uniform responses in any of the investigated outcomes, including apoptosis [18]. In [19], apoptosis was considered together with proliferation to detect possible statistical associations between RF-EMF exposures and cellular response. These responses were significantly associated with cell lines rather than primary cells, but not with other experimental parameters.

Recently, Romeo and co-workers produced a scoping review of the studies investigating the effects of RF-EMF exposure on the apoptotic process, with the aim of systematically mapping the research performed in this area and identifying knowledge gaps. Through a systematic literature search, 121 potentially relevant studies were retrieved, but only 42 complied with basic quality criteria (adequate characterization of exposure conditions, appropriate dosimetry, presence of sham control, temperature monitoring, and a minimum of three independent experiments performed) and were included in the analysis. Most included studies did not find significant alterations in the apoptotic process due to RF-EMF exposure. When a statistically significant effect was observed, it mainly occurred at frequencies above 6 GHz, and for acute (<1h) exposure durations. However, since the number of studies reporting effects was very small, and the considered experimental conditions were highly heterogeneous, the authors highlighted the need for further investigations and replication studies to confirm or refute these results. Moreover, even though the included studies met the basic quality criteria, most of them still presented flaws in the experimental methods (lack of blind analysis and/or positive control, assessment of single endpoints). As a conclusion of the qualitative analysis conducted in the scoping review, the authors stated that the major problems were the low quality of the experimental methodologies adopted in many of the considered studies and that future studies should be performed with an adequate number of experiments to ensure sufficient statistical power [20].

2.2.1.3 Cell proliferation and cell cycle distribution

Cell proliferation is an increase in cell number, which can be stimulated by physiological and pathological conditions and is largely controlled (stimulated or inhibited) by signals from the microenvironment. An excess of stimulators or a deficiency of inhibitors leads to net growth and, in the case of cancer, uncontrolled growth [19]. Cell proliferation is influenced by changes in the cell cycle distribution and rate of DNA synthesis. The cell cycle is divided into four phases: 1) mitotic (M) phase, from the beginning to the completion of a round of cell division; 2) gap 1 (G1) phase, after the completion of cell division and before the beginning of DNA synthesis; 3) DNA synthesizing (S) phase, from the beginning to the completion of DNA synthesis; and 4) gap 2 (G2) phase, after the completion of DNA synthesis and before resumption of cell division [21].

Some reports have shown that RF-EMF exposure decreases cell proliferation and influences cell cycle distribution, but most other studies showed no effect on these parameters. Furthermore, the effects of environmental factors and temperature were not completely excluded in the results, which showed an effect of RF-EMF exposure [21].

In vitro studies addressing the effect of RF-EMF exposure on cellular responses were also reviewed by Simko and co-workers by applying a "grouping approach" to test if any statistical association exists between RF-EMF cell proliferation, considered separately or in association with apoptosis and concerning experimental parameters (cell type, frequency, exposure duration and SAR level) and methodological quality criteria (sham-exposure, appropriate dosimetry, use of positive control, blinded analysis, and temperature control). Cell proliferation was affected in 18% of the extracted experiments, but no association was detected between frequency, exposure duration, or SAR level with cellular responses. Moreover, when quality criteria were considered, the data showed that the lower the methodological quality, the more cellular responses were detected, with a large drop in responses for experiments where all quality criteria were satisfied [19].

2.2.1.4 Genotoxicity

Genotoxicity refers to the ability of chemical or physical agents to damage genetic information within cells. Genotoxic substances can either directly damage DNA or disrupt cellular processes that maintain genetic integrity. If the damage is incorrectly repaired or not repaired at all, it may lead to permanent changes in the DNA that can be transmitted to the next generation of cells. Permanent DNA damage includes gene mutations as well as structural or numerical chromosome aberrations and has been associated with serious adverse human health effects, including cancer.

Genotoxicity, which is mainly investigated by cytogenetic techniques, is assessed by evaluating primary endpoints (chromosomal aberrations, micronuclei, aneuploidy, spindle disturbances, sister chromatid exchanges, mutations) or secondary endpoints (single and double DNA strand breaks, chromatin condensation, and 8-hydroxy-2'-deoxyguanosine adducts).

The most commonly used cytogenetic techniques to assess permanent DNA damage are metaphase cytogenetics and micronucleus assays. In contrast, comet and foci formation assays are used to detect the induction of DNA damage that might still be repaired.

In vitro studies addressing genetic damage in mammalian cells exposed to RF-EMF were the subject of comprehensive reviews [17][22] and meta-analyses [18][23][24][25]. The reviewed body of evidence is characterized by a large variability of the biological and electromagnetic parameters considered. In fact, just observing the biological model adopted, cells of either human or animal origin, primary cells or immortalized cell lines are used in the studies. Among the cells of primary origin, human lymphocytes were mainly employed, but also bovine, canine, and rodent cells, along with spermatozoa, astrocytes, thymocytes, amniotic fluid, brain, adipose, dermal, pulmonary, or epithelial cells, both from human and non-human origin. Similar variability in cell type and origin also applies to healthy cell lines (fibroblasts, trophoblasts, spermatocytes, keratinocytes, stem cells, ovarian, epithelial, umbilical, corneal, etc.), and cancer cells (neuroblastoma, glioma, other types of brain tumour, leukaemia cells, etc.). Concerning the genotoxic endpoints analysed, most of the experiments evaluated the effect of RF exposure on single and/or double DNA breaks, or micronuclei formation. Chromosomal aberrations, sister chromatid exchanges, chromatin condensation, 8-hydroxy-2'-deoxyguanosine adducts, spindle disturbances, mutations, and aneuploidy were also investigated. Concerning the exposure conditions, even though most of the studies applied RF-EMF in the frequency range used for ICT (mobile phone, wi-fi), the other exposure parameters (continuous or pulsed wave, signal modulation, SAR levels, exposure duration and modality) were variable. In particular, SAR levels were below (SAR≤1 W/kg), around (1 W/kg <SAR≤ 2 W/kg) or above (SAR> 2W/kg) exposure limits, and the exposure durations were acute (≤1h), long (>1h to ≤24h) or chronic(>24h).

As for other endpoints, particular attention should be given to the critical role of implementing comprehensive quality control measures in investigations related to genetic damage caused by exposure to RF-EMF.

In the meta-analysis by Vijayalaxmi and Prihoda [25], the impact of the inclusion of quality control measures (namely i) blind analysis; ii) adequate dosimetry; iii) positive control; and iv) sham-exposed controls) on the conclusions of the genotoxicity studies was assessed. The authors found that publications that considered all four quality control measures demonstrated smaller effect sizes compared to those where one or more measures were not considered. Additionally, the number of published studies reporting no significant difference in genetic damage between RF-exposed cells and

control cells increased when an increasing number of quality control measures was employed. This indicates that rigorous methods help minimize spurious findings and enhance the reliability of study outcomes.

In 2024, Romeo et al. published a systematic review on the genotoxic effects on mammalian cell lines due to RF-EMF exposure. Their review found that 80% of the included studies showed no statistically significant genotoxic effects of RF-EMF. Furthermore, most positive (genotoxic) findings came from studies rated as having moderate to low quality, often with bias concerns. Due to inconsistent reporting and high data variability, meta-analysis was not possible and only a qualitative assessment was done. Overall, there is low confidence that RF-EMF causes genotoxic effects in mammalian cells. The review concludes that RF exposure likely does not increase genotoxicity *in vitro* [26].

2.2.1.5 Epigenetics

Epigenetic mechanisms govern the expression of genes without modifying the DNA sequence, shaping unique temporal and spatial patterns of expression specific to cell types. Changes in epigenetic markers have been noted in various diseases, such as cancer and neurological disorders. Accumulating evidence suggests that diverse environmental factors can induce epigenetic changes, potentially impacting the likelihood of developing diseases. Exposure to extremely low-frequency magnetic fields can induce epigenetic dysregulation (such as based on DNA methylation) in various types of cells. Few studies have investigated DNA-methylation modifications at higher frequencies *in vitro* (Table 2). In one study, differences in profiles and organisation of methylated and unmethylated CpG sites in interspersed nuclear elements and ribosomal repeats were observed in three different cell lines exposed to 900 MHz [27]. Another interesting study reported correlations between transcriptomics and epigenetics variations measured in six genes which were differentially expressed and DNA-methylated in HEKa (human epidermal keratinocyte) cells after 1h of exposure at 900 MHz [28]. These genes are involved in critical cell functions such as cell survival and response to damage. For instance, the authors observed hypomethylation and consequential upregulation of DNA damage-inducible 1 homolog 2 (DDI2), which is involved in the control of cancer cell proliferation through the activation of the transcription factors nuclear respiratory factor 1 and 3 (NRF1 and NRF3) [29][30]. More knowledge is needed to verify if similar patterns can be observed for EMF exposure to 4G and 5G signals. Moreover, no study has investigated whether DNA methylation patterns could be affected at different post-exposure timepoints to address transient or longstanding alterations in the epigenomes and to define activated downstream pathways. Some studies have investigated gene expression, cell proliferation, cell viability, oxidative stress and DNA damage of cells exposed to RF immediately and after some resting time following exposure (Table 2), but this was never studied for DNA-methylation. Therefore, more data concerning epigenetic modifications induced by 5G are needed.

The most common method for DNA methylation profiling relies on short-read Next Generation Sequencing (NGS) with the Illumina technology, requiring DNA chemical (bisulfite) or enzymatic pre-treatment and enrichment using arrays. The interpretation of methylation sequencing data plays an important role in investigating differences in methylation levels associated with exposure. These differences are usually investigated at the level of methylation sites (nucleotide positions) or regions (group of CpGs, e.g., in the promoter region of a gene). Then, the statistical significance of these differences is tested. The investigation of methylation differences at the level of regions is usually preferred as this reduces data dimensionality and increases the power of detection by employing nearby CpGs. Furthermore, DNA-methylation alterations that extend to the regional level are thought to be more functionally important (potentially leading to adverse effects, including cancer, as elaborated above) than alterations that affect only isolated sites. In the case of pre-defined regions, the methylation signal is simply summarized over the entire region, and statistical testing, similar to that for single sites, is applied [35].

When considering thresholds to estimate that two sites/regions are differentially methylated, two main approaches are usually used in the literature: 1) investigation of clear and strong differences in the range of 25-30%, as is the case in most cancer-associated methylation markers, or 2) investigation of more subtle differences as low as 5%, as is the case for many exposure-associated methylation markers [36]. For example, in a study analysing aberrant DNA methylation during arsenic-induced HaCat cell transformation (potentially leading to carcinogenesis), differentially methylated CpG sites were considered when they reached at least 20% of difference between arsenic-treated and non-treated cells [37]. In an epigenome-wide study investigating DNA-methylation alterations in cord blood in relation to prenatal perfluoroalkyl substance (PFAS) exposure, a cut-off of 5% was set to compare differentially methylated regions [38]. However, the use of low threshold differences (5%) is more subjected to confounding factors and should be interpreted very carefully, whereas higher threshold differences (25%) are more reliable proofs, which can be linked to biological adverse effect.

Table 2: List of scientific publications investigating the effect of RF exposure on epigenetics.

Authors	Cells	Exposure frequency and SAR	Exposure time	Post-exposure time	Analysis	Results
[28]	HEKa	900 MHz 1.55 x 10^{-3} W/kg	1h	0h 24h[1]	DNA-methylation[2] + transcriptomics +cell viability	Six genes were differentially methylated and expressed. No variation in cell viability.
[27]	HeLa, BE(2)C and SH-SY5Y	900 MHz 1 W/kg	48h	0h	DNA-methylation[3]	Profiles and organization of methylated and unmethylated CpG sites in interspersed nuclear elements-1 and ribosomal repeats were modified in all cell lines studied.
[31]	HDF	2.45 GHz 0.77 W/kg	2h	0h 2h 24h	Transcriptomics	Expression of 6, 39 and 8 genes modified in samples resting during 0h, 2h and 24h, respectively.
[32]	hLEC	1.8 GHz 1, 2, and 3 W/kg	2h	0 min 30 min 60 min 120 min 240 min	DNA damage + cell proliferation + Hsp70 protein quantification and gene expression	3 W/kg induced DNA damage observed 0 and 30 min after exposure, but not later. No difference in cell proliferation. ≥ 2 w/kg for 2h triggered an increased Hsp70 protein level but no corresponding increase of mRNA.
[33]	SH-SY5Y	872 MHz 5 W/kg	1h	0 min 5 min 10 min 15 min 30 min 60 min	DNA damage + cell viability + oxidative stress	RF radiation might enhance chemically induced effects over time, namely ROS production and DNA damage, but no effect on cell viability.
[34]	HTR-8/SV	1.8 GHz 2 W/kg	4h 16h 24h	0 min 30 min 120 min	DNA damage + cell viability	No impact on cell viability. Exposure may affect DNA integrity over time. The effect was reversible after 30 min.

HEKa: human epidermal keratinocytes; HeLa: cervical cancer cells; BE(2)C and SH-SY5Y: human neuroblastoma cells; HDF: human adult fibroblasts; hLEC: human lens epithelial cells; HTR-8/SVneo: human trophoblast HTR-8/SVneo cells.
[1]: only cell viability was investigated 24h after exposure; [2]: whole genome bisulfite sequencing; [3]: targeted bisulfite sequencing

2.2.2 Combined exposure to RF and physical/chemical agents

Given the ubiquity of RF-EMF in indoor and outdoor environments, combined exposures with other chemical and/or physical agents have become inevitable. In the past, it has been speculated that RF-EMF might not have a direct influence on mammalian cell biology, but that it might instead modulate the response of biological systems to other agents *in vitro* and *in vivo*. Combined exposures to RF-EMF and other chemical or physical agents might lead to different cooperative effects:

- additive (the combined effect of two or more agents acting in the same general direction approximates the sum of the effects of the agents administered separately);

- antagonistic (the combined effects of two agents acting in different/opposite directions are smaller than the effect of any one of them in stand-alone mode);
- potentiative (the increased effect of an agent by concurrent action of another agent that does not have a stand-alone effect);
- synergistic (the combined effect of two or more agents is significantly greater than the sum of the effects of each agent administered alone) [39].

The effects strongly depend on the sequence of exposure administration (RF given before, concurrently, or after the other agent) and on the initial functional state of the irradiated biological system.

Synergistic effects of RF-EMF have been detected when given in combination with ionizing radiation [40], chemical substances [41] and UV-radiation [42]. Synergistic interactions between RF-EMF and other environmental factors could potentially impact cancer development or progression.

Alternatively, it has also been suggested that RF-EMF exposure, provided under conditions not inducing damage *per se*, might lead to a decrease in the damage induced by subsequent treatment with other physical or chemical agents (protective effect). This can be considered a particular type of antagonistic effect.

A number of papers have been published reporting that cells or animals that were pre-exposed to RF-EMF (with variable frequency, signal modulation, SAR level, and exposure duration) became resistant to the damage induced by subsequent exposure to a genotoxic agent (ionizing radiation, chemicals). This phenomenon, called an adaptive response, was observed *in vitro* [43][44][45][46][47][48][49] and *in vivo* [43][50][51]. While the adaptive response is manifested by increased resistance of RF-pre-exposed cells to the damaging effects of agents administered to the same cells, the protective effect of RF-EMF has also been detected in the form of a bystander effect, which is the propagation of the effects from cells directly irradiated (targeted cells) to non-irradiated cells (bystander cells) [52][53][54].

The scientific consensus on the outcome of combined exposure to RF-EMF and chemicals/UV radiation in relation to cancer endpoints has not been reached and is subject to ongoing investigation. While some evidence suggests possible synergistic effects, the overall understanding of this complex interaction is limited by methodological challenges and the need for further research. Continued efforts to elucidate the biological mechanisms and assess the health implications of combined exposures are essential for advancing our understanding of environmental carcinogenesis and informing RA strategies.

2.2.3 Exposure to multiple RF frequencies/signals

As a result of the recent advances in wireless communication, the human population is currently exposed to RF-EMF from multiple sources and standards [55], and the question arises regarding the possible effects that could be associated with exposure to RF from multiple sources. This has also been recently highlighted by the ICNIRP [56]. Despite that, the effects of RF exposure to multiple frequencies/signals have been addressed in a few *in vivo* and *in vitro* studies. The studies carried out in the period 2009-2014 deal with several rat models and cell models, exposed for several durations, based on the analysed outcome/endpoint, to frequencies/signals used by 2G and 3G technologies. In particular, the frequencies were in the range 800-2450 MHz, the signals were CDMA and WCDMA, while the SAR levels span from 0.08 W/kg to 4 W/kg. From *in vivo* studies, mainly no statistically significant effects (negative results) were obtained on foetuses of exposed pregnant mice, on the reproductive system, on immunological functions, chronic illness, and total tumours incidence. Negative results on key cellular parameters were also obtained from *in vitro* studies. All these studies have been commented on in [1]. Absence of effect was also detected in more recent studies. Shirai and coworkers (2017) exposed pregnant SD rats for 20 h/day from gestational day 7 to weaning and their delivered offspring 20 h/day up to 6 weeks of age, to eight different RF signals at frequencies between 800 MHz and 5.2 GHz at SARs of 0.08 and 0.4 W/kg. The results did not show any adverse effects on pregnancy and the development of rats [57]. In the *in vitro* study by Lee and co-workers [58], the effect of combined RF radiation on amyloid-beta (Aβ)–induced cytotoxicity in HT22 rat hippocampal neurons, subjected for 2 h to multiple signals (837 MHz CDMA at 2 W/kg plus 1950 MHz W-CDMA at 2 W/kg), was investigated. The results indicated that RF multiple exposure alone did not significantly affect the cell viability, proliferation or ROS production, and did not modify the Aβ-induced decreased cell proliferation, increased ROS production, or cell death.

2.3 *In vivo* effects on *Caenorhabditis elegans* due to RF exposure

2.3.1 Cancer-related endpoints

The nematode *Caenorhabditis elegans (C. elegans)* is a widely used model organism for studying environmental influences on human health. This worm is 1 mm long as an adult and usually lives in soil-associated decaying organic matter. Despite or perhaps because of its simplicity, it is a highly informative animal model widely used in nanotoxicology and biology. Its ease of maintenance, small size, transparency, with a fast life cycle of ~3 days and a life expectancy of ~3 weeks, constant cell number, invariant developmental trajectory, highly conserved and well-annotated genome, differentiated anatomical structures, and easy genetic manipulation renders *C. elegans* a convenient yet powerful model organism (Figure 1). The detailed study of their genetic information revealed that they share 60% of genetic homology with humans [59]. Some of these genes are homologs to human genes involved in diseases [60]. It can be readily cultivated within a laboratory setting, either on agar plates or in a liquid medium utilizing *Escherichia coli* strain OP50 as a food source. *C. elegans* has been used to assess the functional impact of specific gene mutations on tumour development and outcome at the organismal level and to screen for new anticancer drugs [61].

Over the past decade, a growing body of evidence has indicated that autophagy plays a pivotal role in the initiation and progression of various diseases, including cancer. Recent studies have unveiled that autophagy can either impede cancer initiation or facilitate tumour growth in a context-dependent manner [61][62]. Observations that autophagy-deficient mice display elevated levels of ROS suggest that impaired autophagy may induce cellular damage and heightened sensitivity to stress, thereby compromising survival. These mutant animals exhibit increased susceptibility to diseases, including cancer [63]. ROS are broadly considered a two-edged sword for cancer development: they are necessary for cancer progression, yet they can also be utilized to target and eliminate cancer cells specifically. Research on *C. elegans* has illuminated the regulatory mechanisms and impacts of stressful conditions on entire organisms.

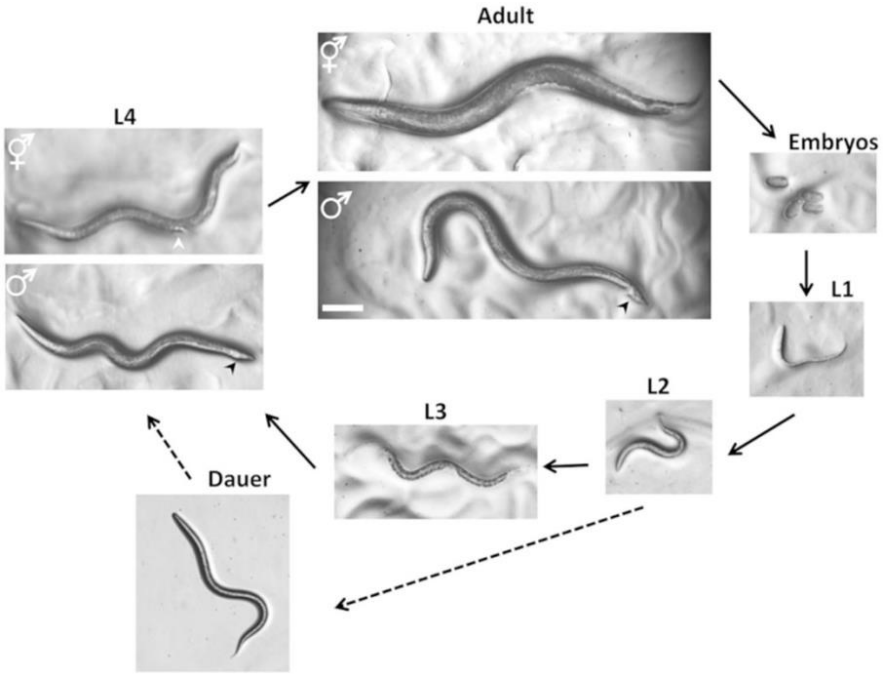


Figure 1: The life cycle of the nematode *C. elegans* comprises an egg, four larval stages (L1 to L4), and one adult stage. Dauer is an alternative developmental stage triggered only under harsh environmental conditions. Bar 0.1 mm. Extracted from [67].

To the best of our knowledge, only a few studies addressing the effects of RF exposure on *C. elegans* are available in the literature. Perhaps the most prominent study addressing the impact of EMF on ROS generation is the one performed by Sun and co-workers [64]. Worms in three adult stages (young adult, egg-laying, and peak egg-laying) were exposed to 50 Hz, 3 mT ELF magnetic field. The findings revealed a significant increase in ATP levels and concurrent upregulation of mitochondrial ATP synthase activity under ELF magnetic field exposure. ROS levels were notably increased in the young adult stage, accompanied by an increase in Total Antioxidant Capacity (TAC) but a decrease in the ROS-TAC score, as indicated by principal component analysis. The ROS-TAC score in humans is

related to infertility. Therefore, a decrease in ROS-TAC score could indicate an impact on worm fertility, although not conclusively stated [65]. However, there were no significant changes in ROS levels and TAC in the worms' egg-laying and peak egg-laying stages. Based on these observations, it was concluded that ELF magnetic field exposure can enhance energy metabolism in worms and induce oxidative stress, characterized mainly by elevated ATP and ROS levels, upregulation of ATP synthase, and a decrease in ROS-TAC score in young adult *C. elegans*. However, it is still inconclusive if the observed rise in ROS can correlate with enhanced susceptibility to cancer. Recently, a review including some *C. elegans* references also tries to summarize the consensus, controversy, limitations, and unsolved issues [66]

2.3.2 Reproduction-related endpoints

The ability to control the development of *C. elegans* in laboratory settings using a synchronization protocol allows reproduction-related endpoints to be performed easily. The synchronization is achieved by incubating *C. elegans* embryos in a saline buffer without food. They cannot develop further than the L1 stage in these conditions if no food is in the media. Once introduced into media with food, worms develop in 15h to the second larval stage (L2) with an average size of ~350 μm. After nine more hours, they reach the third larval stage (L3) with ~500 μm size, and finally, they reach the fourth larval (L4) stage after ten more hours with an average size of ~650 μm. Young adult worms with an average size of ~1 mm are obtained after 48h of transference, and they start laying eggs after ten more hours. Development can be screened by measuring the length of the worms and the progeny counted after 48h of transference to the agar plate.

Shi and co-workers determined that *C. elegans*, at different developmental stages, exposed to a 50 Hz magnetic field at 0.5, 1, 2, and 3 mT showed no significant differences in the total progeny number (brood size), thus concluding that ELF magnetic field exposure did not affect the reproduction rate of *C. elegans* [68].

Bojjawar and co-workers found a decreased fertility in the worms when exposed to nanosecond electric pulses (10 ns pulse width; 0.1 ns rise time, 18 or 62 kV/m electric field), an effect unlikely related to heating. However, they failed to assess the molecular mechanism [69].

Due to the absence of studies investigating RF-EMF effects on *C. elegans* reproduction, it is crucial to define experimental conditions that mimic real scenarios, especially regarding exposure to 5G-EMF.

2.3.3 Developmental effects

RF-EMF has not yet been widely studied for their effects on the development of *C. elegans*. Perhaps the most significant study is the one carried out by Gao and co-workers, who found that worm groups exposed to RF (1750 MHz RF fields at a SAR of 3 W/kg) showed nearly identical developmental rates and similar longevity compared to sham groups. The analysis unveiled 94 up-regulated genes and 17 down-regulated genes in L4-stage worms, 186 up-regulated genes, and 3 down-regulated genes in adult-stage worms. Gene Ontology (GO) analysis indicated that the differentially expressed genes were associated with growth, body morphogenesis, collagen and cuticle-based development, and embryonic and larval development in the offspring. They concluded that no detrimental effects were noted from extended exposure to the development and lifespan of *C. elegans*. Despite detecting some differentially expressed genes following prolonged RF exposure, these differences were attributed to randomly oscillating gene expression patterns [70].

Another study by Fasseas and co-workers examined the influence of electromagnetic radiation emitted by GSM mobile phones, wi-fi (Wireless-Fidelity) routers, and wireless DECT (Digital Enhanced Cordless Telecommunications) phones on the nematode. Likewise, they found no statistically significant differences between the exposed and the sham/control animals in lifespan, fertility, growth, ROS levels, apoptosis rate, or gene expression [71].

As with the effects outlined in previous sections, no studies have addressed the effect of 5G RF-EMF on the development of *C. elegans* to date.

3 Quality criteria in experimental studies

The scientific literature concerning the biological effects of RF-EMF contains a large number of conflicting results, which can be mainly attributed to the lack of standardized methodologies for good-quality experiments on both biological and electromagnetic sides. Several guidelines have been published in the literature providing indications and minimal biological and electromagnetic requirements to obtain reproducible and scientifically valuable results. The main aspects are recalled in the following for *in vitro* studies, which are also suitable for *in vivo* experiments on *C. elegans*.

3.1 Biological requirements

The maintenance of high standards is essential for ensuring the reproducibility, reliability, credibility, acceptance, and proper application of any produced results. Guidance on Good Cell Culture Practice (GCCP) has been developed to promote the maintenance of these standards and to reduce uncertainty in the development and application of animal and human cell and tissue culture procedures and products, by encouraging greater international harmonisation, rationalisation and standardization of laboratory practices, quality control systems, safety procedures, recording and reporting, and compliance with laws, regulations and ethical principles [72].

The standardization of cell culture is a fundamental step which is based on the establishment and maintenance of a sufficient understanding of the *in vitro* system and of the relevant factors that could affect it. Generally, primary cultures (i.e., harvested cells and tissues taken directly from animals and humans) or immortalized cell lines (cells that can divide for extended periods *in vitro* and can be maintained by serial subculture) can be used in experiments. Primary cell cultures generally have a limited lifespan and are known to change their differentiated characteristics with time in culture. They commonly require complex nutrient media supplemented with animal serum and other non-defined or ill-defined components. Primary cultures often represent heterogeneous cell populations and are difficult to standardise and reproduce because of uncontrollable variations between preparations. Immortalized cell lines represent a more stable and reproducible system than primary cells: they are able to multiply for extended periods and can be expanded and cryopreserved as cell bank deposits. It is recommended that authenticated stocks of continuous cell lines be purchased from recognized national and international cell banks [72][73].

Key elements of *in vitro* culture conditions include culture media, supplements and other additives, cultureware, and incubation conditions. The cell culture medium is a defined base solution that includes salts, amino acids, and sugars supplemented with components specific to cell type, which are necessary for obtaining satisfactory cell proliferation and production, or for maintaining a desired differentiation status. The most complex component is the serum that originates from a pool of donations taken from many animals, thus expressing a large variability among different manufacturers. Care should be taken to expose the cells to appropriate environmental conditions (temperature, atmospheric gas composition, pH). Key items of equipment, including incubators, laminar air flow and microbiological safety cabinets, and cryostorage systems, must be set up and used appropriately. Cells should also be periodically checked for contamination or cross-contamination (Mycoplasma is the most common infection) [72].

A wide variety of cell types, ranging from stem cells to highly differentiated tissue-specific cells, can be used. The appropriate cell model must be chosen on the basis of the cellular target investigated. For instance, human lymphocytes represent one of the best-suited cell models for the investigation of the genotoxic effects of chemical and physical agents, including EMF. For each cellular target, it is advisable that more than one endpoint is investigated. Thus, a combination of techniques, confirming and/or complementing each other, is recommended for the reliable detection of effects.

A general requirement for the biological assay in a well-designed *in vitro* experiment is high sensitivity, and particular care must be devoted to setting up accurate experimental control samples. Negative and positive controls provide evidence for controlled experimental conditions, whereas in bioelectromagnetics a sham-exposed sample must be included as a control condition. 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 of controlled experimental conditions and assurance that the assay methodology is responding adequately to a well-known agent. A sham control (a sample placed in an identical applicator to that used to administer the treatment, except for the emission of RF-EMF) guarantees the very same environmental conditions to all experimental groups. Furthermore, it is mandatory

that research personnel are blinded to the study group during exposure and sample analysis to reduce the experimenter bias.

Finally, for an unbiased interpretation of the results, these should be assessed by 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 into account biological variability and measurement error. Appropriate statistical tools must be used when designing a study, 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 must be considered [73].

3.2 Electromagnetic requirements

General guidelines and minimal requirements for designing and developing good-quality exposure setups for bioelectromagnetics *in vitro* have been published [74][75]. The main objective is to obtain well-defined and controlled exposure conditions, which are necessary conditions for appropriate RA. In general, the design and realization of a 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 EMF to the sample, duration of the exposure), which are generally defined based on specific "real life" conditions (exposure to EMF 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 for selecting the hardware and software solutions to be implemented in the experimental setup.

An RF exposure setup is usually made up of the following basic elements:

- a source of RF signal where the main characteristics of the signal (frequency, amplitude, modulation scheme) can be set;
- signal conditioning components (amplifiers or attenuators, couplers, splitters, filters, etc…);
- components to monitor and adjust the signal according to pre-defined requirements (power meters, PC for remote control, etc…);
- RF field applicator (e.g., waveguide, TEM cells, wire patch cells, etc…);
- components to monitor the relevant biological and environmental parameters (temperature, CO_2, humidity, etc…).

RF exposure setups for *in vitro* studies must comply with the basic requirements for cell culture maintenance (temperature, pH, CO_2 concentration and humidity). These can be gained by placing the applicator(s) inside a cell culture incubator or by providing the unit with equipment to maintain appropriate environmental conditions. Generally, cell cultures are placed in Petri dishes or flasks, and different configurations of the RF applicator can be chosen considering 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. On the other hand, any RF exposure system must assure uniform and well-defined exposure conditions for the entire cell population, which can be achieved by means of accurate dosimetry analyses.

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 EMF (frequency, modulation, polarization), the physical and electromagnetic properties (mass density, dielectric properties) 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 dosimetry 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. The performance of the exposure system can be assessed by considering:

- The uniformity of SAR distribution inside the sample must be as high as possible, although an overall standard deviation from the homogeneity of less than 30% is considered acceptable.
- Efficiency, which is defined as the ratio between the average power dissipated in the exposed samples and the input power at the feeding end of the RF applicator, can be increased by optimizing the coupling conditions between the induced EMF and the sample.

A negligible thermal increase in the biological sample, which, in the framework of non-thermal effects of EMF, should be below 1°C. This means that either the SAR level throughout the exposure must be low enough to avoid sample heating, or that the exposure system must be provided with specific thermoregulation tools that counteract the undesired thermal increase, or that a temperature control (*i.e.* a sample subjected to the same temperature increase induced by different methods, such as thermostatic water/oil-bath, or DC current) must be included in the study design.

The quality of experimental methods might impact the outcome of cellular responses, with generally a lower occurrence of alteration of cell responses in studies where quality criteria requirements were satisfied [19][76]. Therefore, it is essential that *in vitro* studies on the effects of EMF are designed in compliance with quality criteria, on both biological and electromagnetic sides. The definition of standard operating procedures or unified "best practice" is highly recommended for a general improvement of the experimental approach.

4 Setup of experimental procedures

4.1 Radiofrequency exposure setups

4.1.1 Exposure setups at CNR

The setup for exposure to 1950 MHz, LTE (Long Term Evolution) signal, represented in Figure 2, is described in [77] and in D4.2.

Briefly, the LTE signal was generated through a code built in-house in the Matlab 2019 (MathWorks) environment, and provided to the RF generator (Agilent, E4432B ESG-D series) via GPIB interface. The signal was sent to a microwave amplifier (MA-LTD, AM38A-0925-40-43), then to a −6 dB power splitter (HP, 11667 A), and conveyed, through a couple of bidirectional power sensors (Rohde & Schwarz, NRTZ43), to two identical, WR430 short-circuited waveguides (WG, 350 mm long), connected to the feeding side by means of coaxial-to-waveguide adapters (Maury Microwave R213A2, VSWR: 1.05). A Labview (National Instruments) program was used to set the exposure parameters (frequency, desired SAR, exposure duration, and starting time), and to remotely control and drive the signal generator and the power sensors. The incident and reflected power levels were continuously monitored throughout the exposure time and adjusted to keep the required SAR constant. The spectral power density of the LTE signal was monitored and acquired through an FPH Spectrum Rider (Rohde&Schwarz, 5 kHz to 31 GHz frequency range) spectrum analyzer.

The WG configuration and sample aspect were optimized through numerical and experimental dosimetry, to ensure high efficiency (>70%) and low nonuniformity degree (coefficient of variation <30%) of SAR distribution in the biological samples, as well as to maximize the number of samples/SAR levels that can be exposed/obtained simultaneously. Indeed, up to four samples can be inserted in a WG by using a vertical, plexiglass stand, and two SAR levels (ratio 1:2 or 1:4 between the central and the distal positions) can be obtained simultaneously by exploiting the symmetry of the TE_{10} propagation mode, as well as that of the samples.

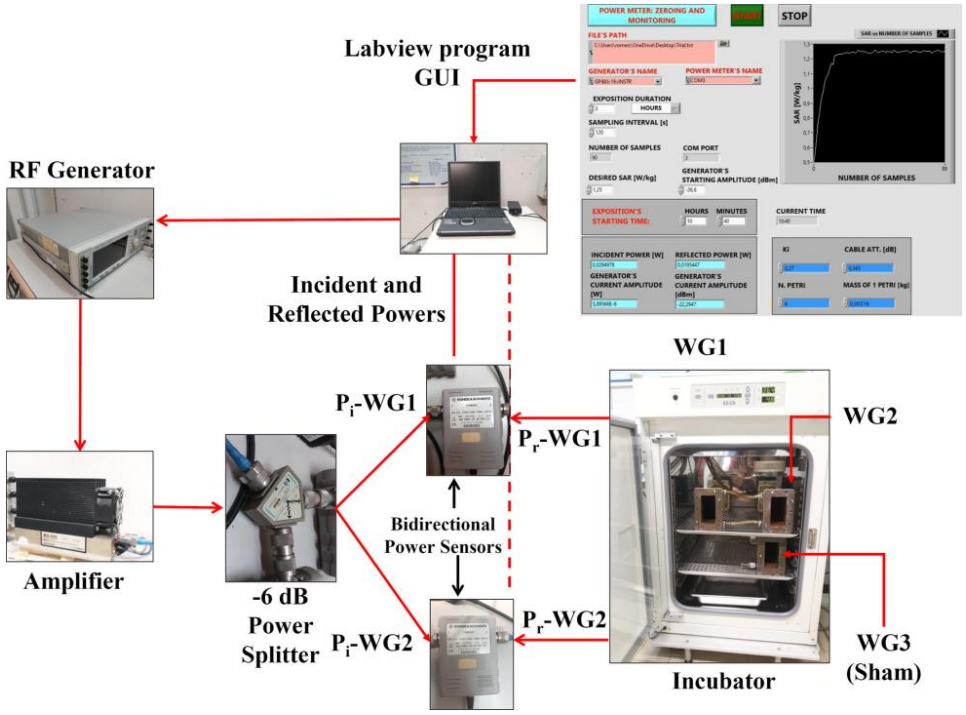


Figure 2: Configuration and components of the setup employed to expose cell cultures to 1950 MHz, LTE electromagnetic field (GUI, graphic user interface; Pi, incident power; Pr, reflected power; WG, waveguide).

The setup for multiple exposures to 4G LTE and WiFi signals, represented in Figure 3, is described in detail in D4.7. Briefly, the LTE signal, generated as described above, and the Wi-Fi signal, generated by a SMM100A RF generator

with IEEE 802.11 option enabled, were sent to the power combiner (PD2020 InSTOCK Wireless components, NJ, USA). The output signal was amplified and sent to one short-circuited waveguide by means of the power sensor. Two waveguides, one for RF and one for sham-exposure, were placed inside a standard cell culture incubator. The Virtual NRT software (Rohde & Schwarz, Munich, Germany) was used to remotely control the power sensor, and the levels of incident and reflected power were recorded throughout the exposure to verify the stability of SAR levels.

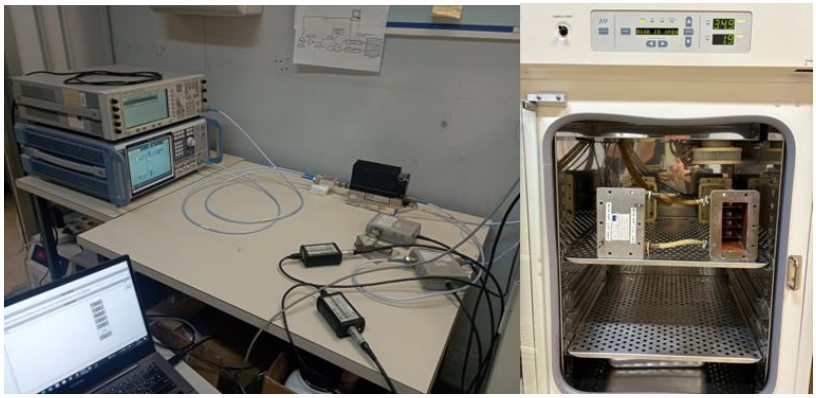


Figure 3: Configuration and components of the setup employed for simultaneous exposures of four samples to 1950 MHz (LTE signal) and 2450 MHz (Wi-Fi) RF-EMF.

Numerical and experimental dosimetry were performed to get high efficiency and uniformity of SAR distribution in the four samples to be simultaneously exposed to 1950 MHz LTE and 2450 MHz Wi-Fi at 2 SAR levels. As a result of the dosimetry evaluation, described in details in D4.7, customized, 30 mm diameter, Pyrex dishes were used for cell cultures, which allowed to reduce the non-uniformity degree to an acceptable level in the case of 2450 MHz.

The setup for exposure of mammalian cells or *C. elegans* cultures to 26.5 GHz, 5G signal, represented in Figure 4 is described in detail in D4.2. Briefly, the field applicator is a reverberation chamber (RC), i.e. an environment with metallic walls whose dimensions are very large compared to the wavelength, so that a large number of modes can be excited, and a spatially uniform power density is obtained by mechanical stirring. The RC was designed by numerical simulations in the CST Microwave Studio (CST, Darmstadt, Germany) platform, to obtain high uniformity of the electric field distribution inside the samples, to maximize the number of samples that can be exposed at the same time, and to be hosted inside standard incubators. Simulations were performed by considering a suitable number of plane waves impinging on the samples (35 mm Petri dish filled with 3 ml of culture medium) with unitary amplitude, both polarizations, and different incoming directions, and by superposing the computed fields. The coefficient of variation was calculated for different slices in a 100 μm-thick bottom layer, corresponding to the cell monolayer, and ranged between 20.2% and 24.3% in the case of 28 waves.

Based on the simulation results, an RC with inner dimensions of 404 mm x 419 mm x 375 mm (compatible with standard incubators) and a wall thickness of 4 mm was made by anticorodal (aluminium-magnesium-silicon alloy). An opening (8 cm diameter) with a metal net (200 μm mesh) was inserted in the top wall to guarantee a constant environment (temperature, relative humidity, and CO_2) when the RC is inserted into the incubator. The stirrers were realized using two rectangular metallic crosses rotating parallel to two perpendicular walls (Figure 4a) at independently controlled velocities (Figure 4b). A stand, opportunely engraved to house Petri dishes, allows a reproducible positioning of samples (Figure 4c). Two WR-28 open-ended waveguides are used as transmitting and receiving antennas. Two identical RCs, one for RF- and one for sham-exposure, were realized and hosted inside two standard, cell culture incubators (Forma Scientific, model 311) for mammalian cell exposure, or in one refrigerated incubator (PHC Biomedical, MIR-554) for exposure of *C. elegans* cultures. The RC used for RF exposure is connected to a Rohde&Schwarz, SMW200A signal generator (Figure 4) with SMW-K144 and SMW-K148 options enabled to generate a 5G compliant signal. The delivered power is measured during the exposure by means of a power meter (Rohde&Schwarz, NRP-Z85) connected to feeding line #2, and power values are monitored and acquired through Rohde&Schwarz Power Viewer software.

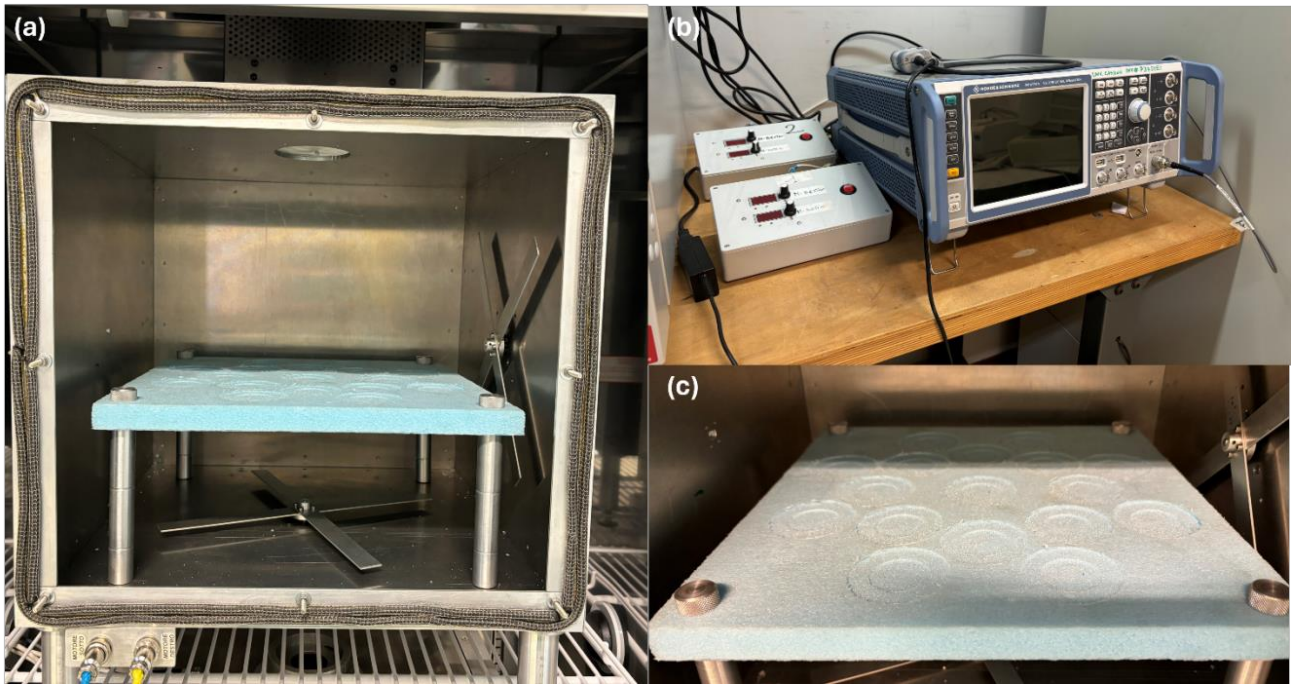


Figure 4: Reverberation chamber-based setup for exposure to 26.5 GHz, 5G signal, electromagnetic field. (a) Open RC hosted inside the incubator, with the stirrers mounted on the right and bottom walls; (b) Rohde&Schwarz, SMW200A signal generator and stirrer controllers; (c) stand for the housing of Petri dishes.

The RC-based exposure system has been characterized by a biological perspective with the aim of assuring that the physiological conditions for biological samples are maintained inside the chambers. To this aim, "sham-sham experiments", using the two RCs in a sham configuration, were performed to evaluate the experimental environment, having the standard cell culture incubator as a reference control. The well-being of cells is the primary condition when a possible cause-and-effect relationship must be identified. In the following, the main procedures of the "sham-sham" experiments and the results are presented.

Evaporation, pH, and temperature were considered as environmental parameters, while cell viability and cell cycle progression were performed to check the metabolic status of cell cultures.

Four samples established with a 3 ml culture medium, handled in parallel, were positioned in each incubator (control, RC1, and RC2). At least three independent experiments were carried out for each parameter.

After 24h incubation, the pH and volume of the culture medium were measured, and no difference among the samples was found, regardless of sample position, for both environmental parameters (data not shown). The temperature inside the RC, measured with a fiber optic thermometer, was about 1 °C lower than in the incubator. Two biological tests were performed to highlight a possible impact on the physiological state of the cell: viability and cell cycle progression. The Resazurin assay to investigate cell viability in terms of metabolic activity [78] and cell staining with PI in permeabilized cells for cell cycle analysis [79] were performed in human keratinocytes (HaCaT), the cell model used for 5G exposure experiments. The same targets were also investigated in a different cell type, human neuroblastoma cells (SH-SY5Y), to consolidate the data. The metabolic activity of HaCaT (Figure 5A) and SH-SY5Y (Figure 5B) cells, hosted for 24h in the RCs, was unaffected. Ethanol treatment for 40 min served as a positive control (6% and 2 % for HaCaT and SH-SY5Y cells, respectively).

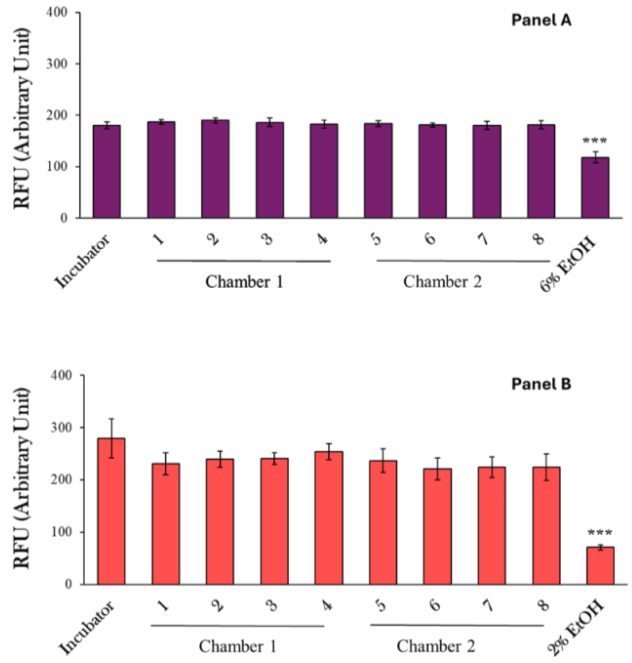


Figure 5: Resazurin assay in HaCaT (panel A) and SH-SY5Y (panel B) cells hosted for 24h in RCs. Relative Fluorescence Unit (RFU) is presented as mean ± SE of 3 independent experiments (***P <0.001, one-way ANOVA for repeated measurements followed by post-hoc Tukey test).

No changes in the percentage of cells in each stage of the cell cycle were observed in any sample (Figure 6), while a significant effect was detected in the positive control samples (500 ng/ml mitomycin-C, MMC, and 0.5% FBS for HaCaT and SH-SY5Y cells, respectively).

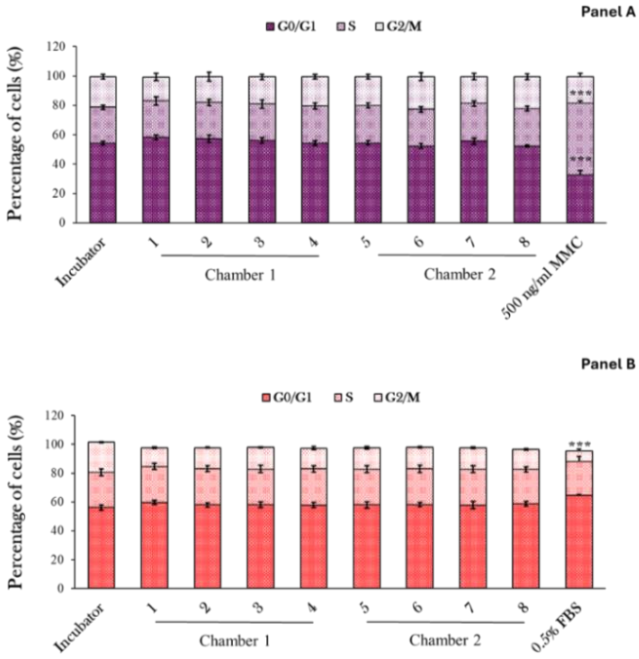


Figure 6: Cell cycle analysis in HaCaT (panel A) and SH-SY5Y (panel B) cells hosted for 24h in RCs. The percentage of cells in each cell cycle stage is presented as mean ± SE of 3 independent experiments (***P <0.001, one-way ANOVA for repeated measurements followed by post-hoc Tukey test).

Overall, the above-presented results of the sham-sham experiments demonstrated that the environmental conditions inside the RCs do not affect the cell physiology of the two cell models. The designed RCs-based exposure setup can be used for the 5G RF exposure experiments without introducing any experimental bias that could alter the reliability of the results.

4.1.2 Exposure setups at SC

For the *in vitro* studies, Sciensano used the sXc3500 exposure system (Figure 7), which was developed by the IT'IS Foundation, and previously described in detail in Deliverable 4.2.

Figure 7: Waveguide-based setup for exposure to a 3.5 GHz, 5G signal electromagnetic field. The waveguides inside the incubator (left) and the signal tower, including the vector signal generator and the amplifier (right).

Briefly, this system is specifically engineered to expose cells *in vitro* to a frequency of 3.5 GHz, incorporating various options for modulation while maintaining a blinded experimental design. For the NextGEM project, the system can generate a 256QAM modulated 5G-NR signal with a bandwidth of 100 MHz and a sub-carrier spacing of 60 kHz.

The system utilizes two R32 rectangular waveguides, one for actual exposure and the other for sham exposure, enabling blinded experiments. Optimized for exposing cell monolayers, the waveguides can accommodate up to six cell cultures in 35 mm Petri dishes in a tray. The system ensures uniform and controlled exposures across the cell cultures with minimal uncertainty. The initial dosimetry for a monolayer at the bottom of these Petri dishes was calculated by the IT'IS Foundation and additional dosimetry calculations for cells in suspension have been performed by UCAS.

The sXc3500 system is employed in Task 4.3 of the project to expose HaCaT cells (keratinocyte-derived immortalized cell line) for the evaluation of various biological endpoints, including genotoxicity (micronucleus and alkaline comet assays), epigenetics, changes in gene expression (TempO-Seq, qPCR), oxidative stress, cell proliferation, and apoptosis. Additionally, the system facilitates experiments with human lymphocytes extracted from volunteer blood samples, wherein cells are exposed *in vitro* and subsequently used for genotoxicity testing and transcriptomics analysis.

4.2 UVB exposure setup

The UV exposure setup, shown in Figure 8a, is made of a wooden box (710 mm long, 260 mm wide, 300 mm deep) with the UV source mounted on the top wall, and the sample holder on the opposite side. The box can be safely closed during UV irradiation. The UV source consists of a board with two parallel strips of six LEDs each (LITE-ON) UVB LED 308 nm, 45 mW, 350 mA, 120°), which are spaced ~5 cm apart on each strip, and ~0.5 cm on the parallel stripes, Figure 8b). The board is powered by a dimmable LED AC/DC power supply (Professionale DALI, TCI srl), a multipower driver supplied with a DIP-switch for the selection of the output current.

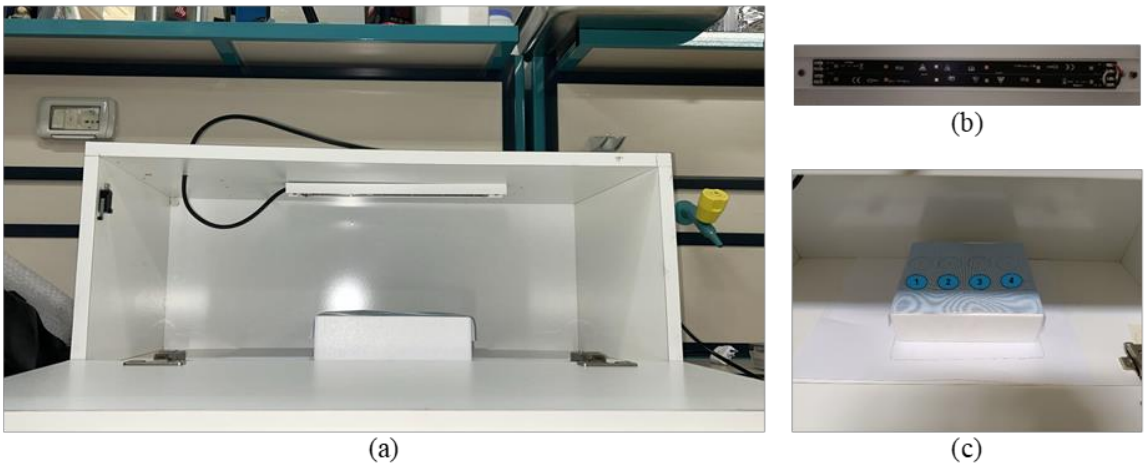


Figure 8: (a) UV exposure setup; (b) LED board; c) irradiance measurement positions

To characterize the UV exposure setup in terms of irradiance, experimental measurements were carried out by using the HD2102.1 (DeltaOhm) radiometer, provided with a UVB probe (LP471UVB, 280-315 nm spectral range). The measurements were carried out in positions 1-4 (Figure 8c) with the UVB probe located on a 6 cm high Styrofoam holder (15 cm from the LED strips).

The measurements were performed 30 min after switching on the LED board, to allow for stabilization of the irradiance levels, and the maximum, minimum, and average levels were acquired over a 15-min time interval. The results indicated that samples in positions 1 - 4 underwent comparable average irradiance values of 4.729, 5.333, 5.268 and 5.016 W/m², respectively and allowed the simultaneous exposure of four cell cultures in the same conditions. The contribution of UVA and UVC was verified and resulted negligible.

The UVB dose (Joule/m²) administered to cell cultures was calculated by multiplying the average irradiance value (W/m²) for the treatment duration (sec).

4.3 Biological procedures –human neuroblastoma SH-SY5Y cells exposed to 4G LTE signal in the absence and in the presence of menadione

4.3.1 Cell cultures, maintenance and 4G RF exposure

SH-SY5Y cells, purchased from ATCC (Cat. No. CRL2266, Manassas, VA, USA), were grown as clusters of neuroblastic cells in DMEM medium supplemented with 10% heat-inactivated FBS, 2 mM GlutaMAX™, 1X Penicillin-Streptomycin solution. Cells were cultured in a commercial incubator (model 311; Forma Scientific) at 37 °C in 95% humidified air with 5% CO_2, supplied with fresh culture medium every 72h, and split once a week by 200 mg/ml trypsin-EDTA treatment. Mycoplasma infection was periodically checked by the DAPI staining test. The same batch of reagents was used to ensure reproducibility among the measurements, and experiments were carried out on cells from passages 3–10.

For the experiments, 1×10^6 cells were seeded in 3 ml complete medium, in 35-mm cell culture dishes (Corning Inc., Corning, NY, USA). 48 hours after seeding, SH-SY5Y cells were exposed for 3h to 1950 MHz, 4G-LTE signal. At the end of the culture period (72h), cells were treated with menadione (MD), a well-known cytotoxic chemical agent. Treatment doses were set downstream of the dose-response curve according to the assay/endpoint.

Three WGs (one disconnected and two connected to the RF feeding and used for sham- and RF- exposures, respectively) are placed inside a cell culture incubator (model 311; Forma Scientific) to ensure a 95% air and 5% CO_2 atmosphere at 37°C. For each experimental run, 12 cultures were handled in parallel: not exposed (incubator), sham-exposed (Sh), RF-exposed at 0.3 W/kg, and RF-exposed at 1.25 W/kg, in the absence and in the presence of two MD doses.

Detailed protocols for the procedures described above are reported in Annexes 1 and 2.

4.3.2 Oxidative stress

The probe 2',7'-Dichlorodihydrofluorescein diacetate (H2DCF-DA) was used to assess the formation of reactive oxygen species (ROS). When H2DCF-DA crosses the cell membrane, it is deacetylated into 2',7'-dichlorodihydrofluorescein (H2DCF), which is oxidized into the fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of ROS.

Preliminary experiments were performed to establish the H2DCF-DA concentration and treatment duration suitable to display ROS formation in SH-SY5Y cells. H2DCF-DA concentrations of 10 and 40 µM were tested for 10 and 30 min in 3 ml absolute DMEM, and 10 min treatment at 10 µM resulted in the most suitable condition. These conditions were used to set up a dose-response curve for MD treatment. Specifically, 5, 10, 20, and 50 µM MD concentrations were administered concurrently with 10 min H2DCF-DA. The results are presented in Figure 9, where a dose-dependent increase in ROS formation was recorded. The concentration of 50 µM MD strongly affected cell viability and was not considered for the analysis. 5 and 20 µM MD were selected as concentrations for RF co-exposures, and cells treated with 20 µm MD also served as positive control.

For the RF exposure experiments, SH-SY5Y cells were treated for 10 min at 37°C with 10 µM H2DCF-DA in 3 ml absolute DMEM, along with MD where required. The cells were harvested by trypsin treatment and washed in cold PBS. DCF fluorescence was measured by acquiring 15000 events by flow cytometry (FACSCalibur™; Becton Dickinson). The FlowJo program was used for data analysis. The results were expressed as the percentage of DCF-positive cells (DCF signal above the background) as an index of ROS formation, setting the threshold value on the background DCF fluorescence of the untreated control.

A detailed protocol for the procedure described above is reported in Annex 3.

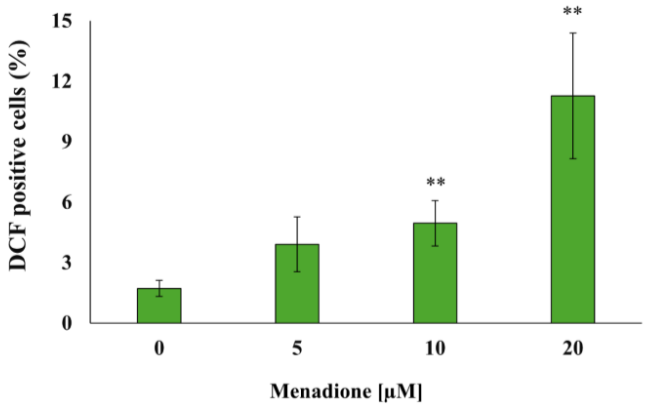


Figure 9: MD dose-response curve for oxidative stress analysis. The percentage of DCF-positive cells (DCF signal above the background in untreated dye-preloaded cells) is presented as mean ± SE of 5 independent experiments. MD treatment induced ROS formation with a statistically significant increase (** $p < 0.01$; Student's t-test).

4.3.3 Apoptosis

Double staining with Annexin V-FITC and propidium iodide (PI) was used to assess apoptosis, the process of programmed cell death. Phosphatidylserine (PS) is a phospholipid that translocates to the external leaflet of the cell membrane bilayer during the early apoptosis stages. The annexin V protein binds PS with high affinity, allowing the detection of early apoptotic cells by flow cytometry when labelled with FITC. In addition, the PI staining enables the discrimination of late apoptotic and necrotic cells.

Samples treated for 30 min with 5, 10, 20, and 30 µM MD were used to set up the dose-response curve. 5 and 20 µM MD were selected as doses for RF co-exposures, and 20 µm MD also served as a positive control. SH-SY5Y cells were treated with MD where required and then harvested by trypsin treatment. 3×10^5 cells were collected by centrifugation, washed in cold PBS, and processed using the annexin V-FITC Apoptosis Detection kit (Leinco Technologies, cod. A432) following the manufacturer's instructions. The fluorescence was measured by acquiring 15000 events by flow cytometry (FACSCalibur™; Becton Dickinson). FlowJo software was used for data analysis by recording the

percentage of cells in each quadrant: apoptotic cells are displayed in the right quadrants of the dot plot, with early apoptotic cells at the bottom (Annexin V- $FITC^+/PI^-$) and late apoptotic cells at the top (Annexin V- $FITC^+/ PI^+$), necrotic cells (Annexin V- $FITC^-/PI^+$) are displayed on the top left. Figure 10 presents the percentage of total apoptotic cells (early+late).

A detailed protocol for the procedure described above is reported in Annex 4.

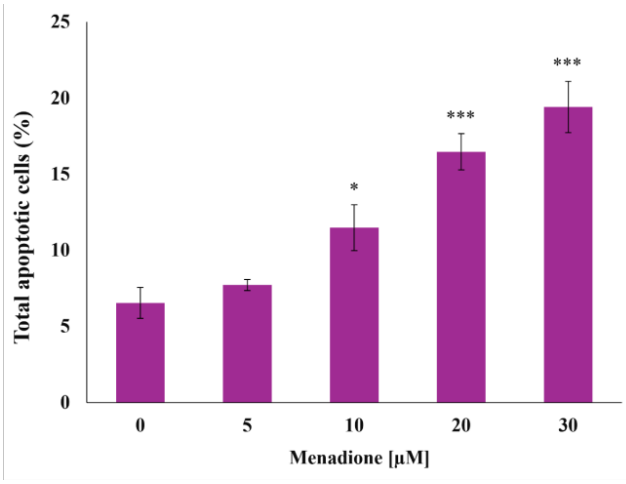


Figure 10: MD dose-response curve for apoptosis analysis. The percentage of total apoptotic cells is presented as mean ± SE of 4 independent experiments. MD treatment induced apoptosis with a statistically significant increase (* $P < 0.05$, *** $P < 0.001$; Student's t-test).

4.3.4 Cell cycle progression

PI staining in permeabilized cells was used for cell cycle analysis using flow cytometry. PI emits fluorescence when it binds to DNA, allowing quantification of cellular DNA content in different phases, i.e., G0/G1, S and G2/M. Normally, cycling cells stained with PI have two separated peaks at G1 and G2, corresponding to cells containing 2N and 4N DNA content, respectively. The cell population between the two peaks represents cells in the S phase.

A 16h treatment with 1 µg/ml Mitomycin-C (MMC), a known cytotoxic agent, was used as a positive control. At the end of the culture period, cells were harvested by trypsin treatment. 5×10^5 cells were collected by centrifugation, washed in cold PBS and treated for 30 min at 4°C with a permeabilizing staining solution (50 µg/mL PI; 33mM sodium citrate, pH 8, and 0.1% Triton X-100) diluted 1:2 in DMEM medium. The fluorescence of PI was detected by acquiring 25000 events by flow cytometry (FACSCalibur™; Becton Dickinson). FlowJo software was used for data analysis. The results were expressed as the percentage of cells in the cell cycle's G0/G1, S, and G2/M stages.

A detailed protocol for the procedure described above is reported in Annex 5.

4.4 Biological procedures – human neuroblastoma SH-SY5Y cells exposed to multiple signals

4.4.1 Cell cultures, maintenance, and multiple RF exposure

The procedures implemented for SH-SY5Y cell cultures and maintenance have been described in 4.3.1, and in Annex 1. For the experiments, 8.5×10^5 cells were seeded in customized Pyrex dishes and grown for 72 h in 1.75 ml of complete medium. The suitability of Pyrex customized dishes to host cell cultures was verified by setting up 4-day-growth curves in Pyrex dishes by using standard Polystyrene dishes as reference control. Cells were simultaneously exposed for 3 h (48-51 h) at 1950 MHz, LTE signal and 2450 MHz, Wi-Fi signal (0.3 or 1.25 W/kg SAR).

Two WGs (one disconnected and one connected to the RF feeding and used for sham- and RF- exposures, respectively) are placed inside a cell culture incubator (model 311; Forma Scientific) to ensure 95% air and 5% CO_2 atmosphere at 37°C. For each experimental run, four cultures were set and handled in parallel: negative control (incubator), sham-exposed (Sh), RF-exposed at 0.3 W/kg (0.3 W/kg) and RF-exposed at 1.25 W/kg (1.25 W/kg). The procedure for the exposure to 4G LTE and WiFi is described in Annex 6

4.4.2 Cell cycle progression

For the analysis of the cell cycle, the procedure described in 4.3.4 and in Annex 5 has been applied.

4.5 Biological procedures – human keratinocyte HaCaT cells at CNR.

4.5.1 Cell cultures, maintenance and 5G RF exposure

HaCaT cells, purchased from CLS (Cat. No. 300493, Eppelheim, Germany), are keratinocytes growing as a monolayer in DMEM medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, and 1X Penicillin-Streptomycin solution. Cells were cultured in a commercial incubator (model 311; Forma Scientific) at 37 °C in 95% humidified air with 5% CO_2, supplied with fresh culture medium every 48 h, and split twice a week by 200 mg/ml trypsin treatment. Mycoplasma infection was periodically checked. The same batch of reagents was used to ensure reproducibility among the measurements, and experiments were carried out on cells from passages 3-20.

For the experiments, 3×10^5 were seeded in 3 ml complete medium, in 35-mm Petri dishes (Corning Inc., Corning, NY, USA). At 48 h after seeding, cells were exposed for 3 or 24 h to 26.5 GHz, 5G signal. After RF exposure, cells were subjected to UVB treatment in PBS and harvested 24 h later to evaluate possible combined effects. For the RF exposure, the signal is sent to a reverberation chamber placed inside a cell culture incubator (model 311; Forma Scientific), while a second reverberation chamber, disconnected from the RF feeding and used for sham exposures, is placed in another identical cell culture incubator. Each chamber is equipped with two stirrers at independently controlled speeds and hosts a sample holder to allow reproducible positioning of the cell culture dishes. For each experimental run, 5 cultures are set up per exposure condition, i.e., not exposed (incubator), not UVB treated but kept on the bench for the duration of UVB treatment (negative control), sham-exposed (Sh) and RF-exposed at 0.4 W/kg or 1 W/kg.

Detailed protocols for the procedures described above are reported in Annexes 7, 8 and 9.

4.5.2 Oxidative stress and apoptosis

The assay methods for ROS and apoptosis measurements adopted here for HaCaT cells are the same ones as described for SH-SY5Y cells under paragraphs 4.3.2 and 4.3.3, respectively, while the experimental procedures were adjusted for this cell model, where needed.

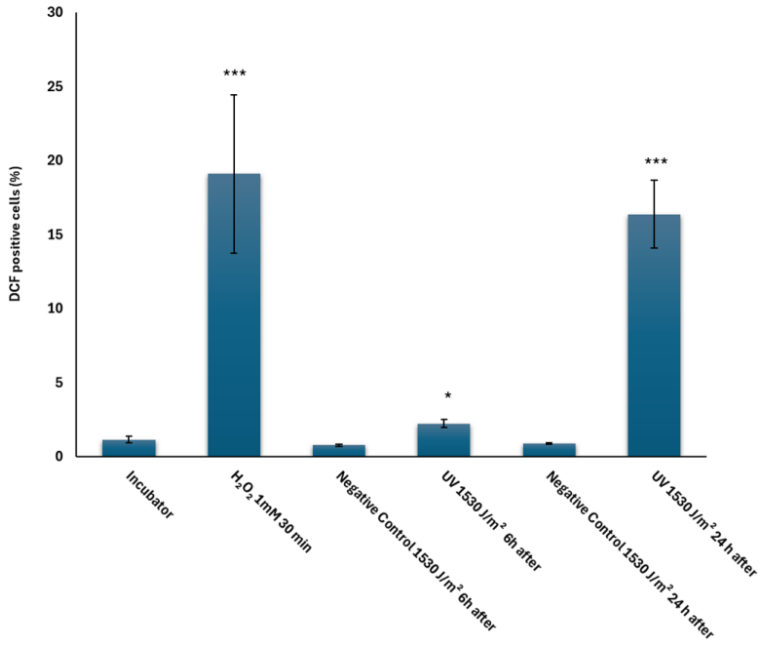


Figure 11: UVB treatments for ROS analysis. The percentage of DCF-positive cells (DCF signal above the background in untreated dye-preloaded cells) is presented as mean ± SE of 6 independent experiments. UVB induced ROS formation with a statistically significant increase (*P<0.05; *** P< 0.001; two tailed unpaired Student's t-test).

Preliminary experiments were performed to establish H2DCF-DA concentration and treatment duration suitable to display ROS formation in HaCaT cells, and 30 min treatment at 10 µM resulted in the most suitable condition. These conditions were used to find out the UVB treatment triggering ROS formation. Results are presented in Figure 11, where the effect of 30 min treatment at 1 mM final concentration H_2O_2 is also presented as a positive control. Treatment with H_2O_2 induced a statistically significant increase in ROS formation. UVB treatment of 1530 J/m^2 induced a slight increase in ROS formation at 6 h post-treatment, and consistent effects at 24 h post-treatment. The latter UVB condition was used for co-exposure experiments.

The same UVB treatment also resulted in inducing statistically significant alterations in apoptosis, and was used for 5G RF co-exposure experiments. Detailed protocols for the procedures described above are reported in Annexes 10 and 11.

4.6 Biological procedures – human keratinocyte HaCaT cells at SC

4.6.1 Cell cultures, maintenance and 5G RF exposure

HaCaT cell lines were obtained from Cell Line Service (CLS) (Cat. No. 300493, Eppelheim, Germany). For the transcriptomics (TempO-Seq), RT-qPCR and epigenetics experiments, HaCaT cells were cultured in DMEM medium supplemented with 10% FBS, 2mM L-Glutamine, and 1X penicillin/streptomycin. To minimize variability in the results, due to differences in experimental conditions, the same batch of cells was used by SC and CNR.

For other tests, including genotoxicity, oxidative stress, cell cycle analysis, and apoptosis, the HaCaT cells were grown in DMEM medium with 10% FBS, 1% Glutamax, 1% gentamycin, 1% non-essential amino acids, 1% sodium pyruvate, and 0.1% amphotericin B.

Cell cultures were maintained in an incubator at 37°C, with 5% CO_2 and approximately 95% humidity. The HaCaT cultures were subcultured twice weekly in T75 cell culture flasks using Trypsin or TrypLE. For lytic assays, such as the TempO-Seq or the epigenetics tests, trypsin was used, but for protocols where isolated cells were more important (such as the comet assay and the micronucleus test), a gentler trypsin alternative, called TrypLE, was used.

At the start of each week, HaCaT cells were seeded for experiments. For the comet assay, TempO-Seq, and epigenetics tests, 3x10^5 cells were seeded per 35 mm Petri dish, whereas for the micronucleus tests, 2.5x10^5 cells were seeded per 35 mm Petri dish.

After seeding, cells were incubated (5% CO_2 and 95% humidity) for at least 24h to allow them to attach to the bottom of the dishes, as the system is optimized for monolayer exposure. In all cases, 3.1 mL cell cultures were established to meet the dosimetry requirement of field uniformity. For the positive control and co-exposure (RF + chemical) conditions, the chemical substance was added 24h after seeding.

For exposures/co-exposures, samples were placed inside waveguides 15 min before starting exposure to stabilize temperature, CO_2 levels, and humidity. Exposure parameters, including dosimetry, duration, and blinding, were set using the sXc3500 control software from IT'IS. The exposure was initiated blinded to prevent researcher bias. After exposure, a logfile was created and decoded only after test analysis to maintain blinding.

In the comet assay, micronucleus assay, TempO-Seq transcriptomics, and epigenetics experiments, exposure was always stopped 48h after seeding, regardless of the exposure time, to ensure uniform cell conditions post-exposure (e.g., for the 3h exposure condition, exposure was started 45h after seeding). Temperature measurements at the sample level were done using a THR-NS-1165B fiber optic temperature sensor from FISO Technologies, recording every second throughout the exposure to ensure that temperature variations were negligible during exposure.

A detailed protocol for the procedures described above is reported in Annex 12.

4.6.2 Genotoxicity

In the NextGem project, the *in vitro* micronucleus test and the *in vitro* comet assay were used to investigate genotoxic effects.

4.6.2.1 Cytokinesis block micronucleus assay

The *in vitro* micronucleus assay is a widely used and OECD standardized method for assessing chromosomal damage induced by various chemical and physical agents [80]. This assay detects the formation of micronuclei in dividing

mammalian cells, i.e., small, extranuclear bodies containing fragments or whole chromosomes that were not incorporated into the main nucleus during cell division. The presence of micronuclei is indicative of chromosome breakage, chromosome loss, or spindle disruption, all of which are associated with genotoxicity and potential carcinogenic effects.

HaCaT cells were exposed/sham-exposed to 5G-NR modulated RF-EMF, at various (0.4 W/kg and 1 W/kg) SAR levels, for 24h. Positive (methyl methanesulfonate, MMS; 150 μM) and negative controls were included in the experiment.

A post-treatment incubation period of 24h with cytochalasin B was included to allow for cell recovery and expression of chromosome damage. Cytochalasin B was added to prevent cytokinesis, the process that divides the cytoplasm of a parental cell into two daughter cells during cell division. As a result, cells that have undergone nuclear division (mitosis) become binucleated, as genetic material is still doubled, but the later stages of cell division are blocked. This allows for the assessment of micronucleus formation in cells that have completed nuclear division. At the end of the incubation period, cells were harvested by trypsinization to prepare a single-cell suspension. The cell suspensions were then fixed and put on glass microscope slides using a methanol-acetic acid fixation method. Next, the fixed cells were stained with DAPI, a DNA-specific dye, to visualize the nuclei and micronuclei. Cells on the stained slides were examined for the presence of micronuclei using an automated system consisting of a Zeiss Axiovert 40 microscope and the Metafer4 imaging platform (Metasystems, Germany). These micronuclei appear as small, round bodies distinct from the main nucleus, often located adjacent to or separate from the main nucleus. A minimum of 5000 binucleated cells for each condition (not pooling duplicates) was scored to have sufficient statistical power to detect small effects induced by the RF-EMF exposure. The cells in which the automated system detected micronuclei were checked manually to exclude artifacts by applying predetermined criteria for micronuclei, including size, shape, and staining intensity. Results were expressed as the number of binucleated cells with micronuclei (e.g., per 1000 binucleated cells) for each treatment condition and control. An increased micronucleus frequency above background levels in treated cells indicates genotoxicity and potential chromosomal damage induced. The cytokinesis-block proliferation index (CBPI) was determined and used as an index of cell cycle progression:

$$CBPI = \frac{((\#\ mononucleated\ cells) + (2\ x\ \#\ Binucleated\ cells) + (3\ x\ \#\ polynucleated\ cells))}{(total\ number\ of\ cells)}$$

This also allows to calculate the percentage cytostasis:

$$\%\ cytostasis = 100 - 100\ x\ \frac{CBPI_T - 1}{CBPI_C - 1}$$

with $CBPI_T$ being the CBPI of the tested condition and $CBPI_C$ being the CBPI of the control.

In conditions with more than 55% cytostasis, assay results should be interpreted with caution.

A detailed protocol for the *in vitro* micronucleus test applied is provided in Annex 13.

4.6.2.2 Alkaline comet assay

The *in vitro* alkaline comet assay, also known as the single-cell gel electrophoresis assay, is a sensitive and widely used method for evaluating DNA damage induced by various chemical and physical agents. More specifically, this assay detects DNA strand breaks and other DNA damage in individual mammalian cells.

In brief, HaCaT cells were cultured under controlled conditions and exposed for 24h to RF-EMF, 5G signal, at 0.4 W/kg and 1 W/kg SAR levels. EMS (200 μM) was used as a positive control. After exposure, cells were embedded in LMP agarose on microscope slides to create a thin gel matrix containing cells with intact nuclei. Cells were then treated with lysis buffer to remove cellular membranes and subjected to alkaline conditions to promote DNA unwinding and denaturation of double-stranded DNA into single-stranded DNA. An electric current was applied after placing the slides in an electrophoresis chamber with an alkaline running buffer. Under these conditions, negatively charged fragmented DNA migrates away from the nucleus towards the anode, forming a comet-like tail shape.

After electrophoresis, the slides were stained with GelRed, a DNA-specific fluorescent dye, and visualized under a fluorescence microscope. Comet images were captured automatically using a Zeiss Axiovert 40 microscope combined with the Metafer4 imaging platform (Metasystems, Germany) and analysed using the specialized Metafer software to quantify parameters such as tail length, tail intensity, and percentage of DNA in the tail. All captured images were also

checked manually to ensure proper discrimination between the head and the tail by the automatic software. A minimum of 100 cells per condition (pooling duplicates) was scored, for a total of 4 gels per condition (two per duplicate) to ensure sufficient statistical power to detect small effects induced by RF-EMF, if any. In practice, the total number of cells scored varied between 200 and 700 cells per condition.

A detailed protocol for the *in vitro* comet assay is provided in Annex 14.

4.6.3 Exploration of epigenetic effects

A literature review was performed (cf. Table 2 in section 2.2.1.5) to select the experimental conditions for the epigenetics study. The design of the experiment was made in close consultation with the transcriptomics experiments (SC and CNR), to have data that can be put in relation with each other, allowing for a global interpretation of the effect of RF on gene expression and regulation, if applicable. Therefore, to evaluate differential gene expression modulated by hyper- or hypo-methylation of corresponding promoters, the epigenetics effect was investigated using similar experimental conditions as done for TempoSeq at SC, i.e. HaCaT cells were exposed to 3.5 GHz (SAR 1 W/kg) for 1h, 3h and 24h (conditions A, B and C, see Table 3). The methylation measurement technology was carefully selected based on the state-of-the-art (cf. human methylome with Twist capture method).

Table 3: Exposure and post-exposure conditions of the epigenetics experiments

Conditions	A	B	C	D	E
Exposure time	1h	3h	24h	3h	3h
Post-exposure time	0h	0h	0h	1h	30h

Immediately after exposure (1h, 3h and 24h), the cells were processed for DNA extraction (0h post-exposure). To assess DNA-methylation modifications after 3h exposure, the analysis was also performed 1h and 30h after exposure (Table 3). For each experiment, 5 cell cultures were included with a mix of the different conditions (Table 4), allowing the obtaining of at least technical triplicates and biological duplicates, both in blinded sham vs. exposed conditions (wave guide 1 (WG1) vs. wave guide (WG2), not knowing which was sham or exposed during the whole analysis).

DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen) (including an RNAse treatment), further purified with MicroCHIP DiaPure columns (Diagenode) and a quality control check was performed for DNA yield (Qubit), purity (Nanodrop), and fragment length (TapeStation). The DNA extraction method was optimized for the experiments and tested successfully, yielding DNA of sufficient quality and quantity, meeting the criteria for methylation measurement.

The extracted DNA of 3 cell cultures per condition (i.e., technical triplicates showing the best DNA quality) was analysed to measure the DNA-methylation level of 3.98 million CpG sites on both strands (9M in total), representing 84% of the total CpGs (genome-wide). This was achieved by using enzymatic methyl-seq (EM-seq) with the Twist human methylome panel, which is based on enzymatic conversion of the DNA followed by hybrid-capture method and sequencing using a Novaseq 6000. No harsh bisulphite treatment was involved. The analysis done with the human methylome panel also covers the promoter regions of all the genes evaluated in the TempoSeq analysis. Therefore, this can allow the comparison between transcriptomics and epigenetics analysis. The remaining samples (biological replicates) were kept for further confirmation of the methylation data using a more targeted method, if required in the future.

Table 4: Organisation of the different conditions for each experiment

		Conditions					
		Well 1	**Well 2**	**Well 3**	**Well 4**	**Well 5**	
Experiments name	**BDE**	B	B	B	D	E	× 2 Sham Vs. Exposed
	DBE	D	D	D	B	E	
	EBD	E	E	E	B	D	
	A1	A	A	A	A	A	
	A2	A	A	A	A	A	
	C1	C	C	C	C	C	
	C2	C	C	C	C	C	

For all the tested conditions, the measured DNA-methylation levels of the investigated CpGs (from the methylome panel) of the sham vs. exposed (which could be either WG1 or WG2, as the analysis was performed blind), allexperiments were compared, to identify positions and regions that were differently hyper- or hypo-methylated in response to RF-EMF exposure.

To do this, the reads were first trimmed and then mapped to the human reference genome using a specialized mapper for methylation short-reads sequencing data. The resulting counts were normalized and filtered and then used to call differentially methylated positions and regions, with a cut-off of at least 25% difference between the compared conditions and significance filtering.

The detailed protocol for the procedure described above is reported in Annex 15.

4.6.4 Exploration of Oxidative stress

Oxidative stress was examined at SC using a Dichlorodihydrofluorescein Diacetate (H2DCF DA) method, measuring the generation of intracellular ROS inside the HaCaT cells. We used a method using a plate reader loosely based on the following protocol [81]. Certain adaptations had to be made in order to satisfy the limitations we have in our setup, mainly the fact that exposure can only happen in dishes and not in multi-well plates. So the assay had to be adapted to satisfy the need that the medium has to be transferred over into a plate before being measured.

For this, a combined H2DCF DA/MTT assay was developed (Figure 12). This allows us to measure the effect of the ROS generation, but it also allows us to normalize it against the total amount of cells present within a sample, allowing us to effectively calculate the signal/cell, which is especially prudent in case there is any cytotoxicity.

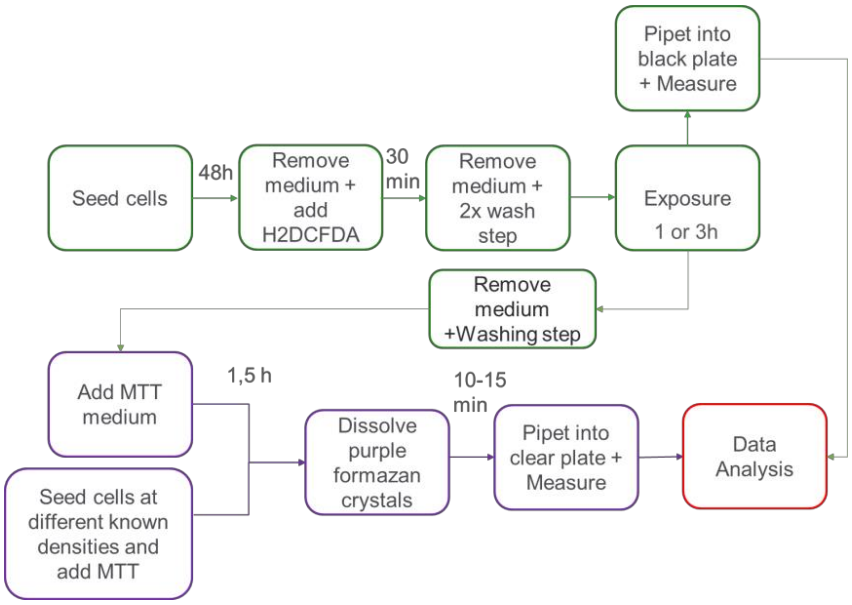


Figure 12: Schematic overview of the combined H2DCF DA/MTT assay

The protocol starts with the addition of non-fluorescent H2DCF-DA, which will be taken up by the cells and meatabolized into the H2DCF molecule. Upon exposure to ROS, or any other oxidizing agent, this H2DCF will be oxidized into the fluorescent DCF molecule, resulting in a signal equivalent to the amount of ROS formed. The remaining H2DCF-DA is removed by a washing step, and the cells are exposed to MD and RF-EMF, according to the protocol, for a period of 1 or 3 hours.

The exposure and measurements are performed in phenol-red-free and serum-free medium, because of the quenching effect these additives have on the fluorescent signal. After exposure, the medium containing DCF is pipetted into a black plate for fluorescence measurement in the plate reader. Fluorescence is measured at 560 nm.

The rest of the medium is removed, and medium containing MTT reagent is added to the dish. In parallel, new cells are seeded in dishes with the same MTT-reagent containing medium at known cell densities. The seeded cells at known densities are used to make a calibration curve, by which we can calculate the number of cells present within our exposed conditions. After 2 hours, the cells will have converted a portion of the MTT into formazan crystals (in a way that is

representative of the number of cells present within the sample These crystals are then dissolved by using DMSO, resulting in a purple substance. This will then be pipetted into a clear plate, and the absorbance will be measured at 550 nm.

In order to test this protocol, a sham-sham experiment was performed, where samples were incubated in the incubator for 3 hours with a known amount of MD and without (control) after 3 hours. No statistically significant effects were observed between the different waveguides and the incubator (Figure 13).

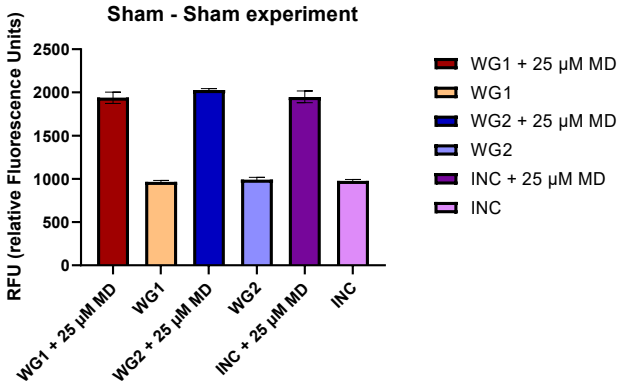
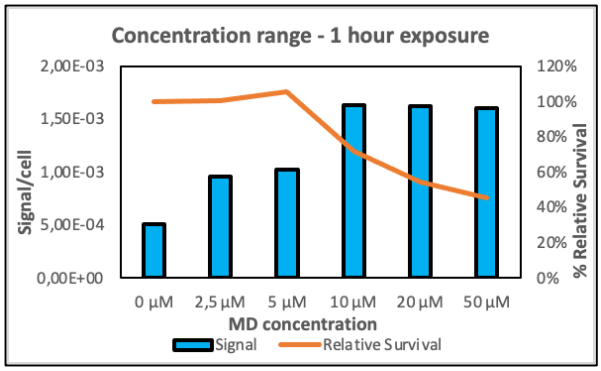


Figure 13: Relative signal from the DCF as a result of MD exposure. Conditions were sham-sham, so no RF-EMF exposure was performed. Each bar represents 3 technical replicates. This shows no interference of the system itself with our assay performance.

In order to determine the optimal concentration of MD to use as a positive control, concentration ranges were used to determine the optimal concentration to optimize for signal and minimize cytotoxicity at 1 hour and 3 hours exposure conditions (Figure 14).

A.



B.

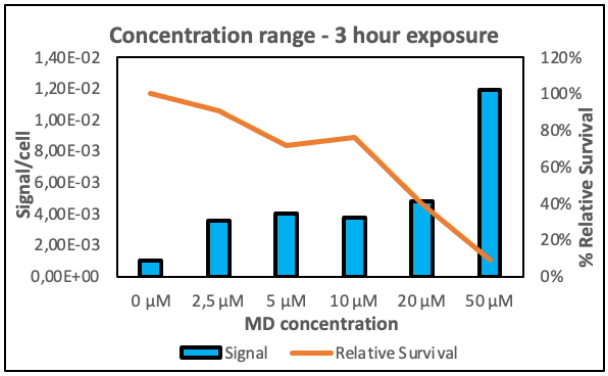


Figure 14: Concentration ranges of MD for both 1 hour (A) and 3 hours exposure (B). The blue bars represent the signal per cell after normalization against the cell count per sample as determined by the MTT assay.

For our experiments, we decided to use a concentration of 5 μM of MD for our positive control, as this optimizes for cytotoxicity, and amount of signal.

A detailed protocol for the procedure described above is reported in Annex 16.

4.7 Biological procedures –human keratinocyte HaCaT cells: joint activities at CNR and SC

4.7.1 Experiments on transcriptomics

CNR and SC optimized a common protocol for transcriptomic experiments in HaCaT cells exposed to 5G signal to explore the possibility that 5G signal operating at different frequencies can induce deregulation in gene expression. 26.5 GHz for 3 or 24h and 3.5 GHz for 1h, 3h and 24h were tested at CNR and SC premises, respectively. In both cases, 0.4 W/Kg and 1 W/kg were tested. For this purpose, the HaCaT cells in use at CNR were provided to SC, and the same batch of reagents were used by the two partners. An overview of the RF exposure conditions adopted in both laboratories can be found in Table 5.

Transcriptomics experiments were performed using the TempO-seq (Templated Oligo assay with Sequencing readout; BioClavis) technology. TempO-Seq (Templated Oligo assay with Sequencing readout) is a high-throughput targeted transcriptome analysis method that measures specific gene sequences directly from RNA in crude lysates or purified RNA. The process involves hybridization and ligating detector oligos (DOs) to target sequences, enzymatic removal of excess DOs, and amplifying and sequencing of ligated DO pairs. This method ensures exceptional specificity, even for highly similar genes, by minimizing mis-ligation and background noise, enabling precise and unbiased measurements. It allows for single-base specificity, facilitating the measurement of expressed variants and the differentiation of homologous genes within or between species.

Samples for the TempO-Seq assay were prepared according to the company's instructions (BioClavis). At both CNR and SC Laboratories, at the end of the exposure, HaCaT cells were washed in PBS and incubated with 1X Enhanced Lysis Buffer for exactly 15 minutes at 37°C to release total RNA while maintaining RNA integrity. The exposure to RF-EMF of the cells is timed so that cell lysis happens 48 hours after the seeding of the cells. Cell lysate samples were transferred into test tubes and stored at -80°C until they were ready for further analysis.

SC collected all the frozen cell lysates, including the ones prepared at CNR. They were gently thawed and transferred to the 96-well plate to be shipped on dry ice to the company for transcriptomics.

Table 5: An overview of the RF exposure conditions adopted for TempO-Seq experiments.

Responsible	SAR (W/kg)	Wavelength (GHz)	Exposure Duration (h)
SC	0.4	3.5	1
SC	0.4	3.5	3
SC	0.4	3.5	24
SC	1	3.5	1
SC	1	3.5	3
SC	1	3.5	24
CNR	0.4	26.5	3
CNR	0.4	26.5	24
CNR	1	26.5	3
CNR	1	26.5	24

The TempO-Seq assay itself was outsourced to BioClavis, which specializes in TempO-Seq Analysis. Samples were sent on dry ice to BioClavis, where synthetic DNA probes complementary to target mRNA transcripts were added to the thawed cell lysates (Human Whole Transcriptome Panel v2.1). These probes contain universal primer sites for subsequent amplification, during which sequencing adaptors and sample-specific barcodes are added. The amplicons are pooled across samples and sequenced through single-end short-read sequencing technology (Illumina). The resulting sequencing reads represent the mRNA transcripts captured and labelled during the TempO-seq assay.

After the assay was completed by BioClavis, the sequencing data were sent to Sciensano for bioinformatics analysis. The reads were filtered, aligned to a modified reference genome based on the used TempO-seq transcriptome panel, and subsequently quantified through a custom bioinformatics framework. The resulting gene expression counts were normalized and investigated for differentially expressed genes (DEGs) between the different treatment conditions using a p-adjusted level of 0.05 and a fold change of 1.5. The DEGs were then subjected to an over-representation analysis to infer their functional annotation and to investigate their association with relevant biological pathways, such as cancer initiation and progression. Data interpretation for this bioinformatics analysis is currently ongoing.

A detailed protocol for the procedure described above is reported in Annex 17.

4.7.2 Experiments on cytogenetics

Experiments performed at Sciensano (5.3.1.) were replicated by CNR. A researcher from Sciensano moved to CNR and performed the experiments at 1W/kg according to the protocol used in Sciensano for both the alkaline comet assay and the *in vitro* micronucleus test.

Methyl methanesulfonate (MMS) and Ethyl methanesulfonate (EMS) were used as a positive control in both sham and RF exposure conditions for the Micronucleus and comet assay, respectively. Exposure to RF-EMF was done using the CNR 26.5 GHz exposure system. Afterwards, the cells were fixed according to the protocol in Methanol-acetic acid for the micronucleus assay. For the comet assay, they were subjected to electrophoresis, before being neutralized and dried with ethanol. Afterwards, these slides were transported to SC for Analysis.

The slides were analyzed in a semi-automated way using the Metafer4 microscope system. This process is described as semi-automated because the analysis is performed by software connected to the microscope, which is connected to a motorized stage. Afterwards, for the micronucleus, the positives (the cells that contain a micronucleus) are checked manually to exclude any artefacts or false positives. For the comet assay, the images are screened for the correct cut-off of the head vs the tail. A minimum of 400 and a maximum of 5000 binucleated cells per slide were scanned for the micronucleus assay. A minimum of 50 cells and a maximum of 500 cells per gel were scanned for the comet assay.

The analysis of the results was performed in a blinded manner. This means that the researcher performing the analysis did not know which condition was exposed or not. The experiments were unblinded and only happened after all analyses had been performed.

4.8 Biological procedures – *C. elegans*

4.8.1 Nematode maintenance and 5G RF exposure

For *C. elegans* RF exposure experiments, the exposure system available at CNR was used, as well as two refrigerated incubators to allow nematode growth at 20°C.

For the RF exposure, a 26.5 GHz, 5G signal is sent to a reverberation chamber placed inside an incubator (PHCbi, MIR-554-PE). A second reverberation chamber, disconnected from the RF feeding and used for sham-exposure, is also located in this incubator. Each chamber is equipped with two stirrers at independently controlled speed and hosts a sample holder to allow for reproducible positioning of the plates. The other incubator is used for non-exposed samples and maintenance of worm cultures. For each experimental run, 4 plates are set up per exposure condition, i.e., not exposed, sham-exposed, and RF-exposed at 1 W/kg.

The aim is to study the biological effects of RF exposure in *C. elegans* at three levels: organismal health, molecular interactions, and gene expression. Figure 15 reports an overview of the experimental endpoints analysed. Furthermore, the wild-type N2 and the cuticle-sensitive CB6055 strains will be compared to determine whether a fragile cuticle allows for stronger effects caused by exposure.

For the experiments performed so far, only the N2 wild-type strain of *C. elegans* has been used, cultivated at 20°C, utilizing *E. coli* strain OP50 (inactivated with 8% paraformaldehyde) as its primary food source [82] and maintained on Nematode Growth Medium (NGM) agar poured into 90-mm Petri dishes (Corning Inc., Corning, NY, USA).

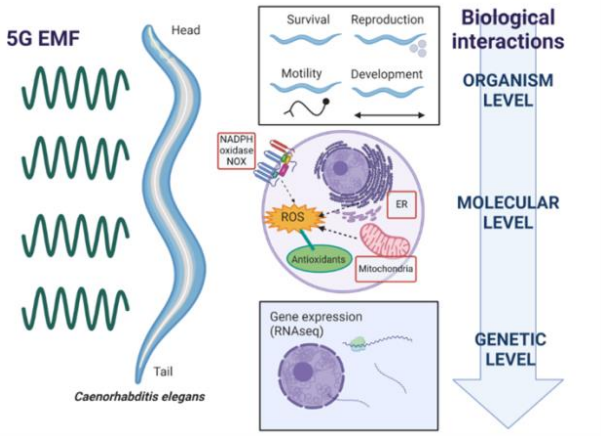


Figure 15: Overview of experimental endpoints planned for *C. elegans*

To study biological interactions at the organism and molecular levels, 600 nematode L1 larvae were seeded in NGM on 35-mm Petri dishes (Corning Inc., Corning, NY, USA). Worms were exposed for 72h to a 26.5 GHz 5G signal, interrupting exposure at the 24h mark to determine early survivability and perform length and motility measurements. After 72h, worms were again counted and measured, the adults collected by washing off with M9 buffer and the plate with laid eggs returned to the incubators. This second generation of worms underwent the same exact procedure, and, once completed, constituted the first of three technical replicates performed.

To study the effects at the genetic level, nematode eggs were seeded on 35-mm Petri dishes and exposed for 72h to a 26.5 GHz 5G signal. Immediately after the RF exposure was completed, the eggs (second generation) were taken, seeded on new plates, and exposed to RF again. This step was repeated, giving rise to a third and final generation.

Detailed protocols for the abovementioned procedure are reported in Annexes 18 and 19.

4.8.2 Toxicity and development

As mentioned previously, the initial evaluation stage is centred around organismal health. Typical assessment parameters include egg hatching rate, nematode survival, length at different stages of the life cycle, and motility as an indicator of altered behaviour.

The first generation of exposed worms begins with L1 larvae, synchronized after hatching through overnight starvation in M9 buffer on an orbital shaker. The worms were seeded and counted to determine the starting population, then placed in their respective incubators and chambers, according to the experimental conditions. After 24 hours, they were retrieved for counting to assess early survival, and again after completing the 72-hour exposure for adult survival. The second generation begins with the laid eggs still on the plate after washing to remove adult worms. They were immediately counted to determine the starting population, and again after 24 (which also provided data for hatching rates) and 72 hours of incubation. Survival rates were calculated for both generations and time points.

Data for first generation hatching was generated separately, by seeding eggs obtained through a bleaching protocol. Similarly, the initial number of eggs was counted and compared against the observable hatched larvae after overnight exposure.

Regarding length as a tool to determine developmental affectations, all stages of the *C. elegans* life cycle are thoroughly characterised, so the expected sizes for each step are well known. Capitalizing on their time away from the incubator, which was necessary for survival, pictures were also taken with a microscope camera of 50 random worms per condition for both time-points. After determining the appropriate scale, the images were analysed with the open-source software ImageJ to obtain length measurements. Similarly, for motility assessments, 2-minute videos were taken of at least 5 worms to allow for later counting of frequency of body bends in their movement. This parameter could be used as an indicator of alterations in the worm's nervous system caused by EMF exposure.

One extra step was performed to assess any effects on reproductive capabilities, by removing through "picking" single worms from each condition onto individual wells on a 12-well plate seeded with OP50. Since worms are hermaphrodites and capable of self-fertilization, one adult worm can give rise to around 300 offspring. After 3 days,

the resulting population was counted to determine any adverse effects caused by RF exposure. A more detailed explanation and the obtained results for this experiment are presented on deliverable 7.1.

A detailed protocol for the procedures described above are reported in Annexes 20 and 21.

4.8.3 Oxidative stress

Immediately after EMF exposure, ROS is quantified using 2',7'-dichlorofluorescein diacetate (H_2DCF-DA) as a probe. This non-polar and non-ionic compound can easily penetrate the cellular membrane; afterwards, it is enzymatically deacetylated by esterases. This biochemical reaction turns H_2DCF-DA into the non-fluorescent compound H_2DCF, which is then rapidly oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of free radical production. Fluorescence intensity measured at 485/535 nm (or under the normal Green Fluorescence Protein GFP channel) is proportional to the amount of ROS.

Yoon and co-workers [83] accurately describe the assay. In brief, it proceeds as follows: after EMF exposure, worms are collected with M9 buffer, washed thrice, and incubated for 1h with H2DCF-DA. After incubation, they are placed on microscope slides (approx. 30 worms/slide), and pictures are taken with a fluorescence microscope using the GFP channel. Fluorescence is quantified afterwards with suitable software (Fiji ImageJ) and normalized against a control.

We tested and compared the ROS levels in *C. elegans* using MD, as it has been used as an oxidant stressor in cell culture experiments. However, not reproducible results were obtained when working with this nematode. Therefore, the use of another oxidant stressor, paraquat was evaluated. Figure 16 shows the fluorescence observed in worms treated with 0.1 mM paraquat in comparison with those treated with 0.2 mM MD. Paraquat gave reproducible results, and we chose it as a positive control in our experiments.

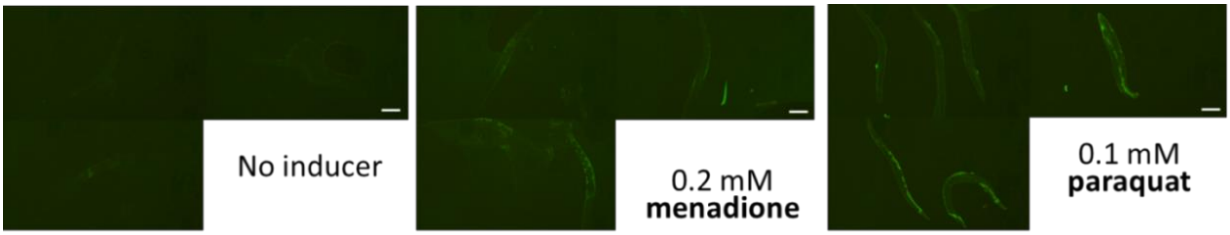


Figure 16: ROS levels in *C. elegans*. Fluorescent images of Control, 0.2 mM MD-treated worms, and 0.1 mM paraquat-treated worms.

Worms will be incubated from eggs (embryos) to L4 on paraquat-containing NGM agar and will serve as a positive control. In parallel, eggs will be exposed to EMF until L4 and ROS levels will be measured following the protocol described.

ROS levels will be complemented with the TAC evaluation. The TAC assay (Abcam 65329) is used in the cell culture experiments. During this period, we optimized and evaluated the TAC assay, which was not previously used in *C. elegans*. We optimized the protocol to use this assay from the L1 larval stage and incubated it for 72h with paraquat in liquid, so we could evaluate the TAC results (Figure 17). We determined the minimum number of worms needed for clear results and good statistics. The number of 600 worms was the most suitable to obtain reproducible results.

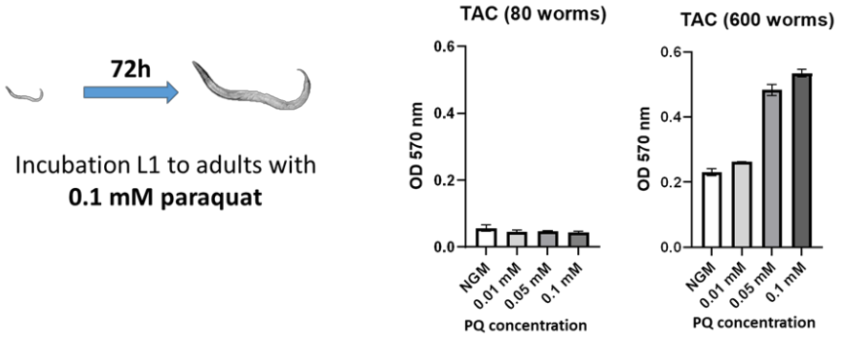


Figure 17: Optimization of TAC assay in *C. elegans* samples

In practice, the oxidative stress experiments on N2 worms could not be carried out with regard to ROS level evaluation. TAC assay evaluation will be performed in the framework of case study 1.

4.8.4 Gene expression (RNAseq)

RNA sequencing (RNAseq) is a powerful tool for detecting subtle changes in gene expression that may not be reflected in survival, reproduction rate, or ROS generation but have a consequence on metabolism.

A typical RNA extraction protocol was followed to perform this experiment. Briefly, around 200 worms per experimental group were collected and homogenized with Trizol and zirconia beads. This mixture served as a starting point for the standard protocol of an RNA extraction kit (PureLink™ RNA Mini Kit (12183018A, Invitrogen by Thermo Fisher). The integrity and amount of extracted RNA were assessed by spectrophotometer before sending the samples to a sequencing facility. Ribosomal mRNA will be subjected to a polyA enrichment pipeline to sequence only the mRNA with a polyA tail selectively.

The company Macrogen will perform sequence annotation. NovaSeqX Plus will produce the stranded mRNA Library (150 x 2 bp) with 30 million total reads per sample. A bioinformatic analysis with mapping and expression profile will be performed. In addition, a differential expression gene analysis will be performed to compare different samples.

RNAseq results must be carefully interpreted, considering that post-transcriptional regulatory mechanisms exist. Nevertheless, they may provide valuable insight and guide the course of future experiments.

A detailed protocol for the procedure described above is reported in Annex 22.

5 Results

5.1 4G LTE RF exposure and co-exposure on cancer-related endpoints in SH-SY5Y cells

5.1.1 Oxidative stress

ROS formation in SH-SY5Y cells was evaluated after 3 hours of RF-EMF exposure to a 1950 MHz, LTE signal, at SAR levels of 0.3 and 1.25 W/kg. Two MD doses, 5 μM (MD1) and 20 μM (MD2), were used for co-exposure experiments.

The results of RF exposure alone are shown in Figure 18. Representative flow cytometry histograms of DCF fluorescence (panel A), and the percentage of DCF-positive cells (DCF signal above the background) as mean ± SE of three independent experiments (panel B) are presented. Sham exposure did not modify the background level of ROS (Sh vs Incubator control). Thus, Sham samples were used as a control in the co-exposure experiments. RF exposure did not induce ROS formation at the investigated SAR levels (Sh vs 0.3 W/kg; Sh vs 1.25 W/kg). The highest dose of MD (MD2) induced a statistically significant increase in ROS formation (MD2 vs Incubator and Sh+MD2 vs Sham) and served as a positive control.

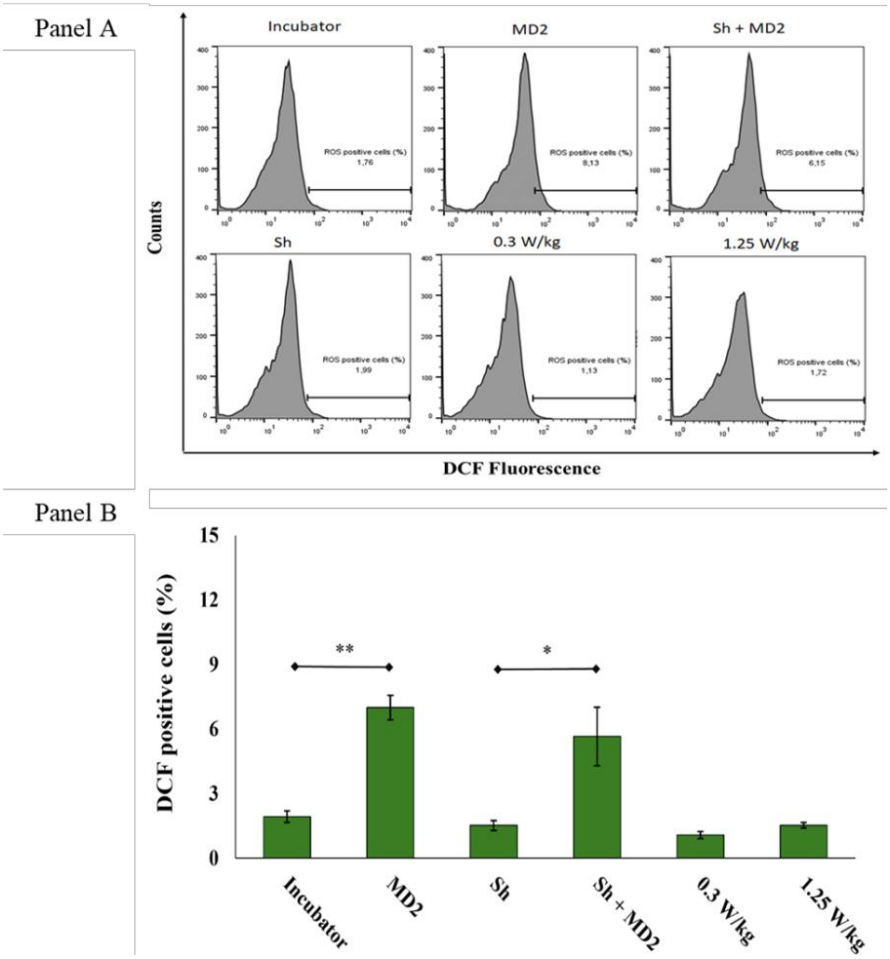


Figure 18: ROS formation in SH-SY5Y cells exposed for 3 h to LTE signal. MD2: 20 μM. Panel A: Representative flow cytometry histograms of DCF fluorescence. Panel B: percentage of DCF-positive cells (DCF signal above the background in untreated dye-preloaded cells) as mean ± SE of three independent experiments. *P<0.05, **P<0.01; two-tailed unpaired Student's t-test.

The results of co-exposure to RF and MD are shown in Figure 19. No combined effect was recorded since RF exposure did not alter the MD-induced effect in any of the conditions tested (Sh+MD1 vs 0.3 W/kg+MD1; Sh+MD1 vs 1.25 W/kg+MD1; Sh+MD2 vs 0.3 W/kg+MD2; Sh+MD2 vs 1.25 W/kg+MD2).

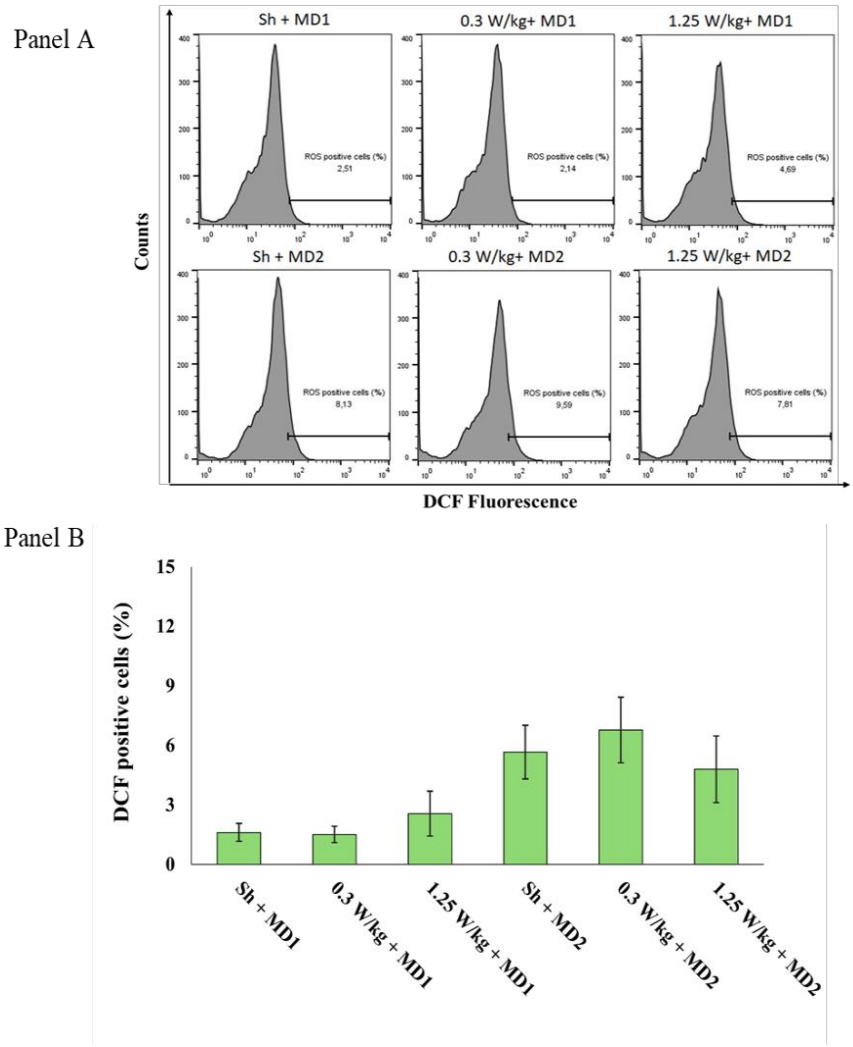


Figure 19: ROS formation in SH-SY5Y cells exposed to RF (3 h, LTE signal) and treated with two MD concentrations. (MD1: 5 μM; MD2: 20 μM). Panel A: Representative flow cytometry histograms of DCF fluorescence. Panel B: percentage of DCF-positive cells (DCF signal above the background in untreated dye-preloaded cells) as mean ± SE of three independent experiments.

5.1.2 Apoptosis

Apoptosis was investigated in SH-SY5Y cells after 3h of RF-EMF exposure to a 1950 MHz, LTE signal, at SAR levels of 0.3 and 1.25 W/kg. Two MD doses, 5 and 20 μM, were used for co-exposure experiments.

The results of RF exposure alone are shown in Figure 20. Representative dot plots showing cell death by apoptosis and/or necrosis using Annexin V-FITC and propidium iodide (PI) stain are reported in panel A, showing the percentage of live cells (Q4), early apoptotic cells (Q3), late apoptotic cells (Q2), and necrotic cells (Q1). The percentage of total apoptotic cells (Q3+Q2) for each experimental condition is represented as mean ± SE of five independent experiments (panel B). Sham exposure did not modify the background level of apoptotic cells (Sh vs Incubator control); thus, sham samples were used as controls in the co-exposure experiments. RF exposure did not induce apoptosis at the investigated SAR levels (Sh vs 0.3 W/kg; Sh vs 1.25 W/kg). The highest dose of MD (MD2) induced a statistically significant increase in total apoptotic cells (MD2 vs Incubator and Sh+MD2 vs Sham) and served as a positive control.

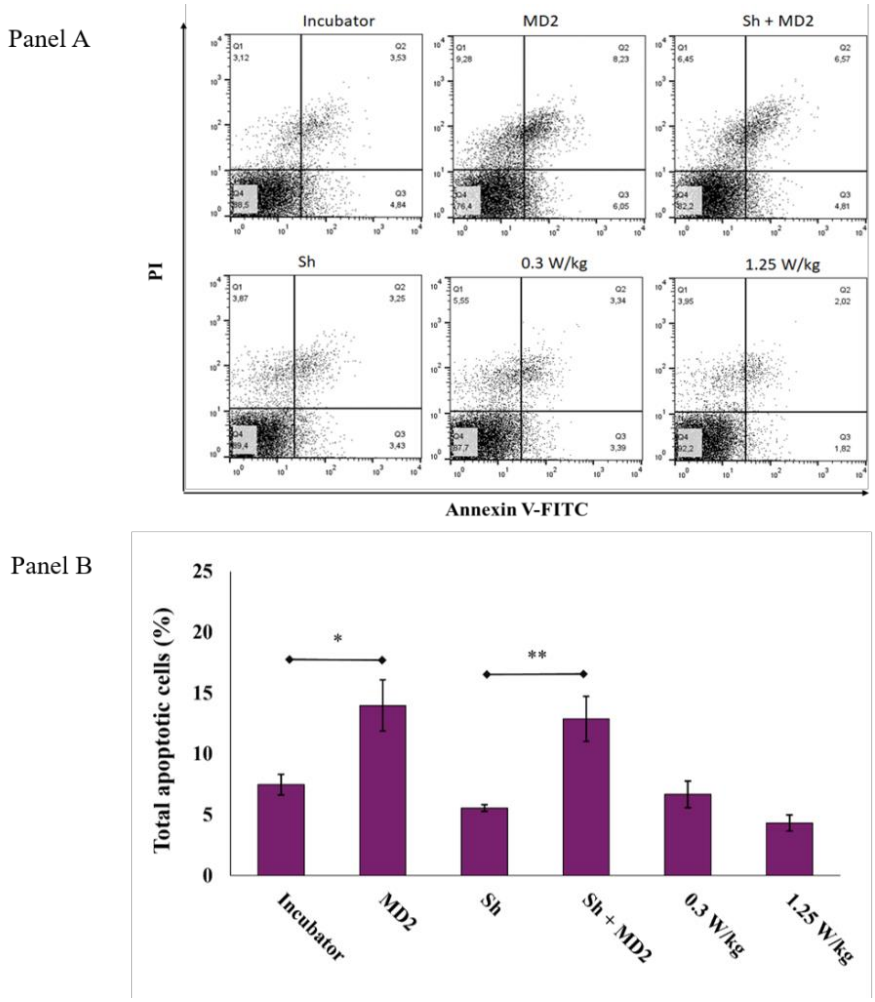


Figure 20: Apoptosis induction in SH-SY5Y cells exposed for 3 h to LTE signal. MD2: 20 µM. Panel A: Representative dot plots for AnnexinV-FITC/ PI analysis; apoptotic cells are displayed in the right quadrants of the dot plot with early apoptotic at the bottom (AnnexinV-FITC+/ PI-, Q3) and late apoptotic at the top (AnnexinV-FITC+/ PI+, Q2). Panel B: percentage of total apoptotic cells (Q3+Q2) for each condition tested (mean ± SE of five independent experiments). *P<0.05, **P<0.01; two-tailed unpaired Student's t-test.

The results of co-exposure to RF and MD are shown in Figure 21. The RF exposure did not modify the MD-induced apoptosis level indicating the absence of combined effect in all the conditions examined (Sh+MD1 vs 0.3 W/kg+MD1; Sh+MD1 vs 1.25 W/kg+MD1; Sh+MD2 vs 0.3 W/kg+MD2; Sh+MD2 vs 1.25 W/kg+MD2).

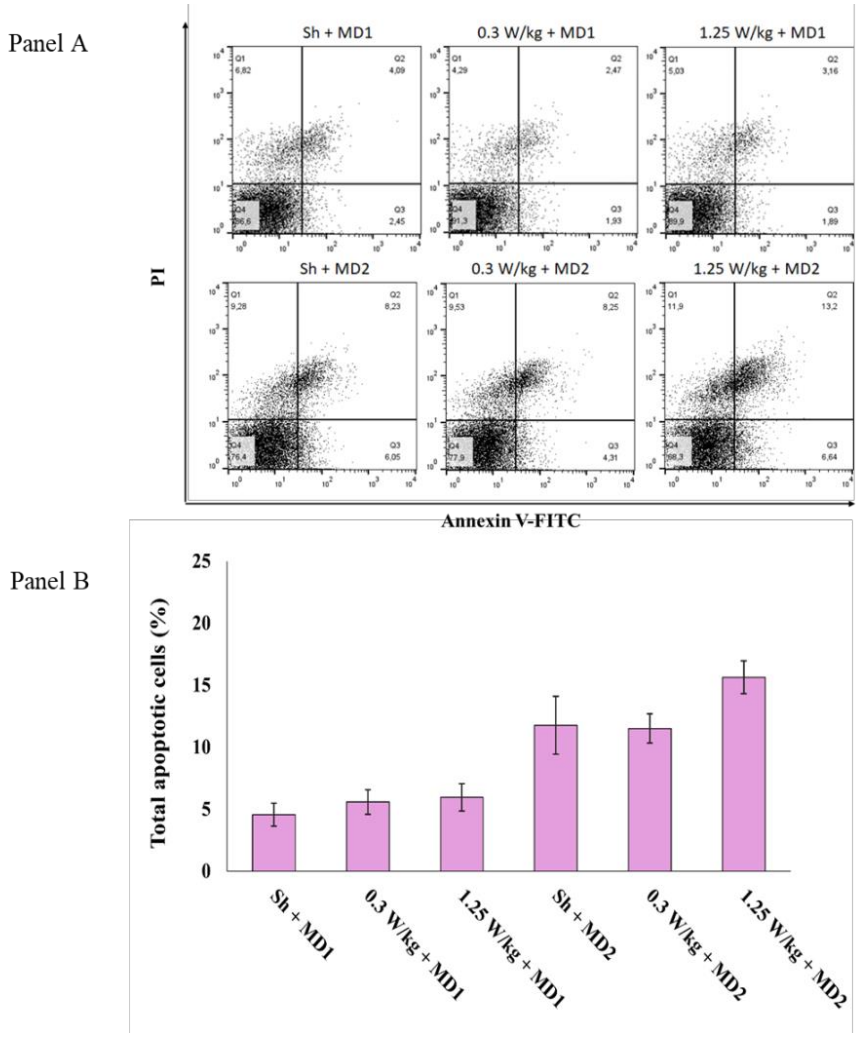


Figure 21: Apoptosis induction in SH-SY5Y cells exposed to RF (3 h, LTE signal) and treated with two MD concentrations. (MD1: 5 μM; MD2: 20 μM). Panel A: Representative dot plots for AnnexinV-FITC/ PI analysis; apoptotic cells are displayed in the right quadrants of the dot plot with early apoptotic cells at the bottom (AnnexinV-FITC$^+$/ PI$^-$) and late apoptotic cells at the top (AnnexinV-FITC$^+$/ PI$^+$). Panel B: percentage of total apoptotic cells for each condition tested (mean ± SD of five independent experiments).

5.1.3 Cell cycle progression

Cell cycle progression was investigated in SH-SY5Y cells after 3 hours of RF-EMF exposure to a 1950 MHz, LTE signal, at SAR levels of 0.3 and 1.25 W/kg.

The results of RF exposure alone are shown in Figure 22. Representative flow cytometry histograms of PI fluorescence (panel A), and the percentage of cells in different stages of the cell cycle as mean ± SE of four independent experiments (panel B) are presented. Sham exposure and RF exposure at both SAR levels did not affect cell cycle progression (Sh vs Incubator control; Sh vs 0.3 W/kg; Sh vs 1.25 W/kg). MMC (1 μg/ml) induced a statistically significant reduction of cells in S phase, likely indicating an arrest at G0/G1 phase (MMC vs Incubator; Sh+MMC vs Sh) and served as a positive control.

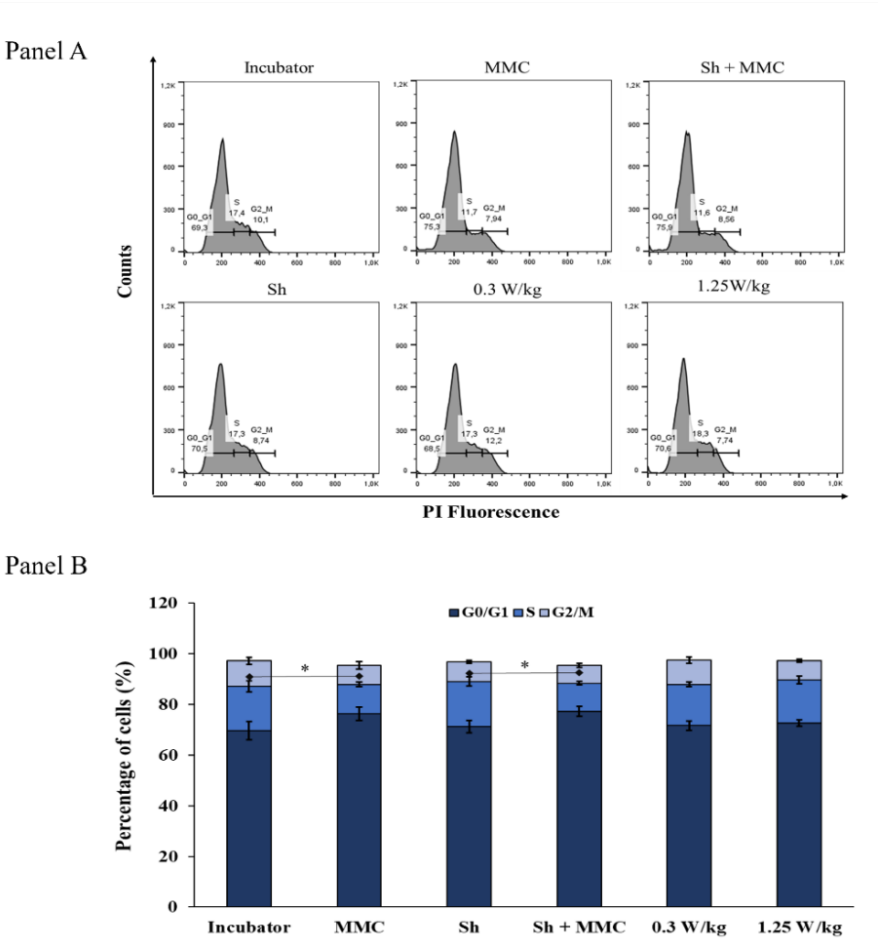


Figure 22: Cell cycle progression in SH-SY5Y cells exposed for 3h to LTE signal. MMC: 1 µg/ml. Panel A: Representative flow cytometry histograms of PI fluorescence. Panel B: percentage of cells in G0/G1, S, and G2/M stages (mean ± SE of four independent experiments). *P<0.05; two-tailed unpaired Student's t-test.

5.2 Multiple RF exposure to 4G LTE and WiFi signals on human neuroblastoma SH-SY5Y cells

Cell cycle progression was investigated in SH-SY5Y cells simultaneously exposed for 3 h to a 1950 MHz 4G, LTE signal and a 2450 MHz, WiFi signal, at SAR levels of 0.3 and 1.25 W/kg.

The results are shown in Figure 23. The percentage of cells in different stages of the cell cycle, as mean ± SE of three independent experiments, is presented. RF exposure at both SAR levels did not affect cell cycle progression (Sh *vs* 0.3 W/kg; Sh *vs* 1.25 W/kg). As expected, MMC treatment (16 h at 1 µg/ml final concentration) induced a statistically significant reduction of cells in S phase, likely indicating an accumulation of cells in G0/G1 phase (MMC vs incubator) and served as a positive control.

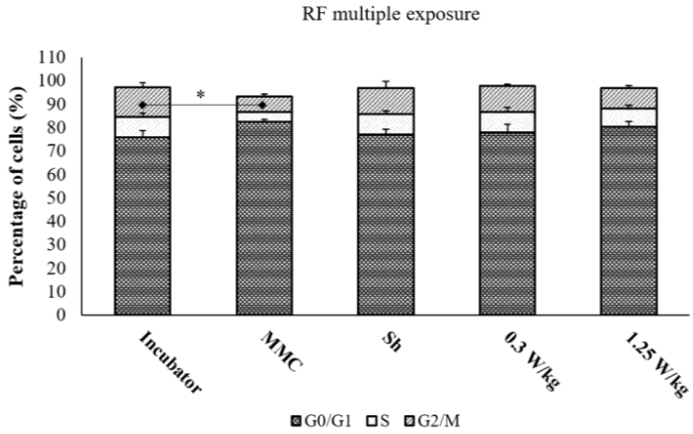


Figure 23: Effects of 3 h multiple exposure to 1950 MHz, LTE signal and 2450 MHz, Wi-Fi signal on cell cycle progression. Mean ± SE of three independent experiments (*P<0.05). MMC: 16 h treatment with 1 µg/ml mitomycin-C

5.3 5G FR1 RF exposure and co-exposure on cancer-related endpoints in HaCaT cells

5.3.1 Genotoxicity: cytokinesis block micronucleus assay

Chromosomal damage was investigated using the *in vitro* micronucleus assay in HaCaT cells after 3.5 GHz exposure for 24h to 0.4 W/kg and 1 W/kg SAR levels. Methyl methanesulfonate (MMS) was used as a positive control in both sham and RF exposure conditions. The results included in this deliverable are not yet final and will be combined with additional data.

The combined results of the 3 independent experiments at 0.4 W/kg and 1 W/kg are shown in Figure 24 and Figure 25, respectively. In each individual experiment, at least 3 slides were scanned for binucleated cells with micronuclei per condition. Moreover, two replicates were included for each condition in the experiments. The mean % micronucleated cells per condition and the associated standard deviation were calculated by combining the data from all slides for the respective condition (thus combining the data from the two replicates; minimum 6 slides in total). Statistical analysis (2-way ANOVA) on data from the three independent experiments showed no significant differences between exposed and non-exposed cells, for both 0.4 W/kg and 1 W/kg, either in RF-EMF only exposed or in RF-EMF and MMS combined conditions. There were no clear signs of cytotoxicity under any of the investigated experimental conditions.

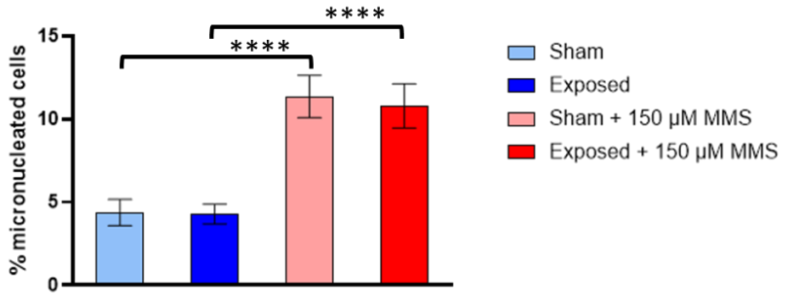


Figure 24: Micronucleated cells after exposure to 0.4 W/kg and/or 150 µM methyl methanesulfonate (MMS). Each bar represents the mean (± SD) of three independent experiments. In each individual experiment, at least 3 slides were scanned until 5000 binucleated cells were obtained per condition. ****P<0.0001; 2-way ANOVA.

Overall, the *in vitro* micronucleus results obtained so far indicate that there is no significant effect of RF-EMF exposure on chromosomal damage under the investigated conditions.

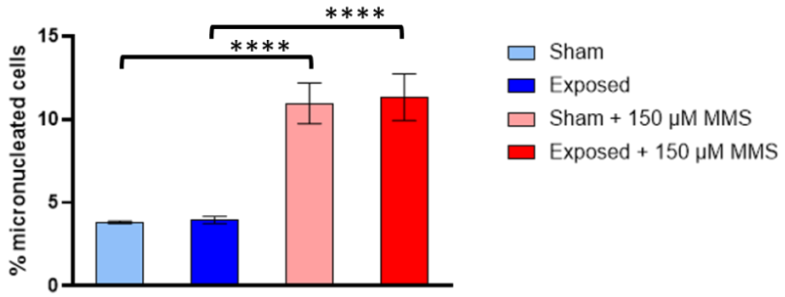


Figure 25: Micronucleated cells after exposure to 1 W/kg and/or 150 µM methyl methanesulfonate (MMS). Each bar represents the mean (± SD) of three independent experiments. In each individual experiment, at least 3 slides were scanned until 5000 binucleated cells were obtained per condition. ****P<0.0001; 2-way ANOVA.

5.3.2 Genotoxicity: alkaline comet assay

The induction of DNA damage was investigated using the *in vitro* alkaline comet assay in HaCaT cells after exposure for 24h to 3.5 GHz, 5G signal, at 0.4 W/kg and 1 W/kg SAR. Ethyl Methanesulfonate (EMS; 200 µM) was used as a positive control in both sham and RF exposure conditions.

Figure 26 and Figure 27 show the combined results of the 3 independent experiments at 0.4 W/kg and 1 W/kg, respectively. Statistical analysis (2-way ANOVA) on data from the three independent experiments showed no significant differences between exposed and sham samples, as well as between exposed + 200µM EMS and sham + 200µM EMS samples, for 0.4 W/kg and 1 W/kg

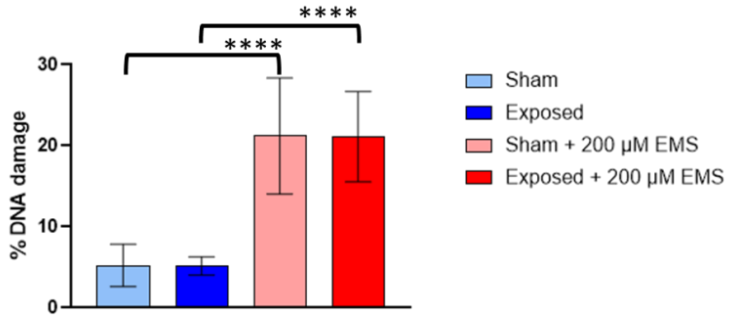


Figure 26: % DNA damage detected in the *in vitro* comet assay in HaCaT cells after exposure to 0.4 W/kg and/or 200 µM ethyl methanesulfonate (EMS). Each bar represents the mean % of DNA damage calculated based on the values of the 3 independent experiments. In each individual experiment, two replicates were included per experimental condition and per replicate, minimum 2 slides were scored. ****P<0.0001; 2-way ANOVA.

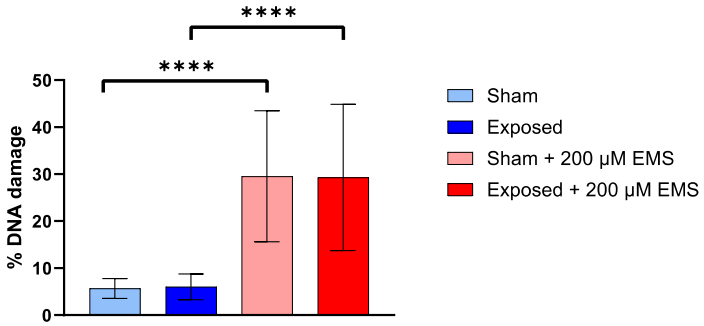


Figure 27: % DNA damage detected in the *in vitro* comet assay in HaCaT cells after exposure to 1 W/kg and/or 200 µM ethyl methanesulfonate (EMS). Each bar represents the mean % of DNA damage calculated based on the values of the 3 independent experiments. In each experiment, two replicates were included per experimental condition, and a minimum of 2 slides were scored per replicate.. ****P<0.0001; 2-way ANOVA.

Detailed information from each independent experiment—including the number of cells scored, the percentage of DNA damage, and the mean DNA damage across experiments—will be provided in an open-access publication in a peer-reviewed journal.

5.3.3 Exploration of epigenetic effects

Exposure of HaCaT cells has been carried out at SC at a frequency of 3.5 GHz and a SAR of 1 W/kg, following the experimental design detailed in section 4.6.3. Three technical replicates (labelled alpha, beta and gamma) per condition (A, B, C, D and E) exposed under wave guide 1 (WG1) or wave guide 2 (WG2) (30 samples), meeting the DNA quality criteria for sequencing (Table 6), were further processed with the Twist methylome panel and enzymatic methylation-sequencing (EM-seq). If needed, the remaining samples (biological replicates and additional technical replicates) were kept for further confirmation of the results with a more targeted methylation analysis method.

Table 6: Mean concentration and purity of the DNA extracted from HaCat cells after the RF-EMF exposure experiment

Measured parameter	Mean*	Minimum*	Maximum*	Quality criteria
Concentration (ng/µL)	108.4	73.2	286	> 5
Purity A260/280	1.9	1.9	2.0	≈ 1.8
Purity A260/230	2.2	1.9	2.3	2.0 – 2.2
Fragment length (bp)	48 541	28 836	> 60 000	No DNA shearing

*: based on a total of 30 technical triplicate samples

After sequencing, an average of 158 M read pairs (min 120 M – max 201 M) were generated per sample, with median Q30 rates of 90.55% and 92.87% before and after trimming, respectively. After mapping the reads to the human reference genome, the targeted CpG regions from the methylome panel had a median depth of 87.5 across samples and conditions, with over 99% of the targeted regions covered. The replicate beta for the condition A exposed with WG2 (A_WG2_beta) was an outlier with only 50x coverage Figure 28. After manual review, this sample also showed problematic mapping results and was therefore excluded from the rest of the analysis.

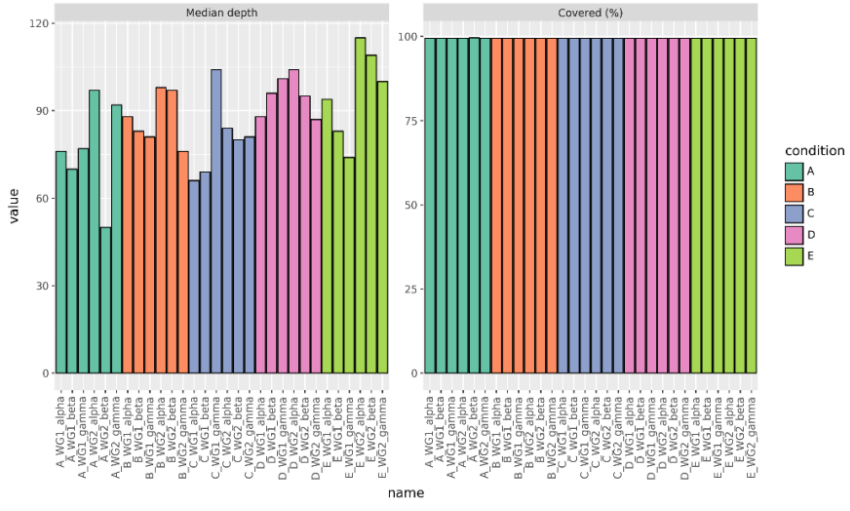


Figure 28: Depth and coverage statistics for the targeted CpG regions of the Twist methylome panel. Bars are colored by condition as indicated in the legend. 'Name' is sample names with following nomenclature [condition]_[wave_guide]_[replicate].

After filtering, a total of 4 840 493 positions (sufficiently covered in all datasets) were found differentially methylated between WG1 and WG2 over all the tested conditions combined. From this total, only 2024 differentially methylated

positions were found to be statistically significant (q-value <0.01), ranging from 245 in condition D to 814 in condition A (Table 7). The ratio of hyper- to hypo-methylated positions was around 50% for all conditions. Of the 2024 positions significantly differentially methylated between WG1 and WG2, across all conditions, only 8 were significant in more than one condition (Table 7). However, these common differences were not replicated in more than two conditions and were located outside the CpG regions from the methylome panel and thus showed a lower coverage. Furthermore, as only single positions were affected here, the consequences on gene expression are thought to be less important than when the methylation profile of an entire region is altered. Nevertheless, some single CpG sites can still be the target of specific key transcription factors, but the way how gene expression will be affected is context dependent and this single site alteration can be compensated by other regulatory elements. Therefore, these 8 differentially methylated positions need to be further investigated before any conclusion on the potential impact on carcinogenesis can be made.

Table 7: Number of significant differentially methylated positions and regions between WG1 and WG2 for all the tested conditions

Condition	Differentially methylated positions (WG1 vs. WG2)	Percentage hyper-methylated (WG1 vs. WG2) (%)	Differentially methylated regions (WG1 vs. WG2)	Percentage hyper-methylated (WG1 vs. WG2) (%)
A: 1h exposure	814	46.15	5	20%
B: 3h exposure	320	42.65	0	n/a
C: 24h exposure	386	51.30	0	n/a
D: 3 h exposure + 3h post-exposure	245	51.18	0	n/a
E: 3h exposure + 30h post-exposure	256	49.34	0	n/a
	Total = 2024		Total = 5	n/a: not applicable

At the region level (i.e., grouped by CpG island as defined in the Twist methylome panel), 91 495 regions were identified differentially methylated between WG1 and WG2 for all the tested conditions, after filtering. From these 91 495 regions, significant results were only found for condition A, where five CpG islands were significantly differentially methylated (q-value <0.01) (Figure 29). Note that for this condition there were only two WG2 replicates instead of three, as replicate beta was a low-quality dataset. The five identified differentially methylated regions were located in upstream or non-coding sequence of genes that were not associated with cancer research in the literature before.

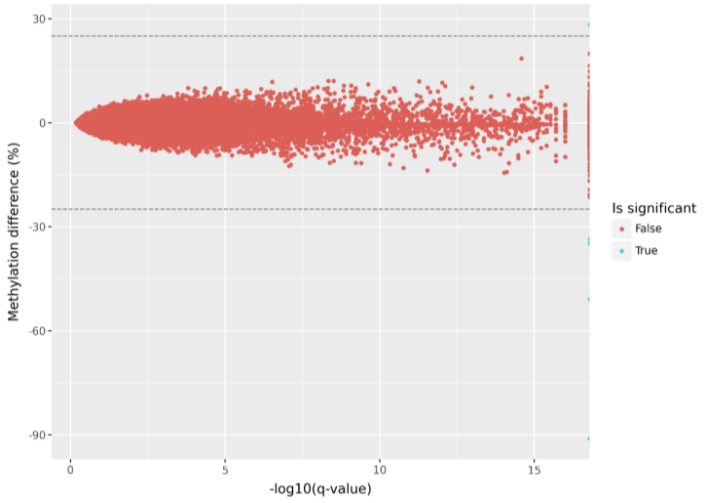


Figure 29: Differentially methylated regions for condition A. All the dots grouped at the right of the figure represent differentially methylated regions with extremely low q-values (not corresponding to the indicated scale). The five blue dots represent the five significant differentially methylated regions with one being hyper-methylated and four being hypo-methylated.

Despite the high quality of the data (except for one technical replicate from condition A, which was excluded from the analysis), the number of statistically significant differentially methylated positions and regions was small. Only a small fraction of the significant positions was significantly hypo- or hyper-methylated, but they were spread over multiple conditions and need further investigation. However, the investigation of methylation differences at the level of CpG regions was preferred as it reduces data dimensionality, increases the power of detection, and is thought to be more functionally important (potentially leading to adverse effects) than alterations that affect only isolated sites. At the level of the CpG regions, significant differential methylation was detected in only five regions for condition A, with none observed in the other conditions. None of the five differentially methylated regions in condition A could be linked to cancer-related alterations.

Amongst the tested conditions, conditions A, B, and C, with exposure of 1h, 3h and 24h, respectively, were designed to investigate a potential epigenetic effect influenced by exposure time. However, this was not observed at the region level, as only a limited number of significantly differentially methylated regions were identified for condition A (shortest exposure time), and no significant effect was detected for the other conditions. Conditions B, D and E included an identical exposure time of 3h but followed by a post-exposure period of 0h, 1h and 30h, respectively. For these conditions, no significant effect was observed at the region level, and post-exposure time's influence could not be investigated either.

During data analysis, positions and regions were considered differentially methylated if at least 25% of difference was reached, a threshold commonly used to detect strong and reliable differences, such as cancer-related methylation markers. However, the epigenetic alterations induced by 5G exposure (3.5 GHz and SAR 1 W/kg) might be more subtle and difficult to clearly distinguish. Therefore, the data analysis will be redone with a difference threshold of 5%, to allow for the detection of potential low differences in methylation levels. However, with this low threshold, subtle differences must be interpreted carefully for their biological relevance, as they might be due to confounding factors and experimental biases. To address this, the focus will be more on the differentially methylated regions rather than positions to identify potentially influenced genes or groups of genes identified as belonging to the same biological pathway after gene set enrichment analysis. In a previous study investigating the effect of RF-EMF exposure (900 MHz for 1h with estimated SAR < 10 mW/kg) on human keratinocytes and applying a differences threshold of 5%, six differentially methylated regions, correlated with differences in gene expression, could be identified [28]. Although the level of exposure and the experimental design were not fully comparable, it will be investigated whether these same six differentially methylated regions could also be detected in our datasets. Until this complementary analysis is done with the new threshold, the result interpretation will remain blind, i.e., not knowing to which wave guide the sham and exposed conditions corresponds, to avoid introducing any bias. Finally, when the new analysis of the data with the 5 % cut-off has been performed, the obtained differentially methylated regions will be compared to the transcriptomics data, to investigate if common genes from the two experiments are influenced by 5G exposure. These new results will be added to the case study 2 report.

5.3.4 Oxidative stress

Intracellular reactive oxygen species (ROS) generation was assessed using a combined H2DCF-DA/MTT assay after a 3-hour exposure to a 5G RF-EMF signal at 1 W/kg. The presented results are based on three independent experimental repetitions, each including three technical replicates. No statistically significant change in intracellular ROS levels was observed following RF-EMF exposure when compared to sham controls. This was confirmed using two-way ANOVA for statistical analysis. The absence of a significant effect suggests that under these specific conditions, short-term (3-hour) exposure at 1 W/kg does not induce detectable oxidative stress in HaCaT cells. MD at 5μM was used as a positive control (Figure 30).

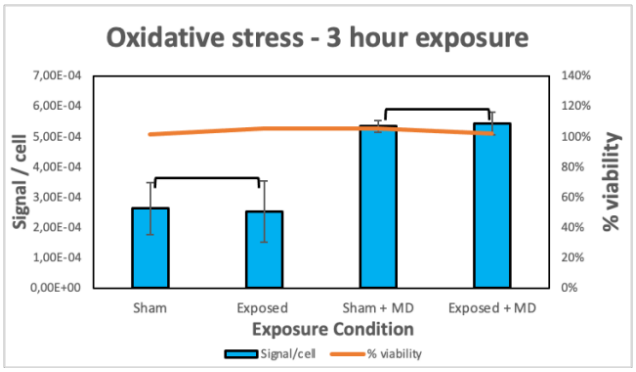


Figure 30 Intracellular ROS levels in HaCaT cells following 3-hour exposure to RF-EMF at 1 W/kg SAR.

Data represent the normalized fluorescence signal per cell, calculated by correcting DCF fluorescence values with MTT-derived cell counts. Results are shown as mean ± standard deviation from three independent experiments, each containing three technical replicates. No statistically significant differences were observed between the RF-exposed and sham-exposed groups (two-way ANOVA). These findings suggest that 3-hour RF-EMF exposure at 1 W/kg does not significantly induce oxidative stress in HaCaT cells under the conditions tested.

Experiments assessing oxidative stress after a one-hour exposure are currently ongoing. At this stage, only preliminary results are available and should be interpreted with caution. These early data are not yet sufficient to draw definitive conclusions and will be complemented by further repetitions in case study two (Figure 31).

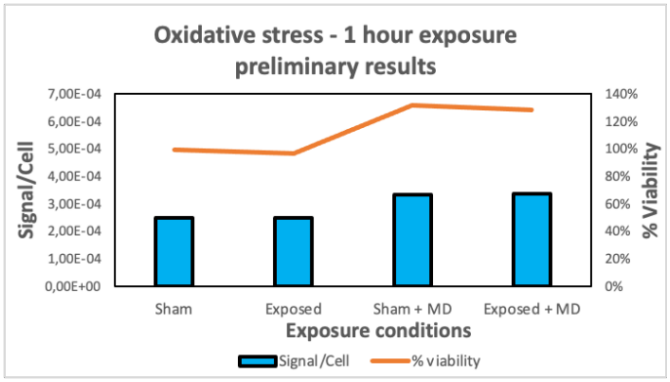


Figure 31 Preliminary assessment of intracellular ROS levels in HaCaT cells following 1-hour exposure to RF-EMF at 1 W/kg SAR. The data shown are from a single experimental run, including three technical replicates.

Results represent the normalized DCF fluorescence signal per cell, adjusted using MTT-derived cell counts. Unexpectedly, an increase in cell viability was observed in the RF-exposed condition compared to the sham control, which may have contributed to a relatively low signal-to-noise ratio in the ROS measurements. As these results are preliminary and based on a single experiment, no conclusions can be drawn at this stage. The experiment will be repeated to confirm findings and improve statistical robustness.

5.4 5G FR2 RF exposure and co-exposure on cancer-related endpoints in HaCaT cells

Exposures of HaCaT cells have been carried out at CNR to a 5 G signal at the frequency of 26.5 GHz, to evaluate the effect of RF exposure alone and in combination with UVB radiation. Currently, the results of 3 h exposure at 0.4 and 1 W/kg SAR are available. The same UVB treatment (1530 J/m²) was used for ROS and apoptosis.

5.4.1 Oxidative stress

Results of ROS analysis, are presented in Figure 32 and Figure 33 for 0.4 and 1 W/kg SAR level, respectively.

RF exposure did not induce ROS formation at any of the SAR values (Sh vs 0.4 W/kg; Sh vs 1 W/kg); UV induced a statistically significant increase in ROS formation (UV *vs* negative control; and Sh+UV *vs* Sh; RF+UV vs RF). RF exposure did not modify UV-induced effect (RF+UV *vs* Sh+UV), indicating an absence of a cooperative effect.

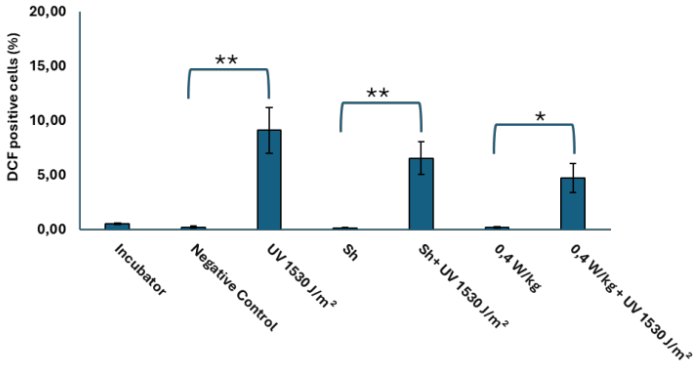


Figure 32: ROS analysis in HaCaT cells exposed for 3h to 26.5 GHz (0.4 W/kg) and co-exposed with 1530 J/m² UV. The percentage of DCF-positive cells (DCF signal above the background in untreated dye-preloaded cells) is presented as mean ± SE of 4 independent experiments. *P<0.05, **p< 0.01; two tailed unpaired Student's *t*-test.

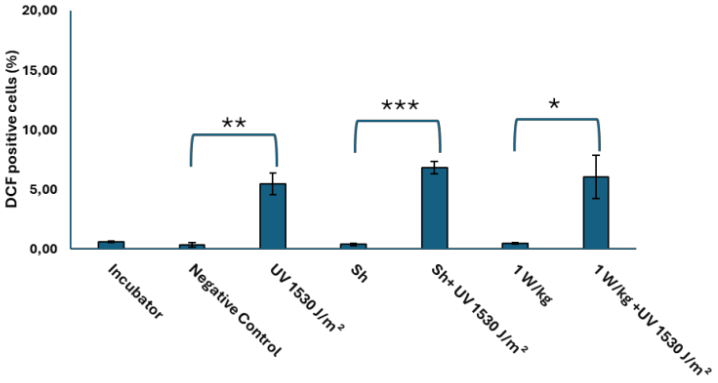


Figure 33: ROS analysis in HaCaT cells exposed for 3h to 26.5 GHz (1 W/kg) and co-exposed with 1530 J/m² UV. The percentage of DCF-positive cells (DCF signal above the background in untreated dye-preloaded cells) is presented as mean ± SE of 3 independent experiments. *P<0.05, **P< 0.01, ***P<0.001; two tailed unpaired Student's *t*-test.

5.4.2 Apoptosis

Results of apoptosis analysis are presented in Figure 34 and Figure 35 for 0.4 and 1 W/kg SAR level, respectively.

In both cases, RF exposure did not induce apoptosis (Sh vs 0.4 W/kg; Sh vs 1 W/kg). UV induced an increase in apoptosis that was statistically significant in all cases, except for two of them, as shown in Figures 30 and 31. This is ascribed to the experimental variability. RF exposure at both SAR levels did not modify UVB-induced effect (RF+UV *vs* Sh+UV), indicating an absence of a cooperative effect.

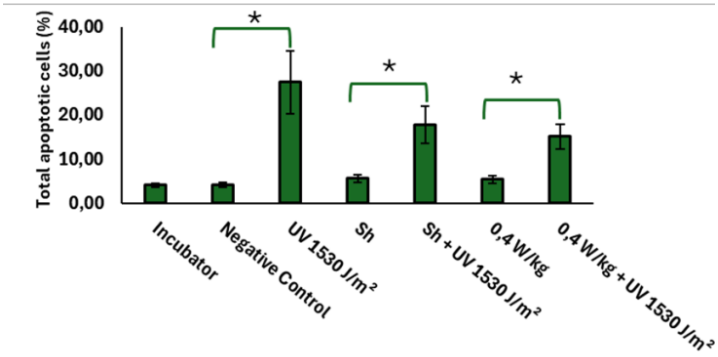


Figure 34: Apoptosis induction in HaCaT cells exposed for 3h to 26.5 GHz (0.4 W/kg) and co-exposed with 1530 J/m² UV. Percentage of total apoptotic cells for each condition tested (mean ± SE of 3 independent experiments). *P<0.05; two tailed unpaired Student's *t*-test.

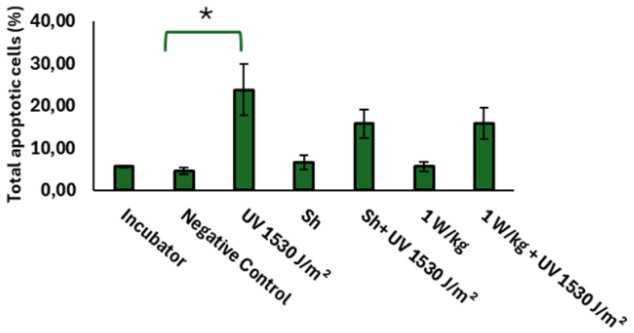


Figure 35: Apoptosis induction in HaCaT cells exposed for 3h to 26.5 GHz (1 W/kg) and co-exposed with 1530 J/m² UV. Percentage of total apoptotic cells for each condition tested (mean ± SE of 3 independent experiments). *P<0.05; two tailed unpaired Student's t-test.

5.5 5G RF exposure on transcriptomics in HaCaT cell: joint activities at CNR and SC

The HaCaT cell samples have been exposed at the frequency of 26.5 GHz (Frequency Range 2, FR2) and at 3.5 GHz (Frequency Range 1, FR1) at CNR and SC, respectively. The exposure conditions have been detailed in the Section 4.6. The cell lysates from both laboratories have been collected and shipped by SC to BioClavis. The bioinformatics analysis and further data interpretation of this data are currently ongoing.

Three replicates per condition (90 samples) were sequenced through the TempO-seq technology, using 22,533 probes targeting 19,682 genes. On average, 9.6 M reads of length 51 bp were generated per sample (min.: 2.3 M; max.: 18.1 M), with an average Q30 rate of 96.6%. The average unique mapping rate was 91.8% (min.: 90.6%; max.: 92.5%).

As data analysis was performed under blinded circumstances, the different treatment conditions were assigned a letter combination ranging from A to AD (SC: A-R, 18 conditions; CNR: S-Z + AA-AD, 12 conditions). Different experimental conditions were compared to each other to identify DEGs, and their comparisons were categorized into three groups, namely 1) sham vs. exposed, 2) exposed vs. exposed (different exposure duration), and 3) exposed vs. exposed (different SAR). Only comparisons between conditions executed in the same center were considered to avoid inflated DEG numbers due to technical differences. The number of DEGs ranged from zero to a maximum of 1,161 (Figure 36: Number of DEGs between the conditions tested at CNR (panel A) and SC (panel B).Figure 36). Excluding the condition comparisons for which zero DEGs were found, no shared DEGs were found for the conditions tested in the SC and CNR centers. Over-representation analysis showed an enrichment of genes involved in immune response and inflammatory response pathways. More in-depth analysis of this data is currently ongoing.

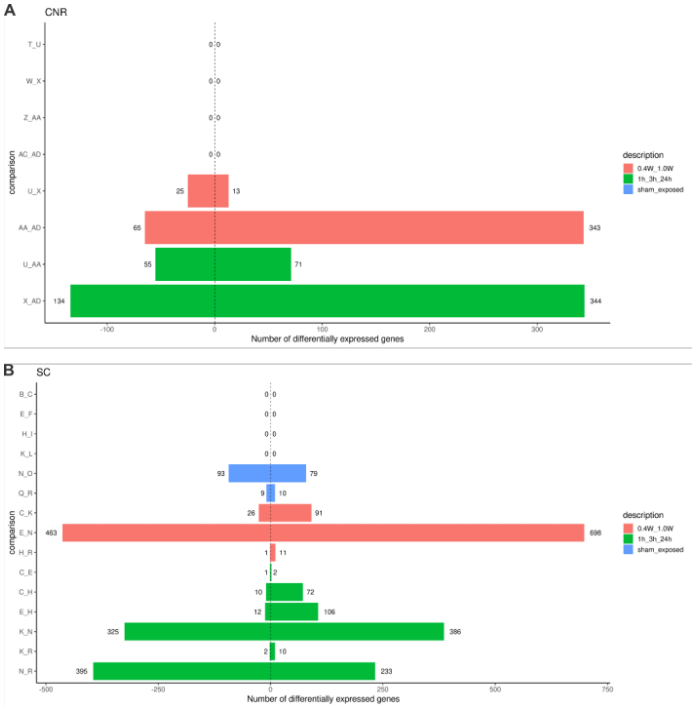


Figure 36: Number of DEGs between the conditions tested at CNR (panel A) and SC (panel B). The condition comparisons are categorized into three groups, namely 1) sham vs. exposed (blue), 2) exposed vs. exposed (different duration; green), and 3) exposed vs. exposed (different SAR; red). The striped black line indicates a DEG number of zero. Bars on the left of this line indicate the number of downregulated DEGs in this condition comparison, while the bars on the right of this line indicate the number of upregulated DEGs in this condition comparison. The number of DEGs is shown on the bars' left and right, respectively.

5.6 5G RF exposure on cytogenetics in HaCaT cell: joint activities at CNR and SC

5.6.1 Alkaline comet assay

We performed additional genotoxicity experiments as described below, with the exception that a frequency of 26.5 GHz was used for a duration of 24 hours at a SAR of 1 W/kg when compared to previous experiments at 3.5 GHz at multiple SARs. The induction of DNA damage was investigated using the *in vitro* alkaline comet assay in HaCaT cells after 24-hour exposure to a 5G signal. Ethyl methanesulfonate (EMS; 200 µM) was used as a positive control under both sham and RF exposure conditions. The combined results of three independent experiments are presented in Figure 37.

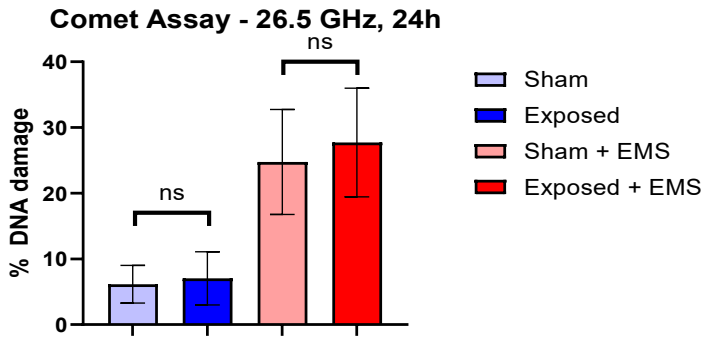


Figure 37: % DNA damage detected in the *in vitro* comet assay in HaCaT cells after exposure to 1 W/kg 26.5 GHz 5G-NR modulated RF-EMF and/or 200 µM ethyl methanesulfonate (EMS). Each bar represents the mean % of DNA damage calculated based on the values of the 3 independent experiments. In each individual experiment, two replicates were included per experimental condition and per replicate, minimum 2 slides were scored.

Statistical analysis using a paired t-test showed no significant differences between exposed and sham samples, and between RF-EMF + 200 µM EMS and sham + 200 µM EMS samples.

5.6.2 *In vitro* cytokinesis block micronucleus assay

Chromosomal damage was assessed using the *in vitro* micronucleus assay in HaCaT cells following 24-hour exposure to a 5G signal at 26.5 GHz, at a SAR level of 1 W/kg. Methyl methanesulfonate (MMS) was used as a positive control under both sham and RF exposure conditions.

The combined results of three independent experiments are presented in Figure 38.

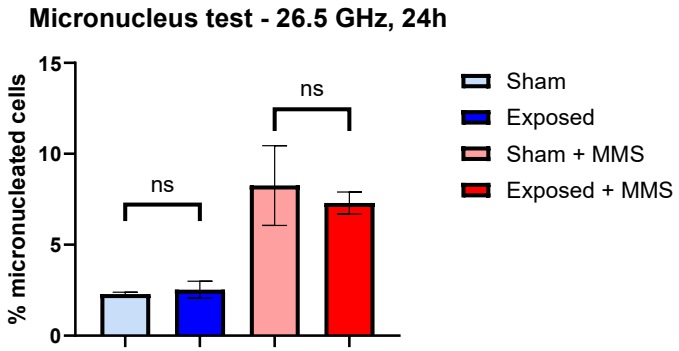


Figure 38: Micronucleated cells after exposure to 1 W/kg and/or 150 µM methyl methanesulfonate (MMS). Each bar represents the mean (± SD) of three independent experiments. In each individual experiment, at least 3 slides were scanned until 5000 binucleated cells were obtained per condition.

In each experiment, at least three slides per condition were analyzed for binucleated cells with micronuclei, and two replicates were included for each condition. The mean percentage of micronucleated cells and associated standard deviation per condition were calculated by pooling data from all slides (minimum of six slides per condition).

Statistical analysis using a paired t-test on the data from the three independent experiments indicated absence of statistically significant difference between exposed and sham-exposed samples, and between sham + MMS and exposed + MMS samples.

5.7 5G RF exposure on health parameters in *C. elegans*

C. elegans were exposed to a 26.5 GHz, 5G signal, at CNR premises. The exposure conditions are detailed in Section 4.8.1, while an explanation of the method used to obtain these results is provided in Section 4.8.2. The following figures (Figure 39 and Figure 40) present the results about hatching rates, survival rates and length measurements of *C. elegans* for the three experimental conditions.

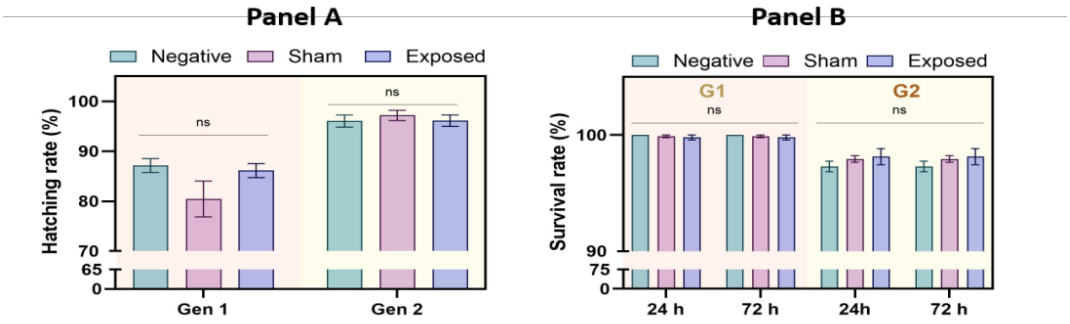


Figure 39: RF toxicity in *C.elegans* after exposure to 26.5 GHz, 5G signal. Panel A)% of hatched eggs after overnight incubation for 3 experimental conditions, across 2 different generations (Gen/G). Each bar represents the mean % of hatched eggs when compared to their respective starting populations, with error bars showing the standard deviation. A total of 3 independent experiments were performed, and in each individual experiment, a minimum of 600 eggs were included per experimental

condition. ns indicates no significant changes between the conditions; 2-way ANOVA. Panel B) % of alive worms after the indicated number of hours (h) for 3 experimental conditions, across 2 different generations. Each bar represents the mean % of live worms when compared to their respective starting populations. A total of 3 independent experiments were performed, and in each individual experiment, a minimum of 600 worms were included per experimental condition. ns indicates no significant changes between the conditions; 2-way ANOVA.

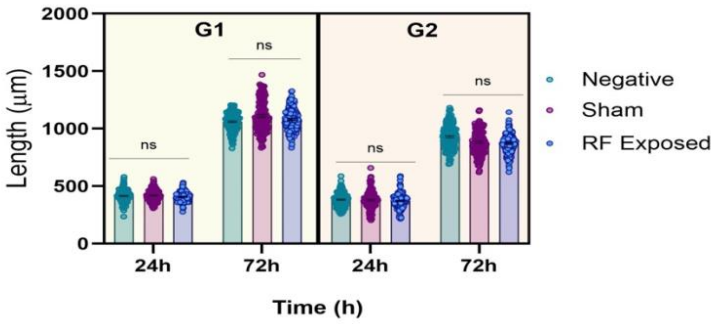


Figure 40: Effect of RF exposure on *C.elegans* length at different time-points and across 2 different generations (G). Each bar represents the average length, with individual values shown as circles and error bars showing the standard deviation. A total of 3 independent experiments were performed, and in each individual experiment, 50 worms were included per experimental condition and time-point. ns indicates no significant changes between the conditions.

5.8 5G RF exposure on transcriptomics in *C. elegans*

C. elegans were exposed to 26.5 GHz, 5G signal, at CNR premises. The exposure conditions are detailed in Section 4.8.1. According to the scheme reported in Figure 41, the worms were collected at different times and shipped to CSIC premises to extract RNA. Samples collected and their quality evaluation are represented in Table 8. RNA extracts were frozen and sent for sequencing to Macrogen company.

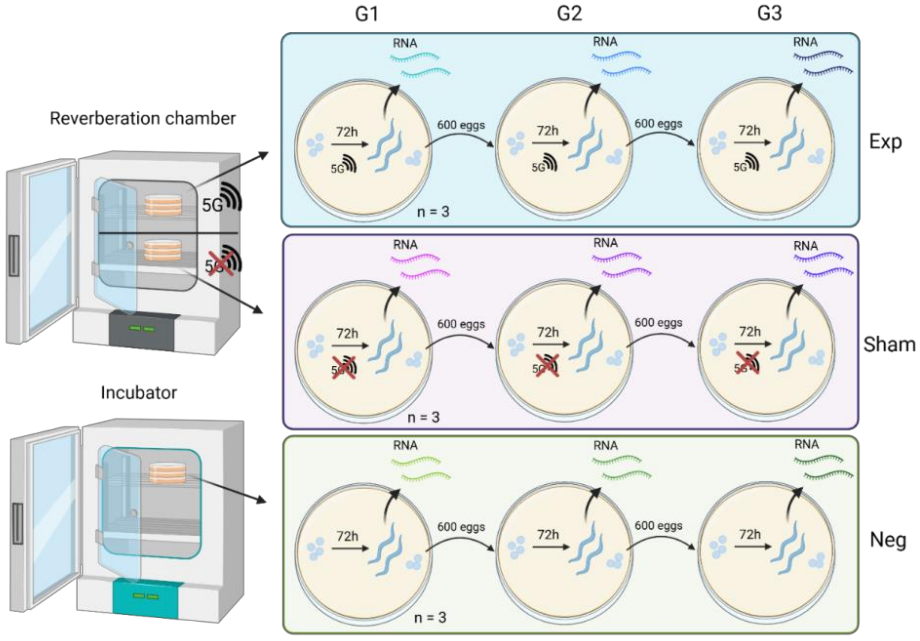


Figure 41: EMF exposure schedule of *C. elegans* for transcriptomics. G: Generation.

Table 8: Quality of the extracted RNA for its sequencing. Neg: Negative control; Exp: RF-exposed; Sham: Sham-exposed.

Samples	Concentration (ng/µl)	A260/A280	A/260/A230	Volume

Neg 1.1	1808.7	2.19	2.28	48
Neg 1.2	2829.2	2.23	2.02	48
Neg 1.3	2241.4	2.23	1.97	48
Neg 2.1	2483.1	2.2	2.55	48
Neg 2.2	2945.4	2.19	1.87	48
Neg 2.3	2787.8	2.18	2.54	48
Neg 3.1	1190.8	2.14	2.56	48
Neg 3.2	3468	2.19	2.54	48
Neg 3.3	4862.3	2.2	2.57	48
Sham 1.1	1964.3	2.19	2.54	48
Sham 1.2	3121.6	2.25	2.37	48
Sham 1.3	2632.7	2.23	2.4	48
Sham 2.1	2188.7	2.2	2.55	48
Sham 2.2	2847.2	2.19	2.39	48
Sham 2.3	2659.1	2.18	2.54	48
Sham 3.1	1851.4	2.13	2.28	48
Sham 3.2	3593.1	2.19	2.34	48
Sham 3.3	4953	2.21	2.58	48
Exp 1.1	2165.8	2.19	2.54	48
Exp 1.2	4794.2	2.26	2.48	48
Exp 1.3	2918.6	2.25	2.04	48
Exp 2.1	1994.7	2.21	2.15	48
Exp 2.2	1788.4	2.19	1.73	46
Exp 2.3	3006.1	2.21	2.21	48
Exp 3.1	1337.3	2.17	1.95	48
Exp 3.2	4027.6	2.19	2.49	48
Exp 3.3	3008.5	2.19	2.31	48

We performed RNA-Seq and differential expression analysis of worms exposed to EMF, with a log2FC threshold of 1 and p-adj value < 0.05. When analyzing the effects of RF exposure on gene expression, we compared all RF-exposed (Exp) samples to Sham control samples and all Exp samples to Negative control (Neg) samples while considering differences between generations.

We also contrasted each generation separately, i.e., G1 Exp samples compared to Sham G1, Exp G1 compared to Neg G1, and the similar comparisons for G2 and G3 samples.

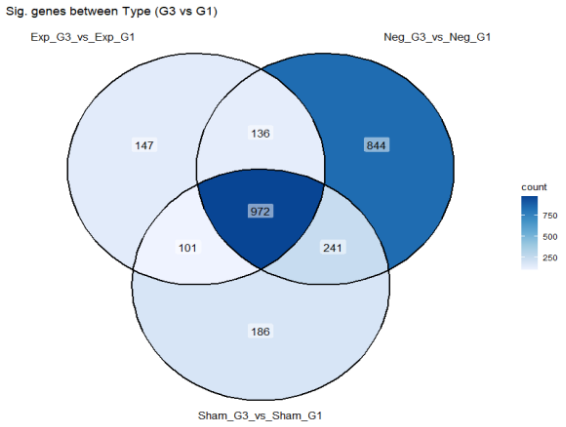


Figure 42: Venn diagram of significant gene lists from contrasts of Exp G3 to Exp G1, Neg G3 to Neg G1, and Sham G3 to Sham G1

Furthermore, we analysed the effects of EMF exposure across generations. For this, we compared later generations to the first generation within the same conditions, i.e., Exp G3 compared to Exp G1, Exp G2 compared to Exp G1, and the exact comparisons for Sham and Neg. Then, the differentially expressed gene lists of the same generation were compared. There were 147 significant genes present only on Exp G3 and 12 significant genes present only on Exp G2.

To assess whether the reverberation chamber affected the worms, we also compared Exp and Sham samples grown in the reverberation chamber to Negative samples grown in a regular incubator, considering generation effects (Figure 42)..

6 Conclusion

Progress in studying the interaction between EMF and biological systems depends on the setting of good-quality *in vitro* and *in vivo* investigations. Strictly controlled experiments for biological and electromagnetic parameters are crucial in generating robust and scientifically valuable data. This is what NextGEM aims to achieve through the activities planned in Task 4.3, to evaluate the effects of RF-EMF exposure alone and in combination with other physical and/or chemical agents on cancer-related endpoints. In such a way, cancer, which is one of the most alarming health effects possibly induced by RF exposure, can be considered in a realistic exposure scenario.

Following up on the initial report, this final report provides a brief overview of the biological effects of RF fields regarding cancer-related endpoints to lay the foundation for the experimental activities planned here. All the experimental procedures presented in this deliverable have been set up to fulfil the biological and electromagnetic requirements for good-quality studies. NextGEM's Standard Operating Procedures have also been developed for the execution of the experiments and included as Annexes. These procedures refer to the experiments on human neuroblastoma (SH-SY5Y) cells, human (HaCaT) keratinocytes, and on the model organism *C. elegans* that were performed to evaluate the effects of RF exposure or co-exposure to frequencies and signals typical of 4G and 5G technologies. No statistically significant effects were detected in the biological models used under any of the experimental conditions analysed so far.

In conclusion, this document presents the results of investigations on cancer-related endpoints of *in vitro* and *in vivo* models exposed to different RF frequencies and signals alone or combined with other chemical/physical agents. The content of this deliverable, with the developed experimental procedures and the results gained so far, represents also the background for the activities currently ongoing in the Case Studies in the framework of WP7, where the generation of new results under more realistic RF exposure scenarios is envisaged.

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Annexes

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Standard Operating Procedure @ CNR- IREA

PROTOCOL	**SH-SY5Y cell culture conditions and handling**
DATE	08/06/2023
AUTHOR(S)	Mariateresa Allocca, Anna Sannino, Stefania Romeo, Maria Rosaria Scarfi, Olga Zeni
REVISED BY	Maria Rosaria Scarfi, Olga Zeni
APPROVED BY	Olga Zeni

Index

1. Purpose

This procedure describes the materials and the protocols used for maintenance and storage of SH-SY5Y cell line.

2. Background

Human neuroblastoma SH-SY5Y cell line was obtained from a metastatic bone tumor of a 4-year-old cancer patient. Frozen cryovial ($6.4x10^6$ cells) was purchased from ATCC (Cat. No. CRL2266, Manassas, VA, USA) and arrived to CNR-IREA on 31 August 2018. Upon arrival, the cells were

amplified, then some stocks were prepared according to the freezing procedure and stored in liquid nitrogen (master bank of cells at passage 3-4). A working bank of SH-SY5Y cells was established from a master bank vial in order to control the number of cell passages for NextGEM experiments.

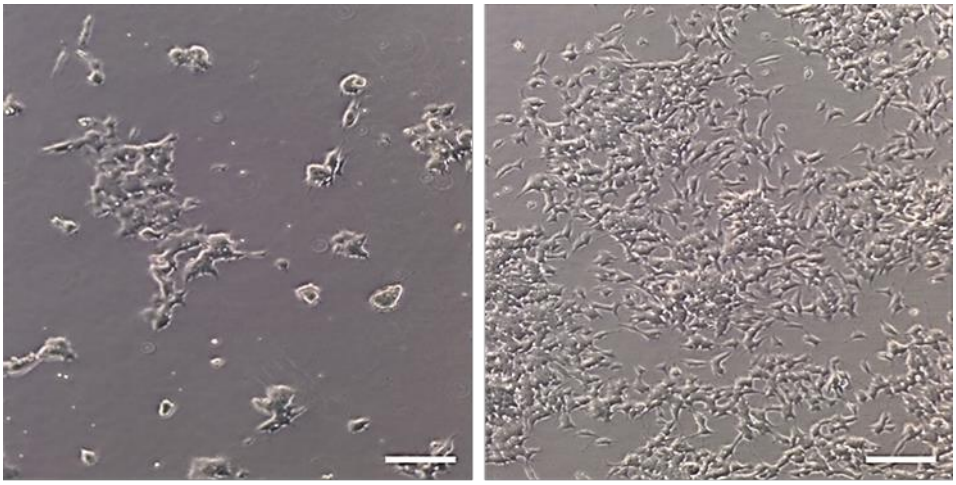

Figure 1: SH-SY5Y cell line as appeared at different growth densities at CNR-IREA lab. Inverted microscope images, scale bar: 100 µm.

The SH-SY5Y cells grow partially in suspension and mostly in adhesion (figure 1), with adherent cells weakly attached. Cultures grow as clusters (figure 2) of neuroblastic cells with multiple, short, fine processes (neurites).

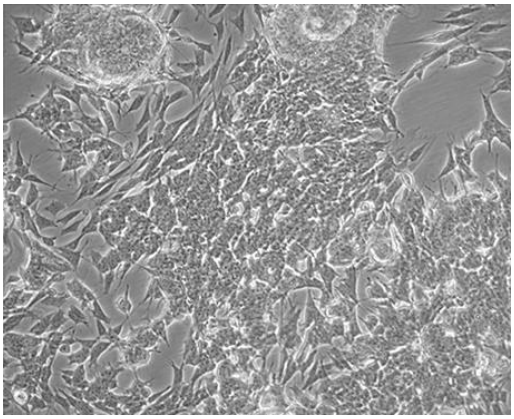

Figure 2: SH-SY5Y cell clumps.

The cell size, measured with Luna II cell counter, varies between 10-11.5 µm. The doubling time depends on the number of cells at seeding and is between 48 and 72 hours.

3. Procedure

The reagents and materials used are sterile and all the procedures are performed under a laminar flow cabinet.

3.1. Equipments

- Cell culture incubator (Thermo Scientific Forma, Model 311)
- Laminar flow cabinet (GELAIRE, BH24TG)
- Water bath (Grant Instruments, J SUB)
- Inverted microscope (Leica, DM IL)
- Refrigerated centrifuge (Thermo Electron, PK 131 R)
- Automated cell counter (Logos Biosystems, Luna II)
- Liquid nitrogen container (MVE XC 47/11-6)

3.2. Materials

- 35 mm cell culture dish (Corning, cod. 430165)
- 100 mm cell culture dish (Corning, cod. 430167)

3.3. Reagents

- DMEM (Dulbecco's Modified Eagle Medium), supplemented with 4.5 g/L glucose, sodium pyruvate and sodium bicarbonate (Microgem, cod. AL007) is stored at +4°C
- Fetal Bovine Serum (Microgem, cod. RM10432) is stored at -20°C
- 200 mM GlutaMAX™ Supplement (Gibco, cod. 35050-038) is stored at +4°C
- 100X Penicillin-Streptomycin solution (Himedia, cod. A001) is stored at -20°C
- 1X PBS (Dulbecco's Phosphate Buffered Saline) w/o Calcium w/o Magnesium (Microgem, cod. TL1006) is stored at +4°C
- Trypsin – EDTA 1X in solution w/o Calcium w/o Magnesium w/ Phenol Red (Microgem, cod. L0930) is stored at -20°C
- DMSO (Dimethylsulphoxide, LABSCAN, cod A3534) is stored at room temperature (RT)
- Trypan blue stain, 0.4% (Logos Biosystems, cod. TB0BJC2301) is stored at RT

3.3.1. Complete medium preparation

The SH-SY5Y culture medium is composed by DMEM supplemented with 10% heat inactivated FBS, 2 mM GlutaMAX™ Supplement, 1X Penicillin-Streptomycin solution.

For 100 ml complete medium: add 10 ml FBS, 1 ml GlutaMAX™ Supplement and 1 ml Penicillin-Streptomycin to 88 ml DMEM. The culture medium can be stored at 4°C for 1-2 weeks.

3.3.2. Cryoprotective medium preparation

The SH-SY5Y cells are frozen in DMEM complete medium with 5% DMSO.

For 10 ml cryoprotective medium: add 0.5 ml DMSO to 9.5 ml complete medium.

3.4. Subculturing procedure

Before splitting:

- Warm trypsin and complete medium to 37°C
- Label tubes and dishes with cell name, passage and date

Splitting:

The following volumes are referred to 100 mm cell culture dish

a. Remove the culture medium and wash the cells with 4 ml PBS

b. Add 2 ml trypsin and incubate for 5 minutes at 37°C

c. Check the detachment of cells and resuspend them in 5 ml complete medium

d. Transfer cells into centrifuge tube and spin at 300 g for 5 minutes

e. Discard the medium and resuspend the cell pellet in 4 ml fresh complete medium

f. Collect an aliquot to count the cells before dispensing the required amount into new dish containing 10 ml of fresh medium

Note:

- For maintenance: split SH-SY5Y cells once a week and seed $5x10^6$ cells in 100 mm cell culture dish
- For the experiments: use SH-SY5Y cells for a maximum of 11 passages

3.5. Freezing procedure

1. When cells are confluent, perform steps a-c described under “splitting”
2. Collect an aliquot to count the cells
3. Centrifuge the cells at 300 g for 5 minutes
4. Resuspend the cells in cryoprotective medium at a concentration of $5x10^6$/ml
5. Aliquot 1 ml of cell suspension in sterile cryovials
6. Place the cells for 1 hour at -20°C, then overnight at -80°C. Finally transfer them into liquid nitrogen for long term storage

Note: perform mycoplasma test (fluorescence DAPI test) before freezing cells

3.6. Thawing procedure

1. Take the cryovial out of liquid nitrogen and quickly thaw by hand

2. Transfer the cells into centrifuge tube containing 5 ml pre-warmed culture medium and spin at 300 g for 5 minutes
3. Discard the medium and resuspend the cell pellet in 5 ml complete medium
4. Transfer the cells to the culture dish and incubate at 37°C and 5% CO_2

Standard Operating Procedure @ CNR- IREA

PROTOCOL	**Cell cultures exposure to 4G LTE signal at 1950 MHz**
DATE	04/09/2023
AUTHOR(S)	Mariateresa Allocca, Stefania Romeo, Anna Sannino, Maria Rosaria Scarfi, Olga Zeni
REVISED BY	Maria Rosaria Scarfi, Olga Zeni
APPROVED BY	Olga Zeni

Index

1. Purpose

This procedure describes the equipment and the protocol used for cell cultures exposure to electromagnetic field (EMF) at 1950 MHz, 4G LTE signal. The aim is to ensure consistency and compliance of the bioelectromagnetic experiments with good laboratory practices.

2. Equipments and Materials

- RF generator (Agilent, E4432B ESG-D series)
- One microwave amplifier (MALTD, AM38A-0925-40-43)
- Two bidirectional power sensors (Rohde & Schwarz, NRTZ43)
- One, −6 dB power splitter (Hewlett-Packard HP11667A)
- PC for remote control through a Labview program (National Instruments)
- One, cell culture incubator (Thermo Scientific Forma, Model 311)
- Three rectangular, short-circuited waveguides (WR430, 350mm long, SAIREM)
- Two coaxial-to-waveguide adapters (Maury Microwave R213A2; VSWR: 1.05)
- Three coaxial cables (SUHNER, SUCOFLEX SN233634 /4)
- 35 mm cell culture dish (Corning, cod. 430165)

- Three, four-layer customized plexiglass stands
- Three metallic slabs
- One plastic spacer

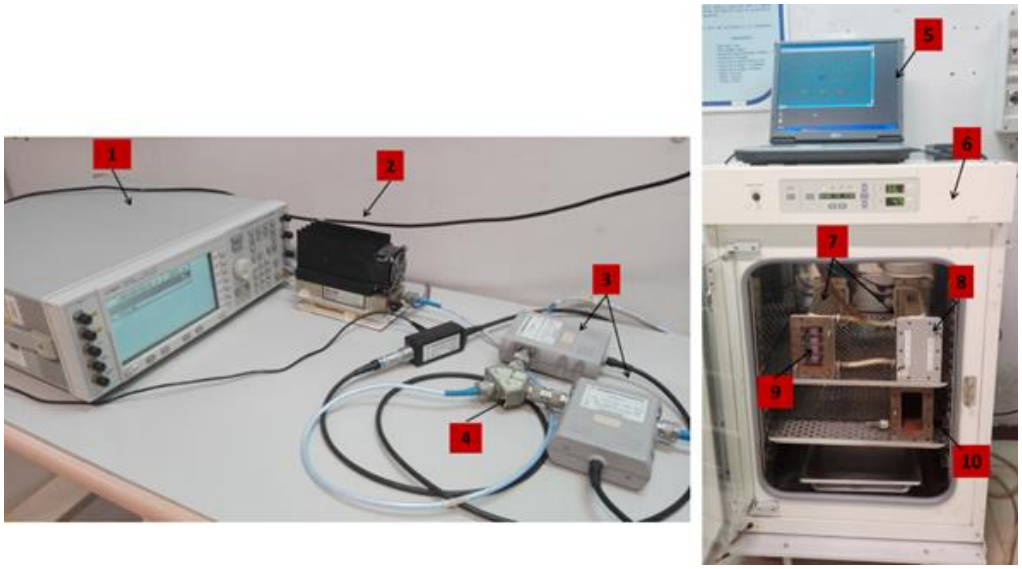

Figure 1. Exposure system setup. (1) RF generator, (2) microwave amplifier, (3) bi-directional power sensors (4) power splitter, (5) PC for remote control, (6) cell culture incubator, (7) waveguide for RF-exposure, (8) metallic slab for short-circuit, (9) four-layer Plexiglas stand loaded with cell cultures, (10) waveguide for sham-exposure.

3. Procedure

3.1. Preparation of cell samples

- Establish the following samples from the same batch of cells: incubator control, sham control, RF exposed at two SAR levels (0.3 and 1.25 W/kg), positive control. Label the cell culture dishes to decode each sample upon completion of the analysis for blind experiments: the operator who performs the analysis is not aware of the sample in hand.
- Locate the dishes on the Plexiglas stands (figure 2), insert the stands in each waveguide at the required distance from the short-circuit by using the plastic spacer and close each waveguide with the metallic slab. Perform this procedure two hours before starting exposure to allow the sample to acclimate in the waveguides.

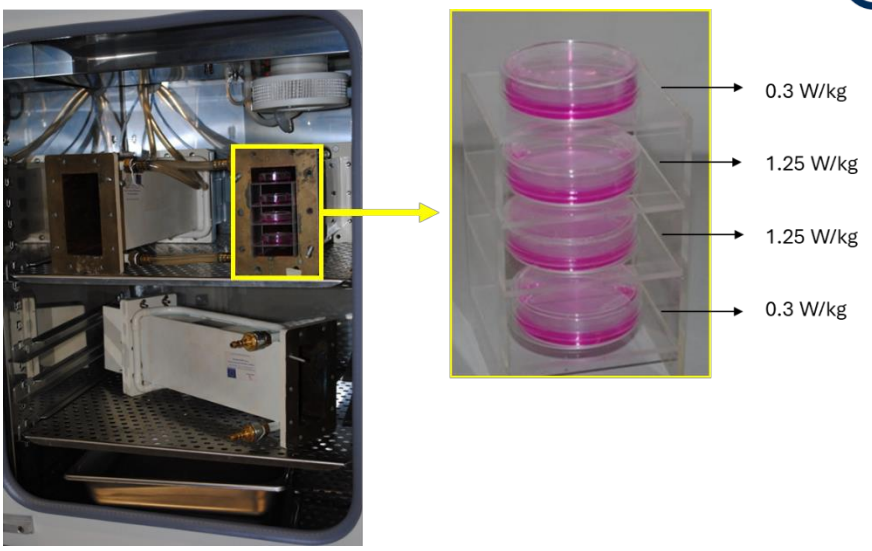

Figure 2. Plexiglas stand hosting cell culture dishes. A higher SAR value is obtained in the central samples with respect to the distal ones (4:1 SAR ratio).

3.2. RF exposure

- Switch on the RF generator and the PC
- (Optional) Open the NI-MAX program to verify that the generator and the two power meters are detected
- Connect to the Wi-Fi network
- Use the Matlab code to generate the LTE signal: launch Matlab 2019 on the PC and open the LTE LOADER program by following the path "Desktop→ Esposizione 4G→ LTE LOADER_ versione_1.0"
- Enter "LTE_LOADER_v1p0" and follow the instructions to build the signal
- Disconnect from the Wi-Fi network
- Start the Labview program for automated exposures by following the path "Desktop→ Esperimento_v4→Esperimento"
- Create a new experiment: select the folder in which the file containing the power measurements will be saved and set the file access mode as "replace or create"
- In the "MAIN" window, set the connection ports GPIBO::19::INSTR and COM3 under “Generator’s name” and “Power meter’s name”, respectively
- Click on the "GENERATOR" field and set the frequency and amplitude, then click "start configuration" and finally "back"
- Click on the " POWER METER: ZEROING AND MONITORING" field, then “start”, then “zero”, then “quit” and finally “back”
- Set up the exposure conditions in the GUI: exposure duration, sampling interval, desired SAR (that of the central positions of the stands) and exposure starting time (figure 3)
- Click on the “START” field
- At the end of the exposure carefully remove the stands with the culture dishes from the waveguides

- Close the “experiment” program, switch off the PC and all the instruments
- Proceed with the harvest and the procedure for the biological assay by following the related SOP

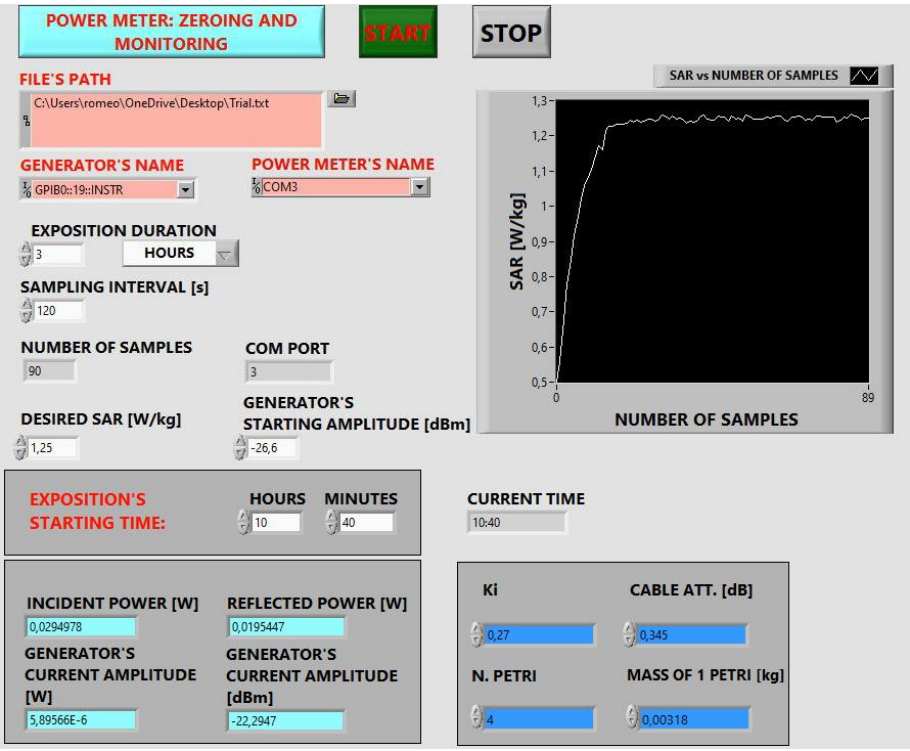

Figure 3. GUI of the Labview program for the control of the exposure setup.

Standard Operating Procedure @ CNR- IREA

PROTOCOL	**Intracellular ROS measurement by flow cytometer in SH-SY5Y cells**
DATE	02/08/2023
AUTHOR(S)	Mariateresa Allocca, Anna Sannino, Stefania Romeo, Maria Rosaria Scarfi, Olga Zeni
REVISED BY	Maria Rosaria Scarfi, Olga Zeni
APPROVED BY	Olga Zeni

Index

1. Purpose

This procedure describes the materials and the protocol used for flow cytometric analysis of ROS formation with H2DCF-DA staining in SH-SY5Y cells.

2. Background

2',7'-Dichlorodihydrofluorescein diacetate (H2DCF-DA) is used to assess the overall oxidative stress. H2DCF-DA crosses the cell membrane and is deacetylate by intracellular esterases, resulting in 2',7'-dichlorodihydrofluorescein (H2DCF). H2DCF reacts with reactive oxygen species (ROS) to give the fluorescent 2',7'-dichlorofluorescein (DCF), which is measured by flow cytometry.

3. Procedure

3.1. Equipments

- Cell culture incubator (Thermo Scientific Forma, Model 311)
- Refrigerated centrifuge (Heraeus Sepatech Minifuge RF)
- FACSCalibur flow cytometer (BD Biosciences) equipped with 488 nm laser

3.2. Materials

- 35 mm cell culture dish (Corning, cod. 430165)
- 5 mL polystyrene round bottom FACS tube (Falcon, cod. 352052)

3.3. Reagents

- DMSO (Dimethylsulphoxide, LABSCAN, cod A3534) is stored at room temperature
- H2DCF-DA (Sigma, cod. 6883): 10 mM stock solution is prepared in DMSO. Aliquots of 100 µl are prepared and stored in the dark at -20°C
- Menadione (Sigma, cod. M5625): 5.8 mM stock solution is prepared in DMSO. Aliquots of 100 µl are prepared and stored in the dark at -20°C
- DMEM (Dulbecco's Modified Eagle Medium), supplemented with 4.5 g/L glucose, sodium pyruvate and sodium bicarbonate (Microgem, cod. AL007) is stored at +4°C
- 1X PBS (Dulbecco's Phosphate Buffered Saline) w/o Calcium w/o Magnesium (Microgem, cod. TL1006) is stored at +4°C

- Trypsin – EDTA 1X in solution w/o Calcium w/o Magnesium w/ Phenol Red (Microgem, cod. L0930) is stored at -20°C

3.4. Experimental procedure

The following procedure has been optimized for SH-SY5Y human neuroblastoma cells (ATCC, Cat. No. CRL2266, Rockville, MD, USA). Procedure for cell maintenance is detailed in "SOP_SH-SY5Y cell maintenance_CNR".

$1x10^6$ cells are seeded in 3 ml complete medium in a 35 mm cell culture dish and the assay is performed after 72 h of growth.

3.4.1. Menadione treatment

Menadione (MD) is a ROS inducer and 20 µm MD is used as positive control.

MD (5 and 20 µm) is used as agent for co-exposure in the 4G LTE radiofrequency experiments (see SOP_4G LTE exposure_CNR).

3.4.2. Sample preparation

1) Remove the culture medium and replace it with 3 ml of DMEM base medium with 10 µM H2DCF-DA (incubation medium), along with menadione where required.

2) Incubate for 10 min at 37°C

3) Collect the incubation medium into a FACS tube

4) Wash the adherent cells with PBS (1 ml) and detach by 3 min trypsin treatment (300 µl) at 37°C

5) Collect the cells by using the incubation medium and add cold PBS (1 ml)

6) Centrifuge (4°C, 1200 RPM, 5 min)

7) Discard the supernatant and wash the cell pellet with cold PBS (2 ml)

8) Centrifuge (4°C, 1200 RPM, 5 min)

8) Resuspend the pellet in 500 µl of cold PBS

9) Analyze by flow cytometer

Note: the whole procedure is performed in the dark.

3.4.3. Sample acquisition

CellQuest software is used for sample acquisition and data storage.

For each sample, 15000 events are acquired. Forward scatter (FSC) and side scatter (SSC) dot plot are selected to identify the cell population. FL1 channel is selected to detect the DCF fluorescence in log scale.

The following graphs are displayed:

1° Dot Plot: Acquisition, FSC (x-axis) and SSC (y-axis)

2° Dot Plot: Acquisition, FL1 (x-axis) and FSC (y-axis)

1° Histogram: Acquisition, FL1

3.4.4. Sample analysis

The DCF fluorescence histograms are analyzed by the Flow Jo analysis program (TreeStar, OR, USA). The percentage of DCF positive cells is quantified considering a threshold fluorescence level (expressed as arbitrary units) set on the basis of the background fluorescence in the control cell population (about 10^2).

NextGEM

Standard Operating Procedure @ CNR- IREA

PROTOCOL	**Apoptosis detection by flow cytometer in SH-SY5Y cells**
DATE	28/08/2023
AUTHOR(S)	Mariateresa Allocca, Anna Sannino, Stefania Romeo, Maria Rosaria Scarfi, Olga Zeni
REVISED BY	Maria Rosaria Scarfi, Olga Zeni
APPROVED BY	Olga Zeni

Index

1. Purpose

This procedure describes the materials and the protocol used for flow cytometric analysis of apoptosis measurement in SH-SY5Y cells.

2. Background

Apoptosis, the physiological process of cell death, is fine-tuned. Phosphatidylserine (PS) is a cell membrane phospholipid that translocates to the outside-facing side of the membrane when apoptosis is initiated. Annexin V, a protein binding PS with high affinity, is used to detect apoptotic cells by flow cytometry when labeled with FITC. If the staining protocol with Annexin V-FITC is combined with propidium iodide (PI), it is also possible to screen late apoptotic and dead cells:

Annexin V-FITC stained cells identify early-stage apoptotic cells, the double stained Annexin V/PI identify late-stage apoptotic cells, while PI stained ones are necrotic.

3. Procedure

3.1. Equipments

- Cell culture incubator (Thermo Scientific Forma, Model 311)
- Refrigerated centrifuge (Heraeus Sepatech Minifuge RF)
- Automated cell counter (Luna II)
- FACSCalibur flow cytometer (BD Biosciences) equipped with 488 nm laser

3.2. Materials

- 35 mm cell culture dish (Corning, cod. 430165)
- 5 mL polystyrene round bottom FACS tube (Falcon, cod. 352052)

3.3. Reagents

- DMSO (Dimethylsulphoxide, LABSCAN, cod A3534) is stored at room temperature (RT)
- Menadione (Sigma, cod. M5625): 5.8 mM stock solution is prepared in DMSO. Aliquots of 100 µl are prepared and stored in the dark at -20°C
- DMEM (Dulbecco's Modified Eagle Medium), supplemented with 4.5 g/L glucose, sodium pyruvate and sodium bicarbonate (Microgem, cod. AL007) is stored at +4°C
- 1X PBS (Dulbecco's Phosphate Buffered Saline) w/o Calcium w/o Magnesium (Microgem, cod. TL1006) is stored at +4°C
- Trypsin – EDTA 1X in solution w/o Calcium w/o Magnesium w/ Phenol Red (Microgem, cod. L0930) is stored at -20°C
- Trypan blue stain, 0.4% (Logos Biosystems, cod. TB0BJC2301) is stored at room temperature (RT)
- Apoptosis Detection kit (Leinco Technologies, cod. A432) containing: i) Binding Buffer, ii) Annexin V-FITC, iii) Propidium Iodide (PI) is stored at 4°C

3.4. Experimental procedure

The Annexin V-FITC Apoptosis Detection kit is used following the manufacturer's instruction and the described procedure has been optimized for SH-SY5Y human neuroblastoma cells (ATCC, Cat. No. CRL2266, Rockville, MD, USA). Procedure for cell maintenance is detailed in Annex 1.

$1x10^6$ cells are seeded in 3 ml complete medium in a 35 mm cell culture dish and the assay is performed after 72 h of growth.

3.4.1. Menadione treatment

Menadione (MD) 20 µm is used as positive control.

MD (5 and 20 µm) is used as agent for co-exposure in the 4G LTE radiofrequency experiments (see SOP_4G LTE exposure_CNR).

3.4.2. Sample preparation

1) Collect cell growth medium into FACS tube

2) Wash the adherent cells with PBS (1 ml) and detach by 3 min trypsin treatment (300 µl) at 37°C

3) Collect the cells by using the growth medium and add cold PBS (500 µl)

4) Centrifuge (4°C, 1200 RPM, 5 min), and remove the supernatant

5) Resuspend the cell pellet with DMEM complete medium (1 ml)

6) Count the cells by trypan blue stain

7) Transfer 300000 cells into a clean FACS tube

8) Add cold PBS (2 ml), centrifuge (4°C, 1200 RPM, 5 min) and remove the supernatant. Repeat this step once

9) Resuspend the pellet in Binding Buffer (100 µl)

10) Add Annexin V-FITC (5 µl), PI (5 µl) and mix by gentle pipetting

11) Incubate for 10 min at RT

12) Add cold PBS (400 µl) and mix by gentle pipetting

13) Analyze by flow cytometer

Note

- Before starting the first experimental run, the calibration of the instrument is required and is achieved by setting up the following samples: i) unstained cells to assess the level of autofluorescence, ii) cells stained only with Annexin V-FITC and iii) cells stained only with PI to define the boundaries of each population.
- Be careful during trypsinization to avoid damaging the cells. Moreover, if trypsin EDTA is used, it is necessary to completely remove the EDTA by washing the cells twice before staining to avoid chelating the calcium needed for Annexin binding.
- Be sure that steps 9 to 13 are performed in the dark.

3.4.3. Sample acquisition

CellQuest software is used for sample acquisition and data storage.

For each sample, 15000 events are acquired. Forward scatter (FSC) and side scatter (SSC) dot plots are selected to identify the cell population. FL1 and FL2 channels are selected to detect the FITC and the PI fluorescence (log scale) respectively.

The following graphs are displayed:

1° Dot Plot: Acquisition, FSC (x-axis) and SSC (y-axis)

2° Dot Plot: Acquisition, FL1 (x-axis) and FL2 (y-axis)

1° Histogram: Acquisition, FL1

2° Histogram: Acquisition, FL2

3.4.4. Sample analysis

FlowJo software (TreeStar, OR, USA) is used for sample analysis.

Apoptotic cells are displayed in the right quadrants of the FL1/FL2 dot plot, with early apoptotic cells at the bottom (Annexin V- FITC positive) and late apoptotic cells at the top (Annexin V- FITC and PI positive). Necrotic cells (PI positive) are displayed on the top left. The percentage of cells in each quadrant is calculated by the software.

Standard Operating Procedure @ CNR- IREA

PROTOCOL	**Cell cycle progression by flow cytometer in SH-SY5Y cells**
DATE	07/02/2024
AUTHOR(S)	Mariateresa Allocca, Anna Sannino, Stefania Romeo, Maria Rosaria Scarfi, Olga Zeni
REVISED BY	Maria Rosaria Scarfi, Olga Zeni
APPROVED BY	Olga Zeni

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1. Purpose

This procedure describes the materials and the protocol used for flow cytometric analysis of cell cycle with propidium iodide staining in SH-SY5Y cells.

2. Background

Propidium iodide (PI) is a fluorescent dye intercalating into the base pairs of double-stranded DNA. PI is used to quantify DNA content in permeabilized cells by flow cytometry. Cell cycle analysis consists in quantifying the percentage of cells in each stage of the cell cycle (G0/G1, S and G2/M).

3. Procedure

3.1. Equipments

- Cell culture incubator (Thermo Scientific Forma, Model 311)
- Refrigerated centrifuge (Heraeus Sepatech Minifuge RF)
- Automated cell counter (Luna II)
- 4°C refrigerator
- FACSCalibur flow cytometer (BD Biosciences) equipped with 488 nm laser

3.2. Materials

- 35 mm cell culture dish (Corning, cod. 430165)
- 5 mL polystyrene round bottom FACS tube (Falcon, cod. 352052)

3.3. Reagents

- Sodium chloride solution 0.9% (Galenica senese, cod A029874385) is stored at room temperature (RT)
- MMC (Mitomycin-C; Sigma, cod. 1001017941): 0.25 mg/ml stock solution is prepared in sodium chloride solution. Aliquots of 500 µl are prepared and stored in the dark at -20°C
- DMEM (Dulbecco's Modified Eagle Medium), supplemented with 4.5 g/L glucose, sodium pyruvate and sodium bicarbonate (Microgem, cod. AL007) is stored at +4°C
    - For complete medium, supplement DMEM with: 10% heat inactivated Fetal Bovine Serum (FBS; Microgem, cod. RM10432), 2 mM GlutaMAX™ Supplement (Gibco, cod. 35050-038), 1X Penicillin-Streptomycin solution (Himedia, cod. A001), and store at 4°C
- 1X PBS (Dulbecco's Phosphate Buffered Saline) w/o Calcium w/o Magnesium (Microgem, cod. TL1006) is stored at +4°C
- Trypsin – EDTA 1X in solution w/o Calcium w/o Magnesium w/ Phenol Red (Microgem, cod. L0930) is stored at -20°C
- Trypan blue stain, 0.4% (Logos Biosystems, cod. TB0BJC2301) is stored at RT
- Triton X-100 solution (Sigma, cod. 93443) is stored at RT
- Sodium citrate dihydrate (Baker, cod. 15598154): 0.75 M stock solution is prepared in H_2O_2, pH 8 and stored at RT
- PI, 1mg/ml (Sigma, cod. P4864) is stored at +4°C
    - For PI solution: 50 µg/ml, 33 mM sodium citrate and 0.1% Triton X-100. It is stored at +4°C

3.4. Experimental procedure

The following procedure has been optimized for SH-SY5Y human neuroblastoma cells (ATCC, Cat. No. CRL2266, Rockville, MD, USA). Procedure for cell maintenance is detailed in Annex 1.

$1x10^6$ cells are seeded in 3 ml complete medium in a 35 mm cell culture dish and the assay is performed after 72 h of growth.

3.4.1. Positive control

Cells treated for 16 h with 1 µg/ml MMC are used as positive control.

3.4.2. Sample preparation

1) Collect cell growth medium into FACS tube
2) Wash the adherent cells with PBS (1 ml) and detach by 3 min trypsin treatment (300 µl) at 37°C
3) Collect the cells by using the growth medium and add cold PBS (500 µl)
4) Centrifuge (4°C, 1200 RPM, 5 min), and remove the supernatant
5) Resuspend the cell pellet with DMEM complete medium (1 ml)
6) Count the cells by trypan blue stain
7) Transfer 500000 cells into a clean FACS tube
8) Add cold PBS (2 ml), centrifuge (4°C, 1200 RPM, 5 min) and remove the supernatant. Repeat this step once
9) Resuspend the cell pellet with 500 µl of cold DMEM base medium and 500 µl of cold PI solution
10) Mix well and vortex
11) Incubate for 30 min at 4°C
12) Analyze by flow cytometer

Note: be sure that steps 7 to 10 are performed in the dark.

3.4.3. Sample acquisition

CellQuest software is used for sample acquisition and data storage.

For each sample, 25000 events are acquired. Forward scatter (FSC) and side scatter (SSC) dot plots are selected to identify the cell population. FL2 channel is selected to detect PI fluorescence in the linear scale.

The following graphs are displayed:

1° Dot Plot: Acquisition, FSC (x-axis) and SSC (y-axis)

2° Dot Plot: Acquisition, FL2W-1024 (x-axis) and FL2-A (y-axis)

1° histogram: Acquisition, FL2-A

2° histogram: Acquisition, FL2-H

Note:

- adjust the sensitivity of photomultiplier tubes for PI staining such that the G0/G1 (2n ploidy) and G2/M (4n ploidy) are centered, respectively, at 200 and 400 (arbitrary units) on the X-axis
- a S-shaped population should be visible in the 2° Dot Plot

3.4.4. Sample analysis

FlowJo software (TreeStar, OR, USA) is used for the analysis. Data are expressed as the relative percentage of cells in different stages of the cell cycle.

Standard Operating Procedure @ CNR- IREA

PROTOCOL	**Cell cultures simultaneous exposure to 4G LTE signal at 1.95 GHz and WiFi signal at 2.45 GHz**
DATE	13/09/2024
AUTHOR(S)	Mariateresa Allocca, Stefania Romeo, Anna Sannino, Valentina Peluso, Maria Rosaria Scarfi, Olga Zeni
REVISED BY	Maria Rosaria Scarfi, Olga Zeni
APPROVED BY	Olga Zeni

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1. Purpose

This procedure describes the equipment and the protocol used for cell cultures simultaneous exposure to electromagnetic field (EMF) at 1950 MHz (4G LTE signal) and at 2450 MHz (WiFi signal). The aim is to ensure consistency and compliance of the bioelectromagnetic experiments with good laboratory practices.

2. Equipment and Materials

- One RF generator (Agilent, E4432B ESG-D series)
- One RF generator (Rohde and Schwarz, SMM100A) with IEEE 802.11 option enabled to generate WiFi signal
- One power combiner (PD2020 InNSTOCK wireless components, NJ, USA)
- One microwave amplifier (MALTD, AM38A-0925-40-43)
- One power sensor (Rohde & Schwarz, NRTZ43)
- Two rectangular, short-circuited waveguides (WR430, 350mm long, SAIREM)
- Two coaxial-to-waveguide adapters (Maury Microwave R213A2; VSWR: 1.05)

- Four coaxial cables (SUHNER, SUCOFLEX SN233634 /4)
- PC for remote control of the power sensor through the R&S virtual NRT program (Rohde & Schwarz)
- One cell culture incubator (Thermo Scientific Forma, Model 311)
- 30 mm customized Pyrex cell culture dish
- Two four-layer customized plexiglass stands
- Two metallic slabs
- One plastic spacer

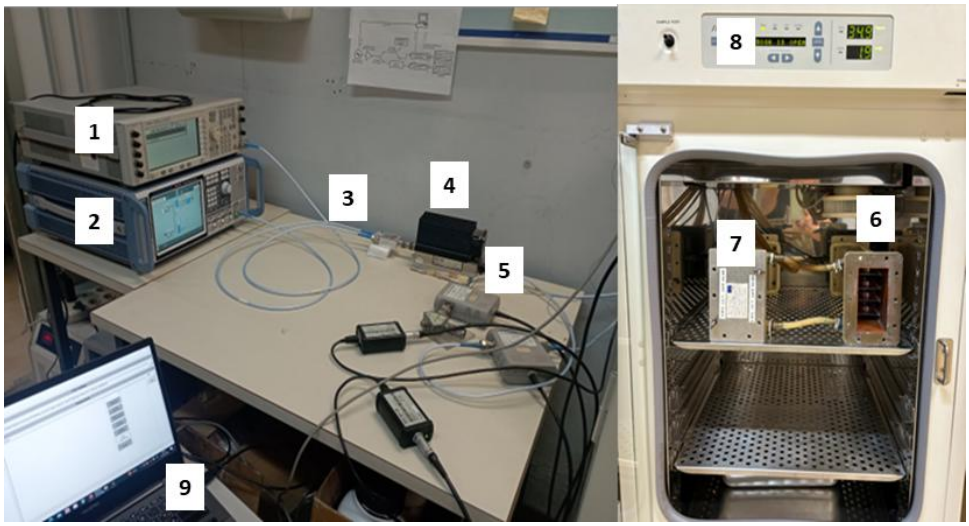

Figure 1. Exposure system setup. (1) Agilent RF generator, (2) Rohde and Schwarz RF generator, (3) power combiner, (4) microwawe amplifier, (5) bi-directional power sensor, (6) waveguide for RF-exposure, (7) metallic slab for short-circuit, (8) cell culture incubator, (9) PC for remote control.

3. Procedure

3.1. Preparation of cell samples

- Establish the following samples from the same batch of cells: incubator control, sham control, RF exposed at two SAR levels (0.3 and 1.25 W/kg), positive control. Label the cell culture dishes to decode each sample upon completion of the analysis for blind experiments: the operator who performs the analysis is not aware of the sample in hand.
- Locate the dishes on the Plexiglas stands (figure 2), insert the stands in each waveguide at the required distance from the short-circuit by using the spacer and close each waveguide with the metallic slab. Perform this procedure two hours before starting exposure to allow the samples to acclimate in the waveguides.

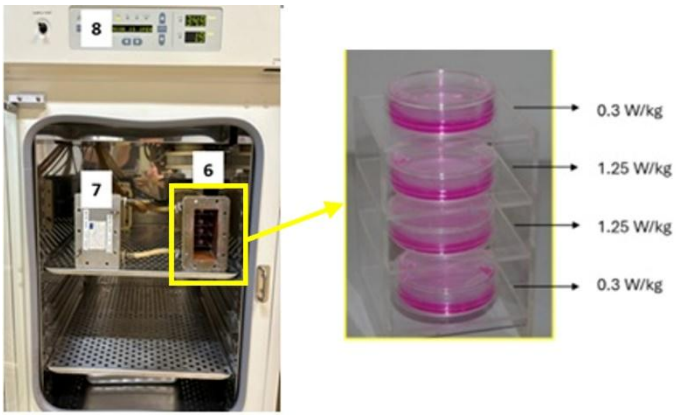

Figure 2. Plexiglas stand hosting cell culture dishes. A higher SAR value is obtained in the central samples with respect to the distal ones (4:1 SAR ratio).

3.2. RF exposure

- Switch on both RF generators and the PC
- (Optional) Open the NI-MAX program to verify that the generators and the power meter are detected
- Connect to the Wi-Fi network
- Use the Matlab code to generate the LTE signal: launch Matlab 2019 on the PC and open the LTE LOADER program by following the path "Desktop→ Esposizione 4G→ LTE LOADER_ versione_1.0"
- Enter "LTE_LOADER_v1p0" and follow the instructions to build the signal
- Disconnect from the Wi-Fi network
- Set the frequency (1.95 GHz) and the amplitude (–32 dBm) on the generator for 4G signal and verify that modulation is on (Mod ON)
- Set the frequency (2.45 GHz) and the amplitude (–32.6 dBm) on the generator for WiFi signal and verify that modulation is on (Mod ON)
- Start the R&S virtual NRT program.
    - Click on the “sensor” field to verify that the right power meter is detected, then click on "ZERO" to start zeroing of the power meter
    - Click on the “option” field, then on “record function” to set the separator (COMMA) and the Index (1)
    - Define the file name and browse the PC folder in which to save it
    - Click on “Auto Trigger” and set the START TIME (5 minutes before starting the RF), the STOP TIME (5 minutes after stopping the RF) and the interval (30 sec). Click on “enter” to verify the set parameters, then select “close”
- Start the RF manually from both the generators
- At the end of the exposure, stop the RF manually from both the generators
- Carefully remove the stands with the culture dishes from the waveguides
- Switch off the PC and all the instruments
- Proceed with the harvest and the procedure for the biological assay by following the related SOP

Standard Operating Procedure @ CNR- IREA

PROTOCOL	**HaCaT cell culture conditions and handling**
DATE	10/07/2023
AUTHOR(S)	Mariateresa Allocca, Anna Sannino, Stefania Romeo, Maria Rosaria Scarfi, Olga Zeni
REVISED BY	Maria Rosaria Scarfi, Olga Zeni
APPROVED BY	Olga Zeni

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1. Purpose

This procedure describes the materials and the protocols used for maintenance and storage of HaCaT cell line.

2. Background

HaCaT cells are *in vitro* spontaneously transformed keratinocyte from the adult human skin of a 62-year-old male. Frozen cryovial ($2x10^6$ cells/ml) was purchased from CLS (Lot. 300493-4820, p32; Eppelheim, Germany) and arrived at IREA on 12 October 2022. Upon arrival, the cells were amplified, then some stocks were prepared according to the manufacturer's instructions and stored in liquid

nitrogen (master bank of cells at passage 3-4). A working bank of HaCaT cells was established from a master bank vial in order to control the number of cell passages for NextGEM experiments.

The HaCaT cells grow as adherent cells in monolayer (figure 1). Their size, measured with Luna II cell counter, varies between 14-19 micrometer. The doubling time depends on the number of cells at seeding and is about 24 hours.

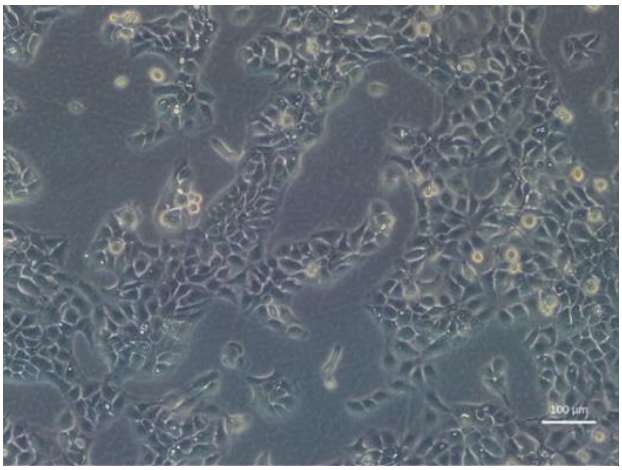

Figure 1: HaCaT cell line at CNR-IREA lab. Inverted microscope images, scale bar: 100 µm.

3. Procedure

The reagents and materials used are sterile and all the procedures are performed under a laminar flow cabinet.

3.1. Equipments

- Cell culture incubator (Thermo Scientific Forma, Model 311)
- Laminar flow cabinet (GELAIRE, BH24TG)
- Water bath (Grant Instruments, J SUB)
- Inverted microscope (Leica, DM IL)
- Refrigerated centrifuge (Thermo Electron, PK 131 R)
- Automated cell counter (Logos Biosystems, Luna II)
- Liquid nitrogen container (MVE XC 47/11-6)

3.2. Materials

- 35 mm cell culture dish (Corning, cod. 430165)
- 100 mm cell culture dish (Corning, cod. 430167)

3.3. Reagents

- DMEM (Dulbecco's Modified Eagle Medium), supplemented with 4.5 g/L glucose, sodium pyruvate and sodium bicarbonate (Microgem, cod. AL007) is stored at +4°C
- Fetal Bovine Serum (Dominique Dutscher, cod. RM10432) is stored at -20°C
- 200 mM L-glutamine (Dominique Dutscher, cod. X0550) is stored at +4°C
- 100X Penicillin-Streptomycin solution (Dominique Dutscher, cod. L0018) is stored at -20°C
- 1X PBS (Dulbecco's Phosphate Buffered Saline) w/o Calcium w/o Magnesium (Dominique Dutscher, cod. L0615 is stored at +4°C
- Trypsin – EDTA 1X in solution w/o Calcium w/o Magnesium w/ Phenol Red (Microgem, cod. L0930) is stored at -20°C
- Cryoprotective media CM-1 (CLS, cod. 800050)
- Trypan blue stain, 0.4% (Logos Biosystems, cod. TB0BJC2301) is stored at RT

3.3.1. Complete medium preparation

The HaCaT culture medium is composed by DMEM supplemented with 10% heat inactivated FBS, 2 mM L-glutamine, 1X Penicillin-Streptomycin solution.

For 100 ml complete medium: add 10 ml FBS, 1 ml L-glutamine and 1 ml Penicillin-Streptomycin to 88 ml DMEM. The culture medium can be stored at 4°C for 1-2 weeks.

3.4. Subculturing procedure

Before splitting:

- Warm trypsin and complete medium to 37°C
- Label tubes and dishes with cell name, passage and date

Splitting:

The following volumes are referred to 100 mm cell culture dish

a. Remove the culture medium and wash the cells with 4 ml PBS

b. Add 2 ml trypsin and incubate for 10 minutes at 37°C

c. Check the detachment of cells and resuspend them in 5 ml complete medium

d. Transfer cells into centrifuge tube and spin at 300 g for 5 minutes

e. Discard the medium and resuspend the cell pellet in 4 ml fresh complete medium

f. Collect an aliquot to count the cells before dispensing the required amount into new dish containing 10 ml of fresh medium

Note:

- For maintenance: split HaCaT cells twice a week and seed $1x10^6$ cells in 100 mm cell culture dish
- For the experiments: use HaCaT cells for a maximum of 16 passages

3.5. Freezing procedure

1. When cells are confluent, perform steps a-d described under “splitting”
2. Resuspend the cells in cryoprotective medium CM-1 at a concentration of $2x10^6$/ml
3. Aliquot 1 ml of cell suspension in sterile cryovials
4. Place the cells for 1 hour at -20°C, then overnight at -80°C. Finally transfer them into liquid nitrogen for long term storage

Note:

- Perform mycoplasma test (fluorescence DAPI test) before freezing cells
- Following the manufacturer's instructions, the day before freezing, change the culture medium to stimulate proliferation
- Use only the cryoprotective media CM-1 purchased from CLS to freeze the HaCaT cells

3.6. Thawing procedure

1. Take the cryovial out of liquid nitrogen and quickly thaw by hand
2. Transfer the cells into centrifuge tube containing 5 ml pre-warmed culture medium and spin at 300 g for 5 minutes
3. Discard the medium and resuspend the cell pellet in 5 ml complete medium
4. Transfer the cells to the culture dish and incubate at 37°C and 5% CO_2

Standard Operating Procedure @ CNR- IREA

PROTOCOL	**Cell cultures exposure to 5G signal at 26.5 GHz**
DATE	01/12/2023
AUTHOR(S)	Mariateresa Allocca, Stefania Romeo, Anna Sannino, Maria Rosaria Scarfi, Olga Zeni
REVISED BY	Maria Rosaria Scarfi, Olga Zeni
APPROVED BY	Olga Zeni

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1. Purpose

This procedure describes the equipment and the protocol used for cell cultures exposure to electromagnetic fields (EMF) at 26.5 GHz, 5G signal. The aim is to ensure consistency and compliance of the bioelectromagnetic experiments with good laboratory practices.

2. Equipments and Materials

- Radiofrequency (RF) generator (Rohde and Schwarz, SMW200A) with SMW-K144 and SMW-K148 options enabled to generate 5G compliant signal
- One wideband power sensor (Rohde and Schwarz, NRP-Z85)
- One directional coupler (Marki Microwave, CA40)
- Two customized stirred reverberation chambers (RCs) characterized by the electromagnetic perspective by University of Cassino (UCAS)
- Two customized stirrer controllers driving 2 stirrers for each RC, positioned on the right (M-Destro) and the bottom (M-Sotto) walls
- Two customized polystyrene stands for cell culture dishes
- Two WR-28 open-ended waveguides

- Two coaxial cables (PL 380P-292M292M-1M)
- PC for remote control through the Power Viewer program (Rohde and Schwarz)
- Two cell culture incubators (Thermo Scientific Forma, Model 311)
- 35 mm cell culture dish (Corning, cod. 430165)

Figure 1. Exposure system setup. The signal is sent to the RC placed inside a cell culture incubator while a second RC, placed inside another cell culture incubator, is used for sham exposure. Each chamber is equipped with two stirrers at independently controlled speed and hosts a sample holder to allow reproducible positioning of the cell culture dishes.

3. Procedure

3.1. Preparation of cell samples

- Prepare the following samples from the same batch of cells: incubator control, sham control, RF exposed, positive control. Label the cell culture dishes to decode each sample upon completion of the analysis for blind experiments: the operator who performs the analysis is not aware of the sample in hand
- Locate the dishes on the stands (figure 2) placed inside the RCs for exposure and sham exposure and close each RC with the metallic slabs. Perform this procedure three hours before starting exposure to allow the sample to acclimate in the RCs

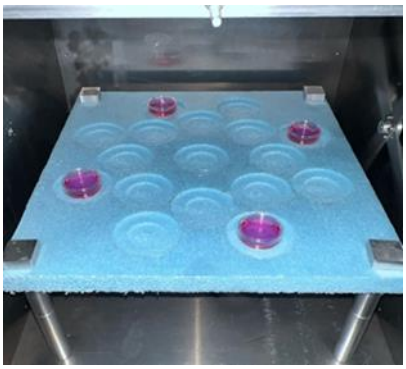

Figure 2. Sample positioning in the RC. The stand is opportunely engraved to allow a reproducible positioning of dishes.

3.2. RF exposure

- Turn on the generator at least 15 minutes before the start of exposure to allow warm-up
- Activate the 5G modulation (Baseband command enabled)
- Set the desired power level in dBm (set 2.66 dBm or 6.64 dBm to expose the samples at SAR 0.4 W/kg or SAR 1 W/kg, respectively)
- Turn on the stirrers in both RCs and set 240 for “M-Destro” and 233.3 for “M-Sotto”
- Turn on the PC and start the Power Viewer
- Connect the power sensor to the USB port of the PC
- Select the "Multi Channel" window. Channel 1 is turned off (Ch1: OFF). Move to channel 2 associated to the power sensor 2 (NRP-Z85, 102535) and set up the exposure conditions in the GUI (figure 3):
    - Frequency: 26.5 GHz
    - Averaging: manual
    - Count: 1048576
- Click on "ZERO" (with RF off) to start zeroing of the power sensor
- Click on the start button (on the top left of the toolbar) to start the visualization of the acquired data
- Select the "Data Log" window and check the following parameters:
    - Source: Multi
    - Ch1: OFF
    - Ch2: Ch 2 [W]
- Tick "Convert to Log Power," define the duration of the acquisition, tick "Log to File," set 500 ms in the "Interval" field and select the PC folder to save the data. Check that the value in the "Signal Frequency" field matches the value set on the generator (26.5 GHz)
- Click on the start button (in data log) to start data logging two minutes before the start of the exposure
- Start the RF manually from the generator
- At the end of the exposure, stop the RF manually from the generator

- Open the RCs
- Carefully remove the culture dishes from the stands
- Close the “experiment” program, switch off the PC and all the instruments
- Proceed with the harvest and the procedure for the biological assay by following the related SOP

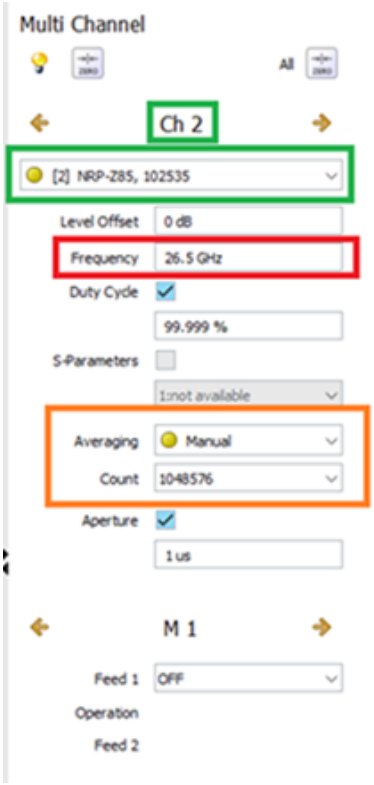

Figure 3. GUI of the Power Viewer program for the control of the exposure setup.

Standard Operating Procedure @ CNR- IREA

PROTOCOL	**Cell cultures exposure to UVB radiation**
DATE	04/03/2025
AUTHOR(S)	Mariateresa Allocca, Valentina Peluso, Stefania Romeo, Anna Sannino, Maria Rosaria Scarfi, Olga Zeni
REVISED BY	Maria Rosaria Scarfi, Olga Zeni
APPROVED BY	Olga Zeni

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1. Purpose

This procedure describes the equipment and the protocol used for cell cultures exposure to UVB radiation. The aim is to ensure consistency and compliance of the experiments with good laboratory practices.

2. Equipments and Materials

- Customized wooden box (710 mm long, 260 mm wide, 300 mm deep)
- UVB LED board with six LEDs each (LITE-ON, part number LTPL-G35UV308GH, UVB LED 308 nm, 45 mW, 350 mA, 120°, ERI Lighting S.R.L)
- LED AC/DC power supply (Professionale DALI, TCI srl)
- Handheld radiometer (DeltaOhm, HD2102.1) and UVB (LP471UVB, 280 – 315 nm) probe

- Styrofoam holder 6 cm high
- 35 mm cell culture dish (Corning, cod. 430165)

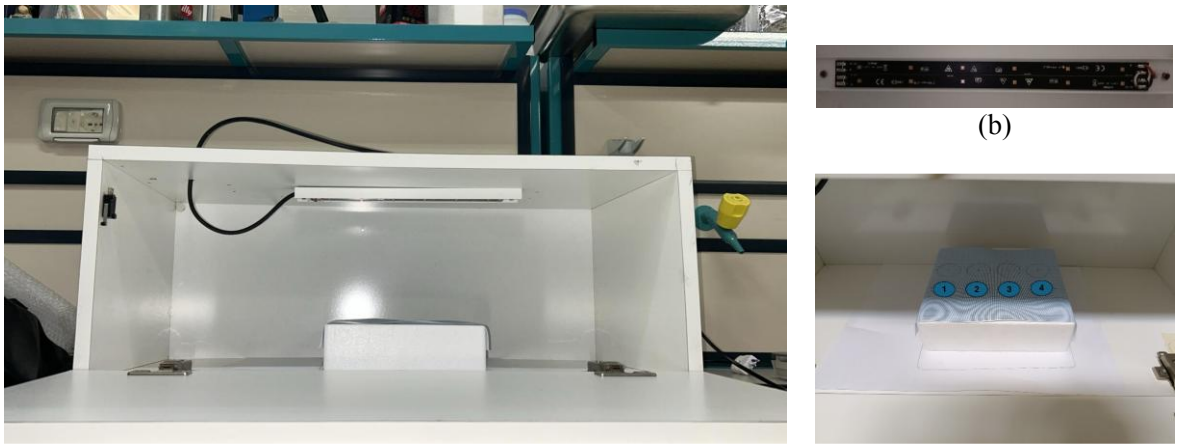

Figure 1. Exposure system setup. (a) UVB exposure set-up; (b) LED board; (c) irradiance measurement positions

3. Procedure

3.1. UVB irradiance measurements

- Wear personal protective equipment (gloves and glasses)
- Turn on the lamp at least 30 minutes before the start of measurement to allow warm-up
- Connect the UVB probe to the HD2102.1 (DeltaOhm) radiometer
- Switch-on the radiometer and wait until the completion of the auto-calibration procedure
- Turn the lamp off to allow placing the probe in the measurement position 1 according to Figure 1(c). STEP 1
- Close the wooden box and turn the lamp on. STEP 2
- Wait 2 min to assure stabilization of the UVB irradiance level. STEP 3
- Acquire the average, minimum and maximum UVB irradiance levels over 2 min time interval. STEP 4
- Repeat STEPS 1-4 for the remaining measurement positions (2, 3 and 4 in Figure 1(c))
- Repeat the whole procedure of UVB irradiance measurements at least three times for each position

- Periodically check the irradiance levels before proceeding with the UVB exposure of cell cultures

3.2. Preparation of cell samples

- Prepare the following samples from the same batch of cells: incubator control, negative control (cell culture kept on the bench outside the wooden box for the whole UVB exposure duration) , UVB exposed, positive control.
- Label the cell culture dishes to decode each sample upon completion of the analysis for blind experiments: the operator who performs the analysis is not aware of the sample in hand

3.3. UVB exposure

- Wear personal protective equipments (gloves and glasses)
- Turn the lamp on at least 30 minutes before the start of exposure to allow warm-up
- Replace the culture medium with 1 ml warmed PBS to avoid the interference of red phenol during the UV exposure
- Locate the dishes on the labelled positions of the styrofoam holder (Figure 1(c)) for UVB exposure and outside the box for negative control
- At the end of the exposure, turn off the lamp
- Open the box and carefully remove the culture dishes
- Proceed with the harvest and the procedure for the biological assay by following the related SOP

Standard Operating Procedure @ CNR- IREA

PROTOCOL	**Intracellular ROS measurement by flow cytometer in HaCaT cells**
DATE	05/03/2025
AUTHOR(S)	Mariateresa Allocca, Valentina Peluso, Anna Sannino, Stefania Romeo, Maria Rosaria Scarfi, Olga Zeni
REVISED BY	Maria Rosaria Scarfi, Olga Zeni
APPROVED BY	Olga Zeni

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1. Purpose

This procedure describes the materials and the protocol used for flow cytometric analysis of ROS formation with H2DCF-DA staining in HaCaT cells.

2. Background

2',7'-Dichlorodihydrofluorescein diacetate (H2DCF-DA) is used to assess the overall oxidative stress. H2DCF-DA crosses the cell membrane and is deacetylate by intracellular esterases, resulting in 2',7'-dichlorodihydrofluorescein (H2DCF). H2DCF reacts with reactive oxygen species (ROS) to give the fluorescent 2',7'-dichlorofluorescein (DCF), which is measured by flow cytometry.

3. Procedure

3.1. Equipment

- Cell culture incubator (Thermo Scientific Forma, Model 311)
- Refrigerated centrifuge (Heraeus Sepatech Minifuge RF)
- FACSCalibur flow cytometer (BD Biosciences) equipped with 488 nm laser

3.2. Materials

- 35 mm cell culture dish (Corning, cod. 430165)
- 5 mL polystyrene round bottom FACS tube (Falcon, cod. 352052)

3.3. Reagents

- H2DCF-DA (Sigma, cod. 6883): 10 mM stock solution is prepared in DMSO. Aliquots of 100 µl are prepared and stored in the dark at -20°C
- H_2O_2 (Sigma, cod. S86968-359): Hydrogen Peroxide 30 wt. % solution in water, 8820 mM stock solution. Aliquots of 2ml are prepared and stored in the dark at +4°C
- DMEM (Dulbecco's Modified Eagle Medium), supplemented with 4.5 g/L glucose, sodium pyruvate and sodium bicarbonate (Microgem, cod. AL007) is stored at +4°C
- 1X PBS (Dulbecco's Phosphate Buffered Saline) w/o Calcium w/o Magnesium (Microgem, cod. TL1006) is stored at +4°C
- Trypsin – EDTA 1X in solution w/o Calcium w/o Magnesium w/ Phenol Red (Microgem, cod. L0930) is stored at -20°C

3.4. Experimental procedure

The following procedure has been optimized for HaCaT human keratinocyte cells (CLS, Cat. No. 300493, Eppelheim, Germany). Procedure for cell maintenance is detailed in Annex 6.

$3x10^5$ cells are seeded in 3 ml complete medium in a 35 mm cell culture dish and the assay is performed after 72 h of growth.

3.4.1. H_2O_2 treatment

H_2O_2 is a ROS inducer and 1 mM for 30 min is used as positive control in setting up the procedure to evaluate ROS formation in HaCaT cells. The aim is to verify the adequacy of the procedure.

3.4.2. UVB exposure

UVB exposure (Annex 8) is used as treatment for co-exposure in the 5G radiofrequency experiments (Annex 7).

3.4.3. Sample preparation

1) Remove the culture medium and replace it with 3 ml of DMEM base medium with 10 µM H2DCF-DA (incubation medium), along with H_2O_2 where required

2) Incubate for 30 min at 37°C

3) Collect the incubation medium into a FACS tube

4) Wash the adherent cells with PBS (1 ml) and detach by 12 min trypsin treatment (300 µl) at 37°C

5) Collect the cells by using the incubation medium and add cold PBS (1 ml)

6) Centrifuge (4°C, 1200 RPM, 5 min)

7) Discard the supernatant and wash the cell pellet with cold PBS (2 ml)

8) Centrifuge (4°C, 1200 RPM, 5 min)

8) Resuspend the pellet in 500 µl of cold PBS

9) Analyze by flow cytometer

Note: the whole procedure is performed in the dark.

3.4.4. Sample acquisition

CellQuest software is used for sample acquisition and data storage.

For each sample, 15000 events are acquired. Forward scatter (FSC) and side scatter (SSC) dot plot are selected to identify the cell population. FL1 channel is selected to detect the DCF fluorescence in log scale.

The following graphs are displayed:

1° Dot Plot: Acquisition, FSC (x-axis) and SSC (y-axis)

2° Dot Plot: Acquisition, FL1 (x-axis) and FSC (y-axis)

1° Histogram: Acquisition, FL1

3.4.5. Sample analysis

The DCF fluorescence histograms are analyzed by the Flow Jo analysis program (TreeStar, OR, USA). The percentage of DCF positive cells is quantified considering a threshold fluorescence level (expressed as arbitrary units) set on the basis of the background fluorescence in the control cell population (about 10^2).

Standard Operating Procedure @ CNR- IREA

PROTOCOL	**Apoptosis detection by flow cytometer in HaCaT cells**
DATE	24/04/2025
AUTHOR(S)	Mariateresa Allocca, Anna Sannino, Valentina Peluso, Stefania Romeo, Maria Rosaria Scarfì, Olga Zeni
REVISED BY	Maria Rosaria Scarfì, Olga Zeni
APPROVED BY	Olga Zeni

1 Index

1. Purpose

This procedure describes the materials and the protocol used for flow cytometric analysis of apoptosis measurement in HaCaT cells.

2. Background

Apoptosis, the physiological process of cell death, is fine-tuned. Phosphatidylserine (PS) is a cell membrane phospholipid that translocates to the outside-facing side of the membrane when apoptosis is initiated. Annexin V, a protein binding PS with high affinity, is used to detect apoptotic cells by flow cytometry when labeled with FITC. If the staining protocol with Annexin V-FITC is

combined with propidium iodide (PI), it is also possible to screen late apoptotic and dead cells: Annexin V-FITC stained cells identify early-stage apoptotic cells, the double stained Annexin V/PI identify late-stage apoptotic cells, while PI stained ones are necrotic.

3. Procedure

3.1. Equipment

- Cell culture incubator (Thermo Scientific Forma, Model 311)
- Refrigerated centrifuge (Heraeus Sepatech Minifuge RF)
- Automated cell counter (Luna II)
- FACSCalibur flow cytometer (BD Biosciences) equipped with 488 nm laser

3.2. Materials

- 35 mm cell culture dish (Corning, cod. 430165)
- 5 mL polystyrene round bottom FACS tube (Falcon, cod. 352052)

3.3. Reagents

- DMSO (Dimethylsulphoxide, LABSCAN, cod A3534) is stored at room temperature (RT)
- DMEM (Dulbecco's Modified Eagle Medium), supplemented with 4.5 g/L glucose, sodium pyruvate and sodium bicarbonate (Microgem, cod. AL007) is stored at +4°C
- Staurosporine 1µM Product code: S6942 (Sigma)
- 1X PBS (Dulbecco's Phosphate Buffered Saline) w/o Calcium w/o Magnesium (Microgem, cod. TL1006) is stored at +4°C
- Trypsin – EDTA 1X in solution w/o Calcium w/o Magnesium w/ Phenol Red (Microgem, cod. L0930) is stored at -20°C
- Trypan blue stain, 0.4% (Logos Biosystems, cod. TB0BJC2301) is stored at room temperature (RT)
- Apoptosis Detection kit (Leinco Technologies, cod. A432) containing: i) Binding Buffer, ii) Annexin V-FITC, iii) Propidium Iodide (PI) is stored at 4°C

3.4. Experimental procedure

The Annexin V-FITC Apoptosis Detection kit is used following the manufacturer's instruction and the described procedure has been optimized for HaCaT human keratinocyte cells (CLS Lot. 300493-4820, p32). Procedure for cell maintenance is detailed in Annex 6.

$3x10^5$ cells are seeded in 3 ml complete medium in a 35 mm cell culture dish and the assay is performed after 72 h of growth.

3.4.1. Staurosporine treatment

Staurosporine 1µM for 1h is used as positive control.

3.4.2. UVB exposure

UVB (Annex 8) is used as agent for co-exposure in the 5G radiofrequency experiments (see Annex 7)

3.4.3. Sample preparation

1) Collect cell growth medium into FACS tube

2) Wash the adherent cells with PBS (1 ml) and detach by 12 min trypsin treatment (500 µl) at 37°C

3) Collect the cells by using the growth medium and add cold PBS (500 µl)

4) Centrifuge (4°C, 1200 RPM, 5 min), and remove the supernatant

5) Resuspend the cell pellet with DMEM complete medium (1 ml)

6) Count the cells by trypan blue stain

7) Transfer 300000 cells into a clean FACS tube

8) Add cold PBS (2 ml), centrifuge (4°C, 1200 RPM, 5 min) and remove the supernatant. Repeat this step once

9) Resuspend the pellet in Binding Buffer (100 µl)

10) Add Annexin V-FITC (5 µl), PI (5 µl) and mix by gentle pipetting

11) Incubate for 10 min at RT

12) Add cold PBS (400 µl) and mix by gentle pipetting

13) Analyze by flow cytometer

Note

Before starting the first experimental run, the calibration of the instrument is required and is achieved by setting up the following samples: i) unstained cells to assess the level of autofluorescence, ii) cells stained only with Annexin V-FITC and iii) cells stained only with PI to define the boundaries of each population.

- Be careful during trypsinization to avoid damaging the cells. Moreover, if trypsin EDTA is used, it is necessary to completely remove the EDTA by washing the cells twice before staining to avoid chelating the calcium needed for Annexin binding.
- Be sure that steps 9 to 13 are performed in the dark.

3.4.4. Sample acquisition

CellQuest software is used for sample acquisition and data storage.

For each sample, 15000 events are acquired. Forward scatter (FSC) and side scatter (SSC) dot plots are selected to identify the cell population. FL1 and FL2 channels are selected to detect the FITC and the PI fluorescence (log scale) respectively.

The following graphs are displayed:

1° Dot Plot: Acquisition, FSC (x-axis) and SSC (y-axis)

2° Dot Plot: Acquisition, FL1 (x-axis) and FL2 (y-axis)

1° Histogram: Acquisition, FL1

2° Histogram: Acquisition, FL2

3.4.5. Sample analysis

FlowJo software (TreeStar, OR, USA) is used for sample analysis.

Apoptotic cells are displayed in the right quadrants of the FL1/FL2 dot plot, with early apoptotic cells at the bottom (Annexin V- FITC positive) and late apoptotic cells at the top (Annexin V- FITC and PI positive). Necrotic cells (PI positive) are displayed on the top left. The percentage of cells in each quadrant is calculated by the software.

Standard Operating Procedure: Sciensano

PROTOCOL	**HaCaT cell culture conditions, and handling**
DATE	13/06/2024
AUTHOR(S)	Seppe Segers, Roel Anthonissen, Birgit Mertens
REVISED BY	Birgit Mertens
APPROVED BY	Birgit Mertens

Table of Contents

1. Objective and Purpose

This procedure describes the storage, culturing and maintenance of the HaCaT cell culture used in the laboratory of experimental toxicology.

2. Definitions et abbreviations

DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
FBS	Foetal Bovine Serum

3. Procedure

3.1. Security

Protective Clothing: Eye-shield, laboratory coat and cryogenic gloves

All manipulations involving the culturing of the cell cultures should be performed in a biosafety cabinet.

3.2. Chemicals

Amphotericin B *(Thermo Fisher Scientific)*
DMEM (Dulbecco's Modified Eagle Medium) *(Thermo Fisher Scientific)*
DMEM (Dulbecco's Modified Eagle Medium) (Microgem)
DMSO (Dimethylsulfoxide) *(Sigma-Aldrich)*
FBS (Foetal Bovine Serum) (Thermo Fisher Scientific)
Gentamycin (Thermo Fisher Scientific)
Glutamax (Thermo Fisher Scientific)
Non Essential Amino Acids (Thermo Fisher Scientific)
PBS pH 7.2 (Thermo Fisher Scientific)
Sodium Pyruvate (Thermo Fisher Scientific)
TrypLE™ (Thermo Fisher Scientific)
Trypsin-EDTA (Thermo Fisher Scientific)

3.3. Solutions

3.3.1. Complete Medium adherent cells (Micronucleus, comet assay, etc.

Add to a 500 ml DMEM bottle:

- 6 ml Gentamycin, Glutamax, Sodium Pyruvate & Non Essential Amino Acids
- 60 ml FBS (2 aliquots)
- 600 µl Amphoterecin B (1 aliquot)
- Label & store at 4°C for maximum 1 month.

3.3.2. Complete Medium adherent cells (TempO-Seq)

The HaCaT culture medium is composed by DMEM (Microgem) supplemented with 10% heat inactivated FBS, 2 mM L-glutamine, 1X Penicillin-Streptomycin solution

3.3.3. Complete Medium adherent cells (Epigenetics)

The HaCaT culture medium is composed by DMEM (Thermo Fisher Scientific) supplemented with 10% heat inactivated FBS, 2 mM L-glutamine, 1X Penicillin-Streptomycin solution and 1mM sodium pyruvate.

3.3.4. 2x freezing DMEM medium

- Add 5 ml DMSO to 45 ml of complete DMEM medium
- Keep on ice
- Prepare freshly and don't store

3.4. Protocol for Passaging Cell Lines

3.4.1. HaCaT Cell Lines

The HaCaT cell density should be maintained between 1.000.000 and 10.000.000 cells in a T75 culture flask. by counting and diluting the cells in a new culture bottle (passaging at the beginning and the end of the week).

Heathy growing cell lines usually are passaged at a cell line specific sub-cultivation ratio before becoming fully confluent, usually at the beginning and the end of the week.

- Aspirate the spent cell culture media from the 75 cm^2 culture flask with a Pasteur pipette
- Wash the cells briefly with minimum 3 ml PBS (enough to cover the cell layer) with a disposable serological pipet
- Aspirate the PBS with a new Pasteur Pipette
- Add 5ml TrypLE. Gently rock the culture flask to obtain complete coverage of the cell layer. Alternatively, trypsin-EDTA can be used, but in general, we prefer to use TrypLE over Trypsin, because we have observed slightly less cell clumping when using TrypLE.
- Incubate the culture flask for 10-15 minutes (37°C, 5% CO2).
- Check for detachment of the cells (visual or under microscope). If necessary, incubate longer (max another 5 minutes) and check every 1-2 minutes for dissociation. Tapping the flask to help cell detachment is also possible although this might cause cell clumping for some cell lines.
- Add 10 ml of pre-warmed growth medium to the detached cells and transfer the cells to a 30 ml Sterelin reservoir. Make sure that all cells are harvested (only if needed pipet over the cell layer surface several times). Use the same disposable serological pipet for this step.
- Centrifuge 5 minutes at 200 x g
- Aspirate the supernatant
- Re-suspend the cell pellet in 1 ml fresh, pre-warmth culture medium with a 1ml micropipette (pipette the suspension 25-30 times up and down). To reduce cell clumping, also use a 100

µL micropipette to pipette the cell suspension up and down repeatedly (20-30 times).

- Label one or more new culture flasks (passaging date, passage number, cell line, splitting rate) and dilute cell suspension to the appropriate volume for sub-culturing (for a total volume of 20 mL) and return the cells to the incubator.

Remark:

- When cells are used to perform a test, the same protocol is followed but cells are counted, diluted to the correct cell number and transferred to the desired well-plate or cell culture dish format.

3.4.2. Passage Number

- All cells stored in the liquid nitrogen are considered to be in passage 1.
- When cells are thawed from the cryogenic storage reservoir they go to passage 2
- Passage number increases when cells are passaged (transferred to a new 75 cm^2 culture flask)
- Cells are used up to maximum passage 18

3.4.3. Population Doubling Time

The population doubling time (PDT) for a cell line is the time taken for the cells in a culture medium to double its cell population. The PDT is specific for each cell line and can be determined experimentally. Cell density should be measured at the beginning and at the end of the incubation time of cells in the log-phase/exponential phase. PDT can be calculated using following formula.

PDT=T ln2/ln(Xe/Xb)

T = incubation time in any units.

Xb = cell number at the beginning of the incubation time.

Xe = cell number at the end of the incubation time.

A very common misuse of calculation of population doubling time (PDT) is frequently seen in many papers: number of cells at seeding time versus number of cells at harvest time. This approach does not account for the lag phase, ie. the time from seeding until the cells begin to proliferate, which span from hours to several days depending on the specific cell line or cell type. Hence, calculation will be reflected by a less steep curve resulting in falsely increased PDT.

The definition of PDT is the average time it takes a cell population to double in the log-phase/exponential phase, ie. during linear growth.

Taken together, proper calculation of PDT should be done by counting cells on say daily basis for e.g. 7 days using 6-wells, T25 or similar. Next, draw a growth curve from the data (number of cells versus time) and calculate the PDT from the linear part of the curve using this equation: (t2 - t1)/3,32 x (log n2 - log n1) where t is time and n number of cells.

3.4.4. Number of Population Doublings

The number of population doublings (PD) of a cell culture refers to the total number of times the cells in the population have doubled since a given time point (eg seeding of cells).

PD = log(Xe/Xb) / log(2)

Xb = cell number at the beginning of the incubation time.

Xe = cell number at the end of the incubation time.

3.5. Cryopreservation cell lines

3.5.1. HaCaT Cell Lines

- Prepare 2x freezing DMEM medium and store at 2-8°C until use.
- Estimate the number of cells (culture flasks) needed to make the cryopreserved cell stock (usually 30-40 cryogenic storage vials with cell density $1*10^6$ cells/ml). Label the cryogenic storage vials (cell line, date).
- Only use pre-confluent healthy cells in the log phase of growth with a passage number as low as possible.
- Detach the cells following the protocol for passaging adherent cell lines
- Collect the cells in fresh DMEM culture medium
- Count the cells with a Bürker haemocytometer
- Calculate the required volume of freezing DMEM medium (final cell density 2-5 10^6 cells/ml)
- Dilute the cells with fresh culture medium to ½ volume of the required volume of freezing DMEM medium (on ice) (cell density 4-10 10^6 cells/ml)
- Slowly add 2x ice cold freezing DMEM medium and dilute the cells to the required freezing DMEM medium volume (on ice)
- Distribute 1 ml cell suspension in each labelled cryogenic storage vials.
- Put the cells overnight in the -80°C freezer
- Put the cryogenic storage vials into a labelled cryogenic storage box and transfer to the liquid nitrogen container
- Complete the cryogenic storage container file with the storage information from the cryopreserved cells (frozen cell stock).

The cell stocks are stored in cryogenic boxes (max 25 cryogenic storage vials) which are fixed in 2 racks (5 storage boxes/rack) that are kept in the liquid nitrogen cryogenic storage container. Each vial from the same cell stock is labelled with the same coloured stopper.

The temperature (-185°C) and liquid nitrogen level (min 40%) from the liquid nitrogen is monitored continuously to assure optimal storage conditions.

3.5.2. Refill Liquid Nitrogen

When the liquid nitrogen level drops to approximately 40% the container should be refilled, usually every 6-7 weeks only on Tuesdays by an external company at the mycology service.

- The day before filling the container should be transported to the mycology service (after informing the people from this service by email).
  Contact person = Yves Bastin (or Sam Roesems in the absence of Yves)
  Yves Bastin Yves.Bastin@sciensano.be
  Sam Roesems Sam.Roesems@sciensano.be
- The transportation always needs to be performed with 2 persons. The container is placed in the elevator with the inscription "Temporary no person transportation" and is send to the -1st floor. The 2 persons take the stairs. It's not allowed to use the elevator together with the container!
- The follow-up FORM (annual calendar) for the liquid nitrogen is completed after the container is filled together with the expected next refill date.
  \\sciensano.be\fs\1442_ExpToxi_Employee\L1 Calibration Material & Products\Cell Stock Liquid Nitrogen
  The most recent new, empty FORM liquid nitrogen tank can be found at the server:
  \\\sciensano.be\fs\1442_ExpToxi_Employee\L1 Calibration Material & Products\kwaliteit labo\FORM Labo\FORM apparaten

3.5.3. Follow-up Cell Stock

Every year an up-to-date excel file for follow-up of the cell stocks in the cryogenic storage container is created.

This file contains

- A visual overview of the content of the liquid nitrogen reservoir (exact place of every cryogenic storage vial in the container)
- The colour of the stopper from each cell stock is displayed
- General information (date of purchase & storage, passage number) from every cell line present in the container is displayed at the bottom of the file

New vials or new cell stocks are added to the file.

Every vial that is taken out of the container is marked (strikethrough) and labelled (date) on the file.

3.5.4. Handling Liquid Nitrogen

Wearing a face mask and cryogenic gloves is obligatory when adding or taking out cryogenic vials into the liquid nitrogen.

Before opening the container be sure where you will add or take out cryogenic vials (check the overview file)

Work as quickly as possible!

- Take out the cryogenic stopper from the container and keep at a safe place
- Take out the correct rack
- Release the desired cryogenic storage box and take the cryogenic vial(s) or add new

vials (*)

- Immediately fix the storage box again into the rack, but the rack back in place and close the container.

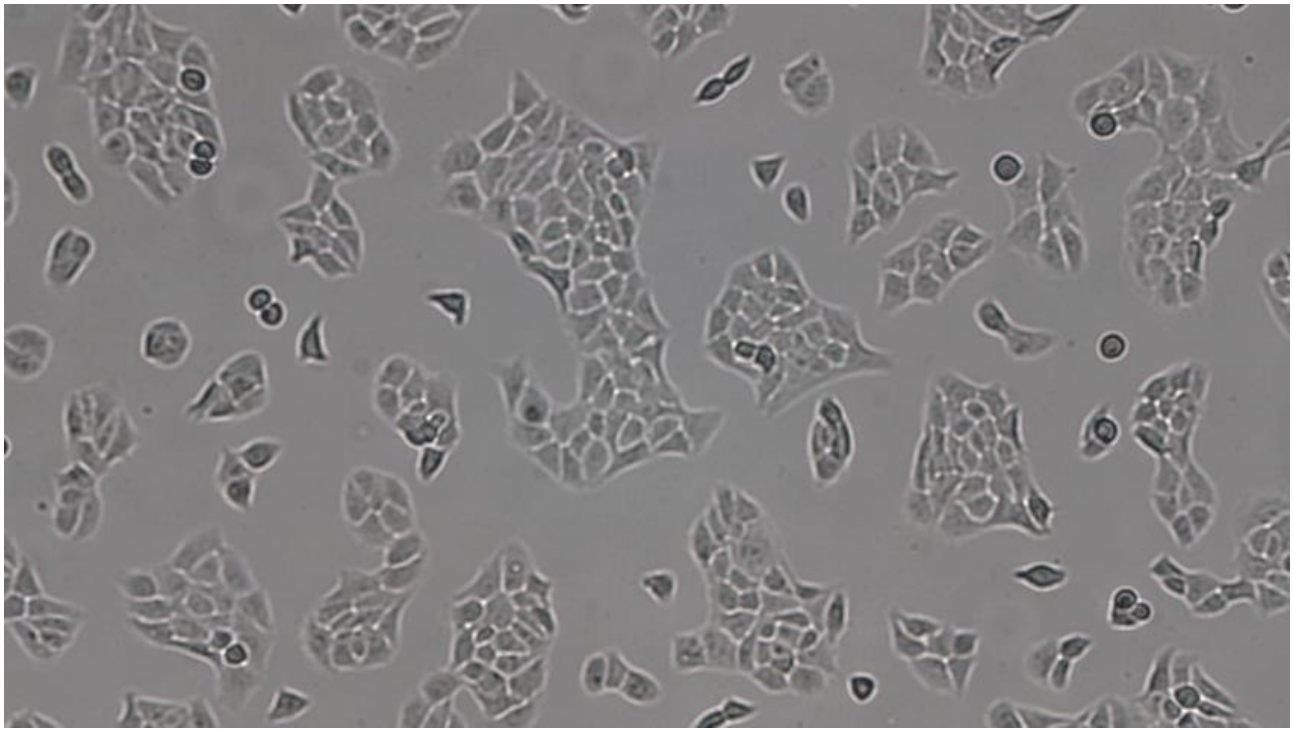
(*) When a new cell stock is added the cryogenic vials are added as quick as possible into the cryogenic box

3.6. Thawing Frozen Cells

- Take one cryogenic storage vial of the desired cell stock out of the liquid nitrogen and indicate this in the cryogenic storage container excel file
- Leave the vial at room temperature for maximum 1 minute.
- Thaw cells for maximum 5 minutes in a 37°C water bath (as quick as possible).
- Transfer the cells carefully to 20 ml freshly pre-warmed of the appropriate culture medium in a 75 cm^2 culture flask
- Optionally: After thawing the cells can be transferred to 10 ml culture medium in a sterile container and centrifuged (5 minutes, 200xg). Gently re-suspend the cell pellet with 1 ml culture medium and transfer to the culture flask.
- Incubate the cells at 37°C and CO2 5%
- Renew the culture medium preferably the day after cell culturing for adherent cell lines

Remark:

- The thawing procedure is stressful to frozen cells and should be performed rapidly.
- New purchased cells should immediately after arrival be stored into the liquid nitrogen container or thawed and cultured following the same protocol.



Standard Operating Procedure @ Sciensano

PROTOCOL	***In vitro* micronucleus test in human keratinocyte HaCaT cells and 5G RF-EMF exposure**
DATE	08/04/2024
AUTHOR(S)	Seppe Segers, Roel Anthonissen
REVISED BY	Birgit Mertens
APPROVED BY	Birgit Mertens

Index

1 Objective and purpose

This procedure describes the method for the detection and automated scoring of micronuclei in HaCaT cells. Other cell types may be used, if justified. Depending on the characteristics of the cell type, modifications to the procedure may be required.

2 Definitions et abbreviations

CytoB	Cytochalasine B
DAPI	4', 6'-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
FBS	Foetal Bovine Serum
KCl	Potassium Chloride
MMS	Methyl Methanesulfonate
PBS	Phosphate Buffered Saline
RO	reverse osmosis

3 Procedure

3.1. Security

- Protective Clothing: Gloves, safety glasses and laboratory coat.
- All manipulations before the fixation of the cells (0) should be performed in a biosafety cabinet. Later manipulations involving chemicals should be performed under a chemical hood.
- All safety measures related to working with potentially carcinogenic chemicals should be respected. Depending on the test products, additional safety measures may be required (see Security sheet).

3.2. Controls

Positive control substances:

- Positive control substances active **without metabolic activation**:
  Methyl Methanesulfonate (MMS) 150 MMS

Alternative positive control substances can be used, if justified.

Negative control substance:

- Treatment medium

3.3. HaCaT Cell Line

HaCaT is a spontaneously immortalized keratinocyte cell line from the adult human skin of a 62-year-old male. Due to their high capacity to differentiate and proliferate in vitro, these cells are extensively utilized in skin biology and differentiation research.

3.4. Chemicals

Acetic Acid (VWR, Heverlee (Leuven), Belgium or equivalent)
Amphotericin B (Thermo Fisher Scientific)
Cytochalasin B (Sigma-Aldrich)
DMEM (Dulbecco's Modified Eagle Medium) (Thermo Fisher Scientific)
DMSO (Dimethylsulfoxide) (Sigma-Aldrich)
Ethanol (denatured) (VWR, Heverlee (Leuven), Belgium or equivalent)
Paraformaldehyde
FBS (Foetal Bovine Serum) (Thermo Fisher Scientific)
Gentamycin (Thermo Fisher Scientific)
Glutamax (Thermo Fisher Scientific)
Methanol (VWR)
MMS (Sigma-Aldrich)
KCl (VWR)
Non Essential Amino Acids (Thermo Fisher Scientific)

PBS pH 7.2 (Thermo Fisher Scientific)
Sodium Pyruvate (Thermo Fisher Scientific)
TrypLETM (Thermo Fisher Scientific)
Vectashield mounting medium containing DAPI (Labconsult, Brussels, Belgium or equivalent)

4 Solutions

4.1. Medium HaCaT cells

Complete medium: Add to a 500 ml DMEM bottle:

- 6 ml Gentamycin, Glutamax, Sodium Pyruvate & Non Essential Amino Acids
- 60 ml FBS (2 aliquots)
- 600 µl Amphoterecin B (1 aliquot)
- Label & store at 4°C for maximum 1 month.

4.2. Cytochalasine B (cytoB)

Stock solution (3.33 mg/ml):

- Dissolve 5 mg cytoB in 1.5 ml DMSO. Make aliquots of 25 µl and store them at -20°C protected from light for maximum 3 years.

Working solution (3 µg/ml):

- For 12 samples (25 ml):

$$C1\ x\ V1 = C2\ x\ V2$$

$$\mathrm{V1} = \frac{\mathrm{C2\ x\ V2}}{\mathrm{C1}}$$

$$\mathrm{v1} = \frac{3\,\frac{\mu g}{ml}\,\mathrm{x}\ 25\ \mathrm{ml}}{3333\ \mu\,\frac{g}{ml}} = 22.5\ \mu\mathrm{l\ stock\ solution\ in\ 25\ ml\ medium + FBS}$$

The working solution should be prepared freshly!

4.3. KCl solution (0.075 M)

- Dissolve 559 mg KCl in 100 ml RO water. Keep in a water bath at 35°C
- *The KCl solution should be prepared freshly!*

4.4. Fixator

4.4.1. Fixator 1

- Fixator 1 (MeOH:CH_3COOH in a ratio 3:1) is prepared just before use and should be kept ice-cold (4°C) throughout the whole fixation
- For 12 samples:
  40 ml CH_3COOH + 120 ml MeOH

  Keep at 4 °C

4.4.2. Fixator 2

- Fixator 2 (MeOH:CH_3COOH in a ratio 9:1) is prepared just before use and should be kept ice-cold (4°C) throughout the whole fixation
- For 12 samples:
  10 ml CH_3COOH + 90 ml MeOH

  Keep at 4 °C

4.5. Propidium Iodide

Stock solution (1 mg/ml)

- Prepare a 1 mg/ml Propidium Iodide solution in RO water and store at 4°C protected from light for maximum 1 year

Working solution (1.5 µg/ml):.

- Dilute the Propidium Iodide stock solution to 1.5 µg/ml with PBS
- The working solution should be prepared freshly
  For example (10 samples): 10 µl stock solution + 990 µl PBS = 10 µg/ml
  100 10 µg/ml + 900 µl PBS = 1.5 µg/ml

4.6. Positive Controls

4.6.1. MMS (200 µM)

Stock solution (184,6 mM):

- The solution should be prepared freshly
- Add 15.4 µl MMS to 984.6 µl of culture medium.

Intermediate solution (10 mM):

- Add 54,17 µl MMS stock solution to 945,83 µl of culture medium.

Working solution (422 µM):

- Dilute 372 µl MMS intermediate solution to 8800 µl with culture medium.
- When 1,1 mL working solution is added to a dish with 2 mL medium, the end concentration will be 150 µM.

5. Experimental procedure

Experiments are performed both in presence and absence of an exogenous metabolising system (i.e. S9 metabolic fraction) in order to detect also compounds requiring metabolic activation.

5.1. Slide cleaning (optional)

- Put slides overnight (or longer) in an acetic acid solution (10%).
- Rinse them in RO water.
- Move them to another jar containing denaturated alcohol for at least one night.
- Dry the slides by polishing them with a medical towel. They are now ready for use.

5.2. Seeding of cells

- Seed 2 ml of a cell suspension containing 200 000 cells/ml in a 35 mm cell culture dish (Nunc, Nunclon™ Surface or equivalent).
- Incubate cells for 24 hours in the incubator (37°C, 5% CO_2).

5.3. Exposure (24 hours after seeding)

5.3.1. Chemicals (positive control substance MMS)

- Add cell medium containing the test substance, the positive control substance (positive control) or the solvent (negative control)
- Incubate the cells for the required time (37°C, 5% CO_2)
  ➔ 24 hours

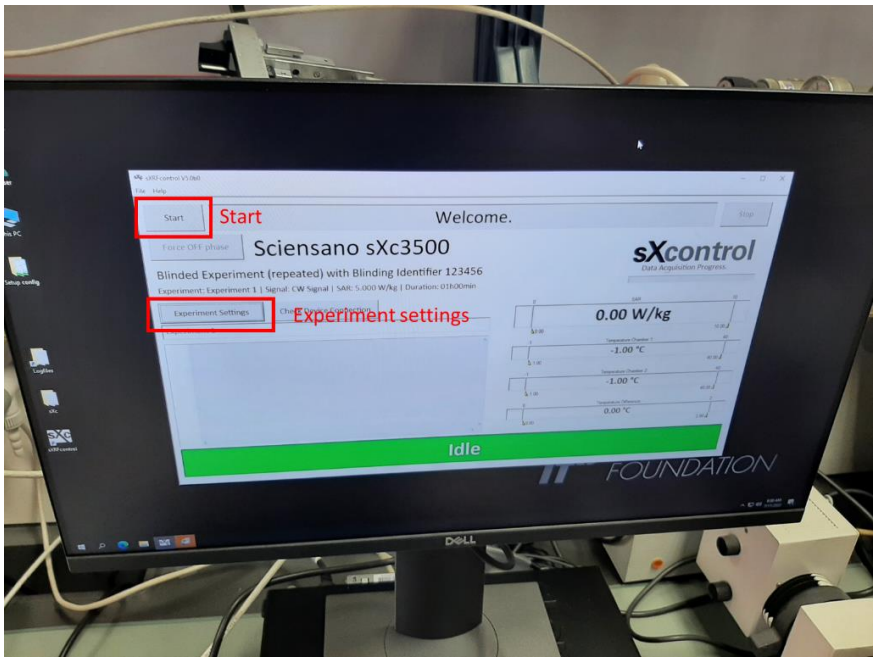
- Remove the treatment medium.
- Rinse with PBS (only for the experiments with S9).
- Add 2 ml of cytoB working solution (3 µg/ml) to each well.
- Incubate for 24 hours (37°C, 5% CO_2).

5.3.2. RF-EMF

- Add cell medium containing the positive control substance (MMS, positive control) or the solvent (negative control).
  Remark:

1. 4 dishes should always be used for the negative control (2 per exposure chamber) and four for the positive control (two per exposure chamber) to ensure the correct amount of samples to achieve statistical significance. Additionally, an incubator control can be added, including additional negative and positive control samples

- Place the cells inside the 5G exposure system (inside the sample holder) and incubate the cells for the required time (37°C, 5% CO_2)



  - For a more in depth description on how to set up the exposure system, see the "sXc 3500 exposure system" guidelines
  - In the "experiment settings" tab, adjustments can be made including SAR, exposure duration and the blinding ID of the experiment
  - Pressing start will enable the 5G EMF to flow inside the exposure chambers and pressing pause will temporarily disable this. It can be enabled again by pressing "start" again.
  - Experiments should always be performed blind, this means that only one of the chambers will be exposed at the same time. This can be decoded by sending the blinding ID to the people at IT'IS (sXc@itis.swiss). This means that the number of samples (2 Negative and 2 Positive) put inside the two exposure chambers should always be identical, since it is unknown which one of the chambers will be exposed beforehand.

5.4. Cytochalasin B treatment

- After the exposure finishes, remove the treatment medium.
- Rinse with PBS.
- Add 2 ml of cytoB working solution (3 µg/ml) to each well.
- Incubate for 24 hours (37°C, 5% CO_2).

- Remove the culture medium with cytoB.
- Add 500 µl of TrypLE to each well.
- Incubate for 10-15 minutes at 37°C. Add 1000 ml of culture medium to each well to block the trypsinisation.
- Add the cell suspension to a 15 ml conical glass centrifuge tube.
- Rinse with 1000mL culture medium to get any remaining cells and add it to the respective glass centrifuge tube.

5.5. Fixation & Slide Preparation

Remark: Prepare the KCl solution (35°C) and the ice-cold fixators 1 and 2 well in advance!

- Remove the culture medium with cytoB.
- Add 500 µl of TrypLE to each well.
- Incubate for 10-15 minutes at 37°C. Add 1000 ml of culture medium to each well to block the trypsinisation.
- Add the cell suspension to a 15 ml conical glass centrifuge tube.
- Rinse with 1000mL culture medium to get any remaining cells and add it to the respective glass centrifuge tube.
- Centrifuge 5 min at 102xg (= 700 rpm)
- Aspirate the supernatant (leave enough supernatant to homogenize the cell pellet).
- Homogenize the cell pellet by tapping (or gently vortexing)
- Slowly add 1 ml of 0.075 M KCl (at 35°C) (drop by drop) while gently vortexing cells (starting with tube nr°1).
- Leave 10 min at room temperature (no longer!!!).
- Slowly add 1 ml (freshly prepared!) ice-cold fixator 1 while gently vortexing cells (start again with tube nr°1).
- Add 1-3 drops of paraformaldehyde to each glass tube for better preservation of the cytoplasm (optional)
- Leave 10 min at room temperature.
- Centrifuge 5 min at 102xg (=700rpm).
- Aspirate the supernatant (leave enough supernatant to homogenize the cell pellet).
- Gently homogenize the cell pellet by vortexing lightly.
- Gently add 1 ml of ice cold fixator 1 in drops by using a pasteur pipette
- Gently add 4 ml of ice-cold fixator 1.
- Centrifuge during 5 min at 102xg (=700rpm).
- Aspirate the supernatant (leave enough supernatant to homogenize the cell pellet).
- Homogenize the cell pellet by tapping (not vortexing).
- Gently add 4 ml of ice-cold fixator 2.

- Centrifuge during 5 min at 102xg (=700rpm).
- Aspirate the supernatant but leave enough fixator (+- 1mL) to obtain an adequate number of cells in suspension for cell spreading.
- Homogenize the cell pellet (gently vortexing).
- Add 2-3 drops of the cell suspension on dry, pre-cleaned slides. Make sure cells are equally spread throughout the slide. Prepare at least 5 slides for each condition (to have +- 5000 cells to analyse).
- Mark the slides with pencil. The following parameters must be marked on the slide:

  Dosimetry and position in the waveguide, absence or presence of positive control subtance and slide number

  Optional: Date, the study number, the experiment number

- Dry slides overnight.

5.6. DAPI staining for MN scoring

- Add 2 drops of Vectashield mounting medium containing DAPI on each slide.
- Put cover (24x50 mm).
- Leave for at least 15 min before starting microscopic analysis of micronuclei.

5.7. Propidium/DAPI staining for CBPI analysis (one slide per condition)

- Pipette 100 µl of the Propidium Iodide working solution on each slide
- Put cover (24x50)
- Leave for 10 minutes in the dark
- Remove cover and rinse with RO water in a 50 ml reservoir
- Rinse a second time with RO water in another 50 ml reservoir
- Air Dry slides for at least 1 hour in the dark
- Add two drops of Vectashield mounting medium containing DAPI on each slide
- Put cover (24x50)
- Leave at least 15 minutes before starting microscopic CBPI analysis (see 3.7.4)

Remarks:

1) Staining is done in the laminar flow
2) If DAPI stained slides are used for PI staining, remove coverslip after microscopic analysis of slides for detection of micronuclei and wash briefly with RO water.

6. Microscopic analysis of slides – detection of micronuclei in binucleated cells

Remark: Switch on the fluorescent lamp at least 15 minutes before analysis.

IMPORTANT: Life span of lamp is limited (300-400 hours).

Preferably use the microscope (lamp) for a continuous longer period instead of multiple smaller periods. Make sure the lamp is switched off after automatic analysis (choose "Shut Down" at "search end" or "continue" if visual analysis of MN is performed immediately).

6.1. Identification of the slides (SET UP)

- Fix slides in a frame:
- Max 8 slides in one frame (see figure 1). The slide in the right hand position is slide No1.
- Up to 10 frames can be used (80 slides).
- If more than 1 frame is analyzed, frames need to be placed in the slide-feeder (frame NR 1 in position 1 etc….).

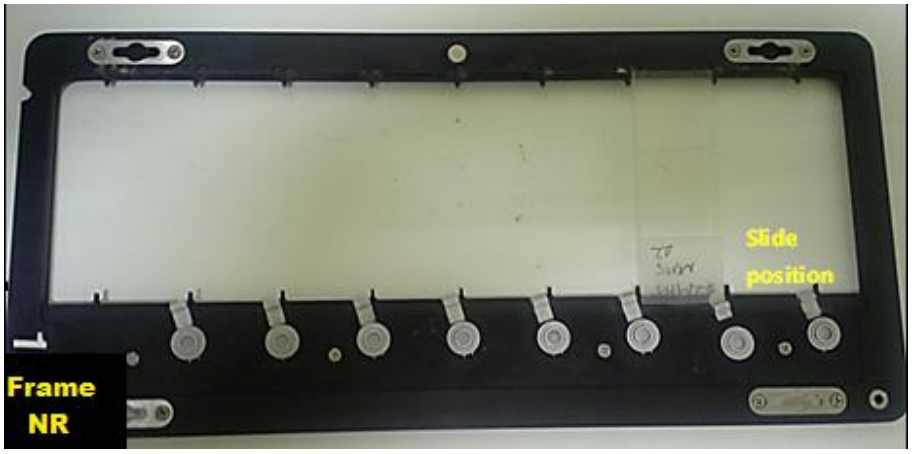
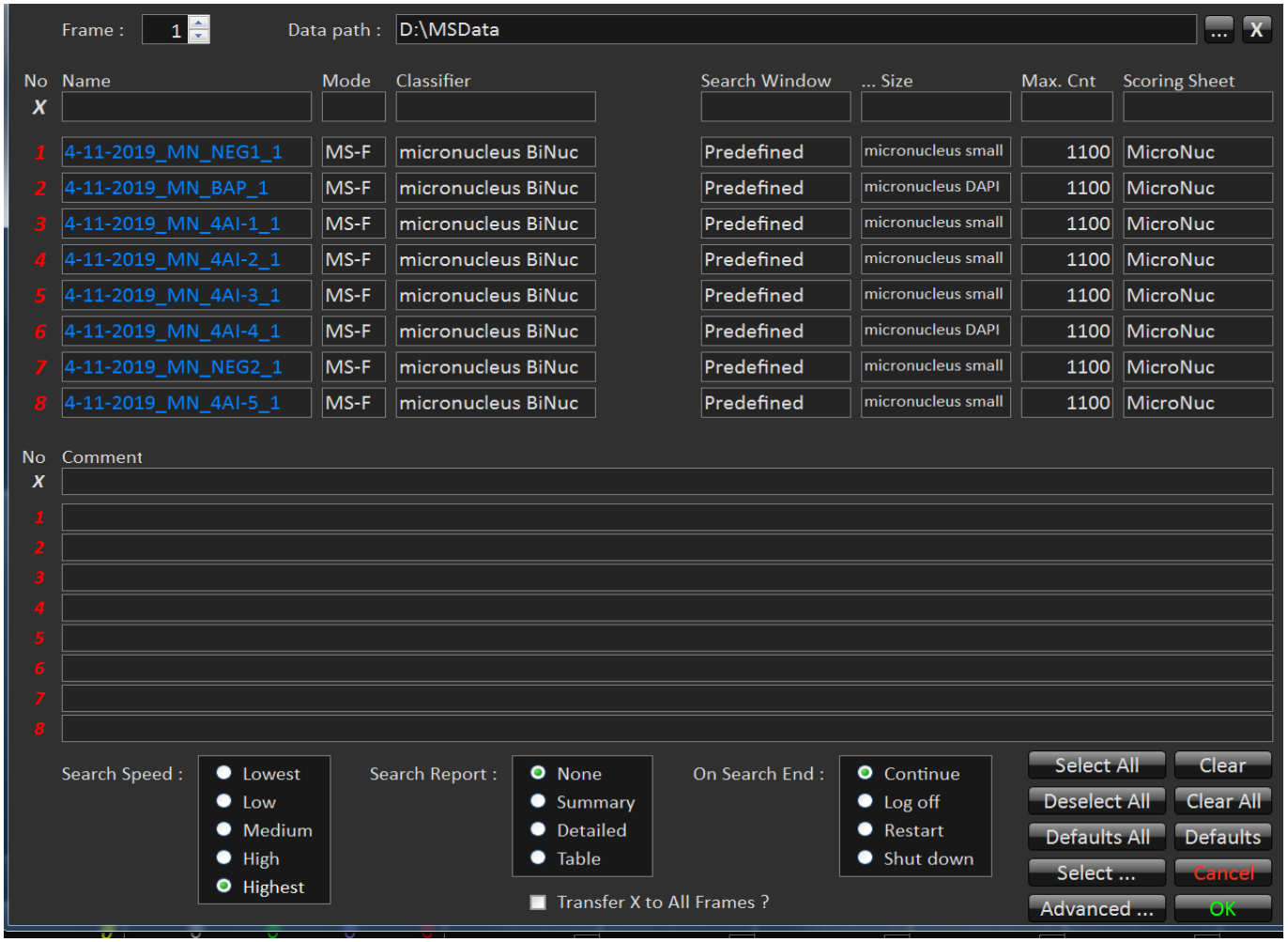


Figure 1: Slide holder

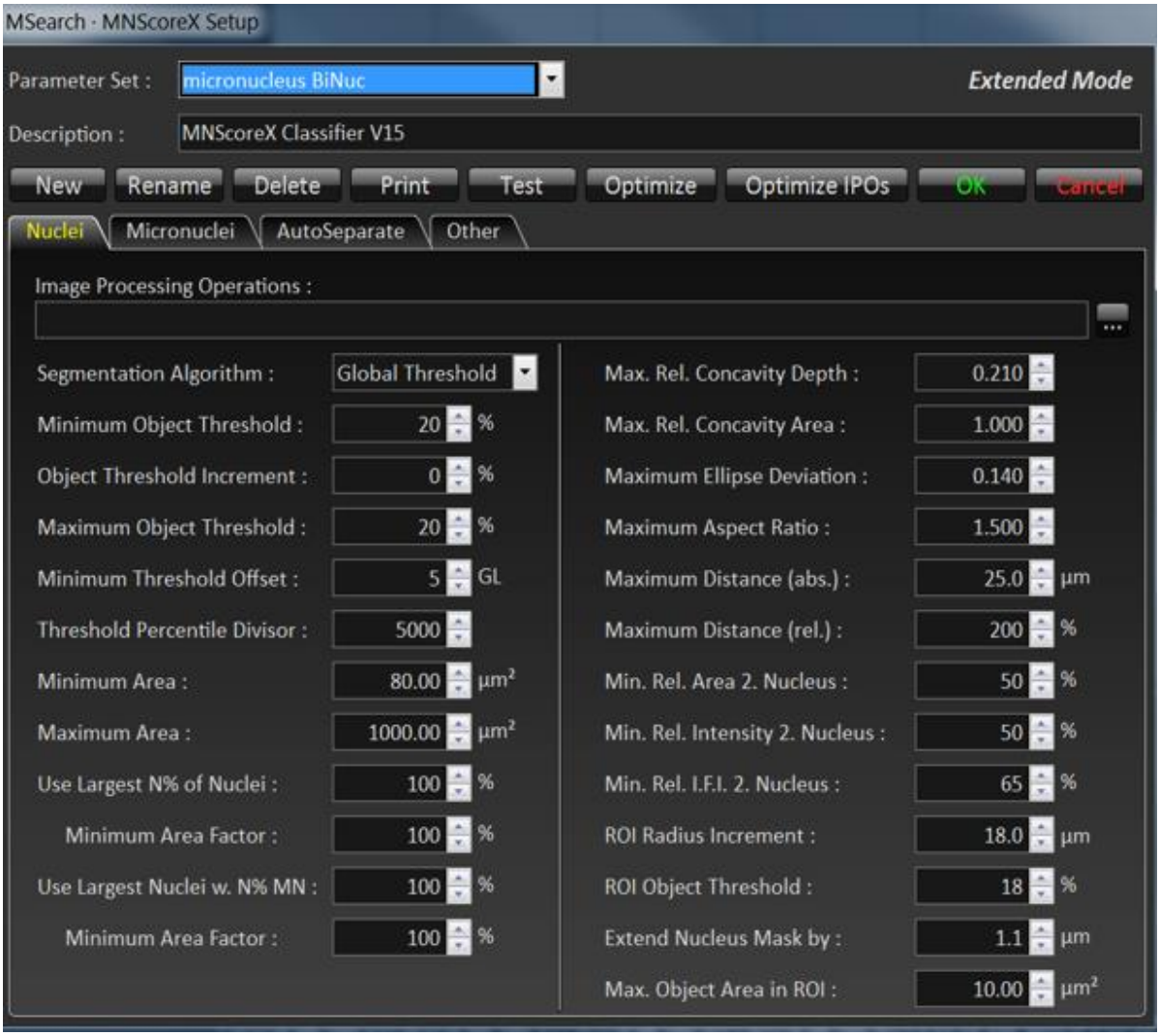
- Open computer
- Open METAFER4 software (double click METAFER4 icon on desktop)
- Open SET UP page
- Choose:
  1) Frame number (1 to 10) (if frame 1 is used also frame 1 should be selected in the SET UP menu)

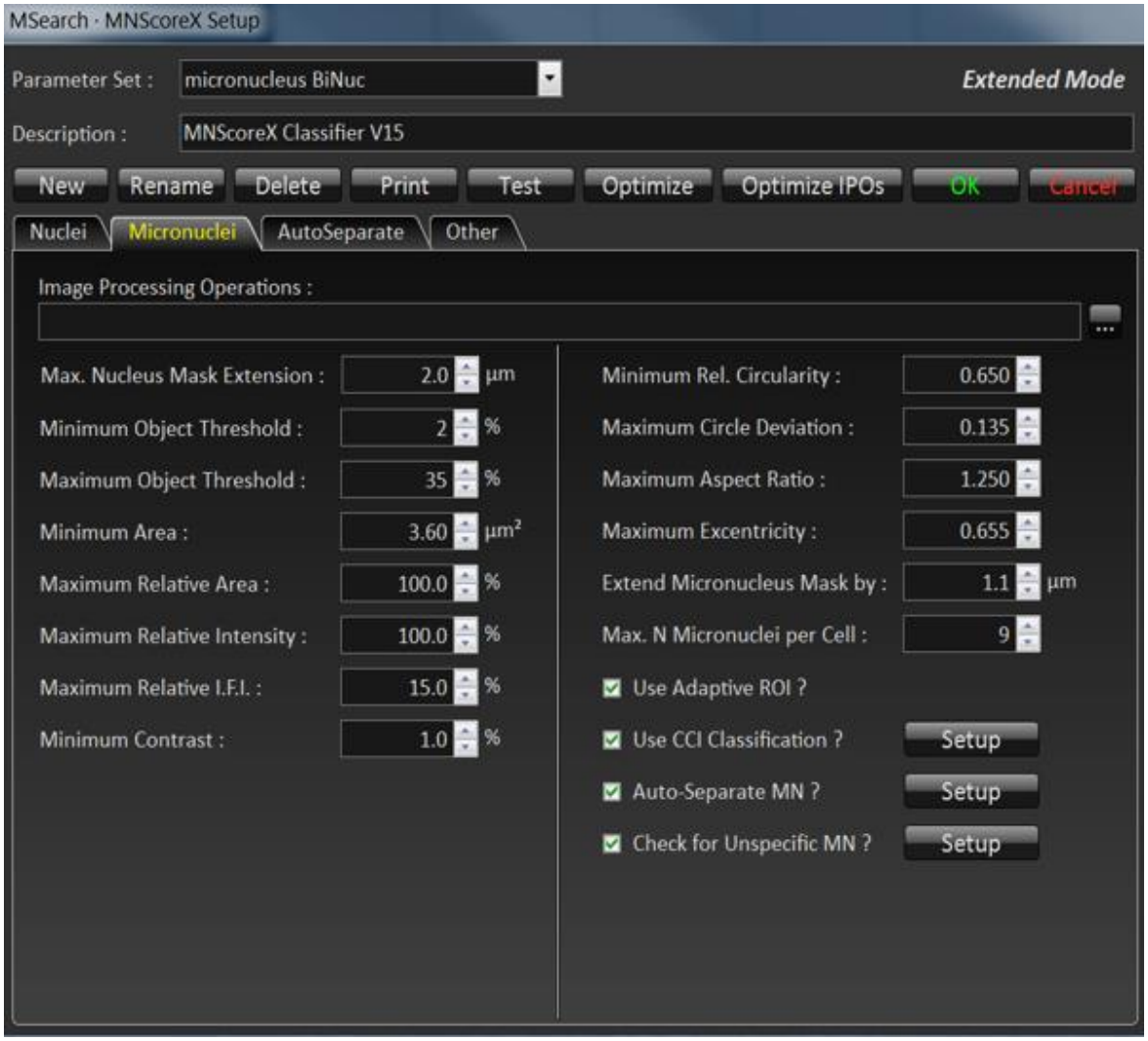
2) Highlight slides that need to be analysed (1 to 8) in red
3) Identify the slides as follows:
   XX-YY-ZZZZ_MN_A_B
   X = day / Y = month / Z = year / A = sample name/ B = slide number
4) Mode MS-F (MSearchFL)
5) Classifier: micronucleus Bi-Nuc
6) Search Window = Predefined
7) …Size
   Micronucleus DAPI or Micronucleus small (smaller window)
8) Max Count
   10000
9) Scoring Sheet: MicrNuc
10) Search Speed: Highest
11) Search Report: None
12) On Search End: Continue OR Shut Down (for an overnight analysis)

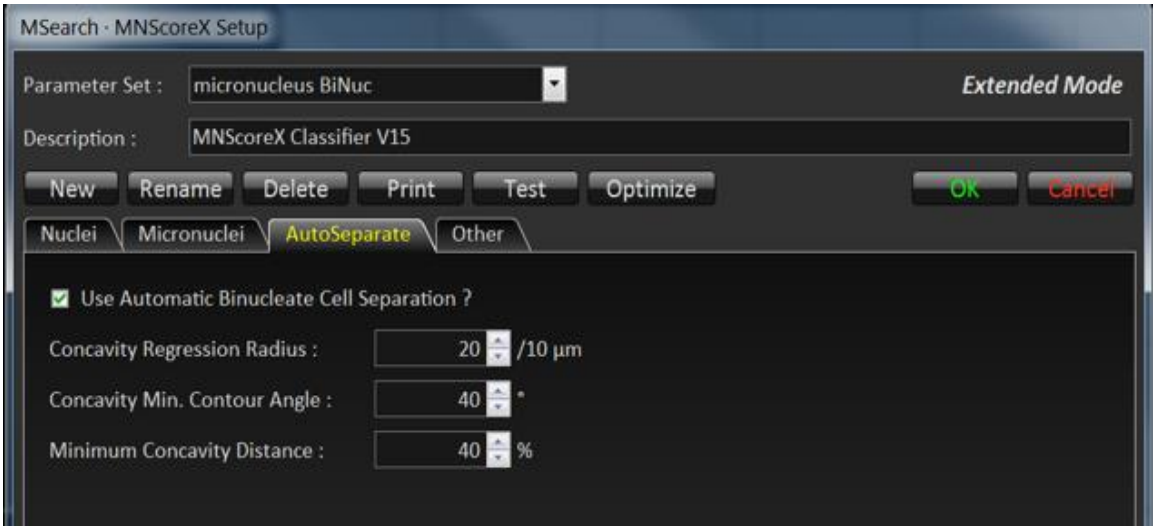
- Click OK

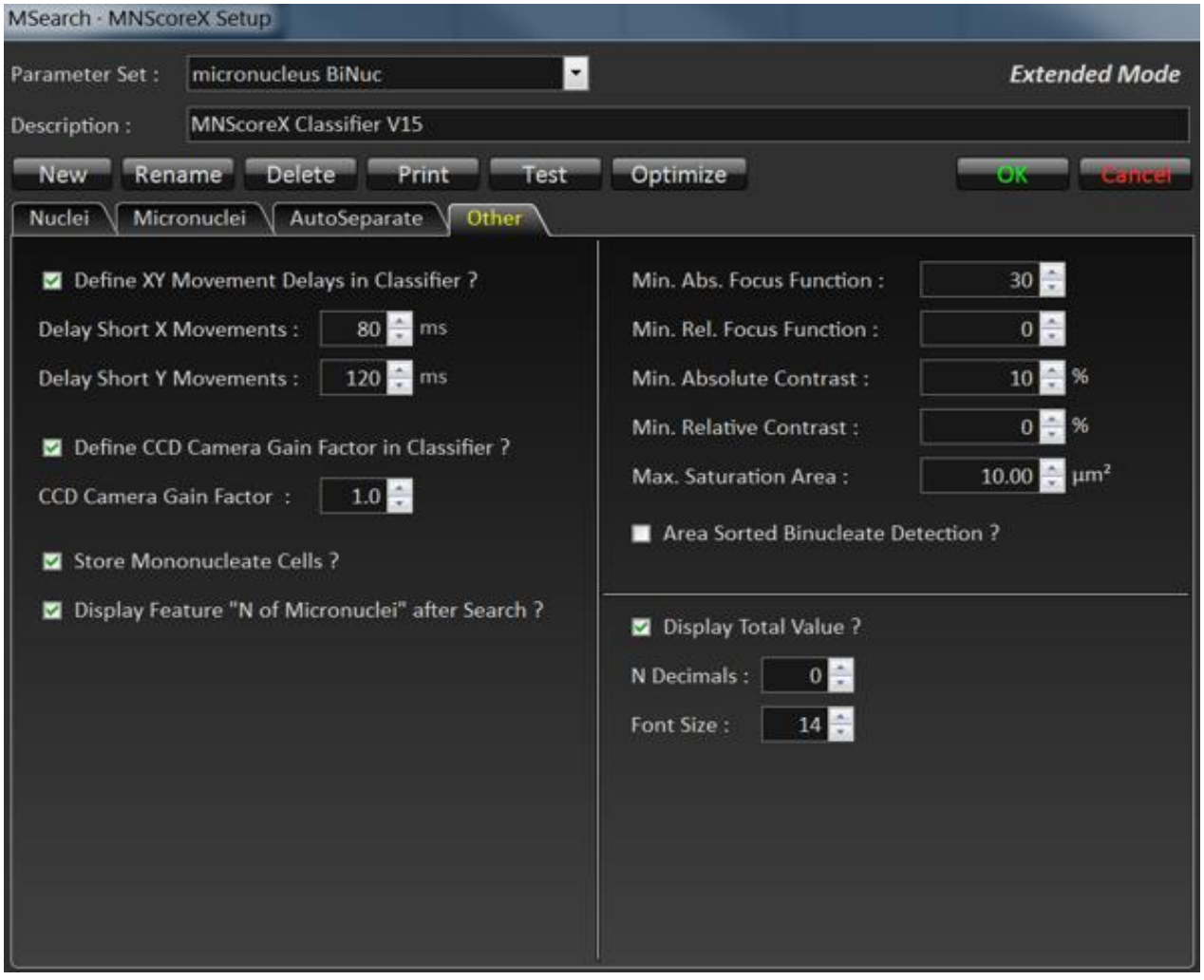


An overview of the parameters of the classifier micronucleus-BiNuc is provided below. These parameters are automatically applied and should not be further adjusted.


MSearch · MNScoreX Setup
Parameter Set : micronucleus BiNuc
Extended Mode
Description : MNScoreX Classifier V15
New
Rename
Delete
Print
Test
Optimize
Optimize IPOs
OK
Cancel
Nuclei
Micronuclei
AutoSeparate
Other
Image Processing Operations :
Segmentation Algorithm : Global Threshold
Minimum Object Threshold : 20 %
Object Threshold Increment : 0 %
Maximum Object Threshold : 20 %
Minimum Threshold Offset : 5 GL
Threshold Percentile Divisor : 5000
Minimum Area : 80.00 µm²
Maximum Area : 1000.00 µm²
Use Largest N% of Nuclei : 100 %
Minimum Area Factor : 100 %
Use Largest Nuclei w. N% MN : 100 %
Minimum Area Factor : 100 %
Max. Rel. Concavity Depth : 0.210
Max. Rel. Concavity Area : 1.000
Maximum Ellipse Deviation : 0.140
Maximum Aspect Ratio : 1.500
Maximum Distance (abs.) : 25.0 µm
Maximum Distance (rel.) : 200 %
Min. Rel. Area 2. Nucleus : 50 %
Min. Rel. Intensity 2. Nucleus : 50 %
Min. Rel. I.F.I. 2. Nucleus : 65 %
ROI Radius Increment : 18.0 µm
ROI Object Threshold : 18 %
Extend Nucleus Mask by : 1.1 µm
Max. Object Area in ROI : 10.00 µm²

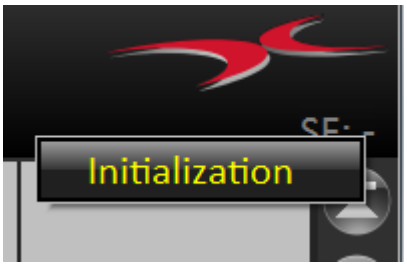

MSearch - MNScoreX Setup
Parameter Set :
micronucleus BiNuc
Extended Mode
Description :
MNScoreX Classifier V15
New
Rename
Delete
Print
Test
Optimize
Optimize IPOs
OK
Cancel
Nuclei
Micronuclei
AutoSeparate
Other
Image Processing Operations :
Max. Nucleus Mask Extension : 2.0 µm
Minimum Object Threshold : 2 %
Maximum Object Threshold : 35 %
Minimum Area : 3.60 µm²
Maximum Relative Area : 100.0 %
Maximum Relative Intensity : 100.0 %
Maximum Relative I.F.I. : 15.0 %
Minimum Contrast : 1.0 %
Minimum Rel. Circularity : 0.650
Maximum Circle Deviation : 0.135
Maximum Aspect Ratio : 1.250
Maximum Excentricity : 0.655
Extend Micronucleus Mask by : 1.1 µm
Max. N Micronuclei per Cell : 9
Use Adaptive ROI ?
Use CCI Classification ?
Setup
Auto-Separate MN ?
Setup
Check for Unspecific MN ?
Setup


MSearch · MNScoreX Setup
Parameter Set :
micronucleus BiNuc
Extended Mode
Description :
MNScoreX Classifier V15
New
Rename
Delete
Print
Test
Optimize
OK
Cancel
Nuclei
Micronuclei
AutoSeparate
Other
Use Automatic Binucleate Cell Separation ?
Concavity Regression Radius :
20
/10 µm
Concavity Min. Contour Angle :
40
°
Minimum Concavity Distance :
40
%


MSearch · MNScoreX Setup
Parameter Set :
micronucleus BiNuc
Extended Mode
Description :
MNScoreX Classifier V15
New
Rename
Delete
Print
Test
Optimize
OK
Cancel
Nuclei
Micronuclei
AutoSeparate
Other
Define XY Movement Delays in Classifier ?
Delay Short X Movements :
80
ms
Delay Short Y Movements :
120
ms
Define CCD Camera Gain Factor in Classifier ?
CCD Camera Gain Factor :
1.0
Store Mononucleate Cells ?
Display Feature "N of Micronuclei" after Search ?
Min. Abs. Focus Function :
30
Min. Rel. Focus Function :
0
Min. Absolute Contrast :
10
%
Min. Relative Contrast :
0
%
Max. Saturation Area :
10.00
µm²
Area Sorted Binucleate Detection ?
Display Total Value ?
N Decimals :
0
Font Size :
14

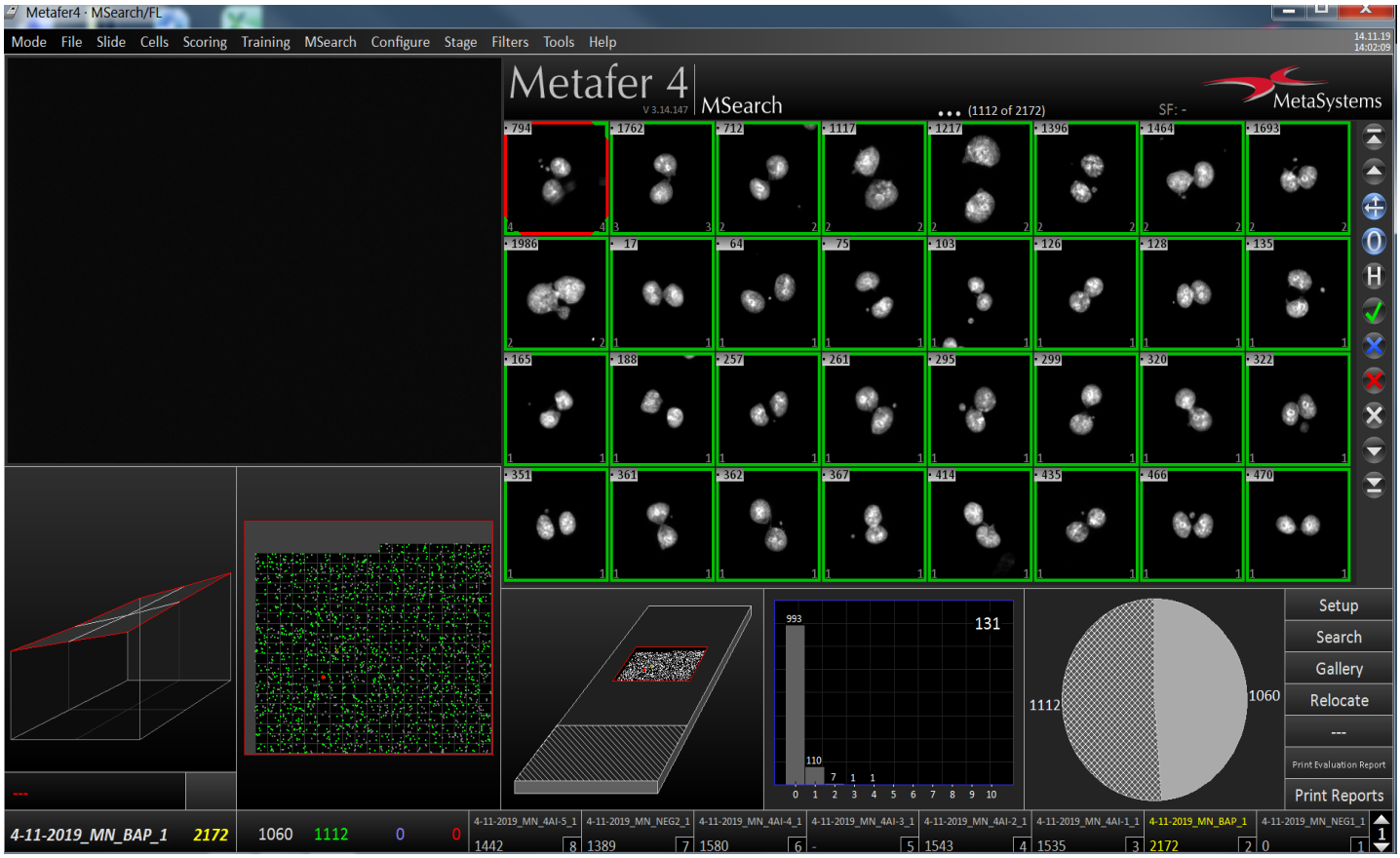
6.2. Analysis (SEARCH)

Remark: If the slide feeder is used, right mouse click on the SF icon to initialize the slide feeder (= initialization).



When only one frame is analysed, put this frame immediately on the slide holder of the microscope (no need to use the slide feeder).

- Click on SEARCH. The first slide from the identified slides in the SET UP menu is automatically selected.
- Focus microscope on a field that contains stained nuclei of binucleated cells representative for the slide and free from unusually bright artifacts or impurities.
- Make sure the camera is open for screen view (screen view/microscope view switch).
- Click OK.
- Automated analysis of all identified slides is performed. Preset number of images (usually 1000 binucleated cells/slide) will be automatically captured per slide and stored into a gallery. For each condition, at least 2 slides are scored in order to obtain min 2000 binucleated cells.
- In the event of an equivocal result, analysis may be extended up to 5000 binucleated cells.
- Mono-nucleated cells from the same field are captured and stored at the same time. Ratio mono/bi nucleated is presented in a pie chart
- Binucleated cells with 0,1,2,3…10 micronuclei are presented in a bar graph



6.3. Visual control of captured MN

After automated analysis, the detected micronuclei are analyzed visually (choose manually Objective 40x and DAPI filter on the microscope) in order to evaluate whether they fulfill the following criteria:

1) Micronuclei are morphologically identical to the main nuclei but they are smaller The diameter of micronuclei varies between 1/16 and 1/3 of the mean diameter of the main nuclei
2) Micronuclei are not linked or connected to the main nuclei.
3) Micronuclei usually have the same staining intensity as the main nuclei but occasionally staining may be more intense (with very small micronuclei) or paler (with larger micronuclei).
4) Micronuclei may touch but not overlap the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary.

remark:
leave slides in the slide holder for visual check! When clicking on a cell from the gallery, the cell will be displayed in the middle of the field visible through the microscope.

6.4. Evaluation of the Cytokinesis-Block Proliferation Index (CBPI)

The Cytokinesis-Block Proliferation Index (CBPI) is determined in order to evaluate cytotoxicity based on the number of mononucleate, binucleate and multinucleate cells.

- Choose manually Objective 20x and DAPI/FITC/Texas Red filter on the microscope.
- Count manually the number of mononucleate, binucleate and multinucleate cells on the acridine orange stained slides (total of 500 cells).
- Calculate CPBI by applying the following formula:

$$CPBI = (M1 + 2M2 + 3Mn)/N$$

with

M1 = number of mononucleated cells

M2 = number of binucleated cells

Mn = number of multinucleated cells

N = total number of cells scored

7. Evaluation of the results

7.1. Study report

For each *in vitro* micronucleus experiment a new MN template (Study Report) should be filled in.

This template contains:

1) Study parameters
2) Exposure Information
3) Results & statistics – one tab for each test item

Remark:

1) Statistical analysis using a Fisher's exact test and chi-square test is separately performed in GraphPad
2) Only the most recent Template should be used.
   \\sciensano.be\fs\1442_ExpToxi_Employee\L1 Calibration Material & Products\kwaliteit labo\Templates Labo\MN

7.2. Historical Data

Negative and positive control values should be added to the historical data file in order to build historical control ranges.

\\sciensano.be\fs\1442_ExpToxi_Employee\L1 Calibration Material & Products\kwaliteit labo\Historische Data negatieve & positive controles\MICRONUCLEUS

7.3. Criteria for a positive and negative result

When evaluating the results, the following criteria should be considered:

- The test is regarded as clearly negative if there is no increase of either statistical or biological significance in the number of micronuclei at any of the concentrations tested compared with concurrent vehicle controls.
- The test is regarded as clearly positive if there is an increase in the number of micronuclei that is of statistical and biological significance and that clearly demonstrates a concentration dependent trend.
- If an increase does not fulfill all criteria for a positive result or in case of an equivocal result scoring additional cells (where appropriate) or performing a repeat experiment possibly using modified experimental conditions (e.g. concentration spacing) could be useful. Importantly, biological relevance has to remain the primary consideration.
- In rare cases, even after further investigations, the data set will not allow a conclusion of positive or negative, and will therefore be concluded as equivocal

8. Norms and references

- Fenech M. 2007. Cytokinesis-block micronucleus cytome assay. Nat Protoc. 2(5):1084-104.
- OECD. 2014. OECD TG487: In vitro Mammalian Cell Micronucleus Test
- Doherty et al. 2016. The in vitro micronucleus assay. In Genetic toxicology testing : a laboratory manual.

Standard Operating Procedure @ Sciensano

PROTOCOL	***In vitro* comet assay in human keratinocyte HaCaT cells and 5G RF-EMF exposure**
DATE	08/04/2024
AUTHOR(S)	Seppe Segers, Roel Anthonissen
REVISED BY	Birgit Mertens
APPROVED BY	Birgit Mertens

Index

1. Objective and purpose

This procedure describes the method for the automated quantitative detection of DNA damage through double strand breaks with the comet assay (single cell electrophoresis assay) in HaCaT cells.

2. Definitions et abbreviations

DMEM = Dulbecco's Modified Eagle Medium
DMSO = Dimethylsulfoxide
FBS = Foetal Bovine Serum
EMS = Ethyl methanesulfonate
PBS = Phosphate Buffered Saline
RO = reverse osmosis

3. Procedure

3.1. Security

Protective clothing:

- Gloves and laboratory coat.
- All manipulations before the collection of the cells should be performed in a biosafety cabinet. Manipulations involving volatile chemicals should be performed under a chemical hood.
- All safety measures related to working with potentially carcinogenic chemicals should be respected. Depending on the test products, additional safety measures may be required (see Security sheet).

3.2. HaCaT Cell Line

HaCaT is a spontaneously immortalized keratinocyte cell line from the adult human skin of a 62-year-old male. Due to their high capacity to differentiate and proliferate in vitro, these cells are extensively utilized in skin biology and differentiation research.

3.3. Controls

3.3.1. Positive control substances

Positive control substances: Ethyl Methane Sulfonate (EMS) 0.2 mM
Alternative positive control substances can be used, if justified.

3.3.2. Negative control substance:

- Treatment medium containing only the solvent for test without S9
- Treatment medium without FBS, 1% S9 and the solvent for test with S9.

3.4. Chemicals

Agarose LMP (*Thermo Fisher Scientific, Gent, Belgium or equivalent)*
Agarose NMP (*Thermo Fisher Scientific, Gent, Belgium or equivalent)*
Amphotericin B (Fungizone) *(Thermo Fisher Scientific)*
HaCaT cell line (LGC Standards, Molsheim, France)
DMEM (Dulbecco's Modified Eagle Medium) *(Thermo Fisher Scientific)*
DMSO (Dimethylsulfoxide) *(Sigma-Aldrich)*
EDTA (Sigma-aldrich)
Ethanol (VWR, Heverlee (Leuven), Belgium or equivalent)
Ethyl Methane Sulfonate (Sigma-Aldrich)

FBS (Foetal Bovine Serum) (Thermo Fisher Scientific)
Gentamycin (Thermo Fisher Scientific)
Gel Red 10000X (VWR)
Glutamax (Thermo Fisher Scientific)
Hydrochloric acid 1N (VWR)
Hydrochloric acid 37% (VWR)
Non Essential Amino Acids (Thermo Fisher Scientific)
PBS pH 7.2 (Thermo Fisher Scientific)
Sodium Chloride (VWR)
Sodium Hydroxide 1N (VWR)
Sodium Hydroxide Pellets (VWR)
Sodium Pyruvate (Thermo Fisher Scientific)
Tris-base (VWR)
Triton X-100 (Sigma-Aldrich)
Trypsine-EDTA (Thermo Fisher Scientific)
TrypLE™ (Thermo Fisher Scientific)
Vectashield mounting medium (VWR)

1.1. Solutions

1.1.1. Medium HaCaT cells

Complete medium:

Add to a 500 ml DMEM bottle:

- 6 ml Gentamycin, Glutamax, Sodium Pyruvate & Non Essential Amino Acids
- 60 ml FBS (2 aliquots)
- 600 µl Amphoterecin B (Fungizone) (1 aliquot)
- Label & store at 4°C for maximum 1 month.

1.1.2. Agarose LMP 0.8%

- Weigh 0,8 g Low Melting Point (LMP) agarose
- Add 100 ml phosphate-buffered saline (PBS)
- Melt in microwave
- Prepare 10 ml aliquots, label and store at room temperature for 1 year

1.1.3. Agarose NMP 1%

- Weigh 2 g Normal Melting Point (NMP) agarose
- Add 200 ml RO water
- Melt in microwave
- This solution is used to immediately coat super frosted slides

1.1.4. Lysing Stock Solution

Prepare at least one day before use.

Dissolve in 600 ml of H2O:

- 146,1 g NaCl = 2,5 M *
- 37,2 g EDTA (Titriplex) = 100 mM *
- 1,2 g TRIS = 10 mM *
- Add 15-20 NaOH pellets
- Adjust to pH 10 by adding NaOH pellets
  If necessary adjust to pH 10 with HCl 1N or NaOH 1N
- Add Water to 890 ml
- Label and store at 4°C for maximum 1 month

* = final concentration in lysing working solution

3.5.5. Lysing working solution

Prepare fresh & ice cold before trypsinisation of cells

100 ml Lysing working solution (max 10slides) contains:

- 89 ml ice cold lysing stock solution Add 10 ml DMSO
- Add 1 ml Triton X-100
- Mix
- Transfer to a 10 slide jar, put immediately in the fridge

Prepare more lysing working solution if more than 10 slides are analyzed (maximum 24 slides in one test), mix and transfer the homogenized solution to 2 or 3 10-slide jars

3.5.6. NaOH 10N for Denaturation/electrophoresis buffer

- Dissolve 100 g NaOH (pellets) in 250 ml H2O
- Label and store at room temperature for maximum 3 months

3.5.7. EDTA 200mM for Denaturation/electrophoresis buffer

- Dissolve 7,44 g EDTA in 100 ml H
- Label and store at room temperature for maximum 1 month

3.5.8. Neutralisation buffer pH 7.5

- Dissolve 48,5 g TRIS-base in 800 ml RO water
- Adjust to pH 7,5 with HCl 37%
- Add RO water to 1000 ml
- Label and store maximum one year at room temperature.

3.5.9. GelRed 3X staining solution

- Add 15 µl GelRed 10000X stock to 50 ml RO water
- Label and store at room temperature (important) in the dark for max 2 year

3.5.10. Positive control solutions

3.5.10.1. EMS 0.2 mM

Intermediate solution (4mM):

- Add **10.3 µl** EMS to **25 ml** of culture medium

Working solution (0.563 mM):

- Add 1.408 ml intermediate solution to 8.592 ml cell culture medium.
- When added to 2 mL cell culture medium, the final concentration will be 200 µM

The intermediate and working solution should be prepared freshly.

3.6. Experimental procedure

3.6.1. Pre-coating of Slides

Only Super Frosted Slides that are pre-coated at least 3 months before use are suited to perform the comet assay.

Coat new slides when only 10 boxes (50 slides/box) are left

- Label top side of Super Frosted slides with pencil
- Immerse slides in Agarose NMP 1%, withdraw the slide and clean the underside
- Dry slides overnight on aluminium foil

3.6.2. Seeding of cells

- Seed 2 ml of a cell suspension containing 150 000 cells/ml in a 35 mm cell culture dish (Nunc, Nunclon™ Surface or equivalent).
- Incubate cells for 24 hours (37°C, 5% CO_2).

3.6.3. Exposure of cells

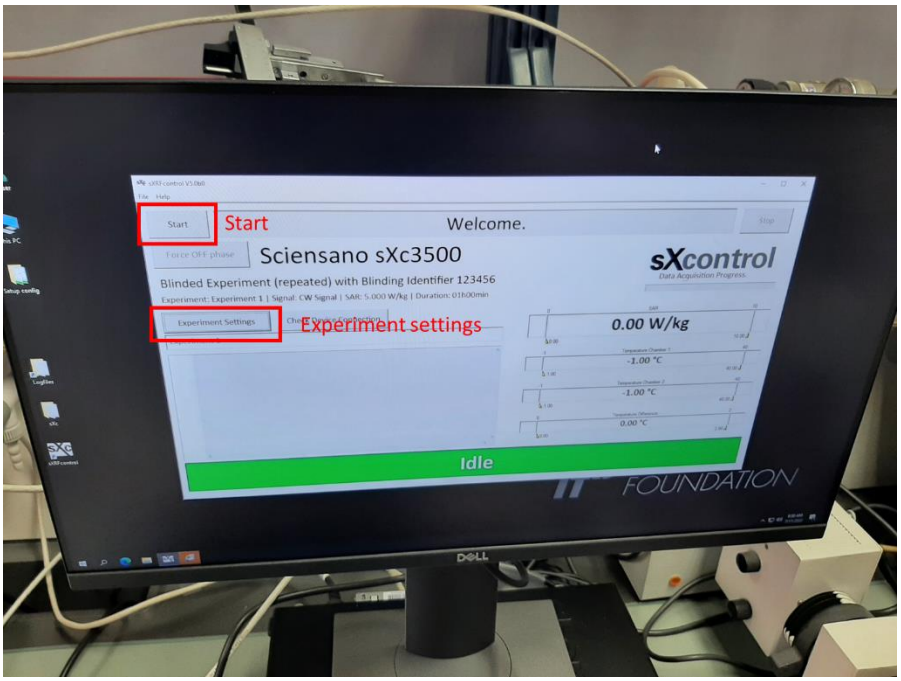
3.6.3.1 Chemicals

- Add 1,1 ml test item to each well for a total of 3.1 mL per dish (MMS for positive conditions and cell culture medium for negative conditions)
- Incubate for 24 hours(37°C, 5% CO_2).

3.6.3.2. RF-EMF

- Add cell medium containing the positive control substance (EMS, positive control) or the solvent (negative control) according to 3.6.3.1
  Remark:

  1. 4 dishes should always be used for the negative control (2 per exposure chamber) and four for the positive control (two per exposure chamber) to ensure the correct amount of samples to achieve statistical significance. Additionally, an incubator control can be added, including additional negative and positive control samples
- Place the cells inside the 5G exposure system (inside the sample holder) and incubate the cells for the required time (37°C, 5% CO_2)



- For a more in depth description on how to set up the exposure system, see the "sXc 3500 exposure system" guidelines
- In the "experiment settings" tab, adjustments can be made including SAR, exposure duration and the blinding ID of the experiment
- Pressing start will enable the 5G EMF to flow inside the exposure chambers and pressing pause will temporarily disable this. It can be enabled again by pressing "start" again.
- Experiments should always be performed blind, this means that only one of the chambers will be exposed at the same time. This can be decoded by sending the blinding ID to the people at IT'IS (sxc.itis@swiss). This means that the number of samples (2 Negative and 2 Positive) put inside the two exposure chambers should always be identical, since it is unknown which one of the chambers will be exposed beforehand.

3.6.4. Collection of Cells

3.6.4.1. Steps before Trypsinisation

<u>Prepare LMP 0.8% aliquots</u>

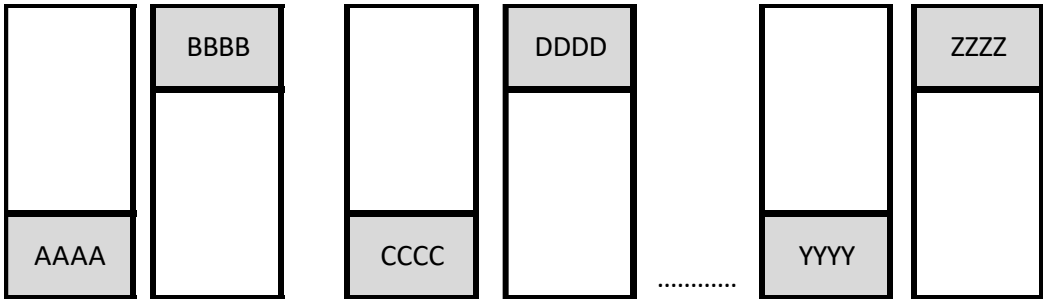
- Put 1.5 ml Eppendorf Tubes (1tube/condition) in a Heating Block at 36°C before melting the 0.8% LMP
- Melt the 0.8% LMP agarose in a water bath (boiling water)

- Pipet 600 µl of 0.8% LMP in each Eppendorf tube (36°C)

Mark Slides

- Mark slides with pencil starting with A up to Z (Z = number of slides)

Denaturation/electrophoresis buffer may erase pencil!!! Therefore mark AAAA etc…



Prepare Lysing Working Solution

- Depending on the number of slides prepare the correct amount of ice cold lysing working solution.
- Distribute lysing working solution to staining jars (1 jar = 10 slides = 100 ml lysing working solution)
- Put jars immediately into the fridge (ice-cold!!!)

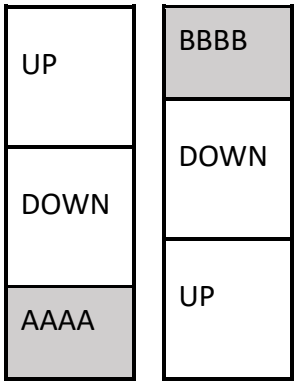
3.6.4.2. Trypsinisation of cells

- Discard solutions
- Rinse cells with PBS (min 300 µl/well)
- Discard PBS
- Add 500 µl TrypLE to each well
- Incubate for 10-15 minutes (37°C, 5% CO_2).
- Add 1500 µl complete medium to each well.
- Transfer cell suspensions in marked Tubes
- Centrifuge 5 minutes at 1000 rpm (micro-centrifuge)
- Discard supernatant
- Re-suspend pellet in 1000 µl ice cold PBS
- Maintain cells on ice (as short as possible)

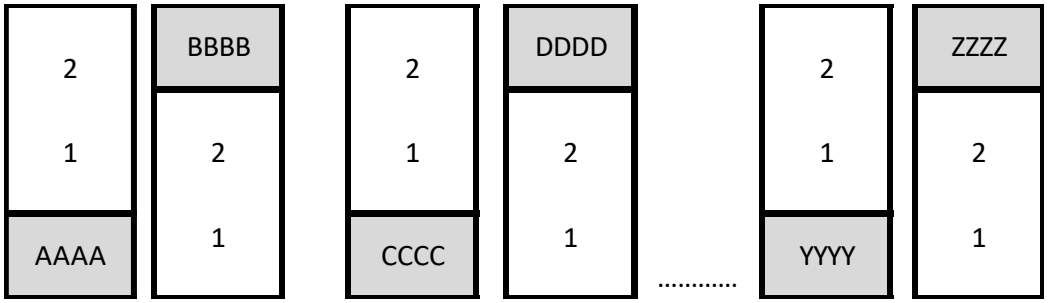
3.6.4.3. Slide Preparation

- Re-suspend cells with micropipette
- Mix 50 µl cell suspension with 600 µl 0.8% LMP (36°C). Avoid air bubbles.

Put 75 µl cell/LMP mix on 2 different pre-coated and marked slides alternating at the DOWN & UP side of a slide (see figure).



- Cover immediately with a 24x24 mm coverslip
- Repeat for all cell suspensions. Put first LMP/Cell Mix on positions n°1 from all slides starting with slide A up to slide Z. Continue with positions n°2 for all slides.



- Put and keep slides for 5 minutes on an ice cold plate at 4°C (fridge)
- Keep plate for 2 minutes at room temperature
- Remove coverslips and distribute slides randomly in jars with ice cold lysing buffer
- Keep slides for at least 1 hour in ice cold lysing buffer (usually overnight).

3.6.6. Denaturation – Electrophoresis – Neutralisation - Drying

3.6.6.1. Preparation of the Denaturation/electrophoresis buffer pH>13

Prepare just before use!

- Transfer approximately 500 ml ice-cold RO water (4°C) in a 1 liter flask
- Add 30 ml NaOH 10N + 5 ml EDTA 200 Mm
- Add room temperature RO water to complete the 1 liter

The temperature of the final denaturation/electrophoresis buffer should be ± 15 -17 °C.

3.6.6.2. Step to step procedure

- Put air-conditioning at lowest temperature possible (18°C)
- Place electrophoresis unit in a stainless steel reservoir
- Check the level (horizontal) of the electrophoresis chamber
- Pour 720-740 ml freshly prepared denaturation/electrophoresis buffer pH 13 (temperature below 17°C) into the electrophoresis chamber (COMET-40, Scie-plas)
- Connect circulating pump to the electrophoresis unit…set speed to 200 rpm and start circulating, once tube is filled with buffer (air bubble free) lower immediately to 15 rpm (lowest speed)
- Connect electrophoresis chamber to the power supply
- Set voltage to 1V/cm = 35V by removing or adding buffer if necessary
- Press view button: Current should be at least 300 mA (preferable 330-360 mA). Adding buffer will increase current, removing buffer will decrease current.
- Disconnect electrophoresis chamber from the power supply
- Rinse slides with denaturation/electrophoresis buffer and put them in the electrophoresis chamber (written part from the slides towards the anode). Maximum capacity of the electrophoresis unit is 40 slides
- Reconnect & restart circulating pump/power supply (as described above)
- Check briefly voltage (and current), if necessary adjust buffer level (voltage should be always 35V)
- Switch off immediately power supply but maintain circulating for 40 minutes (DENATURATION of the DNA).
- Put ice around the electrophoresis unit into the stainless steel reservoir
- Switch power supply on, check voltage (if necessary adjust buffer level) and start electrophoresis for 20 minutes (DNA will migrate towards anode). (ELECTROPHORESIS) Attention: voltage is the driving force and should be constant during electrophoresis
- Switch off power supply & circulating pump
- Remove all slides from electrophoresis chamber (ASAP) and put them in upright position.
- Rinse slides briefly with neutralization TRIS buffer pH 7.5
- Put slides horizontally and cover gels (slides) with neutralization TRIS buffer pH 7.5 for 5 minutes (NEUTRALIZATION)
- Put slides in upright position, put them horizontally again and repeat neutralization step twice
- Put slides into ice cold pro analysis Ethanol in staining jars for 10 minutes at 4°C (DRYING)
- Air-dry slides overnight (room temperature in the dark)

3.6.7. Gel Red Staining

- Pipette 200 µl RO H2O on each slide (re-hydration)
- Put 24x50 coverslip for 10 minutes
- Remove cover slip
- Pipette 100 µl GelRed 3x staining solution on each slide (staining)
- Put 24x50 coverslip for 10 minutes
- Remove cover slip, wash excess Gel Red (rinse slide with RO water)
- Add 2 drops of Vectashield mounting medium, one on each gel, or 200 µl H2O on each slide
- Put slides for at least 15 minutes in the fridge (in a box filled with humidified paper) before analysis

3.6.8. Automated microscoic analysis of slides

Analysis of each sample (gel) is done automatically by the fluorescent microscope (AxioImager.Z2) supplied with a camera and connected to comet imaging software (Automated Scanning System Metafer4 CometScan = Automated system for unattended detection and analysis of Comet assay slides, based on the scanning platform Metafer4).

The automated SlideFeeder makes it possible to analyse up to 80 slides.

A predefined area on each gel is scanned for the presence of single cells with parameters that correspond to those of the settings of a chosen comet assay classifier (program). Images of a preset number of matching cells are captured and various head and tail parameters are determined and measured automatically. Results such as % DNA in the tail are displayed for each cell of the sample in customizable list format and they are saved together with the gallery of all captured images in an individual file for each sample.

3.6.8.1. Fluorescent Lamp

- Switch on the fluorescent lamp at least 15 minutes before analysis
  IMPORTANT: life span of the lamp is limited (300-400 hours).

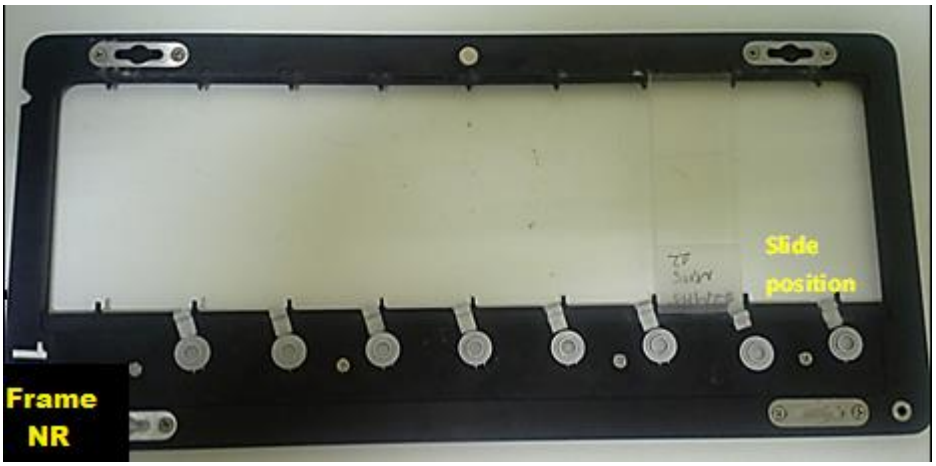
Note:

Preferably use the microscope (lamp) for a continuous longer period instead of smaller periods.

Make sure lamp switches off after automated analysis (choose “Shut Down” at “search end”)

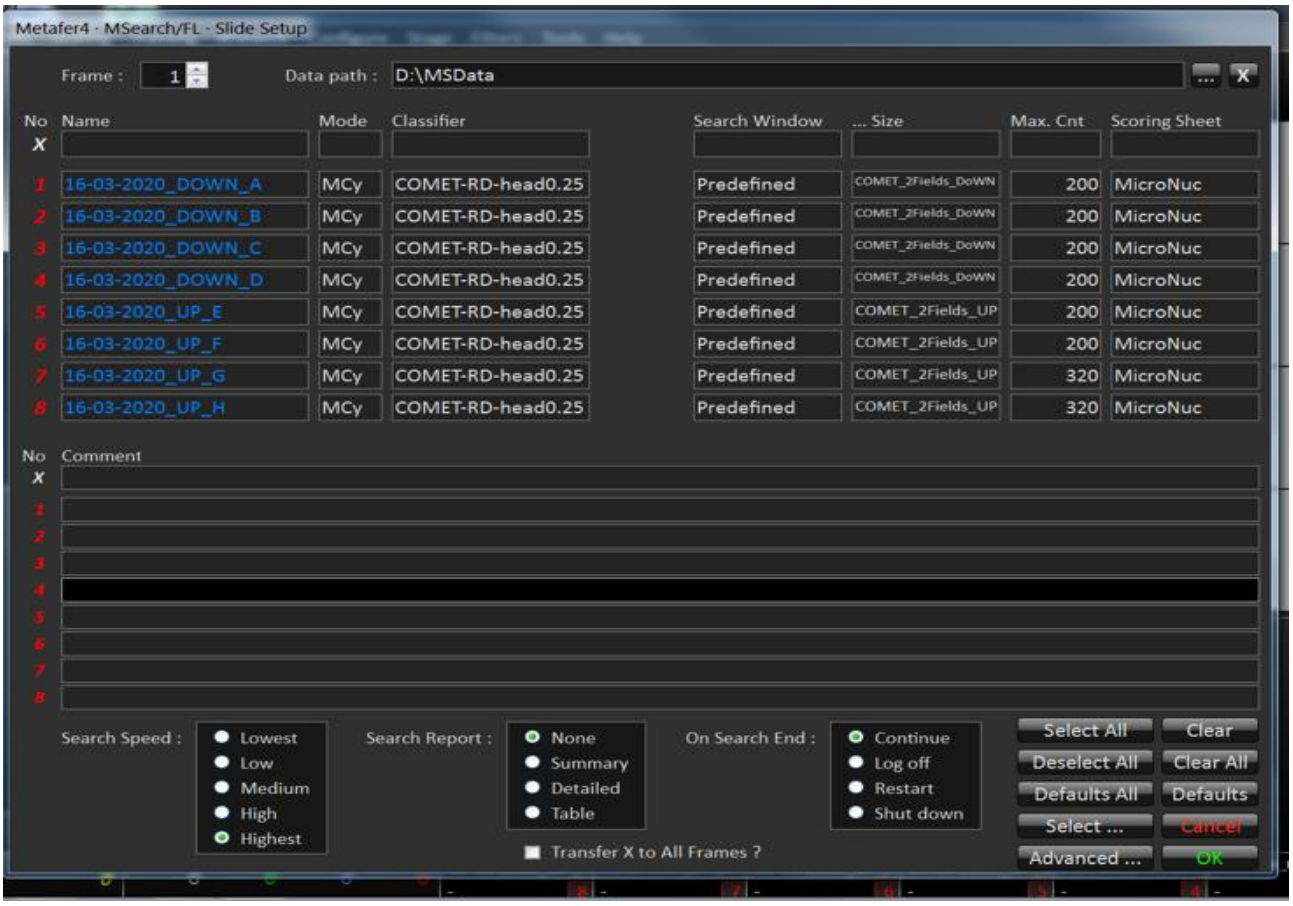
3.6.8.2. Slide fixation

- Slides are fixed alphabetically in a frame starting from the right-hand side from the frame (max 8 slides in one frame)
- Down gels are fixed downwards into the frame
- If more than 1 frame is analysed, frames need to be placed in the slide-feeder (first frame (frame number1) in position1 etc….). Up to 10 frames can be used (80 slides)



3.6.8.3. Slide setup: identification of the slides

- Open computer
- Open METAFER4 software (double click METAFER4 icon on desktop)
- Open SET UP page
- Choose:
  1) Frame number (1 to 10). If frame1 is used also frame 1 should be selected for analysis.
  2) Highlight slides that need to be analysed (1 to 8) in red
  3) Identify the slides:
     UP condition: XX-YY-ZZZZ_UP_A
     DOWN condition: XX-YY-ZZZZ_DOWN_A
     X = day / Y = month / Z = year / A = slide name
  4) Mode: MCy
  5) Classifier: COMET-RD-head0.25
  6) Search Window: predefined
  7) …Size
     UP condition COMET_2Fields_UP
     DOWN condition COMET_2Fields_DOWN
  8) Max Count: 200 (usually, but can be different)
  9) Scoring Sheet: MicroNuc



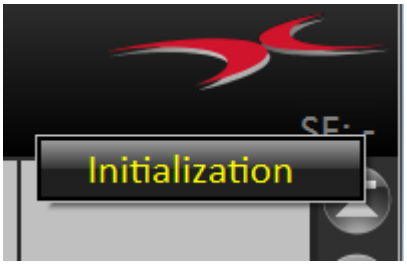
Slides 1-4 = analysis DOWN gel

Slides 5-6 = analysis UP gel

- Search Speed: Highest
- On Search End: “Continue” or “Shut Down” for overnight analysis (with Slide feeder)
- Click OK

3.6.8.4. Slide analysis

If the slide feeder is used for an overnight analysis, right mouse click on the SF icon to initialize the slide feeder (= initialization)

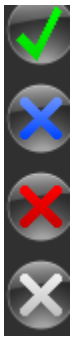


- Click on SEARCH. The first slide from the identified slides in the SET UP menu is automatically selected.
- Focus microscope on a recognizable field containing preferable at least 2 clear comets representative for the sample and free from unusually bright artefacts or impurities.
- Click OK
- Repeat the focus step for all the identified slides accept with OK
- Make sure the camera is open for screen view (screen view/microscope view switch).
- Click OK.
  Automated analysis of all identified slides is performed. Pre-set number of comet images (200) will be automatically captured for each sample and stored into a gallery

3.6.8.5. Selection of cells in the gallery

- Open cell gallery:
- Double click on slide name at the bottom of the SEARCH screen or double click on the slide name in the list with all stored files (this list appears by right mouse clicking on a random slide name at the bottom of the search field).
- Cell gallery appears
- All cells in the gallery are marked as undefined
- Reject images (click on purple cross before clicking
  1) Incorrect cell head selection
  2) Incorrect Tail cut-off
  3) Unclear images
  4) Multi cell images
  5) Artefacts

  Hedgehog comets should also be rejected but a large number of hedgehog cells (especially in control cells) can be the result of problems in the experiment (eg LMP temperature too high)
- Only marked, rejected and undefined images will be stored



= Mark cells

= Reject cells

= Delete cells

= Undefine cells

Remark:

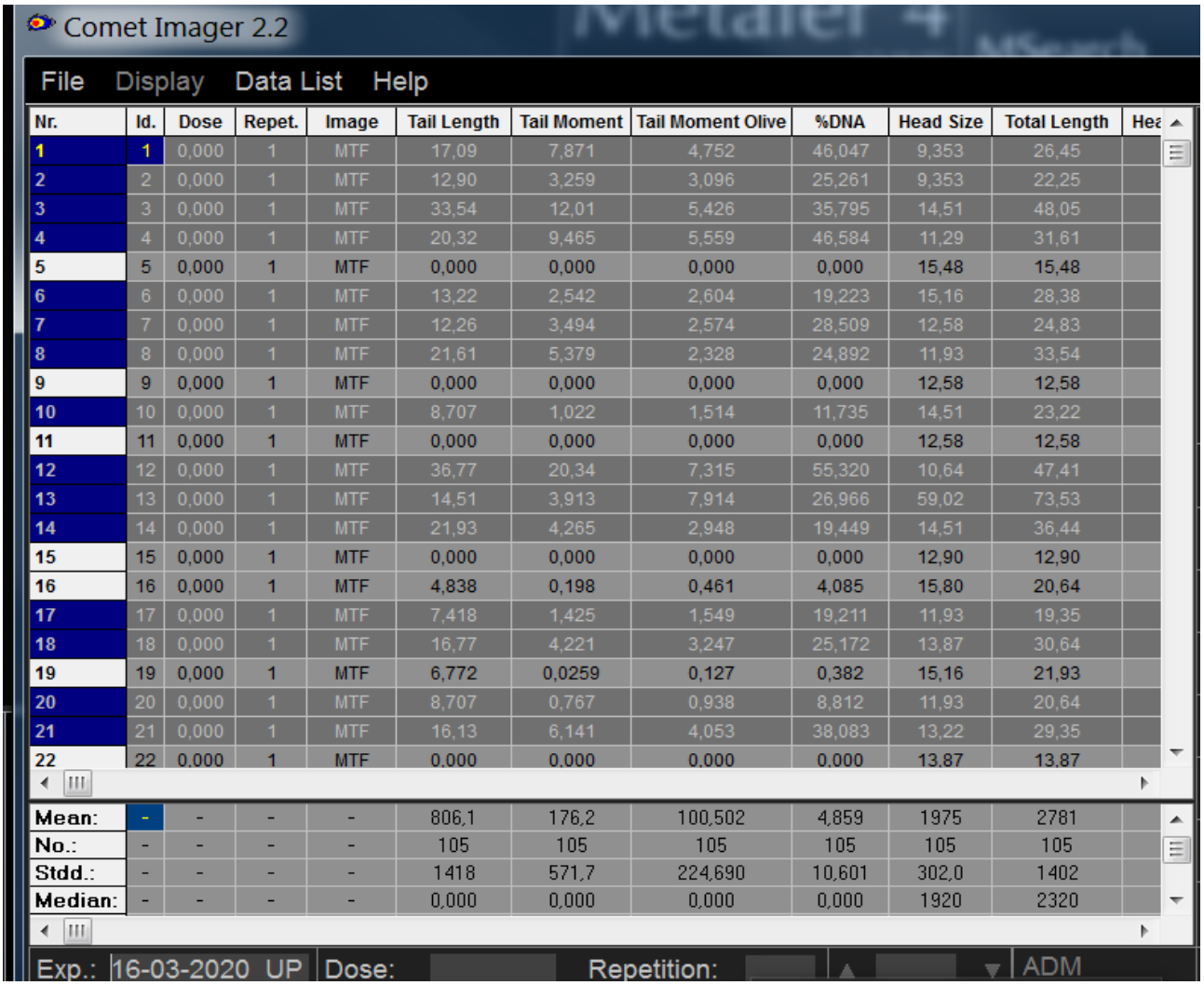
The given slide name in the SET UP menu will also be the filename (is automatically stored after analysis)

3.6.9. Data analysis

3.6.9.1. Export data

Data lists for each sample are stored in Comet Imager 2.2. software

- Open comet imager 2.2 (shortcut on the computer)
- Select FILE/browse/double click on sample name
- Select Data list/long list (contains both rejected (marked in blue) and undefined cells)
- Select FILE/export
- Export file to hard disk (only undefined cells are listed in the files)
- Open the list of the exported Excel Files
- Copy the exported Excel Files to the sciensano server



Nr.	Id.	Dose	Repet.	Image	Tail Length	Tail Moment	Tail Moment Olive	%DNA	Head Size	Total Length	Hea
1	1	0,000	1	MTF	17,09	7,871	4,752	46,047	9,353	26,45	
2	2	0,000	1	MTF	12,90	3,259	3,096	25,261	9,353	22,25	
3	3	0,000	1	MTF	33,54	12,01	5,426	35,795	14,51	48,05	
4	4	0,000	1	MTF	20,32	9,465	5,559	46,584	11,29	31,61	
5	5	0,000	1	MTF	0,000	0,000	0,000	0,000	15,48	15,48	
6	6	0,000	1	MTF	13,22	2,542	2,604	19,223	15,16	28,38	
7	7	0,000	1	MTF	12,26	3,494	2,574	28,509	12,58	24,83	
8	8	0,000	1	MTF	21,61	5,379	2,328	24,892	11,93	33,54	
9	9	0,000	1	MTF	0,000	0,000	0,000	0,000	12,58	12,58	
10	10	0,000	1	MTF	8,707	1,022	1,514	11,735	14,51	23,22	
11	11	0,000	1	MTF	0,000	0,000	0,000	0,000	12,58	12,58	
12	12	0,000	1	MTF	36,77	20,34	7,315	55,320	10,64	47,41	
13	13	0,000	1	MTF	14,51	3,913	7,914	26,966	59,02	73,53	
14	14	0,000	1	MTF	21,93	4,265	2,948	19,449	14,51	36,44	
15	15	0,000	1	MTF	0,000	0,000	0,000	0,000	12,90	12,90	
16	16	0,000	1	MTF	4,838	0,198	0,461	4,085	15,80	20,64	
17	17	0,000	1	MTF	7,418	1,425	1,549	19,211	11,93	19,35	
18	18	0,000	1	MTF	16,77	4,221	3,247	25,172	13,87	30,64	
19	19	0,000	1	MTF	6,772	0,0259	0,127	0,382	15,16	21,93	
20	20	0,000	1	MTF	8,707	0,767	0,938	8,812	11,93	20,64	
21	21	0,000	1	MTF	16,13	6,141	4,053	38,083	13,22	29,35	
22	22	0,000	1	MTF	0,000	0,000	0,000	0,000	13,87	13,87	
Mean:	-	-	-	-	806,1	176,2	100,502	4,859	1975	2781	
No.:	-	-	-	-	105	105	105	105	105	105	
Stdd.:	-	-	-	-	1418	571,7	224,690	10,601	302,0	1402	
Median:	-	-	-	-	0,000	0,000	0,000	0,000	1920	2320	

3.6.9.2. Comet data collection

- Identify the exported excel files
- Copy % DNA data from each file to the study report (2 slides for each condition)
- Mean/No/Stdd/Median is automatically calculated for each condition

3.6.9.3. Mann-Whitney statistic evaluation

- Copy % DNA data results from one sample together with negative and positive control into a data table from GraphPad PRISM file
    1) Copy all results from negative control to the first column
    2) For each concentration of the sample copy results to a separate column in the file (lowest concentration in column 2 and so on)

3) Copy results from positive control to the latest column

- Mann Whitney is calculated for each concentration & positive control versus negative control
- Median %DNA is plotted versus concentration in the PRISM Graph
- Copy Mann Whitney results and Graph to the study report

3.6.9.4. Neural red viabiity data

- Copy Neutral Red cell viability data and Graph for each substance to study report

3.6.10. Study report

For each *comet* experiment a new comet template (Study Report) should be filled in.

This template contains:

1) Study parameters
2) Exposure Information
3) Electrophoresis Information
4) Comet data & Mann-Whitney Statistic Evaluation
5) NRU data
6) Summary (comet, statistics & NRU data from each sample)

Remark:

- Only the most recent Study Report Template should be used.

4. Norms and references

- Collins AR, Azqueta Oscoz A, Brunborg G, Gaivão I, Giovannelli L, Kruszewski M, Smith CC, Stetina R (2008) The comet assay: topical issues. Mutagenesis 23:143–151.
- Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF (2000) Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen 35:206–221.
- Azqueta A. and Collins A. R . (2013) The essential comet assay: a comprehensive guide to measuring DNA damage and repair. Arch. Toxicol ., 87, 949–968

Standard Operating Procedure @ Sciensano

PROTOCOL	**DNA-methylation measurement in HaCaT cells and 5G RF-EMF exposure**
DATE	08/04/2024
AUTHOR(S)	Mathieu Gand, Seppe Segers and Bert Bogaerts
REVISED BY	Sigrid De Keersmaecker
APPROVED BY	Sigrid De Keersmaecker

Index

1. Purpose

This procedure describes the materials and the protocol used for DNA-methylation measurement in human keratinocyte HaCaT cells, including extraction, purification and quality control of DNA for twist hybrid-capture with human methylome panel.

2. Background

The methylation of cytosine–phosphate–guanine (CpG) dinucleotides in the promoter part of genes is responsible of epigenetics regulation: hyper-methylation of CpGs downregulates gene expression by affecting the binding of proteins involved in transcription mechanisms, while hypo-methylation upregulates gene expression. The twist hybrid-capture human methylome panel allows the targeted Next-Generation Sequencing (NGS)-based analysis of 3.98 million CpG sites (at each strand) including 84% of human CpG island sites with high coverage (150X). Prior sequencing, regular cytosines (C) are enzymatically (no bisulfite involved) converted to (U) and then to (T) via DNA amplification, while methylated Cs stay unchanged. The Differentially Methylated Regions (DMR) are detected by comparing the sequencing reads to the reference/control sequence.

3. Procedure

3.1. Equipment

- Incubating mini shaker (VWR)
- Countess 3 automated cell counter (Thermo Fisher)
- sXc3500 exposure system (IT'IS Foundation)
- Centrifuge 5702 (Eppendorf)
- Centrifuge 5417C (Eppendorf)
- VWR MiniStar Microcentrifuge (VWR)
- IKA MS3 Vortexer (IKA)
- 4200 Agilent TapeStation system (Agilent technologies)
- Qubit 4 Fluorometer (Invitrogen)
- Nanodrop 2000 (Thermo Scientific)
- Equipment for human methylome Twist panel, incl. NGS instrument via officially licensed Twist panel provider

3.2. Materials

- Nunclon® 35mm cell culture dishes (Merck)DNA LoBind Tube 1.5 mL (Eppendorf)
- Optical tube strips (8x Strip) (Agilent technologies)
- Optical tube strip caps (8x strip) (Agilent technologies)
- Tube CELLSTAR brown (light protection), 15 ml, PP, 17/120 MM, conical bottom, blue cap, sterile (Greiner Bio)
- Qubit Assay Tubes (Invitrogen)

3.3. Reagents

- DMEM (Dulbecco's Modified Eagle Medium) (Thermo Fisher Scientific)
- FBS (Foetal Bovine Serum) (Thermo Fisher Scientific)

- L-glutamine 200 mM (BIOSIGMA)
- Penicillin-Streptomycin solution 100X (BIOSIGMA)
- Sodium Pyruvate (Thermo Fisher Scientific)
- TrypLE™ (Thermo Fisher Scientific)
- Trypsin-EDTA (Thermo Fisher Scientific)
- DNeasy Blood & Tissue Kit (Qiagen), stored at room temperature (RT) (for storage longer than one year proteinase K, stored at 2-8°C)
- Ethanol absolute for analysis EMSURE (Supelco), stored at RT
- PBS, pH 7.2 (50 mM potassium phosphate, 150 mM NaCl) (Gibco), stored at RT
- Buffer AL (Qiagen), stored at RT
- QIAGEN Proteinase K 2 ml (>600 mAU/ml) (Qiagen), stored at RT (for storage longer than one year proteinase K is stored at 2-8°C)
- RNase 2.5 ml (100 mg/ml; 7000 units/ml) (Qiagen), stored at 2-8°C
- MicroCHIP DiaPure columns (Diagenode), stored at RT
- Qubit dsDNA Quantification High Sensitivity Assay Kit (Invitrogen), stored at 2-8°C
- Genomic DNA ScreenTape (Agilent technologies), stored at 2-8°C
- Genomic DNA Reagents (Agilent technologies), stored at 2-8°C
- Twist methylome panel (via officially licensed Twist provider)

3.4. Solutions

3.4.1. Complete cell culture medium

Add to a 500 ml DMEM bottle:

- 6 ml Sodium Pyruvate, 6 mL pen/Strep and 6mL L-Glutamine
- 60 ml FBS (2 aliquots)
- Label & store at 4°C for maximum 1 month.

3.5. Experimental procedure

After exposure to radiofrequency, the total DNA of the cells is extracted using the DNeasy Blood & Tissue Kit and is further purified with the MicroCHIP DiaPure columns. The DNA quantity and quality is then assessed using Qubit, TapeStation and Nanodrop instruments. DNA samples satisfying the quality criteria required for DNA-methylation analysis are proceeded to NGS with the twist hybrid-capture human methylome panel.

3.5.1. Cell Seeding

- Remove the culture medium and wash the cells with 5 ml PBS
- Add 5 ml trypLE and incubate for 10-15 minutes at 37°C
- Check the detachment of cells and resuspend them in 10 ml complete medium. If the cells aren't properly detached, put the cells back in the incubator for an additional 1-2 min.

- Transfer cells into centrifuge tube and spin at 300 g for 5 minutes
- Discard the media and resuspend the cell pellet in 10 ml of fresh complete medium
- Collect an aliquot to count the cells with the cell countess II automated cell counter
- Seed 3.1 ml of a cell suspension containing 300 000 cells in a 35 mm cell culture dish (Nunc, NunclonTM Surface or equivalent).
- Incubate cells for at least 24 hours in the incubator (37°C, 5% CO2).

Note: each experiment should include:

- Five samples in exposure chamber 1
- Five samples in exposure chamber 2
- In each exposure chamber, 1 sample with just medium should be foreseen as a temperature control. For these samples, a lid with a hole should be used.

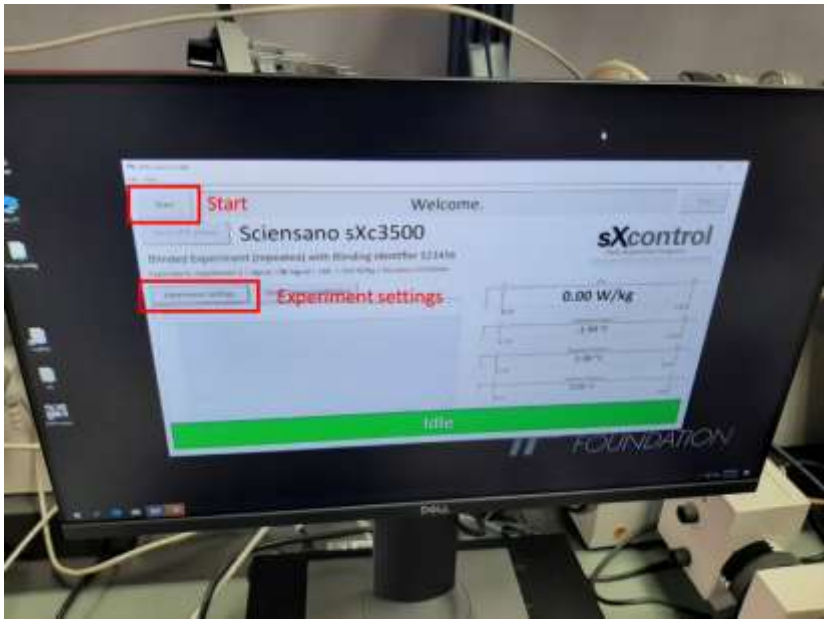
3.5.2. Exposure

The time between cell seeding and the end of exposure should be 48 hours. For a 1 hour exposure time, the cells should be incubated for 47 hours and exposed during the last hour. For 3 hour exposure, the cells should be incubated for 45 hours and exposed during the last three hours and for 24 hour exposure, the cells should be incubated for 24 hours and exposed for 24 hours.

3.5.2.1. RF-EMF

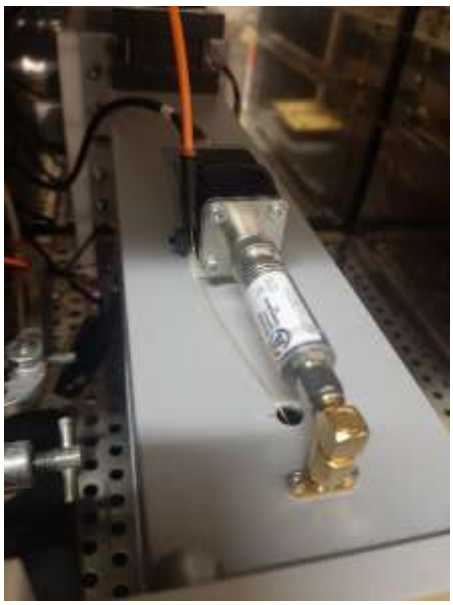
- Place the cells inside the sXc3500 5G exposure system (inside the sample holder)
- incubate the cells for at least half an hour before starting the experiment to allow ambient conditions inside the incubator (37°C, 5% CO2, humidity 90-95%) to return to normal.
- Expose for the required amount of time at the designated SAR.

Note: Experiments should always be performed blind, this means that only one of the chambers will be exposed at the same time. This can be decoded by sending the blinding ID to the people at IT'IS (sxc@itis.swiss). This means that the number of samples put inside the two exposure chambers should always be identical, since it is unknown which one of the chambers will be exposed beforehand.



3.5.2.2. *Temperature measurement*

- Place the temperature sensor through the top hole in the waveguide and through the lid of the temperature control condition.
- As soon as the sensors are put in place, start the measurement in the evolution software by pressing “start” and selecting the path where your files will be stored.



- After the experiment is finished, press “stop” in the “evolution” software.

During data analysis, a correction will be performed on the raw data by equilibrating them to the environmental temperature (as measured by the exposure system), during the time period before exposure. During the time period after exposure, delta T will be made starting from the time of exposure to the end of exposure. We do this to ensure non-thermal conditions.

3.5.3. DNA extraction

The DNA extraction is performed with the DNeasy Blood & Tissue Kit following the manufacturer's instructions with modification concerning cells harvesting and lysis. The modified protocol is included below.

Ensure that ethanol has been added to kit's buffers when indicated by manufacturer's instructions. Preheat a thermal shaker at 56°C before starting.

1) Add 400 µl of PBS, 40 µl of proteinase K and 8 µl of RNase A directly to the well containing the cell culture.
2) Wash the cells by pipetting up and down around 10 times. Incubate for 2 min at room temperature under agitation at 350 rpm.
3) Add 400 µl of buffer AL in each well.
4) Wash the cells by pipetting up and down around 10 times. Incubate for 10 min at 56°C under agitation at 350 rpm.
5) Transfer the lysate into 1.5 ml Eppendorf tube.
6) Add 400 µl of ethanol 96-100% and mix by pipetting up and down around 10 times. It is important to obtain an homogenous solution.
7) Pipet 630 µl of the mixture from step 6) into the DNeasy Mini spin column placed in a 2 ml collection tube (provided). Centrifuge at ≥6000 x g (8000 rpm) for 1 min. Discard flow-through.
8) Repeat previous step with the remaining volume of the mixture. Discard flow-through and collection tube.
9) Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW1, and centrifuge for 1 min at ≥6000 x g (8000 rpm). Discard flow-through and collection tube.
10) Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.

    It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol. If carryover of ethanol occurs, empty the collection tube, then reuse it in another centrifugation for 1 min at 20,000 x g (14,000rpm).

11) Place the DNeasy Mini spin column in a clean 1.5 ml Eppendorf tube, and pipet 100 µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1min, and then centrifuge for 1 min at ≥ 6000 x g (8000 rpm) to elute.

Proceed directly to DNA purification (section 3.5.4.) or alternatively DNA extracts can be stored overnight at 4°C.

3.5.4. DNA purification

The DNA extraction is performed with the MicroCHIP DiaPure columns following the manufacturer's instructions.

1) Add 500 µl ChIP DNA Binding buffer to 100 µl of DNA extract obtained in section 3.4.2.
2) Transfer mixture to a provided Spin column in a Collection tube.
3) Centrifuge at ≥ 10,000 x g for 30 seconds. Discard the flow-through.
4) Add 200 µl DNA Wash buffer to the column. Centrifuge at ≥ 10,000 x g for 30 seconds.
5) Repeat wash step 4).
6) Add 60 µl DNA Elution buffer directly to the column matrix. Transfer the column to a new 1.5 ml Eppendorf tube and centrifuge at ≥ 10,000 x g for 30 seconds to elute the DNA.

The DNA samples are directly processed for DNA quality control (section 3.4.4) or alternatively they can be stored overnight at 4°C.

3.5.5. DNA quality control

The DNA concentration is determined using the Qubit dsDNA quantification high sensitivity assay according to manufacturer's instruction. Briefly, the working solution is prepared by mixing 199 µl of the buffer and 1µl of the reagent, times the number of required reactions (+1 for overestimation). The DNA standards are prepared with 190 µl of working solution and 10 µl of standards 1 and 2. The samples to measure are prepared by mixing 199 µl of working solution and 1 µl of DNA sample from section 3.5.4. The samples are incubated 5 min and read on the Qubit 4 Fluorometer. Samples with concentration higher than 5 ng/µl meet the criteria for NGS with human methylome panel.

The DNA purity is estimated by measuring 1.5 µl of DNA sample from section 3.5.4. with the Nanodrop 2000 after making the blank with the MicroCHIP DiaPure Elution buffer from section 3.5.4. Samples with A260/280 and A260/230 ratios equal to 1.8 – 2.0 and 2.0 – 2.3, respectively, meet the criteria for NGS with human methylome panel.

The DNA integrity is evaluated with the TapeStation system, the Genomic reagents and screen tape according to the manufacturer's instructions. Ten µl of Genomic buffer is mixed with either 1 µL of DNA ladder or 1 µl of DNA sample. If more than 15 samples are analyzed in the same run, 20 µl of buffer and 2 µl of DNA ladder are used. Samples showing absence of DNA shearing with average fragment length above 20 000 bp meet the criteria for NGS with human methylome panel.

After DNA quality control, the DNA samples are stored at -20°C until preparation for NGS with human methylome panel (section 3.5.6.).

3.5.6. Preparation of the sample for NGS with human methylome panel

For each exposure condition, the DNA of triplicate samples (if more than 3 technical replicates are available, samples obtaining the best DNA QC in section 3.4.4. are used) is diluted to obtain 55 µl of

sample at a concentration of 10 ng/µl. Samples are prepared in 96-wells plate and kept at -20°C until analysis. Identify the position of each sample in the ID sheet.

3.5.7. Sample analysis with NGS with human methylome panel

The Twist human methylome analysis was outsourced to a Twist-licensed laboratory who followed the workflow described below for library preparation and sequencing. The raw sequencing data were then sent to SC for further analysis as described in section 3.5.8.

Preparation of human methylome libraries was proceeded as follows:

1) DNA shearing on Bioruptor® Pico (if necessary) with successive profile analysis
2) Library preparation including Enzymatic conversion (NEB)
3) QC of the enzymatically converted libraries (DNA concentration, analysis of the profile)
4) Targeted hybrid-capture using Human Methylome Panel (Twist Bioscience)
5) QC of captured Human Methylome libraries (DNA concentration, analysis of the profile)

The libraries were then further processed for deep sequencing with following settings:

- Samples were sequenced on a Novaseq 6000 (Illumina)
- Paired-end reads were generated with 150bp read length (PE150)
- Minimum 60M raw reads per sample should be generated on average

3.5.8. Data analysis

Reads were first trimmed using fastp v0.23.4 with the 'detect_adapter_for_pe', 'cut_front', 'cut_right' and 'cut_tail' options enabled with the following values: 'cut_front_window_size' and 'cut_tail_window_size' set to 1, 'cut_front_mean_quality' and 'cut_tail_mean_quality' set to 10, 'cut_right_window_size' set to 4, 'cut_right_mean_quality' set to 20 and 'length_required' set to 40.

The trimmed reads were then mapped to the GRCh38 human genome reference (GenBank accession number GCA_000001405.15) using the Bismark v0.24.2 read mapper for bisulfite treated sequencing reads, with default settings. The resulting BAM files were processed using the metyhlKit package v1.24.0 in R v4.2.2. All BAM files were parsed using the 'methRead' function and then first filtered using the 'filterByCoverage' function with the 'lo.count' set to 10, 'hi.count' set to 99.9 and both 'lo.perc' and 'hi.perc' disabled. The resulting counts were then normalized using the 'normalizeByCoverage' function and then combined using the 'unite' function with 'destrand' set to 'TRUE'.

First, differentially methylated positions were called using the 'calculateDiffMeth' function, with the overdispersion parameter set to 'MN'. Statistically significant positions were then extracted using 'getMethylDiff' with the 'difference' option set to 25 and the q-value cut-off to 0.01. Secondly, the analysis was repeated to identify differentially methylated CpG islands. Counts were grouped per region (i.e., per CpG island) using the 'regionCounts' function with the Twist Methylome BED file (i.e. methylome panel) and the 'cov.bases' parameter set to 10. The data was then combined using the

‘unite’ function with the ‘destrand’ option enabled. The same functions and parameters where then used to extract statistically significant differentially methylated CpG islands.

Standard Operating Procedure: Sciensano

PROTOCOL	**Oxidative Stress – H2DCF-DA/MTT Assay in HaCaT cells**
DATE	01/05/2025
AUTHOR(S)	Seppe Segers, Roel Anthonissen, Birgit Mertens
REVISED BY	Seppe Segers
APPROVED BY	Birgit Mertens

Table of Contents

1. Objective and Purpose

This procedure describes the measurement of ROS generation in HaCaT cells using the combined H2DCF-DA and MTT assays used in the laboratory of experimental toxicology. The H2DCF-DA is used to quantify the amount of ROS generated within HaCaT cells, while the MTT is used to normalize this against the number of cells present within the same sample, correcting for changes in growth and cytotoxicity as a result of exposure.

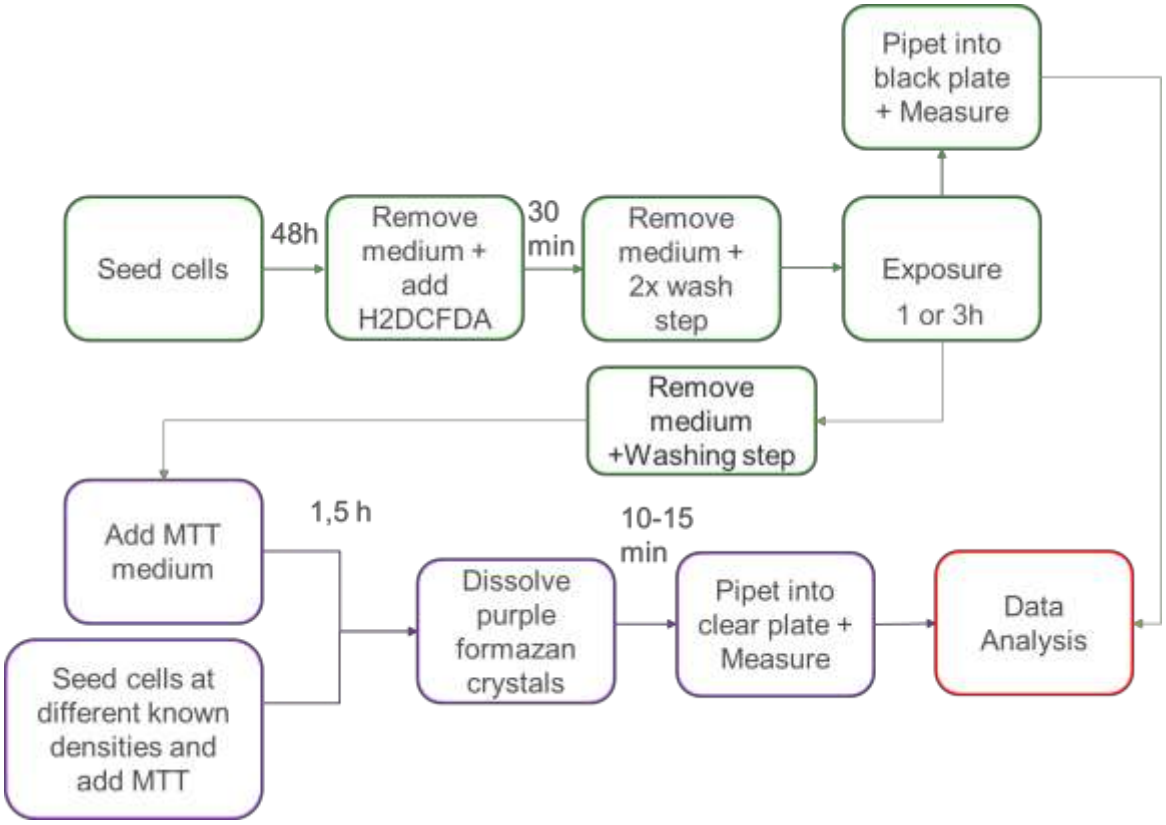


Figure 1: Schematic overview of the combined H2DCF-DA/MTT assay

2. Definitions et abbreviations

DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
H2DCF-DA	2',7'-dichlorodihydrofluorescein diacetate
DCF	2',7'-dichlorofluorescein
FBS	Foetal Bovine Serum
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

3. Procedure

3.1. Principle

- Cell-permeable dye: H2DCF-DA is a non-fluorescent, cell-permeable compound. It easily diffuses into live cells.

- Intracellular deacetylation: Once inside the cell, cellular esterases remove the acetate groups from H2DCF-DA, converting it into 2',7'–dichlorodihydrofluorescein (DCFH), which is still non-fluorescent but now trapped inside the cell.
- Oxidation by ROS: DCFH is oxidized by ROS, especially hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH), and peroxynitrite ($ONOO^-$), to form 2',7'–dichlorofluorescein (DCF).
- Fluorescence emission: DCF is highly fluorescent, and its fluorescence intensity (excitation ~485 nm, emission ~535 nm) is proportional to the amount of ROS present in the cell.

The fluorescent signal measuring ROS generation then gets normalized against the MTT signal, allowing us to calculate an amount of signal vs the amount of cells present within the sample.

- MTT is a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide).
- Uptake and reduction: Viable cells with active mitochondria take up MTT and reduce it using NAD(P)H-dependent oxidoreductase enzymes.
- Formation of formazan: MTT is converted into insoluble purple formazan crystals. This reaction occurs primarily in the mitochondria of metabolically active cells.
- Solubilization: The purple formazan crystals are insoluble in aqueous media, so a solubilizing agent (e.g., DMSO, SDS in acidified isopropanol) is added to dissolve them.
- Quantification: The resulting purple solution is quantified by measuring absorbance at 540–570 nm using a spectrophotometer or plate reader.

More absorbance = more viable/metabolically active cells.

3.2. Security

Protective Clothing: Eye-shield, laboratory coat purple nirtil gloves

All manipulations involving the culturing of the cell cultures should be performed in a biosafety cabinet.

3.3. HaCaT Cell Line

HaCaT is a spontaneously immortalized keratinocyte cell line from the adult human skin of a 62-year-old male. Due to their high capacity to differentiate and proliferate in vitro, these cells are extensively utilized in skin biology and differentiation research.

3.4. Chemicals

Amphotericin B *(Thermo Fisher Scientific)*

Fluorobrite DMEM (Dulbecco's Modified Eagle Medium) *(Thermo Fisher Scientific)*

DMSO (Dimethylsulfoxide) *(Sigma-Aldrich)*

FBS (Foetal Bovine Serum) (Thermo Fisher Scientific)

Gentamycin (Thermo Fisher Scientific)

Glutamax (Thermo Fisher Scientific)

Non Essential Amino Acids (Thermo Fisher Scientific)

PBS pH 7.2 (Thermo Fisher Scientific)

Sodium Pyruvate (Thermo Fisher Scientific)

TrypLE™ (Thermo Fisher Scientific)

Trypsin-EDTA (Thermo Fisher Scientific)

2',7'-dichlorodihydrofluorescein diacetate (Merck)

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Merck)

Menadione (Merck)

H2O2 30% (Merck)

3.5. Solutions

3.5.1. Complete Medium

Add to a 500 ml Fluorobrite DMEM bottle:

- 6 ml Gentamycin, Glutamax, Sodium Pyruvate & Non Essential Amino Acids
- 60 ml FBS (2 aliquots)
- 600 µl Amphoterecin B (1 aliquot)
- Label & store at 4°C for maximum 1 month.

3.5.2. H2DCF-DA stock solution

Dissolve the H2DCF-DA powder in DMSO, so that you have an end concentration of 100 mM.

3.5.3. H2DCF-DA loading medium

For an endconcentration of 40 µM, combine

- 49 mL of base fluorobrite DMEM (without additives!)
- 39.2 µL of H2DCF-DA stock solution.

3.5.4. Menadione stock solution

Prepare a Menadione 5 mM solution in DMSO

3.5.5. Menadione exposure medium

Combine, for an end concentration of 5 µM:

- 50 mL base Fluorobrite DMEM medium
- 50 µL Menadione stock solution

3.5.6. H_2O_2 control medium

Combine 20 µl H_2O_2 (30%) with 6 mL base fluorobrite DMEM medium, for a final concentration of 0.1 % H_2O_2

3.5.7. MTT medium

- Weigh 20 mg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
- Dissolve the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in 4 ml PBS
- Add 36 ml of complete fluorobrite DMEM medium to the solution.

3.6. Experimental procedure for the DCF/MTT assay

3.6.1. Cell seeding

- Seed 2 ml of a cell suspension containing 100 000 cells/ml in a 35 mm cell culture dish (Nunc, NunclonTM Surface or equivalent) in complete fluorobrite DMEM medium. (total of 200 000 cells / dish). Every condition should be performed in triplicate, and include positive control with Menadione and H2O2
- Incubate cells for 24 hours in the incubator (37°C, 5% CO2).

3.6.2. H2DCF-DA Loading

The timing of the loading should be timed, so that the fluorescence will be read out 48 hours after seeding. If exposure will take 3 hours, the loading should be performed 44.5 hours after seeding. For 1 hour exposure, the loading should be performed 46,5 hours after seeding.

- Remove the medium from the cells
- Add 2 ml H2DCF-DA loading medium to the cells
- Incubate cells for 30 minutes in the incubator (37°C, 5% CO2).

3.6.3. Washing step

- Remove the dishes from the incubator and remove medium from the dishes
- Add 2 ml PBS to the dishes
- Remove PBS from the dishes
- Add 2 ml PBS to the dishes
- Remove PBS from the dishes

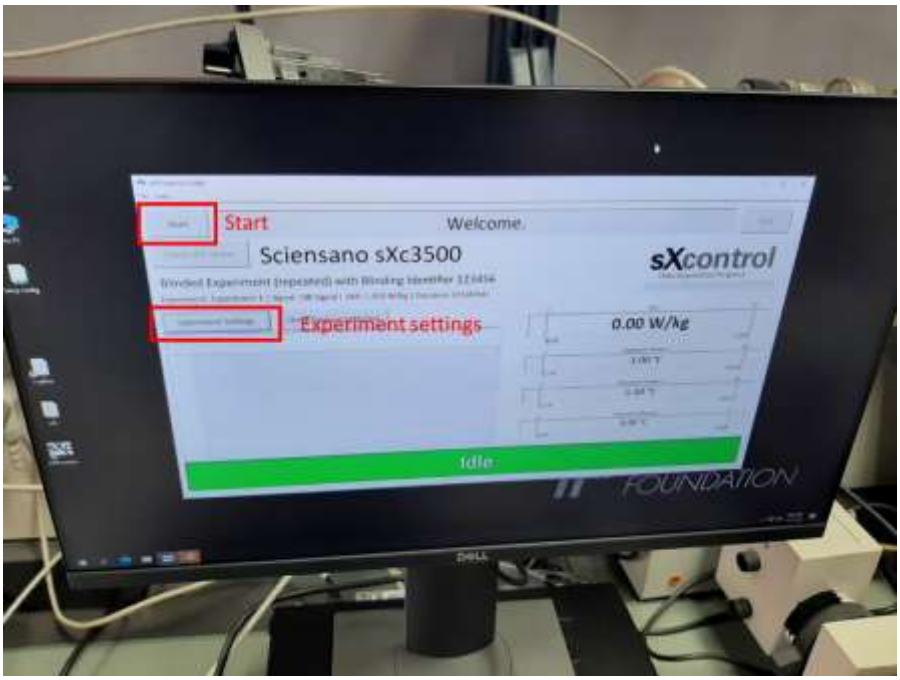
3.6.4. Exposure (RF-EMF)

- Add 3.1 mL cell medium containing the positive control substance (menadione, H2O2) or the solvent (negative control – base fluorobrite DMEM medium).

  Remark:

1. 6 dishes should always be used for the negative control (3 per exposure chamber) and six for the positive control (3 per exposure chamber) to ensure the correct amount of samples to achieve statistical significance. Additionally, an incubator control can be added, including additional negative and positive control samples (both Menadione and H202)

- Place the cells inside the 5G exposure system (inside the sample holder) and incubate the cells for the required time (37°C, 5% CO_2) – either 3 hours or 1 hour, depending on the experiment.



  - For a more in depth description on how to set up the exposure system, see the "sXc 3500 exposure system" guidelines
  - In the "experiment settings" tab, adjustments can be made including SAR, exposure duration and the blinding ID of the experiment
  - Pressing start will enable the 5G EMF to flow inside the exposure chambers and pressing pause will temporarily disable this. It can be enabled again by pressing "start" again.
  - Experiments should always be performed blind, this means that only one of the chambers will be exposed at the same time. This can be decoded by sending the blinding ID to the people at IT'IS (sXc@itis.swiss). This means that the number of samples put inside the two exposure chambers should always be identical, since it is unknown which one of the chambers will be exposed beforehand.

3.6.5. Fluorescence measurement

- After the exposure is finished, take the samples out of the incubator/exposure system.
- Pipet 100 µl into a black 96-well plate. At least 3 wells should be used per sample, in order to account for potential pipetting inconsistencies.

- Measure fluorescence in the Promega GloMax Discover, using excitation at 488 nm and emission at 520 nm.

3.6.6. Washing step

- Remove the DCF medium from the dish and rinse using 2 mL PBS.
- Remove PBS from dishes

3.6.7. Calibration curve cell seeding.

- From a new culture bottle, make a dilution series of cells (600.000 – 300.000 – 150.000 – 75.000 – 37.500 – 0) in an end volume of 1 mL MTT medium.
- Incubate for 2 hours in darkness (covered with alluminum foil).

3.6.8. MTT

- Add 1 mL MTT to the dishes at the same time as doing the calibration curve cell seeding.
- Incubate for 2 hours in darkness (covered with alluminum foil).

3.6.9. Absorbance measurement

- Dissolve the purple crystals in 2 mL DMSO, by putting it on a shaking incubator at 37°C
- Check for complete dissolution of the crystals prior to measurement.
- Pipet 100 µL of of the solution in the dishes into a clear 96-well plate. At least 3 wells should be used per sample, in order to account for potential pipetting inconsistencies.
- Measure absorbance in the Promega GloMax Discover at 560 nm.

4. Data analysis

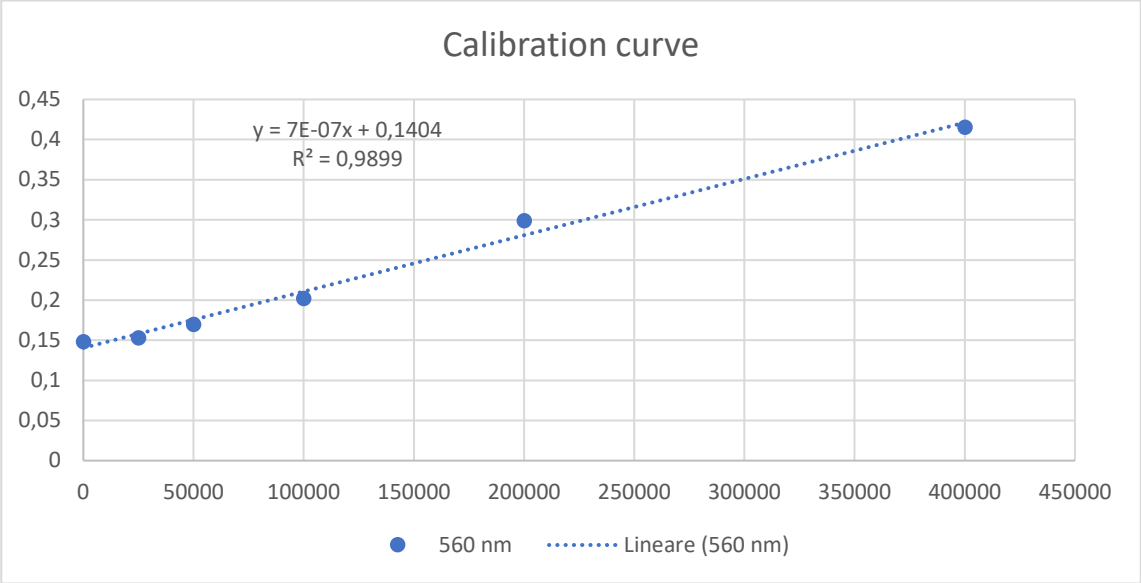
4.1. Fluorescence measurements

Fluorescence measurements calculated against the background (this is an empty well, filled only with the base fluorobrite medium (no cells)

Resulting fluorescence = measured fluorescence – background fluorescence

4.2. MTT calibration curve.

Use the MTT absorbance signal to plot a calibration curve by plotting it against the known number of cells in the sample. Use excel to find the trendline, including the R^2 and the equation.



4.3. MTT signal to cell viability

Use excel to calculate the cell viability:

(% Viability = (Average Absorbance (Treated) – empty well (containing only 1ml MTT medium + 2ml DMSO) / Average Absorbance (Control)) – empty well (containing only 1ml MTT medium + 2ml DMSO) * 100)

4.4. Calculate the amount of cells present in a sample.

Use the equation obtained from the trendline to calculate the amount of cells present in the sample.

If the trendline has the following equation, with

Y = signal

X = amount of cells

$$Y= aX+b$$

This equation can be reformed

$$\frac{Y-b}{a}=X$$

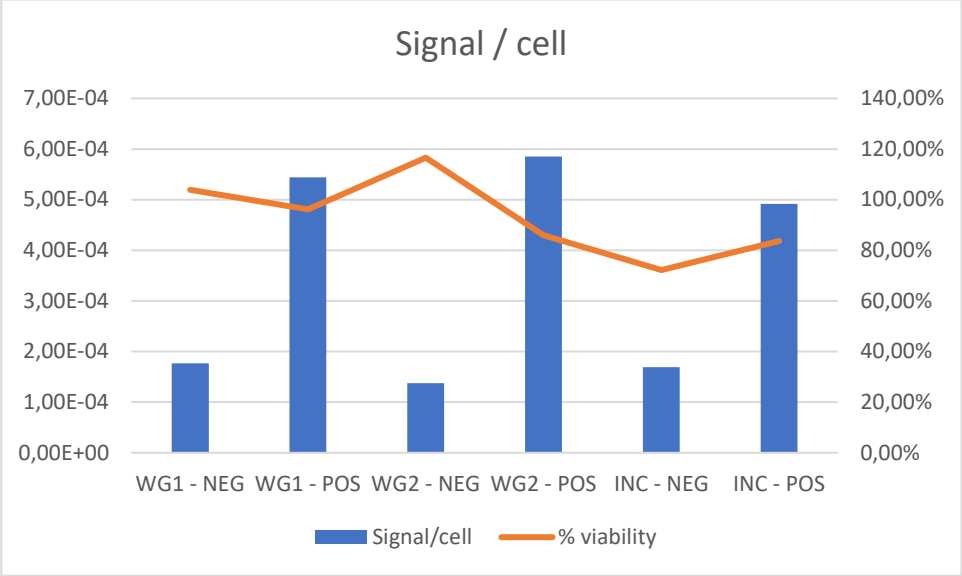
4.5. Calculate signal/ cell

Divide the signal obtained in 4.1. by the amount of cells obtained in 4.4. to obtain the signal/cell

Reported value should be:

Fluorescent signal/cell, averaged over the 3 technical replicate samples.

Ideally, this should be reported on the same graph as the cell viability.


Signal / cell
7,00E-04
6,00E-04
5,00E-04
4,00E-04
3,00E-04
2,00E-04
1,00E-04
0,00E+00
140,00%
120,00%
100,00%
80,00%
60,00%
40,00%
20,00%
0,00%
WG1 - NEG
WG1 - POS
WG2 - NEG
WG2 - POS
INC - NEG
INC - POS
Signal/cell
% viability

Standard Operating Procedure @ Sciensano and CNR-IREA

PROTOCOL	**TempO-Seq sample preparation**
DATE	08/04/2024
AUTHOR(S)	Seppe Segers, Roel Anthonissen, Birgit Mertens
REVISED BY	Mariateresa Allocca, Anna Sannino, Olga Zeni
APPROVED BY	Olga Zeni

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1. Purpose

This procedure describes the method for the sample preparation for TempO-Seq transcriptomics analysis.

2. Background

TempO-Seq (Templated Oligo assay with Sequencing readout) is a high-throughput transcriptome analysis method that measures specific gene sequences directly from RNA in crude lysates or purified RNA. The process involves hybridization and ligation of detector oligos (DOs) to target sequences, enzymatic removal of excess DOs, and amplification and sequencing of ligated DO pairs. This method ensures exceptional specificity, even for highly similar genes, by minimizing mis-ligation and background noise, enabling precise and unbiased measurements. It allows for single-base specificity, facilitating the measurement of expressed variants and the differentiation of homologous genes within or between species.

3. Procedure

3.1. Equipments

- Laminar flow cabinet (Technigen or equivalent)
- Cell culture incubator (Thermo Scientific Forma, Model 311 or equivalent)
- -80°C refrigerator (Thermo Scientific, TSE SERIES Model 933 or equivalent)

3.2. Materials

- 35 mm cell culture dish (Corning, cod. 430165 or Nunclon, with air vent)
- RNAse/DNAse-free cryovial (Smport scientific, T311-1)
- Foil seals (Thermo Fisher Nunc Sealing Tapes, catalog #12-565-398)
- 96-well plate

3.3. Reagents

- PBS (Dulbecco's Phosphate Buffered Saline) w/o Calcium w/o Magnesium (Biosigma, code: L0615 is stored at 4°C.
- TempO-Seq 2X lysis buffer (Bioclavis) is stored at -20°C
    - For 1X lysis buffer, dilute 2X lysis buffer with equal volume of PBS

Note:

- 2X Enhanced Lysis Buffer should be mixed well after thawing by inverting at least 5 times.
- Once thawed, the buffer is stable for up to one day at room temperature (25°C), or for up to one week at 4°C.

- For multiple uses, 2X Enhanced Lysis Buffer can be re-frozen and thawed up to five times. The buffer must be mixed after each thaw.
- If the buffer is to be used over a long period of time, we recommend aliquoting then freezing and thawing individual aliquots as required. => aliquot in 5 mL aliquots
- Do not use the buffer after the indicated expiration date.
- The lot number of the 2X Enhanced Lysis Buffer used should be recorded on the General Information tab of the Sample Submission Form.

3.4. Experimental procedure

The following procedure has been optimized for HaCaT cells (CLS, Lot. 300493-4820, p32; Eppelheim, Germany). Procedures for cell maintenance at CNR and SC premises are detailed in "SOP_HaCaT cell maintenance_CNR" and "SOP_HaCaT cell maintenance_SC", respectively.

$3x10^5$ cells are seeded in complete medium in 35 mm cell culture dish and the assay is performed after 48 h of growth. Note: the amount of medium in the dishes depends on the optimal field disuniformity in the cell culture dishes and is dependent on the exposure system used (3 ml for CNR and 3.1 ml for SC).

3.4.1. Security

- Protective Clothing: gloves, safety glasses and laboratory coat.
- All manipulations before the exposure of the cells should be performed in a biosafety cabinet. Later manipulations involving chemicals should be performed under a chemical hood.
- All safety measures related to working with potentially carcinogenic chemicals should be respected. Depending on the test products, additional safety measures may be required (see Security sheet).

3.4.2. Controls

- Positive control: no positive control substance will be used.
- Negative control: incubator control, not exposed in either of the two waveguides/reverberation chambers (sham).

3.4.3. Sample preparation

- Start three different cultures at the same time and sub culture separately (for independent experiments).
- Seed three experimental conditions (incubator control, sham exposed and RF exposed) from each culture.

- Label samples per subculture, passage number and exposure condition (or with blinding identifier).
- For the exposures follow the procedure detailed in Annexes 7, 10, 11, 12.
- Prepare sufficient volume of 1X TempO-Seq Enhanced Lysis Buffer
- After the exposure finishes, remove the treatment medium and rinse with 2 mL PBS.
- Immediately add 1 ml 1X TempO-Seq Enhanced Lysis Buffer and mix well by nutating or rocking the plate.
- Incubate the lysates for exactly 15 minutes at 37°C.
- Collect with a tip and transfer to the cryovial.
- Store the samples at -80°C.

Note:

➢ Cell lysate sample requirements: 0.25 to 2 million cells/mL of 1X TempO-Seq Enhanced Lysis Buffer.

3.4.4. Storage and shipping

@ CNR: send samples to Sciensano on dry ice.

@ Sciensano: once all the samples are at SC, gently thaw samples and keep on ice.

- Pipette 60 µl will per sample over to a 96-well plate, according to the Sample submission form, which has to be returned to Bioclavis via e-mail prior to shipping the samples.
- Seal plates to prevent cross-contamination between wells with adhesive aluminium foil film RNAse/DNAse-free.
- Before shipping, inspect the frozen plate assembly to ensure that the seals are secure. Do not wrap plates with Parafilm as it will break into fragments when shipped on dry ice.
- Note plate names (matching those recorded in the Sample Submission Form) on the side of each skirted or semi-skirted plate.
- Freeze plates horizontally so that all liquid freezes at the bottom of the wells. If samples are not at the bottom of the well after sealing the plate, centrifuge briefly.
- Send samples on dry ice to Bioclavis for the analysis

3.4.5. Sample analysis

The TempO-Seq assay is outsourced to Bioclavis, a spinout of BioSpyder Technologies, which specializes in TempO-Seq Analysis. Samples are sent on dry ice to Bioclavis, where synthetic DNA probes complementary to target mRNA transcripts are added to the thawed cell lysates. These probes contain universal primer sites for subsequent amplification.

After the assay is performed by Bioclavis, the data from the sequencing reads will be sent to Sciensano for data analysis. The sequencing reads derived from the TempO-Seq libraries represent the mRNA transcripts captured and labeled during the assay. Bioinformatic analysis of the sequencing data involves quantifying gene expression levels, identifying differentially expressed genes, and inferring biological pathways or functional annotations. The data are normalized and statistically analyzed to generate meaningful insights into gene expression patterns.

Standard Operating Procedure @ ICMAB-CSIC

PROTOCOL	***C. elegans* maintenance and handling**
DATE	20/05/2023
AUTHOR(S)	Pol Alonso Pernas, Amanda Muñoz-Juan, Anna Laromaine
REVISED BY	Amanda Muñoz-Juan, Anna Laromaine
APPROVED BY	Anna Laromaine

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1. Purpose

This procedure describes the materials and protocols used for maintaining and storing *Caenorhabditis elegans (C. elegans).*

2. Background

Caenorhabditis elegans (C. elegans) is a 1 mm long free-living nematode postulated as an animal model in 1965 by Sydney Brenner. The ability to grow hundreds of animals on a single Petri dish feeding on bacteria, transparency, rapid life cycle (3 days), short lifespan (2-3 weeks), and facile and inexpensive growth in the lab position the worm as an ideal model. The advances in genetics and molecular biology allowed the identification of all 959 cells of *C. elegans* and its complete genome, demonstrating a high conservation of biological mechanisms between the worm and vertebrates. C. elegans has some limitations as an experimental model since it lacks some specific tissues (i.e., bones), organs (i.e., eyes and ears), and systems (i.e., the circulatory system). However, since its establishment as a model organism, the impact of *C. elegans* has extended from biology to other fields, including chemistry, materials science, and medicine.

C. elegans were obtained from the *Caenorhabditis Genetics Center (CGC)* at the University of Minnesota. Upon arrival, *C. elegans* were expanded in a fresh, clean agar plate with *Escherichia coli* OP50 food. After two generations, cryovials with the *C. elegans* generations were frozen. A working bank of *C. elegans* is maintained at the -80°C freezer at the CSIC-ICMAB premises.

C. elegans´ life cycle is described in the worm atlas book (Figure 1), and the maintenance and handling are considered.

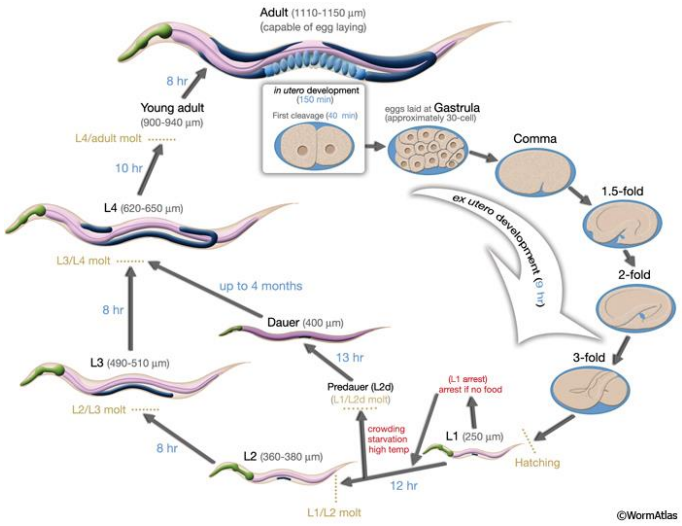

Figure 1 . *C. elegans*'s life cycle. Extracted from Altun, Z.F. and Hall, D.H. 2009. Introduction. In *WormAtlas*

Typically, *C. elegans* is cultivated utilizing *E. coli* strain OP50 as its primary food source and maintained on Nematode Growth Medium (NGM) agar poured into Petri plates. Various sizes of petri plates are available for different purposes, ranging from 35 mm for mattings or when using expensive drugs to 60 mm for general strain maintenance and 100 mm for growing larger quantities of worms. NGM agar can be poured into petri plates using a peristaltic pump, with a constant amount dispensed into each plate, reducing the need for microscope refocusing when switching between plates. Drugs can be added to the NGM solution just before pouring if desired.

3. Procedure

The reagents and materials used are sterile, and all the procedures are performed under a laminar flow cabinet.

C. elegans stocks are best maintained between 16°C and 25°C, typically at 20°C. They can be kept for months between transfers as worms enter the dauer form. *C. elegans* and bacteria stocks can also be stored frozen in liquid nitrogen or at –80°C with good viability. The maintenance of *C. elegans* involves the following steps: i) Preparation of Nematode Growth Media (NGM); ii) Preparation of the bacterial food source; iii) Transference of *C. elegans*.

3.1. Equipments

- *C.elegans* incubator (Memmert ICP110)
- Laminar flow cabinet (ESCO Laminar Flow Cabinet AVC-3D1)
- Stereoscope microscope (Nikon SMZ800N)
- Table centrifugue (J.P. Selecta. S.A. 7002239)
- -80°C Ultrafreezer (Telstar Boreas)
- Vortex (Ibx instrumentos V05 series)3
- Pipet controller (BrandTech™ accu-jet™ Fisher scientific)
- Platform agitator

3.2. Materials

- Eppendorf® tubes
- Falcon tubes
- Autoclavable glass flasks with screw lid
- Metallic spatulas
- Petri dishes (90 mm)
- Petri dishes (35 mm)
- Spreaders
- Inoculation loops
- Platinum wire
- Ethanol Burner
- Glass pasteur pipettes
- Serological pipettes 10 ml
- Serological pipettes 25 ml

3.3. Reagents

- NaCl (S9888, Merck)
- Agar (01916-500G, Merck)
- Peptone (211677 Thermofisher)
- $CaCl_2$ dihydrate (223506 Merck)
- KH_2PO_4 (P0662-500G, Merck)

- K_2HPO_4 (795496-500G, Merck)
- $Na_2HPO_4 \cdot 12\ H_2O$ (Emsure Supelco 1.06579.0500)
- Cholesterol (C8667-5G, Merck)
- Ethanol (99%, 493546-1L, Merck)
- LB broth (12780052, Thermo Fisher)
- Sodium hypochlorite (219250025, Thermo Fisher)
- PFA (158127, Merck)
- NaOH (S5881-500G, Merck)
- $MgSO_4$ (M7506 Merck)
- NGM plates. 30 ml per plate of NGM (3 g/l NaCl, 1 ml/l $MgSO_4$ 1M, 1 ml/l cholesterol 5 mg/ml, 17 g/l agar, 2.5 g/l peptone, 1 ml/l $CaCl_2$ 1M, 25 ml/l K_3PO_4 buffer, MilliQ® water)
- M9 buffer (5g/l NaCl, 1 ml/l $MgSO_4$ 1M, 3 g/l KH_2PO_4, 6 g/l Na_2HPO_4, MilliQ® water)
- K_3PO_4 buffer (108.3 g/l KH_2PO_4, 35.6 g/l K_2HPO_4, MilliQ® water)
- Glycerol (15514011 Thermo Fisher)

3.3.1. NGM agar plates preparation

To prepare NGM plates, mix 3 g NaCl, 17 g agar, and 2.5 g peptone. Add 975 ml H_2O. Autoclave for 20 min at 121°C. Wait until it colds down to 55°C. Add 1 ml 1 M $CaCl_2$, 1 ml 5 mg/ml cholesterol in ethanol, 1 ml 1 M $MgSO_4$, and 25 ml 1 M K_3PO_4 buffer. Swirl to mix well. Using sterile procedures, dispense 30 ml NGM solution into petri plates using a pipet controller.
They are stored at room temperature in a dry ambient for two weeks.

3.3.2. OP50 solution preparation

From a single *E. coli* OP50 colony, inoculate LB broth and allow cultures to grow overnight at 37°C. Afterward, centrifuge to precipitate bacteria and replace the supernatant with M9 buffer (3 g KH2PO4, 6 g Na2HPO4, 5 g NaCl, 1 ml 1 M MgSO4, H2O to 1 l).
E. coli OP50 is inactivated with PFA. This step is essential to avoid the interference of bacteria metabolism with the experiment's final result. *E. coli* OP50 is the usual food source for worms.

1) Inoculate LB culture with one colony of *E. coli* OP50 and incubate overnight (17h) at 30°C.
2) Mix vigorously with 3 ml of PFA 8% for each 45 ml of culture.
3) Incubate for another 20 min more at 30°C.
4) Centrifuge at 14500 rpm for 10 min. Discard the supernatant.
5) Resuspend the pellet in the same volume of PBS buffer.
6) Repeat steps 4 and 5 three times more.
7) Concentrate it 100 times (OP50-100x).
8) Spread 3 drops of 3 µl in an NGM plate. Culture at 30°C to know if it is completely activated.
9) Store it at 4°C until needed.
10) Spread 80 µl 100x OP50 onto 35 mm NGM plates. Store plates in the fridge until they are needed.

3.3.3. OP50 lawn petri dishes preparation

Dispense approximately 0.5 ml of *E. coli* OP50 liquid culture onto 60 mm NGM plates or 0.25 ml onto 35 mm NGM plates using a pipette. *E. coli* OP50 is an uracil auxotroph with restricted growth on NGM plates. Maintaining a limited bacterial lawn is preferable to facilitate easier observation and enhance worm mating.

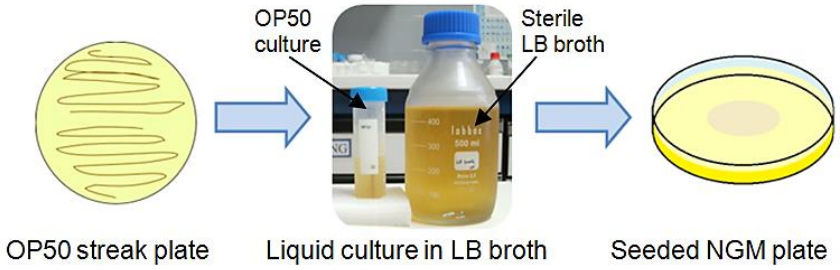

Figure 2 Preparation of the bacterial food source of *C. elegans.*

3.3.4. Worms transference

Worms are transferred from old plates to fresh plates by chunking. 'Chunking' involves moving a chunk of agar from an old plate to a fresh plate using a sterilized spatula. In the new plate, worms crawl out of the chunk and spread onto the bacterial lawn. Chunking is useful for transferring worms when food is scarce and starvation is not desired. The old plates used for chunking must not be contaminated. Otherwise, contaminants will be transferred to the new plate within the chunk (i.e. spores of yeast, bacterial cells).

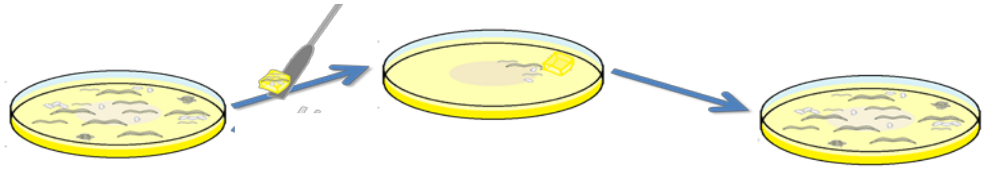

Figure 3. Scheme of the transference of worms. The procedure shows transference by chunking.

3.4. Worms synchronization

This protocol is useful for synchronizing worms and removing contamination. It is done in the laminar flow cabinet.

1. Chunk the worms strain you want two days before the bleaching and culture at 20°.
2. On the bleaching day, check that most of the worms in the gravid worm stage and eggs are on the plate. In addition, check that there is still food on the plates. If not, the worms have suffered from starvation and could grow badly in the next steps.
3. Spread 3 ml M9 buffer onto NGM plates with worms.
4. Using a Pasteur pipette, take the liquid and return it to the plate to remove worms from the NGM plate.
5. Transfer worms to 15 ml falcon tubes.
6. Centrifuge worms at 6000 rpm for 2 min.
7. Remove the supernatant until 5 ml of the tube.
8. Repeat steps d) and e) three times. Adjust the final volume to 5 ml.

9. Prepare the bleaching solution: 2 ml 5M NaOH, 13 ml sodium hypochlorite, 35 ml distilled water.
10. Add 5 ml of bleaching solution.
11. Vortex tubes at 1 min max speed. In this process, the bleaching solution dissolves the worm's tissues.
12. Centrifuge tubes 6000 rpm for 2 min.
13. Remove supernatant until 1 ml of liquid is left on the tube.
14. Add 9 ml of M9
15. Repeat steps 12-14 three times. Finally, worms were resuspended in 5 ml of M9.

3.5. Freezing procedure

Worms can be maintained in the incubator and the freezer. In the incubator, they can survive three months in the same plate, but mutations can appear and change the strain's phenotype. Therefore, preparing a frozen stock when you receive a new strain in the lab is highly recommended. Some protocols suggest freezing the L1-L2 synchronized population, but we have seen that a mixed population can also survive.

1. Prepare two 60 mm-petri dishes with 500 µl *E. coli* OP50 with an optical density at 600 nm (OD600)=1 and put them at 30°C overnight.
2. Do a chunk from a plate with the worms you want to freeze. Put the plate at 20°C (or another temperature where your worms grow) and wait until it is overcrowded with worms (it depends on the growth rate; usually, two days is enough).
3. Recollect worms from plates with M9 as described previously for synchronization.
4. Clean them with M9 to remove bacteria from the solution (at least three times). Centrifuge them at 4400 rpm for one min.
5. Once they are cleaned, worms are resuspended in 2 ml M9. This tube is left in the platform agitator overnight to starve worms.
6. The following day, add an equal volume of glycerol of 30% (Diluted with M9 buffer). Mix the final solution well.
7. Separate the final solution in cryovials (1ml each).
8. Add 4 drops of *E. coli* OP50 with OD600=1.
9. Place them in the freezer at -80°C.
10. Thaw one of the cryovials after one week to see the viability.

3.6. Thawing procedure

1. Remove a vial from the freezer and let it thaw at room temperature.
2. Pour the contents onto one large NGM plate with 500 µl of *E. coli* OP50 OD600=1.
3. Wait until the liquid has dried and incubate the plate overnight at 20°C.
4. The next day, you should check if the worms are alive. If it is, the freezing protocol has worked.
5. Transfer by chunking to a new, fresh plate. Pass worms throw two consecutive generations to avoid any interference from the freezing step.

Standard Operating Procedure @ CNR- IREA

PROTOCOL	***C. elegans* exposure to 5G signal at 26.5 GHz**
DATE	21/03/2024
AUTHOR(S)	Mariateresa Allocca, Stefania Romeo, Anna Sannino, Maria Rosaria Scarfi, Olga Zeni, Anna Laromaine, Pol Alonso Pernas, Amanda Muñoz-Juan
REVISED BY	Maria Rosaria Scarfi, Olga Zeni
APPROVED BY	Olga Zeni

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1. Purpose

This procedure describes the equipment and the protocol used for *C. elegans* exposure to electromagnetic fields (EMF) at 26.5 GHz, 5G signal. The aim is to ensure consistency and compliance of the bioelectromagnetic experiments with good laboratory practices.

2. Equipments and Materials

- Radiofrequency (RF) generator (Rohde and Schwarz, SMW200A) with SMW-K144 and SMW-K148 options enabled to generate 5G compliant signal
- One wideband power sensor (Rohde and Schwarz, NRP-Z85)
- One directional coupler (Marki Microwave, CA40)
- Two customized stirred reverberation chambers (RCs) characterized by the electromagnetic perspective by University of Cassino (UCAS)
- Two customized stirrer controllers driving 2 stirrers for each RC, positioned on the right (M-Destro) and the bottom (M-Sotto) walls
- Two customized polystyrene stands for cell culture dishes
- Two WR-28 open-ended waveguides

- Two coaxial cables (PL 380P-292M292M-1M)
- PC for remote control through the Power Viewer program (Rohde and Schwarz)
- Cooled incubator (PHCbi, MIR-554-PE)
- 60 mm cell culture dish (Corning, cod. 430166)

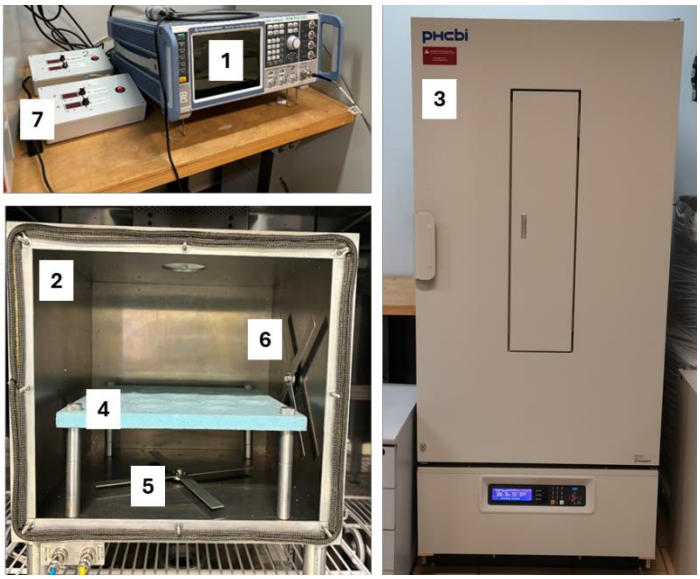

Figure 1. Expsure system setup. The signal from the RF generator (1) is sent to the RC (2) placed inside the incubator (3), together with a second RC used for sham exposure. Each RC hosts a sample holder opportunely engraved to allow reproducible positioning of the *C. elegans* culture dishes (4). The speed of the two stirrers (5,6) located in each chamber is driven by a controller (7).

3. Procedure

3.1. Preparation of *C. elegans* samples

- Prepare the following samples from the same batch of *C. elegans* cultures: incubator control, sham control, RF exposed. Label the culture dishes to decode each sample upon completion of the analysis for blind experiments: the operator who performs the analysis is not aware of the sample in hand
- Locate the dishes on the stands placed inside the RCs for exposure and sham exposure and close each RC with the metallic slabs.

3.2. RF exposure

- Turn on the generator at least 15 minutes before the start of exposure to allow warm-up
- Activate the 5G modulation (Baseband command enabled)
- Set the desired power level in dBm (set 9.70 dBm to expose the samples at SAR 1 W/kg)
- Turn on the stirrers in both RCs and set 240 for “M-Destro” and 233.3 for “M-Sotto”
- Turn on the PC and start the Power Viewer
- Connect the power sensor to the USB port of the PC

- Select the "Multi Channel" window. Channel 1 is turned off (Ch1: OFF). Move to channel 2 associated to the power sensor 2 (NRP-Z85, 102535) and set up the exposure conditions in the GUI (figure 2):
    - Frequency: 26.5 GHz
    - Averaging: manual
    - Count: 1048576
- Click on "ZERO" (with RF off) to start zeroing of the power sensor
- Click on the start button (on the top left of the toolbar) to start the visualization of the acquired data
- Select the "Data Log" window and check the following parameters:
    - Source: Multi
    - Ch1: OFF
    - Ch2: Ch 2 [W]
- Tick "Convert to Log Power," define the duration of the acquisition, tick "Log to File," set 500 ms in the "Interval" field and select the PC folder to save the data. Check that the value in the "Signal Frequency" field matches the value set on the generator (26.5 GHz)
- Click on the start button (in data log) to start data logging two minutes before the start of the exposure
- Start the RF manually from the generator
- At the end of the exposure, stop the RF manually from the generator
- Open the RCs
- Carefully remove the culture dishes from the stands
- Close the “experiment” program, switch off the PC and all the instruments
- Proceed with the harvest and the procedure for the biological assay by following the related SOP

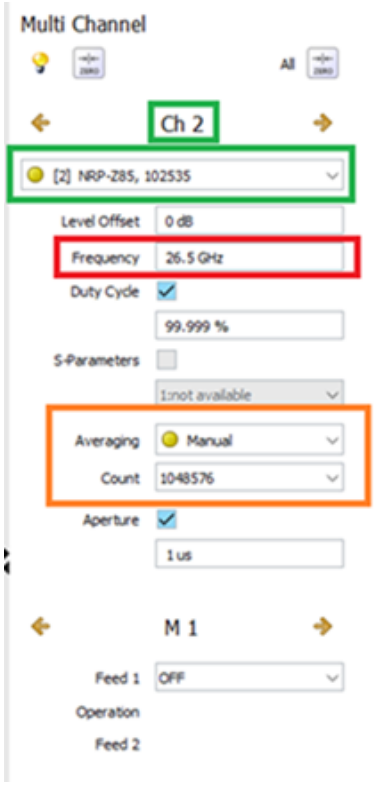

Figure 2. GUI of the Power Viewer program for the control of the exposure setup.

Standard Operating Procedure @ CNR-IREA

PROTOCOL	**Health assessment in *Caenorhabditis elegans* after 5G exposure**
DATE	15/05/25
AUTHOR (S)	Carlos López-Orts, Amanda Muñoz-Juan, Núria Garriga-Alonso, Anna Laromaine
REVISED BY	Anna Laromaine and Carlos López-Orts
APPROVED BY	Anna Laromaine

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1. Purpose

This procedure describes the materials and protocols used to evaluate the health parameters on the model organism *Caenorhabditis elegans* (*C. elegans*) after continued 5G exposure. The health parameters considered are: hatching and survival rates, worm length and motility.

2. Background

C. elegans is a small (1 mm), transparent nematode, widely used in research as a model organism for its short life cycle, extensive characterization, ease of maintenance and genetic homology with humans.

The following procedures were performed on the N2 wild-type strain of *C.elegans*, provided by the Caenorhabditis Genetics Center (CGC, University of Minnesota). For general maintenance,

worms were kept in an incubator at 20°C, and maintained on Nematode Growth Medium (NGM) agar poured into 90-mm Petri, utilizing the OP50 *Escherichia coli* strain (*E. coli*, also provided by the CGC) as its primary food source. *E. coli* was inactivated offsite with paraformaldehyde (PFA), to avoid potential cross-contamination and reduce the variability that could come from the bacteria's metabolism.

3. Health assessment

3.1 Equipment

- Stereomicroscope (EXACTA+ OPTECH SZ-NT)
- Microscope camera
- Tally counter
- Shaker (Labnet rocker S2025-XL-B-230V)
- Cooled Incubator Peltier 46L
- Glass alcohol lamp

3.2 Materials

- Serological pipettes
- Microscope slides
- Pipette tips
- Pipettes

3.3 Reagents

- M9 buffer (see SOP_C. elegans maintenance and handling)

3.4 Software

- ImageJ (FIJI)
- ToupView (ToupTek Photonics)

3.5 Data recording

- 3.5.1 Hatching

Two different procedures were implemented to monitor hatching after exposure to 5G EMF, one for each of the generations studied.

For Generation 1:

1. Perform a worm synchronization (see SOP_C. elegans maintenance and handling).
2. Determine the egg concentration of the resulting solution by pipetting 10 µl onto a microscope slide and counting the number of eggs. Repeat 8 times.
3. Add the egg count results, determine the average, and divide by the total volume used (80 µl) to obtain the estimated egg concentration in the solution.
4. Seed eggs into an NGM plate. The volume depends on the solution's concentration, but should ensure a big enough sample (at least 100 eggs).
5. Manually count the eggs under a microscope to determine the initial population, then place the plate into the respective incubator and leave it overnight.
6. Retrieve the plate and manually count both unhatched eggs and larvae. Ensure the sum of both numbers accounts for the total initial population.

For Generation 2:

1. Start with a 35 mm plate of worms exposed to 5G EMF for 72 hours (see SOP_C. elegans exposure to 5G signal at 26.5 GHz).
2. Collect adult worms by lightly washing with 3 mL of M9 buffer. Laid eggs should remain on the agar.
3. Manually count the eggs to determine the initial population.
4. Let the agar dry, place the plate into the respective incubator and leave for 24 hours.
5. Retrieve the plate and manually count both unhatched eggs and larvae. Ensure the sum of both numbers accounts for the total initial population.

When data from both generations is available, show hatching as a percentage rate by dividing the live population by the initial population and multiplying by 100. Repeat for each exposure group.

- 3.5.2 Survival

Worm survival was determined by observation and, where necessary, manual stimulation with a worm picker. Two different procedures, one for each of the generations studied, were implemented to monitor hatching after exposure to 5G EMF.

For Generation 1:

1. Perform a synchronization procedure (see SOP_ *C. elegans* maintenance and handling).
2. Leave the worm solution overnight on a shaker at 20 °C with agitation to allow for egg hatching and population arrest at the L1 stage.
3. Determine the larval concentration of the resulting solution by pipetting 10 µl onto a microscope slide and counting the number of worms. Repeat 8 times.
4. Add count results, determine the average and divide by the total volume used (80 µl) to obtain the estimated larval concentration in the solution.
5. Seed L1 into an NGM plate. The volume used depends on the solution's concentration, but it should ensure a big enough sample (at least 100 larvae).
6. Manually count the larvae under a microscope to determine the initial population, then place the plate into the respective incubator and leave for 24 hours.
7. Retrieve the plate and manually count both dead and alive larvae. Ensure the sum of both numbers accounts for the total initial population.
8. Return the plate to the incubator and leave it for another 48 h (72 hours of total incubation).
9. Repeat step 7.

For Generation 2:

1. Follow the procedure explained above to obtain hatching data for Generation 2, but do not count unhatched eggs in step 5.
2. Return the plate to the incubator and leave it for another 48 h (72 hours of total incubation).
3. Retrieve the plate and manually count both dead and alive larvae. Ensure the sum of both numbers accounts for the total initial population.

When data from both generations is available, survival is shown as a percentage rate by dividing the live population by the initial population and multiplying by 100. Repeat for each exposure group and time point.

- 3.5.3 Length

Regarding length as a tool to determine developmental effects, all stages of the *C. elegans* life cycle are thoroughly characterized, so the expected sizes for each step are well known. The procedure described below accounts for Generation 1 and Generation 2 worms.

1. Retrieve from the incubator worms exposed for 24 hours to 5G EMF (see SOP_C. elegans exposure to 5G signal at 26.5 GHz).
2. Place the plate under a stereomicroscope and take photos using ToupView software. At least 50 worms per exposure group should be assessed. Record the magnification power used.
3. Return the plate to the incubator and leave it for another 48 hours (72 hours of total incubation).
4. Repeat step 2.
5. Collect adult worms by lightly washing with 3 mL of M9 buffer. Laid eggs should remain on the agar to give rise to Generation 2.
6. Return the plate to the incubator for 24 hours to allow eggs to hatch.
7. Repeat steps 1-4.
8. Using the same magnification power recorded in step 2, place a ruler under the microscope and take a picture showing at least 1 mm.
9. Determine the appropriate scale for length measurements using the software ImageJ.
    1. Open the ruler's image file, select the Line tool, and draw a straight line measuring 1 mm on the image. Use the ruler's notches to guide you.
    2. Analyze>Set Scale will open the scale interface, which assigns the appropriate number of pixels to the measured distance of 1 mm. Make sure the Global option is marked so subsequent images share the scale.
10. Open the worm pictures according to time point and experimental group.
11. Using the Segmented Line tool, draw the worm's shape through the animal's central part. See Figure 1 for an example.
12. Ctrl+M will measure the drawn line, using the scale set in step 9.
13. Repeat steps 11-12 with at least 50 worms.
14. Repeat steps 10-13 with the remaining time points and experimental groups.
15. Data is plotted to a final graph and compared.

Figure 1: Segmented line showing the measured length for an adult *C. elegans*. Scale is shown in the lower left corner as 500 µm.

- 3.5.4 Motility

Videos will be used to study worm motility, as the frequency of bends in the worm's locomotioncan be analyzed to determine abnormal behavior or neurological disorders caused by RF exposure. The procedure described below accounts for Generation 1 and Generation 2 worms.

1. Follow steps 1-7 listed in the Length sub-section above. Rather than taking pictures of 50 worms per exposure group, take 2-minute videos of at least 5 worms per exposure group instead.
2. For each video, count the frequency of body bends in each worm. If a worm is not in the frame for 2 minutes, count how many times it bends while in frame, then extrapolate.
3. Data is plotted to a final graph and compared.

Standard Operating Procedure @ CNR-IREA

PROTOCOL	**Reproductive toxicity in *Caenorhabditis elegans* after 5G exposure**
DATE	16/05/25
AUTHOR (S)	Carlos López-Orts, Amanda Muñoz-Juan, Núria Garriga-Alonso, Anna Laromaine
REVISED BY	Anna Laromaine, Carlos López-Orts
APPROVED BY	Anna Laromaine

Index

1. Purpose

This procedure describes the materials and protocols used to evaluate reproductive toxicity on the model organism *Caenorhabditis elegans* (*C. elegans*) after continued 5G exposure.

2. Background

C. elegans is a small (1 mm), transparent nematode, widely used in research as a model organism for its short life cycle, extensive characterization, ease of maintenance, and genetic homology with humans.

The following procedures were performed on the N2 wild-type strain of *C. elegans*, provided by the Caenorhabditis Genetics Center (CGC, University of Minnesota). For general maintenance, worms were kept in an incubator at 20°C, and maintained on Nematode Growth Medium (NGM) agar poured into 90-mm Petri dishes, utilizing the OP50 Escherichia coli strain (E. coli, also provided by the CGC) as its primary food source. E. coli was inactivated off-site with

paraformaldehyde (PFA) to avoid potential cross-contamination and reduce the variability that could come from the bacteria's metabolism.

To determine the effects of 5G EMF exposure, a brood size (number of laid eggs) assay will be performed, and the percentage of hatched eggs will be quantified as a proxy for reproduction rate.

Brood size will be determined as described previously by Chawla *et al.* in the paper '*Caenorhabditis elegans* glutamylating enzymes function redundantly in male mating' (Biol Open, 2016 Sep 15;5(9):1290-8, doi: 10.1242/bio.017442). In brief, after EMF exposure, 3 randomly picked worms from each exposure group will be transferred individually onto a 12-well plate seeded with a lawn of heat-inactivated OP50. The plate will then be incubated at 20 °C without exposure to EMF, to allow worms to lay eggs. As a positive control, some wells contained fluorodeoxyuridine (FUdR), a known inhibitor of *C. elegans* reproduction that acts by preventing egg hatching (Gandhi, S. et al. A simple method for maintaining large, aging populations of Caenorhabditis elegans (Mech Ageing Dev, 1980 Feb;12(2):137-50.doi: 10.1016/0047-6374(80)90090-1).

3. Brood size assay

3.1 Equipment

- Stereomicroscope (EXACTA+ OPTECH SZ-NT)
- Cooled Incubator Peltier 46L.
- Glass alcohol lamp

3.2 Materials

- Worm picker
- Serological pipettes
- Pipette tips
- Pipettes
- 12-well plates (Corning, cod. 3513)
- Parafilm® sealing film (Merck, cod. 39219090)

3.3 Reagents

- OP50-100x (see SOP_C. elegans maintenance and handling)
- Liquid NGM agar (see SOP_C. elegans maintenance and handling)
- 5-fluorodeoxyuridine (FUdR) (Fischer Scientific, cod. 10144760) stored at 4 °C

3.4 Procedure

- 3.4.1 Plate preparation

1. Dilute FUdR in liquid NGM agar, to a concentration of 50 µM. This step requires hot NGM agar, as the agar solidifies below 42 °C, and that would impede the dilution of FUdR in the medium.
2. Dispense 2 mL of the FUdR-NGM in 3 of the wells of a 12-well plate.
3. Dispense 2 mL of regular NGM in the remaining wells.
4. Wait for the agar to cool down and solidify, then seal the plate with Parafilm and store at room temperature for up to 1 week.
5. Seed with 20 µL of OP50-100x before transferring worms.

- 3.4.2 Worm “picking”

“Picking” is a technique for removing individual worms from a plate and transferring them to a new one. To do so, a platinum wire worm picker was sterilized over an alcohol flame and allowed to cool. While looking under a stereomicroscope, the wire was then used to carefully lift the worm from the agar and immediately deposit it in its new environment. This procedure used a glass alcohol lamp to provide a sterile bubble over the working station.

- 3.4.3 Reproductive toxicity assessment

To assess any effects on reproductive capabilities, single worms from the three exposure conditions were removed through “picking” onto individual wells on a 12-well NGM plate seeded with OP50.

Since worms are hermaphrodites capable of self-fertilization, one adult worm can give rise to around 300 offspring. After 72 hours of incubation at 20°C, the percentage of hatched eggs was assessed by checking each plate. Any unhatched eggs were considered sterile, and thus, a percentage of hatched eggs was calculated based on the total brood size.

The procedure described below accounts for Generation 1 and Generation 2 worms.

1. Retrieve from the incubator worms exposed for 24 hours to 5G EMF (see SOP_C. elegans exposure to 5G signal at 26.5 GHz).
2. “Pick” a single worm into one of the regular NGM wells. Repeat 2 more times to obtain 3 replicates.
3. Repeat step 2 with worms of the remaining exposure groups.
4. “Pick” a single non-exposed worm into one of the FUdR-NGM wells. Repeat 2 more times to obtain 3 replicates.
5. Return the plate to the incubator and leave it for another 48 hours (72 hours of total incubation).
6. Calculate the percentage of hatched eggs based on the total brood size.
7. Repeat steps 2-6, using a fresh 12-well plate.
8. Collect adult worms by lightly washing with 3 mL of M9 buffer. Laid eggs should remain on the agar to give rise to Generation 2.
9. Return the plate to the incubator for 24 hours to allow eggs to hatch.
10. Repeat steps 1-7.

Standard Operating Procedure @ ICMAB-CSIC

PROTOCOL	**RNA extraction from *C. elegans*.**
DATE	20/05/2024
AUTHOR(S)	Amanda Muñoz-Juan, Pol Alonso, Anna Laromaine
REVISED BY	Anna Laromaine
APPROVED BY	Anna Laromaine

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1. Purpose

This procedure describes the materials and protocol used to extract RNA from *C. elegans* after exposure to electromagnetic fields.

2. Background

Environmental changes could alter organisms' genetic expression to adapt to external conditions. We could measure this change by extracting and sequencing the acid ribonucleic (ARN) material. ARN is the translation of specific genes from the organism's acid deoxyribonucleic (DNA) that are transformed into proteins by ribosomes to perform a function. In that way, organisms only produce the proteins required to respond to variable environmental conditions. We previously exposed worms to external electromagnetic waves with their respective control conditions. Worms were exposed for three consecutive generations. They were recollected, cleaned from bacteria and frozen in trizol reagent. Samples were sent from Naples to Barcelona, Spain, for further processing. See

Annex 15 for more information about the exposure conditions. This protocol explains how RNA was extracted and purified for further evaluation.

3. Procedure

3.1. Equipments

- Thermo shaker (Biosan ts-100)
- Table centrifuge (J.P. Selecta. S.A. 7002239)
- Vortex Ibx instruments V05 series)
- -80°C Ultrafreezer (Telstar Boreas)
- Laminar flux hood (ESCO Laminar Flow Cabinet)
- Nanodrop (Nanodrop One C, Thermo Scientific)

3.2. Materials

- Ice
- Polystyrene boxes
- Eppendorf® tubes (Labbox, 1.5 ml, PCRP-015-500)
- Falcon tubes (Labbox, 15 ml, CTPG-E15-050)

3.3. Reagents

- Ethanol (99%) Panreac AppliChem 361086.16153
- N_2 liquid
- Trizol (15596026 Thermo Fisher)
- PureLink™ RNA Mini Kit (12183018A, Invitrogen by Thermo Fisher) Contains spin cartridges with collection tubes, collection tubes, recovery tubes, wash buffer I, wash buffer II, RNAse- and free water.

3.4. Experimental procedure

The experimental procedure is divided into two main steps: worms' lysis and RNA extraction.

3.4.1. Worm's lysis

This step is essential to break all tissues and extract the RNA from the cytoplasm of cells.

1) Defrost worms already in Trizol. The volume of Trizol should be seven times higher than the initial pellet of worms.
2) Vortex for 30 seconds at maximum speed (no units) to resuspend everything.
3) Freeze worms with N2 liquid.
4) Thaw worms in the Thermo shaker at 37°C or in a water bath at 37°C.
5) Repeat steps 3 and 4 five times.
6) Vortex worms for 30 seconds at the maximum speed and let the sample rest for another 30 seconds.
7) Repeat step 6 for five times.
8) Wait 30 seconds at room temperature to allow RNA complexes and proteins to break down.

9) Centrifuge at 2600 g for 5 minutes. Separate the supernatant in sterile falcon tubes. Transfer 1.2 ml of supernatant to these tubes.

Note

- When working with N_2 liquid, take precautions and wear all safety equipment (cold protective gloves, safety glasses, and lab coat). Take a small Polystyrene box and a plastic Eppendorf rack. Remove the Eppendorf rack with tweezers to avoid touching the N_2 liquid.
- For the vortex cycles, divide the tubes into two groups. While one group rests after the vortex, the other is in the vortex. This tip accelerates the process while keeping the optimal conditions.

3.4.2. RNA extraction

After lysis, RNA molecules are mixed with proteins, cellular residues, and other molecules. Therefore, RNA should be purified. PureLink™ RNA Mini Kit is used following the manufacturer's instructions.

1) Add the same volume of 70% Ethanol to these tubes. Add 1.2 ml of 70% EtOH, freshly prepared with sterile water and pure Ethanol.
2) Vortex each tube for 30 seconds to mix everything well to reduce non-homogeneous formation.
3) Transfer 700 µl of the sample (including any remaining precipitate) to the spin cartridge (with the collection tube).
4) Centrifuge at 12000 g for 20 seconds at room temperature. Discard the flow-through and reinsert the spin cartridge into the same collection tube.
5) Repeat steps a-b until the entire sample has been processed.
6) Add 700 µl Wash Buffer I to the spin cartridge.
7) Centrifuge at 12000 g for 20 seconds at room temperature. Discard the flow-through and the collection tube. Insert the spin cartridge into a new collection tube.
8) Add 500 µl of Wash Buffer II to the spin cartridge.
9) Centrifuge at 12000 g for 20 seconds at room temperature. Discard the flow-through, and reinsert the spin cartridge into the same collection tube.
10) Repeat steps 8 and 9 once.
11) Centrifuge at 12000 g for 2 minutes at RT. Discard the flow-through and the collection tube. Insert the cartridge into a recovery tube.
12) Add 50 µl RNAse-free water to the center of the spin cartridge.
13) Incubate at RT for 1 minute.
14) Centrifuge at 12000 g for 2 minutes at RT. Discard the cartridge, close the recovery tube, and store your purified RNA immediately on ice.

Note

- 70% Ethanol was freshly prepared by diluting pure ethanol (99%) with sterile MilliQ® water in the laminar flux cabin.
- Keep samples in ice during this protocol.

- Before using the PureLink™ RNA Mini Kit read carefully the preparation instructions, as you should add pure ethanol to buffer II before using it. Keep them at RT for storage and in the fridge for long periods.
- When centrifuge recovering tubes with the spin cartridge, order them well in the centrifuge to break the tap.

3.4.3. RNA quantification

After purification, RNA quantity should be quantified using a Nanodrop equipment to know the concentration and the purity of these molecules. For quality analysis, two ratios are used:

- Ratio between 260 and 280 nm (A260/A280) represents the contamination by DNA molecules. DNA molecules strongly absorb at 280 whereas DNA molecules absorb at 260 nm. Values between 2-2.2 or higher are considered high-quality levels.
- Ratio between 260 and 230 (A260/A280) represents contamination from chemical reagents used for the extraction procedure. Values around 2.2 or higher indicate good quality.

Procedure

1) Switch on Nanodrop and select the RNA measurement.
2) Clean the measurement area with MilliQ® water and paper.
3) Use the RNA-see free water as a background control for Nanodrop equipment. With sterile tips put 2 µl of water in the area of measurement. Close Nanodrop arm. Measurement starts automatically.
4) Clean the measurement area with MilliQ® water and paper.
5) Introduce the name of the sample you are going to measure.
6) With sterile tips, put 2 µl of the extracted RNA into the measurement area. Close Nanodrop's arm. Measurement starts automatically. Take a picture of the graph on the screen and take notes of the concentration (ng/µl), A260/A280, and A260/230.
7) Repeat steps 4-6 until every sample has been measured.
8) Export data through the pen drive entrance and make a copy of the raw data.
9) Clean the measurement area and switch off Nanodrop.
10) Store your RNA at -80°C until further evaluation.

3.4.4. Sample analysis

Samples should be sent to a company that translates the RNA into complementary DNA (cDNA), amplify the molecules through the PCR technique and sequence each read. Information about the company, how to prepare and send samples, and the quality studies performed by the company.