Project**: SEAWave**

Testing for aneugenicity and disturbances of the spindle apparatus

Work Package:	WP8
Deliverable:	D8.3
Deliverable No.:	D29

Abstract

In Work Package 8, we investigated whether the fifth generation of mobile telephony frequency range 2 (5G FR2) has effects on aneugenicity. Primary epidermal adult and juvenile keratinocytes (NHEK-c and NHEK-f.c., respectively) and adult melanocytes (NHEM) were exposed to 5G FR2 millimeter waves at 27.5 GHz with different power densities (10 W/m², 3.33 W/m², and sham exposure) and were further incubated for 1.5 to 2 cell cycles to allow the formation of micronuclei, before being subjected to centromere and telomere staining using fluorescence in situ hybridization (FISH) with peptide nucleic acid probes and 4',6-diamidino-2-phenylindole (DAPI) as nuclear counterstain. Preliminary data from the micronucleus assay showed that in NHEKs 4 h of exposure to 5G FR2 did not result in any increase in micronucleus frequency. In parallel, images of the telomeres were captured by confocal microscopy for final telomere length measurement. The software for analyzing telomere length was calibrated using the expected highest (sham-exposed NHEK-f.c.) and lowest (senescent NHEK-c.) telomere intensities. As of July 30th, 2025, respective analyses are still ongoing.

Project Details

Project name	SEAWave
Grant number	101057622
Start Date	01 Jun 2022
Duration	42 months
Scientific coordinator	Prof. Th. Samaras, Aristotle University of Thessaloniki (AUTH)

Deliverable Details

Deliverable related number	D8.3
Deliverable No.	D29
Deliverable name	Testing for aneugenicity and disturbances of the spindle apparatus
Work Package number	WP8
Work Package name	Health Risk Studies: Biological Effects of FR2 Exposure on Human-derived Skin Cells
Editors	Dr. A. Bitsch, M. Djuari, Dr. C. Ziemann, ITEM
Distribution	Public
Version	1
Draft/final	Final
Keywords	Aneugenicity, PNA FISH, micronucleus test

Contents

1 Introduction

In work package (WP) 8 of the SEAWave project, we are investigating the biological effects of fifth generation of mobile telephony in frequency range 2 (5G FR2) in *in vitro* settings. One of the main focuses is to examine the carcinogenic potential of 5G FR2 exposure on human skin and thus to estimate potential adversity of electromagnetic fields (EMF) on human health.

In addition to induction of oxidative DNA lesions (oxidative stress) and transcriptomic and epigenetic changes, which were reported in deliverable D8.2, another endpoint to be considered to assess the carcinogenic potential of 5G FR2 in human skin cells is the induction of aneugenicity, which is the main objective of D8.3. Like epigenetic changes, aneugenicity might be involved in cancer development. Aneugenicity can, for example, occur via interactions of insults with the spindle apparatus, specific chromosome structures and regulators of the cell cycle.

Aneugenicity represents a change in chromosome number from the normal diploid or haploid number of chromosomes. The consequence of aneuploidy is a difference in the number of gene copies, also called the gene dose, that are located on the missing or additional chromosome, and thus a difference in the amount of the respective gene products. A multitude of cellular structures may serve as targets for aneugenicity induction. Among these are centromeres and telomeres, kinetochores and chromatid glue proteins, tubulin, microtubule-associated proteins, centrioles and other parts of the spindle apparatus. Furthermore, regulators of the cell cycle including cyclins, cyclin-dependent kinases and p53 as well as the cellular or nuclear membranes are also critical sites for toxic events potentially resulting in aneugenicity, as reviewed by Kirsch-Volders, et al., 2002 and 2019 [1, 2].

To assess above-mentioned changes, micronucleus assays were performed. A micronucleus (MN) represents a broken chromosome fragment or an entire chromosome, which remains in the cytoplasm after cell division. MN can be formed following direct DNA damage (clastogenic event), where a part of a chromosome can be found in the MN. Otherwise, a MN with a whole chromosome can be formed following disturbance of the spindle apparatus (indirect, aneugenic mechanism; see Figure 1) [3, 4].

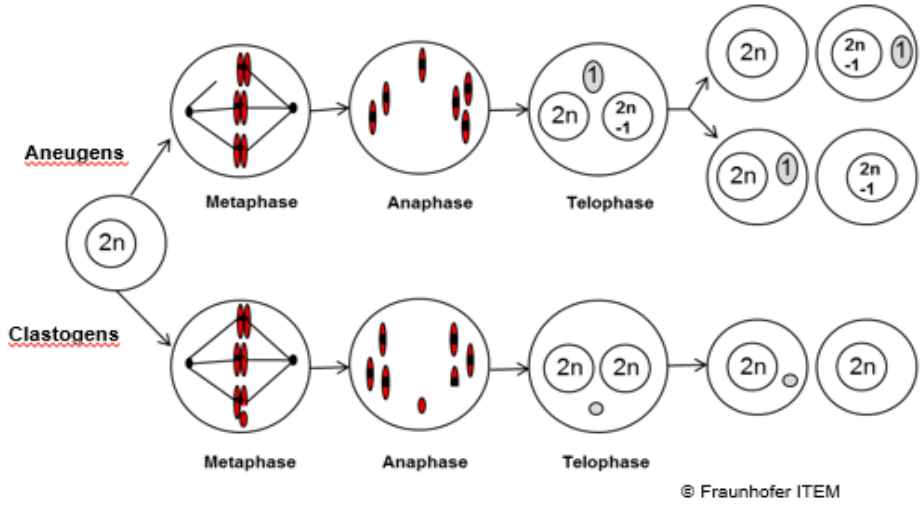

Figure 1. Schematic overview of induction of micronuclei in daughter cells after cell division exposure of parent cells to aneugens and clastogens.

For this assay, the nuclear DNA is initially stained using the fluorescent dye 4',6-diamidino-2-phenylindole (DAPI). To differentiate whether a detected MN after exposure to 5G FR2 results from clastogenic or aneugenic events, centromeres and telomeres were additionally stained using fluorescence in situ hybridization (PNA FISH) with peptide nucleic acid (PNA) probes. PNA-FISH is a highly sensitive and specific method for detecting certain nucleic acid target sequences within fixed cells. The high sensitivity and specificity of PNA probes is based on the strong binding affinity of the probes to DNA. Another advantage of PNA probes is their uncharged, neutral backbone, compared to the negatively charged backbone of DNA, which further stabilizes the bond between the PNA probe and cellular DNA.

In parallel, the PNA-FISH staining also serves for investigation of telomere length, as certain environmental stressors can trigger telomere shortening to a critical length, which can be related to, e.g. metabolic and inflammatory diseases and genomic instability [5].

The fluorescence telomere signal intensity from PNA FISH staining is proportional to telomere length. Therefore, telomere length is measured using an image analyzer, which quantifies the intensity of the signal. The quantification process begins with identifying the nucleus, followed by locating the telomere spots within the nucleus perimeter. The analyzer then calculates the number of telomere spots and the average telomere signal intensity for each nucleus [6].

For aneugenicity testing using the micronucleus test, normal adult or juvenile human epidermal keratinocytes (NHEK-c. and NHEK-f.c., respectively) and normal adult human epidermal melanocytes (NHEM) were exposed to 5G FR2 for 4 and 24 h and then given time to recover and to proliferate (important prerequisite for MN induction) for 1.5 – 2 cell cycles after the end of the

exposure. Centromeres and telomeres were stained using PNA-FISH and the cell nucleus was counterstained with DAPI. A total of 2000 cells were counted to estimate the micronucleus frequency after exposure to 5G FR2 and to differentiate micronuclei with regard to only containing telomeres (clastogen) or also centromeres (aneugen).

2 Methods

2.1 Cell culture

The original vials of keratinocytes and melanocytes from PromoCell (Heidelberg, Germany) were immersed in a 37 °C water bath until the cells detached from the vial wall. Immediately afterwards, the thawed cells were poured into pre-warmed Keratinocytes Growth Medium 3 or Melanocyte Growth Medium M3 (PromoCell) in a T75 cell culture flask and the vial was washed once with the cell suspension. The cells were then incubated for 24 h in an incubator at 37 °C, 5% CO_2, and 95% relative humidity. After 24 h of pre-culture, the medium was replaced with fresh cell culture medium to remove remaining freezing medium and cells were incubated until 90% confluence was reached.

To prepare and freeze working cell batches for a set of 5G FR2 exposures, cells were washed twice with 4 ml of pre-warmed phosphate buffer saline (PBS) and trypsinized using a 0.05 % trypsin/ 0.02 % EDTA solution (PAN Biotech, Aidenbach, Germany) for 5 min at 37 °C. Afterwards, trypsin inhibitor and growth medium were pipetted into the cell suspension to quench trypsin activity. The cell suspension was subsequently transferred into a centrifuge tube and centrifuged at 303 x g for 5 min. After removing the cell supernatant, the cell pellet was resuspended in fresh pre-warmed CryoSFM freezing medium (PromoCell) in the needed cell density, followed by transfer of the cell suspension to cryovials. Cells were finally frozen using a Planer Kryo 10 Series II freezing device (Planer, Sunbury on Thames, United Kingdom) and the cell working batches were then stored in the gas phase of nitrogen tank.

A week prior to 5G FR2 exposure, a vial from the working batch was thawed and incubated until exposure using the method described above. The cells were then pre-incubated for 72 h (NHEK) or 96 h (NHEM) in an incubator at 37 °C, 5% CO_2, and 95% relative humidity prior to cell plating before 5G FR2 exposure.

2.2 Cell exposure to 5G FR2

The cells were exposed to 5G FR2 at 27.5 GHz with 100 MHz bandwidth using the exposure system developed by IT'IS Foundation (Zürich, Switzerland). The system comprises a computer-controlled system rack with radiofrequency (RF) source, power supplies, and data acquisition, as well as three shielded chambers to operate in three different power densities of 10 W/m^2 (high power density or high), 3.33 W/m^2 [low power density (low)] and sham (no 5G RF2) exposure.

The built-in sensors monitor and record the exposure conditions and control exposure, such as temperature and fan current for airflow needed for the cell cultures. Each shielded chamber can cover four T25 flasks inserted directly into an internal support structure.

A pre-check of this exposure system and exposure validation included the measurement of heat development and the effect on survival and growth of cells in culture.

At 24 (NHEK) or 72 h (NHEM) prior to 5G FR2 exposure, the cells were trypsinized using the method described under 2.1 but resuspending the cell pellets in cell growth medium instead of CryoSFM freezing medium. The cells were then plated in at minimum three T25 cell culture flask per exposure condition with cell densities between $0.375 \cdot 10^6$ and 10^6 cells/flask, depending on the assay type. On the day of exposure, the cell culture medium in the cell culture flask was replaced with 8 ml of fresh growth medium.

The cells were then inserted into the exposure chambers. If there were < four flasks with cells to be exposed in each chamber, additional T25 cell culture flasks, filled with 8 ml of cell culture medium without cells, were added to the empty slots in the chambers to ensure accurate exposure to 5G FR2. Afterwards, the exposure settings were defined in the controller software and the exposure was started with 30 min delay to ensure temperature stabilization in the incubator harboring the exposure units. The cells were exposed in a blinded manner, where the power density of each chamber is selected randomly by the computer and hidden during exposure. Decoding of the blinded experiments was done by IT'IS Foundation after finishing exposure analysis.

The exposure duration was chosen based on available publications on *in vitro* studies. Since there were only a few studies available, which investigated biological effects of 5G FR2, we also included *in vitro* publications, examining exposure to frequency range 1 (FR1) microwave. In total 380 *in vitro* publications were found during a search in literature databases, such as EMF Portal, PubMed, Scopus and ScienceDirect. After excluding studies with inadequate exposure setup and reviews, 164 publications were selected for further analyses. In these publications, in total of 14 species and 65 cell types were examined. The examined exposure durations ranged from 5 min to 6 days for neuronal cells, with most publications examining the effects of electromagnetic fields (EMF) for 1, 2, 4 or 24 h. Based on these findings, 4 and 24 h exposure duration was chosen.

2.3 Cell harvest and fixation

After 5G FR2 exposure, the cells were incubated further for another 1.5 – 2 cell cycles to allow for development of micronuclei. The cells were then detached using the methods described in 2.1 without the centrifugation and resuspension steps. The cell suspension was subsequently transferred to 6 x 1.5 ml reaction tubes and centrifuged for 5 min at 106 x g. Cell supernatant was removed and the cells were resuspended in 250 µl PBS. Afterwards, the cell suspensions were dripped onto frozen SuperFrost glass microscope slides (Epredia, Michigan, United States of America) using a Pasteur pipette, followed by a drying step on a hot plate at 65 °C. The slides

were then fixed in a 1:3 mixture of acetic acid (Roth, Karlsruhe, Germany) and methanol (Roth, Karlsruhe, Germany) at room temperature for 5 min. The slides were then stored at -20 °C until further use. As technical positive control for the micronucleus assay, NHEK and NHEM were treated with 9.7 mM (1 µl/ml) ethyl methanesulfonate (EMS, clastogenic compound) for 1 h and incubated further for 1.5 – 2 cell cycles prior to cell harvest and fixation.

2.4 Telomere and micronucleus staining and analysis

The PNA FISH hybridization was performed based on the available protocol from PNABio (California, United States of America) with modifications [7]. Prior to staining, the slides were dehydrated by incubating the slides in 70%, 85% and 100% ethanol (Applichem, Darmstadt, Germany) for 2 min each. The slides were then air-dried for 30 min. Afterwards, the slides and centromere and telomere probe mix [20 mM Tris (Applichem, Darmstadt, Germany), 60% formamide (Roth, Karlsruhe, Germany), 0.5% blocking reagent (Roche, Basel, Switzerland), 500 nM Cy3-labeled telomere probe (Eurogentec, Seraing, Belgium) and 500 nM Alexa488-labeled centromere probe (Eurogentec, Seraing, Belgium)] were pre-heated at 85 °C for 5 min. Subsequently, 50 µl of the probe mix were added to each slide. A plastic stripe was added, and slides were incubated for 10 min at 85 °C, and then at room temperature for 2 h in a wet chamber in the dark. The slides were subsequently washed twice in washing solution [2X SSC (Roth, Karlsruhe, Germany) and 0.1% Tween-20 (Roth, Karlsruhe, Germany) in nuclease-free water (Qiagen, Hilden, Germany)] at 60 °C for 10 min, and then once for 2 min at room temperature.

After hybridization, cellular DNA was counter-stained using a 1:750 dilution of 0.5 mg/ml DAPI solution in 2X SSC (both Roth, Karlsruhe, Germany). The slides were incubated for 10 min at room temperature and washed once in 1 x PBS (Roth, Karlsruhe, Germany) for 2 min. The slides were then dried and 50 µl Antifade Vectashield mounting medium (VectorLabs, California, United States of America) was added before sealing the slides with coverslips.

For analysis of the micronucleus frequency, micronuclei per 2000 cells analyzed were counted manually using a Zeiss Axio Imager 2 fluorescence microscope. When a micronucleus was detected, it was checked for the presence of centromere and telomere signals to determine whether the micronucleus contained an acentric chromosome fragment or a whole chromosome.

For telomere length measurement, images of 40 nuclei per treatment were acquired using a Zeiss Axio Observer.Z1 LSM 890 with Airyscan confocal microscope equipped with a 63x oil objective using a 548 nm (Cy3) and 353 nm (DAPI) laser. Telomere intensity from the images was then analyzed using a specialized software developed in-house by the working group of Prof. Dr. Christian Bär and Dr. Shambhabi Chatterjee (Medicine Highschool Hannover and Fraunhofer ITEM; unpublished yet).

3 Results

As of July 30, 2025, the 5G FR2 exposure for the detection of micronuclei and telomere length measurements has been completed in all primary keratinocytes and melanocytes for both 4 h and 24 h, with 3 independent experiments conducted on 3 different days. We performed PNA FISH and DAPI staining on all fixed cells that were exposed to 5G FR2 for 4 h and captured images for telomere length measurement using a confocal microscope. For the 24 h exposure to 5G FR2, DNA staining and imaging are planned to be completed by the end of August. The analysis of the micronucleus test and telomere length is still ongoing.

3.1 Micronucleus test

In a first step, the sensitivity of the primary keratinocytes and melanocytes to the direct clastogen ethyl methanesulfonate (EMS) as technical positive control was determined. In this experiment, the cells were treated with two different concentrations of EMS, 4.85 mM (0.5 µl/ml) or 9.7 mM (1 µl/ml) for 1 h and incubated further for 1.5 – 2 cell cycles. While NHEM showed a significant EMS-induced increase in micronucleus frequency, the NHEK-c. showed only a slightly higher micronucleus number. Thus, a higher concentration of EMS is needed as technical positive control for NHEK. When comparing the respective negative controls, primary keratinocytes had a significantly lower basal micronuclei frequency per 2000 cells in comparison to NHEM (see Figure 2).

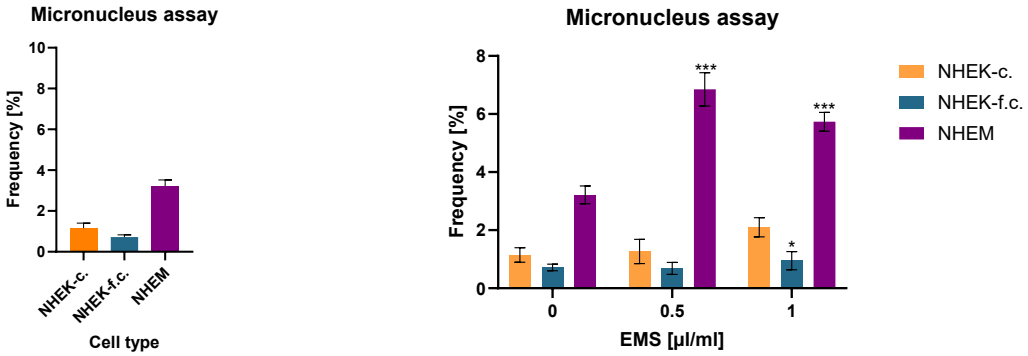

Figure 2. Basal micronucleus frequency (left) and micronucleus frequency after a 1 h treatment with 0.5 µl/ml or 1 µl/ml EMS (right) in NHEK-c., NHEK-f.c. and NHEM. Data represent means ± SD of three independent experiments. Statistics was done using One-Way ANOVA with Dunnett's multiple comparisons test, as post hoc test, compared to untreated slides, where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

After 4 h of exposure to 5G FR2, NHEK-c and NHEK-f.c. showed no increase in micronucleus frequency across all exposure groups, according to preliminary data. In NHEK-c, all detected micronuclei contained whole chromosomes, whereas in NHEK-f.c., a small proportion of the detected micronuclei also contained acentric chromosome fragments. However, analysis of the technical positive control EMS is not yet completed, and thus the overall picture remains unclear.

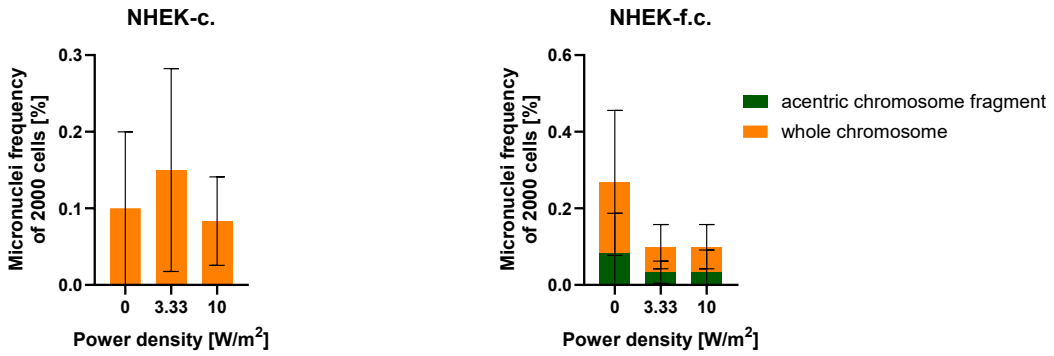

Figure 3: Micronucleus frequency in 2000 cells for NHEK-c (left) and NHEK-f.c. (right) after 4 h of exposure to 5G FR2. The bars show both the frequency of micronuclei, containing acentric chromosome fragments (green) and whole chromosome (orange). The sum of both colors represents the total micronucleus frequency. Data represent means ± SD of three independent experiments (preliminary data).

3.2 Telomere length measurement

The first step for measuring the telomere length is to image successfully stained cells using confocal microscopy, which can deliver images with sufficiently high resolution. Figure 4 shows representative images of nuclei from NHEK-f.c. (upper left) and NHEM (upper right) after 4 h of blinded 5G FR2 exposure. As technical positive controls, cells were used, which were cultured until progression into cellular senescence with the typical bloated morphology (see Figure 4).

The intensity of the telomere (orange) signal at the perimeter of the DAPI-stained nuclei was measured. To set up the measurement, samples from each group with the expected highest and lowest telomere intensities were used to calibrate the specialized in-house software. Preliminary data showed that senescent cells, generated by extended culture over X days/passages exhibited a lower number of telomere spots in the nuclei, compared to healthy, sham-exposed cells. Interestingly, the relative telomere signal intensity of each spot was relatively similar within each group (see Figure 5).

In this preliminary experiment, the analyzer detected up to 200 to 300 telomere spots per nucleus, which exceeded the expected maximum of 92 spots for humans, as human has 2 telomere ends on each of the 46 chromosomes. This discrepancy is primarily due to the image analyzer's inability to distinguish nuclei that are closely adhered to one another, leading the software to combine the telomere spots from all adherent nuclei into a single data point of one nucleus. These errors will be corrected in the final rounds of telomere analysis, measuring 5G FR2 exposed skin cells.

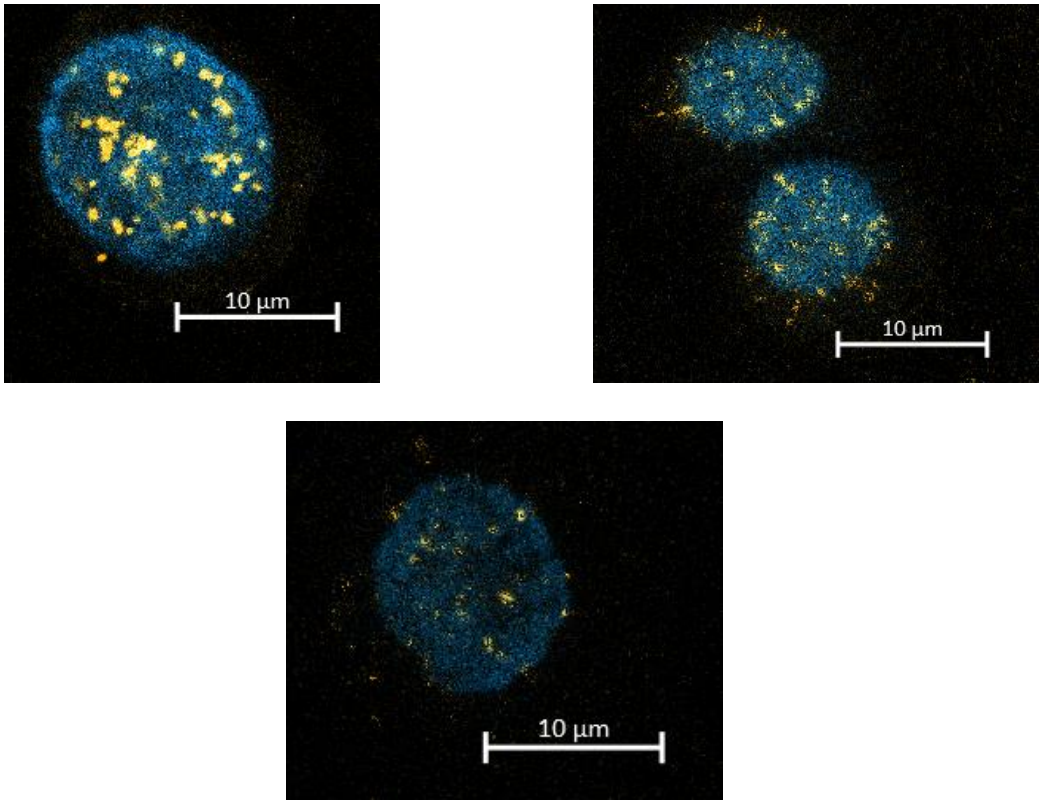

Figure 4. Images of NHEK-f.c. (upper left) and NHEM (upper right) after 4 h of 5G FR2 exposure and further incubation for 1.5 – 2 cell cycles as well as NHEK-c. in passage 6 (below) both stained using PNA-FISH with a Cy3-telomere probe and DAPI staining, and imaged by confocal microscope using 63x magnification.

Figure 5. Preliminary data from telomere length measurements, done by quantification of both telomere signals/spots (left) and averaged telomere signal intensity (right) of each nucleus out of 40 nuclei in total per cell types from 4 h sham-exposed NHEK-f.c. and senescent NHEK-c. (extended culture duration), representing suggested highest and lowest telomere intensities, used to calibrate the in-house analysis software. Each black circle represents one out of 40 nuclei analyzed.

4 Conclusions

In conclusion, preliminary data from the micronucleus assay on NHEK exposed to 5G FR2 for 4 h showed no increase in micronucleus frequency across all exposure groups, thus pointing to no clastogenic or aneugenic potential was present for 5G FR2 exposure, which is in line with negative comet assays, as presented in D8.2. However, the analysis of the positive control is not yet complete, and thus the overall picture remains unclear.

For telomere length measurements, images of PNA-FISH-stained telomeres and DAPI-stained nuclei have been captured and are currently undergoing analysis. The analysis software has been calibrated to ensure accurate measurement. As of July 30, 2025, all cell types have been exposed to 5G FR2 for both 4 h and 24 h. The analyses of both micronuclei and telomere length measurements are still ongoing.

5 References

1. Kirsch-Volders M, Pacchierotti F, Parry EM, Russo A, Eichenlaub-Ritter U, Adler I-D. Risks of aneuploidy induction from chemical exposure: Twenty years of collaborative research in Europe from basic science to regulatory implications. Mutation Research/Reviews in Mutation Research. 2019;779:126-47.
2. Kirsch-Volders M, Vanhauwaert A, De Boeck M, Decordier I. Importance of detecting numerical versus structural chromosome aberrations. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. 2002;504(1):137-48.
3. Beedanagari S. 4.11 - Genetic Toxicology. In: Chackalamannil S, Rotella D, Ward SE, editors. Comprehensive Medicinal Chemistry III. Oxford: Elsevier; 2017. p. 195-203.
4. Fenech M, Kirsch-Volders M, Natarajan AT, Surralles J, Crott JW, Parry J, et al. Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. 2011;26(1):125-32.
5. Kong CM, Lee XW, Wang X. Telomere shortening in human diseases. FEBS J. 2013;280(14):3180-93.
6. O'Sullivan JN, Finley JC, Risques RA, Shen WT, Gollahon KA, Rabinovitch PS. Quantitative fluorescence in situ hybridization (QFISH) of telomere lengths in tissue and cells. Curr Protoc Cytom. 2005;Chapter 12(1):Unit 12 6.
7. PNA Bio. PNA FISH (Fluorescence In Situ Hybridization) Protocol. 25.07.2025. Available from: https://pnabio.com/pdf/FISH_protocol_PNABio.pdf.