Standard Operating Procedure @ CNR-IREA

PROTOCOL	**Reproductive toxicity in *Caenorhabditis elegans* after 5G exposure**
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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.