Standard Operating Procedure @ CNR-IREA

PROTOCOL	**Health assessment in *Caenorhabditis elegans* after 5G exposure**
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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.