Protocol for live-cell imaging during Tumor Treating Fields treatment with Inovitro Live

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Tumor Treating Fields (TTFields) are an FDA-approved anti-cancer treatment using alternating electric fields. Here, we present a protocol to perform live-cell imaging (LCI) of cells during TTFields treatment with the Inovitro Live™ system. The setup we describe dissipates TTFields-related heat production and can be used in conjunction with any LCI-compatible microscope setup. This approach will enable further elucidation of TTFields’ mechanism of action at the molecular level and facilitate the development of promising combination strategies.

Highlights
- Inovitro Live™ allows concurrent live-cell imaging and TTFields treatment
- Suitable for use with both 2D- and 3D-cultured cells
- Describes multiple strategies to dissipate TTFields-associated heat
- Step-by-step description on how to operate the Inovitro Live™ software
Protocol for live-cell imaging during Tumor Treating Fields treatment with Inovitro Live

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SUMMARY

Tumor Treating Fields (TTFields) are an FDA-approved anticancer treatment using alternating electric fields. Here, we present a protocol to perform live-cell imaging (LCI) of cells during TTFields treatment with the Inovitro Live™ system. The setup we describe dissipates TTFields-related heat production and can be used in conjunction with any LCI-compatible microscope setup. This approach will enable further elucidation of TTFields’ mechanism of action at the molecular level and facilitate the development of promising combination strategies.

BEFORE YOU BEGIN

The previously described Inovitro™ system (Porat et al., 2017) allows in vitro application of Tumor Treating Fields (TTFields) of different frequencies (50–500 kHz) and intensities (≤ 3 V/cm). Furthermore, the Inovitro™ system is able to dissipate TTFields-related heat production while maintaining tight control over the medium temperature. Recently, Inovitro Live™ was developed, which allows simultaneous application of TTFields and live-cell imaging (LCI), which is made possible by a transparent bottom. Additionally, Inovitro Live™ can be used in conjunction with any LCI-compatible microscope setup including CO₂ gas supply. The protocol below has been performed with the cancer cell lines U-251MG (RRID:CVCL_0021) and U2OS (RRID:CVCL_0042). However, all steps can be performed with any type of adherent cell line.

Preparations

@ Timing: 30–120 min

△ CRITICAL: Due to TTFields-associated heat production, the Inovitro Live™ setup needs to be able to dissipate heat to maintain stable and robust control over the temperature. This can be achieved with a stage top chamber or by utilizing a cooling coil, as explained below.

1. Setup for temperature regulation:
a. Using a silicone cooling coil (Figure 1A).
   i. Pull silicone tubing (outer ø=0.91 mm) through a small piece (4 mm) of plastic tubing (outer ø=6.35 mm) three times to create a cooling coil.
   ii. Pull the ends of the cooling coil through the bore hole in the Inovitro Live™ insert.
   iii. Connect one end of the cooling coil to a peristaltic pump.
   iv. The speed of the peristaltic pump determines the cooling efficiency.
   v. Connect the other end of the cooling coil to an ice bath.
b. Using a temperature-regulated microscope stage top chamber (Figure 1B)

2. Sterilize a clean Inovitro Live™ insert (Figure 2F) using an autoclave (121°C, 30 min)
3. Connect all Inovitro Live™ devices (Figure 2).
   a. Place the Inovitro Live™ generator (Figure 2A) on a horizontal surface, close to the microscope.
   b. Place the laptop with Inovitro Live™ software within 1.5 m from the generator.
   c. Connect the laptop to the Inovitro Live™ generator by connecting the USB-A to mini B cable (Figure 2G) to the frontal port of the Inovitro Live generator.
   d. Connect the Inovitro Live™ cable (Figure 2C) to the frontal port of the Inovitro Live™ generator in such a manner that the cover heating element and connector are able to reach the Inovitro Live™ insert on the microscope stage.

⚠️ CRITICAL: The cooling efficiency of the cooling coil will differ in each laboratory setup. Therefore, it is important that the Inovitro Live™ setup is first tested at multiple surrounding temperatures to acquire corresponding currents (mA) (explained further below), which can be mimicked with the cooling coil.

4. Perform all preparation steps noted below, but without cultured cells.
5. Start multiple optimization experiments at surrounding temperatures that are going to be used in future experiments.
   a. Maximum TTFields intensity that can be applied before encountering major medium evaporation issues is ≈ 4.6 V/cm pk-pk at a surrounding temperature of 18°C, corresponding to an average current of ≈ 200 mA.
6. For each tested surrounding temperature, evaluate the applied average currents from the log files (Figure 3D).
7. In future experiments, increase the cooling efficiency of the peristaltic pump by increasing its velocity until the applied currents match the average currents from your optimization experiments.
Note: If it is not possible to use either the cooling coil or the stage top chamber, environment cooling can be achieved by decreasing the temperature of the microscope room. However, due to heat production by the microscope, the temperature surrounding the Inovitro Live™ dish has to be closely monitored and optimized if necessary.

Cell culture

© Timing: 30 min

8. Prepare cell suspension for seeding.
   a. Seeding density should be determined by the researcher. Seeding density is dependent on multiple factors, including:
      i. The experiment duration and cell doubling time.
      ii. Cell motility.
      iii. Cell size.
      iv. E.g., for U-251MG, with a doubling time of \( = 18 \) h, 7,500 cells are plated for an experiment that lasts 48 h. Confluency is reached at \( = 30,000 \) cells.
9. Add 300 μL of cell suspension in LCI-medium to an Ibidi 35 mm high wall glass bottom dish (Figure 2E) and incubate in a CO₂ incubator (5%) at 37°C until cells have attached (∼3 h).

10. Fill the Ibidi dish to 3 mL with LCI-medium.

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and virus strains** | | |
| pLenti-PGK-Neo-PIP-Fucci | Grant et al., 2018 | Addgene: 118616 |
| mCherry-BP1-2 pLPC-Puro | PMID 18931659 | |
| **Chemicals, peptides, and recombinant proteins** | | |
| MEM, no glutamine, no phenol red | Gibco | Cat#1200038 |
| Fetal Bovine Serum (FBS) | Gibco | Cat#12657011 |
| Penicillin-Streptomycin | Gibco | Cat#15140122 |
| Sodium Pyruvate | Thermo Fisher Scientific | Cat#11360039 |
| Non Essential Amino Acids | Thermo Fisher Scientific | Cat#11140035 |
| L-Glutamine | Thermo Fisher Scientific | Cat#25030024 |
| SiR-DNA | Spirochrome | Cat#SC007 |
| TMRM | Invitrogen | Cat#I34361 |
| MitoTracker™ Green FM | Invitrogen | Cat#M7514 |
| **Experimental models: cell lines** | | |
| U251 (RRID:CVCL_0021) human GBM cells | Sigma-Aldrich | Cat#09063001 |
| U2OS (RRID:CVCL_0042) human osteosarcoma cells | ATCC | Cat#92022711 |
| **Software and algorithms** | | |
| Inovitro Live™ Software | Novocure | n/a |
| ImageJ | Schneider et al., 2012 | https://imagej.nih.gov/ij/ |
| **Other** | | |
| Inovitro Live™ Insert | Novocure | n/a |
| Inovitro Live™ Generator | Novocure | n/a |
| Inovitro Live™ Cable | Novocure | n/a |
| Laptop with Inovitro Live™ Software | Novocure | n/a |
| USB-A to mini B cable | n/a | n/a |
| Peristaltic Pump PP1300 | VWR | Cat#181-4002 |
| Silicone Tubing (outer ø=0.91 mm) | VWR | Cat#GESSULTRA-C-020-0F |
| Peristaltic Pump Tubing (outer ø=3.2 mm) | VWR | Cat#GESSULTRA-C-062-1F |
| FEP/PTFE tubing (outer ø=6.35 mm) | VWR | Cat#SERSP42083-0014 |
| Water Jacket Chamber | Okolab | H101-K-FRAME |
| K-Frame Insert | Okolab | H101-2x35-M |
| Temperature Controller | Okolab | H101-CRYO-BL-T |
| Syringe PP/PE without needle | Sigma-Aldrich | Cat#2116866 |
| μ-Dish 35 mm, high Glass Bottom | Ibidi | Cat#811158 |
| DIC lid for μ-Dishes | Ibidi | Cat#80050 |
| Andor 505 Dragonfly | Andor | n/a |
| Zyla 4.2+ sCMOS camera | Andor | n/a |

### MATERIALS AND EQUIPMENT

**LCI Medium (store at 4 °C for a maximum duration of 3 months.)**

| Component | Final concentration | Amount |
|-----------|---------------------|--------|
| MEM, no glutamine, no phenol red | n/a | 500 mL |
| Fetal Bovine Serum | 9% | 50 mL |
| Penicillin-Streptomycin | 90 μg/mL | 5 mL |

(Continued on next page)
**STEP-BY-STEP METHOD DETAILS**

**Setting up Inovitro Live™**

© Timing: 30 min

In the following steps, all procedures that have to be performed prior to the start of an experiment are discussed.

1. Place the Ibidi dish with cells and the sterile Inovitro Live™ insert (Figure 2F) in a clean biological hood.
2. Place the Ibidi Glass cover (Figure 2D) on the Inovitro Live™ insert.
3. Insert the Inovitro Live™ insert onto the Ibidi dish.
4. Press down firmly until the insert reaches the bottom (Figure 3A).
   - Stage Top Chamber: Make sure that silicone tubing (Figure 2B) is in place for medium replacement.
   - Cooling Coil: Make sure that cooling coil is in place.
5. Transfer the Ibidi dish with insert horizontally and place it on the microscope stage.
   - Fix it to the stage firmly using a designated connector or glue pads.
6. Connect the Inovitro Live™ cable (Figure 2C) to the insert (Figure 3B).
7. Place the cover heating element over the Inovitro Live™ insert (Figure 3C).
8. Connect CO₂ gas supply to the microscope chamber.
9. Switch on the generator and laptop.
10. Open the Inovitro Live™ software.

**Note:** In this protocol, the microscope chamber is assembled by combining an Okolab H101-K-Frame in conjunction with the Okolab H101-2x35-M insert for 35 mm petri dishes. Although this combination guarantees the Inovitro Live™ setup to fit in the microscope chamber, other microscope chambers with a minimum chamber height of 30 mm may be used.

**Using Inovitro Live™ software**

© Timing: 5 min

In the following steps, all options and functions in the Inovitro Live™ software (Figure 4) are explained.

11. Create a new experiment by pressing the ‘New Experiment’ button (Figure 4A).
   - In the pop-up window, the experiment name, owner, and a short description can be filled in.
     1. Use only regular characters (letters and numbers) for experiment name and owner
   - Disable the “configure session” option.
12. Set the desired parameters correctly (Figure 4B).
   - The frequency of TTFields can be applied between 50 to 500 kHz.
     1. E.g., U251 is treated with 200 kHz.
   - Medium target temperature can be set between 20°C to 40°C.
d. Cover heating element temperature can be set between 37°C to 55°C.
e. Duty cycle A and B represent the frequency of switching between TTFields directions. Set either A or B to ‘0’ to apply TTFields from a single pair of electrodes.

13. Adjust alarm thresholds by pressing the ‘Change Default’ button.
   a. If the medium temperature exceeds the set value, an alarm will be displayed.

14. While the experiment is running (Figure 4C), the following values will be displayed:
   a. Frequency
   b. Target temperature
   c. Temperature (2x)
   d. Cover Temperature
   e. Current (2x)
   f. Resistance (2x)
   g. Vout

15. During an experiment, log graphs (Figure 4D) can be requested by pressing the ‘reports’ button.

Start & end TTF treatment

© Timing: Dependent on experimental setup

16. Set the treatment settings using the Inovitro Live™ software and start the experiment.
17. Depending on the cooling strategy:
   a. Stage top incubator: Set the temperature of the stage top incubator to the desired value, ranging from 18°C-32°C, with lower temperatures leading to higher TTFields intensities.
b. Cooling coil: Start the peristaltic pump at the lowest speed and allow the medium temperature to stabilize. Gradually increase the pump speed until desired current is reached.

c. To prevent disruption of flow of the peristaltic pump:
   i. Check if the silicone tubing is firmly placed in the pump.
   ii. Check if no connections between the silicone tubing are leaking.
   iii. Make sure the cold water inflow tubing is not blocked.

18. Meanwhile, position the dish with cells at the region-of-interest and select the desired microscope settings (channels, exposure times etc.) using the microscope software.

19. Follow the experiment for 20–30 min until the medium temperature has stabilized and, if necessary, adjust the focus of the microscope as temperature changes can affect focusing.

20. To end the experiment, press the stop experiment button in the software (Figure 4).
   a. Allow time for all data to be transferred from the generator to the laptop/computer.

21. Turn off the generator and disconnect all cables from the insert.

22. Remove the Inovitro live™ insert from the dish by pulling vertically.

23. Rinse the insert thoroughly with distilled water and let dry.

24. Sterilize the insert in an autoclave before the next experiment.

**Optional:** When using a stage top incubator in experiments that last longer than 24 h, the medium has to be replaced daily.

25. Connect a sterile 5 mL syringe to the silicone tubing (Figure 2D).

26. Pause the experiment by clicking the pause button in the software (Figure 4).

27. Remove the medium using the syringe.

28. Carefully inject 3 mL new medium with a new, sterile syringe.

**Note:** The cover heating element can become warm. To avoid skin burns, only touch it after sufficient cooling time (>1 min.) after turning off the system.

**Note:** The slightest movement of the dish could impede cell tracking during long experiments. Make sure you are not putting any tension on the dish, cables, or silicone tubing.

**EXPECTED OUTCOMES**

LCI is widely used to obtain a better understanding of biological function through the study of a wide range of cellular processes. The options of Inovitro Live™ application are similarly extensive, with several examples of such applications as high-resolution cell cycle analysis (Figure 5A, Methods video S1), imaging of mitochondrial structure (Figure 5B), cytoskeletal changes (Figure 5C), and DNA damage repair capacity (Figure 5D). The ability to perform TTFields treatment during LCI will greatly benefit research focusing on a better understanding of this treatment modality. Additional to elucidating TTFields’ exact anti-neoplastic properties, LCI of combination strategies can support the translation of promising strategies to clinical implementation.

**LIMITATIONS**

Although Inovitro Live™ is currently the only device that allows investigation of cellular dynamics by microscopy in TTFields treated cells, the system does possess several limitations.

While Inovitro Live™ allows dissipation of excessive heat produced by TTFields while maintaining tight control of the medium temperature, the use of a stage top incubator as a cooling method will result in minimal medium evaporation. Therefore, for experiments studying cellular processes that may be dependent on changes in osmotic parameters, the silicone cooling coil method is preferred.

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Since Inovitro Live™ regulates TTFields intensity according to surrounding temperature, the surrounding temperature has to be homogeneous. As a result, immersion lenses cannot be used. Since the oil/water immersion will be in direct contact with the glass bottom of the culture dish, the heat produced by the microscope will be conducted through the immersion fluid to the culture medium. This will heavily impede TTFields intensities reached by Inovitro Live™. Therefore, only dry lenses can be used, resulting in both lower quality images and limited magnification.

Although peristaltic pumps are very reliable in maintaining constant flow of a liquid through silicone tubing, occasionally the flow is disturbed for a period of time. As a result of disturbed cooling, the TTFields intensity is lowered. After the experiment, the TTFields intensity can be evaluated in the experimental log file.

**TROUBLESHOOTING**

**Problem 1**
Cells will not attach to glass (step 9).

**Potential solution**
The Ibidi μ-Dish 35 mm, high Glass Bottom can be coated with poly-L-lysine, which could increase cell adherence. If this is not sufficient, the μ-dish is also available with a tissue culture-treated polymer coverslip (Cat# 81156), which also improves cell attachment. We do, however, suggest using the
glass coverslip when possible due to higher optical quality. When culturing stem cells, the polymer dishes can be coated with coatings that preserve stem cell-like properties (e.g., laminin, collagen etc.). Additionally, both the glass and polymer μ-Dish can be used for 3D microscopy when using a microscopy-compatible matrix.

**Problem 2**
Out-of-focus images during time-lapse experiments as a result of temperature-related focus drift (step 29).

**Potential solution**
As a result of the temperature gradient, temperature-related focus drift might affect your image quality. To prevent this, several steps can be taken. First, wait until the medium temperature has stabilized at 37°C after start of TTFields treatment. Second, make sure that the ambient temperature in the microscope room and microscope incubator are stable all throughout the day, as air conditioning/heating systems can influence this. Last, use additional hardware (e.g., Zeiss Definite Focus.2) or software-mediated (e.g., Zen2 Software Autofocus) focusing to correct for focus drift.

**Problem 3**
Flow of the peristaltic pump got interrupted, resulting in a drop in TTFields intensity (step 1)

**Potential solution**
Check if the silicone tubing is firmly placed in the pump. Also check if no connections between the silicone tubing are leaking. Make sure the cold water inflow tubing is not blocked.

**Problem 4**
Excessive medium evaporation during the experiment (step 18).

**Potential solution**
Use a gas humidifier to increase the humidity of inflow gasses in the stage top incubator. Else, create a humid chamber using moist towels and/or increase frequency of medium replacement.

**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, [Gerben Borst] (Gerben.borst@nhs.net).

**Materials availability**
This study did not generate new unique reagents.

**Data and code availability**
This paper does not generate any dataset or code.

**SUPPLEMENTAL INFORMATION**
Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101246.

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**AUTHOR CONTRIBUTIONS**
P.S. drafted the manuscript with input from all other authors. Y.P. and M.G. designed and provided the Inovitro Live™ equipment. M.M provided help with the microscope setup. B.v.d.B and K.J.
provided help in designing the dish cooling. M.d.G and O.v.T. provided helpful discussions. G.B. supervised the study.

DECLARATION OF INTERESTS

Y.P. is an employee and shareholder of Novocure, which has applied for a patent for the use of a ‘System for Viewing Cell Cultures under a Microscope Whilst Applying TTFields’ (US20180202991A1).

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