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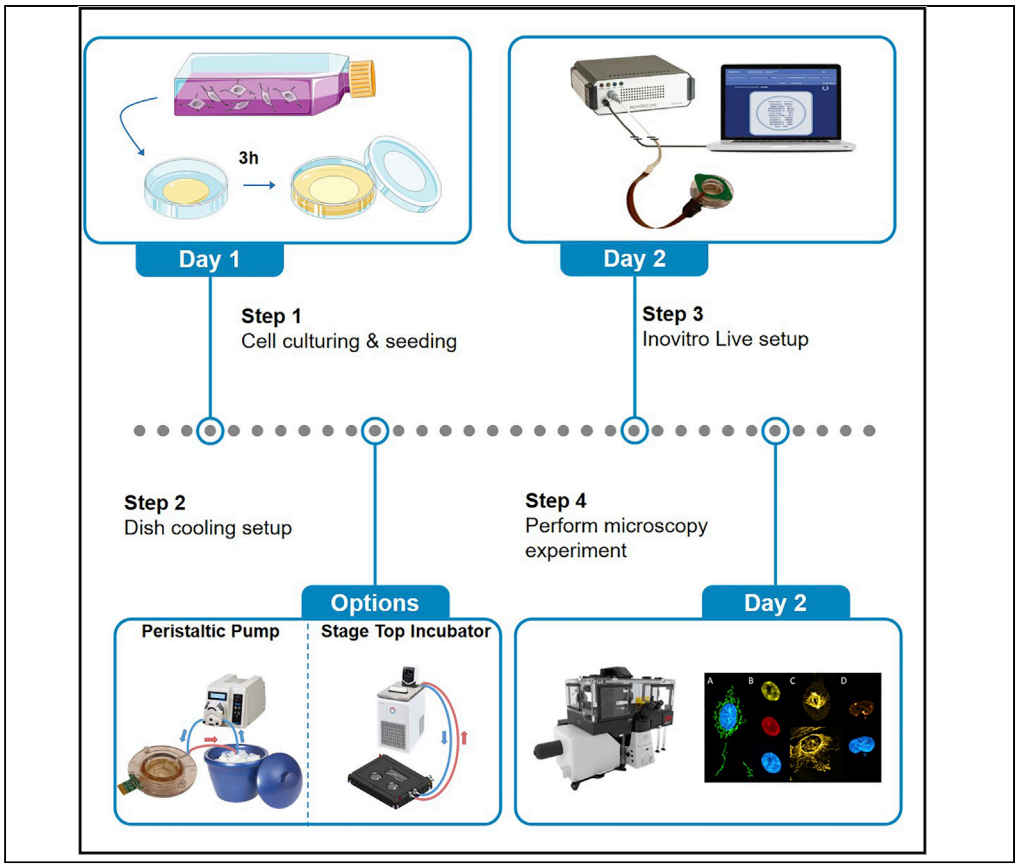
Protocol for live-cell imaging during Tumor Treating Fields treatment with Inovitro Live



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 conjuction 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.

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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

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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 conjuction with any LCI-compatible microscope setup including CO_2 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:

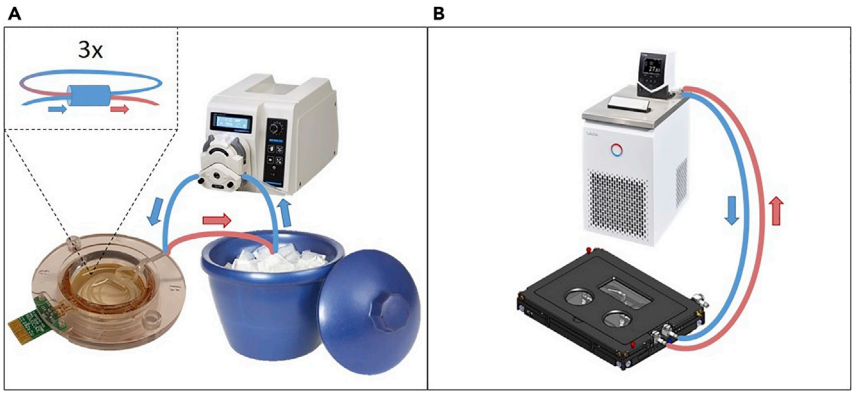

Figure 1. Inovitro Live™ cooling options

(A and B) The peristaltic pump transports ice-cold water through the cooling coil that is placed directly in the culture medium in the Inovitro Live™ insert (A); The temperature controller transports cold water through the water jacket of the stage top incubator (B).

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.

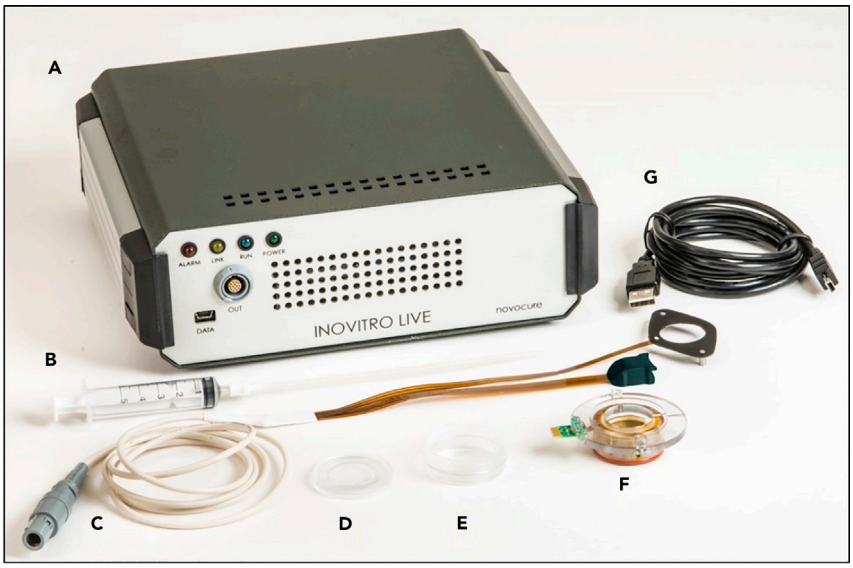

Figure 2. Inovitro Live™ equipment

An overview of the equipment to be used with the Inovitro Live™ setup, consisting of: TTFields generator (A); medium replacement tube (B); Inovitro Live™ cable (C); Glass lid for Ibidi μ-dish (D); Ibidi μ-Dish (E); Inovitro Live™ insert (F); USB cable (G).

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.

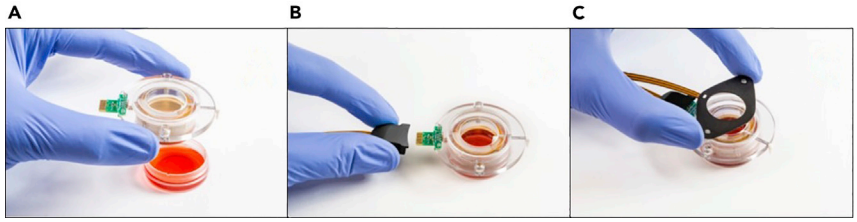

Figure 3. Inovitro Live™ dish preparation

(A–C) Preparing the Inovitro Live™ dish prior to an experiment; Inserting the Inovitro Live™ insert (A); connecting the Inovitro Live™ cable (B); connecting the cover heating element (C).

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 CO_2 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#51200038
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#SCERSP420823-0014
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#Z116866
µ-Dish 35 mm, high Glass Bottom	Ibidi	Cat#81158
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.)		
	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)

Continued

	Final concentration	Amount
Sodium Pyruvate	0.9 mM	5 mL
Non-Essential Amino Acids	0.9×	5 mL
L-Glutamine	1.8 mM	5 mL
Total	n/a	570 mL

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).
   a. Stage Top Chamber: Make sure that silicone tubing (Figure 2B) is in place for medium replacement.
   b. 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.
   a. 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 CO_2 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).
    a. In the pop-up window, the experiment name, owner, and a short description can be filled in.
       i. Use only regular characters (letters and numbers) for experiment name and owner
    b. Disable the ''configure session'' option.
12. Set the desired parameters correctly (Figure 4B).
    a. The frequency of TTFields can be applied between 50 to 500 kHz.
       i. E.g., U251 is treated with 200 kHz.
    c. Medium target temperature can be set between 20°C to 40°C.

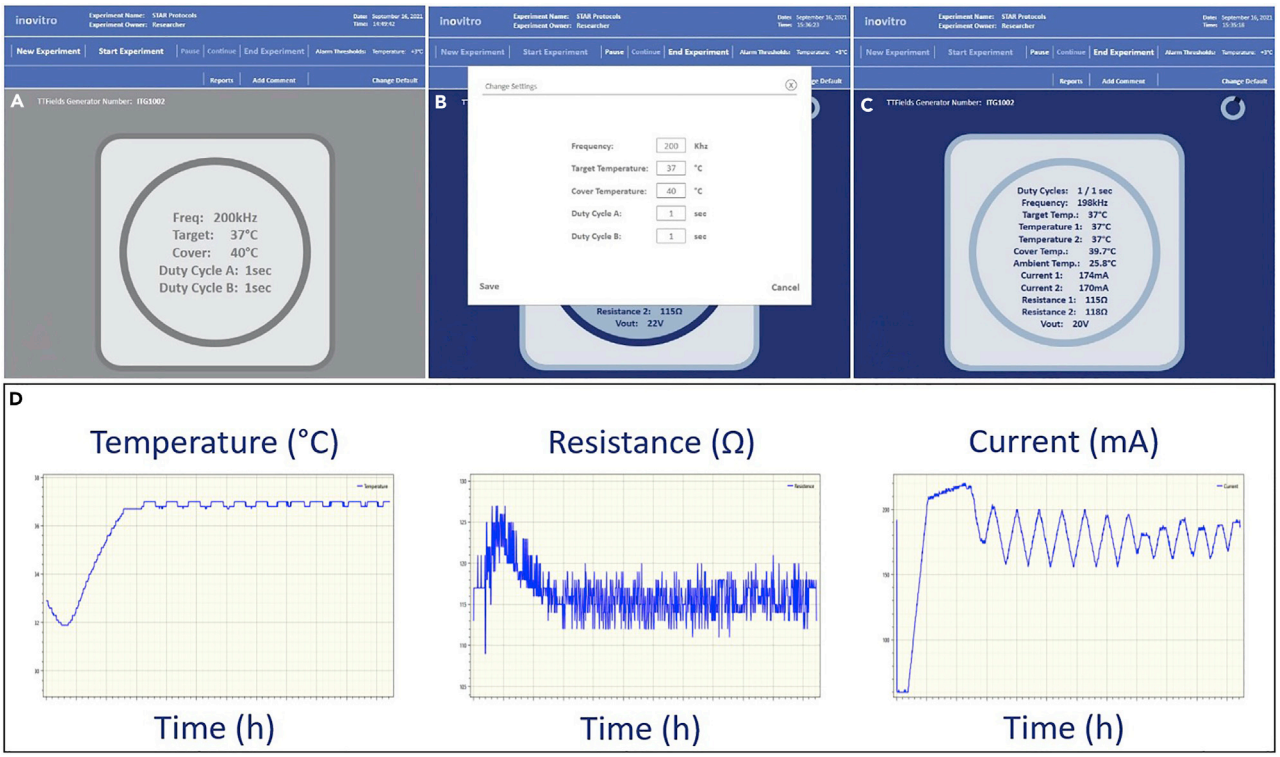

Figure 4. Inovitro Live™ software

A view of the software that is used to control the Inovitro Live™ setup, depicting the main screen (A); panel to change parameter settings (B); ongoing experiment screen displaying all dish parameters (C); graphs of log files over time (D).

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 (2×)
    d. Cover Temperature
    e. Current (2×)
    f. Resistance (2×)
    g. V_{out}
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.

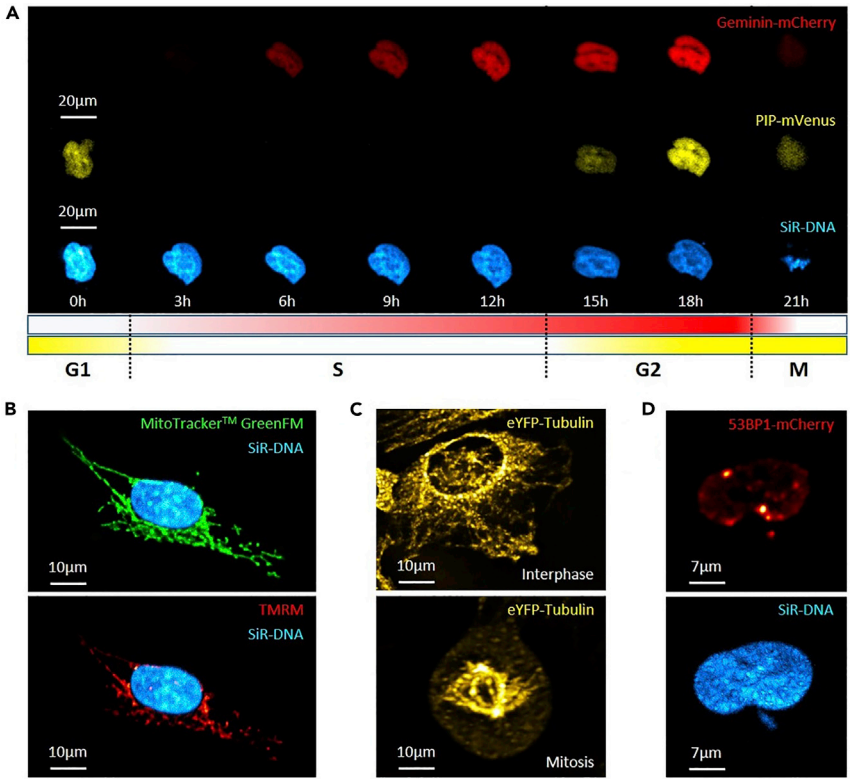

Figure 5. Inovitro Live™ application examples

Cell cycle analysis using PIP-FUCCI; SiR-DNA (cyan), PIP-mVenus (yellow), Geminin-mCherry (red) in U251 cells (A); Determining mitochondrial function;); MitoTracker Green FM (green), TMRM (red) and SiR-DNA (cyan) in U251 cells (B); Investigating cell morphology; eYFP-Tubulin (yellow) in U2OS cells (C); Analysis of DNA damage response; 53BP1-mCherry (red) and SiR-DNA (cyan) in U251 cells (D). Spinning-disk confocal microscopy was performed using an Andor 505 Dragonfly system equipped with a 20× 0.75 NA objective and a Zyla 4.2+, sCMOS camera. Z-stacks were acquired using the 25 µm pinhole disk with a step size of 0.5 µm.

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.

ACKNOWLEDGMENTS

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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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