SARTORIUS

Protocol

Neurite Outgrowth and Cell Health Analysis Protocol

For the Demonstration of Label-Free Neurite Outgrowth and Neuronal Cell Health

This protocol demonstrates Incucyte® label-free measurements of neurite outgrowth multiplexed with real-time measures of neuronal cell health. Incucyte® Nuclight Red Neuro-2a cells are treated with retinoic acid to induce neurite formation and are cultured in the presence of decreasing serum concentrations to generate treatment groups with varying populations of viable and apoptotic cells. Neurite length and cell health are quantified in real time using the Incucyte® Neurotrack Analysis Software Module and Incucyte® Annexin V Green Dye.

Materials Supplied by Sartorius

- Incucyte® Nuclight Red Neuro-2a Cells (Sartorius Cat. No. 4512)
- Incucyte® Annexin V Green Dye (Sartorius Cat. No. 4642)

Materials to be Supplied by Customer

- F-12K Nutrient Mixture (1X) (Thermo Fisher Cat. No. 21127022)
- Fetal Bovine Serum (Sigma Aldrich Cat. No. F2442)
- Puromycin (Thermo Fisher Cat. No. A1113803)
- TrypLE Express Enzyme (Thermo Fisher Cat. No. 12605010)
- D-PBS (without Ca2+, Mg2+) (Thermo Fisher Cat. No. 14190136)
- Retinoic Acid (Sigma Aldrich Cat. No. R2625-50MG)
- 96-well flat-bottom microplate (Corning Cat. No. 3595)

Protocol

Day -3

1. Prepare serum-free and complete media.
   a. Transfer 100 mL of F-12K media into two sterile bottles. Label one bottle "serum-free media". Do not add anything to this bottle as it will be used to prepare the compound plate.
   b. To the second bottle add 10 mL of FBS and 5.5 µL of 10 mg/mL puromycin and label "Neuro-2a complete media" (final media concentrations 10% FBS and 0.5 µg/mL puromycin).
2. Thaw the Nuclight Red Neuro-2a cells.
   a. Warm the complete Neuro-2a media in a water bath prior to use.
   b. Add 20 mL of Neuro-2a complete media to one T75 flask.
   c. Remove one vial of Nuclight Red Neuro-2a cells from liquid nitrogen and thaw in a 37° C water bath for approximately 90 seconds, swirling gently. Remove vial when only a tiny ice crystal remains.
   d. Transfer the cells to the media in the T75 flask using a 5 mL serological pipette.
   e. Rinse the vial with 1 mL of Neuro-2a complete media and transfer to the T75 flask.

Day -2

3. Replace culture media with 20 mL fresh complete Neuro-2a media. Monitor cells in the Incucyte® Live-Cell Analysis System to and allow cells to grow until 70–80% confluent.

Day 0

1. Plate cells at 4,000 cells/well in complete media into a 96-well plate.
   a. Remove medium from the Nuclight Red Neuro-2a cells and gently rinse twice with D-PBS.
      Note: Culture should be at 70–80% confluence in a T75 flask.
   b. Harvest cells and perform a cell count (e.g., trypan bluestaining + hemacytometer).
   c. Dilute cells to 40,000 cells/mL and dispense 100 µL per well in columns 1–4 of a sterile 96-well Corning plate to obtain a density of 4,000 cells/well.
   d. Allow plate to sit at ambient temperature for 30 minutes and place in the incubator 3 hours prior to treatment.
2. Create a 10 mM stock of retinoic acid.
   a. Using a 50 mg ampule of retinoic acid (MW = 300.44) gently tap the ampule to move all powder to the bottom and crack open the ampule. Add 1.664 mL DMSO to the ampule using a 1000 µL micropipette and triturate using a 200 µL micropipette until the retinoic acid is completely in solution (solution will be yellow and transparent), making a 100 mM retinoic acid solution.
   b. Transfer 200 µL of 100 mM retinoic acid from the ampule into a tube containing 1.8 mL DMSO to create a 10 mM retinoic acid stock solution.
3. Prepare assay media.
   a. Make 20 mL of a 25 µM retinoic acid by adding 50 µL of 10 mM retinoic acid stock solution to 19.95 mL serum-free F-12K Nutrient Mixture media in a 50 mL conical tube. This will be your Assay Media for setting up the compound plate with the Annexin V Green Dye.
      Note: Retinoic acid has poor aqueous solubility and should be vortexed thoroughly after adding it to the serum free media.
4. Make compound plate.
   a. Make up 2 mL of 12% FBS by adding 240 µL FBS to 1.760 mL Assay Media. This is 1.5X the final assay concentration of 8%. It will be used as the top concentration for the dilution series.
   b. Add 125 µL of Assay Media to rows B-H in columns 1–4 of a sterile 96-well plate.
   c. Add 250 µL of Assay Media containing 12% FBS to row A of columns 1–4.
   d. Serially dilute the FBS 1:2 by transferring 125 µL from row A into row B and mixing 10X. Repeat until row G. Leave row H untouched, as that will contain 0% FBS.
5. Transfer compound plate to cell plate.
   a. Remove cell plate from incubator and place in hood.
   b. Aspirate all media from the wells and gently wash with 100 µL per well of pre-warmed, serum-free F12K Nutrient Mixture media. Ensure all media is removed from plate after this wash step.
   c. Using a multichannel pipette, gently transfer 100 µL from each well of the compound plate to the cell plate.
6. Addition of Annexin V Green Dye.
   a. To one vial of Annexin V Green Dye add 100 µL of Assay Media. Transfer the contents of the vial to 6.7 mL of Assay Media.
   b. Using a multichannel pipette, add 50 µL of Assay Media containing Annexin V Green Dye to all wells in the cell plate (final assay Annexin V Green Dye dilution 1:200).
7. Remove any bubbles from all wells by gently squeezing a wash bottle (containing 100% ethanol with inner straw removed) to blow vapor over the surface of each well. Keep the tip of the wash bottle approximately 5 cm from the surface of the medium.
8. Position the de-bubbled cell plate in the Incucyte® Live-Cell Analysis System and allow to equilibrate for 20 min prior to the first scan. Schedule 24-hour repeat scanning (10X) for every 4 hours for 3 days.
   a. Objective: 10X.
   b. Vessel Type: Corning 3595.
   c. Scan Mode: Standard.
   d. Scan Pattern: 4 images per well.
   e. Channels: Phase + Green + Red.

Plate Map Set Up

	1	2	3	4	5	6	7	8	9	10	11	12
A	Retinoic Acid 25 µM FBS 8% Neuro-2a (1) 4K cells/well Annexin V Green											
B	Retinoic Acid 25 µM FBS 4% Neuro-2a (1) 4K cells/well Annexin V Green											
C	Retinoic Acid 25 µM FBS 2% Neuro-2a (1) 4K cells/well Annexin V Green											
D	Retinoic Acid 25 µM FBS 1% Neuro-2a (1) 4K cells/well Annexin V Green											
E	Retinoic Acid 25 µM FBS 0.5% Neuro-2a (1) 4K cells/well Annexin V Green											
F	Retinoic Acid 25 µM FBS 0.25% Neuro-2a (1) 4K cells/well Annexin V Green											
G	Retinoic Acid 25 µM FBS 0.13% Neuro-2a (1) 4K cells/well Annexin V Green											
H	Retinoic Acid 25 µM Neuro-2a (1) 4K cells/well Annexin V Green											

Analysis

Two separate Incucyte® analysis jobs must be run to quantify both neurite morphology and neuronal cell health.

1. Neurite Analysis

Neurotrack: Run a Neurotrack analysis job for the phase contrast channel to quantify Neurite Length.

Note: You will have the option to quantify nuclear counts using the Neurotrack analysis job, however, we recommend that the Basic Analyzer is used to quantify both the nuclear counts Annexin V objects to enable easy cross comparison of cell viability data.

Suggested analysis parameters for Phase Neurotrack:

- Segmentation Mode: Texture
- Min Cell Width: 15.0
- Neurite Sensitivity: 0.5
- Neurite Width: 2

2. Quantification of Apoptotic Cells and Viable Cell Count

Basic Analyzer: Run a Basic Analyzer job for both the red and green fluorescent channels. We recommend using Red Object Count to quantify fluorescent nuclei (viable cells), and Green Object Confluence to measure the Annexin V Green response (apoptotic cells).

Note: Retinoic acid may fall out of solution over the course of the assay forming small auto fluorescent green particles. These particles can be removed from the Annexin V Green object analysis by setting a minimum green channel area filter threshold of around 50 µm^2.

Suggested analysis parameters for Annexin V Green processing definition:

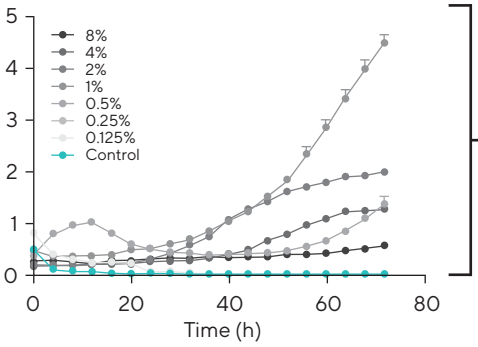
- Top-Hat analysis, radius 100 µm, threshold at 2.0 GCU
- Edge Split On, Sensitivity at -30
- Area Filter, min set at 50 µm^2

Suggested analysis parameters for Red Nuclear Count processing Definition

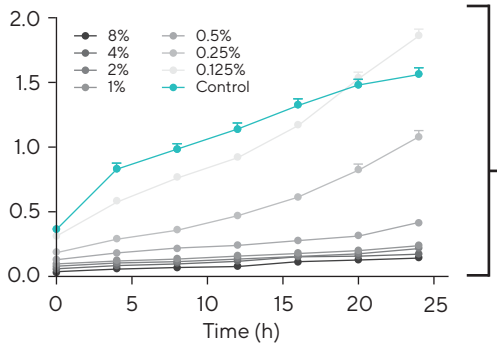
- Top-Hat analysis, Radius 100 µm, threshold at 2.0 RCU

Example Data

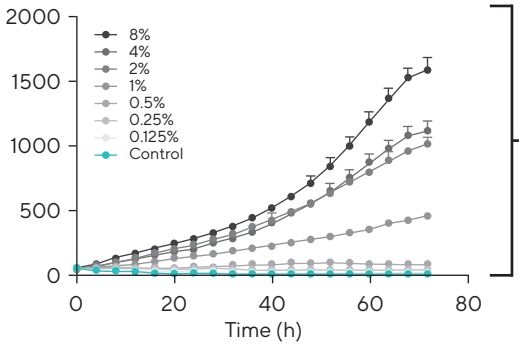
Neurite Length
FBS Concentration Response
Neurite Length (mm/mm²)



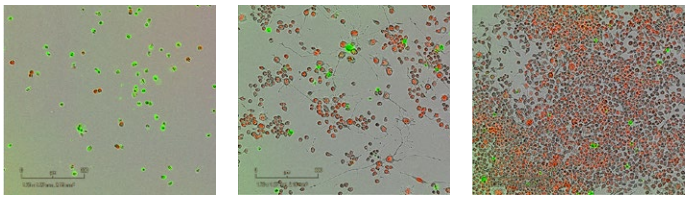
Annexin V Green
FBS Concentration Response
Green Object Confluence (%)



Red Nuclear Count
FBS Concentration Response
Red Object Count (1/mm²)



Incucyte® Images and Metrics at 72 Hours



	0.125 FBS	1% FBS	8% FBS
Neurite Length	0.01 mm/mm²	6.01 mm/mm²	0.55 mm/mm²
Annexin V	2.97%	0.92%	0.67%
Nuclear Count	14.4 nuclei/mm²	439 nuclei/mm²	2069 nuclei/mm²

Expected Results and Interpretation

Neuro-2a cells are a mouse neuroblastoma cell line and proliferate in growth media containing 8 to 10% serum. Neuro-2a cells can be differentiated into a neuronal-like morphology by reducing the serum concentration towards 2% and treating with 25 µM retinoic acid. Differentiated cells will form neurites and cease to proliferate. In the example data shown above maximal neurite length, reduced cell proliferation and minimal cell death were observed at 1% FBS.

Neurite Outgrowth:

- Recommended metric: Neurite Length (mm/mm^2); the total neurite network length in mm expressed per mm^2.
- Decreasing the media serum concentration from 8% to 2% promotes neurite outgrowth. Maximal neurite outgrowth is typically observed at FBS concentrations between 0.5 and 2% in the presence of 25 µM retinoic acid. At serum levels below 0.5% cell viability and neurite outgrowth is impaired.

Annexin V Measurement (Cell Analysis):

- Recommended metric: Green Object Confluence (%); the percentage of the image area occupied by Annexin V labeled objects.
- Serum concentrations below 1 to 0.5% will cause immediate cell death and illicit a concentration dependent Annexin V Green response in the first 24 hours. Please note it is normal to observe an Annexin V Green response for serum concentrations above 1% after approximately 3 days in culture as the cells become confluent and die. We recommend stopping the assay after 72 hours.

Nuclear Count (Viable Cell Analysis):

- Recommended metric: Red Object Count (1/mm^2); the number of Nuclight Red labeled nuclei per mm^2.
- Serum concentrations above 1% will induce Neuro-2a cell proliferation in a concentration dependent manner. FBS levels below 1% will lead to a loss in cell viability and reduced nuclear count.

Combined Metrics:

It is possible to normalize neurite length to either Cell-Body Clusters or per Nucleus if a nuclear labeled cell line is used. This normalization would not be recommended for the FBS optimization experiment described here because the metric can be difficult to interpret when significant cell death is occurring. This normalization would primarily be used to normalize for variations in seeding density when testing compounds that affect neurite outgrowth.

A complete suite of cell health applications is available to fit your experimental needs.

Find more information at www.sartorius.com/incucyte

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