IncuCyte® Annexin V NIR and IncuCyte® NeuroLight Orange Multiplex Protocol

Required materials

- IncuCyte® NeuroPrime Orange Kit (Sartorius Cat. No. 4760), containing:
  - 1X vial IncuCyte® rCortical Neurons (2 x 10^6 cells/vial)
  - 1X vial IncuCyte® rAstrocytes (2 x 10^6 cells/vial)
  - 1X vial IncuCyte® NeuroLight Orange Lentivirus-Synapsin promoter (0.45 mL/vial)
- IncuCyte® Annexin NIR Reagent (Sartorius Cat. No. 4768)

Materials required but not provided

Software:

- IncuCyte® S3 Live-Cell Analysis System for Neuroscience (Cat. No. 4763) with IncuCyte® NeuroTrack Software Module (Cat. No. 9600-0010)

Reagents:

- Poly-D-Lysine—Millipore (Cat. No. A-003-E)
- WFI water—Corning CellGro Mediatech (Cat. No. 25-055-CM)
- Neurobasal Media—Life Technologies (Cat. No. 21103049)
- B-27 Serum Free Supplement—Life Technologies (Cat. No. 17504044)
- GlutaMAX-I Supplement—Life Technologies (Cat. No. 35050061)
- DMEM—Life Technologies (Cat. No. 11965 or 41965)
- Fetal Bovine Serum—Sigma Aldrich (Cat. No. F2442) or Thermo Scientific (Cat. No. SH3007103)
- 5-Fluoro-2'-deoxyuridine—Sigma Aldrich (Cat. No. F0503)
- Uridine—Sigma Aldrich (Cat. No. U3003)
- 70% ethanol w/v

Material:

- Flat bottom tissue culture plate (e.g., Corning Cat. No. 3595)

This protocol provides an overview of the IncuCyte® Apoptosis Assay methodology combined with the IncuCyte® Neurite Analysis Assay.

The IncuCyte Apoptosis Assay uses mix-and-read IncuCyte® Annexin V NIR Reagent (Cat. No. 4768) to detect apoptosis in real time. The highly flexible apoptosis assay format can be combined with your choice of treatments. It is compatible with the IncuCyte® S3 Live-Cell Analysis System for Neuroscience (Cat. No. 4763) configured with the Orange/NIR Optical Module.

The IncuCyte® NeuroPrime Orange Kit (Cat. No. 4760) is a main component of the Neurite Analysis Assay. This kit contains cryopreserved vials of cortical neurons and astrocytes isolated from embryonic stage (E18) Sprague-Dawley rats as well as sufficient IncuCyte® NeuroLight Orange Reagent to perform one 96-well experiment. The lentivirus encodes an orange fluorescent protein regulated by a synapsin promoter that selectively drives expression in neurons and minimizes expression in non-neuronal cell types. After six days in co-culture the neurons form extensive neurite networks enabling the neurotoxic or neuroprotective effects of treatments to be assessed. Dynamic changes in network length and branching are measured using IncuCyte® S3 Live-Cell Analysis System for Neuroscience (Cat No. 4763) and the NeuroTrack Software Module (Cat. No. 9600-0010).

The NeuroPrime Orange Kit and Annexin NIR Reagent are not compatible with instruments configured with the Green/Red Optical Module, e.g. 4647.

Protocol Overview: IncuCyte NeuroPrime Orange Kit

1. Day 0

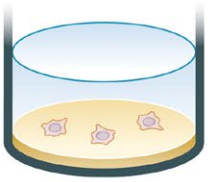
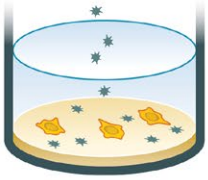


Plate rCortical neurons.

2. Day 0 (+ 4 hours)

Add NeuroLight Orange Reagent.

3. Day 1



95% media replacement. Plate rAstrocytes. Begin IncuCyte® S3 scanning.

4. Day 3

50% media replacement. Add Uridine +5-Fluoro-2'-deoxyuridine.

5. Day 6, 9, 12...

50% media replacement. Treatments at Day 6 and beyond.

Solutions to prepare in advance

Day -1

Poly-D-Lysine

- 100 µg/mL in 12 mL of WFI quality water

Day 0

Neuronal Culture Media—for 50 mL. Recommended: make two batches (total 100 mL) to ensure sufficient volume for Day 1 media replacement

- 48.5 mL Neurobasal Media
- 0.5 mL GlutaMAX-I
- 1 mL B-27 Supplement

Day 1

Astrocyte Culture Media—for 50 mL

- 42.5 mL DMEM
- 7.5 mL FBS

Day 3

2X 5-Fluoro-2'-deoxyuridine and Uridine (FdU/U)

- Dissolve 8 mg of FdU and 28 mg of U in 100 mL Neurobasal to make 10X FdU/U stock solution.
- Dilute to 2X FdU/U in total volume of 12 mL in a 15 mL conical tube.
  - Add 2.4 mL 10X FdU/U
  - Add 9.6 mL NCM
- 10X FdU/U stock solution can be aliquoted and stored at -20° C for future use, at which point it can be thawed on ice.

CRITICAL: Use rigorous aseptic technique at all times. Only open the culture plate and medium bottles within a tissue culture hood.

Protocol

Day -1: Coat 96-well plate with Poly-D-Lysine

1. Coat one 96-well plate with Poly-D-Lysine. Prepare a 1X stock of Poly-D-Lysine (final concentration –100 µg/mL) in WFI quality water and add 100 µL to each well. Replace lid and incubate for 16-20 hours at room temperature in the tissue culture hood.
2. Aspirate and discard the Poly-D-Lysine and rinse the plate twice with 150 µL/well of WFI water. If excess Poly-D-Lysine is not washed away it can impair neurite outgrowth.
3. Leave the plate to dry for at least one hour with lid removed in the tissue culture hood.

Day 0: Thaw and plate neurons

1. Prepare the Neuronal Culture Media (NCM). For 50 mL of complete NCM, add 1 mL of B-27 Serum Free supplement and 0.5 mL GlutaMAX-I to 48.5 mL of Neurobasal media in a 50 mL conical tube. Recommended: make two batches (total 100 mL) to ensure sufficient volume for Day 1 media replacement.
   CRITICAL: Warm NCM to 37° C prior to thawing neurons.
2. Remove the vial of rCortical Neurons from liquid nitrogen storage and thaw in a 37° C water bath until only a tiny ice crystal remains (1 to 2 minutes).
   CRITICAL: Do not agitate the vial during this step.
3. Wipe outside of vial with 70% ethanol.
4. In tissue culture hood, use a P1000 pipettor to pre-wet a tip with 1 mL NCM.
5. Use the pre-wetted tip to transfer the 1 mL volume of thawed neuronal cells to a 50 mL conical tube.
6. Rinse the cryo-vial with 1 mL NCM and transfer the rinse media in a drop-wise fashion to the 50 mL conical tube containing neurons, while gently swirling the 50 mL conical tube.
   CRITICAL: Rapid addition of the media at this point can result in osmotic shock and cell death. The 1 mL addition should take about 30 seconds.
7. In drop-wise fashion, add a further 2 mL pre-warmed NCM to the 50 mL conical tube. The 2 mL addition should take about one minute.

8. Perform a cell count (e.g. Trypan Blue staining with hemocytometer) and dilute neurons to 150,000 cells/mL in pre-warmed NCM. The cell suspension can be transferred to a sterile trough in the tissue culture hood at this point in order to facilitate pipetting of cells in the next step.
9. Using a multichannel handheld pipette, dispense 100 µL of neuronal cell suspension into each well of the Poly-D-Lysine coated 96-well plate (15,000 neurons/well).
   CRITICAL: To ensure proper mixing and uniform seeding of the neurons, mix the cell suspension by gently pipetting up and down 1-2 times between seeding each row of the plate. Rocking the trough is also recommended to ensure equal cell distribution.
10. Let the plate stand at room temperature in the tissue culture hood for 30 minutes and then place inside the incubator.
    CRITICAL: This step ensures the uniform distribution of cells in each well.
11. Allow cells to settle on the plate for 2 to 3 hours before proceeding.

Infect neurons with NeuroLight Orange Reagent

1. Allow the NeuroLight Orange Reagent to thaw on ice (approximately 1 hour).
2. After neurons have adhered for 2-4 hours post-plating, add appropriately diluted NeuroLight Orange Reagent to achieve desired concentration. The final well volume should be 200 µL per well.
   Note: Quality control for the IncuCyte NeuroLight Orange Reagent is the ability to efficiently infect IncuCyte rCortical Neurons to express TagRFP, driven off of the synapsin promotor of the IncuCyte NeuroLight Orange Lentivirus, such that a volume of > 3.2 µL/20,000 neurons results in a neurite length of > 50 mm/mm^2 in a neurite outgrowth assay (rCortical Neurons/rAstrocytes co-culture experiment). We recommend performing a volumetric titration from 100-0.14 µL for each neuronal cell line evaluated. The lowest concentration that results in the highest neurite outgrowth measurement should be selected. Evaluation of neurite dynamics is to be performed on an IncuCyte S3 for Neuroscience.
   CRITICAL: Do not pipette up and down after adding the virus solution as this may result in damage to the plated neurons.

Day 1: Plate astrocytes

1. 16-24 hours after plating and infecting the neurons, warm NCM to 37° C.
2. Carefully remove 190 µL of medium per well using a multi-channel pipettor, and replace immediately with 140 µL of fresh, pre-warmed NCM. Volume should now be 150 µL per well.
3. Prepare 50 mL Astrocyte Culture Media (85% DMEM + 15% FBS; ACM) by adding 7.5 mL FBS to 42.5 mL DMEM and warm to 37° C.
4. Remove the vial of rAstrocytes from liquid nitrogen storage and thaw in a 37° C water bath until only a tiny ice crystal remains (1 to 2 minutes).
5. Wipe vial with 70% ethanol.
6. In tissue culture hood, use a P1000 pipettor to pre-wet a tip with 1 mL ACM.
7. Use the pre-wetted tip to transfer the 1 mL volume of thawed astrocytes to a 50 mL conical tube.
8. Rinse the cryo-vial with 1 mL ACM.
   CRITICAL: Rapid addition of the media to the cell suspension at this point can result in osmotic shock and cell death. Transfer the rinse media in a drop-wise fashion to the 50 mL conical tube containing astrocytes, while gently swirling the 50 mL conical tube.
9. Add 3 mL pre-warmed ACM to the 50 mL conical tube in a drop-wise fashion. The 3 mL addition should be performed slowly, taking at least 1 minute.
10. Centrifuge the astrocytes at 250 x g for 5 min. Carefully aspirate and discard the supernatant and resuspend the cell pellet in 5 mL of ACM. Using a P-1000 handheld pipettor set to 800 µL, triturate the cell suspension by gently aspirating and dispensing 10-15 times to ensure a single cell suspension.
11. Perform a cell count (e.g. Trypan Blue staining with hemocytometer) and dilute cells to 300,000 cells/mL in prewarmed ACM.
12. Using a multichannel handheld pipettor, plate 50 µL of astrocyte cell suspension into each well of the 96-well plate containing the cultured neurons (i.e. 15,000 astrocytes/well) CRITICAL: To ensure proper mixing and uniform seeding of the astrocytes, mix the cell suspension by gently pipetting up and down 1-2 times between seeding each row of the plate. Rocking the trough is also recommended to ensure equal cell distribution.
13. Place plate into the IncuCyte® S3 for Neuroscience and schedule to image every 2 to 12 hours in Phase and Orange image channels. (See IncuCyte User Manual for detailed instructions on setting up an imaging schedule.)

Day 3: Treat plate with 5-Fluoro-2′-deoxyuridine and Uridine
CRITICAL: Addition of 5-Fluoro-2′-deoxyuridine and Uridine (FdU/U) prevents proliferation of non-neuronal cell types.

1. Remove 100 µL of media from each well using a multi-channel pipette and replace with 100 µL fresh NCM containing 2X concentrations of FdU/U to a final assay concentration of 8 µg/mL and 28 µg/mL, respectively.

Feeding cultures

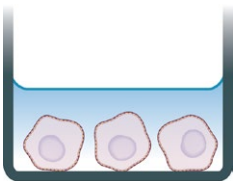
1. Feed cultures with fresh NCM by performing a 50% media change. To do this, remove 100 µL per well and replace with 100 µL of fresh media.
   CRITICAL: Only a single FdU/U treatment is required (Day 3, step 1). Addition of fresh FdU/U is not recommended on following days.
2. Cultures can be stopped at Day 11 or continued for desired length, with 50% media changes occurring every third day.

Days 6, 9, 12 and beyond: Solutions Required

1. Neuronal Culture Media—for 50 mL
   a. 48.5 mL Neurobasal Media
   b. 0.5 mL GlutaMAX I
   c. 1 mL B-27 Supplement

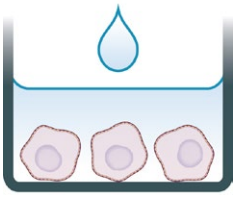
Protocol Overview: IncuCyte Annexin V NIR Reagent

1. Seed cells



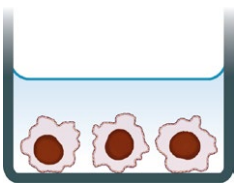
Seed cells (100 µL/well) into a 96-well plate.

2. Prepare apoptosis reagent and treat cells



Prepare the desired treatments at 2X in medium containing IncuCyte® Annexin V Reagent and add treatment.

3. Live-cell fluorescent analysis



Capture images every 2-3 hours (20X or 10X) in the IncuCyte® System. Analyze using integrated software.

Desired day: Annexin V NIR reagent preparation and cell treatment addition

1. Solubilize Annexin V NIR Reagent by adding 100 µL of complete medium or PBS. The reagent may then be diluted in complete medium containing at least 1 mM $CaCl_2$ for a final dilution of 1:100.
   Note: All test agents will be diluted in this reagent-containing medium to 2X concentration, so make up a volume that will accommodate all treatment conditions. The volumes/dilutions added to cells may be varied; however, a volume of 200 µL per well is generally sufficient for the duration of the assay.
2. Take the cell plate from the incubator and remove 100 µL media from wells (50%).
3. Add treatments and controls to appropriate wells of the 96-well plate to achieve a volume 200 µL per well. As solutions were made at 2X, we will have a 1X final dilution of treatments in individual wells (i.e., 1:200 final concentration of Annexin V NIR).
4. Place plate into the IncuCyte S3 for Neuroscience and allow the plate to warm to 37˚C for 30 minutes prior to scanning. Acquire images every 2-3 hours in Phase (optional), Orange, and NIR image channels, adjusting scan schedule if needed.

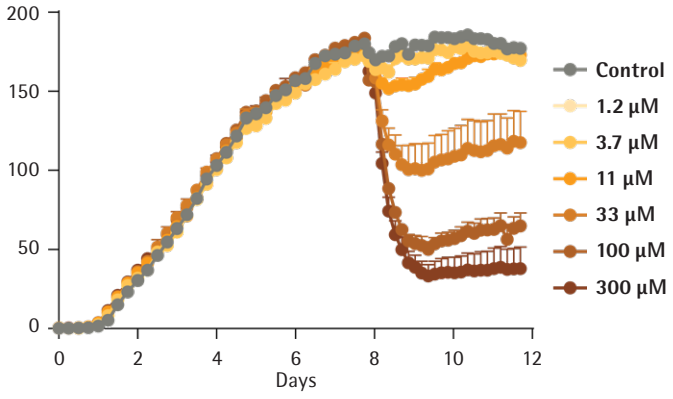
Analysis guidelines

1. Before starting your analysis, first set up the Spectral Unmixing parameters. We recommend removing 2-4% NIR channel from the orange channel.
   Note: Annexin V NIR signal bleeds into the orange channel. Using the Spectral Unmixing feature allows you to produce images that better represent the distribution of the two reagents.
2. Run 2 separate analysis jobs.
   a. For Neurite Analysis Assay:
      i. Analysis Type: NeuroTrack
      ii. Image Channels: Orange to quantify Neurite Length
      iii. Suggested analysis parameters:
         1. Min Cell Width: 15.0
         2. Neurite Fine Sensitivity: 0.5
         3. Neurite Width: 2
   b. For Apoptosis Assay:
      i. Analysis Type: Basic Analyzer
      ii. Image Channels: NIR to quantify apoptotic cells

Example Data

Measurements and images gathered using IncuCyte® S3 for Neuroscience. The figures below above show data and images obtained from a single 96-well plate assay over time.

A. Orange Neurite Length (mm/mm²)



B. NIR Total Fluorescent Area (10^5 mm²/image)

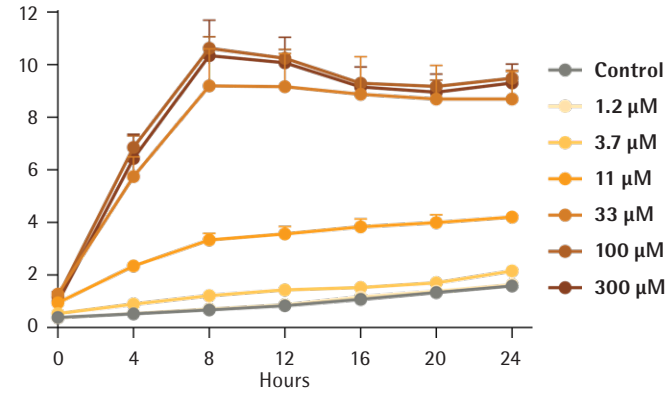
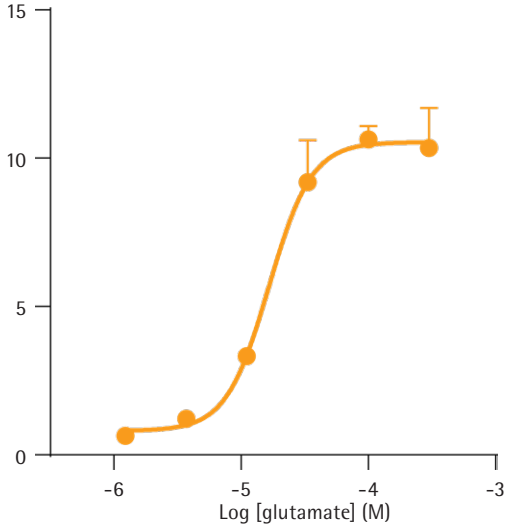
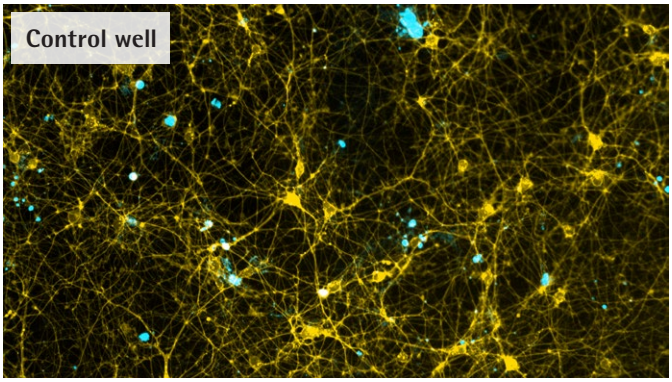
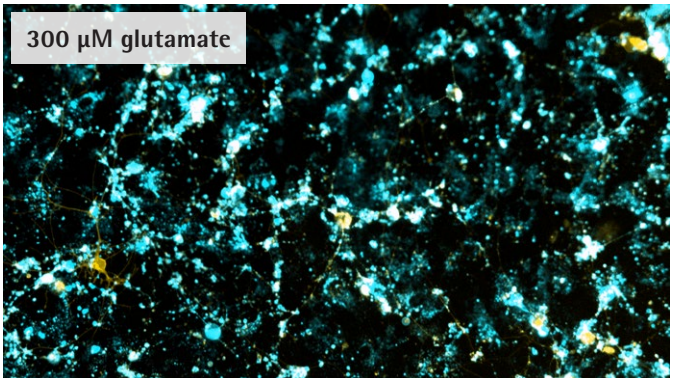


Figure A shows the disruption of Orange Neurite Length with the addition of various concentrations of glutamate at Day 8. Figure B shows the concentration-dependent response of Annexin V NIR Total Area to the glutamate treatment starting at time of addition, indicating neuronal cell death. Figure C is the concentration response curve of NIR Total Area generated with IncuCyte® Software providing an EC_{50} of 13.2 µM.

C. Concentration Response Curve







IncuCyte images showing NeuroLight Orange labeled rCortical Neurons in co-culture with rAstrocytes. The image on the left shows a control well treated with only Annexin V NIR, while the image on the right shows a well treated with Annexin V NIR and 300 µM glutamate. Annexin V NIR and treatments were added on Day 8 and images were taken 8 hours post addition. Cyan color depicts the Annexin V NIR response.

Safety considerations

The backbone of the Lentivirus particles in this system has been modified to improve their safety and minimize their relation to the wild-type, human HIV-1 virus. These modifications include:

1. The lentiviral particles are replication-incompetent and only carry the non-oncogenic gene of interest.
2. A deletion in the 3' LTR (ΔU3) resulting in "self-inactivation" (SIN) of the Lentivirus after transduction and genomic integration of the target cell (Yee et al., 1987; Yu et al., 1986; Zufferey et al., 1998). This alteration renders the lentiviral genome incapable of producing package able virus following host integration.
3. The envelope is psueudotyped with the VSV-G gene from Vesicular Stomatitis Virus of the HIV-1 envelope (Burns et al., 1993; Emi et al., 1991; Yee et al., 1994).

Replication-defective lentiviral vectors, such as the 3rd generation vector provided in this product, are not known to cause any diseases in humans or animals. However, lentivirus particles still pose some biohazardous risk because they can transduce primary human cells and can integrate into the host cell genome thus posing some risk of insertional mutagenesis. For this reason, **we highly recommend that you treat lentiviral stocks as Biosafety Level 2 (BSL-2, BL-2) organisms and strictly follow all published BL-2 guidelines with proper waste decontamination.**

For more information about the BL-2 guidelines and Lentivirus handling, we recommend referring to local documentation based on geography. The Essen BioScience 3rd generation HIV-based lentiviruses meet BL-2 requirements based on the criteria in the document, "Biosafety in Microbiological and Biomedical Laboratories", 5th Edition, published by the Centers for Disease Control (CDC). This document may be downloaded at **http://www.cdc.gov/biosafety/publications/bmbl5/index.htm**

Institutional Guidelines: Safety requirements for use and handling of lentiviruses may vary at individual institutions. We recommend consulting your institution's health and safety guidelines and/or officers prior to implementing the use of these reagents in your experiments.
A detailed discussion of lentiviral vectors is provided in Pauwels, K. et al (2009). **State-of-the-art lentiviral vectors for research use: Risk assessment and biosafety recommendations.** *Curr. Gene Ther.* 9: 459-474.

Biohazard note

The rCortical Neurons and rAstrocytes contain cells of rodent origin. Although the cells test negative for mycoplasma, bacteria and fungi, no test procedure can guarantee the absence of known and unknown infectious agents. Consequently, all products should always be considered potentially biohazardous and appropriate precautions should be taken. Use good laboratory practice and aseptic technique at all times.

FOR RESEARCH USE ONLY. NOT FOR THERAPEUTIC OR DIAGNOSTIC USE.

Licences and warranty

Essen BioScience warrants that this product performs as described on the product label and in all literature associated with the sale of said product when used in accordance with the described protocol. This limited warranty is the sole warranty. No other warranties exist that extend beyond this warranty, either expressed or implied. Essen BioScience disclaims any implied warranty of merchantability or warranty of fitness for a particular purpose. Essen BioScience disclaims any responsibility for injury or damage and shall not be liable for any proximate, incidental or consequential damages in connection with this product. If it is proven to the satisfaction of Essen BioScience that this product fails to meet performance specifications, Essen BioScience's sole obligation, at the option of Essen BioScience, is to replace the product or provide the purchaser with credit at or below the original purchase price. This limited warranty does not extend to anyone other than the purchaser. Notice of suboptimal performance must be made to Essen BioScience within 30 days of receipt of the product.
This Essen BioScience product contain proprietary nucleic acid(s) coding for proprietary fluorescent protein(s) being, including its derivatives or modifications, the subject of pending patent applications and/or patents owned by Evrogen JSC (hereinafter "Evrogen Fluorescent Proteins").
The purchase of Essen BioScience products incorporating these fluorescent proteins conveys to the buyer the non-transferable right to use Evrogen Fluorescent Proteins only for research conducted by the buyer. No rights are conveyed to modify or clone the gene encoding fluorescent protein contained in this product or to use Evrogen Fluorescent Proteins for commercial purposes. The right to use Evrogen Fluorescent Proteins specifically excludes the right to validate or screen compounds for commercial purposes. For information on commercial licensing, contact Evrogen Licensing Department, email: license@evrogen.com.

Essen Bioscience,
a Sartorius Company
300 West Morgan Road
Ann Arbor, Michigan, 48108
USA

www.sartorius.com/incucyte
Email: AskAScientist@sartorius.com

USA +1.734.769.1600
UK +44.1707.358688
Japan, Australia and other countries
around the world: +65.6872.3966