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Applying IncuCyte® Apoptosis Assay for the Fluorescent Detection of Caspase-3/7 Activation or Phosphatidylserine Externalization

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Introduction

This protocol describes the IncuCyte® Apoptosis Assay methodology that enables real-time detection of apoptosis using mix-and-read IncuCyte® Caspase 3/7 or Annexin V Reagents. The method can be used with the IncuCyte® Live-Cell Analysis System using any type of treatment and cell type.

The assay format is highly flexible and can be integrated with Essen BioScience's range of IncuCyte® NucLight red nuclear labeling reagents or labeled cell lines, for multiplexed measurements of apoptosis and proliferation in the same well.

Required materials

The following materials were used:

- IncuCyte® Annexin V Red Reagent (EssenBioScience Cat #4641) or IncuCyte® Caspase- 3/7 Apoptosis Reagent (EssenBioscience Cat #4440) or IncuCyte® Annexin V Green Reagent (Essen BioScience Cat #4642)
- Fibronectin (Sigma A7906) – optional, for non-adherent cells
- Poly-L-ornithine (Sigma P4957) – optional, for non-adherent cells
- Flat bottom tissue culture plate (e.g., Corning 3595)

General guidelines

Medium with low levels of riboflavin is recommended to reduce the green fluorescence background. RPMI and DMEM have high riboflavin (>0.2 mg/L). Eagles MEM, F12-K, and EBM have low riboflavin (<0.2 mg/L).

Once cell seeding is done, the plates should be placed at ambient temperature

for 45 minutes in the case of non-adherent cell lines and 15 minutes in the case of adherent cell lines. This ensures uniform cell settling.

Bubbles should be removed from all the wells by lightly squeezing a wash bottle that contains 70-100% ethanol with the inner straw detached. This is done to blow vapor across the surfaces of all wells.

Once the plate placed in the IncuCyte® Live-Cell Analysis System, it should be allowed to warm to 37 °C for a period of 30 minutes before scanning.

When observing apoptosis in primary neuronal cultures, the IncuCyte® Annexin V Red reagent is recommended so as to remove the risk of green channel excitation problems in these sensitive cell lines.

Adherent cell line protocol

Seed cells

Seed cells (100 µL/well, 1,000 — 5,000) into a 96-well plate and incubate overnight.

Prepare apoptosis reagent and treat cells

Prepare the desired treatments at 1x in medium containing IncuCyte® Caspase-3/7 or Annexin V Reagents. Aspirate media from wells and add treatment (100 µL/well).

Live-cell fluorescent analysis

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

Day 0: 1. Seed effector cells

Cells of any type (100 µL per well) should be seeded at a suitable density into a 96-well plate, so that the cell confluence is around 30% by day 1.

For the individual cell line used, the seeding density has to be optimized. But, it was observed that 1,000 to 5,000 cells per well (10,000 – 50,000 cells/mL seeding stock) are viable starting points.

Using the IncuCyte® system, cell growth should be monitored to capture phase contrast images every 2 hours and these images can be examined using the integrated confluence algorithm.

Day 1: 2. Apoptosis reagent preparation and cell treatment addition

Apoptosis reagents should be diluted in preferred medium formulations.

- If Caspase-3/7 is used, the reagent should be diluted to a final concentration of 5 µM (1:1000 dilution)
- If Annexin V reagents are used, Annexin V should be solubilized by adding 100 µL of PBS or complete medium. The reagents can be subsequently diluted in complete medium that contains a minimum of 1 mM $CaCl_2$ for a final dilution of 1:200

Note: Test agents have to be diluted in this reagent-containing medium. Therefore a sufficient volume should be prepared to accommodate all treatment conditions. While the volumes/dilutions added to cells may be different, a volume of 100 µL per well is usually adequate for the assay duration.

The cell plate should be removed from the incubator and the growth medium should be removed by aspiration.

Treatments and controls should be added to appropriate wells of the 96-well plate.

3. Live-cell imaging of apoptosis

Once the cell plate is placed into the IncuCyte® Live-Cell Analysis System, it should be allowed to warm to 37 °C for a period of 30 minutes before scanning.

Channel selection: Phase Contrast and Green (+ "Red" if fluorescent label or an extra cell health reagent is used)

- Objective: 10x or 20x
- Scan type: Standard (2 to images per well)
- Scan interval: Usually, every 2 hours, until the experiment is completed

Non-adherent cell line protocol

Coat plate

Coat plate with 0.01% poly-L-ornithine solution or 5 µL/mL fibronectin diluted in 0.1% BSA.

Prepare IncuCyte® apoptosis reagent and treatment

Dilute apoptosis reagent in medium and prepare cell treatments.

Seed cells and add treatment

Seed cells (100 µL/well, 5,000 – 25,000 cells) into the coated 96-well plate. Immediately add apoptosis reagent ± treatments and triturate.

Live-cell fluorescent analysis

Capture images every 2-3 hours (20x or 10x) in the IncuCyte® system.

Day 2: 1. Coat plate

A suitable coating matrix should be used to coat a 96-well flat bottom plate. Coating can be done with 50 µL of 5 µg/mL fibronectin (Sigma A7906) or 0.01% poly-L-ornithine solution (Sigma P4957) diluted in 0.1% BSA.

The plates should be coated for 1 hour at ambient temperature, and this is followed by removing the solution from the wells and allowing the plates to dry for 30 to 60 minutes before cell addition.

2. Prepare apoptosis reagent and treatments

Before cell seeding, apoptosis reagents should be diluted in a preferred medium formulation.

- If Caspase-3/7 is used, the reagent should be diluted to a final concentration of 5 µM (1:1000 dilution)
- If Annexin V reagents are used, Annexin V should be solubilized by adding 100 µL of PBS or complete medium. The reagents can then be diluted in complete medium that contains 1 mM $CaCl_2$ for a final dilution of 1:200

Note: Test agents have to be diluted in this reagent-containing medium, and therefore a sufficient volume has to be prepared to accommodate all treatment conditions. While the volumes/dilutions added to cells may be changed, a volume of 200 µL per well is usually adequate for the assay duration.

Cell treatments should be prepared at 2x final assay concentration in sufficient cell culture medium containing Annexin V or Caspase-3/7 to acquire a volume of 100 µL per well.

3. Seed cells and add prepared treatments

Cells of any type (100 µL per well) should be seeded at an appropriate density into a 96-well plate in medium containing Caspase-3/7 or Annexin V.

For the individual cell line used, the seeding density have to be optimized. But, it was observed that 5,000 to 25,000 cells per well (50,000 – 250,000 cells/mL seeding stock) are viable starting points.

Treatments and controls should be instantly added to suitable wells of the 96-well plate containing cells. The wells should be triturated to appropriately mix the treatment and allow cell exposure at 1x.

4. Live-cell imaging of apoptosis

After placing the cell plate into the IncuCyte® Live-Cell Analysis System, it should be allowed to warm to 37 °C for a period of 30 minutes before scanning.

- Channel selection: Phase Contrast and Green (+ "Red" if fluorescent label or an extra cell health reagent are used)
- Objective: 10x or 20x
- Scan type: Standard (2 to 4 images per well)
- Scan interval: Usually, every 2 hours, until the experiment is completed

Related products and applications

Essen Bioscience offers a wide range of cell health and fluorescent nuclear labeling reagents that can be used in the IncuCyte® Live-Cell Analysis System to perform multiplexed measurements of proliferation, cytotoxicity and apoptosis. A complete range of cell health applications is available from Essen Bioscience to suit specific experimental requirements.

Product	Cat No.	Amount
IncuCyte® NucLight Red BacMam 3.0 Reagent for nuclear labeling	4621	1 mL

IncuCyte® NucLight Green BacMam 3.0 Reagent for nuclear labeling	4622	1 mL
IncuCyte® NucLight Green Lentivirus Reagent (EF-1 α, Puro) for nuclear labeling	4624	0.2 mL
IncuCyte® NucLight Red Lentivirus Reagent (EF-1 α, Puro) for nuclear labeling	4625	0.2 mL
IncuCyte® NucLight Green Lentivirus Reagent (EF-1 α, Bleo) for nuclear labeling	4626	0.2 mL
IncuCyte® NucLight Red Lentivirus Reagent (EF-1 α, Bleo) for nuclear labeling	4627	0.2 mL
IncuCyte® NucLight Green Lentivirus Reagent (EF-1 α, Puro) for nuclear labeling	4475	0.6 mL
IncuCyte® NucLight Red Lentivirus Reagent (EF-1 α, Puro) for nuclear labeling	4476	0.6 mL
IncuCyte® NucLight Green Lentivirus Reagent (EF-1 α, Bleo) for nuclear labeling	4477	0.6 mL
IncuCyte® NucLight Red Lentivirus Reagent (EF-1 α, Bleo) for nuclear labeling	4478	0.6 mL
IncuCyte® Cytotox Red Reagent for counting dead cells	4632	5 µL x 5
IncuCyte® Cytotox Green Reagent for counting dead cells	4633	5 µL x 5
IncuCyte® Annexin V Red Reagent for apoptosis	4641	100 tests
IncuCyte® Annexin V Green Reagent for apoptosis	4642	100 tests
IncuCyte® Caspase-3/7 Green Reagent for apoptosis	4440	20 µL

IncuCyte® Caspase-3/7 Red Reagent for apoptosis	4704	20 µL

About Essen BioScience

Essen BioScience enables scientists and researchers to better understand live-cell behaviors and functions, and conduct live-cell experiments more efficiently using continuous live-cell imaging and quantitative data analysis methods.

Founded in Ann Arbor, Michigan in early 90's, Essen BioScience specializes in developing and manufacturing instruments, software, reagents and consumables for real-time live-cell imaging and data analysis.

Essen BioScience has a long history of innovation, including FLIPR and IonWorks, two laboratory instruments that revolutionized industrial screening approaches to G-protein coupled receptor and ion channel target drug discovery.

In 2006, Essen launched a third ground breaking platform technology, the IncuCyte® System, a real-time quantitative live-cell imaging and analysis platform enables visualization and quantification of cell behavior over time (from hours to weeks) by automatically gathering and analyzing images around the clock within a standard laboratory incubator.

The system allows researchers to make time-lapsed fully kinetic measurements from living cells over days and weeks thus providing insight into active biological processes in real time.

The IncuCyte® System, with its continuous live-cell imaging and quantitative data analysis capabilities allows researchers to conduct a variety of live-cell assays in a faster and automated way.

Their live-cell imaging and analysis tools are used in academic and industrial

laboratories throughout the world. You can view the publications using IncuCyte's® live-cell imaging and analysis HERE.

Whether you need to evaluate common processes such as cell proliferation and viability or measure functionally-specific activities such as T cell killing, chemotaxis, migration & invasion or phagocytosis, their R&D scientists and product specialists can offer consultative support that is customized to your research needs.

Their goal is to enable advancements in life science research through more informative cell-based assay solutions.

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