Protocol

IncuCyte® Live-Cell Immunocytochemistry Assay

For cell surface marker analysis

This protocol describes a solution for measuring immunocytochemistry in live cells expressing a surface antigen of interest. The method utilizes the IncuCyte® FabFluor-488 Antibody Labeling Reagent in combination with IncuCyte Opti-Green and the IncuCyte® S3 Live-Cell Analysis System for image-based fluorescent measurement enabling live-cell immunocytochemistry.

Required materials

- IncuCyte® FabFluor-488 Antibody Labeling Reagent plus IncuCyte® Opti-Green suppressor (Sartorius Cat #4745 mouse IgG1, Sartorius Cat# 4743 mouse IgG2a or Sartorius Cat#4744 mouse IgG2b).
- Test antibody of interest (at known concentration) containing mouse Fc region.
  - Recommend using Azide-free antibodies when available.
- Target cells of interest.
- Target cell growth media
- Effector cell culture media
- PBS (w/o Ca^{2+}/Mg^{2+}, Life Tech 14190).
- 96-well flat bottom microplate (e.g. Corning® 3595) for imaging
- 96-well round round bottom plate (e.g. Corning® 3799) or amber microtube (e.g. Cole parmer® MCT-150-X) for conjugation step.

Additional material for non-adherent cell types

- Poly-L-ornithine, PLO (Sigma P4957)

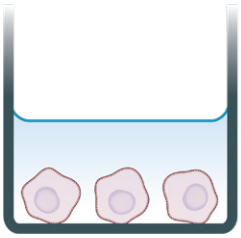
Recommended materials

It is strongly recommended to run both a positive and negative control alongside test antibodies and cell lines. CD71 (anti transferrin receptor) marker is recommended as a positive control for the mouse Fab IgG1 or 2a. Isotype matched IgG are recommended as negative controls.

- Anti-CD71, clone MEM-189, IgG1 e.g. Sigma SAB4700520-100UG
- Anti-CD71, clone CYG4, IgG2a e.g. Biolegend 334102
- Isotype controls, depending on isotype being studied
  - Mouse IgG1, e.g. R&D Systems, MAB002 or Biolegend 400124
  - Mouse IgG2a e.g. Biolegend 401501
  - Mouse IgG2b e.g. Biolegend 400322

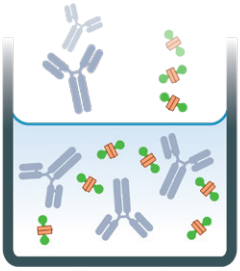
Quick Guide

1. Seed cells



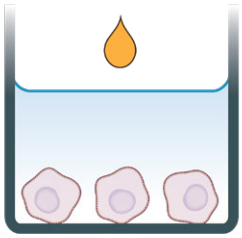
Cell seeding
Seed cells (50 µl/well, 5-30K/well) into 96-well plate.
NOTE: for non-adherent cell types, PLO coat plate prior to cell seeding.

2. Label test antibody



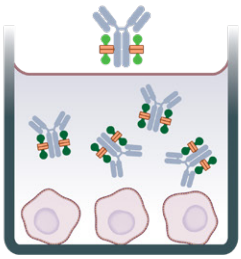
Labeling of test antibody with IncuCyte® FabFluor-488 reagent
Mix antibody and FabFluor-488 reagent at a molar ratio of 1:3 in media, 3x final concentration. Incubate for 15 minutes to allow conjugation.

3. Add Incucyte® Opti-Green



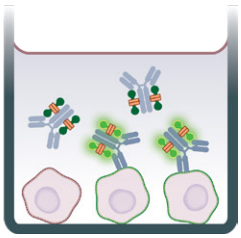
IncuCyte® Opti-Green background suppressor addition
Add 50 µl/well, (3x final concentration).

4. Add labeled AB



IncuCyte® FabFluor-488-labeled antibody addition
Add antibody-FabFluor mix (50 µl/well) to cell plate. Non-adherent cells – spin plate

5. Live-cell fluorescent imaging



Automated imaging and quantitative analysis
Capture images, (time span and objective depends on assay and cells type, 10x or 20x) in IncuCyte® S3 Live-Cell Analysis System.

IncuCyte® Live-cell Immunocytochemistry Assay Methodology

1a. Seed target cells of interest - Adherent cell

1.1. Harvest cells of interest and determine cell concentration (e.g. trypan blue + hemocytometer).

1.2. Prepare cell seeding stock in target cell growth media to achieve 40-50% confluence after 2-6 h. Suggested starting range 5,000-20,000 cells/well (depends on cell type used).

NOTE: Seeding density must be optimized for each cell type.

1.3. Using a multi-channel pipette, seed cells (50 µL per well) into a 96-well flat bottom microplate. Lightly tap plate side to ensure even liquid distribution in well.

1.4. Remove bubbles from all wells by gently squeezing a wash bottle (containing 70-100% ethanol with the inner straw removed) to blow vapor over the surface of each well.

1.5. Allow cells to settle on a level surface for 30 minutes at room temperature, then place in IncuCyte® S3 Live-Cell Analysis System to monitor cell confluence.

NOTE: Depending on cell type, plates can be used in assay once cells have adhered to plastic and achieved normal cell morphology e.g. 2-3 hr for HT1080. Some cell types may require overnight incubation.

1b. Non-adherent cells

NOTE: For this assay, non-adherent cells will be the last addition to the plate (prepare suspension during the antibody conjugation step).

1.1. Coat a 96-well flat bottom plate with relevant coating matrix. We recommend coating with 50 µL of either 0.01% poly-L-ornithine solution (Sigma P4957) or 5 µg/mL fibronectin (Sigma P4957) diluted in 0.1% BSA. Coat for 1 hour at ambient temperature, remove solution from wells, and then allow plates to dry for 30-60 minutes prior to cell addition.

NOTE: Some optimization of plate coatings may be required.

1.2. Count cells of interest and determine cell concentration (e.g. trypan blue + hemocytometer).

1.3. Prepare cell seeding stock in target cell growth media, suggest starting range of 20,000 – 40,000 cells/well in 50 µL (depends on cell type used).

1.4. Using a multi-channel pipette, seed cells (50 µL per well) into a 96-well flat bottom microplate.

1.5. Remove bubbles from all wells by gently squeezing a wash bottle (containing 70-100% ethanol with the inner straw removed) to blow vapor over the surface of each well.

1.6. Allow cells to settle on a level surface for 30 minutes at room temperature then place in IncuCyte® S3 Live-Cell Analysis System to monitor cell confluence.

NOTE: To reduce settling time cell plates can be centrifuged for 1 minute at 50 g.

2. Labeling of test antibody

NOTE: It is recommend to use low azide or azide-free antibodies (e.g. LEAF™ from Biolegend). Effects on cell growth from high concentrations of azide have been observed in some cell types. If this is of concern, the buffer can be exchanged using a simple desalting column (e.g. Zeba from Thermo Scientific).

2.1. Rehydrate IncuCyte® FabFluor-488 Antibody Labeling reagent with 100 µL sterile water (final concentration = 0.5 mg/mL).

NOTE: A 1:3 molar ratio of test antibody to IncuCyte® FabFluor-488 reagent is recommended. The labeling reagent is a third of the size of a standard antibody. Therefore, equal mg/mL quantities produce a 1:3 molar ratio of test antibody to labeling Fab.

NOTE: Reagent is light sensitive, keep in amber or foil wrapped tubes. Remaining re-hydrated reagent can be aliquoted and stored at -80°C (avoid freezing and thawing, stable for > year).

2.2. Mix test antibody with dilute IncuCyte® FabFluor-488 Antibody Labeling reagent and target cell growth media in a round bottom microplate or amber tube to protect from light. Prepare sufficient quantity to enable 50 µL/well at 3X final assay concentration.

NOTE: WE strongly recommend using both a negative and positive control antibody (see **Recommended Materials** above)

a. Add test antibody at 3X the final antibody concentration. Recommendation: A final concentration of <1.5 µg/mL of test antibody. A reasonable starting concentration is 1 µg/mL (e.g. 3X working concentration = 3 µg/mL).

b. Add IncuCyte® FabFluor-488 Antibody Labeling reagent at a 1:3 (test antibody:FabFluor) molar ratio. See **Example calculations** below.

c. Add media to dilute to 3X final assay concentration. Triturate to mix.

d. Incubate for 15 minutes at 37°C in the dark to allow for conjugation.

Example calculations of antibody labeling using positive control anti-CD71 at 1 mg/mL stock concentration

1. Required final assay concentration of test antibody — 1 µg/mL for anti-CD71 is recommended for positive control wells. Working concentration = 3X, or 3 µg/mL.
2. Determine volume of labeled antibody required at 3X final assay concentration (i.e. dilution of 1:3 recommended upon addition to cells): [# wells] x 50 µL (plus additional required to prepare dilution series if desired).

E.g. For 8 replicates of 1:3 dilution of labeled test antibody:

8 x 50 µL= 400 µL minimum (500µL used for this example)

3. Calculate volumes of test antibody, IncuCyte FabFluor reagent, and media required to provide 3X final assay concentration of labeled test antibody.

a. Determine volume of test antibody:

[Total volume] µL x [Working concentration test antibody] µg/mL / [Stock concentration test antibody] mg/mL /1000

500 µL x 3 µg/mL / 1 mg/mL / 1000 = 1.5 µL

b. Determine volume of IncuCyte FabFluor:

[Volume of test antibody] µL X [Stock concentration of test antibody] mg/mL / [Stock concentration of FabFluor] mg/mL

1.5 µL x 1 mg/mL / 0.5 mg/mL = 3.0 µL

NOTE: IncuCyte FabFluor reagent is a third of the molecular weight of a standard antibody. Therefore, equal volumes of equal mg/mL quantities produce a 1:3 molar ratio of test antibody to FabFluor as MW of a typical antibody is ~3x of FabFluor. In this case, the stock concentration in mg/mL of test antibody is twice that of FabFluor. Therefore, 2X volume of FabFluor is required.

c. Determine volume of media: [Total volume] – [Test antibody volume] – [FabFluor volume]

500 µL – 1.5 µL – 3.0 µL = 495.5 µL

3. Dilution of IncuCyte Opti-Green background suppressor

3.1. Dilute Opti-Green stock in complete growth media for a final assay concentration of 0.5 mM or 1:200 dilution of stock (see calculations below).

NOTE: A final assay concentration of 0.5 mM has proven to be suitable across a range of cell types, however some optimization may be required to assess cell proliferation in the presence of Opti-Green. For lower expressed markers, Opti-green may be increased in some cell types (1 mM or 1:100 dilution of stock final assay concentration), allowing for a higher concentration of test antibody to be added (<3 µg/mL). A full list of pre-assessed cell types is included at the end of this protocol.

Example calculations for Opti-Green background suppressor

1. Required final assay concentration of Opti-Green Background Suppressor — 0.5 mM. Working concentration = 3X, or 1.5 mM.

2. Determine volume of Opti-Green Background Suppressor required at 3X final assay concentration (i.e. dilution of 1:3 recommended upon addition to cells): [# wells] x 50 µL (plus additional required to prepare dilution series if desired).

   E.g. For 96 replicates of 1:3 dilution of Opti-Green Background Suppressor:

   96 x 50 µL= 4800 µL minimum (5000 µL used for this example)

3. Calculate volume of Opti-Green Background Suppressor required to provide 3X final assay concentration.

   a. Determine volume of Opti-Green Background Suppressor: [Total volume] µL x [Working concentration Opti-Green Background Suppressor] mM / [Stock concentration Opti-Green Background Suppressor] mM 5000 µL x 1.5 mM / 100 mM = 75 µL

   b. Determine volume of media:[Total volume] – [Opti-Green Background Suppressor]

   5000 µL – 75 µL = 4925 µL

4. Add IncuCyte FabFlour-488 test antibody and Opti-Green to Cells

Adherent cells

4.1 Remove cell plate from incubator.

4.2 Using a multi-channel pipette:

a. Add 50 µL of diluted Opti-Green to wells
b. Add 50 µL of labeled antibody to required test wells.
c. Remove any bubbles and place plate in IncuCyte® S3 Live-Cell Analysis System.

4.3 Place plate in IncuCyte® S3 Live-Cell Analysis System.

Non-adherent cells

4.1 Add reagents to matrix coated plate:

a. Add 50 µL of diluted Opti-green
b. Add 50 µL of labelled antibody to required test wells.
c. Add 50 µL of cell suspension to wells.
d. Remove any bubbles

4.2 Allow the plate to sit for 30 minutes at room temperature to allow even settling, or centrifuge at 50 g for 1 minute.

4.3 Place plate in IncuCyte® S3 Live-Cell Analysis System.

5. Aquire images and analyze

5.1 Using IncuCyte® Software, schedule 24-hour repeat scanning for every 2-3 h.

a. Scan on schedule, Standard.
b. Channel selection: select "phase" and "green"
c. Objective: 10x or 20x depending on cell types used. Generally, 10x is recommended for adherent cells, and 20x for non-adherent or smaller cells.

5.2 To generate the metrics, user must create an Analysis Definition suited to the cell type, assay conditions and magnification selected.

5.3 Select images from a well containing a positive signal and an isotype control well (negative signal) at a time point where staining is visible.

5.4 In the Analysis Definition:

a. Set mask for phase confluence measure with green channel turned off.
b. Turn green channel on and mask green objects. Exclude background fluorescence using the background subtraction feature. The feature "Top-Hat" will subtract local background from brightly fluorescent objects within a given radius; applicable for analyzing objects which change in fluorescence intensity over time.
   i. The radius chosen should reflect the size of fluorescent objects but contain enough background to reliably estimate background fluorescence in the image; 20-30 µm is often a useful starting point.
   ii. The threshold chosen will ensure that objects below a fluorescence threshold will not be masked.
   iii. Choose a threshold in which green objects are masked in the positive response image but low numbers in the isotype control, negative response well.

NOTE: For both cell types, individual cell identification can be enabled with the use of the IncuCyte Cell-by-Cell Analysis Software Module (PN 9600 0031). This enables the subsequent classification into subpopulations based on properties including fluorescence intensity, size and shape. For further details of this analysis module and it's application see: **www.essenbioscience.com/cell-by-cell**

Analysis Guidelines

Staining of surfaced expressed protein will appear as a green ring followed by intracellular green signal as there will be internalization of the signal over time (time depends on cell type studied). Suggested metrics for data analysis are shown below:

1. Quantification of fluorescence area ("total object area" or "green object confluence"). Suggested metric: Analyze using "Total green Object Area (µm²/well)".
2. Quantification of intensity, integrated over the area of detectable fluorescence (i.e. "Total Integrated Intensity"). Suggested metric: Analyze using "Total green Object Integrated Intensity (GCU x µm²/ well)" metrics.
3. To correct for cell proliferation, it is advisable to normalize the area measurement for cell coverage (e.g. "green object confluence"/"phase confluence").

NOTE: If using Cell-by-Cell Analysis, post classification the data can be displayed as either % of cells expressing green fluorescence or mean intensity of positive green objects.

Table 1. IncuCyte® Opti-Green concentration recommendations

Cell Type	Cell Line	Type	Concentration Range Tested	Recommended Concentration
Adherent	HT1080 NR	Epithelial (fibrosarcoma)	0.25mM-30mM	1mM
	A549 NR	Lung		
	MDA-MB-231 NR	Breast		
	MCF-7 NR	Breast		
	SKOV-3 NR	Ovary		
Non-adherent	Jurkat	T cell leukaemia	0.125mM-10mM	0.5mM
	CCRF-CEM	T cell leukaemia		
	Raji	B cell lymphoma		
	Ramos	B cell lymphoma		
	THP-1	Monocytic		
	HL-60	Pre-myelocytic		
	PBMCs	Peripheral blood cells		

A complete suite of cell health applications is available to fit your experimental needs. Find more information at **essenbioscience.com**

For additional product or technical information, please e-mail us at **AskAScientist@sartorius.com**
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