Fluorescent Dye Optimization

The IncuCyte™ ZOOM Live Content Imaging system is configured to quantitatively measure biology in three imaging channels: Phase contrast, Green, and Red. The dual color filter module [Model: 4459] found within the IncuCyte™ ZOOM gantry houses the optical system (lenses, filters, etc.) as well as the light-emitting diodes (LEDs) used to excite fluorescent samples within the tissue culture vessel. The spectral characteristics of the IncuCyte™ ZOOM, and the older IncuCyte™ FLR Live Content imaging systems can be found in the graphic at right. Several commercially available fluorescent dyes are compatible with the IncuCyte™ FLR and ZOOM. Optimization of these fluorescent dyes is recommended to determine the concentration which gives the best signal without being toxic to cell health. This document outlines protocols to optimize membrane permeable and impermeable dyes to be used with the IncuCyte™ FLR and ZOOM.

IncuCyte™ ZOOM
Green:
Excitation Wavelength: 460 nm; Passband: [440,480] nm
Emission Wavelength: 524 nm; Passband: [504,544] nm
Red:
Excitation Wavelength: 585 nm; Passband: [565,605] nm
Emission Wavelength: 635 nm; Passband: [625,705] nm

IncuCyte™ FLR
Green:
Excitation Wavelength: 470 nm; Passband: [450,490] nm
Emission Wavelength: 515 nm; Passband: [500,430]

Membrane Permeable dsDNA Dyes

Membrane permeable double stranded (dsDNA) dyes are inert fluorescent dyes which freely cross the cell membrane and fluoresce green or red when bound to nucleic acid. As a result, membrane permeable dsDNA dyes are ideal for obtaining a total nuclear count at a single time point, but cannot be used for long term kinetic assays as most of these types of dyes intercalate dsDNA and are detrimental to cell health. In this report, four membrane permeable dyes were tested using the IncuCyte™ ZOOM (Table 1). Our studies indicate that Vybrant® DyeCycle™ Green (Life Technologies) and Nuclear-ID™ Red (ENZO) provide the optimal signal in green and red channels, respectively. The red dsDNA dyes, Vybrant® DyeCycle™ Ruby (Life Technologies) and Draq5® (Cell Signaling Technology), were also tested and found to either produce high background fluorescence or have spectral characteristics that are not ideal for image acquisition on the IncuCyte™ ZOOM.

Table 1: IncuCyte ZOOM Compatibility Properties of Membrane Permeable Dyes

Dye	Company	Channel	Excitation	Emission	Spectral Unmixing	Recommended Concentration**	Incubation Time
Vybrant® Dye Cycle™ Green*	Life Technologies	Green	488	520	None	1µM	1-4h
Nuclear-ID™ Red*	Enzo	Red	566	650	14.5% Red from Green	2.5-5µM	1-4h
Vybrant® Dye Cycle™ Ruby	Life Technologies	Red	638	686	None	2.5µM-10µM	10-20h
Draq5®	Cell Signaling Technology	Red	633	680	None	>10µM	12-18h

*Best Endpoint Dyes to use with IncuCyte™ ZOOM and/or FLR
**Recommended concentration based on the minimum concentration which gives the best signal:noise ratio

In completing this analysis, several commercially available dyes were not evaluated either based on non-ideal spectral properties, or because they appeared to be rarely used. However, using these dyes for the right application may be of interest to IncuCyte users. When using this strategy to count nuclei, determining an optimal staining protocol is necessary to ensure the best signal, and to facilitate segmentation and quantitation using the integrated image processing software on the IncuCyte™. The following protocol outlines steps to find the optimal concentration and incubation time for any membrane permeable dye with any cell type on the IncuCyte™ ZOOM or FLR.

Optimization Protocol for Membrane Permeable dsDNA Dyes on IncuCyte™ FLR or ZOOM

1. Seed a 96-well microplate with cells at a density of 5,000-7,500 cells/well so that cells are 40-50% confluent at time of dye addition and incubate overnight at 37°C 5% CO_2.
2. Prepare a range of concentrations that include the manufacturer's recommendations. For example, ENZO recommends that Nuclear-ID™ Red DNA stain be added to adherent cells at a final concentration of 2.5-5.0µM. In this case, test a range of 2-fold dilutions from 0.16-10µM.
3. Add the diluted dye directly to wells containing media and cells (n=3).
4. Place the plate into an IncuCyte™ ZOOM or FLR and image every 30-60 minutes for 2-3 hours.
5. Visually observe which dye concentrations give the best signal:noise ratio.
6. Determine spectral unmixing properties and create a processing definition.
7. Run an analysis job and determine the minimal concentration necessary to properly segment the images.
8. Validate this concentration: Seed a range of densities and compare this optimized concentration to the results of a known membrane permeable dye (Figure 1).

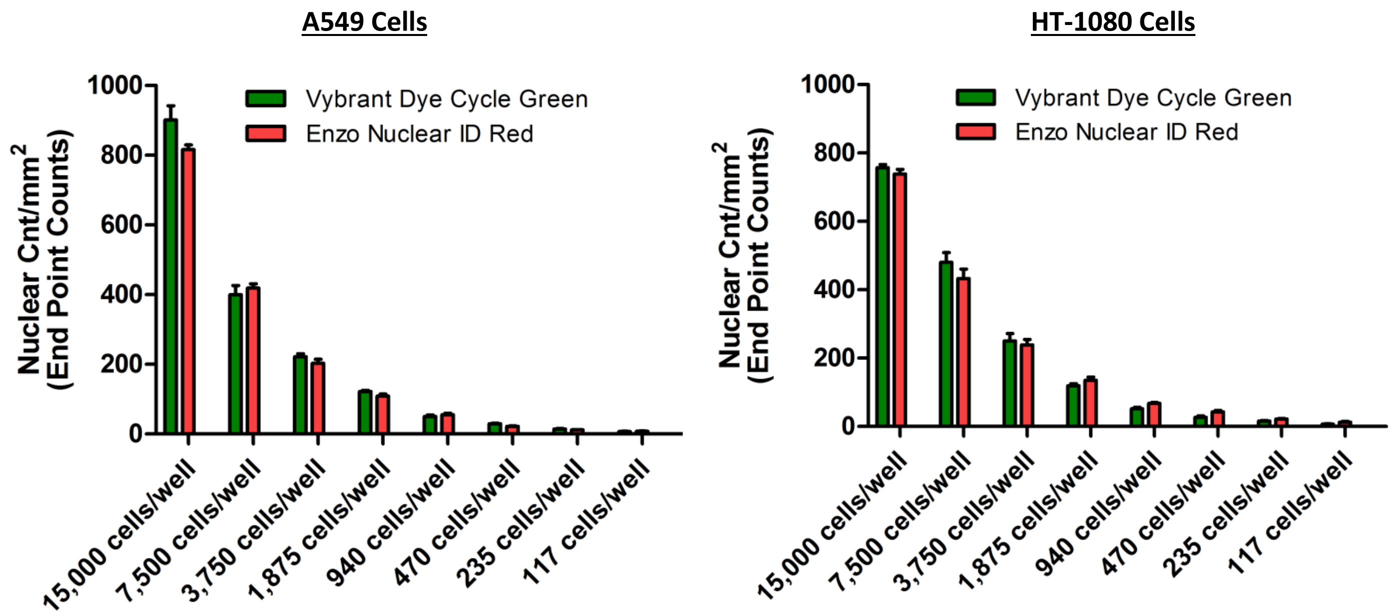

Figure 1: A549 and HT-1080 cells were seeded in 96-well plates at various densities and incubated overnight. The following day, Vybrant Dye Cycle Green and Nuclear ID Red were added directly to cells at final concentrations of 1µM and 5µM, respectively. Cells were incubated at 37°C for 1h before being imaged and analyzed by IncuCyte™ ZOOM. Both membrane permeable dyes produced similar cell counts.

Membrane Impermeable dsDNA Dyes

Membrane impermeable dsDNA dyes are inert fluorescent dyes which do not freely cross intact cellular membranes. When the membrane is compromised, membrane impermeable dyes enter the cell and bind to nucleic acid resulting in a fluorescent signal. At the right concentration, these dyes are non-perturbing to cell biology, and are ideal for monitoring cell viability in long term kinetic assays. Several commercially available membrane impermeable dyes, and other common stains (Annexin V) have been tested on the IncuCyte™ ZOOM and FLR (Table 2). CellTox™ Green (Promega) is the preferred green membrane impermeable dye because it has a high signal to noise ratio, it is not toxic to cell health, and the signal lasts for long term kinetic assays. Likewise, YOYO®-3 (Life Technologies) is the recommended red membrane impermeable dye. YOYO®-3 gives a high signal to noise ratio compared to YO-PRO®-3 (Life Technologies), which has the same spectral properties but produces a lot of background. Membrane impermeable dyes which are not listed below can be tested on the IncuCyte™ ZOOM following the optimization protocol.

Table 2: Membrane Impermeable Dyes

Dye	Company	Channel	Excitation	Emission	Spectral Unmixing	Recommended Concentration
YOYO®-1	Life Technologies	Green	491	509	None	100-250nM
YOYO®-3*	Life Technologies	Red	612	631	5% Red from Green	125-500nM
YO-PRO®-3	Life Technologies	Red	612	631	5% Red from Green	250-500nM
CellTox™ Green*	Promega	Green	513	532	None	1:4000
Sytox® Green Nucleic Acid	Life Technologies	Green	504	523	None	125nM
Sytox® Green Dead Cell	Life Technologies	Green	488	530	3% Green from Red	20nM
Sytox® Red Dead Cell	Life Technologies	Red	640	658	20% Red from Green	60nM
7-AAD	Biotium	Red	543	655	~65% Red from Green	Not Recommended**
Propidium Iodide	Biotium	Red	535	617	>80% Red from Green	500nM
RedDot™2	Biotium	Red	665	695	~20% Red from Green	0.25x
EthD-III	Biotium	Red	528	617	90% Red from Green (5% Green from Red)	2uM
GreenDot™2	Biotium	Green	N/A	N/A	0.05% Green from Red	0.0078x
Annexin V, CF488A	Biotium	Green	490	515	None	125ng/ml
Annexin V, CF594	Biotium	Red	593	614	~6% Red from Green	125ng/ml
Draq7™	Cell Signaling	Red	599	678	N/A	3µM

Technologies

*Best membrane impermeable dyes to use with IncuCyte™ ZOOM and/or FLR
** Not recommended for kinetic assays due to toxic effects on proliferation and migration

Optimization Protocol for Membrane Impermeable dsDNA Dyes on IncuCyte™ FLR and/or ZOOM

1. Seed a 96-well microplate with cells at a density of 2,500-5,000 cells/well so that cells are 20-40% confluent at time of dye addition and incubate cells overnight at 37°C 5% CO_2.
2. Prepare a range of dye concentrations in the presence or absence of a cytotoxic compound (e.g. camptothecin).
3. Add the diluted dye directly to cells (n=3).
4. Incubate the cells immediately and begin imaging in the IncuCyte™ FLR or ZOOM 30 minutes after incubation every 2h for 48h.
5. Use phase contrast images and analyses to determine if any of the dye concentrations are toxic to cell proliferation, indicated by a decrease in cell growth or changes in morphology (Figure 2a).
6. Using fluorescent images, determine the dye concentration that provides the best signal:noise in the presence of the cytotoxic compound.
7. Determine spectral unmixing properties and run an analysis job to compare results obtained using different concentrations of dye (Figure 2b).
8. Determine the minimal concentration necessary to give a positive signal on the IncuCyte™ FLR or ZOOM.
9. Validate the chosen dye concentration: Test a range of concentrations of a known cytotoxic compound and measure pharmacology using the area under the curve (Figure 2c-d).

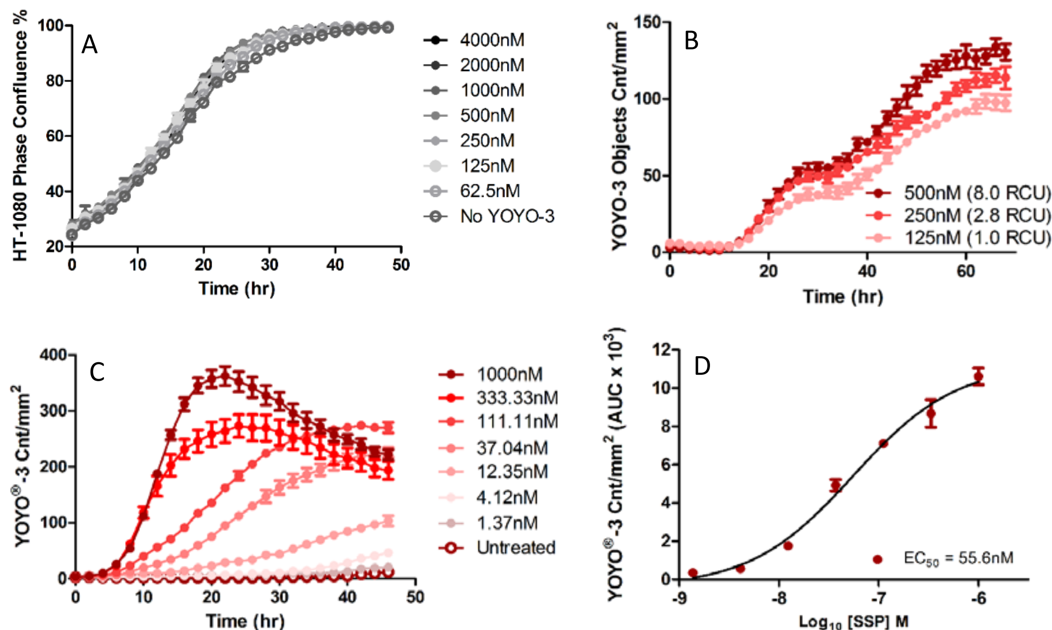

Figure 2: A) HT-1080 cells were grown in the presence of increasing dilutions of YOYO®-3. Confluence was monitored over time. YOYO®-3 did not have an effect on proliferation at any concentration tested. B) HT-1080 cells were treated with 150nM camptothecin in the presence of varying dilutions of YOYO®-3. The ideal concentration is around 250-500nM YOYO®-3. The threshold programmed into the processing definition used to identify fluorescent objects is indicated in parentheses. C) HT-1080 cells were treated with varying concentrations of staurosporine in the presence of 250nM YOYO®-3. D) Area under the curve of YOYO®-3 object counts/mm^2 was used to calculate an EC_{50} value of 55.6nM.