



Fluorescent cell-labeling strategies for IncuCyte® live-cell analysis

Why? What ? And when?

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In the pursuit of more relevant and translational cell-based assays, researchers are increasingly turning to IncuCyte® live-cell analysis for their studies. IncuCyte® provides cell biologists with greater insight and productivity by enabling quantification of cell function over hours, days and weeks via time-lapse image analysis, all automatically and within the controlled environment of the incubator.

Many cell functions, such as proliferation, migration and neurite outgrowth, can be quantified in simple mono-cultures without fluorescent cell labels by analyzing features of phase-contrast images. This 'label-free' approach is desirable and user friendly since it avoids complications associated with cell labels. These include delivery, stability and toxicity of the reagent as well as the inevitable reagent cost. However, there are limitations to what can be achieved without cell labels. For example, it is difficult to extract true cell count metrics from phase-contrast images in dense cultures, and in co-culture models cell labels are absolutely required to identify different cell types to observe their interactions.

Accordingly, a range of different cell-labeling strategies for IncuCyte® analysis have been developed. In this article, we review the details and pros and cons of these label- and label-free strategies and provide user guidance as to when and how these should be used.

1. Label-free assays

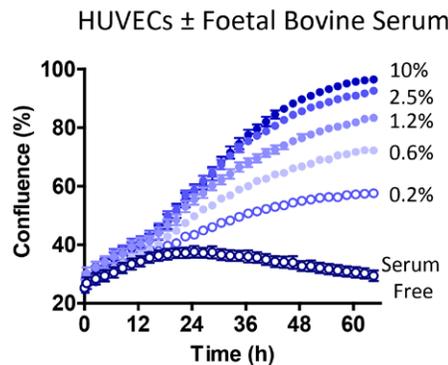
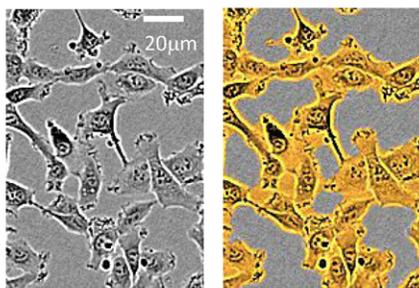
The simplest and most common IncuCyte® application is monitoring of morphology and proliferation over time based on phase-contrast image analysis. For quantification, a software mask is applied that differentiates 'cell' from 'background' (plastic) to return a percentage confluence (area)

metric for each image. As cells proliferate this metric increases to a maximum of 100% confluence, where the entire image is occupied by cells. Confluence is generally a good measure for proliferation and has been successfully used with hundreds of different cell types, including immortalized cell lines,

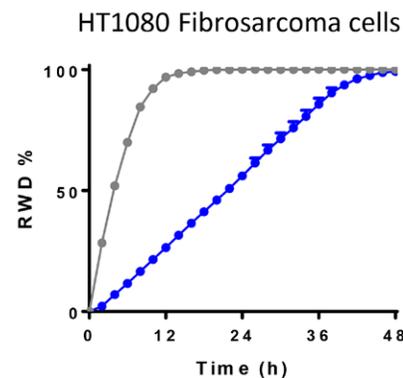
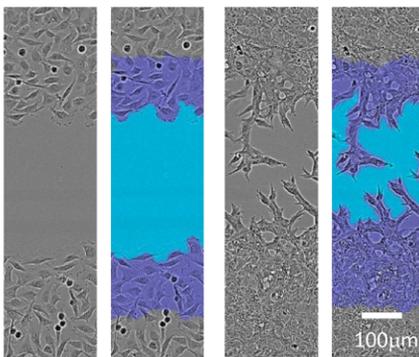
primary cells, stem cells and both adherent and non-adherent cells. With whole-well viewing and 20x magnification researchers can visualize and quantify proliferation arising from a single cell. Even with rapidly dividing cells (e.g. 4-6h doubling times) it is possible to follow up to 5-6 proliferation cycles in microplates by plating at low starting cell confluence (e.g. 5%). For slower growing cultures proliferation can be measured over many (>7) days using the confluence metric. In 3D cell spheroids grown in ultra-low attachment plates, IncuCyte® bright-field (label-free) analysis is useful for proliferation and size measurements.

Under certain conditions the linear relationship between confluence and cell number can deteriorate. For example, at high densities cells begin to pack in or grow on top of each other and true cell number is underestimated. Similarly, if cells undergo significant morphological change during the experiment (e.g. flattening, shrinkage, differentiation) then confluence values may become inaccurate. Perhaps most problematic is when cells die - the confluence metric can be tuned to ignore some cell debris but is generally unable to distinguish the morphology of living from dying

A Cell Proliferation



B Migration Invasion



C Neurite Outgrowth

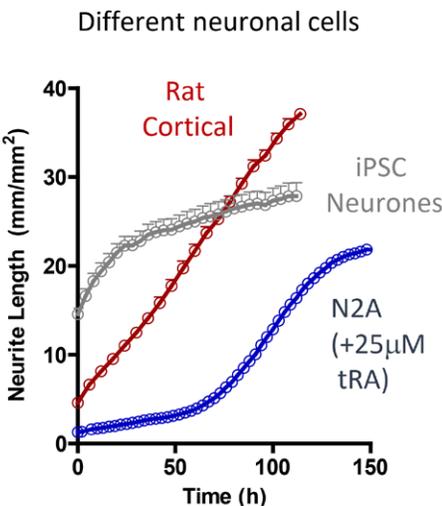
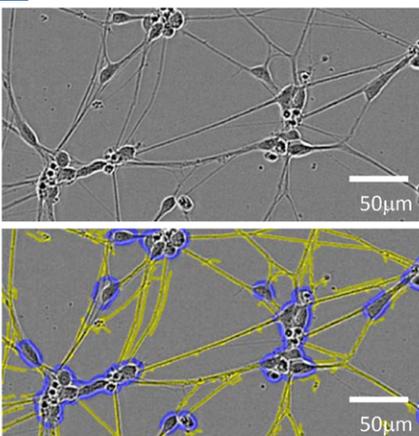


Figure 1.

IncuCyte Phase Contrast Imaging for label free measurements of (A) cell proliferation based on confluence (human umbilical vein vascular endothelial cells), (B) cell migration and invasion through Matrigel (HT1080 cells), and (C) Neurite Outgrowth (iPS-derived neurons). HD Phase images are shown in the absence and presence of the corresponding image mask, alongside quantified timecourse data. In panel B the initial wound mask (t=0) is shown in sky blue and the mask at t=6h (migration) and 18h (invasion) in purple.

cells. As mentioned earlier, label free measurements are of limited use in co-culture models, if it is important to isolate the properties of one cell type from the other. In these situations a fluorescent cell label is required.

2. Fluorescent Proteins (FPs)

Protein-based fluorophores, such as GFP and RFP, have been used for several decades to label cells¹ and are ideally suited to real-time live-cell analysis. FPs provide excellent long term and highly stable cell labeling, are generally non-toxic and not depleted by repeated fluorescent excitation. By using specific targeting sequences in the expression constructs, proteins can be directed to label different cell types or organelles. Via this approach, we have developed a range of different vectors and delivery methods: these including nuclear-targeted FPs ('NuLight'), cytoplasmic FPs ('CytoLight') and neuronal-specific FPs ('NeuroLight'). These are packaged for delivery with either lentiviral or baculoviral (BacMam) expression constructs. In most cases both green (GFP) and red (mKate) FP products are available. These proteins have excitation/emission spectra that are compatible with IncuCyte optics, and provide choice and flexibility for duplexing assays with a second color.

IncuCyte® NuLight FPs

NuLight FPs are retained within the nuclear envelope of the cell, and do not distribute into the cytosol. New NuLight protein is synthesized and expressed during mitosis so there is no signal dilution or drop off in dividing cells. Importantly, NuLight proteins differ from other nuclear-restricted proteins in that they do not tag cell histones – this is a common alternative approach to nuclear targeting but can impair cell division. By directly comparing proliferation rates measured using NuLight FP nuclei counting and phase confluence we can demonstrate that NuLight FP expression does not interfere with cell replication. This combination of non-perturbance, nuclear restriction and labeling of cell progeny make NuLight FPs ideal for long-term cell proliferation assays based on true cell count. Nuclear counting affords several advantages over phase confluence measurements: (1) it enables true cell doubling time analysis (2) it is not subject to artefacts arising from changes in cell shape or area (3) proliferation can be still be quantified beyond 100% confluence, and (4)

only living cells are quantified since the FP label is lost on degradation of the nuclear envelope during cell death. The most common application of NuLight FPs is to monitor the growth of cancer cell lines, either in mono- or co-culture systems. Indeed, we consider this a gold-standard method inasmuch as the absolute live-cell number is counted directly and over time, without removing cells from the incubator. Proliferation curves can be fitted to exponential growth functions to yield true cell doubling times. This contrasts with other approaches which are surrogate, indirect endpoints made from cells on the bench (e.g. ATP, LDH, MTT). In co-cultures, the impact of one cell type on the growth of a second can be quantified using NuLight labeling. This has proved particularly useful in stromal/cancer cells studies and immuno-oncology research.

To use NuLight FPs, they must first be transfected or transduced into cells. Our favored approach for immortalized cells is to make stable cell lines using IncuCyte lentiviral products. These products integrate the NuLight plasmid, which includes an antibiotic selection marker cassette (puromycin, bleomycin or neomycin), into the DNA of the cell. As a one off exercise, cells are exposed to the lentivirus for 24h, washed to remove excess virus and then grown out in antibiotic to eliminate non-expressing cells. Depending on the growth rate of the cells it typically takes 10-14 days to create a line in which expression is stable, consistent and homogeneous – after this time the line should express the NuLight for 10-100s passages and can be banked and/or scaled up for larger assays. In the cell lines that we have made and tested (e.g. MDA-MB231, A549, HT1080), the number of residual viral particles is below detectable limits (GP120 assay) such that these lines can be used in low-level safety category 1 biohazard laboratories. Lentiviral transduction of NuLight FPs is effective in a wide range of cell types, including adherent and non-adherent tumor cell lines, epithelia cells and macrophages, but works less well in primary immune cells and certain neurons.

When only a small number of experiments are foreseen or when working with larger numbers of different cell lines, NuLight FPs may be delivered transiently using BacMam. Similar to lentivirus, the baculoviral expression method involves adding viral particles to cells in media, waiting for 24h and then washing off excess virus. Protein expression is generally rapid and

NuLight can be viewed within 4-6h. The BacMam plasmid does not integrate into the cell DNA so the expression of the protein is more short-lived than with lentivirus, typically lasting 3-7 days post transduction. The BacMam system generally expresses protein more rapidly than lentivirus and can be handled under category 1 containment conditions.

IncuCyte® CytoLight FPs

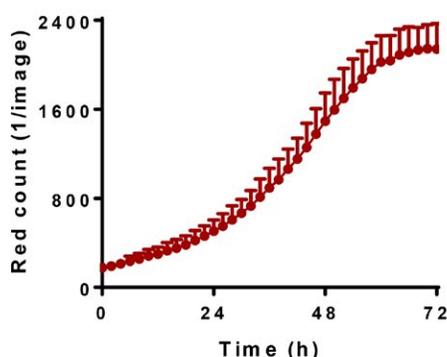
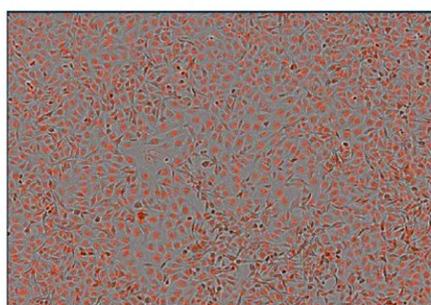
IncuCyte® CytoLight FPs share all features of the NuLight FPs, with the exception that the protein is expressed throughout the cell rather than nuclear-restricted. These reagents are designed for applications where cell area analysis is valuable, as opposed to direct cell counting (Figure 4). As an example, CytoLight Green is used to label human vascular endothelial cells (HUVEC) that are co-cultured with human dermal fibroblasts in a cell model of tube formation (AngioKit). The tube-like structures formed by the HUVECs are discriminated from the supporting dermal cells using the CytoLight FP green fluorescence signal, and measured with a custom IncuCyte analysis algorithm. A second application that benefits from CytoLight FPs is spheroid cell health. While bright-field analysis is helpful for size measurements (see above), CytoLight cells can be used as reporters of spheroid cell health (dying cells lose fluorescence) and in co-cultures with immune or stromal cells.

IncuCyte® NeuroLight FPs

Neurons are known to be difficult to transfect and are more susceptible to reagent and environmental toxicity. IncuCyte® NeuroLight Red is designed to label neurons for use in co-cultures

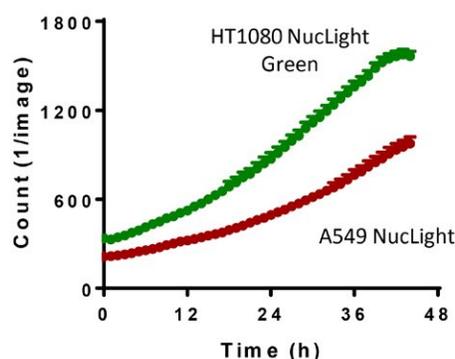
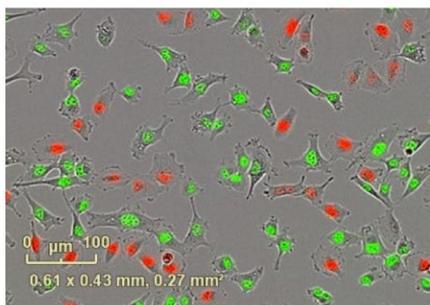
A Counting in mono-culture

HT1080 + NuLight Rapid Red



B Counting in co-culture

A549 NuLight Red & HT1080 NuLight Green



C Immune cell killing

SKOV3 NuLight Red & human PBMCs

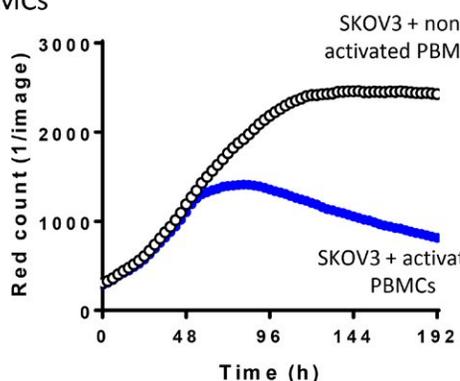
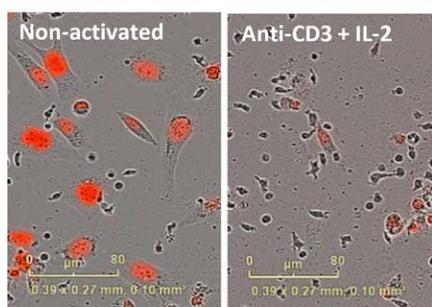


Figure 2.

IncuCyte Imaging for Fluorescence measurements of (A) cell proliferation based on cell count in a HT1080 mono-culture including the NuLight Rapid Red reagent (B) cell proliferation based on count in a co-culture of HT1080 NuLight Green and A549 NuLight Red cells and (C) Immune cell killing of SKOV3 NuLight Red cells by activated PBMCs. Blended phase and fluorescent images are shown, alongside quantified time-course data. In panel C images of SKOV3 cells in the presence of non-activated or anti-CD3/IL-2 activated PBMCs are shown.

e.g. with astrocytes or glia, and expresses a membrane-targeted mKate2 fluorescent protein via a neuronal-specific synapsin promoter. A green FP equivalent is not available since the necessary blue excitation light is detrimental to cell health. The recommended protocol for NeuroLight Red, validated across various primary, immortalized and iPS-derived neurons is (1) plate neurons and after 4h add NeuroLight Red, (2) wait 24h and then refresh media including astrocytes, (3) on day 3 replace 50% media with fresh media containing Uridine & 5 FD

Uridine to minimize astrocyte proliferation (4) replace 50% media every 3rd day thereafter. Using this method, neuronal labeling takes 48-72h to stabilize and then remains stable for > 28 days in culture. We observe no detrimental effects of NeuroLight on cell health and the neurons retain their ability to send out neurites and form synaptic networks. The expression levels and brightness of NeuroLight are such that even fine neuronal projections can be quantified with red fluorescence and the IncuCyte Neurotrack analysis software.

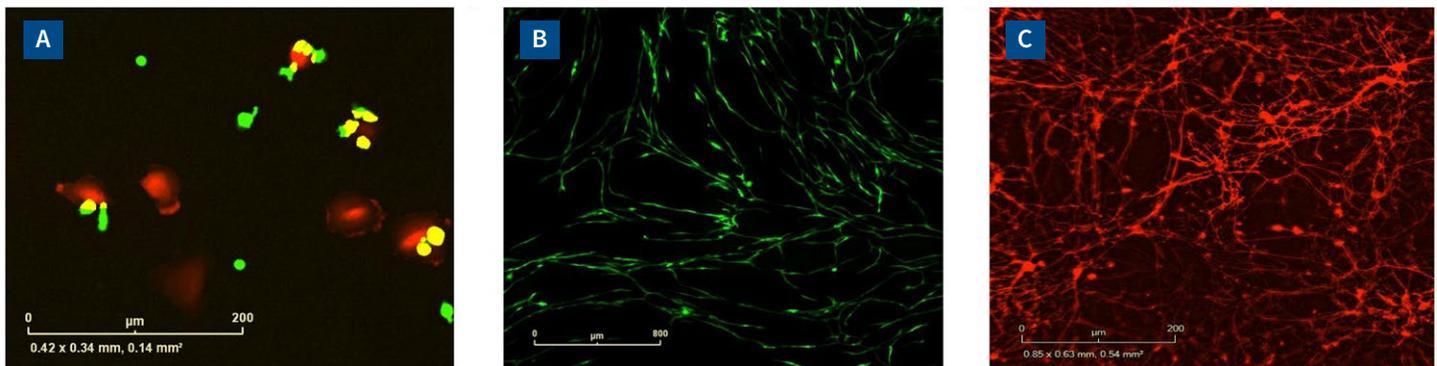


Figure 4.

Cytoplasmic and neuronal membrane-labeling IncuCyte live-cell applications. **(A)** Immune-cell proximity. Peripheral blood mononuclear cells (PBMCs) were activated and labeled with IncuCyte CytoLight Rapid Green Dye and then added to tumor cells stably expressing a red fluorescent protein (CytoLight Red Lentivirus Reagent). Immune cells in close proximity to tumor cells are masked as 'coincident' (co-localized) objects (yellow) **(B)** Angiogenesis in co-culture. Human Vascular Endothelial Cells (HUVECs) were labeled with IncuCyte CytoLight Green FP and co-cultured with (unlabeled) human dermal fibroblasts. HUVEC vascular networks (green fluorescence) are quantified for length, area and branch points as measures of angiogenesis. **(C)** Neurite dynamics in co-culture. Rat primary forebrain neurons are labeled with IncuCyte NeuroLight Red and co-cultured with (unlabeled) rat astrocytes. Neurite networks (red fluorescence) are quantified for length and branch points. IncuCyte images taken with 20x (A) and 10x (B,C) objectives, scale bars as shown.

3. Fluorescent Cell Labeling Dyes

In certain scenarios it may be difficult or undesirable to transfect cells with fluorescent proteins. Examples include immune cells that have a limited life span in vitro, and neurons and patient specific tumour cells that may be challenging to genetically manipulate. In these cases, cells can be labeled with CytoLight Rapid cytoplasmic or NucLight Rapid nuclear binding fluorescent dyes. When used correctly, these chemical probes are non-perturbing to cells and produce uniform and homogeneous labeling. These dyes have very different properties and use protocols to each other, and to the protein labels, and are best aligned with different applications.

IncuCyte® CytoLight Rapid

CytoLight Rapid dyes produce cytoplasmic fluorescent labeling of cells via a simple, 'load and wash' protocol. The

dye is added to cells, incubated for a short time period to allow uptake, and then washed to remove excess. Almost all adherent and non-adherent cells can be labeled with this method. The excitation and emission spectra of these dyes are tuned to IncuCyte optics, yielding bright, discernable and homogeneous cellular fluorescence. Importantly, and with optimization, dye concentrations can be identified that do not perturb cells, as evidenced by unmodified cell morphology and proliferation rates. CytoLight Rapid dyes do not transfer from cell to cell upon contact, which is critical to their use in co-culture systems.

In our hands, CytoLight Rapid dyes exhibit acceptably stable fluorescence for up to 24-48h post-labeling and are suitable for live-cell analysis over this time period.

Beyond this, signal loss becomes problematic. If cells are rapidly dividing, the partitioning (dilution) of dye between daughter cells exacerbates this problem. The reduction in cellular fluorescence over time presents challenges with long term cell quantification, since the IncuCyte object masking approach depends on setting threshold (background) levels, above or below which masked objects will fall as their fluorescence drops. This can yield under or over-estimates of true cell area. Since the dye labels the entire cytoplasm it is only possible to count individual cells at low densities using Cytolight Rapid dyes – at higher cell densities or when cells cluster it is not possible to resolve connected cells. For these reasons, we generally find that Cytolight Rapid labeling offers little value over phase metrics for measuring cell proliferation in rapidly dividing mono-cultures, and is therefore not recommended for this purpose.

Rather, the most appropriate use is for visualizing and quantifying labeled cells in co-cultures, where the dye can be used to identify cells of interest. As an example, for immuno-oncology T-cell killing assays, either the effector T-cells or target tumor cells can be labeled before creation of the co-culture. In this case, by proximity analysis it's possible to verify the 'attack' of the tumor cells by the labeled immune cells. Loss of CytoLight Rapid fluorescence can also be used as a specific measure of tumor cell death. This approach should prove particularly useful for profiling CAR-T cells and for killing assays on patient-specific primary tumor samples.

4. Summary & Wrap up

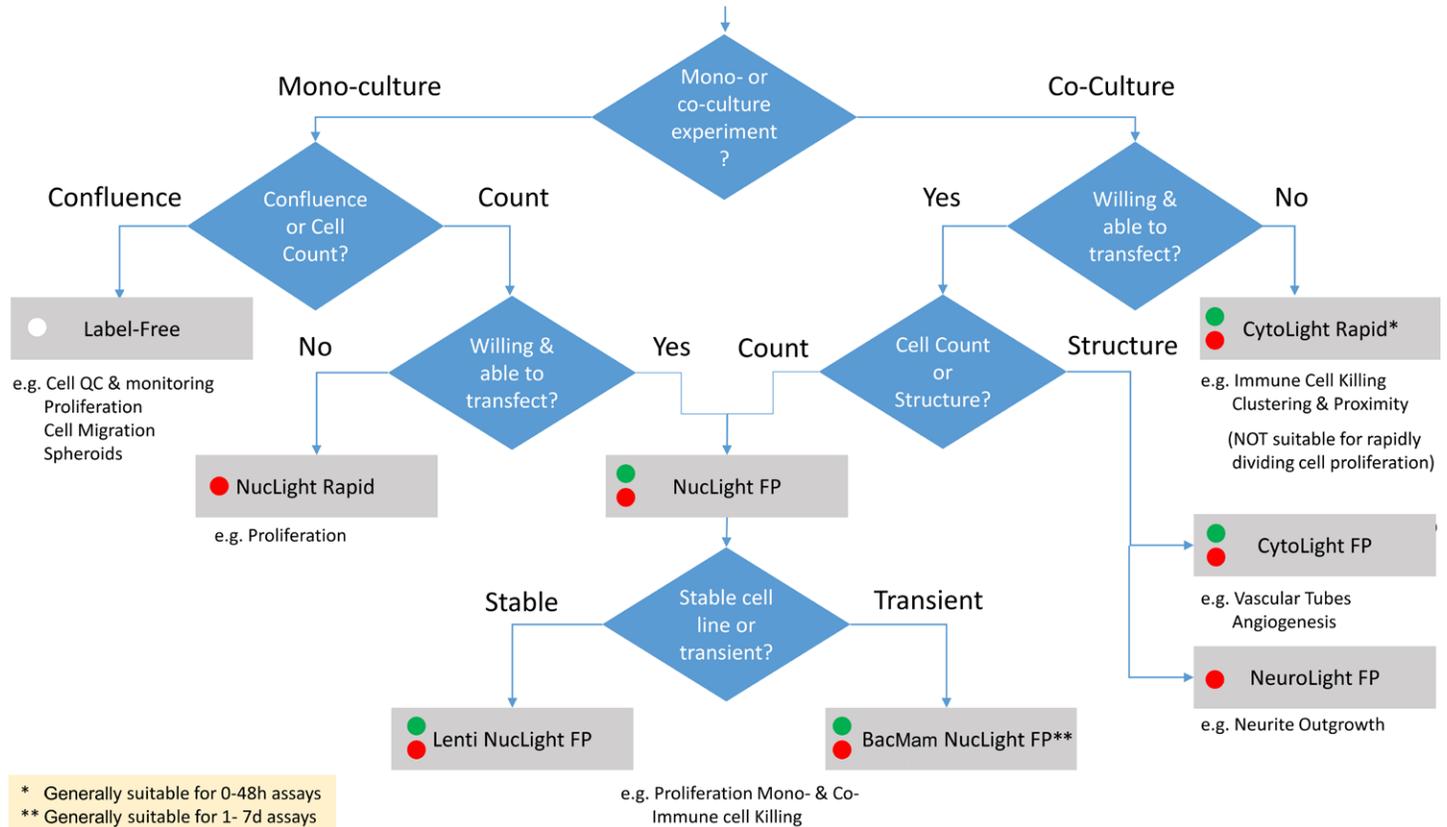
In summary, label-free, fluorescent protein and dye-labeling approaches each afford distinct and valuable opportunities for IncuCyte® live-cell analysis. Label-free methods are technically most straightforward, unequivocally non-perturbing to the biology and well suited to mono-culture proliferation assays in monolayer and spheroid models. NuLight FPs provide the gold-standard solution for true cell counting and proliferation, and for co-culture models where transfection is possible. The choice of lentivirus or baculovirus delivery depends on the importance of long term, stable expression and bio-safety considerations. CytoLight and NeuroLight FPs have specialized uses for specific phenotypic measurements

IncuCyte® NuLight Rapid

NuLight Rapid is a novel, cell permeant DNA binding fluorescent dye. As with NuLight FPs, it labels just the nucleus of the cell rather than the cytoplasm. The labeling method involves continuous exposure of cells to the dye when added to the culture media, as opposed to the 'load and wash' method used for CytoLight Rapid. Via this approach, the progeny of dividing cells are also labeled by the NuLight Rapid, affording true cell (nuclei) counting over time. Since labeling is generally uniform and consistent, nuclear masking is straightforward. At selected concentrations, NuLight Rapid is non-perturbing to cells and long-term measurements for >1 week are possible.

One interesting feature of NuLight Rapid is that, depending upon mechanism of cell death, the DNA may remain labeled even after a cell dies, presumably as the DNA remains relatively intact long after the cell membrane integrity is compromised. The labeling of the DNA of dead cells versus live cells has strikingly different fluorescent intensities, enabling the ability for a true kinetic cell death index (% of dead cells) to be determined. This contrasts with NuLight FP where the nuclear signal is lost.

in angiogenesis, spheroids and neurobiology assays. CytoLight Rapid dyes allow identification and quantification of cells in co-culture assays, and are useful when it is difficult to transfect cells. However, they are limited to short-term (up to 48h) measurements and are not recommended for proliferation measurements. NuLight Rapid dye enables true kinetic cell counting, but only in mono-cultures. Understanding these different characteristics and design features is key to selecting the most appropriate experimental strategy. Together, these methods and reagents enable a powerful and flexible suite of IncuCyte live-cell analysis applications.



* Generally suitable for 0-48h assays
 ** Generally suitable for 1- 7d assays

Confluence or Cell Count?

Both confluence (area) and cell count metrics are useful for live-cell analysis of cell growth, but which one to use and when? There are few hard and fast rules, but in most cases:

- (1) Cell Confluence is the simpler metric to determine, either label-free or with Cytolight labels, but has more theoretical and practical caveats if used as an index of cell number. This is based upon complications that may arise from changes in cell size and shape over time.
- (2) Cell counting is the superior metric and is easiest to understand, but other than at low cell densities, absolutely requires the use of a fluorescent NuLight label (protein or dye).

The selection is determined through pragmatic consideration of the experimental question and cell system. In our view, for QC, basic monitoring and comparison of cell growth rates, label-free area (confluence) analysis is appropriate. In this case any (minor) limitation in the assumptions made by the area analysis are far outweighed by the obviated need to label

the cells. In contrast, for co-culture assays a fluorescent label is required de facto, in which case the researcher may as well benefit from using the superior nuclear count analysis. The grey region lies in mono-culture cell proliferation assays for compound, antibody or genetic library screens. Here, there is no doubt that cell labeling and direct cell counting affords value over the confluence proxy, particularly when test agents produce profound morphological changes that can upset an area analysis. Combining cell counts with cell health reporters (e.g. IncuCyte Cytotox Red, Caspase 3/7 or Annexin V probes) can yield powerful live/dead analyses such as apoptotic index, and discrimination of cytostatic and cytotoxic profiles. However, that's not to say that area analysis for proliferation is not valid or valuable. In our hands, conclusions from pharmacological analyses are remarkably resilient to imperfections in area/cell number assumptions. The final decision rests with the experimenter and their perspective on 'fit for purpose'.

IncuCyte Product	Label	Delivery Method	Localisation	Live/Dead	Dilution in Daughter Cells	Longevity	Fluorescence Channel	IncuCyte Product Codes
NuLight FP	Protein	Lentivirus (or BacMam)	Nuclear	Live	No	Weeks	Red - mKate	4625, 4627, 4621, 4476, 4478
							Green - GFP	4624, 4626, 4622, 4475, 4477
CytoLight FP	Protein	Lentivirus (or BacMam)	Cytoplasmic	Live	No	Weeks	Red - mKate	4482
							Green - GFP	4513
NeuroLight FP	Protein	Lentivirus	Neuronal Membrane	Live	-	Weeks	Red - mKate	4584
NuLight Rapid	Dye	Mix & Read	Nuclear	Live & Dead	No	0-7d	Red	4717
CytoLight Rapid	Dye	Load & Wash	Cytoplasmic	Live	Yes	0-48h	Red	4705
							Green	4706

Table 1.

Comparison of attributes of IncuCyte live-cell analysis labeling reagents. Live/Dead refers to whether the product labels living or both living & dying/dead cells. Dilution in daughter cells refers to whether the fluorescent signal is diluted between cells during mitosis.

Labelling Strategy	Mono-culture		Co-culture	
	Cell Proliferation	Specialised Assays	Cell Proliferation	Specialised Assays
Label-Free (Phase)	Confluence	Migration, Neurite Outgrowth, Immune Cell Clustering, Clonal Dilution, Stem Cell monitoring	-	-
NuLight FP	True Cell Counting	Cell Health, Spheroids	True Cell Counting	Immune Cell Killing, Cell Health, Spheroids
CytoLight FP	Confluence	Migration	Confluence	Immune Cell Killing
NeuroLight FP	-	-	-	Neurite Outgrowth
NuLight Rapid	True Cell Counting	-	-	-
CytoLight Rapid	(Not recommended)	(Not recommended)	(Not recommended for rapidly proliferating cells)	Immune Cell Killing (up to 48h), visualising cells of interest

Table 2.

Live-cell analysis applications of label-free (phase) and IncuCyte cell-labeling approaches.

Citations

- Misteli T, Spector DL (1997). **Applications of the green fluorescent protein in cell biology and biotechnology.** *Nat Biotechnol.* 10:961-4.

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