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Live-Cell Immunocytochemistry

Correlating Surface Marker Expression with Morphological and Functional Changes in Living Cells Over Time

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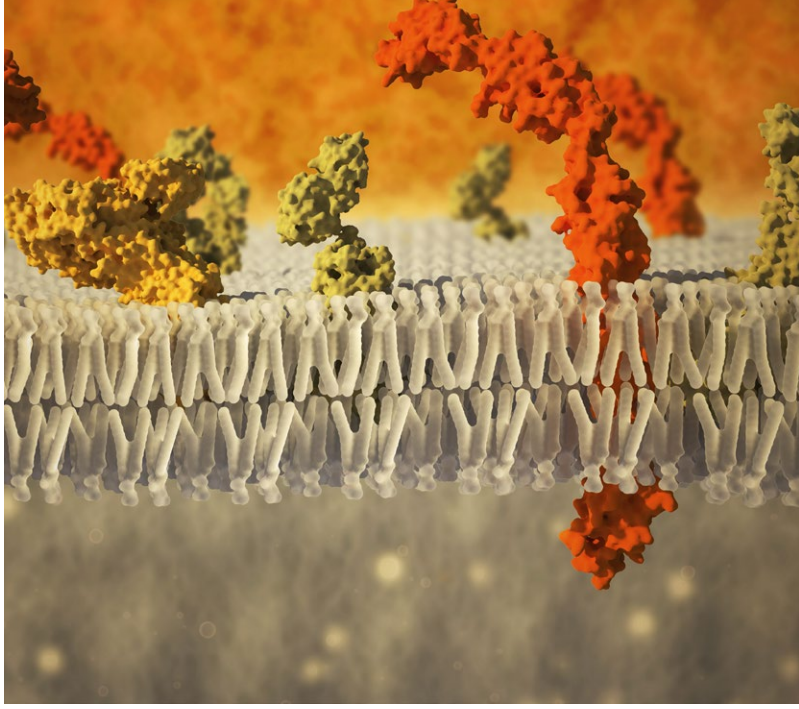
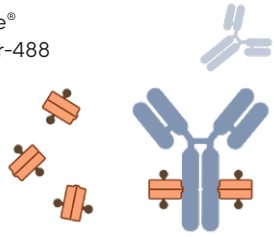
Immunocytochemistry (ICC) is a powerful laboratory imaging technique for visualizing the location of specific proteins in cells using targeted fluorescently-labeled antibodies ('immunofluorescence'). With cell fixation protocols and specialized microscopes, spatial resolution down to the nanometer level can be achieved. Where conventional ICC is less useful, however, is in studying changes in protein expression over time, as may occur as cells differentiate, interact, or respond to external stimuli. Indeed, there is a strong unmet need for technical solutions that provide temporal tracking of the distribution and abundance of proteins in living cells, and link these to cell morphology and function.

In this article, we introduce an integrated solution for 'live-cell' ICC, based on Incucyte® live-cell imaging and analysis and fluorescently-labeled antibody fragments. The methodology is amenable to any protein expressed on the cell surface to which there is a corresponding specific antibody. In brief, the antibody is first tagged with an isotype-matched Fc-targeted

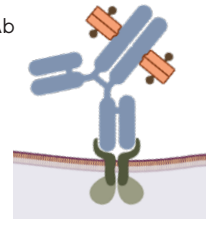
fluorescently-labeled antigen binding fragment (Fab) via a simple mix, no wash protocol. This complex is then added directly to living cells and used to visualize the expression of proteins on cells. Using Incucyte® Live-Cell Analysis System, time-lapse images of cellular fluorescence can be gathered over hours and days, and automatically analyzed to provide an index of the levels and pattern of expression over time (Figure 1). Importantly, this approach can be combined with analyses of other morphological and phenotypic parameters, such as cell health, neurite outgrowth and phagocytosis, to temporally associate surface protein expression changes with cell function. Moreover, cell-cell interactions and proximity relationships between motile cells can be observed and quantified in ways that hitherto have been inaccessible.



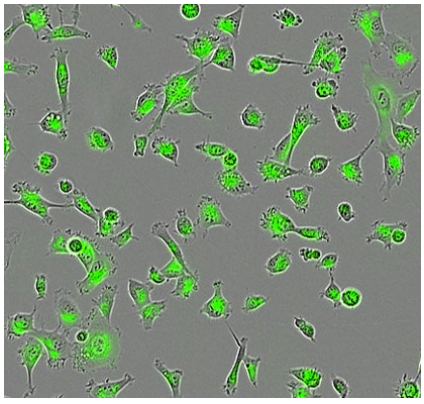
Incucyte®
Fabfluor-488
Dye



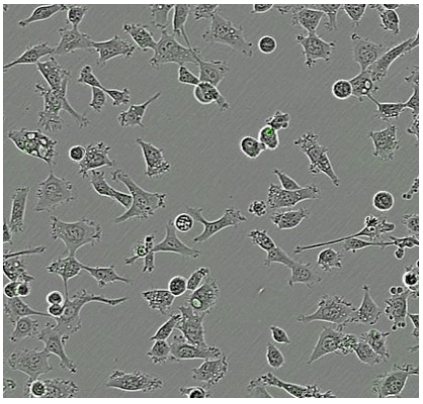
Mix Fabfluor-488-Ab
complex with cells.



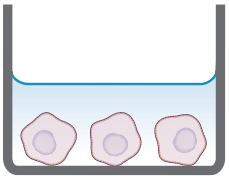
CD71-Fabfluor-488: HT-1080 cells



IgG-Fabfluor-488: HT-1080 cells

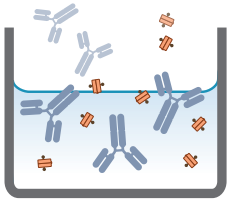


1. Seed cells



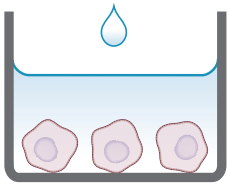
Seed cells (50 µL/well, 5–30K/well) into a 96-well plate.
Note: For non-adherent cell types, PLO coat plate prior to cell seeding.

2. Label test antibody



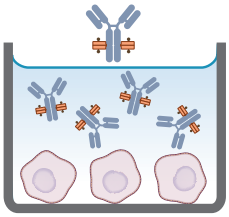
Mix antibody and Fabfluor-488 Dye at a molar ratio of 1:3 in media, 3X final concentration. Incubate for 15 minutes to allow conjugation.

3. Add Incucyte® Opti-Green



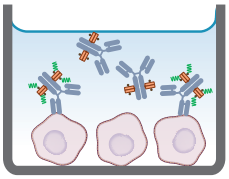
Add 50 µL/well, 3X final concentration.

4. Add labeled AB



Add antibody-Fabfluor mix (50 µL/well) to cell plate. For non-adherent cells, spin plate.

5. Live-cell fluorescent imaging



Capture images (time span and objective depend on assay and cell type, 10X or 20X) in Incucyte® Live-Cell Analysis System.

Figure 1: Incucyte® live-cell immunocytochemistry: concept and protocol. Images shown are Incucyte® 20X magnification.

Method Validation

There are a number of key considerations and validation requirements for this approach (Figure 2). We confirmed the signal specificity of the method by labeling Ramos B-lymphocytes with a fluorescent Fab (Incucyte® Fabfluor-488) complexed with antibodies to either CD20, CD45, CD3 or IgG. Experiments were conducted in the presence of Incucyte® Opti-Green, a background suppressor dye, to help resolve cellular fluorescence. As anticipated, Ramos cells were labeled only by the B cell marker CD20 and the general lymphocyte marker CD45, but not the T cell marker CD3 or isotype control. Both fluorescence intensity and area measurements were useful metrics for protein labeling. In cell mixture experiments with Ramos (B cells) and Jurkats (T cells), the extent of specific labeling followed the proportions of cells in the mix with the anticipated ratios. For the temporal analysis to be valuable, it is essential that the Fab | Ab complex is stable over the period of the cellular analysis. To verify this, we monitored cellular fluorescence for > 72 h in the continued presence of the label. Under these conditions stable fluorescence was observed over the full time course, with no evidence of signal drop off even after 3 days. Finally, and critically, we determined that the Incucyte® Fabfluor-488 and Incucyte® Opti-Green suppressor had no overt detrimental effects to cell health under the experimental conditions, by monitoring cell proliferation, vitality, and morphology. While it is possible that certain Abs may have target specific toxicity (e.g., rituximab targeting CD20), in our hands this is generally not the case. Similar observations regarding specificity, longevity of labeling and non-perturbance were made with a range of target antibodies (e.g., CD45, CD8, CD56, CD4, etc.) in a range of cell types (e.g., immune cells, neurons, tumor cell lines, etc.). Together, we consider these properties of our labeling method to be suitable for long term live-cell analysis. We further validate and illustrate the value of this approach with four examples.

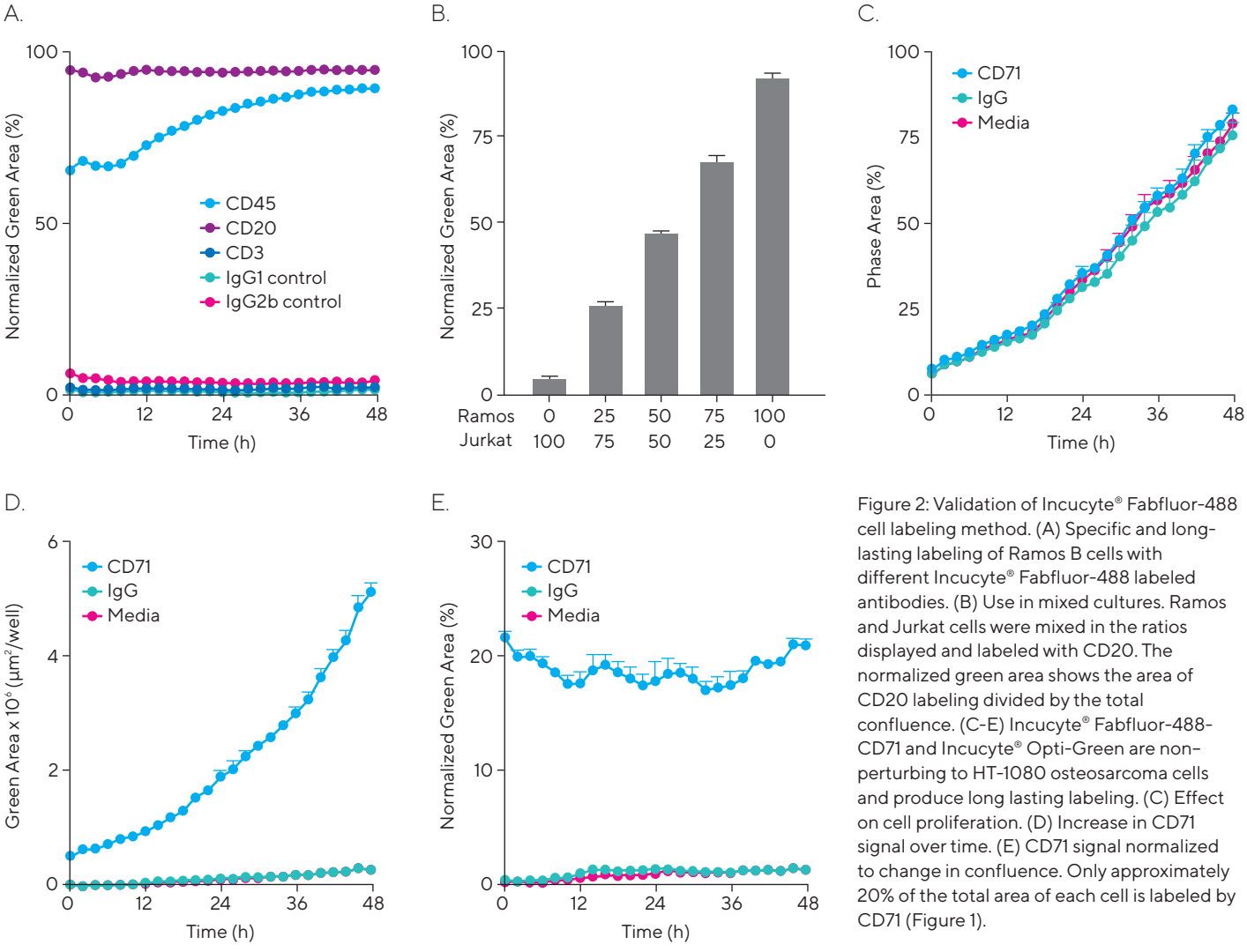


Figure 2: Validation of Incucyte® Fabfluor-488 cell labeling method. (A) Specific and long-lasting labeling of Ramos B cells with different Incucyte® Fabfluor-488 labeled antibodies. (B) Use in mixed cultures. Ramos and Jurkat cells were mixed in the ratios displayed and labeled with CD20. The normalized green area shows the area of CD20 labeling divided by the total confluence. (C-E) Incucyte® Fabfluor-488-CD71 and Incucyte® Opti-Green are non-perturbing to HT-1080 osteosarcoma cells and produce long lasting labeling. (C) Effect on cell proliferation. (D) Increase in CD71 signal over time. (E) CD71 signal normalized to change in confluence. Only approximately 20% of the total area of each cell is labeled by CD71 (Figure 1).

1. Surface Protein Expression Dynamics

First, we studied the regulation of the checkpoint protein, Programmed Death-Ligand 1 (PD-L1), in MDA-MB-231 breast cancer cells in response to the cytokine interferon-γ (IFNγ: Figure 3). PD-L1 is a transmembrane protein that plays a key role in tumor suppression of the immune system, and is the target for a number of immuno-modulatory anticancer treatments in the clinic. IFNγ has known tumorigenic effects, possibly through its regulatory effects on the PD-L1 pathway.[1] Following treatment with IFNγ, a time-dependent increase in PD-L1 labeling was observed over 72 h, which at the highest concentration was 3-fold greater than the vehicle control. The IFNγ response was also concentration-dependent, with an EC_{50} value of 9 ng ml^{-1}. IFNγ had no effect on the growth rate of MDA-MB-231, indicating that the protein response was a specific upregulation. These data illustrate how dynamic changes in cell surface checkpoint proteins can be quantified in living cells in response to an inflammatory stimulus and may prove a useful format for further studies on the regulation of immune-cell signaling pathways in tumors.

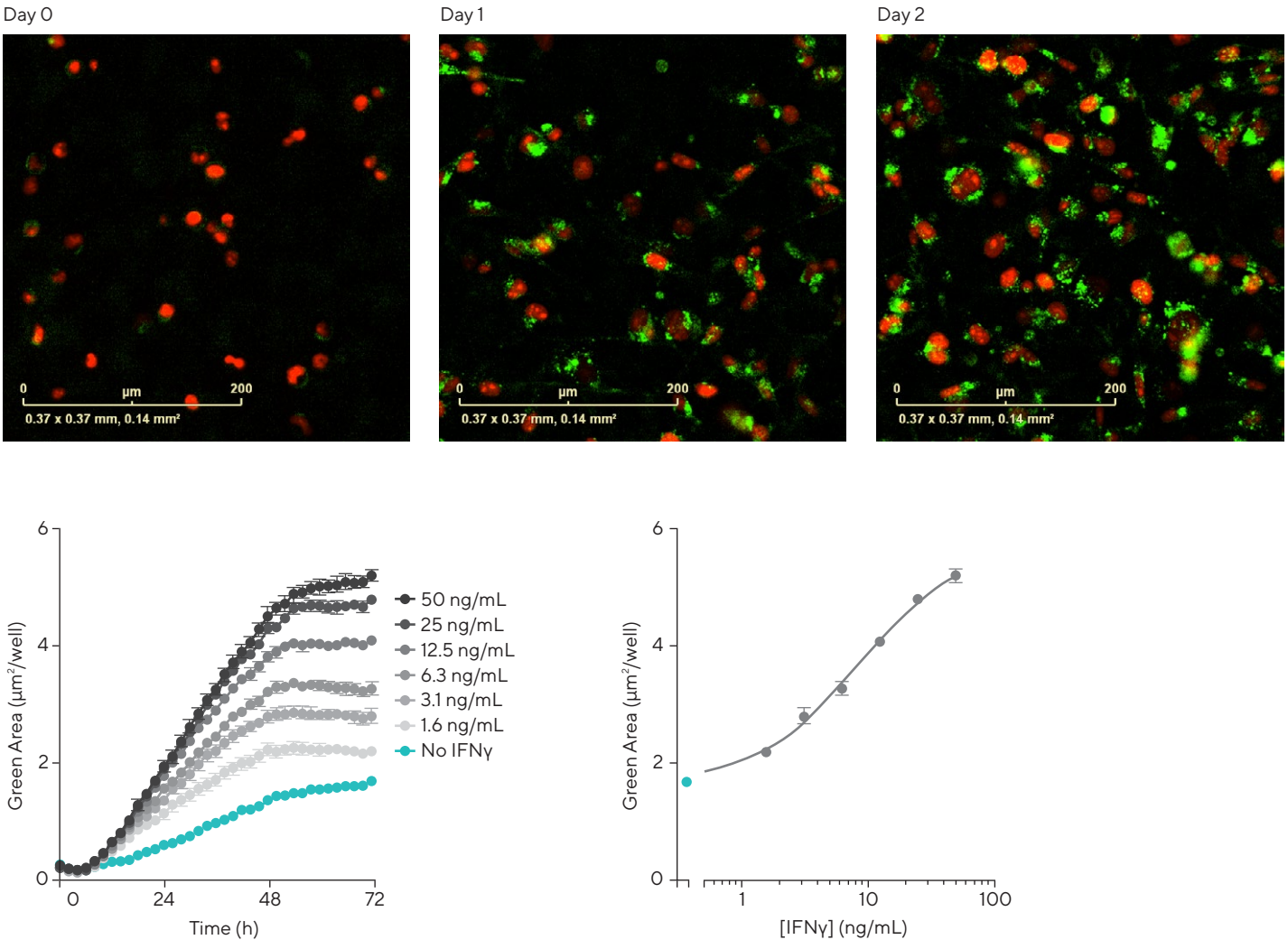


Figure 3: Upregulation of PD-L1 checkpoint protein in tumor cells in response to IFNγ. Fabfluor-488 was conjugated to anti-PD-L1 Ab (BioLegend) and added to Incucyte® Nuclight Red MDA-MB-231 breast cancer cells in the absence and presence of IFNγ (+ Incucyte® Opti-Green background suppressor). Quantification of the green fluorescent area shows that IFNγ induces a time- and concentration-dependent increase in PD-L1 expression, with a mean EC_{50} value of 9.0 ng/mL. Values are shown are mean ± SEM, from four wells.

2. Coupling Protein Dynamics to Morphological Changes

In the second example, we coupled measurements of cell surface markers to morphological changes in human neuroblastoma SH-SY-5Y cells (Figure 4). Under control conditions, SH-SY-5Y cells proliferate rapidly and display heavily clustered, neuroblast-like, non-polarized cell bodies with few, truncated processes. With Incucyte® live-cell ICC, low levels of surface expression for the homophilic binding glycoprotein NCAM (CD56, neural cell adhesion molecule) and CD71 (transferrin receptor) were initially observed. Over extended periods in culture (> 4 days), the fluorescent signal for CD71, but not CD56, increased over time.

Measurement of neurite length indicated little or no maturation into a neuronal phenotype. When cells were differentiated with the vitamin A derivative all trans-retinoic acid (atRA, 50µM), cell proliferation slowed and the expression of CD56 increased, coincident with the differentiation of cells into more pyramidal shaped soma with marked extensions that are reminiscent of dendrites and/or axons. There was a clear association between the change in NCAM and the increase in neurite length over time, supporting the notion that NCAM promotes the development of neuronal lineages from precursor cells.[2]

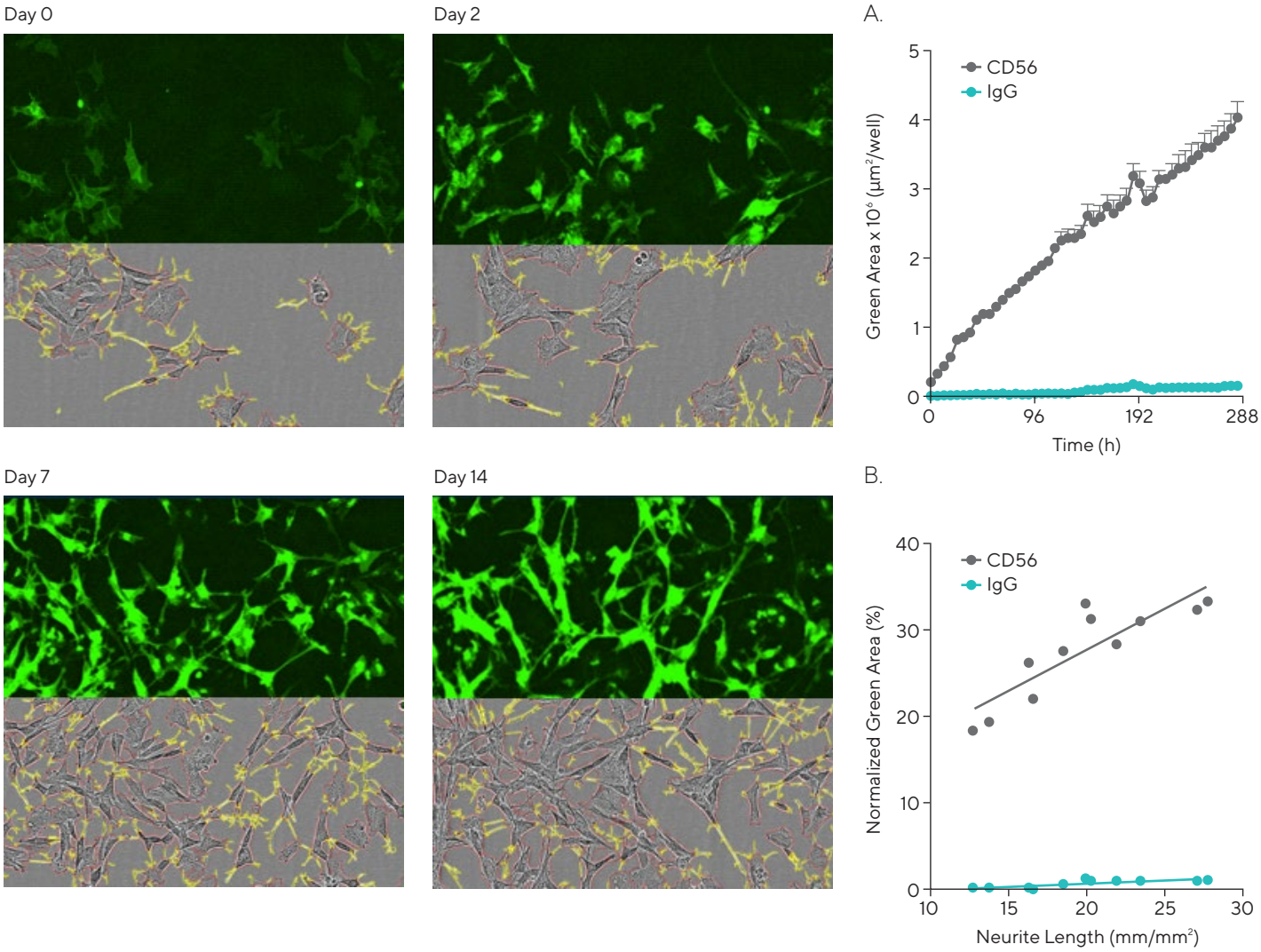
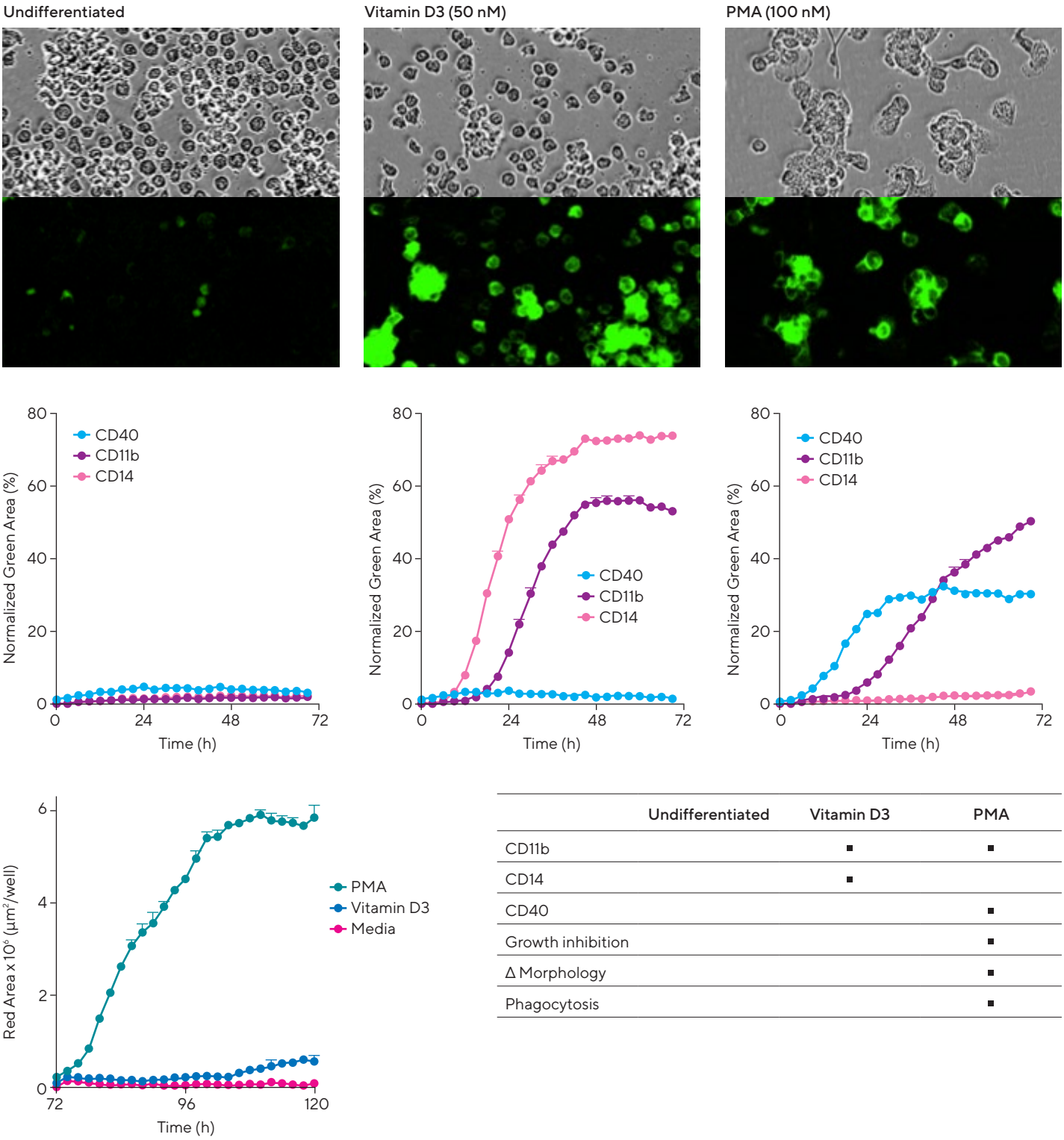


Figure 4: Dynamics of neurite outgrowth and CD56 expression in at RA-differentiated SH-SY-5Y cells. Cells were incubated with Fabflour-488 conjugated anti-CD56 and treated with at RA (50 µM) at t = 0. Blended images show green fluorescence (CD56) and phase morphology. Neurites are masked in yellow (Incucyte® Neurotrack Analysis Software Module). The time-course of upregulation of CD56 (mean values ± SEM, 4 wells) and the relationship between CD56 and neurite outgrowth (normalized to confluency) are shown in panels A and B, respectively. In panel B, each symbol represents data taken at different time-points throughout the experiment. Note the time-dependent increase in neurite length and associated CD56 signal.

3. Coupling Protein Dynamics to Cell Function

In the example on the next page, live-cell ICC was used to probe the differentiation of human monocytic THP-1 cells into macrophage-like cells[3] (Figure 5). Untreated hTHP-1 cells expressed the ubiquitous immune cell surface marker CD45 (and CD71) throughout the 3 day time-course of the experiment and acted as a stable 'housekeeping' protein to validate the Incucyte® Fabfluor-488 | Ab complex as a rapid and long lasting label. The expression of CD45 was largely unchanged by a range of differentiation stimuli, including vitamin D3, IFNγ + mCSF, IFNγ + LPS and Phorbol Myristate Acetate (PMA). hTHP-1 cells showed little expression of CD11b (integrin αM, part of the complement C3 receptor), CD14 (an endotoxin receptor) and CD40 (a co-stimulatory protein) for up to 72 h under control conditions. All three CD markers could be upregulated to different extents over 12–72 h with specific differentiation agents. Most notably, PMA upregulated CD11b and CD40, but not CD14, while vitamin D3 increased expression of CD11b and CD14 in a time-dependent manner. Vitamin D3 did not upregulate CD40. From close inspection of the Incucyte® images, only PMA produced a marked change in morphology, yielding large, flattened, adherent 'macrophage-like' cells. To correlate these observations with function, we measured the ability of differentiated THP-cells to phagocytose Incucyte® pHrodo®-labeled apoptotic Jurkat cells. Only those cells treated with PMA were phagocytic. While these data do not provide definitive evidence of a causative and direct link between marker changes and function, they illustrate how protein measurements can be associated to the functional properties of cells.



	Undifferentiated	Vitamin D3	PMA
CD11b		■	■
CD14		■	
CD40			■
Growth inhibition			■
Δ Morphology			■
Phagocytosis			■

Figure 5: Differentiation of hTHP-1 monocytes: surface marker and functional changes. hTHP-1 cells were exposed to media (undifferentiated), vitamin D3 (50 nM) or PMA (100 nM) in the presence of Incucyte® Fabfluor-488 Dye complexed to CD11b, CD14 or CD40 Abs (+ Incucyte® Opti-Green). Incucyte® images (Top, 20X, every 3 h) were analyzed for marker expression (green fluorescent area), normalized for confluency (Center: mean ± SEM, 4 wells). In the lower panel the phagocytic effects were determined by exposing the cells to pHrodo®-labeled apoptotic Jurkat cells (1 x 10^5 per well) at 72 h and measuring red fluorescence. Note the association of the CD40 marker expression with the functional profiles (orange = +ve).

4. Monitoring Cell–Cell Interactions

Finally, we used live-cell ICC to study the dynamic interactions of immune cells and tumor cells in a co-culture model system. Incucyte® Cytolight Red A549 lung cancer cells were cultured with human PBMCs in the presence of either Incucyte® Fabfluor-488-tagged CD45 or CD8 antibody, to label either all leukocytes or CD8+ cytotoxic T cells (Figure 6). By creating expanded image masks around the RFP signal, and quantifying the overlap with this and the green (immune-cell) image mask, a metric describing the extent of interaction or 'proximity' of immune cells to tumor cells was determined. Activation of the PBMCs with anti-CD3 | IL-2 significantly increased their interaction with tumor cells, compared to non-activated control PBMCs. The labeling approach highlighted the size increase and shape change of the immune cells over time (increase in green area) which could be accounted for and isolated from the interaction metric. Close inspection of the Incucyte® time-lapse movies highlighted how individual and sometimes multiple immune cells associate with the target cell, and remain attached even as the tumor cells move. With the CD8-labeling Ab, we could confirm that the subset of CD8+ cytotoxic T-lymphocytes specifically engaged with the tumor cells. Interestingly, the CD8-labeling confirmed a polarity to these cells, where the CD8+ region of the effector cell appeared to contact the target. Together, these data illustrate how subsets of living cells can be identified in living cultures over time with Incucyte® live-cell ICC, and how interactions between motile cells can be observed and quantified.

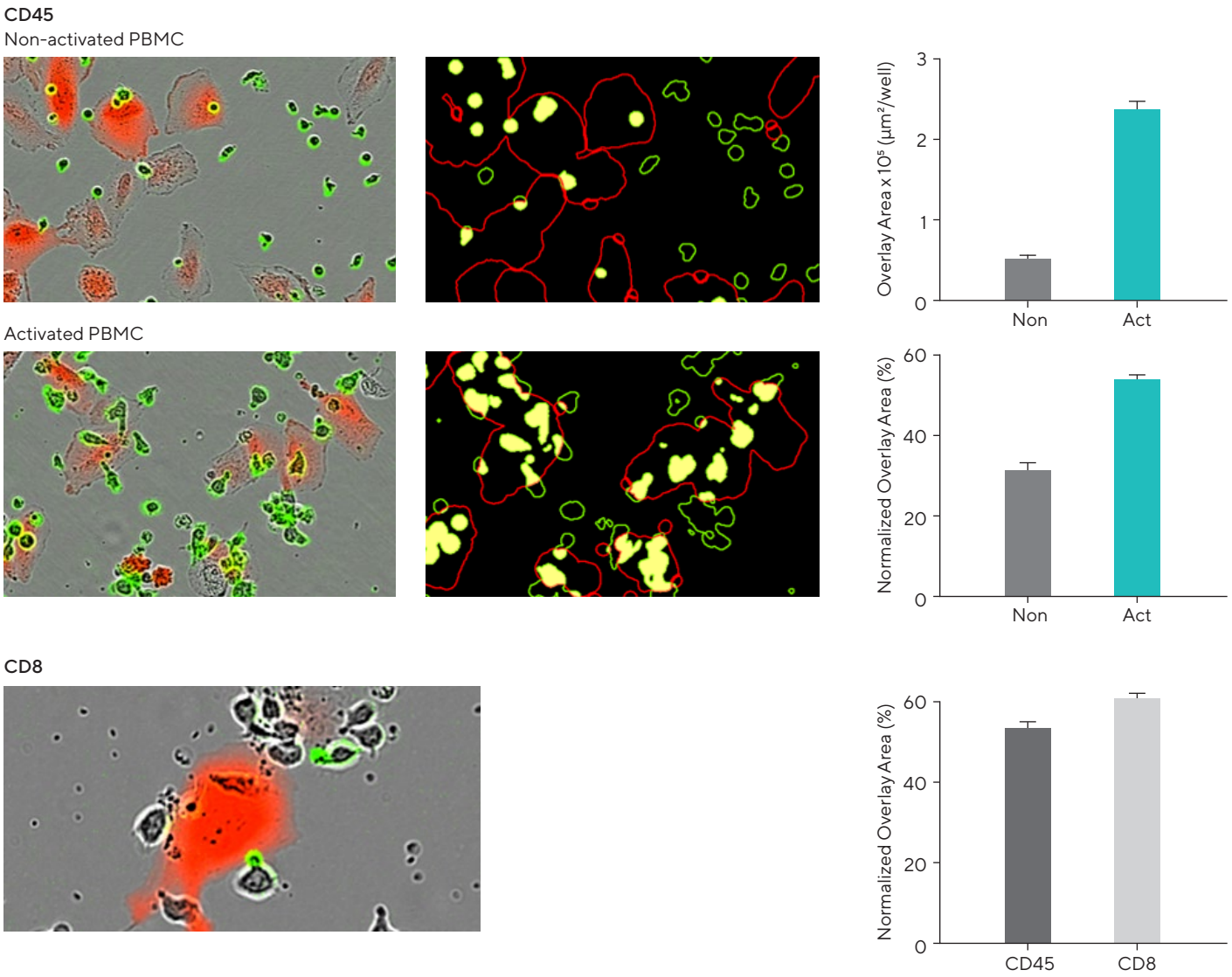


Figure 6: Visualization and quantification of cell-cell interactions. Incucyte® Cytolight Red A549 tumor cells were mixed with either pre-activated (anti-CD3 | IL-2) or non-activated PBMC's in the presence of Incucyte® Fabfluor-488-α-CD45 (upper) or CD8 (lower panel). The masked image (center) illustrates the overlay (yellow) of the immune cells (green) and tumor cells (red). The overlay area metric shows the increase in interaction between the two cell types upon activation, either without (upper) or with (middle) normalization to the green area. For CD8, note the clear polarity of the protein signal in the lymphocyte, and formation of the CD8 | immune cell 'synapse'.

Summary and Perspective

From the validation studies and examples above, we have shown how ICC can be extended from the traditional 'fix and stain' endpoint approach to a method that allows for dynamic monitoring of surface proteins in living cells over several days.[4]

The Incucyte® live-cell labeling protocol and workflow is simple and robust—the Incucyte® Fabfluor-488 Dye can be used to easily label any corresponding primary Ab in a single step, and the resulting conjugate is then added directly to living cells in complete media. This approach obviates the need for researchers to source Abs with an appropriate fluorescent tag, and ensures compatibility with the excitation | emission green channel of Incucyte® Live-Cell Analysis System and the background suppressor (Incucyte® Opti-Green). Washing and cell fixation protocols are not required. Images (up to 20X) are then taken automatically over time with the Incucyte® live–cell system, which resides inside a cell incubator to provide full environmental control (temperature, O_2 | CO_2, humidity) from the start to the end of the experiment. Simultaneous experiments in up to six 384-well microplates are possible at once, providing sufficient throughput for the analysis of multiple Abs, cell types and/or treatment combinations. Using automated analysis algorithms, Incucyte® Live-Cell Analysis System returns real-time quantification of Ab labeling and cell function | morphology as the studies unfold. The method is only amenable to cell surface, and not intracellular, protein targets since the Fab | Ab complex does not readily cross the plasma membrane.

This technique opens up new possibilities for connecting long-term dynamic changes in protein abundance and distribution in cells with morphology and function. We have demonstrated this with studies on checkpoint protein regulation in tumor cells and differentiation of precursor cells to mature neuronal and phagocytic phenotypes. Excitingly, we have used live-cell ICC to observe and quantify the interplay of cells in a complex tumor | immune cell co-culture, to provide insight into the extent of interactions between different subtypes of cells. These findings support the addition of Incucyte® live-cell ICC to the armory of the cell biologist as a powerful and enabling tool for cellular analysis.

	Incucyte® Live-Cell ICC	Fix and Stain ICC
Suitable For	Cell surface proteins	Both intracellular and cell surface proteins
Optimized For	Protein dynamics and linking changes to function and morphology. Cell identification and cell-cell interactions in motile systems	In-depth structural	morphological analysis, subcellular distribution, organelles, protein trafficking and redistribution
Reagents	Primary antibody + Incucyte® Fabfluor-488 Dye + Opti-Green background suppressor	F-labeled primary or secondary Ab
Hardware	Incucyte® Live-Cell Analysis System	Fluorescent microscopes, high-content imagers (Incucyte®)
Protocols	Mix and read (one hour prep time)	Fix, wash and stain (6–24 hours)
Resolution	Cellular, up to 20X magnification	Subcellular > 100X possible with oil immersion, etc.
Cell Status	Living cells in media	serum	Dead	dying cells post fixation
Imaging Paradigm	Repeated imaging over several days, time-lapse movies	Single time point ("end point")

Table 1: Comparison of Incucyte® Live-Cell ICC with conventional 'fix and stain' ICC

References

1. Abiko K, Matsumura N, Hamanishi J, *et al.* **IFN-γ from lymphocytes induces PD-L1 expression and promotes progression of ovarian cancer.** *Br J Cancer,* 112;1501–1509 (2015)
2. Shin MH, Lee EG, Lee SH, Lee YS, Son H. **Neural cell adhesion molecule (NCAM) promotes the differentiation of hippocampal precursor cells to a neuronal lineage, especially to a glutamatergic neural cell type.** *Exp Mol Med,* 34(6);401-410 (2002)
3. Aldo PB, Craveiro V, Guller S, Mor G. **Effect of culture conditions on the phenotype of THP-1 monocyte cell line.** *Am J Reprod Immunol,* 70(1);80–86 (2013)
4. Eilken H , Rieger M, Hoppe P , *et al.* **Continuous long-term detection of live cell surface markers by 'in culture' antibody staining.** *Protocol Exchange,* (2011) doi:10.1038/protex.2011.205

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