Use of fluorescent Fab/Ab complexes and IncuCyte® live-cell analysis to dynamically track cell surface markers and cell populations in mixed cultures

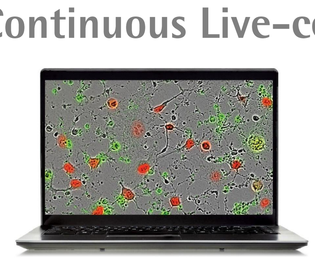
N. Bevan, V. Blancheteau, H. Campwala, N. Dana, N. Holtz, E. Endsley, T. Dale, C. Szybut, L. Kelsey, G. Lovell, B. O'Clair, & D. Trezise
Essen BioScience, Welwyn Garden City, AL7 3AX UK & Ann Arbor, MI, 48108, USA

Summary & Impact

- Fluorescently-labelled antibodies are widely used for visualising cell-surface protein expression and immuno-phenotyping (e.g. immunocytochemistry & flow cytometry).
- However, their applications are largely confined to end-point or short-term (min-h) detection, and the cell processing and labelling steps that are required often perturb the biology of interest.
- To enable longer-term, fully dynamic applications of cell surface protein markers in living cells, we have developed a novel strategy based on fluorescently-labelled antibody fragments (Fabs) and IncuCyte live-cell analysis.
- An anti-mouse Fc-targeted Fab fragment conjugated to a green fluoroprobe (IncuCyte® FabFluor-488) was used to tag Abs to surface markers (e.g. CD4, CD20) via a simple, one-step no-wash protocol.
- Addition of the FabFluor-488-Ab complex to living cells produces a long-lasting, specific and stable fluorescence and does not perturb cell morphology or growth.
- This methodology enables long-term tracking and quantification of protein expression and the ability to identify cell subsets in living cultures over time.
- This approach should prove powerful in analyses on complex and advanced heterogeneous cell models.

IncuCyte® System for Continuous Live-cell Analysis: Methodology

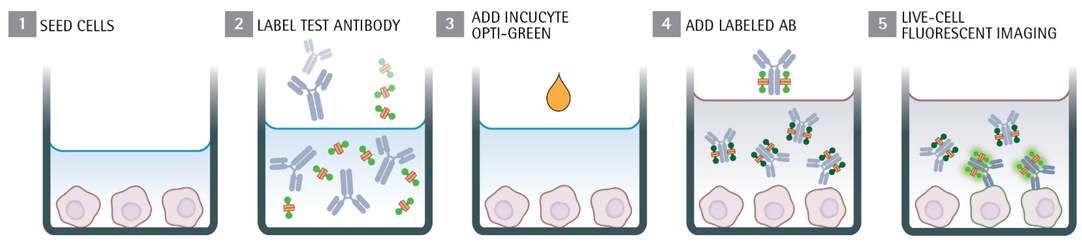
IncuCyte® S3 Live-Cell Analysis System
A fully automated phase contrast and two-color fluorescence imager that resides within a standard cell incubator for optimal cell viability. Designed to scan plates and flasks repeatedly over time.



IncuCyte® Software
Fast, flexible and powerful control hub for continuous live-cell analysis comprising image acquisition, processing and data visualization.

IncuCyte® Reagents and Consumables
A suite of non-perturbing cell labeling and reporter reagents. Includes nuclear-targeted GFP and RFPs for cell counting plus no-wash cell health reagents for apoptosis and cytotoxicity.

Quick Guide



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.

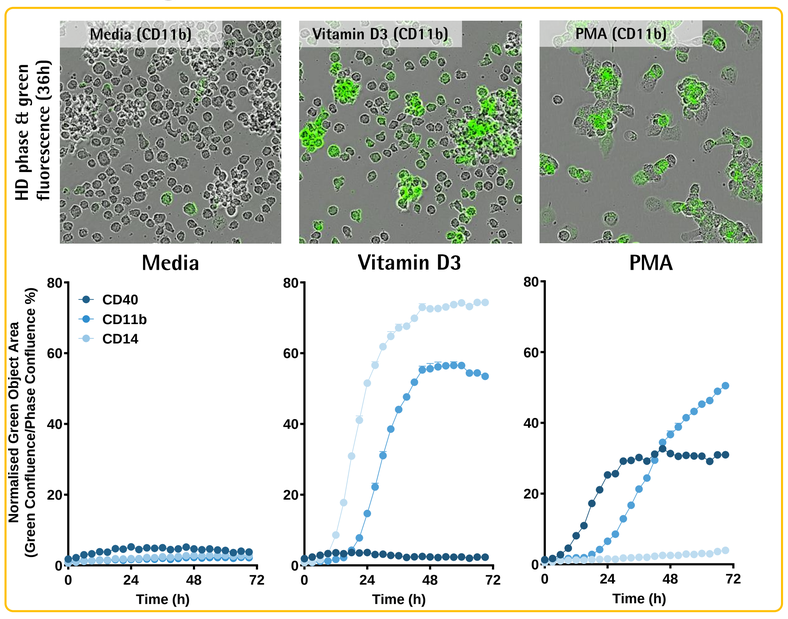
Labeling of test antibody with IncuCyte® FabFluor-488 reagent
Mix antibody and FabFluor-488 reagent at a molar ratio of 1:3 in media, 3x final concentration. Incubate for 15 minutes to allow conjugation.

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

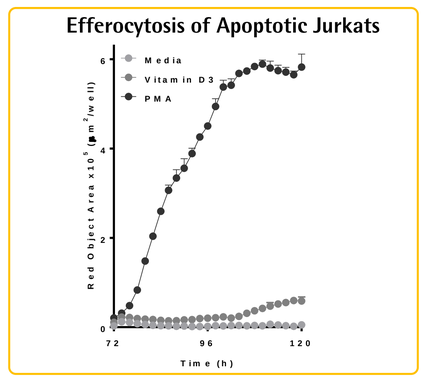
IncuCyte® FabFluor-488-labeled antibody addition
Add antibody-FabFluor mix (50 µl/well) to cell plate. Non-adherent cells – spin plate

Automated imaging and quantitative analysis
Capture images, (time span and objective depends on assay and cells type, 10x or 20x) in IncuCyte.

Coupling Protein Expression Dynamics to Cell Differentiation

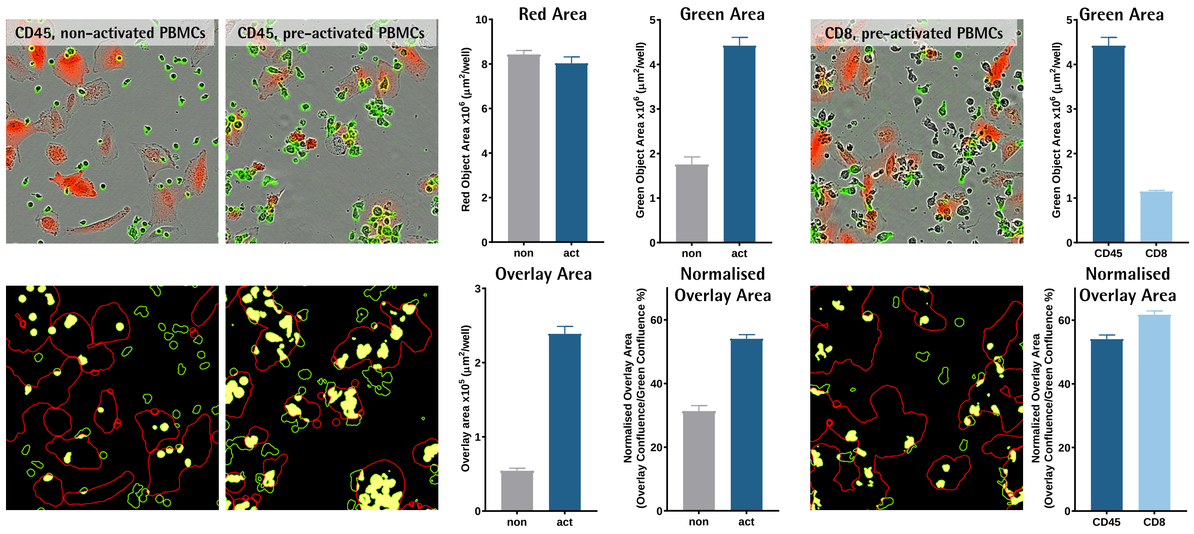


- THP-1 cells (30K/well) were incubated with Opti-Green, FabFluor-488-Ab and either Vitamin D3 (50 nM) or phorbol myristate acetate (PMA; 100 nM) to induce a macrophage-like phenotype.
- Phase and fluorescence images were captured (20x objective).



- THP-1 cells treated with Vitamin D3 or PMA show differences in morphology, protein expression and function (efferocytosis).
- Both treatments enhance CD11b expression but only Vitamin D3 upregulates CD14, whilst only PMA induces CD40.
- PMA-treated THP-1 cells exhibit a macrophage-like morphology, proliferate at a slower rate, and display efferocytic potential.

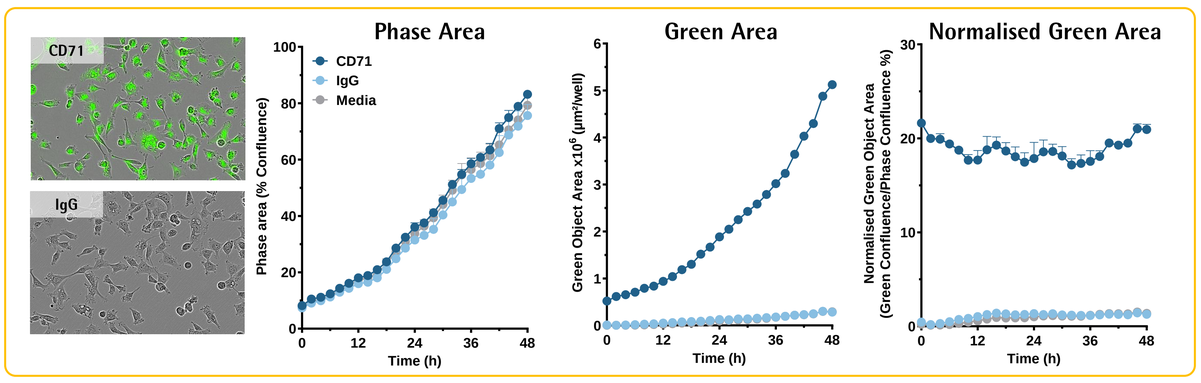
Monitoring Cell-to-Cell Interactions: Immune Cell Killing



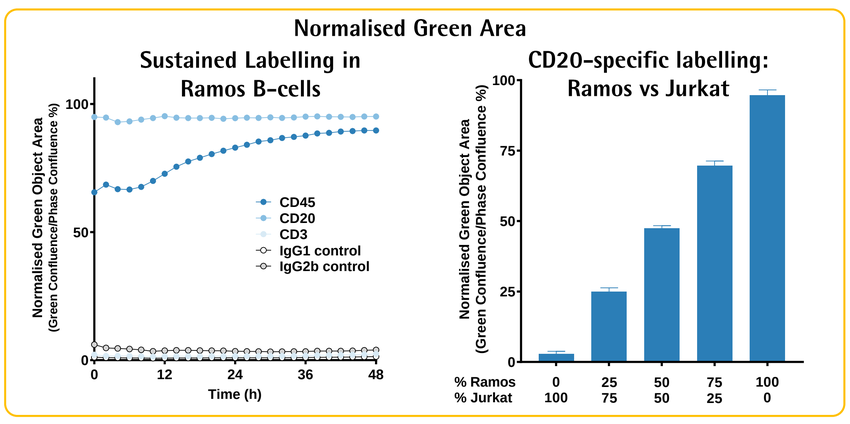
- A549 CytoLight™ Red (adenocarcinoma) cells (5K/well) were incubated with pre-activated PBMCs (anti-CD3/IL2, 4 d) in the presence of Opti-Green and CD45 or CD8 Abs labelled with FabFluor-488.
- Images were captured 2 h post-set-up (20x objective).
- Pre-activated PBMCs (CD45-positive), show a greater level of interaction (overlay area) with A549 target cells than non-activated PBMCs. Normalised overlay area suggests that CD8-positive cells may interact more with A549 cells than the general CD45-positive PBMC population.

Validation: Non-perturbing, Sustained, and Specific Labelling

- HT-1080 fibrosarcoma cells (5K/well) were incubated with Opti-Green Background Suppressor (0.5 mM) in the presence of media or antibodies to CD71 (transferrin receptor) and IgG (isotype control) labeled with FabFluor-488 reagent.



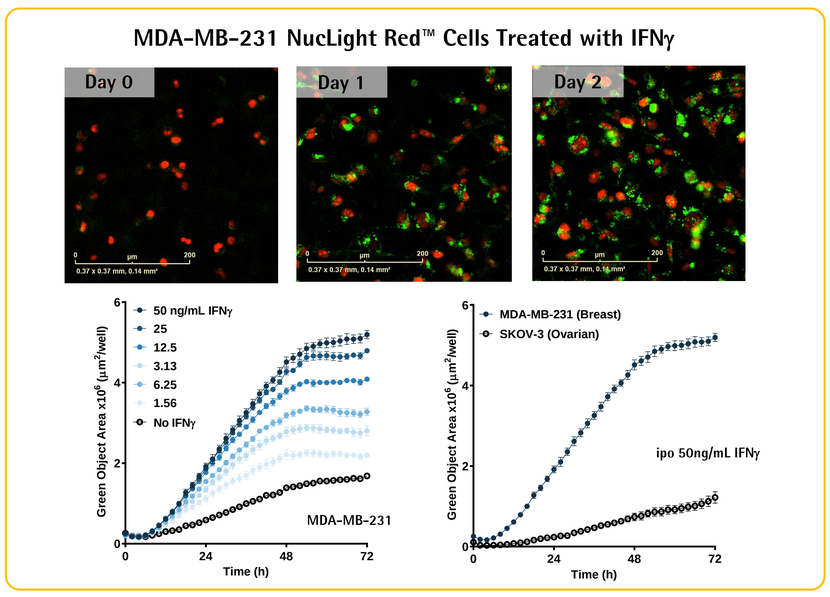
- CD71 labels the entire HT-1080 cell population. No cell labelling was observed with IgG.
- CD71 FabFluor-488-Ab complex in the presence of Opti-Green does not impair cell proliferation (Phase Data)
- CD71 expression increases over time, in line with cell proliferation.
- Normalised green/phase area metric confirms the stability and longevity of CD71 labelling



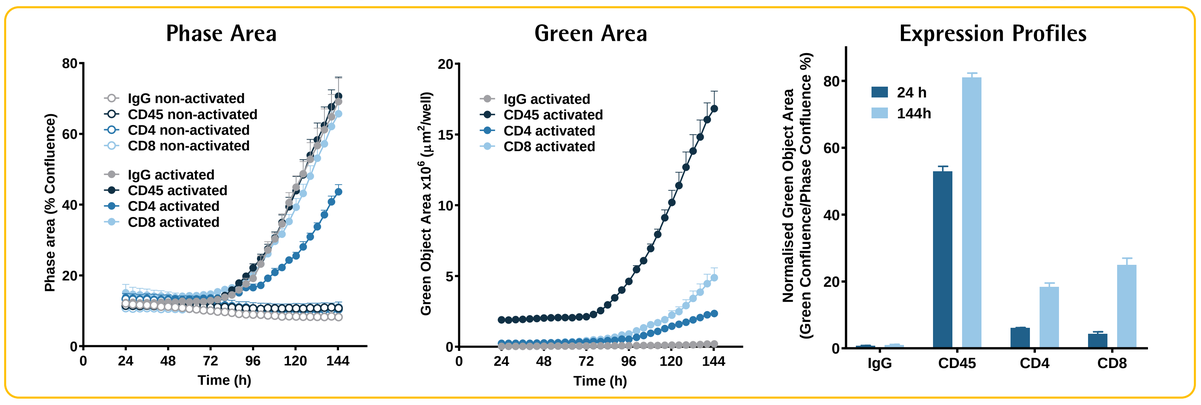
- Ramos (B-lymphoma) cell labelling with CD45 (common lymphocyte antigen), CD20 (B-lymphocyte antigen), but not CD3 Ab complex (T-cell co-receptor).
- CD20 labelling area follows anticipated proportions in mixed cultures (Jurkat T-cells are CD20 negative)

IFNγ-induced Upregulation of PD-L1 Checkpoint Protein

- MDA-MB-231 (breast) or SKOV-3 (ovarian) cancer cells (5K/well) were incubated with IFNγ in the presence of Opti-Green and FabFluor-488-PD-L1 Ab.
- Quantification of the green fluorescence area shows that IFNγ induces a time- and concentration-dependent increase in PD-L1 expression in MDA-MB-231 cells.
- Differential PD-L1 expression in MDA-MB-231 (high expresser) and SKOV-3 (medium expresser) cells can be visualised using this methodology.



Live-Cell Immunocytochemistry: PBMC Immuno-phenotyping



- PBMCs (30K/well) were incubated with or without anti-CD3/IL2 (10 ng/ml), in the presence of Opti-Green and various FabFluor-488-Abs.
- IgG, CD45 and CD8, had no effect on PBMC proliferation. The CD4 Ab produced a significant reduction.
- Green fluorescence area provides an index of the increase in the specific subpopulations – CD45, CD8 and CD4 all increased.
- Normalised green area estimates the expression profiles of individual cell sub-populations. The true expression profile is underestimated as the green area is smaller than the phase area within each cell.