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Antibody internalization assays for cancer drug discovery

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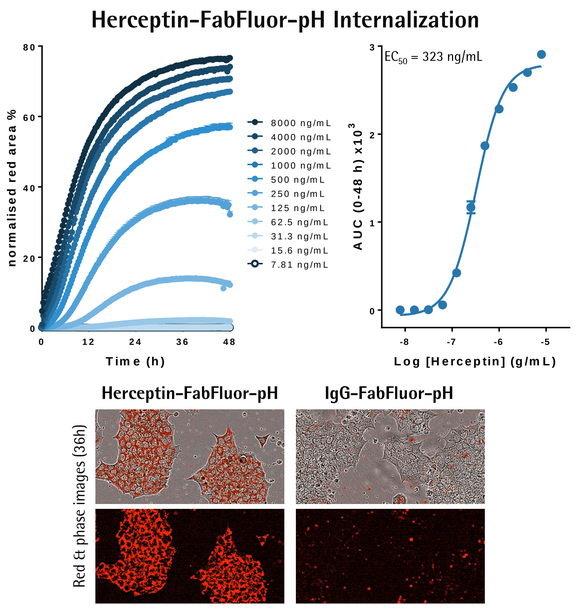
Summary & Impact

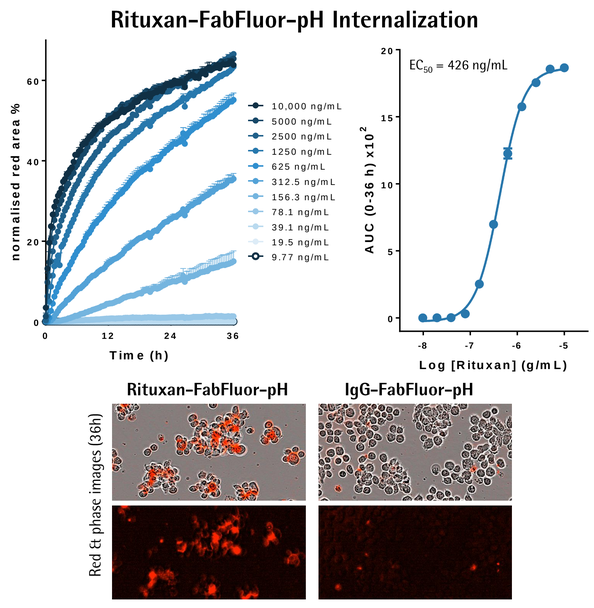
- Monoclonal antibodies (mAb) and antibody-drug conjugates are widely used biological therapeutics.
- A key property is the extent and rate of internalization, governing efficacy, safety and PD profile, thus quantifying and comparing Ab internalization is a critical step in the biopharmaceutical process.
- Here, we describe an automated, novel and enabling cell-based Ab internalization assay that is turnkey, medium throughput and geared toward industrial biologics discovery.
- Internalization measurements are made over time on 96-well microplates using live-cell analysis (IncuCyte®).
- An antibody-binding fragment coupled to a pH-sensitive dye (FabFluor-pH) was used to label and test mAbs using a single-step, no-wash protocol.
- As expected, an increase in fluorescence signal was observed as the mAb complex was internalized into the acidic lysosome.
- This approach has been validated for use in pharmacological and temporal characterisation of mAb.
- Proof of concept as part of a screening cascade to compare Ab characteristics and specificity of signal across cell lines and epitopes.

Concentration-dependent internalization of clinical antibodies

Herceptin-FabFluor-pH Internalization



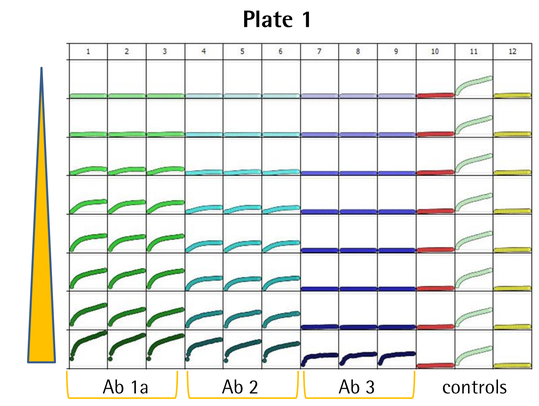
Rituxan-FabFluor-pH Internalization

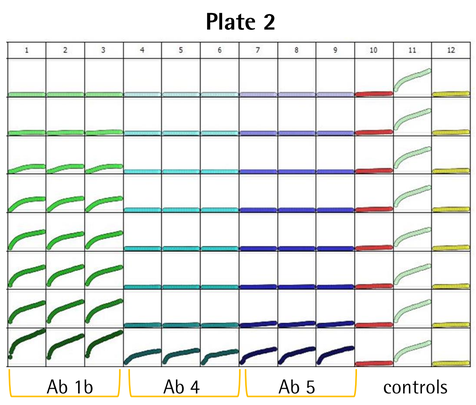


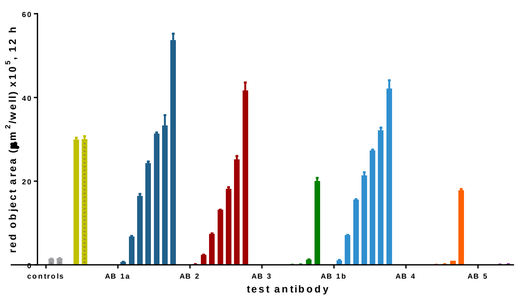
- BT-474 Her2-positive breast cancer cells (10K/well) were treated with IncuCyte FabFluor-pH labeled-Herceptin or IgG, isotype control.
- Red fluorescence and phase images were taken (10x) every 30 min over 48 h.
- Quantification of the red fluorescence area, normalized for phase area (confluence) demonstrates a rapid, concentration-dependent increase over time with labeled Hercpetin but no signal with IgG.

- Raji cells (30K/well) were treated with either IncuCyte FabFluor-pH labeled-Rituxan or IgG, isotype control.
- Red fluorescence and phase images were taken (20x) every 30 min over 36 h.
- Quantification of the red fluorescence, normalized for phase area (confluence) demonstrates a rapid, concentration dependent increase in signal over time with labeled Rituxan but no signal with IgG.

Drug discovery screening application



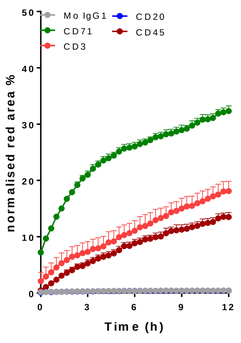




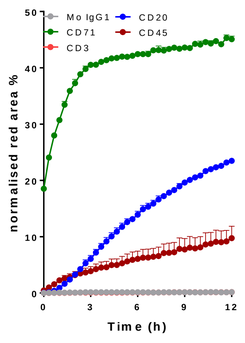
- Multiple anti-CD71 Abs were labeled and internalization properties compared (triplicate wells, CRC) in HT1080 fibrosarcoma cells (8K/well).
- Positive control wells (α-CD71) display a marked increase in fluorescence, whereas little or no signal was observed in the negative controls (IgG isotype); Z' values of 0.75 & 0.87.
- Of the 6 Abs, 3 (Ab 1a, 2 & 1b) produced a large signal and were detected at low concentrations (<0.05 µg/mL).
- Ab 1a & 1b were the Ab clone from different suppliers and as expected produced similar responses.
- Abs 3, 4 & 5 were internalized only at higher test concentrations.

Receptor expression specificity

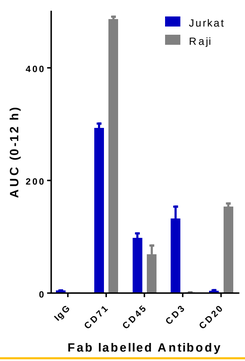
Jurkat (T cell like)



Raji (B cell like)



Expression Profile



- Various FabFluor-pH-Abs were added to Jurkat or Raji cells (4 µg/mL).
- Both cell lines internalized CD71 and CD45 (general lymphocyte markers) but only Jurkat internalized CD3 (T cell marker) and not CD20, with the converse in Raji cells (B cells).
- Data correlates with known CD surface marker expression in these cell lines providing strong confidence in the signal specificity and generic utility of the method.

Continuous Live-Cell Analysis: Methodology

IncuCyte® S3 Live-Cell Analysis System

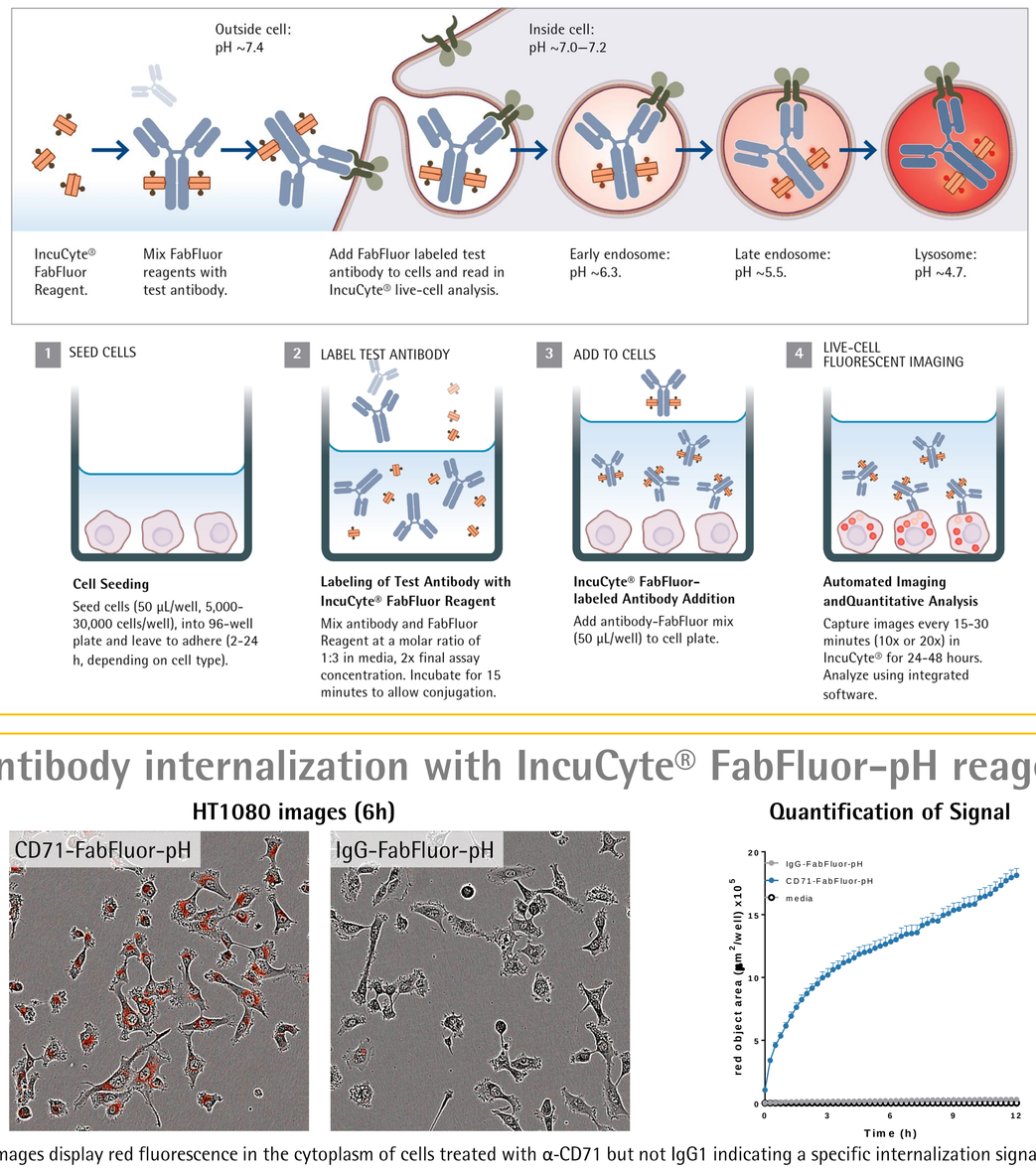
A flexible assay platform that sits inside a standard tissue culture incubator. IncuCyte automatically and continuously acquires and analyzes HD phase and fluorescent images of living cells cultured in microplates, dishes, or flasks.

IncuCyte® Software

Fast, flexible, and powerful control hub for continuous live-cell analysis comprising image acquisition, processing, and date visualization.

IncuCyte® Reagents & Consumables

A suite of non-perturbing cell labeling and reporter reagents. Includes nuclear-targeted GFP and RFPs for cell counting, no-wash caspase 3/7 substrate for apoptosis, and cell kits for angiogenesis.



Cell Seeding

Seed cells (50 µL/well, 5,000-30,000 cells/well), into 96-well plate and leave to adhere (2-24 h, depending on cell type).

Labeling of Test Antibody with IncuCyte® FabFluor Reagent

Mix antibody and FabFluor Reagent at a molar ratio of 1:3 in media, 2x final assay concentration. Incubate for 15 minutes to allow conjugation.

IncuCyte® FabFluor-labeled Antibody Addition

Add antibody-FabFluor mix (50 µL/well) to cell plate.

Automated Imaging andQuantitative Analysis

Capture images every 15-30 minutes (10x or 20x) in IncuCyte® for 24-48 hours. Analyze using integrated software.

Antibody internalization with IncuCyte® FabFluor-pH reagent

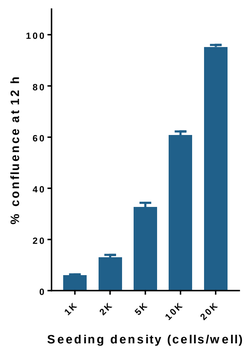
HT1080 images (6h)

Quantification of Signal

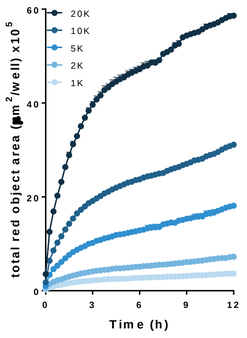
- Images display red fluorescence in the cytoplasm of cells treated with α-CD71 but not IgG1 indicating a specific internalization signal.
- Quantification of the red signal (fluorescence area) demonstrates a rapid increase in red object area over time with labeled α-CD71 only.

Signal amplitude is cell number-dependent

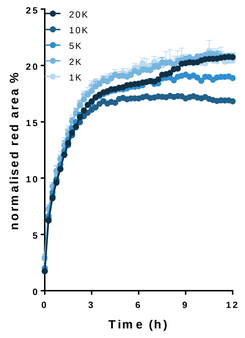
Phase confluence



Red object area



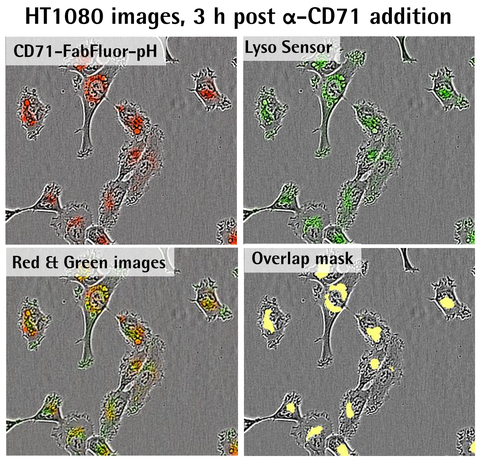
Normalized red area



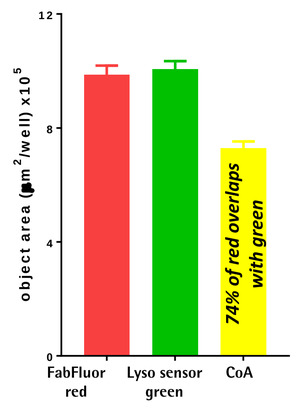
- An increasing density of HT1080 cells were treated with FabFluor-pH-α-CD71 (4 µg/mL).
- Time-course data shows an increase in the internalization signal size with increasing cell density.
- When the signal area was normalized for cell density (phase area) the responses overlay showing a similar rate of internalization.
- The data exemplifies the dependence of the signal size on cell number but not the rate of internalization.

Lysosomal localization of internalization signal

HT1080 images, 3 h post α-CD71 addition



Quantification of Images



- Internalization of FabFluor-pH-α-CD71 (4 µg/mL) was established before Lyso Sensor® green was added (Thermo, 0.25 µM).
- Images show the cellular localization of the internalization signal (red), the lysosome marker (green), overlaid images and masking of the co-localized signal.
- Quantification shows a similar area for both red & green signal in the cells.
- Using the coincident object analysis (COA) feature it was estimated that 74% of the red area was co-localized with the green.
- The data suggests the antibody has been internalized into lysosomes.