A High-Throughput, Multiplexed Antibody Internalization Assay

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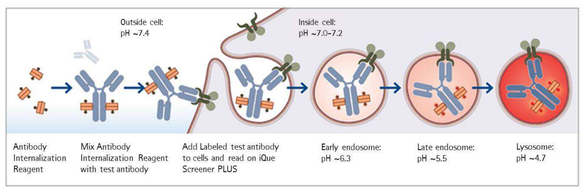
Introduction:

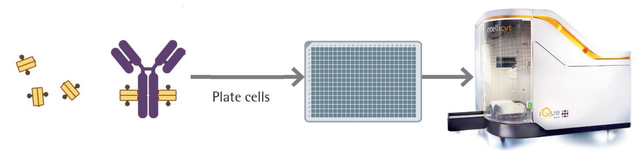
We describe a high-throughput, multiplexed, no-wash assay that measures antibody internalization, antibody specificity, and cell health from a single, 10uL sample. This assay is amenable to profiling large numbers of human or mouse antibodies in 96- and 384-well plate formats. Antibodies are easily labeled with the novel, pH sensitive Intellicyt Antibody Internalization Reagent. The labeled antibodies have little fluorescence at neutral pH, but become highly fluorogenic at low pH when they are internalized and processed through the acidic lysosome/endosome pathway. Cell viability can also be measured using MultiCyt Cell Membrane Integrity Dye and, using the same sample, cell specificity can be characterized using encoding dyes (cell lines) or directly conjugated fluorescent antibodies (cells). The mouse and human versions of the Antibody Internalization Reagent provide the flexibility to assay antibodies at different stages in the engineering workflow. Data acquisition using the Intellicyt iQue Screener PLUS platform combines high-throughput sampling, flow cytometry detection, and on-board analysis with plate level-analytics that deliver rich content with sampling times of less than 20 minutes for a 384-well plate. The integrated ForeCyt software enables data analysis and visualization.

The Intellicyt platform is an integrated solution that rapidly profiles antibody internalization and other critical antibody characteristics from small sample volumes and multiple cell types within the same well. The combination of non-perturbing and validated reagents for multiplexing, no-wash protocols, high-throughput capabilities, and integrated software for multi-parametric data analysis and visualization facilitates accelerated antibody discovery, development, and screening of antibody drug candidates.



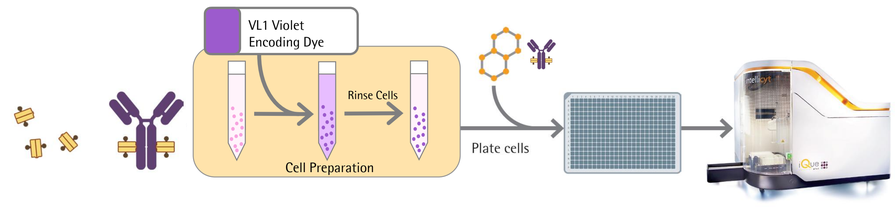
Antibody Internalization Reagent Principles

Antibody Internalization Reagent Workflow



1. Label antibodies with Antibody Internalization Reagent and prepare dilutions
2. Plate cells, add labeled antibody, and incubate 2hrs
3. Acquire data

Multiplexed Viability/Encoding Antibody Internalization Reagent Workflow



1. Label antibodies with Antibody Internalization Reagent and prepare dilutions
2. Encode cell populations with Violet Encoding Dye and plate encoded cells
3. Add labeled antibody and Membrane Integrity Dye, incubate 2hrs
4. Acquire data

Multiplexing with Encoding and Viability Dye:

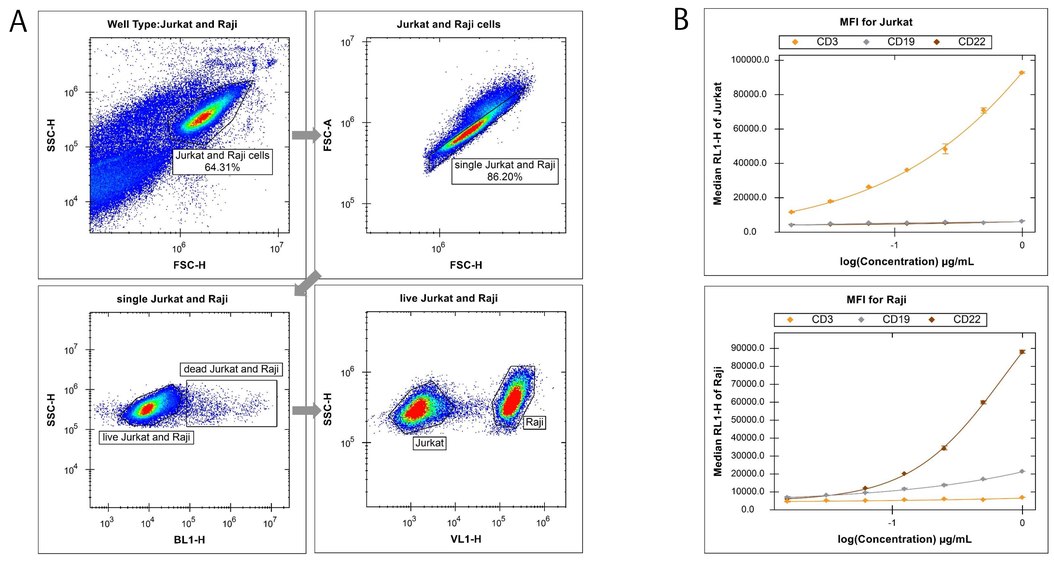


Figure 4. Multiplexing with Encoding Dye. Violet-encoded Raji cells and unstained Jurkat cells in the same sample well with a 8-point serial dilution of Mouse Antibody Internalization Reagent-labeled specificity antibodies with a top concentration of 1 µg/mL, MFI at a single, 2 hour time point. **(A)** Gating strategy used to separate the two cell types. **(B)** Antibody internalization for mixture of cell types shows the same specificity and relative MFI as when run separately.

Methods:

Cell Culture and Reagents: Jurkat cells (human T-cell line) and Raji cells (human B-cell line) (ATCC) were grown in RPMI-1640 + 10% FBS (Life Technologies). Test mouse antibodies were purchased from BioLegend (anti-human CD3, CD19, CD22, and CD45) and Sigma (anti-human CD20, CD71, and control mouse IgG1). Test human antibody Truxima (anti-human CD20) was a generous gift from BioOutsource, and control human IgG1 was purchased from Absolute Antibody. Mouse and Human Antibody Internalization Reagent, MultiCyt Membrane Integrity Dye (BL1, B/Green), and Violet Encoding Dye (VL1) were from Intellicyt. Mouse FabFluor-488 Green labeling reagent (IncuCyte) and Dynole® 2-24 (Abcam) were used for internalization inhibition experiments. Human fluorescent CD3 and CD20 antibodies (BioLegend) were used for the immunophenotyping studies.

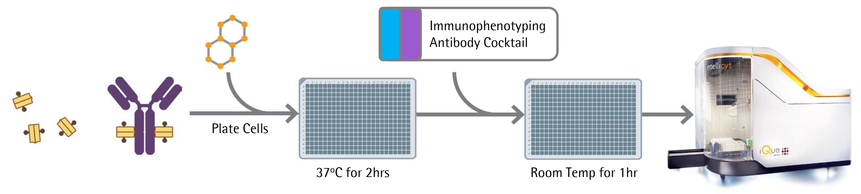
Antibody Labeling and Reagent Preparation: The mouse or human test antibodies (3X final assay concentration) were labeled at a molar ratio of 1:3 in growth media with the respective species Antibody Internalization Reagent. For temperature inhibition experiments, antibodies were labeled with Mouse FabFluor-488 Green and incubated for 15 minutes at 37° C. An 8 point, 2-fold dilution series was created for validation assays with a top antibody concentration of 1 µg/mL. For additional studies, a 12-point, 2-fold dilution series was used with a top antibody concentration of 1 µg/mL. MultiCyt Membrane Integrity Dye was prepared at a final assay concentration of 3X. For encoding studies, Raji cells (2 million/mL in PBS) were added 1:1 to Violet Encoding Dye and incubated for 15 minutes at room temperature. Cells were washed, centrifuged and re-suspended at 3X concentration in media. Unstained Jurkat cells were prepared at a 3X concentration in media.

Antibody Internalization Experiments: Cells were added into 384-well, v-bottom plates at a final concentration of 10^6 cells/mL along with Antibody Internalization Reagent-labeled antibodies and Membrane Integrity Dye in a final volume of 30 µl. All samples were done in triplicate. Data were acquired 2 hours after addition of labeled antibodies. For temperature inhibition experiments, cells and Mouse Antibody Internalization Reagent or Mouse FabFluor-488 Green-labeled CD71 antibody were cooled to 4° C before incubation and held at 4° C for 2 hours prior to data acquisition. For chemical inhibition experiments, cells were re-suspended in serum-free media before treatment with either 1% DMSO (vehicle control) or 10 µM Dynole 2-24 in 1% DMSO. The Mouse Antibody Internalization Reagent-conjugated CD71 antibody was then added and incubated at 37° C for 2 hours prior to data acquisition.

Data Acquisition and Analysis: Data were acquired on the Intellicyt® iQue Screener PLUS using 1 second sips corresponding to approximately 2000 cells/well. Dose response curves and EC_{50} calculations were automatically generated using ForeCyt software. Antibody internalization was assessed in the RL1 channel (675/30 nm), cell viability in the BL1 channel (530/30 nm) and encoded cells in the VL1 channel (445/45 nm). This panel was chosen to eliminate the need for color compensation in this assay. The BL1 channel was also used for measuring Mouse FabFluor-488 Green-labeled antibody in the temperature inhibition experiments.

Primary Cell Specificity: PBMCs (Astarte Biosciences) were cultured overnight in RPMI-1640 with 10% FBS, 1% Non-Essential Amino Acids, 1% Sodium Pyruvate, and 1% Pen/Strep. Cells were prepared at a 4x concentration before assay. A 12-point dilution series of mouse Internalization Reagent-conjugated antibodies (mIgG1, CD45, or CD22) was prepared with a top concentration of 4 µg/mL. Ten microliters of cells, Internalization Reagent-conjugated antibodies, and MultiCyt Membrane Integrity Dye were added to each well for a total volume of 30 µL. The plate was centrifuged briefly to ensure liquid was at the bottom of the well, mixed for 10 seconds at 3000 rpm, and incubated at 37°C. After 2 hours, 10 µL of an optimized cocktail of fluorescently labeled antibodies were added to the cells to identify T-cells (CD3) and B-cells (CD20), for a final volume of 40 µL. The cells were incubated for an additional hour at room temperature before data acquisition on the Intellicyt iQue Screener PLUS.

Primary Cell Antibody Internalization Reagent Workflow



1. Label antibodies with Antibody Internalization Reagent and prepare dilutions
2. Plate PBMCs, add labeled antibody and Membrane Integrity Dye, incubate 2hrs
3. Add Immunophenotyping antibody cocktail, incubate 1hr
4. Acquire data

Proof of Concept:

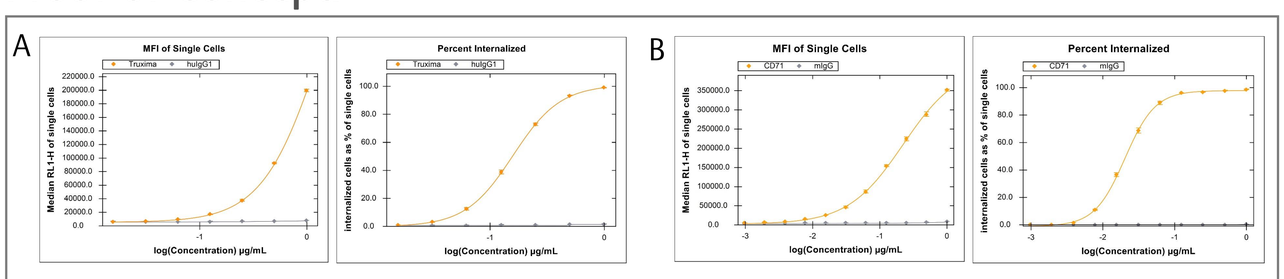


Figure 1. Human and Mouse Antibody Internalization Controls. MFI and percent positive at a single, 2-hour time point. **(A)** Dose response curves for an 8-point serial dilution of Human Antibody Internalization Reagent-conjugated human IgG1 (negative) or Truxima (anti-CD20, positive) with a top concentration of 1 µg/mL in Raji cells. **(B)** Dose response curves for a 12-point serial dilution of Mouse Antibody Internalization Reagent-conjugated mouse IgG1 (negative) or anti-CD71 (positive) with a top concentration of 1 µg/mL in Jurkat cells. For both Raji and Jurkat cells, dose dependent internalization was observed for the positive but not the isotype control antibody.

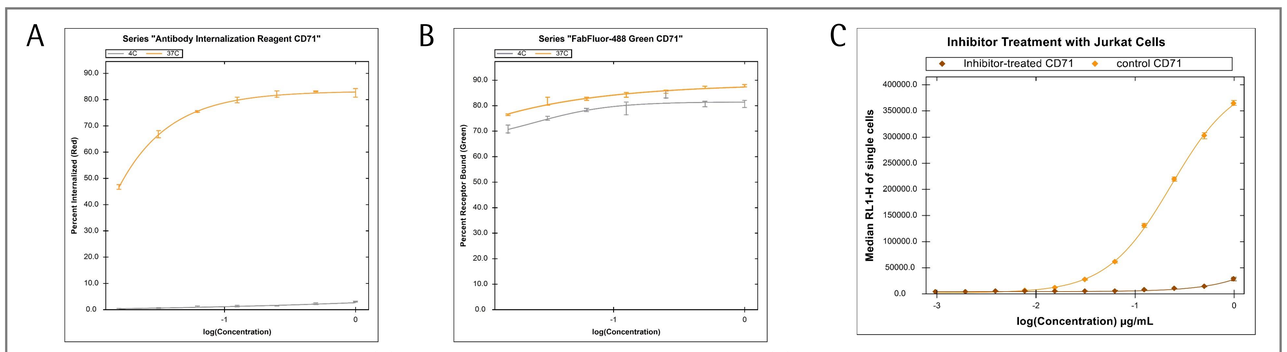


Figure 2. Antibody Internalization Inhibition. (A) Mouse Antibody Internalization Reagent- and **(B)** Mouse FabFluor-488 Green-labeled anti-CD71 incubated with Jurkat cells in 4°C and 37°C conditions, and **(C)** Dynole 2-24 treated and control Jurkat cells. An 8-point serial dilution with a top concentration of 1µg/mL of Mouse Antibody Internalization Reagent-labeled CD71 antibody demonstrated a dose-dependent increase in the percent cells internalized for the 37°C condition only. The Mouse FabFluor-488 Green labeled CD71 antibody showed no difference in the percent receptor bound between 4°C and 37°C conditions, indicating receptor binding independent of internalization. Jurkat cells treated with the endocytosis inhibitor Dynole 2-24 showed near-complete inhibition of internalization of the Mouse Antibody Internalization Reagent-labeled anti-CD71 as compared with cells treated with the vehicle control.

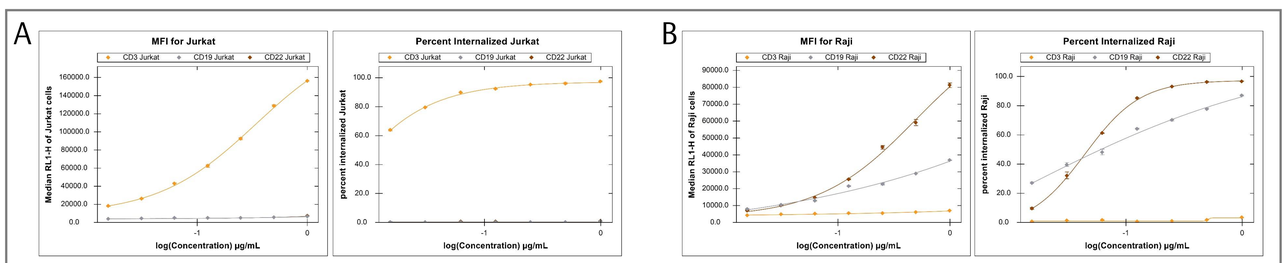


Figure 3. Antibody Internalization Specificity. MFI and percent positive at a single, 2 hour time point. Dose response curves for an 8-point serial dilution of Mouse Antibody Internalization Reagent-labeled anti-CD3 (T-cell marker), anti-CD19 or anti-CD22 (B-cell markers) with a top concentration of 1 µg/mL. **(A)** Jurkat cells showed a dose-dependent increase in internalization of anti-CD3, but not the two B-cell markers. **(B)** Raji cells showed a dose-dependent increase in internalization of anti-CD19 and anti-CD22, but not anti-CD3.

Primary Cell Internalization:

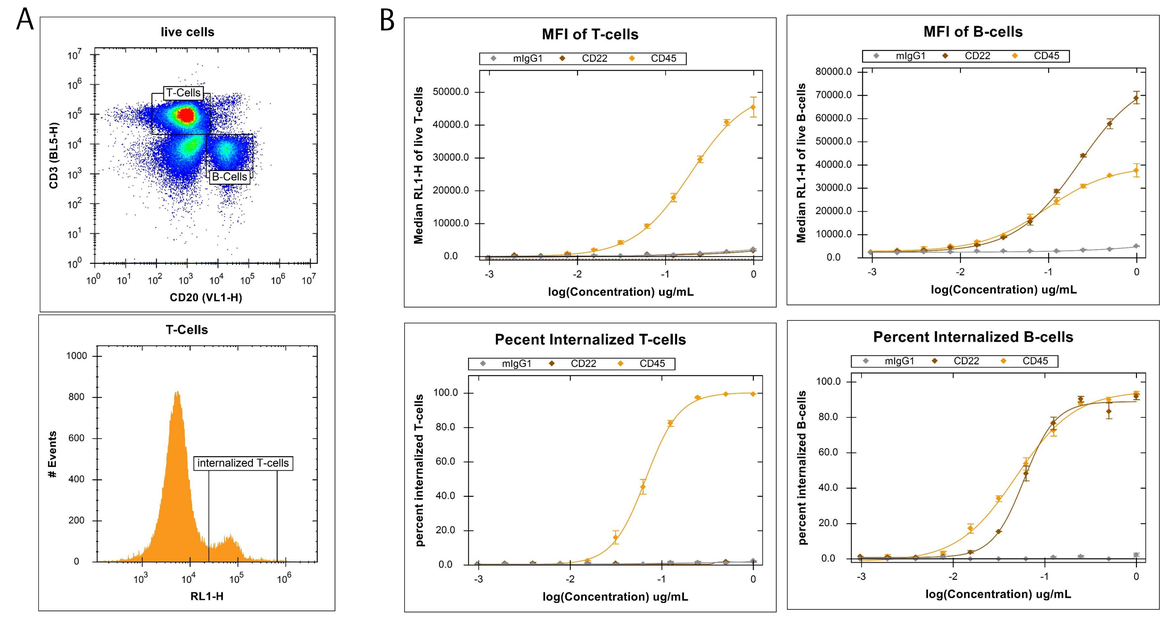


Figure 5. Primary Cell (PBMC) Specificity. PBMCs were incubated for 2 hours with a 12-point dilution series of Internalization Reagent-conjugated mouse IgG1 (negative control), anti-CD45 (positive control), or anti-CD22 (B-cell specific), and Membrane Integrity Dye. A cocktail of fluorescently labeled anti-CD3 (T-cell positive, BL5) and anti-CD20 (B-cell positive, VL1) was added to the cells to identify the different cell types and incubated for another hour at room temperature before acquisition on the iQue Screener PLUS. **(A)** Gating strategy used to separate the cell types. After gating on viable cells, cell types were separated using fluorescence and antibody internalization was assessed. **(B)** Dose response curves were generated for each cell type, and cell type specific antibody internalization was observed, consistent with previous experiments.

Conclusions:

- Intellicyt's Antibody Internalization Reagent is a novel, pH sensitive dye that identifies antibody internalization in a simple, plate-based format.
- This assay enables the rapid (2-3 hours), simultaneous measurement of important attributes, such as antibody internalization and viability using 10 µL sample volumes.
- Specificity can be demonstrated with either encoding dye or immunophenotyping antibodies.
- The assay provides the flexibility to combine with other validated reagents (such as QBeads) for multiplexed, no-wash, high-throughput capabilities.
- The Intellicyt iQue Screener PLUS platform and integrated ForeCyt software provide data analysis and visualization with plate-level analytics.