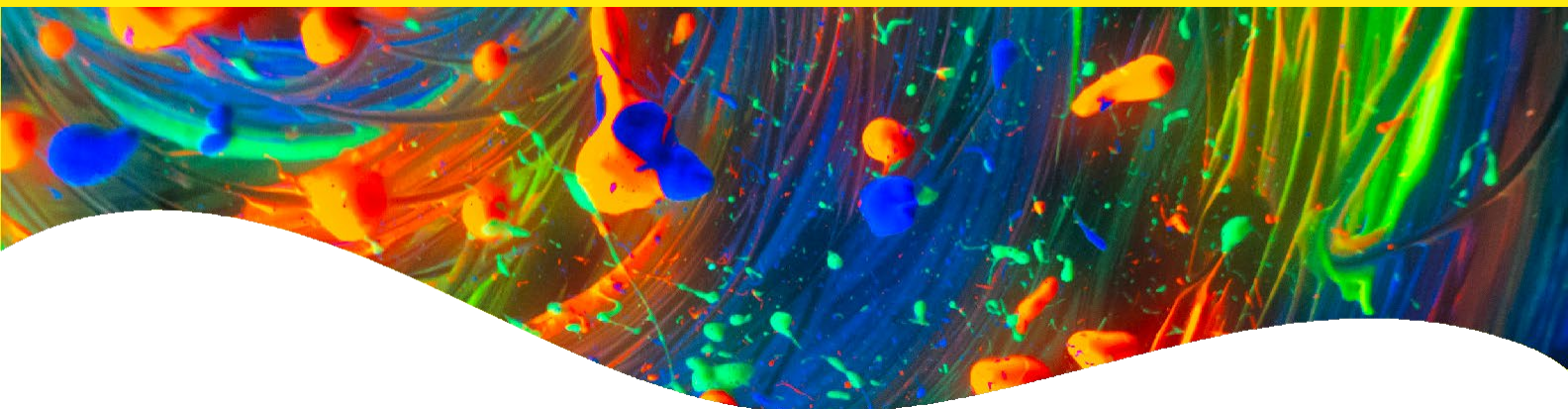




PhenoVue Human IgG Antibody Internalization Kit (Fluor 515, pH sensitive) (2 x 96-well and 10 x 96-well plates)



Overview

Antibody-mediated internalization is increasingly studied, particularly in immuno-oncology. When an antibody binds to its target receptor, the antibody-receptor complex is endocytosed and trafficked through acidic vesicles. Depending on the receptor pathway, the complex may recycle to the plasma membrane or progress toward lysosomal degradation. These mechanisms are central to the mode of action of antibody-drug conjugates (ADCs), where internalization and lysosomal trafficking support payload release. Accurate and efficient evaluation of antibody internalization is therefore critical for the development of ADCs and related biologics.

The PhenoVue™ Human IgG Antibody Internalization Kit (Fluor 515, pH-sensitive) provides a ready-to-use and reproducible method for no-wash live-cell monitoring of antibody and ADC internalization. The kit contains PhenoVue Fluor 515 pH-sensitive Fab anti-human IgG antibody, which binds with high affinity to the Fc region of human IgG antibodies, whether unconjugated or conjugated, including ADCs. Its fluorescence remains low at neutral extracellular pH but increases as the Fab-antibody complex traffics into acidic intracellular compartments.

PhenoVue Fluor 515 pH-sensitive Fab anti-human IgG antibody combines high photostability with a lower pKa (~5.7), helping to reduce fluorescence at neutral and mildly acidic pH and preferentially report antibody trafficking into strongly acidic intracellular compartments. This profile makes it particularly well suited to repeated time-lapse acquisitions and kinetic profiling of deep internalization. When combined with PhenoVue 633 Lysosomal Stain and PhenoVue Hoechst 33342 Nuclear Stain, the kit enables no-wash live-cell monitoring of antibody trafficking toward acidic endosomal and lysosomal compartments.

For low-target-expression models, weak internalization signals, or imaging systems not optimized for Fluor 515 detection, the complementary PhenoVue Human IgG Antibody Internalization Kit (Fluor 647, pH-sensitive) offers a far-red detection option with broad instrument compatibility.

Product information

Product name	Part no.	Number of vials per unit	Shipping conditions
PhenoVue human IgG antibody internalization kit (Fluor 515, pH sensitive) - 2 x 96 wells*	PHAINTG14	3	Dry ice
PhenoVue human IgG antibody internalization kit (Fluor 515, pH sensitive) - 10 x 96 wells**	PHAINTG15	11	Dry ice

* PhenoVue human IgG antibody internalization kit (Fluor 515, pH sensitive) PHAINTG14 provides sufficient reagents to stain 2 x 96-well plates or 1 x 384-well plate, following the suggested concentrations and volumes.

** PhenoVue human IgG antibody internalization kit (Fluor 515, pH sensitive) PHAINTG15 provides sufficient reagents to stain 10 x 96-well plates or 5 x 384-well plates, following the suggested concentrations and volumes.

Part no.	Kit contents (For 2 x 96-well plates)	Format	Packaging	Storage
PHAINTG14	PhenoVue Hoechst 33342 nuclear stain (also available as spare part CP71)	Liquid (H ₂ O)	1 vial (70 µL, 10000x)	2-8 °C or below. Protect from light
	PhenoVue 633 lysosomal stain (also available as spare part CP10DR1)	Desiccated	1 vial (5,5µg)	-16 °C or below. Protect from light.
	PhenoVue Fluor 515 pH sensitive Fab anti-human IgG antibody (also available as spare part 2HFAB515PH1)	Lyophilized	1 vial (90µg)	2-8 °C or below. Protect from light

Part no.	Kit contents (For 10 x 96-well plates)	Format	Packaging	Storage
PHAINTG15	PhenoVue Hoechst 33342 nuclear stain (also available as spare part CP71)	Liquid (H ₂ O)	1 vial (70 µL, 10000x)	2-8 °C or below. Protect from light
	PhenoVue 633 lysosomal stain (also available as spare part CP10DR1)	Desiccated	5 vials (5,5µg)	-16 °C or below. Protect from light.
	PhenoVue Fluor 515 pH sensitive Fab anti-human IgG antibody (also available as spare part 2HFAB515PH1)	Lyophilized	5 vials (90µg)	2-8 °C or below. Protect from light

Storage and stability

- For convenience, store the kit at ≤ -16 °C. However, each reagent can be stored separately between ≤ -16 °C and 2-8 °C, as indicated in the table above. Avoid repeated freeze-thaw cycles. After reconstitution, aliquoted reagents must be stored at -16 °C or below and are stable for 6 months.
- Allow the reagents to warm up to room temperature for 30 min before opening the vials and reconstitution.
- The stability of these products is supported until the expiration date provided in the certificate of analysis, when stored as provided and protected from light.

Safety information

Chemical reagents are potentially harmful, please refer to the Safety Data Sheet (SDS) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Other materials and reagents not provided

Reagents or consumables	Usage
Complete cell culture medium	Diluent for PhenoVue stains and compounds.
IgG antibody isotype controls	Available as spare parts PAHIGG1CT1 or PAHIGG4CT1
PhenoPlate™ 96 or 384-well microplates	Cell plating, stimulation, staining and imaging

Live- and fixed-cell compatibility

Product name	Live cell staining	Fixation/permeabilization steps post live-cell staining	Fixed-cell staining
PhenoVue human IgG antibody internalization kit (Fluor 515, pH sensitive)	Yes	No*	No

* PhenoVue Fluor 515 pH sensitive Fab anti-human IgG antibody can be fixed, while the PhenoVue 488 lysosomal stain cannot.

Spectral and pH-sensitive properties of the PhenoVue human IgG antibody internalization kit

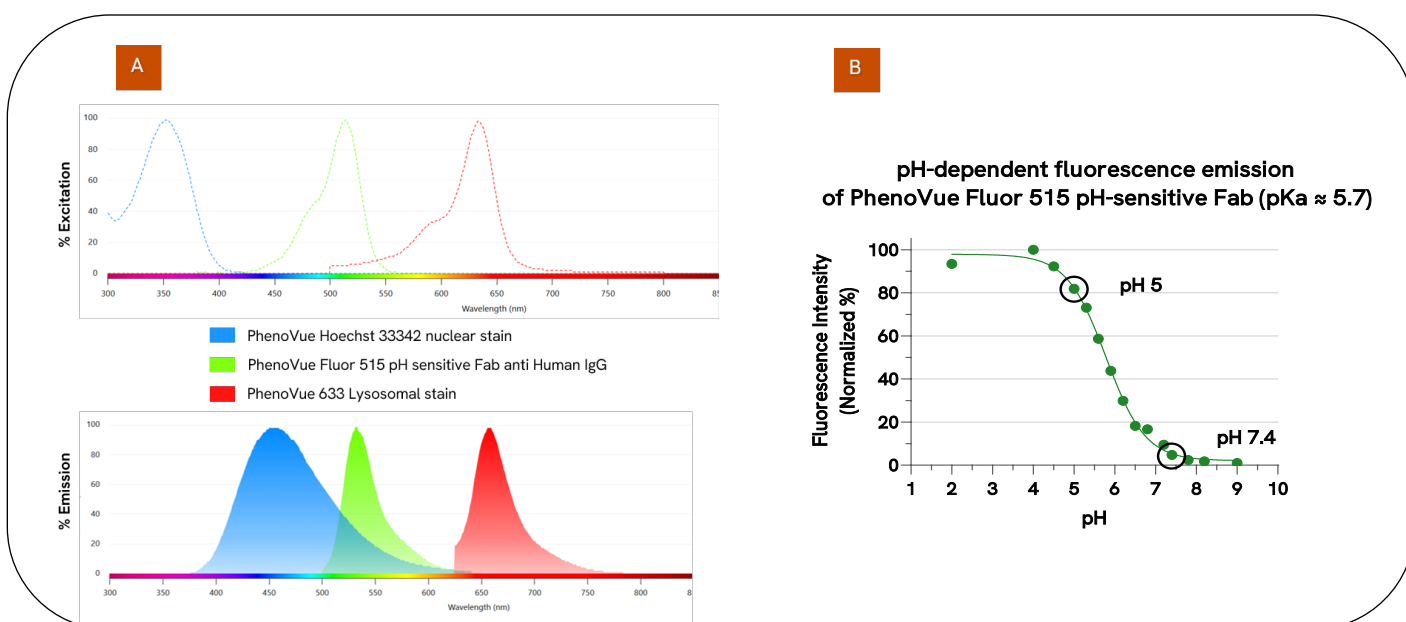


Figure 1: Spectral and pH-sensitive properties of the PhenoVue human IgG antibody internalization kit (Fluor 515, pH-sensitive). (A) Excitation and emission spectra of the kit components: PhenoVue Hoechst 33342 nuclear stain, PhenoVue 633 lysosomal stain, and PhenoVue Fluor 515 pH-sensitive Fab anti-human IgG antibody. (B) pK_a curve of the PhenoVue Fluor 515 pH-sensitive Fab anti-human IgG antibody, showing a pK_a around 5.7, with strong fluorescence at acidic pH (e.g. pH 5) and reduced signal at neutral pH (e.g. pH 7.4).

Reagent reconstitution and preparation of staining solution

1. Ready-to-use stock solutions

Reagents	PhenoVue human IgG antibody internalization kit 2 x 96-well / 10 x 96-well
PhenoVue Hoechst 33342 nuclear stain	Ready-to-use stock solution at 1 mg/mL (10,000x)
PhenoVue 633 lysosomal stain	Reconstitution using 100 μ L dH ₂ O gives a stock concentration of 1000x
PhenoVue Fluor 515 pH-sensitive Fab anti-human IgG antibody	Reconstitution using 100 μ L dH ₂ O gives a stock concentration of 18 μ M

Aliquot stock solutions and store at -20 °C for up to 3 months. Avoid repeated freeze-thaw cycles.

2. Preparation of staining solutions

Reagents	Preparation of staining solutions (100 μ L/well)	Example volumes
Staining Solution 1	Prepare in 24.744 mL cell culture medium	25 mL total
PhenoVue Hoechst 33342 nuclear stain	Step 1: Dilute stock solution 1:100 in culture medium (intermediate solution). Step 2: Dilute this intermediate solution 1:100 in culture medium (final 1x solution).	Add 250 μ L from Step 2
PhenoVue 633 lysosomal stain	Dilute stock solution 1:4000 in culture medium (final 0.25x solution).	6.25 μ L
Staining Solution 2	Prepare in 10 mL of Staining Solution 1	10 mL total
Human IgG antibody or human IgG antibody isotype controls (<i>not provided</i>)	Dilute in Staining Solution 1 to obtain a final concentration of 60 nM* (2x).	Adjust volume depending on antibody concentration
PhenoVue Fluor 515 pH-sensitive Fab anti-human IgG antibody	Dilute in Staining Solution 1 to obtain a final concentration of 180 nM (2x).	100 μ L

*The suggested 60 nM antibody concentration is provided as a guideline and may be adjusted according to experimental needs. At this concentration, the PhenoVue Fluor 515 pH-sensitive Fab anti-human IgG antibody provides sufficient reagent to stain either two 96-well plates or one 384-well plate

Important: Discard staining solutions after use. Do not store or reuse.

Example preparation of staining solution

The following example describes the preparation of 25mL staining solution 1 and 10mL staining solution 2 - sufficient for 2 x 96-well plates.

Staining solution 1 uses cell culture medium and contains:

- PhenoVue Hoechst 33342 Nuclear Stain
- PhenoVue 633 - Lysosomal stain



25 mL staining solution 1 (protect from light)
To 24.744 mL of **complete culture medium** :

- Add 250 μ L of pre-diluted PhenoVue **Hoechst 33342**
- Add 6.25 μ L of PhenoVue **633 - Lysosomal**

Staining solution 2 uses Staining solution 1 and contains:

- Your Human IgG antibody
- PhenoVue Fluor 515 pH sensitive Fab anti Human IgG
- PhenoVue Hoechst 33342 Nuclear Stain
- PhenoVue 633 - Lysosomal stain



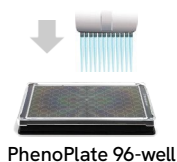
10 mL staining solution 2
Start with **9.9 mL - Vx** of **Staining Solution 1**.

- Add a volume **Vx** of your Human IgG Antibody to obtain a final concentration of **60 nM**.
- Add 100 μ L of **PhenoVue Fluor 515 pH-sensitive Fab anti-Human IgG** to reach a final concentration of **180 nM**.

Incubate for 30 minutes at RT.

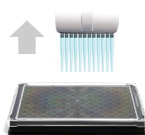
Experimental workflow

Cell seeding
(100-200 μ L / well)

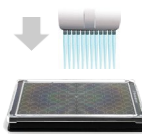


PhenoPlate 96-well

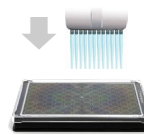
Harvest
cell culture medium



Staining
Solution 1
(50 μ L/well)



Staining
Solution 2*
(50 μ L/well)



Time-lapse
acquisition



1h @ RT

Time points

*Prior to use, the prepared staining solution 2 should be pre-incubated for 30 minutes at RT.

Protocol for 96-well imaging plate

Cell culture

Seed cells in PhenoPlate 96-well imaging microplates* or any other suitable cell culture vessel. Incubate under appropriate conditions (typically 37 °C, 5% CO₂) until cells reach 50–70% confluency.

*A PhenoPlate 384-well microplate may also be used; in that case, adjust the cell seeding density appropriately and halve all reagent volumes.

Live cell staining protocol

1. Cell preparation

Carefully remove the culture medium by gentle aspiration.

2. Add 50 µL of Staining Solution 1 per well.

Incubate for 1 hour at 37 °C, 5% CO₂.

3. Preparation of Staining Solution 2

During the pre-staining step, prepare Staining Solution 2 and allow it to equilibrate for 30 minutes at room temperature (RT). Maintain an antibody:Fab molar ratio of 1:3.

4. Add 50 µL of Staining Solution 2 per well.

Incubate at 37 °C, 5% CO₂.

Note: If additional compounds are used (e.g. Bafilomycin A), adapt the protocol accordingly. Ensure that the final concentration of staining solutions and the Ab:Fab ratio remain consistent with the suggested conditions.

5. Dose-response assays (optional)

For dose-response experiments, serially dilute the pre-incubated PhenoVue Fluor 515 pH-sensitive Fab-antibody complex in cell culture medium to cover the desired concentration range.

Include a Fab-only control (PhenoVue Fluor 515 pH-sensitive Fab anti-human IgG antibody without test antibody) at the final concentrations tested. This control enables calculation of the specific internalization signal:

- Mean Signal (test antibody) – Mean Signal (Fab-only control).
- This correction is particularly important for accurate EC₅₀ determination.

Image acquisition

- Transfer the microplates to the imaging system and begin acquisition (e.g. for time-lapse imaging).
- An optional acquisition point can be included between steps 2 and 3.
- Refer to the following section for guidance on acquisition settings.

Image analysis

For image analysis using Revvity high-content imaging instruments, refer to the dedicated section below.

Tips

- **No-wash protocol:** Do not remove culture medium containing PhenoVue stains, as washing steps reduce signal intensity.
- **Live-cell only:** The PhenoVue human IgG antibody internalization kit (Fluor 515, pH-sensitive) is intended for live-cell imaging only. The detected compartments are not preserved after fixation.
- **Optimized conditions:** Pre-staining reagent concentrations are optimized for reliable signals over 24 h of live-cell imaging. For longer experiments, adjust concentrations or acquisition settings, particularly for the PhenoVue 633 lysosomal stain.
- **Ab ratio optimization:** An Ab ratio of 1:3 is a good starting condition. However, because PhenoVue Fluor 515 pH-sensitive Fab anti-human IgG antibody has a relatively low pKa (≈5.7), resulting in lower background, performance may be further improved by increasing the Ab ratio and testing a broader range of antibody concentrations. For challenging cell models, such as those with low receptor expression or high background, both the Ab ratio and antibody concentration should be optimized.
- **Cytotoxicity caution:** Concentrations are optimized for high signal while reducing cytotoxicity. Exceeding suggested concentrations may impair cell viability.
- **Light protection caution:** PhenoVue 633 lysosomal stain is particularly light-sensitive especially when stored at neutral pH. Thus, Staining solution 1 must be protected from the light.
- **Nuclear stain:** PhenoVue Hoechst 33342 is used at a low concentration (100 ng/mL) to limit effects on proliferation. While non-toxic, it may slightly slow growth depending on the cell type.
- **Controls:** Signal dynamics vary by cell type. Include an IgG antibody isotype control in your experiment.
- **Acquisition mode:** Confocal acquisition is suggested for higher signal-to-background ratios compared to widefield mode.
- **Reduce phototoxicity and photobleaching:** The brightness of the dyes means that unnecessarily high exposure times or laser powers can be avoided. Limit timepoints to 4–5 per experiment in time-lapse mode to reduce risk of phototoxicity or photobleaching. Alternatively, change acquisition fields to reduce repeated exposure of the same cells.

Guidance for acquisition settings

PhenoVue Fluor 515 pH-sensitive Fab anti-human IgG antibody is compatible with standard FITC-channel configurations. When available, a 510-515 nm excitation / 525-580 nm emission configuration is suggested for better performance. On systems using a standard FITC configuration, acquisition settings should be optimized for the instrument, objective, and cell model. Potential crosstalk into the 555 channel should be considered during multiplex design, particularly on imaging systems where spectral separation is less favorable.

HCS instruments		PhenoVue Hoechst 33342	PhenoVue 488 or PhenoVue 510	PhenoVue 555 (free channel)	PhenoVue 647
Opera Phenix™ Plus 5 lasers	Excitation laser (nm)	375	488	561	640
	Emission filters (nm)	435-480	500-550	570-630	650-760
Opera Phenix Plus 4 lasers	Excitation laser (nm)	405	488	561	640
	Emission filters (nm)	435-480	500-550	570-630	650-760
Operetta™ CLS 8 LED	Excitation LED filters (nm)	370 (355-385)	475 (460-490)	550 (530-560)	630 (615-645)
	Emission filters (nm)	430-500	500-550	570-650	655-760
Operetta™ CLS 4 LED	Excitation LED filters (nm)	370 (355-385)	*510 (489-514)	550 (530-560)	630 (615-645)
	Emission filters (nm)	430-500	*525-580	570-650	655-760

**Performances of PhenoVue Fluor 515 pH-sensitive Fab anti-human IgG antibody are highly increased with this configuration.*

The PhenoVue human IgG antibody internalization kit (Fluor 515, pH-sensitive) enables simultaneous multiplexing of three fluorescent channels. To achieve improved signal intensity and image quality on Revvity high-content screening systems, we suggest the following:

Channel configuration :

- Three channels are dedicated to the kit reagents.
- An additional free channel may be used for another compatible marker. For best spectral separation, we suggest selecting a fluorescent dye with properties similar to PhenoVue Fluor 555.

Imaging mode :

- Use confocal mode to obtain higher signal-to-background ratios and sharper localization of intracellular structures.

Z-stacks :

- Acquire 2-3 z-planes per field and apply a maximum intensity projection to capture internalization events while reducing acquisition time.

Live-cell considerations :

- Thanks to the high brightness of PhenoVue dyes, high exposure times or laser powers can be avoided.
- Limit the number of timepoints in time-lapse experiments to a maximum of 4-5 per acquisition series. This reduces the risk of phototoxicity or photodamage, particularly in short acquisition cycles. Under these conditions, reduced cell stress and improved image quality have been observed.

Guidance for image analysis using Revvity's Harmony™ or Signals Image Artist™ software

The following analysis workflow can be considered a basic protocol for quantifying antibody internalization intensity within acidic compartments.

Flexibility of the method: depending on the cell type used, the method and specific building block parameters may require adjustments to optimize segmentation and measurement accuracy.

Configurable: additional building blocks can be included if further parameters need to be evaluated, such as number of spots, spot size, degree of overlap, or co-localization metrics.

This modular approach allows the analysis pipeline to be tailored to experimental needs while maintaining a reproducible basis for quantifying internalization.

1. Input Image

Acquire 2 or 3 z-planes and apply a "maximum projection" to better account for cellular variability and morphology over time. Set Flatfield correction to "Basic" or "Advanced".

2. Find Nuclei

Use the PhenoVue Hoechst channel for nuclei segmentation.

3. Filter Image

Use the PhenoVue 633 lysosomal channel to create a new and smoothed PhenoVue 633 lysosomal image.

4. Find Cytoplasm

Apply the "find cytoplasm" building block on smoothed PhenoVue 633 lysosomal image (channel) and the nuclei selected (with removed border objects).

5. Select Population to remove border cells

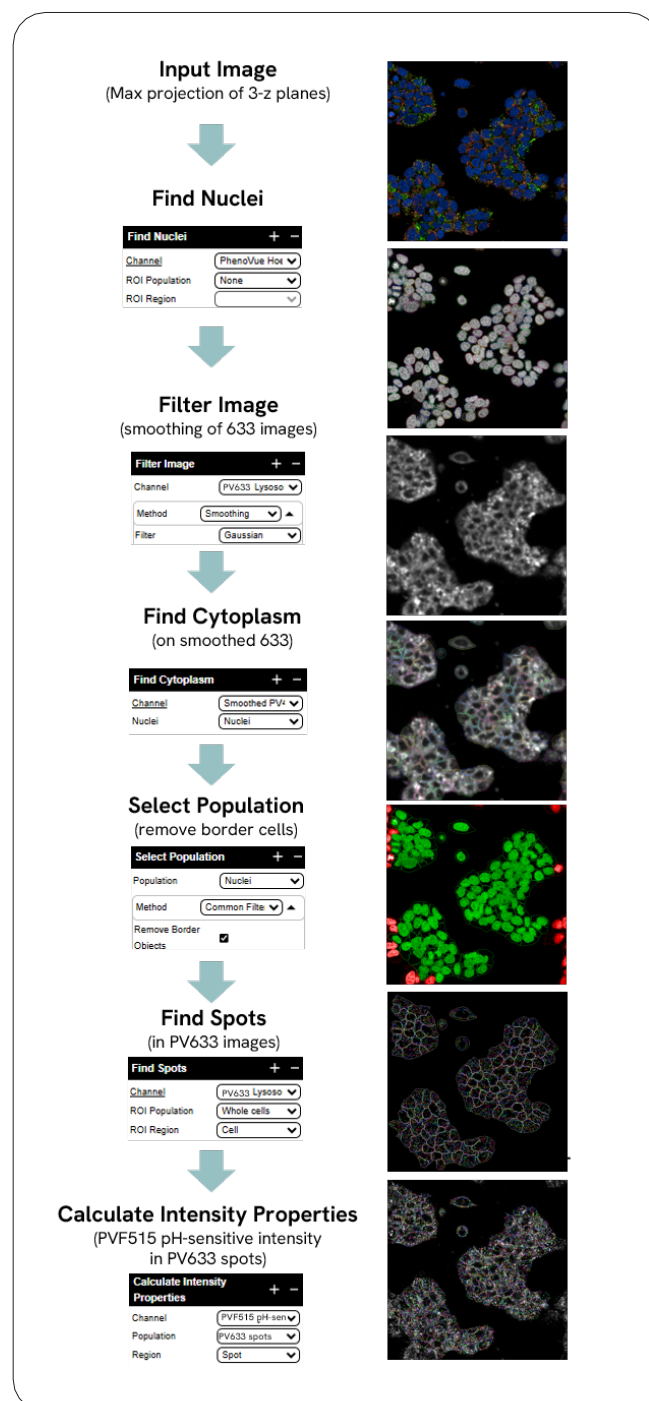
Apply the "Select Population" building block on the nuclei population with common filters method after selection of "Cell" Region to keep only whole cells.

6. Find Spot

Apply the "find Spots" building block on PhenoVue 633 lysosomal channel and select the appropriate method to detect the PhenoVue 633 spots corresponding to acid pH vesicles

7. Calculate Intensity Properties

Apply the "calculate Intensity Properties" building block to quantify the PhenoVue 515-pH sensitive intensity in the PhenoVue 633 spots



Assay validation

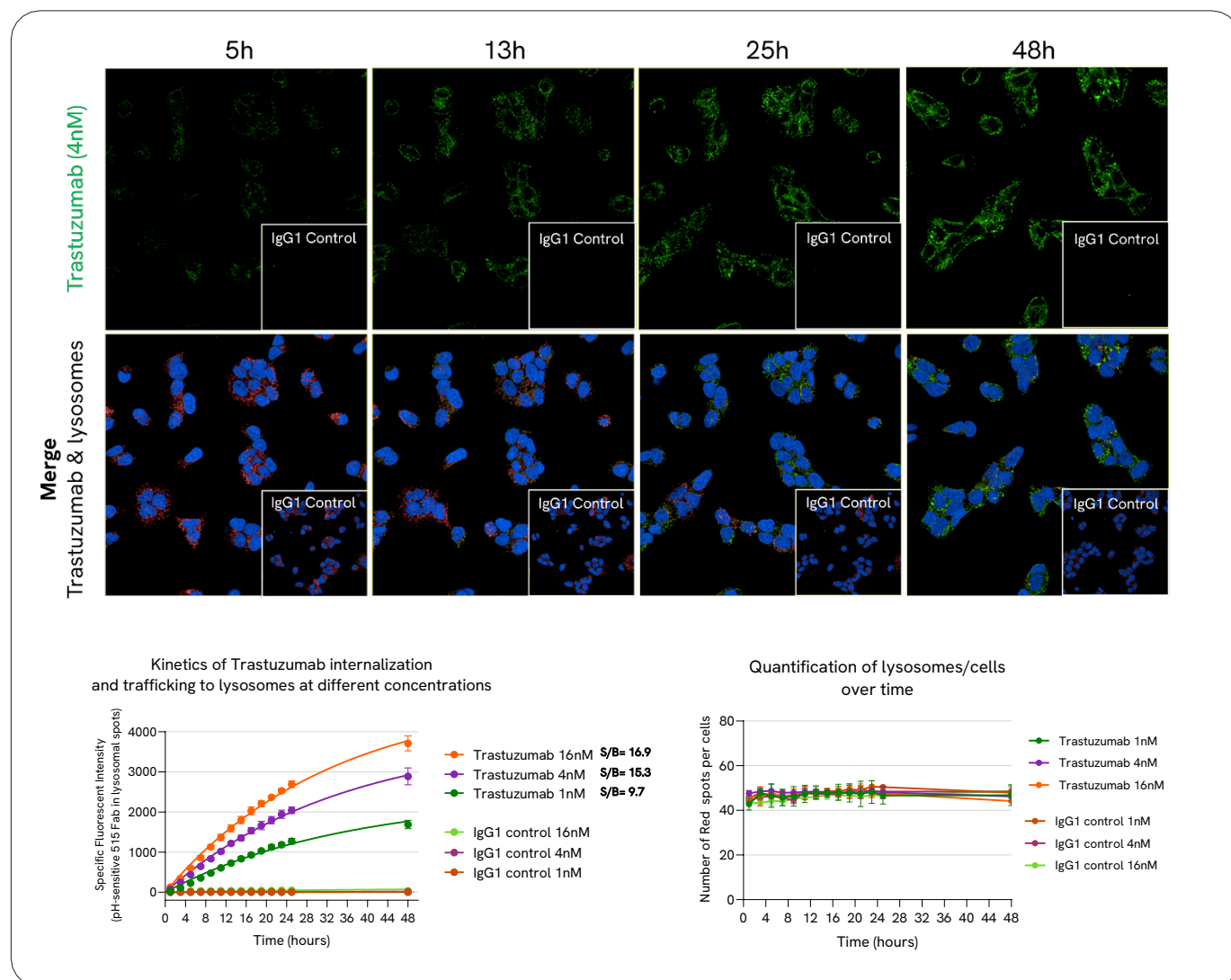


Figure 2. Long-term kinetics of Trastuzumab internalization and trafficking to lysosomes in BT-474 cells monitored with the PhenoVue Human IgG Antibody Internalization Kit (Fluor 515, pH-sensitive) in a 384-well format.

BT-474 cells were seeded in PhenoPlate 384-well microplates and incubated overnight at 37 °C, 5% CO₂. Cells were stained following the suggested live-cell protocol and treated with Trastuzumab (anti-HER2) or the PhenoVue Human IgG1 antibody isotype control at 1, 4, and 16 nM, maintaining an antibody:Fab molar ratio of 1:3. Fab-only controls at the corresponding concentrations were included to define background signal.

Time-lapse imaging was performed for up to 48 h. Representative images obtained with 4 nM Trastuzumab are shown at 5, 13, 25, and 48 h. Specific internalization was quantified as the PhenoVue Fluor 515 pH-sensitive Fab intensity within PhenoVue 633-positive lysosomal spots, after subtraction of the corresponding Fab-only background.

Trastuzumab showed a clear concentration- and time-dependent increase in internalization, reaching S/B values of approximately 9.7, 15.3, and 16.9 at 48 h for 1, 4, and 16 nM, respectively. In parallel, the number of lysosomal spots per cell remained stable over time, supporting that the increase in Fluor 515 signal reflects progressive antibody internalization and trafficking rather than changes in lysosome abundance.

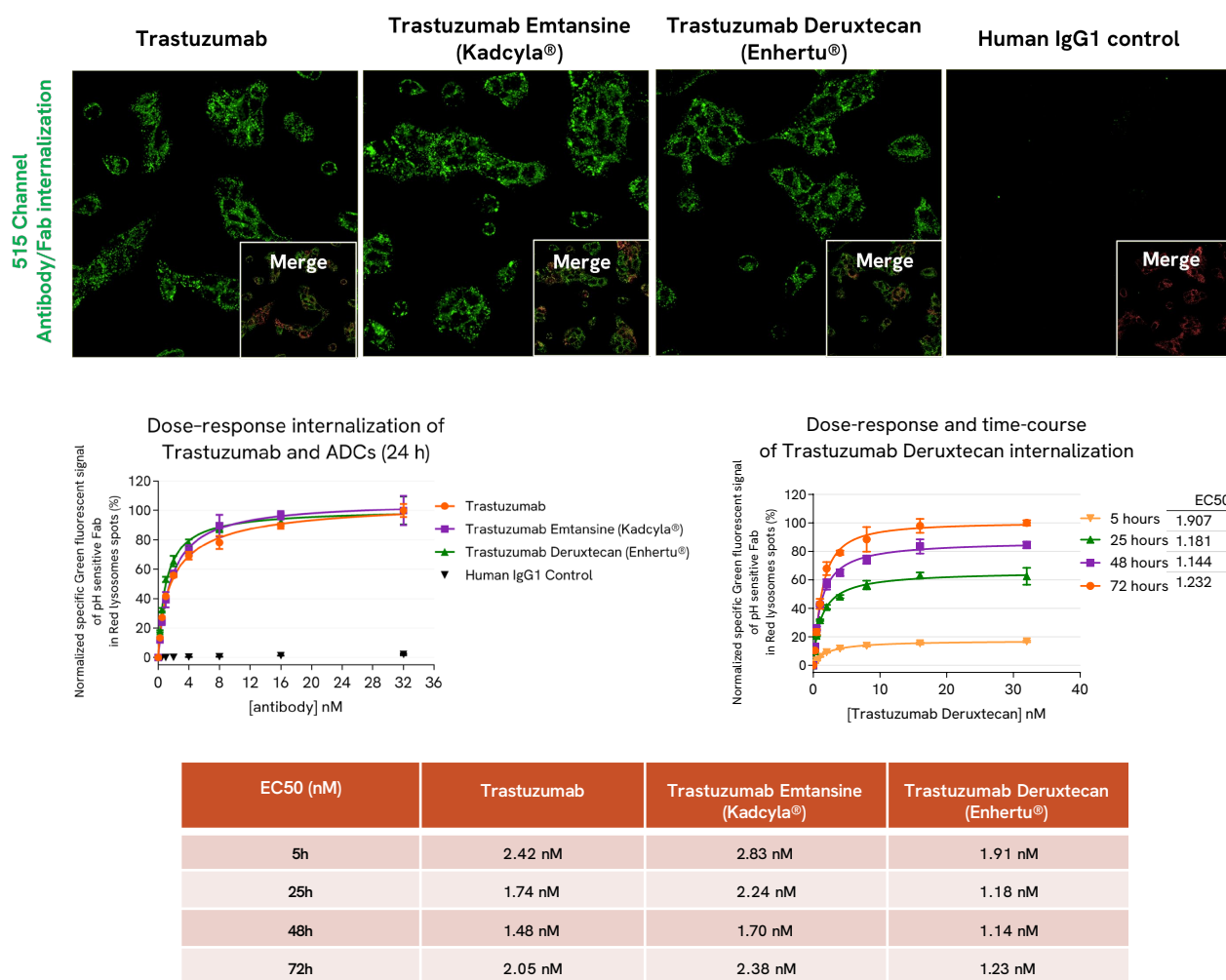


Figure 3. Long-term dose-response internalization of Trastuzumab and associated ADCs monitored with the PhenoVue Human IgG Antibody Internalization Kit (Fluor 515, pH-sensitive) in a 384-well format.

BT-474 cells were seeded in PhenoPlate 384-well microplates and incubated overnight at 37 °C, 5% CO₂. Trastuzumab, Trastuzumab Emtansine (Kadcyla®), Trastuzumab Deruxtecan (Enhertu®), and the PhenoVue Human IgG1 antibody isotype control were evaluated over a concentration range of 0.25–16 nM, with an antibody:Fab molar ratio of 1:3. Parallel Fab-only controls were included to determine background signal.

Internalization was monitored for up to 72 h without washing. Representative images show strong Fluor 515 pH-sensitive signals for Trastuzumab and both ADCs, with minimal signal from the IgG1 antibody isotype control. Dose-response analysis generated robust concentration-dependent internalization curves and reproducible EC₅₀ values over time.

EC₅₀ values ranged from 1.48–2.42 nM for Trastuzumab, 1.70–2.83 nM for Trastuzumab Emtansine, and 1.14–1.91 nM for Trastuzumab Deruxtecan between 5 and 72 h. The stability of these values demonstrates that the Fluor 515 pH-sensitive format supports reliable quantitative comparison of naked antibodies and ADCs during extended live-cell experiments.

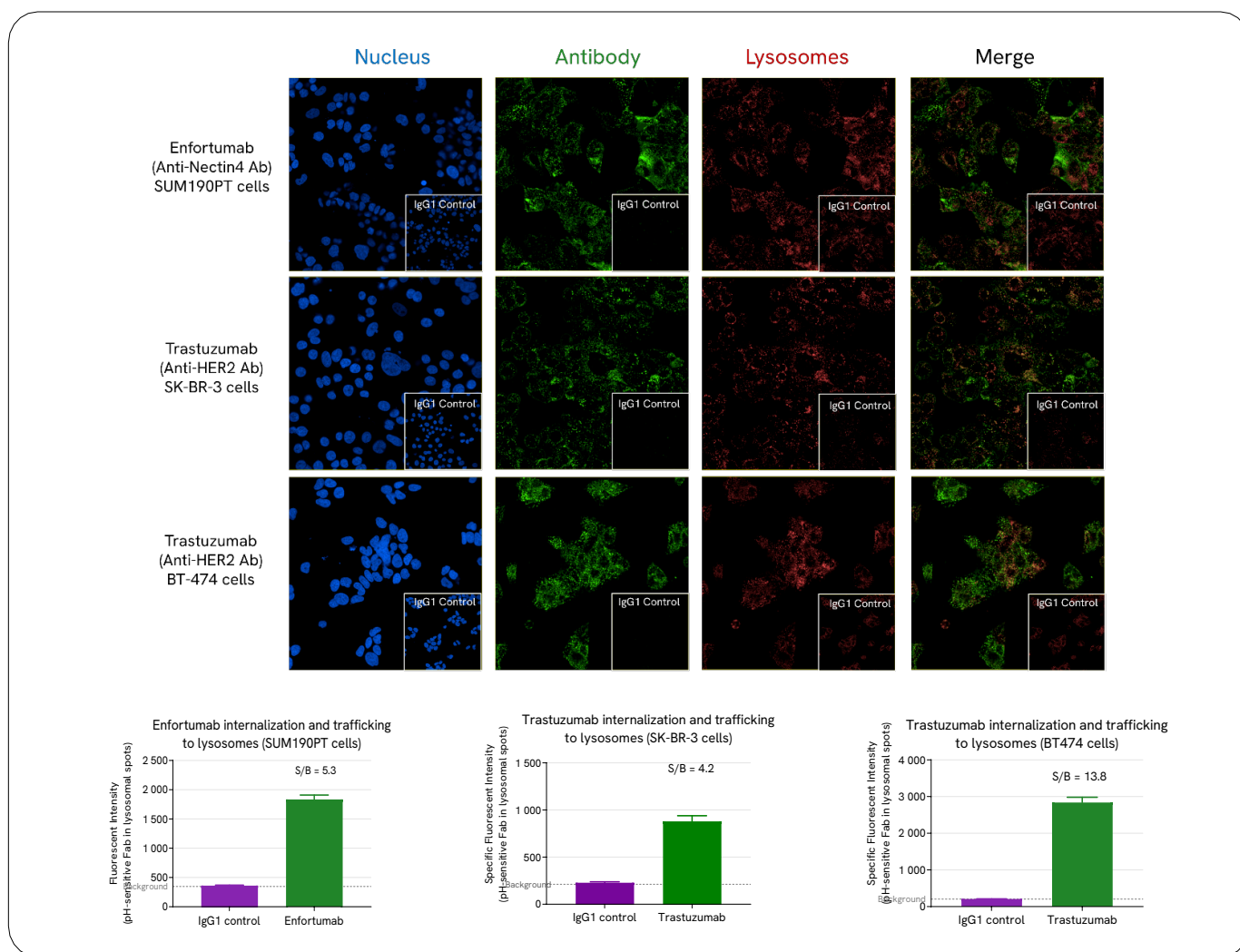
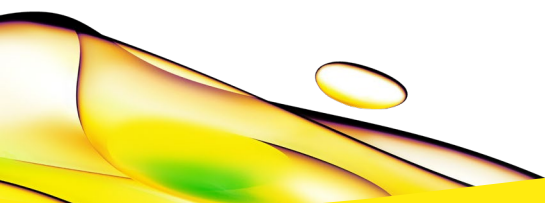


Figure 4. Antibody internalization monitored with the PhenoVue Human IgG Antibody Internalization Kit (Fluor 515, pH-sensitive) across different antibody targets and cell models.

The performance of the Fluor 515 pH-sensitive internalization assay was evaluated across three receptor-positive cell models: Enfortumab (anti-Nectin-4) in SUM190PT cells and Trastuzumab (anti-HER2) in SK-BR-3 and BT-474 cells. The PhenoVue Human IgG1 antibody isotype control was included in parallel to assess non-specific background.

In all three models, antibody treatment produced a strong PhenoVue Fluor 515 pH-sensitive signal associated with PhenoVue 633-positive lysosomal compartments, whereas IgG1 antibody control samples showed low background.

Quantification demonstrated clear discrimination between specific antibody internalization and the isotype control, with S/B values of 5.3 for Enfortumab in SUM190PT cells, 4.2 for Trastuzumab in SK-BR-3 cells, and 13.8 for Trastuzumab in BT-474 cells. These results demonstrate robust detection and quantification of antibody internalization across different antibody targets, antibodies, and cellular backgrounds, supporting the broad applicability of the Fluor 515 pH-sensitive assay.



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