Monitoring Antibody Internalization Using NYONE® and CELLAVISTA®

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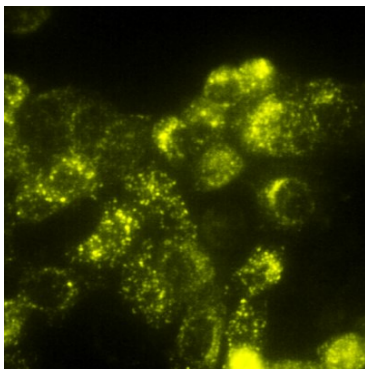
Introduction



Antibody Internalization is a key parameter for protein-based pharmaceuticals. Either in regards to antibody-drug conjugates (ADC) that have to be internalized to deliver cytotoxic small-molecule effectors to specific cells expressing a certain antigen[1]. Or in regards to antibodies that have recruiting functions via their Fc-part to elicit ADCC[2] or other cell based functions, where rapid internalization is not desirable, to allow time for effector cell recruitment. Also it is key to monitor internalization of antibodies that mediate receptor endocytosis to potentially downregulate receptor function and thus inhibit growth of tumor cells. The use of pH-sensitive fluorescent probes like pHAb[3] (Promega) is a valuable application to monitor internalization of molecules that are labeled with the dye. The antibody or ADC to be tested is labeled with pHAb that is not fluorescent above pH 7 and is only fluorescent in acidic pH environments. Upon target binding and internalization of mAb-target complexes into endosomes and lysosomes the pH is shifted to acidic and the dye becomes fluorescent. This method in combination with NYONE® and CELLAVISTA® can be used in an endpoint assay or kinetic measurements over time to monitor internalization rates in high throughput applications. We conducted a series of experiments to show the applicability of this assay to NYONE® and CELLAVISTA® by applying the method to three cell lines that express different amounts of the targeted antigen Her2. MDA-MB-468[4] is a Her2-negative breast cancer cell line, that was used as a negative control and a positive control upon stable Her2-overexpression[5] (Her2^{OX}). Caco-2 is a heterogeneous human epithelial colorectal adenocarcinoma cell line that is known to express small amounts of Her2.

Keywords: Internalization Assay, ADC, Internalization, Antibody Discovery, Cell Confluence, Fluorescence Imaging, Cell Morphology, MDA-MB-468, Caco-2, Cancer Research

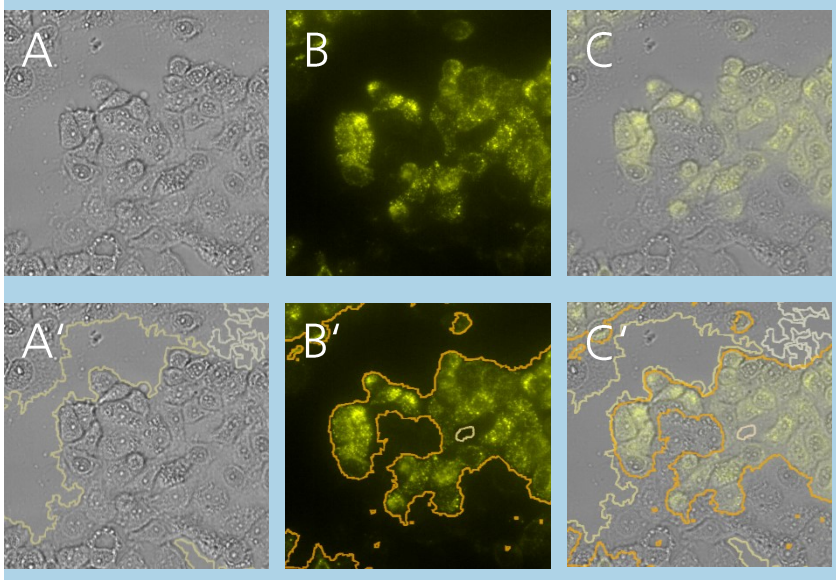


Fig. 1: MDA-MB-468 Her2^{OX} cells after Trastuzumab-Internalization.
A: brightfield image, **B:** fluorescence image, **C**: brightfield and fluorescence overlay, **A'-C'**: respective images with detection.

Material and Methods

Materials:

- Adherent epithelia-forming cells, here Caco-2 and MDA-MB-468
- DMEM/Ham's F12 1:1 with 3 % fetal bovine serum (PAN-Biotech)
- Phosphate Buffered Saline (w & w/o Ca^{2+} & Mg^{2+}) (PAN-Biotech)
- Trypsin 0.05 %/EDTA 0.02 % (PAN-Biotech)
- One of SYNENTEC's imaging systems, e.g. NYONE® or CELLAVISTA®
- Black walled 384 well plates (Corning 3712, TC-treated)
- pHAb Amine Reactive Dye (Promega, G9841)
- CentriPure P2 desalting column (Genaxxon Bioscience, CP-0110-2)

Procedure

The Her2-targeting monoclonal antibody Trastuzumab was labeled with pHAb Amine reactive dye according to the manufacturers instructions. Excess dye was removed using a CentriPure P2 desalting column (Genaxxon Bioscience, Ulm). Cell lines were grown overnight in 384 well plates (Corning 3712 in standard conditions) until a confluence of 30 % was reached. Cells were treated for 2 h with increasing concentrations of labeled Trastuzumab in growth media to induce internalization. The process was monitored using NYONE® equipped with a 20x UPlanSAPO NA: 0.75 Olympus Objective in full well scanning mode. Brightfield and fluorescent images (NYONE® HE4, 8 bit, blue/amber, 200 ms, 30 % gain) were captured and analyzed using **Confluence 1F** image processing. The images were repeatedly captured over a time course of 24h. Graphpad Prism 7 was used to analyze the data from the automatically performed image processing results. One result parameter **(BF AND Fluo Area) / BF Area [%]** quantifies the fluorescence of internalized and acidified pHAb::Trastuzumab with respect to cell confluence. Prior to imaging, 50 µL DMEM/Ham's F12 containing 3 % FBS was replaced with 20 µL PBS^{++} to remove background fluorescence of FBS.

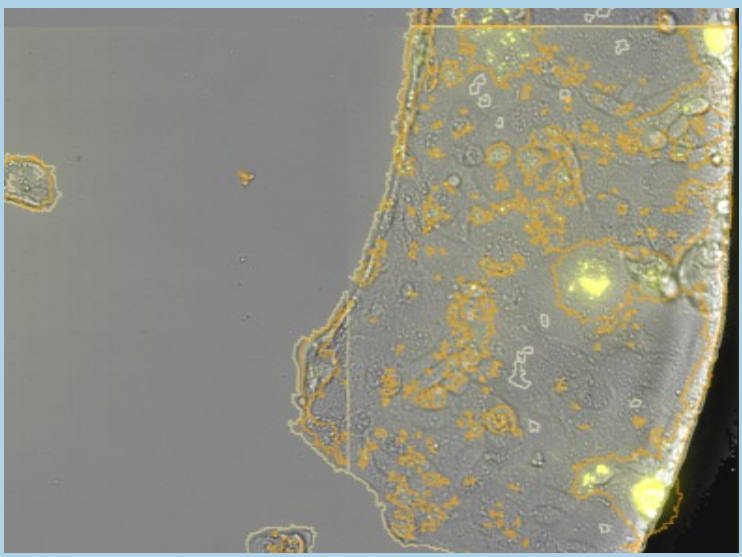


Fig. 2: Caco-2 cells in brightfield/fluorescence overlay image. Caco-2 cells are detected correctly albeit their low contrast in brightfield.

Results

Internalized mAb / Confluence was plotted against the concentration of Trastuzumab. The data was aquired 24h after treatment. MDA-MB-468 (fig. 5, bottom) cells show no response to treatment, whereas MDA-MB-468 Her2^{OX} (fig. 4) show strong internalization of Trastuzumab. Caco-2 (fig. 5, top) cells show low, but clear internalization of Her2::Trastuzumab::pHAb (fig.3).

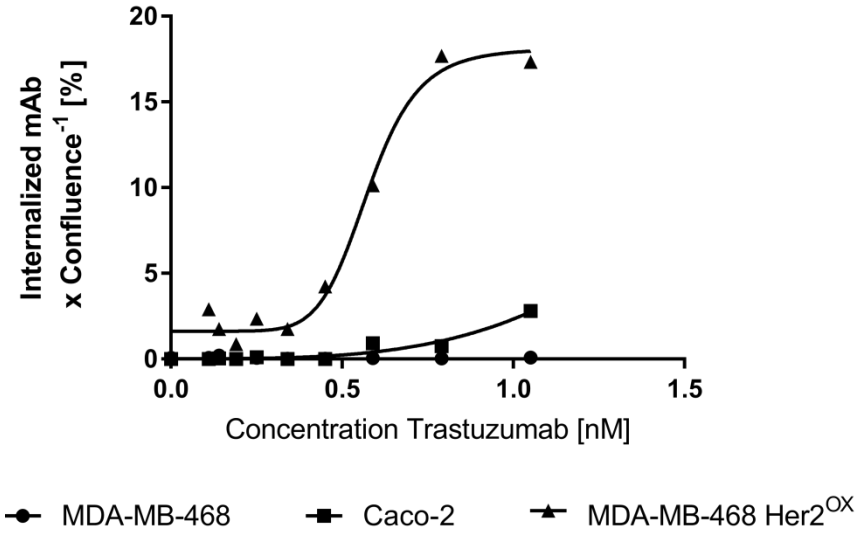


Fig. 3: Dose dependency of internalization signals 24h after treatment.

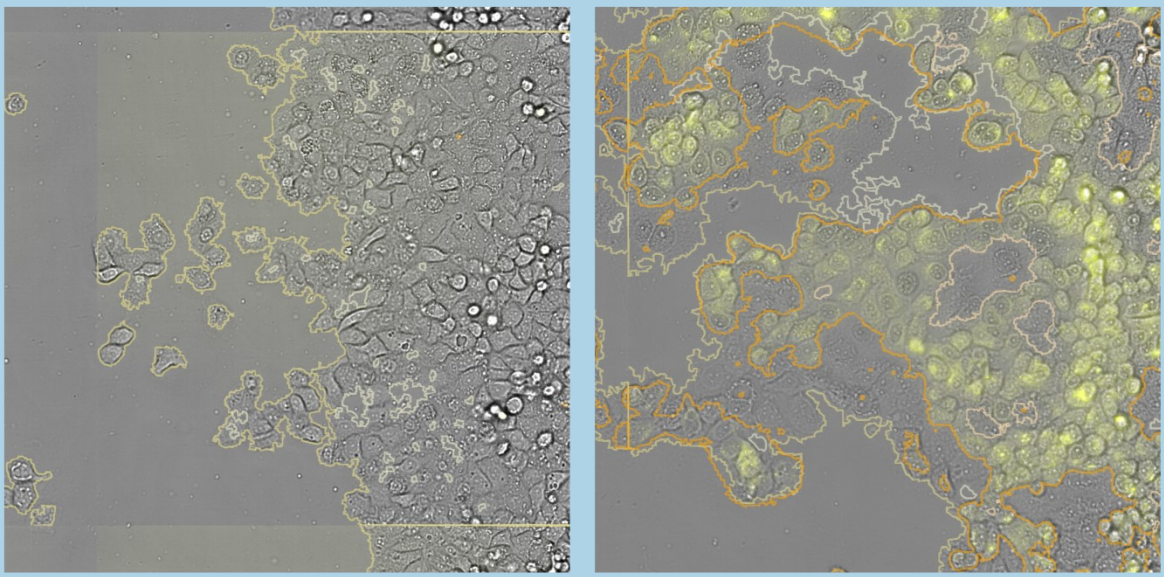


Fig 4: MDA-MB-468 Her2^{OX} cells before (left) treatment with labeled Trastuzumab and 24h after (right) removal of labeled antibody. The results of Confluence 1F image processing mark area covered with cells (yellow) and fluorescent signals from internalized Trastuzumab (orange).

Results

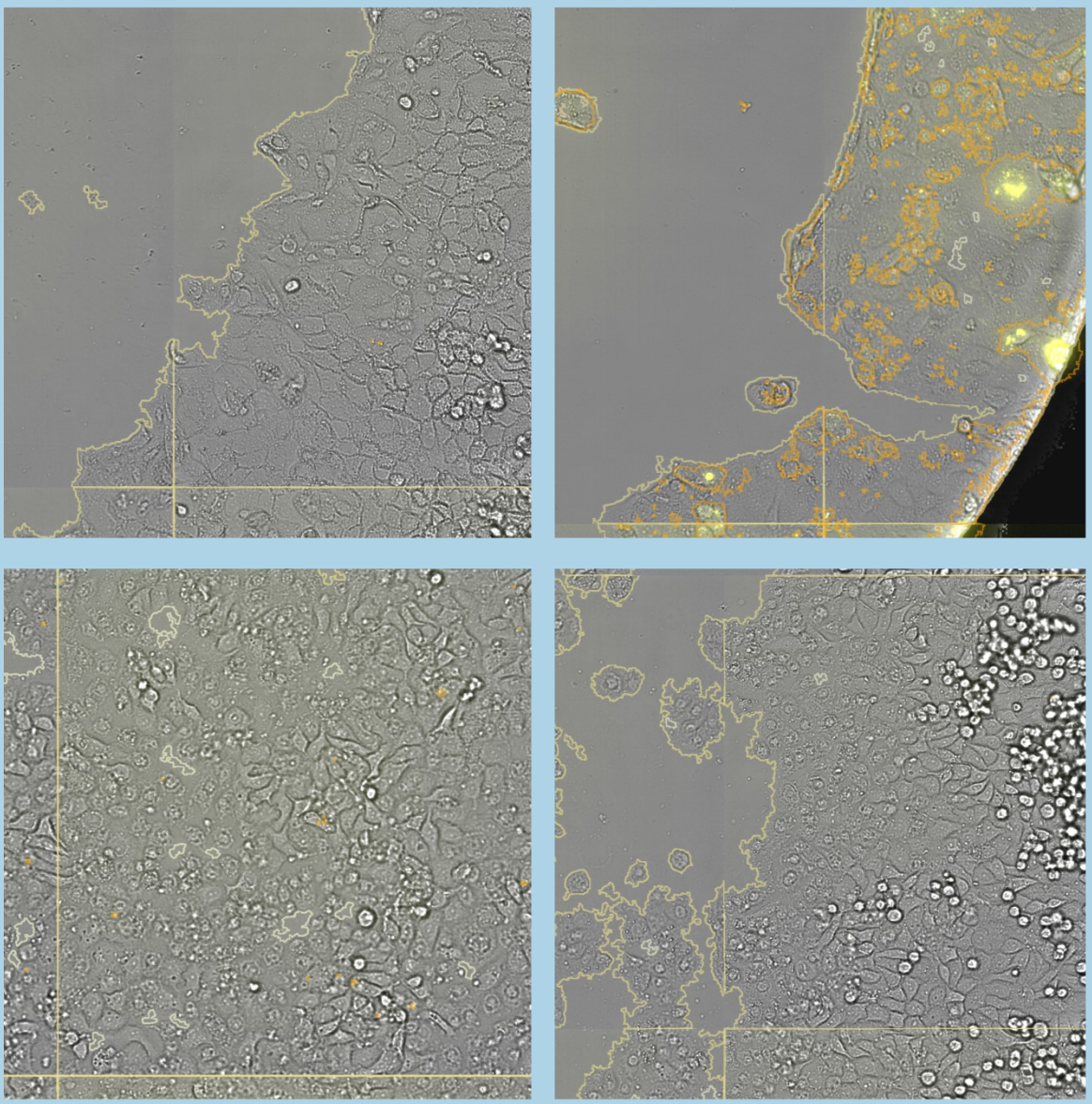


Fig. 5: Caco-2 cells before (top-left) treatment with pHAb-labeled Trastuzumab and 24h after (top-right) removal of labeled antibody. MDA-MB-468 (bottom images) cells are used as a negative control and show no internalization of Trastuzumab as the cell line is Her2-negative.
The results of **Confluence 1F** image processing quantify area covered with cells also when a cell line is used that has only low contrast in Brightfield images.

Results

Internalized mAb / Confluence was plotted as internalization over time, to demonstrate the time-dependent internalization of Trastuzumab (fig. 6, right). The data was aquired starting 2 h after treatment, growth medium was used as a negative control. MDA-MB-468 cells show no response to treatment as these cells do not express Her2, whereas Her2-overexpressing MDA-MB-468 Her2^{OX} show strong internalization of Trastuzumab in a concentration and time-dependent manner. Caco-2 cells show low, but clear internalization of Her2::Trastuzumab::pHAb, a slow decrease over time is recorded. Caco2 is known to express low amounts of Her2, which is seemingly internalized completely within 2 h after treatment, before the first measurement. Finally, the internalization rates for Her2-overexpressing MDA-MB-468 Her2^{OX} could be calculated and also show a dependency on treatment concentration (fig. 6, left).

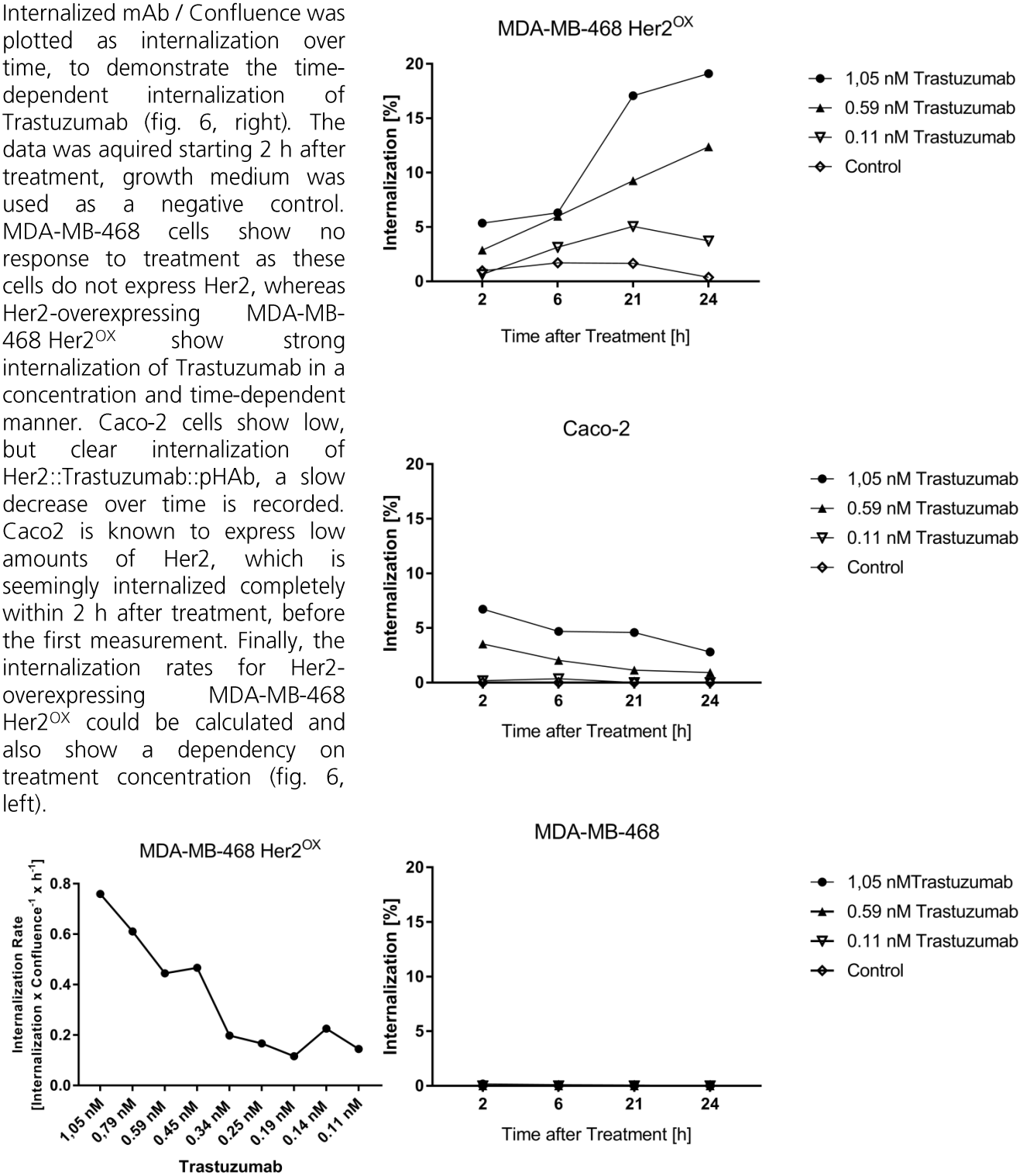


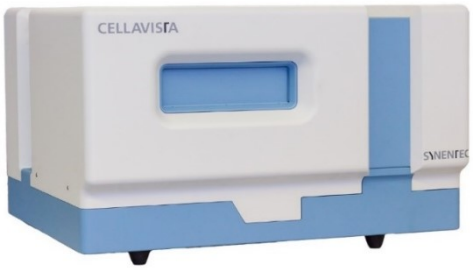
Fig. 6: Results from time course of internalization (right) and calculated internalization rates of MDA-MB-468 Her2^{OX} (left).

CONCLUSION

NYONE® and CELLAVISTA® are high throughput, automatable imaging systems that can be of high value to laboratories that are working in fields like cell line development, antibody discovery or optimization and target discovery. In this short application note, we could show that the imagers can be used to monitor antibody internalization. The assay can be used in an endpoint format or measurements can be repeatedly conducted to monitor assay kinetics.

In an endpoint assay, a nuclear dye could be used to use **Virtual Cytoplasm** image processing with the same sensitive image analysis in the internalization-channel to actually count adherent cells using the nuclear stain to generate data in accounting for actual cell number rather than confluence, which is not accounting for different cell sizes. The use of suspension cell lines is applicable using **Suspension Cell Count** in brightfield in the **Suspension Cell Count-Ab-Binding** image processing.

The soon to be launched Scientific line of NYONE® and CELLAVISTA® will use highly sensitive sCMOS camera chips that are, combined with high numerical aperture lenses, even more capable of performing assays due to the 16 bit nature of the camera, aided by the highly sensitive optics that are shipped with this new line of instruments.



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