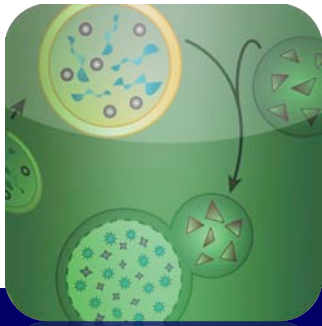

Using Cellometer Vision and Cyto-ID Stain for Autophagy Detection in Living Cells

WHITE PAPER

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Introduction

Our understanding of autophagy has expanded tremendously in recent years, largely due to the identification of the many genes involved in the process, and the use of GFP-LC3 fusion proteins to visually monitor autophagosomes and autophagic activity both biochemically and microscopically [1, 2]. Recently, a novel fluorescent probe, Cyto-ID® Green autophagy dye, has been developed to facilitate the investigation of the autophagic process [3-5]. In this study, a novel method was performed using the Cellometer image cytometry in combination with Cyto-ID Green autophagy dye for detecting autophagy in live cells. First, Cyto-ID Green autophagy dye was validated by observing co-localization of the dye and RFP-LC3 in HeLa cells using fluorescence microscopy. Next, image- based and flow cytometry-based methods are benchmarked for measuring macroautophagic signals in nutrient-starved Jurkat cells. Autophagic signals of starved Jurkat cells induced with an autophagy inhibitor were also quantified and compared using the two instrument platforms [6]. In order to establish the feasibility of employing the imaging-based workflow for drug discovery applications, a time-course study of the induction of autophagy in Jurkat (suspension) and PC-3 (adherent) cells treated with rapamycin was undertaken [7, 8], demonstrating the ability to detect autophagy with a similar sensitivity as the starvation model. Finally, direct dose-response comparisons of two small molecule autophagy inducers, rapamycin and tamoxifen, were performed [9].

Materials and Methods

Image-Based Cytometry Instrumentation and Disposable Counting Chamber

The Cellometer Vision instrumentation has been described previously [10]. The system utilizes bright-field (BR) and dual-fluorescent (FL1 and FL2) imaging modes to quantitatively analyze and measure the fluorescence intensities of target cells. Bright-field imaging uses a white light- emitting diode (LED) and fluorescent imaging uses two different monochromatic LEDs (470 and 525 nm) as the excitation light sources. The monochromatic LEDs are combined with two specific filter optics modules VB-535-402 (475/535 nm) and VB-595- 502 (525/595 nm). The software analyzes three image channels and generates a fluorescent data set that is automatically exported to FCS Express 4 Flow Cytometry. In this work, FL1 and FL2 images were specifically analyzed for Cyto-ID Green autophagy dye and propidium iodide fluorescence, respectively.

Cell lines and reagents preparation

The Jurkat cell line was cultured and grown to log phase in RPMI medium. PC-3 cells were cultured and grown to 70% confluence in F-12K medium in a 24-well plate. HeLa cells were

cultured in Eagle's MEM with low glucose. The cell culture was maintained in an incubator at 37°C and 5% CO_2.

Cyto-ID Green autophagy dye was provided as a component of a kit. The probe is a cationic amphiphilic tracer (CAT) dye that rapidly partitions into cells in a similar manner as many cationic drugs. The dye is taken up by passive diffusion across the plasma membrane bilayer and does not require protein binding or transporter activity. The excitation and emission maxima of the dye are 463 and 534 nm, respectively. Propidium iodide was used as is for fluorescently staining nonviable Jurkat cells, in order to exclude the necrotic cells during data analysis.

For performance of starvation and recovery experiments, Earle's balanced salts solution was used as the nutrient-deprived media. For autophagic flux experiments, chloroquine (CQ) and dimethyl sulfoxide (DMSO) were obtained, and the CQ was diluted directly in the DMSO before use. For drug treatment, the rapamycin was diluted directly into ethanol to a final concentration.

Cyto-ID Green autophagy dye staining procedure for autophagy detection

Cyto-ID Green solution was prepared by mixing the dye with 1× assay buffer. The cell sample was centrifuged, and the dye was pipetted in for image-based or flow cytometry, respectively. The sample was shielded from direct light and incubated for 30 min at 37°C, followed by a wash and resuspension in 1× assay buffer before imaged-based or flow cytometric analysis. A slight modification was made in the standard protocol in that propidium iodide was added to the protocol.

Starvation and recovery experiments

Jurkat cells were collected from the culture media and centrifuged. The control tube was resuspended in RPMI, while the cells in the second tube were incubated with EBSS media to induce amino acid starvation. The cells in both tubes were transferred to cell culture flasks. After 2 h, each cell sample was collected and stained following the procedure described above. The stained cells were then analyzed by image-based and flow cytometry. Nutrient-starved Jurkat cells were then resuspended in RPMI media for a 1-h recovery period. After recovery, the staining procedure was repeated and the cells were analyzed again by image-based and flow cytometry.

Time-course measurement of rapamycin-induced autophagy

In order to show the capability of image-based cytometry for dose-response analysis, rapamycin was selected to induce autophagy in Jurkat cells. Five rapamycin solutions were prepared using RPMI media from 0.01 to100 µM final concentration. The Jurkat cells were resuspended in each rapamycin solution, including a control with only RPMI medium. Each sample was incubated in cell culture flasks before Cyto-ID Green and propidium iodide staining. Analysis was performed at 4, 8 and 18 h using the image-based cytometer.

To demonstrate the capability of image-based cytometry for autophagy detection in adherent cells, PC-3 cells were treated with rapamycin at 1, 10 and 100 µM. After 4 h treatment,

rapamycin-containing media was aspirated off and trypsinized. Cells were then resuspended in the Cyto-ID Assay buffer before performing autophagy staining. Fluorescence measurements were performed using the image-based cytometer.

Comparison of dose-response profiles of rapamycin and tamoxifen

Five solutions were prepared using RPMI media from 0.01 to 100 μM final concentration of rapamycin and tamoxifen. The Jurkat cells were resuspended in each solution, including controls with only RPMI medium supplemented with ~1% ethanol or DMSO (vehicle controls for rapamycin or tamoxifen, respectively). Each sample was incubated in cell culture flasks before Cyto-ID Green and propidium iodide staining. Fluorescence analysis was performed after 18 h of incubation using image-based cytometry.

Image-based cytometric analysis

VB-535-402 and VB-595-502 were used to detect Cyto-ID Green autophagy dye and propidium iodide, respectively. Each sample analysis was performed in duplicate.

Flow cytometric analysis

Flow cytometry was used for comparison with the image-based cytometry method for autophagy detection. Experiments were performed using a FACS Calibur bench- top flow cytometer equipped with a blue (488 nm) and violet (407 nm) laser. Cyto-ID Green fluorescence was measured in the FL1 channel (530 nm) with blue laser excitation. The flow cytometry data was exported and analyzed in FCS Express 4 Flow Cytometry software and each sample was analyzed in duplicate.

Results

Figure 1. Data analysis for autophagy detection.

- (A and B) The fluorescent and bright-field images showed bright green fluorescent spots inside the Jurkat cells that represented Cyto-ID Green-stained autophagosomes/autolysosomes. Samples were also stained with propidium iodide, shown in the fluorescent image as orange signal, which allowed for the exclusion of dead cells from data analysis.
- (C) The fluorescence of propidium iodide was plotted with respect to Cyto-ID Green, and the propidium iodide-positive Jurkat cell signals were removed to allow analysis of only the viable cells in the Cyto-ID Green fluorescence histogram (D).

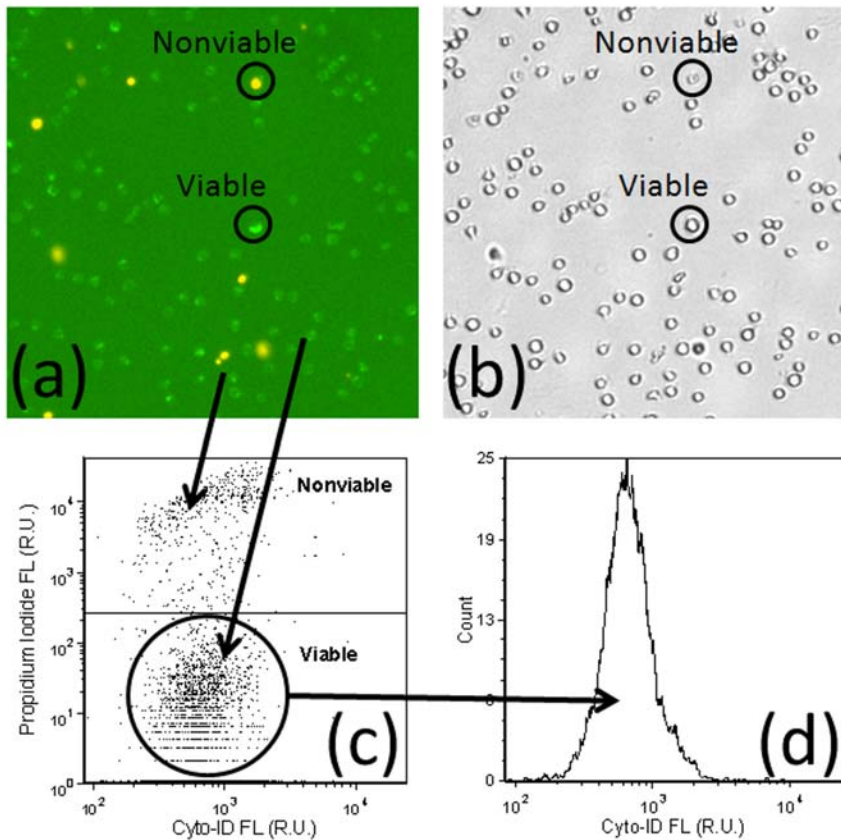


Figure 2. Validation of Cyto-ID Green autophagy dye using fluorescence microscopy.

- The starvation experiment with HeLa cells stained with Cyto-ID and Hoechst showed an obvious increase in the Cyto-ID fluorescence signals. Importantly, little to no staining of lysosomes in the control cells was observed.
- For the rapamycin treatment experiment, the control and the sample with 3-MA displayed low green fluorescence as expected, while the sample treated with rapamycin in the absence of 3-MA showed an increase in green fluorescence, indicating higher levels of autophagy.
- HeLa cells were transfected with RFP-LC3 and treated with tamoxifen. Under fluorescence microscopy, the green fluorescence was found to be associated with punctate structures that co-localized with the red fluorescence of RFP-LC3. Note that RFP-LC3 was not uniformly expressed in all cells, due to the limited transfection efficiency.

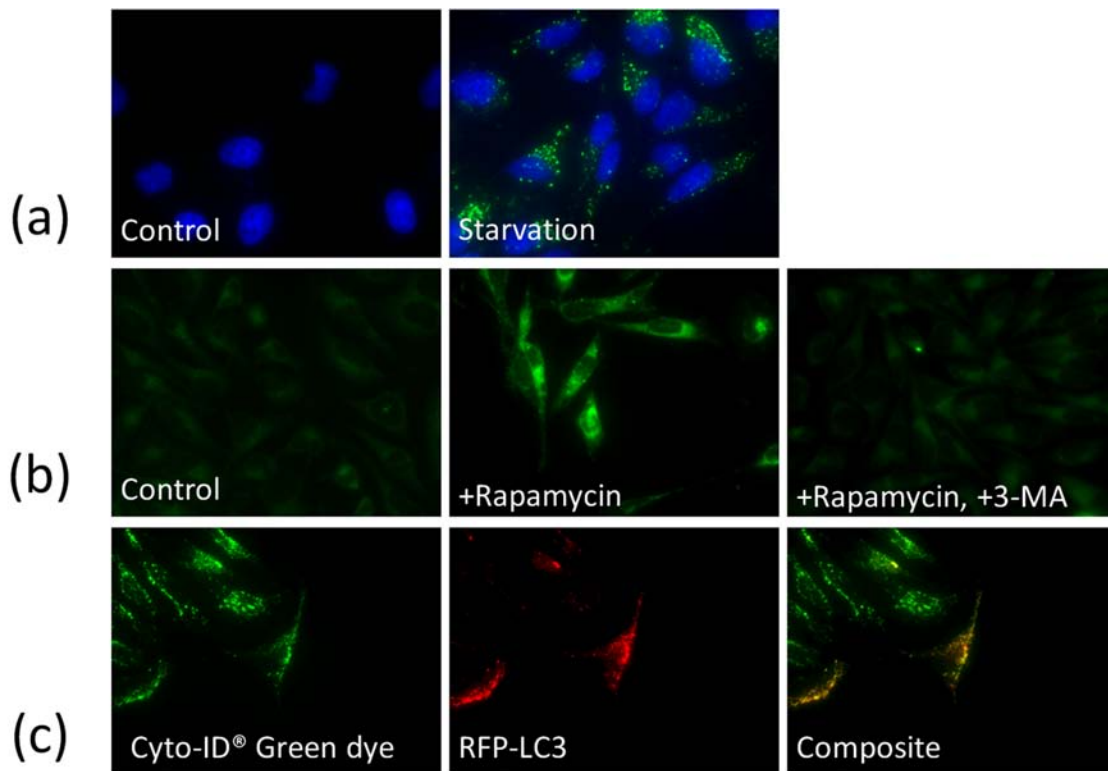


Figure 3. Comparison of starvation and recovery assay using image-based and flow cytometry.

- Starvation was induced by incubating Jurkat cells in EBSS media for 2 h. Here, bright-field and fluorescent images of control, recovery and nutrient-starved Jurkat cells are shown. The fluorescent images show strong fluorescence in the nutrient-starved Jurkat cells compared with the control and recovered samples. However, it was difficult to visually distinguish the fluorescence intensities between the control and recovered samples without examining the fluorescence histograms.
- A comparison of fluorescence histogram results obtained with the Cellometer and FACS Calibur cytometers is shown here. Profiles from the three samples were overlaid on the same histogram plot to facilitate changes in fluorescence. The peaks in the plot showed comparable response trends using both instrumentations, where the control group showed the lowest fluorescent intensity, followed by the recovery group, while the nutrient-starved group displayed the highest fluorescence intensity values.

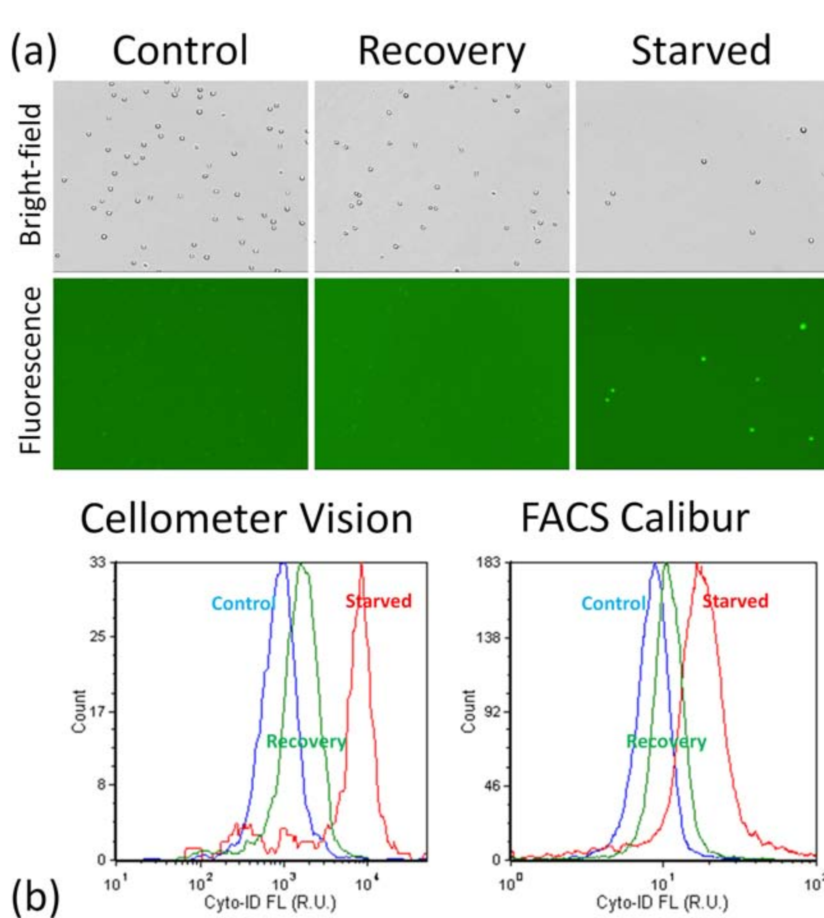


Figure 4. Comparison of rapamycin and tamoxifen dose response effects.

- (5A) Calculated AAF values for drug dose response effects in Jurkat cells. The calculated AAF values for rapamycin showed approximately 20% increase in autophagy at 18 h incubation. The 4 and 8 h samples were comparable, indicating rapamycin required more than 8 h of incubation in order to induce noticeable autophagy.
- (5B) Both visually and analytically, rapamycin showed higher autophagic signals than tamoxifen after 18 h incubation. It was interesting to note that tamoxifen induced cytotoxicity at the highest concentration in contrast to rapamycin.

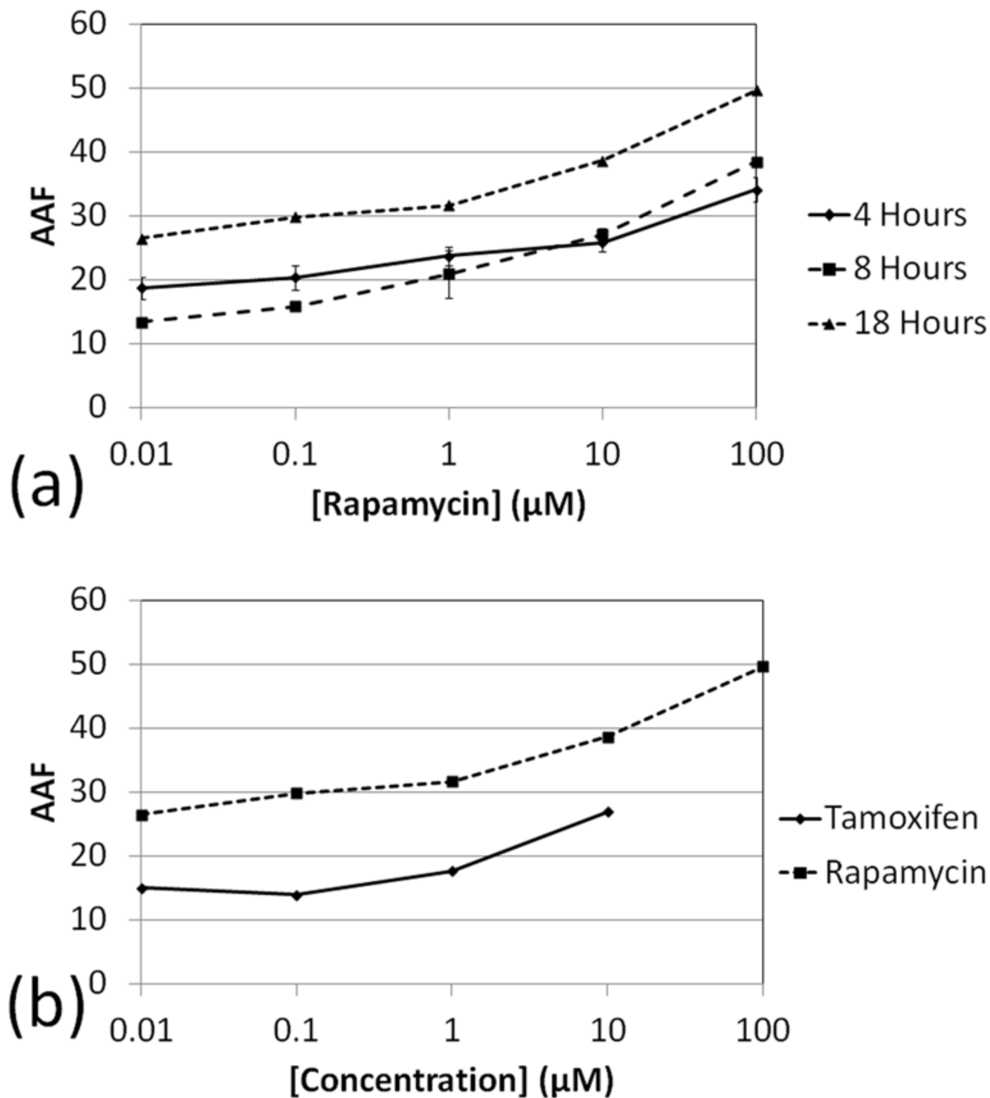
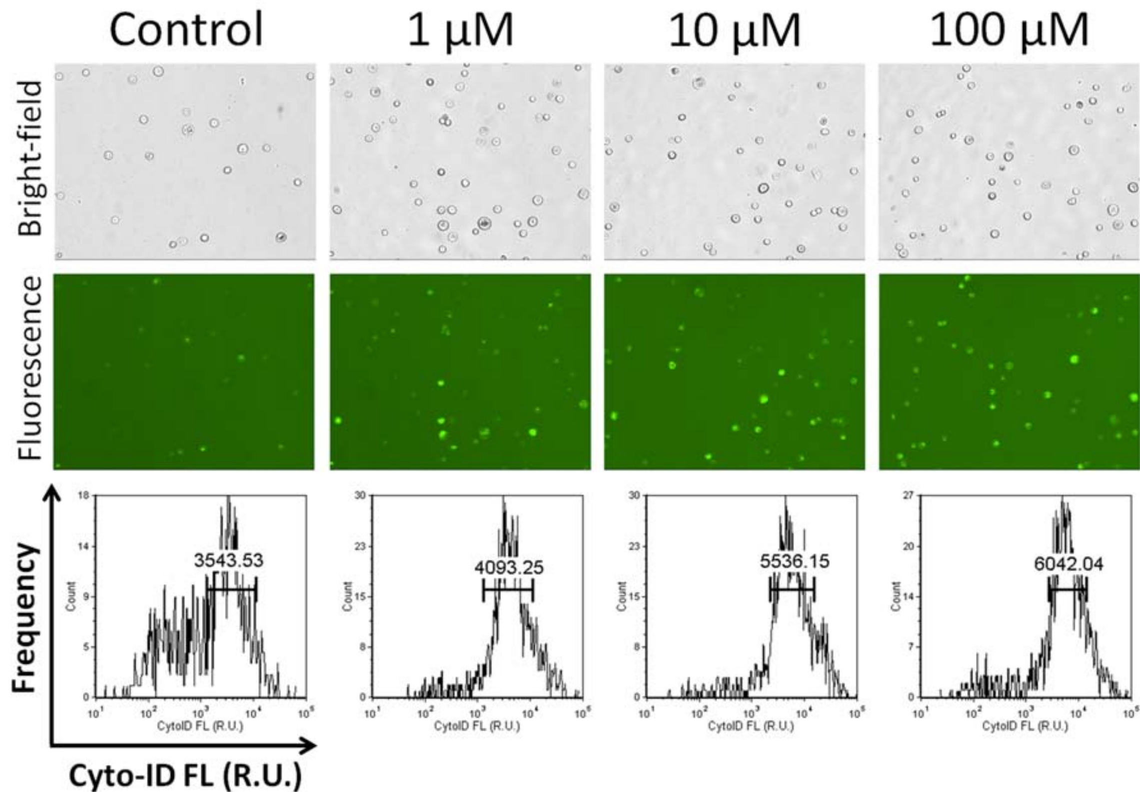


Figure 5. Time-dependent dose response effects in rapamycin-induced autophagy.

- (6) Rapamycin-induced autophagy in PC-3 cells. Bright-field (top) and fluorescent (middle) images of PC-3 cells induced with 0, 1, 10 and 100 μM of rapamycin for 4 h. The fluorescent images clearly showed the increase in fluorescence and population of Cyto-ID Green autophagy dye-stained PC-3 cells, confirmed by the fluorescence histogram (bottom), showing increase in average fluorescence intensity as rapamycin concentration increased.



Conclusion

- The ability to efficiently measure and analyze autophagy in living cells is of particular importance when screening for compounds that can potentially modify a disease state.
- Here, Cyto-ID Green was validated using fluorescence microscopy to demonstrate the colocalization of RFP-LC3 and Cyto-ID Green in a starvation model using HeLa cells.
- In order to develop the novel autophagy detection method, image-based cytometry was compared with conventional flow cytometry in the measurement of autophagy in nutrient-starved Jurkat cells. Both methods showed a strong increase in autophagy in nutrient-starved cells, which decreased for cells that had been allowed to recover by returning them to standard media.
- An essential aspect in developing a rapid autophagy detection method is to demonstrate its ability to analyze samples under drug discovery conditions. This capability was established to measure autophagic levels of Jurkat cells induced with rapamycin at various concentrations. Since rapamycin required at least 12 h of incubation in order to observe autophagy induction, the goal was to demonstrate the ability to measure dose response effects as a function over a relatively long time period. Overall, image-based cytometry was able to detect the differences in autophagic levels across the various incubation periods.
- Since many autophagy studies involve the use of adherent cells, the human prostate cancer cell line, PC-3 was selected to benchmark the capability of image-based cytometry. The resolution of Cellometer Vision was sufficient to image and measure fluorescent autophagosomes (puncta), as indicated in both fluorescent images captured by the system and fluorescence intensity histograms generated.
- In addition to measuring time-dependent dose response of rapamycin, it was also important to demonstrate the ability to compare autophagic effects of multiple compounds, which can prove useful in a drug discovery campaigns. Tamoxifen was employed as an alternative to rapamycin in this context. The analysis revealed that at the same concentration, rapamycin induced a higher level of autophagy than tamoxifen. However, 100 μM tamoxifen actually proved to be somewhat cytotoxic, leading to Jurkat cell death after 18 h incubation.
- In this experiment, image-based cytometry was able to verify the cytotoxicity effect of tamoxifen at high concentration, which proved to be useful in eliminating uncertainties from results that only plotted as scatter plots or histograms.
- Image-based cytometry has been shown to generate comparable results as standard flow cytometry for fluorescent-based cellular analysis [10, 12].
- Image-based cytometry may offer certain advantages in detection and analysis of response in cell-based assays:
    - For example, cell sample volume requirements typically ranges from 10 to 40 μl for image-based cytometers, which means the number of cells used are significantly reduced compared with a typical flow cytometer, which requires volumes of 300 to 500 μl.

- The ability to capture images allows researchers to visually inspect acquired fluorescent data, such as data generated from starvation and recovery experiments. This can prove useful in order to identify cytotoxicity as a complicating side effect of a drug treatment regime.
- Since the disposable counting slides are plastic, autofluorescence can occur with the excitation and emission of Cyto-ID Green autophagy dye, which may give rise to high background signals. However, the software can automatically remove the background signal to obtain the actual target fluorescence.
- While the fluorescence exposure times used with the Cellometer Vision are longer than instruments using high power lasers or LEDs, fluorescence photo bleaching becomes less of an issue when performing cell-based assays.

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