High Content Imaging is Now High Throughput: End-to-End High Content Imaging and Subcellular Analysis of Autophagy in Minutes

Matthew Boisvert and Josh Kieler
Araceli Biosciences 7405 SW Tech Center Drive Suite A160 Tigard, Oregon 97223 U.S.A.

Introduction: Measuring Autophagic Flux at High Throughput Speeds

- Cells eliminate intracellular waste and defective components through autophagy ("self-eating")
- A key therapeutic target for aging-related dysfunction, autophagy is implicated in neurodegeneration and cancer
- Autophagic flux can be measured by quantifying autophagic vesicles, visualized through examining marker protein LC3b or dyes activated by vesicle-specific conditions
- High content imaging (HCI) allows for cell-level resolution, visualizing aggregates and defining cell borders, quantifying spots per cell
- Resolving aggregated autophagosomes is essential, cell lines often have baseline levels of autophagy, generally non-aggregated
- Screening large compound libraries for autophagic flux requires speed, low variability and accurate detection
- Using the Araceli Endeavor® HCI Platform this assay demonstrates:
  - **Assay validation with four mechanistically different compounds**
  - **Submicron resolution to accurately visualize aggregates**
  - **Elimination of variability with whole well imaging**
  - **<10-minute scan times for 96, 384 and 1536-well high content plates**
  - **Flexibility: equivalent results with immunohistochemistry (IHC) and cell dye-based workflows**

Materials and Methods

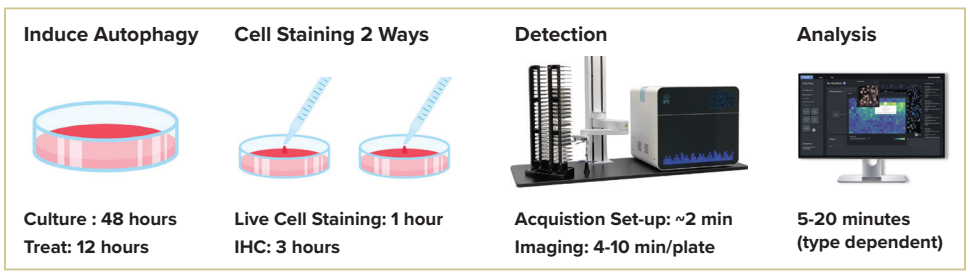


Figure 1: High content assay, imaging, and analysis workflow for a single plate.

Assay: Human bone carcinoma (U2OS) cells were plated at 12500 cells/well in 96 well Grenier CellView plates and cultured for 48 hours in standard conditions (complete media, 5% CO, 37°C). Cells were treated with compound or DMSO in a 1:2 dilution series, 3 replicates/plate, for 12-16 hours.

Treatments: DMSO: vehicle control; [hydroxy]chloroquine: autophagosome degradation inhibitor; verapamil: autophagy inducer via Ca2+ inhibition; MG-132: autophagosome arrest via proteasome inhibitor; torin-1: autophagy inducer via mTOR inhibition

Immunohistochemistry-based detection and analysis: Cells were fixed in 10% formalin for 15 minutes, permeabilized with 0.1% Triton x-100, then stained and counterstained for 60min/step in 1% BSA, with 1:1000 Rb anti-LC3b (L10382) and 1:1000 Gt anti-Rb Alexa 488 with 1:750 Hoechst 33342 (nuclei), and 1:1000 iFluor Phallodin-647 (actin). Cells were washed 4x between steps and all solutions are in PBS pH 7.4.

Dye-based detection and analysis: Cells incubated at 37°C with 1:500 dilution of cationic amphiphilic tracer (CAT) CYTO-ID® Autophagy detection kit 2.0 in complete media, then fixed (10% formalin for 15 minutes) before staining with 1:750 Hoechst 33342.

Imaging: The 96-well plate was imaged on Araceli Endeavor®, in 2 channels (live cell) or 3 channels (IHC), with full well (4.5mm x 4.5mm/well), submicron resolution (.27µm pixel size). Data (3072-7200 images) collected for the whole 96-well plate in 4-10 minutes.

Analysis: Araceli internal analysis tool used for all analysis. For both assays, machine vision template match used to quantify nuclei and autophagic vesicles, with a larger template used for CytoID, as staining appeared more aggregate-like. For IHC data, nuclei used to seed cell, thresholded actin staining used to define cell, and cytoplasmic vesicles counted per cell (10-20 minutes). Live stain data analyzed segmentation-free, normalized to nuclear count (5-10 min).

Full Well, Full Plate High Resolution Scans in under 10 minutes

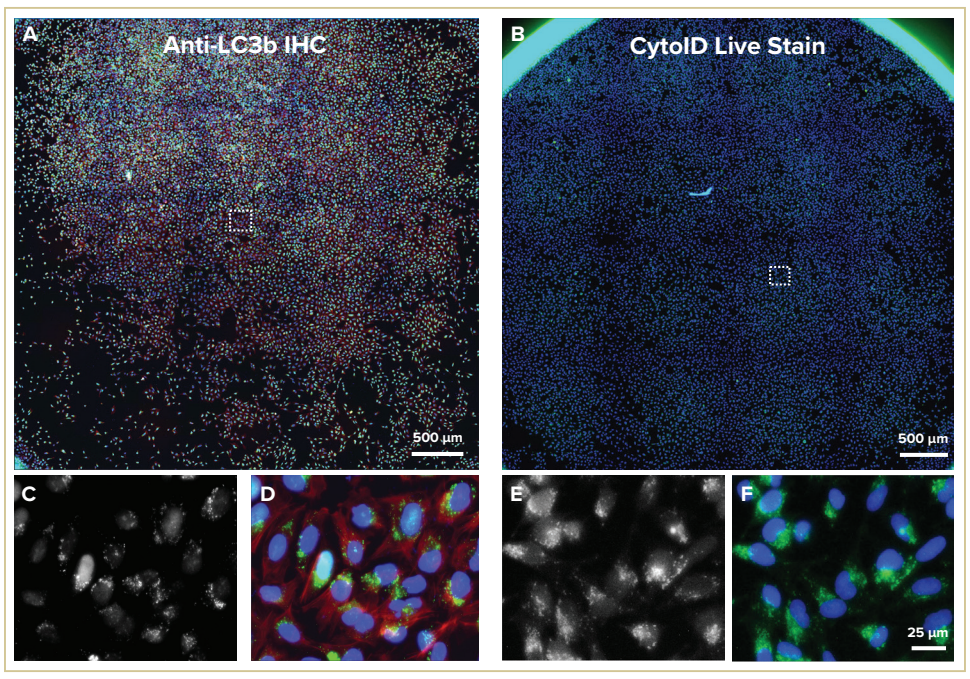


Figure 2: 96 well plates imaged at 0.27µm/pixel with full wells (>85% of well area) collected. 10µM chloroquine treatment shown. **A)** 3000x3000 pixel images with 5x5 fields of view/well for anti-LC3b antibody staining (green, actin in red and nuclei blue); **B)** 4400 x 4400 pixel images with 4x4 FOV/well taken for CytoID live stain (green, nuclei in blue). Zoom in as indicated by box with **(C, E)** autophagy stain and **(D, F)** merged image with counterstain.

Autophagic Flux Consistently Detected with Both Immunohistochemical and Live Cell Dye Assays

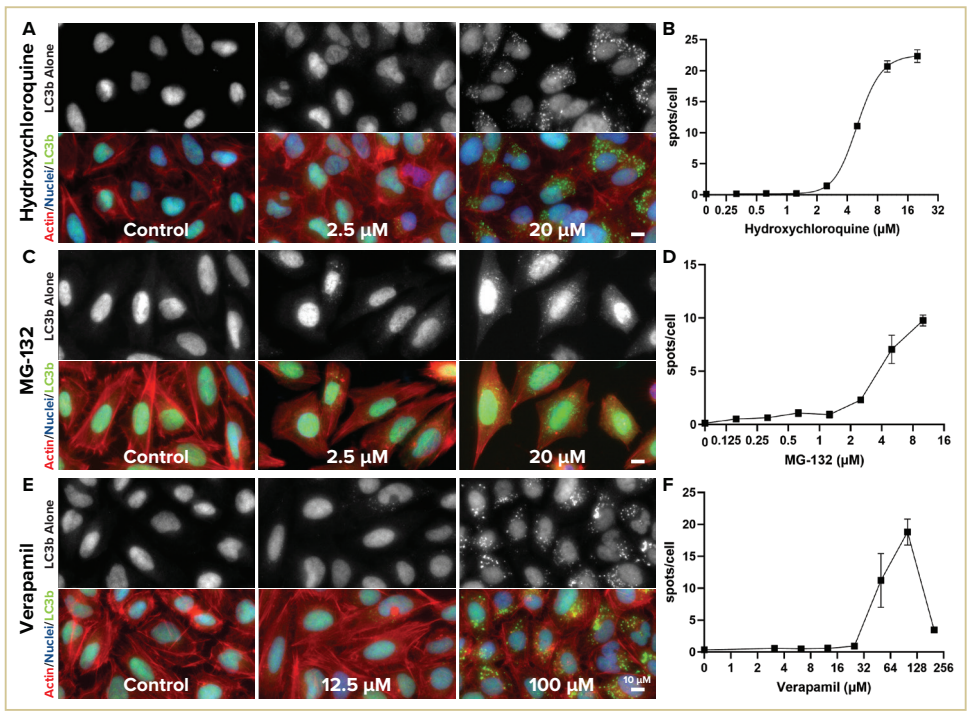


Figure 3: Immunohistological detection of Autophagic flux with LC3b antibody.

Autophagic flux consistently detected with both immunohistochemical **(Figure 3)** and live cell dye **(Figure 4)** assays. Representative images **(A, C, E)** of high, medium and control (no) dosages shown with autophagic vesicle staining (gray, upper panel) and merged with nuclear (both stainings) and actin counterstains (IHC only). Graphs of quantified staining **(B, D, F)** with nonlinear fit calculated for [hydroxy]chloroquine **(B)** and torin 1 **(4D)** with R2>0.98; EC50 for chloroquine is 5 µM in IHC and 3.5 µM for live dye experiment, Torin 1 EC50=0.1 µM (GraphPad Prism). Error bars +/- standard deviation. Verapamil **(3E, F)** is cytotoxic at high dosage.

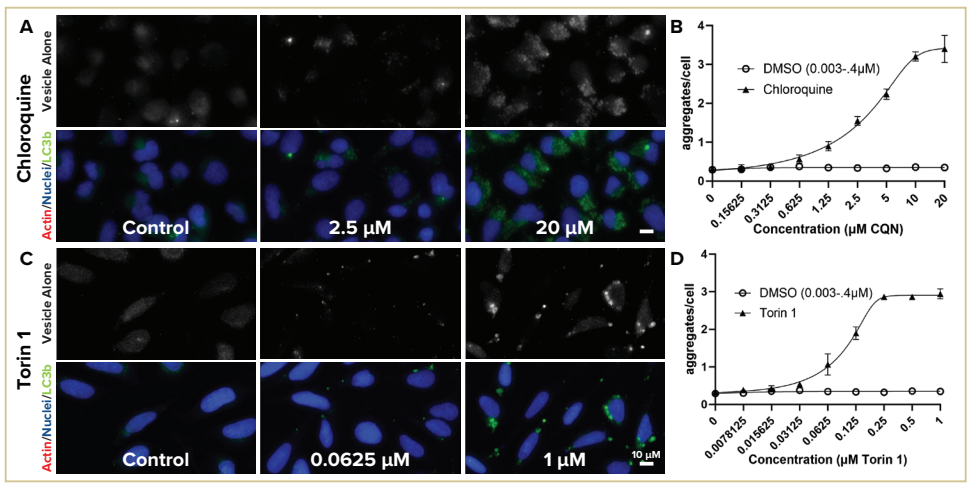
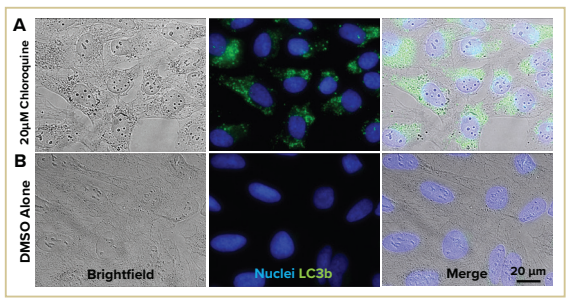


Figure 4: Detecting autophagic flux with live cell CAT dye.

Z' SCORE	Chloroquine	MGL132	Verapamil	Torin 1
LC3b IHC	0.850297	0.834196	0.632496	Not Tested
CytoID Live	0.63359	Not Tested	Not Tested	0.824062

Table 1: Z' from screen data (above) using maximal response; IHC uses hydroxychloroquine.

Figure 5: Morphological changes after autophagic flux visualized in brightfield. Adding only 2-3 minutes to a full well scan, changes in vesicle morphology and nucleoli visible in brightfield **(i)** after chloroquine treatment **(A)** compared to control **(B)**. Darker, larger vesicles seen in treatment group, corresponding to LC3b stain **(ii, iii)**.



Conclusion: High Throughput High Content Imaging at Submicron Resolution with Subcellular Analysis

- Autophagic flux reliably detected using distinct modulators in dilution
- Consistent Z'>0.5 indicates robust effect size, minimal variability
- Plate to results delivered in under 30 minutes
- Assay reliability and flexibility at speed: both immunohistochemistryand live cell dyes yield consistent results with similar EC50 values
- 0.27µm pixel size allows for spot detection at submicron scale
- Autophagic vesicles visible in brightfield, allowing label-free detection
- Assay broadly generalizable to other quantitative spot-based HCI assays such as: subcellular protein localization, plaque formation, aggregation, FISH, phagocytosis

References

Djajadikerta A, Keshri S, Pavel M, Prestil R, Ryan L, Rubinsztein DC. Autophagy Induction as a Therapeutic Strategy for Neurodegenerative Diseases. J Mol Biol. 2020 Apr 3;432(8):2799-2821. doi: 10.1016/j.jmb.2019.12.035.

Joachim J, Jiang M, McKnight NC, Howell M, Tooze SA. High-throughput screening approaches to identify regulators of mammalian autophagy. Methods. 2015 Mar;75:96-104. doi: 10.1016/j.ymeth.2015.02.002

Sharifi MN, Mowers EE, Drake LE, Macleod KF. Measuring autophagy in stressed cells. Methods Mol Biol. 2015;1292:129-50. doi: 10.1007/978-1-4939-2522-3_10.

Marinković M, Šprung M, Buljubašić M, Novak I. Autophagy Modulation in Cancer: Current Knowledge on Action and Therapy. Oxid Med Cell Longev. 2018 Jan 31;2018:8023821. doi: 10.1155/2018/8023821