Tools and protocols for high-content imaging and analysis

Integrating hardware, software, and fluorescent labels for optimized HCA assay development.

- Chambers KM, Mandavilli BS, Dolman NJ, Janes MS (2018) General staining and segmentation procedures for high-content imaging and analysis. *Methods Mol Biol* 1683:21–31.
- Mandavilli BS, Aggeler RJ, Chambers KM (2018) Tools to measure cell health and cytotoxicity using high-content imaging and analysis. *Methods Mol Biol* 1683:33–46.
- Mandavilli BS, Yan M, Clarke S (2018) Cell-based high-content analysis of cell proliferation and apoptosis. *Methods Mol Biol* 1683:47–57.
- Dolman NJ, Samson BA, Chambers KM, Janes MS, Mandavilli BS (2018) Tools to measure autophagy using high-content imaging and analysis. *Methods Mol Biol* 1683:59–71.

High-content imaging and analysis transforms fluorescence microscopy into a high-throughput, quantitative tool for investigating spatial and temporal aspects of cell biology [1]. Automation—not only of the image acquisition but also of the analysis—allows millions of cells to be analyzed and reveals the heterogeneity of responses that exist within cell populations. These cellular responses can then be assessed across a range of manipulations, whether they are genome-wide screens or small-molecule library analyses [2].

To achieve a seamless high-content workflow, automation is required at every step, from image capture to the hardware for scanning microtiter plates and integrating with robotic plate-handling systems. Furthermore, analysis of the acquired images must also be automated; with Thermo Scientific™ HCS Studio™ software, this analysis can occur immediately after capture, producing visible data outputs even as the microplate is scanned.

For those entering the field of high-content imaging, the hardware, software, and reagent considerations can be overwhelming. In a series of recently published reviews in *Methods in Molecular Biology*, Mandavilli and colleagues introduce the essential elements of the high-content imaging and analysis process [3-6]. These reviews provide direction for hardware considerations and software settings, as well as optimized protocols for labeling cells with fluorescent probes. A range of fluorescent probes are discussed, from dyes for labeling cell structures that underpin segmentation to reagents that form the basis of the most commonly used functional assays.

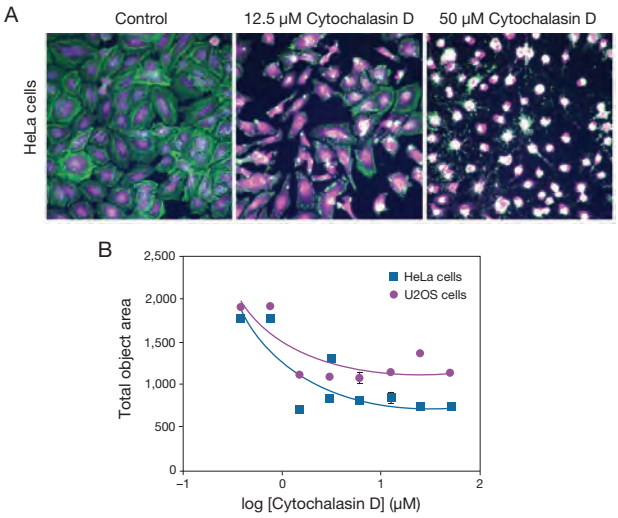

Figure 1. Cytochalasin D disrupts actin filaments and reduces the total area of actin in cells. (A) HeLa or U2OS (images not shown) cells were plated on a 96-well plate at a density of 5,000 cells/well. The cells were treated with different doses of cytochalasin D, from 0.375 µM to 50 µM, for 4 hr. The cells were then fixed, permeabilized, and stained with anti-tubulin antibody (using an Invitrogen™ Alexa Fluor™ 594 secondary antibody) and Invitrogen™ Alexa Fluor™ 488 phalloidin (Cat. No. A12379). After washing, the cells were stained with Invitrogen™ HCS CellMask™ Near-IR stain and Hoechst™ 33342 (Cat. No. H3570), imaged, and analyzed on a Thermo Scientific™ CellInsight™ CX7 LZR High-Content Analysis Platform using a 20x objective. The mean fiber area (actin) was plotted against the cytochalasin D dose.

Segmentation: The cornerstone of high content

High-content imaging and analysis provides automated quantitation of images captured on a fluorescence microscope. Even before the first image is acquired, the high-content analysis (HCA) system must recognize a cell in the field, and this recognition starts with segmentation. Segmentation—the identification of specific elements of the cell—can be achieved through imaging cells stained with fluorescent dyes that selectively label either the nucleus (e.g., Invitrogen™ HCS NuclearMask™ stains) or the entire cell (Invitrogen™ HCS CellMask™ stains). Once the nucleus or cell is recognized as an object to analyze, the software can quantify additional fluorescent reporters for various cellular processes. →

Segmentation based on nuclear labeling enables the HCA software to identify what is or is not a cell. Moreover, the central location of the nucleus within a cell allows cytoplasmic segmentation to be routinely performed without additional labels in the majority of cell types. Chambers et al. [3] provide protocols for labeling cells with the HCS NuclearMask stains, along with optimization approaches based on the types of algorithms selected within HCS Studio software (Table 1). While essential for segmentation, these probes can also offer insight into biological processes. For example, DNA-binding dyes can report DNA content, providing a basis for cell cycle analysis. In addition, the HCS CellMask stains can report changes in cell shape as a function of compound treatment (Figure 1).

HCA assays for cell health and cytotoxicity

One of the most common applications of high-content imaging and analysis is to provide a multiparametric readout of cell health and cytotoxicity. Overall viability assessed using Invitrogen™ LIVE/DEAD™ reagents can be combined with readouts requiring a spatial element, such as probes for mitochondrial membrane potential. Mandavilli et al. describe protocols and trouble-shooting steps for implementing multiparametric probe sets for viability and mitochondrial health, as well as fluorescent probes for determining reactive oxygen species and phospholipidosis/steatosis within cells [4]. For example, the Invitrogen™ HCS Mitochondrial Health Kit provides the reagents required for simultaneous measurement of cell number (blue-fluorescent dye), mitochondrial membrane potential (orange-fluorescent dye), and cell viability (green-fluorescent dye). This approach provides important data when screening for compound toxicity, and even prelethal toxicity. Loss of mitochondrial membrane potential is a common precursor of cell death and is therefore a useful indicator of drug cytotoxicity.

Naturally, some studies require additional assessment of cytotoxicity, and for this reason Mandavilli et al. also provide a detailed discussion regarding the use of Invitrogen™ CellROX™ reagents and Invitrogen™ HCS LipidTox™ stains for measuring reactive oxygen species generation and phospholipidosis/steatosis, respectively, in high-content imaging and analysis applications [4].

Table 1. Nuclear, whole cell, and plasma membrane stains for segmentation.

Segmentation tool	Ex/Em (nm)	Target	Cat. No.
HCS NuclearMask Blue stain	350/461	Nucleus	H10325
HCS NuclearMask Red stain	622/645	Nucleus	H10326
HCS NuclearMask Deep Red stain	638/686	Nucleus	H10294
Hoechst 33342 dye	350/461	Nucleus	H3570
HCS CellMask Blue stain	346/442	Whole cell	H32720
HCS CellMask Green stain	493/516	Whole cell	H32714
HCS CellMask Orange stain	556/572	Whole cell	H32713
HCS CellMask Red stain	588/612	Whole cell	H32712
HCS CellMask Deep Red stain	650/655	Whole cell	H32721
CellTracker Blue CMAC stain	353/466	Whole cell	C2110
CellTracker Blue CMF_2HC stain	371/464	Whole cell	C12881
CellTracker Blue CMHC stain	372/470	Whole cell	C2111
CellTracker Violet BMQC stain	415/516	Whole cell	C10094
CellTracker Green CMFDA stain	492/517	Whole cell	C7025
CellTracker Green BODIPY stain	522/529	Whole cell	C2102
CellTracker Orange CMTMR stain	541/565	Whole cell	C2927
CellTracker Orange CMRA stain	548/576	Whole cell	C34551
CellTracker Red CMTPX stain	577/602	Whole cell	C34552
CellTracker Deep Red stain	630/660	Whole cell	C34565
CellMask Green plasma membrane stain	522/535	Plasma membrane	C37608
CellMask Orange plasma membrane stain	554/567	Plasma membrane	C10045
CellMask Deep Red plasma membrane stain	649/666	Plasma membrane	C10046

HCA assays for cell proliferation, apoptosis, and autophagy

In concert with a suite of advanced fluorescent probes, high-content imaging and analysis can be used to explore mechanistic aspects of cell health, including cell proliferation, apoptosis [5], and autophagy [6]. Proliferation and apoptosis are two key readouts for assessing cell health and are included in established probe combinations for measuring cytotoxicity during compound development and screening [7].

Mandavilli et al. describe the use of a fluorogenic apoptosis probe, Invitrogen™ CellEvent™ Caspase-3/7 Green Reagent, to monitor the early stages of apoptosis [5]. The CellEvent reagent comprises the DEVD peptide—which contains the recognition site for caspase-3 and -7—conjugated to a nucleic acid–binding dye. Because the DEVD peptide inhibits the ability of the dye to bind DNA, CellEvent Caspase-3/7 Green Reagent is intrinsically nonfluorescent. In the presence of activated caspase-3/7, the dye is cleaved from the DEVD peptide and free to bind DNA, producing a bright green-fluorescent signal

(fluorescence emission maximum ~520 nm) indicative of apoptosis (Figure 2A). An important advantage of a fluorogenic probe is that cells do not need to be washed (to remove unbound or unincorporated probe) following incubation. This protocol simplification not only allows preservation of the entire apoptotic population, including fragile cells, which can be lost during wash steps, but also facilitates time-lapse imaging studies, as cells can be imaged in the presence of the probe.

In addition, HCA protocols are described for measuring cell proliferation by labeling cells with the thymidine analog 5-ethynl-2′-deoxyuridine (EdU) and subsequent detection by click chemistry of EdU incorporated into newly synthesized DNA [5], as well as for monitoring the induction of autophagy by immunolabeling cells with an antibody to the autophagosomal marker LC3B [6]. Autophagy is a key pro-survival mechanism implicated in a variety of disease states, including lysosomal storage disorders, neurodegenerative diseases, cancers, and Parkinson's disease [8]. Dolman et al. describe protocols for immunolabeling cells and quantifying LC3B-positive puncta that appear after either blockade of autophagic flux with compounds such as chloroquine or bafilomycin A1, or induction of autophagy through nutrient deprivation or mTOR inhibition (Figure 2B) [6]. Approaches for validating the specificity of autophagosomal labeling using genetic knockout of critical autophagy genes (by CRISPR-Cas9 genome editing) are discussed.

More content about high content

High-content imaging and analysis instruments from Thermo Fisher Scientific—which include the Thermo Scientific™ CellInsight™ CX5 and CellInsight™ CX7 platforms—build on a 20-year legacy of HCA instrument and software development. To find out more about our high-content instrument platforms, software, applications, and analysis reagents, or to request an in-lab demonstration of one of our HCA instruments, visit thermofisher.com/hcabp77. ■

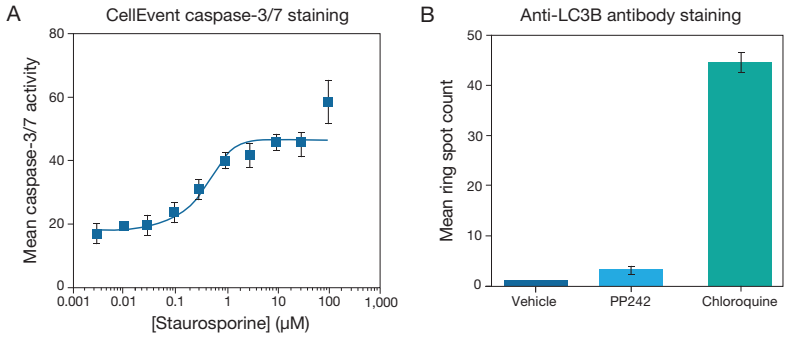

Figure 2. High-content analysis of apoptosis and autophagy. (A) U2OS cells were treated with a range of staurosporine concentrations for 4 hr and then labeled with Hoechst™ 33342 (Cat. No. H3570) and Invitrogen™ CellEvent™ Caspase-3/7 Green Detection Reagent (Cat. No. C10423). The fluorogenic CellEvent reagent reports a dose-dependent increase in induction of apoptosis. **(B)** U2OS cells were treated with either 20 µM chloroquine (to block autophagic flux) or 1 µM PP242 (to stimulate autophagy through mTOR inhibition) and subsequently processed for immunocytochemistry using an antibody against the autophagosomal marker LC3B. Both induction of autophagy and blockade of flux cause a significant increase in the number of autophagosomes detected (LC3B spots). Analysis was carried out using a Thermo Scientific™ CellInsight™ CX5 High-Content Screening Platform.

References

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3. Chambers KM, Mandavilli BS, Dolman NJ et al. (2018) *Methods Mol Biol* 1683:21–31.
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5. Mandavilli BS, Yan M, Clarke S (2018) *Methods Mol Biol* 1683:47–57.
6. Dolman NJ, Samson BA, Chambers KM et al. (2018) *Methods Mol Biol* 1683:59–71.
7. Towne DL, Nicholl EE, Comess KM et al. (2012) *J Biomol Screen* 17:1005–1017.
8. Jiang P, Mizushima N (2014) *Cell Res* 24:69–79.

Product	Quantity	Cat. No.
CellInsight™ CX5 High-Content Screening Platform	1 each	CX51110
CellInsight™ CX7 High-Content Analysis Platform	1 each	CX7A1110
CellInsight™ CX7 LZR High-Content Analysis Platform	1 each	CX7A1110LZR
CellEvent™ Caspase-3/7 Green Detection Reagent	100 µL	C10423
CellROX™ Green Reagent	5 x 50 µL	C10444
CellROX™ Orange Reagent	5 x 50 µL	C10443
CellROX™ Deep Red Reagent	5 x 50 µL	C10422
CellROX™ Reagent Variety Pack	1 kit	C10448
Click-iT™ EdU Alexa Fluor™ 488 HCS Assay	2 x 96-well plates	C10350
Click-iT™ EdU Alexa Fluor™ 555 HCS Assay	2 x 96-well plates	C10352
Click-iT™ EdU Alexa Fluor™ 594 HCS Assay	2 x 96-well plates	C10354
Click-iT™ EdU Alexa Fluor™ 647 HCS Assay	2 x 96-well plates	C10356
HCS LipidTOX™ Phospholipidosis and Steatosis Detection Kit	1,200 assays	H34158
HCS LipidTOX™ Green Phospholipidosis Detection Reagent	10 x 96-well plates	H34350
HCS LipidTOX™ Red Phospholipidosis Detection Reagent	10 x 96-well plates	H34351
HCS LIVE/DEAD™ Green Kit	1 kit	H10290
HCS Mitochondrial Health Kit	1 kit	H10295