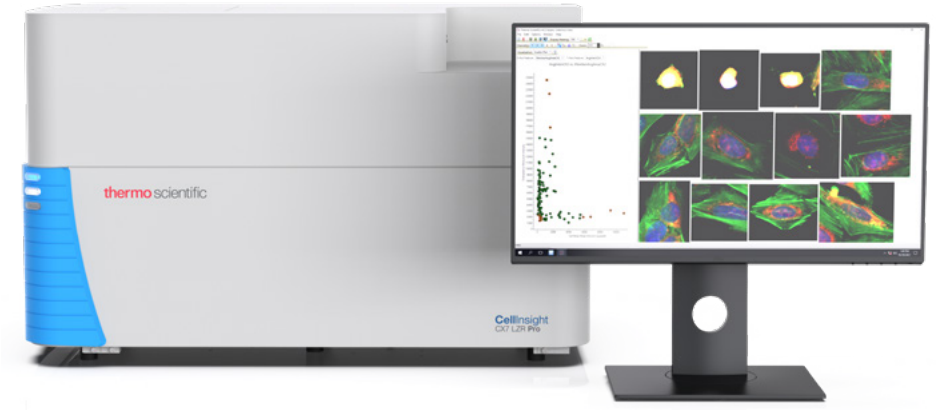
Cell analysis

Cell painting high-content screening assay

Keywords
Cell painting, morphological profiling, multiparameter screening, high-content screening, CellInsight CX7 Pro, CellInsight CX7 LZR Pro, Image-iT Cell Painting Kit



History and introduction

Cell painting was invented by Anne Carpenter and colleagues from the Broad Institute to provide an image-based profiling tool amendable to the development of drug discovery assays and multiparameter screening campaigns. As originally described by Carpenter and colleagues, approximately 1,500 measurements can be extracted from each cell based on changes in size, shape, texture, and fluorescence intensity [1]. These measurements can be used to detect subtle changes in phenotype.

The power of cell painting as a phenotypic measuring tool is best characterized by its comprehensive ability to extract data-rich information gained from studying multiparametric cellular biology at the cytoskeletal, plasma membrane, and organelle levels. In contrast, conventional high-throughput screening applications evaluate only a few measurements for the purposes of scale, and have limited breadth to detect diverse phenotypic changes caused by compound exposure. While morphological changes to cellular structure are central to cell painting, several organelle-based measurements are also evaluated, including those of the nucleus, nucleolus, endoplasmic reticulum, Golgi apparatus, and mitochondria. Single-cell measurements can be extracted from this imaging-based technology, enabling the identification of cells most sensitive to compound exposure relative to the overall population. While originally adopted for drug discovery applications [2,3], cell painting has since expanded its utility to drug safety [4], environmental toxicology [5], and multiomics applications that include the prediction of lung cancer variants [6]. Cell painting assays can enable identification of hits in initial discovery screens and can provide mechanistic insights unavailable through traditional high-throughput assays.

Cell painting methods

Cell painting multiparameter overview

The multiplex panel for cell painting consists of eight organelle and morphological markers. These include:

- Invitrogen™ Hoechst™ 34580 dye to label the nucleus
- Invitrogen™ Concanavalin A, Alexa Fluor™ 488 Conjugate, to label the endoplasmic reticulum
- Invitrogen™ SYTO™ 14 Green Fluorescent Nucleic Acid Stain to label both the nucleoli and the cytoplasmic RNA
- Invitrogen™ Wheat Germ Agglutinin (WGA), Alexa Fluor™ 555 Conjugate, to label both the Golgi apparatus and the plasma membrane
- Invitrogen™ Alexa Fluor™ 568 Phalloidin to label the actin cytoskeleton
- Invitrogen™ MitoTracker™ Deep Red FM Dye to label the mitochondria and measure the mitochondrial membrane potential proportional to intensity

Collectively, this panel requires an image acquisition configuration of at least five fluorescence channels to capture the eight markers. Figure 1 shows a representative cell painting image of U2OS cells labeled with the Invitrogen™ Image-iT™ Cell Painting Kit, acquired using the Thermo Scientific™ CellInsight™ CX7 LZR Pro instrument with a 20x 0.7 NA objective.

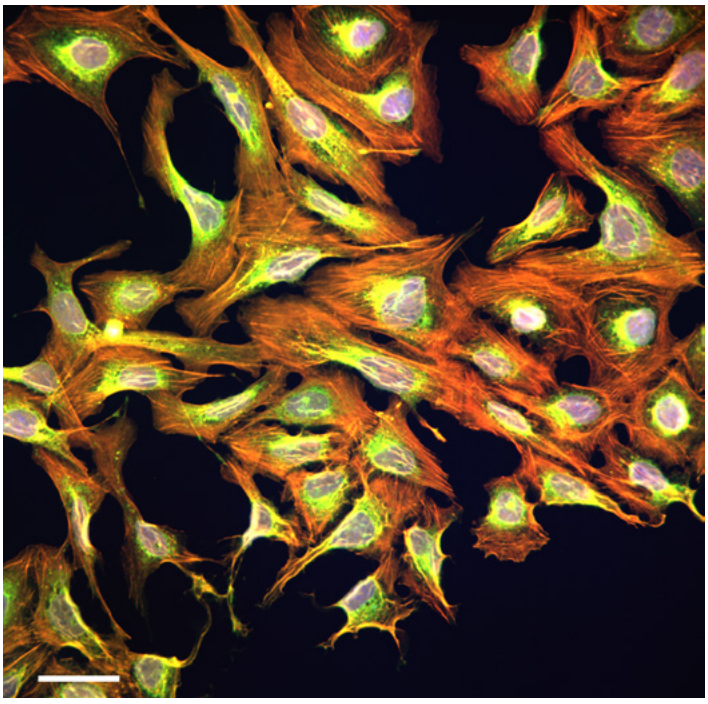


Figure 1. Cell painting image of U2OS cells obtained using the CellInsight CX7 LZR Pro instrument. The following markers were labeled using the Image-iT Cell Painting Kit: nucleus (channel 1); endoplasmic reticulum (channel 2); nucleoli and cytoplasmic RNA (channel 3); actin, Golgi apparatus, and plasma membrane (channel 4); and mitochondria (channel 5). The resulting red, green, and blue (RGB) image of all 5 channels is shown with pseudocoloring.

To help accelerate development of the cell painting assay, we have recently released the Image-iT Cell Painting Kit in conjunction with the Cell Painting Assay in the Thermo Scientific™ HCS Studio™ 5.0 Cell Analysis Software.

Labeling with the Image-iT Cell Painting Kit

Prepare stock solutions:

- SYTO 14 Green Fluorescent Nucleic Acid Stain is supplied as a 5 mM solution (1,666X) and does not require preparation.
- Dissolve 5 mg of Hoechst 34580 dye in 5 mL of ddH_2O to make a 1 mg/mL solution (2,000X).
- Dissolve 5 mg of Concanavalin A, Alexa Fluor 488 Conjugate, in 1 mL of 0.1 M sodium bicarbonate (~pH 8.3) to make a 5 mg/mL solution (50X).
- Dissolve 5 mg of Wheat Germ Agglutinin, Alexa Fluor 555 Conjugate, in 5 mL of ddH_2O to make a 1 mg/mL solution (666X).
- Dissolve 300 units of Alexa Fluor 568 Phalloidin in 150 µL of dimethyl sulfoxide (DMSO) to make a 66 µM solution (400X).
- Dissolve the contents of one vial of MitoTracker Deep Red FM Dye in 91.98 µL of DMSO to make a 1 mM solution (2,000X).

Label cells:

- Seed cells into a 384-well plate in growth medium (40 µL cell suspension/well) at a density of 1,000 cells/well. For 96-well plates, use a 100 µL cell suspension/well at a density of 4,000 cells/well.
- Incubate the plate for 60 minutes at room temperature, followed by incubation for ~20 hours at 37°C, 5% CO_2, and ~95% relative humidity to allow overnight recovery and growth of the plated cells.
  Note: These conditions are adapted for U2OS cells. For other cell types, the conditions can be modified for optimal results.
- On the following day, prepare 10X test compounds in DMSO and dispense at 1:10 dilution to each well to achieve a 10 µM assay concentration, then incubate the plates for 24–48 hours at 37°C, 5% CO_2, and ~95% relative humidity. To avoid DMSO vehicle effects, be sure to keep the final DMSO concentration below 0.5%. **Important:** Do not remove the growth medium.
- Prepare a 10X MitoTracker Deep Red staining solution by adding 50 µL of the 1 mM stock solution to 10 mL of complete medium. Dispense this medium at a 1:10 dilution to each well to achieve a final concentration of 0.5 µM.
- Incubate the plate for 30 minutes at 37°C, 5% CO_2, and 95% relative humidity.
- Prepare an 8% paraformaldehyde (PFA) fixation solution (without methanol), and dispense a volume equal to that already in each well, to achieve a final concentration of 4% PFA.

- Incubate the plate for 15 minutes at room temperature.
- Remove the supernatant and replace it with Invitrogen™ Triton™ X-100 (1%) (Cat. No. HFH10), diluted to a working concentration of 0.1%.
- Incubate the plate for 15 minutes at room temperature.
- Remove the supernatant and replace it with the Alexa Fluor 568 phalloidin, concanavalin A, Hoechst 34580, wheat germ agglutinin, and SYTO 14 staining solutions diluted in 1X Hanks' Balanced Salt Solution (HBSS) + 1% bovine serum albumin (BSA) according to Table 1.
- Incubate the plate for 30 minutes at room temperature.
- Discard the solution and wash the wells twice with 1X HBSS.
- Fill the wells with 1X HBSS/0.05% sodium azide to prevent bacterial growth.
- Tightly seal the plate with an adhesive seal. The plate is now ready for screening.

CellInsight CX7 LZR Pro platform and cell painting application configuration

The cell painting application has been developed for the Thermo Scientific™ CellInsight™ CX7 Pro series instruments, including the light-emitting diode (LED)-based CellInsight™ CX7 Pro instrument and the laser-based CellInsight CX7 LZR Pro instrument. Image acquisition on either instrument is performed using the back-illuminated CMOS camera for optimum detection capability of the 8-target screen. Note that the CellInsight CX7 LZR Pro instrument will provide superior fluorescence specificity due to the nature of laser fluorescence compared to an LED. Scanning is completed in an automated format, including laser-based autofocusing performed on every well. Both instruments also leverage the real-time analysis capabilities of HCS Studio software that enable simultaneous image acquisition and analysis; a sufficient number of fields are acquired using the 20x objective so that at least 500 cells are measured. Additional cells can be monitored by increasing the "intra well stop" criterion to the desired number.

Quantitative analysis of the cell painting image sets is conducted using the Thermo Scientific™ Cellomics™ Compartmental Analysis bioapplication designed to extract measurements based on both "circ" (nuclear) and "ring" (cytoplasmic to cell membrane) regions, including puncta spot–level detection. An overview of the circ and ring regions of interest, including how spot puncta are determined in each region, is shown in Figure 2.

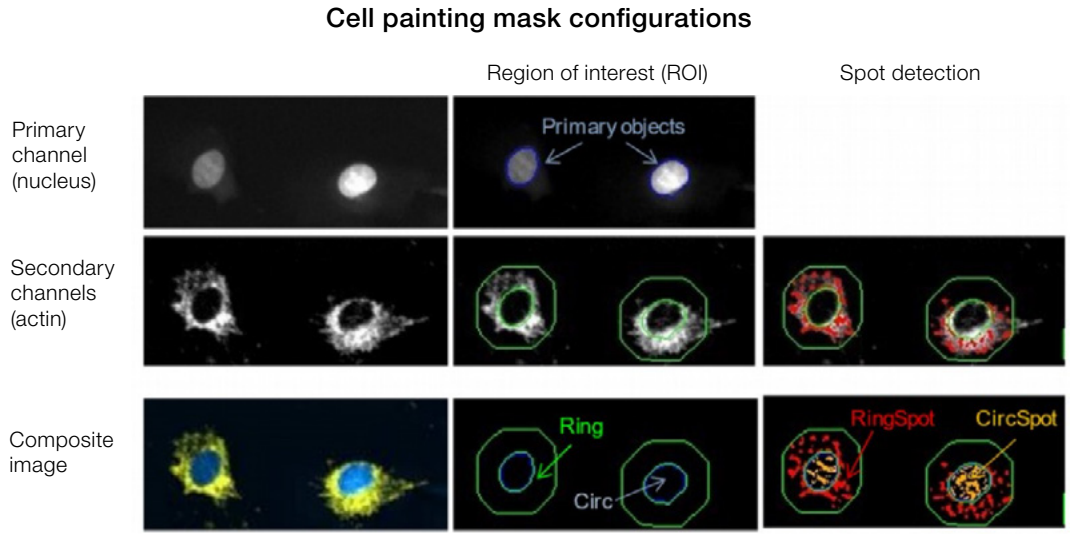


Figure 2. An overview of the circ and ring regions of interest, including how spot puncta are determined in each region.

Table 1. Concentration of staining solutions for cell painting assay.

	Hoechst 34580 dye (1 mg/mL)	Concanavalin A, Alexa Fluor 488 conjugate (5 mg/mL)	SYTO 14 stain (5 mM)	Wheat germ agglutinin, Alexa Fluor 555 conjugate (1 mg/mL)	Alexa Fluor 568 phalloidin (66 µM)
Dilution from stock solution	1:2,000	1:50	1:1,666	1:666	1:400
Final concentration in well	0.5 µg/mL	0.1 mg/mL	3 µM	1.5 µg/mL	165 nM
Diluent buffer	1X HBSS + 1% BSA				

Total cell number and nuclear measurements, including nuclear area, shape, texture, and nuclear intensity, are derived from channel 1. Channel 2 is compartmentalized into nuclear and cytoplasmic regions of interest, such that RNA puncta quantifications (spot area, intensity) are measured within the nuclear and perinuclear regions, while the endoplasmic reticulum organelle measurements are derived from the cytoplasmic ring region of interest. Similarly, channel 3 is also compartmentalized into cytoplasmic and outer whole-cell regions, such that the Golgi apparatus is measured within the cytoplasm only and the cell membrane is measured over the outer whole-cell region. Channel 4 is used to image and analyze the mitochondrial membrane potential and number of puncta within the cytoplasm. Channel 5 is used to image and analyze the actin whole-cell marker. Quality control is conducted on all detected U2OS cells to help ensure that accurate single cell–level data are collected. Time-to-data estimates using these protocols is approximately 4 minutes per 96-well plate using the CellInsight CX7 LZR Pro instrument, and 6 minutes per 96-well plate for the CellInsight CX7 Pro instrument. Representative cell painting primary endpoints are given in Tables 2–5.

Representative cell painting primary endpoints

Measurement	Cell painting compartmental analysis endpoint
Total cell number	Valid object count
Nuclear area	Mean_ObjectAreaCh1
Nuclear shape	Mean_ObjectShapeP2ACh1 Mean_ObjectShareLWRCh1 Mean_ObjectSizeCh1
Nuclear intensity	Mean_ObjectTotalIntenCh1 Mean_ObjectAvgIntenCh1
Nuclear texture	Mean_ObjectVarIntenCh1
Nucleoli + perinuclear RNA ROI intensity	Mean_CircTotalIntenCh2 Mean_CircAvgIntenCh2
Nucleoli + perinuclear RNA puncta count	Mean_CircSpotCountCh2
Nucleoli + perinuclear RNA puncta area	Mean_CircSpotTotalAreaCh2 Mean_CircSpotAvgAreaCh2
Nucleoli + perinuclear RNA puncta intensity	Mean_CircSpotTotalIntenCh2 Mean_CircSpotAvgIntenCh2

Measurement	Cell painting compartmental analysis endpoint
Endoplasmic reticulum cytoplasmic ROI intensity	Mean_RingTotalIntenCh2 Mean_RingAvgIntenCh2
Endoplasmic reticulum puncta cytoplasmic count	Mean_RingSpotCountCh2
Endoplasmic reticulum puncta cytoplasmic area	Mean_RingSpotTotalAreaCh2 Mean_RingSpotAvgAreaCh2
Endoplasmic reticulum puncta cytoplasmic intensity	Mean_RingSpotTotalIntenCh2 Mean_RingSpotAvgIntenCh2
Golgi apparatus cytoplasmic ROI intensity	Mean_CircTotalIntenCh3 Mean_CircAvgIntenCh3
Golgi apparatus puncta count	Mean_CircSpotCountCh3
Golgi apparatus puncta area	Mean_CircSpotTotalAreaCh3 Mean_CircSpotAvgAreaCh3
Golgi apparatus puncta intensity	Mean_CircSpotTotalIntenCh3 Mean_CircSpotAvgIntenCh3

Measurement	Cell painting compartmental analysis endpoint
Plasma membrane ROI intensity	Mean_RingTotalIntenCh3 Mean_RingAvgIntenCh3
Plasma membrane ROI puncta count	Mean_RingSpotCountCh3
Plasma membrane ROI puncta area	Mean_RingSpotTotalAreaCh3 Mean_RingSpotAvgAreaCh3
Plasma membrane ROI puncta intensity	Mean_RingSpotTotalIntenCh3 Mean_RingSpotAvgIntenCh3
Mitochondrial membrane potential intensity	Mean_RingTotalIntenCh4 Mean_RingAvgIntenCh4
Mitochondrial organelle puncta count	Mean_RingSpotCountCh4
Mitochondrial organelle puncta area	Mean_RingSpotTotalAreaCh4 Mean_RingSpotAvgAreaCh4
Mitochondrial organelle puncta intensity	Mean_RingSpotTotalIntenCh4 Mean_RingSpotAvgIntenCh4

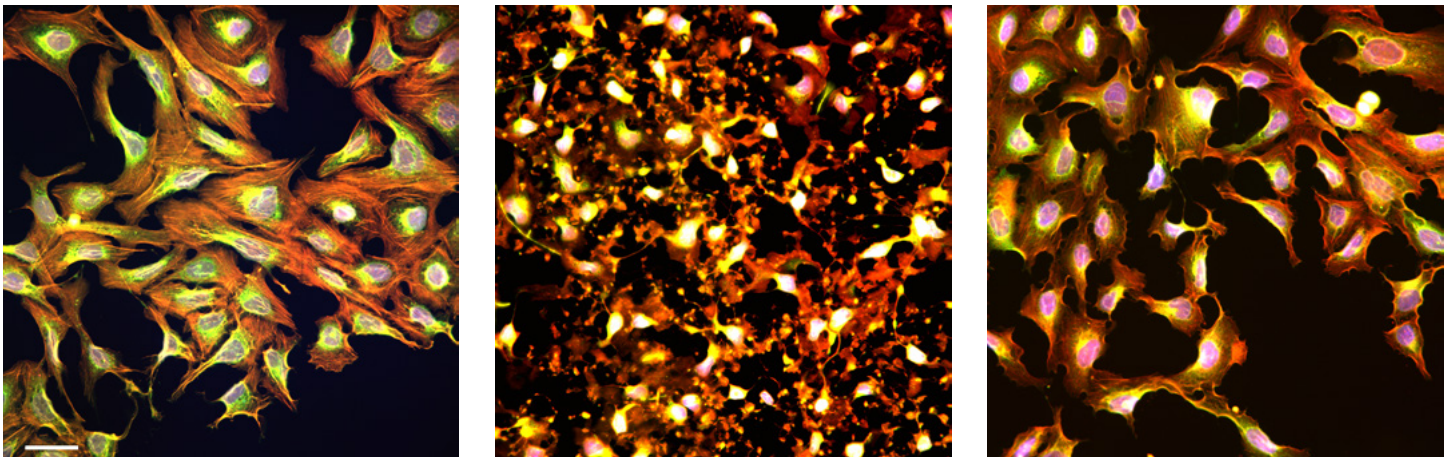
Measurement	Cell painting compartmental analysis endpoint
Actin whole cell ROI intensity	Mean_RingTotalIntenCh5 Mean_RingAvgIntenCh5
Actin whole cell ROI puncta count	Mean_RingSpotCountCh5
Actin whole cell ROI puncta area	Mean_RingSpotTotalAreaCh5 Mean_RingSpotAvgAreaCh5
Actin whole cell ROI puncta intensity	Mean_RingSpotTotalIntenCh5 Mean_RingSpotAvgIntenCh5

Cell painting results

Conventional cell painting results in U2OS cells

The data generated from the cell painting application can be evaluated at the cell population level as mean averages, or the single-cell measurements of every validated U2OS cell can be evaluated. For additional quality control considerations, the cell-level cutouts of each cell displayed on the scatterplot can be shown. Phenotypic changes in untreated vs. pharmacologically treated controls are displayed by cell painting in Figure 3.

Figure 4 is a representative example displaying fluorescence intensity of the endoplasmic reticulum compared to area measurements of the cellular body as calculated by the distribution of actin filaments. Note: Connection to a Thermo Scientific™ Store database is needed for the displayed cell-level cutout QC functionality.



Untreated | 10 µM staurosporine | 1 µM Taxol

Figure 3. Phenotype comparisons of untreated vs. pharmacological control exposure in U2OS cells. Cells were treated with compounds of interest at 1–100 µM final concentrations for 48 hours in 96-well imaging plates. After treatment with the compounds, cells were immediately labeled using the Image-iT Cell Painting Kit and analyzed using the CellInsight CX7 LZR Pro instrument.

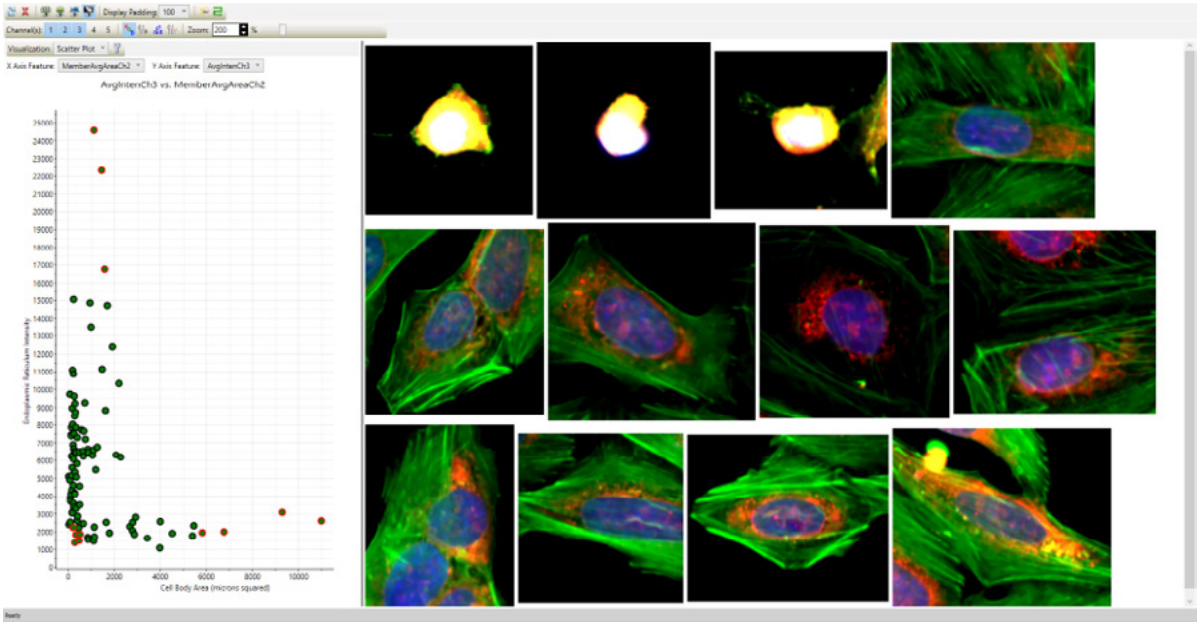


Figure 4. Cell painting for single cell–level assessment of responses. U2OS cells were seeded at 2,500 cells/well in a 96-well plate format. Two hours after cell adherence, the cells were exposed to either the 0.1% DMSO vehicle or pharmacological positive controls. The cells were immediately labeled using the using the Image-iT Cell Painting Kit and analyzed with the CellInsight CX7 LZR Pro instrument.

Customizations to cell painting

The cell painting assay can be customized to accommodate a different range of targets or to accommodate a different type of cell. An example of such a customization is shown in Figure 5 for cardiomyocytes instead of U2OS cells. In this case, cell number, nuclear area, cardiomyocyte cell body hypertrophy, mitochondrial membrane potential, cytochrome c release indicative of apoptosis, phosphorylation of p53 within the nucleus for DNA damage, dihydroethidium oxidation for detection of reactive oxygen species (ROS), and fatty acid accumulation for steatosis were measured, and the resulting population-level data were reported. All compounds were screened in a 12-point dose response curve in triplicate. Best-fit curves were calculated, and the resulting IC_{50} values for potency considerations were reported.

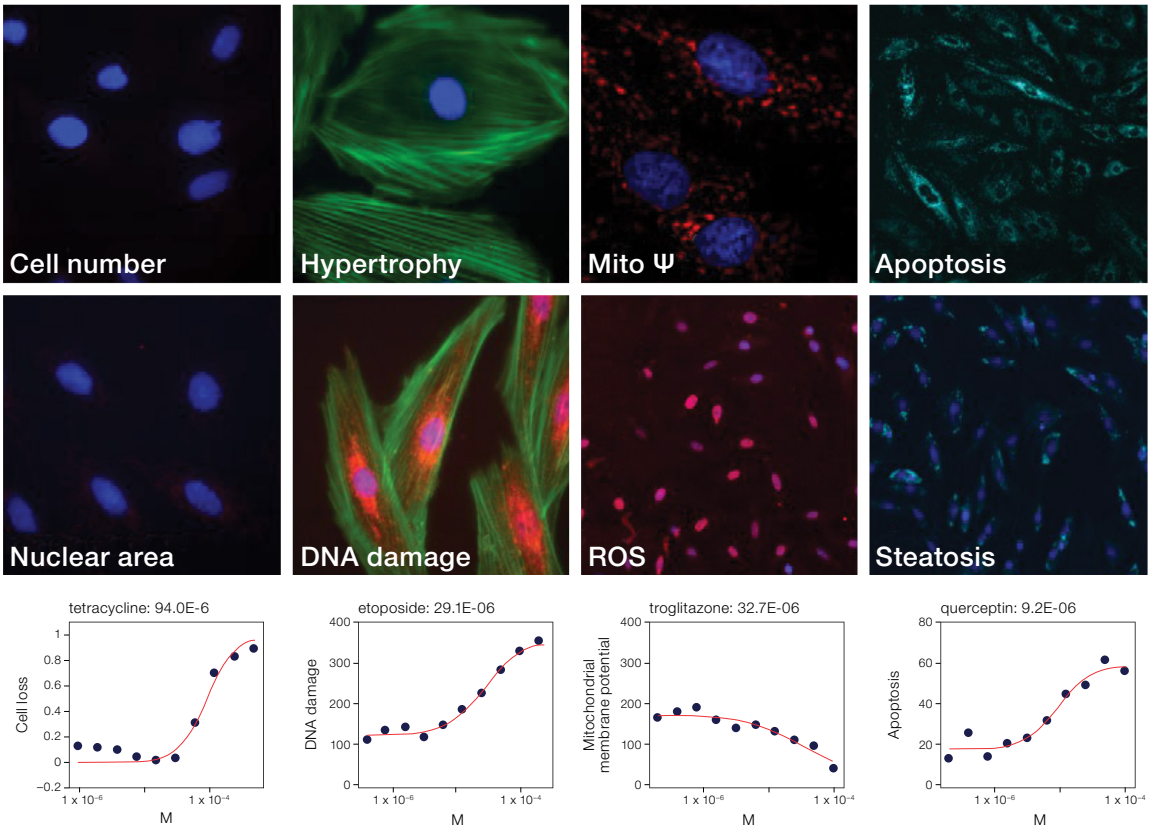


Figure 5. Evaluation of cardiomyocyte mechanism of action. H9c2 cells were seeded at 2,000 cells per well in 96-well plates. Two hours after seeding, the cells were treated with the listed compounds for 4, 24, or 96 hours before immunofluorescent labeling and analysis using the Cellomics Compartmental Analysis bioapplication.

Conclusions

The CellInsight CX7 LZR Pro instrument powered by HCS Studio 5.0 Cell Analysis Software and the Image-iT Cell Painting Kit are preconfigured for multiparameter cell painting to help enable the development of next-generation drug discovery and safety mechanism of actions. Cell painting using the CellInsight CX7 Pro series and HCS Studio 5.0 software allows you to collect all five channels of data required by the cell painting assay while using either software or laser autofocusing, to populate all image collection settings and analysis parameters, and to collect images and analyze your data in parallel for the fastest available time-to-data, simplifying your cell painting workflow and supercharging your data.

Ordering information

Description	Quantity	Cat. No.
CellInsight CX7 Pro HCS Platform	1 instrument	HCSDCX7LEDPRO
CellInsight CX7 LZR Pro HCS Platform	1 instrument	HCSDCX7LZRPRO
Image-iT Cell Painting Kit	1 kit	I64000
Nunc Microwell 96-Well Optical-Bottom Plate	Pack of 10	165305
Nunc 384-Well Optical-Bottom Plate	Pack of 10	142761

References

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