General Guidelines and Protocol

General Guidelines

ChromaLive dye dilution and preparation

- Warm up the *ChromaLive* dye tube to room temperature before use to avoid condensation to form and water to get into the anhydrous dye solution
- Gently spin the tube before use to collect any dye solution that may remain near the cap
- Dilute *ChromaLive* dye in preferred culture medium (1:1000 the provided solution). Vortex thoroughly.

Cell culture protocol and compound testing, with ChromaLive dye

- Seed cells at desired density (typically to achieve 70-80% confluence) on imaging support, in previously prepared culture medium with *ChromaLive* dye
- Add control compounds, test compounds and negative controls for phenotypes of interest.
- NOTE: *ChromaLive* dye can also be added after cell seeding and compound addition. In that case, incubate dye for at least 3h at 37°C, 5% CO_2 before imaging.

Imaging

- NOTE: Keep *ChromaLive* dye in solution while imaging, no need for a washing step.
- NOTE: Nuclear staining can be added for cell segmentation during image analysis. Check manufacturer's guidelines.
- Imaging conditions: the supplied *ChromaLive* dye needs to be imaged at 3 different wavelengths which can be identified in the following manners:
  - ChromaLive488_Yellow: excitation at 488nm, image acquisition between 550-630nm
  - ChromaLive488_Red: excitation at 488nm, image acquisition between 630-750nm
  - ChromaLive561: excitation at 561nm, image acquisition between 590-630nm
- NOTE: more information regarding imaging and filter sets is available at info@saguarobio.com, where our team can check set-up compatibility.

Standard Protocol (for kinetic, 2D, live-cell assay)

MCF-7 cells, with standard compounds for apoptosis, necroptosis, ER stress and autophagy

ChromaLive dye dilution and preparation (day0):

- Warm up the *ChromaLive* dye tube to room temperature before use to avoid condensation to form and water to get into the anhydrous dye solution
- Gently spin the tube before use to collect any dye solution that may remain near the cap
- Dilute 10µL *ChromaLive* dye in 10mL culture medium (1:1000 the provided solution). Vortex thoroughly.
- NOTE: Culture medium here is RPMI 1640 complemented with 10% FBS and 1% Penicillin/Streptomycin

Cell culture protocol with ChromaLive dye (day0):

- Harvest MCF-7 cells and resuspend in prepared culture medium with *ChromaLive* dye at 80,000 cells / mL
- Seed 96 well plate with 100µL cell suspension per well, for a final amount of 8,000 cells per well
- Incubate overnight at 37°C, 5% CO_2.

Hoechst labeling of nucleus, for cell segmentation (day1)

- OPTIONAL: Dilute Hoechst 33342 solution at 1µg/mL in culture medium, add 12.5µL per well for a final concentration of 100ng/mL. Incubate for at least 3h, at 37°C, 5% CO_2, before imaging.

Compound preparation and testing (day1)

- Prepare dose response curves with 10x concentrations, maintaining constant vehicle solvent concentration
- Prepare negative controls with vehicle solvent (here, 0.1% DMSO)
- Distribute 12.5µL of test compounds or controls per well.

Imaging and data acquisition (day1-3)

- Image 96 well plate at 3h, 6h, 24h and 48h after addition of test compounds

Materials:

- 96-well plate: Greiner Bio-One Black µClear 96-well cell-culture treated plate, ref: 655090
- Nuclear stain: Hoechst 33342 (Invitrogen, ref: H1399)
- Vehicle: DMSO (Fisher BioReagents, ref: BP231-100)
- Culture medium: RPMI 1640 (ATCC modified) (Gibco, ref: A1049101), complemented with 10% FBS and 1% Penicillin/Streptomycin

Additional Information

Acquisition channels and examples of filter settings

- ChromaLive488_Yellow
  - Excitation: 488nm laser OR 475/34nm excitation filter
  - Acquisition: 593/40nm emission filter
- ChromaLive488_Red
  - Excitation: 488nm laser OR 475/34nm excitation filter
  - Acquisition: 692/40nm emission filter
- ChromaLive561
  - Excitation: 561 laser OR 560/32nm excitation filter
  - Acquisition: 593/40nm emission filter
- Optional: DAPI
  - Excitation: 405nm laser OR 377/54nm excitation filter
  - Acquisition: 447/60nm emission filter

Control Compound Examples (doses and duration for MCF-7 cells)

Cell death mechanism	Control compounds	End-point	Time points
Apoptosis	TNF-α (3ng/mL), with Cycloheximide (3µg/mL) Actinomycin D (10ng/mL) Staurosporine (100nM)	TNF-α + Cycloheximide: 12h (to be confirmed) Actinomycin D: 48h Staurosporine: 24h	3h, 6h, 12h, 24h 3h, 6h, 12h, 24h, 48h 3h, 6h, 12h, 24h
ER stress	Tunicamycin (200ng/mL) Thapsigargin (1µM)	Tunicamycin: 24h Thapsigargin: 12h (to be confirmed)	3h, 6h, 12h, 24h
Autophagy	Rapamycin (1µM)	Rapamycin: 24h	3h, 6h, 12h, 24h

*Only provided as examples. Controls require validation.
**Images could be collected more frequently with the appropriate equipment, especially for time-lapse imaging (controlled temperature and CO_2, auto-focusing, etc.)

Storage
Store kit at 4°C and protect from light.

Intended Use
For research use only. Not for use in diagnostics or therapeutic procedures.