

Multiplex Cytotoxicity High Content Screen

1. Rationale/Aim

The multiplex cytotoxicity high content screen is used to investigate the cytotoxicity of compounds in different cell lines. This assay detects cell viability, proliferation and different cell death mechanisms by staining living cells with different fluorescent dyes. The assay has been established for high content screening by an automated microscope of cells treated with different compound libraries to detect toxic effects using a kinetic measurement every 12h for 72h. Cell sub-populations are gated by a machine-learning-guided algorithm of the CellPathfinder Software (Yokogawa). The cell toxicity of each compound is described by parameters like the number of healthy, early apoptotic, late apoptotic, necrotic and lysed cells as well as the growth rate of the cells. Further also the state nucleus is assessed and divided into healthy, fragmented nuclei and pyknotic nuclear sub-populations.

2. Experimental conditions

2.1 Key Requirement:

Instruments and Materials

Material	Description	Manufacturer
Multidrop	dispenser	ThermoScientific
BioRad TC20 automated cell counter	Cell counter	BioRad
ECHO*	Robot to transfer liquids	Labcyte
CQ1	Confocal microscope	Yokogawa
Momentum automatization system	Automatization system to transfer assay plates	Momentum
384 nunc optical bottom plate	Cell culture microplate 384 well, PS, F-bottom, μ CLEAR, black, sterile (Ref 781091)	Greiner
Multichannel pipette	Multichannel pipette 30-300 μ L	Thermo Scientific

*for smaller amount of compounds, the compounds can also be pipetted manually and volumes adjusted accordingly

Experimental parameters:

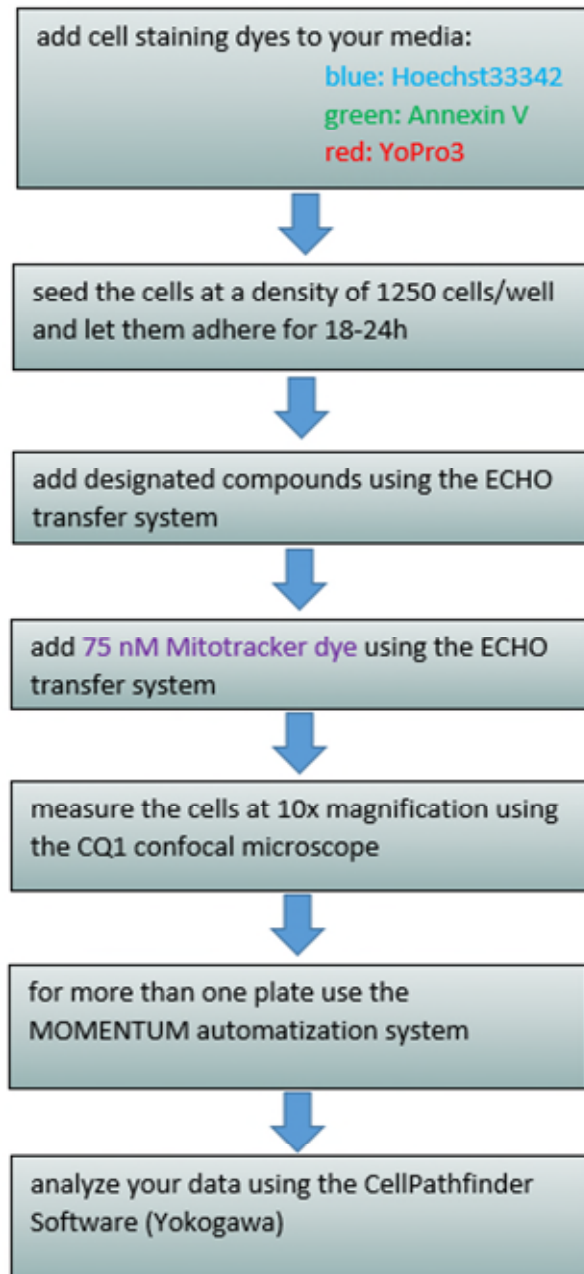
Cell staining dye	Light Source	Emission	Exposure	concentration
Annexin V Alexa Fluor TM 488 conjugate	488nm 40%	525/50nm	50 ms	0,3 µL/well
YoPro TM-3 iodide	561nm 40%	617/73nm	100 ms	1 µM
Hoechst 33342	405nm 50%	447/60nm	500 ms	75 nM
Mitotracker Far Red	640nm 20%	685/40nm	50 ms	75 nM
Brightfield	100%		300 ms	

2.2 Key resources table:

Material	Remarks	Supplier	Order No.
EMEM	Media	ATCC	ATCC 30-2003
DMEM	Media	Gibco	11500416
Penicillin/ Streptomycin	Supplement for media	Invitrogen	15140
Fetal Bovine Serum	Supplement for media	Gibco	11523387
Compounds		diverse	
Annexin V Alexa Fluor TM 488 conjugate	Cell staining dye	Invitrogen	REF A13201
YoPro TM-3 iodide	Cell staining dye	Invitrogen	REF Y3607
Hoechst 33342	Cell staining dye	ThermoScientific	Prod #62249
Mitotracker Far Red	Cell staining dye	Invitrogen	Ref M22426

3. Protocol

3.1 Workflow



3.2 Protocol:

1. Add the cell staining dyes (Annexin V, Alexa Fluor™, 680 conjugate Lot 2130531, Ref A35109 (Life Technologies Corporation) 0,3 µL per well; YoPro 3 iodidide Lot 1974549, Ref Y3607 (Life Technologies Corporation) 1µM and Hoechst 33342 Prod #62249, Lot #TE2568051 (Thermo) 20mM) to your media
2. 24h prior to measurement, seed the cells on a 384 well plate (nunc-plate, flat-bottom, µclear) at a density of 1250 cells/well in 50 µL per well
3. Incubate the plate on bench for 30 minutes at room temperature and then overnight in an incubator at 37°C and 5% CO₂.
4. After 24h centrifuge the plate gently at 100g for 1 minute
5. Add the designated compounds using the ECHO transfer system (Labcyte)
6. Add mitotracker dye (MitoTracker Deep Red FM, Lot 2161838, Ref M22426, invitrogen 75nM) to every well
7. Measure the plate at the CQ1 confocal microscope using the MOMENTUM automatization system (automatization system is used when measuring more than one plate)
8. For measurement use the following parameters:
 - 10x magnification
 - Using five channels:
 - CH1: Hoechst staining: Ex 405nm at 50%, Emission 447/60
 - CH2: Annexin-V staining: Ex 488nm at 40%, Emission 525/50, exp 50ms
 - CH3: Yo-Pro3 staining: Ex 561nm at 40%, Emission 617/73, exp 100ms
 - CH4: Mitotracker staining: Ex 640nm at 40%, Emission 685/40, exp 100ms
 - CH5: Bright field: transmitted light 100%, 50ms
 - no autofocus: 7 Z-stacks

9. Analysis using the Pathfinder software, fixed machine learning (ML)-guided protocols for each cell line thought with images of the following cytotoxic reference compounds with different known mode of actions:

1. Digitonin (membran integrity)
2. Staurosporine (poptosis)
3. Cis platin (Apoptosis)
4. HgCl₂ (apoptosis, necrosis)
5. Milcilib (cell cycle arrest)
6. Torin (cell cycle arrest, autophagy)
7. Paclitaxel (mitotic spindle inhibition)
8. Ricolinostat (HDAC inhibitor)
9. JQ1 (apoptosis)
10. VE-822 (strongly fluorescent control)
11. Captopthecin (cell cycle arrest)

Parameters used for machine learning algorithm (Cell/Nuclei):

(Nucleus) Area
(Nucleus) X-CoordinateInWell
(Nucleus) Y-CoordinateInWell
(Nucleus) Diameter
(Nucleus) Circularity
(Nucleus) Compactness
(Nucleus) Anisometry
(Nucleus) TotalIntensity CH1
(Nucleus) TotalIntensity CH2
(Nucleus) TotalIntensity CH3
(Nucleus) MeanIntensity CH1
(Nucleus) MeanIntensity CH2
(Nucleus) MeanIntensity CH3
(Nucleus) TotalPeak CH1
(Nucleus) TotalPeak CH2
(Nucleus) TotalPeak CH3
(Nucleus) MeanPeak CH1
(Nucleus) MeanPeak CH2
(Nucleus) MeanPeak CH3
(Cytoplasm) Area
(Cytoplasm) Diameter
(Cytoplasm) Circularity
(Cytoplasm) Compactness
(Cytoplasm) Anisometry
(Cytoplasm) TotalIntensity CH4
(Cytoplasm) TotalIntensity CH5
(Cytoplasm) MeanIntensity CH4
(Cytoplasm) MeanIntensity CH5
(Cytoplasm) TotalPeak CH4
(Cytoplasm) TotalPeak CH5
(Cytoplasm) MeanPeak CH4
(Cytoplasm) MeanPeak CH5

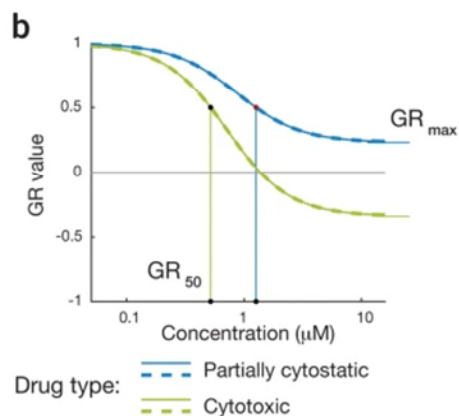
10. Data reporting:

All data is reported in Scarab: *SGC/Biophysics & Chemical Biology/Multiplex/Multiplex*

The following parameters are reported for each tested compound:

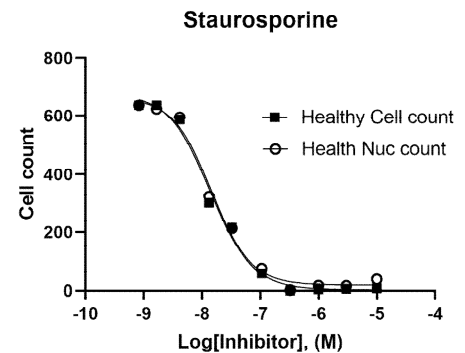
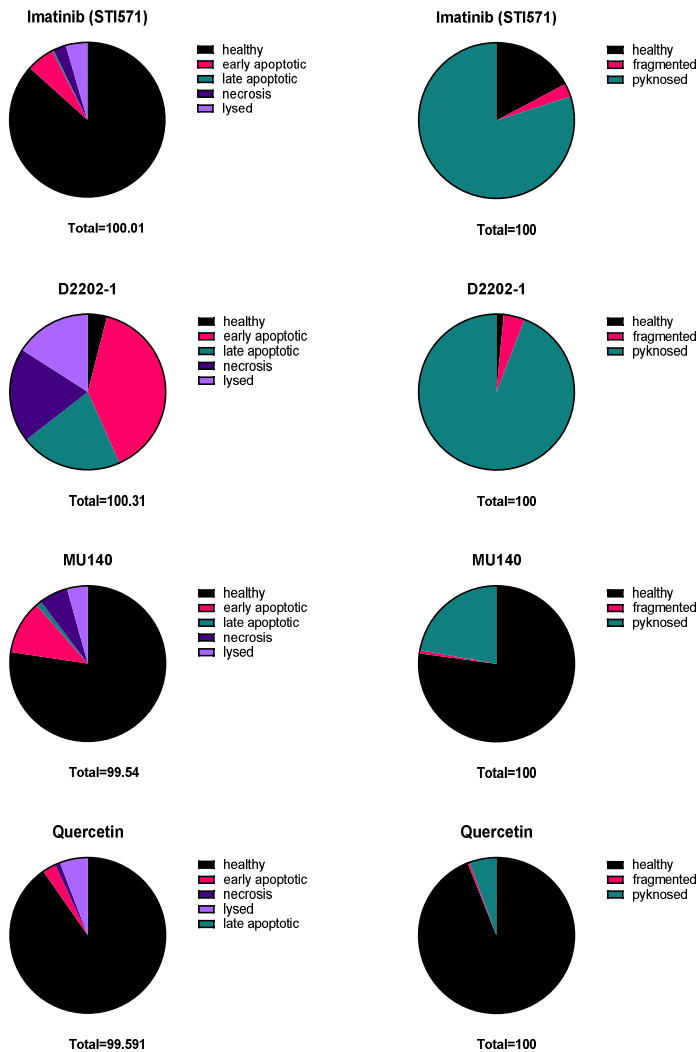
growth rate (GR)
 total cell count
 total cell count normalized vs DMSO %
 healthy cell count normalized against DMSO healthy cell count %
 population healthy %
 population early apoptotic %
 population late apoptotic %
 population necrotic %
 population lysed %
 total nuc count normalized against DMSO %
 healthy nuc count normalized against DMSO healthy nuc count %
 population healthy nuc %
 population nuc fragmented %
 population nuc pyknosed %
 DMSO cell count average
 DMSO healthy cell count average %
 DMSO early apoptotic cell count average %
 DMSO late apoptotic cell count average %
 DMSO necrotic cell count average %
 DMSO lysed cell count average %
 DMSO healthy nuc count average %
 DMSO fragment nuc count average %
 DMSO pyknosed nuc count average %

Compounds are defined as cytotoxic if threshold of GR of compound is $<0,5$



Hafner M, Niepel M, Sorger PK. (2017) Alternative drug sensitivity metrics improve preclinical cancer pharmacogenomics. *Nat Biotech*, vol. 35(6), 500-2. doi: [10.1038/nbt.3882](https://doi.org/10.1038/nbt.3882).

Reported data can be visualized as following examples:



The protocol can be adapted to other high content microscopes as described in <https://journals.sagepub.com/doi/full/10.1177/2472555220923979>