



Live Cell Assay Automation



Abstract

Affordable live cell automation is an enigma in the realm of drug discovery biomedical research. Modular robotic systems, historically designed with high throughput screening as their focus, often consist of a crane or articulating arm that picks and places microplates from a number of process specific instruments to carry out the workflow of assays. By their nature these systems are generally quite large and expensive, requiring specialized rooms or custom chambers with HEPA filters to maintain sterility when running live cell assays. Compact automated low throughput systems have also been developed primarily with ELISA reactions in mind. These systems generally are limited in regards to assay flexibility, with minimal capacity for reagent addition and fixed incubation temperatures and no environmental gas or humidity control. While suitable for ELISA they make a poor choice for automated live cell assays.

To address the need of an assay workstation that is both flexible and affordable, yet provides the means to run live cell assays BioTek has developed the BioSpa™ 8 Automated Incubator. The BioSpa 8 links BioTek readers or imagers together with washers and dispensers for a full workflow automation of up to 8 microplates. Real time control and continuous temperature, CO₂/O₂ and humidity level monitoring; along with lid handling ensure an ideal environment for cell cultures during all experiment stages, with minimal manual intervention. BioSpa 8 software, which features customizable text or email notifications and alerts, also relieves the need for onsite monitoring. The software's session timelines and environmental reports allow quick scrutiny of the process and system status. BioSpa 8 automates assay workflows by repeated manipulation and storage of microplates containing live cells or temperature sensitive reagents. Its size is such that it, along with a liquid handler and reader/imager, can be placed inside a conventional 6-foot biosafety cabinet.

Cell Fixing and

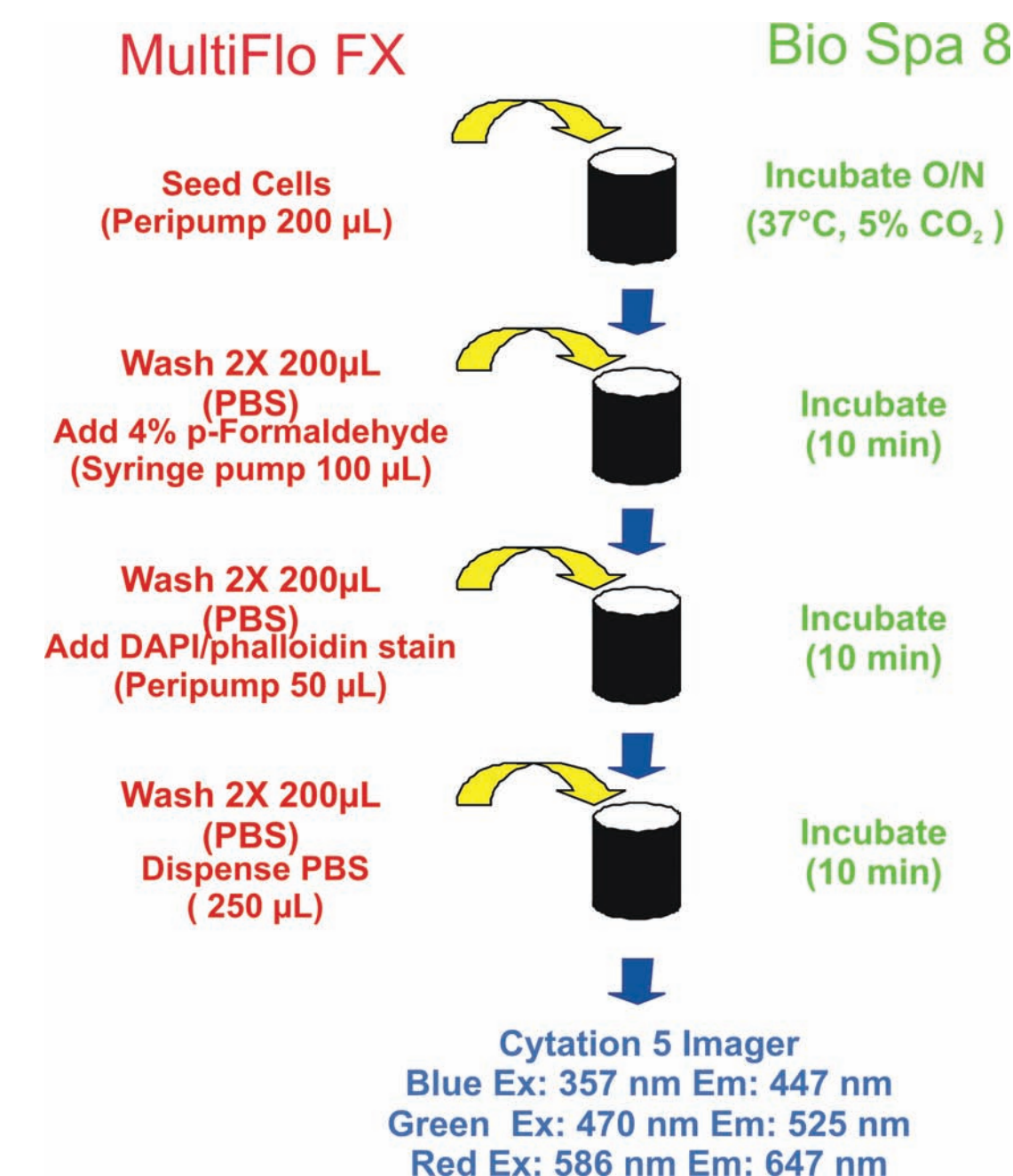


Figure 1. Automated Cell Seeding, Fixing and Staining. NIH3T3 cells were seeded at various cell densities and allowed to attach overnight in the BioSpa 8 Automated Incubator. The following day the cells were fixed and stained. All cell washes and reagent additions for cell fixation and fluorescent staining were carried out using the MultiFlo™ FX Multi-Mode Dispenser. Cells were washed two times with 200 μ L of PBS. After which 100 μ L of 4% paraformaldehyde (PFA) solution was added using the syringe pump dispenser manifold. Cells were fixed for 10 minutes at room temperature followed by 2 washes of 200 μ L using PBS. Using the peripump dispenser, actin and nuclear DNA were then stained using 50 μ L of a working solution of Texas Red phalloidin and DAPI respectively. Cells were stained for 10 minutes followed by a two-cycle wash with 200 μ L of PBS. After aspiration 300 μ L of PBS was added to all wells and the plates were imaged using a Cytation™ 5 Cell Imaging Multi-Mode Reader.

Cell Fixing and Staining

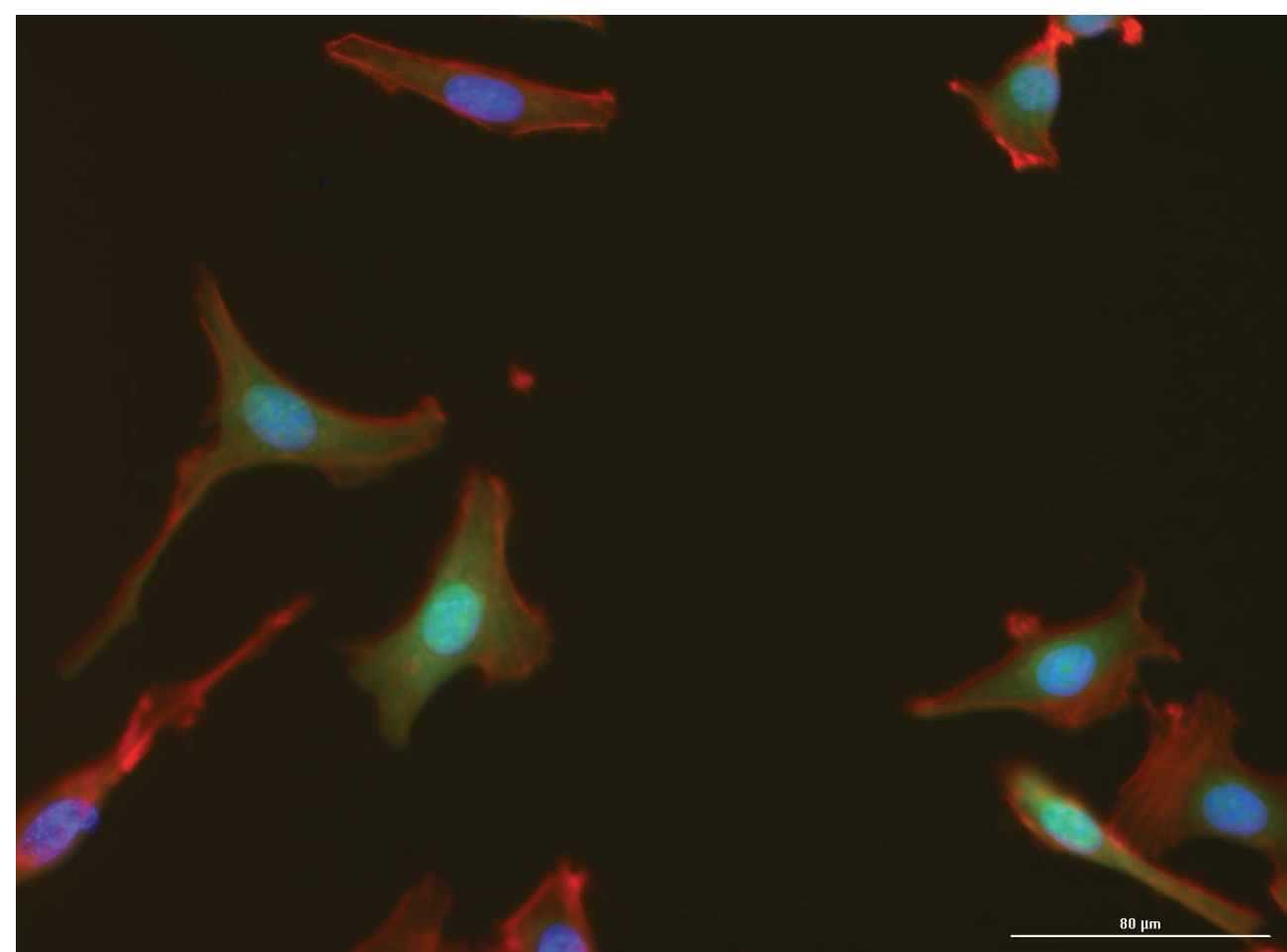


Figure 2. Representative 20X image of fixed and stained NIH3T3 cells expressing GFP stained with DAPI and Texas red phalloidin. Fixed and stained cultures were imaged using Cytation 5 configured with DAPI, GFP and Texas Red light cubes. The imager uses a combination of LED light sources in conjunction with band pass filters and dichroic mirrors to provide sensitive fluorescence detection. The DAPI light cube uses a 337/50 excitation filter and a 447/60 emission filter, GFP light cube uses a 469/35 excitation filter and a 525/39 emission filter, while the TR light cube uses a 586/15 excitation and 647/57 emission filters.

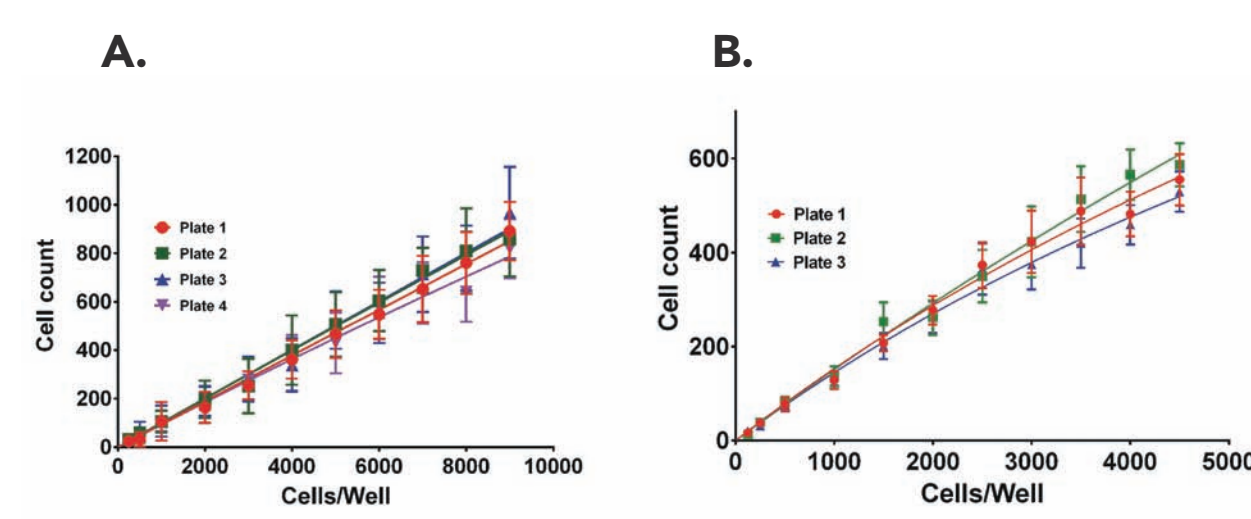


Figure 3. Repeatability of Cell Counts after seeding and fixation. NIH3T3 cells were seeded into multiple 96-well microplates at titrating cell densities on two separate experiments. (A) Linear titration of cells on four plates with the starting cell density of 10,000 cells per well. (B) Linear titration of cells on three plates with the starting cell density of 5,000 cells per well. Cell counts were made using object counting image analysis of DAPI stained nuclei from 4X objective images.

Cytotoxicity Assay

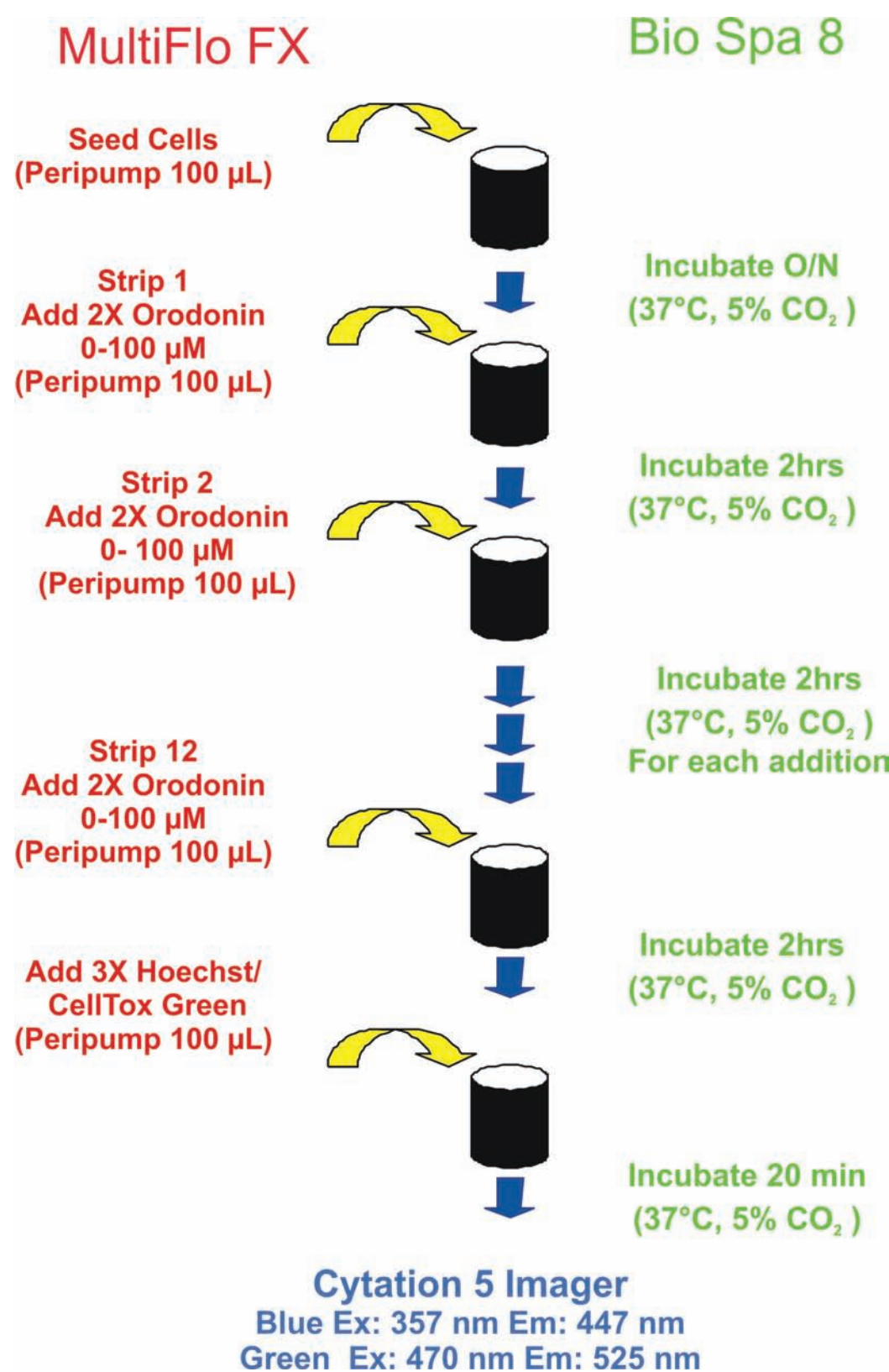


Figure 4. Assay Process steps for a Cytotoxicity Assay. A series of strip dispense routines were carried out with the MultiFlo FX to add various concentrations (0-100 μ M) of Oridonin every 2 hours to U-2 OS cells in 4 separate plates. Plate are incubated in the BioSpa 8 at 37 $^{\circ}$ C, with a humidified 5% CO₂ atmosphere between reagent additions. After 24 hours Hoechst 3342 and CellTox[®] Green stains are added and the plates imaged in the DAPI and GFP channels with a Cytation 5 Cell Imager multi-mode reader.

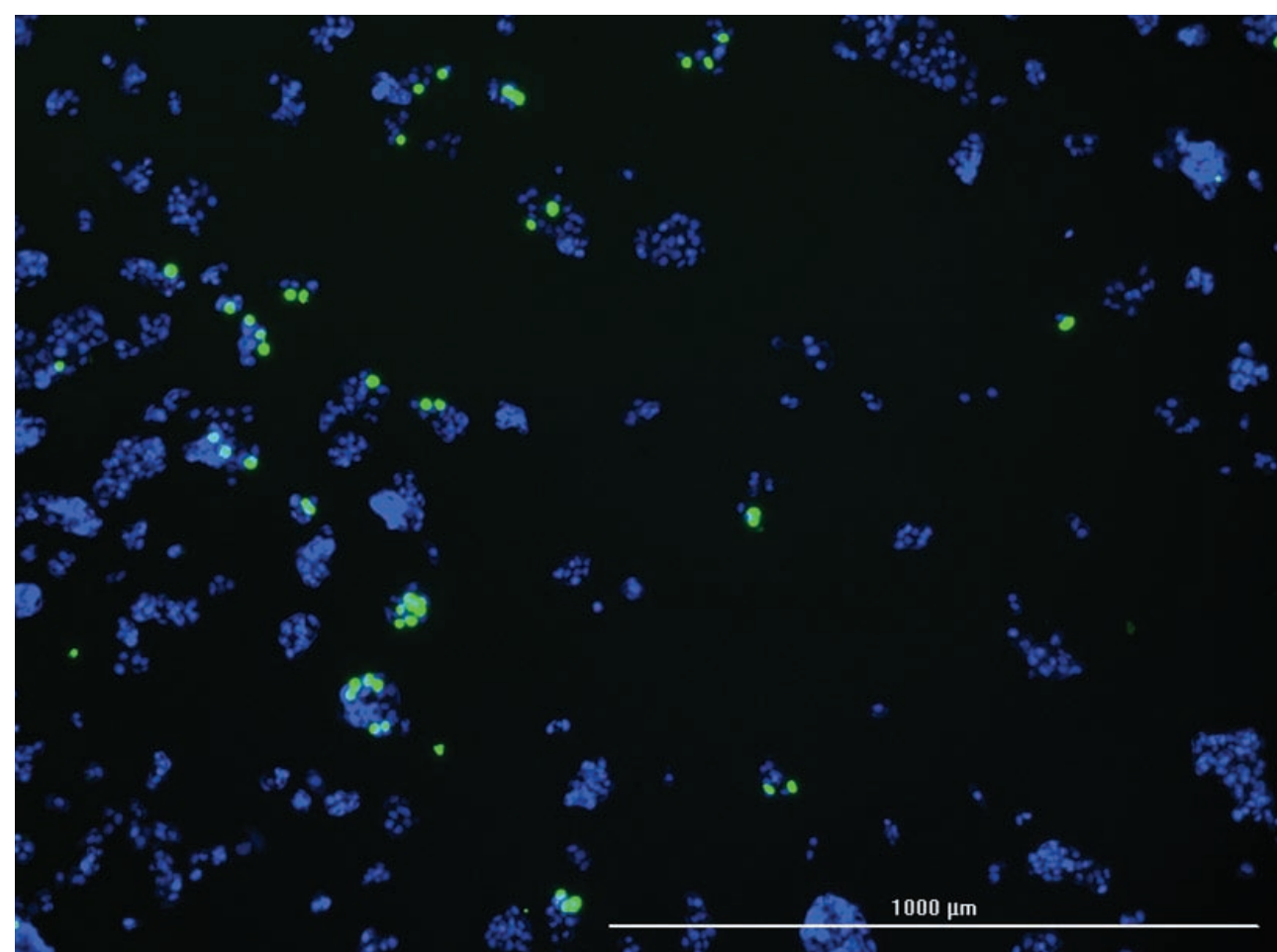
 Cytotoxicity Assay

Figure 5. Representative 4X image of U-2 OS cells stained with Hoechst 33342 and CellTox™ Green. Cells were treated with oridonin for various amounts of time, then stained with Hoechst 33342 (1 µg/ml) and CellTox™ green for 30 minutes. Digital microscopic images (4X) were made using Cytation 5. Hoechst 33342 is a membrane permeable dye that will bind nucleic acids of live and dead cells, while CellTox™ green can only stain nucleic acids from membrane incompetent dead cells.

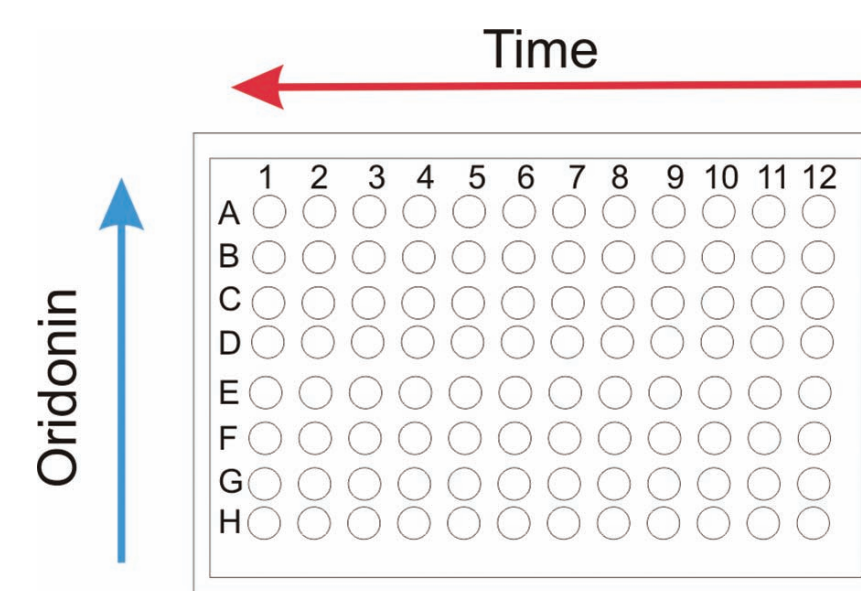


Figure 6. Plate map configuration of Oridonin cytotoxicity experiments. Oridonin is added to the plate using the Multiflo FX peripump such that different concentrations of drug are added with each of the eight separate dispense tubes in rows A-H. Drug is added at different times with each strip 1-12. Well A1 would have the highest drug concentration and exposure time, while well H12 would have the lowest drug concentration and the least amount of exposure time.

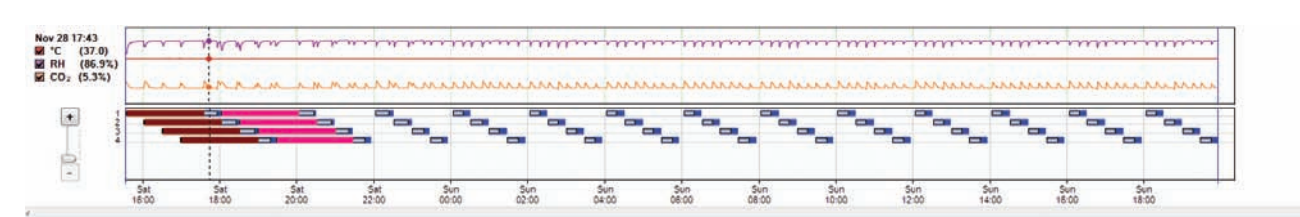


Figure 7. Gantt chart of a BioSpa 8 cytotoxicity assay session. A series of strip dispense routines are carried out with the MultiFlo FX to add various concentrations (0-100 μ M) of Oridonin every 2 hours to U-2 OS cells in 4 separate plates. Plate are incubated in the BioSpa 8 at 37 $^{\circ}$ C, with a humidified 5% CO₂ atmosphere between reagent additions. After 24 hours, the plates are stained and imaged.

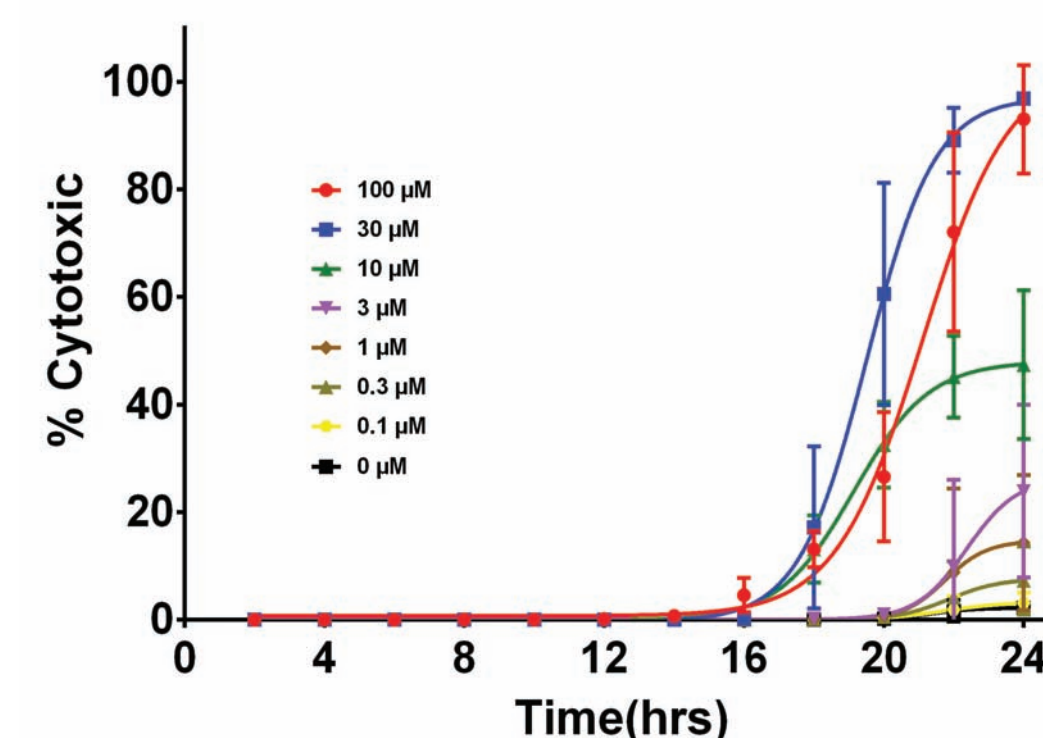


Figure 8. Effect of Exposure time of Oridonin on Cytotoxicity. U-2 OS cells were treated with various concentrations of oridonin and assayed for cytotoxicity after different exposure times. Results are expressed as a percent of the total number of cells counted from imaged based object counting of Hoechst 33342 stained nuclei. Positive cell nuclei exhibit a mean green fluorescence greater than 40,000.

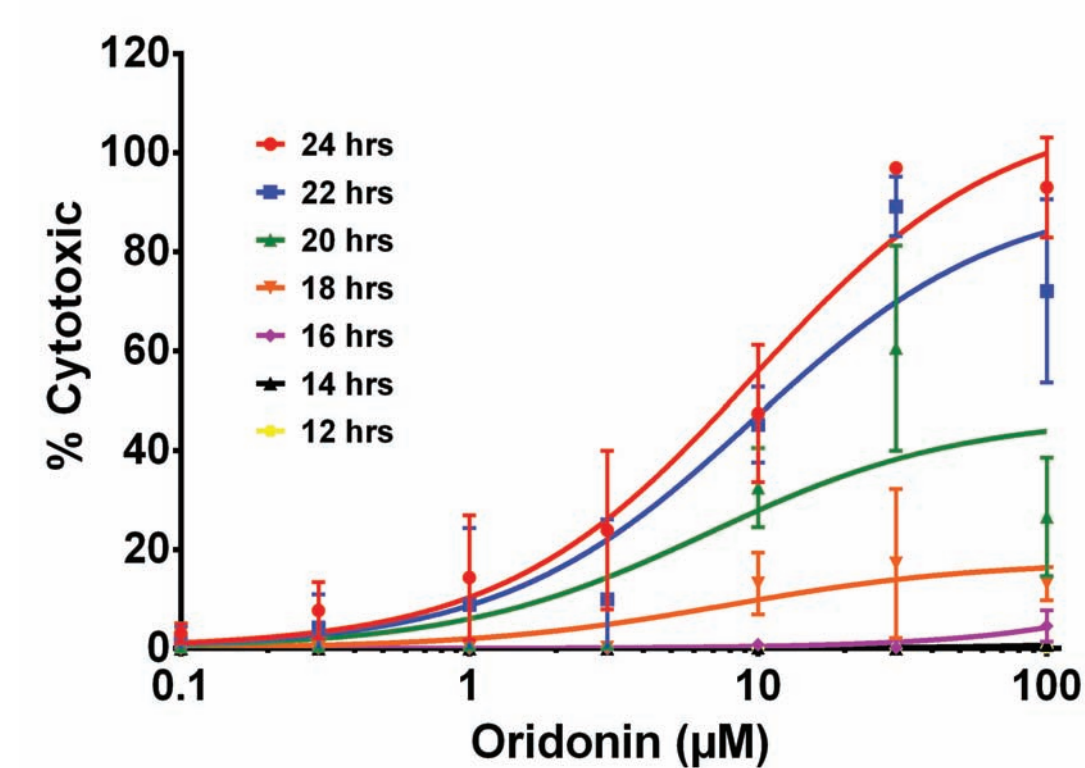


Figure 9. Effect of Oridonin Concentration on Cytotoxicity. U-2 OS cells were treated with various concentrations of oridonin and assayed for cytotoxicity after different exposure times. Results are expressed as a percent of the total number of cells counted from imaged based object counting of Hoechst 33342 stained nuclei. Positive cell nuclei exhibit a mean green fluorescence greater than 40,000.

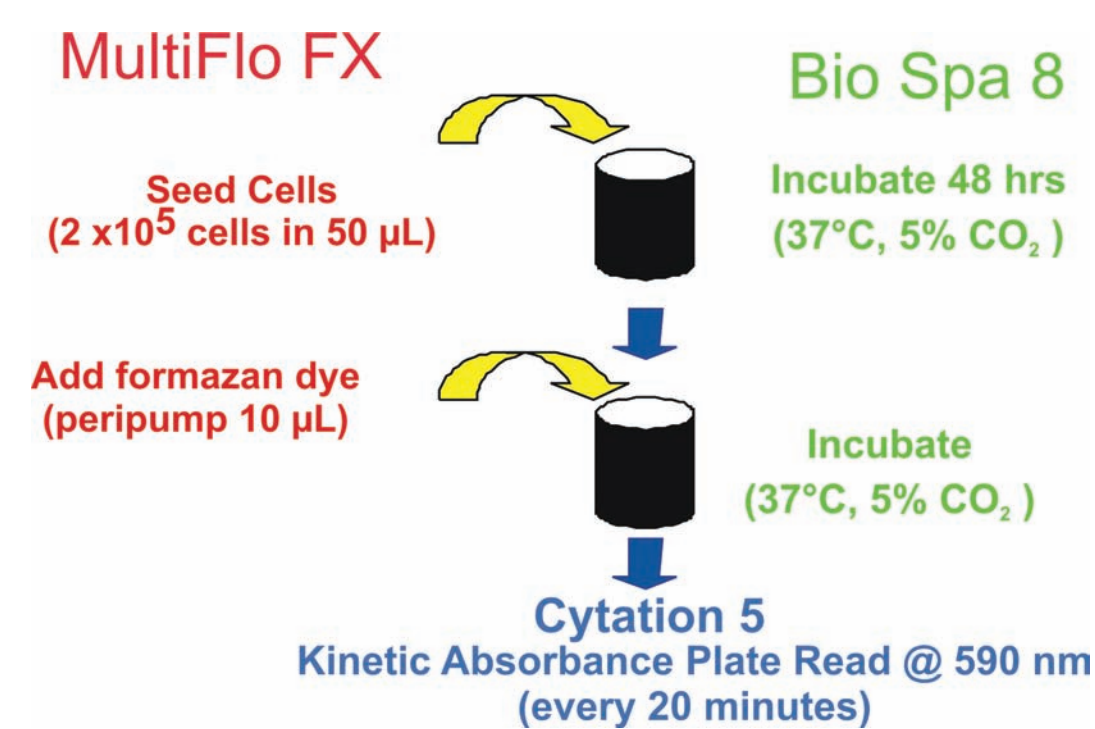
 Biology Phenotype

Figure 10. Biolog Phenotypic Characterization Microarray[®] Procedure. U-2 OS, HEK 293, NIH3T3, and MDA-MG-231 cell lines were harvested by treatment with trypsin and suspended at 400,000 cells per ml in Biolog's IF-M1 medium supplemented with 5% FCS, 0.3 mM Gln and 1X Pen Strep. The cells were dispensed into Phenotypic Microarray panels M1 to M4 (50 µL, 20,000 cells per well) using a MultiFlo FX and incubated at 37 °C under humidified 5% CO₂ for 24 h. Biolog Redox Flex Mix (AA 10 µL) was added with the MultiFlo FX to achieve a final concentration and the absorbance was measured at 590 nm (750 nm reference) using Cytation 5. Plates were maintained at 37 °C and 5% CO₂, while in the plate reader and stored in the BioSpa 8 incubator at 37 °C, with a humidified 5% CO₂ atmosphere when not being read.

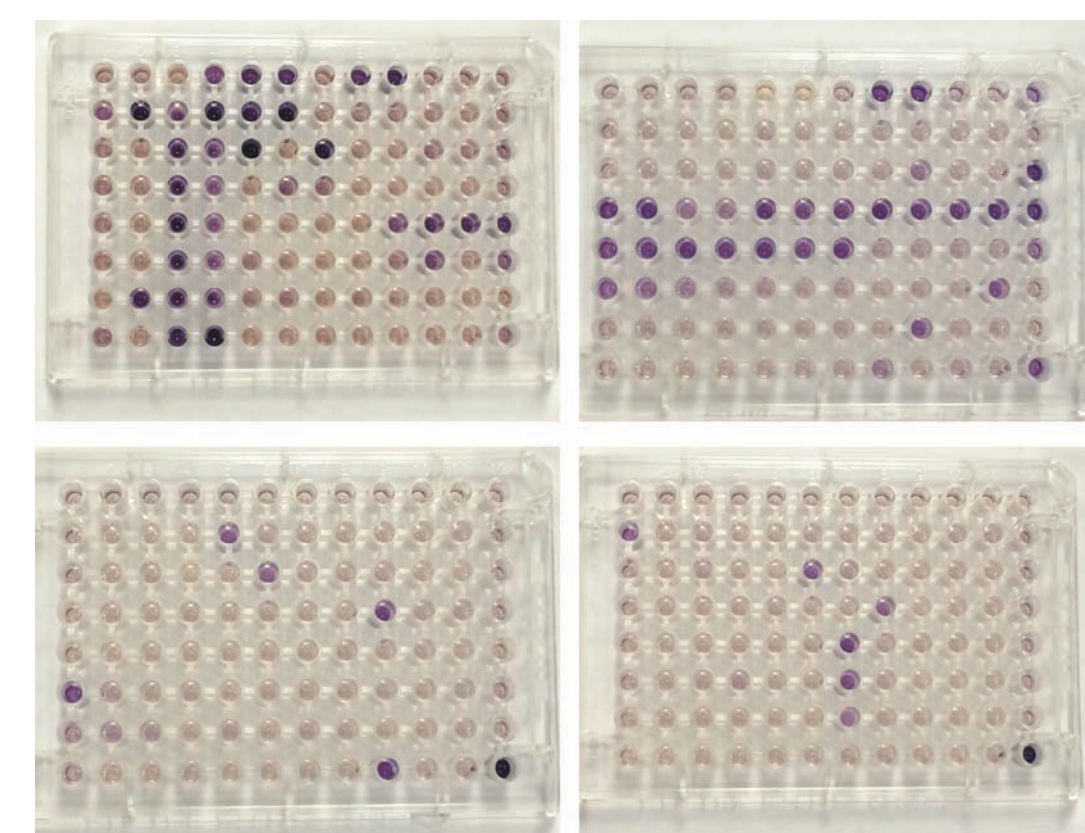


Figure 11. Metabolic phenotype patterns of U-2 OS cells. Cultured U-2 OS grown in Biolog microarray panels for 44 hours and compared. Cells were incubated for 12 hr at 37 °C under humidified 5% CO₂ - 95% air before plates were photographed.

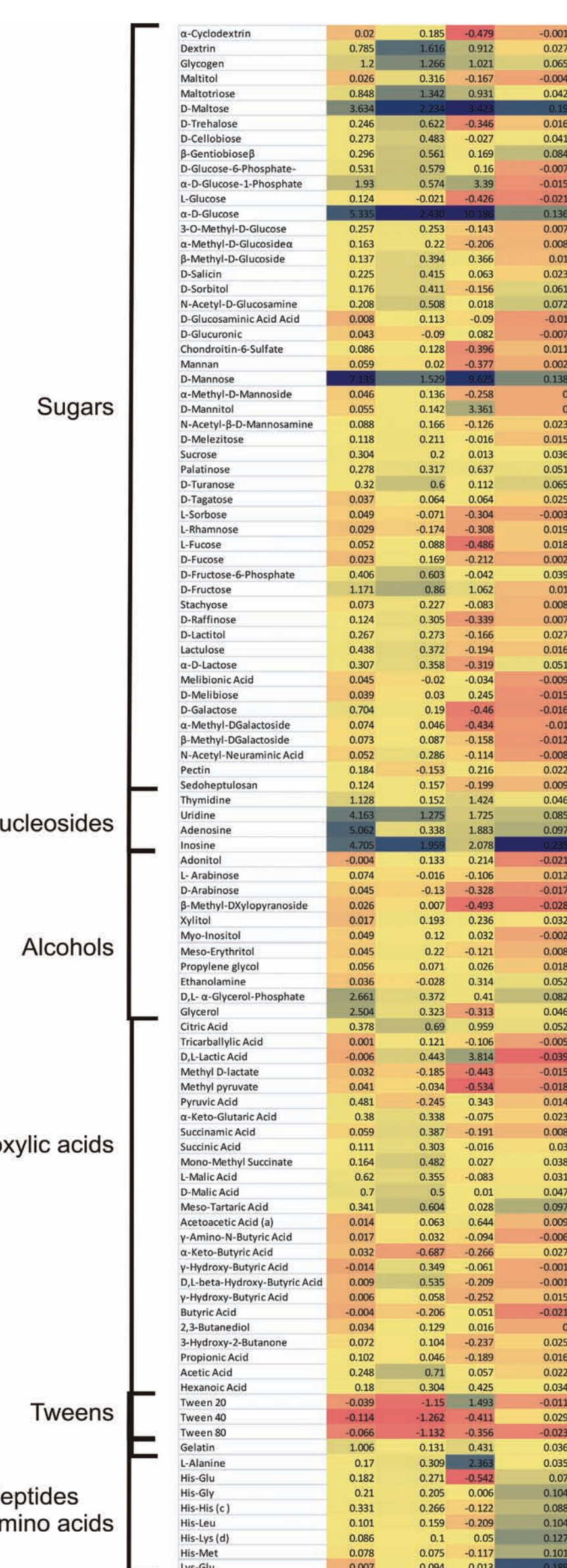


Figure 12. Substrate growth phenotype comparison. Four different cultured cell lines were grown on Biolog phenotype microarray plates M1-4 and the rate of formazan dye formation as measured by change in absorbance at 590 nm over the first 2 hours were compared. Highest rates are indicated with blue coloration, while lowest rates depicted with red coloration.

 | Instrumentation

Figure 13. BioSpa 8 Automated Incubator. The BioSpa 8 is a microplate incubator that can interface a BioTek microplate liquid handling device with a BioTek Microplate reader/imager. The BioSpa 8 maintains temperature and humidity, as well as providing CO₂ and O₂ gas control for up to 8 microplates.



Figure 14. MultiFlo FX Multi-Mode Dispenser.
The MultiFlo FX is a modular upgradable reagent dispenser that can have as many as two peri-pump (8 tube dispensers), two syringe pump dispensers and a strip washer. The syringe and washer manifolds can be configured for plate densities from 6- to 384-well.



Figure 15. Cytation 5 Cell Imaging Multi-Mode Reader. Cytation 5 is a modular, upgradable multi-mode reader that combines automated digital microscopy and conventional microplate detection. Cytation 5 includes both filter- and monochromator-based detection; the microscopy module provides up to 60x magnification in fluorescence, brightfield, color brightfield and phase contrast. Incubation to 65° and plate shaking are standard features. The imaging module uses a turret to hold up to 6 objectives. Excitation and emission wave lengths for the microscopy module are provided using LED light cubes in combination with specific band pass filters and dichroic mirrors. The imaging module holds up to 4 LED cubes. In conjunction with the multi-mode reader, Gen5™ software, which controls reader function, also provides image analysis and data reduction.

Conclusions

- BioSpa 8 Automated Incubator is capable of performing a multitude of different live cell assays
 - Plate handling
 - Walk-away system
 - Environmental controls
 - Temperature
 - Gas (CO₂ & O₂)
 - Humidity
- Cytotoxic effects of oridonin are demonstrated
 - Exposure time
 - Drug concentration
- Phenotypic differences in metabolic pathways in cultured cell lines
- Repeatability of cell seeding titrations
- MultiFlo FX Multi-Mode Dispenser
 - Automates the liquid handling tasks necessary for live cell assays
- Cytation 5 Cell Imaging Multi-Mode Reader has a number of features that enable live cell assays
 - Imaging
 - Auto-focus and auto-exposure
 - Multiple color imaging capabilities
 - Multi-mode reading
- Quantitative image analysis using Gen5 Software
 - Population analysis