Put the Attune NxT Autosampler to work for high-throughput screening

Multiparametric flow cytometry assays for determining pharmacological effects.

High-throughput screening (HTS) is an extremely effective method for identifying potential candidates for therapeutics. Initial compound screening is often performed on a microplate reader because multiwell plate assays tend to be easy to perform, cost-effective, and robust, and they enable the screening of a large number of compounds in a single run. Another useful platform for HTS is flow cytometry, which offers the additional benefit of cell-by-cell analysis. A flow cytometer paired with an autosampler—such as the Invitrogen™ Attune™ NxT Flow Cytometer equipped with the Invitrogen™ Attune™ NxT Autosampler—provides an extremely effective platform for performing HTS assays using multiwell plates (Figure 1). The Attune NxT Flow Cytometer is up to 10 times faster than traditional flow cytometers and has 16 detection channels available, allowing analysis of up to 35,000 events per second. These features greatly benefit studies of protein–protein interactions, metabolic activity, and DNA content in a single or multiparametric assay format.

Figure 1. Setup of Attune NxT Flow Cytometer with Autosampler for high-throughput screening experiments. (A) Invitrogen™ Attune™ NxT Flow Cytometer with Invitrogen™ Attune™ NxT Autosampler. **(B)** Plate heat-mapping is available on Invitrogen™ Attune™ NxT Software to monitor results as they are acquired.

HTS on the Attune NxT Flow Cytometer

Multiparametric flow cytometry provides a means of analyzing large numbers of individual cells for several cell health parameters in a short amount of time. The ability to perform multiple secondary and tertiary assays at the same time as the initial compound screening assay helps to minimize sample collection and preparation steps while also allowing for the robust analysis of cell health in a population. Multiparametric analyses provide a more complete picture of the complex effects on cell function that result from a particular compound treatment.

The toxicity of a pharmaceutical compound can be assessed in less than 45 minutes per plate (acquisition time, with real-time visualization as results are collected) on the Attune NxT Flow Cytometer with Autosampler. When running multiple samples, Invitrogen™ Attune™ NxT Software allows easy transitions between tube and plate format using a one-click command; no disassembly is required. The many advantages to running the Attune NxT Flow Cytometer with Autosampler in high-throughput mode include:

- Compatibility with a variety of plate types, including 96- and 384-well, deep-well, and round- and V-bottom plates
- Optimized mixing to enable maximal sample throughput with minimal effects on cell viability
- Live heat maps for rapid screening and the ability to monitor results while the instrument acquires data
- Real-time numeric overlay text for assay feedback in live heat maps
- Consistent data with minimal variation, regardless of sampling method and collection rate
- Automated compensation calculations

Initial screening for compounds that affect cell viability

In a typical initial screening of potential pharmaceutical drugs, "hit" compounds—defined as molecules that have the desired activity based on the mechanistic readout of the functional assay used—are identified and then further analyzed using a variety of cell health assays and compound concentrations to establish potency. In the HTS flow cytometry experiment shown in Figure 2, we used a cell viability assay to initially identify hit compounds that affected the health of Ramos cells (B cells) and Jurkat cells (T cells, data not shown) cultured at different oxygen levels. In this example, the viability was determined based on membrane integrity using the Invitrogen™ LIVE/DEAD™ Fixable →

Aqua Dead Cell Stain, a membrane-impermeant amine-reactive dye that only enters cells with compromised membranes. Thus, in dead cells with compromised membranes, the dye reacts with free amines both in the cell interior and on the cell surface, yielding intense fluorescent staining; in live cells, the dye's reactivity is limited to cell-surface amines, resulting in less-intense fluorescence.

Cells were cultured at both normoxic (≥19% O_2) and hypoxic (1% O_2) conditions to mimic the variable oxygen levels found in tumors. Figure 2 shows an example of the results of this initial screening, with a few compounds marked as hits. In this experiment, a hit is defined as a compound that produces different effects on the viability of cells exposed to the two different oxygen levels. These hit compounds were then tested further to assess their potency, including determining the drug dose response and their apparent EC_{50}.

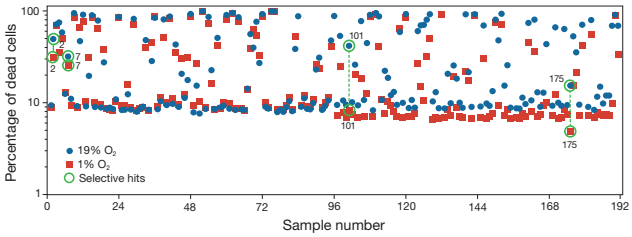

Figure 2. High-throughput screening of Ramos cells stained with LIVE/DEAD Fixable Aqua Dead Cell Stain using the Attune NxT Flow Cytometer with Autosampler. Identical plates containing Ramos cells (40,000 cells per well) were treated with 10 µM of the different drugs in the Killer Collection (MicroSource Discovery Systems Inc.) and cultured under standard conditions with either hypoxic (1% O_2) or normoxic (19% O_2) levels using a Thermo Scientific™ Heracell™ VIOS 160i CO_2 Incubator. Cells were stained with Invitrogen™ LIVE/DEAD™ Fixable Aqua Dead Cell Stain (for 405 nm excitation, Cat. No. L34957) and analyzed on the Invitrogen™ Attune™ NxT Flow Cytometer with Autosampler. Cell viability is expressed as percentage of dead cells (which are >50-fold more fluorescent than live cells); ~Z′ = 0.8.

Secondary screening to define mechanism of action

Once initial hit compounds were identified, secondary assays were performed to help define the mechanism of action of each compound on the cell model. To better characterize these compounds, we determined the drug dose dependency using assays for cell cycle phase (DNA content), membrane integrity, mitochondrial function, and apoptosis. For example, here we show the secondary screening of the hit compound amsacrine, a potent chemotherapeutic drug known to inhibit DNA replication, in both Ramos cells (data not shown) and Jurkat cells (Figures 3 and 4) at both normoxic and hypoxic oxygen conditions. To perform this battery of cell health assays, we incubated cells with a dye cocktail containing four different fluorescent cell function probes, using a single-step, multiplex high-throughput workflow.

The drug dose response can be visualized on a heat map that is generated by Attune NxT Software and referenced to any statistic such as cell count, cell percentage, or cell concentration in each well of the microplate. To obtain statistically relevant data sets, each sample (and well) should contain the same number of cells, and the same number of events across all samples should be collected to avoid sample-to-sample variation that can result from differences in cell number and fluorescence signal.

To monitor the effect of amsacrine on cell health, the dye cocktail included Hoechst™ 33342 dye, a cell-permeant nucleic acid stain that can be used to measure DNA content and to distinguish the cell cycle phase of each cell by flow cytometry (Figure 3A). Because data are acquired at the single-cell level, histogram plots generated during this

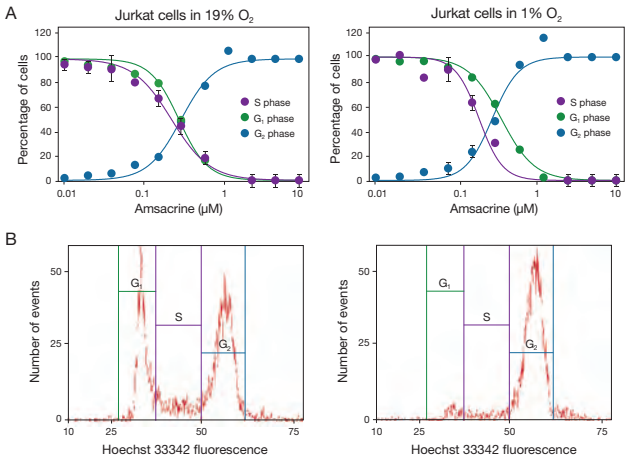

Figure 3. Determination of the effective dose response to pharmaceutical drugs in each phase of the cell cycle by staining with Hoechst 33342 dye. (A) Using Jurkat cells stained with Invitrogen™ Hoechst™ 33342 Ready Flow™ Reagent (Cat. No. R37165), EC_{50} values can be calculated for each phase of the cell cycle. There are no significant differences in the cell cycle for Jurkat cells treated with amsacrine at either 19% or 1% O_2. **(B)** The G_1, S, and G_2/M phases are observed in healthy replicating cells (left). Cells treated with 2.5 µM amsacrine become apoptotic and accumulate in the G_2 phase (right).

flow cytometry analysis also allow visualization of the distribution of cells in each cell cycle stage at a given amsacrine concentration (Figure 3B).

To investigate the effect of amsacrine on membrane integrity and mitochondria, Invitrogen™ SYTOX™ Red Dead Cell Stain and tetramethylrhodamine methyl ester (TMRM) were also included in the dye cocktail. SYTOX Red stain is a cell-impermeant nucleic acid stain that easily

penetrates cells with compromised membranes, where it becomes highly fluorescent upon binding DNA, but that will not cross the membranes of live cells. TMRM is a cell-permeant, red-orange–fluorescent dye that accumulates in functioning mitochondria with intact membrane potentials; as mitochondrial membranes depolarize, fluorescent signals decrease. Jurkat cells exposed to either 19% or 1% oxygen and treated with increasing concentrations of amsacrine showed a similar response, with no significant differences in EC_{50} values between the two oxygen levels (Figure 4A) for cell viability and mitochondrial health.

Similarly, oxygen levels had no effect on apoptosis, which was detected using Invitrogen™ CellEvent™ Caspase-3/7 Green Detection Reagent (Figure 4B), also included in the dye cocktail. The cell-permeant CellEvent reagent comprises the four–amino acid peptide DEVD—which contains the recognition site for caspases 3 and 7—conjugated to a nucleic acid–binding dye. Because the DEVD peptide inhibits the ability of the dye to bind to DNA, CellEvent Caspase-3/7 Green reagent is intrinsically nonfluorescent. In the presence of activated caspases 3 and 7, key markers in the early stages of apoptosis, the dye is cleaved from the DEVD peptide and free to bind DNA, producing a bright green-fluorescent signal indicative of apoptosis.

Benefits of multiparametric flow cytometry screening

Using the Attune NxT Flow Cytometer with Autosampler in combination with a dye cocktail to assess cell health, we assayed several cell health parameters simultaneously in multiple samples in a multiwell plate. The Attune NxT Autosampler provides the functionality to run a drug dose response assay across a 96-well plate and obtain six independent EC_{50} values at two different oxygen levels, all in under 45 minutes (Table 1, Figure 5). See our comprehensive suite of products for flow cytometry, from instruments and standards to antibodies and cell function reagents, at **thermofisher.com/flowcytometry**. ■

Product	Quantity	Cat. No.
Attune™ NxT Autosampler	1 each	4473928
Attune™ NxT Flow Cytometer, blue/red/violet6/yellow	1 each	A29004
CellEvent™ Caspase-3/7 Green Flow Cytometry Assay Kit	100 assays	C10427
Hoechst™ 33342 Ready Flow™ Reagent	120 assays	R37165
LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit, for 405 nm excitation	80 assays 200 assays 400 assays	L34965 L34957 L34966
SYTOX™ Red Dead Cell Stain, for 633 or 635 nm excitation	1 mL	S34859
MitoProbe™ TMRM Assay Kit for Flow Cytometry	100 assays	M20036

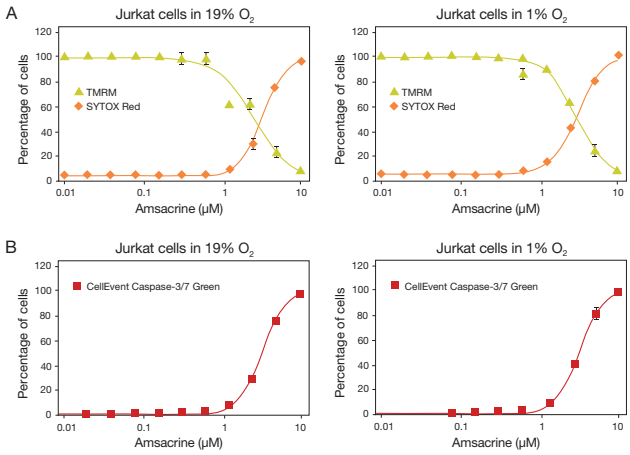

Figure 4. Secondary assays to examine cellular responses to amsacrine treatment. (A) Jurkat cells were exposed to a range of amsacrine concentrations overnight and then stained with Invitrogen™ SYTOX™ Red Dead Cell Stain (for 633 or 635 nm excitation, Cat. No. S34859) and tetramethylrhodamine methyl ester (Invitrogen™ MitoProbe™ TMRM Assay Kit for Flow Cytometry, Cat. No. M20036). As the amsacrine concentration increases, the SYTOX Red signal intensifies, indicating an increase in cell death as the cell membrane integrity is lost, while the TMRM signal decreases, indicating a loss in mitochondrial membrane potential. **(B)** In Jurkat cells stained with the Invitrogen™ CellEvent™ Caspase-3/7 Green Flow Cytometry Assay Kit (Cat. No. C10427), an increase in CellEvent Caspase-3/7 Green signal, indicative of apoptosis, is seen in response to increasing concentrations of amsacrine.

Table 1. Summary of EC_{50} values in cell function assays using Jurkat cells.

Fluorescent probe	Cell function detected	EC_{50} (µM) in 1% O_2	EC_{50} (µM) in 19% O_2
Hoechst 33342	S phase	0.20	0.24
	G_1 phase	0.37	0.30
	G_2 phase	0.28	0.31
CellEvent Caspase-3/7 Green	Apoptosis	2.84	3.30
TMRM	Mitochondrial health	2.89	2.60
SYTOX Red	Membrane integrity	2.93	3.48

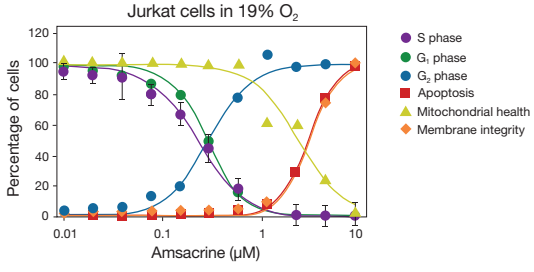

Figure 5. Summary of multiplex staining of Jurkat cells in a range of amsacrine concentrations under normoxic (19% O_2) conditions. Jurkat cells were stained with a cocktail containing four different cell health indicator dyes, and six EC_{50} values were generated. At >0.3 µM (EC_{50}) amsacrine, Jurkat cells are arrested in the G_2 phase and no longer replicate. Measurements of mitochondrial health and caspase activity based on amsacrine treatment correlate with one another, supporting the role of mitochondria in the apoptosis pathway.