Rapid Apoptosis Monitoring Using Annexin V and Orflo's Moxi GO II – Next Generation Flow Cytometer

Introduction/Background

Cellular apoptosis is a sophisticated mechanism employed by cells to carefully control death in response to cell injury. Commonly referred to as "programmed cell death," apoptosis progresses through a systematic signaling cascade that results in characteristic, directed morphological and biochemical outputs in the cell. The overall outcome is a highly regulated cell death process that minimizes trauma to the cell's extracellular environment. At a systemic level, the critical role of apoptosis is easily highlighted through its implication in hearing loss[1], cardiovascular failure[2], cancer progression[3], neurodegenerative disorders[4], and numerous other pathologies[5]. Correspondingly, scientific research has intently focused on revealing the underlying pathways of apoptosis, defining its external triggers,

Figure 1 – Orflo's Moxi GO II – Next Generation Flow Cytometer. Image shows user loading a sample into the two-test disposable flow cell. The Moxi GO II is a configurable flow cytometer with a 488nm laser and up to two fluorescence recording channels (2 PMT's with emission filters at 525/45nm filter and 561nm/LP, respectively).

identifying therapeutic interventions, and further exploring the role of apoptosis in numerous pathologies. Furthermore, the monitoring of apoptosis has also been established as a key indicator in identifying the "biocompatibility" of pharmaceutics and other environmental conditions for cell systems.

Concurrent with the increased interest in apoptosis monitoring, numerous assays have been created to quantify the expression of associated molecular targets such as phosphatidylserine (PS) externalization in the plasma membrane and caspase enzyme activation as well as other apoptotic characteristics including mitochondrial potential changes, chromatin condensation, and DNA fragmentation. However, these assays often require advanced technical expertise, costly instrumentation, and time-consuming analysis using techniques such as western blot, ELISA, and flow cytometry. Here, we present the Orflo Moxi GO II system as a simple, rapid, and effective flow cytometric approach to the study of apoptosis using Annexin V.

Orflo's newest, "Next Generation Flow Cytometer", the Moxi GO II (Figure 1), is ideally and uniquely suited to fulfilling researchers needs for apoptosis monitoring. The system combines the Coulter Principle, the recognized gold standard for precise cell sizing and counts, with simultaneous fluorescent measurements using a 488nm laser, coupled with two PMT detection channels, one filtered to detect at 525/45nm and the other at with 561nm/LP. This fluorescence configuration is ideal for many of the most common fluorophores including phycoerythrin (PE, immunolabeling, Annexin V), propidium iodide (PI for viability), Calcein-AM (Cell Health), FITC (immunolabeling, Annexin V), and GFP (transfection efficiency). The Moxi GO II utilizes a disposable flow-cell architecture, does not require warm-up, runs test in under 10 seconds, and does not require cleaning/shutdown procedures. Its low cost and very small footprint make it easy for researchers' to acquire one for their own lab, placing it on the bench-top or even the cell culture hood. This enables researchers to easily acquire temporal flow data over short or long periods. The result is

an affordable flow cytometer that delivers "Assays on Demand," including apoptosis monitoring with Annexin V.

Example Data – Results and Discussion

One of the most well-characterized, early-stage markers in the cellular apoptosis progression is the translocation of the membrane phospholipid, phosphatidylserine (PS), from the inner to the outer leaflet of the plasma membrane. The detection of PS translocation is achieved through the use of Annexin V, a cellular protein with a high affinity for PS. Because Annexin V is cell-impermeant, only cells with apoptosis-related exposure of PS, or cells with compromised membranes (necrotic or late-stage apoptotic cells), are labeled with this probe. Consequently, by conjugating Annexin V to a suitable fluorophore (e.g. FITC for the Moxi GO II), cells can be labeled for PS in order to distinguish Annexin V+ (apoptotic) from Annexin V- (healthy) populations. Figure 2a provides a representative, user-generated screenshot for a FITC-Annexin V labeled Jurkat sample that was processed on the Moxi GO II. The large fluorescence shifts for the Annexin V+ vs. Annexin V- and PI+ vs. PI- cell populations are clearly resolvable in the Moxi GO II output. In this case, the cell sample was pre-treated through a 4 hour incubation in 20µM camptothecin, a cell toxin known to interfere with DNA replication and, as result, induce apoptosis. That population was then mixed with 50% heat-killed Jurkats to generate a necrotic population for more complete graphical representations of the possible apoptosis states. The resulting mixture reflects the 46.6% late-stage/necrotic cells (upper-right, mostly heat-killed), the 38% healthy cells (lower-right, no stain), and the 14.7% early-stage apoptotic cells (top-left, camptothecin-treatment induced apoptosis). Figure 2b also presents the quadrant-gated data in tabular and bar-chart format for easier interpretation/display of the results. Both the quadrant chart and table chart can be user-printed in BMP format to generate document images (as was done for this app note).

The Moxi GO II has built-in functionality for rapidly comparing the results of two assays from both
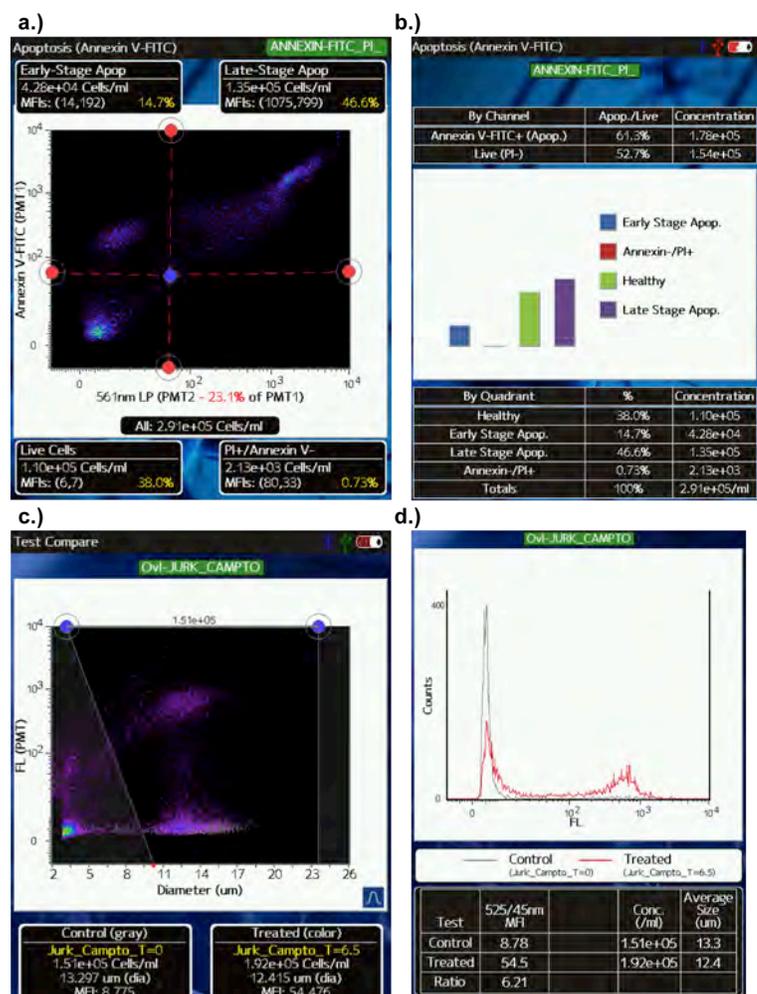
Figure 2 – *a.)* Typical Moxi GO II apoptosis (FITC – Annexin V) scatter plot (FITC vs. PI) output for Jurkat cells treated with camptothecin (20µM, 4hours) mixed with 50% heat-killed cells. The quadrant plots shows the three expected states for the culture: Live, Early-Stage Apoptosis, and Late-Stage Apoptosis. *b.)* Data can be presented in tabular/bar chart format for easy interpretation of states *c.)* The system is capable of generating comparison/overlays of data for two tests. Shown is an overlay of the Annexin V fluorescence vs. cell size scatter plots for a camptothecin-treated (separate from above) Jurkat sample at the t=0 (gray scatter plot) and t=6.5 hour (colored scatter plot) time points *d.)* This data can also be displayed as FITC fluorescence histograms for the t=0 hour (gray curve) and t=6.5 hour (red curve) samples.

visual and quantitative perspectives.   Figure 2c provides an example of the comparison of the Annexin V+ measurements of the Jurkat cells when first exposed to camptothecin (t=0 hour) and after prolonged (t=6.5 hour) camptothecin exposure. Specifically, Figure 2c shows the overlay of the fluorescence (FITC-Annexin V) vs. size scatter plots for the two samples with the initial reading (t=0 hour) shown in gray and the colored scatter plot showing the cells after 6.5 hour of camptothecin treatment. Figure 2d provides the corresponding fluorescence (FITC-Annexin V) histogram overlay for both samples (gray curve is in the initial reading, red curve is the 6.5 hour post-treatment reading). The data here visually shows the clear increase in Annexin V+ cell percentages for the treated sample as well as the corresponding decrease in the mean cell size, a well-defined characteristic of apoptotic cells. This discrimination of the cell size shift is uniquely enabled on the Moxi GO through the precise Coulter Principle sizing of the cells, a feature not available on any other flow cytometers.

Because of the ease-of-use, rapid test and operation time, and the convenience of the Moxi GO II, it is uniquely suited to collection of time-course data. Figure 3 shows the application of the Moxi GO II to the measurement of the time-course of camptothecin-induced apoptosis through measurements of Annexin V expression and PI-based viability.   The green markers represent the calculated (Annexin V+ % minus PI+ %) early stage apoptosis percentages (Annexin V+/PI- %) of the camptothecin-treated sample vs. the negative control (treated with DMSO-only) as a function of the elapsed drug-exposure time. Plotting the data in this manner allows for curve-fitting (sigmoid, dashed green line) to extract the apoptosis activation kinetics (base=5.9%, max=34.2%, rate=35.7%/hr, half time=4hr) of these cells in response to the applied camptothecin dose. The individual PI-based viability measurements (Figure 3 inset) were used to quantify the late-stage
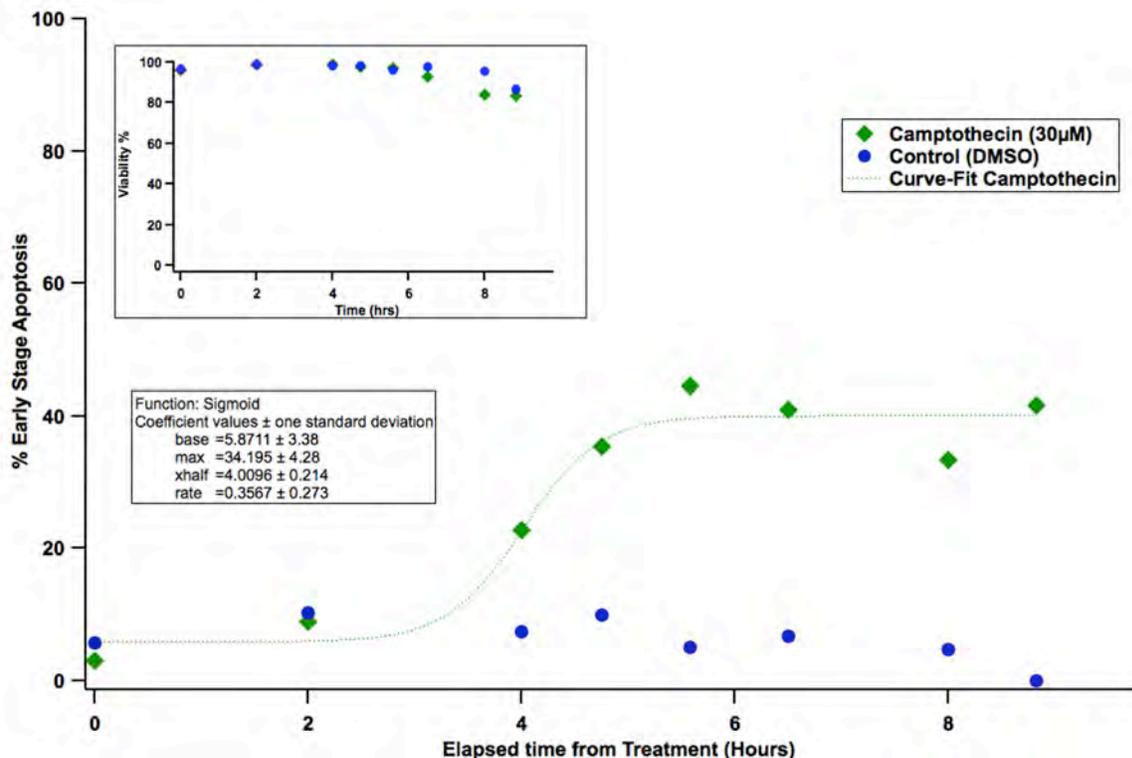
Figure 3 - Time-course of apoptosis induction Jurkat cells. Apoptosis (FITC-Annexin V) was measured on the Moxi GO II at eight discrete time points for a Camptothecin-treated (30μM) sample and a control (DMSO only) sample. (***Inset)*** Shows corresponding viability measurement (PI-permeability) of each sample over the same time course. The data for the treated sample was fit with a sigmoid in order to quantify the camptothecin activation kinetics.

apoptosis/necrotic cell percentages as well as to confirm the viability of both samples over the course of the Annexin V measurements.   The convenience of the Moxi GO II readily enables this type of prolonged time-based analysis, an approach that can be prohibitively expensive (both cost and time) in labs that are not otherwise equipped with continually operated flow instrumentation.

<u>Conclusions</u>

The data in this study shows the versatility of the Moxi GO II in monitoring and quantifying apoptosis. Specifically researchers can measure the apoptosis state of cells by monitoring the translocation of PS from the inner to the outer leaflet of the plasma membrane using Annexin V. Cell viability can be correspondingly assessed using PI. Furthermore, the system provides the capability for monitoring the characteristic size shift of the apoptotic cells (through precise Coulter Principle-based measurements). One of the most powerful features of the Moxi GO II instrument is the ease-of-use and convenience in the collection of data. With an ability to run tests without the need for system warm-up, maintenance, or shutdown procedures, the Moxi GO II is ideally suited to time course experiments, including the monitoring of apoptosis.   Finally, as the Moxi GO II touchscreen GUI is designed to make even the most complex flow analysis accessible to researchers, regardless of their flow expertise. These features should make the Moxi GO II indispensable in any lab performing apoptosis measurements or other cell-based flow cytometry techniques.

References

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Methods

Cell Culture

Jurkat E6-1 (ATCC) were cultured (37°C, 5% CO_2) in RPMI-1640 supplemented with 10% FBS, 1mM Sodium Pyruvate, and 10mM HEPES (all Life Tech.).   For apoptosis induction, 3µL of 10mM camptothecin (Tocris) stock (in DMSO) was added per ml of culture media (30µM final camptothecin concentration). For the negative control, DMSO an equivalent volume of DMSO was substituted for the camptothecin treatment. For generation of a necrotic cell population, healthy Jurkat cells were heat-killed by placing a 15ml centrifuge vials with cells incubation in a 60°C water bath for 10min+. The necrotic population was mixed 1:1 with a Camptothecin treated (20µM, 4hr) population to generate a "three quadrant" Apoptosis example.

FITC-Annexin V Assay

Dual-label data (Figure 2a and 2b) were labeled with FITC-Annexin V (BioLegend, Cat #640905) and Propidium Iodide (2µg/ml) following Orflo's "Moxi GO – Early Stage Apoptosis Monitoring with Annexin V" protocol (below).   After preparation, cells were run on the Moxi GO II system (Orflo Cat#MXG102). For the time course data and overlay data a 1 PMT/channel version of the Moxi GO II system was used. Other than the PMT configuration, the system has the same architecture as the Moxi GO II so performance would be equivalent.   For those samples, a separate prep was simultaneously stained with PI to get the corresponding viability data. For the time-course data, cells were assayed on the Moxi GO (1 PMT version) at eight discrete time points over a ~9 hour period. Annexin V and PI measurements were made at each time point for both the camptothecin-treated and negative control samples.

Screenshots & Data Analysis

Screenshots were all generated directly from the using the built-in system screenshot functionality (exported screenshots appear on system drive as BMPs). Data comparisons/overlays were all performed on-unit using the built-in system functionality for comparing saved tests. Final image cropping and arrangement was performed using Photoshop (Adobe) and Illustrator (Adobe). Time course data was extracted by loading the Moxi GO FCS files into FlowJo 10.2 (TreeStar), running on Mac OSX 10.11. Summary data for apoptosis and viability percentages were loaded into the IGOR Pro (v6.37, Wavemetrics, Inc) analysis package for curve-fitting and final graph generation.

Instrument/Cassettes:
- Orflo Moxi GO II Next Generation Flow Cytometer (Orflo Cat #MXG102)
- Compatible Orflo Cassette (any of the following would work):
  o Type CST (Orflo Cat# MXC040)
  o MF-S (Orflo Cat# MXC020)
  o MF-S+ (MXC030)

Reagents/Components:
- FITC-Annexin V conjugate (e.g. BioLegend, cat#6409075)
- Annexin V Binding Buffer (e.g. BioLegend, cat#422201)
- Propidium Iodide (PI) staining solution (100μg/ml in PBS) – This can be made by diluting Thermo P3566 (1mg/ml PI) 10x with Purified water (e.g. Sigma W4502)
- *Optional/Recommended: Orflo Flow Reagent (Orflo Cat #MXA080)*

Protocol:
Notes:

- *For comparison and compensation purposes, it can be useful to generate a positive control by inducing apoptosis with a pharmacological agent (e.g. 20μM Camptothecin treated, 4+ hours, 37°C for Jurkat cells).*
- *Process a sample of healthy, untreated, cells for use as a negative control.*

1. Isolate cells to a single-cell suspension. (If necessary use a protease and/or pipette trituration to break apart the clusters)
2. Wash cells twice in PBS (2.5mL volume, 300 x *g*, 5 minutes).
3. Re-suspend pellet to 1 x 10^6 cells/ml in Annexin V Binding Buffer (verify counts with the Moxi GO II instrument).
4. Aliquot 100 μl of cells per tube ($\sim$1x10^5 total cells). Mix well before aliquoting. For compensation, prepare three tubes:
   a. PI only sample
   b. FITC - Annexin V only sample
   c. FITC - Annexin V and PI Sample
5. Add 1-5μL of of manufacturer recommended test volume of FITC - Annexin V conjugate (i.e. 2μL for BioLegend Annexin V listed above). *Note: Titration of the Annexin dose might be necessary. Recommended Mfg. volumes are typically 5μL.*
6. Gently vortex (3-4 setting) the cells and incubate for 15 minutes at room temperature (25°C), protected from light.
7. *Optional: To lower the background (improve signal to noise ratios) for bright samples, a 1-2x wash (300xg, 5min) with binding buffer will remove the excess FITC – Annexin conjugate.*
8. Add 300μL of Annexin V Binding Buffer to all tubes.
9. Add 2.5 μl of 100μg/ml Propidium Iodide (PI) to appropriate tubes (target final concentration of 1μg/ml PI). Incubate those tubes for 5 additional minutes.
10. *Optional: Add 8μL of Orflo Flow Reagent to sample (20μL flow reagent / ml of sample)*
11. Run on Moxi GO II using the "*Apoptosis (Annexin V - FITC & PI)*" app within 15 minutes of staining, protect from light.
    a. Adjust size gates to define the cell population.
    b. Touch "X/Y" to select a PMT vs PMT display of the FITC Annexin (PMT1) vs. PI (PMT2) fluorescence.
    c. Adjust for spillover as appropriate using the "Comp" button functionality.