The Effect of Doxorubicin on the U2OS Cell Cycle

by

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ABSTRACT

Treatment of U2OS cells with the chemotherapeutic drug Doxorubicin results in either apoptosis or cellular senescence. The pathway the cell takes is dependent upon the dosage of Doxorubicin administered to the cells. When a 10 μM dose is administered Topoisomerase II is inhibited resulting in double stranded DNA breaks because the DNA is unable to religate during synthesis. This is shown by lower levels of synthesis after analysis with Bromo-2-deoxyuridine (BrdU) and Propidium Iodide (PI) staining. The cells are unable to recover from the severity of this damage and become apoptotic. When a 2 μM dose is applied to the cells, a G2 arrest occurs. This is shown by lower levels of Cyclin B in the G2 phase during flow cytometry analysis and staining with PI. Apoptosis levels are monitored using cleaved Caspase 3 and cleaved PARP. The percentage of 10 μM cells undergoing apoptosis increased steadily over 48 hours, while the 2 μM and untreated cells maintained constant low levels of apoptosis. Both cellular senescence and apoptosis put a halt to cell proliferation.

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Introduction

Cancer is one of the most common causes of death in the world. In the United States cancer kills close to 1,500 Americans each day (Medical News Today). Cancerous tumors arise from actively dividing cells. The high rates of proliferation for these cells put them at a high risk for developing mutations - a main cause of cancer (Campisi, 2003). Cancer is commonly treated with chemotherapy (National Cancer Institute). A commonly used chemotherapeutic agent is Doxorubicin. This particular drug acts by inhibiting the DNA ligase activity of Topoisomerase II (Jarvinen). As such, during replication, the topoisomerase II can cut the DNA double helix but not re-ligate. This creates damage within the cell when the DNA cannot recoil resulting in double stranded breaks. One outcome of DNA damage is apoptosis (programmed cell death). The other outcome is cell cycle arrests. These arrests occur in the cell cycle to allow the DNA to repair before DNA synthesis and mitosis (Dipaola). Our goal is to experimentally determine where these arrests occur. Using flow cytometry we will measure the proteins responsible for controlling the cell cycle and determine how their levels change after treatment with Doxorubicin. This allows us to understand the relationship between the dosage of Doxorubicin administered and the resulting cell cycle arrests and apoptosis.

The cell cycle is comprised of four different stages – G1, G2, S and M. During these phases DNA is replicated and mitosis occurs, creating a new cell. Cyclins and cyclin dependent kinases (cdks), control the progression through the cell cycle (Senderowicz). Kinases transfer phosphate groups from ATP to protein substrates. This post-translational modification alters the activity of the kinase substrate. Cyclin dependent kinases are activated by cyclins and proceed by phosphorylating serine and

threonine residues. During G1, the target of the cdks is the retinoblasta protein (pRb), which monitors transcription (Schafer). The cdks work with four cyclins involved in the cell cycle – cyclin D, cyclin E, cyclin A, and cyclin B. Cyclin D acts with cdk 4, E and A with cdk 2 and B with cdk1. This can be seen in Figure 1 below.

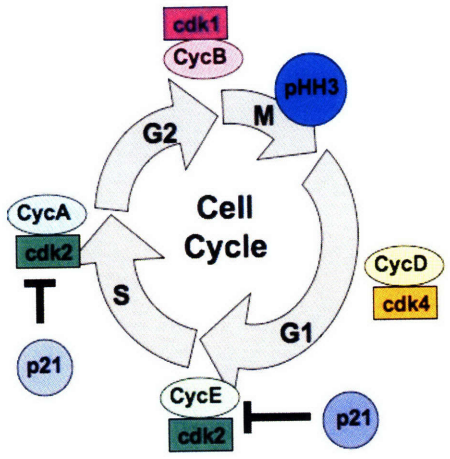

Figure 1. Cell Cycle – The cell cycle is comprised of four stages: G1, S, G2 and M (mitosis). The cyclin-CDK complexes drive the cell through the different phases of the cell cycle as indicated. P21 inhibits the cyclin-CDK interaction. pHH3 acts as a marker for mitosis.

Each cyclin is also responsible for a different cell cycle transition. Cyclin D activates the G0-G1 transition, cyclin E promotes the transition between G1 and S, cyclin A advances the transition between S and G2 and cyclin B the progression from G2 to mitosis.

Alterations in cell cycle progression result in unregulated cell growth known as cancer (Kastan). When the enzymatic activity is too high the cdks detach from their cyclin. The cyclin and cdk complex activity is modulated by the cell cycle inhibiting proteins p21, p27 and Cdc25. The first stage of the cycle, G1, is regulated by p21 and p27, which bind to cyclin D/cdk 4 complexes and cause a G0/G1 arrest (Vermeulen). The p21 protein also moderates the S phase and the G2/M transition. The phosphatase Cdc25 controls entry and progression through the S phase by removing cdc2 inhibitory

phosphates (DiPaola, 3311). Many cancers show an over expression of Cdc25 (Kristjansdottir).

Levels of p21 and p27 are controlled by p53 (Maki). p53 is a transcription factor responsible for regulating the cell cycle. Because of its inhibitory qualities it is also called a tumor suppressor (IMCB). In response to DNA damage in G1, p53 causes a G1/S phase arrest allowing the DNA to repair before replication. If the damage is too significant to be fixed the cell becomes apoptotic (Schafer). Many human cancers have mutations in p53. When p53 is suppressed or inactivated cells grow at higher rates and are not checked to ensure proper configurations (Moreno). This causes rapid cell proliferation, often inducing tumors that result in cancer. In order to ensure that the cell does not enter mitosis with DNA damage present the checkpoint homologs, Chk1 and Chk2, are used to stop the cycle until the damage has been repaired (Kastan).

Sometimes the DNA damage present in the G2 phase is too significant to be repaired so the cells remain in an arrested state and do not enter mitosis. This cell cycle arrest leads to non-dividing cells containing double the amount of DNA (4N). These arrested cells are called senescent. Senescence is similar to apoptosis because it is a stress response. Unlike apoptosis it does not result in death, instead halting the cell cycle to keep cells from proliferating (Serrano). DNA damage, like that occurring during chemotherapeutic treatments is thought to be a possible cause of cellular senescence (Campisi, 2005).

One of the chemotherapeutic treatments for cancer is Doxorubicin. This drug works by inhibiting the protein topoisomerase II (Jarvinenen). Topoisomerase II cuts both strands of DNA during replication in order to relieve topological stress caused by

the unwinding of the DNA double helix. Doxorubicin stabilizes the topoisomerase after it has broken the DNA strands, preventing them from re-ligating. This stops DNA replication from occurring and generates DNA strand breaks. Doxorubicin can cause both cell cycle arrest and apoptosis when used in different dosages depending on the severity of the DNA damage (Zhou). Apoptosis can be caused by severe DNA damage like double stranded breaks while lesser amounts of damage result in cell cycle arrests.

Our goal is to determine how Doxorubicin affects the U2OS cell cycle. Our U2OS cells come from the human osteosarcoma U2OS cell line. This is one of the most commonly used cell lines in biology research because its protein expression is well known (Niforou). Flow cytometry will be used to monitor the progression of treated cells through the cell cycle. PI-BrdU staining of the U2OS cells shows progression through the cell cycle by monitoring DNA synthesis. Bromo-2'-deoxyuridine (BrdU) is incorporated into the DNA during replication (S-phase) (Darzynkiewicz). When these cells are stained with propidium iodide (PI) and run through flow cytometry the percentage of cells falling into the G1, S and G2/M phases is determined. PI is a marker for nucleic acids (Molecular Probes). After DNA synthesis the number of nucleic acids in the cell doubles and this size change is detected and used to monitor cell cycle progression. This tells us the fraction of replicating cells. We will also monitor cell cycle arrests using flow cytometric analysis. The amounts of Cyclin B present during each stage of the cell cycle determines if cells are actively progressing. Cyclin B is needed to advance from G2 to Mitosis. High Cyclin B levels show cells in G2, while low levels represent G1. Cyclin B also monitors senescence. Cells undergoing mitosis are marked by the presence of pHH3 (phospho-histone H3) (Colman).

We will determine whether the cell has entered apoptosis by simultaneously monitoring cleaved caspase 3 and cleaved PARP antibodies, both of which are markers for programmed cell death (Boulares). Monitoring for apoptosis will occur at three time points (12, 24 and 48 hours). The cell cycle's progression will be monitored at eight different time points over the span of 48 hours (2,4,8,12,16,24,36,48). This determines how the DNA damage caused by different dosage levels of Doxorubicin affects the cell cycle over a fixed set of time. After we have determined where the cell cycle arrests occur and where apoptosis is most prevalent it will be possible to predict how different treatments will affect cell cycle arrests and induce cell death.

This research will create a model of treated U20S cells during the cell cycle by monitoring the cell's progression through each stage. Since we are treating with Doxorubicin the cell's reaction to the drug will be used to simulate a model of how the cell reacts. As the dosages are altered and the progression of the cells monitored it is possible to determine how the level of Doxorubicin affects when the cell decides to arrest or enter apoptosis. While the cell cycle has been studied in detail this model of the system allows us to predict how the cell will react to a given treatment before it is actually administered.

Materials and Methods

Plating

U2OS cells were grown in Dulbecco's Modified Eagle's media supplemented with 10% Fetal Bovine Serum, 1% Penicillin, 1% Streptomycin and 2 mM Glutamine. Cells were incubated at 37°C in 5% CO2. For the experiment 4x10^6 cells were plated per 15 cm plate. Each time point corresponded to one 15 cm plate.

Doxorubicin Treatment

24 of the plates were used for treatment with 1uM Doxorubicin with time points at 2,4,8,12,16,24,36 and 48 hours. The treatments were applied in three doses 2uM, 10uM and Untreated (UN) split evenly among the plates. Four hours into the time course the media was swapped for DME + 1% FBS, 1% Pen/Strep and 1% Glutamine.

9 plates were used for apoptosis analysis at 12,24 and 36 hour time points. Three doses of doxorubicin were applied in 2uM, 10uM and Untreated increments to three plates each.

Ethanol Fixing

The plated cells were harvested and the media was stored on ice in a 50mL Falcon tube. The cells were washed with 10mL cold PBS and added to the tube of media. 2mL of trypsin were added to the plate and it was incubated at 37°C for 5 to 10 minutes. Using the PBS/media mixture the trypsinized cells were washed from the plate and added to the Falcon tube. The tube was spun down for 5 minutes at 1500 RPM in the swinging bucket rotor. The supernatant was removed and the cells washed with 1mL PBS and transferred to eppendorfs. The cells were washed again and then resuspended in 80% EtOH. The tubes are stored at -20°C.

Methanol Fixing

The plated cells were harvested and the media was stored on ice in a 50mL Falcon tube. 10mL of PBS was used to wash the cells and added to the Falcon tube. The cells were trypsinized with 2mL of trypsin and incubated at 37°C for 5-10 minutes. The trypsinized cells were washed with the PBS/media solution and added to the Falcon tube. The tube was spun down for 5 minutes at 5000 RPM in the swinging bucket rotor. The

supernatant was removed and the cells washed with 1mL of PBS then transferred to eppendorfs. The cells were rewashed with cold PBS and resuspended in 200uL 4% formaldehyde and incubated for 15 minutes at room temperature. The cells are washed with PBS and resuspended in 200uL of 100% methanol. The cells are stored at -20°C.

Cell Titer Glo – Viability Testing

100 μL of ethanol harvested cells were placed in 96 well plates for testing and equilibrated to room temperature for 30 minutes. The CellTiter-Glo Luminescent Viability Assay was used. The CellTiter-Glo Buffer was thawed to room temperature. 10mL of Buffer was transferred to the CellTiter-Glo Substrate, reconstituting the enzyme/substrate mixture, creating the reagent and mixed by vortexing. 100μL of reagent was added to each well, and the contents mixed on an orbital shaker for 2 minutes. Plate stabilized at room temperature for 10 minutes. Luminescence was recorded.

BrdU/PI Flow Cytometry

20uL of 10mM BrdU was added to the plated cells one hour before harvesting. After the time course was complete 200uL aliquots of all 24 time points were put into eppendorfs and spun down for 1 minute at 5000 RPM. The supernatant was removed and the cells washed with 1mL of a PBS-OBB solution containing 50%PBS and 50% Odyssey Blocking Buffer (OBB). The cells were spun down and incubated in 1mL of 2M HCL/0.5%Triton X-100 for 30 minutes at room temperature. The HCl was removed and 1mL of 0.1M $Na_2B_4O_7$ pH 8.5 added to each eppendorf and incubated at room temperature for 2 minutes. The $Na_2B_4O_7$ is removed and the cells were washed with 1mL of PBST-OBB containing 50% PBS-Tween and 50% OBB. A primary antibody solution of 1.3 mL PBST-OBB and 13uL mouse anti-BrdU antibody was made. 50uL of this

solution is added to each eppendorf. The samples are incubated overnight at 4°C. A secondary antibody solution is made with 1.3mL PBST-OBB and 13uL Goat anti-Mouse 647 antibody and incubated for 1 hour. The cells are washed twice with PBST and then 50uL of the secondary solution was added. The cells incubated 1 hour at room temperature and were then washed twice. The Propidium Iodide (PI) solution was made with 10mL PBS, 100uL of 10mg/mL PI and 10uL Sigma RNAse A. 400uL of the PI solution was added to each tube and they were transferred to flow cytometry tubes and stored on ice.

Cleaved Caspase 3/ Cleaved PARP Flow Cytometry

Aliquots of 200uL of the apoptosis time points were put in eppendorfs. The cells were spun down at 5000 RPM for 1 minute and the supernatant removed. The cells were washed with 200uL PBST. A primary antibody solution was made with 900uL PBST-OBB, 4.5uL rabbit cleaved caspase3 (BD 559565) and 4.5uL of mouse cleaved PARP AlexaFlour 647. 50uL of the primary antibody solution was added to each of the eppendorfs and the samples were incubated overnight at 4°C. The cells were washed with 200uL PBST. A secondary antibody solution was prepared with 900uL PBST-OBB, 4.5uL goat anti-rabbit 488 IgG (Invitrogen A11008). 50uL of the secondary solution was added and the cells were incubated overnight at 4°C. The cells were washed, resuspended in 200uL PBST and transferred to Flow Cytometry tubes.

Cyclin B/ pHH3 Flow Cytometry Analysis

Aliquots of 200 µL of the ethanol fixed U2OS cells were added to eppendorfs. The cells were spun down at 5000 RPM for 1 minute and the supernatant was removed. The cells were washed with 200 µL PBST. A primary antibody solution was made with

26 μL rabbit Cyclin B antibody (SC-752) added to 1.3mL PBST-OBB containing 50% PBS-Tween and 50% OBB. 50 μL of the primary antibody solution was added to each eppendorf and the cells were incubated overnight at 4°C. A secondary antibody solution was prepared with 13μL goat anti-rabbit 488 IgG (Invitrogen A11008) and 1.3mL PBST-OBB . 50 μL of this solution was added to the eppendorfs and incubated for 1 hour at room temperature. The cells were washed and resuspended in 200 μL PBST. The Propidium Iodide (PI) solution was made with 10mL PBS, 100uL of 10mg/mL PI and 10uL Sigma RNAse A. 400uL of the PI solution was added to each tube and they were transferred to flow cytometry tubes and stored on ice.

Flow Cytometry

Flow Cytometry is used to analyze cells suspended in a fluid stream. A beam of light is passed through the liquid and numerous detectors are aimed at it in order to analyze the cells in suspension. One is in line with the beam to detect for forward scatter (FSC) and others are located perpendicular to the flow and used to analyze the side scatter (SSC). Also numerous fluorescent detectors are present to analyze fluorescent emissions from staining in the sample. Analyzing the emission peaks from the detectors relates specific information about the cell. The FSC is used to determine the cell volume and the SSC determines inner complexities, such as area (BD Biosciences).

A flow cytometer is used to analyze cells at a rapid rate. Using a high-throughput system the cells are passed through the light beam and monitored by detectors to analyze the cell's progression through the cell cycle. Antibodies marked by fluorescence are added to and incorporated into the cell. When the cells are passed through the light beam the antibody levels are detected. By using antibodies responsible for integral parts of the

cell cycle, i.e. the transition between cell cycle stages or markers for each stage, it is possible to tell how far progressed each cell is (Flow Cytometry Principles).

The flow cytometry results are then analyzed using a program called FlowJo. This allows us to change the axes and sort the cells appropriately. The intact cells are sorted by graphing the SSC vs. FSC. To determine which of the intact cells are single cells and not groups, the area and the height are compared. The cells can then be analyzed to determine the specifics of the cell cycle progression.

Results

Treating U20S cells with Doxorubicin in three dosage amounts produces two distinct results. Analysis after harvesting the cells at eight time points over forty-eight hours shows a noticeable trend in both the 2 μM and 10 μM treated cells While the untreated cells grow and divide becoming more confluent the treated cells respond differently to Doxorubicin. Instead of proliferating the 10 μM U2OS cells become apoptotic, and the 2 μM cells enter a G2 phase cell cycle arrest (Figure 2).

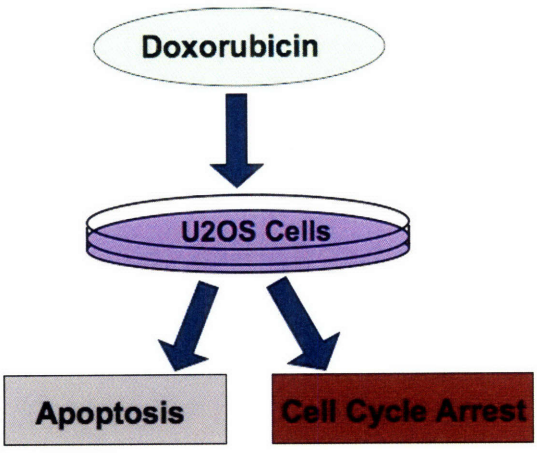

Figure 2. Cellular Outcome after Doxorubicin Treatment – Treating U2OS cells with doxorubicin results in either cell cycle arrest or cell apoptosis in a dose dependent manner.

Apoptosis

U2OS cells apoptose in response to Doxorubicin (Igney). Apoptosis is monitored in two ways; by using a cell titer glow assay to determine viability and also by using the cleavage of Casp3 and PARP as markers of apoptosis. The graph in Figure 3 shows the results of the Cell Titer-Glo assay.

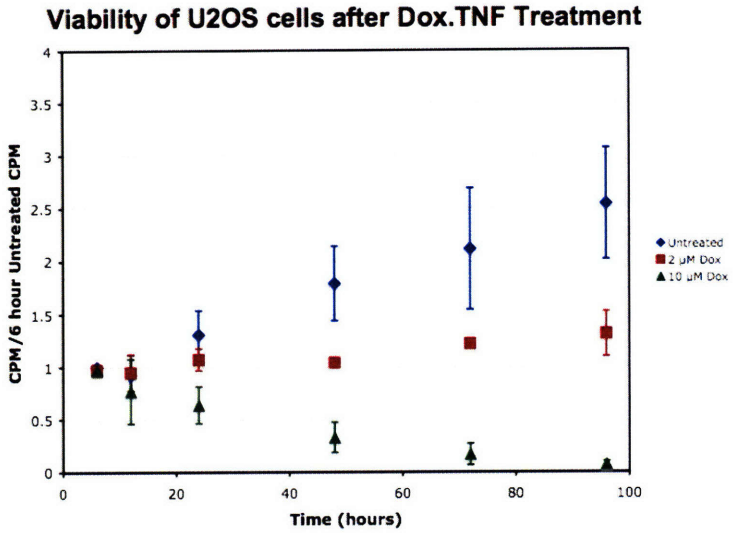

Figure 3. Cell Viability Using Cell Titer Glo Assays
This graph compares the cell viability of Untreated, 2 μM treated and 10 μM treated Doxorubicin cells over a 48 hour time period.

Over the duration of the time course the percent of untreated viable cells grew. This shows the cells were still proliferating. The viability levels of the 10 μM treated Doxorubicin cells had a trend opposite to the untreated, with almost no viable cells after 48 hours. Instead of undergoing mitosis and replicating the high dosage of Doxorubicin causes apoptosis in 10 μM treated U2OS cells. The 2 μM treated cells did not have a distinct growth or decline in cell viability. This suggests that the cells under went a cell cycle arrest.

To determine the percentage of cells undergoing apoptosis after treatment with 2 μM and 10 μM doxorubicin compared to untreated cells, flow cytometry analysis was done using Caspase 3 and PARP (Poly ADP ribose polymerase). Apoptosis levels were monitored using the cleavage of Caspase 3 and PARP. Both Caspase 3 and PARP are markers for programmed cell death or apoptosis (Boulares). The methanol fixed U2OS cells (12, 24 and 48 hours) were marked with cleaved Caspase 3 (cCasp3) and cleaved PARP (cPARP). Figure 4 shows the flow cytometry results at 24 hours (entire time course in appendix 1).

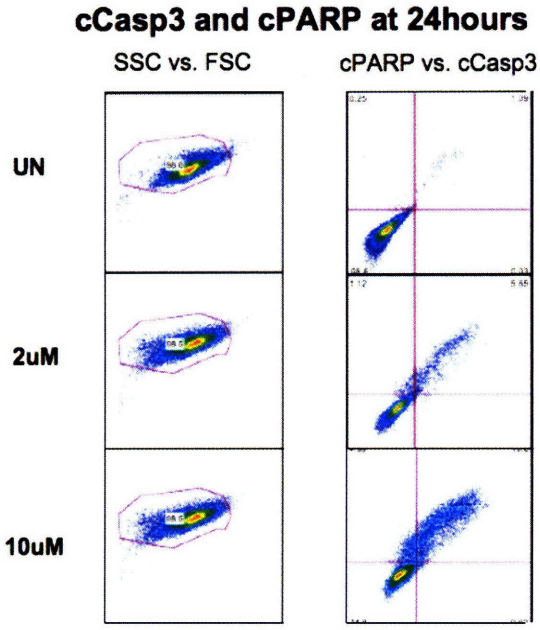

Figure 4. U2OS cells apoptose after treatment with doxorubicin.
Cleavage of caspase 3 and PARP was monitored by flow cytometry. The left panel shows the side scatter vs. forward scatter plot. Gating of intact cells is shown (circle). The right panel shows the cleaved PARP vs. cleaved caspase 3 plots. Cells positive for both cCasp3 and cPARP (upper right quadrant) are apoptotic.

As the dosage of Doxorubicin increased the number of cells positive for both cleaved Casp3 and cleaved PARP increased, as can be seen in the upper right quadrant of the cPARP vs. cCasp3.

The increased percentage of cells entering apoptosis can be seen in Figure 5.

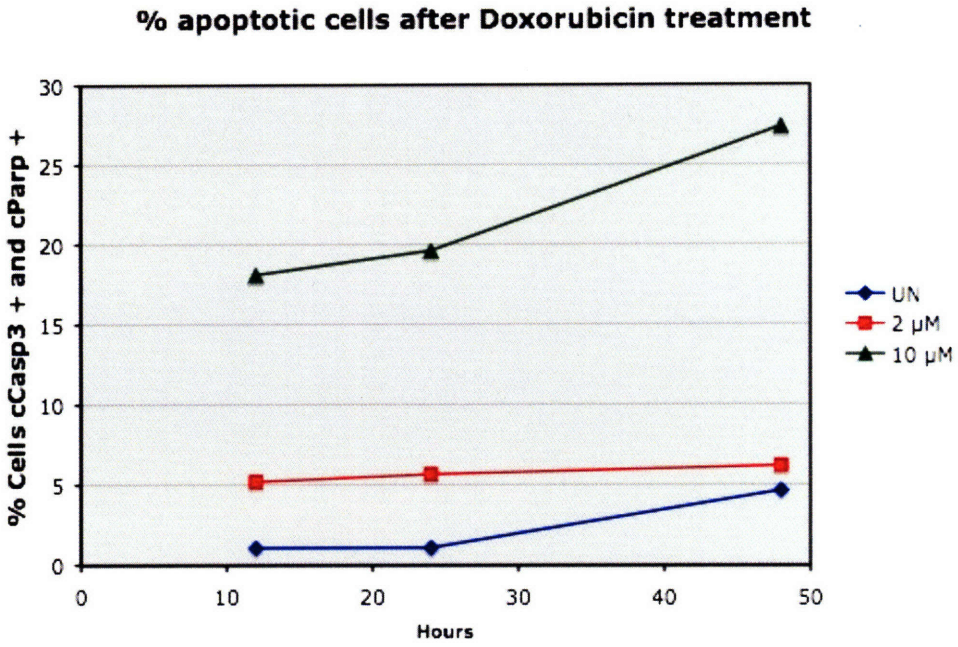

Figure 5. Graph of cCasp3 and cPARP over 48 hours – This graph compares the amount of cell death at 12, 24 and 48 hour time points for the three dosage amounts – UN, 2uM and 10uM.

The readings were taken at three time points – 12, 24 and 48 hours. The untreated cells have the lowest percentage of apoptosis with only 5% of cells becoming apoptotic after 48 hours. This could be due to overcrowding on the plates when the cells became too confluent. The 2µM cells show little change over the 48-hour time course. This suggests that the cells undergo some sort of arrest, preventing them from replicating but resulting in a fate other than cell death. The 10 µM cells show a constant increase in the percentage of apoptosed cells. From 12 hours on the percentage of cells positive for both cCasp3 and cPARP is over 17%. This is three times higher than the other treatments. This leads us to believe that U2OS cells are not able to recover from such a high dosage of Doxorubicin.

Cell Cycle Arrest

U2OS cells treated with 2μM Doxorubicin do not only apoptose, but also cease replicating because of a cell cycle arrest. To monitor the progression of the cells through each phase of the cell cycle the U2OS cells were treated with BrdU one hour before harvesting to allow for incorporation. The level of incorporation after this addition was monitored by flow cytometry analysis of the harvested cells using PI staining. PI staining monitors nucleic acid levels in the cell (Molecular Probes). As cells go through the cell cycle and DNA is synthesized, the amount of nucleic acid present doubles. Staining with PI shows this growth and allows grouping of the cells based upon cell cycle phase. This progression of the cells and the gating for analysis at 24 hours is seen in Figure 6 (entire time course in appendix 2).

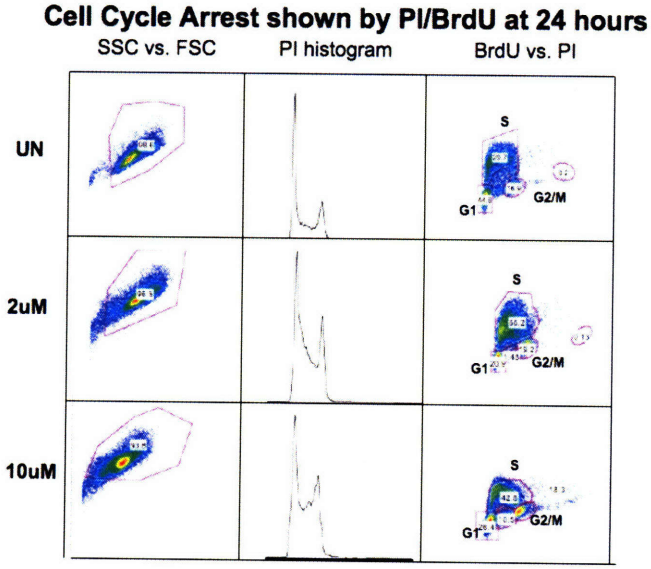

Figure 6. Cell Cycle Arrest shown by PI-BrdU analysis at 24 hours.
To monitor progression through the cell cycle cells incorporation of BrdU and DNA content were monitored by flow cytometry. The left panel shows gating (circle) for intact cells. The center panel shows the levels of PI incorporation. The right panel shows the percent of cells in each phase of the cell cycle. Data for cells harvested 24 hours after treatment is shown. Cells treated with 2 μM Doxorubicin exhibit a pronounced arrest in the G2 phase of the cell cycle.

The left panel shows the selection of intact cells similar to the gating in Figure 4. This gating allows us to separate apoptosed cell particulates from the intact cells still progressing through the cell cycle. The right hand panel shows the cells' progression.

BrdU is incorporated during the S phase, so cells still undergoing DNA synthesis show increased BrdU levels. All three of the dosage amounts show an uptake of BrdU during the S phase, but the 10 μM cells have a lower percentage of cells actively synthesizing new DNA. While the untreated cells show a normal progression between the phases, the 2 μM cells exhibit a pronounced arrest during the G2 phase. The cells are undergoing synthesis, as can be seen by high levels of BrdU incorporation, but are not dividing. The cell cycle arrest over time is shown in the graphs in Figure 7.

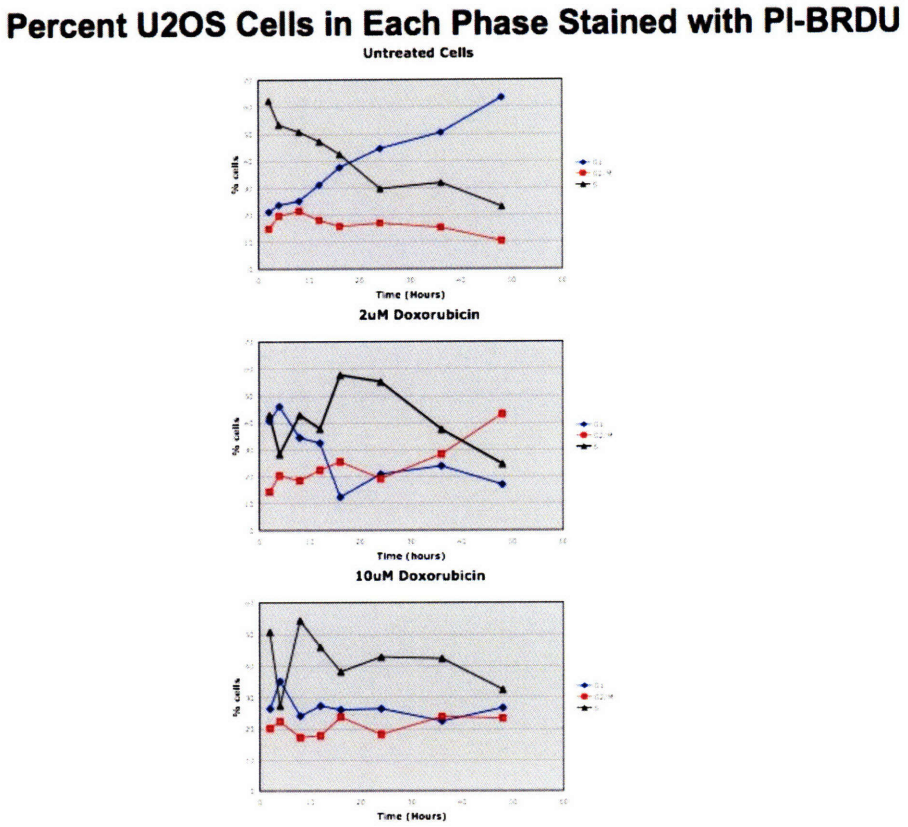

Figure 7. U2OS cell cycle dynamics after doxorubicin treatment.
Untreated cells (upper panel) rise in G1 with increasing time. 2 μM cells (center panel) rise in G2. 10 μM cells show no significant growth or declines after 8 hours.

The top graph shows the percentage of untreated cells in each phase of the cell cycle over the 48 hour time period. The number of cells in G1 increases as time goes on, showing that cells have replicated, divided and are reentering the cell cycle. The 2 μM cells in the center panel exhibit the G2 arrest starting at 24 hours, where the percentage of cells in G2 increases at a constant rate, while the percentage of cells in G1 declines slightly and S declines at a rapid pace. The cells are reaching the G2 stage and undergoing a cell cycle arrest. This is caused because there is enough DNA damage to halt the cell's progression through the cycle, but the damage is not severe enough to result in apoptosis. This also shows that the 2 μM treatment's effect on the cell cycle is less harsh than the 10 μM, shown in the bottom graph. At this dosage the cells remain constant in G1 and G2 with a lower percentage in S because the cells are too damaged to be repaired and enter apoptosis.

G2 Phase and Mitosis

Cyclin B works with CDK1 to progress the cell from the G2 phase to mitosis. High cyclin B levels show that the cell is currently in the G2 phase. Analysis by flow cytometry using the ethanol fixed U2OS cells with Cyclin B as a marker for G2 determines what stage of the cell cycle the cells are in. This is shown for the 24 hour treatment in Figure 8 (entire time course in appendix 3).

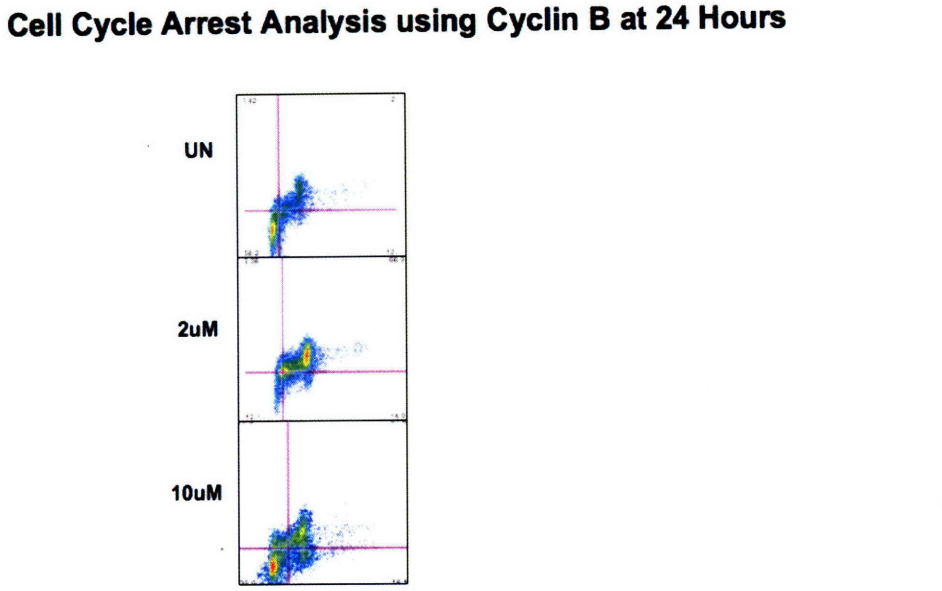

Figure 8. Cyclin B levels drop in U2OS cells arrested in G2 after 2 μM Doxorubicin treatment. The upper panel shows Cyclin B level plotted against PI staining for untreated U2OS cells. Cyclin B is low in G1, increases through S phase and peaks in G2. Cells arrested after treatment with 2 μM doxorubicin develop a population that is 4N but low in cyclin B. Cells treated with 10 μM doxorubicin do not show the normal progression through the cell cycle, with a large portion below the G1 phase.

The lower left quadrant shows low Cyclin B levels, which is expected since progression through G1 is controlled by Cyclins D and E. As the cell cycle continues into S and G2, the Cyclin B levels increase, as can be seen in the upper right hand quadrant. This shows more cells being trapped in the G2 phase. The percentage of cells in G2 is much higher in the 2 μM Doxorubicin treated cells than the untreated. The 10 μM cells have a lower percentage of cells positive for Cyclin B with most cells accumulating below the G1 phase. These cells are apoptotic.

The 2 μM treated cells show an accumulation of cells in the G2 phase that are not positive for Cyclin B. This lack of Cyclin B means the cells will not progress into mitosis completing the cell cycle, instead remaining in the G2 stage as 4N cells. This halt in progressions is called senescence. When cells become senescent they enter a permanent cell cycle arrest. In this study the cells enter senescence because the 2 μM

Doxorubicin has caused significant DNA damage, but unlike the 10 μM treatment is not severe enough to result in apoptosis.

Another marker to show that Doxorubicin treated U2OS cells fail to enter mitosis is shown using pHH3 as a marker during flow cytometry. Positive levels of pHH3 indicate the cells are mitotic (Colman). Figure 9 shows the 24 hour untreated, 2 μM treated and 10 μM treated cells after analysis with pHH3 (entire time course in appendix 4).

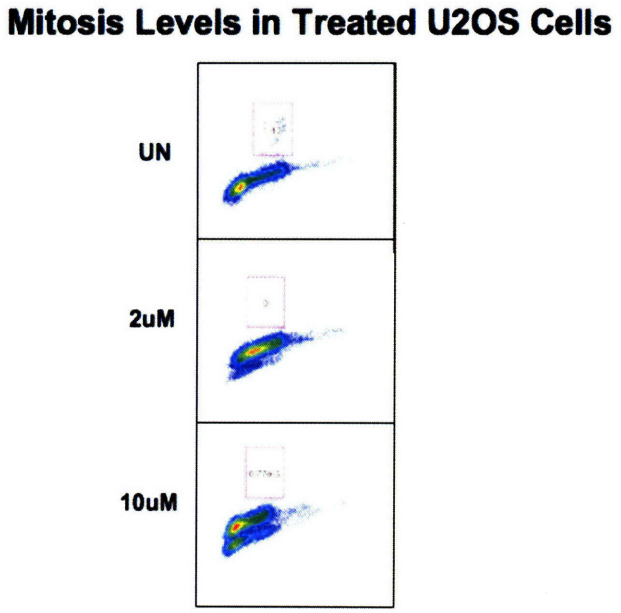

Figure 9. pHH3 Levels Identify Cells in Mitosis at 24 hours.
pHH3 is a marker in the cell cycle for cells going through mitosis. The top box shows the number of untreated cells in mitosis (pink ring). The middle box shows 2 μM cells and the lower box 10μM cells, neither of which show signs of mitosis.

While the untreated cells have a small percentage of cells in mitosis they show a steady cell progression compared to the 2 μM and 10 μM cells. The Doxorubicin treated cells' lack of pHH3 expression shows that advancement through the cell cycle has ceased. The 2 μM cells have entered cellular senescence while the 10 μM cells have undergone apoptosis.

Treatment with Doxorubicin in different dosages has two possible responses in U2OS cells. These different results are shown in Figure 10.

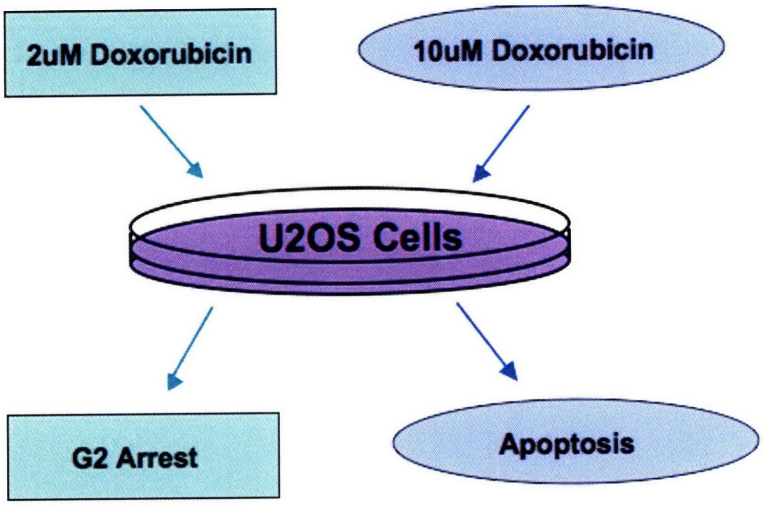

Figure 10. Doxorubicin Treatment Results – Treating U2OS with 2uM Doxorubicin results in a permanent cell cycle arrest in the G2 phase. Treating cells with 10uM Doxorubicin results in cell apoptosis.

When the cells are treated with a low (2 μM) dosage of Doxorubicin, they exhibit a G2 phase cell cycle arrest. The low Cyclin B levels seen in these cells show cellular senescence. The 10 μM treated cells suffer too much DNA damage to simply undergo a G2 arrest. The PI/BrdU staining shows a decrease in synthesis levels for these cells. Cells treated with a high dosage of Doxorubicin (10 μM) are no longer able to replicate their DNA. Instead these cells become apoptotic. For both the 2 μM and 10 μM treatments the cells are no longer capable of proliferation.

Discussion

U2OS cells treated with Doxorubicin either apoptose or enter a cell cycle arrest. The cell's outcome depends on the level of Doxorubicin administered. Cells treated with the higher level, 10 μM, cease replicating and enter apoptosis. These cells have sustained

a level of DNA damage too high to repair. A lower dosage of 2 μM does not cause cell death, instead resulting in a G2/M cell cycle arrest. In 2 μM cells this arrest is permanent and is referred to as cellular senescence. Senescent cells do not apoptose and instead are removed by natural killing cells (Serrano).

Analysis using cCasp3 and cPARP shows a significant increase in apoptosis for cells treated with 10 μM Doxorubicin over the 48 hour time period. The untreated and 2 μM treated cells do not have this high percentage of cell death. Instead of entering mitosis the cells undergo programmed cell death. This is caused by increased amounts of DNA damage. The data also shows a decreased level of synthesis in the 10 μM treated cells. This is seen in Figure 6's flow cytometry analysis of the BrdU levels versus PI staining. The lower number of cells going through synthesis means that the Doxorubicin is inhibiting Topoisomerase II (Jarvinen). Topoisomerase II is responsible for religating the two strands of DNA and when inhibited synthesis is stopped. When Doxorubicin is administered in high dosages the cells cannot repair the damage caused by the inactivation of Topoisomerase II and enter apoptosis.

The 2 μM synthesis levels were lower than the untreated cells but the cells were not damaged severely enough to become apoptotic. Instead, the cells enter an arrested state during the G2 phase of the cell cycle. The G2 phase is one of the checkpoints for cellular progression as monitored by Chk1 and Chk 2. If the cells exhibit DNA damage, replication is halted allowing time for DNA repair to occur. In the 2 μM treated cells, damage is not repaired and the process is permanently halted. Arrested cells accumulate in the G2 phase as seen in the BrDU vs. PI graphs shown in Figure 7. The cells are too damaged to go through mitosis and replicate, but not damaged enough to apoptose.

Analysis using Cyclin B vs PI staining gives a clearer view of how the 2 μM cells arrest. Cyclin B is responsible for the cell's progression through the G2 phase and into mitosis. The cell's location in the cell cycle is shown by analysis with Cyclin B antibodies, the cell's place in the cell cycle is shown, as shown in Figure 8. The untreated cells show a normal progression, with cells starting with low levels of Cyclin B in G1 that rise as the cells enter G2 and mitosis. The 2 μM cells have a different progression. The earlier time points show the cell's normal advancement from G1 to G2 with an elevated incorporation of Cyclin B in the G2 phase. As the time points progress, the cells enter the G2 phase but lack the positive Cyclin B levels.

Decreased Cyclin B levels mean that the cell is unable to progress further into mitosis. These cells are not apoptotic, instead stuck in a permanent G2 arrest. This arrest has cells with double the amount of DNA (4N) and the cells are referred to as senescent. Senescent cells are a stress response to DNA damage. Our data shows that 10 μM treatments are too high to cause cellular senescence, but the 2 μM treatments cause enough DNA damage to result in a permanent cell cycle arrest but avoid apoptosis. Senescence, like apoptosis, is an effective method of treating cancer. When cells become senescent tumor growth ceases. When progression stops, the tumor cells are attacked by neutrophils, macrophages and other killer cells within the body. These eventually destroy the halted tumor cells (Serrano). This means that higher dosages of Doxorubicin are not necessarily needed in chemotherapeutic treatments.

The PI vs BrdU flow cytometry analysis showed the levels of synthesis over the 48 hour time course, allowing comparisons between the different treatment levels. Similar to the 10 μM treatments the 2 μM cells also show a decrease in synthesis levels

over time, just declining at a slower rate than the stronger dosage. The FlowJo gating grouped the cells into the different cell cycle phases. This shows accumulation of untreated cells in G1, 2 μM in G2 and a low level of synthesis for the 10 μM treated cells.

Treatment resulting in apoptosis and cell cycle arrest means the cells are not progressing through mitosis. The percentage of cells still replicating is shown using pHH3, and after Doxorubicin is added the treated cells show little to no mitotic cells. This reinforces that both dosages of Doxorubicin are successful in halting the cell's progression by inhibiting replication of damaged cells. While the 2 μM and 10 μM treatments aid in tumor regression they do so via different methods. During apoptosis the cells die quickly, and the cell's contents are cross-linked and removed by scavenging cells. This means that inflammatory reactions do not occur (Campisi, 339). As previously stated, cells in a fixed arrest state are consumed by macrophages and other killer cells.

Cancer cell proliferation and growth occurs when damaged cells are able to replicate in large amounts (Campisi, 2005). Doxorubicin is able to control how the cells progress through the cell cycle and even inhibit growth and replication. Over the 48-hour time period, U2OS cells treated with 10 μM Doxorubicin went from a level of high confluence at the start of treatment, to low viability with over 25% of the cells positive for cCasp3 and cPARP, signaling apoptosis. The synthesis levels were also significantly reduced during 10 μM treatments. The high levels of apoptosis and low levels of synthesis show that at high dosage levels cells cannot properly function or repair DNA because the damage is too severe.

While the high treatment levels resulted in apoptosis, other inhibitory effects caused by Doxorubicin occurred after the 2 μM treatments. U2OS cells treated with this lower dosage amount were seen to undergo a permanent G2 arrest called cellular senescence. These senescent cells have 4N DNA, as shown by PI staining, but do not have positive levels of Cyclin B, the normal marker for G2 progression. With a lack of Cyclin B the cells are unable to leave the G2 phase and enter mitosis. This inability to enter mitosis is further validated by the pHH3 data where little to no cells progressed through mitosis. Cellular senescence is an effective break on tumor progression, because naturally occurring cells remove the cancerous ones, and the permanent halt acts as a successful stop to proliferation (Serrano).

While the processes of apoptosis and cellular senescence are very different, they are controlled through the same regulatory mechanisms. Both processes are monitored by the p53 and Rb pathways (Campisi, 2003). The p53 pathway is responsible for regulation by starting the transcription of important genes. The Rb pathway is actually responsible for controlling the cell cycle's progression and therefore regulates cellular senescence. Because the Rb and p53 pathways overlap at times, Rb is also responsible for apoptosis. The p53 protein is also very important in sensing stress (Serrano). Since tumors are stressful environments the p53 regulatory system is needed to monitor cellular stress and ensuing DNA damage.

These results show that it is possible to stop cellular progression through the cell cycle using two different methods to halt cell proliferation. The two responses are controlled by the dosage of Doxorubicin applied to the cells. 2 μM treatments are capable of preventing the cells from replicating by holding them in the G2 phase. These

senescent cells lack Cyclin B, which allows them to enter into mitosis, and therefore remain in a stable arrested state. When the dosage level is increased to an amount of 10 μM a significant level of DNA damage occurs, resulting in apoptosis. While these different treatment options both halt the cell's progression they use different mechanisms of action to produce this same result. Cells that apoptose have undergone programmed cell death and lost all cell viability. Senescent cells are intact cells in the 4N stage that are unable to enter mitosis and proliferate. Scavenging cells take care of the dead apoptotic cells while Natural killer cells consume the senescent cells.

U2OS cells treated with Doxorubicin in either 2 μM or 10 μM dosages experience a stop in cell proliferation. For the 2 μM cells, the DNA damage is not severe enough to cause apoptosis and the cells enter a permanent G2 phase arrest called senescence. These senescent cells lack Cyclin B and are unable to enter mitosis. Administering a 10 μM dose of Doxorubicin to U2OS cells inhibits the ligase activity of Topoisomerase II and causes apoptosis. Both treatments stop cellular replication causing a halt in tumor growth and therefore might act as successful stops in cancer development.

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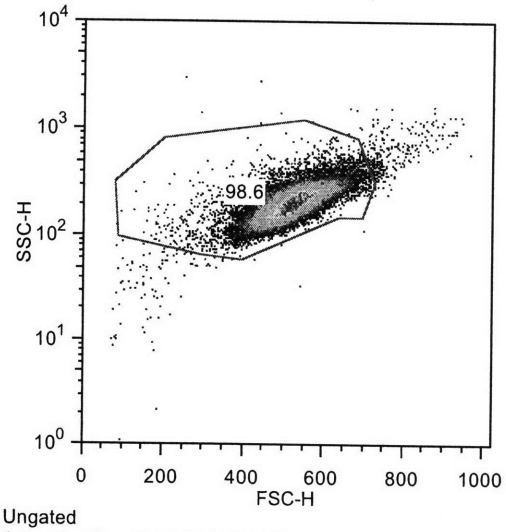
APPENDICES



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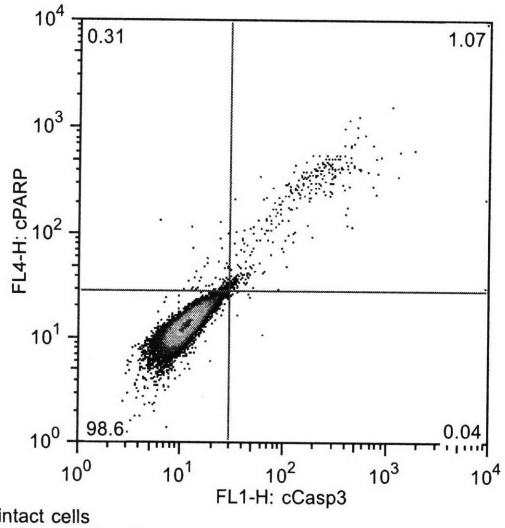

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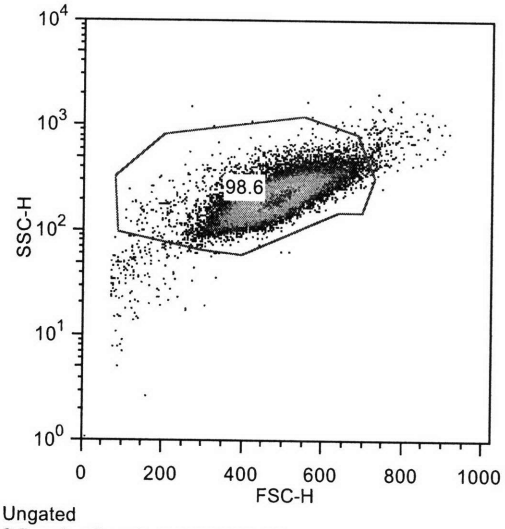

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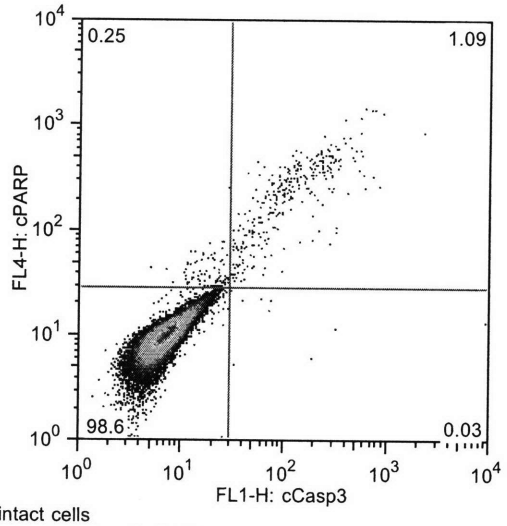

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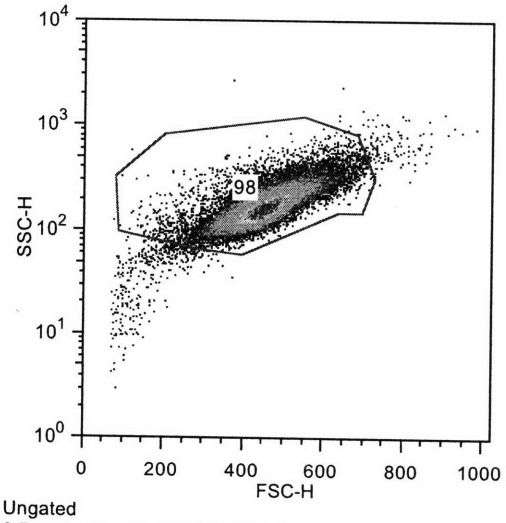

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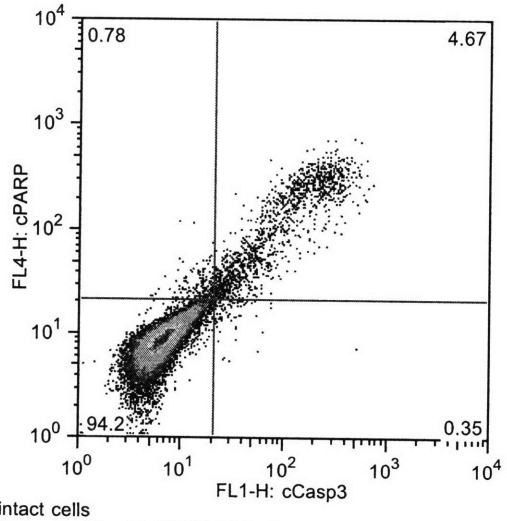

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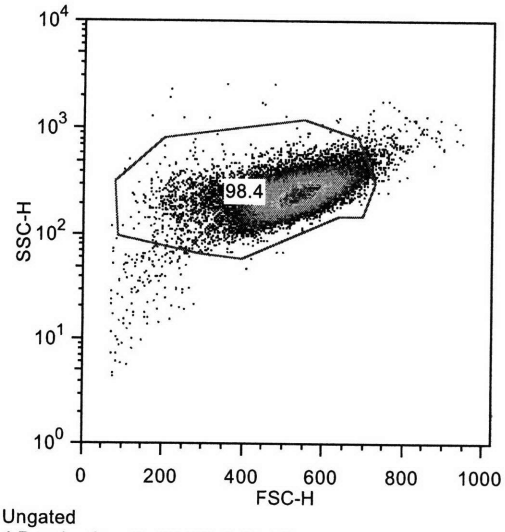

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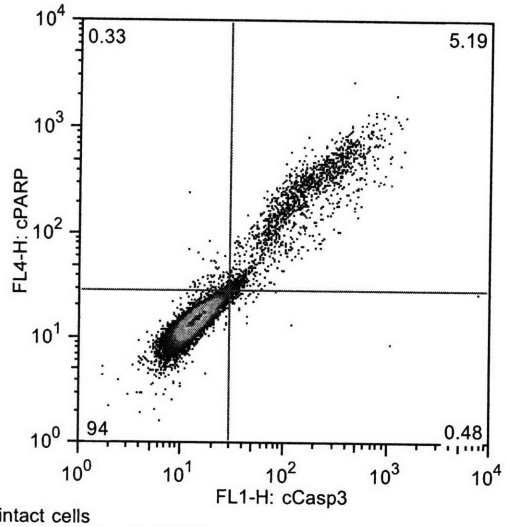

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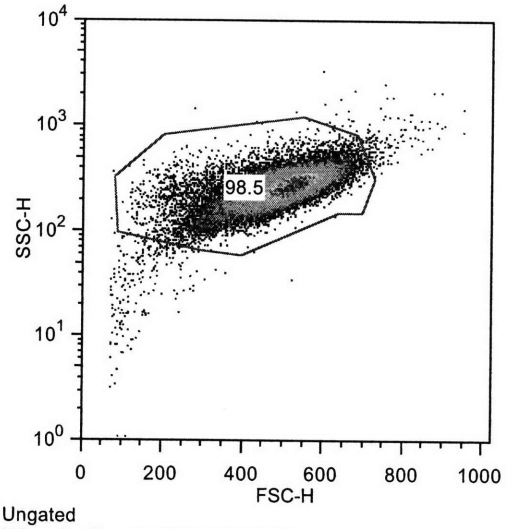

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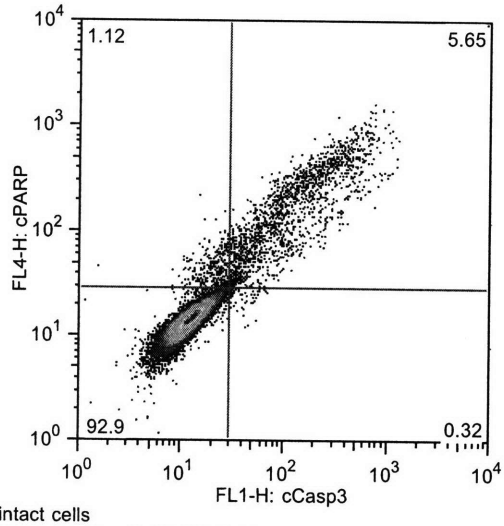

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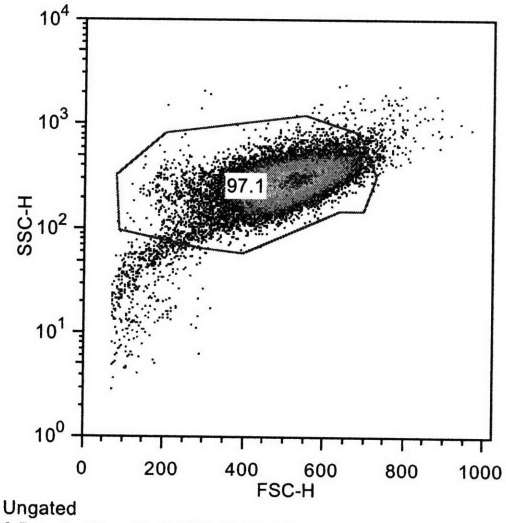

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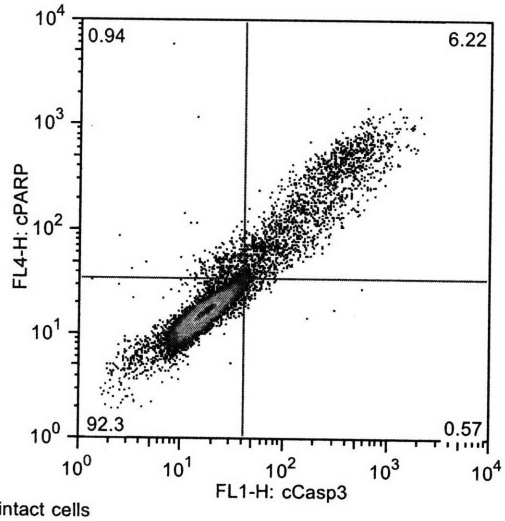

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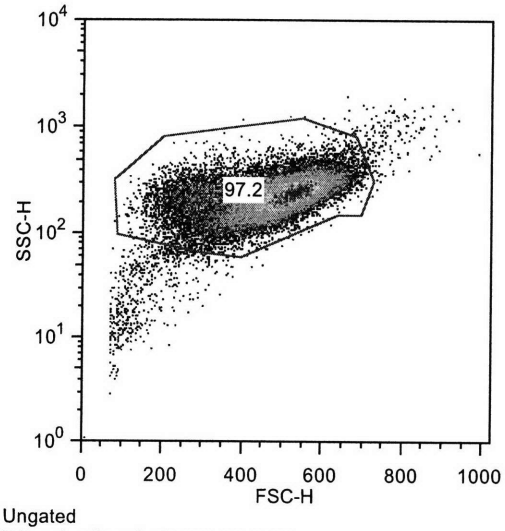

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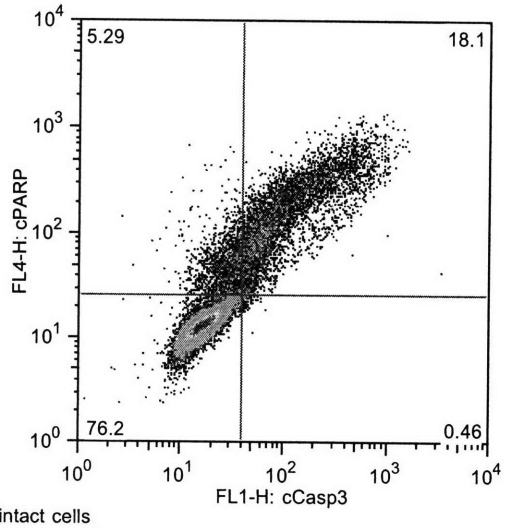

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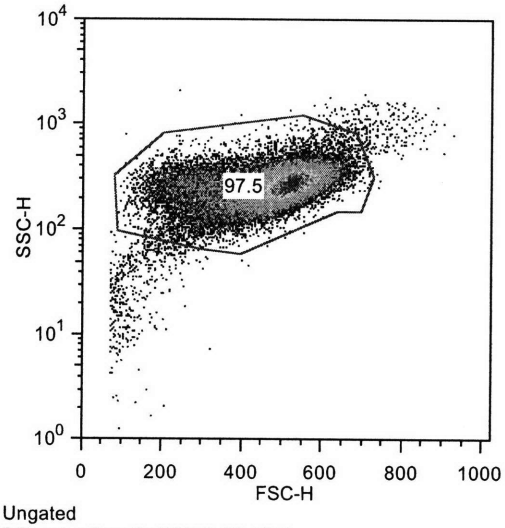

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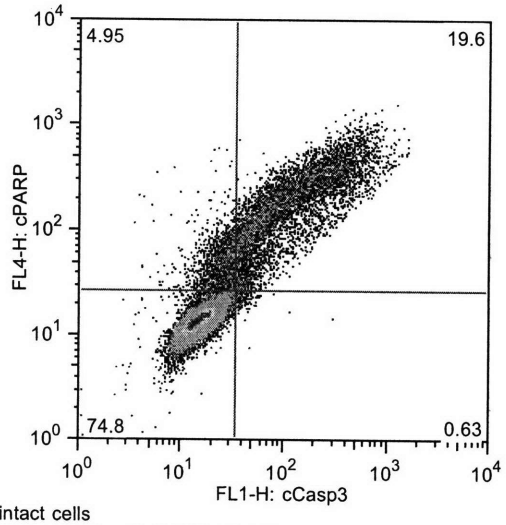

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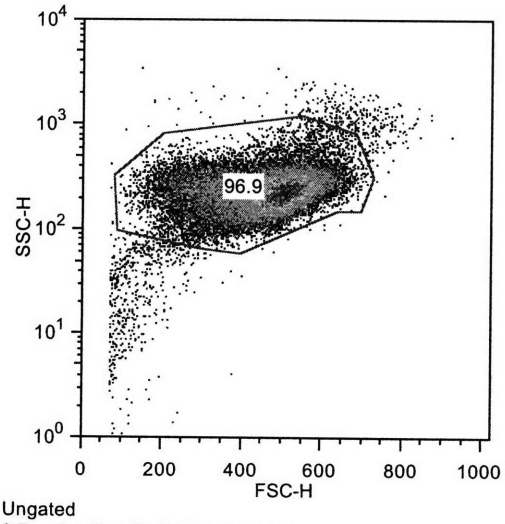

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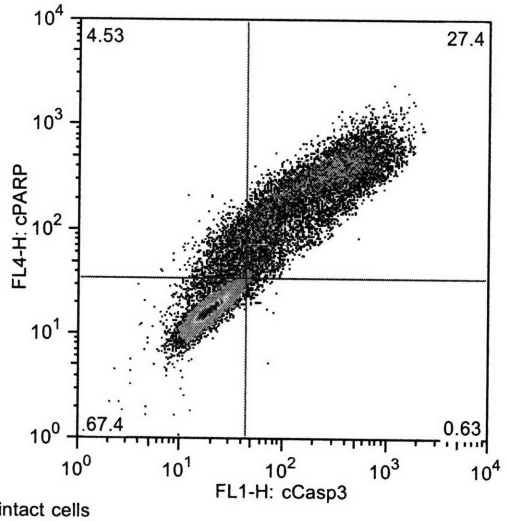

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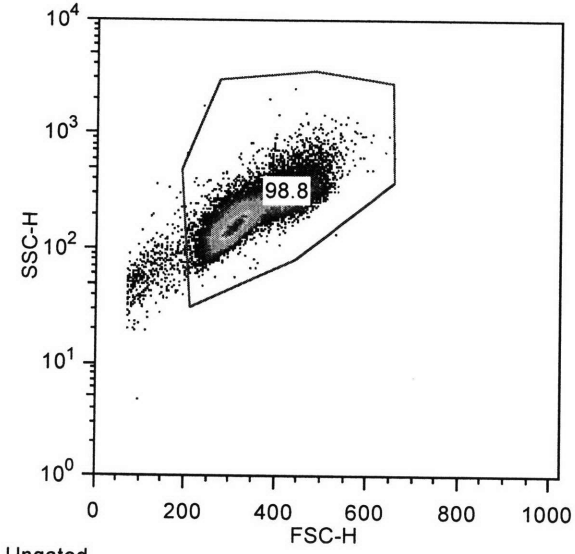

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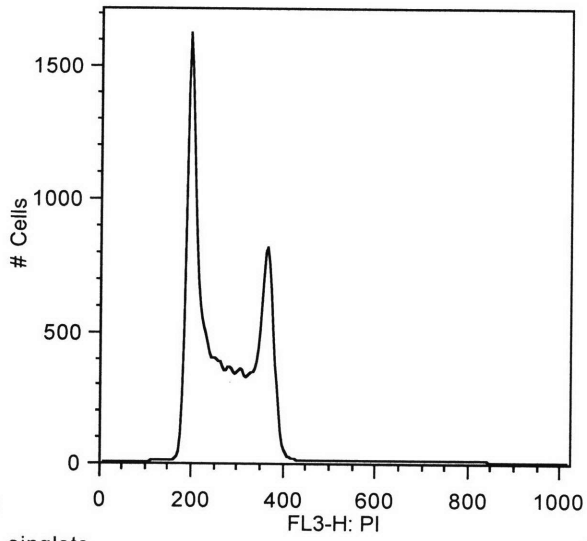

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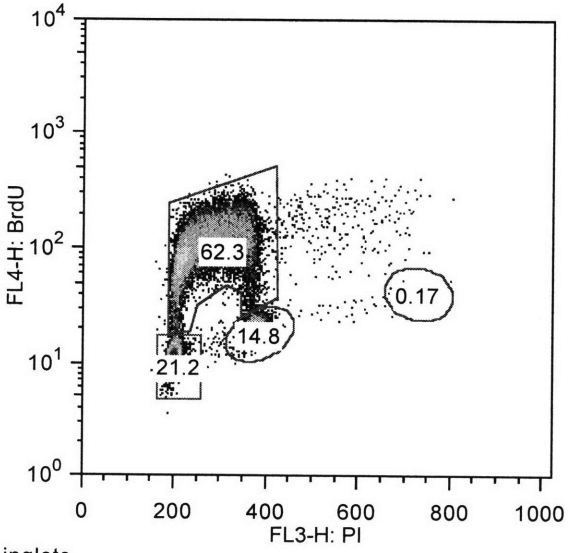

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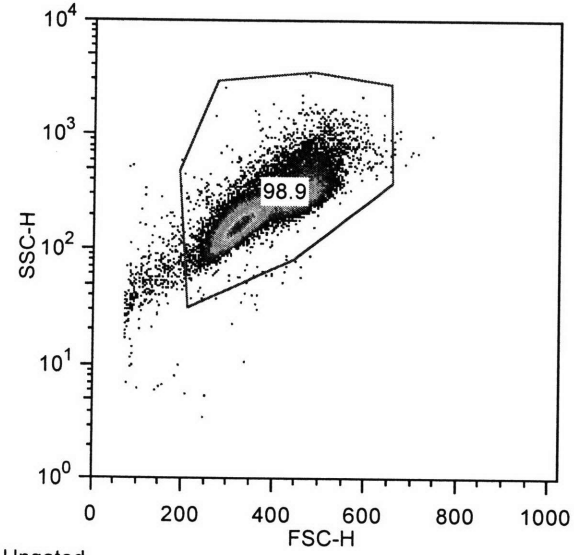

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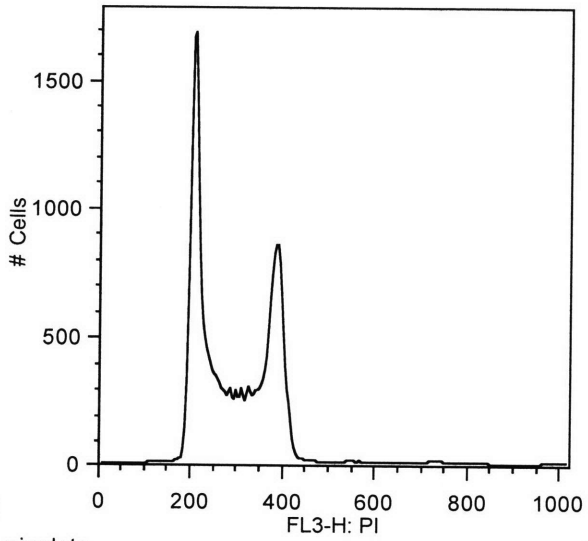

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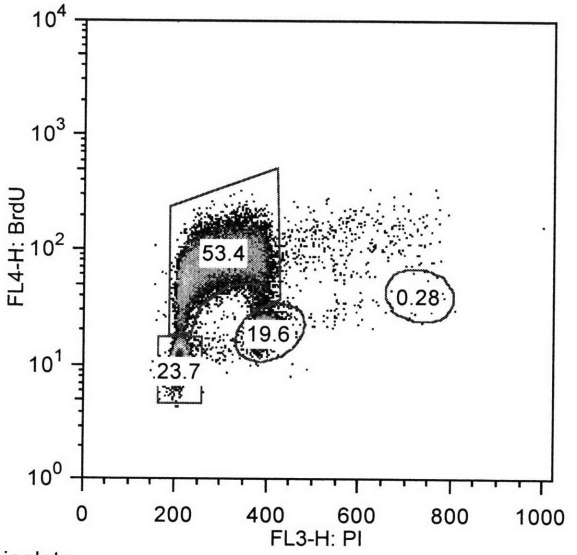

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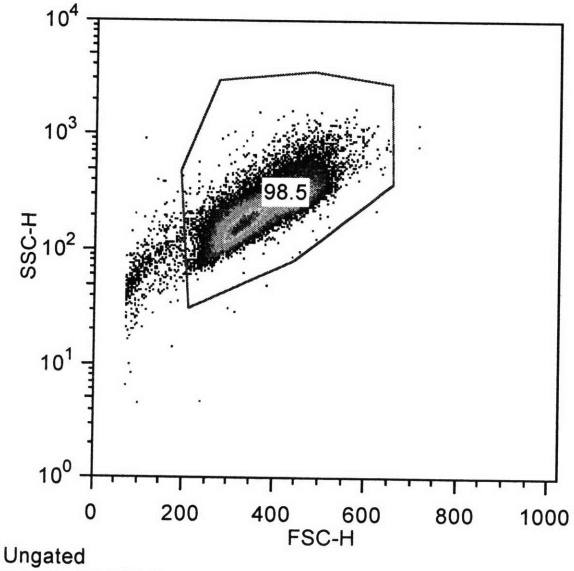

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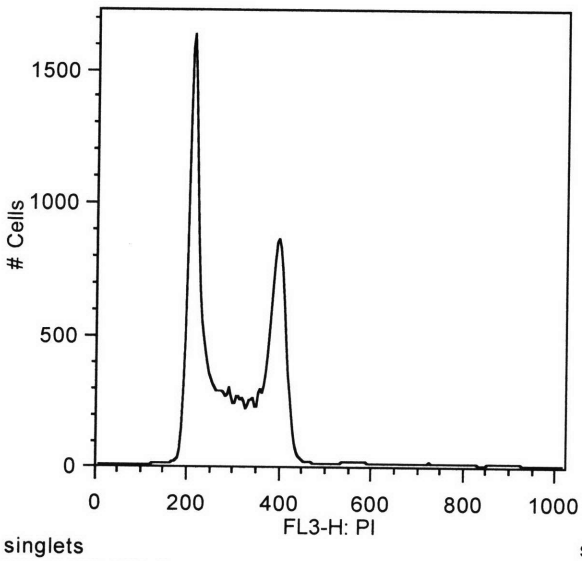

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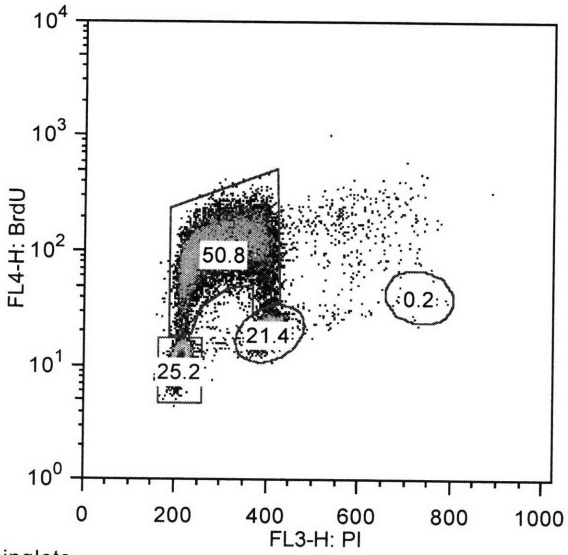

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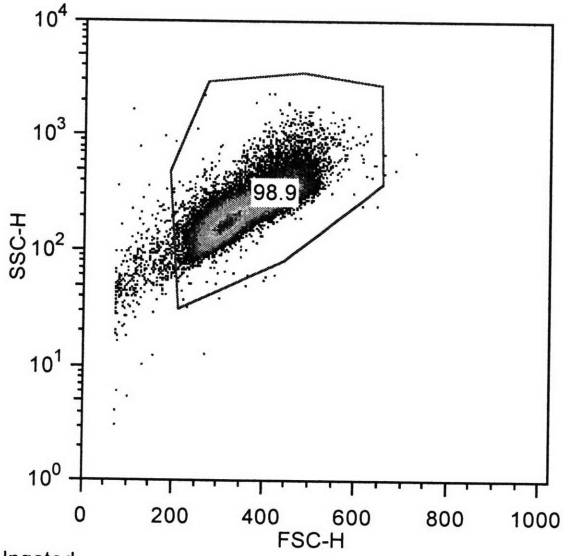

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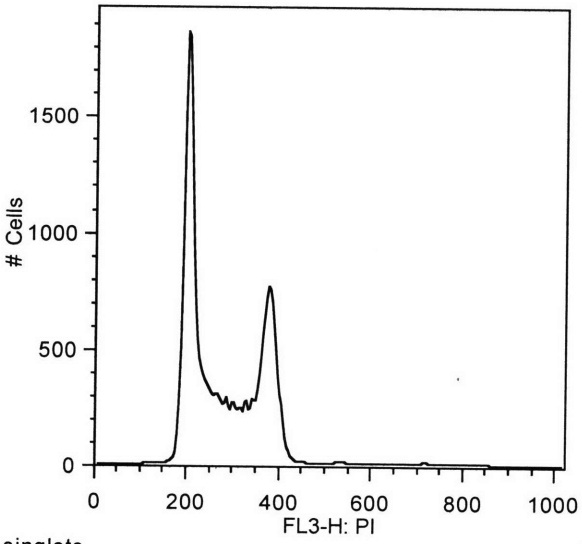
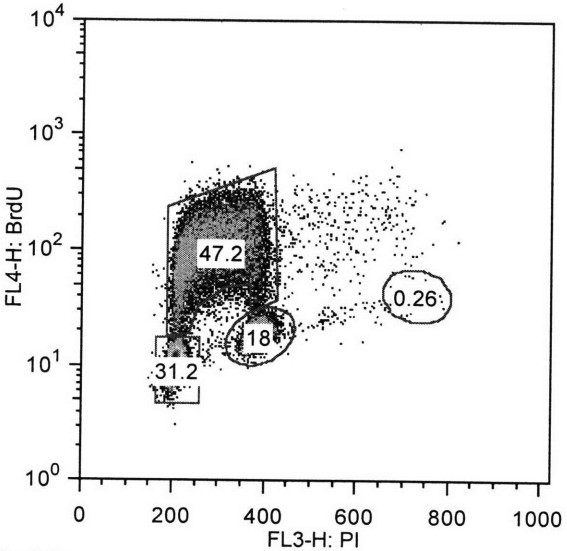
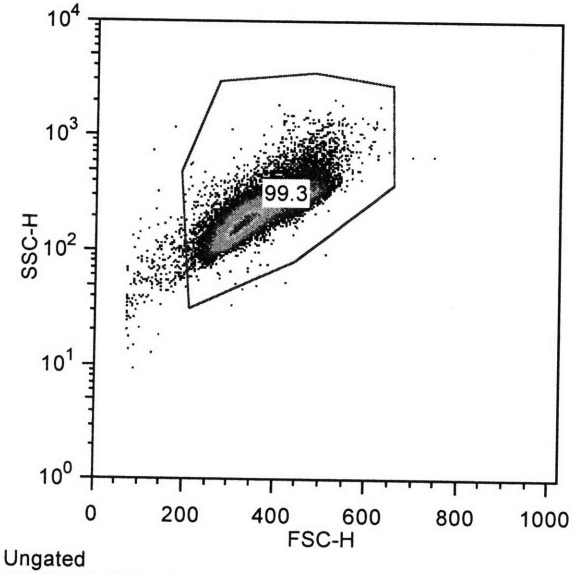


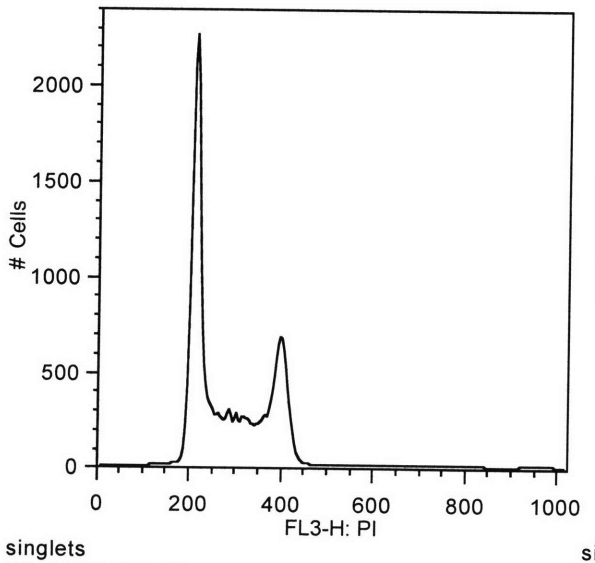
singlets
4.BrdU.PI_UN_12
Event Count: 29985



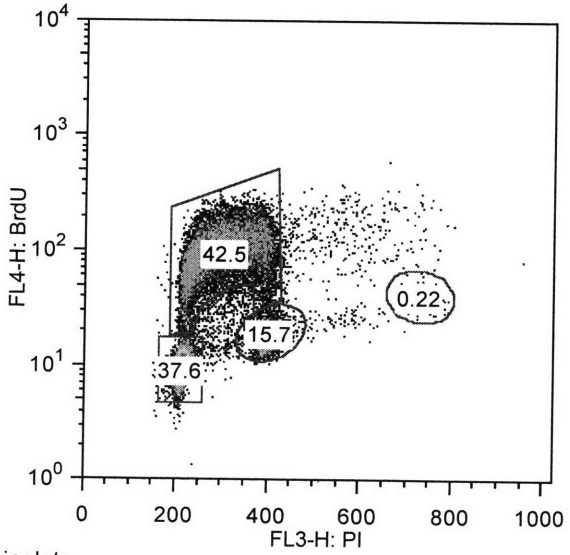
singlets
4.BrdU.PI_UN_12
Event Count: 29985



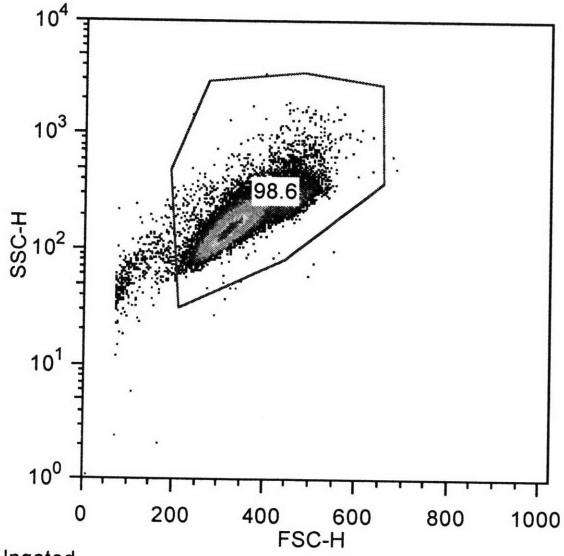
Ungated
5.BrdU.PI_UN_16
Event Count: 36818



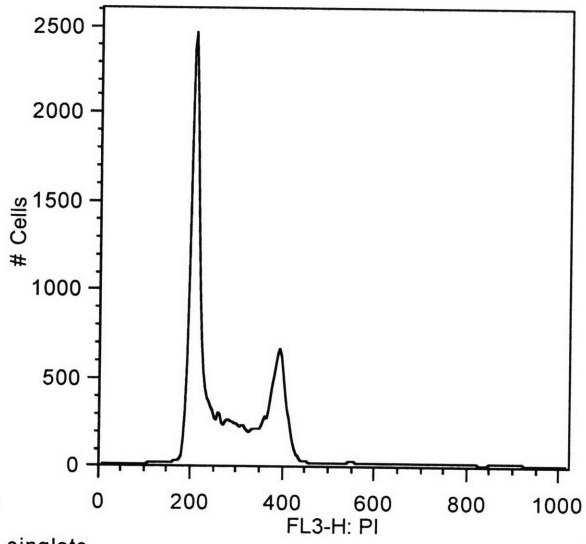
singlets
5.BrdU.PI_UN_16
Event Count: 30018



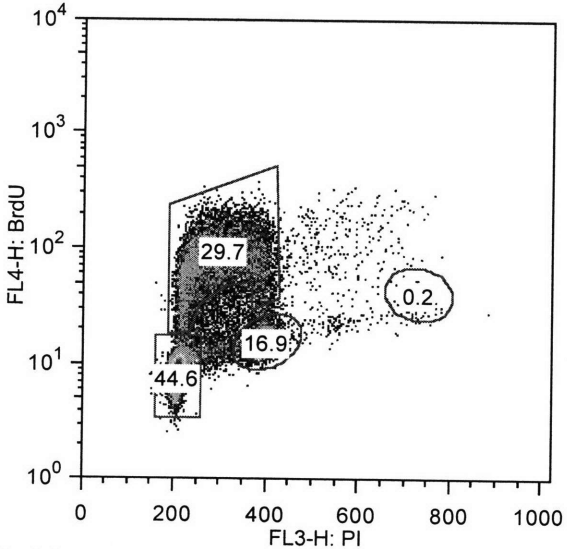
singlets
5.BrdU.PI_UN_16
Event Count: 30018



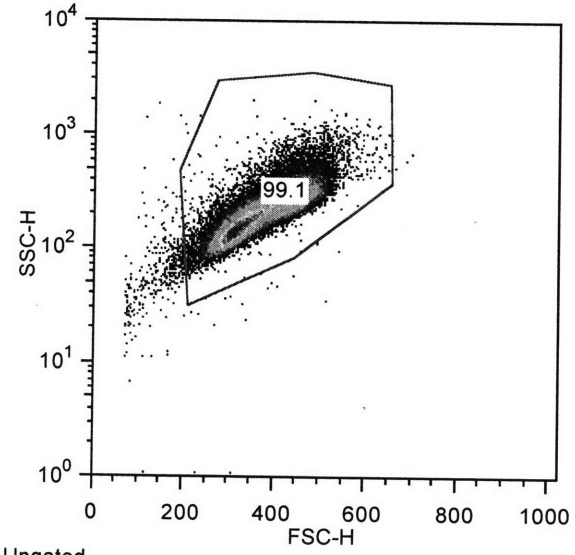
Ungated
6.BrdU.PI_UN_24
Event Count: 34729



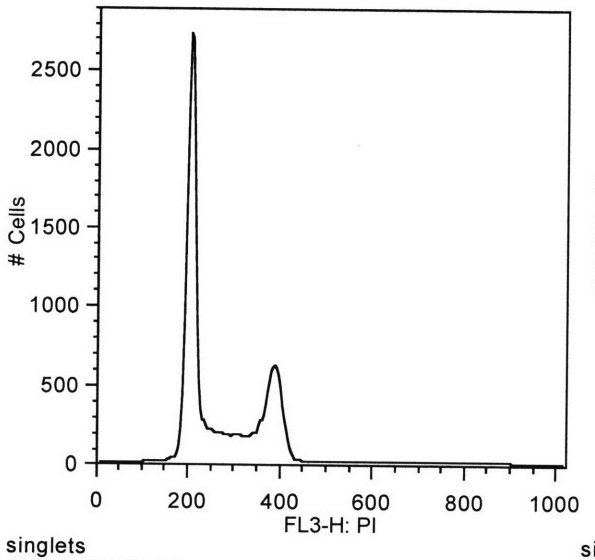
singlets
6.BrdU.PI_UN_24
Event Count: 29979



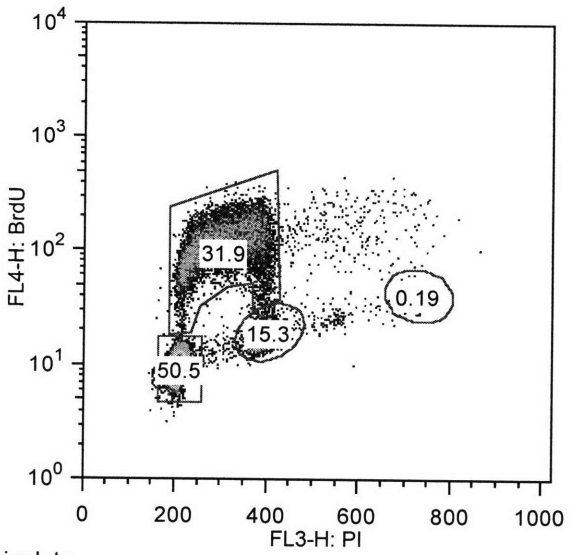
singlets
6.BrdU.PI_UN_24
Event Count: 29979



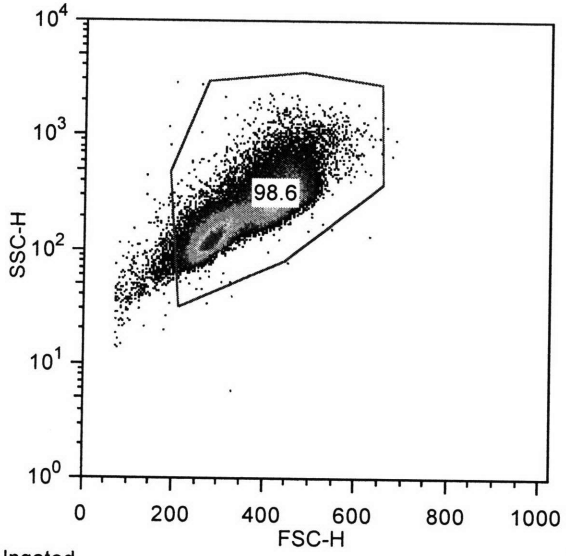
Ungated
7.BrdU.PI_UN_36
Event Count: 46358



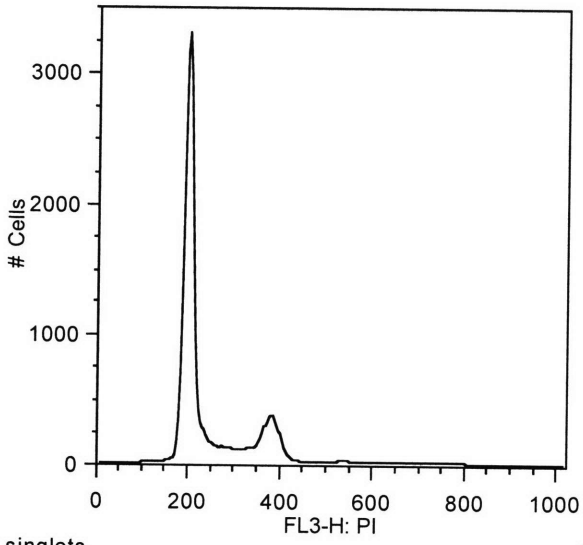
singlets
7.BrdU.PI_UN_36
Event Count: 29954



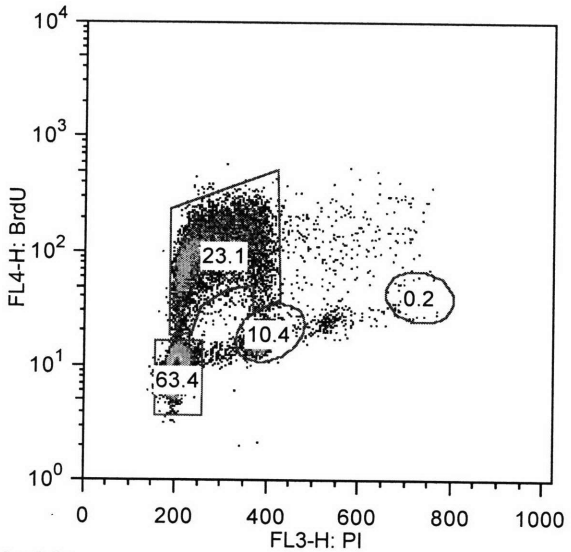
singlets
7.BrdU.PI_UN_36
Event Count: 29954



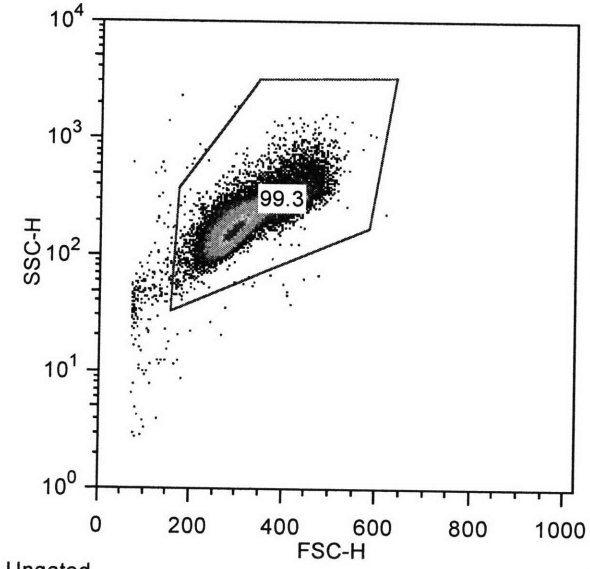
Ungated
8.BrdU.PI_UN_48
Event Count: 52500



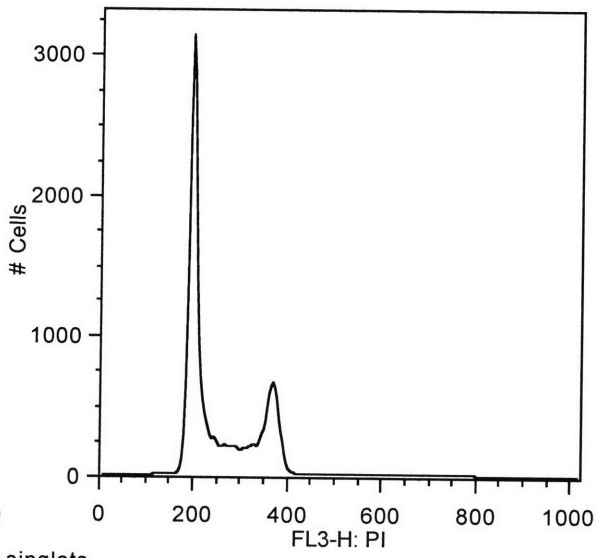
singlets
8.BrdU.PI_UN_48
Event Count: 29920



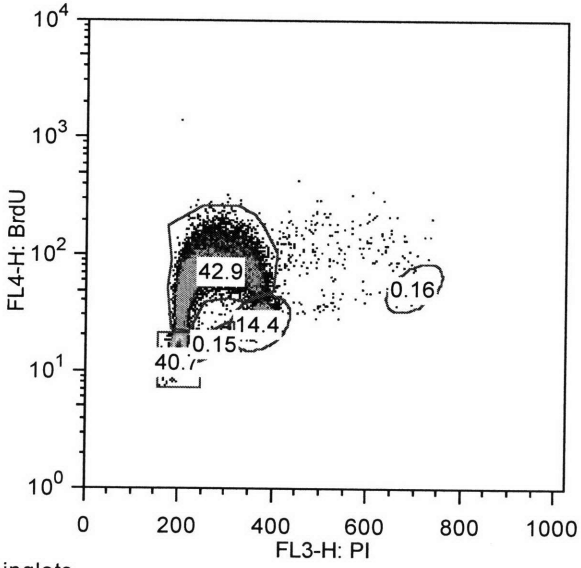
singlets
8.BrdU.PI_UN_48
Event Count: 29920



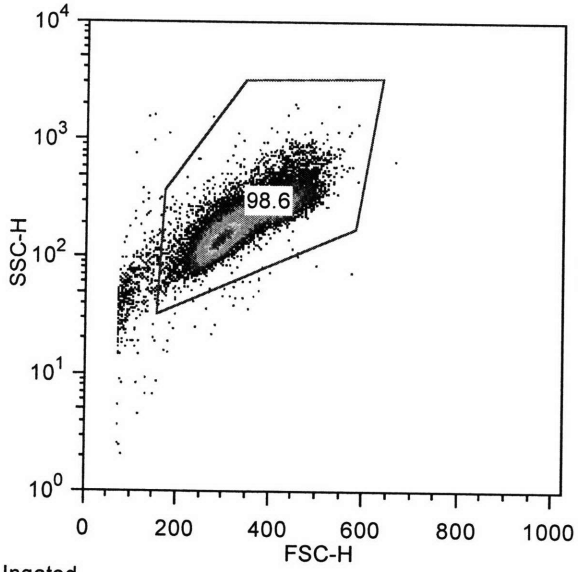
Ungated
1.Dox.TNF_BrdU.PI_2uM_2
Event Count: 36435



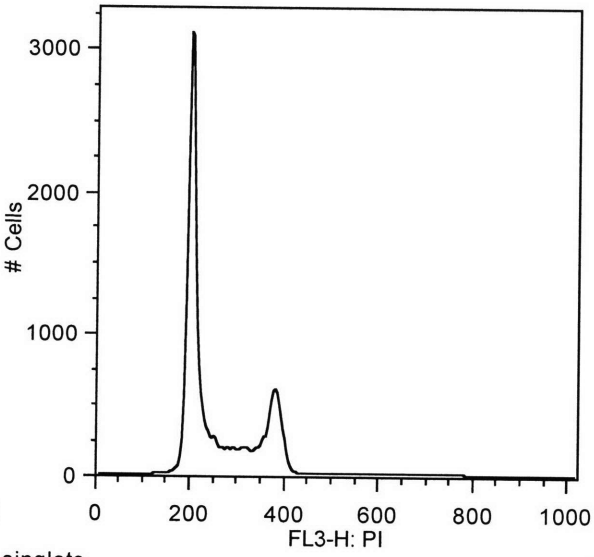
singlets
1.Dox.TNF_BrdU.PI_2uM_2
Event Count: 30058



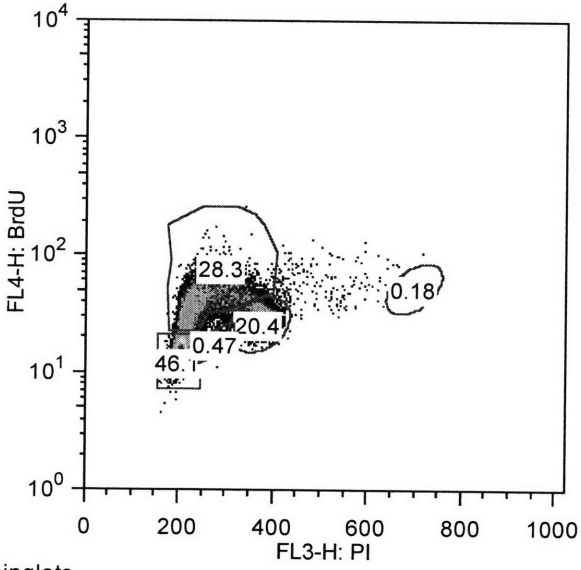
singlets
1.Dox.TNF_BrdU.PI_2uM_2
Event Count: 30058



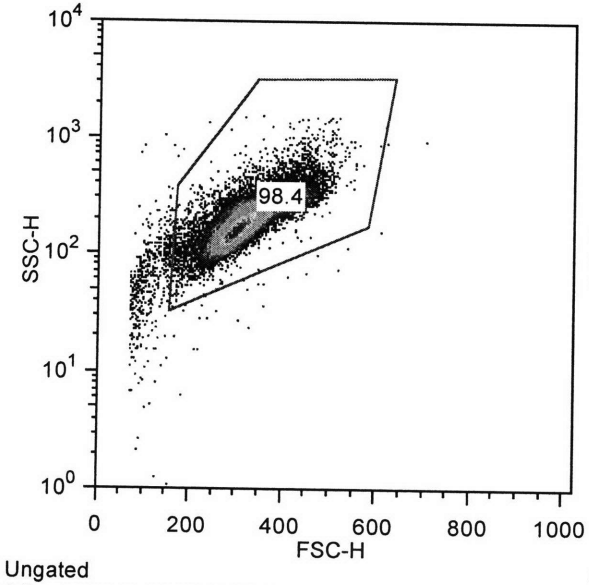
Ungated
2.Dox.TNF_BrdU.PI_2uM_4
Event Count: 37423



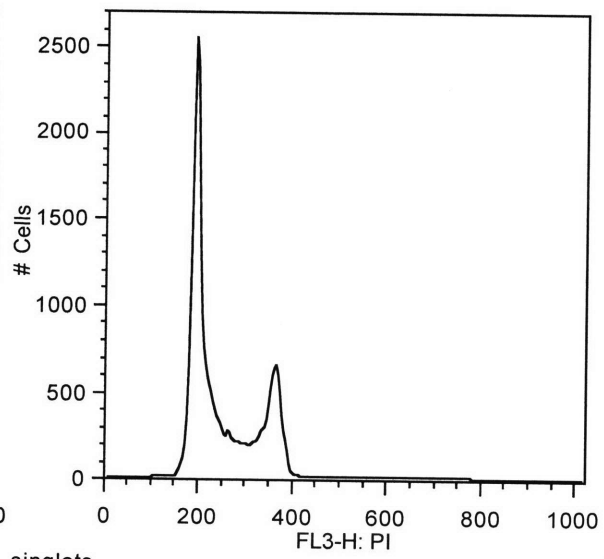
singlets
2.Dox.TNF_BrdU.PI_2uM_4
Event Count: 30037



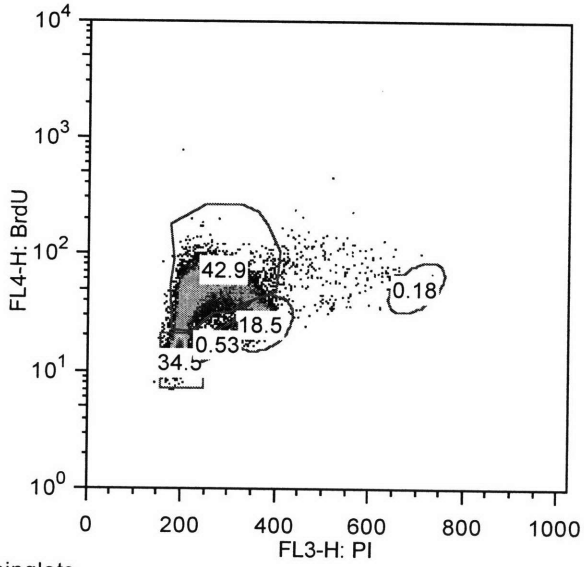
singlets
2.Dox.TNF_BrdU.PI_2uM_4
Event Count: 30037



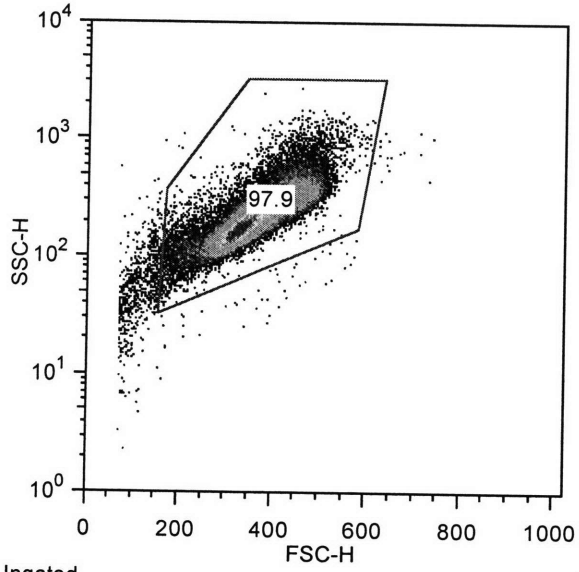
Ungated
3.Dox.TNF_BrdU.PI_2uM_8
Event Count: 34964



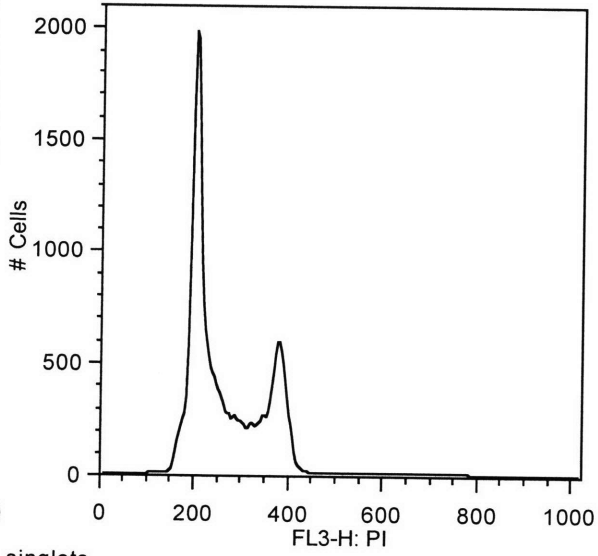
singlets
3.Dox.TNF_BrdU.PI_2uM_8
Event Count: 29914



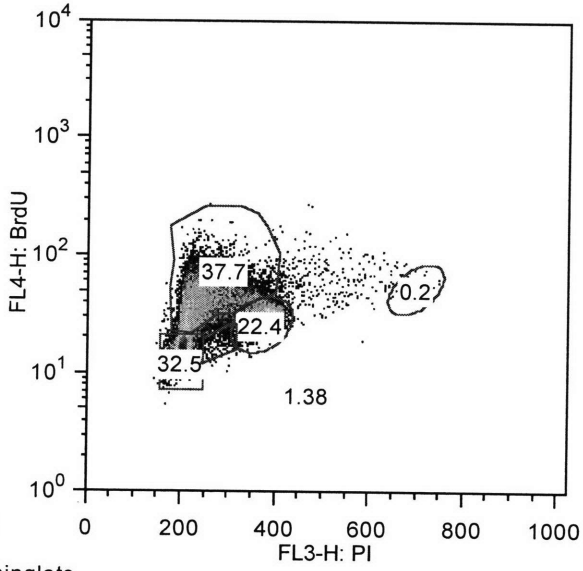
singlets
3.Dox.TNF_BrdU.PI_2uM_8
Event Count: 29914



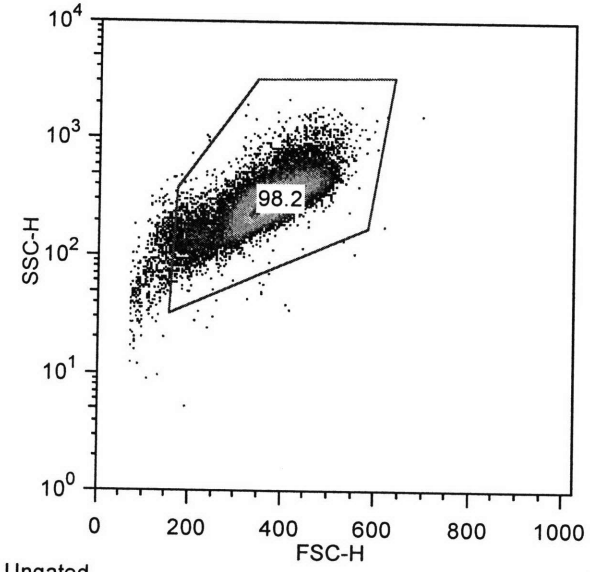
Ungated
4.Dox.TNF_BrdU.PI_2uM_12
Event Count: 42284



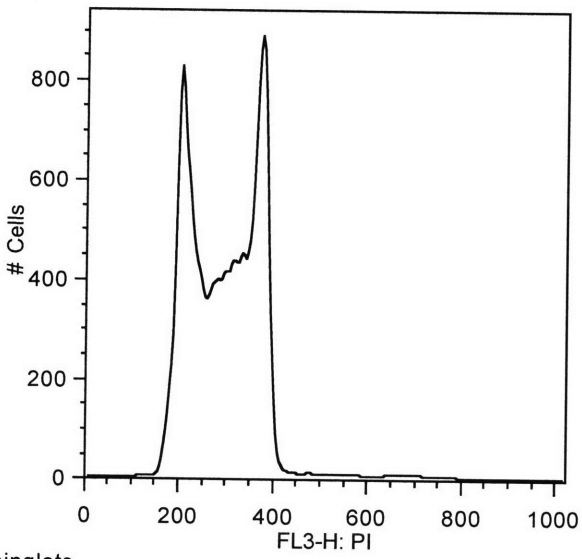
singlets
4.Dox.TNF_BrdU.PI_2uM_12
Event Count: 30022



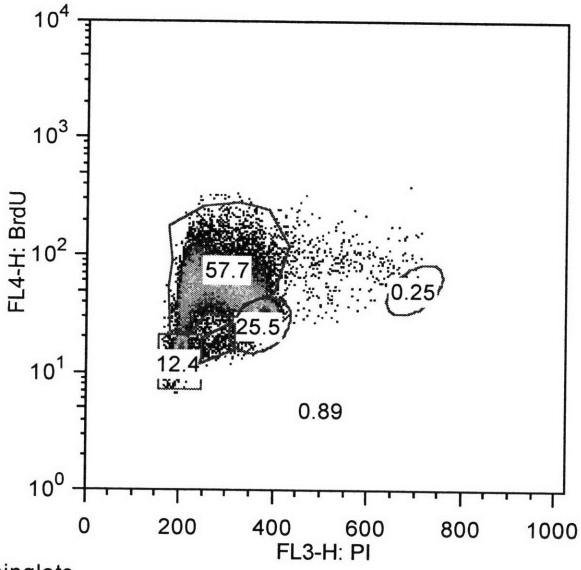
singlets
4.Dox.TNF_BrdU.PI_2uM_12
Event Count: 30022



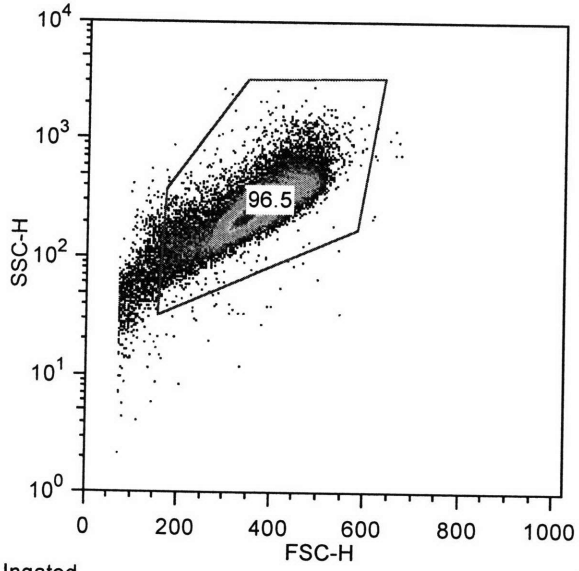
Ungated
5.Dox.TNF_BrdU.PI_2uM_16
Event Count: 38418



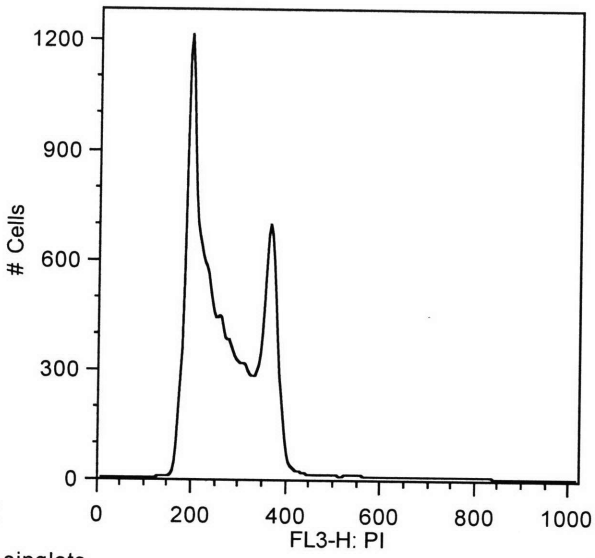
singlets
5.Dox.TNF_BrdU.PI_2uM_16
Event Count: 29165



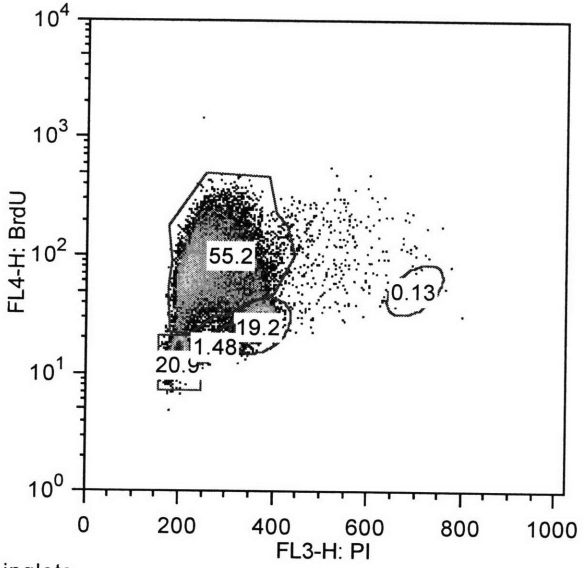
singlets
5.Dox.TNF_BrdU.PI_2uM_16
Event Count: 29165



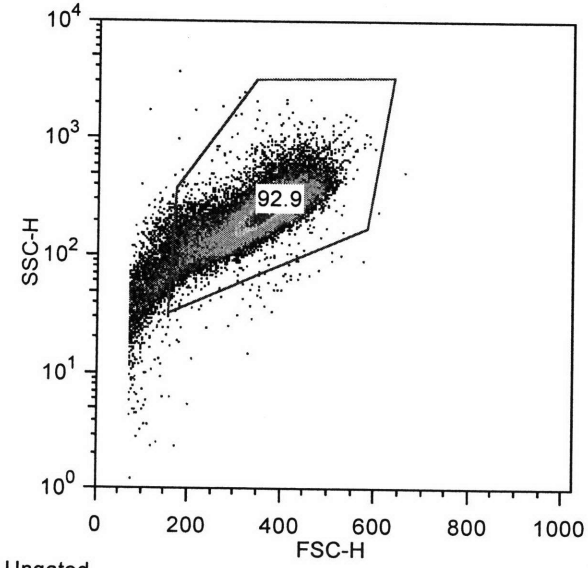
Ungated
6.Dox.TNF_BrdU.PI_2uM_24
Event Count: 40114



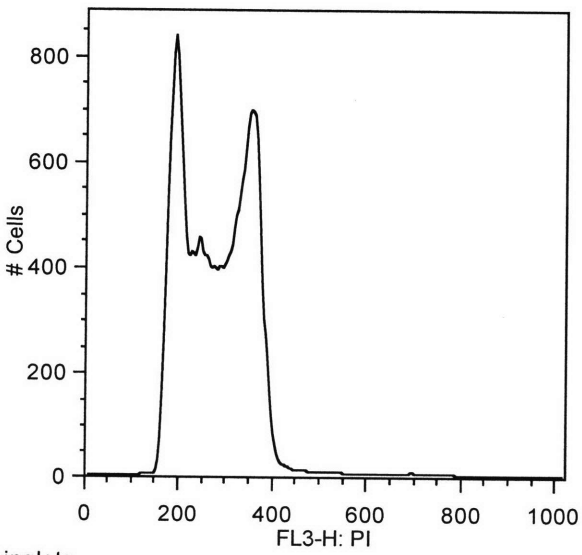
singlets
6.Dox.TNF_BrdU.PI_2uM_24
Event Count: 28547



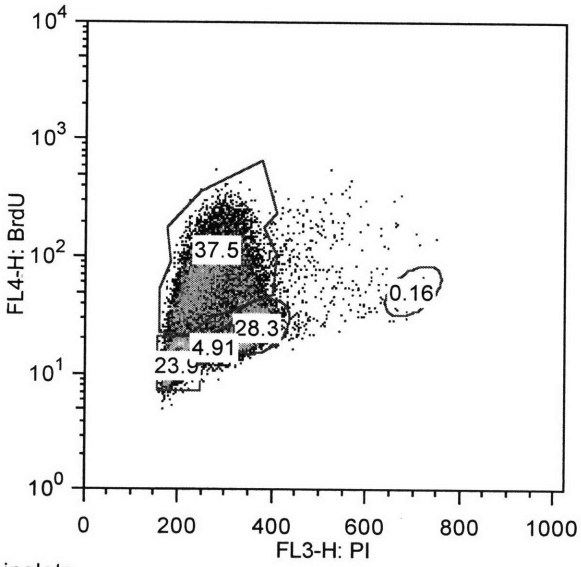
singlets
6.Dox.TNF_BrdU.PI_2uM_24
Event Count: 28547



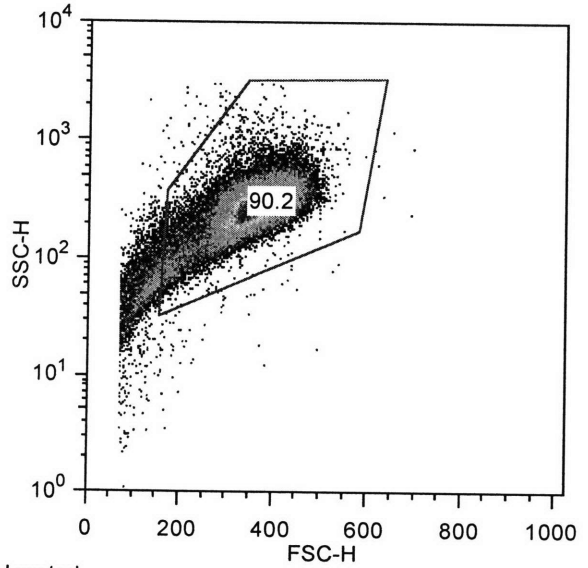
Ungated
7.Dox.TNF_BrdU.PI_2uM_36
Event Count: 39907



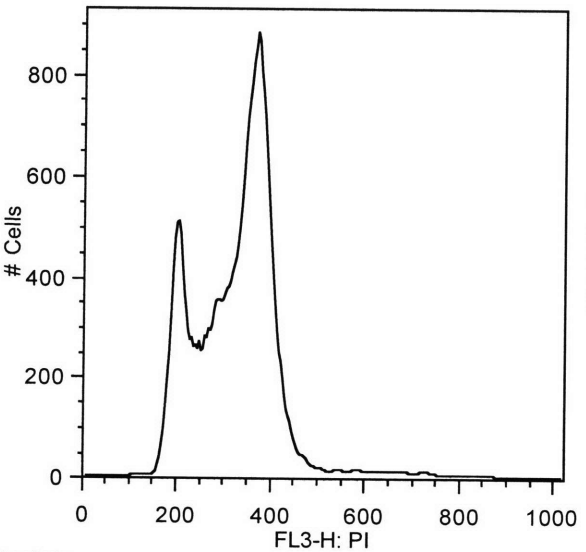
singlets
7.Dox.TNF_BrdU.PI_2uM_36
Event Count: 29783



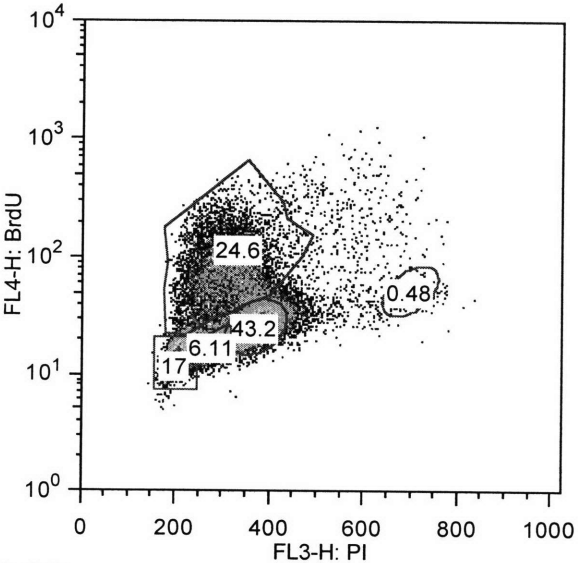
singlets
7.Dox.TNF_BrdU.PI_2uM_36
Event Count: 29783



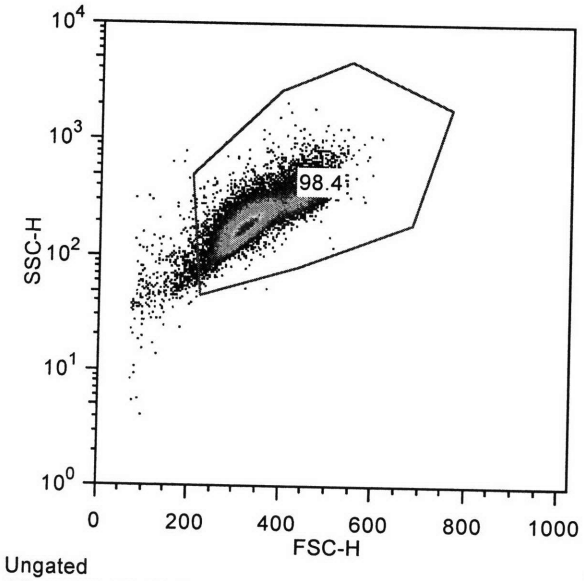
Ungated
8.Dox.TNF_BrdU.PI_2uM_48
Event Count: 41584



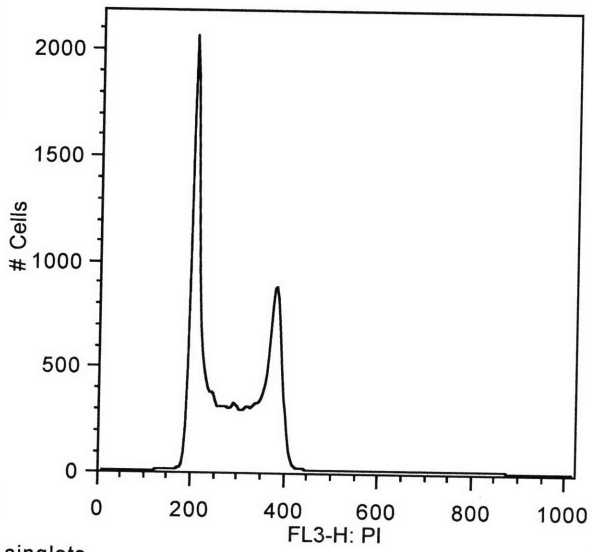
singlets
8.Dox.TNF_BrdU.PI_2uM_48
Event Count: 30085



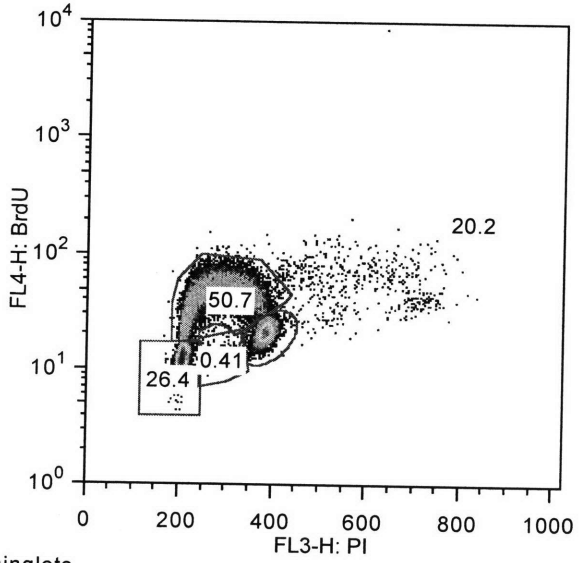
singlets
8.Dox.TNF_BrdU.PI_2uM_48
Event Count: 30085



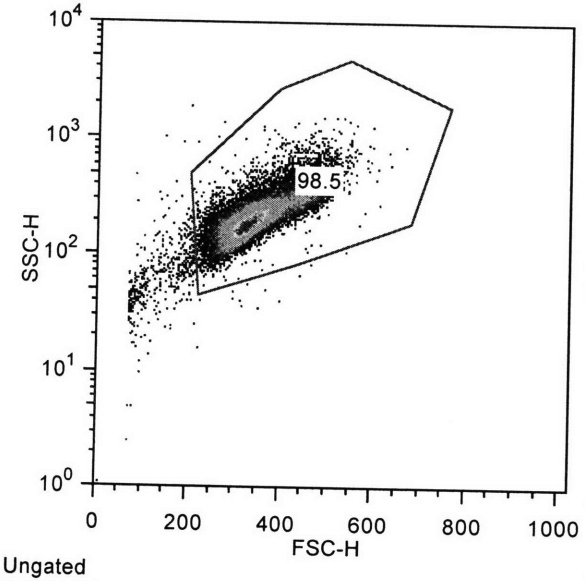
Ungated
1.BrdU.PI_10uM_2
Event Count: 35540



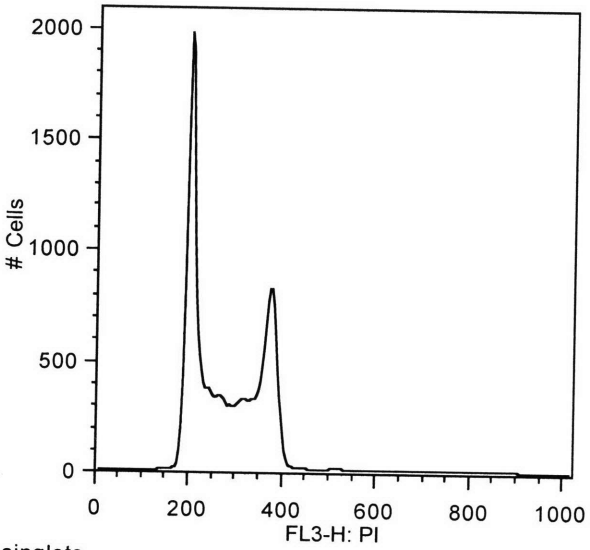
singlets
1.BrdU.PI_10uM_2
Event Count: 30044



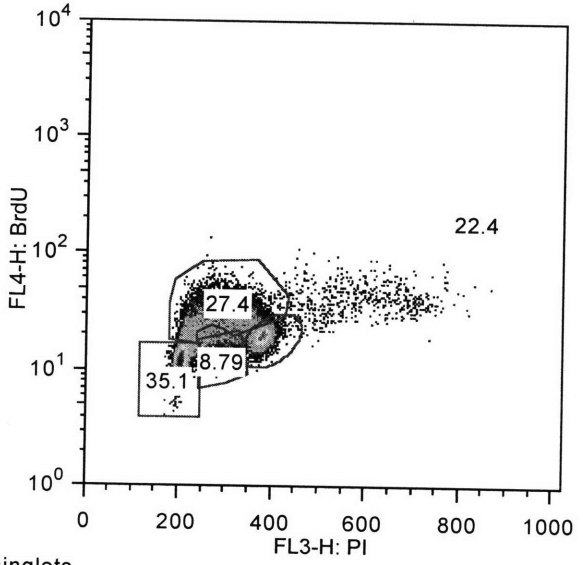
singlets
1.BrdU.PI_10uM_2
Event Count: 30044



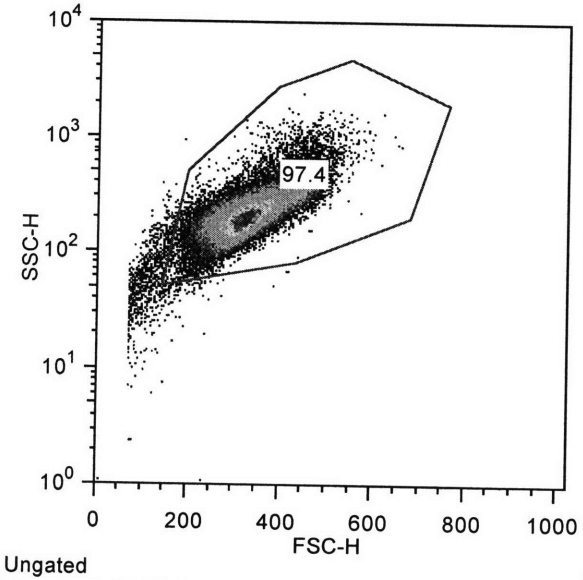
Ungated
2.BrdU.PI_10uM_4
Event Count: 36062



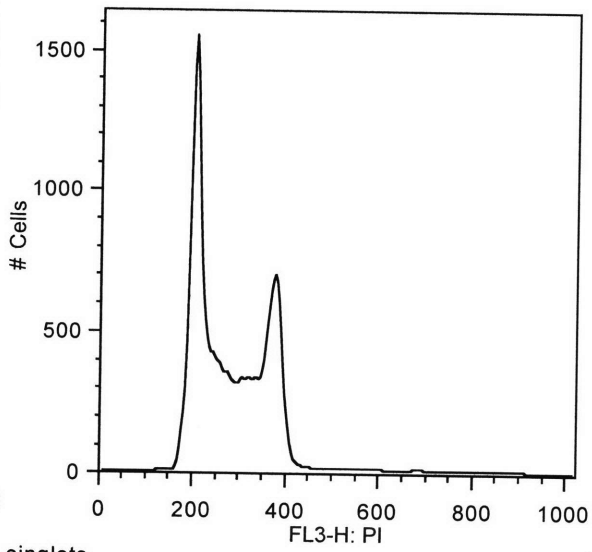
singlets
2.BrdU.PI_10uM_4
Event Count: 30149



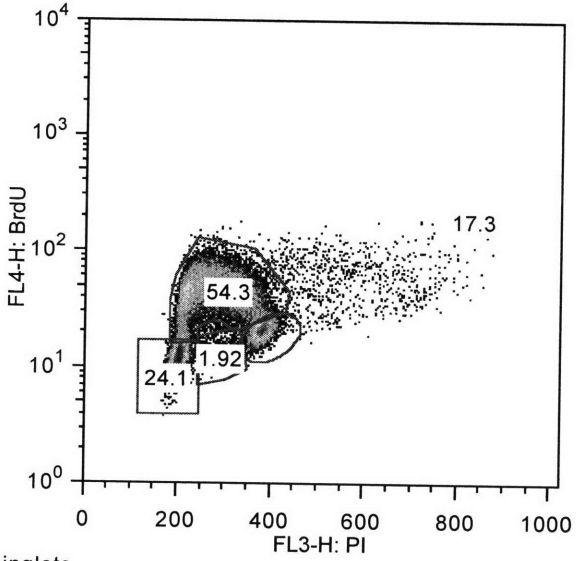
singlets
2.BrdU.PI_10uM_4
Event Count: 30149



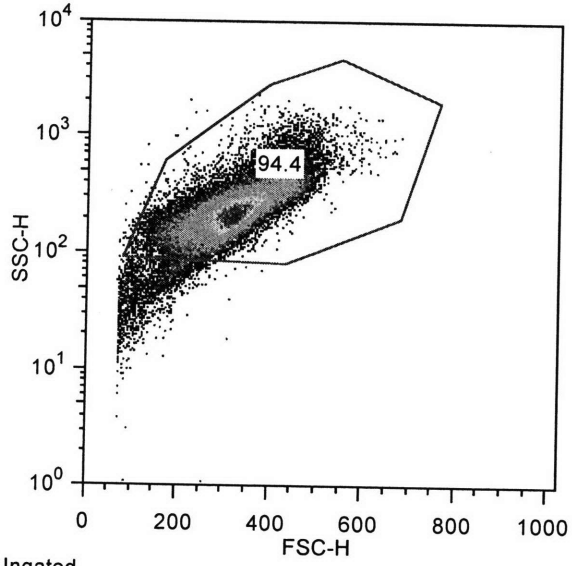
Ungated
3.BrdU.PI_10uM_8
Event Count: 38844



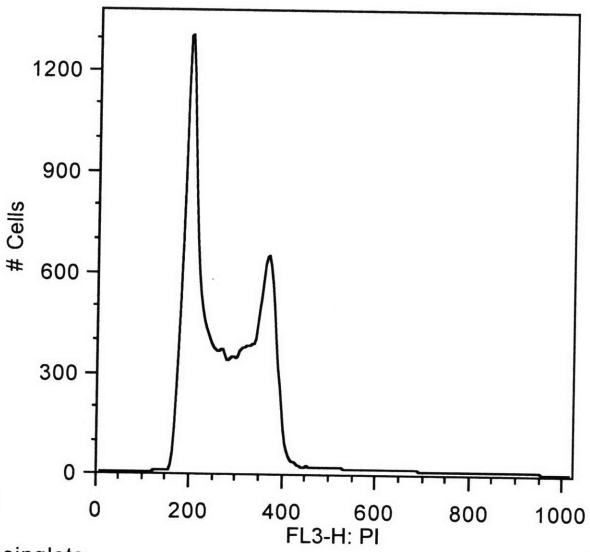
singlets
3.BrdU.PI_10uM_8
Event Count: 30466



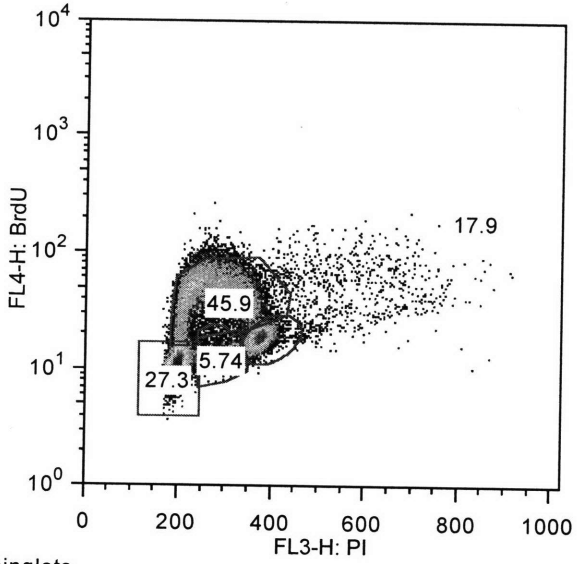
singlets
3.BrdU.PI_10uM_8
Event Count: 30466



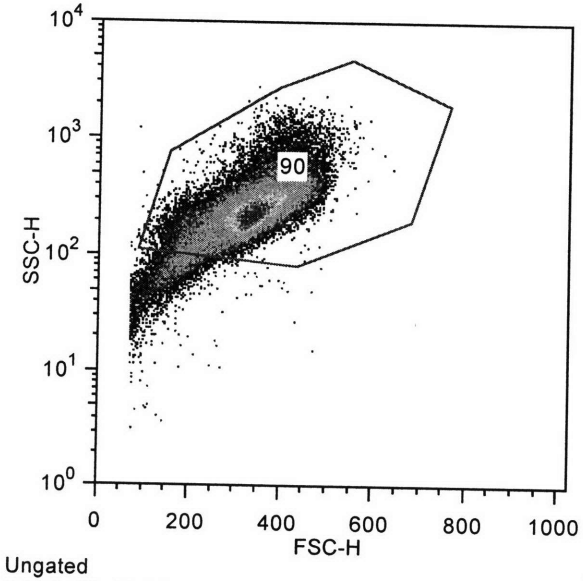
Ungated
4.BrdU.PI_10uM_12
Event Count: 43307



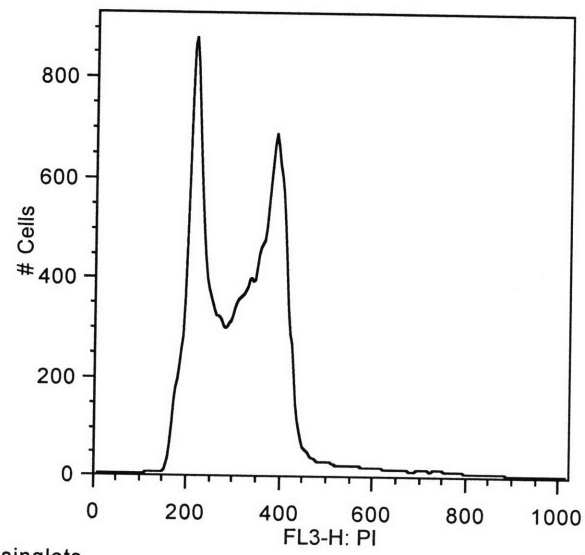
singlets
4.BrdU.PI_10uM_12
Event Count: 30907



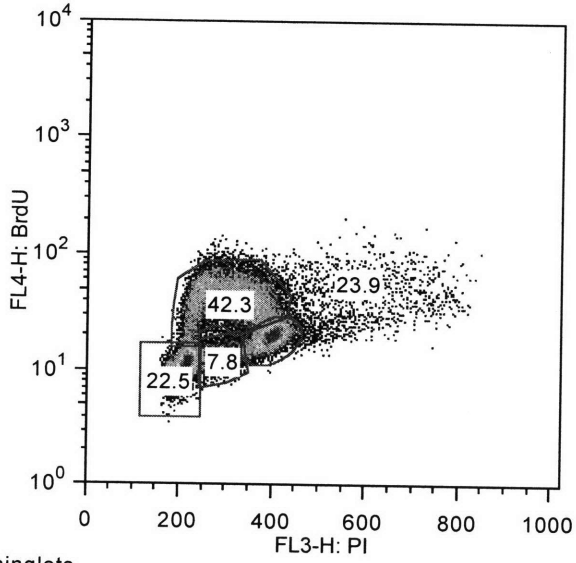
singlets
4.BrdU.PI_10uM_12
Event Count: 30907



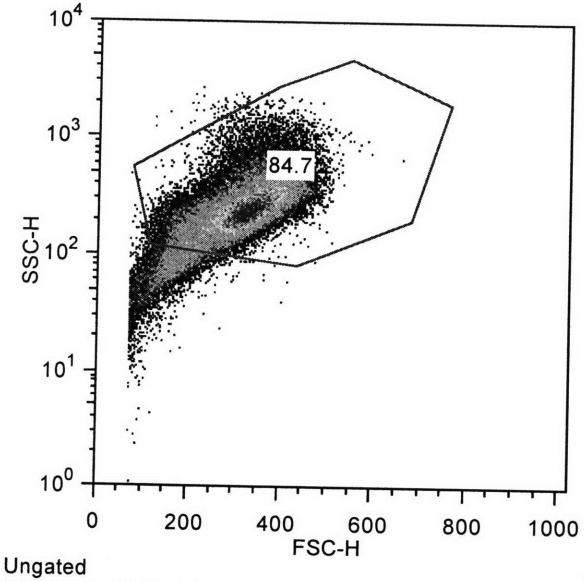
Ungated
7.BrdU.PI_10uM_36
Event Count: 46942



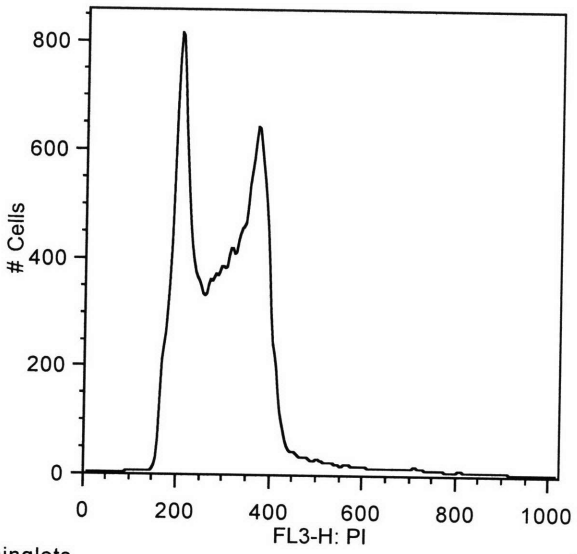
singlets
7.BrdU.PI_10uM_36
Event Count: 30709



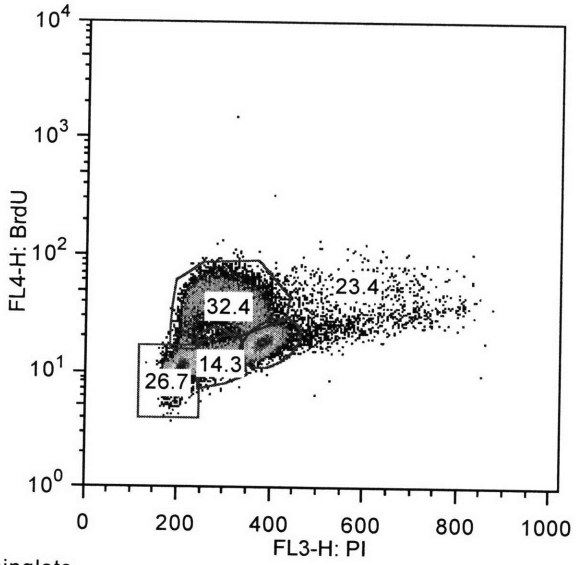
singlets
7.BrdU.PI_10uM_36
Event Count: 30709



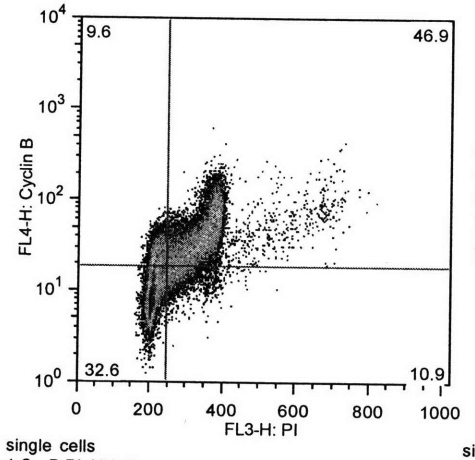
Ungated
8.BrdU.PI_10uM_48
Event Count: 52345



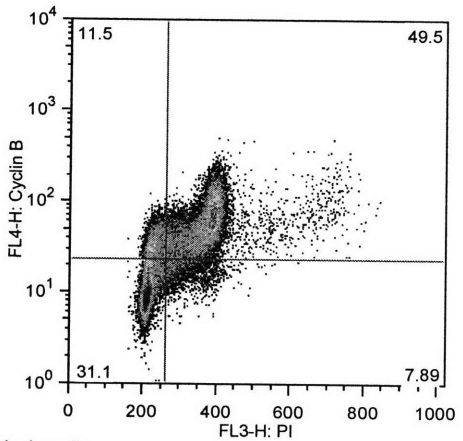
singlets
8.BrdU.PI_10uM_48
Event Count: 30344



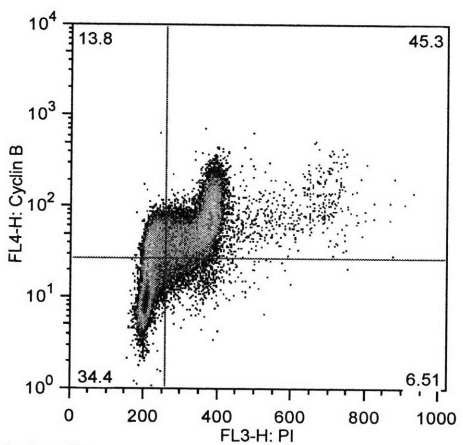
singlets
8.BrdU.PI_10uM_48
Event Count: 30344



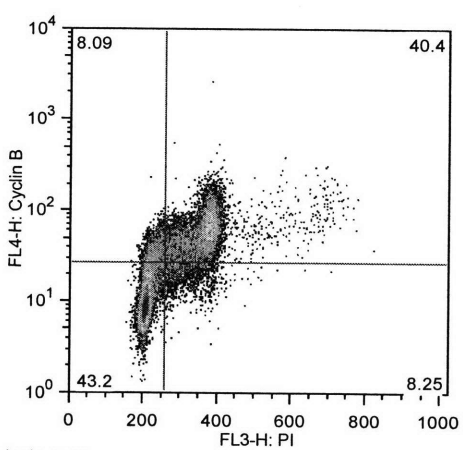
single cells
1.CycB.PI_UN_2
Event Count: 29862



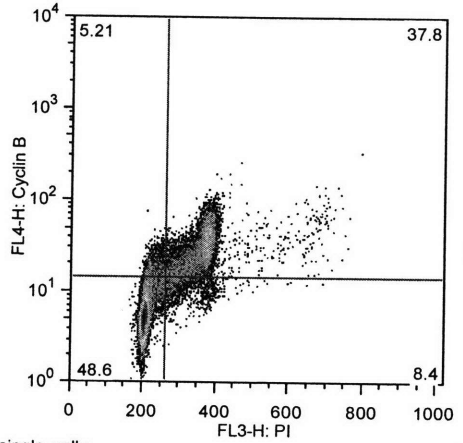
single cells
2.CycB.PI_UN_4
Event Count: 29918



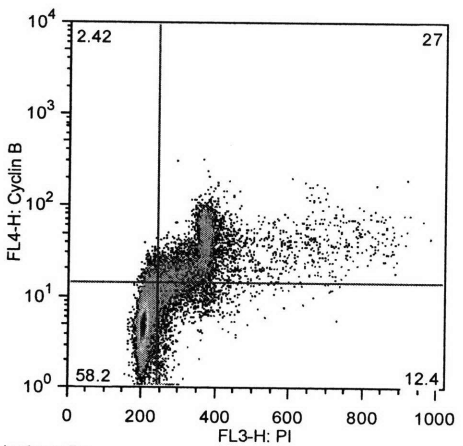
single cells
3.CycB.PI_UN_8
Event Count: 29909



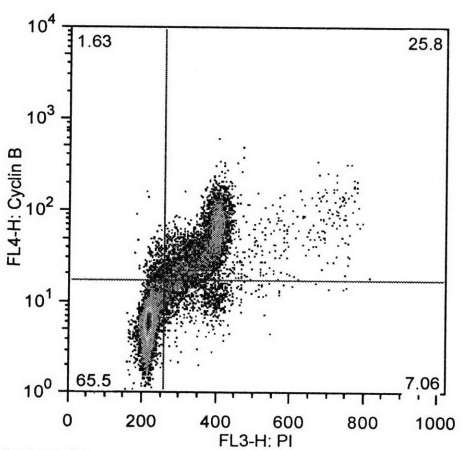
single cells
4.CycB.PI_UN_12
Event Count: 19902



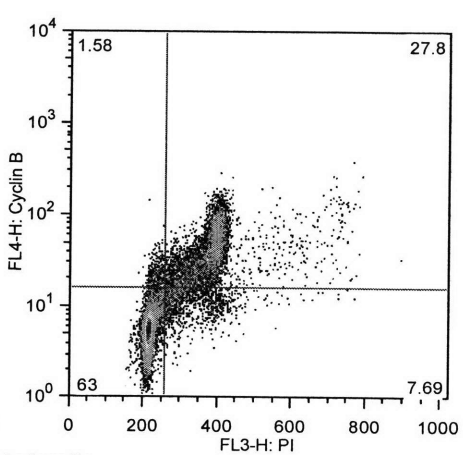
single cells
5.CycB.PI_UN_16
Event Count: 19855



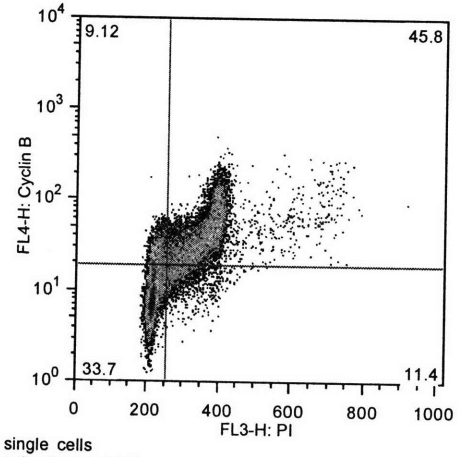
single cells
6.CycB.PI_UN_24
Event Count: 19965



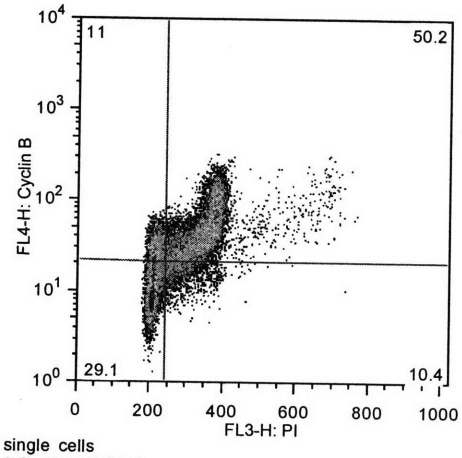
single cells
7.CycB.PI_UN_36
Event Count: 19798



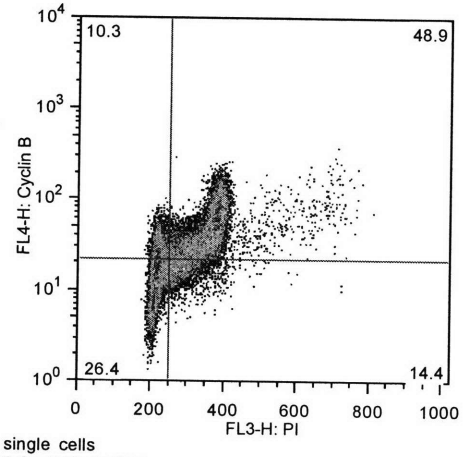
single cells
8.CycB.PI_UN_48
Event Count: 19819



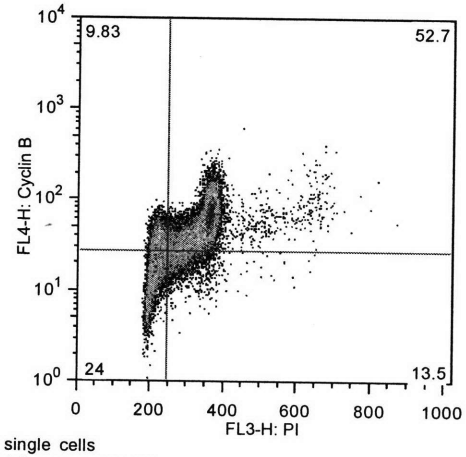
single cells
1.CycB.PI_2uM_2
Event Count: 19769



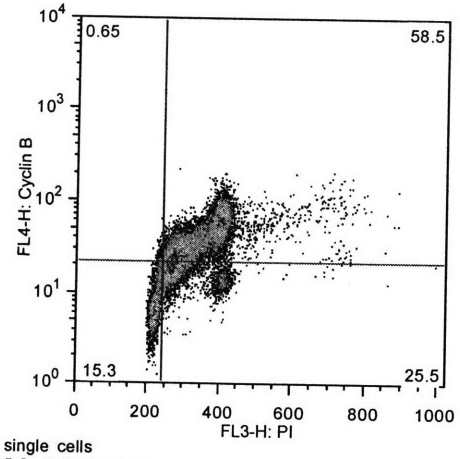
single cells
2.CycB.PI_2uM_4
Event Count: 19847



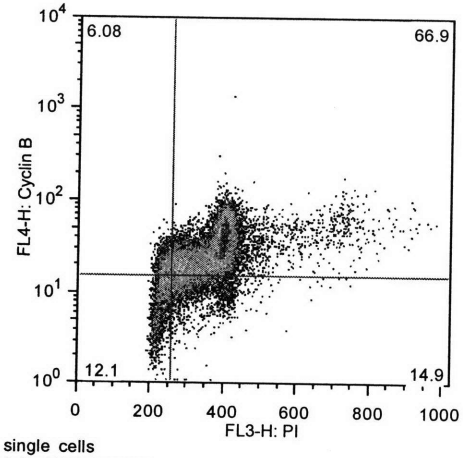
single cells
3.CycB.PI_2uM_8
Event Count: 19896



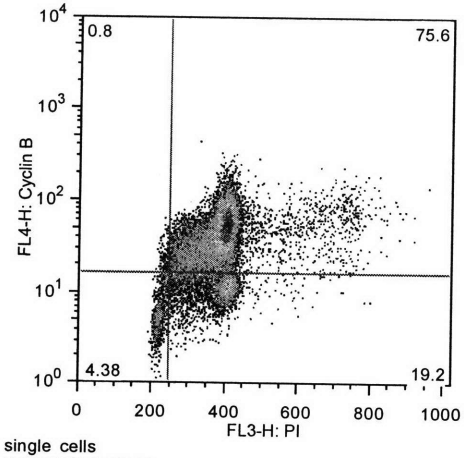
single cells
4.CycB.PI_2uM_12
Event Count: 19837



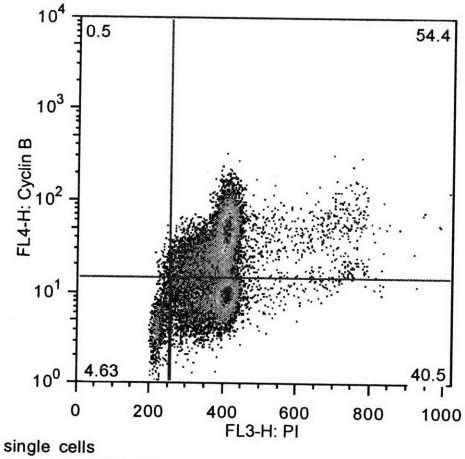
single cells
5.CycB.PI_2uM_16
Event Count: 19968



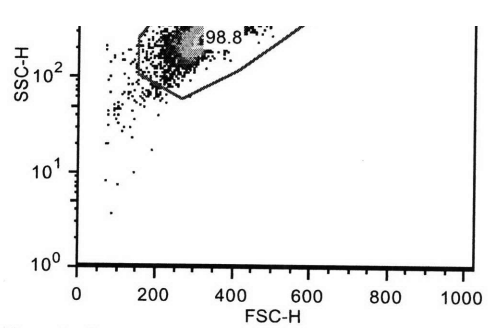
single cells
6.CycB.PI_2uM_24
Event Count: 20372



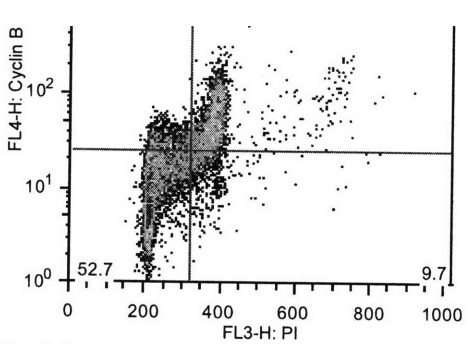
single cells
7.CycB.PI_2uM_36
Event Count: 20641



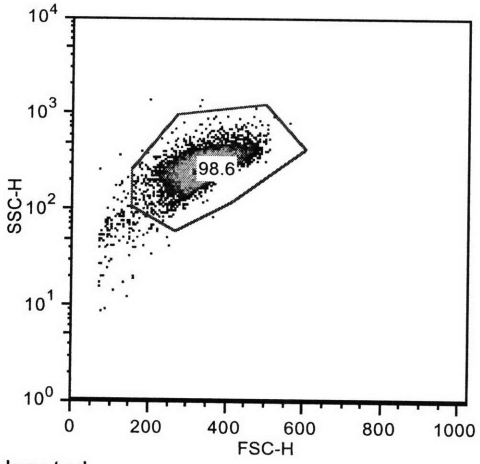
single cells
8.CycB.PI_2uM_ 48
Event Count: 20490



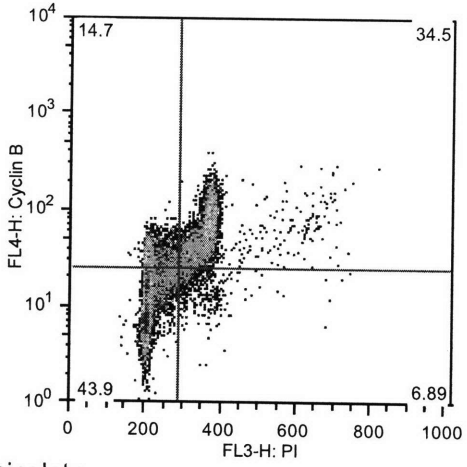
Ungated
1.CycB.PI_10uM_ 2
Event Count: 21918



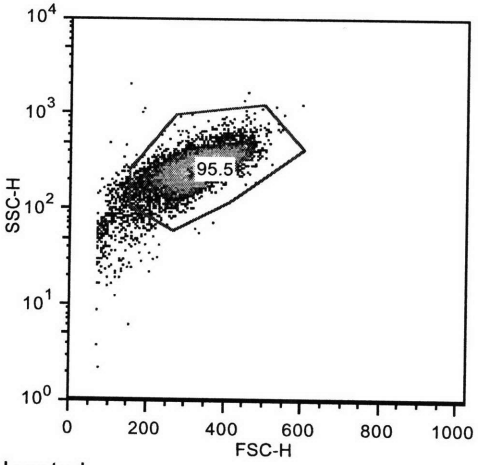
singlets
1.CycB.PI_10uM_ 2
Event Count: 19926



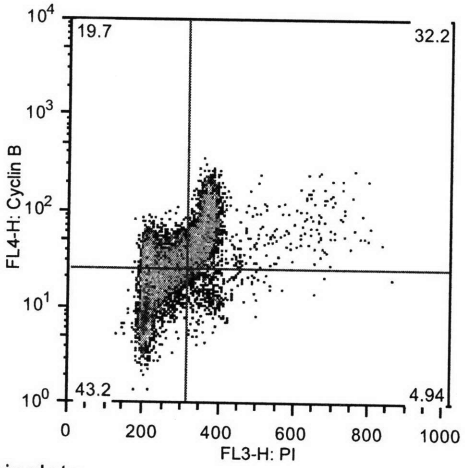
Ungated
2.CycB.PI_10uM_ 4
Event Count: 21799



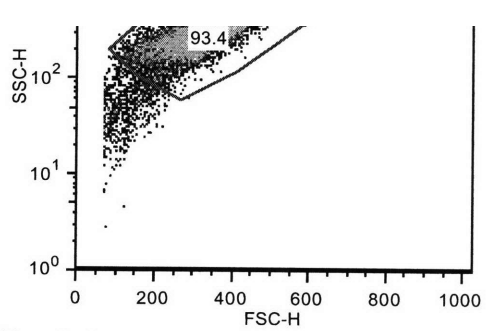
singlets
2.CycB.PI_10uM_ 4
Event Count: 19960



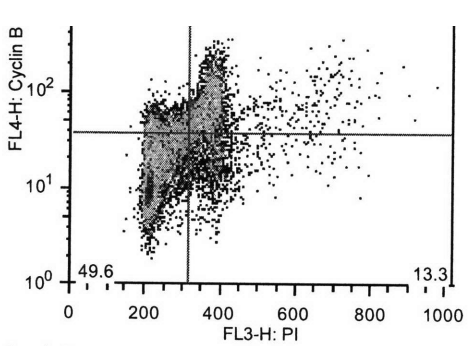
Ungated
3.CycB.PI_10uM_ 8
Event Count: 22745



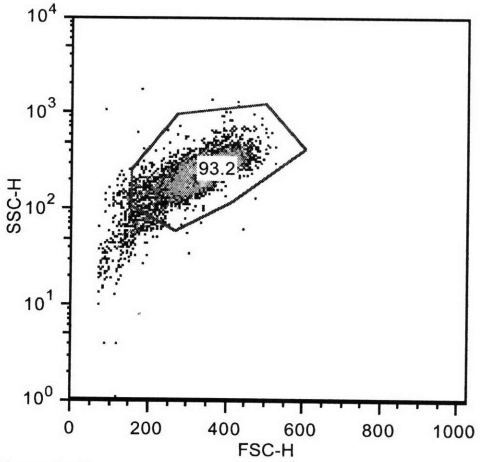
singlets
3.CycB.PI_10uM_ 8
Event Count: 19701



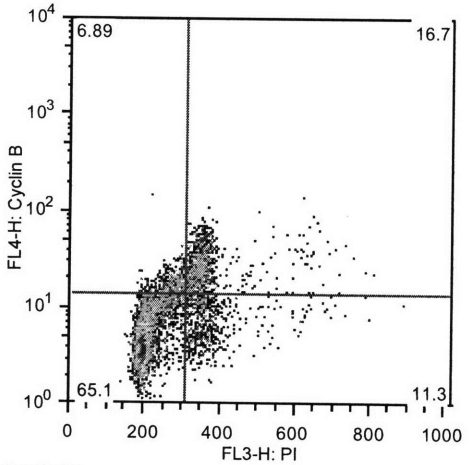
Ungated
4.CycB.PI_10uM_ 12
Event Count: 24189



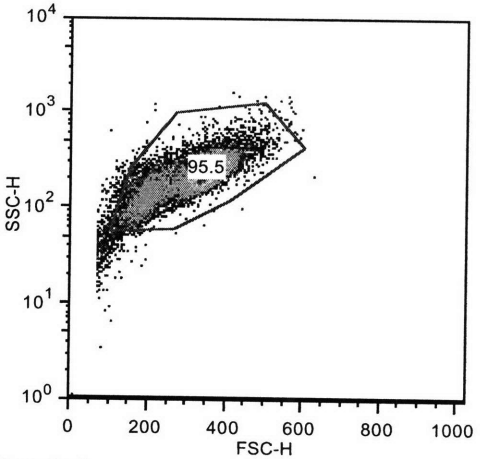
singlets
4.CycB.PI_10uM_ 12
Event Count: 19692



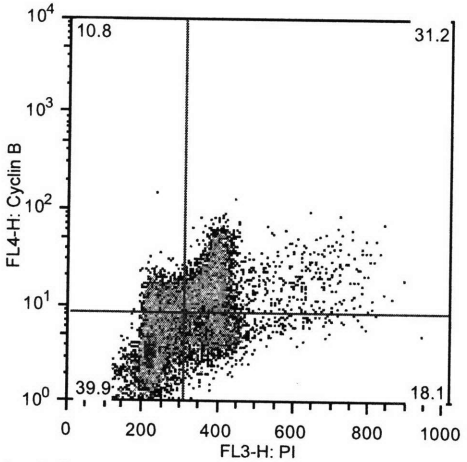
Ungated
5.CycB.PI_10uM_16
Event Count: 11850



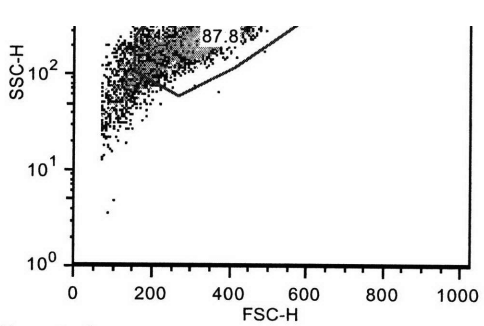
singlets
5.CycB.PI_10uM_16
Event Count: 9776



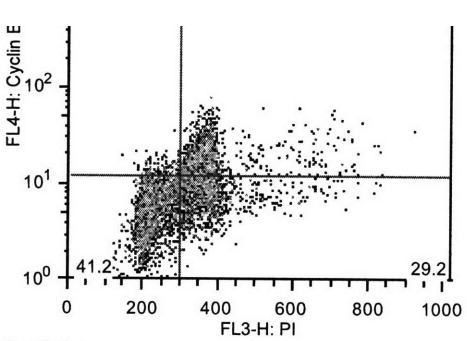
Ungated
6.CycB.PI_10uM_24
Event Count: 22759



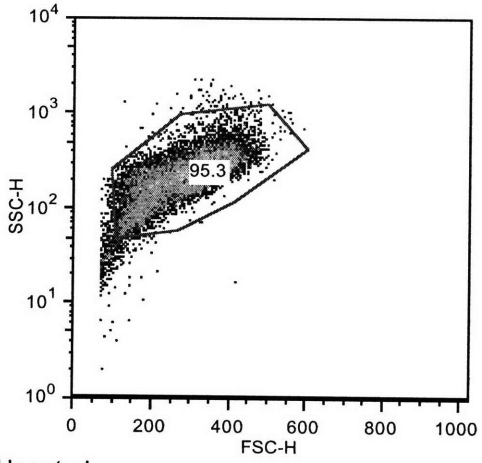
singlets
6.CycB.PI_10uM_24
Event Count: 19675



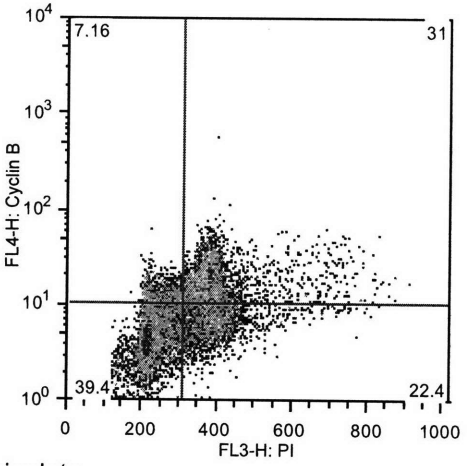
Ungated
7.CycB.PI_10uM_36
Event Count: 11880



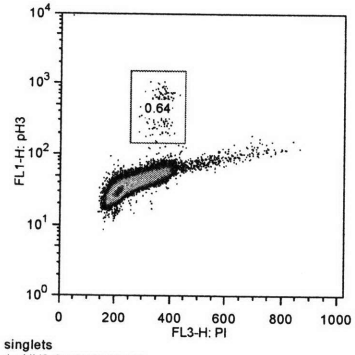
singlets
7.CycB.PI_10uM_36
Event Count: 9339



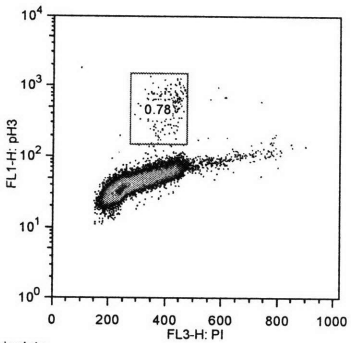
Ungated
8.CycB.PI_10uM_48
Event Count: 24194



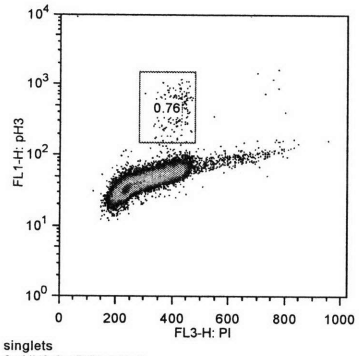
singlets
8.CycB.PI_10uM_48
Event Count: 18637



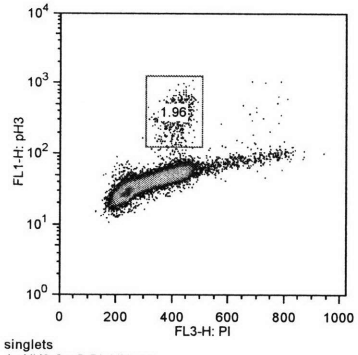
singlets
1.pHH3.CycB.PI_UN_2
Event Count: 30043



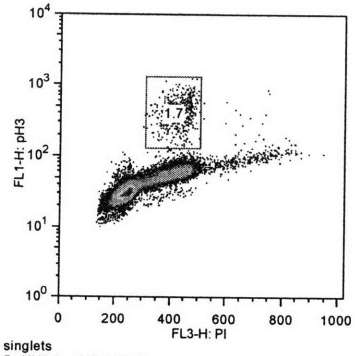
singlets
2.pHH3.CycB.PI_UN_4
Event Count: 30039



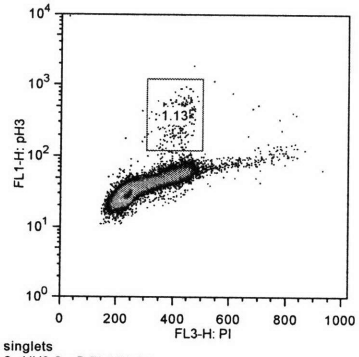
singlets
3.pHH3.CycB.PI_UN_8
Event Count: 30063



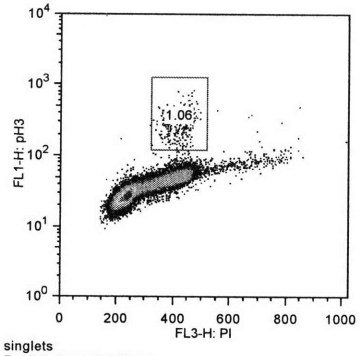
singlets
4.pHH3.CycB.PI_UN_12
Event Count: 30105



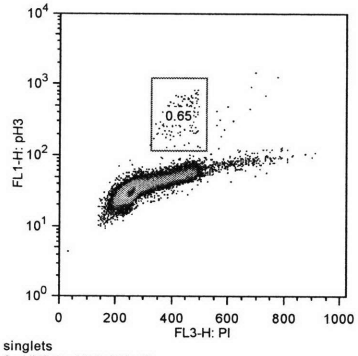
singlets
5.pHH3.CycB.PI_UN_16
Event Count: 29881



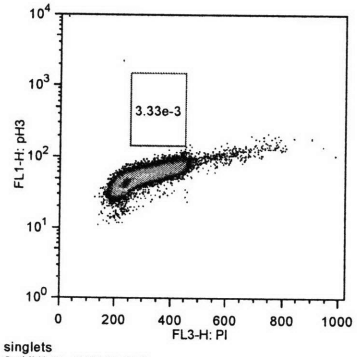
singlets
6.pHH3.CycB.PI_UN_24
Event Count: 30039



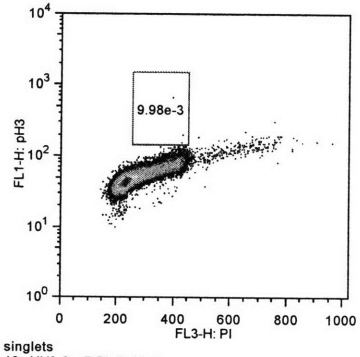
singlets
7.pHH3.CycB.PI_UN_36
Event Count: 30031



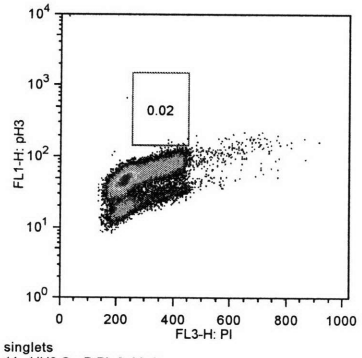
singlets
8.pHH3.CycB.PI_UN_48
Event Count: 29964



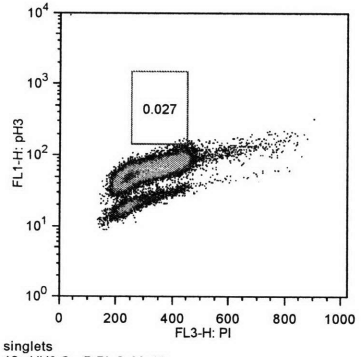
singlets
9.pHH3.CycB.PI_2μM_2
Event Count: 30034



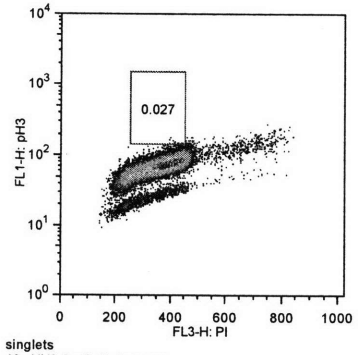
singlets
10.pHH3.CycB.PI_2μM_4
Event Count: 30058



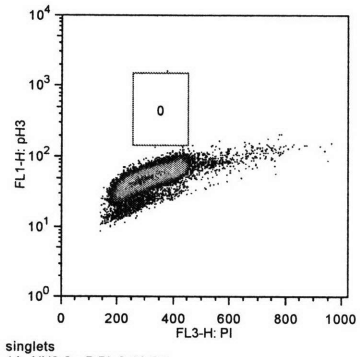
singlets
11.pHH3.CycB.PI_2μM_8
Event Count: 30046



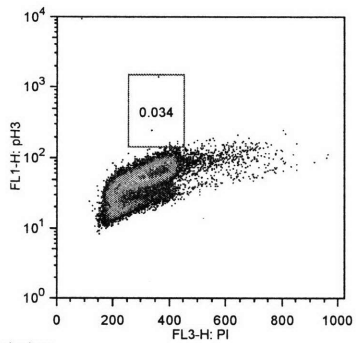
singlets
12.pHH3.CycB.PI_2μM_12
Event Count: 30131



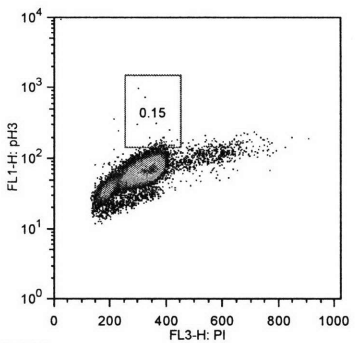
singlets
13.pHH3.CycB.PI_2μM_16
Event Count: 30183



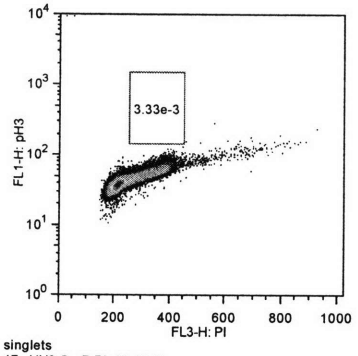
singlets
14.pHH3.CycB.PI_2μM_24
Event Count: 30245



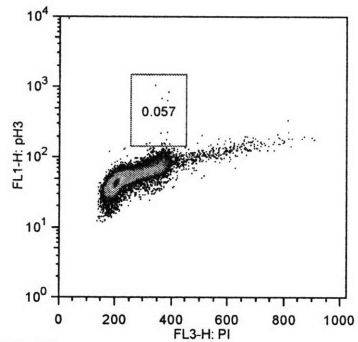
singlets
15.pHH3.CycB.PI_2μM_36
Event Count: 29625



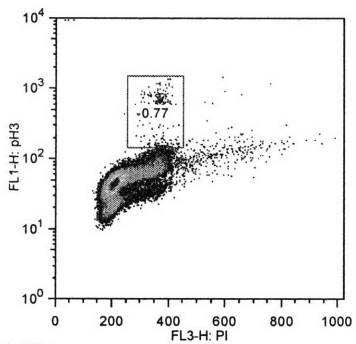
singlets
16.pHH3.CycB.PI_2μM_48
Event Count: 29367



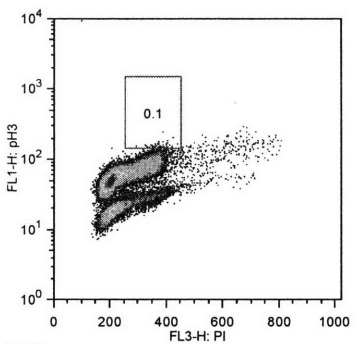
singlets
17.pHH3.CycB.PI_10μM_2
Event Count: 29997



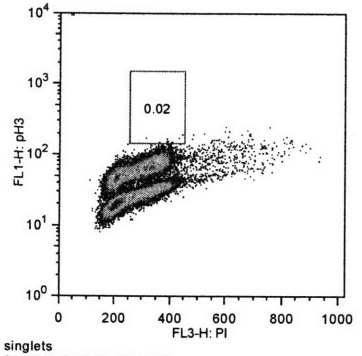
singlets
18.pHH3.CycB.PI_10μM_4
Event Count: 30033



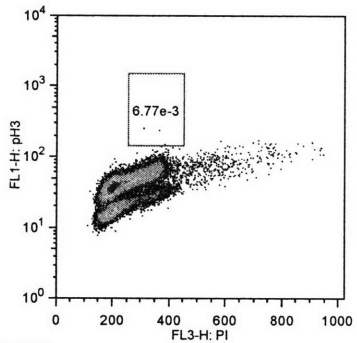
singlets
19.pHH3.CycB.PI_10μM_8
Event Count: 30068



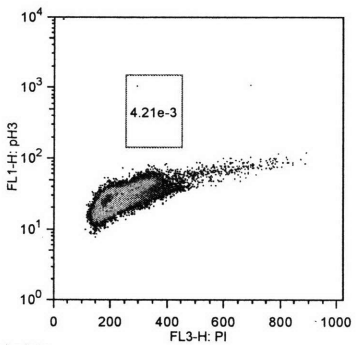
singlets
20.pHH3.CycB.PI_10μM_12
Event Count: 30091



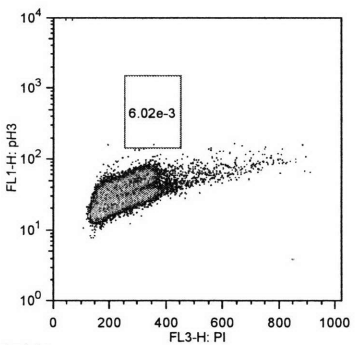
singlets
21.pHH3.CycB.PI_10μM_16
Event Count: 30137



singlets
22.pHH3.CycB.PI_10μM_24
Event Count: 29533



singlets
23.pHH3.CycB.PI_10μM_36
Event Count: 23763



singlets
24.pHH3.CycB.PI_10μM_48
Event Count: 16599