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TITLE:

Use of the Pyrimidine Analog, 5-Iodo-2'-Deoxyuridine (IdU) with Cell Cycle Markers to establish Cell Cycle Phases in a Mass Cytometry Platform

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SUMMARY:
This protocol adapts cell cycle measurements for use in a mass cytometry platform. With the multi-parameter capabilities of mass cytometry, direct measurement of iodine incorporation allows identification of cells in S-phase while intracellular cycling markers enable characterization of each cell cycle state in a range of experimental conditions.

ABSTRACT:
The regulation of cell cycle phase is an important aspect of cellular proliferation and homeostasis. Disruption of the regulatory mechanisms governing the cell cycle is a feature of a number of diseases, including cancer. Study of the cell cycle necessitates the ability to define the number of cells in each portion of cell cycle progression as well as to clearly delineate between each cell cycle phase. The advent of mass cytometry (MCM) provides tremendous potential for high throughput single cell analysis through direct measurements of elemental isotopes, and the development of a method to measure the cell cycle state by MCM further extends the utility of MCM. Here we describe a method that directly measures 5-iodo-2'-deoxyuridine (IdU), similar to 5-bromo-2´-deoxyuridine (BrdU), in an MCM system. Use of this IdU-based MCM provides several advantages. First, IdU is rapidly incorporated into DNA during its synthesis, allowing reliable measurement of cells in the S-phase with incubations as short as 10-15 minutes. Second, IdU is measured without the need for secondary antibodies or the need for DNA degradation. Third, IdU staining can be easily combined with measurement of cyclin B1, phosphorylated retinoblastoma protein (pRb), and phosphorylated histone H3 (pHH3), which collectively provides clear delineation of the five cell cycle phases. Combination of these cell cycle markers

with the high number of parameters possible with MCM allow combination with numerous other metrics.

INTRODUCTION:

Mass cytometry enables detection of approximately 40 parameters by taking advantage of the high resolution and quantitative nature of mass spectroscopy. Metal-labeled antibodies are used instead of fluorophore conjugated antibodies that allow for a higher number of channels and produce minimal spillover[1,2]. MCM has advantages and disadvantages in regard to cell cycle analysis in comparison to flow cytometry. One major advantage of MCM is that the large number of parameters enables the simultaneous measurement of cell cycle state across a large number of immunophenotypically distinct T-cell types in highly heterogeneous samples. MCM has been successfully used to measure the cell cycle state during normal hematopoiesis in human bone marrow[3] and transgenic murine models of telomerase deficiency[4]. Analysis of cell cycle state in acute myeloid leukemia (AML) showed that cell cycle correlated to known responses to clinical therapies, providing an in vivo insight into functional characteristics that can inform therapy selections[5]. A second advantage of mass cytometric cell cycle analysis is the ability to measure a large number of other functional markers that may be correlated with cell cycle state. Recent work has been able to correlate protein and RNA synthesis with cell cycle state through the use of IdU and metal tagged antibodies to BRU and rRNA[6]. This kind of highly parametric analysis measuring cell cycle state across numerous populations in a continuum of differentiation would be nearly impossible with current flow cytometry technology. The major disadvantage of MCM is the lack of comparable DNA or RNA stains as those used in fluorescent flow cytometry (e.g., DAPI, Hoechst, Pyronin Y, etc.). Fluorescent dyes can give relatively precise measurements of DNA and RNA content, but this precision is only possible due to the changes in the fluorescent properties of these dyes that occur upon intercalation between nucleotide bases. MCM analysis is thus unable to measure DNA or RNA content with similar precision. Instead, mass cytometric cell cycle analysis relies on measurements of proteins related to cell cycle state such as cyclin B1, phosphorylated retinoblastoma protein (pRb), and phosphorylated Histone H3 (pHH3) combined with direct measurement of the iodine atom from IdU incorporation into S-phase cells. These two measurement approaches yield highly similar results during normal cellular proliferation, but can potentially be discordant when cell cycle progression is disrupted.

Measurement of the number of cells in each cell cycle phase is important in understanding normal cell cycle development as well as cell cycle disruption, which is commonly observed in cancers and immunological diseases. MCM provides reliable measurement of extracellular and intracellular factors using metal-tagged antibodies; however, measurement of the S-phase was limited as the iridium-based DNA intercalator was unable to differentiate between 2N and 4N DNA. In order to define cell cycle phases, Behbehani developed a method that utilizes IdU with a mass of 127, which falls within the range of the mass cytometer and allows direct measurement of cells in S-phase[3]. This direct measurement circumvents the need for secondary antibodies or use of DNA denaturing agents such as acid or DNase. In conjunction with intracellular cycling markers, it allows high resolution of cell cycle distribution in experimental models.

This protocol adapts cell cycle measurements from common flow cytometry protocols for MCM. Our methods provide a convenient and simple way to include cell cycle parameters. IdU incorporation of in vitro samples requires only 10 to 15 minutes of incubation at 37 °C, which is shorter than most BrdU staining protocols that recommend incubation times of several hours[3,7]. IdU and BrdU incorporated samples can be fixed using a proteomic stabilizer and then stored for some time in a -80 °C freezer. This allows large numbers of IdU stained samples to be archived for batch analysis without reduction in sample quality.

PROTOCOL:

1. Preparation of IdU stocks

1.1. Dissolve 5-iodo-2'-deoxyuridine (IdU) in DMSO to a concentration of 50 mM. Sterile filter, aliquot into 10-50 µL tubes, and store at -80 °C

1.2. Remove IdU from the freezer, and thaw at room temperature. Dilute IdU in RPMI-1640 to make a working solution at a final concentration of 1 mM. Pipette up and down or vortex to mix.

1.2.1. Typically, dilute the concentrated IdU into the media in which the cells are being cultured (e.g. DMEM, IMDM, etc.) or have diluted it into PBS for addition directly to peripheral blood or bone marrow aspirate samples. This pre-dilution step facilitates mixing of the DMSO with the aqueous media of the cells of interest.

NOTE: The final concentration of IdU during incubation should be 10 µM; a solution of 1 mM will can be added at a ratio of 10 µL to every 1 mL of media.

2. IdU incubation and sample preservation

2.1. Maintain samples in a humidified 37 °C incubator. Remove the sample from the incubator and move the sample into a biosafety hood.

2.2. Add 10 µL of 1 mM IdU to every 1 mL of sample.

2.2.1. For a 6-well plate, add 30 µL of 1 mM IdU to the 3 mL of culture media in each well. IdU can also be used directly in bone marrow aspirates as well as murine studies[4].

2.3. Place the sample back into the incubator at 37 °C for 10-15 min. Maintain cells under the optimal growth conditions of interest during IdU exposure in order to get the most accurate measurement of S-phase.

2.4. After the IdU incubation, remove the sample and transfer to a conical tube.

2.5. Spin the sample at 400 x *g* for 10 min at room temperature.

2.6. Aspirate the supernatant and resuspend in 200 µL of PBS.

2.6.1. If needed, perform a live/dead stain (using cisplatin) at this step to mark dead cells before fixation and freezing.

NOTE: Rhodium live/dead staining does not perform well after methanol permeabilization, so it is not recommended for use in cell cycle analyses.

2.7. Add 18.75 µL of 16% paraformaldehyde (PFA) to the PBS for a final concentration of 1.5% PFA. Incubate at room temperature for 10 min. Spin the sample down at 400 x *g* for 10 min at room temperature.

2.8. Aspirate the PBS/PFA solution and re-suspend the sample in 500 µL of Cell Staining Media (CSM; 1x PBS with 0.5% BSA and 0.02% sodium azide) + 10% DMSO prior to freezing.

2.8.1. If using commercial Proteomic Stabilizer, add 280 µL of Proteomic Stabilizer to sample re-suspended in 200 µL of PBS (1:1.4). Incubate samples at room temperature for 10 min and then place directly into the -80 °C.

NOTE: IdU has been shown to incorporate effectively within 10-15 min incubation at 37 °C. IdU incubations longer than 10-15 min will progressively reduce resolution of the S and G2-phase populations, as IdU-labeled cells leave S phase and progress to G2 or M phase. We have also observed that long-term incubation with IdU can cause cell death and cell cycle artifacts. The subsequent cell processing and antibody staining following IdU incorporation is sufficient to wash away residual IdU that was not incorporated into S-phase cells. We have not observed significant Iodine background when using the in vitro protocol described here; however, we have very rarely observed iodine contamination in clinical samples. This may occur from medical procedures, such as iodine contrast in a CT scan, or from iodine-containing pharmaceuticals. Should large amounts of IdU background be observed, the sample should not be run to avoid damage to the mass cytometer's detector.

3. Staining samples for mass cytometry

3.1. Remove the samples from the -80 °C and allow to thaw before surface staining.

3.1.1. If using the SmartTube method of fixation, thaw the samples at 0-4 °C to avoid additional fixation as the samples warm up.

3.2. After the samples are thawed, transfer approximately 1-2 million cells into a 5 mL FACS tube.

3.3. Centrifuge the FACS tube at 600 x *g* for 5 min, and fill the FACS tube with cell staining media (CSM) to wash the cells. Repeat one additional time.

3.3.1. If cells are known to stick together, add 400 U/mL of heparin to CSM washes in order to prevent cell to cell contact but this is not strictly necessary.

3.4. Incubate the cells with FC-blocking agent, 5 µL of the agent per 100 µL of cells, for 10 min at room temperature.

3.5. Prepare a mixture of antibodies that will stain the surface, or extracellular portion, of the cells. The total staining mixture will amount to 100 µL per 1-2 million cells in each test. The staining mixture will be balanced accordingly with CSM and heparin in the cocktail and FACS tube.

NOTE: Addition of CSM at this step will also reduce nonspecific staining artifacts[8]. This staining mixture is entirely dependent on targets of interest and surface phenotype (e.g., a study involving T-cells will use a surface mixture of CD45, CD3, CD4, CD8, etc.). A detailed protocol of sample processing and staining can be found in Behbehani and McCarthy et al.[9,10].

3.6. Add the surface staining mixture to the cells and incubate at room temperature with continuous shaking for 30-60 min.

3.7. After staining, fill the FACS tube with CSM, and spin down at 600 x *g* for 5 min.

3.8. Wash two more times with CSM, spinning the sample at 600 x *g* for 5 min and aspirating each CSM wash.

3.9. Fix the extracellular antibodies by adding 1 mL of PBS with 10% CSM and 1.5% PFA.

3.10. Fill the FACS tube containing the PBS/CSM/PFA mixture with CSM. Spin down at 600 x *g* for 5 min and aspirate the supernatant.

3.11. Add methanol at -20 °C.

3.12. Vortex the sample for 1-2 min to achieve a single cell suspension and verify that all cell clumps have been re-suspended.

3.13. While the sample is vortexing slowly, rapidly add 1 mL of ice-cold methanol using a 1,000 µL pipette with a filter tip.

3.14. Hold the FACs tube up to the light and make sure there are no visible clumps; cloudiness is to be expected. Any clumps will render the sample unusable for subsequent MCM analysis.

3.15. Store the sample at -20 °C for 10-20 min.

3.16. Prepare the intracellular staining mixture during this time. The intracellular staining mixture will be dependent on targets of interest. For cell cycle analysis, include CyclinB1, pRb, Ki67, and pHH3 in this staining mixture, but other intracellular markers can be added as needed.

3.17. After 10-20 min at -20 °C, remove the sample, add 1.5 mL of PBS and fill the remainder with CSM.

3.18. Centrifuge the sample 600 x *g* for 5 min, and aspirate the supernatant.

3.19. Wash two more times with CSM, spinning the sample down at 600 x *g* for 5 min and aspirating the supernatant each time.

3.20. After the last CSM wash, centrifuge the sample and leave a residual volume of approximately 50 µL.

3.21. Add the prepared antibody mixture (typically add 50 µL of antibody staining cocktail to achieve a final staining volume of 100 µL) to the sample and incubate on a shaking platform for 30 to 60 min at room temperature.

3.22. After staining add CSM and centrifuge at 600 x *g* for 5 min.

3.23. Aspirate the CSM, wash again with CSM, spinning at 600 x *g* for 5 min, aspirating the CSM, then add PBS.

3.24. After the completion of intracellular staining, place cells into an intercalator solution that fixes the antibodies to the cells and stains the DNA of each cells to enable identification. The intercalator solution contains nonisotopically pure iridium intercalator (pentamethylcyclopentadienyl-Ir(III)-dipyridophenazine) added from the manufacturer's stock solution at a concentration of 500 µM. Dilute the Iridium stock 1:4000 in a solution of PBS and 1.5% PFA. Add the iridium intercalator solution at 100-200 µL per million cells in order to stain evenly and prevent overstaining.

NOTE: The iridium in this intercalator solution is intended to identify cells for singlet gating, it should not be used for live/dead stains. If live/dead stains are desired they need to be performed before fixation as noted above and in McCarthy et al.[9].

3.25. Store the samples in intercalator solution in a 4 °C refrigerator for up to two weeks before sample acquisition on the CyTOF.

4. Mass cytometer operation

NOTE: Mass cytometry operation can be machine specific. It is always advisable to check the CyTOF user's manual before operation. Additionally, there are currently two JoVE articles dealing with machine start up and maintenance[9,11].

4.1. Check the nebulizer for any clogs, cracks, and other irregularities before operating the mass cytometer.

4.2. Connect the nebulizer to the cytometer and begin the warmup procedure. Do not start the mass cytometer without the nebulizer in place.

4.3. Run water through the sample lines once the mass cytometer has finished warming up. The spray chamber needs to reach approximately 200 °C before performing tuning or sample analysis.

4.4. Run water for 5-10 min. After 5-10 min, load the tuning solution and select the tuning manager. The tuning solution is a solution containing fixed concentrations of metals and used to optimize the mass cytometer before sample acquisition

4.5. In the tuning manager, select **Preview** once the tuning solution has reached a steady state hit record to begin the automated tuning process.

4.6. Once tuning is finished load the sampler with water and allow water to run through the sample lines during sample processing. Detailed protocol for daily cytometer operation and tuning can be found at Leipold[11].

4.7. Occasionally, the automated tuning will not tune to optimal machine performance. Repeat the tuning procedure to correct this.

4.8. Wash the sample with CSM once and with pure deionized water twice before sample acquisition. Washing with water is important to remove residual salt from the PBS/CSM.

4.9. Check the sensitivity and sample flow using manufacturer supplied equilibration beads, polystyrene beads loaded with known metal concentrations.

4.10. Change the acquisition mode from tuning to event capture mode. Set the time limit to stop acquisition at 120 s. Wait 45 s before selecting **Record**.

4.11. The mass cytometer will stop sample acquisition automatically after 120 seconds. Use the rain plot viewer to check Eu151 and Eu153 intensity.

4.12. Dilute equilibration beads in pure deionized water at a 1:20 ratio.

4.13. Before sample acquisition check the experiment manager. Use the experiment manager to assign names to channels and to add channels to be recorded.

4.13.1. Make sure the 127-I channel is added if using IdU.

4.13.2. Note that it is essential to set the mass cytometer to measure the needed parameters (e.g., IdU) prior to sample acquisition. If channels are not selected in advance, data will not be collected from any unselected channel and cannot be recovered.

4.14. Dilute the cells to a concentration of approximately 1-2 x 10^6/mL using the 1:20 pure deionized water and equilibration bead mixture. Pass the cells through the filter topped FACS tube in order to remove any residual clumps.

4.15. Load the sample and change the acquisition time.

4.16. Press **Preview** and wait for event count per second to stabilize.

4.16.1. Do not run events in excess of 400 events per second, this will lead to significant amounts of doublets and debris. We typically collect at least 20,000 to 50,000 cell events, but the optimal number will depend on the experimental design. Staining of up to 2 million cells will typically yield 300,000 to 400,000 cell events. Note that not all events will be cells (there will be debris and bead events included in the event count).

4.17. Once sample acquisition is done load a washing solution, start sample induction and run for 5-10 minutes. After 5-10 minutes stop sample induction and run water for 10-20 minutes. Wash solution is a weak solution of hydrofluoric acid designed to strip residual metal from the sample lines.

4.18. Shut down the mass cytometer and remove the nebulizer. The nebulizer will be hot, take care during handling.

5. Data analysis

5.1. In order to remove beads and also to correct for signal drift during sample acquisition, normalize the FCS files using Fluidigm software or the application developed by Finck[12].

5.2. Upload the FCS to Cytobank or other flow cytometry analysis software. FCS files can be used in any compatible software, for the purposes of this protocol all gating and further analysis has been done in Cytobank[13].

5.3. Before cell cycle gates can be drawn, exclude any doublets or cell debris from downstream analysis, this can be done by using the biaxial plot of Event Length vs 191-Ir (**Figure 1a**). Cells will form a distinct, bright population Ir^{high} that can be used to exclude doublets and debris. This is the singlet gate. This gating method typically removes about 50-60% of doublet cell events, so additional strategies may be required to remove remaining doublet cell events.

5.3.1. Change the event length scale (minimum and maximum) to make the cells appear more prominent to aid in singlet gating.

5.3.2. Further remove doublets and debris by using Gaussian parameters, Residual and Offset. A higher Residual with lower Offset is also debris and doublets, and gating around this population can further remove doublets and debris (**Figure 1b, c**).

5.4. S-phase gating – S-phase is the easiest gate to draw but also the most important. Draw this gate using a biaxial plot of IdU vs pRb, Ki67, or cyclinB1. S-phase IdU^{+} cells will form a distinct population when looking at these biaxial plots (**Figure 2b**).

5.5. G0/G1-phase, G2/M-phase gating – Establish the G0/G1 and G2/M phase gates on the IdU vs CyclinB1 plot and the use of IdU incorporation is crucial to establish the boundary between the G0/G1 and G2/M phase gates. G0/G1-phase will be $CyclinB1^{low}/IdU^{-}$ and G2/M-phase will be $CyclinB1^{high}/IdU^{-}$. Good CyclinB1 staining will show a natural population between the G0-G1 and G2-M populations; however, this will vary across sample and cell types under experimental conditions. In experimental conditions where the cell cycle distribution may be affected and there is less separation between the CyclinB1 G0/G1-phase and G2/M-phase utilizing the S-phase will allow consistent gating for particular cell types in each specific experiment. This method is detailed below.

5.5.1. Plot only the S-phase cells on the CyclinB1 vs IdU to help establish the separation between G0/G1-phase and G2/M-phase. Draw a gate on the $CyclinB1^{high}$ population and adjust until approximately the top 5% of the S-phase population is inside the gate (**Figure 2c**). This establishes the breakpoint between the G0/G1 and G2/M phase gates (**Figure 2e-f**). The active population will be changed to the population of interest and the portion residing inside the previous gate will be the G2/M-phase population while the remainder will be the G0/G1-phase population.

5.6. G0-phase gating – Establish the G0-phase on the pRb vs IdU plot. The G0-phase will be represented by a pRb^{low}/IdU^{-} population. The active cycling population will have high expression of pRb and IdU incorporation, the G0-phase gate can be drawn on this boundary as it typically expresses at two distinct populations (**Figure 2g-i**).

5.6.1. Define the G0-phase by making the S-phase population drawn previously (**Figure 2b**) the active population and drawing a gate incorporating the top 90-100% of the pRb^{high} population. This is the pRb+ cycling population (**Figure 2i**), the pRb^{low} population outside this gate is the G0 population (**Figure 2j**).

5.6.2. If pRb is unavailable or not able to be recorded, use Ki67 vs IdU to establish the G0-phase population. Drawing a gate representing the majority of the S-phase population and using that gate as the boundary for the Ki67 vs IdU the remainder of the population will be the G0-phase (**Figure 3a**).

5.7. M-phase gating – Establish the M-phase in the IdU vs pH3 biaxial plot. The M-phase represents a very small fraction of cells and is gated on the $pH3^{high}$ population (**Figure 2d**).

5.8. IdU incorporation failed or was not possible – If IdU is unavailable, define cell cycling and not cycling fractions using Ki67 and pRb. Ki67 and pRb, in normal conditions, form two distinct populations a $Ki67^{high}/pRb^{high}$ and a $Ki67^{low}/pRb^{low}$. The double positive population represents the active cycling population, correlating to G1-phase, S-phase, G2-phase, and M-phase. The double low population represents the not cycling population, correlating to the G0-phase (**Figure 3b**).

NOTE: It is not possible to delineate each individual phase using the Ki67 vs pRb but experimental effects on the relative cycling/not cycling populations can be determined.

5.9. Cell cycle analysis – Once the gates have been established, export the numerical values from the gates for further analysis. The percentages in each cycle can be achieved by subtracting the single populations from the combined populations. The G0-phase, drawn on the $pRb^{low}IdU^{low}$, gate percentage can be subtracted from the G0/G1-phase, drawn on $CyclinB1^{low}IdU^{neg}$, to find the G1-phase percentage. Similarly the G2-phase percentage is derived from the subtraction of the M-phase gate from the G2/M-phase gate. This will generate numerical values for each individual cell cycle phase; G0, G1, S, G2, and M. The numerical values generated for each individual cell cycle phase can be used for further analysis such as graphing and statistical analysis.

REPRESENTATIVE RESULTS:

Utilizing HL-60 cells and a human bone marrow aspirate it is possible to show how experimental conditions can affect cell cycle distribution and analysis. First, the gating strategy must be established to demonstrate how the cell cycle phases are derived. In **Figure 1** we show the establishment of the singlet gate, which is important in separating cellular debris and doublets, establishing a single cell population. For cell lines the singlet gate is all that is needed to move onto cell cycle analysis (**Figure 2a**). For human samples immunophenotypic populations typically need to be established prior to cell cycle analysis, since the exact boundaries of each cell cycle gate can vary across different cell types. Once the populations have been established (usually by gating on surface markers that define that population) the cell cycle gates will then need to be established. **Figure 2b** demonstrates the establishment of the S-phase on the IdU vs CyclinB1 biaxial plot. This plot is also used to establish the boundary of the G2/M-phase gate (**Figure 2f**). Once the G2/M-phase is established the remainder is the G0/G1-phase gate (**Figure 2f**). The IdU vs pRb is used to establish the pRb^{+} cycling population first by establishing a gate on IdU incorporating cells (**Figure 2g-i**). The pRb^{+}/IdU^{neg} population outside this gate is the G0-phase (**Figure 2j**). M-phase is established on the IdU vs pHH3 where M-phase cells express high levels of pHH3 and exhibit no IdU incorporation (**Figure 2k**). In the event that pRb is not included the G0-phase can be replicated using Ki67 in a similar way to the method described above (**Figure 3a**). If IdU incorporation failed or was not performed it is still possible to determine relative cycling fractions using Ki67 and pRb. By using the Ki67 and pRb biaxial two distinct populations form, a $pRb^{+}/Ki67^{+}$ double positive and a $pRb^{low}/Ki67^{low}$ population. The double positive population represents cells in cycle, while the low represents cells not in cycle (**Figure 3b**). Using IdU incorporating cells and pRb^{low} cells with no IdU incorporation we show that the S-phase is primarily in the $pRb^{+}/Ki67^{+}$ population while the G0-phase is primarily in the $pRb^{low}/Ki67^{low}$ population.

Cell cycle analysis relies on good experimental technique especially during the IdU incubation step. While IdU incorporation is flexible (being applicable in cell culture, bone marrow aspirates, and even murine studies), it necessary to perform IdU incorporation and fixation without disrupting the cell cycle state of experimental interest. IdU labeling and thus downstream cell

cycle analysis can significantly be affected by time and temperature as indicated by **Figure 4**. Cells that remain too long in enclosed vessels or at that might be encountered in sample shipment or sample transport between locations, will have reduced S-phase fraction and not be accurate for cell cycle analysis (**Figure 4a**). Short time periods though, those under an hour in total, will have normal cell cycle distribution indicating that quick transport may not negatively cell cycle analysis (**Figure 4b**). Another important modifier is cryopreservation that is routinely used in most laboratories. When examining cell cycle state in cryopreserved cells a long equilibration period may be required before cells return to active cell cycling, which may still not reflect the pre-cyropreservation cell cycle state (**Figure 4c**).

Primary human samples are often composites of multiple different cell types, these different cell types can have different sensitivities to processing leading to different cell cycle gating. In two bone marrow aspirates that were IdU labeled immediately, stored for 30 minutes before IdU labeling, or cryogenically stored after Ficoll separation there are differences between each sample and population (**Figure 5a-b**). Two immunophenotypic populations were examined for differences in IdU incorporation; T-cells ($CD45^{high}/CD3^{high}$) and monoblasts ($CD33^{+}$, $HLADR^{+}$, $CD11b^{low}$, $CD14^{neg}$). With the correct combination of surface marks, it is possible to examine further immunophenotypic populations. In marrow #2 there was a noticeable T-cell activation effect after 30 minutes storage that was not seen in the monoblasts from the same patient (**Figure 5b**). Like cultured cells there were noticeable changes in IdU labeling after cryogenic storage that was also dependent on population. Marrow #1 had reduction in the T-cell population but increase in monoblasts IdU labeled fractions when compared to baseline (**Figure 5a-b**), Marrow #2 showed reduction in both T-cells and monoblasts when compared to baseline (**Figure 5a-b**). Frozen cells then require a notable incubation period before returning to normal cell cycle state and this can influence studies that rely on modifying cell cycle state or cell cycle state as a metric of drug or experimental effect.

Another benefit of MCM is the ability to discriminate cells in cell cycle arrest or that have abnormal cell cycle distribution. While DNA dyes commonly used in flow cytometry are able to discriminate between 2N and 4N DNA content, they are very bright, which can greatly complicate measuring other parameters from that laser. IdU, however, only takes one mass channel and has minimal spill over allowing for other markers to be used in cell cycle determination. MOLM13 cells that were irradiated show decreased IdU incorporation and decrease in M-phase when compared to control cells (**Figure 6**). Disruption of the normal cell cycle checkpoints might alter the apparent cell cycle state by MCM. Looking at pH2AX and cPARP populations in the non-irradiated cells the $pH2AX^{low}$ and $cPARP^{low}$ population shows normal cell cycle distribution while cells expressing higher levels of pH2AX or cPARP localize mainly in the G0/G1-phase which is expected (**Figure 6a**). In the irradiated cells the $pH2AX^{low}$ and $cPARP^{low}$, however, the cells are almost entirely localized in the G0-phase, while the $pH2AX^{high}$ and $cPARP^{low}$ cells show a cell cycle arrest phenotype with IdU incorporation and localization to the G0/G1-phase and G2-phase with an absence of M-phase. The $pH2AX^{high}$ and $cPARP^{high}$ cells also show cells incorporating some IdU and localizing to the G0/G1-phase indicative of radiation damage (**Figure 6b**).

FIGURE LEGENDS:

Figure 1. Establishing the singlet gates using 191-Ir by Event Length and also Gaussian parameters, Residual and Offset. The differences in T-cell ($CD45^+/CD3^+$) and S-phase (IdU^+) between an ungated sample (**a**), an event length vs 191-Ir singlet gate (**b**), or a singlet gate combined with Gaussian parameters, residual and offset (**c**). Singlet gating removes debris, doublets, and beads shown in the loss of the pRb^{high} population on the right corner of the biaxial. This singlet gate can be further optimized by including Gaussian parameters such as residual and offset, removing more debris.

Figure 2. The gating schema for establishing cell cycle gates for G0, G1, S, G2, and M phases using IdU, CyclinB1, pRb, and pHH3. The singlet gate is established to remove doublets and debris (**a**). The S-phase must be established (**b**), once the S-phase is established the IdU^+ population can be used to establish the G2/M-phase boundary (**c,d**). The establishment of the G2/M-phase boundary establishes the bounds of the G0/G1-phase population (**f**). The pRb^+ and G0-phase population are established on the IdU vs pRb biaxial. The IdU^+ cells (**h**) are used to establish the boundary for the pRb^+ population (**i**). The boundary of the pRb^+ population establishes the boundary of the G0-phase population (**j**). The M-phase is established on $pHH3^+$ cells that are IdU^- (**k**).

Figure 3. Establishing cell cycle gates without the use of pRb or without the use of IdU incorporation. Drawing the G0-phase can also be done using Ki-67 following the same gating strategy that was used with pRb, if pRb is not included in the experiment (**a**). If IdU incorporation failed or was not performed it possible to still recover the relative cell cycling fractions through the use of Ki67 and pRb. Ki67 and pRb double positive expression is correlative to cells in cycle as evidenced by demonstrating the IdU^+ cells are found primarily in the double positive population (**b**). The Ki67 and pRb low population correlates with the G0-phase or not cycling population demonstrated by pRb^{low}/IdU^{neg} cells being found in the Ki67 and pRb low population. This method cannot discriminate individual cell cycle phases but can still be used to determine relative cycling fractions in experimental conditions.

Figure 4. Representative Figures of the effect of different storage conditions on the cell cycle distribution of HL-60 cells. Cells were incubated for an hour followed by an hour rest at the stated temperature conditions in sealed tubes (**a**). There are noticeable effects on cell cycle distribution when compared to the control. HL60 cells were kept in sealed tubes at room temperature for 30 minutes, a situation that may occur in a clinical setting, before IdU incorporation (**b**). Sealed tubes kept at room temperature for 30 minutes did not show appreciable cell cycle differences. The effect of cryogenic storage was investigated on cell cycle in HL60 cells, where a sample was taken before cryogenic storage and a sample taken after an hour rest following a week in cryogenic storage (**c**). One hour post thaw the cell cycle distribution is affected and cell cycle distribution does not return to normal until approximately a week following thaw.

Figure 5. Processing can have an effect on patient samples and representative images are shown between two different patients showing the (a) T-cells ($CD45^{high}/CD3^{high}$) and (b) Monoblasts ($CD33^+$, $HLADR^+$, $CD11b^{low}$, $CD14^{neg}$). Between marrow one and marrow two it is

clear that in marrow 2 there was a T-cell activation effect during the 30 minutes rest whereas marrow one had no such effect. Marrow one showed possible activation effect in the monoblasts population after 30 minutes while marrow two did not. In both marrows, however, it was clear that cryogenic storage impacted cell cycle distribution regardless of cell type.

Figure 6. MOLM13 cells were either left as controls or irradiated using an X-ray irradiator at 10Gy. MOLM13 cells show the cell cycle distribution in four different populations of pH2AX and cPARP expression. Control cells show minimal pH2AX and cPARP staining, with normal cell cycle characteristics being shown in the $pH2AX^{low}$ and $cPARP^{low}$ population (**a**). While the irradiated cells show an abnormal cell cycle distribution with the majority of cycling cells being located in the $pH2AX^{high}$ and $cPARP^{high}$, indicating cell cycle disruption (**b**). The non-damaged, $pH2AX^{low}$ and $cPARP^{low}$, cells show a lack of cycling characteristics are primarily found in the G0-phase. Without these markers these cells would appear as 4N and 2N cells in normal flow cytometry possibly confounding downstream cell cycle analysis.

DISCUSSION:

The examples presented here demonstrate how to use an MCM platform to analyze cell cycle distribution. It has also been demonstrated that cell cycle analysis is sensitive to experimental conditions such as time and temperature, which is an important consideration researchers must take when considering MCM for their cell cycle analysis[14]. Samples left in storage for a short period of time, no longer than an hour, will have IdU incorporation comparable to their normal state. Samples in a closed system for long periods of time, approximately 2 hours, will have reduced IdU incorporation, however, the relative cycling and non-cycling fractions will not change allowing coarse cell cycle analysis. Cryogenic storage and subsequent thawing are disruptive to normal cell cycle distribution for a significant period of time. Cryogenic storage has been noted previously to disrupt protein and RNA distribution but has only recently been shown to disrupt cell cycle[14-17]. Taken together these indicate that if long storage times are anticipated or cryogenic preservation of samples it would be better to stain the cells with IdU before storage or cryogenic storage, fix the cells and store them until analysis can be done. As cell cycle analysis by MCM does not require live cells this would enable researchers to bank precious samples and perform accurate downstream cell cycle analysis.

Cell cycle analysis by MCM is a robust system capable of deep interrogation of experimental models. Due to MCM high parameter count, approximately 40-50 mass channels, cell cycle analysis can be compounded with other intracellular or extracellular markers bypassing the need for sorting of cells based on immunophenotype which may cause significant cell cycle effects to be lost. The high parameter nature of MCM lends itself to examining effects in high-dimensional mapping applications such as SPADE and viSNE. While SPADE and viSNE are typically used to define immunophenotypic populations which can then be examined for cell cycle changes it would also be possible to map on cell cycle markers. Depending on experimental conditions mapping cell cycle markers in a high-dimensional space can show cell cycle correlation with drug effect or what immunophenotypic populations may be localizing to each cycle state[3,5]. While MCM may be limited by the lack of DNA binding fluorescent dyes this is compensated by direct IdU incorporation during S-phase cells and intracellular cell cycle proteins can be used to

determine cell cycle state. These cell cycle proteins can also help discriminate between stages of cell cycle arrest that would otherwise appear as 3N or 4N in traditional flow using DNA binding dyes. Such highly parametric systems are not without disadvantages however, and it is sensitive to disruptions in cell cycle. We have shown that long storage times and cryogenic storage can significantly affect cell cycle distribution. This is especially important when trying to rationalize experimental effects for drugs that may affect cell cycle distribution. Treatment of drugs designed to affect cell cycle on frozen primary samples may give false data on cell cycle effect when used immediately after thawing from cryogenic storage. MCM is a versatile technology for cell cycle analysis that can be applied to a number of experimental models and is especially suited to deep profiling of heterogeneous systems. As with other highly parametric methods it is necessary to have a carefully designed experiment with appropriate considerations for how processing and experimental effects will affect cell cycle analysis.

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DISCLOSURES:

Dr. Behbehani receives travel support from Fluidigm. Fluidigm has also purchased reagents and materials for lab use.

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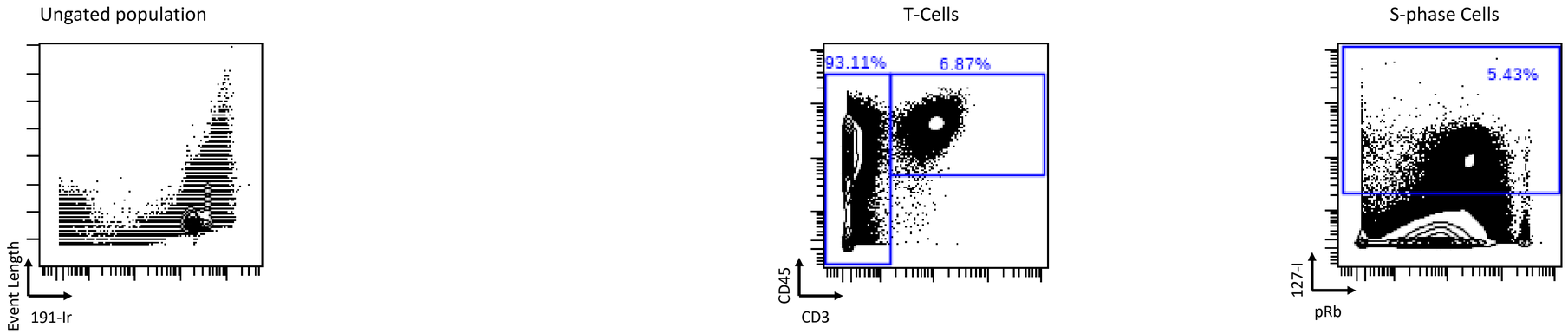

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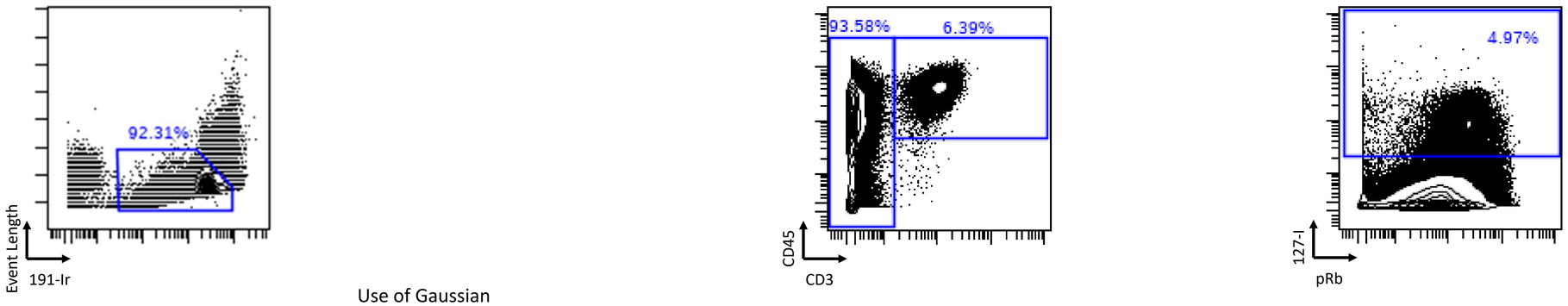

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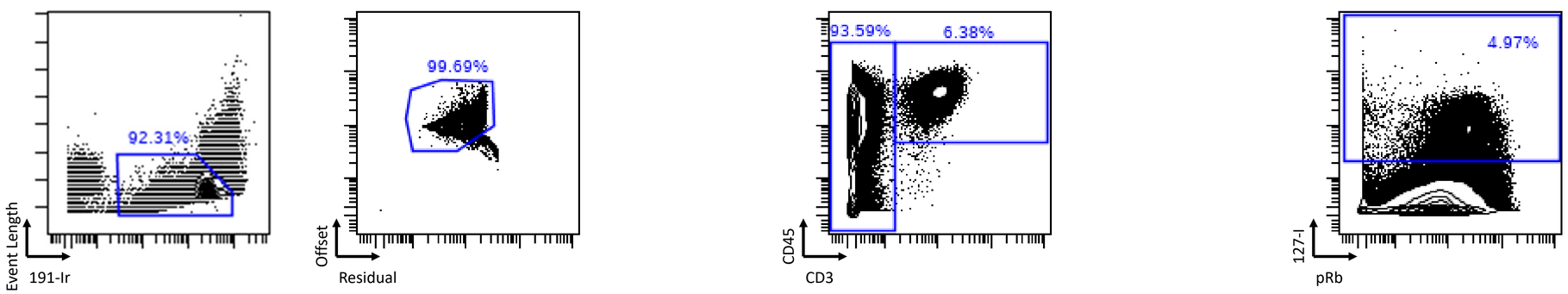

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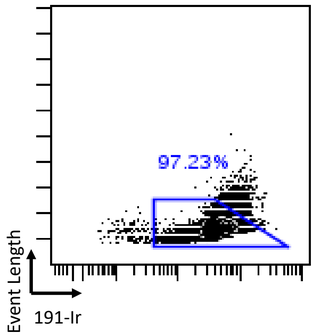
Singlet gate removes debris and doublets and used for all further gates



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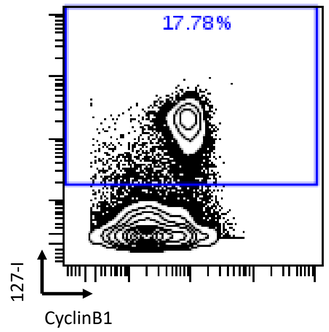
Establishing S-phase



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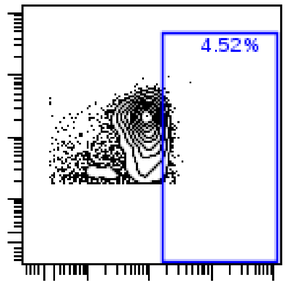

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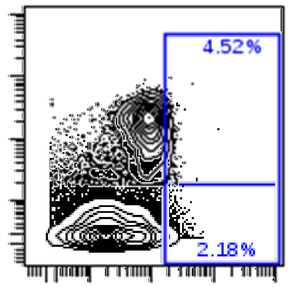
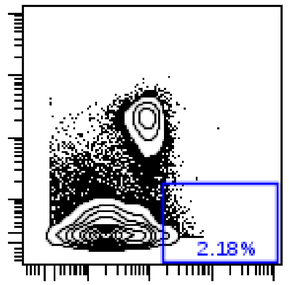
Establishing G2/M gate on the S-phase population



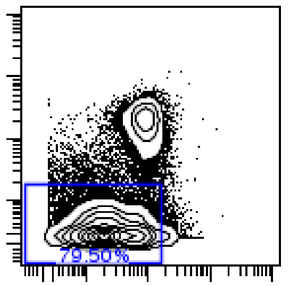
e.)

The boundary of previous gate is the G2/M gate

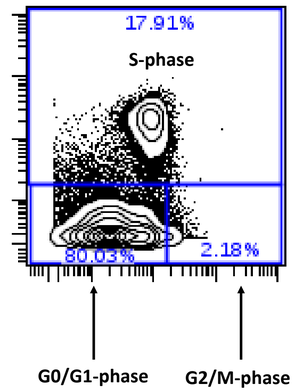


f.)

G0/G1 population is the remainder of CyclinB1, IdU^{low} population



Three major cell cycle gates shown at once



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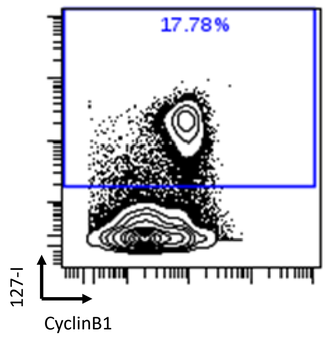
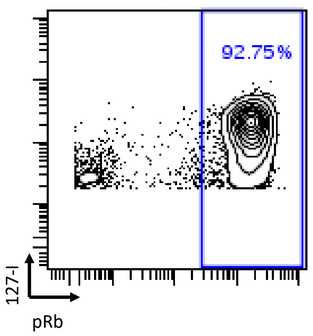


h.)

Establishing pRb^{+} gate on the S-phase population



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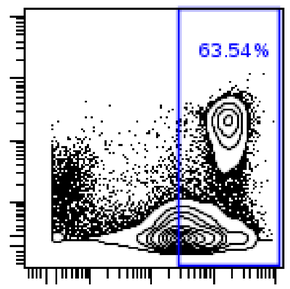
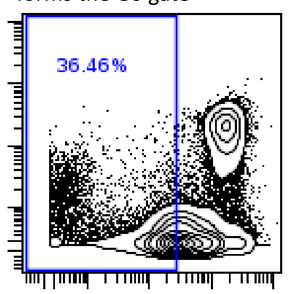
The boundary of the gate is the pRb^{+} population

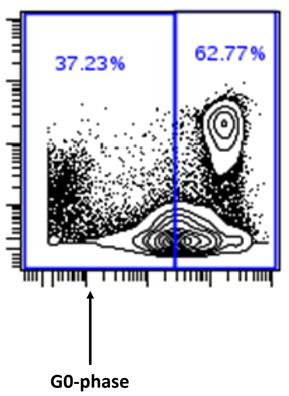


j.)

The pRb^{low} population forms the G0 gate

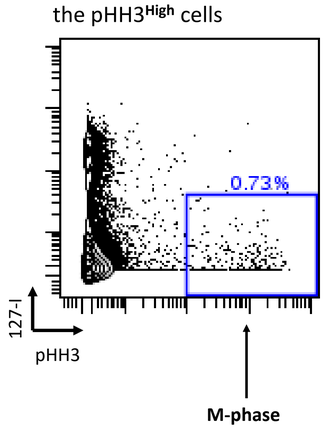


Both gates shown at once

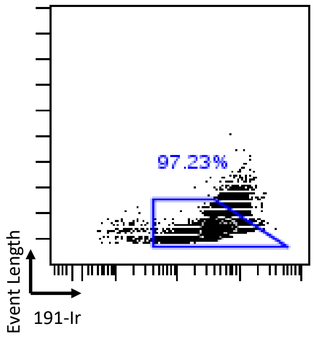


k.)

M-phase is established on the pHH3^{High} cells



Singlet gate removes debris and doublets and used for all further gates



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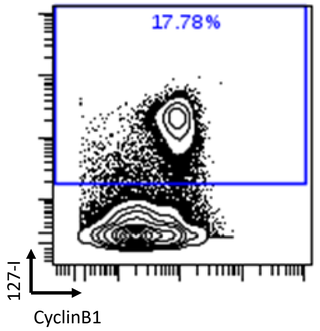
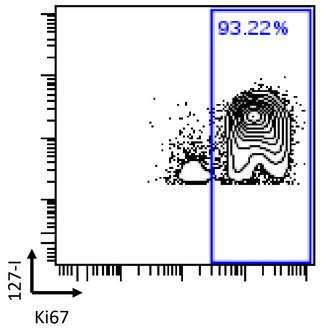
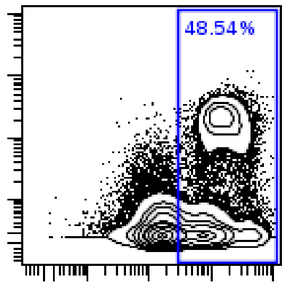


Establishing Ki67⁺ gate on the S-phase population



The boundary of the gate is the Ki67⁺ population. The remaining population is the G0 population.



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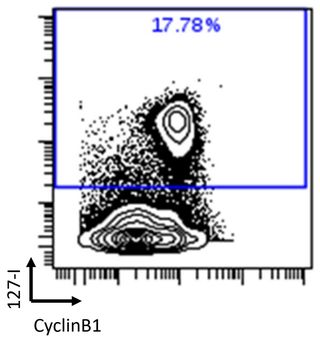
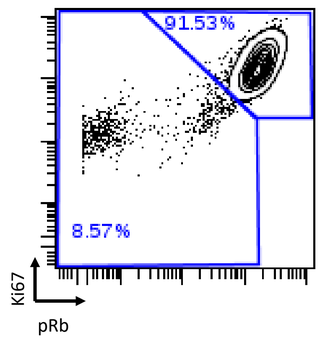
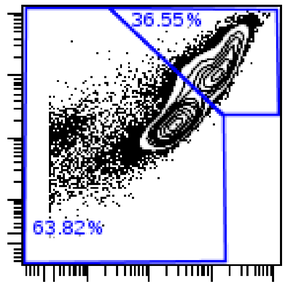


The S-phase population is double positive for Ki67 and pRb.



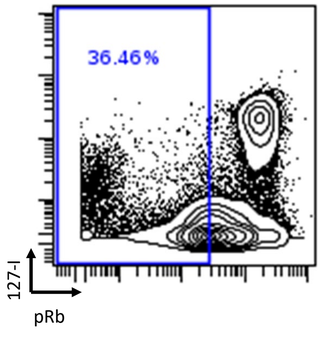
Two populations are evident in Ki67 and pRb which can be used to demonstrate cycling and not-cycling if IdU is not available

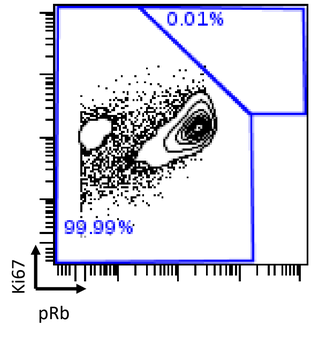


Cycling population

Not cycling population

The G0-phase population shows lower expression for Ki67 and pRb.





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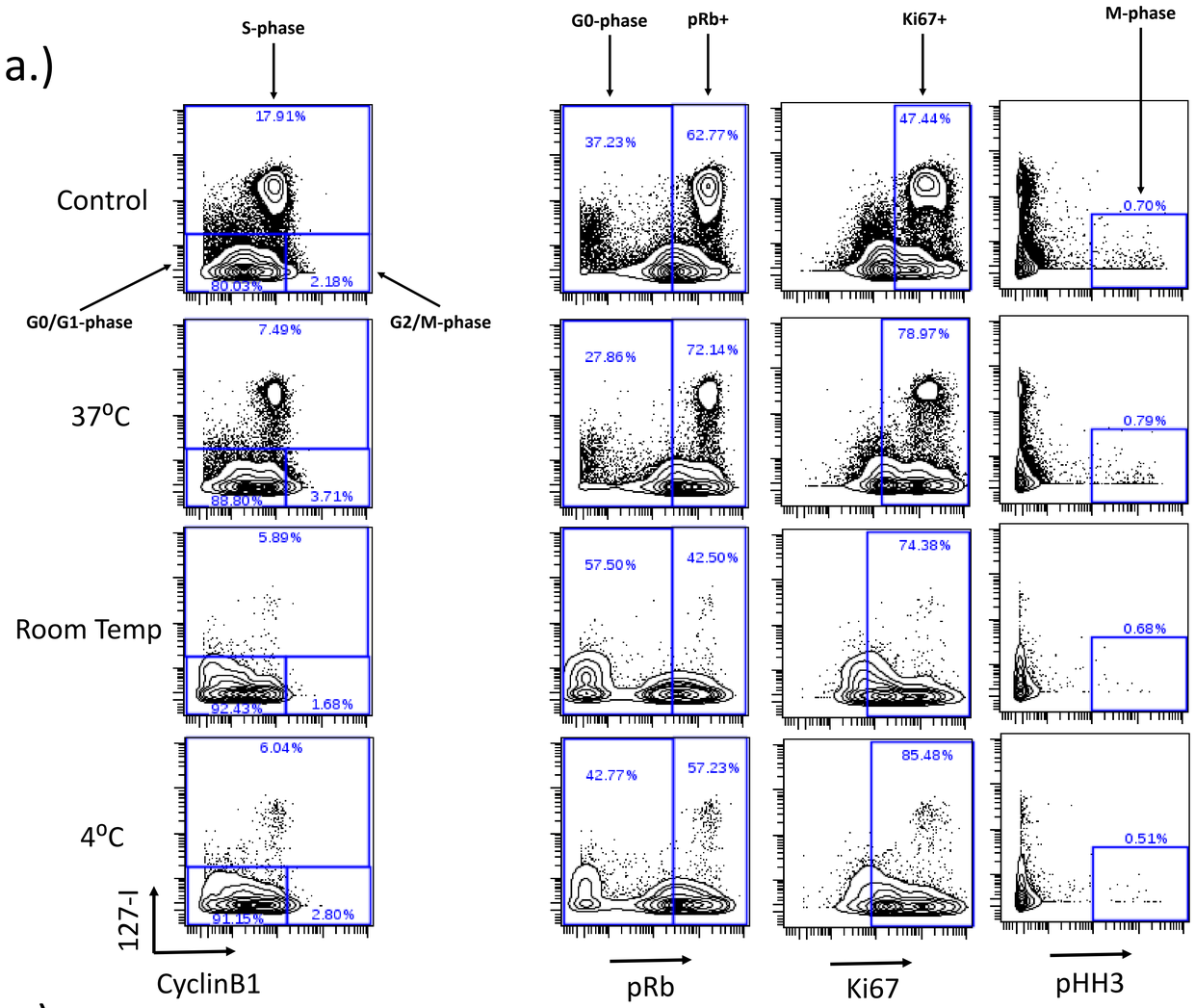

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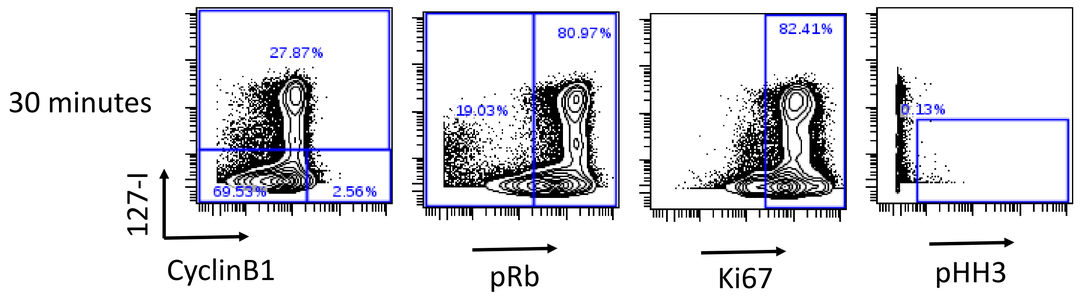

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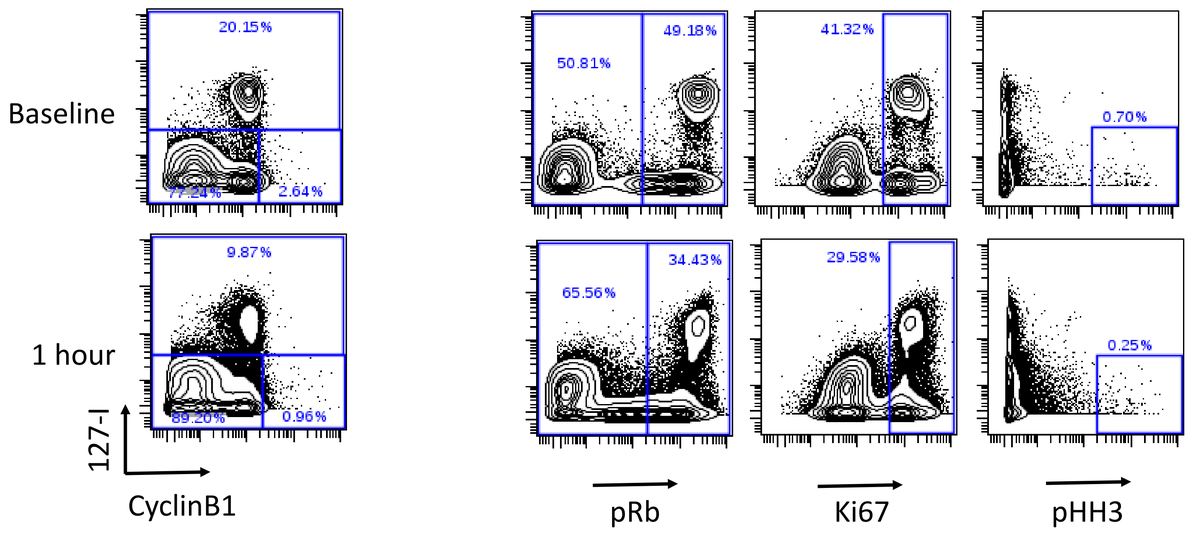


Figure 5

Click here to access/download;Figure;JoVE_Figure5.pdf

a.)

T-Cells

Marrow 1

Baseline

1.71% 1.28% 97.01%

92.97% 6.65%

27.46%

0.00%

30 min

1.68% 7.49% 0.88%

93.92% 5.65%

54.03%

0.03%

Post thaw

0.47% 0.19% 99.34%

98.92% 1.12%

45.88%

0.00%

Marrow 2

Baseline

0.64% 0.45% 98.91%

98.37% 1.63%

2.73%

0.03%

30 min

2.24% 1.52% 96.23%

92.50% 7.28%

20.37%

0.09%

Post thaw

0.30% 0.12% 99.58%

97.87% 2.13%

3.90%

0.01%

127-I

CyclinB1

pRb

Ki67

pHH3

b.)

Monoblasts

Baseline

3.37% 1.22% 95.41%

77.51% 23.24%

48.23%

0.00%

30 min

12.59% 1.54% 85.87%

53.57% 46.71%

77.20%

0.14%

Post thaw

6.02% 0.64% 93.34%

78.24% 22.21%

67.28%

0.00%

Marrow 2

Baseline

6.02% 1.43% 92.55%

84.90% 15.71%

41.84%

0.00%

30 min

6.02% 1.43% 92.55%

91.12% 6.91%

69.74%

0.00%

Post thaw

4.64% 0.35% 95.02%

88.65% 11.00%

49.76%

0.00%

127-I

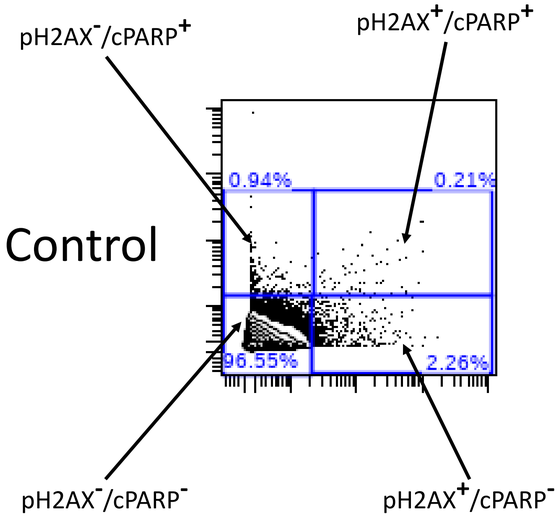
CyclinB1

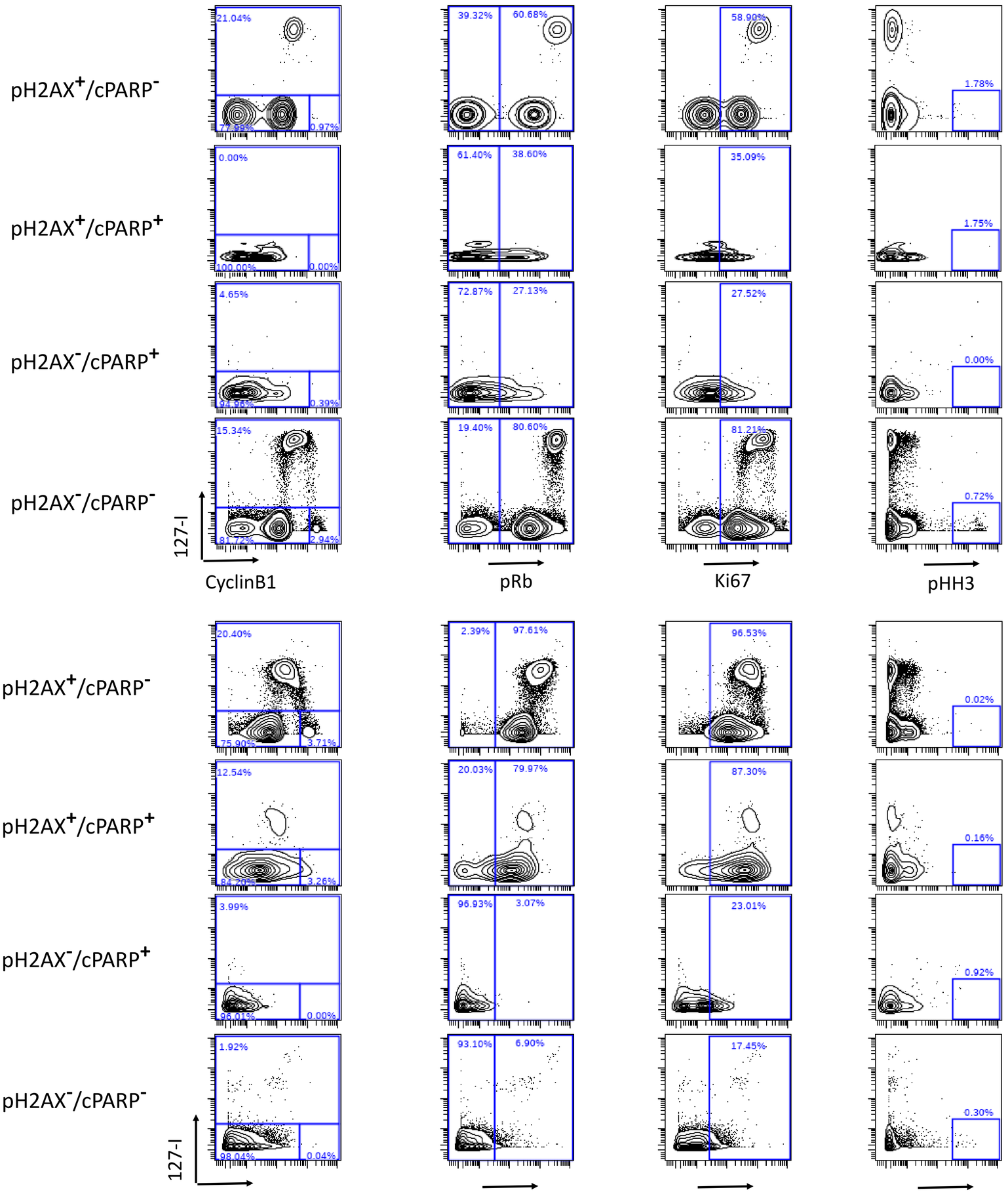
pRb

Ki67

pHH3

a.)





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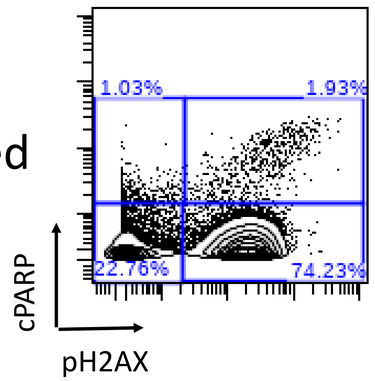

Table of Materials

Name of Material/ Equipment	Company	Catalog Number
Bovine Serum Albumin (BSA)	Sigma	A3059
Centrifuge	Thermo Scientific	75-217-420
Cleaved-PARP (D214)	BD Biosciences	F21-852
Cyclin B1	BD Biosciences	GNS-1
Dimethylsulfoxide (DMSO)	Sigma	D2650
EQ Four Element Calibration Beads	Fluidigm	201078
FACS Tube w/ mesh strainer	Corning	08-771-23
Fetal Bovine Serum (FBS)	VWR	97068-085
Helios	Fluidigm	
Heparin	Sigma	H3393
IdU (5-Iodo-2'-deoxyuridine)	Sigma	I7125
Ki-67	eBiosciences	SolA15
MaxPar Multi Label Kit	Fluidigm	201300
Microplate Shaker	Thermo Scientific	88880023
Paraformaldehyde (PFA)	Electron Microscopy Services	15710
pentamethylcyclopentadienyl-Ir(III)-dipyridophenazine	Fluidigm	201192
p-H2AX (S139)	Millipore	JBW301
p-HH3 (S28)	Biolegend	HTA28
Phosphate Buffered Saline (PBS)	Gibco	14190-144
p-Rb (S807/811)	BD Biosciences	J112906
Proteomic Stabilizer	SmartTube Inc	PROT1
RPMI 1640	Gibco	21870-076
Sodium Azide	Acros Organics	AC447810250

Comments/Description

Component of CSM
Sample centrifugation
Identification of apoptotic cells
G2 Resolution
Cryopreservative

Internal metal standard for CyTOF performance
Cell strainer to remove clumps/debris before CyTOF run
Cell culture growth supplement
CyTOF System/Platform
Staining additive to prevent non-specific staining
Incorporates in S-phase
Confirmation of G0/G1
Metal labeling kit, attaches metals to antibodies
Mixing samples during staining

Fixative

Cell identification during CyTOF acquisition
Detection of DNA damage
M-phase Resolution

Wash solution for cell culture and component of fixative solution
G0/G1 Resolution
Sample fixative
Cell culture growth medium
Component of CSM/Antibody buffer, biocide

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Title of Article: Use of the pyrimidine analog, 5'-iodo-2'-deoxyuridine (IdU), Cyclin B1, phosphorylated retinoblastoma protein (pRb), and phosphorylated histone H3 (p-HH3), to establish cell cycle phases in a mass cytometry system.

Author(s): Raymond D. Devine, Gregory K. Behbehani

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7-Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In this methods article written by Devine and Behbehani, the authors outline a protocol to use IdU incorporation to measure cell cycle states in actively dividing cells. The authors extrapolate from their previous work (Behbehani et al Cytometry A), to outline the proper procedure to stain samples with IdU for mass cytometry as well as outlining technical considerations including the effects of freeze/thaw, resting conditions, and cellular perturbations (e.g. radiation) on cell cycle. This is a well-designed protocol and will be well-received in the field.

Major Concerns:

1) The Table of reagents should be referenced within the protocol and expanded to include types of media, BSA used in the CSM, DMSO etc. Reagents included in the reagent list are not mentioned within the text or figures (e.g. Cyclin A, Cycline E, pCDK1, PCNA).
    a. We have removed antibodies we are not currently referencing in the protocol and included the reagents and chemicals referenced in the paper in the table of contents. In instances where we make things in lab (i.e. CSM) we have listed the component reagents where applicable.
2) The protocol requires more specific details in order to make it "fool proof". For instance in the "Preparation of IdU Stocks" section the authors suggest diluting in pre-warmed media, or use the term "tissue-culture vessel" in subsection 2. More discrete terms should be used as this is intended to be a specific protocol. The exact protocol should either be more specific to specify a type of optimal media, use an example, or outline what the authors used in their sample data and technical notes on types of media or culture vessels can be added.
    a. We originally left this intentionally open ended as the IdU can be used in multiple formats. We have used IdU in RPMI 1640, DMEM, SFEM II, PBS, as well as IdU added directly to bone marrow aspirates. We have made the protocol more specific for the cultured cells we used and have included in a note that IdU can be used in multiple media formats.
3) The note at the end of subsection 1 is confusing and should be clarified.
    a. We have clarified the end of subsection 1
4) In subsection 3.5 the authors indicate that cells should be stained with a surface staining cocktail, yet do not mention a previous step of making a cocktail.
    a. We have clarified this section concerning setting up a staining reaction and we have also cited a JoVE article from McCarthy et al concerning sample preparation for mass cytometry including staining.
5) In section 3.19 the authors should specify which intercalator (cisplatin, iridium, rhodium)? It may be advantageous to highlight the pros/cons of each.

    a. We have clarified we are referring to iridium when we discuss intercalator solution. We have also included in a note section the applicability of live/dead stains and referenced relevant articles.
6) In subsection 4, the authors may want to include a diagram as a figure on the cytometer and different parts of the CyTOF unit (e.g. when you say "Connect the nebulizer line to the gas port on the side arm of the nebulizer and insert the capillary line to the inlet line on the nebulizer", include a diagram pointing to the nebulizer, capillary line, and inlet).
    a. We have removed the overly technical jargon concerning the cytometer and have discussed in more general terms about cytometer set up and included references to a JoVE article published by Leipold et al which deals with daily cytometer operation
7) It would be helpful to include technical notes in the protocol regarding "good" and "bad" techniques. What happens when the tuning fails? What can you do, if anything if you acquire "bad data". What would "bad data" look like?
    a. We have discussed in the data acquisition what a good representation of the experiment might be and have made a new figure detailing a method to still determine cycling and not cycling cells if IdU incorporation was not done properly. Though this method will not be able to discriminate each individual cell cycle it can approximate relative fraction of in cycle and not in cycle. We've added a note about CyTOF operation and ensuring proper operation. We have included references to a JoVE publication from Leipold et al concerning tuning and daily operation of the cytometer since it is outside the scope of this protocol to go in depth about cytometer operation.
8) In the Acquisition portion of the protocol, it may be beneficial to include a primer on setting up and applying templates.
    a. We have included in the mass cytometer operation a description on adding a channel to make sure the 127 Iodine channel is included.
9) In Section 5, "Data Analysis", the authors use an Event Length vs. 191-Ir gate. It would be informative to include or discuss this strategy as well as Fluidigm's gating strategy based on Gaussian parameters as well.
    a. We have included a figure of a normal human marrow showing the differences between ungated, Event Length vs 191-Ir gate, and Event Length vs 191-Ir combined with Residual vs Offset gate. We have shown T-cells and an IdU vs pRb biaxials to show how these populations may change with the inclusion of each gate.
10) In the gating procedure outlined in the Data Analysis section, it would be helpful to reference the figures in order to better sense of context within the protocol.
    a. We have included figure references as appropriate in context with the protocol.
11) In Subsection 5.7, the authors state that "Once the gates have been established it will be necessary to separate out each individual phase and this can be done with any computing software". It is unclear what the authors mean by this statement.
    a. We have clarified this section. It was originally intended to mean exporting the percent in gate as the M-phase and G1-phase need to be derived by the subtraction of the M-phase from the G2/M-phase and G0-phase from the G0/G1-phase gate respectively.
12) As cell cycle is ubiquitous across all conditions and cell types, it would be informative to the reader if the authors included a discussion of proper controls for IdU and the other cell cycle markers as well as technical considerations for iodine contamination in the water supply,

reagents, or at any point in during sample collection at the instrument and how this may impact interpretation of results.

   a. We have included this as a note in sample preparation and data analysis. Anecdotally we have not noticed significant IdU contamination from any human samples with the exception of 1 from a possible CT scan. While IdU will be present in the media during incubation due to processing this IdU is removed well before sample acquisition.

13) The authors focus on using biaxial plots to define the different stages of the cell cycle. As the cell cycle is carefully regulated, the cell cycle is not synchonized prior to analysis, and whether a cell is "G0", "G1", "G2", "S", or "M" could highly depend on the gating strategy and the tightness of the gates, could the authors incorporate the technical considerations into the discussion as well as consideration of the importance of scales, scale factors, over/understaining for IdU or other cell cycle markers etc. In the same vein, as the authors focus on biaxial gating, could they also include a figure or discussion of implementing cell cycle parameters in multidimensional space (i.e. using UMAP or tSNE on 40+ parameters at once).

   a. We have subdivided the figures in order to be understood better. We have also tried to make figure 1 clearer. The IdU population is primarily used to discriminate between the different phases and by gating on the IdU population until approximately the top 5% population is in gate it removes some of the variability inherent to gating. While this requires a discrete IdU population it is possible to backgate the IdU population from the cycling population as described in the new figure. By using the Ki67+/pRb+ population it is possible to identify the IdU positive population if there is under staining. In the discussion section we have included looking at cell cycle parameters in multi-dimensional space referencing Behbehani et al.

Minor Concerns:

1) Line 74-75 needs correction: "..with the amount of amount of bound antibody labeled…"
   a. We have corrected this typo
2) Plots in Figure 1 are difficult to interpret as their in no clear, intuitive gating schema. Perhaps Figure 1 could be subdivided into panels A-D?
   a. We have subdivided this figure and referenced each subdivision when necessary
3) Adding Gate labels to Figures 1 and 2 will be beneficial so that the reader can clearly understand the different phases of the cell cycle.
   a. We have added arrows pointing to each gate with the appropriate gate label to help the reader track the gate labels.
4) Panel 2D is not present in the Figure, yet is mentioned in the text (line 328-329). Similarly, Figure 3 is labeled with 2 panels (A and B), yet is referenced in the text as having 4 panels (A-D).
   a. We have corrected this error and referenced the sub panels appropriately
5) In the representative results described Figure 3, the authors highlight an experiment using bone marrow-derived T cells and monoblasts. Could the authors describe what markers and/or pregating steps were used to define these populations?

a. In the text description we have added "T-cells ($CD45^{high}/CD3^{high}$) and monoblasts ($CD33^{+}$, $HLADR^{+}$, $CD11b^{low}$, $CD14^{neg}$)" to help the reader understand our derivation of each population. These populations were chosen as a mature and immature population and the known effects of sample processing on T-cell stimulation

6) In Figure 5, the authors use radiation damage as an example of a cellular perturbation that influences cell cycle using H2AX and cPPAR as readouts for apoptosis/genotoxic stress. It would be informative, and emphasize their point, it the authors compared IdU and cell cycle readouts in H2AX or PPAR positive cells versus H2AX,or PPAR negative cells.
   a. We have modified Figure 5, now Figure 6, to demonstrate the cell cycle differences between a range of pH2AX and cPARP expression we hope will be clearer to the reader. In summary even with concurrent pH2AX expression cPARP positive cells show minimal or no IdU incorporation or are primarily found in G0-phase using normal gating strategies which is what is typically expected as cells commit to apoptosis. This is also functionally different from necrosis, which is why we have referenced the cisplatin staining protocols as mentioned above since that would be more sensitive to necrosis rather than programmed cell death.

Reviewer #2:

Manuscript Summary:

This manuscript details the method of using IdU labeling for cell cycle phase discrimination using mass cytometry. The value of this method has been previously demonstrated by this group in two excellent prior publications. This method is of high interest and utility for the broader scientific community.

Major Concerns:

none

Minor Concerns:

the style of writing was very informal which impact the clarity of the writing. There are some sentences that were difficult to understand.

We have reformatted the style of the writing to be more in line with JoVE guidelines which will be more imperative and formal which should help with understanding.

Reviewer #3:

Manuscript Summary:

Devine and Behbehani present a thorough and careful illustration of cell cycle analysis by IdU incorporation. The work is of fundamental interest to those studying cancer or immune cells via mass cytometry, allowing a accurate analysis of cell cycle state using IdU along with cyclin B1, pRB, pHH3. These four markers are able to delineate the 5 cell cycle states with few of the issues one would have with fluorescence flow cytometry (spillover in particular). This protocol builds on previous work by Behbehani originally in 2012, developed further to profile acute myeloid leukemia in 2015. The protocol is straightforward and clear and will make an informative and useful video for the journal's readers/viewers.

Major Concerns:

none

Minor Concerns:

I have only a few minor things to note, but they need not delay acceptance can be addressed easily in final submission:

- There is no mention of viability stains (eg cisplatin or rhodium). A line addressing the compatibility of cisplatin in particular with the use of IdU would be helpful to viewers.

We have included in the discussion the use of cisplatin and rhodium stains for viability. We have also referenced another JoVE article from McCarthy et al which discusses sample preparation and includes cisplatin staining protocol.

- Ki67 is included in the gating figures and in the gating strategies but its importance or necessity is unclear.

We have included a figure discussing the applicability of Ki67 for use in cell cycle analysis and also how Ki67 and pRb can be used to get relative in cycle and not in cycle when IdU is either not available or the incorporation was not successful.

- How long can one store IdU incorporated samples in CSM + DMSO (step after IdU incorporation)? indefinitely?

As the IdU is incorporated directly into the DNA it should be detectable until there is breakdown of the DNA and cell. While there is probably a theoretical limit we are still able to discriminate cell cycle of marrow aspirates collected approximately seven years ago. Below we have included the same marrow aspirate run two years apart. The difference between the two runs is that in 2019 the lab began using the wide bore (WB) injector for its experiments. The main populations are still easily discernable and the percentages consistent across multiple different runs.