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screening solutions for life

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Measurement of cell proliferation is a valuable endpoint in studies of cell growth and differentiation. The ability to proliferate is often a key indicator of cell health or a measure of a cell's ability to produce a functional response. When used to assess cell health, proliferation assays allow evaluation of compound toxicity or, in the example of tumor inhibition, drug efficacy.

Proliferation measurements have a long history in studies related to the immune system and hematopoiesis; both require cell proliferation to properly function. An adaptive immune response, for example, requires rapid proliferation of helper T-cells upon presentation of foreign antigen. A measure of the extent of T-cell proliferation often serves as a functional readout for the immune-modulatory effects of a treatment.

Proliferation measurements can be made in a number of ways. Simple, but crude, methods involve quantification of total cell number. Cell numbers are typically estimated by staining for total DNA content or monitoring the increase of a metabolic activity. These techniques only approximate proliferation because they do not capture information about divisional history or the identity of proliferated cells. More sophisticated techniques allow confirmation that proliferation occurred, even if the population size does not change due to turnover, by detection of new DNA synthesis. These techniques, however, are not suitable to long-term studies that span many generations. Importantly, many methods are not amenable to high throughput screening due to complex workflows, numerous wash steps, and large volume requirements.

These issues are solved by the MultiCyt Cell Proliferation Dye Panel. The dye panel currently features two dyes that enable simplified workflows for long-term tracking of cell proliferation. The dye family was carefully assembled to maximize the availability of fluorescence channels on IntelliCyt screening systems, enabling more multiplexed assay combinations. Multiplexing with other reagents, like MultiCyt cell health and cell function dyes, allows more information to be generated from a single assay well, increasing confidence in results by providing a deeper understanding of the relevant biology, all at lower cost than running individual assays.

When used with IntelliCyt's high throughput flow systems, such as iQue® Screener, the Cell Proliferation dyes offer unique advantages. A wide dynamic range allows simultaneous detection of cells that span several generations – an impossible task for high content imaging systems which have difficulty detecting dim and bright signals in a single exposure. In contrast to standard microplate readers, which can only detect a single average proliferation signal per well, the iQue Screener allows cell-by-cell detection of four fluorescent signals and label free measurements of cell size and complexity. The combination of an IntelliCyt system and the Cell Proliferation dyes offer the following advantages:

- Positive identification of proliferated cells in long term assays, including their generation number, ≥ 6 generations;
- Extremely bright staining with minimal cytotoxicity;
- Fast, linear response. Stable staining means minimal dye loss and allows differentiation between generation 1 fluorescence and generation 2 fluorescence in short-term studies;
- Dye panel is optimized for multiplexing. Each dye has been specifically titrated for robust signal stability, while maintaining at least two open channels for additional endpoints;
- Demonstrated ability to measure proliferation in suspension and adherent cells – with or without fixation.

This application note explains the principles behind the MultiCyt Cell Proliferation dyes. Their key advantages are demonstrated, including several examples that illustrate the value of multiplexing with other endpoints.

Identification of Proliferation by Dye Dilution

The MultiCyt Cell Proliferation Dye Panel is comprised of two spectrally distinct, proprietary fluorescent dyes. The proliferation dyes are fluorescent molecules that are cell permeable. Once the dye enters a cell, it binds to intracellular targets. Cell proliferation is detected and quantified based on the halving or "dilution" of loaded dye after each round of cell division. Daughter cells will retain half of the original fluorescence intensity. Subsequent rounds of division will further halve the signal (Figure 1).

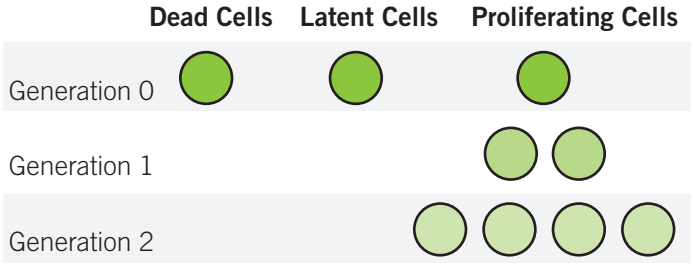

Figure 1. Cell Proliferation dyes function by first staining the cells that will be used in an assay (Generation 0). As cells divide, the dye is equally distributed between daughter cells, halving the fluorescence intensity with each new generation. When assay plates are read on IntelliCyt systems, rapid fluorescence measurements are made on an individual cell basis for each sample.

Multiplexing

The Cell Proliferation dye panel can be multiplexed with other biological endpoints, like those measured by other MultiCyt reagents. The dye family is an especially useful way to multiplex proliferation assays because the two dyes fluoresce at opposite ends of the spectrum. The FL1 Proliferation dye occupies FL1 and FL2 channels and the FL4 Proliferation dye occupies just FL4. Between the two dyes, it is always possible to measure proliferation and leave the appropriate channel open for your other multiplexed reagents.

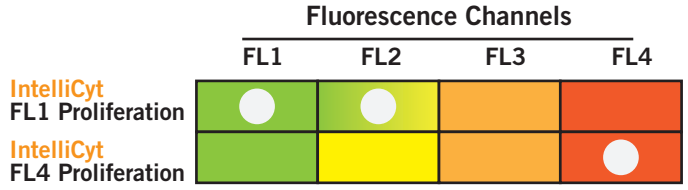

Figure 2. The Cell Proliferation dyes have spectral characteristics that leave at least two fluorescence channels open, creating additional flexibility in the development of multiplexed assays. The FL1 Cell Proliferation dye occupies the FL1 and FL2 channels, leaving FL3 and FL4 open. The FL4 Cell Proliferation dye occupies only the FL4 channel, leaving FL1, FL2, and FL3 open. These open fluorescence channels can be occupied by other fluorescent probes that measure different readouts.

Assay Workflow

The Cell Proliferation dye panel offers a simplified protocol for batch staining of cells. After staining, cells are dispensed into microplates and can be used in standard high throughput screening (Figure 3A). After an incubation length suitable for your assay, plates are directly read on the iQue Screener – no additional assay processing is necessary. Additional multiplexed dyes or antibodies can be added after the desired plate treatment and incubation length (Figure 3B). If multiplexing with other MultiCyt reagents, most require only addition of dye to the plate, no washing, and a short incubation period before reading the plate.

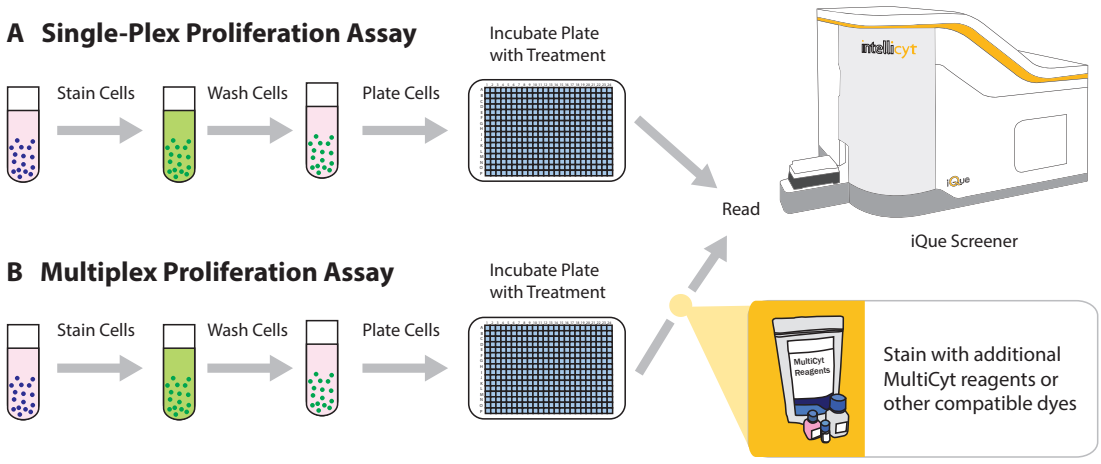

Figure 3. IntelliCyt Cell Proliferation Dye workflow. (A) Cells are first batch stained with a MultiCyt Cell Proliferation dye. After staining, cells are plated for treatment and incubation. At the end of the incubation, plates are directly read on the system with no additional assay processing necessary. (B) If additional endpoints are desired, plates can be stained with other fluorescent probes or antibodies just before reading the plate on the iQue Screener.

MultiCyt Cell Proliferation dyes produces a linear response in dividing cell lines, ≥ 6 generations

To demonstrate the ability of Cell Proliferation dyes to identify multiple generations of dividing cells, Jurkat cells were stained with either the FL1 or FL4 proliferation dyes. Starting on the day cells were seeded (day 0), cells were sampled daily and analyzed on the iQue Screener. On each successive day, the median fluorescence intensity decreased. When the fluorescence intensity distributions of cells are overlaid, distinct separation is observed between daily measurements up to day 6 (Figures 4A and 4C). Summarizing the data as median fluorescence intensity and plotting on a log-log scale shows a linear response, as is expected for cultures dividing at a constant rate (approximately 24 hours for Jurkat cells) and with dye dilution of 50% at each generation. Median fluorescence intensities from day 7 to day 9 continued to decrease at the expected rate although peak separation becomes less visually distinct at the lower intensity values (Figures 4B and 4D).

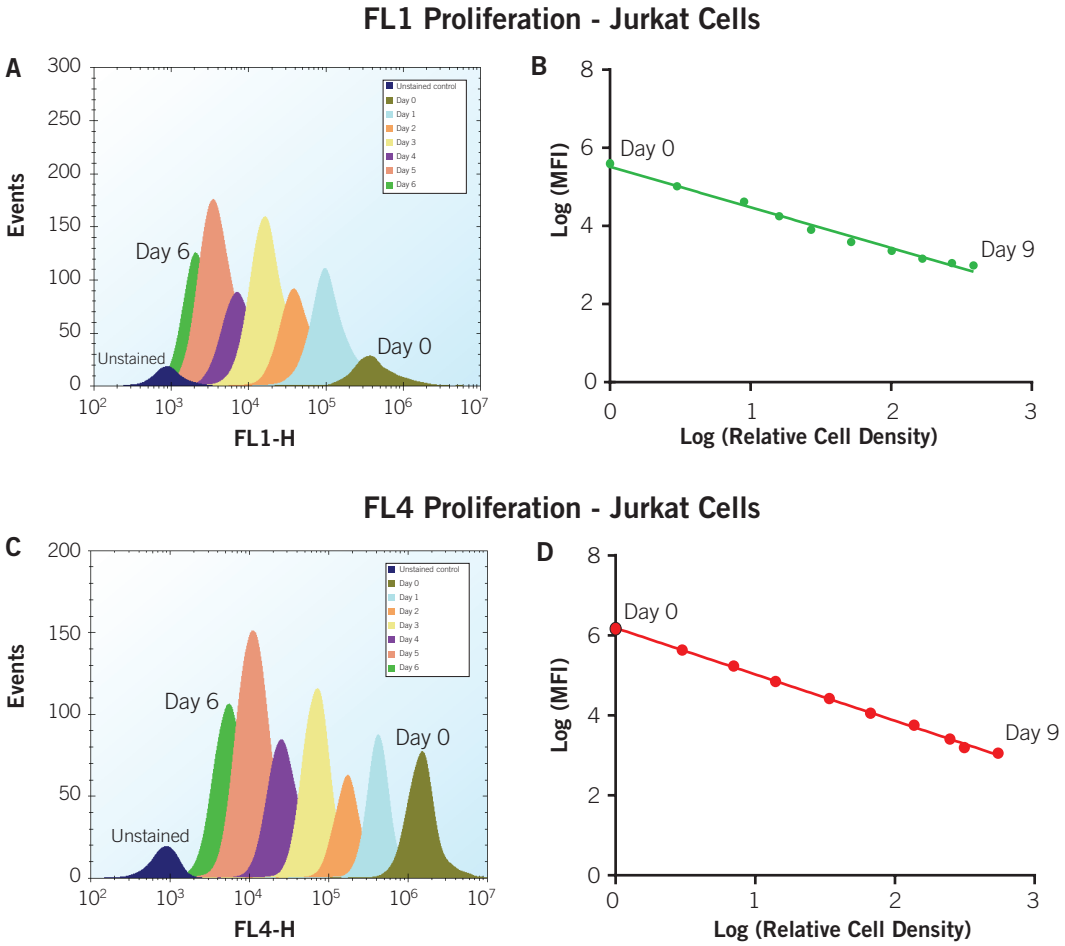

Figure 4. Jurkat cells stained with either the FL1 proliferation dye (A and B) or the FL4 proliferation dye (C and D) were sampled for up to 9 consecutive days, passaging cells on days 2,4, 6, and 8 to keep cells in log growth phase. Both dyes are able to distinguish the differing extent of proliferation between each day with visually distinct peak separation up to 6 days (A and B). The relationship between cell density and median fluorescence intensity is linear (C and D) up to 9 days, even as the peak separation becomes less distinct at the lower intensity values. Relative cell density was calculated as follows: cell density was normalized against the density at day 0 and adjusted for the dilution factor on days that cells were passaged.

MultiCyt Cell Proliferation dyes perform similarly in unfixed and fixed cells

Similar responses were measured when Jurkat cells were fixed after incubation with Cell Proliferation dye on day 3 (data not shown). Three fixation methods were tested: 4% paraformaldyde, 1% Formalin, and Cytofix/Cytoperm (BD Biosciences). Median fluorescence intensities of cells measured 10 minutes after fixation and 24 hours after fixation closely matched the results from unfixed cells.

Proliferation Dyes are compatible with suspension, adherent, and primary cells

The FL1 and FL4 Cell Proliferation dyes were also tested for function on A549 cells and primary blood mononuclear cells (PBMCs). A549 are epithelial carcinoma cells and were chosen as an example of an adherent cell line. PBMCs are a frequently used immunological model and are an example of a complex mixture of primary cells. After three days in culture, A549 cells showed a clear decrease in median fluorescence compared to cells that were growth-inhibited by treatment with Mitomycin C (Figure 5A). The fluorescence decrease was also observed for PBMCs that were stimulated with the mitogen phytohaemagglutinin (PHA) relative to PBMCs that did not receive PHA treatment (Figure 5B). These data, along with the response seen with Jurkat cells in Figure 4, demonstrate the ability of the FL1 and FL4 Cell Proliferation dyes to properly function across a wide range of cell classes: suspension, adherent, and primary cells.

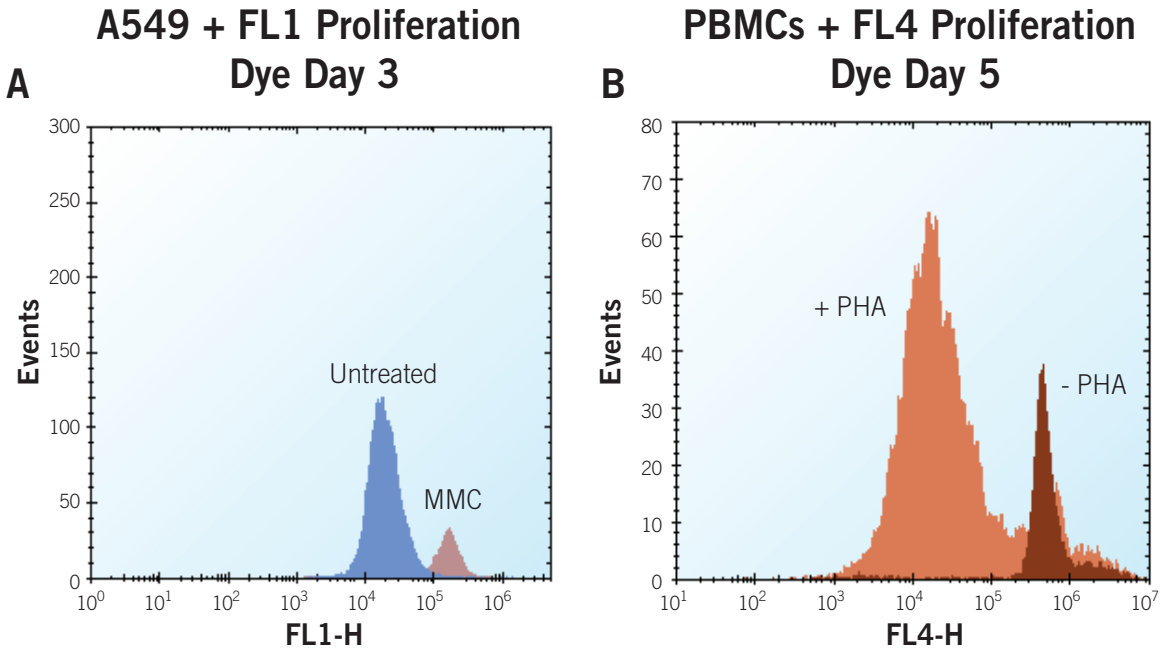

Figure 5. Proliferation measurements using A549 cells (adherent) and PBMCs (suspension). (A) A549 cells stained with FL1 Proliferation dye demonstrated a decrease in median fluorescence intensity after a three day culture, indicating proliferation. A549 cells treated with the growth inhibitor Mitomycin C (MMC) did not proliferate. (B) In PBMCs stained with the FL4 Proliferation dye, proliferation was only observed in cells treated with the mitogen PHA. Untreated PBMCs did not proliferate, due to the lack of a stimulatory signal.

Multiplexing MultiCyt Cell Proliferation and Cell Health Dyes: Membrane Integrity and Cell Cycle

The spectral characteristics of the proliferation dyes, as well as the option to choose among multiple fluorescent colors, allows multiplexing with MultiCyt dyes for other cell health indicators. Using PHA-stimulated PBMCs, membrane integrity and cell cycle status were measured in conjunction with proliferation.

Membrane integrity and proliferation were multiplexed by using the FL1 Cell Proliferation dye in conjunction with the FL4 Membrane Integrity dye, which stains cells with compromised membranes. PBMCs were first stained with proliferation dye, then stimulated with PHA over a five day incubation. The plates were then stained with the FL4 Membrane Integrity dye for one hour before reading on the iQue Screener, as depicted in Figure 3B. The extent of proliferation was then assessed for just the viable cells (Figures 6A and 6B). This highlights an important benefit of multiplexing IntelliCyt dyes on the iQue Screener: multiplexed readouts are made on the same cells in the same assay, preserving the context between multiple measurements and allowing for more sophisticated analysis. In this case, restricting the proliferation measurement to viable cells provides a more accurate proliferation readout by eliminating cells that may have lost proliferation dye due to compromised membranes. These cells would have falsely been included in the analysis as proliferated.

A second multiplexed proliferation assay was done by combining the FL1 Cell Proliferation dye with the MultiCyt Cell Cycle dye. In this case, PHA-stimulated PBMCs were stained with cell cycle dye one hour before reading the plate. The cell cycle status was assessed for just the proliferating cells (Figures 6C and 6D). In this example, proliferation is used to identify the relevant population for further analysis with the multiplexed cell cycle dye.

Multiplexing Proliferation with Immunophenotyping

The positive identification of the type of a proliferated cell, or non-proliferated cell, can be done by multiplexing immunophenotyping antibodies with a Cell Proliferation dye. Here, PHA-stimulated PBMCs were stained with a fluorescent antibody cocktail containing anti-CD3 and anti-CD8. In this example, the extent of proliferation was measured on cytotoxic T-cells, which were CD3 and CD8 positive (Figure 6E and 6F). Of course, cells of any other combination of surface markers can be included or excluded from the analysis, which in this case would allow comparison between the various T-cell subsets.

Common to all three multiplexed assays, a MultiCyt dye or antibody cocktail was simply added at the end of the proliferation incubation period (Figure 3B). After a short (typically 30 min.–1 hour) incubation with the newly added stain, the plates were directly read on the iQue Screener without the need for a wash step. This workflow illustrates one of the key advantages of MultiCyt dyes and the iQue Screener—easily multiplexed assays in no-wash formats.

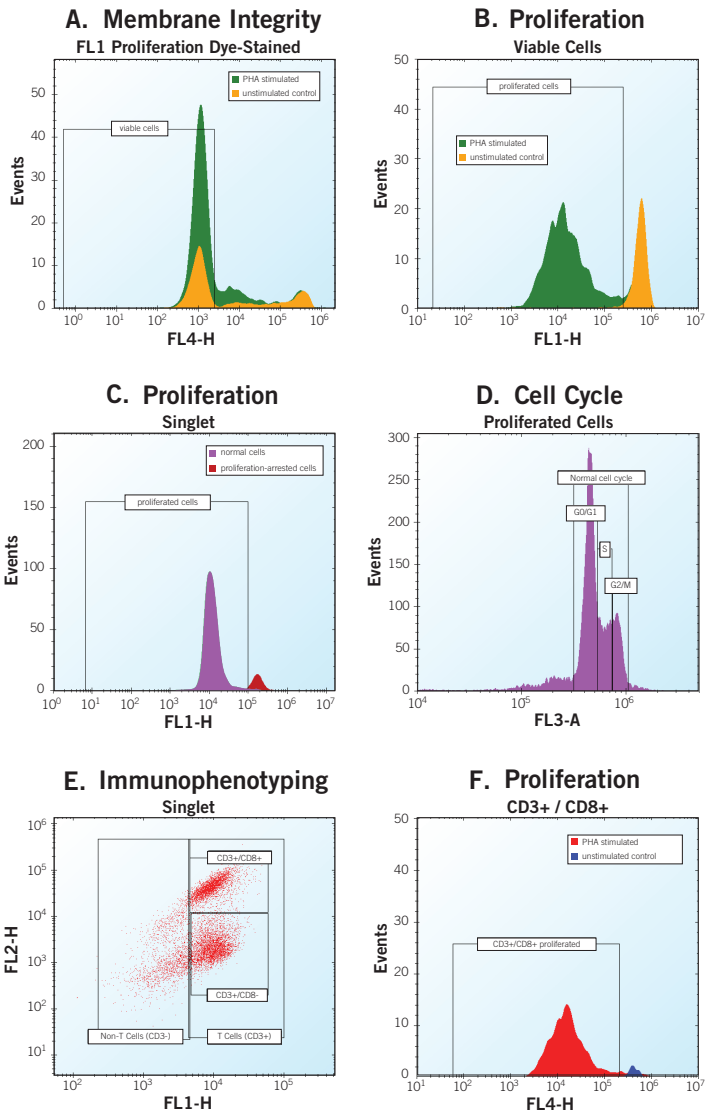

Figure 6. Multiplexing MultiCyt Proliferation dyes with other dyes or antibodies. PBMCs were stained with MultiCyt Cell Proliferation dye before stimulation with PHA. After a five day incubation, various MultiCyt dyes and antibodies were used to stain for other biological endpoints. (A) Cell viability was determined by staining with the MultiCyt FL4 Membrane Integrity dye. Applying a gate on viable cells allowed analysis of proliferation for just live cells. (C) Proliferated cells were interrogated for cell cycle status (D) using the MultiCyt Cell Cycle dye. (E) Staining with a cocktail of 2 antibodies against CD3 and CD8 allowed immunophenotyping antibodies against CD3, CD4, and CD8, and positive identification of various T-cell subsets. (F) Gating CD3+/CD8+ cells allowed proliferation analysis specifically of the cytotoxic T-cells.

The Cell Proliferation dyes measure proliferation with simple workflows, perfect for high-throughput screening. When used with an IntelliCyt system, the MultiCyt Cell Proliferation dyes allow multiplexing with a wider range of dyes that stain for a wider range of biological endpoints. Multiplexing can improve confidence in proliferation results and provides a richer description of the effects of your treatments, all while reducing costs.

In sum, the MultiCyt Cell Proliferation dyes offer:

- Accurate measurements of proliferation, easily discriminating up to 6 generations of cells
- Easy multiplexing with a wide range of dyes
- Workflows optimized for high-throughput screening

Cell Culture

Jurkat cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum and 2 mM L-glutamine. A549 cells were cultured in DMEM and 10% FBS. PBMCs were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 10% FBS, 1% Non-Essential Amino Acids, 1% Sodium Pyruvate, and 1% Penicillin / Streptomycin.

Proliferation Staining and Measurement

Proliferation of Jurkat cells was measured by staining with IntelliCyt FL1 or FL4 Proliferation Dye according to the kit protocol (workflow shown in figure 3). Cells were seeded at a density of 0.2 million / ml. In order to maintain cultures in log-growth phase, the cells were passaged using a 1:3 dilution on days 2,4, and 6 post-seeding. On each day of the experiment, an aliquot of the culture was analyzed on the iQue Screener by measuring the median fluorescence intensity (MFI) of cells in the FL1 or FL4 channel, depending on which Proliferation dye was used. A549 and PBMC cells were similarly stained, but fluorescence was measured at a single time point: day 3 for A549 cells and day 5 for PBMCs. No cell density adjustments were made for these cultures.

Cell Treatments

In experiments where cells were treated with either the mitogen phytohaemagglutinin (Sigma) or the growth inhibitor Mitomycin C (Sigma), the PHA concentration was 20 µg/mL and the Mitomycin C concentration was 10 µg/mL.

Multiplexed Measurement of Viability, Cell Cycle, and Immunophenotype

For multiplexed measurements of proliferation and other MultiCyt dyes, peripheral blood mononuclear cells (PBMCs) were first stained with FL1 or FL4 Proliferation dye, depending on which stain was used for downstream multiplexing: the FL1 Proliferation dye was used when multiplexed with cell cycle or viability, the FL4 Proliferation dye was used for multiplexing with immunophenotyping antibodies. After staining PBMCs with proliferation dye, cells were seeded at a concentration of 0.2 million / mL, treated with PHA, and allowed to incubate for five days. After incubation, 10 µL aliquots of cells were transferred into three new 384-well plates for multiplexed staining.

To one plate, MultiCyt FL4 Membrane Integrity dye was added according to the kit protocol. To the second plate, MultiCyt Cell Cycle dye was added, also according to the kit protocol. To the final plate, Immunophenotyping of PBMCs was performed by staining cells with CD3-FITC (BD) and CD8-PE-Cy5 (BD) antibodies at a dilution factor of 1:40 for 30 minutes at room temperature.

Notes

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