Analysis of proliferation dynamics in primary and immortalized human cells
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ABSTRACT
Cell proliferation and the characterization of agents that affect proliferation dynamics are extremely important areas of research. A variety of fluorescent tools and techniques are available for evaluating and quantifying the fraction of eukaryotic cells in each phase of the cell cycle. Using a variety of human cell types and fluorescent reporters we characterized cell cycle profiles and their sensitivity to anti-proliferative agents, compounds commonly used to treat cancer. The fraction of cells in each stage of the cell cycle was measured using a DNA fluorescent dye in conjunction with a fluorescent protein ubiquitination cell cycle indicator expressed by transfecting cells. Surviving DNA during the S phase was detected using a 532 nm laser (A) reaction to fluorescently label 3'-deoxy-3'-thymidine (dCTdU), a thymidine analog incorporated into replicating DNA. Generational analysis was performed using a novel violet-avidin-conjugate cell proliferation tracking dye (A). A comparison of primary human cells and immortalized cells derived from tumors (cervical carcinoma, alveolar epithelium, and osteosarcoma) demonstrated that immortalized cells have an increased senescence to microtubule perturbation. Moreover, non-immortalized cells show a significantly enriched G0 population, compared to immortalized cells, reflecting a decreased proliferative index.

INTRODUCTION
Here we present an overview of several techniques used to characterize the growth of primary and immortalized human cells. One technique uses the fluorescent ubiquitination cell cycle indicator, PremoTM Fucci (Life Technologies). This is a fluorescent protein-based sensor that employs a Red fluorescent Protein (RFP) and a Green fluorescent Protein (GFP), each of which is fused to one of two different regulators of the cell cycle, Geminin and Cdt1 and serves as an intrinsic reporter of the cell cycle. Ubiquitination of each protein results in the biophase labeling of the cell cycle and GFP/Fruit fly through the cycle (A). Another technique uses flow cytometry to measure the number of fluorescent cells. A fluorescent emission signal is proportional to DNA mass. Flow cytometric analysis of these stained populations is then used to produce a histogram that reveals the various phases of the cell cycle. A third technique uses the incorporation of the fluorescent protein ubiquitin into newly synthesized DNA using a Click-iT® labeling reaction (B). Image-based proliferation can be analyzed using fluorescent dye CellTrace™ Violet. When cells such as primary T-cells are stained with CellTrace™ Violet, gain in culture, and evaluated on a flow cytometer, the resulting fluorescence histogram contains several peaks, each of which corresponds to a discrete generation of proliferating cells (B).

MATERIALS AND METHODS
Human alveolar epithelial cells (A549) and cervical carcinoma cells (HeLa) were transduced with PremoTM Fucci constructs at a multiplicity of infection (MOI) of 20. Cells were stained with the DNA content dye FxCycle™ Violet and the viability dye LIVE/DEAD™ Fixable Red Dead Cell Stain. HeLa cells were also treated with 200 mM thidium for 24 hours. (Figure 1)

Human Primary Dermal Fibroblasts, cervical carcinoma cells (HeLa), and human alveolar epithelial cells (A549) were treated with 500 nM Taxol for 16 hours or left untreated. Microtubules were visualized with anti-tubulin goat anti-mouse Alexa Fluor® 555 (red). Nuclei were counterstained with Hoechst 33342 (blue). (Figure 2)

RESULTS

Figure 1. Fluorescent protein-based cell cycle interruption

The Fucci system utilizes the ubiquitous degradation of two cell cycle regulators, Geminin and Cdt1, to monitor cell proliferation. Geminin is a cell cycle regulator that binds to dCTdU and triggers cell cycle arrest. Cdt1 is a replication licensing factor that plays a key role in the initiation of DNA replication. In Cdt1-deficient cells, the premature degradation of Cdt1 allows for the initiation of DNA replication at the cell cycle. Figure 1A, shows the progression of the cell cycle. Figure 1B, demonstrates the progression of the cell cycle in a cell line transfected with Click-iT® EdU Alexa Fluor® 488 dye.

Figure 2. Multiparameter Analysis of Cell Cycle Perturbation

Figure 3. Generational analysis of proliferating primary T-cells

Figure 4. Multiparameter proliferation analysis of stimulated primary T-cells

Figure 5. Evaluation of sensitivity to microtubule perturbation

CONCLUSIONS
Cell cycle information provided by the fluorescent protein-based PremoTM Fucci Cell Cycle Sensor correlates well with flow cytometry data provided by the nuclear acid binding dyes Fucci™ Violet. Cell cycle dysfunction due to microtubule perturbation can be quantified using a dual-parameter plot to determine the DNA content information from Fucci® Violet with the S-phase analysis provided by Click-iT® EdU. Individual generations of proliferating lymphocytes can be identified using a histogram of Cdt1-MFP Fluorescence intensity. Further information of proliferation dynamics can be obtained by combining the Premo™ Fucci 3.0 with Click-iT® EdU to identify the proportion of cells in each generation that have recently synthesized DNA. These data can also be combined with a DNA content dye such as Fucci™ Violet for distinct quiescent cell populations in each generation from those that are actively progressing through cell division.

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