Research Techniques Made Simple: Techniques to Assess Cell Proliferation

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Cell proliferation is commonly assayed in the laboratory for research purposes, but is increasingly used clinically to gauge tumor aggressiveness and potentially guide care. Therefore, both researchers and clinicians should have a basic understanding of techniques used to assess cell proliferation. Multiple cell proliferation assays exist, and the choice of method depends on the laboratory resources available, the types of cells/tissues to be studied, and the specific experimental goals. In this article, we identify four overarching categories of cell proliferation assays that signify various stages of the cell cycle: nucleoside-analog incorporation, cell cycle-associated protein detection, use of cytoplasmic proliferation dyes, and indirect measures of cell proliferation. Each method has strengths and limitations that should guide the dermatology investigator's choice of assay.

Journal of Investigative Dermatology (2016) **136,** e1-e7; doi:10.1016/j.jid.2015.11.020

INTRODUCTION

Cell proliferation is defined as an increase in cell number secondary to cell growth and division (reviewed in Schafer, 1998). Assessing cell proliferation is a cornerstone of basic, translational, and clinical research and of clinical medicine. Given this pervasiveness, it is important for the clinical dermatologist and dermatology researcher alike to have a basic comprehension of cell proliferation and the assays most commonly used to measure it.

A number of methods exist to measure cell proliferation, and they vary in regard to which phase of cellular growth and division they assay, the equipment and expertise required, whether additional studies can be performed in parallel or in series, and what types of cells/tissues can be studied by that assay. To simplify this topic, this article outlines the principal approaches used to assess cell proliferation based on three aspects of cell division: nucleoside-analog incorporation during DNA synthesis, cell cycle-associated proteins, and cytoplasmic proliferation dyes. A fourth section briefly reviews indirect methods of assessing cell proliferation via cell counting and viability and metabolic activity assays.

BENEFITS

- Commonly utilized in laboratory research and increasingly in clinical practice.
- Broad range of assays available to assess various stages of the cell cycle.
- Some assays can be performed in conjunction with other stains, and some techniques maintain viable cells for follow-up experimentation.
- Many methods are technically straightforward and utilize commonly available equipment.

LIMITATIONS

- Some assays require specialized equipment and/or materials.
- Available sample(s) may be incompatible with certain techniques (eg, cytoplasmic dyes and thymidine incorporation cannot be used on formalin-fixed clinical skin specimens).
- Some assays are lethal to cells, so further experimentation cannot be performed.

THE CELL CYCLE

Cellular proliferation results from progression through the cell cycle (Figure 1). The cell cycle has two major phases: interphase and mitosis (reviewed in Schafer, 1998). A cell spends most of its life in interphase, which is divided into three stages: Gap 1 (G_1), Synthesis (S), and Gap 2 (G_2). During interphase, the cell is growing and preparing for division. Cellular division occurs during mitosis, or (M) phase, which actually consists of both mitosis and cytokinesis. Mitosis refers to division of the nucleus resulting in equal separation of chromosomes and is subdivided into phases: prophase, prometaphase, metaphase, anaphase, and telophase. Cytokinesis is the equal division of the cell membrane, cytoplasm, and organelles. M phase results in two daughter cells identical to their parent cell. A fourth stage, Gap zero (G_0), describes resting cells and cells that rarely or never divide.

The stages of the cell cycle can be identified based on specific characteristics. For example, nucleosides are incorporated into replicating DNA exclusively during S phase, and histone protein H3 is only phosphorylated during M phase. Many proliferation assays take advantage of these unique characteristics of each cell cycle phase.

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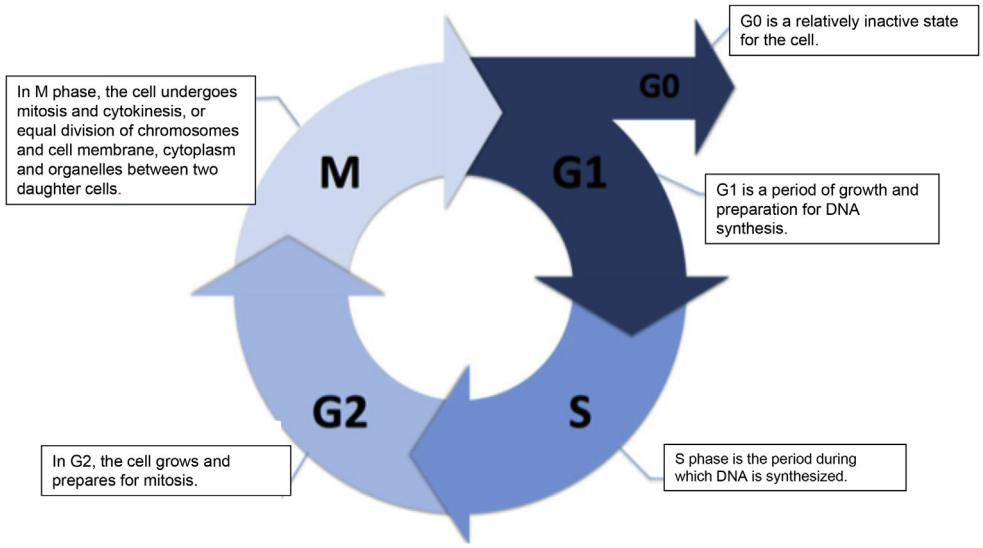

Figure 1. Schematic of the cell cycle depicting the phase detected by different proliferation assays. G_0 is a relatively inactive state for the cell. G_1 is a period of cell growth and preparation for DNA synthesis, or the S phase. Cell growth continues during G_2 as the cell prepares for mitosis (M) phase. M phase consists of equal division of chromosomes and cytoplasmic components (termed cytokinesis) between two daughter cells. Cytoplasmic proliferation dyes are internalized during all phases of the cell cycle including G_0, but proliferation is only appreciated once cytokinesis has occurred and the fluorescence intensity of the dye is halved in the daughter cells. Ki-67 is expressed during active phases of the cell cycle (G_1-M). Nucleoside-analog incorporation assays (ie, [^{3}H]TdR and BrdU) and proteins such as PCNA are specific to the S phase. BrdU, 5-bromo-2′-deoxyuridine; [^{3}H]TdR, tritiated thymidine; PCNA, proliferating cell nuclear antigen.

NUCLEOSIDE-ANALOG INCORPORATION ASSAYS

During the S phase of the cell cycle, genome replication, DNA polymerases incorporate nucleosides (eg, deoxyadenosine, deoxyguanosine, deoxycytidine, and thymidine) into new strands of DNA. Nucleoside-analog incorporation assays introduce chemically or radioactively labeled nucleosides into the sample of interest, which are incorporated into newly synthesized DNA during S phase.

A classic example of this strategy is the tritiated thymidine ([^{3}H]TdR) incorporation assay whereby excess radiolabeled thymidine is added to cell cultures and allowed to incubate for multiple days. Excess [^{3}H]TdR is then washed away and incorporated. [^{3}H]TdR is measured using a liquid scintillation counter. The assay can be performed in vitro or ex vivo, but not in vivo. This method quantifies overall division compared with a control and is commonly regarded as reliable and accurate. Assays should be run in triplicate and using varying numbers of cells to generate a proliferation curve (Figure 2a). Drawbacks are that radioactive reagents must be handled and disposed of with caution, thymidine is measured by the scintillation counter per well of cells rather than per individual cell so the assay reveals nothing about an individual cell's division history, and no additional assays can be performed with or after [^{3}H]TdR incorporation (it is an endpoint assay) because the assay extracts DNA from cells that are then washed away in the process. A common use for [^{3}H]TdR incorporation is to assess T-cell proliferation, for example, in response to therapeutic dendritic cells, as shown in Figure 2a (Divito et al., 2010).

Another common nucleoside-analog incorporation assay employs 5-bromo-2′-deoxyuridine (BrdU), also a thymidine analog. Incorporated BrdU is detected by a BrdU-specific monoclonal antibody that may be bound directly to a fluorescent tag or measured indirectly via a secondary antibody. Fluorescence can then be measured via a flow cytometer or fluorescence microscopy (Figure 2b). Alternatively, chromogenic tagging (colored, rather than fluorescent) can be performed that requires only a standard light microscope for inspection. An added benefit of the assay is that DNA can be "captured" via the antibody (ie, bound by the antibody, then purified by immunoprecipitation, and subsequently sequenced). Further, BrdU can be used not only in vitro but also in vivo; it can be injected into an animal or added to the animal's drinking water and ingested. BrdU is stably incorporated into DNA, so it persists for several months. Additional stains can be included in this assay, for example, to identify the particular type of cell that is proliferating. Lastly, there is no handling of radioactive material. Two negatives of this method are that it is an endpoint assay because staining requires cell membrane permeabilization and fixation, and it cannot identify cells that have undergone numerous rounds of division (reviewed in Lyons et al., 2013).

CELL CYCLE-ASSOCIATED PROTEIN ASSAYS

As cells progress through the cell cycle, phase-specific proteins are generated and can be detected using antibodies. Example phase-specific proteins include topoisomerase II alpha, phosphorylated-histone H3, and proliferating cell nuclear antigen, though there are many others. Topoisomerase II alpha expression begins in late S phase and peaks in the G_2 and M phases (Woessner et al., 1991). Histone H3 becomes phosphorylated only during the M phase of the cell cycle (reviewed in Hans and Dimitrov, 2001). Proliferating cell nuclear antigen expression increases during late G_1 and peaks during S phase (Kurki et al., 1987). The most commonly assayed cell cycle-associated protein is Ki-67, although it does not actually allow delineation of the different cell cycle phases because it is expressed in G_1, S, G_2, and M, but absent in the resting phase, G_0.

The major advantage of assaying cell cycle-associated proteins is that multiple techniques can be used: formalin-fixed paraffin-embedded and frozen tissue samples by