International Journal of Research and Review
Vol.7; Issue: 2; February 2020
Website: www.ijrrjournal.com
Review Article *E-ISSN: 2349-9788; P-ISSN: 2454-2237*

Methods of Detecting Cell Proliferation

Sayeeda Sadaf Hashmath[1], Hari Aparna Laxmi Gayatri[1], Pyata Vamshikrishna[1], Charu Suri[2], Shravan Kumar[3]

[1]Post Graduate Student, [2]Professor, [3]Reader,
Department of Oral and Maxillofacial Pathology, Sri Sai College of Dental Surgery, Vikarabad

Corresponding Author: Sayeeda Sadaf Hashmath

ABSTRACT

A cell is the structural and functional unit of life. Every cell has to undergo a series of events, called the cell cycle, in order to proliferate and become a part of the cell population and to be functional. There are various mechanisms in our body which distinguish between healthy and diseased cells. Over the years, many methods have been have evolved in order to determine the cell proliferation. Some of them are tedious yet reliable and cost effective while others are advanced and highly accurate but expensive. Though Hematoxylin and Eosin staining method is considered to be gold standard in interpreting the pathology of the lesion, evolution of various methods for detecting cell proliferation, starting from counting the nuecleolar organising regions to determining the number of proliferating cells by imaging method using label free photonic crystal biosensors, have made the work more easier. In this review, different methods of determining cell proliferation have been explained.

Keywords: Cell cycle, Cell division, Cell proliferation

INTRODUCTION

The cells in the cell cycle need to be assessed in order to ensure the prognosis of the disease. It serves as an important hallmark to differentiate between healthy state and disease condition.[1] The cell cycle, or cell-division cycle, is the series of events that take place in a cell leading to its division and duplication of its DNA (DNA replication) to produce two daughter cells. The cell cycle is divided into three periods: interphase, the mitotic (M) phase, and cytokinesis. During interphase, the cell grows, accumulates nutrients needed for mitosis, preparing the cell for its division and duplicating its DNA. During the M phase, the chromosomes separate. During the final stage, cytokinesis, the chromosomes and cytoplasm separate into two new daughter cells. To ensure the proper division of the cell, there are control mechanisms known as cell cycle checkpoints[2]. Over the years, various methods have been developed in order to detect the cell proliferation like by using special stains which help in determining the nuclear organizer regions, radioautography, flow cytometry using cytoplasmic proliferation dyes, immunohistochemistry and imaging methods using biosensors. Knowledge of these methods is essential for providing a better, easier and accurate diagnosis.

MITOTIC COUNT AND MITOTIC INDEX

Mitotic activity is a widely used criterion for assessing cancers in animals and humans. The value of counting mitotic figures in a histologic section to estimate mitotic activity has withstood the test of time and new techniques. It is a common component of most tumor grading systems. Mitotic activity can be estimated using mitotic count and mitotic indices. Mitotic count is a very cheap technique. Mitosis is

assessed as the number of mitotic figures per high power field, with the mean number of mitoses from 10 consecutive high power fields in the most cellular area of the tumor being measured. This method does not take into account differences in cellularity or cell size from one tumor to another.[3]

Mitotic indices are the fraction of the nuclei counted in mitosis. In this method, instead of enumerating mitoses at a single point of time, the rate at which cells enter mitosis can be quantified. The rate of entry into mitosis can also be determined by the 'metaphase arrest technique'.The technique uses agents such as vincristine to prevent dividing cells from completing the cell cycle. By counting the accumulation of the cells arrested at metaphase in each interval, an estimate of the cohort of cells entering mitosis can be made.[4]

NUCLEOLAR ORGANISING REGIONS (NOR)

The proliferating cells contain electrolucent areas during the interphase which can be seen at the ultrastructural level. These structures can be seen in the light microscope by using the AgNOR method which is a special stain. They are involved in ribosome production and also in potentially qualitative or quantitative changes taking place during the interphase. NORs may be visible in relation to proliferative activity or transformation and could aid in diagnosis or prognostication.[5]

AgNOR correlates with the rate of proliferation along with the percentages of the S phase cells and the mitotic cells. The interphasic NORs can be clearly visualized at the light microscopical level by using a silver reaction which stains the acidic proteins of the NORs on routinely prepared histopathological and cytological samples. After silver staining, the AgNORs can be identified as black dots throughout the nucleolar area. In quantitative terms, the number of AgNORS per nucleus suggests it to be a marker of the proliferative activity of the cell. Qualitatively (based on the shape, size and the pattern of distribution), AgNOR acts as a marker of pre-malignant or malignant change. In retrospective studies the samples can be destained and restained with silver. It can guide to a diagnosis when extra unstained slides are unavailable and also in doubtful cases with no corresponding histological specimens.[6]

RADIOAUTOGRAPHY

The basic principle of this method is that, in the biological specimens the location of radioactivity is observed through dark spots on an emulsion film. Also known as autoradiography, is a method where the proliferating cells are labeled in vitro with thymidine and then the tissue is processed with paraffin embedding.. The thymidine labeled cells usually corresponds to the "S phase". The cells are counted per 2000 tumor cell nuclei and expressed as thymidine labeling index. This method is employed as prognostic marker in breast carcinoma. During the S phase of the cell cycle, genome replication takes place. DNA polymerases incorporate nucleosides like thymidine into new strands of DNA. Thymidine is measured as per wall of cells rather than per individual cell. The assay reveals nothing about an individual cell's division history.[7]

DNA SYNTHESIS ANALYSIS WITHIN CELLS

Macallan and colleagues, in 1998, developed a method for measuring DNA synthesis and analyzing the same within the cells by administering Glutamate gated chloride channels (Glc) in the proliferating cells. In this method genomic DNA was isolated first and later was enzymatically hydrolyzed to free deoxyribonucleosides and prepared for Gas chromatography – mass spectrometry (GC-MS) analysis of deoxyriboadenosine (dA) or deoxyriboguanosine (dG) isotopic enrichments, or both. Comparison of dA or dG to extracellular Glc enrichment revealed the fraction of newly synthesized DNA.

The technique was different from the widely used [3H]thymidine or Bromodeoxyturidine (BrdU) techniques .

This method can be applied for both in- vitro (cell culture) and in-vivo (animal model). It has several advantages over previously available techniques for measuring the cell turnover as it does not involve radioactivity or potentially toxic metabolites, and is suitable for use in humans.

The availability of a reliable and safe method for measuring cell proliferation in humans opens up a number of fundamental questions to direct experimental testing, including basic problems related to cancer, Acquired immuno deficiency syndrome (AIDS) etc.[8]

CYTOPLASMIC PROLIFERATION DYES

Flow cytometry is used to assess cell proliferation in this method as it allows visualization of each round of division. It is a technique used to detect and measure physical and chemical characteristics of a population of cells or particles. A sample containing cells or particles is suspended in a fluid and injected into the flow cytometer instrument. The sample is focused to ideally flow one cell at a time through a laser beam and the light scattered is characteristic to the cells and their components. Cells are often labeled with fluorescent markers so that light is first absorbed and then emitted in a band of wavelengths. Tens of thousands of cells can be quickly examined and the data gathered are processed by a computer.[9] Flow cytometric analysis can be performed using different types of lights, by using multiparametric analysis and by using different dyes. The use of cytoplasmic dyes is the latest advancement in flow cytometry.[10]

Cytoplasmic proliferation dyes can be used both in vitro and in vivo for assessing cell proliferation. These dyes are fluorescent chemicals that permeate the cell membrane and covalently bind to cellular cytosolic components. After each cell division, the dye is evenly distributed between the two daughter cells, as a result of cytokinesis, which then exhibit half the fluorescence intensity of their parent cell. Although cytoplasmic dyes can be viewed under microscopy, but it does not easily allow quantification of dye and therefore is not typically a reliable method of measuring division.[11,12]

IMMUNOHISTOCHEMISTRY (IHC)

The cell cycle checkpoints monitors and dictates the progression of the cell through the cell cycle. This system acts like a timer, or a clock, which sets a fixed amount of time for the cell to spend in each phase of the cell cycle, while at the same time it also responds to information received from the processes it controls. The cell cycle checkpoints play an important role in the control system by sensing defects that occur during essential processes such as DNA replication or chromosome segregation, and inducing a cell cycle arrest in response until the defects are repaired.

The nuclear antigen specific for cell growth and division is stained by immunohistochemical method and then positive cells are counted under the microscope or by image analyser. Such proliferation markers include Ki67, PCNA and cyclins.[13] IHC is a relatively simple alternative to the more complex techniques. The major advantage of assaying cell cycle-associated proteins is that it can be used in multiple techniques like, formalin-fixed paraffin-embedded and frozen tissue samples by microscopy, single cell suspensions by flow cytometry, and cell lysates by western blot.

Different IHC markers are used to detect cell proliferation in various stages of cell cycle. Some of them are as follows:

Table : 1 IHC markers for cell proliferation[14]

Method	Marker	Use and benefits
DNA synthesis	BrdU	1. Immunoassay to quantify cells in G1, S, and G2/M. 2. Trace cell cycle kinetics
	Iodo-deoxyuridine (IdU) and Chloro-deoxyuridine (CldU)	Immunoassay to study DNA replication fork progression rates, stability or origin firing. 1. Two dyes (against IdU and CldU) allow more complex experiments than with a single dye
	Ethiny-ldeoxyuridine (EdU)	1. Immunoassay to quantify cells in G1, S, and G2/M 2. Trace cell cycle kinetics 3. Simple protocol, without DNA denaturation
Cellular metabolism	3-(4,5- dimethylthiazol-2-yl)-2,5- diphenyletetrazolium bromide (MTT)	1. Biochemical assay to indirectly quantify proliferating (respiring) cells 2. Simple method
	(2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT)	1. Biochemical assay to indirectly quantify proliferating (respiring) cells 2. Simple method 3. More sensitive than MTT
	Water soluble tetrazolium salts (WST 1)	1. Biochemical assay to indirectly quantify proliferating (respiring)cells 2. Simple method 3. More sensitive than MTT and XTT
Proliferation proteins	PCNA	1. Immunoassay to detect cells mainly in late G1 and S phases 2. Prognostic value in some cancers
	Ki 67	Immunoassay to detect cells in G1, S, G2 and M 1. Prognostic and diagnostic value in some cancers 2. Huge body of supporting evidence
	MCM 2	1. Immunoassay to detect cells in G1, S, G2 and M 2. Prognostic and diagnostic value in some cancers

LABEL-FREE PHOTONIC CRYSTAL BIOSENSOR IMAGING METHOD

A variety of assays for measuring necrosis, apoptosis, and cell proliferation are currently in widespread usage. These methods are time-consuming and laborious, and it is not practical for large numbers of samples, particularly for establishing growth curves for cell populations. Therefore, indirect analysis of cell proliferation is done by measuring the DNA synthesis rate through the incorporation of labeled DNA precursors during cell division. As with apoptosis assays, each proliferation assay requires staining of the cells with proprietary reagents (resulting in cell death), followed by removal of cells from their culture environment which is a multi-step assay protocol, followed by detection of the cells by label free photonic crystal biosensor imaging method.

This imaging system is based upon the unique properties of photonic crystal biosensors and a high resolution imaging detection instrument which is described to be capable of quantifying cytotoxicity and proliferation. The sensor is incorporated within standard microplates, and allows the same cells to be measured many times without removing them from their liquid environment. Firstly, an imaging instrument is used to detect the attachment of cells to the surface of a photonic crystal biosensor. An imaging detection instrument is used to determine the spatial distribution of attached cells by mapping the shift in between the cells which is reflected by the resonant wavelength as a function of position.

The capability for image-based label-free detection of cell attachment to the sensor is used to visualize and quantify the rates of cancer cell proliferation and subsequent cell apoptosis induced by the introduction of a cytotoxic chemical compound. [15]

CONCLUSION

There has been a revolution in the methods that are used to determine the cell proliferation. Starting from tedious, time consuming yet reliable methods like mitotic index to the evolution of accurate but expensive methods such as IHC marker, all the methods have their own advantages and disadvantages. Nevertheless, these methods have been a great help to the pathologist in distinguishing between normal and diseased conditions by helping them in determining the aggressiveness of the lesion. These

methods have made the work of the pathologist more easier.

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How to cite this article: Sayeeda Sadaf Hashmath SS, Gayatri HAL, Vamshikrishna P et.al. Methods of detecting cell proliferation. International Journal of Research and Review. 2020; 7(2): 402-406.

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