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CHROMATIN DETECTION METHODS-DIAGNOSTIC SCOPE IN ORAL PATHOLOGY

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ABSTRACT

Study of chromatin from the earliest stages of embryonic development to senescence are important for understanding the basis of normal gene expression in health and the causes of gene dysregulation in human diseases. With the availability of newer technologies and a highly sophisticated environment, this review provides unique opportunities to know the various methods to study chromatin architecture such as Cellular strains, Micropipette aspiration, Atomic force microscopy, Particle-tracking microrheology, Fractal dimension of chromatin, Chromatin Immunoprecipitation and MiniChIP-chip.

KEYWORDS: Chromatin, Atomic Force Microscopy, Fractal Dimension, Immunoprecipitataion.

INTRODUCTION

Chromatin, also called epigenome is the complex of DNA, histone proteins and associated macromolecules. Chromatin is finely granular and almost invisible in normal cell, whereas in a neoplastic cell, chromatin forms well defined irregular clumps with variable size, shapes and sharp pointed projections. For example, lower grades of OSCC show delicate strands and homogenous pattern of chromatin. While higher grades exhibits coarse clumped chromatin arranged in heterogeneous pattern. Earlier chromatin was considered as an inert structure. However, extensive research in this field has revealed that, chromatin has key role in controlling the gene activity and there by transforming normal cell to malignant cell.[1]

This review explains the various methods to study chromatin patterning normal cell and neoplastic cell.

Cellular Strain Experiments

It is an experimental approach to study the nuclear mechanics and to observe nuclear deformations in cells subjected to uniform substrate strain. Banes et al. 1985, first developed controlled uniaxial or biaxial strain to cultured cells to study sensitivity of these cells to mechanical stress.[2 Barbee et al. 1994, have combined strain experiments with microscopic observations to check out subcellular mechanical properties.[3 Uniaxial strain was applied to bovine aortic endothelial cells to give clear evidence of mechanical coupling between the extracellular matrix and nucleus was given by Caille et al. 1998.[4] Lammerding et al ,used this strain method to study the role of nuclear envelope proteins such as lamins and emerins and to study physical properties of nucleus.[5]

METHOD

In this method, cells are placed on transparent silicone membrane dishes coated with fibronectin or other cellular matrix proteins to provide firm cell adhesion. Cells are collected and serum starved to minimize fraction of cells in mitosis to focus on interphase nuclei. Nuclei are labeled with cell permeable DNA-intercalating fluorescent dye and cells are kept in culture media or Hanks buffer solution with calcium and magnesium. Silicone membrane dishes are then mounted on a custom-built strain device and placed on an inverted fluorescence microscope. Phase contrast pictures of silicone membrane and images of nucleus should be taken. The fluorescent images of nucleus taken before strain application.[6] Later at each stage of procedure pictures of several fields should be taken to view number of cells in single experiment. Care should be taken not to strain cells for extended periods to minimize cell damage to the strain by cytoskeletal remodeling.[7]

Advantages

The properties of cells can be measured in situ. Many cells can be simultaneously strained. This method is can also be used to study nuclear mechanics & nuclear deformations.[8]

Atomic Force Microscopy

Atomic force microscopy (AFM) is a measurement which typically probe nucleus on smaller scale length with surface morphology at nanometer scale. Advantage

of this method is, it can measure intermolecular forces and can be used as a nonsurgical tool. Imaging can be done in physiological solutions, no fixation is needed. The greater complexity of the biological structure is achieved at lower resolution.[9]

METHOD

Atomic force microscopy method uses a cantilever with a pyramidal or spherical probe tip which must be pressed against the sample surface to assess the surface morphology. Underlying materials used would determine the resolution due to plasticity of material. Higher resolution of this method will help to study nuclear membrane proteins, e.g. nuclear pore complexes, on an isolated nuclear membrane patch spread on mica or glass compared to those on the total nucleus.[10]

Forces are measured in a range of nano newtons while indentations are in the range of 50 nm to several micrometres. Measurement of nuclear rheology is done in isolated nuclei rather than in cells which are intact. The cells obtained are placed on rigid substrate which promotes nuclear adhesion e.g. poly-L-lysine[11], poly-HEMA.[12]

Currently, poly-L-lysine is used in balancing concentrations that promote adhesion while avoiding nuclear aberrations due to excessive adhesion and surface interactions.[13] Probe is selected depending on type of experiment performed which should have small radius of curvature i.e. the ideal probe for studying the morphology of larger surface area is spherical microbeaded type of 25 nm or larger. Smaller tip probes can be used which provide higher spatial resolutions and could measure local deformations at small scale length.[14] For all types othe probes, the applied force is determined only by the stiffness of the cantilever and the deflection of the cantilever arm from the resting (zero-force) value. Vertical displacement of the cantilever from the point of contact with subtraction of the cantilever deflection measures indentation of the nucleus. In contact mode AFM probe is brought onto the nucleus with constant velocity maintained at given degree of indentation. In intact nuclei contact mode method is generally used, using micro-bead probe tips. AFM has been especially useful for the study of chromatin fibers.[15]

Partially unfolded chromatin fibers are studied owing to the tendency for chromatin condensation which coincides with loss of resolution at the level of nucleosomes.[16]

AFM is used to study nuclear mechanics in living cells like fibroblasts in which the nucleus is positioned close to cell membrane and which is easily accessible to the AFM probe.[17]

Advantages

AFM studies on intact nuclei is that they only probe the mechanical response of nuclei to compression, while many of the physiological mechanical stimuli are thought to exert tensile forces on the nucleus.[18]

Particle-Tracking Microrheology

Particle-tracking microrheology (PTM) provides highest resolutions i.e. nanometers in cellular probing mechanics. This method defines the rheological properties of the cytoskeleton and the nucleus. Method gives complete description of the elastic and viscous properties of the material. Single, double and multiple particle-tracking methods are used to determine the rheological properties.[19]

Particle-tracking microrheology studies on the intranuclear region have shown that this region is much stiffer than the cytoplasm and more elastic than viscous, indicating that this region displays an unexpectedly strong solid-like behavior.

Advantages

Highest spatial resolution, Causes minor disturbances to the cell/nucleus.[19] LIMITATION: Particles have to be delivered into the cell/nucleus. PTM results depend on particle size and mesh size of probed cellular structure.[19]

Micropipette Aspiration Experiments

Direct technique to study the physical properties of intact nuclei is micropipette aspiration. Method was adapted by Evans and colleagues as a tool to study the viscoelastic properties of membranes and thin-shelled vesicles.[20] Used to study the mechanical behavior of leukocytes, blood cells and various other cell types. Micropipette aspiration of nuclei has been used to transplant nuclei from somatic cells into oocytes.[21] Recently, technique is used to study physical properties of isolated nuclei.[22]

METHOD

Micropipette aspiration method, nuclei are partially aspirated into a micropipette with opening of 2-10 mm by applying a particular controlled aspiration pressure in the range of 100-10000 Pa.[23] Pressure applied can be measured by transducer and nuclear deformation can be observed in high power objective on a inverted microscope. Isolation of nuclei can be obtained by treatment with detergent which causes mechanical disruption of osmotically swollen cells in a Dounce homogenizer. Isolated nuclei are further purified by centrifugation process through a sucrose gradient which removes excessive membranes from the endoplasmic reticulum or other kinds of cytoplasmic contamination.[24] This micropipette aspiration can be done on nuclei within living cells that are treated with cytoskeleton-disrupting drugs such as latrunculin A, which would inhibit actin polymerization.[25] Fluorescent labeling of nuclear components such as nuclear lamina or nucleoli by fluorescent dyes enables detailed study of

deformations in these components by fluorescence/ confocal images. The experiment would provide a diagnostic tool to study physical properties of nuclei. This technique requires isolation of nuclei in living cells. The results on isolated nuclei are sensitive to variations in composition of buffers used in experiment. Isolation of nuclei done by detergent or mechanical disruption would result in damage of nuclei during studying disease models which have increased nuclear fragility, so to avoid this care should be taken to confirm quality of isolated nuclei by performing micropipette aspiration in intact cells. Sometimes it alters cytoskeletal interactions and would disturb the normal environment of cell. In contrast, in adherent cell, nucleus is in a pre-stained configuration, so disruption would produce nuclear collapse which would lead to more spheroidal appearance of nucleus.

Advantages
This method is powerful tool to study physical properties of intact nuclei in living cells.[26]

Limitation
Requires isolation of nuclei or disruption of cytoskeleton in living cells.[26]

Chromatin Immunoprecpitation
Gold standard approach for investigation of epigenetic chromatin. As epigenetic modifications like methylation of DNA or histones are linked with transcriptional output of genes, they play important role in genome functioning. This method is considered as best method to study the detailed characterization of various chromatin. This method is used globally in a context of analyzing singe genome.[27] The comparison of chromatin profiles between different tissue and different individuals can be done, such groups are intra group analysis and inter group analysis. The goal of intra individual variation is to detect the chromatin modifications with that of healthy individuals.[28] Analysis can be carried out on well-defined groups of oncology patients i.e. comparing cancerous patients tissues with normal tissue from healthy individuals (Esteller, 2008).[29] By this method chromatin difference between one or more sample can be detected. Each sample being presented by one tiling arrayor sequencing experiment chromatin which have to be analysed are marked on sample.[30]

Advantages
Powerful method that directly permits the measurement of in vivo DNA Protein interact action. When combined with microarray (ChIP-chip) and high through-put sequencing (ChIP-Seq) and miniChIP-chip, help to uncover novel targets.

Chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) experiments Method is used to determine entire genomes, can determine sites of any protein of interest, including, eg., transcription factors, RNA polymerases, histones with or without various modifications. This method determines the occupancy sites within one cell type and under one condition.Comparision of various cell type, tissues and conditions can be done by using this method. Uses spike adjustment procedure(SAP)which uses single batch of chromatin of any foreign genome and is added to the experiment genome. This method would reveal similarity between replicates and biological differences including global and largely uniform changes.[31]

Method: Chromatin with in intact cells treated with formaladehyde which is a cross linking agent. The crosslinked chromatin is isolated and is fragmented by sonication .Material obtained is used as starting material for immunoprecipitation with antibodies directed against the factors of interest. The immunoprecipate containing protien is targeted by antibody and DNA is cross linked to it. This is heated to reverse crosslinks are then the obtaied DNA is purified, amplified and presented to deep sequencing which generates sequence ‘‘tags’’ of commonly 35 to 100 nucleotides (nt), are then aligned onto the genome. This method has confirmed impressively powerful in characterizing chromatin organization,i.e.,in identifying sites to which they bound, eg.., transcription factors, by histones carrying (or not) specific modifications, or by RNA polymerases.[32]

It identifies regions of factor occupancy within a single chromatin sample, ChIP-seq is invaluable for comparing the level of occupancy at a set of loci between different chromatin samples from various cell types or tissues, from cells which are submitted to different conditions, or from cells at different developmental stages, etc.[33]

Fractal Dimension of Chromatin
Fractal analysis is used to study single cells, by which one can describe fractal features of cells.[34] Study of chromatin structure can be done in grey level images of cell nuclei. Fractal analysis can be helpful in detecting changes in nuclear components in ultra-structural level in pathologic tissue cells. Carcinogenesis would involve alterations in normal gene showing increased amount of percentage of chromatin as well as difference in structure. A study done by Danielsenet.al on liver cell nuclei in normal liver and compared it with that of hepatocellular carcinomas noted very much discriminating variables. Fractal features can easily be estimated in digitalized microscopic images and are helpful for diagnosis and prognosis of neoplasias. During carcinogenesis and tumor progression, the fractal dimension of chromatin usually increases.[35]

Fractal features are anticipated in microscopic digital images which would be helpful in diagnosing cancers. In cancer, increased fractal dimension (FD) of stained nuclei have been observed in oral squamous cell carcinoma, intraepithelial lesions of cervix, and in adenocarcinomas of pancreas. Increased FD in oral squamous cell carcinoma makes it unfavorable prognosis.

Advantages
This method will help in determining hetero and euchromatin of single cell in fractal globule.

Importance of chromatin in cancer and its remodeling
In human's uncontrolled proliferation of immature blood cells by using histone deacetylase would cause acute myeloid leukemia (AML) and promyelocytic leukemia (PML) these disorders are associated with chromosomal translocations. Cell cycle pathways are often mutated in human cancer, some of this include retinoblastoma protein (RB), cyclin dependent kinase inhibitors p16/ink4A. These genes are often silenced by DNA methylation. Checkpoint proteins depend on chromatin remodeling to silence genes that would otherwise continue the proliferation. So, association between histone deacetylase with DNA methyltransferase provides pathway. The proper recognition and selective inhibition of this chromatin remodeling pathway could give best therapeutic window in cancer biology.[36]

What happens to chromatin cancer
In cancer, genes expression plays an important role by allowing cancer cells to acquire their characteristics so instability in gene expression i.e alterations in these genes would promote carcinogenesis. There is frequent mutation in genes encoding chromatin regulatory factors and histone proteins in human cancer thus denoting that these are major mediators in pathogenesis of malignancies and solid tumors.

Chromatin proteins mutated in cancer are histones H3 N-terminal tail, methyltransferase, demethylase, deacetylase.[37]

CONCLUSION
Number of experimental techniques have been developed in recent years to quantify the chromatin. These methods help in early diagnosis and treatment. With the availability of newer technologies and a highly sophisticated environment, this review provides fresh and unique opportunities to study the chromatin architecture in live cells by various methods.Thus gold standard method for detection of chromatin is immunoprecipitation.

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