Original Article

Laser Capture Microdissection - A Breakthrough Technology in Head and Neck Diagnostics

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Abstract:

Laser capture microdissection (LCM) is a technique in which, from a heterogenous tissue specimen some pure cell populations are isolated. This procedure can capture and target the desired number of cells that are required. A low energy laser pulse is used in this technique. Apart from systemic applications LCM can also be applied in the field of dentistry and pathology.

Keywords: Laser capture microdissection, dentistry, LOH analysis, genome, tumor cells.

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Introduction:

Modern LCM technology is used to separate pure cell populations from a heterogeneous tissue samples. It can accurately target and seize the target cells for a variety of downstream assays (Angen Liu 2010). LCM is based on the idea that cells and tissue fragments that have been visually targeted will only adhere to a thermoplastic membrane when it has been activated by a low energy laser pulse.5 Tumor cell line culturing is used to study genetic changes in neoplasia and carcinogenesis. This method produces high-quality DNA, RNA and protein, only drawback is that the procedure is lengthy, costly and it's not successful always. Tumor cells are selectively isolated and it's precursor lesion also need a method in which the tumor cells are separated from primary lesion. In LCM a subpopulation of specific cells are isolated and they use direct visualization of the cells for culturing live cells & cytological preparation from a diverse background of cell types. In 1970s, requirement for microdissection was understood when Lowry and Passonneau introduced a method in which lyophilized tissue sections taken from specific cell types were microdissected by using quantitative biochemical analysis. This was a free hand procedure in which dissecting microscope was used having flexible bristle to which splinters of razor blade are mounted. A small piece of human hair was used to lift & transfer the dissected part and mounted to a glass holder of pencil-shaped. Approximately 15 - 20µm of tissue samples are dissected.[1]

Three main categories of recent LCM approaches exist:

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desired foci extracted manually, selective removal of undesirable portions, and laser capture microdissection, which uses laser pulses to capture cells of interest. Manual method was reported by **Goelz et al (1985)** [2], in which desired tissue were removed from the wax block by direct visual examination, tissue was taken for removal of DNA. Genomic material taken from this method can be used for PCR amplification and sequence analysis. DNA was extracted manually from lesions < 1 mm using modified Pasteur pipettes or tungsten wire. Disadvantage with this method is they are both tedious and operator dependent, we need to prevent contamination from minor air currents while transferring tissue from pipette or needle tip to microcentrifuge tube. Adhesive tape was used to improve upon manual extractions method which can avoid loss of microdissected material from air current or tremor. With contrast to the above method, UV radiation was used by selective ultraviolet radiation fractionation (SURF) to damage unwanted areas, after which molecular analysis is done by manually scrapping off the portions of tissue which is left behind. **Shibata et al (1998)** [3] first described this method. **Becker et al (1997)** [4] described the analogous technique in which they removed the non-neoplastic components using a manual micromanipulator and UV laser. Ablative method can also be used for formalin fixed samples.[1]

LCM comprises of a solid state near infrared laser diode, an inverted microscope, a CCD camera a joy sticks controlled microscope stage with a vacuum chuck for slide immobilisation, a colour monitor, a CCD camera and a laser control unit. A PC is connected to LCM microscope, which helps in image recording & laser controlling. For tissue processing a thermoplastic membrane (approx. 6 mm diameter) mounted on an optically clear cap, is used to transfer selected cells, that fits on 0.5 ml microcentrifuge tubes.[5]

Recently 7.5 µm is the minimum diameter and 30 µm is the maximum diameter in laser beam LCM microscope. Maximum energy is absorbed by membrane. Activation of Laser is in the range of 90°C. Photochemical effects which are hypothetically damaged is avoided by less energy infrared laser.[1]

Benefits and limitations of LCM:

Speed combined with precision and versatility is the main benefit of LCM. Within a few minutes thousands of cells are collected, which depends on size of laser spot, architectural features of the tissues and desired precision of the microdissection. In comparison with micromanipulator based microdissection method LCM is quicker. LCM is a "no touch" method in which manual dexterity is not needed. Risk of tissue loss is decreased because sequestered cells are tightly attached to cap of microscope.[5]

Many tissue components from both normal and neoplastic cells are sampled in one slide since LCM causes less damage to nearby tissues & is quicker. After removal of coverslip successfully stained sections can be used.

Along with the advantages, LCM also has few limitations which reproduces the complications of microdissection. Routinely stained dehydrated tissue sections (without coverslip) has less optical resolution, which makes accurate cell dissection from complex tissues with absence of architectural characteristics like infiltrating carcinomas and lymphoid tissues.[5]

Small cells with diameter less than 7.5 µm are problematic to separate without contaminating fragments of nearby cells. Risk of contamination increases due to larger area of contact of tissues with cap.

The major drawback of LCM is that it cannot remove the selected cells from the slide, and this leads to difficulty in attaching cells to the membrane. Increased adherence of slide to section prevents the deletion of cells of interest, seen in frozen tissue sections if dried for a longer time, in paraffin wax section no special treatment required.

Laser microbeam microdissection is an advanced version of laser microdissection. (PALM; Mikrolaser Technologie, Bernried, Germany) is other commercially available technology. In comparision with LCM, UV laser beam along with a motorised stage is used to remove surrounding structures. Tissue is removed by laser pressure catapulting or by a needle tip. This leads to free contact in tissue collection in microcentrifuge tube cap.15 Laser beam helps to remove tissue sections (mounted on thin supporting membrane) that leads to retrival of fragments that are intact with larger tissues having similar morphology.

LCM & LMM having an important development like laser pressure catapulting in microdissection, future advances improves both systems performance. Effortless handling, superior control, high speed, records of resected tissue makes LCM a perfect techique for faster gathering of more samples of tissue and single cells of more numbers are pooled. Greater skills and more time is required for LMM. Absence of larger surface area instead of smaller laser beam diameter, motorised stage, contact of tissue with cap of LCM is more suitable.[5]

Implementation of LCM in Various Fields :

There are 3 different types of biomolecules that is analyzed in LCM specimens: Proteins, RNA, DNA. DNA and RNA analyses need less material than protein analyses. Genomic analyses can be performed on samples derived from one single cell, but in recent generation of proteomic tests this may not be possible for proteins.[1]

Cancer Genome Anatomy Project in Cancer Resarch:

The National Cancer Institute's Cancer Genome Anatomy Project (CGAP) uses microdissection to track the molecular evolution of cancer. It also compares and contrasts expression profiles from microdissected regions in the same patients with different genetic makeups between normal, preneoplastic, and cancer tissues. Recent studies have shown that LCM is useful for creating expression libraries that are specific to different tissues. For instance, it was originally believed that contaminating T cells in the prostatic interstitium were responsible for the highly expressed T cell receptor discovered in the prostate, but subsequent investigations showed that the transcript actually originated from prostate epithelial cells. In near future, we can anticipate similar outcomes for additional tissues. To yield useful data, superior quality primary tissues are required. [1]

Analyses of genome from Microdissected Materials :

Solid tumors in many cases develops in a multistep pathway. Accumulation of continuous genetic aberrations leads to malignant transformation. Two hit hypothesis by Knudson's explains the function of tumor suppressor gene, deletion of one parental allele and second is inactivated by mutation. In malignant tissue, normal allele is lost (loss of heterozygosity). Since a long time LOH (loss of heterozygosity) is used in cancer research. Analysis of LOH needs preneoplastic foci or tumor cell pure population. Microdissection used in cancer study made a striking difference in the use of LOH analysis

The investigation of sequential genetic alterations in pre-neoplastic lesions, the location of potential chromosomal "hot spots," and the mapping of tumour suppressor genes (Tags) have all benefited greatly from the use of LOH analysis. Many priceless samples had to be abandoned because the requisite purity could not be reached, before microdissection techniques were widely used. It was nearly impossible to analyse genetic losses in preneoplastic lesions. In many ways, microdissection had completely changed how LOH research was conducted. This investigation was first made possible by it. It has also been shown that LOH frequencies in non-microdissected specimen frequently underestimate the real incidence of genetic alterations and heterogeneity within a single tumour where some genetic modifications frequently starts, such as in histologically "normal" or slightly abnormal tissue.

In addition to analysis of LOH, microdissected samples may also be used for other genome analyses, including X-chromosome inactivation pattern analysis to determine clonality, single strand conformation polymorphism (SSCP) and restriction fragment length polymorphism (RFLP) analysis to determine whether critical genes like P53 and Ki-ras have undergone mutation, most recently, the analysis of promoter hypermethylation. A frequent and early occurrence in the development of cancer is hypermethylation of the promoter regions of tumour suppressor genes. It is also utilised for trails in chemoprevention as a substitute biomarker and also reversible. Microdissected primer extension preamplification (PEEP) and whole genome amplification (WGA) are combined and it has become feasible for detailed examination in preneoplastic lesions, which has opened up a completely new area of research in cancer. For instance, until recently, the comparative genomic hybridization (CGH) method required substantial

volumes of DNA taken from tumour samples. CGH may now be carried out on microdissected cells (20-100) from paraffin tissue blocks and less in material and derived from methanol like fixative agents, thanks to improved technology. The most enthusiasm has been generated by microarrays, also referred to as "gene chips," of all the expression technologies currently in use, particularly when combined with LCM. All of these techniques can use archival tissue that has been treated in formalin. The sample size for each PCR should not be more than 50–100 dissected cells, materials from frozen specimens in methanol or cryostat slices can also be used.[1]

Gene Expression Investigation of Microdissected Material:

Degree of difference gene expression is a good indicator of how cancers differ from the normal tissues they are formed. Gene expression pattern discovery linked with inflammation, repair of tissue and neoplastic transformation has profound effects on prognostic and diagnostic research, preventative medicine, and the development of novel therapeutics for certain genetic abnormalities. Several techniques, including degree of difference shows representation of ESTs, difference analysis (RDA), SAGE and differential gene chips are available for studying gene expression. Issue of contamination with inflammatory and stromal cells in the study of gene expression is similar to that in DNA-based investigations. Because HICK or in situ hybridization requires tedious, time-consuming procedures, it has become more necessary to adopt microdissection techniques in expression research as well. Since mRNA in situ hybridization is insensitive to detecting low-abundance transcripts, the demand for single-cell LCM has increased as well.[1]

Analyses of Proteimic in Microdissected Material:

Proteins have an essential role in functioning of cells. DNA sequence do not aid in the synthesis of corresponding protein, its function and cellular locations. It has no role in giving information about sulphatation, glycosylation and phosphorylation. Proteomics plays role in gene expression and provides information about post-translational remodelling. This determines the proteomes which is also a proteins set, which are essential in cellular physiology and changed due to malignancy. Other methods like western blotting, 2-D PAGE (high resolution two dimensional polyacrylamide gel electro- phoresis), sequence of peptide and mass spectrometry peptide also used. While doing analysis of protein in samples of LCM factors like tissue type, molecules, downstream analysis should be considered. In this method steps of amplification for protein analysis is not present. Protein is first separated by charge and dimension with the help of 2-D PAGE. RNA is less stable than protein. It has a negative effect for measuremening the molecules in clinical samples and biological materials.[1]

Proteomic analysis may be affected by cellular heterogeneity, which also effects DNA and RNA analysis. In newer methods tissue homogenization is required and has no contribution for measuring protein content.

LCM is recently used for studying alteration of protein in tumors and preneoplastic lesions, this also aids in management and involvement of strategies. Prostate specific antigen (PSA) levels may be calculated with quantitative chemo- luminescent assay rapidily, sensitivitively in microdissected benign and malignant tissues. In normal and tumor cells this method analyzed a variety of proteins. Using SELDI biochip, **Paweletz et. al. (2000)**[6] used SELDI biochip successfully and classified the population according to their molecular weight and showed diverse expression of protein patterns in premalignant and malignant lesions in human tissue obtained by LCM.

Diagnostics Applications of LCM:

In tumor sporadic gene mutations aids in therapeutic responses & prognosis. Gene mutation should be detected in classification, pathological diagnosis and treatment of tumors. LCM has a major role in analysis of sporadic gene mutations, in this process prior to molecular analysis captured tumor tissues are enriched in tumor-associated genetic alterations. This method reduces time taking methods in mutational analysis, which leads to speedy and effective tumor genotyping.

LCM also helps in routine diagnosis of various dermatological lesions like cutaneous B- or T-cell lymphomas, as diagnosis of these lesions are challenging. **Yazdi et. al.** in the year **(2004)**[7] introduced LCM-based clonality assay to rule out the

difficulty in diagnosis of these lesions. Rearrangement of clonal T-cell receptor (TCR) gene that detected results from few dermatological conditions that are inflammatory in origin and also in lymphoma.[1]

Cancer Chemoprevention:

IEB also known asIntermediate endpoint biomarker may be used to check success rate of chemoprevention of cancer. IEBs example for malignancy are CIN (cervical carcinoma), PIN (prostate carcinoma), adenomas in colorectal cancer. Patients with high risk for cancer development are selected by this biomarker, efficacy of novel chemopreventive agents is also evaluated, that decreases cost of clinical trials. It's difficult to evaluate the efficacy of chemoprevention in dysplasia along, so genotypic alterations in "normal" epithelium near dysplasia also required to be evaluated. **Hong et. al.** in **(2000)**[8] had done a study on chemoprevention of oral leucoplakia in which 54% patients who were treated with 13-cis-retinoic acid showed a decrease in dysplastic features. In 50% of patients after discontinuiation of drug therapy relapsed is seen, malignant clone was also present in them. A follow up study was conducted in which biopsy was taken from oral premalignant lesions, which showed 89% of patients are having perseverance of LOH at the 9p21 (p16 locus). The above data concluded, that epithelial tissues are showing a time difference between genotypic & phenotypic reaction.

Biomarker Discovery:

LCM is applicable to a variety of human tissue types and organ systems. as a biomarker. It is also used in evaluation of forensic examination of hair follicles and preserved cell samples, tumor microenvironment, animal model xenografting, plant cell biology,infectious disease biology, xenografting, researches in developmental biology and embryology, spermatogenesis. [1]

Applications of LCM in various fields:

LCM has it's application in various fields. Some of the systemic applications are prefertilization genetic diagnosis, organ transplantation, blood vessel analysis & analysis of keratinocytes from wounds. It also has applications in pathology like separation of tumor & normal cells, gene expression, molecular characterization of cancer cells and single cell mutation analysis. LCM plays a vital role in dentistry and aids in oral cancer diagnosis, to study pulp biology, PDL, tooth eruption & tooth development.

Conclusion:

To harvest a pure population of cell, from a biopsy specimen or any other sample a novel and specific tool is available to use i.e; LASER CAPTURE MICRODISSECTION

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