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Exploring the Potential of Laser Capture Microdissection Technology in Integrated Oral BioSciences

Running Title: Laser Capture Microdissection in Oral Biosciences

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Abstract

Laser capture microdissection (LCM) is a high end research and diagnostic technology that helps in obtaining pure cell populations for the purpose of cell or lesion specific genomic and proteomic analysis. Literature search on the application of LCM in oral tissues was made through PUBMED. There is ample evidence to substantiate the utility of LCM in understanding the underlying molecular mechanism involving an array of oral physiological and pathological processes, including odontogenesis, taste perception, eruptive tooth movement, oral microbes, and cancers of the mouth and jaw tumors. This review is aimed at exploring the potential application of LCM in oral tissues as a high-throughput tool for integrated oral sciences. The indispensable application of LCM in the construction of lesion specific genomic libraries with emphasis on some of the novel molecular markers thus discovered is also highlighted.

Keywords: laser capture microdissection, oral squamous cell carcinoma, oral embryology, oral physiology

Introduction

The prudent application of molecular diagnostics in understanding many of the body's physiological and pathological processes is acknowledged and well accepted. With the emergence of high-throughput methods such as DNA microarrays, Differential Display - Comparative Genomic Hybridization (DD-CGH), sequential elongation processes and

proteomics, over the last decade, molecular biology has refined the basic understanding of these biological processes and broadened our approach in dealing with the disease. The process of acquiring the pertinent cell or tissue is, however shrouded by an assorted background of stromal and inflammatory cells at the tissue level, thus raising questions about the precision of these tools.(Curran et al., 2000, Domazet et al., 2008, Chokechanachaisakul et al., 2010) The molecular understanding of pathologic processes in the oral cavity is further compounded as the surgical specimens provided are small and often contain an admixture of various cells. In general, a paraffin embedded tissue of squamous cell carcinoma (SCC) may contain less than 5% of the true representative tumor cells. The rest includes normal epithelial cells, fat cells, endothelial cells, fibroblasts, lymphocytes, e.t.c.(Domazet et al., 2008, Fend & Raffeld, 2000) This is even more for preneoplastic lesions that may be spatially different from the tumor, and quite often, sparse in quantity. These challenges in molecular diagnostics are overcome by obtaining a pure population of cells, by simply eliminating the heterogeneous background.(Hunt & Finkelstein, 2004) This uncontaminated procurement of cells is best achieved with Laser Capture Microdissection (LCM).(Hunt & Finkelstein, 2004)

Such purity might be vital for either gauging the genetic changes or for quantifying them. For example, in gauging the probable loss of heterozygosity (LOH) of tumor suppressor genes (TSGs), normal contaminating cells may artifactually even out the allelic balance, leading to erroneous conclusion tumor cells contain normal DNA.(Hunt & Finkelstein, 2004) Furthermore, LCM might be vital for sorting out the bits of the suspected floaters from the rest of the tissue sample.(Hunt & Finkelstein, 2004, Hunt et al., 2003) This article will focus on the principle and technology behind LCM and how it has been used to further our knowledge of the oral milieu, from the perspective of an oral pathologist.

Historical perspective

The necessity for microdissection was realized in the early 1970s, when Lowry and Passonneau introduced a method of quantitative biochemical analysis of the exact cell type's microdissected from lyophilized tissue sections.(Lowry & Passonneau, 1971, Domazet et al., 2008) The procedure was done freehand under a dissecting microscope with pieces of a razor blade mounted on a flexible bristle.(Domazet et al., 2008) The dissected portions were then picked up and transferred to a short piece of human hair mounted to a pencil-shaped glass holder.(Lowry & Passonneau, 1971, Domazet et al., 2008) Although this procedure was used for isolating specific cell types, it was laborious, crude and a considerable amount of tissue was destroyed in the process.(Xu, 2010)

In the late nineteenth century the application of UV radiation was explored as it would destroy the surrounding unwanted regions around the islands of tissue that could be manually scraped off for molecular analysis.(Becker et al., 1997, Shibata, 1998) Shibata coined the term selective ultraviolet radiation fractionation (SURF) for this method.(Shibata, 1998) Microdissection utilizing of laser pulses to capture cells of interest referred to as LCM was developed at the National Cancer Institute (NCH) of the National Institutes of Health (NIH), Bethesda, in the mid 1990 by Lance Liotta, Emmert-Buck and co-workers.(Emmert-Buck et al., 1996)

Principle and working of LCM

The fundamental principles of the laser microdissection process are: 1) Visualization of the cells via microscopy; 2) Transfer of laser energy to the area of interest and removal of the cells of interest from the heterogeneous tissue surround; 3) Collecting the cells of interest into a microtube/device.

A typical LCM setup consists of an inverted microscope, a laser diode (near infrared wavelength), a laser control unit, a joystick controlled microscope stage with provision for slide immobilization via a vacuum chuck, a Charged Couple Device (CCD) based camera, and a color monitor.(Hunt & Finkelstein, 2004) Diagrammatic representation of a typical LCM setup is shown in Figure 1. The thermoplastic membrane utilized for the transport of selected cells has a diameter of about 6 mm. This membrane is then mounted on a cap, which is optically clear. This cap fits on standard 0.5 ml microcentrifuge tubes and can be taken for further tissue processing. It is usually impregnated with a dye that helps with visualization and absorbing the laser energy to prevent damage to the cellular areas. The cap is then suspended on a mechanical transport arm and positioned in the requisite area of the dehydrated tissue section.(Domazet et al., 2008) The cap is then lowered in a precise juxtaposition to the requisite area,(Domazet et al., 2008) and since it is optically clear, it allows the laser to be centred on the same plane as the tissue section. The calibre of the laser beam of the LCM microscope can range from a minimum of 7.5 μm to a maximum diameter 30 μm.(Datta et al., 2015)

Once the desired area has been visualized and set in place, the laser is activated. Laser activation raises temperature to 90°C which causes localized momentary melting of the ethylene vinyl acetate (EVA) membrane. The polymer melts only in the vicinity of the laser pulse, and fills the tremendously minute gaps present in the tissue. The area of polymer melting corresponds quite closely to the laser spot size, under standard working conditions. On cooling down, the polymer solidifies within milliseconds and forms a polymer-cell composite that buries the cells into the plastic membrane.(Domazet et al., 2008) The selected tissue fragments can be collected by simply peeling off, of the polymer from the tissue surface, allowing the shearing off, of the embedded cells from the requisite area.(Domazet et al., 2008) Diagramatically represented in Figure 1a. An Eppendorf tube, 0.5ml in volume

containing lysis buffer is used for storing the cap with the isolated cells.(Domazet et al., 2008) The isolated cells are released into the solution through the breakdown of long chain polymers within the EVA film by the lysis buffer.(Domazet et al., 2008)

Various modifications of this basic working have been developed. The PixCell system (Arcturus, MDS Analytical Technology, CA, U.S.A.) uses a dual laser system - the ultraviolet (UV) laser to incise and infrared (IR) laser to gather cells.(Chokechanachaisakul et al., 2010) The Pixcell LCM system is the most preferred LCM system in use.(Chokechanachaisakul et al., 2010) Zeiss's PALM system (a unit of Carl Zeiss Micro Imaging, Jana, Germany) uses a UV laser to incise the tissues via inverted microscope and gather cells by photonic pressure.(Chokechanachaisakul et al., 2010) Diagramatically represented in Figure 1b Leica AS LMD system (Mannheim, Germany) uses a UV laser to incise, and then the dismembered cells fall into a collecting tube by gravity.(Chokechanachaisakul et al., 2010) Diagramatically represented in Figure 1c. The detailed working of each has been described elsewhere.(Chokechanachaisakul et al., 2010)

General considerations for LCM

As most LCM cells are useful in molecular analysis, regular precautions while grossing the specimens should be taken, the workbench and instruments should be thoroughly cleaned and disposable gloves and blades should be changed between samples.(Hunt & Finkelstein, 2004) Fixatives must be vigilantly selected if molecular techniques are to be used.(Hunt & Finkelstein, 2004) Fixatives that have a low pH (Bouin's fluid (contains picric acid) or decalcifying solutions) should be avoided. Also, fixatives that contain heavy metals like mercury should be avoided.(Hunt & Finkelstein, 2004)

Formalin fixed paraffin embedded tissue block (FFPE)

Most pathology laboratories store tissues in the form of FFPE tissue blocks. Stained and unstained tissue sections can both be used for LCM but care should be taken to utilize glass slides specific to the type of LCM machine used. Most of the times, specific uncharged and uncoated glass slides are utilized for LCM, as charged and coated slides can hinder the transfer of tissue from the slide onto the cap (e.g. Poly-Ethylene Naphthalate (PEN)-coated membrane slides (Carl Zeiss Micro Imaging, LLC, Thornwood, IL).(Liu, 2010, Accurso et al., 2011)

The optimal tissue section thickness for LCM is 4–15 μm.(Liu, 2010) Slides must be used as soon as possible or stored at room temperature. Before microdissection is attempted, the slides for LCM must be deparaffinized by standard sequence of washes in alcohol and xylene.(Hunt & Finkelstein, 2004) If the tissue is dehydrated too much, it may drop off in big fragments rather than as single isolated cells.(Hunt & Finkelstein, 2004) If the tissue is partly dehydrated, however, it may stick too firmly to the slide and will not be isolated during microdissection.(Hunt & Finkelstein, 2004)

It should be noted that most LCM studies prefer using frozen tissues in comparison to FFPE sections as cross-linking during fixation impedes the recovery of RNA and protein from FFPE tissue.(Liu, 2010) FFPE tissues cannot be used for protein extraction, however, DNA extraction requires incubation with proteinase K for a minimum of 16 hours.(Liu, 2010)

Frozen tissue

Frozen tissue allows exceptional preservation of RNA, DNA, and proteins and thus is considered as the optimum technique; for their analysis. However, it is inconvenient for handling and storage and also lacks histological details.(Liu, 2010) When frozen sections are

to be used, the tissue should be collected under sterile conditions.(Hunt & Finkelstein, 2004) For storage, wrap in aluminum foil and store in a -70˚C freezer until ready to use.(Hunt & Finkelstein, 2004) For long-term storage, cryopreservation in liquid nitrogen may be necessary.(Hunt & Finkelstein, 2004) Most LCM based studies involved in understanding the dynamics of tooth development and other aspects of oral biology employ wholly frozen mandibles of rats.

Mineralized tissue (dentin and enamel)

The common obstacle, in mineralized tissue research, is the difficulty in preparing intact unfixed, undecalcified sections of highly mineralized tissues such as dentin and enamel. Another impediment is the means to obtain high-quality RNA from such tissues.(Sun et al., 2012) A modified procedure of sectioning unfixed and undecalcified frozen mouse mandibles, for microarray processing and subsequent gene expression analyses has been described in recent times to be specifically used for dental hard tissue associated cells.(Sun et al., 2012)

Identification of cells

A key aspect of LCM is the specific visualization of the correct cell to be studied in question. Routine hematoxylin and eosin are sufficient for easy localization. Some of the stains can however interfere with DNA and RNA extraction after microdissection and should be avoided e.g. Weigert iron hematoxylin or Delafield and Mayer alum.(Hunt & Finkelstein, 2004) Other stains that help in visualization and also preserve nucleic acids are methyl green and nuclear fast red.(Hunt & Finkelstein, 2004) Microdissection of tissue sections that are unstained should be coupled with a hematoxylin-eosin–stained scout section.(Hunt & Finkelstein, 2004)

As RNA/protein quality will degrade swiftly following staining, LCM must be performed and completed within 1 hour.(Liu, 2010) It has been hypothesized that the basis of RNA degradation is the activation RNAase due to the existence of water in staining solutions.(Clément-Ziza et al., 2008) Thus, utilization of RNAase inhibitor with aqueous staining solutions is expected to extend the working time.(Wang et al., 2006a) There are, however two other methods by which this time slot can be extended by another 0.5 hours. One method consists of performing LCM under an argon atmosphere, thus averting tissue rehydration; moreover it is also compatible with all existing microdissection protocols. The second one is a novel fixation and staining method utilizing ethanol as solvent in all preparatory steps to LCM that improves fixation and dehydration of samples.(Clément-Ziza et al., 2008)

A final point to remember while equipping a microscope visualization support for an LCM is the fact that a coverslip cannot be placed over the tissue section.(Wang et al., 2006a, Suarez-Quian et al., 2000) This leads to diminution in the image quality and makes targeting decisions difficult.(Suarez-Quian et al., 2000) The underlying reason for the degradation in image quality is the presence of air spaces within the dehydrated tissue that causes the scattering of light and ensuing refractive contrast obscures intricate details.(Suarez-Quian et al., 2000) Placement of a light diffuser (presently obtainable from Arcturus Engineering Inc) into the light path of the microscope and construction of a digital map of the targeted image easily overcomes this limitation.(Suarez-Quian et al., 2000)

Post LCM procedures

Following the completion of microdissection, the cap with adhered target cells is placed into a tube that contains an appropriate buffer for extraction of DNA, RNA, or protein.(Liu, 2010) The quality assessment of the extracted bio-molecule from an entire tissue section is usually

necessary and also recommended prior to LCM procedure, which can be done via manual gel or automatic bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).(Liu, 2010) For RNA quality check, two peaks of ribosomal RNA (rRNA), 28S and 18S, and high value of RNA integrity number (RIN) should be obtained in the bioanalyzer profile. The high value of RIN indicates a successful RNA preparation.(Liu, 2010)

Once quality check is done, the DNA/RNA is usually amplified and then further molecular analysis like qRT-PCR, cDNA microarrays, DNA sequencing, Western blots, mass spectrometry, etc. can be carried out.(Liu, 2010)

Validation of LCM technique for use in oral tissues

A study evaluating the plasminogen activator (PA) in oral squamous cell carcinoma (OSCC) concluded that with the use of LCM, the PA content could be tested in as little as 0.5µg protein lysate.(Curino et al., 2004) High-throughput Surface Enhanced Laser Desorption/Ionization-Time Of Flight-Mass Spectrometry (SELDI-TOF-MS) and LCM have been used in OSCC and oral leukoplakia samples in order to obtain their proteomic profile.(He et al., 2009) The authors in above study concluded that LCM usually guarantees approximately 95% purity of the correct cell population from tissues while maintaining the integrity of tissues.(He et al., 2009)

Several studies have established the importance of LCM to generate sufficient genomic material for analysis.(Ohyama et al., 2002, Hoffmann et al., 2001, Sun et al., 2012, Salmon et al., 2012) Indeed, Single Nucleotide Polymorphism (SNP) array analysis of genotype, copy number, and LOH on the DNA content obtained from microdissected areas of FFPE samples

of oral cancer has been successfully accomplished, however it is still not precise enough to be used for diagnosis or treatment planning.(Stokes et al., 2011) See Table 1 for details of these studies.

LCM for integrated oral biosciences:

Neural crest cells differentiate into various specific populations in the oral region to give rise to dental hard tissues derived from ameloblasts, odontoblasts and cementoblasts. Bhattacherjee et al., (2007) separated neural crest- and mesoderm-derived mesenchymal cells by indelibly marking the neural crest cells and their derivatives with expressing enhanced green fluorescent protein (EGFP) which was visualized and photographed using an Arcturus PixCell IIe LCM system equipped with epifluorescence optics (excitation - 470-490nm & emission - 525–550nm; Arcturus Engineering, Mountain View, CA).(Bhattacherjee et al., 2007)

The use of LCM in procuring specific cells to study the genomic changes occurring in cell transition and development as it goes through various embryological phases has been implicated. LCM has also been used to study dentinogenesis,(Hoffmann et al., 2001) cementogenesis,(Paula-Silva et al., 2010, Date et al., 2012) odontogenesis(Jumlongras et al., 2012) and ectomesenchyme transition in palatogenesis.(Huang et al., 2011, Nakajima et al., 2007, Nakajima et al., 2010, Nawshad et al., 2004) The intact cells from cervical loop and the enamel knot were microdissected from the developing tooth germ utilizing the LCM. Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) and Proliferating Cell Nuclear Antigen (PCNA) were then utilized to immunolabel the microdissected cells and were subsequently sorted via a flow cytometer.(Matalova et al., 2010) This novel technique of obtaining pure microdissected cells by combining flow cytometry along with LCM has

revealed that varying rates of apoptosis is an important step in tooth patterning.(Matalova et al., 2010) LCM has also been used to decipher the entire transcriptomic profile of human "ameloblast" and "odontoblast" referred to as the "dentome". This may provide newer insights into the complex mechanism controlling biomineralization and odontogenesis. Furthermore, in future studies of Odontogenic pathology, dentome can be utilized as the ideal reference tissue (biologically relevant tissue) for comparisons of data (Discussed later).(Hu et al., 2015)(Table 2).

Oral physiological processes are unique and diverse owing to the specific embryological pattern of differentiation of cells and the unique functions of the oral cavity. LCM technology has been used to elucidate the molecular basis of taste sensation,(Suzuki et al., 2007, Moyer et al., 2009, Hevezi et al., 2009, Neira et al., 2001, Gao et al., 2009) eruption(Wise et al., 2004, Wise & Yao, 2006, Liu et al., 2006, Werner et al., 2007, Yao et al., 2007, Yao et al., 2004, Oikawa et al., 2011) and oral bone healing and osseointegration.(Lin et al., 2011) LCM has been utilized to build the gene expression atlas of salivary glands, which has given valuable insights into the molecular mechanism of branching morphogenesis during normal salivary gland development.(Musselmann et al., 2011) A novel chemokine ligand referred to as Mucosa associated Epithelial Chemokine (MEC) / Chemokine (C-C motif) ligand 28 (CCL28) has been discovered through LCM.(Pan et al., 2000) MEC has been shown to facilitate homing of cells expressing chemokine receptors CCR3 (eosinophils) and CCR10 (T and B lymphocytes) to mucosal epithelial surfaces in salivary glands and other diverse tissues.(Pan et al., 2000, Kunkel et al., 2003, John et al., 2005) Thus, MEC may have an important role in oral mucosal immunity.(Pan et al., 2000) (Table 3)

The junctional epithelium is a key feature of innate immunity against periodontitis. This has been authenticated by experiments done to gauge the inflammatory response of the junctional epithelium in a germ-free condition and in the presence of commensal bacteria.(Tsukamoto et

al., 2012) LCM studies involving the junctional epithelium have yielded important molecules involved in the pathogenesis of periodontal infections. Keratinocyte growth factor -1 (KGF-1) controls the homeostasis normal epithelium and was found to be significantly increased in periodontitis thus hypothesizing that up-regulation of KGF-1 and KGF Receptor (KGFR) protein associated with disease regulates onset and progression of periodontal pocket formation.(Li et al., 2005) Similarly Transforming Growth Factor - β_1 (TGF-β_1)(Nakamura et al., 2007) and Claudin-1(CLDN-1)(Fujita et al., 2012) have been implicated in periodontitis. The loss of CLDN-1, which is a major structural protein of tight junctions, was seen as an important factor in the initiation of periodontitis.(Fujita et al., 2012) LCM studies in understanding oral microflora, however, have been limited. It has been used to isolate specific microorganism implicated in the periodontal disease,(Guyodo et al., 2012) understanding the oral candidal interaction(Westwater & Schofield, 2012) and proposing oral viral latency model in oral papillomas.(Maglennon et al., 2011) (Table 4)

LCM has found a formidable role in creating specific genomic and proteomic libraries implicating novel genes and pathways in the pathogenesis of oral cancer.(Patel et al., 2008, Leethanakul et al., 2000b, Alevizos et al., 2001, Knezevic et al., 2001, Zhu et al., 2008) The notch and wnt pathways were for first time implicated in the pathogenesis of OSCCs via LCM.(Leethanakul et al., 2000a) LCM generated cDNA arrays have been instrumental in studying differentially expressed genes in head and neck/oral carcinogenesis like Neuromedin U (NMU), osteonectin, ferritin, cathepsin L and oncofetal trophoblast glycoprotein (5T4) that are not yet functionally characterized or studied with traditional methods.(Alevizos et al., 2001) cDNA libraries thus created were used to contribute to the head and neck cancer genome anatomy project (HNCGAP) in their gene discovery efforts and systematic cataloguing of genes. In the process, Monocyte Chemotactic Protein-3 (MCP-3), a chemokine known to induce the production of Matrix Metallo-Proteinase-9 (MMP-9) was discovered.

Additionally, markers required for normal epithelial differentiation like cornifin B and calgranulin A, were identified.(Patel et al., 2008) See table 5 for details of these studies.

LCM configured upregulation of various genes like ferritin heavy polypeptide I (FTH1), urokinase plasminogen activator (uPA), ATP-binding cassette (ABC) transporter, interleukin-1 (IL-1) receptor antagonist and keratin 4 (K4),(Alevizos et al., 2001) keratin-13 (K13),(Ida-Yonemochi et al., 2012) keratin-19 (K19) and keratin-21 (K21),(Jiang et al., 2012) keratin-15 (K15),(Khanom et al., 2012) Interferon-stimulated gene 15 (ISG-15),(Sumino et al., 2013) miR-372 and miR-373(Tu et al., 2015) have been implicated in oral cancer pathogenesis. All these findings suggest that the potential use of LCM could be considered as a forefront technology for early detection of OSCC.(Xiao et al., 2015, Jensen et al., 2015, Khanom et al., 2012, He et al., 2009)

The variable genetic makeup between primary intraosseous carcinoma (PIOC) and OSCCs has been studied via LCM.(Alevizos et al., 2002) It revealed numerous, verifiable upregulated (n=102) and downregulated (n=99) genetic events unique to PIOC. Some upregulated genes were namely Nuclear Factor-116 (NF116), tyrosine phosphatase CIP2 and oncofetal antigen 5T4. ARF-activated phosphatidylcholine-specific phospholipase D1a, zinc finger protein and Forkhead, Drosophila, Homolog Like-15 (FKHL-15) were some of the downregulated genes studied.(Alevizos et al., 2002)

The biological behavior of oral cancer is extremely variable owing to its heterogeneity. The continuing disparity associated with heterogeneity can only be validated with further LCM based studies to examine specific pure cell populations and their behavior in the Tumor Microenvoirment (TME). LCM has been used to study tumor heterogeneity of oral cancer by picking up cell populations from the tumor invasive front and the central region of the same tumor island.(Wang et al., 2006b) It has been reported that there is substantial upregulation of

Tumor Protein-53 (TP53) and Ribosomal Protein S6 (RPS6) in the tumor invasive front than the centre.(Wang et al., 2006b) However, another study reported no difference in the expression of molecular markers like p14, p53, cyclin D1 and Rb in the SCCs arising from various sites like the larynx, oropharynx, hypopharynx and the oral cavity. It has been proposed that, other factors like viral or chemical agents might be responsible for the observed clinical heterogeneity in these subtypes of SCCs.(Weinberger et al., 2009)

Extensive proteonomic profiling of the TME via mircoarrays has demonstrated that intricate molecular crosstalk between cancer cells and stroma is vital for the progression of oral cavity cancers.(Knezevic et al., 2001) However, only a few studies have scrutinized the role of stromal cells in remodelling TME and in pathogenesis of OSCCs.(Weber et al., 2007, Rosenthal et al., 2004a, Horvath et al., 2005) Fibroblasts of normal oral mucosa and cancer tissue were selectively ablated and studied,(Rosenthal et al., 2004a) where TGF-β_1, the primary growth factor secreted by carcinoma associated fibroblasts in the TME would act in a paracrine and/ or autocrine fashion to modulate tumor growth and invasion. In a separate study it was reported that Membrane Type 1 Matrix Metallo-Proteinase (MT1-MMP) is the chief protease augmented in the stromal cells of head and neck tumors correlating with poor patient outcome and lymph node metastasis (LMN).(Rosenthal et al., 2004b) Some novel biomarkers in stromal fibroblasts implicated in oral cancer pathogenesis are nei endonuclease VIII-like 3 (NEIL3), fanconi anemia group D2 (FANCD2), Fanconi Anemia Zinc Finger (FAZF) and Excision Repair Cross Complementation Group 2 (ERCC2)/ Xennoderma Pigmentosum group D (XPD).(Weber et al., 2007) Since, all these are involved in DNA repair, downregulation of these markers make the stromal fibroblasts more prone to accumulate genetic aberrations (Genetic Instability) which in turn reciprocally increase the ability of invasion and metastasis of the epithelial tumor component.(Weber et al., 2007) In

fact, among all these novel stromal markers, the downregulation of NEIL3 can be considered as the initial event that precedes cascade of genetic alterations in the stroma.(Weber et al., 2007) Furthermore, the combined downregulation of all these biomarkers has a synergistic effect in promoting genetic instability in stroma.(Weber et al., 2007)

The process of metastasis is associated with the differential expression of numerous genes.(Chang et al., 2011, Toruner et al., 2004) A specific genomic profile map has been created for OSCC that demonstrate prospective genes governing the metastasis of OSCCs.(Toruner et al., 2004) The upregulation of neuregulin 1 (NRG1) and some of its alternatively spliced isoforms were shown to promote metastasis. The downregulated genes included K4, sciellin (SCEL), CLDN-17 and Desmoglein 1 (DSG1). The downregulation of these genes resulted in dedifferentiation of epithelial cells and loss of cellular cohesion. There was also an upregulation of MMP-1 gene, which encodes for collagenase 1. All these changes are consistent with the process of tumor cell invasion into the connective tissue, and their subsequent metastasis.(Toruner et al., 2004) Additionally, downregulation of transglutaminase-3 (TGM-3), keratin-16 (K-16) and the upregulation of Ribosomal Protein 27 (RPS27) have also been reported to be consistent features of OSCCs with positive LNM.(Méndez et al., 2007) It has also been reported that anti-apoptotic protein B-cell lymphoma 2 (Bcl-2) is highly expressed in tumor-associated endothelial cells of OSCC patients with LNM, while it is undetectable in the endothelial cells of OSCCs patients that did not present with LMN.(Tarquinio et al., 2012) Novel proteins specific for metastatic cells of OSCCs, peroxiredoxin 4 (PRDX4) and prolyl 4-hydroxylase subunit alpha-2 (P4HA2) have been discovered using LCM.(Chang et al., 2011)

b) Salivary gland tumors

The expression of cyclooxygenase-2(COX-2), Bcl2 and Ki67 protein in cells of pleomorphic adenoma has been examined.(Aoki et al., 2004) The upregulated expression of COX-2 and anti-apoptotic protein Bcl2 in the luminal cells of pleomorphic adenoma was linked to tumor pathogenesis. A study was undertaken to analyze gene expression in adenoid cystic carcinoma (AdCC) to correlate it to perineural invasion by microdissecting cancer cells close to the nerve and distant from the nerve.(Chen et al., 2007) This study implicated several novel genes responsible for perineural invasion in AdCC like melanoma cell adhesion molecule (MCAM), amphiregulin (AREG), meningioma expressed antigen 6 (MGEA6), N-terminal caspase recruitment domain 12 (CARD12), peripheral myelin protein 22 (PMP22), taxol resistance associated gene 3 (TRAG3), MMP-7 and nuclear transport factor 4 (NTF4) gene.(Chen et al., 2007) The authors found that melanoma associated gene (MAGE) family proteins (A2, A3, A4 and A9) and TRAG3 which cause DNA demethylation, are downregulated in AdCC with perineural invasion.(Chen et al., 2007)

c) Odontogenic cysts and tumors

LCM technology is crucial in the study of tumors that lack solid growth pattern, but rather are categorized by an infiltrative and cystic component that often leads to contamination of tumor tissue via conventional bulk tissue analysis.(DeVilliers et al., 2011) Gene expression profile has been recently formed for LCM procured ameloblastoma cells in order to identify genes or gene products that may have diagnostic, prognostic, or therapeutic potential.(DeVilliers et al., 2011) DeVilliers et al., (2011) in their study of the microgenomics of ameloblastomas found that most of the overexpressed genes were typical of an ameloblastoma lineage.(DeVilliers et al., 2011) Among the overexpressed genes characterizing ameloblastic lineage were, the wingless-type mouse mammary tumor virus (MMTV) integration site family, member 10A,

MyoD family inhibitor (MDFI), Protein patched homolog (PTCH) and B-TFIID TATA-box binding protein associated factor 1 (BTAF1) RNA polymerase II transcription factor.(DeVilliers et al., 2011) The genes that were downregulated were Ras related protein 31 (RAB31), T-cell acute lymphocytic leukemia 1 (TAL-1) and suppression of tumorigenicity-13 (ST-13).(DeVilliers et al., 2011) LCM in conjunction with Immunohistochemistry (IHC), In-Situ-Hybridization (ISH) and Polymerase Chain Reaction (PCR) has been utilized to rule out the role of HPV in etiopathogenesis of ameloblastoma.(Migaldi et al., 2005)

Long Interspersed Element-1 (LINE-1) methylation levels in ameloblastomas and keratocystic odontogenic tumors (KCOT) were studied in order to understand the epigenetic changes occurring in their formation.(Kitkumthorn & Mutirangura, 2010) The study demonstrated a significant reduction of the methylation level of LINE-1 elements in ameloblastoma compared with KCOT. As LINE-1 hypomethylation is considered a progressive process during tumor development, it might be an important epigenetic event leading to the aggressiveness of ameloblastomas.(Kitkumthorn & Mutirangura, 2010)

Hu, et al., (2016) compared the transciptome of KCOT utilizing whole genome microarray with the "dentome" and identified two different molecular subtypes operative in non syndromic KCOTs. In one molecular subtype the Phosphoinositide 3 Kinase/AK Strain Transforming (PI3K/AKT) pathway was functional and in the other Mitogen Activated Protein Kinase (MAPK) pathway was active. The former has a gene expression profile similar to secretory ameloblast (s-KCOT), while the latter had a gene expression profile resembling odonotoblast (o-KCOT). The common down regulated genes in these distinct molecular subtypes were PCTH-1 and GLI-1.(Hu et al., 2016) The authors concluded that even though the phenotype was same (both were KCOT), yet their response to therapy may

be different (based on molecular pathway operative i.e. they their genotypes were different). This underscores the importance of techniques like LCM, in helping to formulate precise and molecularly targeted therapy.

d) Miscellaneous

LCM can help in isolating particular regions or cell populations from intricate tissues like oral mucosa and help in analyzing them in their native tissue context. Indeed, a study utilizing LCM has questioned the notation of oral lichen planus as a pre-malignant lesion.(Accurso et al., 2011) Zhou et al., (2005) examined allelic imbalances in oral pre-malignant dysplasias(Zhou et al., 2005) and constructed phage display peptide library for delivery of intravenously administered drugs to exclusively target tumor cells.(Lu et al., 2004)

Evaluation of the expression of transcription factors and cytokines in the infiltrating lymphocytes through LCM, have led to interesting insights in the evolution and development of the Sjögrens syndrome.(Maehara et al., 2012) It was demonstrated that the expression of T helper (Th^1- and Th^{17}-) related molecules in infiltrating lymphocytes with ectopic germinal centres (GC) was lower than those without ectopic GC. In contrast, the expression of Th^2 and T follicular helper-related molecules in infiltrating lymphocytes was lower in non ectopic GC when compared to ectopic GC. It was proposed that Th^2 cells might be involved in the succession of the disease, predominantly in the commencement and growth of ectopic GC.

Godfraind et al., (2013) in order to resolve the question whether oral pyogenic granuloma (OPG) is a reactive process or a tumor, performed genome-wide transcriptional profiling of laser-captured vessels from OPG and compared it with richly vascularized tissue, placenta, proliferative and involutive haemangioma. Their study recognized a gene signature specific

to OPG, by the means of which they designated it as a reactive lesion. They concluded that OPG results from tissue injury followed by an impaired wound healing response, during which vascular growth is driven by Fms-related tyrosine kinase 4 (FLT4) and the nitric oxide (NO) pathways.(Godfraind et al., 2013)

Future Directions:

LCM technology has significantly advanced, since its advent in the 1990s. The technology has overcome several challenges to address the requirement of large quantities of biomolecules and an absolute need for accuracy. In the future, development of image analysis software utilizing advanced algorithms may facilitate automatic dissection of the cells of interest. The development of automated LCM systems will significantly enhance the precision and pace at which cells can be microdissected and acquired for molecular analysis besides facilitate large scale molecular analysis. Expression LCM is a relatively new development which allows operator independent selection of individual cells via molecular targeting, leading to eradication of inconsistency caused by individual operators.

Summary

As the era of genomic and proteomic analysis continues to evolve at a fast rate, the need of the hour is to be highly specific in our approach to studying the humble cell. LCM is a technology that accomplishes this feat and isolates pure cell populations from their natural habitat or TME. With this technology, cDNA libraries can be constructed for understanding the underlying molecular dynamics and discovering novel gene targets for diagnostic and therapeutic purposes. By this review, we conclude that although limited, it has been used to study various aspects of embryology, physiology and pathology, thus opening new insights

into a variety of unknown processes. LCM is an advanced technology that can aid an oral pathologist in many ways and we support and urge the use of this equipment in further scientific research.

Figure legends

Figure 1: Cell of interest is retrieved with the help of a polymer membrane that attaches to the cell after it has been microdissected with the help of an IR/ UV laser in the Pixcell system (a). Cell is microdissected by a UV laser from the bottom via an inverted microscope onto a specimen mounted on a PEN (Poly-Ethylene Naphtalate) foiled slide and retrieved via photonic pressure in the Zeiss PALM system (b). Tissue is mounted on a PEN foiled slide and placed upside down on the stage. The laser dissects the cells of interest which fall down into a collecting cap via simple gravitational forces in the Leica system

Conflicts of Interest

None to Declare

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Table 1: Studies validating the use of LCM in oral tissues

S.no	Application	Specimen	Processing	LCM used	Reference
1	Preparation of cRNA	Microdissected Odontoblasts	Frozen and FFPE	P.A.L.M. laser microdissection system (P.A.L.M. Microlaser Technologies GmbH, Germany)	Hoffmann et al.,[22] 2001
2	High-density oligonucleotide microarray analysis	Oral cancer tissue	-	-	Ohyama et al.,[21] 2000
3	Identification of candidate genes and molecular profiling	OSCC	Frozen sections	-	Alevizos et al.,[61] 2001
4	Phage peptide library screening	SCC cells	Frozen sections	PixCell II apparatus (Arcturus, Moutain View, CA)	Lu et al.,[90] 2004
5	Gene expression analyses	Developing teeth	Frozen sections	Veritas Microdissection system (Arcturus Bioscience, Mountain View, CA, USA)	Sun et al.,[15] 2012
6	Gene expression analysis	Dental hard tissues		-	Salmon et al.,[23] 2012

Table 2: Application of Laser Capture Microdissection Application in oral embryology

No	Application	Process	Sample	LCM used	Reference
1	Gene expression	Odontoblasts/ Stages of Dentinogenesis	Frozen and FFPE mouse embryo mandible	P.A.L.M. laser microdissection system (P.A.L.M. Microlaser Technologies GmbH, Germany).	Hoffmann et al.,[22] 2001
2	Gene expression	Palatogenesis/ Epithelial Mesenchymal Transitions (EMT)	Frozen sections	Leica Laser Microdissection (Leica Microsystems, Inc., Bannockburn, IL)	Nawshad et al.,[32] 2004
3	Role of Macrophage Colony Stimulating Factor (CSF-1)	Teeth in eruption *in vivo*/Endogenous matrix formation	Frozen sections	PixCell II (Arcturus, Mountain View, CA)	Werner et al.,[43] 2007
4	Gene expression profiles	Embryonic cell lineages/ Orofacial development	Frozen sections	PixCell II (Arcturus, Mountain View, CA)	Bhattacherjee et al.,[25] 2007
5	Role of Transforming Growth Factor-β3	Palatal fusion	Frozen sections	Arcturus engineering (Mountain View, CA)	Nakajima et al.,[30] 2007
6	Role of mesenchymal stem cell marker Stro-1	Cementogenesis	FFPE sections	Leica AS/LMD; Leica Microsystems, Wetzlar, Germany	Paula-Silva et al.,[26] 2010
7	Proliferation and apoptosis	Enamel knot and cervical loop of a tooth germ	Frozen sections	PALM@ROBO, V2.2, Olympus	Matalova et al.,[33] 2010
8	Role of TGF-β3 and MMP13	Palatogenesis/EMT	Frozen sections	Arcturus engineering (Mountain View, CA)	Nakajima et al.,[31] 2010
9	Gene expression database	Mechanism of branching and morphogenesis of mammalian salivary glands	Frozen sections	ArcturusXT Microdissection System (Applied Biosystems, Carlsbad, CA, USA)	Musselmann et al.,[48] 2011
10	Patterning	Lateral and medial nasal processes/ Palatogenesis	Frozen sectionS	Leica Laser Microdissection (Leica Microsystems)	Huang et al.,[29] 2011
11	Role of Cementoblast-specific gene, ZFH/Chd3	In the initiation and termination of apical root extension	Frozen sections	PixCell II (Arcturus Engineering, Mountain View, CA)	Date et al.,[27] 2012
12	Expression of Bmp4	Incisor development	Frozen sections	Leica laser capture microdissection (LCM) LMD 6000 microscope	Jumlongras et al.,[28] 2012

Table 3: Application of Laser Capture Microdissection in oral physiology

S.no	Application	Process	Sample	LCM used	Reference
1	Discovery of novel chemokine ligand mucosae-associated epithelial chemokine (MEC)	Oral mucosal physiological functions		Pixcell II (Arcturus Engineering, Mountain View, CA)	Pan et al.,[49] 2000
2	Novel gene (rmSTG) expression	Taste cells			Neira et al.,[38] 2001
3	RANKL expression	In the follicle for tooth eruption	Frozen sections	Arcturus engineering (Mountain View, CA)	Yao et al.,[45] 2004
4	Regulation of osteoprotegrin	Dental follicle cells during tooth eruption	Frozen sections	Arcturus engineering (Mountain View, CA)	Wise et al.,[40] 2004
5	IL-10 gene expression	Dental follicle and association with osteoclastogenesis and tooth eruption	Frozen sections	PALM LCM System	Liu et al.,[42] 2006
6	Parathyroid hormone-related protein (PTHrP)	Tooth eruption	Frozen sections	PALM LCM system	Yao et al.,[44] 2007
7	Regional difference in the expression of BMP2 and RANKL	Associated with alveolar bone resorption and tooth eruption	Frozen sections	PALM LCM system	Wise et al.,[41] 2006
8	Numerous neurotrophic factors	physiology of taste sensation	Frozen sections	Leica AS LMD system (Leica microsystems, Tokyo)	Suzuki et al.,[35] 2007
9	Role of voltage-gated sodium channels	Taste bud physiology	Frozen sections	MMI Cellcut laser microdissection system	Gao et al.,[39] 2009
10	Construction of first genome-wide survey of gene expression	In taste buds from a primate	Frozen sections	MMI Cellcut laser microdissection system	Hevezi et al.,[37] 2009
11	Identified specific gene expression patterns	In discrete taste cell populations;	Frozen sections	MMI Cellcut laser microdissection system	Moyer et al.,[36] 2009
12	Investigate the role of TGF-beta2 and 3	Vertical alveolar bone growth and rearrangement of the PDL	Frozen and FFPE sections	PALM LCM system	Oikawa et al.,[46] 2011
13	The expression of osteogenesis-related growth factors and chemokines	Bone healing and ossointegration were studied	FFPE sections	Not mentioned	Lin et al.,[47] 2011

Table 4: Application of Laser Capture Microdissection to study microbes in periodontology

S.no	Application	Process/Function	Sample	LCM used	Reference
1	KGF-1 protein expression	Upregulated in periodontitis	Frozen sections	Arcturus engineering (Mountain View, CA)	Li et al.,[53] 2005
2	TGF-beta 1	Maintenance of healthy periodontium			Nakamura et al.,[54] 2007
3	Oral papillomavirus latency	A model described in rabbits		Not mentioned	Maglennon et al.,[58] 2011
4	Co-localization of P. gingivalis with immune cells	Periodontal disease			Guyodo et al.,[56] 2012
5	Claudin-1 gene and protein in periodontitis	Significantly reduced at the junctional epithelium	Frozen sections	Zeiss P.A.L.M.	Fujita et al.,[55] 2012
6	Infection linked alterations inCandida albicans gene expression	Understanding host-fungus interaction			Westwater et al.,[57] 2012
7	Critical role of junctional epithelium in	Periodontal homeostasis and disease	Frozen sections	PALM-MB (Carl Zeiss, Tokyo, Japan)	Tsukamoto et al.,[52] 2012

Table 5: Utilization of Laser Capture Microdissection Application in oral pathology

S.no	Application	Process	Sample	LCM used	Reference
1	Construction of gene expression profiles constructed	Oral squamous cell carcinoma	Frozen sections	Arcturus Engineering, Mountain View, CA	Leethanakul et al.,[60] 2000
2	Identification of candidate genes and molecular profiling	Oral squamous cell carcinoma	Frozen sections		Alevizos et al.,[61] 2001
3	Construction of specific proteomic profile	Oral squamous cell carcinoma			Knezevic et al.,[62] 2001
4	Difference in the genomic profile	Primary intra-osseous carcinoma squamous cell carcinoma	Frozen sections	PixCell II (Acturus Engineering, Mountain View, CA)	Alevizos et al.,[72] 2002
5	Cancer associated fibroblasts	Tumor cell proliferation and invasion	Frozen sections	PixCell I (Arcturus Engineering, Mountain View, CA)	Rosenthal et al.,[76, 78] 2004
6	Inter relationship of COX2 and BCl2	Pleomorphic adenoma pathogenesis	Frozen sections	Arcturus Engineering, Mountain View	Aoki et al.,[83] 2004

7	Gene expression profile	Oral squamous cell carcinoma invasion	Frozen sections	Arcturus Engineering, Mountain View, CA	Toruner et al.,[80] 2004
8	Plasminogen Activator	Role in tumor prognosis	Frozen sections	PixCell II apparatus (Arcturus, Moutain View, CA)	Curino et al.[19] 2004
9	Allelic imbalance of 8p, 11q22.2 approximately q22.3, and 9p21	Oral premalignant dysplasia	Frozen sections		Zhou et al.,[89] 2005
10	Genetic heterogeneities of TP53 and RPS6	At the invasive tumour front and and center of the same tumor	Frozen sections	PALM Company, Germany	Wang et al.,[73] 2006
11	Gene expression profiles of stromal component for prediction of clinical outcome	Head and Neck Squamous cell carcinoma	FFPE sections	PixCell II (Arcturus, Mountain View, CA)	Weber et al.,[75] 2007
12	Tumor-specific differential gene expression	Metastatic and non-metastatic tumor cells	Frozen sections	Arcturus Engineering (Mountain View, CA)	Mendez et al.,[81] 2007
13	Identified several novel genes	Adenoid cystic carcinoma cells showing peri-neural invasion	Frozen sections	PixCell II (Arcturus, Mountain View, CA)	Chen et al.,[84] 2007
14	Devised a novel proteomics platform	Global detection of expressed proteins	FFPE sections	Pixcell IIe microdissection equipment (MDS Analytical Technologies)	Patel et al.,[59] 2008
15	Role of immunoglobulin mRNA	human oral epithelial tumor cells	Frozen sections		Zhu et al.,[63] 2008
16	Proteomic analysis	Sub sites within the head and neck region	FFPE sections	PixCell II (Arcturus, Mountain View, CA)	Weinberger et al.,[74] 2009
17	Protein extraction for protein fingerprint	early diagnosis of OSCC	Frozen sections	Arcturus, Mountain View, CA	He et al.,[20] 2009
17	Methylation level of LINE-1 elements.	Reduced in ameloblastoma compared with KCOT.	FFPE sections	Not mentioned	Kitkumthorn et al.,[87] 2010
18	Allelic imbalance in oral lichen planus	Assessment of its classification as a premalignant condition	FFPE sections	PALM MicroBeam IV (Carl Zeiss Group, Bernried, Germany)	Accurso et al.,[14] 2011
19	Confirmation of oral premalignant status	In oral lichen planus	FFPE sections	PALM MicroBeam IV (Carl Zeiss Group, Bernried, Germany)	Accurso et al.,[14] 2011

20	Identification of PRDX4 and P4HA2	Metastasis related prognosis markers	Frozen sections		Chang et al.,[79] 2011
21	Gene profiling	Ameloblastoma	FFPE sections		DeVilliers et al.,[85] 2011
22	Signalling pathway for regulation of lymphangiogenesis and metastatic spread	Oral Cancer	FFPE sections	Not mentioned	Tarquinio et al.,[82] 2012
23	Loss of Keratin-13	Oral carcinoma *in situ*	FFPE sections	Leica Microsystems GmbH, Wetzlar, Germany	Ida-Yonemochi et al.,[65] 2012
24	Expression of CD21, CK19 and EBV RNA in	oral dysplasias and squamous cell carcinomas	FFPE sections	PixCell IIe LCM system (Arcturus Engineering Inc., Mountain View, CA)	Jiang et al.,[66] 2012
25	Expression of basal cell keratin 15 and keratin 19	Oral squamous cell carcinoma	Frozen sections		Khanom et al.,[67] 2012
26	Various populations of lymphocytes in understanding	Pathobiology of Sjogren's syndrome	Frozen and FFPE sections	Leica Microsystems Japan (ASLMD; Leica Microsystems Japan, Tokyo, Japan)	Maehara et al.,[91] 2012