Research Techniques Made Simple: Laser Capture Microdissection in Cutaneous Research

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In cutaneous research, we aim to study the molecular signature of a diseased tissue. However, such a study is met with obstacles due to the inherent heterogeneous nature of tissues because multiple cell types reside within a tissue. Furthermore, there is cellular communication between the tissue and the neighboring extracellular matrix. Laser capture microdissection is a powerful technique that allows researchers to isolate cells of interest from any tissue using a laser source under microscopic visualization, thereby circumventing the issue of tissue heterogeneity. Target cells from fixed preparations can be extracted and examined without disturbing the tissue structure. In live cultures, a subpopulation of cells can be extracted in real time with minimal disturbance of cellular communication and molecular signatures. Here we describe the basic principles of the technique, the different types of laser capture microdissection, and the subsequent downstream analyses. This article will also discuss how the technique has been employed in cutaneous research, as well as future directions.

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Description: This article, designed for dermatologists, residents, fellows, and related healthcare providers, seeks to reduce the growing divide between dermatology clinical practice and the basic science/current research methodologies on which many diagnostic and therapeutic advances are built.

Objectives: At the conclusion of this activity, learners should be better able to:
- Recognize the newest techniques in biomedical research.
- Describe how these techniques can be utilized and their limitations.
- Describe the potential impact of these techniques.

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PRINCIPLES OF LCM

Emmert-Buck et al. developed laser capture microdissection (LCM) in 1996 at the National Institutes of Health to support the Cancer Genome Anatomy Project (Emmert-Buck et al., 1996). The goal of the Cancer Genome Anatomy Project was to develop a high caliber expression library of human cancers and precancerous lesions. Such an undertaking called for an isolation of specific tumor cells from solid tumors without disturbing the integrity of biomolecules (DNA, RNA, protein) within the collected cells. To accomplish this task, the team developed a microscope-based microdissection platform, now known as LCM.

LCM is a technology used to isolate a single cell or a specific cell population from a heterogeneous tissue section, cytological preparation, or live cell culture by direct visualization of the cells (Emmert-Buck et al., 1996). There are two main classes of laser capture microdissection systems: infrared LCM (IR-LCM) and ultraviolet LCM (UV-LCM). IR-LCM instruments are available as manual or

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Abbreviations: EVA, ethylene-vinyl acetate; FFPE, formalin-fixed paraffin-embedded; IHC, immunohistochemical; IR-LCM, infrared LCM; LCM, laser capture microdissection; UV-LCM, ultraviolet LCM

WHAT LCM DOES

- LCM is a technique that isolates cells of interest or even a single cell from a heterogeneous tissue specimen using a laser source under microscopic visualization.
- Cells isolated by LCM contain intact DNA, RNA, and proteins for downstream molecular analysis.
- LCM is capable of isolating diseased cells from the primary lesion without altering their molecular signatures.
- LCM can be applied to a wide variety of tissue and cellular preparations.

LIMITATIONS

- In the absence of a cover slip, the optical resolution of complex tissues may be limited.
- The reliance on visual identification of target cells creates room for human error.
- Unlike IR-LCM, UV-LCM is limited by the potential to induce UV damage in the circumferential cells, which may be subsequently collected for analysis.

automated systems. Other available platforms include an IR/UV combined system. Regardless of the platform used, the principal steps of LCM are the visualization of cells by microscopy, the transfer of laser energy to isolated cells of interest, and the collection of cells of interest from the tissue section (Espina et al., 2006).

LCM can be applied to a variety of preparations including histological specimens (formalin-fixed paraffin-embedded [FFPE] or fresh-frozen sections) and cytology preparations (direct smears, touch preps, or cell block). Samples can be stained, unstained, or tagged by immunohistochemistry. Frozen tissue effectively preserves RNA, DNA, and proteins, but may distort histologic differentiation. The standard method for preservation of tissue morphology is FFPE. However, it causes unwanted crosslinking between proteins and nucleic acids and proteins are not extractable from FFPE samples (Liu, 2010).

INFRARED LCM

This technique uses a lower energy laser in the IR spectrum of 810 ηm to activate a 100-μm, transparent, and thermosensitive film containing ethylene-vinyl acetate (EVA) saturated with a dye that absorbs IR laser energy. The thermosensitive film is positioned over a stained frozen or FFPE tissue section, which can be visualized with an inverted microscope. The microscope is connected to a computer for laser control and image archiving. A laser beam is directed at the cells of interest, but only the thermosensitive film absorbs the energy of the laser. Consequently, there is no damage to the underlying cells or biomolecules within the cells. The focused pulse from the IR laser produces a conformational change in the EVA polymer, which becomes fixed to the cells of interest underneath. The adhesive force of cells to the film exceeds the adhesive force to the slide, enabling selective removal of cells (Figure 1). Once removed, the cells are transferred to a microcentrifuge tube containing DNA, RNA, or enzyme buffer where the cellular material detaches from the film (Emmert-Buck et al., 1996).

ULTRAVIOLET LCM

The LCM technique using an ultraviolet cutting laser is also known as laser microbeam microdissection. Laser microbeam microdissection uses a high-energy UV laser (355 ηm) capable of cutting tissues. The laser is used to cut around the cells of interest, in contrast to IR capture that focuses the laser on the cells. In UV-LCM, the surrounding unwanted tissue is photoablated whereas the desired cells remain intact (Schutze and Lahr, 1998). Target cells are retrieved through a variety of methods depending on the instrument. The cells can be collected by photonic pressure from a second laser shot that catapults them into a collection cap (PALM/Zeiss system, Oberkochen, Germany), by gravity that deposits them into a collection cap (Leica Microsystems, Wetzlar, Germany), or by a sticky cap to which they are glued after LCM (MMI Instruments, Eching, Germany) (Espina et al., 2006; Liu et al., 2014).

IMMUNO-LCM

Immuno-LCM uses immunohistochemical (IHC) staining to identify and isolate a specific cell population that is challenging to discern visually. For instance, cells that are morphologically similar but immunologically distinct such as B and T lymphocytes can be distinguished using IHC staining for a type-specific antigen before LCM. The common IHC reagents do not adversely affect downstream analysis using assays such as PCR (Fend et al., 1999). RNA degradation from IHC staining can be prevented by prelabeling cells, for example, by injecting animals with a fluorogold label before harvesting the tissue (Yao et al., 2005). However, immuno-LCM is not optimal for studying protein expression, as the protein of interest is bound by antibodies (both primary and secondary) during IHC staining. These bound antibodies can interfere with downstream methods such as polyacylamide gel electrophoresis, western blotting, and mass spectrometry.

DOWNSTREAM ANALYSIS

Once collected, DNA can be subjected to sequencing, DNA methylation assays, and loss of heterozygosity studies. RNA can be used for sequencing and constructing a cDNA library, as well as in gene expression arrays, real-time RT-PCR, and quantitative PCR. Protein can be studied with western blotting, 2D gel electrophoresis, mass spectrometry, and reverse-phase protein microarray. It is important to note that proteomic tests require more material than DNA and RNA analyses (Espina et al., 2006).

ALTERNATIVE METHODS

An alternative approach to isolate and concentrate cells of interest is by cell sorting techniques such as FACS and magnetic-activated cell sorting. These methods require that cells be processed in fluid suspensions, which are suitable for the analysis of hematopoietic and circulating cells but not ideal in the analysis of solid tissue.

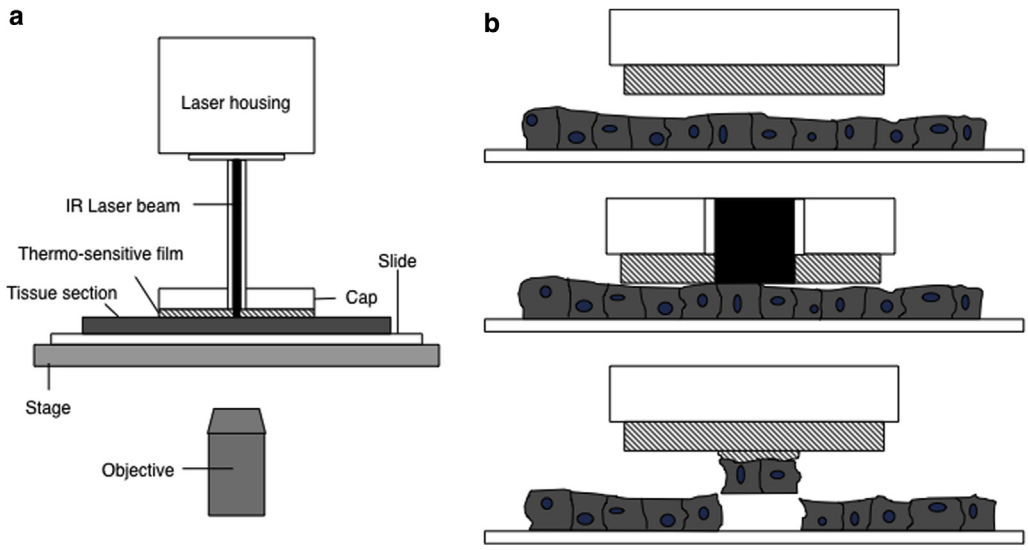

Figure 1. Schematic representation of infrared laser capture microdissection (IR-LCM). (**a**) The IR-LCM setup includes an inverted microscope, an infrared laser, a cap with thermosensitive film on the bottom surface, and a tissue section on a slide without a cover slip. (**b**) Thermosensitive ethylene-vinyl acetate (EVA) film under the cap. An IR laser melts the EVA film. Cells of interest are captured by polymer-cell adhesion. Reprinted from Espina et al. (2006) with permission from Macmillan Publishers Ltd, and from Fend and Raffeld (2000) with permission from BMJ Publishing Group Ltd.

Intercellular adhesions and the extracellular matrix prevent the disaggregation of cells. Disruption of intact tissue alters gene and protein expression and renders the subsequent interpretation of molecular studies difficult (Holle et al., 2016). In this regard, LCM is advantageous because it is capable of isolating diseased cells from the primary lesion without altering molecular signatures.

ADVANTAGES AND LIMITATIONS

Advantages of LCM include speed, precision, ease of use, and versatility. With the IR-LCM, because laser pulses are delivered through an optical cap, the pulses can be repeated across the cap surface to collect thousands of cells per cap. IR-LCM can also be used to collect cells sequentially from the same tissue section, as the directed pulse does not alter adjacent cells. In contrast, the high-energy UV laser is useful for microdissection of thick specimens (up to 200 μm thickness). The UV-LCM laser has a finer beam diameter (0.5 μm) compared with that of the IR-LCM laser (7.5 μm), making UV-LCM more precise in microdissection of a single cell. However, UV-LCM is more time consuming. Additionally, UV-LCM can induce UV damage in neighboring cells, which may limit analysis of sequentially collected cells (Espina et al., 2006).

There are a few limitations that apply to both IR-LCM and UV-LCM. Both require the use of noncoverslip slides to allow physical access to the tissue surface for microdissection. In the absence of a cover slip, the tissue section has a limited optical resolution, which can make precise dissection of complex tissues very difficult. Staining the cell population to be isolated or avoided is a common way to address this issue. Another major limitation of LCM is the reliance on visual discrimination of the target cells. For large-scale molecular profiling projects that involve lesions or cells that are difficult to discern, consultation with a trained pathologist may be required. Further limitations or errors may result from the perishability of tissue specimens, as well as tissue staining protocols and fixation techniques that are not compatible with the downstream analysis. For instance, unwanted crosslinking of nucleic acids and proteins in FFPE tissue sections limits downstream analysis of proteins and RNA (Espina et al., 2006, Fend and Raffeld, 2000).

LCM IN CUTANEOUS RESEARCH

LCM was employed by Masterson et al. (2014) to identify prognostic biomarkers of the rare and poorly understood cutaneous malignancy, Merkel cell carcinoma. IR-LCM and UV-LCM were used to isolate tumor cells for subsequent RNA expression analysis using Affymetrix GeneChip arrays. In addition to the 191 genes demonstrating differential expression, *A2 group X*, *kinesin family member 3A*, *tumor protein*

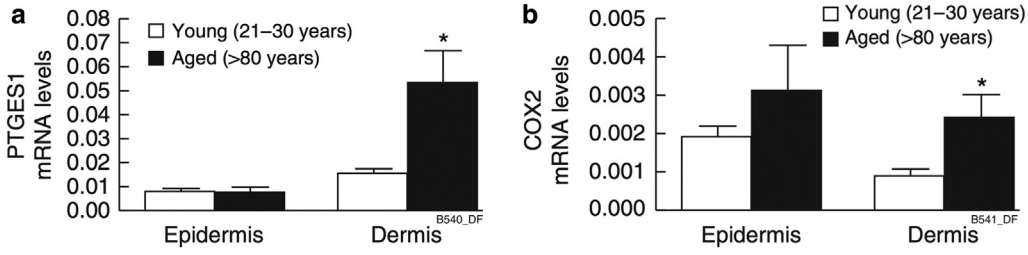

Figure 2. Quantitation of mRNA level by real-time PCR after laser capture microdissection. In both young and aged skin, the epidermis and the dermis were separated and collected by laser capture microdissection. The levels of mRNA for both *PTGES1* (**a**) and *COX2* (**b**) are increased in the dermis of the aged skin compared with that of the young skin, whereas there was no difference between the young and old skin in expression of the two genes in the epidermis. $*P < 0.05$. Reprinted from Li et al. (2015) with permission from Elsevier.

D52, *mucin 1*, and *KIT* were identified as novel genes upregulated in the tumor cells of the patients with poor prognoses. New clinical prognostic markers and therapeutic modalities may be discovered with continued investigation of these promising targets (Masterson et al., 2014).

Wouters et al. (2014) used LCM technology, cDNA library construction, and qRT-PCR to examine the molecular phenotype of melanoma cells undergoing metastatic transformation. Fibronectin 1 is an epithelial-to-mesenchymal transition marker of melanoma. Fibronectin 1 high melanoma cells were found to reside in hypoxic environments such as melanoma lesions with ischemic necrosis. This association suggests that the hypoxic tumor microenvironment may induce melanoma cells to become migratory and more invasive (Wouters et al., 2014).

Li et al. (2015) used LCM and subsequent qPCR to study changes in aging skin. Simply comparing the tissue samples between young and old skin did not initially yield any significant differences. However, using LCM to separate the dermis from the epidermis, followed by qPCR to assess gene expression, Li et al. showed that dermal expression of *PTGES1* and *COX2* genes was significantly higher in aged skin (Figure 2). *PTGES1* and *COX2* contribute to aging skin by increasing levels of PGE2, which inhibits collagen production leading to thinning of the skin. Notably, the therapeutic inhibition of PGE2 may help combat age-associated collagen decrease in human skin (Li et al., 2015).

Goldstein et al. (2015) used LCM, RNA amplification, and qRT-PCR to better understand the effectiveness of narrow band UVB in the treatment of vitiligo. Narrow band UVB treatment was correlated with an increase in gene transcription and subsequent protein expression of certain markers of melanocyte differentiation in treated skin. The examination of molecular changes in activated and mobilized melanocytes is essential for understanding the mechanism of this autoimmune condition as well as for the development of more evidence-based therapies (Goldstein et al., 2015).

MULTIPLE CHOICE QUESTIONS

1. What does LCM do?
   - A. Sorts cells based on morphology (size, granularity, density)
   - B. Sorts cells with either IR or UV laser technology
   - C. Collects cells of interest with laser technology
   - D. Photoablates cells of interest with laser technology

2. What is the thinnest diameter of the UV-LCM laser beam?
   - A. 0.5 μm
   - B. 5.0 μm
   - C. 7.5 μm
   - D. 30 μm

3. Which technique uses a thermosensitive EVA film to sequester cells of interest?
   - A. IR-LCM
   - B. UV-LCM
   - C. Laser microbeam microdissection
   - D. FACS

4. In the absence of a cover slip, the optical resolution of complex tissues may be limited. This issue can be addressed by:
   - A. Using a temporary cover slip
   - B. Increasing the thickness of the tissue section
   - C. Decreasing the thickness of the tissue section
   - D. Staining with immunohistochemistry

5. Which of the following statements regarding the LCM technique is NOT true?
   - A. UV-LCM is better suited for single cell microdissection
   - B. IR-LCM is more time consuming than UV-LCM
   - C. The EVA membrane undergoes conformational change when exposed to IR laser energy
   - D. The downstream analysis of proteins is limited in FFPE tissue sections due to undesirable protein and nucleic acid crosslinking

SUMMARY AND FUTURE DIRECTIONS

LCM is used in cutaneous research to study molecular profiles of a specific cell or population within heterogeneous tissue. Future directions for improvement of the technique include automation to increase efficiency and ease of use, integration of cell recognition software to reduce human errors, and optimization of protocols for sample preparation to aid microdissection itself and to preserve the integrity of the biomolecules for subsequent studies. In the near future, we can anticipate the use of LCM in clinical and research settings with a wide range of applications.

CONFLICT OF INTEREST

The authors state no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to this paper. Teaching slides are available as supplementary material.

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