Laser Microdissection Microscopy and its Applications in Molecular Biology

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1. Introduction

Molecular profiling of tissue samples forms the basis of research in all fields of medicine and is increasingly used for diagnosis, prognosis and treatment.

Tissues are complex structures composed of multiple cell types with different functions. The molecular profile of a tissue sample is a synopsis of the multitude of interactions between its cellular components. In pathological conditions, injured tissues are rendered even more heterogeneous by inflammatory infiltrates, fibrosis, and regenerative processes. Neoplastic transformation may add to the structural and molecular complexity. The molecular profile of a “blind” sample from an injured tissue could be equally representative, for example, of a scar, a spot of angiogenesis, a cluster of regenerative epithelium, a small abscess, or an area of tumour necrosis.

Microdissection techniques have been developed in recent years to obviate this limitation. Specific cell populations can be visualised with a microscope and isolated for further molecular analysis. Early isolation techniques were based on the use of a scalpel blade, a fine stainless steel needle attached to a micromanipulator, irradiation of manually ink-stained sections to destroy unwanted genetic material, or touch preparations of frozen sections specimens. Fluorescent activated cell sorting (FACS) flow cytometry separates cell populations from cell suspensions using one or more markers specific for each cell type, but does not apply well to solid tissues.

Laser-based technology has revolutionised microdissection. Sections of frozen or fixed tissue, cytology preparations or even live cells are laid on polymer coated glass slides, framed polymer membranes or specially designed Petri dishes and visualised with an inverted or upright microscope using bright field, differential interference contrast (DIC), phase contrast or epifluorescence illumination. Specific cell populations are selected manually, or by image recognition software, with or without immunohistochemistry. The selected cells are separated from the surrounding tissue by a laser and collected in a tube for subsequent molecular analysis. Laser microdissection (LMD) is now used worldwide in a variety of fields including pathology, cancer research, neuroscience, cardiology, cell biology, forensics, prenatal diagnostics, plant and reproduction science. In the field of hepatology, LMD has been applied in various areas including hepatocellular carcinoma, cholangiocarcinoma, primary biliary cirrhosis and chronic hepatitis.

In this chapter we report our experience in LMD with emphasis on liver tissue and the technical aspects on tissue preparation, correlation between sample size and quality and quantity of DNA, RNA and protein yield, and their suitability for further molecular biology analysis such as fingerprinting, real-time PCR (rt-PCR) and ELISA.

2. Types of laser based microdissection microscopes

To our knowledge, the first laser based microdissection system was developed at the institute of cancer research in Beteshda in collaboration with Arcturus Engineering. This system is based on an inverted bright-field light microscope. A cap coated with a transparent ethylene vinyl acetate layer is placed over the cells of interest laid on a glass slide and a low power infrared laser beam is directed at these target cells. Fixed position, short duration, focused pulse from an infra-red laser passes through the cap causes the thermoplastic film to adhere to the target cells and, on removal of the cap from the slide, the chosen tissue remains adherent to the cap.

The whole process of melting and cooling of the plastic following each laser pulse is very rapid, in the range of a few milliseconds, and can be repeated many times using the same membrane and section of tissue, allowing for multiple cells to be captured onto a single membrane. Once the microdissection is complete, the membrane with the microdissected cells attached can be removed and used for molecular biology analysis. The power (1–100 mW) of the laser beam and its diameter (7.5, 15 and 30 µm) can both be adjusted, as formalin fixed paraffin embedded (FFPE) sections usually need higher power compared with either unfixed tissue sections or cytological preparations.

Other systems (cutting or cold LMD) are based on a narrow beam ultraviolet-A lasers used to draw around and cut out the cells of interest from surrounding tissue. Laser light is focused through an objective lens of the microscope to a diameter of about a micron or less, and only the cells within the small laser focus is ablated leaving the surrounding tissue intact. Sections are mounted on membrane slides like polyethylene naphthalate (PEN), polyester (POL) or membrane coated glass slides. In contrast to laser capture microdissection, this system is a
non contact system that avoids heating and cooling, therefore the theoretical risk of tissue damage due to melting and solidifying of plastic and potential contamination of tissue are both negligible.

The way microdissected cells are deposited into a collection tube varies depending on the system used. For example the PALM Microlaser Technologies LMD system, is based on catapulting upward the dissected cells into a microtube positioned above the sample by a laser pulse (Laser Microdissection and Pressure Catapulting (LMPC) technology). The Leica AS LMD platform is an upright microscope, in which the dissected sample is transferred into a microfuge tube cap solely by gravity, without any mechanical contact or laser pulse. All the laser cutting systems use a very narrow diameter laser beam (less than 1 µm in diameter), with great precision of laser cutting suitable for dissecting small number of cells.

3. LMD Applications

Various factors can affect the efficiency and efficacy of LMD, and include tissue type, tissue preparation including freezing techniques, storage, fixation, and staining, quantity of tissue dissected, duration of cutting sessions, LMD technique and downstream molecular analysis.

Generally, fresh, frozen tissue is preferred for isolating RNA and proteins, as RNA degradation by ubiquitous RNases is minimised. A long interval between section preparation and microdissection can however affect molecular integrity. FFPE sections are of limited use for RNA and protein extraction mainly due to cross links between formalin, proteins and nucleic acids. Formalin fixation is not immediate in deeper tissue layers which may suffer from protein and RNA degradation. Alternative fixatives and special methods of extraction and amplification of RNA, and proteins from whole FFPE tissue sections have been described recently. DNA can be prepared from both frozen and FFPE microdissected material.

The necessary cell yield varies depending on the downstream application. The recent review by Espina et al provides a useful list of recommended cell yields for different types of downstream analysis.

Valuable information for example on optimal slide temperature, fixation, staining, and dehydration for RNA, DNA and protein extraction can be found in various papers, but these refer specifically to testis, and brain. Other tissues with different function, structure and patterns of injury may need specifically adapted LMD protocols.

4. Applications in hepatology

LMD of liver tissue is usually targeted to hepatocytes, biliary epithelium, inflammatory infiltrates or tumours. Table 1 gives examples of published literature to date and includes the type of downstream molecular application used. To our knowledge, benchmark methodology publications on LMD on liver tissue are scanty.

Our experience is mainly based on a Leica LMD 6000 upright microscope set up for bright field, DIC and epifluorescence illumination. We usually carry out LMD on 10µm thick frozen or FFPE sections. FFPE sections have the advantage of offering access to archival material. We use standard fixation and embedding procedures. We follow the LMD microscope manufacturer protocol to prepare FFPE sections for LMD. Haematoxylin and eosin (H&E) is our stain of choice.

Frozen tissue sections (FTS) in our laboratory are obtained from tissue samples laid into OCT medium, snap-frozen in an isopentane bath and stored at -80°C. As for FFPE sections, FTS are stained following the manufacturer protocol. Both FFPE and FTS sections need to be dried thoroughly after stain, to allow proper separation of the polymer membrane and attached tissue section from the glass slide after cutting.

Liver tissue is usually well visualised at low or high magnification. All structures are easily recognised as in routine diagnostic H&E preparations, despite the thickness of the sections, absence of cover slip and inverted position of the slide. Depending on the cell population of interest, LMD can be carried out at low or high magnification. Large sheets of liver parenchyma (i.e. hepatocyte plates including stellate, sinusoidal endothelial, Kupffer cells and intrasinusoidal lymphocytes) can be easily cut at low magnification in a few minutes. LMD of hepatocytes either individually or in small clusters is possible at high (400x) magnification, but takes longer. In a previous study based on LMD of hepatocytes for DNA extraction and short tandem repeat analysis from FS, we cut up to 100 spots of hepatocyte clusters, each session lasting between 1 and 2 hours. Biliary epithelium can be microdissected very easily, at high speed and low magnification for large parahilar biliary structures, and vice versa for smaller interlobular and septal peripheral bile ducts (unpublished).

Of note, LMD of unstained liver tissue sections, and in particular unstained frozen sections (FU) is possible due to the particular microscopic structure of the liver, its high content in fluorocromes which provide a natural contrast under epifluorescence illumination, and the particular pattern of some types of liver injury. These properties coupled with the use of different microscopy modalities (e.g. bright field, phase contrast, epifluorescence) allow the pathologist to identify specific cell populations or areas of injury without the need of conventional staining. Examples are shown in figure 1. The advantage of this approach is that tissue integrity is maximised, with no interference by dyes, or other chemicals used during staining.
Table 1: Review of some LMD literature in hepatology

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PBC: primary biliary cirrhosis; HCC: hepatocellular carcinoma, RT-PCR: Real-Time PCR, FFPE: formalin fixe paraffin embedded section.

The built in morphometry software within the Leica system allows the instantaneous calculation of the surface area selected for LMD. The surface area is expressed in “µm²”. This is a useful function as it allows comparison between quantity of tissue cut and subsequent yield. We have standardised our protocols using an arbitrary area of 500,000 µm² (0.5 mm²) of liver parenchyma (see above) as reference unit. In our experience, a quantity of microdissected liver parenchyma of 5-10 times the reference unit (2.5-5mm²) is sufficient for most of the molecular applications in use in our laboratory. Here below we describe in more details our experience with reference to RNA, protein and DNA extraction.
Figure 1. Comparison between different liver tissue preparations of ALF sample (10µm section). (a) Section shows perivenular/midzonal confluent hepatocyte necrosis following POD. Unstained frozen section; bright-field, and (b) same field under differential interference contrast (DIC). (c) Epifluorescence from the same field using a “blue” filter [Excitation filter 420/30, dichromatic mirror 415, suppression filter 465/20; supplied by Leica Microsystems Ltd., Milton Keynes, UK], and (d) same sections stained with H&E and slide repositioned to photograph the same field using positioning memory software (Leica LMD 6000). The H&E section shows the centrilobular and midzonal confluent hepatocyte necrosis on the left hand side of the picture. Epifluorescence microscopy shows that the autofluorescence of the tissue allows identification of the various structures including portal tracts and areas of necrosis. The area of necrosis appears to contain substance, which fluoresces red visible also using red and green filters (not shown), with a rim of degenerate hepatocytes fluorescing green. The area of necrosis, portal tract and viable parenchyma are also recognizable using bright-field and DIC microscopy.

RNA

In our experience, a good RNA yield is obtained using TRIZOL® for RNA extraction according to manufacturer protocol (Invitrogen Ltd. Paisley, UK) with some modifications. RNA yield is proportional to the amount of tissue microdissected, using up to fifteen multiples of the reference unit. Further increase in microdissected surface area results in a yield plateau. This is likely due to excess of tissue interfering with the RNA extraction protocol.

RNA extracted from frozen sections is suitable for downstream rt-PCR and microarray analysis. However, RNA obtained from FFPE sections tend to be degraded and may not be easily amplified by rt-PCR. Samples containing as low as five multiples of the reference unit are of sufficient quantity (Figure 2), and quality for performing reverse transcription followed by rt-PCR. Analysis of normal liver parenchyma from five different liver specimen using all the three preparation techniques showed that the mean for the 260nm/280nm ratio was of high quality in all groups; FU (mean1.9, SEM±0.07), frozen stained (FS) (2.0±0.08) and FFPE (1.9±0.09).
RNA extracted from small LMD areas is suitable for rt-PCR and microarray analysis without the need of RNA amplification kits. RNA can be reverse transcribed into cDNA using any of the commercially available kits. We used Omniscript® Reverse Transcriptase kit (Qiagen, West Sussex, UK) according to the manufacturer’s protocol, with some modifications. Levels of albumin gene expression using relative quantification rt-PCR were higher in the FS samples by 3.6 to 45.5 folds higher than the FU baseline sample. In FFPE samples, albumin gene expression was undetectable (Figure 3). The poor results of FFPE samples probably reflects RNA degradation as described in tissues other than liver.\(^{(28)}\)

**Figure 2.** Concentration of total RNA extracted from LMD areas, data presented as mean and SEM.

**Figure 3.** A representative example of rt-PCR runs for albumin gene expression in LMD areas. [FU = frozen unstained, FS = frozen stained, and FFPE = formalin fixed paraffin embedded]

**Protein**

In our experience, it is possible to obtain a protein lysate using RIPA buffer from LMD sections similar to that reported for LMD cells from nervous tissue,\(^{(30)}\) with some modifications. LMD of surface areas of viable or necrotic liver
parenchyma ranging from $8 \times 10^6$ to $10 \times 10^6 \, \mu \text{m}^2$ generated 330-439ug/ml (total volume was 25-30ul) as per modified Lowry method for protein estimation, following extraction based on PMSF (10µl/ml), Aprotinin (30µl/ml), Na orthovanadate (100mM) - 10µl/ml, for proteome array (Aushon Byosystems).

We believe that liver tissue microdissected for protein analysis is best obtained from FU sections. In four out of five specimens, the levels of albumin measured by ELISA were higher in samples microdissected from FU sections (mean 92.3±SEM 37.6ng/ml) compared to samples of similar size from FS (50.3± 27.7) (Figure 4). Albumin level measured using ELISA from tissue lysate was undetectable in microdissected samples obtained from FFPE sections.

**Figure 4.** Mean albumin concentration level in frozen unstained (FU) vs frozen stained (FS) samples analysed by ELISA, data presented as mean and SEM.

Comparison of results using different methods can generate some interesting observations. We have noted for example a discrepancy between albumin synthesis and albumin gene expression in microdissected areas of necrosis following paracetamol-induced injury. Albumin levels (ELISA) in necrotic areas of necrosis were higher (367.1 ± 23.9 ng/ml) compared to viable parenchyma (164.6 ±51.4 ng/ml), (Figure 5). In contrast, albumin gene expression level in necrotic areas was either undetectable or very low (125 folds lower) compared to viable tissue. A possible explanation for this paradox is that albumin produced and released by hepatocytes is ubiquitous, but albumin gene expression remains confined to viable and functioning hepatocytes.

**Figure 5.** Albumin concentration in LMD areas of hepatocytes vs. necrosis, using ELISA, data presented as mean and SEM.
DNA

Short tandem repeat polymorphism analysis was successful on samples obtained by LMD on FS from three livers.\(^{32}\) In each case LMD was carried out at high magnification, to obtain up to 100 clusters made of 2 to 10 hepatocytes. When LMD was repeated on serial paraffin sections containing 30–50 hepatocytes entrapped into portal thrombi the quantity of DNA obtained was not sufficient to be amplified by PCR.

5. Conclusions

Laser microdissection microscopy is a practical and reliable tool for the rapid and efficient isolation of specific populations of cells, free of contaminants and amenable to a variety of molecular analyses, with a multitude of potential applications particularly in the field of hepatology. Tissue preparation is crucial for laser microdissection, the specific modality depending on the planned downstream molecular application. Our experience in LMD on liver tissue is that unstained frozen sections are best for protein analysis, and H&E stained frozen sections for RNA work. The effect of temperature variation during the different phases of snap-freezing, storage, retrieval, cryostat cutting and microdissection needs to be investigated further. Despite some support in the literature, FFPE sections are of limited use.

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References


