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Needle in a haystack: microdissecting the proteome of a tissue

Review Article

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Summary. Laser-assisted microdissection is a recent technology that enables cells to be harvested from tissue sections. Proteins can be extracted from the dissected cells for molecular analysis. This enables the analysis of proteins in specific cell types in an *in vivo* system. Although quantities of protein obtained from the dissected material can be small, it is possible to use established methods such as Western Blotting and 2D-PAGE, as well as newer technologies such as SELDI-MS, to analyse the proteins. This review describes the applications and technical considerations for using laser-assisted dissected cells in proteomics research.

Keywords: Laser capture microdissection – Laser-assisted microdissection – Proteomics

1 Introduction

Identifying and measuring the expression of proteins can reveal important information regarding a biological process or disease. Particularly in the field of cancer proteomics there has been much focus on identifying biomarkers of tumourigenesis. It is hoped that these will aid in the detection and diagnosis of cancer as well as in the development of therapies. One obstacle in identifying tissue biomarkers is the complex nature of many samples. It is difficult to identify proteins whose expression is specific to certain cells when expression profiles are generated from the whole tissue sample. Identification of biomarkers for tumourigenesis may require separate analysis of protein expression in the tumour cells and surrounding tissue and perhaps even differentiating the grades of the lesion. Immunohistochemistry can be used to measure and localise protein expression, but this is difficult to quantify and the method is not amenable to large scale analysis or the identification of novel biomarkers. A better approach may be to physically isolate cells, extract proteins and subject them to analysis. This can be achieved by creating a suspension of cells from tissues and then isolating a subset by their density or cell marker expression. However it may be difficult to verify that the selected cells are the cells of interest. In the last few years, laser-assisted microdissection techniques have been developed that allow the isolation of cells from tissues for the study of DNA, RNA and, more recently, protein. The advantages of laser-assisted microdissection are that cells can be identified by their morphology and, using the appropriate protocols, high quality proteins and nucleic acids can be extracted.

The feasibility of coupling protein analysis methods to microdissection depends primarily on the sensitivity of the technique, as the amount of protein retrieved from the dissected tissue can be very small. Researchers working with nucleic acids have the advantage of using amplification methods to overcome the problem of small amounts of material, an option that is not available with proteins. However, as protein expression does not always correlate with mRNA levels, the investment in time needed for the capturing of larger amounts of material required for protein analysis may be very worthwhile. Additionally the activity of a protein may be regulated by post-translational modifications, which requires analysis of the proteins. Despite the difficulties of working with small amounts of protein, researchers have successfully utilised established methods such as two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and Western blotting as well as newer technologies based on

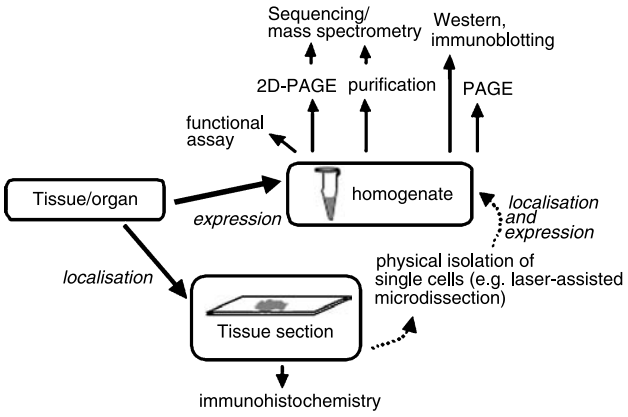

Fig. 1. Approaches for analysing proteins from tissues

mass spectrometry to analyse proteins from microdissected tissue.

2 Laser-assisted microdissection technologies

Several systems are available for laser-assisted microdissection. Using these systems it is possible to quickly dissect an area of tissue, e.g. a tumour, from the surrounding tissue section. Alternatively, it is also possible to collect a quantity of smaller structures, e.g. single cells, that are scattered around a tissue section although this can take considerably more time to obtain a similar amount of material. The PixCell II Laser Capture Microdissection (LCM) system (Arcturus, Mountain View, CA, USA) utilises an inverted microscope and a low energy infra red laser beam. The laser beam focuses through a plastic disposable cap onto the tissue section (Emmert-Buck et al., 1996). The cap has small rails (12 μm) to prevent its surface from making contact with the section. The laser beam (7.5, 15 or 30 μm in diameter) is aligned with a cell and firing it causes thermoplastic film on the surface of the cap to melt and drop to the surface of the section. The melted plastic makes contact with the tissue and bonds to it at that spot. When the cap is lifted away, the attached cells are removed from the surrounding tissue. A disposable well former is placed over the cap, excluding the rails (which may have loose material stuck to them) and the cells can be lysed for further molecular analysis.

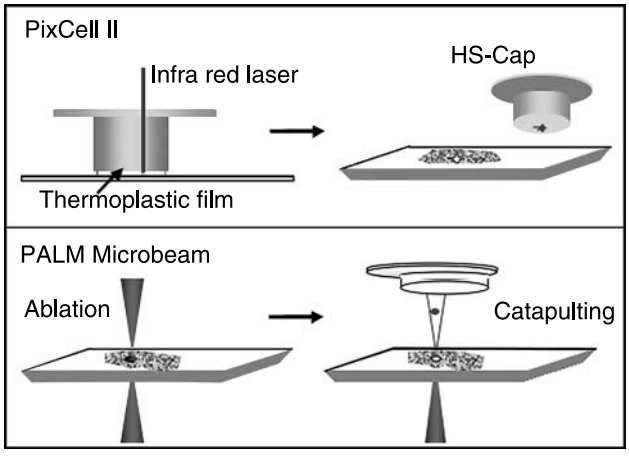

Fig. 2. A schematic representation of the approaches of laser-assisted microdissection using the PixCell II microscope and the PALM microbeam (not drawn to scale)

Another approach is to dissect cells using a laser to sever or ablate the surrounding tissue. The PALM Microbeam (P.A.L.M Microlaser Technologies AG, Benried, Germany) uses a UV laser to ablate material in the focus of the beam. This can be used to dissect around an area of interest. The laser energy is then able to increase the pressure beneath the selected area, catapulting it into the cap of a tube. The Leica AS LMD system (Leica Microsystems, Wetzlar, Germany) uses a similar approach with a UV laser ablating tissue. However, due to the orientation of the slide, the dissected material falls by force of gravity into a collection vessel.

Both the PALM microbeam and PixCell II are specific enough to allow dissection of a single cell, but the ablation approach can dissect smaller sections of tissue compared with the PixCell system. The caps in the PixCell system make contact with the tissue section so there are strict requirements for section preparation, i.e. dehydration through ethanols and xylene. As the PALM Microbeam and Leica AS LMD systems do not make contact with the tissue there is greater flexibility with section preparation, including even the isolation of live cells. The morphology of the dissected cells is easily visible with the PixCell system, but this is lost with catapulting unless sections are prepared on a membrane (Cornea and Mungenast, 2002). The PixCell II, PALM Microbeam and Leica AS LMD systems were reported to provide a similar quantity and quality of RNA retrieval in a comparative study (Cornea and Mungenast, 2002) although they have not been directly compared with regard to protein retrieval. The majority of proteomic studies to date have utilised the PixCell II system.

3 Practical considerations

It is commonly found that the dissection process itself does not significantly affect downstream protein analysis (Banks et al., 1999; Xu et al., 2002), however the steps

preceding dissection can have a significant effect on protein retrieval and quality.

3.1 Tissue preparation

Many protocols recommend fresh frozen tissue for optimal protein recovery. Fresh frozen tissue preserves protein integrity but morphology may be poor, making dissection difficult. There is an abundance of formalin-fixed and paraffin embedded archive material available. However, though morphology is preserved, these sections are not compatible with protein analysis due to cross linking of proteins (Ahram et al., 2003). A compromise may be ethanol (70%) fixation, which has been demonstrated to maintain histological structure along with good protein quality (although reduced in quantity) for proteomic analysis (Ahram et al., 2003).

3.2 Staining

Whichever microdissection system is used, accurate dissection requires that the cells of interest can be distinguished from the surrounding tissue. This can mean using a histological stain or a more complex procedure such as immunostaining. It is important that these detection methods do not interfere with downstream protein analysis. In addition, when using the PixCell II system, the staining method must be compatible with being processed through ethanol and xylene. In most protocols, protease inhibitors are included in staining solutions to minimise protein degradation during the process.

Some commonly used stains have a deleterious effect on 2D-PAGE protein profiles. For example, staining sections with eosin dramatically reduced the number of spots visible on 2D PAGE gels in studies of prostate and brain tissue (Mouledous et al., 2002; Ahram et al., 2003) but had a lesser effect on the profiles obtained from kidney tissue (Craven et al., 2002). The better result with the kidney tissue was suggested to be due to lower concentrations of stain and longer stain differentiation (Craven et al., 2002), though this modified staining protocol did not provide good cell differentiation on all tissues (Mouledous et al., 2002). Cresyl violet and toluidine blue both had deleterious effects on 2D-PAGE profiles (Mouledous et al., 2002) although, again, the latter stain had a milder effect at a lower concentration (Craven et al., 2002). There was no effect on protein separation after Methyl green staining (Craven et al., 2002). Histological stains can have variable effects on other protein analyses. For example, haemotoxylin and eosin had a deleterious effect on matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS) spectral profiles in one study (Xu et al., 2002) but has not been found to interfere with immunoblotting (Casciola-Rosen and Nagaraju, 2002).

Immunostaining can be used to identify specific cell types for dissection. One method using immunogold staining allowed recovery of 40% of protein and had a similar 2D PAGE profile compared with unstained tissue (Craven et al., 2002). A fluorescent detection method allowed greater protein recovery and subsequent 2D PAGE profiles and identification of proteins via MALDI-MS was comparable to unstained tissue (Mouledous et al., 2003b).

An alternative approach to avoid staining methods affecting protein integrity is to use a stained section to navigate around an adjacent unstained section (Mouledous et al., 2003a). This method was used to dissect specifically-defined brain regions but may not be appropriate for dissecting smaller areas of tissue.

The accuracy of dissection or purity of the dissected population can be assessed in two ways. With the PixCell system, the captured material is easily visualised and the morphology of the cells can be assessed. Once cells have been catapulted with the PALM Microbeam, the morphology is lost unless the sections are prepared on a membrane. Alternatively the purity of the captured material could be assessed by comparing the expression of cell specific markers in the dissected cells versus a whole section homogenate (Ball et al., 2002).

3.3 Downstream analysis

The amount of material dissected for analysis is reported in a number of ways including the estimation of the number of cells dissected, the quantity of protein extracted and, for the PixCell system, the number of captures using a particular laser spot size. Analyses using mass spectrometry are able to detect and identify proteins from less than a hundred cells. Methods more suited to quantifying protein expression, e.g. 2D PAGE and Western Blots, require the dissection of thousands of cells. To date, most studies have utilised surface-enhanced laser desorption/ionisation mass spectrometry (SELDI-MS), Western Blotting or 2D-PAGE for analysis of proteins from dissected tissue.

MALDI-MS has been used to identify proteins separated by 2D-PAGE. However, it can also be used to profile proteins directly from dissected cells. Cells captured using the PixCell system are fused to thermoplastic film attached to a plastic disposable cap. The film is removed

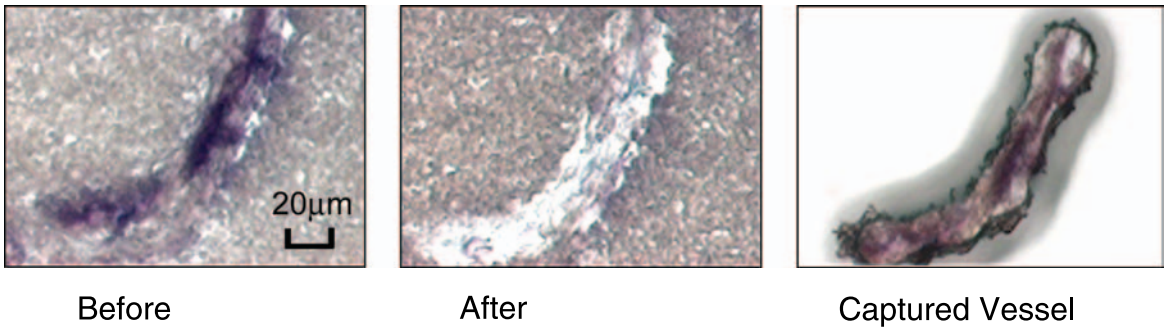

Fig. 3. Dissection of a blood vessel from mouse brain using the PixCell II system

and placed on the MALDI plate and matrix added to the cells. The spectra can then be collected. This has been used to generate distinct spectral profiles of dissected cells representing normal epithelia and breast ductal carcinoma cells (Palmer-Toy et al., 2000; Xu et al., 2002). A reproducible spectral profile was generated from as little as 10 mouse colon crypt cells (Xu et al., 2002).

One problem with MALDI-MS is that the contaminating buffers or cellular components can inhibit the ionisation of proteins. SELDI-MS avoids this problem by capturing proteins based on their affinity for various binding surfaces and washing away unbound material. It is possible that this washing step might make SELDI-MS more compatible with histological stains as hematoxylin and eosin have been used in conjunction with SELDI-MS whereas they were found to have a deleterious effect in MALDI-MS (von Eggeling et al., 2000; Xu et al., 2002). SELDI-MS is a separation and purification method that requires a fraction of the material needed for 2D-PAGE, though SELDI-MS is more suited to analysis of lower molecular weight proteins so the two techniques may be complementary. SELDI-MS has been used to measure the expression of several known markers of prostatic carcinoma in tumour cells as well as identifying a number of unknown proteins that were not present in the surrounding tissue (Wright Jr et al., 1999). It now has been applied to many dissected tumour samples to obtain profiles of carcinomas from the head, neck, skin, kidney, cervix, prostate, breast, oesophagus, ovary, colon and liver as well as trophoblast cells from complete mole placentas (Paweletz et al., 2000a; von Eggeling et al., 2000; von Eggeling et al., 2001; Wellmann et al., 2002; Batorfi et al., 2003). It was found that as few as 25 cells were sufficient to generate a reproducible profile (Paweletz et al., 2000a).

Another approach using mass spectrometry was able to identify numerous proteins in samples of approximately 10,000 cells obtained by LCM (Wu et al., 2003). Solubilised protein was digested with trypsin and the mix of peptide fragments was separated by reverse phase HPLC then analysed by mass spectrometry (LC-MS/MS).

Immunoblotting can be used to detect protein obtained from several thousand dissected cells, depending on the cell type, abundance of protein and sensitivity of detection methods. Dissection followed by immunoblotting has two major advantages compared to immunohistochemistry. It is easier to quantitate expression by Western blot analysis and also the molecular weight of the immunoreactive protein can be determined. This provides important additional information about the protein's identity or state. For example, a subset of histologically diverse neoplasms express CD34 by immunohistochemistry, however it is not clear if they actually express the CD34 protein or another protein that shares epitopes with CD34. Tumour cells were dissected from surrounding normal tissue and, by immunoblotting, it was shown that the immunoreactive protein was the same size (110 kDa) as CD34, indicating that the protein was very likely to be CD34 (Natkunam et al., 2000). Expression of the protein could not be detected in lysates of the dissected normal cells. Another example where the molecular weight of the immunoreactive protein provided useful information was in determining the state (free or bound) of prostate specific antigen (PSA) in normal epithelium and prostatic adenocarcinoma (Ornstein et al., 2000a).

Microdissection followed by immunoblotting also is used to quantify protein expression in a particular subset of cells. The authors of a study on calcitrenin expression wished to measure expression of the protein in granule cells of the cerebellum in two strains of mice (Nahm et al., 2002). However a confounding factor was that one strain of mice had fewer granule cells than the other. To overcome this, an equal number of granule cells were captured from each strain of mice and calcitrenin expression was measured by immunoblotting in the captured material.

Reverse phase protein lysate arrays are suggested to be a more accurate way to quantitate protein compared with Western Blot analysis (Jones et al., 2002; Herrmann et al., 2003). Serial dilutions of lysates are arrayed on nitrocellulose membrane and the membranes are probed with antibody, similar to a Western Blot. The use of serial dilutions ensures that the proteins are measured in the linear dynamic range. Approximately 2,000 cells microdissected from prostatic and ovarian tissue were required to measure the expression of several proteins in normal and malignant cells (Jones et al., 2002; Herrmann et al., 2003). An alternative approach is to immobilise antibodies on a physical support and detect soluble proteins. Microparticles coated with antibody were used to quantify PSA in dissected normal epithelium, prostatic intraepithelial neoplasia and invasive carcinoma (Simone et al., 2000). In a larger scale analysis, a membrane array of several hundred antibodies measured protein expression in lysates of approximately 3,000 cells dissected from squamous cell carcinomas (SCC) of the oral cavity (Knezevic et al., 2001).

2D-PAGE is used to simultaneously measure the expression of hundreds of proteins separated according to their charge and size. It requires the dissection of at least 50,000 cells for over 500 proteins to be visible by silver staining. This is less than the number of proteins generally visualised with larger tissue samples, indicating that low abundance proteins may not be detectable in dissected tissue. A 2D-PAGE analysis comparing dissected normal epithelium from the esophagus with SCC cells estimated that proteins in the abundance range of 50,000 to 100,000 copies per cell were detectable (Emmert-Buck et al., 2000). To increase the sensitivity of the 2D-PAGE technique, proteins from microdissected tissue have been labelled with fluorescent Cy3 and Cy5 dyes before electrophoresis. This resulted in the detection of over 1,500 proteins from less than 6.6 μg of protein obtained from a 1 mm^2 area of dissected tissue (Kondo et al., 2003). As well as having a broader dynamic range compared to silver stains, the use of fluorescent dyes enables two samples to be compared in the one gel. Differential in-gel electrophoresis (DIGE), using Cy3 and Cy5 dyes, enabled the protein profiles from microdissected normal epithelium and SCC cells of the esophagus to be compared on the same gel (Zhou et al., 2002). Another advantage of a fluorescent dye such as SYPRO Ruby is that it is both sensitive and compatible with the subsequent identification of proteins by MALDI-MS (Berggren et al., 2000). This could be important in studies using microdissected cells where there may be only enough material available to run a single gel.

Despite having a smaller dynamic range compared with fluorescent dyes, silver staining has been successfully used to detect differential protein expression in microdissected tissue. The profiles of the normal epithelium and SCC cells from the esophagus were found to be very similar, but 17 proteins showed differential expression in the two populations (Emmert-Buck et al., 2000). Two of the differentially expressed proteins were excised and identified by mass spectrometry as being cytokeratin I and annexin I. Obtaining sufficient material for identification of proteins is difficult, therefore it was suggested that another gel with larger amounts of protein from undissected tissue could be run in parallel (Emmert-Buck et al., 2000). The second gel would contain all the proteins present in the dissected material and could be used to obtain proteins for identification. The loss of the annexin I protein was further confirmed by microdissection followed by Western blot analysis in a larger set of esophageal SCC and prostatic adenocarcinoma cases (Paweletz et al., 2000b).

Another study comparing normal epithelium and adenocarcinoma cells dissected from prostate specimens likewise found a very high degree of similarity between the matched samples, and even between patients (Ornstein et al., 2000b). In contrast, the epithelial cell profiles from the prostate and esophagus specimens are very different to the profiles generated from dissected cells of a different lineage and phenotype or stromal cells (Emmert-Buck et al., 2000; Ornstein et al., 2000b). In both cases the differentially-expressed proteins from the normal epithelium and carcinoma cells were not present in the stromal cells. These studies demonstrate that microdissection is not only important for isolating the malignant cells but for obtaining an enriched population of the appropriate matched control cells, e.g. normal epithelium. Interestingly, the protein profiles from dissected adenocarcinoma cells of the prostate differed significantly from the profiles of matched cells that had been cultured *in vitro*. This highlights the usefulness of microdissection for analysing protein expression in specific cell types *in vivo* rather than in cells that may have undergone significant changes *in vitro*.

The effects of microdissection on protein expression profiles may vary according to the composition of the tissue and the type of cell dissected from the tissue. For example, dissection of proximal tubules from a normal kidney produced a protein profile by 2D-PAGE that was not significantly different from that derived from whole tissue (Craven et al., 2002). In contrast, analysis of the protein profiles from dissected cervical epithelium compared with the whole tissue indicated there was significant

enrichment (Craven et al., 2002). It was suggested that microdissection may be particularly important in dissecting normal epithelium for comparison with carcinoma cells as epithelial cells be a minor constituent of some tissues (Wulfkuhle et al., 2003).

4 Conclusion

Tissues are a complex collection of different cell types and studying pools of proteins from tissue homogenates can greatly dilute the information obtained about the proteome of a specific subset of cells within the tissue. Microdissection is a powerful technique for focusing proteome analysis on the cells of interest and excluding extraneous contributions from other cells.

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